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# Investigating the role of androgens in myometrial biology during pregnancy

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**Thesis submitted to the University of Edinburgh**

**for the degree of Doctor of Philosophy**




**October 2014**

# Declaration

I declare that the work presented in this thesis is my own and has been generated by me as the result of my own original research.

Where I have quoted the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.

I acknowledge the Histology Facility of SuRF (Mike Millar) for the histological process (embedding) of all collected tissues and Dr. Sara Rinaldi for performing the stitching of the mice after surgery.

The work described in this thesis has not been previously accepted for or is currently being submitted for another degree or qualification.

Finally, I confirm that this work was done wholly while in candidature for a research degree at the University of Edinburgh.

Signed:

Date:

# Abstract

Understanding the physiology of pregnancy enables effective management of pregnancy complications that could otherwise be life threatening for both mother and fetus. A functional uterus (a) retains the fetus in utero during pregnancy without initiating stretch-induced contractions and (b) is able to dilate the cervix and contract the myometrium at term to deliver the fetus. The onset of labour is associated with successful cervical remodelling and contraction of myometrium, arising from concomitant activation of uterine immune and endocrine systems. A large body of evidence suggest that the action of local sex hormones may drive changes occurring in the uterine microenvironment at term. Although there have been a number of studies considering the potential role(s) played by progesterone and estrogens at the time of parturition, the role of androgens has received less scrutiny.

The overarching aim of this thesis was to investigate the potential roles of androgens in myometrial biology at the time of pregnancy. We examined both the gene-transcription dependent (genomic) and independent (non-genomic) action of androgens on the uterine smooth muscle, employing *in vitro*, *ex vivo*, and *in vivo* approaches.

We found that the androgen receptor (AR) mRNA was significantly increased in the myometrium during labour when compared to the term non-labouring myometrium. Our gene expression studies revealed that ligand-dependent AR signalling in the myometrium might play a role in regulation of uterine smooth muscle cell contractility. We explored the effect of androgens on contraction of uterine smooth muscle strips obtained from both human myometrial biopsies collected at term and murine uterine horns. We found that testosterone (T) and dihydrotestosterone (DHT) in a range of 10-100 μM concentrations rapidly relaxed spontaneous and oxytocin-initiated contractions. The relaxant effect was not mediated by the classical intracellular AR nor was cell-surface initiated as shown by experiments employing a specific AR antagonist (flutamide) and a cell-surface impermeable androgen (TBSA). We investigated whether the relaxant effect was specific to androgens or a generic effect of sex hormones. We demonstrated that both estradiol (E2) and progesterone (P4) were also capable of relaxing the human and murine myometrium at the same

dose range. In addition, a sex hormone "cocktail" (all four sex hormones combined at 10 μM dose each) mimicked the relaxant effect that each individual sex hormone elicited at a 40 μM dose, implying that the effect was possibly attributable to the steroid structure of the sex hormones.

To study the underlying molecular events that mediate the relaxant effect of sex hormones observed *ex vivo*, we employed two human myometrial cell lines namely PHM1-41s and UtSMCs. We demonstrated that the androgen-induced relaxation *in vitro* was not induced by cell death but was mediated by a physiological mechanism whereby incubation with the androgen impaired the stimulated-$Ca^{2+}$ entry into the uterine myocytes, which in turn resulted in poor phosphorylation of myosin light chain protein.

Finally, we conducted a pilot study to explore the hypothesis that administration of androgen could relax the uterine muscle *in vivo*. We utilised a mouse model of infection-induced preterm labour, where infection was induced by intrauterine administration of liposaccharide (LPS) on day 17 of murine pregnancy. Our preliminary data showed that intrauterine administration of DHT on day 17 did not significantly reduce the rate of LPS-induced preterm birth in the doses tested in this study.

In conclusion, the androgen-induced *in vitro* tocolysis appears to be sex hormone-specific rather than androgen-specific. Therefore, sex hormones might have the potential to be used for effective *in vivo* tocolysis to inhibit premature-initiated contractions. Our investigation of the androgen-dependent signalling in the myometrium contributed to the development of novel hypotheses regarding the role of androgens in the regulation of the phenotypic transition of MSMCs during pregnancy. These hypotheses remain to be confirmed in future studies.

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# Dedication

Dedicated to my grandmother Zara Totrova, who passed away in July 2014, and my grandfather Konstantin Tolparov, who passed away in November 2013.

Their words of wisdom have inspired me to make the best out of life.



# Presentations and publications relating to this thesis

## Presentations

**Oral:**

**Makieva S., Saunders P.T.K., Norman J.E.** Androgens action and androgen receptor (AR) expression in myometrial smooth muscle cells (MSMCs) at term pregnancy. *Presidential New Investigator Plenary Session, 61st Annual Meeting of the Society for Gynecologic Investigation 26-29 March 2014, Florence Italy*

**Poster:**

**Makieva S., Saunders P.T.K., Norman J.E.** The relaxing effect of dihydrotestosterone on myometrial smooth muscle contraction is mediated via blockade of intracellular calcium and inhibition of myosin light chain phosphorylation. *World Congress of Reproductive Biology 2-4 September 2014, Edinburgh UK*

**Makieva S., Saunders P.T.K., Norman J.E.** Sex hormone cocktail drug - a possible therapeutic agent for inhibition of preterm contractions? *Perinatal Medicine Meeting 9-11 June 2014, Harrogate UK*

## Awards

The Giorgio Pardi foundation president's plenary research award for research presented by junior investigator. *61st Annual Meeting of the Society for Gynecologic Investigation 26-29 March 2014, Florence Italy*

## Publications

**Makieva S., Saunders P.T.K., Norman J.E. (2014)**. Androgens in pregnancy: roles in parturition. *Hum Reprod Update. 2014 Jul-Aug; 20(4): 542-59* (Appendix 3)

# List of abbreviations

| | |
|---|---|
| A.U.C | Area under the curve |
| A4 | Androstenedione |
| ACTH | Adrenocorticotropic hormone |
| ADRB2 | Beta 2-adrenergic receptor |
| AP-1 | Activator protein 1 |
| AR | Androgen receptor |
| ARE | Androgen response elements |
| AREs | Androgen response elements |
| ART | Assisted reproductive technology |
| ASM | Airway smooth muscle |
| ANOVA | Analysis of variance |
| BCL-2 | B-cell lymphoma 2 gene |
| BCP | Bromo-3-chloropropane |
| BKCa | Large conductance KCa |
| BSA | Bovine serum albumin |
| $Ca^{2+}$ | Calcium |
| C.I | Confidence interval |
| CAPs | Contraction-associated proteins |
| CCK 8 | Cholecystokinin |
| Con-43 | Connexin 43 |
| COX-1 | Cyclo-oxygenase-1 |
| COX-2 | Cyclo-oxygenase-2 |
| cPLA2 | Cytoplasmic phospholipase A2 |
| CS | Calcium-sensitisation |

| | |
|---|---|
| CYP19 | Aromatase |
| DHEA | Dehydroepiandrosterone |
| DHEAS | Dehydroepiandrosterone sulfate |
| DHT | Dihydrotestosterone |
| DLS | Dye loading solution |
| DMEM | Dulbecco's Modified Eagle Medium |
| DRD2 | Dopamine receptor D2 |
| E1 | Estrone |
| E3 | Estriol |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| ER-β | Estrogen receptor alpha |
| FAD | Fetal aromatase deficiency |
| FBS | Fetal Bovine Serum |
| fHPA | Fetal hypothalamic-pituitary-adrenal axis |
| fT | Free testosterone |
| GOI | Gene of interest |
| GPCR | G protein coupled receptors |
| hCG | Human chorionic gonadotropin |
| HL | Hyperreactio luteinalis |
| HRP | Horseradish Peroxidase Streptavidin |
| ICW | In cell Western |
| IGF-1 | Insulin-like growth factor 1 |
| IGF-1R | IGF-1 receptor |
| IGFBP-1 | Insulin-like growth factor-binding protein 1 |
| IGFBP-2 | Insulin-like growth factor-binding protein 2 |

| | |
|---|---|
| IGFBP-3 | Insulin-like growth factor-binding protein 3 |
| IGFBP-5 | Insulin-like growth factor-binding protein 5 |
| IGFBP-6 | Insulin-like growth factor-binding protein 6 |
| IGJC | Intracellular gap junction communication |
| IKCa | Intermediate conductance KCa |
| IL-1 | Interleukin 1 |
| IL-1Rα | IL-1R antagonist |
| IL-1β | Interleukin 1beta |
| IL-4 | Interleukin 4 |
| IL-6 | Interleukin 6 |
| IL-8 | Interleukin 8 |
| IL-10 | Interleukin 10 |
| IL1R | Interleukin receptor |
| IL6R | Interleukin 6 receptor |
| IP3 | Inositol triphosphate |
| IUGR | Growth restriction in utero |
| IVF | In vitro fertilisation |
| KATP | $K^+$ channels sensitive ATP |
| KCa | $K^+$ channels sensitive calcium, |
| KO | Knock out |
| K | Potassium |
| Kv | $K^+$ channels sensitive to voltage |
| LPS | Lipopolysaccharides |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MCR | Metabolic clearance rate |
| miRNAs | Micro RNAs |

| | |
|---|---|
| MLCK | Myosin light chain kinase |
| MLC | Myosin light chain |
| MLCP | Myosin light chain phosphatase |
| MPA | Medroxyprogesterone acetate |
| MSMCs | Myometrial smooth muscle cells |
| NBF | Neutral-buffered formalin |
| NCX | $Na^{+}/Ca^{2+}$ exchange pump |
| NF-kB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NGS | Normal goat serum |
| NRS | Normal rabbit serum |
| NSS | Normal swine serum |
| NSAIDS | Non-steroidal anti-inflammatory drugs |
| NSS | Normal swine serum |
| OBB | Odyssey blocking buffer |
| OVX | Ovariectomised |
| OXT | Oxytocin |
| OXTR | Oxytocin receptor |
| P4 | Progesterone |
| PAK1 | p21-activated protein kinase |
| PAK1IP1 | p21-activated protein kinase-interacting protein 1 |
| PBSA | Progesterone conjugated to BSA |
| PCNA | Proliferating cell nuclear antigen |
| PCOS | Polycystic ovarian syndrome |
| PE | Preeclampsia |
| PGE2 | Prostaglandin E2 |
| PGF2 | Prostaglandin F2 |

| | |
|---|---|
| PGL | Phase Lock Gel heavy tubes |
| PGs | Prostaglandins |
| PHM1-41s | Pregnant human myometrial cell line |
| PIP2 | Phosphatidylinositol 4,5-bisphosphate |
| PKA | Protein kinase A |
| PLC | Phosphoinositide phospholipase C |
| PMCA | $Ca^{2+}$ ATPase |
| PMLC | Phosphorylated myosin light chain |
| POF | Premature ovarian failure |
| PPROM | Preterm premature rupture of membranes |
| PR-A | Progesterone receptor A |
| PR-B | Progesterone receptor B |
| PR | Progesterone receptor |
| PTGER2 | Prostaglandin E receptor 2 |
| PTGES | Prostaglandin E synthase |
| PTGFR | Prostaglandin F receptor |
| PTL | Preterm labour |
| PTB | Preterm birth |
| R.Q | Relative quantification |
| RhoA | Ras homolog gene family, member A |
| RIPA | Radioimmunoprecipitation assay |
| ROCCs | Receptor-operated $Ca^{2+}$ channels |
| ROK | Rho-associated kinase |
| RPE | Retinal pigment epithelial cells |
| RPMI | Rosewell Park Memorial Institute 1640 medium |
| SACs | Stretch-activated channels |

| | |
|---|---|
| SEM | Standard error of the mean |
| SHBG | Sex-hormone binding globulin |
| SKCa | Small conductance KCa |
| SLC | Small luteal cells |
| SMCs s | Smooth muscle cells |
| SOCs | Store-operated channels |
| SR | Sarcoplasmic reticulum |
| TBSA | Testosterone 3-(O-carboxymethyl)oxime:BSA |
| TBS | Tris-buffered saline |
| TGF-β2 | Transforming growth factor-beta 2 |
| TLR-4 | Toll-like-Receptor 4 |
| TLRs | Toll-like receptors |
| TNF-α | Tumour necrosis factor alpha |
| TNFR1 | Tumour necrosis factor receptor 1 |
| TNFR2 | Tumour necrosis factor receptor 2 |
| T | Testosterone |
| tT | Total testosterone |
| UtSMCs | Uterine smooth muscle cells |
| VAPA | Vesicle-associated membrane protein-associated protein A |
| VOCCs | Voltage-operated $Ca^{2+}$ channels |
| ZEB1 | Zinc finger E-box binding home box 1 |
| ZEB2 | Zinc finger E-box binding home box 2 |
| α-SMA | Alpha smooth muscle actin |
| β-hCG | High beta-human chorionic gonadotropin |
| $[Ca^{2+}]$ | Intracellular $Ca^{2+}$ |

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# Chapter 1: Literature review

## 1.1 Uterus

The human uterus is a hollow organ surrounded by a thick uterine wall. It is composed of three layers; the endometrium (or decidua during pregnancy), which is the glandular inner lining of the uterus, the muscular layer consisting predominantly of smooth muscle cells (SMCs) known as the myometrium, and the outer epithelial layer that covers the uterus called perimetrium (**Figure 1.1**).

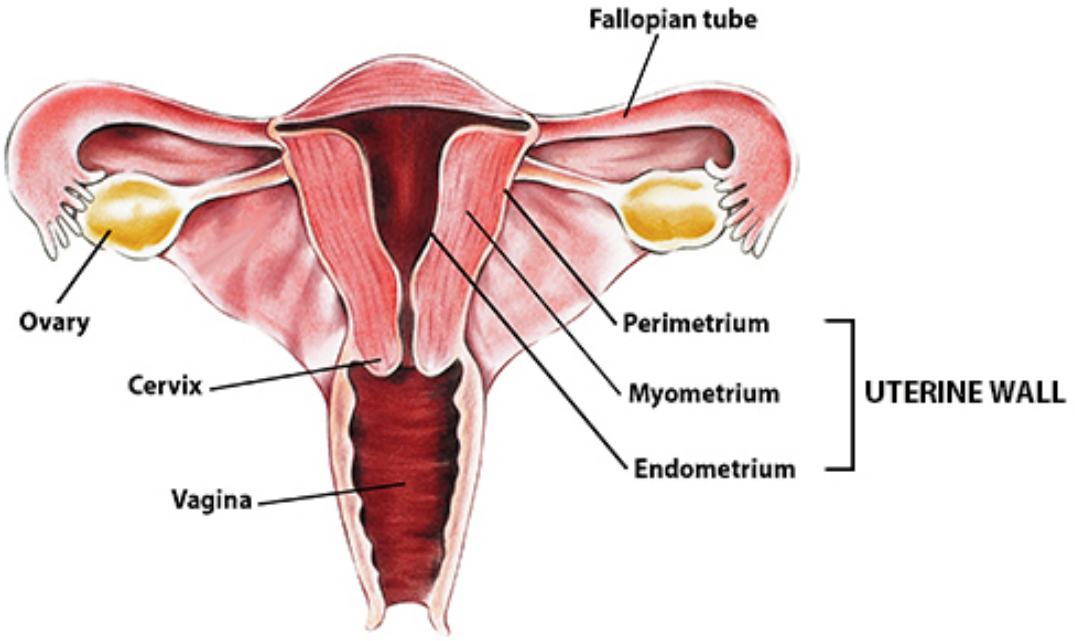



**Figure 1.1:** The anatomy of the human uterus.
Image adapted from http://www.alaskadock.com/female-uterus-anatomy-diagram/.

During pregnancy the uterus undergoes dramatic changes that are required for successful pregnancy and labour. The endometrium adapts to accommodate the needs of the developing fetus and forms part of the placenta. The myometrial smooth muscle cells (MSMCs) undergo hypertrophy and hyperplasia to thicken and strengthen the uterine muscle in readiness for labour. During labour the myometrium develops powerful contractions to help expel both the fetus and the placenta (Young and Hession, 1999). These preparatory changes result in significant enlargement of the uterus, which at term will weigh approximately 20 times its non-pregnant weight

(Gray's Anatomy, 1995). Following delivery, the uterine layers undergo rapid remodelling via phagocytosis and autolysis to help return the uterus to its pre-pregnant size (Matsuzaki et al., 1971).

## 1.2 Parturition

Parturition is the process whereby the fetus and the placenta, including the fetal membranes, are expelled from the genital tract.

### 1.2.1 Term parturition

Normal parturition occurs at 'term', which is anytime between 37-42 weeks of gestation, and is considered to be spontaneous in onset and low-risk for both mother and fetus. Although the process is continuous, it is divided into three stages. The first stage of parturition is subdivided into the latent and the active phase. During the latent phase, the cervix shortens and dilates to 3-4 cm reaching its full dilation at the end of the active phase of the first stage of labour due to the initiation of regular uterine contractions (Byrne and Edmonds, 1990). The second stage of parturition is subdivided into the passive and the expulsive phase. The passive phase commences at the time of full dilatation of the cervix and ends when the head of the fetus descends from the maternal pelvis. The expulsive stage involves active maternal pushing until delivery of the fetus. The second stage of parturition is considered to normally last no more than three hours; if prolonged, it may result in fetal distress and maternal urinary tract damage (Kopas, 2014). The process is concluded at the third stage with the delivery of the placenta.

### 1.2.2 Mediators of parturition

Although the subject of decades of research, the exact mechanism(s) that trigger the onset of labour are still not known. However, we do know that labour is a multifactorial process where inflammatory and endocrine pathways orchestrate the transition of the uterus from a quiescent to an active stage. These pathways favour the transition to an active uterus by promoting the expression of a cassette of contraction-associated proteins (CAPs) and the concomitant activation of contraction cascades in the term myometrium (Smith et al., 2012).

#### *1.2.2.1 Endocrine mediators*

There is robust evidence to suggest that labour is driven by activation of fetal hypothalamic-pituitary-adrenal axis (fHPA), whereby an increase in fetal cortisol increases the placental availability of specific enzymes that favour the conversion of C21 steroids (i.e. progesterone) to C18 steroids (estrogens) (Smith et al., 2002). Hence, we would expect that an increase in cortisol at term would result in a decrease in progesterone (P4) and an increase in estrogens in the maternal circulation. Conversely, in women, concentrations of both sex hormones increase in circulation throughout gestation with no notable change occurring at onset of labour (Tulchinsky D, 1980). This is in contrast to the significant drop in circulating P4 concentration, the so-called P4 withdrawal, observed shortly before the onset of labour in rodents and many other animals [see (Mesiano and Welsh, 2007) for review]. Although human labour is not preceded by a fall in P4, it is hypothesised that a 'functional P4 withdrawal' at this time enables a decrease of P4 activity and promotes initiation of labour (Mesiano et al., 2002). It has been proposed that a decrease in P4 activity may be achieved through a range of mechanisms, including antagonism of progesterone receptor (PR) from other molecules, expression of different isoforms of PR in gestational tissues depending on the gestational time, altered PR cofactor expression and local metabolism of P4 to estrogens (Condon et al., 2003; Mesiano et al., 2002). The importance of P4 signalling in parturition has been highlighted in reports where administration of the PR antagonist RU486 induced softening of the cervix and increased the sensitivity of uterus to uterotonic agents (Hapangama and Neilson, 2009). Other studies have revealed that the two isoforms of the PR, the PR-A and PR-B, are differentially expressed in various gestational tissues (Pieber et al., 2001a; Pieber et al., 2001b). In particular, there is evidence that the binding of P4 to the myometrial PR-B promotes uterine quiescence whereas an epigenetically-regulated switch in predominance of PR-A at term is thought to trigger the expression of CAPs and activate pathways that lead to contraction (Chai et al., 2012; Conneely et al., 2001; Mesiano, 2004; Mesiano et al., 2002). Therefore, a high PR-A/PR-B ratio in the myometrium is associated with the onset of labour. Studies in the placenta and fetal membranes have also demonstrated

an increase in PR-A/PR-B ratio with the onset of labour (Merlino et al., 2009; Merlino et al., 2007; Ziyan et al., 2010).

In contrast to P4, which favours uterine quiescence via PR-B-dependent signalling, estrogens are thought to promote uterine activation by binding to estrogen receptor alpha (ERα) (Mesiano et al., 2002). As mentioned above, the concentration of estrogen in maternal circulation does not change with the onset of labour. However, an increase in ERα expression, detected in the human myometrium at term, is hypothesised to result in 'functional estrogen activation'. Numerous studies, which investigated the role of estrogens in the myometrium at term, have shown that estrogen-dependent signalling drives the expression of CAPs, including oxytocin receptor (OXTR) and connexin 43 (con-43) and, therefore, plays a crucial role in the mechanism of labour onset [see (Welsh et al., 2012) for review].

Recent studies looking at the expression profile of micro RNAs (miRNAs) in the mouse myometrium have identified a family of miRNAs that are induced at term (Renthal et al., 2010). The miR-200 family of miRNAs repress the expression of Zinc finger E-box binding homeobox 1 and 2 (ZEB1 and ZEB2) transcription factors, which are thought to promote myometrial quiescence through inhibition of OXTR and con-43 expression (Williams et al., 2012a). The levels of ZEB1 and ZEB2 drop with the onset of both normal and preterm labour and this may 'fine-tune' the actions of both P4 and estrogens (Renthal et al., 2010; Williams et al., 2012a).

In addition to the sex hormones, oxytocin (OXT) and prostaglandins are thought to play key roles in the regulation of parturition. OXT is a neurohypophysial hormone secreted by the posterior pituitary gland and is believed to be necessary for stimulation and maintenance of myometrial contractions. The binding of OXT to the OXTR, has an excitatory effect on myometrial smooth muscle (Arrowsmith and Wray, 2014). In human myometrium, the concentration of OXTR is low during most of the pregnancy but increases 12-fold at term (Fuchs et al., 1984). This increase is thought to enhance the sensitivity of myometrium to OXT (Soloff et al., 1979). Similar to P4 and estrogen, circulating OXT concentration does not change with labour onset (Thornton et al., 1992). However, a reported local (uterine) OXT surge

at term is thought to regulate myometrial function (Blanks et al., 2003; Mitchell et al., 1998). Contradictory to the studies suggesting that the OXT-dependent signalling is essential for the onset of labour, OXTR knock out (KO) studies in mice demonstrated that null mice undergo normal labour at term (Nishimori et al., 1996; Young et al., 1996). In line with that study, pregnant women who lack OXT due to pituitary gland dysfunction deliver normally (Phelan et al., 1978). It is however plausible that the loss of OXT is compensated by other mechanisms, so that labour is initiated and progresses normally.

Prostaglandins (PGs) are hormone-like lipid compounds derived from arachidonic acid and are well characterised for their roles as central mediators in regulation of myometrial smooth muscle excitation (Arulkumaran et al., 2012; Pawelec et al., 2013). Following synthesis in gestational tissues, including the fetal membranes and decidua, PGs travel to target tissues where they are metabolised to various end products depending on the availability of relevant enzymes (Gaffney et al., 1990; Romero et al., 1996). PGs bind to a number of PG receptor subtypes (EP1-4 and FP), which reportedly increase in the fetal membranes and placenta with labour (Unlugedik et al., 2010). Studies have demonstrated that the primary prostaglandin 2 (PGE2) is significantly increased in amniotic fluid at term and further increased with labour (Dray and Frydman, 1976; Olson et al., 1985). PGE2 is synthesised in the fetal membranes from where it diffuses to the decidua, and reaches the myometrium to stimulates local myometrial PGs production, which act in an autocrine manner to stimulate myometrial contraction (Kandola et al., 2014; Slater et al., 1999; Smith, 2007). Another PG, Prostaglandin F2α (PGF2α) has been reported to favour the expression of PR-A in myometrium, consistent with the role of PR-A in induction of labour (Madsen et al., 2004).

#### *1.2.2.2 Inflammatory mediators*

In addition to the endocrine mediators, a growing body of literature suggests that inflammation is a key feature of human parturition (Challis et al., 2009; Norman et al., 2007). A number of studies have observed an influx of inflammatory cells into the myometrium, cervix and fetal membranes at the onset of labour (Bollapragada et al., 2009; Osman et al., 2003; Thomson et al., 1999). The invading leucocytes have

been shown to increase the production of pro-inflammatory cytokines, such as Interleukin 6/8 and 1β (IL-6; IL-8; IL-1β) in both cervix and myometrium (Elliott et al., 2000; Osman et al., 2003; Young et al., 2002). Notably, IL-1β has been shown to induce calcium transients and, hence, promote excitation of MSMCs (Tribe et al., 2003). In addition to its direct effect on calcium entry, IL-1β has been reported to promote arachidonic acid and cyclo-oxygenase-2 (COX-2) production in the myometrium, resulting in an increase in PGs synthesis and concomitant augmentation of myometrial contraction (Belt et al., 1999; Molnar et al., 1993). Similar effects have been reported for tumour necrosis factor alpha (TNF-α) (Fitzgibbon et al., 2009; Molnar et al., 1993). Besides the roles of pro-inflammatory cytokines in regulation of myometrial contraction, these cytokines have roles in cervical remodelling via stimulation of cervical ripening (Roh et al., 2000; Sennstrom et al., 2000). The NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) protein complex has also been shown to play an important role in the labour cascade (Cookson and Chapman, 2010). There is evidence that the activity of NF-kB in myometrium and fetal membranes increases at term and regulates labour via numerous mechanism, including induction of IL-8, upregulation of CAPs, upregulation of a cassette of genes involved in immunity and inflammation and antagonism of the relaxant effect of P4 on the myometrium (Allport et al., 2001; Allport et al., 2000; Chapman et al., 2004; Khanjani et al., 2011; Khanjani et al., 2012; Lim et al., 2012). The pro-inflammatory role of NF-kB in the myometrium suggests that MSMCs are not only contractile but also immune cells, supporting the notion that the activation of myometrial contractile apparatus is an inflammation-associated response.

Although numerous studies have suggested ways in which endocrine and inflammatory mediators may drive labour, the exact sequence of these events remains poorly understood. A model of ‘modular accumulation’ has been suggested to explain parturition, whereby a number of interlinking processes occur in parallel until a critical threshold is achieved, which leads to labour (Mitchell and Taggart, 2009). The disruption of one or more of these processes may be associated with impaired parturition, which encompasses spontaneous PTB.

### 1.2.3 Preterm parturition

Preterm labour (PTL), defined as anomalous parturition occurring spontaneously before 37 weeks of gestation, is a common pregnancy complication accounting for approximately 11% of recorded births worldwide (Blencowe et al., 2012). Preterm birth (PTB) is a growing clinical challenge and the major cause of neonatal morbidity and mortality in the developed countries (Norman, 2007). The aetiology of PTB is complex with possible contributions from the environment and both maternal and fetal genetics (York et al., 2009). With respect to genetics, various studies to date have investigated and compared the genomes of mothers and infants between preterm and term birth. These studies have analysed a multitude of cascades that are believed to trigger labour and identified polymorphisms in numerous candidate genes that are likely to be associated with the incidence of PTB. Some of these genes include inflammatory mediators such as TNF-α and its receptors TNFR1/TNFR2, IL-4, IL-6 and its receptor IL6R, IL-10 and IL-1 receptor (IL1R) (Menon et al., 2006a; Menon et al., 2006b; Plunkett and Muglia, 2008). In addition, polymorphisms associated with PTL have been detected in genes critical for the excitation of the myometrium, for example the $\beta_2$-adrenergic receptor (ADRB2) (Landau et al., 2002). In contrast, no differences were observed in genes encoding the dopamine receptor D2 (DRD2), prostaglandin E receptor 2 (PTGER2), prostaglandin E synthase (PTGES) and prostaglandin F receptor (PTGFR) (Hao et al., 2004). Although these reports contribute to a better understanding of genetic predisposition to PTB, a concrete link between polymorphisms and the incidence of PTB has not been established, as the results from these studies either did not provide compelling evidence or failed to be replicated.

The majority of preterm births have been attributed to intrauterine infections ascending into the tract via various routes; the mechanisms of infection-induced PTB has been extensively studied (Pawelec et al., 2013). A number of infectious microorganisms arrive into the uterine cavity and are recognised by Toll-Like Receptors (TLRs), which activate inflammatory pathways that then lead to increased production of cytokines and NF-kB and, thus, labour onset (Abrahams et al., 2013; Ganu et al., 2013). Intrauterine infection in cases of PTL has been confirmed by the presence of inflammation in the decidua, the amniotic fluid, the fetal membranes or

the fetus itself (Goldenberg et al., 2008). Clinical studies and animal models have demonstrated the detrimental effect of intrauterine infection on the fetus (Burdet et al., 2014). The presence of inflammation in the uterine cavity during the initiation of PTL complicates the management of PTB, as any possible treatment to retain the fetus in utero until term would not be necessarily beneficial. Although our understanding of the underlying mechanisms surrounding the onset of PTB is still inadequate, studies employing various animal models, both to elucidate these mechanisms and to test interventions to stop PTL, have provided significant insight into the regulation of term and preterm parturition [see (Ratajczak et al., 2010) for review].

### 1.2.4 Animal models of preterm parturition

It is generally accepted that the relevance of animal models of PTB to the clinical scenario of women giving birth preterm is limited. However, given the practical and ethical concerns surrounding a) the research that uses human biological material obtained during pregnancy, and b) the testing of potential treatments for PTL on pregnant women, these animal models have been widely used.

The longest established animal model for studying timing to birth and the effect of an intrauterine infection on the fetus is the sheep (Grigsby et al., 2003; Liggins, 1968; Schlafer et al., 1994). Sheep and human pregnancy share a number of similarities, including gestational length, number of offspring and the fact that the placenta is the main source of P4, in contrast to the predominantly ovarian source of P4 during rodent pregnancy. In addition, the size of both mother and fetus in sheep is closer to that of humans than that of mouse or rat, making experimental manipulation easy. On the other hand, sheep labour is characterised by overt P4 withdrawal in contrast to the functional P4 withdrawal occurring at human parturition. Studies on sheep from the 1960s were the first to show that the onset of labour depends on fetal regulation through the fHPA axis (Liggins, 1968). In these studies, infusion of either adrenocorticotropic hormone (ACTH) or cortisol during pregnancy induced PTB (Liggins, 1968). In contrast, glucocorticoids do not seem to play a trigger role in human labour, limiting the usefulness of sheep for modelling human preterm parturition (Elovitz and Mrinalini, 2004; Mitchell and Taggart, 2009; Ratajczak et al., 2010).

Perhaps the most appropriate animal model for the study of human parturition is the non-human primate. In contrast to the Old and New World monkeys, the rhesus macaques do not undergo systemic P4 withdrawal at labour, which makes them a physiologically representative model to study human labour. The hypothesis that inflammation is key to initiation of human labour was confirmed in studies where intra-amniotic administration of bacteria, such as Lipopolysaccharides (LPS) or pro-inflammatory cytokines (IL-1β and TNF-α), induced PTL in pregnant rhesus macaques (Gravett et al., 1994; Sadowsky et al., 2006). These infection-induced PTB models were utilised in studies testing possible therapeutic agents to stop premature labour. In these studies, few agents effectively inhibited inflammation-stimulated premature myometrial contractions, including TLR-4 inhibitors, which antagonised the action of LPS, the anti-inflammatory dexamethasone and IL-10, and OXTR antagonists (Adams Waldorf et al., 2008; Reinheimer, 2007; Sadowsky et al., 2003).

Since the use of sheep and non-human primates is often less practical due to high maintenance costs and long gestational periods, the use of rodent models has been popular. The most commonly used animal model to study term and preterm birth is the mouse. The mouse genome can be easily manipulated to produce animals with specific gene deletions critical for the timing of birth (Hirsch and Wang, 2005). Studies of transgenic mice have highlighted a critical role of some genes and a less important role of other genes in the process of parturition. For example, mice lacking genes that encode enzymes necessary for PGs synthesis, such as cytoplasmic phospholipase A2 ($cPLA_2$) and Cyclo-oxygenase-1 (COX-1), or proteins critical for initiation of myometrial contraction, such as con-43, have delayed parturition (Bonventre et al., 1997; Doring et al., 2006; Langenbach et al., 1995). On the other hand, mice with no OXT or OXTR undergo normal labour at term (Nishimori et al., 1996; Takayanagi et al., 2005; Young et al., 1996). In addition, mice null for IL-10 give birth preterm, in contrast to IL-1R and IL-1β KO animals, which have normal labour at term (Hirsch et al., 2002; Reznikov et al., 1999). Inflammatory agents like LPS, TNF-α and IL-1β evidently induce premature labour when given to pregnant mice via various routes, making the infection-induced PTB mouse model a cost effective tool to test possible treatments for PTL (Elovitz and Mrinalini, 2004; Sadowsky et al., 2006; Silver et al., 1994). A number of studies employing the above

models have documented that administration of agents like TLR-4 antagonist, anti-TNF-α antibody, IL-1R antagonist (IL-1Rα), NF-kB inhibitors, IL-10, medroxyprogesterone acetate (MPA) and prostaglandin 15d-PGJ2 could all prevent inflammation-induced PTL (Chang et al., 2011; Elovitz and Wang, 2004; Li et al., 2010; Nath et al., 2010). Studies utilising the inflammation-induced PTB model on transgenic mice have identified genes involved in inflammation-induced PTL. For example, TLR-4 KO mice do not deliver preterm when administered intrauterine bacteria, suggesting that TLR-4 is a crucial mediator of the inflammatory cascades in utero (Wang and Hirsch, 2003). Furthermore, IL-1Ra overexpression in mice failed to prevent the expected IL-1β-induced PTB (Yoshimura and Hirsch, 2005). When combined with previous studies on IL-1 and IL-1R, the latter study suggests that IL-1 might be an important but not an essential mediator for initiation of inflammatory cascades in inflammation-induced PTB. Although a useful animal model to study the mechanisms that lead to term and preterm parturition, the relevance of mouse labour to human is limited, firstly, because the major source of P4 in mouse pregnancy is the ovary as opposed to placenta and, secondly, because mice undergo systemic P4 withdrawal just before the onset of labour (Mitchell and Taggart, 2009).

Finally, although not as commonly used as mice, the guinea pig is a promising model in parturition research. The pregnancy of guinea pig is very similar to human because the placenta is the major source of P4 after 4 weeks of gestation and because P4 withdrawal is not required for initiation of labour (Nnamani et al., 2013). Furthermore, compared to mice, guinea pig has a longer gestational period and its fetuses are developmentally similar to human fetuses when born at term (Owen et al., 2005). The only disadvantage in using the guinea pig in parturition research is the fact that their genome is not fully sequenced and, therefore, the experimental reagents available for this species are limited.

Although the currently available animal models to study term and preterm parturition have their benefits, the inter-species variations should be taken into consideration when the findings from these studies are translated for the purposes of drug development for the management and prevention of human PTB.

## 1.3 Current treatments for preterm birth

Despite the considerable advances in parturition research, concrete causal links between the activation of specific molecular pathways and preterm onset of labour have not been established. The biggest challenge for management of PTB has been to develop a treatment that will effectively prolong pregnancy and improve neonatal outcome at the same time. In addition, it is important to ensure that the drugs aiming to improve pregnancy duration have a beneficial effect on neonatal morbidity and mortality and are not associated with long-term impairment of the offspring. Currently, there is no single treatment shown to effectively improve pregnancy duration and long-term outcome in women presenting PTL. Instead, the available treatments for PTL are largely focused on stopping preterm uterine contractions with tocolytic agents (Haas et al., 2012). Tocolysis with calcium channel blockers, beta mimetics, OXTR antagonist, non-steroidal anti-inflammatory drugs (NSAIDS) and nitric oxide donors have each been shown to successfully delay PTB by up to 7 days (Duckitt et al., 2014; Flenady et al., 2014; Haas et al., 2014; King et al., 2003; Neilson et al., 2014; Sykes et al., 2014; van Vliet et al., 2014). However, there is insufficient information to confirm a definite beneficial effect on the fetus (Jorgensen et al., 2014). Of note, these agents target the end-point in the onset of the labour cascade, when the mechanism is already activated and hard to reverse. Hence, drugs that target the underlying trigger for labour are required. In this context, a few drugs have been proposed as potential therapeutic agents for management of PTB, including antibiotics, aimed to treat intrauterine infection, and P4, aimed to maintain uterine quiescence (Brocklehurst et al., 2013; Dodd et al., 2013; Kenyon et al., 2010; Manuck et al., 2014; Seelbach-Goebel, 2013). Data from clinical trials using these agents have been highly informative. Erythromycin administration to women with PPROM (Preterm Premature Rupture of Membranes) in ORACLE I trial prolonged pregnancy and reduced neonatal morbidity (Kenyon et al., 2001a). However, the same antibiotic had no such effect when administered to women experiencing spontaneous PTB in the absence of PPROM and infection in ORACLE II trial (Kenyon et al., 2001b). What is more, follow-up studies revealed an association of the antibiotic treatment with the incidence of cerebral palsy in the offspring in ORACLE II, implying that exposure to antibiotics may be detrimental during the

neurological development of the fetus (Kenyon et al., 2010; Kenyon et al., 2008; Marlow et al., 2012). In light of the evidence that PR antagonists, such as mifepristone and RU486, can induce cervical ripening and initiate labour in humans and animals, P4 therapy for prevention of PTL is believed to have enormous potential for the management of PTB (Clark et al., 2006; Hapangama and Neilson, 2009; Yellon et al., 2013). Consequently, several clinical trials were initiated to explore whether different P4 derivatives, which were delivered in different dosages and via different routes, would reduce the incidence of PTB. To date, P4 derivatives have been shown to be effective in prolonging pregnancy only in women with previous PTB, short cervix or ART (assisted reproductive technology) pregnancy (Aflatoonian et al., 2013; Choudhary et al., 2014; Conde-Agudelo et al., 2013; DeFranco et al., 2007; Dodd et al., 2008; Mackenzie et al., 2006; McKay et al., 2014; Schmouder et al., 2013). Conversely, P4 derivative administration does not prolong pregnancy in women with arrested PTL, multiple pregnancies or women with PPROM (Briery et al., 2014; Briery et al., 2011; Combs et al., 2010; Norman et al., 2009; O'Brien et al., 2007a; O'Brien et al., 2009; Senat et al., 2013; Wood et al., 2012). In view of the lack of evidence on the long-term effects of P4 administration, an on-going study, which will follow the offspring until the age of 2 years (OPPTIMUM) is underway in order to shed some light on the effects of P4 exposure on offspring outcome (Norman et al., 2012).

Overall, these studies highlight that the current management with tocolytics, antibiotics and P4 has been proven ineffective in most cases of PTL and there is little evidence of long-term beneficial effects of these agents for the baby (Hosli et al., 2014). It is therefore important to continue developing novel therapeutic approaches that target molecular pathways involved in the initiation of PTL. To achieve this goal we need to broaden our understanding of the physiology of labour and identify novel pathways involved in the regulation of parturition. The discussed roles of P4 and estrogens in the regulation of term labour and the use of P4 derivatives as preventative therapy in some cases of PTL inspired the studies described in this thesis, which aimed to explore the understudied roles of androgens in the biology of parturition. In addition to P4 and estrogens, androgens also increase with normal pregnancy. Their role in female physiology has been an active area of investigation

for several decades. Although their impact on women's health has been investigated particularly in the context of polycystic ovarian disease, the role they play during pregnancy remains less well described. Evidence for the importance of androgens in the biology of pregnancy and, particularly, parturition are discussed.

## 1.4 Androgens in the physiology of pregnancy and parturition

To date only a few studies have explored androgenic actions in systems critical for pregnancy reaching term, such as myometrial contractility and cervical remodelling. Further understanding of the roles of androgens in pregnancy and parturition will facilitate management of potentially life-threatening complications for both the mother and the fetus. The following sections focus on reviewing what is currently known about a) the biosynthesis, clearance and availability of androgens during pregnancy, b) the correlation of androgens with adverse pregnancy outcomes, c) the role of androgens in physiology of cervical remodelling and d) the roles of androgens in the physiology of the myometrial function.

### 1.4.1 Androgen biology in female physiology

Androgens are critical for development of the fetal male reproductive tract and maintenance of male secondary sexual characteristics in adulthood (Macleod et al., 2010; Scott et al., 2007; Welsh et al., 2008). In addition, they can act as pro-hormones for biosynthesis of estrogens in both males and females (Purohit and Foster, 2012; Rivas et al., 2002; Traish et al., 2011). In women, androgens are synthesised by cells within the ovaries, the adrenal glands and also in fat, where they act in an endocrine, paracrine or intracrine manner. The major androgens synthesised in women are dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAS), androstenedione (A4), testosterone (T) and dihydrotestosterone (DHT) (Burger, 2002; Labrie et al., 2011; Rothman et al., 2011). Besides having key roles in the regulation of reproductive tissues such as the ovary and endometrium, androgens have functional impact on the liver, kidney, bone and muscle (Brenner et al., 2003; Sen and Hammes, 2010; Walters et al., 2008). In women, excess production of androgens, for example in association with conditions such as polycystic ovarian syndrome (PCOS), can result in acne, hirsutism and infertility as well as increase the risk of other complications, such as high blood pressure, cardiovascular disease and

type 2 diabetes mellitus (Dewailly, 2010; Escobar-Morreale et al., 2012; Rosmond, 2006). Since the 1960s, it has been generally hypothesised that, during pregnancy, androgens act as a substrate for estrogen formation in the placenta (Edman et al., 1981; Pion et al., 1965; Siiteri and MacDonald, 1966; Smith, 2007). According to this hypothesis, DHEAS, produced by fetal adrenals, and to a smaller extent from the maternal adrenals, enters the placenta, where it is metabolised to A4 and T, which is then converted into E1 (estrone) (Strauss et al., 1996). E1 is in turn converted into E2 (estradiol), which enters the fetal circulation where is taken up by the liver and converted into E3 (estriol) (Hirano, 1961; Schwarzel et al., 1973). E3 can then pass to the maternal circulation and be cleared in maternal urine (Willows, 1966).

The importance of the androgen receptor (AR) in mediating the effects of androgens is well documented. For example, mutations or deletions in the AR gene located on the X-chromosome are associated, in both human and mouse, with failure to masculinize. This results in 'testicular feminisation', a condition where a male with 46XY karyotype is phenotypically female (Charest et al., 1991; Hughes et al., 2012; Lubahn et al., 1988). The AR is a member of a superfamily of ligand-activated transcription factors; out of all androgens, only T and DHT bind to the AR with high specificity and affinity (Pereira de Jesus-Tran et al., 2006). Two isoforms of the AR (A and B) have been identified (**Figure 1.2**). Ligand binding to the receptor within the cytoplasm, where the AR resides in its unbound state, results in a conformational change in the receptor protein, dimerization, nuclear translocation, association with co-factors and ultimately interaction with specific regions of the genome known as androgen response elements (AREs) (Denayer et al., 2010). The net result of these changes is an upregulation or downregulation in gene expression. Such a response is termed "genomic" as it involves gene transcription. In addition to their genomic effects, androgens are also able to exert their effects in a "non-genomic" manner, independent of DNA binding or specific induction of protein synthesis (Lang et al., 2013). Non-genomic effects of androgens may involve interaction with the cell membrane via a number of signalling molecules, including membrane receptors, ion channels or enzyme-linked receptors and cytoplasmic regulatory proteins (Foradori et al., 2008). These non-genomic effects are rapid and can be observed within

seconds, in contrast to genomic effects, which may take hours or even days to manifest.

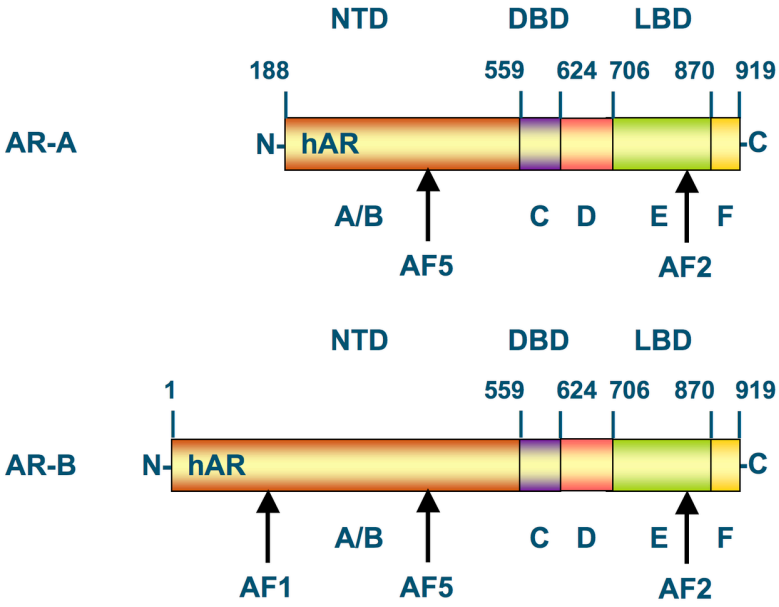



**Figure 1.2:** The two isoforms of the AR. Figure shows the structure of the human AR-A and AR-B. The numbers above the bars represent the amino acid residues that separate the domains of the AR, starting from the N-terminus (left) to C-terminus (right). NTD = N-terminal domain; DBD = DNA binding domain; LBD = ligand binding domain; AF = activation function. Image adapted from http://en.wikipedia.org/wiki/Androgen_receptor.

### 1.4.2 Androgen biosynthesis during pregnancy

The biosynthetic conversion of cholesterol to androgens involves several steps and enzymes. A fundamental step of this process is the synthesis of pregnenolone. Only certain cells types in the human are capable of pregnenolone biosynthesis and these include testicular Leydig cells, ovarian theca and corpus luteum cells, placental trophoblast cells, adrenal cortex cells and some specific cells in the brain [reviewed in (Ghayee and Auchus, 2007)]. Pregnenolone is metabolised to DHEA, which is further metabolised to A4, the precursor of T. T can be reduced to a number of androgens, such as DHT (5α-DHT), 5β-DHT, 5β-androstandiol, 3α-Adiol, 3β-Adiol or further metabolised to estrogens. The reduction of T to DHT and the conversion of T to estrogens is catalysed by the metabolising enzymes 5α-reductase (SRD5A) and aromatase (CYP19) respectively **(Figure 1.3)**. Androgen biosynthesis has been recently extensively reviewed by Penning and colleagues who have identified a number of potential pathways (Penning, 2010).

In the non-pregnant premenopausal woman, approximately 50% of all DHEA is secreted by the adrenal glands, 20% from the ovaries and 30% from the peripheral tissues (Abraham, 1974). In contrast, the adrenals and ovaries produce equal amounts of A4 (Longcope, 1986). Approximately 50% of T is equally synthesised in the ovaries and adrenals and the other half is reported to be produced from A4 in the peripheral tissues (Piltonen et al., 2002). Finally, DHT is not secreted by the endocrine glands but this potent androgen is synthesised from T in the peripheral androgen-target tissues (Ito and Horton, 1971; Marchetti and Barth, 2013). During pregnancy additional sources of androgens include the fetus and the placenta. In particular, the developing fetus is the source of placental 16α-hydroxy-DHEA, a product of 16α-hydroxylation of DHEAS in the fetal liver (Cantineau et al., 1985). In a similar way to its non-16α-hydroxylated form, 16α-hydroxy-DHEA can be metabolised to 16α-hydroxy-A4. The latter is the predominant source of E3 (Milewich et al., 1986). See **Figure 1.3** for summary of these pathways.

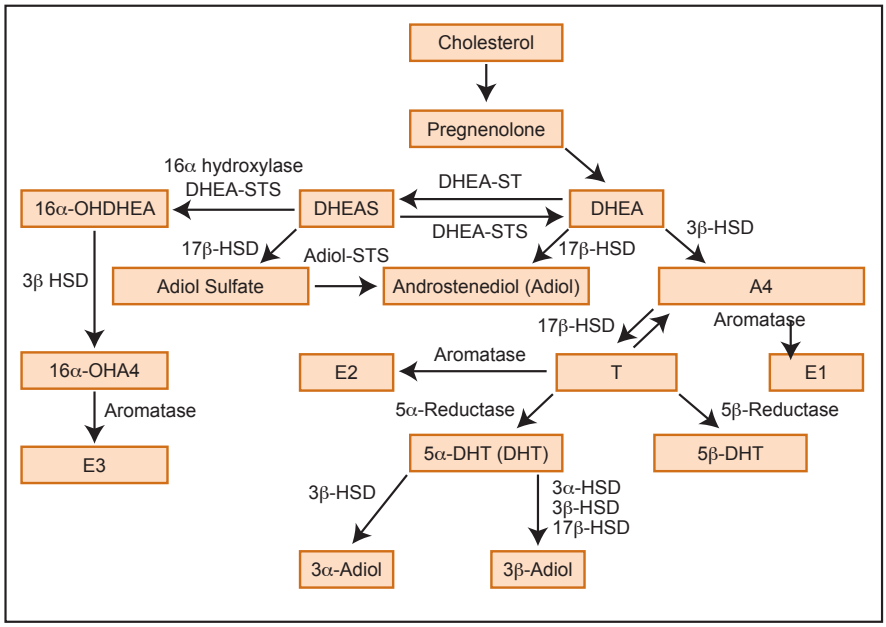



**Figure 1.3:** Testosterone metabolism during pregnancy. DHEA = dehydroepiandrosterone; DHEAS = dehydroepiandrosterone sulphate; A4 = androstenedione; T = testosterone; E1 = estrone; E2 = estradiol; E3 = estriol; 3α-HSD = 3-α-hydroxysteroid dehydrogenase/Δ-5-4 isomerase; 3β-HSD = 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase; 17β-HSD = 17β-hydroxysteroid dehydrogenase; 3α-Adiol = 5α-androstane-3α, 17β-diol; 3β-Adiol = 5α-androstane-3β, 17β-diol; DHT = dihydrotestosterone, STS = sulfatase, ST = sulfotransferase; 16α-OH = 16α-hydroxy. Image adapted from (Makieva et al., 2014).

Once synthesised, androgens enter the maternal circulation, where they can be measured. Usually the circulating concentration of T is reported as either total (tT) or 'free' (fT) with an important distinction between them - the latter is unbound to plasma proteins, such as sex hormone binding globulin (SHBG), and therefore able to pass freely across the plasma membrane to interact with the AR, whereas bound T is unable to penetrate the cell membrane (Mendel, 1989). The percentage binding to SHBG is greater for DHT (59%) than for T (44%) and E2 (20%) and is very low for A4, DHEA, E1 and E3 (Avvakumov et al., 2010). The affinity of each androgen for SHBG is dependent on their structural characteristics. For example, a 17β-OH group and a 5α-hydrogen atom increase the affinity for SHBG binding whereas androgens with either a double bond in ring A or an aromatic ring A are less SHBG-potent (Siiteri and Simberg, 1986). Therefore, DHT, which has both the 17β-OH group and a 5α-hydrogen atom, has two-fold greater affinity for SHBG than T, which has a double bond in ring A (Siiteri and Simberg, 1986).

#### *1.4.2.1 Concentration of androgens during pregnancy*

A number of studies have reported elevated concentration of some, but not all, circulating androgens during normal pregnancy (Bammann et al., 1980; Buster et al., 1979; Dawood and Saxena, 1977; Mizuno et al., 1968; Rivarola et al., 1968; Saez et al., 1972). A notable increase in tT is observed during the first trimester of pregnancy and further elevations are reported towards term (Bammann et al., 1980; Berger et al., 1984; Saez et al., 1972). However, circulating fT only increases significantly at the third trimester of pregnancy (Dawood and Saxena, 1977). In addition, serum concentration of A4 is significantly elevated between 37-42 weeks of pregnancy compared to its non-pregnant concentration, although the relative increase of T during pregnancy is greater than the increase in A4 (Mizuno et al., 1968). In contrast, maternal circulating concentration of DHEAS falls across gestation to approximately 50% of its non-pregnant concentration (Milewich et al., 1978). Finally, circulating SHBG increases during the first trimester of pregnancy and continues to increase dramatically throughout mid and late gestation (Wilke and Utley, 1987). **Table 1.1** summarises the maternal serum concentration of different androgens during pregnancy and **Figure 1.4** is a graphical representation of this information.

**Table 1.1:** Concentration of androgens in maternal serum. Table shows the concentration of each androgen as given in the references quoted. tT = total testosterone; fT = free testosterone; SHBG = sex hormone binding globulin; DHEA = dehydroepiandrosterone; DHEAS = dehydroepiandrosterone sulphate; A4 = androstenedione; DHT = dihydrotestosterone.

| Androgen | Non-pregnant | 1st Trimester | 2nd Trimester | 3rd Trimester | Reference |
|---|---|---|---|---|---|
| fT (pmol/L) | 6.2 | 11.1 | 7.5 | 13.3 | *(Wilke and Utley, 1987)* |
| tT (mmol/L) | 0.21 - 2.98 | 0.90 - 7.32 | 1.20 - 8.40 | 2.20 - 10.70 | *(O'Leary et al., 1991)* |
| A4 (ng/mL) | 1.0 - 2.0 | 2.5 - 3.5 | 0.6 - 7.8 | 1.6 - 14.0 | *(Carlsen et al., 2006; Castracane et al., 1998)* |
| DHT (ng/mL) | 0.022 - 0.107 | 0.113 | 0.18 | 0.1 - 0.3 | *(Buster et al., 1979; Dawood and Saxena, 1977)* |
| SHBG (nmol/L) | 42.2 | 68.1 | 279.3 | 246.1 | *(Wilke and Utley, 1987)* |
| DHEA (nmol/L) | 1.0 - 40.0 | 10.0 - 60.0 | 5.0 - 50.0 | 5.0 - 50.1 | *(Tagawa et al., 2004)* |
| DHEAS (nmol/L) | 2000-4000 | 2000-4000 | 500-2000 | 500-200 | *(Tagawa et al., 2004)* |

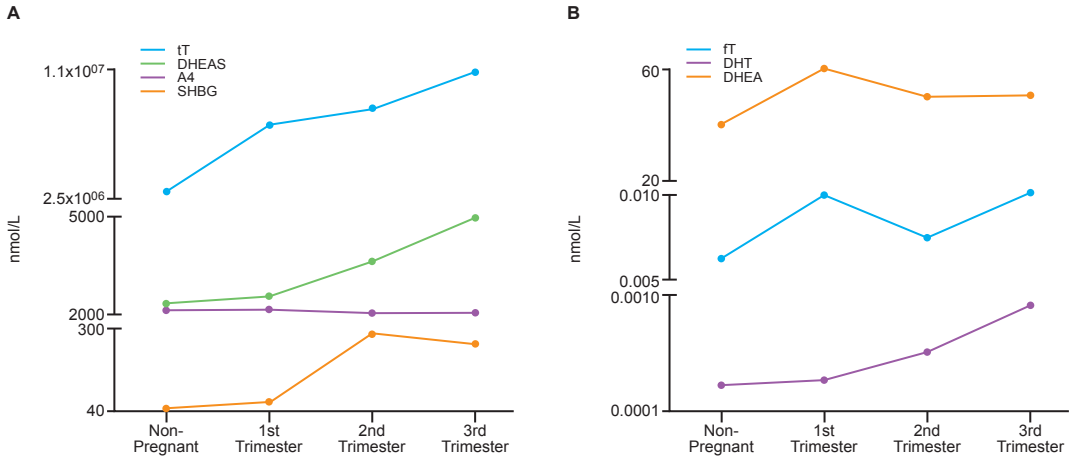



**Figure 1.4:** Graphical presentation of the highest (**A**) and lowest (**B**) androgens in maternal serum throughout gestation. tT = total testosterone; fT = free testosterone; SHBG = sex hormone binding globulin; DHEA = dehydroepiandrosterone; DHEAS = dehydroepiandrosterone sulphate; A4 = androstenedione; DHT = dihydrotestosterone. Adapted from (Makieva et al., 2014).

In the fetus, circulating concentrations of some androgens are dependent on the fetal sex and gestation. In fetal blood, T concentration is higher in males than females

(Rodeck et al., 1985). This contrasts with DHT concentration, which is similar in both sexes (Diez d'Aux and Pearson Murphy, 1974; Rodeck et al., 1985). In male fetuses, serum concentration of T increases until the end of first trimester, reaching a peak of 150 ng/dL at the end of week 12 of gestation, after which it declines by 70% to a nadir at 23 weeks (Rodeck et al., 1985). In female fetuses, T remains generally low throughout first and second trimester (50 ng/dL) and declines rapidly at term (Diez d'Aux and Pearson Murphy, 1974). In addition to T, DHEA concentration is higher in the cord blood of male fetuses compared to females whereas A4 is similar (Keelan et al., 2012). Labour is associated with increased cord blood A4, DHEA and SHGB and decreased tT and fT (Keelan et al., 2012). In the amniotic fluid, T and A4 are higher in male-bearing pregnancies (Carson et al., 1982). On the other hand, there is no notable difference between the concentration of DHEA in the amniotic fluid in male and female fetuses (Robinson et al., 1977). Although T is higher in the fetal compartment of male fetuses, there is no correlation between fetal sex and maternal serum concentrations of any androgen (Rivarola et al., 1968). The correlation between fetal sex and androgen concentration (T or A4) in the fetal compartment, including serum and amniotic fluid, is consistent with androgen biosynthesis within Leydig cells of the fetal testis (Scott et al., 2009). Therefore, increased T and A4 in the amniotic fluid of male fetuses may reflect androgens excreted in fetal urine (Mitchell and Shackleton, 1969). Notably, the studies measuring androgen concentrations during pregnancy in maternal and gestational tissues were performed a few decades ago and, thus, employed traditional radioimmunoassays (RIAs). Nowadays, there are numerous novel methods for measuring the concentration of steroids in serum or homogenised tissue, such as mass spectrometry, which could be utilised to update the current knowledge.

The tissue origin and cause of the increase in androgens in maternal circulation during pregnancy remains uncertain but is likely to involve production from ovary or placenta as discussed below.

#### *1.4.2.2 Tissue origin of androgen synthesis during pregnancy*

Within the ovary, androgens are synthesised in small luteal cells (SLC) of the corpus luteum (former theca cells of ovarian follicle) (Sanders et al., 1996). Once pregnancy

occurs, it is plausible that stimulation of SLC by human chorionic gonadotropin (hCG), which is elevated in first trimester, results in the reported increase in circulating concentration of T at this time (Braunstein et al., 1976; Liu and Hsueh, 1986). However, hCG concentration reaches a peak at the end of first trimester and then declines, in contrast to T, which increases steadily, suggesting either an alternative regulation of androgen production by the corpus luteum or an alternative source of androgens at the end of the first trimester (Braunstein et al., 1976). Studies on women with premature ovarian failure (POF), who became pregnant following in-vitro fertilisation (IVF) with donor egg transfer, indicate that the ovary is the major contributor to the circulating concentrations of T and A4 during pregnancy (Castracane and Asch, 1995). The authors of that study reported that those patients had significantly lower circulating T and A4 when compared to pregnant women without POF. Perhaps not surprisingly, this study is yet to be replicated by other research groups.

In addition to the maternal ovary, the maternal adrenal is an important source of androgens throughout pregnancy. Indeed, maternal and fetal virilisation due to androgen excess have been reported in cases of adrenal adenomas (Fuller et al., 1983). Studies in baboons showed that E2 suppressed the maternal adrenal production of DHEA and DHEAS during pregnancy, which is in line with the reported decrease in DHEAS concentration in the maternal circulation throughout pregnancy (Albrecht and Pepe, 1995; Tagawa et al., 2004; Umezaki et al., 2001). These studies demonstrated that removal of the fetus but not the placenta (fetectomy) induced a decline in E2 and an increase in DHEA and DHEAS concentrations in the maternal circulation (Albrecht et al., 1980; Albrecht and Pepe, 1995). The decrease in E2 was probably attributed to withdrawal of fetal precursors of E2 biosynthesis. Interestingly, the authors showed that exogenous E2 could inhibit the fetectomy-induced increase in DHEA and DHEAS. The observation that the metabolic clearance rates of DHEA and DHEAS remained unchanged before and after E2 administration, led the authors to conclude that the estrogen-induced decline in maternal DHEA and DHEAS reflected a decrease in maternal adrenal production of these androgens.

Despite the prevailing belief formulated in the studies from the 1960s that the placenta does not have the metabolic capacity to synthesise androgens *de novo* (Pion et al., 1965; Siiteri and MacDonald, 1966) and, thus, relies solely on fetal androgens, a recent study has demonstrated that placental syncytiotrophoblasts (but not cytotrophoblast) can synthesise androgens (Escobar et al., 2011). Specifically, the syncytiotrophoblast expresses both the mRNA and the protein encoded by the *CYP17* gene, the enzyme that converts C21 steroids (such as P4) to C19 steroids (such as T). The syncytiotrophoblasts were also shown to have CYP17 activity *in vitro* (Escobar et al., 2011).

The non-pregnant human myometrium evidently possess the metabolic capacity to convert A4 to T and DHT *in vitro* –but whether this occurs in situ during pregnancy is unknown (Jasonni et al., 1982). Consistent with this study, a more recent study has reported that the myometrium, derived from non-pregnant and early pregnant pig uteri, can synthesise A4 and T *in vitro* (Franczak, 2008). Whether the pregnant cervix has the metabolic capacity to synthesise androgens is currently unknown. **Figure 1.5** summarises the possible sites for androgen production in the materno-placental unit.

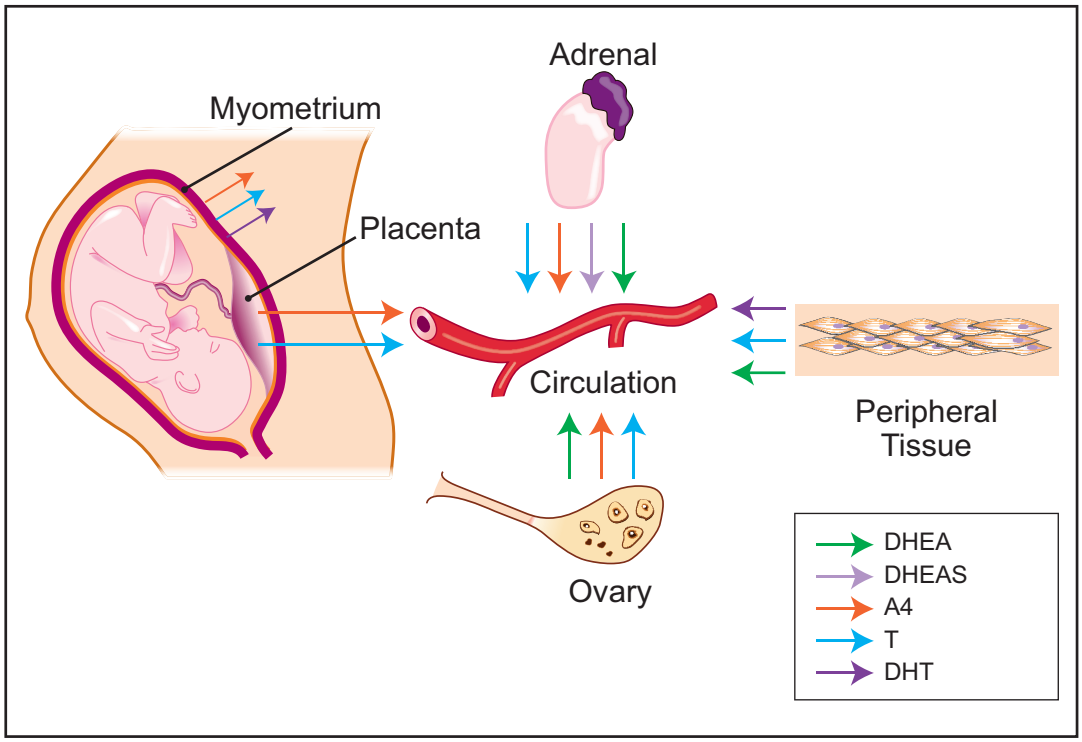



**Figure 1.5:** Maternal sites of androgen biosynthesis. The maternal adrenal secretes DHEA, DHEAS, A4 and T into the maternal circulation, from which androgens get distributed to peripheral tissues. The placenta is an additional site for de novo biosynthesis of A4 and T. Ovaries can also produce and secrete T, A4 and DHEA into the maternal circulation and the myometrium can synthesise A4, T and DHT. Finally DHEA and T can be generated from precursors by androgen metabolising enzymes. DHEA = dehydroepiandrosterone; T = testosterone; DHT = dihydrotestosterone; DHEAS = dehydroepiandrosterone sulphate; A4 = androstenedione. Adapted from (Makieva et al., 2014).

#### *1.4.2.3 Clearance*

The concentration of androgens measurable in blood (Pc) are also influenced by their metabolic clearance rate (MCR) – the volume of blood cleared of a steroid per unit time (Baird et al., 1969). The blood production rate of an androgen (Pb) is the amount of androgen entering the circulation from all possible sources (endocrine glands and periphery). The blood production rate can be calculated according to the formula Pb = MCR x Pc and it represents an approximation of the daily production rate of the steroid (Gurpide, 1990). The MCR of androgens, which represents the summation of the individual organ clearance rates, is significantly influenced by the amount of circulating SHBG, which reduces peripheral breakdown of androgens (Vermeulen and Ando, 1979).

In women, soon after implantation, the production of maternal P4, E2 and androgens by the corpus luteum increases significantly (Brannian and Stouffer, 1991; Elbaum and Keyes, 1976; Fisch et al., 1989; Laherty et al., 1985; Richardson and Masson, 1981; Stocco et al., 2007; Stouffer et al., 1977; Webley and Hearn, 1987). Corpus luteum-derived E2 in turn stimulates liver SHBG synthesis leading to an increase in circulating concentration of SHBG (Joseph, 1994; Wilke and Utley, 1987). The increase in SHBG should decrease the bioavailability of fT and therefore lower the clearance of T and DHT. Indeed, it has been reported that the MCR of T and DHT during pregnancy is lower during the first trimester compared to the non-pregnant state; however, the blood production rates of DHT and T are unchanged (Saez et al., 1972). These finding are in agreement with a study in guinea pigs, where pregnancy induced a two-fold decrease in the MCR of T (Despres et al., 1982).

In contrast to the MCR of T and DHT, the MCR of DHEA and DHEAS increases two- and five-fold respectively in pregnancy, starting at mid-pregnancy (Belisle et al., 1977; Belisle et al., 1980b; Gant et al., 1971). This phenomenon may reflect the metabolism of these steroids in the feto-placental unit, as both steroids are important as estrogen precursors (Goodyer and Branchaud, 1981; Longcope, 1996). In addition to the increase in the MCR of DHEAS, its production rate by the maternal adrenals increases in pregnancy (Milewich et al., 1978). The net result is lower DHEAS concentration in the maternal serum at the end of first trimester in comparison to non-pregnant state (Tagawa et al., 2004). For A4, although pregnancy does not induce changes in the MCR, the blood production rate is increased two-fold, explaining the observed increase in the circulating concentration of A4 (Belisle et al., 1980a; Castracane et al., 1998).

In conclusion, changes in the production rate of androgens (produced predominantly in the ovary and to a lesser extent in the adrenals and periphery) and their MCRs occurring during pregnancy may all impact the detected concentration of androgen in the circulation. Of note, changes in the total concentration of circulating androgens may not reflect the bioavailability of these steroids in target tissues such as the myometrium and the cervix. The functional role of the increase in androgen concentration during pregnancy has been understudied, although a number of adverse

pregnancy outcomes have been correlated with impaired maternal androgen levels. These studies are discussed below.

### 1.4.3 Androgens and adverse pregnancy outcomes

Considering the adverse effects of high circulating androgens in non-pregnant women, such as in PCOS cases, one would assume that the physiological pregnancy-induced increase in androgen concentrations could potentially have similar detrimental effects. However, it is generally accepted that pregnancy-specific mechanisms are activated to protect both mother and fetus from the pregnancy-induced androgen excess (Hensleigh et al., 1975). In the absence of these "protective" mechanisms, exposure to androgens can cause hirsutism and/or virilisation of both mother and any female fetus. The maternal "protective" mechanisms include a) a physiological increase in maternal circulating SHBG, which binds and inactivates androgens (Hammond, 2011) b) a pregnancy-induced rapid elevation of P4, which can compete for binding to the AR (Birrell et al., 2007; Slayden et al., 2001) and c) the affinity of P4 for 5α-reductase resulting in inhibition of conversion of T to the more potent AR ligand DHT (Cabeza et al., 1999; Hodgins, 1982). Due to these mechanisms, pregnancy itself may reduce clinical manifestation of pre-existing or pathological pregnancy-induced hyperandrogenic conditions (Crisosto et al., 2012; Phelan and Conway, 2011). The commonest hyperandrogenic conditions are known as "non-tumour ovarian hyperandrogenism" and include PCOS, hyperreactio luteinalis (HL) and pregnancy luteoma (Kanova and Bicikova, 2011). Cases of HL, occurring predominantly during the second or third trimester and caused by high β-human chorionic gonadotropin (β-hCG) levels, have been reported (Amoah et al., 2011; Haimov-Kochman et al., 2004; Van Holsbeke et al., 2009). Such studies revealed that, in spite of the proposed protective mechanisms, a small fraction of women with HL exhibited virilisation and/or hirsutism but, fortunately, their female fetuses were hardly ever virilised (Abe et al., 2011; Amoah et al., 2011; Angioni et al., 2007; Annamalai et al., 2011; Foulk et al., 1997; Hensleigh et al., 1975; Holt et al., 2005; Van Holsbeke et al., 2009; Veleminsky, 2010). Although most maternal hyperandrogenic symptoms of HL resolve postpartum, HL is strongly associated with adverse pregnancy outcomes such as preeclampsia (PE) and PTB (Atis et al., 2010; Gatongi et al., 2006; Grgic et al.,

2008; Haq, 2010; Lynn et al., 2013; Masuyama et al., 2009; Simsek et al., 2012). Other hyperandrogenic disorders, which are generally very rare, include fetal-induced hyperandrogenism, which is caused by fetal aromatase deficiency (FAD), iatrogenic hyperandrogenism, caused by exogenous androgen administration, and adrenal tumour hyperandrogenism (Dahl et al., 2008).

The fetal "protective" mechanism includes the metabolism of androgens in the placenta (Siiteri and MacDonald, 1966; Simpson et al., 1994). Specifically, the aromatase complex within the placenta plays critical role in protecting the fetus from the mother's androgens as this enzyme rapidly converts T or A4 to E1 and E2 respectively (see **Figure 1.3**) (Bardin et al., 1983; Edman et al., 1981; Hall et al., 1987; Pasanen and Pelkonen, 1994; Simpson et al., 1994). The efficacy of this placental barrier to androgens is evidenced by studies showing that women with hyperandrogenic disorders during pregnancy, such as PCOS, do not necessarily deliver virilised female fetuses (Escobar-Morreale, 2010; Sir-Petermann et al., 2002). On the other hand, the few women identified with placental aromatase deficiency can deliver virilised fetuses, again demonstrating the importance of placental aromatase (Ludwig et al., 1998; Shozu et al., 1991). Taken together these studies show that pregnancy is associated with the development of protective mechanisms, which ameliorate both maternal and fetal effects of the increase in androgens that is associated with normal physiology of pregnancy.

Numerous studies have also explored the association between an abnormal androgen increase during pregnancy with factors such as maternal age, the sex of the baby and pregnancy outcome. Women with PCOS, who have higher concentration of androgens compared to healthy pregnant women, have reportedly an approximately 6% higher risk of delivering preterm compared to women without PCOS (Sir-Petermann et al., 2005; Yamamoto et al., 2012). However, androgen excess might not be the only reason for early birth in these women, as PCOS is also associated with an increase in many inflammatory mediators, which may contribute to PTL (Diamanti-Kandarakis et al., 2006). A study investigating the association of birth size of the offspring with the concentration of maternal androgens has indicated that elevated T is positively associated with in utero growth restriction (Carlsen et al.,

2006). In addition, women pregnant with male fetuses are in greater risk of delivering preterm, a phenomenon sometimes attributed to the fact that pregnancies with male fetuses have higher concentration of androgens in the fetal compartment compared to female fetuses (McGregor et al., 1992; Zeitlin et al., 2002). However, there is no direct evidence that the relationship between higher androgen concentration in the fetal compartment and PTB is causal. In contrast to the positive association between androgen concentration (at least in the fetal compartment) and the presence of a male fetus and PTB, the increasing maternal age (which is negatively associated with [maternal] androgen concentration in pregnancy) is also known to be a risk factor for preterm deliveries (Astolfi and Zonta, 1999; Carlsen et al., 2003). Recently, a report identified changes in the fetal *AR* gene to be associated with a predisposition to PTB (Karjalainen et al., 2012). In their linkage analysis study they reported that a repeat sequence in exon-1 of *AR* in the offspring, who were born preterm, was longer in cases of PTB than controls and implicated the *AR* as a fetal susceptibility gene for PTB (Karjalainen et al., 2012). Abnormal increases in androgens during pregnancy have also been correlated with the development of gestational diabetes, which is also associated with PTB (Abbott et al., 2010; Ackerman et al., 2013; Espinos et al., 1992; Morisset et al., 2013; Vejrazkova et al., 2014). The latter is probably due to the impact of androgens on modulation of insulin secretion from the pancreas and inhibition of glucose uptake in fat and muscle cells (Corbould, 2008; Rao et al., 2013). The mechanism linking the two hormonal axes is poorly understood and, interestingly, this has not been considered or explored during pregnancy. Increased T in the maternal circulation during the third trimester have been positively associated with the incidence of PE, suggesting T as a predictive marker of PE (Ghorashi and Sheikhvatan, 2008; Lorzadeh and Kazemirad, 2012; Sharifzadeh et al., 2012; Simsek et al., 2012). In this context, placentae from preeclamptic pregnancies have higher expression of AR in both male and female fetuses (Sathishkumar et al., 2012). Therefore, it is possible that the AR-dependent signalling in the placenta contributes to the pathology of PE; a notion that deserves further investigation.

A correlation between androgen concentration and preterm delivery has been studied in few primate studies. For example, continuous administration of A4 to pregnant

rhesus monkeys either from early gestation or for a period of 48 hours during third trimester resulted in preterm initiation of myometrial contractions and cervical dilatation that lead to preterm delivery (Figueroa et al., 1989; Mecenas et al., 1996). In these studies, preterm delivery was associated with an increase in E2 in maternal circulation, suggesting conversion of A4 to E2 in the placenta. Nathanielsz et al later supported this idea by showing that the preterm delivery could be prevented by direct administration of an aromatase inhibitor (Nathanielsz et al., 1998). However, in other studies, direct administration of E2 or E1 to pregnant primates failed to induce preterm delivery (Albrecht et al., 1989; Novy and Walsh, 1983). Therefore, it is possible that the normal physiological increase in androgens in the maternal circulation that occurs from the start to the end of pregnancy may hold the key to maintenance of pregnancy. In support of this notion, abnormal changes in androgen concentrations at the start of pregnancy have been associated with spontaneous abortions or recurrent miscarriages. For example, spontaneous abortion occurred in women whose circulating androgens failed to increase at the beginning of pregnancy (Bammann et al., 1980). On the other hand, women with recurrent miscarriages had significantly higher circulating T, A4 and higher T/SHBG ratio compared to women without recurrent miscarriages (Okon et al., 1998).

Although these studies suggest that the normal increase in androgen concentrations might be pivotal for maintenance of pregnancy and development of the fetus, they fail to show a consistent relationship between either androgen excess or androgen deficiency and pregnancy problems. What is more, the mechanism by which androgens may impact pregnancy outcomes has not been fully understood. Below we discuss the functions of androgens in tissues, such as the cervix and the myometrium, focusing on their effects on cervical remodelling and myometrial contractility, both key processes in determining the timing of delivery.

### 1.4.4 The role of androgens in the cervix

#### *1.4.4.1 Structure of cervix*

The cervical stroma is composed of fibroblasts, SMCs, epithelial cells and immune cells (Leppert, 1995). These cells secrete an extracellular matrix (ECM), which consists primarily of collagen, glycosaminoglycans, and proteoglycans. Hormones

including progestogens and E2, relaxin and prostaglandins can alter the composition of ECM and affect the mechanical strength of the cervix (Ekman-Ordeberg et al., 2003; Ghule et al., 2012; Ji et al., 2011; Simon and Einspanier, 2009). The consequence of this process is remodelling of the cervix, which can be divided into four stages: softening (first stage), ripening (second stage), dilatation (third stage) and reconstitution of the non-pregnant cervix postpartum (Read et al., 2007). Each stage is characterised by a distinct composition of collagens and proteoglycans as the tissue re-organises and prepares for the next steps in the parturition process (Word et al., 2007). Cervical ripening is characterised by a 60% decrease in collagen and proteoglycan concentration that occurs in parallel with an increase in collagenase activity, which itself is involved in collagen catabolism and disturbance of collagen bundles (Ekman et al., 1986; Norman et al., 1993). However, collagen synthesis increases in the cervix at term, due to collagen turnover being balanced between production and degradation (Ekman et al., 1986). Structural abnormalities of the cervix have been associated with an increased risk of PTB, thus a better understanding of the physiology of cervical remodelling is crucial [reviewed by (Norman, 2007)].

#### *1.4.4.2 The role of androgen signalling in cervical remodelling*

Numerous human and animal studies have explored the role of androgens in the cervix, particularly during pregnancy (El Maradny et al., 1996; Ji et al., 2008; Kanayama et al., 1998; Maradny et al., 1996; Mochizuki et al., 1978a; Mochizuki et al., 1978b; Mochizuki and Maruo, 1985; Sakyo et al., 1986, 1987; Sasaki et al., 1982; Takahashi et al., 1984; Yamashita et al., 1991). These studies suggest that androgens regulate cervical remodelling, particularly cervical ripening at term. **Table 1.2** summarises the key findings from these studies. Collectively these reports provide compelling evidence that the fetal androgen DHEAS promotes cervical ripening by enhancing collagenase activity and, thus, decreasing fibril collagen organisation. However, it is noteworthy that the majority of these reports are of a few decades of age and, particularly, the *in vivo* studies are characterised by a small number of patients employed for those studies.

**Table 1.2:** Studies on androgen administration in the cervix. HCF = human cervical fibroblast, CCs = cervical cells.

| Androgen | Dose | Species/tissue | Key findings | Reference |
|---|---|---|---|---|
| DHEAS | 0.01 mg/ml<br>0.1 mg/ml | Human<br>*In vitro*: HCF derived from term pregnancy | ↑ IL-8 in conditioned medium<br>↑ IL receptor in HCF | (Kanayama et al., 1998) |
| DHEAS | 200 mg | Human<br>*In vivo*: Term pregnant women (>38 weeks) | ↑ Bishop score<br>↑ Collagenase activity | (Mochizuki et al., 1978b) |
| DHEAS | 200 mg | Human<br>*In vivo*: Term pregnant women (38-42 weeks) | ↑ Bishop score<br>↑ Collagenase activity<br>↑ E2 in maternal serum | (Mochizuki and Maruo, 1985) |
| DHEAS | 50-100 mg | Human<br>*In vivo*: Term pregnant women (38-42 weeks) | ↑ Bishop score<br>↓ Time to delivery<br>No side effects | (Mochizuki et al., 1978a) |
| DHEAS | 100 mg | Human<br>*In vivo*: Term pregnant multiparous and nulliparous women (>38 weeks) | ↑ Bishop score<br>↓ Duration of labour in nulliparous women<br>↓ Time to delivery in nulliparous<br>No side effects | (Sasaki et al., 1982) |
| DHEAS | 50-200 mg | Human<br>*In vivo*: Term pregnant singleton and twin pregnancies (>39 weeks) | ↑ Bishop score (Higher correlation of Bishop score with twin pregnancies)<br>↑ E2 levels in maternal serum (Higher correlation of E2 levels with twin pregnancies)<br>No change in E1, E3 levels in maternal serum. | (Takahashi et al., 1984) |
| DHEAS | 1 μM | Rabbit<br>*In vitro*: CCs derived from term pregnancy | ↑ Collagenase levels<br>No Effect of E2 or DHEA treatment | (Sakyo et al., 1986) |
| DHEAS | 1 μM | Rabbit<br>*In vitro*: CCs derived from term pregnancy | ↑ Collagenase levels<br>No effect on collagen levels | (Sakyo et al., 1987) |
| DHEAS | 10 mg | Rabbit<br>*In vivo*: term pregnancy | ↑ Collagenase activity<br>↓ Collagen content | (Maradny et al., 1996) |

| | | | | |
|---|---|---|---|---|
| | | | Combined DHEAS + IL-8 (100 ng) treatment induced neutrophil infiltration<br><br>Combined treatment produced maximal decrease in collagen content | |
| DHEAS | 100 mg | Rat<br>*In vivo*: term pregnancy (day 16) | ↓ Collagen content<br><br>↑ Collagenase activity | (Yamashita et al., 1991) |
| DHEA | 0.01 mg /ml<br>0.1 mg/ml | Human<br>*In vitro*: HCF derived from term pregnancy | Slight + IL-8 in conditioned medium<br><br>Slight + IL receptor in HCF | (Kanayama et al., 1998) |
| DHT | 2 mg | Rat<br>*In vivo*: term pregnancy (day 15) | ↓ Cervical resistance<br><br>↓ Proteoglycan mRNA<br><br>Flutamide treatment (10 mg)<br>↑ Cervical resistance<br>↑ Proteoglycan mRNA | (Ji et al., 2008) |

The mechanisms by which androgens promote cervical ripening are not fully understood. In support of a physiological effect of DHEAS on cervical ripening, one study has reported that the DHEAS concentration in serum is significantly correlated with favourable Bishop outcome; i.e. higher DHEAS in women with a successful (spontaneous) cervical ripening compared to women in need of induction of labour (Koyuncu et al., 1995). DHEAS administration has also been positively correlated with an increase in E2 levels in the maternal serum (Takahashi et al., 1984). Therefore, it has been argued that DHEAS may be metabolised to estrogens within the term cervix and it is the estrogens that initiate the cervical ripening. However, other studies have reported that administration of estrogens alone fails to ripen the cervix (Dasgupta and Singh, 2012; Larmon et al., 2002; Thiery et al., 1979). Notably DHT, which cannot be metabolised to estrogens, also promotes ripening of the cervix in rats, implying an androgen-specific effect (Ji et al., 2008). Interestingly, mice with ablation of the gene that encodes 5α-reductase type 1 develop an abnormal cervix

that fails to ripen at labour (Mahendroo et al., 1999). The 5α-reductase type 1 enzyme converts T to DHT and is the predominant enzyme expressed in cervix at term (Mahendroo and Russell, 1999). Administration of a 5α-reduced androgen, a metabolite of DHT- namely 5alpha-androstane-3alpha, 17β-diol (Adiol) rescues the impaired phenotype of 5α-reductase type 1 KO animals, allowing normal cervical ripening (Mahendroo et al., 1996). Although the latter finding suggests the local conversion of androgens to other (more potent) androgen metabolites to be the underlying mechanism for the androgen-induced cervical ripening, it is important to note that Adiol can also bind estrogen receptor beta (ERβ) (Saijo et al., 2011).

Although poorly explored, there is some evidence that the mechanism of the observed DHEAS-induced collagenase increase is indirect and involves DHEAS-stimulated neutrophil secretion of proteolytic enzymes (El Maradny et al., 1996; Maymon et al., 2000). Notably, the action of DHEAS on cervical ripening has been correlated with increased concentration of IL-8, a cytokine involved in chemotaxis of neutrophils that are reportedly recruited to the cervix towards term (Kanayama et al., 1998; Maymon et al., 2000).

The involvement of the AR in mediation of cervical ripening by androgens has been recently examined in pregnant rats (Ji et al., 2008). In that study, the antagonism of AR by flutamide inhibited events associated with ripening of the cervix, such as a decrease in collagen fibril organisation, a decrease in cervical resistance and a decrease in proteoglycan synthesis. On the other hand, these events were promoted by intravaginal administration of DHT on day 16 of rat pregnancy (Ji et al., 2008). Although that study did not examine the expression of AR in the cervix, other immunohistochemical studies have reported cervical localisation of AR in pregnant and non-pregnant human (Ji et al., 2008; Vermeirsch et al., 2002; Vladic-Stjernholm et al., 2009; Wilson and McPhaul, 1996). In addition, it has been reported that androgens upregulate AR expression in the human cervix (van der Kwast et al., 1994).

In summary, the reports discussed above suggest that DHEA and DHT might promote cervical ripening via a pathway that alters collagenase synthesis and activity. Although the precise mechanism is not well established, there is some

evidence that it may be mediated via metabolism of androgens to reduced androgens and/or genomic AR-dependent signalling. However, this notion needs more investigation.

### 1.4.5 The role of androgens in the myometrium

#### *1.4.5.1 The MSMCs*

The MSMCs are specialised contractile cells that, in contrast to skeletal and other smooth muscle cells, exhibit cellular plasticity, which enables reversible differentiation into different phenotypes (Gabbiani et al., 1981; Tomiyasu et al., 1988). Studies in the rat myometrium during pregnancy have demonstrated that the MSMCs undergo five phenotypic changes throughout gestation (Shynlova et al., 2009b). Briefly, these are: a proliferative stage, a synthetic stage, a contractile stage, a labour stage and a postpartum involution phase. Early in pregnancy, following implantation of the conceptus, the MSMCs proliferate rapidly and become hyperplastic. Hormones and growth factors, including IGF-1 (insulin-like growth factor 1) and EGF (epidermal growth factor) are key regulators of the proliferative phase [reviewed in (Shynlova et al., 2007)]. Following proliferation, the MSMCs undergo an intermediate synthetic phase that starts on day 14 of rat pregnancy (Lye et al., 2001). The synthetic phenotype is characterised by MSMCs hypertrophy and increased synthesis and deposition of ECM (Shynlova et al., 2009b). On day 21 of rat gestation, the myocytes switch to a contractile phenotype (Fata et al., 2000; Nishinaka and Fukuda, 1991; Shynlova et al., 2004). The switch to the contractile phenotype is predominantly triggered by mechanical pathways that get activated in response to uterine stretch (Loudon et al., 2004; Manabe et al., 1981; Sooranna et al., 2004; Terzidou et al., 2005). At the end of pregnancy, the MSMCs adopt a labour phenotype (Shynlova et al., 2009b). At this point the myometrium develops synchronous contractions and expresses a number of CAPs, such as con-43, OXTR and PGF receptor (Erkinheimo et al., 2000; Fuchs et al., 1984; Rehman et al., 2003; Slater et al., 1999; Sparey et al., 1999). The return of the MSMCs to a non-pregnant state involves rapid apoptosis, which is characteristic of the postpartum involution (O'Brien et al., 2007b; Roh et al., 2000; Shynlova et al., 2009b). Each stage of

MSMCs differentiation is characterised by a distinct gene and protein expression profile, which is outlined in **Figure 1.6**.

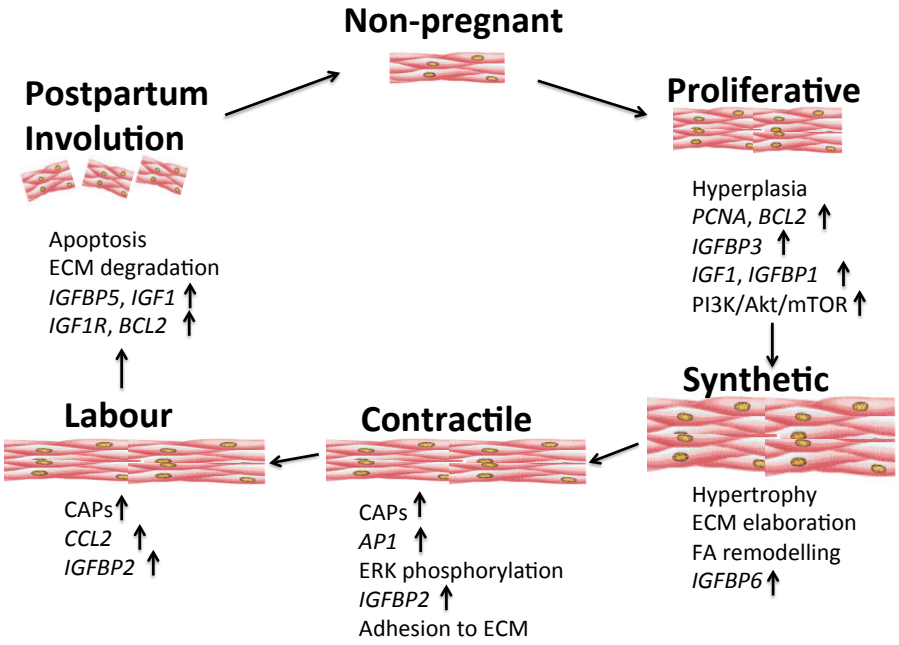



**Figure 1.6:** The MSMCs undergo phenotypic changes during pregnancy. The IGF family members have a distinct gene expression profile depending on the phase of myometrial differentiation. *IGF1*, *IGFBP1* and *IGFBP3* genes are all upregulated during the proliferative phase of MSMCs. *IGFBP6* is elevated during the synthetic phase. *IGFBP2* is increased during the contractile phase, and *IGF1* and *IGFBP5* induced during postpartum involution. The proliferating cell nuclear antigen (PCNA) gene, which is essential for DNA replication, is upregulated during the proliferative phase and the PI3K/Akt/mTOR pathway is induced. Genes involved in focal adhesion (FA) remodelling are induced during the synthetic phase. The contractile phase is characterised by increased expression of CAPs, upregulation of the gene encoding the activator protein 1 (AP-1) transcription factor and phosphorylation of ERK. The labouring phenotype is characterised by upregulation of *CCL2* gene, which encodes the chemokine CCL-2. B-cell lymphoma 2 gene (*BCL2*) is increased during the proliferative and postpartum phase, where it has anti-apoptotic and pro-apoptotic roles respectively. Image modified from (Shynlova et al., 2009b).

#### *1.4.5.2 The role of androgen signalling in MSMCs*

A recent study, conducted in rats, reported that the AR protein is most abundant in the myometrium during the proliferative stage and progressively declines towards the end of pregnancy, suggesting a role (at least in this species) in establishment of myometrial growth at the early stages of pregnancy (Liu et al., 2013). Liu et al additionally showed that ablation of the AR gene from MSMCs *in vitro* reduced the stability of IGF-1 receptor (IGF-1R), which in turn downregulated the proliferative cascades downstream of IGF-1, including P13K/Act (Liu et al., 2013). Besides rats, a significant decrease in the expression of the AR protein reportedly occurs in the porcine myometrium three weeks prior to labour (Slomczynska et al., 2008). A human microarray study reported that the AR mRNA concentration was downregulated in the myometrium during preterm (0.4-fold) and term labour (0.3-fold) compared to non-labouring term myometrium; protein expression was not determined (Bethin et al., 2003). Based on these studies, it would appear that the AR-dependent signalling is likely to be regulating MSMCs proliferation, which is pivotal for the establishment of a functional myometrium that will be able to contract and expel the fetus at term. Besides its genomic actions, the non-genomic actions of androgens in the myometrium have been explored. Robson was the first to suggest that androgens could relax myometrial contraction in a rapid non-genomic (transcription-independent) manner (Robson, 1937). Robson administered 12 mg of testosterone propionate (a small fast acting ester of testosterone) for 4-days to ovariectomised (OVX) non-pregnant rabbits and examined the uterine contractile response to 1 unit of injected OXT both *in vivo* and *ex vivo*. He observed that T reduced the OXT-stimulated response but hardly affected the spontaneously-initiated uterine contractions (Robson, 1937). Later studies demonstrated similar 'relaxing' effects of various androgens including T, DHT, A4 and DHEAS, which were tested on myometrium from non-pregnant rats and non-pregnant and pregnant women (Kubli-Garfias et al., 1980; Perusquia et al., 1991a; Perusquia et al., 1991b; Perusquia et al., 2005). The findings from these studies are summarised in **Table 1.3**. Interestingly, these studies are the outcome of the work of a single research group over few decades and, further studies are required to replicate their findings. Notably,

the relaxant effect of androgens occurs only when used in pharmacological concentrations (micromolar range) (Anderson et al., 2009; Perusquia et al., 1990; Tica et al., 2011). A logical assumption would be that the observed effect does not occur physiologically as the physiological concentrations of androgens are in a nanomolar range. However, understanding the non-genomic pathways employed by androgens to relax the uterine muscle could help develop new tocolytic agents to manage preterm initiated myometrial contractions.

**Table 1.3:** Studies on the effect of androgens on myometrial contractions. DHEA = dehydroepiandrosterone; T = testosterone; DHT = dihydrotestosterone; DHEAS = dehydroepiandrosterone sulphate; A4 = androstenedione.

| Androgen | Dose | Species/Tissue | Key Findings | Reference |
|---|---|---|---|---|
| T, DHEA, Androstanediol, Androsterone, A4, 5α-DHT, 5β-DHT | 10-100 μM | Rat (non-pregnant) *ex vivo*: Myometrial strips | Spontaneous contractions inhibited rapidly (<30 min) by all androgens<br><br>Potency: Androstanediol, Androsterone > 5β-DHT > T=A4=DHEA=5α-DHT | (Kubli-Garfias et al., 1980) |
| T, DHEA, Androstanediol, Androsterone, 5β-DHT | 3-100 μM | Rat (non-pregnant) *ex vivo*: Myometrial strips | Tonic contractions (KCl-induced) inhibited rapidly (<30 min) by all androgens<br><br>Potency: Androsterone= Androstanediol > 5β DHT > T | (Perusquia et al., 1990) |
| Androstanediol, Androsterone, 5α-DHT, 5β-DHT | 3-100 μM | Rat (non-pregnant) *ex vivo*: Myometrial strips | Serotonin-induced contractions inhibited rapidly (<30 min) by all androgens | (Perusquia et al., 1991a) |
| Androstanediol, Androsterone, 5α-DHT, 5β-DHT | 3-100 μM | Rat (non-pregnant) *ex vivo*: Myometrial strips | Acetylcholine-induced contractions inhibited rapidly (<30 min) by all androgens | (Perusquia et al., 1991b) |
| Androstanediol, Androsterone, 5α-DHT, 5β-DHT | 3-100 μM | Rat (non-pregnant) *ex vivo*: Myometrial strips | Oxytocin-induced contractions inhibited rapidly (<30 min) by all androgens | (Perusquia, 1991) |
| T, DHEA, DHT, 5α-DHT, 5β-DHT, Androstanediol, Androsterone | 3-100 μM | Human (term pregnant, non-pregnant) *ex vivo*: Myometrial strips | Spontaneous contractions and tonic contractions (KCl-induced) inhibited rapidly (<30 min) by all androgens<br><br>Potency: 5β-DHT > Androsterone=DHEA=T> 5α-DHT=Androstanediol | (Perusquia et al., 2005) |

## 1.5 Uterine smooth muscle contraction

The MSMCs are myogenic, which means that they can contract spontaneously (Tomiyasu et al., 1988). The membrane potential in MSMCs is not stable.

Spontaneous depolarisation of membrane potential, fired by alterations in membrane currents and ion channels, occurs during pregnancy. A decrease in membrane potential triggers myocyte contractile activity and leads to spontaneous contractions - conversely amplification of membrane potential to a resting -50 mV maintains the uterus in a quiescent state (Nakajima, 1971; Pressman et al., 1988). The upstroke of MSMCs membrane action potential (i.e. the initial decrease in membrane potential) predominantly occurs due to calcium ($Ca^{2+}$) entry, whereas repolarisation occurs as a result of blockade of $Ca^{2+}$ channels (Nakajima, 1971). The key biochemical event in spontaneous contraction of MSMCs is an increase in the concentration of intracellular $Ca^{2+}$ ($[Ca^{2+}]$) from $10^{-7}$M to $10^{-6}$M (Horowitz et al., 1996). This increase is the result of $Ca^{2+}$ influx from the extracellular space and/or release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR). Extracellular $Ca^{2+}$ enters the cell via a variety of $Ca^{2+}$ channels, classified into voltage-operated $Ca^{2+}$ channels (VOCCs) and voltage-independent $Ca^{2+}$ channels [including receptor-operated $Ca^{2+}$ channels (ROCCs), store-operated channels (SOCs) and stretch-activated channels (SACs)] (Floyd and Wray, 2007; Noble et al., 2009; Wray et al., 2005; Wray et al., 2003). ROCCs are regulated by agonist-receptor interactions (Guibert et al., 2008). Receptors that interact with ROCCs upon ligand binding are G protein-coupled receptors (GPCRs), for example the OXTR. GPCRs can activate the inositol triphosphate (IP3) pathway that leads to release of $Ca^{2+}$ from the SR via activation of phosphoinositide phospholipase C (PLC) and hydrolysis of $PIP_2$ (phosphatidylinositol 4,5-bisphosphate) (Meldrum et al., 1991). The resulting increase in $[Ca^{2+}]$ leads to formation of a complex between calmodulin and myosin light chain kinase (MLCK), which then phosphorylates light chains on myosin (MLC) (Ito and Hartshorne, 1990). The latter allows binding of actin to myosin leading to cross-bridge cycling, which results in contraction (Dillon et al., 1981). Myosin light chain phosphatase (MLCP) counters MLCK and inhibits contraction by dephosphorylating light chains on MLC. The increase in MLCK/MLCP ratio leads to increased MLC phosphorylation and force of contraction for a given $[Ca^{2+}]$ concentration, a phenomenon known as calcium-sensitisation (CS) (Arthur et al., 2007). It is now known that the RhoA/ROK pathway promotes CS. Activation of GPCRs can activate small monomeric G proteins, such as RhoA, which in turn

activate Rho-associated kinase (ROK). ROK is involved in inhibition of MLCP and, therefore, helps sustain the phosphorylation of MLC promoting the contractile phenotype (Arthur et al., 2007). Extrusion of $Ca^{2+}$ from MSMCs can occur by a reverse process mediated by two major transporters; the $Ca^{2+}$ ATPase (PMCA) and the $Na^{+}/Ca^{2+}$ exchange pump (NCX) (Shmigol et al., 1998). Studies examining the mechanisms by which $Ca^{2+}$ ions are removed from the cytoplasm in isolated rat MSMCs showed that PMCA accounted for 85% of the $Ca^{2+}$ efflux (Shmigol et al., 1998). The mechanism of MSMCs contraction is illustrated at **Figure 1.7.**

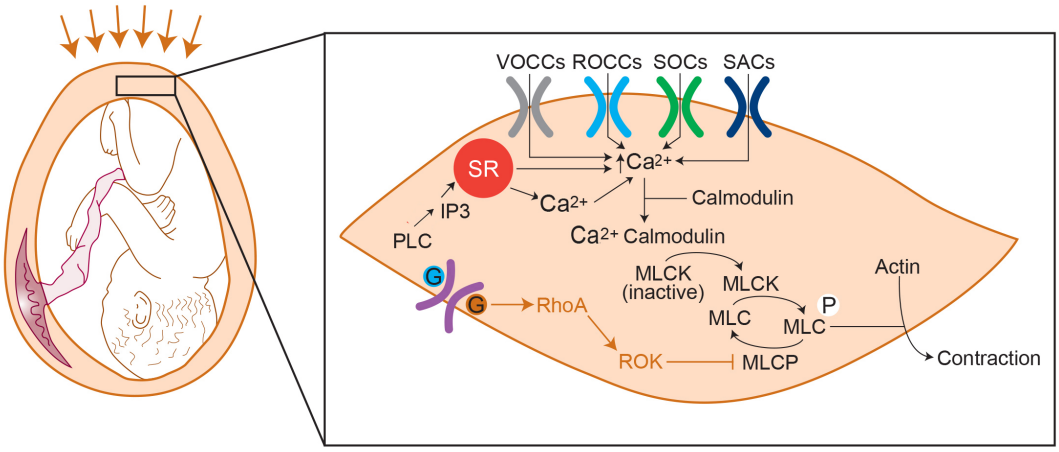



**Figure 1.7:** The mechanism of MSMCs contraction. $Ca^{2+}$ enters the cell from the extracellular space through VOCCs, ROCCS, SOCs and SACs or is released from the SR. One of the mechanisms to induce the release of $Ca^{2+}$ from the SR is activation of specific GPCRs coupled to specific G-proteins. When activated, the GPCR activates the PLC pathway. The PLC generates IP3, which enters the SR and promotes the release of $Ca^{2+}$. The [$Ca^{2+}$] molecules form a complex with calmodulin, which activates MLCK. The active MLCK phosphorylates myosin (MLC), allowing myosin interaction with actin and generation of contraction. Dephosphorylation of MLC by MLCP results in relaxation. The activity of MLCP is regulated by the RhoA/ROK pathway, which is involved in CS but is independent of $Ca^{2+}$ influx/efflux. GPCRs, coupled to alternative G-proteins, activate RhoA, which in turn activates ROK and inhibits MLCP. VOCCs = voltage operated $Ca^{2+}$ channels; ROCCs = receptor operated $Ca^{2+}$ channels, SOCs = store-operated channels; SACs = stretch-activated channels; SR = sarcoplasmic reticulum; GPCR = G protein coupled receptors; IP3 = inositol triphosphate; PLC = phosphoinositide phospholipase C; MLCK = myosin light chain kinase; MLC = myosin; MLCP = myosin light chain phosphatase; RhoA = Ras homolog gene family, member A; ROK = RhoA kinase.

### 1.5.1 Voltage-operated $Ca^{2+}$ channels

Two types of VOCCs have been identified in the non-pregnant and pregnant human myometrium; the L-type and the T-type VOCCs (Wray et al., 2003). Studies using selective blockers for L-type (Nifedipine) and T-type VOCCs have demonstrated that it is the L-type channels, and not the T-type channels, that primarily mediate the excitation-contraction process in uterine myocytes (Wray et al., 2001). Although not

playing a major role, T-type channels are thought to be important for the initiation of action potential in MSMCs and are involved in the generation of the frequency of $Ca^{2+}$ transients (Lee et al., 2009; Young and Zhang, 2005). The latter was confirmed in studies where a specific T-type blocker (Nickel) reduced the frequency of spontaneous human myometrial contractions (Blanks et al., 2007). The L-type $Ca^{2+}$ channel exists in three states; resting or closed, activated or open and inactivated. The transition from a closed to an open state is regulated by membrane depolarisation and the opening of the channel lasts for approximately 0.67 ms before returning to a closed state (Kreuzberg et al., 2000). The gating kinetics and the time in open/closed states are highly influenced by phosphorylation of the channel subunits by numerous regulatory enzymes. In addition, hormones have been shown to modulate the myometrial L-type channel function during guinea pig and rat pregnancy (Collins et al., 2000; Mershon et al., 1994; Tezuka et al., 1995). P4 treatment in physiological concentration downregulated the expression of L-type channel subunits in the rat myometrium and similar effects has been documented in human immortalised myometrial cells (Soloff et al., 2011; Tezuka et al., 1995). It has been suggested that the P4/estrogen ratio is an important regulator of the expression of different L-type channel subunits (Helguera et al., 2002). Both hormones given at high pharmacological concentrations have shown to decrease spontaneous myometrial contractility in different species (Anderson et al., 2009; Perusquia, 2001). Albeit the reports on the relaxant effects of androgens on myometrial contractions, their effects on VOCCs have been unexplored. However, Perusquia's studies showed that the androgen-induced relaxation of the myometrium is likely to be mediated via blockade of L-type VOCCs (Perusquia et al., 2005). High $K^+$ in the extracellular space can induce cell membrane depolarisation, which in turn results in opening of VOCCs and an increase in $[Ca^{2+}]$. Persuquia and colleagues reported that, in addition to the relaxant effect of androgens on spontaneous myometrial contraction, 5β-DHT rapidly relaxed tonic myometrial contraction (induced by high $K^+$ in the extracellular space) and decreased the concentration of $[Ca^{2+}]$ in MSMCs; removal of the androgen reversed the effect (Perusquia et al., 2005). Based on those findings, the authors suggested that the androgen inhibited L-type VOCCs on the

membrane of MSMCs. However, it is not clear whether the effect of androgen involves physical interaction with VOCCs or is indirect.

### 1.5.2 Potassium ($K^+$) channels

A mentioned above, action potentials in MSMCs result from activation of VOCCs and the presence of high $K^+$ in the extracellular space. Inactivation of VOCCs and concurrent activation of outward $K^+$ currents can reverse membrane depolarisation and decrease the concentration of [$Ca^{2+}$]. There are several types of $K^+$ channels in the myometrium; $K^+$ channels sensitive to voltage ($K_v$), calcium ($K_{Ca}$), ATP ($K_{ATP}$) and the inwardly-rectifying channels ($K_{IR}$) (Brainard et al., 2007). The $K_{Ca}$ channels can be further divided into large conductance $K_{Ca}$ ($BK_{Ca}$), intermediate conductance $K_{Ca}$ ($IK_{Ca}$) and small conductance $K_{Ca}$ ($SK_{Ca}$) (Brainard et al., 2007). The channel that is predominantly expressed in the non-pregnant and pregnant human myometrium is the $BK_{Ca}$, which is also a voltage sensitive $K^+$ channel (Chanrachakul et al., 2003; Gao et al., 2009; Moczydlowski, 2006; Tritthart et al., 1991). The expression of $BK_{Ca}$ is decreased by 50% with the onset of labour and studies of $BK_{Ca}$ in the myometrium demonstrated that it plays an important role in modulation of uterine quiescence by regulation of membrane potential and the availability of [$Ca^{2+}$] (Chanrachakul, 2006; Matharoo-Ball et al., 2003; Xu et al., 2011). In support of this idea, a few reports have reported that blockade of $BK_{Ca}$ channels with selective blockers can augment myometrial contractions in humans (Anwer et al., 1993; Khan et al., 1997). In contrast, activation of $BK_{Ca}$ channels can lead to relaxation of the human myometrium (Doheny et al., 2003; Doheny et al., 2007). The bioavailability of $BK_{Ca}$ in the myometrium is modulated by a number of factors, including posttranslational modifications, alternative splicing and hormonal sensitivity. Hormones, including estrogens and relaxin, evidently alter the activity of the channel and induce loss of $Ca^{2+}$ sensitivity of $BK_{Ca}$ (Benkusky et al., 2002; Meera et al., 1995). Other factors, such as adenylyl cyclase and nitric oxide have similar effects upon $BK_{Ca}$ (Okawa et al., 2000; Okawa et al., 1999; Shimano et al., 2000). Taken together, these studies suggest that the $BK_{Ca}$ channels remain open for the duration of gestation to promote myometrial quiescence but the decrease in their expression at term contributes to the increase in the concentration of [$Ca^{2+}$], which then leads to contraction.

The role of the other $K^+$ channels is less clear. $SK_{Ca}$ channels have been investigated predominantly in mice because $BK_{Ca}$ channels do not seem to an important regulator of uterine contraction as they are in humans (Aaronson et al., 2006). Mice genetically modified to overexpress subunits of $SK_{Ca}$ demonstrated uterine dysfunction and delayed parturition, suggesting that this channel is needed for generation of uterine contractions (Pierce et al., 2008). A later study showed than inhibition of $SK_{Ca}$ channels using selective blockers significantly decreased the outward current, caused depolarisation of the membrane in previously quiescent cells and augmented the amplitude of myometrial contractions (Noble et al., 2010). That study suggested that, at least in mice, the functional effect of $SK_{Ca}$ inhibition is larger than that of $BK_{Ca}$. There is one study to suggest that IKCa channels do not have a major role in regulation of rat myometrial contractility (Pistilli et al., 2012). Interestingly there are no other studies on the IKCa channels in the myometrium, although their important roles in the placenta have been recently highlighted (Diaz et al., 2014). In addition, there is not sufficient evidence of the importance of $K_v$ channel in modulation of myometrial contractility. One study of the $K_V$ in the mouse myometrium during pregnancy indicated that changes in the expression and function of the channel influenced uterine contractility (Smith et al., 2007). In addition, there is currently not enough convincing evidence to support the existence of $K_{ATP}$ channels in the myometrium, although some studies have reported that sulphonylureas can blunt the relaxant action of $K^+$ channel-openers, implying a potential role for $K_{ATP}$ channel in the myometrial contraction (Khan et al., 1998; Morrison et al., 1993).

### 1.5.3 Propagation of contraction

During most of pregnancy the contraction of the MSMCs is local and not synchronous, contributing to maintenance of quiescence. At term, the force and frequency of single cell contraction increases and a mechanism whereby adjacent MSMCs interact to achieve synchronous contractile activity is induced (Garfield and Hayashi, 1981). This mechanism involves gap junction proteins, which are transmembrane channels that link the cytoplasmic compartments of neighbouring cells (Boittin et al., 2013). Gap junctions allow propagation of action potentials between adjacent MSMCs and facilitate the transfer of ions and small molecules from one cell to another in order to create an electrical syncytium (Brading and

Brain, 2011). A gap junction is composed of two hemichannels or connexons, each of which consists of six connexin proteins (Roomans et al., 1993). In the human myometrium, four connexins (con-) have been detected; con-26, con-40, con-43 and con-45, which have distinct expression patterns throughout gestation (Brading and Brain, 2011). For example, con-26 is highly expressed at term but significantly decreases prior to the onset of labour (Orsino et al., 1996). Con-45 is expressed in the non-pregnant myometrium but its expression declines following implantation (Albrecht et al., 1996). Con-40 is expressed in the pregnant uterus; however, there is not sufficient evidence to support its importance in pregnancy (Kilarski et al., 1998). Finally, con-43 is induced at term pregnancy with a significant increase just before the onset of labour, making con-43 the most relevant to parturition research and perhaps the most investigated connexin in the myometrium (Chow and Lye, 1994; Ou et al., 1997). It has been well documented that electrical and metabolic coupling is greatly influenced by the expression of gap junctions and myometrial contractility (Garfield et al., 1978; Miller et al., 1989; Sims et al., 1982). For example, during labour, the increase in the amount of con-43 enhances intracellular gap junction communication (IGJC), which in turn promotes increased propagation of the action potential in the myometrium (Miyoshi et al., 1996; Sakai et al., 1992). Con-43 expression is believed to be regulated by mechanical stretch and hormones. P4 and estrogens have been reported to have opposing effects, with the latter enhancing and the former inhibiting con-43 expression, consistent with the accepted roles of these hormones in regulation of uterine quiescence (Lye et al., 1993; MacKenzie and Garfield, 1985). The importance of con-43 in the biology of parturition has been demonstrated in mice using a MSMCs-specific KO of con-43 (Doring et al., 2006). Mice null for myometrial con-43 exhibited delayed parturition and intrauterine fetal death. Consistent with these findings, another study, which utilised a mouse with a mutation in con-43 gene (*gja1*), showed reduced intracellular coupling and delayed onset of labour in the mutants (Tong et al., 2009). Interestingly, the same study showed that uterine strips obtained from these mice developed significantly weaker contractions compared to wild type animals.

## 1.6 Summary

Premature birth, the outcome of premature labour, is a major cause of maternal and perinatal morbidity and mortality occurring in approximately 11% of recorded live births worldwide ranging from 5% in developed to 18% in undeveloped countries (Blencowe et al., 2012). The exact cause of PTL is unknown although concomitant activation of endocrine and immune pathways is believed to drive events that result in spontaneous initiation of uterine contractions and cervical ripening (Norman et al., 2007; Romero et al., 2007; Smith et al., 2012). There is no fully effective agent for prevention of PTL. P4, used to maintain uterine quiescence, and cervical cerclage, used to prevent premature cervical ripening have some efficacy in terms of reducing PTB, but there is little evidence of long-term beneficial effects for the baby (Alfirevic et al., 2012; Likis et al., 2012). Short- but not long-term outcomes were also demonstrated in a recent network analysis of tocolytics (Haas et al., 2012). Thus, new strategies are needed.

This chapter discussed the evidence that androgens, which increase throughout gestation in maternal circulation, might play functional roles in the physiology of pregnancy in parallel with the well-established roles of progestins and estrogens. *In vivo* and *in vitro* studies have demonstrated that androgens play roles in two critical systems in the biology of pregnancy and parturition - cervical remodelling and myometrial function. In particular, androgens seem to promote cervical ripening and, thus, assist initiation of labour. On the other hand, androgens appear to be effective in *ex vivo* tocolysis of myometrial contractions. In addition, there is evidence that the AR-dependent signalling regulates MSMCs proliferation, which is critical for establishment of a functional myometrium that will effectively contract and expel the fetus at term. Further investigations are required to fully understand the molecular events regulated by androgens in the cervix and myometrium and their interplay with other signalling pathways. These investigations could utilise animal models of adverse pregnancy outcomes, where the outcome of a targeted administration of androgens to either cervix or myometrium could be studied.

In conclusion, research into the roles of androgens in cervical and myometrial function during pregnancy and parturition has the potential to inform new therapeutic strategies for management of pregnancy complications, including PTB.

## 1.7 Hypothesis and aims

The overarching hypothesis of this thesis was that androgens play diverse roles in myometrial biology, which could potentially influence the timing of birth. To address this hypothesis we aimed to

- Explore the role of AR signalling in myometrium during pregnancy
- Examine the effect of androgens on myometrial contractility
- Gain insights into the mechanism by which androgens regulate myometrial contractions
- Investigate the potential of androgen administration to delay PTL in a mouse model of inflammation-induced PTB.

# Chapter 2: Materials and methods

The aim of this chapter is to describe the materials and methods that were used for the experiments presented in this thesis. A detailed version of the experimental design will be given in the materials and methods section of the relevant result chapters. The source or supplier of a reagent or chemical used in the experiments described is enclosed in parentheses or detailed in tables within this chapter. The suppliers of the chemicals that are not included in this chapter are listed in **Table 9.1** (Appendix 1).

## 2.1 Tissue

Human myometrium and murine uterine horns were used for functional assays both to assess the contractile activity of the uterine muscle and in gene/protein expression studies to examine the physiology of the myometrium.

### 2.1.1 Human myometrium

A biopsy of myometrium was obtained from the upper margin of the lower segment of myometrium of women undergoing elective or emergency caesarean section at the Simpson's Centre for Reproductive Health at the Royal Infirmary of Edinburgh, following informed written consent. All biopsies were removed after delivery of the fetus and before syntocinon administration. The ethical approval for recruitment of all pregnant women was granted by the West of Scotland Research Ethics Committee to the Edinburgh Reproductive Tissue BioBank (ERTBB), which is partly funded by the Tommy's Baby Charity. All biopsies were collected from lean women ($19 < BMI < 25$) delivering at term (>37 weeks of gestation) either during labour (term labouring; TL) or prior to the initiation of labour (term non-labouring; TNL). The indication for elective caesarean section in TNL women was breech presentation or previous caesarean section. In TL deliveries, emergency caesarean sections were performed due to fetal distress and failure to progress.

We operated the following exclusion criteria:

- Gestational diabetes
- Steroid medication
- Twin pregnancy
- Pregnancy complications (i.e. preeclampsia, hypertension, intrauterine growth restriction)
- History of cancer or other disease (i.e. HIV, hepatitis)
- Smoking

The recovered biopsies were rinsed in ice-cold Rosewell Park Memorial Institute 1640 medium (RPMI-1640; Cat. No 11879 020, Gibco) and transported to the laboratory where they were kept under refrigeration (4°C) and used either fresh for contractile activity bioassays or placed in RNAlater (Cat. No R0901, Sigma) for RNA extraction. Other biopsies were either snap-frozen for protein extraction or placed in 10% neutral-buffered formalin (NBF; Cat. No HT501128, Sigma) for immunohistochemical examination.

### 2.1.2 Murine uterine horns

Experimental procedures were licensed (PPL 60/4241; PIL 60/13875) under the UK Home office Animals (Scientific Procedures) Act (1986). Uterine horns were obtained from 8-week old non-pregnant C57BL/6 mice supplied by Charles River (Kent, London, UK) for contractile activity bioassays. All animals were sacrificed by lethal exposure to $CO_2$. Uterine horns were harvested and kept under refrigeration (4°C) in ice-cold RPMI medium prior to the initiation of the experiment.

## 2.2 Contractile activity bioassay

Following collection, TNL myometrial biopsies were stored overnight in RPMI (4°C) and used for the assay the following morning. Previous studies have reported that the storage of myometrial biopsies for up to 18 hours post collection in physiological saline does not affect the contractile behaviour or the response of tissue following exposure to various agents, when compared to fresh myometrium (Hillock and Crankshaw, 1999; Popat and Crankshaw, 2001; Senchyna and Crankshaw,

1999). The biopsies were rinsed in Dulbecco's Phosphate-Buffered Saline (PBS; Cat. No 21300-058, Gibco) and dissected in small strips (2 x 8 mm) parallel to the muscle fibber bundles. Murine horns were harvested and transported to the laboratory were they where immediately processed for contraction studies. Each murine uterine horn was approximately 2 cm-long and was prepared into two strips (1 cm-long each). Thus, a maximum of 4 uterine strips was obtained from each mouse. To measure the contractile activity of these strips, an organ bath system (Panlab ADInstruments, UK) was utilised and the effect of different compounds on the contraction was analysed with LabChart7 software (ADInstruments, UK).

### 2.2.1 Organ bath

Myometrial or uterine strips were attached by silk suture (Mersilk 3-0, Cat. No W329, Ethicon Inc) to a force transducer (ML0186/10 Panlab ADInstruments, UK) and stretched under passive resting tension of 20 mN in 10 ml Krebs Buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $NaH_2PO_4$, 1.2 mM $Na_2SO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM Glucose, pH 7.4) equilibrated with 95% $O_2$, 5% $CO_2$ at 37°C. The suture attaching the strip to the transducer was placed in line with the transducer avoiding the bath walls. Strips were allowed to develop spontaneous rhythmic contractile activity for a maximum of 2 hours before applying any treatment. Following this equilibration period, we determined the effect of various experimental compounds on spontaneous contraction. Cumulative or non-cumulative treatments were applied on each strip in a range of concentrations for a fixed period of time depending on the experimental design. At the end of each experiment, the strips were either treated with a stimulatory dose of KCl (55 mM; prepared in Krebs buffer) to verify tissue viability or Krebs buffer was removed and replaced with treatment-free fresh Krebs buffer in order to examine tissue recovery.

### 2.2.2 Data acquisition

The force generated by the myometrial or uterine strip was converted into an electrical signal by an isometric force transducer and detected using the LabChart7 data acquisition software. The complete set up of the organ bath system is illustrated

in **Figure 2.1**. Isometric measurements were measured as mN. A weight of 1 g was suspended onto the force transducer to calibrate it and convert the force measured by the recording signal to mN. This was achieved by using the equation: $N=Kg.m^{-2}$, where 1 Kg=9.8 N. Importantly, prior to the recording of each experiment, a number of parameters were set through the software. In order to make a good quality recording, the parameters were optimised for the size of the force signal being recorded. The sampling rate was set to 1 k/s and the output range of the transducer was optimised to avoid cropping the maximum response. In addition, motion artefacts (background noise) were dampened by the application of an analogue filter, which removed unwanted frequencies. The filter used for all recordings in this study was a "Mains Filter", which is an adaptive filter that tracks and removes noise from the mains (electrical equipment or power supply).

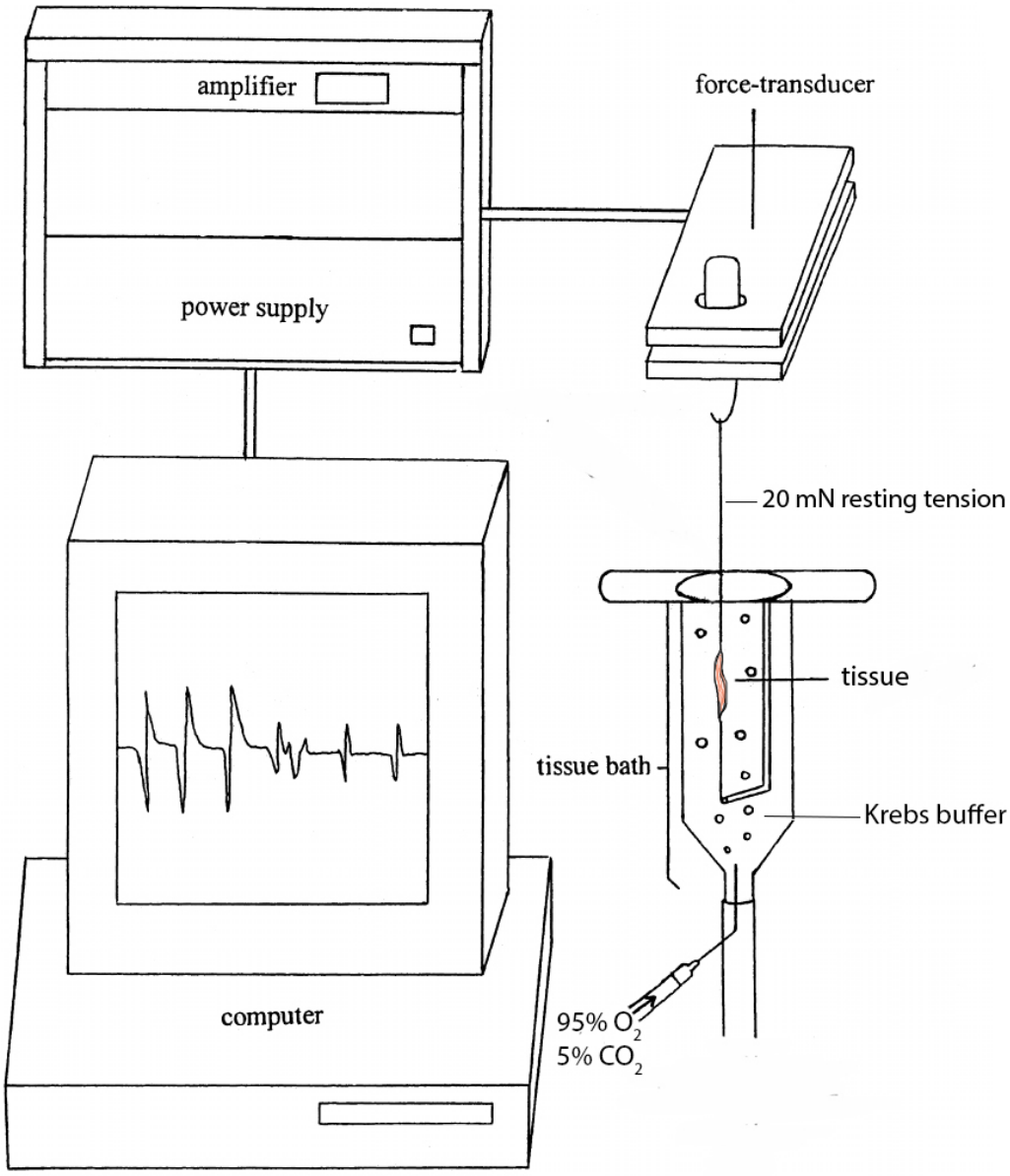



**Figure 2.1:** The organ bath system was used to record uterine contractions. One end of the tissue was tied to a support rod and the other was attached to the force transducer. The force transducer was connected to a power source and an amplifier, which was linked to a computer based data acquisition system. Image adapted from (Krajniak, 2001).

### 2.2.3 Contractile parameters

In order to determine the effect of each treatment on spontaneous contraction, we examined three contractile parameters (**Figure 2.2**): frequency, amplitude and integral or area under the curve (A.U.C). Compounds were added to the organ bath following a period of contraction stabilisation, whereby the frequency and amplitude of consecutive contractions were stable over a period of 30 minutes (for human tissue) or 10 minutes (for murine tissue). Labchart 7 software was used to generate the frequency, amplitude and A.U.C values. Contractile parameter values were generated before and after the addition of each compound and compared. The frequency (Hz) was calculated as a number of peaks occurring per second over a set period of time; 30 minutes or 10 minutes for human or murine tissue respectively. The amplitude (mN) of each contraction was calculated as the magnitude of the difference between the contraction wave's baseline (set to 0 mN) and the peak. The mean amplitude of consecutive contractions occurring over a set period of time was used for the analysis. The A.U.C (mN s) of each contraction was calculated as the integral of the selected area. The mean A.U.C value of consecutive contractions occurring over a set period of time was used for the analysis.

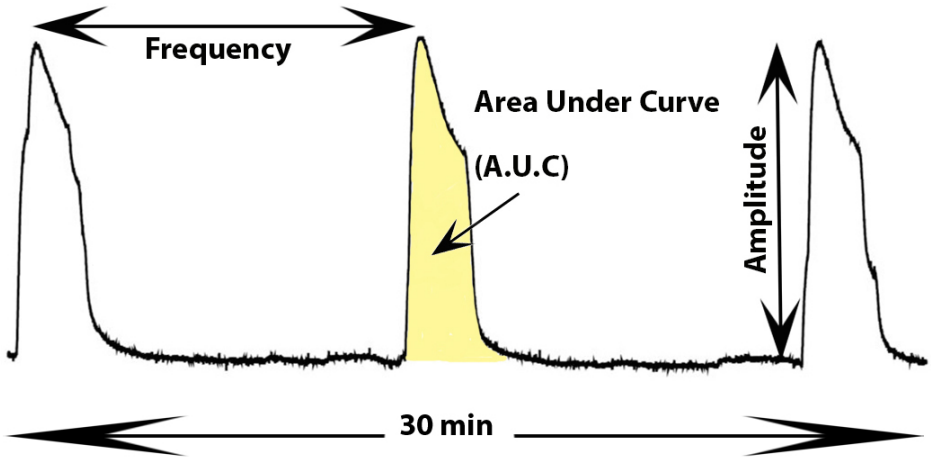



**Figure 2.2:** Contractile parameters measured. The figure shows a representative 30 min readout obtained from contraction of a human myometrial strip.

### 2.2.4 Contractile parameters analysis

The LabChart7 data acquisition software enables the user to perform a number of calculations on each recording channel. These calculations allow display of calculated values from the raw signal in each channel. For the analysis of spontaneous contraction recordings, we used the output of "Cyclic Measurements", which can be used to analyse periodic waveforms. "Cyclic Measurements" pre-processed the signal, detected cycles in the waveform and used the detected cycles to perform various calculations, such as frequency and amplitude. The A.U.C was calculated using the Peak Analysis module of the software. Data sets from each channel were transferred from the LabChart7 DataPad into Excel sheets. The effect of each experimental compound on each contractile parameter was expressed as the percentage of the original value obtained prior to the addition of the compound. For statistical analysis, percentage data was transformed to proportions, which were further arcsin-transformed and analysed using one/two-way ANOVA with Tukey's post hoc test in GraphPad v6.0 software.

## 2.3 Cell and tissue culture

Two human myometrial cell lines, the UtSMCs and the PHM1-41s were used for a) collagen contractility assays, b) in-cell Westerns, c) calcium assays and d) gene expression analysis. In addition, human myometrial explants were cultured in conditions explained later in this chapter and a PCR array profiling the gene expression of "Human Androgen Receptor Signalling Targets" (Cat. No PAHS 142ZA, Qiagen) was performed.

### 2.3.1 Myometrial cell lines

UtSMCs (Uterine Smooth Muscle Cells) are a commercially available primary cell line derived from a single non-pregnant donor (Cat. No CC-2562, Lonza Walkersville Inc, USA). PHM1-41s (Pregnant Human Myometrium) are a myometrial immortalized cell line obtained from a single late-term pregnant donor, which was generously provided by Dr. Barbara Sanborn (Colorado State University, Fort Collins, CO). PHM1-41s are infected with a replication-defective adenovirus

and are selected through resistance to Geneticin as detailed in Monga's study (Monga et al., 1996).

### 2.3.2 Maintaining and passaging cells

#### *2.3.2.1 UtSMCs*

The UtSMCs were cultured in phenol red-free high glucose (4.5 g/ L) Dulbecco's Modified Eagle Medium (DMEM; Cat. No 21063-029, Gibco), supplemented with 10% (v/v) Fetal Bovine Serum (FBS; Cat. No 10500, Gibco) and 1% penicillin-streptomycin/L-Glutamine (500 IU/ ml penicillin and 500 µg/ ml streptomycin; PAA laboratories, Pasching, Austria). Cells required medium to be changed every 3 days and were passaged approximately every 10 days, once 80-90% confluence was reached. To passage, the cells were rinsed with pre-warmed PBS prior to incubation with 0.05% trypsin-EDTA (Cat. No BE17-161E, Lonza) for 5 minutes in 37$^{o}$C (2 ml/ T75 or 4 ml/ T162 flask). Following detachment, cells were resuspended in fresh pre-warmed medium to neutralise trypsin-EDTA action and counted using a haemocytometer. Briefly, 20 µl of resuspended cells were mixed with 20 µl of 0.4% Trypan Blue Solution (Cat. No 15250-061, Gibco) and 20 µl of the 40 µl mixture was placed onto the haemocytometer to assess both cell concentration and viability. Cells were then centrifuged at 300 g for 5 minutes in an Eppendorf centrifuge (5840R, Eppendorf AG, Hamburg, Germany), resuspended in fresh pre-warmed medium and seeded in new flasks at a density of 3 x $10^6$ cells/T75 flask (Cat. No 430641, Corning) or at a density of 4 x $10^6$ cells/T162 flask (Cat. No EK-680031, Corning) and maintained at 37°C (5% $CO_2$) in an incubator. The UtSMCs were used up to passage 13 for all the experiments described in this thesis.

#### *2.3.2.2 PHM1-41s*

The PHM1-41s were cultured in phenol red free high glucose (4.5 g/ L) DMEM supplemented with 10% (v/v) FBS, 1% penicillin-streptomycin/L-Glutamine, 0.1 mg/ml G418 sulfate (Cat. No 345812, Life Technologies) and 100 µg/ ml Normocin (Cat. No ant-nr-1, InvivoGen). Normocin is a broad-spectrum antibiotic against mycoplasma and Gram-positive and Gram-negative bacteria. The cells were seeded at a density of 1-3 x $10^6$ cells/ T75 flask or at a density of 2-4 x $10^6$

cells/ T162 flask and maintained at 37°C (5% $CO_2$) in an incubator. The cells required medium to be changed every other day and were passaged every 4 days, when they reached 60-80% confluence. During passaging, the medium was removed and cells were rinsed with pre-warmed PBS. To dislodge cells from the bottom of the flask, cells were incubated with trypsin-EDTA for 3-5 minutes at 37°C (5% $CO_2$). Fresh pre-warmed DMEM was added to inactivate trypsin and the cells were counted. The viability was assessed with Trypan Blue Solution as described above. The cells were then centrifuged at 300 g for 5 minutes, resuspended in fresh pre-warmed DMEM and seeded in new flasks. The PHM1-41s were used up to passage 20 for all the experiments described in this thesis.

As a general rule, G418 sulfate and Normocin were omitted from the DMEM medium one passage prior to the cells being seeded for an experiment, and the percentage of FBS was adjusted depending on the requirements of each experiment.

### 2.3.3 Myometrial explant culture

Myometrial biopsies from TNL women where collected in RPMI medium during caesarean section as described above. Following transport to the laboratory, the biopsies were washed in PBS and dissected into small pieces (1 x 1 mm). Four pieces were placed into each well of a 24-well plate (Cat. No 3524, Corning) in 2 ml phenol red-free and FBS-free RMPI medium (Cat. No 11835063, Gibco) supplemented with 1% penicillin-streptomycin/L-Glutamine. The tissue was left overnight to rest at 37$^{o}$C. The following morning, the medium was removed from the wells and the tissue was rinsed with pre-warmed PBS. 1 ml of fresh RPMI was added to each well and the explants were immediately exposed to various treatments including a) nil (control), b) vehicle (ethanol) and c) dihydrotestosterone (DHT) for 6, 24 and 48 hours. Following each time point, the medium was removed and the tissue pieces were rinsed in PBS and transferred into sterile 2 ml Eppendorf tubes on dry ice. The samples were stored at -80$^{o}$C.

## 2.4 RNA extraction

### 2.4.1 RNA extraction from myometrial cell lines

RNA was extracted from myometrial cell lines using the RNeasy®Mini Kit (Cat. No 74106, Qiagen) according to supplier's protocol. Briefly, UtSMCs and PHM1-41s, which had been seeded into 6-well plates, were washed twice in PBS and lysed in 300 µl RTL buffer prepared with β-mercaptoethanol (10 µl β-ME per 1 ml of RTL buffer). The lysates were passed through a 20 g needle (0.9 mm; Cat. No 304827, BD Microlance) attached to a 1 ml syringe (Cat. No 309659, BD Plastipak), transferred to 1.5 ml Eppendorf tubes and stored at -80$^{o}$C. At the day of extraction, the samples were thawed on ice and one volume of 70% ethanol was added to each sample. The samples were then transferred to the RNeasy spin columns provided with the kit and centrifuged at 10000 rpm for 30 seconds (Eppendorf centrifuge 5415R). The RNA was purified by the addition of ethanol-based buffers. Initially, 350 µl of RW1 buffer, which removed carbohydrates and proteins, was added into each column and samples were centrifuged at 10000 rpm for 30 seconds. On-column DNase I digestion was performed for 15 minutes at room temperature by addition of a mixture of 10 µl DNase I and 70 µl of RDD buffer (Cat. No 79254, Qiagen) to each sample. Columns were then washed with 350 µl RW1 and then with 500 µl RPE, which is a buffer that removed salt residues from the samples, and centrifuged at 10000 rpm for 30 seconds. Another centrifugation in 500 µl RPE at 10000 rpm for 2 minutes was performed to dry the columns. A final centrifugation, without addition of any buffer, was performed for 1 minute at maximum speed to remove excess buffer. The RNA was eluted by direct addition of 30 µl of RNase free water to the column matrix and centrifugation at 10000 rpm for 30 seconds. The samples were collected into sterile 1.5 Eppendorf tubes and stored at -80$^{o}$C.

### 2.4.2 RNA extractions from human myometrial samples

In order to extract RNA from human myometrial biopsies, the samples were thawed on ice and homogenised in 2 ml Eppendorf tubes containing a single sterile 5 mm ball bearing and 1 ml TRI reagent (Cat. No T9424, Sigma) using a Retsch TissueLyser (Qiagen) at 25 Hz for 3 x 4 minutes. Following tissue homogenisation,

the samples were allowed to incubate for 15 minutes at room temperature and were centrifuged at 14000 rpm for 10 minutes at 4°C (Eppendorf centrifuge 5415R). The supernatants were placed into Phase Lock Gel heavy tubes (PLG; Cat. No 2302830, 5Prime), and 200 µl of 1-bromo-3-chloropropane (BCP; Cat. No B9673, Sigma) was added to each tube to eliminate protein contamination. PLG tubes were vigorously agitated by shaking for 15 minutes and left to incubate on the bench for 10 minutes. Following incubation, the PLG tubes were centrifuged at 14000 rpm for 10 minutes at 4°C. The organic phase and the interphase materials were effectively trapped below the barrier of the gel. The aqueous phase, containing the RNA, was collected into pre-chilled 1.5 ml Eppendorf tubes. Following addition of one volume of 70% ethanol, the samples were transferred onto RNeasy Mini Spin Columns (Qiagen) and RNA was extracted using RNeasy®Mini Kit, with on-column DNase I digestion as described in section 2.4.1. The RNA samples were stored at -80°C.

### 2.4.3 Determination of RNA quantity and quality

The concentration and quality of the extracted RNA was determined with a Nanodrop 1000 spectrophotometer (ThermoScientific, DE, USA). The Agilent 2100 BioAnalyzer (Agilent Technologies, CA, USA) was additionally used to assess the purity of RNA extracted from human myometrial explants.

### 2.4.4 Nanodrop

All RNA extracts were initially assessed using a Nanodrop spectrophotometer. Briefly, the system was calibrated using RNase free water and 2 µl of each sample was loaded onto the apparatus. The absorbency peaks were measured at 260 nm and 280 nm and the concentrations were given at ng/ µl. The purity was determined by 260:280 and 260:230 ratios, which ideally had values between 1.7 and 2.1. Ratios outside this range indicated sample contamination with phenol or protein and those samples were considered not suitable for subsequent evaluation.

### 2.4.5 BioAnalyzer

The Agilent RNA 6000 Nano Kit (Cat. No 5067-1511, Agilent Technologies) was

used to assess the purity of RNA extracts obtained from myometrial explants, which were used to conduct a PCR Array (see section 2.6.4). The samples and a "reference" ladder were loaded onto a 12-well chip according to the company's protocol. Briefly, a gel-dye mix was prepared in 0.5 ml RNase free tube by mixing 65 µl of sterile filtered RNA 6000 Nano gel matrix with 1 µl of RNA 6000 Nano dye concentrate. The solution was vortexed and centrifuged at 13000 g for 10 minutes at room temperature and 9 µl of the solution was pipetted in the wells marked as G on the chip. To each well, 5 µl of RNA 6000 Nano marker and 1 µl of each sample (or 1 µl of ladder) were added. The chip was vortexed on the IKA vortexer (Agilent) for 1 minute at 2400 rpm and then placed on the BioAnalyser for analysis. The purity of the sample was determined on the basis of the sample RIN score (ratio of integrity), which represents the ratio of 28S rRNA to 18s rRNA. RNA was deemed of high quality when the RIN was greater than 7. Only such samples were used for the PCR array described in section 2.6.4.

## 2.5 Complementary DNA (cDNA) synthesis

### 2.5.1 CDNA synthesis from cell/tissue RNA extracts

The cDNA was synthesised using the High Capacity cDNA Synthesis Kit (Cat. No 4368813-Applied Biosystems, US). RNA extracted from UtSMCs and PHM1-41s was diluted in RNase free water to a concentration of 200 ng/ 10 µl and 20 µl of the sample was added to a 20 µl of mastermix to make up a final reaction volume of 40 µl. The RNA extracted from TNL and TL myometrial biopsies was diluted in RNase free water to a concentration of 600 ng/ 10 µl and 20 µl of the sample was added to 20 µl of mastermix. A Reverse Transcriptase (RT) Enzyme Mix-free (-RT) reaction was included in order to assess any residual genomic DNA contamination. The details for mastermix preparation are detailed in **Table 2.1**. Reactions were incubated in a PTC-100 Thermal Cycler (GRI G-Storm, UK) as detailed in **Table 2.2** and stored at -20$^{o}$C. The cDNA samples from cells and explants were preceded for the RT-PCR protocol described in section 2.6.2 and 2.6.3 respectively.

**Table 2.1:** Reagents for cDNA synthesis mixture per reaction

| Reagent | +RT x 1 (µl) | -RT x 1 (µl) |
|---|---|---|
| 10 x RT Buffer | 4 | 4 |
| 25 x DNTPs | 1.6 | 1.6 |
| 10 x Random Primers | 4 | 4 |
| Reverse Transcriptase (RT) | 2 | - |
| RNase Inhibitors (1U/ µl) | 2 | 2 |
| RNase free $dH_2O$ | 6.4 | 8.4 |
| Sample | 20 | 20 |
| Total reaction volume | 40 | 40 |

**Table 2.2:** Thermal cycler programme settings

| Temperature ($^oC$) | Time (min) |
|---|---|
| 25 | 10 |
| 37 | 120 |
| 85 | 5 |
| 4 | Hold |

### 2.5.2 CDNA synthesis from RNA extracted from explant cultures

The $RT^2$ First Strand Kit (Cat. No 330401, Qiagen) was used to prepare cDNA from RNA extracts from cultured myometrial explants using the manufacturer's instructions. Briefly, each RNA sample was prepared in $dH_2O$ to a concentration of 400 ng/ 8 µl, and 8 µl of this sample was then mixed with 2 µl of Buffer GE, provided with the kit, to make up a final volume of 10 µl of genomic DNA elimination mix. This mix was incubated at 42$^o$C for 5 minutes and the plate was immediately placed on ice for 1 minute. The reverse-transcription mastermix for each reaction was prepared as shown in **Table 2.3**.

**Table 2.3:** Reverse-transcription mastermix per reaction for cDNA synthesis

| Component | Volume (µl) x 1 |
|---|---|
| 5 x Buffer BC3 | 4 |
| Control P2 | 1 |
| RE Reverse Transcriptase Mix | 2 |
| $dH_2O$ | 3 |
| Total | 10 |

To each 10 µl of genomic DNA elimination mix, 10 µl of mastermix was added to make up a reaction of 20 µl, and the reactions were incubated at 42$^o$C for 15 minutes and then at 95$^o$C for 5 minutes. To each reaction, 91 µl of $dH_2O$ was added and the samples were preceded for the RT-PCR protocol described in section 2.6.4.

## 2.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

### 2.6.1 Methology

Taqman qRT-PCR was performed to determine the concentrations of mRNAs endoced by genes of interest in cultured UtSMCs, PHM1-41s and myometrial biopsies collected from TNL and TL pregnancies. Two different methods were employed depending on the experimental design. The ΔΔCt method was used to assess gene expression in UtSMCs and PHM1-41s. The standard curve method was used to examine the expression of genes in TNL and TL myometrial samples. All primer/probe pre-validated assays used in this thesis had FAM-labelled (fluorophore) probes and were purchased from Applied Biosystems (listed in **Table 2.4**).

**Table 2.4:** Taqman primer/probe assays supplied by Applied Biosystems

| Full gene name | Gene | Assay ID |
|---|---|---|
| B-cell lymphoma 2 | *BCL2* | Hs00608023_m1 |
| Insulin-like growth factor 1 | *IGF1* | Hs01547656_m1 |
| Insulin-like growth factor binding protein 1 | *IGFBP1* | Hs00236877_m1 |
| Insulin-like growth factor binding protein 3 | *IGFBP3* | Hs00365742_g1 |
| Mammalian target of rapamycin | *MTOR* | Hs00234508_m1 |
| Insulin-like growth factor binding protein 6 | *IGFBP6* | Hs00181853_m1 |
| Elastin | *ELN* | Hs00355783_m1 |
| Fibronectin 1 | *FN1* | Hs00365052_m1 |
| Oxytocin receptor | *OXTR* | Hs00168573_m1 |
| Insulin-like growth factor binding protein 2 | *IGFBP2* | Hs01040719_m1 |
| Chemokine (C-C motif) ligand 2 | *CCL2* | Hs00234140_m1 |
| Insulin-like growth factor binding protein 5 | *IGFBP5* | Hs00181213_m1 |
| Prostaglandin-endoperoxide synthase 2 | *PTGS2* | Hs00153133_m1 |
| Matrix metalloproteinase-2 | *MMP2* | Hs01548727_m1 |
| Matrix metalloproteinase-9 | *MMP9* | Hs00234579_m1 |
| Zinc finger E-box-binding homeobox 1 | *ZEB1* | Hs03680602_m1 |
| Interleukin 6 | *IL6* | Hs02621719_u1 |
| Interleukin 8 | *IL8* | Hs01553824_g1 |
| Androgen receptor | *AR* | Hs04260217_m1 |

### 2.6.2 ΔΔCt method

To examine the expression of a gene of interest (GOI) in each sample, a singleplex Taqman qRT-PCR was performed using the Taqman Mastermix (Cat. No H4440040, Applied Biosystems). A mastermix containing either the primer/probe mix for the GOI or the primer/probe for the VIC-labelled 18S housekeeping gene (Cat. No 4308329, Applied Biosystems) was prepared. The 18S primer/probe was prepared

according to the kit instructions and used in 1:2 working dilution. 14 µl of each mastermix was prepared as shown in **Table 2.5** and added to each well of a 384-microwell plate (Cat. No 4309849, Applied Biosystems). 1 µl of cDNA was added to each well and the plate was sealed using an adhesive film (Cat. No 4311971, Applied Biosystems) and centrifuged at 1000 rpm for 1 minute. All qRT-PCR reactions were performed in technical duplicate and three control samples were included on each plate (-RT, -RT $dH_2O$ and $H_2O$).

**Table 2.5:** Reagents for Taqman qRT-PCR per well

| Reagent | Volume (µl) x 1 |
|---|---|
| Taqman Mastermix | 7 |
| Primer/probe assay | 0.7 |
| RNase free $dH_2O$ | 6.3 |
| Total reaction volume | 14 |

The reaction was carried out using the 7900HT Fast Real-Time PCR System with SDS v2.4 software (Applied Biosystems) set on the following setting: 50$^{o}$C for 2 minutes, 95$^{o}$C for 10 minutes, repeats of 95$^{o}$C for 15 seconds and 60$^{o}$C for 1 minute for 40 cycles. Data was analysed using the ΔΔCt method. Initially, the thresholds for the GOI and 18S were set in the linear phase of the exponential region of the amplification curves. The cycle number at which the PCR signal crossed a set threshold was used to determine relative gene expression. These comparative cycle threshold (Ct) values were extracted to Excel sheets and the average Ct value for the GOI and 18S was calculated initially from the technical replicates and then experimental replicates. The average 18S Ct value was subtracted from the GOI to calculate ΔCt and the number was normalized to the control tissue sample (ΔΔCt). The fold change was calculated using the formula 2^(-ΔΔCt). ΔΔCt values were used for statistical analysis and data was plotted as fold change (2^(-ΔΔCt)) compared to the relevant control sample.

### 2.6.3 Standard curve method

A standard curve qRT-PCR method was conducted to quantify transcript levels of genes of interest in the experimental samples (TNL and TL myometrium). An equal amount of each experimental sample's cDNA was pooled to make a "reference" sample. Several dilutions of the "reference" sample (1:4, 1:8, 1:16, 1:32, 1:64, 1:128,

1:256, 1:512) were prepared and used to obtain a standard curve for each GOI. Each experimental sample and -RTs controls (-RT sample and -RT $dH_2O$) were diluted 1:20 in $dH_2O$. 2 µl of each of the "reference" standards or the experimental samples was dispensed into each well of a 384-microwell plate. An 8 µl reaction mixture containing the primer/probe assay for the GOI was prepared and added to each well to make up a final reaction volume of 10 µl as detailed in **Table 2.6.**

**Table 2.6:** Reagents for standard curve qRT-PCR per well

| Reagent | Volume (µl) x 1 |
|---|---|
| Taqman Mastermix | 5 |
| Primer/probe assay | 0.5 |
| RNase free $dH_2O$ | 2.5 |
| Sample (1:20) | 2 |
| Total reaction volume | 10 |

RT-PCR was performed on both the experimental samples and "reference" standards. Relative value for the abundance of GOI in each experimental sample was extrapolated from the standard curve generated from the "reference" standard. All RT-PCR reactions were performed in triplicate (standards) or duplicates (samples). Standard curve RT-PCR was carried out for the housekeeping gene 18S under the same conditions to enable comparison between samples. The reaction was carried out using the 7900HT Fast Real-Time PCR System with SDS v2.4 software set on the following setting: 50°C for 2 minutes, 95°C for 10 minutes, repeats of 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. The expression levels were calculated from a linear regression of the standard curve as GOI expression compared to 18S expression using arbitrary units. Acceptable standard curves had a slope between -3.3 and -3.7 ($R^2 > 0.99$).

### 2.6.4 PCR array

A $RT^2$ Profiler PCR Array, which was designed with probes directed against genes known to be regulated by AR-dependent signalling, was used to examine the effect of activation of AR on gene expression in the human myometrial explants. The array was supplied in 384-well [4 x 96] format (Cat. No 330231 PAHS-142ZA, Qiagen). Following preparation of cDNA as described in section 2.5.2, a PCR mastermix was prepared as shown in **Table 2.7** using components provided by the company. The

384EZLoad Cover plates provided with the kit were used to assist loading of the 384-well plate and 10 µl of PCR mastermix were added into each well. The plates were then sealed and centrifuged at 1000 g for 1 minute prior to being run on the 7900HT Fast Real-Time PCR System with SDS v2.4 software set on the following setting: 95$^{o}$C for 10 minute, repeats of 95$^{o}$C for 15 seconds and 60$^{o}$C for 1 minute for 40 cycles. The Ct values were calculated according to the supplier's instructions and exported to an Excel sheet for use with the SABiosciences PCR Array Web Data Analysis Software. The software automatically performed quantification using the ΔΔCt method and results were presented in tables detailing fold changes and p values for each gene.

**Table 2.7:** PCR Array mastermix components

| Component | Volume (µl) x 1 |
|---|---|
| 2 x RT$^2$ SYBR Green Mastermix | 650 |
| cDNA | 102 |
| $dH_2O$ | 548 |
| Total reaction volume | 1300 |

## 2.7 Protein extraction from human myometrium

Snap frozen TNL and TL myometrial biopsies were thawed on ice and homogenised in 600 µl of Radioimmunoprecipitation assay (RIPA) lysing buffer (Cat. No R0278, Sigma) containing protease inhibitor cocktail tablet (1 tablet/ 10 ml RIPA buffer; Cat. No 11697498001, Roche) using a TissueLyser at 25 Hz for 3 x 4 minutes. Following tissue homogenisation, the lysates were allowed to incubate for 15 minutes on ice and were then centrifuged at 14000 rpm for 10 minutes to pellet cell debris. Supernatants were collected and aliquots of 100 µl were stored at -80$^{o}$C. Protein concentration was determined with the DC Protein Assay Kit (Cat. No 500-0120, Bio-Rad) as described below.

### 2.7.1 Determination of protein concentration (BCA assay)

Following protein extraction the amount of protein in each sample was quantified using the DC Protein Assay Kit (Cat. No 500-0116, Biorad), which is a colorimetric assay for protein quantification similar to the Lowry protein assay. A standard curve

of bovine serum albumin (BSA 1.37 mg/ ml; Cat. No 500-0112, Biorad) was created in RIPA buffer and 5 µl of each standard was added into a nunc 96-well immunoplate (Cat. No 439454, Thermo Scientific) in triplicate. The protein samples were diluted 1 in 10 and 5 µl of each sample was added into the plate in triplicate. The reagent A' buffer was prepared as per supplier's instructions; reagent A' buffer = 20 µl reagent S buffer + 1 ml reagent A buffer. A volume of 25 µl of reagent A' buffer was then added to each well. A multichannel pippete was used to dispense 200 µl of reagent B buffer to each well and the plate was left to incubate for 15 minutes at room temperature in order to develop a characteristic blue colour. The colour was a result of the reaction of protein with an alkaline copper tartrate solution and Folin reagent. In particular, the reaction between protein and cooper in an alkaline solution induced reduction of Folin reagent, which resulted in production of reduced species with a blue colour and maximum absorbance at 750 nm. The absorbance was measured using a spectrophotometer and the protein concentration of each sample was extrapolated from the standard curve.

## 2.8 Western analysis

### 2.8.1 Western gel analysis

Western immunoblotting was used to quantify and compare the concentration of total AR protein between TNL and TL myometrial samples. Briefly, samples were prepared as described in **Table 2.8** and heated to 70$^{o}$C for 10 minutes to make 20 µl of sample, which contained 20 µg of reduced protein extract. Reduced samples were loaded into individual wells on 10% Bis-Tris (1.0 mm x 15 well) precast NuPAGE gels (Cat. No NP0303BOX, Invitrogen) and subjected to electrophoresis at 180 V for 1 hour on ice in MOPS running buffer (Cat. No NP0001, Invitrogen) with 500 µl NuPAGE antioxidant (Cat. No NP0005, Invitrogen) in the upper chamber of the XCell SureLock™ Mini-Cell Electrophoresis System (Invitrogen). Following separation, the proteins were transferred onto an Immobilon-FL PVDF membrane (Cat. No P/N IPFL00010, Millipore), which was pre-activated by a 30 seconds incubation in 10% methanol, using a Mini-PROTEAN II electrophoresis cell system (Biorad) equilibrated in 1 x transfer buffer (3 gr Tris, 14.4 gr Glycine, 810 ml $dH_2O$, 100 ml methanol; pH 8.3) at 100 V for 90 minutes. The membrane was blocked for

1 hour at room temperature in Odyssey Blocking Buffer (OBB; Cat. No 92740000, LI-COR Biosciences). Following blocking, the membrane was incubated overnight at 4$^{o}$C with primary antibodies diluted in OBB (detailed in **Table 2.9**) to detect the AR and alpha-smooth muscle actin (α-SMA) protein. The membrane was initially washed for 5 minutes in Tris Buffered Saline (TBS) supplemented with 0.05% (v/v) Tween 20 (TBST; pH 7.4) and then 3 x 5 minutes in TBS (Cat. No T6664, Sigma). Thereafter the membrane was incubated with secondary antibodies (detailed in **Table 2.9**) diluted in OBB for 1 hour at room temperature in the dark. Following incubation, the membrane was washed once for 5 minutes in TBST and 3 x 5 minutes in TBS and scanned using the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences) using the following parameters: Resolution; 84 µm, Quality; high, Focus offset; 3.0 mm, Intensity; 700 – 7.0 and 800 - 7.0. The bands were quantified using Odyssey analysis software (LI-COR Image Studio v2.1) and the raw values were exported to Excel sheets.

**Table 2.8:** Reagents per reduced sample

| Reagent | 1 x |
|---|---|
| 4 x LDS Sample Buffer | 7.5 µl |
| 10 x Reducing agent | 3 µl |
| Protein (20 µg) | 9.5 µl |
| Total | 20 µl |

**Table 2.9:** Antibodies used for Western gel analysis

| Antibody | Dilution | Supplier |
|---|---|---|
| Primary 1: Monoclonal rabbit anti-AR | 1:200 | Spring Bioscience (SP107) |
| Primary 2: Monoclonal mouse anti-α-SMA | 1:10000 | Sigma (A2547) |
| Secondary 1: Polyclonal donkey anti-rabbit (IgG H+L) 800CW | 1: 10000 | LI-COR Biosciences (926-32213) |
| Secondary 2: Polyclonal donkey anti-mouse (IgG H+L) 680CW | 1:10000 | LI-COR Biosciences (926-68072) |

### 2.8.2 In-cell Western

An in-cell Western for quantification of phosphorylated myosin light chain (PMLC) protein in PHM1-41s and UtSMCs was performed according to previously published

protocol (Aguilar et al., 2010; Hutchinson et al., 2014). **Figure 2.3** shows a schematic representation of the protocol detailed below.

Cells were collected and centrifuged as described in section 2.3.2 and cell pellets were resuspended in charcoal stripped 5% (v/v) FBS DMEM to a concentration of 17600 cells/ 200 µl. Black optically clear bottom tissue culture treated 96-well plates (Cat. No 6005182, PerkinElmer) were used for this assay and 200 µl of the cell suspension was added to each well (550 cells/ $mm^2$). Plates were left at room temperature for 1 hour to prevent cell clump formation and placed in the incubator (37$^oC$) for an overnight incubation. Following incubation the medium was removed from the plates by inversion onto a paper towel and fresh pre-warmed 100 µl charcoal stripped 5% (v/v) FBS DMEM was added into each well. The desired treatments were prepared in pre-warmed FBS-free DMEM and added to triplicate wells using a multichannel pipette. The details of the treatments are discussed in the methods section of Chapter 5. Following incubation, the cells were fixed by addition of 10 µl of 37% (v/v) formaldehyde (Cat. No F1635, Sigma) for 30 minutes at room temperature. The fixative was removed from the plate by inversion onto a paper towel and cells were washed with 1% (v/v) Triton X-100 (Cat. No T8532, Sigma) in PBS (3 x 5 minutes). Cells were blocked for 1 hour in OBB at room temperature. Primary antibodies to detect the PMLC and the loading control α-Tubulin were diluted in OBB, the blocking buffer was removed and 50 µl of antibody was added into each well for an overnight incubation at 4$^oC$. The following day, plates were washed in PBS (3 x 5 minutes) and incubated for 1 hour with secondary antibodies (50 µl) diluted in OBB at room temperature protected from light. The details of primary and secondary antibodies used in this protocol are detailed in **Table 2.10**. The plate was rinsed with PBS (3 x 5 minutes) and scanned using the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences). The LI-COR Odyssey Infrared Imaging System was set at the following parameters: Resolution; 84 µm, Quality; high, Focus offset; 3.0 mm, Intensity; 700 – 7.0; 800 - 7.0. The intensity of fluorescence was analysed using the LI-COR Image Studio software and the fluorescence intensity measurement for PMLC and α-Tubulin in each well were used

as readout. The raw measurements were exported into Excel sheets where PMLC was calculated relative to α-Tubulin and compared between treatments.

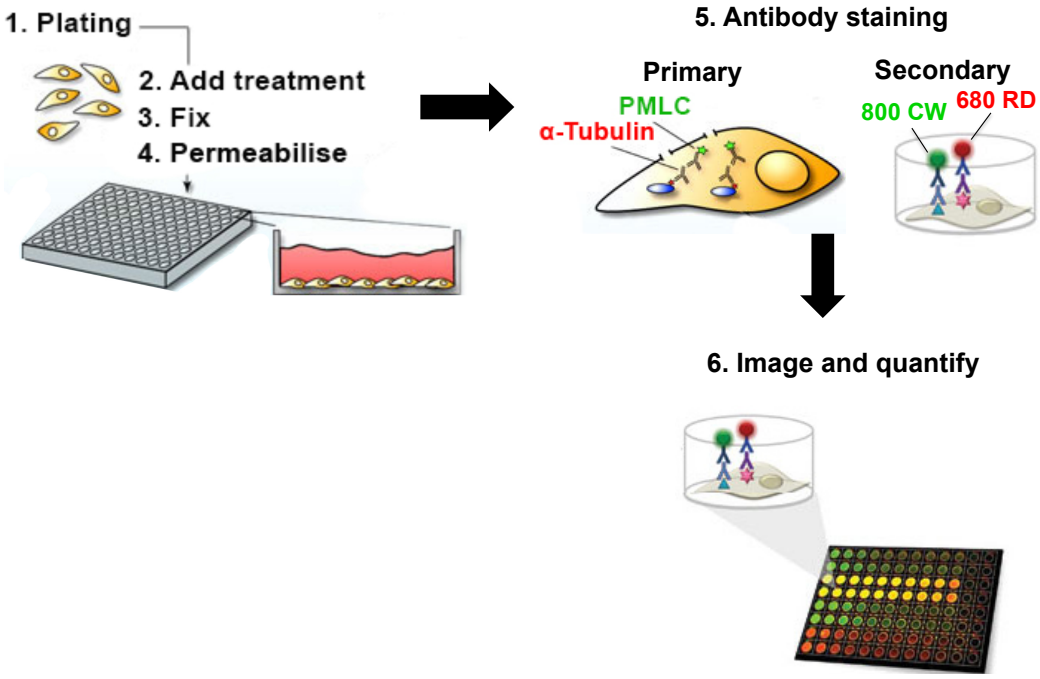



**Figure 2.3:** Schematic representation of the ICW protocol. The PHM1-41s and UtSMCs were seeded into 96-well plates, incubated with different experimental compounds and fixed in the wells. The fixed cells were permeabilised and incubated with primary antibodies against PMLC and α-Tubulin protein and secondary LICOR antibodies (800 CW and 680 RD). The plates were scanned using LI-COR Odyssey Infrared Imaging System and the fluorescence intensity in each channel was quantified. Images modified from LI-COR BIOSCIENCES.

**Table 2.10:** Details of antibodies used for in-cell Western assay

| Antibody | Dilution | Supplier |
|---|---|---|
| Primary 1: Polyclonal rabbit anti-PMLC 2 (Ser19) | 1:50 | Cell-Signalling (3671) |
| Primary 2: Monoclonal mouse anti-α-Tubulin | 1:1000 | Sigma (T9026) |
| Secondary 1: Polyclonal donkey anti-rabbit (IgG H+L) 800CW | 1: 10000 | LI-COR Biosciences (926-32213) |
| Secondary 2: Polyclonal donkey anti-mouse (IgG H+L) 680RD | 1:10000 | LI-COR Biosciences (926-68072) |

## 2.9 Collagen contractility assay

The UtSMCs and PHM1-41s were embedded in collagen gels to determine the effect of various experimental compounds on myometrial smooth muscle tone as described in Hutchinson et al methods (Hutchinson et al., 2014).

### 2.9.1 Preparation of collagen

Collagen I was prepared from adult rat tails provided by Dr Alex Henke (University of Edinburgh MRC Centre for Reproductive Health, UK) according to methods detailed in Bell et al (Bell et al., 1979). In order to obtain the collagen, the tails, which were frozen immediately after collection, were thawed for 20 minutes in 70% ethanol and the tendons were removed, minced and incubated for 48 hour in dilute acetic acid (250 ml/ tail), under refrigeration with constant agitation. The mixture was centrifuged at 14000 g for 1 hour and the supernatant, which was a crude collagen extract, was stored at $4^{o}$C; the solution was incubated with 0.1 M of sodium hydroxide (6:1 ratio) to neutralise the acetic acid and precipitate the collagen. Collagen was recovered by centrifugation at 300 g for 5 minutes, the supernatant was discarded and the pellet was resolubilised in an equal volume of acetic acid. The final collagen mixture was stored at $4^{o}$C and used for the contraction assays described below.

### 2.9.2 Gel casting and contraction assay

PHM1-41s and UtSMCs were collected from confluent flasks as described in section 2.3.2. Following the centrifugation step, cell pellets were resuspended at $1 \times 10^{6}$ cells/ ml and 2.4 ml of this suspension was transferred to a 50 ml falcon tube on ice with addition of 5.6 ml medium, to make a total 8 ml of cell suspension. The medium used was phenol red free high glucose (4.5 g/ L) DMEM supplemented with 10 (v/v) FBS and 1% penicillin-streptomycin/L-Glutamine. The following steps were conducted as rapidly as possible and on ice. A further 1.2 ml of 0.1 M NaOH, which was prepared in sterile filtered $dH_2O$, was added to the cell suspension and the final volume was divided into two 50 ml falcon tubes on ice (2 x 4.6 ml). The collagen (2 ml) was added initially to one of the two tubes and resuspended by gentle

pipetting. A 500 µl volume of the collagen/cell suspension was added into each well of a 24-well plate (Cat. No 3738, Corning). Once half of the plate was filled, 2 ml of collagen was added to the other tube of cells and the procedure was repeated to fill the entire 24-well plate. The collagen/cell suspension was allowed to polymerase overnight (~18 hours) at 37$^{o}$C. The gels were serum-starved for 2 hours at 37$^{o}$C by addition of 500 µl of FBS-free high glucose (4.5 g/ L) DMEM supplemented with 1% penicillin-streptomycin/L-Glutamine. Thereafter the medium was carefully removed from each well and replaced with 500 µl DMEM supplemented with 5% (v/v) charcoal stripped FBS. The same medium was used to prepare all treatments, which were applied in quadruplicate or greater. Treatment details are discussed in the methods section of Chapter 5. To perform the assay, the gels were detached using a pipette tip around the gel edge and the plates were placed into the incubator. Untreated gels developed a basal contraction, which was manifested as a decrease in gel area.

### 2.9.3 Gel analysis

Gels were measured at three time points: 0, 24 and 48 h. A Leica MZ6 light microscope/ camera (Mayfair, UK) using Leica Firecam software on the ICD 2 x 2 colour binning setting was used to photograph the gels and the images were analysed using Adobe Photoshop CS6 (Mountain View, CA, USA). To analyse the gel area, images were loaded in extended view and the gel area was selected by the Quick Selection tool. The pixel number of each gel area was used as readout. The readout from each gel at 24 and 48 hours was expressed as percentage of the readout of the same gel at the 0-hour time point.

### 2.9.4 Cell viability at the end of contraction assay

The viability of cells embedded in gels was assessed using the CellTitre 96 AQueousOne Solution Cell Proliferation Assay kit (Cat. No G3580, Promega). This assay works by measuring the amount of formazan in each well, which is the product of reagent MTS tetrazolium reduction. Living cells are able to metabolise the reagent into coloured formazan and, thus, the quantity of colour is proportional to the number

of viable cells. Briefly, the gels were transferred to a new 24-well plate and 1 ml of fresh FBS free DMEM supplemented with 1% penicillin-streptomycin/L-Glutamine was added to each well. The same medium was used to prepare the AqueousOne reagent at a concentration of 20% (v/v) and the gels were incubated with 500 µl of the reagent for 2 hours at 37$^{o}$C. Following incubation, total 100 µl of reagent/well in triplicate was transferred to a 96-well plate. The Thermomax Microplate Reader (Molecular Devices, UK) was used to read the optical density at 490 nm. The average measurement was calculated for each treatment and compared between treatments to assess cell viability.

## 2.10 Immunohistochemistry

Immunolocalisation of the AR in human and mouse myometrium was investigated using either 3,3-diaminobenzidine tetrahydrochloride (DAB) staining or immunofluorescence (IFL) depending on the experimental design.

### 2.10.1 Tissue fixation, embedding and sectioning

Tissues were fixed in 10% NBF for 24 hours and transfer to 70% ethanol. The tissues were processed and embedded in paraffin according to standard protocols. Serial sections of 5 µm thicknesses were cut on a microtome (RM2235, Leica), mounted on electrostatically charged glass slides (Leica) and dried overnight in a 50$^{o}$C incubator. Prior to the immunohistochemical analysis, gross morphology was assessed by haematoxylin and eosin (H&E) staining using standard protocols.

### 2.10.2 DAB staining for AR

Slides were dewaxed in xylene (2 x 5 minutes), rehydrated in graded alcohol (100%, 90%, 70% for 20 seconds each), washed in $dH_2O$ and underwent heat induced antigen retrieval in a decloaking chamber (Biocare Medical, CA, USA) with 0.01 M citrate buffer (citrate acid 1.92 g, 1 ml $dH_2O$, pH 6.0). After washing in $dH_2O$ (3 x 5 minutes) slides were incubated in 3% (v/v) $H_2O_2$ (Cat. No H1009, Sigma) in methanol for 30 minutes to inhibit endogenous peroxidase activity. Slides were further washed in $dH_2O$ (3 x 5 minutes) and transferred into TBS (50 mM Tris,

0.85% NaCl, pH 7.4) for 2 x 5 minutes. Sections were blocked for 30 minutes with normal goat serum (NGS) prepared 4:1 TBS to NGS with 5% (w/v) BSA (Cat. No A3294, Sigma). Endogenous biotin was blocked using the streptavidin (0.01 M for 15 minutes)/ biotin (0.01 M for 15 minutes) blocking kit (Cat. No SP2001, Vector). The primary antibody was diluted in NGS and applied for overnight incubation at 4$^{o}$C. The slides were washed twice in TBS for 5 minutes each and then incubated with the secondary antibody diluted in NGS for 30 minutes in room temperature. The slides were washed twice in TBS for 5 minutes each and a last incubation with Horseradish Peroxidase Streptavidin (HRP) diluted in TBS was performed for 30 minutes in room temperature. Details of all antibodies and dilutions used in the present protocol are shown in **Table 2.11**. DAB (Cat. No SK-4105, Dako) was used for colorimetric detection, and samples were counterstained with haematoxylin followed by dehydration and mounting. The experiment included a negative control (primary antibody absent) and images were acquired on Provis BX2 microscope (Olympus America Inc. Center Valley, PA, USA) using a Canon DS126131 camera and EOS image capture software (Canon, Woodhatch, Surrey, UK). Scale bars were inserted using AxioVision software v4.8.0.0 (Carl Zeiss Imaging Solutions, Germany).

**Table 2.11:** Antibodies used for DAB staining

| Antibody | Dilution | Supplier |
|---|---|---|
| Primary: Monoclonal rabbit anti-AR | 1:400 | Spring Bioscience (SP107) |
| Secondary: Goat anti-rabbit biotinylated | 1:500 | Vector (BA 1000) |
| HRP | 1:500 | Vector (SA 5004) |

### 2.10.3 Immunofluorescence

For co-localisation studies, the expression of the AR and α-SMA protein was examined by dual immunofluorescence. Slides were dewaxed, rehydrated, antigen retrieved and incubated in 3% (v/v) $H_2O_2$ in methanol as described in section 2.10.2. Following two TBS washes for 5 minutes each, slides were blocked with normal swine serum (NSS; Cat. No B0625, Biosera), prepared 4:1 TBS to NSS with 5%

(w/v) BSA, for 30 minutes. Slides were then washed in TBS for 5 minutes and incubated with the first primary rabbit anti-AR antibody overnight at 4$^{o}$C. Following one wash with TBST for 5 minutes and 2 x 5 minutes TBS washes, the slides were incubated with the secondary anti-rabbit antibody diluted in NSS for 1 hour at room temperature. Slides were washed once with TBST for 5 minutes and then 2 x 5 minutes with TBS, and incubated for 10 minutes with Red Tyramide (Cy3; Tyramide Signal Amplification kit TSA- Perkin Elmer) diluted 1:50 in its own substrate. Two washes with TBS were performed and the slides were initially microwaved in 0.01 M citrate buffer (pH 6.0) for 4 minutes in full power until the buffer boiled and then for additional 2.5 minutes. Slides were left for 30 minunes to cool down, washed in tap water for 5 minutes and TBS for 5 minutes, and blocked with normal rabbit serum (NRS; Cat. No GO-605/500, Biosera), prepared 4:1 TBS to NRS with 5% (w/v) BSA, for 30 minutes at room temperature. Following a 5 minutes wash with TBS, sections were incubated overnight at 4$^{o}$C with mouse anti-α-SMA prepared in NRS. The slides were washed with TBST for 5 minutes and TBS twice for 5 minutes and incubated with rabbit anti-mouse Peroxidase IgG2a for 1 hour on the bench. Additional wash steps with TBST and TBS were performed and sections were incubated for 10 minutes with Green Tyramide (Fluorescein, TSA kit, Perkin Elmer) prepared 1:50 in its own substrate. Slides were washed 3 x 5 minutes in PBS and counterstained with Dapi (Cat. No D9542, Sigma). A final wash step with PBS 3 x 5 minutes was performed and slides were mounted with Permafluor (Cat. No TA-030-FM, Thermo Scientific). Details of the primary and secondary antibodies used in this protocol can be found in **Table 2.12**. Slides were visualised on a Zeiss LSM 710 confocal microscope (Carl Zeiss, UK) and the images were obtained using the ZEN2009 software (Carl Zeiss MicroImaging GmbH, UK).

**Table 2.12:** Antibodies used for the double immunofluorescence protocol

| Antibody | Dilution | Supplier |
|---|---|---|
| Primary: Monoclonal rabbit anti-AR (N-20) | 1:1000 | Santa Cruz (Sc 816) |
| Primary: Monoclonal mouse anti-AMSA | 1:10000 | Sigma (A2547) |
| Swine anti-rabbit peroxidase | 1:500 | DAKO (P0399) |
| Rabbit anti-mouse peroxide | 1:200 | Zymed/ Cambridge Bioscience (61-0220) |

## 2.11 Calcium ($Ca^{2+}$) assay

The BD™ Calcium Assay Kit (Cat. No 640176, BD Biosciences) was employed to evaluate changes in the concentration of intracellular $Ca^{2+}$ induced by the action of experimental compounds in PHM1-41s and UtSMCs. This high-throughput assay involves loading of cells with a non-fluorescent acetoxymethyl ester (Fluo-4 AM), which is cleaved inside the living cell to give the free $Ca^{2+}$-sensitive Fluo-4. The principle of this assay is illustrated in **Figure 2.4.**

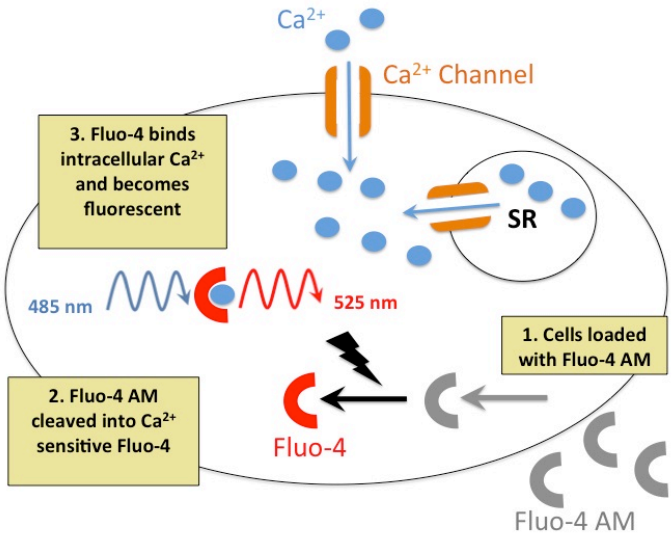



**Figure 2.4:** The principle of the Fluo-4 calcium assay. The cell membrane-permeable acetoxymethyl ester (Fluo-4 AM) enters the cell. Once inside the cell, Fluo-4 AM is cleaved into Fluo-4, which is then capable of binding intracellular $Ca^{2+}$. The concentration of intracellular $Ca^{2+}$ is elevated either by entry of $Ca^{2+}$ from the extracellular space via the membrane $Ca^{2+}$ channels or by release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR). The binding of Fluo-4 to intracellular $Ca^{2+}$ results in fluorescence, which can be detected at 525 nm.

### 2.11.1 Cell preparation

The calcium assay was performed as per Li et al methods (Li et al., 2008). The PHM1-41s and UtSMCs were seeded on white 96-well plates with a clear bottom (Cat. No 6005181, Perkin Elmer). The cells were seeded in 100 µl/well charcoal stripped 5% (v/v) FBS phenol red-free DMEM and at a density of 3 x $10^4$ cells/well

to form a confluent monolayer. The plates were left 1 hour in room temperature to prevent formation of cell clumps in the middle of the wells. The plates were then placed in the incubator (37$^{o}$C) for an overnight incubation to allow cells to attach to the bottom of the wells.

### 2.11.2 Dye-loading

The dye-loading solution (DLS) was prepared according to the supplier's instructions. Briefly, the loading solution was prepared by mixing 1 ml of 10 x Signal Enhancer with 9 ml 1 x Calcium Assay Buffer. The calcium indicator (Fluo-4 AM) was reconstituted in 100% Dimethyl sulfoxide (DMSO; 100 µl DMSO/ vial) and 10 µl of the dye was added to 10 ml of loading solution to make the DLS. Unused dye was stored in 10 µl aliquots at -20$^{o}$C protected from light. Following an overnight incubation, the plates were removed from the incubator and 100 µl of DLS was added into each well. The plates were placed back to the incubator for 1 hour to allow 'loading' of the dye. The cells were then incubated with different experimental compounds in quadruplicate for various time points. The details of these treatments and time points are given in the materials and methods section of Chapter 5.

### 2.11.3 Calcium flux assay and analysis

Following exposure to various treatments, the plates were placed on the NOVOstar plate reader (BMG Labtech, Germany). The NOVOstar reader has built-in injectors, which can be loaded with various compounds that can be injected directly into the wells. In this study, OXT (100 nM, Alliance Pharmaceuticals, UK) was used as a positive control to promote $Ca^{2+}$ flux, as previous studies reported that PHM1-41s respond to OXT by increasing the concentration of intracellular $Ca^{2+}$ (Monga et al., 1996). The effect of each treatment on the OXT-stimulated increase in intracellular $Ca^{2+}$ was assessed. Each well's fluorescence was monitored and recorded for 20 seconds prior to and 40 seconds post injection of OXT. The highest fluorescence measurement recorded (peak) following injection of OXT was used as readout and the average of the highest value was calculated and compared between treatments.

All raw data was recorded using MARS Data Analysis Software (BMG Labtech, Germany) and exported to Excel datasheets.

## 2.12 Mouse model of PTL

The experiments described in this method were approved by the UK Home Office license number 60/4241 (PPL) hold by Professor Jane Norman. Pregnant time-mated CD57BL/6 mice were purchased from Charles River (Kent, London, UK) on day 10 (D10) of gestation (plug day was considered as D1 of gestation) and were allowed to acclimatise for a week prior to the start of the experiment.

### 2.12.1 Induction of PTL

The surgery was performed on D17 of pregnancy as previously described in Rinaldi's et al study (Rinaldi et al., 2014). Briefly, mice were anaesthetised with 5% Isoflurane-Vet (Merial Animal Health Ltd, UK) in oxygen and 2.5% Isoflurane was used for maintenance of anaesthesia during a lower abdomen mini-laparotomy. Both uterine horns were exposed through the incision and the number of viable fetuses in each horn was recorded. The mice received an intrauterine injection of either 20 μg LPS (E.Coli 0111:B4; Sigma, UK) or sterile filtered PBS in a 25 μl volume using a 33-g Hamilton syringe (Sigma, UK). The treatment was infused into the uterine lumen of the horn that had the most fetuses between the first and second anterior fetuses and care was taken not to inject the amniotic cavity (**Figure 2.5**). The time of injection was recorded. Sterile filtered PBS was applied on the horns to help return them to the abdomen. The incision was then closed with a continuous stitch using 5/0 sutures (Cat. No W580, MidMeds Ltd) and the skin was closed with interrupted stitches using 4/0 sutures (Cat. No W9443, MidMeds Ltd). The average time of procedure/animal was 30 minutes. Following surgery, the mice were subcutaneously injected with 60 μl of 0.03 mg/ ml Vetergesic (Alstoe Ltd) analgesia and allowed to recover on heated pads (30$^{o}$C) prior to being transferred to individual cages for monitoring.

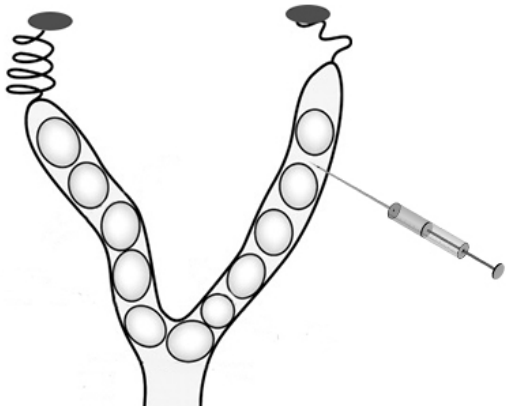


**Figure 2.5:** The site of intrauterine injection. Treatments were injected into the uterine lumen of the horn with the most fetuses between the first and second anterior fetuses. Image adapted from (Elovitz et al., 2003).

### 2.12.2 Time to delivery

A mini colour camera (SKC160IR, Sunkwang Electronics Co. Ltd, UK) connected to a digital recording software (AverMedia Technologies Inc, UK) was attached to each cage to monitor the mice and to record the time of delivery. The time to delivery was calculated as the hours from the time of intrauterine injection to the delivery of first pup. A normal C57BL/6 mouse pregnancy is normally completed between D19 and D21 of gestation. For our studies, preterm delivery was defined as delivery before 36 hours post injection and the rate of preterm delivery was calculated and compared between treatment groups by dividing the mice that delivered preterm by the total number of mice in each treatment group. Following completion of labour (delivery of all pups), the number of live pups in the cage of each mouse was recorded. The proportion of live pups per dam was calculated within 24 hours of delivery and compared between treatment groups by dividing the number of live pups recorded in the cage by the number of viable fetuses recorded at the time of injection. Mice and pups were culled by cervical dislocation at the end of each experiment. Mice were dissected to confirm delivery of all pups and any live pups found in the uterine horns were not considered as live born pups.

### 2.12.3 The use of model in this study

The mouse model of PTL was used to explore the hypothesis that administration of DHT on D17 of mouse pregnancy could prevent PTL. The experimental design of this work is thoroughly detailed in Chapter 6.

## 2.13 Experimental compounds

The suppliers of the experimental compounds used in this thesis are listed in **Table 2.13.**

**Table 2.13:** Details of experimental compounds

| Compound | Supplier |
|---|---|
| DHT | A8380 (Sigma) |
| T | T1500 (Sigma) |
| E2 | E8875 (Sigma) |
| P4 | P0130 (Sigma) |
| Flutamide | F9397 (Sigma) |
| TBSA | T3392 (Sigma) |
| OXT | Syntocinon 10 IU/ml capsules ALLIANCE pharmaceuticals) |
| Nifedipine | N7634 (Sigma) |
| LPS | L4391-1MG (Sigma) |

## 2.14 Statistical analysis

All analysis was conducted using the GraphPad Prism v6.0 (La Jolla, USA). An initial analysis to assess whether the data were of a Gaussian distribution was performed using the either D'Agostino-Pearson ($n<5$) or Kolmogorov–Smirnov ($n\geq5$) normality test. N denotes the experimental unit. Specifically, in human or mouse studies, n represents the number of individual patients or mice utilised for the study. In the *in vitro* studies, the n number denotes the number of times the experiment was replicated rather than the amount of experimental replicates in each experiment. The amount of experimental replicates in each experiment for the *in vivo*, *ex vivo* and *in vitro* studies detailed in this thesis are clarified appropriately in the methods section of each experimental chapter or the figure legends. Normally distributed data were analysed with t-test or analysis of variance (ANOVA) with post-hoc tests depending on the comparisons. Not normally distributed data were

analysed with the non-parametric Kruskal-Wallis test with Dunn's post hoc test. For statistical analysis, all percentage-presented data were initially changed to proportions and arcsine-transformed. The arcsine-transformed data were then analysed as described above. Unless otherwise stated, experiments were conducted a minimum of three times and were represented as the mean and standard error of the mean (SEM) with significance considered to be $p<0.05$ or greater.

# Chapter 3: Investigating AR expression and AR-dependent signalling in the human myometrium

## 3.1 Introduction

Premature initiation of myometrial contractions results in PTL and early delivery of the baby, which may cause neonatal neurodevelopmental and respiratory complications (Blencowe et al., 2012). Over the last decades, experts in the field of parturition research have been trying to deduce which molecular events within the myometrium lead to the preterm initiation of contractions with the aim of identifying new targets for development of effective tocolytic agents to prevent preterm contractions and, consequently PTL. It is now established that two separate but integrated pathways regulate the timing of contraction initiation; endocrine cascades, involving the fHPA axis and the placenta, and mechanical signals arising from uterine stretch in response to the growing fetus (Shynlova et al., 2009b; Smith et al., 2012). Both pathways regulate the expression of numerous contractile mediators within the myometrium, including CAPs (Aguilar and Mitchell, 2010). Once the contractile apparatus is activated and labour has been initiated, an effective tocolysis can be challenging. To overcome this challenge, it is important to understand the events that occur in the MSMCs before they commit to a contractile phenotype. Studies in rats have revealed that the MSMCs undergo phenotypic transitions during pregnancy, whereby they progress through five distinct phenotypic stages (Shynlova et al., 2009b). At the start of rat pregnancy, which lasts 21 days, the MSMCs proliferate and become hyperplastic to accommodate the growing fetus (Shynlova et al., 2006). Characteristic of the proliferative phase is the upregulation of genes that encode for proteins associated with cell growth such as PCNA and the anti-apoptotic factor Bcl-2 (Lye et al., 2001). Interestingly, these genes are upregulated in both gravid and non-gravid horns, suggesting endocrine rather than mechanical control. Consistent with this hypothesis, estrogen-regulated growth factors including IGF-1, EGF and the IGF binding proteins IGFBP1/3 are highly expressed in the proliferative MSMCs (Shynlova et al., 2007). Notably, IGF-1 is activator of the PI3K-Akt-mTOR pathway, which contributes to muscle growth (Glass, 2010). The expression of mTOR mRNA is induced in the proliferative MSMCs, where it has been shown to

mediate estrogen-induced hyperplasia; inhibition of mTOR at the proliferative stage inhibited proliferation (Jaffer et al., 2009). At day 14 of rat pregnancy, the MSMCs switch from a hyperplastic to a hypertrophic phenotype (Shynlova et al., 2009b). During the phase of the hypertrophic phenotype, the expression of anti-apoptotic and proliferative markers declines and genes associated with ECM synthesis and cellular hypertrophy are upregulated (Shynlova et al., 2006). These include collagenases, elastin (*ELN*), fibronectin (*FN1*) and *IGFBP6* (Shynlova et al., 2004; Shynlova et al., 2007). In contrast to the proliferative phase, the synthetic phase is regulated by both endocrine and mechanical signals. No hypertrophy was observed in non-gravid horns of pregnant rats (Lye et al., 2001) and exogenous administration of P4 promoted matrix synthesis, inhibited CAPs and upregulated *IGFBP6* (Shynlova et al., 2009b). On the other hand, P4 antagonism downregulated *IGFBP6* and blocked hypertrophy in gravid rat horns (Shynlova et al., 2004). On day 21 of pregnancy, the MSMCs enter a contractile phenotype in preparation for labour (Shynlova et al., 2009b). Expression of laminin β2, collagen IV and numerous other matrix proteins is induced and there is evidence that the fall in plasma P4 drives these events in rats (Shynlova et al., 2009a). A P4-independent 99-fold increase in *IGFBP2* is observed at this stage (Shynlova et al., 2007). A final phenotypic switch occurs at the time of labour when MSMCs become fully contractile. Characteristic of the "labour" phase is the induction of CAPs, including OXTR and con-43 (Ou et al., 1998). During this stage, the contribution of MSMCs in generation and control of uterine inflammation is critical: they actively synthesise pro-inflammatory cytokines IL-6 and IL-8 and chemokines, including CCL-2 (Esplin et al., 2005; Tattersall et al., 2008). P4 is thought to negatively regulate CCL-2 in the rat myometrium during pregnancy (Shynlova et al., 2008). P4 has also reported to upregulate the expression of ZEB-1, which has been recently identified as a CAPs suppressor in MSMCs (Renthal et al., 2013; Williams et al., 2012a). Studies in mice and humans showed that ZEB-1 was dramatically decreased during term and preterm labour, highlighting the potential importance of this factor in molecular events associated with initiation of labour (Renthal et al., 2010). At the end of labour, the MSMCs undergo apoptosis to return to the non-pregnant state (Shynlova et al., 2006). A number of metalloproteinases (MMPs) are induced in the postpartum rat myometrium, which is associated with

ECM degradation (Shynlova et al., 2009b). Notably, the expression of *IGFBP5*, which is a key player in apoptotic pathways of different cell types (Allan et al., 2004; Firth and Baxter, 2002; Ning et al., 2007), is also increased in the rat MSMCs that undergo postpartum involution (Shynlova et al., 2007).

Although the roles of estrogen and P4 signalling in the regulation of myometrial progression during pregnancy have been the subject of studies in several species, including humans, the role of androgen signalling is poorly explored. The AR has been detected in the human non-pregnant myometrium (Mertens et al., 2001). Immunohistochemical studies of the porcine uterus have showed that the AR staining was strong in the myometrium throughout most of the pregnancy but its expression was limited to the nuclei of few cells at the last day of pregnancy (Slomczynska et al., 2008). Recently, a study in rat myometrium during pregnancy has reported that the AR protein is highly expressed in the beginning of pregnancy where it is reportedly regulating proliferation of MSMCs but gradually declines thereafter (Liu et al., 2013). The authors of that study additionally showed that the AR-dependent signalling interacted with IGF-1 and EGF pathways and regulated proliferation-specific gene transcription. This is in line with studies in skeletal muscle where IGF-1 activation resulted in PI3K/Akt-mediated AR phosphorylation, which enhanced the AR activity (Kim and Lee, 2009a, b; Lee, 2009). Surprisingly, to our knowledge, there are no other studies that examine the expression of AR in the human myometrium during pregnancy and more evidence is required to elucidate the role of AR signalling in the progression of MSMCs through different phenotypes.

### 3.1.1 Specific aims

In this chapter, studies were designed to:

- Examine the expression of the human AR in the myometrium during term pregnancy
- Deduce whether AR expression is regulated by sex hormones, including E2, P4, DHT and T
- Explore the role of AR signalling in changes in MSMCs phenotype during pregnancy
- Investigate the effect of DHT treatment on the expression of AR-responsive genes in the human myometrium.

## 3.2 Materials and Methods

### 3.2.1 Cell lines

For the purposes of the experiments described in this chapter we used two myometrial cell lines; PHM1-41s derived from a single pregnant donor and UtSMCs derived from the myometrium of a single non-pregnant donor. Details about these cell lines can be found in Chapter 2 section 2.3.1. Cells were cultured as described in section 2.3.2. For the experiments of this chapter, PHM1-41s were used in passages (p) 15-20 and UtSMCs were used in p 5-10.

### 3.2.2 Cell culture

PHM1-41s ($2x10^5$ cells/well) and UtSMCs ($3x10^5$ cells/well) were seeded in 6-well plates in a volume of 2 ml of phenol-free DMEM (4.5 g/ L glucose) supplemented with 1% penicillin-streptomycin/L-Glutamine and 10% (v/v) FBS. The cells were left overnight at 37$^o$C in order to attach to the bottom of the well. The cells were then serum-starved for 24 hours in phenol-free DMEM (4.5 g/ L glucose) supplemented with 1% penicillin-streptomycin/L-Glutamine and 5% (v/v) charcoal stripped FBS.

E2, P4, DHT and T were purchased from Sigma, reconstituted in absolute ethanol (etOH) as per supplier's instructions and stock dilutions were stored in -20$^o$C in the dark. Details about these drugs can be found in **Table 2.13**. Working dilutions were prepared in phenol-free DMEM (4.5 g/ L glucose) supplemented with 1% penicillin-streptomycin/L-Glutamine and 5% (v/v) charcoal stripped FBS.

Following starvation, the medium was replaced with fresh 5% (v/v) charcoal stripped FBS DMEM. Treatments were applied in triplicate in 2 ml final volume/well for 8, 24 or 48 hours depending on the experimental design.

PHM1-41s (n=6) were incubated with E2, P4, DHT, T ($10^{-8}$M and $10^{-10}$M) or E2+P4 for 24 and 48 hours. The vehicle used for each time point was etOH.

In another experiment, PHM1-41s (n=6) and UtSMCs (n=6) were incubated with DHT ($10^{-8}$M and $10^{-10}$M) for 8 or 24 hours. The vehicle used for each time point was etOH.

### 3.2.3 Human tissue

Myometrial biopsies from TNL and TL women where obtained from the upper margin of the lower segment at the time of caesarean section as described in section 2.1.1. The biopsies were collected either in ice-cold RPMI medium for the purposes of tissue culture or in 10% NFB for histology processing. For RNA and protein extraction, the biopsies were snap-frozen immediately after collection.

### 3.2.4 Tissue culture

TNL biopsies (n=6) were washed in PBS and dissected into small explants. The explants were placed into 24-well plates in phenol red- and FBS-free RMPI medium supplemented with 1% penicillin-streptomycin/L-Glutamine and were left overnight to rest at 37$^{o}$C as described in section 2.3.3. The medium was removed and the explants were washed with PBS prior to be exposed to the treatments.

All treatments were prepared in phenol red- and FBS-free RPMI medium supplemented with 1% penicillin-streptomycin/L-Glutamine. DHT ($10^{-8}$M) was applied in triplicate in a volume of 1 ml/ well for 6 or 24 hours. An untreated control and a vehicle (etOH) were included.

Following each time point, the medium was removed and the explants were rinsed in PBS and immediately transferred to 2 ml Eppendorf tubes on dry ice. The samples were stored at -80$^{o}$C.

### 3.2.5 RNA extraction and cDNA synthesis

RNA was extracted from

- Cultured UtSMCs and PHM1-41s
- Frozen biopsies collected from TNL and TL myometrium
- Myometrial explants

***Cultured PHM1-41s and UtSMCs***

Following incubation, the medium was removed and the cells were rinsed with PBS and RNA was extracted using the RNeasy®Mini Kit as described in section 2.4.1. The concentration and purity of extracted RNA, was assessed with Nanodrop 1000 spectrophotometer as described in section 2.4.4.

The cDNA was synthesised (10 ng RNA/µl) using the High Capacity cDNA Synthesis Kit as described in section 2.5.1.

***Frozen biopsies collected from TNL and TL myometrium***

The samples were thawed on ice, homogenised in TRI reagent using a Retsch TissueLyser and processed in PLG tubes as described in section 2.4.2. Briefly, the aqueous phase in PLG tubes, which contained the RNA, was collected in pre-chilled 1.5 ml Eppendorf tubes. The samples were then transferred onto RNeasy Mini Spin Columns and the RNA was extracted using the RNeasy®Mini Kit. The concentration and purity of RNA was determined with Nanodrop 1000 spectrophotometer as described in section 2.4.4. The cDNA was synthesised (30 ng RNA/µl) using the High Capacity cDNA Synthesis Kit as described in section 2.5.1.

***Myometrial explants***

The samples were thawed on ice, homogenised in TRI reagent using a Retsch TissueLyser and processed in PLG tubes as described in section 2.4.2. The aqueous phase in the PLG tubes, which contained the RNA, was collected in pre-chilled 1.5 ml Eppendorf tubes. The samples where then transferred onto RNeasy Mini Spin Columns and the RNA was extracted using the RNeasy®Mini Kit. The concentration and purity of RNA was determined with Nanodrop 1000 spectrophotometer as described in section 2.4.4 and the integrity of RNA was assessed with Agilent 2100 BioAnalyzer as described in section 2.4.5.

The RNA was used for cDNA synthesis, which was used for either a PCR array or a qRT-PCR for the validation of the array.

The $RT^2$ First Strand Kit was used to prepare the cDNA for the PCR array as described in section 2.5.2.

The High Capacity cDNA Synthesis Kit was used to prepare the cDNA (30 ng RNA/µl) for the array validation as described in section 2.5.1.

### 3.2.6 QRT-PCR

QRT-PCR was performed to quantify transcript levels of genes of interest in

- Cultured UtSMCs and PHM1-41s
- Frozen biopsies collected from TNL and TL myometrium
- Myometrial explants (validation of the array)

***Cultured UtSMCs and PHM1-41s***

Taqman singleplex qRT-PCR was performed as described in detail in section 2.6.2. Briefly, a mastermix containing either the primer/probe mix for the gene of interest (GOI) or the primer/probe for the housekeeping gene 18S was prepared and 1 µl of cDNA was added to each reaction. All reactions were performed in technical duplicate and the reaction was carried using the 7900HT Fast Real-Time PCR System. Data was analysed using the ΔΔCt method: the thresholds for GOI and 18S were set in the linear phase of the exponential region of the amplification curves. The cycle number at which the PCR signal crossed a set threshold was used to determine relative gene expression. These comparative Ct values were extracted to Excel sheets and the average Ct for GOI and 18S was calculated initially from the technical replicates and then experimental replicates. The average 18S Ct value was subtracted from the GOI to calculate ΔCt and the number was normalised to the control tissue sample (ΔΔCt). The fold change was calculated using the formula 2^(-ΔΔCt). ΔΔCt values were used for statistical analysis and data was plotted as fold change (2^(-ΔΔCt)) compared to the relevant control sample. Pre-validated primer/probe assays were purchased from Applied Biosystems and are listed in **Table 3.1.**

**Table 3.1:** Taqman primer/probe assay list

| Gene | Assay ID |
|---|---|
| *PCNA* | Hs00427214_g1 |
| *BCL2* | Hs00608023_m1 |
| *IGF1* | Hs01547656_m1 |
| *IGFBP1* | Hs00236877_m1 |
| *IGFBP3* | Hs00365742_g1 |
| *MTOR* | Hs00234508_m1 |
| *IGFBP6* | Hs00181853_m1 |
| *ELN* | Hs00355783_m1 |
| *CON43* | Hs00748445_s1 |
| *FN1* | Hs00365052_m1 |
| *OXTR* | Hs00168573_m1 |
| *IGFBP2* | Hs01040719_m1 |
| *CCL2* | Hs00234140_m1 |
| *IGFBP5* | Hs00181213_m1 |
| *MMP2* | Hs01548727_m1 |
| *MMP9* | Hs00234579_m1 |
| *ZEB1* | Hs03680602_m1 |
| *IL6* | Hs02621719_u1 |
| *IL8* | Hs01553824_g1 |
| *AR* | Hs04260217_m1 |

***Frozen biopsies collected from TNL and TL myometrium***

A standard curve qRT-PCR method was conducted to quantify transcript levels of genes of interest in TNL and TL myometrium as described in detail in section 2.6.3. Briefly, a 'reference' cDNA sample (cDNA pool), consisting of equal volumes of all the experimental samples, was created and a standard curve for each GOI was obtained using several dilutions of the pooled cDNA (1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512). The cDNA of each experimental sample was diluted 1:20 in $dH_2O$; 2 µl of the pooled cDNA or the experimental sample cDNA was added to the qRT-PCR reaction mixture, which contained the primer/probe assay for the GOI. RT-PCR was performed on both the experimental samples and "reference" standards. The aim was to have the diluted samples in the linear part of the standard curve in order to achieve accurate comparison of relative values. All qRT-PCR reactions were performed in triplicate (standard curves) or duplicates (samples). Standard curve qRT-PCR was carried out for the housekeeping gene 18S under the same conditions to enable comparison between samples. The reaction was carried out using the 7900HT Fast Real-Time PCR System. The expression levels were calculated from a linear regression of the standard curve as GOI expression compared to 18S expression. Acceptable standard curves had a slope between -3.3 and -3.7

($R^2 > 0.99$). Primer/probe assays were purchased from Applied Biosystems and are listed in **Table 3.2.**

**Table 3.2:** Taqman primer/probe assay list

| Gene | Assay ID |
|---|---|
| *PTGS2* | Hs00153133_m1 |
| *AR* | Hs04260217_m1 |

***Myometrial explants***

Taqman singleplex qRT-PCR using the ΔΔCt method was used to validate the changes observed with the PCR array. The method was similar to the method described above for the qRT-PCR for cultured cells. All experimental replicates were included in the validation (n=6). Primer probes were purchased from Applied Biosystems and are listed in **Table 3.3.**

**Table 3.3:** Taqman primer/probe assay list

| Gene | Assay ID |
|---|---|
| *VAPA* | Hs00427749_m1 |
| *AR* | Hs04260217_m1 |
| *PAK1IP1* | Hs00215515_m1 |

### 3.2.7 PCR array

The $RT^2$ Profiler PCR Array [384-Well (4 x 96) Format] for Human Androgen Receptor Signalling Targets was used to examine the effect of DHT treatment ($10^{-8}$M) on the expression of androgen-responsive genes in the human myometrium at term. The array was performed on 4 experimental replicates (n=4). The vehicle but not the untreated control was included. The method is described in detail in section 2.6.4. Briefly, cDNA and PCR mastermix were prepared according to the company's instructions and a mixture of cDNA/mastermix was added into each well. The reactions were run on the 7900HT Fast Real-Time PCR System with SDS. Ct values were calculated and exported to an Excel sheet for use with the SABiosciences PCR Array Web Data Analysis Software. The software automatically performed quantification using the ΔΔCt method and compared the treatments to the vehicle. The results were presented in tables indicating fold regulation and p values for each gene.

### 3.2.8 Double immunofluorescence

The immunolocalisation of AR in the TNL and TL myometrial biopsies was investigated using a double immunofluorescence protocol for AR and the smooth muscle cytoplasmic marker α-SMA. The TNL and TL biopsies were fixed and processed for histology as described in section 2.10.1. Double immunofluorescent staining was performed as described in detail in section 2.10.3. Briefly, the slides were dewaxed, rehydrated, antigen retrieved and incubated in 3% (v/v) $H_2O_2$ in methanol as described in section 2.10.2. The slides were then blocked with NSS and incubated with the first primary rabbit anti-AR antibody overnight at 4$^{o}$C. Following washes with TBST (1 x 5 minutes) and TBS (2 x 5 minutes), the slides were incubated with the secondary anti-rabbit antibody for 1 hour at room temperature. Following washes with TBST and TBS, the slides were incubated for 10 minutes with Red Tyramide (Cy3) and microwaved in 0.01 M citrate buffer (pH 6.0) for 4 minutes in full power until the buffer boiled and then for additional 2.5 minutes. The slides were then blocked with NRS for 30 minutes at room temperature and incubated overnight at 4$^{o}$C with mouse anti-α-SMA antibody. The slides were washed and incubated with rabbit anti-mouse Peroxidase IgG2a antibody for 1 hour in room temperature prior to be incubated for 10 minutes with Green Tyramide. Finally the slides were counterstained with Dapi, washed and mounted with Permafluor. **Table 3.4** shows the details of the primary and secondary antibodies used in this protocol. The slides were visualised on a Zeiss LSM 710 confocal microscope and images were obtained using the ZEN2009 software.

**Table 3.4:** Antibodies used for the double immunofluorescence protocol

| Antibody | Dilution | Supplier |
|---|---|---|
| Primary 1: Monoclonal rabbit anti-AR (N-20) | 1:1000 | Santa Cruz (Sc 816) |
| Primary 2: Monoclonal mouse anti-α-SMA | 1:10000 | Sigma (A2547) |
| Swine anti-rabbit peroxidase | 1:500 | DAKO (P0399) |
| Rabbit anti-mouse peroxide | 1:200 | Zymed/ Cambridge Bioscience (61-0220) |

### 3.2.9 Western Immunoblotting

Protein was extracted from frozen TNL and TL biopsies as described in detail in section 2.7. Briefly, the tissue was homogenised in RIPA buffer using a TissueLyser. The lysates were then centrifuged to pellet the cell debris and the supernatants, which contained the protein, were collected and stored at -80$^{o}$C. Protein concentration was determined with the DC Protein Assay Kit as described in section 2.7.1.

Western gel analysis was used to quantify and compare the expression of total AR protein between TNL and TL myometrial samples as described in detail in section 2.8.1. Briefly, 20 µg of protein per sample was resolved on 10% Bis-Tris precast NuPAGE gel (180 V for 1 hour) on ice in MOPS running buffer. The protein was transferred using a wet-transfer system (100 V for 90 minutes) to an Immobilon-FL PVDF membrane. The membrane was blocked with OBB and incubated overnight at 4$^{o}$C with primary antibodies (**Table 3.5**) diluted in OBB. Following washes with TBST (1 x 5 minutes) and TBS (3 x 5 minutes) the membrane was incubated with secondary LICOR antibodies (**Table 3.5**) diluted in OBB for 1 hour at room temperature in darkness. The membrane was washed in TBS and TBST and scanned using the LI-COR Odyssey Infrared Imaging System. The bands were quantified using the Odyssey analysis software and the raw values were exported to Excel sheets where the AR values were calculated relative to the loading control α-SMA values.

**Table 3.5:** Antibodies used for Western immunoblotting

| Antibody | Dilution | Supplier |
|---|---|---|
| Primary 1: Monoclonal rabbit anti-AR | 1:200 | Spring Bioscience (SP107) |
| Primary 2: Monoclonal mouse anti-α-SMA | 1:10000 | Sigma (A2547) |
| Secondary 1: Polyclonal donkey anti-rabbit 800CW | 1: 10000 | LI-COR Biosciences (926-32213) |
| Secondary 2: Polyclonal donkey anti-mouse 680RD | 1:10000 | LI-COR Biosciences (926-68072) |

### 3.2.10 Statistical analysis

The qRT-PCR and relative protein quantification data were tested for normality using the Kolmogorov–Smirnov test. Normally distributed data were analysed using two-tailed unpaired t-test. Not normally distributed data were analysed with Kruskal-Wallis with Dunn's post-hoc test. Unless otherwise stated all data is represented as the mean and standard error of the mean (SEM) with significance considered to be $p<0.05$.

## 3.3 Results

### 3.3.1 The expression of the human AR in the myometrium at term pregnancy

The total concentration of *AR* mRNA was investigated in the human TNL and TL myometrium with qRT-PCR standard curve method (**Figure 3.1**). The *AR* mRNA was significantly decreased with labour (p=0.006; **Figure 3.1 A**). PTGS2 was used as a positive control; the concentration of *PTGS2* mRNA was significantly increased with labour (p=0.024; **Figure 3.1 B**).

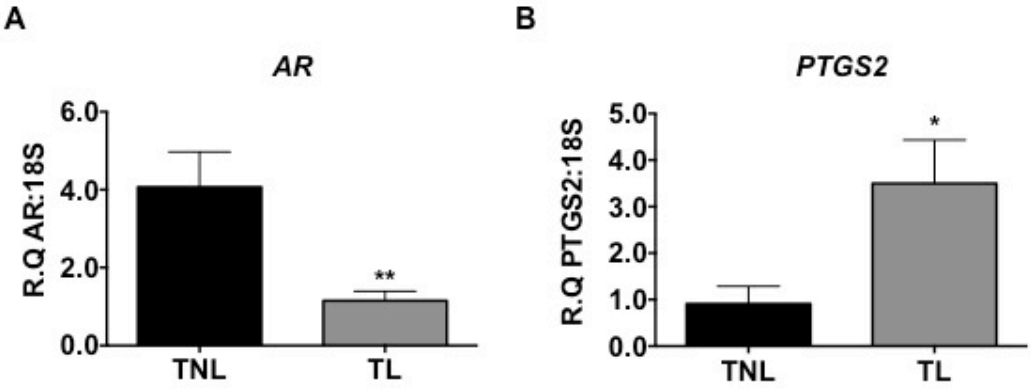



**Figure 3.1:** The expression of AR and PTGS2 mRNAs in the TL (n=7) and TNL (n=7) human myometrium. (**A**) AR mRNA was significantly downregulated in TL myometrium **p=0.006. (**B**) PTGS2 mRNA was significantly upregulated in TL samples *p=0.024. Data were analysed using a two-tailed unpaired t-test.

The localisation of AR was explored in the TNL and TL myometrium with double immunofluorescent staining; the slides were double-stained for AR and α-SMA (cytoplasmic SMC marker). The AR protein was clearly localised to the nuclei of multiple MSMCs in fixed tissue sections of both TNL (**Figure 3.2**, **Figure 3.3**) and TL (**Figure 3.4**, **Figure 3.5**) myometrium.

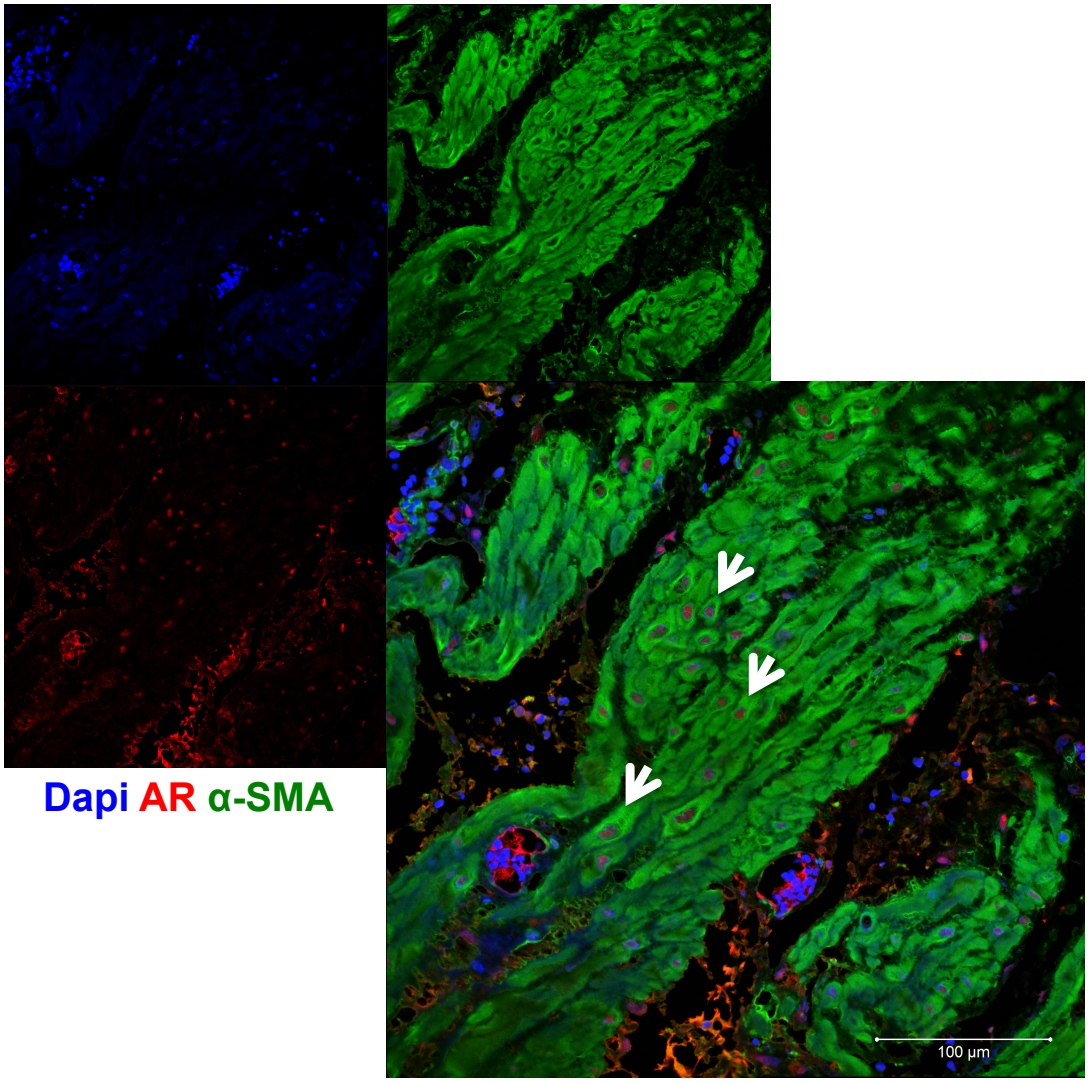



**Figure 3.2:** The localisation of AR in the human TNL myometrium. Immunofluorescent double staining for AR and α-SMA in one representative (n=5) TNL myometrial sample. White arrows indicate AR/α-SMA double positive staining in MSMCs. The AR was localised to the nuclei of MSMCs. AR=red, α-SMA=green, counterstain (Dapi)=blue. Scale bar=100 µm.

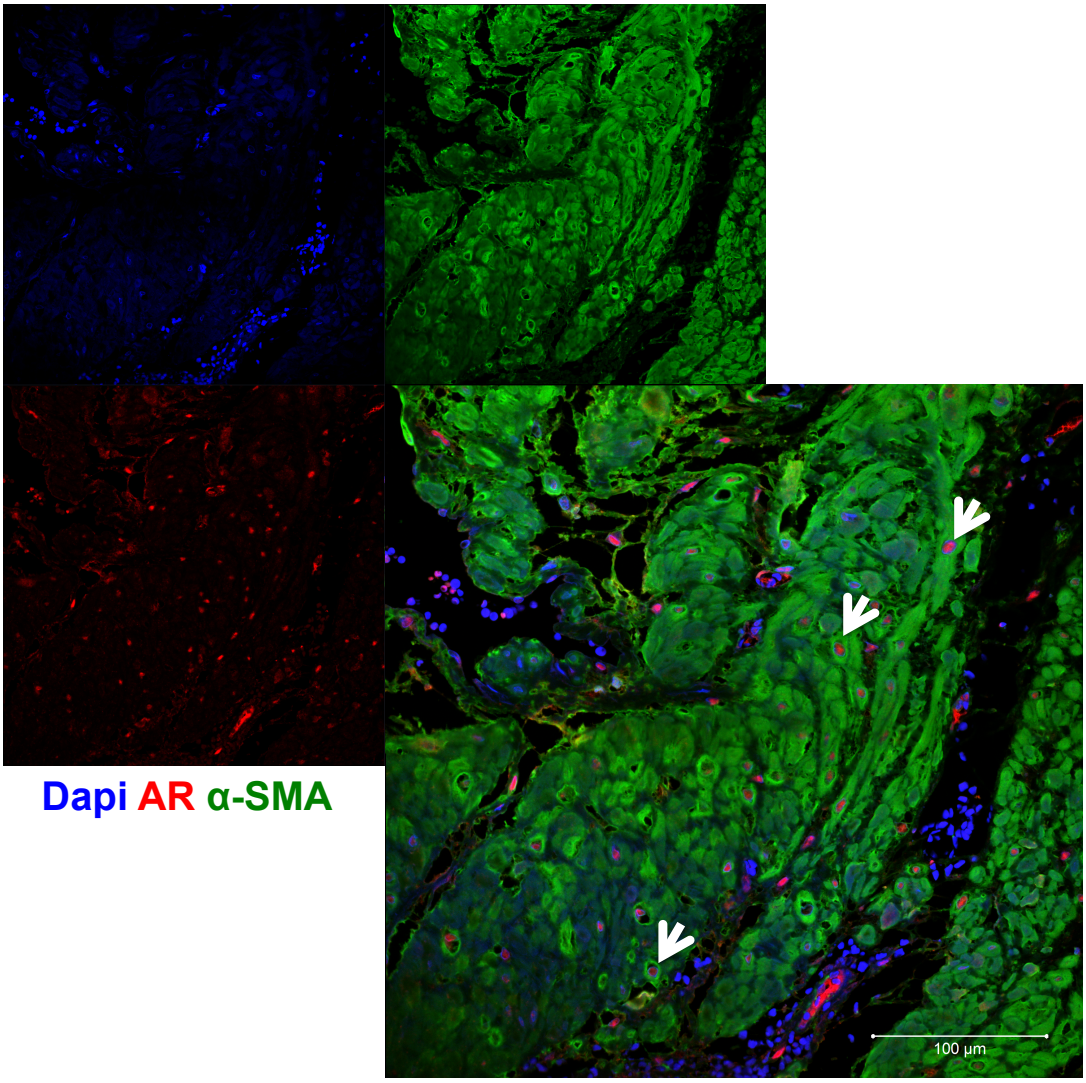



**Figure 3.3:** The localisation of AR in the human TNL myometrium. Immunofluorescent double staining for AR and α-SMA in a second representative (n=5) TNL myometrial sample. White arrows indicate AR/α-SMA double positive staining in MSMCs. The AR was localised to the nuclei of MSMCs. AR=red, α-SMA=green, counterstain (Dapi)=blue. Scale bar=100 µm.

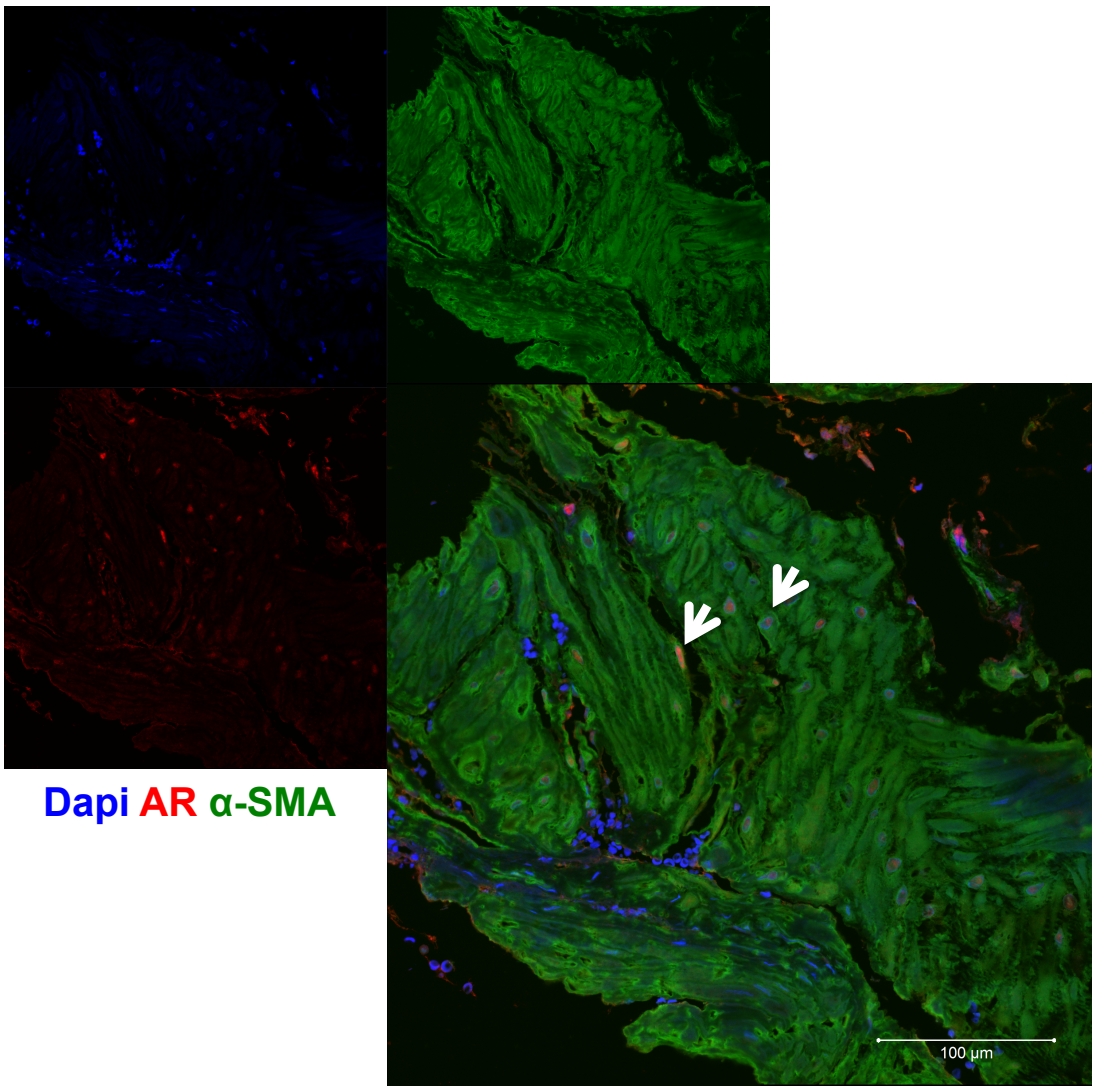



**Figure 3.4:** The localisation of AR in the human TL myometrium. Immunofluorescent double staining for AR and α-SMA in one representative (n=5) TL myometrial sample. White arrows indicate AR/α-SMA double positive staining in MSMCs. The AR was localised to the nuclei of MSMCs. AR=red, α-SMA=green, counterstain=blue. Scale bar=100 µm.

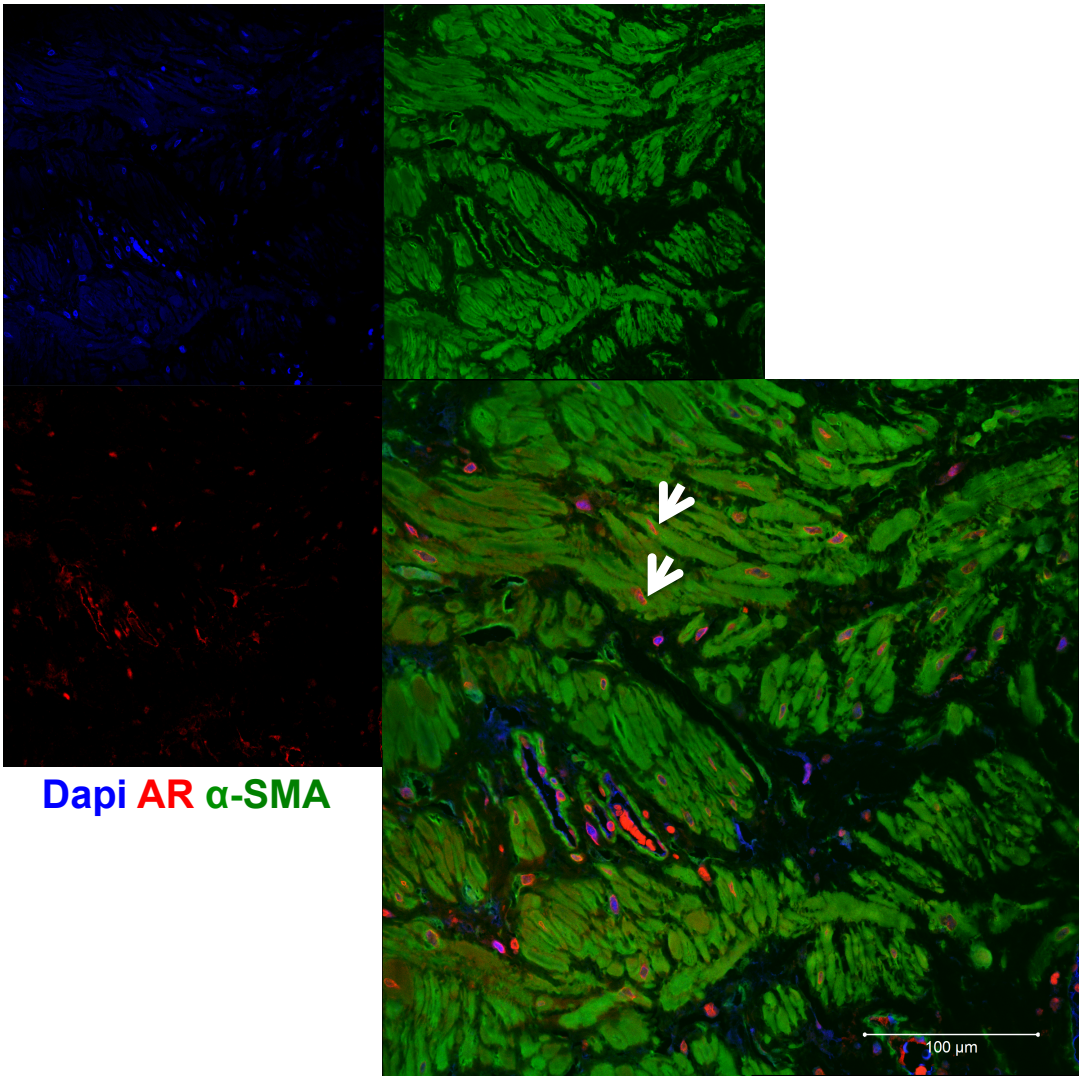



**Figure 3.5a:** The localisation of AR in the human TL myometrium. Immunofluorescent double staining for AR and α-SMA in a second representative (n=5) TL myometrial sample. White arrows indicate AR/α-SMA double positive staining in MSMCs. The AR was localised to the nuclei of MSMCs. AR=red, α-SMA=green, counterstain (Dapi)=blue. Scale bar=100 µm.

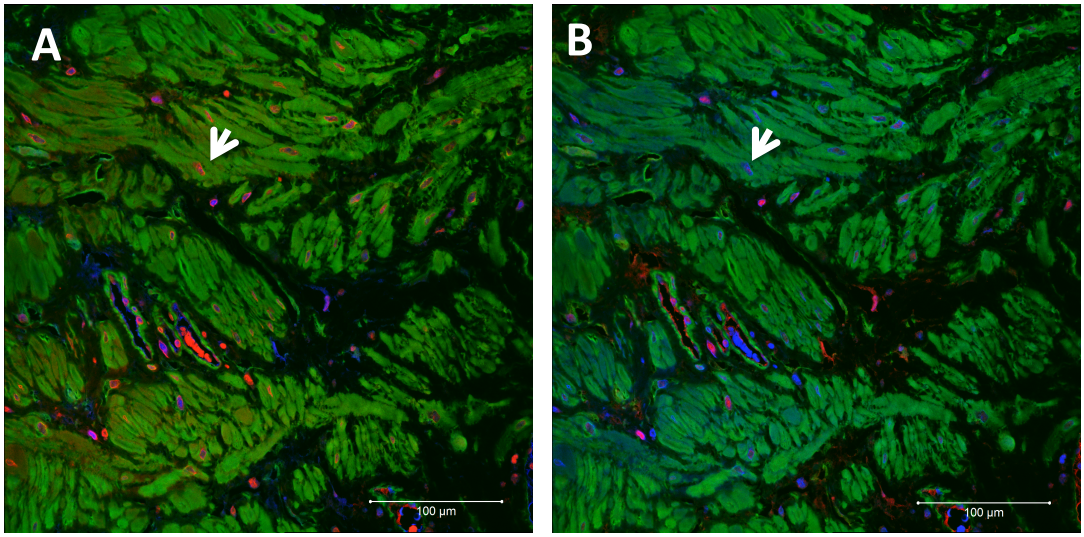



**Figure 3.5b:** The specificity of AR antibody. (**A**) Positive AR staining is localised to the nuclei of α-SMA positive cells in the TL human myometrium (**B**) Negative control shows the absence of AR positive staining in α-SMA positive cells. White arrow in A points a positive MSMC for AR cell and white arrow in B points the same nuclei in the negative control to indicate AR antibody specificity. AR=red, α-SMA=green, counterstain (Dapi)=blue. Scale bar=100 µm.

The total tissue content of AR protein was quantified in the TNL and TL myometrium using Western immunoblotting. In contrast to the mRNA results, no significant difference in AR protein concentration was observed between the two groups, although there was a trend towards decrease in the TL group (**Figure 3.6**).

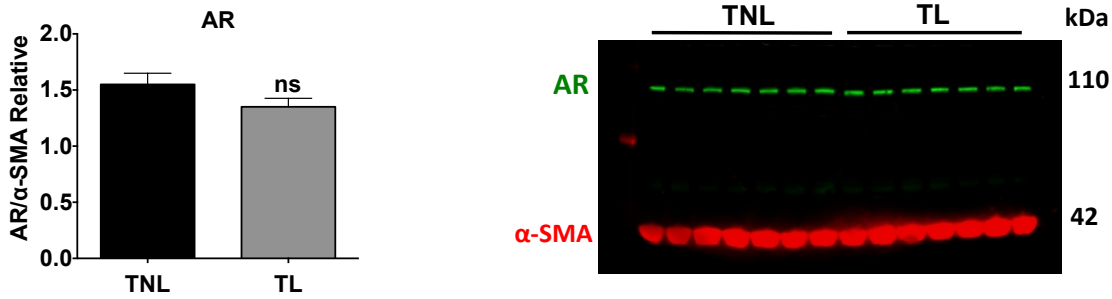



**Figure 3.6:** The expression of AR protein in the TNL and TL human myometrium. Western immunoblotting using a monoclonal anti-AR antibody showed that there was no significant difference in the content of AR protein between the two groups (TNL: n=7, TL: n=7). Data were analysed using a two-tailed unpaired t-test.

### 3.3.2 The regulation of AR mRNA expression by sex hormones in human myometrium at term pregnancy

To explore the hypothesis that the expression of AR mRNA is regulated by sex hormones in the human MSMCs, we incubated PHM1-41s with a low ($10^{-10}$M) and a high ($10^{-8}$M) dose of E2, P4, DHT, T and E2+P4 together (at $10^{-8}$M each) for 24 and 48 hours. We found that *AR* was 2.3 fold (p=0.024) upregulated in PHM1-41s following 24-hour incubation with the high dose of DHT (**Figure 3.7 A**). After 48 hours, the combined E2+P4 treatment induced a 2.5 fold (p=0.009) and a 1.8 fold (p=0.045) increase in *AR* mRNA at the highest and lowest dose respectively (**Figure 3.7 B**)

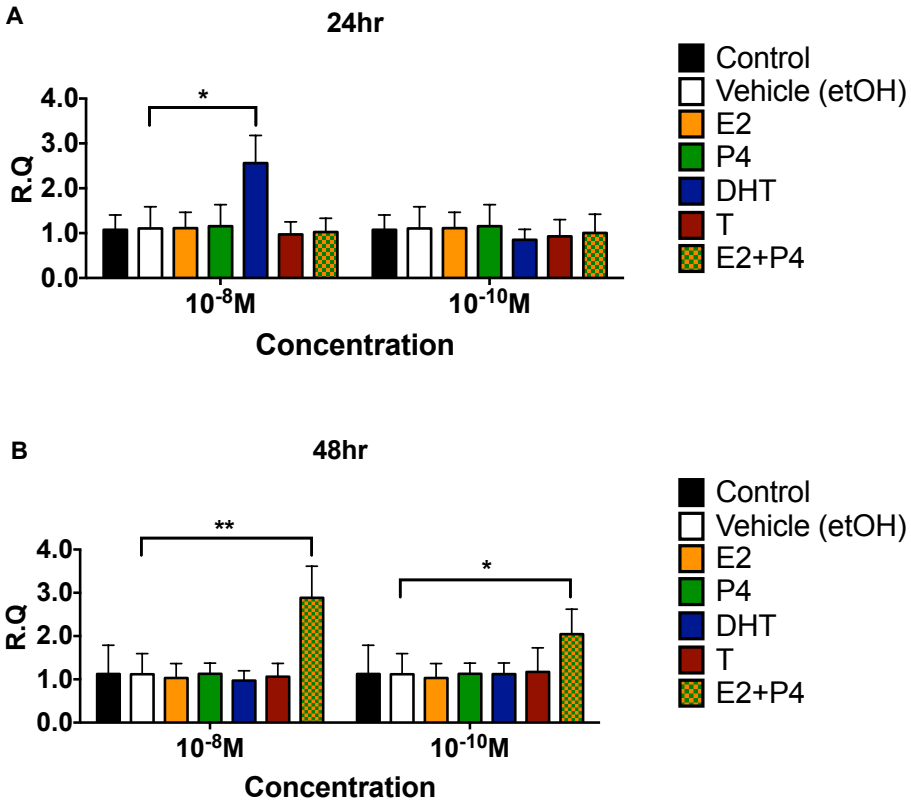



**Figure 3.7:** Sex steroid regulation of AR mRNA expression in PHM1-41s. PHM1-41s were incubated for 24 (**A**) or 48 (**B**) hrs with vehicle, E2, P4, DHT, T or E2+P4 ($10^{-8}$M or $10^{-10}$M) and the expression of AR mRNA was quantified with qRT-PCR. All treatments were applied in triplicate. (**A**) *AR* expression was significantly upregulated in PHM1-41s following 24 hr incubation with DHT ($10^{-8}$M) when compared to the vehicle (*p=0.024, n=6). (**B**) The expression of *AR* in PHM1-41s was significantly upregulated following 48-hr incubation with E2+P4 treatment compared to the vehicle (*p=0.045, **p=0.009, n=6). Data were analysed using Kruskal–Wallis with Dunn's post-hoc test.

### 3.3.3 The role of AR signalling on the myometrial phenotypic transition during pregnancy

We explored the hypothesis that AR-dependent signalling is involved in the regulation of the expression of genes associated with different myometrial phenotypes during pregnancy. PHM1-41s and UtSMCs were incubated with DHT ($10^{-8}$M or $10^{-10}$M) for 8 or 24 hours. All treatments were applied in triplicate. The expression of genes that have key roles in the regulation of the proliferative, synthetic, contractile/labour and postpartum phenotype of MSMCs during pregnancy were quantified with qRT-PCR.

Proliferative phenotype (**Figures 3.8-3.11**)

Following 8-hour incubation with DHT, the expression of the genes *PCNA*, *BCL2, IGFBP1*, *IGF1*, *IGFBP3* and *mTOR* did not change in PHM1-41s (**Figure 3.8**) and UtSMCs (**Figure 3.9**). Similar responses occurred at the 24-hour time point in PHM1-41s (**Figure 3.10**) and UtSMCs (**Figure 3.11**).

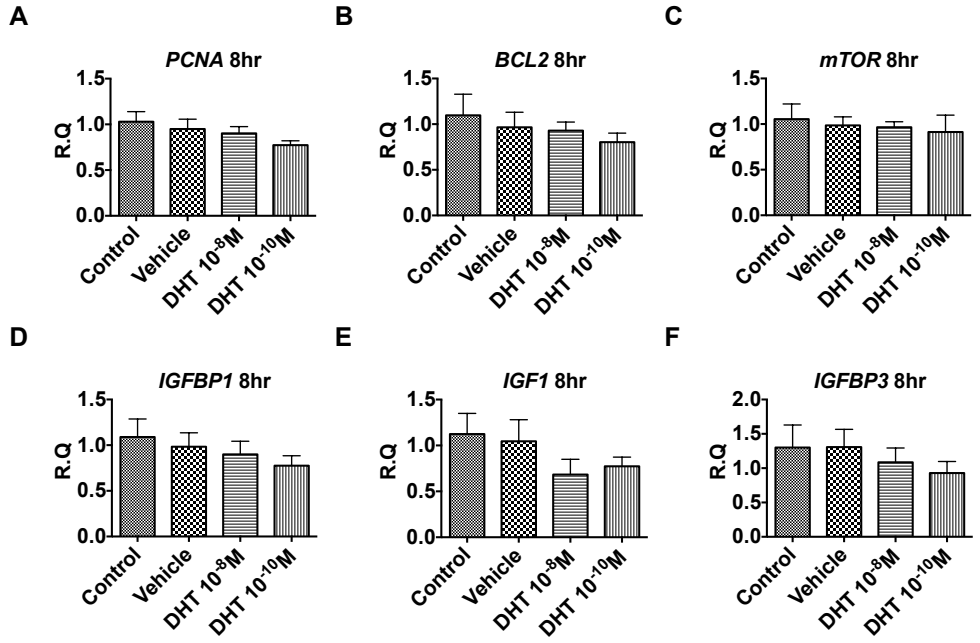



**Figure 3.8:** The effect of 8 hr DHT treatment on the expression of genes associated with the proliferative phenotype of MSMCs in PHM1-41s. PHM1-41s were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 8 hrs and the mRNA of *PCNA* (**A**), *BCL2* (**B**), *mTOR* (**C**), *IGFBP1* (**D**), *IGF1* (**E**) and *IGFBP3* (**F**) was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

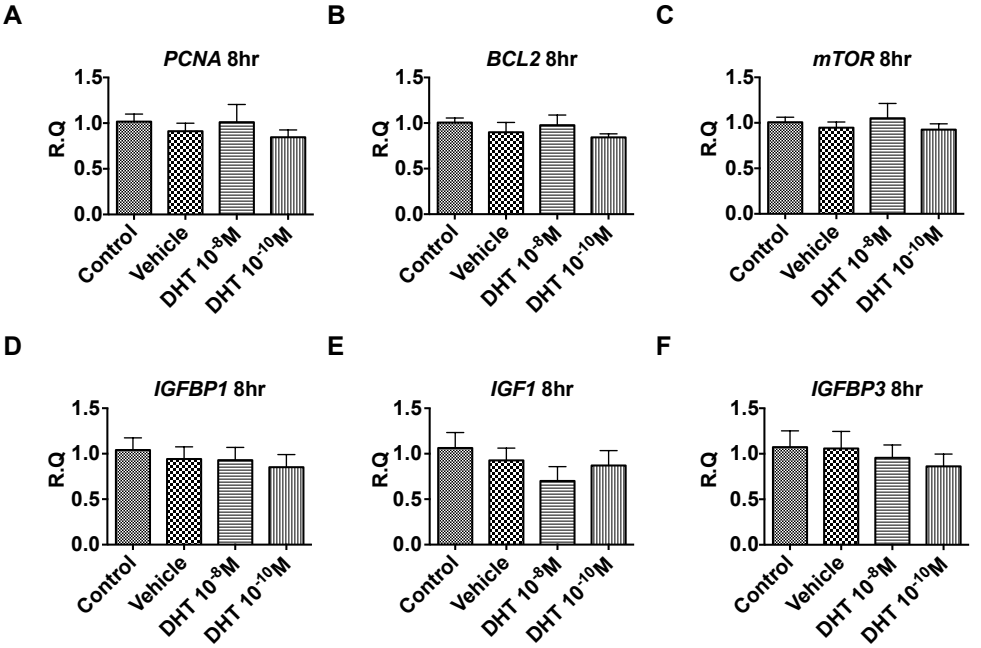



**Figure 3.9:** The effect of 8 hr DHT treatment on the expression of genes associated with the proliferative phenotype of MSMCs in UtSMCs. UtSMCs were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 8 hrs and the mRNA of *PCNA* (**A**), *BCL2* (**B**), *mTOR* (**C**), *IGFBP1* (**D**), *IGF1* (**E**) and *IGFBP3* (**F**) was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

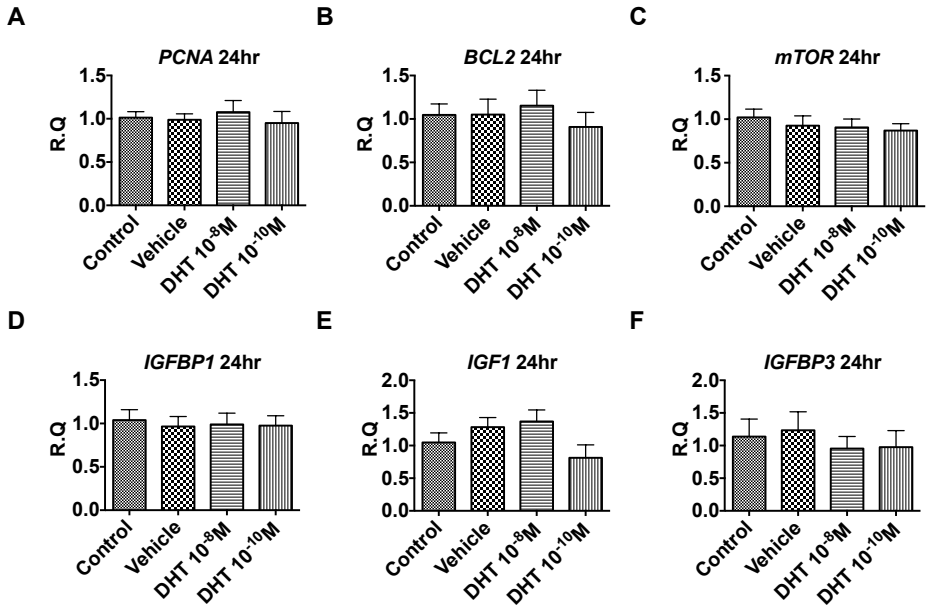



**Figure 3.10:** The effect of 24 hr DHT treatment on the expression of genes associated with the proliferative phenotype of MSMCs in PHM1-41s. PHM1-41s were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 24 hrs and the mRNA of *PCNA* (**A**), *BCL2* (**B**), *mTOR* (**C**), *IGFBP1* (**D**), *IGF1* (**E**) and *IGFBP3* (**F**) was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

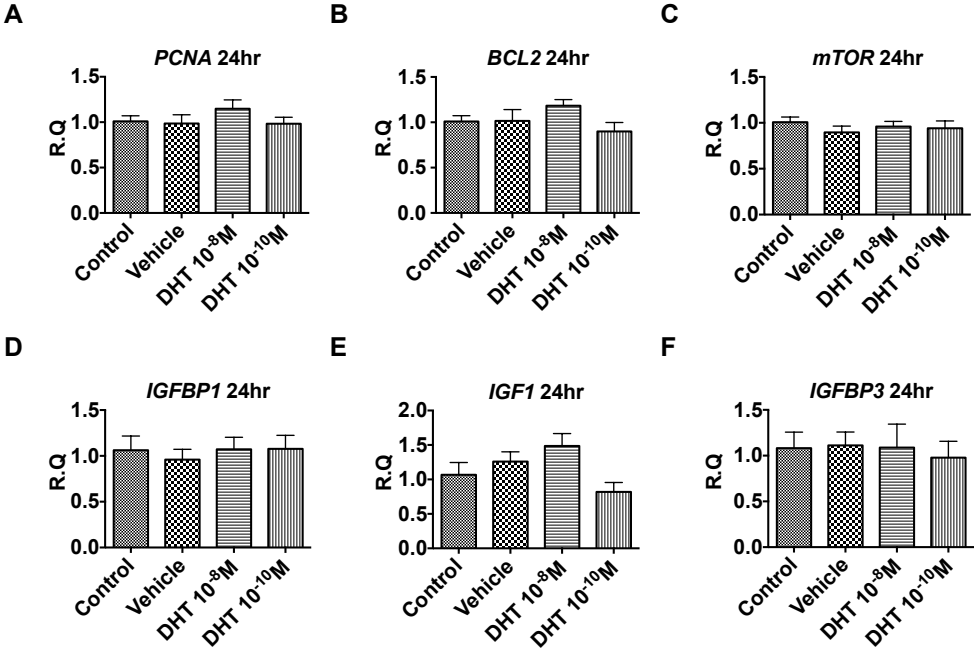



**Figure 3.11:** The effect of 24 hr DHT treatment on the expression of genes associated with the proliferative phenotype of MSMCs in UtSMCs. UtSMCs were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 24 hrs and the mRNA of *PCNA* (**A**), *BCL2* (**B**), *mTOR* (**C**), *IGFBP1* (**D**), *IGF1* (**E**) and *IGFBP3* (**F**) was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

Synthetic phenotype (**Figures 3.12-3.15**)

The expression of the genes *IGFBP6*, *FN1* and *ELN* did not change following 8-hour incubation of PHM1-41s (**Figure 3.12**) and UtSMCs (**Figure 3.13**) with DHT ($10^{-8}$M or $10^{-10}$M). Similar responses were observed at the 24-hour time point in PHM1-41s (**Figure 3.14**) and UtSMCs (**Figure 3.15**).

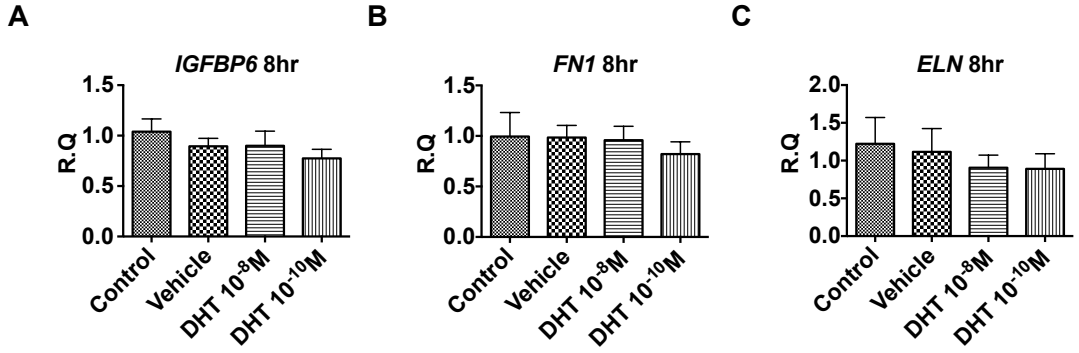



**Figure 3.12:** The effect of 8 hr DHT treatment on the expression of genes associated with the synthetic phenotype of MSMCs in PHM1-41s. PHM1-41s were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 8 hrs and the mRNA of *IGFBP6* (**A**), *FN1* (**B**) and *ELN* (**C**) was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

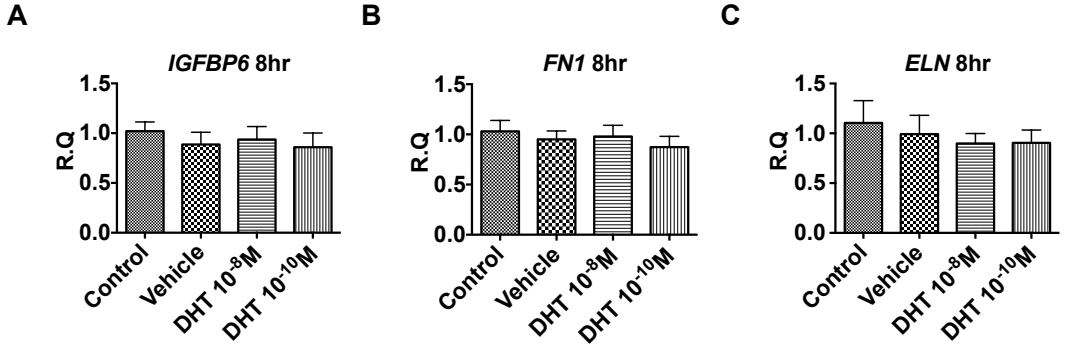



**Figure 3.13:** The effect of 8 hr DHT treatment on the expression of genes associated with the synthetic phenotype of MSMCs in UtSMCs. UtSMCs were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 8 hrs and the mRNA of *IGFBP6* (**A**), *FN1* (**B**) and *ELN* (**C**) was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

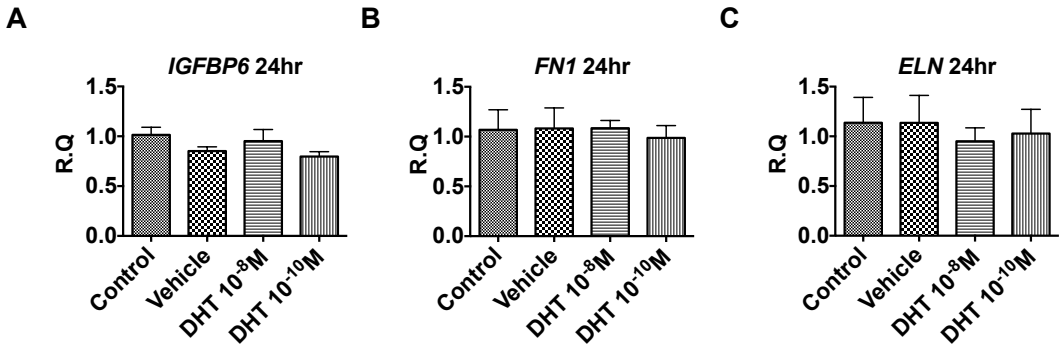



**Figure 3.14:** The effect of 24 hr DHT treatment on the expression of genes associated with the synthetic phenotype of MSMCs in PHM1-41s. PHM1-41s were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 24 hrs and the mRNA of *IGFBP6* (**A**), *FN1* (**B**) and *ELN* (**C**) was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

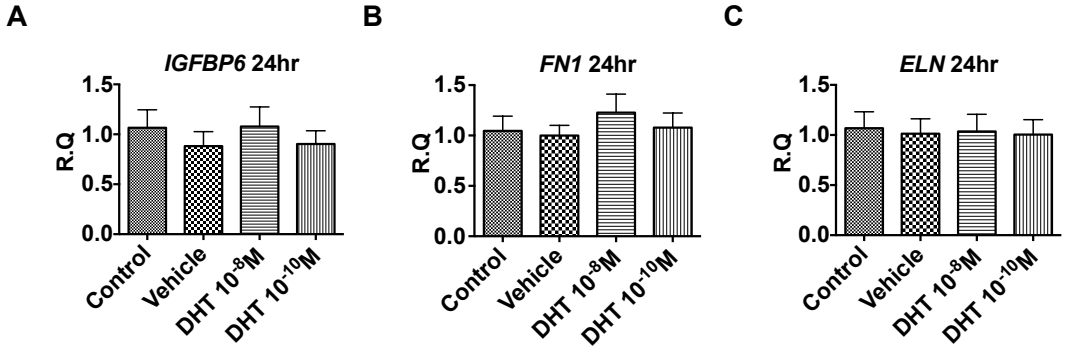



**Figure 3.15:** The effect of 24 hr DHT treatment on the expression of genes associated with the synthetic phenotype of MSMCs in UtSMCs. UtSMCs were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 24 hrs and the mRNA of *IGFBP6* (**A**), *FN1* (**B**) and *ELN* (**C**) was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

Contractile/labour phenotype (**Figures 3.16-3.19**)

Following 8-hour incubation with DHT ($10^{-8}$M or $10^{-10}$M), the expression of the genes *IGFBP2*, *IL6*, *IL8*, *CCL2, ZEB1* and *OXTR* did not change in PHM1-41s (**Figure 3.16**) and UtSMCs (**Figure 3.17**). In addition, no changes were observed in the expression of these genes at the 24-hour time point in PHM1-41s (**Figure 3.18**) and UtSMCs (**Figure 3.19**).

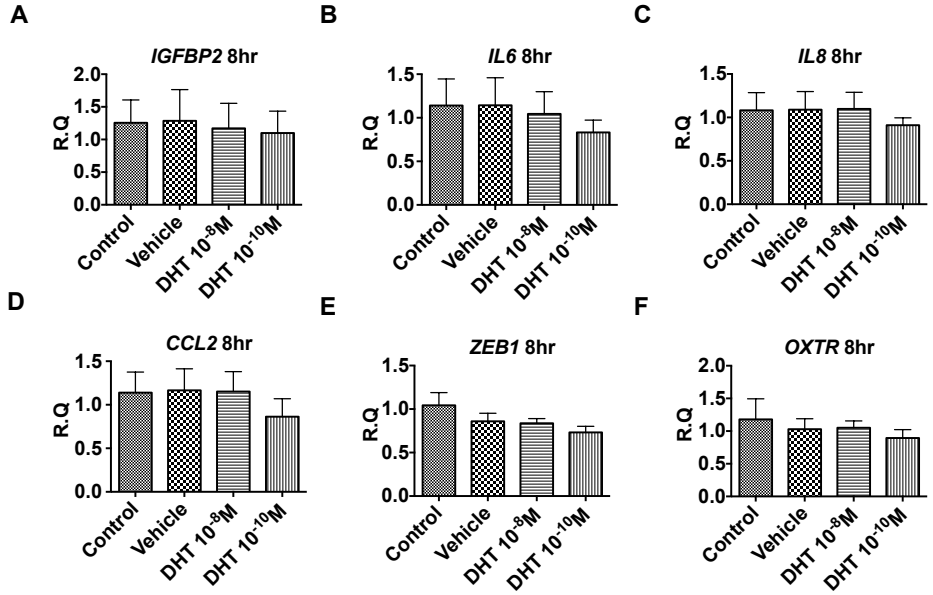



**Figure 3.16:** The effect of 8 hr DHT treatment on the expression of genes associated with the contractile/labour phenotype of MSMCs in PHM1-41s. PHM1-41s were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 8 hrs and the mRNA of *IGFBP2* (**A**), *IL6* (**B**), *IL8* (**C**), *CCL2* (**D**), *ZEB1* (**E**) and *OXTR* (**F**) was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

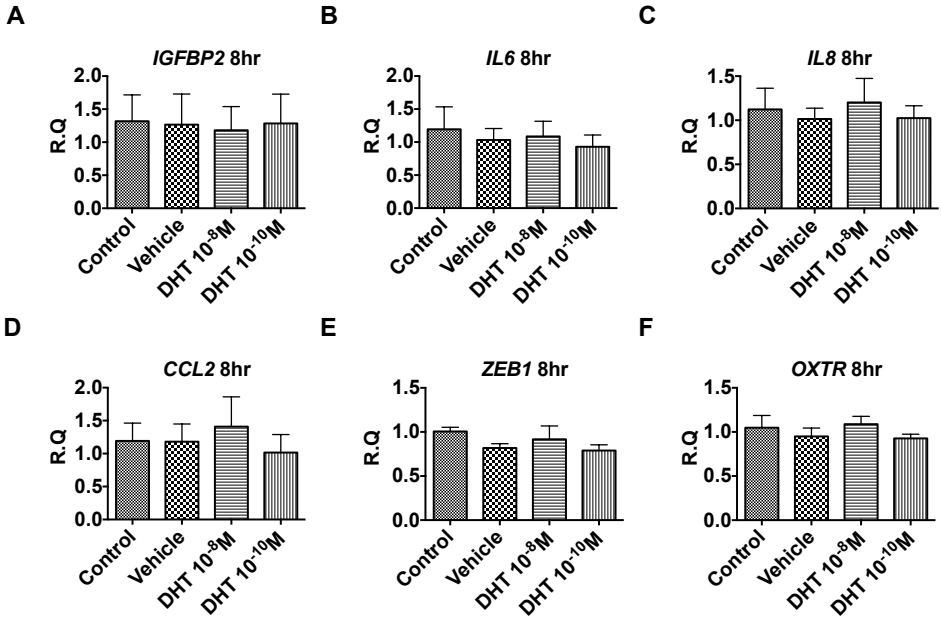



**Figure 3.17:** The effect of 8 hr DHT treatment on the expression of genes associated with the contractile/labour phenotype of MSMCs in UtSMCs. UtSMCs were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 8 hrs and the mRNA of *IGFBP2* (**A**), *IL6* (**B**), *IL8* (**C**), *CCL2* (**D**), *ZEB1* (**E**) and *OXTR* (**F**) was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

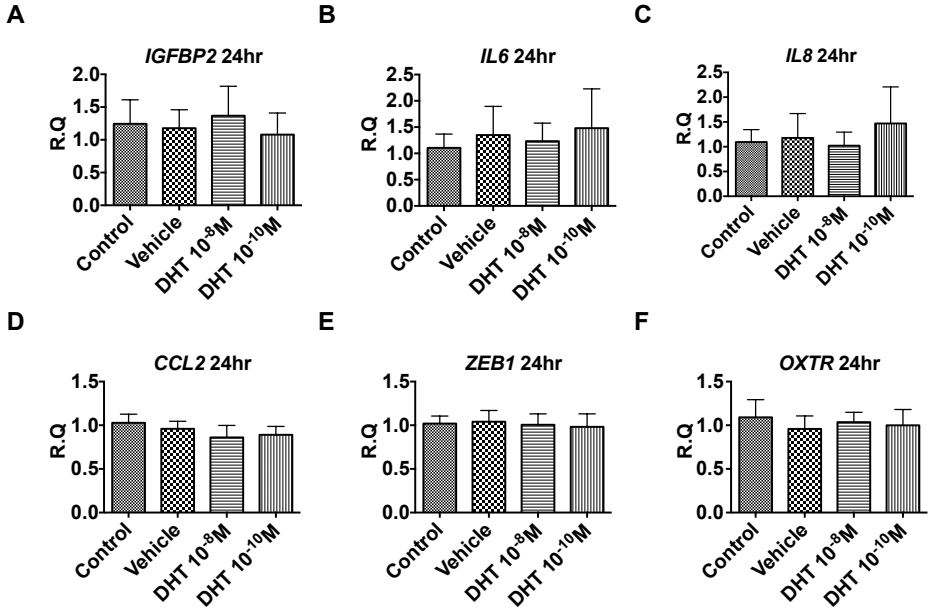



**Figure 3.18:** The effect of 24 hr DHT treatment on the expression of genes associated with the contractile/labour phenotype of MSMCs in PHM1-41s. PHM1-41s were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 24 hrs and the mRNA of *IGFBP2* (**A**), *IL6* (**B**), *IL8* (**C**), *CCL2* (**D**), *ZEB1* (**E**) and *OXTR* (**F**) was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

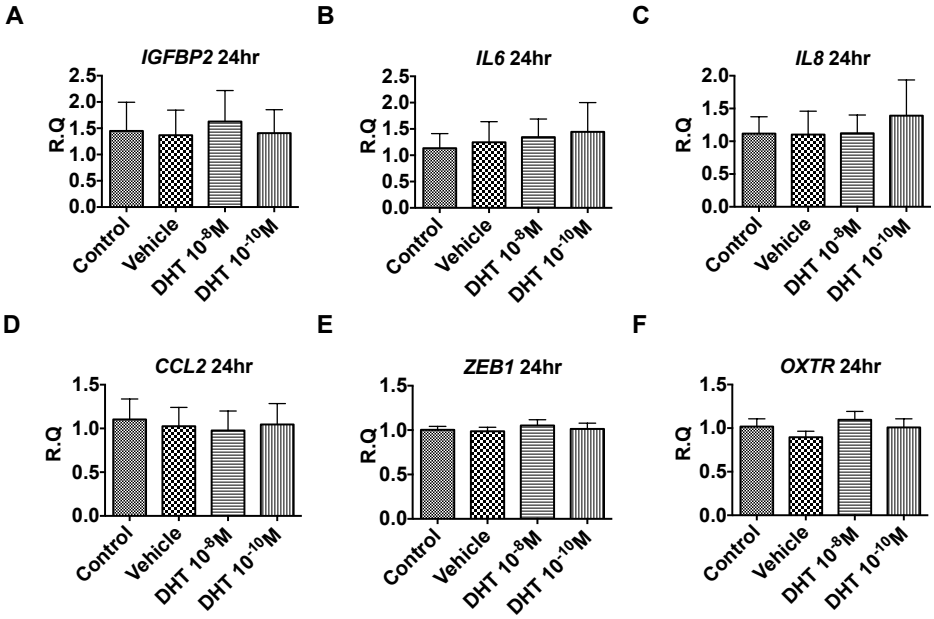



**Figure 3.19:** The effect of 24 hr DHT treatment on the expression of genes associated with the contractile/labour phenotype of MSMCs in UtSMCs. UtSMCs were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 24 hrs and the mRNA of *IGFBP2* (**A**), *IL6* (**B**), *IL8* (**C**), *CCL2* (**D**), *ZEB1* (**E**) and *OXTR* (**F**) was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

Postpartum phenotype (**Figures 3.20-3.23**)

The expression of the genes *IGFBP5*, *MMP2* and *MMP9* did not change following 8-hour incubation of PHM1-41s (**Figure 3.20**) and UtSMCs (**Figure 3.21**) with DHT ($10^{-8}$M or $10^{-10}$M). Similar responses were observed at the 24-hour time point in PHM1-41s (**Figure 3.22**) and UtSMCs (**Figure 3.23**).

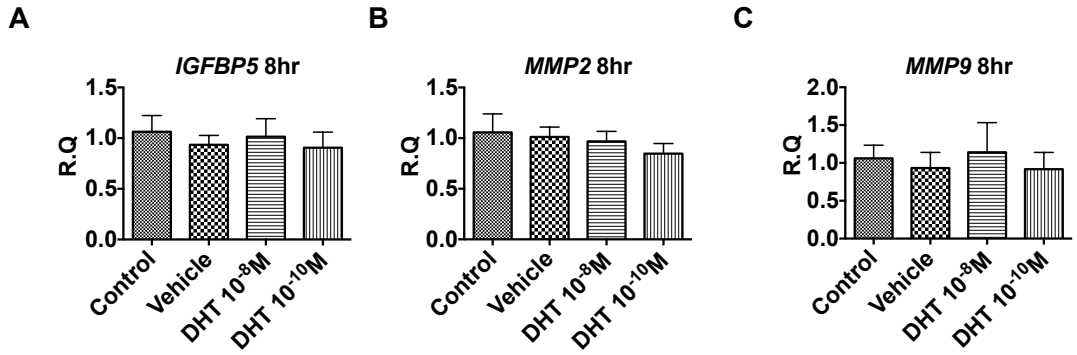



**Figure 3.20:** The effect of 8 hr DHT treatment on the expression of genes associated with the postpartum phenotype of MSMCs in PHM1-41s. PHM1-41s were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 8 hrs and the mRNA of *IGFBP5* (**A**), *MMP2* (**B**) and *MMP9* was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

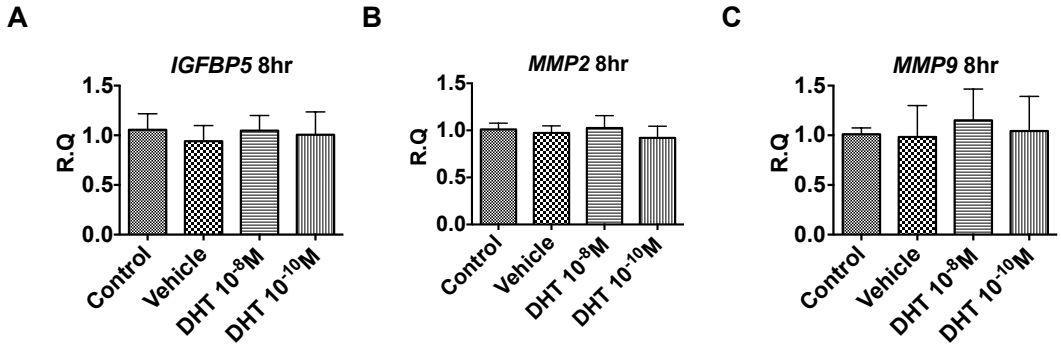



**Figure 3.21:** The effect of 8 hr DHT treatment on the expression of genes associated with the postpartum phenotype of MSMCs in UtSMCs. UtSMCs were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 8 hrs and the mRNA of *IGFBP5* (**A**), *MMP2* (**B**) and *MMP9* was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

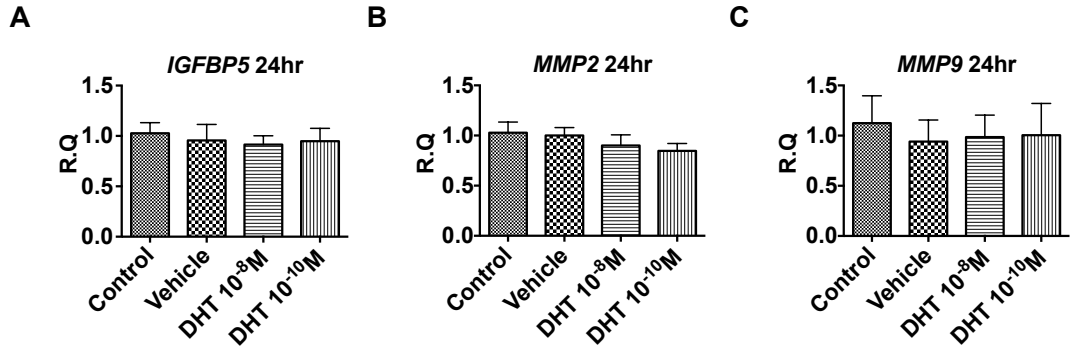



**Figure 3.22:** The effect of 24 hr DHT treatment on the expression of genes associated with the postpartum phenotype of MSMCs in PHM1-41s. PHM1-41s were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 24 hrs and the mRNA of *IGFBP5* (**A**), *MMP2* (**B**) and *MMP9* was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

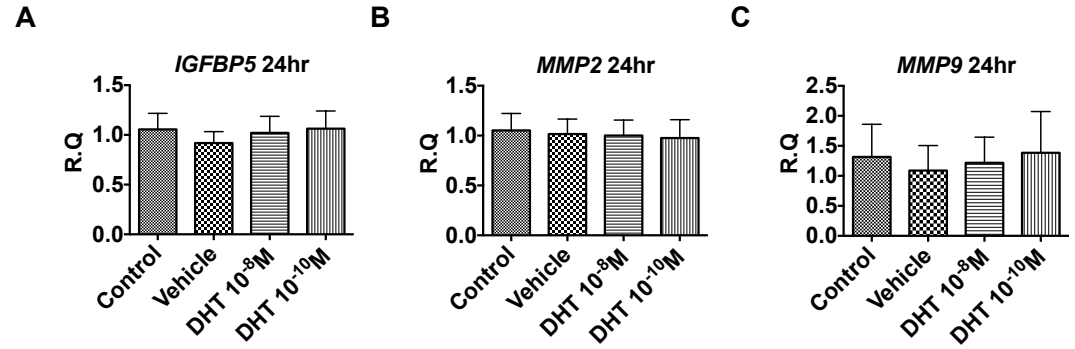



**Figure 3.23:** The effect of 24 hr DHT treatment on the expression of genes associated with the postpartum phenotype of MSMCs in UtSMCs. UtSMCs were incubated with DHT ($10^{-8}$M, $10^{-10}$M) for 24 hrs and the mRNA of I *IGFBP5* (**A**), *MMP2* (**B**) and *MMP9* was quantified with qRT-PCR. No significant changes were observed for any of the genes when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

Positive control (**Figure 3.24)**

Following the observation that none of the genes examined changed in response to the DHT treatment, we thought to examine the expression of *AR* mRNA in PHM1-41s and UtSMCs to verify the uptake of DHT by the cells. Following 24-hour treatment with DHT ($10^{-8}$M) the *AR* mRNA was significantly increased in PHM1-41s (2.3 fold, p=0.031; **Figure 3.24 A**) and UtSMCs (2.6 fold, p=0.025; **Figure 3.24 B**) when compared to the vehicle treatment.

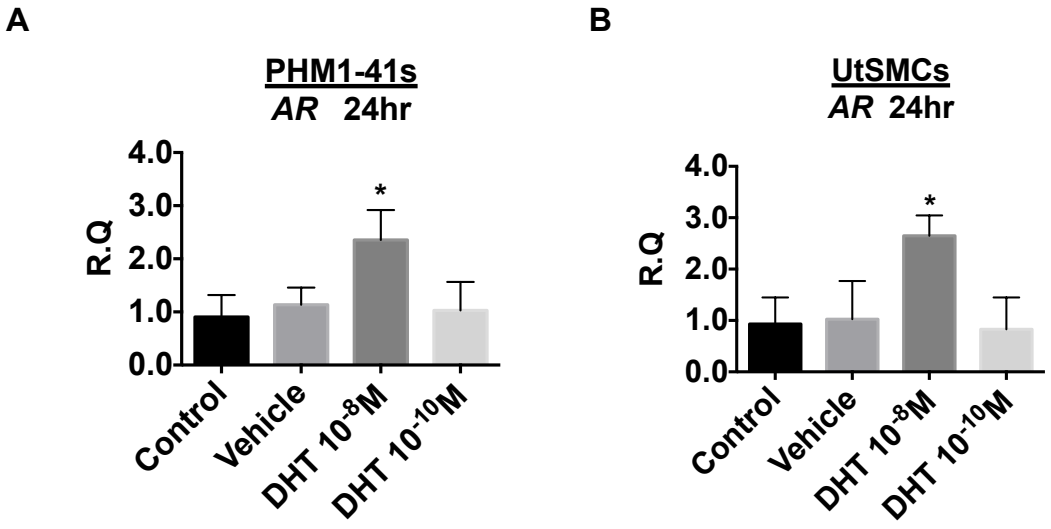



**Figure 3.24:** The expression of AR mRNA in PHM1-41s (**A**) and UtSMCs (**B**) following incubation with DHT ($10^{-8}$M or $10^{-10}$M) for 24 hours. AR mRNA was significantly increased in PHM1-41s (*p=0.031; **A**) and UtSMCs (*p=0.025) **B**) following DHT treatment ($10^{-8}$M) when compared to vehicle. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6.

### 3.3.4 The role of AR signalling on the expression of AR-dependent genes in the human myometrium at term pregnancy

We explored the effect of DHT treatment on the expression of androgen-responsive genes in human TNL myometrium using a human AR signalling-targets PCR array. Myometrial explants (n=4) were treated with DHT ($10^{-8}$M) for 6 or 24 hours in triplicate and the expression of 84 genes (see **Table 9.2** in **Appendix 2**) was assessed.

Following 6-hour incubation with DHT, 12 genes were apparently upregulated and 16 genes were downregulated (**Table 3.6**). Out of these genes, only the change in *PAK1IP1* mRNA reached significance (**Table 3.6**).

Following 24-hour incubation with DHT, 28 genes were upregulated and 4 genes were downregulated (**Table 3.7**). Out of these genes, only the changes in *AR* and *VAPA* mRNAs reached statistical significance (**Table 3.7**).

Array validation

To validate the results of the array, the concentration of *PAK1IP1* mRNA was quantified in human TNL myometrium (n=6) following 6-hour incubation with DHT ($10^{-8}$M) in triplicate. The DHT treatment induced a significant 1.9 fold increase (p=0.009) in the expression of *PAK1IP1* mRNA compared to the vehicle treatment.

The expression of *AR* and *VAPA* mRNAs was also quantified in human TNL myometrium (n=6) following 24-hour incubation with DHT ($10^{-8}$M). The DHT treatment induced a 3.6 fold significant increase (p=0.021) in the expression of *AR* mRNA compared to the vehicle treatment. In addition, the DHT treatment induced a 4.3 fold increase (p=0.036) in the expression of *VAPA* mRNA compared to the vehicle treatment.

**Table 3.6:** Gene regulation at 6 hrs. Table shows the AR-responsive genes that were upregulated and downregulated in human term non-labouring myometrium in response to 6-hr DHT ($10^{-8}$M) treatment (n=4 patient samples, n=3 experimental replicates).

| 6 hr | Gene | Fold regulation | p value |
|---|---|---|---|
| **Upregulation** | ***AR*** | 3.654 | >0.05 |
| | ***FAM105A*** | 3.0426 | >0.05 |
| | ***GUCY1A3*** | 2.3617 | >0.05 |
| | ***IGFBP5*** | 2.1106 | >0.05 |
| | ***MT2A*** | 2.4425 | >0.05 |
| | ***NFKB2*** | 2.172 | >0.05 |
| | ***PAK1IP1*** | 3.4481 | **0.0461** |
| | ***PIAS1*** | 3.7925 | >0.05 |
| | ***SMS*** | 2.6976 | >0.05 |
| | ***STK39*** | 8.5991 | >0.05 |
| | ***TSC22D1*** | 4.6063 | >0.05 |
| | ***VAPA*** | 6.2227 | >0.05 |
| **Downregulation** | ***ERRFI1*** | 3.4461 | >0.05 |
| | ***FKBP5*** | 6.8716 | >0.05 |
| | ***IGF1R*** | 2.1994 | >0.05 |
| | ***JUN*** | 4.2902 | >0.05 |
| | ***LRIG1*** | 3.1191 | >0.05 |
| | ***MAF*** | 5.7271 | >0.05 |
| | ***NFKB1*** | 2.5549 | >0.05 |
| | ***PMEPA1*** | 4.5522 | >0.05 |
| | ***PPAP2A*** | 2.8441 | >0.05 |
| | ***RAB4A*** | 3.2773 | >0.05 |
| | ***SEC22C*** | 2.5798 | >0.05 |
| | ***SLC26A2*** | 2.484 | >0.05 |
| | ***SNAI2*** | 2.4004 | >0.05 |
| | ***SORD*** | 6.4291 | >0.05 |
| | ***SP1*** | 2.9268 | >0.05 |
| | ***SRF*** | 2.4962 | >0.05 |
| | ***TIPARP*** | 2.5708 | >0.05 |
| | ***WIPI1*** | 2.1316 | >0.05 |
| | ***ZBTB16*** | 2.6329 | >0.05 |

**Table 3.7:** Gene regulation at 24 hrs. Table shows the AR-responsive genes that were upregulated and downregulated in human term non-labouring myometrium in response to 24-hr DHT ($10^{-8}$M) treatment (n=4 patient samples, n=3 experimental replicates).

| 24 hr | Gene | Fold regulation | p value |
|---|---|---|---|
| **Upregulation** | ***AR*** | 6.779 | **0.0123** |
| | ***ELK1*** | 2.149 | >0.05 |
| | ***ELL2*** | 2.3999 | >0.05 |
| | ***ERRFI1*** | 2.1017 | >0.05 |
| | ***FAM105A*** | 7.264 | >0.05 |
| | ***FOS*** | 3.1016 | >0.05 |
| | ***GUCY1A3*** | 4.2423 | >0.05 |
| | ***HERC3*** | 3.1913 | >0.05 |
| | ***IRS2*** | 8.8554 | >0.05 |
| | ***JUN*** | 2.7275 | >0.05 |
| | ***KRT8*** | 3.2475 | >0.05 |
| | ***LIFR*** | 3.7436 | >0.05 |
| | ***LRRFIP2*** | 5.8115 | >0.05 |
| | ***MAP7D1*** | 3.6697 | >0.05 |
| | ***MME*** | 2.415 | >0.05 |
| | ***MYC*** | 4.2811 | >0.05 |
| | ***NFKB2*** | 2.5846 | >0.05 |
| | ***PAK1IP1*** | 3.2597 | >0.05 |
| | ***REL*** | 5.2505 | >0.05 |
| | ***RHOU*** | 4.6365 | >0.05 |
| | ***SRF*** | 2.7492 | >0.05 |
| | ***VAPA*** | 8.6761 | **0.02554** |
| | ***ZBTB10*** | 4.062 | >0.05 |
| | ***ZNF189*** | 5.3365 | >0.05 |
| | ***FKBP5*** | 2.3801 | >0.05 |
| | ***LRIG1*** | 2.4067 | >0.05 |
| | ***MAF*** | 2.6049 | >0.05 |
| | ***NKX3-1*** | 2.7157 | >0.05 |
| **Downregulation** | ***RELA*** | 3.5261 | >0.05 |
| | ***SLC26A2*** | 5.1733 | >0.05 |
| | ***TSC22D3*** | 8.7675 | >0.05 |
| | ***HPRT1*** | 3.0783 | >0.05 |

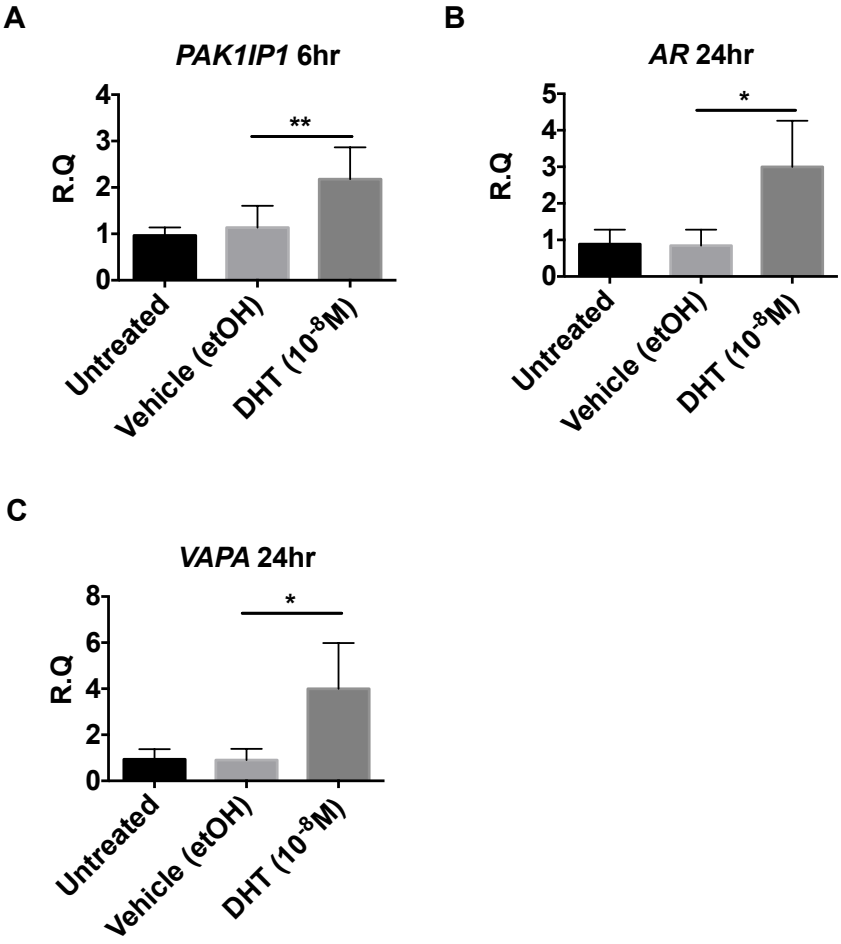



**Figure 3.25:** Validation of the PCR array. Following 6-hr treatment the expression of the gene *PAK1IP1* (**A**) was significantly upregulated in human TNL myometrium treated with DHT ($10^{-8}$M) when compared to vehicle treatment (**p=0.009). Following 24-hr treatment the expression of the genes *AR* (**B;** *p=0.021) and *VAPA* (**C;** *p=0.036) was significantly upregulated in the DHT-treated group when compared to the expression in the vehicle group. Data were analysed using Kruskal–Wallis with Dunn's post-hoc test, n=6 patient samples, n=3 experimental replicates.

## 3.4 Discussion

In contrast to the well-established roles of the PR and ER signalling, the potential that the AR signalling in the myometrium may play a role during pregnancy has been poorly investigated. The AR protein is reportedly expressed in porcine and rat myocytes throughout gestation (Liu et al., 2013; Slomczynska et al., 2008) and in human non-pregnant myometrium (Mertens et al., 2001). Surprisingly, the expression of the AR in the human myometrium during pregnancy has not been explored. We report for the first time that the AR is expressed and localised to the nuclei of MSMCs prior to- and during human labour. We did not quantify the immunofluorescence data in order to assess the ratio of AR positive to AR negative cells. However, porcine studies have demonstrated reduced number of AR positive MSMCs in the labouring myometrium compared to mid-pregnant myometrium (Slomczynska et al., 2008). In line with these findings, the expression of AR in the rat myometrium was strong at early and mid-gestation but weakened with labour (Liu et al., 2013). What is more, Western blotting analysis of the total AR protein in the myometrium of rats during pregnancy revealed gradual loss of expression as pregnancy progressed (Liu et al., 2013).

We found a significant decline of the total concentration of AR mRNA in the TL myometrium compared with the TNL, which did not coincide with a parallel decrease in AR protein. However, there are many processes between transcription and translation that can influence the availability and stability of the AR protein. For example, the AR is subject to posttranscriptional modifications (PTM) including acetylation, SUMOylation, ubiquititination and phosphorylation all of which influence its activity and stability (Gioeli and Paschal, 2012). It is plausible that the translated product of AR mRNA in the non-labouring myometrium was modified in a way that reduced its turnover at the time of labour, despite the fact that the *AR* gene was already downregulated. PTMs that regulate AR protein turnover include ubiquititination of lysine residues on the C-terminus of the AR, phosphorylation of serine 515 and lysine 534 and SUMOylation of lysine 386 and 520 (Coffey et al., 2013; Gioeli and Paschal, 2012; Lin et al., 2002). A close investigation of the expression and activity of hydrolases (SENP1, Usp10, Usp26) and/or transferases

(Ubc9, MAPK, Src) that mediate these PTMs would help examine the above hypothesis. Of note, the rate of mRNA transcription is considerably lower compared to the rate of protein translation (2 mRNAs/hr versus dozens of proteins/mRNA), which could also explain the observed disparity between mRNA and protein data (Vogel and Marcotte, 2012).

Liu *et al* studies have shown that the gradual decrease in total uterine AR protein throughout rat pregnancy occurred only in the gravid horns or horns stretched by laminaria cylinders but not in the non-gravid horns or horns where non-expandable plastic cylinder was inserted, suggesting that the expression of AR was stretch-regulated (Liu et al., 2013). In addition, they explored the effect of E2 and E2+P4 administration on the expression of AR protein in the uterus of OVX rats. Their Western blotting studies showed that the AR was decreased in the E2-treated uterus but the decrease was not significant compared to vehicle-treated uterus. However, the combined E2 and P4 treatment significantly increased AR protein compared to the E2 treatment alone, suggesting that a decrease in P4:E2 attenuated the expression of AR in the myometrium (Liu et al., 2013). Similar responses were observed in the uterus of rhesus monkeys where a combined E2 and P4 but not E2 alone treatment increased AR mRNA expression in the myometrium of OVX animals (Adesanya-Famuyiwa et al., 1999). Upregulation of the AR mRNA and protein by a combined E2 and P4 treatment has been additionally reported in airway smooth muscle cells isolated from OVX female rats (Zarazua, 2011). We also found that the AR mRNA was upregulated in PHM1-41s following a 2 day-incubation with E2+P4 but not E2, P4, DHT or T alone. In addition, DHT but not T treatment augmented the expression of AR mRNA after 24 hours. This is in line with findings from studies in the human cervix, where the AR protein is evidently increased in stromal cells of the endocervix and ectocervix in response to DHT treatment (van der Kwast et al., 1994). The regulation of AR expression by androgens is cell type and tissue dependent. For example, DHT treatment increased AR expression in some but not all prostate cancer cell lines (Cai et al., 2011). Furthermore, a DHT-induced upregulation of AR expression has been previously reported in human skeletal and rat smooth muscle cells (Lin et al., 1993; Sinha-Hikim et al., 2004). Interestingly, studies in porcine myometrium showed that a 4-day course administration of E2 and E2+P4, but not

P4, DHT or T, positively regulated the transcription of AR mRNA (Cardenas and Pope, 2003). Taken together these findings suggest that the fine-tuning of AR expression in the myometrium is likely to be modulated by the local availability of sex hormones and the stretch of the uterus. It is plausible that the functional P4 withdrawal, which triggers the onset of human labour, is contributing to the observed AR mRNA downregulation. Although the concentrations of P4, E2 and DHT are increasing in the maternal circulation throughout gestation, future studies examining the concentrations of these sex hormones in the myometrium before and during labour would help explore possible correlations with receptor levels.

The biological significance of the gradual loss of the AR in the myometrium throughout gestation is not understood. However, the role of the ligand-activated AR in stimulation of prostate cancer cell proliferation is well described (Berry et al., 2008) and the anabolic effects of androgens on skeletal muscle well established (Dubois et al., 2012; O'Connell and Wu, 2014). We explored the role of AR-activation by DHT on the expression of a panel of proliferative markers in cells derived from pregnant (PHM1-41s) or non-pregnant (UtSMCs) myometrium. The proliferative phenotype of MSMCs is associated with induction of genes that encode IGF-1 and its binding proteins IGFBP1/3 as well as PCNA, Bcl-2 and mTOR. Our findings suggest that the DHT-induced AR activation did not promote the expression of these genes at the time points investigated. However, previous *in vitro* studies have demonstrated that ligand-activated AR induced an increase in IGF-1 expression in prostate cancer cells (Culig et al., 1994). It is plausible that the AR signalling regulates proliferation of MSMCs in a ligand independent manner. In support of this hypothesis, Liu and colleagues have showed that the AR-independent signalling favours MSMCs hyperplasia through stabilisation of IGF-1 receptor (IGF-R1), highlighting the critical role of the AR in the normal progression of pregnancy (Liu et al., 2013). Their *in vitro* studies demonstrated that the knockdown of AR in MSMCs significantly reduced proliferation but the incubation of AR-expressing MSMCs with DHT did not promote proliferation, suggesting that the AR regulated MSMCs proliferation in a ligand independent manner (Liu et al., 2013). Although the knockdown of AR decreased proliferation of MSMCs, it did not affect the expression of IGF-1 protein. On the contrary, it significantly reduced IGF-1R protein content

and inhibited the IGF-1R downstream signalling events, such as phosphorylation of JNK kinase (Liu et al., 2013). Ligand-independent effect of AR activation on cell proliferation has been previously described in *in vitro* studies of skeletal muscle. In these studies, the activation of IGF1-R resulted in MAPK- and PI3K/Akt - mediated phosphorylation of the AR (Kim et al., 2009; Kim and Lee, 2009a, b). Overall, these studies highlight that AR activation can promote cell proliferation in a ligand independent manner.

We also examined the effect of DHT-induced AR activation on the expression of genes associated with the other phenotypes that the MSMCs adopt during pregnancy. Rat studies have shown that *IGFBP6*, *FN1* and *ELN* genes are highly expressed during the synthetic phenotype (Shynlova et al., 2009b). Although previous studies have shown that *IGFBP6*, *FN1* and *ELN* are androgen-responsive (DePrimo et al., 2002; Nelson et al., 2002; Rooman et al., 2005; Wolinsky, 1972), we did not find an effect of DHT treatment on the expression of these genes in the human cell lines we used. In addition, the effect of DHT treatment on the expression of genes associated with the labouring phenotype of MSMCs was examined. Genes that encode the pro-inflammatory markers IL-6 and IL-8, the chemokine CCL-2 and the OXTR are all upregulated with labour (Osman et al., 2003; Shynlova et al., 2008; Shynlova et al., 2009b). The transcription factor ZEB-1 has been recently shown to be decreased in term and preterm labouring myometrium where its expression is P4 and E2 regulated (Renthal et al., 2013; Williams et al., 2012b). The regulation of ZEB-1 expression by androgens in the myometrium has not been explored, but AREs have been identified on the promoter of *ZEB1*, suggesting that its transcription might be androgen-regulated (Anose and Sanders, 2011; Mooney et al., 2014). We did not find an effect of DHT treatment on the expression of any of the above genes. Finally, we examined the hypothesis that the apoptosis of MSMCs during postpartum involution is androgen-regulated. Genes that encode the IGFBP-5 and the metalloproteinases MMP2 and MMP9 are upregulated in the apoptotic MSMCs at the end of pregnancy (Shynlova et al., 2009b). Previous studies have confirmed the presence of AREs on the promoter of genes encoding metalloproteinases (Li et al., 2007) and the regulation of IGFBP-5 expression by androgens has been demonstrated in the male reproductive tract (Ha et al., 2013; Xu et al., 2007) and fibroblasts (Yoshizawa and

Ogikubo, 2006). The studies described in this chapter failed to detect any androgen-dependent changes in the concentration of mRNAs encoding these genes in the human myometrium.

Collectively, despite the fact that the AR mRNA was upregulated by the DHT treatment, we did not find a direct effect of DHT on the expression of genes associated with the proliferative, synthetic, contractile/labour or apoptotic phenotype of MSMCs. Albeit the upregulation of the AR mRNA in PHM1-41s following the DHT treatment, it is possible that the AR protein remained unchanged. Given that the knockdown of AR and, thus, reduction in AR protein, reduced cell proliferation of MSMCs in previous studies (Liu et al., 2013), it is plausible that the DHT treatment did not exert an effect on AR protein and thus did not upregulate the expression of proliferative markers. Examination of the AR protein following DHT treatment in future studies would help verify this notion.

In order to identify additional genes that might be altered by AR signalling in the human myometrium at term, we conducted a PCR array exploring the effect of DHT treatment on the expression of 84 previously validated AR-responsive genes. Although a number of genes were upregulated or downregulated, only changes in *VAPA*, *PAK1IP1* and *AR* encoded mRNAs were statistically significant. The small sample size (n=4) and the variation between the human tissues might have contributed to this result. The *VAPA* gene encodes the VAPA membrane protein (vesicle-associated membrane protein-associated protein A). VAPAs reside on the plasma membrane and on intracellular vesicles where they are involved in protein trafficking, protein complex assembly, cell motility, stress-signalling system, cytoskeletal organisation and protein fusion. The VAPA proteins are predominantly researched in the context of neuroscience where they are involved in the pathophysiology of mental disease (Fatemi et al., 2009; Lohoff et al., 2008). However, VAPAs are also involved in membrane trafficking in cardiac and skeletal muscle (Schwenk et al., 2010; Takahashi et al., 2013). In particular, VAPA proteins are required for insulin-dependent glucose transporter 4 (GLUT4) both in muscle and fat (Foster and Klip, 2000; Foster et al., 2000; Kioumourtzoglou et al., 2014). There is evidence that during rat skeletal muscle contraction, VAPA co-localises with

GLUT4 on the cell surface, suggesting that VAPAs translocate to the cell membrane in response to skeletal muscle contraction to assist GLUT4 uptake (Rose et al., 2009). It is possible that VAPAs facilitate protein trafficking during myometrial contraction and androgen-dependent signalling mediates these events. However, our findings are preliminary and there is not yet enough evidence to show that VAPAs have major roles in myometrial contraction.

The PAK1IP1 (p21-activated protein kinase-interacting protein 1) is an enzyme that is known to interact with the serine/threonine-protein kinase PAK1. The p21-activated kinase (PAK) family is implicated in cytoskeletal reorganisation, cell motility, gene transcription and apoptosis (Kumar et al., 2006). The roles of PAK1 in development and progression of prostate cancer are well established and PAKs are known to favour proliferation of AR-responsive prostate cancer cells (Goc et al., 2013). Studies in colon cancer cells reported that PAK1 phosphorylation, important for cytoskeleton reorganisation, is downstream AR signalling (Gu et al., 2013). In vasculature, PAK1 is driving smooth muscle cell migration, proliferation and remodelling (Hinoki et al., 2010; Weber et al., 2004; Woolfolk et al., 2005). Interestingly, PAKs may affect SMC contraction through phosphorylation of key proteins in the contraction cascade, such as MLCK, MLCP and caldesmon, which is a calmodulin binding protein. For example, phosphorylation of MLCK by PAK1 inhibits MLCK activity and, consequently, contraction of skinned smooth muscle (Wirth et al., 2003). Attenuation of contraction by PAKs has also been demonstrated in the human myometrium (Moore et al., 2000). In that study, PAK1 protein, which was expressed in the pregnant but not non-pregnant myometrium, inhibited MLCK activity, suggesting a role for PAK1 in maintenance of uterine quiescence (Moore et al., 2000). It is tempting to hypothesise that androgen-dependent signalling might play a role in inhibition of contraction by affecting PAKs. In order to further investigate these events we would need to examine the effect of androgen-induced AR activation on proteins involved in the activation of the contractile apparatus.

In conclusion, we demonstrated for the first time that the AR is expressed in the term human myometrium where its mRNA is downregulated with onset of labour. Our PCR data suggest that the ligand activated AR is unlikely to be involved in MSMCs

proliferation or apoptosis but might have a role in inhibition of contraction throughout pregnancy. More appropriate PCR arrays on DHT-treated myometrium, examining the gene expression profile of the molecules involved in cell-to-cell and cell-to-matrix interactions (ECM) or genes involved in the contraction of MSMCs would help further explore the above hypotheses.

# Chapter 4: The effect of androgens on myometrial contraction

## 4.1 Introduction

The maintenance of uterine quiescence is critical for pregnancy reaching term and the initiation of myometrial contractions at term is pivotal for successful expulsion of the fetus. The concentration of sex hormones, such as P4 and estrogens, rise in maternal circulation as pregnancy progresses and this event is thought to modulate the transition of the myometrium from a quiescent to a contractile state in a gene transcription-dependent way (Buster et al., 1979; Mesiano et al., 2002; Smith et al., 2002). According to this genomic theory, P4 is thought to bind to the myometrial PR and promote quiescence and, estrogens via the ER are believed to favour myometrial activation (Mesiano, 2004; Mesiano and Welsh, 2007; Winkler et al., 2002). Besides their genomic mechanism of action, rapid non-genomic relaxant effects of P4 and estrogens on the myometrial contraction have been described in studies employing various pharmacological screening tools to access concentration-response (Perusquia, 2001). These studies have demonstrated the ability of P4 to relax spontaneous contractions of rat, pig, horse and human myometrium when administered in non-genomic (at the micromolar range) concentrations *ex vivo* (Anderson et al., 2009; Chanrachakul et al., 2005; Kurowicka et al., 2005; Ousey et al., 2000; Perusquia et al., 1990; Perusquia and Jasso-Kamel, 2001; Perusquia and Kubli-Garfias, 1994; Ruddock et al., 2008; Sanchez Aparicio et al., 1992). In addition to its effect on spontaneous stretch-induced contractions, P4 has been shown to relax OXT-initiated contractions (Kurowicka et al., 2005; Ousey et al., 2000). Myometrial relaxant effect has been observed for many P4 metabolites, such as 5-α and 5-β reduced progestins (Perusquia et al., 1991b; Perusquia and Jasso-Kamel, 2001) and studies showed that the P4 derivative 17-hydroxyprogesterone (17OHP) either stimulates (Ruddock et al., 2008) myometrial contractions or has no effect (Anderson et al., 2009; Sexton et al., 2004). The roles of estrogens on myometrial contractions have been explored using similar methodology. For example, non-genomic doses of E2 have been shown to relax myometrial contractions stimulated by stretch or other stimulants like OXT or KCl in rats and humans *ex vivo*

(Fernandez et al., 1993; Fu et al., 1996; Gutierrez et al., 1998; Ma et al., 2013; Perusquia and Navarrete, 2005; Tsai et al., 2003; Vedernikov et al., 2003; Yin et al., 2012). This is in contrast with the genomic action of estrogens to promote uterine activation (Welsh et al., 2012). The genomic and non-genomic action of the sex steroids is believed to be mediated via separate pathways; in contrast to the genomic pathway, the outcome of which is evident after hours or days, the non-genomic pathways are gene transcription-independent and, therefore, rapid in onset. The mechanism of the non-genomic action of P4 and estrogens is not fully understood, but is thought to involve blockade of $Ca^{2+}$ flux into the cell, which is a key event to initiation of muscle contraction. Few studies have explored how estrogens and P4 might block $Ca^{2+}$ entry into the cell (Perusquia, 2001). It is now thought that the relaxant effect of estrogens and P4 may be mediated via binding to a myometrial membrane steroid receptor or direct binding to $Ca^{2+}$ channels on the cell surface (Duval et al., 1983; Perusquia, 2001; Perusquia et al., 1990; Tica et al., 2011). In addition, micromolar concentrations of estrogens have shown to impair uterine smooth muscle IGJC and reduce the density of myometrial caveolae, which are lipid raft invaginations of the cell membrane that can regulate the function of $Ca^{2+}$ channels and, thus, $Ca^{2+}$ signalling (Tsai et al., 1998; Turi et al., 2001). Finally, studies that tested the involvement of PR and ER in the mediation of myometrial relaxation have demonstrated that antagonism of these receptors with RU486 and tamoxifen respectively failed to inhibit the actions of P4 or E2 (Perusquia and Kubli-Garfias, 1994; Perusquia and Navarrete, 2005).

In contrast to the well-studied non-genomic action of P4 and estrogens in the myometrium, the effects of androgens have received less scrutiny. Perusquia and colleagues explored the non-genomic action of various androgens on rat and human myometrium and demonstrated that, similarly to P4 and estrogens, androgens inhibit stretched-induced contractions with DHT having a more potent relaxant effect than other androgen metabolites (Perusquia et al., 1990; Perusquia et al., 2005). To explore the underlying mechanism of androgenic action, their studies also examined the involvement of the classical AR in mediating myometrial relaxation. They reported that AR antagonism with flutamide did not prevent the androgen-induced relaxation. These studies concluded that androgens relax the uterine muscle in an

AR-independent manner (Perusquia et al., 2005). Based on their additional observation that androgens relax KCl-stimulated tonic contraction, they hypothesised that the relaxant effect was mediated via blockade of L-typed $Ca^{2+}$ channels (VOCCs) (Perusquia et al., 2005). Notably, other possible mechanisms that facilitate the myometrial effects of androgens have not been investigated and more studies are necessary to understand the underlying events of their relaxant action.

Based on this work, we sought to further explore the role of androgens on myometrial contractility using human myometrial and mouse uterine tissue in order to elucidate the mechanism of the relaxant effect and, importantly, deduce whether the effect is specific or generic to all sex hormones.

### 4.1.1 Specific aims

In this chapter we specifically aimed to:

- Investigate the effect of the androgens DHT and T on contractions of both human and mouse myometrium
- Examine whether any effects are mediated via the AR or a cell-surface receptor for AR
- Examine whether any effects of DHT and T are specific or whether they reflect a generic (steroid) effect.

## 4.2 Materials and Methods

### 4.2.1 Human and mouse tissue

Human myometrial biopsies were collected from women (n=26 patients; 150 strips) during elective caesarean section before the initiation of labour as described in Chapter 2 section 2.1.1 and stored overnight at 4$^{o}$C in RPMI medium. Murine uterine horns were obtained from non-pregnant 8-week old C57BL/6 mice (n=10 mice; 40 strips) as described in section 2.1.2 and used directly for the organ bath studies.

### 4.2.2 Organ bath

The human myometrial biopsies and the murine uterine horns were cut into strips as described in section 2.2 and placed under tension in Krebs buffer in an organ bath system as described in section 2.2.1. Strips were left to develop rhythmic spontaneous contractions before application of any treatment.

For human tissue, rhythmic stable spontaneous activity was defined as approximately 3-4 consecutive contractions occurring within a period of 30 minutes with approximately similar frequency and amplitude. The average time required for a human myometrial strip to start contracting in a rhythmic manner was 2 hours.

For mouse tissue, rhythmic stable spontaneous activity was defined as approximately 5-12 consecutive contractions occurring within a period of 10 minutes with approximately similar frequency and amplitude. The average time required for a mouse uterine strip to start contracting in a rhythmic manner was approximately 1 hour.

#### *4.2.2.1 Treatments of human myometrial and murine uterine strips*

**Treatments of stretched-initiated contractions**

Initially, human myometrial strips were treated with DHT and/or T in a non-cumulative manner, so that each strip was exposed to one concentration of each androgen. The concentrations applied were 10 pM, 1 nM, 1 μM and 100 μM. Following the observation that DHT and T relaxed myometrial contractions only at the high dose of 100 μM, we determined a dose response from 10 μM to 100 μM whereby treatments were applied in a cumulative manner on human and murine

strips. In a similar manner, the effects of P4 and E2 on human myometrial and mouse uterine contraction were also examined. All steroids were purchased from Sigma and reconstituted according to the supplier's instructions in absolute ethanol (etOH); the stocks were kept in sterile glass vials at -20$^{o}$C in the darkness and working concentrations were prepared in Krebs buffer at the time of the experiment.

The vehicle was etOH diluted in equivalent doses to the dose of experimental compounds.

Testosterone 3-(O-carboxymethyl)oxime:BSA (TBSA), which is a T conjugate to BSA and, thus, a cell-membrane impermeable molecule, was reconstituted in PBS at 2 mg/ml at the day of the experiment. The conjugation ratio was 30 molecules T: 1 molecule BSA. The molecular weight of TBSA was 77274.4 Daltons and the molecular weight of T [Testosterone 3-(O-carboxymethyl)oxime] was 361 Daltons. Hence reconstitution of TBSA at 2 mg/ml gave a stock of 25.8 μM of TBSA in which T was in a concentration of 5.5 mM. Myometrial strips were treated with TBSA in a 0.5 μM concentration (100 μM dose of T). The vehicle control in this experiment was PBS.

Flutamide was reconstituted at the time of the experiment in etOH; working dilutions were prepared in Krebs, and flutamide was used at a final concentration of 100 μM. The vehicle control was etOH.

The steroid cocktail was prepared as shown below and added to the organ bath on stretched-induced contractions.

| 40 μM Cocktail = 10 μM DHT + 10 μM T + 10 μM E2 + 10 μM P4 |
|---|

Human myometrial strips and murine uterine strips were exposed to each treatment for 30 minutes and 10 minutes respectively.

**Treatments of OXT-initiated contractions**

OXT was prepared in distilled water ($dH_2O$) and used at a 10 μM concentration to stimulate myometrial contraction. Each one of the sex hormones (DHT, T, E2 and P4) was applied 10 minutes following stimulation with OXT. When applied on OXT-

stimulated contractions, the sex hormones were in a concentration of 50 μM. This concentration was selected based on the IC50 values generated from the effects of the individual sex hormones on stretched-induced contractions. As the IC50 values were slightly different for each sex hormone, the individual IC50 values were averaged to give the same concentration for all sex hormones to be used with OXT. The vehicle control in these experiments was $dH_2O$.

**Tissue recovery and viability**

At the end of each experiment, the strips were flushed with fresh pre-warmed Krebs and stimulated with 40 mM of KCl to induce a tonic contraction and, thus, confirm tissue viability. Data from strips that did not respond to KCl treatment were not included as that suggested that the experimental compound induced a terminal effect and the tissue was no longer viable (**Figure 4.1b**).

Details of all the experimental compounds used in this chapter are shown in **Table 4.1**.

**Table 4.1:** Details of experimental compounds

| Compound | Supplier |
|---|---|
| DHT | A8380 (Sigma) |
| T | T1500 (Sigma) |
| E2 | E8875 (Sigma) |
| P4 | P0130 (Sigma) |
| Flutamide | F9397 (Sigma) |
| TBSA | T3392 (Sigma) |
| OXT | Syntocinon 10 IU/ml capsules (ALLIANCE pharmaceuticals) |
| KCl | P9541 (Sigma) |

### 4.2.3 Data acquisition and statistical analysis

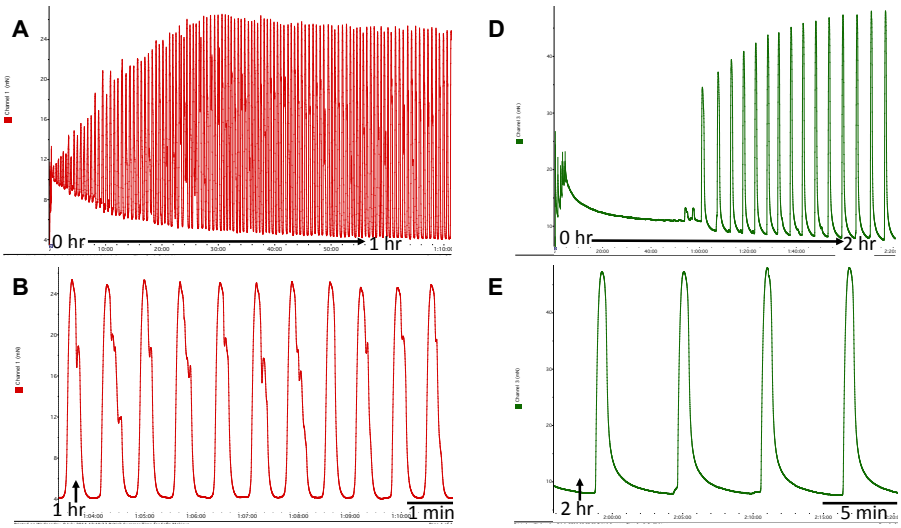
The organ bath data was acquired with LabChart7 data acquisition software as described in section 2.2.2 and the contraction frequency, amplitude and A.U.C were all calculated by the same software as described in sections 2.2.3 and 2.2.4. The effect of each experimental compound on each contractile parameter was expressed as the percentage of the original value obtained prior to the addition of the compound. Percentage data were transformed to proportions, which were arcsine-transformed to perform statistical analysis. Data were tested for normality using the Kolmogorov–Smirnov test and analysed using one-way ANOVA with Tukey's post

hoc test in GraphPad v6.0 software. Individual response curves were analysed using nonlinear regression (Graphpad Prism) and the inhibitory concentration that produced half of the minimum contraction was presented as an IC50 value. Unless otherwise stated, error bars denote ±SEM.

## 4.3 Results

### *4.3.1* Human myometrial and mouse uterine spontaneous contractions *ex vivo*

Human myometrial strips and murine uterine strips were prepared and placed under tension in order to develop stable spontaneous contractions. The required time to develop rhythmic activity was approximately 1 hour for mouse tissue (**Figure 4.1a B**) and 2 hours for human tissue (**Figure 4.1a E**). Following the equilibration period, the human myometrial strips contracted in a stable manner with frequency of 7.6 ± 0.05 contraction/ hour and of amplitude 45 ± 1.77 mN (**Figure 4.1a F**). Murine uterine strips contracted with frequency of 45.9 ± 6.1 contraction/ hour and of amplitude 23.67 ± 0.96 mN (**Figure 4.1a C**).




C

| Frequency (contraction/hr) | Amplitude (mN) |
|---|---|
| 45.9 ± 6.1 | 23.67 ± 0.96 |

F

| Frequency (contraction/hr) | Amplitude (mN) |
|---|---|
| 7.6 ± 0.5 | 45 ± 1.77 |

**Figure 4.1a:** Representative contractility traces of murine (**A-B**) uterine strips and human (**D-E**) myometrial strips. Murine uterine strips and human myometrial strips were placed under tension and allowed to equilibrate for a period of 1 hr (**A**) and 2 hrs (**D**) respectively to develop stable spontaneous contractions. After 1 hr equilibration for mouse tissue (**B**) and 2 hrs for human tissue (**E**), the strips contracted in a rhythmic manner. The average frequency and amplitude of the stable spontaneous contractions following the equilibration time was calculated for mouse (**C**; n=6 mice/ 24 strips) and human (**F**; n=14 patients/ 70 strips) tissue.

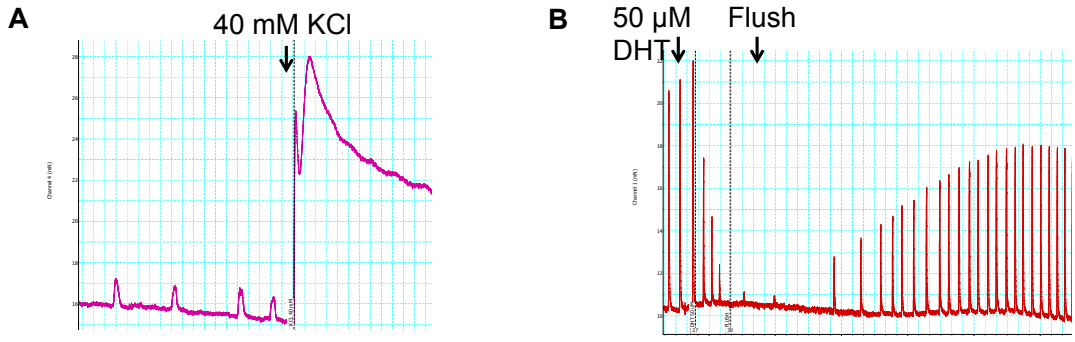



**Figure 4.1b:** Tissue viability and recovery following an organ bath experiment. At the end of each experiment, the strips were flushed with fresh pre-warmed Krebs buffer (**B**) to test tissue recovery and then stimulated with 40 mM KCl (**A**) to induce tonic contraction to confirm tissue viability.

### 4.3.2 DHT and T relaxed spontaneous human myometrial contractions

DHT administration in non-cumulative doses of 10 pM, 1 nM and 1 μM did not change the frequency, amplitude or A.U.C of spontaneous human myometrial contractions (**Figure 4.2 A**). However, when a dose of 100 μM was applied, the frequency decreased to 3.5% ± 1.8, the amplitude to 2.5% ± 1.5 and the A.U.C to 1.5% ± 0.7 of the original value (**Figure 4.2 B**). These effects were significant when compared to the effect of vehicle ($p < 0.0001$). Similarly, T administration in non-cumulative doses of 10 pM, 1 nM and 1 μM had no effect on the frequency, amplitude or A.U.C of contraction (**Figure 4.3 A**). The higher dose of 100 μM, however, decreased the frequency to 4% ± 2.1, the amplitude to 3.1% ± 1.9 and the A.U.C to 3% ± 1.1 of the original value ($p < 0.0001$ compared to vehicle) (**Figure 4.3 B**).

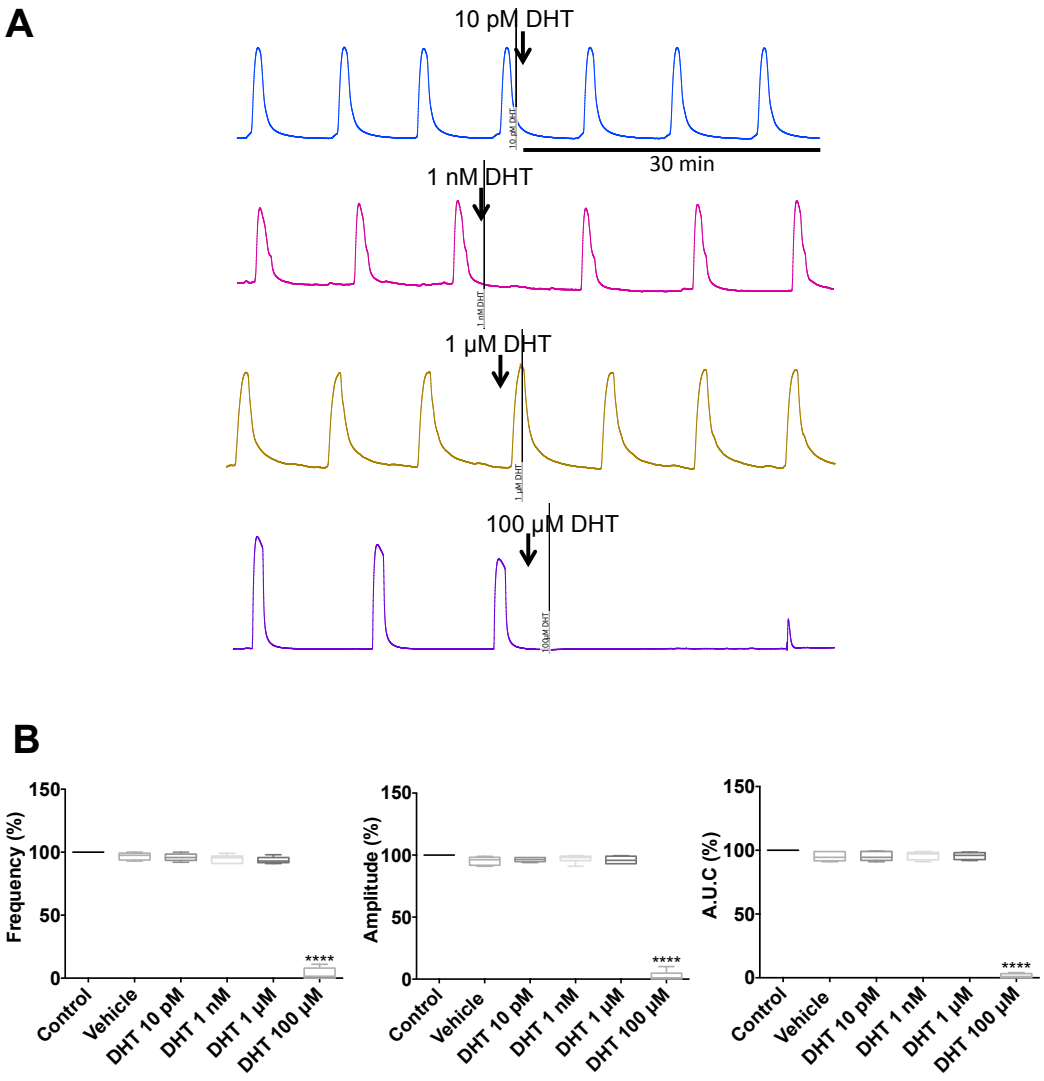



**Figure 4.2:** The effect of non-cumulative doses of DHT on spontaneous human myometrial contractions. (**A**) Representative recordings of myometrial contractions following application of DHT in 10 pM, 1 nM, 1 µM and 100 µM. (**B**) At 100 µM, DHT significantly reduced the frequency, amplitude and the A.U.C of contraction. ****p<0.0001 compared to vehicle, n=6 patients/1 strip per treatment. Data were analysed using one-way ANOVA with Tukey's post-hoc test.

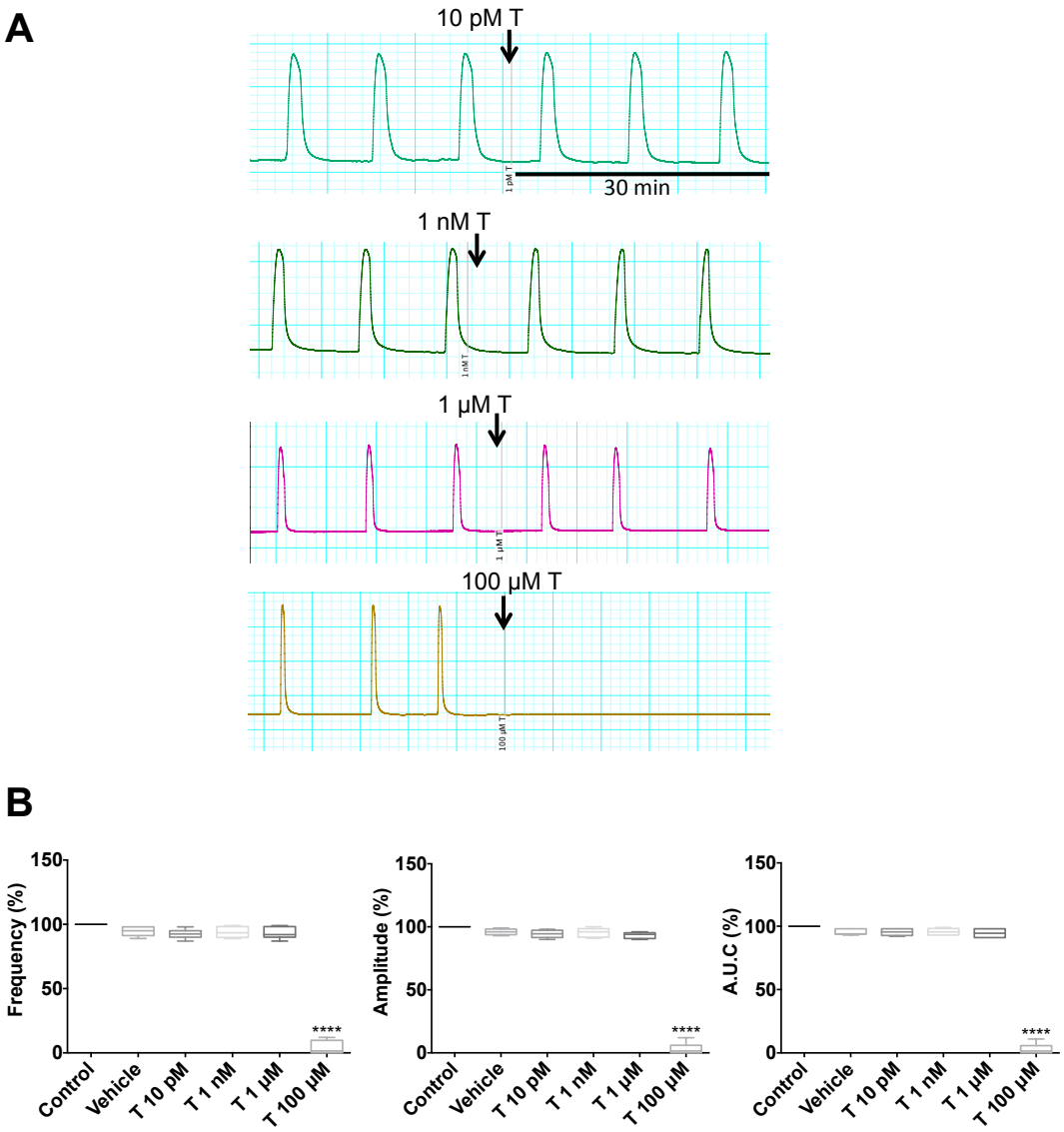


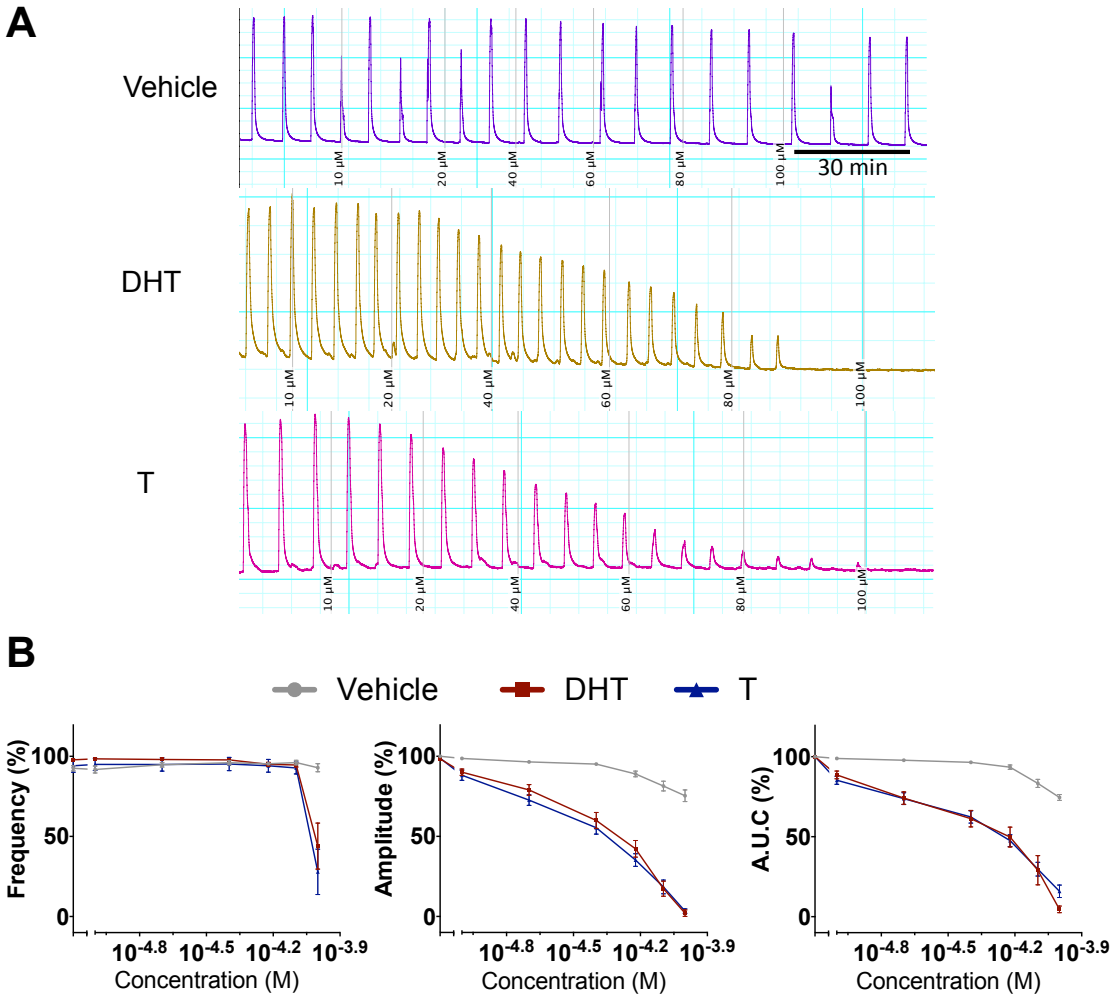

**Figure 4.3:** The effect of non-cumulative doses of T on spontaneous human myometrial contractions. **(A)** Representative recordings of myometrial contractions following application of T in 10 pM, 1 nM, 1 µM and 100 µM. **(B)** At 100 µM, T significantly reduced the frequency, the amplitude and the A.U.C of contraction. ****p<0.0001, n=6 patients, 1 strip per treatment. Data were analysing using one-way ANOVA with Tukey's post-hoc test.

Following the observation that DHT and T relaxed spontaneous human myometrial contractions only at a high dose (100 μM), the effect of doses ranging from 10 μM to 100 μM was explored. Doses of 10 μM, 20 μM, 40 μM, 60 μM, 80 μM and 100 μM of DHT or T were added in a cumulative manner to each myometrial strip for 30 minutes each (**Figure 4.4 A**). Equivalent doses of vehicle (etOH) were applied in the same range; the minimum concentration of etOH was 0.03% (at 10 μM) and the maximum was 0.3% (at 100 μM) (**Figure 4.4 A**). The effect of DHT and T was then compared to the effect of vehicle at the relevant doses.

The DHT inhibited the amplitude and the A.U.C of contraction in a dose-dependent manner (**Figure 4.4 B**). DHT did not change the frequency of contraction in a dose range from 10 μM to 80 μM, but significantly decreased the frequency to 43.9% ± 14.3 of the original value at the 100 μM dose ($p<0.0001$ compared to vehicle). The minimum significant inhibition of amplitude and A.U.C of contraction was observed at the 20 μM dose; the amplitude was reduced to 78.9% ± 3.3 and the A.U.C to 74.1% ± 3.1 of the original value (**Figure 4.4 C**). The maximum inhibition was observed at the 100 μM dose; the amplitude was reduced to 2% ± 2 and the A.U.C was reduced to 4.5% ± 2 of the original value (**Figure 4.4 C**). The IC50 of DHT for amplitude of contraction was 39 μM [32 μM to 47 μM 95% confidence interval (C.I)] and the IC50 of DHT for A.U.C was 43 μM (35 μM to 54 μM 95% C.I) (**Figure 4.4 C**).

In a similar manner to DHT, T inhibited the amplitude and the A.U.C of contraction in a dose-dependent manner (**Figure 4.4 B**). T did not change the frequency of contraction at any dose tested from 10 μM to 80 μM, but significantly decreased the frequency to 27.8% ±14.1 of the original value at the 100 μM dose ($p<0.0001$ compared to vehicle) (**Figure 4.4 B**). The minimum significant inhibition of the amplitude and A.U.C was observed at the 20 μM dose; the amplitude was reduced to 72.6% ± 3.3 and the A.U.C to 73.7% ± 3.6 of the original values (**Figure 4.4 C**). The maximum inhibition was observed at the 100 μM dose; the amplitude was reduced to 3.3% ± 1.3 and the A.U.C was reduced to 15.8% ± 3.8 (**Figure 4.4 C**). The IC50 of T for amplitude of contraction was 34 μM (28 μM to 40 μM 95% C.I) and the IC50 of T for A.U.C was 45 μM (39 μM to 53 μM 95% C.I) (**Figure 4.4 C**). There were

no significant changes between the effects of DHT and T on the inhibition of human myometrial contractions.




**C**

**Amplitude**

| Compound | | | | Concentration | | | IC50 |
|---|---|---|---|---|---|---|---|
| | 10 µM | 20 µM | 40 µM | 60 µM | 80 µM | 100 µM | |
| DHT | ns | 78.9±3.3% (p<0.0001) | 59.9±5% (p<0.0001) | 42.2±5.1% (p<0.0001) | 17.3±4.6% (p<0.0001) | 2±2% (p<0.0001) | 39 µM (32 to 47 95% C.I.) |
| T | ns | 72.6±3.3% (p<0.0001) | 55.4±3.9% (p<0.0001) | 35.2±3.9% (p<0.0001) | 18.3±4.3% (p<0.0001) | 3.3±1.3% (p<0.0001) | 34 µM (28 to 40 95% C.I.) |

**A.U.C**

| Compound | | | | Concentration | | | IC50 |
|---|---|---|---|---|---|---|---|
| | 10 µM | 20 µM | 40 µM | 60 µM | 80 µM | 100 µM | |
| DHT | ns | 74.1±3.8% (p<0.0001) | 61.2±5.1% (p<0.0001) | 49.7±6.2% (p<0.0001) | 29±9.1% (p<0.0001) | 4.5±2% (p<0.0001) | 43 µM (35 to 54 95% C.I.) |
| T | ns | 73.7±3.6% (p<0.0001) | 62.3±3.8% (p<0.0001) | 47.5±3.7% (p<0.0001) | 29.6±4.2% (p<0.0001) | 15.8±3.8% (p<0.0001) | 45 µM (39 to 53 95% C.I.) |

**Figure 4.4:** The effect of DHT and T on human spontaneous myometrial contractions. (**A**) Representative recordings following addition of cumulative doses of DHT and T. (**B**) Concentration response curves showing the effect of DHT, T and vehicle on frequency, amplitude and A.U.C of contraction. The frequency of contraction significantly decreased with 100 µM DHT and T ($p<0.0001$ compared to vehicle). The amplitude and A.U.C decreased in a dose-dependent manner. (**C**) Tables show the percentage (%) of the original value to which the amplitude and A.U.C decreased following treatment with DHT and T in each concentration (p values and ns=non-significant are compared to vehicle). The last column of the table shows the IC50 values of DHT and T; the range in brackets is expressed as 95% confidence interval (C.I). Data were analysed using one-way ANOVA with Tukey's post-hoc test (n=9 patients, 1 strip per treatment).

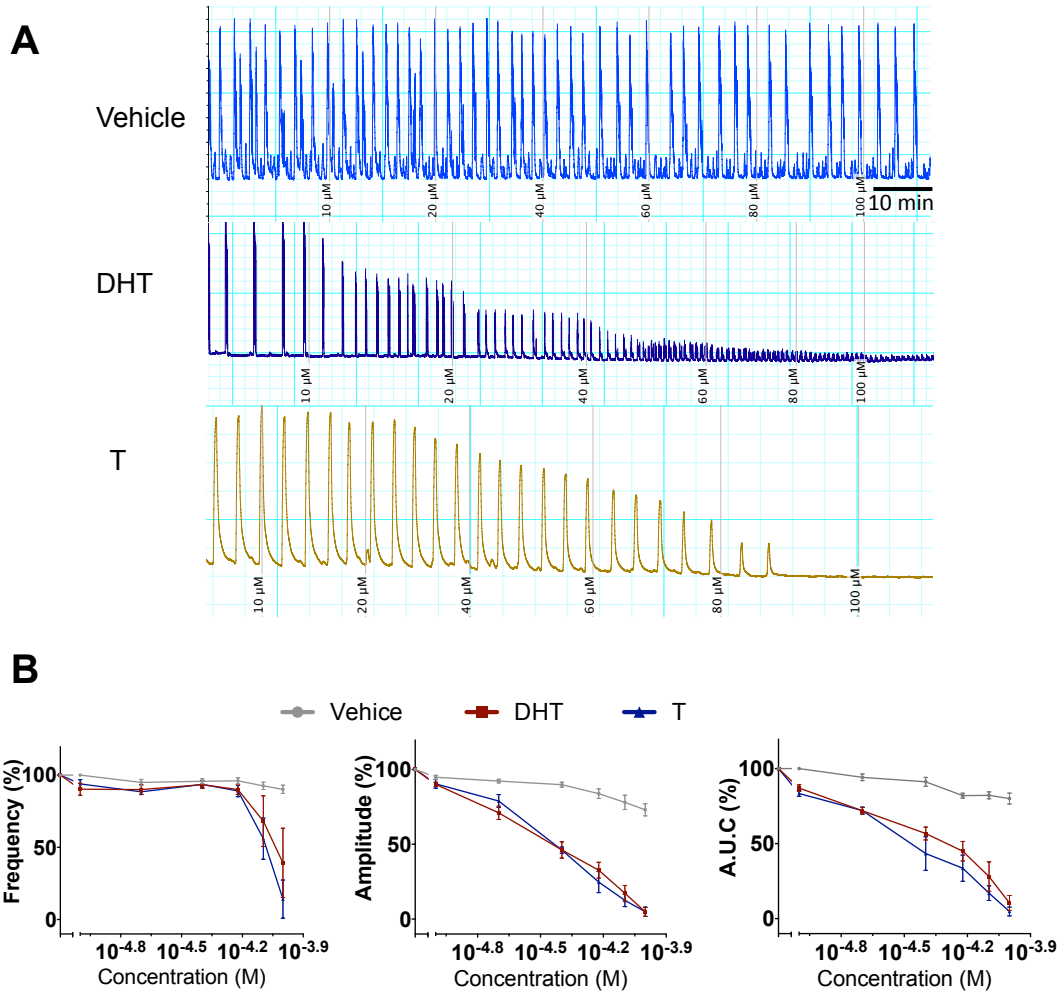
### 4.3.3 DHT and T relaxed murine spontaneous uterine contractions

The effect of DHT and T on spontaneous contractions was additionally examined using uterine horns obtained from non-pregnant mice. The uterine horns were prepared into strips and each strip was incubated with cumulative doses of 10 μM, 20 μM, 40 μM, 60 μM, 80 μM and 100 μM of DHT or T each for 10 minutes (**Figure 4.5 A**). Equivalent doses of etOH (vehicle) were applied in the same range (minimum 0.03% etOH and maximum 0.3% etOH) (**Figure 4.5 A**). The effect of DHT and T was then compared to the effect of vehicle at the relevant doses.

The DHT inhibited the amplitude and the A.U.C of murine uterine contraction in a dose-dependent manner (**Figure 4.5 B**). DHT did not change the frequency of contraction at any dose from 10 μM to 80 μM, but significantly decreased the frequency to 39.2% ± 24 of the original value at the 100 μM dose ($p<0.001$ compared to vehicle). The minimum significant inhibition of amplitude and A.U.C of contraction was observed at the 20 μM dose; the amplitude was reduced to 71% ± 4.4 and the A.U.C to 71.9% ± 2.1 (**Figure 4.5 C**). The maximum inhibition was observed at the 100 μM dose; the amplitude was reduced to 4.8% ± 3 and the A.U.C was reduced to 10.4% ± 5 (**Figure 4.5 C**). The IC50 of DHT for amplitude of contraction was 30.5 μM (24 μM to 37 μM 95% C.I) and the IC50 of DHT for A.U.C was 40 μM (32 μM to 51 μM 95% C.I) (**Figure 4.5 C**).

In a similar manner to DHT, T inhibited the amplitude and the A.U.C of uterine contraction in a dose-dependent manner (**Figure 4.5 B**). T did not change the frequency of contraction at any dose from 10 μM to 60 μM, but significantly decreased the frequency to 55.9 ± 14.3 of the original value ($p<0.001$ compared to vehicle) at the 80 μM dose and 14.1 ± 13.2 ($p<0.0001$) at the 100 μM dose (**Figure 4.5 B**). The minimum significant inhibition of amplitude and A.U.C of contraction was observed at the 20 μM dose; the amplitude was reduced to 78.8% ± 4.3 and the A.U.C to 72% ± 2.3 (**Figure 4.5 C**). The maximum inhibition was observed at the 100 μM dose; the amplitude was reduced to 4.9% ± 3 and the A.U.C was reduced to 4.8% ± 2.9 (**Figure 4.5 C**). The IC50 of T for amplitude of contraction was 40 μM (32 μM to 51 μM 95% C.I.) and the IC50 of T for A.U.C was and 30 μM (28 μM to 38 μM 95% C.I.) (**Figure 4.5 C**).

There were no significant differences between the effects of DHT and T on the inhibition of murine uterine contractions.




**C**

**Amplitude**

| Compound | Concentration | | | | | | IC50 |
|---|---|---|---|---|---|---|---|
| | 10 µM | 20 µM | 40 µM | 60 µM | 80 µM | 100 µM | |
| DHT | ns | 71±4.4% (p<0.01) | 46.2±5.4% (p<0.0001) | 32.6±5.2% (p<0.0001) | 17.2±5.1% (p<0.0001) | 4.8±3% (p<0.0001) | 30.5 µM (24 to 37 95% C.I.) |
| T | ns | 78.8±4.3% (p<0.01) | 46±2% (p<0.0001) | 24.7±7% (p<0.0001) | 12.5±4.1% (p<0.0001) | 4.9±3% (p<0.0001) | 30.7 µM (23 to 39 95% C.I.) |

**AUC**

| Compound | Concentration | | | | | | IC50 |
|---|---|---|---|---|---|---|---|
| | 10 µM | 20 µM | 40 µM | 60 µM | 80 µM | 100 µM | |
| DHT | ns | 71.9±2.1% (p<0.01) | 56.7±4.3% (p<0.001) | 44.9±6.4% (p<0.0001) | 28±9.8% (p<0.0001) | 10.4±5% (p<0.0001) | 40 µM (32 to 51 95% C.I.) |
| T | ns | 72±2.3% (p<0.01) | 43.4±11.2% (p<0.0001) | 33.6±8.7% (p<0.0001) | 17±4.9% (p<0.0001) | 4.8±2.9% (p<0.0001) | 30 µM (28 to 38 95% C.I.) |

**Figure 4.5:** The effect of DHT and T on spontaneous contractions of mouse uterine strips. (**A**) Representative recordings following cumulative administration of DHT and T. (**B**) Concentration response curves showing the effect of DHT, T and vehicle on frequency, amplitude and A.U.C of contraction. 100 µM of DHT significantly decreased the frequency of contraction ($p<0.001$ compared to vehicle). For T, the frequency was significantly reduced at both 80 µM ($p<0.001$ compared to vehicle) and 100 µM ($p<0.0001$ compared to vehicle) doses. (**C**) Tables show the percentage (%) of the original value to which the amplitude and A.U.C decreased following treatment with DHT and T in each concentration (p values and ns=non-significant are compared to vehicle). The last column of the table shows the IC50 values of DHT and T; the range is indicated in brackets as 95% C.I. Data were analysed using one-way ANOVA with Tukey's post-hoc test (n=5 mice, 1 strip per treatment).

### 4.3.4 DHT and T relaxed OXT-induced human myometrial contractions

In addition to the effect of DHT and T on stretch-induced myometrial contractions, their effects on OXT-stimulated contractions were explored. OXT administration (10 nM) induced a 41.8 % ± 3 increase in the frequency of contraction (**Figure 4.6 B**) and a 13.17% ± 0.56 increase in the amplitude of contraction (**Figure 4.6 C**). The OXT-stimulated increase in amplitude was significantly reduced to 4.9% ± 0.8 and 6.1% ± 0.6 of the increased value following treatment with DHT (50 μM) or T (50 μM) respectively (**Figure 4.6 C**). The OXT-induced increase in frequency was not affected by treatment with either DHT or T (**Figure 4.6 B**).

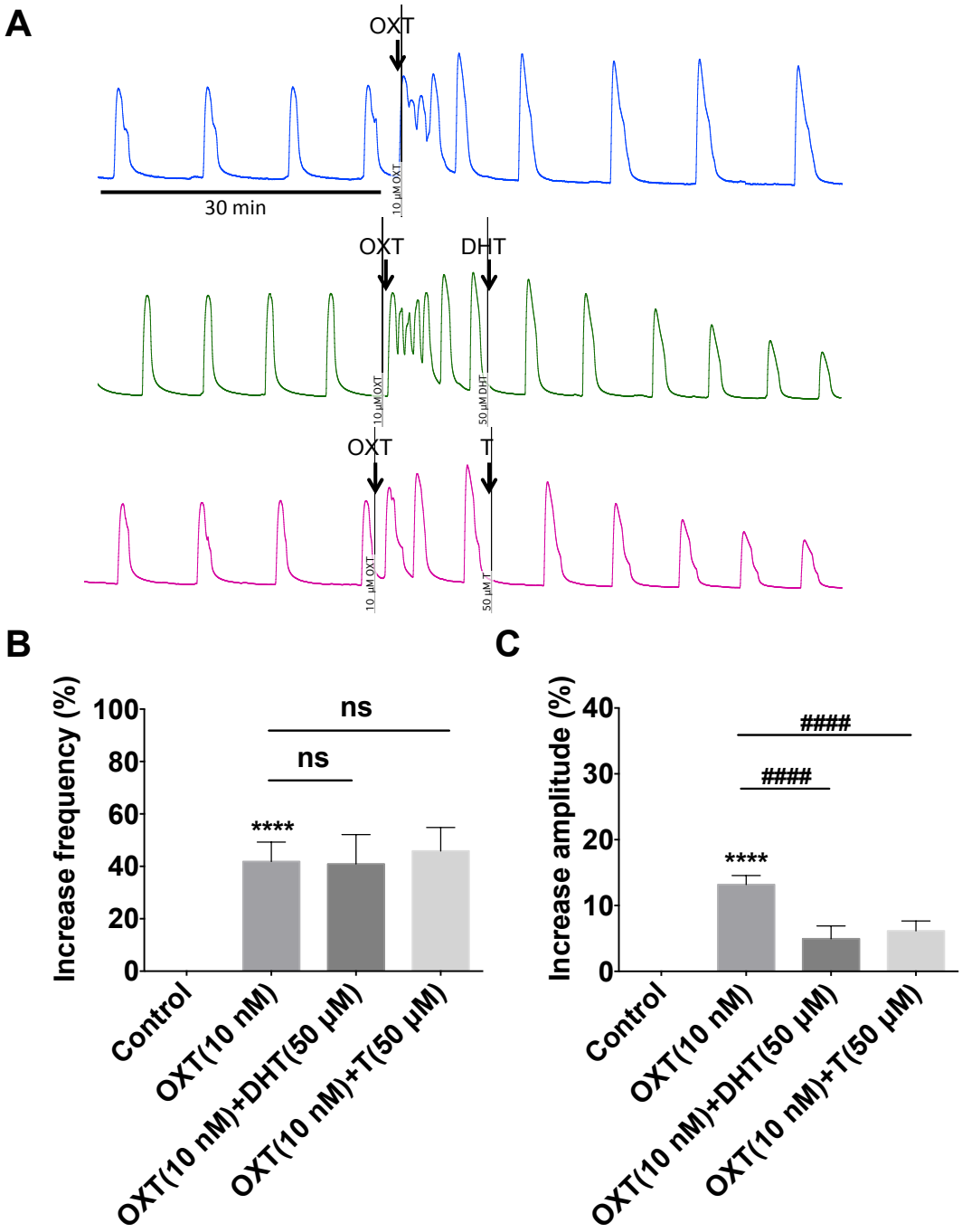



**Figure 4.6:** The relaxant effect of DHT and T on OXT-stimulated human myometrial contractions. (**A**) Representative recordings of human myometrial contractions following administration of OXT (10 nM) alone or in combination with DHT (50 µM) or T (50 µM) applied 10 min after OXT. (**B**) OXT treatment induced a significant increase in the frequency of contraction when compared to the effect of vehicle control ($dH_2O$) (****p<0.0001). Treatments with DHT and T did not decrease the OXT-induced increase in frequency (ns=non-significant). (**C**) OXT treatment induced a significant increase in the amplitude of contraction when compared to control (****p<0.0001). The OXT-stimulated increase in amplitude was significantly reduced following treatment with DHT and T (####p<0.0001). Data were analysed using one-way ANOVA with Tukey's post-hoc test (n=6 patients, 1 strip per treatment).

### 4.3.5 Androgen receptors are unlikely to mediate the relaxant effect of androgens

In order to elucidate whether the observed relaxant effect of androgens on myometrium is mediated via the classical cytoplasmic AR or a membrane AR (mAR), two different approaches were employed. To test the involvement of a mAR in the relaxant effect, human myometrial strips were treated with TBSA, an agent that is unable to penetrate through the cell membrane. TBSA (0.5 μM) did not have a significant effect, suggesting that penetration of T into the cell membrane is a necessary event in mediation of myometrial relaxation by T (**Figure 4.7 A**). To examine whether the AR, which typically resides in the cell cytoplasm, is utilised to mediate the relaxant effect of DHT on the myometrium, a potent AR antagonist namely flutamide (100 μM) was used to block the AR prior to treatment with DHT (100 μM) (**Figure 4.7 B**). However, flutamide pre-treatment did not inhibit the 5.2 ± 3.2% decrease in A.U.C induced by the DHT (100 μM) treatment (**Figure 4.7 B**). Taken together these data suggest that the relaxant effect of DHT possibly requires the agent to penetrate through the cell membrane but it does not require binding to the classical AR.

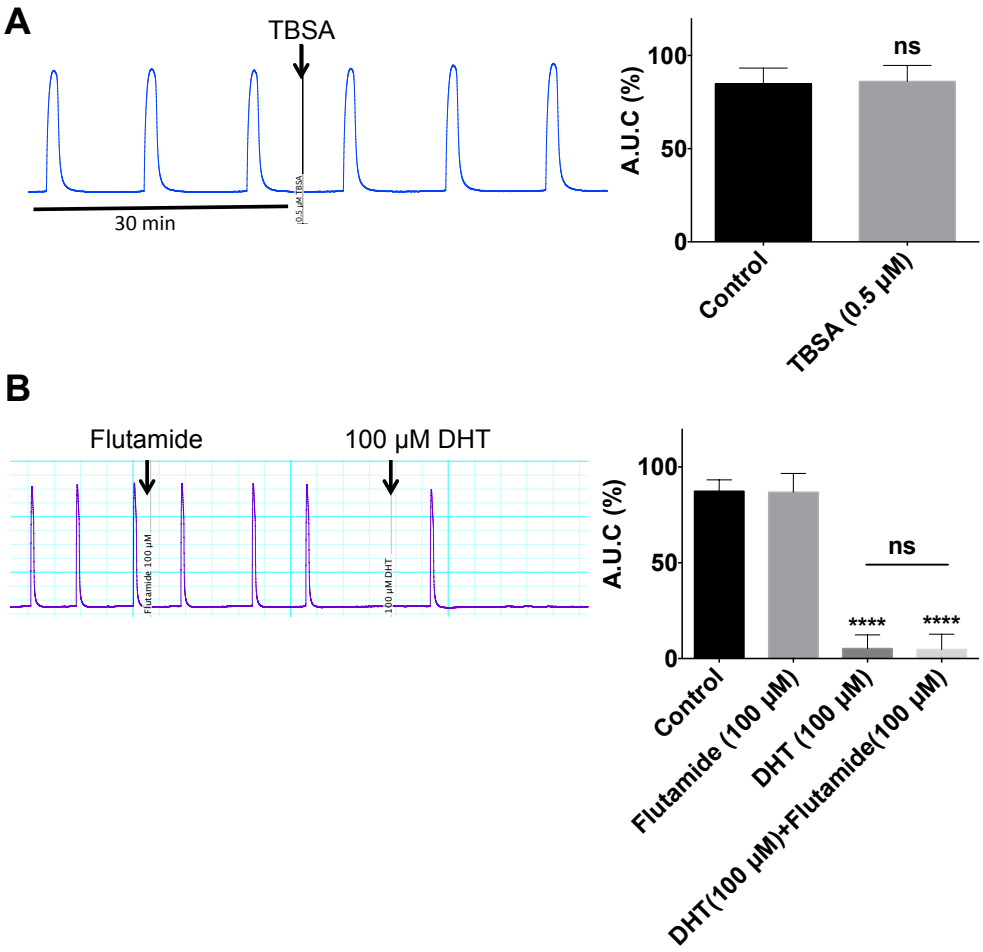



**Figure 4.7:** The effect of TBSA on human spontaneous myometrial contractions and the effect of flutamide pre-treatment on DHT-induced effect. (**A**) TBSA (0.5 µM) treatment did not induce a significant effect on the A.U.C of contraction when compared to the effect of vehicle control (PBS). (**B**) Flutamide (100 µM) pre-treatment did not inhibit the decrease in A.U.C induced by the DHT (100 µM) treatment [****p<0.0001, compared to vehicle control (etOH)]. ns=non-significant. Data were analysed with one-way ANOVA with Tukey's post-hoc test (n=5 patients, 1 strip per treatment).

### 4.3.6 E2 and P4 relaxed human spontaneous myometrial contractions

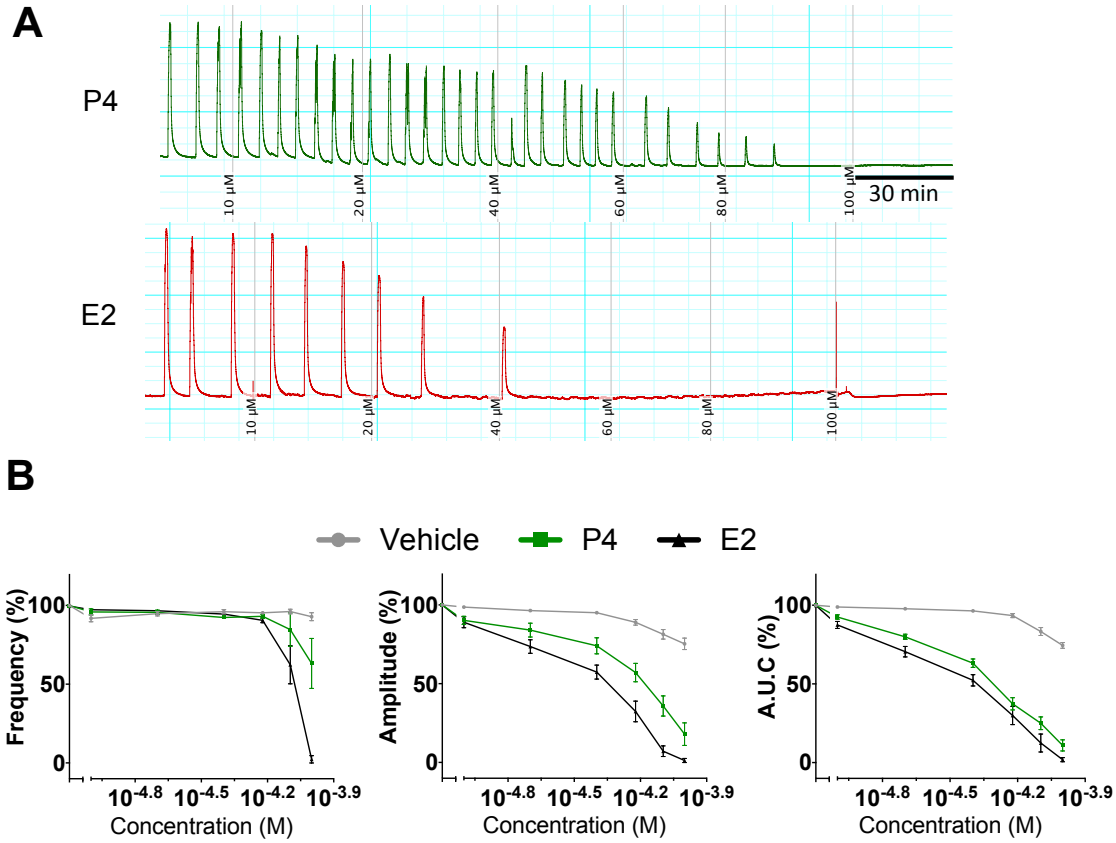
Following the observation that androgens relax spontaneous human myometrial contractions, the effect of E2 and P4 in micromolar doses ranging from 10 μM to 100 μM was explored. Cumulative doses of 10 μM, 20 μM, 40 μM, 60 μM, 80 μM and 100 μM of E2 or P4 were applied on each myometrial strip for 30 minutes (**Figure 4.8 A**). Equivalent doses of vehicle (etOH) were applied in the same range (**Figure 4.8 A**). The effect of E2 and P4 was then compared to the effect of vehicle at the relevant doses.

For E2, the frequency of contraction was significantly decreased to 62.3% ± 12 of the original value ($p<0.0001$ compared to vehicle) at the 80 μM dose and to 2.3% ± 2 of the original value ($p<0.0001$ compared to vehicle) at the 100 μM dose. E2 and P4 both inhibited the amplitude and the A.U.C of myometrial contraction in a dose-dependent manner (**Figure 4.8 B**). The minimum significant inhibition of amplitude and A.U.C of contraction was observed at the 20 μM dose; the amplitude was reduced to 73.7% ± 4.2 and the A.U.C to 70.5% ± 3.3 of the original values (**Figure 4.8 C**). The maximum inhibition was observed at the 100 μM dose; the amplitude was reduced to 1.3% ± 1.3 and the A.U.C was reduced to 1.8% ± 1.2 of the original values (**Figure 4.8 C**). The IC50 of E2 for amplitude of contraction was 32.2 μM (29 μM to 40 μM 95% C.I) and the IC50 of E2 for A.U.C was 30 μM (25 μM to 37 μM 95% C.I) (**Figure 4.8 C**).

In a similar manner to E2, P4 inhibited the amplitude and the A.U.C of myometrial contraction in a dose-dependent manner (**Figure 4.8 B**). P4 did not change the frequency of contraction at any dose tested from 10 μM to 80 μM, but significantly decreased the frequency to 63.3% ± 15.8 ($p<0.01$) of the original value at the 100 μM dose (**Figure 4.8 B**). The minimum significant inhibition of amplitude and A.U.C of contraction was observed at the 20 μM dose; the amplitude was reduced to 84% ± 4.4 and the A.U.C to 79.9% ± 1.6 of the original values (**Figure 4.8 C**). The maximum inhibition was observed at the 100 μM dose; the amplitude was reduced to 18% ± 7.1 and the A.U.C was reduced to 10.9% ± 3.5 of the original values (**Figure 4.8 C**). The IC50 of P4 for amplitude of contraction was 62.5 μM (50 μM to 78 μM

95% C.I) and the IC50 of P4 for A.U.C was 43 μM (36 μM to 50 μM 95% C.I) (**Figure 4.8 C**).

The effect of E2 on the amplitude of contraction was significantly different compared to that of P4 at 20 μM ($p<0.05$), 40 μM ($p<0.01$), 60 μM ($p<0.01$) and 80 μM ($p<0.01$) (**Table 4.2**). Additionally, the effect of E2 on A.U.C was significantly different compared to that of P4 at 20 μM ($p<0.05$) and 40 μM ($p<0.05$) doses (**Table 4.2**). The effect on the frequency of contraction was significantly different between E2 and P4 at the 80 μM ($p<0.001$) and 100 μM ($p<0.0001$) dose (**Table 4.2**). Overall, the effect of E2 was greater than the effect of P4.




C

**Amplitude**

| Compound | | | | Concentration | | | IC50 |
|---|---|---|---|---|---|---|---|
| | 10 µM | 20 µM | 40 µM | 60 µM | 80 µM | 100 µM | |
| P4 | ns | 84±4.4% (p<0.0001) | 74±5.1% (p<0.0001) | 57.1±5.8% (p<0.0001) | 35.8±6.4% (p<0.0001) | 18±7.1% (p<0.0001) | 62.5 µM (50 to 78 95% C.I.) |
| E2 | ns | 73.7±4.2% (p<0.0001) | 57±4.4% (p<0.0001) | 32.4±6.5% (p<0.0001) | 7.2±3.4% (p<0.0001) | 1.3±1.3% (p<0.0001) | 32.2 µM (29 to 40 95% C.I.) |

**AUC**

| Compound | | | | Concentration | | | IC50 |
|---|---|---|---|---|---|---|---|
| | 10 µM | 20 µM | 40 µM | 60 µM | 80 µM | 100 µM | |
| P4 | ns | 79.9±1.6% (p<0.0001) | 63.2±2.7% (p<0.0001) | 37.3±3.8% (p<0.0001) | 25±4% (p<0.0001) | 10.9±3.5% (p<0.0001) | 43 µM (36 to 50 95% C.I.) |
| E2 | ns | 70.5±3.3% (p<0.0001) | 52.3±3.5% (p<0.0001) | 29.9±5.8% (p<0.0001) | 12.4±5.7% (p<0.0001) | 1.8±1.3% (p<0.0001) | 30 µM (25 to 37 95% C.I.) |

**Figure 4.8:** The effect of P4 and E2 on human spontaneous myometrial contractions. (**A**) Representative recordings following addition of cumulative doses of P4 and E2. (**B**) Concentration response curves for frequency, amplitude and A.U.C of contraction. At 100 µM dose of P4, the frequency of contraction was reduced ($p<0.01$ compared to vehicle). For E2, the frequency of contraction was significantly decreased at 80 µM and at 100 µM dose ($p<0.0001$ compared to vehicle) (**C**) Tables show the percentage (%) of the original value to which the amplitude and A.U.C decreased following incubation with P4 or E2 in each concentration (p values and ns=non-significant are compared to vehicle). The last column of the table shows the IC50 values of P4 and E2; the range is indicated in brackets as 95% C.I. Data were analysed using one-way ANOVA with Tukey's post-hoc test (n=9 patients, 1 strip per treatment).

**Table 4.2:** Comparison between the effect of E2 and P4 on human myometrial contractions. E2 had a greater effect when compared to the effect of P4. Data were analysed using one-way ANOVA with Tukey's post-hoc test, n=9.

| Comparison E2 vs P4 | Concentration | | | | | |
|---|---|---|---|---|---|---|
| | 10 µM | 20 µM | 40 µM | 60 µM | 80 µM | 100 µM |
| Frequency | ns | ns | ns | ns | p<0.001 | p<0.0001 |
| Amplitude | ns | p<0.05 | p<0.01 | p<0.01 | p<0.01 | ns |
| A.U.C | ns | p<0.05 | p<0.05 | ns | ns | ns |

### 4.3.7 E2 and P4 relaxed murine spontaneous uterine contractions

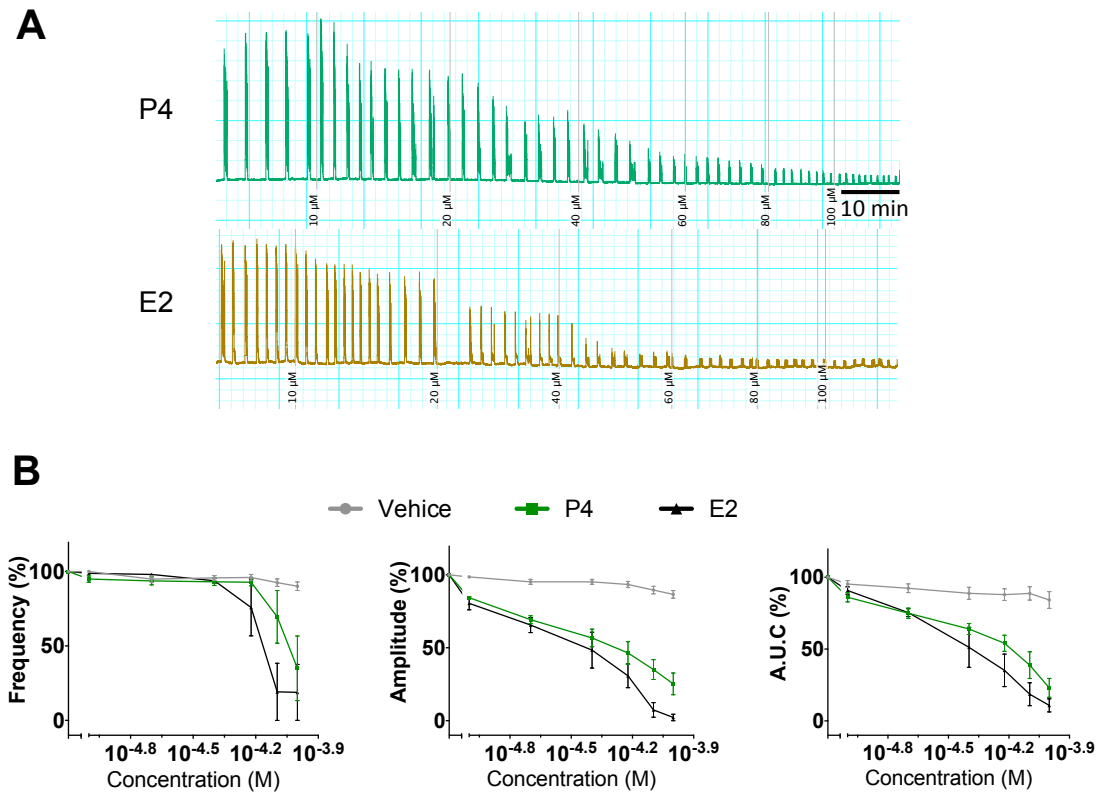
In addition to the human spontaneous myometrial contractions, E2 and P4 relaxed spontaneous contractions of murine uterine strips in micromolar doses ranging from 10 µM to 100 µM. Cumulative doses of 10 µM, 20 µM, 40 µM, 60 µM, 80 µM and 100 µM of E2 or P4 were applied on each uterine strip for 30 minutes (**Figure 4.9 A**). Equivalent doses of vehicle were applied in the same range (**Figure 4.9 A**). The effect of E2 and P4 was then compared to the effect of vehicle.

For E2, the frequency of contraction was significantly decreased to 19.2% ± 19 of the original value ($p<0.0001$ compared to vehicle) at the 80 µM dose and 18.8% ± 18 ($p<0.0001$) at the 100 µM. E2 and P4 inhibited in a dose-dependent manner the amplitude and the A.U.C of myometrial contractions (**Figure 4.9 B**). The minimum significant inhibition of amplitude and A.U.C of contraction was observed at the 20 µM dose; the amplitude was reduced to 65.6% ± 5.1 and the A.U.C to 75% ± 2.7 of the original values (**Figure 4.9 C**). The maximum inhibition was observed at the 100 µM dose; the amplitude was reduced to 2.2% ± 2.2 and the A.U.C was reduced to 10.7% ± 4.5 (**Figure 4.9 C**). The IC50 of E2 for amplitude of contraction was 27 µM (20 µM to 35 µM 95% C.I) and the IC50 of E2 for A.U.C was 36 µM (26 µM to 49 µM 95% C.I) (**Figure 4.9 C**).

In a similar manner to E2, P4 inhibited the amplitude and the A.U.C of myometrial contractions in a dose-dependent manner (**Figure 4.9 B**). P4 did not change the frequency of contraction at any dose tested from 10 µM to 80 µM, but significantly decreased the frequency to 35% ± 21% of the original value ($p<0.01$ compared to vehicle) at a 100 µM dose (**Figure 4.9 B**). The minimum significant inhibition of the amplitude and A.U.C of contraction was observed at the 20 µM dose; the amplitude was reduced to 69.2% ± 2.7 and the A.U.C to 74.9% ± 3.5 (**Figure 4.9 C**). The

maximum inhibition was observed at the 100 μM dose; the amplitude was reduced to 25.2% ± 7.4 and the A.U.C was reduced to 22.9% ± 6.5 (**Figure 4.9 C**). The IC50 of P4 for amplitude of contraction was 46 μM (38 μM to 56 μM 95% C.I) and the IC50 of P4 for A.U.C was 55 μM (45 μM to 68 μM 95% C.I) (**Figure 4.9 C**).

The effect of E2 on the amplitude of contraction was significantly different compared to that of P4 at 80 μM ($p<0.01$) and 100 μM ($p<0.05$) doses (**Table 4.3**). In addition, the effect of E2 on frequency was significantly different compared to that of P4 at the 80 μM ($p<0.01$) dose (**Table 4.3**). Overall the effect of E2 was greater compared to the effect of P4.




C

**Amplitude**

| Compound | Concentration | | | | | | IC50 |
|---|---|---|---|---|---|---|---|
| | 10 µM | 20 µM | 40 µM | 60 µM | 80 µM | 100 µM | |
| P4 | ns | 69.2±2.7% (p<0.0001) | 56.7±6% (p<0.0001) | 46.4±7.7% (p<0.0001) | 35±6.8% (p<0.0001) | 25.2±7.4% (p<0.0001) | 46 µM (38 to 56 95% C.I.) |
| E2 | ns | 65.6±5.1% (p<0.0001) | 48.4±12.2% (p<0.0001) | 30.8±8.1% (p<0.0001) | 7.4±4.9% (p<0.0001) | 2.2±2.2% (p<0.0001) | 27 µM (20 to 35 95% C.I.) |

**AUC**

| Compound | Concentration | | | | | | IC50 |
|---|---|---|---|---|---|---|---|
| | 10 µM | 20 µM | 40 µM | 60 µM | 80 µM | 100 µM | |
| P4 | ns | 74.9±3.5% (p<0.01) | 64±3.8% (p<0.001) | 54±5.6% (p<0.0001) | 38±9.2% (p<0.0001) | 22.9±6.5% (p<0.0001) | 55 µM (45 to 68 95% C.I.) |
| E2 | ns | 75±2.7% (p<0.01) | 51.2±13.9% (p<0.0001) | 35.1±11.3% (p<0.0001) | 18.5±7.9% (p<0.0001) | 10.7±4.5% (p<0.0001) | 36 µM (26 to 49 95% C.I.) |

**Figure 4.9:** The effect of P4 and E2 on murine spontaneous uterine contractions. (**A**) Representative recordings following addition of cumulative doses of P4 and E2. (**B**) Concentration response curves for frequency, amplitude and A.U.C of contraction. At 100 µM dose of P4, the frequency of contraction was significantly reduced ($p<0.01$ compared to vehicle). For E2, the frequency of contraction was significantly decreased at 80 µM and at 100 µM dose ($p<0.0001$ compared to vehicle). (**C**) Tables show the percentage (%) of the original value to which the amplitude and A.U.C decreased following P4 and E2 in each concentration (p values and ns=non-significant are compared to vehicle). The last column of the table shows the IC50 values of P4 and E2; the range is indicated in brackets as 95% C.I. Data were analysed using one-way ANOVA with Tukey's post hoc test (n=5 mice, 1 strip per treatment).

**Table 4.3:** Comparison between the effect of E2 and P4 on murine myometrial contractions. The effect of E2 was greater than the effect of P4. Data were analysed using one-way ANOVA with Tukey's post-hoc test, n=5.

| Comparison E2 vs P4 | Concentration | | | | | |
|---|---|---|---|---|---|---|
| | 10 µM | 20 µM | 40 µM | 60 µM | 80 µM | 100 µM |
| Frequency | ns | ns | ns | ns | p<0.01 | ns |
| Amplitude | ns | ns | ns | ns | p<0.01 | p<0.05 |
| A.U.C | ns | ns | ns | ns | ns | ns |

### 4.3.8 P4 and E2 relax OXT-stimulated human spontaneous myometrial contractions

Likewise DHT and T, P4 and E2 relaxed OXT-stimulated human myometrial contractions. OXT administration (10 nM) induced a 41.8 % ± 3 increase in the frequency of contraction (**Figure 4.10 B**) and a 13.17% ± 0.56 increase in the amplitude of contraction above baseline (**Figure 4.10 C**). The OXT-stimulated increase in amplitude was significantly reduced to 6.4% ± 0.9 and 4.4% ± 0.7 of the original increase following treatment with P4 (50 µM) and E2 (50 µM) respectively (**Figure 4.10 C**). The OXT-induced increase in frequency was not affected by P4 or E2 treatment (**Figure 4.10 B**).

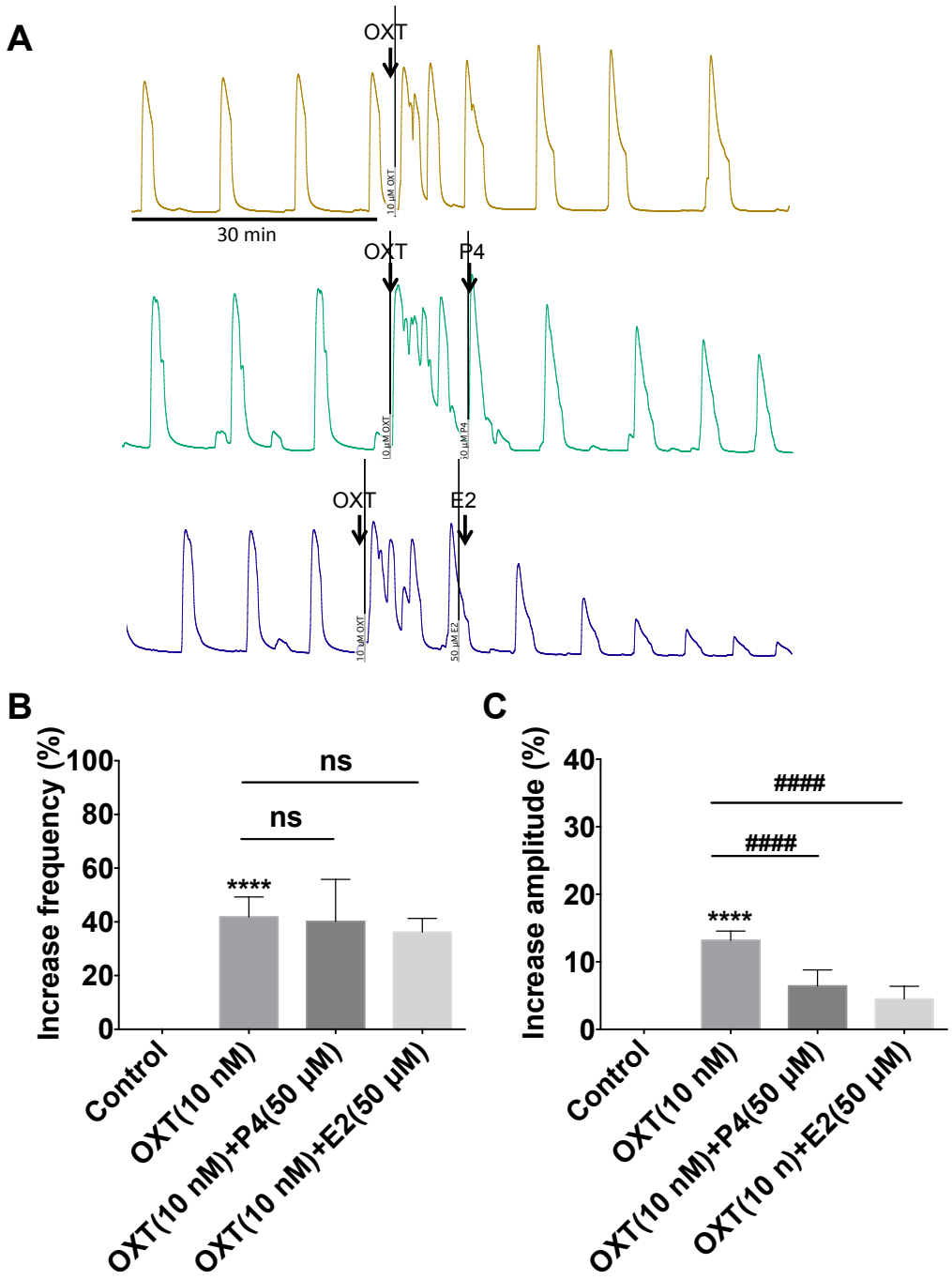



**Figure 4.10:** The effect of P4 and E2 on OXT-stimulated human myometrial contractions. (**A**) Representative recordings of human myometrial contractions following administration of OXT (10 nM) alone or in combination with P4 (50 µM) or E2 (50 µM) added 10 min after OXT. (**B**) OXT treatment alone induced an increase in the frequency of contraction when compared to the effect of vehicle control ($dH_2O$) (****$p < 0.0001$). Treatments with P4 or E2 did not decrease the OXT-induced increase in frequency (ns=non-significant). (**C**) OXT treatment induced an increase in the amplitude of contraction when compared to control (****$p < 0.0001$). The OXT-stimulated increase in amplitude was significantly reduced following treatment with P4 and E2 (####$p<0.0001$). Data were analysed using one-way ANOVA with Tukey's post-hoc test (n=6 patients, 1 strip per treatment).

### 4.3.9 The relaxant effect of DHT, T, P4 and E2 is generic and attributable to their steroid nature.

Following the observation that P4 and E2 relaxed the human myometrium and the mouse uterus in a similar manner to DHT and T, we sought to examine whether the relaxant effect was generic and, therefore, attributable to the common (steroid) nature of these hormones. A cocktail of steroid (40 μM) was prepared as described in section 4.2.2.1 of this chapter and its effect on spontaneous human and murine uterine contraction was compared to the effect induced by 40 μM of P4, E2, DHT or T.

In human, the A.U.C of the myometrial contraction was significantly decreased to 58.8% ± 5.4 of the original value ($p<0.0001$ compared to vehicle) following administration of 40 μM of steroid cocktail (**Figure 4.11 B**). The decrease induced by the cocktail was not significantly different from the decreases induced by the individual sex hormones on human spontaneous myometrial contraction at the 40 μM dose (**Figure 4.11 B**).

In mouse, the A.U.C of the uterine contraction was significantly decreased to 53.6% ± 3.9 of the original value ($p<0.0001$ compared to vehicle) following administration of 40 μM of steroid cocktail (**Figure 4.12 B**). The decrease induced by the cocktail was not significantly different from the decreases induced by the individual sex hormones on mouse spontaneous uterine contraction at the 40 μM dose (**Figure 4.12 B**).

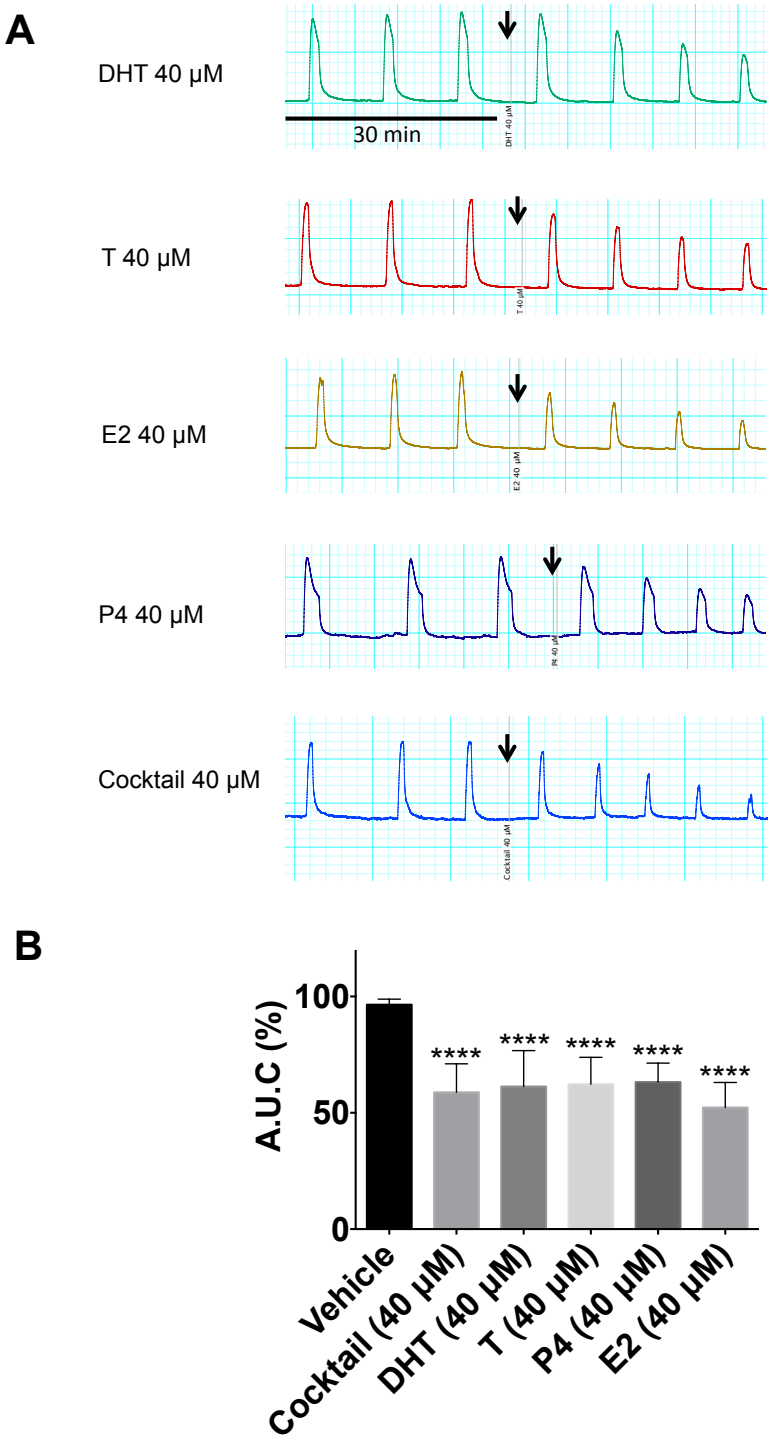



**Figure 4.11:** A steroid cocktail consisting of DHT, T, P4 and E2 relaxed spontaneous human myometrial contractions. **(A)** Representative recordings showing the effect induced by each sex hormone (40 µM). Arrows indicate addition of the compound. The last trace shows the relaxant effect induced by the steroid cocktail (40 µM). (**B**) The A.U.C of the contraction was significantly decreased following administration of steroid cocktail (40 µM) when compared to the effect of vehicle (etOH). The decrease induced by the cocktail was not significantly different from the decreases induced by the individual steroids in the same concentration (****$p<0.0001$ compared to the effect of vehicle). Data were analysed using one-way ANOVA with Tukey's post-hoc test (n=5 patients, 1 strip per treatment).

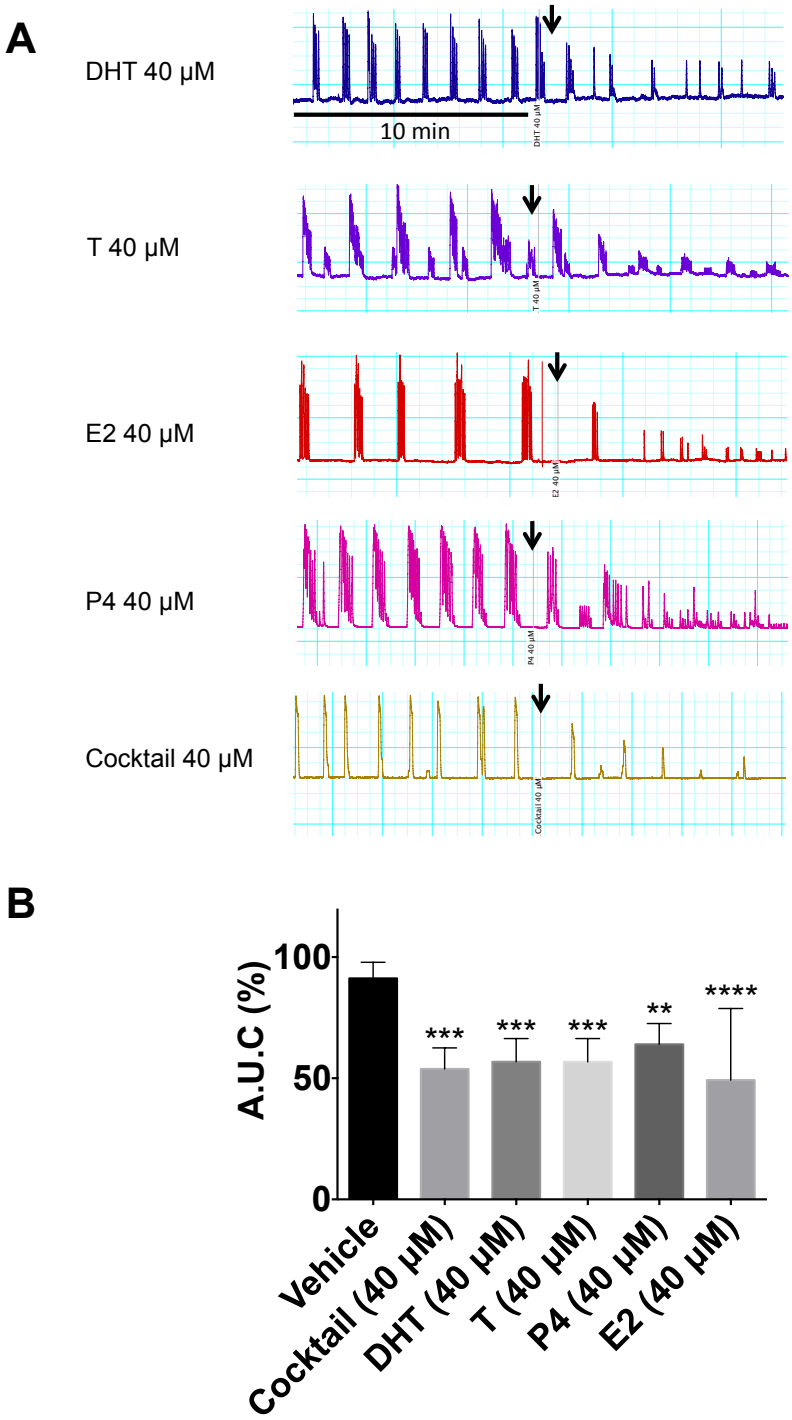



**Figure 4.12:** A steroid cocktail consisting of DHT, T, P4 and E2 relaxed spontaneous murine uterine contractions. **(A)** Representative recordings showing the relaxant effect of each sex hormone (40 µM) on uterine contractions. Arrows indicate addition of compound. The last trace shows the effect steroid cocktail (40 µM) on uterine contraction. (**B**) The A.U.C of the contraction was significantly decreased following administration of steroid cocktail when compared to the vehicle (etOH). The decrease induced by the cocktail was not significantly different from the decreases induced by each steroid in the same concentration (****p<0.0001, ***p<0.001, **p<0.01 compared to the effect of vehicle). Data were analysed using one-way ANOVA with Tukey's post-hoc test (n=5 mice, 1 strip per treatment).

## 4.4 Discussion

There is growing evidence that androgens, the concentrations of which increase throughout gestation in the maternal blood, might play a functional role in the physiology of pregnancy in parallel with the well-established roles of progestins and estrogens. Previous studies have demonstrated that high pharmacological concentrations of androgens can relax stretched-induced or compound-stimulated myometrial contractions in rabbit, rat and human (Kubli-Garfias et al., 1980; Perusquia et al., 1991b; Perusquia et al., 1990; Perusquia et al., 2005; Perusquia, 1991; Robson, 1937). However, the mechanism that mediates the relaxant effect of androgens has been poorly explored.

In this chapter we studied the effects of two androgens on the contraction of uterine muscle in term pregnant women and non-pregnant mice; T and the non-aromatisable androgen DHT. There were two reasons for employment of murine tissue in our contractility studies. First and foremost, we wanted to see if the effect that androgens had on the myometrium was conserved in human and mouse. Secondly, we wanted to generate the data on wild type C57BL/6 mice in light of our future plans to use C57BL/6 AR knockout mice and their uterus for contractility studies. We found that high (but not low) micromolar concentrations of DHT and T rapidly relaxed myometrial contractions in humans and mice in a dose-dependent manner. In particular, the amplitude and A.U.C of contraction where dose-dependently decreased following administration of both androgens. Of note, higher doses of the androgen (100 μM), induced complete relaxation of the contraction in some but not all cases, explaining the significant decrease in frequency at this dose; the frequency remained unchanged for all lower doses. The minimum concentration of DHT and T to elicit a significant relaxant effect was 20 μM. The IC50 values for the androgens indicated that there was no significant difference in the potency of T and DHT, which is in contrast to Perusquia's finding that DHT was significantly more potent compared to T in relaxing human myometrial contractions (Perusquia et al., 2005). In the latter study, the authors investigated a role for AR in inhibition of myometrial contraction and demonstrated that flutamide-induced AR antagonism failed to inhibit the relaxant effect of DHT (Perusquia et al., 2005). Notably, in the Perusquia study,

flutamide was administered at a much lower concentration (10 μM) compared to the dose of DHT (100 μM). Given that flutamide should be administered at least at the same concentration with the agonist to induce an effective antagonism of AR, it was not clear whether AR was involved in the relaxant effect. In our study, we observed that even a 100 μM dose of flutamide did not inhibit the DHT-mediated (100 μM) relaxant effect, suggesting that the effect is unlikely to be mediated via the classical AR, which resides in the cell cytoplasm.

In contrast to the data on myometrial smooth muscle described here, studies on airway SMCs showed that, treatments with membrane impermeable T conjugated to BSA (TBSA) could also inhibit muscle contraction (in addition to T itself), suggesting that T exerted its effects on the outer aspect of the membrane rather than in the cytoplasm, and implying involvement of a membrane AR (mAR) (Kouloumenta et al., 2006). This type of receptor has been initially identified in fish (Thomas et al., 2006) and is believed to be either a classic AR lacking a DNA binding site or a GPCR similar to the membrane receptors for ER (GPR30) (Thomas et al., 2005) and PR (mPR) (Zhu et al., 2003). Recently, Thomas and colleagues identified and characterised the human analogue of the mAR in (human) prostate cancer cells (Thomas et al., 2014). No effect of TBSA on spontaneous human myometrial contractions in our study, suggests that the androgen-induced relaxant effect is unlikely to be mediated via such membrane receptor. However, our TBSA experiments require further investigation, as a positive control was not utilised to prove the bioactivity of this compound in our hands. Future work employing airway SMCs to show that the TBSA used in our study is bioactive could provide an appropriate positive control to backup our data and arrive to the correct conclusion.

To further explore the mechanism of action of androgens, we sought to examine whether their action on myometrial contraction is similar to that observed for progestins and estrogens. Several studies have shown that pharmacological concentrations (at the micromolar range) of P4 and E2 can induce relaxation of uterine muscle in rats, pigs and humans (Anderson et al., 2009; Chanrachakul et al., 2005; Fu et al., 1996; Kurowicka et al., 2005; Ma et al., 2013; Sanchez Aparicio et al., 1992). Consistent with these studies, our work showed that E2 and P4 induced

rapid relaxation of human and mouse uterine smooth muscle. Minimum relaxation was observed at a dose of 20 μM for P4 and E2, when the amplitude and A.U.C of contraction was significantly decreased. Both parameters continued decreasing with increasing doses of the drug; the frequency, however, remained unchanged until higher doses of P4 and E2 were applied. IC50 values revealed that E2 was significantly more potent in induction of relaxation when compared to P4.

Oxytocin, acetylcholine and serotonin receptors are GPCRs. Their activation leads to subsequent activation of extracellular $Ca^{2+}$ influx via ROCCs and/or $Ca^{2+}$ efflux from the SR via the IP3 pathway (Large, 2002; Thorneloe and Nelson, 2005). To gain more mechanistic insight we explored whether sex hormones could inhibit OXT-induced contractions and, hence, interfere with ROCCs. We observed that DHT, T, E2 and P4 relaxed OXT-stimulated human myometrial contractions. Our results are in line with previous studies showing relaxation of OXT-stimulated myometrial contractions by P4 and E2 in horses, rats, pigs and humans (Fernandez et al., 1993; Fu et al., 1996; Kurowicka et al., 2005; Ousey et al., 2000). Androgens have also been shown to relax uterine contractions induced by serotonin, OXT and acetylcholine in rats (Perusquia et al., 1991a; Perusquia, 1991). It is possible that androgens, estrogens and progestins exert their effects on OXT-induced contraction via blockade of the IP3 pathway and release of $Ca^{2+}$ from the SR. However, inhibition of $Ca^{2+}$ release from the SR is not sufficient for complete blockade of the total $Ca^{2+}$ flux and, thus, not sufficient to reduce the concentration of intracellular $Ca^{2+}$ ($[Ca^{2+}]$). (Kupittayanant et al., 2002). Therefore, a logical assumption would be that the steroids block the ROCCs associated with $Ca^{2+}$ influx rather than components of IP3 pathway. This notion is supported by the inability of T, in another study, to inhibit both caffeine- and carbachol-induced $Ca^{2+}$ release (activators of IP3-pathway) in coronary SMC (Murphy and Khalil, 1999). Identical findings were reported in isolated rat thoracic aortic strips and porcine coronary arteries, where T could inhibit $K^{+}$-induced but not caffeine-induced contractions (Crews and Khalil, 1999a, b).

Following the observation that all four sex hormones relaxed the uterine muscle, we sought to investigate whether this effect is a generic steroid effect and, hence, not

specific to DHT, T, E2 or P4. We prepared a mixture of 10 μM of each steroid (DHT, T, E2 and P4) to make a 40 μM steroid cocktail mix. Although the application of DHT, T, E2 and P4 in a single 10 μM dose did not induce significant relaxation of spontaneous myometrial contractions, these doses combined in a cocktail significantly inhibited the contraction of uterine smooth muscle in human and mouse. The inhibition induced by the 40 μM dose of cocktail was not statistically different to the inhibition induced by 40 μM of single dose of DHT, T, P4 or E2. Therefore, we suggest that the relaxant effect is possibly generic and attributable to the steroid nature of sex hormones.

The fact that the amplitude of myometrial contraction decreased with the steroid treatment but the frequency remained unchanged might suggest that the steroid mechanism of action is via limitation of the availability of [$Ca^{2+}$] but not the recruitment of the modulators involved in the events, which initiate the contraction. Therefore it is possible that the mechanism of steroid action involves direct or indirect inhibition of $Ca^{2+}$ entry. Inhibition of $Ca^{2+}$ entry to the MSMCs may involve interaction of the steroid with ROCCs, as discussed above, VOCCs, the lipid bilayer or the gap junctions.

High $K^+$ in the extracellular space induces cell membrane depolarisation, which in turn results in opening of $Ca^{2+}$ channels (VOCCs) and an increase in the concentration of [$Ca^{2+}$] in the cell. In addition to the relaxant effect of androgens on spontaneous myometrial contractions, DHT rapidly relaxed tonic contractions (induced by high $K^+$) and decreased the concentration of [$Ca^{2+}$]; removal of the androgen reversed the effect (Perusquia et al., 2005). E2 has also been demonstrated to relax KCl-induced tonic contraction (Gutierrez et al., 1998). Therefore, it is reasonable to hypothesise that the relaxant effect on KCl-stimulated contraction in both cases would involve inhibition of VOCCs; however, it is not clear whether the effect involves physical or indirect interaction with these channels.

Besides VOCCs and ROCCs, $Ca^{2+}$ flux is highly influenced by the MSMCs cell membrane fluidity. A proton NMR spectroscopy study investigating various parameters of myometrial membrane fluidity at pregnancy, including phospholipid double bonds, fatty acid chain length and the cholesterol/phospholipid ratio, reported

that the cell membrane fluidity of MSMCs is increased with labour (Pulkkinen et al., 1998). Cell membrane fluidity is modulated by changes in concentration of membrane cholesterol (Shmygol et al., 2007). Cholesterol is a component of the lipid bilayer, which integrates into the membrane by placing its steroid ring next to the hydrocarbon chains and locating its hydroxyl group close to the head of the phospholipid (Ikonen, 2008; Simons and Vaz, 2004; van Meer, 1989). High cholesterol results in a stiff membrane, which can, reduce fluidity, whereas low cholesterol promotes membrane fluidity. Decreased membrane fluidity is characterised by $Ca^{2+}$ channel inhibition, such as VOCCs and ROCCs (Jennings et al., 1999; Shmygol et al., 2007). Administration of cholesterol *in vitro* decreased the concentration of [$Ca^{2+}$] in MSMCs and inhibited phasic contractions in rat and human studies (Smith et al., 2005; Zhang et al., 2007). On the other hand, depletion of cholesterol from the membranes of MSMCs with methyl-beta-cyclodextrin (MCD) treatment induced an increase in cell excitability and contraction in rats (Jennings et al., 1999; Shmygol et al., 2007). High cholesterol has been correlated with increased activities of NCX and PMCA, which is consistent with their roles in promoting removal of $Ca^{2+}$ from the cell (Kutryk and Pierce, 1988; Ortega and Mas-Oliva, 1984; Verbist et al., 1991). In a similar manner to cholesterol, it is plausible that cholesterol metabolites (sex hormones) mediate non-genomic responses via direct penetration into the cell membrane of MSMCs, which in turn results in reduction in membrane fluidity. For example, hydrophobic androgens, such as DHT and T, have been shown to interact with the phospholipid bilayer of the negatively charged membrane (Duval et al., 1983; Van Bommel et al., 1987). Such interaction has been shown to impair $Ca^{2+}$ homeostasis due to an increase in the activity of PMCA. Regulation of PMCA by androgens is documented in different tissues. For example, in synaptosomal plasma membranes, PMCA activity increased 95% following treatment with T (10 μM) (Deliconstantinos, 1988). In addition, PMCA activity in kidney was increased following administration of T (10 μM), as measured by *in vitro* assays where ATP-dependent $Ca^{2+}$ flux was determined by comparison of $Ca^{2+}$ transport (implying PMCA activity) with and without exogenous addition of ATP (Dick et al., 2003). In line with the latter study, androgen deprivation induced by castration altered the immunochistochemical localisation of PMCA in prostate

epithelial cells with no changes in mRNA or protein levels (Coviello et al., 2006). Additionally, the regulation of $Ca^{2+}$-ATPase activity by T has been explored in ventricular muscle of orchidectomised (ORX) rats (Witayavanitkul et al., 2013). In that study, T treatment managed to prevent ORX-induced decrease in $Ca^{2+}$-ATPase activity. Collectively, an alternative mechanism for the non-genomic action of steroids in MSMC relaxation is proposed whereby penetration into the lipid bilayer decreases membrane fluidity and results in an increase in PMCA activity and $Ca^{2+}$ efflux to the extracellular space. However, the effect of these steroids on PMCA activity in the myometrium has not yet been explored and, thus, this hypothesis remains to be confirmed in future studies.

There is some evidence that steroids may inhibit myometrial contraction by impairing IGJC. An increase in the concentration of [$Ca^{2+}$] of a single smooth muscle cell may initiate a synchronised increase in the concentration of [$Ca^{2+}$] in adjacent cells, a process mediated by gap junctions (Boittin et al., 2013; Brading and Brain, 2011; Loch-Caruso et al., 2003). Gap junction proteins increase the ability of MSMCs to generate synchronous contractions and mount a synchronous response to hormones/agents in the microenvironment of uterus during labour (Garfield and Hayashi, 1981; Ikeda et al., 1987). Connexins are gap junction trans-membrane proteins that assemble to form a gap junction; con-43 is the predominant connexin involved in initiation of contractions in the term myometrium (Chow and Lye, 1994). A study in pregnant rats on high cholesterol diet showed that their diet was associated with decrease in con-43 protein in the myometrium, suggesting possible implications for uterine contractility (Elmes et al., 2011). A study in isolated rat cardiac myocytes demonstrated that disturbance of IGJC, which was achieved either by silencing the gene that encodes con-43 or by addition of a specific drug (heptanol), induced a significant reduction in $Ca^{2+}$ transients, suggesting that con-43 may be involved in regulation of basal $Ca^{2+}$ signalling in these cells (Li et al., 2012). Interestingly, an estrogenic compound 4-OH-TCB has been shown to act in a similar manner to heptanol and significantly inhibit IGJC in rat myometrium (Tsai et al., 1998). What is more, the same study showed that 4-OH-TCB could reduce contractions of rat myometrial strips, suggesting that the relaxant effect of 4-OH-TCB is mediated through blockade of IGJC. There is evidence that androgens can

influence IGJC in isolated rat cardiac myocytes and Sertoli cells (Pluciennik et al., 1996). In that study, a dose dependent (1-25 μM) inhibition of IGJC by T propionate was reversed by withdrawal of T but was unaffected by pre-incubation with AR antagonist, implying a non-genomic effect of T (Pluciennik et al., 1996). Based on these studies, it is tempting to hypothesise that sex hormone steroids may induce relaxation of myometrium via direct interaction with the membrane proteolipidic structure, which might alter directly or indirectly the function of gap junction channels; however, more evidence in needed and this hypothesis could be investigated in future studies.

In conclusion, our organ bath studies showed that DHT, T, E2 and P4 can rapidly relax *ex vivo* spontaneous and OXT-stimulated contractions of the uterine muscle in humans and mice. The mechanism possibly did not involve recruitment of an intracellular or a cell-surface AR and was likely mediated by non-genomic pathways. Further studying of these effects could help develop a better understanding of the alternative mechanisms that can induce myometrial relaxation, which could help identify novel targets for development of new tocolytic agents.

# Chapter 5: Investigating the mechanism of sex hormone-induced relaxation of uterine smooth muscle

## 5.1 Introduction

Sex hormones have been documented to relax the uterine smooth muscle *ex vivo* in different species (Anderson et al., 2009; Chanrachakul et al., 2005; Kurowicka et al., 2005; Ousey et al., 2000; Perusquia, 2001; Perusquia et al., 1991b; Perusquia et al., 1990; Perusquia and Navarrete, 2005; Perusquia et al., 2005). The mechanism by which steroids induce relaxation is an active area of investigation and there is now evidence to suggest that it is gene transcription-independent. For example, inhibitors of transcription or translation fail to inhibit androgen- and progestin-induced relaxation in human non-pregnant and pregnant myometrium (Perusquia, 2001; Perusquia et al., 2009; Perusquia et al., 2005) and estrogen-induced relaxation in rat uterus (Perusquia and Navarrete, 2005). In addition, antagonism of nuclear sex hormone receptors, such as AR, PR and ER, does not prevent the sex hormone-induced relaxation of uterine smooth muscle, indicating that the action of sex steroids is not mediated by nuclear receptors (Perusquia, 2001). A possible toxic effect of steroids at the micromolar range has also been postulated – in other words that the inhibitory effect of steroids on smooth muscle contraction is due to cell toxicity. However, our own and other studies have observed that the relaxant effect is fully reversible following incubation with a steroid-free medium (Perusquia et al., 1990; Perusquia et al., 2005; Perusquia and Villalon, 1996). Hence, it is likely that the pharmacological effect of sex hormones is a non-toxic effect that is associated with a non-genomic physiological mechanism of action. It has been hypothesised that sex hormones act on the cell membrane by blocking $Ca^{2+}$ channels and, thus, directly interfere with the contraction pathway (Perusquia, 2001). In support of this hypothesis, the KCl-stimulated increase in the concentration of [$Ca^{2+}$] was reduced following incubation with DHT at the micromolar range, suggesting a DHT-induced blockade of L-type $Ca^{2+}$ channel in human myometrium (Perusquia et al., 2005). Several studies have demonstrated the effects of estrogens and progestins on $Ca^{2+}$ flux in MSMCs employing whole cell-current and voltage clamp methods. For example, E2 at micromolar doses could depolarise the cell membrane, attenuate the

generation of action potential and influence the inward/outward currents in rat myometrium (Inoue et al., 1999). A phytoestrogen, Genistein, has been shown to reduce L-type $Ca^{2+}$ currents of rat myocytes in a dose-dependent (micromolar) manner (Kusaka and Sperelakis, 1995). Another study showed that both P4 and E2 could inhibit $Ca^{2+}$ entry into rat myocytes (Batra and Bengtsson, 1978). Porcine studies also revealed that incubation with P4 at micromolar levels reduced OXT-stimulated increase in the concentration of $[Ca^{2+}]$ in myometrial cells derived at late pregnancy (Franczak et al., 2006). Similarly, the synthetic variant of P4 namely MPA could attenuate OXT-stimulated increase in $[Ca^{2+}]$ in human myocytes *in vitro* (Fomin et al., 1999).

The biochemical basis for the development of myometrial contraction is the interaction of actin and myosin (Aguilar and Mitchell, 2010). This process is regulated by the myosin light chain kinase (MLCK), which phosphorylates myosin light chain (MLC) at Ser 19 to activate and prepare it for interaction with actin. On the other hand, dephosphorylation of MLC by myosin phosphatase (MLCP) causes relaxation. The activity of MLCK is dependent on the availability of $[Ca^{2+}]$. Therefore, the rise in internal $Ca^{2+}$ is critical for MLC phosphorylation. Administration of ovarian steroids during pregnancy has been demonstrated to control the availability of phosphorylated MLC (PMLC) in uterine myocytes through regulation of MLCK gene expression (genomic manner) (Badia et al., 1986; Matsui et al., 1983; Soloff et al., 2011). Surprisingly, the non-genomic effects of sex hormones on MLC phosphorylation have never been explored in MSMCs; thus, it is unknown whether the observed sex hormone-induced relaxation results from poor MLC phosphorylation in uterine myocytes.

In Chapter 4, we demonstrated that DHT and T, likewise P4 and E2, could inhibit spontaneous and OXT-induced myometrial contractions of mouse and human. Herein, we examined the mechanism of the sex hormone-induced relaxation focusing on rapid sex hormone-mediated effects on $Ca^{2+}$ transients and phosphorylation of MLC. Given that the relaxant effect of sex hormones was evidently generic, rather than specific to DHT, T, P4 or E2, we performed the experiments described in this chapter using DHT and T.

### 5.1.1 Specific aims

The specific aims of this chapter were to:

- Deduce whether the myometrial cell lines available were suitable for the study of the mechanism of androgen-induced relaxation by embedding PHM1-41s and UtSMCs into collagen gels and confirming inhibition of contractility in response to DHT and T.
- Confirm that the DHT-induced relaxation is not attributable to a concentration associated toxic effect upon the myometrium but is associated with a physiological mechanism of action, whereby DHT incubation impairs $Ca^{2+}$ transients and MLC phosphorylation.

## 5.2 Materials and Methods

### 5.2.1 Cell culture

Two human myometrial cell lines, PHM1-41s and UtSMCs, were used for the *in vitro* work described in this chapter. Details about these cell lines are available in Chapter 2 (section 2.3.1). PHM1-41s and UtSMCs were cultured as described in Chapter 2 (section 2.3.2) and used for collagen contraction assays, calcium assays and in-cell Westerns (ICW). PHM1-41s were used in passages (p) 12-20 and UtSMCs were used in p 4-13.

### 5.2.2 Collagen contraction assay

A collagen contraction assay was used to deduce whether PHM1-41s and UtSMCs responded to DHT and T *in vitro* as the myometrial tissue *ex vivo*.

UtSMCs and PHM1-41s were embedded in collagen gels ($10^5$ cells/gel) and left to polymerase overnight at 37$^o$C as per methods described in Hutchinson et al (Hutchinson et al., 2014) and detailed in Chapter 2 (section 2.9.2). Briefly, following the overnight incubation, gels were serum-starved for 2 hours by addition of FBS-free phenol red-free DMEM (4.5 g/ L glucose) supplemented with 1% penicillin-streptomycin/L-Glutamine. The medium was then replaced with phenol red-free DMEM (4.5 g/ L glucose) supplemented with 1% penicillin-streptomycin/L-Glutamine and 5% (v/v) charcoal stripped FBS. Treatments were prepared in the same medium and added to the gels. Following addition of the treatments, gels were detached using a pipette tip around the gel edge and photographed using a Leica MZ6 light microscope/ camera (0-hour time point). The gels were additionally photographed at 24-hour and 48 hour time points; the plates were kept at 37$^o$C in between these time points.

#### *5.2.2.1 Treatments of collagen gels*

DHT, T, TBSA and flutamide were purchased from Sigma and OXT was purchased from ALLIANCE pharmaceuticals (details of drugs can be found in **Table 2.13**). DHT, T and flutamide were reconstituted in etOH, TBSA in PBS and OXT in $dH_20$ according to supplier's instructions (see section 4.2.2.1 in Chapter 4 for more details). Six replicates were used for each treatment. Only flutamide was added to the

gels for 30 minutes prior to the treatment with DHT. The gels were detached following addition of the last treatment.

In experiments with DHT, T and flutamide the vehicle was etOH. In experiments with TBSA+T the vehicle was etOH+PBS. In experiments with OXT the vehicle was $dH_20$. In experiments with OXT+DHT the vehicle was etOH+$dH_20$.

PHM1-41s and UtSMCs were used in p 12-19 and 4-11 respectively and each experiment was repeated between 4 and 7 times (n=4-7).

#### *5.2.2.2 Gel analysis*

A decrease in collagen gel area equated to an increase in contraction. Gels were photographed and photos were analysed using Adobe Photoshop CS6 as described in section 2.9.3. The pixel number of each gel area was used as readout. The gel area at 24 and 48 hours was expressed as the percentage of the original gel area (0-hour time point).

#### *5.2.2.3 Cell viability*

We assessed the viability of PHM1-41s (p 15-18; n=4) and UtSMCs (p 8-11; n=4) embedded in gels and incubated with DHT and T (100 μM) for 48 hours using the CellTiter 96® AQueous One Solution Cell Proliferation Assay as described in detail in section 2.9.4.

### 5.2.3 ICW

The phosphorylation of MLC in PHM1-41s and UtSMCs was explored. Due to the rapid oscillations between the phosphorylated and dephosphorylated states of MLC and in order to accurately capture the cell transient contractile state, we utilised ICW instead of classical Western gel analysis (Aguilar et al., 2010; Hutchinson et al., 2014); described in detail in section 2.8.2. Briefly, PHM1-41s and UtSMCs were resuspended in phenol-free DMEM (4.5 g/ L glucose) supplemented with 1% penicillin-streptomycin/L-Glutamine and 5% (v/v) charcoal stripped FBS and seeded onto black 96-well plates with a clear bottom to a density of 17600 cells/ well in a volume of 200 μl. Plates were initially left for 1 hour in room temperature to prevent cell clump formation and then placed into the incubator (37$^{o}$C) overnight. The

medium was replaced with 100 µl fresh DMEM and treatments were applied as described below.

#### *5.2.3.1 Treatments for ICW*

All treatments were prepared in phenol-free DMEM (4.5 g/ L glucose) supplemented with 1% penicillin-streptomycin/L-Glutamine and 5% (v/v) charcoal stripped FBS.

In order to assess the effect of OXT on phosphorylation of MLC, PHM1-41s (p 15-20; n=6) and UtSMCs (p 4-9; n=6) were incubated with either OXT (10 nM and/or 100 nM) or vehicle ($dH_2O$) for 30 or 60 seconds. Treatments were applied using a multichannel pipette in triplicate.

Nifedipine, which is a L-Type $Ca^{2+}$ channel blocker, was used as a positive control as it has been shown to inhibit OXT-induced effect on phosphorylation of MLC in myometrial cells (Aguilar et al., 2010). Nifedipine was purchased from Sigma (N7634) and reconstituted in DMSO as per the supplier's instructions. The cells were pre-treated with nifedipine (50 µM) or vehicle (DMSO) for 15 minutes prior to acute (30 seconds) stimulation with OXT (100 nM). These experiments were performed using PHM1-41s (p 16-20; n=5) and UtSMCs (p 5-9; n=5).

PHM1-41s (p 16-20; n=5) and UtSMCs (p 5-9; n=5) were additionally pre-incubated with DHT (50 µM) for 15 minutes prior to acute (30 seconds) OXT (100 nM) stimulation.

The cells were then fixed, blocked and incubated with primary and secondary antibodies against PMLC and α-Tubulin as described in section 2.8.2. The details of the antibodies used for the ICW protocol are shown in **Table 2.10** (Chapter 2).

#### *5.2.3.2 Analysis of ICW*

The plates were scanned using the LI-COR Odyssey scanner as described in section 2.8.2. The intensity of fluorescence was measured using the LI-COR Image Studio software and the fluorescent intensity in each well was used as readout. The raw fluorescent intensities for PMLC and α-Tubulin were exported into Excel, were the intensity of PMLC in each well was calculated relative to the intensity of α-Tubulin

in the same well. Statistics were performed on these relative values of PMLC without further normalisation.

### 5.2.4 Calcium assay

The BD$^{TM}$ Calcium Assay kit was used to measure intracellular $Ca^{2+}$ in PHM1-41s and UtSMCs as described in section 2.11.

PHM1-41s and UtSMCs were resuspended in phenol-free DMEM (4.5 g/ L glucose) supplemented with 1% penicillin-streptomycin/L-Glutamine and 5% (v/v) charcoal stripped FBS and seeded onto sterile white 96-well plates with a clear bottom ($3x10^4$ cells/well) in a volume of 100 µl. Plates were left for 1 hour at room temperature to prevent formation of cell clumps in the middle of the well and placed into the incubator ($37^oC$) for an overnight incubation. The dye-loading solution (DLS), containing the $Ca^{2+}$ indicator, was prepared as described in 2.11.2 and added into each well for 1 hour. Treatments were applied following the dye-loading step.

#### *5.2.4.1 Treatments and calcium assay*

Following incubation with DLS, the plates were placed onto a fluorometric imaging plate reader (FLIRP)-NOVOstar as described in section 2.11.3.

To examine the effect of OXT on $Ca^{2+}$ flux, the injector of the NOVOstar was loaded with OXT (10 nM and/or 100 nM) or vehicle ($dH_20$). The basal concentration of [$Ca^{2+}$] was recorded for 20 seconds and then the treatment was injected directly into the well.

To examine to effect of DHT pre-treatment on OXT-induced effect on the concentration of [$Ca^{2+}$], the cells were incubated with DHT (100 nM, 300 nM, 800 nM and 50 µM) or vehicle (etOH) for 10 minutes at room temperature following incubation with DLS. DHT and vehicle treatments were prepared in phenol-free DMEM (4.5 g/ L glucose) supplemented with 1% penicillin-streptomycin/L-Glutamine and 5% (v/v) charcoal stripped FBS. Following incubation with DHT or vehicle, plates were placed onto the NOVOstar plate reader and injected with OXT (10 nM). In addition, untreated cells were injected with either DHT or vehicle ($dH_20$ or etOH).

All treatments (and injections) were performed in quadruplicate. The PHM1-41s and UtSMCs were used in p 14-20 and 4-9 respectively and each experiment was repeated 6 times (n=6).

#### *5.2.4.2 Analysis of calcium assay*

Each well's fluorescence was monitored and recorded for a total of 1 minute as described in section 2.11.3. The highest fluorescence measurement recorded (peak) following injection of treatment was used as readout and analysed using MARS Data Analysis Software. The readouts were exported into Excel sheets and data between different treatments were compared.

### 5.2.5 Statistical analysis

All data in this chapter were analysed using Graphpad prism v6.0. Data were first assessed for normality as described in methods section 2.14 of Chapter 2.

All collagen gel assay data (percentage of the original gel area at 0-hour time point) was initially changed to proportions and arcsine transformed. The arcsine-transformed data were analysed using one-way ANOVA with Tukey's correction.

For ICW, all data were analysed using one-way ANOVA with Tukey's post-hoc test.

Calcium assay data were analysed using one-way ANOVA with either Tukey's (comparison between all groups) or Sidak's (comparison between selected groups) correction. Unless otherwise stated, error bars denote ±SEM and significance was considered to be $p<0.05$.

## 5.3 Results

### 5.3.1 Establishing an *in vitro* model to study steroid-induced inhibition of uterine muscle contraction

We tested whether PHM1-41s and/or UtSMCs were suitable *in vitro* model to study the mechanism of steroid-induced relaxation. In particular, we examined the effects of androgens DHT and T. The effects of P4 and E2 were not investigated, as the data from Chapter 2 suggested that the action of sex hormones on myometrial contraction was generic.

#### *5.3.1.1 DHT and T inhibited PHM1-41s and UtSMCs contraction*

PHM1-41s and UtSMCs were embedded in collagen gels. Untreated gels developed a spontaneous (basal) contraction, which was evident by the time-dependent decrease in the gel area (**Figure 5.0**).

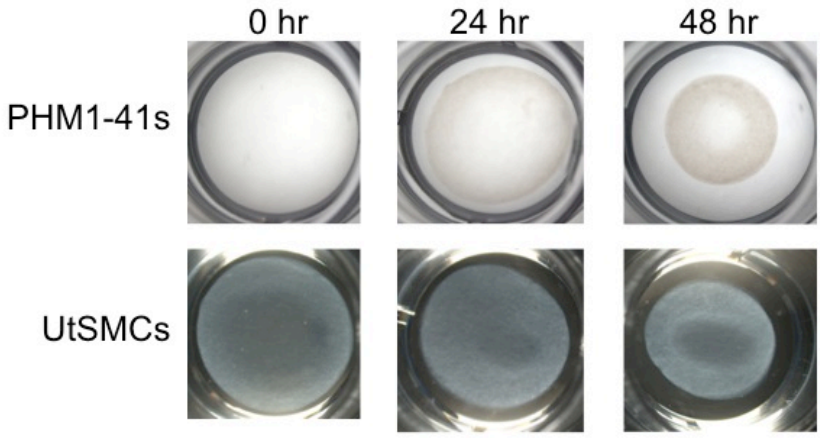



**Figure 5.0:** PHM1-41s and UtSMCs embedded in collagen gels develop a basal contraction, which is evident by the decrease in the gel area following 24 and 48 hrs.

We sought to examine the effects of DHT and T on the basal contraction of the gel. Therefore, PHM1-41s and UtSMCs were embedded in collagen gels and the gels were incubated with vehicle (etOH) or DHT (1 μM, 50 μM, 100 μM) or T (1 μM, 50 μM, 100 μM) for 24 and 48 hours. The above doses were chosen on the basis of our organ bath data, which showed that only the concentrations above 10 μM of DHT and T inhibit the contraction of myocytes. The 1 μM dose was chosen as a negative control. The gel area of cells treated with DHT or T was compared to the

gel area of cells treated the vehicle at both time points. The gel area was expressed as the percentage of the gel area recorded at the 0-hour time point.

In PHM1-41s, the DHT treatment inhibited gel's basal contraction (vehicle) at 24 and 48 hours when the DHT was applied at 50 μM and 100 μM but not 1 μM dose (**Figure 5.1 B, C**). Specifically, at 24 hours (**Figure 5.1 B**) the gel area was decreased to 77.28% ± 3.4 with the vehicle treatment, to 94.18% ± 1.6 with the DHT-50 μM treatment ($p<0.01$) and 95% ± 1.2 with the DHT-100 μM treatment ($p<0.001$). At 48 hours (**Figure 5.1 C**), the gel area was decreased to 65.23% ± 3.7 with the vehicle treatment, to 87.66% ± 2.5 with the DHT-50 μM treatment ($p<0.01$) and to 82% ± 6.4 with the DHT-100 μM treatment ($p<0.05$). Similarly, all doses of T but the 1 μM inhibited PHM1-41s basal gel contraction at 24 and 48 hours (**Figure 5.1 D, E)**. Specifically, at 24 hours (**Figure 5.1 D**), the gel area was decreased to 77.44% ± 3.3 with the vehicle treatment, to 94.53% ± 1.8 with the T-50 μM treatment ($p<0.001$) and to 94.9% ± 1.2 with the T-100 μM treatment ($p<0.001$). At 48 hours (**Figure 5.1 E**), the gel area was decreased to 61.4% ± 3.7 with the vehicle treatment, to 81% ± 3 with the T-50 μM treatment ($p<0.05$) and to 87.7% ± 5.5 with the T-100 μM treatment ($p<0.001$).

Similar effects were observed for UtSMCs (**Figure 5.2**). Treatment with DHT and T at 50 μM and 100 μM but not 1 μM dose inhibited the gel's basal contraction at 24 and 48 hours. In particular, at 24 hours (**Figure 5.2 B**), the gel area was decreased to 76.3% ± 3 with the vehicle treatment, to 94.5% ± 1.7 with the DHT-50 μM treatment ($p<0.0001$) and to 95.7% ± 1 with the DHT-100 μM treatment ($p<0.0001$). At 48 hours (**Figure 5.2 C**), the gel area was decreased to 57.7% ± 4 with the vehicle treatment, to 79% ± 4 with the DHT-50 μM treatment ($p<0.001$) and to 88.4% ± 1.7 with the DHT-100 μM treatment ($p<0.0001$). When UtSMCs were treated with T for 24 hours, the gel area was only decreased to 94% ± 2.7 with the T-50 μM treatment ($p<0.0001$) and to 95.7% ± 1.4 with the T-100 μM treatment ($p<0.0001$) as opposed to 76.2% ± 3 with the vehicle treatment (**Figure 5.2 D**). At 48 hours (**Figure 5.2 E**), the gel area of UtSMCs was decreased to 87.7% ± 3 with the T-50 μM treatment ($p<0.0001$) and to 87% ± 4.4 with the T-100 μM treatment ($p<0.001$).

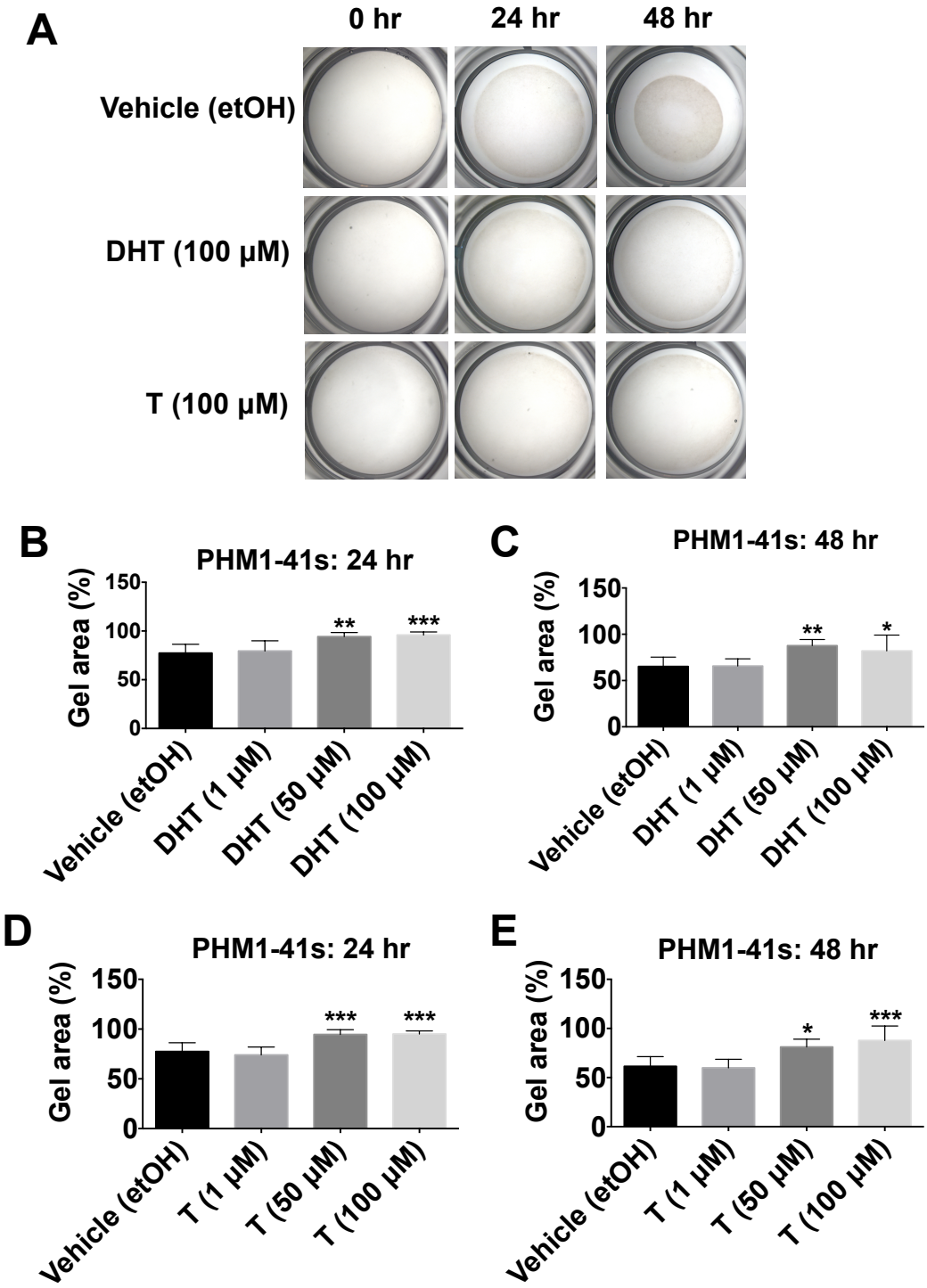



**Figure 5.1:** DHT and T inhibited PHM-41s contraction. PHM1-41s were embedded in collagen gels in 24-well plates and incubated with vehicle (etOH) or DHT (1 µM, 50 µM, 100 µM; **B**, **C**) or T (1 µM, 50 µM, 100 µM; **D**, **E**). The gel area was measured after 24 and 48 hrs. (**A**) Representative photographs of wells showing the reduction in gel area at 24 and 48 hrs. The embedded cells developed a basal contraction, which was evident by the time-dependent decrease in gel area following incubation with the vehicle. DHT and T treatments significantly inhibited the basal gel contraction. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to vehicle, n=7/ each treatment in 6 replicates. Data were analysed using one-way ANOVA with Tukey's post-hoc test.

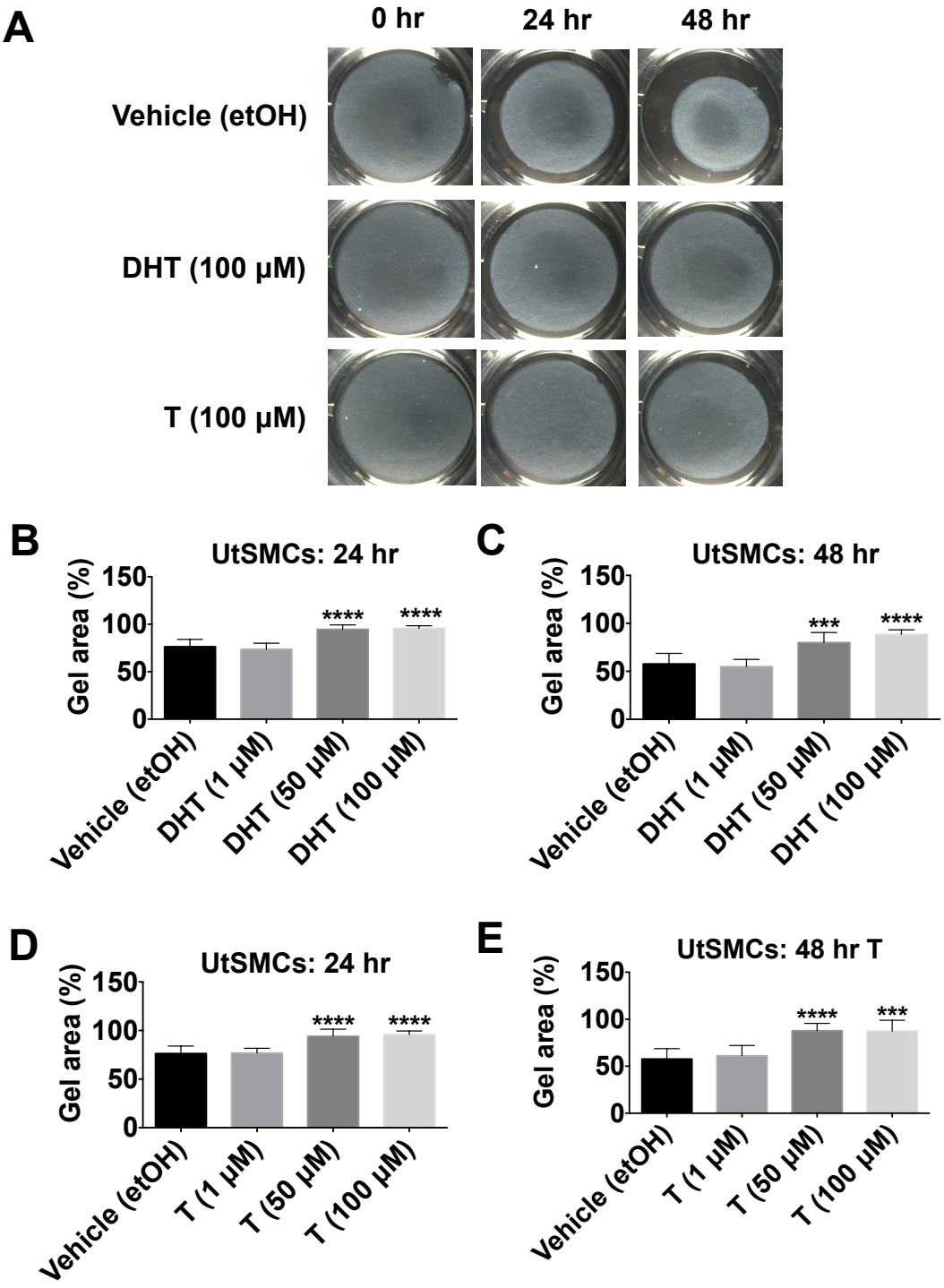



**Figure 5.2:** DHT and T inhibited UtSMCs contraction. UtSMCs were embedded in collagen gels in 24-well plates and incubated with vehicle (etOH) or DHT (1 µM, 50 µM, 100 µM; **B**, **C**) or T (1 µM, 50 µM, 100 µM; **D**, **E**). The gel area was measured after 24 and 48 hrs. (**A**) Representative photographs of wells showing the reduction in gel area at 24 and 48 hrs. The embedded cells developed a basal contraction, which was evident by the time-dependent decrease in gel area following incubation with the vehicle. DHT and T treatments significantly inhibited the basal gel contraction. *p<0.05, **p<0.01, ***p<0.001 compared to vehicle, n=7/ each treatment in 6 replicates. Data were analysed using one-way ANOVA with Tukey's post-hoc test.

#### *5.3.1.2 The action of DHT and T on PHM1-41s and UtSMCs contraction was not receptor-mediated*

Following the observation that DHT and T could prevent the basal contraction of PHM1-41s and UtSMCs when embedded in collagen gels, we sought to examine whether the AR was mediating that effect. PHM1-41s and UtSMCs gels were pre-treated with the AR antagonist-flutamide (100 μM) for 30 minutes prior to exposure to DHT (100 μM). The gels were photographed at 24 and 48 hours. Pre-treatment with flutamide failed to inhibit the effect that DHT had on the contraction of PHM1-41s (**Figure 5.3**) or UtSMCs (**Figure 5.4**) at both time points.

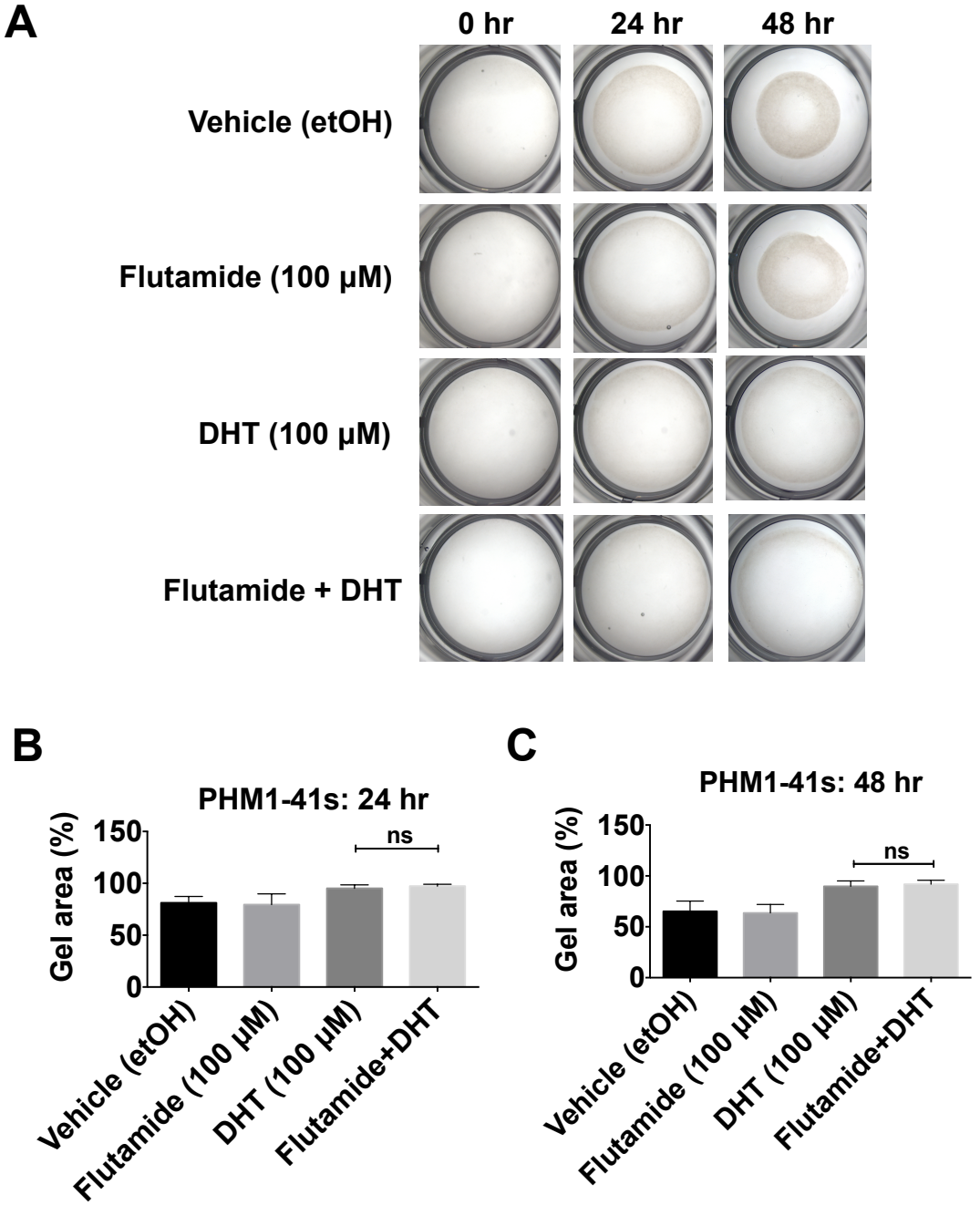



**Figure 5.3:** Flutamide pre-treatment did not prevent the DHT-induced effect on the basal contraction of PHM1-41s gel-embedded cells. PHM1-41s were embedded in collagen gels in 24-well plates and incubated with vehicle (etOH) or flutamide (100 µM) or DHT (100 µM) or flutamide+DHT (flutamide was added 30 min prior to DHT). The gel area was measured after 24 and 48 hrs. (**A**) Representative photographs of wells showing the reduction in gel area at 24 and 48 hrs. The embedded cells developed a basal contraction, which was evident by the time-dependent decrease in gel area following incubation with the vehicle. Pre-treatment with flutamide did not significantly affect the action of DHT. ns=non-significant comparison between DHT and flutamide+DHT groups, n=5/ each treatment in 6 replicates. Data were analysed using one-way ANOVA with Tukey's post-hoc test.

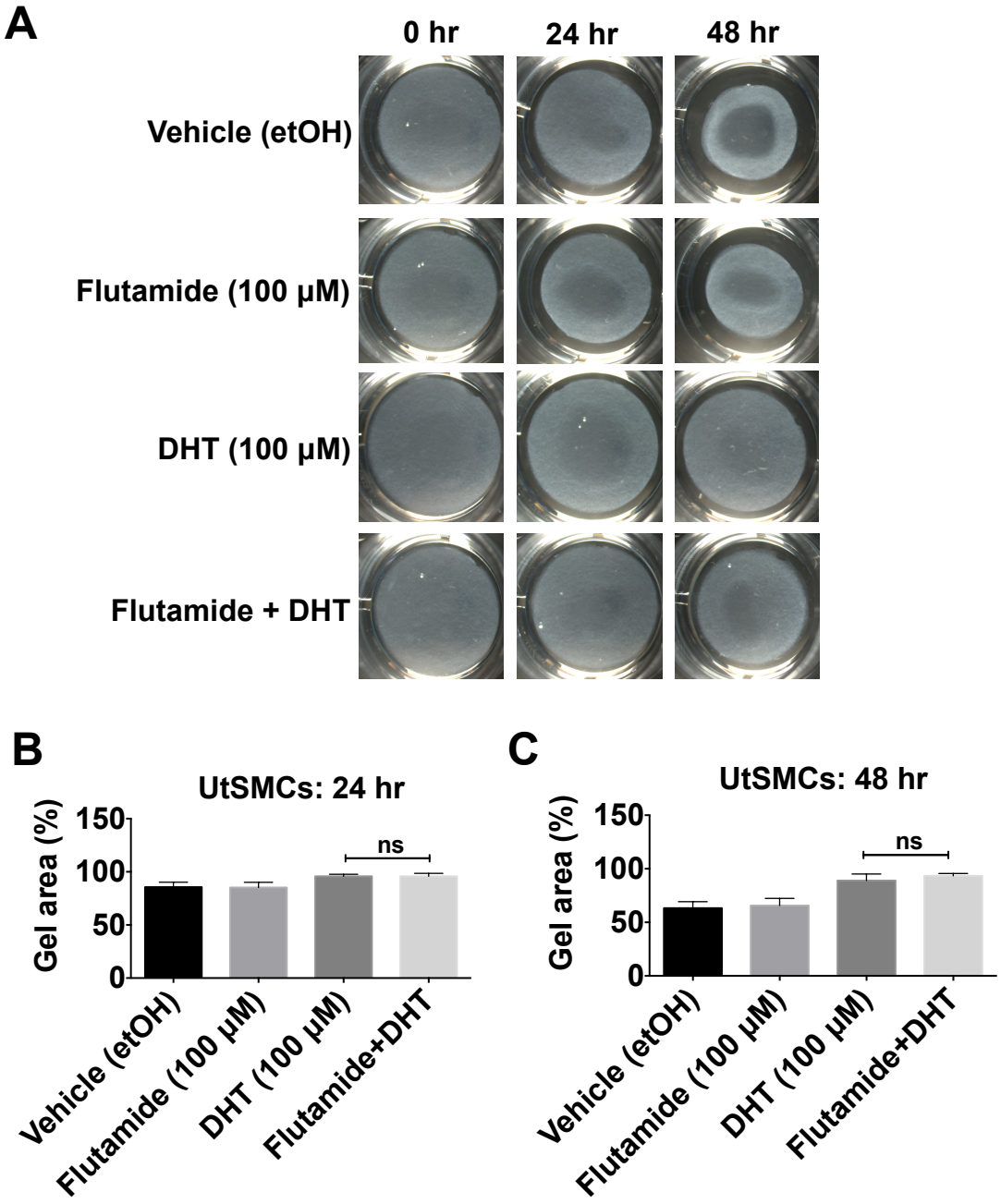



**Figure 5.4:** Flutamide pre-treatment did not prevent the DHT-induced effect on the basal contraction of UtSMCs gel-embedded cells. UtSMCs were embedded in collagen gels in 24-well plates and incubated with vehicle (etOH) or flutamide (100 µM) or DHT (100 µM) or flutamide+DHT (flutamide was added 30 min prior to DHT). The gel area was measured after 24 and 48 hrs. (**A**) Representative photographs of wells showing the reduction in gel area at 24 and 48 hrs. The embedded cells developed a basal contraction, which was evident by the time-dependent decrease in gel area following incubation with the vehicle. Pre-treatment with flutamide did not significantly affect the action of DHT. ns=non-significant comparison between DHT and flutamide+DHT groups, n=5/ each treatment in 6 replicates. Data were analysed using one-way ANOVA with Tukey's post-hoc test.

In addition, we tested the hypothesis that the action of DHT and T was mediated via a cell-surface receptor. PHM1-41s and UtSMCs gels were treated with vehicle (etOH+PBS), T (100 μM) or the cell impermeable T (TBSA; 0.5 μM) for 24 and 48 hours. The gel area of cells treated with T or TBSA was compared to the gel area of cells treated with vehicle at both time points.

In PHM1-41s (**Figure 5.5**), the gel area at 24 hours (**Figure 5.5 B**) was decreased to 85.6% ± 2 with the vehicle treatment, to 85.2% ± 2.1 with the TBSA treatment and to 95.8% ± 0.8 with the T treatment ($p<0.01$). At 48 hours (**Figure 5.5 C**), the gel area was decreased to 63.2% ± 2.7 with the vehicle treatment, to 65.6% ± 3 with the TBSA treatment and to 89% ± 2.7 with the T treatment ($p<0.0001$).

In UtSMCs (**Figure 5.6**), the gel area at 24 hours (**Figure 5.6 B**) was decreased to 82.4% ± 1.9 with the vehicle treatment, to 83.6% ± 3.2 with the TBSA treatment and to 96% ± 1.2 with the T treatment ($p<0.01$). At 48 hours (**Figure 5.6 C**), the gel area was decreased to 62.2% ± 4 with the vehicle treatment, to 60.8% ± 5 with the TBSA treatment and to 85% ± 2.3 with the T treatment ($p<0.01$).

The gel area following TBSA treatment was not significantly different to the gel area following the vehicle treatment at any time point examined in PHM1-41s and UtSMCs, suggesting that TBSA did not have similar effect to T.

Collectively, these findings might suggest that neither the AR nor a cell-surface receptor was required for the mediation of the relaxant effects of DHT and T on the uterine muscle *in vitro*.

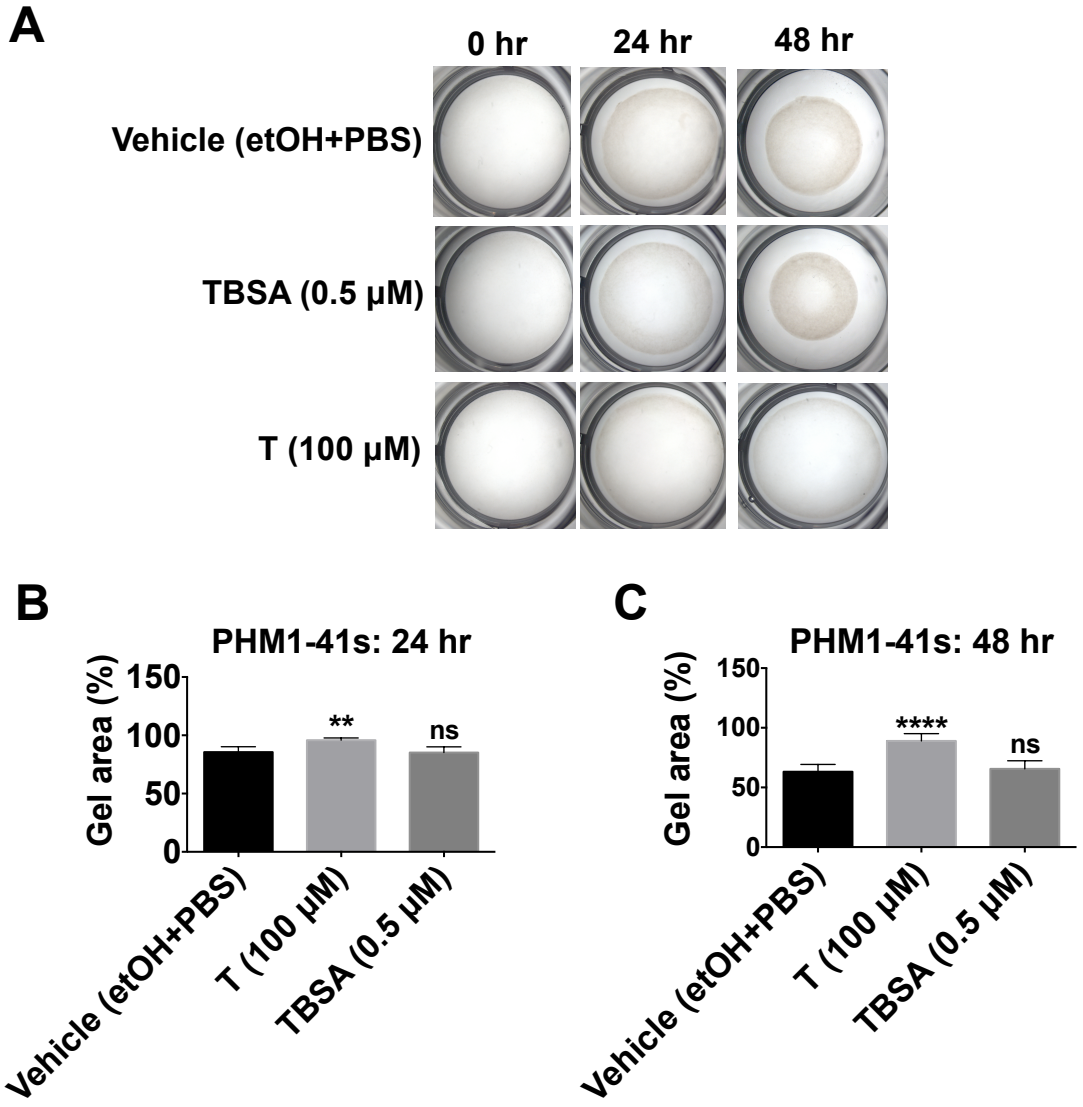



**Figure 5.5:** TBSA did not inhibit PHM1-41s contraction. PHM1-41s were embedded in collagen gels in 24-well plates and incubated with vehicle (etOH+PBS) or T (100 µM) or TBSA (0.5 µM). The gel area was measured after 24 and 48 hrs. (**A**) Representative photographs of wells showing the reduction in gel area at 24 and 48 hrs. The embedded cells developed a basal contraction, which was evident by the time-dependent decrease in gel area following incubation with the vehicle. T but not TBSA significantly inhibited the basal gel contraction. **p<0.01, ****p<0.001, ns=non-significant compared to vehicle, n=5/ each treatment in 6 replicates. Data were analysed using one-way ANOVA with Tukey's post-hoc test.

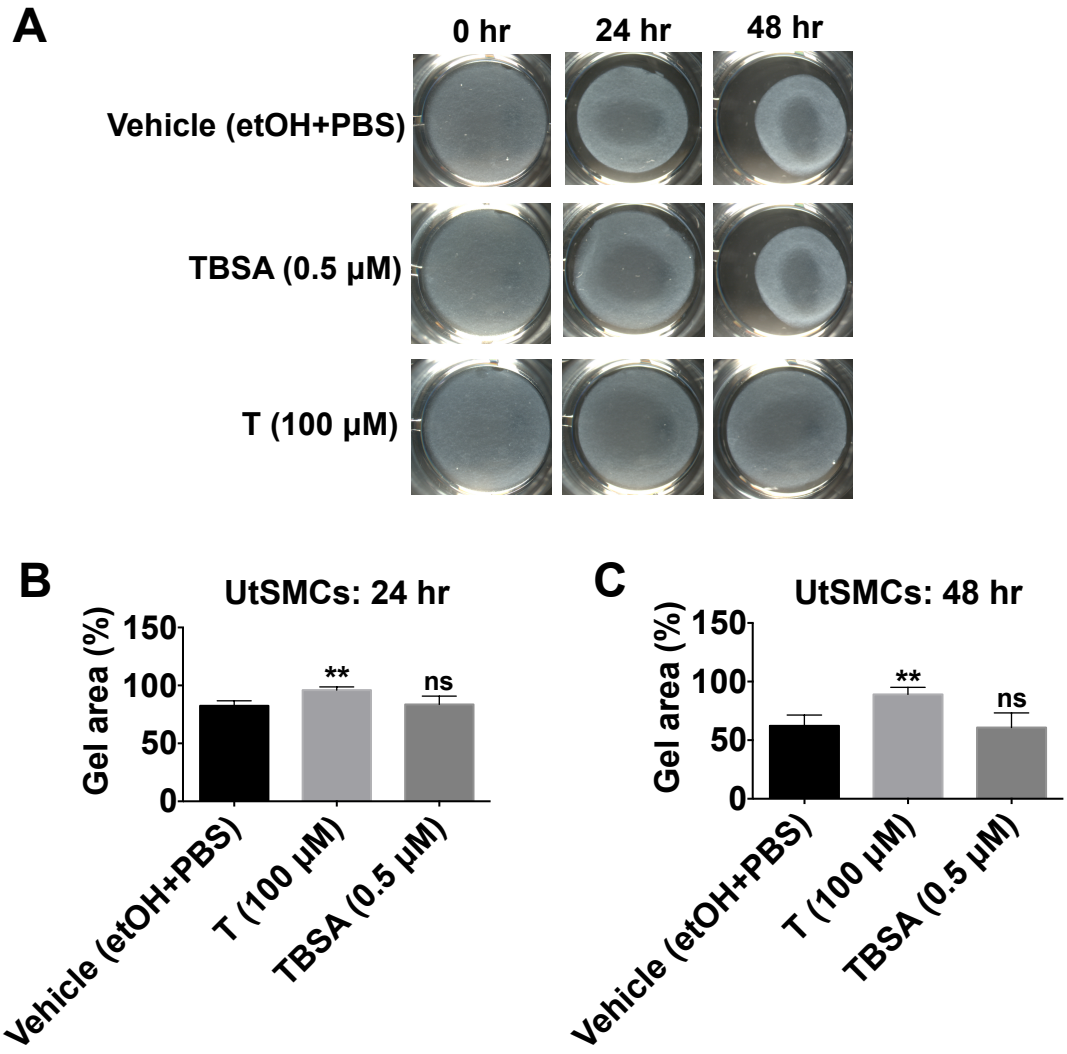



**Figure 5.6:** TBSA did not inhibit UtSMCs contraction. UtSMCs were embedded in collagen gels in 24-well plates and incubated with vehicle (etOH+PBS) or T (100 µM) or TBSA (0.5 µM). The gel area was measured after 24 and 48 hrs. (**A**) Representative photographs of wells showing the reduction in gel area at 24 and 48 hrs. The embedded cells developed a basal contraction, which was evident by the time-dependent decrease in gel area following incubation with the vehicle. T but not TBSA significantly inhibited the basal gel contraction. **p<0.01, ns=non-significant compared to vehicle, n=5/ each treatment in 6 replicates. Data were analysed using one-way ANOVA with Tukey's post-hoc test.

#### *5.3.1.3 DHT inhibited the OXT-stimulated contraction of PHM1-41s and UtSMCs*

Initially, we examined the effect of OXT treatment on the basal contraction of PHM1-41s and UtSMCs. Cells were embedded in collagen gels and treated with either vehicle ($dH_2O$) or OXT (10 nM or 100 nM) for 24 hours. The gel area of OXT treated cells was compared with the gel area of vehicle treated cells.

In PHM1-41s (**Figure 5.7 A**), the gel area was decreased to 85.8% ± 3.2 with vehicle treatment, to 60.4% ± 6 with OXT-10 nM treatment ($p<0.01$) and to 65% ± 4 with OXT-100 nM treatment ($p<0.05$). In UtSMCs (**Figure 5.8 A**), the gel area was decreased to 78.8% ± 2.7 with vehicle treatment, to 57.8% ± 5.6 with OXT-10 nM treatment and to 63.8% ± 2.4 ($p<0.01$) with OXT-100 nM treatment ($p<0.05$).

Following confirmation that OXT could effectively stimulate PHM1-41s and UtSMCs contraction, we tested whether DHT would inhibit the OXT-stimulated contraction in a similar way that it inhibited the basal contraction. PHM1-41s and UtSMCs were embedded in gels and treated with vehicle ($dH_2O$+etOH) or OXT (10 nM) or DHT (100 μM) or OXT+DHT for 24 hours. The gel area of OXT alone treated cells was compared with the gel area of OXT+DHT treated cells.

In PHM1-41s (**Figure 5.7 B**), the gel area was decreased to 83.4% ± 6.9 with vehicle treatment, to 66% ± 1.9 with OXT-10 nM treatment and to 82.8% ± 2.8 with OXT+DHT treatment. The gel area of OXT treated cells was significantly smaller compared with the gel area of OXT+DHT treated cells ($p<0.01$).

In UtSMCs (**Figure 5.8 A**), the gel area was decreased to 81.6% ± 3 with vehicle treatment, to 64% ± 2.7 with OXT-10 nM treatment and to 84.8% ± 1.8 with OXT+DHT treatment. The gel area of OXT treated cells was significantly smaller compared with the gel area of OXT+DHT treated cells ($p<0.001$).

The above findings demonstrated that DHT could inhibit both basal and OXT-stimulated contraction of uterine myocytes *in vitro*.

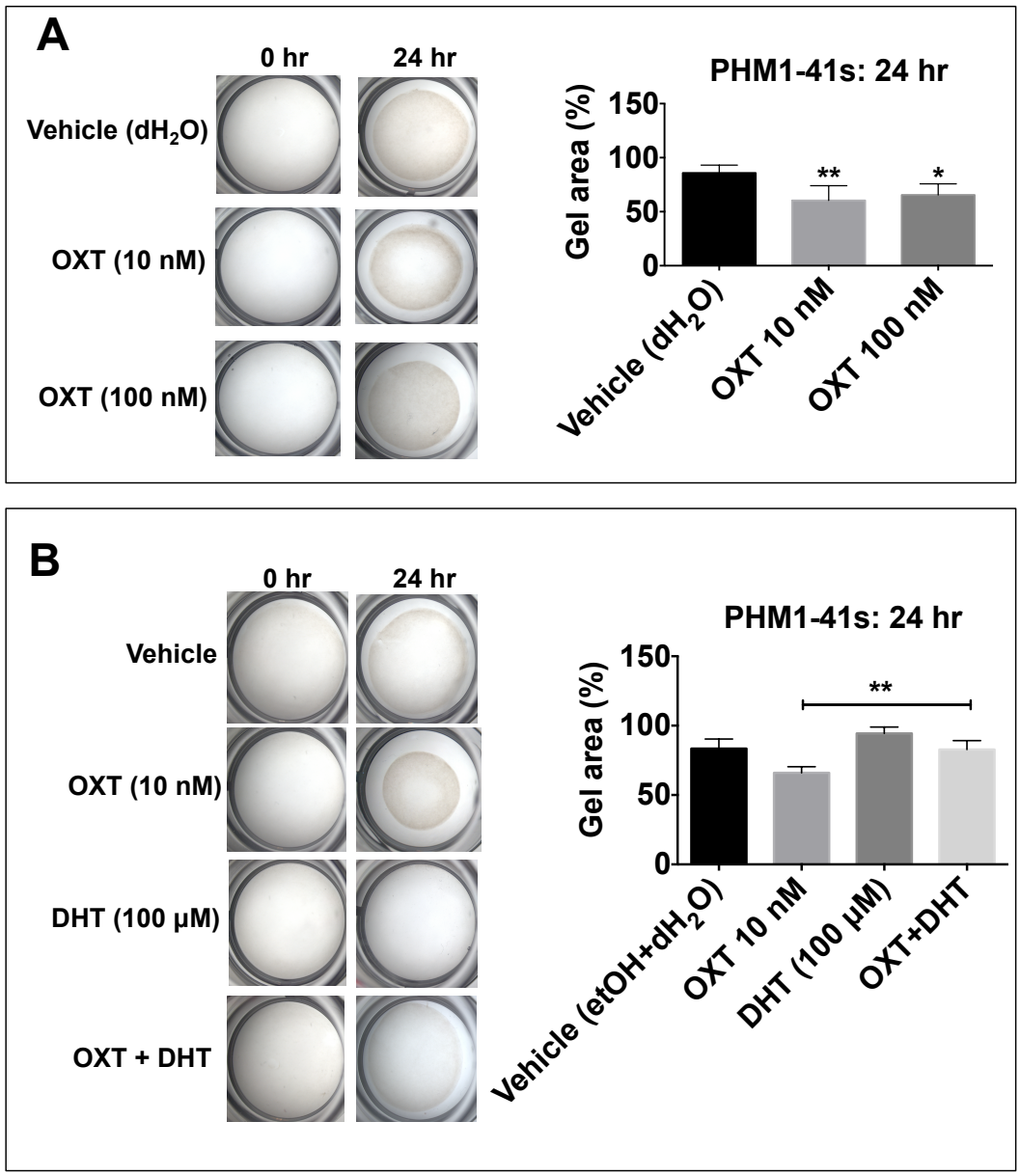



**Figure 5.7:** DHT inhibited the OXT-stimulated contraction of PHM1-41s. PHM1-41s were embedded in collagen gels in 24-well plates. (**A**) Cells were initially incubated with vehicle ($dH_2O$) or OXT (10 nM or 100 nM) for 24 hrs. The gel area was significantly smaller following incubation with OXT when compared to the gel area following vehicle treatment (*$p<0.05$, **$p<0.01$ compared to vehicle). (**B**) Cells were incubated with vehicle ($dH_2O$+etOH) or OXT (10 nM) or DHT (100 µM) or OXT+DHT for 24 hrs. The gel area was significantly smaller following OXT treatment when compared to the gel area following DHT+OXT treatment (**$p<0.01$ comparison between OXT and OXT+DHT groups), n=5/ each treatment in 6 replicates. Data were analysed using one-way ANOVA with Tukey's post-hoc test.

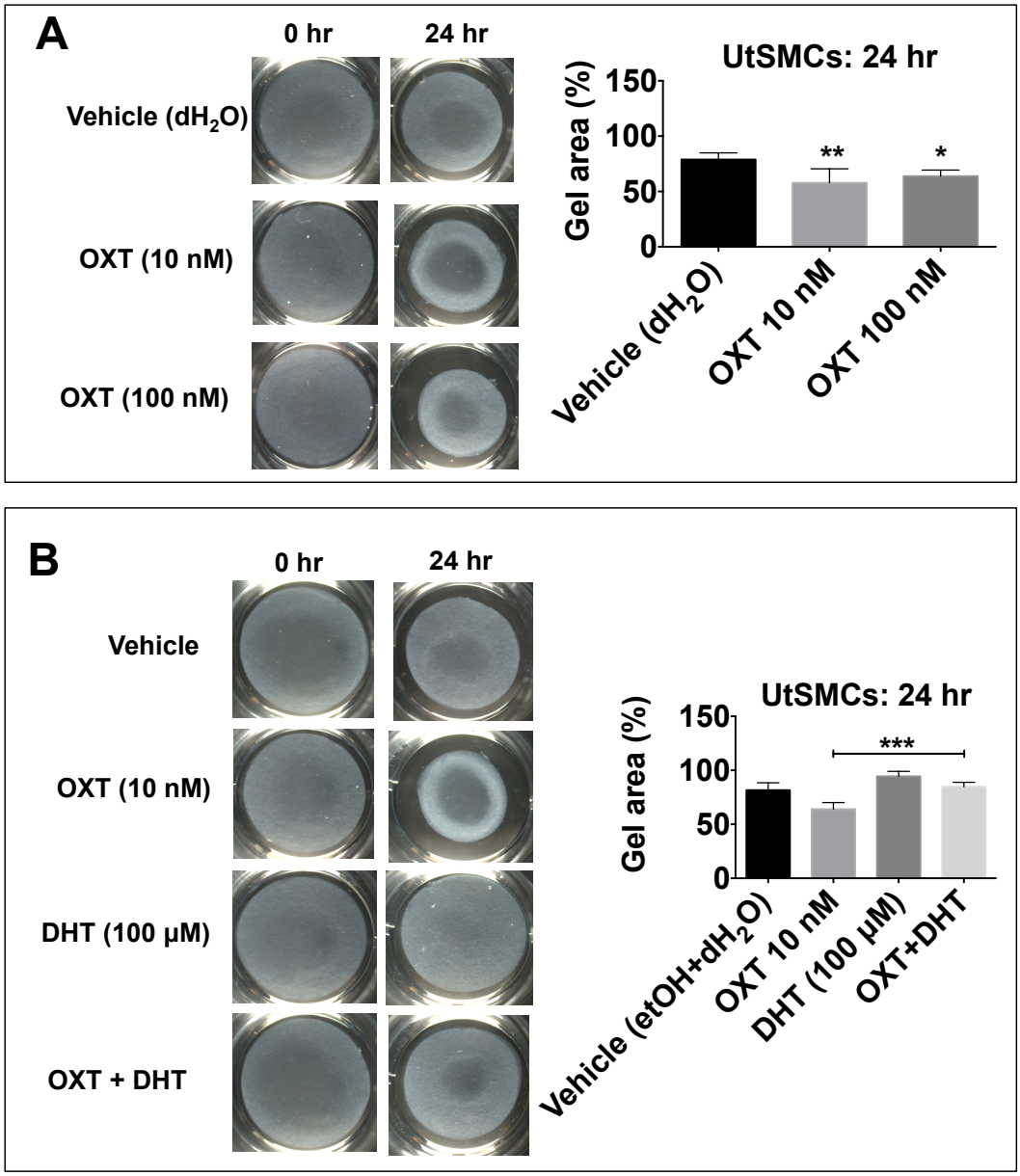



**Figure 5.8:** DHT inhibited the OXT-stimulated contraction of UtSMCs. UtSMCs were embedded in collagen gels in 24-well plates. (**A**) Cells were initially incubated with vehicle ($dH_2O$) or OXT (10 nM or 100 nM) for 24 hrs. The gel area was significantly smaller following incubation with OXT when compared to the gel area following vehicle treatment (*$p<0.05$, **$p<0.01$ compared to vehicle). (**B**) Cells were incubated with vehicle ($dH_2O$+etOH) or OXT (10 nM) or DHT (100 µM) or OXT+DHT for 24 hrs. The gel area was significantly smaller following OXT treatment when compared to the gel area following DHT+OXT treatment (***$p<0.001$ comparison between OXT and OXT+DHT groups), n=5/ each treatment in 6 replicates. Data were analysed using one-way ANOVA with Tukey's post-hoc test.

### *5.3.1.4 DHT and T treatments did not affect cell-viability*

The viability of embedded in gels PHM1-41s and UtSMCs was assessed 48 hours post DHT and T treatment (**Figure 5.9**). Gels were treated with vehicle (etOH) or DHT (100 μM) or T (100 μM) for 48 hours and the viability was assessed with a colorimetric assay. The viability of PHM1-41s (**Figure 5.9 A**) and UtSMCs (**Figure 5.9 B**) following DHT and T treatment was not statistically different from the viability of cells exposed to the vehicle treatment.

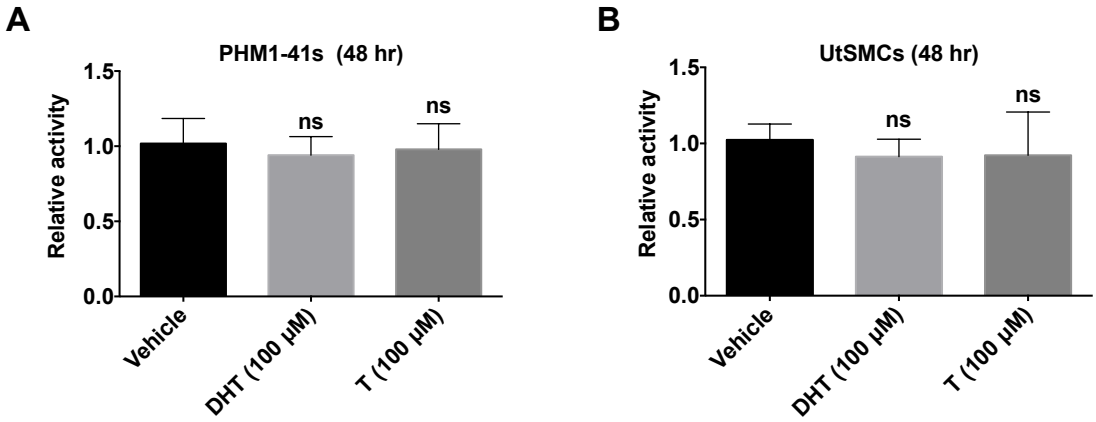



**Figure 5.9:** Viability of gel-embedded PHM1-41s (**A**) and UtSMCs (**B**) following incubation with DHT and T (100 μM) for 48 hrs. Treatments with DHT and T did not affect the cell viability, ns=non-significant compared to vehicle (etOH), n=4. Data were analysed using Kruskal-Wallis with Dunn's post-hoc test.

Taken together, all the above findings confirmed that PHM1-41s and UtSMCs responded to DHT and T *in vitro* in a similar way to myometrial strips *ex vivo*, suggesting that PHM1-41s and UtSMCs could be utilised for further studies to investigate the underlying mechanism of the DHT-induced relaxation of the uterine smooth muscle.

### 5.3.2 The effect of DHT on phosphorylation of myosin light chain (MLC)

We aimed to deduce whether the DHT-induced relaxation of the uterine smooth muscle was mediated via a physiological mechanism, which resulted in reduction of the available intracellular concentration of phosphorylated MLC (PMLC).

#### *5.3.2.1 OXT induced rapid phosphorylation of MLC in PHM1-41s and UtSMCs*

Initially we tested the effect of OXT treatment on the concentration of intracellular PMLC protein in PHM1-41s and UtSMCs. The ICW method was utilised for these studies.

PHM1-41s and UtSMCs were seeded into 96-well plates, treated with either OXT (10 nM or 100 nM) or vehicle ($dH_2O$) for 30 or 60 seconds and fixed. The PMLC and α-Tubulin (loading control) proteins were detected in the same well. The concentration of intracellular PMLC following stimulation with OXT was compared with the concentration of PMLC following treatment with the vehicle at both time points.

In PHM1-41s (**Figure 5.10 A**) treatments with OXT-10 nM and OXT-100 nM for 30 seconds induced a significant increase in the concentration of PMLC ($p<0.0001$). Similarly, in UtSMCs (**Figure 5.10 B**), OXT-10 nM and OXT-100 nM induced a significant increase in the concentration of PMLC ($p<0.0001$). The 100 nM dose of OXT was significantly more effective in stimulating the phosphorylation of MLC compared to the 10 nM dose in PHM1-41s ($p<0.0001$; **Figure 5.10 A**) and UtSMCs ($p<0.01$; **Figure 5.10 B**). The effect of OXT on PMLC was not significant at the 60-seconds time point in both cell types (**Figure 5.10 C, D)**.

The more effective dose of OXT (100 nM) and stimulation for 30 seconds were chosen as optimum for the purposes of the next experiments.

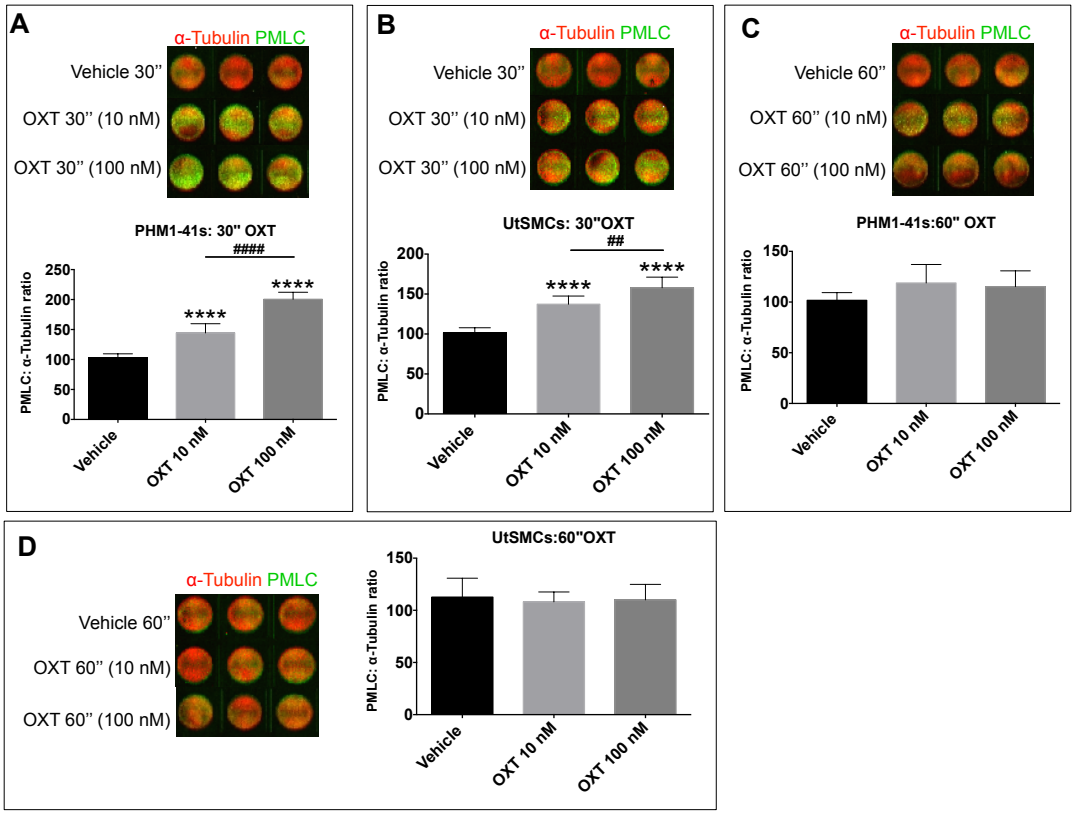



**Figure 5.10:** OXT stimulated phosphorylation of MLC in PHM1-41s and UtSMCs. PHM1-41s and UtSMCs were treated with either OXT (10 nM or 100 nM) or vehicle ($dH_2O$) for 30 or 60 sec. Representative scans of 96-well plates show PMLC (green) and α-Tubulin (red) in the same well (in triplicate/treatment) in PHM1-41s (**A, C**) and UtSMCs (**B, D**). The concentration of PMLC was significantly higher in wells treated with OXT (10 nM and 100 nM) for 30 sec in PHM1-41s (**A**) and UtSMCs (**B**). OXT treatment for 60 sec was not effective in stimulation of MLC phosphorylation in PHM1-41s (**C**) and UtSMCs (**D**). ****$p<0.0001$ compared to vehicle, ##$p<0.01$, ####$p<0.0001$ comparison between OXT-10 nM and OXT-100 nM groups, n=6/ treatments in triplicate. Data were analysed using two-way ANOVA with Tukey's post-hoc test.

#### *5.3.2.2 Nifedipine blunted the effect of OXT on PMLC in PHM1-41s and UtSMCs*

We used a $Ca^{2+}$ channel blocker (nifedipine) as a positive control to inhibit OXT-induced stimulation of MLC phosphorylation (Aguilar et al., 2010).

PHM1-41s (**Figure 5.11 A**) and UtSMCs (**Figure 5.11 B**) were seeded into 96-well plates and treated with nifedipine (50 μM) or vehicle (DMSO) for 15 minutes. The cells were then exposed to acute OXT (100 nM) treatment (30 seconds). The concentration of PMLC following stimulation with OXT in the DMSO pre-treated group was compared to the concentration of PMLC following stimulation with OXT in the nifedipine pre-treated group.

In PHM1-41s (**Figure 5.11 A**), acute treatment with OXT induced a 1.6 fold increase in the concentration of PMLC in the DMSO pre-treated cells ($p<0.0001$). The concentration of PMLC was significantly higher in the DMSO pre-treated cells compared to concentration of PMLC in the nifedipine pre-treated cells following acute treatment with OXT ($p<0.001$).

Similarly, in UtSMCs (**Figure 5.11 B**), acute treatment with OXT induced a 1.4 fold increase in the concentration of PMLC in the DMSO pre-treated cells ($p<0.01$). The concentration of PMLC was significantly higher in the DMSO pre-treated cells compared to concentration of PMLC in the nifedipine pre-treated cells following acute treatment with OXT ($p<0.001$).

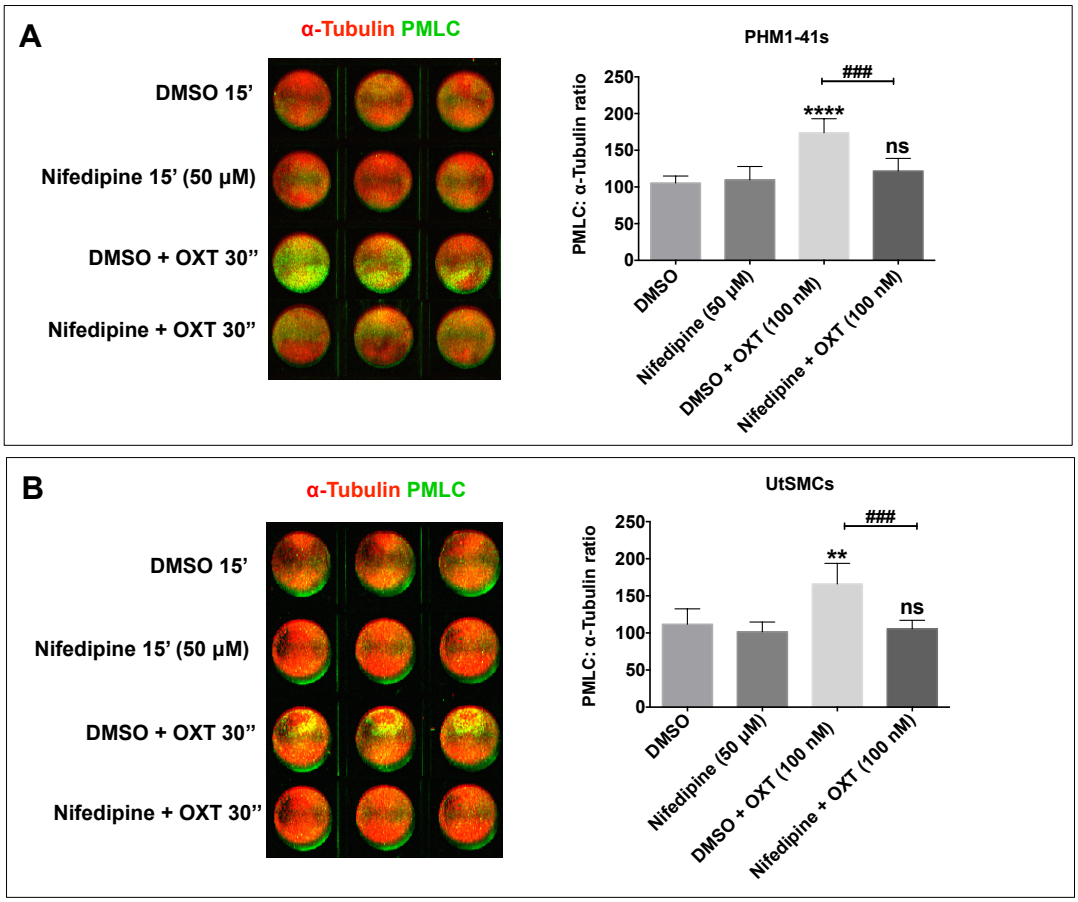



**Figure 5.11:** Nifedipine pre-treatment blunts the effect of OXT on PMLC in PHM1-41s (**A**) and UtSMCs (**B**). Cells were seeded into 96-well plates and pre-treated with either vehicle (DMSO) or nifedipine (50 μM) prior to being exposed to acute (30 sec) treatment with OXT (100 nM). The concentration of PMLC in the DMSO pre-treated cells following treatment with OXT was higher compared to the concentration of PMLC in the nifedipine pre-treated cells following exposure to OXT. **p<0.01, ****p<0.0001 compared to DMSO alone. ns=non-significant compared to nifedipine alone. ###p<0.001 comparison between the DMSO+OXT and nifedipine+OXT groups, n=5/ treatments in triplicate. Data were analysed using one-way ANOVA with Tukey's post-hoc test.

#### *5.3.2.3 DHT blunted the effect of OXT on PMLC in PHM1-41s and UtSMCs*

PHM1-41s (**Figure 5.12 A**) and UtSMCs (**Figure 5.12 B**) were seeded into 96-well plates and treated with DHT (50 μM) or vehicle (etOH) for 15 minutes. The cells were then exposed to acute OXT (100 nM) treatment (30 seconds). The concentration of PMLC following stimulation with OXT in the etOH pre-treated group was compared to the concentration of PMLC following stimulation with OXT in the DHT pre-treated group.

In PHM1-41s (**Figure 5.12 A**), acute treatment with OXT induced a 1.7 fold increase in the concentration of PMLC in the etOH pre-treated cells ($p<0.0001$). The concentration of PMLC was significantly higher in the etOH pre-treated cells compared to the concentration of PMLC in the DHT pre-treated cells following acute treatment with OXT ($p<0.001$).

Similarly, in UtSMCs (**Figure 5.12 B**), acute treatment with OXT induced a 1.6 fold increase in the concentration of PMLC in the etOH pre-treated cells ($p<0.01$). The concentration of PMLC was significantly higher in the etOH pre-treated cells compared to the concentration of PMLC in the DHT pre-treated cells following acute treatment with OXT ($p<0.01$).

Taken together, these findings suggested that the action of DHT was mediated via a physiological mechanism similar to nifedipine that resulted in decrease in MLC phosphorylation, which can impact contraction.

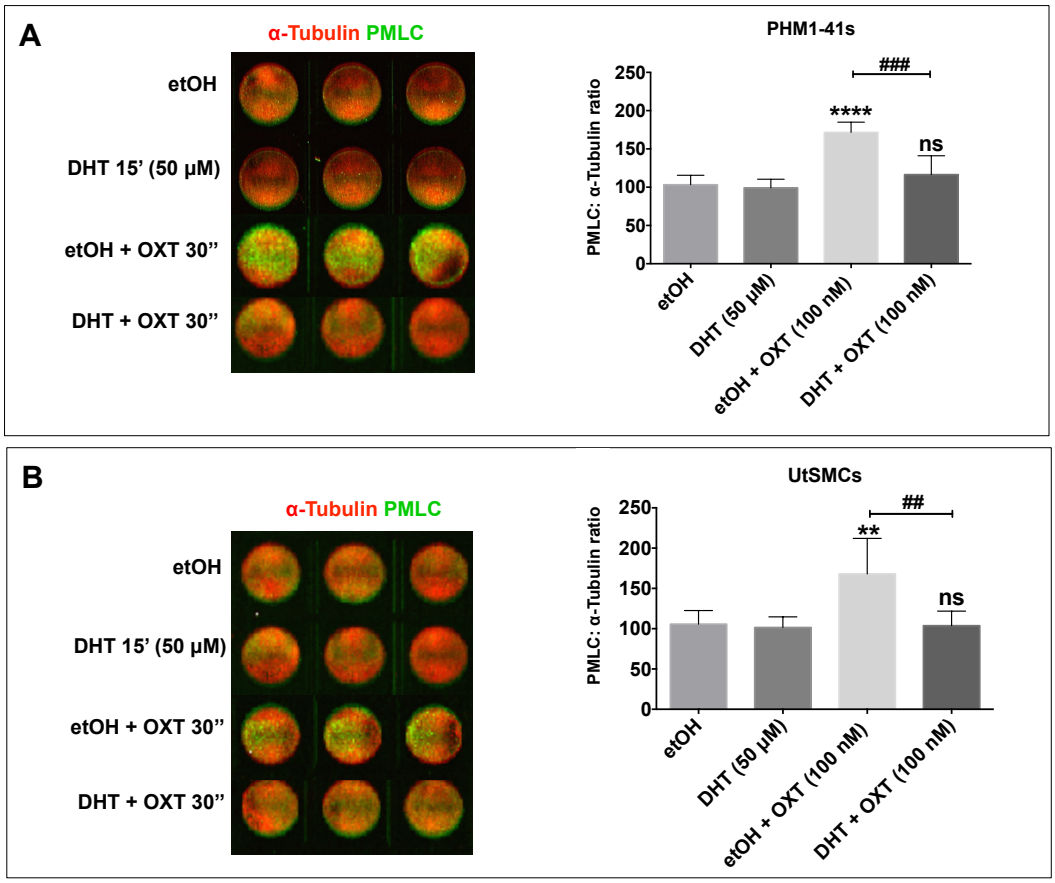



**Figure 5.12:** DHT pre-treatment blunts the effect of OXT on PMLC in PHM1-41s (**A**) and UtSMCs (**B**). Cells were seeded into 96-well plates and pre-treated with either vehicle (etOH) or DHT (50 μM) prior to being exposed to acute (30 sec) treatment with OXT (100 nM). The concentration of PMLC in the etOH pre-treated cells following treatment with OXT was higher compared to the concentration of PMLC in the DHT pre-treated cells following exposure to OXT. **p<0.01, ****p<0.0001 compared to etOH alone. ns=non-significant compared to DHT alone. ##p<0.001, ###p<0.001 comparison between the etOH+OXT and DHT+OXT groups, n=5/ treatments in triplicate. Data were analysed using one-way ANOVA with Tukey's post-hoc test.

### 5.3.3 The effect of DHT on $Ca^{2+}$ flux

We examined whether the demonstrated effect of DHT pre-treatment on the concentration of PMLC resulted from blockade of a mechanism, which induces increase in the concentration of intracellular $Ca^{2+}$ in response to a stimuli. OXT was used as stimuli to induce an increase in intracellular $Ca^{2+}$ in PHM1-41s and UtSMCs (Monga et al., 1999; Monga et al., 1996). A calcium assay was performed to record the concentration of intracellular $Ca^{2+}$ in response to OXT.

#### *5.3.3.1 OXT treatment increased the concentration of [$Ca^{2+}$] in PHM1-41s and UtSMCs*

PHM1-41s and UtSMCs were seeded into 96-well plates, incubated with DLS (which contained the $Ca^{2+}$ indicator) and placed on a microplate fluorometer. The baseline fluorescence generated by the indicator, which denoted the basal concentration of [$Ca^{2+}$], was recorded for 20 seconds and then the cells were injected with either OXT (10 nM or 100 nM) or vehicle ($dH_2O$). The injection of OXT (10 nM, 100 nM) induced an increase in the concentration of [$Ca^{2+}$] as was evident by the increase in fluorescence intensity. The injection of vehicle did not increase the concentration of [$Ca^{2+}$]. To quantify these effects we used two readouts (fluorescence intensity); the highest readout (recorded after the injection) and the readout recorded before the injection (baseline). These raw readouts were used without normalisation.

In PHM1-41s (**Figure 5.13 C**), the injection of OXT-10 nM induced a 1.25 fold increase ($p<0.0001$) in the concentration of [$Ca^{2+}$] above baseline; the effect of OXT-10 nM on the concentration of [$Ca^{2+}$] was significantly greater when compared to the effect of vehicle injection ($p<0.001$). Injection of OXT-100 nM induced a 1.7 fold increase ($p<0.0001$) in the concentration of [$Ca^{2+}$] compared to baseline; the effect of OXT was significantly greater when compared to the vehicle-induced effect ($p<0.0001$) on the concentration of [$Ca^{2+}$].

In UtSMCs (**Figure 5.13 D**), the injection of OXT-10 nM induced a 1.5 fold increase ($p<0.0001$) in the concentration of [$Ca^{2+}$] above baseline; the effect of OXT was greater compared with the effect that the vehicle injection had on the concentration of [$Ca^{2+}$] ($p<0.001$). In addition, injection of OXT-100 nM induced a 1.9 fold

increase ($p<0.0001$) in concentration of [$Ca^{2+}$] above baseline, which was also significantly greater compared to the effect induced by the vehicle injection ($p<0.0001$).

The effect of OXT-10 nM injection on [$Ca^{2+}$] was not significantly different to the effect induced by OXT-100 nM injection. Therefore, OXT-10 nM was chosen thereafter as optimum for the next experiments.

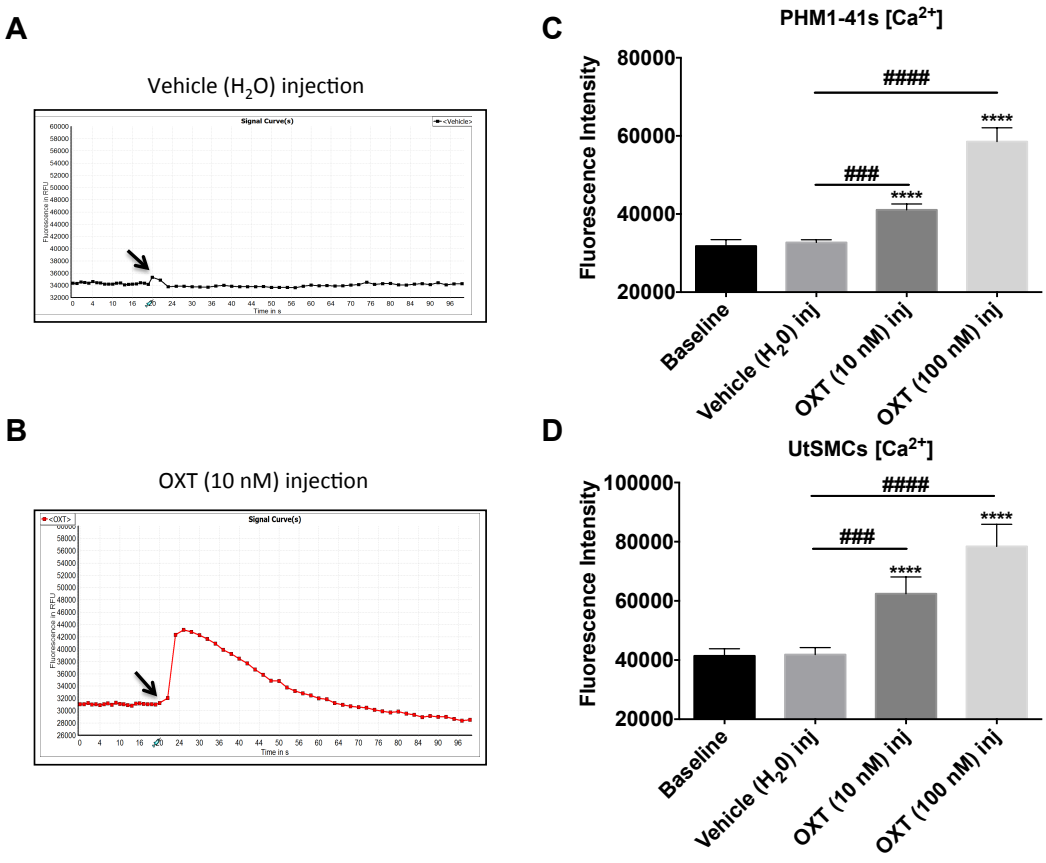



**Figure 5.13:** OXT stimulation increased the concentration of [$Ca^{2+}$] in PHM1-41s (**C**) and UtSMCs (**D**). (**A, B**) Representative readouts from the microplate fluorometer software show the effect of vehicle (**A**) or OXT (**B**) injection on [$Ca^{2+}$] in PHM1-41s. The injection of OXT to PHM1-41s (**C**) and UtSMCs (**D**) significantly increased the concentration of [$Ca^{2+}$] above baseline as was evident by the increase in fluorescence intensity. The effect of OXT was significantly greater compared to the effect induced by injection of the vehicle ($dH_2O$ inj). ****$p<0.0001$ compared to baseline, ###$p<0.001$, ####$p<0.0001$ comparison between OXT and vehicle groups, n=6/ 4 replicates per treatment. Data were analysed using one-way ANOVA-with Tukey's post-hoc test.

#### *5.3.3.2 DHT blunted the effect of OXT on [$Ca^{2+}$] in PHM1-41s and UtSMCs*

We examined whether DHT pre-treatment would affect the response of PHM1-41s and UtSMCs to OXT.

PHM1-41s and UtSMCs were seeded into 96-well plates and following incubation with the $Ca^{2+}$ indicator for 1 hour, cells were incubated with either vehicle (10 min; etOH) or DHT (10 min) in a range of concentrations (100 nM, 300 nM, 800 nM and 50 μM). The cells were then injected with OXT (10 nM). The effect of OXT on the concentration of [$Ca^{2+}$] was compared between the groups pre-treated with vehicle (10 min) or DHT (10 min).

In PHM1-41s (**Figure 5.14**), the effect of OXT on the concentration of [$Ca^{2+}$] was greater in the vehicle pre-treated group compared to the DHT pre-treated group for all concentrations of DHT tested (**Figure 5.14 B, C, D**) but the 100 nM (**Figure 5.14 A**). Specifically, the effect of OXT was 1.3 fold greater ($p<0.0001$) in the vehicle pre-treated group compared to the DHT-300 nM pre-treated group (**Figure 5.14 B**). In addition, the effect of OXT on the vehicle pre-treated group was 1.4 fold ($p<0.0001$) and 1.6 fold ($p<0.0001$) greater compared with the DHT-800 nM (**Figure 5.14 C**) and DHT-50 μM (**Figure 5.14 D**) pre-treated groups respectively.

Similarly, in UtSMCs, the effect of OXT on the concentration of [$Ca^{2+}$] was greater in the vehicle pre-treated group compared to the DHT pre-treated group for all concentrations of DHT tested (**Figure 5.14 B, C, D**) but the 100 nM (**Figure 5.14 A**).

These findings might suggest that DHT blocked $Ca^{2+}$ entry from the extracellular space into PHM1-41s and UtSMCs.

Combining the findings from the *in vitro* work on PHM1-41s and UtSMCs described in this chapter, it is plausible that DHT acts via a physiological mechanism whereby it blocks $Ca^{2+}$ entry into the cell, which in turn results in decrease in the phosphorylation of MLC and, consequently, decrease in contraction of uterine myocytes.

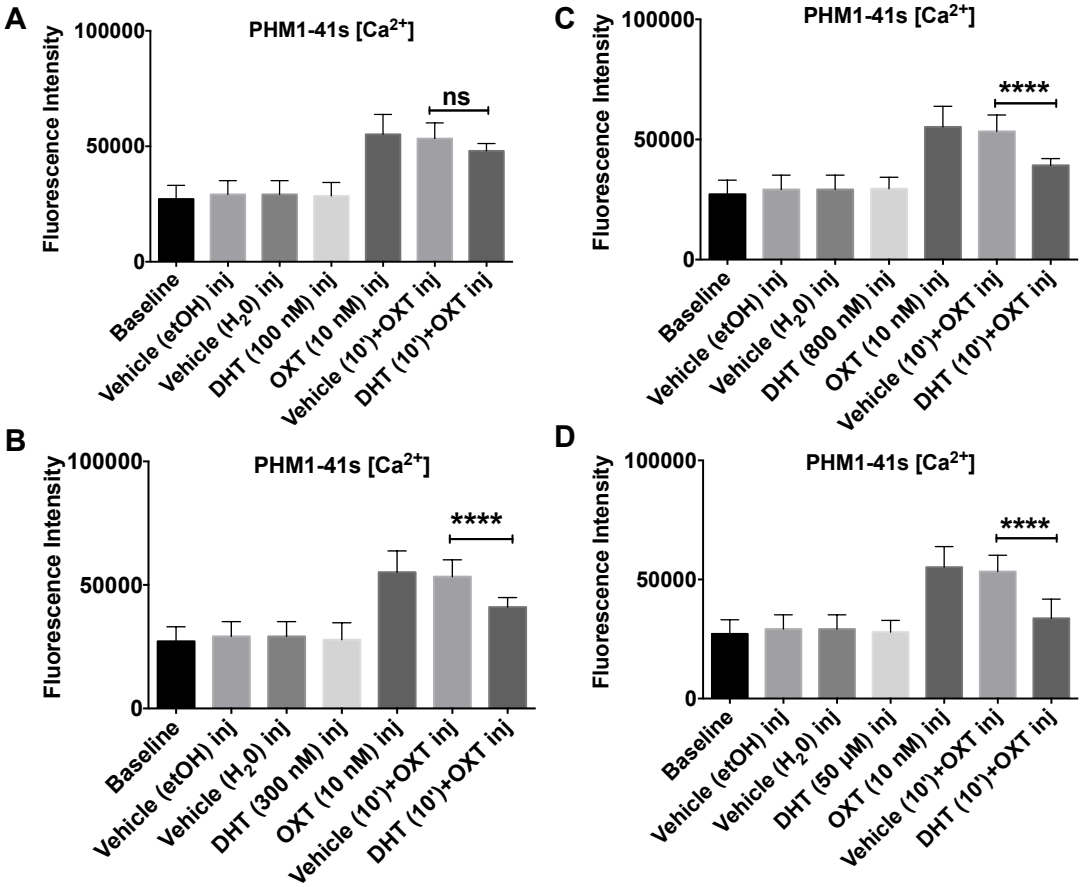



**Figure 5.14:** DHT pre-treatment reduced the response of PHM1-41s to OXT. PHM1-41s were incubated with vehicle (etOH; 10 min) or DHT (10 min) in a range of concentrations; 100 nM (**A**), 300 nM (**B**), 800 nM (**C**) and 50 µM (**D**). The cells were then injected with OXT (10 nM) and the concentration of [$Ca^{2+}$] was compared between the vehicle pre-treated and DHT pre-treated groups. The DHT pre-treatment (300 nM **B**, 800 nM **C**, 50 µM **D**) significantly reduced the effect induced by OXT injection on the concentration of [$Ca^{2+}$] in vehicle pre-treated group ****$p<0.001$ or ns=non-significant comparison between the groups vehicle+OXT and DHT+OXT, n=6/ 4 replicates per treatment. Data were analysed using one-way ANOVA with Sidak's correction.

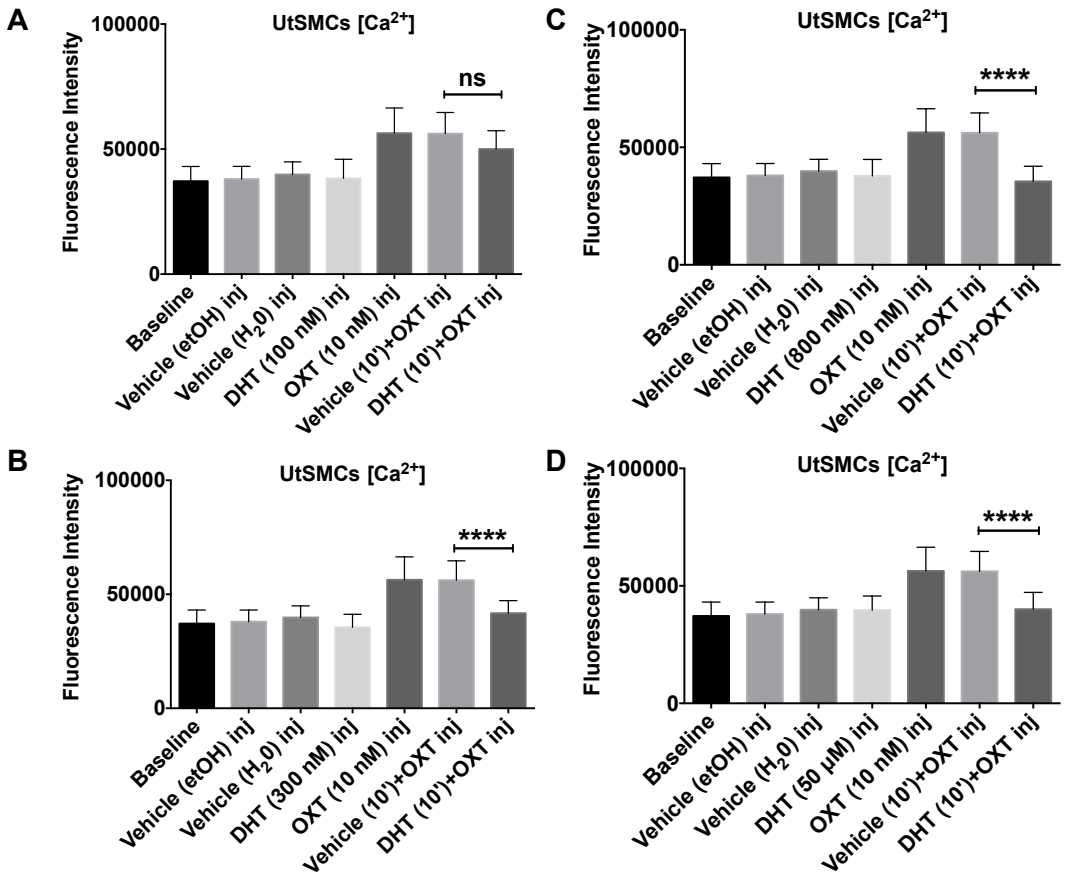



**Figure 5.15:** DHT pre-treatment reduced the response of UtSMCs to OXT. Cells were incubated with vehicle (etOH; 10 min) or DHT (10 min) in a range of concentrations; 100 nM (**A**), 300 nM (**B**), 800 nM (**C**) and 50 µM (**D**). The cells were then injected with OXT (10 nM) and the concentration of $[Ca^{2+}]$ was compared between the vehicle pre-treated and DHT pre-treated groups. The DHT pre-treatment (300 nM **B**, 800 nM **C**, 50 µM **D**) significantly reduced the effect induced by OXT injection on the concentration of $[Ca^{2+}]$ in vehicle pre-treated group ****$p<0.001$ or ns=non-significant comparison between the groups vehicle+OXT and DHT+OXT, n=6/ 4 replicates per treatment. Data were analysed using one-way ANOVA with Sidak's correction.

## 5.4 Discussion

Sex hormone-induced relaxation of myometrium has been reported both by us (Chapter 4) and in other studies in humans, mice, rats, pigs, horses and rabbits (Kurowicka et al., 2005; Ousey et al., 2000; Perusquia, 2001; Perusquia and Navarrete, 2005; Perusquia et al., 2005). However, fundamental questions remain regarding the mechanism of their action, which is generally accepted to be non-genomic. Studies, which investigated possible non-genomic interactions of sex hormones with the contractile machinery, suggested that sex hormones might induce relaxation via direct or indirect blockade of $Ca^{2+}$ entry into the uterine smooth muscle; however, this notion has been poorly explored. We hypothesised that the action of sex hormones is mediated via a physiological mechanism, whereby one or more steps in the contraction pathway are inhibited. In particular, we explored the impact of DHT treatment on two critical events in the contraction cascade; the phosphorylation of MLC and the increase in the concentration of intracellular $Ca^{2+}$.

For these studies, we utilised two well-characterised cell lines; the commercially available primary UtSMCs and the immortalised PHM1-41s. The ability of uterine smooth muscle cells to contract when embedded in collagen gels is well established and provides a useful tool to assess the impact of numerous agents on contraction (Fitzgibbon et al., 2009; Hutchinson et al., 2014; Ivanov et al., 2006; Waite et al., 2014; Williams et al., 2012b). We confirmed that both DHT and T inhibited UtSMCs and PHM1-41s gel basal and OXT-stimulated contraction, which was in line with our organ bath findings (Chapter 4). Recently, an estrogenic compound namely 4-tert-octylphenol (OP) has also been shown to inhibit the basal contraction of uterine cells isolated from rats and embedded in collagen gels (An et al., 2013). In addition, E2 and P4 have been reported to inhibit TGF-β2-induced-contraction of retinal pigment epithelial cells (RPE) embedded in collagen gels (Kimura et al., 2014). In these and in our own studies, the effect of sex hormone was observed only after 24 hours, which was in contrast to the rapid responses observed in the organ bath. However, investigation of rapid responses with this assay was impossible due to the fact that the basal contraction of gels was only significantly observed at 24 hours and not earlier; a 6-hour time point was investigated but the basal contraction was not

significant at that time point (data not shown). Similar to the effect demonstrated in the organ bath, flutamide pre-incubation did not prevent the effect of DHT on the contraction of PHM1-41s and UtSMCs, contributing to the theory that the relaxant effect of sex hormones is unlikely to be mediated via nuclear steroid receptors (Perusquia et al., 2009; Perusquia et al., 2005; Sanchez Aparicio et al., 1992). The involvement of membrane steroid receptors in mediation of myometrial contraction has been poorly investigated. The membrane progesterone receptor (mPR) has been identified on the cell membrane of human myometrial cells at term pregnancy (Karteris et al., 2006). Only two recent studies have looked at the role of mPR in mediation of P4-induced relaxation in uterine smooth muscle. The first study demonstrated that inhibition of a specific mPR namely PGRMC1 prevented the P4-induced relaxation of spontaneous myometrial contractions (Wu et al., 2011). A later study, which examined the relaxing properties of P4 and a progestin (R5020) with a low affinity for mPR, found that R5020 did not relax human spontaneous myometrial contractions, implying that the P4-induced relaxation was mPR mediated (Gökdeniz, 2013). The involvement of a mER namely GPR30 has also been investigated. In contrast to mPR, studies in humans and rats have showed that activation of GPR30 using two different agonists resulted in stimulation rather than inhibition of myometrial contractions (Maiti et al., 2011; Tica et al., 2011). However, the function of GPR30 as an extranuclear ER has been challenged (Kang et al., 2010). The human mAR has been recently identified (Thomas et al., 2014) but there are no studies examining its role in the mediation of androgen-induced relaxation in the myometrium. Notably, if a mAR was mediating the relaxant effect of androgens in the myometrium, the cell-surface impermeable T (TBSA) would have also relaxed the uterine smooth muscle. However, our studies demonstrated that the TBSA failed to elicit such effect not only in myometrial strips *ex vivo* (Chapter 4) but also in PHM1-41s and UtSMCs *in vitro*, suggesting that the action of the androgen was probably cell-surface receptor -independent.

The cell viability assay performed on PHM1-41s and UtSMCs embedded in gels, confirmed that the relaxant effect was not attributable to cell death, providing evidence that the DHT and T treatment at the micromolar range (100 μM) was not toxic. This is in line with our observations that the effect induced by the sex

hormones is reversible in the organ bath following flushing of myometrial strips with a steroid-free Krebs buffer and that KCl stimulation an the end of each experiment induced a tonic contraction.

We showed that pre-incubation with DHT prevented MLC phosphorylation in OXT-stimulated PHM1-41s and UtSMCs. Kimura et al has also reported that pre-incubation with micromolar doses of E2 and P4 decreased TGF-β2-stimulated MLC phosphorylation in RPE cells (Kimura et al., 2014). Another study, which examined the impact of steroid hormones on motility of human colon muscle cells, has shown that administration of P4 ($10^{-5}$M - $10^{-7}$M) significantly reduced the cholecystokinin CCK8-induced MLC phosphorylation (Cheng et al., 2008). In contrast to P4, treatments with P4 conjugated to BSA (PBSA) have been previously reported to increase PMLC in human myocytes, suggesting a cell-surface initiated response (Karteris et al., 2006). Our finding suggests that DHT might target one or more steps in the cascade, which regulates MLC phosphorylation in uterine myocytes. It is plausible that DHT promoted the dephosphorylation of MLC by MLCP in uterine myocytes, through interaction and inhibition of the RhoA/Rho kinase pathway, which is a negative regulator of MLCP activity. Indeed, studies in rat vasculature have reported that E2 attenuated SMC contraction via inhibition of RhoA/Rho kinase pathway, which resulted in poor MLC phosphorylation (Yang et al., 2009). However, the interaction of sex hormones with the RhoA/Rho kinase pathway in the myometrium is unexplored. Therefore, this notion remains to be investigated in future studies.

Based on our findings, it is reasonable to hypothesise that the incubation with DHT essentially impaired the entry of extracellular $Ca^{2+}$ into the uterine myocytes. It is generally accepted that reduction of total [$Ca^{2+}$] in uterine myocytes results in reduction of MLCK activity and, thus, poor phosphorylation of MLC (Wray et al., 2001). We examined whether DHT could impair $Ca^{2+}$ transients in uterine myocytes. Indeed, we demonstrated that short incubation with DHT decreased the level of OXT-stimulated increase in [$Ca^{2+}$] in Fluo-4-loaded PHM1-41s and UtSMCs. Few studies in SMCs have reported poor $Ca^{2+}$ flux in response to stimuli following incubation with sex hormones. For example, short incubation with E2 inhibited the

histamine-induced increase in [$Ca^{2+}$] in Fura-2-loaded airway smooth muscle (ASM) cells (Townsend et al., 2010). In addition, DHT treatment of Fura-2-loaded isolated rat vas defens cells blunted the KCl-induced elevation in [$Ca^{2+}$] (Lafayette et al., 2008). The mechanism by which sex hormones regulate $Ca^{2+}$ transients in smooth muscle has been investigated in several tissues using patch clamp studies and selective inhibitors of various $Ca^{2+}$ and $K^+$ channels. These studies examined the hypothesis that sex hormones could directly or indirectly prevent depolarisation or induce hyperpolarisation of the cell membrane via closure of $Ca^{2+}$ channels or opening of $K^+$ channels respectively to induce relaxation. For example, studies of smooth muscle of canine basilar artery have demonstrated that T-induced relaxation was mediated via $Ca^{2+}$ channel blockade but not $K^+$ channel activation (Ramirez-Rosas et al., 2011). Consistently, the DHT-induced relaxation of rat vas defens smooth muscle was not prevented by numerous $K^+$ blockers, suggesting that the observed blockade of $Ca^{2+}$ flux in these cells was mediated via $Ca^{2+}$ rather than $K^+$ channels (Lafayette et al., 2008). Incubation with $K_{ATP}$ blockers did not inhibit the T-induced relaxation in human umbilical artery (Cairrao et al., 2008) and corporal SMCs (Han et al., 2008), also implying no involvement of $K^+$ channels in the mediation of relaxation. In addition, Anderson and colleagues explored the implication of $K_v$, $K_{ATP}$ and BKCa channels in the mediation of P4-induced relaxation in human myometrium (Anderson et al., 2009). Their studies showed that the P4 relaxing action was resistant to incubation with selective $K^+$ channel blockers. On the other hand, few studies have reported a reduced response to sex hormones following incubation with selective $K^+$ channel blockers. For example, the relaxant effect of T was attenuated following blockade of $K_v$ and $K_{ATP}$ channels in human SMCs isolated from umbilical artery (Cairrao et al., 2008). Similarly, a $K_{ATP}$ blocker inhibited T-induced relaxation of SMCs isolated from human spermatic vein (Seyrek et al., 2011). Finally, blockade of the BKCa channel reportedly decreased the relaxing action of T on vascular SMCs in different species (Cairrao et al., 2008; Deenadayalu et al., 2012; Han et al., 2008; Unemoto et al., 2007). Collectively, whether $K^+$ channels are involved in mediation of non-genomic effects of sex hormones on SMCs is a subject of controversy. However, $K^+$ channels are widely

expressed in the human myometrium where their roles as mediators of contraction are well established (Knock et al., 2001).

An alternative mechanism, whereby sex hormones elicit their non-genomic effects on smooth muscle by increasing the intracellular levels of cAMP has been proposed. According to this hypothesis, activation of β-receptors on the SMC membrane induces a rapid increase in cAMP which in turn activates protein kinase A (PKA) resulting in decrease in [$Ca^{2+}$] and, thus, decrease in MLCK activity (Catterall, 2000). It has been hypothesised that sex hormones induce relaxation in a similar way to β2-adrenergic agonists, such as β-mimetics, well known for their tocolytic action. Consistent with this hypothesis, short administration of E2 increased cAMP in SMCs derived from rat pulmonary artery (Farhat et al., 1996), mesenteric artery (Keung et al., 2011) and porcine coronary artery (Keung et al., 2005). Townsend et al, additionally, showed that inhibition of PKA prevented the E2-induced decrease in [$Ca^{2+}$] in histamine-stimulated ASM cells, suggesting that E2-induced relaxation was mediated via β-receptors (Townsend et al., 2012). However, inhibition of PKA in another study did not prevent the T-induced relaxation in SMCs derived from porcine gallbladder (Kline and Karpinski, 2008). E2 additionally induced an increase in cAMP content in SMCs of porcine coronary artery (Teoh and Man, 2000). In another study, micromolar concentrations of P4 rapidly increased cAMP in human myometrial cells (Fu et al., 1998). Increased accumulation of cAMP in response to P4 and E2 treatments has been reported in porcine myometrial SMCs *in vitro* (Kisielewska et al., 1997). Interestingly, older *in vivo* studies have revealed that administration of E2 and P4 to pregnant rabbits increased MLCK activity in the myometrium (Matsui et al., 1983), which is in contrast to the previously discussed effects of E2 and P4 on intracellular cAMP. A logical assumption to explain this disparity would be that the effects observed in that latter study, in contrast to the previous studies, were gene-transcription dependent. Notably, there is no published evidence of a direct effect of steroid hormones on MLCK activity in the uterine muscle and it is currently unclear whether the observed effects of sex hormones on $Ca^{2+}$ transients and MLC are mediated via a β-receptor pathway.

An interaction of sex hormones with ROCCs in mediation of relaxation has also been proposed. In our studies, DHT inhibited the OXT-induced increase in [$Ca^{2+}$] in UtSMCs and PHM1-41s. OXT is known to stimulate both capacitive and non-capacitive $Ca^{2+}$entry in PHM1-41s (Monga et al., 1999). OXT promotes $Ca^{2+}$ entry through ROCCs and/or internal stores (SR) via the IP3 pathway (Large, 2002; Thorneloe and Nelson, 2005). It is reasonable to suggest that the DHT treatment in our study inhibited the IP3 pathway and, consequently, the release of $Ca^{2+}$ from the SR into the cytoplasm, which manifested as a decrease in total concentration of [$Ca^{2+}$]. However, this is unlikely as T in another study failed to inhibit both caffeine- and carbachol-induced (activators of IP3-pathway) $Ca^{2+}$ release from the SR in coronary SMCs (Murphy and Khalil, 1999). What is more, studies in isolated rat thoracic aortic strips and porcine coronary arteries also showed that T did not inhibit caffeine-induced contractions (Crews and Khalil, 1999b). Thus, it is likely that androgens block the ROCCs-associated $Ca^{2+}$ influx rather than components of the IP3 pathway. In future studies it would be worth examining the DHT-induced effects on $Ca^{2+}$ transients using a $Ca^{2+}$-depleted medium to elucidate whether the action of DHT is mediated via inhibition of capacitive or non-capacitive $Ca^{2+}$entry. The possibility of a non-capacitive entry is supported by studies showing that steroids can relax the KCl-induced tonic contraction, implying a VOCCs-mediated effect (Hidalgo et al., 1996; Perusquia et al., 1990; Perusquia et al., 2005; Perusquia and Villalon, 1996). This notion is further supported by the observation that the relaxant effect of sex hormones mimics the effect elicited by typical $Ca^{2+}$-entry blockers suggesting a $Ca^{2+}$-antagonistic property (Perusquia, 2001). We also observed that the action of DHT in our study was similar to that of an L-type $Ca^{2+}$ channel blocker namely nifedipine. In line with the hypothesis of a VOCCs-mediated relaxant effect, other findings have shown that sex hormone-induced relaxation was reversed by $Ca^{2+}$ ionophores (A-23187 and X- 537A) (Perusquia et al., 1990).

In conclusion, the findings in this chapter demonstrated a physiological mechanism for the DHT-induced relaxation of uterine muscle, whereby DHT might impair $Ca^{2+}$ entry and, consequently, impact phosphorylation of MLC in uterine myocytes. Based on the published literature on the non-genomic actions of sex hormones and our findings, it is tempting to suggest that sex hormones in micromolar concentrations

act on the myometrial cell membrane and block $Ca^{2+}$ entry either via direct blockade of $Ca^{2+}$ channels or indirectly via penetration into the cell membrane as discussed in Chapter 4. The non-genomic mechanism of sex hormone action on myometrium warrants further investigation and the elucidation of these events could contribute towards the discovery of novel targets for development of tocolytic agents.

# Chapter 6: Investigating the effect of intrauterine DHT administration for prevention of preterm labour in a mouse model of inflammation-induced preterm birth

## 6.1 Introduction

Despite the considerable advances in parturition research the precise molecular mechanisms underlying the onset of preterm labour (PTL) remain elusive. Tocolytic agents, which target components of the uterine contraction apparatus and induce myometrial relaxation, are currently the only available treatments for PTL (Duckitt et al., 2014; Flenady et al., 2014; Haas et al., 2014; Haas et al., 2012; King et al., 2003; Neilson et al., 2014; Sykes et al., 2014; van Vliet et al., 2014). Notably, these agents target the end-points in the onset of the labour cascade, when the mechanism is already activated and hard to reverse. This may explain the inability of tocolytics to prolong pregnancy for more than 7 days (King et al., 2003). Antibiotic therapy, aimed at treatment of intrauterine infection during pregnancy, and P4, aimed at maintaining uterine quiescence, have been reported to prevent some but not all cases of PTL (Brocklehurst et al., 2013; Dodd et al., 2013; Hapangama and Neilson, 2009; Kenyon et al., 2010; Manuck et al., 2014; Seelbach-Goebel, 2013; Yellon et al., 2013). To date, P4 has been shown to be effective in prolonging pregnancy only in women with previous PTB, short cervix or ART pregnancy (Aflatoonian et al., 2013; Choudhary et al., 2014; Conde-Agudelo et al., 2013; DeFranco et al., 2007; Dodd et al., 2008; Mackenzie et al., 2006; McKay et al., 2014; Schmouder et al., 2013). Antibiotics are associated with adverse long-term effects in women with intact fetal membranes (Kenyon et al., 2008) and the long-term effect on the offspring following P4 administration during pregnancy is yet to been reported (Norman et al., 2012) Hence, despite the wide usage of tocolytics, antibiotics and P4 for management of PTL, these agents are not fully effective and there is little evidence of long-term beneficial effects for the baby (Hosli et al., 2014; Jorgensen et al., 2014; Kenyon et al., 2010; Kenyon et al., 2008; Marlow et al., 2012). Therefore, it is important to develop novel therapeutic approaches that target molecular pathways involved in the initiation of PTL.

Animal models have been utilised in PTB research to investigate the physiology of labour and test the ability of several agents to prevent or stop PTL (Hirsch and Wang, 2005; Mitchell and Taggart, 2009). Inflammatory agents like LPS, TNF-α and IL-1β have shown to induce premature labour when given to pregnant mice via various routes, making the infection-induced PTB mouse model a cost effective tool to test possible interventions for PTL (Elovitz and Mrinalini, 2004; Sadowsky et al., 2006; Silver et al., 1994). A number of studies employing the above models have reported that administration of agents like TLR-4 antagonist, anti-TNF-α antibody, IL-1R antagonist, NF-kB inhibitors, IL-10, medroxyprogesterone acetate (MPA), prostaglandin 15d-PGJ2, phosphodiesterase (PDE4) inhibitor and P4 could all block inflammation-induced PTL (Chang et al., 2011; Elovitz and Wang, 2004; Gonzalez et al., 2011; Li et al., 2010; Nath et al., 2010; Schmitz et al., 2007). However, the mechanism by which these agents act is not fully understood and some of these findings did not stand up to replication attempts. Hence their potential as drugs that will prevent PTL in humans is unclear. Studies in our and other laboratories have previously reported that intrauterine administration of LPS on day 17 of murine pregnancy results in preterm delivery and pup mortality (Elovitz et al., 2003; Rinaldi et al., 2014). The mechanism of LPS action in the uterus is likely TLR-4 mediated (Li et al., 2010). Our group has recently demonstrated that the TLR-4-mediated LPS signalling promoted myometrial contraction by sensitising MSMCs to $Ca^{2+}$ entry via the Rho/ROCK pathway *in vitro* (Hutchinson et al., 2014). In that study, the effect of LPS on the contraction of collagen embedded MSMCs was reversed by incubation of gels with a ROCK inhibitor. We have showed earlier in this thesis that non-genomic doses of DHT can induce relaxation of uterine horns *ex vivo* (Chapter 4) and that the action of DHT is probably mediated via blockade of $Ca^{2+}$ entry into the human MSMCs *in vitro* (Chapter 5). These findings combined would suggest that DHT might inhibit the LPS-induced preterm-initiated myometrial contractions in a mouse model of LPS-induced PTL and, thus, prevent the incidence of PTL. In support of this hypothesis, human studies have showed that pregnancies characterised by a significantly lower concentration of maternal androgens, compared to the reported levels during normal pregnancy, resulted in recurrent miscarriages, suggesting that androgens may play key role in maintenance of pregnancy (Bammann et al., 1980;

Okon et al., 1998). Notably, intrauterine infections are positively associated with the incidence of spontaneous abortion and recurrent miscarriage (Howie et al., 2011). A link between intrauterine infection and the availability of androgens in the microenvironment of the pregnant uterus has been previously documented. In these studies, the concentration of androgens in the amniotic fluid was reportedly greater in cases of intrauterine infection-induced PTL when compared to the cases of PTL with no presence of infection (Gravett et al., 2000). Besides the amniotic fluid, abnormally high concentration of androgens has been demonstrated in the maternal serum in some cases of PTL in humans (Furui et al., 2007). Collectively, it is possible that infection-induced inflammatory cascades stimulate androgen biosynthesis, suggesting that androgens might have anti-inflammatory properties.

Based on the above evidence, it was tempting to hypothesise that intrauterine administration of DHT would prevent the LPS-induced PTL in a mouse model of inflammation-induced PTB.

### 6.1.1 Specific aims

In order to test the above hypothesis we aimed to

- Confirm that the AR is expressed in the mouse myometrium on day 17 of gestation
- Perform a pilot study to explore the effect of intrauterine administration of DHT in a mouse model of LPS-induced PTL.

## 6.2 Materials and Methods

### 6.2.1 DAB immunostaining for Ar in the C57BL/6 myometrium

The presence of the murine AR (Ar) in the myometrium of C57BL/6 mice on D17 of gestation was investigated using immunohistochemistry. Uterine tissue was collected from C57BL/6 mice on D17 of pregnancy and processed for histology as described in section 2.10.1. The DAB immunostaining was performed as described in section 2.10.2. Briefly, slides were dewaxed and rehydrated prior to being antigen retrieved in citrate buffer (pH 6) according to standard protocols. Slides were incubated in methanol supplemented with 3% v/v $H_2O_2$ for 30 minutes and blocked with NGS (5% w/v BSA). Sections were additionally blocked using the avidin/ biotin blocking kit as per supplier's (Vector Labs) instructions and incubated overnight at 4$^{o}$C with the primary anti-AR antibody diluted in NGS. The secondary biotin-conjugated antibody diluted in NGS was applied (30 minutes) and a last incubation with HRP diluted in TBS was performed (30 minutes). All washes between incubations were performed with TBS. The DAB HRP detection system was used as per supplier's instructions (Vector Labs) and the slides were counterstained with haematoxylin. The experiments included a negative control (primary antibody absent) and images were acquired on Provis BX2 microscope using a Canon DS126131 camera and EOS image capture software. Details of all antibodies and dilutions used in the present protocol are shown in **Table 6.1**.

**Table 6.1:** Antibodies used for AR detection with DAB detection system.

| Antibody | Dilution | Supplier |
|---|---|---|
| Primary: Monoclonal rabbit anti-AR | 1:400 | Spring Bioscience (SP107) |
| Secondary: Goat anti-rabbit biotinylated | 1:500 | Vector (BA 1000) |
| HRP | 1:500 | Vector (SA 5004) |

### 6.2.2 Intrauterine administration of DHT to a mouse model of LPS-induced PTL

#### *6.2.2.1 Animals*

Pregnant time-mated CD57BL/6 mice (n=35) were purchased from Charles River on day 10 of gestation (plug day was considered as day 1 of gestation) and were allowed to acclimatise for a week prior to the start of the experiment. The experiments were conducted under a UK Home Office licence and were in line with the Animal Scientific Procedure Act (1986).

#### *6.2.2.2 Surgery*

Surgery was performed as described in detail in section 2.12.1. Briefly, on day 17 of pregnancy (D17) the mice were anaesthetised with Isoflurane (5%) and a lower abdomen mini-laparotomy was performed, whereby both uterine horns were exposed through the incision and the number of viable fetuses/horn was recorded. All mice received two intrauterine injections, which varied between the different treatment groups (**Table 6.2**). The first treatment was infused into the uterine lumen of the horn that had the most fetuses between the first and second anterior fetuses using a 33-gauge Hamilton syringe (**Figure 2.5**, Chapter 2). The second treatment was infused 60 seconds after the first treatment and the time of injection was recorded. The incision was then closed with a continuous stitch using 5/0 sutures and the skin was closed with interrupted stitches using 4/0 sutures. The average time of procedure/animal was 30 minutes. Following surgery the mice were subcutaneously injected with 60 µl of 0.03 mg/ ml vetergesic analgesia and allowed to recover on heated pads (30$^{o}$C) prior to being transferred to individual cages for monitoring. The doses of drugs were selected on the basis of their effectiveness in previous published studies or our own organ bath studies. In particular, LPS has been showed to induce PTL in a dose of 20 µg in the same model (Rinaldi et al., 2014). Intrauterine administration of DHT has not been attempted before. The high dose of DHT (non-genomic/pharmacological) was selected based on the IC50 values from our organ bath studies; 290 ng/animal was equivalent to the 40 µM dose. The low dose of DHT (genomic/physiological) was selected following assessment of the systemic (i.p or s.c) doses used in previously published studies where DHT was administered to

pregnant mice and taking into consideration that local (intrauterine) administration of DHT should be lower to avoid unwanted toxic effects; 22.5 pg/animal.

**Table 6.2:** Intrauterine injections administered per group on D17 of gestation

| Group | Intrauterine injection | Volume | Animals |
|---|---|---|---|
| Vehicle | PBS + etOH | 25 µl + 25 µl | 7 |
| DHT 22.5 pg | PBS + DHT (22.5 pg) | 25 µl + 25 µl | 4 |
| DHT 290 ng | PBS + DHT (290 ng) | 25 µl + 25 µl | 5 |
| LPS | etOH + LPS (20 µg) | 25 µl + 25 µl | 7 |
| LPS+DHT 22.5 pg | LPS (20 µg) + DHT (22.5 pg) | 25 µl + 25 µl | 6 |
| LPS+DHT 290 ng | LPS (20 µg) + DHT (290 ng) | 25 µl + 25 µl | 5 |

#### *6.2.2.3 Time to delivery and pup mortality*

The monitoring of the mice was performed as described in detail in section 2.12.2. Briefly, a mini colour camera was attached to each cage to record time of delivery, which was calculated as the hours from the time of intrauterine injection to the delivery of first pup. Preterm delivery was defined as delivery before 36 hours post injection. The rate of preterm delivery was calculated and compared between treatment groups by dividing the mice that delivered preterm by the total number of mice in each treatment group and was expressed as a percentage. Following completion of labour (delivery of all pups), the number of live pups in the cage of each mouse was recorded. The proportion of live pups per dam was calculated within 24 hours of delivery and compared between treatment groups by dividing the number of live pups recorded in the cage by the number of viable fetuses recorded at the time of injection. Mice and pups were culled by cervical dislocation. Mice were dissected to confirm delivery of all pups and any live pups found in the uterine horns were not considered as live born pups.

#### *6.2.2.4 Statistical analysis*

All analysis was conducted using the GraphPad Prism v6.0 software. The time to delivery data was log-transformed and analysed using one-way ANOVA with Tukey's post-hoc test. The pup mortality data was expressed as proportion of live born pups, arcsin transformed and analysed using one-way ANOVA with Tukey's post-hoc test. Significance was considered to be $p<0.05$ or greater. All error bars denote ±SEM.

## 6.3 Results

### 6.3.1 The expression of Ar in the myometrium of the C57BL/6 mice on D17 of pregnancy

Intense immunopositive staining for Ar was detected in the myometrium of pregnant mice on D17 of gestation and localised to the nuclei of MSMCs (**Figure 6.1a**). No cytoplasmic or perinuclear staining was observed. Although most cells in the myometrium expressed Ar at that time of gestation, a small number of MSMCs were Ar negative (**Figure 6.1 B**).

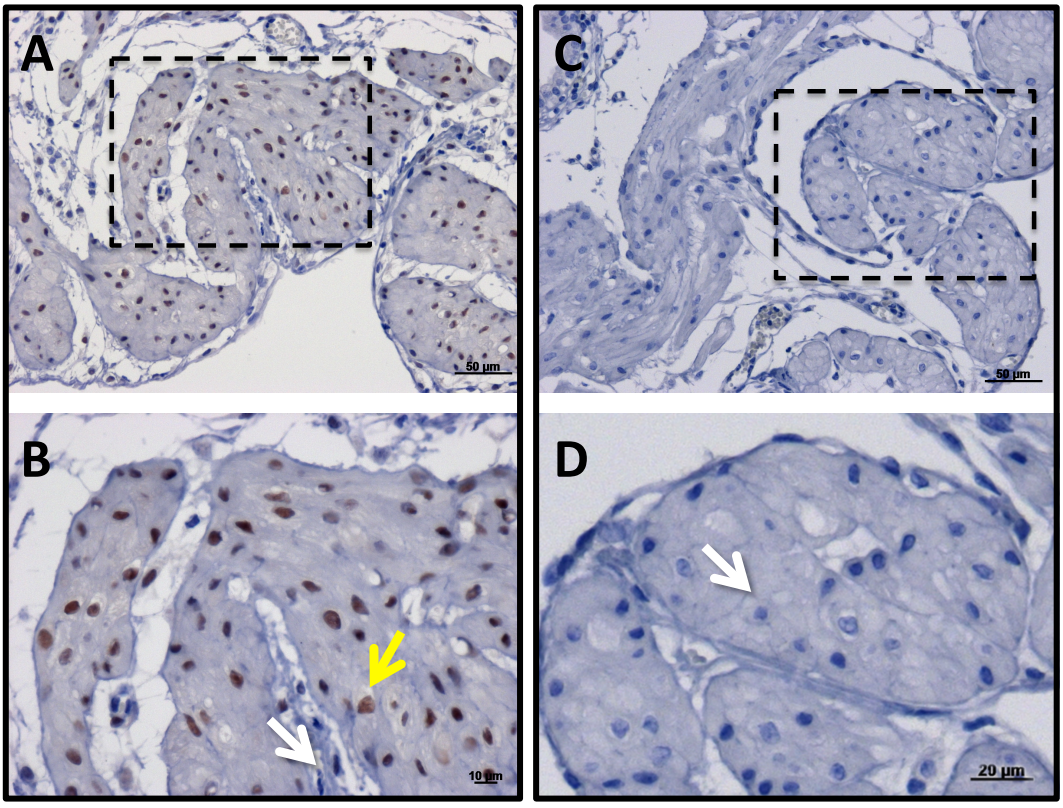



**Figure 6.1:** The Ar is present in the myometrium on D17 of C57BL/6 mouse pregnancy. The dashed area indicated in (**A**) is magnified in (**B**). (**B**) The Ar is localised to the nuclei of some but not all MSMCs. The dashed area indicated in (**C**; negative control) is magnified in (**D**). Yellow arrow points an Ar positive MSMC nucleus. White arrow points an Ar negative MSMC nucleus. Scale bars: 50 µm (**A**, **C**) or 100 µm (**B**).

### 6.3.2 Intrauterine injection of DHT did not prevent preterm labour in the mouse model of preterm birth

PTB was considered birth before 36 hours following injection. The PTB rate in the vehicle group (n=7) was 28.5% and the average time to delivery following injection was 59.74±9.4 hours (**Figure 6.2 A**). For the mice treated with the LPS alone, the rate of PTB was 100% and the animals delivered on average 22.74±1.3 hours after the injection (n=7). For the mice treated with LPS+DHT, the rate of PTB was 66.6% and 80% for LPS+DHT (22.5 pg) and LPS+DHT (290 ng) respectively (**Figure 6.2 A**). Average time to delivery was 40.33±12.43 (n=6) and 36.05±13.79 (n=5) hours following injection of LPS+DHT (22.5 pg) or LPS+DHT (290 ng) respectively (**Figure 6.2 A**). The average values suggest that the mice injected with LPS+DHT delivered their first pup after the 36-hour time point post injections, which was no longer considered preterm. However, comparison between PTB rates between the LPS alone and the LPS+DHT groups did not show a statistical significance, suggesting that DHT did not prevent the LPS-induced PTL phenotype. The PTB rates for DHT (22.5 pg) and DHT (290 ng) treated animals were 0% and 20% respectively. The pup mortality rate was assessed at the end of labour (delivery of last pup). None of the pups born from LPS alone-injected mothers was alive (**Figure 6.2 B**). The pup mortality rate induced by LPS alone was statistically significant when compared to the pup mortality rate induced by the vehicle-injection ($p<0.05$). The DHT treatments did not change the LPS-induced pup mortality rate (**Figure 6.2 B**).

It is noteworthy that administration of a higher dose of DHT (20 μg) in combination with LPS or alone induced a severe phenotype to the pregnant mice characterised by protruded uterus and inability to successfully deliver their first pup. These mice were culled due to distress.

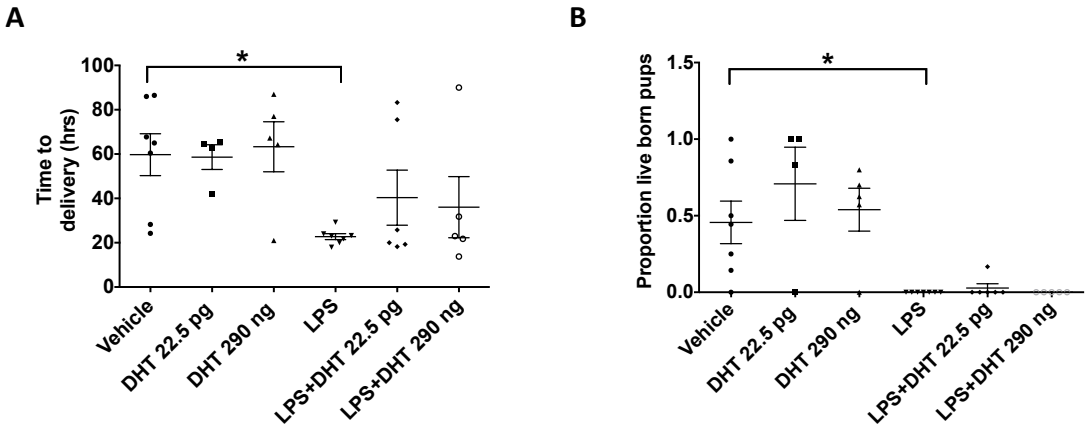



**Figure 6.2:** Intrauterine injection of DHT did not prevent the LPS-induced preterm labour in C57BL/6 mice. Intrauterine injection of LPS on D17 of mouse gestation significantly reduced time to delivery (**A**) and proportion of pups born alive (**B**) when compared to vehicle (PBS+etOH). Intrauterine administration of DHT (290 ng or 22.5 pg) did not prevent the LPS-induced effect on time to delivery (**A**) or live born pup proportion (**B**). *$p<0.05$, n=4-7. Data was analysed using one-way ANOVA with Tukey's post-hoc test.

## 6.4 Discussion

Some androgens, including DHT, are continuously increasing in the maternal blood as human pregnancy progresses [reviewed in (Makieva et al., 2014)]. In contrast, studies in rat suggest that the expression of AR is gradually decreasing as the myocytes progress through a series of phenotypic phases (Liu et al., 2013). It has been postulated that the increase in circulating androgens and the decrease in the myometrial AR might be important for successful pregnancy and timing of parturition. Indeed, there is evidence that ligand independent-AR signalling plays a key role in myometrial proliferation, suggesting a role in establishment of a functional uterus, which will accommodate the growing fetus and rhythmically contract to expel it at term (Liu et al., 2013). Interestingly, the role of the ligand-dependent AR signalling on other myometrial functions, including contraction, has not been studied. Earlier in this thesis (Chapter 3) we reported that the DHT-stimulated AR signalling upregulated the expression of *PAK1IP1* gene in the human myometrial explants. PAK1IP1 protein is known to interact with the PAK1 enzyme, which is a negative modulator of the excitation-contraction coupling in the human myometrium (Moore et al., 2000). Hence, the ligand-dependent AR signalling might favour uterine quiescence. In addition, we found that DHT could induce relaxation of uterine horns derived from non-pregnant mice in an AR-independent way (Chapter 4). From the above studies we hypothesised that genomic and/or non-genomic doses of DHT could possibly inhibit preterm contraction of the myometrium and prevent premature labour. To explore this hypothesis we utilised a mouse model of PTL, whereby PTL was induced by intrauterine administration of LPS (Rinaldi et al., 2014). Previous studies from our laboratory showed the binding of LPS to myometrial TLR-4 activated the Rho/ROCK pathway and promoted $Ca^{2+}$ entry into the cells, which stimulated contraction of human myocytes *in vitro* (Hutchinson et al., 2014). In addition, we observed that DHT treatment could block $Ca^{2+}$ entry into the myocytes and consequently inhibit MLC phosphorylation (Chapter 5). Hence, it was reasonable to hypothesise that intrauterine DHT administration to pregnant LPS-treated mice, would inhibit the LPS-induced preterm-initiated contraction of the myometrium and rescue the PTL phenotype.

The administration of androgens during pregnancy has been a subject of controversy for several decades. Studies performed in 1950s demonstrated that prenatal exposure to T during the guinea pig masculinisation window resulted in female offspring with hermaphrodism and masculinised behaviour in adulthood (Phoenix et al., 1959). Follow up studies in the next decades established that continuous administration of DHT to pregnant rodents resulted in abnormal gonadal and brain development and affected the metabolic function of the offspring, which was further associated with the development of PCOS in females at puberty (Dean et al., 2012; Lazic et al., 2011; Sullivan and Moenter, 2004; Witham et al., 2012; Wu et al., 2010; Yan et al., 2013). It is noteworthy that in order to achieve the described adverse effects on the fetus or the offspring, the DHT was administered systemically (i.p or s.c) from D16 to D19 of rat pregnancy or from D16 to D18 of murine pregnancy in extremely high doses (at the mg range). Notably, in spite of the high concentration, the systemic administration of DHT did not seem to effect pregnancy duration, litter size or pup survival in the studies in which these parameters were investigated. Only one study to date has attempted to deliver intravaginal DHT (2 mg) on D15 of rat pregnancy in order to explore its effect on cervical resistance (Ji et al., 2008). Unfortunately, the pregnancy duration was not studied in these experiments as the animals were sacrificed on D16 for tissue collection purposes (Ji et al., 2008). We performed a pilot study whereby intrauterine DHT was injected on D17 of mouse pregnancy in significantly lower doses compared to previous studies (22.5 pg and 250 ng). Therefore, following critical assessment of all the published evidence to date, we did not expect to observe a deleterious effect of DHT to the mother or the offspring under our experimental conditions. Of note, if DHT was to be used in the future as a tocolytic agent, it would be necessary to address any adverse effects on both future pregnancies of the mother and the development of the fetus. Our preliminary findings suggested that none of the doses of DHT used could effectively reduce the rates of LPS-induced PTL in mice. However, we observed that the PTL rates were trending towards a decrease following DHT injection to LPS-treated animals and that the average time to delivery was extended above 36 hours post injection of LPS. Given that this was a pilot study, we cannot exclude the possibility that these trends would be statistically significant if more animals were included or higher doses of DHT

were tested. However, a power calculation based on these preliminary data suggests that in order to prolong pregnancy for as long as 36 hours (power of 0.8) following administration of DHT to LPS-injected mice, over 300 animals would have to be used.

There is little evidence to support the hypothesis that DHT could inhibit the LPS induced responses in the mouse uterus. Previous studies utilising the mouse model of LPS-induced PTL have shown that P4 could prevent PTL only when given intravaginally (Gonzalez et al., 2011) but not when injected directly into the uterus (Elovitz and Wang, 2004). On the other hand, intrauterine administration of the synthetic progesterone-MPA (2 mg) or 17-alpha hydroxyprogesterone caproate (2 mg) as well as the PDE4 inhibitor prevented the LPS-induced PTL (Elovitz and Wang, 2004; Elovitz and Mrinalini, 2006; Schmitz et al., 2007). It is well established that the action of LPS in the mouse-model of PTL is mediated via the myometrial TLR-4 receptor, which is consistent with evidence that TLR-4 mutant mice do not deliver preterm (Elovitz et al., 2003; Li et al., 2010; Wang and Hirsch, 2003). TLR-4 activation in the myometrium has been proposed to activate the NF-kB pathway, whereby NF-kB directs transcription of chemo-attractants, cell adhesion molecules and pro-inflammatory cytokines and their receptors (Chang et al., 2012; Sykes et al., 2014). An increase in numerous pro-inflammatory mediators, including COX-2, IL-1beta, IL-6 and TNF-alpha, has been reported in the maternal serum and amniotic fluid of mice exposed to intrauterine LPS (Burd et al., 2010). MPA is reported to suppress transcription of genes that encode pro-inflammatory mediators and CAPs in response to LPS in the mouse uterus (Elovitz and Mrinalini, 2005). In addition, administration of a PDE4 inhibitor initiated an anti-inflammatory response, involving inhibition of NF-kB pathway and downregulation of numerous inflammatory cytokines (Schmitz et al., 2007). Therefore, it is tempting to hypothesise that higher doses of DHT (i.e 2 mg) might significantly prevent PTL by activating a similar mechanism to MPA or PDE4. Indeed, the anti-inflammatory properties of DHT have been previously documented (Osterlund et al., 2010; Vignozzi et al., 2012). For example, studies in vascular smooth muscle cells, demonstrated that DHT could reduce the LPS-initiated COX-2 synthesis (Osterlund et al., 2010). Notably, in that study the action of DHT was estrogen receptor beta (ERβ)- rather than AR-

mediated. In line with these findings, DHT could also attenuate the expression of inflammatory markers in microvascular endothelial cells via a mechanism involving local metabolism of DHT to 5α-androstane-3β,17β-diol, capable of binding to ERβ but not AR (Zuloaga et al., 2012). The ability of DHT to suppress NF-kB activation, and consequently decrease the synthesis of several inflammatory factors, such as IL-8 and IL-6, has been demonstrated in prostatic hyperplasia stromal cells (Vignozzi et al., 2012). Human studies indicated that the intrauterine infection-induced PTL, but not spontaneous PTL, was associated with increased content of androgens in the amniotic fluid, suggesting that an increase in local androgen biosynthesis could play a role in infection-induced in utero inflammation during pregnancy (Gravett et al., 2000). Notably, abnormally low levels of circulating androgens have been associated with the incidence of spontaneous abortion and recurrent miscarriage (Bammann et al., 1980; Okon et al., 1998), both of which are additionally associated with the presence of intrauterine infection (Howie et al., 2011; Kitaya, 2011; Ugwumadu, 2010). Based on the above evidence, it is plausible that in our study the DHT-stimulated AR signalling in the myometrium might have inhibited the LPS-initiated inflammatory cascades; however, the doses of DHT used might not have been in the correct range to induce a statistically significant effect on PTL. Of note, the response elicited by LPS administered via laparotomy is extremely severe and in many cases irreversible unless very high doses of drugs are applied. Notably, the amount of MPA that prevented the LPS response in the mouse model of PTL was at 2 mg/dose, which was approximately $2 \times 10^6$ times greater that the higher dose of DHT used in our study (Elovitz and Wang, 2004). Interestingly, MPA is reported to be a potent AR agonist that binds to the AR with similar affinity to that of DHT but requires a 100 fold greater concentration for a compatible induction of transcription (Bentel et al., 1999; Kemppainen et al., 1999). The latter would suggest that higher dose of DHT in future experiments might potentially effectively prevent the LPS associated PTL phenotype.

Besides its potential anti-inflammatory roles, there is some evidence to suggest that DHT might also be involved in the regulation of uterine ripening, which involves the degradation of ECM prior to labour. The myometrium contains a substantial amount of ECM, which connects the muscle bundles (Schwalm and Dubrauszky, 1966).

Remodelling of the myometrial ECM takes place during pregnancy and it has been reported that the main fibrillar (collagens) and non-fibrillar (proteoglycans) components of ECM decrease in the term myometrium compared to the non-pregnant tissue. In particular, there is evidence that the two main proteoglycans namely decorin and biglycan are dramatically decreased in the human myometrium during labour (Hjelm et al., 2001). The critical role of the timing of the decorin and biglycan decrease has been recently highlighted in studies where mice with a double KO for both proteoglycans exhibited a PTL phenotype, which was rescued by the presence of at least two biglycan or decorin wild type alleles (Calmus et al., 2011). Hormonal control of these components has been previously described in the mouse endometrium but has not yet been explored in the myometrium during pregnancy (Salgado et al., 2011; San Martin et al., 2003). In contrast to humans, in the sheep, the decorin concentration in the myometrium is increased with the onset of labour (Wu et al., 2000). The expression of decorin in the sheep myometrium is under E2 control, suggesting that an additional pro-contractile property of E2 is to promote myometrial ECM degradation (Wu et al., 2000).

The degradation of ECM is under the control of matrix metalloproteinases, including MMP2 and MMP9, which are secreted from immune cells that infiltrate the myometrium at term to promote initiation of myometrial contraction (Roh et al., 2000). P4 has been shown to reduce the activity of MMPs and decrease ECM degradation in the myometrium of pregnant bitches (Kanca et al., 2011) and rats (Jeffrey et al., 1990; Wilcox et al., 1992). Incubation of human myometrial explants with LPS, which were obtained at term pregnancy, induced an increase in MMP2 and MMP9 activity and ECM degradation (Wendremaire et al., 2013). DHT has been additionally documented to downregulate the expression of MMPs in vascular SMCs (Mountain et al., 2013). Whether the AR-dependent signalling has a regulatory role in uterine ECM remodelling has yet to be explored. However, AR null mice exhibit longer uterine horns with thinner uterine smooth muscle layer compared to wild type mice (Hu et al., 2004; Walters et al., 2009). These findings might indicate that the AR-dependent signalling plays a key role in uterine muscle proliferation, which is positively correlated with increased expression of ECM components (Nagler et al., 1997). Therefore, it is possible that the AR-dependent signalling either directly

regulates the expression of these components in the pregnant myometrium or favours their deposition in the ECM and, thus, promotes uterine quiescence. Testing the hypothesis that DHT interferes with MMPs action in the myometrium following exposure to LPS and inhibits ECM degradation would help address the discussed notions. In support of a role of AR in the control of ECM remodelling, studies in the rat cervix during pregnancy showed that administration of DHT promoted ECM degradation and decreased cervical resistance, proposing a pro-labour role for AR-dependent signalling (Ji et al., 2008). We cannot exclude the possibility that the DHT in our study descended from the uterus to the cervix to promote cervical ripening. We observed that some LPS-treated mice did not deliver preterm. A possible explanation would be that in these mice the DHT did not reach the cervix but remained in the uterine microenvironment where it prevented the previously discussed LPS-initiated cascades. Future experiments utilising a fluorescent-labelled DHT would help explore the localisation of DHT in the reproductive tract following the intrauterine injection.

In summary, the results from our pilot study indicated that significant lower rates of PTL were not observed in the groups of animals treated with both DHT and LPS compared to the LPS alone group and, thus, it is not conclusive whether DHT is effective in prevention of inflammation-induced PTL. Future studies including alternative doses of DHT would help conclude whether DHT has the potential to be used as a tocolytic agent to prevent inflammation-induced PTL.

# Chapter 7: General discussion

The role of androgens in pregnancy and parturition has been highlighted in numerous human and animal studies and they are proposed to regulate myometrial and cervical function [reviewed in (Makieva et al., 2014)]. In this thesis we investigated the role of androgens in myometrial biology during pregnancy. The key novel findings from our experiments are summarised and discussed in this chapter.

## 7.1 Summary of findings

- The AR protein was expressed in the human myometrium at term and localised to the nuclei of some, but not all, MSMCs. The AR mRNA in myometrial biopsies obtained during active labour was significantly lower compared to myometrial biopsies obtained at term but before the onset of labour. However, the total concentration of the AR protein, derived from term myometrial homogenates, did not show a significant decrease with labour.
- The expression of the AR mRNA in human myometrial cell line derived at term pregnancy (PHM1-41s) was upregulated by its ligand DHT and following exposure to co-treatment with E2+P4 but not E2 or P4 alone.
- Treatment of human myometrial cell lines (PHM1-41s and UtSMCs) with DHT did not alter the expression of genes that are associated with the process of phenotypic transition of MSMCs with pregnancy. However, in human myometrial explants, DHT significantly upregulated the expression of the *PAK1IP1* gene, which might be involved in the regulation of human myometrial contraction.
- Both DHT and T rapidly, and dose-dependently, relaxed the spontaneous contraction of human myometrial strips obtained at term pregnancy and murine uterine horns obtained from non-pregnant C57BL/6 mice. The DHT and T additionally relaxed OXT-stimulated contraction of human myometrial strips at term pregnancy.
- The membrane-impermeable T (TBSA) did not have a relaxant effect and pre-incubation with an AR-antagonist (flutamide) did not prevent the relaxant

effect of DHT. Hence, it was concluded that DHT and T did not recruit a membrane or a classical AR to induce their relaxant effects.

- A sex hormone cocktail, consisting of P4, E2, DHT and T, was as effective in induction of relaxation as each individual steroid in the equivalent dose, suggesting that the effect was not specific to one sex hormone and was perhaps attributable to their steroid structure.
- DHT and T could inhibit contraction of myometrial cells (UtSMCs and PHM1-41s) embedded in collagen gels. Investigation of the mechanism of the DHT-induced relaxation using these cells revealed that DHT possibly attenuated $Ca^{2+}$ entry into the MSMCs, which consequently affected phosphorylation of MLC.
- A pilot *in vivo* study in which DHT was administered into the uterine muscle of a mouse model of LPS-induced PTL on D17 of pregnancy suggested that DHT might lower the rates of PTL; however at the doses that the DHT was used in this study these effects were not significant and further studies are required.

## 7.2 The role of the genomic androgen signalling in the myometrium during pregnancy

Prior to the current study, only one study explored the expression and the role of AR in the myometrium during pregnancy and that was conducted in rats (Liu et al., 2013). That study reported two key findings; a) that the AR protein was decreasing in the myometrium as pregnancy progressed and b) that the AR-dependent signalling increased MSMCs proliferation. Both findings suggest that the AR signalling might be important at the start of pregnancy, where it could be involved in establishment of a functional uterine muscle that will grow to accommodate the growing fetus and contract at labour to expel the baby. We sought to extend these findings by examining the role of AR-dependent signalling in the myometrium during pregnancy utilising human myometrial tissue derived at term pregnancy and two myometrial cell lines; the PHM1-41s, which were obtained from a single pregnant donor at term, and the UtSMCs, which were obtained from a single non pregnant donor.

The expression of the AR in the human myometrium during pregnancy has not been previously explored. Our new findings suggest that the AR protein is localised to the nuclei of MSMCs at term before the initiation of labour and during active labour, suggesting that it could act as a transcription factor to regulate gene expression. Interestingly, we observed that AR mRNA concentrations were significantly decreased in the labouring myometrium when compared to non-labouring tissue. However, we did not observe a similar trend in the amount of AR protein in myometrial homogenates from term non-labouring and term-labouring biopsies. A logical assumption to explain such disparity between the expression of mRNA and protein would be that the AR was subjected to one or more PTMs (posttranslational modifications). Indeed a number of PTMs have been reported for the human AR, including AR phosphorylation and ubiquitination (Gioeli and Paschal, 2012). Both PTMs could result in stabilisation of the AR protein and a reduction in its turnover even if the transcription of the *AR* gene was already programmed to decrease. There are two possible reasons why an overall decrease in the AR in the myometrium during labour might be important. Firstly, the decrease in AR might play a role in initiation of contraction in the labouring myometrium, as the AR-dependent signalling might be involved in suppression of pro-contractile genes. Secondly, the decrease in AR might be important for successful postpartum involution of the myometrium, as AR-dependent signalling is thought to promote MSMCs proliferation.

We decided to investigate the impact of AR-dependent signalling on myometrial proliferation, contraction and apoptosis. Based on a report that during rat pregnancy the MSMCs undergo a phenotypic transition characterised by a phenotype-specific gene expression profile (Shynlova et al., 2009b), we examined the expression of some of these genes following exposure of human myometrial cell lines to DHT. The expression of genes reported to be involved in the regulation of proliferative, synthetic, contractile and apoptotic phenotype of MSMCs was studied. Although our findings suggest that concentrations of DHT in a physiological range did not regulate the expression of any of the genes we examined, we cannot exclude the possibility that AR-dependent signalling is involved in regulation of myometrial events during pregnancy because we only examined candidate genes. A whole human gene

expression microarray might shed some light on the role of AR activation in the human myometrium during pregnancy. Instead of conducting a whole genome array, we decided to conduct a pilot study during which we used a PCR array-examining 84 pre-selected validated androgen-responsive genes with androgen response elements (ARE) on their promoter. Notably, that array has been widely used in cancer research, particularly in studies on prostate cancer, where AR-signalling is key regulator of cancer cell proliferation (Bennett et al., 2009; Cai et al., 2011; Sadar, 1999; Yang et al., 2011). The "AR-signalling targets PCR array" was performed on human myometrial biopsies derived at term before labour, which were incubated with DHT. Previously in this thesis we established that the expression of AR mRNA in PHM1-41s was under DHT-control and that the ligand upregulated AR mRNA expression following 24 hours incubation. For the PCR array study, we only used the samples that were treated with the concentration of DHT that induced upregulation of the AR mRNA in the PHM1-41s ($10^{-8}$M). The results from four experimental replicates indicated that out of 84 genes examined, some genes were apparently upregulated and some were apparently downregulated at 6 and 24 hours post DHT treatment. However, due to the low experimental size and the high variability between the samples, we were unable to confirm that all of these were statistically significant. The gene *PAK1IP1* was of one of the few genes that were significantly induced by the DHT treatment. PAK1IP1 is an enzyme that interacts with PAK1 protein. Notably, there is already evidence to suggest that PAKs might be involved in maintenance of pregnancy through inhibition of cascades associated with the initiation of uterine contraction (Moore et al., 2000; Wirth et al., 2003). Thus, it is possible that AR-dependent signalling might be involved in the regulation of myometrial quiescence. Additional PCR arrays, which would examine the transcriptional profile of key mediators in the contraction cascade following exposure to DHT, could help explore this notion. In addition, it would be interesting to explore the effect of the DHT dependent AR-activation on the remodelling of myometrial ECM during pregnancy. There is some evidence to suggest that the sex hormone receptor-mediated signalling is involved in ECM degradation in the myometrium and cervix during pregnancy in different species (Ekman-Ordeberg et al., 2003; Ji et al., 2011; Salgado et al., 2011; San Martin et al., 2003; Shynlova et al.,

2004; Simon and Einspanier, 2009). To date, only one study has examined the role of the AR activation by DHT in cervical ECM remodelling (Ji et al., 2008). That study reported that DHT promoted cervical ripening by inducing EMC degradation. The role of AR-signalling in uterine ECM remodelling is currently unexplored but could potentially become an exciting novel area of investigation in future studies.

Taken together, our findings revealed that the human AR was expressed in the myometrium at term pregnancy and was localised to the nuclei of MSMCs, suggesting transcriptional activity. In addition, our *in vitro* studies suggested that *AR* gene transcription was upregulated by its ligand DHT. The latter finding, combined with the nuclear localisation of AR protein, further suggests that the AR in MSMCs at term is ligand activated and transcriptionally active. However, Liu studies of the AR in the rat myometrium reported that AR-mediated MSMCs proliferation was ligand-independent (Liu et al., 2013), so this idea may be oversimplistic. An ARE reporter assay would help explore whether DHT regulates AR transcriptional activity in MSMCs (Bombail et al., 2010).

We also believe that the androgen transcription-dependent signalling in the MSMCs might be involved in the return of myometrium from a pregnant to a non-pregnant state. We did some very preliminary studies using mice with a conditional Ar KO in the myometrium and we observed that these mice had impaired parturition, which was characterised by the inability of pregnant mothers to expel their pups at labour (n=4). These data are not shown in this thesis because we observed considerable phenotype variations in this small group of animals, which we believe to be a consequence of highly variable levels of Ar expression in the myometrium. For example, some mice appeared to be myometrial-mosaic instead of myometrial-null for the Ar. Interestingly, previous studies have reported that the Ar null female mice (total KO) had normal parturition although their uterine muscle was thinner compared to the wild type (Walters et al., 2009). Notably, in that study, the Ar KO animals were mated only once and the effect of muscle ablation on future pregnancies and their parturition was not examined. In our pilot study we did not record the number of pregnancies for each myometrial-Ar KO female. It is tempting to speculate that the impaired parturition phenotype we observed was attributable to

the fact that the uterine muscle did not effectively undergo post-partum involution in the previous pregnancies these mice had due to the ablation of AR in MSMCs. This aspect could be examined in future studies employing a fully validated myometrium-specific Ar KO mouse model and has the potential to inform whether androgen genomic signalling is involved in the regulation of postpartum involution.

## 7.3 The role of the non-genomic androgen signalling in the myometrium during pregnancy

Our organ bath studies demonstrated that DHT and T both could induce a dose-dependent relaxant effect on strips of uterine smooth muscle obtained from pregnant women and non-pregnant mice. The doses used to induce these effects were at the pharmacological (μM) range. Our findings are in line with findings from other laboratories, which reported that androgens induced *ex vivo* relaxation of uterine muscle derived from rat, rabbit and non-pregnant human when applied in pharmacological doses (Perusquia et al., 1991a; Perusquia et al., 1991b; Perusquia et al., 2005; Robson, 1937). The muscle relaxant effect of androgens has been extensively studied in vascular, respiratory, gastrointestinal and skeletal muscle in numerous species (Bordallo et al., 2008; Chou et al., 1996; Costarella et al., 1996; Gupte et al., 2002; Kouloumenta et al., 2006; Kubli-Garfias et al., 1987; Witayavanitkul et al., 2013). The mechanism of the relaxant effect is not fully understood but it was proposed to be androgen-specific, transcription-independent (due to its rapid nature) and not AR-mediated. However, an understanding of the underlying mechanism by which androgens induce relaxation of the uterine smooth muscle could help discover novel targets for development of new tocolytic agents for prevention of preterm-initiated contractions. We decided to study the mechanism of androgen-induced relaxation of myometrial contraction. Our findings suggested that the observed relaxant effect was not androgen-specific but was also achieved using μM concentrations of P4 and E2. In addition, our studies employing a specific AR antagonist (flutamide) or the conjugated androgen TBSA, that is unable to cross the cell membrane, showed that the classical AR or the hypothesised membrane AR were not involved in this androgen-induced relaxation. Notably, we also examined the effect of DHT treatment on uterine strips derived from one myometrium-specific

Ar KO female mouse and observed that the ablation of the *Ar* gene did not prevent the relaxing action of DHT (data not shown in this thesis). We also discovered that a sex hormone "cocktail" mixture (40 μM), consisting of DHT, T, E2 and P4 (each at 10 μM), was as effective in induction of relaxation as each individual sex hormone in the equivalent dose (40 μM). Taken together we suggested that the ability of sex hormones to induce relaxation of MSMCs was perhaps attributable to their molecular structure. Besides relaxing spontaneous myometrial contractions, all the sex hormones tested in our study could also inhibit contractions stimulated with OXT. The inhibition of OXT-initiated contraction was either mediated via direct blockade of OXTR or inhibition of other components of the contractile apparatus, which are involved in the regulation of $Ca^{2+}$ entry into the MSMCs. The latter is more likely, as there is published evidence to suggest that sex hormones can inhibit serotonin-, acetylcholine- and KCl-stimulated uterine contractions, implying that the effect is generic and, hence, does not involve blockade of a single neurotransmitter receptor or potassium channel (Perusquia et al., 1991a; Perusquia et al., 1991b; Perusquia et al., 2005). It is tempting to suggest that the action of sex hormones is mediated via direct or indirect blockade of $Ca^{2+}$ channels (VOCCs/ROCCs). Indeed, our *in vitro* studies, utilising PHM1-41s and UtSMCs, confirmed that incubation with DHT could block OXT-initiated $Ca^{2+}$ escalations in a similar way to the $Ca^{2+}$ channel blocker nifedipine. Thus, DHT appears to induce myometrial relaxation via a physiological mechanism, whereby it attenuates the availability of intracellular $Ca^{2+}$, which in turn results in lower rate of MLC phosphorylation. Incubation of MSMCs with a 100 μM dose of DHT and T for 48 hours did not affect cell viability, therefore we believe that the relaxant effect of sex hormones on the MSMCs was not a result of toxicity.

Taken together these findings support the hypothesis that steroid hormones inhibit myometrial contraction through inhibition of one or more components of the mechanisms that are involved in the generation of intracellular $Ca^{2+}$. Possible targets include $Ca^{2+}$ channels (VOCCs/ROCCs), the plasma membrane $Ca^{2+}$ ATPase (PMCA), which removes $Ca^{2+}$ from the cell, or the gap junctions (Wray et al., 2003). Notably, all these targets reside on the cell membrane and, hence, could all be affected by a sudden change in the membrane permeability.

Sex hormones are metabolites of cholesterol, which is a natural component of the lipid bilayer. Cholesterol helps strengthen the lipid bilayer, decreases its permeability and regulates the activity of numerous membrane proteins (Ikonen, 2008). It is possible that, when applied in the doses within the μM range, sex hormones can penetrate into the cell membrane and interact with the lipid bilayer in a similar way to cholesterol; therefore, impairing MSMCs fluidity. Notably, hydrophobic androgens, such as DHT and T, have been shown to interact with the phospholipid bilayer of the negatively charged membrane (Duval et al., 1983; Van Bommel et al., 1987). Perhaps the penetration of a sex hormone into the lipid bilayer increased PMCA activity, which in turn promoted $Ca^{2+}$ efflux to the extracellular space and resulted in myometrial relaxation. Indeed, there is considerable evidence to suggest that androgen treatment can influence PMCA localisation and activity (Coviello et al., 2006; Deliconstantinos, 1988; Dick et al., 2003; Witayavanitkul et al., 2013). Besides PMCA, interaction of sex hormones with the cell membrane bilayer could also affect the function of trans-membrane gap junctions, which increase smooth muscle $Ca^{2+}$ transients and, thus, promote generation of synchronous contractions. A study in pregnant rats on a high cholesterol diet showed that high cholesterol was associated with decrease in myometrial connexin 43, which is a key connexin involved in the assemble of a gap junction (Elmes et al., 2011). Non-genomic concentrations (μM) of sex hormones have been previously shown to decrease IGJC in rat cardiac myocytes and Sertoli cells (Pluciennik et al., 1996). Therefore, following critical assessment of ours and others findings, we propose that sex hormones induce myometrial relaxation via a mechanism involving direct interaction with the membrane proteolipidic structure, which then alters the function of key components in the regulation of $Ca^{2+}$ homeostasis, such as VOCCs, PMCA and gap junctions. The schematic representation of this hypothesis is shown in **Figure 7.1**.

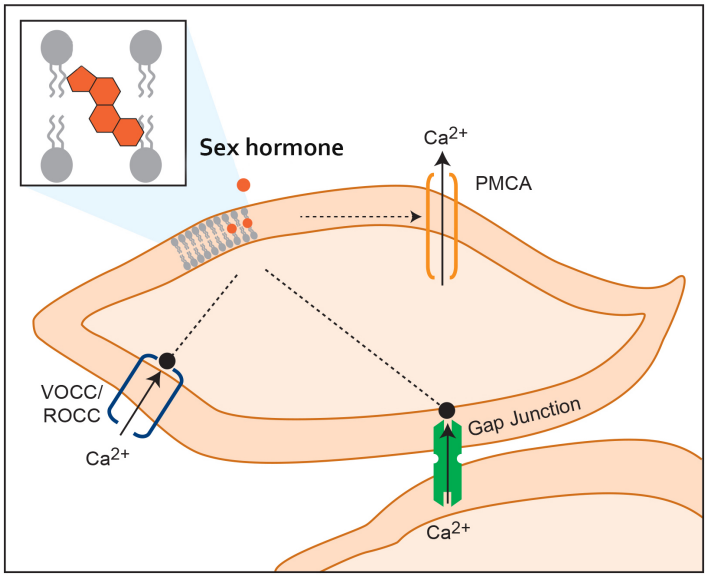



**Figure 7.1:** Schematic summary of the proposed non-genomic mechanism underlying the sex hormone-mediated relaxation in MSMCs. The sex hormone penetrates into the cell lipid bilayer, which may impairs IGJC and/or block VOCCs/ROCCs and/or promote PMCA activity. These events contribute to the decrease in the concentration of intracellular $Ca^{2+}$, which in turn results in relaxation of MSMCs. Image adapted from (Makieva et al., 2014).

Future studies should be performed to explore this hypothesis. For example, MSMCs membranes could be isolated following incubation with sex hormones to validate their steroid content. In addition, electron microscopy studies of MSMCs treated with sex hormones could help deduce whether membrane structures, such as caveolae, which meditate signalling cascades, are affected by incubation with μM doses. Furthermore, the activity of PMCA could be examined *in vitro* following incubation of MSMCs with sex hormones. Pre-incubation of myometrial strips with a PMCA pump blocker (Amarjargal et al., 2008) prior to treatment with sex hormones in the organ bath, could answer whether PMCA is directly involved in the mediation of relaxation. The effect of sex hormones on IGJC could be determined using a scrape-loading dye transfer system (Albrecht et al., 1996), whereby a small molecular weight fluorescent dye is loaded into the intracellular place and its transfer through gap junctions into contiguous cells can be studied. The latter experiment would inform whether the sex hormone-induced relaxation is a consequence of an impaired IGJC in MSMCs.

## 7.4 DHT for prevention of PTL

We showed that steroid hormones relaxed the uterine smooth muscle when applied in high pharmacological doses. Additionally we showed that DHT could block a stimulated increase in intracellular $Ca^{2+}$ in MSMCs. Therefore, it was reasonable to hypothesise that administration of DHT *in vivo* could also inhibit uterine contraction. To examine this hypothesis we utilised a mouse model of PTB, whereby PTL was induced by LPS intrauterine injection on D17 of pregnancy. Besides stimulating pro-inflammatory gene expression, LPS has been recently showed to promote myometrial contraction via a pathway that sensitises the MSMCs to $Ca^{2+}$ influx (Hutchinson et al., 2014). We sought to explore whether DHT could inhibit the pro-contractile properties of LPS in the mouse model of PTB and consequently prevent PTL. We conducted a pilot study whereby DHT was injected at the same time with LPS on D17 of murine pregnancy. Due to limited time, only two doses of DHT were tested. A high dose (290 ng) that was equivalent to the 40 μM organ bath dose, which effectively induced relaxation of murine uterine contraction *ex vivo*, and a lower dose (22.5 pg) that was likely to stimulate a genomic response. Both doses of DHT did not significantly prolong time to delivery when injected in combination with LPS. Some pregnant animals that received the combined DHT and LPS treatment did reach term. However, at this point it is still unclear whether this result is attributed to the inhibitory action of DHT or it is the outcome of the LPS treatment not inducing preterm birth effectively. If the former is the case, the action of DHT could be mediated via two distinct pathways depending on the dose used. For example, the high dose might have induced uterine relaxation in a non-genomic way, whereby the DHT interacted with the cell surface of MSMCs and inhibited intracellular $Ca^{2+}$ escalations. In contrast, the lower DHT dose, which was notably more effective compared to the high dose, was likely to have induced a genomic-response, whereby the DHT-AR complex could have initiated the transcription of a cassette of anti-inflammatory genes. As discussed in Chapter 6, the anti-inflammatory properties of DHT have been documented in numerous tissues, including the smooth muscle (Osterlund et al., 2010; Vignozzi et al., 2012). To test the hypothesis of an anti-inflammatory action of DHT in our study, we would have to investigate the expression of different anti-inflammatory mediators in the uterus

following exposure to DHT in genomic range of doses. In addition, it would be interesting to examine whether the AR-dependent signalling might have affected uterine ECM remodelling in the pregnant mouse uterus. We might speculate that the action of the lower dose of DHT was mediated via inhibition of the LPS-induced degradation of myometrial ECM. Previous studies have showed that incubation of human myometrial explants with LPS resulted in a decrease in total collagen content, indicating ECM remodelling (Wendremaire et al., 2013). It would be interesting to explore these events in our mouse model by characterising the effects of LPS and DHT on myometrial ECM remodelling. In support of a potential role for DHT in uterine ECM remodelling, Ar null mice exhibit thinner uterine muscle and elongated uterine horns that could be a consequence of poor proliferation of MSMCs, which itself is correlated with impaired ECM organisation (Walters et al., 2009). It is noteworthy that the DHT-dependent signalling appears to promote cervical ECM degradation at term pregnancy and thus be a key regulator of cervical ECM remodelling in rats (Ji et al., 2008). However, it is possible that AR-dependent signalling may have opposing roles in the cervix and the myometrium, as it is common for single sex hormones to have tissue-specific opposing effects.

An aspect that deserves attention is the distribution of DHT following the intrauterine injection. We cannot exclude the possibility that following injection, the DHT descended into the cervix, where it could have accelerated the effect of LPS and accounted for the variability in PTL incidence in our study. A logical assumption would be that the DHT was maintained in the uterine microenvironment of mice that did not deliver preterm following DHT+LPS injection, but descended to the cervix in the mice that delivered preterm. Studies utilising a fluorescent-labelled DHT would help verify or exclude this notion. On the other hand, we cannot exclude the possibility that the LPS-injected mice that delivered at term, were simply LPS-non responsive. Notably, some of our vehicle controls exhibited a PTL phenotype; an effect possibly attributable to the invasive nature of the method we used. Consequently, a replication of these experiments using a less invasive method in order to administer intrauterine compounds would be recommended. Recently, a non-invasive non-surgical procedure has been developed in our laboratory, whereby compounds were injected into the uterus of pregnant mice using ultrasound (Rinaldi,

unpublished). Besides its usage for testing possible interventions for PTL in the mouse model, this method could help study the physiology of androgen signalling in the mouse myometrium during normal pregnancy. It would be interesting to explore the effect of DHT-initiated AR signalling on the myometrial function *in vivo*. In these future studies, an ultrasound guided intrauterine injection of DHT would reduce any "falsely" observed pro-inflammatory gene expression cascades, which are potentially induced following a surgical procedure.

Utilising a mouse model of PTB, whereby PTL is induced by administration of LPS, makes the testing for possible interventions challenging, as the action of LPS in the uterus is difficult to revert. We used a 20 μg dose of LPS, which was effective in inducing PTL in our laboratory in two strains of mice; CD1 (Rinaldi et al., 2014) and C57BL/6 (Frew et al., unpublished). Since the establishment of this mouse model on CD1 mice in 2003 (Elovitz et al., 2003), only a few agents have been showed to effectively revert the action of LPS and prevent the incidence of PTL. One of them was the synthetic P4 namely MPA, which was believed to blunt the LPS-induced effect via activation of anti-inflammatory cascades and inhibition of the expression of genes encoding CAPs (Elovitz and Wang, 2004). Interestingly, MPA, which is a potent AR ligand when applied in high doses, when administered into the uterus at 2 mg/dose was shown to induce a significant effect (Elovitz and Wang, 2004). Given that P4 did not have the same effect as MPA, it is possible that the action of MPA was mediated through the AR rather than PR. We tried a 1 mg and 0.2 mg dose of DHT (data not included) and observed an impaired parturition phenotype whereby animals exhibited protruded uteruses during active labour and were unable to deliver a single pup; consequently all these animals were culled. We believe that this dose probably induced a terminal relaxant effect to the mouse uterus resulting in null contractile activity. In the MPA study, in which MPA was injected together with LPS, PTL was defined as delivery of first pup before 36 hours following the injection. The MPA+LPS injected animals had 0% PTL rate, as they did not deliver a pup before the 36-hour time point. Surprisingly, these animals were killed 48 hours following the injection and it was not reported whether the MPA-treated animals managed to deliver a single pup. In our study, we monitored the animals that were administered the DHT in extremely high doses (1 and 0.2 mg) beyond 48 hours post

injection and concluded that these animals were unable to deliver a pup. Therefore, it is tempting to suggest that the MPA in the Elovitz and Wang study did not have a physiological or pharmacological but rather exhibited a toxic effect to the uterus of the mice.

In summary, in our pilot study we did not see a significant decrease in the rate of PTL following administration of DHT to an inflammation induced-mouse model of PTB. However, the decreasing trend in PTL rate that we observed is promising. Therefore more studies using a wider dose range, more animals and the ultrasound guided method rather than the laparotomy would answer the question whether DHT could be used for prevention of PTL. More *in vivo* studies investigating the effect of DHT exposure on fetal development and maternal ability to have a future successful pregnancy would address the suitability of DHT to be administered during pregnancy.

## 7.5 Conclusions and future directions

We performed *in vitro*, *ex vivo* and *in vivo* studies to explore the understudied role of androgens in myometrial biology during pregnancy. Our findings suggest that the androgen-induced *in vitro* and *ex vivo* tocolysis is sex hormone-specific rather than androgen-specific. Therefore, sex hormones hold the potential to be used for effective *in vivo* tocolysis to prevent premature initiated contractions. Developing an agent similar to the cocktail steroid mixture in our studies could eliminate the plausible and unwanted side effects of each individual steroid, when administered as a preventative tocolytic agent throughout pregnancy. This agent would consist of lower doses of each individual hormone and, thus, would reduce the potential adverse effects that a high dose of a single sex hormone could have on the fetus or the mother. More research is necessary to understand the mechanism by which sex hormones inhibit $Ca^{2+}$ entry into the uterine myocytes. If the molecular structure of a sex hormone is key to the relaxant effect, synthetic agents with the same structure, but without the receptor binding sites, could perhaps elicit similar effects. Our *in vitro* studies of the androgen-dependent signalling in the myometrium contributed to the development of new hypotheses regarding the role of AR-dependent signalling in the regulation of the phenotypic transition of MSMCs during pregnancy. These hypotheses (summarised in **Figure 7.2**) deserve further exploration in order to elucidate the physiological role(s) played by androgens in the myometrium during pregnancy, which has the potential to inform new therapeutic strategies for management of numerous pregnancy complications, including PTB.

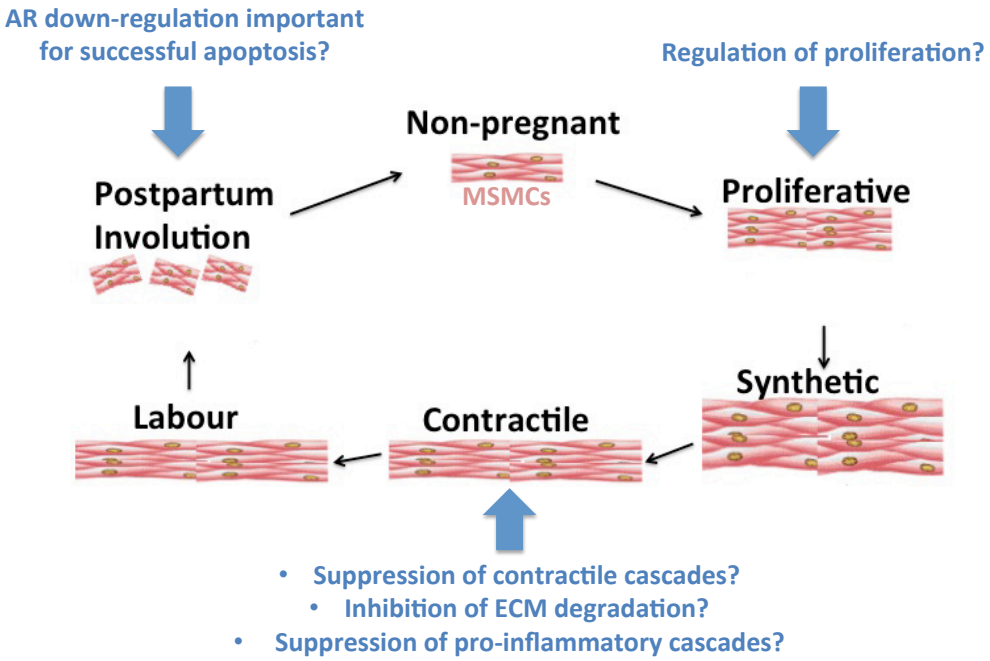



**Figure 7.2:** The hypothetical roles of AR-dependent signalling in the myometrium during pregnancy. Ligand dependent or independent AR signalling might play roles in successful phenotypic transition of MSMCs throughout gestation. AR-dependent signalling might be promoting MSMCs proliferation. In addition, it might have roles in maintenance of uterine quiescence via a) transcriptional suppression of genes encoding pro-contractile proteins, b) inhibition of ECM degradation and/or c) transcriptional suppression of genes encoding pro-inflammatory mediators. Finally, the decrease in AR mRNA during labour might suggest that AR-dependent signalling is not required in the post-labour myometrium. It is perhaps important to decrease the availability of AR for successful MSMCs apoptosis and ECM degradation, both of which occur during postpartum involution. MSMCs=Myometrial Smooth Muscle Cells.

# References

Aaronson, P.I., Sarwar, U., Gin, S., Rockenbauch, U., Connolly, M., Tillet, A., Watson, S., Liu, B., and Tribe, R.M. (2006). A role for voltage-gated, but not Ca2+-activated, K+ channels in regulating spontaneous contractile activity in myometrium from virgin and pregnant rats. Br J Pharmacol *147*, 815-824.

Abbott, D.H., Bruns, C.R., Barnett, D.K., Dunaif, A., Goodfriend, T.L., Dumesic, D.A., and Tarantal, A.F. (2010). Experimentally induced gestational androgen excess disrupts glucoregulation in rhesus monkey dams and their female offspring. Am J Physiol Endocrinol Metab *299*, E741-751.

Abe, T., Ono, S., Igarashi, M., Akira, S., Watanabe, A., and Takeshita, T. (2011). Conservative management of hyperreactio luteinalis: a case report. J Nippon Med Sch *78*, 241-245.

Abraham, G.E. (1974). Ovarian and adrenal contribution to peripheral androgens during the menstrual cycle. J Clin Endocrinol Metab *39*, 340-346.

Abrahams, V.M., Potter, J.A., Bhat, G., Peltier, M.R., Saade, G., and Menon, R. (2013). Bacterial modulation of human fetal membrane Toll-like receptor expression. Am J Reprod Immunol *69*, 33-40.

Ackerman, C.M., Lowe, L.P., Dyer, A.R., Hayes, M.G., Metzger, B.E., Lowe, W.L., Urbanek, M., and Group, H.S.C.R. (2013). Maternal testosterone levels are associated with C-peptide levels in the Mexican American subset of the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study cohort. Horm Metab Res *45*, 617-620.

Adams Waldorf, K.M., Persing, D., Novy, M.J., Sadowsky, D.W., and Gravett, M.G. (2008). Pretreatment with toll-like receptor 4 antagonist inhibits lipopolysaccharide-induced preterm uterine contractility, cytokines, and prostaglandins in rhesus monkeys. Reprod Sci *15*, 121-127.

Adesanya-Famuyiwa, O.O., Zhou, J., Wu, G., and Bondy, C. (1999). Localization and sex steroid regulation of androgen receptor gene expression in rhesus monkey uterus. Obstet Gynecol *93*, 265-270.

Aflatoonian, A., Amouzegar, H., and Dehghani Firouzabadi, R. (2013). Efficacy of 17alpha- hydroxy progestrone on decreasing preterm labor in ART pregnancies: A randomized clinical trial. Iranian journal of reproductive medicine *11*, 785-790.

Aguilar, H.N., and Mitchell, B.F. (2010). Physiological pathways and molecular mechanisms regulating uterine contractility. Hum Reprod Update *16*, 725-744.

Aguilar, H.N., Zielnik, B., Tracey, C.N., and Mitchell, B.F. (2010). Quantification of rapid Myosin regulatory light chain phosphorylation using high-throughput in-cell Western assays: comparison to Western immunoblots. PLoS One *5*, e9965.

Albrecht, E.D., Crenshaw, M.C., Jr., and Pepe, G.J. (1989). The effect of estrogen on placental delivery after fetectomy in baboons. Am J Obstet Gynecol *160*, 237-241.

Albrecht, E.D., Haskins, A.L., and Pepe, G.J. (1980). The influence of fetectomy at midgestation upon the serum concentrations of progesterone, estrone, and estradiol in baboons. Endocrinology *107*, 766-770.

Albrecht, E.D., and Pepe, G.J. (1995). Suppression of maternal adrenal dehydroepiandrosterone and dehydroepiandrosterone sulfate production by estrogen during baboon pregnancy. J Clin Endocrinol Metab *80*, 3201-3208.

Albrecht, J.L., Atal, N.S., Tadros, P.N., Orsino, A., Lye, S.J., Sadovsky, Y., and Beyer, E.C. (1996). Rat uterine myometrium contains the gap junction protein connexin45, which has a differing temporal expression pattern from connexin43. Am J Obstet Gynecol *175*, 853-858.

Alfirevic, Z., Stampalija, T., Roberts, D., and Jorgensen, A.L. (2012). Cervical stitch (cerclage) for preventing preterm birth in singleton pregnancy. Cochrane Database Syst Rev *4*, CD008991.

Allan, G.J., Beattie, J., and Flint, D.J. (2004). The role of IGFBP-5 in mammary gland development and involution. Domestic animal endocrinology *27*, 257-266.

Allport, V.C., Pieber, D., Slater, D.M., Newton, R., White, J.O., and Bennett, P.R. (2001). Human labour is associated with nuclear factor-kappaB activity which mediates cyclo-oxygenase-2 expression and is involved with the 'functional progesterone withdrawal'. Mol Hum Reprod *7*, 581-586.

Allport, V.C., Slater, D.M., Newton, R., and Bennett, P.R. (2000). NF-kappaB and AP-1 are required for cyclo-oxygenase 2 gene expression in amnion epithelial cell line (WISH). Mol Hum Reprod *6*, 561-565.

Amarjargal, N., Mazurek, B., Haupt, H., Andreeva, N., Fuchs, J., and Gross, J. (2008). Effects of SERCA and PMCA inhibitors on the survival of rat cochlear hair cells during ischemia in vitro. Physiol Res *57*, 631-638.

Amoah, C., Yassin, A., Cockayne, E., and Bird, A. (2011). Hyperreactio luteinalis in pregnancy. Fertil Steril *95*, 2429 e2421-2423.

An, B.S., Ahn, H.J., Kang, H.S., Jung, E.M., Yang, H., Hong, E.J., and Jeung, E.B. (2013). Effects of estrogen and estrogenic compounds, 4-tert-octylphenol, and bisphenol A on the uterine contraction and contraction-associated proteins in rats. Mol Cell Endocrinol *375*, 27-34.

Anderson, L., Martin, W., Higgins, C., Nelson, S.M., and Norman, J.E. (2009). The effect of progesterone on myometrial contractility, potassium channels, and tocolytic efficacy. Reprod Sci *16*, 1052-1061.

Angioni, S., Portoghese, E., Milano, F., Melis, G.B., and Fulghesu, A.M. (2007). Hirsutism and hyperandrogenism associated with hyperreactio luteinalis in a singleton pregnancy: a case report. Gynecol Endocrinol *23*, 248-251.

Annamalai, A.K., Hoveyda, F., Williams, R.M., Patterson, A., and Simpson, H.L. (2011). Hyperreactio luteinalis. QJM *104*, 807-808.

Anose, B.M., and Sanders, M.M. (2011). Androgen Receptor Regulates Transcription of the ZEB1 Transcription Factor. Int J Endocrinol *2011*, 903918.

Anwer, K., Oberti, C., Perez, G.J., Perez-Reyes, N., McDougall, J.K., Monga, M., Sanborn, B.M., Stefani, E., and Toro, L. (1993). Calcium-activated K+ channels as modulators of human myometrial contractile activity. Am J Physiol *265*, C976-985.

Arrowsmith, S., and Wray, S. (2014). Oxytocin: its mechanism of action and receptor signalling in the myometrium. J Neuroendocrinol *26*, 356-369.

Arthur, P., Taggart, M.J., and Mitchell, B.F. (2007). Oxytocin and parturition: a role for increased myometrial calcium and calcium sensitization? Front Biosci *12*, 619-633.

Arulkumaran, S., Kandola, M.K., Hoffman, B., Hanyaloglu, A.C., Johnson, M.R., and Bennett, P.R. (2012). The roles of prostaglandin EP 1 and 3 receptors in the control of human myometrial contractility. J Clin Endocrinol Metab *97*, 489-498.

Astolfi, P., and Zonta, L.A. (1999). Risks of preterm delivery and association with maternal age, birth order, and fetal gender. Hum Reprod *14*, 2891-2894.

Atis, A., Cifci, F., Aydin, Y., Ozdemir, G., and Goker, N. (2010). Hyperreactio luteinalis with preeclampsia. J Emerg Trauma Shock *3*, 298.

Avvakumov, G.V., Cherkasov, A., Muller, Y.A., and Hammond, G.L. (2010). Structural analyses of sex hormone-binding globulin reveal novel ligands and function. Mol Cell Endocrinol *316*, 13-23.

Badia, E., Nicolas, J.C., Haiech, J., and Crastes de Paulet, A. (1986). Effect of steroid hormones on the regulation of uterine contractility. Pflugers Arch *407*, 670-676.

Baird, D.T., Horton, R., Longcope, C., and Tait, J.F. (1969). Steroid dynamics under steady-state conditions. Recent Prog Horm Res *25*, 611-664.

Bammann, B.L., Coulam, C.B., and Jiang, N.S. (1980). Total and free testosterone during pregnancy. Am J Obstet Gynecol *137*, 293-298.

Bardin, C.W., Brown, T., Isomaa, V.V., and Janne, O.A. (1983). Progestins can mimic, inhibit and potentiate the actions of androgens. Pharmacol Ther *23*, 443-459.

Batra, S., and Bengtsson, B. (1978). Effects of diethylstilboestrol and ovarian steroids on the contractile responses and calcium movements in rat uterine smooth muscle. J Physiol *276*, 329-342.

Belisle, S., Lehoux, J.G., and Brault, J. (1980a). The metabolism of androstenedione in human pregnancy: the use of constant infusion of unlabeled steroid to assess its metabolic clearance rate, its production rate, and its conversion into androgens and estrogens. Am J Obstet Gynecol *136*, 1030-1035.

Belisle, S., Osathanondh, R., and Tulchinsky, D. (1977). The effect of constant infusion of unlabeled dehydroepiandrosterone sulfate on maternal plasma androgens and estrogens. J Clin Endocrinol Metab *45*, 544-550.

Belisle, S., Schiff, I., and Tulchinsky, D. (1980b). The use of constant infusion of unlabeled dehydroepiandrosterone for the assessment of its metabolic clearance rate, its half-life, and its conversion into estrogens. J Clin Endocrinol Metab *50*, 117-121.

Bell, E., Ivarsson, B., and Merrill, C. (1979). Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proc Natl Acad Sci U S A *76*, 1274-1278.

Belt, A.R., Baldassare, J.J., Molnar, M., Romero, R., and Hertelendy, F. (1999). The nuclear transcription factor NF-kappaB mediates interleukin-1beta-induced expression of cyclooxygenase-2 in human myometrial cells. Am J Obstet Gynecol *181*, 359-366.

Benkusky, N.A., Korovkina, V.P., Brainard, A.M., and England, S.K. (2002). Myometrial maxi-K channel beta1 subunit modulation during pregnancy and after 17beta-estradiol stimulation. FEBS Lett *524*, 97-102.

Bennett, N., Hooper, J.D., Lee, C.S., and Gobe, G.C. (2009). Androgen receptor and caveolin-1 in prostate cancer. IUBMB Life *61*, 961-970.

Bentel, J.M., Birrell, S.N., Pickering, M.A., Holds, D.J., Horsfall, D.J., and Tilley, W.D. (1999). Androgen receptor agonist activity of the synthetic progestin, medroxyprogesterone acetate, in human breast cancer cells. Mol Cell Endocrinol *154*, 11-20.

Berger, N.G., Repke, J.T., and Woodruff, J.D. (1984). Markedly elevated serum testosterone in pregnancy without fetal virilization. Obstet Gynecol *63*, 260-262.

Berry, P.A., Maitland, N.J., and Collins, A.T. (2008). Androgen receptor signalling in prostate: effects of stromal factors on normal and cancer stem cells. Mol Cell Endocrinol *288*, 30-37.

Bethin, K.E., Nagai, Y., Sladek, R., Asada, M., Sadovsky, Y., Hudson, T.J., and Muglia, L.J. (2003). Microarray analysis of uterine gene expression in mouse and human pregnancy. Mol Endocrinol *17*, 1454-1469.

Birrell, S.N., Butler, L.M., Harris, J.M., Buchanan, G., and Tilley, W.D. (2007). Disruption of androgen receptor signaling by synthetic progestins may increase risk of developing breast cancer. FASEB J *21*, 2285-2293.

Blanks, A.M., Vatish, M., Allen, M.J., Ladds, G., de Wit, N.C., Slater, D.M., and Thornton, S. (2003). Paracrine oxytocin and estradiol demonstrate a spatial increase in human intrauterine tissues with labor. J Clin Endocrinol Metab *88*, 3392-3400.

Blanks, A.M., Zhao, Z.H., Shmygol, A., Bru-Mercier, G., Astle, S., and Thornton, S. (2007). Characterization of the molecular and electrophysiological properties of the T-type calcium channel in human myometrium. J Physiol *581*, 915-926.

Blencowe, H., Cousens, S., Oestergaard, M.Z., Chou, D., Moller, A.B., Narwal, R., Adler, A., Vera Garcia, C., Rohde, S., Say, L.*, et al.* (2012). National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet *379*, 2162-2172.

Boittin, F.X., Alonso, F., Le Gal, L., Allagnat, F., Beny, J.L., and Haefliger, J.A. (2013). Connexins and M3 muscarinic receptors contribute to heterogeneous Ca(2+) signaling in mouse aortic endothelium. Cell Physiol Biochem *31*, 166-178.

Bollapragada, S., Youssef, R., Jordan, F., Greer, I., Norman, J., and Nelson, S. (2009). Term labor is associated with a core inflammatory response in human fetal membranes, myometrium, and cervix. Am J Obstet Gynecol *200*, 104.e101-111.

Bombail, V., Collins, F., Brown, P., and Saunders, P.T. (2010). Modulation of ER alpha transcriptional activity by the orphan nuclear receptor ERR beta and evidence for differential effects of long- and short-form splice variants. Mol Cell Endocrinol *314*, 53-61.

Bonventre, J.V., Huang, Z., Taheri, M.R., O'Leary, E., Li, E., Moskowitz, M.A., and Sapirstein, A. (1997). Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2. Nature *390*, 622-625.

Bordallo, J., de Boto, M.J., Meana, C., Velasco, L., Bordallo, C., Suarez, L., Cantabrana, B., and Sanchez, M. (2008). Modulatory role of endogenous androgens on airway smooth muscle tone in isolated guinea-pig and bovine trachea; involvement of beta2-adrenoceptors, the polyamine system and external calcium. Eur J Pharmacol *601*, 154-162.

Brading, A.F., and Brain, K.L. (2011). Ion channel modulators and urinary tract function. Handb Exp Pharmacol, 375-393.

Brainard, A.M., Korovkina, V.P., and England, S.K. (2007). Potassium channels and uterine function. Semin Cell Dev Biol *18*, 332-339.

Brannian, J.D., and Stouffer, R.L. (1991). Progesterone production by monkey luteal cell subpopulations at different stages of the menstrual cycle: changes in agonist responsiveness. Biol Reprod *44*, 141-149.

Braunstein, G.D., Rasor, J., Danzer, H., Adler, D., and Wade, M.E. (1976). Serum human chorionic gonadotropin levels throughout normal pregnancy. Am J Obstet Gynecol *126*, 678-681.

Brenner, R.M., Slayden, O.D., Nayak, N.R., Baird, D.T., and Critchley, H.O. (2003). A role for the androgen receptor in the endometrial antiproliferative effects of progesterone antagonists. Steroids *68*, 1033-1039.

Briery, C.M., Klauser, C.K., Martin, R.W., Magann, E.F., Chauhan, S.P., and Morrison, J.C. (2014). The use of 17-hydroxy progesterone in women with arrested preterm labor: a randomized clinical trial. J Matern Fetal Neonatal Med.

Briery, C.M., Veillon, E.W., Klauser, C.K., Martin, R.W., Magann, E.F., Chauhan, S.P., and Morrison, J.C. (2011). Women with preterm premature rupture of the membranes do not benefit from weekly progesterone. Am J Obstet Gynecol *204*, 54.e51-55.

Brocklehurst, P., Gordon, A., Heatley, E., and Milan, S.J. (2013). Antibiotics for treating bacterial vaginosis in pregnancy. Cochrane Database Syst Rev *1*, Cd000262.

Burd, I., Bentz, A.I., Chai, J., Gonzalez, J., Monnerie, H., Le Roux, P.D., Cohen, A.S., Yudkoff, M., and Elovitz, M.A. (2010). Inflammation-induced preterm birth alters neuronal morphology in the mouse fetal brain. Journal of neuroscience research *88*, 1872-1881.

Burdet, J., Rubio, A.P., Salazar, A.I., Ribeiro, M.L., Ibarra, C., and Franchi, A.M. (2014). Inflammation, Infection and Preterm Birth. Current pharmaceutical design.

Burger, H.G. (2002). Androgen production in women. Fertil Steril *77 Suppl 4*, S3-5.

Buster, J.E., Chang, R.J., Preston, D.L., Elashoff, R.M., Cousins, L.M., Abraham, G.E., Hobel, C.J., and Marshall, J.R. (1979). Interrelationships of circulating maternal steroid concentrations in third trimester pregnancies. II. C18 and C19 steroids: estradiol, estriol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, delta 5-androstenediol, delta 4-androstenedione, testosterone, and dihydrotestosterone. J Clin Endocrinol Metab *48*, 139-142.

Byrne, D.L., and Edmonds, D.K. (1990). Clinical method for evaluating progress in first stage of labour. Lancet *335*, 122.

Cabeza, M., Gutierrez, E., Miranda, R., Heuze, I., Bratoeff, E., Flores, G., and Ramirez, E. (1999). Androgenic and anti-androgenic effects of progesterone derivatives with different halogens as substituents at the C-6 position. Steroids *64*, 413-421.

Cai, C., He, H.H., Chen, S., Coleman, I., Wang, H., Fang, Z., Chen, S., Nelson, P.S., Liu, X.S., Brown, M.*, et al.* (2011). Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. Cancer cell *20*, 457-471.

Cairrao, E., Alvarez, E., Santos-Silva, A.J., and Verde, I. (2008). Potassium channels are involved in testosterone-induced vasorelaxation of human umbilical artery. Naunyn-Schmiedeberg's archives of pharmacology *376*, 375-383.

Calmus, M.L., Macksoud, E.E., Tucker, R., Iozzo, R.V., and Lechner, B.E. (2011). A mouse model of spontaneous preterm birth based on the genetic ablation of biglycan and decorin. Reproduction *142*, 183-194.

Cantineau, R., Kremers, P., De Graeve, J., Gielen, J.E., and Lambotte, R. (1985). 15- and 16-hydroxylations of androgens and estrogens in the human fetal liver: a critical step in estetrol biosynthesis. J Steroid Biochem *22*, 195-201.

Cardenas, H., and Pope, W.F. (2003). Distribution and changes in amounts of the androgen receptor in the pig uterus during the estrous cycle, early pregnancy and after treatment with sex steroids. J Endocrinol *177*, 461-469.

Carlsen, S.M., Jacobsen, G., and Bjerve, K.S. (2003). Androgen levels in pregnant women decrease with increasing maternal age. Scand J Clin Lab Invest *63*, 23-26.

Carlsen, S.M., Jacobsen, G., and Romundstad, P. (2006). Maternal testosterone levels during pregnancy are associated with offspring size at birth. Eur J Endocrinol *155*, 365-370.

Carson, D.J., Okuno, A., Lee, P.A., Stetten, G., Didolkar, S.M., and Migeon, C.J. (1982). Amniotic fluid steroid levels. Fetuses with adrenal hyperplasia, 46,XXY fetuses, and normal fetuses. Am J Dis Child *136*, 218-222.

Castracane, V.D., and Asch, R.H. (1995). Testosterone and androstenedione in premature ovarian failure pregnancies: evidence for an ovarian source of androgens in early pregnancy. Hum Reprod *10*, 677-680.

Castracane, V.D., Stewart, D.R., Gimpel, T., Overstreet, J.W., and Lasley, B.L. (1998). Maternal serum androgens in human pregnancy: early increases within the cycle of conception. Hum Reprod *13*, 460-464.

Catterall, W.A. (2000). Structure and regulation of voltage-gated Ca2+ channels. Annual review of cell and developmental biology *16*, 521-555.

Chai, S.Y., Smith, R., Zakar, T., Mitchell, C., and Madsen, G. (2012). Term myometrium is characterized by increased activating epigenetic modifications at the progesterone receptor-A promoter. Mol Hum Reprod *18*, 401-409.

Challis, J.R., Lockwood, C.J., Myatt, L., Norman, J.E., Strauss, J.F., 3rd, and Petraglia, F. (2009). Inflammation and pregnancy. Reprod Sci *16*, 206-215.

Chang, E.Y., Zhang, J., Sullivan, S., Newman, R., and Singh, I. (2011). N-acetylcysteine attenuates the maternal and fetal proinflammatory response to intrauterine LPS injection in an animal model for preterm birth and brain injury. J Matern Fetal Neonatal Med *24*, 732-740.

Chang, E.Y., Zhang, J., Sullivan, S., Newman, R., and Singh, I. (2012). N-acetylcysteine prevents preterm birth by attenuating the LPS-induced expression of contractile associated proteins in an animal model. J Matern Fetal Neonatal Med *25*, 2395-2400.

Chanrachakul, B. (2006). Ion channels: new targets for the next generation of tocolytics agents. Journal of the Medical Association of Thailand = Chotmaihet thangphaet *89 Suppl 4*, S178-183.

Chanrachakul, B., Broughton Pipkin, F., Warren, A.Y., Arulkumaran, S., and Khan, R.N. (2005). Progesterone enhances the tocolytic effect of ritodrine in isolated pregnant human myometrium. Am J Obstet Gynecol *192*, 458-463.

Chanrachakul, B., Matharoo-Ball, B., Turner, A., Robinson, G., Broughton-Pipkin, F., Arulkumaran, S., and Khan, R.N. (2003). Immunolocalization and protein expression of the alpha subunit of the large-conductance calcium-activated potassium channel in human myometrium. Reproduction *126*, 43-48.

Chapman, N.R., Europe-Finner, G.N., and Robson, S.C. (2004). Expression and deoxyribonucleic acid-binding activity of the nuclear factor kappaB family in the human myometrium during pregnancy and labor. J Clin Endocrinol Metab *89*, 5683-5693.

Charest, N.J., Zhou, Z.X., Lubahn, D.B., Olsen, K.L., Wilson, E.M., and French, F.S. (1991). A frameshift mutation destabilizes androgen receptor messenger RNA in the Tfm mouse. Mol Endocrinol *5*, 573-581.

Cheng, L., Pricolo, V., Biancani, P., and Behar, J. (2008). Overexpression of progesterone receptor B increases sensitivity of human colon muscle cells to progesterone. American journal of physiology Gastrointestinal and liver physiology *295*, G493-502.

Chou, T.M., Sudhir, K., Hutchison, S.J., Ko, E., Amidon, T.M., Collins, P., and Chatterjee, K. (1996). Testosterone induces dilation of canine coronary conductance and resistance arteries in vivo. Circulation *94*, 2614-2619.

Choudhary, M., Suneja, A., Vaid, N.B., Guleria, K., and Faridi, M.M. (2014). Maintenance tocolysis with oral micronized progesterone for prevention of preterm birth after arrested preterm labor. Int J Gynaecol Obstet *126*, 60-63.

Chow, L., and Lye, S.J. (1994). Expression of the gap junction protein connexin-43 is increased in the human myometrium toward term and with the onset of labor. Am J Obstet Gynecol *170*, 788-795.

Clark, K., Ji, H., Feltovich, H., Janowski, J., Carroll, C., and Chien, E.K. (2006). Mifepristone-induced cervical ripening: structural, biomechanical, and molecular events. Am J Obstet Gynecol *194*, 1391-1398.

Coffey, K., Rogerson, L., Ryan-Munden, C., Alkharaif, D., Stockley, J., Heer, R., Sahadevan, K., O'Neill, D., Jones, D., Darby, S.*, et al.* (2013). The lysine demethylase, KDM4B, is a key molecule in androgen receptor signalling and turnover. Nucleic acids research *41*, 4433-4446.

Collins, P.L., Moore, J.J., Lundgren, D.W., Choobineh, E., Chang, S.M., and Chang, A.S. (2000). Gestational changes in uterine L-type calcium channel function and expression in guinea pig. Biol Reprod *63*, 1262-1270.

Combs, C.A., Garite, T., Maurel, K., Das, A., and Porto, M. (2010). Failure of 17-hydroxyprogesterone to reduce neonatal morbidity or prolong triplet pregnancy: a double-blind, randomized clinical trial. Am J Obstet Gynecol *203*, 248.e241-249.

Conde-Agudelo, A., Romero, R., Nicolaides, K., Chaiworapongsa, T., O'Brien, J.M., Cetingoz, E., da Fonseca, E., Creasy, G., Soma-Pillay, P., Fusey, S.*, et al.* (2013). Vaginal progesterone vs. cervical cerclage for the prevention of preterm birth in women with a sonographic short cervix, previous preterm birth, and singleton gestation: a systematic review and indirect comparison metaanalysis. Am J Obstet Gynecol *208*, 42.e41-42.e18.

Condon, J.C., Jeyasuria, P., Faust, J.M., Wilson, J.W., and Mendelson, C.R. (2003). A decline in the levels of progesterone receptor coactivators in the pregnant uterus at term may antagonize progesterone receptor function and contribute to the initiation of parturition. Proc Natl Acad Sci U S A *100*, 9518-9523.

Conneely, O.M., Mulac-Jericevic, B., Lydon, J.P., and De Mayo, F.J. (2001). Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. Mol Cell Endocrinol *179*, 97-103.

Cookson, V.J., and Chapman, N.R. (2010). NF-kappaB function in the human myometrium during pregnancy and parturition. Histology and histopathology *25*, 945-956.

Corbould, A. (2008). Effects of androgens on insulin action in women: is androgen excess a component of female metabolic syndrome? Diabetes Metab Res Rev *24*, 520-532.

Costarella, C.E., Stallone, J.N., Rutecki, G.W., and Whittier, F.C. (1996). Testosterone causes direct relaxation of rat thoracic aorta. J Pharmacol Exp Ther *277*, 34-39.

Coviello, A.D., Lakshman, K., Mazer, N.A., and Bhasin, S. (2006). Differences in the apparent metabolic clearance rate of testosterone in young and older men with gonadotropin suppression receiving graded doses of testosterone. J Clin Endocrinol Metab *91*, 4669-4675.

Crews, J.K., and Khalil, R.A. (1999a). Antagonistic effects of 17 beta-estradiol, progesterone, and testosterone on Ca2+ entry mechanisms of coronary vasoconstriction. Arterioscler Thromb Vasc Biol *19*, 1034-1040.

Crews, J.K., and Khalil, R.A. (1999b). Gender-specific inhibition of Ca2+ entry mechanisms of arterial vasoconstriction by sex hormones. Clin Exp Pharmacol Physiol *26*, 707-715.

Crisosto, N., Echiburu, B., Maliqueo, M., Perez, V., Ladron de Guevara, A., Preisler, J., Sanchez, F., and Sir-Petermann, T. (2012). Improvement of hyperandrogenism and hyperinsulinemia during pregnancy in women with polycystic ovary syndrome: possible effect in the ovarian follicular mass of their daughters. Fertil Steril *97*, 218-224.

Culig, Z., Hobisch, A., Cronauer, M.V., Radmayr, C., Trapman, J., Hittmair, A., Bartsch, G., and Klocker, H. (1994). Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res *54*, 5474-5478.

Dahl, S.K., Thomas, M.A., Williams, D.B., and Robins, J.C. (2008). Maternal virilization due to luteoma associated with delayed lactation. Fertil Steril *90*, 2006 e2017-2009.

Dasgupta, E., and Singh, G. (2012). Vaginal Misoprostol vs Vaginal Misoprostol With Estradiol for Labor Induction: A Prospective Double Blind Study. J Obstet Gynaecol India *62*, 47-51.

Dawood, M.Y., and Saxena, B.B. (1977). Testosterone and dihydrotestosterone in maternal and cord blood and in amniotic fluid. Am J Obstet Gynecol *129*, 37-42.

Dean, A., Smith, L.B., Macpherson, S., and Sharpe, R.M. (2012). The effect of dihydrotestosterone exposure during or prior to the masculinization programming window on reproductive development in male and female rats. Int J Androl *35*, 330-339.

Deenadayalu, V., Puttabyatappa, Y., Liu, A.T., Stallone, J.N., and White, R.E. (2012). Testosterone-induced relaxation of coronary arteries: activation of BKCa channels via the cGMP-dependent protein kinase. Am J Physiol Heart Circ Physiol *302*, H115-123.

DeFranco, E.A., O'Brien, J.M., Adair, C.D., Lewis, D.F., Hall, D.R., Fusey, S., Soma-Pillay, P., Porter, K., How, H., Schakis, R.*, et al.* (2007). Vaginal progesterone is associated with a decrease in risk for early preterm birth and improved neonatal outcome in women with a short cervix: a secondary analysis from a randomized, double-blind, placebo-controlled trial. Ultrasound Obstet Gynecol *30*, 697-705.

Deliconstantinos, G. (1988). Structure activity relationship of cholesterol and steroid hormones with respect to their effects on the Ca2+-stimulated ATPase and lipid fluidity of synaptosomal plasma membranes from dog and rabbit brain. Comp Biochem Physiol B *89*, 585-594.

Denayer, S., Helsen, C., Thorrez, L., Haelens, A., and Claessens, F. (2010). The rules of DNA recognition by the androgen receptor. Mol Endocrinol *24*, 898-913.

DePrimo, S.E., Diehn, M., Nelson, J.B., Reiter, R.E., Matese, J., Fero, M., Tibshirani, R., Brown, P.O., and Brooks, J.D. (2002). Transcriptional programs activated by exposure of human prostate cancer cells to androgen. Genome biology *3*, Research0032.

Despres, G., Rigaudiere, N., and Delost, P. (1982). Changes in the metabolic clearance rate and plasma production rate of testosterone in the pregnant guinea-pig. Steroids *39*, 703-710.

Dewailly, D. (2010). Androgen excess in women: not just a cosmetic problem. Ann Endocrinol (Paris) *71*, 1.

Diamanti-Kandarakis, E., Alexandraki, K., Piperi, C., Protogerou, A., Katsikis, I., Paterakis, T., Lekakis, J., and Panidis, D. (2006). Inflammatory and endothelial markers in women with polycystic ovary syndrome. Eur J Clin Invest *36*, 691-697.

Diaz, P., Wood, A.M., Sibley, C.P., and Greenwood, S.L. (2014). Intermediate conductance Ca2+-activated K+ channels modulate human placental trophoblast syncytialization. PLoS One *9*, e90961.

Dick, I.M., Liu, J., Glendenning, P., and Prince, R.L. (2003). Estrogen and androgen regulation of plasma membrane calcium pump activity in immortalized distal tubule kidney cells. Mol Cell Endocrinol *212*, 11-18.

Diez d'Aux, R.C., and Pearson Murphy, B.E. (1974). Androgens in the human fetus. J Steroid Biochem *5*, 207-210.

Dillon, P.F., Aksoy, M.O., Driska, S.P., and Murphy, R.A. (1981). Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. Science *211*, 495-497.

Dodd, J.M., Flenady, V.J., Cincotta, R., and Crowther, C.A. (2008). Progesterone for the prevention of preterm birth: a systematic review. Obstet Gynecol *112*, 127-134.

Dodd, J.M., Jones, L., Flenady, V., Cincotta, R., and Crowther, C.A. (2013). Prenatal administration of progesterone for preventing preterm birth in women considered to be at risk of preterm birth. Cochrane Database Syst Rev *7*, Cd004947.

Doheny, H.C., Houlihan, D.D., Ravikumar, N., Smith, T.J., and Morrison, J.J. (2003). Human chorionic gonadotrophin relaxation of human pregnant myometrium and activation of the BKCa channel. J Clin Endocrinol Metab *88*, 4310-4315.

Doheny, H.C., O'Reilly, M.J., Sexton, D.J., and Morrison, J.J. (2007). THG113.31, a specific PGF2alpha receptor antagonist, induces human myometrial relaxation and BKCa channel activation. Reprod Biol Endocrinol *5*, 10.

Doring, B., Shynlova, O., Tsui, P., Eckardt, D., Janssen-Bienhold, U., Hofmann, F., Feil, S., Feil, R., Lye, S.J., and Willecke, K. (2006). Ablation of connexin43 in uterine smooth muscle cells of the mouse causes delayed parturition. J Cell Sci *119*, 1715-1722.

Dray, F., and Frydman, R. (1976). Primary prostaglandins in amniotic fluid in pregnancy and spontaneous labor. Am J Obstet Gynecol *126*, 13-19.

Dubois, V., Laurent, M., Boonen, S., Vanderschueren, D., and Claessens, F. (2012). Androgens and skeletal muscle: cellular and molecular action mechanisms underlying the anabolic actions. Cell Mol Life Sci *69*, 1651-1667.

Duckitt, K., Thornton, S., O'Donovan, O.P., and Dowswell, T. (2014). Nitric oxide donors for treating preterm labour. Cochrane Database Syst Rev *5*, Cd002860.

Duval, D., Durant, S., and Homo-Delarche, F. (1983). Non-genomic effects of steroids. Interactions of steroid molecules with membrane structures and functions. Biochim Biophys Acta *737*, 409-442.

Edman, C.D., Toofanian, A., MacDonald, P.C., and Gant, N.F. (1981). Placental clearance rate of maternal plasma androstenedione through placental estradiol formation: an indirect method of assessing uteroplacental blood flow. Am J Obstet Gynecol *141*, 1029-1037.

Ekman, G., Malmstrom, A., Uldbjerg, N., and Ulmsten, U. (1986). Cervical collagen: an important regulator of cervical function in term labor. Obstet Gynecol *67*, 633-636.

Ekman-Ordeberg, G., Stjernholm, Y., Wang, H., Stygar, D., and Sahlin, L. (2003). Endocrine regulation of cervical ripening in humans--potential roles for gonadal steroids and insulin-like growth factor-I. Steroids *68*, 837-847.

El Maradny, E., Kanayama, N., Halim, A., Maehara, K., Sumimoto, K., and Terao, T. (1996). Biochemical changes in the cervical tissue of rabbit induced by interleukin-8, interleukin-1beta, dehydroepiandrosterone sulphate and prostaglandin E2: a comparative study. Hum Reprod *11*, 1099-1104.

Elbaum, D.J., and Keyes, P.L. (1976). Synthesis of 17beta-estradiol by isolated ovarian tissues of the pregnant rat: aromatization in the corpus luteum. Endocrinology *99*, 573-579.

Elliott, C.L., Slater, D.M., Dennes, W., Poston, L., and Bennett, P.R. (2000). Interleukin 8 expression in human myometrium: changes in relation to labor onset and with gestational age. Am J Reprod Immunol *43*, 272-277.

Elmes, M.J., Tan, D.S., Cheng, Z., Wathes, D.C., and McMullen, S. (2011). The effects of a high-fat, high-cholesterol diet on markers of uterine contractility during parturition in the rat. Reproduction *141*, 283-290.

Elovitz, M., and Wang, Z. (2004). Medroxyprogesterone acetate, but not progesterone, protects against inflammation-induced parturition and intrauterine fetal demise. Am J Obstet Gynecol *190*, 693-701.

Elovitz, M.A., and Mrinalini, C. (2004). Animal models of preterm birth. Trends Endocrinol Metab *15*, 479-487.

Elovitz, M.A., and Mrinalini, C. (2005). Can medroxyprogesterone acetate alter Toll-like receptor expression in a mouse model of intrauterine inflammation? Am J Obstet Gynecol *193*, 1149-1155.

Elovitz, M.A., and Mrinalini, C. (2006). The use of progestational agents for preterm birth: lessons from a mouse model. Am J Obstet Gynecol *195*, 1004-1010.

Elovitz, M.A., Wang, Z., Chien, E.K., Rychlik, D.F., and Phillippe, M. (2003). A new model for inflammation-induced preterm birth: the role of platelet-activating factor and Toll-like receptor-4. Am J Pathol *163*, 2103-2111.

Erkinheimo, T.L., Saukkonen, K., Narko, K., Jalkanen, J., Ylikorkala, O., and Ristimaki, A. (2000). Expression of cyclooxygenase-2 and prostanoid receptors by human myometrium. J Clin Endocrinol Metab *85*, 3468-3475.

Escobar, J.C., Patel, S.S., Beshay, V.E., Suzuki, T., and Carr, B.R. (2011). The human placenta expresses CYP17 and generates androgens de novo. J Clin Endocrinol Metab *96*, 1385-1392.

Escobar-Morreale, H.F. (2010). Diagnosis and management of hirsutism. Ann N Y Acad Sci *1205*, 166-174.

Escobar-Morreale, H.F., Carmina, E., Dewailly, D., Gambineri, A., Kelestimur, F., Moghetti, P., Pugeat, M., Qiao, J., Wijeyaratne, C.N., Witchel, S.F.*, et al.* (2012). Epidemiology, diagnosis and management of hirsutism: a consensus statement by the Androgen Excess and Polycystic Ovary Syndrome Society. Hum Reprod Update *18*, 146-170.

Espinos, J.J., Corcoy, R., Roca, B., Rodriguez-Espinosa, J., and Calaf, J. (1992). [Serum insulin and androgen concentrations in women with histories of gestational diabetes]. Med Clin (Barc) *98*, 445-448.

Esplin, M.S., Peltier, M.R., Hamblin, S., Smith, S., Fausett, M.B., Dildy, G.A., Branch, D.W., Silver, R.M., and Adashi, E.Y. (2005). Monocyte chemotactic protein-1 expression is increased in human gestational tissues during term and preterm labor. Placenta *26*, 661-671.

Farhat, M.Y., Abi-Younes, S., Dingaan, B., Vargas, R., and Ramwell, P.W. (1996). Estradiol increases cyclic adenosine monophosphate in rat pulmonary vascular smooth muscle cells by a nongenomic mechanism. J Pharmacol Exp Ther *276*, 652-657.

Fata, J.E., Ho, A.T., Leco, K.J., Moorehead, R.A., and Khokha, R. (2000). Cellular turnover and extracellular matrix remodeling in female reproductive tissues: functions of metalloproteinases and their inhibitors. Cell Mol Life Sci *57*, 77-95.

Fatemi, S.H., Reutiman, T.J., and Folsom, T.D. (2009). The role of lithium in modulation of brain genes: relevance for aetiology and treatment of bipolar disorder. Biochemical Society transactions *37*, 1090-1095.

Fernandez, A., Cantabrana, B., and Hidalgo, A. (1993). Estrogen and antiestrogen non-genomic effect in rat uterus contraction in calcium-free solution. General pharmacology *24*, 391-395.

Figueroa, J.P., Honnebier, M.B., Binienda, Z., Wimsatt, J., and Nathanielsz, P.W. (1989). Effect of a 48-hour intravenous delta 4-androstenedione infusion on the pregnant rhesus monkey in the last third of gestation: changes in maternal plasma estradiol concentrations and myometrial contractility. Am J Obstet Gynecol *161*, 481-486.

Firth, S.M., and Baxter, R.C. (2002). Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev *23*, 824-854.

Fisch, B., Margara, R.A., Winston, R.M., and Hillier, S.G. (1989). Cellular basis of luteal steroidogenesis in the human ovary. J Endocrinol *122*, 303-311.

Fitzgibbon, J., Morrison, J.J., Smith, T.J., and O'Brien, M. (2009). Modulation of human uterine smooth muscle cell collagen contractility by thrombin, Y-27632, TNF alpha and indomethacin. Reprod Biol Endocrinol *7*, 2.

Flenady, V., Reinebrant, H.E., Liley, H.G., Tambimuttu, E.G., and Papatsonis, D.N. (2014). Oxytocin receptor antagonists for inhibiting preterm labour. Cochrane Database Syst Rev *6*, Cd004452.

Floyd, R., and Wray, S. (2007). Calcium transporters and signalling in smooth muscles. Cell Calcium *42*, 467-476.

Fomin, V.P., Cox, B.E., and Word, R.A. (1999). Effect of progesterone on intracellular Ca2+ homeostasis in human myometrial smooth muscle cells. Am J Physiol *276*, C379-385.

Foradori, C.D., Weiser, M.J., and Handa, R.J. (2008). Non-genomic actions of androgens. Front Neuroendocrinol *29*, 169-181.

Foster, L.J., and Klip, A. (2000). Mechanism and regulation of GLUT-4 vesicle fusion in muscle and fat cells. Am J Physiol Cell Physiol *279*, C877-890.

Foster, L.J., Weir, M.L., Lim, D.Y., Liu, Z., Trimble, W.S., and Klip, A. (2000). A functional role for VAP-33 in insulin-stimulated GLUT4 traffic. Traffic (Copenhagen, Denmark) *1*, 512-521.

Foulk, R.A., Martin, M.C., Jerkins, G.L., and Laros, R.K. (1997). Hyperreactio luteinalis differentiated from severe ovarian hyperstimulation syndrome in a spontaneously conceived pregnancy. Am J Obstet Gynecol *176*, 1300-1302; discussion 1302-1304.

Franczak, A. (2008). Endometrial and myometrial secretion of androgens and estrone during early pregnancy and luteolysis in pigs. Reprod Biol *8*, 213-228.

Franczak, A., Kurowicka, B., Oponowicz, A., Petroff, B.K., and Kotwica, G. (2006). The effect of progesterone on oxytocin-stimulated intracellular mobilization of Ca2+ and prostaglandin E2 and F2alpha secretion from porcine myometrial cells. Prostaglandins & other lipid mediators *81*, 37-44.

Fu, X., Backstrom, T., and Ulmsten, U. (1998). Progesterone increases cAMP release and accumulation in isolated term human myometrium. Gynecol Obstet Invest *45*, 242-246.

Fu, X., Moberg, C., Backstrom, T., and Ulmsten, U. (1996). Effects of estradiol and interaction with progesterone or oxytocin on term human myometrial contractile activity. Acta Obstet Gynecol Scand *75*, 13-18.

Fuchs, A.R., Fuchs, F., Husslein, P., and Soloff, M.S. (1984). Oxytocin receptors in the human uterus during pregnancy and parturition. Am J Obstet Gynecol *150*, 734-741.

Fuller, P.J., Pettigrew, I.G., Pike, J.W., and Stockigt, J.R. (1983). An adrenal adenoma causing virilization of mother and infant. Clin Endocrinol (Oxf) *18*, 143-153.

Furui, T., Imai, A., and Ohno, T. (2007). Pre-term labour in cases with high maternal testosterone levels. J Obstet Gynaecol *27*, 155-156.

Gabbiani, G., Schmid, E., Winter, S., Chaponnier, C., de Ckhastonay, C., Vandekerckhove, J., Weber, K., and Franke, W.W. (1981). Vascular smooth muscle cells differ from other smooth muscle cells: predominance of vimentin filaments and a specific alpha-type actin. Proc Natl Acad Sci U S A *78*, 298-302.

Gaffney, R.C., Rice, G.E., and Brennecke, S.P. (1990). Is human labour triggered by an increase in the rate of synthesis of prostaglandin G/H synthase? Reproduction, fertility, and development *2*, 603-606.

Gant, N.F., Hutchinson, H.T., Siiteri, P.K., and MacDonald, P.C. (1971). Study of the metabolic clearance rate of dehydroisoandrosterone sulfate in pregnancy. Am J Obstet Gynecol *111*, 555-563.

Ganu, R.S., Ma, J., and Aagaard, K.M. (2013). The role of microbial communities in parturition: is there evidence of association with preterm birth and perinatal morbidity and mortality? Am J Perinatol *30*, 613-624.

Gao, L., Cong, B., Zhang, L., and Ni, X. (2009). Expression of the calcium-activated potassium channel in upper and lower segment human myometrium during pregnancy and parturition. Reprod Biol Endocrinol *7*, 27.

Garfield, R.E., and Hayashi, R.H. (1981). Appearance of gap junctions in the myometrium of women during labor. Am J Obstet Gynecol *140*, 254-260.

Garfield, R.E., Sims, S.M., Kannan, M.S., and Daniel, E.E. (1978). Possible role of gap junctions in activation of myometrium during parturition. Am J Physiol *235*, C168-179.

Gatongi, D.K., Madhvi, G., Tydeman, G., and Hasan, A. (2006). A case of hyperreactio luteinalis presenting with eclampsia. J Obstet Gynaecol *26*, 465-467.

Ghayee, H.K., and Auchus, R.J. (2007). Basic concepts and recent developments in human steroid hormone biosynthesis. Rev Endocr Metab Disord *8*, 289-300.

Ghorashi, V., and Sheikhvatan, M. (2008). The relationship between serum concentration of free testosterone and pre-eclampsia. Endokrynol Pol *59*, 390-392.

Ghule, V.V., Gray, C., Galimberti, A., and Anumba, D.O. (2012). Prostaglandin-induced cervical remodelling in humans in the first trimester is associated with increased expression of specific tight junction, but not gap junction proteins. J Transl Med *10*, 40.

Gioeli, D., and Paschal, B.M. (2012). Post-translational modification of the androgen receptor. Mol Cell Endocrinol *352*, 70-78.

Glass, D.J. (2010). PI3 kinase regulation of skeletal muscle hypertrophy and atrophy. Curr Top Microbiol Immunol *346*, 267-278.

Goc, A., Al-Azayzih, A., Abdalla, M., Al-Husein, B., Kavuri, S., Lee, J., Moses, K., and Somanath, P.R. (2013). P21 activated kinase-1 (Pak1) promotes prostate tumor growth and microinvasion via inhibition of transforming growth factor beta expression and enhanced matrix metalloproteinase 9 secretion. J Biol Chem *288*, 3025-3035.

Gökdeniz, R. (2013). Progesterone Inhibits Human Myometrial Contractions by its Action on Membrane Receptors. Çukurova Üniversitesi Tıp Fakültesi Dergisi *38*, 92-102.

Goldenberg, R.L., Culhane, J.F., Iams, J.D., and Romero, R. (2008). Epidemiology and causes of preterm birth. Lancet *371*, 75-84.

Gonzalez, J.M., Franzke, C.W., Yang, F., Romero, R., and Girardi, G. (2011). Complement activation triggers metalloproteinases release inducing cervical remodeling and preterm birth in mice. Am J Pathol *179*, 838-849.

Goodyer, C.G., and Branchaud, C.L. (1981). Regulation of hormone production in the human feto-placental unit. Ciba Found Symp *86*, 89-123.

Gravett, M.G., Hitti, J., Hess, D.L., and Eschenbach, D.A. (2000). Intrauterine infection and preterm delivery: evidence for activation of the fetal hypothalamic-pituitary-adrenal axis. Am J Obstet Gynecol *182*, 1404-1413.

Gravett, M.G., Witkin, S.S., Haluska, G.J., Edwards, J.L., Cook, M.J., and Novy, M.J. (1994). An experimental model for intraamniotic infection and preterm labor in rhesus monkeys. Am J Obstet Gynecol *171*, 1660-1667.

Grgic, O., Radakovic, B., and Barisic, D. (2008). Hyperreactio luteinalis could be a risk factor for development of HELLP syndrome: case report. Fertil Steril *90*, 2008 e2013-2006.

Grigsby, P.L., Hirst, J.J., Scheerlinck, J.P., Phillips, D.J., and Jenkin, G. (2003). Fetal responses to maternal and intra-amniotic lipopolysaccharide administration in sheep. Biol Reprod *68*, 1695-1702.

Gu, S., Kounenidakis, M., Schmidt, E.M., Deshpande, D., Alkahtani, S., Alarifi, S., Foller, M., Alevizopoulos, K., Lang, F., and Stournaras, C. (2013). Rapid activation of FAK/mTOR/p70S6K/PAK1-signaling controls the early testosterone-induced actin reorganization in colon cancer cells. Cell Signal *25*, 66-73.

Guibert, C., Ducret, T., and Savineau, J.P. (2008). Voltage-independent calcium influx in smooth muscle. Prog Biophys Mol Biol *98*, 10-23.

Gupte, S.A., Tateyama, M., Okada, T., Oka, M., and Ochi, R. (2002). Epiandrosterone, a metabolite of testosterone precursor, blocks L-type calcium channels of ventricular myocytes and inhibits myocardial contractility. J Mol Cell Cardiol *34*, 679-688.

Gurpide, E. (1990). Experimental designs used to estimate rates of steroid production and metabolism in vivo and in vitro. Ann N Y Acad Sci *595*, 165-172.

Gutierrez, M., Fernandez, A.I., Revuelta, M.P., Cantabrana, B., and Hidalgo, A. (1998). Partial contribution of polyamines to the relaxant effect of 17 alpha-estradiol in rat uterine smooth muscle. General pharmacology *30*, 71-77.

Ha, S., Iqbal, N.J., Mita, P., Ruoff, R., Gerald, W.L., Lepor, H., Taneja, S.S., Lee, P., Melamed, J., Garabedian, M.J.*, et al.* (2013). Phosphorylation of the androgen receptor by PIM1 in hormone refractory prostate cancer. Oncogene *32*, 3992-4000.

Haas, D.M., Benjamin, T., Sawyer, R., and Quinney, S.K. (2014). Short-term tocolytics for preterm delivery - current perspectives. International journal of women's health *6*, 343-349.

Haas, D.M., Caldwell, D.M., Kirkpatrick, P., McIntosh, J.J., and Welton, N.J. (2012). Tocolytic therapy for preterm delivery: systematic review and network meta-analysis. BMJ *345*, e6226.

Haimov-Kochman, R., Yanai, N., Yagel, S., Amsalem, H., Lavy, Y., and Hurwitz, A. (2004). Spontaneous ovarian hyperstimulation syndrome and hyperreactio luteinalis are entities in continuum. Ultrasound Obstet Gynecol *24*, 675-678.

Hall, P.F., Chen, S., Nakajin, S., Shinoda, M., and Shively, J.E. (1987). Purification and characterization of aromatase from human placenta. Steroids *50*, 37-50.

Hammond, G.L. (2011). Diverse roles for sex hormone-binding globulin in reproduction. Biol Reprod *85*, 431-441.

Han, D.H., Chae, M.R., Jung, J.H., So, I., Park, J.K., and Lee, S.W. (2008). Effect of testosterone on potassium channel opening in human corporal smooth muscle cells. J Sex Med *5*, 822-832.

Hao, K., Wang, X., Niu, T., Xu, X., Li, A., Chang, W., Wang, L., Li, G., Laird, N., and Xu, X. (2004). A candidate gene association study on preterm delivery: application of high-throughput genotyping technology and advanced statistical methods. Human molecular genetics *13*, 683-691.

Hapangama, D., and Neilson, J.P. (2009). Mifepristone for induction of labour. Cochrane Database Syst Rev, Cd002865.

Haq, A.N. (2010). Hyperreactio luteinalis associated with pregnancy induced hypertension. J Coll Physicians Surg Pak *20*, 137-139.

Helguera, G., Olcese, R., Song, M., Toro, L., and Stefani, E. (2002). Tissue-specific regulation of Ca(2+) channel protein expression by sex hormones. Biochim Biophys Acta *1569*, 59-66.

Hensleigh, P.A., Carter, R.P., and Grotjan, H.E., Jr. (1975). Fetal protection against masculinization with hyperreactio luteinalis and virilization. J Clin Endocrinol Metab *40*, 816-823.

Hidalgo, A., Suzano, R.C., Revuelta, M.P., Sanchez-Diaz, C., Baamonde, A., and Cantabrana, B. (1996). Calcium and depolarization-dependent effect of pregnenolone derivatives on uterine smooth muscle. General pharmacology *27*, 879-885.

Hillock, C.J., and Crankshaw, D.J. (1999). Inhibitory prostanoid EP receptors in human non-pregnant myometrium. Eur J Pharmacol *378*, 99-108.

Hinoki, A., Kimura, K., Higuchi, S., Eguchi, K., Takaguri, A., Ishimaru, K., Frank, G.D., Gerthoffer, W.T., Sommerville, L.J., Autieri, M.V.*, et al.* (2010). p21-activated kinase 1 participates in vascular remodeling in vitro and in vivo. Hypertension *55*, 161-165.

Hirano, M. (1961). Formation and biological activity of estriol in placenta. Tohoku J Exp Med *75*, 89-102.

Hirsch, E., Muhle, R.A., Mussalli, G.M., and Blanchard, R. (2002). Bacterially induced preterm labor in the mouse does not require maternal interleukin-1 signaling. Am J Obstet Gynecol *186*, 523-530.

Hirsch, E., and Wang, H. (2005). The molecular pathophysiology of bacterially induced preterm labor: insights from the murine model. J Soc Gynecol Investig *12*, 145-155.

Hjelm, A., Ekman-Ordeberg, G., Barchan, K., and Malmstrom, A. (2001). Identification of the major proteoglycans from human myometrium. Acta Obstet Gynecol Scand *80*, 1084-1090.

Hodgins, M.B. (1982). Binding of androgens in 5 alpha-reductase-deficient human genital skin fibroblasts: inhibition by progesterone and its metabolites. J Endocrinol *94*, 415-427.

Holt, H.B., Medbak, S., Kirk, D., Guirgis, R., Hughes, I., Cummings, M.H., and Meeking, D.R. (2005). Recurrent severe hyperandrogenism during pregnancy: a case report. J Clin Pathol *58*, 439-442.

Horowitz, A., Menice, C.B., Laporte, R., and Morgan, K.G. (1996). Mechanisms of smooth muscle contraction. Physiol Rev *76*, 967-1003.

Hosli, I., Sperschneider, C., Drack, G., Zimmermann, R., Surbek, D., and Irion, O. (2014). Tocolysis for preterm labor: expert opinion. Arch Gynecol Obstet *289*, 903-909.

Howie, S.E., Horner, P.J., and Horne, A.W. (2011). Chlamydia trachomatis infection during pregnancy: known unknowns. Discovery medicine *12*, 57-64.

Hu, Y.C., Wang, P.H., Yeh, S., Wang, R.S., Xie, C., Xu, Q., Zhou, X., Chao, H.T., Tsai, M.Y., and Chang, C. (2004). Subfertility and defective folliculogenesis in female mice lacking androgen receptor. Proc Natl Acad Sci U S A *101*, 11209-11214.

Hughes, I.A., Davies, J.D., Bunch, T.I., Pasterski, V., Mastroyannopoulou, K., and MacDougall, J. (2012). Androgen insensitivity syndrome. Lancet *380*, 1419-1428.

Hutchinson, J.L., Rajagopal, S.P., Yuan, M., and Norman, J.E. (2014). Lipopolysaccharide promotes contraction of uterine myocytes via activation of Rho/ROCK signaling pathways. Faseb j *28*, 94-105.

Ikeda, M., Shibata, Y., and Yamamoto, T. (1987). Rapid formation of myometrial gap junctions during parturition in the unilaterally implanted rat uterus. Cell Tissue Res *248*, 297-303.

Ikonen, E. (2008). Cellular cholesterol trafficking and compartmentalization. Nat Rev Mol Cell Biol *9*, 125-138.

Inoue, Y., Okabe, K., and Soeda, H. (1999). Augmentation and suppression of action potentials by estradiol in the myometrium of pregnant rat. Can J Physiol Pharmacol *77*, 447-453.

Ito, M., and Hartshorne, D.J. (1990). Phosphorylation of myosin as a regulatory mechanism in smooth muscle. Prog Clin Biol Res *327*, 57-72.

Ito, T., and Horton, R. (1971). The source of plasma dihydrotestosterone in man. J Clin Invest *50*, 1621-1627.

Ivanov, V., Roomi, M.W., Kalinovsky, T., Niedzwiecki, A., and Rath, M. (2006). Natural nutrient mixture effectively reduces collagen matrix contraction driven by human uterine smooth muscle cells. J Obstet Gynaecol Res *32*, 23-31.

Jaffer, S., Shynlova, O., and Lye, S. (2009). Mammalian target of rapamycin is activated in association with myometrial proliferation during pregnancy. Endocrinology *150*, 4672-4680.

Jasonni, V.M., Bonavia, M., Lodi, S., Preti, S., Bulletti, C., and Flamigni, C. (1982). Androstenedione metabolism in human uterine tissues: endometrium, myometrium and leiomyoma. J Steroid Biochem *17*, 547-551.

Jeffrey, J.J., Roswit, W.T., and Ehlich, L.S. (1990). Regulation of collagenase production by steroids in uterine smooth muscle cells: an enzymatic and immunologic study. J Cell Physiol *143*, 396-403.

Jennings, L.J., Xu, Q.W., Firth, T.A., Nelson, M.T., and Mawe, G.M. (1999). Cholesterol inhibits spontaneous action potentials and calcium currents in guinea pig gallbladder smooth muscle. Am J Physiol *277*, G1017-1026.

Ji, H., Dailey, T.L., Long, V., and Chien, E.K. (2008). Androgen-regulated cervical ripening: a structural, biomechanical, and molecular analysis. Am J Obstet Gynecol *198*, 543 e541-549.

Ji, H., Long, V., Briody, V., and Chien, E.K. (2011). Progesterone modulates integrin {alpha}2 (ITGA2) and {alpha}11 (ITGA11) in the pregnant cervix. Reprod Sci *18*, 156-163.

Jorgensen, J.S., Weile, L.K., and Lamont, R.F. (2014). Preterm labor: current tocolytic options for the treatment of preterm labor. Expert opinion on pharmacotherapy *15*, 585-588.

Joseph, D.R. (1994). Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. Vitam Horm *49*, 197-280.

Kanayama, N., El Maradny, E., Goto, J., and Terao, T. (1998). Effect of dehydroepiandrosterone sulfate on interleukin-8 receptor during cervical ripening. Eur J Endocrinol *138*, 587-593.

Kanca, H., Walter, I., Miller, I., Schafer-Somi, S., Izgur, H., and Aslan, S. (2011). Expression and activity of matrix metalloproteinases in the uterus of bitches after spontaneous and induced abortion. Reprod Domest Anim *46*, 197-204.

Kandola, M.K., Sykes, L., Lee, Y.S., Johnson, M.R., Hanyaloglu, A.C., and Bennett, P.R. (2014). EP2 receptor activates dual G protein signaling pathways that mediate contrasting proinflammatory and relaxatory responses in term pregnant human myometrium. Endocrinology *155*, 605-617.

Kang, L., Zhang, X., Xie, Y., Tu, Y., Wang, D., Liu, Z., and Wang, Z.Y. (2010). Involvement of estrogen receptor variant ER-alpha36, not GPR30, in nongenomic estrogen signaling. Mol Endocrinol *24*, 709-721.

Kanova, N., and Bicikova, M. (2011). Hyperandrogenic states in pregnancy. Physiol Res *60*, 243-252.

Karjalainen, M.K., Huusko, J.M., Ulvila, J., Sotkasiira, J., Luukkonen, A., Teramo, K., Plunkett, J., Anttila, V., Palotie, A., Haataja, R.*, et al.* (2012). A potential novel spontaneous preterm birth gene, AR, identified by linkage and association analysis of X chromosomal markers. PLoS One *7*, e51378.

Karteris, E., Zervou, S., Pang, Y., Dong, J., Hillhouse, E.W., Randeva, H.S., and Thomas, P. (2006). Progesterone signaling in human myometrium through two novel membrane G protein-coupled receptors: potential role in functional progesterone withdrawal at term. Mol Endocrinol *20*, 1519-1534.

Keelan, J.A., Mattes, E., Tan, H., Dinan, A., Newnham, J.P., Whitehouse, A.J., Jacoby, P., and Hickey, M. (2012). Androgen concentrations in umbilical cord blood and their association with maternal, fetal and obstetric factors. PLoS One *7*, e42827.

Kemppainen, J.A., Langley, E., Wong, C.I., Bobseine, K., Kelce, W.R., and Wilson, E.M. (1999). Distinguishing androgen receptor agonists and antagonists: distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone. Mol Endocrinol *13*, 440-454.

Kenyon, S., Boulvain, M., and Neilson, J.P. (2010). Antibiotics for preterm rupture of membranes. Cochrane Database Syst Rev, Cd001058.

Kenyon, S., Pike, K., Jones, D.R., Brocklehurst, P., Marlow, N., Salt, A., and Taylor, D.J. (2008). Childhood outcomes after prescription of antibiotics to pregnant women with preterm rupture of the membranes: 7-year follow-up of the ORACLE I trial. Lancet *372*, 1310-1318.

Kenyon, S.L., Taylor, D.J., and Tarnow-Mordi, W. (2001a). Broad-spectrum antibiotics for preterm, prelabour rupture of fetal membranes: the ORACLE I randomised trial. ORACLE Collaborative Group. Lancet *357*, 979-988.

Kenyon, S.L., Taylor, D.J., and Tarnow-Mordi, W. (2001b). Broad-spectrum antibiotics for spontaneous preterm labour: the ORACLE II randomised trial. ORACLE Collaborative Group. Lancet *357*, 989-994.

Keung, W., Chan, M.L., Ho, E.Y., Vanhoutte, P.M., and Man, R.Y. (2011). Non-genomic activation of adenylyl cyclase and protein kinase G by 17beta-estradiol in vascular smooth muscle of the rat superior mesenteric artery. Pharmacological research : the official journal of the Italian Pharmacological Society *64*, 509-516.

Keung, W., Vanhoutte, P.M., and Man, R.Y. (2005). Acute impairment of contractile responses by 17beta-estradiol is cAMP and protein kinase G dependent in vascular smooth muscle cells of the porcine coronary arteries. Br J Pharmacol *144*, 71-79.

Khan, R.N., Morrison, J.J., Smith, S.K., and Ashford, M.L. (1998). Activation of large-conductance potassium channels in pregnant human myometrium by pinacidil. Am J Obstet Gynecol *178*, 1027-1034.

Khan, R.N., Smith, S.K., Morrison, J.J., and Ashford, M.L. (1997). Ca2+ dependence and pharmacology of large-conductance K+ channels in nonlabor and labor human uterine myocytes. Am J Physiol *273*, C1721-1731.

Khanjani, S., Kandola, M.K., Lindstrom, T.M., Sooranna, S.R., Melchionda, M., Lee, Y.S., Terzidou, V., Johnson, M.R., and Bennett, P.R. (2011). NF-kappaB regulates a cassette of immune/inflammatory genes in human pregnant myometrium at term. Journal of cellular and molecular medicine *15*, 809-824.

Khanjani, S., Terzidou, V., Johnson, M.R., and Bennett, P.R. (2012). NFkappaB and AP-1 drive human myometrial IL8 expression. Mediators of inflammation *2012*, 504952.

Kilarski, W.M., Dupont, E., Coppen, S., Yeh, H.I., Vozzi, C., Gourdie, R.G., Rezapour, M., Ulmsten, U., Roomans, G.M., and Severs, N.J. (1998). Identification of two further gap-junctional proteins, connexin40 and connexin45, in human myometrial smooth muscle cells at term. European journal of cell biology *75*, 1-8.

Kim, B.J., Rho, Y.K., Lee, H.I., Jeong, M.S., Li, K., Seo, S.J., Kim, M.N., and Hong, C.K. (2009). The effect of calcipotriol on the expression of human beta defensin-2 and LL-37 in cultured human keratinocytes. Clin Dev Immunol *2009*, 645898.

Kim, H.J., and Lee, W.J. (2009a). Insulin-like growth factor-I induces androgen receptor activation in differentiating C2C12 skeletal muscle cells. Molecules and cells *28*, 189-194.

Kim, H.J., and Lee, W.J. (2009b). Ligand-independent activation of the androgen receptor by insulin-like growth factor-I and the role of the MAPK pathway in skeletal muscle cells. Molecules and cells *28*, 589-593.

Kimura, K., Orita, T., Fujitsu, Y., Liu, Y., Wakuta, M., Morishige, N., Suzuki, K., and Sonoda, K.H. (2014). Inhibition by female sex hormones of collagen gel contraction mediated by retinal pigment epithelial cells. Invest Ophthalmol Vis Sci *55*, 2621-2630.

King, J.F., Flenady, V., Papatsonis, D., Dekker, G., and Carbonne, B. (2003). Calcium channel blockers for inhibiting preterm labour; a systematic review of the evidence and a protocol for administration of nifedipine. The Australian & New Zealand journal of obstetrics & gynaecology *43*, 192-198.

Kioumourtzoglou, D., Gould, G.W., and Bryant, N.J. (2014). Insulin stimulates syntaxin4 SNARE complex assembly via a novel regulatory mechanism. Mol Cell Biol *34*, 1271-1279.

Kisielewska, J., Zezula-Szpyra, A., Bilinska, B., Flint, A.P., and Ziecik, A.J. (1997). Effect of estradiol and progesterone on growth of porcine myometrial smooth muscle cells and phospholipase C and adenylate cyclase signalling systems in vitro. Folia Histochem Cytobiol *35*, 143-149.

Kitaya, K. (2011). Prevalence of chronic endometritis in recurrent miscarriages. Fertil Steril *95*, 1156-1158.

Kline, L.W., and Karpinski, E. (2008). Testosterone and dihydrotestosterone inhibit gallbladder motility through multiple signalling pathways. Steroids *73*, 1174-1180.

Knock, G.A., Tribe, R.M., Hassoni, A.A., and Aaronson, P.I. (2001). Modulation of potassium current characteristics in human myometrial smooth muscle by 17beta-estradiol and progesterone. Biol Reprod *64*, 1526-1534.

Kopas, M.L. (2014). A review of evidence-based practices for management of the second stage of labor. Journal of midwifery & women's health *59*, 264-276.

Kouloumenta, V., Hatziefthimiou, A., Paraskeva, E., Gourgoulianis, K., and Molyvdas, P.A. (2006). Non-genomic effect of testosterone on airway smooth muscle. Br J Pharmacol *149*, 1083-1091.

Koyuncu, F., Aksoy, S., Karanfil, C., Demir, N., Bozkurt, K., and Tuncay, G. (1995). [The effect of dehydroepiandrosterone sulfate on cervical maturation during pregnancy]. J Gynecol Obstet Biol Reprod (Paris) *24*, 630-633.

Krajniak, K.G. (2001). The Use of an Isolated Earthworm Crop-Gizzard Preparation as a Model for Teaching Smooth Muscle Physiology. THE AMERICAN BIOLOGY TEACHER *63*, 59-65.

Kreuzberg, U., Theissen, P., Schicha, H., Schroder, F., Mehlhorn, U., de Vivie, E.R., Boknik, P., Neumann, J., Grohe, C., and Herzig, S. (2000). Single-channel activity and expression of atrial L-type Ca(2+) channels in patients with latent hyperthyroidism. Am J Physiol Heart Circ Physiol *278*, H723-730.

Kubli-Garfias, C., Lopez-Fiesco, A., Pacheco-Cano, M.T., Ponce-Monter, H., and Bondani, A. (1980). In vitro effects of androgens upon the spontaneous rat uterine contractility. Steroids *35*, 633-641.

Kubli-Garfias, C., Medina-Jimenez, M., Garcia-Yanez, E., Vazquez-Alvarez, A., Perusquia, M., Gomez-Garcia, N., Almanza, J., Ibanez, R., and Rodriguez, R. (1987). Relaxant action of androgens, progestins and corticosteroids on the isolated ileum of the guinea pig. Acta Physiol Pharmacol Latinoam *37*, 357-364.

Kumar, R., Gururaj, A.E., and Barnes, C.J. (2006). p21-activated kinases in cancer. Nature reviews Cancer *6*, 459-471.

Kupittayanant, S., Luckas, M.J., and Wray, S. (2002). Effect of inhibiting the sarcoplasmic reticulum on spontaneous and oxytocin-induced contractions of human myometrium. BJOG *109*, 289-296.

Kurowicka, B., Franczak, A., Oponowicz, A., and Kotwica, G. (2005). In vitro contractile activity of porcine myometrium during luteolysis and early pregnancy: effect of oxytocin and progesterone. Reprod Biol *5*, 151-169.

Kusaka, M., and Sperelakis, N. (1995). Inhibition of L-type calcium current by genistein, a tyrosine kinase inhibitor, in pregnant rat myometrial cells. Biochim Biophys Acta *1240*, 196-200.

Kutryk, M.J., and Pierce, G.N. (1988). Stimulation of sodium-calcium exchange by cholesterol incorporation into isolated cardiac sarcolemmal vesicles. J Biol Chem *263*, 13167-13172.

Labrie, F., Martel, C., and Balser, J. (2011). Wide distribution of the serum dehydroepiandrosterone and sex steroid levels in postmenopausal women: role of the ovary? Menopause *18*, 30-43.

Lafayette, S.S., Vladimirova, I., Garcez-do-Carmo, L., Monteforte, P.T., Caricati Neto, A., and Jurkiewicz, A. (2008). Evidence for the participation of calcium in non-genomic relaxations induced by androgenic steroids in rat vas deferens. Br J Pharmacol *153*, 1242-1250.

Laherty, R.F., Rotten, D., Yamamoto, M., and Jaffe, R.B. (1985). Effects of oestradiol and prolactin on progesterone production by rhesus monkey luteal cells in vitro. Acta Endocrinol (Copenh) *108*, 266-272.

Landau, R., Xie, H.G., Dishy, V., Stein, C.M., Wood, A.J., Emala, C.W., and Smiley, R.M. (2002). beta2-Adrenergic receptor genotype and preterm delivery. Am J Obstet Gynecol *187*, 1294-1298.

Lang, F., Alevizopoulos, K., and Stournaras, C. (2013). Targeting membrane androgen receptors in tumors. Expert Opin Ther Targets *17*, 951-963.

Langenbach, R., Morham, S.G., Tiano, H.F., Loftin, C.D., Ghanayem, B.I., Chulada, P.C., Mahler, J.F., Lee, C.A., Goulding, E.H., Kluckman, K.D.*, et al.* (1995). Prostaglandin synthase 1 gene

disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. Cell *83*, 483-492.

Large, W.A. (2002). Receptor-operated Ca2(+)-permeable nonselective cation channels in vascular smooth muscle: a physiologic perspective. J Cardiovasc Electrophysiol *13*, 493-501.

Larmon, J.E., Magann, E.F., Dickerson, G.A., and Morrison, J.C. (2002). Outpatient cervical ripening with prostaglandin E2 and estradiol. J Matern Fetal Neonatal Med *11*, 113-117.

Lazic, M., Aird, F., Levine, J.E., and Dunaif, A. (2011). Prenatal androgen treatment alters body composition and glucose homeostasis in male rats. J Endocrinol *208*, 293-300.

Lee, S.E., Ahn, D.S., and Lee, Y.H. (2009). Role of T-type Ca Channels in the Spontaneous Phasic Contraction of Pregnant Rat Uterine Smooth Muscle. The Korean journal of physiology & pharmacology : official journal of the Korean Physiological Society and the Korean Society of Pharmacology *13*, 241-249.

Lee, W.J. (2009). Insulin-like growth factor-I-induced androgen receptor activation is mediated by the PI3K/Akt pathway in C2C12 skeletal muscle cells. Molecules and cells *28*, 495-499.

Leppert, P.C. (1995). Anatomy and physiology of cervical ripening. Clin Obstet Gynecol *38*, 267-279.

Li, B.Y., Liao, X.B., Fujito, A., Thrasher, J.B., Shen, F.Y., and Xu, P.Y. (2007). Dual androgen-response elements mediate androgen regulation of MMP-2 expression in prostate cancer cells. Asian journal of andrology *9*, 41-50.

Li, C., Meng, Q., Yu, X., Jing, X., Xu, P., and Luo, D. (2012). Regulatory effect of connexin 43 on basal Ca2+ signaling in rat ventricular myocytes. PLoS One *7*, e36165.

Li, L., Kang, J., and Lei, W. (2010). Role of Toll-like receptor 4 in inflammation-induced preterm delivery. Mol Hum Reprod *16*, 267-272.

Li, X., Llorente, I., and Brasch, M. (2008). Improvements in live cell analysis of G protein coupled receptors using second generation BD calcium assay kits. Current chemical genomics *2*, 10-15.

Liggins, G.C. (1968). Premature parturition after infusion of corticotrophin or cortisol into foetal lambs. J Endocrinol *42*, 323-329.

Likis, F.E., Edwards, D.R., Andrews, J.C., Woodworth, A.L., Jerome, R.N., Fonnesbeck, C.J., McKoy, J.N., and Hartmann, K.E. (2012). Progestogens for preterm birth prevention: a systematic review and meta-analysis. Obstet Gynecol *120*, 897-907.

Lim, S., MacIntyre, D.A., Lee, Y.S., Khanjani, S., Terzidou, V., Teoh, T.G., and Bennett, P.R. (2012). Nuclear factor kappa B activation occurs in the amnion prior to labour onset and modulates the expression of numerous labour associated genes. PLoS One *7*, e34707.

Lin, H.K., Wang, L., Hu, Y.C., Altuwaijri, S., and Chang, C. (2002). Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. Embo j *21*, 4037-4048.

Lin, M.C., Rajfer, J., Swerdloff, R.S., and Gonzalez-Cadavid, N.F. (1993). Testosterone down-regulates the levels of androgen receptor mRNA in smooth muscle cells from the rat corpora cavernosa via aromatization to estrogens. J Steroid Biochem Mol Biol *45*, 333-343.

Liu, L., Li, Y., Xie, N., Shynlova, O., Challis, J.R., Slater, D., Lye, S., and Dong, X. (2013). Proliferative action of the androgen receptor in human uterine myometrial cells--a key regulator for myometrium phenotype programming. J Clin Endocrinol Metab *98*, 218-227.

Liu, Y.X., and Hsueh, A.J. (1986). Synergism between granulosa and theca-interstitial cells in estrogen biosynthesis by gonadotropin-treated rat ovaries: studies on the two-cell, two-gonadotropin hypothesis using steroid antisera. Biol Reprod *35*, 27-36.

Loch-Caruso, R.K., Criswell, K.A., Grindatti, C.M., and Brant, K.A. (2003). Sustained inhibition of rat myometrial gap junctions and contractions by lindane. Reprod Biol Endocrinol *1*, 62.

Lohoff, F.W., Weller, A.E., Bloch, P.J., Nall, A.H., Ferraro, T.N., and Berrettini, W.H. (2008). Association between polymorphisms in the vesicle-associated membrane protein-associated protein A (VAPA) gene on chromosome 18p and bipolar disorder. Journal of neural transmission (Vienna, Austria : 1996) *115*, 1339-1345.

Longcope, C. (1986). Adrenal and gonadal androgen secretion in normal females. Clin Endocrinol Metab *15*, 213-228.

Longcope, C. (1996). Dehydroepiandrosterone metabolism. J Endocrinol *150 Suppl*, S125-127.

Lorzadeh, N., and Kazemirad, S. (2012). The effects of fetal gender on serum human chorionic gonadotropin and testosterone in normotensive and preeclamptic pregnancies. J Pregnancy *2012*, 874290.

Loudon, J.A., Sooranna, S.R., Bennett, P.R., and Johnson, M.R. (2004). Mechanical stretch of human uterine smooth muscle cells increases IL-8 mRNA expression and peptide synthesis. Mol Hum Reprod *10*, 895-899.

Lubahn, D.B., Joseph, D.R., Sullivan, P.M., Willard, H.F., French, F.S., and Wilson, E.M. (1988). Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science *240*, 327-330.

Ludwig, M., Beck, A., Wickert, L., Bolkenius, U., Tittel, B., Hinkel, K., and Bidlingmaier, F. (1998). Female pseudohermaphroditism associated with a novel homozygous G-to-A (V370-to-M) substitution in the P-450 aromatase gene. J Pediatr Endocrinol Metab *11*, 657-664.

Lye, S.J., Mitchell, J., Nashman, N., Oldenhof, A., Ou, R., Shynlova, O., and Langille, L. (2001). Role of mechanical signals in the onset of term and preterm labor. Front Horm Res *27*, 165-178.

Lye, S.J., Nicholson, B.J., Mascarenhas, M., MacKenzie, L., and Petrocelli, T. (1993). Increased expression of connexin-43 in the rat myometrium during labor is associated with an increase in the plasma estrogen:progesterone ratio. Endocrinology *132*, 2380-2386.

Lynn, K.N., Steinkeler, J.A., Wilkins-Haug, L.E., and Benson, C.B. (2013). Hyperreactio luteinalis (enlarged ovaries) during the second and third trimesters of pregnancy: common clinical associations. J Ultrasound Med *32*, 1285-1289.

Ma, Q., Li, H.F., Jin, S., Dou, X.C., Zhang, Y.F., Zhang, L.X., and Du, Z.R. (2013). [Inhibitory effects of 17beta-estradiol on spontaneous and activated contraction of rat uterus smooth muscle]. Zhongguo ying yong sheng li xue za zhi = Zhongguo yingyong shenglixue zazhi = Chinese journal of applied physiology *29*, 305-309.

MacKenzie, L.W., and Garfield, R.E. (1985). Hormonal control of gap junctions in the myometrium. Am J Physiol *248*, C296-308.

Mackenzie, R., Walker, M., Armson, A., and Hannah, M.E. (2006). Progesterone for the prevention of preterm birth among women at increased risk: a systematic review and meta-analysis of randomized controlled trials. Am J Obstet Gynecol *194*, 1234-1242.

Macleod, D.J., Sharpe, R.M., Welsh, M., Fisken, M., Scott, H.M., Hutchison, G.R., Drake, A.J., and van den Driesche, S. (2010). Androgen action in the masculinization programming window and development of male reproductive organs. Int J Androl *33*, 279-287.

Madsen, G., Zakar, T., Ku, C.Y., Sanborn, B.M., Smith, R., and Mesiano, S. (2004). Prostaglandins differentially modulate progesterone receptor-A and -B expression in human myometrial cells: evidence for prostaglandin-induced functional progesterone withdrawal. J Clin Endocrinol Metab *89*, 1010-1013.

Mahendroo, M.S., Cala, K.M., and Russell, D.W. (1996). 5 alpha-reduced androgens play a key role in murine parturition. Mol Endocrinol *10*, 380-392.

Mahendroo, M.S., Porter, A., Russell, D.W., and Word, R.A. (1999). The parturition defect in steroid 5alpha-reductase type 1 knockout mice is due to impaired cervical ripening. Mol Endocrinol *13*, 981-992.

Mahendroo, M.S., and Russell, D.W. (1999). Male and female isoenzymes of steroid 5alpha-reductase. Rev Reprod *4*, 179-183.

Maiti, K., Paul, J.W., Read, M., Chan, E.C., Riley, S.C., Nahar, P., and Smith, R. (2011). G-1-activated membrane estrogen receptors mediate increased contractility of the human myometrium. Endocrinology *152*, 2448-2455.

Makieva, S., Saunders, P.T., and Norman, J.E. (2014). Androgens in pregnancy: roles in parturition. Hum Reprod Update.

Manabe, Y., Sakaguchi, M., and Nakajima, A. (1981). Initiation of uterine contractions by purely mechanical stretching of the uterus at midpregnancy. Int J Biol Res Pregnancy *2*, 63-69.

Manuck, T.A., Watkins, W.S., Moore, B., Esplin, M.S., Varner, M.W., Jackson, G.M., Yandell, M., and Jorde, L. (2014). Pharmacogenomics of 17-alpha hydroxyprogesterone caproate for recurrent preterm birth prevention. Am J Obstet Gynecol *210*, 321.e321-321.e321.

Maradny, E., Kanayama, N., Maehara, K., Kobayashi, T., and Terao, T. (1996). Dehydroepiandrosterone sulfate potentiates the effect of interleukin-8 on the cervix. Gynecol Obstet Invest *42*, 191-195.

Marchetti, P.M., and Barth, J.H. (2013). Clinical biochemistry of dihydrotestosterone. Ann Clin Biochem *50*, 95-107.

Marlow, N., Pike, K., Bower, E., Brocklehurst, P., Jones, D., Kenyon, S., Kurinczuk, J.J., Taylor, D., and Salt, A. (2012). Characteristics of children with cerebral palsy in the ORACLE children study. Developmental medicine and child neurology *54*, 640-646.

Masuyama, H., Tateishi, Y., Matsuda, M., and Hiramatrsu, Y. (2009). Hyperreactio luteinalis with both markedly elevated human chorionic gonadotropin levels and an imbalance of angiogenic factors subsequently developed severe early-onset preeclampsia. Fertil Steril *92*, 393 e391-393.

Matharoo-Ball, B., Ashford, M.L., Arulkumaran, S., and Khan, R.N. (2003). Down-regulation of the alpha- and beta-subunits of the calcium-activated potassium channel in human myometrium with parturition. Biol Reprod *68*, 2135-2141.

Matsui, K., Higashi, K., Fukunaga, K., Miyazaki, K., Maeyama, M., and Miyamoto, E. (1983). Hormone treatments and pregnancy alter myosin light chain kinase and calmodulin levels in rabbit myometrium. J Endocrinol *97*, 11-19.

Matsuzaki, M., Udo, S., and Fukumoto, N. (1971). Changes of elastic fibers in the uterine wall during pregnancy and after parturition. Kumamoto Igakkai zasshi The Journal of the Kumamoto Medical Society *45*, 741-751.

Maymon, E., Romero, R., Pacora, P., Gomez, R., Athayde, N., Edwin, S., and Yoon, B.H. (2000). Human neutrophil collagenase (matrix metalloproteinase 8) in parturition, premature rupture of the membranes, and intrauterine infection. Am J Obstet Gynecol *183*, 94-99.

McGregor, J.A., Leff, M., Orleans, M., and Baron, A. (1992). Fetal gender differences in preterm birth: findings in a North American cohort. Am J Perinatol *9*, 43-48.

McKay, L.A., Holford, T.R., and Bracken, M.B. (2014). Re-analysis of the PREGNANT trial confirms that vaginal progesterone reduces the rate of preterm birth in women with a sonographic short cervix. Ultrasound Obstet Gynecol *43*, 596-597.

Mecenas, C.A., Giussani, D.A., Owiny, J.R., Jenkins, S.L., Wu, W.X., Honnebier, B.O., Lockwood, C.J., Kong, L., Guller, S., and Nathanielsz, P.W. (1996). Production of premature delivery in pregnant rhesus monkeys by androstenedione infusion. Nat Med *2*, 443-448.

Meera, P., Anwer, K., Monga, M., Oberti, C., Stefani, E., Toro, L., and Sanborn, B.M. (1995). Relaxin stimulates myometrial calcium-activated potassium channel activity via protein kinase A. Am J Physiol *269*, C312-317.

Meldrum, E., Parker, P.J., and Carozzi, A. (1991). The PtdIns-PLC superfamily and signal transduction. Biochim Biophys Acta *1092*, 49-71.

Mendel, C.M. (1989). The free hormone hypothesis: a physiologically based mathematical model. Endocr Rev *10*, 232-274.

Menon, R., Velez, D.R., Simhan, H., Ryckman, K., Jiang, L., Thorsen, P., Vogel, I., Jacobsson, B., Merialdi, M., Williams, S.M*., et al.* (2006a). Multilocus interactions at maternal tumor necrosis factor-alpha, tumor necrosis factor receptors, interleukin-6 and interleukin-6 receptor genes predict spontaneous preterm labor in European-American women. Am J Obstet Gynecol *194*, 1616-1624.

Menon, R., Velez, D.R., Thorsen, P., Vogel, I., Jacobsson, B., Williams, S.M., and Fortunato, S.J. (2006b). Ethnic differences in key candidate genes for spontaneous preterm birth: TNF-alpha and its receptors. Human heredity *62*, 107-118.

Merlino, A., Welsh, T., Erdonmez, T., Madsen, G., Zakar, T., Smith, R., Mercer, B., and Mesiano, S. (2009). Nuclear progesterone receptor expression in the human fetal membranes and decidua at term before and after labor. Reprod Sci *16*, 357-363.

Merlino, A.A., Welsh, T.N., Tan, H., Yi, L.J., Cannon, V., Mercer, B.M., and Mesiano, S. (2007). Nuclear progesterone receptors in the human pregnancy myometrium: evidence that parturition involves functional progesterone withdrawal mediated by increased expression of progesterone receptor-A. J Clin Endocrinol Metab *92*, 1927-1933.

Mershon, J.L., Mikala, G., and Schwartz, A. (1994). Changes in the expression of the L-type voltage-dependent calcium channel during pregnancy and parturition in the rat. Biol Reprod *51*, 993-999.

Mertens, H.J., Heineman, M.J., Theunissen, P.H., de Jong, F.H., and Evers, J.L. (2001). Androgen, estrogen and progesterone receptor expression in the human uterus during the menstrual cycle. Eur J Obstet Gynecol Reprod Biol *98*, 58-65.

Mesiano, S. (2004). Myometrial progesterone responsiveness and the control of human parturition. J Soc Gynecol Investig *11*, 193-202.

Mesiano, S., Chan, E.C., Fitter, J.T., Kwek, K., Yeo, G., and Smith, R. (2002). Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium. J Clin Endocrinol Metab *87*, 2924-2930.

Mesiano, S., and Welsh, T.N. (2007). Steroid hormone control of myometrial contractility and parturition. Semin Cell Dev Biol *18*, 321-331.

Milewich, L., Gomez-Sanchez, C., Madden, J.D., Bradfield, D.J., Parker, P.M., Smith, S.L., Carr, B.R., Edman, C.D., and MacDonald, P.C. (1978). Dehydroisoandrosterone sulfate in peripheral blood of premenopausal, pregnant and postmenopausal women and men. J Steroid Biochem *9*, 1159-1164.

Milewich, L., MacDonald, P.C., Guerami, A., Midgett, W.T., Lassiter, W.L., and Carr, B.R. (1986). Human fetal liver estrogen 16 alpha-hydroxylase: precursor specificity, kinetic parameters, and in vitro regulation. J Clin Endocrinol Metab *63*, 180-191.

Miller, S.M., Garfield, R.E., and Daniel, E.E. (1989). Improved propagation in myometrium associated with gap junctions during parturition. Am J Physiol *256*, C130-141.

Mitchell, B.F., Fang, X., and Wong, S. (1998). Oxytocin: a paracrine hormone in the regulation of parturition? Rev Reprod *3*, 113-122.

Mitchell, B.F., and Taggart, M.J. (2009). Are animal models relevant to key aspects of human parturition? Am J Physiol Regul Integr Comp Physiol *297*, R525-545.

Mitchell, F.L., and Shackleton, C.H. (1969). The investigation of steroid metabolism in early infancy. Advances in clinical chemistry *12*, 141-215.

Miyoshi, H., Boyle, M.B., MacKay, L.B., and Garfield, R.E. (1996). Voltage-clamp studies of gap junctions between uterine muscle cells during term and preterm labor. Biophysical journal *71*, 1324-1334.

Mizuno, M., Lobotsky, J., Lloyd, C.W., Kobayashi, T., and Murasawa, Y. (1968). Plasma androstenedione and testerone during pregnancy and in the newborn. J Clin Endocrinol Metab *28*, 1133-1142.

Mochizuki, M., Honda, T., Deguchi, M., Morikawa, H., and Tojo, S. (1978a). A study on the effect of dehydroepiandrosterone sulfate on so-called cervical ripening. Acta Obstet Gynecol Scand *57*, 397-401.

Mochizuki, M., Honda, T., and Tojo, S. (1978b). Collagenolytic activity and steroid levels after administration of dehydroepiandrosterone sulfate. Int J Gynaecol Obstet *16*, 248-253.

Mochizuki, M., and Maruo, T. (1985). Effect of dehydroepiandrosterone sulfate on uterine cervical ripening in late pregnancy. Acta Physiol Hung *65*, 267-274.

Moczydlowski, E.G. (2006). The maxi K+ channel of human myometrium reveals a split personality. J Physiol *573*, 286.

Molnar, M., Romero, R., and Hertelendy, F. (1993). Interleukin-1 and tumor necrosis factor stimulate arachidonic acid release and phospholipid metabolism in human myometrial cells. Am J Obstet Gynecol *169*, 825-829.

Monga, M., Campbell, D.F., and Sanborn, B.M. (1999). Oxytocin-stimulated capacitative calcium entry in human myometrial cells. Am J Obstet Gynecol *181*, 424-429.

Monga, M., Ku, C.Y., Dodge, K., and Sanborn, B.M. (1996). Oxytocin-stimulated responses in a pregnant human immortalized myometrial cell line. Biol Reprod *55*, 427-432.

Mooney, S.M., Parsana, P., Hernandez, J.R., Liu, X., Verdone, J.E., Torga, G., Harberg, C.A., and Pienta, K.J. (2014). The presence of Androgen Receptor Elements regulates ZEB1 expression in the absence of Androgen Receptor. Journal of cellular biochemistry.

Moore, F., Da Silva, C., Wilde, J.I., Smarason, A., Watson, S.P., and Lopez Bernal, A. (2000). Up-regulation of p21- and RhoA-activated protein kinases in human pregnant myometrium. Biochem Biophys Res Commun *269*, 322-326.

Morisset, A.S., Dube, M.C., Drolet, R., Pelletier, M., Labrie, F., Luu-The, V., Tremblay, Y., Robitaille, J., John Weisnagel, S., and Tchernof, A. (2013). Androgens in the maternal and fetal circulation: association with insulin resistance. J Matern Fetal Neonatal Med *26*, 513-519.

Morrison, J.J., Ashford, M.L., Khan, R.N., and Smith, S.K. (1993). The effects of potassium channel openers on isolated pregnant human myometrium before and after the onset of labor: potential for tocolysis. Am J Obstet Gynecol *169*, 1277-1285.

Mountain, D.J., Freeman, B.M., Kirkpatrick, S.S., Beddies, J.W., Arnold, J.D., Freeman, M.B., Goldman, M.H., Stevens, S.L., Klein, F.A., and Grandas, O.H. (2013). Androgens regulate MMPs and the cellular processes of intimal hyperplasia. The Journal of surgical research *184*, 619-627.

Murphy, J.G., and Khalil, R.A. (1999). Decreased [Ca(2+)](i) during inhibition of coronary smooth muscle contraction by 17beta-estradiol, progesterone, and testosterone. J Pharmacol Exp Ther *291*, 44-52.

Nagler, A., Miao, H.Q., Aingorn, H., Pines, M., Genina, O., and Vlodavsky, I. (1997). Inhibition of collagen synthesis, smooth muscle cell proliferation, and injury-induced intimal hyperplasia by halofuginone. Arterioscler Thromb Vasc Biol *17*, 194-202.

Nakajima, A. (1971). Action potential of human myometrial fibers. Am J Obstet Gynecol *111*, 266-269.

Nath, C.A., Ananth, C.V., Smulian, J.C., and Peltier, M.R. (2010). Can sulfasalazine prevent infection-mediated pre-term birth in a murine model? Am J Reprod Immunol *63*, 144-149.

Nathanielsz, P.W., Jenkins, S.L., Tame, J.D., Winter, J.A., Guller, S., and Giussani, D.A. (1998). Local paracrine effects of estradiol are central to parturition in the rhesus monkey. Nat Med *4*, 456-459.

Neilson, J.P., West, H.M., and Dowswell, T. (2014). Betamimetics for inhibiting preterm labour. Cochrane Database Syst Rev *2*, Cd004352.

Nelson, P.S., Clegg, N., Arnold, H., Ferguson, C., Bonham, M., White, J., Hood, L., and Lin, B. (2002). The program of androgen-responsive genes in neoplastic prostate epithelium. Proc Natl Acad Sci U S A *99*, 11890-11895.

Ning, Y., Hoang, B., Schuller, A.G., Cominski, T.P., Hsu, M.S., Wood, T.L., and Pintar, J.E. (2007). Delayed mammary gland involution in mice with mutation of the insulin-like growth factor binding protein 5 gene. Endocrinology *148*, 2138-2147.

Nishimori, K., Young, L.J., Guo, Q., Wang, Z., Insel, T.R., and Matzuk, M.M. (1996). Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. Proc Natl Acad Sci U S A *93*, 11699-11704.

Nishinaka, K., and Fukuda, Y. (1991). Changes in extracellular matrix materials in the uterine myometrium of rats during pregnancy and postparturition. Acta Pathol Jpn *41*, 122-132.

Nnamani, M.C., Plaza, S., Romero, R., and Wagner, G.P. (2013). Evidence for independent evolution of functional progesterone withdrawal in primates and guinea pigs. Evolution, medicine, and public health *2013*, 273-288.

Noble, K., Floyd, R., Shmygol, A., Shmygol, A., Mobasheri, A., and Wray, S. (2010). Distribution, expression and functional effects of small conductance Ca-activated potassium (SK) channels in rat myometrium. Cell Calcium *47*, 47-54.

Noble, K., Matthew, A., Burdyga, T., and Wray, S. (2009). A review of recent insights into the role of the sarcoplasmic reticulum and Ca entry in uterine smooth muscle. Eur J Obstet Gynecol Reprod Biol *144 Suppl 1*, S11-19.

Norman, J.E. (2007). Preterm labour. Cervical function and prematurity. Best Pract Res Clin Obstet Gynaecol *21*, 791-806.

Norman, J.E., Bollapragada, S., Yuan, M., and Nelson, S.M. (2007). Inflammatory pathways in the mechanism of parturition. BMC Pregnancy Childbirth *7 Suppl 1*, S7.

Norman, J.E., Mackenzie, F., Owen, P., Mactier, H., Hanretty, K., Cooper, S., Calder, A., Mires, G., Danielian, P., Sturgiss, S.*, et al.* (2009). Progesterone for the prevention of preterm birth in twin pregnancy (STOPPIT): a randomised, double-blind, placebo-controlled study and meta-analysis. Lancet *373*, 2034-2040.

Norman, J.E., Shennan, A., Bennett, P., Thornton, S., Robson, S., Marlow, N., Norrie, J., Petrou, S., Sebire, N., Lavender, T.*, et al.* (2012). Trial protocol OPPTIMUM-- does progesterone prophylaxis for the prevention of preterm labour improve outcome? BMC Pregnancy Childbirth *12*, 79.

Norman, M., Ekman, G., and Malmstrom, A. (1993). Changed proteoglycan metabolism in human cervix immediately after spontaneous vaginal delivery. Obstet Gynecol *81*, 217-223.

Novy, M.J., and Walsh, S.W. (1983). Dexamethasone and estradiol treatment in pregnant rhesus macaques: effects on gestational length, maternal plasma hormones, and fetal growth. Am J Obstet Gynecol *145*, 920-931.

O'Brien, J.M., Adair, C.D., Lewis, D.F., Hall, D.R., Defranco, E.A., Fusey, S., Soma-Pillay, P., Porter, K., How, H., Schackis, R.*, et al.* (2007a). Progesterone vaginal gel for the reduction of recurrent preterm birth: primary results from a randomized, double-blind, placebo-controlled trial. Ultrasound Obstet Gynecol *30*, 687-696.

O'Brien, J.M., Defranco, E.A., Adair, C.D., Lewis, D.F., Hall, D.R., How, H., Bsharat, M., and Creasy, G.W. (2009). Effect of progesterone on cervical shortening in women at risk for preterm birth: secondary analysis from a multinational, randomized, double-blind, placebo-controlled trial. Ultrasound Obstet Gynecol *34*, 653-659.

O'Brien, M., O'Shaughnessy, D., Ahamide, E., Morrison, J.J., and Smith, T.J. (2007b). Differential expression of the metalloproteinase MMP3 and the alpha5 integrin subunit in human myometrium at labour. Mol Hum Reprod *13*, 655-661.

O'Connell, M.D., and Wu, F.C. (2014). Androgen effects on skeletal muscle: implications for the development and management of frailty. Asian journal of andrology *16*, 203-212.

O'Leary, P., Boyne, P., Flett, P., Beilby, J., and James, I. (1991). Longitudinal assessment of changes in reproductive hormones during normal pregnancy. Clin Chem *37*, 667-672.

Okawa, T., Longo, M., Vedernikov, Y.P., Chwalisz, K., Saade, G.R., and Garfield, R.E. (2000). Role of nucleotide cyclases in the inhibition of pregnant rat uterine contractions by the openers of potassium channels. Am J Obstet Gynecol *182*, 913-918.

Okawa, T., Vedernikov, Y.P., Saade, G.R., Longo, M., Olson, G.L., Chwalisz, K., and Garfield, R.E. (1999). Roles of potassium channels and nitric oxide in modulation of uterine contractions in rat pregnancy. Am J Obstet Gynecol *181*, 649-655.

Okon, M.A., Laird, S.M., Tuckerman, E.M., and Li, T.C. (1998). Serum androgen levels in women who have recurrent miscarriages and their correlation with markers of endometrial function. Fertil Steril *69*, 682-690.

Olson, D.M., Lye, S.J., Skinner, K., and Challis, J.R. (1985). Prostanoid concentrations in maternal/fetal plasma and amniotic fluid and intrauterine tissue prostanoid output in relation to myometrial contractility during the onset of adrenocorticotropin-induced preterm labor in sheep. Endocrinology *116*, 389-397.

Orsino, A., Taylor, C.V., and Lye, S.J. (1996). Connexin-26 and connexin-43 are differentially expressed and regulated in the rat myometrium throughout late pregnancy and with the onset of labor. Endocrinology *137*, 1545-1553.

Ortega, A., and Mas-Oliva, J. (1984). Cholesterol effect on enzyme activity of the sarcolemmal (Ca2+ + Mg2+)-ATPase from cardiac muscle. Biochim Biophys Acta *773*, 231-236.

Osman, I., Young, A., Ledingham, M.A., Thomson, A.J., Jordan, F., Greer, I.A., and Norman, J.E. (2003). Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. Mol Hum Reprod *9*, 41-45.

Osterlund, K.L., Handa, R.J., and Gonzales, R.J. (2010). Dihydrotestosterone alters cyclooxygenase-2 levels in human coronary artery smooth muscle cells. Am J Physiol Endocrinol Metab *298*, E838-845.

Ou, C.W., Chen, Z.Q., Qi, S., and Lye, S.J. (1998). Increased expression of the rat myometrial oxytocin receptor messenger ribonucleic acid during labor requires both mechanical and hormonal signals. Biol Reprod *59*, 1055-1061.

Ou, C.W., Orsino, A., and Lye, S.J. (1997). Expression of connexin-43 and connexin-26 in the rat myometrium during pregnancy and labor is differentially regulated by mechanical and hormonal signals. Endocrinology *138*, 5398-5407.

Ousey, J.C., Freestone, N., Fowden, A.L., Mason, W.T., and Rossdale, P.D. (2000). The effects of oxytocin and progestagens on myometrial contractility in vitro during equine pregnancy. Journal of reproduction and fertility Supplement, 681-691.

Owen, D., Banjanin, S., Gidrewicz, D., McCabe, L., and Matthews, S.G. (2005). Central regulation of the hypothalamic-pituitary-adrenal axis during fetal development in the Guinea-pig. J Neuroendocrinol *17*, 220-226.

Pasanen, M., and Pelkonen, O. (1994). The expression and environmental regulation of P450 enzymes in human placenta. Crit Rev Toxicol *24*, 211-229.

Pawelec, M., Palczynski, B., Krzemieniewska, J., Karmowski, M., Korys, J., Latkowski, K., and Karmowski, A. (2013). Initiation of preterm labor. Advances in clinical and experimental medicine : official organ Wroclaw Medical University *22*, 283-288.

Penning, T.M. (2010). New frontiers in androgen biosynthesis and metabolism. Curr Opin Endocrinol Diabetes Obes *17*, 233-239.

Pereira de Jesus-Tran, K., Cote, P.L., Cantin, L., Blanchet, J., Labrie, F., and Breton, R. (2006). Comparison of crystal structures of human androgen receptor ligand-binding domain complexed with various agonists reveals molecular determinants responsible for binding affinity. Protein Sci *15*, 987-999.

Perusquia, M. (2001). Nongenomic action of steroids in myometrial contractility. Endocrine *15*, 63-72.

Perusquia, M., Campos, G., Corona, J.L., and Kubli-Garfias, C. (1991a). Antagonism by 5-reduced steroids of the tonic and phasic contractions induced by serotonin in the isolated rat uterus. Proc West Pharmacol Soc *34*, 395-398.

Perusquia, M., Corona, J.L., and Kubli-Garfias, C. (1991b). Inhibitory effect of 5-reduced androgens and progestins on the uterine contraction induced by acetylcholine. Proc West Pharmacol Soc *34*, 89-92.

Perusquia, M., Espinoza, J., and Navarrete, E. (2009). Nongenomic uterine relaxing effect of RU 486 (mifepristone) prior to its antiprogesterone activity in the human pregnancy. Steroids *74*, 825-831.

Perusquia, M., Garcia-Yanez, E., Ibanez, R., and Kubli-Garfias, C. (1990). Non-genomic mechanism of action of delta-4 and 5-reduced androgens and progestins on the contractility of the isolated rat myometrium. Life Sci *47*, 1547-1553.

Perusquia, M., and Jasso-Kamel, J. (2001). Influence of 5alpha- and 5beta-reduced progestins on the contractility of isolated human myometrium at term. Life Sci *68*, 2933-2944.

Perusquia, M., and Kubli-Garfias, C. (1994). Progesterone-like relaxant effect of RU 486 in the rat myometrium. Life Sci *54*, 1501-1506.

Perusquia, M., and Navarrete, E. (2005). Evidence that 17alpha-estradiol is biologically active in the uterine tissue: antiuterotonic and antiuterotrophic action. Reprod Biol Endocrinol *3*, 30.

Perusquia, M., Navarrete, E., Jasso-Kamel, J., and Montano, L.M. (2005). Androgens induce relaxation of contractile activity in pregnant human myometrium at term: a nongenomic action on L-type calcium channels. Biol Reprod *73*, 214-221.

Perusquia, M., and Villalon, C.M. (1996). The relaxant effect of sex steroids in rat myometrium is independent of the gamma-amino butyric acid system. Life Sci *58*, 913-926.

Perusquia, M.C., G (1991). Inhibitory effect of androgens and progestins on the contraction induced by oxytocin in the rat myometrium. Med Sci Res *19*, 177–179.

Phelan, J.P., Dainer, M.J., and Cowherd, D.W. (1978). Pregnancy complicated by thoracolumbar scoliosis. Southern medical journal *71*, 76-78.

Phelan, N., and Conway, G.S. (2011). Management of ovarian disease in pregnancy. Best Pract Res Clin Endocrinol Metab *25*, 985-992.

Phoenix, C.H., Goy, R.W., Gerall, A.A., and Young, W.C. (1959). Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology *65*, 369-382.

Pieber, D., Allport, V.C., and Bennett, P.R. (2001a). Progesterone receptor isoform A inhibits isoform B-mediated transactivation in human amnion. Eur J Pharmacol *427*, 7-11.

Pieber, D., Allport, V.C., Hills, F., Johnson, M., and Bennett, P.R. (2001b). Interactions between progesterone receptor isoforms in myometrial cells in human labour. Mol Hum Reprod *7*, 875-879.

Pierce, S.L., Kresowik, J.D., Lamping, K.G., and England, S.K. (2008). Overexpression of SK3 channels dampens uterine contractility to prevent preterm labor in mice. Biol Reprod *78*, 1058-1063.

Piltonen, T., Koivunen, R., Morin-Papunen, L., Ruokonen, A., Huhtaniemi, I.T., and Tapanainen, J.S. (2002). Ovarian and adrenal steroid production: regulatory role of LH/HCG. Hum Reprod *17*, 620-624.

Pion, R., Jaffe, R., Eriksson, G., Wiqvist, N., and Diczfalusy, E. (1965). STUDIES ON THE METABOLISM OF C-21 STEROIDS IN THE HUMAN FOETO-PLACENTAL UNIT. I. FORMATION OF A BETA-UNSATURATED 3-KETONES IN MIDTERM PLACENTAS PERFUSED IN SITU WITH PREGNENOLONE AND 17-ALPHA-HYDROXYPREGNENOLONE. Acta Endocrinol (Copenh) *48*, 234-248.

Pistilli, M.J., Petrik, J.J., Holloway, A.C., and Crankshaw, D.J. (2012). Immunohistochemical and functional studies on calcium-sensing receptors in rat uterine smooth muscle. Clin Exp Pharmacol Physiol *39*, 37-42.

Pluciennik, F., Verrecchia, F., Bastide, B., Herve, J.C., Joffre, M., and Deleze, J. (1996). Reversible interruption of gap junctional communication by testosterone propionate in cultured Sertoli cells and cardiac myocytes. J Membr Biol *149*, 169-177.

Plunkett, J., and Muglia, L.J. (2008). Genetic contributions to preterm birth: implications from epidemiological and genetic association studies. Annals of medicine *40*, 167-195.

Popat, A., and Crankshaw, D.J. (2001). Variable responses to prostaglandin E(2) in human non-pregnant myometrium. Eur J Pharmacol *416*, 145-152.

Pressman, E.K., Tucker, J.A., Jr., Anderson, N.C., Jr., and Young, R.C. (1988). Morphologic and electrophysiologic characterization of isolated pregnant human myometrial cells. Am J Obstet Gynecol *159*, 1273-1279.

Pulkkinen, M.O., Nyman, S., Hamalainen, M.M., and Mattinen, J. (1998). Proton NMR spectroscopy of the phospholipids in human uterine smooth muscle and placenta. Gynecol Obstet Invest *46*, 220-224.

Purohit, A., and Foster, P.A. (2012). Steroid sulfatase inhibitors for estrogen- and androgen-dependent cancers. J Endocrinol *212*, 99-110.

Ramirez-Rosas, M.B., Cobos-Puc, L.E., Munoz-Islas, E., Gonzalez-Hernandez, A., Sanchez-Lopez, A., Villalon, C.M., Maassenvandenbrink, A., and Centurion, D. (2011). Pharmacological evidence that Ca(2)+ channels and, to a lesser extent, K+ channels mediate the relaxation of testosterone in the canine basilar artery. Steroids *76*, 409-415.

Rao, P.M., Kelly, D.M., and Jones, T.H. (2013). Testosterone and insulin resistance in the metabolic syndrome and T2DM in men. Nat Rev Endocrinol *9*, 479-493.

Ratajczak, C.K., Fay, J.C., and Muglia, L.J. (2010). Preventing preterm birth: the past limitations and new potential of animal models. Dis Model Mech *3*, 407-414.

Read, C.P., Word, R.A., Ruscheinsky, M.A., Timmons, B.C., and Mahendroo, M.S. (2007). Cervical remodeling during pregnancy and parturition: molecular characterization of the softening phase in mice. Reproduction *134*, 327-340.

Rehman, K.S., Yin, S., Mayhew, B.A., Word, R.A., and Rainey, W.E. (2003). Human myometrial adaptation to pregnancy: cDNA microarray gene expression profiling of myometrium from non-pregnant and pregnant women. Mol Hum Reprod *9*, 681-700.

Reinheimer, T.M. (2007). Barusiban suppresses oxytocin-induced preterm labour in non-human primates. BMC Pregnancy Childbirth *7 Suppl 1*, S15.

Renthal, N.E., Chen, C.C., Williams, K.C., Gerard, R.D., Prange-Kiel, J., and Mendelson, C.R. (2010). miR-200 family and targets, ZEB1 and ZEB2, modulate uterine quiescence and contractility during pregnancy and labor. Proc Natl Acad Sci U S A *107*, 20828-20833.

Renthal, N.E., Williams, K.C., and Mendelson, C.R. (2013). MicroRNAs--mediators of myometrial contractility during pregnancy and labour. Nat Rev Endocrinol *9*, 391-401.

Reznikov, L.L., Fantuzzi, G., Selzman, C.H., Shames, B.D., Barton, H.A., Bell, H., McGregor, J.A., and Dinarello, C.A. (1999). Utilization of endoscopic inoculation in a mouse model of intrauterine infection-induced preterm birth: role of interleukin 1beta. Biol Reprod *60*, 1231-1238.

Richardson, M.C., and Masson, G.M. (1981). Stimulation by human chorionic gonadotrophin of oestradiol production by dispersed cells from human corpus luteum: comparison with progesterone production; utilization of exogenous testosterone. J Endocrinol *91*, 197-203.

Rinaldi, S.F., Catalano, R.D., Wade, J., Rossi, A.G., and Norman, J.E. (2014). Decidual neutrophil infiltration is not required for preterm birth in a mouse model of infection-induced preterm labor. J Immunol *192*, 2315-2325.

Rivarola, M.A., Forest, M.G., and Migeon, C.J. (1968). Testosterone, androstenedione and dehydroepiandrosterone in plasma during pregnancy and at delivery: concentration and protein binding. J Clin Endocrinol Metab *28*, 34-40.

Rivas, A., Fisher, J.S., McKinnell, C., Atanassova, N., and Sharpe, R.M. (2002). Induction of reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbestrol: evidence for importance of the androgen-estrogen balance. Endocrinology *143*, 4797-4808.

Robinson, J.D., Judd, H.L., Young, P.E., Jones, O.W., and Yen, S.S. (1977). Amniotic fluid androgens and estrogens in midgestation. J Clin Endocrinol Metab *45*, 755-761.

Robson (1937). Reactions of the uterine muscle and endometrium of rabbit to testosterone. Quart J Exp Physiol *35*, 355-363.

Rodeck, C.H., Gill, D., Rosenberg, D.A., and Collins, W.P. (1985). Testosterone levels in midtrimester maternal and fetal plasma and amniotic fluid. Prenat Diagn *5*, 175-181.

Roh, C.R., Oh, W.J., Yoon, B.K., and Lee, J.H. (2000). Up-regulation of matrix metalloproteinase-9 in human myometrium during labour: a cytokine-mediated process in uterine smooth muscle cells. Mol Hum Reprod *6*, 96-102.

Romero, R., Espinoza, J., Goncalves, L.F., Kusanovic, J.P., Friel, L., and Hassan, S. (2007). The role of inflammation and infection in preterm birth. Semin Reprod Med *25*, 21-39.

Romero, R., Munoz, H., Gomez, R., Parra, M., Polanco, M., Valverde, V., Hasbun, J., Garrido, J., Ghezzi, F., Mazor, M.*, et al.* (1996). Increase in prostaglandin bioavailability precedes the onset of human parturition. Prostaglandins, leukotrienes, and essential fatty acids *54*, 187-191.

Rooman, R.P., De Beeck, L.O., Martin, M., van Doorn, J., Mohan, S., and Du Caju, M.V. (2005). Ethinylestradiol and testosterone have divergent effects on circulating IGF system components in adolescents with constitutional tall stature. Eur J Endocrinol *152*, 597-604.

Roomans, G.M., Kilarski, W.M., Ciray, N., Persson, B.E., Backstrom, T., and Ulmsten, U. (1993). Structural and functional studies of gap junctions in human myometrium. Eur J Morphol *31*, 55-59.

Rose, A.J., Jeppesen, J., Kiens, B., and Richter, E.A. (2009). Effects of contraction on localization of GLUT4 and v-SNARE isoforms in rat skeletal muscle. Am J Physiol Regul Integr Comp Physiol *297*, R1228-1237.

Rosmond, R. (2006). Androgen excess in women--a health hazard? Med Hypotheses *67*, 229-234.

Rothman, M.S., Carlson, N.E., Xu, M., Wang, C., Swerdloff, R., Lee, P., Goh, V.H., Ridgway, E.C., and Wierman, M.E. (2011). Reexamination of testosterone, dihydrotestosterone, estradiol and estrone

levels across the menstrual cycle and in postmenopausal women measured by liquid chromatography-tandem mass spectrometry. Steroids *76*, 177-182.

Ruddock, N.K., Shi, S.Q., Jain, S., Moore, G., Hankins, G.D., Romero, R., and Garfield, R.E. (2008). Progesterone, but not 17-alpha-hydroxyprogesterone caproate, inhibits human myometrial contractions. Am J Obstet Gynecol *199*, 391.e391-397.

Sadar, M.D. (1999). Androgen-independent induction of prostate-specific antigen gene expression via cross-talk between the androgen receptor and protein kinase A signal transduction pathways. J Biol Chem *274*, 7777-7783.

Sadowsky, D.W., Adams, K.M., Gravett, M.G., Witkin, S.S., and Novy, M.J. (2006). Preterm labor is induced by intraamniotic infusions of interleukin-1beta and tumor necrosis factor-alpha but not by interleukin-6 or interleukin-8 in a nonhuman primate model. Am J Obstet Gynecol *195*, 1578-1589.

Sadowsky, D.W., Novy, M.J., Witkin, S.S., and Gravett, M.G. (2003). Dexamethasone or interleukin-10 blocks interleukin-1beta-induced uterine contractions in pregnant rhesus monkeys. Am J Obstet Gynecol *188*, 252-263.

Saez, J.M., Forest, M.G., Morera, A.M., and Bertrand, J. (1972). Metabolic clearance rate and blood production rate of testosterone and dihydrotestosterone in normal subjects, during pregnancy, and in hyperthyroidism. J Clin Invest *51*, 1226-1234.

Saijo, K., Collier, J.G., Li, A.C., Katzenellenbogen, J.A., and Glass, C.K. (2011). An ADIOL-ERbeta-CtBP transrepression pathway negatively regulates microglia-mediated inflammation. Cell *145*, 584-595.

Sakai, N., Tabb, T., and Garfield, R.E. (1992). Modulation of cell-to-cell coupling between myometrial cells of the human uterus during pregnancy. Am J Obstet Gynecol *167*, 472-480.

Sakyo, K., Ito, A., and Mori, Y. (1986). Effects of dehydroepiandrosterone sulphate on the production of collagenase and gelatinolytic metalloproteinase by rabbit uterine cervical cells in primary cultures. J Pharmacobiodyn *9*, 276-286.

Sakyo, K., Ito, A., and Mori, Y. (1987). Dehydroepiandrosterone sulfate stimulates collagenase synthesis without affecting the rates of collagen and noncollagen protein syntheses by rabbit uterine cervical fibroblasts. Biol Reprod *36*, 277-281.

Salgado, R.M., Favaro, R.R., and Zorn, T.M. (2011). Modulation of small leucine-rich proteoglycans (SLRPs) expression in the mouse uterus by estradiol and progesterone. Reprod Biol Endocrinol *9*, 22.

San Martin, S., Soto-Suazo, M., De Oliveira, S.F., Aplin, J.D., Abrahamsohn, P., and Zorn, T.M. (2003). Small leucine-rich proteoglycans (SLRPs) in uterine tissues during pregnancy in mice. Reproduction *125*, 585-595.

Sanchez Aparicio, J.A., Cantabrana, B., and Hidalgo, A. (1992). Effects of antiandrogens and progesterone on isolated rat uterus. General pharmacology *23*, 643-647.

Sanders, S.L., Stouffer, R.L., and Brannian, J.D. (1996). Androgen production by monkey luteal cell subpopulations at different stages of the menstrual cycle. J Clin Endocrinol Metab *81*, 591-596.

Sasaki, K., Nakano, R., Kadoya, Y., Iwao, M., Shima, K., and Sowa, M. (1982). Cervical ripening with dehydroepiandrosterone sulphate. Br J Obstet Gynaecol *89*, 195-198.

Sathishkumar, K., Balakrishnan, M., Chinnathambi, V., Chauhan, M., Hankins, G.D., and Yallampalli, C. (2012). Fetal sex-related dysregulation in testosterone production and their receptor expression in the human placenta with preeclampsia. J Perinatol *32*, 328-335.

Schlafer, D.H., Yuh, B., Foley, G.L., Elssaser, T.H., Sadowsky, D., and Nathanielsz, P.W. (1994). Effect of Salmonella endotoxin administered to the pregnant sheep at 133-142 days gestation on fetal oxygenation, maternal and fetal adrenocorticotropic hormone and cortisol, and maternal plasma tumor necrosis factor alpha concentrations. Biol Reprod *50*, 1297-1302.

Schmitz, T., Souil, E., Herve, R., Nicco, C., Batteux, F., Germain, G., Cabrol, D., Evain-Brion, D., Leroy, M.J., and Mehats, C. (2007). PDE4 inhibition prevents preterm delivery induced by an intrauterine inflammation. J Immunol *178*, 1115-1121.

Schmouder, V.M., Prescott, G.M., Franco, A., and Fan-Havard, P. (2013). The rebirth of progesterone in the prevention of preterm labor. The Annals of pharmacotherapy *47*, 527-536.

Schwalm, H., and Dubrauszky, V. (1966). The structure of the musculature of the human uterus--muscles and connective tissue. Am J Obstet Gynecol *94*, 391-404.

Schwarzel, W.C., Kruggel, W.G., and Brodie, H.J. (1973). Studies on the mechanism of estrogen biosynthesis. 8. The development of inhibitors of the enzyme system in human placenta. Endocrinology *92*, 866-880.

Schwenk, R.W., Dirkx, E., Coumans, W.A., Bonen, A., Klip, A., Glatz, J.F., and Luiken, J.J. (2010). Requirement for distinct vesicle-associated membrane proteins in insulin- and AMP-activated protein kinase (AMPK)-induced translocation of GLUT4 and CD36 in cultured cardiomyocytes. Diabetologia *53*, 2209-2219.

Scott, H.M., Hutchison, G.R., Mahood, I.K., Hallmark, N., Welsh, M., De Gendt, K., Verhoeven, G., O'Shaughnessy, P., and Sharpe, R.M. (2007). Role of androgens in fetal testis development and dysgenesis. Endocrinology *148*, 2027-2036.

Scott, H.M., Mason, J.I., and Sharpe, R.M. (2009). Steroidogenesis in the fetal testis and its susceptibility to disruption by exogenous compounds. Endocr Rev *30*, 883-925.

Seelbach-Goebel, B. (2013). Antibiotic Therapy for Premature Rupture of Membranes and Preterm Labor and Effect on Fetal Outcome. Geburtshilfe und Frauenheilkunde *73*, 1218-1227.

Sen, A., and Hammes, S.R. (2010). Granulosa cell-specific androgen receptors are critical regulators of ovarian development and function. Mol Endocrinol *24*, 1393-1403.

Senat, M.V., Porcher, R., Winer, N., Vayssiere, C., Deruelle, P., Capelle, M., Bretelle, F., Perrotin, F., Laurent, Y., Connan, L.*, et al.* (2013). Prevention of preterm delivery by 17 alpha-hydroxyprogesterone caproate in asymptomatic twin pregnancies with a short cervix: a randomized controlled trial. Am J Obstet Gynecol *208*, 194.e191-198.

Senchyna, M., and Crankshaw, D.J. (1999). Operational correlates of prostanoid TP receptor expression in human non-pregnant myometrium are unaffected by excision site or menstrual cycle status of the donor. Br J Pharmacol *128*, 1524-1528.

Sennstrom, M.B., Ekman, G., Westergren-Thorsson, G., Malmstrom, A., Bystrom, B., Endresen, U., Mlambo, N., Norman, M., Stabi, B., and Brauner, A. (2000). Human cervical ripening, an inflammatory process mediated by cytokines. Mol Hum Reprod *6*, 375-381.

Sexton, D.J., O'Reilly, M.W., Friel, A.M., and Morrison, J.J. (2004). Functional effects of 17alpha-hydroxyprogesterone caproate (17P) on human myometrial contractility in vitro. Reprod Biol Endocrinol *2*, 80.

Seyrek, M., Irkilata, H.C., Vural, I.M., Yildirim, I., Basal, S., Yildiz, O., and Dayanc, M. (2011). Testosterone relaxes human internal spermatic vein through potassium channel opening action. Urology *78*, 233.e231-235.

Sharifzadeh, F., Kashanian, M., and Fatemi, F. (2012). A comparison of serum androgens in pre-eclamptic and normotensive pregnant women during the third trimester of pregnancy. Gynecol Endocrinol *28*, 834-836.

Shimano, M., Nakaya, Y., Fukui, R., Kamada, M., Hamada, Y., Maeda, K., and Aono, T. (2000). Activation of Ca2+-activated K+ channels in human myometrium by nitric oxide. Gynecol Obstet Invest *49*, 249-254.

Shmigol, A., Eisner, D.A., and Wray, S. (1998). Carboxyeosin decreases the rate of decay of the [Ca2+]i transient in uterine smooth muscle cells isolated from pregnant rats. Pflugers Arch *437*, 158-160.

Shmygol, A., Noble, K., and Wray, S. (2007). Depletion of membrane cholesterol eliminates the Ca2+-activated component of outward potassium current and decreases membrane capacitance in rat uterine myocytes. J Physiol *581*, 445-456.

Shozu, M., Akasofu, K., Harada, T., and Kubota, Y. (1991). A new cause of female pseudohermaphroditism: placental aromatase deficiency. J Clin Endocrinol Metab *72*, 560-566.

Shynlova, O., Chow, M., and Lye, S.J. (2009a). Expression and organization of basement membranes and focal adhesion proteins in pregnant myometrium is regulated by uterine stretch. Reprod Sci *16*, 960-969.

Shynlova, O., Mitchell, J.A., Tsampalieros, A., Langille, B.L., and Lye, S.J. (2004). Progesterone and gravidity differentially regulate expression of extracellular matrix components in the pregnant rat myometrium. Biol Reprod *70*, 986-992.

Shynlova, O., Oldenhof, A., Dorogin, A., Xu, Q., Mu, J., Nashman, N., and Lye, S.J. (2006). Myometrial apoptosis: activation of the caspase cascade in the pregnant rat myometrium at midgestation. Biol Reprod *74*, 839-849.

Shynlova, O., Tsui, P., Dorogin, A., Langille, B.L., and Lye, S.J. (2007). Insulin-like growth factors and their binding proteins define specific phases of myometrial differentiation during pregnancy in the rat. Biol Reprod *76*, 571-578.

Shynlova, O., Tsui, P., Dorogin, A., and Lye, S.J. (2008). Monocyte chemoattractant protein-1 (CCL-2) integrates mechanical and endocrine signals that mediate term and preterm labor. J Immunol *181*, 1470-1479.

Shynlova, O., Tsui, P., Jaffer, S., and Lye, S.J. (2009b). Integration of endocrine and mechanical signals in the regulation of myometrial functions during pregnancy and labour. Eur J Obstet Gynecol Reprod Biol *144 Suppl 1*, S2-10.

Siiteri, P.K., and MacDonald, P.C. (1966). Placental estrogen biosynthesis during human pregnancy. J Clin Endocrinol Metab *26*, 751-761.

Siiteri, P.K., and Simberg, N.H. (1986). Changing concepts of active androgens in blood. Clin Endocrinol Metab *15*, 247-258.

Silver, R.M., Lohner, W.S., Daynes, R.A., Mitchell, M.D., and Branch, D.W. (1994). Lipopolysaccharide-induced fetal death: the role of tumor-necrosis factor alpha. Biol Reprod *50*, 1108-1112.

Simon, C., and Einspanier, A. (2009). The hormonal induction of cervical remodeling in the common marmoset monkey (Callithrix jacchus). Reproduction *137*, 517-525.

Simons, K., and Vaz, W.L. (2004). Model systems, lipid rafts, and cell membranes. Annu Rev Biophys Biomol Struct *33*, 269-295.

Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C.R., Michael, M.D*., et al.* (1994). Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev *15*, 342-355.

Sims, S.M., Daniel, E.E., and Garfield, R.E. (1982). Improved electrical coupling in uterine smooth muscle is associated with increased numbers of gap junctions at parturition. The Journal of general physiology *80*, 353-375.

Simsek, Y., Celen, S., Ustun, Y., Danisman, N., and Bayramoglu, H. (2012). Severe preeclampsia and fetal virilization in a spontaneous singleton pregnancy complicated by hyperreactio luteinalis. Eur Rev Med Pharmacol Sci *16*, 118-121.

Sinha-Hikim, I., Taylor, W.E., Gonzalez-Cadavid, N.F., Zheng, W., and Bhasin, S. (2004). Androgen receptor in human skeletal muscle and cultured muscle satellite cells: up-regulation by androgen treatment. J Clin Endocrinol Metab *89*, 5245-5255.

Sir-Petermann, T., Hitchsfeld, C., Maliqueo, M., Codner, E., Echiburu, B., Gazitua, R., Recabarren, S., and Cassorla, F. (2005). Birth weight in offspring of mothers with polycystic ovarian syndrome. Hum Reprod *20*, 2122-2126.

Sir-Petermann, T., Maliqueo, M., Angel, B., Lara, H.E., Perez-Bravo, F., and Recabarren, S.E. (2002). Maternal serum androgens in pregnant women with polycystic ovarian syndrome: possible implications in prenatal androgenization. Hum Reprod *17*, 2573-2579.

Slater, D.M., Dennes, W.J., Campa, J.S., Poston, L., and Bennett, P.R. (1999). Expression of cyclo-oxygenase types-1 and -2 in human myometrium throughout pregnancy. Mol Hum Reprod *5*, 880-884.

Slayden, O.D., Nayak, N.R., Burton, K.A., Chwalisz, K., Cameron, S.T., Critchley, H.O., Baird, D.T., and Brenner, R.M. (2001). Progesterone antagonists increase androgen receptor expression in the rhesus macaque and human endometrium. J Clin Endocrinol Metab *86*, 2668-2679.

Slomczynska, M., Duda, M., Burek, M., Knapczyk, K., Czaplicki, D., and Koziorowski, M. (2008). Distribution of androgen receptor in the porcine uterus throughout pregnancy. Reprod Domest Anim *43*, 35-41.

Smith, R. (2007). Parturition. N Engl J Med *356*, 271-283.

Smith, R., Mesiano, S., and McGrath, S. (2002). Hormone trajectories leading to human birth. Regulatory peptides *108*, 159-164.

Smith, R., Paul, J., Maiti, K., Tolosa, J., and Madsen, G. (2012). Recent advances in understanding the endocrinology of human birth. Trends Endocrinol Metab *23*, 516-523.

Smith, R.C., McClure, M.C., Smith, M.A., Abel, P.W., and Bradley, M.E. (2007). The role of voltage-gated potassium channels in the regulation of mouse uterine contractility. Reprod Biol Endocrinol *5*, 41.

Smith, R.D., Babiychuk, E.B., Noble, K., Draeger, A., and Wray, S. (2005). Increased cholesterol decreases uterine activity: functional effects of cholesterol alteration in pregnant rat myometrium. Am J Physiol Cell Physiol *288*, C982-988.

Soloff, M.S., Alexandrova, M., and Fernstrom, M.J. (1979). Oxytocin receptors: triggers for parturition and lactation? Science *204*, 1313-1315.

Soloff, M.S., Jeng, Y.J., Izban, M.G., Sinha, M., Luxon, B.A., Stamnes, S.J., and England, S.K. (2011). Effects of progesterone treatment on expression of genes involved in uterine quiescence. Reprod Sci *18*, 781-797.

Sooranna, S.R., Lee, Y., Kim, L.U., Mohan, A.R., Bennett, P.R., and Johnson, M.R. (2004). Mechanical stretch activates type 2 cyclooxygenase via activator protein-1 transcription factor in human myometrial cells. Mol Hum Reprod *10*, 109-113.

Sparey, C., Robson, S.C., Bailey, J., Lyall, F., and Europe-Finner, G.N. (1999). The differential expression of myometrial connexin-43, cyclooxygenase-1 and -2, and Gs alpha proteins in the upper and lower segments of the human uterus during pregnancy and labor. J Clin Endocrinol Metab *84*, 1705-1710.

Stocco, C., Telleria, C., and Gibori, G. (2007). The molecular control of corpus luteum formation, function, and regression. Endocr Rev *28*, 117-149.

Stouffer, R.L., Nixon, W.E., Gulyas, B.J., and Hodgen, G.D. (1977). Gonadotropin-sensitive progesterone production by rhesus monkey luteal cells in vitro: a function of age of the corpus luteum during the menstrual cycle. Endocrinology *100*, 506-512.

Strauss, J.F., 3rd, Martinez, F., and Kiriakidou, M. (1996). Placental steroid hormone synthesis: unique features and unanswered questions. Biol Reprod *54*, 303-311.

Sullivan, S.D., and Moenter, S.M. (2004). Prenatal androgens alter GABAergic drive to gonadotropin-releasing hormone neurons: implications for a common fertility disorder. Proc Natl Acad Sci U S A *101*, 7129-7134.

Sykes, L., MacIntyre, D.A., Teoh, T., and Bennett, P. (2014). Anti-inflammatory prostaglandins for the prevention of preterm labour. Reproduction.

Tagawa, N., Hidaka, Y., Takano, T., Shimaoka, Y., Kobayashi, Y., and Amino, N. (2004). Serum concentrations of dehydroepiandrosterone and dehydroepiandrosterone sulfate and their relation to cytokine production during and after normal pregnancy. Clin Chim Acta *340*, 187-193.

Takahashi, K., Ikeno, N., Hoshiai, H., and Suzuki, M. (1984). The effect of dehydroepiandrosterone sulfate on serum steroid concentration in singleton and twin pregnancies and its effect on cervical canal-ripening in singleton pregnancies. Tohoku J Exp Med *142*, 289-298.

Takahashi, M., Tajika, Y., Khairani, A.F., Ueno, H., Murakami, T., and Yorifuji, H. (2013). The localization of VAMP5 in skeletal and cardiac muscle. Histochemistry and cell biology *139*, 573-582.

Takayanagi, Y., Yoshida, M., Bielsky, I.F., Ross, H.E., Kawamata, M., Onaka, T., Yanagisawa, T., Kimura, T., Matzuk, M.M., Young, L.J.*, et al.* (2005). Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice. Proc Natl Acad Sci U S A *102*, 16096-16101.

Tattersall, M., Engineer, N., Khanjani, S., Sooranna, S.R., Roberts, V.H., Grigsby, P.L., Liang, Z., Myatt, L., and Johnson, M.R. (2008). Pro-labour myometrial gene expression: are preterm labour and term labour the same? Reproduction *135*, 569-579.

Teoh, H., and Man, R.Y. (2000). Enhanced relaxation of porcine coronary arteries after acute exposure to a physiological level of 17beta-estradiol involves non-genomic mechanisms and the cyclic AMP cascade. Br J Pharmacol *129*, 1739-1747.

Terzidou, V., Sooranna, S.R., Kim, L.U., Thornton, S., Bennett, P.R., and Johnson, M.R. (2005). Mechanical stretch up-regulates the human oxytocin receptor in primary human uterine myocytes. J Clin Endocrinol Metab *90*, 237-246.

Tezuka, N., Ali, M., Chwalisz, K., and Garfield, R.E. (1995). Changes in transcripts encoding calcium channel subunits of rat myometrium during pregnancy. Am J Physiol *269*, C1008-1017.

Thiery, M., De Gezelle, H., Van Kets, H., Voorhoof, L., Verheugen, C., Smis, B., Gerris, J., Derom, R., and Martens, G. (1979). The effect of locally administered estrogens on the human cervix. Z Geburtshilfe Perinatol *183*, 448-452.

Thomas, P., Dong, J., Berg, A.H., and Pang, Y. (2014). Identification and characterization of membrane androgen receptors in the ZIP9 zinc transporter subfamily: II. Role of human ZIP9 in testosterone-induced prostate and breast cancer cell apoptosis. Endocrinology, en20141201.

Thomas, P., Dressing, G., Pang, Y., Berg, H., Tubbs, C., Benninghoff, A., and Doughty, K. (2006). Progestin, estrogen and androgen G-protein coupled receptors in fish gonads. Steroids *71*, 310-316.

Thomas, P., Pang, Y., Filardo, E.J., and Dong, J. (2005). Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology *146*, 624-632.

Thomson, A.J., Telfer, J.F., Young, A., Campbell, S., Stewart, C.J., Cameron, I.T., Greer, I.A., and Norman, J.E. (1999). Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. Hum Reprod *14*, 229-236.

Thorneloe, K.S., and Nelson, M.T. (2005). Ion channels in smooth muscle: regulators of intracellular calcium and contractility. Can J Physiol Pharmacol *83*, 215-242.

Thornton, S., Davison, J.M., and Baylis, P.H. (1992). Plasma oxytocin during the first and second stages of spontaneous human labour. Acta Endocrinol (Copenh) *126*, 425-429.

Tica, A.A., Dun, E.C., Tica, O.S., Gao, X., Arterburn, J.B., Brailoiu, G.C., Oprea, T.I., and Brailoiu, E. (2011). G protein-coupled estrogen receptor 1-mediated effects in the rat myometrium. Am J Physiol Cell Physiol *301*, C1262-1269.

Tomiyasu, B.A., Chen, C.J., and Marshall, J.M. (1988). Comparison of the activity of circular and longitudinal myometrium from pregnant rats: co-ordination between muscle layers. Clin Exp Pharmacol Physiol *15*, 647-656.

Tong, D., Lu, X., Wang, H.X., Plante, I., Lui, E., Laird, D.W., Bai, D., and Kidder, G.M. (2009). A dominant loss-of-function GJA1 (Cx43) mutant impairs parturition in the mouse. Biol Reprod *80*, 1099-1106.

Townsend, E.A., Sathish, V., Thompson, M.A., Pabelick, C.M., and Prakash, Y.S. (2012). Estrogen effects on human airway smooth muscle involve cAMP and protein kinase A. Am J Physiol Lung Cell Mol Physiol *303*, L923-928.

Townsend, E.A., Thompson, M.A., Pabelick, C.M., and Prakash, Y.S. (2010). Rapid effects of estrogen on intracellular Ca2+ regulation in human airway smooth muscle. Am J Physiol Lung Cell Mol Physiol *298*, L521-530.

Traish, A.M., Kang, H.P., Saad, F., and Guay, A.T. (2011). Dehydroepiandrosterone (DHEA)--a precursor steroid or an active hormone in human physiology. J Sex Med *8*, 2960-2982; quiz 2983.

Tribe, R.M., Moriarty, P., Dalrymple, A., Hassoni, A.A., and Poston, L. (2003). Interleukin-1beta induces calcium transients and enhances basal and store operated calcium entry in human myometrial smooth muscle. Biol Reprod *68*, 1842-1849.

Tritthart, H.A., Mahnert, W., Fleischhacker, A., and Adelwohrer, N. (1991). Potassium channels and modulating factors of channel functions in the human myometrium. Zeitschrift fur Kardiologie *80 Suppl 7*, 29-33.

Tsai, M.L., Cesen-Cummings, K., Webb, R.C., and Loch-Caruso, R. (1998). Acute inhibition of spontaneous uterine contractions by an estrogenic polychlorinated biphenyl is associated with disruption of gap junctional communication. Toxicol Appl Pharmacol *152*, 18-29.

Tsai, M.L., Chang, C.C., Lee, C.L., and Huang, B.Y. (2003). The differential effects of tamoxifen and ICI 182,780 on the reduction of Na+/K+ ATPase activity and spontaneous oscillations by 17beta-estradiol. The Chinese journal of physiology *46*, 55-62.

Tulchinsky D, R.K. (1980). Maternal-Fetal Endocrinology. Philadelphia: WB Saunders.

Turi, A., Kiss, A.L., and Mullner, N. (2001). Estrogen downregulates the number of caveolae and the level of caveolin in uterine smooth muscle. Cell Biol Int *25*, 785-794.

Ugwumadu, A. (2010). Chorioamnionitis and mid-trimester pregnancy loss. Gynecol Obstet Invest *70*, 281-285.

Umezaki, H., Hess, D.L., Valenzuela, G.J., and Ducsay, C.A. (2001). Fetectomy alters maternal pituitary-adrenal function in pregnant rhesus macaques. Biol Reprod *65*, 1616-1621.

Unemoto, T., Matsushita, M., Tamura, K., Tanaka, Y., Koike, K., and Kogo, H. (2007). Role of BK channels in testosterone-induced relaxation of the aorta in spontaneously hypertensive rats. Biological & pharmaceutical bulletin *30*, 1477-1480.

Unlugedik, E., Alfaidy, N., Holloway, A., Lye, S., Bocking, A., Challis, J., and Gibb, W. (2010). Expression and regulation of prostaglandin receptors in the human placenta and fetal membranes at term and preterm. Reproduction, fertility, and development *22*, 796-807.

Van Bommel, T., Marsen, T., and Bojar, H. (1987). Effects of high-dose medroxyprogesterone acetate and various other steroid hormones on plasma membrane lipid mobility in CAMA-1 mammary cancer cells. Anticancer Res *7*, 1217-1223.

van der Kwast, T.H., Dommerholt, H.B., van Vroonhoven, C.C., and Chadha, S. (1994). Androgen receptor expression in the cervix of androgen-treated female-to-male transsexuals: association with morphology and chain-specific keratin expression. Int J Gynecol Pathol *13*, 133-138.

Van Holsbeke, C., Amant, F., Veldman, J., De Boodt, A., Moerman, P., and Timmerman, D. (2009). Hyperreactio luteinalis in a spontaneously conceived singleton pregnancy. Ultrasound Obstet Gynecol *33*, 371-373.

van Meer, G. (1989). Lipid traffic in animal cells. Annu Rev Cell Biol *5*, 247-275.

van Vliet, E.O., Boormans, E.M., de Lange, T.S., Mol, B.W., and Oudijk, M.A. (2014). Preterm labor: current pharmacotherapy options for tocolysis. Expert opinion on pharmacotherapy *15*, 787-797.

Vedernikov, Y.P., Hartke, J.R., de Long, M.A., Saade, G.R., and Garfield, R.E. (2003). Sex hormone effects in non-pregnant rat and human myometrium. Eur J Obstet Gynecol Reprod Biol *108*, 59-66.

Vejrazkova, D., Vcelak, J., Vankova, M., Lukasova, P., Bradnova, O., Halkova, T., Kancheva, R., and Bendlova, B. (2014). Steroids and insulin resistance in pregnancy. J Steroid Biochem Mol Biol *139*, 122-129.

Veleminsky, M., Jr. (2010). Hyperreactio luteinalis in spontaneous singleton pregnancy. Neuro Endocrinol Lett *31*, 304-305.

Verbist, J., Gadella, T.W., Jr., Raeymaekers, L., Wuytack, F., Wirtz, K.W., and Casteels, R. (1991). Phosphoinositide-protein interactions of the plasma-membrane Ca2(+)-transport ATPase as revealed by fluorescence energy transfer. Biochim Biophys Acta *1063*, 1-6.

Vermeirsch, H., Van den Broeck, W., Coryn, M., and Simoens, P. (2002). Immunohistochemical detection of androgen receptors in the canine uterus throughout the estrus cycle. Theriogenology *57*, 2203-2216.

Vermeulen, A., and Ando, S. (1979). Metabolic clearance rate and interconversion of androgens and the influence of the free androgen fraction. J Clin Endocrinol Metab *48*, 320-326.

Vignozzi, L., Cellai, I., Santi, R., Lombardelli, L., Morelli, A., Comeglio, P., Filippi, S., Logiodice, F., Carini, M., Nesi, G.*, et al.* (2012). Antiinflammatory effect of androgen receptor activation in human benign prostatic hyperplasia cells. J Endocrinol *214*, 31-43.

Vladic-Stjernholm, Y., Vladic, T., Blesson, C.S., Ekman-Ordeberg, G., and Sahlin, L. (2009). Prostaglandin treatment is associated with a withdrawal of progesterone and androgen at the receptor level in the uterine cervix. Reprod Biol Endocrinol *7*, 116.

Vogel, C., and Marcotte, E.M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nature reviews Genetics *13*, 227-232.

Waite, S.L., Gandhi, S.V., Khan, R.N., and Chapman, N.R. (2014). The effect of trichostatin-A and tumor necrosis factor on expression of splice variants of the MaxiK and L-type channels in human myometrium. Frontiers in physiology *5*, 261.

Walters, K.A., Allan, C.M., and Handelsman, D.J. (2008). Androgen actions and the ovary. Biol Reprod *78*, 380-389.

Walters, K.A., McTavish, K.J., Seneviratne, M.G., Jimenez, M., McMahon, A.C., Allan, C.M., Salamonsen, L.A., and Handelsman, D.J. (2009). Subfertile female androgen receptor knockout mice exhibit defects in neuroendocrine signaling, intraovarian function, and uterine development but not uterine function. Endocrinology *150*, 3274-3282.

Wang, H., and Hirsch, E. (2003). Bacterially-induced preterm labor and regulation of prostaglandin-metabolizing enzyme expression in mice: the role of toll-like receptor 4. Biol Reprod *69*, 1957-1963.

Weber, D.S., Taniyama, Y., Rocic, P., Seshiah, P.N., Dechert, M.A., Gerthoffer, W.T., and Griendling, K.K. (2004). Phosphoinositide-dependent kinase 1 and p21-activated protein kinase mediate reactive oxygen species-dependent regulation of platelet-derived growth factor-induced smooth muscle cell migration. Circ Res *94*, 1219-1226.

Webley, G.E., and Hearn, J.P. (1987). Local production of progesterone by the corpus luteum of the marmoset monkey in response to perfusion with chorionic gonadotrophin and melatonin in vivo. J Endocrinol *112*, 449-457.

Welsh, M., Saunders, P.T., Fisken, M., Scott, H.M., Hutchison, G.R., Smith, L.B., and Sharpe, R.M. (2008). Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. J Clin Invest *118*, 1479-1490.

Welsh, T., Johnson, M., Yi, L., Tan, H., Rahman, R., Merlino, A., Zakar, T., and Mesiano, S. (2012). Estrogen receptor (ER) expression and function in the pregnant human myometrium: estradiol via ERalpha activates ERK1/2 signaling in term myometrium. J Endocrinol *212*, 227-238.

Wendremaire, M., Mourtialon, P., Goirand, F., Lirussi, F., Barrichon, M., Hadi, T., Garrido, C., Le Ray, I., Dumas, M., Sagot, P.*, et al.* (2013). Effects of leptin on lipopolysaccharide-induced remodeling in an in vitro model of human myometrial inflammation. Biol Reprod *88*, 45.

Wilcox, B.D., Rydelek-Fitzgerald, L., and Jeffrey, J.J. (1992). Regulation of collagenase gene expression by serotonin and progesterone in rat uterine smooth muscle cells. J Biol Chem *267*, 20752-20757.

Wilke, T.J., and Utley, D.J. (1987). Total testosterone, free-androgen index, calculated free testosterone, and free testosterone by analog RIA compared in hirsute women and in otherwise-normal women with altered binding of sex-hormone-binding globulin. Clin Chem *33*, 1372-1375.

Williams, K.C., Renthal, N.E., Condon, J.C., Gerard, R.D., and Mendelson, C.R. (2012a). MicroRNA-200a serves a key role in the decline of progesterone receptor function leading to term and preterm labor. Proc Natl Acad Sci U S A *109*, 7529-7534.

Williams, K.C., Renthal, N.E., Gerard, R.D., and Mendelson, C.R. (2012b). The microRNA (miR)-199a/214 cluster mediates opposing effects of progesterone and estrogen on uterine contractility during pregnancy and labor. Mol Endocrinol *26*, 1857-1867.

Willows, R.L. (1966). Urinary estriol estimations in normal and abnormal pregnancies. Can Med Assoc J *94*, 1098-1101.

Wilson, C.M., and McPhaul, M.J. (1996). A and B forms of the androgen receptor are expressed in a variety of human tissues. Mol Cell Endocrinol *120*, 51-57.

Winkler, M., Kemp, B., Classen-Linke, I., Fischer, D.C., Zlatinsi, S., Neulen, J., Beier, H.M., and Rath, W. (2002). Estrogen receptor alpha and progesterone receptor A and B concentration and localization in the lower uterine segment in term parturition. J Soc Gynecol Investig *9*, 226-232.

Wirth, A., Schroeter, M., Kock-Hauser, C., Manser, E., Chalovich, J.M., De Lanerolle, P., and Pfitzer, G. (2003). Inhibition of contraction and myosin light chain phosphorylation in guinea-pig smooth muscle by p21-activated kinase 1. J Physiol *549*, 489-500.

Witayavanitkul, N., Woranush, W., Bupha-Intr, T., and Wattanapermpool, J. (2013). Testosterone regulates cardiac contractile activation by modulating SERCA but not NCX activity. Am J Physiol Heart Circ Physiol *304*, H465-472.

Witham, E.A., Meadows, J.D., Shojaei, S., Kauffman, A.S., and Mellon, P.L. (2012). Prenatal exposure to low levels of androgen accelerates female puberty onset and reproductive senescence in mice. Endocrinology *153*, 4522-4532.

Wolinsky, H. (1972). Effects of androgen treatment on the male rat aorta. J Clin Invest *51*, 2552-2555.

Wood, S., Ross, S., Tang, S., Miller, L., Sauve, R., and Brant, R. (2012). Vaginal progesterone to prevent preterm birth in multiple pregnancy: a randomized controlled trial. J Perinat Med.

Woolfolk, E.A., Eguchi, S., Ohtsu, H., Nakashima, H., Ueno, H., Gerthoffer, W.T., and Motley, E.D. (2005). Angiotensin II-induced activation of p21-activated kinase 1 requires Ca2+ and protein kinase C{delta} in vascular smooth muscle cells. Am J Physiol Cell Physiol *289*, C1286-1294.

Word, R.A., Li, X.H., Hnat, M., and Carrick, K. (2007). Dynamics of cervical remodeling during pregnancy and parturition: mechanisms and current concepts. Semin Reprod Med *25*, 69-79.

Wray, S., Burdyga, T., and Noble, K. (2005). Calcium signalling in smooth muscle. Cell Calcium *38*, 397-407.

Wray, S., Jones, K., Kupittayanant, S., Li, Y., Matthew, A., Monir-Bishty, E., Noble, K., Pierce, S.J., Quenby, S., and Shmygol, A.V. (2003). Calcium signaling and uterine contractility. J Soc Gynecol Investig *10*, 252-264.

Wray, S., Kupittayanant, S., Shmygol, A., Smith, R.D., and Burdyga, T. (2001). The physiological basis of uterine contractility: a short review. Experimental physiology *86*, 239-246.

Wu, W., Shi, S.Q., Huang, H.J., Balducci, J., and Garfield, R.E. (2011). Changes in PGRMC1, a potential progesterone receptor, in human myometrium during pregnancy and labour at term and preterm. Mol Hum Reprod *17*, 233-242.

Wu, W.X., Zhang, Q., Unno, N., Derks, J.B., and Nathanielsz, P.W. (2000). Characterization of decorin mRNA in pregnant intrauterine tissues of the ewe and regulation by steroids. Am J Physiol Cell Physiol *278*, C199-206.

Wu, X.Y., Li, Z.L., Wu, C.Y., Liu, Y.M., Lin, H., Wang, S.H., and Xiao, W.F. (2010). Endocrine traits of polycystic ovary syndrome in prenatally androgenized female Sprague-Dawley rats. Endocrine journal *57*, 201-209.

Xu, C., Gao, L., You, X., Dai, L., Li, Y., Gu, H., Slater, D.M., Olson, D.M., and Ni, X. (2011). CRH acts on CRH-R1 and -R2 to differentially modulate the expression of large-conductance calcium-activated potassium channels in human pregnant myometrium. Endocrinology *152*, 4406-4417.

Xu, C., Graf, L.F., Fazli, L., Coleman, I.M., Mauldin, D.E., Li, D., Nelson, P.S., Gleave, M., Plymate, S.R., Cox, M.E*., et al.* (2007). Regulation of global gene expression in the bone marrow microenvironment by androgen: androgen ablation increases insulin-like growth factor binding protein-5 expression. The Prostate *67*, 1621-1629.

Yamamoto, M., Feigenbaum, S.L., Crites, Y., Escobar, G.J., Yang, J., Ferrara, A., and Lo, J.C. (2012). Risk of preterm delivery in non-diabetic women with polycystic ovarian syndrome. J Perinatol *32*, 770-776.

Yamashita, A., Oshima, S., Matsuo, K., Ito, K., Ito, A., and Mori, Y. (1991). [Pharmacological studies of intravaginally applied dehydroepiandrosterone sulfate (DHA-S)]. Nihon Yakurigaku Zasshi *98*, 31-39.

Yan, X., Dai, X., Wang, J., Zhao, N., Cui, Y., and Liu, J. (2013). Prenatal androgen excess programs metabolic derangements in pubertal female rats. J Endocrinol *217*, 119-129.

Yang, E., Jeon, S.B., Baek, I., Chen, Z.A., Jin, Z., and Kim, I.K. (2009). 17beta-estradiol attenuates vascular contraction through inhibition of RhoA/Rho kinase pathway. Naunyn-Schmiedeberg's archives of pharmacology *380*, 35-44.

Yang, X., Guo, Z., Sun, F., Li, W., Alfano, A., Shimelis, H., Chen, M., Brodie, A.M., Chen, H., Xiao, Z*., et al.* (2011). Novel membrane-associated androgen receptor splice variant potentiates proliferative and survival responses in prostate cancer cells. J Biol Chem *286*, 36152-36160.

Yellon, S.M., Dobyns, A.E., Beck, H.L., Kurtzman, J.T., Garfield, R.E., and Kirby, M.A. (2013). Loss of progesterone receptor-mediated actions induce preterm cellular and structural remodeling of the cervix and premature birth. PLoS One *8*, e81340.

Yin, Z., Sada, A.A., Reslan, O.M., Narula, N., and Khalil, R.A. (2012). Increased MMPs expression and decreased contraction in the rat myometrium during pregnancy and in response to prolonged stretch and sex hormones. Am J Physiol Endocrinol Metab *303*, E55-70.

York, T.P., Strauss, J.F., 3rd, Neale, M.C., and Eaves, L.J. (2009). Estimating fetal and maternal genetic contributions to premature birth from multiparous pregnancy histories of twins using MCMC and maximum-likelihood approaches. Twin research and human genetics : the official journal of the International Society for Twin Studies *12*, 333-342.

Yoshimura, K., and Hirsch, E. (2005). Effect of stimulation and antagonism of interleukin-1 signaling on preterm delivery in mice. J Soc Gynecol Investig *12*, 533-538.

Yoshizawa, A., and Ogikubo, S. (2006). IGF binding protein-5 synthesis is regulated by testosterone through transcriptional mechanisms in androgen responsive cells. Endocrine journal *53*, 811-818.

Young, A., Thomson, A.J., Ledingham, M., Jordan, F., Greer, I.A., and Norman, J.E. (2002). Immunolocalization of proinflammatory cytokines in myometrium, cervix, and fetal membranes during human parturition at term. Biol Reprod *66*, 445-449.

Young, R.C., and Hession, R.O. (1999). Three-dimensional structure of the smooth muscle in the term-pregnant human uterus. Obstet Gynecol *93*, 94-99.

Young, R.C., and Zhang, P. (2005). Inhibition of in vitro contractions of human myometrium by mibefradil, a T-type calcium channel blocker: support for a model using excitation-contraction coupling, and autocrine and paracrine signaling mechanisms. J Soc Gynecol Investig *12*, e7-12.

Young, W.S., 3rd, Shepard, E., Amico, J., Hennighausen, L., Wagner, K.U., LaMarca, M.E., McKinney, C., and Ginns, E.I. (1996). Deficiency in mouse oxytocin prevents milk ejection, but not fertility or parturition. J Neuroendocrinol *8*, 847-853.

Zarazua, A., Guerra, Oropeza, Campos M (2011). Expression And Regulation Of Estrogen, Progesterone And Androgen Receptors In Airway Smooth Muscle Cells In An Allergic Asthma Model. Am J Respir Crit Care Med *183*.

Zeitlin, J., Saurel-Cubizolles, M.J., De Mouzon, J., Rivera, L., Ancel, P.Y., Blondel, B., and Kaminski, M. (2002). Fetal sex and preterm birth: are males at greater risk? Hum Reprod *17*, 2762-2768.

Zhang, J., Bricker, L., Wray, S., and Quenby, S. (2007). Poor uterine contractility in obese women. BJOG *114*, 343-348.

Zhu, Y., Rice, C.D., Pang, Y., Pace, M., and Thomas, P. (2003). Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci U S A *100*, 2231-2236.

Ziyan, J., Huaibin, R., Xiaotian, M., Guangtong, S., Xiaoqing, C., Zijiang, D., Ziyue, J., We, D., and Lizhou, S. (2010). Regulation of progesterone receptor A and B expression in human preterm, term, and postterm placental villi. Acta Obstet Gynecol Scand *89*, 705-711.

Zuloaga, K.L., Swift, S.N., Gonzales, R.J., Wu, T.J., and Handa, R.J. (2012). The androgen metabolite, 5alpha-androstane-3beta,17beta-diol, decreases cytokine-induced cyclooxygenase-2, vascular cell adhesion molecule-1 expression, and P-glycoprotein expression in male human brain microvascular endothelial cells. Endocrinology *153*, 5949-5960.

# Appendices

## Appendix 1

**Table 8.1:** List of chemicals and suppliers

| Chemical | Supplier |
|---|---|
| NaCl | 746398 Sigma |
| KCl | P9541 Sigma |
| $MgCl_2$ | M-8266 Sigma |
| $NaH_2PO_4$ | S-3139 Sigma |
| $Na_2SO_4$ | 239313 Sigma |
| $CaCl_2$ | 22313,294 VWR PROLABO |
| $NaHCO_3$ | 10247 AnalaR BDH |
| Glucose | G8270 Sigma |
| Glycine | 410225 Sigma |
| Methanol | M/4000/17 Fisher Chemicals |
| Ethanol | 20821 VWR PROLABO |
| Citrate acid | C-8532 Sigma |
| Acetic acid | A6283 Sigma |
| DMSO | D2438 Sigma |
| Tween-20 | H5152 Promega |
| TBS tablets | T6664 Sigma |
| PBS tablets | S24650 Sigma |
| Xylene | X/0200/17 Fisher Chemicals |

## Appendix 2

**Table 8.2:** List of genes profiled with the Human Androgen Receptor Signalling Targets RT² Profiler PCR Array

| Symbol | Description |
|---|---|
| ABCC4 | ATP-binding cassette, sub-family C (CFTR/MRP), member 4 |
| ABHD2 | Abhydrolase domain containing 2 |
| ACSL3 | Acyl-CoA synthetase long-chain family member 3 |
| ADAMTS1 | ADAM metallopeptidase with thrombospondin type 1 motif, 1 |
| ALDH1A3 | Aldehyde dehydrogenase 1 family, member A3 |
| APPBP2 | Amyloid beta precursor protein (cytoplasmic tail) binding protein 2 |
| AR | Androgen receptor |
| CAMKK2 | Calcium/calmodulin-dependent protein kinase kinase 2, beta |
| CENPN | Centromere protein N |
| CITED2 | Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 |
| ACKR3 | Chemokine (C-X-C motif) receptor 7 |
| CYP2U1 | Cytochrome P450, family 2, subfamily U, polypeptide 1 |
| DBI | Diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein) |
| DHCR24 | 24-dehydrocholesterol reductase |
| EAF2 | ELL associated factor 2 |
| ELK1 | ELK1, member of ETS oncogene family |
| ELL2 | Elongation factor, RNA polymerase II, 2 |

| | |
|---|---|
| ENDOD1 | Endonuclease domain containing 1 |
| ERRFI1 | ERBB receptor feedback inhibitor 1 |
| FAM105A | Family with sequence similarity 105, member A |
| FKBP5 | FK506 binding protein 5 |
| FOS | FBJ murine osteosarcoma viral oncogene homolog |
| FZD5 | Frizzled family receptor 5 |
| GUCY1A3 | Guanylate cyclase 1, soluble, alpha 3 |
| HERC3 | Hect domain and RLD 3 |
| HPGD | Hydroxyprostaglandin dehydrogenase 15-(NAD) |
| IGF1R | Insulin-like growth factor 1 receptor |
| IGFBP5 | Insulin-like growth factor binding protein 5 |
| IRS2 | Insulin receptor substrate 2 |
| JUN | Jun proto-oncogene |
| KLK2 | Kallikrein-related peptidase 2 |
| KLK3 | Kallikrein-related peptidase 3 |
| KLK4 | Kallikrein-related peptidase 4 |
| KRT8 | Keratin 8 |
| LIFR | Leukemia inhibitory factor receptor alpha |
| LRIG1 | Leucine-rich repeats and immunoglobulin-like domains 1 |
| LRRFIP2 | Leucine rich repeat (in FLII) interacting protein 2 |
| MAF | V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian) |
| MAP7D1 | MAP7 domain containing 1 |
| MME | Membrane metallo-endopeptidase |
| MT2A | Metallothionein 2A |
| MYC | V-myc myelocytomatosis viral oncogene homolog (avian) |
| NCAPD3 | Non-SMC condensin II complex, subunit D3 |
| NDRG1 | N-myc downstream regulated 1 |
| NFKB1 | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 |
| NFKB2 | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100) |
| NFKBIA | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha |
| NKX3-1 | NK3 homeobox 1 |
| ORM1 | Orosomucoid 1 |
| ORM2 | Orosomucoid 2 |
| PAK1IP1 | PAK1 interacting protein 1 |
| PGC | Progastricsin (pepsinogen C) |
| PIAS1 | Protein inhibitor of activated STAT, 1 |
| PIK3R3 | Phosphoinositide-3-kinase, regulatory subunit 3 (gamma) |
| PMEPA1 | Prostate transmembrane protein, androgen induced 1 |
| PPAP2A | Phosphatidic acid phosphatase type 2A |
| RAB4A | RAB4A, member RAS oncogene family |
| REL | V-rel reticuloendotheliosis viral oncogene homolog (avian) |
| RELA | V-rel reticuloendotheliosis viral oncogene homolog A (avian) |
| RHOU | Ras homolog gene family, member U |
| SEC22C | SEC22 vesicle trafficking protein homolog C (S. cerevisiae) |
| SGK1 | Serum/glucocorticoid regulated kinase 1 |
| SLC26A2 | Solute carrier family 26 (sulfate transporter), member 2 |
| SLC45A3 | Solute carrier family 45, member 3 |
| SMS | Spermine synthase |
| SNAI2 | Snail homolog 2 (Drosophila) |
| SORD | Sorbitol dehydrogenase |
| SP1 | Sp1 transcription factor |

| | |
|---|---|
| SPDEF | SAM pointed domain containing ets transcription factor |
| SRF | Serum response factor (c-fos serum response element-binding transcription factor) |
| STEAP4 | STEAP family member 4 |
| STK39 | Serine threonine kinase 39 |
| TIPARP | TCDD-inducible poly(ADP-ribose) polymerase |
| TMPRSS2 | Transmembrane protease, serine 2 |
| TPD52 | Tumor protein D52 |
| TRIB1 | Tribbles homolog 1 (Drosophila) |
| TSC22D1 | TSC22 domain family, member 1 |
| TSC22D3 | TSC22 domain family, member 3 |
| VAPA | VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa |
| VIPR1 | Vasoactive intestinal peptide receptor 1 |
| WIPI1 | WD repeat domain, phosphoinositide interacting 1 |
| ZBTB10 | Zinc finger and BTB domain containing 10 |
| ZBTB16 | Zinc finger and BTB domain containing 16 |
| ZNF189 | Zinc finger protein 189 |

# Appendix 3

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human reproduction update

## Androgens in pregnancy: roles in parturition

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  - The role of androgens in myometrial function
- Conclusions

**BACKGROUND:** Understanding the physiology of pregnancy enables effective management of pregnancy complications that could otherwise be life threatening for both mother and fetus. A functional uterus (i) retains the fetus *in utero* during pregnancy without initiating stretch-induced contractions and (ii) is able to dilate the cervix and contract the myometrium at term to deliver the fetus. The onset of labour is associated with successful cervical remodelling and contraction of myometrium, arising from concomitant activation of uterine immune and endocrine systems. A large body of evidence suggests that actions of local steroid hormones may drive changes occurring in the uterine microenvironment at term. Although there have been a number of studies considering the potential role(s) played by progesterone and estrogen at the time of parturition, the bio-availability and effects of androgens during pregnancy have received less scrutiny. The aim of this review is to highlight potential roles of androgens in the biology of pregnancy and parturition.

**METHODS:** A review of published literature was performed to address (i) androgen concentrations, including biosynthesis and clearance, in maternal and fetal compartments throughout gestation, (ii) associations of androgen concentrations with adverse pregnancy outcomes, (iii) the role of androgens in the physiology of cervical remodelling and finally (iv) the role of androgens in the physiology of myometrial function including any impact on contractility.

**RESULTS:** Some, but not all, androgens increase throughout gestation in maternal circulation. The effects of this increase are not fully understood; however, evidence suggests that increased androgens might regulate key processes during pregnancy and parturition. For example, androgens are believed to be critical for cervical remodelling at term, in particular cervical ripening, via regulation of cervical collagen fibril organization. Additionally, a number of studies highlight potential roles for androgens in myometrial relaxation via non-genomic, AR-independent pathways critical for the pregnancy reaching term. Understanding of the molecular events leading to myometrial relaxation is an important step towards development of novel targeted tocolytic drugs.

**CONCLUSIONS:** The increase in androgen levels throughout gestation is likely to be important for establishment and maintenance of pregnancy and initiation of parturition. Further investigation of the underlying mechanisms of androgen action on cervical remodelling and myometrial

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contractility is needed. The insights gained may facilitate the development of new therapeutic approaches to manage pregnancy complications such as preterm birth.

**Key words:** androgen / pregnancy / labour / cervix / myometrium

## Introduction

Preterm birth (PTB), the outcome of preterm labour (PTL), is a major cause of maternal and perinatal morbidity and mortality, occurring in ~11% of recorded live births worldwide ranging from 5% in developed countries to 18% in developing countries (Blencowe *et al.*, 2012). The exact cause of PTL is unknown, although the concomitant activation of endocrine and immune pathways are believe to drive events, resulting in spontaneous initiation of uterine contractions and cervical ripening (CR) (Norman *et al.*, 2007; Romero *et al.*, 2007; Smith *et al.*, 2012). There is no fully effective agent for prevention of PTL. Progesterone (P4) to maintain uterine quiescence, and cervical cerclage to prevent premature CR have some efficacy in terms of reducing PTB, but there is little evidence of long-term beneficial effects for the baby (Alfirevic *et al.*, 2012; Likis *et al.*, 2012). Similarly, short term, but not long-term, benefits were demonstrated in a recent network analysis of tocolytics (Haas *et al.*, 2012). Thus, new strategies are needed.

Sex hormone steroids, including androgens, increase with normal pregnancy. The role of androgens in female physiology has been an active area of investigation for several decades. The major androgens synthesized in women are dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAS), androstenedione (A4), testosterone (T) and dihydrotestosterone (DHT) (Burger, 2002; Labrie *et al.*, 2011; Rothman *et al.*, 2011) (Fig. 1). Although their impact on women's health has been investigated, particularly in the context of polycystic ovarian disease, the role they play during pregnancy remains less well described. A number of studies have explored androgenic actions in systems critical for pregnancy reaching term, and further understanding of their roles will facilitate management of potentially life-threatening complications for the mother and the fetus.

Such key systems are cervical remodelling and myometrial contractility. In this review we discuss (i) the biosynthesis, clearance and availability of androgens during pregnancy, (ii) the associations of androgens with adverse pregnancy outcomes, (iii) the role of androgens in physiology of cervical remodelling and (iv) the roles of androgens in the physiology of the myometrial function with a focus on the crosstalk between androgens and myometrial contractile machinery.

## Methods

### Search strategy

We searched Pubmed using the keywords 'androgens' and 'pregnancy' to conduct to our knowledge the first narrative review decribing what is known about androgens during pregnancy. The initial Pubmed search identified 5976 manuscripts. Although the focus of this report was manuscripts

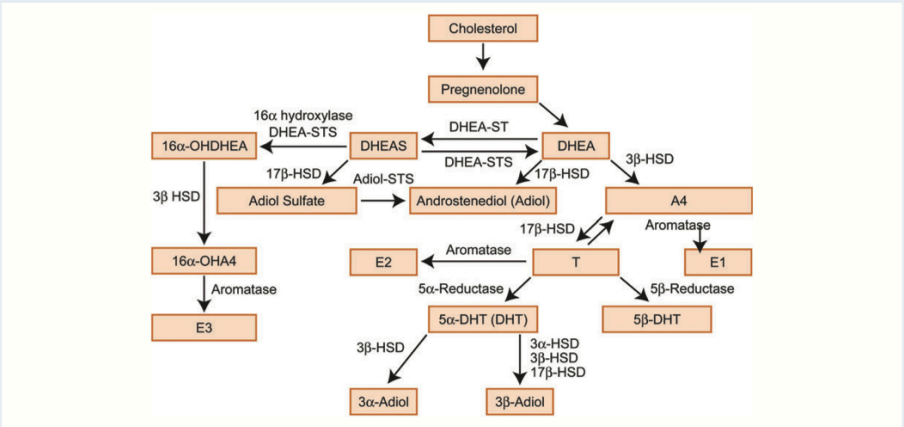


**Figure 1** Testosterone metabolism. DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate, A4, androstenedione; T, testosterone; E1, estrone; E2, estradiol; E3, estriol; 3α-HSD, 3-α-hydroxysteroid dehydrogenase/Δ-5-4 isomerase; 3β-HSD, 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3α-Adiol, 5α-androstane-3α, 17β-diol; 3β-Adiol, 5α- androstane-3β,17β-diol; DHT, dihydrotestosterone; STS, sulphatase; ST, sulfotransferase; 16α-OH, 16α-hydroxy.

published between 1980 and 2013, seminal references from 1930 to 1980 were included. For a study to be included in this review, it had to focus on potential roles for androgens in adverse pregnancy outcomes and processes involved in parturition, such as cervical remodelling and myometrial contractility. In addition, studies on androgen metabolism and papers documenting levels of androgens during pregnancy were included. Reports on the role of androgens on fetal gonadal development, fetal lung development and fetal immune system were not included. Following these inclusion and exclusion criteria, ~500 studies were judged as relevant and used to form the basis of this review.

# Results

## Androgen biology during pregnancy

Herein we introduce androgens in female physiology focusing on androgen biosynthesis and androgen metabolic clearance at the time of pregnancy. Concentrations of androgens in the feto-maternal unit throughout gestation are reviewed. Afterwards, we highlight studies correlating androgen levels in the feto-maternal unit with adverse pregnancy outcomes.

### *Androgens in female physiology*

Androgens are critically important for the development of the male reproductive tract during fetal life and the maintenance of male secondary sexual characteristics in adulthood (Scott *et al.*, 2007; Welsh *et al.*, 2008; Macleod *et al.*, 2010). Androgens can also act as pro-hormones for biosynthesis of estrogens in both sexes (Rivas *et al.*, 2002; Traish *et al.*, 2011; Purohit and Foster, 2012). In women, androgens are synthesized by cells within the ovaries, the adrenal glands, and also in fat, acting in an endocrine, paracrine or intracrine manner. In addition to playing key roles in the regulation of reproductive tissues such as the ovary and endometrium, androgens have a functional impact on the liver, kidney, bone and muscle (Brenner *et al.*, 2003; Walters *et al.*, 2008; Sen and Hammes, 2010). An excess production of androgens in women, for example in association with conditions such as polycystic ovarian syndrome (PCOS), can result in acne, hirsutism and infertility as well as to an increase in the risk of other complications, such as high blood pressure, cardiovascular disease and type 2 diabetes mellitus (Rosmond, 2006; Dewailly, 2010; Escobar-Morreale *et al.*, 2012). Since the 1960s, it has been generally hypothesized that, during pregnancy, androgens act as substrates for estrogen formation in the placenta (Pion *et al.*, 1965; Siiteri and MacDonald, 1966; Edman *et al.*, 1981; Smith, 2007). According to this hypothesis, DHEAS, produced by the fetal adrenals, and to a smaller extent from the maternal adrenals, enters the placenta, where it is metabolized to A4 and T, which is then converted into E1 (estrone) (Strauss *et al.*, 1996). E1 is in turn converted into E2 (estradiol), which enters the fetal circulation where it is taken up by the liver and converted into E3 (estriol) (Hirano, 1961; Schwarzel *et al.*, 1973). E3 can then pass to maternal circulation and be cleared in maternal urine (Willows, 1966). Androgen biosynthesis during pregnancy is extensively discussed later in this review and shown in Fig. 1.

### *Androgen-dependent signalling*

The importance of the androgen receptor (AR) in mediating the effects of androgens is well documented. For example, mutations or deletions in the *AR* gene located on the X chromosome are associated in both man and mouse with failure to masculinize. This results in 'testicular feminization', a condition where a male with a 46XY karyotype is phenotypically female (Lubahn *et al.*, 1988; Charest *et al.*, 1991; Hughes *et al.*, 2012). The AR is a member of a superfamily of ligand-activated transcription factors; only T and DHT bind AR with high specificity and affinity (Pereira de Jesus-Tran *et al.*, 2006). Ligand binding within the cytoplasm, where the classic AR resides, results in a conformational change in receptor protein, dimerization, nuclear translocation, association with co-factors and ultimately interaction with specific regions of the genome known as androgen response elements (Denayer *et al.*, 2010). The net result of these changes is an up- or down-regulation in gene expression. Such a response is termed 'genomic' as it involves gene transcription. In addition to their genomic effects, androgens are also able to exert their effects in a 'non-genomic' manner, independent of gene transcription or protein synthesis (Lang *et al.*, 2013). Non-genomic effects of androgens involve interaction with the cell membrane via a number of signalling molecules, including membrane receptors, ion channels or enzyme-linked receptors and cytoplasmic regulatory proteins (Foradori *et al.*, 2008). These non-genomic effects are rapid and can be observed within seconds, in contrast to genomic effects, which may take hours or even days.

### *Androgen biosynthesis*

The biosynthetic conversion of cholesterol (Ch) to androgens involves several steps and enzymes. A fundamental step in this process is the synthesis of pregnenolone. Only certain cell types in humans are capable of pregnenolone manufacture, including testicular Leydig cells, ovarian theca and corpus luteum cells, placental trophoblast cells, adrenal cortex cells and some specific cells in the brain (reviewed in Ghayee and Auchus, 2007). Pregnenolone is metabolized to DHEA, which is further metabolized to A4, the precursor of T. T can be reduced to a number of androgens, such as 5α-DHT (DHT), 5β-DHT, 5β-androstandiol, 3α-Adiol, 3β-Adiol or further metabolized to estrogens. The reduction of T to DHT and the conversion of T to estrogens is catalysed by the metabolising enzymes 5α-reductase and aromatase (CYP19), respectively (Fig. 1). Androgen biosynthesis has been extensively reviewed by Penning (2010).

In the non-pregnant premenopausal woman, ~50% of all DHEA is secreted by the adrenal glands, 20% from the ovaries and 30% from the peripheral tissues (Abraham, 1974). In contrast, the adrenals and ovaries produce equal amounts of A4 (Longcope, 1986). Regarding T, ~50% is equally synthesized in the ovaries and adrenals and the other half is produced from A4 in the peripheral tissues (Piltonen *et al.*, 2002). Finally, DHT is not secreted by the endocrine glands, and this androgen is synthesized from T in the peripheral androgen target tissues (Ito and Horton, 1971; Marchetti and Barth, 2013). During pregnancy, an additional source of androgens is the fetus and the placenta. In particular, the developing fetus is the source of placental 16α-hydroxy-DHEA, a product of 16α-hydroxylation of DHEAS in the fetal liver (Cantineau *et al.*, 1985). In a similar way to its non-16α-hydroxylated form, 16α-hydroxy-DHEA can be metabolized to 16α-hydroxy-A4. The latter is the predominant source of E3 (Milewich *et al.*, 1986) (Fig. 1).

Once synthesized, androgens enter the maternal circulation, where their levels can be detected. Usually T levels are reported as either total (tT) or 'free' (fT) with an important distinction between them; the latter are unbound to plasma proteins, such as sex-hormone-binding globulin (SHBG), and therefore able to pass freely across plasma membranes to interact with AR, whereas bound T is unable to penetrate

the cell membrane (Mendel, 1989). The percentage binding of SHBG is greater for DHT (59%) than for T (44%) and E2 (20%) and is very low for A4, DHEA, E1 and E3 (Avvakumov *et al.*, 2010). The affinity of each androgen for SHBG is dependent on their structural characteristics. For example, a 17β-OH group and a 5α-hydrogen moeity increase the affinity for SHBG binding, whereas androgens with either a double bond in ring A or an aromatic ring A are less SHBG potent (Siiteri and Simberg, 1986). Therefore, DHT, which has both the 17β-OH group and a 5α-hydrogen atom, has 2-fold greater affinity for SHBG than T, which has a double bond in ring A (Siiteri and Simberg, 1986).

### *Concentrations of androgens during pregnancy*

A number of studies have reported elevated levels of some, but not all, circulating androgens during normal pregnancy (Mizuno *et al.*, 1968; Rivarola *et al.*, 1968; Saez *et al.*, 1972; Dawood and Saxena, 1977; Buster *et al.*, 1979; Bammann *et al.*, 1980).

A notable increase in tT is observed from the first trimester of pregnancy and further elevations are reported towards term (Saez *et al.*, 1972; Bammann *et al.*, 1980; Berger *et al.*, 1984). However, circulating fT levels only increase significantly at the third trimester of pregnancy (Dawood and Saxena, 1977). In addition, serum A4 is significantly elevated between 37–42 weeks of pregnancy compared with non-pregnant levels, but the relative increase of T during pregnancy is greater than the increase in A4 (Mizuno *et al.*, 1968). In contrast, maternal circulating DHEAS levels fall across gestation to ~50% of the non-pregnant levels (Milewich *et al.*, 1978). Finally, SHBG levels increase during the first trimester of pregnancy and continue to increase dramatically throughout mid and late gestation (Wilke and Utley, 1987). Table I summarizes the maternal serum levels of different androgens during pregnancy and Fig. 2 is a graphical representation of this information.

In the fetus, levels of some androgens are dependent on fetal sex and gestation. In fetal blood, T levels are higher in males (Rodeck *et al.*, 1985). This contrasts with DHT levels, which are similar in both sexes (Diez d'Aux and Pearson Murphy, 1974; Rodeck *et al.*, 1985). In male fetuses, serum concentrations of T increase until the end of first trimester, reaching a peak of 150 ng/dL at the end of week 12 of gestation, after which they decline by 70% to a nadir at 23 weeks (Rodeck *et al.*, 1985). In female fetuses, T levels remain generally lower throughout first and

**Table I Levels of androgens in maternal serum.**

| Androgen | Non-pregnant | First trimester | Second trimester | Third trimester | References |
|---|---|---|---|---|---|
| fT (pmol/l) | 6.2 | 11.1 | 7.5 | 13.3 | Wilke and Utley (1987) |
| tT (mmol/l) | 0.21–2.98 | 0.90–7.32 | 1.20–8.40 | 2.20–10.70 | O'Leary *et al.* (1991) |
| A4 (ng/ml) | 1.0–2.0 | 2.5–3.5 | 0.6–7.8 | 1.6–14.0 | Castracane *et al.* (1998), Carlsen *et al.* (2006) |
| DHT (ng/ml) | 0.022–0.107 | 0.113 | 0.18 | 0.1–0.3 | Buster *et al.* (1979), Dawood and Saxena (1977) |
| SHBG (nmol/l) | 42.2 | 68.1 | 279.3 | 246.1 | Wilke and Utley (1987) |
| DHEA (nmol/l) | 1.0–40.0 | 10.0–60.0 | 5.0–50.0 | 5.0–50.1 | Tagawa *et al.* (2004) |
| DHEAS (nmol/l) | 2000–4000 | 2000–4000 | 500–2000 | 500–200 | Tagawa *et al.* (2004) |

The table shows the mean concentrations of androgens as given in references quoted. tT, total testosterone; fT, free testosterone; SHBG, sex hormone binding globulin; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; A4, androstenedione; DHT, dihydrotestosterone.

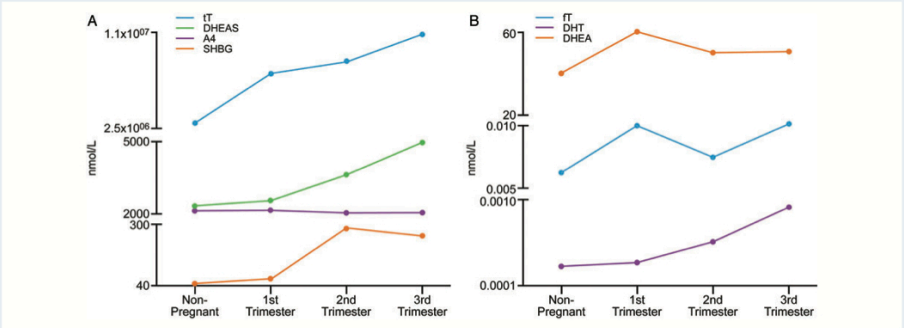


**Figure 2** Graphical presentation of the highest (**A**) and lowest (**B**) levels of androgens in maternal serum throughout gestation. tT, total testosterone; fT, free testosterone; SHBG, sex hormone-binding globulin; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; A4, androstenedione; DHT, dihydrotestosterone.

second trimester (50 ng/dL) and decline rapidly at term (Diez d'Aux and Pearson Murphy, 1974). In addition to T, DHEA levels are higher in cord blood of male fetuses compared with females, whereas A4 levels are similar (Keelan et al., 2012). Labour is associated with increased levels of cord blood A4, DHEA and SHGB and decreased levels of tT and fT (Keelan et al., 2012). In amniotic fluid, T and A4 are higher in male-bearing pregnancies (Carson et al., 1982). On the other hand, there is no notable difference in amniotic fluid DHEA levels between male and female fetuses (Robinson et al., 1977). Although T levels are higher in the *fetal* compartment of male fetuses, there is no association between fetal sex and *maternal* serum concentrations of any androgen (Rivarola et al., 1968). The association between fetal sex and androgen levels, T or A4, in the fetal compartment, including serum and amniotic fluid, is consistent with androgen biosynthesis within Leydig cells of the fetal testis (Scott et al., 2009). Therefore, increased T and A4 levels in the amniotic fluid of male fetuses may reflect androgens excreted in fetal urine (Mitchell and Shackleton, 1969). The tissue origin and cause of the increase in androgens in maternal circulation during pregnancy remains uncertain but is likely to involve production from the ovary or placenta as discussed below.

### *Tissue origin of androgen synthesis during pregnancy*

Within the ovary, androgens are synthesized in small luteal cells (SLC) of the corpus luteum (former theca cells of ovarian follicle) (Sanders et al., 1996). Once pregnancy occurs, it is plausible that SLC stimulation by human chorionic gonadotrophin (hCG), which is elevated in first trimester, results in the reported increase in T levels at this time (Braunstein et al., 1976; Liu and Hsueh, 1986). However, hCG levels reach a peak at the end of first trimester and then decline, in contrast to T, which increases steadily, suggesting either an alternative regulation of androgen production by the corpus luteum or an alternative source of androgen increase following the end of the first trimester (Braunstein et al., 1976). Studies on women with premature ovarian failure (POF) who become pregnant following *in vitro* fertilization (IVF) with donor oocyte transfers suggest that the ovary is the major contributor to circulating concentrations of T and A4 during pregnancy (Castracane and Asch, 1995). Such women were shown to have significantly lower levels of T and A4 when compared with pregnant women without POF. Perhaps not surprisingly, such studies are yet to be replicated by other research groups.

In addition to the maternal ovary, the maternal adrenal is an important source of androgen production throughout pregnancy. Indeed maternal and fetal virilization due to androgen excess have been reported in cases of adrenal adenomas (Fuller et al., 1983). Studies in baboons showed that the maternal adrenal production of DHEA and DHEAS is suppressed during pregnancy by E2; this is in line with a reported decrease in DHEAS in maternal circulation throughout pregnancy (Albrecht and Pepe, 1995; Umezaki et al., 2001; Tagawa et al., 2004). These studies demonstrated that removal of the fetus but not the placenta (fetectomy), in order to eliminate a fetal source of DHEA/DHEAS, induced a decline in E2 and an increase in DHEA/DHEAS levels in the maternal circulation (Albrecht et al., 1980; Albrecht and Pepe, 1995). The decrease in E2 is probably due to withdrawal of fetal precursors for E2 formation. Interestingly, the authors showed that exogenous E2 could inhibit the fetectomy-induced increase in DHEA/DHEAS. The observation that metabolic clearance rate of DHEA/DHEAS remained unchanged before and after E2 administration, led authors to conclude that the estrogen-induced decline in maternal DHEA/DHEAS levels reflected a decrease in maternal adrenal production of these androgens.

Despite the prevailing belief from studies from 1960s that the placenta does not hold the metabolic capacity to synthesize androgens de novo, but relies solely on fetal androgens, a recent study has demonstrated that placental syncytiotrophoblast (but not cytotrophoblast) can synthesize androgens (Pion et al., 1965; Siiteri and MacDonald, 1966; Escobar et al., 2011). Specifically, syncytiotrophoblast expresses both mRNA and protein of the metabolising enzyme CYP17, which converts C21 steroids (such as P4) to C19 steroids (such as T). The syncytiotrophoblast has been confirmed to have CYP17 activity *in vitro* (Escobar et al., 2011).

The non-pregnant human myometrium has been shown to possess the metabolic capacity to convert A4 to T and DHT *in vitro*, but whether this system is functional in pregnancy is unknown (Jasonni et al., 1982). Consistent with this, a more recent study has reported that myometrium, derived from non-pregnant and early pregnant pig uteri, can synthesize A4 and T *in vitro* (Franczak, 2008). Whether the cervix during pregnancy has the metabolic capacity to synthesize androgens is unknown.

Figure 3 summarizes possible sites for androgen production in the materno-placental unit.

### *Androgen clearance during pregnancy*

The concentrations of androgens measurable in blood (Pc) are also influenced by their metabolic clearance rate (MCR), the volume of blood cleared of a steroid per unit time (Baird et al., 1969). The blood production rate of an androgen (Pb) is the amount of androgen entering circulation from all possible sources (endocrine glands and periphery). This can be calculated according to the formula Pb = MCR x Pc and it represents an approximation of daily production rate of the steroid (Gurpide, 1990). The MCR of androgens, which represents the summation of individual organ clearance rates, is highly influenced by the amounts of circulating SHBG, which reduces the peripheral breakdown of androgens (Vermeulen and Ando, 1979).

Soon after implantation, the production of maternal P4, E2 and androgens by the corpus luteum increases significantly (Elbaum and Keyes, 1976; Stouffer et al., 1977; Richardson and Masson, 1981; Laherty et al., 1985; Webley and Hearn, 1987; Fisch et al., 1989; Brannian and Stouffer, 1991; Stocco et al., 2007). Corpus luteum-derived E2 stimulates liver SHBG synthesis leading to an increase in SHBG levels (Wilke and Utley, 1987; Joseph, 1994). The increase in SHBG should decrease the availability of fT and therefore lower the clearance of T and DHT. Indeed, it has been reported that the MCR of T and DHT is lower during the first three months of pregnancy compared with the non-pregnant state; however, DHT and T blood production rates are unchanged (Saez et al., 1972). These finding are in agreement with a study in guinea-pigs, where pregnancy induced a 2-fold decrease in MCR of T (Despres et al., 1982).

In contrast to T and DHT, the MCR of DHEA and DHEAS increases 2- and 5-fold, respectively, starting at mid-pregnancy (Gant et al., 1971; Belisle et al., 1977; Belisle et al., 1980b). This phenomenon may reflect the metabolism of these steroids in the feto-placental unit, as both steroids are important as estrogen precursors (Goodyer and Branchaud, 1981; Longcope, 1996). In addition to the increase in the MCR of DHEAS, its production rate by maternal adrenals increases in pregnancy (Milewich et al., 1978). The net result is lower DHEAS levels in the

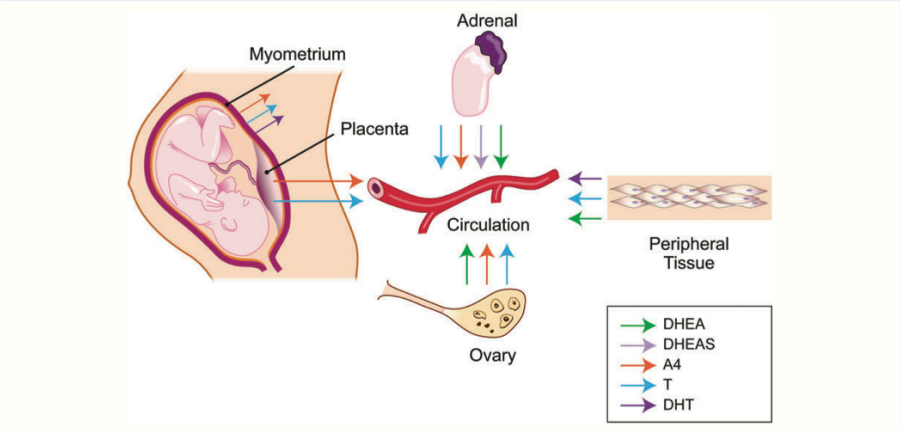


**Figure 3** Maternal sites of androgen synthesis. The maternal adrenal gland secretes DHEA, DHEAS, A4 and T to the maternal circulation, from which androgens get distributed to peripheral tissues. Placenta is an additional site for *de novo* synthesis of A4 and T synthesis. Ovaries can also produce and secrete T, A4 and DHEA into maternal circulation. Myometrium can also synthesize A4, T and DHT. Finally, DHEA and T can be generated from precursors by androgen metabolizing enzymes. DHEA, dehydroepiandrosterone; T, testosterone; DHT, dihydrotestosterone; DHEAS, dehydroepiandrosterone sulphate; A4, androstenedione.

maternal serum at the end of first trimester in comparison with the non-pregnant state (Tagawa *et al.*, 2004). For A4, although pregnancy does not induce changes in the MCR, the production rate is increased 2-fold, explaining the observed increase in A4 in the blood (Belisle *et al.*, 1980a, b; Castracane *et al.*, 1998).

In conclusion from the above studies, it is understood that changes in the production of androgens (predominantly in the ovary and to a lesser extent in the adrenals and periphery) and the MCR occurring during pregnancy may all have an impact on androgen concentrations in the circulation. Of note, changes in concentrations of circulating androgens may not reflect the bioavailability of these steroids in target tissues and therefore their potential to play a role in the physiology of tissues such as the myometrium, cervix and placenta. The role of the increase in androgen levels during pregnancy has been understudied, although a number of adverse pregnancy outcomes have been correlated with impaired maternal androgen levels. These studies are discussed below.

## Androgens and adverse pregnancy outcomes

Considering the adverse effects of increased circulating androgens in non-pregnant women, such as in PCOS, one would assume that the physiological pregnancy-induced increase in androgen levels could potentially have similar detrimental effects. However, it is generally accepted that pregnancy-specific mechanisms are activated to protect both the mother and fetus from pregnancy-induced androgen excess (Hensleigh *et al.*, 1975). In the absence of these 'protective' mechanisms, high levels of androgens would cause hirsutism and/or virilization of both mother and female fetus.

Maternal 'protective' mechanisms include (i) the physiological increase of maternal circulating SHBG, which binds and inactivates elevated androgens (Hammond, 2011) (ii) the pregnancy-induced rapid elevation of P4, which competes for AR binding (Slayden *et al.*, 2001; Birrell *et al.*, 2007) and (iii) P4 having an affinity for 5α-reductase resulting in an inhibition of the conversion of T to the more potent DHT (Hodgins, 1982; Cabeza *et al.*, 1999). Due to these mechanisms, pregnancy itself may reduce the clinical manifestation of pre-existing or pathological pregnancy-induced hyperandrogenic conditions (Phelan and Conway, 2011; Crisosto *et al.*, 2012). The most common hyperandrogenic conditions are known as 'non-tumour ovarian hyperandrogenism' and include PCOS, hyperreactio luteinalis (HL) and pregnancy luteoma (Kanova and Bicikova, 2011). Numerous cases of HL, occurring predominantly during the second or third trimester and caused by high β-human chorionic gonadotrophin (β-hCG) levels, have been reported (Haimov-Kochman *et al.*, 2004; Van Holsbeke *et al.*, 2009; Amoah *et al.*, 2011). These studies highlight that, in spite of the protective mechanisms, a small fraction of women with HL exhibit virilization and/or hirsutism, although their female fetuses are hardly ever virilized (Hensleigh *et al.*, 1975; Foulk *et al.*, 1997; Holt *et al.*, 2005; Angioni *et al.*, 2007; Van Holsbeke *et al.*, 2009; Veleminsky, 2010; Abe *et al.*, 2011; Amoah *et al.*, 2011; Annamalai *et al.*, 2011). Although most maternal hyperandrogenic symptoms of HL resolve post-partum, HL is highly associated with adverse pregnancy outcomes such as pre-eclampsia (PE) and PTB (Gatongi *et al.*, 2006; Grgic *et al.*, 2008; Masuyama *et al.*, 2009; Atis *et al.*, 2010; Haq, 2010; Simsek *et al.*, 2012; Lynn *et al.*, 2013). Other hyperandrogenic disorders, which are generally very rare, include fetal-induced hyperandrogenism, due to fetal aromatase deficiency

(FAD), iatrogenic hyperandrogenism, caused by exogenous androgen administration and adrenal tumour hyperandrogenism (Dahl *et al.*, 2008).

Fetal 'protective' mechanisms largely include the metabolism of androgens in the placenta (Siiteri and MacDonald, 1966; Simpson *et al.*, 1994). Specifically, the aromatase complex within the placenta plays a critical role in the protection of fetus from the mother's androgens as this enzyme rapidly converts T or A4 to E1 and E2, respectively (Edman *et al.*, 1981; Bardin *et al.*, 1983; Hall *et al.*, 1987; Pasanen and Pelkonen, 1994; Simpson *et al.*, 1994). The efficacy of this placental barrier to androgens is evidenced by studies showing that women with hyperandrogenic disorders during pregnancy, such as PCOS, do not necessarily deliver virilized female fetuses (Sir-Petermann *et al.*, 2002; Escobar-Morreale, 2010). On the other hand, women with rare FAD can deliver virilized fetuses, again demonstrating the crucial importance of placental aromatase (Shozu *et al.*, 1991; Ludwig *et al.*, 1998). Taken together, these studies show that pregnancy is associated with protective mechanisms, which ameliorate both maternal and fetal effects of the androgen increase associated with normal pregnancy physiology.

Besides the physiological androgen increase, numerous studies have explored the association of impaired androgen increases during pregnancy, and factors such as maternal age and sex of the baby, with pregnancy outcome. Women with PCOS, who have higher androgen levels than healthy pregnant women, are reported to have an ~6% higher risk of delivering preterm than women without PCOS (Sir-Petermann et al., 2005; Yamamoto *et al.*, 2012). However, androgen excess might not be the only reason for early birth in these women, as PCOS is also associated with an increase in many inflammatory mediators, which may contribute to PTL (Diamanti-Kandarakis *et al.*, 2006). A study investigating the association of birth size of the offspring with maternal androgens has shown that elevated T is positively associated with *in utero* growth restriction (Carlsen *et al.*, 2006). In addition, women pregnant with male fetuses are at greater risk of delivering preterm (McGregor *et al.*, 1992; Zeitlin *et al.*, 2002), a phenomenon sometimes attributed to the fact that pregnancies with male fetuses have higher levels of androgens in the fetal compartment compared with female fetuses. However, there is no evidence that the relationship between higher androgen levels in the fetal compartment and PTB is causal. In contrast to the positive association between androgen levels (at least in the fetal compartment) and the presence of a male fetus and PTB, increasing maternal age (which is negatively associated with maternal androgen levels in pregnancy) is known to be a risk factor for preterm deliveries (Astolfi and Zonta, 1999; Carlsen *et al.*, 2003). Recently, a specific change in the sequence of the fetal *AR* gene has been associated with predisposition to PTB (Karjalainen *et al.*, 2012). In particular, a linkage analysis study revealed that the exon-1 of *AR* of the offspring who was born preterm had more repetitions of a sequence encoding for a site in the domain involved in ligand activation (Karjalainen *et al.*, 2012). Abnormal increase in androgens during pregnancy has been additionally correlated with the development of gestational diabetes, which is further associated with PTB (Espinos *et al.*, 1992; Abbott *et al.*, 2010; Vejrazkova *et al.*, 2014; Ackerman *et al.*, 2013; Morisset *et al.*, 2013). This is probably due to the role of androgens in modulation of insulin secretion from the pancreas and inhibition of glucose uptake in fat and muscle cells (Corbould, 2008; Rao *et al.*, 2013). The mechanism linking the two hormonal axes is poorly understood and, interestingly, has not been considered or explored during pregnancy. Increased T levels in the maternal circulation at third trimester have been associated with the incidence of PE, suggesting T as a predictive marker (Ghorashi and Sheikhvatan, 2008; Lorzadeh and Kazemirad, 2012; Sharifzadeh *et al.*, 2012; Simsek *et al.*, 2012). In this context, placentae from preeclamptic pregnancies have higher expression levels of AR in both male and female fetuses (Sathishkumar *et al.*, 2012). Therefore, it is possible that AR signalling in the placenta contributes to the pathology of PE.

The association of androgen levels with preterm delivery has been studied in a few primate studies. For example, continuous administration of A4 to pregnant rhesus monkeys from early gestation or for a period of 48 h during third trimester resulted in preterm initiation of myometrial contractions and cervical dilatation leading to preterm delivery (Figueroa *et al.*, 1989; Mecenas *et al.*, 1996). In these studies, preterm delivery was associated with an increase in E2 in maternal circulation, suggesting conversion of A4 to E2 in the placenta. Nathanielsz *et al.* (1998) later supported that notion by showing that the preterm phenotype could be rescued by administration of an aromatase inhibitor. However, in other studies, direct administration of E2 or E1 to pregnant primates failed to induce preterm delivery (Novy and Walsh, 1983; Albrecht *et al.*, 1989). Therefore, it is likely that a physiological increase in androgen levels in maternal circulation from the start to end of pregnancy holds the key to term pregnancy. In support of this notion, abnormal changes in androgens levels at the start of pregnancy have been associated with spontaneous abortions or recurrent miscarriages. For example, spontaneous abortion was shown to occur in women whose androgen levels failed to increase at the beginning of pregnancy (Bammann *et al.*, 1980). On the other hand, women with recurrent miscarriages had significantly higher levels of T, A4 and T/SHBG ratio compared with women without recurrent miscarriages (Okon *et al.*, 1998).

Although these studies emphasize how the normal increase in androgen levels might be pivotal for maintenance of pregnancy and development of the fetus, they fail to show a consistent relationship between either androgen excess or androgen deficiency and pregnancy problems. What is more, the mechanism by which androgens impact pregnancy outcomes has not been fully understood. Below we discuss the effect of androgens on cervical remodelling and myometrial function, the key processes in determining the timing of delivery. Particular focus is paid to myometrial smooth muscle cell (MSMC) physiology and the ability of androgens to interact with the contractile machinery of the cell.

## The role of androgens in CR

### *Structure of cervix during pregnancy*

The cervical stroma is composed of fibroblasts, smooth muscle cells (SMCs), epithelial cells and immune cells (Leppert, 1995). These cells secrete an extracellular matrix (ECM), which consists primarily of collagen, glycosaminoglycans and proteoglycans. Hormones including progestogens and E2, relaxin and prostaglandin can alter ECM composition and affect the mechanical strength of the cervix (Ekman-Ordeberg *et al.*, 2003; Simon and Einspanier, 2009; Ji *et al.*, 2011; Ghule *et al.*, 2012). The consequence of this process is remodelling of the cervix, which can be divided into four stages: softening (first trimester), ripening (second trimester), dilatation (third trimester) and reconstitution of the non-pregnant cervix post-partum (Read *et al.*, 2007). Each stage has a characteristic collagen and proteoglycan composition as the tissue re-organizes and prepares for the next steps in the parturition process (Word *et al.*, 2007). CR is characterized by a 60% decrease

in collagen and proteoglycan levels and occurs in parallel with an increase in collagenase activity, which itself is involved in collagen catabolism and disturbance of collagen bundles (Ekman *et al.*, 1986; Norman *et al.*, 1993). However, collagen synthesis increases in the cervix at term due to collagen turnover being balanced between production and degradation (Ekman *et al.*, 1986). Structural abnormalities of the cervix have been associated with an increased risk of PTB, thus a better understanding of the physiology of cervical remodelling is crucial (reviewed by Norman, 2007).

### *The role of androgens in CR*

Numerous human and animal studies have explored the role of androgens in the cervix, particularly during pregnancy (Mochizuki *et al.*, 1978a, b; Sasaki *et al.*, 1982; Mochizuki and Maruo, 1985; Takahashi *et al.*, 1984; Sakyo *et al.*, 1986, 1987; Yamashita *et al.*, 1991; El Maradny *et al.*, 1996; Kanayama *et al.*, 1998; Ji *et al.*, 2008). These studies formed the basis of a hypothesis, whereby androgens regulate cervical remodelling, particularly CR at term. Table II summarizes key findings from these studies. Collectively, these reports provide enough evidence that the fetal androgen, DHEAS, promotes CR by enhancing collagenase activity and thus decreasing fibril collagen organization.

### *Mechanism of androgen effect on CR*

The physiology or pharmacology of androgen effects on CR is not fully understood. In support of a physiological effect of DHEAS on CR, one study has reported that DHEAS levels are significantly correlated with favourable outcome according to the Bishop score, i.e. higher levels of DHEAS in women with successful (spontaneous) CR compared with women in need of induction of labour (Koyuncu *et al.*, 1995). DHEAS administration has been additionally positively correlated with increases in E2 levels in maternal serum (Takahashi *et al.*, 1984). Therefore, it has been argued that DHEAS is metabolized to estrogens in term cervix, which in turn initiates CR. However, studies examining such effects showed that administration of estrogens alone fails to ripen the cervix (Thiery *et al.*, 1979; Larmon *et al.*, 2002; Dasgupta and Singh, 2012). Notably DHT, which cannot be metabolized to estrogens, also promotes CR, implying an androgen-specific effect (Ji *et al.*, 2008). Interestingly, mice with knockout (KO) of 5α-reductase type 1 develop an abnormal cervix that fails to ripen at labour (Mahendroo *et al.*, 1999). 5α-reductase type 1 converts T to DHT and is the predominant enzyme expressed in cervix at term (Mahendroo and Russell, 1999). Administration of a 5α-reduced androgen, a metabolite of DHT, 5alpha-androstane-3alpha, 17β-diol, rescues the impaired phenotype of 5α-reductase type 1 KO animals, allowing normal CR (Mahendroo *et al.*, 1996). The latter finding suggests local conversion of androgens to other (more potent) androgen metabolites as the underlying mechanism in the initiation of CR.

Although poorly explored, there is some evidence to suggest that the mechanism of DHEAS action on the collagenase increase is indirect and is mediated via DHEAS-stimulated neutrophil secretion of the proteolytic enzyme (El Maradny *et al.*, 1996; Maymon *et al.*, 2000). Indeed, DHEAS action on CR has been correlated with increased levels of IL-8 cytokine involved in chemotaxis of neutrophils recruited in cervix towards term (Kanayama *et al.*, 1998; Maymon *et al.*, 2000).

The involvement of AR in mediation of CR by androgens has been recently examined (Ji *et al.*, 2008). In that study, antagonism of AR by flutamide inhibited events associated with CR, such as a decrease in collagen fibril organization, a decrease in cervical resistance and a decrease in proteoglycan synthesis in pregnant rats pre-treated with DHT on Day 16, suggesting genomic signalling (Ji *et al.*, 2008). Although this study did not examine AR expression in the cervix, other immunohistochemical studies in non-pregnant and pregnant humans and canine cervix have demonstrated cervical expression of AR (Wilson and McPhaul, 1996; Vermeirsch *et al.*, 2002; Ji *et al.*, 2008; Vladic-Stjernholm *et al.*, 2009). In addition, it has been reported that androgens up-regulate AR expression in the human cervix (van der Kwast *et al.*, 1994).

All the above reports suggest that DHEA and DHT might promote CR via a pathway impacting collagenase synthesis and/or activity. Although the mechanism of this action is not well established, there is some evidence that it is possibly mediated via metabolism of androgens to reduced androgens and/or genomic signalling through AR. This notion needs more investigation. In addition, the role of androgens and androgen signalling in the cervical endothelial/epithelial cells and cervical SMCs in terms of cervical remodelling throughout pregnancy is surprisingly unexplored.

## The role of androgens in myometrial function

### *MSMCs changes during pregnancy*

MSMCs are specialized contractile cells that, in contrast to skeletal and SMCs, exhibit cellular plasticity, which enables reversible differentiation into different phenotypes (Gabbiani *et al.*, 1981; Tomiyasu *et al.*, 1988). Studies of myometrium during rat pregnancy have demonstrated that MSMCs undergo four phenotypic changes throughout gestation (Shynlova *et al.*, 2009). Briefly, these are a proliferative stage, a synthetic stage, a contractile stage and, finally, a labour stage. In early pregnancy, following implantation of the conceptus, MSMCs proliferate rapidly and become hyperplastic. Hyperplasia is believed to be mediated by hormone signalling and growth factors, such as IGF-1 and EGF, have been shown to be involved in induction of the so-called proliferative phenotype (Shynlova *et al.*, 2007). Following the proliferative phase, MSMCs undergo an intermediate synthetic phase starting on Day 14 of rodent pregnancy (Lye *et al.*, 2001). The synthetic phenotype is characterized by increased synthesis of ECM and cellular hypertrophy of MSMCs (Shynlova *et al.*, 2009). On Day 21 of rodent gestation, uterine myocytes switch to a contractile phenotype (Nishinaka and Fukuda, 1991; Fata *et al.*, 2000; Shynlova *et al.*, 2004). Differentiation into a contractile phenotype is largely attributed to mechanical stretch (Manabe *et al.*, 1981; Loudon *et al.*, 2004; Sooranna *et al.*, 2004; Terzidou *et al.*, 2005). MSMCs undergo a final phase of differentiation following initiation of labour, the so-called labour phenotype (Shynlova *et al.*, 2009). At this point, the myometrium develops synchronous contractions and expresses a number of contraction-associated proteins (CAPs), such as connexin 43 (Con43), oxytocin receptor (OXTR) and prostaglandin F receptor (Fuchs *et al.*, 1984; Slater *et al.*, 1999; Sparey *et al.*, 1999; Erkinheimo *et al.*, 2000; Rehman *et al.*, 2003). Returning MSMCs to a non-pregnant state involves rapid apoptosis (Roh *et al.*, 2000; O'Brien *et al.*, 2007; Shynlova *et al.*, 2009).

A recent study, conducted in rats, reported that AR is highly expressed in MSCMs during the proliferative stage and progressively declines towards the end of pregnancy, suggesting a role (at least in this species) in establishment of myometrial growth at early stages of pregnancy (Liu *et al.*, 2013). Liu *et al.* additionally showed that the AR

**Table II Studies on the effect of androgen administration on CR.**

| Androgen | Dose | Species/tissue | Key findings | Reference |
|---|---|---|---|---|
| DHEAS | 0.01 mg/ml<br>0.1 mg/ml | Human<br>*In vitro*: HCF derived from term pregnancy | ↑ IL-8 in conditioned medium<br>↑ IL receptor in HCF | Kanayama *et al.* (1998) |
| DHEAS | 200 mg | Human<br>*In vivo*: term pregnant women (>38 weeks) | ↑ Bishop score<br>↑ Collagenase activity | Mochizuki *et al.* 1978a, b |
| DHEAS | 200 mg | Human<br>*In vivo*: term pregnant women (38–42 weeks) | ↑ Bishop score<br>↑ Collagenase activity<br>↑ E2 in maternal serum | Mochizuki and Maruo (1985) |
| DHEAS | 50–100 mg | Human<br>*In vivo*: term pregnant women (38–42 weeks) | ↑ Bishop score<br>↓ Time to delivery<br>No side effects | Mochizuki *et al.* 1978a, b |
| DHEAS | 100 mg | Human<br>*In vivo*: term pregnant multiparous and nulliparous women (>38 weeks) | ↑ Bishop score<br>↓ Duration of labour in nulliparous women<br>↓ Time to delivery in nulliparous<br>No side effects | Sasaki *et al.* (1982) |
| DHEAS | 50–200 mg | Human<br>*In vivo*: term pregnant singleton and twin pregnancies (>39 weeks) | ↑ Bishop score<br>(higher correlation of Bishop score with twin pregnancies)<br>↑ E2 levels in maternal serum<br>(higher correlation of E2 levels with twin pregnancies)<br>No change in E1, E3 levels in maternal serum. | Takahashi *et al.* (1984) |
| DHEAS | 1 μM | Rabbit<br>*In vitro*: CCs derived from term pregnancy | ↑ Collagenase levels<br>No effect of E2 or DHEA treatment | Sakyo *et al.* (1986) |
| DHEAS | 1 μM | Rabbit<br>*In vitro*: CCs derived from term pregnancy | ↑ Collagenase levels<br>No effect on collagen levels | Sakyo *et al.* (1987) |
| DHEAS | 10 mg | Rabbit<br>*In vivo*: term pregnancy | ↑ Collagenase activity<br>↓ Collagen content<br>Combined DHEAS + IL-8 (100 ng) treatment-induced neutrophil infiltration<br>Combined treatment produced maximal decrease in collagen content | Maradny *et al.* (1996) |
| DHEAS | 100 mg | Rat<br>*In vivo*: term pregnancy (day 16) | ↓ Collagen content<br>↑ Collagenase activity | Yamashita *et al.* (1991) |
| DHEA | 0.01 mg/ml<br>0.1 mg/ml | Human<br>*In vitro*: HCF derived from term pregnancy | Increase of IL-8 in conditioned medium<br>Increase of IL receptor in HCF | Kanayama *et al.* (1998) |
| DHT | 2 mg | Rat<br>*In vivo*: term pregnancy (15 day) | ↓ Cervical resistance<br>↓ Proteoglycan mRNA<br>Flutamide treatment (10 mg)<br>↑ Cervical resistance<br>↑ Proteoglycan mRNA | Ji *et al.* (2008) |

DHEAS, dehydroepiandrosterone sulphate; DHT, dihydrotestosterone; HCF, human cervical fibroblast; CCs, cervical cells.

decrease itself affects IGF-1 receptor (IGF-1R) stability and thus down-regulates downstream cascades that IGF-1 is involved in (including P13K/Akt, which is highly important in proliferative pathways) (Liu *et al.*, 2013). Besides rats, a significant decrease in AR expression occurs in porcine myometrium 3 weeks prior to labour (Slomczynska *et al.*, 2008). A human microarray study also showed AR gene down-regulation in the myometrium during preterm (0.4-fold) and term labour (0.3-fold) compared with non-labouring myometrium, but the protein expression was not determined (Bethin *et al.*, 2003). Thus, androgen signalling in MSMCs is likely to be involved in proliferative pathways pivotal for 'building' of the myometrium in the early stages of pregnancy.

*The role of androgens in myometrial contractility*

Androgens have been demonstrated to relax SMCs, such as rabbit and pig trachea, rat mesenteric arterial bed, rat thoracic aorta and human coronary artery (Perusquia *et al.*, 1991a, b; Chou *et al.*, 1996; Costarella *et al.*, 1996; Rosano *et al.*, 1999; Tep-areenan *et al.*, 2002; Perusquia *et al.*, 2005; Kouloumenta *et al.*, 2006; Bordallo *et al.*, 2008; Montano *et al.*, 2008; Chevalier *et al.*, 2012). Robson (1937) was the first to suggest that androgens may have an inhibitory action on uterine muscle contraction. Following a 4-day administration of 12 mg of testosterone propionate (a small fast-acting ester of testosterone) to ovariectomized non-pregnant rabbits, he examined the contractile response of their

uterus to 1 unit of injected oxytocin both *in vivo* and *in vitro*. The uterus of T-treated rabbits did not respond to oxytocin administration in contrast to the uterus of control rabbits. However, the spontaneous contractile activity of the uterus was only slightly affected (Robson, 1937). Following Robson, some other studies have demonstrated similar relaxing effects of various androgens including T, DHT, A4 and DHEAS on the myometrium of non-pregnant rats and non-pregnant and pregnant humans (Kubli-Garfias *et al.*, 1980; Perusquia *et al.*, 1991a, b; Perusquia *et al.*, 2005). The findings from these studies are summarized in Table III. The relaxing effect occurs only in pharmacological concentrations (micromolar) and is rapid, suggesting non-genomic mechanism of action (Perusquia *et al.*, 1990; Anderson *et al.*, 2009; Tica *et al.*, 2011). A logical assumption would be that the observed effect of androgens might not occur physiologically because physiological androgen concentrations are in a nanomolar range. However, understanding the molecular pathways employed by androgens in modulation of myometrial relaxation could help in the development of new tocolytic agents to manage preterm spontaneous contractions. We discuss below the current evidence for possible molecular mechanisms by which androgens might elicit relaxing effects in myometrium.

### *Mechanism of MSMC contraction*

MSMCs are myogenic, which means that they can contract spontaneously (Tomiyasu *et al.*, 1988). The membrane potential in MSMCs is not stable. Spontaneous depolarization of membrane potential, fired by alterations in membrane currents and ion channels, occurs during pregnancy. A decrease in membrane potential triggers myocyte contractile activity and leads to spontaneous contractions; conversely, amplification of membrane potential to a resting −50 mV maintains the uterus in a quiescent state (Nakajima, 1971; Pressman *et al.*, 1988). The upstroke of MSMCs membrane action potential (i.e. the initial decrease in membrane potential) is predominantly due to calcium ($Ca^{2+}$) entry, whereas repolarization occurs as a result of blockage of $Ca^{2+}$ channels (Nakajima, 1971). The key biochemical event in spontaneous MSMC contraction is an increase in intracellular $Ca^{2+}$ ($[Ca^{2+}]$) from $10^{-7}$ to $10^{-6}$ M (Horowitz *et al.*, 1996). This increase is the outcome of extracellular influx of $Ca^{2+}$ and/or release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR). The entry of $Ca^{2+}$ is mediated via a variety of $Ca^{2+}$ channels classified into voltage-operated $Ca^{2+}$ channels (VOCCs) and voltage-independent $Ca^{2+}$ channels (including receptor-operated $Ca^{2+}$ channels, ROCCs) (Wray *et al.*, 2003, 2005; Floyd and Wray, 2007; Noble *et al.*, 2009). ROCCs are regulated by an agonist-receptor interaction (Guibert *et al.*, 2008). Receptors that interact with ROCCs upon ligand binding are G protein coupled receptors (GPCRs), such as the OXTR. GPCRs can activate the inositol triphosphate (IP3) pathway leading to release of $Ca^{2+}$ from SR via activation of phosphoinositide phospholipase C and hydrolysis of PIP2 (Meldrum *et al.*, 1991). The resulting increase in $[Ca^{2+}]$ leads to formation of a complex between calmodulin and myosin light chain kinase, which then phosphorylates light chains on myocin (MLC) (Ito and Hartshorne, 1990). The latter event allows binding of actin to myocin leading to cross-bridge cycling, which then causes contraction (Dillon *et al.*, 1981). Extrusion of $[Ca^{2+}]$ in MSMCs can occur by reverse processes and is mediated by two major transporters: plasma membrane $Ca^{2+}$ ATPase (PMCA) and the $Na^+/Ca^{2+}$ exchange pump (NCX) (Shmigol *et al.*, 1998). Studies examining mechanisms by which $Ca^{2+}$ ions are removed from the cytoplasm in isolated rat MSMCs show that PMCA accounts for 85% of the $Ca^{2+}$ efflux (Shmigol *et al.*, 1998).

Besides VOCCs, ROCCs, PMCA and NCX, $Ca^{2+}$ flux is highly influenced by MSMC membrane fluidity. A proton nuclear magnetic resonance (NMR) spectroscopy study investigating various parameters of myometrial membrane fluidity at pregnancy, including phospholipid double bonds, fatty acid chain length and the Ch/phospholipid ratio, reported that MSMC membrane fluidity is increased with labour (Pulkkinen *et al.*, 1998). Cell membrane fluidity is modulated by changes in the concentration of membrane Ch (Shmygol *et al.*, 2007). Ch is a component of the lipid bilayer, which integrates into the membrane by placing its steroid ring next to the hydrocarbon chains and locating its hydroxyl group close to the head of phospholipid (van Meer, 1989; Simons and Vaz, 2004; Ikonen, 2008). High Ch results in a stiff membrane and thus reduces membrane fluidity, whereas low Ch promotes membrane fluidity. Decreased membrane fluidity is characterized by inhibition of $Ca^{2+}$ channels such as VOCCs and ROCCs (Jennings *et al.*, 1999; Shmygol *et al.*, 2007). Administration of Ch *in vitro* decreases $[Ca^{2+}]$ and inhibits the phasic contractions in MSMCs of rats and humans (Smith *et al.*, 2005; Zhang *et al.*, 2007). On the other hand, depletion of Ch from the rat MSMC membrane with methyl-beta-cyclodextrin treatment causes an increase in cell excitability and contraction (Jennings *et al.*, 1999; Shmygol *et al.*, 2007). High Ch has been correlated with increased activities of NCX and PMCA, which is consistent with their roles in promoting $Ca^{2+}$ efflux (Ortega and Mas-Oliva, 1984; Kutryk and Pierce, 1988; Verbist *et al.*, 1991).

### *Non-genomic interactions of androgens with the contractile machinery*

Possible interactions of androgens with the MSMC contractile machinery, including interactions with VOCCs, ROCCs, the lipid bilayer, gap junctions and membrane receptors, which may result in the inhibition of contraction, are discussed below.

*Androgens and VOCCs.* High $K^+$ in the extracellular space induces cell membrane depolarization, which in turn results in opening of $Ca^{2+}$ channels (VOCCs) and an increase in $[Ca^{2+}]$. In addition to the relaxing effect of androgens on spontaneous myometrial contractions, 5β-DHT rapidly relaxes tonic contractions (induced by high $K^+$) and decreases single MSMC $[Ca^{2+}]$, while removal of the androgen reverses the effect (Perusquia *et al.*, 2005). A logical explanation of the mechanism of the relaxing effect of the androgen in that case would be via inhibition of VOCCs. However, it is not clear whether the effect of androgens involves a physical interaction with VOCCs or is indirect.

*Androgens and ROCCs.* Besides VOCCs, there is some evidence that androgens may be able to block ROCCs. For example, androgens can relax rat uterine contractions induced by serotonin, oxytocin and acetylcholine *in vitro* (Perusquia, 1991; Perusquia *et al.*, 1991a, b). Oxytocin, acetylcholine and serotonin receptors are GPCRs. Their activation leads to subsequent activation of extracellular $Ca^{2+}$ influx via ROCCs and/or $Ca^{2+}$ influx from SR via the IP3 pathway (Large, 2002; Thorneloe and Nelson, 2005). It is possible that androgens exert their effects on oxytocin-induced contraction via blockage of IP3 pathway and release of SR $Ca^{2+}$. However, inhibition of the SR $Ca^{2+}$ release is not sufficient for complete blockage of the overall $Ca^{2+}$ influx (Kupittayanant *et al.*, 2002). Therefore, a logical assumption would be that androgens block the ROCC-associated $Ca^{2+}$ influx rather than components of IP3 pathway. This notion is supported by the inability of T in another study to inhibit both caffeine- and carbachol- induced $Ca^{2+}$ release (activators of IP3-pathway) in coronary SMC (Murphy and Khalil, 1999). Identical

**Table III Studies on the effect of androgen on myometrial contractions.**

| Androgen | Dose | Species/tissue | Key findings | References |
|---|---|---|---|---|
| T, DHEA, androstanediol, androsterone, A4, 5α-DHT, 5β-DHT | 10–100 μM | Rat (non-pregnant) *Ex vivo*: myometrial strips | Spontaneous contractions inhibited rapidly (<30 min) by all androgens Potency: androstanediol, androsterone >5β-DHT > T = A4 = DHEA = 5α-DHT | Kubli-Garfias *et al.* (1980) |
| T, DHEA, androstanediol, androsterone, 5β-DHT | 3–100 μM | Rat (non-pregnant) *Ex vivo*: myometrial strips | Tonic contractions (KCl induced) inhibited rapidly (<30 min) by all androgens Potency: androsterone = androstanediol >5β-DHT > T | Perusquia *et al.* (1990) |
| androstanediol, androsterone, 5α-DHT, 5β-DHT | 3–100 μM | Rat (non-pregnant) *Ex vivo*: myometrial strips | Serotonin-induced contractions inhibited rapidly (<30 min) by all androgens | Perusquia *et al.* (1991a, b) |
| androstanediol, androsterone, 5α-DHT, 5β-DHT | 3–100 μM | Rat (non-pregnant) *Ex vivo*: myometrial Strips | Acetylcholine-induced contractions inhibited rapidly (<30 min) by all androgens | Perusquia *et al.* (1991a, b) |
| androstanediol, androsterone, 5α-DHT, 5β-DHT | 3–100 μM | Rat (non-pregnant) *Ex vivo*: myometrial strips | Oxytocin-induced contractions inhibited rapidly (<30 min) by all androgens | Perusquia (1991) |
| T, DHEA, DHT, 5α-DHT, 5β-DHT, androstanediol, androsterone | 3–100 μM | Human (term pregnant, non-pregnant) *Ex vivo*: myometrial strips | Spontaneous contractions and tonic contractions (KCl induced) inhibited rapidly (<30 min) by all androgens Potency: 5β-DHT > androsterone = DHEA = T > 5α-DHT = androstanediol | Perusquia *et al.* (2005) |

DHEA, dehydroepiandrosterone; T, testosterone; DHT, dihydrotestosterone; DHEAS, dehydroepiandrosterone sulphate; A4, androstenedione.

findings were reported in isolated rat thoracic aortic strips and porcine coronary arteries, where T could inhibit $K^+$-induced but not caffeine-induced contractions (Crews and Khalil, 1999a, b). There are no data on relaxing effect of androgens on a GPCR agonist-stimulated contractions (such as oxytocin and prostaglandin FP) induced contraction in human myometrium.

*Androgens and the lipid bilayer.* In a similar manner to Ch, it is plausible that Ch metabolites (including androgens) mediate non-genomic responses via direct penetration into the cell membrane and reduction in MSMC membrane fluidity. For example, hydrophobic androgens, such as DHT and T, have been shown to interact with the phospholipid bilayer of the negatively charged membrane (Duval *et al.*, 1983; Van Bommel *et al.*, 1987). Such interactions may impair $Ca^{2+}$ homeostasis due to an increase in the activity of PMCA. Regulation of PMCA by androgens is documented in different tissues. For example, in synaptosomal plasma membranes, PMCA activity increases 95% following 10 μM of T treatment (Deliconstantinos, 1988). In addition, PMCA activity in kidney is increased following administration of 10 μM of T, as measured by *in vitro* assays where the ATP-dependent $Ca^{2+}$ flux was determined by comparison of $Ca^{2+}$ transport (implying PMCA activity) with and without exogenous addition of ATP (Dick *et al.*, 2003). In line with the latter study, androgen deprivation induced by castration alters the immunochistochemical localization of PMCA in prostate epithelial cells with no changes in mRNA or protein levels (Coviello *et al.*, 2006). Additionally, the regulation of $Ca^{2+}$-ATPase in SR activity by T has been explored in ventricular muscle of orchidectomized (ORX) rats (Witayavanitkul *et al.*, 2013). In that study, T treatment managed to prevent an ORX-induced decrease in $Ca^{2+}$-ATPase activity. Collectively, an alternative mechanism for the non-genomic action of androgens in MSMC relaxation is proposed, whereby androgens penetrate the lipid bilayer and decrease membrane fluidity, resulting in an increase in PMCA activity and $Ca^{2+}$ efflux. However, the effect of androgens on PMCA activity in the myometrium has not yet been explored and, thus, this hypothesis remains to be confirmed in this tissue.

*Androgens and gap junctions.* Increases in single SMC [$Ca^{2+}$] initiates a synchronized increase in [$Ca^{2+}$] in adjacent cells, a process mediated by gap junctions (Loch-Caruso *et al.*, 2003; Brading and Brain, 2011; Boittin *et al.*, 2013). Gap junction proteins increase the ability of MSMCs to generate synchronous contractions and mount a synchronous response to hormones or agents in the microenvironment of uterus at labour (Garfield and Hayashi, 1981; Ikeda *et al.*, 1987). Connexins are gap junction transmembrane proteins that assemble to form a gap junction; Con43 is the predominant connexin involved in the initiation of MSMC contractions at term (Chow and Lye, 1994). A study in pregnant rats on a high Ch diet showed that high Ch is associated with a decrease in Con43 protein in the myometrium, suggesting possible implications for uterine contractility (Elmes *et al.*, 2011). A study in isolated rat cardiac myocytes demonstrated that gap junctional intracellular communication (GJIC) disturbance, by either silencing the gene for Con43 or by addition of specific drugs, such as heptanol, induced a significant reduction in $Ca^{2+}$ transients, suggesting that Con43 may be involved in the regulation of basal $Ca^{2+}$ signalling in these cells (Li *et al.*, 2012). Interestingly, an estrogenic compound

4-OH-TCB has been shown to act in a similar manner to heptanol and significantly block GJIC in rat myometrium (Tsai *et al.*, 1998). What is more, the same study showed that 4-OH-TCB could reduce contractions of rat myometrial strips, suggesting that the relaxing effect of 4-OH-TCB is mediated through blockage of GJIC. There is evidence that androgens can influence GJIC in isolated rat cardiac myocytes and Sertoli cells (Pluciennik *et al.*, 1996). One study demonstrated a dose-dependent (1–25 μM) inhibitory effect of T propionate on GJIC, which was reversed by T withdrawal and was unaffected by pre-incubation with AR antagonist, again implying a non-genomic effect of T (Pluciennik *et al.*, 1996). Based on these studies, it is tempting to hypothesize that androgens may induce relaxation of myometrium via direct interaction with the membrane proteolipidic structure, which might alter directly or indirectly the function of gap junction channels; however, more evidence in needed.

*Androgens and ARs.* A role for ARs in inhibition of myometrial contraction has been investigated (Perusquia *et al.*, 2005). Flutamide-induced AR antagonism failed to inhibit the relaxing effect of 5β-DHT (Perusquia *et al.*, 2005). Notably, in that study, flutamide was administered at a significantly lower concentration (10 μM) than the concentration of 5β-DHT (100 μM), thus it is not clear whether nuclear AR is involved in the relaxing effect. However, the rapid action of androgens on myometrial relaxation makes a genomic mechanism unlikely. On the other hand, a few reports have now demonstrated that AR can be recruited in non-genomic actions of androgens (reviewed in Hammes and Levin, 2011). This mechanism involves the largely unexplored membrane AR (mAR). The mAR is hypothesized to be either a GPCR or a classic AR lacking a DNA-binding site; the latter is thought to have greater affinity for androgens compared with nAR (Konoplya and Popoff, 1992; Foradori *et al.*, 2008; Yang *et al.*, 2011). A study on airway SMCs showed that in addition to T, treatments with membrane impermable T conjugated to BSA (TBSA) could inhibit contraction, suggesting that T exerts its effects on the outer aspect of the membrane rather than in the cytoplasm (Kouloumenta *et al.*, 2006). However, the downstream mechanism of mAR activation in that study was unclear. The structure of mAR is yet to be characterized in mammals and it is unknown whether it is utilized by androgens to inhibit MSMC contraction (Thomas *et al.*, 2006).

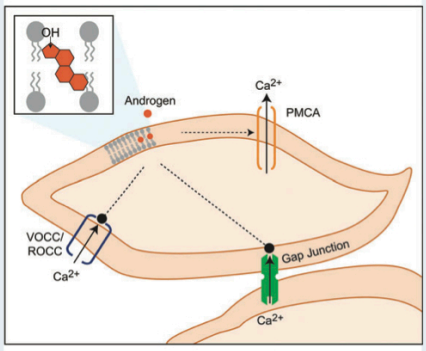


**Figure 4** Hypothetical targets of androgens in modulation of MSMC relaxation. Androgens are hypothesized to interact with contractile machinery of MSMCs via penetration into the lipid bilayer. This might promote PMCA to induce rapid $Ca^{2+}$ efflux, block VOCCs and ROCCs and impair IGJC via effects on the gap junctions. PMCA, $Ca^{2+}$ ATPase; VOCC, voltage-operated $Ca^{2+}$ channels; ROCC, receptor-operated $Ca^{2+}$ channels; IGJC, intercellular gap junctional communication.

Collectively, the above studies allow us to hypothesize that androgen-induced MSMC relaxation is mediated via penetration of the androgen into the lipid bilayer where it affects molecules critical for generation of $Ca^{2+}$ transients, including VOCCs, ROCCs, gap junctions and PMCA (Fig. 4).

## Conclusions

There is growing evidence that androgens, which increase throughout gestation, might play a functional role in the physiology of pregnancy in parallel with the well-established roles of progestins and estrogens. Herein we reviewed what is currently known about roles played by androgens in two critical systems in biology of pregnancy and parturition, cervical remodelling and myometrial function.

There is evidence from *in vivo* and *in vitro* studies that androgens promote CR and, thus, assist initiation of labour. On the other hand, androgens appear to be effective in *in vitro* tocolysis of myometrial contractions. Although the action of androgens on cervical remodelling is possibly mediated via classical activation of genomic signalling via AR, it is unlikely that the same mechanism mediates androgen effects in myometrial contractility. In contrast, there is more evidence that AR acting within the nucleus is involved in the proliferation of MSMCs and, thus, may be important for the growth of myometrium at the beginning of pregnancy. Further investigations are required to fully understand the molecular events regulated by androgens in cervix and myometrium and their interplay with other signalling pathways. These investigations should potentially utilize animal models of adverse pregnancy outcomes, where targeted administration of androgens to either cervix or myometrium could be highly informative. In conclusion, research into the roles of androgens in cervical and myometrial function during pregnancy and parturition has the potential to inform new therapeutic strategies for management of pregnancy complications, such as PTL and PTB.

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## Authors' roles

S.M., P.T.K.S. and J.E.N. made substantial contributions to the conception, design, analysis, drafting and revision of the article and approved the final version to be published. The authors declare that there are no conflicts of interest.

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## Conflict of interest

None declared.

## References


Abbott DH, Bruns CR, Barnett DK, Dunaif A, Goodfriend TL, Dumesic DA, Tarantal AF. Experimentally induced gestational androgen excess disrupts glucoregulation in rhesus monkey dams and their female offspring. *Am J Physiol Endocrinol Metab* 2010;**299**:E741–E751.

Abe T, Ono S, Igarashi M, Akira S, Watanabe A, Takeshita T. Conservative management of hyperreactio luteinalis: a case report. *J Nippon Med Sch* 2011; **78**:241–245.

Abraham GE. Ovarian and adrenal contribution to peripheral androgens during the menstrual cycle. *J Clin Endocrinol Metab* 1974;**39**:340–346.

Ackerman CM, Lowe LP, Dyer AR, Hayes MG, Metzger BE, Lowe WL, Urbanek M, Group HSCR. Maternal testosterone levels are associated with C-peptide levels in the Mexican American subset of the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study cohort. *Horm Metab Res* 2013;**45**:617–620.

Albrecht ED, Haskins AL, Pepe GJ. The influence of fetectomy at midgestation upon the serum concentrations of progesterone, estrone, and estradiol in baboons. *Endocrinology* 1980;**107**:766–770.

Albrecht ED, Crenshaw MC Jr, Pepe GJ. The effect of estrogen on placental delivery after fetectomy in baboons. *Am J Obstet Gynecol* 1989;**160**:237–241.

Albrecht ED, Pepe GJ. Suppression of maternal adrenal dehydroepiandrosterone and dehydroepiandrosterone sulfate production by estrogen during baboon pregnancy. *J Clin Endocrinol Metab* 1995;**80**:3201–3208.

Alfirevic Z, Stampalija T, Roberts D, Jorgensen AL. Cervical stitch (cerclage) for preventing preterm birth in singleton pregnancy. *Cochrane Database Syst Rev* 2012; **4**:CD008991.

Amoah C, Yassin A, Cockayne E, Bird A. Hyperreactio luteinalis in pregnancy. *Fertil Steril* 2011;**95**:2429, e2421–2423.

Anderson L, Martin W, Higgins C, Nelson SM, Norman JE. The effect of progesterone on myometrial contractility, potassium channels, and tocolytic efficacy. *Reprod Sci* 2009;**16**:1052–1061.

Angioni S, Portoghese E, Milano F, Melis GB, Fulghesu AM. Hirsutism and hyperandrogenism associated with hyperreactio luteinalis in a singleton pregnancy: a case report. *Gynecol Endocrinol* 2007;**23**:248–251.

Annamalai AK, Hoveyda F, Williams RM, Patterson A, Simpson HL. Hyperreactio luteinalis. *QJM* 2011;**104**:807–808.

Astolfi P, Zonta LA. Risks of preterm delivery and association with maternal age, birth order, and fetal gender. *Hum Reprod* 1999;**14**:2891–2894.

Atis A, Cifci F, Aydin Y, Ozdemir G, Goker N. Hyperreactio luteinalis with preeclampsia. *J Emerg Trauma Shock* 2010;**3**:298.

Avvakumov GV, Cherkasov A, Muller YA, Hammond GL. Structural analyses of sex hormone-binding globulin reveal novel ligands and function. *Mol Cell Endocrinol* 2010;**316**:13–23.

Baird DT, Horton R, Longcope C, Tait JF. Steroid dynamics under steady-state conditions. *Recent Prog Horm Res* 1969;**25**:611–664.

Bammann BL, Coulam CB, Jiang NS. Total and free testosterone during pregnancy. *Am J Obstet Gynecol* 1980;**137**:293–298.

Bardin CW, Brown T, Isomaa VV, Janne OA. Progestins can mimic, inhibit and potentiate the actions of androgens. *Pharmacol Ther* 1983;**23**:443–459.

Belisle S, Osathanondh R, Tulchinsky D. The effect of constant infusion of unlabeled dehydroepiandrosterone sulfate on maternal plasma androgens and estrogens. *J Clin Endocrinol Metab* 1977;**45**:544–550.

Belisle S, Lehoux JG, Brault J. The metabolism of androstenedione in human pregnancy: the use of constant infusion of unlabeled steroid to assess its metabolic clearance rate, its production rate, and its conversion into androgens and estrogens. *Am J Obstet Gynecol* 1980a;**136**:1030–1035.

Belisle S, Schiff I, Tulchinsky D. The use of constant infusion of unlabeled dehydroepiandrosterone for the assessment of its metabolic clearance rate, its half-life, and its conversion into estrogens. *J Clin Endocrinol Metab* 1980b; **50**:117–121.

Berger NG, Repke JT, Woodruff JD. Markedly elevated serum testosterone in pregnancy without fetal virilization. *Obstet Gynecol* 1984;**63**:260–262.

Bethin KE, Nagai Y, Sladek R, Asada M, Sadovsky Y, Hudson TJ, Muglia LJ. Microarray analysis of uterine gene expression in mouse and human pregnancy. *Mol Endocrinol* 2003;**17**:1454–1469.

Birrell SN, Butler LM, Harris JM, Buchanan G, Tilley WD. Disruption of androgen receptor signaling by synthetic progestins may increase risk of developing breast cancer. *FASEB J* 2007;**21**:2285–2293.

Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, Adler A, Vera Garcia C, Rohde S, Say L *et al.*. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet* 2012; **379**:2162–2172.

Boittin FX, Alonso F, Le Gal L, Allagnat F, Beny JL, Haefliger JA. Connexins and M3 muscarinic receptors contribute to heterogeneous Ca(2+) signaling in mouse aortic endothelium. *Cell Physiol Biochem* 2013;**31**:166–178.

Bordallo J, de Boto MJ, Meana C, Velasco L, Bordallo C, Suarez L, Cantabrana B, Sanchez M. Modulatory role of endogenous androgens on airway smooth muscle tone in isolated guinea-pig and bovine trachea; involvement of beta2-adrenoceptors, the polyamine system and external calcium. *Eur J Pharmacol* 2008;**601**:154–162.

Brading AF, Brain KL. Ion channel modulators and urinary tract function. *Handb Exp Pharmacol* 2011;**202**:375–393.

Brannian JD, Stouffer RL. Progesterone production by monkey luteal cell subpopulations at different stages of the menstrual cycle: changes in agonist responsiveness. *Biol Reprod* 1991;**44**:141–149.

Braunstein GD, Rasor J, Danzer H, Adler D, Wade ME. Serum human chorionic gonadotropin levels throughout normal pregnancy. *Am J Obstet Gynecol* 1976; **126**:678–681.

Brenner RM, Slayden OD, Nayak NR, Baird DT, Critchley HO. A role for the androgen receptor in the endometrial antiproliferative effects of progesterone antagonists. *Steroids* 2003;**68**:1033–1039.

Burger HG. Androgen production in women. *Fertil Steril* 2002;**77**(Suppl. 4):S3–S5.

Buster JE, Chang RJ, Preston DL, Elashoff RM, Cousins LM, Abraham GE, Hobel CJ, Marshall JR. Interrelationships of circulating maternal steroid concentrations in third trimester pregnancies. II. C18 and C19 steroids: estradiol, estriol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, delta 5-androstenediol, delta 4-androstenedione, testosterone, and dihydrotestosterone. *J Clin Endocrinol Metab* 1979;**48**:139–142.

Cabeza M, Gutierrez E, Miranda R, Heuze I, Bratoeff E, Flores G, Ramirez E. Androgenic and anti-androgenic effects of progesterone derivatives with different halogens as substituents at the C-6 position. *Steroids* 1999;**64**:413–421.

Cantineau R, Kremers P, De Graeve J, Gielen JE, Lambotte R. 15- and 16-hydroxylations of androgens and estrogens in the human fetal liver: a critical step in estetrol biosynthesis. *J Steroid Biochem* 1985;**22**:195–201.

Carlsen SM, Jacobsen G, Bjerve KS. Androgen levels in pregnant women decrease with increasing maternal age. *Scand J Clin Lab Invest* 2003;**63**:23–26.

Carlsen SM, Jacobsen G, Romundstad P. Maternal testosterone levels during pregnancy are associated with offspring size at birth. *Eur J Endocrinol* 2006;**155**:365–370.

Carson DJ, Okuno A, Lee PA, Stetten G, Didolkar SM, Migeon CJ. Amniotic fluid steroid levels. Fetuses with adrenal hyperplasia, 46,XXY fetuses, and normal fetuses. *Am J Dis Child* 1982;**136**:218–222.

Castracane VD, Asch RH. Testosterone and androstenedione in premature ovarian failure pregnancies: evidence for an ovarian source of androgens in early pregnancy. *Hum Reprod* 1995;**10**:677–680.

Castracane VD, Stewart DR, Gimpel T, Overstreet JW, Lasley BL. Maternal serum androgens in human pregnancy: early increases within the cycle of conception. *Hum Reprod* 1998;**13**:460–464.

Charest NJ, Zhou ZX, Lubahn DB, Olsen KL, Wilson EM, French FS. A frameshift mutation destabilizes androgen receptor messenger RNA in the Tfm mouse. *Mol Endocrinol* 1991;**5**:573–581.

Chevalier M, Gilbert G, Lory P, Marthan R, Quignard JF, Savineau JP. Dehydroepiandrosterone (DHEA) inhibits voltage-gated T-type calcium channels. *Biochem Pharmacol* 2012;**83**:1530–1539.

Chou TM, Sudhir K, Hutchison SJ, Ko E, Amidon TM, Collins P, Chatterjee K. Testosterone induces dilation of canine coronary conductance and resistance arteries in vivo. *Circulation* 1996;**94**:2614–2619.

Chow L, Lye SJ. Expression of the gap junction protein connexin-43 is increased in the human myometrium toward term and with the onset of labor. *Am J Obstet Gynecol* 1994;**170**:788–795.

Corbould A. Effects of androgens on insulin action in women: is androgen excess a component of female metabolic syndrome? *Diabetes Metab Res Rev* 2008; **24**:520–532.

Costarella CE, Stallone JN, Rutecki GW, Whittier FC. Testosterone causes direct relaxation of rat thoracic aorta. *J Pharmacol Exp Ther* 1996;**277**:34–39.

Coviello AD, Lakshman K, Mazer NA, Bhasin S. Differences in the apparent metabolic clearance rate of testosterone in young and older men with gonadotropin suppression receiving graded doses of testosterone. *J Clin Endocrinol Metab* 2006; **91**:4669–4675.

Crews JK, Khalil RA. Antagonistic effects of 17 beta-estradiol, progesterone, and testosterone on Ca2+ entry mechanisms of coronary vasoconstriction. *Arterioscler Thromb Vasc Biol* 1999a;**19**:1034–1040.

Crews JK, Khalil RA. Gender-specific inhibition of Ca2+ entry mechanisms of arterial vasoconstriction by sex hormones. *Clin Exp Pharmacol Physiol* 1999b;**26**:707–715.

Crisosto N, Echiburu B, Maliqueo M, Perez V, Ladron de Guevara A, Preisler JSanchez F, Sir-Petermann T. Improvement of hyperandrogenism and hyperinsulinemia during pregnancy in women with polycystic ovary syndrome: possible effect in the ovarian follicular mass of their daughters. *Fertil Steril* 2012;**97**:218–224.

Dahl SK, Thomas MA, Williams DB, Robins JC. Maternal virilization due to luteoma associated with delayed lactation. *Fertil Steril* 2008;**90**:2006, e2017–2009.

Dasgupta E, Singh G. Vaginal misoprostol vs vaginal misoprostol with estradiol for labor induction: a prospective double blind study. *J Obstet Gynaecol India* 2012;**62**:47–51.

Dawood MY, Saxena BB. Testosterone and dihydrotestosterone in maternal and cord blood and in amniotic fluid. *Am J Obstet Gynecol* 1977;**129**:37–42.

Deliconstantinos G. Structure activity relationship of cholesterol and steroid hormones with respect to their effects on the Ca2+-stimulated ATPase and lipid fluidity of synaptosomal plasma membranes from dog and rabbit brain. *Comp Biochem Physiol B* 1988;**89**:585–594.

Denayer S, Helsen C, Thorrez L, Haelens A, Claessens F. The rules of DNA recognition by the androgen receptor. *Mol Endocrinol* 2010;**24**:898–913.

Despres G, Rigaudiere N, Delost P. Changes in the metabolic clearance rate and plasma production rate of testosterone in the pregnant guinea-pig. *Steroids* 1982; **39**:703–710.

Dewailly D. Androgen excess in women: not just a cosmetic problem. *Ann Endocrinol (Paris)* 2010;**71**:1.

Diamanti-Kandarakis E, Alexandraki K, Piperi C, Protogerou A, Katsikis I, Paterakis T, Lekakis J, Panidis D. Inflammatory and endothelial markers in women with polycystic ovary syndrome. *Eur J Clin Invest* 2006;**36**:691–697.

Dick IM, Liu J, Glendenning P, Prince RL. Estrogen and androgen regulation of plasma membrane calcium pump activity in immortalized distal tubule kidney cells. *Mol Cell Endocrinol* 2003;**212**:11–18.

Diez d'Aux RC, Pearson Murphy BE. Androgens in the human fetus. *J Steroid Biochem* 1974;**5**:207–210.

Dillon PF, Aksoy MO, Driska SP, Murphy RA. Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. *Science* 1981;**211**:495–497.

Duval D, Durant S, Homo-Delarche F. Non-genomic effects of steroids. Interactions of steroid molecules with membrane structures and functions. *Biochim Biophys Acta* 1983;**737**:409–442.

Edman CD, Toofanian A, MacDonald PC, Gant NF. Placental clearance rate of maternal plasma androstenedione through placental estradiol formation: an indirect method of assessing uteroplacental blood flow. *Am J Obstet Gynecol* 1981;**141**:1029–1037.

Ekman G, Malmstrom A, Uldbjerg N, Ulmsten U. Cervical collagen: an important regulator of cervical function in term labor. *Obstet Gynecol* 1986;**67**:633–636.

Ekman-Ordeberg G, Stjernholm Y, Wang H, Stygar D, Sahlin L. Endocrine regulation of cervical ripening in humans—potential roles for gonadal steroids and insulin-like growth factor-I. *Steroids* 2003;**68**:837–847.

Elbaum DJ, Keyes PL. Synthesis of 17beta-estradiol by isolated ovarian tissues of the pregnant rat: aromatization in the corpus luteum. *Endocrinology* 1976;**99**:573–579.

El Maradny E, Kanayama N, Halim A, Maehara K, Sumimoto K, Terao T. Biochemical changes in the cervical tissue of rabbit induced by interleukin-8, interleukin-1beta, dehydroepiandrosterone sulphate and prostaglandin E2: a comparative study. *Hum Reprod* 1996;**11**:1099–1104.

Elmes MJ, Tan DS, Cheng Z, Wathes DC, McMullen S. The effects of a high-fat, high-cholesterol diet on markers of uterine contractility during parturition in the rat. *Reproduction* 2011;**141**:283–290.

Erkinheimo TL, Saukkonen K, Narko K, Jalkanen J, Ylikorkala O, Ristimaki A. Expression of cyclooxygenase-2 and prostanoid receptors by human myometrium. *J Clin Endocrinol Metab* 2000;**85**:3468–3475.

Escobar JC, Patel SS, Beshay VE, Suzuki T, Carr BR. The human placenta expresses CYP17 and generates androgens de novo. *J Clin Endocrinol Metab* 2011; **96**:1385–1392.

Escobar-Morreale HF. Diagnosis and management of hirsutism. *Ann N Y Acad Sci* 2010; **1205**:166–174.

Escobar-Morreale HF, Carmina E, Dewailly D, Gambineri A, Kelestimur F, Moghetti P, Pugeat M, Qiao J, Wijeyaratne CN, Witchel SF *et al.*. Epidemiology, diagnosis and management of hirsutism: a consensus statement by the Androgen Excess and Polycystic Ovary Syndrome Society. *Hum Reprod Update* 2012;**18**:146–170.

Espinos JJ, Corcoy R, Roca B, Rodriguez-Espinosa J, Calaf J. Serum insulin and androgen concentrations in women with histories of gestational diabetes. *Med Clin (Barc)* 1992; **98**:445–448.

Fata JE, Ho AT, Leco KJ, Moorehead RA, Khokha R. Cellular turnover and extracellular matrix remodeling in female reproductive tissues: functions of metalloproteinases and their inhibitors. *Cell Mol Life Sci* 2000;**57**:77–95.

Figueroa JP, Honnebier MB, Binienda Z, Wimsatt J, Nathanielsz PW. Effect of a 48-hour intravenous delta 4-androstenedione infusion on the pregnant rhesus monkey in the last third of gestation: changes in maternal plasma estradiol concentrations and myometrial contractility. *Am J Obstet Gynecol* 1989;**161**:481–486.

Fisch B, Margara RA, Winston RM, Hillier SG. Cellular basis of luteal steroidogenesis in the human ovary. *J Endocrinol* 1989;**122**:303–311.

Floyd R, Wray S. Calcium transporters and signalling in smooth muscles. *Cell Calcium* 2007;**42**:467–476.

Foradori CD, Weiser MJ, Handa RJ. Non-genomic actions of androgens. *Front Neuroendocrinol* 2008;**29**:169–181.

Foulk RA, Martin MC, Jerkins GL, Laros RK. Hyperreactio luteinalis differentiated from severe ovarian hyperstimulation syndrome in a spontaneously conceived pregnancy. *Am J Obstet Gynecol* 1997;**176**:1300–1302; discussion 1302–1304.

Franczak A. Endometrial and myometrial secretion of androgens and estrone during early pregnancy and luteolysis in pigs. *Reprod Biol* 2008;**8**:213–228.

Fuchs AR, Fuchs F, Husslein P, Soloff MS. Oxytocin receptors in the human uterus during pregnancy and parturition. *Am J Obstet Gynecol* 1984;**150**:734–741.

Fuller PJ, Pettigrew IG, Pike JW, Stockigt JR. An adrenal adenoma causing virilization of mother and infant. *Clin Endocrinol (Oxf)* 1983;**18**:143–153.

Gabbiani G, Schmid E, Winter S, Chaponnier C, de Ckhastonay C, Vandekerckhove J, Weber K, Franke WW. Vascular smooth muscle cells differ from other smooth muscle cells: predominance of vimentin filaments and a specific alpha-type actin. *Proc Natl Acad Sci USA* 1981;**78**:298–302.

Gant NF, Hutchinson HT, Siiteri PK, MacDonald PC. Study of the metabolic clearance rate of dehydroisoandrosterone sulfate in pregnancy. *Am J Obstet Gynecol* 1971; **111**:555–563.

Garfield RE, Hayashi RH. Appearance of gap junctions in the myometrium of women during labor. *Am J Obstet Gynecol* 1981;**140**:254–260.

Gatongi DK, Madhvi G, Tydeman G, Hasan A. A case of hyperreactio luteinalis presenting with eclampsia. *J Obstet Gynaecol* 2006;**26**:465–467.

Ghayee HK, Auchus RJ. Basic concepts and recent developments in human steroid hormone biosynthesis. *Rev Endocr Metab Disord* 2007;**8**:289–300.

Ghorashi V, Sheikhvatan M. The relationship between serum concentration of free testosterone and pre-eclampsia. *Endokrynol Pol* 2008;**59**:390–392.

Ghule VV, Gray C, Galimberti A, Anumba DO. Prostaglandin-induced cervical remodelling in humans in the first trimester is associated with increased expression of specific tight junction, but not gap junction proteins. *J Transl Med* 2012;**10**:40.

Goodyer CG, Branchaud CL. Regulation of hormone production in the human feto-placental unit. *Ciba Found Symp* 1981;**86**:89–123.

Grgic O, Radakovic B, Barisic D. Hyperreactio luteinalis could be a risk factor for development of HELLP syndrome: case report. *Fertil Steril* 2008;**90**:2008, e2013–2006.

Guibert C, Ducret T, Savineau JP. Voltage-independent calcium influx in smooth muscle. *Prog Biophys Mol Biol* 2008;**98**:10–23.

Gurpide E. Experimental designs used to estimate rates of steroid production and metabolism in vivo and in vitro. *Ann N Y Acad Sci* 1990;**595**:165–172.

Haas DM, Caldwell DM, Kirkpatrick P, McIntosh JJ, Welton NJ. Tocolytic therapy for preterm delivery: systematic review and network meta-analysis. *BMJ* 2012; **345**:e6226.

Haimov-Kochman R, Yanai N, Yagel S, Amsalem H, Lavy Y, Hurwitz A. Spontaneous ovarian hyperstimulation syndrome and hyperreactio luteinalis are entities in continuum. *Ultrasound Obstet Gynecol* 2004;**24**:675–678.

Hall PF, Chen S, Nakajin S, Shinoda M, Shively JE. Purification and characterization of aromatase from human placenta. *Steroids* 1987;**50**:37–50.

Hammes SR, Levin ER. Minireview: recent advances in extranuclear steroid receptor actions. *Endocrinology* 2011;**152**:4489–4495.

Hammond GL. Diverse roles for sex hormone-binding globulin in reproduction. *Biol Reprod* 2011;**85**:431–441.

Haq AN. Hyperreactio luteinalis associated with pregnancy induced hypertension. *J Coll Physicians Surg Pak* 2010;**20**:137–139.

Hensleigh PA, Carter RP, Grotjan HE Jr. Fetal protection against masculinization with hyperreactio luteinalis and virilization. *J Clin Endocrinol Metab* 1975; **40**:816–823.

Hirano M. Formation and biological activity of estriol in placenta. *Tohoku J Exp Med* 1961;**75**:89–102.

Hodgins MB. Binding of androgens in 5 alpha-reductase-deficient human genital skin fibroblasts: inhibition by progesterone and its metabolites. *J Endocrinol* 1982; **94**:415–427.

Holt HB, Medbak S, Kirk D, Guirgis R, Hughes I, Cummings MH, Meeking DR. Recurrent severe hyperandrogenism during pregnancy: a case report. *J Clin Pathol* 2005;**58**:439–442.

Horowitz A, Menice CB, Laporte R, Morgan KG. Mechanisms of smooth muscle contraction. *Physiol Rev* 1996;**76**:967–1003.

Hughes IA, Davies JD, Bunch TI, Pasterski V, Mastroyannopoulou K, MacDougall J. Androgen insensitivity syndrome. *Lancet* 2012;**380**:1419–1428.

Ikeda M, Shibata Y, Yamamoto T. Rapid formation of myometrial gap junctions during parturition in the unilaterally implanted rat uterus. *Cell Tissue Res* 1987; **248**:297–303.

Ikonen E. Cellular cholesterol trafficking and compartmentalization. *Nat Rev Mol Cell Biol* 2008;**9**:125–138.

Ito M, Hartshorne DJ. Phosphorylation of myosin as a regulatory mechanism in smooth muscle. *Prog Clin Biol Res* 1990;**327**:57–72.

Ito T, Horton R. The source of plasma dihydrotestosterone in man. *J Clin Invest* 1971; **50**:1621–1627.

Jasonni VM, Bonavia M, Lodi S, Preti S, Bulletti C, Flamigni C. Androstenedione metabolism in human uterine tissues: endometrium, myometrium and leiomyoma. *J Steroid Biochem* 1982;**17**:547–551.

Jennings LJ, Xu QW, Firth TA, Nelson MT, Mawe GM. Cholesterol inhibits spontaneous action potentials and calcium currents in guinea pig gallbladder smooth muscle. *Am J Physiol* 1999;**277**:G1017–G1026.

Ji H, Dailey TL, Long V, Chien EK. Androgen-regulated cervical ripening: a structural, biomechanical, and molecular analysis. *Am J Obstet Gynecol* 2008;**198**:543, e541–549.

Ji H, Long V, Briody V, Chien EK. Progesterone modulates integrin {alpha}2 (ITGA2) and {alpha}11 (ITGA11) in the pregnant cervix. *Reprod Sci* 2011;**18**:156–163.

Joseph DR. Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. *Vitam Horm* 1994;**49**:197–280.

Kanayama N, El Maradny E, Goto J, Terao T. Effect of dehydroepiandrosterone sulfate on interleukin-8 receptor during cervical ripening. *Eur J Endocrinol* 1998; **138**:587–593.

Kanova N, Bicikova M. Hyperandrogenic states in pregnancy. *Physiol Res* 2011; **60**:243–252.

Karjalainen MK, Huusko JM, Ulvila J, Sotkasiira J, Luukkonen A, Teramo K, Plunkett J, Anttila V, Palotie A, Haataja R *et al.* A potential novel spontaneous preterm birth gene, AR, identified by linkage and association analysis of X chromosomal markers. *PLoS One* 2012;**7**:e51378.

Keelan JA, Mattes E, Tan H, Dinan A, Newnham JP, Whitehouse AJ, Jacoby P, Hickey M. Androgen concentrations in umbilical cord blood and their association with maternal, fetal and obstetric factors. *PLoS One* 2012;**7**:e42827.

Konoplya EF, Popoff EH. Identification of the classical androgen receptor in male rat liver and prostate cell plasma membranes. *Int J Biochem* 1992;**24**:1979–1983.

Kouloumenta V, Hatziefthimiou A, Paraskeva E, Gourgoulianis K, Molyvdas PA. Non-genomic effect of testosterone on airway smooth muscle. *Br J Pharmacol* 2006;**149**:1083–1091.

Koyuncu F, Aksoy S, Karanfil C, Demir N, Bozkurt K, Tuncay G. [The effect of dehydroepiandrosterone sulfate on cervical maturation during pregnancy. *J Gynecol Obstet Biol Reprod (Paris)* 1995;**24**:630–633.

Kubli-Garfias C, Lopez-Fiesco A, Pacheco-Cano MT, Ponce-Monter H, Bondani A. In vitro effects of androgens upon the spontaneous rat uterine contractility. *Steroids* 1980;**35**:633–641.

Kupittayanant S, Luckas MJ, Wray S. Effect of inhibiting the sarcoplasmic reticulum on spontaneous and oxytocin-induced contractions of human myometrium. *BJOG* 2002;**109**:289–296.

Kutryk MJ, Pierce GN. Stimulation of sodium-calcium exchange by cholesterol incorporation into isolated cardiac sarcolemmal vesicles. *J Biol Chem* 1988; **263**:13167–13172.

Labrie F, Martel C, Balser J. Wide distribution of the serum dehydroepiandrosterone and sex steroid levels in postmenopausal women: role of the ovary? *Menopause* 2011;**18**:30–43.

Laherty RF, Rotten D, Yamamoto M, Jaffe RB. Effects of oestradiol and prolactin on progesterone production by rhesus monkey luteal cells in vitro. *Acta Endocrinol (Copenh)* 1985;**108**:266–272.

Lang F, Alevizopoulos K, Stournaras C. Targeting membrane androgen receptors in tumors. *Expert Opin Ther Targets* 2013;**17**:951–963.

Large WA. Receptor-operated Ca2(+)-permeable nonselective cation channels in vascular smooth muscle: a physiologic perspective. *J Cardiovasc Electrophysiol* 2002;**13**:493–501.

Larmon JE, Magann EF, Dickerson GA, Morrison JC. Outpatient cervical ripening with prostaglandin E2 and estradiol. *J Matern Fetal Neonatal Med* 2002;**11**:113–117.

Leppert PC. Anatomy and physiology of cervical ripening. *Clin Obstet Gynecol* 1995; **38**:267–279.

Li C, Meng Q, Yu X, Jing X, Xu P, Luo D. Regulatory effect of connexin 43 on basal Ca2+ signaling in rat ventricular myocytes. *PLoS One* 2012;**7**:e36165.

Likis FE, Edwards DR, Andrews JC, Woodworth AL, Jerome RN, Fonnesbeck CJ, McKoy JN, Hartmann KE. Progestogens for preterm birth prevention: a systematic review and meta-analysis. *Obstet Gynecol* 2012;**120**:897–907.

Liu YX, Hsueh AJ. Synergism between granulosa and theca-interstitial cells in estrogen biosynthesis by gonadotropin-treated rat ovaries: studies on the two-cell, two-gonadotropin hypothesis using steroid antisera. *Biol Reprod* 1986;**35**:27–36.

Liu L, Li Y, Xie N, Shynlova O, Challis JR, Slater D, Lye S, Dong X. Proliferative action of the androgen receptor in human uterine myometrial cells—a key regulator for myometrium phenotype programming. *J Clin Endocrinol Metab* 2013;**98**:218–227.

Loch-Caruso RK, Criswell KA, Grindatti CM, Brant KA. Sustained inhibition of rat myometrial gap junctions and contractions by lindane. *Reprod Biol Endocrinol* 2003; **1**:62.

Longcope C. Adrenal and gonadal androgen secretion in normal females. *Clin Endocrinol Metab* 1986;**15**:213–228.

Longcope C. Dehydroepiandrosterone metabolism. *J Endocrinol* 1996;**150**(Suppl.): S125–S127.

Lorzadeh N, Kazemirad S. The effects of fetal gender on serum human chorionic gonadotropin and testosterone in normotensive and preeclamptic pregnancies. *J Pregnancy* 2012;**2012**:874290.

Loudon JA, Sooranna SR, Bennett PR, Johnson MR. Mechanical stretch of human uterine smooth muscle cells increases IL-8 mRNA expression and peptide synthesis. *Mol Hum Reprod* 2004;**10**:895–899.

Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science* 1988;**240**:327–330.

Ludwig M, Beck A, Wickert L, Bolkenius U, Tittel B, Hinkel K, Bidlingmaier F. Female pseudohermaphroditism associated with a novel homozygous G-to-A (V370-to-M) substitution in the P-450 aromatase gene. *J Pediatr Endocrinol Metab* 1998;**11**:657–664.

Lye SJ, Mitchell J, Nashman N, Oldenhof A, Ou R, Shynlova O, Langille L. Role of mechanical signals in the onset of term and preterm labor. *Front Horm Res* 2001; **27**:165–178.

Lynn KN, Steinkeler JA, Wilkins-Haug LE, Benson CB. Hyperreactio luteinalis (enlarged ovaries) during the second and third trimesters of pregnancy: common clinical associations. *J Ultrasound Med* 2013;**32**:1285–1289.

Macleod DJ, Sharpe RM, Welsh M, Fisken M, Scott HM, Hutchison GR, Drake AJ, van den Driesche S. Androgen action in the masculinization programming window and development of male reproductive organs. *Int J Androl* 2010;**33**:279–287.

Mahendroo MS, Russell DW. Male and female isoenzymes of steroid 5alpha-reductase. *Rev Reprod* 1999;**4**:179–183.

Mahendroo MS, Cala KM, Russell DW. 5 alpha-reduced androgens play a key role in murine parturition. *Mol Endocrinol* 1996;**10**:380–392.

Mahendroo MS, Porter A, Russell DW, Word RA. The parturition defect in steroid 5alpha-reductase type 1 knockout mice is due to impaired cervical ripening. *Mol Endocrinol* 1999;**13**:981–992.

Manabe Y, Sakaguchi M, Nakajima A. Initiation of uterine contractions by purely mechanical stretching of the uterus at midpregnancy. *Int J Biol Res Pregnancy* 1981; **2**:63–69.

Maradny E, Kanayama N, Maehara K, Kobayashi T, Terao T. Dehydroepiandrosterone sulfate potentiates the effect of interleukin-8 on the cervix. *Gynecol Obstet Invest* 1996;**42**:191–195.

Marchetti PM, Barth JH. Clinical biochemistry of dihydrotestosterone. *Ann Clin Biochem* 2013;**50**:95–107.

Masuyama H, Tateishi Y, Matsuda M, Hiramatrsu Y. Hyperreactio luteinalis with both markedly elevated human chorionic gonadotropin levels and an imbalance of angiogenic factors subsequently developed severe early-onset preeclampsia. *Fertil Steril* 2009;**92**:393, e391–393.

Maymon E, Romero R, Pacora P, Gomez R, Athayde N, Edwin S, Yoon BH. Human neutrophil collagenase (matrix metalloproteinase 8) in parturition, premature rupture of the membranes, and intrauterine infection. *Am J Obstet Gynecol* 2000; **183**:94–99.

McGregor JA, Leff M, Orleans M, Baron A. Fetal gender differences in preterm birth: findings in a North American cohort. *Am J Perinatol* 1992;**9**:43–48.

Mecenas CA, Giussani DA, Owiny JR, Jenkins SL, Wu WX, Honnebier BO, Lockwood CJ, Kong L, Guller S, Nathanielsz PW. Production of premature delivery in pregnant rhesus monkeys by androstenedione infusion. *Nat Med* 1996; **2**:443–448.

Meldrum E, Parker PJ, Carozzi A. The PtdIns-PLC superfamily and signal transduction. *Biochim Biophys Acta* 1991;**1092**:49–71.

Mendel CM. The free hormone hypothesis: a physiologically based mathematical model. *Endocr Rev* 1989;**10**:232–274.

Milewich L, Gomez-Sanchez C, Madden JD, Bradfield DJ, Parker PM, Smith SL, Carr BR, Edman CD, MacDonald PC. Dehydroisoandrosterone sulfate in peripheral blood of premenopausal, pregnant and postmenopausal women and men. *J Steroid Biochem* 1978;**9**:1159–1164.

Milewich L, MacDonald PC, Guerami A, Midgett WT, Lassiter WL, Carr BR. Human fetal liver estrogen 16 alpha-hydroxylase: precursor specificity, kinetic parameters, and in vitro regulation. *J Clin Endocrinol Metab* 1986;**63**:180–191.

Mitchell FL, Shackleton CH. The investigation of steroid metabolism in early infancy. *Adv Clin Chem* 1969;**12**:141–215.

Mizuno M, Lobotsky J, Lloyd CW, Kobayashi T, Murasawa Y. Plasma androstenedione and testerone during pregnancy and in the newborn. *J Clin Endocrinol Metab* 1968; **28**:1133–1142.

Mochizuki M, Maruo T. Effect of dehydroepiandrosterone sulfate on uterine cervical ripening in late pregnancy. *Acta Physiol Hung* 1985;**65**:267–274.

Mochizuki M, Honda T, Deguchi M, Morikawa H, Tojo S. A study on the effect of dehydroepiandrosterone sulfate on so-called cervical ripening. *Acta Obstet Gynecol Scand* 1978a;**57**:397–401.

Mochizuki M, Honda T, Tojo S. Collagenolytic activity and steroid levels after administration of dehydroepiandrosterone sulfate. *Int J Gynaecol Obstet* 1978b; **16**:248–253.

Montano LM, Calixto E, Figueroa A, Flores-Soto E, Carbajal V, Perusquia M. Relaxation of androgens on rat thoracic aorta: testosterone concentration dependent agonist/antagonist L-type Ca2+ channel activity, and 5beta-dihydrotestosterone restricted to L-type Ca2+ channel blockade. *Endocrinology* 2008;**149**:2517–2526.

Morisset AS, Dube MC, Drolet R, Pelletier M, Labrie F, Luu-The V, Tremblay Y, Robitaille J, John Weisnagel S, Tchernof A. Androgens in the maternal and fetal circulation: association with insulin resistance. *J Matern Fetal Neonatal Med* 2013; **26**:513–519.

Murphy JG, Khalil RA. Decreased [Ca(2+)](i) during inhibition of coronary smooth muscle contraction by 17beta-estradiol, progesterone, and testosterone. *J Pharmacol Exp Ther* 1999;**291**:44–52.

Nakajima A. Action potential of human myometrial fibers. *Am J Obstet Gynecol* 1971; **111**:266–269.

Nathanielsz PW, Jenkins SL, Tame JD, Winter JA, Guller S, Giussani DA. Local paracrine effects of estradiol are central to parturition in the rhesus monkey. *Nat Med* 1998; **4**:456–459.

Nishinaka K, Fukuda Y. Changes in extracellular matrix materials in the uterine myometrium of rats during pregnancy and postparturition. *Acta Pathol Jpn* 1991; **41**:122–132.

Noble K, Matthew A, Burdyga T, Wray S. A review of recent insights into the role of the sarcoplasmic reticulum and Ca entry in uterine smooth muscle. *Eur J Obstet Gynecol Reprod Biol* 2009;**144**(Suppl. 1):S11–S19.

Norman JE. Preterm labour. Cervical function and prematurity. *Best Pract Res Clin Obstet Gynaecol* 2007;**21**:791–806.

Norman M, Ekman G, Malmstrom A. Changed proteoglycan metabolism in human cervix immediately after spontaneous vaginal delivery. *Obstet Gynecol* 1993; **81**:217–223.

Norman JE, Bollapragada S, Yuan M, Nelson SM. Inflammatory pathways in the mechanism of parturition. *BMC Pregnancy Childbirth* 2007;**7**(Suppl. 1):S7.

Novy MJ, Walsh SW. Dexamethasone and estradiol treatment in pregnant rhesus macaques: effects on gestational length, maternal plasma hormones, and fetal growth. *Am J Obstet Gynecol* 1983;**145**:920–931.

O'Brien M, O'Shaughnessy D, Ahamide E, Morrison JJ, Smith TJ. Differential expression of the metalloproteinase MMP3 and the alpha5 integrin subunit in human myometrium at labour. *Mol Hum Reprod* 2007;**13**:655–661.

Okon MA, Laird SM, Tuckerman EM, Li TC. Serum androgen levels in women who have recurrent miscarriages and their correlation with markers of endometrial function. *Fertil Steril* 1998;**69**:682–690.

O'Leary P, Boyne P, Flett P, Beilby J, James I. Longitudinal assessment of changes in reproductive hormones during normal pregnancy. *Clin Chem* 1991;**37**:667–672.

Ortega A, Mas-Oliva J. Cholesterol effect on enzyme activity of the sarcolemmal (Ca2+ + Mg2+)-ATPase from cardiac muscle. *Biochim Biophys Acta* 1984; **773**:231–236.

Pasanen M, Pelkonen O. The expression and environmental regulation of P450 enzymes in human placenta. *Crit Rev Toxicol* 1994;**24**:211–229.

Penning TM. New frontiers in androgen biosynthesis and metabolism. *Curr Opin Endocrinol Diabetes Obes* 2010;**17**:233–239.

Pereira de Jesus-Tran K, Cote PL, Cantin L, Blanchet J, Labrie F, Breton R. Comparison of crystal structures of human androgen receptor ligand-binding domain complexed with various agonists reveals molecular determinants responsible for binding affinity. *Protein Sci* 2006;**15**:987–999.

Perusquia MC, G. Inhibitory effect of androgens and progestins on the contraction induced by oxytocin in the rat myometrium. *Med Sci Res* 1991;**19**:177–179.

Perusquia M, Garcia-Yanez E, Ibanez R, Kubli-Garfias C. Non-genomic mechanism of action of delta-4 and 5-reduced androgens and progestins on the contractility of the isolated rat myometrium. *Life Sci* 1990;**47**:1547–1553.

Perusquia M, Campos G, Corona JL, Kubli-Garfias C. Antagonism by 5-reduced steroids of the tonic and phasic contractions induced by serotonin in the isolated rat uterus. *Proc West Pharmacol Soc* 1991a;**34**:395–398.

Perusquia M, Corona JL, Kubli-Garfias C. Inhibitory effect of 5-reduced androgens and progestins on the uterine contraction induced by acetylcholine. *Proc West Pharmacol Soc* 1991b;**34**:89–92.

Perusquia M, Navarrete E, Jasso-Kamel J, Montano LM. Androgens induce relaxation of contractile activity in pregnant human myometrium at term: a nongenomic action on L-type calcium channels. *Biol Reprod* 2005;**73**:214–221.

Phelan N, Conway GS. Management of ovarian disease in pregnancy. *Best Pract Res Clin Endocrinol Metab* 2011;**25**:985–992.

Piltonen T, Koivunen R, Morin-Papunen L, Ruokonen A, Huhtaniemi IT, Tapanainen JS. Ovarian and adrenal steroid production: regulatory role of LH/HCG. *Hum Reprod* 2002;**17**:620–624.

Pion R, Jaffe R, Eriksson G, Wiqvist N, Diczfalusy E. Studies on the metabolism of c-21 steroids in the human foeto-placental unit. I. Formation of a beta-unsaturated 3-ketones in midterm placentas perfused in situ with pregnenolone and 17-alpha-hydroxypregnenolone. *Acta Endocrinol (Copenh)* 1965;**48**:234–248.

Pluciennik F, Verrecchia F, Bastide B, Herve JC, Joffre M, Deleze J. Reversible interruption of gap junctional communication by testosterone propionate in cultured Sertoli cells and cardiac myocytes. *J Membr Biol* 1996;**149**:169–177.

Pressman EK, Tucker JA Jr, Anderson NC Jr, Young RC. Morphologic and electrophysiologic characterization of isolated pregnant human myometrial cells. *Am J Obstet Gynecol* 1988;**159**:1273–1279.

Pulkkinen MO, Nyman S, Hamalainen MM, Mattinen J. Proton NMR spectroscopy of the phospholipids in human uterine smooth muscle and placenta. *Gynecol Obstet Invest* 1998;**46**:220–224.

Purohit A, Foster PA. Steroid sulfatase inhibitors for estrogen- and androgen-dependent cancers. *J Endocrinol* 2012;**212**:99–110.

Rao PM, Kelly DM, Jones TH. Testosterone and insulin resistance in the metabolic syndrome and T2DM in men. *Nat Rev Endocrinol* 2013;**9**:479–493.

Read CP, Word RA, Ruscheinsky MA, Timmons BC, Mahendroo MS. Cervical remodeling during pregnancy and parturition: molecular characterization of the softening phase in mice. *Reproduction* 2007;**134**:327–340.

Rehman KS, Yin S, Mayhew BA, Word RA, Rainey WE. Human myometrial adaptation to pregnancy: cDNA microarray gene expression profiling of myometrium from non-pregnant and pregnant women. *Mol Hum Reprod* 2003;**9**:681–700.

Richardson MC, Masson GM. Stimulation by human chorionic gonadotrophin of oestradiol production by dispersed cells from human corpus luteum: comparison with progesterone production; utilization of exogenous testosterone. *J Endocrinol* 1981;**91**:197–203.

Rivarola MA, Forest MG, Migeon CJ. Testosterone, androstenedione and dehydroepiandrosterone in plasma during pregnancy and at delivery: concentration and protein binding. *J Clin Endocrinol Metab* 1968;**28**:34–40.

Rivas A, Fisher JS, McKinnell C, Atanassova N, Sharpe RM. Induction of reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbestrol: evidence for importance of the androgen-estrogen balance. *Endocrinology* 2002; **143**:4797–4808.

Robinson JD, Judd HL, Young PE, Jones OW, Yen SS. Amniotic fluid androgens and estrogens in midgestation. *J Clin Endocrinol Metab* 1977;**45**:755–761.

Robson . Reactions of the uterine muscle and endometrium of rabbit to testosterone. *Quart J Exp Physiol* 1937;**35**:355–363.

Rodeck CH, Gill D, Rosenberg DA, Collins WP. Testosterone levels in midtrimester maternal and fetal plasma and amniotic fluid. *Prenat Diagn* 1985;**5**:175–181.

Roh CR, Oh WJ, Yoon BK, Lee JH. Up-regulation of matrix metalloproteinase-9 in human myometrium during labour: a cytokine-mediated process in uterine smooth muscle cells. *Mol Hum Reprod* 2000;**6**:96–102.

Romero R, Espinoza J, Goncalves LF, Kusanovic JP, Friel L, Hassan S. The role of inflammation and infection in preterm birth. *Semin Reprod Med* 2007;**25**:21–39.

Rosano GM, Leonardo F, Pagnotta P, Pelliccia F, Panina G, Cerquetani E, della Monica PL, Bonfigli B, Volpe M, Chierchia SL. Acute anti-ischemic effect of testosterone in men with coronary artery disease. *Circulation* 1999;**99**:1666–1670.

Rosmond R. Androgen excess in women—a health hazard? *Med Hypotheses* 2006; **67**:229–234.

Rothman MS, Carlson NE, Xu M, Wang C, Swerdloff R, Lee P, Goh VH, Ridgway EC, Wierman ME. Reexamination of testosterone, dihydrotestosterone, estradiol and estrone levels across the menstrual cycle and in postmenopausal women measured by liquid chromatography-tandem mass spectrometry. *Steroids* 2011; **76**:177–182.

Saez JM, Forest MG, Morera AM, Bertrand J. Metabolic clearance rate and blood production rate of testosterone and dihydrotestosterone in normal subjects, during pregnancy, and in hyperthyroidism. *J Clin Invest* 1972;**51**:1226–1234.

Sakyo K, Ito A, Mori Y. Effects of dehydroepiandrosterone sulphate on the production of collagenase and gelatinolytic metalloproteinase by rabbit uterine cervical cells in primary cultures. *J Pharmacobiodyn* 1986;**9**:276–286.

Sakyo K, Ito A, Mori Y. Dehydroepiandrosterone sulfate stimulates collagenase synthesis without affecting the rates of collagen and noncollagen protein syntheses by rabbit uterine cervical fibroblasts. *Biol Reprod* 1987;**36**:277–281.

Sanders SL, Stouffer RL, Brannian JD. Androgen production by monkey luteal cell subpopulations at different stages of the menstrual cycle. *J Clin Endocrinol Metab* 1996;**81**:591–596.

Sasaki K, Nakano R, Kadoya Y, Iwao M, Shima K, Sowa M. Cervical ripening with dehydroepiandrosterone sulphate. *Br J Obstet Gynaecol* 1982;**89**:195–198.

Sathishkumar K, Balakrishnan M, Chinnathambi V, Chauhan M, Hankins GD, Yallampalli C. Fetal sex-related dysregulation in testosterone production and their receptor expression in the human placenta with preeclampsia. *J Perinatol* 2012; **32**:328–335.

Schwarzel WC, Kruggel WG, Brodie HJ. Studies on the mechanism of estrogen biosynthesis. 8. The development of inhibitors of the enzyme system in human placenta. *Endocrinology* 1973;**92**:866–880.

Scott HM, Hutchison GR, Mahood IK, Hallmark N, Welsh M, De Gendt K, Verhoeven G, O'Shaughnessy P, Sharpe RM. Role of androgens in fetal testis development and dysgenesis. *Endocrinology* 2007;**148**:2027–2036.

Scott HM, Mason JI, Sharpe RM. Steroidogenesis in the fetal testis and its susceptibility to disruption by exogenous compounds. *Endocr Rev* 2009;**30**:883–925.

Sen A, Hammes SR. Granulosa cell-specific androgen receptors are critical regulators of ovarian development and function. *Mol Endocrinol* 2010;**24**:1393–1403.

Sharifzadeh F, Kashanian M, Fatemi F. A comparison of serum androgens in pre-eclamptic and normotensive pregnant women during the third trimester of pregnancy. *Gynecol Endocrinol* 2012;**28**:834–836.

Shmigol A, Eisner DA, Wray S. Carboxyeosin decreases the rate of decay of the [Ca2+]i transient in uterine smooth muscle cells isolated from pregnant rats. *Pflugers Arch* 1998;**437**:158–160.

Shmygol A, Noble K, Wray S. Depletion of membrane cholesterol eliminates the Ca2+-activated component of outward potassium current and decreases membrane capacitance in rat uterine myocytes. *J Physiol* 2007;**581**:445–456.

Shozu M, Akasofu K, Harada T, Kubota Y. A new cause of female pseudohermaphroditism: placental aromatase deficiency. *J Clin Endocrinol Metab* 1991;**72**:560–566.

Shynlova O, Mitchell JA, Tsampalieros A, Langille BL, Lye SJ. Progesterone and gravidity differentially regulate expression of extracellular matrix components in the pregnant rat myometrium. *Biol Reprod* 2004;**70**:986–992.

Shynlova O, Tsui P, Dorogin A, Langille BL, Lye SJ. Insulin-like growth factors and their binding proteins define specific phases of myometrial differentiation during pregnancy in the rat. *Biol Reprod* 2007;**76**:571–578.

Shynlova O, Tsui P, Jaffer S, Lye SJ. Integration of endocrine and mechanical signals in the regulation of myometrial functions during pregnancy and labour. *Eur J Obstet Gynecol Reprod Biol* 2009;**144**(Suppl. 1):S2–10.

Siiteri PK, MacDonald PC. Placental estrogen biosynthesis during human pregnancy. *J Clin Endocrinol Metab* 1966;**26**:751–761.

Siiteri PK, Simberg NH. Changing concepts of active androgens in blood. *Clin Endocrinol Metab* 1986;**15**:247–258.

Simon C, Einspanier A. The hormonal induction of cervical remodeling in the common marmoset monkey (Callithrix jacchus). *Reproduction* 2009;**137**:517–525.

Simons K, Vaz WL. Model systems, lipid rafts, and cell membranes. *Annu Rev Biophys Biomol Struct* 2004;**33**:269–295.

Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD *et al.* Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr Rev* 1994;**15**:342–355.

Simsek Y, Celen S, Ustun Y, Danisman N, Bayramoglu H. Severe preeclampsia and fetal virilization in a spontaneous singleton pregnancy complicated by hyperreactio luteinalis. *Eur Rev Med Pharmacol Sci* 2012;**16**:118–121.

Sir-Petermann T, Maliqueo M, Angel B, Lara HE, Perez-Bravo F, Recabarren SE. Maternal serum androgens in pregnant women with polycystic ovarian syndrome: possible implications in prenatal androgenization. *Hum Reprod* 2002;**17**:2573–2579.

Sir-Petermann T, Hitchsfeld C, Maliqueo M, Codner E, Echiburu B, Gazitua R, Recabarren S, Cassorla F. Birth weight in offspring of mothers with polycystic ovarian syndrome. *Hum Reprod* 2005;**20**:2122–2126.

Slater DM, Dennes WJ, Campa JS, Poston L, Bennett PR. Expression of cyclo-oxygenase types-1 and -2 in human myometrium throughout pregnancy. *Mol Hum Reprod* 1999;**5**:880–884.

Slayden OD, Nayak NR, Burton KA, Chwalisz K, Cameron ST, Critchley HO, Baird DT, Brenner RM. Progesterone antagonists increase androgen receptor expression in the rhesus macaque and human endometrium. *J Clin Endocrinol Metab* 2001; **86**:2668–2679.

Slomczynska M, Duda M, Burek M, Knapczyk K, Czaplicki D, Koziorowski M. Distribution of androgen receptor in the porcine uterus throughout pregnancy. *Reprod Domest Anim* 2008;**43**:35–41.

Smith R. Parturition. *N Engl J Med* 2007;**356**:271–283.

Smith RD, Babiychuk EB, Noble K, Draeger A, Wray S. Increased cholesterol decreases uterine activity: functional effects of cholesterol alteration in pregnant rat myometrium. *Am J Physiol Cell Physiol* 2005;**288**:C982–C988.

Smith R, Paul J, Maiti K, Tolosa J, Madsen G. Recent advances in understanding the endocrinology of human birth. *Trends Endocrinol Metab* 2012;**23**:516–523.

Sooranna SR, Lee Y, Kim LU, Mohan AR, Bennett PR, Johnson MR. Mechanical stretch activates type 2 cyclooxygenase via activator protein-1 transcription factor in human myometrial cells. *Mol Hum Reprod* 2004;**10**:109–113.

Sparey C, Robson SC, Bailey J, Lyall F, Europe-Finner GN. The differential expression of myometrial connexin-43, cyclooxygenase-1 and -2, and Gs alpha proteins in the upper and lower segments of the human uterus during pregnancy and labor. *J Clin Endocrinol Metab* 1999;**84**:1705–1710.

Stocco C, Telleria C, Gibori G. The molecular control of corpus luteum formation, function, and regression. *Endocr Rev* 2007;**28**:117–149.

Stouffer RL, Nixon WE, Gulyas BJ, Hodgen GD. Gonadotropin-sensitive progesterone production by rhesus monkey luteal cells in vitro: a function of age of the corpus luteum during the menstrual cycle. *Endocrinology* 1977;**100**:506–512.

Strauss JF 3rd, Martinez F, Kiriakidou M. Placental steroid hormone synthesis: unique features and unanswered questions. *Biol Reprod* 1996;**54**:303–311.

Tagawa N, Hidaka Y, Takano T, Shimaoka Y, Kobayashi Y, Amino N. Serum concentrations of dehydroepiandrosterone and dehydroepiandrosterone sulfate and their relation to cytokine production during and after normal pregnancy. *Clin Chim Acta* 2004;**340**:187–193.

Takahashi K, Ikeno N, Hoshiai H, Suzuki M. The effect of dehydroepiandrosterone sulfate on serum steroid concentration in singleton and twin pregnancies and its effect on cervical canal-ripening in singleton pregnancies. *Tohoku J Exp Med* 1984; **142**:289–298.

Tep-areenan P, Kendall DA, Randall MD. Testosterone-induced vasorelaxation in the rat mesenteric arterial bed is mediated predominantly via potassium channels. *Br J Pharmacol* 2002;**135**:735–740.

Terzidou V, Sooranna SR, Kim LU, Thornton S, Bennett PR, Johnson MR. Mechanical stretch up-regulates the human oxytocin receptor in primary human uterine myocytes. *J Clin Endocrinol Metab* 2005;**90**:237–246.

Thiery M, De Gezelle H, Van Kets H, Voorhoof L, Verheugen C, Smis B, Gerris J, Derom R, Martens G. The effect of locally administered estrogens on the human cervix. *Z Geburtshilfe Perinatol* 1979;**183**:448–452.

Thomas P, Dressing G, Pang Y, Berg H, Tubbs C, Benninghoff A, Doughty K. Progestin, estrogen and androgen G-protein coupled receptors in fish gonads. *Steroids* 2006; **71**:310–316.

Thorneloe KS, Nelson MT. Ion channels in smooth muscle: regulators of intracellular calcium and contractility. *Can J Physiol Pharmacol* 2005;**83**:215–242.

Tica AA, Dun EC, Tica OS, Gao X, Arterburn JB, Brailoiu GC, Oprea TI, Brailoiu E. G protein-coupled estrogen receptor 1-mediated effects in the rat myometrium. *Am J Physiol Cell Physiol* 2011;**301**:C1262–C1269.

Tomiyasu BA, Chen CJ, Marshall JM. Comparison of the activity of circular and longitudinal myometrium from pregnant rats: co-ordination between muscle layers. *Clin Exp Pharmacol Physiol* 1988;**15**:647–656.

Traish AM, Kang HP, Saad F, Guay AT. Dehydroepiandrosterone (DHEA)—a precursor steroid or an active hormone in human physiology. *J Sex Med* 2011; **8**:2960–2982; quiz 2983.

Tsai ML, Cesen-Cummings K, Webb RC, Loch-Caruso R. Acute inhibition of spontaneous uterine contractions by an estrogenic polychlorinated biphenyl is associated with disruption of gap junctional communication. *Toxicol Appl Pharmacol* 1998;**152**:18–29.

Umezaki H, Hess DL, Valenzuela GJ, Ducsay CA. Fetectomy alters maternal pituitary-adrenal function in pregnant rhesus macaques. *Biol Reprod* 2001; **65**:1616–1621.

Van Bommel T, Marsen T, Bojar H. Effects of high-dose medroxyprogesterone acetate and various other steroid hormones on plasma membrane lipid mobility in CAMA-1 mammary cancer cells. *Anticancer Res* 1987;**7**:1217–1223.

van der Kwast TH, Dommerholt HB, van Vroonhoven CC, Chadha S. Androgen receptor expression in the cervix of androgen-treated female-to-male transsexuals: association with morphology and chain-specific keratin expression. *Int J Gynecol Pathol* 1994;**13**:133–138.

Van Holsbeke C, Amant F, Veldman J, De Boodt A, Moerman P, Timmerman D. Hyperreactio luteinalis in a spontaneously conceived singleton pregnancy. *Ultrasound Obstet Gynecol* 2009;**33**:371–373.

van Meer G. Lipid traffic in animal cells. *Annu Rev Cell Biol* 1989;**5**:247–275.

Vejrazkova D, Vcelak J, Vankova M, Lukasova P, Bradnova O, Halkova T, Kancheva R, Bendlova B. Steroids and insulin resistance in pregnancy. *J Steroid Biochem Mol Biol* 2014;**139**:122–129.

Veleminsky M Jr. Hyperreactio luteinalis in spontaneous singleton pregnancy. *Neuro Endocrinol Lett* 2010;**31**:304–305.

Verbist J, Gadella TW Jr, Raeymaekers L, Wuytack F, Wirtz KW, Casteels R. Phosphoinositide-protein interactions of the plasma-membrane Ca2(+)-transport ATPase as revealed by fluorescence energy transfer. *Biochim Biophys Acta* 1991;**1063**:1–6.

Vermeirsch H, Van den Broeck W, Coryn M, Simoens P. Immunohistochemical detection of androgen receptors in the canine uterus throughout the estrus cycle. *Theriogenology* 2002;**57**:2203–2216.

Vermeulen A, Ando S. Metabolic clearance rate and interconversion of androgens and the influence of the free androgen fraction. *J Clin Endocrinol Metab* 1979; **48**:320–326.

Vladic-Stjernholm Y, Vladic T, Blesson CS, Ekman-Ordeberg G, Sahlin L. Prostaglandin treatment is associated with a withdrawal of progesterone and androgen at the receptor level in the uterine cervix. *Reprod Biol Endocrinol* 2009;**7**:116.

Walters KA, Allan CM, Handelsman DJ. Androgen actions and the ovary. *Biol Reprod* 2008;**78**:380–389.

Webley GE, Hearn JP. Local production of progesterone by the corpus luteum of the marmoset monkey in response to perfusion with chorionic gonadotrophin and melatonin in vivo. *J Endocrinol* 1987;**112**:449–457.

Welsh M, Saunders PT, Fisken M, Scott HM, Hutchison GR, Smith LB, Sharpe RM. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest* 2008;**118**:1479–1490.

Wilke TJ, Utley DJ. Total testosterone, free-androgen index, calculated free testosterone, and free testosterone by analog RIA compared in hirsute women and in otherwise-normal women with altered binding of sex-hormone-binding globulin. *Clin Chem* 1987;**33**:1372–1375.

Willows RL. Urinary estriol estimations in normal and abnormal pregnancies. *Can Med Assoc J* 1966;**94**:1098–1101.

Wilson CM, McPhaul MJ. A and B forms of the androgen receptor are expressed in a variety of human tissues. *Mol Cell Endocrinol* 1996;**120**:51–57.

Witayavanitkul N, Woranush W, Bupha-Intr T, Wattanapermpool J. Testosterone regulates cardiac contractile activation by modulating SERCA but not NCX activity. *Am J Physiol Heart Circ Physiol* 2013;**304**:H465–H472.

Word RA, Li XH, Hnat M, Carrick K. Dynamics of cervical remodeling during pregnancy and parturition: mechanisms and current concepts. *Semin Reprod Med* 2007; **25**:69–79.

Wray S, Jones K, Kupittayanant S, Li Y, Matthew A, Monir-Bishty E, Noble K, Pierce SJ, Quenby S, Shmygol AV. Calcium signaling and uterine contractility. *J Soc Gynecol Investig* 2003;**10**:252–264.

Wray S, Burdyga T, Noble K. Calcium signalling in smooth muscle. *Cell Calcium* 2005; **38**:397–407.

Yamamoto M, Feigenbaum SL, Crites Y, Escobar GJ, Yang J, Ferrara A, Lo JC. Risk of preterm delivery in non-diabetic women with polycystic ovarian syndrome. *J Perinatol* 2012;**32**:770–776.

Yamashita A, Oshima S, Matsuo K, Ito K, Ito A, Mori Y. Pharmacological studies of intravaginally applied dehydroepiandrosterone sulfate (DHA-S). *Nihon Yakurigaku Zasshi* 1991;**98**:31–39.

Yang X, Guo Z, Sun F, Li W, Alfano A, Shimelis H, Chen M, Brodie AM, Chen H, Xiao Z *et al.* Novel membrane-associated androgen receptor splice variant potentiates proliferative and survival responses in prostate cancer cells. *J Biol Chem* 2011; **286**:36152–36160.

Zeitlin J, Saurel-Cubizolles MJ, De Mouzon J, Rivera L, Ancel PY, Blondel B, Kaminski M. Fetal sex and preterm birth: are males at greater risk? *Hum Reprod* 2002; **17**:2762–2768.

Zhang J, Bricker L, Wray S, Quenby S. Poor uterine contractility in obese women. *BJOG* 2007;**114**:343–348.