THE UNIVERSITY *of* EDINBURGH

Novel regulators of human gonadal development

Sharon Lynn Eddie

BSc, Iowa State University

MSc, University of Edinburgh

Centre for Reproductive Health

Queen's Medical Research Institute

47 Little France Crescent

Edinburgh EH16 4TJ

Thesis submitted to the University of Edinburgh for the Degree of Doctor of Philosophy

October 2011

Declaration

The studies undertaken in the thesis were the sole work of the author, except where acknowledgement is made by reference. The work described in this thesis has not been previously accepted for, or is currently being submitted for another degree or qualification at the University of Edinburgh or any other institution.

Sharon Eddie

October 2011

Acknowledgements

Although this thesis bears my name, the work within would not have been possible without the assistance of several people along the way.

First and foremost, I'd like to thank my supervisors, Profs Richard Anderson and Henry Jabbour, for their help over the past three years. I cannot thank them enough for their support and guidance, and more importantly their patience with my American English! I appreciate the time and effort they have spent helping me launch my scientific career. Although not officially my supervisor, I would also like to deeply thank Dr. Andrew Childs (IMoS), whose tireless hours of work both in the lab and at the desk, have been invaluable. You have truly moulded me into a better scientist and have taught me so much. I only hope I can pass on to others the mentorship you provided me.

I'd also like to thank the other members of the Anderson lab who helped me with my work over the years:

Firstly, to Anne Saunderson and Joan Creiger, I could not have asked for better research nurses—I always look forward to your calls in the morning. Also, many thanks to Rosey Bayne who helped me throughout my PhD and kick started my work by performing the PGE_2 cultures, which became the basis of my initial work. Additional thanks to Hazel Kinnell, who also assisted in some of my work, spending hours on the stereologer performing the e17.5 paracetamol counts. I'd also like to thank Craig Collins and Jing He for their help and technical support in the lab. I'd like to make a special note of thanking my two lovely students: Karen Kilcoyne, who helped with the PROK testis work, and Lenka Hrabalkova, who helped with the initial paracetamol work. I hope I wasn't too much of a slave driver! You were both great students and in teaching you, I learnt a lot myself.

In addition, I would like to thank members of the old histology gang: Sheila MacPherson, Mike Millar, Aranzta Esnal, and Marion Walker. You've taught me all the basic skills I needed; from immunohistochemistry to stereology, and when things became more than difficult, you were always there to help me troubleshoot novel protocols and provide me with such amazing expertise. In addition, I would like to thank Dr. Pam Brown for her help and expertise in the creation of plasmid utilised to stably transfect the T-Cam2 cells with *PROKR1*. You were always patient with me and had time for my questions, no matter how simple the answer.

Outwith the lab, I'd also like to thank my scientific colleagues and close friends for their support. We all know there are days when the PhD is not 'smooth-sailing', and you've all provided an assisting hand or a kind ear to keep me motivated. I'd especially like to thank Laura, Shalini, Joao, Juile, Kirsten, Afshan, Matt, Michelle, Karen, Hazel, Abdi and Uma. You guys are absolutely amazing friends (not to mention scientists). A massive **THANK YOU** for all you've done over the years.

Finally, I'd like to thank my fiancé, Gareth for being overwhelmingly supportive, as well as my soon to be in-laws for their patience with my incessant working on holidays and weekends. And last but not least, I'd like to thank my family-- without you I would never have taken the plane over to Edinburgh four years ago to start on this crazy path. I'd especially like to thank my mother, Peggy, who sacrificed a lot for me over the years and has always just been a phone call away when I needed.

Abstract

The production of viable germ cells during human embryonic development determines adult reproductive success. This is particularly true for females, as development of germ cells (GCs) into primordial follicles before birth is imperative for future fertility. During fetal development GCs migrate to the genital ridge to form the gonad, after which several tightly regulated events, including proliferation, differentiation, and association with somatic cells, must occur to form a functional gonad. In the ovary these processes also include the initiation and subsequent arrest of meiosis. These developmental processes are orchestrated by local autocrine and paracrine factors, many of which remain to be identified in the human.

In order to decipher further the pathways by which the gonad and GCs develop, potential regulators including prostaglandin (PG) E_2, the interleukin (IL)6-type cytokines, and the prokinetecins (PROKs), were examined in the human fetal ovary and PROKs in the human fetal testis. Patterns of gene expression, protein localisation, function, and interaction of the potential mediators throughout human development (8-20 weeks gestation) were determined. Primary fetal tissue was investigated, in addition to immortalized GCs (T-Cam2 cells) and a murine model of fetal ovarian development.

PGE_2 interacts with known regulators of GC development in non-reproductive organs. It was postulated PGE_2 may regulate GC progression by modulating these factors. Examination of PGE_2 receptors and precursor enzymes in the fetal ovary revealed that all were present and some were developmentally regulated, with mRNA expression increasing with gestation. These developmentally regulated components were localised to the GCs. The PGE_2 receptors were among those differentially expressed, with one localised solely to mature GCs. Culture of human fetal ovary confirmed that PGE_2 regulates known regulators of GC development, increasing expression of survival and anti-apoptotic factors. To test the hypothesis that PGE_2 is necessary for female GC development, paracetamol, an inhibitor of PGE_2 precursor enzymes, was utilised in a murine model of fetal exposure. Fetal ovaries from this experiment displayed disruption of normal development.

The IL6-type cytokines are also postulated to be involved in early gonad development, and are known to regulate proliferation and differentiation of mouse embryonic stem and GCs *in vitro.* A significant increase in transcript levels of the shared receptor components was determined in second trimester human ovaries, as well as developmental increases of several of the IL6-type ligands. Both common receptor components were located specifically in the GCs identifying them as the target of IL6 action in the human fetal ovary.

The PROKs regulate cell migration, proliferation and differentiation, and modulate secretion of PGE_2 and expression of some IL6-type cytokines. To-date, PROKs have not been examined in the human fetal gonad. Transcript levels were higher in the fetal testis compared to the ovary, with receptor and ligand components increasing with gestation. Most components also increased with gestation in the ovary. However, location of PROK components was strikingly different between the two tissues, with GCs being the primary target of PROK action in the fetal ovary, and Leydig and interstitial cells being the target in the testis. PROKs interaction with other regulators of gonad development was examined utilising a GC line in the case of the ovary and primary interstitial cell cultures in the case of the testis.

These studies have identified new factors involved in human fetal gonad development, and how they interact with known regulatory pathways of development.

Presentations relating to this thesis

Oral Presentations

Prokineticins Exert Anti-Apoptotic Effects in the Human Fetal Testis
Society for the Study of Reproduction Portland, OR, USA 2011

Identifying the Targets of IL-6 Ligand Action in the Human Fetal Ovary

Society for Reproduction and Fertility Oxford, UK 2009
Awarded SRF Student Prize for 2009

Poster Presentations

Maternal Paracetamol Administration Affects Fetal Ovarian Germ Cell Development

Society for Reproduction and Fertility Brighton, UK 2011

Maternal Acetaminaphen Affects on Fetal Ovarian Germ Cell Development

Society for Gynecological Investigation Miami, FL, USA 2011

Prokineticins in the Human Fetal Testis

Society for Reproduction and Fertility Nottingham, UK 2010

Prokineticins in the Human Fetal Ovary
National Ovarian Workshop Cambridge, UK 2009

Novel Effects of Prostaglandin E2 in the Developing Human Ovary

Society for Gynecological Investigation Glasgow, UK 2008

Publications relating to this thesis

Bayne R, **Eddie S**, *et al*. Prostaglandin E2 as a regulator of germ cells during ovarian development. *JCEM* **2009** 10:4053-60.

Eddie, S, *et al*. Developmentally-regulated IL6-type cytokines signal to germ cells in the human fetal ovary. *Mol Hum Reprod* **2011** DOI: 10.1093/molehr/gar061

Full text can be found in the appendices.

Table of Contents

List of Figures

List of Tables

Commonly Used Abbreviations

AMH	anti-Müllerian hormone
BAD	BCL-2 associated agonist of cell death
BAX	BCL-2 associate X protein
BCL-2	B-cell lymphoma/leukemia-2
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BMP	bone morphogenic protein
bp	base pair
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
C°	degrees Celsius
CC3	cleaved caspase-3
cDNA	Complementary Deoxyribonucleic Acid
c-Kit	Kit ligand receptor
CNTF	ciliary neurotrophic factor
CNTFR	ciliary neurotrophic factor receptor
COX	cyclooxygenase
CXCR4	chemokine receptor 4
Dazl	deleted in azoospermia like
DKK1	dikkopf homolog 1
e	embryonic day
ECM	extracellular matrix
EP	PGE2 receptor
ER	estrogen receptor
ES	embryonic stem (cell)
FIGLA	factor in the germline alpha
FOXL2	forkhead box L2
FOXO3a	forkhead box O3a
GC	germ cell
GDF9	growth differentiation factor 9
GFP	green flouroscent protein
gp130	glycoprotein-130
H_2O	water
HRP	horse radish peroxidase
ID	inhibitor of differentiation
IL6	interleukin 6
IL6R	interleukin 6 receptor
Jak	Janus kinases
KL	Kit ligand
l	liter
LHX8	LIM homeobox 8
LIF	leukemia inhibitory factor
LIFR	leukemia inhibitory factor receptor
MAPK	Mitogen-Activated Protein Kinase
Mcl-1	myeloid leukemia sequence-1
min	minute
MIS	Müllerian inhibiting substance
MOF	multiple oocyte follicle
MVH	mouse VASA homologue
Nobox	newborn ovary homeobox
NS	normal serum
NSAID	non-steroidal anti-inflammatory drug
NT4	neurotrophin-4
OSM	oncostatin-M
OSMR	oncostatin-M receptor
PBS	phosphate buffered saline
PG	pre-granulosa cell
PGC	primordial germ cell
PHH3	phospho-histone H3
pnd	postnatal day
POI	premature ovarian insufficiency
PROK	prokineticin
PROKR	prokineticin receptor
PTGES	prostaglandin E synthase
RA	retinoic acid
RAR	retinoic acid receptor
rpm	revolutions per minute
RSPO1	roof plate-specific spondin-1
SDF1	stromal derived factor 1
sec	second
SF1	steroidogenic factor 1
SM	somatic cell
Socs	suppresor of cytokine signaling
SOHLH	spermatogenesis and oogenesis specific basic helix-loop-helix
Stat	signal transducer and activator of transcription
Stra8	stimulated by retinoic acid
TBS	tris buffered saline
TGFβ	transforming growth factor β
TNAP	tissue non-specific alkaline phosphatase
TRK	tropomyosin-related kinase
VEGF	vascular endothelial growth factor
WNT4	wingless-type MMTV integration site 4
wpf	weeks post fertilisation

Chapter 1

Literature Review

Chapter 1. Literature Review

1.1 Introduction

Fetal ovarian development is comprised of many complex processes, initiating with germ cell specification and migration to form the bi-potential gonad, followed by sex determination and entry to meiosis, culminating in primordial follicle formation (Edson *et al*, 2009). Proper development of the ovary and primordial follicles during fetal life will define a female's future reproductive capacity (McGee & Hsueh, 2000; Adhikari & Liu, 2010; Reddy *et al*, 2010).

Fetal ovarian development is regulated by the germ cell niche; a milieu of autocrine, juxtacrine and paracrine factors of both germ cell and somatic cell origin that govern the processes necessary for fetal ovarian function. The germ cell niche is not only important for regulation of the ovarian processes described above, but also oocyte survival and proliferation, in concert with germ cell atresia. In the human fetal ovary, germ cell numbers reach 7 million at 20 weeks (Baker, 1963), followed by rapid depletion of the oocyte population via apoptosis, such that ~85% of oocytes are lost by birth and over 99% by puberty (Baker, 1963; Forabosco *et al*, 1991) (Figure 1.1). This dynamic interplay between survival and apoptosis regulates which oocytes are available for future ovulation, disruption of which can lead to premature ovarian insufficiency (POI) or infertility.

Although some factors have been identified as regulators of these processes, their interaction with other regulators and the overall mechanisms governing fetal ovarian development remain unclear. For this reason, examination of the established factors involved in fetal ovarian development, and their interactions with possible novel mediators is essential for further understanding of this critical developmental window.

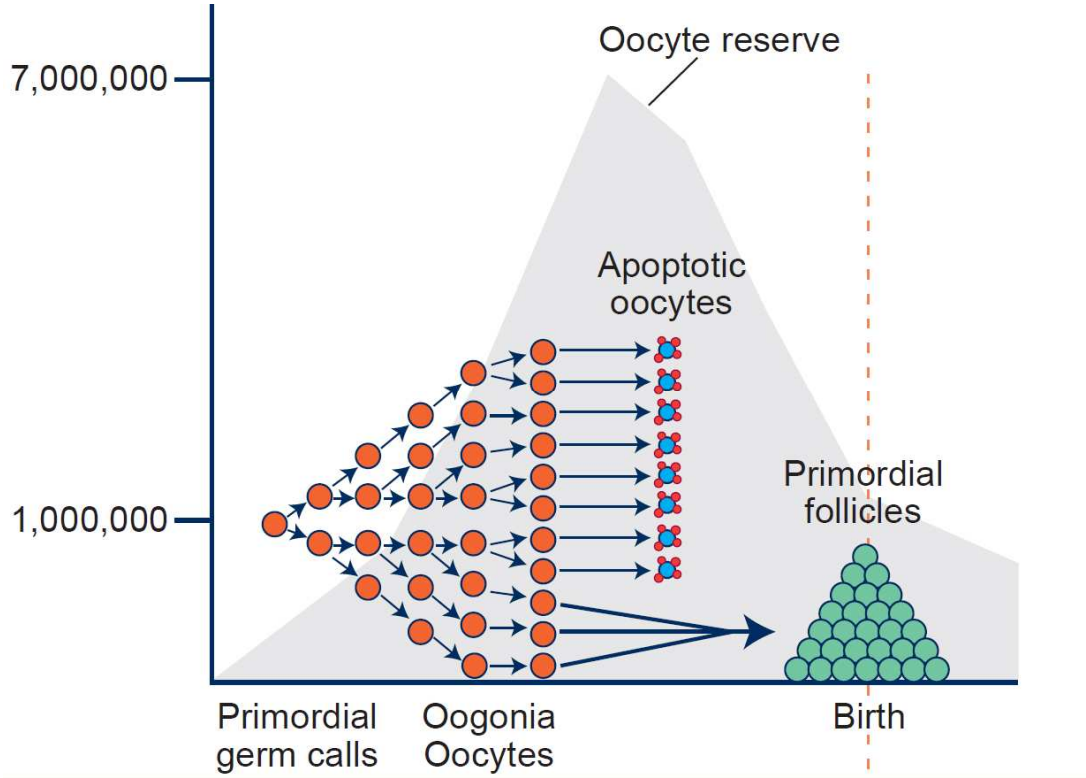

Figure 1.1 Schematic of human ovarian development.

Demonstrating germ cell number changes before birth leading up to follicle formation. Figure adapted from (Kaipia & Hsueh, 1997).

1.2 Fetal Gonad Development

The following sections will focus predominantly on human data; however due to limitations in availability and age range of human fetal tissue, most factors are initially examined in murine models before being characterised in human tissue. Therefore, key processes and known regulators of early ovarian development will be discussed as characterised in the mouse with further more limited information in the human fetal ovary. In addition, although mouse ovarian development and primordial follicle formation involves similar processes to those in the human, there are key species-specific differences. Notably, mouse gestation is ~20 days, with primordial follicle formation occurring postnatally and nest breakdown being governed by estrogen, whereas human primordial follicle formation happens prenatally and

steroidogenic cues have not been identified-although it remains possible that they contribute. Table 1.1 summarises the key stages of ovarian development and their timing in both species.

Estimation of gestational age is done in one of two ways in the human; it is either estimated as weeks post fertilisation (wpf) or gestational age as determined from the last menstrual period (gestation) (Lynch & Zhang, 2007). Fertilisation generally occurs 2 weeks after the last menstrual period in a women with a regular 28 day cycle, thus development deemed gestational age is 2 weeks later than wpf age.

Table 1.1 Gestational comparison of ovarian development between the human and mouse

(Abbreviations: e, embryonic day; pnd, postnatal day; wpf, weeks post fertilization)

Event	Human	Human Reference	Mouse	Mouse Reference
GC Specification	≤3 w gestation	(Falin, 1969)	e 5.5	(Saitou, 2009)
GC Migration	4-5 w gestation	(Fujimoto *et al*, 1977)	e 7.5	(Anderson *et al*, 2000)
Colonisation of the Gonad	4 wpf (6 w gestation)	(Francavilla *et al*, 1990)	e10.5	(Molyneaux *et al*, 2001)
Sex Determination	6-7 wpf (8-9 w gestation)	(Moore, 1967)	e 12.5	(Adams & McLaren, 2002)
Initiation of Meiosis	11 w gestation	(Motta *et al*, 1997)	e 13.5	(Hilscher *et al*, 1974)
Primordial Follicle Formation	18 w gestation	(Hertig & Adams, 1967)	pnd 0	(Epifano & Dean, 2002)
Length of Gestation	40 w gestation	(Guerrero & Florez, 1969)	20-21 d	(Lanman & Seidman, 1977)

1.2.1 Germ Cell Specification

During mammalian fetal development, precursor cells which will develop into primordial germ cells (PGCs) originate in the proximal epiblast (Chiquoine, 1954a; Lawson & Hage, 1994), and are induced by signals from the adjacent stroma to become the germline, a process which is deemed epigenesis and occurs between embryonic days (e)5.5-7.5 in the mouse (Saitou, 2009).

Key to initiation of PGC specification is signalling from the bone morphogenic proteins (Bmp) 2, 4, and 8. Bmp4 and 8b signal from the extra embryonic ectoderm (Lawson *et al*, 1999; Ying *et al*, 2000) whereas Bmp2 signals from the visceral endoderm (Ying & Zhao, 2001). These factors signal to the proximal epiblast via their transducer proteins Smad1 and 5 which are translocated to the cell nucleus via the mediator protein Smad4 in order to interact with other transcriptional activators and modulate gene expression. Bmp signalling promotes expression of further PGC factors, which as a whole interact to suppress somatic genes and initiate the PGC pathway in a subset of cells at ~e5.5 in the mouse (Lawson *et al*, 1999). The importance of Bmp signalling was confirmed with targeted disruption of *Bmp4* and *Bmp8* in rodent models: these have a reduced number of PGCs which are unable to migrate or proliferate as a result of disruption of specification (Lawson *et al*, 1999; Ying *et al*, 2000). Mouse models with disruptions of *Smad1* or *5* also display reduced numbers of PGCs (Chang & Matzuk, 2001; Tremblay *et al*, 2001), as does disruption of their intracellular mediator *Smad4* (Chu *et al*, 2004).

Bmp signalling in precursor PGCs promotes expression of several PGC factors, most notably; interferon-inducible gene 3 (Ifitm3/*fragilis*), PR domain-containing 1(Prdm1/*Blimp1*), and POU domain 5f1 (*Pou5f1*/Oct4) (Ying & Zhao, 2001; Saitou *et al*, 2002; Tanaka & Matsui, 2002; Ohinata *et al*, 2005; Vincent *et al*, 2005; Okamura *et al*, 2008). Bmp4 expression up-regulates *fragilis*, which was hypothesised to be key for PGC specification, as cells expressing *fragilis* at high levels initiate expression the PGC marker *Stella* and repress somatic genes such as *Hoxa1, Hoxb1, Lim1 and Evx1* (Saitou *et al*, 2002). However, expression of *fragilis* does not confine a cell to the PGC fate pathway (Saitou *et al*, 2002; Tanaka & Matsui, 2002) and deletion of *fragilis* from embryos does not result in a phenotype

(Lange *et al*, 2008). Further, four other related genes (*fragilis2-5)* have been identified, of which *fragilis2* and *3* are also implicated in PGC specification (Saitou *et al*, 2003).

Bmp signalling also regulates Blimp1, a post-transcriptional repressor which functions in precursor PGCs to suppress somatic gene expression (Ohinata *et al*, 2005; Yabuta *et al*, 2006; Hayashi *et al*, 2007). Blimp1 expression commits the fate of a cell, and is therefore thought to be the earliest PGC-specific marker (Ohinata *et al*, 2005). Along with repression of somatic genes, Blimp1 also promotes maintenance and/or resumption of pluripotency associated genes, such as *Sox2* and *Nanog* (Ohinata *et al*, 2005; Yamaguchi *et al*, 2005; Yabuta *et al*, 2006). This role was confirmed by deletion of *Blimp1 in vitro*, which resulted in lack of somatic gene repression, as well as only 50% of PGC-related gene induction (Saitou *et al*, 2005). Further, systemic deletion of *Blimp1* in mouse embryos resulted in a small cohort of PGC-like cells which were unable to migrate or proliferate (Ohinata *et al*, 2005; Robertson *et al*, 2007). Prdm14, a Blimp1 related factor, has also recently been deemed essential for PGC specification (Yabuta *et al*, 2006; Yamaji *et al*, 2008). In addition to the pluripotency- associated factors up-regulated by Blimp1, Oct4 expression is maintained in cells destined to become the germline; becoming restricted solely to these cells by e7.5 in the mouse (Scholer *et al*, 1990; Ohmura *et al*, 2004) and is also essential for PGC specification (Okamura *et al*, 2008).

1.2.2 Primordial Germ Cell Migration

Once PGCs have been specified they begin their journey to the genital ridge to form the gonad. This process is well documented in the mouse, with PGC migration initiating in the primitive streak at e7.5 (Clark & Eddy, 1975; Anderson *et al*, 2000), after which the PGCs move through the hindgut at e8.0-9.5, and arrive at the genital ridge at e10.5-e11.5 (Lawson & Hage, 1994; Molyneaux *et al*, 2001). In the human migration occurs at 4-5 week gestation (Fujimoto *et al*., 1977). Migration is controlled by several factors including chemoattractants, guiding the cells, and regulators, initiating expression of motility factors within the PGC population. Early studies hypothesised that PGCs responded to a signal or trigger that initiated migration (Godin *et al*, 1990) and change in morphology of the PGCs prior to

initiation of migration was noted, with the cells becoming polar with cytoplasmic protrusions allowing for motility (Anderson *et al*, 2000). It is thought this trigger might be expression of Kit ligand (KL/Scf), a somatic cue necessary for PGC motility (Runyan *et al*, 2006; Gu *et al*, 2009), which functions via a tyrosine kinase receptor, c-kit, located on the PGCs themselves to promote migratory competence. Absence of KL results in a dramatic decrease of PGC migration (Godin *et al*, 1991; Manova *et al*, 1990). Bmp proteins are thought to regulate KL expression, as inhibition of Bmp signalling by the antagonist Noggin results in decreased KL expression and disruption of migration (Dudley *et al*, 2007).

Stromal derived factor 1 (Sdf1/Cxcl12) is a chemoattractant expressed by the genital ridge, which signals through the chemokine receptor 4 (Cxcr4) expressed by PGCs and is thought to guide the PGCs to the gonad (Ara *et al*, 2003; Molyneaux *et al*, 2003b; Stebler *et al*, 2004). Targeted disruption of *Cxcr4* results in a failure of PGC migration (Molyneaux *et al*, 2003b). The PGCs also utilise several components of the extracellular matrix (ECM) to aid progression of migration toward the gonad including collagen III-V, fibronectin, and laminin (Ffrenchconstant *et al*, 1991; GarciaCastro *et al*, 1997; Anderson *et al*, 1999; Soto-Suazo *et al*, 2004), all of which PGCs are able to adhere to, suggesting the ECM aids in progression of the cells. It has been suggested the ECM may be involved in concentrating other growth factors which directly regulate PGC migration (Bendel-Stenzel *et al*, 1998).

Finally, it is further postulated that cell-cell interaction is able to promote migration and survival of the PGCs. Linkage of PGCs to form a network during migration has been noted in the fetal mouse (Gomperts *et al*, 1994; Molyneaux *et al*, 2001). This hypothesis is supported by the expression of e-cadherin, Pecam1, and β1-integrin by PGCs during migration, which are factors necessary for cell-cell adhesion and interaction (Bendel-Stenzel *et al*, 1998; Anderson *et al*, 1999; Di Carlo & De Felici, 2000; Wakayama *et al*, 2003). This may promote not only interaction with adjacent PGCs but also the surrounding stromal cells.

In addition to promotion of motility and guidance of the PGCs, several factors have been implicated in survival of the PGC cohort during migration including nanos homolog 3 (Nanos3) and dead end homolog 1 (Dnd1) (Tsuda *et al*, 2003; Youngren *et al*, 2005; Suzuki *et al*, 2009).

1.2.3 Proliferation and Colonisation of the Gonad

PGCs proliferation occurs during migration and continues upon arrival to the gonad, such that germ cells number between ~10,000-25,000 at 6 wpf in the human (8 weeks gestation) (Monk & McLaren, 1981; Byskov, 1986; Lawson & Hage, 1994; Bendsen *et al*, 2006). This proliferation is regulated by several factors implicated in migration including KL (Dolci *et al*, 1991; Buehr *et al*, 1993; Pesce *et al*, 1993a) and SDF1, but also by the fibroblast growth factors (FGFs) (Resnick *et al*, 1992; Kawase *et al*, 2004) and possibly the interleukin-6 (IL6)-type cytokines (Defelici & Dolci, 1991; Matsui *et al*, 1991; Pesce *et al*, 1993a) which will be further discussed later in this chapter.

Upon arrival in the gonad the PGCs lose their ability to migrate (Defelici *et al*, 1992; Gomperts *et al*, 1994; GarciaCastro *et al*, 1997) and their morphology changes to a larger, rounded profile (Donovan *et al*, 1986). In addition, the PGCs lose expression of several pluripotency-associated genes (Chiquoine, 1954b; Tsuda *et al*, 2003) and genome-wide demethylation occurs (Lopes *et al*, 2008). However, expression of some pluripotency-associated genes is maintained, including *Oct4* (Scholer *et al*, 1990) , but this may be because Oct4 is necessary for later function in germ cell development (Kehler *et al*, 2004). In contrast, developing germ cells begin to express a new set of gene markers including deleted in azoospermia like (Dazl) (Selgman and Page) and mouse-vasa homologue (Mvh/VASA) (Toyooka *et al*, 2000). This change in expression of germ cell markers is also observed in human fetal ovaries (Anderson *et al*, 2007), although the change in expression of OCT4 to VASA or DAZL occurs later in ovarian development in the human.

1.2.4 Sex Determination

Following germ cell migration, the gonad remains bipotential with little initial organisation of the somatic, interstitial or germ cells (Figure 1.2, page 8). In the human the process of sex determination is initiated by expression of the sex-determining region-Y (*SRY*) gene positioned on the Y chromosome in male offspring at ~6-7 weeks gestation (Gubbay *et al*, 1990; Sinclair *et al*, 1990; Tilmann & Capel, 1999; Tilmann & Capel, 2002). If an embryo has inherited a Y chromosome from the paternal donor, the *SRY* gene it carries prompts a signalling cascade resulting in a male phenotype including testis and secondary sex organs including Wolffian ducts and penis. Proof that the *SRY* gene is critical for sex determination was provided by Koopman *et al*, who demonstrated that a male phenotype results if the *SRY* gene is expressed in embryonic XX fetuses (Koopman *et al*, 1991).

SRY expression in Sertoli precursor cells triggers sex determination, although the exact mechanism by which this happens has yet to be fully elucidated (Albrecht & Eicher, 2001; Wilhelm *et al*, 2005). SRY prompts expression of downstream transcription factors and cytokines which induce testis formation. More specifically, SRY is likely to promote the up-regulation of a chemoattractant that allows for the mesonephroic cells to enter the testis for the formation of seminiferous tubules (Capel *et al*, 1999).

Some of SRY's downstream targets have been identified. Of known importance are the genes *SOX9* and steroidogenic factor-1 (*SF1*) (Ramkissoon & Goodfellow, 1996). SOX9 is expressed slightly after SRY in XY gonads (Koopman *et al*, 2001; Kobayashi *et al*, 2005), and is therefore likely to be downstream of SRY. It is thought the increase in *SOX9* expression is via direct regulation of a *SOX9* enhancer by SRY (Sekido & Lovell-Badge, 2008). However, XX genotypes expressing an additional copy of the *SOX9* gene develop testes; expression of SOX9 alone can promote a male phenotype without the expression of SRY (Huang *et al*, 1999). SOX9 and SF1 promote masculinisation of the Sertoli and Leydig cells in the testis, thereby promoting the expression of Anti-Müllerian hormone (AMH, also known as Müllerian inhibiting substance (MIS)) and testosterone, preventing the development

of the Müllerian ducts and promoting the formation of seminiferous tubules respectively (Shen *et al*, 1994; Arango *et al*, 1999).

Until recently it was considered that the female/ovarian fate pathway was a 'default' based upon Alfred Jost's experiments in the late 1940s (Jost, 1947). He noted that regardless of chromosomal sex (XX or XY), castrated fetal rabbits would develop female secondary sex organs (Wolffian ducts and external genitalia). However, the idea of a 'default setting' is becoming less favoured, with the consensus shifting towards the hypothesis that active signalling is necessary for both testis and ovarian development (Yao *et al*, 2002).

Starting as early as the 1980s several factors have been identified as opposing forces to the male SRY signalling pathway. This derives from the finding that XY individuals expressing two copies of an active gene on the X chromosome developed ovarian structures (Bernstein *et al*, 1980; Bardoni *et al*, 1994). This gene was identified as dosage-sensitive sex reversal-congenital adrenal hypoplasia critical region on the X chromosome protein-1 (*DAX1*) and appears to work in an antagonistic fashion against the male pathway by limiting *SF1* expression (Nachtigal *et al*, 1998; Swain *et al*, 1998). Additionally, the wingless-type MMTV integration site 4 (*Wnt4*) gene has been proven to be essential for ovarian development. Female mice homozygous null for *Wnt4* fail to form ovaries, and the structures that do form express AMH and testosterone production enzymes (Vainio *et al*, 1999). Moreover, *Wnt4*$^{-/-}$ mice have demonstrated the protein is able to regulate follistatin expression to inhibit the formation of the male coelomic vessel (Yao *et al*, 2004).

WNT4 signals via canonical β-catenin signalling, along with another ovarian regulating factor, roof plate-specific spondin 1 (RSPO1; (Tevosian & Manuylov, 2008). *Rspo1*-null XX gonads are partially sex reversed (Chassot *et al*, 2008; Tomizuka *et al*, 2008), as are human female patients with mutations in the *RSPO1* gene (Parma *et al*, 2006). Rspo1 may be up-stream or regulate *Wnt4*, as up-regulation of *Wnt4* during early ovarian development is not seen in *Rspo1*-null mice (Chassot *et al*, 2008; Tomizuka *et al*, 2008). *Wnt4* expression is down-regulated after the onset of SRY expression in the testes, denoting there is indirect regulation

of WNT4 by SRY, further promoting the male pathway (Bernard *et al*, 2008). Part of this down-regulation is due to the expression of Dickkopf homolog 1 (*DKK1*) (Manuylov *et al*, 2008), as this factor has been shown to repress Wnt4/Rspo1 β-catenin signalling (Manuylov *et al*, 2008) and is up-regulated specifically in the testes (Combes *et al*, 2011).

Another factor known to be essential for ovarian development is forkhead box L2 protein (FOXL2), as it is capable of repressing expression of *SOX9* and its downstream pathway (Ottolenghi *et al*, 2005; Wilhelm *et al*, 2009). Over-expression of FOXL2 in XY individuals results in disrupted testicular formation and mass disorganisation (deemed ovo-testes) (Ottolenghi *et al*, 2007). FOX factors are also able to interact with estrogen receptor genes (ESRs) (Carroll *et al*, 2005), which are actively involved in gonad development in other species (Crisponi *et al*, 2001; Cocquet *et al*, 2002a; Loffler *et al*, 2003) and have recently been characterised in the human fetal ovary (Fowler *et al*, 2011).

With these mediators in mind (summarised in Figure 1.2), it can be stated that ovarian development is not a default as suggested previously, but rather both testes and ovaries require active signalling for functional gonad development, as well as the ability to repress the alternative developmental pathway.

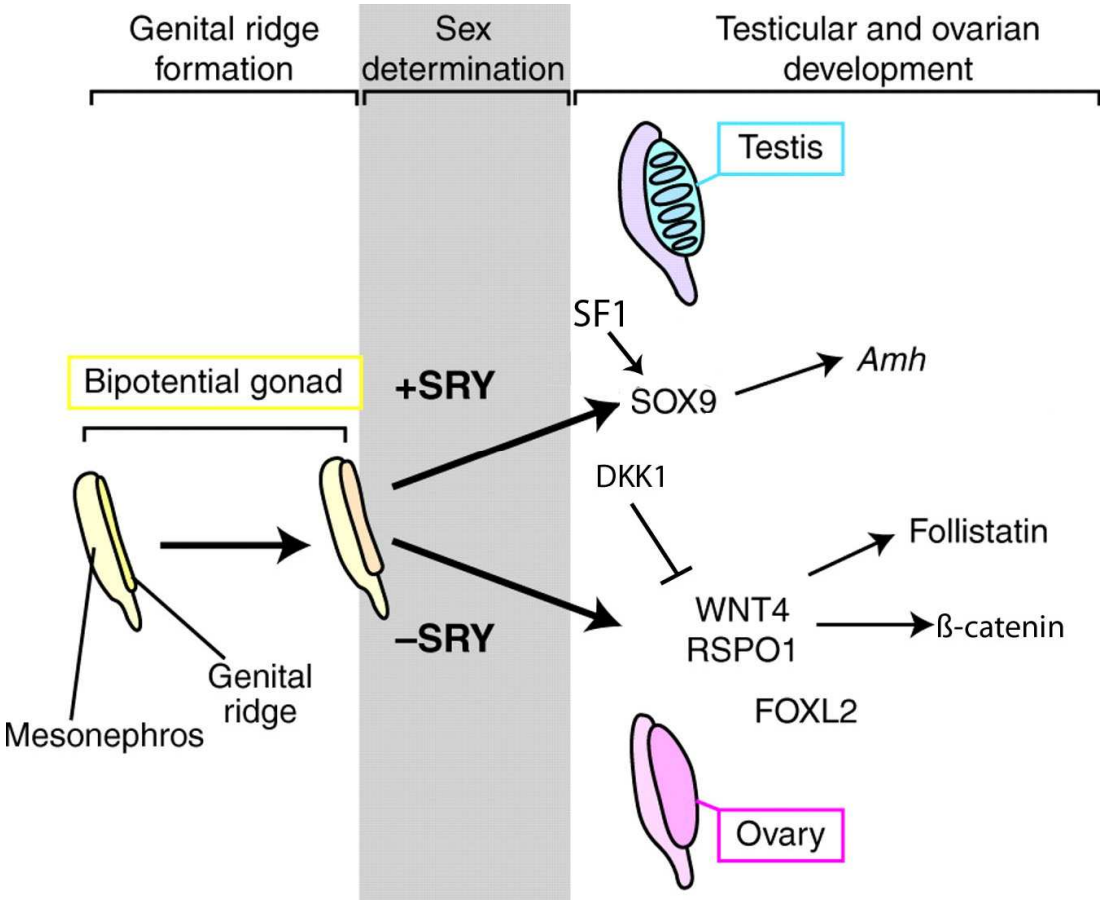

Figure 1.2 Summary of sex determination

Summary of major signalling factors involved in sex determination as discussed in the text. Figure adapted from (Kashimada & Koopman, 2010).

1.3 Fetal Ovarian Development

As the majority of this thesis focuses on ovarian development, further discussion of the literature will be limited to ovarian development and maturation, whereas discussion of testis development will be reserved for later chapters.

1.3.1 Germ Cell Proliferation

Although there is some argument regarding when the population of germ cells in the gonad become oogonia as opposed to PGCs, for this sake of clarity once sex determination has occurred (prior to meiosis) the germ cells in the ovary will be referred to as oogonia in this literature review. The difference is both morphological and based on activity; PGCs are irregular in shape, making them less distinct from interstitial and stromal cells (Gondos *et al*, 1986) and are smaller in size than oogonia (Gosden, 1995; Gougeon, 1996). Mitotically active PGCs express OCT4, rather than the more mature germ cell marker VASA which is expressed from ~12 weeks gestation in the human (Stoop *et al*, 2005; Anderson *et al*, 2007). Oogonia are rounded and can also be identified by globular mitochondria associated with mitotic division (Gondos *et al*, 1986). These oogonia undergo mass proliferation, and by late first trimester, the number of germ cells has increased by ten-fold from the original cohort of PGCs that migrated to the gonad (11 weeks gestation ~240,000; (Bendsen *et al*, 2006)). The oogonia undergo incomplete cytokinesis upon mitotic division (Gondos & Zamboni, 1969), creating clusters of oogonia termed 'germ cell nests' or syncytia (de Cuevas *et al*, 1997; Skinner, 2005) connected by cyotoplasmic bridges between cells (de Cuevas *et al*, 1997; McNatty *et al*, 2000) which can be seen as early as 10 weeks gestation (Gondos *et al*, 1986) (Figure 1.3). These cytoplasmic bridges allow for the exchange of nutrients between cells and may also be involved in synchronous division (Pepling & Spradling, 1998; Epifano & Dean, 2002). Outwith the germ cell nests, the gonad also contains somatic cell populations which remain fairly unorganised until roughly 12 weeks gestation (Byskov, 1986).

The two main populations of somatic cells in the fetal ovary include the pre-granulosa cells and the mesenchymal stromal cells (in addition to the epithelial lining of the ovary and endothelial cells lining blood vessels). During first trimester, somatic cells and germ cells are arranged in an apparently chaotic manner. However, during proliferation of oogonial nests, the pre-granulosa cells integrate with germ cells becoming part of the oogonial nests and the mesenchymal stromal cells form separate channels or streams amongst the nests (Figure 1.3) (Gondos & Zamboni, 1969; Wartenberg, 1982).

1.3.2 Entry into Meiosis

In late first trimester ~11 weeks gestation, some oogonia progress from mitotic division to meiotic division (Baker & Franchi, 1967; Motta *et al*, 1997), a process which is asynchronous in the human, and occurs progressively through second and third trimester, with oogonia in the interior of the ovary initiating meiosis while oogonia near the epithelium continue to proliferate via mitosis (Skrzypczak *et al*, 1981; Anderson *et al*, 2007). However, in mice this process is more synchronised, with initiation in an anterior-posterior wave occurring between e13.5-15.5 (Menke *et al*, 2003).

One of the first factors implicated in regulation of meiosis was Dazl, expression of which is required for germ cells to become meiotically competent (Lin *et al*, 2008). Human studies have identified *DAZL* mutations associated with subfertility in females (Hertig & Adams, 1967; Lynch & Zhang, 2007) and that another closely related gene BOULE may also be involved (Kee *et al*, 2009). Dazl up-regulates protein levels of factors necessary for meiosis, including Synaptonemal Complex Protein 3 (Sycp3) (Reynolds *et al*, 2007), a component of the synaptonemal complex necessary for the first phase of meiosis (Costa & Cooke, 2007), which also functions in the repair of double-strand breaks during meiotic recombination (Pittman *et al*, 1998; Yoshida *et al*, 1998).

Dazl is also thought to be upstream of stimulated by retinoic acid (*Stra8*), as Dazl $^{-/-}$ gonocytes have reduced *Stra8* expression (Lin *et al*, 2008). *Stra8* regulates initiation of meiosis I and is required for pre-meiotic DNA replication (Baltus *et al*, 2006).

Stra8 is activated by retinoic acid (RA) secreted from the mesonephros in the mouse (Di Carlo *et al*, 2000; Bowles *et al*, 2006; Koubova *et al*, 2006; Anderson *et al*, 2008), however recent data indicates *STRA8* expression in the human is reliant on ovarian RA regulation rather than extrinsic sources (Le Bouffant *et al*, 2010; Childs *et al*, 2011). After initiation, prophase I of meiosis progresses through leptotene (chromosome condensation); zygotene (chromosomal pairing); pachytene (initiation of recombination) and diplotene (chromosomes begin to separate but remain attached) before arresting (Baillet, 2011). Arrival at diplotene stage of meiosis I is required for oocyte arrest and the formation of primordial follicles (Hilscher, 1991). Proliferation of all ovarian cell types occurs during early second trimester prior to meiosis, after which oocytes cannot undergo further mitotic replication leaving somatic cells to proliferate further (Fulton *et al*, 2005).

1.3.3 Apoptosis and germ cell nest breakdown

Following nest breakdown all oogonia/oocytes enter one of two fate pathways; they either degrade or they continue to mature and associate with stromal cells to form primordial follicles (Vaskivuo *et al*, 2001). Around the time of meiotic entry, oogonia/oocytes also begin to degrade in a process termed atresia (Kaipia & Hsueh, 1997). Although the exact timing of this event is uncertain, it is thought to initiate between 13-14 weeks gestation (Vaskivuo *et al*, 2001; Hussein, 2005). The number of germ cells that go through degradation prior to birth is great (~80%) resulting in a drastic reduction throughout the remainder of gestation (Baker, 1963; De Pol *et al*, 1997). It is proposed the major mechanism of germ cell degradation is programmed cell death, apoptosis (De Pol *et al*, 1997; Costa & Cooke, 2007). The peak of apoptosis is reported to occur around the initiation of primordial follicle formation (Baker, 1963; Fulton *et al*, 2005) with one to two thirds of oocytes being lost during nest breakdown prior to this process (Pepling & Spradling, 2001; Bristol-Gould *et al*, 2006). It is hypothesised this clearing of germ cells acts as a quality control measure, eliminating oogonia exhibiting chromosomal or mitochondrial defects (Tilly, 2001). This is supported by high levels of germ cell apoptosis in fetuses with chromosomal abnormalities (Modi *et al*, 2003; Di Giacomo *et al*, 2005; Hunt & Hassold, 2008) or mitochondrial deficiencies (Perez & Sanville, 2000).

During human fetal ovarian development, the majority of the cells detected undergoing apoptosis are indeed germ cells (Vaskivuo *et al*, 2001; Abir *et al*, 2002; Hussein, 2005), with four main factors identified in the regulation of apoptosis; BCL-2 associated X protein (BAX) and BCL-2 associated agonist of cell death (BAD) as pro-apoptotic factors, and B-cell lymphoma/leukemia-2 (BCL-2) and myeloid leukaemia sequence-1 (MCL-1) as anti-apoptotic (Boise *et al*, 1993; Tilly, 1996). BAX and BAD are expressed in somatic and germ cell populations; whereas BCL-2 and MCL-1 were exclusively located in the somatic and germline respectively (Hartley *et al*, 2002). *BCL-2* expression is seen as early as 6 weeks gestation (Quenby *et al*, 1999) and is maintained until roughly 18 weeks gestation (Albamonte *et al*, 2008), at which time, *MCL-1* is highly expressed and may play a role in the inhibition of cell death after follicle assembly (Hartley *et al*, 2002). These factors interact with the pro-apoptotic factors to determine cell fate (Hsu *et al*, 1997). The pro-apoptotic factor *BAX* is expressed from week 12 of gestation onwards, and with expression not restricted like that of *BCL-2* (Vaskivuo *et al*, 2001; Hartley *et al*, 2002; Albamonte *et al*, 2008). Protein expression of BAX as determined via immunohistochemistry was also seen to be more intense and stable than that of BCL-2 after initiation of meiosis, which is consistent with the onset of apoptosis (Albamonte *et al*, 2008).

It is thought the pro-apoptotic members BAX and BAD function via dimerization, increasing mitochondrial permeability and forming large channels within targeted cells, resulting in release of cytochrome c from the mitochonidria and activating caspases (Cory & Adams, 2002). This is supported by the detection of cleaved caspase-3 (the active form of this pro-apoptotic caspase) in the human fetal ovary in oocytes before primordial follicle assembly, but is absent in oocytes within primordial follicles (Fulton *et al*, 2005). Observations from mice lacking functional *Bax* also support its role in oocyte apoptosis and nest breakdown, as these animals have three times as many oocytes as their wild-type counterparts (Perez *et al*, 1999). Disruption of *caspase-2* also results in increased primordial follicle number (Bergeron *et al*, 1998).

It is postulated that the purpose of generating of so many 'unnecessary' oocytes is to produce additional nutrients and organelles that are then donated to the surviving oocytes via autophagy (Baum *et al*, 2005). This is supported by the finding of mitochondria between oogonia within cytoplasmic bridges (Pepling & Spradling, 2001). The hypothesis behind this mechanism suggests the oocytes remaining after germ cell atresia will have additional cellular resources and are therefore best suited to withstand the decades of meiotic arrest. The autophagy theory is supported by evidence that apoptosis, although likely to be the main cause of germ cell death, cannot account for the large number of germ cells lost during fetal development (Lobascio *et al*, 2007; De Felici *et al*, 2008). Consistent with this theory, various mouse models generated with disruption of autophagy genes display marked reduction in primordial follicle numbers at birth (Gawriluk *et al*, 2011). These data not only support the conclusion that germ cell death is necessary for primordial follicle formation but also that autophagy is a vital pathway for normal fetal ovarian development. Apart from apoptosis and autophagy, clearing of germ cells has also been demonstrated via exfoliation from the ovarian surface epithelium (Motta *et al*, 1997).

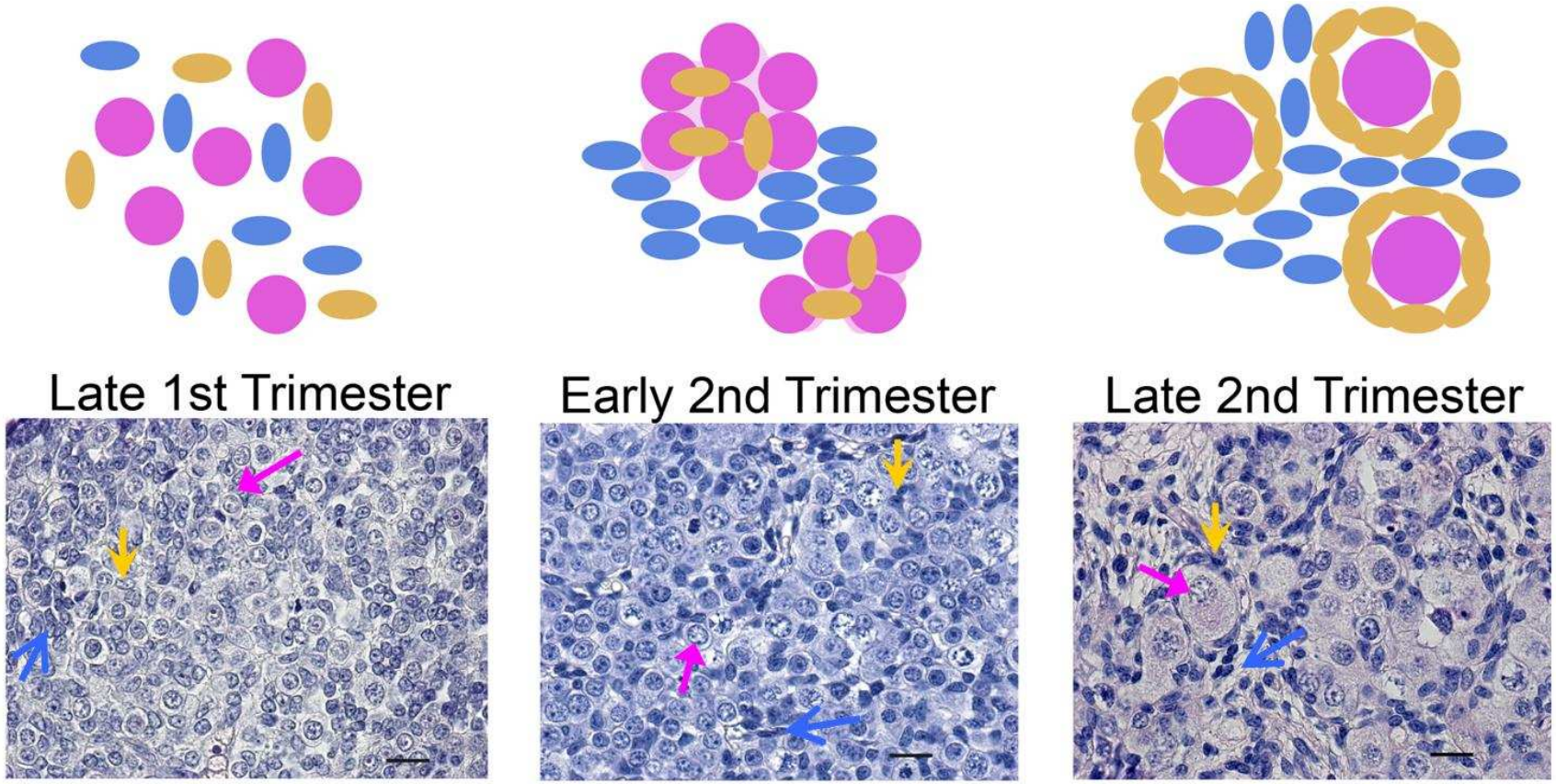

Figure 1.3 Schematic of fetal human ovarian development

(1st trimester) Once germ cells (pink; at this point deemed PGCs) enter the ovary they integrate with mesenchymal stromal cells (blue) and pre-granulosa interstitial cells (orange) in an unorganised fashion. **(Early 2nd trimester)** During a mass mitotic event the germ cells (now deemed oogonia) undergo incomplete division to form germ cell nests with pre-granulosa cells intertwined, after which entering meiosis (now deemed oocytes). **(Late 2nd trimester)** Oocytes grow in diameter as they mature and begin to break away from germ cell nests (as a majority of the oocytes degrade) and associate with pre-granulosal cells to form primordial follicles.

1.3.4 Primordial Follicle Assembly

The breakdown of germ cell nests allows the surviving oocytes (~20%) to closely associate with intermixed pre-granulosal cells (Pepling & Spradling, 2001) (Figure 1.3). During nest breakdown the cytoplasmic bridges are either retracted or degraded via protease action of the pre-granulosa cells (Tingen *et al*, 2009), which form a protective layer around the remaining oocytes. Oocytes that do not associate with pre-granulosa cells to form primordial follicles are lost (Byskov, 1986; McLaren, 1991; Coucouvanis *et al*, 1993; De Pol *et al*, 1997; Pepling & Spradling, 2001). The maturing oocyte with somatic cell association is called a primordial follicle, and although initiation of follicle assembly in the human is reported at different stages in second trimester (Abir *et al*, 2002; Sforza *et al*, 2003), our laboratory reports the onset of primordial follicle formation to occur around 18 weeks gestation (Fulton *et al*, 2005). The primordial follicle complex is then enclosed in an individual basement membrane (Rajah *et al*, 1992) separating it from the mesenchymal stromal cell environment (Figure 1.3).

In the mouse fetal ovary, breakdown of germ cell nests allowing for primordial follicle formation is governed by estrogenic signalling (Iguchi *et al*, 1986). During gestation the fetal oocytes are exposed to maternal estrogen which keeps oocytes within germ cell nests (Kezele & Skinner, 2003; Chen *et al*, 2009), at parturition this signalling is removed signalling for the nests to breakdown which allows for primordial follicle formation via the integration of pre-granulosa cells into germ cell nests allowing for association with oocytes. Exposure to estrogenic signalling postnatally results in multiple oocyte follicles or MOFs (Jefferson *et al*, 2002), which are created by the disruption of nest breakdown; whereas premature removal from estrogenic signalling results in premature nest breakdown (Chen *et al*, 2007b). However, in the human, although estrogen receptors are present (Fowler *et al*, 2011) primordial follicle formation occurs during mid-gestation, and thus removal of maternal estrogen is not possible as a signalling cue for germ cell nest breakdown highlighting another species-specific difference during ovarian development.

However, the non-human primate, like the human, initiates primordial follicle formation during mid-gestation, and thus has served as a good model for identifying possible estrogenic roles in fetal follicle formation. A recent baboon model of estrogen deprivation during *in utero* development, resulted in a significant reduction in primordial follicle number, suggesting estrogen does play a role during fetal ovarian development (Zachos *et al*, 2002). Further studies demonstrated estrogen regulates several factors important for primordial follicle formation, including the ratio of Activin : Inhibin expression, development of oocyte microvilli, and expression of the transferrin receptor (Billiar *et al*, 2003; Billiar *et al*, 2004; Zachos *et al*, 2004; Zachos *et al*, 2008). Further, estrogen is able to directly regulate baboon oocytes via estrogen receptor β (ERβ), but estrogen receptor α (ERα) is not expressed (Bocca *et al*, 2008).

Arrested oocytes within primordial follicles remain quiescent until recruitment for growth and ultimately ovulation later in life, and are reliant on stromal support to maintain the follicle throughout the female's reproductive lifespan, which in the human lasts some 3 decades (Pincus & Enzmann, 1935; Borum, 1961; Maheshwari & Fowler, 2008).

1.4 Alternate sources of follicles during adulthood?

It is widely accepted that the number of follicles a female produces during fetal life will determine the number of follicles that she will have for ovulation during adulthood (Morita & Tilly, 1999a). There are however, reports purporting the existence of female germ-line stem cells in the adult ovary (Johnson *et al*, 2004), from which primordial follicles could potentially be derived postnatally. In these reports, it is postulated that ovarian germline stem cells could be located in the surface epithelium of the ovary or in bone marrow (Johnson *et al*, 2004; Johnson *et al*, 2005). Subsequent studies from other groups have demonstrated bone marrow is not involved in the formation of functional oocytes (Eggan *et al*, 2006), but factors from the bone marrow may regulate the growth of new oocytes especially following ovarian damage (Lee *et al*, 2007).

More recently, cells purported to be germline stem cells have been isolated from the ovarian epithelium of adult and prepubertal mice via immune-magnetic isolation of MVH/VASA positive cells (Zou *et al*, 2009). The isolated cells were cultured for ~6 months, allowing for all non-proliferating (meiotic) cells to perish leaving only MVH positive mitotic cells; the cells were then infected with a GFP virus and transplanted into the ovaries of sterilised recipient female mice. Upon further analysis, recipients had GFP positive oocytes within follicles at all stages and bore offspring, 28% of which were GFP positive (Zou *et al*, 2009).

Although, this research is exciting, as it suggests a pool of oogonia which could maintain fertility outwith the finite cohort of primordial follicles formed at birth, it seems unlikely this is the full story, as several animal models (as referenced in (Skinner, 2005) and women (Telfer *et al*, 2005) fail to demonstrate any replenishment of this cohort of follicles. Indeed, initial studies reported isolated ovarian stem cells to be few per ovary (Johnson *et al*, 2004; Tilly *et al*, 2009). Additionally, it is unclear how these studies in rodent models equate to human physiology. Therefore, the relevance of ovarian stem cells remain to be further elucidated. Additionally the existence of functional stromal cells including granulosa stem cells *in vivo* would need to be considered, as follicle formation would require more than just an oocyte in cases of severe ovarian damage (Tilly & Telfer, 2009).

1.5 Repercussions of fetal follicle formation

Consistent with the currently accepted theory that primordial follicles determine the cohort of oocytes ovulated in adulthood, failure to form sufficient primordial follicles during fetal life results in premature ovarian insufficiency (POI) or infertility (Mattison *et al*, 1983; Byskov, 1986; Byskov *et al*, 2005). Although the field is progressing, POI remains relatively common, occurring in 1% of women (Coulam, 1986; Goswami, 2005). Primordial follicle regulation and preservation are also of interest in current cancer therapies, as intensive courses of either radio- or chemotherapy can result in the loss of primordial follicles leading to infertility (Sonmezer & Oktay, 2004). Understanding the process of primordial follicle formation and ovarian development during fetal life could provide insight into what factors influence the cell fate decisions of apoptosis vs. survival, which in turn could be manipulated for clinical use.

1.6 Known Regulation of Fetal Germ Cell Development

Various factors have been identified as important for germ cell development leading to primordial follicle formation. The following section will briefly discuss both secreted factors that effect ovarian cells via classical ligand-receptor signalling deemed growth factors, as well as transcription factors, which regulate ovarian development via modulation of transcription of other genes. These two groups of regulators work in concert to coordinate early ovarian development leading to primordial follicle formation.

1.7 Growth factors

Growth factors are signalling molecules which function via receptor binding to activate intracellular signalling cascades in order to regulate critical cellular functions including; proliferation, differentiation, cell survival and apoptosis (Bianco *et al*, 2006). Several growth factors have been established as regulators of fetal ovarian development and will be described in the following section (Figure 1.4).

1.7.1 Kit ligand (KL)

As previously discussed, KL via its receptor c-kit, regulates PGCs migration and survival during gonad formation as demonstrated in the mouse (Godin *et al*, 1991; Pesce *et al*, 1993a). PGCs express c-kit before they leave the hindgut (Runyan *et al*, 2006; Gu *et al*, 2009), and allows for direct KL regulation after germ cell specification. It is thought KL functions as a chemoattractant during this process, guiding PGCs to the gonads (Farini *et al*, 2007). This role was confirmed in the homozygous null murine models for either *KL* or *c-kit*, which result in disrupted migration and complete loss of the PGC cohort ((Mintz & Russell, 1957; McCoshen & McCallion, 1975) as discussed in (Edson *et al*, 2009)).

Although early data regarding KL's role in regulation of migration is not available in the human fetal ovary, it is known after gonad formation KL is expressed by mesenchymal stromal cells and the pre-granulosa cells closely associated with oogonia (Hoyer *et al*, 2005). It is hypothesised KL acts to prevent precocious entry into meiosis at this point in ovarian development (Coutts *et al*, 2008; Childs & Anderson, 2009), allowing for continued proliferation via mitosis, which is consistent with the down-regulation of c-Kit expression around the initiation of meiosis entry. Expression of c-Kit is resumed around initiation of follicle formation, and thus it is further thought to maintain meiotic arrest until follicle activation (Vainio *et al*, 1999; Jaaskelainen *et al*, 2010). *In vitro* study of rodent oocytes demonstrated that KL is able to up-regulate PI3/AKT, which functions to supress Foxo transcription factors (such as Foxo3a which will be discussed in a subsequent section) (Reddy *et al*, 2005). Foxo3A is known to regulate follicle activation, and

thus KL's regulation of this factor may orchestrate follicle development as well as maintenance of follicle arrest (Castrillon *et al*, 2003).

Expression of c-Kit is down-regulated upon germ cell nest formation but is up-regulated in oocytes after meiotic division (Robinson *et al*, 2001; Stoop *et al*, 2005). This expression pattern is consistent with the hypothesis that KL signalling is important for regulation of oocytes later in ovarian development. In order to study KL effects, without perturbing initial migration and survival of PGCs, an anti-murine c-kit antibody, ACK2, was injected to female mice at different points during gametogenesis to inhibit c-kit function and to elucidate possible KL functions in later gestation (Yoshida *et al*, 1997) . This study determined blockade of KL/c-kit interaction at birth (initiation of primordial follicle formation) did not result in a disruption of primordial follicle formation, rather it affected granulosa cell proliferation, thereby affecting further follicular development. However, conflicting data gathered from *in vitro* culture of fetal murine ovaries demonstrates KL treatment is able to accelerate primordial follicle formation (Wang & Roy, 2004). Thus KL's role in fetal ovarian development post-migration remains unclear.

The human fetal ovary expresses c-Kit in a similar fashion to that established in the mouse, with localisation restricted to mitotic oogonia, diminished in germ cell nests undergoing meiosis, and renewed expression in oocytes within primordial follicles (Robinson *et al*, 2001; Stoop *et al*, 2005). Recent evidence demonstrates *KL* is inhibited by Activin A in the human fetal ovary, which may prevent precocious primordial follicle formation (Figure 1.5), further suggesting a role for KL in later gestation and demonstrating processes in the fetal ovary are highly regulated by interaction of several growth factors (Childs & Anderson, 2009).

.

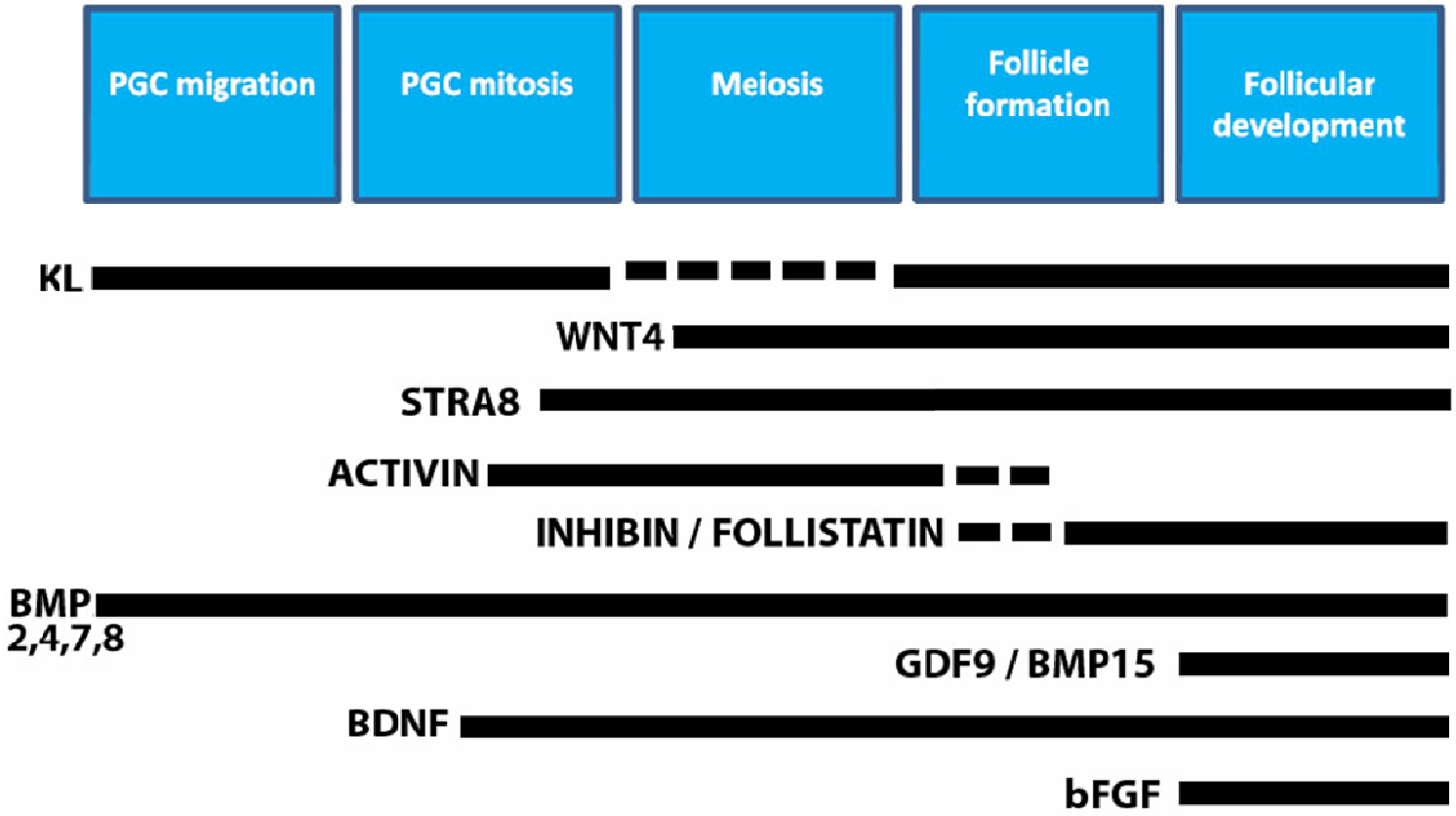

Figure 1.4 Growth factors important for germ cell maturation and primordial follicle formation

Several growth factors are identified in regulation of early human ovarian development leading up to primordial follicle formation and beyond, including KL/c-Kit, WNT4, Stra8, Activin, Inhibin, Follistatin, BMP2, 4, 7, 8 and 15, GDF9, BDNF, and bFGF. Expression patterns of each are demonstrated above via a continued line through the gestational period. Repression/down-regulation is depicted as a dashed line. Figure adapted from (Jagarlamudi & Rajkovic, 2011).

1.7.2 Wnt4

Wnt4 action is essential for sex determination, but it has also been demonstrated to regulate other processes in the fetal ovary. Wnt4 expression in the fetal female mouse is restricted to pre-granulosa cells of the developing ovary and is also expressed in the mesonephros (Vainio *et al*, 1999; Jaaskelainen *et al*, 2010). Wnt4's role in reproductive development was investigated in female mice homozygous null for the *Wnt4* allele, which were found to be partially sex reversed with male-like steroidogeneic cells (Vainio *et al*, 1999; Heikkila *et al*, 2005). In addition *Wnt4*$^{-/-}$ ovaries also had a distinct coelomic vessel, similar to that seen in the testis (Jeays-Ward *et al*, 2003) with associated Wolffian rather than Müllerian ducts (Vainio *et al*, 1999). Thus Wnt4 represses the male pathway, later demonstrated to be via downstream β-catenin and follistatin signalling (Yao *et al*, 2004).

However, in addition to the clearly defined role in sex determination, *Wnt4*$^{-/-}$ ovaries also had marked reduction in oocyte number after e14.5 (post-migration, proliferation, and meiosis), which was not seen in *Wnt4*$^{-/-}$ males (Heikkila *et al*, 2005), demonstrating *Wnt4* plays a role in oocyte-specific survival and development. Further investigation demonstrated a rapid depletion of ~90% of oocytes around e16 (Yao *et al*, 2004) and that remaining follicle structures displayed disrupted morphology (Vainio *et al*, 1999). Further, *Wnt4* expression is notably heightened in fetal ovaries around initiation of primordial follicle formation, a timepoint which is also associated with a decline in apoptosis (Vaskivuo *et al*, 2001), suggesting a role in the regulation of oocyte survival. However, investigation of this hypothesis demonstrated no change in expression of apoptotic factors in *Wnt4*$^{-/-}$ ovaries, suggesting regulation of oocyte survival via Wnt4 is may be downstream of another mechanism (Jaaskelainen *et al*, 2010).

In the human fetal ovary, WNT4 is expressed by the pre-granulosa cells, but is also expressed by oocytes, with increased expression during initiation of primordial follicle formation (Jaaskelainen *et al*, 2010). Despite this difference in localisation, loss of function mutation of *WNT4* in humans mimics the sex reversal seen in the *Wnt4*$^{-/-}$ mouse model, with excess androgens noted as well as masculinisation of reproductive organs (Biason-Lauber *et al*, 2004); demonstrating Wnt4's role in gonad development is likely conserved between species.

1.7.3 Retinoic Acid

Retinoic acid (RA) is an active derivative of vitamin A , and functions via the nuclear hormone receptors RAR-α, -β, -γ, and RXR-α, -β, -γ (Bowles *et al*, 2006; Koubova *et al*, 2006), which are localised primarily to the germ cells of both male and female gonads in the mouse and human (Morita & Tilly, 1999b; Le Bouffant *et al*, 2010; Childs *et al*, 2011). RA is able to promote the onset of meiosis during fetal ovarian development (Bowles *et al*, 2006; Koubova *et al*, 2006), and functions by up-regulating *STRA8*, which is required for DNA replication prior to initiation of meiosis (Baltus *et al*, 2006; Anderson *et al*, 2008). *STRA8* expression increases in both the human and mouse ovary with the onset of meiosis (Menke *et al*, 2003; Houmard *et al*, 2009; Le Bouffant *et al*, 2010).

Although RA is produced by both sexes, data from mice fetal testes homozygous null for a retinoid degrading enzyme *Cyp26b1*, demonstrated this factor is up-regulated in the testis compared to the ovary and is able to metabolise RA (Bowles *et al*, 2006). Based upon this data it was hypothesised that *Cyp26b1* functions to shield male germ cells from RA, thus preventing *Stra8* expression in the testes and the initiation of meiosis. *In vitro* culture of e11.5 murine fetal testes treated with RA seemed to support this, with induced *Stra8* expression and initiation of meiosis (Trautmann *et al*, 2008). However, a recent murine model homozygous null for the RA synthesis enzyme *Raldh2*, which renders the animal unable to synthesise or signal via RA demonstrates these suggested roles may be poorly understood (Kumar *et al*, 2011). *Raldh2*$^{-/-}$ ovaries express *Stra8* and undergo meiosis, and inhibition of *Cyp26b1* in *Raldh2*$^{-/-}$ testes initiates meiosis, all of which occur independently of RA interaction (Kumar *et al*, 2011). These data suggest that although Stra8 is important for

regulation of meiosis, RA may be unnecessary for its production. In addition the degrading enzyme Cyp26b1 is needed for prevention of meiosis in the testis, but is likely to degrade an RA–like substance rather than RA itself.

Investigation in the human fetal gonad determined RA alone is not able to promote meiosis in fetal testes, again suggesting RA either functions in concert with other factors to initiate meiosis or that it is not involved in this process (Childs *et al*, 2011). However, it is likely meiosis is differentially regulated in the human compared to the mouse, as initiation of meiosis is asynchronous in the human and may require more specific regulation (Childs *et al*, 2011). This hypothesis is supported by the heightened expression of *CYP26B1* in the human fetal ovary compared to the fetal testis (Le Bouffant *et al*, 2010; Childs *et al*, 2011), suggesting this factor may play a different role in the human or may be involved in inhibiting meiosis entry in some cells during ovarian development.

While the roles of RA, Stra8, and Cyp26b1 in meiosis remain to be fully understood, data suggest RA may regulate other functions in the fetal gonad, namely proliferation (Morita & Tilly, 1999b).

1.7.4 TGF-β signalling

The members of the TGFβ superfamily have been verified as ovarian regulators in various species. More specifically, the actions of Activin, Inhibin, and the BMPs are essential for fetal ovarian development (Aaltonen *et al*, 1999; Galloway *et al*, 2002; Nilsson & Skinner, 2003; Ross *et al*, 2007b). These cytokines function via serine/threonine kinase receptors to induce intracellular signalling via the SMAD family of proteins (Attisano & Wrana, 1996; Kawabata *et al*, 1999) (Figure 1.5).

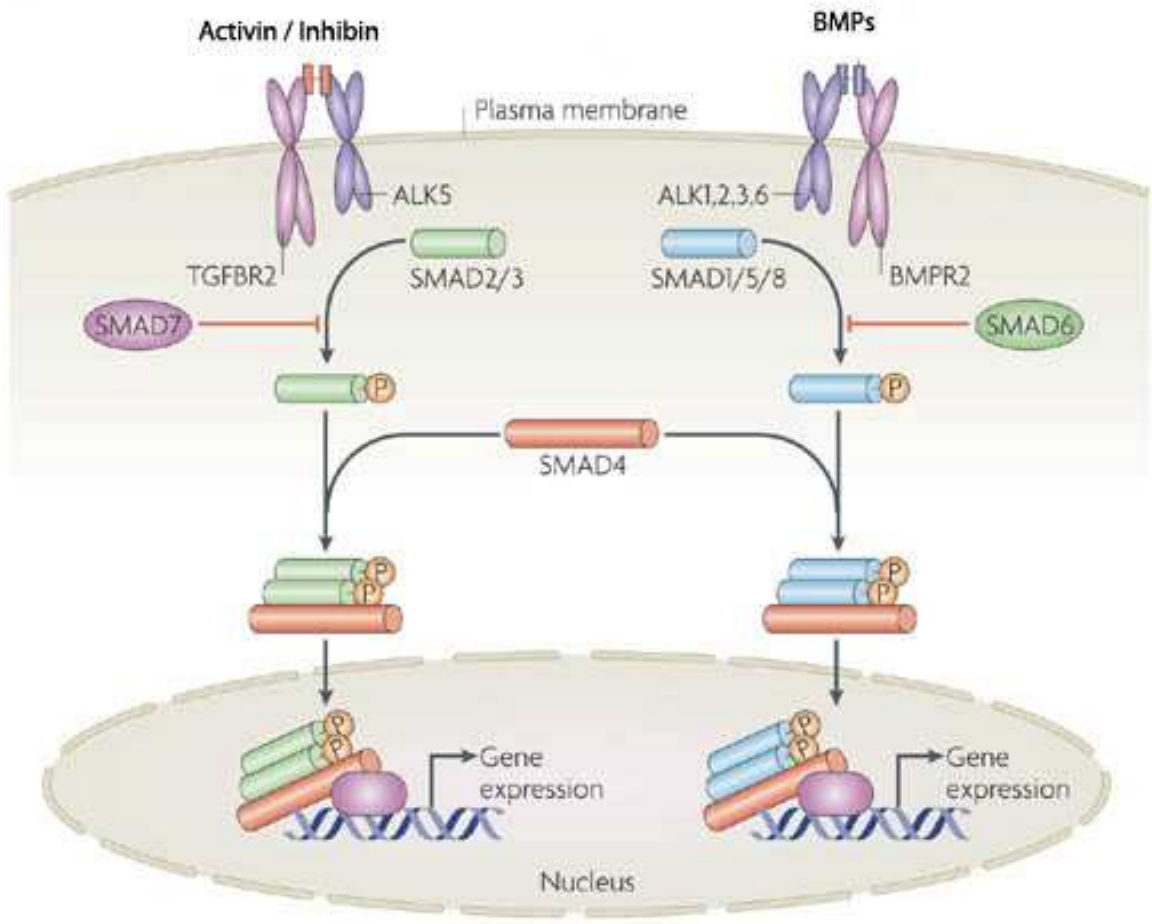

Figure 1.5 Schematic of TGF-β signalling

TGF-β superfamily members signal via Ser/Thr kinase receptors, with Activin and Inhibin utilising TGFβR2 and ALK5 and the BMPs utilising BMPR2 and ALK-1, -2, 3-, and -6. Activated receptors then induce phosphorylation of receptor regulated SMADs, with Activin/Inhibin inducing SMADs 2/3 and BMPs inducing SMADs 1/5/8. Activated receptor SMADs then form heteromeric complexes with the co-transporter SMAD (4) and translocate to the nucleus to regulate gene expression. Phosphorylation of receptor SMADs can be inhibited by inhibitory SMADs (6 and 7), allowing for cell specific regulation of TGF-β signalling. Figure from (ten Dijke & Arther, 2007).

Activin and Inhibin

The Activin/Inhibin family of growth factors are made up of homo- or hetero-dimeric complexes of α and β subunits (Norton *et al*, 1998; Muttukrishna *et al*, 2004), with two isoforms of the β subunit; βA and βB (Trombly *et al*, 2009b). Inhibin is formed by the disulphide-linkage of an α subunit and either β subunit, creating Inhibin A or Inhibin B respectively. Alternatively, Activin formation does not require an α subunit, rather is the homo- or hetero-dimerisation of two β subunits, creating Activin A, Activin B, or Activin AB (Trombly *et al*, 2009b). Inhibin is able to antagonise Activin function via competitive binding of the Activin receptors (Lewis *et al*, 2000), and utilisation of β subunits (McMullen *et al*, 2001). The two components bind Activin receptors which in turn modulate gene expression via Smad2/3 activation and the MAPK pathway (Massague, 1998; Mulder, 2000). However, murine models, created to further elucidate Activin/Inhibin function have been largely inconclusive due to embryonic lethality and overlapping functions of the TGFβ family; therefore research in human primary tissue has been vital for determination of Activin/Inhibin function during fetal ovarian development (Matzuk *et al*, 1995; Burns & Matzuk, 2002).

The Activin/Inhibin subunits are expressed by the germ and somatic cell compartments of the human fetal ovary (Rabinovici *et al*, 1991; Harkness & Baird, 1997; Martins da Silva *et al*, 2004). However, the βA subunit is localised solely in the germ cells with increasing expression in larger germ cell (diameter is indicative of oocyte maturation) but expression ceases once oocytes form primordial follicles (Martins da Silva *et al*, 2004). The βB subunit is localised in both germ and stromal cells (Martins da Silva *et al*, 2004) and is expressed in relatively low levels compared to the βA subunit (Rabinovici *et al*, 1991). In contrast, transcript and protein for the α subunit was not detected in the human fetal ovary, determining Activin A and B to be the active Inhibin/Activin regulators during human fetal ovarian development (Martins da Silva *et al*, 2004). However, other studies have demonstrated Inhibin expression in granulosa cells later in follicular development coincident with the reduction in Activin expression (Davis *et al*, 1987). Several of the Activin receptors (ActRIIA, ActRIIB, ALK2, and ALK4) have been localised in the human fetal

ovary, with expression determined in both germ and somatic cells (Coutts *et al*, 2008), demonstrating Activin is able to act in both an autocrine and paracrine fashion.

In support of a role for Activin A in fetal ovarian regulation are data demonstrating regulation of germ cell survival and proliferation in human ovarian culture via downstream SMAD 2/3 signalling (Martins da Silva *et al*, 2004). It is possible that Activin A regulates proliferation and survival via the anti-apoptotic factor MCL-1, as the two factors display similar localisation in the human fetal ovary, but this mechanism has yet to be confirmed (Martins da Silva *et al*, 2004). In addition, Activin A is able to regulate brain-derived neurotrophic factor (BDNF) action, in order to promote germ cell survival via downstream paracrine signalling from pre-granulosa cells (Childs *et al*, 2010a).

Further studies utilising injection of Activin A into neonatal mice resulted in a 30% increase in primordial follicle number (Bristol-Gould *et al*, 2006), suggesting a role in initiation of follicle formation as well as proliferation/survival. Although functional studies regarding Activin A's role in follicle formation have yet to be performed in the human fetal ovary, a possible mechanism has been demonstrated, as Activin A is able to inhibit membrane bound KL expression (Coutts *et al*, 2008; Childs & Anderson, 2009). From this data it is hypothesised that Activin A is able to limit cross-talk between somatic and germ cells by inhibiting KL, thus inhibiting precocious nest breakdown and follicle formation (Figure 1.6) (Childs & Anderson, 2009).

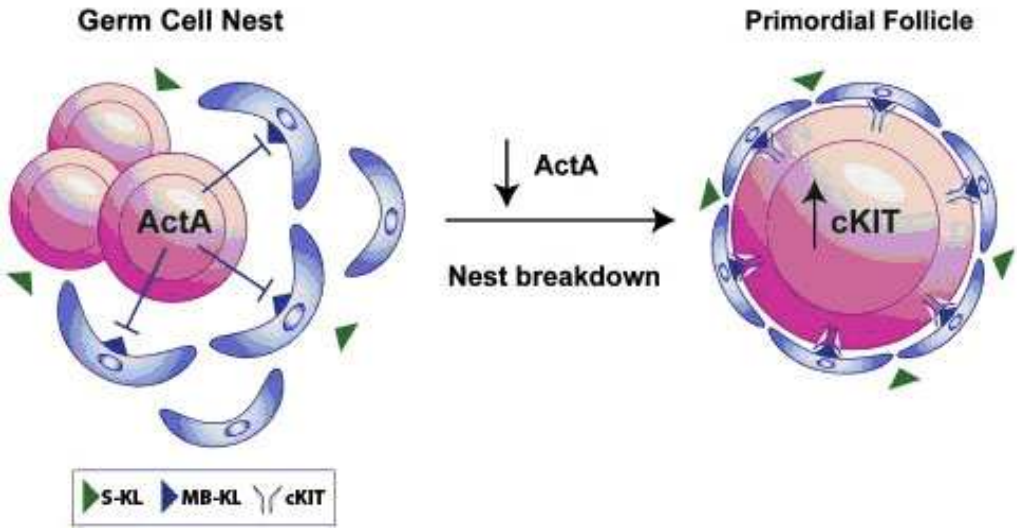

Figure 1.6 Schematic of proposed mechanism of Activin A

Activin A (ActA) is thought to regulate initiation of primordial follicle formation via repression of membrane bound Kit ligand (MB-KL), while allowing signalling via soluble Kit ligand (S-KL). As ActA expression decreases with nest breakdown, this allows for signaling via MB-KL increasing c-KIT downstream signalling and promoting initiation of primordial follicle formation. Figure adapted from (Childs & Anderson, 2009).

Follistatin

Although not a TGFβ superfamily member itself, Follistatin mediates some of its ovarian function via interaction with Activin (Hillier & Miro, 1993; Muttukrishna *et al*, 2004). *Follistatin* is expressed by somatic cells in in the murine fetal ovary initiating at e11.5 and peaking at e12.5 concomitant with sex determination (Yao *et al*, 2004). Although unrelated to Activin, Follistatin has affinity for and binds Activin repressing its biological effects (Nakamura *et al*, 1990; Schneyer *et al*, 1994). The bound complex is then targeted by cell surface proteoglycans for endocytosis and is degraded (Hashimoto *et al*, 1997). This antagonistic mechanism is noted *in vivo* in several species with Activin expression decreased with increase of Follistatin expression (Schneyer *et al*, 2000; Knight & Glister, 2001), and was confirmed *ex vivo* in the human fetal ovary, with up-regulation of SMAD2/3 signalling with Activin A treatment and down-regulation of this pathway with Follistatin treatment (Coutts *et al*, 2008). Further, three isoforms of Follistatin exist *in vivo* with differing levels of affinity for proteoglycan degradation (Inouye *et al*, 1992; Sugino, 1993). Specific deletion of the *Follistatin* isoform with highest affinity for intracellular degradation in a murine model leads to both reduced litter size and frequency, denoting Follistatin mediated Activin degradation may be important for ovarian biology (Kimura *et al*, 2010).

Follistatin has also been implicated in interaction with another subset of the TGFβ superfamily, the BMPs. Although, few data are available regarding this interaction in the fetal ovary, data from various species and tissues suggests Follistatin is able to inhibit the action of BMP2, 4, 7, and 15 (Fainsod *et al*, 1997; Otsuka *et al*, 2001; Glister *et al*, 2004). However, this mechanism remains to be demonstrated in the fetal ovary.

BMPs

The BMPs are also TGFβ superfamily factors, of which, BMP2, 4, 7, 8, 15 and growth differentiation factor 9 (GDF9) are of particular interest during fetal ovarian development. Unlike Activin/Inhibin signalling, BMP factors utilise SMADs 1/5/8 for intracellular signalling rather than SMAD 2/3 (Lin *et al*, 2003).

BMP2

Bmp2 expression is restricted to the somatic cells of the fetal ovary post-sex determination in the mouse, which is consistent with the hypothesis it is driven by Wnt4 signalling (Yao *et al*, 2004). *Bmp2* is up-regulated around the time of germ cell nest formation and initiation of meiosis (Kashimada *et al*, 2011), with *in vitro* study of Bmp2 and Foxl2 in an ovarian cell line suggesting these factors may work in cooperation to suppress Follistatin (Kashimada *et al*, 2011).

However, it appears *BMP2* may play a different role in the human fetal ovary. Although function and localisation of BMP2 in the human has yet to be investigated, it is known to signal solely to the germ cells in the human fetal ovary and is developmentally up-regulated prior to primordial follicle formation (Childs *et al*, 2010b) rather than during nest formation and meiosis. It is likely that species-specific differences are involved in the differential regulation seen with several of the Bmp factors.

BMP4

Bmp4 is important for PGC specification and migration. Further studies have identified that inhibition of *Bmp4* in neonatal ovarian culture led to progressive loss of PGCs and that mice homozygous null for *Bmp4* have no germ cells (Lawson *et al*, 1999; Fujiwara *et al*, 2001; Nilsson & Skinner, 2003), it was hypothesised *Bmp4* might also be important for early germ cell survival post-migration (Dudley *et al*, 2007). However, this role was not supported by further work in the mouse (Ross *et al*, 2003) or in the human, where culture of fetal ovary in the presence of BMP4 promoted germ cell apoptosis (Childs *et al*, 2010b). Bmp4 regulation of apoptosis is also evident in development of other organs (Graham *et al*, 1994; Trousse *et al*, 2001). Therefore, it is likely BMP4 regulates germ cell loss in fetal ovarian development rather than survival.

In addition, BMP4 may play a role in inhibition of differentiation, in later ovarian biology, via regulation of the inhibitor of differentiation (*ID*) genes, which are known to regulate apoptosis, proliferation, and differentiation in other tissues (Miyazono & Miyazawa, 2002). Although few published data are available regarding the BMP4/ID pathway in the fetal ovary, some reports suggest this pathway may mediate adult ovarian function and that the Ids are localised to the granulosa cell compliment (Haugen & Johnson, 2010; Hogg *et al*, 2010) .

BMP7

Bmp7 is expressed in the fetal ovary from e10.5 in the mouse, with homozygous mutation resulting in a reduction in PGC number in both sexes (Ross *et al*, 2007b). Additionally Bmp7 appears to be involved in the primordial to primary follicle transition in the rodent (Lee *et al*, 2001; Lee *et al*, 2004). However, *BMP7*'s role in human fetal development has not been investigated, and levels of BMP7 transcript are relatively low compared to both *BMP2* and *4*, suggesting it may not be important for ovarian development in the human (Childs *et al*, 2010b).

BMP8

As previously discussed, Bmp8b is involved in PGC specification in both sexes, with homozygous deletion resulting in a reduced PGC compliment (Zhao *et al*, 1996; Ying *et al*, 2000). However, there are few data clarifying any post-migratory role for BMP8 during fetal ovarian development.

BMP15 and GDF9

Bmp15 and Gdf9 are postulated to regulate the ovary in collaboration and display similar structure and expression patterns in the fetal ovary (McNatty *et al*, 2005; Edson *et al*, 2009). However, it is uncertain if their effects are important during fetal development, as expression of both factors is thought to be restricted to oocytes in primary and growing oocytes, with function identified in later stage follicles in cumulus expansion (Dong *et al*, 1996; Aaltonen *et al*, 1999; Nilsson & Skinner, 2002; McNatty *et al*, 2005).

However, both GDF9 and BMP15 expression is detected as early as 16 weeks gestation in the human fetal ovary (Sun *et al*, 2010), suggesting there may be a role for these factors earlier in development. In addition, there have been several reports of mutations in either *GDF9* or *BMP15* in women with POI (Dixit *et al*, 2005; Dixit *et al*, 2006). There is also evidence that Gdf9 may interact with other growth factors involved in fetal ovarian development, including Kitl (Joyce *et al*, 2000; Wang & Roy, 2004). Therefore further evaluation of BMP15 and GDF9 during fetal ovarian development is needed.

Noggin

Noggin, like Follistatin, is not a member of the TGFβ superfamily, rather it functions via repression of Bmp signalling (Zimmerman *et al*, 1996; Groppe *et al*, 2002). Although Noggin is not well characterised *in vivo* in either the fetal mouse or human ovary; recent *in vitro* studies utilising granulosa cell culture have demonstrated Noggin is able to suppress Bmp2 and 4 signalling (Pierre *et al*, 2005; Haugen & Johnson, 2010). Consistent with this hypothesis, mutations in *NOG* (gene encoding Noggin) have been reported in women with POI, suggesting Noggin may affect the primordial follicle pool (Kosaki *et al*, 2004).

1.7.5 Neurotrophins: BDNF and NT4

The neurotrophins are a family of growth factors known to regulate human ovarian development, doing so via their tropomyosin-related kinase (Trk) receptors and the common p75 receptor (Bibel & Barde, 2000). The neurotrophins were initially characterised in the nervous system, where they mediate survival, proliferation and differentiation (Davies, 2000; Bernd, 2008). Consistent with this role, several of the neurotrophin signalling components are expressed in the human ovary, and are developmentally regulated during fetal development (Anderson *et al*, 2002b; Childs *et al*, 2010a).

One of the neurotrophin receptors, TrkB, has increased transcript expression concomitant with primordial follicle formation in mouse (Spears *et al*, 2003), and is localised exclusively to the germ cells of the fetal ovary in both the mouse and the human (Dissen *et al*, 1995; Anderson *et al*, 2002b; Spears *et al*, 2003; Paredes *et al*, 2004). Targeted homozygous disruption of *Ntrk2*, which encodes the TrkB receptor results in reduced germ cell survival and primordial follicle number (Spears *et al*, 2003; Kerr *et al*, 2009), as well as arrest of follicles later in development during the primary to secondary transition (Paredes *et al*, 2004). These results led researchers to investigate further the two main ligands which utilise TrkB for signal transduction, in order to elucidate the ligand that might be regulating these ovarian functions; brain derived neurotrophic factor (BDNF) and nerotrophin-4 (NT4).

In the rodent ovary, both *Bdnf* and *Ntf5* (which encodes Nt4), are expressed across early gestation, with increased *Ntf5* expression compared to *Bdnf*, as well as an increase in *Ntf5* expression around the time of primordial follicle formation (Dissen *et al*, 1995; Childs *et al*, 2010a). Increased expression of *Ntf5* suggests this is the dominant neurotrophin ligand in the mouse fetal ovary; a hypothesis which is supported by unsuccessful attempts to localise Bdnf in the fetal mouse ovary, suggesting, as with transcript level, Bdnf expression is low (Childs *et al*, 2010a). However, homozygous NT4-deficiency does not result in reduced fertility suggesting it may not be a key regulator of follicle formation (Conover *et al*, 1995).

In the human, *BDNF* and *NFT4* (which encodes NT4) are expressed across gestation in the fetal ovary, with increasing *BDNF* leading to primordial follicle formation (similar to the pattern reported for rodent *Ntf5* expression) (Dissen *et al*, 1995; Childs *et al*, 2010a). BDNF is localised to the somatic cells of human fetal ovary from 9 weeks gestation onwards, and is more strongly expressed by pre-granulosa cells associating with larger oocytes, with a similar expression pattern noted for NT4 (Anderson *et al*, 2002b; Childs *et al*, 2010a). As TrkB receptors are expressed by the germ cells this suggests neurotrophin ligands regulate the oocyte by a paracrine mechanism.

As discussed earlier, Activin A is able to regulate *BDNF* expression in the human fetal ovary, but does not change the expression of *NFT4*; conversely, in the mouse fetal ovary Activin A up-regulates *Nt5* and does not affect *Bdnf* (Childs *et al*, 2010a). Based upon these results and differing transcript expression patterns, it is hypothesised the two neurotrophin ligands BDNF and NT4 may have reversed roles in the rodent and human (Childs *et al*, 2010a). Despite this difference, it appears the role for neurotrophin signalling in regulation of germ cell survival in the mouse (Spears *et al*, 2003; Kerr *et al*, 2009) may also be relevant in the human, as a recent study in the human fetal ovary suggests NT4 increases the number of primordial follicles (Farhi *et al*, 2011). However, this study did not assess the number of naked oocytes in addition to assembled primordial follicles, and therefore it is not possible to elucidate the exact effect of NT4, as increased primordial follicle number could be the result of several mechanisms (such as precocious activation, proliferation and/or survival of oocytes). Therefore, further investigation is necessary to truly understand the roles of the neurotrophins in the human fetal ovary; although the mechanism is likely to be similar to that seen in the mouse, as Activin A, which up-regulates *BDNF* also increases proliferation and survival in the human fetal ovary (Martins da Silva *et al*, 2004).

As functional roles of NT4 and BDNF have yet to explain the phenotype of *TrkB*$^{-/-}$ mice, two other neurotrophins have been examined for their possible reproductive roles: neurotrophin-3 (NT3) and nerve growth factor (NGF), which are expressed along with their receptors in the human fetal ovary (Anderson *et al*, 2002b). However, data from the mouse determined these factors are not likely to be the ligands signalling via TrkB to regulate fetal ovarian development, as Nt3 regulates primordial to primary transition (Nilsson *et al*, 2009) and that Ngf plays a role in theca cell differentiation (Dissen *et al*, 1995; Dissen *et al*, 2001), with no effects noted in primordial follicles. However, as data from BDNF and NT4 suggests there may be differential regulation of the neurotrophins in the human compared to the rodent, it would be interesting to see if these factors play roles in human fetal ovarian development.

1.7.6 Basic fibroblast growth factor (bFGF)

Basic FGF is of the fibroblast growth factor (FGF) family of growth factors, and is well characterised as a mitogenic factor in the mature ovary, where it is localised to the epithelial and granulosa cells (Gospodarowicz *et al*, 1989; Koos & Olson, 1989; Nilsson *et al*, 2001; Ben-Haroush *et al*, 2005). The function of bFGF during fetal ovarian development has been hypothesised based upon *in vitro* culture of rodent fetal ovaries, which indicate bFGF is able to promote primordial to primary follicle transition (Nilsson *et al*, 2001). Further *in vitro* data suggests bFGF is able to do this via up-regulation of KL (Nilsson & Skinner, 2004).

The function of bFGF remains to be elucidated in the human fetal ovary. However, localisation of bFGF in the human fetal ovary is restricted to oocytes (Koike & Noumura, 1994; Ben-Haroush *et al*, 2005) and transcript expression has been reported across early gestation along with bFGF receptors (Yeh & Osathanondh, 1993; Ben-Haroush *et al*, 2005). The expression of bFGF in primordial follicles is down-regulated upon activation and transition to the primary stage (Quennell *et al*, 2004), suggesting function before this stage. However, it was determined as bFGF expression declines in oocytes, it is up-regulated in the surrounding granulosa and theca cells of primary follicles (Yamamoto *et al*, 1997), suggesting a bi-phasic role for bFGF as the follicle matures.

Although there are more growth factors involved in early ovarian development and germ cell differentiation, the factors described here are the most well described in terms of function and human expression (summarised in Table 1.2).

Table 1.2 Summary of Growth Factor Regulation

Summary of fetal ovarian phenotype from murine models (or function where noted) of key growth factors

Growth Factor	Localisation	Phenotype	Model	Reference
Kit-ligand	GC	Loss of GCs and infertility	Steel mutant mice (homozygous mutation at S1 locus)	(McCoshen & McCallion, 1975)
WNT4	SM	Disruption of sex determination and loss of oocytes	Homozygous null mice	(Heikkila *et al*, 2005)
Retinoic Acid	SM	No phenotype (although Stra8 and Cyp26b1 both important for regulation of meiosis)	Homozygous null mice	(Kumar *et al*, 2011)
Activin	GC	Function -GC proliferation, survival and initiation of primordial follicle formation	Human fetal ovarian culture	(Coutts *et al*, 2008; Childs & Anderson, 2009)
Follistatin	SM	Loss of GCs and subfertility	FST288-only homozygous null mice	(Kimura *et al*, 2010)
BMP2	GC	Embryonic lethality – reduced PGCs	Homozygous null mice	(Ying & Zhao, 2001)
BMP4	GC	Embryonic lethality – no PGCs	Homozygous null mice	(Lawson *et al*, 1999)
BMP7	GC	Function in GC proliferation	Homozygous null mice	(Ross *et al*, 2007a)
BMP8	GC	Embryonic lethality – reduced PGCs	Bmp8b homozygous null mice	(Ying *et al*, 2000)
BMP15	GC	Subfertility due to ovulation defect	Homozygous null mice	(Yan *et al*, 2001)
GDF9	GC	Infertile- defect in cumulous expansion	Homozygous null mice	(Yan *et al*, 2001)
(Neurotrophins) TrkB	GC-TrkB SM- BDNF/NT4	GC loss at primordial stage	TrkB Homozygous null mice	(Spears *et al*, 2003)
bFGF	SM	Function- activation of follicles past primordial stage	Mouse and bovine ovarian culture	(Nilsson *et al*, 2001)

1.8 Transcription Factors

Transcription factors work in concert with growth factors to regulate developmental processes in the fetal ovary. Growth factors regulate cell function via ligand-receptor signalling followed by an intracellular cascade, which often modulates transcription factor/s in order to exert function. In turn, transcription factors affect cell function via direct up- or down-regulation of target genes. Transcription factors known to regulate fetal ovarian development are described in detail below (Figure 1.7).

1.8.1 OCT4

OCT4 is a transcription factor from the POU domain family, and is expressed by germ cells in both human and mouse, as early as PGC specification and migration (Rosner *et al*, 1990; Scholer *et al*, 1990; Pesce *et al*, 1998a). OCT4 expression is also found later in gestation, in oocytes undergoing primordial follicle formation; however, its expression is repressed in between these events, with down-regulation occurring around meiosis (Pesce *et al*, 1998a; Pesce *et al*, 1998b; Rajpert-De Meyts *et al*, 2004). It is postulated OCT4 repression may be the result of antagonism by meiotic-specific factors including retinoic acid or its downstream targets (Schoorlemmer *et al*, 1994; Dann *et al*, 2008). It is further hypothesised that Oct4 acts in fetal germ cells as a transcriptional repressor, and may be down-regulated at this time to allow for heighten transcription in the oocytes during the initiation of meiosis (Pesce *et al*, 1998b).

OCT4 is well characterised for its role in maintaining pluripotency in embryonic stem cells (ES cells) (Nichols *et al*, 1998) (Pesce & Scholer, 2000). As ESCs have many phenotypic similarities to the PGC population, it is understandable that OCT4 regulation is also important in germ cells *in vivo* (Pesce *et al*, 1998a; Pesce & Scholer, 2000); however, there are distinct differences between these two cell types and the role of OCT4 in each. In ES cells, OCT4 is characterised for its role in maintenance of pluripotency (Pan *et al*, 2002). However, the PGC- specific deletion of Oct4, utilising CRE/loxP targeting of tissue non-specific alkaline phosphatase ((TNAP) PGC specific marker before e10.5 (Lomeli *et al*, 2000)) in a murine model,

leads to PGC apoptosis (Kehler *et al*, 2004), demonstrating Oct4 regulates survival in PGCs rather than differentiation. The exact functions of OCT4 later in gestation and in the human fetal ovary remain unknown. It has, however been demonstrated that disruption of *OCT4* is associated with gonadal tumours, highlighting actions in the maintenance of gonadal function in the human (Looijenga *et al*, 2003; Rajpert-De Meyts *et al*, 2004).

1.8.2 NANOG

NANOG, like OCT4, is a pluripotency-associated factor in ES cells, and is expressed by PGCs in both human and mouse (Matsui *et al*, 1991; Chambers *et al*, 2003; Mitsui *et al*, 2003; Hart *et al*, 2004; Yamaguchi *et al*, 2005). However unlike OCT4, NANOG is not expressed past the initiation of meiosis (Wang *et al*, 2003a; Yamaguchi *et al*, 2005; Kerr *et al*, 2008). PGC specific deletion of *Nanog*, (utilising TNAP CRE/loxP cross as described for *Oct4*, in addition to a second line utilising an estrogen receptor-CRE) resulted in loss of PGC founder cells, with the remaining PGCs dying during migration (Yamaguchi *et al*, 2009). These results demonstrate *Nanog* is necessary for regulation of PGC survival prior to and during migration, and similarly to Oct4, Nanog does not regulate differentiation as demonstrated in ES cells.

It is postulated Nanog's function in survival of germ cells may be due in part to its regulation of TIA1 cytotoxic granule-associated RNA binding protein-like 1(*Tial1)*, a regulator of apoptosis (Taupin *et al*, 1995; Beck *et al*, 1998), oogonia are absent in mice lacking *Tial1* (Beck *et al*, 1998). Nanog may also function via regulation of the Ids (as discuss previously for Bmp4), as Nanog deficient mice demonstrate a down-regulation of *Id1* (Yamaguchi *et al*, 2009), which is known to regulate transcription in ES cells (Kim *et al*, 2008). Finally, although the germ cell-specific *Nanog*$^{-/-}$ murine model (Yamaguchi *et al*, 2009) demonstrated no disruption of migration, recent studies in zebrafish suggest Nanog may regulate PGC expression of Cxcr4, which aids in guiding PGCs to the genital ridge (Sanchez-Sanchez *et al*, 2010), suggesting a role in migration as well as survival. However, it remains to be seen if Nanog regulates CXCR4 in the mouse or human.

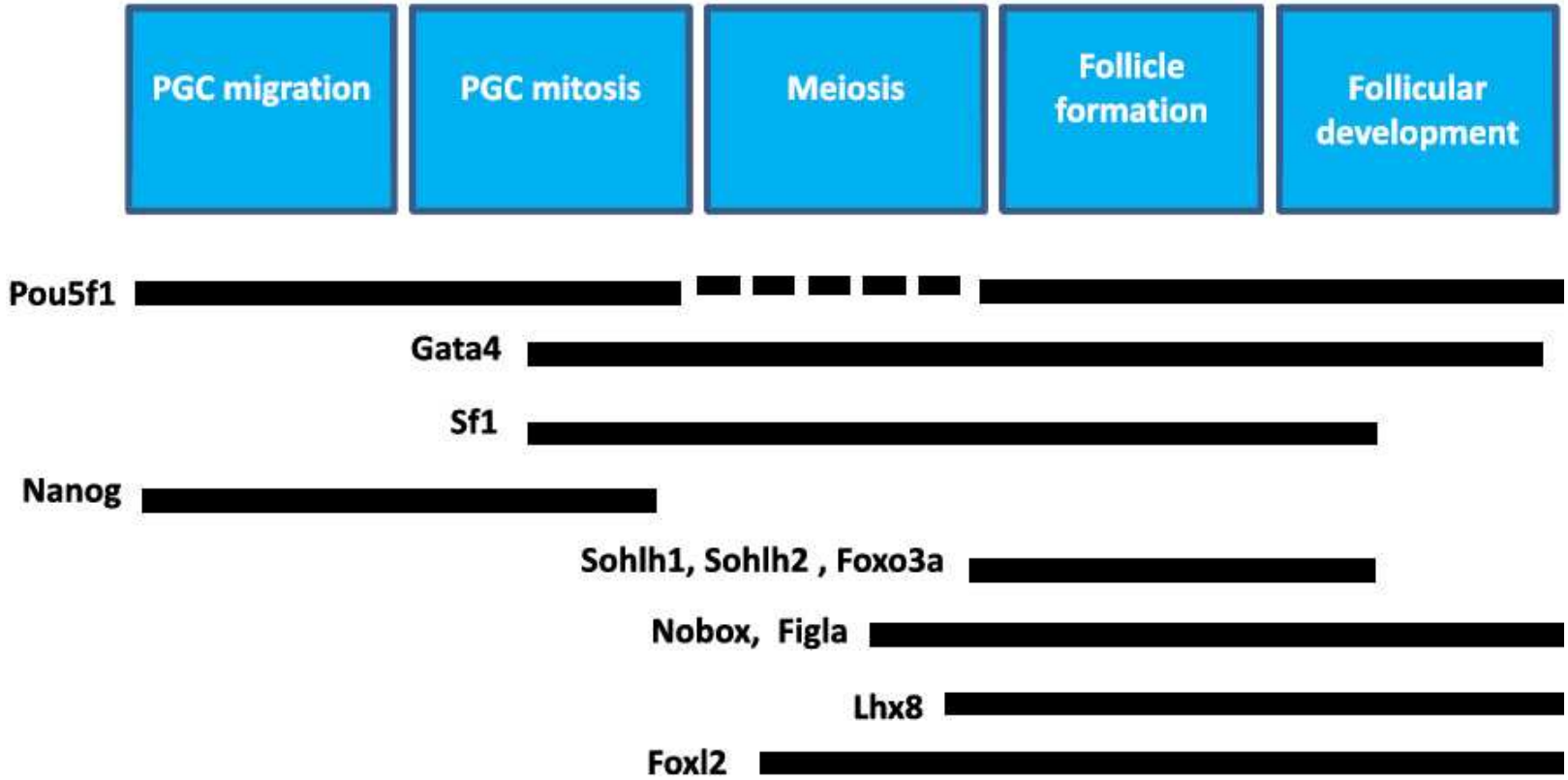

Figure 1.7 Transcription factors important for ovarian development

Several transcriptional regulators are known to be involved in early human ovarian development leading up to primordial follicle formation and beyond, including Pou5f1 (OCT4), GATA4, SF1, NANOG, SOHLH1-2, FOXO3A, NOBOX, FIGLA, LHX8 and FOXL2. Expression patterns of each are demonstrated above via a continued line through the gestational period. OCT4 is repressed during meiosis and thus is depicted as a dashed line during this period. Figure adapted from (Jagarlamudi & Rajkovic, 2011).

1.8.3 Newborn ovary homeobox (NOBOX)

NOBOX is an oocyte-specific homeodomain transcription factor, which is expressed by both the mouse and human in germ cell nests and in oocytes within primordial follicles (Suzumori *et al*, 2002; Huntriss *et al*, 2006). In the mouse, homozygous deletion of *Nobox*, results in germ cell nests which fail to breakdown properly, and rather than forming primordial follicles at birth, entire syncytia undergo atresia (Rajkovic *et al*, 2004; Lechowska *et al*, 2011). *Nobox*$^{-/-}$ ovaries appear to have an occasional primordial follicle, but these oocytes never grow beyond 20 µm and do not recruit the required granulosa cell compliment necessary for later activation (Rajkovic *et al*, 2004; Lechowska *et al*, 2011). Complete repression of *Gdf9*, *Bmp15*, and *Oct4* are noted in *Nobox*$^{-/-}$ homozygous null mutants, demonstrating Nobox regulates expression of these genes and possibly others in order to orchestrate its function in ovarian development (Rajkovic *et al*, 2004; Choi & Rajkovic, 2005; Choi *et al*, 2010a). Although direct functional analysis has not been performed in the human, NOBOX is up-regulated during primordial follicle formation (Fowler *et al*, 2009), and mutations in *NOBOX* has also been identified in patients with POI (Qin *et al*, 2007).

1.8.4 LIM homeobox 8 (LHX8)

LIM homeobox 8 (Lhx8) is a LIM homeodomain protein expressed by oocytes in germ cell nests and primordial follicles in the fetal mouse ovary from e13.5 onwards (Pangas *et al*, 2006). Similar to *Nobox*$^{-/-}$ ovaries, homozygous deletion of *Lhx8* results in infertility, with loss of oocytes around initiation of primordial follicle formation (Pangas *et al*, 2006; Choi *et al*, 2008a). Examination at postnatal day (pnd) 0 and pnd7 demonstrated there was no difference in oocyte number in *Lhx8*$^{-/-}$ ovaries compared to wild-type ovaries at birth; although there were significantly fewer primordial follicles, with the majority of oocytes lost in *Lhx8*$^{-/-}$ ovaries before pnd7 (Choi *et al*, 2008a). Expression of *Gdf9*, *Oct4*, and *Nobox* is abolished in *Lhx8*$^{-/-}$ ovaries (Choi *et al*, 2008a), which leads to the suggestion that *Lhx8* might exert some of its effects via direct regulation of *Nobox* (Pangas *et al*, 2006). However, further transcriptome analysis of *Nobox*$^{-/-}$ and *Lhx8*$^{-/-}$ ovaries determined that although several factors were similarly missexpressed, there were distinct regulatory

targets that were not shared between the factors (Choi *et al*, 2008a). One factor distinctly down-regulated in *Lhx8*$^{-/-}$ ovaries was *Kit* and its ligand *Kitl*, which may explain the more severe phenotype when compared to *Nobox*$^{-/-}$ ovaries. However, it is yet to be seen if *LHX8* is important in human primordial follicle formation and oocyte survival, as the *LHX8* mutations identified thus far in human patients have not been associated with a reproductive phenotype (Qin *et al*, 2008).

1.8.5 GATA4

Gata4 is a zinc finger transcription factor involved in sex determination in the gonads, and is normally expressed in the somatic cells of the fetal ovary (Heikinheimo *et al*, 1997; Viger *et al*, 1998; Tevosian *et al*, 2002). In the mouse *Gata4* is expressed from e11.5 onwards, with homozygous ablation resulting in embryonic lethality (Kuo *et al*, 1997; Tevosian *et al*, 2002). It was determined that Gata4 interacts with another somatic factor, Friend of GATA2 (Fog2), to promote the female pathway during sex determination (Tevosian *et al*, 2002). Homozygous deletion of *Fog2* also ends in embryonic lethality (Molkentin *et al*, 1997), but this can be partially rescued by expression of Fog2 in the myocardium, which allows fetuses to develop to ~e17.5 (Tevosian *et al*, 2000). This murine line, in addition to a model utilising a *Gata4* knock-in allele which prevents interaction between Gata4 and Fog2 (Crisponi *et al*, 2001), were utilised to examine Gata4/Fox2 regulation of fetal ovarian development (Manuylov *et al*, 2008). Both murine lines led to disruption of ovarian development in a similar fashion, with PGC depletion, suggesting interaction with Gata4 is the sole cause for the phenotype seen in Fog2 disrupted ovaries (Tevosian *et al*, 2002; Manuylov *et al*, 2008). In addition, *Follistatin* and *Foxl2* are down-regulated and *Inha* and *Dkk1* are up-regulated in both mutant lines suggesting these are likely targets of Gata4/Fog2 action (Manuylov *et al*, 2008).

In the human GATA4 mRNA and protein are localised to the granulosa cells of the fetal ovary, expression of which decreases with gestation (Vaskivuo *et al*, 2001). This pattern of expression has led to the hypothesis GATA4 is likely to function in the human fetal ovary both in sex determination, as well as a protective factor in the

granulosa cells of follicles. However, further investigation is necessary to determine function of GATA4 in the human.

1.8.6 Steroidogenic factor 1 (SF1)

SF1 (NR5A1) is an orphan nuclear receptor expressed by both granulosa and mesenchymal stromal cells of the human and murine ovary (Ikeda *et al*, 1994; Tajima *et al*, 2003). Sf1 expression is noted as early as e9.5 in the fetal mouse ovary and is essential for fetal gonad development and sex determination (Parker & Schimmer, 1997). It is thought Sf1 may regulate these processes via its known targets including *Amh* (Shen *et al*, 1994), *Cyp11a1* and *3βhsd* (Parker & Schimmer, 1994; LeersSucheta *et al*, 1997). Sf1's role in gonad development was determined via systemic homozygous deletion, which resulted in a lack of gonads and adrenal glands in both male and female offspring (Ingraham *et al*, 1994; Luo *et al*, 1994; Sadovsky *et al*, 1995).

However, in order to dissect ovarian-specific function, Sf1 was disrupted via CRE/loxP mutatgenesis utilising the *Amhr-2*/CRE line (limiting deletion to cells of granulosa origin) (Jeyasuria *et al*, 2004; Pelusi *et al*, 2008). Granulosa-specific disruption of *Sf1* led to a decrease in primordial follicle number and a defect in ovulation, rendering the females infertile. However, follicles of all stages were present demonstrating Sf1 plays a role in ovarian development after primordial follicle formation (Jeyasuria *et al*, 2004; Pelusi *et al*, 2008). It was also noted that several proliferative factors (p27 and cyclin D2), aromatase and Amh were down-regulated in Sf1conditional ovaries suggesting these factors are downstream of Sf1 and may be responsible for the defects in follicle number and/or ovulation. However further investigation is necessary to fully determine Sf1's role during fetal ovarian development, as the *Amhr-2*/CRE conditional model disrupted only granulosa Sf1, with continued expression in interstitial/theca cells which may mask overall Sf1 ovarian function (Jeyasuria *et al*, 2004; Pelusi *et al*, 2008).

In addition, the role of SF1 in the human fetal ovary is unclear. Although several cases of *SF1* disruption have been reported in regards to disruption of male sex differentiation (Coutant *et al*, 2007; Reuter *et al*, 2007; Koehler *et al*, 2008), only one

case of female disruption of *SF1* has been reported, with no change in ovarian function (Biason-Lauber & Schoenle, 2000). However, it has been hypothesised a similar orphan nuclear receptor, liver receptor homolog-1 (LRH-1/NR5A2), may also be able to regulate SF1 targets, and thus masks SF1's function in ovarian biology (Weck & Mayo, 2006).

1.8.7 SOHLH1 and 2

Spermatogenesis and oogenesis specific basic helix-loop-helix 1 and 2 (Sohlh1 and 2) are basic helix-loop-helix (bHLH) transcription factors, which are expressed in germ cell syncitia and oocytes within primordial follicles in the female, as well as in spermatagonia in the male (Ballow *et al*, 2006; Pangas *et al*, 2006). *Sohlh1* was initially identified along with *Nobox* in an array identifying genes expressed preferentially in oocytes (Rajkovic *et al*, 2001), with *Sohlh2* subsequently identified as a bHLH factor sharing high homology with *Sohlh1* (Ballow *et al*, 2006) .

The murine model of homozygous ablation of *Sohlh1* has a similar phenotype to *Nobox*$^{-/-}$ ovaries, losing all follicles by 3 weeks postnatal age (Choi *et al*, 2008b; Toyoda *et al*, 2009). This phenotype is the result of a defect in primordial follicle development rather than formation, as follicles do not progress to the primary stage as a result of disrupted proliferation of pre-granulosa cells (Pangas *et al*, 2006). As a result follicles are lost, which is accompanied by down-regulation of *Gdf9*, *Bmp15*, *Lhx8*, *Kit* and *Nobox* (Pangas *et al*, 2006). Gene expression patterns and phenotype were notably similar to that of *Nobox*$^{-/-}$ ovaries, which led to the hypothesis that *Sohlh1* regulates *Nobox*, as *Nobox* is affected in *Sohlh1*$^{-/-}$ ovaries but no change of Sohlh1 was determined in *Nobox*$^{-/-}$ ovaries (Pangas *et al*, 2006). Similar comparison of phenotypes was performed in other models leading to the hypothesis that *Sohlh1* also regulates *Lhx8* and *Figla* to varying degrees (Pangas *et al*, 2006; Choi *et al*, 2008a).

Sohlh2 homozygous null ovaries also demonstrate a loss of follicles postnatally; however the phenotype is less severe than seen in either *Sohlh1*$^{-/-}$ or *Nobox*$^{-/-}$ ovaries, with a few follicles progressing to a stage with multi-layered granulosa cells (Choi *et al*, 2008b; Toyoda *et al*, 2009). *Sohlh2*$^{-/-}$ ovaries also demonstrate similar down-

regulation of follicle-associated genes to that seen in *Sohlh1*$^{-/-}$ ovaries, which is not surprising as Sohlh1 and 2 are able to form heterodimers, and thus may exert their functions in cooperation (Choi *et al*, 2008b; Toyoda *et al*, 2009).

Data regarding SOHLH1 or 2 in the human fetal ovary are limited. Data from azoospermic patients demonstrates the *SOHLH1* human orthologue does play a role in germ cell function (Choi *et al*, 2010b). In addition, up-regulation of *SOHLH1* in mid-gestational fetal ovaries has been determined (Fowler *et al*, 2009). Therefore the role of SOHLH1 and 2 in the human fetal ovary with regards to primordial follicle formation and development, along with possible mutations in POI patients remain of interest.

1.8.8 Factor in the germline alpha (FIGLA)

*FIGL*A is also a basic helix-loop-helix (bHLH) transcription factor, restricted to the germ cells within the ovary in the mouse and human (Liang *et al*, 1997; Soyal *et al*, 2000; Huntriss *et al*, 2002; Bayne *et al*, 2004). *Figla* was originally characterised as a regulatory factor in the adult ovary via its interaction with the zona pelucida (ZP) proteins (Liang *et al*, 1997; Dean, 2002), which function later in ovarian biology in regulation of plasma membrane formation and fertilisation (Bleil & Wassarman, 1980). However, a murine model of systemic ablation of *Figla* created to investigate this role further demonstrated the resulting homozygous females were unable to form primordial follicles after birth resulting in oocytes loss and infertility (Soyal *et al*, 2000) suggesting an important role for *Figla* in both fetal and adult ovarian biology.

Further investigation determined *Figla* expression peaks at initiation of nest breakdown and follicle formation (Soyal *et al*, 2000; Joshi *et al*, 2007). This increase in expression co-insides with the up-regulation of both *Oct4* and NACHT domain, leucine-rich-repeat domain, and pyrin domain-containing protein 5 *(Nalp5)*, which Figla is hypothesised to regulate (Soyal *et al*, 2000; Joshi *et al*, 2007). As previously discussed, Oct4 is highly involved in early PGC development, and is also thought to regulate primordial follicle formation. Function of Nalp5 in the fetal ovary has yet to be determined, although it expressed specifically by oocytes and is up-regulated with initiation of primordial follicle formation in the mouse (Joshi *et al*, 2007) and human

(Fowler *et al*, 2009). Studies in other tissues suggest Nalp5 may regulate caspase-3 activation and apoptosis, which is consistent with the hypothesis Figla functions to regulate germ cell nest breakdown (Lo *et al*, 2008). In addition to up-regulation of oocytes specific factors, Figla appears to repress testis specific factors and thus may also play a protective role during early ovarian development (Joshi *et al*, 2007; Hu *et al*, 2010).

Up-regulation of *FIGLA* prior to primordial follicle formation was also noted in the human fetal ovary (Huntriss *et al*, 2002; Bayne *et al*, 2004). FIGLA is thought to have a similar function in the human fetal ovary, as demonstrated in the mouse, as it is able to regulate the *ZP* genes in a similar fashion (Bayne *et al*, 2004; Fowler *et al*, 2009) to that noted in the mouse, and there are reports of POI patients with mutations in *FIGLA* (Zhao *et al*, 2008).

1.8.9 Forkhead box O3a (FOXO3A)

Foxo3a is a forkhead transcription factor, which is expressed primarily by oocytes within primordial follicles, with further expression in granulosa cells surrounding activated follicles (Liu *et al*, 2007). A murine model homozygous null for *Foxo3* was generated as it was hypothesised the Foxo family of transcription factors were involved in development (Castrillon *et al*, 2003). The resulting animals had several minor physiological abnormalities including anaemia and decreased rate of glucose uptake; however the main phenotypic difference with systemic deletion of *Foxo3a* was a rapid reduction in fertility in female mice, which were completely sterile by 15 weeks of age (Castrillon *et al*, 2003). Further examination of *Foxo3a*$^{-/-}$ females determined no change in ovarian physiology compared to wildtype mice at pnd3, but determined a rapid decline in oocyte number after primordial follicle formation, the result of premature activation of follicles (Castrillon *et al*, 2003).

However, as both germ cell and somatic cells express Foxo3, a tamoxifen-inducible, germ cell-specific *Vasa*-CRE was utilised to further study Foxo3 function specifically in oocytes (John *et al*, 2008). This conditional model developed a similar phenotype to that of the *Foxo3a*$^{-/-}$ ovaries, with global activation of follicles after tamoxifen treatment. It was further demonstrated that *Foxo3a* is activated by

the PTEN-PI3K-AKT signalling pathway (as it is in other systems (Tran *et al*, 2003)), as a similar *Vasa*-CRE conditional deletion of PTEN demonstrated the same phenotype of *Foxo3a* conditional deletion (John *et al*, 2008). An additional murine model was designed to further confirm Foxo3a function in oocytes via constitutively activating *Foxo3a* under control of the *Zp3* promoter which is expressed after the primary stage of folliculogenesis (Liu *et al*, 2007). Constitutively active *Foxo3a* results in loss of all follicles at the primary and secondary stage and oocytes display impaired growth (Liu *et al*, 2007). These data determined Foxo3a regulates signalling allowing for primordial follicle arrest, and is likely downstream of PTEN-PI3K-AKT signalling as well as Kit (as previously discussed) (Castrillon *et al*, 2003; Hosaka *et al*, 2004; Liu *et al*, 2007; John *et al*, 2008). However, study of the human ortholog of *FOXO3a* in patients with POI has yet to identify a similar function (Watkins *et al*, 2006; Gallardo *et al*, 2008; Vinci *et al*, 2008) and little is known about its expression in the human fetal ovary.

1.8.10 Forkhead box L2 (FOXL2)

Foxl2 is important during sex determination, in the repression of male specific factors (Uhlenhaut *et al*, 2009). FOXL2 is expressed by the pre-granulosa and granulosa cells in the fetal and adult ovary respectively in both the human and mouse (Crisponi *et al*, 2001; Cocquet *et al*, 2003; Duffin *et al*, 2009). Transcript expression of *Foxl2* is increased in mid-gestation in the mouse around the initiation of sex determination, consistent with its established function at this time (Cocquet *et al*, 2003; Duffin *et al*, 2009). Further, functional analysis was performed utilising female mice homozygous null for *Foxl2,* which are infertile as a result of impaired pre-granulosa cell differentiation and basal lamina formation, thereby preventing primordial follicle formation (Schmidt *et al*, 2004; Uda *et al*, 2004). This disruption also leads to a reduction in Activin βa and Amh expression (Schmidt *et al*, 2004; Uda *et al*, 2004).

Conditional deletion of *Foxl2* utilising a tamoxifen inducible CRE affecting both granulosa and theca cells (*R26CreERT2*) in the adult mouse ovary resulted in immediate differentiation of these cells to Sertoli and Leydig-like cells, and increased expression of testis markers including *Sox9* and steroidogenic acute regulatory gene (*StAR*) (Pisarska *et al*, 2004; Uhlenhaut *et al*, 2009). This data further demonstrates Foxl2 acts to repress the male pathway even after initial sex determination and promotes differentiation of granulosa cells necessary for primordial follicle formation.

Interestingly, *FOXL2* is one of the few factors of the germ cell niche originally identified in the human, with mutation identified in humans patients with blepharophimosis ptosis epicanthus inversus syndrome (BPES I and II), which includes ovarian failure or subfertility (Crisponi *et al*, 2001; Prueitt & Zinn, 2001; Cocquet *et al*, 2002b; Cocquet *et al*, 2003). Non-BPES mutations of *FOXL2* have also been reported with ovarian dysfunction (Harris *et al*, 2002). Both FOXL2 localisation and gene expression patterns in the human fetal ovary are comparable to that seen in the mouse, with initiation of F*OXL2* expression before 8 weeks gestation and up-regulated at 14-15 weeks gestation (Duffin *et al*, 2009). However, FOXL2 function in the human fetal ovary has yet to be confirmed.

This is a summary of the established transcription factors (Table 1.3) identified as regulators in the fetal ovary and female germ cell development.

Table 1.3 Summary of Transcription Factor Regulation

Summary of fetal ovarian phenotype from murine models (or function where noted) of key transcription factors

Transcription Factor	Localisation	Mouse Phenotype	Mouse Model	Reference
Oct4	GC	PGC loss - infertility	PGC-specific CRE/Lox	(Kehler *et al*, 2004)
Gata4	SM	PGC loss - infertility	$Gata4^{ki}$ – inhibits Gata4/Fog2 interaction	(Manuylov *et al*, 2008)
Sf1	SM	PGC loss - infertility	*FTz-F1* disruption which codes for Sf1 protein	(Luo *et al*, 1994)
Nanog	GC	PGC loss - infertility	PGC-specific CRE/Lox	(Yamaguchi *et al*, 2009)
Sohlh1 and Sohlh2	GC	Primordial to primary block –sterile	Homozygous null	(Pangas *et al*, 2006; Choi *et al*, 2008b)
Foxo3a	GC	Global activation of follicles –sterile	Homozygous null	(Castrillon *et al*, 2003)
Nobox	GC	Primordial to primary block –sterile	Homozygous null	(Rajkovic *et al*, 2004)
Figla	GC	Primordial follicle block - infertility	Homozygous null	(Soyal *et al*, 2000)
Lhx8	GC	Primordial to primary block –sterile	Homozygous null	(Choi *et al*, 2008a)
Foxl2	SM	Loss of follicles due to somatic fault –sterile	Homozygous null	(Uda *et al*, 2004)

1.8.11 Germ cell niche and other factors

Growth factors (illustrated in Tables 1.2 and Table 1.4) extracellular matrix proteins, and hormones, allow for crosstalk between the germ cells and neighbouring cells in the human fetal ovary, which in turn initiates intracellular signalling and modulation of transcription factors (Table 1.3) in order to regulate ovarian biology and germ cell development.

The paracrine, juxtacrine and autocrine support provided via these factors creates the microenvironment necessary for germ cell development and function and is termed the 'germ cell niche' (McLaren, 1991; De Felici, 2000). This term refers to the signalling milieu that is necessary to sustain the germ cell progression allowing for normal fertility in adulthood. However, the full cohort of growth and transcription factors involved in ovarian development remain unknown. Primordial follicle formation is important for future fertility, thereby making the determination of the factors within the germ cell niche and their interactions essential.

Table 1.4 Other factors that may be involved in fetal ovarian development

Summary of fetal ovarian phenotype from murine models (or function where noted) of other factors possibly involved in fetal ovarian development

Factor	Hypothesised function	Model	Reference
Notch	Regulates follicle formation	Mouse organ culture	(Trombly *et al*, 2009a)
Nalp5	Involved in primordial follicle formation	Abscent in Figla$^{-/-}$ mice Up-regulated in 2nd trimester human fetal ovary	(Joshi *et al*, 2007) (Fowler *et al*, 2009)
Wt1	Regulates gonad formation	Homozygous null mice	(Kreidberg *et al*, 1993)
Zglp1	Regulates GC survival	ZGLP-1 (lacZ) null mice	(Li *et al*, 2007)
Lhx9	Regulates PGC survival	Homozygous null mice	(Birk *et al*, 2000)
Pod1	Regulates of gonad structure	Homozygous null mice	(Cui *et al*, 2004)
Aromatase	Regulates follicle formation	Homozygous null mice	(Britt *et al*, 2004)
AMH	Regulates primordial follicle activation	AMH levels in humans relates to follicle number Mouse organ culture	(de Vet *et al*, 2002; Durlinger *et al*, 2002)
Aryl-hydrocarbon receptor	Regulates primordial follicle number	Homozygous null mice	(Benedict *et al*, 2000; Robles *et al*, 2000)

1.9 Possible regulators of early ovarian development

Several questions remain regarding the established factors of the germ cell niche and whether other unidentified factors are involved during fetal ovarian development. Several factors are known to regulate similar mechanisms in other tissues (proliferation, differentiation, apoptosis, etc), and interact with established ovarian regulators. In addition, these factors are known to be vital to adult ovarian function. However, their roles during fetal ovarian development and interaction with the germ cell niche are unknown. The following sections will describe these regulatory factors postulated to be involved during fetal ovarian function.

1.9.1 Prostaglandin E2

The prostaglandins (PGs) are lipid mediators classically characterised as regulators of inflammation, immunity, and vascular homeostasis (Murakami *et al*, 1993). Their signalling is known to promote angiogenesis, inhibit apoptosis, and increase proliferation and migration (Sales & Jabbour, 2003a), with regulation determined in various species ranging from invertebrates to humans denoting their roles are generally evolutionarily conserved (Cha *et al*, 2006). PGs came into the limelight in the clinical world in the early 1970's, with non-steroidal anti-inflammatory drugs (NSAIDs) which reduce inflammation symptoms including swelling, pain and fever by inhibiting PG synthesis (Vane, 1971).

PGs are synthesised by the conversion of arachadonic acid by cyclooxygenase enzymes (COX1 and COX2). In a physiological sense, COX1 is traditionally thought of to be the ubiquitously expressed "maintenance" enzyme, whereas COX2 is important for cytokine mediated specific events. This is supported by constitutive expression of COX1 in several organs (kidney, lung, stomach, colon) and the inability to stimulate COX1 levels *in vitro* (Kargman *et al*, 1996), whereas COX2 expression is not constitutively expressed in most tissues but can be rapidly induced by cytokine or growth factor expression (Cha *et al*, 2006).

The COX enzymes produce prostaglandin H_2 (PGH_2), which is further converted to prostaglandin D_2, E_2, $F_{2\alpha}$, thromboxane (TXA_2 or TXB_2) or prostacyclin (PGI_2) by specific terminal prostanoid synthase enzymes, such as prostaglandin E synthase (PTGES) (As shown in Figure 1.8). In addition, although the mechanism remains unclear, genetically modified murine models have demonstrated the COX enzymes may preferentially synthesize specific prostaglandins (as further described below). It is also notable that the PGs and COX2 are able to signal in both an autocrine and paracrine fashion that results in a positive-self-sustaining feedback loop (Sales & Jabbour, 2003b).

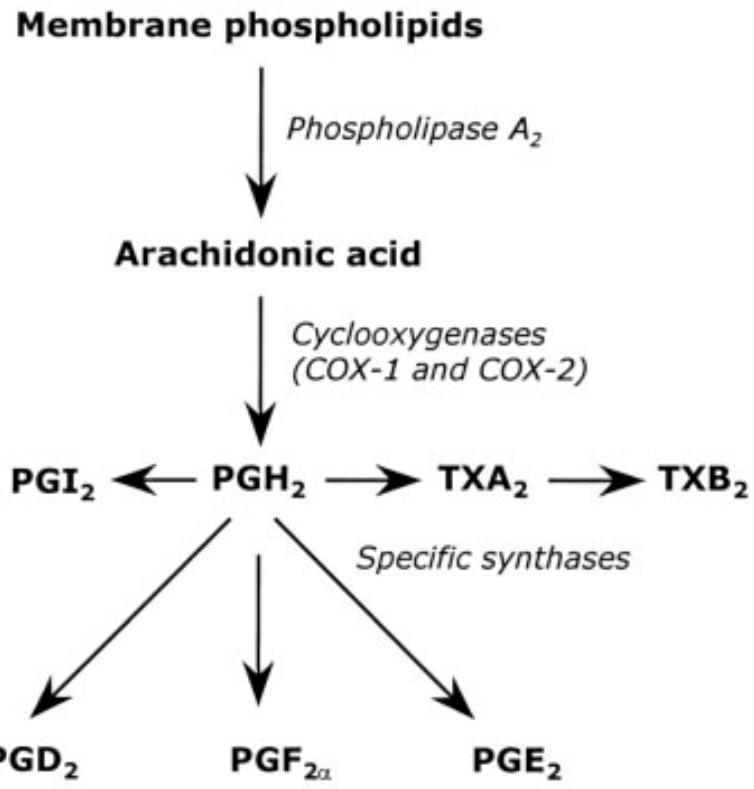

Figure 1.8 Arachadonic acid to prostaglandin pathway

As fully described in the text, PGE2 is synthesised from a stepwise enzymatic pathway initiating with breakdown of phospholipids and resulting in several prostaglandins (D2, F2α, and E2), thromboxane (TXA2 and TXB2) and prostacyclin PGI2).

Even before PGs were identified, several scientific groups had determined there was a lipid mediator in semen (now known to be PGs) that causes contraction of the uterine smooth muscle and reduced blood pressure (Cha *et al*, 2006). For this reason, the PGs were isolated and characterised as regulators of reproductive inflammatory events, including parturition, ovulation, menstruation and endometriosis, as well as fertilisation, implantation and decidualisation (Behrman, 1979; Oates *et al*, 1988a; Oates *et al*, 1988b; Baird *et al*, 1996; Sales & Jabbour, 2003b).

Studies using animal models have further defined these roles, with homozygous ablation of *COX1* in female mice resulting in prolonged gestation and parturition, with normal ovulation and fetal development (Langenbach *et al*, 1995). The reproductive phenotype could also be rescued with administration of $PGF_{2\alpha}$ (Gross *et al*, 1998), suggesting this may be the dominate PG product of COX1. In contrast, *COX2* homozygous null females result in a more severe reproductive phenotype, including failure to ovulate, fertilise, implant, and decidualise (Dinchuk *et al*, 1995; Lim *et al*, 1997). However, the ovarian phenotype was rescued with the administration of PGE_2 (Lim *et al*, 1997). Despite both precursor enzymes catalysing the same reaction and some level of redundancy confirmed (Wang & Roy, 2004), it is apparent from these models that the COX enzymes may catalyse different amounts of each prostaglandin leading to different downstream outcomes. Unfortunately, overall effects of complete ablation of the COX enzymes cannot be investigated as double-knockout mice die shortly after birth due to patent ductus arteriosus (Loftin *et al*, 2001).

However, specific $COX^{-/-}$ models have identified PGE_2 and $PGF_{2\alpha}$ as the two main PGs responsible for of the above reproductive phenotypes, and therefore the most involved in reproductive function. Further investigation led to the ablation of the $PGF_{2\alpha}$ receptor, which resulted in a mouse model that loses parturition function, whereas ablation of one of the PGE_2 receptors results in disruption of ovulation (Hizaki *et al*, 1999; Kennedy *et al*, 1999; Tilley *et al*, 1999; Narumiya & FitzGerald, 2001). From this evidence it is apparent that PGE_2 may be the more important PG in ovarian function.

PGE_2 functions in the adult ovary in initiation of ovulation and fertilisation of the oocyte (Ojeda *et al*, 1982; Hizaki *et al*, 1999), doing so via four G-coupled protein receptors (GPCRs), EP1-4. Each of these signals via a different downstream pathway; EP1 signals via Gq to up-regulate intracellular calcium; EP2 and EP4 are 'stimulatory' receptors and signal via Gαs to increase cyclic adenosine monophosphate (cAMP), with EP4 also able to act via the PI3/AKT pathway; and EP3 is regarded as 'inhibitory' and signals via Gi to decrease cAMP (Alfranca *et al*, 2006) (Figure 1.9). This variance in downstream signalling, coupled with varying localisation to different cell types, allows a single prostanoid to exert multiple functions in a single tissue.

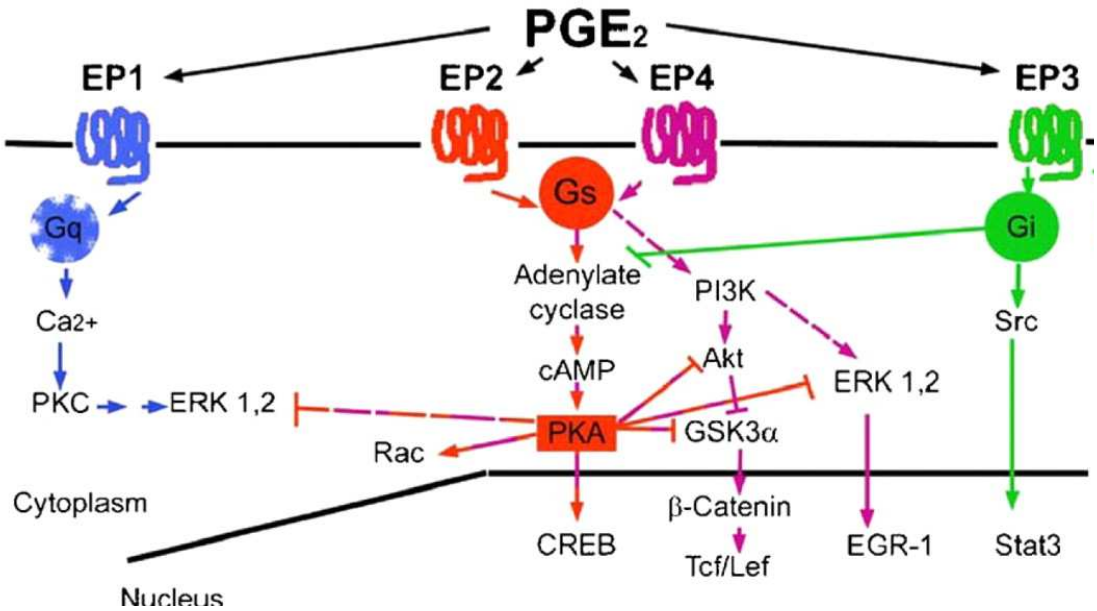

Figure 1.9 Specific downstream signalling of the four EP receptors

PGE_2 is able to signal via 4 G-coupled protein receptors (EP1-4), each of which signals via a distinct downstream intracellular cascade. EP1 signals via Gq to increase intracellular calcium, EP2 and EP4 via Gs to induce adenylate cyclase activity (with EP4 also inducing the PI3K/AKT pathway), and EP3 via Gi inhibits adenylate cyclase activity and induces Stat signalling. Figure adapted from (Alfranca *et al*, 2006).

In addition to classical ligand receptor action, there is also evidence that the EP receptors can be internalised to the nucleus to directly regulate gene transcription (Bhattacharya *et al*, 1998; Bhattacharya *et al*, 1999; Desai *et al*, 2000). Of the EP receptors, EP2 is thought to be the most involved in ovarian function, determined by the ovulatory defects and failure of fertilisation in *Ep2* homozygous null mice, whereas inactivation of the remaining Ep receptors lead to no major reproductive dysfunction (Hizaki *et al*, 1999; Kennedy *et al*, 1999; Tilley *et al*, 1999). It was determined that gonadotropins increase both *Cox2* and *Ep2* levels in the adult ovary leading to cumulus expansion (Hizaki *et al*, 1999), which is sustained by the positive feedback capabilities of the PGE_2 system. It is likely the $Ep2^{-/-}$ phenotype may be a result of inhibition of this signalling pathway.

Apart from reproductive function, PGE_2 mediates development of other organs, most critically closure of the ductus arteriosus, which allows for postnatal circulation, and is the main cause of fatality in $COX1/2^{-/-}$ doubly ablated mice (Nguyen *et al*, 1997; Loftin *et al*, 2001). PGE_2 regulation of fetal angiogenesis is also well characterised (Majima *et al*, 1997), and is likely to be mediated by the angiogenic factors up-regulated by PGE_2 including bFGF and vascular endothelial growth factor (VEGF) (Cheng *et al*, 1998). Additionally, PGE_2 is postulated to be required for blastocyst development (Dey *et al*, 1980; Davis *et al*, 1983), as inhibition of the COX enzymes during pregnancy results in degradation of murine blastocysts (Cha *et al*, 2006). This key role in development is also noted in the inhibition of PGE_2 in the zebrafish, where it regulates cell migration necessary for gastrulation (Cha *et al*, 2006).

With roles in both the adult ovary and development of other organ systems, PGE_2 is an attractive candidate for possible regulation of fetal ovarian development. PGE_2 has also been shown to interact with established germ cell niche factors. PGE_2 is known to be up-regulated during human placental development, when the hypoxic environment activates Activin A (Petraglia *et al*, 1993; Jenkin *et al*, 2001; Supramaniam *et al*, 2004), it is also demonstrated to regulate *BMP2*, *4*, and *7* in osteoid cells (Virdi *et al*, 1998; Takiguchi *et al*, 1999; Paralkar *et al*, 2002), and induces *BDNF* expression in astrocytes (Toyomoto *et al*, 2004; Hutchinson *et al*, 2009) In addition, an recent exploratory gene expression assay revealed PGE_2 as a

potent regulator of the neurotrophins in female reproductive tissues (Jabbour, unpublished). However, none of these interactions/relationships have been confirmed in the human fetal ovarian environment.

1.9.2 The IL6-type cytokines

The IL6-type cytokines are secreted, pro/anti-inflammatory cytokines which demonstrate overlapping function and may, like PGE_2, be involved in fetal ovarian development. The IL6-type cytokine family is composed of several cytokines which signal via receptor complexes consisting of shared components, and includes IL6, IL11, leukaemia inhibitory factor (LIF), oncostatin-M (OSM), cardiotropin-1 and ciliary neurotrophic factor (CNTF) (Heinrich *et al*, 1998). The IL-6 type cytokines signal via receptor complexes made up of hetero- or homo- dimers or trimers, with a common receptor component gp130 in each receptor complex (Figure 1.10). As reviewed in detail (Heinrich *et al*, 1998); each receptor complex contains one or more shared signal transducing components, and some complexes also include an α-receptor subunit, which although not required for intracellular signalling is necessary for specific ligand binding. LIF and OSM share the same signalling complex consisting of a heterodimer of LIF receptor (LIFR) and gp130 (Gearing & Bruce, 1992). OSM is also able to signal via a heterodimer of a newly characterised OSM receptor (OSMR) and gp130 (Ichihara *et al*, 1997). Comparable to LIF and OSM, CNTF also utilises the LIFR/gp130 heterodimer, but also requires a specific CNTF receptor (CNTFR) α subunit for signalling (Gearing & Bruce, 1992; Davis *et al*, 1993). Finally, IL6 ligand utilises an α-receptor subunit, IL6 receptor in combination with a homodimer of gp130 (IL6R) (Taga *et al*, 1989). Several studies have demonstrated a high degree of redundancy in IL6-type cytokine function as a result of shared receptor components and similar intracellular signalling (Koshimizu *et al*, 1996; Liu *et al*, 2000).

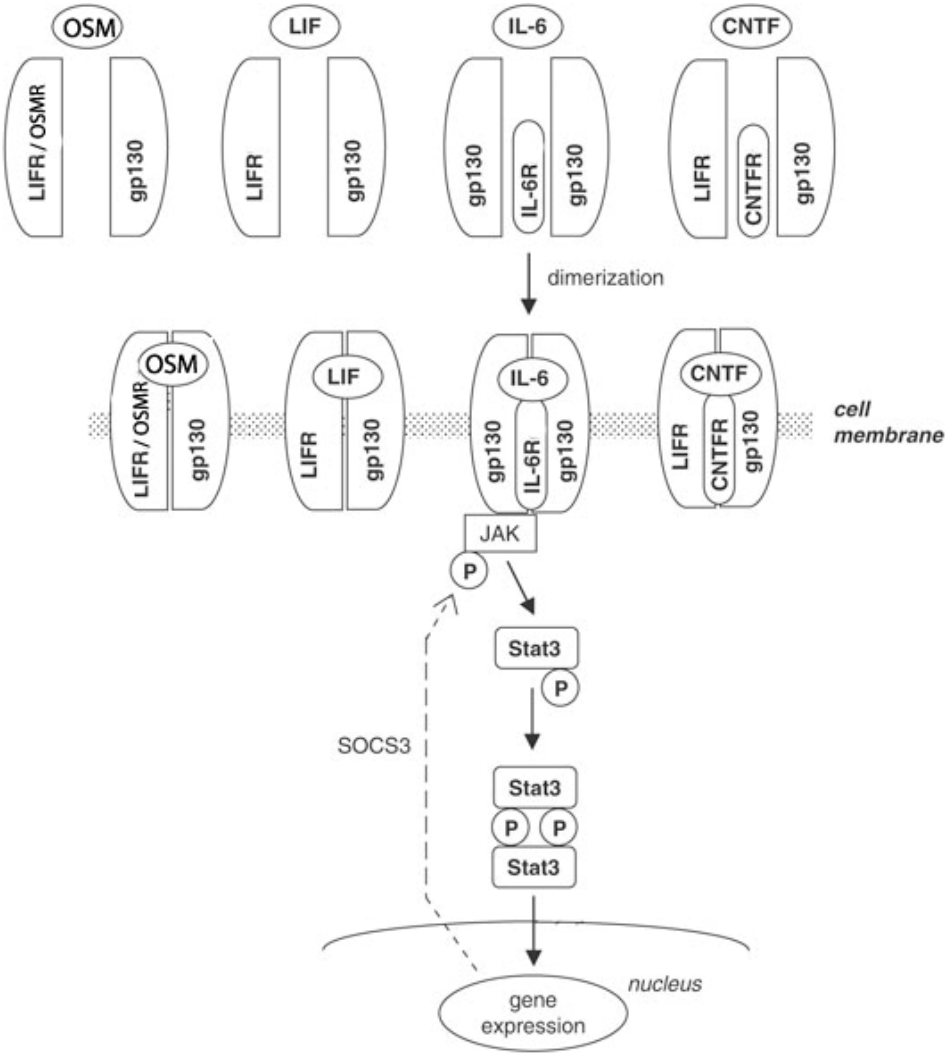

Figure 1.10 IL6-type cytokine signalling

As described fully in the text, the IL6 type cytokines signal via formation of hetero- or homo- dimers or trimers of various shared receptor components to intracellularly signal via the Jak/STAT pathway in order to modulate gene expression. This signalling can be inhibited via SOCS3, which is also a downstream target of IL6-type signalling and thus part of a negative feedback loop. Figure adapted from (Suzuki *et al*, 2009).

The IL6-type cytokine receptor complexes signal via activation of the Janus kinases (Jaks) and the signal transducer and activator of transcription (STAT) transcription factor (Jak/STAT) pathway (Lutticken *et al*, 1994; Stahl *et al*, 1994). The activation of this signalling pathway via various phyosphorylation events is able regulate target gene expression via phosphoylated dimerisation of STATs which is required for DNA binding (Shual *et al*, 1993). However, Jak/STAT regulation is known to be transient and thus effective mediators of the system, specifically suppressor of cytokine signalling 3 (SOCS3), have been identified (Nicholson *et al*, 2000; Schmitz *et al*, 2000). STAT dimers are able to up-regulate various transcripts, including the SOCS inhibitory factors themselves (Heinrich *et al*, 1998), which in turn act as negative feedback inhibitors by acting on activated IL6 receptors or Jaks to inhibit further signalling. Both IL6 and LIF signalling are known to induce SOCS3 expression, suggesting these factors are able to promote their own negative feedback loop, limiting signalling and allowing for acute regulation (Endo *et al*, 1997). This acute regulation allows the IL6-type cytokines to regulate specific events in various tissues, including the ovary. In the adult ovary, IL6-type cytokines are involved in various processes including cumulus expansion and oocyte competence (Liu *et al*, 2009).

As with Oct4 and Nanog, the IL-6 type cytokine family was originally highlighted as a potential regulator of germ cell development based upon various *in vitro* experiments using stem cells, embryonic germ (EG) cells, and PGCs (Williams *et al*, 1988; Defelici & Dolci, 1991; Matsui *et al*, 1991; Resnick *et al*, 1992; Cheng *et al*, 1994; Koshimizu *et al*, 1996). LIF ligand in particular has been well characterised in the maintenance of ES cells and PGCs in culture in combination with KITL and bFGF (Matsui *et al*, 1991; Resnick *et al*, 1992), thus demonstrating regulation of proliferation and survival. Further studies demonstrated LIF function could be replicated by addition of IL6, CNTF or OSM to the culture media, demonstrating compensatory action (Cheng *et al*, 1994; Koshimizu *et al*, 1996). Further, culture of mouse PGCs demonstrated reduction in apoptosis with LIF treatment (Pesce *et al*, 1993a), and that the common signalling component gp130 is able to regulate initiation of meiosis (Chuma & Nakatsuji, 2001). Based upon this accumulation of

in vitro data, it was hypothesised the IL6-type cytokines had a role in germ cell function *in vivo*, specifically during early development and maturation.

However, as discussed for both Oct4 and Nanog, regulatory roles demonstrated in ES cells and/or PGCs *in vitro* often differ from their physiological roles *in vivo*. To date, several genetically engineered murine models have been generated in attempts to recapitulate the regulation of IL6-type cytokines seen *in vitro* in the fetal gonad (Stewart *et al*, 1992; Escary *et al*, 1993; Ware *et al*, 1995; Yoshida *et al*, 1996; Molyneaux *et al*, 2003a) with little success. Systemic deletion of LIF was the first model to be investigated, which did result in infertility in female offspring, but it was identified this was the result of an ovulatory defect rather than an effect on germ cell differentiation (Stewart *et al*, 1992). As the IL6-type cytokines are known to have overlapping function, several models focused on shared signalling components, in order to prevent compensation by other IL6 cytokines which might mask function (Ware *et al*, 1995; Yoshida *et al*, 1996; Takeda *et al*, 1997). However, homozygous deletion of the shared receptor components gp130 or LIFR or the common downstream target *STAT3* resulted in a lethal phenotype, either embryonically or shortly after birth (Ware *et al*, 1995; Yoshida *et al*, 1996; Takeda *et al*, 1997), preventing any elucidation of systemic blockage of IL6-type cytokine signalling. A *gp130* germ cell specific knockout was also generated utilising the *TNAP*-CRE, as previously described for Oct4 and Nanog (Molyneaux *et al*, 2003a). This line resulted in no change in ovarian phenotype during fetal and early postnatal life (although there was an ovulation defect in adult female and a reduction in PGCs in male pups (Molyneaux *et al*, 2003a).

Although several attempts have been made to elucidate IL6-type cytokine function *in vivo*, their role during fetal gonad development has not been established. In addition little is known about the IL6-type cytokines and their receptors in the human fetal ovary, despite strong evidence for these factors as regulators of differentiation and proliferation *in vitro*. For these reasons, the IL6-type cytokines were hypothesised to function as part of the germ cell niche during ovarian development.

1.9.3 The Prokineticins

A final group of regulators were also hypothesised to regulate fetal ovarian development; the prokineticins (PROK) 1 and 2, which are recently described proteins known to regulate angiogenesis and inflammation (Catalano *et al*, 2010; Monnier & Samson, 2010). The name prokineticin reflects how the proteins were originally characterised, as potent regulators of smooth muscle contractility in the gastrointestinal tract (Li *et al*, 2001) (eg. pro- kinetic). The PROKs have similar function to vascular endothelial growth factor (VEGF) in promotion of angiogenesis, and PROK1 was initially termed endocrine gland VEGF (EG-VEGF) (LeCouter *et al*, 2001). In addition, PROK1 was also named venom protein A (VPRA) and mamba intestinal toxin1 (MIT1), as the PROK1 orthologue was initially identified as a non-toxic protein derived from the venom of the back mamba snake (*Dendroaspis polylepis*) (Joubert, 1980; Schweitz *et al*, 1990). PROK2 was also identified in a different species by a different name; Bv8, derived from the skin secretions of the yellow-bellied toad (*Bombina variegata*) (Mollay *et al*, 1999). Despite various aliases and nomenclatures, the human orthologous of the PROKs were identified and termed PROK1 and PROK2, as they will be referred to in this report.

The PROKs signal via two prokineticin receptors (PROKR1 and PROKR2) (Masuda *et al*, 2002) which share 87% homology (Soga *et al*, 2002). Both PROK ligands are able to signal interchangeably via the two GPCRs; however, PROK2 has a slightly higher affinity for both receptors (Lin *et al*, 2002; Soga *et al*, 2002). The PROKRs then signal via either Gi or Gq signalling, which in turn increases intracellular calcium concentration and provokes rapid activation of p44/p42 MAP kinase signalling as well as activation of STAT signalling (Lin *et al*, 2002; Masuda *et al*, 2002; Soga *et al*, 2002).

Although initially characterised in endothelial cells, PROK receptors and ligands are expressed in various cell types throughout the body. PROK1 is expressed in B and T cells (Dorsch *et al*, 2005) and PROK2 in bone marrow, blood cells, immune cells, and neuronal tissue (LeCouter *et al*, 2004). In these systems PROKs are shown to promote proliferation, differentiation and survival (Melchiorri *et al*, 2001; LeCouter *et al*, 2004). Additionally, both ligands are highly expressed in the steroidogenic

reproductive tissues, such as the gonads (Wechselberger *et al*, 1999; Ferrara *et al*, 2003; Kisliouk *et al*, 2003; LeCouter *et al*, 2003; Samson *et al*, 2004), the placenta (LeCouter *et al*, 2001; Hoffmann *et al*, 2006) the endometrium (Evans *et al*, 2008; Evans *et al*, 2009) and the adrenal glands (Lin *et al*, 2002). Although relatively little systemic data is available for these recently characterised proteins, each ligand has been well characterised in a distinct organ system. PROK1, as alluded to previously, is extensively described in its role in smooth muscle contractility, with evidence for regulation of most of the GI tract in various species (Li *et al*, 2001; Hoogerwerf, 2006; Wade *et al*, 2010). However this role is not shared with PROK2, which demonstrates that, although similar, the two ligands share distinct roles in different systems (Bassil *et al*, 2005). PROK1 and 2 are able to induce proliferation and differentiation in the central nervous system (CNS) (Ngan *et al*, 2007; Ngan *et al*, 2008; Ruiz-Ferrer *et al*, 2011), but only PROK2 is able to regulate more specific CNS processes such as circadian rhythm, pain sensation, appetite, locomotor activity and thermoregulation (Cheng *et al*, 2002; Negri *et al*, 2002; Cheng *et al*, 2005; Prosser *et al*, 2007; Jethwa *et al*, 2008; Ren *et al*, 2011).

Despite the elucidation of PROK function in other organ systems, little is known their roles in the gonads, despite being localised in the interstitial cells of both the testis and ovary (Wechselberger *et al*, 1999; Ferrara *et al*, 2003; Kisliouk *et al*, 2003; LeCouter *et al*, 2003; Samson *et al*, 2004). Homozygous ablation of *Prok2* or *Prokr2* using rodent models resulted in the atrophy of the reproductive system (male or female) similar to the phenotype seen in human Kallmann syndrome (Dode *et al*, 2006; Matsumoto *et al*, 2006; Pitteloud *et al*, 2007); however it was determined this was likely due to *Prok2/Prokr2* effects on gonadoropin-releasing hormone (GnRH) expression, rather than direct regulation in the gonads (Matsumoto *et al*, 2006; Pitteloud *et al*, 2007). Further evidence of this regulation was found in women who suffer reversible hypothalamic amenorrhea (predisposition to GnRH deficiency/disruption after exposed to stressors), which found several of these women had mutations in PROKR2 (Caronia *et al*, 2011). Although not yet defined, it has been postulated PROK1 does have a direct role in the ovary and is localised to the theca cells in adult tissues (Ferrara *et al*, 2003; Kisliouk *et al*, 2003). This

hypothesis is based upon the observation that PROK1 transcript expression increases with development of the corpus luteum (Ferrara *et al*, 2003; Fraser *et al*, 2005). However, further mechanistic investigation and localisation of the PROK receptors has yet to be performed.

However, two recent transcriptome studies have demonstrated the *PROK*s are expressed and may play a role in the fetal ovary. One array demonstrated increased expression of *Prok1* in the fetal ovary comparative to the fetal testis (Houmard *et al*, 2009). The other detected novel expression of *PROK2* in the human fetal ovary which increased with gestation leading to primordial follicle formation, suggesting a possible role for the ligand in ovarian development (Fowler *et al*, 2009). Further, there is evidence from other reproductive tissues, that the PROKs may regulate signalling components of the other candidate regulators (PGE_2 and the IL6-type cytokines) (Denison *et al*, 2008; Evans *et al*, 2008; Evans *et al*, 2009; Cook *et al*, 2010).

In addition to the hypothesis that the PROKs may be active in regulating the human fetal ovary, it is also postulated the PROKs may play a role in fetal testis development, as several studies have highlighted increased expression of PROK2 and PROKR2 in the fetal testis compared to both the fetal ovary, and other tissues, including the brain where PROK2 and PROKR2 are known to be highly active (Wechselberger *et al*, 1999; LeCouter *et al*, 2003; Samson *et al*, 2004). These studies also highlighted a potential role for PROK regulation of steroidogenesis, which is integral to sex differentiation and further testis development. With this data in mind, the expression of PROKs in the human fetal ovary and testis were further characterised, to explore both PROK regulation during fetal gonad development, but also differential regulation between the sexes.

1.10 Aims of PhD

Human fetal ovarian development is critical, as primordial follicle development at this stage underpins adult fertility in later life. The fetal ovary and germ cells within are regulated by the germ cell niche, which regulates progression from PGC specification to primordial follicle formation. Despite the importance of the germ cell niche, few of its contributing factors have been identified, leaving the regulation of early ovarian development poorly understood. It is likely the germ cell niche is more similar to a web than a single cascade, allowing for positive and negative feedback loops/checks and balances in the system to prevent disruption. Therefore, the question remains: What factors make up the germ cell niche, and how do these factors interact to regulate fetal ovarian development?

In order to further answer this question, several factors implicated in regulation of reproductive function later in life, which were known to interact with established factors of the germ cell niche were identified; PGE_2, the IL-6 type cytokines, and the PROKs. It was postulated that these regulatory factors (or families of factors), were involved in fetal ovarian development and primordial follicle formation.

The specific aims of this research were to:

1. Examine expression and localisation of signalling PGE_2 components across human gestation (8-20 weeks), and further investigate transcriptional and functional effects of PGE_2 treatment *ex vivo* in human fetal ovarian samples.

2. Further determine the relationship between PGE_2 signalling and germ cell development *in vivo*, utilizing a fetal rat model of COX inhibition via paracetamol exposure.

3. Explore the roles of the IL6-type cytokines in the human fetal ovary to further clarify in vivo roles during germ cell development, and to further clarify if regulation seen in ESC culture *in vitro* is relevant to PGCs and oocytes *in vivo*.

4. Characterise PROK expression and localisation in the human fetal ovary, and further investigate function via the creation of a germ cell line stably expressing PROKR1, to determine signalling pathways regulated by PROK1-PROKR1 interaction.

Secondary to the focus on the ovarian germ cell niche is interest in how ovarian and testicular development differ, and how the same regulatory factors have differing roles in the two systems. As the PROKs are differentially expressed in the testis and the ovary in both the human and the mouse, it was hypothesised that PROKs might have differing regulation in each system, and thus were also investigated in the fetal testis:

5. Compare PROK expression and localisation in the human fetal testis to that demonstrated in the human fetal ovary, and further identify sex-specific function by culturing interstitial cells isolated from human fetal testes.

Chapter 2

General Materials and Methods

Chapter 2. General Materials and Methods

2.1 Human Dissection and Tissue Collection

Human fetuses (8-20 wks) were obtained as previously described in (Coutts *et al*, 2008). Informed consent was given, and the study approved by the Lothian Research Ethics Committee (LRC08/S1101/1). Surgical terminations were also obtained after administration of misoprostol (200mg per vaginam) via vacuum aspiration. General aesthetic was used during the procedure.

It is recognised misoprostol treatment may interfere with parts of this study that focus on prostaglandin interaction in fetal tissue. However, as tissue collected responded to PGE_2 treatment, it is unlikely that the gonads are greatly affected by maternal misoprostol treatment (see Chapter 3).

All fetuses were gestationally aged based on ultrasound scan. This gives an 'obstetric' gestation based on last menstrual period rather than post-conception dating. After the specimens were obtained this gestation was confirmed utilising either crown-rump length in first trimester specimens or foot length in second trimester specimens.

After obtaining samples, tissue was transported to the laboratory for further dissection. Micro-dissection to remove the gonads was performed in sterile conditions and further tissue taken for SRY polymerase chain reaction (PCR) as explained below (Section 2.3) in order to sex first trimester fetal gonads. Gonads to be used for RNA/protein analysis or tissue culture were carefully separated from all extraneous material including the mesonephros using sterile needles (BD).

2.2 Rat Treatment, Dissection and Tissue Collection

Female Wister rats were maintained according to UK Home Office guidelines and fed soy-free breeding diet (RM3 (E) soya free, SDS). Rats were kept in controlled lighting housing (on at 0700h, off at 1900h) at a temperature of 19-21º C with LITASPEN standard bedding (SPPS). Timed matings were established by visible vaginal plug and deemed embryonic day (e) 0.5. At e13.5 of gestation, dams were fed via gavage 350 mg/kg paracetamol (Sigma) diluted in corn oil (Mazola). A dosage of 350 mg/kg paracetamol was utilised as previous studies had determined this concentration was high enough to affect rodent fetuses without causing maternal morbity (including liver necrosis) (Kristensen *et al*, 2011). A higher dosage than that which a pregnant woman would use was utilised as rats have both an increased basal metabolic rate (~7x that of the human) and drug clearance rate (acetaminophen/paracetamol ~8.6x that of the human) comparative to humans (Choiu *et al*, 1998). Gavage delivery of paracetamol was utilised to mimic oral delivery of paracetamol in humans, with one large bolus rather than multiple doses to reduce stress to the animals which may have obscured effects of the drug itself.

Control dams received corn oil via gavage only. This was repeated every morning for 7 days (e20.5), followed by culling of the dams via cervical dislocation at e21.5. Pups were recovered and weighed before being culled via decapitation. Ovaries were removed via micro-dissection and one was snap frozen at -80° C for further analysis via enzyme linked immunosorbent assay (ELISA), while the other was fixed in Bouins solution (Clintech) for 1 h and processed as described in section 2.6.

2.3 SRY Genotyping

PCR amplification of the sex-determination gene (*SRY*) was performed to determine the sex of human first trimester specimens, as gender cannot be visually determined (Friel *et al*, 2002). The sample of specimen retained for DNA extraction was added to 100 µl of 25 mM NaOH/0.2 mM EDTA. The solution was heated at 95° C for 20 min and then neutralised in 100 µl of 40 mM Tris.HCl on ice.

The samples are vortexed for 1 min and 5 µl of the sample added to the reaction mix (Table 2.1). Remaining sample can be stored at -20°C.

Table 2.1 SRY RT-PCR Reaction Mix

Reagent	Amount Used	Manufacturer
ImmoMix Red	12.5 µl	Bioline
25 µM SRY Forward Primer	0.5 µl	Eurogentech
25 µM SRY Reverse Primer	0.5 µl	Eurogentech
H_2O	6.5 µl	Ambion

The reaction mix was run on a thermocycler (PTC-100, MJ Research Inc) as in Table 2.2. Each sample was run with a 100 bp ladder (Promega) via electrophoresis on a 1% agarose gel (Sigma) containing Gel Red (Biotium) for visualisation. A product of 300 bp confirms the specimen as male, absence of the band is determined as female. Known positive and negative controls were utilised to ensure reaction success.

Table 2.2 SRY PCR Thermocycler Programme

Step	Temperature	Time
1	95°C	10 min
2	95°C	30 sec
3	58°C	30 sec
4	72°C	45 sec
Repeat steps 2-4 for 35 cycles		
5	72°C	10 min

2.4 cDNA synthesis and RT-PCR

Snap frozen samples (-80º C) were homogenised and RNA extracted using the RNeasy Micro Kit (Qiagen) with on-column DNase digestion according to manufacturer's instructions. Reverse transcription was performed using the Superscript VILO kit (Invitrogen) according the manufacturer's instructions, 0.5 μg of RNA was used in each reaction. Negative control samples (RT-) lacking transcription enzyme were also synthesised to identify contamination in further experiments.

Target-specific PCR was performed to determine presence or absence of specific transcripts using 1 μl of the RT+ or RT- cDNA reaction in a reaction volume of 25 μl (Table 2.3). Products were run on agarose gels (1-2.5%, Sigma) with a 100 bp ladder (Promega) using electrophoresis. Primer pairs can be found in Table 2.4.

Table 2.3 RT-PCR Reaction Mix

Reagent	Amount Used	Manufacturer
ImmoMix Red	12.5 μl	Bioline
25 μM Forward Primer	0.5 μl	Eurogentech
25 μM Reverse Primer	0.5 μl	Eurogentech
H_2O	10.5 μl	Ambion

Table 2.4 PCR Primer Pairs

Gene Target	Forward Primer	Reverse Primer	Product Size
3β-HSD	GCGGCTAATGGGTGGAATCTA	CTGGCAGAAAGGAATGGGCC	101 bp
AMH	ACATCAGGCCCAGCTCTATCAC	TGTTTGTGCAGGACAGACCC	151 bp
BDNF	AACAATAAGGACGCAGACTT	TGCAGTCTTTTTGTCTGCCG	222 bp
CNTF	CAGGGCCTGAACAAGAACAT	CTAAGAGCCTGGCCAACAAA	151 bp
COX-1	TGTTCGGTGTCCAGTTCCAATA	ACCTTGAAGGAGTCAGGCATGAG	95 bp
COX-2	CCTTCCTCCTGTGCCTGATG	ACAATCTCATTTGAATCAGGAAGCT	82 bp
CYP11A1	CCAGACCTGTTCCGTCTGTT	GAAGTTCTGGGTGTATATGTCAGC	99 bp
DAZL	GAAGGCAAAATCATGCCAAACAC	CTTCTGCACATCCACGTCATTA	186 bp
DESMIN	GGAGATTGCCACCTACCG	GGTCTGGATGGGGAGATTG	67 bp
DKK1	GCGGGAATAAGTACCAGACCAT	GGGACTAGCGCAGTACTCATCAGT	92 bp
EP1	AGATGGTGGGCCAGCTTGT	GCCACCAACACCAGCATTG	73 bp
EP2	GACCGCTTACCTGCAGCTGTAC	TGAAGTTGCAGGCGAGCA	71 bp
EP3	GACGGCCATTCAGCTTATGG	TTGAAGATCATTTTCAACATCATTATCA	87 bp
EP4	ACGCCGCCTACTCCTACATG	AGAGGACGGTGGCGAGAAT	64 bp
GAPDH	GACATCAAGAAGGTGGTGAAGC	GTCCACCACCCTGTTGCTGTAG	212 bp
GP130	CTGAATGGGCAACACACAAGTT	CCAGACTTCAATGTTGACAAAATACA	107 bp
ID3	CTCTTCAGGCCACAAGTTCAC	GTGTCCTGACACCTCCAGAAC	192 bp
IL6	GCCGCCCCACACAGACA	CCGTCGAGGATGTACCGAAT	71 bp
IL6R	AGCTCAGATATCGGGCTGAA	GGACTCCTGGATTCTGTCCA	202 bp
INHBA	GGCAAGTTGCTGGATTATAGTG	CCACATACCCGTTCTCCCCGAC	272bp
LIF	TGGTGGAGCTGTACCGCATA	TGGTCCCGGGTGATGTTG	64 bp
LIFR	TCAAAGGGGCCTGATACTTG	CTGCTTTGTGCTGAGGATCA	178 bp
MCL1	ATCTCTCGGTACCTTCGGGAGC	GCTGAAAACATGGATCATCACTCG	221 bp
NTF5	QUANTITECT PRIMAR Assay Hs_NTF5_1_SG (QIAGEN)		96 bp
OCT4	ACATCAAAGCTCTGCAGAAAGAAC	CTGAATACCTTCCCAAATAGAACCC	126 bp
OSM	ACAGAGGACGCTGCTCAGTC	AGGAGTCTGCTGGTGTCCTG	150 bp
OSMR	TTGACAACCCGGAAGAAAAG	TGAATCAGCATCGAGGAGTG	157 bp
PROK1	GTGCCACCCCGGCAG	AGCAAGGACAGGTGTGGTGC	65 bp
PROKR1	TCTTACAATGGCGGTAAGTCCA	CTCTTCGGTGGCAGGCAT	69 bp
PROK2	TTGGGCGGAGGATGCA	AAATGAAGTCCGTAAACAGGCC	65 bp
PROKR2	GCTCTGTGCCTCCGTCAACT	CCAGCAAGGCATTGGTGG	65 bp
PTGES	GAAGAAGGCCTTTGCCAAC	GGGTTAGGACCCAGAAAGGA	168 bp
RPL32	CATCTCCTTCTCGGCATCA	AACCCTGTTGTCAATGCCTC	100 bp
SMAD2	TTTCAGTTCCGCCTCCAATCGC	CGTGAATGGCAAGATGGACG	208 bp
SMAD3	TGAGGCTGTCTACCAGTTGACC	CTAAGACACACTGGAACAGCGG	199 bp
StAR	GGCATCCTTAGCAACCAAGA	ACTTTGTCCCCATTGTCCTG	62 bp
VASA	AAGAGAGGCGGCTATCGAGATGGA	CGTTCACTTCCACTGCCACTTCTG	238 bp

2.5 Quantitative RT- PCR

Quantitative real time PCR (qRT-PCR) was performed to determine mRNA transcript levels using Applied Biosystems 7500Fast or 7900Fast instruments (specified in each chapter). Standard curves for all targets were performed as described (Coutts *et al*, 2008) using either late second trimester fetal ovarian or endometrial cDNA to ensure proper reaction efficiency was achieved for each target. All measurements were performed in duplicate 10 µl reactions.

For relative comparison, PowerSYBR Green Master Mix (ABI) was utilised and targets were analysed alongside a ubiquitously expressed 'housekeeping' gene (*GAPDH* or *RPL32*) and calculated as relative expression. Each reaction was performed in a single well of a 96-well reaction plate (Applied Biosystems) as described in Table 2.5.

Table 2.5 Sybr Green Reaction Mix

Reagent	Amount Used	Manufacturer
Sybr Green	5 µl	Invitrogen
25 µM Forward Primer	0.2 µl	Eurogentech
25 µM Reverse Primer	0.2 µl	Eurogentech
H_2O	3.6 µl	Ambion
Sample (diluted 1/10 in H_2O)	1 µl	--

Some primers were designed and donated by the Henry Jabbour lab for Taqman multiplexing comparison using Express qPCR Super Mix with Premixed ROX (Invitrogen) (targets used in this manner are listed in Table 2.8). Similarly, some primers were designed and donated by the Philippa Saunders lab and utilised for multiplexing comparision using the Universal Probe Library (Roche). These primers alongside specifically designed probes were utilised with internal-well comparison marker 18s (ribosomal RNA control, Applied Biosystems). Each target was calculated and presented as relative expression to this internal control in a reaction mixes are as described in Table 2.6 and 2.7.

Table 2.6 Taqman Reaction Mix

Reagent	Amount Used	Manufacturer
Super Mix	6.3 µl	Invitrogen
25 µM Forward Primer	0.2 µl	Eurogentech
25 µM Reverse Primer	0.2 µl	Eurogentech
5 µM Probe	0.5 µl	Eurogentech
18s	0.2 µl	Applied Biosystems
H_2O	4.6	Ambion
Sample (diluted 1/10 in H_2O)	1 µl	--

Table 2.7 Universal Probe Taqman Reaction Mix

Reagent	Amount Used	Manufacturer
Super Mix	7.5 µl	Invitrogen
200 nM Forward Primer	0.15 µl	Eurogentech
200 nM Reverse Primer	0.15 µl	Eurogentech
100 nM Universal Probe	0.15 µl	Roche
18s	0.1125 µl	Applied Biosystems
H_2O	5.4375 µl	Ambion
Sample (diluted 1/10 in H_2O)	1.5 µl	--

Table 2.8 PCR Probes

Gene Target	Probe
CYP11A1	Universal Probe Library (Roche) – Probe 11
DESMIN	Universal Probe Library (Roche) - Probe 55
DKK1	TACCAGCCGTACCCGTGCGCAG
PROKR1	TGCAGACCTGGACCTCAAGACAATTGG
PROK2	CACTTGCCCATGTCTGCCAGGCT
PROKR2	CCTGCGCACCGTCTCCCTCTACG
StAR	Universal Probe Library (Roche) – Probe 11

Data analysis was performed using GraphPad Prism version 5 (GraphPad Software Inc). One-way ANOVA and t-tests were performed with post-testing as described for each chapter.

2.6 Tissue Fixation, Processing, and Slide Prep

Tissue was fixed in Bouins solution (Clintec) for 2-3 hours and then transferred to 70% ethanol (VWR) for immunohistochemistry.

Fixed tissue was processed in an automated Leica TP1050 processor and embedded in paraffin wax. Paraffin blocks were cut via microtome into 5 µm thick sections and floated in a warm water bath to promote smooth tissue transfer. Sections were mounted on electrostatically charged glass slides (pink slides for human tissue, Thermo-scientific; white slides for animal tissue, Leica). The slides were kept at 50°C overnight in a slide oven to ensure tissue adhered to the slide surface.

2.7 Haematoxylin and Eosin Staining

2.7.1 Dewaxing and Rehydration

Samples from each specimen were taken every 10^{th} slide to determine tissue quality and orientation. Slides were dewaxed and rehydrated through a series of alcohol gradients. The slides are first put in xylene (VWR) twice for 5 min, then moved to absolute alcohol twice for 20 sec, 90% alcohol for 20 sec, 80% alcohol for 20 sec, 70% alcohol for 20 sec, and finally in tap water.

2.7.2 Counterstaining

Slides were immersed in haematoxylin (Leica), a blue nuclear counterstain, for 30-60 sec. Slides were washed in water to remove excess stain and immersed in acid alcohol (section 2.13) to remove non-specific cytoplasmic staining. Slides were rinsed again in water to remove the acid alcohol and further immersed in Scott's tap water (section 2.13) for 30 sec to allow the haematoxylin to develop. Slides were then immersed in Eosin, (BD) a red cytoplasmic stain, for 1 min, and rinsed in tap water.

2.7.3 Dehydration and Mounting

After counterstaining the slides were dehydrated in preparation for mounting. This is done by immersing the slides in 70% ethanol for 20 sec, 85% ethanol for 20 sec, 95% ethanol for 20 sec, and finally 100% ethanol twice for 20 sec each. The slides were then immersed in xylene twice for 5 min.

After dehydration the slides are mounted using glass coverslips (VWR) and Pertex (Histolab), mounting solvent based glue. Air pockets were pushed away from the tissue section with pressure applied by forceps, and the slides left to dry. The slides were analysed as described below and stored in dry storage at room temperature.

2.8 Immunohistochemistry

Immunohistochemistry was used to for localisation and visualisation of target proteins in tissues of interest.

2.8.1 Dewaxing and Rehydration

Dewaxing and rehydration were performed as described above, moving the slides to antigen retrieval after the first series of alcohol immersions.

2.8.2 Antigen Retrieval

In order to ensure detection of proteins that may have been masked during the fixation process, the tissue is exposed to high temperature and pH change (Shi *et al*, 1993). Antigen retrieval was performed in 0.01 M citrate buffer with a pH of 6.0 in a standard domestic pressure cooker. The slides were added to 2000 ml of boiling citrate buffer (section 2.13) and the pressure cooker sealed for 5 min. The pressure cooker was then taken off the heat source and the pressure released. The slides were left to stand for 20 min, after which they were cooled with water.

2.8.3 Non-Specific Blocking

To prevent non-specific detection by the primary or secondary antibody, the slides are blocked using peroxide blocking solution (section 2.13) rocking for 30 min (all rocking is performed at 20-40 rpm). The slides are then washed in tris buffered saline (TBS) twice for five min.

After peroxidase blocking, the slides are blocked for endogenous biotin, which is known to be prevalent in human tissue. This is done via a strepavidin/biotin blocking kit (Vector). The strepavidin solution is applied neat to the tissue for 15 min incubation in a humidity chamber, and then the slides are washed in TBS twice for five min. The biotin solution is applied as previously described for the strepavidin solution and the slides washed after incubation.

To further prevent non-specific binding of the secondary antibody, the slides are incubated for 30 min in a blocking buffer consisting of normal serum (NS)(Biosera) diluted in TBS (1:4) containing 5% bovine serum albumin (NS/TBS/BSA) in a humidity chamber. Normal goat serum (NGS) was used unless the primary antibody was raised in goat, in which case normal rabbit serum was used in the blocking buffer.

2.8.4 Primary Antibody

The blocking serum (NS/TBS/BSA) was then replaced with primary antibody diluted in blocking serum to an optimised concentration. Concentration was determined for each antibody by titering the dilution of the antibody on standard tissue to find the maximum level of detection lacking non-specific background staining. The primary antibody is left on the tissue overnight at 4° C. The table below (Table 2.9) describes the antibodies used and optimum concentrations for fetal tissue.

Appropriate negative and positive controls were included as appropriate. Negative controls were done in two ways (as outlined in each chapter); adding no primary antibody to the serum for the overnight incubation, or adding antibody that was incubated with peptide to block binding and reveal any non-specific binding that may have occurred.

Table 2.9 Antibodies used for Immunohistochemistry

Primary Antibody	Dilution	Species Raised	Secondary	Manufacturer
BrdU	1:1000	Sheep	Rabbit Anti-Sheep	Fitzgerald
COX1	1:50	Goat	Rabbit Anti-Goat	Santa Cruz
COX2	1:50	Goat	Rabbit Anti-Goat	Santa Cruz
CC3	1:75	Rabbit	Rabbit Anti-Goat	Cell Signaling
EP1	1:700	Rabbit	Goat Anti-Rabbit	Cayman
EP2	1:100	Rabbit	Goat Anti-Rabbit	Cayman
EP3	1:2000	Rabbit	Goat Anti-Rabbit	Cayman
EP4	1:250	Rabbit	Goat Anti-Rabbit	Cayman
GP130	1:1000	Rabbit	Goat Anti-Rabbit	Santa Cruz
LIFR	1:100	Rabbit	Goat Anti-Rabbit	Santa Cruz
PHH3	1:3000	Rabbit	Goat Anti-Rabbit	Upstate
PROK1	1:250	Rabbit	Goat Anti-Rabbit	Phoenix
PROKR1	1:250	Rabbit	Goat Anti-Rabbit	Caltag
PROKR1	1:100	Rabbit	Polymer	Sigma
PROK2	1:50	Goat	Rabbit Anti-Goat	Santa Cruz
PROKR2	1:50	Rabbit	Goat Anti-Rabbit	MBL
PROKR2	1:250	Rabbit	Goat Anti-Rabbit	Lifespan
PTGES	1:50	Rabbit	Goat Anti-Rabbit	Cayman
TRA-98	1:25	Rat	Goat Anti-Rat	Abcam

2.8.5 Secondary Antibody

After washing off the primary antibody in TBS twice for five min, biotinylated secondary antibody (diluted 1:500 in blocking serum) was applied to the tissue and incubated in a humidity chamber for 30 min. The secondary antibodies are raised against a species-specific sequence on the primary antibody. Therefore the type of secondary used depends on both the primary antibody and the NS used previously. Table 2.8 outlines which secondary was used with each antibody, and Table 2.9 describes the secondary antibodies.

For antibodies that were not readily detectable with standard secondary antibodies, a signal amplification polymer was used (Immpress reagent, Vector). This polymer was used undiluted on slides in place of a secondary antibody and strepavidin (see Tabel 2.10), followed by standard detection.

Table 2.10 Secondary Antibodies

Antibody	Manufacturer
Goat Anti-Rabbit	Vector
Goat Anti-Rat	Vector
Rabbit Anti-Goat	Dako
Rabbit Anti-Sheep	Vector

2.8.6 Visualization of Antibody

The secondary antibody was removed by rocking in TBS twice for five min. Streptavidin-horseradish peroxidase (HRP) (Vector) was diluted 1:1000 in TBS and applied to the slides for a 30 min incubation in the humidity chamber before being removed using TBS.

Some antibodies with low detectability levels were incubated with Streptavidin-ABC (Vector) in place of HRP (as outlined in each chapter). The complex was incubated for 30 min in an Eppendorf tube before usage as per manufacturer's instructions. The Streptavidin-ABC was dropped onto slides and incubated for 30 min as in the above protocol for HRP.

Antibody localisation was determined using 3,3-diaminobenzidine (liquid DAB+, DAKO). DAB chromagen was diluted in its buffer, according to the manufacturers' instructions. Sections were then incubated with DAB until staining was optimally detected (determined via use of light microscope). This process usually took ~60 sec, but possibly up to 5 min in some cases. The reaction was stopped by immersing the sections in water.

2.8.7 Counterstaining, Dehydration, and Slide Mounting

After antigen detection, the slides were counterstained using haematoxylin (no eosin) and mounted as described previously.

2.9 Fluorescent Immunohistochemistry

Fluorescent immunohistochemistry allows for more specific protein localisation within a tissue. It also allows antigens to be co-localised within the target tissue, so comparisons can be made. The general protocol for fluorescent immunohistochemistry is similar to that of DAB Immunohistochemistry (section 2.7) with some exceptions as described below.

2.9.1 Washes and Blocking Serum

All washes between treatments were performed in phosphate-buffered saline (PBS) instead of TBS for fluorescent immunohistochemistry. This also affects the blocking serum as it is changed to NS/PBS/BSA.

2.9.2 Primary Antibody

Concentration of primary antibody was adjusted as fluorescent detection is more sensitive. See the table for the dilutions used for fluorescent immunohistochemistry.

Table 2.11 Antibodies used for Fluorescent Immunohistochemistry

Primary Antibody	Dilution	Species Raised	Secondary	Manufacturer
3β-HSD	1:5000	Rabbit	Goat Anti-Rabbit	Gift from Prof. Ian Mason
EP2	1:4000	Rabbit	Goat Anti-Rabbit	Cayman
EP4	1:2000	Rabbit	Goat Anti-Rabbit	Cayman
OCT4	1:150	Goat	Rabbit Anti-Goat	Santa Cruz
PROKR1	1:2000	Rabbit	Polymer	Sigma
PROKR2	1:500	Rabbit	Goat Anti-Rabbit	Lifespan
SMA	1:500	Mouse	Goat Anti-Mouse	Sigma
VASA	1:300	Rabbit	Goat Anti-Rabbit	Abcam

2.9.3 Secondary Antibody

The secondary antibodies used for fluorescence were peroxidase-conjugated (Table 2.12) rather than biotinylated as they were retrieved in a slightly different way. Peroxidase antibodies were used at 1:500 and were diluted in NS/PBS/BSA.

Table 2.12 Secondary Antibodies

Antibody	Manufacturer
Goat Anti-Rabbit	Vector
Goat Anti-Mouse	Vector

2.9.4 Visualisation of Antibody

Rather than using Strepavidin detection, fluorescent immunohistochemistry utilises tyramide detection (TSA-Plus cyanine 3 system, Perkin Elmer). After the peroxidase secondary was washed off, a tyramide enhancement was applied diluted 1:50 in its own buffer as described by the manufacturer. The slides were then incubated in a dark humidity chamber for 10 min. The tyramide was removed with PBS in an opaque container to prevent any bleaching of the fluorescence.

2.9.5 Counterstain

The counterstain used for fluorescence varied depending on the colour of detection used for the primary antibodies (Table 2.13). They were diluted 1:1000 in PBS and applied to the slides for a10 min incubation in a dark humidity chamber.

Table 2.13 Secondary Antibodies

Counterstain	Manufacturer
Propidium Iodide (Red)	Sigma
Sytox Green (Green)	Invitrogen
Dapi (Turquoise)	Sigma

2.9.6 Dehydration and Mounting

Fluorescent immunohistochemical slides do not need to be dehydrated. Instead they are mounted using Permaflur (Lab Vision Corp), a mounting medium for fluorescence. The slides are then stored at 4°C in the dark.

2.10 Co-localisation by Fluorescent Immunohistochemistry

In order to visualise two antigens in the same tissue the previous protocol (section 2.8) was followed by additional citrate retrieval in a microwave oven for 2 min boiling followed by the slides resting in the warm buffer for 20 min. Following the citrate retrieval, the above section (2.8) was repeated starting from the streptavidin/biotin blocking stage. Slides were kept in the dark as much as possible to preserve fluorescence, and the strongest primary antibody (one used at the highest dilution) was used first.

2.11 Microscopy

2.11.1 Light microscopy

Cellular sites of expression of target proteins were determined and slides photographed using a Provis AX70 (Olympus) microscope fitted with a camera (AxioCam HRc, Zeiss). Scale bars were generated using measurement slides and Adobe Photoshop CS5. This program was also used to compile photographic figures seen in the text.

2.11.2 Fluorescent microscopy

Slides were visualised using a Zeiss 510 META confocal microscope. Scale bars were embedded using the Zeiss software and images compiled into figures using Photoshop CS5.

2.11.3 Stereology / image analysis

Image analysis was performed on histological sections to quantify differences between treatment groups in various experiments. Several forms of image analysis were utilised as described below with the assistance of an Imager.A1 microscope (Zeiss) fitted with a Qicam Fast 1394 camera (Qimaging) and a ProScan II (Prior) image tiling device. Image-Pro Plus software (Media Cybernetics) was used to quantify images and calibrations were regularly checked.

Positive cells per area

For germ cell, apoptosis, and proliferative counts cells were stained for appropriate markers and the number of positive cells directly quantified. The area of the organ or tubule was determined. The positive cell count was then divided by the area to get an index of positive cells per area (Figure 2.1).

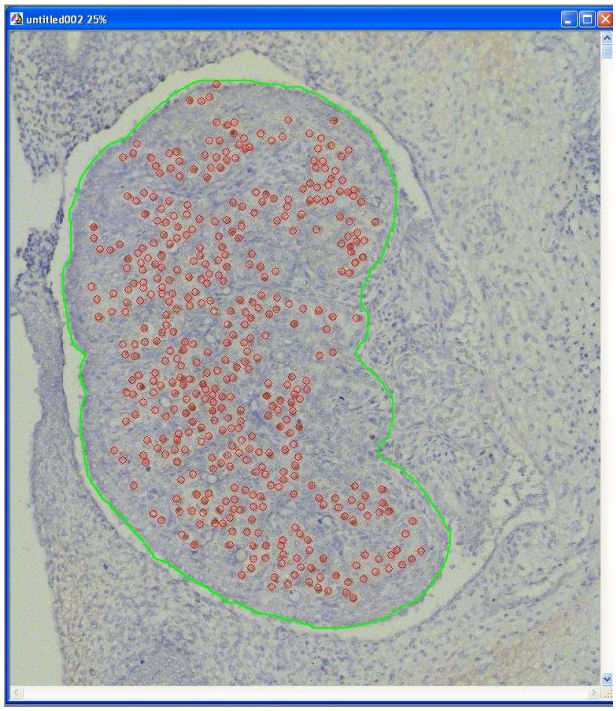

Figure 2.1 Screen shot of germ cells per area count

Fetal rat ovaries were analysed using Image-Pro Plus software, that allowed for positively stained celsl to be quantified (red circles) and the area of the ovary to be measured (green outline) on the same piece of tissue.

Ovarian volume

Ovarian volumes were determined for e21.5 fetal rat ovaries by H&E staining every 10^{th} slide of the serial sectioned ovary. A start and end point of the ovary was then determined and area taken from every slide containing ovarian tissue. These areas were then averaged and multiplied by the number of slides between the start and end of the ovary to determine volume.

Germ cell nuclear diameter

Quantification of nuclear diameter was performed as a measure of germ cell maturity. This measure was taken by tri-secting the nucleus of a cell and the subsequent measurement of each diameter. In order to get an accurate measurement of the variation of nuclear diameters in each specimen, multiple germ cells and slides were quantified as outlined in the following chapters.

2.12 Protein Extraction and Quantification

Proteins were extracted from primary tissue or cells with 50 µl RIPA buffer for primary tissue or 350 µl for cells (Section 2.13). Cellular samples were then snap frozen (-80 ºC) for future use if not immediately needed. Before use, samples were homogenised with an electronic pestle (Sigma) with disposable tips (Sigma). This ensured maximum lysis of extracts.

2.12.1 Bradford Assay

Protein extracts were quantified by Bradford assay to determine concentration for further use. This was performed with the DC Protein Assay Kit (Bio-Rad) according to manufacturer's instructions, utilising a 96-well microplate (Corning). After 15 min incubation plates were scanned on a Multiskan EX microplate reader (Labsystems) at 590 nm. Specimens were then compared to a BSA standard curve run on the same microplate to determine protein concentration.

2.12.2 Western Blot

Protein samples were diluted in dH_2O to 30 mg in 13 µl. Thereafter, 5 µl of 4x SDS loading buffer and 2 µl of reducing agent (2-Mercaptoethanol, Sigma Aldrich) were added to each sample before denaturing at 85° C for 5 min on a Thermomixer (Eppendorf). Samples were cooled on ice and spun down (Eppendorf 5415D centrifuge) before loading into a 12% tris-HEPES-SDS Precise Protein gel (Thermo Scientific) alongside SeeBlue Plus2 protein standard (Invitrogen). Gels were run in a protein electrophoresis system (Bio-Rad) with running buffer (tris-HEPES SDS Buffer, Thermo Scientific) at 125v utilising a Powerpac (Bio-Rad) for 45-60 min or until the loading dye had run to the bottom of the gel.

Gels were then removed and washed in dH_2O for 10 min, then washed in transfer buffer (fast semi-dry buffer, Thermo Scientific) for 10 min. PVDF membrane (Millipore) was cut to the appropriate size for the gel and prepared for transfer by washing 1 min in methanol, 1 min in dH_2O, and 5 min in transfer buffer. Filter paper was prepared by soaking in transfer buffer. The gel and membrane were loaded with the filter paper into a semi-dry blotter as per manufacturer's instructions and run at 25v for 7-9 min depending on protein size.

The membrane was collected after transfer and washed in dH_2O, before being blocked in blocking buffer (1:1 PBS tween: Li-cor blocking buffer (Odyssey)). Primary antibodies were diluted in blocking buffer and the membrane was left to incubate at 4 °C overnight.

Membranes were washed in PBS tween the following morning for 15 min, then twice for 5 min, before being incubated with fluorescently labelled secondary antibodies diluted in blocking buffer for 1 hr at room temperature in the dark. Membranes were then washed x4 for 5 min in PBS tween, again in the dark, before detection on the Li-Cor Infrared Imaging System (Odyssey).

2.13 Cell / tissue culture

2.13.1 Stable transfection of PROKR1 T-Cam2 cells

In order to fully examine PROK1 signalling via PROKR1, the T-Cam2 germ cell line was stably transfected with the PROKR1 receptor. The creation of the PROKR1 construct was performed by Dr. Pamela Brown as follows:

The PROKR1 gene was subcloned from an Ishikawa vector (Evans *et al*, 2008) into a pLENTI6-derived destination vector containing a prolactin (PRL) peptide to ensure the receptor was expressed. A sequence for Blasticidin resistance was also part of the construct and used for later selection.

Cells were transfected alongside a similar construct containing GFP, as a positive control for transfection.

Linearisation

Both constructs (GFP and PROKR1) were linearised using the restriction enzyme NaeI (New England Bio) as per manufacturer's instructions before transfection, in order to increase the number of cells integrating the plasmid in their DNA. Cells were also transfected with the plasmid uncut (super-coiled), as this increased the number of plasmids to enter the cells initially. A 2 µl sample of the linearised plasmid and supercoiled plasmid were run on a gel to verify the linearised sample was fully cut before transfection.

Ethanol Precipitation and Quantification of DNA

Digested plasmid was then purified and concentrated using ethanol precipitation. This was performed by adding 100 µl dH_2O to the plasmid, followed by 20 µl Sodium Acetate (Sigma) (3M, pH 5.2) and 3 volumes of pure ethanol (660 µl). This was mixed and incubated at -20º C for 20 min to speed up precipitation of DNA. The sample was then centrifuged at full speed for 10 min at 4 ºC and the supernatant carefully removed. Then 500 µl 70% ethanol was added to remove salt and further spun at 4 ºC for 5 min. The supernatant was removed and the tube left open for the remaining ethanol to evaporate. The pellet was re-suspended in 20 µl TE and left at 4 ºC overnight. Plasmids were quantified using a Nanodrop spectrophotometer (Thermo Scientific).

Transfection

Cells were plated in 150mm diameter cell culture plates (Corning) 2 days prior to transfection to ensure maximum transfection efficiency. 15 µg of DNA from each plasmid was incubated with 60 µl TransIT –LT1 transfection reagent (Mirus) for 20 min in an Eppendorf tube. The solution containing the plasmid was then dropped onto the plates with newly added media and mixed by gentle rocking.

The T-Cam2 cells were left overnight with the transfection solution in the media. The next morning medium was changed on the plates and a selection medium was applied, consisting of normal T-Cam2 medium with 10 µg/ml Blastacidin (Invivogen). This killed off any cells that had not been transfected, or in which the plasmid was not correctly integrated. This selection medium was used for 12 days

post transfection and the medium was changed every 2 days to ensure dead cells were removed promptly (prevented transfected cells from being adversely affected by dying cells).

PROKR1 T-Cam2 cells were then trypsinised as in normal passaging (section 2.10.11), and kept in a 25 cm^2 flasks. Standard maintenance medium as described in section 2.13 was then used (with only 2 µg/ml Blasticidin). At this stage linearised transfections were abandoned as transfection was low if at all successful. All experiments were done using the supercoiled plasmid for PROKR1. Cells were frozen periodically for stock in 1.5 ml Bambanke freezing medium (Lymphotec). Heightened expression of PROKR1 was confirmed via qRT-PCR and Western blot analysis.

2.13.2 Stable cell culture maintenance

T-Cam2 wild-type and PROKR1 transfected cells were kept in T-Cam2 medium as described below (Section 2.14) at 37 °C with 5% CO_2. The cells were maintained in 162cm^2 flasks and passaged every 3-4 days using 0.5% Trypsin/EDTA (Gibco). Trypsin was added for 5 min and cells were put back in the incubator to accelerate their detachment. Cells were then split 1:4 in the case of wild-type or 1:3 in the case of PROKR1 cells.

Ishikawa cells stably transfected with the PROKR1 gene (Evans *et al*, 2008) were used as a positive control. They were maintained in the media described in Section 2.14 in the same culture conditions as the T-Cam2 cells. They were split every 3-4 days at a ratio of 1:5.

2.13.3 Organ culture

For organ culture analysis of fetal gonads, tissue was micro-dissected as described above (Section 2.1). First trimester gonads were halved, with one piece of each gonad going into the treatment group and the other into control (Table 2.13). Second trimester gonads were dissected into small pieces which were randomised and put into treatment groups. Small pieces of tissue were taken from each specimen as a 'time 0' control to determine tissue quality before being put into culture.

Droplets of 30 µl of organ culture media (as described in section 2.14) were placed on the lid of a 10cm^2 square dish (Corning). Each piece of gonad was delicately placed into the droplets using needles. The bottom of the dishes was filled with 10ml dPBS (Gibco) to keep tissue hydrated. The lid was then quickly turned and placed on top of the bottom of the dish, suspending the organ pieces in the media. This technique was devised to mimic *in vivo* surface tension and prevent necrotic effects of direct exposure to culture plates and/or filters, and has been utilised successfully for fetal gonad culture in several organisms, thus potentially proving a better model for human fetal culture possibly resulting in better tissue quality post-culture (Zhang *et al*, 2008; Szczepny *et al*, 2009).

Tissue was removed from the media droplets after the appropriate amount of time (Table 2.14) and snap frozen (-80 °C) or fixed for further analysis.

Table 2.14 Organ culture treatments

Treatment	Manufacturer	Amount	Tissue/Purpose	Culture Time
PGE_2	Sigma	100nM	Ovary/Histology	8 or 24 hours (as specified)
PROK1	Peprotech	40nM	Ovary and Testis/Culture	24 hours

2.13.4 Testicular interstitial cell culture

Fetal testes were removed from early second trimester fetal specimens and extraneous material removed via micro-dissection. The testes were then transferred into a glass dimple slide containing 100 µl Collagenase IV (Sigma) (1mg/ml in HBSS (Sigma)) and teased apart using needles until pieces were small enough to pipette. Tissue was then transferred into a 1.5 ml tube (Eppendorf) and the slide washed 4 times with 100 µl Collagenase each time adding wash to the tube (making the total volume 500 µl).

The sample was incubated at 37 ºC for 5 min, mixing at 650 rpm (Thermomixer, Eppendorf). The sample was mixed via pipetting to ensure a one-cell suspension and incubated for a further 5 min. DNase I (Sigma) (10 µl of 10mg/ml in HBSS) was added to the suspension to reduce viscosity and samples were then incubated for a further 5 min with this addition.

To remove the Collagenase, the cells were centrifuged at 800g for 5 min. The supernatant was discarded and the sample re-suspended in 1 ml HBSS. This wash process was repeated for a second time, and a small portion of cells removed for a 'time 0' control. Cells were then washed in testis culture medium (Section 2.13) spun down a final time, and re-suspended in 1 ml medium. Cells were run through a 70 µm cell strainer to ensure a single-cell suspension, and plated into 2 wells of a 6-well plate (500 µl each well) that had been coated with 0.1% sterile gelatine (Sigma).

Cells were allowed to adhere to the plate surface overnight. Medium was changed in the morning, with one well getting 40 nM PROK treatment and the other receiving the dH2O vector control. Cells were left to incubate for 24 hours, and then lysed in RLT buffer for mRNA analysis.

2.14 Commonly Used Solutions

Acid Alcohol

Acid alcohol is prepared using 70% ethanol with 1% concentrated HCl.

Blocking Serum

Normal serum (Biosera) was diluted 1:4 in TBS or PBS as appropriate with 5% bovine serum albumen (BSA) (Sigma).

Citrate Buffer

42.02g Citric acid monohydrate was diluted in 900 ml distilled water, and brought to pH 6.0 using NaOH.

Freezing Medium

Storage of cell lines was performed by freezing cells down in Bambanker freezing media (Lymphotec).

Ishikawa Medium

PROKR1 transfected Ishikawa cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) with Glutamax (Gibco), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco), 200 µg/ ml G418 (Invivogen).

Ishikawa Starving Medium

PROKR1 transfected Ishikawa cells were starved in DMEM Glutamax, with no supplementation.

Peroxide Blocking Solution

3% by volume hydrogen peroxide (Fisher Scientific) (30mls of 30%) added to methanol (270 mls) (Fisher Scientific).

NP40 / RIPA Lysis Buffer

Lysis buffer was prepared with 150 mM NaCl (Sigma), 50 mM Tris.HCl, pH7.5 (Sigma), 1% NP40 and 0.25% sodium deoxycholate brought to pH 7.4 with HCl. A protein inhibitor mini tablet (Roche) and 20 µl sodium vanadate (New England Biolabs) per 10 ml of solution was added immediately before use.

Organ Culture Media

Fetal testis and ovary samples were cultured whole or in pieces ($1mm^2$) in αMEM (Gibco), supplemented with 3% BSA, 1% 100x Non-Essential Amino Acids (NEAA) (Gibco), 1% 200mM L-glutamine, 1% Penicillian-strepromycin (Lonza), 1% Sodium pyruvate (Gibco), and 0.002% ITS (Lonza). Media was filter sterilised before use.

Phosphate-Buffered Saline (PBS)

PBS used for fluorescent immunohistochemistry and western blotting was prepared by adding 1 PBS tablet (Sigma) consisting of 0.01M phosphate buffer, 0.0027 M potassium choloride, and 0.137 M NaCl with a pH of 7.4 to 200 ml distilled water.

Scott's tap water

Scott's tap water was prepared by adding 10 g Potassium hydrogen carbonate and 100 g Magnesium sulphate to 5000 ml tap water.

T-Cam2 Medium

T-Cam2 cells were cultured in RPMI medium, supplemented with 10% heat inactivated FBS and 1% 100x L-glutamine. Blasticidin (2 µg/ml) was added to PROKR1 transfected T-Cams only.

T-Cam2 Starving Medium

T-Cam2 cells were starved in RPMI medium, supplemented with 2mM L-glutamine.

Testicular Interstitial Culture Medium

Interstitial cell cultures derived from fetal testis tissue, were maintained in phenol-red free DMEM, supplemented with 10% FBS, 1x NEAA2mM L-glutamine, 1% Penicillian-strepromycin, 1% amphotericin (Sigma), and 1% D-(+)-Glucose (45% solution; Sigma). In addition, 10 IU of hCG was added to each ml of medium to ensure normal steroidogenesis was promoted. Medium was filter-sterelised before use.

Tris-Buffered Saline (TBS)

TBS used for DAB immunohistochemistry was prepared by adding 121.1g Tris base (Sigma) and 170g NaCl to 2000 ml distilled water, and brought to pH 7.4 with HCl.

Chapter 3

Prostaglandin E_2 in fetal ovarian development

Chapter 3. Prostaglandin E2 in fetal ovarian development

3.1 Introduction

PGE_2 is a potent mediator of cellular migration, proliferation, and vascularisation (Diazflores *et al*, 1994; Wang & DuBois, 2006). Within female reproduction, PGE_2 plays well-recognised and important roles in tissues such as the ovary, endometrium, myometrium, and cervix (Oates *et al*, 1988b; Oates *et al*, 1988a; Sales & Jabbour, 2003a). PGE_2 is a well-characterised regulator of ovulation and fertilisation but its role in early ovarian development or function has not been investigated. Despite this lack of information, it is known that PGE_2 and other prostaglandins such as PGD_2 can induce expression of the neurotrophins, known regulators of early ovarian development (Anderson *et al*, 2002b; Spears *et al*, 2003; Kerr *et al*, 2009; Childs *et al*, 2010a), in astrocyte cultures (Toyomoto *et al*, 2004). In addition to Toyomoto's work, recent studies conducted by colleagues at the University of Edinburgh have also identified PGE_2 as a mediator of the neurotrophins in the human female reproductive tract (Jabbour, unpublished). This link suggests PGE_2 may play an up-stream role in the pathway controlling human ovarian development and possibly primordial follicle formation.

PGE_2 is derived step-wise from arachadonic acid by the cyclooxygenase enzymes (COX1 and 2), and the PGE_2 specific synthase, PTGES (Figure 3.1) (Funk, 2001). COX1 and 2 play differing roles in the production of downstream prostanoids, as COX1 is constitutively expressed in many cell types, whereas COX2 is an inducible enzyme synthesised in response to cytokine action (Sales & Jabbour, 2003b). PTGES, unlike the COX enzymes, is a specific terminal prostanoid synthesis enzyme which controls conversion of PGH_2, a prostanoid intermediate, to PGE_2 (Forsberg *et al*, 2000). After synthesis, PGE_2 signals through one of four GPCRs (EP1-4) to elicit its targeted response (Narumiya *et al*, 1999). The EP receptors are able to regulate differing downstream intracellular cascades (Section 1.10; (Alfranca *et al*, 2006)), allowing PGE_2, to mediate various functions in the same tissue (Coleman *et al*, 1994). Additionally, as PGE_2 is a lipid mediator with a short half-life, causing its action to be restricted to cells close to sites of PG secretion, this allows for paracrine or autocrine regulation, and thus cell specific control, as compared to hormones which can act systemically (Negishi *et al*, 1995).

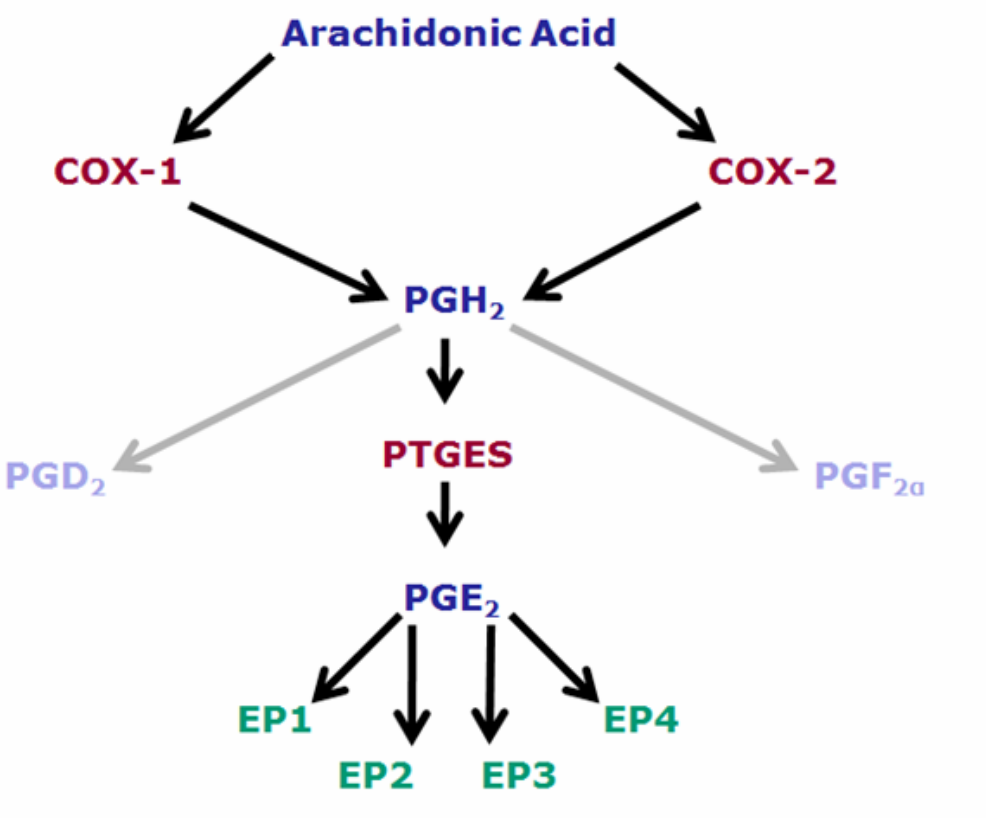

Figure 3.1 Prostaglandin E2 synthesis and signalling pathway.

PGE_2 is synthesised from arachidonic acid by the action of the cyclo-oxygenase enzymes (COX1 or 2) to produce the intermediate PGH_2. This intermediate is further converted by specific terminal enzymes such as PTGES (in the case of PGE_2) to form prostanoids including PGD_2, PGE_2 and $PGF_{2\alpha}$. After synthesis PGE_2 is able to signal via four G-coupled protein receptors (EP1-4). Synthesis enzymes are noted in red, lipids in blue and receptors in green.

In order to fully explore what role PGE_2 might play in early ovarian development, the expression of its precursor enzymes (COX1, COX2, and PTGES) as well as its receptors (EP1-4) were characterised. These enzyme and receptor targets were examined across the first and second trimester, with both transcript levels and protein localisation identified. Receptors which were deemed to have discreet germ cell localisation were further inspected to determine if their expression was limited to a specific subset of oogonia. In addition, human fetal ovarian tissue was cultured with PGE_2 and analysed for alteration in expression of germ cell markers and known regulatory factors. Subsequent cultures were performed to identify histological changes to further identify a function for PGE_2 in the developing ovary.

3.2 Materials and Methods

Tissue collection

Human fetal tissue used in the following experiments was obtained and dissected as described previously (Section 2.1), with subsequent SRY genotyping to determine sex of first trimester specimens (Section 2.3).

RNA extraction and cDNA synthesis

Sample tissue was then processed to extract RNA and subsequently cDNA synthesised for genomic analysis as previously discussed (Section 2.4).

Quantitative RT-PCR

In order to determine if mRNA transcripts encoding PGE_2 precursor enzymes (COX1, COX2, and PTGES) and receptors (EP1-4) varied across gestation, qRT-PCR was performed using the ABI 7500Fast system and either SYBR Green or Taqman analysis as described in Section 2.5.

Quantitative RT-PCR analysis of human fetal cultures was also performed using the ABI 7900HTFast system and SYBR Green analysis to determine any changes in germ cell marker expression (OCT4, VASA, DAZL) and known ovarian regulators (BDNF, NTF5, MCL-1, INHBA, SMAD2, SMAD3); details of this protocol are also outlined in Section 2.5.

Immunohistochemistry

Human fetal ovarian samples were used to localise PGE_2 precursor enzymes (COX1, COX2, and PTGES) and receptors (EP1-EP4) to determine cell-specific sites of PGE_2 synthesis and action. Varying germ cell maturity markers were also utilised to determine if specific receptors were developmentally related (VASA, OCT-4). Finally, immunohistochemistry was also utilised to determine changes in apoptosis (CC3) and proliferation (BrdU). Protocols for these localisations can be found in Section 2.8.

Human fetal ovarian culture

Second trimester fetal ovaries (n= 6, 15-17 weeks gestation) were cultured as small explants on culture inserts as described previously (Martins da Silva *et al*, 2004). Tissue was cultured using organ culture media as described in Section 2.13. Tissue was split into 3 treatment groups: no treatment, 3 µg/ml indomethacin (Sigma) with vehicle (ethanol), or 3 µg/ml indomethacin with 100nM PGE_2. Indomethacin was utilised to reduce endogenous PG production which may have masked effects of the exogenous PGE_2 treatment. Tissue cultured for transcript expression analysis was cultured for 8 hours, subsequently snap-frozen (-80 °C) followed by RNA extraction as described in Section 2.4. These cultures were performed by Dr. Rosey Bayne.

A second series of experiments were performed for histological analysis (n=5, 15-17 weeks gestation). Tissue was cultured in hanging drops with treatment as above for 24 hours with BrdU and subsequently fixed in Bouins solution and processed as in Section 2.5.

Stereology/Image Analysis

Histological changes after PGE_2 treatment in the human fetal ovary were determined via image analysis using various markers as described in section 2.10.9.

Measurements of proliferation were performed using 5 slides from each sample at least 10 sections apart (50 microns). Positive cells (BrdU) were counted and ovarian area taken (necrotic tissue as the result of the culturing process was not taken in to account in this analysis, and the amount of necrotic tissue was not seen to vary between treatment groups). This method was deemed to be more than necessary for accurate quantification, with subsequent apoptotic counts (CC3) performed on 3 slides from each sample rather than 5 slides.

Statistical Analysis

Quantitative RT-PCR data were analysed with GraphPad Prism version 4 statistical software (GraphPad Software Inc). Gestational comparision data were analysed using one-way ANOVA, data were then either analysed as described or log-transformed for further analysis. Log-transformation was performed in instances where data sets did not fit a Gaussian distribution. The Kolmogorov-Smirnov normality test was used for this purpose.

Data were then analysed utilising the Newman-Keuls Multiple Comparison post-test to determine significant changes between gestational values. This post-test was chosen as has more strength than a Tukey post-test and the risk of type I error does not occur with only three groups (as in our study). Some data was also analysed using a post-test for linear trend, this test was performed when data were in a natural order (ie across gestation).

Gene expression changes identified using qRT-PCR after PGE_2 treatment of human fetal ovaries were also analysed using GraphPad Prism as above. Data were analysed using paired t-tests on log-transformed data comparing PGE_2 treated tissue to indomethacin-only treated tissue (as the appropriate control).

Stereology/image analysis data were analysed using the above statistical software, with Student's t-test to determine difference between treatment groups.

3.3 Results

3.3.1 Precursor enzymes of PGE2 are up-regulated at initiation of primordial follicle formation

To determine the presence and pattern of expression of mRNA transcripts encoding PGE_2 precursor enzymes during human fetal ovarian development, qRT-PCR was performed for *COX1, COX2* and *PTGES* across a range of gestations. Ovarian specimens were grouped into three gestational stages to broadly reflect the key developmental events of early to mid-gestation ovarian development, namely the proliferation of undifferentiated PGCs (8-11 weeks gestation), the formation of germ cell nests and entry of the first germ cells into meiosis (13-16 weeks), and on-going meiotic entry and the onset of primordial follicle formation (17-20 weeks).

Transcripts encoding all precursor enzymes were detected in the human fetal ovary, as shown in Figure 3.2. Expression of transcripts encoding COX2 increased across gestation, being expressed at low levels at 8-11 weeks gestation ($0.5\pm0.2 \times 10^{-7}$, relative to the housekeeping gene *GAPDH)*, increasing at 13-16 weeks gestation ($3.9\pm1.0 \times 10^{-7}$ relative to *GAPDH)* and again at 17-20 weeks gestation ($5.4\pm2.2 \times 10^{-7}$ relative to *GAPDH*, linear trend p=0.02, n=5-7 per group, Figure 3.2B). Additionally, the PGE_2 specific enzyme *PTGES* was significantly decreased at early second trimester compared with both first and late second trimester (2.3 ± 0.2 and 1.8 ± 0.2 compared to $0.8\pm0.1 \times 10^{-1}$ relative to *GAPDH*, p= 0.002 and 0.008 respectively, Figure 3.2C). However, no change was seen in the transcripts encoding the constitutively expressed precursor enzyme COX1 (p= 0.79, Figure 3.2A). These data reveal transcripts encoding the PGE_2 precursor enzymes COX2 and PTGES are up-regulated in late second trimester coincident with initiation of primordial follicle formation. In addition, PTGES, which specifically synthesises PGE_2, displays a bi-phasic pattern as it is heightened in first trimester as well as late second trimester.

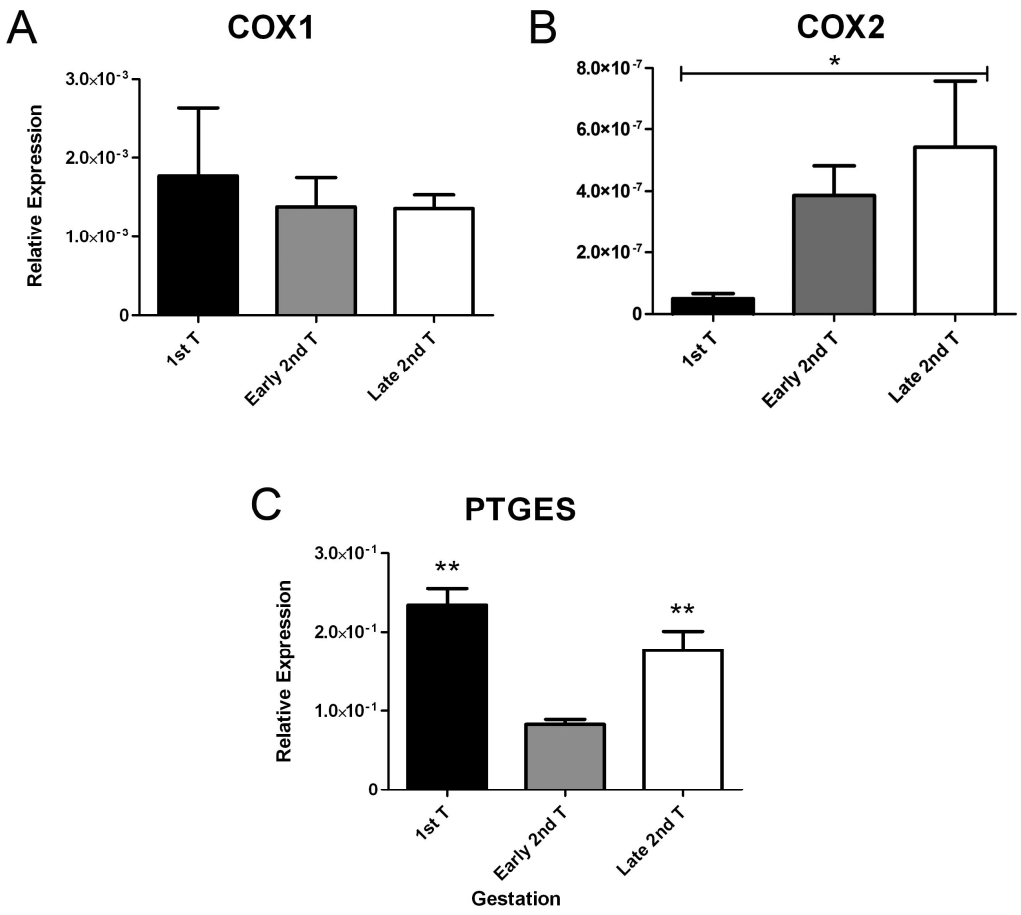

Figure 3.2 Genes encoding the PGE_2 precursor enzymes are up-regulated at initiation of primordial follicle formation.

Expression of the three enzymes necessary to synthesise PGE_2, was analysed by qRT-PCR across gestation and split into age groups concordant with gestational events; PGC proliferation in 8-11 weeks (1st T), meiosis and germ cell nest formation in 13-16 weeks (Early 2nd T), and primordial follicle formation in 17-20 weeks (Late 2nd T; n= 5-7 samples for each gestation). (A) Expression of COX1 did not change during early ovarian development. (B) COX2 expression was significantly up-regulated at late 2nd trimester (*=$p<0.05$ determined by test for linear trend). (C) Transcript levels for PTGES were significantly increased at both 1st and late 2nd trimester, compared with expression at early 2nd trimester gestation (**=$p<0.01$). All data are expressed as a relative expression, normalised to the housekeeping gene *GAPDH*.

3.3.2 EPs required for PGE_2 signalling are expressed and developmentally regulated in the fetal ovary

Further examination was performed to determine the expression of transcripts encoding the four GPCRs necessary for PGE_2 signalling using the gestational range outlined above. EP1, 2, and 4 displayed similar expression patterns (Figure 3.3 A, C, D), increasing transcript expression with increasing gestational age. However, only EP4 showed a significant change across gestation. Expression of EP4 was low at 8-11 weeks gestation ($0.5\pm0.3 \times 10^{-3}$ relative to *GAPDH*), increased at 13-16 weeks ($1.1\pm0.3 \times 10^{-3}$ relative to *GAPDH*) and again at 17-20 weeks gestation ($1.6\pm0.3 \times 10^{-3}$ relative to *GAPDH*, linear trend p= 0.3, Figure 3.3D). EP1 and EP2 displayed a similar trend, however this did not reach significance (p=0.16 and 0.35 respectively). EP3 did not show a similar trend to the other receptors, displaying high variability in early 2nd trimester, with no significant changes in transcript expression across gestation (p=0.49, Figure 3.3B). Outwith expression pattern, it is also notable that EP3 and EP4 are expressed at higher levels than that of EP1 and EP2. These data suggest EP3 and EP4 may play a more predominant role during human fetal ovarian development, and that the role of EP4 increases with development leading to primordial follicle formation.

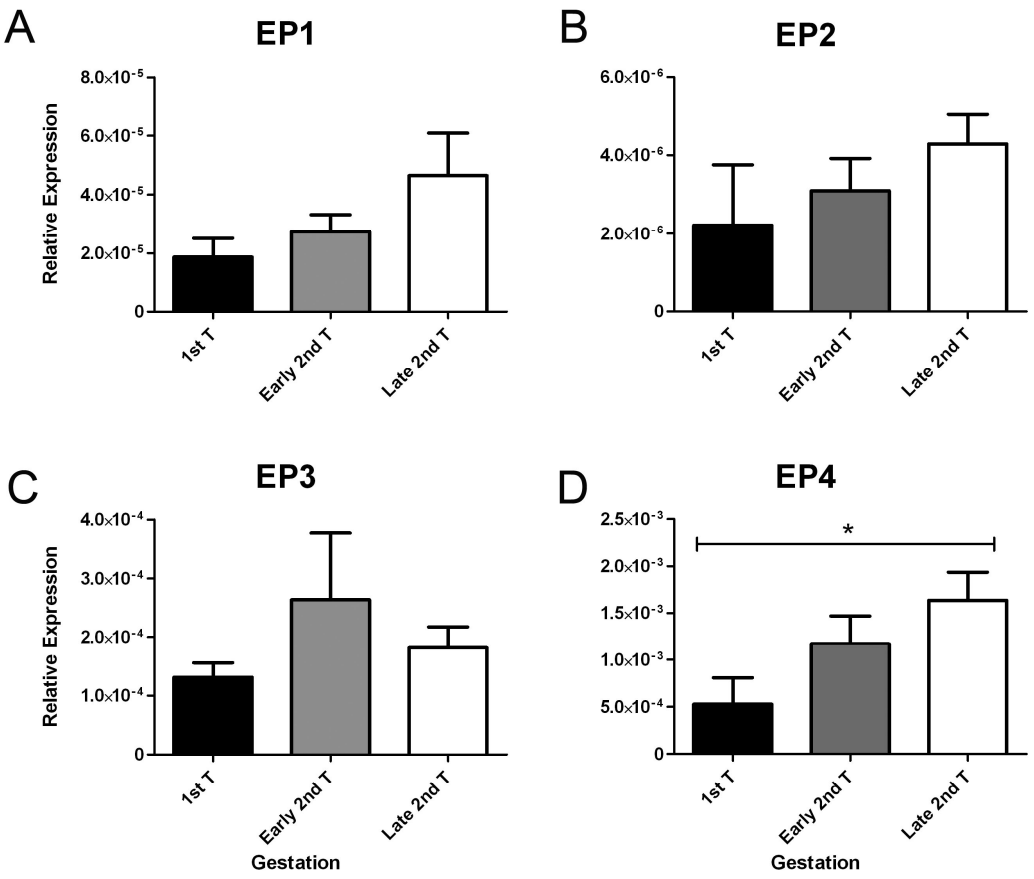

Figure 3.3 PGE_2 receptor mRNA expression across gestation

Expression of the four receptors (EP1-4) necessary for PGE_2 signalling, was analysed by qRT-PCR across gestation and split into age groups concordant with gestational events; PGC proliferation in 8-11 weeks (1st T), meiosis and germ cell nest formation in 13-16 weeks (Early 2nd T), and primordial follicle formation in 17-20 weeks (Late 2nd T; n= 4-6 samples for each gestation). Expression of (A) EP1 (B) EP2 and (C) EP3 did not change during early ovarian development. (D) Transcript levels for EP4 are significantly up-regulated across gestation (*=$p<0.05$, determined by test for linear trend). All data are expressed as a relative expression, normalised to the housekeeping gene *GAPDH*.

3.3.3 Developmentally-regulated precursor enzymes are germ cell specific

To determine the site of PGE_2 production, immunohistochemistry was performed using human fetal ovarian tissue to detect the precursor enzymes required for PGE_2 synthesis. COX1 protein was localised to the pre-granulosa cells closely associated with germ cells within the germ cell nests. No expression was seen in the germ cells or the interstitial cells within stromal cell streams (Figure 3.4A-B). Conversely, COX2 was not seen in pre-granulosa cells or stromal cell streams; rather being expressed in a germ cell-specific manner with varying intensity of expression seen in specific germ cells and distinct staining of primordial follicles (Figure 3.4C-D). PTGES, the PGE_2 specific enzyme, was also germ cell-specific with consistent expression throughout the germ cells (Figure 3.4E-F). The expression patterns noted for the PGE_2 precursor enzymes determines that PGs in the ovary can be derived from the breakdown of arachadonic acid by COX1 in the pre-granulosa cells or by COX2 in the germ cells, but that final conversion into functional PGE_2 occurs exclusively in the germ cells within the human fetal ovary.

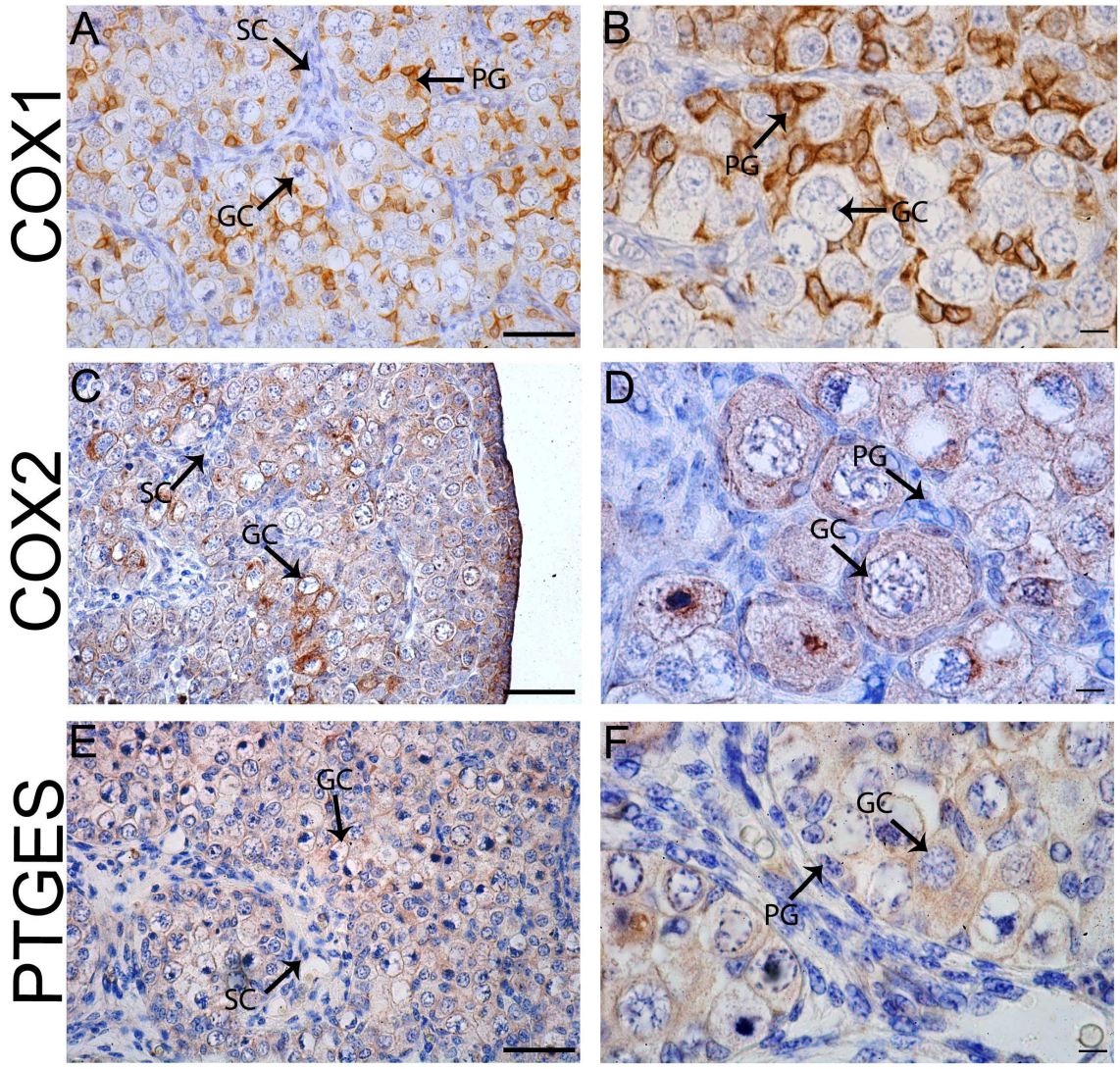

Figure 3.4 Immunohistochemical detection of the PGE_2 precursor enzymes in the human fetal ovary.

Prostaglandin synthesis enzymes were localised in second trimester human fetal ovarian tissue tissue using 3,3′-diaminobenzidine tetrahydrochloride staining (brown). (A-B) COX1 is pre-granulosa cell specific, staining somatic cells within the germ cell nests. (C-D) COX2 is germ cell specific, with no staining detected in stromal cell streams or pre-granulosa cells. Additionally, intensity of COX2 staining varied between neighbouring germ cells. (E-F) PTGES was also localised to germ cell nests but expression was consistent across germ cells. Scale bars equal 50 microns (A, C, and E) and 10 microns (B, D, and F). Images are a composite of several stained tissues, with each antibody utilised on at least three separate tissues from specimens of varied ages to confirm localisation and identified any differences in expression across gesetation.

3.3.4 PGE_2 receptor expression is primarily localised to the germ cells in the human fetal ovary

In order to determine the cell-specific sites of PGE_2 action in the human fetal ovary, immunolocalisation was performed to detect each of the four EP receptors. EP2 and EP4 displayed similar sites of expression, with the germ cells appearing to be the sole location for both receptors (Figure 3.5C, D, G, and H). However, EP2 and EP4 localisation was discrete, with EP2 staining only expressed in the more central germ cells (ie the most mature) with distinct staining of primordial follicles, whereas EP4 staining was primarily localised to the immature germ cell population near the periphery of the ovary. EP3 displayed expression in both pre-granulosa cells and germ cells within germ cell nests, as well as strong expression in the epithelial layer of the ovary (Figure 3.5 E-F). EP1 expression was notably weaker than that of the other EP receptors, with expression in cells lining blood vessels and the blood cells within, but no notable expression in surrounding ovarian tissue (Figure 3.5A-B). These data demonstrate that three of the EP receptors are capable of directly regulating the germ cells, and germ cells appear to be the sole site of expression of EP2 and EP4. Additionally, these data also highlight the possibility that EP2 and EP4 may play a role in distinct subsets of germ cells.

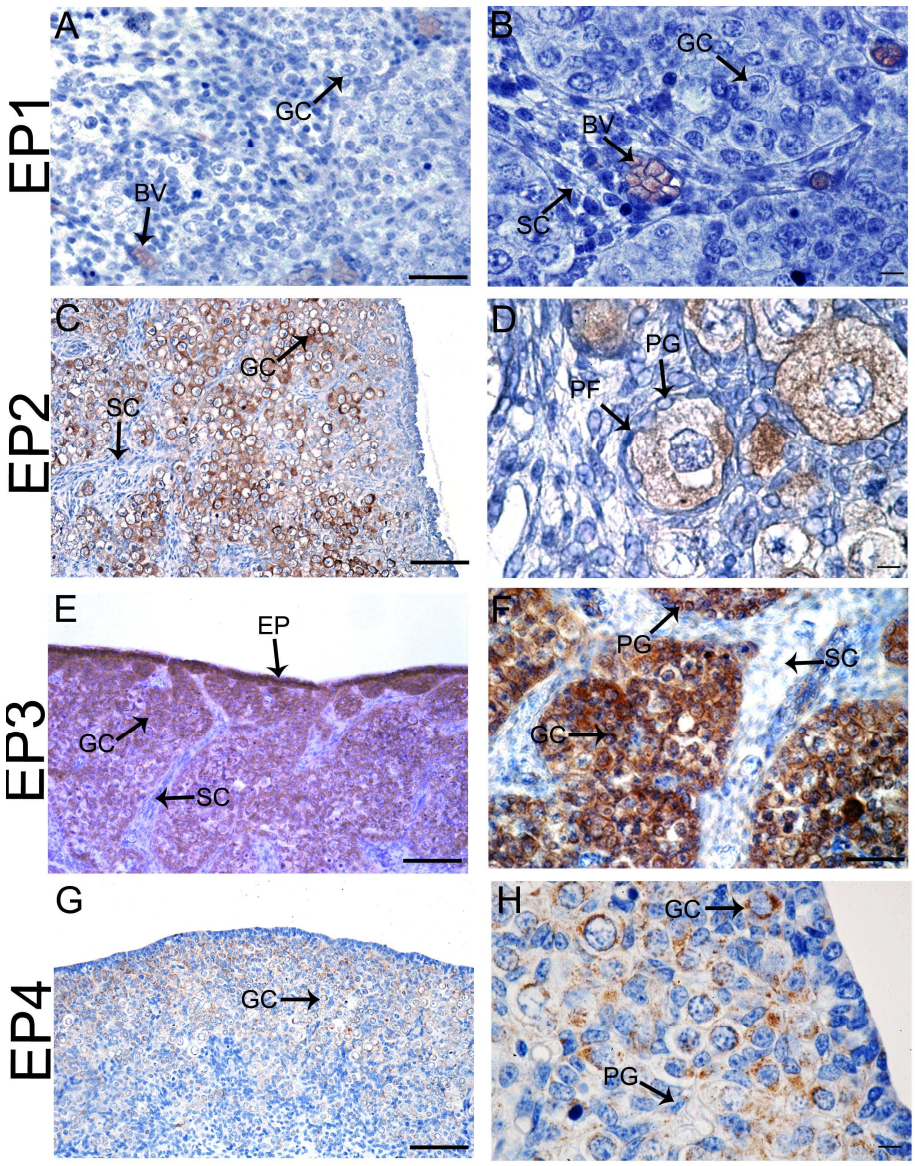

Figure 3.5 Immunohistochemical detection of the PGE_2 receptors in the human fetal ovary.

The PGE_2 receptors EP1-4 were localised in second trimester ovarian tissue using 3,3′-diaminobenzidine tetrahydrochloride staining (brown). (A-B) EP1 was limited to the blood vessels (BV) with no staining of germ cells (GC). (C-D) EP2 was localised to a discreet population of GC and primordial follicles (PF) with no staining seen near the periphery of the ovary, in somatic cells (SC) or pre-granulosa (PG) populations. (E-F) EP3 was the most ubiquitously expressed PGE_2 receptor with clear staining throughout the GCs and PGs, with additional staining in the epithelial layer (EP). (G-H) EP4 was localised to the GCs, and appeared to display more expression near the periphery of the ovary and less in the medulla. Scale bars equal 100 microns (E, G, and C), 50 microns (A and F) and 10 microns (B,D, and H). Images are a composite of at least three separate tissues from specimens of varied ages.

3.3.5 EP2 and EP4 rarely co-localise in the human fetal ovary

To further determine site-specific roles of EP2 and EP4, co-localisation was performed in second trimester ovarian tissue. Both EP2 and EP4 were expressed as previously demonstrated in Figure 3.5; EP2 was expressed discreetly in the centre of the ovary, whereas EP4 expression was more widespread but concentrated at the periphery (Figure 3.6). Low magnification images suggest little co-localisation of the two receptors (Figure 3.6A); however, higher magnification shows that the receptors are expressed in the same cells near the middle of the ovary (Figure 3.6B). Expression of the receptors on co-localised cells was often not interspersed, rather one side of the cell expressed EP2 and the other EP4. These images demonstrate that although EP2 and EP4 receptors are primarily expressed in differing germ cell populations, their sites of action are not mutually-exclusive. Additionally, they often display a expression on opposite sides of a germ cell when co-localised.

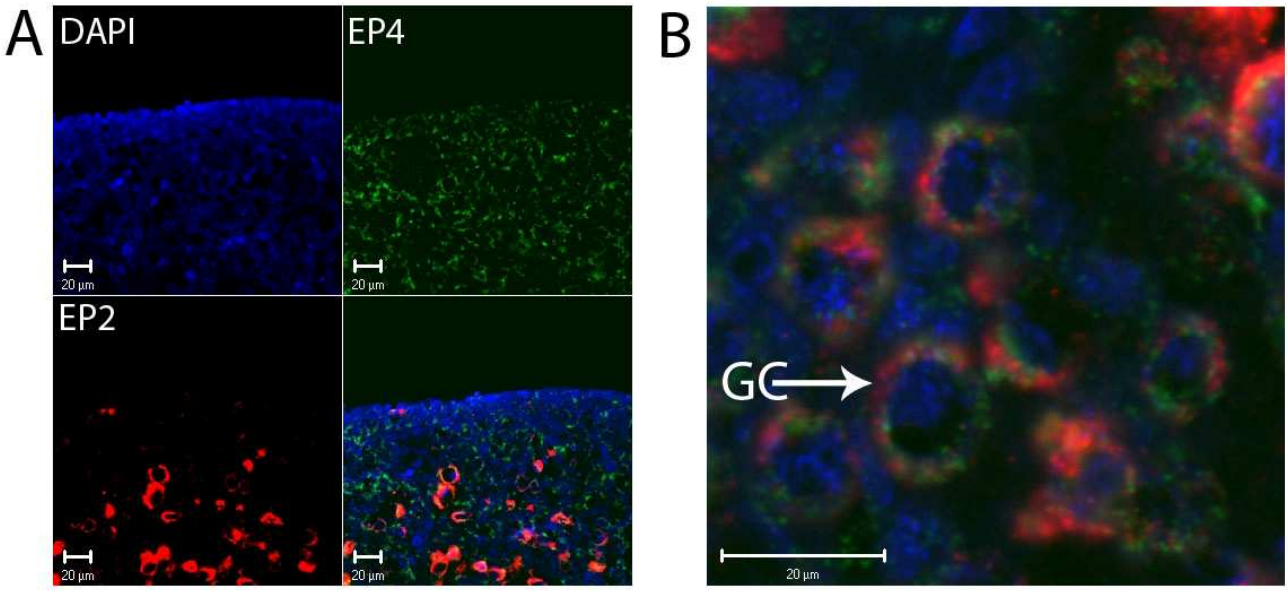

Figure 3.6 Co-localisation of EP2 and EP4 in the human fetal ovary.

The germ cell-specific receptors EP2 and EP4 were examined in 17 week gestation human fetal ovarian tissue. (A) EP2 (red) and EP4 (green) were expressed in the germ cells (GC) of the human fetal ovary with rare co-localisation (yellow). (B) Cells in which both receptors were co-expressed showed polar localisation of the two receptors with EP2 on one side and EP4 on the other (arrow). Dapi counterstain (blue) was utilised and scale bars equal 20 microns. Images are representative of staining performed on three separate second trimester tissues.

3.3.6 EP2 expression is limited to more mature oogonia and oocytes within primordial follicles

As EP2 expression appeared to be restricted to maturing germ cells, co-localisation with the immature germ cell (PGC) marker OCT4 was performed, alongside co-localisation with a mature germ cell marker VASA (Anderson, 2007). No co-localisation of EP2 and OCT4 was determined in the human fetal ovary (Figure 3.7). OCT4 was seen to stain the immature PGCs on the periphery of the ovary, whereas EP2 was localised to the centre of the ovary (Figure 3.7A). There was some interspersion of OCT4 positive cells nearer to the centre of the ovary but none of these cells expressed EP2 (Figure 3.7B).

VASA expression was shown in most germ cells in the human fetal ovary, bar the PGC layer near the epithelium of the ovary (Figure 3.8A). EP2 was also lacking in these PGCs, but EP2 expression was restricted to an even more mature population than that of VASA, with a distinct band of VASA positive germ cells nearer the periphery that were EP2 negative. In addition, in the centre of the ovary, primordial follicles could be identified which contained oocytes positive for both VASA and EP2. These data demonstrate the PGE_2 receptor EP2 is exclusively located on the more mature germ cells in the human fetal ovary, including oocytes within primordial follicles.

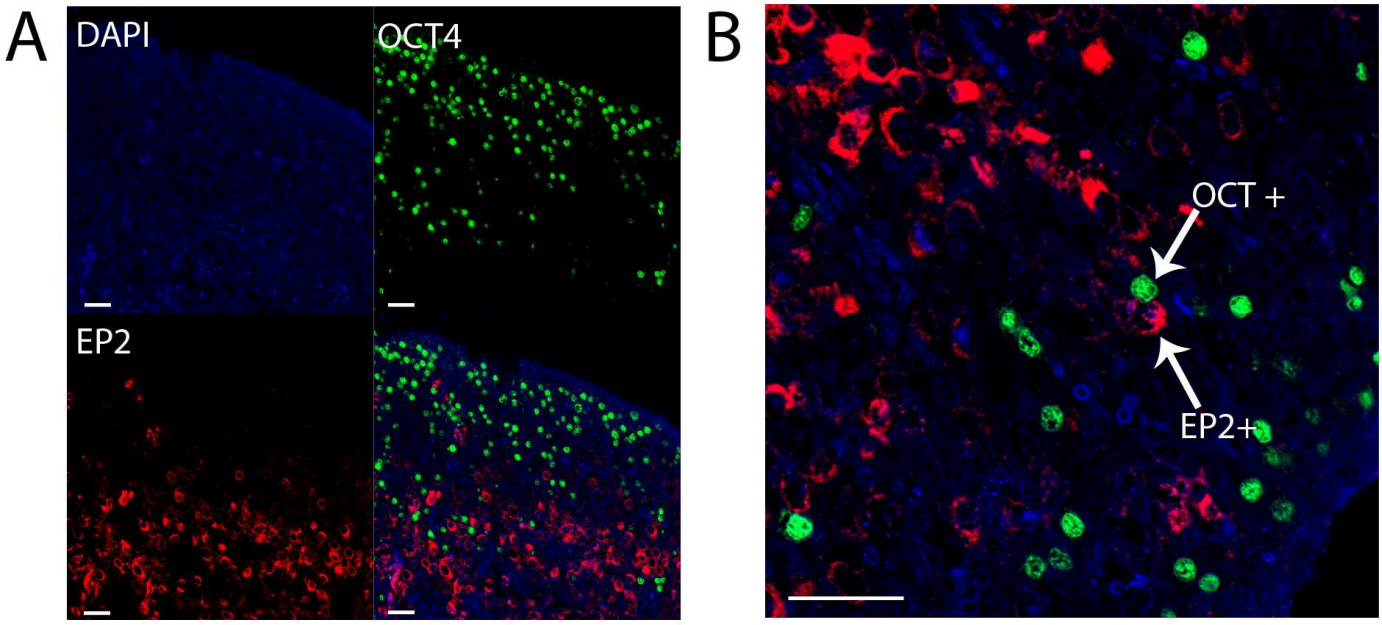

Figure 3.7 EP2 is not expressed by PGCs in the human fetal ovary

EP2 (red) and the immature germ cell marker OCT4 (green) were dual expressed in 19 week human fetal ovarian tissue. (A) OCT4 was restricted to a band of PGCs near the epithelium of the ovary, whereas EP2 expression was concentrated in the more mature germ cells. (B) Some OCT4 positive cells were more centrally localised, but no co-localisation of EP2 was seen in these immature cells (noted by neighbouring cells (arrows) exclusively expressing one marker). Dapi (blue) was used for counterstain and scale bars equal 20 microns. Images are representative of staining performed on three separate second trimester tissues.

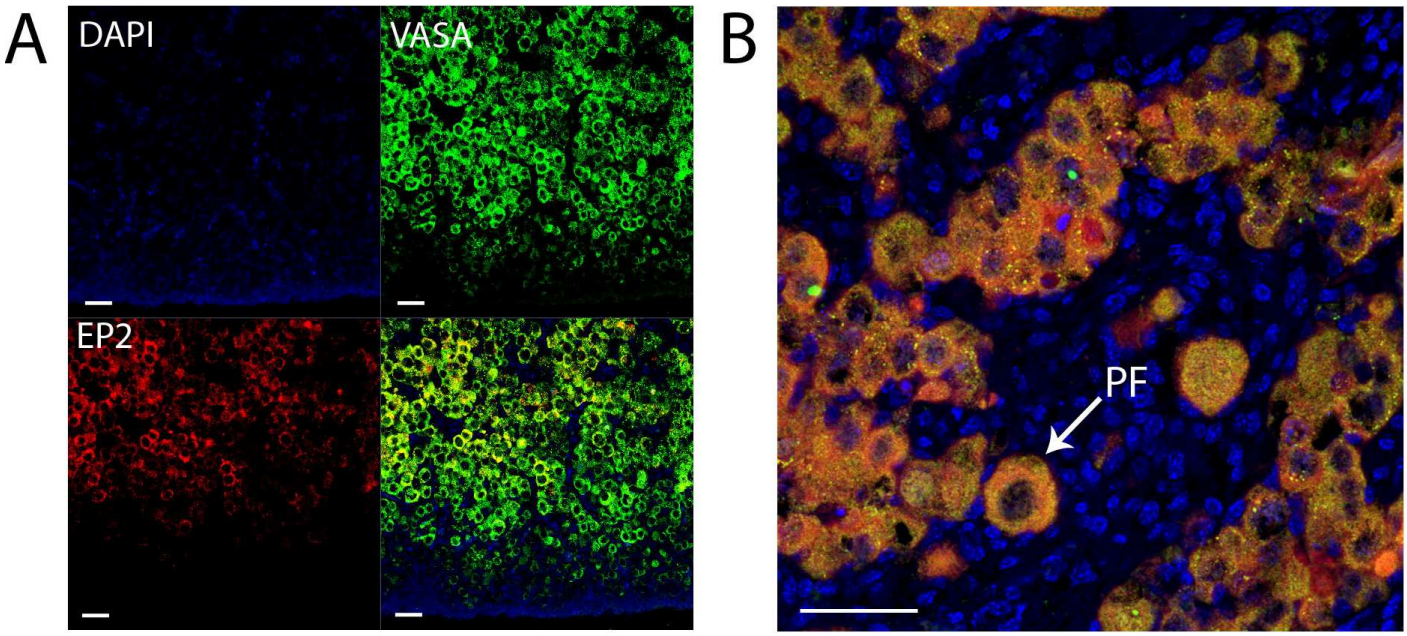

Figure 3.8 EP2 co-localises with VASA in mature oocytes.

EP2 (red) and the mature germ cell marker VASA (green) were dual expressed in 18 week human fetal ovarian tissue. (A) VASA expression is germ cell specific but does not stain immature PGCs on the periphery of the ovary. EP2 shows a similar expression pattern but is restricted to even more mature germ cells, as displayed by the more central localisation of expression. (B) Co-localisation of the VASA and EP2 (orange/yellow) in the centre of the ovary demonstrates both proteins are located in mature germ cell nests as well as oocytes within primordial follicles (PF). Dapi (blue) was used for counterstain and scale bars equal 20 microns. Images are representative of staining performed on three separate second trimester tissues.

3.3.7 EP4 is not restricted to primordial germ cells

Co-localisation with EP2 and EP4 demonstrated that the two PGE_2 receptors displayed differing patterns of expression in the human fetal ovary. After confirmation that the EP2 receptor was restricted to maturing oocytes and oocytes within primordial follicles, immunolocalisation was used to further clarify whether EP4 was solely expressed by immature PGCs in the human fetal ovary. The immature marker OCT4 was again utilised for this purpose. PGCs were identified co-expressing both OCT4 (nuclear) and EP4 (membranous) (Figure 3.9A-B); however, co-localisation also revealed EP4 is not germ cell specific, which became more apparent in later gestational tissue, rather EP4 is also expressed by somatic cells and epithelium at the periphery of the ovary (Figure 3.9B). Expression of EP4 was primarily restricted to the edge of the human fetal ovary with less expression seen centrally. These data demonstrate EP4 is not germ cell specific, and is also expressed by interstitial and epithelial cells at the edge of the ovary.

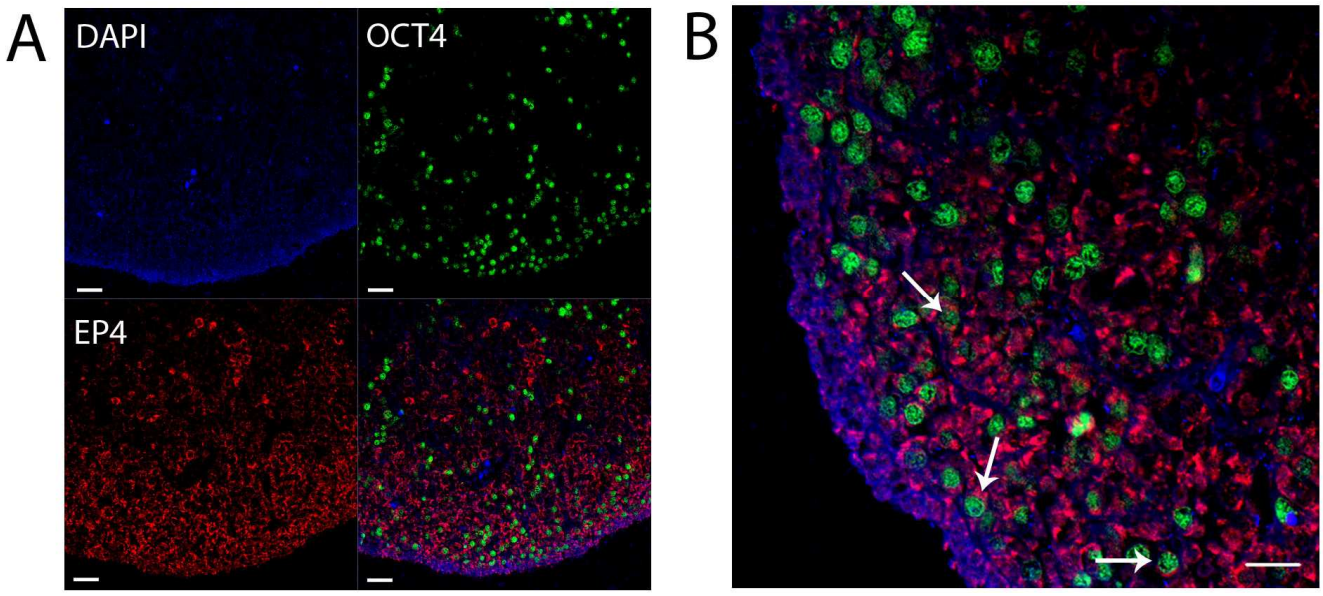

Figure 3.9 EP4 is not germ cell specific but is more concentrated near the periphery of the ovary.

EP4 (red) and the immature PGC marker OCT4 (green) were dual expressed in 19 week human fetal ovarian tissue. (A) EP4 is not germ cell-specific and is expressed by the somatic and epithelial cells at the edge of the fetal ovary. (B) EP4 is expressed by some fetal germ cells as co-localisation between OCT4 (nuclear) and EP4 (cytoplasmic) is displayed (white arrows). Dapi (blue) was used for counterstain and scale bars equal 20 microns. Images are representative of staining performed on three separate second trimester tissues.

3.3.8 Determination of PGE_2 function in the human fetal ovary

To determine a possible function for PGE_2 in the human fetal ovary, early second trimester human fetal ovarian cultures were performed in the presence of PGE_2 for 8 hours (n=6), with subsequent qRT-PCR analysis to determine modulation of germ cell genes or known ovarian regulators. Culture tissue was split into three treatment groups; untreated control, indomethacin control (indomethacin was utilised in culture to prevent endogenous PG production, allowing effects of exogenous PG treatment to be investigated), and indomethacin plus PGE_2 treatment.

No change in expression was determined in any of the germ cell markers investigated when comparing indomethacin control against PGE_2 treated tissue (*OCT4 (*p=0.62), *VASA* (p=0.87) or *DAZL* (p=0.63), Figure 3.10A-C). As predicted in the previously discussed array (Jabbour, unpublished) the neurotrophin, *BDNF* was up-regulated by PGE_2 (0.8±0.3 vs 1.3±0.6 relative to *GAPDH*, p=0.004, Figure 3.10D). However, another neurotrophin *NTF5* (which codes for NT4) was not affected by PGE_2 treatment (p=0.22, Figure 4.9E). Another known regulator of ovarian development, *INHBA* (which encodes for the protein Activin A), was also investigated and shown to significantly increase in response to PGE_2 treatment (2.0±0.2 vs 4.2±1.1 $x10^{-3}$ relative to *GAPDH*, p=0.04, Figure 3.10G). In contrast, the two Activin signalling factors *SMAD2* and *SMAD3* were not changed (p=0.26 and 0.25 respectively; Figure 3.10H and I). In addition, the anti-apoptotic factor *MCL-1* was also up-regulated with PGE_2 treatment (3.8±0.2 vs 4.4±0.5 $x10^{-2}$ relative to *GAPDH*, p=0.04, Figure 3.10F).

These data demonstrate PGE_2 is capable of regulating expression of *BDNF* and *INHBA*, which are known regulators of ovarian development. PGE_2 also up-regulated *MCL-1*, an anti-apoptotic factor. However, PGE_2 does not modulate germ cell marker expression, the neurotrophin *NTF5*, or the downstream signalling components of Activin.

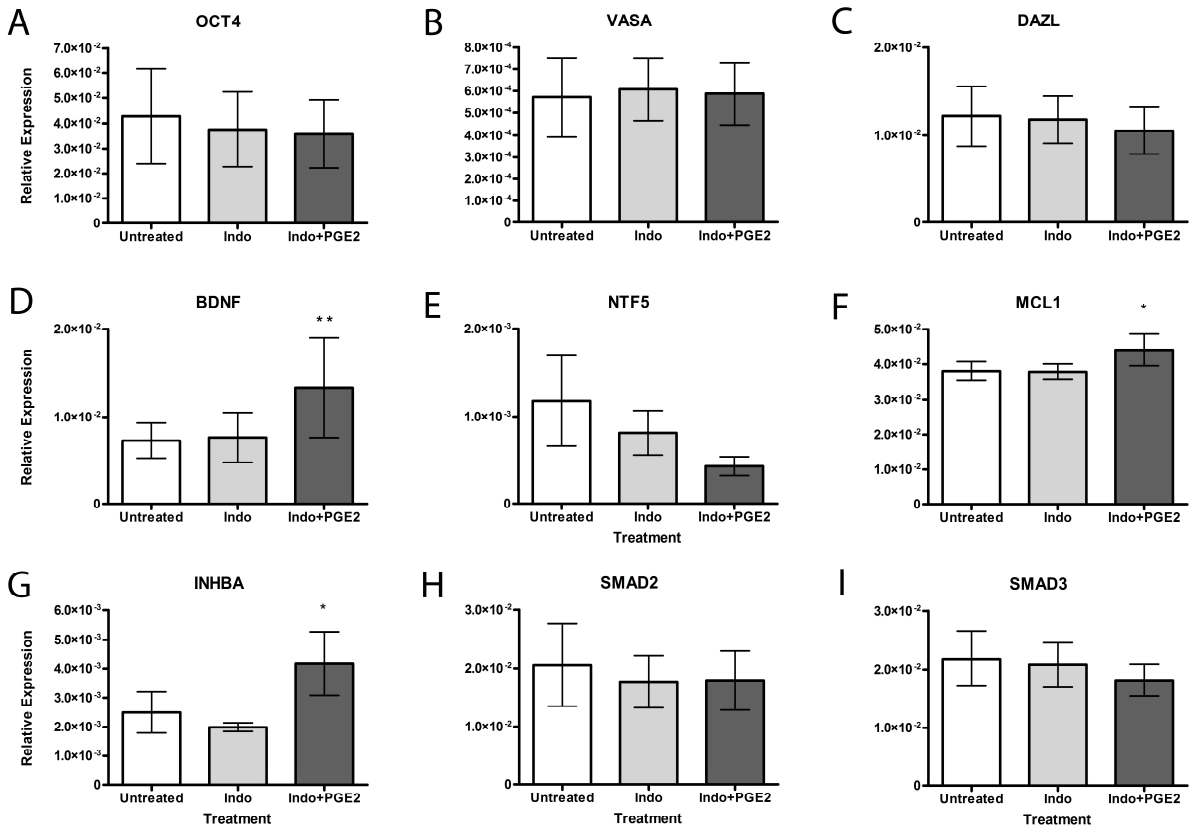

Figure 3.10 qRT-PCR analysis of genomic changes with PGE_2 treatment in the human fetal ovary.

Human fetal ovarian explants were cultured with no treatment (untreated), in the presence of 3µg/ml indomethacin (Indo), or indomethacin and 100nM PGE_2 (Indo+PGE_2). No change was seen in the germ cell markers investigated; (A) OCT4, (B) VASA, or (C) DAZL. (D) The neurotrophin BDNF was significantly up-regulated with PGE_2 treatment, but another neurotrophin, NTF5 was not changed. (F) The anti-apoptotic factor MCL-1 was also seen to increase with PGE_2 treatment, as was the TGF-β superfamily member, (G) INHBA. However, neither of the inhibin/activin downstream signalling molecules were altered with PGE_2 treatment (SMAD2 and 3 (H and I)). All data are expressed as relative expression, normalised to the housekeeping gene *GAPDH*. These cultures were performed by Dr. Rosey Bayne.

3.3.9 PGE_2 treatment does not lead to gross changes in apoptosis or proliferation

To further characterise the functional effects of PGE_2 treatment, cultures were repeated in a similar fashion with an extended treatment period of 24 hours and examined for histological changes via the immunohistochemical markers 5-bromo-2'-deoxyuridine (BrdU) which marks proliferation, and cleaved caspase-3 (CC3) which marks apoptosis (n=5). No changes were determined in proliferation (Figure 3.11A and B) of somatic ($p=0.81$) or germ cells ($p=0.98$) in the human fetal ovary. Similarly, no changes were seen in the number of cells undergoing programmed cell death (somatic cell apoptosis ($p=0.87$) and germ cell apoptosis ($p=0.60$), Figure 3.11C and D). These data demonstrate that treatment with 100 nM PGE_2 for 24 hours does not lead to any gross changes in ovarian cell turnover.

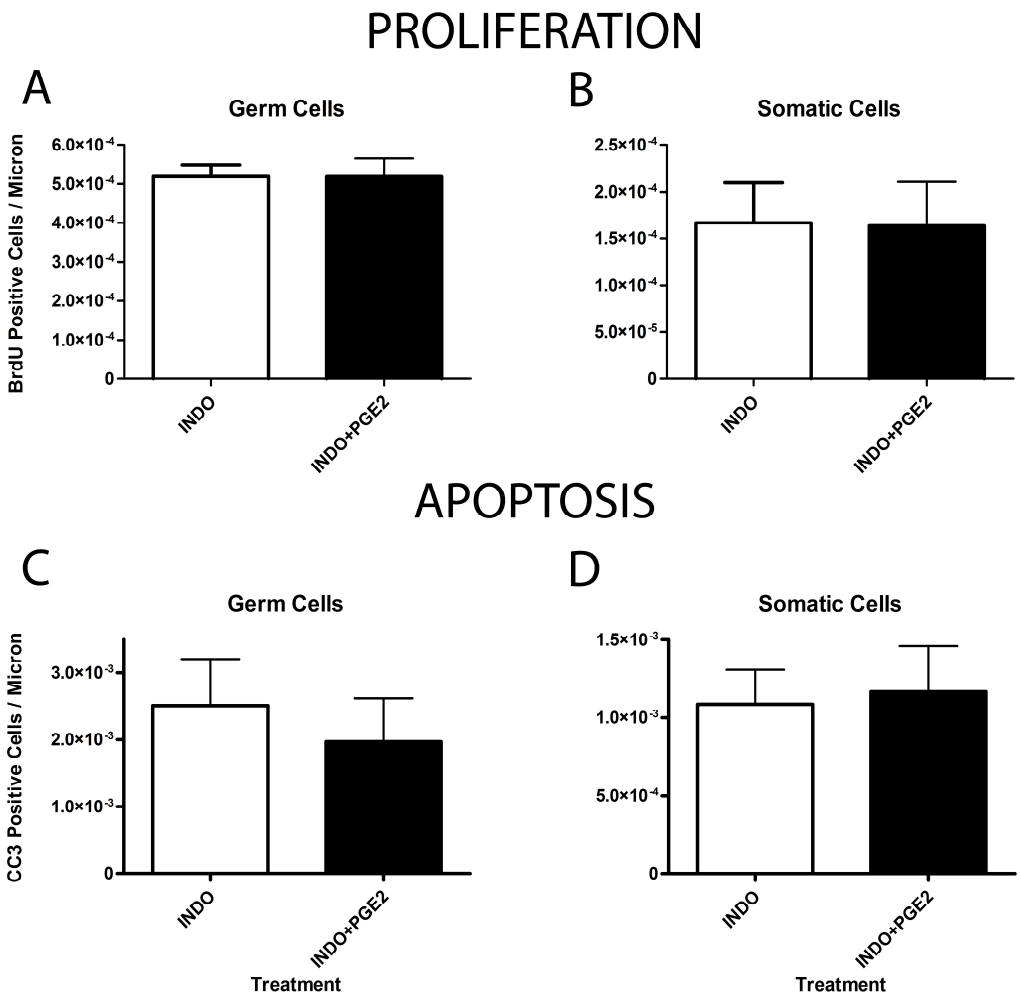

Figure 3.11 No change in proliferation or apoptosis is detected after 24 hour treatment with 100nM PGE_2

Second trimester fetal ovarian tissue (15-17 weeks, n=5), was treated with 3µg/ml indomethacin (INDO), or indomethacin and 100nM PGE_2 (INDO+PGE2) for 24 hours. Proliferation was observed via positive 5-bromo-2'-deoxyuridine (BrdU) staining and was not seen to change in either the (A) germ cell or (B) somatic cell populations. Apoptosis was also assessed via the marker cleaved caspase-3 (CC3) and did not change in (C) germ cells or (D) somatic cells. These data are expressed as positively stained cells per micron of tissue.

3.4 Discussion

Fetal ovarian development and germ cell maturation are vital for adult fertility. PGE_2 has been well-characterised as a potent regulator of ovarian function in the adult (Oates *et al*, 1988a; Oates *et al*, 1988b), but few data exist relating to its function during ovarian development. Additionally, PGE_2 has been identified as a regulator of the neurotrophins in tissue outwith the ovary (Toyomoto *et al*, 2004; Jabbour, unpublished). The neurotrophins are known regulators of early ovarian development leading to the hypothesis that PGE_2 may also play a functional role in the ovary at this time. The data presented here confirm that all precursor enzymes necessary for PGE_2 production (COX1, COX2, and PTGES) are expressed in the human fetal ovary, as are the receptors required for PGE_2 signalling (EP1-4). Furthermore, the expression of several of these components is restricted to the germ cells, denoting PGE_2 is synthesised in the germ cells and can target the germ cells directly in the developing human ovary. Treatment of human fetal ovary with PGE_2 *in vitro* demonstrated increase in expression of germ-cell expressed genes previously identified as important regulators of early ovarian development, providing evidence that PGE_2 may play an important role in developmental signalling in the human fetal ovary.

Expression of two of the precursor enzymes (COX2 and PTGES) required for PGE_2 synthesis was developmentally-regulated. *COX2* expression was increased linearly across gestation, with highest expression in late second trimester concomitant with the initiation of primordial follicle formation. *PTGES* showed a significant drop in expression in early second trimester compared with expression in first and late second trimester, possibly indicating biphasic PGE_2 production in the fetal ovary. Protein of both developmentally-regulated enzymes was found to be exclusively expressed by the fetal germ cells at all gestations (data not shown), revealing germ cells to be the sole site of PGE_2 synthesis in the human fetal ovary. Unlike the other enzymes involved in PGE_2 production, *COX1* was consistently expressed across gestation and was localised to the somatic cells at the periphery of the human fetal

ovary. This enhanced role for COX2 over COX1 in ovarian function is supported by rodents deficient for each of the enzymes; $COX1^{-/-}$ females show no disruption of adult ovarian function, whereas $COX2^{-/-}$ females suffer multiple reproductive disruptions including ovulation, fertilisation and implantation (Dinchuk *et al*, 1995; Lim *et al*, 1997). It is also noted that protein expression of COX2 seemed to be heightened in specific germ cells within germ cell nests, together with its heighted expression in late second trimester. This may suggest that COX2 is involved in survival or degradation of specific germ cells leading up to primordial follicle formation.

Expression of the PGE_2 receptors was varied in the human fetal ovary. *EP1*, *2*, and *3* showed no significant change in transcript expression across gestation, whereas *EP4* displayed a significant increase similar to that of *COX2*. This pattern was consistent with protein expression, where EP4 was heightened in later gestations at the periphery of the ovary. This increase in protein expression was not exclusive to the germ cells as first postulated, but rather EP4 is also expressed by somatic, epithelial and germ cells near the periphery of the ovary. The increase seen in EP4 and its location may indicate a role in maintenance of the immature PGC population. In contrast, EP2 was expressed in a more mature subset of oocytes, with little overlap in expression between EP2 and EP4 determined. EP2 was solely expressed by mature germ cells, also positive for VASA, in germ cell nests and oocytes within primordial follicles; this may denote a specific signalling role for EP2 in mature germ cells. EP3 was expressed by germ cells and pre-granulosa cells within germ cell nests. Conversely, EP1 displayed limited expression in the human fetal ovary, with weak staining in blood vessels revealing it is not likely to be involved in fetal ovarian/germ cell development. Based upon these findings it is likely that PGE_2 action in the human fetal ovary is via EP2, 3, or 4. Distinct expression of each of these receptors provides evidence for multiple roles for PGE_2 as it interacts with the differing receptors during ovarian development and germ cell maturation. Of the EP receptors, EP2 is the only one to be shown to be essential in adult ovarian function, as loss of EP2 function in a murine model resulted in impaired ovulation (Hizaki *et al*, 1999; Kennedy *et al*, 1999; Tilley *et al*, 1999). However, it is probable that PGE_2

action in the fetal ovary is orchestrated via multiple receptors to exert differing effects on distinct subsets of germ cells. With PGE_2 synthesised in germ cells, receptor localisation demonstrates this PGE_2 function can be via autocrine (germ cell) or paracrine (somatic cell) action.

It is possible the developmental changes in mRNA expression of the enzymes and receptors are a result of changes in the tissue composition as the fetal ovary develops, rather than increased expression as a result of increased PGE_2 signalling. As discussed in the introduction, in the human fetal ovary the germ cells proliferate greatly between first and second trimester, increasing their proportion compared to the somatic environment. This may account for some of the increase in expression seen in *COX2*, *PTGES* and *EP4*. However, if this were the only cause for increases determined, expression of *PTGES* should also increase. In addition, as expression of the germ-cell specific receptor *EP2* is not increased in a significant fashion and conversely the somatic-specific enzyme *COX1* is not significantly decreased with gestation, it does not appear that the significant changes in expression levels can be solely due to changes in ovarian composition.

Subsequent culture analysis partially illuminates PGE_2 function during early development. As the PGE_2 receptors were expressed primarily by germ cells in the human fetal ovary, tissue was assessed for any changes in expression of germ cell markers (*OCT4*, *VASA*, and *DAZL*), whose expression is also associated with differing stages of germ cell development (Anderson *et al*, 2007). No significant changes were seen that might suggest a change in germ cell development, which provides further evidence for an upstream effect on germ cell growth factors rather than a direct action on germ cells survival. However, it is possible the short timecourse of 8 hours was not long enough to determine developmental effects.

As speculated, the neurotrophin *BDNF* was up-regulated by PGE_2 treatment. However, another neurotrophin *NTF5* was not affected by PGE_2 treatment. The receptor for these neurotrophins, the TrkB receptor, has been well characterized as an important mediator of development in the mammalian ovary (Dissen *et al*, 1995; Spears *et al*, 2003; Paredes *et al*, 2004; Childs *et al*, 2010a). Both BDNF and NT4

are expressed primarily in the somatic cells in the developing human ovary (Coutts *et al*, 2008; Childs *et al*, 2010a); however, *BDNF* expression is also present in the germ cells at later gestations. Given that PGE_2 receptors are primarily expressed by the germ cells, it is possible this difference in regulation is due to *BDNF* mRNA up-regulation specifically in the germ cells, which may explain the lack of effect on *NTF5*.

Supporting this germ cell specific effect hypothesis, is the up-regulation of *MCL-1* and *INHBA*, which are also expressed solely by the larger, more mature germ cells, including oocytes within primordial follicles (Hartley *et al*, 2002; Martins da Silva *et al*, 2004). *INHBA* is another known regulator of human fetal ovarian development, which exhibits increased transcript levels in late second trimester (Martins da Silva *et al*, 2004) and may regulate germ cell development via regulation of kit ligand/c-KIT expression (Coutts *et al*, 2008; Childs *et al*, 2010a). As PGE_2 is able to up-regulate *INHBA* expression, it is possible it plays an up-stream role in this regulation. *MCL-1*, an anti-apoptotic factor (Kozopas *et al*, 1993), was also up-regulated by PGE_2 treatment. This up-regulation may be a direct action or possibly secondary to the up-regulation of *INHBA*, as *MCL-1* can be regulated by Activin in other systems (Fukuchi *et al*, 2001). In contrast, other downstream targets of Activin action, SMAD2 and *3*, were not affected by PGE_2 treatment, but as with *NTF5*, these factors are located in the somatic cell population.

In order to further determine the functional effects of these expression changes, the PGE_2 cultures were repeated with an extended culture period of 24 hours. No changes were determined in the rate of proliferation or apoptosis in either somatic or germ cell populations. This observation and the inability of PGE_2 to modulate expression of germ cell genes suggest that PGE_2 may not have a direct role in germ cell survival, rather regulating other factors in ovarian development, although the lack of functional changes in histology may also be due to the short duration of the culture (24 hours). Further investigation, using either an *in vivo* model, such as the mouse, or an extended *in vitro* culture system capable of sustaining tissue survival for extended periods of time would be beneficial for determining PGE_2 function.

In conclusion, the necessary components for PGE_2 signalling are present in the fetal ovary, and treatment with PGE_2 can increase transcript levels for germ-cell specific factors known to be important for oocyte survival and maturation. These data provide novel evidence for PGE_2 as a regulator of early human ovarian signalling and thereby germ cell development. Future research should establish the direct function PGE_2 may play during germ cell development.

Chapter 4

Effects of maternal paracetamol usage on fetal ovarian development

Chapter 4. Effects of maternal paracetamol usage on fetal ovarian development

4.1 Introduction

Studies in the human fetal ovary determined a possible function for PGE_2 in regulation of oocyte survival and proliferation, but were unable to determine its mechanism of action; therefore, it was determined a longer duration, in vivo model of PG disruption would be beneficial in order to investigate further PG function (specifically PGE_2) during fetal ovarian development. Maternal administration of paracetamol (acetaminophen) during pregnancy in a rodent model was utilised for this purpose, as treatment with PGE_2 would likely induce early parturition (Kelly *et al*, 2009), administration of other PG inhibitors (indomethacin, NSAIDs, etc) causes maternal morbidity (Wilcox *et al*, 1997; Sigthorsson *et al*, 2000), and a model of maternal exposure to paracetamol is clinically relevant.

More than 50% of women in the Western world use analgesics during pregnancy, with paracetamol being most widely used (Werler *et al*, 2005). Paracetamol is similar to the NSAID family of analgesics, with both drug types functioning via the inhibition of the COX enzymes to prevent inflammatory signalling. However paracetamol is a weak anti-inflammatory with poor anti-platelet activity compared to the NSAIDs (Lages & Weiss, 1989; Niemi *et al*, 2000). This difference is likely due to differing mechanisms of action. NSAIDs directly inhibit the COX enzymes both in the central nervous system (CNS) and peripheral systems (Ayoub *et al*, 2006). Conversely, paracetamol, despite being on the market for over 50 years, does not have a well-characterised mechanism of action, although it has been established it interacts with the COX enzymes (Graham & Scott, 2005). It was briefly hypothesised that paracetamol functioned via inhibition of a third, yet to be characterised COX enzyme (COX3) (Chandrasekharan *et al*, 2002) but this hypothesis has since been rejected (Dinchuk *et al*, 2003; Schwab *et al*, 2003) Schwab, 2003). Recent evidence suggests the primary target of paracetamol action in the human is COX2, as it exhibits comparable COX2 inhibition to other NSAIDs and COX2-specific inhibitors, but is unable to suppress COX1 to therapeutic levels

comparable to the NSAIDs (Hinz *et al*, 2008). There may also be other targets of paracetamol action, and its direct mechanism of inhibiting COX2 remains unknown.

Specific COX2 inhibitors, including paracetamol, are commonly prescribed for chronic pain and/or inflammation management, as COX2 specific inhibitors do not induce gastrointesitinal bleeding, which is a side effect of long-term inhibition of COX1 in NSAIDs (Wilcox *et al*, 1997; Sigthorsson *et al*, 2000). It is also thought COX2 inhibitors are less likely to cause renal complications when compared to traditional NSAIDs (Zhao *et al*, 2001; Whelton *et al*, 2002). However, COX2 inhibitors have been shown to heighten risk of cardiovascular complications with long term use (Bombardier, 2002; Bresalier *et al*, 2005).

Despite this, paracetamol has been considered safe, with no reported feto-toxic effects when taken within the therapeutic range (Rathmell *et al*, 1997). However, recent studies from Denmark have disputed the safety of prenatal exposure to analgesics, suggesting that maternal usage of these drugs, including paracetamol, results in increased risk of cryptorchidism in male offspring (Kristensen *et al*, 2011). This was determined via a meta-study of maternal usage of analgesics; paracetamol and NSAIDs (non-steroidal anti-inflammatory drugs; aspirin and ibuprofen), noting duration of use and what point in gestation the treatment was taken. This study determined use of analgesics was associated with congenital cryptorchidism in a dose-dependent manner, with increased risk in mothers who used analgesics during second trimester. These data were further supported by a rodent model that demonstrated paracetamol produced anti-androgenic effects in offspring leading to impaired masculinisation (as demonstrated by reduced ano-genital distance; (Kristensen *et al*, 2011)).

As previously discussed COX enzymes, which are targeted by paracetamol and NSAIDs, synthesise PGs and other inflammatory mediators. Outwith the Kristensen *et al.* study, historical literature from Gupta and colleagues has also demonstrated inhibition of the Cox enzymes and indeed direct inhibition of the Pgs is able to disrupt male development in rodents (Gupta & Goldman, 1986; Gupta, 1989). However, the effect of maternal analgesic usage on female development has not been investigated.

Current knowledge of PG involvement in gonad development is limited. PGD_2 is thought to be involved in early sex determination, and based upon data gathered in the previous chapter, it is postulated that PGE_2 plays a regulatory role during ovarian development. Inhibition of PG synthesis by analgesics during pregnancy would affect these pathways and might lead to disruption of gonad development in offspring. To test this hypothesis, as well as further examine the function of PGE_2 during fetal ovarian development, the rat model used in the Danish study was duplicated (Kristensen *et al*, 2011). This model utilises maternal gavage (oral administration) also allowing the study to remain clinically relevant to human administration of analgesics during pregnancy. Resulting female pups were examined for gross morphological and gonadal differences compared to pups exposed to vehicle control (corn oil). Further investigation of the male pups and testis development was also investigated by collaborators, but will not be discussed in this chapter.

4.2 Materials and Methods

Tissue collection and dissection

Pregnant Wister rats were either treated with 350 mg/kg paracetamol in oil for 7 days of gestation or oil control alone, from day e13.5 to e20.5. Fetal rat ovaries were collected at embryonic day (e)21.5 of gestation. In a second series of studies, paracetamol treatment was the same but with exposure for only 3 days with ovaries collected at day e17.5. 5 litters of each treatment group were analysed in the following experiments (with at least 3 animals per litter analysed). Full details are in Section 2.2.

Gross morphological measurements

Body mass and ano-genital distance (AGD) were measured at dissection using a micro-balance (Sartorius Handy model #110) and digital callipers (Faithfull Tools). Body mass was measured before decapitation to detect possible systemic changes. AGD was measured from the start of the anus to the start of the phallus in both sexes as an index of masculinsation (Figure 4.1). Scales and calipers were re-calibrated before use.

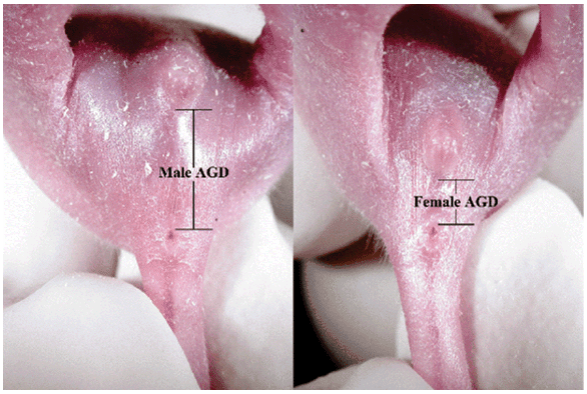

Figure 4.1 Ano-genital distance measurement

Ano-genital distance (AGD) was measured as indicated above, with a clear difference between male and female animals. AGD was utilised as an early indicator of disruption of masculinisation (Ostby & Gray, 2004)

AGD was also adjusted for body mass, as AGD changes in proportion to body size. The equation is as described below and the adjusted measurement referred to as ano-genital index (AGI).

$$\textbf{AGI = AGD (mm) / body mass}^{1/3}\textbf{ (grams)}$$

Immunohistochemistry

In order to determine if rodent expression of Pge_2 factors was similar to that of the human fetal ovary, Cox1, Cox2, Ptges, and Ep1-4 were localised in the rat ovary using the same concentrations of primary antibody as in the human. Further to determine changes in germ cell number, both Tra-98 and Vasa staining were utilised, with additional staining for proliferation marker phosphohistone-H3 (PHH3) and apoptosis marker cleaved caspase-3 (CC3) to identify any changes in ovarian cell turnover. Details of immunohistochemical protocols can be found in Section 2.8.

Stereology

All stereology was performed using the equipment described in Section 2.11.3. All counts were performed blind to prevent inadvertent bias.

Ovarian volumes

Fetal rat ovaries were serial sectioned, with 2 sections placed on each slide. Every tenth slide was stained with hematoxylin and eosin (as in Section 2.7) and the ovarian area measured. An average ovarian area and the number of slides containing complete ovarian sections was then used to extrapolate volume using the equation below and as depicted in Figure 4.2.

$$\textbf{ovarian volume} = \textbf{average ovarian area x (number of slides containing ovary x 10 µm)}$$

(Each section is 5 µm, and each slide contains two sections making each slide 10 µm).

This calculation is based upon Calvalieri's principle, which postulates the area of 2D sections multiplied by the distance from end to end of an object is an accurate calculation for volume of the object, regardless of shape. This principle has been verified as an accurate way to discern tissue volume, as long as the sections examined are of equal interval (Mayhew & Olsen, 1991).

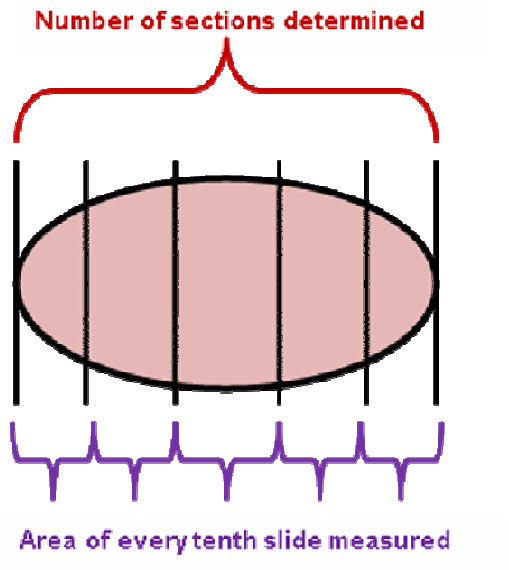

Figure 4.2 Schematic of determination of ovarian volume.

Fetal rat ovaries were serial sectioned. Area of every tenth slide was determined using image analysis and the area averaged before multiplying by the depth of ovary as determined by number of slides and thickness of the sections on the slides.

Germ cells per area

The germ cell marker Tra-98 was used to mark germ cells in the fetal rat ovary, and stereology was performed to count number of germ cells using two slides per animal (one from the centre of the tissue as determined from ovarian area, and another at least fifty microns from the centre). The number of germ cells quantified was then adjusted for ovarian area determined from the same section and presented as germ cells per micron^2. A figure depicting this analysis can be found in Section 2.11.

Germ cell nuclear diameter

Germ cell nuclear diameter (GCND) can be used as an index of oogonia/oocyte maturation, with more mature germ cells having larger nuclear diameters. Germ cell nuclear diameter was measured using the 'nucleator' function of the above software, which tri-sects the germ cell and each of 6 radii are measured. These radii are then averaged to provide an accurate measurement of nuclear diameter of the cell. Fifty germ cells per section were randomly measured in this fashion, and two sections (the same slides were utilised for both germ cell number quantification and GCND measurement) from each animal were quantified. Some of the paracetamol treated animals did not have 50 germ cells in a section. In these cases, all germ cells were measured.

Apoptosis

Apoptosis was measured via the marker CC3, as in the previous chapter. CC3 positive cells were counted and adjusted for ovarian area and presented as apoptotic cells per micron^2.

Proliferation

Proliferation was measured via the marker PHH3, similarly to the protocol used in the previous chapter for BrdU. PHH3 positive cells were counted and adjusted for ovarian area and presented as proliferating cells per micron^2.

Statistical Analysis

Gross and stereological measurements were analysed with GraphPad Prism version 4 (GraphPad Software Inc). Student's unpaired t-test was utilised for determination of significant changes between treatment groups, following analysis of each measurement for normality utilising the Kolmogorov-Smirnov test.

Data were analysed both on an individual basis and as litter average. This was performed by averaging the individual animal data for each animal from the same litter/dam.

4.3 Results

Fetal rat ovaries were collected after paracetamol or control treatment for 7 days of pregnancy at e21.5 gestation, and the following analyses were performed. Gross morphological data presented (body mass, AGD, and AGI) is based upon all female animals collected (n=7 litters for both treatment, consisting of 35 control pups and 36 treated pups). The subsequent stereological data was only performed on a subset of female animals chosen randomly, from 5 litters from both control and treated groups examined and at least 3 animals from each litter analysed (n=16 control animals and 16 treated animals). Only 5 of the 7 litters obtained were chosen for further stereological analysis. One litter from each of the control and treated groups was omitted for having either fewer than 3 female pups or having an abnormally small litter. All data were analysed and presented both as individual animal data and litter mean data to reveal any possible 'litter effects' which can be caused if animals from one or more litter are above or below average, resulting in skewed data.

4.3.1 Fetal body mass is not altered by paracetamol exposure

Body mass was taken before decapitation of all fetal rats collected. Although there was a non-significant trend for paracetamol rats to be heavier when analysed as individual animals (p=0.23, Figure 4.3A), this trend was not seen when animals were analysed by litter means (p=0.73, Figure 4.3B). These data establish there are no major changes in pup body mass with maternal usage of paracetamol.

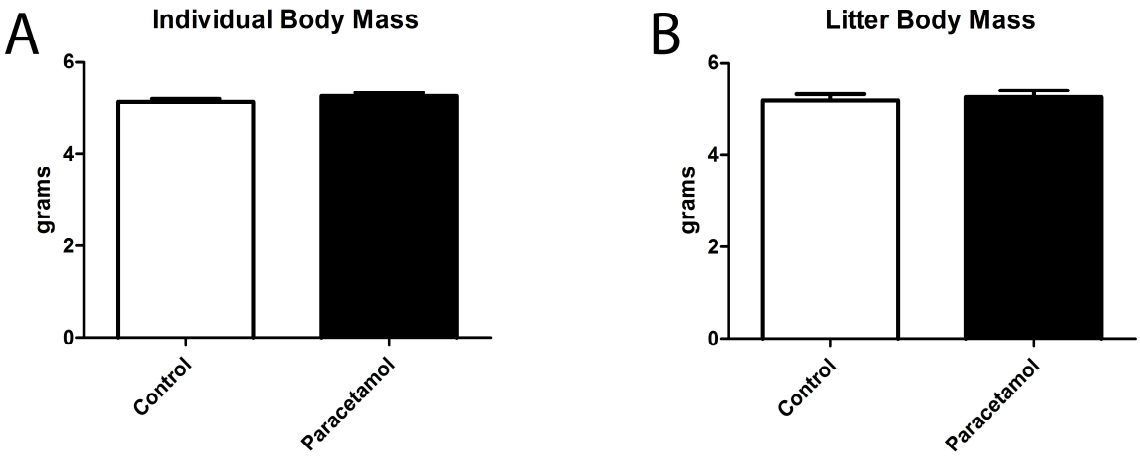

Figure 4.3 Female pup body mass does not change after fetal paracetamol exposure.

Body mass was taken for e21.5 fetal female rats treated with 350mg/kg paracetamol (paracetamol) for 7 days of gestation or vehicle control (control). (A) No change was determined in body weight between paracetmol exposed and control individuals. (B) This is consistent when analysed as litter mean, with no significant difference seen between treatment groups.

4.3.2 Masculinisation as determined by AGD and AGI is not changed with maternal usage of paracetamol

AGD is classically used as an early indicator of masculinisation during fetal development (Edwards, 2006). As data from previous studies suggest paracetamol exposure may affect early androgen signalling thus affecting male development, both AGD and AGI were examined. Analysis of individual animal's AGD resulted in a trend for increased AGD in paracetamol exposed animals as compared to control ($p=0.06$), suggesting treated animals may have increased androgenic signalling compared to control animals (Figure 4.4A). However, this trend was lost when analysed using litter mean data ($p=0.61$, Figure 4.4B). As a slight change was detected in body mass, AGI was also utilised and confirmed there was no change in masculinisation of the female pups on both the individual basis or by litter mean data ($p=0.10$ and $p=0.67$, Figure 4.5A and B respectively).

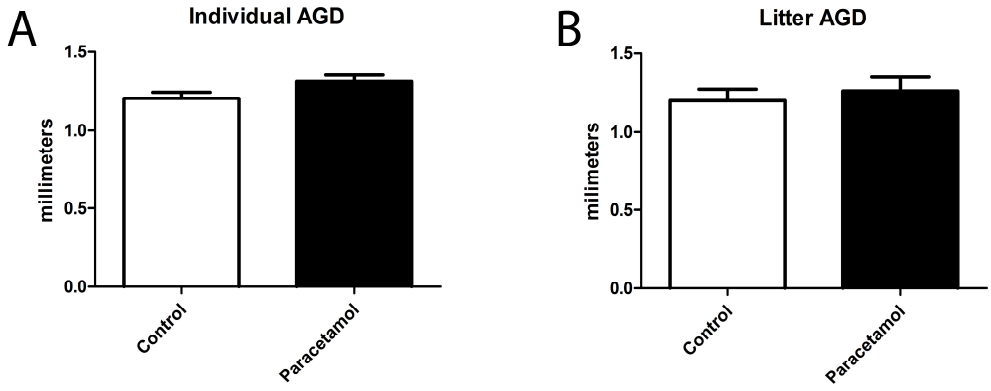

Figure 4.4 Ano-genital distance is not affected in female pups exposed to paracetamol.

AGD of e21.5 fetal female rats was measured in both treated (350mg/kg paracetamol for 7 days of gestation (paracetamol)) and control (control) animals. (A) A trend for increased AGD was seen in paracetamol treated animals but was not deemed significant. (B) This trend was not seen when data was analysed using litter means suggesting individual animal varience may have been responsible for the trend seen when analysed as individual animals.

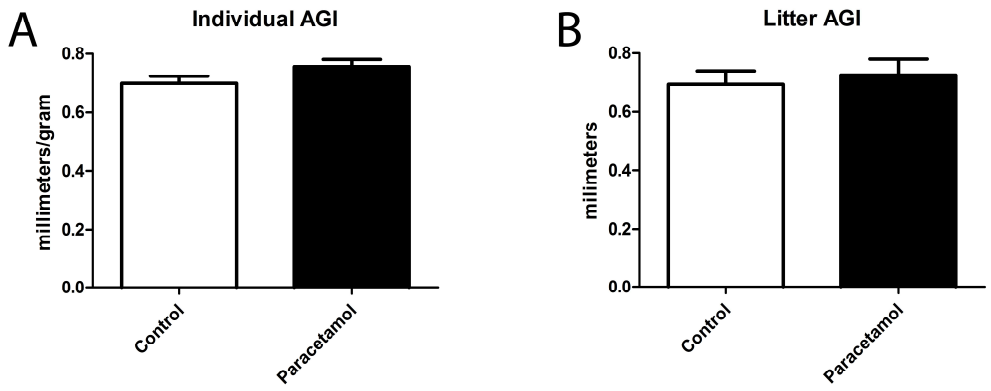

Figure 4.5 Ano-genital index is not affected in female pups exposed to paracetamol.

AGI of e21.5 fetal female rats was measured in both treated (350mg/kg paracetamol for 7 days of gestation (paracetamol)) and control (control) animals. (A) No change was seen in AGI when analysed using individual animal measurements or (B) using litter mean measurements.

4.3.3 Ovaries from paracetamol treated pups are significantly smaller than control ovaries

Fetal rat ovaries at e21.5 are relatively small compared to fetal rat testes or human fetal ovaries, and are enclosed in a thin layer of tissue referred to as the ovarian capsule. These two features restricted initial measurement of ovarian mass, as removal of the capsule increased risk of damage to the ovarian tissue underneath and acquiring an accurate measurement of such small tissue was difficult. Therefore, ovaries were removed and fixed with capsule intact and further examination was performed on serial sections of tissue. Initial investigation of the ovaries was performed using the germ cell markers Tra-98 and Vasa. Visual evaluation of morphology indicated there might be differences in both size and number of germ cells in the ovaries of paracetamol exposed tissue compared to control tissue (Figure 4.6). Therefore further quantification was performed.

Ovarian volume was measured via stereology to determine potential differences in ovarian size between treatment groups. Quantification determined the ovaries of paracetamol treated rats were smaller than control ovaries when analysed on both an individual (2.6 ± 0.4 vs 4.2 ± 0.3 x10^7 micron^3, $p=0.0017$, Figure 4.7A) and litter mean basis (2.7 ± 0.5 vs 4.3 ± 0.3 x10^7 micron^3, $p=0.03$, Figure 4.7B). These data suggest paracetemol exposure *in utero* effects ovarian size. This decrease in mass is not seen in body weight, suggesting the difference may be due to a loss of cells within the ovary.

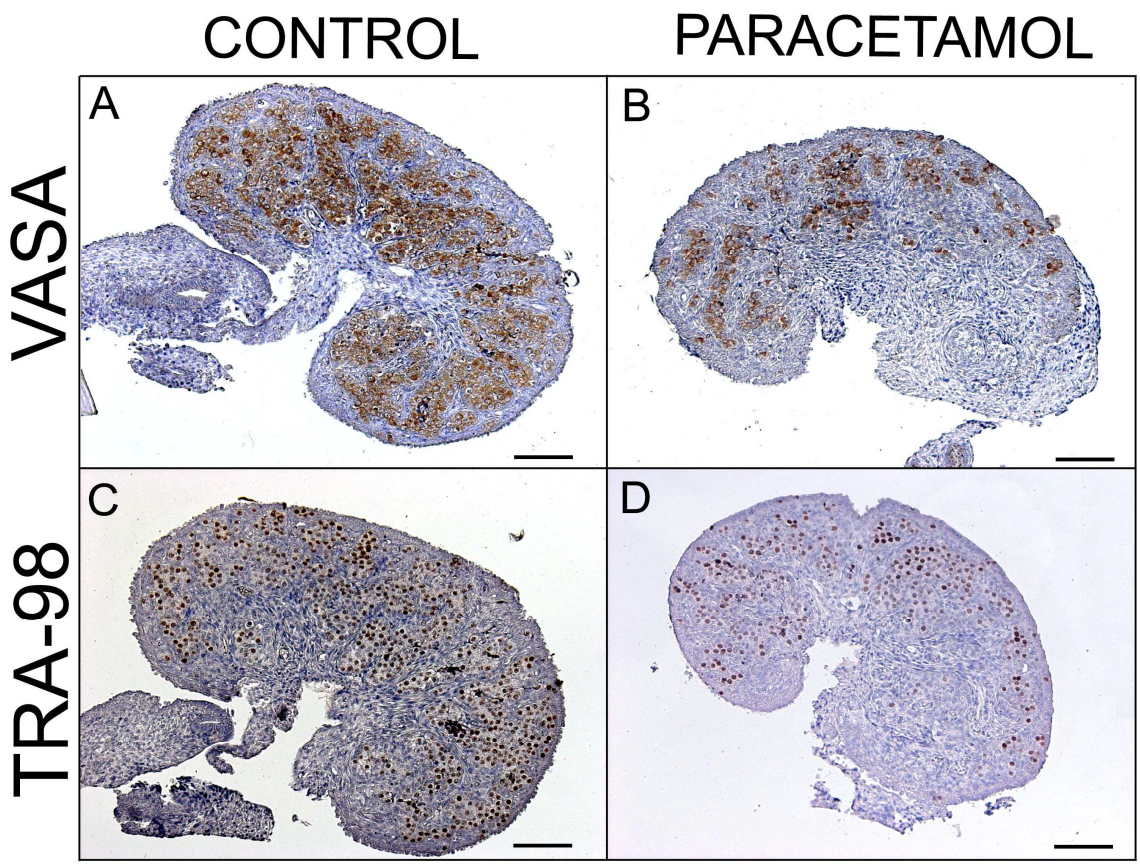

Figure 4.6 Morphological comparison of paracetamol exposed ovaries to control ovaries.

Serial sections from control (A and C) and paracetamol exposed (B and D) e21.5 fetal rat ovaries. Immunolocalisation (brown) for cytoplasmic germ cell marker Vasa (A and B) and nuclear marker Tra-98 (C and D) demonstrating visual observations of smaller ovarian size as well as fewer germ cells in the paracetamol exposed ovaries. Scale bars equal 50 microns.

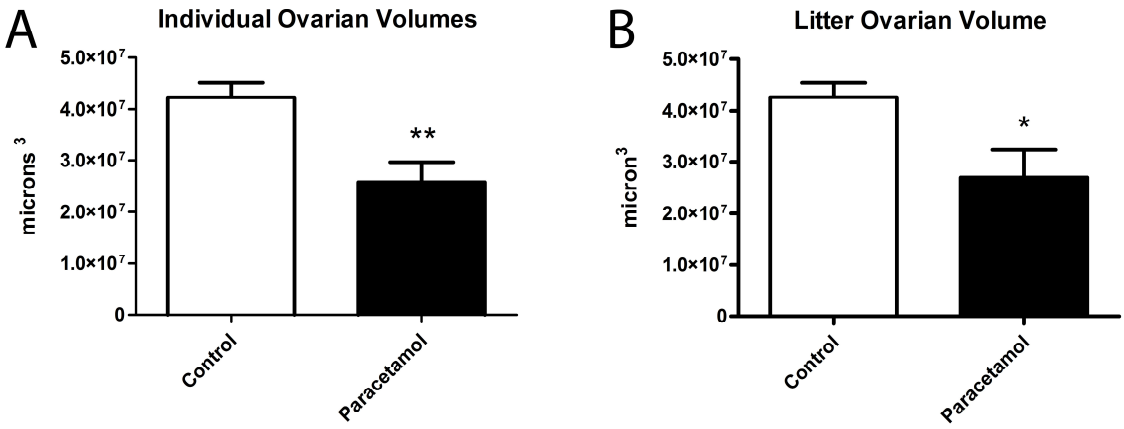

Figure 4.7 Ovarian volumes from e21.5 fetal rats.

Fetal ovarian volumes were determined using stereological methods. (A) Paracetamol treated pups (paracetamol) had significantly smaller ovaries than control animals (control) when analysed by individual animals (n=16 control and treated animals, **=$p<0.01$). (B) Paracetamol treated ovaries also retained significance when analysed as litter means (n=5 litters for both treated and control, *= $p<0.05$).

4.3.4 Fetal exposure to maternal paracetamol results in a decreased number of germ cells

As it was hypothesised the decrease in ovarian volume might be the result of a loss of a specific cell type within the ovary and based upon visual observation of decreased germ cell staining, germ cell number was quantified in control and paracetamol treated ovaries. Germ cell counts were adjusted for ovarian area as paracetamol ovaries were deemed to be significantly smaller, in order to reveal if germ cell number was independently altered with fetal paracetamol exposure. Paracetamol treated ovaries displayed a significant reduction in germ cells per micron^2 of ovary in comparison to controls when analysed as individual animals (1.0±0.1 vs 1.8±0.2 x10^{-3}, p=0.004, Figure 4.8A). When analysed as litter means the loss of germ cells remained consistently significant between paracetamol and control litters (1.0±0.1 vs 1.8±0.2 x10^{-3}, p=0.01, Figure 4.8B). These data suggest factor/s altered by paracetamol exposure lead to germ cell loss or possibly decreased proliferation in fetally exposed ovaries.

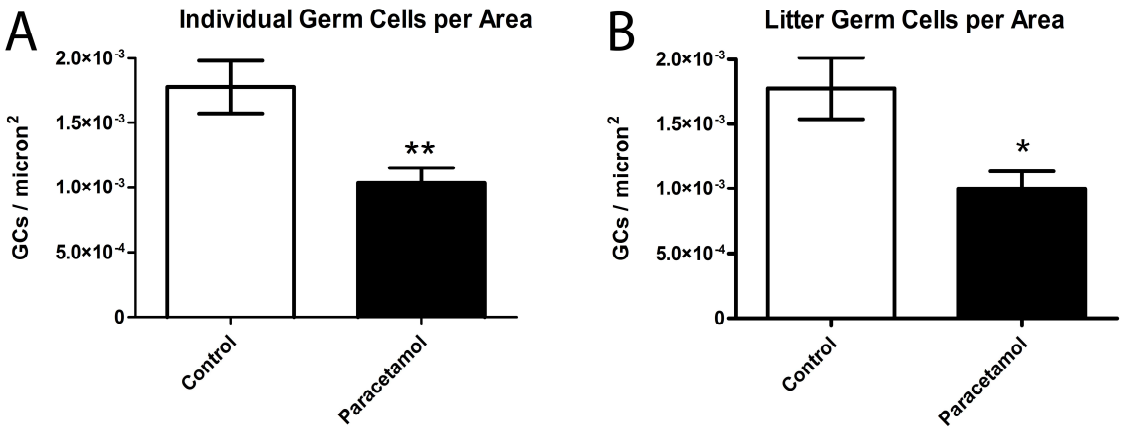

Figure 4.8 Ovarian germ cell number is decreased with maternal usage of paracetamol.

Germ cell number was determined using the nuclear germ cell marker Tra-98 via stereological methods and adjusted for ovarian size. (A) Ovaries from paracetamol treated pups (paracetamol) had significantly fewer germ cells per micron^2 when compared to control ovaries (control) (n=16 for both groups, **=$p<0.01$). (B) This reduction in germ cell number was also seen when germ cell number was analysed using litter means (n=5 for both groups, *= $p<0.05$).

4.3.5 Reason for reduction in germ cell number is not apparent at e21.5.

In order to identify a possible mechanism for germ cell loss in paracetamol treated ovaries, the rate of apoptosis and proliferation in treated and control ovaries was assessed. Rate of programmed cell death, as determined by the apoptotic marker CC3, was not significantly different between control and paracetamol exposed ovaries on an individual or litter basis ($p= 0.54$ and 0.21, Figure 4.9A and B respectively). Additionally, no significant change was seen in proliferation when using the mitotic marker PHH3, on either an individual or a litter mean basis ($p=0.22$ and 0.25, Figure 4.10A and B respectively). These rates are demonstrated as a percentage of positively stained (CC3 (Figure 4.9C-D) or PHH3 cells (Figure 4.10C-D)) compared to total germ cell number (as collected from previous data). These data determine that paracetamol exposure is not affecting normal ovarian cell turnover at e21.5, although the decrease in germ cell number may be a result of a developmental arrest.

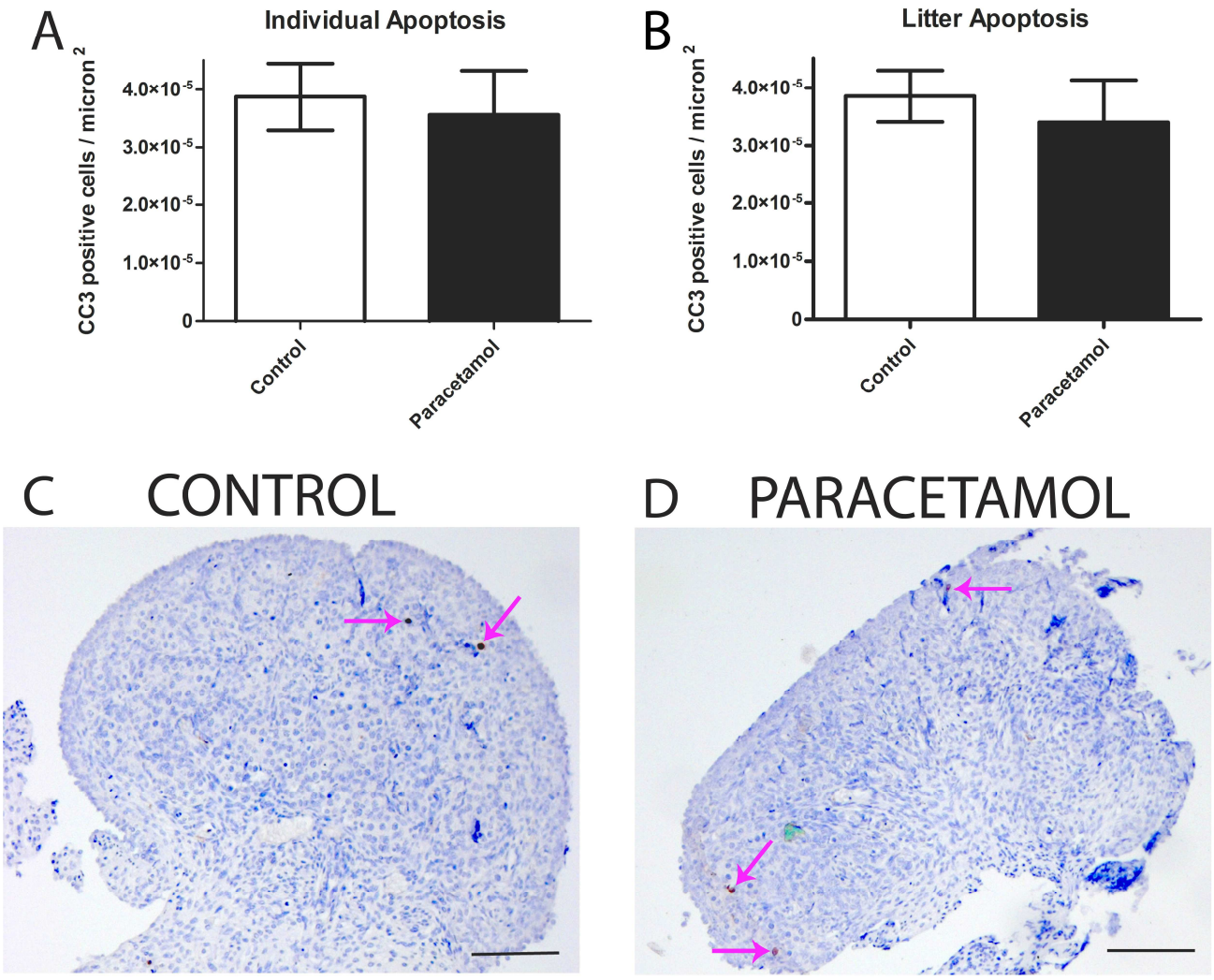

Figure 4.9 Germ cell reduction in paracetamol ovaries is not the result of increased apoptosis.

Rates of germ cell apoptosis were determined using the apoptotic marker cleaved capsase-3 (CC3) via stereological methods and adjusted for total number of germ cells, in order to demonstrate the proportion of CC3 positive germ cells per animal or litter. (A) No change was seen in ovarian apoptosis with fetal paracetamol exposure when examining changes in individual animals (n=16 animals per treatment). (B) This finding was confirmed using litter means, however there was a trend of increased apoptosis in paracetamol treated pups (n=5 litters per treatment). (C) Control and (D) paracetamol treated ovaries stained for CC3 (pink arrows). Scale bars equal 50 microns.

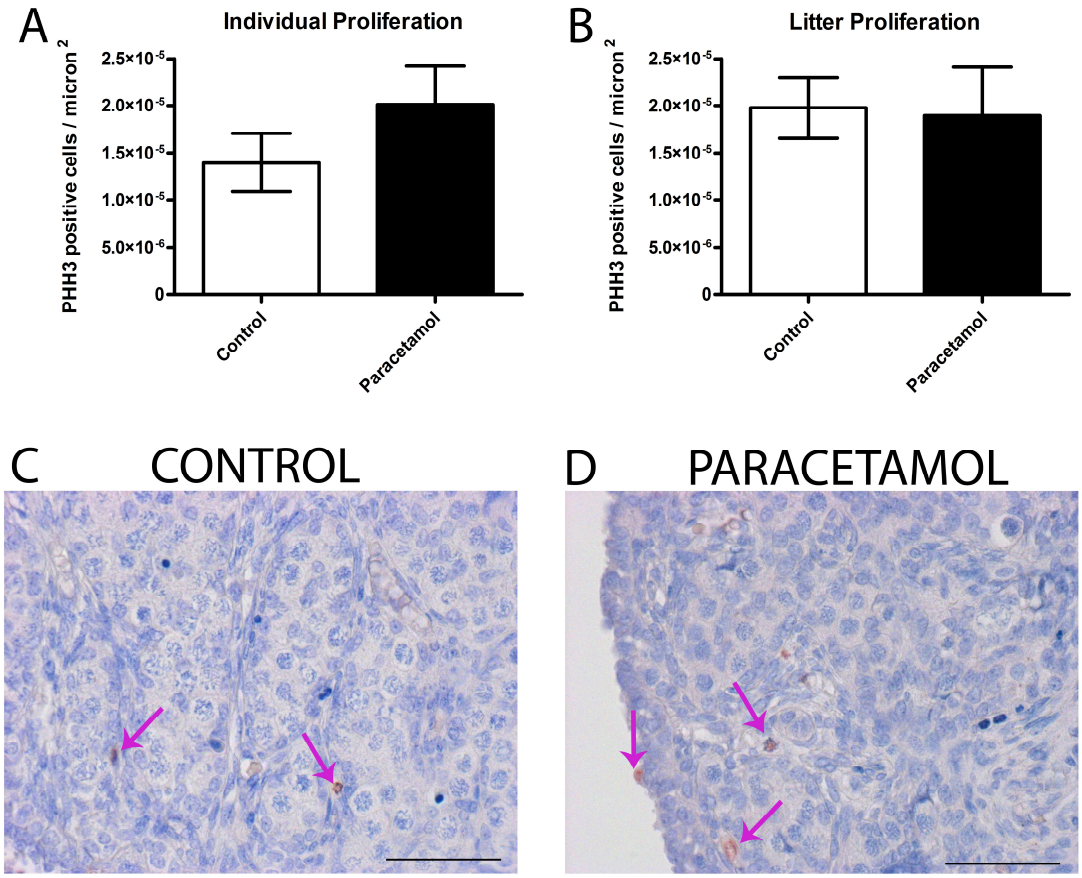

Figure 4.10 Germ cell reduction in paracetamol ovaries is not the result of decreased proliferation.

Proliferation rate was examined using the marker phospho-hisotone H3 (PHH3) via stereological methods and adjusted for total number of germ cells. The number of PHH3 germ cells per area was then adjusted for total germ cell number to demonstrate the proportion of PHH3 positive germ cells per animal or litter. (A) A trend was seen for more proliferation in individual paracetamol exposed ovaries when compared to control animals but was not significant (n=16 ovaries per treatment). (B) This trend was also seen using litter mean but was also not significant (n=5 litter per treatment). (C) Control and (D) paracetamol treated ovaries stained for PHH3 (pink arrows). Scale bars equal 100 microns.

4.3.6 Fetal paracetamol exposure delays germ cell progression and may affect germ cell synchronicity in the rat ovary

GCND was examined as a measurement of germ cell maturity in both control and treated ovaries, in order to distinguish if paracetamol exposure had any effects on germ cell development. When individual animal GCND means were calculated, germ cells from paracetamol animals were found to have significantly smaller GCND ie be less mature than those from control ovaries (7.4±0.1 vs 6.8±0.2microns, p=0.03, Figure 4.11A). However, when GCNDs were analysed as a litter mean, significance was lost, although the trend remained (p=0.07, Figure 4.11B). The rat ovary is comparatively synchronous at e21.5, composed of germ cell nests of roughly the same developmental stage; therefore, GCND data was further analysed to determine the proportion of cells at each diameter to detect subtle changes in maturation and changes in synchronicity.

Analysis of the total population of germ cell measured for each treatment determined there was a significant difference in the distribution of development between control and paracetamol treated germ cells, with a larger population of smaller, immature germ cells in the paracetamol ovaries when compared with control (7.4±0.03 vs 7.0±0.04 microns, n= 1385 and 1166, p=0.0001, Figure 4.11C). In addition, paracetamol ovaries displayed a lack of synchronicity, compared with control germ cells, of which ~80% remained within a variance of only 3 µm compared to a 4 µm variance in the same proportion of cells from paracetamol exposed ovaries. However, the paracetamol exposed germ cells were still able to reach the same maturity levels as seen in control germ cells (albeit fewer cells reached these stages), demonstrating a complete arrest of development was not occurring in paracetamol exposed ovaries, rather a developmental delay.

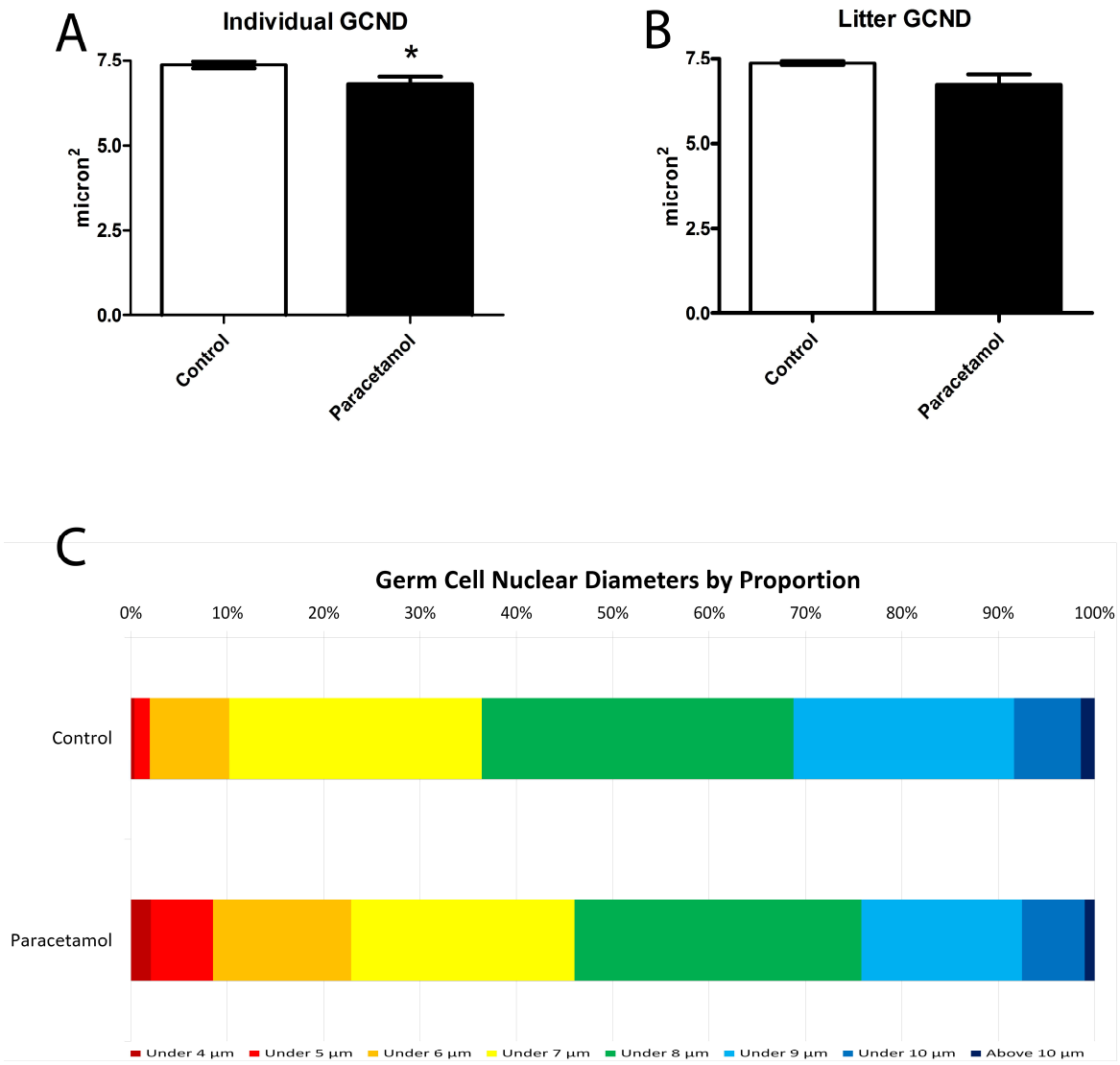

Figure 4.11 Development of germ cells in paracetamol exposed ovaries is delayed and less synchronous when compared to control germ cells.

Germ cell nuclear diameter (GCND) was determined using the nuclear germ cell marker Tra-98 via stereological methods. (A) A significant reduction in germ cell nuclear diameter can be seen upon comparison of individual animal GCND means (*= $p<0.05$, n=16 control and treated animals). (B) This trend was consistent when comparing litter mean data, but was not significant (n=5 litters per group). (C) Total germ cell population from all animals was further analysed to determine subtle changes in maturation and changes in synchronicity (n=1385 control germ cells and 1166 paracetamol exposed germ cells).

4.3.7 Paracetamol effects on fetal rats at e17.5

Although we determined exposure to paracetamol over 7 days *in utero* affects fetal rat ovaries when examined at e21.5, no clear mechanism for these effects was determined. It was postulated the changes in ovarian size, germ cell number, and GCND may be the result of paracetamol effects earlier in gestation. In order to examine possible changes in apoptosis or proliferation prior to e21.5 pups were exposed to paracetamol as before, but were dissected at e17.5 after only 3 days of *in utero* exposure.

Gross morphological examination upon dissection demonstrated control animals were significantly smaller than paracetamol pups (0.62 ± 0.03 vs 0.81 ± 0.01, $p<0.0001$, Figure 4.12A). However, when analysed on a litter mean basis this reduction in body mass is no longer significant ($p=0.15$, Figure 4.12B). AGD or AGI were not examined at e17.5 as little variance is seen at this age and no change had been determined at e21.5. These data demonstrate there is no gross morphological difference between control or paracetamol exposed fetal female rats at e17.5.

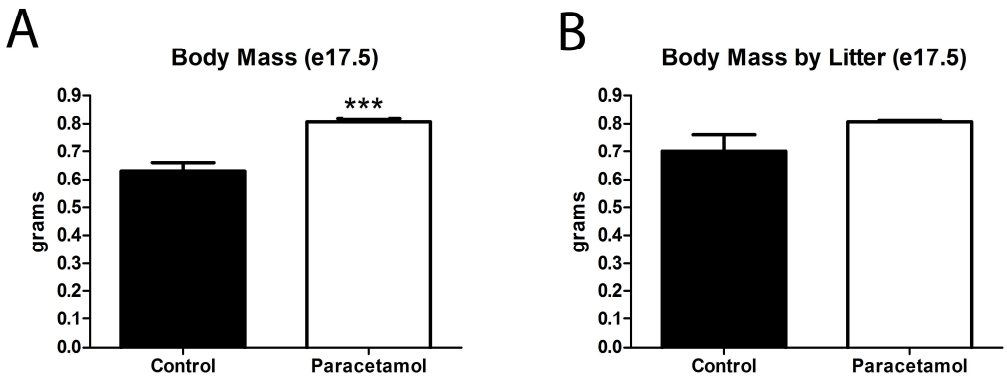

Figure 4.12 No difference in body mass of e17.5 fetal rats exposed to paracetamol

Body mass was recorded from e17.5 fetal female rats treated with 350mg/kg paracetamol (paracetamol) for 4 days of gestation or vehicle control (control). (A) Paracetamol exposed female were significantly larger than control animals when analysed as individuals. (B) When analysed as litter mean data, there was no significant difference seen between treatment groups (*** $p<0.0001$).

4.3.8 No change in ovarian volume in e17.5 paracetamol exposed fetal rats

Ovarian volume was calculated as above for e21.5, to determine if a similar decrease in ovarian size could be detected at e17.5. A trend for increased ovarian size was seen in paracetamol exposed animals but this was not significant (p=0.13, Figure 4.13A). Analysis of ovarian volumes utilizing litter mean data also demonstrated no significant difference between control and paracetamol exposed pups (p=0.36, Figure 4.13B). These data demonstrate the significant change in ovarian volume seen at e21.5 is a result of action between e17.5 and e21.5 or requires the longer duration of exposure.

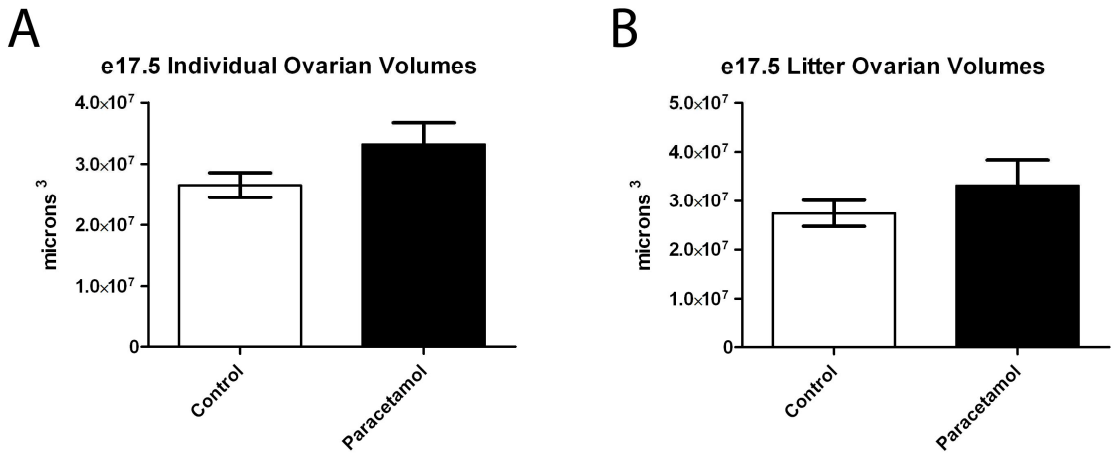

Figure 4.13 No change in fetal ovarian volume at e17.5

Fetal ovarian volumes were determined using stereological methods. (A) No change in ovarian volume was determined with paracetamol exposure (paracetamol) on an individual animal basis compared to control animals (control, n=15 control and treated animals). (B) Analysis using litter mean data also demonstrates no change with paracetamol treatment (n=5 litters for both treated and control).

4.3.9 No change in germ cell number with paracetamol exposure at e17.5

Although no change in ovarian volume was determined at e17.5, germ cell number per micron^2 was further evaluated at this age in order to determine any subtle changes in germ cell number. No change in germ cell density was determined on either the individual (p=0.84, Figure 4.14A) or on a litter mean basis (p=0.82, Figure 4.14B). This further demonstrates the significant change seen at e21.5 must be a result of effects between e17.5 and e21.5.

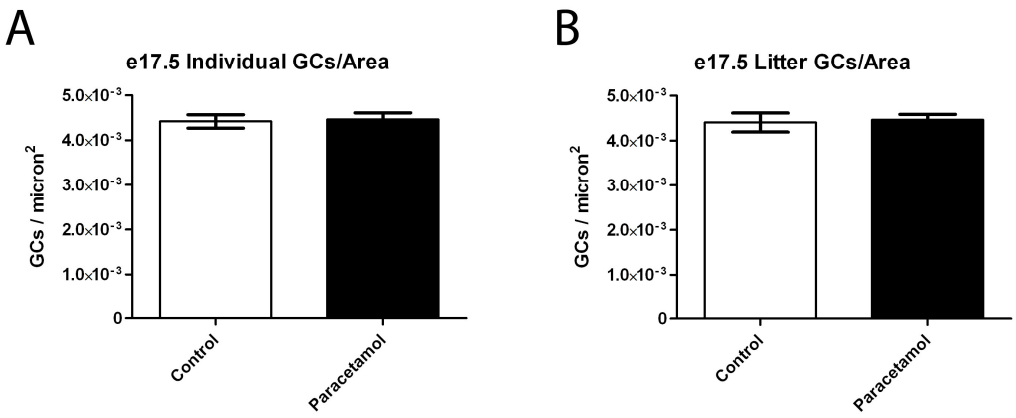

Figure 4.14 No change in germ cells per micron^2 with paracetamol exposure at e17.5

Germ cell number was determined using the nuclear germ cell marker VASA via stereological methods and adjusted for ovarian size. (A) Germ cell number in paracetamol treated pups (paracetamol) were not significantly different than that of control animals (control; n=15 for both groups). (B) No significant change was seen with paracetamol exposure when data was analysed using litter mean analysis (n=5).

4.3.10 No demonstration of altered apoptotic or proliferative rates at e17.5

No change in apoptosis or proliferation was seen at e21.5, which would indicate a cause for the demonstrated change in germ cell number. In order to determine if this change in germ cell number was a result of earlier change in cell turnover, both apoptosis and proliferation rates were examined in e17.5 ovaries. These rates were determined as previously described for e21.5, with CC3 or PHH3 positive cells per micron^2 adjusted for total number of germ cells per micron^2. No change was seen in apoptotic rate on either an individual ($p=0.87$, Figure 4.15A) or litter mean basis ($p=0.95$, Figure 4.15B). Additionally, no change was seen in proliferation rate on either an individual ($p=0.21$, Figure 4.16A) or litter mean basis ($p=0.40$, 4.16B). These results demonstrate there is no change in cell turnover at e17.5, suggesting either the change in cell turnover is acute between e17.5 and e21.5 or that the change in germ cell number may be due to loss via another mechanism.

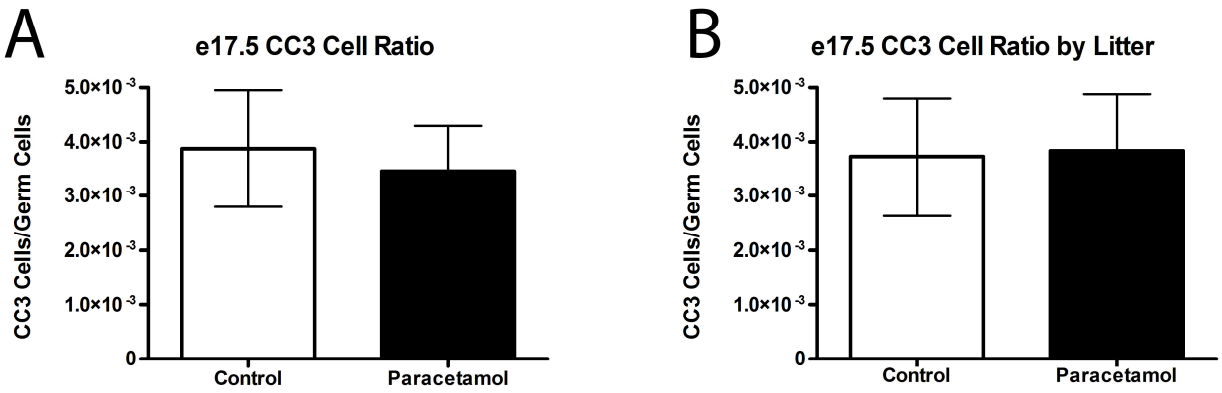

Figure 4.15 Germ cell reduction in paracetamol ovaries not the result of increased apoptosis at 17.5.

Rates of germ cell apoptosis were determined using the apoptotic marker cleaved capsase-3 (CC3) via stereological methods and adjusted for ovarian size. The number of CC3 germ cells per area was then adjusted for total germ cell number to demonstrate the proportion of CC3 positive germ cells per animal or litter. (A) No change was seen in ovarian apoptosis with fetal paracetamol exposure when examining changes in individual animals (n=15 animals per treatment). (B) This finding was confirmed using litter mean data (n=5 litters per treatment).

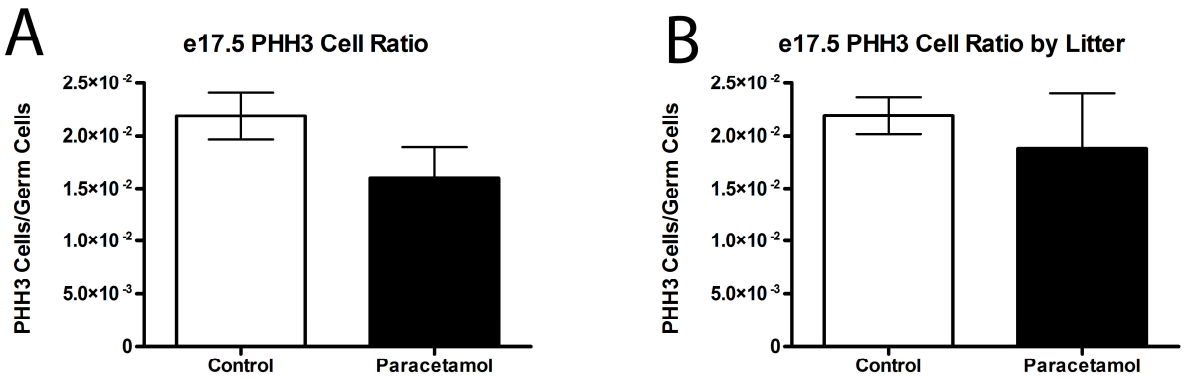

Figure 4.16 Germ cell reduction in paracetamol ovaries is not the result of decreased proliferation.

Proliferation rate was examined using the marker phospho-hisotone H3 (PHH3) via stereological methods and adjusted for ovarian size. The number of PHH3 germ cells per area was then adjusted for total germ cell number to demonstrate the proportion of PHH3 positive germ cells per animal or litter. (A) A trend was seen for less proliferation in individual paracetamol exposed ovaries when compared to control animals but was not significant (n=15 ovaries per treatment). (B) This trend was lost when analysed as litter mean data (n=5 litter per treatment).

4.3.11 Prostaglandin synthesis in the fetal rat ovary

In an effort to further elucidate possible causes of paracetamol exposure effects, further investigation of Cox and downstream prostaglandin signalling was investigated in the fetal rat ovary. The localisation of the Cox enzymes was identified in wild type e21.5 fetal rat ovaries. In addition, as Pge_2 was linked to germ cell survival in the human fetal ovary (as previously discussed in Chapter 3) (Bayne *et al*, 2009), Ptges, which is downstream of the Cox enzymes and responsible for Pge_2 specific synthesis, was also investigated.

Unlike the human, little Cox1 expression was seen in the fetal rat ovary (Figure 4.17A-B), although expression can be seen in blood cells. However, this expression is minimal, as shown by comparison with positive control tissue from the human endometrium (Figure 4.17C). Conversely, Cox2 expression was very similar to that seen in the human fetal ovary, displaying germ cell-specific localisation with variability between germ cells within the same germ cell nests (Figure 4.17D-F). Ptges expression was also similar to that seen in humans, with the majority of protein localised to the germ cells of the fetal ovary (Figure 4.17G-I). However, in the rat, unlike the human, expression is also seen in the pre-granulosa and somatic cells of the ovary. Distinct absence of Ptges expression can be seen in the capsule tissue surrounding the ovary, suggesting Ptges immunolocalisation in the fetal ovary is specific. These data demonstrate the primary site of Cox synthesis enzyme expression to be the germ cells in the fetal rat ovary, suggesting these are the targets of paracetamol action.

.

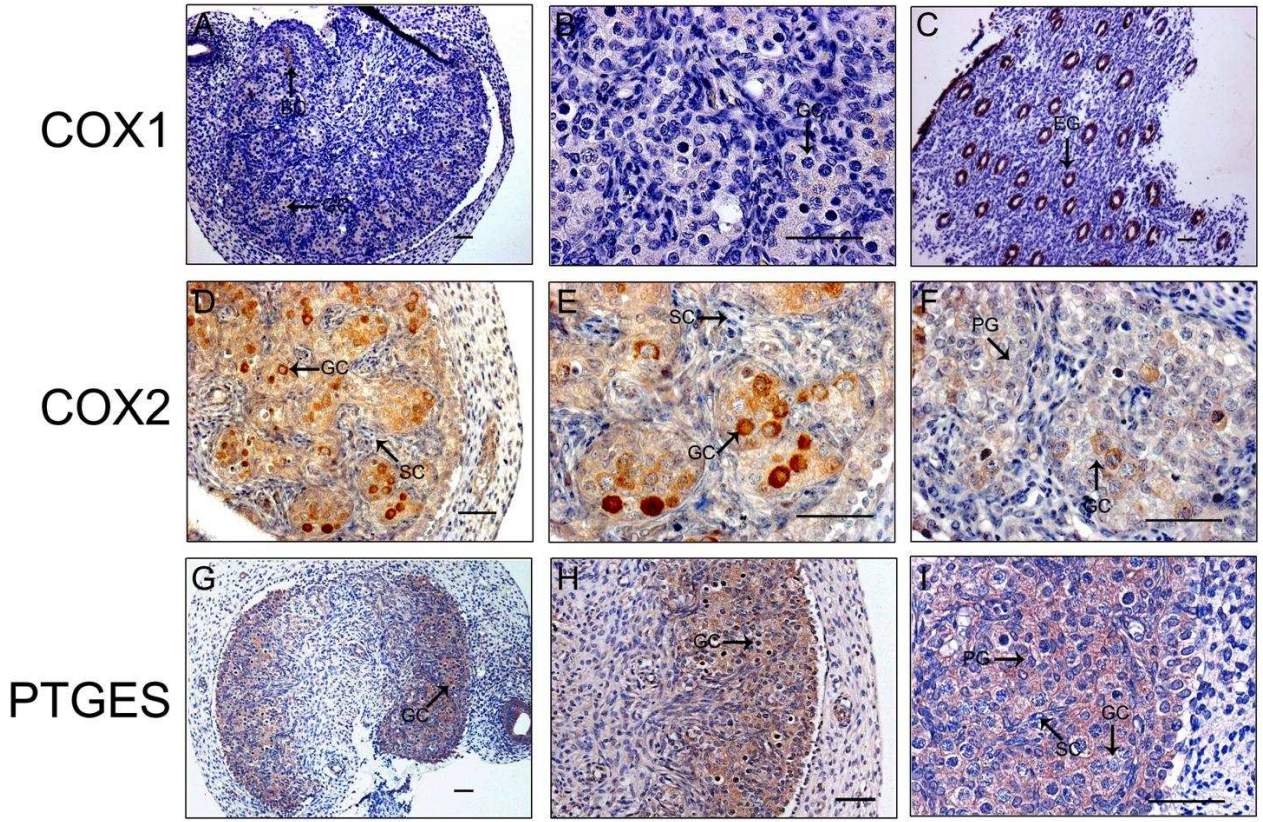

Figure 4.17 Cox and Ptges expression in the wild type fetal rat ovary

The Cox enzymes and Ptges were immunolocalised in the e21.5 fetal rat ovary. (A-B) Cox1 was mostly expressed in the blood cells (BC) of the fetal rat ovary with minimal expression in the germ cells (GC) compared to (C) glands in human endometrial tissue used as a positive control (EG). (D-F) Cox2 expression was exclusive to the germ cells of the rat fetal ovary with somatic cells (SC) and pre-granulosa cells (PG) remaining immune-negative. (G-I) Ptges was localized to all fetal ovarian cells in the rat, including GCs, SCs, and PGs. Scale bars equal 50 microns in all panels. Images are representative of staining performed on three separate e21.5 ovaries.

4.3.12 Pge2 receptor expression in the fetal rat ovary

To determine the targets of the Cox product Pge_2, its four receptors (Ep1-4) were localised in the fetal rat ovary and compared to previous data from investigation in the human fetal ovary. Ep1 expression was similar to that of expression in the human ovary, with little expression seen outside of the blood cells within vessels, although slight expression was seen in some germ cells (Figure 4.18A-B). Ep2 expression was also similar to that seen in the human fetal ovary and was localised specifically to rat fetal germ cells (Figure 4.18C-D). Ep3 is also primarily expressed by fetal germ cells, although some Ep3 expression was seen in pre-granulosal cells, (Figure 4.18E-F). Ep4, unlike the other Pge_2 receptors, was localised to the nucleus of cells (Figure 4.18G-H), with primary localisation in somatic nuclei, but expression could also be seen in select germ and pre-granulosal cells. This expression was different to that seen in the human study, where EP4 expression was membranous. These results determine Pge_2 is capable of targeting various cell types in the rat fetal ovary, including the germ cells.

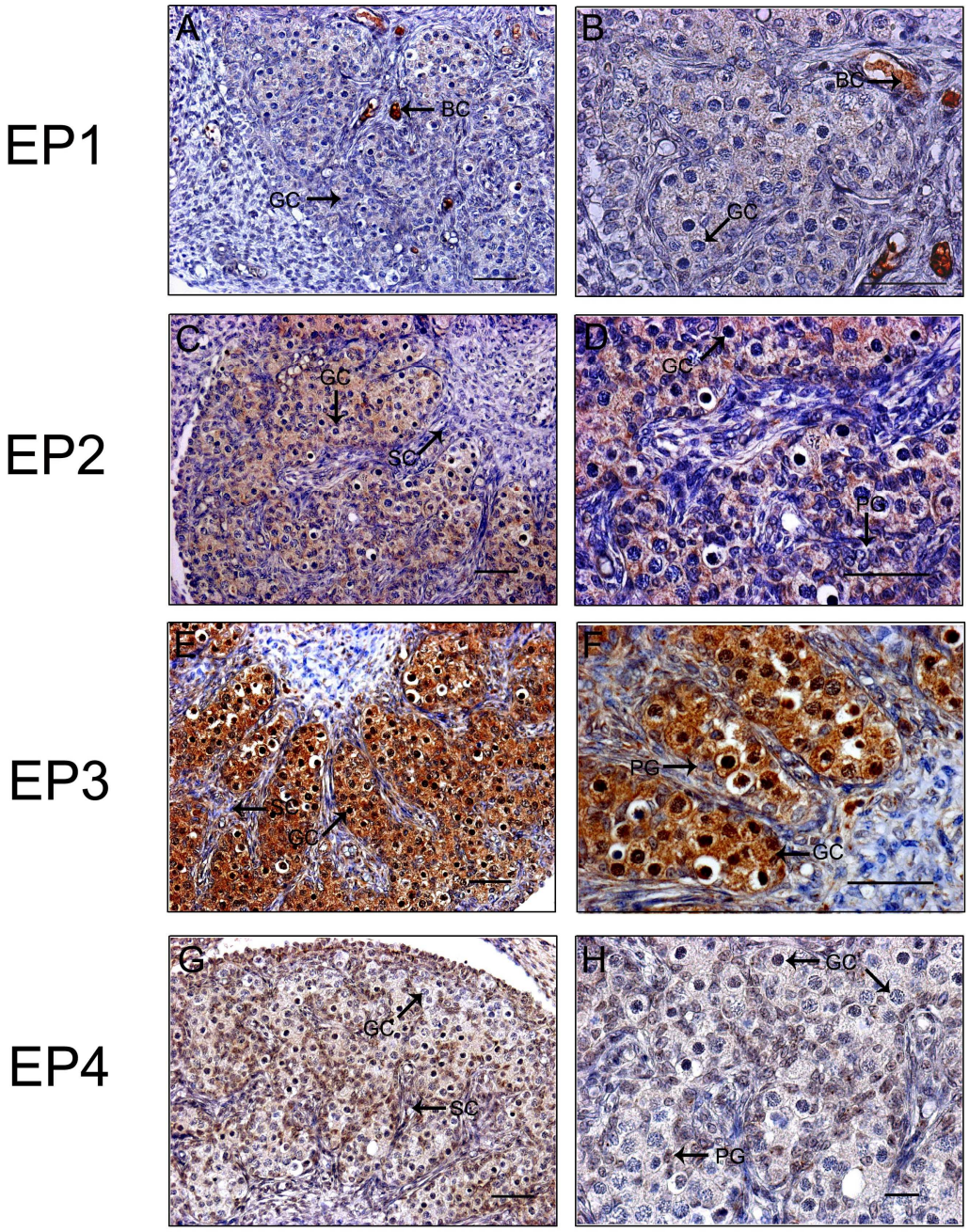

Figure 4.18 Pge_2 receptor expression in the rat fetal ovary

Ep receptors were immunolocalised in the e21.5 fetal rat ovary to identify targets of Pge_2 action. (A-B) Ep1 was expressed minimally by the germ cells (GC) but was predominately expressed by blood cells (BC) in the fetal rat ovary. (C-D) Ep2 expression was exclusive to the GC with no expression seen in somatic (SC) or pre-granulosa cells (PG). (E-F) Ep3 was localised to both the GC and PG of the germ cell nests in the rat fetal ovary, with negative SC streams. (G-H) Ep4 was localised to the nucleus of most cells in the ovary, predominately the SC, but also seen in the PG and some GC. Scale bars equal 50 microns. Images are representative of staining performed on three separate e21.5 ovaries.

4.4 Discussion

Germ cell development during fetal life is essential for adult fertility in the female. Disruption of early ovarian development can lead to premature ovarian insufficiency (POI) or infertility. Previous studies in the human fetal ovary identified PGE_2 as a possible regulator of this early germ cell development (outlined in Chapter 3 (Bayne *et al*, 2009)) but were unable to identify PGE_2 function due to the constraints of using human tissue. The rodent model of maternal paracetamol exposure utilised in this study, circumvents these issues, and provides further evidence for a role for Pge_2 in fetal ovarian development, as disruption of the Cox enzymes results in disruption of germ cell survival and maturation in female offspring. This disruption was quantified using the germ cell marker Tra-98, which is a nuclear marker similar to germ cell nuclear antigen (GCNA; (Bagheri-Fam *et al*, 2011)) which we were unable to obtain commercially. Tra-98 was utilised over other traditional germ cell markers because of its nuclear localisation, which allowed us to count both germ cell number as well as GCND using the same sections of ovary.

The reduction in germ cell number is likely to be the cause for the significantly smaller ovarian volumes observed, as no change was observed in overall body size of the treated pups. It was hypothesised this reduction in germ cell number might be the result of reduced proliferation or increased cell death. However, after quantifying markers for both proliferation and apoptosis, neither were found to be significantly altered in paracetamol treated ovaries compared to vehicle treated controls. It was further speculated that no change was seen in cell turnover at e21.5 as the alteration in germ cell number was a result of changes occurring earlier in gestation; however this theory was not supported by data collected from an earlier gestational group (e17.5). Additionally, e17.5 litters demonstrated no change in ovarian size or germ cell number with paracetamol exposure, suggesting that late gestational treatment (e17.5-e21.5) immediately before parturition in the rat is possibly a period susceptible to developmental disruption, and effects seen in e21.5 rats are due to change during this time. This hypothesis is supported by human meta-analysis data, indicated developmental effects of paracetamol and NSAIDs were heightened if exposure occurred during the second trimester in humans (Kristensen *et*

al, 2011), which is equivalent to late gestation in the rodent. However, it is also possible germ cells are lost via another unknown mechanism (eg autophagy).

It was further noted that remaining germ cells from paracetamol exposed pups at e21.5 had a reduced GCND, indicating the germ cells were developmentally delayed compared to control germ cells. However, the paracetamol treated germ cells did span the same diameters as compared to control germ cells ruling out complete arrest or specific loss of a subset of germ cells, but diameter was less synchronous in paracetamol exposed pups. These data indicated Cox products may play a role not only in germ cell survival but also maturation and possibly synchronicity. In further support of this theory, although not significant, both proliferation and apoptosis were heightened in paracetamol exposed germ cells at e21.5. These changes in cell turnover are only possible in pre-meiotic germ cells thus further suggesting the paracetamol exposed germ cells are developmentally delayed.

Based upon data collected from the human fetal ovary, it is postulated that inhibition of Pge_2 synthesis is likely to be at least one of the factors underpinning the germ cell effects seen with paracetamol exposure. To demonstrate Pge_2 is capable of targeting the germ cells in the fetal rat ovary, the Cox enzymes; Pge_2 specific enzyme, Ptges; and the Pge_2 receptors were immunolocalised in the wild-type rat fetal ovary. It is unlikely Cox1 plays a role in fetal ovarian development, as it was weakly expressed and is not greatly affected by paracetamol action (Hinz *et al*, 2008). In contrast, Cox2 was highly expressed in the germ cells of the rat fetal ovary, with variable expression between germ cells. This intense expression of Cox2 in specific germ cells may account for the partial loss of germ cells seen with paracetamol exposure, rather than a complete loss. Additionally, the Pge_2 specific synthesis enzyme Ptges is also expressed in germ cells; however, unlike in the human Ptges is also expressed in stromal and pre-granulosa cells of the rat fetal ovary. These results demonstrate that paracetamol is most likely to specifically affect Cox2 in the germ cells in the fetal ovary.

Secreted Pge2 can then signal via one of the four Ep receptors, which were found to have specific, diverging expression patterns in the rat fetal ovary, as in the human. Expression of Ep1-3 was similar to that of the human fetal ovary. Conversely, Ep4 expression was not similar to that seen in the human, with primary localisation seen in somatic cell streams with variable expression in germ and pre-granulosal cells, although all expression was nuclear in the rat, unlike the membranous expression expected from a classical GPCR. This staining is not seen in negative control tissue, and likely to be specific. Both EP4 and EP2 have been reported to translocate intracellularly in some instances to act as transcriptional activators rather than membrane bound receptors, allowing development of a positive feedback loop (Figure 4.19) (Tjandrawinata & Hughes-Fulford, 1997; Inoue *et al*, 2000; Faour *et al*, 2008; Steinert *et al*, 2009). It is possible this localisation indicates Ep4 is acting directly to regulate transcription and in turn, increase Cox2 expression and Pge_2 synthesis. In support of this theory, Ep4 is only selectively expressed in the nuclei of a subset of germ cells as is heighted expression of Cox2. Although further evidence is needed for this mechanism, these results determine direct regulation of fetal germ cells in the rat ovary by Pge_2 is likely via Ep2, 3, or 4, with possible paracrine action mediated through Ep3 or 4, as Ep1 is minimally expressed. Although these data do not provide evidence for Pge_2 inhibition as the sole mediating factor affecting germ cells in paracetamol treated ovaries, they do provide further support for the role of Pge_2 during early fetal ovarian development.

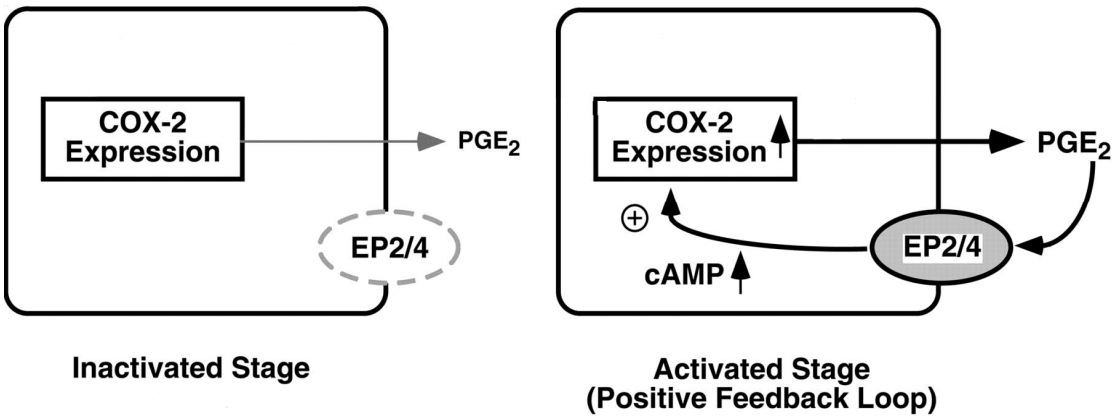

Figure 4.19 Positive feedback loop exerted via EP2/4 receptors during PGE_2 signalling.

Positive feedback loop mechanism utilized by PGE_2 via the two GPCRs EP2 and EP4. When inactivated, the cell expresses COX2 which in turn promotes secretion of PGE_2 and signaling via membrane bound GPCRs on neighboring cells. In an activated stage, the PGE_2 signals back to the cell via EP2 or 4 which then translocate inside the cell to promote sustained expression of the precursor enzyme COX2 via cAMP signaling maintaining a positive feedback system. Abbreviations: cAMP, cyclic AMP; COX2, cyclooxygenase-2; EP2/4, prostaglandin E receptor 2/4GPCR, g-coupled protein receptor; PGE_2, prostaglandin E2. Figure adapted from (Inoue *et al*, 2000).

Alternative factors possibly affected by paracetamol exposure have been suggested, including anti-androgenic signalling, as suggested in the previous studies of paracetamol exposure in male offspring (Gupta & Goldman, 1986; Gupta, 1989; Kristensen *et al*, 2011). Although no change in AGD/AGI was observed in the female pups of this study, there remains to be evidence this measure is indicative of steroid signalling in female offspring. However, male counterparts did show a significant fall in inter-testicular testosterone, as well as a reduction in AGD/AGI, consistent with a reduction in fetal androgens (data not shown). This fall in testosterone was also paired with a reduction in male germ cell number and an evident delay in maturation of germ cells with paracetamol exposure, similar to that seen in the female pups. Studies in mouse, rat and human fetal ovaries have demonstrated enzymes and receptors necessary for steroid synthesis and signalling are expressed during early ovarian development preceding primordial follicle formation suggesting these pathways are active

(Weniger, 1993; Chen *et al*, 2007b; Chen *et al*, 2009; Fowler *et al*, 2011). Further, Chen *et al*. demonstrated progesterone/estrogenic signalling is capable of regulating both germ cell nest breakdown and primordial follicle formation in the mouse (Chen *et al*, 2007b). Thus altered steroid signalling may lead to early nest breakdown which would result in germ cell loss. However, there is little evidence for this as a possible mechanism as estrogen receptor null mice do not display any disruption of fetal ovarian development and establish a normal cohort of primordial follicles (Dupont *et al*, 2000; Couse *et al*, 2001). In addition, a progesterone receptor-null model was also examined and deemed to develop phenotypically normal ovaries (Lydon *et al*, 1995). Alternatively, as in the male, testosterone might be the steroid altered by COX inhibition. Although little is known about testosterone's role in the fetal ovary, it is known that the receptor by which it signals, androgen receptor (AR), is important for ovarian function. This was determined in an AR knockout (ARKO) mouse model, which demonstrated premature ovarian insufficiency at 8 weeks of age, despite what was deemed a normal compliment of follicles after birth (Shiina *et al*, 2006). It is clear from these results, testosterone does play a role in ovarian biology; however lack of functional data regarding its role during fetal ovarian development obscures further postulation of what functions might be disrupted by COX inhibition at this time.

Another factor to be considered is maternal metabolism, with a possible metabolite of paracetamol or a change in liver response due to the additional metabolic effects of paracetamol resulting in downstream fetal effects. Paracetamol is almost entirely metabolised into other substances before being excreted (~98%), only one of these resulting metabolites, accounting for ~4% of total paracetamol metabolism, is known to be potentially toxic (Prescott, 1980). This toxic metabolite is the highly reactive alkylating agent, benzoquinoneimine, which in normal rodent and human metabolism is swiftly inactivated by conjugation with glutathione in the liver (Prescott, 1980). However, in cases of paracetamol toxicity, the amount of benzoquinonemine exceeds the glutathionine produced, allowing for the excess metabolite to bind to liver cells causing acute necrosis (Mitchell *et al*, 1977). As no liver necrosis was observed in the dams, it is thought this is not the cause of the fetal effects of paracetamol;

however, further studies investigating rate of paracetamol clearance would demonstrate if even minute paracetamol toxicity is occurring, as rate of clearance is slowed in animals experiencing paracetamol poisoning (Prescott & Wright, 1973).

Additionally, paracetamol's direct mechanism of action remains unknown, making it possible for an alternative unidentified signalling pathway (not a result of Cox inhibition) to be the cause of the developmental disruptions. In order to rule out these two possibilities, *in vitro* culture of fetal rat ovaries using the same gestational ages (e13.5-e20.5) were attempted with treatment of paracetamol and indomethacin (a COX specific inhibitor with known mechanism) (data not shown). These cultures would provide evidence for an ovarian specific effect outwith maternal and fetal metabolism; and if effects seen with indomethacin mimicked that of paracetamol, that effects were a result of Cox inhibition. However, these cultures were unsuccessful, as tissue recovered was highly distorted. This distortion was likely due to being harvested close to sex determination and/or the length of culture, as tubular structures were identified in both sexes as well as a lack of proliferating germ cells. An additional attempt was made using gonads from slightly later in gestation (e15.5) with culture treatment for a shorter time period but this too resulted in widespread apoptosis in all treatment groups.

In conclusion, although further study is required to determine if Pge2 disruption is the cause of germ cell loss and delay seen in e21.5paracetamol exposed fetal ovaries, this study has demonstrated there is an effect on fetal ovarian development with the use of the common analgesic paracetamol which inhibits the Cox enzymes upstream of Pge_2 synthesis. Additionally, components necessary for Pge_2 signalling are present in the fetal rat ovary with most components expressed in a similar localisation to that of the human fetal ovary, in which Pge_2 signalling has been demonstrated to regulate factors involved in proliferation and survival. Absence of this regulation might result in germ cell loss or developmental disruption, the two main effects seen in paracetamol exposed fetal ovaries.

Overall, these data demonstrate fetal exposure to paracetamol affects fetal germ cell number, and may thereby affect the number of primordial follicles formed. This would further affect the number of follicles available for ovulation and thus may lead

to POI in foetuses exposed to sustained paracetamol during *in utero* development. Further, investigation is necessary to determine if there are effects seen postnatally in paracetamol exposed female (and male) pups. As paracetamol is one of the most common drugs to take during pregnancy in the human, this may have relevant clinical implications (Werler *et al*, 2005). Additionally, incidence of POI, most of which is unexplained, remains high, this may further the understanding of mechanisms predisposing women to early follicle loss (Coulam, 1986).

Chapter 5

IL6-type cytokines in the human fetal ovary

Chapter 5. IL6-type cytokines in the human fetal ovary

5.1 Introduction

The IL6-type cytokines, which include IL6, leukaemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF), are well characterized regulators of the immune response. IL6-type cytokines also play key roles in the regulation of cell proliferation, differentiation and survival during embryonic development (Heinrich *et al*, 1998). LIF has a well characterized role in the maintenance of pluripotency in mouse embryonic stem (ES) cells (Williams *et al*, 1988), and consistent with this can (in the presence of basic fibroblast growth factor (bFGF) and KL) promote the conversion of PGCs into pluripotent ES cell-like Embryonic Germ (EG) cells (Matsui *et al*, 1991; Resnick *et al*, 1992). LIF can also increase isolated mouse PGC numbers *in vitro* (Pesce *et al*, 1993b; Cheng *et al*, 1994), as does OSM, CNTF, or IL6 (when cultured in the presence of the soluble isoform of its receptor) (Resnick *et al*, 1992; Cheng *et al*, 1994; Koshimizu *et al*, 1996). It has been suggested this results from an anti-apoptotic action of IL6-type cytokines on germ cells, rather than through the promotion of proliferation (Pesce *et al*, 1993a). LIF (and IL6 in conjunction with soluble receptor) also inhibits the spontaneous entry of mouse primordial germ cells into meiosis (Chuma & Nakatsuji, 2001), and has been shown to promote the primordial to primary follicle transition *in vitro* (Nilsson *et al*, 2002).

The IL6-type cytokines signal by forming dimers or trimers of various receptor components including two shared components glycoprotein-130 (gp130) and LIF receptor (LIFR) (reviewed in Heinrich *et al*, 1998 and depicted in Figure 5.1). LIF and OSM signal via heterodimeric complexes of gp130 in combination with LIFR, and additionally, OSM can signal through an alternative receptor complex consisting of gp130 and an OSM specific receptor (OSMR). CNTF utilizes a specific heterotrimeric complex of gp130, LIFR and a specific α-receptor subunit CNTF receptor (CNTFR). IL6 also signals via a trimeric complex consisting of a gp130 homodimer combined with its specific α-receptor subunit IL6 receptor (IL6R). The

α-receptor subunits (IL6R and CNTFR), although necessary for formation of specific receptor complexes, do not themselves transduce signal, whereas gp130, LIFR, and OSMR trigger phosporylation of Janus (JAK) Kinases, in order to activate the Jak/STAT signalling cascade (Heinrich *et al*, 1998). As a direct result of utilizing similar receptor complexes, the IL6-type cytokines have overlapping biological activity (Taga & Kishimoto, 1997), and this biological redundancy has hindered efforts to elucidate the function of specific ligands. Thus *in vivo* results from genetically engineered animal models with target disruptions of IL6-type ligands and receptors have not recapitulated the effects of IL6-type cytokines on germ cells *in vitro* (Stewart *et al*, 1992; Escary *et al*, 1993; Ware *et al*, 1995; Yoshida *et al*, 1996; Molyneaux *et al*, 2003a).

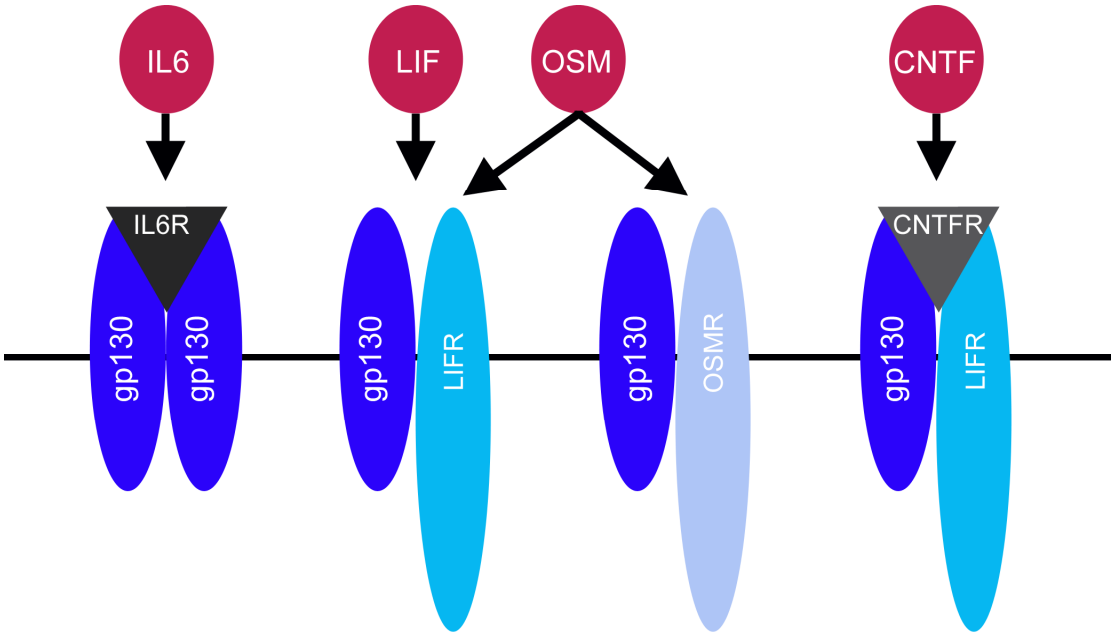

Figure 5.1 IL6-type cytokines signal via receptor complexes.

IL6-type cytokine complexes are made of shared signal transducing components (light blue) and α-receptor subunits (black and grey) that are necessary for specific ligand binding. The OSM ligand can bind a receptor complex consisting of gp130 homodimerised to LIFR or OSMR (reviewed in Heinrich *et al*, 1998).

Transcripts encoding LIF, gp130, and LIFR are expressed in the late fetal and adult human, and receptors are localized to oocytes (Abir *et al*, 2004). However, the expression of these factors during the key developmental window encompassing PGC proliferation, the initiation of meiosis and the formation of primordial follicles in the human fetal ovary has not been previously reported. We therefore examined the expression of *LIF*, *IL6*, *OSM*, and *CNTF*, as well as their receptor components in the human fetal ovary across increasing gestation from 8 to 20 weeks. We also investigated the expression and localization of gp130 and LIFR proteins to determine the cellular target(s) of IL6-type signalling, as a basis for understanding the roles that these cytokines might play *in vivo* in the human.

5.2 Materials and Methods

Tissue collection and dissection

Human fetal tissue used in the following experiments was obtained and dissected as described previously (Section 2.1), with subsequent SRY genotyping to determine sex of first trimester specimens (Section 2.3).

RNA extraction and cDNA synthesis

Sample tissue was then processed to extract RNA and subsequently synthesis cDNA for genomic analysis as previously discussed (Section 2.4).

Quantitative RT-PCR

In order to determine if mRNA transcripts encoding IL6-type cytokine ligands (IL6, LIF, OSM and CNTF) and receptors (gp130, IL6R, LIFR, OSMR) varied across gestation, qRT-PCR was performed using the ABI 7500Fast system and both Sybr Green and Taqman analysis as described in Section 2.5.

Statistical Analysis

Quantitative RT-PCR data were analysed with GraphPad Prism version 4 statistical software (GraphPad Software Inc). Gestational comparision data were analysed using one-way ANOVA, data were then either analysed as described or log-transformed for further analysis. Log-transformation was performed in instances where data sets did not fit a Gaussian distribution. The Kolmogorov-Smirnov normality test was used for this purpose.

Data were then analysed utilising the Newman-Keuls Multiple Comparison post-test to determine significant changes between gestational values. This post-test was chosen as has more strength than a Tukey post-test and the risk of type I error does not occur with only three groups (as in our study). Some data was also analysed using a post-test for linear trend, this test was performed when data looked to be in a natural order (increasing or decreasing across gestation).

Immunohistochemistry

In order to determine the cell-specific sites of IL6-type cytokine signalling, the common receptor components gp130 and LIFR were immunolocalised across gestation as outlined in Section 2.8.

5.3 Results

5.3.1 Genes encoding IL6 ligands are developmentally-regulated

To determine presence and pattern of expression of transcripts encoding IL6-type cytokines during human fetal ovarian development, we performed qRT-PCR for *IL6*, *LIF*, *OSM*, and *CNTF* across a range of gestations. Ovarian specimens were grouped into three gestational stages to broadly reflect the key developmental events of early to mid-gestation ovarian development, namely; the proliferation of undifferentiated PGCs (8-11 weeks gestation; first trimester), the formation of germ cell nests and entry of the first germ cells into meiosis (13-16 weeks; early second trimester) and on-going meiotic entry and the onset of primordial follicle formation (17-20 weeks; late second trimester).

Transcripts encoding all four ligands investigated were detected in the human fetal ovary. Expression of transcripts encoding IL6 increased by a modest but significant amount between first and early second trimester ($1.6\pm0.3 \times 10^{-4}$ vs $3.0\pm0.4 \times 10^{-4}$ relative to expression of the housekeeping gene *GAPDH*, p=0.03, n=5-6 per group) weeks gestation, but declined subsequently, such that expression at late second trimester ($1.9\pm0.2 \times 10^{-4}$ relative to *GAPDH*) was significantly lower than early second trimester (but not significantly different from first trimester (p=0.04)) (Figure 5.2A) . *OSM* expression increased significantly with increasing gestation, starting at a borderline detectible level at first trimester ($2.3\pm0.5 \times 10^{-5}$ relative to *GAPDH*), increasing at early second trimester ($9.2\pm3.1 \times 10^{-5}$ relative to *GAPDH*), and again at late second trimester ($1.9\pm0.8 \times 10^{-4}$ relative to *GAPDH*, linear trend p=0.04, Figure 5.2B). In contrast, the expression of *LIF* and *CNTF* did not change significantly across the gestational range examined (p=0.69 and 0.17 respectively, Figure 5.2C and D). Transcript levels for CNTF were variable. These data reveal transcripts encoding IL6 and OSM are developmentally-regulated but expression of *LIF* and *CNTF* is not.

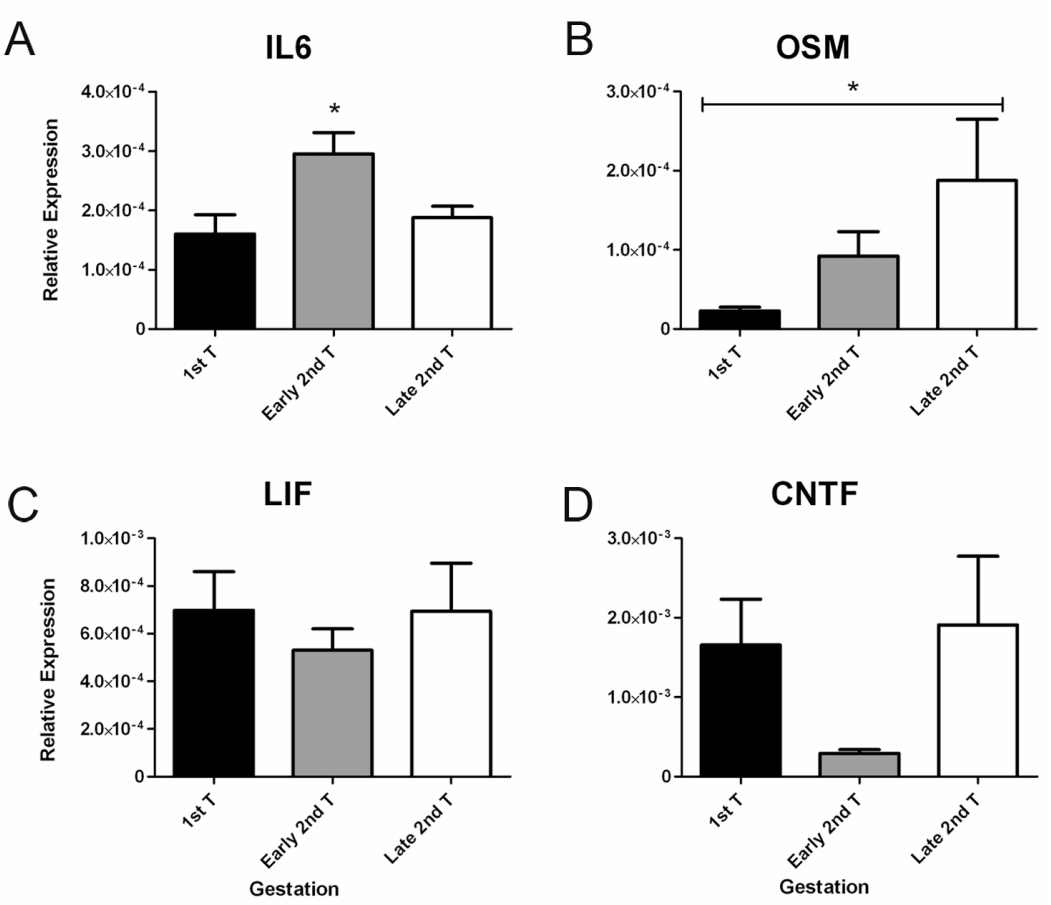

Figure 5.2 Genes encoding the IL6-type ligands are developmentally regulated in the human fetal ovary.

Genes encoding the IL6-type ligands are developmentally regulated in the human fetal ovary. Expression of the four ligands (IL6, LIF, OSM and CNTF) was analysed by qRT-PCR across gestation and split into age groups concordant with gestational events; PGC proliferation in 8-11 weeks (1st trimester), meiosis and germ cell nest formation in 13-16 weeks (early 2nd trimester), and primordial follicle formation in 17-20 weeks (late 2nd trimester); n= 4-6 samples for each gestation). (A) Expression of *IL6* increased between 1st and early 2nd trimester, and was subsequently decreased at late 2nd trimester (*=$p<0.05$). (B) *LIF* expression did not change during early ovarian development. (C) Transcript levels for OSM are significantly up-regulated across gestation (*=$p<0.05$, determined by test for linear trend). (D) *CNTF* expression was variable but no significant change across gestation was determined. Values denote mean ± standard error of the mean relative to the housekeeping gene *GAPDH*.

5.3.2 Expression of transcripts encoding IL6-type cytokine receptors are developmentally-regulated

We next examined the expression of transcripts encoding the IL6-type cytokine receptors gp130, LIFR, OSMR, and IL6R across the gestational range outlined above (n=5-6 per gestational group). Expression of transcripts encoding both of the common signalling receptor components (gp130 and LIFR) increased between first and second trimester. Messenger RNA expression of gp130 was significantly greater at early second trimester (2.1±0.3 $x10^{-2}$ relative to *GAPDH*, p=0.02) and late second trimester (2.1±0.3 $x10^{-2}$ relative to *GAPDH*, p=0.05, Figure 5.3A) than at first trimester (1.0±0.1 $x10^{-2}$ relative to *GAPDH*). The mRNA expression of LIFR displayed a similar pattern, with expression at first trimester (1.3±0.2 $x10^{-2}$ relative to *GAPDH*) significantly lower than that at both early (5.9±1.2 $x10^{-2}$ relative to *GAPDH*, p=0.01) and late second trimester (7.4±1.6 $x10^{-2}$ relative to *GAPDH*, p=0.01, Figure 5.3B). *IL6R*, which is required for the IL6 signalling complex, but does not actively signal itself, was higher at late second trimester (8.8±0.9 $x10^{-2}$ relative to *GAPDH*) compared to both first (3.5±0.6 $x10^{-2}$ relative to *GAPDH*, p=0.002) and early second trimester(1.8±0.7 $x10^{-2}$ relative to *GAPDH*, p=0.0003, Figure 5.3C). Expression of *OSMR*, which is also conducts intracellular signalling, displayed a similar trend to that of transcripts encoding gp130 and LIFR (i.e. increasing with gestation) but showed no significant changes across the gestational range examined (Figure 5.3D). These data demonstrate the two common receptor components and the IL6 specific component increase with gestation.

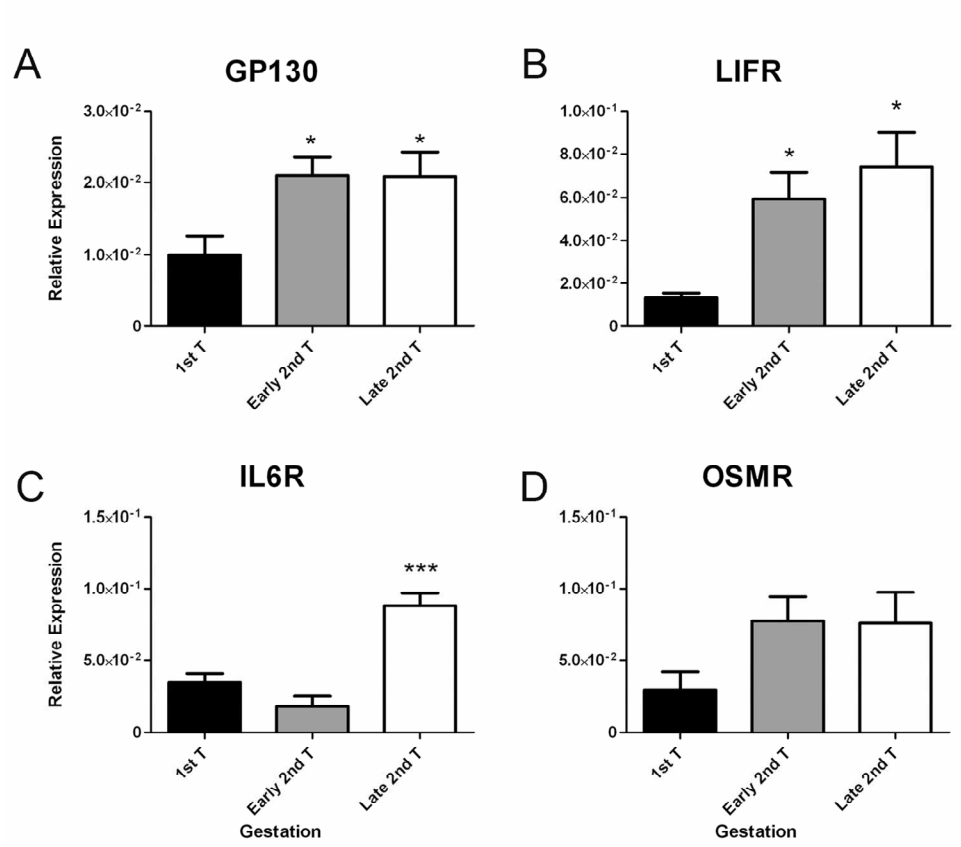

Figure 5.3 Expression of the common receptor components for the IL6-type cytokines increases with gestation in the human fetal ovary.

Expression of the common receptor components for the IL6-type cytokines increases with gestation in the human fetal ovary. Expression of receptor components necessary for IL6-type signalling was analysed by qRT-PCR across gestation. (A) Expression of *IL6ST* which encodes for gp130, was significantly higher in early and late 2nd trimester fetal ovaries than in 1st trimester samples (*=p<0.05). (B) *LIFR* showed a similar pattern of expression, with a significant increase in transcript levels at 1st trimester compared to early and late 2nd trimester (*=p<0.05). (C) *IL6R* was increased at late 2nd trimester compared to 1st and early 2nd trimester, concomitant with initiation of primordial follicle formation (***=p<0.001). (D) Although sharing a similar trend to that of gp130 and *LIFR* expression, *OSMR* expression did not change across gestation. Values denote mean ± standard error of the mean relative to the housekeeping gene *GAPDH*.

5.3.3 gp130 and LIFR are expressed exclusively by the germ cells in the human fetal ovary

To determine the cellular target(s) of IL6-type cytokine signalling in the human fetal ovary, we performed immunohistochemistry to detect the functional receptor components gp130 and LIFR across the developmental window outlined above. At 8 weeks gestation, gp130 expression was detected in germ cells, but staining was weak and sporadic and many germ cells displayed no staining (Figure 5.4A). In contrast, from 14 weeks gestation, expression was stronger and restricted to germ cells, with stromal cell streams and pre-granulosa cells not showing any expression (Figure 5.4B and C), a pattern maintained in later gestations (Figure 5.4D and E). Staining was also detected in oocytes in primordial follicles at 20 weeks gestation (Figure 5.4E inset), revealing that germ cells continue to express gp130 following nest breakdown and follicle assembly.

The expression pattern of LIFR in the human fetal ovary was similar to that of gp130, with staining detected exclusively in germ cells from 8 weeks of gestation onwards (Figure 5.5A-D). Notably, germ cells displayed variability in the intensity of LIFR staining even within close proximity (Figure 5.5E). At 20 weeks gestation, oocytes within primordial follicles expressed LIFR (Figure 5.5E inset). The expression pattern of the two shared, functional signalling components indicates that IL6-type cytokines are able to signal to germ cells in the fetal ovary; indeed germ cells appear the sole cell type involved.

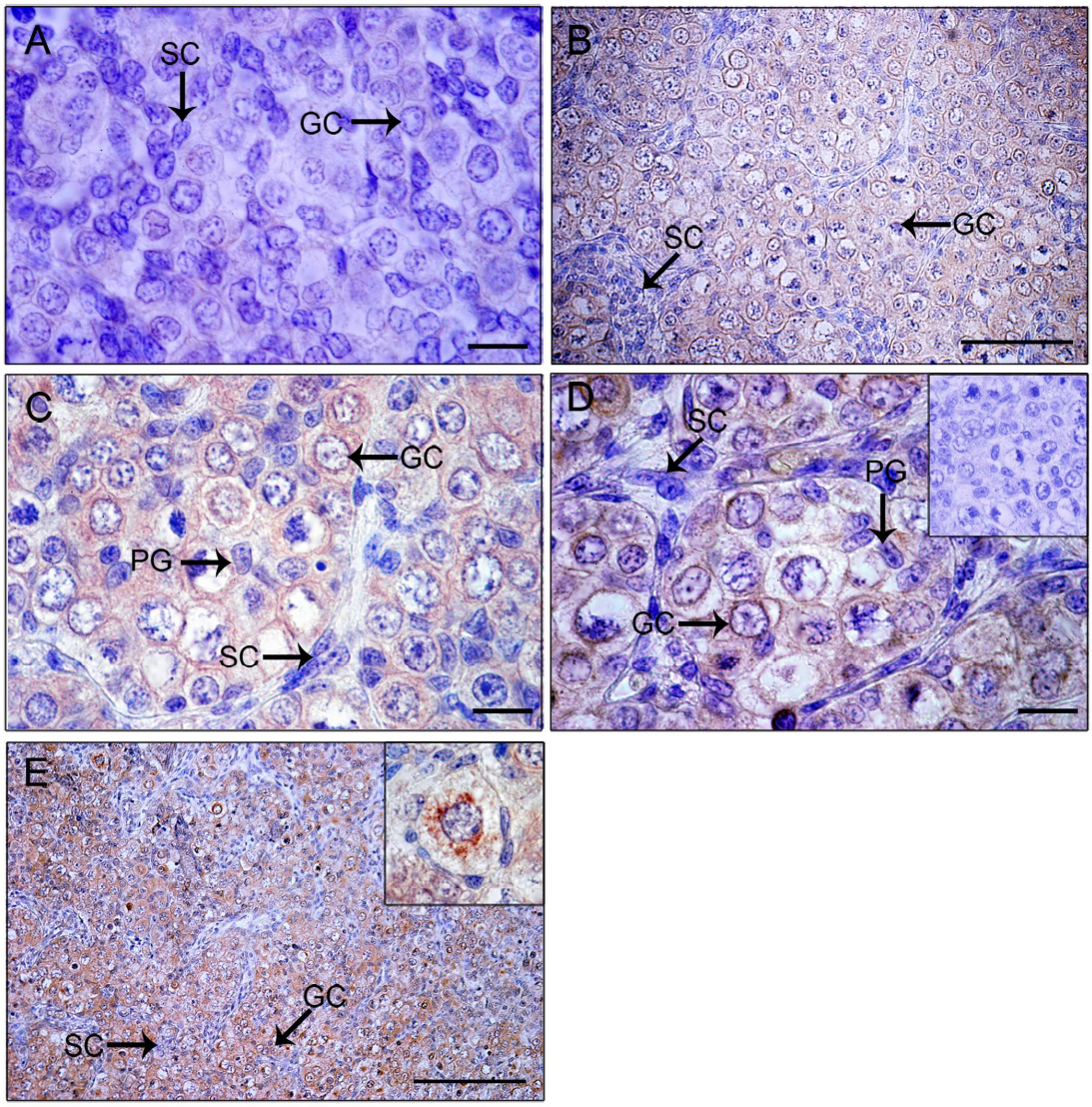

Figure 5.4 Immunohistochemical detection of gp130 in the fetal ovary.

Immunohistochemical detection of gp130 in the human fetal ovary across gestation. (A) At 8 weeks gestation, faint gp130 staining is detectable in some germ cells (GCs), but the majority show no expression. At 14 weeks (B and C) and 17 weeks (D), clear gp130 expression can be seen in the GC clusters, whilst stromal somatic cell streams (SC) and pre-granulosal cells (PG) remain immuno-negative. (D, Inset) Negative control sample, lacking primary antibody, is absent of any non-specific staining. (E) At 20 weeks gestation, gp130 staining is more distinct and with immuno-positive GCs (E Inset) including oocytes within primordial follicles. Scale bars equal 10 µm in (A) (C) and (D), 25 µm (B), and 50 µm in (E). Images are representative of staining performed on at least 2 specimens at roughly the same gestation.

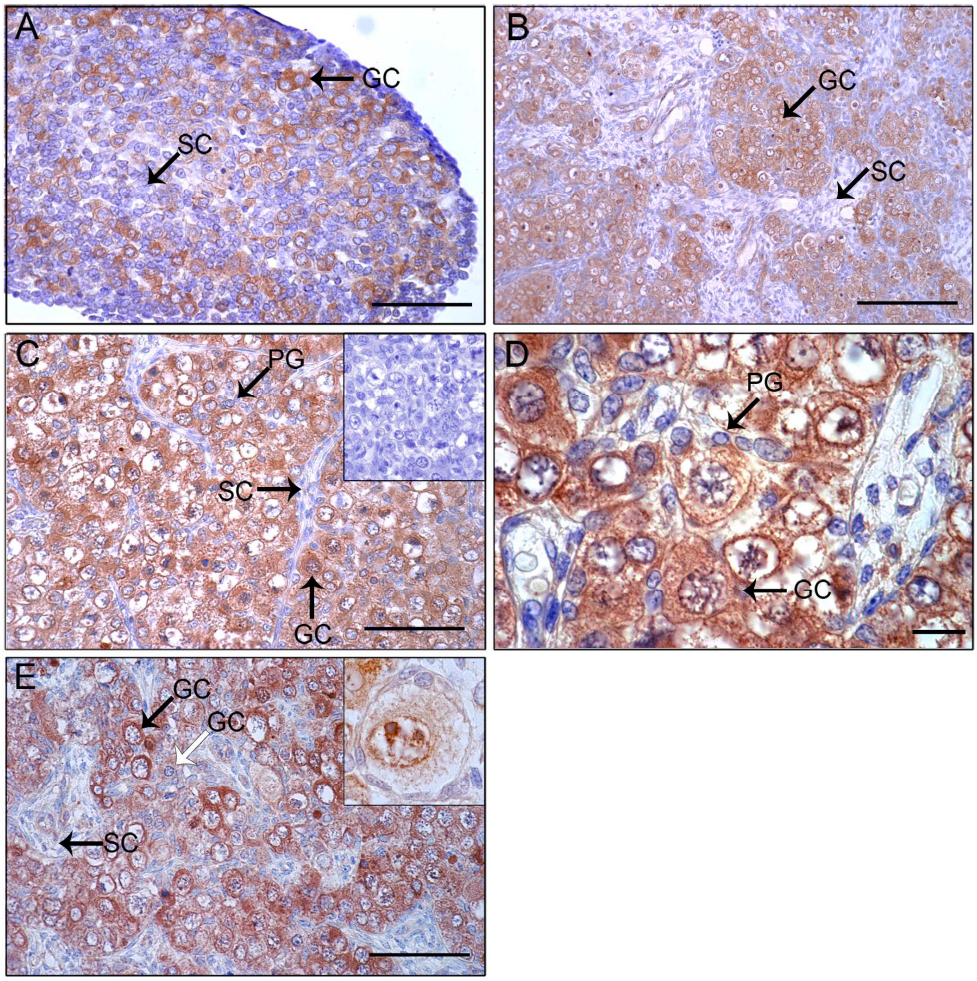

Figure 5.5 Immunohistochemical detection of LIFR in the fetal ovary

Immunohistochemical detection of LIFR, is expressed in the human fetal ovary. (A) At 8 weeks gestation, germ cell (GC)-specific staining for LIFR is detectable. (B) GC clusters at 14 (B) and 17 (C and D) weeks express LIFR, with no staining detected in stromal somatic cell streams (SC) and pre-granulosal cells (PG) interspersed within germ cell clusters. (C, Inset) No immunostaining was detected in the negative control, demonstrating antibody specificity. (E) LIFR receptor expression at 20 weeks was localized specifically to germ cells, but variations in the intensity of staining between neighbouring germ cells is apparent (white and black arrows denote germ cells with weak or strong LIFR expression respectively). (E, Inset) oocytes in primordial follicles also expressed LIFR. Scale bars equal 10 µm in D, 25 µm in (A) and (C), and 50 µm in (B) and (E). Images are representative of staining performed on at least 2 specimens at roughly the same gestation.

5.4 Discussion

Germ cell development and primordial follicle formation are vital to female fertility. Extensive *in vitro* studies have demonstrated roles for LIF and other IL6-type cytokines in the regulation of germ cell development (Matsui *et al*, 1991; Resnick *et al*, 1992; Cheng *et al*, 1994; Koshimizu *et al*, 1996; Chuma & Nakatsuji, 2001; Nilsson *et al*, 2001). However, little evidence has been provided regarding their potential roles during human fetal ovarian development. The data presented here confirm that all IL6-type cytokines previously demonstrated to regulate murine germ cell development *in vitro* (LIF, IL6, OSM, and CNTF) are expressed in the human fetal ovary, as are their common receptor components. Furthermore, the expression of the latter is confined to germ cells, revealing these cells to be the exclusive targets of IL6-type signalling in the human fetal ovary.

Expression of the genes encoding IL6-type signalling components was found to be developmentally-regulated, a finding consistent with the immunohistochemistry data also presented here. Unexpectedly, the finding that gp130 expression is low or undetectable in primordial germ cells in the first trimester human fetal ovary suggests that these cells have little or no capacity to receive and/or transduce IL6-type cytokine signals, and implies that, as in rodents (Molyneaux *et al*, 2003a), gp130 signalling activity is not required for the survival of human PGCs, or their maintenance in an undifferentiated state *in vivo*. The mRNA and protein levels of both gp130 and LIFR appear to be up-regulated in the early second trimester, however. This coincides with the entry of the first germ cells into meiosis (Gondos *et al*, 1986; Hibi *et al*, 1990), and indicates that IL6-type signalling may be important in the regulation of this critical event. As both LIF and IL6 are reported to inhibit the spontaneous initiation of meiosis of isolated mouse PGCs in culture (Chuma & Nakatsuji, 2001), it is possible that up-regulation of receptor expression functions to prevent precocious entry into meiosis, maintaining an immature cell population that can still undergo further proliferation. The absence of signalling in first trimester germ cells, and the lack of an apparent restriction of gp130 and LIFR expression to undifferentiated, pre-meiotic germ cells found at the periphery of the second trimester human fetal ovary (Anderson *et al*, 2007) casts doubt on this hypothesis.

More likely, IL6-type cytokines play a role in promoting the survival of differentiating/meiotic germ cells. Consistent with this, a modest anti-apoptotic effect was reported when LIF treatment was applied to germ cells in 3-day cultures of fetal mouse ovaries isolated from e13.5 mice (a development stage comparable to that of the early second trimester (13-16 weeks gestation) specimens reported here) (Morita *et al*, 1999). Of particular note is the striking variability in expression of LIFR in between germ cells, particularly at later gestations, similar to that we have reported recently for brain derived neurotrophic factor (BDNF) (Childs *et al*, 2010a). Given the anti-apoptotic role of IL6-type cytokines for germ cells at this developmental stage (Morita *et al*, 1999), it is possible that the germ cells expressing the highest level of LIFR may gain a selective survival advantage during the wave of oocyte death that accompanies nest breakdown and follicle formation. The increased expression of *IL6R* specifically in the late second trimester (as nest breakdown and primordial follicle formation commence) is also consistent with this hypothesis.

Expression patterns of the IL6-type ligand transcripts are more varied. No changes were detected in the expression of *LIF*, which is postulated to be involved in ovarian development (Nilsson *et al*, 2002), or *CNTF* across the gestational range examined (although the high variability in CNTF expression between specimens may mask such a change). Each of these ligands utilizes the same LIFR/gp130 receptor complex, however, only *OSM*, displays increasing expression with gestation similar to that of the two receptor components. The expression of both *IL6* and its specific receptor *IL6R* are also increased in second trimester, albeit at slightly different developmental stages. Together these data demonstrate that multiple components of the IL6-type cytokine signalling machinery are up-regulated in the second trimester human fetal ovary, suggesting potential roles for these factors in the later stages of germ cell development and primordial follicle formation.

Signalling by IL6-type cytokines has been reported to interact with other key signalling pathways that regulate mammalian ovarian development. OSM up-regulates the growth factor bFGF *in vitro* (Wijelath *et al*, 1997), which promotes primordial follicle formation (Nilsson *et al*, 2001). Similarly, KL expression is

regulated by LIF in rodent ovarian culture and is able to activate differentiation and promote survival in germ cells (Nilsson *et al*, 2002). It has thus been proposed that bFGF, KL, and the IL6-type cytokines have similar functions and work synergistically *in vivo* in the ovary (Kezele *et al*, 2002).

In addition to interactions with bFGF and KL, the IL6-type cytokines have been shown to interact in non-reproductive organs with cytokines known to regulate human ovarian development. LIF and BMP2, signalling through STAT3 and SMAD1 respectively, are known to act synergistically in promoting the differentiation of astrocytes from cultures of fetal neuronal precursor cells (Nakashima *et al*, 1999). We have recently reported increased expression of *BMP2* and *SMAD1* in the second trimester human fetal ovary, and identified germ cells to be the exclusive targets of BMP signalling in this tissue (Childs *et al*, 2010b); thus the apparatus for such a synergistic interaction between IL6-type cytokine and BMP signalling also exists in human fetal ovarian germ cells. LIF also acts synergistically with the neurotrophin BDNF to promote cell survival and proliferation in the developing rodent brain (Marzella *et al*, 1999). This is similar to the known role for BDNF in the rodent ovary (Spears *et al*, 2003; Paredes *et al*, 2004; Childs *et al*, 2010a).

To date, animal models have failed to determine *in vivo* roles for the IL6-type cytokines during ovarian development. Female $Lif^{-/-}$ mice do manifest an infertility phenotype, but as a result of an implantation defect, rather than a disruption of germ cell development (Stewart *et al*, 1992; Escary *et al*, 1993). Successful ovulation of viable oocytes was confirmed, but quantity of follicles and long term fertility were not examined. Interestingly, LIF-deficient mice display a significant loss of stem cell populations in the spleen and bone marrow (Escary *et al*, 1993). To bypass the difficulty of IL6 ligand redundancy, mouse models with targeted disruptions of the genes encoding the gp130 and LIFR receptors have been generated, however, both models were either embryonically or early postnatally lethal (Ware *et al*, 1995; Yoshida *et al*, 1996), precluding a detailed analysis of their fertility. A mouse model in which gp130 is conditionally disrupted only in germ cells ($gp130^{\Delta/\Delta}$) has been also

been generated (Molyneaux *et al*, 2003a). Embryonic day (e) 13.5 gp130$^{\Delta/\Delta}$ males showed significantly fewer PGCs, but mature males were fertile. Embryonic females had no difference in germ cell number; adult homozygous recessive females were infertile or produced small litters due to defects in ovulation or late oocyte maturation. In addition gp130$^{\Delta/\Delta}$ females also had fewer primary follicles, but no difference in the number of primordial follicles.

Although this study does not explore the possible functions of the IL6-type cytokines, it does confirm they signal solely to the germ cells in the human fetal ovary at the gestations examined. Both the shared signalling components gp130 and LIFR, one or both of which are necessary for formation of all IL6-type signalling complexes, were immunohistochemically localized in germ cells and primordial follicles, suggesting any role the IL6-type cytokines would be via direct regulation of the germ cells. Previous reports regarding LIF localization in the human ovary suggest this is via juxtacrine signalling from pre-granulosa stromal cells or autocrine signalling from the germ cells themselves (Abir *et al*, 2004).

In conclusion, these data demonstrate that IL6-type cytokines are expressed in the human fetal ovary during germ cell development, and that germ cells are the sole target of IL6-type action in the fetal ovary. Additionally, transcripts for both ligands and receptors are developmentally regulated, increasing with gestation, suggesting that the IL6-type cytokines are important for *in vivo* development of the human ovary, predominantly regulating the transition of differentiating germ cells from the formation of germ cell nests, through the entry into meiosis and the formation of primordial follicles.

Chapter 6

Prokineticins in human fetal ovarian development

Chapter 6. Prokineticins in fetal ovarian development

6.1 Introduction

PROK1 and 2 are recently described proteins that are well characterised for their role in regulation of angiogenesis and inflammation ((Sales & Jabbour, 2003b; Ngan & Tam, 2008) as fully described in Chapter 1). The PROK ligands signal interchangeably via two GPCR receptors (PROKR1 and PROKR2; (Lin *et al*, 2002; Soga *et al*, 2002); Figure 6.1), and function similarly to vascular endothelial growth factor (VEGF) but are restricted to steroidogenic organs (adrenals, placenta, gonads, etc).

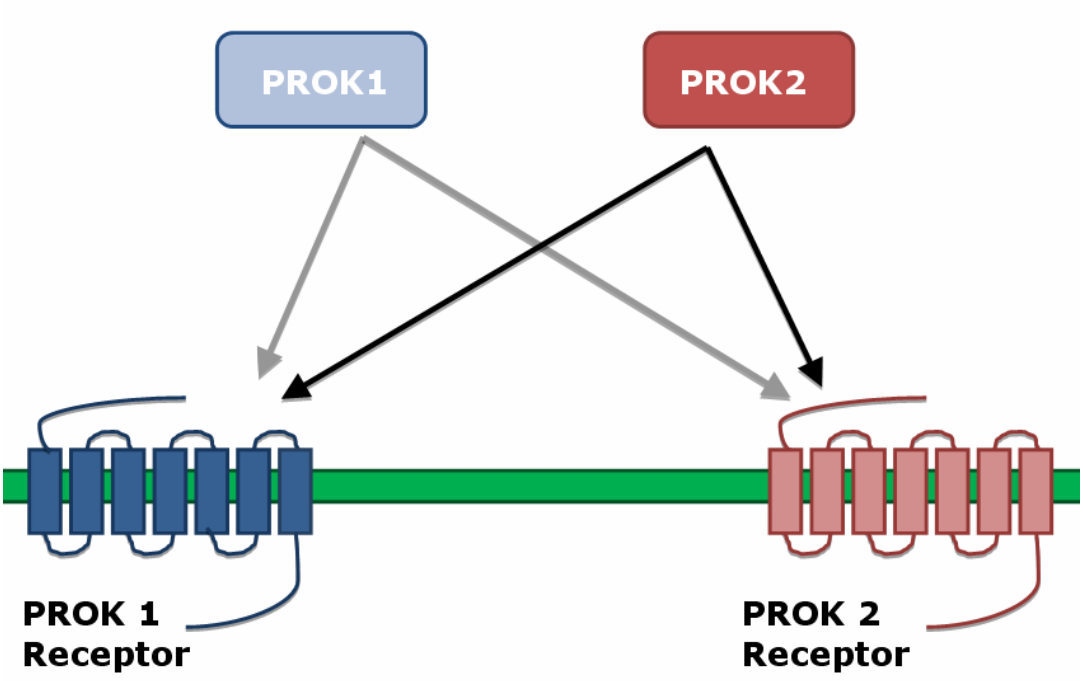

Figure 6.1 PROK signaling components

PROK1 and 2 signal via two shared g-coupled protein receptors (GPCRs); PROK receptor 1 (PROKR1) and 2 (PROKR2). Although the ligands are able to signal interchangeably, PROK2 has a higher affinity for both receptors compared to PROK1.

Angiogenesis is a key process in the cyclical activity of the normal adult ovary, as well as ovarian disorders including polycystic ovary syndrome (PCOS) and ovarian cancer (Bamberger & Perrett, 2002; Ferrara *et al*, 2003). For this reason the PROKs have been characterised in the adult ovary. In the human ovary, PROK1 mRNA is expressed highly in granulosa cells surrounding primordial and primary follicles, but expression declines sharply as the follicles mature to form secondary and antral follicles (Ferrara *et al*, 2003). PROK1 is also highly expressed in the remaining theca of atretic follicles and for this reason it has been suggested to function in remodeling and proliferation required for ovarian repair post-ovulation (Ferrara *et al*, 2003). In support of this hypothesis, studies in a bovine model have also suggested a role for PROK1 in proliferation and survival of cells within the corpus luteum (Kisliouk *et al*, 2003; Fraser *et al*, 2005).

Little is known about the other PROK signaling components in the ovary. It has been confirmed the two PROK receptors are expressed in the adult human ovary, however further localisation has not been performed (Lin *et al*, 2002; Soga *et al*, 2002). Additionally, PROK2 mRNA was not detectible in the human adult ovary in previous studies (Ferrara *et al*, 2003). Interestingly, homozygous deletion of the PROKR2 gene in a rodent model results in severe atrophy of the reproductive system including the ovary (Matsumoto *et al*, 2006; Pitteloud *et al*, 2007). However, it was determined that this was not due to direct action on the reproductive tract, rather via PROKR2's role in GnRH neuron development in the hypothalamus. Further, the *PROKR2*$^{-/-}$ phenotype is distinctly similar to that of humans with Kallmann syndrome, and subsequent studies have identified PROK2 and PROKR2 mutations in several Kallmann patients (Dode *et al*, 2006).

Further characterisation studies of the PROKs and their role in the human fetal ovary during development have not been performed. However, two recent genetic array studies have examined the PROKs in the fetal ovary, with intriguing results. One transcriptome study suggested an increased role for PROK1 in the fetal mouse ovary compared to the fetal testis, as a result of increased transcript expression (Houmard *et al*, 2009). Another study detected developmentally-regulated expression of PROK2 in the fetal ovary (Fowler *et al*, 2009).

Outwith the ovary PROK1 has been demonstrated to regulate several factors known to be expressed and/or involved in ovarian development including LIF, the IL6-type ligand and COX2 the prostaglandin synthase (Evans *et al*, 2008; Evans *et al*, 2009). With this data in mind, it was hypothesised that PROKs might play a role in regulating these factors, as well as others to promote proliferation, differentiation and possibly angiogenesis during the development of the fetal ovary. In order to determine further PROK function, the two PROK ligands and their shared receptors were characterised across early gestation in the human fetal ovary. Functional investigation was then performed utilising the TCam-2 cell line, generated from adult (35 yrs) human testicular germ cell tumour (Mizuno *et al*, 1993).

TCam-2 cells expresses several pluripotency and developmental genes similarly to PGCs (Eckert *et al*, 2008; Young *et al*, 2011; Childs *et al*, unpublished) (Figure 6.2). These PGC markers include NANOG, OCT4, KIT, and VASA, which are classically associated with early germ cells (roughly comparable to the first trimester specimens utilised in this study (8-12 weeks)). The later germ cell marker DAZL, which is increasingly expressed in second trimester human fetal ovary (Anderson *et al*, 2007), is not detected in the TCam-2 cells at either the transcript or protein level supporting the comparability of these cells to PGCs. The TCam-2 line was utilised for further functional exploration of possible connections between PROK signalling and the other factors discussed in this thesis, as well as regulators known to be involved in germ cell proliferation and development.

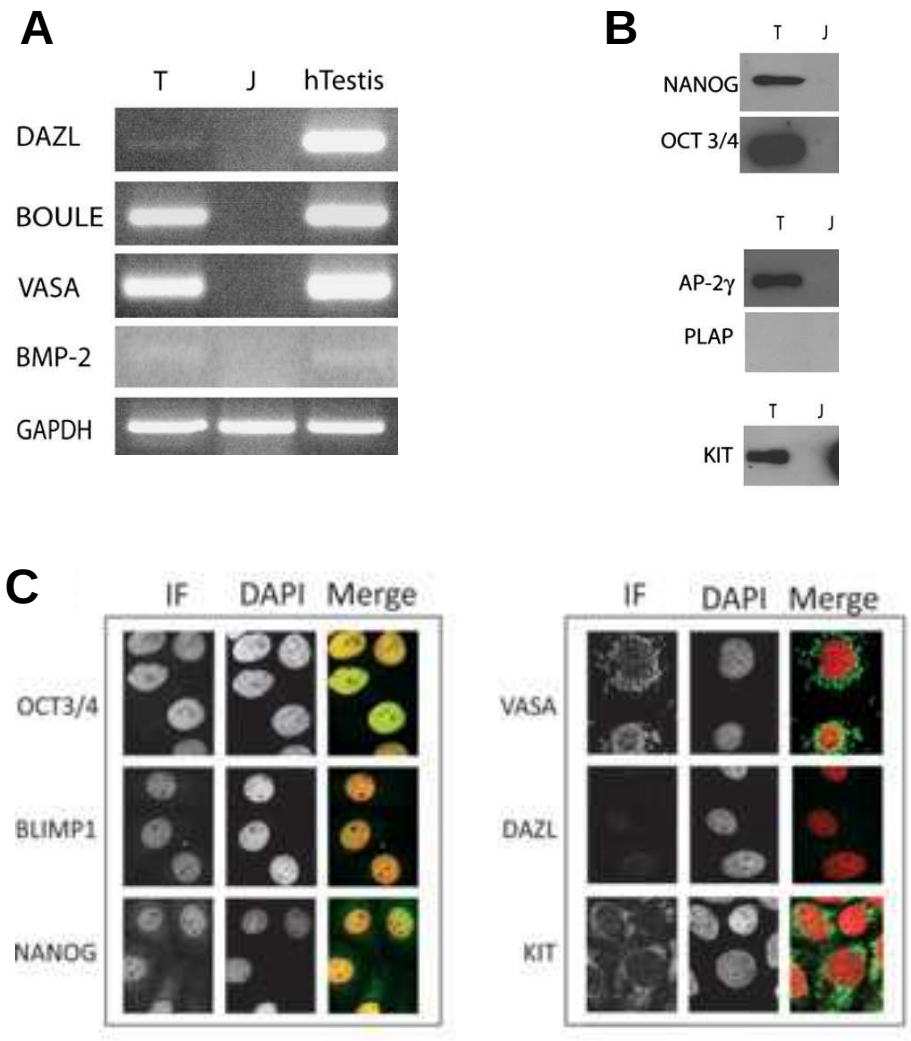

Figure 6.2 Previous characterization of the TCam-2 cell line

TCam-2 cells (T) express several pluripotent and early germ cell markers similar to primordial germ cells. (A-B) RT-PCR comparison of several factors with another seminoma line JKT-1 (J) and human testis (hTestis) demonstrate the TCam-2 expression profile closely mimics that of early germ cells. (C) TCam-2 cells also express pluripotent/germ cell markers at the protein level, as determined via immuno-fluorescence. Figure compiled (A) from (Childs *et al*, unpublished) (B) (Eckert *et al*, 2008) (C) (Young *et al*, 2011).

6.2 Materials and Methods

Tissue collection

Human fetal tissue used in the following experiments was obtained and dissected as described previously (Section 2.1), with subsequent SRY genotyping to determine sex of first trimester specimens (Section 2.3).

RNA extraction and cDNA synthesis

Sample tissue was then processed to extract RNA and subsequently synthesis cDNA for genomic analysis as previously discussed (Section 2.4).

Quantitative RT-PCR

In order to determine if mRNA transcripts encoding PROK signalling targets (PROK1, PROK2, PROKR1 and PROKR1) expression varied across gestation, qRT-PCR was performed using the ABI 7500Fast system and both Sybr Green and Taqman analysis as described in Section 2.5.

Quantitative RT-PCR analysis of PROKR1 transfected T-Cam2 cells was also performed using the ABI 7900HTFast system and Sybr Green analysis to determine gene expression changes in possible PROK targets (LIF, OSM, IL6, CNTF, PTGES, ID3, BDNF, MCL-1) and Taqman analysis was also performed for targets with primer/probe sequences (COX2), details of this protocol are also outlined in Section 2.5.

Immunohistochemistry

Human fetal ovarian samples were used to localise PROK ligands (PROK1 and 2) and receptors (PROKR1 and 2) to determine cell-specific sites of PROK synthesis and action. Protocols are described in detail in Section 2.8.

PROKR1 transfected T-Cam2 cell cultures

T-Cam2 cells stably transfected with the PROKR1 receptor were created as described in previously in Section and cultured in media as described in Section 2.13. Cells were cultured under standard procedures as described, and plated for treatment with vehicle control or 40nM PROK1 over a 24 hour time course, with cells harvested at 0, 2, 4, 6, 8, 12, and 24 hours after treatment to determine down-stream gene regulation. Cell cultures were collected in RLT buffer following culture and snap frozen for further RNA extraction and cDNA synthesis.

Statistical Analysis

Quantitative RT-PCR data were analysed with GraphPad Prism version 4 statistical software (GraphPad Software Inc). Gestational comparision data were analysed using one-way ANOVA, data were then either analysed as described or log-transformed for further analysis. Log-transformation was performed in instances where data sets did not fit a Gaussian distribution. The Kolmogorov-Smirnov normality test was used for this purpose.

Data were then analysed utilising the Newman-Keuls Multiple Comparison post-test to determine significant changes between gestational values. This post-test was chosen as has more strength than a Tukey post-test and the risk of type I error does not occur with only three groups (as in our study). Some data was also analysed using a post-test for linear trend, this test was performed when data looked to be in a natural order (increasing or decreasing across gestation).

Gene expression changes identified using qRT-PCR after PROK1 treatment of T-Cam2 cells were also analysed using GraphPad Prism as above. Data were analysed using paired t-tests comparing PROK1 treated cells to dH_2O treated cells (as the appropriate control).

6.3 Results

6.3.1 PROK signalling components are developmentally regulated in the human fetal ovary

To determine presence and pattern of expression of transcripts encoding PROK signalling components during human fetal ovarian development, qRT-PCR was performed for the two PROK ligands (*PROK1* and *2*) and the two PROK receptors (*PROKR1* and *2*) across a range of gestations. Ovarian specimens were grouped into three gestational stages, as in previous chapters to reflect broadly key developmental events; the proliferation of undifferentiated PGCs (8-11 weeks gestation; first trimester), the formation of germ cell nests and entry of the first germ cells into meiosis (13-16 weeks; early second trimester) and on-going meiotic entry and the onset of primordial follicle formation (17-20 weeks; late second trimester).

Transcripts encoding PROK ligands and receptors were expressed in the human fetal ovary across all gestations investigated. Expression of *PROK1* increased significantly in late second trimester compared to both first and early second trimester (2.0±0.4 vs 0.4±0.1 and .9±0.3 $x10^{-2}$ expression relative to the housekeeping gene *GAPDH*, p=0.02 and 0.05 respectively, Figure 6.3A). *PROK2* also increased with gestation, with a significant increase in expression between first and early second trimester (0.1±0.03 vs 0.6±0.2 $x10^{-6}$ relative to *GAPDH*, p=0.02). A further significant increase in *PROK2* expression was demonstrated at late second trimester compared to both first and early second trimester (1.2±0.2 vs 0.1±0.03 and 0.6±0.2 $x10^{-6}$ relative to *GAPDH*, p=0.0001 and 0.03 respectively, Figure 6.3B). *PROKR1* displayed a similar expression pattern to that of *PROK1* with a significant increase specifically in late second trimester in comparison to both first and early second trimester (1.8±0.3 vs 0.7±0.1 and 0.8±0.1 $x10^{-7}$ relative to *GAPDH*, p=0.008 and 0.007 respectively, Figure 6.3C). Conversely, no change gestational change in expression was determined for *PROKR2* (p=0.4, Figure 6.3D). These data demonstrate that both ligands and the PROKR1 receptor are developmentally regulated in the human fetal ovary.

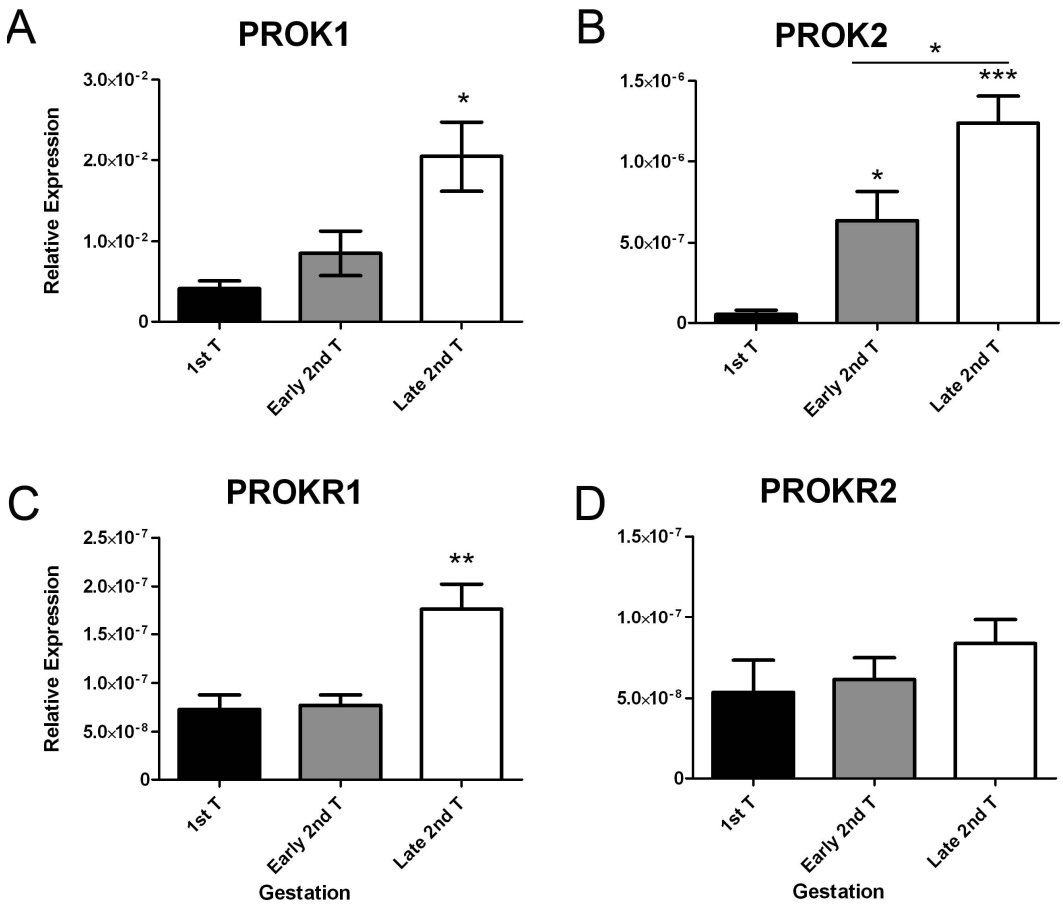

Figure 6.3 PROK ligand and receptor expression is up-regulated across fetal ovarian development.

Expression of the two ligands (*PROK1* and *2*) and their two shared receptors (*PROKR1* and *2*) was analysed by qRT-PCR across gestation and split into age groups; 8-11 weeks (1st T), 14-16 weeks (Early 2nd T), and 17-20 weeks (Late 2nd T; n= 4-6 samples for each gestation). (A) Expression of PROK1 increased at late 2nd trimester compared to both 1st and early second trimester. (B) PROK2 expression was up-regulated significantly at each gestation, with late 2nd trimester significantly increased compared to both 1st and early 2nd trimester. (C) PROKR1 was significantly up-regulated specifically in late 2nd trimester, but (D) no change was seen in PROKR2 expression. All data are expressed relative to the housekeeping gene *GAPDH*. Significance is determined by one-way ANOVA with Newman-Keuls post-test (p*<0.05, p**<0.01, and p*** <0.001).

6.3.2 PROK ligands localised to the germ cell nests in developing human fetal ovaries

Further characterisation of the PROK ligands was performed via immunohistochemical localisation in the human fetal ovary across a range of gestations. Expression was barely detectible for both ligands in first trimester (data not shown), with increased expression seen in second trimester. PROK1 expression was germ cell-specific, with pairs or groups of germ cells staining intensely compared to other germ cells within the same nest (Figure 6.4A-C). This expression pattern was seen throughout second trimester, with primordial follicles also demonstrating positive PROK1 expression (Figure 6.3 A inset), although not as intense as seen in the earlier germ cells). PROK2 expression was weaker than that of PROK1 with weak expression seen in germ cell nests, including both germ and pre-granulosa cells (Figure 6.4D-F). This expression pattern was seen across second trimester, although definitive staining in primordial follicles was not seen. Negative control tissue incubated without primary antibody confirmed the specificity of the PROK staining, with no positive immunostaining determined (Figure 6.4D inset). These results confirm the protein expression of the PROK ligands mimics that of mRNA, with increasing expression with gestation. The specific intensity of PROK1 in pairs or groups of germ cells suggests it might play a heighted role in these cells.

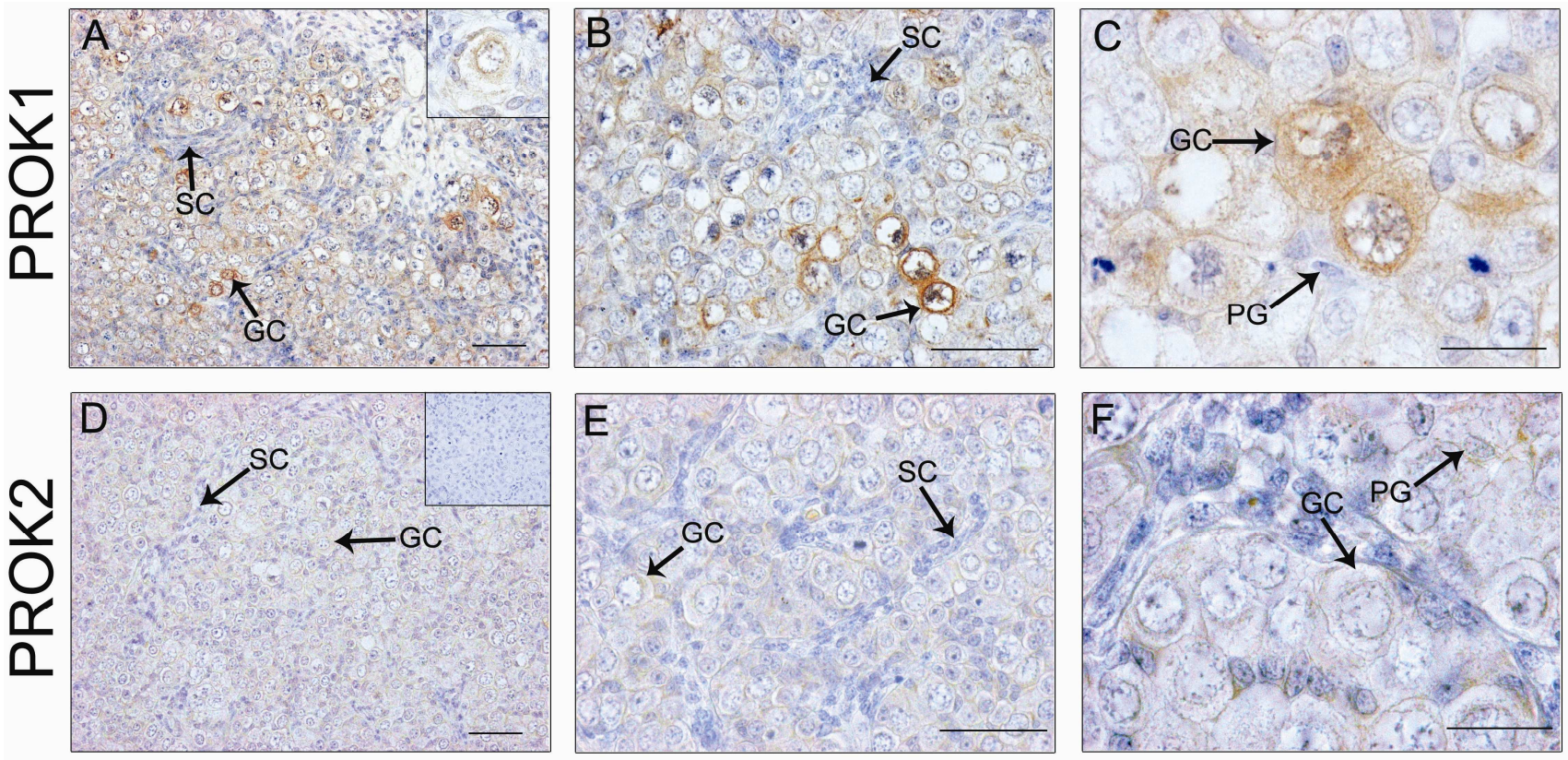

Figure 6.4 PROK ligands localised to the germ cell nests in the human fetal ovary

PROK1 and 2 were immunolocalised in the human fetal ovary (14 weeks) using 3,3′-diaminobenzidine tetrahydrochloride staining (brown). (A-C) PROK1 expression was germ cell (GC) specific in the human fetal ovary, with pairs or multiple closely associated germ cells displaying heightened PROK1 expression compared to others nearby. (A inset) PROK1 was also seen in primordial follicles (17 week). (D-F) PROK2 expression was considerably weaker than PROK1 with staining localised to both germ cells and pre-granulosal cells (PG) within germ cell nests, with no staining seen in stromal cell streams (SC). (D inset) No immunolocalisation was seen in negative control tissue in which primary antibody was omitted. Sections were counterstained with hematoxylin and scale bars equal 50 microns in A,B,D, and E; and 20 microns in C and F. Images are representative of staining performed on at least three specimens at roughly the same gestation.

6.3.3 PROK receptors expressed by germ cells of the fetal ovary

In order to determine the targets of PROK action in the human fetal testis the PROK receptors were immunohistochemically located in human fetal ovarian tissue. PROKR1 was found to be strongly expressed by the germ cells of the fetal ovary, with no staining seen in somatic cell streams or in negative control tissue incubated without primary antibody (Figure 6.5A). Upon closer examination it was apparent that the blood vessels of the human fetal ovary were also positive for PROKR1 (in keeping with its role in angiogenesis) as were some pre-granulosa cells (Figure 6.5B). PROKR2 protein was less distinctly stained, in keeping with lower PROKR2 mRNA compared to PROKR1 (Figure 6.5C). However, staining was apparent in germ cells, with immuno-negative somatic and pre-granulosa cells (Figure 6.5D). These data suggests PROK signalling primarily targets the germ cells of the human fetal ovary, with additional targeting of the blood vessels by PROKR1.

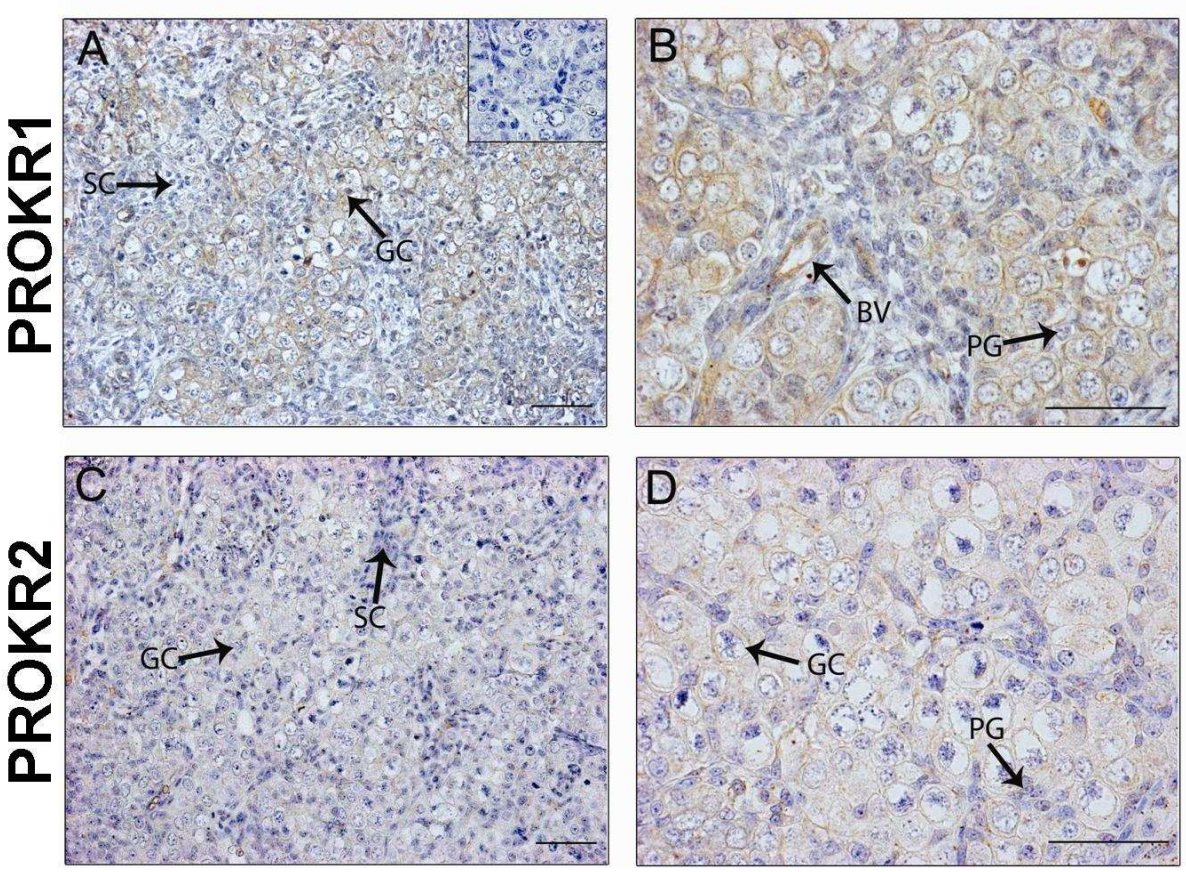

Figure 6.5 PROK receptor expression in the human fetal ovary

PROK receptors were immunolocalised in human fetal ovarian tissue (14wk) using 3,3′-diaminobenzidine tetrahydrochloride staining (brown). (A-B) PROKR1 is primarily expressed by fetal germ cells (GC) as well as blood vessels (BV) in the fetal ovary, with some staining of pre-granulosa cells (PG). However, no somatic cell (SC) streams were immuno-negative, as was negative control tissue (A-inset). (C-D) PROKR2 was not intensely expressed by the human fetal ovary, but was apparent in the germ cells. Sections were counterstained with hematoxylin and scale bars equal 50 microns. Images are representative of staining performed on at least three specimens at roughly the same gestation.

6.3.4 T-Cam2 cells stably transfected with PROKR1 as a model for fetal germ cells

As germ cells were determined as the primary target of PROK action, a cell model was created for further investigation of PROK regulation. The TCam-2 cell line have been characterised as a model of fetal germ cells (Young *et al*, 2011), and thus were utilised for this purpose. The cells were initially characterised for expression of the PROK ligands and receptor, but were found to express the PROK receptors at much lower levels than was seen in either the human fetal testis or ovary (as demonstrated later in figure 6.7). For this reason, the TCam-2 cell line was stably transfected with the PROKR1 receptor, as similarly to what was performed previously in the Ishikawa cell line to investigate PROK signalling in endometrial carcinoma (Evans *et al*, 2008).

Initial transfection was performed with both supercoiled and linearised plasmid, as it was unclear which protocol would be most successful in the TCam-2 cells (Figure 6.6A). Positive control plasmid, containing green fluorescent protein (GFP), was simultaneously transfected in order to visually determine positive colonies, which were only seen in cells transfected with supercoiled plasmid (Figure 6.6B-C). These cells were further characterised for continued experimentation.

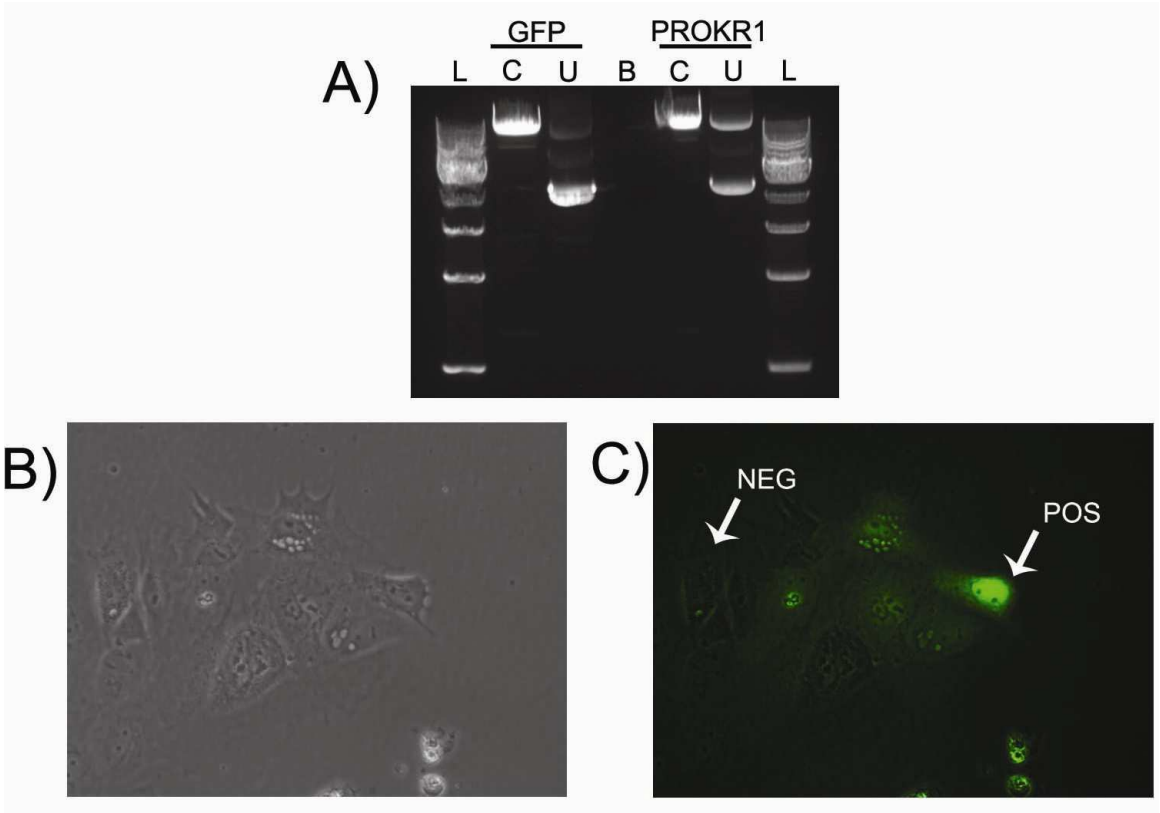

Figure 6.6 Transfection of TCam-2 cells with PROKR1

(A) Plasmid containing the PROKR1 gene or GFP was enzymatically cut (C, linearised) or left uncut (U, supercoiled) for transfection of the TCam-2 cell line, and run via electrophoresis to detect successful linearisation as determined by a single high molecular weight band. Uncut plasmid was run alongside, displaying a high molecular weight band (spontaneous linearisation) along with two other bands demonstrating circular and supercoiled plasmid. GFP transfected positive control cells were examined 24 hours post transfection to determine successful transfection. (B) Darkfield microscopy depicting a colony of transfected T-Cam2 cells. (C) The same colony via fluorescent microscopy depicting successful transfection in the form of positively transfected cells (pos) compared to cells negative for GFP (neg).

6.3.5 Characterisation of PROKR1 TCam-2 cells

Before further experimentation, the PROKR1 TCam-2 line was fully characterised to ensure successful transfection. RT-PCR confirmation of increased transcription of *PROKR1* in transfected TCam-2 cells (Figure 6.7A), was further supported by qRT-PCR comparison of PROKR1 TCam-2 cells with untransfected TCam-2 cells, PROKR1 transfected Ishikawa cells and primary human gonadal tissue (Figure 6.7B). Increased expression in TCam-2 cells was also seen at the protein level, as determined via immuno-blotting (Figure 6.7C); this was comparable to levels seen in primary tissue. A second band was seen in the immuno-blot, approximately double the size expected for PROKR1. Although homodimerisation of PROKR2 has been confirmed (Marsango *et al*, 2010), it has only been hypothesised for PROKR1, although this is the likely cause of the larger band, also seen in testis extracts demonstrating the band is physiologically relevant and not an artefact of the cell line. These data determine the transfected TCam-2 cells express *PROKR1* at a heightened level compared to untransfected cells at both the transcript and protein level. This characterization also demonstrates that although *PROKR1* transcript expression in the PROKR1 transfected T-Cam2 line is increased compared to primary tissue, and the protein level expression is comparable to that seen in human fetal gonads.

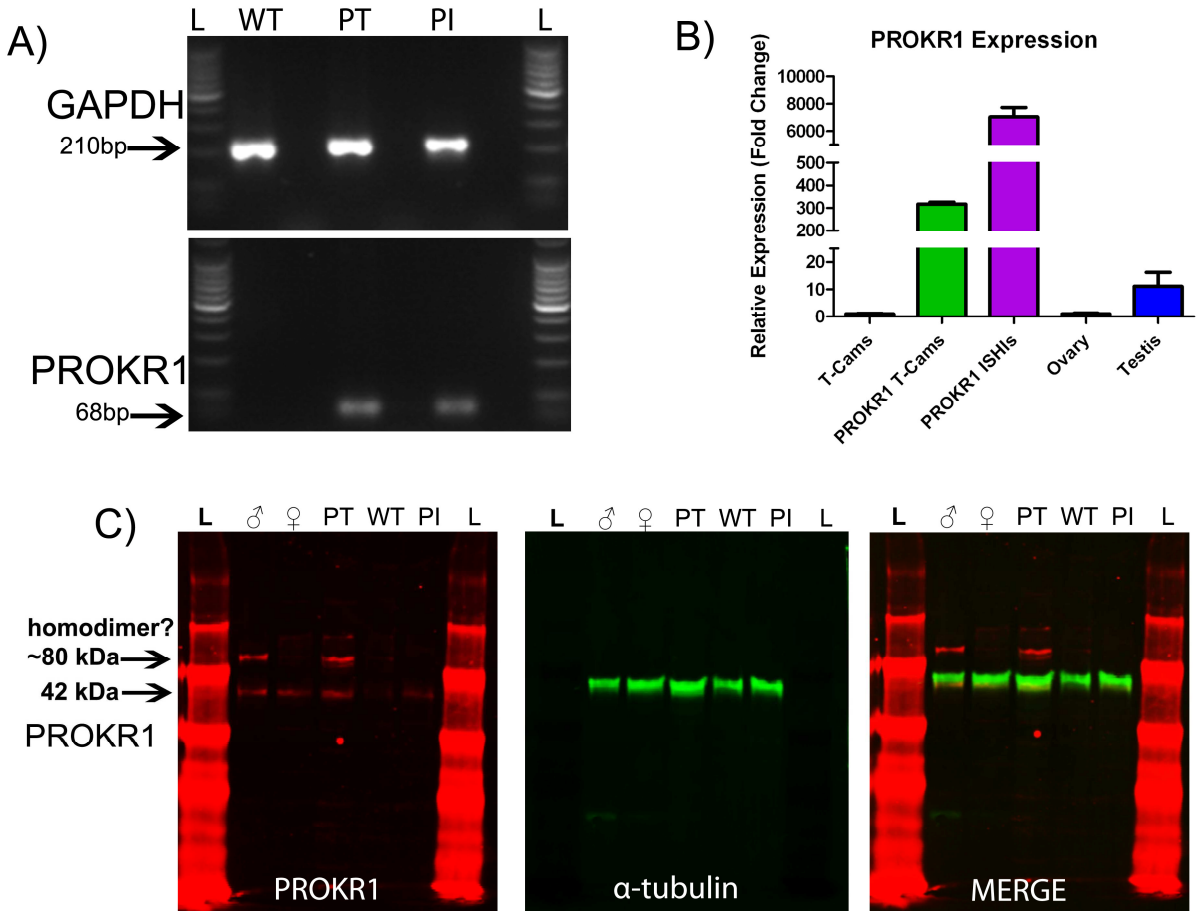

Figure 6.7 Confirmation of transfection of PROKR1 into TCam-2 cells

RT-PCR confirmed increased expression of PROKR1 in PROKR1 transfected TCam-2 cells (PT) compared to wild-type T-Cams (WT). PROKR1 transfected Ishikawa (PI) cells were run alongside as a positive control. B) Quantitative RT-PCR further distinguished this heightened expression in comparison to primary tissue. C) Protein expression was also heightened compared to fetal ovarian (♀) and testicular (♂) tissue. Additionally, a band double the size of PROKR1 was determined in both PROKR1 TCam-2 cells and human fetal testis.

6.3.6 PROK regulation in model germ cells

Previous studies of PROK signalling in the endometrium have suggested PROK is able to regulate both the PGs and the IL-6 type cytokines. Initial investigation of this relationship in fetal germ cells was performed in untransfected/wild-type and PROKR1 transfected TCam-2 cells treated with 40nM PROK1 for 12 hours (n=6). No change was seen in any of the genes examined in untransfected cells, consistent with low expression of the PROK receptors before transfection.

PROKR1 transfected TCam-2 cells displayed a significant increase in *COX2* transcript levels compared to untransfected cells (2.4±0.8 vs 6.9±2.4 $x10^{-6}$ relative percentage compared to the housekeeping gene *RPL32*, p= 0.011, Figure 6.8A). However, no subsequent increase in the PGE_2 specific enzyme *PTGES* was seen (Figure 6.8B). Despite evidence in other reproductive tissues, no significant up-regulation of any of the IL6-type cytokines was found (Figure 6.8C-F). In contrast, both *IL6* and *LIF* in transfected cells showed a trend for reduced expression, however this was not significant. These data confirm PROK1 via PROKR1 is able to regulated *COX2* expression in an *in vitro* model of fetal germ cells. Additionally, unlike regulation seen in other tissues, the IL6-type cytokines do not respond to PROK1 treatment.

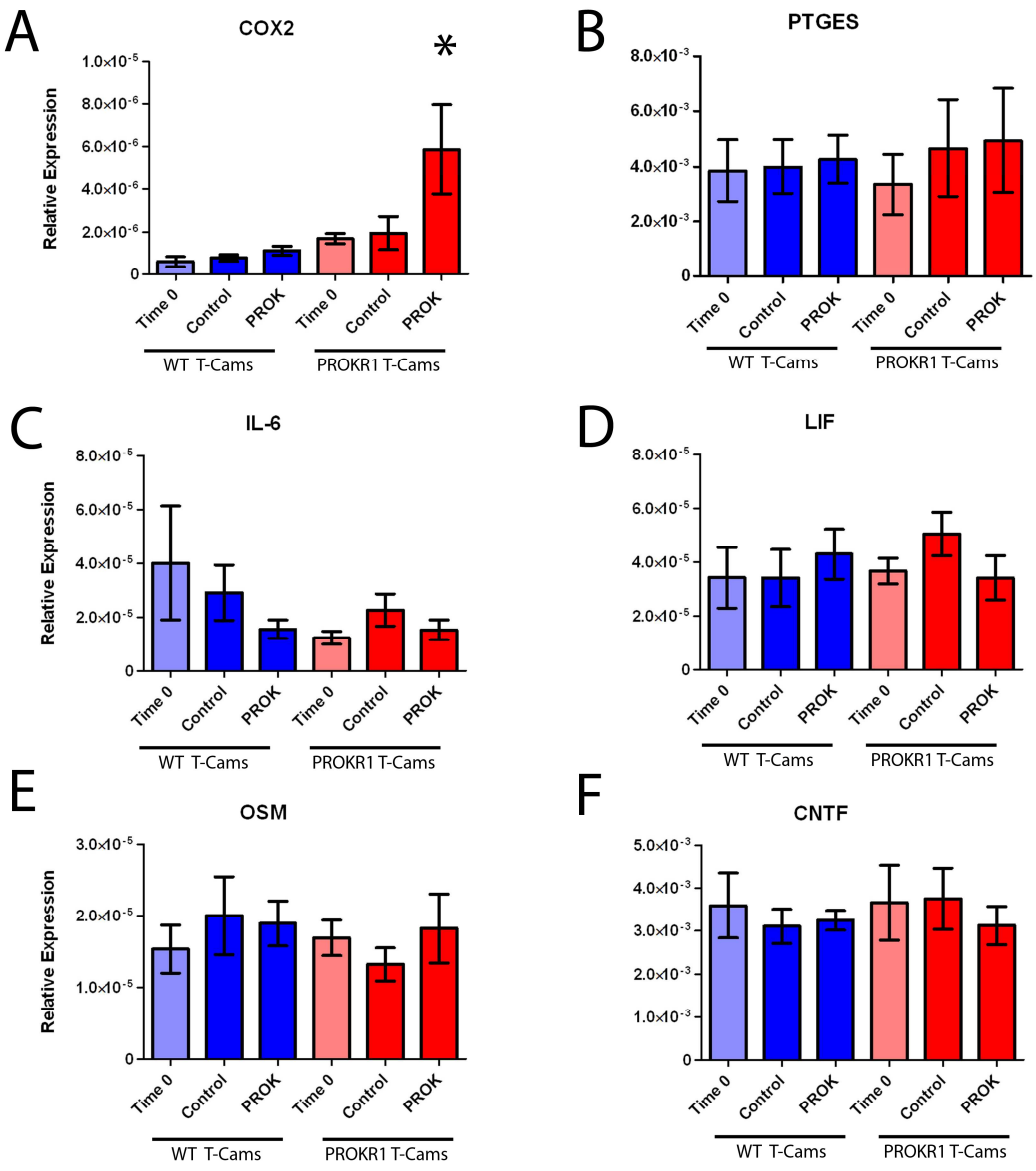

Figure 6.8 Gene expression changes in PROKR1 transfected TCam-2 cells with PROK1 treatment.

Wild-type TCam-2 cells (WT T-Cams) and TCam-2 cells stably transfected with the PROKR1 gene (PROKR1 T-Cams) were treated for 12h with (PROK) or without (Control) 40nM PROK1 and gene expression changes evaluated compared to control (Time 0). (A) The prostaglandin precursor enzyme COX2 was significantly up-regulated when compared to PROK1 treated WT T-Cams, PROKR1 Time 0, and PROKR1 Control (12h untreated). No significant change was seen with treatment in (B) PTGES, (C) IL6, (D) LIF, (E) OSM, or (F) CNTF. (*=$p<0.05$)

6.3.7 PROK1 regulation of PG signalling components

As initial treatment of cells with PROK1 led to a significant change in COX2 at 12 hours, a full time course was performed on several PG pathway components to identify when up-regulation of gene expression was occurring and what other signalling components might be regulated. In particular, the receptors localised to human fetal germ cells were investigated, as some of the EP receptors have been shown to work in a positive feedback loop with COX2. PROK1-treated (40nM) and untreated PROKR1 transfected TCam-2 cells were collected 2, 4, 8, 12, and 24 hours after treatment along with time 0 control (n=6).

In addition to confirming *COX2* up-regulation at 12 hours, further timecourse analysis determined significant up-regulation at 4 and 8 hour time points (4 hours: 1.6±0.6 vs 3.2 ± 0.9 $x10^{-6}$ relative to *RPL32*, p=0.02, 8 hours: 2.6 ± 0.6 vs 5.0 ± 1.6 $x10^{-6}$ relative to *RPL32*, p=0.03, Figure 6.9A). None of the other PG precursor enzymes (*COX1* and *PTGES*) were significantly up-regulated with PROK1 treatment (Figure 6.9B-C). Similarly, the three PGE_2 receptors localised to fetal germ cells; *EP2*, *3* and *4*, were not significantly up-regulated with PROK1 treatment (Figure 6.9D-E). Interestingly, it does appear that *EP3* may be up-regulated in response to continued serum starvation (Figure 6.9E). These data determine PROK1 is able to up-regulate *COX2* specifically, and does not initiate a positive feedback loop of PGE_2 signalling in this system modelling human fetal germ cells.

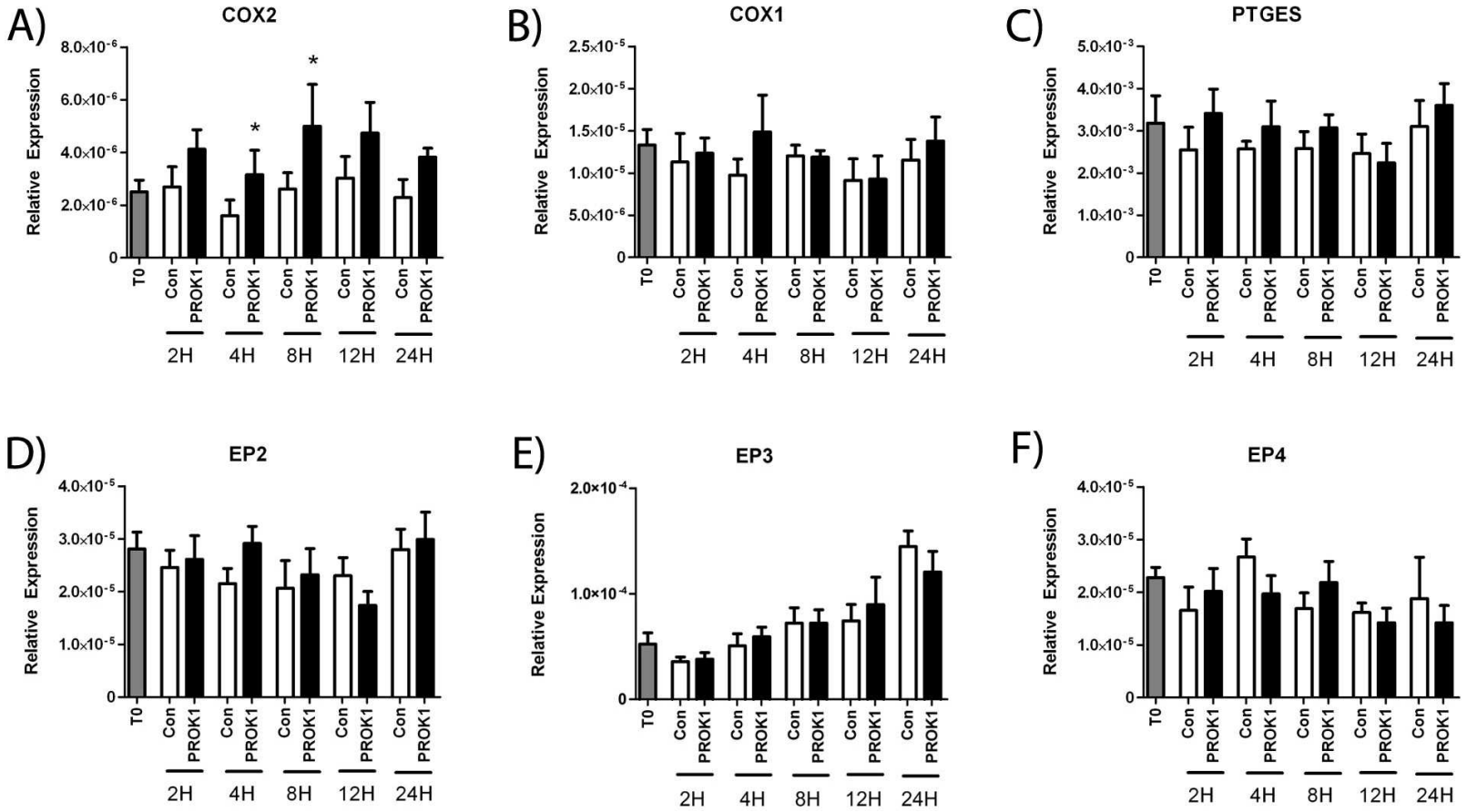

Figure 6.9 Specific up-regulation of COX2 compared with other prostaglandin signalling components

Timecourse analysis of gene expression changes of prostaglandin signaling components after 40nM PROK1 treatment, at time 0 (T0), 2, 4, 8, 12, and 24 hours (H). (A) *COX2* is significantly up-regulated with PROK1 treatment after 4 and 8 hours compared to control. No change is seen in the other prostaglandin precursor enzymes (B) *COX1* and (C) *PTGES*. Additionally, no change is seen in the PGE_2 receptors expressed by the germ cells (D) *EP2* (E) *EP3* or (F) *EP4*. *p<0.05.

6.4 Discussion

Identifying novel regulators of fetal ovarian development is essential for further understanding of the processes which occur leading up to primordial follicle formation. Fetal ovarian development is critical for adult fertility, thus making regulators of these processes are key to female reproduction. The PROKs are recently identified proteins which have been demonstrated to regulate various other components of the female reproductive system, including the endometrium (Battersby *et al*, 2004; Evans *et al*, 2008; Evans *et al*, 2009), placenta (Hoffmann *et al*, 2006; Hoffmann *et al*, 2007; Denison *et al*, 2008), and fallopian tube (Shaw *et al*, 2010a; Shaw *et al*, 2010b; Shaw *et al*, 2011). Additionally, studies have identified that PROKs may play a role in regulation of the granulosa cells in the adult ovary (Kisliouk *et al*, 2003; Fraser *et al*, 2005).

However, this study is the first to examine possible roles for the PROKs during fetal ovarian development. In doing so, it has been determined that all PROK ligands and receptors are expressed in the human fetal ovary and that expression of both *PROK1* and *2* ligands and *PROKR1* are up-regulated with human ovarian development leading to primordial follicle formation. Unlike in the adult ovary, where most PROK expression is restricted to the granulosa cells (Kisliouk *et al*, 2003; Fraser *et al*, 2005), PROK ligands and receptors were predominantly localised to the fetal germ cells. Additional expression was seen in vascular endothelial cells, which is unsurprising based upon PROK's well identified role in angiogenesis (LeCouter *et al*, 2001; LeCouter & Ferrara, 2003; Tanaka *et al*, 2006). With primary expression of the PROK components in germ cells it is likely that signalling is autocrine or paracrine between other germ cells.

As PROK signalling is predominately restricted to one cell type, we further examined function of the PROKs using a model cell line for primordial germ cells, the T-Cam2 cells. Despite expression of several classical pluripotent and germ cell markers, the T-Cam2 cells only endogenously expressed low amounts of either PROK receptors. In order to circumvent this, the T-Cam2 line was stably transfected with the *PROKR1* gene, as this receptor was previously shown to be both developmentally regulated and more highly expressed in the fetal ovary in

comparison to *PROKR2*. This stably transfected germ cell line further identified PROK1 via PROKR1 signalling is able to up-regulated the prostaglandin precursor enzyme *COX2* specifically, and that this ligand-receptor signalling does not regulate IL6-type cytokine expression in the germ cells, as was shown previously in other cells and tissues (Evans *et al*, 2009; Cook *et al*, 2010).

PROK1 signalling via PROKR1 was able to induce COX2 signalling but did not further affect any of the other prostaglandin enzymes or receptors investigated. This is in contrast to a study which was able to discern up-regulation of EP4 with PROK1 treatment (Wade *et al*, 2010). These data suggest that PROK1, by selectively signalling to COX2 over COX1, may be involved specific events in the fetal ovary rather than homeostatic function, as COX2 is the inducible enzyme which is expressed in response to cytokine signalling (Otto & Smith, 1995; Herschman, 1996). Further, COX2 seems to be important for germ cell development, as demonstrated in the previous study regarding paracetamol exposure during fetal development (as paracetamol is thought to act primarily via inhibition of COX2, Chapter 4). However, the exact downstream effects of COX2 upon up-regulation by PROK1 remains unknown, with data presented here suggesting function downstream of PROK1 signalling may not be via PGE_2 in fetal germ cells. However, it is also possible that significant up-regulation of PGE_2 components was not detected in this study due to limitations of this cell line, and further exploration in primary tissue would be informative.

Additionally, it is possible that the PROK1 may regulate a different COX2-related prostaglandin other than PGE_2, such as $PGF_{2\alpha}$ or PGD_2. There is evidence that PGD_2 plays a role in early gonad development during sex determination (Malki *et al*, 2005; Wilhelm *et al*, 2005); however, little is known about the localisation or function of PGD_2 or $PGF_{2\alpha}$ in regards to germ cell development. Data from other systems also suggests PROK1 is also able to regulate other downstream prostanoids, such as thromboxanes or prostocylin (Wade *et al*, 2010) which may be downstream of PROK1 and COX2 up-regulation in fetal germ cells.

Interestingly, PROK1 was not shown to up-regulate the IL6-type cytokines in this study. Previous investigation in the human reproductive tract demonstrated a regulatory relationship between PROK1 and LIF specifically (Evans *et al*, 2009). In addition, although protein localisation of the other IL6-type cytokines investigated in this study remains unknown in the human fetal ovary (IL6, OSM, CNTF), there is data to suggest LIF is expressed by the fetal oocytes in the human (Abir *et al*, 2004). This demonstrates PROK1 regulation of LIF in germ cell cultures is physiologically relevant. However, PROK1 did not regulate any of the IL6 type cytokines in the PROKR1 T-Cam2 cells, suggesting this signalling pathway may not play a role in early germ cell development.

Several other possibilities may explain the lack of PROK1 regulation of these factors in the PROKR1 T-Cam2 cells. Firstly, LIF localisation has only been performed in human fetal ovaries from 19 weeks gestation onwards (Abir *et al*, 2004). Previous study of the T-Cam2 cells has identified that they more closely resemble germ cells from first trimester specimens (Eckert *et al*, 2008; Young *et al*, 2011; Childs *et al*, unpublished), which may not express LIF protein or the other IL6 type cytokines at physiologically relevant levels (although transcript expression of each was confirmed). Further, although T-Cam2 cells do express early PGC markers, they were derived from male origin and thusly may not respond in the same fashion as germ cells generated from female specimens. No data have been collected regarding IL6-type cytokine expression in the testis, so it is reasonable to conclude there may be gender differences in expression and function of these factors. Additionally, as functional data was gathered from a germ cell specific line, it is possible that PROK1 regulates these factors in another cell type in the fetal ovarian tissue, namely vascular endothelial cells.

In conclusion, this study has examined the newly characterised regulatory proteins PROK1 and 2 in the human fetal ovary across early gestation. We have determined that both ligands and one of their shared receptors, *PROKR1*, are developmentally up-regulated leading to primordial follicle formation and that all components are primarily expressed by the fetal germ cells suggesting autocrine signalling or paracrine signalling between germ cells. However, there is also evidence for

expression in the endothelial cells of the vasculature suggesting PROKs may play a role in angiogenesis, for which they are well characterised in other tissues. Further functional characterisation was performed on a novel PROKR1 stably transfected human germ cell line, in order to determine downstream targets of PROK1 signalling and possible function in germ cells. These studies determined that PROK1 is able to consistently regulate the prostaglandin precursor enzyme COX2, which is implicated in germ cell survival in previous chapters (Chapter 3 and 4). Together these data suggest a novel role for PROKs in the human fetal ovary in the regulation of COX2 and possible downstream targets to promote pathways such as survival and/or angiogenesis. However, additional investigation is needed in primary tissue to further discern further exact function of the PROK ligands during human fetal ovarian development.

Chapter 7

Prokineticins in fetal testis development

Chapter 7. Prokineticins in fetal testis development

7.1 Introduction

PROKs function to promote angiogenesis, proliferation, and migration in various reproductive organs (reviewed in (Maldonado-Perez *et al*, 2007) and discussed in detail in Section 6.1) but have yet to be described in the fetal testis. Early characterisation of the PROKs identified they were active in steroidogenic organs of the body, including the prostate and the testis (Wechselberger *et al*, 1999; LeCouter & Ferrara, 2003). In fact, in the mouse and rat, the testis is described to have the highest level of *Prok2* expression in the body, although Prok2's best characterised role is in the regulation of the hypothalamus (LeCouter *et al*, 2003). Brief characterisation of PROK expression was also performed in the human testes when examining their possible function in testicular cancer (Samson *et al*, 2004). These studies determined PROK1 ligand is expressed in the Leydig cells of the adult testis rather than in the germ cells as demonstrated in the ovary in the previous chapter (LeCouter *et al*, 2003; Samson *et al*, 2004). Further, gene expression array analysis of gonads from both male and female mice determined there were differential expression patterns of some of the PROK signalling components during ovarian and testicular development (Menke & Page, 2002). This differential localisation and transcript expression, along with the heightened expression of PROK2 in the testis compared to the rest of the body, led to the hypothesis that the PROKs might play a role during early testicular development.

In the fetal testis, like the fetal ovary, several developmental processes take place in a complex and highly organised fashion in order to form a functional gonad for future adult fertility. Male sex determination (as described in Section 1.3) occurs early in gestation, with testis differentiation becoming evident at ~week 8 of gestation in the human (Anderson *et al*, 2002a). Testis development is initiated after Sertoli cell differentiation, which has been hypothesised to be the rate limiting step in further differentiation of other testicular cell types (Wilhelm *et al*, 2007). Developing Sertoli cells appear to regulate a cascade of developmental and morphological changes in the testis via several growth factors. One such factor is Anti-Müllerian

hormone (AMH), which is expressed by the differentiating Sertoli cells subsequent to SRY expression (Hacker *et al*, 1995) at ~8 weeks gestation in the human (Rajpert-De Meyts *et al*, 1999; Barbara *et al*, 2000). Sertoli cells then migrate and associate with germ cells to form sex cords (Hughes, 2001). These cords provide the basis for future seminiferous tubules, being the first sign of testis specific differentiation, and after which point the germ cells are referred to gonocytes and pre-spermatagonia (as opposed to oogonia and oocytes in the ovary) (Cooke & Saunders, 2002; Gaskell *et al*, 2004).

AMH released from the Sertoli cells promotes migration of cells from the mesonephros to further colonise the testis (Bezard *et al*, 1987; Behringer, 1995). However, AMH is not thought to be the only factor involved in mesonephros migration (Ross *et al*, 2003). FGF9 is also postulated to aid in regulation of cell migration (Colvin *et al*, 2001). Although the signalling mechanisms governing cell migration to the testis from the mesonephros are poorly understood, it is a process which appears to be essential for further testis cord formation in the rodent (Tilmann & Capel, 1999). It is postulated the migratory cells then differentiate and give rise to the peri-tubular myoid (PTM) cells, peri-vascular cells, and a sub-set of the Leydig cells (Merchantlarios *et al*, 1993; Martineau *et al*, 1997; Merchant-Larios & Moreno-Mendoza, 1998; Nishino *et al*, 2001).

Leydig cells are evident in the human fetal testis from ~8 weeks gestation, and are formed from two distinct sub-sets of progenitors (Codesal *et al*, 1990; Ostrer *et al*, 2007; DeFalco *et al*, 2011). As described above, one of the cohorts of Leydig cell progenitors migrates from a specialised region between the gonad and mesonephros border; while the other cohort of progenitor cells migrates from the coleomic ephithelium. Sertoli cells then function in a paracrine fashion, secreting factors to promote Leydig cell differentiation (Karl & Capel, 1998; Ge *et al*, 2006; DeFalco *et al*, 2011). Pdgfα, is one such factor secreted by the Sertoli cells, which signals to the interstitial cells in the fetal testis, and interruption of Pdgfα signalling leads to Leydig cell disruption (Brennan *et al*, 2003). Another factor with similar function is Desert hedgehog (Dhh) (Bitgood *et al*, 1996). In addition to Leydig cell disruption, $Dhh^{-/-}$ mice also display poor segregation of the tubules from the interstitial compartment

(Yao & Capel, 2002), suggesting Dhh might be involved outwith Leydig cell differentiation. Mutations in DHH in humans are associated with fertility disorders, most often gonadal dysgenesis and infertility (Umehara *et al*, 2000; Canto *et al*, 2004; Canto *et al*, 2005), and fetal testes collected from smoking mothers also displayed lowered DHH levels (Fowler *et al*, 2008).

Leydig cells regulate further masculinisation of the testis and secondary sex organs via the secretion of androgens (Clark *et al*, 2000; Hu *et al*, 2002; Welsh *et al*, 2007). Testosterone is produced via a several step enzymatic process from cholesterol (as depicted in Figure 7.1). Enzymes produced by the Leydig cells (eg StAR, CYP11A1), necessary for androgen production, and testosterone itself are detected around the start of Leydig cell differentiation and peak at early second trimester in the human (Tapanainen *et al*, 1981; O'Shaughnessy *et al*, 2007; Scott *et al*, 2009). In addition to androgens, the Leydig cells also produce insulin-like3 (INSL3), a factor initially characterised in regulation of testicular descent (Ivell & Hartung, 2003; Klonisch *et al*, 2004). In addition, INSL3 regulates germ cell survival directly via its receptor leucine-rich repeat-containing G-protein-coupled receptor 8 (LGR8) (Kawamura *et al*, 2003).

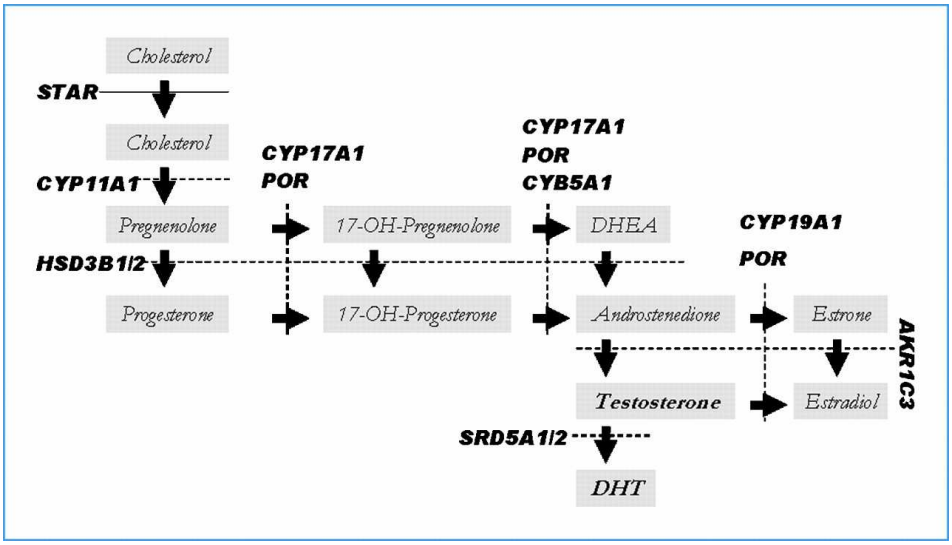

Figure 7.1 Steroid synthesis pathway in the human testis.

The human fetal Leydig cells produce testosterone via the degradation of cholesterol via several enzymatic steps as demonstrated briefly above. Figure from (Hofland *et al*, 2010).

After initial differentiation, the Leydig cells in the fetal testis undergo a proliferative/hyperplastic event from 14-18 weeks, increasing vastly in number, which also correlates with increased steroidogenesis during this time (Codesal *et al*, 1990; Murray *et al*, 2000).

Other cell types to migrate from the mesenphros to the testes are the PTM cells and the perivascular cells (Martineau *et al*, 1997; Nishino *et al*, 2001). Both of these cell types become evident in the human fetal testis at ~12 weeks gestation (Ostrer *et al*, 2007). Once settled in the gonad, the PTM cells differentiate and migrate to surround the germ and Sertoli cell aggregations to form a flattened cell layer providing infrastructure to the seminiferous tubules (Wilhelm *et al*, 2007). In the human, the PTM layer displays tight junctions similar to other connective tissues (Ross & Long, 1966) and demonstrates contractile properties later in adulthood aiding in release of elongated spermatids. Perivascular cells also migrate from the mesenephros to the fetal testis (Nishino *et al*, 2001) and are important for neoangiogenesis. Blood vessel formation is heighted during fetal testis development and aids in trafficking of hormones within the testis and to other organs.

In summary, early fetal testis differentiation is highly organised and composed of several processes including: Sertoli cell differentiation, cord formation and AMH release; migration of multiple cell types to the testis; Leydig cell differentiation, proliferation and initiation of steroidogenesis; PTM differentiation and seminiferous tubule enclosure; and vascular formation (as summarised in Figure 7.2). All of these events are important for the development and support of the gonocytes for future spermatogenesis during adulthood.

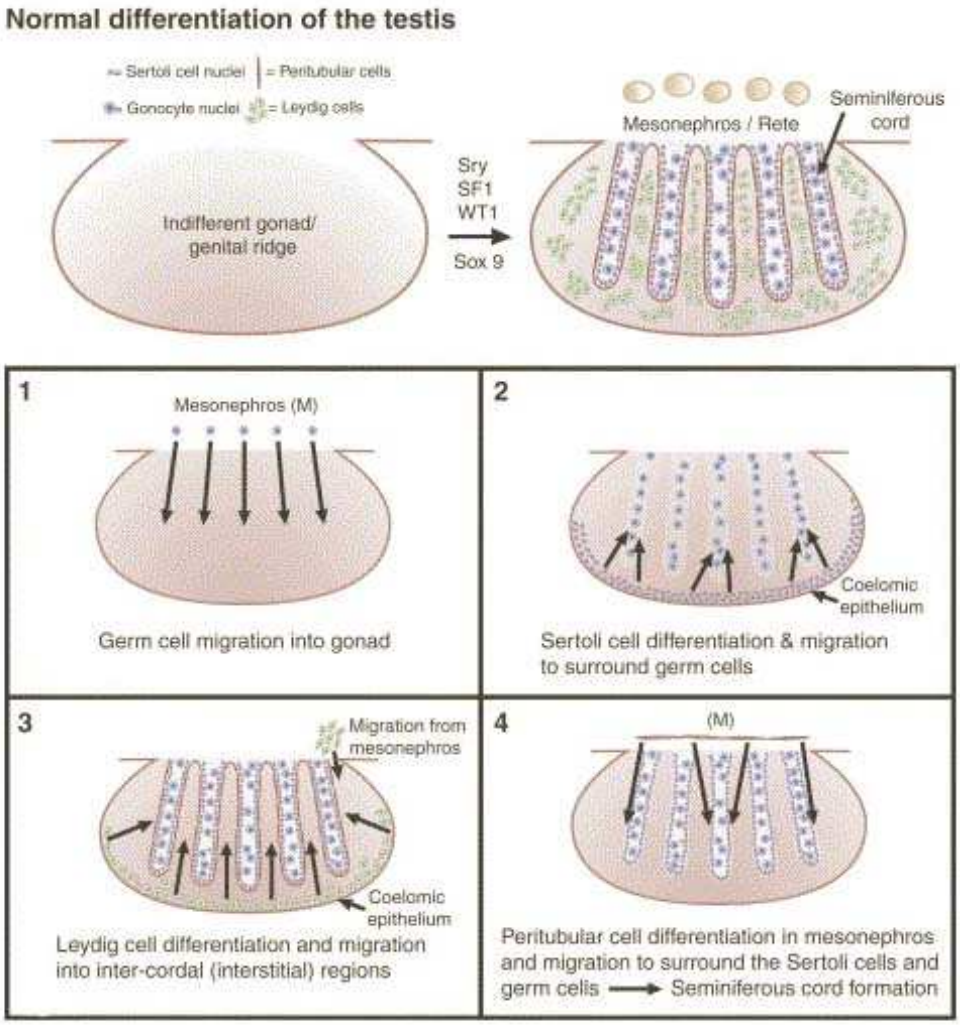

Figure 7.2 Summary of human fetal testis development.

Gonad formation initiates with the 1) migration of the primordial germ cells to the genital ridge. At this point the gonad is bi-potential, but signaling from the SRY gene promotes 2) Sertoli cell differentiation and migration. This is followed by 3) migration of the Leydig cells to the testis from the mesonephros and the coelomic epithelium and promote further testis formation via secretion of androgens. 4) Further migration of peritubular myoid cells and peri-vascular cells form the infrastructure of the fetal testis. Figure adapted from (Sharpe *et al*, 2006).

As in the ovary, little is known about the developmental pathways that regulate testicular formation. The PROKs have been postulated by other groups to be involved in angiogenesis and steroidogenesis, which are two main functions required for testicular growth and development (Wechselberger *et al*, 1999; Samson *et al*, 2004; LeCouter *et al*, 2003). For this reason, the PROK ligands and receptors were characterised in the human fetal testis across early gestation, as was performed in the fetal ovary in the previous chapter. Further dual immunohistochemical staining and cultures were performed in order to extrapolate a possible function for PROK signalling during early male development.

In addition, PROK1 regulation of several factors was investigated, to determine changes in transcript expression. These factors include components from previous studies (the PGE_2 and IL6-type cytokine pathways), components of the steroidogenesis pathway (CYP11A1 and StAR), and factors found to be regulated by PROK1 in previous studies which are thought to be involved in testis development (DKK1 and ID3) (Macdonald *et al*, 2010).

7.2 Materials and Methods

Tissue collection

Human fetal tissue used in the following experiments was obtained and dissected as described previously (Section 2.1), with subsequent SRY genotyping to determine sex of first trimester specimens (Section 2.3).

RNA extraction and cDNA synthesis

Sample tissue was then processed to extract RNA and subsequently synthesis cDNA for genomic analysis as previously discussed (Section 2.4).

Quantitative RT-PCR

In order to determine if mRNA transcripts encoding PROK signalling targets (PROK1, PROK2, PROKR1 and PROKR1) were expressed differentially compared to the fetal ovary and to determine variation across gestation, qRT-PCR was performed using the ABI 7500Fast system and both Sybr Green and Taqman analysis as described in Section 2.5.

Quantitative RT-PCR analysis of fetal interstitial cultures was also performed using the ABI 7900HTFast system and Sybr Green analysis to determine any gene expression changes in possible PROK targets (*PTGES, BDNF, MCL-1, LIF, OSM, IL6, CYP11A1, StAR,* and *ID3*) and Taqman analysis was also performed for targets with primer/probe sequences (*COX2,* and *DKK1*), details of this protocol are also outlined in Section 2.5.

Immunohistochemistry

Human fetal testis samples were used to localise PROK ligands (PROK1 and 2) and receptors (PROKR1 and 2) to determine cell-specific sites of PROK synthesis and action. Further staining was then performed with cell specific markers 3β-HSD (Leydig) and SMA (blood vessels), to confirm cell specificity of the PROK receptors. Finally, immunohistochemistry was also utilised to determine changes in apoptosis (CC3) and proliferation (BrdU). Protocols for these localisations can be found in Section 2.8.

Human fetal testicular culture

Second trimester fetal testes (n= 6, 14-17 weeks gestation) were cultured as small explants in hanging-drop culture as described previously (Zhang *et al*, 2008; Szczepny *et al*, 2009). Tissue was cultured using organ culture media as described in Section 2.13. Tissue was split into two treatment groups; vehicle control (H_2O) or 40nM PROK1. Tissue was cultured for 24 hours for histological analysis and subsequently fixed in Bouins solution and processed as in Section 2.6.

Further examination to determine gene expression changes resulting from PROK1 treatment were performed by differential plating to isolate fetal interstitial cells for culture (n= 6, 14-16 weeks gestation). Testes were disaggregated and cultured overnight in 6-well plates with 0.01% gelatin coating to promote adhesion of interstitial cells. Germ, Sertoli, and Leydig cells did not adhere and were removed 12 hours post-plating, at which time the remaining interstitial cells were treated with 40 nM PROK1 for 24 hours. This allowed for additional isolation of the cells responding to PROK1 treatment, allowing for selective investigation of specific cell response. Interstitial cell cultures were lysed in RLT buffer following culture and snap frozen for further RNA extraction and cDNA synthesis.

Stereology/Image Analysis

Histological changes after PROK1 treatment in the human fetal testis were determined via image analysis using various markers as described in Section 2.11.3.

Measurements of proliferation and apoptosis were performed using two slides from each sample at least 10 sections apart (50 microns). Each slide contained sections from 4-6 pieces of hanging drop tissue. Positive cells (BrdU or CC3) were counted and designated intra- or extra- tubular based upon their localisation within the tissue. Random frames were generated and all cells were counted within the frame until a quantity designated as significant (as determined based upon area of the tissue) by the image analysis software was achieved. These counts were then corrected for area of the whole tissue and tubular area.

Statistical Analysis

Quantitative RT-PCR data were analysed with GraphPad Prism version 4 statistical software (GraphPad Software Inc). Gestational comparision data were analysed using one-way ANOVA, data were then either analysed as described or log-transformed for further analysis. Log-transformation was performed in instances where data sets did not fit a Gaussian distribution. The Kolmogorov-Smirnov normality test was used for this purpose.

Data were then analysed utilising the Newman-Keuls Multiple Comparison post-test to determine significant changes between gestational values. This post-test was chosen as has more strength than a Tukey post-test and the risk of type I error does not occur with only three groups (as in our study). Some data was also analysed using a post-test for linear trend, this test was performed when data looked to be in a natural order (increasing or decreasing across gestation).

Gene expression changes identified using qRT-PCR after PROK1 treatment of human fetal testes were also analysed using GraphPad Prism as above. Data were analysed using paired t-tests comparing PROK1 treated tissue to dH_2O treated tissue (as the appropriate control).

7.3 Results

7.3.1 Genes encoding PROK signalling components are developmentally-regulated in the fetal testis

To determine presence and pattern of expression of the two PROK ligands and their shared receptors during human fetal testis development, qRT-PCR was performed across a range of gestations. Testis samples were split in three gestational groups, similar to what was performed for ovarian gestational analysis in previous chapters. In the testis these ages also equate to developmental events: 1st trimester (8-10 weeks), Sertoli cell differentiation; early second trimester (14-16 weeks), Leydig cell migration, differentiation, and testosterone secretion; and late second trimester (17-21 weeks) PTM and peri-vascular cell differentiation and Leydig and germ cell proliferation.

Transcripts encoding all four PROK signalling components were detected in the human fetal testis across all gestations examined. Expression of *PROK1* was significantly increased in early and late second trimester compared to first trimester (4.1±1.2 and 3.9±1.4 vs 0.4±0.1 relative expression compared to the housekeeping gene *GAPDH*, p=0.009 and 0.04 respectively, Figure 7.3A). *PROK2* expression increased linearly across gestation, with increased expression between first to early second trimester (0.2±0.04 vs 1.7±0.5 x10^{-2} relative to *GAPDH*) and again between early second and late second trimester (1.7±0.5 vs 3.3±0.7 x10^{-2} relative to *GAPDH*, linear trend p=0.005, Figure 7.3B). Messenger RNA expression of both PROK receptors (PROKR1 and 2) displayed similar expression patterns across gestation, with significant increases in expression specifically at late second trimester compared to both first and early second trimester (PROKR1= 0.7±0.1 and 0.6±0.1 vs 1.3±0.1 x10^{-2} relative to *GAPDH*, p=0.009 and 0.007 respectively, Figure 7.3C) (PROKR2= 2.4±0.7 and 1.5±0.6 vs 9.6±2.0 x10^{-3} relative to *GAPDH*, p=0.01 for both comparisons, Figure 7.3D). These data demonstrate that mRNA expression of both PROK ligands and receptors is increased with testis development.

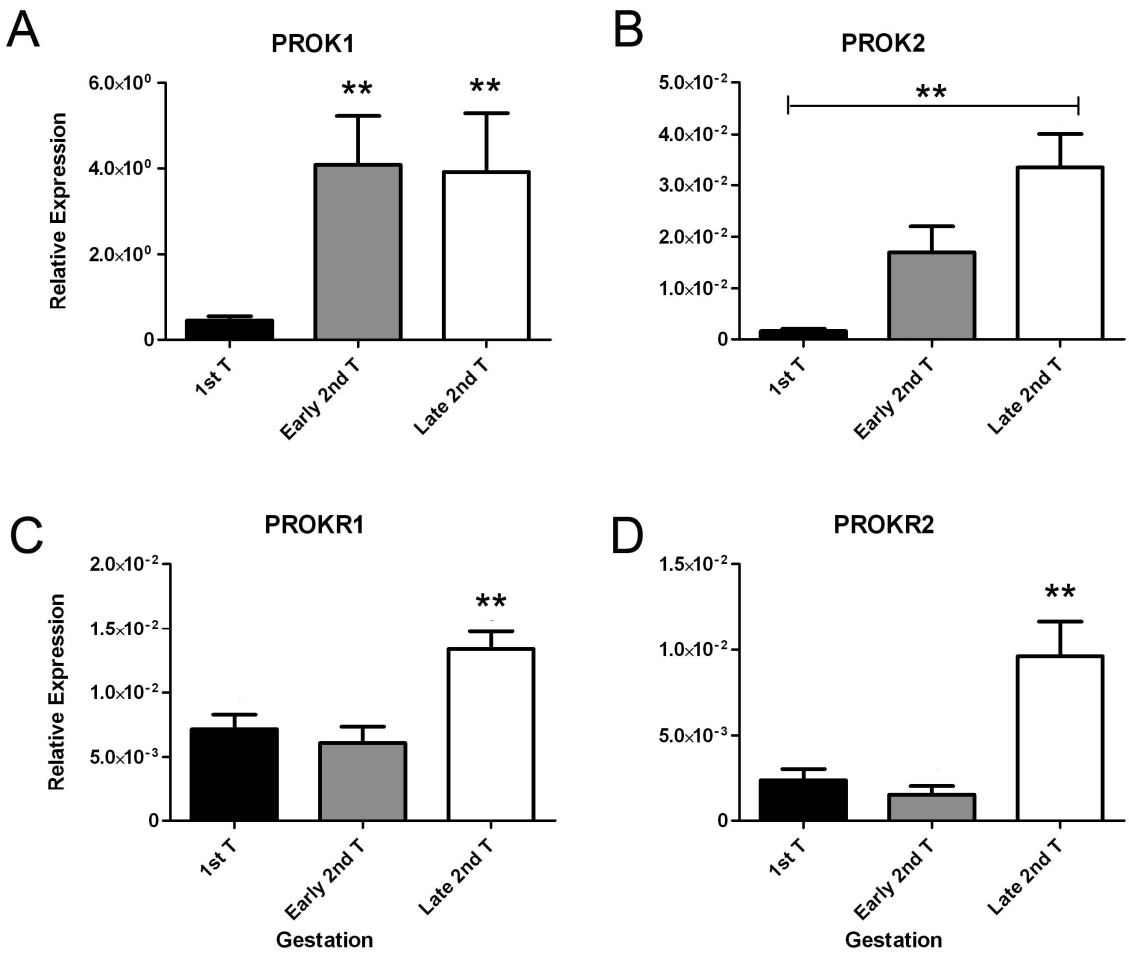

Figure 7.3 Messneger RNA expression of PROK components is up-regulated with development in the fetal testis

Expression of the two ligands (PROK1 and 2) and their two shared receptors (PROKR1 and 2) was analysed by qRT-PCR across gestation and split into age groups; 8-10 weeks (1st T), 14-16 weeks (Early 2nd T), and 17-21 weeks (Late 2nd T; n= 4-6 samples for each gestation). (A) Expression of PROK1 increased between 1st trimester and both early and late 2nd trimester (**=p<0.01). (B) PROK2 expression was significantly up-regulated across gestation (**=p<0.01, determined by test for linear trend). (C) PROKR1 and (D) PROKR2 expression were significantly up-regulated specifically in late 2nd trimester (**=p<0.01). All data are expressed relative to the housekeeping gene *GAPDH*. Significance is determined by one-way ANOVA with Newman-Keuls post-test, except in the case of PROK2 which was determined by post-test for linear trend.

7.3.2 PROK ligands are expressed in most cell types of the human fetal testis

DAB immunohistochemical detection was performed to determine sites of PROK ligand protein expression in the human fetal testis. PROK1 expression was diffuse with expression seen both within the seminiferous cords and in the interstitial compartment of the fetal testis (Figure 7.4A and B). PROK1 expression was also seen in blood vessels of the human fetal testis. PROK1 expression does appear to be specific, as control tissue from the endometrium was run with the same concentration of primary antibody and clearly displays immuno-negative stromal cells along with immuno-positive glandular tissue (Figure 7.4 inset). PROK2 expression was more restricted, with the interstitial compartment being the primary site of PROK2 expression (Figure 7.4C and D). Weak staining was also seen within seminiferous cords but was not seen in the epithelium. Negative control tissue incubated without primary antibody was run alongside both PROK1 and 2 and detected no non-specific staining. In comparison to second trimester, tissue from first trimester specimens did no readily express either ligand (data not shown), suggesting PROK protein expression is lower in earlier ages, similar to that of PROK transcript expression. These data suggest PROK1 is highly expressed in the human fetal testis and is not restricted to a specific cell type, whereas PROK2 is also highly expressed, but is primarily expressed by Leydig and interstitial cells in the interstitial compartment of the fetal testis.

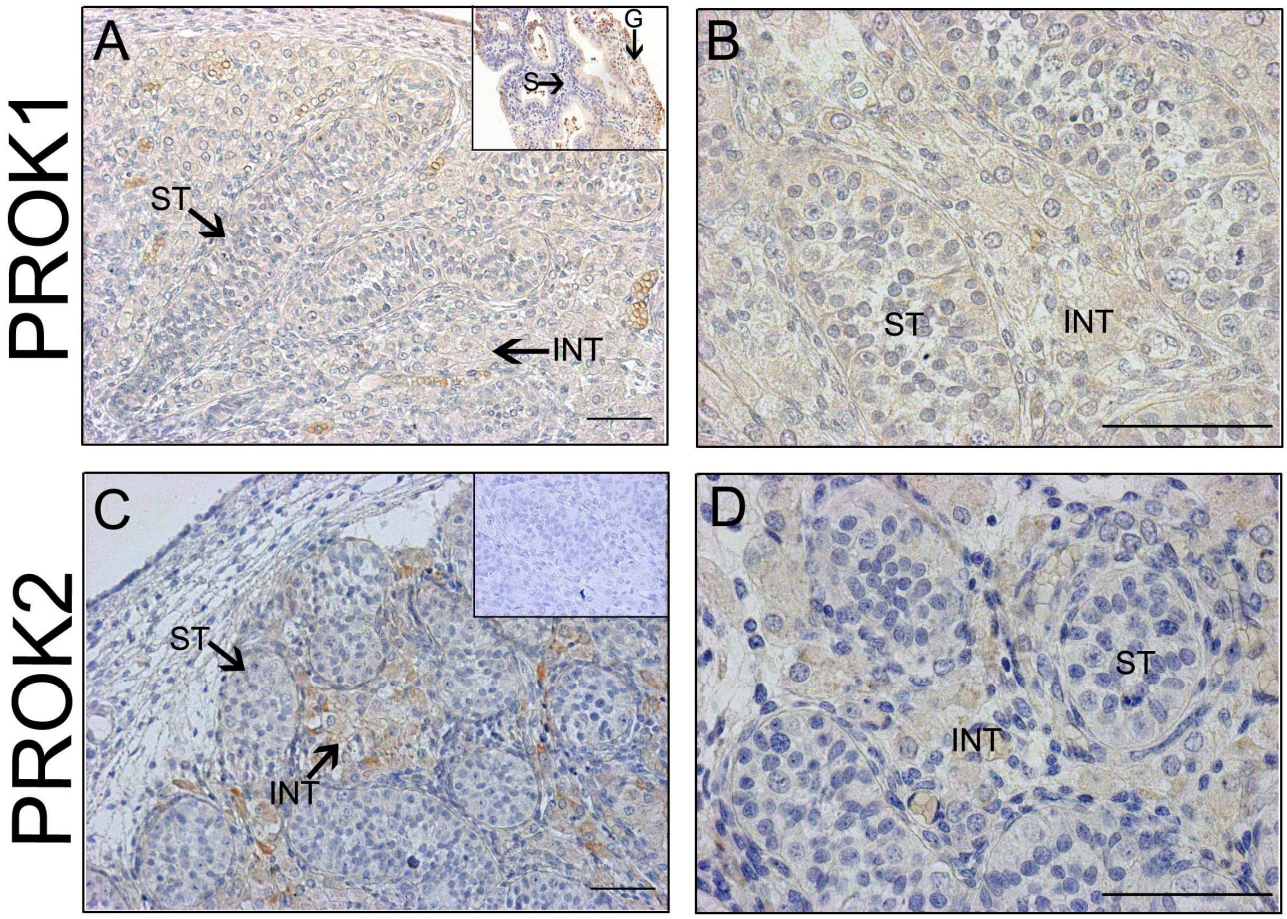

Figure 7.4 PROK1 and 2 are highly expressed in the human fetal testis

Immunohistochemistry was performed for PROK1 and 2 in 15 week human fetal testis tissue. (A-B) 3,3′-diaminobenzidine tetrahydrochloride staining (brown) determined PROK1 expression was diffuse throughout the fetal testis, localised both in the seminiferous cords (ST) as well as the interstitial compartment (INT). (A inset) This localisation appears to be specific as the same concentration of primary antibody stained glandular tissue (G) but not stroma (S) in endometrial control tissue. (C) PROK2 was primarily localised to the interstitial compartment of the human fetal testis (D) with weak expression seen within the seminiferous cords. (C inset) No staining was determined in the negative control testis tissue incubated without primary antibody. All scale bars equal 50 microns. Images are representative of staining performed on at least three specimens at roughly the same gestation.

7.3.3 PROK receptors localised to the interstitial compartment of the human fetal testis

Fluorescent immunohistochemistry was utilised to determine the protein expression of the two PROK receptors (PROKR1 and 2) in the human fetal testis. Both receptors displayed similar sites of expression, with marked expression within the interstitial compartment of testis but no expression within the cords (Figure 7.5 A-B). However, it was noted that not all cells within the interstitial compartment were immuno-positive for either PROKR1 or PROKR2. These results suggest the two receptors may be specific to certain cell type, most likely being either Leydig or other interstitial cells.

In order to determine the site specific expression of the two receptors, each was fluorescently co-localised with the Leydig cell marker 3β-HSD (an enzymes utilised in steroid synthesis). PROKR1 did not display any co-localisation with 3β-HSD, demonstrating its primary site of localisation in the human fetal testis to be the non-Leydig interstitial cells (Figure 7.6). Conversely, PROKR2 was highly co-localised with 3β-HSD, signifying PROKR2 is primarily expressed by the Leydig cells of the human fetal testis (Figure 7.6). These data indicate the PROK receptors are both expressed within the interstitial compartment of the fetal testis making this the primary site of PROK ligand action; however, the receptors are expressed on differing cell types, with PROKR1 targeting non-Leydig interstitial cells and PROKR2 targeting the Leydig cells. In addition, expression of both receptors increased with gestation, in keeping with transcript expression data.

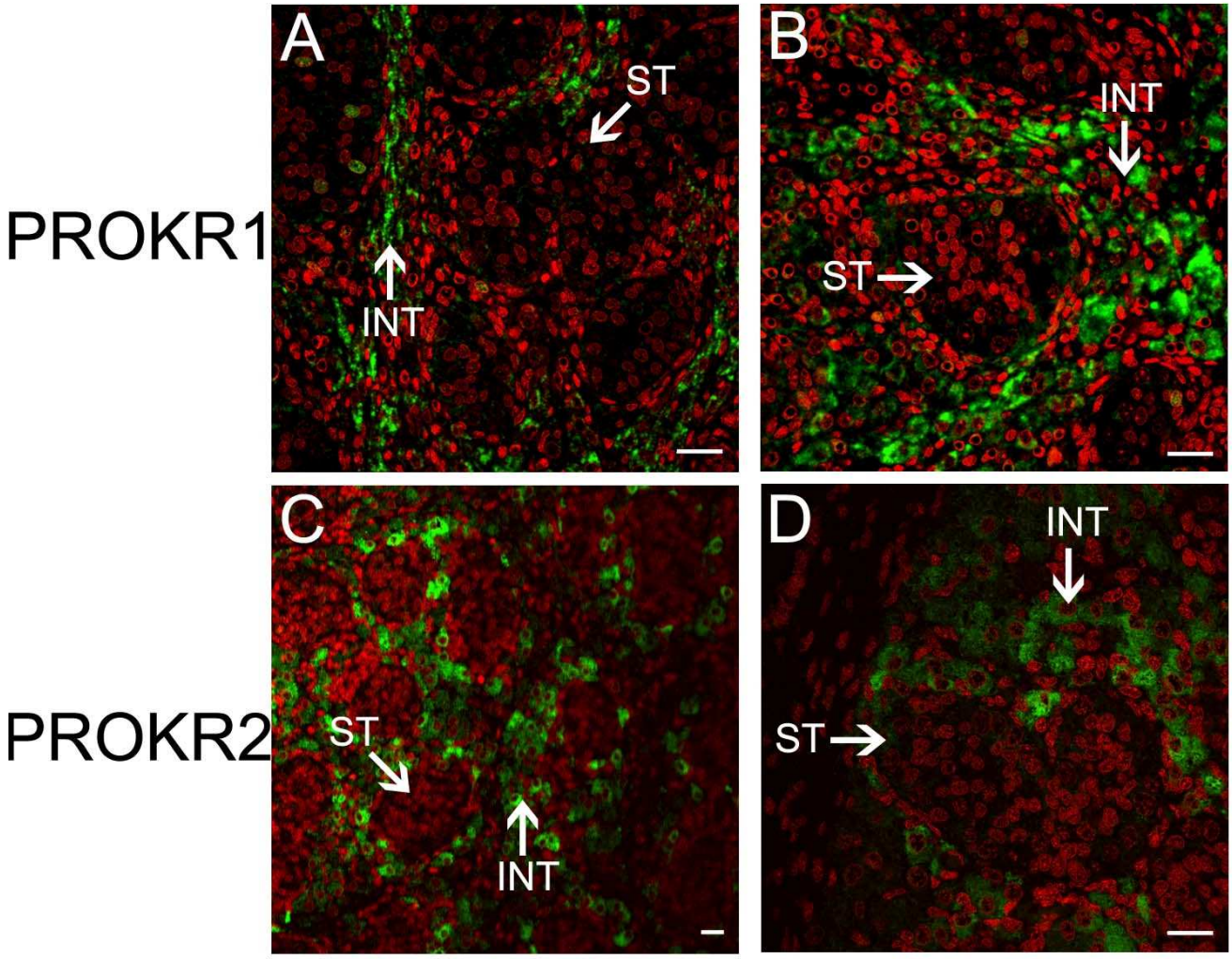

Figure 7.5 PROK receptor expression restricted to interstitial compartment of the human fetal testis

Fluorescent immunohistochemical detection of the PROK receptors (green) in 18 week human fetal testis tissue. (A-B) PROKR1 is restricted to the interstitial compartment (INT) of the human fetal testis with no expression determined in the semineferous tubules (ST) as is (C-D) PROKR2. Not all cells in the INT are expressing the receptors suggesting a possible cell-specific target. Propidium iodide used as a nuclear counterstain (red). Scale bars equal 20 microns. Images are representative of staining performed on at least three specimens at roughly the same gestation.

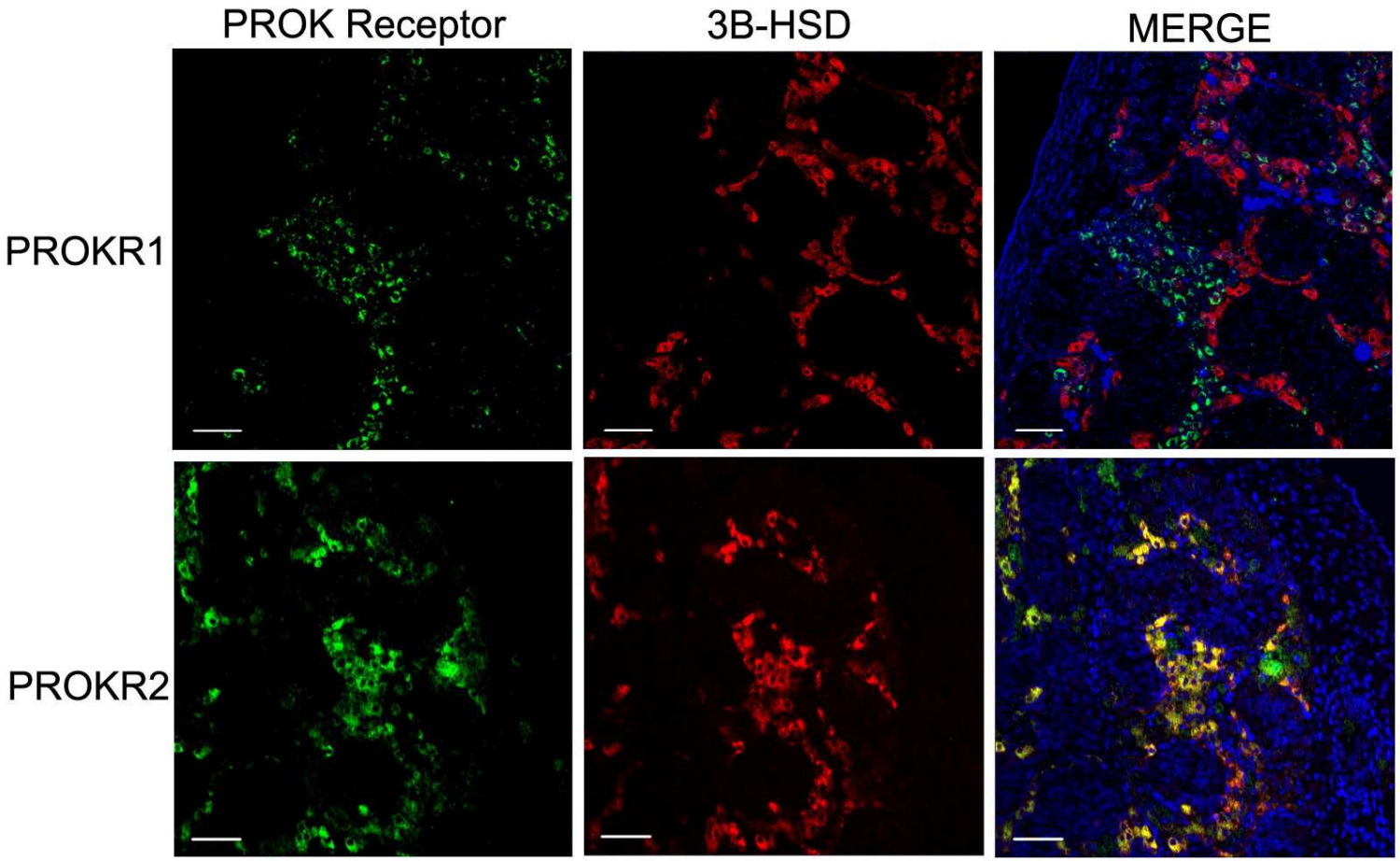

Figure 7.6 Co-localisation of PROK receptors with Leydig cell marker 3β-HSD

Fluorescent immunohistochemical localisation in 18 wk human fetal testis of PROK receptors (green) with Leydig cell marker 3β-HSD (red). (A) PROKR1 does not co-localise with 3β-HSD, and is therefore restricted to the non-Leydig cells of the human fetal testis. (B) PROKR2 does co-localise with 3β-HSD, demonstrating Leydig cells are the primary site of PROKR2 signalling. DAPI counterstain (blue) was utilised. Scale bars equal 50 microns. Images are representative of staining performed on at least three specimens at roughly the same gestation.

7.3.4 PROK receptors expressed by fetal testicular vasculature

PROK receptor localisation was also investigated in fetal vasculature. The vascular marker α-smooth muscle actin (SMA) was utilised to identify blood vessels within human fetal testis tissue. Both PROKR1 and 2 displayed distinct co-localisation with SMA throughout later gestations (distinct vasculature is not apparent until late second trimester, Figure 7.7). These data determine PROK signalling may play a classical role in neo-angiogenesis in the testis as well as a possible role with interstitial cells in the human fetal testis.

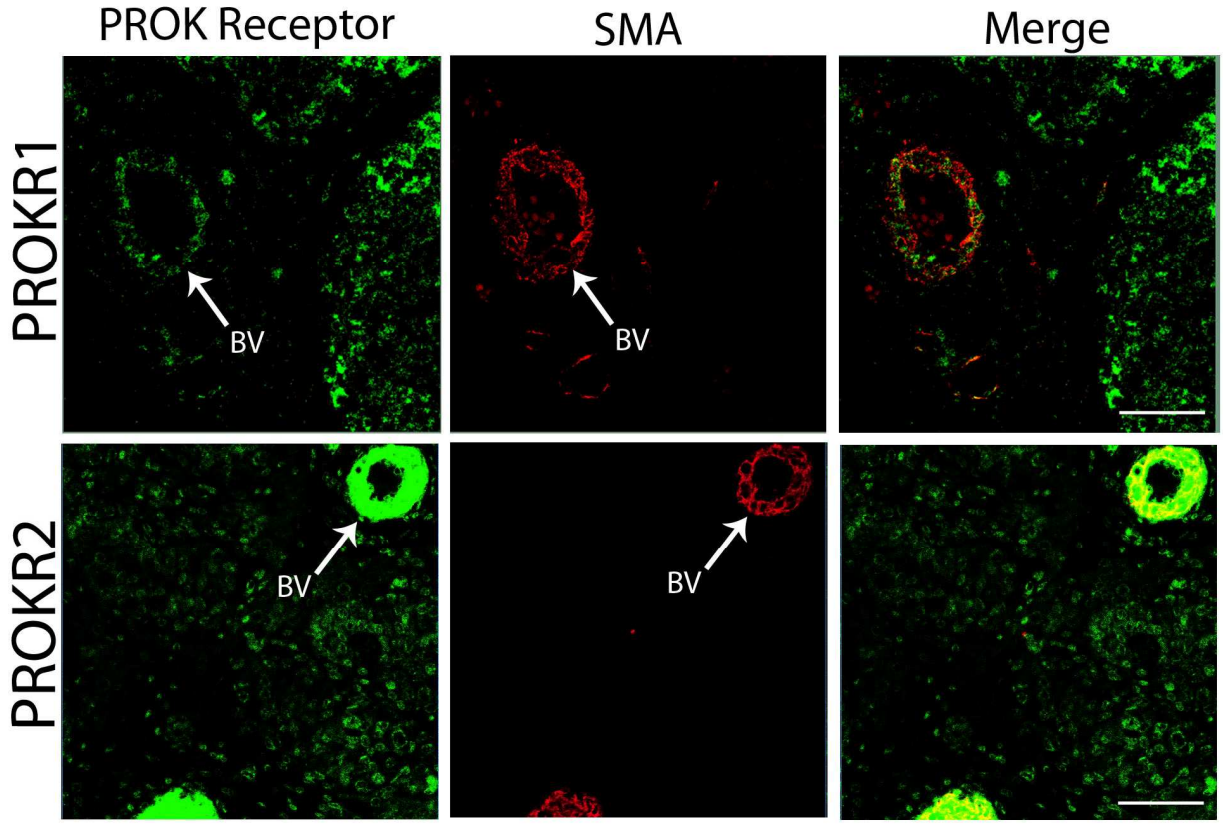

Figure 7.7 PROK receptors are co-expressed with SMA in fetal testicular vasculature.

The PROK receptors (green) were co-immunolocalised with the vascular marker α-smooth muscle actin (SMA, red) in human fetal testicular tissue (18 wks). Both (A) PROKR1 and (B) PROKR2 co-localised with SMA in the blood vessels (BV) of the fetal testis. Scale bars equal 50 microns. Images are representative of staining performed on at least three specimens at roughly the same gestation.

7.3.5 PROK1 functions as pro-survival factor in intratubular cells in the human fetal testis

As PROK1 mRNA expression was higher than that of PROK2 and protein expression was also heightened in the human fetal testis compared to the endometrium, where it is known to function, the role of PROK1 was investigated during development in the human fetal testis. PROK1 function was examined via hanging drop culture (as in chapter 3) of fragments of fetal testis (14-17 weeks, n=6) with 40 nM PROK1 or vehicle control for 24 hours. The testis tissue was then examined for histological changes in apoptosis or proliferation as determined by the markers CC3 or BrdU respectively (Figure 7.8 and 7.9). Positively stained cells were quantified and categorised as either extratubular (interstitial) cells or intratubular (within seminiferous tubules), to narrow function to a specific compartment.

As PROK receptor localisation determined the primary site of PROK action was the interstitial compartment of the human fetal testis (both Leydig and non-Leydig cells), the extratubular compartment was postulated to be the main site of PROK1 action. However, upon quantification of apoptosis and proliferation in this compartment, no change in cell apoptosis or proliferation was determined (Figure 7.8).

Apoptosis and proliferation were also quantified in the intratubular (germ and Sertoli cell) population of the human fetal testis. Although no change was seen in proliferation of these cells, there was a significant difference in the rate of apoptosis, with a significant decrease in apoptosis with PROK1 treatment (5.7±4.5 vs 3.6±2.3 $x10^{-4}$, p=0.03, Figure 7.9). These data suggest PROK1 does not affect the rate of cell turn over in the extratubular compartment which is composed of PROK target cells. However, PROK1 treatment does affect cell turn over in the intratubular compartment suggesting PROK1 signals to the Leydig and/or non-Leydig interstitial cells to produce a paracrine factor that in turn promotes survival in the intratubular compartment consisting of Sertoli and germ cells.

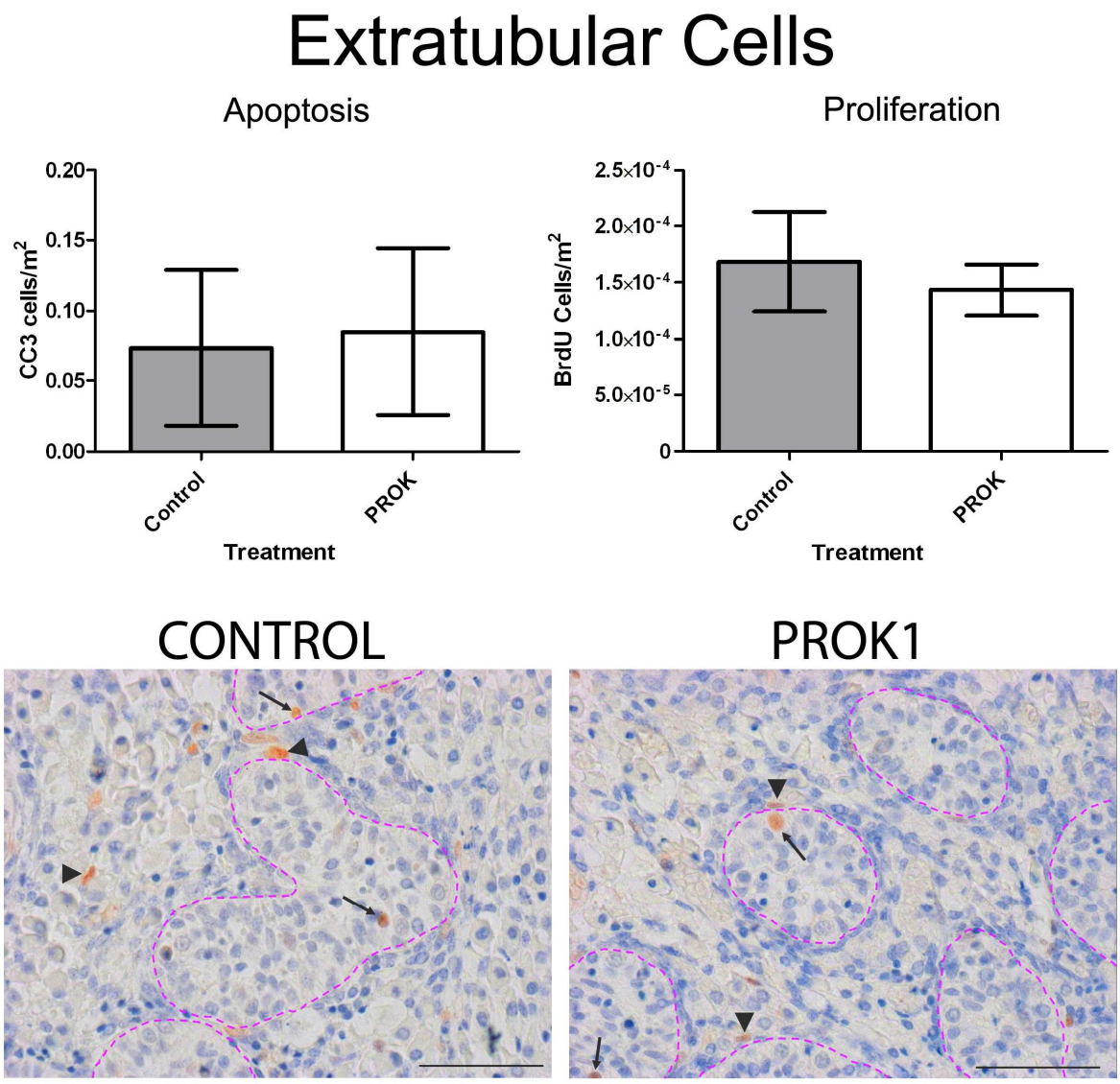

Figure 7.8 No change in apoptosis or proliferation in the extratubular cells of the human fetal testis with PROK1 treatment

Apoptosis and proliferation in the extratubular (outwith the seminferous tubules outlined by dashed line) compartment of the human fetal testis was quantified with the apoptotic marker cleaved caspase-3 (CC3) and the proliferative marker BrdU (14-17 weeks gestation, n=6). Positive cells were quantified and adjusted for extratubular area. This determined PROK1 treatment did not induce a change in cell turnover in the extratubular compartment. Sample BrdU staining from control and PROK1 treated testis, demonstrating no change in extratubular (arrowhead) staining. Intratubular staining marked with arrows. Scale bars equal 100 microns.

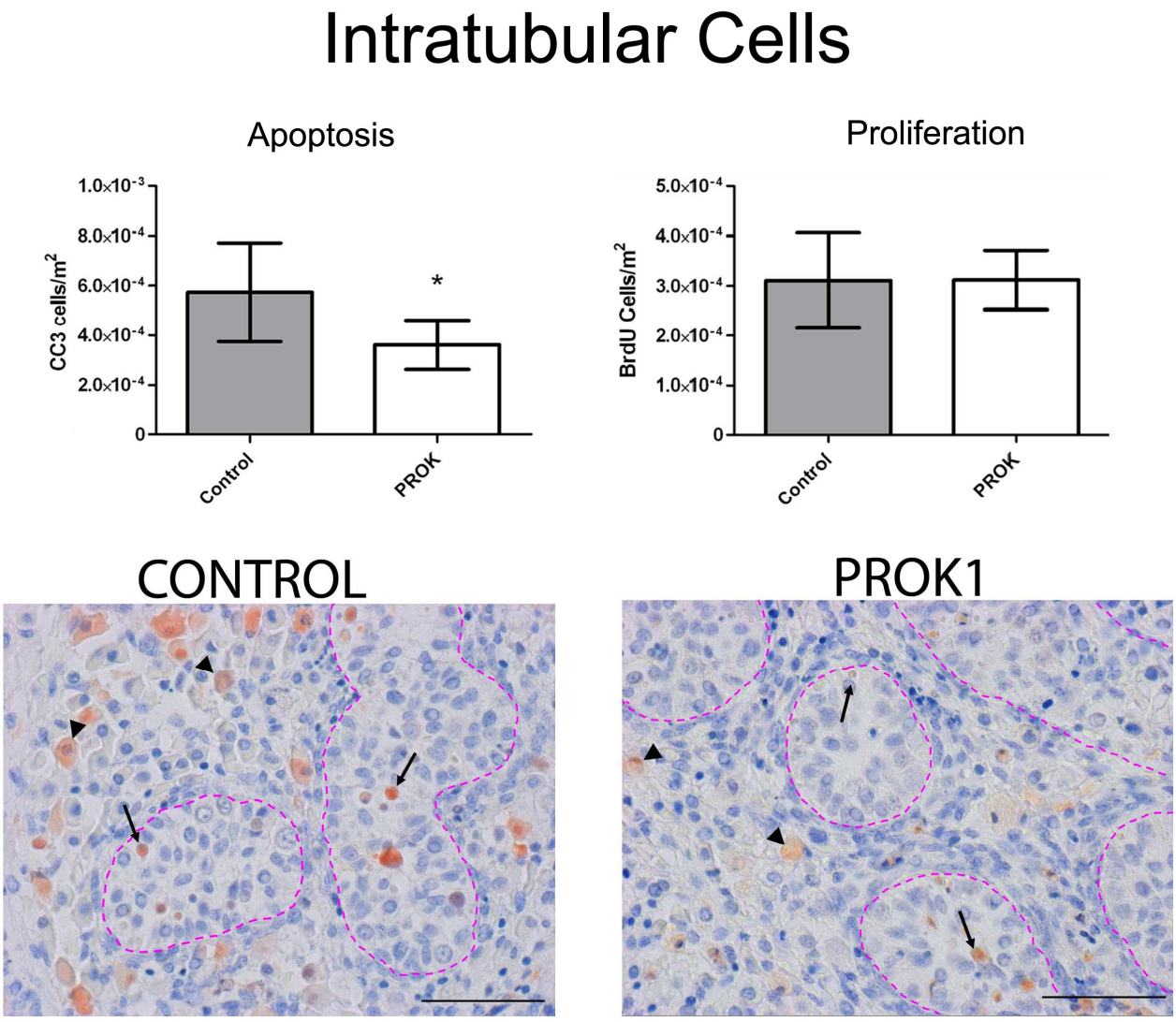

Figure 7.9 Significant reduction of apoptosis in intratubular cells with PROK1 treatment in the human fetal testis

Apoptosis and proliferation in the intratubular compartment (seminferous tubules outlined by dashed line) of the human fetal testis was quantified with the apoptotic marker cleaved caspase-3 (CC3) and the proliferative marker BrdU (14-17 weeks gestation, n=6). Positive intratubular cells were quantified and adjusted for tubular area. This determined PROK1 treatment significantly reduced apoptosis of intratubular cells (arrows) (*p=0.05) unlike in extratubular cells (arrowheads), but did not alter proliferation rate in either cell type. Scale bars equal 100 microns.

7.3.6 COX2 and DKK1 expression are up-regulated by PROK1 signalling via PROKR1

PROK1 cultures suggest a paracrine downstream factor regulates intratubular cell survival in the human fetal testis. Further investigation was performed to identify possible pathways affected by PROK1 signalling via differential plating of dissociated human fetal testes (n=6, 14-17 weeks gestation), which allowed for the segregation of the interstitial cell compliment from the intratubular cells and steroidogenic cells (germ, Sertoli, and Leydig cells do not adhere/survive after dissociation; Figure 7.10). To determine true differential plating was successful, a sample was taken after dissociation of cells and compared to untreated control cells after passage and culture.

A significant loss of germ, Sertoli and Leydig cells after passage was confirmed by significant reduction in cell-specific transcript expression. The germ cell-specific marker *VASA* demonstrated the most significant loss after passage (3.3±1.8 vs 0.1±0.05 x 10^{-3} relative to *RPL32*, p=0.0006), with both the Sertoli cell marker, *AMH* (7.3±2.7 vs 2.0±1.0 x10^{-3} relative to *RPL32*, p=0.02) and the Leydig cell marker *3βHSD* also reduced after passage (1.3±0.3 vs 0.2±0.1 x10^{-2} relative to *RPL32*, p=0.02). There was no change in the non-Leydig interstitial cell marker Desmin demonstrating these cells were retained in culture (p=0.9). This segregation allowed for further culture and treatment of PROKR1 target cells specifically, rather than the whole testis.

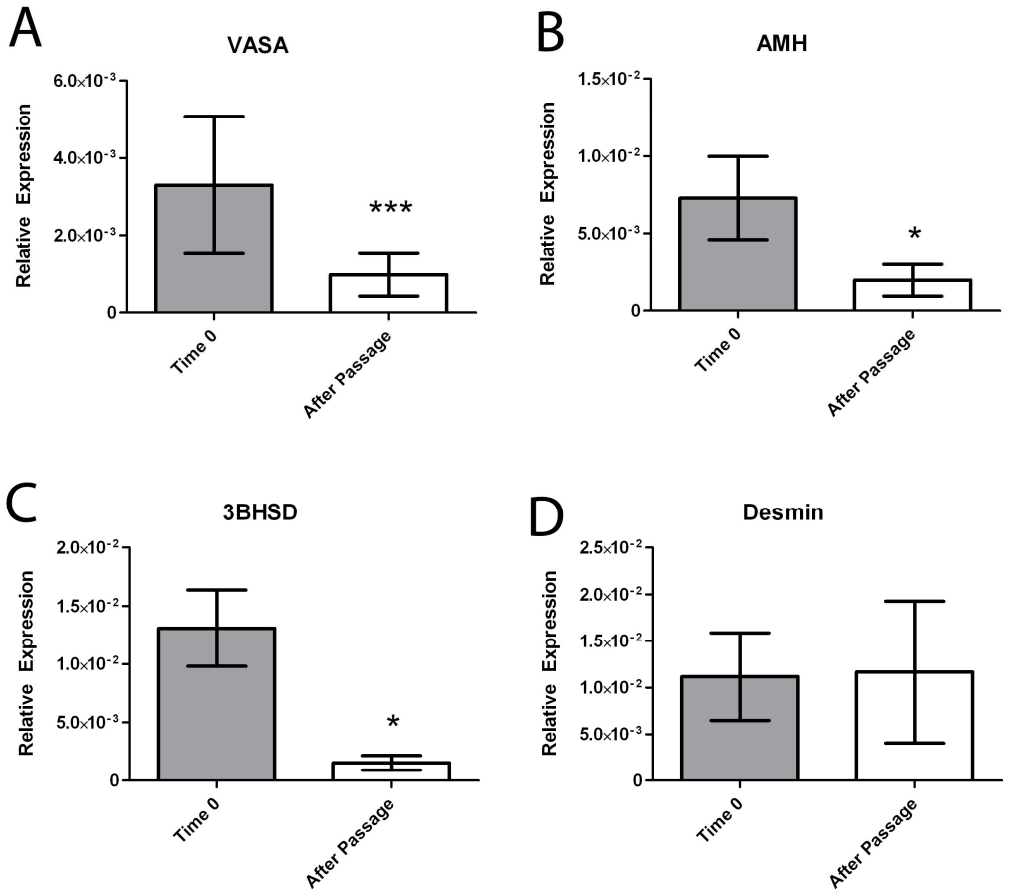

Figure 7.10 Characterisation of the testis interstitial cell cultures

Differential plating of disaggregated fetal testis (n=6, 14-17 weeks gestation) were performed in order to culture PROK specific target cells from the human fetal testis. qRT-PCR analysis of cells before plating and after passage (untreated for 24 hours) was performed to confirm which cell types were sustained. (A) The germ cell gene VASA, (B) Sertoli cell gene AMH and (C) Leydig cell gene 3βHSD were all significantly downregulated post-differential plating determining these cells did not adhere/survive to be PROK1 treated. (D) No change was seen in the interstitial cell marker Desmin demonstrating these cells were able to adhere in culture. * $p \leq 0.05$, *** $p \leq 0.001$.

Several downstream pathways were investigated, including the PG pathway, which has been demonstrated to have pro-survival effects on germ cells in the fetal gonads (ovarian data in chapter 3 and 4, testis data not shown). The PG precursor enzyme *COX2* was significantly up-regulated with PROK1 treatment compared to control (9.7±3.8 vs 3.9±1.5 $x10^{-5}$ relative percentage compared to the housekeeping gene *RPL32*, p=0.05, Figure 7.11A). However, the PGE_2 specific precursor enzyme PTGES (Figure 7.11B) and known downstream targets of PGE_2 (identified by human fetal cultures in Chapter 3) were not affected (7.11C-D).

Changes in the IL6-type cytokines were also investigated, as it has been postulated they target germ cells and in the endometrium data suggests they are direct targets of PROK1 action (Evans *et al*, 2008; Evans *et al*, 2009). However, no change in expression levels after 24h treatment with PROK1 was identified in *IL6*, *LIF*, *OSM* or *CNTF* (Figure 7.11E-H).

Additionally, the steroidogenic enzymes *CYP11A1* and *StAR* were investigated, as it was postulated PROK1 could be affecting the steroidogenesis pathway either directly or via COX2 regulation, thereby affecting the intratubular cells. However, no change was seen in either target (Figure 7.11I-J).

Finally, two genes identified in an array identifying possible PROK1 targets (Evans, 2008), and known to function in human gonad development were investigated; *DKK1* and *ID3*. *DKK1* was significantly up-regulated with PROK1 treatment compared to control (4.5±1.1 vs 3.6±1.0$x10^{-4}$ relative to *RPL32*, p=0.03, Figure 7.11K), but no change was seen in *ID3* (Figure 7.11L).

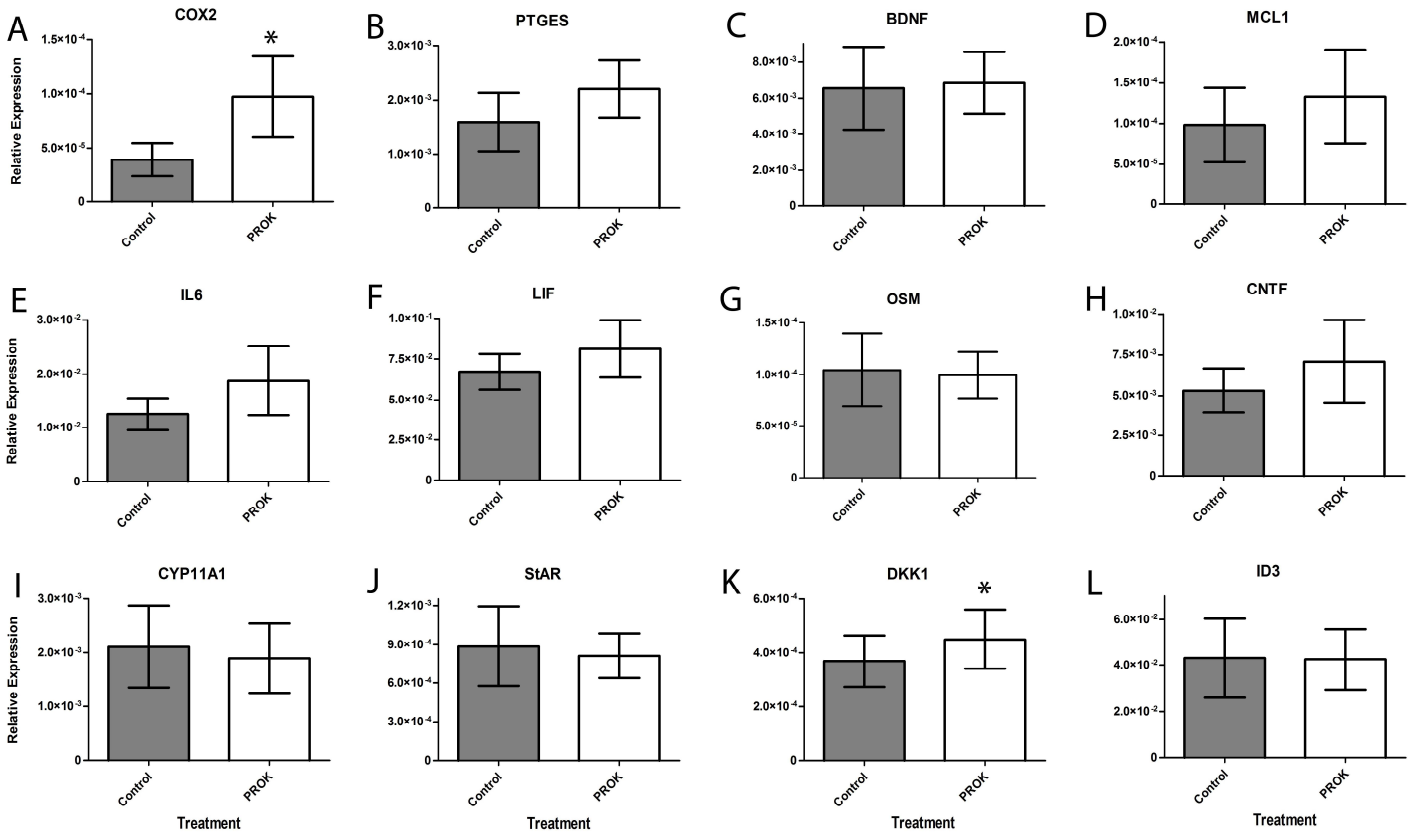

Figure 7.11 Gene expression changes of fetal testis interstitial cells once cultured with PROK1

Interstitial cells collected from human fetal testis samples (14-17 wks) were cultured with 40 nM PROK1 for 24h, resulting in (A) a significant up-regulation of the prostaglandin (PG) pre-cursor enzyme COX2 (*=p≤0.05) but no change (B) in the PGE_2 specific enzyme PTGES or (C-D) BDNF and MCL-1 known to be regulated by PGE_2. (E-H) No significant change was detected in the IL-6 type cytokines. (I-J) No change was seen in steroidogenic enzymes. (K) Significant up-regulation of DKK1 (*=p<0.05) was detected but no change was seen in ID3 which is known to interact with DKK1 in the fetal gonad. All data are expressed relative to the housekeeping gene *RPL32*. Significance is determined by log transformation (COX2 only) and paired t-test.

7.4 Discussion

As in the ovary, early development of the testis is imperative for adult fertility. The PROK ligands are two recently described proteins involved in angiogenesis and proliferation in other tissues; however despite array data demonstrating that PROK2 expression is high in the fetal testis compared to the ovary (LeCouter *et al*, 2003), PROK function has not been elucidated. This study confirms that PROK ligands and receptors are expressed in the human fetal testis and all components are developmentally-regulated. Additionally, it identifies the interstitial compartment as the site of PROK action in fetal testis, with primary expression of receptors on Leydig and non-Leydig cells as well as vasculature. Notably, this study also identifies a novel function for PROK1 as a pro-survival factor in the fetal testis, in the intratubular cell population consisting of germ and Sertoli cells. Localisation of the PROK receptors suggests this pro-survival effect is due to downstream paracrine signalling. Dissociation and culture of the interstitial cell portion of fetal testes was utilised to determine changes in gene expression and further identify paracrine pathways contributing to increased survival. These cultures identified key components of two pathways which were up-regulated with PROK1 treatment; *COX2* a PG precursor enzyme and *DKK1* an antagonist of the WNT pathway.

As previously demonstrated, inhibition of COX2 via paracetamol exposure leads to germ cell loss in rodent fetal gonads (Chapter 4). Additionally, human fetal ovarian cultures determined that PGE_2 signalling downstream of COX2 is able to up-regulate known differentiation factors (Bayne *et al*, 2009) (Chapter 3). Therefore, up-regulation of *COX2* by PROK1 may account for the pro-survival effects seen. COX2 regulation of steroidogenesis in Leydig cells has also been demonstrated, with inhibition of the enzyme resulting in increased testosterone production (Wang *et al*, 2003b; Wang *et al*, 2005; Chen *et al*, 2007a). Therefore, it was hypothesised that PROK1 regulation of *COX2* might modulate expression of either PGE_2 or steroidogenic components in order to exert pro-survival effects. Factors from both downstream pathways were investigated; however, no significant regulation of either pathway was identified in this study. As these alterations would be secondary to the up-regulation of *COX2* by PROK1 it is possible that subtle changes would not be

detected without additional sample numbers or at later time points. Nevertheless, these data confirm that *COX2* is up-regulated by PROK1 treatment, and that this treatment promotes intratubular survival further supporting the possible role of COX2 as an anti-apoptotic factor in the fetal gonads.

DKK1 was also up-regulated by PROK1 treatment. *DKK1* (as briefly discussed in Chapter 1) functions in early gonad development by modulating WNT signalling necessary for ovarian development (Vainio *et al*, 1999; Jeays-Ward *et al*, 2003). DKK1 binds to low-density lipoprotein receptor-related protein 6 (LRP6) a necessary component for WNT signalling (Bafico *et al*, 2001; Mao *et al*, 2001; Semenov *et al*, 2001), making cells less responsive to WNT signalling and preventing activation of downstream β-catenin signalling necessary for ovarian development (Bafico *et al*, 2001; Mao *et al*, 2001; Semenov *et al*, 2001). *Dkk1* is sex-specifically increased in the fetal testis during development (Manuylov *et al*, 2008; Combes *et al*, 2011) and is thought to prevent inappropriate WNT signalling. Loss of Wnt4 signalling using a homozygous null rodent model produced XX gonads with features normally associated with testis development (Yao *et al*, 2004). However, $Dkk1^{-/-}$ XY gonads do not show up-regulation of Wnt signalling nor are they feminised (Combes *et al*, 2011) leading to the hypothesis that *Dkk1* plays a supportive role in repression of ovarian development, rather than being a sole protective factor. The fetal testis interstitial culture data support this protective theory, as up-regulation of DKK1 with PROK1 treatment is concomitant with survival of intratubular cells.
Interestingly, the role for PROK1 in angiogenesis as demonstrated in the adult testis in previous studies (LeCouter *et al*, 2003; Samson *et al*, 2004), was not supported in the fetal testis, as no change was seen in proliferation of the extratubular cell population (including vascular endothelial cells). However, previous angiogenic studies in the testis were performed over 1 week (LeCouter *et al*, 2003). It is possible the cultures utilised in this study did not identify changes in vasculature due to the short period of culture (24h) or due to the stage of testis development, as vascularisation of the testis initiates at the beginning of second trimester with migration of endothelial cells (Nishino *et al*, 2001; Ostrer *et al*, 2007), but would not be as advanced as in later gestations or in the adult.

In addition to functional aspects of PROK1 in the human fetal testis, this study also demonstrates that PROK components are more highly expressed in the human fetal testis than in the ovary at similar gestations (Chapter 6), with developmental increases of PROK signalling components in both sexes, suggesting an increasingly important role for PROK during second trimester in both sexes. The fetal testis is quite dynamic during early development, with several processes occurring simultaneously. Therefore, pinpointing which processes PROK might be involved in based upon expression levels is not possible. However, PROK1 seems to be up-regulated around the initiation of Leydig differentiation and initiation of steroidogenesis (Codesal *et al*, 1990; Murray *et al*, 2000; Gaskell *et al*, 2004); whereas both receptors are up-regulated in late second trimester which is mostly associated with an increase in proliferation, differentiation, and vascular formation (Nishino *et al*, 2001). It is likely, based upon culture and localisation data, that PROK signalling is involved in regulation of some of these processes.

In addition to differential transcript expression between the sexes, there is also a discrepancy in PROK protein localisation. PROK targets the germ cells in the human fetal ovary, whereas in the testis the interstitial compartment is the site of PROK action. The two PROK receptors also display a striking difference in localisation, with PROKR1 primarily expressed in the non-Leydig interstitial cells and PROKR2 expressed by the Leydig cells. Although both receptors were expressed by the testicular vasculature, this drastic difference in primary site of localisation suggests the two receptors play differing roles during testis development. As PROK1 is able to signal interchangeably via either PROK receptor, it is not known which receptor is responsible for demonstrated pro-survival effects. Regulation of *COX2* and *DKK1* in cell lines transfected with the PROKR1 gene (Chapter 6) (Evans *et al*, 2008; Evans *et al*, 2009; Macdonald *et al*, 2010) support this mechanism for regulation in the testis. However, PROK1 treatment of PROKR1 transfected cells has a negative effect on proliferation in an endometrial epithelial cell line *in vitro* (Macdonald *et al*, 2010). Further investigation utilising receptor-specific antagonists would be beneficial for dissecting specific roles for PROKR1 and 2 in the fetal testis.

In conclusion, these data demonstrate that PROK ligands and receptor transcripts are developmentally-up-regulated and are expressed at higher levels in the human fetal testis than ovary. Additionally, in the fetal testis PROKs primarily target the Leydig and non-Leydig interstitial cells with additional expression in the vasculature. Despite this localisation, it was determined that PROK1 was able to promote survival of intratubular cells, inferring paracrine signalling to these cells, which may be via *DKK1, COX2*, or another downstream target yet to be identified. These data outline a novel role for PROK function in the human fetal testis and demonstrate it functions as a pro-survival factor during development.

Chapter 8

General Discussion

Chapter 8. General Discussion

8.1 Introduction

Fetal ovarian development encompasses several highly regulated events, from initial germ cell specification and migration to the proximal epiblast in formation the gonad, followed by sex determination, entry into meiosis and subsequent arrest, and concluding in germ cell nest breakdown and association of oocytes with granulosa cells to form primordial follicles. In addition to these developmental processes, germ cell number is also highly regulated across fetal ovarian development, the few germ cells generated at initial specification proliferate vastly, only for their number to be drastically reduced prior to primordial follicle formation. The regulation of both germ cell survival and development leads to the establishment of a primordial follicle pool, which defines the number of oocytes a woman will have for ovulation in adulthood, and thus determine her future fertility. Regulation of germ cell differentiation is provided by autocrine, paracrine, and juxtacrine signalling from both the germ cells themselves and their somatic environment, collectively termed the germ cell niche. Although identified as such, the factors which comprise the germ cell niche remain to be fully elucidated. This thesis encapsulates several studies that aimed to identify novel regulators of ovarian development, in an effort to further clarify what governs germ cell development and survival.

Three signalling pathways were initially hypothesised to regulate human fetal ovarian development, and thereby be part of the germ cell niche: prostaglandins (and specifically PGE_2), IL6-type cytokines, and the PROKs. These postulated regulators were identified as germ cell niche candidates for several key reasons (interaction with established factors of the germ cell niche, regulation of the adult ovary, and/or differentially regulated during early ovarian development) and were further investigated in order to establish the role of each factor, how they interact with established factors of the germ cell niche, and other postulated regulators.

8.2 Novel interactions in the germ cell niche

The experimental studies which constitute this thesis have demonstrated several novel factors and mechanisms within the germ cell niche (summarised in blue in Figure 8.4). These data provide further understanding of the inter-relationships between factors involved in germ cell development, as well as providing additional hypothesises for further interaction.

These data demonstrated that PROK1 and PROK2 signal in an autocrine manner to both PROKR1 and R2 receptors which are solely expressed by germ cells in the human fetal ovary (Figure 8.1A). Further, PROK1 via PROKR1 is able to up-regulate *COX2* expression (Chapter 6, Figure 8.1B), thereby demonstrating interaction between two of the postulated pathways.

Outwith the characterisation of novel components of the germ cell niche in the ovary, the work included in this thesis has also identified differential roles for the PROKs in the human fetal testis (as compared to the ovary, Chapter 7). These studies identified that not unlike in the ovary, PROK1 via PROKR1 is able to up-regulate *COX2* and promote survival of cells (Figure 8.1D). However, unlike the ovarian studies, PROK ligands were shown to solely target interstitial cells in the human fetal testis (Leydig and non-Leydig) and act in a paracrine fashion affecting intratubular cells rather than in an autocrine manner (Figure 8.1C). It was further demonstrated PROK1 is also able to regulate *DKK1*, a factor involved in sex determination, and thus may further promote survival via a male-specific pathway. As in the ovary, it is postulated PROK1 via PROKR1 up-regulation of *COX2* may be protective via interaction with the steroidogenesis pathway, whereby COX2 increases testosterone production promoting intratubular survival.

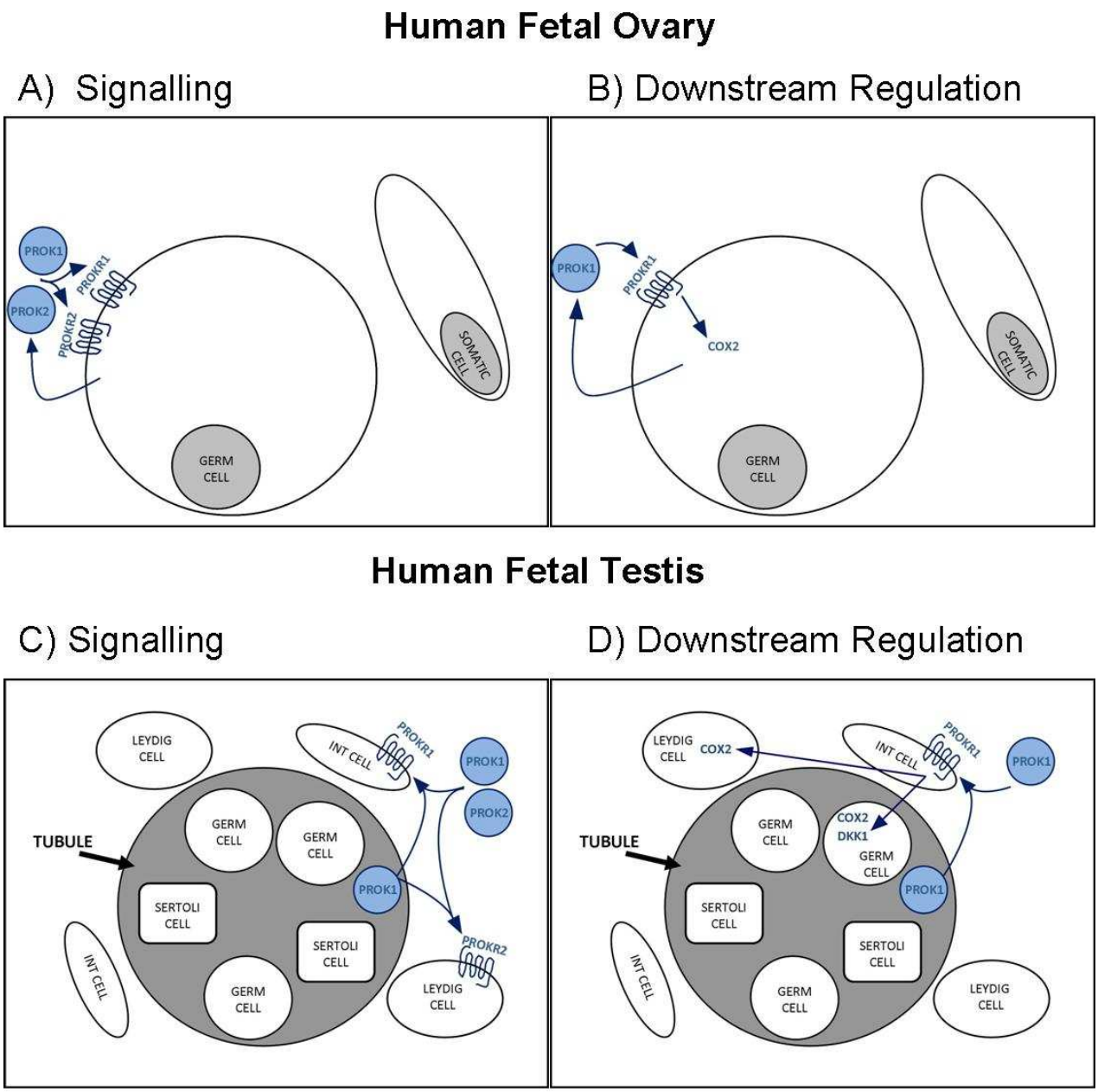

Figure 8.1 Prokineticin signalling in the fetal gonads

Summary of PROK signalling differences in the human fetal gonads as determined by studies this thesis. A) In the ovary the PROK ligands are expressed by the germ cells as are the PROK receptors demonstrating an autocrine signalling mechanism, B) with further data demonstrating PROK1 via the PROKR1 receptor is able to regulate *COX2* in the germ cells. C) Conversely, in the fetal testis ligand expression is more diffuse and the PROK receptors are localised to interstitial cells. D) However, as in the ovary, PROK1 via PROKR1 is able to regulate *COX2* expression as well as *DKK1*, a factor involved in sex-determination, both of which are expressed in other cells demonstrating paracrine downstream signalling. (Abbreviations: INT-interstitial, TUBULE- seminiferous tubule).

However, it is unlikely that all of the COX2 produced in the fetal ovary is a direct result of PROK1 signalling, as *COX2* expression increases linearly with gestation; whereas *PROK1* up-regulation is later in late second trimester. Further, it is possible that signalling by PROK2 via the same receptor (PROKR1) may have an additive effect; as PROK signalling has the potential for redundancy. In addition, *PROK2* unlike *PROK1* displays a similar pattern of expression to that of *COX2* in the ovary. Despite the possibility of an additive effect of PROK signalling, it is unlikely this is the only regulatory pathway up-stream of COX2 production in the ovary. Data from mice homozygous null for *PROKR1* and *PROKR2* support this hypothesis, as neither demonstrate any alteration in ovarian function ((Negri *et al*, 2006; Matsumoto *et al*, 2006) unlike *COX2*$^{-/-}$ and *EP2*$^{-/-}$ females, which are unable to ovulate normally (Dinchuk *et al*, 1995; Hizaki *et al*, 1999)). These data suggest further regulators outwith the PROKs, are responsible for some of the COX2 production in the human fetal gonads, particularly in earlier gestation (14-16 weeks), at which time *COX2* expression is up-regulated but *PROK1* expression is not.

Following up-regulation via PROK1 and other possible pathways, COX2 and subsequently, PTGES synthesize and release PGE_2 from fetal germ cells in the human fetal ovary, which then acts in an autocrine (via receptors EP2, 3, or 4, Figure 8.2A) or paracrine fashion (via EP3 or 4, Figure 8.2B) in order to regulate downstream targets including Activin A (*INHBA*), *BDNF*, and *MCL-1* (Chapter 3, Figure 8.2C).

It is hypothesised that regulation of Activin A is downstream of PGE_2 signalling via the EP2 receptor (Figure 8.2C), as this receptor is solely expressed by more mature germ cells, which are the site of Activin A expression and regulation, whereas EP3 and 4 are expressed by both somatic and germ cells. It therefore appears that PGE_2 promotes the role of Activin A in germ cell survival and proliferation (Martins da Silva *et al*, 2004). Further, data from this thesis suggest that PGE_2 promotes the inhibition of membrane-bound KL via increased expression of Activin A, which was previously demonstrated in the human ovary (Childs *et al*, 2010a), thereby preventing precocious primordial follicle formation.

BDNF expression is also stimulated by PGE_2 in the human fetal ovary, and may do so via two pathways. As the PGE_2 receptors EP3 and EP4 are expressed by somatic cells, the site of *BNDF* expression, PGE_2 may directly up-regulate expression of *BDNF* in the somatic cells (Figure8.2C). However, previous data have suggested a second mechanism, as *BDNF* is known to be regulated by Activin A in the human fetal ovary (Childs *et al*, 2010a) suggesting regulation may be via an indirect mechanism rather than via direct PGE_2 action (Figure 8.2D). Further, it is possible both mechanisms are functional in the regulation of *BDNF* expression. In this regard, it would be beneficial to perform interstitial-only ovarian cultures and compare with whole ovarian cultures to determine the route of regulation. However, as prostaglandins were initially identified as regulators of the neurotrophins in the endometrium (Jabbour, unpublished), a tissue which lacks a germ cell component, it is likely *BDNF* is up-regulated directly via PGE_2 signalling to the somatic cells or via additive direct and indirect signalling. Regardless of the initial mechanism, BDNF is then able signal via the TrkB receptor, which is known to be essential for oocyte survival at this time (Spears *et al*, 2003; Kerr *et al*, 2009).

Data from this study has also identified PGE_2 regulation of the anti-apoptotic factor MCL-1. As with *BDNF*, increased expression of *MCL-1* is postulated to be either direct via PGE_2 regulation (Figure 8.2C) or indirect (Figure 8.2D) downstream of regulation via Activin A (Hartley *et al*, 2002; Martins da Silva *et al*, 2004). Data from lung adenocarcinoma cultures also demonstrate MCL-1 regulation downstream of COX2 signalling is a pathway that is conserved in several tissues (Lin *et al*, 2001; Chen *et al*, 2010). Activation of this pathway further supports the hypothesis that PGE_2 acts as a pro-survival factor during human fetal ovarian development, a hypothesis that was supported by studies inhibiting COX2 expression (and thereby PGE_2 production) in fetal rat ovaries *in vivo* (Chapter 4). However, this thesis has demonstrated fetal COX2 inhibition lead to significant oocyte loss that was not via increased apoptosis or decreased proliferation. It is hypothesised that part of this phenotype may be the result of decreased Activin A, BDNF and MCL-1 production via inhibition of PGE_2 signalling, and that this signalling in turn may have some effect on maturation/development of germ cells, instead of affecting apoptosis.

However, an alternative hypothesis is that the reduction in germ cell number is a result of COX2 regulation of steroidogenesis (Figure 8.2E), which has been demonstrated in adult tissue (Wang *et al*, 2003b; Wang *et al*, 2005; Chen *et al*, 2007a). Testosterone production is indeed perturbed in the male counterparts exposed to COX2 inhibition in this study. Therefore, it is suggested the loss of oocytes might be due to altered steroidogenesis in the ovary. Although little is known about the role of steroid regulation in the human fetal ovary, estrogen has been established as a key factor necessary for germ cell nest breakdown in the mouse thereby allowing for primordial follicle formation (Iguchi *et al*, 1986; Kezele & Skinner, 2003; Chen *et al*, 2009). If steroidogenesis is altered by COX2 inhibition, thereby reducing testosterone or if indeed estrogen is directly affected by COX2 inhibition, it is possible germ cell nests are precociously broken down, resulting in germ cell atresia out-with programmed cell death. It is also possible that alteration of testosterone alone could affect germ cell survival, as ARKO female mice undergo early ovarian failure (Shiina *et al*, 2006).

Further, data from a non-human primate model suggests a similar role for estrogen (Zachos *et al*, 2002) and has determined that estrogen may regulate germ cell nest breakdown via up-regulation of microvilli expression, ECM proteins, and altering the Activin : Inhibin ratio (Billiar *et al*, 2003; Billiar *et al*, 2004; Zachos *et al*, 2004; Bocca *et al*, 2008; Zachos *et al*, 2008). These data suggest a similar mechanism is also likely in the human, where expression of steroid receptors and synthesis enzymes have been confirmed (Fowler *et al*, 2011).

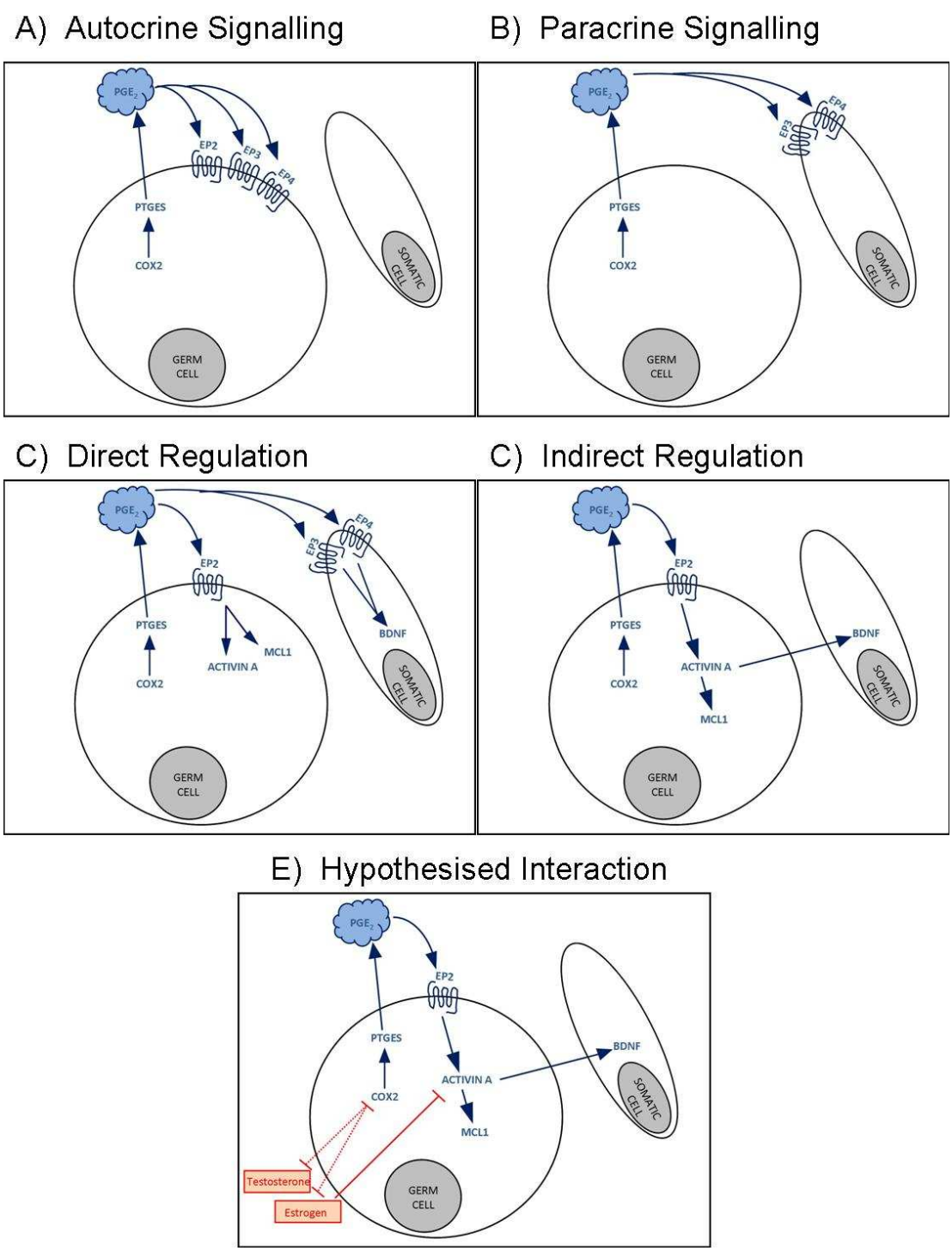

Figure 8.2 Prostaglandin signalling in the fetal ovary

Data from this thesis has determined that PGE_2 is able to signal in both an A) autocrine and B) paracrine manner, with downstream up-regulation of Activin A, MCL1 and BDNF, which maybe via either C) direct or D) indirect mechanisms. In addition, it is hypothesised that this pathway might also interact with the steroid hormone pathway to further regulate human ovarian development.

Another further group of factors has further been characterised in the human fetal ovary, the IL6-type cytokines (Chapter 5). Although function of these factors has not been investigated in this thesis, the present data demonstrate that they solely target germ cells in the human fetal ovary, as their shared receptor components gp130 and LIFR are exclusively localised to these cells (Figure 8.3A). Investigation of the IL6-type cytokines has further demonstrated developmentally-regulated transcript expression of the shared receptor components and the OSM ligand in the period leading up to primordial follicle formation. Based on these data, it is hypothesised that the IL6-type cytokines are likely to function in later oocyte development. As LIF has previously been shown to regulate the primordial to primary transition in the rodent ovarian culture, it may play a similar role in the human (Nilsson *et al*, 2002; Nilsson & Skinner, 2004). Both KL and bFGF, which are well-established in cooperation with LIF in the maintenance of stem cell culture, also function alongside LIF to promote follicle activation in these rodent ovarian cultures. In addition, similar up-regulation of *bFGF* and *KL* (as compared to the IL6-type receptors) is noted in the human (Figure 8.3B) (Robinson *et al*, 2001; Quennell *et al*, 2004; Stoop *et al*, 2005) suggesting a mechanism of bFGF/LIF/KL interaction may be important in the human as well as in the rodent for follicle transition, however this remains speculative. Further study of IL6-type cytokine interaction with bFGF and KL, as well other established regulators of follicle activation including FOXO3a, GDF9 and BMP15, would further clarify the roles of these factors in the human fetal ovary.

.

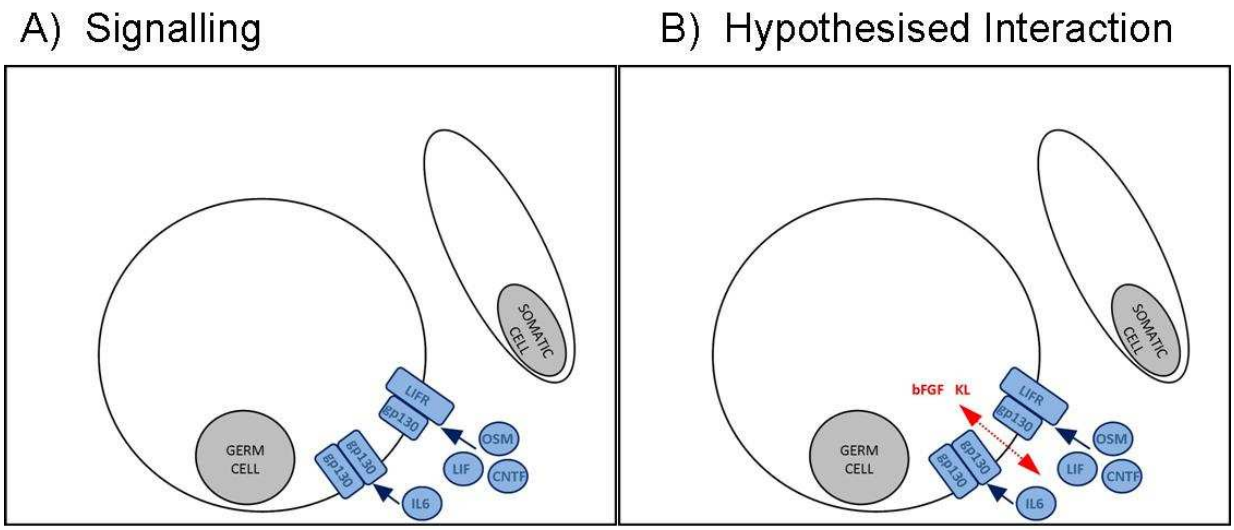

Figure 8.3 IL6-type cytokine signalling in the fetal ovary

This thesis has determined A) the IL6-type cytokines signal solely to the germ cells in the human fetal ovary as their two shared receptors (gp130 and LIFR), necessary for all IL6-type signalling, are located on these cells. In addition, mRNA expression patterns suggest the IL6-type cytokines may play a role in later follicular development, B) leading to the hypothesis that the IL6-type cytokines may interact with bFGF and KL, which are important for later germ cell development *in vivo* and are known to interact with the IL6-type cytokine *in vitro*.

8.3 Conclusions

In conclusion, this thesis has demonstrated PGE_2, the IL6-type cytokines, and the PROKs are all expressed in the human fetal ovary, and signal directly to the germ cells (summarised in Figure 8.4). Additionally, functional studies have identified PROK1 and PGE_2 are up-stream of pro-survival and proliferation pathways and thereby play a role in regulating fetal oocyte development as part of the germ cell niche. PROK1 also plays a similar pro-survival role in the fetal testis. However, in both the fetal testis and the fetal ovary, it is likely that COX2 downstream of PROK1 plays a larger role pro-survival/anti-apoptotic function, as COX2 is able to directly modulate these pathways via several downstream targets including Activin A, BDNF, and MCL-1, as well as a likely link with steroidogenesis. In addition, this thesis further speculates that the COX2 produced by the fetal ovary is not solely the result of increased PROK1 signalling, and that other up-stream regulatory pathways have yet to be identified.

These studies have contributed to the understanding of fetal ovarian development in the human, and as such have provided further knowledge regarding what factors influence germ cell death and survival prior to primordial follicle formation. Disruption of these developmental events can result in POI or infertility, and thus understanding these pathways is essential for women's reproductive health.

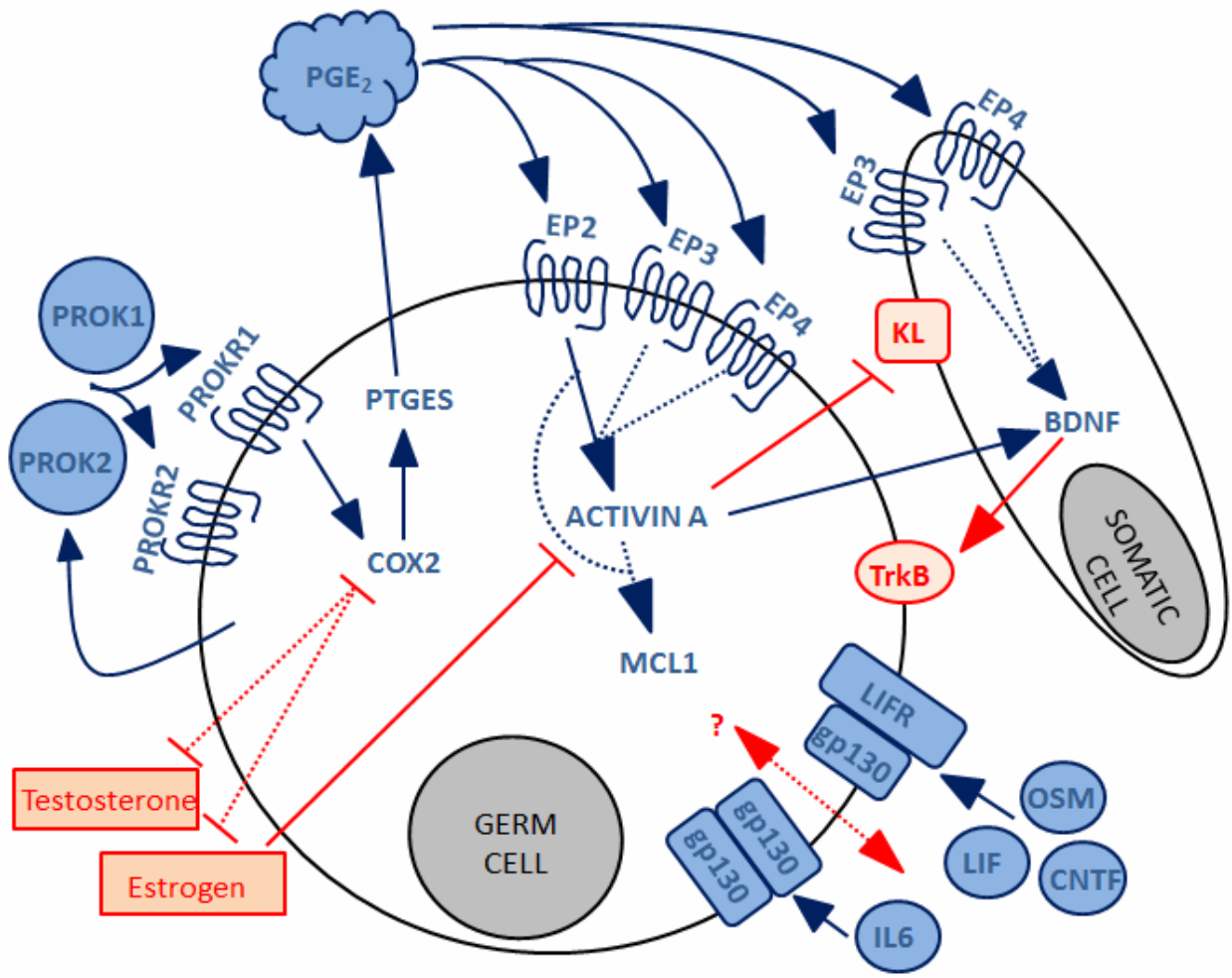

Figure 8.4 Summary of the novel pathways involved in the germ cell niche and their likely interactions with established pathways

Schematic representation of the newly characterised factors of the germ cell niche and their downstream interactions (blue), in corporation with suggested interactions with established signalling pathways essential for early germ cell survival and development (red). Established pathways are demonstrated in solid lines whereas those hypothesised are represented by dashed lines. In the case of EP2 regulation of Activin A, this pathway is not established, but is most likely.

8.4 Future Work

Although this thesis initially established function for novel factors in the human fetal ovary and testis, further work is necessary to fully understand the mechanisms and overall function of these factors throughout ovarian biology. Follow-up studies of particular importance are as follows:

8.4.1 Paracetamol Exposure Study

The paracetamol study is of particular interest for further work, as data from e17.5 rats has demonstrated there is no change in germ cell number in early gestation, and there is no apparent mechanism for loss by e21.5 (no change in apoptosis or proliferation). Therefore, further analysis of ovaries at this stage is necessary in order to establish possible mechanisms of action. Data from the male paracetamol exposed pups has noted a change in steroidogenesis, suggesting it will be important to investigate levels of steroid expression in the female pups, as well as levels of PGE_2 to identify the likely downstream targets of paracetamol action resulting in reduced germ cell number. To that end, the levels of steroids and PGE_2 will also be investigated in the dam to see how the maternal system is affected. As discussed previously, estrogen is known to function in nest breakdown and alteration of the estrogen levels in the dam could possibly perturb this process in the pups.

In addition, as a reduction in germ cell number is likely to impact on primordial follicle formation and thus affect adult fertility, future studies are planned to track effects of paracetamol exposure on primordial follicle formation in offspring, and throughout postnatal life in order to identify possible premature follicle depletion in aged paracetamol-exposed females.

8.4.2 PROK1 effects in the human fetal testis

Although it was determined that PROK1 functions to reduce apoptosis in intratubular cells of the human fetal testis, further study is necessary to determine if this increased survival is of Sertoli or germ cells (or both). Identification of which cell type is being affected will aid in understanding downstream mechanism of PROK action, and will be performed by stereological analysis of dual detection for a germ cell marker and cleaved caspase-3.

References

Aaltonen J, Laitinen MP, Vuojolainen K, Jaatinen R, Horelli-Kuitunen N, Seppa L, Louhio H, Tuuri T, Sjoberg J, Butzow R, Hovatta O, Dale L, Ritvos O (1999) Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. *Journal of Clinical Endocrinology & Metabolism* **84:** 2744-2750

Abir R, Orvieto R, Dicker D, Zukerman Z, Barnett M, Fisch B (2002) Preliminary studies on apoptosis in human fetal ovaries. *Fertility and Sterility* **78:** 259-264

Abir R, Fisch B, Jin S, Barnet M, Freimann S, Van den Hurk R, Feldberg D, Nitke S, Krissi H, Ao A (2004) Immunocytochemical detection and RT-PCR expression of leukaemia inhibitory factor and its receptor in human fetal and adult ovaries. *Molecular Human Reproduction* **10:** 313-319

Adams IR, McLaren A (2002) Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. *Development* **129:** 1155-1164

Adhikari D, Liu K (2010) mTOR signaling in the control of activation of primordial follicles. *Cell Cycle* **9:** 1673-1674

Albamonte MS, Willis MA, Albamonte MI, Jensen F, Espinosa MB, Vitullo AD (2008) The developing human ovary: immunohistochemical analysis of germ-cell-specific VASA protein, BCL-2/BAX expression balance and apoptosis. *Human Reproduction* **23:** 1895-1901

Albrecht KH, Eicher EM (2001) Evidence that Sry is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. *Developmental Biology* **240:** 92-107

Alfranca A, Iniguez MA, Fresno M, Redondo JM (2006) Prostanoid signal transduction and gene expression in the endothelium: Role in cardiovascular diseases. *Cardiovascular Research* **70:** 446-456

Anderson EL, Baltus AE, Roepers-Gajadien HL, Hassold TJ, de Rooij DG, van Pelt AMM, Page DC (2008) Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proceedings of the National Academy of Sciences of the United States of America* **105:** 14976-14980

Anderson R, Fassler R, Georges-Labouesse E, Hynes RO, Bader BL, Kreidberg JA, Schaible K, Heasman J, Wylie C (1999) Mouse primordial germ cells lacking beta 1 integrins enter the germline but fail to migrate normally to the gonads. *Development* **126:** 1655-1664

Anderson R, Copeland TK, Scholer H, Heasman J, Wylie C (2000) The onset of germ cell migration in the mouse embryo. *Mechanisms of Development* **91:** 61-68

Anderson RA, Cambray N, Hartley PS, McNeilly AS (2002a) Expression and localization of inhibin alpha, inhibin/activin beta A and beta B and the activin type II and inhibin beta-glycan receptors in the developing human testis. *Reproduction* **123:** 779-788

Anderson RA, Robinson LLL, Brooks J, Spears N (2002b) Neurotropins and their receptors are expressed in the human fetal ovary. *Journal of Clinical Endocrinology & Metabolism* **87:** 890-897

Anderson RA, Fulton N, Cowan G, Coutts S, Saunders PTK (2007) Conserved and divergent patterns of expression of DAZL, VASA and OCT4 in the germ cells of the human fetal ovary and testis. *BMC Developmental Biology* **7:** 136

Ara T, Nakamura Y, Egawa T, Sugiyama T, Abe K, Kishimoto T, Matsui Y, Nagasawa T (2003) Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). *Proceedings of the National Academy of Sciences of the United States of America* **100:** 5319-5323

Arango NA, Lovell-Badge R, Behringer RR (1999) Targeted mutagenesis of the endogenous mouse Mis gene promoter: In vivo definition of genetic pathways of vertebrate sexual development. *Cell* **99:** 409-419

Attisano L, Wrana JL (1996) Signal transduction by members of the transforming growth factor-beta superfamily. *Cytokine and Growth Factor Reviews* **7:** 327-339

Ayoub SS, Colville-Nash PR, Willoughby DA, Botting RM (2006) The involvement of a cyclooxygenase 1 gene-derived protein in the antinociceptive action of paracetamol in mice. *European Journal of Pharmacology* **538:** 57-65

Bafico A, Liu GZ, Yaniv A, Gazit A, Aaronson SA (2001) Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nature Cell Biology* **3:** 683-686

Bagheri-Fam S, Argentaro A, Svingen T, Combes AN, Sinclair AH, Koopman P, Harley VR (2011) Defective survival of proliferating Sertoli cells and androgen receptor function in a mouse model of the ATR-X syndrome. *Human Molecular Genetics* **20:** 2213-2224

Baillet AM-P, B (2011) Mammalian ovary differentiation- A focus on female meiosis. *Molecular and Cellular Endocrinology* DOI:10.1016/j.mce.2011.09.029

Baird DT, Cameron ST, Critchley HOD, Drudy TA, Howe A, Jones RL, Lea RG, Kelly RW (1996) Prostaglandins and menstruation. *European Journal of Obstetrics Gynecology and Reproductive Biology* **70:** 15-17

Baker TG (1963) A quantitative and cytological study of germ cells in the human ovaries. *Proceedings of the Royal Society of London Series B-Biological Sciences* **158:** 417-433

Baker TG, Franchi LL (1967) The fine structure of oogonia and oocytes in human ovaries. *Journal of cell science* **2:** 213-224

Ballow DJ, Xin Y, Choi Y, Pangas SA, Rajkovic A (2006) Sohlh2 is a germ cell-specific bHLH transcription factor. *Gene Expression Patterns* **6:** 1014-1018

Baltus AE, Menke DB, Hu YC, Goodheart ML, Carpenter AE, de Rooij DG, Page DC (2006) In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nature Genetics* **38:** 1430-1434

Bamberger ES, Perrett CW (2002) Angiogenesis in epithelian ovarian cancer. *Journal of Clinical Pathology-Molecular Pathology* **55:** 348-359

Barbara PD, Moniot B, Poulat F, Berta P (2000) Expression and subcellular localization of SF-1, SOX9, WT1, and AMH proteins during early human testicular development. *Developmental Dynamics* **217:** 293-298

Bardoni B, Zanaria E, Guioli S, Floridia G, Worley KC, Tonini G, Ferrante E, Chiumello G, McCabe ERB, Fraccaro M, Zuffardi O, Camerino G (1994) A dosage sensitive locus at chromosome xp21 is involved in male to female sex reversal. *Nature Genetics* **7:** 497-501

Bassil AK, Dass NB, Murray CD, Muir A, Sanger GJ (2005) Prokineticin-2, motilin, ghrelin and metoclopramide: Prokinetic utility in mouse stomach and colon. *European Journal of Pharmacology* **524:** 138-144

Battersby S, Critchley HOD, Morgan K, Millar RP, Jabbour HN (2004) Expression and regulation of the prokineticins (endocrine gland-derived vascular endothelial growth factor and Bv8) and their receptors in the human endometrium across the menstrual cycle. *Journal of Clinical Endocrinology & Metabolism* **89:** 2463-2469

Baum JS, St George JP, McCall K (2005) Programmed cell death in the germline. *Seminars in Cell & Developmental Biology* **16:** 245-259

Bayne RA, Eddie SL, Collins CS, Childs AJ, Jabbour HN, Anderson RA (2009) Prostaglandin E2 as a regulator of germ cells during ovarian development. *The Journal of Clinical Endocrinology and Metabolism* **94:** 4053-4060

Bayne RAL, da Silva SJM, Anderson RA (2004) Increased expression of the FIGLA transcription factor is associated with primordial follicle formation in the human fetal ovary. *Molecular Human Reproduction* **10:** 373-381

Beck ARP, Miller IJ, Anderson P, Streuli M (1998) RNA-binding protein TIAR is essential for primordial germ cell development. *Proceedings of the National Academy of Sciences of the United States of America* **95:** 2331-2336

Behringer RR (1995) The Mullerian inhibitor and mammalian sexual development. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **350:** 285-288

Behrman HR (1979) Prostaglandins in hypothalamo-pituitary and ovarian function. *Annual Review of Physiology* **41:** 685-700

Ben-Haroush A, Abir R, Ao A, Jin S, Kessler-Icekson G, Feldberg D, Fisch B (2005) Expression of basic fibroblast growth factor and its receptors in human ovarian follicles from adults and fetuses. *Fertility and Sterility* **84:** 1257-1268

Bendel-Stenzel M, Anderson R, Heasman J, Wylie C (1998) The origin and migration of primordial germ cells in the mouse. *Seminars in Cell & Developmental Biology* **9:** 393-400

Bendsen E, Byskov AG, Andersen CY, Westergaard LG (2006) Number of germ cells and somatic cells in human fetal ovaries during the first weeks after sex differentiation. *Human Reproduction* **21:** 30-35

Benedict JC, Lin TM, Loeffler IK, Peterson RE, Flaws JA (2000) Physiological role of the aryl hydrocarbon receptor in mouse ovary development. *Toxicological Sciences* **56:** 382-388

Bergeron L, Perez GI, Macdonald G, Shi LF, Sun Y, Jurisicova A, Varmuza S, Latham KE, Flaws JA, Salter JCM, Hara H, Moskowitz MA, Li E, Greenberg A, Tilly JL, Yuan JY (1998) Defects in regulation of apoptosis in caspase-2-deficient mice. *Genes & Development* **12:** 1304-1314

Bernard P, Sim H, Knower K, Vilain E, Harley V (2008) Human SRY inhibits beta-catenin-mediated transcription. *International Journal of Biochemistry & Cell Biology* **40:** 2889-2900

Bernd P (2008) The Role of Neurotrophins During Early Development. *Gene Expression* **14:** 241-250

Bernstein R, Jenkins T, Dawson B, Wagner J, Dewald G, Koo GC, Wachtel SS (1980) Female phenotype and multiple abnormalities in sibs with a Y-chromosome and partial X-chromosome duplication-H-Y-Antigen and XG blood-group findings. *Journal of Medical Genetics* **17:** 291-300

Bezard J, Vigier B, Tran D, Mauleon P, Josso N (1987) Immunocytochemical study of anti-Mullerian hormone in sheep ovarian follicles during fetal and postnatal development. *Journal of Reproduction and Fertility* **80:** 509-516

Bhattacharya M, Peri KG, Almazan G, Ribeiro-Da-Silva A, Shichi H, Durocher Y, Abramovitz M, Hou X, Varma DR, Chemtob S (1998) Nuclear localization of prostaglandin E-2 receptors. *Proceedings of the National Academy of Sciences of the United States of America* **95:** 15792-15797

Bhattacharya M, Peri K, Ribeiro-da-Silva A, Almazan G, Shichi H, Hou X, Varma DR, Chemtob S (1999) Localization of functional prostaglandin E-2 receptors EP3 and EP4 in the nuclear envelope. *Journal of Biological Chemistry* **274:** 15719-15724

Bianco RB, Melisi D, Ciardiello F, Tortora G (2006) Key cancer cell signal transduction pathways as therapeutic targets. *European Journal of Cancer* **42:** 290-294

Biason-Lauber A, Schoenle EJ (2000) Apparently normal ovarian differentiation in a prepubertal girl with transcriptionally inactive steroidogenic factor 1 (NR5A1/SF-1) and adrenocortical insufficiency. *American Journal of Human Genetics* **67:** 1563-1568

Biason-Lauber A, Konrad D, Navratil F, Schoenle EJ (2004) A WNT4 mutation associated with Mullerian-duct regression and virilization in a 46,XX woman. *New England Journal of Medicine* **351:** 792-798

Bibel M, Barde YA (2000) Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes & Development* **14:** 2919-2937

Billiar RB, Zachos NC, Burch MG, Albrecht ED, Pepe GJ (2003) Up-regulation of alpha-inhibin expression in the fetal ovary of estrogen-suppressed baboons is associated with impaired fetal ovarian folliculogenesis. *Biology of Reproduction* **68:** 1989-1996

Billiar RB, St Clair B, Zachos NC, Burch MG, Albrecht ED, Pepe GJ (2004) Localization and developmental expression of the activin signal transduction proteins Smads 2, 3, and 4 in the baboon fetal ovary. *Biology of Reproduction* **70:** 586-592

Birk OS, Casiano DE, Wassif CA, Cogliati T, Zhao LP, Zhao YG, Grinberg A, Huang SP, Kreidberg JA, Parker KL, Porter FD, Westphal H (2000) The LIM homeobox gene Lhx9 is essential for mouse gonad formation. *Nature* **403:** 909-913

Bitgood MJ, Shen LY, McMahon AP (1996) Sertoli cell signaling by Desert hedgehog regulates the male germline. *Current Biology* **6:** 298-304

Bleil JD, Wassarman PM (1980) Mammalian sperm-egg interaction - identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell* **20:** 873-882

Bocca SM, Billiar RB, Albrecht ED, Pepe GJ (2008) Oocytes of baboon fetal primordial ovarian follicles express estrogen receptor beta mRNA. *Endocrine* **33:** 254-260

Boise LH, Gonzalezgarcia M, Postema CE, Ding LY, Lindsten T, Turka LA, Mao XH, Nunez G, Thompson CB (1993) Bcl-X, a Bcl-2-related gene that functions as a dominant regulator of apoptotic cell-death. *Cell* **74:** 597-608

Bombardier C (2002) An evidence-based evaluation of the gastrointestinal safety of coxibs. *American Journal of Cardiology* **89:** 3D-9D

Borum K (1961) Oogenesis in mouse - a study of meiotic prophase. *Experimental Cell Research* **24:** 495-507

Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, Yashiro K, Chawengsaksophak K, Wilson MJ, Rossant J, Hamada H, Koopman P (2006) Retinoid signaling determines germ cell fate in mice. *Science* **312:** 596-600

Brennan J, Tilmann C, Capel B (2003) Pdgfr-alpha mediates testis cord organization and fetal Leydig cell development in the XY gonad. *Genes & Development* **17:** 800-810

Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgan K, Lines C, Riddell R, Morton D, Lanas A, Konstam MA, Baron JA, Investigators APT (2005) Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *New England Journal of Medicine* **352:** 1092-1102

Bristol-Gould SK, Kreeger PK, Selkirk CG, Kilen SM, Mayo KE, Shea LD, Woodruff TK (2006) Fate of the initial follicle pool: Empirical and mathematical evidence supporting its sufficiency for adult fertility. *Developmental Biology* **298:** 149-154

Britt KL, Saunders PK, McPherson SJ, Misso ML, Simpson ER, Findlay JK (2004) Estrogen actions on follicle formation and early follicle development. *Biology of Reproduction* **71:** 1712-1723

Buehr M, Gu S, McLaren A (1993) Mesonephric contribution to testis differentiation in the fetal mouse. *Development* **117:** 273-281

Burns KH, Matzuk MM (2002) Minireview: Genetic models for the study of gonadotropin actions. *Endocrinology* **143:** 2823-2835

Byskov AG (1986) Differentiation of mammalian embryonic gonad. *Physiological Reviews* **66:** 71-117

Byskov AG, Faddy MJ, Lemmen JG, Andersen CY (2005) Eggs forever? *Differentiation* **73:** 438-446

Canto P, Soderlund D, Reyes E, Mendez JP (2004) Mutations in the Desert hedgehog (DHH) gene in patients with 46,XY complete pure gonadal dysgenesis. *Journal of Clinical Endocrinology & Metabolism* **89:** 4480-4483

Canto P, Vilchis F, Soderlund D, Reyes E, Mendez JP (2005) A heterozygous mutation in the desert hedgehog gene in patients with mixed gonadal dysgenesis. *Molecular Human Reproduction* **11:** 833-836

Capel B, Albrecht KH, Washburn LL, Eicher EM (1999) Migration of mesonephric cells into the mammalian gonad depends on Sry. *Mechanisms of Development* **84:** 127-131

Caronia LM, Martin C, Welt CK, Sykiotis GP, Quinton R, Thambundit A, Avbelj M, Dhruvakumar S, Plummer L, Hughes VA, Seminara SB, Boepple PA, Sidis Y, Crowley WF, Jr., Martin KA, Hall JE, Pitteloud N (2011) A Genetic Basis for Functional Hypothalamic Amenorrhea. *New England Journal of Medicine* **364:** 215-225

Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoute J, Shao WL, Hestermann EV, Geistlinger TR, Fox EA, Silver PA, Brown M (2005) Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* **122:** 33-43

Castrillon DH, Miao LL, Kollipara R, Horner JW, DePinho RA (2003) Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science* **301:** 215-218

Catalano RD, Lannagan TRM, Gorowiec M, Denison FC, Norman JE, Jabbour HN (2010) Prokineticins: novel mediators of inflammatory and contractile pathways at parturition? *Molecular Human Reproduction* **16:** 311-319

Cha YI, Solnica-Krezel L, DuBois RN (2006) Fishing for prostanoids: Deciphering the developmental functions of cyclooxygenase-derived prostaglandins. *Developmental Biology* **289:** 263-272

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113:** 643-655

Chandrasekharan NV, Dai H, Roos KLT, Evanson NK, Tomsik J, Elton TS, Simmons DL (2002) COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: Cloning, structure, and expression. *Proceedings of the National Academy of Sciences of the United States of America* **99:** 13926-13931

Chang H, Matzuk MM (2001) Smad5 is required for mouse primordial germ cell development. *Mechanisms of Development* **104:** 61-67

Chassot AA, Gregoire EP, Magliano M, Lavery R, Chaboissier MC (2008) Genetics of Ovarian Differentiation: Rspo1, a Major Player. *Sexual Development* **2:** 219-227

Chen HL, Luo LD, Liu J, Zirkin BR (2007a) Cyclooxygenases in rat Leydig cells: Effects of luteinizing hormone and aging. *Endocrinology* **148:** 735-742

Chen Y, Jefferson WN, Newbold RR, Padilla-Banks E, Pepling ME (2007b) Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary in vitro and in vivo. *Endocrinology* **148:** 3580-3590

Chen Y, Breen K, Pepling ME (2009) Estrogen can signal through multiple pathways to regulate oocyte cyst breakdown and primordial follicle assembly in the neonatal mouse ovary. *Journal of Endocrinology* **202:** 407-417

Chen W, Bai L, Wang X, Xu S, Belinsky SA, Lin Y (2010) Acquired activation of the Akt/cyclooxygenase-2/Mcl-1 pathway renders lung cancer cells resistant to apoptosis. *Molecular Pharmacology* **3:** 416-423

Cheng L, Gearing DP, White LS, Compton DL, Schooley K, Donovan PJ (1994) Role of leukemia inhibitory factor and its receptor in mouse primordial germ cell growth. *Development* **120:** 3145-3153

Cheng MY, Bullock CM, Li CY, Lee AG, Bermak JC, Belluzzi J, Weaver DR, Leslie FM, Zhou QY (2002) Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. *Nature* **417:** 405-410

Cheng MY, Bittman EL, Hattar S, Zhou QY (2005) Regulation of prokineticin 2 expression by light and the circadian clock. *BMC Neuroscience* **6:** 17

Cheng T, Cao W, Wen R, Steinberg RH, LaVail MM (1998) Prostaglandin E-2 induces vascular endothelial growth factor and basic fibroblast growth factor mRNA expression in cultured rat Muller cells. *Investigative Ophthalmology & Visual Science* **39:** 581-591

Childs AJ, Anderson RA (2009) Activin A selectively represses expression of the membrane-bound isoform of Kit ligand in human fetal ovary. *Fertility and Sterility* **92:** 1416-1419

Childs AJ, Bayne RAL, Murray AA, Da Silva SJM, Collins CS, Spears N, Anderson RA (2010a) Differential Expression and Regulation by Activin of the Neurotrophins BDNF and NT4 During Human and Mouse Ovarian Development. *Developmental Dynamics* **239:** 1211-1219

Childs AJ, Kinnell HL, Collins CS, Hogg K, Bayne RAL, Green SJ, McNeilly AS, Anderson RA (2010b) BMP Signaling in the Human Fetal Ovary is Developmentally Regulated and Promotes Primordial Germ Cell Apoptosis. *Stem Cells* **28:** 1368-1378

Childs AJ, Cowan G, Kinnell HL, Anderson RA, Saunders PTK (2011) Retinoic Acid Signalling and the Control of Meiotic Entry in the Human Fetal Gonad. *PLoS One* **6:** DOI:10.1371/jounal.pone.0010149

Childs AJ, He J, Anderson RA (unpublished) T-Cam2 cell work.

Chiou WL, Robbie G, Chung SM, Wu T, Ma C (1998) Correlation of plasma clearance of 54 extensively metabolized drugs between humans and rats: mean allometric coefficient of 0.66. *Pharmaceutical Research* **15:** 1474-1479

Chiquoine AD (1954a) Distribution of alkaline phosphomonoesterase in the central nervous system of the mouse embryo. *Journal of Comparative Neurology* **100:** 415-439

Chiquoine AD (1954b) The identification, origin, and migration of the primordial germ cells in the mouse embryo. *Anatomical Record* **118:** 135-146

Choi M, Lee O-H, Jeon S, Park M, Lee DR, Ko J-J, Yoon TK, Rajkovic A, Choi Y (2010a) The oocyte-specific transcription factor, Nobox, regulates the expression of Pad6, a peptidylarginine deiminase in the oocyte. *Febs Letters* **584:** 3629-3634

Choi Y, Rajkovic A (2005) Nobox deficiency and gene expression in newborn ovaries. *Biology of Reproduction***:** 93-93

Choi Y, Ballow DJ, Xin Y, Rajkovic A (2008a) Lim homeobox gene, Lhx8, is essential for mouse oocyte differentiation and survival. *Biology of Reproduction* **79:** 442-449

Choi Y, Yuan D, Rajkovic A (2008b) Germ Cell-Specific Transcriptional Regulator Sohlh2 Is Essential for Early Mouse Folliculogenesis and Oocyte-Specific Gene Expression. *Biology of Reproduction* **79:** 1176-1182

Choi Y, Jeon S, Choi M, Lee M-h, Park M, Lee DR, Jun K-Y, Kwon Y, Lee O-H, Song S-H, Kim J-Y, Lee K-A, Yoon TK, Rajkoyic A, Shim SH (2010b) Mutations in SOHLH1 Gene Associate with Nonobstructive Azoospermia. *Human Mutation* **31:** 788-793

Chu GC, Dunn NR, Anderson DC, Oxburgh L, Robertson EJ (2004) Differential requirements for Smad4 in TGF beta-dependent patterning of the early mouse embryo. *Development* **131:** 3501-3512

Chuma S, Nakatsuji N (2001) Autonomous transition into meiosis of mouse fetal germ cells in vitro and its inhibition by gp130-mediated signaling. *Developmental biology* **229:** 468-479

Clark AM, Garland KK, Russell LD (2000) Desert hedgehog (Dhh) gene is required in the mouse testis for formation of adult-type Leydig cells and normal development of peritubular cells and seminiferous tubules. *Biology of Reproduction* **63:** 1825-1838

Clark JM, Eddy EM (1975) Fine-structural observations on origin and associations of primordial germ-cells of mouse. *Developmental Biology* **47:** 136-155

Cocquet J, DeBaere E, Jaubert F, Xuhua X, Messiaen L, Pailhoux E, Cotinot C, Fellous M, Veitia R (2002a) FOXL2: Evolution and expression. *European Journal of Human Genetics* **10:** 167-167

Cocquet J, Pailhoux E, Jaubert F, Servel N, Xia X, Pannetier M, De Baere E, Messiaen L, Cotinot C, Fellous M, Veitia RA (2002b) Evolution and expression of FOXL2. *Journal of Medical Genetics* **39:** 916-921

Cocquet J, De Baere E, Gareil M, Pannetier M, Xia X, Fellous M, Veitia RA (2003) Structure, evolution and expression of the FOXL2 transcription unit. *Cytogenetic and Genome Research* **101:** 206-211

Codesal J, Regadera J, Nistal M, Regaderasejas J, Paniagua R (1990) Involution of human fetal Leydig-cells - an immunohistochemical, ultrastructural and quantitative study. *Journal of Anatomy* **172:** 103-114

Coleman RA, Smith WL, Narumiya S (1994) International union of pharmacology classification of prostanoid receptors - properties, distribution, and strusture of the receptors and their subtypes. *Pharmacological Reviews* **46:** 205-229

Colvin JS, Green RP, Schmahl J, Capel B, Ornitz DM (2001) Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell* **104:** 875-889

Combes AN, Bowles J, Feng CW, Chiu HS, Khoo PL, Jackson A, Little MH, Tam PPL, Koopman P (2011) Expression and Functional Analysis of Dkk1 during Early Gonadal Development. *Sexual Development* **5:** 124-130

Conover JC, Erickson JT, Katz DM, Bianchi LM, Poueymirou WT, McClain J, Pan L, Helgren M, Ip NY, Boland P, Friedman B, Wiegand S, Vejsada R, Kato AC, Dechiara TM, Yancopoulos GD (1995) Neuronal deficits, not involving motor-neurons, in mice lacking BDNF and/or NT4. *Nature* **375:** 235-238

Cook IH, Evans J, Maldonado-Perez D, Critchley HO, Sales KJ, Jabbour HN (2010) Prokineticin-1 (PROK1) modulates interleukin (IL)-11 expression via prokineticin receptor 1 (PROKR1) and the calcineurin/NFAT signalling pathway. *Molecular Human Reproduction* **16:** 158-169

Cooke HJ, Saunders PTK (2002) Mouse models of male infertility. *Nature Reviews Genetics* **3:** 790-801

Cory S, Adams JM (2002) The BCL2 family: Regulators of the cellular life-or-death switch. *Nature Reviews Cancer* **2:** 647-656

Costa Y, Cooke HJ (2007) Dissecting the mammalian synaptonemal complex using targeted mutations. *Chromosome Research* **15:** 579-589

Coucouvanis EC, Sherwood SW, Carswellcrumpton C, Spack EG, Jones PP (1993) Evidence that the mechanism of prenatal germ-cell death in the mouse is apoptosis. *Experimental Cell Research* **209:** 238-247

Coulam CASA, JF (1986) Incidence of premature ovarian failure. *Obstetrics and Gynecology* **4:** 604-606.

Couse JF, Dixon D, Yates M, Moore AB, Ma L, Maas R, Korach KS (2001) Estrogen receptor-alpha knockout mice exhibit resistance to the developmental effects of neonatal diethylstilbestrol exposure on the female reproductive tract. *Developmental Biology* **238:** 224-238

Coutant R, Mallet D, Lahlou N, Bouhours-Nouet N, Guichet A, Coupris L, Croue A, Morel Y (2007) Heterozygous mutation of steroidogenic factor-1 in 46,XY subjects may mimic partial androgen insensitivity syndrome. *Journal of Clinical Endocrinology & Metabolism* **92:** 2868-2873

Coutts SM, Childs AJ, Fulton N, Collins C, Bayne RA, McNeilly AS, Anderson RA (2008) Activin signals via SMAD2/3 between germ and somatic cells in the human fetal ovary and regulates kit ligand expression. *Developmental Biology* **314:** 189-199

Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S, Ristaldi MS, Marzella R, Rocchi M, Nicolino M, Lienhardt-Roussie A, Nivelon A, Verloes A, Schlessinger D, Gasparini P, Bonneau D, Cao A, Pilia G (2001) The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. *Nature Genetics* **27:** 159-166

Cui SY, Ross A, Stallings N, Parker KL, Capel B, Quaggin SE (2004) Disrupted gonadogenesis and male-to-female sex reversal in Pod1 knockout mice. *Development* **131:** 4095-4105

Dann CT, Alvarado AL, Molyneux LA, Denard BS, Garbers DL, Porteus MH (2008) Spermatogonial Stem Cell Self-Renewal Requires OCT4, a Factor Downregulated During Retinoic Acid-Induced Differentiation. *Stem Cells* **26:** 2928-2937

Davies AM (2000) Neurotrophins: Neurotrophic modulation of neurite growth. *Current Biology* **10:** R198-R200

Davis DL, Pakrasi PL, Dey SK (1983) Prostaglandins in swine blastocysts. *Biology of Reproduction* **28:** 1114-1118

Davis S, Aldrich TH, Stahl N, Pan L, Taga T, Kishimoto T, Ip NY, Yancopoulos GD (1993) LIFR beta and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science* **260:** 1805-1808

Davis SR, Krozowski Z, McLachlan RI, Burger HG (1987) Inhibin gene-expression in the human corpus-luteum. *Journal of Endocrinology* **115:** R21-R23

de Cuevas M, Lilly MA, Spradling AC (1997) Germline cyst formation in Drosophila. *Annual Review of Genetics* **31:** 405-428

De Felici M (2000) Regulation of primordial germ cell development in the mouse. *International Journal of Developmental Biology* **44:** 575-580

De Felici M, Lobascio AM, Klinger FG (2008) Cell death in fetal oocytes - Many players for multiple pathways. *Autophagy* **4:** 240-242

De Pol A, Vaccina F, Forabosco A, Cavazzuti E, Marzona L (1997) Apoptosis of germ cells during human prenatal oogenesis. *Human Reproduction* **12:** 2235-2241

de Vet A, Laven JSE, de Jong FH, Themmen APN, Fauser B (2002) Antimullerian hormone serum levels: a putative marker for ovarian aging. *Fertility and Sterility* **77:** 357-362

Dean J (2002) Oocyte-specific genes regulate follicle formation, fertility and early mouse development. *Journal of Reproductive Immunology* **53:** 171-180

DeFalco T, Takahashi S, Capel B (2011) Two distinct origins for Leydig cell progenitors in the fetal testis. *Developmental Biology* **352:** 14-26

Defelici M, Dolci S (1991) Leuakemia inhibitory factor sustains the survival of mouse primoridal germ-cells cultured on TM4 feeder layers. *Developmental Biology* **147:** 281-284

Defelici M, Dolci S, Pesce M (1992) Cellular and molecular aspects of mouse primordial germ-cell migration and proliferation in culture. *International Journal of Developmental Biology* **36:** 205-213

Denison FC, Battersby S, King AE, Szuber M, Jabbour HN (2008) Prokineticin-1: A novel mediator of the inflammatory response in third-trimester human placenta. *Endocrinology* **149:** 3470-3477

Desai S, April H, Nwaneshiudu C, Ashby B (2000) Comparison of agonist-induced internalization of the human EP2 and EP4 prostaglandin receptors: Role of the carboxyl terminus in EP4 receptor sequestration. *Molecular Pharmacology* **58:** 1279-1286

Dey SK, Chien SM, Cox CL, Crist RD (1980) Prostaglandin synthesis in the rabbit blastocyst. *Prostaglandins* **19:** 449-453

Di Carlo A, De Felici M (2000) A role for E-cadherin in mouse primordial germ cell development. *Developmental Biology* **226:** 209-219

Di Carlo A, Travia G, De Felici M (2000) The meiotic specific synaptonemal complex protein SCP3 is expressed by female and male primordial germ cells of the mouse embryo. *International Journal of Developmental Biology* **44:** 241-244

Di Giacomo M, Barchi M, Baudat F, Edelmann W, Keeney S, Jasin M (2005) Distinct DNA-damage-dependent and -independent responses drive the loss of oocytes in recombination-defective mouse mutants. *Proceedings of the National Academy of Sciences of the United States of America* **102:** 737-742

Diazflores L, Gutierrez R, Varela H (1994) Angiogenesis - an update. *Histology and Histopathology* **9:** 807-843

Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffee BD, Covington MB, Contel NR, Eng VM, Collins RJ, Czerniak PM, Gorry SA, Trzaskos JM (1995) Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase-II. *Nature* **378:** 406-409

Dinchuk JE, Liu RQ, Trzaskos JM (2003) COX-3: in the wrong frame in mind. *Immunology Letters* **86:** 121-121

Dissen GA, Hirshfield AN, Malamed S, Ojeda SR (1995) Expression of neurotrophins and their receptors in the mammalian ovary is developmentally-regulated - changes at the time of folliculogenesis. *Endocrinology* **136:** 4681-4692

Dissen GA, Romero C, Hirshfield AN, Ojeda SR (2001) Nerve growth factor is required for early follicular development in the mammalian ovary. *Endocrinology* **142:** 2078-2086

Dixit H, Rao LK, Padmalatha V, Kanakavalli M, Deenadayal M, Gupta N, Chakravarty B, Singh L (2005) Mutational screening of the coding region of growth differentiation factor 9 gene in Indian women with ovarian failure. *Menopause-the Journal of the North American Menopause Society* **12:** 749-754

Dixit H, Rao LK, Padmalatha VV, Kanakavalli M, Deenadayal M, Gupta N, Chakrabarty B, Singh L (2006) Missense mutations in the BMP15 gene are associated with ovarian failure. *Human Genetics* **119:** 408-415

Dode C, Teixeira L, Levilliers J, Fouveaut C, Bouchard P, Kottler M-L, Lespinasse J, Lienhardt-Roussie A, Mathieu M, Moerman A, Morgan G, Murat A, Toublanc J-E, Wolczynski S, Delpech M, Petit C, Young J, Hardelin J-P (2006) Kallmann syndrome: Mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. *PLoS Genetics* **2:** 1648-1652

Dolci S, Williams DE, Ernst MK, Resnick JL, Brannan CI, Lock LF, Lyman SD, Boswell HS, Donovan PJ (1991) Requirement for mast-cell growth-factor for primodial germ-cell survival in culture. *Nature* **352:** 809-811

Dong JW, Albertini DF, Nishimori K, Kumar TR, Lu NF, Matzuk MM (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383:** 531-535

Donovan PJ, Stott D, Cairns LA, Heasman J, Wylie CC (1986) Migratory and postmigratory mouse primordial germ-cells behave differentialy in culture *Cell* **44:** 831-838

Dorsch M, Qiu YB, Soler D, Frank N, Duong T, Goodearl A, O'Neil S, Lora J, Fraser CC (2005) PK1/EG-VEGF induces monocyte differentiation and activation. *Journal of Leukocyte Biology* **78:** 426-434

Dudley BM, Runyan C, Takeuchi Y, Schaible K, Molyneaux K (2007) BMP signaling regulates PGC numbers and motility in organ culture. *Mechanisms of Development* **124:** 68-77

Duffin K, Bayne RAL, Childs AJ, Collins C, Anderson RA (2009) The forkhead transcription factor FOXL2 is expressed in somatic cells of the human ovary prior to follicle formation. *Molecular Human Reproduction* **15:** 771-777

Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M (2000) Effect of single and compound knockouts of estrogen receptors alpha (ER alpha) and beta (ER beta) on mouse reproductive phenotypes. *Development* **127:** 4277-4291

Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JTJ, Grootegoed JA, Themmen APN (2002) Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology* **143:** 1076-1084

Eckert D, Nettersheim D, Heukamp LC, Kitazawa S, Biermann K, Schorle H (2008) TCam-2 but not JKT-1 cells resemble seminoma in cell culture. *Cell and Tissue Research* **331:** 529-538

Edson MA, Nagaraja AK, Matzuk MM (2009) The Mammalian Ovary from Genesis to Revelation. *Endocrine Reviews* **30:** 624-712

Eggan K, Jurga S, Gosden R, Min IM, Wagers AJ (2006) Ovulated oocytes in adult mice derive from non-circulating germ cells. *Nature* **441:** 1109-1114

Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, Matsumoto A, Tanimura S, Ohtsubo M, Misawa H, Miyazaki T, Leonor N, Taniguchi T, Fujita T, Kanakura Y, Komiya S, Yoshimura A (1997) A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* **387:** 921-924

Epifano O, Dean J (2002) Genetic control of early folliculogenesis in mice. *Trends in Endocrinology and Metabolism* **13:** 169-173

Escary JL, Perreau J, Dumenil D, Ezine S, Brulet P (1993) Leaukemia inhibitory factor is necessary for maintenance of hematopoietic stem-cells and thymocyte stimulation. *Nature* **363:** 361-364

Evans J, Catalano RD, Morgan K, Critchley HO, Millar RP, Jabbour HN (2008) Prokineticin 1 signaling and gene regulation in early human pregnancy. *Endocrinology* **149:** 2877-2887

Evans J, Catalano RD, Brown P, Sherwin R, Critchley HO, Fazleabas AT, Jabbour HN (2009) Prokineticin 1 mediates fetal-maternal dialogue regulating endometrial leukemia inhibitory factor. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **23:** 2165-2175

Fainsod A, Deissler K, Yelin R, Marom K, Epstein M, Pillemer G, Steinbeisser H, Blum M (1997) The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. *Mechanisms of Development* **63:** 39-50

Falin LI (1969) Development of genital glands and origin of germ cells in human embryogenesis. *Acta Anatomica* **72:** 195-232

Faour WH, Gomi K, Kennedy CRJ (2008) PGE(2) induces COX-2 expression in podocytes via the EP(4) receptor through a PKA-independent mechanism. *Cellular Signalling* **20:** 2156-2164

Farhi J, Fisch B, Garor R, Peled Y, Pinkas H, Abir R (2011) Neurotrophin 4 enhances in vitro follicular assembly in human fetal ovaries. *Fertility and Sterility* **95:** 1267-1271

Farini D, La Sala G, Tedesco M, De Felici M (2007) Chemoattractant action and molecular signaling pathways of Kit ligand on mouse primordial germ cells. *Developmental Biology* **306:** 572-583

Ferrara N, Frantz G, LeCouter J, Dillard-Telm L, Pham T, Draksharapu A, Giordano T, Peale F (2003) Differential expression of the angiogenic factor genes vascular endothelial growth factor (VEGF) and endocrine gland-derived VEGF in normal and polycystic human ovaries. *American Journal of Pathology* **162:** 1881-1893

Ffrenchconstant C, Hollingsworth A, Heasman J, Wylie CC (1991) Response to fibronectin of mouse primordial germ-cells before, during and after migration. *Development* **113:** 1365-1373

Forabosco A, Sforza C, Depol A, Vizzotto L, Marzona L, Ferrario VF (1991) Morphometric study of the human neonatal ovary. *Anatomical Record* **231:** 201-208

Forsberg L, Leeb L, Thoren S, Morgenstern R, Jakobsson PJ (2000) Human glutathione dependent prostaglandin E synthase: gene structure and regulation. *FEBS Letters* **471:** 78-82

Fowler PA, Cassie S, Rhind SM, Brewer MJ, Collinson JM, Lea RG, Baker PJ, Bhattacharya S, O'Shaughnessy PJ (2008) Maternal smoking during pregnancy specifically reduces human fetal desert hedgehog gene expression during testis development. *Journal of Clinical Endocrinology & Metabolism* **93:** 619-626

Fowler PA, Flannigan S, Mathers A, Gillanders K, Lea RG, Wood MJ, Maheshwari A, Bhattacharya S, Collie-Duguid ES, Baker PJ, Monteiro A, O'Shaughnessy PJ (2009) Gene expression analysis of human fetal ovarian primordial follicle formation. *The Journal of Clinical Endocrinology and Metabolism* **94:** 1427-1435

Fowler PA, Anderson RA, Saunders PT, Kinnell H, Mason JI, Evans DB, Bhattacharya S, Flannigan S, Franks S, Monteiro A, O'Shaughnessy PJ (2011) Development of Steroid Signaling Pathways during Primordial Follicle Formation in the Human Fetal Ovary. *Journal of Clinical Endocrinology & Metabolism* **96:** 1754-1762

Francavilla S, Cordeschi G, Properzi G, Concordia N, Cappa F, Pozzi V (1990) Ultrastructure of fetal human gonad before sexual-differentiation and during early testicular and ovarian development. *Journal of Submicroscopic Cytology and Pathology* **22:** 389-400

Fraser HM, Bell J, Wilson H, Taylor PD, Morgan K, Anderson RA, Duncan WC (2005) Localization and quantification of cyclic changes in the expression of endocrine gland vascular endothelial growth factor in the human corpus luteum. *Journal of Clinical Endocrinology & Metabolism* **90:** 427-434

Friel A, Houghton JA, Glennon M, Lavery R, Smith T, Nolan A, Maher M (2002) A preliminary report on the implication of RT-PCR detection of DAZ, RBMY1, USP9Y and Protamine-2 mRNA in testicular biopsy samples from azoospermic men. *International Journal of Andrology* **25:** 59-64

Fujimoto T, Miyayama Y, Fuyuta M (1977) Origin, migration and fine morphology of human primordial germ-cells. *Anatomical Record* **188:** 315-329

Fujiwara T, Dunn NR, Hogan BLM (2001) Bone morphogenetic protein 4 in the extraembryonic mesoderm is required for allantois development and the localization and survival of primordial germ cells in the mouse. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 13739-13744

Fukuchi Y, Kizaki M, Yamato K, Kawamura C, Umezawa A, Hata J, Nishihara T, Ikeda Y (2001) Mcl-1, an early-induction molecule, modulates activin A-induced apoptosis and differentiation of CML cells. *Oncogene* **20:** 704-713

Fulton N, da Silva SJM, Bayne RAL, Anderson RA (2005) Germ cell proliferation and apoptosis in the developing human ovary. *Journal of Clinical Endocrinology & Metabolism* **90:** 4664-4670

Funk, CD (2001) Prostalgnadins and leukotrienes: advances in eicosanoid biology. *Science* **294**: 1871-1875.

Gallardo TD, John GB, Bradshaw K, Welt C, Reijo-Pera R, Vogt PH, Touraine P, Bione S, Toniolo D, Nelson LM, Zinn AR, Castrillon DH (2008) Sequence variation at the human FOXO3 locus: a study of premature ovarian failure and primary amenorrhea. *Human Reproduction* **23:** 216-221

Galloway SM, Gregan SM, Wilson T, McNatty KP, Juengel JL, Ritvos O, Davis GH (2002) Bmp15 mutations and ovarian function. *Molecular and Cellular Endocrinology* **191:** 15-18

GarciaCastro MI, Anderson R, Heasman J, Wylie C (1997) Interactions between germ cells and extracellular matrix glycoproteins during migration and gonad assembly in the mouse embryo. *Journal of Cell Biology* **138:** 471-480

Gaskell TL, Esnal A, Robinson LLL, Anderson RA, Saunders PTK (2004) Immunohistochemical profiling of germ cells within the human fetal testis: Identification of three subpopulations. *Biology of Reproduction* **71:** 2012-2021

Gawriluk TR, Hale AN, Flaws JA, Dillon CP, Green DR, Rucker EB, III (2011) Autophagy is a cell survival program for female germ cells in the murine ovary. *Reproduction (Cambridge)* **141:** 759-765

Ge RS, Dong QA, Sottas CM, Papadopoulos V, Zirkin BR, Hardy MP (2006) In search of rat stem Leydig cells: Identification, isolation, and lineage-specific development. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 2719-2724

Gearing DP, Bruce AG (1992) Oncostatin-M binds the high-affinity leukemia inhibitory factor receptor. *New Biologist* **4:** 61-65

Glister C, Kemp CF, Knight PG (2004) Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4,-6 and-7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. *Reproduction* **127:** 239-254

Godin I, Wylie C, Heasman J (1990) Genital ridges exert long-range effects on mouse primordial germ-cell numbers and direction of migration in culture. *Development* **108:** 357-363

Godin I, Deed R, Cooke J, Zsebo K, Dexter M, Wylie CC (1991) Effects of the steel gene-product on mouse primordial germ-cells in culture. *Nature* **352:** 807-809

Gomperts M, Garciacastro M, Wylie C, Heasman J (1994) Interactions between primoridal germ-cells play a role in their migration in mouse embryos. *Development* **120:** 135-141

Gondos B, Zamboni L (1969) Ovarian development - functional importance of germ cell interconnections. *Fertility and Sterility* **20:** 176-189

Gondos B, Westergaard L, Byskov AG (1986) Initiation of oogenesis in the human-fetal ovary - ultrastructural and squash preparation study. *American Journal of Obstetrics and Gynecology* **155:** 189-195

Gosden RG (1995) *Ovulation 1: Oocyte development throughout life*. In Grudzinskas JG, Yovich JL (eds) **Gametes-The Oocyte.** Cambridge Reviews in Human Reproduction. Cambridge University Press, Cambridge, 119-149

Gospodarowicz D, Plouet J, Fujii DK (1989) Ovarian germinal epithelial- cells respond to basic fibroblast growth-factor and express its gene - implications for early folliculogenesis. *Endocrinology* **125:** 1266-1276

Goswami DC, GS (2005) Premature ovarian failure. *Human Reproduction Update* **4:** 391-410

Gougeon A (1996) Regulation of ovarian follicular development in primates: Facts and hypotheses. *Endocrine Reviews* **17:** 121-155

Graham A, Franciswest P, Brickell P, Lumsden A (1994) The signaling molecule Bmp4 mediates apoptosis in the rhombencephalic neural crest. *Nature* **372:** 684-686

Graham GG, Scott KF (2005) Mechanism of action of paracetamol. *American Journal of Therapeutics* **12:** 46-55

Groppe J, Greenwald J, Wiater E, Rodriguez-Leon J, Economides AN, Kwiatkowski W, Affolter M, Vale WW, Belmonte JCI, Choe S (2002) Structural basis of BMP signalling inhibition by the cystine knot protein Noggin. *Nature* **420:** 636-642

Gross GA, Imamura T, Ludeke C, Vogt SK, Olson LM, Nelson DM, Sadovsky Y, Muglia LJ (1998) Opposing actions of prostaglandins and oxytocin determine the onset of murine labor. *Proceedings of the National Academy of Sciences of the United States of America* **95:** 11875-11879

Gu Y, Runyan C, Shoemaker A, Surani A, Wylie C (2009) Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantois, and provides a continuous niche throughout their migration. *Development* **136:** 1295-1303

Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, Vivian N, Goodfellow P, Lovellbadge R (1990) A gene-mapping to the sex-determining region of the mouse Y-chromosome is a member of a novel family of embryonically expressed genes. *Nature* **346:** 245-250

Guerrero R, Florez PE (1969) The duration of pregnancy. *Lancet* **2:** 268-269

Gupta C, Goldman AS (1986) The arachidonic-acid cascade is involved in the masculinizing action of testosterone on embryonic external genitalia in mice. *Proceedings of the National Academy of Sciences of the United States of America* **83:** 4346-4349

Gupta C (1989) The role of prostaglandins in masculine differentiation - modulation of prostaglandin levels in the differentiating genital tract of the fetal mouse *Endocrinology* **124:** 129-133

Hacker A, Capel B, Goodfellow P, Lovellbadge R (1995) Expression of SRY, the mouse sex-determining gene. *Development* **121:** 1603-1614

Harkness LM, Baird DT (1997) Morphological and molecular characteristics of living human fetuses between Carnegie stages 7 and 23: Developmental stages in the post-implantation embryo. *Human Reproduction Update* **3:** 3-23

Harris SE, Chand AL, Winship IM, Gersak K, Aittomaki K, Shelling AN (2002) Identification of novel mutations in FOXL2 associated with premature ovarian failure. *Molecular Human Reproduction* **8:** 729-733

Hart AH, Hartley L, Ibrahim M, Robb L (2004) Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. *Developmental Dynamics* **230:** 187-198

Hartley PS, Bayne RAL, Robinson LLL, Fulton N, Anderson RA (2002) Developmental changes in expression of myeloid cell leukemia-1 in human germ cells during oogenesis and early folliculogenesis. *Journal of Clinical Endocrinology & Metabolism* **87:** 3417-3427

Hashimoto O, Nakamura T, Shoji H, Shimasaki S, Hayashi Y, Sugino H (1997) A novel role of follistatin, an activin-binding protein, in the inhibition of activin action in rat pituitary cells - Endocytotic degradation of actin and its acceleration by follistatin associated with cell-surface heparan sulfate. *Journal of Biological Chemistry* **272:** 13835-13842

Haugen MJ, Johnson AL (2010) Bone morphogenetic protein 2 inhibits FSH responsiveness in hen granulosa cells. *Reproduction* **140:** 551-558

Hayashi K, Lopes S, Surani MA (2007) Germ cell specification in mice. *Science* **316:** 394-396

Heikinheimo M, Ermolaeva M, Bielinska M, Rahman NA, Narita N, Huhtaniemi IT, Tapanainen JS, Wilson DB (1997) Expression and hormonal regulation of transcription factors GATA-4 and GATA-6 in the mouse ovary. *Endocrinology* **138:** 3505-3514

Heikkila M, Prunskaite R, Naillat F, Itaranta P, Vuoristo J, Leppaluoto J, Peltoketo H, Vainio S (2005) The partial female to male sex reversal in Wnt-4-deficient females involves induced expression of testosterone biosynthetic genes and testosterone production, and depends on androgen action. *Endocrinology* **146:** 4016-4023

Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L (1998) Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochemical Journal* **334:** 297-314

Herschman HR (1996) Prostaglandin synthase 2. *Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism* **1299:** 125-140

Hertig AT, Adams EC (1967) Studies on human oocyte and its follicle I ultrastructural and histochemical observations on primordial follicle stage. *Journal of Cell Biology* **34:** 647-675

Hibi M, Murakami M, Saito M, Hirano T, Taga T, Kishimoto T (1990) Molecular-cloning and expressin of an IL-6 signal transducer, GP130. *Cell* **63:** 1149-1157

Hillier SG, Miro F (1993) Inhibin, activin, and follistatin - potential roles in ovarian physiology. *Annals of the New York Academy of Sciences* **687:** 29-38

Hilscher B, Hilscher W, Bulthoffohnolz B, Kramer U, Birke A, Pelzer H, Gauss G (1974) Kinetics of gametogenesis 1. Comparative histological and autoradiographic stuides of oocvytes and transitional prospermatogonia during oogenesis and perspermatogenesis. *Cell and Tissue Research* **154:** 443-470

Hilscher W (1991) The genetic-control and germ-cell kinetics of the female and male germ line in mammals including man. *Human Reproduction* **6:** 1416-1425

Hinz B, Cheremina O, Brune K (2008) Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man. *FASEB Journal* **22:** 383-390

Hizaki H, Segi E, Sugimoto Y, Hirose M, Saji T, Ushikubi F, Matsuoka T, Noda Y, Tanaka T, Yoshida N, Narumiya S, Ichikawa A (1999) Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP2. *Proceedings of the National Academy of Sciences of the United States of America* **96:** 10501-10506

Hoffmann P, Feige JJ, Alfaidy N (2006) Expression and oxygen regulation of endocrine gland-derived vascular endothelial growth factor/prokineticin-1 and its receptors in human placenta during early pregnancy. *Endocrinology* **147:** 1675-1684

Hoffmann P, Feige JJ, Alfaidy N (2007) Placental expression of EG-VEGF and its receptors PKR1 (prokineticin receptor-1) and PKR2 throughout mouse gestation. *Placenta* **28:** 1049-1058

Hofland J, van Weerden WM, Dits NFJ, Steenbergen J, van Leenders G, Jenster G, Schroder FH, de Jong FH (2010) Evidence of Limited Contributions for Intratumoral Steroidogenesis in Prostate Cancer. *Cancer Research* **70:** 1256-1264

Hogg K, Etherington SL, Young JM, McNeilly AS, Duncan WC (2010) Inhibitor of Differentiation (Id) Genes Are Expressed in the Steroidogenic Cells of the Ovine Ovary and Are Differentially Regulated by Members of the Transforming Growth Factor-beta Family. *Endocrinology* **151:** 1247-1256

Hoogerwerf WA (2006) Prokineticin 1 inhibits spontaneous giant contractions in the murine proximal colon through nitric oxide release. *Neurogastroenterology and Motility* **18:** 455-463

Hosaka T, Biggs WH, Tieu D, Boyer AD, Varki NM, Cavenee WK, Arden KC (2004) Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proceedings of the National Academy of Sciences of the United States of America* **101:** 2975-2980

Houmard B, Small C, Yang L, Naluai-Cecchini T, Cheng E, Hassold T, Griswold M (2009) Global gene expression in the human fetal testis and ovary. *Biology of Reproduction* **81:** 438-443

Hoyer PE, Byskov AG, Mollgard K (2005) Stem cell factor and c-Kit in human primordial germ cells and fetal ovaries. *Molecular and Cellular Endocrinology* **234:** 1-10

Hsu YT, Wolter KG, Youle RJ (1997) Cytosol-to-membrane redistribution of Bax and Bcl-X-L during apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **94:** 3668-3672

Hu MC, Hsu NC, El Hadj NB, Pai CI, Chu HP, Wang CKL, Chung BC (2002) Steroid deficiency syndromes in mice with targeted disruption of Cyp11a1. *Molecular Endocrinology* **16:** 1943-1950

Hu W, Gauthier L, Baibakov B, Jimenez-Movilla M, Dean J (2010) FIGLA, a Basic Helix-Loop-Helix Transcription Factor, Balances Sexually Dimorphic Gene Expression in Postnatal Oocytes. *Molecular and Cellular Biology* **30:** 3661-3671

Huang B, Wang SB, Ning Y, Lamb AN, Bartley J (1999) Autosomal XX sex reversal caused by duplication of SOX9. *American Journal of Medical Genetics* **87:** 349-353

Hughes IA (2001) Minireview: Sex differentiation. *Endocrinology* **142:** 3281-3287

Hunt PA, Hassold TJ (2008) Human female meiosis: what makes a good egg go bad? *Trends in Genetics* **24:** 86-93

Huntriss J, Gosden R, Hinkins M, Oliver B, Miller D, Rutherford AJ, Picton HM (2002) Isolation, characterization and expression of the human Factor In the Germline alpha (FIGLA) gene in ovarian follicles and oocytes. *Molecular Human Reproduction* **8:** 1087-1095

Huntriss J, Hinkins M, Picton HM (2006) cDNA cloning and expression of the human NOBOX gene in oocytes and ovarian follicles. *Molecular Human Reproduction* **12:** 283-289

Hussein MR (2005) Apoptosis in the ovary: molecular mechanisms. *Human Reproduction Update* **11:** 162-178

Hutchinson AJ, Chou C-L, Israel DD, Xu W, Regan JW (2009) Activation of EP(2) prostanoid receptors in human glial cell lines stimulates the secretion of BDNF. *Neurochemistry International* **54:** 439-446

Ichihara M, Hara T, Kim H, Murate T, Miyajima A (1997) Oncostatin M and leukemia inhibitory factor do not use the same functional receptor in mice. *Blood* **90:** 165-173

Iguchi T, Takei T, Takase M, Takasugi N (1986) Estrogen participation in induction of cerviovaginal adenosis-like lesions in immature mice exposed prenatally to diethystilbestrol *Acta Anatomica* **127:** 110-114

Ikeda Y, Shen WH, Ingraham HA, Parker KL (1994) Developmental expression of mouse steroidogeneic factor-1, an essential regulator of the steroid hydroxlases *Molecular Endocrinology* **8:** 654-662

Ingraham HA, Lala DS, Ikeda Y, Luo XR, Shen WH, Nachtigal MW, Abbud R, Nilson JH, Parker KL (1994) The nuclear receptor steroidogenic factor-1 acts at multiple levels of the reproductive axis. *Genes & Development* **8:** 2302-2312

Inoue H, Tanabe T, Umesono K (2000) Feedback control of cyclooxygenase-2 expression through PPAR gamma. *Journal of Biological Chemistry* **275:** 28028-28032

Inouye S, Ling N, Shimasaki S (1992) Localization of the heparin binding-site of follistatin. *Molecular and Cellular Endocrinology* **90:** 1-6

Ivell R, Hartung S (2003) The molecular basis of cryptorchidism. *Molecular Human Reproduction* **9:** 175-181

Jaaskelainen M, Prunskaite-Hyyrylainen R, Naillat F, Parviainen H, Anttonen M, Heikinheimo M, Liakka A, Ola R, Vainio S, Vaskivuo TE, Tapanainen JS (2010) WNT4 is expressed in human fetal and adult ovaries and its signaling contributes to ovarian cell survival. *Molecular and Cellular Endocrinology* **317:** 106-111

Jabbour HN (unpublished) Gene array for changes with PGE2 treatment in the human endometrium.

Jagarlamudi K, Rajkovic A (2011) Oogenesis: Transcriptional regulators and mouse models. *Moecularl and Cellular Endocrinoogy* DOI: 10.1016/j.mce.2011.07.049

Jeays-Ward K, Hoyle C, Brennan J, Dandonneau M, Alldus G, Capel B, Swain A (2003) Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad. *Development* **130:** 3663-3670

Jefferson WN, Couse JF, Padilla-Banks E, Korach KS, Newbold RR (2002) Neonatal exposure to genistein induces estrogen receptor (ER)alpha expression and multioocyte follicles in the maturing mouse ovary: Evidence for ER beta-mediated and nonestrogenic actions. *Biology of Reproduction* **67:** 1285-1296

Jenkin G, Ward J, Hooper SB, O'Connor AE, de Kretser DM, Wallace EM (2001) Feto-placental hypoxemia regulates the release of fetal activin A and prostaglandin E-2. *Endocrinology* **142:** 963-966

Jethwa PH, I'Anson H, Warner A, Prosser HM, Hastings MH, Maywood ES, Ebling FJP (2008) Loss of prokineticin receptor 2 signaling predisposes mice to torpor. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **294:** R1968-R1979

Jeyasuria P, Ikeda Y, Jamin SP, Zhao LP, De Rooij DG, Themmen APN, Behringer RR, Parker KL (2004) Cell-specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function. *Molecular Endocrinology* **18:** 1610-1619

John GB, Gallardo TD, Shirley LJ, Castrillon DH (2008) Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth. *Developmental Biology* **321:** 197-204

Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL (2004) Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature* **428:** 145-150

Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT, Tilly JL (2005) Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell* **122:** 303-315

Joshi S, Davies H, Sims LP, Levy SE, Dean J (2007) Ovarian gene expression in the absence of FIGLA, an oocyte-specific transcription factor. *BMC Developmental Biology* **7:** 67

Jost A (1947) *Recherches sur la differenciation sexuelle de lembryon de lapin. *Archives D Anatomie Microscopique Et De Morphologie Experimentale* **36:** 151-315

Joubert FJ (1980) Snake-venom - the amino-acid sequqence of protein-A from dendroapis - polylepis-polylepis (Black mamba) venom *Hoppe-Seylers Zeitschrift Fur Physiologische Chemie* **361:** 1787-1794

Joyce IM, Clark AT, Pendola FL, Eppig JJ (2000) Comparison of recombinant growth differentiation factor-9 and oocyte regulation of KIT ligand messenger ribonucleic acid expression in mouse ovarian follicles. *Biology of Reproduction* **63:** 1669-1675

Kaipia A, Hsueh AJW (1997) Regulation of ovarian follicle atresia. In *Annual Review of Physiology*, Hoffman JF (ed), Vol. 59, pp 349-363

Kargman S, Charleson S, Cartwright M, Frank J, Riendeau D, Mancini J, Evans J, Oneill G (1996) Characterization of prostaglandin G/H synthase 1 and 2 in rat, dog, monkey, and human gastrointestinal tracts. *Gastroenterology* **111:** 445-454

Karl J, Capel B (1998) Sertoli cells of the mouse testis originate from the coelomic epithelium. *Developmental Biology* **203:** 323-333

Kashimada K, Koopman P (2010) Sry: the master switch in mammalian sex determination. *Development* **137:** 3921-3930

Kashimada K, Pelosi E, Chen H, Schlessinger D, Wilhelm D, Koopman P (2011) FOXL2 and BMP2 Act Cooperatively to Regulate Follistatin Gene Expression during Ovarian Development. *Endocrinology* **152:** 272-280

Kawabata M, Imamura T, Inoue H, Hanai JI, Nishihara A, Hanyu A, Takase M, Ishidou Y, Udagawa Y, Oeda E, Goto D, Yagi K, Kato M, Miyazono K (1999) Intracellular signaling of the TGF-beta superfamily by Smad proteins. In *Anticancer Molecules: Structure, Function, and Design*, Maruta H (ed), Vol. 886, pp 73-82.

Kawamura K, Sato N, Fukuda J, Kodama H, Kumagai J, Tanikawa H, Murata M, Tanaka T (2003) The role of leptin during the development of mouse preimplantation embryos. *Molecular and Cellular Endocrinology* **202:** 185-189

Kawase E, Hashimoto K, Pedersen RA (2004) Autocrine and paracrine mechanisms regulating primordial germ cell proliferation. *Molecular Reproduction and Development* **68:** 5-16

Kee K, Angeles VT, Flores M, Nguyen HN, Pera RAR (2009) Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation. *Nature* **462:** 222-295

Kehler J, Tolkunova E, Koschorz B, Pesce M, Gentile L, Boiani M, Lomeli H, Nagy A, McLaughlin KJ, Scholer HR, Tomilin A (2004) Oct4 is required for primordial germ cell survival. *EMBO Reports* **5:** 1078-1083

Kelly AJ, Malik S, Smith L, Kavanagh J, Thomas J (2009) Vaginal prostaglandin (PGE2 and PGF2a) for induction of labour at term. *Cochrane Database Systematic Review* **7**: DOI: 10.1002/14651858.CD003101.pub2

Kennedy CRJ, Zhang YH, Brandon S, Guan YF, Coffee K, Funk CD, Magnuson MA, Oates JA, Breyer MD, Breyer RM (1999) Salt-sensitive hypertension and reduced fertility in mice lacking the prostaglandin EP2 receptor. *Nature Medicine* **5:** 217-220

Kerr B, Garcia-Rudaz C, Dorfman M, Paredes A, Ojeda SR (2009) NTRK1 and NTRK2 receptors facilitate follicle assembly and early follicular development in the mouse ovary. *Reproduction* **138:** 131-140

Kerr CL, Hill CM, Blumenthal PD, Gearhart JD (2008) Expression of pluripotent stem cell markers in the human fetal ovary. *Human Reproduction* **23:** 589-599

Kezele P, Skinner MK (2003) Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: Endocrine model of follicle assembly. *Endocrinology* **144:** 3329-3337

Kezele PR, Nilsson EE, Skinner MK (2002) Insulin but not insulin-like growth factor-1 promotes the primordial to primary follicle transition. *Molecular and Cellular Endocrinology* **192:** 37-43

Kim J, Chu J, Shen X, Wang J, Orkin SH (2008) An extended transcriptional network for pluripotency of embryonic stem cells. *Cell* **132:** 1049-1061

Kimura F, Sidis Y, Bonomi L, Xia Y, Schneyer A (2010) The Follistatin-288 Isoform Alone Is Sufficient for Survival But Not for Normal Fertility in Mice. *Endocrinology* **151:** 1310-1319

Kisliouk T, Levy N, Hurwitz A, Meidan R (2003) Presence and regulation of endocrine gland vascular endothelial growth factor/prokineticin-1 and its receptors in ovarian cells. *Journal of Clinical Endocrinology & Metabolism* **88:** 3700-3707

Klonisch T, Fowler PA, Hombach-Klonisch S (2004) Molecular and genetic regulation of testis descent and external genitalia development. *Developmental Biology* **270:** 1-18

Knight PG, Glister C (2001) Potential local regulatory functions of inhibins, activins and follistatin in the ovary. *Reproduction* **121:** 503-512

Kobayashi A, Chang H, Chaboissier MC, Schedl A, Behringer RR (2005) Sox9 in testis determination. In *Testicular Cell Dynamics and Endocrine Signaling*, ed Hardy **1061:** 9-17.

Koehler B, Lin L, Ferraz-de-souza B, Wieacker P, Heidemann P, Schroeder V, Biebermann H, Schnabel D, Grueters A, Achermann JC (2008) Five novel mutations in sterolidogenlic factor 1 (SF1, NR5A1) iin 46,XY patients with severe underandrogenization but without adrenal insufficiency. *Human Mutation* **29:** 59-64

Koike S, Noumura T (1994) Cell-specific and stage-specific expression of basic FGF in the developing rat gonads. *Growth Regulation* **4:** 77-81

Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovellbadge R (1991) Male development of chromosomally female mice transgenic for Sry. *Nature* **351:** 117-121

Koopman P, Bullejos M, Bowles J (2001) Regulation of male sexual development by Sry and Sox9. *Journal of Experimental Zoology* **290:** 463-474

Koos RD, Olson CE (1989) Expression of basic fibroblast growth-factor in the rat ovary - detection of messenger-RNA using reverse transcription-polymerase chain reaction amplification. *Molecular Endocrinology* **3:** 2041-2048

Kosaki K, Sato S, Hasegawa T, Matsuo N, Suzuki T, Ogata T (2004) Premature ovarian failure in a female with proximal symphalangism and Noggin mutation. *Fertility and Sterility* **81:** 1137-1139

Koshimizu U, Taga T, Watanabe M, Saito M, Shirayoshi Y, Kishimoto T, Nakatsuji N (1996) Functional requirement of gp130-mediated signaling for growth and survival of mouse primordial germ cells in vitro and derivation of embryonic germ (EG) cells. *Development* **122:** 1235-1242

Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC (2006) Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 2474-2479

Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW (1993) MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proceedings of the National Academy of Sciences of the United States of America* **90:** 3516-3520

Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R (1993) WT-1 is required for early kidney development. *Cell* **4:** 679-691

Kristensen DM, Hass U, Lesne L, Lottrup G, Jacobsen PR, Desdoits-Lethimonier C, Boberg J, Petersen JH, Toppari J, Jensen TK, Brunak S, Skakkebaek NE, Nellemann C, Main KM, Jegou B, Leffers H (2011) Intrauterine exposure to mild analgesics is a risk factor for development of male reproductive disorders in human and rat. *Human Reproduction* **26:** 235-244

Kumar S, Chatzi C, Brade T, Cunningham TJ, Zhao X, Duester G (2011) Sex-specific timing of meiotic initiation is regulated by Cyp26b1 independent of retinoic acid signalling. *Nature Communications* **2:** 151

Kuo CT, Morrisey EE, Anandappa R, Sigrist K, Lu MM, Parmacek MS, Soudais C, Leiden JM (1997) GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes & Development* **11:** 1048-1060

Lages B, Weiss HJ (1989) Inhibition of human-platelet fuction-invitro and exvivo by acetaminophen. *Thrombosis Research* **53:** 603-613

Lange UC, Adams DJ, Lee C, Barton S, Schneider R, Bradley A, Surani MA (2008) Normal germ line establishment in mice carrying a deletion of the Ifitm/Fragilis gene family cluster. *Molecular and Cellular Biology* **28:** 4688-4696

Langenbach R, Morham SG, Tiano, HF, Loftin CD, Ghanayem BI, Chulada PC, Mahler JF, Lee CA, Goulding EH, Kluckman KD, Kim HS, Smithies O (1995) Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. Cell **83:** 483-492

Lanman JT, Seidman L (1977) Length of gestation in mice under a 21-hour day. *Biology of Reproduction* **17:** 224-227

Lawson KA, Hage WJ (1994) Clonal analysis of the origin of primordial germ-cells in the mouse. *Germline Development* **182:** 68-84

Lawson KA, Dunn NR, Roelen BAJ, Zeinstra LM, Davis AM, Wright CVE, Korving J, Hogan BLM (1999) Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes & Development* **13:** 424-436

Le Bouffant R, Guerquin MJ, Duquenne C, Frydman N, Coffigny H, Rouiller-Fabre V, Frydman R, Habert R, Livera G (2010) Meiosis initiation in the human ovary requires intrinsic retinoic acid synthesis. *Human Reproduction* **25:** 2579-2590

Lechowska A, Bilinski S, Choi Y, Shin Y, Kloc M, Rajkovic A (2011) Premature ovarian failure in nobox-deficient mice is caused by defects in somatic cell invasion and germ cell cyst breakdown. *Journal of Assisted Reproduction and Genetics* **28:** 583-589

LeCouter J, Kowalski J, Foster J, Hass P, Zhang ZM, Dillard-Telm L, Frantz G, Rangell L, DeGuzman L, Keller GA, Peale F, Gurney A, Hillan KJ, Ferrara N (2001) Identification of an angiogenic mitogen selective for endocrine gland endothelium. *Nature* **412:** 877-884

LeCouter J, Ferrara N (2003) EG-VEGF and Bv8: A novel family of tissue-selective mediators of angiogenesis, phenotype, endothelial and function. *Trends in Cardiovascular Medicine* **13:** 276-282

LeCouter J, Lin R, Tejada M, Frantz G, Peale F, Hillan KJ, Ferrara N (2003) The endocrine-gland-derived VEGF homologue Bv8 promotes angiogenesis in the testis: Localization of Bv8 receptors to endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 2685-2690

LeCouter J, Zlot C, Tejada M, Peale F, Ferrara N (2004) Bv8 and endocrine gland-derived vascular endothelial growth factor stimulate hematopoiesis and hematopoietic cell mobilization. *Proceedings of the National Academy of Sciences of the United States of America* **101:** 16813-16818

Lee HJ, Selesniemi K, Niikura Y, Niikura T, Klein R, Dombkowski DM, Tilly JL (2007) Bone marrow transplantation generates immature oocytes and rescues long-term fertility in a preclinical mouse model of chemotherapy-induced premature ovarian failure. *Journal of Clinical Oncology* **25:** 3198-3204

Lee WS, Otsuka F, Moore RK, Shimasaki S (2001) Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. *Biology of Reproduction* **65:** 994-999

Lee WS, Yoon SJ, Yoon TK, Cha KY, Lee SH, Shimasaki S, Lee S, Lee KA (2004) Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. *Molecular Reproduction and Development* **69:** 159-163

LeersSucheta S, Morohashi K, Mason JI, Melner MH (1997) Synergistic activation of the human type II 3 beta-hydroxysteroid dehydrogenase/Delta(5)-Delta(4) isomerase promoter by the transcription factor steroidogenic factor-1/adrenal 4-binding protein and phorbol ester. *Journal of Biological Chemistry* **272:** 7960-7967

Lewis KA, Gray PC, Blount AL, MacConnell LA, Wiater E, Bilezikjian LM, Vale W (2000) Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. *Nature* **404:** 411-414

Li M, Bullock CM, Knauer DJ, Ehlert FJ, Zhou QY (2001) Identification of two prokineticin cDNAs: Recombinant proteins potently contract gastrointestinal smooth muscle. *Molecular Pharmacology* **59:** 692-698

Li SR, Lu MM, Zhou DY, Hammes SR, Morrisey EE (2007) GLP-1: A novel zinc finger protein required in somatic cells of the gonad for germ cell development. *Developmental Biology* **301:** 106-116

Liang LF, Soyal SM, Dean J (1997) FIG alpha, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. *Development* **124:** 4939-4947

Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK (1997) Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91:** 197-208

Lin MT, Lee RC, Yang PC, Ho FM, Kuo ML (2001) Cyclooxygenase-2 inducing Mcl-1-dependant survival mechanism in human lung adneocarinoma CL1.0 cells. Involvment of phosphatidylinositol 3-kinase/Akt pathway. *Journal of Biological Chemistry* **52:** 48997-489002

Lin DC, Bullock CM, Ehlert FJ, Chen JL, Tian H, Zhou QY (2002) Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticins/endocrine gland vascular endothelial growth factor. *The Journal of Biological Chemistry* **277:** 19276-19280

Lin HY, Wang HM, Zhu C (2003) The Smad pathway in transforming growth factor-beta signaling. *Science in China Series C-Life Sciences* **46:** 449-463

Lin YF, Gill ME, Koubova J, Page DC (2008) Germ Cell-Intrinsic and -Extrinsic Factors Govern Meiotic Initiation in Mouse Embryos. *Science* **322:** 1685-1687

Liu L, Rajareddy S, Reddy P, Du C, Jagarlamudi K, Shen Y, Gunnarsson D, Selstam G, Boman K, Liu K (2007) Infertility caused by retardation of follicular development in mice with oocyte-specific expression of Foxo3a. *Development* **134:** 199-209

Liu ZJ, Sakamoto T, Yokomuro S, Ezure T, Subbotin V, Murase N, Contrucci S, Demetris AJ (2000) Acute obstructive cholangiopathy in interleukin-6 deficient mice: compensation by leukemia inhibitory factor (LIF) suggests importance of gp-130 signaling in the ductular reaction. *Liver* **20:** 114-124

Liu ZL, de Matos DG, Fan HY, Shimada M, Palmer S, Richards JS (2009) Interleukin-6: An Autocrine Regulator of the Mouse Cumulus Cell-Oocyte Complex Expansion Process. *Endocrinology* **150:** 3360-3368

Lo CF, Ning X, Gonzales C, Ozenberger BA (2008) Induced expression of death domain genes NALP1 and NALP5 following neuronal injury. *Biochemical and Biophysical Research Communications* **366:** 664-669

Lobascio AM, Klinger FG, Scaldaferri ML, Farini D, De Felici M (2007) Analysis of programmed cell death in mouse fetal oocytes. *Reproduction* **134:** 241-252

Loffler KA, Zarkower D, Koopman P (2003) Etiology of ovarian failure in blepharophimosis ptosis epicanthus inversus syndrome: FOXL2 is a conserved, early-acting gene in vertebrate ovarian development. *Endocrinology* **144:** 3237-3243

Loftin CD, Trivedi DB, Tiano HF, Clark JA, Lee CA, Epstein JA, Morham SG, Breyer MD, Nguyen M, Hawkins BM, Goulet JL, Smithies O, Koller BH, Langenbach R (2001) Failure of ductus arteriosus closure and remodeling in neonatal mice deficient in cyclooxygenase-1 and cyclooxygenase-2. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 1059-1064

Lomeli H, Ramos-Mejia V, Gertsenstein M, Lobe CG, Nagy A (2000) Targeted insertion of Cre recombinase into the TNAP gene: Excision in primordial germ cells. *Genesis* **26:** 116-117

Looijenga LHJ, Stoop H, de Leeuw H, Brazao CAD, Gillis AJM, van Roozendaal KEP, van Zoelen EJJ, Weber RFA, Wolffenbuttel KP, van Dekken H, Honecker F, Bokemeyer C, Perlman EJ, Schneider DT, Kononen J, Sauter G, Oosterhuis JW (2003) POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. *Cancer Research* **63:** 2244-2250

Lopes S, Hayashi K, Shovlin TC, Mifsud W, Surani MA, McLaren A (2008) X chromosome activity in mouse XX primordial germ cells. *PLoS Genetics* **4:** e30

Luo XR, Ikeda YY, Parker KL (1994) A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation *Cell* **77:** 481-490

Lutticken C, Wegenka UM, Yuan JP, Buschmann J, Schindler C, Ziemiecki A, Harpur AG, Wilks AF, Yasukawa K, Taga T, Kishimoto T, Barbieri G, Pellegrini S, Sendtner M, Heinrich PC, Horn F (1994) Association of transcription factor APRF and protein-kinase JAK1 with the interleukin-6 signal transducer gp130 *Science* **263:** 89-92

Lydon JP, Demayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Shyamala G, Conneely OM, Omalley BW (1995) Mice lacking progesterone-receptor exhibit pleiotropic reproductive abnormalities. *Genes & Development* **9:** 2266-2278

Lynch CD, Zhang J (2007) The research implications of the selection of a gestational age estimation method. *Paediatric and Perinatal Epidemiology* **21:** 86-96

Macdonald LJ, Catalano RD, Sales KJ, Critchley HOD, Jabbour HN (2010) Prokineticin-1 Regulates Dickkopf-1, an Inhibitor of Wnt Signaling, in the Human Endometrium and during Early Pregnancy. *Reproductive Sciences* **17:** 245A-245A

Maheshwari A, Fowler PA (2008) Primordial follicular assembly in humans - revisited. *Zygote* **16:** 285-296

Majima M, Isono M, Ikeda Y, Hayashi I, Hatanaka K, Harada Y, Katsumata O, Yamashina S, Katori M, Yamamoto S (1997) Significant roles of inducible cyclooxygenase (COX)-2 in angiogenesis in rat sponge implants. *Japanese Journal of Pharmacology* **75:** 105-114

Maldonado-Perez D, Evans J, Denison F, Millar RP, Jabbour HN (2007) Potential roles of the prokineticins in reproduction. *Trends in Endocrinology and Metabolism* **18:** 66-72

Malki S, Nef S, Notarnicola C, Thevenet L, Gasca P, Mejean C, Berta P, Poulat F, Boizet-Bonhoure B (2005) Prostaglandin D2 induces nuclear import of the sex-determining factor SOX9 via its cAMP-PKA phosphorylation. *EMBO Journal* **24:** 1798-1809

Manova K, Nocka K, Besmer P, Bachvarova RF (1990) Gonadal expression of c-kit encoded at the w-locus of the mouse. *Development* **110:** 1057-1069

Manuylov NL, Smagulova FO, Leach L, Tevosian SG (2008) Ovarian development in mice requires the GATA4-FOG2 transcription complex. *Development* **135:** 3731-3743

Mao BY, Wu W, Li Y, Hoppe D, Stannek P, Glinka A, Niehrs C (2001) LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature* **411:** 321-325

Marsango S, Bonaccorsi di Pattie MC, Barra D, Miele R (2011) Evidence that prokineticin receptor 2 exsits as a dimer in vivo. *Cellular and Molecular Life Sceinces* **68:** 2919-2929

Martineau J, Nordqvist K, Tilmann C, LovellBadge R, Capel B (1997) Male-specific cell migration into the developing gonad. *Current Biology* **7:** 958-968

Martins da Silva SJ, Bayne RA, Cambray N, Hartley PS, McNeilly AS, Anderson RA (2004) Expression of activin subunits and receptors in the developing human ovary: activin A promotes germ cell survival and proliferation before primordial follicle formation. *Developmental Biology* **266:** 334-345

Marzella PL, Gillespie LN, Clark GM, Bartlett PF, Kilpatrick TJ (1999) The neurotrophins act synergistically with LIF and members of the TGF-beta superfamily to promote the survival of spiral ganglia neurons in vitro. *Hearing Research* **138:** 73-80

Massague J (1998) TGF-beta signal transduction. *Annual Review of Biochemistry* **67:** 753-791

Masuda Y, Takatsu Y, Terao Y, Kumano S, Ishibashi Y, Suenaga M, Abe M, Fukusumi S, Watanabe T, Shintani Y, Yamada T, Hinuma S, Inatomi N, Ohtaki T, Onda H, Fujino M (2002) Isolation and identification of EG-VEGF/prokineticins as cognate ligands for two orphan G-protein-coupled receptors. *Biochemical and Biophysical Research Communications* **293:** 396-402

Matsui Y, Toksoz D, Nishikawa S, Nishikawa SI, Williams D, Zsebo K, Hogan BLM (1991) Effect of steel factor and leukemia inhibitory factor on murine primoridal germ-cells in culture. *Nature* **353:** 750-752

Matsumoto S, Yamazaki C, Masumoto KH, Nagano M, Naito M, Soga T, Hiyama H, Matsumoto M, Takasaki J, Kamohara M, Matsuo A, Ishii H, Kobori M, Katoh M, Matsushime H, Furuichi K, Shigeyoshi Y (2006) Abnormal development of the olfactory bulb and reproductive system in mice lacking prokineticin receptor PKR2. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 4140-4145

Mattison DR, Nightingale MS, Shiromizu K (1983) Effects of toxic-substances on female reproduction. *Environmental Health Perspectives* **48:** 43-52

Matzuk MM, Kumar TR, Bradley A (1995) Different phenotypes for mice deficient in either activins or activin receptor-type-II. *Nature* **374:** 356-360

Mayhew TM, Olsen DR (1991) Magnetic-resonance-imaging (MRI) and model-free estimates of brain volume determined using the cavalieri principle. *Journal of Anatomy* **178:** 133-144

McCoshen JA, McCallion DJ (1975) Study of primordial germ-cells during their migratory phase in steel mutant mice. *Experientia* **31:** 589-590

McGee EA, Hsueh AJW (2000) Initial and cyclic recruitment of ovarian follicles. *Endocrine Reviews* **21:** 200-214

McLaren A (1991) Development of the mammalian gonad - the fate of the supporting cell lineage. *Bioessays* **13:** 151-156

McMullen ML, Cho BN, Yates CJ, Mayo KE (2001) Gonadal pathologies in transgenic mice expressing the rat inhibin alpha-subunit. *Endocrinology* **142:** 5005-5014

McNatty KP, Fidler AE, Juengel JL, Quirke LD, Smith PR, Heath DA, Lundy T, O'Connell A, Tisdall DJ (2000) Growth and paracrine factors regulating follicular formation and cellular function. *Molecular and Cellular Endocrinology* **163:** 11-20

McNatty KP, Juengel JL, Reader KL, Lun S, Myllymaa S, Lawrence SB, Western A, Meerasahib MF, Mottershead DG, Groome NP, Ritvos O, Laitinen MPE (2005) Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function. *Reproduction* **129:** 473-480

Melchiorri D, Bruno V, Besong G, Ngomba RT, Cuomo L, De Blasi A, Copani A, Moschella C, Storto M, Nicoletti F, Lepperdinger G, Passarelli F (2001) The mammalian homologue of the novel peptide Bv8 is expressed in the central nervous system and supports neuronal survival by activating the MAP kinase/PI-3-kinase pathways. *European Journal of Neuroscience* **13:** 1694-1702

Menke DB, Page DC (2002) Sexually dimorphic gene expression in the developing mouse gonad. *Gene Expression Patterns* **2:** 359-367

Menke DB, Koubova J, Page DC (2003) Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave. *Developmental Biology* **262:** 303-312

Merchant-Larios H, Moreno-Mendoza N (1998) Mesonephric stromal cells differentiate into Leydig cells in the mouse fetal testis. *Experimental Cell Research* **244:** 230-238

Merchantlarios H, Morenomendoza N, Buehr M (1993) The role of the mesonephros in cell-differentiation and morphogenesis of the mouse fetal testis. *International Journal of Developmental Biology* **37:** 407-415

Mintz B, Russell ES (1957) Gene-induced embryological modification sof primordial germ cells in the mouse. *Journal of Experimental Zoology* **134:** 207-237

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113:** 631-642

Mitchell JR, McMurtry RJ, Statham CN, Nelson SD (1977) Molecular basis for several drug-induced nephropathies. *American Journal of Medicine* **62:** 518-526

Miyazono K, Miyazawa K (2002) Id: a target of BMP signaling. *Science's STKE : Signal Transduction Knowledge Environment* **2002:** 40

Mizuno Y, Gotoh A, Kamidono S, Kitazawa S (1993) Establishment and characterization of a new human testicular germ cell tumur cell line (TCam-2). *Nihon Naika Gakkai zasshi The Journal of the Japanese Society of Internal Medicine* **84:** 1211-1218

Modi DN, Sane S, Bhartiya D (2003) Accelerated germ cell apoptosis in sex chromosome aneuploid fetal human gonads. *Molecular Human Reproduction* **9:** 219-225

Molkentin JD, Lin Q, Duncan SA, Olson EN (1997) Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes & Development* **11:** 1061-1072

Mollay C, Wechselberger C, Mignogna G, Negri L, Melchiorri P, Barra D, Kreil G (1999) Bv8, a small protein from frog skin and its homologue from snake venom induce hyperalgesia in rats. *European Journal of Pharmacology* **374:** 189-196

Molyneaux KA, Stallock J, Schaible K, Wylie C (2001) Time-lapse analysis of living mouse germ cell migration. *Developmental Biology* **240:** 488-498

Molyneaux KA, Schaible K, Wylie C (2003a) GP130, the shared receptor for the LIF/IL6 cytokine family in the mouse, is not required for early germ cell differentiation, but is required cell-autonomously in oocytes for ovulation. *Development* **130:** 4287-4294

Molyneaux KA, Zinszner H, Kunwar PS, Schaible K, Stebler J, Sunshine MJ, O'Brien W, Raz E, Littman D, Wylie C, Lehmann R (2003b) The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. *Development* **130:** 4279-4286

Monk M, McLaren A (1981) X chromosome activity in fetal germ cells of the mouse. *Journal of Embryology and Experimental Morphology* **63:** 533-537

Monnier J, Samson M (2010) Prokineticins in angiogenesis and cancer. *Cancer Letters* **296:** 144-149

Moore KL (1967) Sex determination sexual differentiation and intersex development. *Canadian Medical Association Journal* **97:** 292-295

Morita Y, Manganaro TF, Tao XJ, Martimbeau S, Donahoe PK, Tilly JL (1999) Requirement for phosphatidylinositol-3 '-kinase in cytokine-mediated germ cell survival during fetal oogenesis in the mouse. *Endocrinology* **140:** 941-949

Morita Y, Tilly JL (1999a) Oocyte apoptosis: like sand through an hourglass. *Developmental Biology* **213:** 1-17

Morita Y, Tilly JL (1999b) Segregation of retinoic acid effects on fetal ovarian germ cell mitosis versus apoptosis by requirement for new macromolecular synthesis. *Endocrinology* **140:** 2696-2703

Motta PM, Makabe S, Nottola SA (1997) The ultrastructure of human reproduction .1. The natural history of the female germ cell: origin, migration and differentiation inside the developing ovary. *Human Reproduction Update* **3:** 281-295

Mulder KM (2000) Role of Ras and Mapks in TGF beta signaling. *Cytokine & Growth Factor Reviews* **11:** 23-35

Murakami M, Hibi M, Nakagawa N, Nakagawa T, Yasukawa K, Yamanishi K, Taga T, Kishimoto T (1993) IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. *Science* **260:** 1808-1810

Murray TJ, Fowler PA, Abramovich DR, Haites N, Lea RG (2000) Human fetal testis: Second trimester proliferative and steroidogenic capacities. *Journal of Clinical Endocrinology & Metabolism* **85:** 4812-4817

Muttukrishna S, Tannetta D, Groome N, Sargent I (2004) Activin and follistatin in female reproduction. *Molecular and Cellular Endocrinology* **225:** 45-56

Nachtigal MW, Hirokawa Y, Enyeart-VanHouten DL, Flanagan JN, Hammer GD, Ingraham HA (1998) Wilms' tumor 1 and DAX-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. *Cell* **93:** 445-454

Nakamura T, Takio K, Eto Y, Shibai H, Titani K, Sugino H (1990) Activin-binding protein from rat ovary is follistatin. *Science* **247:** 836-838

Nakashima K, Yanagisawa M, Arakawa H, Kimura N, Hisatsune T, Kawabata M, Miyazono K, Taga T (1999) Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. *Science* **284:** 479-482

Narumiya S, Sugimoto Y, Ushikubi F (1999) Prostanoid receptors: Structures, properties, and functions. *Physiological Reviews* **79:** 1193-1226

Narumiya S, FitzGerald GA (2001) Genetic and pharmacological analysis of prostanoid receptor function. *Journal of Clinical Investigation* **108:** 25-30

Negishi M, Sugimoto Y, Ichikawa A (1995) Molecular mechanisms of diverse actions of prostanoid receptors. *Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism* **1259:** 109-119

Negri L, Lattanzi R, Giannini E, Metere A, Colucci M, Barra D, Kreil G, Melchiorri P (2002) Nociceptive sensitization by the secretory protein Bv8. *British Journal of Pharmacology* **137:** 1147-1154

Ngan ESW, Lee KY, Sit FYL, Poon HC, Chan JKY, Sham MH, Lui VCH, Tam PKH (2007) Prokineticin-1 modulates proliferation and differentiation of enteric neural crest cells. *Biochimica Et Biophysica Acta-Molecular Cell Research* **1773:** 536-545

Ngan ESW, Shum CKY, Poon H-C, Sham M-H, Garcia-Barcelo M-M, Lui VCH, Tam PKH (2008) Prokineticin-1 (Prok-1) works coordinately with glial cell line-derived neurotrophic factor (GDNF) to mediate proliferation and differentiation of enteric neural crest cells. *Biochimica Et Biophysica Acta-Molecular Cell Research* **1783:** 467-478

Ngan ESW, Tam PKH (2008) Prokineticin-signaling pathway. *International Journal of Biochemistry & Cell Biology* **40:** 1679-1684

Nguyen MT, Camenisch T, Snouwaert JN, Hicks E, Coffman TM, Anderson PAW, Malouf MN, Koller BH (1997) The prostaglandin receptor EP4 triggers remodelling of the cardiovascular system at birth. *Nature* **390:** 78-81

Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith A (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95:** 379-391

Nicholson SE, De Souza D, Fabri LJ, Corbin J, Willson TA, Zhang JG, Silva A, Asimakis M, Farley A, Nash AD, Metcalf D, Hilton DJ, Nicola NA, Baca M (2000) Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130. *Proceedings of the National Academy of Sciences of the United States of America* **97:** 6493-6498

Niemi TT, Backman JT, Syrjala MT, Viinikka LU, Rosenberg PH (2000) Platelet dysfunction after intravenous ketorolac or propacetamol. *Acta Anaesthesiologica Scandinavica* **44:** 69-74

Nilsson E, Parrott JA, Skinner MK (2001) Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. *Molecular and Cellular Endocrinology* **175:** 123-130

Nilsson E, Dole G, Skinner MK (2009) Neurotrophin NT3 promotes ovarian primordial to primary follicle transition. *Reproduction* **138:** 697-707

Nilsson EE, Kezele P, Skinner MK (2002) Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. *Molecular and Cellular Endocrinology* **188:** 65-73

Nilsson EE, Skinner MK (2002) Growth and differentiation factor-9 stimulates progression of early primary but not primordial rat ovarian follicle development. *Biology of Reproduction* **67:** 1018-1024

Nilsson EE, Skinner MK (2003) Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biology of Reproduction* **69:** 1265-1272

Nilsson EE, Skinner MK (2004) Kit ligand and basic fibroblast growth factor interactions in the induction of ovarian primordial to primary follicle transition. *Molecular and Cellular Endocrinology* **214:** 19-25

Nishino K, Yamanouchi K, Naito K, Tojo H (2001) Characterization of mesonephric cells that migrate into the XY gonad during testis differentiation. *Experimental Cell Research* **267:** 225-232

Norton JD, Deed RW, Craggs G, Sablitzky F (1998) Id helix-loop-helix proteins in cell growth and differentiation. *Trends in Cell Biology* **8:** 58-65

O'Shaughnessy PJ, Baker PJ, Monteiro A, Cassie S, Bhattacharya S, Fowler PA (2007) Developmental changes in human fetal testicular cell numbers and messenger ribonucleic acid levels during the second trimester. *Journal of Clinical Endocrinology & Metabolism* **92:** 4792-4801

Oates JA, FitzGerald GA, Branch RA, Jackson EK, Knapp HR, Roberts LJ, 2nd (1988a) Clinical implications of prostaglandin and thromboxane A2 formation (1). *The New England Journal of Medicine* **319:** 689-698

Oates JA, FitzGerald GA, Branch RA, Jackson EK, Knapp HR, Roberts LJ, 2nd (1988b) Clinical implications of prostaglandin and thromboxane A2 formation (2). *The New England Journal of Medicine* **319:** 761-767

Ohinata Y, Payer B, O'Carroll D, Ancelin K, Ono Y, Sano M, Barton SC, Obukhanych T, Nussenzweig M, Tarakhovsky A, Saitou M, Surani MA (2005) Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* **436:** 207-213

Ohmura M, Yoshida S, Ide Y, Nagamatsu G, Suda T, Ohbo K (2004) Spatial analysis of germ stem cell development in Oct-4/EGFP transgenic mice. *Archives of Histology and Cytology* **67:** 285-296

Ojeda SR, Negrovilar A, McCann SM (1982) Evidence for involvement of alpha-adrenergic receptors in norepinephrine-induced prostaglandin-E2 and lutenizing- hormone-releasing hormone-release from the median-eminence. *Endocrinology* **110:** 409-412

Okamura D, Tokitake Y, Niwa H, Matsui Y (2008) Requirement of Oct3/4 function for germ cell specification. *Developmental Biology* **317:** 576-584

Ostby JS, Gray LE, Jr. (2004) Transgenerational (in utero/lactational) exposure to investigate the effects of endocrine disrupting compounds (EDCS) in rats. *Current protocols in toxicology / editorial board, Mahin D Maines (editor-in-chief)* **Chapter 16** Unit 16.8

Ostrer H, Huang HY, Masch RJ, Shapiro E (2007) A cellular study of human testis development. *Sexual Development* **1:** 286-292

Otsuka F, Moore RK, Iemura S, Ueno N, Shimasaki S (2001) Follistatin inhibits the function of the oocyte-derived factor BMP-15. *Biochemical and Biophysical Research Communications* **289:** 961-966

Otto JC, Smith WL (1995) Prostaglandin endoperoxidase synthase-1 and synthase-2. *Journal of Lipid Mediators and Cell Signalling* **12:** 139-156

Ottolenghi C, Omari S, Garcia-Ortiz JE, Uda M, Crisponi L, Forabosco A, Pilia G, Schlessinger D (2005) Foxl2 is required for commitment to ovary differentiation. *Human Molecular Genetics* **14:** 2053-2062

Ottolenghi C, Pelosi E, Tran J, Colombino M, Douglass E, Nedorezov T, Cao A, Forabosco A, Schlessinger D (2007) Loss of Wnt4 and Foxl2 leads to female-to-male sex reversal extending to germ cells. *Human Molecular Genetics* **16:** 2795-2804

Pan GJ, Chang ZY, Scholer HR, Pei DQ (2002) Stem cell pluripotency and transcription factor Oct4. *Cell Research* **12:** 321-329

Pangas SA, Choi Y, Ballow DJ, Zhao YG, Westphal H, Matzuk MM, Rajkovic A (2006) Oogenesis requires germ cell-specific transcriptional regulators Sohlh1 and Lhx8. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 8090-8095

Paralkar VM, Grasser WA, Mansolf AL, Baumann AP, Owen TA, Smock SL, Martinovic S, Borovecki F, Vukicevic S, Ke HZ, Thompson DD (2002) Regulation of BMP-7 expression by retinoic acid and prostaglandin E-2. *Journal of Cellular Physiology* **190:** 207-217

Paredes A, Romero CN, Dissen GA, DeChiara TM, Reichardt L, Cornea A, Ojeda SR, Xu BJ (2004) TrkB receptors are required for follicular growth and oocyte survival in the mammalian ovary. *Developmental Biology* **267:** 430-449

Parker KL, Schimmer BP (1994) The role of nuclear receptors in steroid-hormones production. *Seminars in Cancer Biology* **5:** 317-325

Parker KL, Schimmer BP (1997) Steroidogenic factor 1: A key determinant of endocrine development and function. *Endocrine Reviews* **18:** 361-377

Parma P, Radi O, Vidal V, Chaboissier MC, Dellambra E, Valentini S, Guerra L, Schedl A, Camerino G (2006) R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nature Genetics* **38:** 1304-1309

Pelusi C, Ikeda Y, Zubair M, Parker KL (2008) Impaired Follicle Development and Infertility in Female Mice Lacking Steroidogenic Factor 1 in Ovarian Granulosa Cells. *Biology of Reproduction* **79:** 1074-1083

Pepling ME, Spradling AC (1998) Female mouse germ cells form synchronously dividing cysts. *Development* **125:** 3323-3328

Pepling ME, Spradling AC (2001) Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Developmental Biology* **234:** 339-351

Perez GI, Robles R, Knudson CM, Flaws JA, Korsmeyer SJ, Tilly JL (1999) Prolongation of ovarian lifespan into advanced chronological age by Bax-deficiency. *Nature Genetics* **21:** 200-203

Perez RP, Sanville H (2000) BAXzeta a novel BAX variant, lacks BH3 domain and promotes cell death. *Proceedings of the American Association for Cancer Research Annual Meeting* **6:** 153-154

Pesce M, Farrace MG, Piacentini M, Dolci S, De Felici M (1993a) Stem cell factor and leukemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis). *Development* **118:** 1089-1094

Pesce M, Farrace MG, Piacentini M, Dolci S, Defelici M (1993b) Stem-cell factor and leukemia inhibitory factor promote primordial germ-cell survival by suppressing programmed cell-death (apoptosis). *Development* **118:** 1089-1094

Pesce M, Gross MK, Scholer HR (1998a) In line with our ancestors: Oct-4 and the mammalian germ. *Bioessays* **20:** 722-732

Pesce M, Wang XY, Wolgemuth DJ, Scholer H (1998b) Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. *Mechanisms of Development* **71:** 89-98

Pesce M, Scholer HR (2000) Oct-4: Control of totipotency and germline determination. *Molecular Reproduction and Development* **55:** 452-457

Petraglia F, Anceschi MM, Calza L, Garuti GC, Fusaro P, Giardino L, Genazzani AR, Vale W (1993) Inhibin and activin in human fetal membranes - evidence for a local effect on prostaglandin release. *Journal of Clinical Endocrinology & Metabolism* **77:** 542-548

Pierre A, Pisselet C, Monget P, Monniaux D, Fabre S (2005) Testing the antagonistic effect of follistatin on BMP family members in ovine granulosa cells. *Reproduction Nutrition Development* **45:** 419-425

Pincus G, Enzmann EV (1935) The comparative behavior of mammalian eggs in vivo and in vitro I. The activation of ovarian eggs. *Journal of Experimental Medicine* **62:** 665-U664

Pisarska MD, Bae J, Klein C, Hsueh AJW (2004) Forkhead L2 is expressed in the ovary and represses the promoter activity of the steroidogenic acute regulatory gene. *Endocrinology* **145:** 3424-3433

Pitteloud N, Zhang C, Pignatelli D, Li J-D, Raivio T, Cole LW, Plummer L, Jacobson-Dickman EE, Mellon PL, Zhou Q-Y, Crowley WF, Jr. (2007) Loss-of-function mutation in the prokineticin 2 gene causes Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 17447-17452

Pittman DL, Cobb J, Schimenti KJ, Wilson LA, Cooper DM, Brignull E, Handel MA, Schimenti JC (1998) Meiotic prophase arrest with failure of chromosome synapsis in mice deficient for Dmc1, a germline-specific RecA homolog. *Molecular Cell* **1:** 697-705

Prescott LF, Wright N (1973) The effects of hepatic and renal damage on paracetamol metabolism and excretion following overdosage. A pharmacokinetic study. *British Journal of Pharmacology* **49:** 602-613

Prescott LF (1980) Kinetics and metabolism of paracetamol and phenacetin. *British Journal of Clincial Pharamacology* **10:** 291S-298S

Prosser HM, Bradley A, Chesham JE, Ebling FJP, Hastings MH, Maywood ES (2007) Prokineticin receptor 2 (Prokr2) is essential for the regulation of circadian behavior by the suprachiasmatic nuclei. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 648-653

Prueitt RL, Zinn AR (2001) A fork in the road to fertility. *Nature Genetics* **27:** 132-134

Qin Y, Choi Y, Zhao H, Simpson JL, Chen Z-J, Rajkovic A (2007) NOBOX homeobox mutation causes premature ovarian failure. *American Journal of Human Genetics* **81:** 576-581

Qin Y, Zhao H, Kovanci E, Simpson JL, Chen Z-J, Rajkovic A (2008) Analysis of LHX8 mutation in premature ovarian failure. *Fertility and Sterility* **89:** 1012-1014

Quenby SM, Gazvani MR, Brazeau C, Neilson J, Lewis-Jones DI, Vince G (1999) Oncogenes and tumour suppressor genes in first trimester human fetal gonadal development. *Molecular Human Reproduction* **5:** 737-741

Quennell JH, Stanton JAL, Hurst PR (2004) Basic fibroblast growth factor expression in isolated small human ovarian follicles. *Molecular Human Reproduction* **10:** 623-628

Rabinovici J, Goldsmith PC, Roberts VJ, Vaughan J, Vale W, Jaffe RB (1991) Localization and secretion of inhibin activin subunits in the human and subhuman primate fetal gonads. *Journal of Clinical Endocrinology & Metabolism* **73:** 1141-1149

Rajah R, Glaser EM, Hirshfield AN (1992) The changing architecture of the neonatal rat ovary during histogenesis *Developmental Dynamics* **194:** 177-192

Rajkovic A, Yan CN, Klysik M, Matzuk M (2001) Discovery of germ cell-specific transcripts by expressed sequence tag database analysis. *Fertility and Sterility* **76:** 550-554

Rajkovic A, Pangas SA, Ballow D, Suzumori N, Matzuk MM (2004) NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. *Science* **305:** 1157-1159

Rajpert-De Meyts E, Jorgensen N, Graem N, Muller J, Cate RL, Skakkebaek NE (1999) Expression of anti-Mullerian hormone during normal and pathological gonadal development: Association with differentiation of Sertoli and granulosa cells. *Journal of Clinical Endocrinology & Metabolism* **84:** 3836-3844

Rajpert-De Meyts E, Hanstein R, Jorgensen N, Graem N, Vogt PH, Skakkebaek NE (2004) Developmental expression of POU5F1 (OCT-3/4) in normal and dysgenetic human gonads. *Human Reproduction* **19:** 1338-1344

Ramkissoon Y, Goodfellow P (1996) Early steps in mammalian sex determination. *Current Opinion in Genetics & Development* **6:** 316-321

Rathmell JP, Viscomi CM, Ashburn MA (1997) Management of nonobstetric pain during pregnancy and lactation. *Anesthesia and Analgesia* **85:** 1074-1087

Reddy P, Shena LJ, Ren C, Boman K, Lundin E, Ottander U, Lindgren P, Liu YX, Sun QY, Liu K (2005) Activation of Akt (PKB) and suppression of FKHRL1 in mouse and rat oocytes by stem cell factor during follicular activation and development. *Developmental Biology* **281:** 160-170

Reddy P, Zheng W, Liu K (2010) Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. *Trends in Endocrinology and Metabolism* **21:** 96-103

Ren P, Zhang H, Qiu F, Liu Y-Q, Gu H, O'Dowd DK, Zhou Q-Y, Hu W-P (2011) Prokineticin 2 regulates the electrical activity of rat suprachiasmatic nuclei neurons. *PLoS One* **6:** e20263

Resnick JL, Bixler LS, Cheng LZ, Donovan PJ (1992) Long-term proliferation of mouse primorial germ-cells in culture. *Nature* **359:** 550-551

Reuter AL, Goji K, Bingham NC, Matsu M, Parker KL (2007) A novel mutation in the accessory DNA-binding domain of human steroidogenic factor I causes XY gonadal dysgenesis without adrenal insufficiency. *European Journal of Endocrinology* **157:** 233-238

Reynolds N, Collier B, Bingham V, Gray NK, Cooke HJ (2007) Translation of the synaptonemal complex component Sycp3 is enhanced in vivo by the germ cell specific regulator Dazl. *Rna-a Publication of the RNA Society* **13:** 974-981

Robertson EJ, Charatsi I, Joyner CJ, Koonce CH, Morgan M, Islam A, Paterson C, Lejsek E, Arnold SJ, Kallies A, Nutt SL, Bikoff EK (2007) Blimp1 regulates development of the posterior forelimb, caudal pharyngeal arches, heart and sensory vibrissae in mice. *Development* **134:** 4335-4345

Robinson LLL, Gaskell TL, Saunders PTK, Anderson RA (2001) Germ cell specific expression of c-kit in the human fetal gonad. *Molecular Human Reproduction* **7:** 845-852

Robles R, Morita Y, Mann KK, Perez GI, Yang S, Matikainen T, Sherr DH, Tilly JL (2000) The aryl hydrocarbon receptor, a basic helix-loop-helix transcription factor of the PAS gene family, is required for normal ovarian germ cell dynamics in the mouse. *Endocrinology* **141:** 450-453

Rosner MH, Vigano MA, Ozato K, Timmons PM, Poirier F, Rigby PWJ, Staudt LM (1990) A POU-domain transcription factor in early stem-cells and germ-cells of the mammalian embryo. *Nature* **345:** 686-692

Ross A, Munger S, Capel B (2007a) Bmp7 regulates germ cell proliferation in mouse fetal gonads. *Sexual Development* **1:** 127-137

Ross A, Munger S, Capel B (2007b) Bmp7 regulates germ cell proliferation in mouse fetal gonads. *Sexual development : genetics, molecular biology, evolution, endocrinology, embryology, and pathology of sex determination and differentiation* **1:** 127-137

Ross AJ, Tilman C, Yao H, MacLaughlin D, Capel B (2003) AMH induces mesonephric cell migration in XX gonads. *Molecular and Cellular Endocrinology* **211:** 1-7

Ross MH, Long IR (1966) Contractile cells in human seminiferous tubules. *Science* **153:** 1271-1273

Ruiz-Ferrer M, Torroglosa A, Nunez-Torres R, Carlos de Agustin J, Antinolo G, Borrego S (2011) Expression of PROKR1 and PROKR2 in Human Enteric Neural Precursor Cells and Identification of Sequence Variants Suggest a Role in HSCR. *Plos One* **6** DOI: 10.1371/journal.pone.0023475

Runyan C, Schaible K, Molyneaux K, Wang ZQ, Levin L, Wylie C (2006) Steel factor controls midline cell death of primordial germ cells and is essential for their normal proliferation and migration. *Development* **133:** 4861-4869

Sadovsky Y, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte LM, Simburger K, Milbrandt J (1995) Mice deficient in the orphan receptor steroidogenic factor-1 lack adrenal- glands and gonads but express p450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids *Proceedings of the National Academy of Sciences of the United States of America* **92:** 10939-10943

Saitou M, Barton SC, Surani MA (2002) A molecular programme for the specification of germ cell fate in mice. *Nature* **418:** 293-300

Saitou M, Payer B, Lange UC, Erhardt S, Barton SC, Surani MA (2003) Specification of germ cell fate in mice. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **358:** 1363-1370

Saitou M, Payer B, O'Carroll D, Ohinata Y, Surani MA (2005) Blimp1 and the emergence of the germ line during development in the mouse. *Cell Cycle* **4:** 1736-1740

Saitou M (2009) Germ cell specification in mice. *Current Opinion in Genetics & Development* **19:** 386-395

Sales KJ, Jabbour HN (2003a) Cyclooxygenase enzymes and prostaglandins in pathology of the endometrium. *Reproduction* **126:** 559-567

Sales KJ, Jabbour HN (2003b) Cyclooxygenase enzymes and prostaglandins in reproductive tract physiology and pathology. *Prostaglandins & Other Lipid Mediators* **71:** 97-117

Samson M, Peale FV, Frantz G, Rioux-Leclercq N, Rajpert-De Meyts E, Ferrara N (2004) Human endocrine gland-derived vascular endothelial growth factor: Expression early in development and in Leydig cell tumors suggests roles in normal and pathological testis angiogenesis. *Journal of Clinical Endocrinology & Metabolism* **89:** 4078-4088

Sanchez-Sanchez AV, Camp E, Leal-Tassias A, Atkinson SP, Armstrong L, Diaz-Llopis M, Mullor JL (2010) Nanog Regulates Primordial Germ Cell Migration Through Cxcr4b. *Stem Cells* **28:** 1457-1464

Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC, Treier M (2004) The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development* **131:** 933-942

Schmitz J, Weissenbach M, Haan S, Heinrich PC, Schaper F (2000) SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment site of gp130. *Journal of Biological Chemistry* **275:** 12848-12856

Schneyer AL, Rzucidlo DA, Sluss PM, Crowley WF (1994) Characterization of unique binding-kinetics of follistatin and activin or inhibin in serum. *Endocrinology* **135:** 667-674

Schneyer AL, Fujiwara T, Fox J, Welt CK, Adams J, Messerlian GM, Taylor AE (2000) Dynamic changes in the intrafollicular inhibin/activin/follistatin axis during human follicular development: Relationship to circulating hormone concentrations. *Journal of Clinical Endocrinology & Metabolism* **85:** 3319-3330

Scholer HR, Ruppert S, Suzuki N, Chowdhury K, Gruss P (1990) New type of POU domain in germ line-specific protein Oct-4. *Nature* **344:** 435-439

Schoorlemmer J, Vanpuijenbroek A, Vandeneijnden M, Jonk L, Pals C, Kruijer W (1994) Characterization of a negative retinoic acid response element in the murine Oct4 promoter. *Molecular and Cellular Biology* **14:** 1122-1136

Schwab JM, Schluesener HJ, Laufer S (2003) COX-3: just another COX or the solitary elusive target of paracetamol? *Lancet* **361:** 981-982

Schweitz H, Bidard JN, Lazdunski M (1990) Purification and phrmacological characerization of peptide toxins from the Black Mamba (Dendroaspis-polylepis) venom *Toxicon* **28:** 847-856

Scott HM, Mason JI, Sharpe RM (2009) Steroidogenesis in the Fetal Testis and Its Susceptibility to Disruption by Exogenous Compounds. *Endocrine Reviews* **30:** 883-925

Sekido R, Lovell-Badge R (2008) Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. *Nature* **453:** 930-934

Semenov MV, Tamai K, Brott BK, Kuhl M, Sokol S, He X (2001) Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. *Current Biology* **11:** 951-961

Sforza C, Vizzotto L, Ferrario VF, Forabosco A (2003) Position of follicles in normal human ovary during definitive histogenesis. *Early Human Development* **74:** 27-35

Sharpe RM, Veeramachaneni DNR, Skakkebaek NE, McLachlan J (2006) Germ cell atypia in undescended testes hinges on the aetiology of cryptorchidism but not the abdominal location per se - Discussion. *International Journal of Andrology* **29:** 239-240

Shaw JLV, Denison FC, Evans J, Durno K, Williams AR, Entrican G, Critchley HOD, Jabbour HN, Horne AW (2010a) Evidence of prokineticin dysregulation in fallopian tube from women with ectopic pregnancy. *Fertility and Sterility* **94:** 1601-U1601

Shaw JLV, Oliver E, Lee K-F, Entrican G, Jabbour HN, Critchley HOD, Horne AW (2010b) Cotinine Exposure Increases Fallopian Tube PROKR1 Expression via Nicotinic AChR alpha-7 A Potential Mechanism Explaining the Link between Smoking and Tubal Ectopic Pregnancy. *American Journal of Pathology* **177:** 2509-2515

Shaw JLV, Wills GS, Lee K-F, Horner PJ, McClure MO, Abrahams VM, Wheelhouse N, Jabbour HN, Critchley HOD, Entrican G, Horne AW (2011) Chlamydia trachomatis Infection Increases Fallopian Tube PROKR2 via TLR2 and NF kappa B Activation Resulting in a Microenvironment Predisposed to Ectopic Pregnancy. *American Journal of Pathology* **178:** 253-260

Shen WH, Moore CCD, Ikeda Y, Parker KL, Ingraham HA (1994) Nuclear receptor steroidogenic factor-1 regulates the mullerian-inhibiting substance gene - a link to the sex determination cascade. *Cell* **77:** 651-661

Shi SR, Chaiwun B, Young L, Cote RJ, Taylor CR (1993) Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society* **41:** 1599-1604

Shiina H, Matsumoto T, Sato T, Igarashi K, Miyamoto J, Takemasa S, Sakari M, Takada I, Nakamura T, Metzger D, Chambon P, Kanno J, Yoshikawa H, Kato S (2006) Premature ovarian failure in androgen receptor-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 224-229

Shual K, Ziemiecki A, Wilks AF, Harpur AG, Sadowski HB, Gilman MZ, Darnell JE (1993) Polypeptide signaling to the nucleus through tyrosine phosphorylation of JAK and STAT proteins. *Nature* **366:** 580-583

Sigthorsson G, Simpson R, Bjarnason I (2000) Concomitant inhibition of COX-1 and 2 underlies the intestinal toxicity of NSAIDs. *Arthritis and Rheumatism* **43:** S299-S300

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovellbadge R, Goodfellow PN (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* **346:** 240-244

Skinner MK (2005) Regulation of primordial follicle assembly and development. *Human Reproduction Update* **11:** 461-471

Skrzypczak J, Pisarski T, Biczysko W, Kedzia H (1981) Evaluation of germ-cells development in gonads of human fetuses and newborns. *Folia Histochemica Et Cytochemica* **19:** 17-24

Soga T, Matsumoto S, Oda T, Saito T, Hiyama H, Takasaki J, Kamohara M, Ohishi T, Matsushime H, Furuichi K (2002) Molecular cloning and characterization of prokineticin receptors. *Biochimica et Biophysica Acta* **1579:** 173-179

Sonmezer M, Oktay K (2004) Fertility preservation in female patients. *Human Reproduction Update* **10:** 251-266

Soto-Suazo M, San Martin S, Zorn TMT (2004) Collagen and tenascin-C expression along the migration pathway of mouse primordial germ cells. *Histochemistry and Cell Biology* **121:** 149-153

Soyal SM, Amleh A, Dean J (2000) FIG alpha, a germ cell-specific transcription factor required for ovarian follicle formation. *Development* **127:** 4645-4654

Spears N, Molinek MD, Robinson LL, Fulton N, Cameron H, Shimoda K, Telfer EE, Anderson RA, Price DJ (2003) The role of neurotrophin receptors in female germ-cell survival in mouse and human. *Development* **130:** 5481-5491

Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S, Ihle JN, Yancopoulos GD (1994) Association and activation of JAK-TYK kinases by CNTF-LIF-OSM-IL-6 beta-receptor components. *Science* **263:** 92-95

Stebler J, Spieler D, Slanchev K, Molyneaux KA, Richter U, Cojocaru V, Tarabykin V, Wylie C, Kessel M, Raz E (2004) Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. *Developmental Biology* **272:** 351-361

Steinert D, Kueper C, Bartels H, Beck F-X, Neuhofer W (2009) PGE(2) potentiates tonicity-induced COX-2 expression in renal medullary cells in a positive feedback loop involving EP2-cAMP-PKA signaling. *American Journal of Physiology-Cell Physiology* **296:** C75-C87

Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, Abbondanzo SJ (1992) Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. *Nature* **359:** 76-79

Stoop H, Honecker F, Cools M, de Krijger R, Bokemeyer C, Looijenga LHJ (2005) Differentiation and development of human female germ cells during prenatal gonadogenesis: an immunohistochemical study. *Human Reproduction* **20:** 1466-1476

Sugino H (1993) Follistatin and its role as an activin-binding protein. In *International Congress Symposium and Seminar Series; Progress in endocrinology*, ed Mornex **3**: 608-610

Sun RZ, Lei L, Cheng L, Jin ZF, Zu SJ, Shan ZY, Wang ZD, Zhang JX, Liu ZH (2010) Expression of GDF-9, BMP-15 and their receptors in mammalian ovary follicles. *Journal of Molecular Histology* **41:** 325-332

Supramaniam VG, Jenkin G, Wallace EM, O'Connor AE, de Kretser DM, Miller SL (2004) Effect of graded hypoxia on activin A, prostaglandin E-2 and cortisol levels in the late-pregnant sheep. *Reproduction Fertility and Development* **16:** 625-632

Suzuki H, Sada A, Yoshida S, Saga Y (2009) The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins Nanos2 and Nanos3. *Developmental Biology* **336:** 222-231

Suzumori N, Yan CN, Matzuk MM, Rajkovic A (2002) Nobox is a homeobox-encoding gene preferentially expressed in primordial and growing oocytes. *Mechanisms of Development* **111:** 137-141

Swain A, Narvaez S, Burgoyne P, Camerino G, Lovell-Badge R (1998) Dax1 antagonizes Sry action in mammalian sex determination. *Nature* **391:** 761-767

Szczepny A, Hogarth CA, Young J, Loveland KL (2009) Identification of Hedgehog Signaling Outcomes in Mouse Testis Development Using a Hanging Drop-Culture System. *Biology of Reproduction* **80:** 258-263

Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, Hirano T, Kishimoto T (1989) Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell* **58:** 573-581

Taga T, Kishimoto T (1997) gp130 and the interleukin-6 family of cytokines. *Annual Review of Immunology* **15:** 797-819

Tajima K, Dantes A, Yao Z, Sorokina K, Kotsuji F, Seger R, Amsterdam A (2003) Down-regulation of steroidogenic response to gonadotropins in human and rat preovulatory granulosa cells involves mitogen-activated protein kinase activation and modulation of DAX-1 and steroidogenic factor-1. *Journal of Clinical Endocrinology & Metabolism* **88:** 2288-2299

Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, Akira S (1997) Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proceedings of the National Academy of Sciences of the United States of America* **94:** 3801-3804

Takiguchi T, Kobayashi M, Nagashima C, Yamaguchi A, Nishihara T, Hasegawa K (1999) Effect of prostaglandin E-2 on recombinant human bone morphogenetic protein-2-stimulated osteoblastic differentiation in human periodontal ligament cells. *Journal of Periodontal Research* **34:** 431-436

Tanaka N, Ikawa M, Mata NL, Verma IM (2006) Choroidal neovascularization in transgenic mice expressing prokineticin 1: An animal model for age-related macular degeneration. *Molecular Therapy* **13:** 609-616

Tanaka SS, Matsui Y (2002) Developmentally regulated expression of mil-1 and mil-2, mouse interferon-induced transmembrane protein like genes, during formation and differentiation of primordial germ cells. *Mechanisms of Development* **119:** S261-S267

Tapanainen J, Kellokumpulehtinen P, Pelliniemi L, Huhtaniemi I (1981) Age-related-changes in endogenous steroids of human-fetal testis during early and mid-pregnancy. *Journal of Clinical Endocrinology & Metabolism* **52:** 98-102

Taupin JL, Tian QS, Kedersha N, Robertson M, Anderson P (1995) The RNA-binding protein TIAR is translocated from the nucleus to the cytoplasm during FAS-mediated apoptotic cell-death. *Proceedings of the National Academy of Sciences of the United States of America* **92:** 1629-1633

Telfer EE, Gosden RG, Byskov AG, Spears N, Albertini D, Andersen CY, Anderson R, Braw-Tal R, Clarke H, Gougeon A, McLaughlin E, McLaren A, McNatty K, Schatten G, Silber S, Tsafriri A (2005) On regenerating the ovary and generating controversy. *Cell* **122:** 821-822

ten Dijke P, Arthur HM (2007) Extracellular control of TGFbeta signalling in vascular development and disease. *Nature Reviews in Molecular Cell Biology* **8**: 857-869

Tevosian SG, Deconinck AE, Tanaka M, Schinke M, Litovsky SH, Izumo S, Fujiwara Y, Orkin SH (2000) FOG-2, a cofactor for GATA transcription factors, is essential for heart morphogenesis and development of coronary vessels from epicardium. *Cell* **101:** 729-739

Tevosian SG, Albrecht KH, Crispino JD, Fujiwara Y, Eicher EM, Orkin SH (2002) Gonadal differentiation, sex determination and normal Sry expression in mice require direct interaction between transcription partners GATA4 and FOG2. *Development* **129:** 4627-4634

Tevosian SG, Manuylov NL (2008) To beta or Not to beta: Canonical beta-Catenin Signaling Pathway and Ovarian Development. *Developmental Dynamics* **237:** 3672-3680

Tilley SL, Audoly LP, Hicks EH, Kim HS, Flannery PJ, Coffman TM, Koller BH (1999) Reproductive failure and reduced blood pressure in mice lacking the EP2 prostaglandin E(2) receptor. *Journal of Clinical Investigation* **103:** 1539-1545

Tilly JL (1996) The molecular basis of ovarian cell death during germ cell attrition, follicular atresia, and luteolysis. *Frontiers in Bioscience (online)* **1:** D1-11

Tilly JL (2001) Commuting the death sentence: How oocytes strive to survive. *Nature Reviews Molecular Cell Biology* **2:** 838-848

Tilly JL, Niikura Y, Rueda BR (2009) The Current Status of Evidence for and Against Postnatal Oogenesis in Mammals: A Case of Ovarian Optimism Versus Pessimism? *Biology of Reproduction* **80:** 2-12

Tilly JL, Telfer EE (2009) Purification of germline stem cells from adult mammalian ovaries: a step closer towards control of the female biological clock? *Molecular Human Reproduction* **15:** 393-398

Tilmann C, Capel B (1999) Mesonephric cell migration induces testis cord formation and Sertoli cell differentiation in the mammalian gonad. *Development* **126:** 2883-2890

Tilmann C, Capel B (2002) Cellular and molecular pathways regulating mammalian sex determination. *Recent Progress in Hormone Research, Vol 57* **57:** 1-18

Tingen C, Kim A, Woodruff TK (2009) The primordial pool of follicles and nest breakdown in mammalian ovaries. *Molecular Human Reproduction* **15:** 795-803

Tjandrawinata RR, Hughes-Fulford M (1997) Up-regulation of cyclooxygenase-2 by product-prostaglandin E-2. In *Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Radiation Injury 3* **407**: 163-170.

Tomizuka K, Horikoshi K, Kitada R, Sugawara Y, Iba Y, Kojima A, Yoshitome A, Yamawaki K, Amagai M, Inoue A, Oshima T, Kakitani M (2008) R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. *Human Molecular Genetics* **17:** 1278-1291

Toyoda S, Miyazaki T, Miyazaki S, Yoshimura T, Yamamoto M, Tashiro F, Yamato E, Miyazaki J-i (2009) Sohlh2 affects differentiation of KIT positive oocytes and spermatogonia. *Developmental Biology* **325:** 238-248

Toyomoto M, Ohta M, Okumura K, Yano H, Matsumoto K, Inoue S, Hayashi K, Ikeda K (2004) Prostaglandins are powerful inducers of NGF and BDNF production in mouse astrocyte cultures. *FEBS Letters* **562:** 211-215

Toyooka Y, Tsunekawa N, Takahashi Y, Matsui Y, Satoh M, Noce T (2000) Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development. *Mechanisms of Development* **93:** 139-149

Tran H, Brunet A, Griffith EC, Greenberg ME (2003) The many forks in FOXO's road. *Science STKE* **172:** re5.

Trautmann E, Guerquin M-J, Duquenne C, Lahaye J-B, Habert R, Livera G (2008) Retinoic acid prevents germ cell mitotic arrest in mouse fetal testes. *Cell Cycle* **7:** 656-664

Tremblay KD, Dunn NR, Robertson EJ (2001) Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation. *Development* **128:** 3609-3621

Trombly DJ, Woodruff TK, Mayo KE (2009a) Suppression of Notch Signaling in the Neonatal Mouse Ovary Decreases Primordial Follicle Formation. *Endocrinology* **150:** 1014-1024

Trombly DJ, Woodruff TK, Mayo KE (2009b) Roles for Transforming Growth Factor Beta Superfamily Proteins in Early Folliculogenesis. *Seminars in Reproductive Medicine* **27:** 14-23

Trousse F, Esteve P, Bovolenta P (2001) BMP4 mediates apoptotic cell death in the developing chick eye. *Journal of Neuroscience* **21:** 1292-1301

Tsuda M, Sasaoka Y, Kiso M, Abe K, Haraguchi S, Kobayashi S, Saga Y (2003) Conserved role of nanos proteins in germ cell development. *Science* **301:** 1239-1241

Uda M, Ottolenghi C, Deiana M, Kimber W, Forabosco A, Cao A, Schlessinger D, Pilia G (2004) Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development. *Human Molecular Genetics* **13:** 1171-1181

Uhlenhaut NH, Jakob S, Anlag K, Eisenberger T, Sekido R, Kress J, Treier A-C, Klugmann C, Klasen C, Holter NI, Riethmacher D, Schuetz G, Cooney AJ, Lovell-Badge R, Treier M (2009) Somatic Sex Reprogramming of Adult Ovaries to Testes by FOXL2 Ablation. *Cell* **139:** 1130-1142

Umehara F, Tate G, Itoh K, Yamaguchi N, Douchi T, Mitsuya T, Osame M (2000) A novel mutation of desert hedgehog in a patient with 46,XY partial gonadal dysgenesis accompanied by minifascicular neuropathy. *American Journal of Human Genetics* **67:** 1302-1305

Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP (1999) Female development in mammals is regulated by Wnt-4 signalling. *Nature* **397:** 405-409

Vane JR (1971) Inhibition of prostaglandin sythesis as mechanism of action for asprin-like drugs. *Nature-New Biology* **231:** 232-235

Vaskivuo TE, Anttonen M, Herva R, Billig H, Dorland M, te Velde ER, Stenback F, Heikinheimo M, Tapanainen JS (2001) Survival of human ovarian follicles from fetal to adult life: Apoptosis, apoptosis-related proteins, and transcription factor GATA-4. *Journal of Clinical Endocrinology & Metabolism* **86:** 3421-3429

Viger RS, Mertineit C, Trasler JM, Nemer M (1998) Transcription factor GATA-4 is expressed in a sexually dimorphic pattern during mouse gonadal development and is a potent activator of the Mullerian inhibiting substance promoter. *Development* **125:** 2665-2675

Vincent SD, Dunn NR, Sciammas R, Shapiro-Shalef M, Davis MM, Calame K, Bikoff EK, Robertson EJ (2005) The zinc finger transcriptional repressor Blimp1/Prdm1 is dispensable for early axis formation but is required for specification of primordial germ cells in the mouse. *Development* **132:** 1315-1325

Vinci G, Christin-Maitre S, Pasquier M, Bouchard P, Fellous M, Veitia RA (2008) FOXO3a variants in patients with premature ovarian failure. *Clinical Endocrinology* **68:** 495-497

Virdi AS, Cook LJ, Oreffo ROC, Triffitt JT (1998) Modulation of bone morphogenetic protein-2 and bone morphogenetic protein-4 gene expression in osteoblastic cell lines. *Cellular and Molecular Biology* **44:** 1237-1246

Wade PR, Palmer JM, Mabus J, Saunders PR, Prouty S, Chevalier K, Gareau MG, McKenney S, Hornby PJ (2010) Prokineticin-1 evokes secretory and contractile activity in rat small intestine. *Neurogastroenterology and Motility* **22:** e152-e161

Wakayama T, Hamada K, Yamamoto M, Suda T, Iseki S (2003) The expression of platelet endothelial cell adhesion molecule-1 in mouse primordial germ cells during their migration and early gonadal formation. *Histochemistry and Cell Biology* **119:** 355-362

Wang D, DuBois RN (2006) Prostaglandins and cancer. *Gut* **55:** 115-122

Wang J, Roy SK (2004) Growth differentiation factor-9 and stem cell factor promote primordial follicle formation in the hamster: modulation by follicle-stimulating hormone. *Biology of Reproduction* **70:** 577-585

Wang SH, Tsai MS, Chiang MF, Li H (2003a) A novel NK-type homeobox gene, ENK (early embryo specific NK), preferentially expressed in embryonic stem cells. *Gene Expression Patterns* **3:** 99-103

Wang XJ, Dyson MT, Jo Y, Stocco DM (2003b) Inhibition of cyclooxygenase-2 activity enhances steroidogenesis and steroidogenic acute regulatory gene expression in MA-10 mouse Leydig cells. *Endocrinology* **144:** 3368-3375

Wang XJ, Shen CL, Dyson MT, Eimerl S, Orly J, Hutson JC, Stocco DM (2005) Cyclooxygenase-2 regulation of the age-related decline in testosterone biosynthesis. *Endocrinology* **146:** 4202-4208

Ware CB, Horowitz MC, Renshaw BR, Hunt JS, Liggitt D, Koblar SA, Gliniak BC, McKenna HJ, Papayannopoulou T, Thoma B, Cheng LZ, Donovan PJ, Peschon JJ, Bartlett PF, Willis CR, Wright BD, Carpenter MK, Davison BL, Gearing DP (1995) Targeted disruption of the low-affinity leukemia inhibitory factor-receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. *Development* **121:** 1283-1299

Wartenberg H (1982) Development of the early human-ovary and role of the mesonephros in the differentiation of the cortex. *Anatomy and Embryology* **165:** 253-280

Watkins WJ, Umbers AJ, Woad KJ, Harris SE, Winship IM, Gersak K, Shelling AN (2006) Mutational screening of FOXO3A and FOXO1A in women with premature ovarian failure. *Fertility and Sterility* **86:** 1518-1521

Wechselberger C, Puglisi R, Engel E, Lepperdinger G, Boitani C, Kreil G (1999) The mammalian homologues of frog Bv8 are mainly expressed in spermatocytes. *FEBS Letters* **462:** 177-181

Weck J, Mayo KE (2006) Switching of NR5A proteins associated with the inhibin alpha-subunit gene promoter after activation of the gene in granulosa cells. *Molecular Endocrinology* **20:** 1090-1103

Welsh M, Saunders PTK, Sharpe RM (2007) The critical time window for androgen-dependent development of the Wolffian duct in the rat. *Endocrinology* **148:** 3185-3195

Weniger JP (1993) Estrogen production by fetal rat gonads. *Journal of Steroid Biochemistry and Molecular Biology* **44:**459-462.

Werler MM, Mitchell AA, Hernandez-Diaz S, Honein MA, Natl Birth Defects P (2005) Use of over-the-counter medications during pregnancy. *American Journal of Obstetrics and Gynecology* **193:** 771-777

Whelton A, Fort JG, Puma JA, Normandin D, Bello AE, Verburg KM, Grp SVS (2002) Cyclooxygenase-2-specific inhibitors and cardiorenal function: A randomized, controlled trial of celecoxib and rofecoxib in older hypertensive osteoarthritis patients (Reprinted from Lippincott Williams & Wilkins). *American Journal of Managed Care* **8:** S371-S382

Wijelath ES, Carlsen B, Cole T, Chen J, Kothari S, Hammond WP (1997) Oncostatin M induces basic fibroblast growth factor expression in endothelial cells and promotes endothelial cell proliferation, migration and spindle morphology. *Journal of Cell Science* **110:** 871-879

Wilcox CM, Alexander LN, Cotsonis GA, Clark WS (1997) Nonsteroidal antiinflammatory drugs are associated with both upper and lower gastrointestinal bleeding. *Digestive Diseases and Sciences* **42:** 990-997

Wilhelm D, Martinson F, Bradford S, Wilson MJ, Combes AN, Beverdam A, Bowles J, Mizusaki H, Koopman P (2005) Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. *Developmental Biology* **287:** 111-124

Wilhelm D, Palmer S, Koopman P (2007) Sex determination and gonadal development in mammals. *Physiological Reviews* **87:** 1-28

Wilhelm D, Washburn LL, Truong V, Fellous M, Eicher EM, Koopman P (2009) Antagonism of the testis- and ovary-determining pathways during ovotestis development in mice. *Mechanisms of Development* **126:** 324-336

Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, Wagner EF, Metcalf D, Nicola NA, Gough NM (1988) Myeloid-leukemia inhibitory factor maintains the developmental potential of embryonic stem-cells. *Nature* **336:** 684-687

Yabuta Y, Kurimoto K, Hinata Y, Seki Y, Saitou M (2006) Gene expression dynamics during germline specification in mice identified by quantitative single-cell gene expression profiling. *Biology of Reproduction* **75:** 705-716

Yamaguchi S, Kimura H, Tada M, Nakatsuji N, Tada T (2005) Nanog expression in mouse germ cell development. *Gene Expression Patterns* **5:** 639-646

Yamaguchi S, Kurimoto K, Yabuta Y, Sasaki H, Nakatsuji N, Saitou M, Tada T (2009) Conditional knockdown of Nanog induces apoptotic cell death in mouse migrating primordial germ cells. *Development* **136:** 4011-4020

Yamaji M, Seki Y, Kurimoto K, Yabuta Y, Yuasa M, Shigeta M, Yamanaka K, Ohinata Y, Saitou M (2008) Critical function of Prdm14 for the establishment of the germ cell lineage in mice. *Nature Genetics* **40:** 1016-1022

Yamamoto S, Konishi I, Nanbu K, Komatsu T, Mandai M, Kuroda H, Matsushita K, Mori T (1997) Immunohistochemical localization of basic fibroblast growth factor (bFGF) during folliculogenesis in the human ovary. *Gynecological Endocrinology* **11:** 223-230

Yan CN, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ, Matzuk MM (2001) Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Molecular Endocrinology* **15:** 854-866

Yao HHC, Capel B (2002) Sertoli cell-derived desert hedgehog signaling specifies fetal Leydig cell fate in testis organogenesis. *Biology of Reproduction* **66:** 191-192

Yao HHC, Tilmann C, Zhao GQ, Capel B (2002) The battle of the sexes: opposing pathways in sex determination. in The Genetics and BIology of Sex Determination: Novartis Foundation Symposia 244 Chadwick D, Goode J. John Wiley & Sons, Ltd, Chichester, UK. DOI: 10.1002/0470868732.ch15.

Yao HHC, Matzuk MM, Jorgez CJ, Menke DB, Page DC, Swain A, Capel B (2004) Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. *Developmental Dynamics* **230:** 210-215

Yeh J, Osathanondh R (1993) Expression of messenger ribonucleic-acids encoding for basic fibroblast growth-factor (FGF) and alternatively spliced FGF receptor in human fetal ovary and uterus. *Journal of Clinical Endocrinology & Metabolism* **77:** 1367-1371

Ying Y, Liu XM, Marble A, Lawson KA, Zhao GQ (2000) Requirement of Bmp8b for the generation of primordial germ cells in the mouse. *Molecular Endocrinology* **14:** 1053-1063

Ying Y, Zhao GQ (2001) Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse. *Developmental Biology* **232:** 484-492

Yoshida H, Takakura N, Kataoka H, Kunisada T, Okamura H, Nishikawa S (1997) Stepwise requirement of c-kit tyrosine kinase in mouse ovarian follicle development. *Developmental Biology* **184:** 122-137

Yoshida K, Taga T, Saito M, Suematsu S, Kumanogoh A, Tanaka T, Fujiwara H, Hirata M, Yamagami T, Nakahata T, Hirabayashi T, Yoneda Y, Tanaka K, Wang WZ, Mori C, Shiota K, Yoshida N, Kishimoto T (1996) Targeted disruption of gp130, a common signal transducer for the interleukin 6 family of cytokines, leads to myocardial and hematological disorders. *Proceedings of the National Academy of Sciences of the United States of America* **93:** 407-411

Yoshida K, Kondoh G, Matsuda Y, Habu T, Nishimune Y, Morita T (1998) The mouse RecA-like gene Dmc1 is required for homologous chromosome synapsis during meiosis. *Molecular Cell* **1:** 707-718

Young JC, Jaiprakash A, Mithraprabhu S, Itman C, Kitazawa R, Looijenga LHJ, Loveland KL (2011) TCam-2 seminoma cell line exhibits characteristic foetal germ cell responses to TGF-beta ligands and retinoic acid. *International Journal of Andrology* **34:** E204-E217

Youngren KK, Coveney D, Peng XN, Bhattacharya C, Schmidt LS, Nickerson ML, Lamb BT, Deng JM, Behringer RR, Capel B, Rubin EM, Nadeau JH, Matin A (2005) The Ter mutation in the dead end gene causes germ cell loss and testicular germ cell tumours. *Nature* **435:** 360-364

Zachos NC, Billiar RB, Albrecht ED, Pepe GJ (2002) Developmental regulation of baboon fetal ovarian maturation by estrogen. *Biology of Reproduction* **67:** 1148-1156

Zachos NC, Billiar RB, Albrecht ED, Pepe GJ (2004) Regulation of oocyte microvilli development in the baboon fetal ovary by estrogen. *Endocrinology* **145:** 959-966

Zachos NC, Burch MG, Billiar RB, Li C, Albrecht ED, Pepe GJ (2008) Regulation of Expression of Microvillus Membrane Proteins by Estrogen in Baboon Fetal Ovarian Oocytes. *Biology of Reproduction* **79:** 1160-1168

Zhang Z, Hill J, Holland M, Kurihara Y, Loveland KL (2008) Bovine Sertoli cells colonize and form tubules in muirine hosts following transplantation and grafting procedures. *Journal of Andrology* **29:** 418-430

Zhao GQ, Deng K, Labosky PA, Liaw L, Hogan BLM (1996) The gene encoding bone morphogenetic protein 8B is required for the initiation and maintenance of spermatogenesis in the mouse. *Genes & Development* **10:** 1657-1669

Zhao H, Chen Z-J, Qin Y, Shi Y, Wang S, Choi Y, Simpson JL, Rajkovic A (2008) Transcription factor FIGLA is mutated in patients with Premature Ovarian Failure. *American Journal of Human Genetics* **82:** 1342-1348

Zhao SZ, Reynolds MW, Lefkowith J, Whelton A, Arellano FM (2001) A comparison of renal-related adverse drug reactions between rofecoxib and celecoxib, based on the World Health Organization Uppsala Monitoring Centre safety database. *Clinical Therapeutics* **23:** 1478-1491

Zimmerman LB, DeJesusEscobar JM, Harland RM (1996) The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86:** 599-606

Zou K, Yuan Z, Yang ZJ, Luo HC, Sun KJ, Zhou L, Xiang J, Shi LJ, Yu QS, Zhang Y, Hou RY, Wu J (2009) Production of offspring from a germline stem cell line derived from neonatal ovaries. *Nature Cell Biology* **11:** 631-U424

Appendix 1: Manufacturers' Details:

Company	City	Country
Abcam	Cambridge	UK
Adobe	San Jose, CA	USA
Ambion	Austin, TX	USA
Applied Biosystems	Foster City, CA	USA
BD	Franklin Lakes, NJ	USA
Bioline	London	UK
Bio-Rad	Hercules, CA	USA
Biotium	Hayward, CA	USA
Caltag	Buckingham	UK
Cayman	Ann Arbour, MI	USA
Clintech	Guilford	UK
Corning	Corning, NY	USA
CoStar	Schipol-Rijk	Netherlands
Dako	Glostrup	Denmark
Eppendorf	Hamburg	Germany
Eurogentec	Liege	Belguim
Faithfull Tools	Kent	UK
Fisher Scientific	Loughborough	UK
Fitzgerald	Acton, MA	USA
Gibco	Carlsbad, CA	USA
GraphPad Software	La Jolla, CA	USA
Histolab	Gotheburg	Sweden
Invitrogen	Carlsbad, CA	USA
Invivogen	San Diego, CA	USA
Lab Vision Corp	Now owned by Thermo Scientific (see below)	
Labsystems	Vienna, VA	USA
Leica	Wetzlar	Germany
Li-Cor	Lincoln, NE	USA
Lifespan	Seattle, WA	USA
Lonza	Basel	Switzerland
Lymphotec	Tokyo	Japan
Mazola	Memphis, TN	USA
MBL	Woburn, MA	USA
Media Cybernetics	Bethesda, MD	USA
Millipore	Billerica, MA	USA
Mirus	Madison, WI	USA
MJ Research	Waltham, MA	USA
New England Biolabs	Ipswich, MA	USA
Olympus	Essex	UK
Qiagen	Hamburg	Germany
Qimaging	Surrey	Canada
Perkin Elmer	Waltham, MA	USA
Phoneix	Burlingham, CA	USA
Prior	Cambridge	UK
Promega	Madison, WI	USA
Roche	Basel	Switzerland

Company	City	Country
Santa Cruz	Santa Cruz, CA	USA
SDS	Dundee	UK
Sigma	St Louis, MO	USA
SPPS	Argenteuil	France
Thermo Scientific	Waltham, MA	USA
Upstate	Now owned by Millipore (see above)	
Vector	Burlingame, CA	USA
VWR	West Chester, PA	USA
Zeiss	Oberkochen	Germany

Appendix 2: Publications relating to this thesis