

Regulation of FOXO Transcription Factors by Gonadotropin-Releasing Hormone

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Αυτή η διατριβή είναι αφιερωμένη τους γονείς μου,
Σταύρο Σταύρου και Ελένη Δημάκη

This thesis is dedicated to my parents,
Stavro Stavrou and Eleni Dimaki

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DECLARATION

I hereby declare that the work presented within this thesis was carried out by myself during the course of my PhD and that it has not been submitted for any other degree or qualification. Where I have used the work of others, the sources of information have been detailed clearly in the presentation.

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ABSTRACT

G protein-coupled receptors (GPCRs) are a large family of trans-membrane receptors that transmit signals from extracellular stimuli to target intracellular signal transduction pathways. The gonadotropin-releasing hormone receptor (GnRH-R) is a GPCR which binds the decapeptide GnRH. In the pituitary gonadotrope, GnRH stimulates gonadotropin (LH and FSH) biosynthesis and secretion to regulate reproduction. GnRH and the GnRH-Rs are also present in many extra-pituitary tissues, although their role at these sites remains largely undetermined. GnRH-Rs are known to recruit a diverse array of signalling pathway mediators in different cell-types. These include; $G_{q/11}$ -PLC β -IP $_3$ /DAG-Ca $^{2+}$ /PKC signalling, monomeric G-proteins and integrins to mediate cell adhesion and migration, the activation of the major members of the mitogen-activated protein kinase (MAPK) super-family (extracellular signal-regulated kinase (ERK), c-Jun N-terminal Kinase (JNK) and p38MAPK), and β -catenin and other mediators of the canonical Wnt signalling pathway.

This thesis describes the regulation of Forkhead Box O (FOXO) transcription factors by GnRH. The mammalian FOXO transcription factors, FOXO1, FOXO3a and FOXO4, are emerging as an important family of proteins that modulate the expression of genes involved in cell-cycle regulation, induction of apoptosis, DNA damage repair and response to oxidative stress. In this thesis, emphasis is placed on delineating the novel role of FOXO transcription factors in mediating two important and widely-researched areas of GnRH biology. Firstly, the role of FOXO transcription factors in mediating cell-growth inhibition in response to GnRH

treatment is assessed in a heterologous HEK293/GnRH-R expressing cell line. Secondly, the role of transcription factors in regulating luteinising hormone- β (LH β)-subunit expression is investigated in the L β T2 gonadotrope cell line.

Activation of the GnRH-R can inhibit cell proliferation and induce apoptosis in certain tumour-derived cell lines. Several studies have reported that these events can occur as a result of changes in the expression profiles of specific cell-cycle regulatory and apoptotic genes, many of which are FOXO-target genes, including *GADD45*, *FasL*, *p21Cip1* and *p27Kip1*. In this thesis, a role for FOXOs in targeting the expression of several of these genes in response to GnRH is assessed, highlighting a specific role for FOXO3a in mediating GADD45 and FasL expression. The signalling mechanisms through which FOXO3a regulates GADD45 expression in response to GnRH is also described. Finally, a stable FOXO3a-knock-down cell line was generated in order to further examine FOXO3a involvement in GnRH-induced cell-growth inhibition.

GnRH is an essential regulator of the reproductive process by stimulating the synthesis of LH and FSH in pituitary gonadotropes, thereby regulating gametogenesis and steroidogenesis. Diverse signalling pathways have been reported to regulate LH β -subunit expression in response to GnRH, including the ERK/JNK/p38MAPK cascades and factors such as Egr1, SF1 and β -catenin. In the second part of this thesis, the role of FOXOs in regulating LH β -subunit expression in response to GnRH is described. The data presented suggests that GnRH can regulate LH β -subunit expression through both indirect and direct FOXO3a-mediated mechanisms. Firstly, FOXO3a was found to regulate Egr1 expression to indirectly

target *LHβ*-promoter activity. Secondly, a role for β -catenin as a FOXO3a co-factor to directly regulate LH β -subunit expression, together with Egr1 and SF1, is also proposed. FOXO3a expression and sub-cellular localisation was assessed and demonstrated in L β T2 cells and in adult human male pituitary sections.

The research presented in this thesis adds to the diversity of signalling pathways and mediators that GnRH can target in different cellular backgrounds in order to mediate a variety of cellular processes. The antiproliferative and apoptotic effects of GnRH on tumour-derived cell lines are well-documented, and this research highlights a novel role for FOXO3a in mediating these events. The regulation of gonadotropin synthesis remains an important topic of research, and the novel implication of FOXO3a in mediating LH β -subunit expression adds further complexity to gonadotrope physiology.

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ABBREVIATIONS

AFX	<u>ALL1 Fused gene from chromosome X</u>
AML	<u>Acute Myeloid Leukemia</u>
APAF1	<u>Apoptotic Activating Factor 1</u>
AVPV	<u>Anteroventral Periventricular Nucleus</u>
BMK	<u>Big MAPK</u>
BPE	<u>Bovine Pituitary Extract</u>
BSA	<u>Bovine Serum Albumin</u>
cAMP	<u>cyclic Adenosine Monophosphate</u>
CArG	<u>CC(A/T)6GG</u>
cDNA	<u>complementary DNA</u>
CK1	<u>Casein Kinase 1</u>
ChIP	<u>Chromatin Immuno-Precipitation</u>
CREB	<u>cAMP Response Element-Binding</u>
CsA	<u>Cyclosporin A</u>
DAB	<u>Diaminobenzidine</u>
DAG	<u>Diacylglycerol</u>
DBD	<u>DNA Binding Domain</u>
DBE	<u>DAF-16 family protein-Binding Element</u>
ΔCT	<u>Deletion of trans-activation domain</u>
ΔDB	<u>Deletion of DNA Binding domain</u>
DEPC	<u>Diethylpyrocarbonate</u>
DGK	<u>DAG kinase</u>
DMEM	<u>Dulbecco's Modified Eagle Medium</u>
DMSO	<u>Dimethyl Sulfoxide</u>
DNA	<u>Deoxyribonucleic acid</u>
DPBS	<u>Dulbeccos Phosphate Buffered Saline</u>
DYRK1A	<u>Dual-specificity Regulated Kinase 1A</u>
ECL	<u>Extracellular Loop</u>
EDTA	<u>Ethylenediaminetetraacetic Acid</u>
Egr1	<u>Early Growth Response factor 1</u>
ERK1/2	<u>Extracellular signal-Regulated Kinases 1/2</u>
FADD	<u>FAS-Associated Death Domain</u>
FasL	<u>Fas Ligand</u>

FHRE	<u>F</u> ork <u>h</u> ead <u>R</u> esponsive <u>E</u> lement
FGF	<u>F</u> ibroblast <u>G</u> rowth <u>F</u> actor
FKHR	<u>F</u> ork <u>h</u> ead in <u>R</u> habdomyosarcoma/ <u>F</u> OXO1
FKHRL1	<u>F</u> ork <u>h</u> ead in <u>R</u> habdomyosarcoma like 1/ <u>F</u> OXO3a
FOX	<u>F</u> orkhead <u>B</u> ox
FOXO	<u>F</u> OX the “ <u>O</u> ” sub-group
FSH	<u>F</u> ollicle <u>S</u> timulating <u>H</u> ormone
FSH β	<u>F</u> ollicle <u>S</u> timulating <u>H</u> ormone <u>beta</u> (β)
GADD45	<u>G</u> rowth <u>A</u> rrest and <u>D</u> NA <u>D</u> amage protein 45
GATA2	<u>G</u> ATA-binding protein 2
GDP	<u>G</u> uanosine <u>d</u> iphosphate
GFP	<u>G</u> reen <u>F</u> luorescent <u>P</u> rotein
GnRH	<u>G</u> onadotropin- <u>R</u> eleasing <u>H</u> ormone
GnRH-R	<u>G</u> onadotropin- <u>R</u> eleasing <u>H</u> ormone- <u>R</u> eceptor
GPCR	<u>G</u> -protein <u>C</u> oupled <u>R</u> eceptor
GPR54	<u>G</u> -protein-coupled membrane <u>R</u> eceptor 54
GTP	<u>G</u> uanosine-5'- <u>t</u> risphosphate
GSE	<u>G</u> onadotrope- <u>S</u> pecific <u>E</u> lements
HEK293	<u>H</u> uman <u>E</u> mbryonic <u>K</u> idney 293 cells
HEPES	4-(2- <u>H</u> ydroxyethyl)-1- <u>P</u> iperazineethanesulfonic acid
ICL	<u>I</u> ntracellu <u>l</u> ar <u>L</u> oop
IEG	<u>I</u> mmEDIATE <u>E</u> ARLY <u>G</u> ENE
IGF1	<u>I</u> nsulin-like <u>G</u> rowth <u>F</u> actor 1
IL-3	<u>I</u> nterleukin 3
InsP ₃ or IP3	<u>I</u> nositol 1,4,5-triphosphate
IP	<u>I</u> nositol <u>P</u> hosphate
IKK	<u>I</u> κ <u>B</u> <u>k</u> inase
JNK	c- <u>J</u> un <u>N</u> -terminal <u>k</u> inase
KDa	<u>K</u> ilo <u>D</u> alton
Kiss1	<u>K</u> isspeptin 1
Kiss1r	<u>K</u> isspeptin 1 <u>R</u> eceptor
LA	<u>L</u> euprolide <u>A</u> cetate
LB-agar	<u>L</u> uria- <u>B</u> ertani <u>B</u> roth-agar
LH	<u>L</u> uteinising <u>H</u> ormone
LH β	<u>L</u> uteinising <u>H</u> ormone <u>beta</u> (β)

LH-R	<u>L</u> uteinising <u>H</u> ormone- <u>R</u> eceptor
LHX3	<u>L</u> IM <u>H</u> omeobox gene <u>3</u>
LiSS	<u>L</u> igand- <u>I</u> nduced <u>S</u> elective <u>S</u> ignalling
MAPK	<u>M</u> itogen- <u>A</u> ctivated <u>P</u> rotein <u>K</u> inase
MAP2K	<u>M</u> itogen- <u>A</u> ctivated <u>P</u> rotein <u>K</u> inase <u>K</u> inase
MAP3K	<u>M</u> itogen- <u>A</u> ctivated <u>P</u> rotein <u>K</u> inase <u>K</u> inase <u>K</u> inase
MAPK8	<u>M</u> itogen- <u>A</u> ctivated <u>P</u> rotein <u>K</u> inase <u>8</u>
mRNA	<u>m</u> essenger <u>R</u> NA
NAD	<u>N</u> icotinamide <u>A</u> denine <u>D</u> inucleotide
NES	<u>N</u> uclear <u>E</u> xport <u>S</u> equence
NFAT	<u>N</u> uclear <u>F</u> actor of <u>A</u> ctivated <u>T</u> -cells
NF- κ B	<u>N</u> uclear <u>F</u> actor <u>K</u> appa-light-chain-enhancer of activated <u>B</u> cells
NF-Y	<u>N</u> uclear transcription <u>F</u> actor- <u>Y</u>
NGF	<u>N</u> erve <u>G</u> rowth <u>F</u> actor
NGS	<u>N</u> ormal <u>G</u> oat <u>S</u> erum
NLS	<u>N</u> uclear <u>L</u> ocalisation <u>S</u> ignal
p21Cip1	<u>p</u> 21 <u>CDK</u> - <u>I</u> nteracting <u>P</u> rotein <u>1</u>
p38MAPK	<u>p</u> 38 <u>mitogen</u> - <u>A</u> ctivated <u>P</u> rotein <u>K</u> inases
PA	<u>P</u> hosphatidic <u>A</u> cid
PARP	<u>P</u> oly(<u>ADP</u> - <u>R</u> ibose) <u>P</u> olymerase
PCR	<u>P</u> olymerase <u>C</u> hain <u>R</u> eaction
PDGF	<u>P</u> latelet- <u>D</u> erived <u>G</u> rowth <u>F</u> actor
PK1	<u>3</u> '- <u>P</u> hosphoinositide- <u>D</u> ependent <u>K</u> inase- <u>1</u>
PGF _{2α}	<u>P</u> rostaglandin <u>F</u> 2 α
PH	<u>P</u> leckstrin <u>H</u> omology domain
PIP ₂	<u>P</u> hosphatidylinositol 4,5-bisphosphate
PI3K	<u>P</u> hosphatidylinositol <u>3</u> - <u>K</u> inase
Pitx1	<u>P</u> ituitary <u>H</u> omeobox <u>1</u>
PKB	<u>P</u> rotein <u>K</u> inase <u>B</u>
PKC	<u>P</u> rotein <u>K</u> inase <u>C</u>
PLC	<u>P</u> hospholipase <u>C</u>
PTK1	<u>P</u> rotein <u>T</u> yrosine <u>K</u> inase <u>1</u>
PVDF	<u>P</u> olyvinylidene <u>F</u> luoride
qRT-PCR	<u>q</u> uantitative <u>R</u> eal <u>T</u> ime <u>P</u> CR
rEGF	<u>R</u> ecombinant <u>E</u> pidermal <u>G</u> rowth <u>F</u> actor

RGS	<u>Regulators of G protein Signalling</u>
RLU	<u>Relative Light Units</u>
RNA	<u>Ribonucleic Acid</u>
RNase	<u>Ribonuclease</u>
RSB	<u>RNA Storage Buffer</u>
RTKs	<u>Receptor Tyrosine Kinases</u>
SDS-PAGE	<u>SDS-Polyacrylamide Gel Electrophoresis</u>
SDS	<u>Sodium Dodecyl Sulfate</u>
SEM	<u>Standard Error of the Mean</u>
shRNA	<u>short hairpin RNA</u>
SF1	<u>Steroidogenic Factor 1</u>
SGK	<u>Serum and Glucocorticoid inducible Kinase</u>
SIRT1	<u>Sirtuin (silent mating type information regulation 2 homolog) 1</u>
SMAD	<u>Sma and Mad related proteins</u>
S.O.C	<u>Super Optimal broth with Catabolite repression</u>
SRB	<u>Sulforhodamine B</u>
STAT5	<u>Signal Transducer and Activator of Transcription 5</u>
TBS	<u>Tris Buffered Saline</u>
TCA	<u>Trichloroacetic acid</u>
TCF	<u>T cell Factor</u>
TGF β	<u>Transforming Growth Factor β</u>
TM	<u>Transmembrane</u>
TRIR	<u>Total RNA Isolation Reagent</u>
TSH	<u>Thyroid-Stimulating Hormone</u>
UV	<u>Ultraviolet Light</u>
α GSU	<u>Glycoprotein hormone α-Subunit</u>

CHAPTER ONE

LITERATURE REVIEW

In the first part of this Chapter, a brief description of the hypothalamic-pituitary axis, and GnRH/GnRH-R structure/function will be given. This will be followed by a review of GnRH-induced signalling pathways, among them the PI3K/Akt pathway. Next, the mechanisms through which GnRH induces cell-growth inhibition will be reviewed. In the second part of this Chapter, the signalling pathways that mediate LH β -subunit expression in response to GnRH will be outlined. In addition, a number of transcription factors that are required to maximal *LH β* promoter transcriptional activity will be discussed in some detail. Finally, the third part of this Chapter will provide an overview of FOXO transcription factors. This will be followed by a review of the FOXO-target genes and the role of FOXOs in cancers.

1.1 OVERVIEW

The G protein-coupled receptor (GPCR) super-family represents the largest group of cell-surface membrane receptors. They are seven-transmembrane domain receptors that are activated by extracellular signals, leading to the induction of intracellular signal transduction pathways. GPCRs are located within the plasma membrane and contain an extracellular N-terminus which is followed by seven transmembrane (TM) domains connected by three intracellular (ICL), and three extracellular (ECL) loops, and an intracellular C-terminal tail. While GPCRs are common drug targets, only a small proportion have been targeted for therapeutic purposes. GPCRs are classified

into five major families; the Rhodopsin-like family, the Secretin family, the Adhesion family, the Glutamate family and the Frizzled/Taste family (Millar and Newton, 2010). Gonadotropin-Releasing Hormone Receptors (GnRH-Rs) belong to the Rhodopsin-like family of the GPCRs, and are activated following the binding of Gonadotropin-Releasing Hormone (GnRH).

The hypothalamic neuropeptide GnRH is synthesised and released by GnRH neurons in the hypothalamus. GnRH stimulates pituitary gonadotrope cells to synthesise and release the gonadotropins, Luteinising Hormone (LH) and Follicle Stimulating Hormone (FSH) (Naor, 2009). Binding of GnRH to the GnRH-R on the surface of gonadotropes in the anterior pituitary initiates a variety of signalling pathways that leads to the synthesis the gonadotropins, and their release into the blood-stream (Ferris and Shupnik, 2006; Lim *et al.*, 2009; Seeburg *et al.*, 1987; Vale *et al.*, 1977).

Forkhead Box O (FOXO) proteins constitute a large family of transcription factors that mediate diverse cellular responses. These proteins have a characteristic/conserved DNA-binding motif, known as the “forkhead domain”. Although, they contain this conserved DNA-binding domain, they show differences between their C-terminal and N-terminal trans-activation domains (Burgering, 2008). Over the past 15 years, a number of FOX subclasses have been identified with different biological roles. Among the FOX family is the “O” sub-group (FOXO) which consists of four members, three of which are mammalian, namely FOXO1 (FKHR), FOXO3a (FKHRL1) and FOXO4 (AFX). FOXOs share similar DNA-binding domains, and were originally thought to regulate the expression of the same set of genes (Obsil and Obsilova, 2008). However, their specificity is now known to

be determined by post-translational modification and/or the action of co-factors. FOXOs are also involved in a number of cellular processes such as cell proliferation, apoptosis and cell metabolism, and are of great interest because they play a pivotal role in cell-fate decision and in tumour suppression in a variety of cancers (Fu and Tindall, 2008).

1.2 THE HYPOTHALAMIC-PITUITARY AXIS

The reproductive axis consists of three endocrine organs; the hypothalamus, pituitary and gonads. The hypothalamus is located at the base of the brain. It contains the hypothalamic areas or nuclei consisting of a relatively compact cluster of neurons (Sisk and Foster, 2004). GnRH is produced by specialised neurons (parvocellular neurons), whose cell bodies reside in the parvocellular nuclei. The axons of GnRH neurons terminate at the median eminence of the basal hypothalamus, where they secrete pulses of GnRH (Johnson, 2007). GnRH enters the hypothalamo-pituitary portal vessels, and travels to the anterior pituitary to stimulate the synthesis and secretion of LH and FSH. LH and FSH in turn regulate gonadal steroidogenesis and gametogenesis in both sexes (**Fig. 1-1**) (Sisk and Foster, 2004).

The pituitary gland or hypophysis is an endocrine gland that is located posterior to the hypothalamus at the base of the brain. It is composed of two main lobes, the anterior and the smaller posterior regions. Gonadotropes are located in the anterior lobe of the pituitary. The anterior pituitary contains a variety of cells types, including gonadotropes, lactotrophs, somatotrophs, thyrotrophs and corticotrophs (Johnson,

2007; Ooi *et al.*, 2004). Approximately, 50% of the anterior pituitary cells are somatotropes, 10–20% are lactotropes, 10–20% are thyrotropes, 10–20% are corticotropes and 10–20% are gonadotropes (Horváth and Palkovits, 1988).

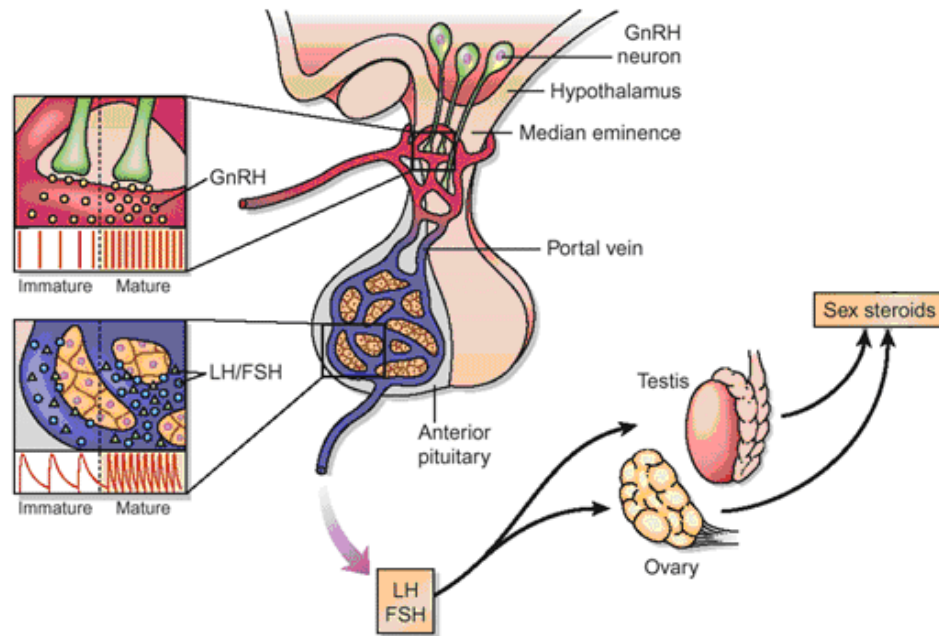


Figure 1-1: The hypothalamic-pituitary-gonadal axis.

GnRH is synthesised by specialised neurons in the hypothalamus and packaged into storage granules. GnRH is secreted from nerve terminals located at the median eminence of the basal hypothalamus. GnRH enters the hypothalamo-pituitary portal vasculature and is transported to the anterior pituitary. In the anterior pituitary, GnRH stimulates the synthesis and secretion of LH and FSH from gonadotropes, which stimulate the testis and ovaries to mediate steroidogenesis and gametogenesis. Figure reproduced from Sisk and Foster, 2004.

1.2.1 Kisspeptin (Kiss1) and kisspeptin receptor (Kiss1r)

Kisspeptin receptor (Kiss1r; also known as GPR54) is a G protein-coupled receptor which binds the peptide hormone kisspeptin. Although this thesis will focus on the signalling pathways downstream of the GnRH-R, a brief introduction on kisspeptin will be given, since Kiss1 and Kiss1r have recently been described as major

neuroendocrine regulators of reproduction through stimulation of GnRH neurons (Oakley *et al.*, 2009).

Kiss1r is a GPCR, with three glycosylation sites at the N-terminus (Clements *et al.*, 2001). Kisspeptin (Kiss1) is the product of the *Kiss1* gene. The initial *Kiss1* product is a 145-amino acid peptide, from which is cleaved a 54-amino acid protein, which can be further truncated to 14, 13 or 10 amino acid carboxyl-terminal fragments, all known as kisspeptins. These N-terminal truncated peptides belong to a large family of peptides known as RFamides which all share a common Arg–Phe–NH₂ motif at their C-terminus (Roseweir and Millar, 2009). Kiss1 and its receptor are highly involved in the regulation of the reproduction. Indeed, mutations in the *Kiss1r* gene are associated with the idiopathic hypothalamic hypogonadism. Moreover, Kiss1 stimulates GnRH secretion and regulates gonadotropin production (**Fig. 1-2**) (Oakley *et al.*, 2009).

Neurons that express Kiss1r and Kiss1 are found in discrete nuclei in the hypothalamus, and in other brain regions in many vertebrates. Kiss1r and Kiss1 are also expressed in other tissues including pancreas, adipocytes, ovary, placenta and pituitary (Oakley *et al.*, 2009).

As discussed above, Kiss1 regulates GnRH release from GnRH neurons in the hypothalamus. GnRH then regulates gonadotropin production, at the level of the pituitary to stimulate LH and FSH secretion (Oakley *et al.*, 2009). Double immunofluorescence labelling for LH β and Kiss1, or Kiss1r has been demonstrated in gonadotropes (Richard *et al.*, 2008), as has Kiss1 secretion into the hypophysial portal circulation (Richard *et al.*, 2009). These findings proposed the action of Kiss1

at the pituitary level. In contrast, other studies have reported that Kiss1 does not act at the pituitary level (Reviewed by Oakley *et al.* (2009)). The stimulatory effect of Kiss1 was blocked by a GnRH antagonist. This finding suggests that the effect of Kiss1 is restricted at the level of GnRH secretion (Reviewed by Oakley *et al.* (2009)).

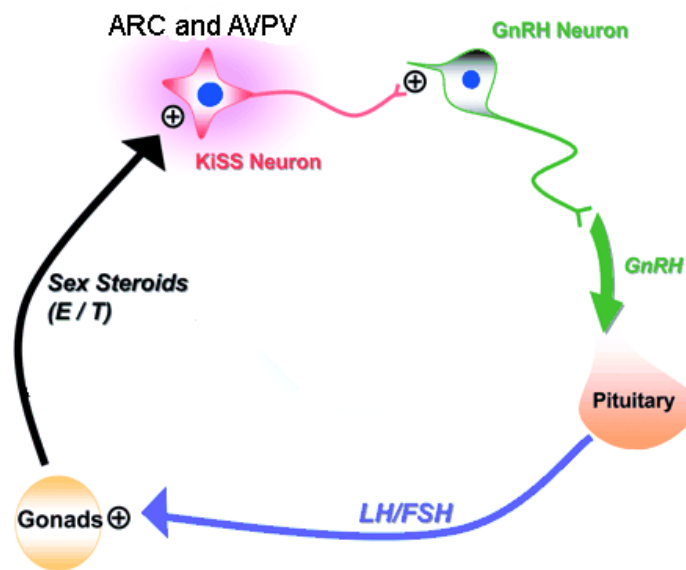


Figure 1-2: Regulation of the reproductive axis by kisspeptin in mouse.

Kisspeptin is released from Kisspeptin neurons mainly in the arcuate nucleus (ARC) and in the anteroventral periventricular nucleus (AVPV) to stimulate GnRH release from GnRH neurons, which in turn stimulates the secretion of LH and FSH from the anterior pituitary. LH and FSH stimulate steroidogenesis and gametogenesis, which feed-back to regulate the activity of Kisspeptin neurons. Figure reproduced from Gottsch *et al.*, 2006.

1.2.2 Gonadotropin-Releasing Hormone (GnRH)

GnRH is the central regulator of the reproductive system downstream of Kiss1 and exists as 23 isoforms in vertebrates. In most vertebrates, there are three forms of GnRH; GnRH I, GnRH II and GnRH III. The mammalian hypophysiotropic form, GnRH I, is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) and is

primarily involved in the synthesis and secretion of LH and FSH from gonadotropes (discussed in Section 1.5). The second form is GnRH II. It is ubiquitously expressed, conserved in structure, and was first isolated from chicken brain (Miyamoto *et al.*, 1984). GnRH II is widely distributed in discrete regions of the central and peripheral nervous system and in non-neural tissues (Millar, 2003). Finally, a third form of GnRH, GnRH III, has been isolated in many vertebrates species (Millar, 2003, 2005; Millar and Newton, 2010; Pawson *et al.*, 2003).

1.2.3 The Gonadotropin-Releasing Hormone Receptor (GnRH-R)

The GnRH-R is a member of the GPCR super-family, and belongs to the Rhodopsin-like family. In mammals, there are two forms of GnRH-R, the Type I and Type II GnRH-Rs.

The Type I GnRH-R is expressed in pituitary gonadotropes and regulates gonadotropin synthesis. It is also expressed in other tissues, including prostate, breast and ovary (Dubois *et al.*, 2002; Millar, 2005; Millar and Newton, 2010). Structurally, the mammalian GnRH-Rs differ from other GnRH-Rs in vertebrates, in that they completely lack the cytoplasmic C-terminal tail (**Fig. 1-3**). The cytoplasmic C-terminal tail is involved in receptor desensitisation, and the mammalian Type I GnRH-Rs which lack a cytoplasmic C-terminal tail do not undergo rapid desensitisation (Davidson *et al.*, 1994; McArdle *et al.*, 1999).

mRNAs of *Type II GnRH-Rs* are expressed in many parts of the human and marmoset brain and peripheral tissues. However, *Type II GnRH-R* mRNAs in human and some other mammals (e.g. chimp, sheep and bovine) are not translated to a full-

length and functional GPCR due to the presence of various mutations including a frame-shift and internal stop codon (Faurholm *et al.*, 2001; Millar, 2003).

GnRH I binds both Type I and Type II GnRH-Rs, however, it binds the Type I GnRH-R with higher affinity. GnRH I binds to and activates Type I GnRH-R inducing a number of signalling pathways. Despite the lack of a full-length functional Type II GnRH-Rs in human, GnRH II is able to bind the Type I GnRH-R with high affinity. The ability of GnRH II to bind to the Type I GnRH-R suggests that the Type I GnRH-R is able to take over the role of the Type II GnRH-R in humans (Millar *et al.*, 2004). GnRH I or GnRH II binding to the Type I GnRH-R recruits different intracellular signalling proteins to produce different phenotypic effects in different cells, a phenomenon called ligand-induced selective signalling (LiSS) (Millar and Newton, 2010). In particular, GnRH I binding to the Type I GnRH-R is more potent at generating G_q -coupled inositol phosphate production, which is the predominant signalling pathway in gonadotropes. In contrast, binding of GnRH II to the Type I GnRH is more potent at activating antiproliferative signalling pathways in cancer-derived cell lines (Millar and Newton, 2010).

GnRH-Rs are primarily localised within the plasma membrane, however a number of studies have demonstrated that GnRH-Rs are also localised on the nuclear membrane (Janovick *et al.*, 2003; Millar *et al.*, 1983; Re *et al.*, 2010). More recently, the nuclear localisation of functional human Type I GnRH-Rs was confirmed (Re *et al.*, 2010). The nuclear membrane localisation of GnRH-Rs suggests the potential for novel intracellular signalling pathways with important physiological roles (Re *et al.*, 2010).

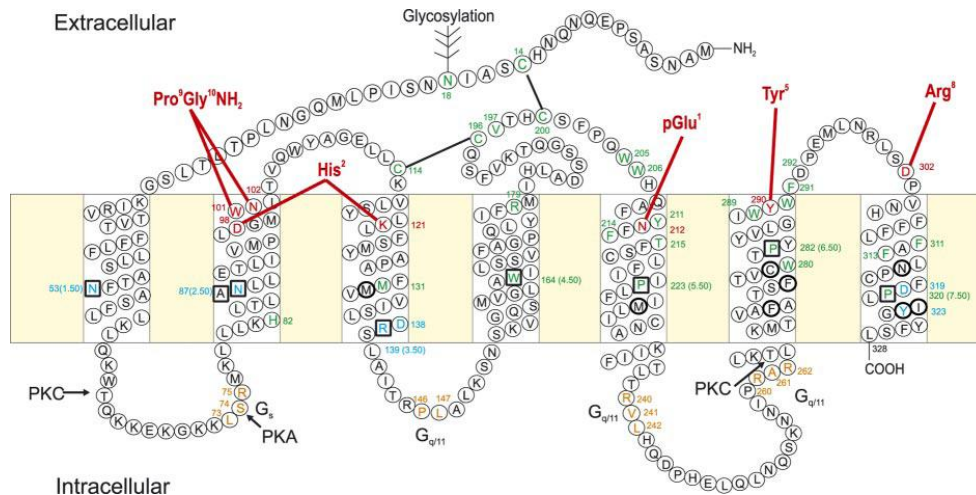


Figure 1-3: The Human Gonadotropin-Releasing Hormone Receptor.

The GnRH-R consists of seven trans-membrane domains which are connected by three intracellular and extracellular loops. Figure reproduced from Millar and Newton, 2010.

1.3 GnRH-R SIGNALLING

1.3.1 Coupling to G-proteins

Activation of the GnRH-R induces a conformational change leading to coupling and activation of heterodimeric G-proteins. G-proteins consist of α -, β - and γ -subunits, which transduce signals from GPCRs to intracellular mediators (Naor *et al.*, 2000). The α -subunit is a 37-42KDa protein, which contains a guanine nucleotide-binding pocket with intrinsic GTPase activity. The 35KDa β -subunit and the 8-11KDa γ -subunit are tightly associated with each other (Goldsmith and Dhanasekaran, 2007). There are over 20 $G\alpha$ -subunit which are divided into four major families; $G\alpha_{q11}$ which activates phospholipase C (PLC), $G\alpha_s$ which activates adenylyl cyclase, $G\alpha_{i/o}$ which inhibits adenylyl cyclase and $G\alpha_{12/13}$ which activates small GTPases (Naor, 2009; Naor *et al.*, 2000). Activation of GnRH-Rs catalyses the exchange of the

bound guanosine-5-diphosphate (GDP) to GTP guanosine-5-triphosphate (GTP) on the α -subunit. The GTP-bound α -subunit in turn dissociates from the receptor and $\beta\gamma$ -subunit, and initiates distinct signalling pathways (Goldsmith and Dhanasekaran, 2007). The $G\beta\gamma$ -subunit is also capable of activating other effector systems (Naor, 2009). The amino acid residues of GnRH-R which are essential for G-protein activation are conserved among mammalian and non-mammalian GnRH-Rs (Naor, 2009). In particular, GnRH-R coupling to $G\alpha_{q/11}$ occurs through the intracellular loops 2 and 3, while coupling to G_s occurs through intracellular loop 1 (Millar *et al.*, 2004). In the remainder of this Section 1.3, only the mediators activated by GnRH-R coupling to G_q will be reviewed

1.3.2 Phospholipase C

PLC is a soluble multi-domain protein found in eukaryotes. Four β , two γ and four δ isoforms have been described in mammals (Rebecchi and Pentylala, 2000).

The PLC β isoforms are regulated by the coupling of $G\alpha_q$ - and $G\beta\gamma$ -proteins. Following GnRH-R activation, $G\alpha_q$ family proteins and $G\beta\gamma$ subunits activate PLC β , which hydrolyses the highly phosphorylated lipid phosphatidylinositol 4,5-bisphosphate [PIP₂], generating two intracellular products, inositol 1,4,5-trisphosphate (InsP₃ or IP₃), a calcium-mobilising second messenger, and diacylglycerol (DAG), an activator of protein kinase C (PKC) (Naor, 2009; Rebecchi and Pentylala, 2000) (**Fig. 1-4**).

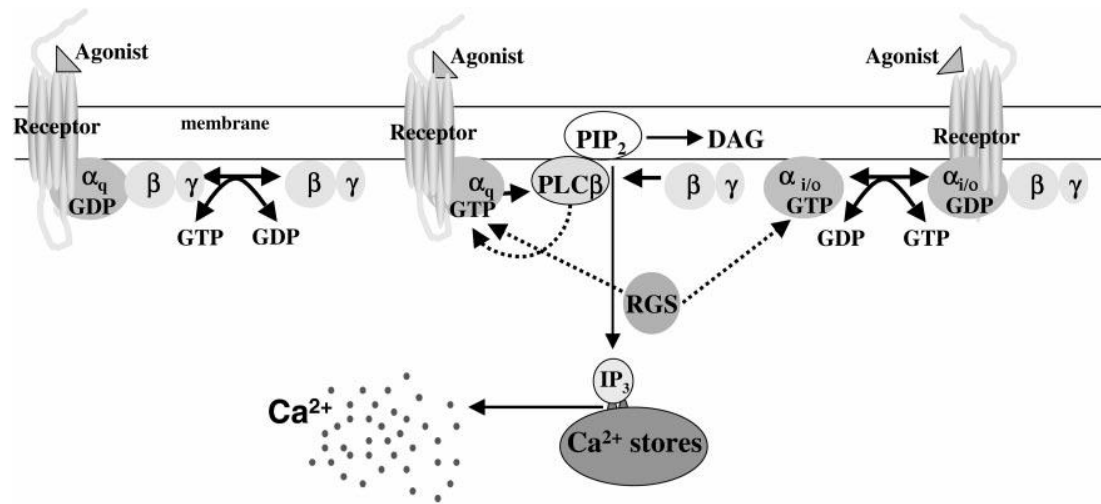


Figure 1-4: Regulation of PLCβ by GPCRs.

The G α_q -subunit activates PLC β causing hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) generates diacylglycerol (DAG) and IP₃, which in turn releases calcium from internal stores. G $\beta\gamma$ -subunits released from G α_i/o also activate PLC β (RGS: Regulators of G protein signalling). Figure reproduced from Bercchi, 2000.

1.3.3 IP₃ and Ca²⁺

When IP₃ is released into the cytosol it binds to IP₃ receptors located on the endoplasmic reticulum. The activation of IP₃ receptors stimulates rapid Ca²⁺ mobilisation from internal IP₃-sensitive stores, followed by external Ca²⁺ influx via Ca²⁺ channels (**Fig. 1-4**) (Berridge, 2009; Naor, 2009). The IP₃/Ca²⁺ pathway is a versatile signalling system that regulates many processes including exocytosis, cell proliferation, apoptosis and cell metabolism. The IP₃/Ca²⁺ pathway also mediates gonadotropin exocytosis in gonadotropes. The involvement of Ca²⁺ in cell proliferation is well illustrated in lymphocytes responding to antigen, where Ca²⁺ functions in conjunction with other signalling pathways such as the MAPK and PI3K pathways (Berridge *et al.*, 2000). In general, Ca²⁺ controls cell proliferation by activating transcription factors, either in the cytoplasm (NF- κ B) or within the nucleus

(cAMP response element-binding(CREB)). These transcription factors then mediate expression of a number of genes that are involved in cell proliferation, including *interleukin 2* and Fas ligand (*FasL*) (Reviewed by Berridge *et al.* (2000)).

1.3.4 Diacylglycerol (DAG) and phosphatidic acid (PA)

As previously mentioned, DAG is formed from [PIP₂] by the action of PLC β . DAG regulates a number of cellular functions through the binding to and activation of PKC. DAG is also phosphorylated by DAG kinase (DGK) to phosphatidic acid (PA). PA in turn regulates a number of signalling proteins, including the atypical PKCs, the Raf-1 kinase and the mammalian target of rapamycin (mTOR). Notably, the list of PA-dependent protein members is still growing (Sakane *et al.*, 2007).

1.3.5 Protein Kinase C

PKC is a serine/threonine kinase lipid activated kinase, and consists of at least 10 related isoforms. PKC has a regulatory domain and a carboxyl-terminal catalytic domain. DAG binds to the regulatory domain of the PKC leading to activation. Activation of PKC by DAG leads to the activation of major members of the mitogen-activated protein kinase (MAPK) family including extracellular signal-regulated kinase (ERK), c-Jun N-terminal Kinase (JNK) and p38 mitogen-activated protein kinases (p38MAPK) (Dobkin-Bekman *et al.*, 2006). Activation of PKC contributes to the activation of the MAPK cascade through directly phosphorylating and stimulating c-Raf. c-Raf is a member of the Raf kinase family of serine/threonine-specific protein kinases (Goldsmith and Dhanasekaran, 2007). PKC also exerts a

negative feedback on PLC β activity, and therefore regulates IP $_3$ and Ca $^{2+}$ signalling (Naor, 2009).

1.3.6 Mitogen-activated protein kinase (MAPK)

GnRH-R activation initiates diverse signalling cascades including the MAPK pathways. Four major MAPK cascades are activated by GnRH-R; ERK1/2, c-Jun N-terminal kinase, p38MAPK and the big MAPK (BMK) (Naor, 2009). The MAPK cascade consists of at least three distinct kinases, the upstream mitogen-activated protein (MAP) kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K) and a downstream MAP kinase (MAPK). Therefore, the MAPK cascade proceeds through sequential phosphorylation of the MAP3K, MAP2K and MAPK (Goldsmith and Dhanasekaran, 2007).

The main characteristic of the MAPK family is their ability to translocate to the nucleus and activate a variety of transcription factors (Dobkin-Bekman *et al.*, 2006), including early growth response factor 1 (Egr1), c-Fos, c-Jun and STAT (Naor, 2009).

1.3.7 The phosphatidylinositol 3-Kinase (PI3K)/Akt signalling pathway

PI3K is a minor inositol lipid kinase that is involved in many cellular processes, including cell survival, proliferation and differentiation. PI3K is activated by receptor tyrosine kinases (RTKs) and by GPCRs, and transduces signals by generating phospholipids. There are three classes of PI3K, however only Class I is activated by GPCRs. PI3K is a heterodimer and consists of a p110 catalytic subunit

and a p85 regulatory subunit. Class I PI3Ks activated by RTKs and GPCRs, phosphorylate the inositol ring of inositol phospholipids (Foster *et al.*, 2003; Liu *et al.*, 2009; Vanhaesebroeck and Alessi, 2000). These phospholipids then bind to and activate Akt.

Akt (also known as PKB) is a 57KDa serine/threonine protein kinase which contains a pleckstrin homology (PH) domain and is activated by the PI3K. PH domains are globular protein domains which are found in over 150 proteins, and are activated by binding phospholipids with high affinity (Vanhaesebroeck and Alessi, 2000). The PH domain of Akt binds to the phospholipids which are produced by PI3K activation, and Akt is subsequently activated. Akt is primarily located in the cytoplasm in unstimulated cells and translocates to the plasma membrane following PI3K activation, where it binds phospholipids via its PH domain to become activated (Scheid and Woodgett, 2003).

Another PH domain-containing serine/threonine kinase is 3'-phosphoinositide-dependent kinase-1 (PDK1). Like Akt, PDK1 is primarily located in the cytoplasm in unstimulated cells and translocates to the plasma membrane following PI3K activation, where it binds phospholipids via its PH domain and phosphorylates Akt (Vanhaesebroeck and Alessi, 2000). Akt translocation to the plasma membrane causes a conformational change that allows PDK1 to phosphorylate it at the Thr³⁰⁸ and Ser⁴⁷³ phosphorylation sites. Phosphorylation of Akt at these sites is required for its full activation (Liu *et al.*, 2009; Vanhaesebroeck and Alessi, 2000). A number of studies have also demonstrated PI3K-independent activation of Akt via a variety of mechanisms, including heat-shock response or increases in intracellular Ca²⁺ or

cyclic adenosine monophosphate (cAMP). However, although different studies reported a heat-shock-dependent activation of Akt, their results are contradictory in relation to PI3K-dependent activation (Vanhaesebroeck and Alessi, 2000). Once Akt is phosphorylated and activated, it phosphorylates a diverse array of signalling mediators including the I κ B kinase (IKK) (discussed in Section 1.6.2.1.2) and FOXO transcription factors (discussed in Section 1.6.2.1.1) (Liu *et al.*, 2009).

1.4 GnRH-INDUCED ANTIPROLIFERATION AND APOPTOSIS

As described above, GnRH-Rs are not only expressed in gonadotropes, but also in extra-pituitary tissues, including breast, prostate, placenta, endometrium and ovary. In addition, GnRH has been found in extra-pituitary tissues such as in placenta, ovary, mammary gland and lymphoid cells (Kraus *et al.*, 2006; Millar, 2005; Millar *et al.*, 2004). While GnRH acts at its receptor in gonadotropes to regulate the expression of the gonadotropins (LH and FSH), numerous studies have demonstrated that GnRH-R activation in extra-pituitary cell types can mediate inhibition of cell proliferation¹ or lead to a programmed cell-death (apoptosis). *In vitro* and tumour xenograft studies have demonstrated that GnRH treatment causes cell-growth inhibition² in cell lines that express the GnRH-R (Cheng and Leung, 2005; Everest *et al.*, 2001; Franklin *et al.*, 2003; Kraus *et al.*, 2006; Limonta *et al.*, 2003; Maudsley *et al.*, 2004; Miles *et al.*, 2004; Morgan *et al.*, 2008). In addition, *in vitro* and *in vivo* studies have demonstrated that treatment of cancer cells with GnRH analogues results in a reduction of cell proliferation (Kraus *et al.*, 2006). GnRH analogues are

¹**Cell proliferation:** refers to the increase of cells number

²**Cell growth inhibition:** refers to the cell division /growth of cell populations

widely used for cancer treatment. They were first used to treat prostate cancer (Tolis *et al.*, 1982) and uterine leiomyomas (Carr *et al.*, 1993). GnRH analogues are now more widely used in the treatment of ovarian cancer (Bifulco *et al.*, 2004), uterine cancer (Hofstra *et al.*, 1999), breast cancer (Klijn and de Jong, 1982) and endometrial cancer (Mizutani *et al.*, 1998).

The antiproliferative effects of GnRH have been shown to be dependent on GnRH-R expression levels and different cellular environments. GnRH initiates distinct signalling pathways in various cancer-derived cell lines depending on different cellular environments and GnRH-R expression levels (Fister *et al.*, 2009; Kraus *et al.*, 2006; Morgan *et al.*, 2008). A number of signalling pathways mediate the antiproliferative effects of GnRH. Several of these will be reviewed below.

As described above, a number of studies have examined the signalling pathways via which GnRH mediates its antiproliferative or apoptotic effect in a variety of model cell lines and cancer-derived cell lines. A common feature for these signalling pathways, is that the intracellular signalling activated by the GnRH-R is mediated through coupling to the G_q protein (Emons *et al.*, 1998; Imai *et al.*, 1997; Limonta *et al.*, 2001; Lopez de Maturana *et al.*, 2008; White *et al.*, 2008a).

A variety of downstream mediators have been implicated in the antiproliferative effects of GnRH, in different cell types. Elevated levels of cleaved poly (ADP-ribose) polymerase (PARP), a substrate of caspase 3 was observed in HEK293/rat GnRH-R expressing cells in response to GnRH (Lopez de Maturana *et al.*, 2008). The role of PKC and caspases to completely or partially rescue the cell-growth inhibitory effect of GnRH in HEK293/rat GnRH-R expressing cells has also been

examined (Morgan *et al.*, 2008). The involvement of JNK and PI3K/Akt in the apoptotic process in prostate cancer cell lines was examined. Inhibition of JNK rescued prostate cancer cells from the apoptotic effect of GnRH, whereas inhibition of PI3K/Akt increased the apoptotic effect of GnRH in prostate cancer cells (Kraus *et al.*, 2004). The role of ERK in the antiproliferative effect of GnRH was also assessed in Caov-3 human ovarian cancer cells, and was suggested to be mediated by coupling to the $G\alpha_i$ protein (Kimura *et al.*, 1999). GnRH also induces activation of JunD, which negatively regulates proliferation, and affects cell-cycle regulation and DNA synthesis in primary human ovarian cancer cells and EFO-21 and EFO-27 human ovarian cancer cell lines (Gunthert *et al.*, 2002). The involvement of ERK1/2 and p38MAPK pathways in the antiproliferative effects of GnRH in ECC-1 endometrial cancer cells was also demonstrated. Cells pre-treated with MAPK inhibitors were rescued from GnRH II-mediated cell-growth inhibition (Wu *et al.*, 2009). A reduction of PI3K/Akt activity by the GnRH analogue leuprolide acetate (LA) in uterine leiomyomas has been demonstrated to mediate the reduction of leiomyoma volume (Bifulco *et al.*, 2004). GnRH also induced apoptosis in tissues derived from women with endometriosis, adenomyosis and uterine myoma, while activated caspase 3 was demonstrated in response to GnRH (Khan *et al.*, 2010). Treatment of MCF-7 and triple-negative MDA-MB-231 human breast-cancer cells (triple-negative breast cancer does not express estrogen and progesterone receptors, and no overexpression/amplification of the HER2-neu gene occurs (Grundker *et al.*, 2010)) with a GnRH II antagonist results in apoptotic cell death *in vitro* via activation of stress-activated p38MAPK and loss of mitochondrial membrane potential. Interestingly, the GnRH II antagonist [Ac-D2Nal1, D-4Cpa2, D-3Pal3, 6, Leu8, D-

Ala10] also induced activation of caspase 3 in MDA-MB-231 human breast-cancer cells (Grundker *et al.*, 2010).

The effect on cell-cycle regulation following GnRH stimulation has been examined in a number of studies (Gunthert *et al.*, 2002; Kim *et al.*, 1999; Miles *et al.*, 2004). GnRH treatment of HEK293 cells expressing the rat GnRH-R induced an accumulation of cells in G2/M phase, whereas no G1 block was observed (Miles *et al.*, 2004). GnRH also blocked cell-cycle progression in the G0/G1 phase without inducing apoptosis in OVCAR-3 and SKOV-3 human ovarian cancer cells (Kim *et al.*, 1999). Finally, treatment of primary human ovarian cancer cells and EFO-21 and EFO-27 human ovarian-cancer cell lines with the GnRH agonist Triptorelin caused an increase in the proportion of cell found in G0/G1 phase, and a decrease of cells in G2/S phase of the cell-cycle (Gunthert *et al.*, 2002).

1.4.1 *The role of FasL and GADD45 in GnRH-induced apoptosis*

GnRH-induced cell-growth inhibition is also mediated via the pro-apoptotic protein FasL and the growth arrest and DNA damage (GADD45) protein (Imai *et al.*, 1998b; Wu *et al.*, 2009). FasL induces apoptosis in a variety of cell types via activation of the Fas receptor (discussed in Section 1.6.3.2), while GADD45 is a regulator of cell-cycle progression, DNA repair and apoptosis (discussed in Section 1.6.3.4). The role of FasL in GnRH-induced cell-growth inhibition was first revealed in a study by Imai *et al.* (1998). An increase in the levels of FasL mRNA and protein, following GnRH stimulation has been demonstrated in the endometrial and ovarian carcinoma cells expressing the GnRH-R (Imai *et al.*, 1998a). The speculation that GnRH-induced FasL expression may mediate the antiproliferative action of GnRH analogues was

confirmed in a follow-up study by the same group, where the antiproliferative effect of GnRH analogues on cells bearing Fas receptor compared with Fas receptor-negative cells was examined. Indeed, the antiproliferative effect of GnRH analogues was demonstrated only on cells expressing the Fas receptor, and not in Fas receptor-negative cells (Imai *et al.*, 1998b). In contrast, treatment of uterine leiomyomas with a GnRH analogue failed to increase apoptosis, and moreover, lead to a decrease in *FasL* expression (Huang *et al.*, 2002).

The involvement of GADD45 in GnRH-induced cell-growth inhibition was demonstrated in a recent study conducted by Wu *et al.* (2009). The mechanism by which GnRH II induced apoptosis of human endometrial cancer cells was examined. Binding of GnRH II to the Type I GnRH receptor was demonstrated to induce apoptosis of human endometrial cancer cells by activating the expression of *GADD45a* gene (Wu *et al.*, 2009).

Although FasL and GADD45 mediate GnRH-induced cell-growth inhibition, the exact mechanisms involved has not yet been completely characterised.

1.5 THE GONADOTROPINS

1.5.1 Luteinising Hormone

Reproduction in males and females is dependent on the physiological function of the hypothalamic-pituitary-gonadal axis, and the synthesis and release of several hormones including LH and FSH. LH binds to Luteinising Hormone receptors (LH-

Rs) on Leydig cells in the testis for spermatogenesis, and on granulosa and theca cells in the ovary for follicular maturation and ovulation (Pierce and Parsons, 1981). In order to identify the role of LH in reproduction, knock-out mice lacking the *LH β* (Luteinizing hormone beta)- subunit gene have been generated. Mutations of the *LH β* gene that lead to its inactivation are very rare in nature (Kumar, 2007). *LH β* -null mice were viable with postnatal defects in gonadal growth and infertility, and *LH β* -null male mice were hypogonadal with decreased size of testis and accessory glands, and showed decreased serum and intra-testicular testosterone level. *LH β* -null female mice were also hypogonadal and had low serum of estradiol and progesterone levels (Kumar, 2007; Ma *et al.*, 2004).

LH and FSH are heterodimer glycoproteins which are composed of common α -glycoprotein (α GSU) and hormone-specific β -subunits. The *LH β* -subunit confers LH binding specificity to LH-Rs, which are GPCRs found in the ovary, testis and extragonadal organs like the uterus (Thackray *et al.*, 2010). Each subunit of the LH protein is produced by a separate gene, and both subunits are glycosylated and assembled non-covalently as heterodimers in secretory granules where they are stored (Jorgensen *et al.*, 2004; Pierce and Parsons, 1981; Thackray *et al.*, 2010).

1.5.2 Follicle-Stimulating Hormone

FSH is a heterodimer glycoprotein which is composed of a common α GSU and hormone-specific β -subunit (Pierce and Parsons, 1981). In females, FSH stimulates ovarian follicle growth and maturation, while in males it stimulates androgen production (Johnson, 2007).

As previously mentioned, the main regulator of FSH expression is GnRH. The mechanism through which GnRH stimulates *FSH β* (Follicle-Stimulating Hormone beta)- subunit transcription includes several signalling pathways and transcription factors. GnRH stimulates *FSH β* -subunit expression via the ERK1/2 cascade; however the involvement of ERK1/2 is not yet well established (Bliss *et al.*, 2009; Kanasaki *et al.*, 2005). Several *in vivo* and *in vitro* studies have shown that although GnRH stimulates the p38MAPK and JNK cascades, a definitive role for these pathways in GnRH-induced regulation of FSH expression has not yet been established (Reviewed in Bernard *et al.* (2010)). A number of transcription factors, including the steroidogenic factor 1 (SF1), pituitary homeobox 1 (Pitx1), the LIM homeobox gene 3 (LHX3) and the GATA-binding protein 2 (GATA2) have also been implicated in *FSH β* expression. However, their role in GnRH-induced *FSH β* expression has not been definitively demonstrated (Bernard *et al.*, 2010).

Another important regulator for *FSH β* production is activin, a member of the transforming growth factor β (TGF β) super-family (Vale *et al.*, 1988). Activin is involved in the up-regulation of *FSH β* levels via the regulatory proteins SMAD2 and SMAD3. However, the role of activin in GnRH-induced *FSH β* expression is not entirely clear due to the contradictory effect of activin action on *FSH β* -expression following acute and chronic administration of GnRH (Xia and Schneyer, 2009).

1.5.3 *LH β* promoter structure

All the important DNA elements of the *LH β* promoter essential for its transcription are located within 500bp of the *LH β* promoter, which consists of the proximal and

the distal promoter domains. The proximal promoter region (140bp) is highly conserved across all mammals (Jorgensen *et al.*, 2004), with small differences in the nucleotide sequence between species, and comprises of two DNA binding sites for Egr1 (Halvorson *et al.*, 1998; Lee *et al.*, 1996; Topilko *et al.*, 1998) and SF1 (Halvorson *et al.*, 1998; Halvorson *et al.*, 1996; Keri and Nilson, 1996), and one binding site for Pitx1 (Quirk *et al.*, 2001) which is flanked by the Egr1 and SF1 binding sites (**Fig. 1-5A**).

Regulatory elements have been also found on the distal domain of the *LH β* promoter. Gonadotrope-specific elements (GSEs) that bind the transcription factor Sp1 (Kaiser *et al.*, 1998; Weck *et al.*, 2000), and a CC(A/T)6GG motif (CArG) (Weck *et al.*, 2000) have been found in the distal rodent promoters, and binding sites for the nuclear transcription factor-Y (NF-Y) have been found in the distal bovine promoter (Keri *et al.*, 2000) (**Fig. 1-5B**).

1.5.4 Regulation of LH β expression – Transcription factors

In addition to gonadotropes, there are other cell types that express and secrete hormones in the anterior pituitary which also indirectly regulate the synthesis of the LH. Thus, gene regulation and the biosynthesis of LH and FSH in gonadotropes are also regulated by multiple factors that are expressed by nearby cells (Kaiser *et al.*, 2000). In this section, the transcription factors that mediate GnRH-induced LH β expression will be reviewed. The main transcription factors dealt with are the Egr1, SF1, Pitx1 and Sp1.

1.5.4.1 *Egr1*

Egr1, a member of the immediate early gene (IEG) family of proteins, is a zinc-finger transcription factor that is rapidly induced in many cell lines by growth, differentiation and apoptotic stimuli (Lee *et al.*, 1996; Salisbury *et al.*, 2008). GnRH treatment up-regulates the expression of *Egr1* via a PKC-mediated pathway (Tremblay and Drouin, 1999; Wolfe and Call, 1999). A study by Tremblay *et al.* (1999) reported that one hour after GnRH treatment *Egr1* mRNA increased transiently to a maximal level compared to the non-stimulated α T3-1 and L β T2 gonadotrope cells. *Egr1* binds to the *LH β* promoter in gonadotropes at two *Egr1* DNA binding sites. Over-expression of *Egr1* up-regulates the expression of the LH β -subunit through action at both *Egr1* binding sites (Fortin *et al.*, 2009; Halvorson *et al.*, 1998; Kaiser *et al.*, 2000; Lee *et al.*, 1996). Several studies have shown that GnRH-induced LH β expression is mediated by *Egr1* (Kaiser *et al.*, 2000; Tremblay and Drouin, 1999; Wolfe and Call, 1999), and mutations of the *Egr1* binding sites reduced GnRH-induced *LH β* gene expression (Kaiser *et al.*, 2000).

1.5.4.2 *SF1*

SF1 is an orphan member of the nuclear receptor super-family, initially identified as a transcription factor that controls expression of the cytochrome P-450 steroid hydroxylase genes in gonads and adrenal cortex (Ikeda *et al.*, 1993). SF1 also binds to and regulates the transcription of the *LH β* gene. A study by Ingraham *et al.* (1994) first demonstrated expression of SF1 in pituitary tissues by *in situ* hybridisation (Ingraham *et al.*, 1994). *In vitro* studies revealed that SF1 directly regulates the expression of the *LH β* gene (Dorn *et al.*, 1999; Halvorson *et al.*, 1998; Halvorson *et*

al., 1996; Keri and Nilson, 1996; Tremblay and Drouin, 1999; Wolfe and Call, 1999). Indeed, over-expression of SF1 increases LH β expression (Halvorson *et al.*, 1996; Kaiser *et al.*, 2000; Keri and Nilson, 1996). However, GnRH treatment does not up-regulate *SF1* gene expression (Tremblay and Drouin, 1999). While SF1-/- mice exhibited decreased LH β gene expression (Ingraham *et al.*, 1994; Shinoda *et al.*, 1995; Zhao *et al.*, 2001b), GnRH treatment increased LH β expression (Ikeda *et al.*, 1993). In contrast, an *in vivo* study by Kaiser *et al.* (1998) demonstrated that LH β promoter activity is not increased in response to GnRH in transgenic mice with a mutation of the SF1 binding site (Kaiser *et al.*, 1998). Another study by the same group, demonstrated that over-expression of LH β promoter with a mutation at the SF1 binding site reduced the LH β promoter activity in rat pituitary adenoma GH₃ cells in response to GnRH. However, over-expression of LH β promoter with a mutation on the SF1 binding site did not reduce the LH β promoter activity in L β T2 cells in response to GnRH (Kaiser *et al.*, 2000). Therefore, the role of SF1 in the GnRH-induced LH β expression is not yet completely clear.

1.5.4.3 *Pitx1*

Pitx1 is a transcription factor that is expressed in gonadotropes (Tremblay *et al.*, 1998). Pitx1 activates LH β gene expression in addition to the transcription of a large number of gonadotrope genes, including *FSH β* and *GnRH-R* (Tremblay *et al.*, 1998). Pitx1 regulates the LH β gene expression via direct binding to the LH β promoter (Quirk *et al.*, 2001), however *Pitx1* mRNA expression is not regulated by GnRH (Tremblay and Drouin, 1999). A study by Quirk *et al.* (2001) demonstrated that transient co-expression of the LH β promoter bearing mutations at the Pitx1 binding

site resulted in reduced *LHβ* promoter activity compared to the wild type *LHβ* promoter in response to GnRH. However, following GnRH treatment, the *LHβ* promoter activity was similar when either *LHβ* promoter bearing mutations at the Pitx1 binding site or the wild type *LHβ* promoter were over-expressed (Quirk *et al.*, 2001), indicating that Pitx1 protein is critical for the basal but not for the GnRH-induced LHβ expression.

A.



B.

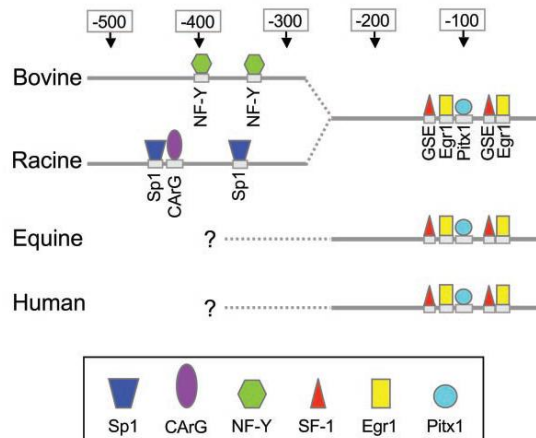


Figure 1-5: Structure of *LHβ*-subunit gene promoter.

(A) The transcription factors binding sites on the rat *LHβ* promoter. Figure reproduced from Thackray *et al.*, 2010. (B) DNA binding elements on the proximal and distal regions of the *LHβ* promoter of different species. DNA binding elements of the proximal region (0/-140bp) are highly conserved among the species. Distal regions are less well characterised. (NF-Y: Nuclear transcription factor Y, Sp1: selective promoter factor 1 or specificity protein 1, CArG: CC(A/T)6GG, GSE: Gonadotropin-specific element) (Figure reproduced from Jorgensen *et al.*, 2004).

1.5.4.4 *Sp1*

Sp1 is a member of the zinc finger family of transcription factors (Berg, 1992). Sp1 binds to the *LHβ* promoter at two binding sites, and regulates its transcriptional activity. These two binding sites are located in positions -490/-352 and -207/-82 on the rat *LHβ* promoter. Mutations of these binding sites reduce the basal level of *LHβ* promoter activity, and also reduce GnRH-induced *LHβ* expression (Kaiser *et al.*, 2000; Kaiser *et al.*, 1998; Weck *et al.*, 2000).

A number of *in vivo* studies have been conducted in mice with targeted deletion of additional proteins which bind to and regulate *LHβ* promoter in order to determine their biological role. These are summarised in **Table 1-1**.

1.5.4.5 *Transcription factor synergism at the LHβ promoter*

The transcription factors described above act synergistically to regulate maximal activity of the *LHβ* promoter. Co-expression of Egr1 and SF1 results in maximal *LHβ* protein expression (Dorn *et al.*, 1999; Halvorson *et al.*, 1998; Kaiser *et al.*, 2000; Tremblay and Drouin, 1999; Wolfe and Call, 1999). In addition, the physical interaction of Egr1 with Ptx1 and SF1, and the resultant synergy between Egr1, SF1 and Pitx1 leads to maximal Egr1-mediated GnRH-induced *LHβ* expression (Halvorson *et al.*, 1998; Tremblay and Drouin, 1999).

Gene/Species	Effect on LH β expression	Biological effect	Reference
NGFI ^{-/-} ⁽¹⁾ male mice	Decreased	Fertile	(Lee <i>et al.</i> , 1996)
NGFI ^{-/-} ⁽¹⁾ female mice	Undetectable	Unfertile	(Lee <i>et al.</i> , 1996)
Krox-24 ^{-/-} ⁽¹⁾ male/female	Absence of LH	Sterile	(Topilko <i>et al.</i> , 1998)
Ftz-F ⁽²⁾ 1-disrupted mice	Undetectable	No data	(Ingraham <i>et al.</i> , 1994)
mFtz-FI ⁽²⁾ -disrupted mouse	Decreased	No data	(Shinoda <i>et al.</i> , 1995)
Pituitary-specific SF1 knockout mice	Markedly decreased	Infertile	(Zhao <i>et al.</i> , 2001a)

⁽¹⁾ *Egr1* (*Krox-24*, *NGFI-A*, *Zif268*)

⁽²⁾ *SF1* (*Ftz-FI*)

Table 1-1: Effect on LH β expression and fertility in mice by knocking-down transcription factors known to regulate the LH β promoter activity.

A mechanism of GnRH-induced regulation of LH β promoter activity has been proposed by Kaiser *et al.* (2000) (**Fig. 1-6**). An interaction between the proximal and the distal domains of the rat LH β promoter in response to GnRH was proposed. When an LH β promoter bearing a inactivating mutation at the Sp1 binding site was over-expressed in GH₃ cells, GnRH-induced LH β expression was decreased, suggesting that Sp1 is involved in the regulation of the LH β promoter activity (Kaiser *et al.*, 2000). Overall, this study proposed a mechanism by which GnRH treatment increases Egr1 protein level, which together with Sp1 and SF1 binds to an unidentified transcription factor (co-factor). This complex which includes Egr1, Sp1, SF1 with the co-factor causes a conformational change in the promoter that leads to enhanced LH β promoter activity (**Fig. 1-6**).

A recent study by Walsh *et al.* (2009) also demonstrated a proteasome-based regulation of the LH β gene in response to GnRH. This study reported that two of the main transcription factors acting at the LH β promoter, Egr1 and SF1, are targets of

the ubiquitin proteasome system. Fluctuation in Egr1 and SF1 levels leads to their cyclic association with the *LHβ* promoter, thus allowing the *LHβ* gene to respond to pulsatile stimulation of GnRH *in vivo* (Walsh and Shupnik, 2009).

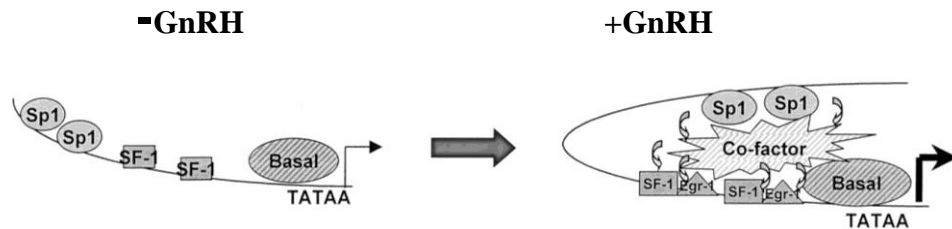


Figure 1-6: Model for interaction of SF1, SP1 and Egr1 on the promoter of *LHβ* gene in response to GnRH.

All three transcription factors interact with an unidentified transcription co-factor to mediate the full response to GnRH at the *LHβ* promoter activity (Basal indicates a possible transcription factors that is important in the transcription in this complex). Figure reproduced from Kaiser *et al.*, 2000.

1.5.4.6 *β-catenin*

β -catenin is a transcription co-activator, and a member of the canonical Wnt signalling pathway. There is a well-established role for β -catenin in the formation of the active complex required for the *LHβ* expression (Salisbury *et al.*, 2008). This was first demonstrated in a study by Salisbury *et al.* (2007) where reduction of β -catenin levels resulted in a decrease in GnRH-induced *LHβ* gene expression. β -catenin was demonstrated to interact with SF1, and this interaction is a requirement for the formation of an active complex between the SF1 and Egr1 following GnRH treatment, resulting in maximal GnRH-induced *LHβ* gene expression (Salisbury *et al.*, 2007). Following GnRH treatment β -catenin associates with the proximal region of the *LHβ* promoter in a complex comprising of SF1 and Egr1 to maximise *LHβ* expression levels (Gardner *et al.*, 2007; Gardner *et al.*, 2010; Salisbury *et al.*, 2007).

In contrast, in the absence of GnRH treatment, a complex between β -catenin, SF1 and Pitx1 maintains the *LH β* gene expression at basal levels (**Fig. 1-7**) (Salisbury *et al.*, 2008).

1.5.5 Regulation of Luteinising Hormone expression – signalling pathways

Induction of LH β expression by GnRH is dependent on a number of mediators, including members of the MAPK cascade (Reviewed by Thackray *et al.* (2010)). The involvement of the three sub-families of the MAPKs, ERK1/2, p38MAPK and JNK in the GnRH-induced LH β expression has been reported (Liu *et al.*, 2002a).

Several studies have reported the involvement of ERK1/2 in GnRH-induced LH β expression (Call and Wolfe, 1999; Harris *et al.*, 2002; Liu *et al.*, 2002a; Maudsley *et al.*, 2007; Yamada *et al.*, 2004), while others have found no role for ERK1/2 in the transcriptional regulation of the *LH β* gene by GnRH (Weck *et al.*, 1998; Yokoi *et al.*, 2000). In GnRH-induced LH β expression pathway, ERK1/2 activation is mediated by coupling to $G\alpha_q$ proteins (Liu *et al.*, 2002b). In addition, elevated levels of LH β protein expression has been demonstrated with over-expression of a constitutively active $G\alpha_q$ construct, while expression of inhibitory peptides of $G\alpha_q$ and $G\alpha_s$, inhibited the GnRH-induced ERK1/2 activation and GnRH-stimulated LH β production (Liu *et al.*, 2002b). A more specific role for ERK1/2 in the GnRH-induced LH β expression was reported in a study conducted by Maudsley *et al.* (2007). The formation of a proline-rich tyrosine kinase 2 (Pyk2)-dependent multi-protein complex was demonstrated following GnRH-R activation. This complex consists of c-Src, Grb2 and mSOS, which mediates ERK1/2 translocation into the

nucleus in L β T2 cells. Activated ERK1/2 regulates LH β expression via the regulation of *Egr1* expression. The *Egr1* promoter is also activated by p38MAPK, but not by JNK (Maudsley *et al.*, 2007). In addition, the involvement of JNK in the stimulation of LH β production has also been demonstrated (Harris *et al.*, 2002; Yokoi *et al.*, 2000).

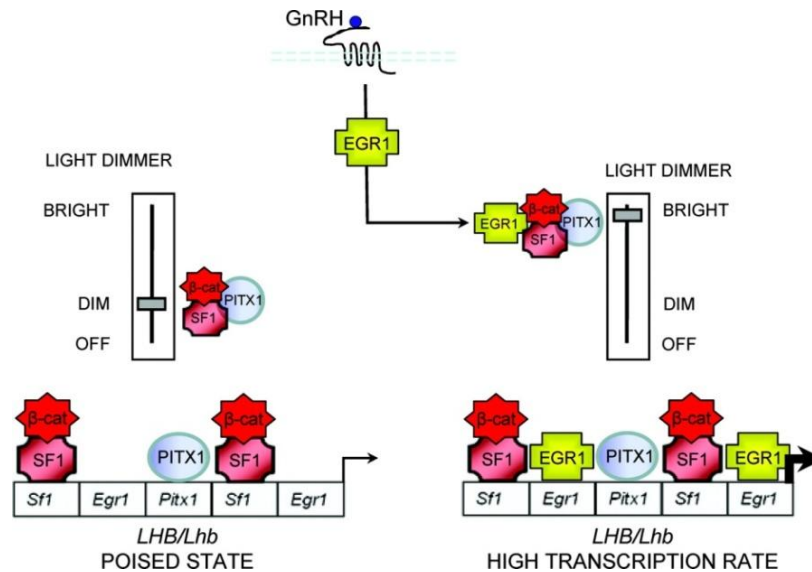


Figure 1-7: A potential mechanism describing an involvement of β -catenin in GnRH-induced LH β gene expression.

In the absence of GnRH treatment, *Egr1* protein levels are extremely low, and a complex comprising of β -catenin, SF1 and Pitx1 maintains LH β gene expression at basal levels. GnRH treatment increases *Egr1* levels, and *Egr1* forms a complex with β -catenin, SF1 and Pitx1 increasing the transcriptional activity of LH β promoter. Figure reproduced from Salisbury *et al.*, 2008.

The role of PKC in LH β production has also been examined, with conflicting results being reported. In particular, a positive role of PKC in this pathway has been demonstrated in several studies (Call and Wolfe, 1999; Harris *et al.*, 2002; Saunders *et al.*, 1998; Vasilyev *et al.*, 2002), while one study has reported that PKC is not involved in this pathway (Yokoi *et al.*, 2000).

The role of calcium (Ca^{2+}) in the GnRH-induced LH β expression pathway has also been reported. Weck *et al.* (1998) demonstrated that GnRH regulation of LH β expression is mediated by an elevation of Ca^{2+} levels (Weck *et al.*, 1998), however a number of other studies have reported conflicting results (Call and Wolfe, 1999; Saunders *et al.*, 1998; Yokoi *et al.*, 2000) The opposing results regarding the involvement of the above mediators on GnRH-induced LH β expression can be explained by the use of different cell types and promoter constructs used in each study. All the above signalling pathways are summarised in **Figure 1-8**.

Finally, the role of other receptors in the regulation of LH β expression has been assessed (Naor *et al.*, 2007). For example, reciprocal stimulation of GnRH-R and prostaglandin receptors regulate LH β expression. In particular, activation of prostaglandin receptors via prostaglandin F $_{2\alpha}$ (PGF $_{2\alpha}$) reduced GnRH-R-stimulated LH β expression in L β T2 cells (Naor *et al.*, 2007). While many factors have been reported to be involved in GnRH-induced LH β expression in gonadotropes, the mechanisms, signalling pathways and mediators involved are not yet completely understood (Thackray *et al.*, 2010).

1.5.6 Pulsatile regulation of LH and FSH expression by GnRH

The frequency of GnRH release influences the differential regulation of LH and FSH expression in gonadotropes of the pituitary. Low GnRH pulses (>120-240min) favour the expression of FSH, while high pulses (<1hour) favour the expression of LH (Ferris and Shupnik, 2006). However, to date it is not clear how the pulses frequency of GnRH differentially regulates the expression of LH and FSH. The

mechanism by which this occurs appears to include signalling pathways that are stimulated or suppressed, transcription factors that are bound and released from the promoter of these genes, or proteins (such as transcription factors) that are degraded (ubiquitinated) during pulsative GnRH stimulation (Ferris and Shupnik, 2006; Tsutsumi and Webster, 2009). Two recent studies by Armstrong *et al.* (2009 and 2010) examined the possible mediators that could mediate the pulsatile effect of GnRH on gonadotropin expression. The involvement of ERK in FSH β - and Egr1-promoter luciferase activity (Armstrong *et al.*, 2010), and of Ca⁺²/calmodulin/calcineurin/NFAT pathways on LH β -, FSH β - and α GSU-promoter luciferase activity (Armstrong *et al.*, 2009) following pulsatile GnRH treatment were demonstrated.

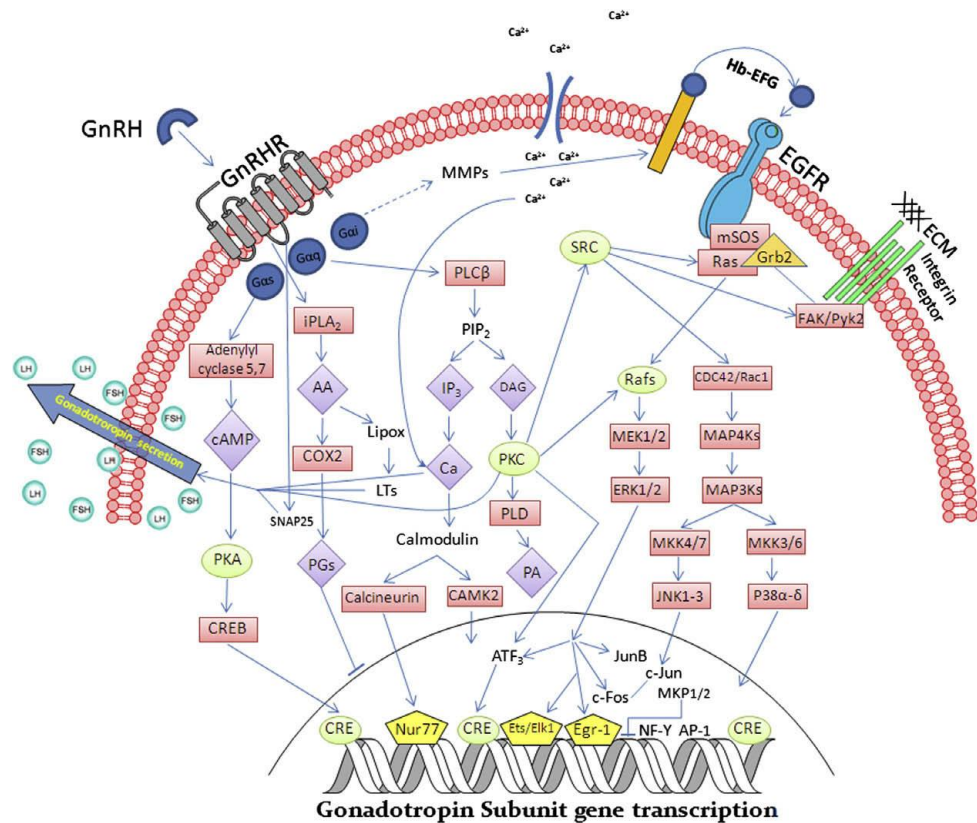


Figure 1-8: Schematic representation of the signalling pathways initiated by GnRH-R or EGFR resulting in the synthesis and secretion of the gonadotropins. (PKA: Protein Kinase A, AA: AA, arachidonic acid, PLD, phospholipase D, iPLA₂:calcium-independent phospholipase A₂, COX2: Cyclooxygenase-2, SNAP25: Synaptosomal-associated protein 25, PGs: Prostaglandins, PLCβ: Phospholipase beta, MMPs: Matrix metalloproteinases, Hb-EFG: Heparin-binding EGF-like growth factor, ECM: extracellular matrix, Ras: RA Sarcoma, Grb2: Growth factor receptor-bound protein 2, CAMK2: Calcium/calmodulin-dependent Protein Kinase II, CDC42: Cell division control protein 42 homolog, Rac1: Ras-related C3 botulinum toxin substrate 1, Elk1: Ets-like transcription factor-1, MEK1/2: MAP Kinase Kinase, MAP3Ks: MAP kinase kinase kinase, CRE: cAMP response elements Figure reproduced from Naor., 2009.

1.6 FORKHEAD TRANSCRIPTION FACTORS

The FOXO family of transcription factors consists of four members, three of which are mammalian, namely FOXO1 (FKHR), FOXO3a (FKHRL1) and FOXO4 (AFX) (Tran *et al.*, 2002). FOXOs are of great interest because they have been implicated in

a number of biological processes, including cell-fate decision and tumour suppression in a variety of cancers (Fu and Tindall, 2008). FOXOs are generally found in their active form in the nucleus where they bind to the promoters of genes and modulate their transcriptional activity. FOXOs also constitute an important downstream target of the PI3K/Akt and IKK signalling pathways which regulate FOXO localisation and transcriptional activity (Hu *et al.*, 2004; van der Horst and Burgering, 2007). This regulation is mainly determined by post-translational modification such as phosphorylation, acetylation and ubiquitination. FOXO regulation is also determined by interaction with co-factors such as β -catenin (Burgering, 2008; Gardner *et al.*, 2010; Obsil and Obsilova, 2008).

1.6.1 FOXO structure and function

Each member of the FOXO family consists of four domains; a highly conserved DNA binding domain (DBD), a nuclear localization signal (NLS) domain, a nuclear export sequence (NES) and a C-terminal trans-activation domain (Obsil and Obsilova, 2008) (**Fig. 1-9**).

FOXO1 and FOXO3a proteins contain approximately 650 amino-acids, and FOXO4 contains about 500 amino-acids. Analysis of FOXO sequences revealed that several regions on FOXO proteins show high levels of similarity (Obsil and Obsilova, 2008).

The DNA binding domain, also called “the winged helix/forkhead domain”, contains 100 amino acids, and its structure constitutes three α -helices, three small β -strands, one wing and the C-terminal α -helix (Obsil and Obsilova, 2008). FOXOs bind via their DBDs to the 5'-GTAAACAA-3' consensus sequence. Tsai *et al.* (2007) have

demonstrated that the highly conserved helix H3 among the FOXO DBDs acts as the main DNA recognition site, while other regions, such as the C-terminal region, the N-terminal region and the wing W1 region are also important for DNA-DBD binding specificity and stability (Tsai *et al.*, 2007) (**Fig. 1-10**).

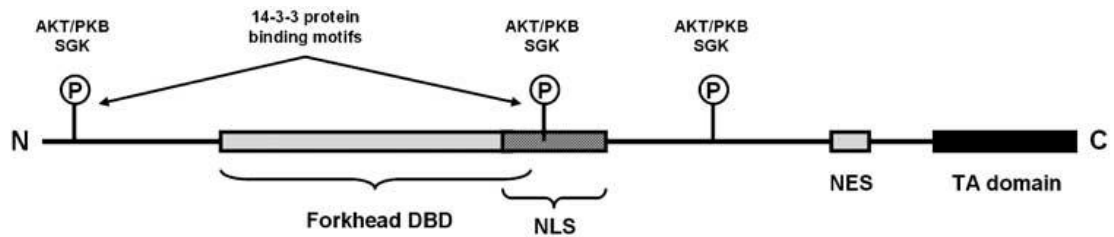


Figure 1-9: Structure of FOXO proteins.

Conserved FOXO domains are shown. Figure reproduced from Obsil and Obsilova, 2008.

The NES, which is involved in the translocation of FOXOs from the nucleus to the cytoplasm, consists of a leucine-rich sequence. 14-3-3, a 30kDa protein with a U-shaped structure, is member of the cellular chaperones that interact with phosphorylated residues on proteins, and shuttles them out of the nucleus. 14-3-3 recognises and binds to Akt-phosphorylated serine and threonine residues of FOXOs resulting in the translocation of FOXO/14-3-3 complex into the cytoplasm (Van Der Heide *et al.*, 2004).

The NLS domain contains another Akt phosphorylation site. Phosphorylation of this site also allows binding of 14-3-3 protein. By masking the NLS domain, 14-3-3 prevents FOXO re-import from the cytoplasm to the nucleus. The localisation and transcriptional activity of FOXOs are also affected by exposure of their domains to acetylation and ubiquitination (discussed in Sections 1.6.2.2 and 1.6.2.3) (Obsil and Obsilova, 2008).

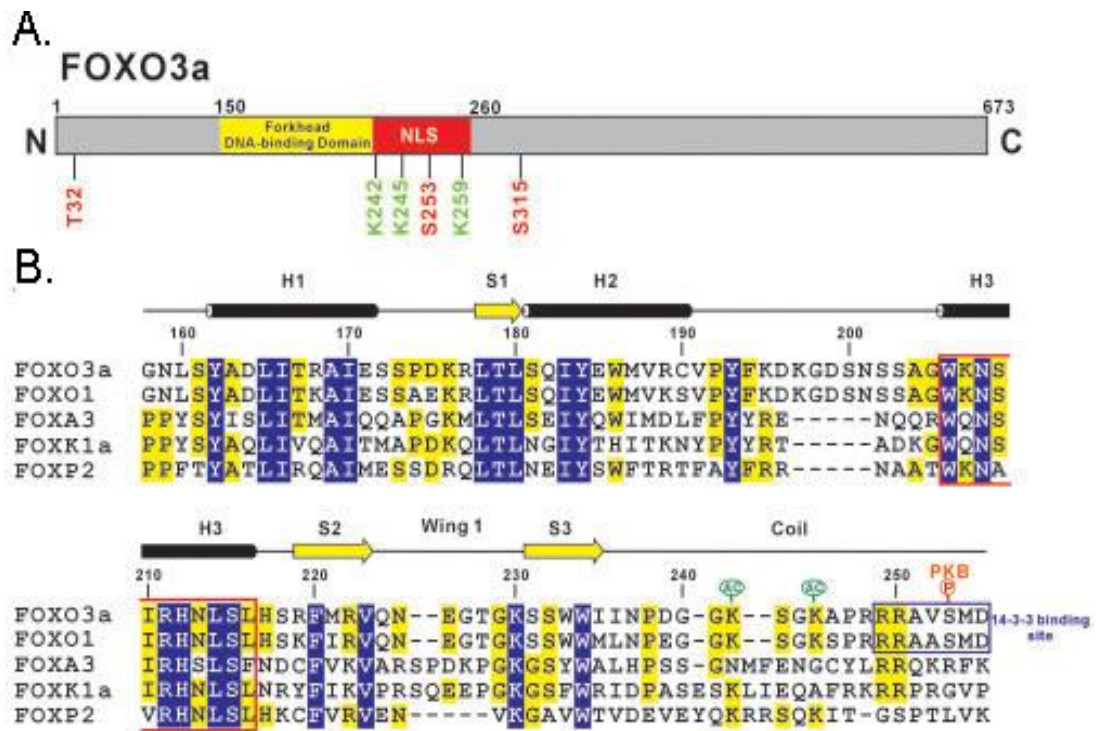


Figure 1-10: Structure of FOXO3a and sequence alignment with other FOX proteins.

(A) Akt/PKB phosphorylation (red) and acetylation (green) sites in FOXO3a. The DBD is coloured yellow. (B) Sequence alignment of the DBDs of several FOX proteins (α -helices, black, β -strands, yellow). Figures reproduced from Tsai *et al.*, 2007.

1.6.2 Regulation of FOXO transcription factors

1.6.2.1 Phosphorylation

FOXO transcriptional activity is regulated by phosphorylation via a variety of signalling pathways. The main regulators of FOXO phosphorylation are the PI3K/Akt and IKK signalling pathways. However, other kinases such as the protein kinase casein kinase 1 (CK1), serum and glucocorticoid inducible kinase (SGK), and dual-specificity regulated kinase 1A (DYRK1A) can also phosphorylate FOXOs (Van Der Heide *et al.*, 2004). The phosphorylation, acetylation and ubiquitination sites on FOXO1/3a/4 are shown in **Figure 1-11**. In this section, the role of the PI3K/Akt and IKK signalling pathways in FOXO phosphorylation will be reviewed.

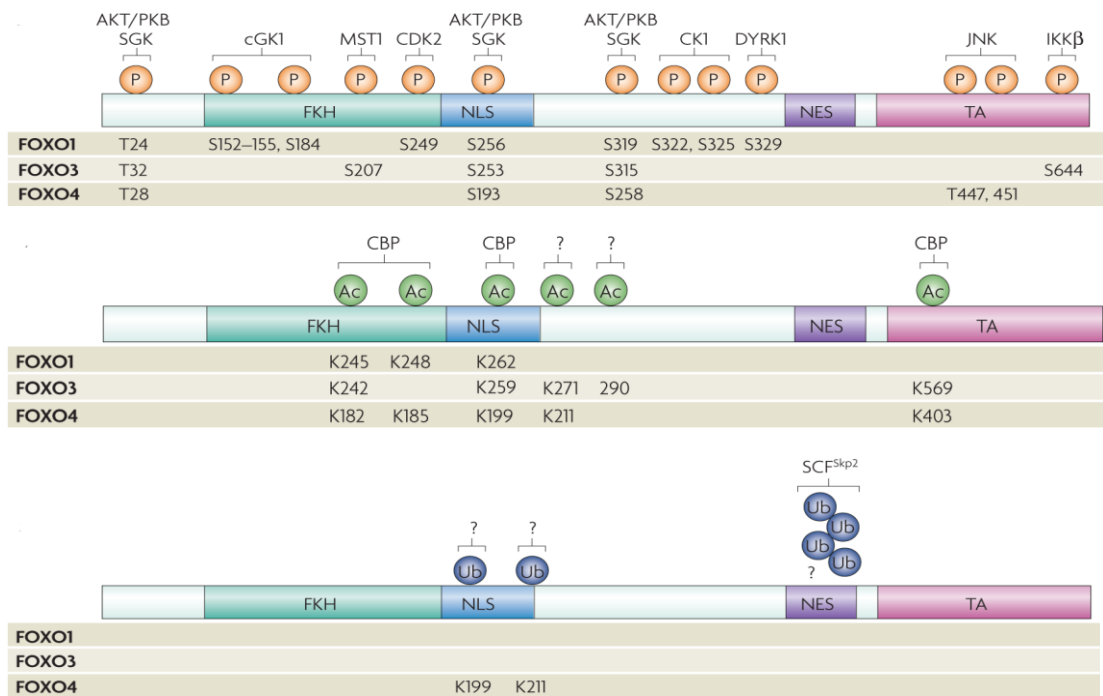


Figure 1-11: Post-translational modifications of FOXOs.

The phosphorylation (P: orange), acetylation (Ac: green) and ubiquitination (Ub: blue) sites on FOXO1/3a/4 transcription factors (see for details sections 1.3.3.1, 2 and 3). (SGK: serum- and glucocorticoid-inducible kinase, cGKI: cGMP-activated kinase, MST1: mammalian sterile 20-like kinase 1, DYRK1: Dual specificity tyrosine-phosphorylation-regulated kinase 1, CBP: (CREB)-binding protein. Figure reproduced from van der Horst *et al.*, 2007.

1.6.2.1.1 *The PI3K/Akt signalling pathway*

FOXOs are generally found in their active form in the nucleus where they bind to promoters and modulate expression. Changes in FOXO transcriptional activity are induced by their phosphorylation status at specific sites. In particular, FOXOs constitute an important downstream target of the PI3K/Akt signalling pathway. Akt phosphorylates FOXOs at specific amino acids (**Fig. 1-11**), which leads to their retention in the cytoplasm, and therefore a significant reduction in their transcriptional activity (van der Horst and Burgering, 2007). The transcriptional

activity induced by a FOXO3a triple mutant, in which the three Akt-phosphorylation sites, at Ser²⁵³, Ser³¹⁵ and Thr³² have been mutated to Alanine residues, was significantly higher when compared with the transcriptional activity induced by wild type FOXO3a (Brunet *et al.*, 1999). This clearly indicates that these Akt phosphorylation sites are responsible for the translocation of FOXOs from the nucleus to the cytoplasmic, thus reducing their transcription activity (Tran *et al.*, 2002).

Each of the Akt phosphorylation sites has a specific role in how it affects FOXO function (Obsil and Obsilova, 2008). Active Akt phosphorylates FOXOs thereby generating two consensus binding sites for the 14-3-3 chaperone protein. The binding of 14-3-3 to FOXOs results in their release from the DNA. However, binding of FOXOs to 14-3-3 is not sufficient for their translocation to cytoplasm. FOXOs also contain the NLS region which is responsible for their localisation to the nucleus. Thus, Akt phosphorylation may regulate the translocation of FOXOs to the cytoplasm by disrupting the function of the NLS region due to phosphorylation at specific sites (**Fig. 1-12**) (Huang and Tindall, 2007; Tran *et al.*, 2003).

Several kinases including Akt, PKC and JNK also phosphorylate 14-3-3. Phosphorylation of 14-3-3 by JNK is able to release FOXO from 14-3-3, even when FOXO3a is phosphorylated by Akt. The translocation of FOXOs from the cytoplasm to the nucleus is mediated by their de-phosphorylation at the Akt sites, however the protein phosphatases that are responsible for this have not yet been identified (Calnan and Brunet, 2008). The localisation and consequently function of FOXOs

are therefore modulated by two different mechanisms which are opposed to each other (Obsil and Obsilova, 2008; Sunayama *et al.*, 2005).

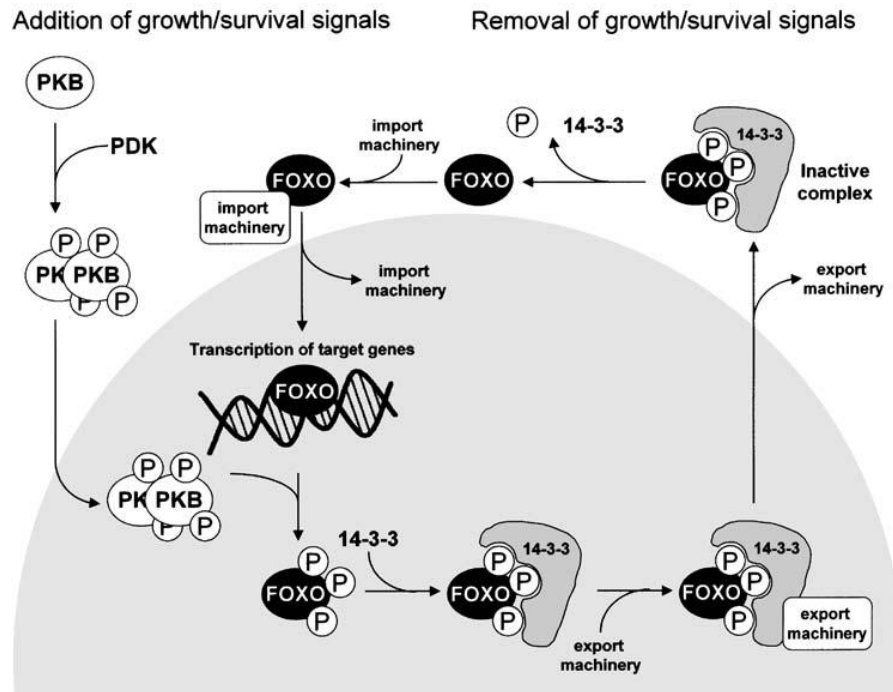


Figure 1-12: FOXO movement between nucleus and cytoplasm in response to growth signals. The mechanism by which FOXOs translocate from the nucleus to the cytoplasm following Akt phosphorylation. 14-3-3 proteins have been postulated to play a direct role in nuclear export of FOXO. Following FOXO de-phosphorylation, 14-3-3 is released and FOXO translocates into the nucleus. Figure reproduced from Birkenkamp and Coffey, 2003.

1.6.2.1.2 *The IKK signalling pathway*

FOXO3a can also be phosphorylated by the IKK (Hu *et al.*, 2004). IKK belongs to the IKK/NF- κ B pathway which appears to be involved in the regulation of FOXO3a activity. Hu *et al.* (2004) demonstrated that FOXO3a was found in the cytoplasm in breast tumour cells in the absence of active Akt. This was shown to occur due to FOXO3a phosphorylation by IKK β at Ser⁶⁴⁴, resulting its translocation to the cytoplasm, and reduction in its transcriptional activity (Hu *et al.*, 2004). A more

recent study, conducted by Chapuis *et al.* (2010) has also demonstrated IKK-dependent FOXO3a nuclear exclusion in acute myeloid leukemia (AML) cells. FOXO3a nuclear localisation, which was also independent of Akt phosphorylation, resulted in its transcriptional inactivation (Chapuis *et al.*, 2010).

IKK is phosphorylated by both the PI3K/Akt and PI3K/PDK1 signalling pathways (**Fig. 1-13**) (Salminen and Kaarniranta, 2010; Tanaka *et al.*, 2005). In particular, Akt activates the IKK α kinase by phosphorylating it at Thr²³ inducing NF- κ B activity (Ozes *et al.*, 1999), while PDK1, which is activated by Akt, phosphorylates IKK β at Ser¹⁸¹, inducing the nuclear translocation of NF- κ B complexes, resulting in the expression of NF- κ B-dependent genes (Tanaka *et al.*, 2005).

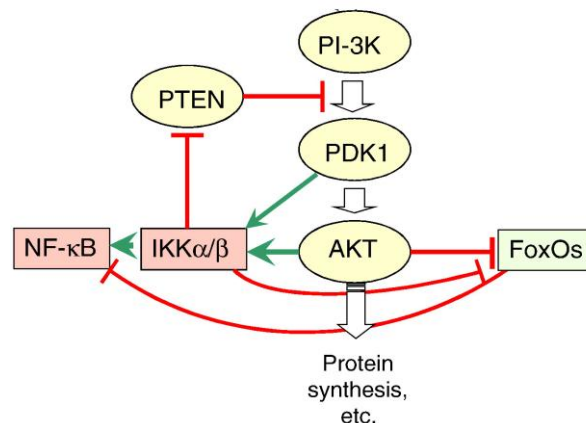


Figure 1-13: The PI3K-PDK1-IKK-FOXO and PI3K-PDK1-Akt-FOXO signalling pathways.

FOXO activity is inhibited by the PI3K/PDK1/Akt, or PI3K/PDK1/IKK α/β signalling pathways. FOXOs inhibit NF- κ B signalling. Figure reproduced from Salminen and Kaarniranta, 2010.

1.6.2.2 Acetylation

FOXO function and translocation is also regulated by acetylation by histone acetyltransferases such as SIRT1. SIRT1 is an NAD-dependent de-acetylase that de-

acetylates a variety of proteins (Giannakou and Partridge, 2004). Acetylation of FOXOs by SIRT1 leads to their nuclear translocation. Thus, SIRT1 modulates FOXO transcriptional activity. Interestingly, the interaction of FOXO and SIRT1 appears to require the presence of 14-3-3. Indeed, a *Caenorhabditis elegans* (*C. elegans*) 14-3-3 protein mediates the interaction of SIR-2.1 (the *C. elegans* ortholog of SIRT1) with DAF-16/FOXO (Wang *et al.*, 2006). There are conflicting reports with regard to whether de-acetylation of FOXO by SIRT1 increases or decreases FOXO activity (Obsil and Obsilova, 2008). The majority of published studies suggest that FOXO de-acetylation by SIRT1 enhances its transcriptional activity, and increased FOXO-mediated apoptosis or cell-cycle arrest (Daitoku *et al.*, 2004; van der Horst *et al.*, 2004). In contrast, de-acetylation has also been reported to suppress the transcriptional activity of the three mammalian FOXOs (FOXO1/3a/4) (Motta *et al.*, 2004). Overall, although the majority of studies indicate that FOXO de-acetylation enhances its transcriptional activity, SIRT1 has been proposed to have a dual role in FOXO activity (Daitoku *et al.*, 2004), and it is therefore apparent that the exact role of FOXO acetylation/de-acetylation requires further investigation (Obsil and Obsilova, 2008).

1.6.2.3 *Ubiquitination*

Another post-translational modification that regulates the transcription of FOXO transcription factors is ubiquitination. Ubiquitination is an enzymatic, protein post-translational modification process which leads to protein degradation by proteosomes. A polyubiquitin chain is covalently linked to the protein, and it enters the proteolytic chamber of the proteasome where it is degraded (Sorokin *et al.*,

2009). The level of FOXO protein in mammalian cells has been demonstrated to be regulated by PI3K/Akt-dependent poly-ubiquitylation and proteasome degradation processes (Matsuzaki *et al.*, 2003; Plas and Thompson, 2003). For example, in cells treated with insulin, FOXO1 levels are reduced due to mono-ubiquitination via a Skp-ubiquitin ligase (Vogt *et al.*, 2005). In addition, FOXO3a phosphorylation at Ser⁶⁴⁴ by IKK leads to its translocation to the cytoplasm where it is ubiquitinated and degraded (Hu *et al.*, 2004). More recently, the MDM2, an E3 ubiquitin ligase, was demonstrated to bind to FOXO1 and FOXO3a and promotes their ubiquitination and degradation (Fu *et al.*, 2009).

The signalling pathways that regulate FOXO movement between nucleus and cytoplasm, and FOXO ubiquitination and degradation are still not well understood. It appears that there are competitive signalling pathways that determine the activation and inactivation of FOXO transcriptional activity. Reduction of their activity via ubiquitination/proteasome-mediated degradation appears to be the mechanism that mediates their inactivation (Vogt *et al.*, 2005).

1.6.2.4 FOXO co-factors

FOXOs interact with a variety of co-factors which are involved in regulating their transcriptional activity. For example, FOXOs interact with the Runt domain-containing transcription factor (RUNX3), SMAD3 and SMAD4, STAT5, androgen receptor, estrogen receptor (ER α) and β -catenin, resulting in the regulation of FOXO trans-activation potential (Reviewed in Calnan and Brunet. 2008). In this section, only the role of β -catenin in regulating FOXO activity will be reviewed.

The first indication that FOXOs bind and interact with accessories proteins came from a study by Ramaswamy *et al.* (2002), which demonstrated that a mutant of FOXO1, in which the DBD was disrupted, was still able to effectively regulate expression of a specific number of the genes that wild-type FOXO could regulate (Ramaswamy *et al.*, 2002). An active interaction between FOXO3a and FOXO4 with β -catenin that enhances the transcriptional activity of FOXO4 has been demonstrated (Essers *et al.*, 2005). Interestingly, this interaction has a dual role (Hooigeboom *et al.*, 2008). Binding of β -catenin to FOXOs enhance their transcriptional activity and inhibits the T cell factor (TCF) transcriptional activity, since FOXO competes with TCF for binding to β -catenin. Interaction between FOXO4 and β -catenin, and competition between FOXO4 and TCF for β -catenin binding has also been observed in a recent study in various colon cell lines (Kwon *et al.*, 2010). To date, an interaction of β -catenin with a number of transcription factors, and among them the FOXOs, has been demonstrated, with β -catenin/FOXO interaction resulting in enhanced FOXO activity (Hooigeboom and Burgering, 2009).

1.6.2.5 FOXO auto-regulation

FOXO transcriptional activity is regulated by growth factors via the regulatory mechanisms described above. However, growth factors also regulate FOXO activity by targeting *FOXO* gene expression (Essaghir *et al.*, 2009). Although the mechanism for the regulation of *FOXO* gene expression is still not clear, transcription factors such as E2F1 has been demonstrated to induce the expression of endogenous FOXO1 and FOXO3a (Nowak *et al.*, 2007), while growth factors such as PDGF and FGF

decrease the expression of FOXO1, FOXO3a and FOXO4 (Essaghir *et al.*, 2009). In addition, FOXO binding sites on the *FOXO1* promoter have been identified (Essaghir *et al.*, 2009). In particular, FOXO3a regulates the mRNA expression of *FOXO1* and *FOXO4*, and FOXO1 is able to regulate its own expression (Essaghir *et al.*, 2009).

1.6.3 FOXO-target genes

FOXOs bind the promoters of a variety of genes that are involved in apoptosis (*FasL*, *BIM*), cell-cycle regulation (*p21Cip1*, *p27Kip1*) and oxidative stress resistance (*GADD45*) (**Table 1-2**). The role of FOXOs in cell function, and several of the FOXO-target genes that mediate these functions will be reviewed in this section.

In mammalian cells, the apoptotic response can be mediated through caspase activation, via either an intrinsic or extrinsic pathway, depending on the origin of the stimuli. There are two types of caspases; the initiator and the effectors caspases (Riedl and Shi, 2004). Following external or internal death stimuli, initiator caspases are activated, and in turn activate the effectors caspases. The effector caspases are then responsible for the proteolytic cleavage of a broad spectrum of cellular targets, which subsequently leads to apoptosis (Riedl and Shi, 2004). FOXO activation, which leads to nuclear localisation, has been shown to promote apoptosis and cell-growth inhibition in several cell lines including cerebellar granule neurons, Jurkat T lymphocytes, CCL39 fibroblasts and mouse pre-B cell line (Brunet *et al.*, 1999; Dijkers *et al.*, 2002; Roy *et al.*, 2010; Srinivasan *et al.*, 2005; Zheng *et al.*, 2002). In contrast, activation of FOXO3a has been demonstrated to be required for neuron

survival (Srinivasan *et al.*, 2005). However, the majority of studies have reported that FOXOs mediate apoptosis via regulating the expression of a number of apoptotic genes, including *BIM* and *FasL* (Dijkers *et al.*, 2000; Gilley *et al.*, 2003; Kops *et al.*, 2002a; Peng *et al.*, 2010; Sunter *et al.*, 2003). These studies are summarised in **Tables 1.3 and 1.4.**

	Gene	Function	References
Apoptosis	BIM	Pro-apoptotic activity	(O'Connor <i>et al.</i> , 1998)
Apoptosis	FasL	Triggers apoptosis via Fas receptor	(Imai <i>et al.</i> , 1998b)
G1 cell-cycle arrest	p21Cip1	Cell-cycle arrest at G1/S	(Seoane <i>et al.</i> , 2004)
G1 cell-cycle arrest	p27Kip1	Cell-cycle arrest at G1/S	(Nakamura <i>et al.</i> , 2000)
G2 cell-cycle arrest	GADD45	Mediates G2/M arrest	(Tran <i>et al.</i> , 2002)
DNA repair	GADD45	Mediates DNA repair	(Tran <i>et al.</i> , 2002)

Table 1-2: FOXO-target genes and their functions.

FOXOs bind to and regulate the promoters of genes that are involved in cell proliferation, either by regulating cell-cycle progression or by promoting apoptosis.

1.6.3.1 *BIM*

BIM is a pro-apoptotic member of the Bcl-2 family which includes anti- and pro-apoptotic factors (O'Connor *et al.*, 1998). *BIM* contains a BH3 domain which allows the formation of hetero-dimers with other anti-apoptotic Bcl-2 members, neutralising their function (Dijkers *et al.*, 2000). Following activation, *BIM* induces the release of apoptogenic factors (cytochrome c, Smac/Diablo homolog, Omi) from the mitochondria, which in turn, activate caspase 9. Caspase 9 then triggers a cascade of caspase activation leading to apoptosis (**Fig. 1-14**) (Fesik and Shi, 2001). *BIM*

therefore participates in the intrinsic apoptotic pathway that is mediated by mitochondria (Riedl and Shi, 2004).

1.6.3.2 *FasL*

The pro-apoptotic *FasL* gene, which participates in the extrinsic apoptotic pathway, is a trans-membrane protein that induces apoptosis via the binding to the Fas receptor (Brunet *et al.*, 1999; Fu and Tindall, 2008) (**Fig. 1-14**). This binding recruits further cytoplasmic factors, including the FAS-associated death domain (FADD) and caspase 8, forming a death-inducing signalling complex. Formation of this complex leads to the activation of caspase 8, which then cleaves and activates caspase 3.

1.6.3.3 *p21Cip1 and p27Kip1*

FOXOs are involved in cell-cycle regulation, since over-expression of FOXO1 and FOXO3a induce cell-cycle arrest at the G1/S and G2 phases (Dijkers *et al.*, 2000; Medema *et al.*, 2000; Ramaswamy *et al.*, 2002; Schmidt *et al.*, 2002). FOXOs mediate cell-cycle progression via the regulation of cyclin dependent kinases (Cdks). The Cdk family includes the cell-cycle activators, cyclin D1 and cyclin D2, that promote cell-cycle progress, and which inhibit the cell-cycle inhibitors, *p27Kip1* and *p21Cip1* which block cell-cycle progress (**Table 1-5**). FOXOs regulate cell-cycle arrest at G1/S either by repressing cell-cycle activators, cyclin D1 and cyclin D2 or by activating the cell-cycle inhibitors, *p21Cip1* and *p27Kip1* (Dijkers *et al.*, 2000; Katayama *et al.*, 2008; Medema *et al.*, 2000; Nakamura *et al.*, 2000).

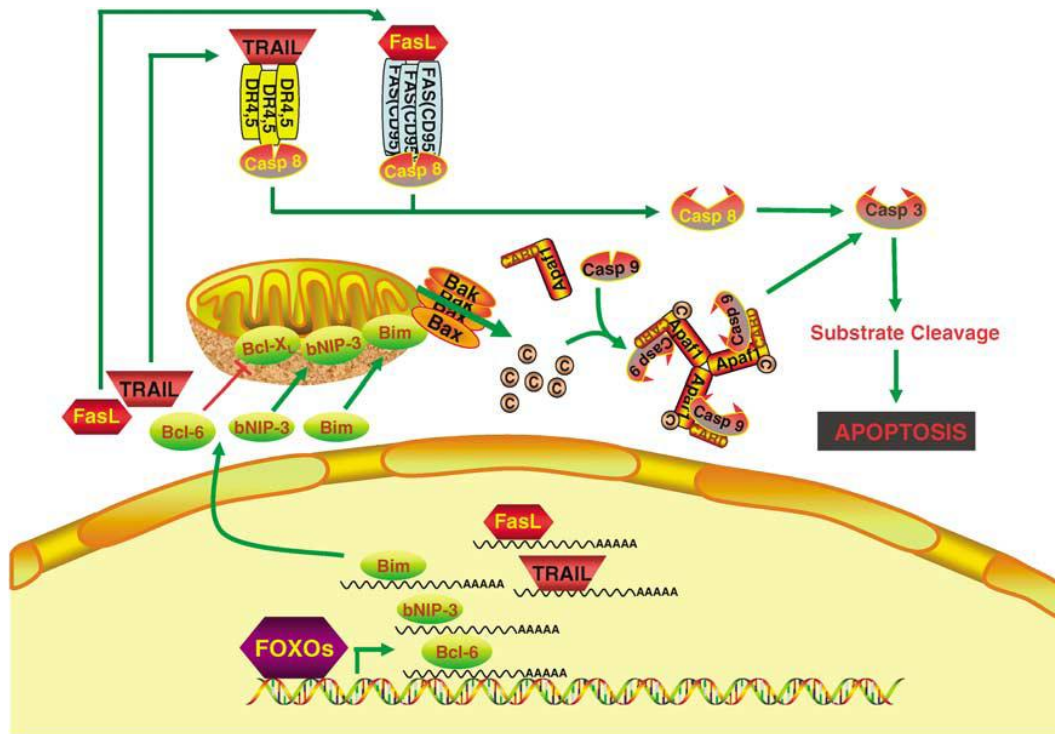


Figure 1-14: FOXO-induced apoptosis by FasL and BIM.

FOXOs bind to the *FasL* promoter and up-regulates its expression. FasL then binds to the Fas receptor which leads to the formation of a complex which includes FADD and caspase 8. Activated caspase 8 activates caspase 3 and subsequently induces apoptosis. FOXOs also bind to the promoter of the *BIM* gene and up-regulate its expression. This leads to increased mitochondria permeability and several proteins are released into the cytoplasm, including cytochrome c. Cytochrome c binds and activates protein apoptotic protease activating factor 1 (Apaf1), and forms the apoptosome which mediates the activation of caspase-9. (TRAIL: [TNF-related apoptosis inducing ligand, DR4,5: Death Receptor 4,5, Bcl-X_L: B-cell lymphoma-X_L, bNIP-3: BCL2/adenovirus E1B 19 kDa protein-interacting protein 3, Apaf1: Apoptotic protease activating factor 1, CARD: caspase recruitment domain) Figure reproduced from Fu and Tindall, 2008.

FOXO	Cell type	Effect	In response to	Biological role	Reference
FOXO3a	Ba/F3 cells	Increases BIM mRNA level and protein expression	IL-3 ⁽¹⁾ deprivation, BIM expression increased steadily	In lymphocytes, BIM is the dominant transducer of death signals by controlling its expression level via cytokine-mediated regulation of forkhead transcription factors	(Dijkers <i>et al.</i> , 2000)
FOXO3a	Sympathetic neurons cells	FOXO3a directly activates the BIM promoter	NGF ⁽²⁾ , causes FOXO3a inactivation via the PI3K/Akt signalling pathway and therefore reduction of BIM mRNA expression	FOXO transcriptional activity contributes to NGF withdrawal-induced death in sympathetic neurons	(Gilley <i>et al.</i> , 2003)
FOXO3a	MCF-7 and MDA MB-231 cells	FOXO3a directly activates the BIM promoter in MCF-7 but has a minor effect in MDA MB-231	Paclitaxel, there is an up-regulation of FOXO3a activation which can result in increased levels of BIM mRNA and protein	Paclitaxel-induced FOXO3a transcriptional activity increases BIM expression which could result in apoptosis in MCF-7 but not in MDA-231 breast cancer cells	(Sunters <i>et al.</i> , 2003)
FOXO3a	DL23 and Ba/F3 cells	Activation of FOXO3a in DL23 cells does not up-regulate BIM expression, but does in Ba/F3 cells	No data	No data	(Kops <i>et al.</i> , 2002b)
FOXO3a	Cells from the neural region of zebrafish embryo	Loss-of-function of FOXO3a induces BIM expression	No data	Loss-of-function of FOXO3a causes an increase of apoptotic cells in the neural region of zebrafish embryo	(Peng <i>et al.</i> , 2010)

Table 1-3: FOXO3a and FOXO1 regulate the expression of *BIM* gene in different cellular environments.

This table summarises the effect of FOXO over-expression on BIM expression in different cell lines. The effect on FOXO transcriptional activity in response to different stimuli, and the biological significance of FOXOs in different cellular environments are also shown.

FOXO	Cell type	Effect	In response to	Biological role	Reference
FOXO3a	Post-mitotic neurons and PC12 cells	FOXO3a induces FasL expression	IGF1 ⁽³⁾ , the expression of the FasL-promoter luciferase reporter gene was significantly reduced by FOXO3a, via of PI3K/Akt signalling pathway	FOXO3a induces apoptosis by induction of FasL pathway in CCL39 cells (little effect on Jurkat cells)	(Brunet <i>et al.</i> , 1999)
FOXO1	Glioma cells	FOXO1 induces FasL expression	CsA ⁽⁴⁾ , expression of FasL-promoter luciferase reporter gene may be regulated by FOXO1, via PI3K/Akt signalling pathway	Inhibition of Akt signaling may be critical for Forkhead-dependent up-regulation of FasL expression in glioma cells, but activation of other transcription factors is required to induce FasL expression and cell death	(Ciechomska <i>et al.</i> , 2003)
FOXO3a	293T cells	FOXO3a induces FasL promoter activity	No data	IKK-dependent repression of FOXO3a can promote cell growth and tumorigenesis	(Hu <i>et al.</i> , 2004)

⁽¹⁾IL-3 : Interleukin 3

⁽²⁾NGF : Nerve growth factor

⁽³⁾IGF1 : Insulin-like growth factor 1

⁽⁴⁾CsA : cyclosporin A

Table 1-4: FOXO3a and FOXO1 regulate the expression of *FasL* gene in different cellular environments.

This table summarises the effect of FOXO over-expression on FasL expression in different cell lines. The effect on FOXO transcriptional activity in response to different stimuli, and the biological significance of FOXOs in different cellular environments are also shown.

FOXO	Cell type	Effect	In response to	Biological role	Reference
FOXO1	786-O cells(PTE N-null tumor cell)	FOXO1 Increases p27Kip1 mRNA level and prolonged the half life of p27Kip1 protein	No data	Low level of p27Kip1 in PTEN-null cells may arise as a consequence of a lack of FOXO1 which may be important for tumorigenesis	(Nakamura <i>et al.</i> , 2000)
FOXO3a	Ba/F3 cells	FOXO3a increases <i>p27Kip1</i> promoter activity	IL-3, there is inhibition of p27Kip1 transcription through PI3K-induced FOXO3a phosphorylation	Repression of p27Kip1 levels through Akt-mediated FOXO3a phosphorylation may be necessary for cytokine-mediated survival and proliferation	(Dijkers <i>et al.</i> , 2000)
FOXO3a	NIH 3T3 cells	FOXO3a increases p27Kip1 expression	No data	No data	(Martinez-Gac <i>et al.</i> , 2004)
FOXO3a	293T	FOXO3a increases <i>p27Kip1</i> promoter activity	No data	No data	(Hu <i>et al.</i> , 2004)
FOXO3a	FRTL5 cells	Nuclear accumulation of FOXO3a increases p27Kip1 expression	Insulin, serum or growth media, the mRNA expression level of <i>p27kip1</i> was significantly reduced (compared with treatment with the starving control medium) TSH ⁽⁵⁾ , mRNA expression of <i>p27kip1</i> did not significantly alter compared with starved controls	No data	(Karger <i>et al.</i> , 2009)

Table 1-5: FOXO3a and FOXO1 regulate the expression of *p27Kip1* gene in different cellular environments.

This table summarises the effect of FOXO over-expression on p27Kip1 expression in different cell lines. The effect on FOXO transcriptional activity in response to different stimuli, and the biological significance of FOXOs in different cellular environments are also shown.

FOXO	Cell type	Effect	In response to:	Biological role	Reference
FOXO3a	Mouse myoblasts C2C12	FOXO3a increases GADD45 expression	oxidative stress, FOXOs increases GADD45 (in HeLa cells)	FOXOs play a key role in the regulation of G2 checkpoint by inducing GADD45 (in HeLa cells)	(Furukawa-Hibi <i>et al.</i> , 2002)
FOXO3a		FOXO3a increases GADD45 expression	No data	No data	(Tran <i>et al.</i> , 2002)
FOXO3a	FRTL-5	Role of FOXO3a-induced <i>GADD45A</i> mRNA expression in Thyroid cancer cells	oxidative stress, activates FOXO3a in thyrocytes with JNK (MAPK8)-mediated nuclear accumulation of FOXO3a and increased expression of GADD45A TSH, there is nuclear accumulation of FOXO3a, together with a decrease in <i>GADD45A</i> mRNA expression insulin, there is a cytoplasmic accumulation of FOXO3a in line with increased expression of GADD45A	No data	(Karger <i>et al.</i> , 2009)

Table 1-6: FOXO3a and FOXO1 regulate the expression of *GADD45* gene in different cellular environments.

This table summarises the effect of FOXO over-expression on GADD45 expression in different cell lines. The effect on FOXO transcriptional activity in response to different stimuli, and the biological significance of FOXOs in different cellular environments are also shown.

⁽⁵⁾TSH : Thyroid-stimulating hormone

1.6.3.4 *GADD45*

FOXO3a also induces expression of the *GADD45* gene (Furukawa-Hibi *et al.*, 2002; Tran *et al.*, 2002). *GADD45* belongs to the family of growth arrest and DNA damage (GADD) proteins which comprises of five members, the *GADD45 α* , *GADD45 β* , *GADD45 γ* , *GADD34* and *GADD153* (Reddy *et al.*, 2008). However, *GADD45 α* (also referred as *GADD45*) is the most well-characterised member, and its induction and subsequent effects will be reviewed in this section.

GADD45 is a 18.4KDa protein which mediates G2/M arrest, DNA repair and apoptosis. Following exposure to UV or ionising radiation, *GADD45* gene expression is induced by p53-independent or p53-dependent mechanisms respectively (Yang *et al.*, 2009). When the *GADD45* promoter is activated by p53, *GADD45* interacts with and activates MTK1, a MAP3K with a unique N-terminal regulatory domain, which in turn activates the JNK and p38MAPK to induce cell-growth inhibition and apoptosis (Sheikh *et al.*, 2000; Yang *et al.*, 2009). In addition, following cellular stress response, *GADD45* gene expression is induced by FOXO3a resulting in the initiation of DNA repair pathways (Tran *et al.*, 2002). *GADD45* also binds other regulatory proteins in order to exert its function. Specifically, p21Cip1 binds to the central region of *GADD45*. The biological significance of this interaction remains unclear, however this interaction suggests a cross-talk between these two important cell-cycle regulators that act at different stages of the cell-cycle (Yang *et al.*, 2009). *GADD45* also binds to Cdc2, and inhibits the activity of Cdc2/Cyclin B1 complex, which results in cell-cycle arrest at G2/M (Taylor and Stark, 2001). Finally, *GADD45* acts as a downstream target of NF- κ B. NF- κ B

activation down-regulates the expression of GADD45, via the regulation of another transcription factor, c-myc. Thus *c-myc* gene expression is activated by NF- κ B, however the mechanism by which c-myc represses *GADD45* gene expression is not yet well understood (Rosemary Siafakas and Richardson, 2009; Yang *et al.*, 2009).

1.6.4 Cell-type specific FOXO activity

Activation of FOXOs mediates different biological effects dependent on intracellular environment (these different biological effects are summarised in **Tables 1-3, 1-4, 1-5** and **1-6**). For example, although activation of FOXOs induces apoptosis in some cell lines (Brunet *et al.*, 1999), they can induce cell-cycle arrest in others (Furukawa-Hibi *et al.*, 2002). In addition, activation of FOXOs differentially effects the regulation of specific target genes in a variety of cell types. For example, FOXO3a activation increases *BIM* mRNA expression in Ba/F3, MCF-7 and MDA MB-231 cell lines (Dijkers *et al.*, 2000; Gilley *et al.*, 2003), whereas decreases *BIM* expression in cells derived from neural region of zebrafish embryo (Peng *et al.*, 2010). A mechanism of gene regulation by FOXO transcription factors has been suggested, which could explain the differences in the effect of FOXOs at specific genes (Ramaswamy *et al.*, 2002). In particular, utilising gene expression profile analysis, FOXOs were found to regulate two types of genes; genes that require FOXO binding, and genes that are regulated independently of FOXO binding to their promoters. In the latter case, FOXOs interact with other transcription factors to modulate their activities. Therefore, the cellular environment is important for determining the functional effect of FOXOs in different cell types (Birkenkamp and Coffey, 2003; Ramaswamy *et al.*, 2002).

1.6.5 The role of FOXOs in cancer progression

FOXO phosphorylation by PI3K/Akt, IKK or ERK signalling pathways, leads to FOXO inactivation by their exclusion from the nucleus, and subsequently their degradation. Inactivation of FOXOs, either by chromosomal deletion of the *FOXO* gene or by cytoplasmic localisation, is associated with a variety of cancers, including breast cancer, prostate cancer and leukemia (Yang and Hung, 2009). Indeed, FOXO3a activation not only promotes apoptosis *in vitro* but also restricts tumour growth *in vivo*, in breast cancer cells (Yang and Hung, 2009). Furthermore, constitutively active FOXO4 reduces tumour size (Greer and Brunet, 2005).

Thus, activation of FOXOs could be a potential anticancer therapeutic strategy. An advantage for targeting FOXO activation is that FOXO3a mutations have not been found in human cancers. Therefore, activation of functional FOXO3a could be sufficient to target tumour suppression. A number of FOXO3a-activating target drugs have been developed, including paclitaxel for breast cancer treatment and KP372-1 for treating acute myeloid leukemia (Ho *et al.*, 2008; Yang and Hung, 2009).

1.6.6 FOXO knock-down mice

Although *in vitro* studies have revealed many functions for FOXOs in diverse cell lines, animal studies have also been conducted in order to further understand the role of the specific FOXO sub-types. FOXO null mice, FOXO knock-down mice targeted in specific tissues, transgenic mice that over-express particular type of FOXO, or mice with disrupted FOXO genes in somatic cells have been generated. The phenotype observed in these FOXO-deficient mice are summarised in **Table 1-7**.

FOXO knock-down	Phenotype	Reference
FOXO1^{-/-} null mice	Embryonic lethal phenotype due to incomplete vascular development	(Nakae <i>et al.</i> , 2002)
FOXO1^{+/-} mice	Has a role in a negative regulator of insulin sensitivity	(Nakae <i>et al.</i> , 2002)
FOXO3a-null mice	Show age-dependent infertility and suppressed follicular activation	(Castrillon <i>et al.</i> , 2003)
FOXO3a-null mice	Increased lympho-proliferation and wide-spread organ inflammation	(Lin <i>et al.</i> , 2004)
FOXO4-null mice	No histological abnormalities and normal in appearance	(Hosaka <i>et al.</i> , 2004)
FOXO1/3/4 deletion	Show metastatic cancer	(Paik <i>et al.</i> , 2007)

Table 1-7: The phenotype observed in FOXO knock-down in mice

1.6.7 FOXO transcription factors and LH

A role for FOXO3a in regulating LH levels was demonstrated *in vivo* in a study conducted by Castrillon *et al* (2003) in which female FOXO3a knock-down mice (FOXO3a^{-/-}) were generated. The FOXO3a^{-/-} mice were viable and showed age-dependent infertility with no histological abnormalities of the pituitary tissues. However, serum LH levels were lower compared to control FOXO3a^{+/+} mice. Therefore, the effect of FOXO3a knock-down with regards to mice infertility was assessed. Since FOXO3a knock-down was not specific to the pituitary, the infertility of FOXO3a^{-/-} mice could be due to the effect of FOXO3a knock-down on ovarian function. However, administration of gonadotropins induced ovulation and restored fertility, suggesting that FOXO3a is not required for gonadotropin responsiveness at the ovary (Castrillon *et al.*, 2003).

Thesis aims and objectives:

In Chapter two, the regulation of FOXO transcriptional activity following GnRH-R activation is investigated. Since the transcriptional activity of FOXO is determined by post-translational modifications and the action of co-factors, such as β -catenin, the role of these post-translational modifications and the involvement of β -catenin are characterised. Finally, the role of FOXOs in targeting the expression of several FOXO-target genes is assessed. In Chapter three, the role of FOXO3a in the GnRH-induced cell-growth inhibition was examined, by targeting genes that are involved in apoptosis and cell proliferation, such as GADD45 and FasL.

In Chapter five, the role of FOXO1 and FOXO3a in GnRH-induced expression of LH β , FSH β and the GnRH-R in gonadotropes is examined. The expression of FOXO3a and FOXO in human pituitary gonadotropes is also examined, and a role for FOXO3a in the regulation of LH β expression in response to GnRH treatment is highlighted. In addition, the regulation of Egr1 expression via a FOXO3a-dependent signalling pathway was demonstrated. Chromatin immunoprecipitation (ChIP) assays were used to assess FOXO3a recruitment to Egr1 and LH β promoters. FOXO3a targets Egr1 expression to, at least in part, indirectly regulate LH β promoter activity. These findings suggest that GnRH regulates LH β -subunit expression through one or more FOXO3a-mediated mechanisms.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MAMMALIAN CELL CULTURE

2.1.1 Cell Media

HEK293 embryonic kidney cells (originally obtained from ATCC) stably expressing the rat Type I GnRH-R (designated SCL60 (Stable Cell Lines)) were generated within our laboratory (Anderson *et al.*, 1995). Cells were maintained in complete medium (as described in the table below) supplemented with G418 (Sigma) (unless otherwise specified, all materials utilised in this study, were supplied from Sigma) and plasmocin (all cell lines were grown at 37°C in a humidified 5% CO₂ atmosphere unless otherwise indicated).

LβT2 mouse gonadotrope cells (obtained from P. Mellon, University of California) were maintained on complete medium. LβT2 cells were grown on Matrigel (BD Biosciences) coated plastic flasks.

WPE1-NB26-3 prostate cancer cells (*WPE1-NB26* cell line originally obtained from ATCC and clone 3 was made by Dr. Kevin Morgan) stably expressing the rat Type I GnRH-R were generated in our laboratory. These cells were maintained in serum-free keratinocyte medium (Invitrogen) (Keratinocyte-SFM containing BPE (bovine pituitary extract) (25µg/ml), rEGF (Rat Epidermal Growth Factor) (0.1-0.2ng/ml) and G418 sulphate (500µg/ml)).

Complete Media:

- Dulbecco's modified Eagles medium (DMEM)
- 10% fetal calf serum
- 2% glutamine
- 1% penicillin (10,000 units/ml) / streptomycin (10,000 units/ml)

Starving Media (Serum Free Media):

- Dulbecco's modified Eagles medium (DMEM)
- 2% glutamine
- 1% penicillin (10,000 units/ml) / streptomycin (10,000 unit/ml)

2.1.2 Sub-culturing of cells

SCL60 and L β T2 cells were grown until they were 80-90% confluent. The medium was removed and cells washed twice with Dulbeccos Phosphate Buffered Saline (PBS). 2ml of 1x trypsin was added to each 162cm² flask and the flask return to the incubator (for 3-5 minutes at 37°C) until the cell monolayer detached. Trypsin was inactivated by adding 8ml of complete medium. The cells then were plated on a 162cm² plate at a ratio of 1:5. Cells were counted in a haemocytometer and were seeded in plates for experiments and then maintained at 37°C.

PWE-1-NB26-3 cells were grown until they were 80-90% confluent. The medium then was removed and washed twice with PBS. 2ml of 1x trypsin was added to each 162cm² flask and the flask returned to the incubator (for 10minutes at 37°C) until the cell monolayer detached. Trypsin was inactivated by adding 8ml (0.1% in PBS) trypsin inhibitor (Invitrogen). The cell suspension was centrifuged at 500 x g for 2 minutes at room temperature. The cell pellet was gently re-suspended in 8ml of serum-free keratinocyte medium containing BPE (25 μ g/ml) and rEGF (0.1-0.2 ng/ml). Then cells were seeded in plates for experiments and then maintained at 37°C.

2.1.3 Cryopreservation of cells

Cells from a confluent (~80% confluent) dish were trypsinised and the trypsin inhibited as described above. Cells were then pelleted at 500 x g for 2 minutes to remove residual trypsin. The resulting pellet was re-suspended in cryoprotectant (10% dimethylsulphoxide (DMSO) (v/v) in fetal calf serum). Cells were transferred to cyrotubes and frozen at -80°C. The tubes were then transferred to liquid nitrogen after 48 hours.

2.1.4 Recovery of frozen cells

Cells were removed from liquid nitrogen storage and thawed rapidly at 37°C. They were re-suspended in complete medium, and then were pelleted at 500 x g for 2 minutes. The cell suspension was added to 9ml of complete medium and seeded into an appropriate culture vessel.

2.1.5 Generation of stable FOXO3a knock-down SCL60 and PWE-1-NB26-3 lines, and LBT2 transient FOXO3a knock-down cell lines

In order to examine the effect of FOXO3a in different cellular processes in response to GnRH, stable and transient FOXO3a knock-down cell lines were generated. A lentiviral-based delivery system was utilised. Mission shRNA Plasmid DNA (pLKO.1-pure vector system; the mission vector has a U6 promoter) (<http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/shc001bul.Par.0001.File.tmp/shc001bul.pdf>) was used, and were transfected into lentivirus using the Mission Packaging Mix (<http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/shp001bul.Par.0001.File.tmp/shp001bul.pdf>) by Dr. Pamela

Brown (MRC HRSU Unit, Edinburgh). Using this system allowed efficient lentiviral infection and integration of the specific shRNA construct into cells mediating life-long gene specific silence. This happens because the lentivirus used was a non-replicating virus. The basic principle is that these viruses after a single round of infection ensure stable integration of the transgene with only a minimal component of viral genome, so that the transgene (in this case the shRNA) continuously expresses but the infectious virus is not generated in order to infect the cells again. This is achieved by selectively splitting the different components of the viral genome into 3-4 separate plasmid vectors which are needed in order for a new virus to be generated (Manjunath *et al.*, 2009).

Before using the lentiviral shRNA system, pilot experiments were performed whether that lentiviral infection caused any change in cell number. Cells were seeded at density of 1×10^6 in 60mm plates with 5ml complete medium. The next day, medium was removed and cells were infected with different titres of GFP-lentivirus and were incubated for 24 hours at 37°C in a 5% CO₂ humidified incubator. The next day, medium with the GFP-lentivirus was removed and was replaced with fresh complete medium. After 1,2,3,4 and 5 days of incubation cells was visualised with a Zeiss Axioskop microscope. The results showed that lentiviral infection caused no change in cell number. The most effective ratio of virus:cells for maximal transduction was also assessed.

Stable and transient FOXO3a knock-downs using the shRNA lentivirus system were generated as follows:

1×10^6 cells were seeded in complete medium in a 6-well plate and were incubated for 24 hours. Complete medium was removed and 2ml of shRNA lentivirus or scrambled shRNA lentivirus in DMEM (negative control) (1:2 cells/virus) plus 3ml of complete medium was added to the cells, and cells were incubated for a further 24 hours at 37°C. Virally transduced cells were trypsinised and re-seeded into 60mm plates with fresh complete medium followed by a further 24 hour incubation at 37°C.

Transient knock-down: (LBT2 cells) Cells were harvested and were assayed for interference of the target genes over 1, 2, 4 and 5 days. Western Blot analysis was performed in order to detect clones with significant reduction of FOXO3a protein level. Cells with the higher knock-down (i.e. with the most reduced target protein level) were used for further experiments.

Stable knock-down: (SCL60 and PWE-1-NB26-3 cells) The medium was removed and replaced with fresh medium containing puromycin (3µg/ml) for selection of transduced cells. The medium was replaced with fresh, puromycin-containing medium every 3-4 days until resistant colonies could be identified. The colonies were picked using glass cloning cylinders, and these cells were seeded in a new plate with fresh puromycin containing complete medium. Generally, 10-12 days after colony selection, when plates were about 80% confluent, cells were trypsinised and split into two plates. Cells from the first plate were allowed to grow to confluency and were cryopreserved. Cells from the second plate were assessed for FOXO3a protein level by Western Blot analysis. Cells with the higher knock-down (i.e. with the most reduced target protein level) were used for further experiments. The shRNAs used to knock-down human and mouse FOXO3a are listed in **Table 2-1**:

Gene	shRNA target	Sequence
Human Forkhead 3a (FOXO3a)	sh1566	CCGGGTCACTGCATAGTCGATTCATCTCGAGATGA ATCGACTATGCAGTGACTTTTTTG
	sh2208	CCGGCTCCTTTAACAGCACGGTGTCTCGAGAACACCGT GCTGTAAAGGAGTTTTTG
	sh2864	CCGGCCACACAGAATGTTGTTGGTTCTCGAGAACCAA CAACATTCTGTGTGGTTTTTG
	sh2978	CCGGCAGACCCTCAAACCTGACACAACCTCGAGT TGTGTCAGTTTGAGGGTCTGTTTTTG
	sh1687	CCGGACAATAGCAACAAGTATACCCTCGAGGGTATAC TTGTTGCTATTGCTTTTTTG
Mouse Forkhead 3a (FOXO3a)	sh841	CCGGCCCGGACAAACGGCTCACTTTCTCGAGAAAGT GAGCCGTTTGTCCGGGTTTTTG
	sh1441	CCGGCGGCACCATGAATCTGAATGACTCGAGTCA TTCAGATTCATGGTGCCGTTTTTG
	sh1624	CCGGGTTTGGACCTTCGTCTCTGAACTCGAGTT CAGAGACGAAGGTCCAAACTTTTTTG
	sh2195	CCGGGAGTCCATCATCCGTAGTGAACCTCGAGT TCACTACGGATGATGGACTCTTT

Table 2-1: shRNA sequences against human and mouse FOXO3a gene

2.1.6 DNA transfection of mammalian cells

2.1.6.1 *Transfection with Superfect® reagent*

SCL60 were transfected with Superfect® Transfection reagent (Qiagen) using 6µl of Superfect® per 1µg of plasmid DNA. The day before transfection 2×10^6 cells were seeded into 100mm dishes in complete medium and were incubated overnight. Cells were transfected with the following mixture (per 100mm dish):

incubated at room temperature for 30mins prior to transfection	DMEM	1.5ml
	Renilla	33ng
	DNA	10µg
	Superfect®	60µl

Table 2-2: Mixture for Superfect® transfection

Prior to transfection, medium was removed and cells washed twice with PBS. After, DNA/Superfect complexes had formed, the transfection mixture was added to the cells with complete medium in a total volume of 3ml. The transfection mix was left on the cells in the incubator for 3 hours. Subsequently, cells were washed twice with PBS, and fresh complete medium was added. Cells were then incubated at 37°C overnight before experiments were carried out.

2.1.6.2 Transfection with Fugene® reagent

LβT2 cells were transfected with Fugene® reagent (Roche) using 6µl of Fugene® reagent per 1µg of plasmid DNA. 2×10^6 cells were seeded into 100mm dishes and incubated overnight. The following day cells were transfected with the following mixture (per 100mm dish):

incubated at room temperature for 30mins prior to transfection	Optimem serum free medium (Gibco)	1.5ml
	Renilla	33ng
	DNA	10µg
	Fugene®	60µl

Table 2-3: Mixture for Fugene® transfection

Prior to transfection, medium was removed and cells were washed twice with PBS. After DNA/Fugene complexes had formed, the transfection mixture was added to the cells with complete medium in a total volume of 3ml. The transfection mix was left

on the cells in the incubator for 3 hours, then 7mls of complete medium was added to the cells and they were left for overnight incubation before experiments were carried out.

2.1.6.3 *Dual light reporter gene luciferase assay*

Luciferase assay is used in order to assess the transcriptional activity of a gene. In particular, cells are transfected with a construct containing the luciferase gene under the control of a promoter of interest. The detected luciferase activity is used in order to assess the transcriptional activity of the gene of interest.

Transient transfections with luciferase reporter constructs, or co-transfection with various mutant constructs or vector controls where appropriate, as well as with a renilla luciferase construct to control for transfection efficiency, was performed using Superfect® reagent for SCL60 cells and Fugene® reagent for LβT2 cells, as described above (Sections 2.1.6.1 and 2.1.6.2). Transfected cells from 100mm plates were seeded into 12-well plates and after 6 hours the complete medium was removed and replaced by starving medium with or without ligand as dictated by the length of ligand stimulation. For stimulations of 8 hours or less complete medium was replaced by starving medium and cells were serum starved overnight. The following day starving medium was replaced by starving medium containing the appropriate concentration of ligand for cell treatment.

Luciferase assays were performed with Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions using a FLUOStar Optima luminometer (BMG Lab technology). Luciferase activity was expressed in arbitrary

units relative to the activity observed in the non-stimulated control cells and was normalized for Renilla luciferase activity.

2.2 IMMUNOFLUORESCENCE CONFOCAL MICROSCOPY

SCL60 cells were plated in eight-well Chamber Slides (Lab-TEK) at a density of 2×10^5 cells per well. Cells were cultured in starving medium for 24 hours before cell stimulation with $1 \mu\text{M}$ of GnRH I. After stimulation, cell monolayers were washed twice with ice cold PBS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) and then fixed in $200 \mu\text{l}$ of 100% methanol (MeOH) at -20°C for 10 minutes. Following fixing, monolayers were washed in PBS and incubated for 30 minutes in an NP-40 based cell permeabilisation buffer (PBS, 10% fetal calf serum, 1% bovine serum albumin, 0.2% NP-40) at room temperature. After permeabilisation, fixed cells were blocked in a PBS-based blocking solution (PBS, 10% fetal calf serum, 1% bovine serum albumin) for 1 hour at room temperature or 16 hours at 4°C . To visualise the proteins, $100 \mu\text{l}$ of primary antibody (1:100) per well was added and slides were incubated overnight at 4°C . The next day, cells were washed twice with PBS and were visualised using an Alexafluor (Molecular Probes, Invitrogen) Goat-Anti-Rabbit (488nm) secondary antibody (1:200 dilution). After incubation for 1 hour, slides were washed twice with PBS and incubated with 4',6-diamidino-2-phenylindole (DAPI 1:2000) for 5 min and then washed three times with PBS. Slides were mounted in Permafluor fixative (Immunotech). Confocal laser microscopy was performed on a Zeiss LSM510 laser scanning microscope (Carl Zeiss).

2.3 DNA MANIPULATION

2.3.1 Plasmid constructs

Plasmids used in this study are listed in **Table 2-4**:

Plasmid	Vector	Reference/source
FLAG-FOXO1 (pCMV5)	pCMN5	Prof. Domenico Accili (Kitamura YI et al., 2005)
FLAG-FOXO1ADA (pCMV5)	pCMV5	Prof. Domenico Accili (Kitamura YI et al., 2005)
HA-FOXO3a-WT	pECE	Prof. Michael Greenberg (Brunet A et al., 1999)
HA-FOXO3a-TM	pECE	Prof. Michael Greenberg (Brunet A et al., 1999)
HA-FOXO3a T32A	pECE	Prof. Michael Greenberg (Brunet A et al., 1999)
HA-FOXO3a S253A	pECE	Prof. Michael Greenberg (Brunet A et al., 1999)
HA-FOXO3a S315A	pECE	Prof. Michael Greenberg (Brunet A et al., 1999)
HA-FOXO3a-TM delta CT	pECE	Prof. Michael Greenberg (Tran H et al., 2002)
HA-FOXO3a-WT delta CT	pECE	Prof. Michael Greenberg (Tran H et al., 2002)
HA-FOXO3a-TM delta DB	pECE	Prof. Michael Greenberg (Tran H et al., 2002)
HA-FOXO3a-WT DBM (H212R)	pECE	Prof. Michael Greenberg (Tran H et al., 2002)
GADD45 WT-luc	pGL3 basic	Prof. Michael Greenberg (Tran H et al., 2002)
Flag-SIRT1	pECE	Prof. Michael Greenberg (Brunet A et al., 2004)
Flag-SIRT1 H363Y	pECE	Prof. Michael Greenberg (Brunet A et al., 2004)
FHRE-luc	pGL3 basic	Prof. Michael Greenberg (Brunet A et al., 1999)
6xDBE-luc	pGL3 basic	Tatsuo Furuyama, University of Osaka (Furuyama <i>et al.</i> , 2000)
pcDNA3.1	pECE	Invitrogen
LH7 sequence (-692, -136bp)	pcDNA3.1	Dr. Pamela Brown, MRC HRSU Unit, Edinburgh
Flag-Egr-1	pCRII	Dr. Pamela Brown, MRC HRSU Unit, Edinburgh
Egr1-luc	pCRII	Dr. Pamela Brown, MRC HRSU Unit, Edinburgh
mdEgr1-luc	pCRII	Dr. Pamela Brown, MRC HRSU Unit, Edinburgh
mpEgr1-luc	pCRII	Dr. Pamela Brown, MRC HRSU Unit, Edinburgh
mdpEgr1-luc	pCRII	Dr. Pamela Brown, MRC HRSU Unit, Edinburgh
LH β	pA3	Dr. Pamela Brown, MRC HRSU Unit, Edinburgh (Larder <i>et al.</i> , 2006)
FSH β	pBluescript II SK+	W. Miller, North Carolina States University, Raleigh (Huang <i>et al.</i> , 2001)
GnRHR-luc	pCAT	J.N.Laverriere, Universite Pierre et Marie Curie, Paris (Pincas <i>et al.</i> , 2001)

Table 2-4: Plasmids used for this study

2.3.2 Transformation of competent E.Coli

10ng of DNA was mixed with OneShotTop10[®] E.coli for 30 minutes on ice. Then, bacteria were shocked at 42°C for 30 seconds and immediately returned to ice for 2-3 minutes. 200µl of S.O.C (Super Optimal broth with Catabolite repression) medium was added and the mix was incubated at 37°C for approximately one hour. 10µl of this medium was spread out on Luria Broth (LB)-agar plates containing the selective antibiotic (Ampicillin - stock solution 50mg/ml) and incubated over-night (maximum incubation 16 hours). If DNA plasmid has already been transformed into bacteria and stored as a glycerol stock, then recovery of bacteria from that glycerol stock is required. To recover the bacteria, a small amount of the bacteria were streaked out on a LB-agar plate containing selective antibiotic and incubated overnight at 37°C (maximum incubation 16 hours).

2.3.3 Extraction and purification of plasmid DNA

A single bacterial colony was picked from agar plates and incubated in 15ml of LB medium containing the appropriate selective antibiotic, at 37°C overnight with shaking (maximum incubation 16 hours). After overnight incubation 5ml of this culture was used to inoculate 250ml of fresh antibiotic containing LB medium, for overnight growth at 37°C with shaking (maximum incubation 16 hours). Plasmid DNA was purified using Quiagen maxi-preparation columns and eluted using TE (Tris-EDTA) buffer according to the manufacturer's instruction (QIAGEN[®] Plasmid Maxi Kit).

2.3.4 DNA quantification

DNA concentration and quantity was determined using the Nanodrop (Nanodrop Technologies, Delaware).

2.3.5 Preparation of glycerol stocks of transformed bacteria

500µl of the bacterial culture (which had grown overnight at 37°C) was mixed with 500µl of 80% glycerol. Mix was stored at -80°C.

2.4 RNA MANIPULATION

2.4.1 Total RNA extraction

Cells were seeded at density of 2×10^6 per 60mm dish and were incubated overnight. Next day, cells were stimulated for the appropriate time with ligand (GnRH I or D-Tpr⁶-GnRH I). After treatment, medium was removed and RNA was extracted with “Total RNA Isolation Reagent” (TRIR; Abgene) according to the manufacturer’s instructions. Briefly, 1ml of TRIR was added to each plate. TRIR was left on the plate on ice for 5 minutes to lyse cells completely. Lysed cells were transferred to a MaXtractTM High Density Gel tube (Qiagen) with 200µl of chloroform, vortexed and incubated on ice for 10 minutes. The MaXtractTM was centrifuged at 8000 x g for 15 minutes at 4°C allowing the gel to create a solid barrier between the lower phenol layer and the upper aqueous phase, which contains the nucleic acid. The aqueous phase was transferred to a clean tube and 0.5ml of iso-propanol was added to each tube. The tubes were vortexed and RNA allowed to precipitate in ice for up to one hour. Then, the samples were centrifuged at 8000 x g for 15 minutes at 4°C and the

supernatant was removed carefully so as not to dislodge the RNA pellet. 0.2ml of 70% ethanol was added to each tube and the samples were centrifuged at 8000 x g for 5 minutes at 4°C. Ethanol was removed and the RNA pellet, which was formed, was allowed to dry. The pellet was re-suspended in an appropriate volume of RSB (RNA Storage Buffer). RNA was stored at -80°C.

2.4.2 Quantitative Real Time PCR (qRT-PCR)

2.4.2.1 *Reverse Transcription for quantitative RT-PCR - cDNA synthesis*

The single strand of RNA that was isolated, was used as a template in order to produce cDNA. RNA was diluted to 50ng/μl in RNase free water. Then, 4μl of the diluted RNA was added to a 200μl thin-walled PCR tube (Adgene). 6μl of the mastermix (**Table 2-5**) was added to each sample and placed in the BioRad DNA Engine Thermal Cycler” and the following thermal cycles were performed:

Thermal cycles	<i>Step1</i>	<i>10min/25°C</i>
	<i>Step2</i>	<i>120min/37°C</i>
	<i>Step3</i>	<i>5sec/85°C</i>
	<i>Step4</i>	<i>4°C hold</i>

Samples were then stored long term at -20°C. For each PCR reaction two types of controls were used; one in which the reverse transcriptase was not added in order to check for genomic DNA contamination and a second in which water (H₂O) was added to the Reverse Transcriptase PCR reaction instead of RNA to check for contamination of Reverse Transcriptase reagents.

Mastermix (to give a 20µl reaction mix):

5X Vilo Reaction Mix (Invitrogen)	4µl
10X Superscript [®] Enz Mix (Invitrogen)	2µl
RNA	2µl (500ng/µl)
DEPC water	12µl

Table 2-5: Mastermix for RT-PCR reaction

2.4.2.2 *Designing primers for qRT-PCR*

The primers and probes sequences are listed in the **Table 2-6** in the following page.

Primer Express (ABI) program was used to design the primers/probes sets.

Primers	Forward (Sequence 5'-3')	Reverse(Sequence 5'-3')	Probe (FAM-TAMRA*)
human GADD45-F	TCTCGGCTGGAGAGCAGAAG	TGGATCTGCAGAGCCACATC	CCGAAAGCGACCCCGATAACGTG
human FASL-F	GCATCATCTTTGGAGAAGCAAA	CCTGAGTTGGACTTGCTGTAAA	CACCCCAGTCCACCCCTGAAAAA
human BIM-F	CCATGAGTTGTGACAAATCAACAC	TGGAAGCCATTGCACTGAGA	AACCCCAAGTCCTCCTTGCCAGGC
human p27kip1-F	CCGACGATTCTTCTACTCAAACA	GGGCGTCTGCTCCACAGA	ATGTTTCAGACGGTTCCCAAATGCC
human p21Cip1-F	CCCGTGAGCGATGGAAC	CGTCCCAGGCGAAGTC	CGACTTTGTCACCGAGACACCACTGGA
human FOXO3a-F	AAGGATAAGGGCGACAGCAA	CCGCATGAATCGACTATGCA	TGGAAGAACTCCATCCGGCACAACC
human FOXO1-F			
18S-F	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT	TGCTGGCACCAGACTTGCCCTC
LH β promoter (F)	CTTGTTTCCCGTGCTTCCA	CCAGTGAATTGGCCTCACACT	TCAGCTAAGCCCTGACACCTGGGC
LH β (distal upstream region of the LH β gene) (F)	CCCAGTCTGCATCACCTTCA	TTGGCCCGCTCACCAT	CCGGCTACTGTCCTAG
Egr1 promoter (F)	GGAGGTGCCACCACTCTT	TCCGCCGTGACGTACATG	CTTCACGTCACTCCGGGTCCTCCC
Egr1 (distal upstream region of the Egr1 gene) (F)	AGTTTCACGTCTTGGTGCCTT	CCCTCACATCTGCGCATGT	TGACACGCCTTGCCGATGGCT

Table 2-6: Primers and probes used for PCR amplification of DNA

* FAM: 5-Carboxyfluorescein, TAMRA: Tetramethyl-6-Carboxyrhodamine

2.4.2.3 *qRT-PCR*

DEPC water was added to the cDNA to give 8ng/ μ l equivalent input starting RNA. The stock solutions of primers and probes were made up with sterile water at concentrations of 100 μ M and 50 μ M respectively. The primers and probes were then diluted in sterile water before use to 250nM and 900nM respectively. Then, a “primers and probe mix” was used in order to make the mastermix for the RT-PCR reaction:

2x master mix	10 μ l
Primers/probe mix	1 μ l
18S mix	0.4 μ l
Water	6.1 μ l
cDNA	2.5 μ l

Table 2-7: Mastermix used for the RT-PCR reaction

The samples were mixed and 19 μ l was added in triplicate to a 96-well plate (Applied Biosystems). The expression of each gene was normalized for RNA loading for each sample using 18S rRNA as an internal standard. Data was analysed using the $\Delta\Delta$ CT method.

2.5 PROTEIN ANALYSIS

2.5.1 Total protein extraction

Following ligand stimulation, cell monolayers were placed on ice, washed once with cold PBS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) and lysed in an NP40-based solubilisation buffer (250mM NaCl, 50mM HEPES, 0.5% Nonidet P-40, 10% glycerol, 2mM EDTA pH 8.0, supplemented with 1mM sodium orthovanadate, 1mM phenylmethylsulphonyl

fluoride and 1mg/ml leupeptin). Total proteins were extracted from solubilised lysates by centrifugation at 20,000 x g for 12 minutes.

2.5.2 Immunoblotting

Total protein extracts were mixed with an equal volume of 2x Laemmli Sample buffer (LSB) (10% (w/v) SDS, 5% (v/v) β -mercaptoethanol, 10% (v/v) glycerol, 2.5% (v/v) 1M Tris (pH 7.0), 2.5% (v/v) distilled H₂O), and were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred to PVDF membrane (NEN Life Sciences) for protein immunoblotting. The amount of proteins loaded on the gel lanes, within any single experiment were constant. However, the loading volumes of homogenized protein were varied according to what was appropriate for cell number and protein abundance in each different experiment. PVDF membranes were blocked in 4% bovine serum albumin, 50mM Tris-HCl, pH 7.0, 0.05% Tween-20 and 0.05% NP-40 blocking solution for one hour at room temperature prior to incubation with primary antibodies at the appropriate dilution, at 4°C, with gentle shaking over-night. β -actin was detected with a 1:1000 dilution of the antibody. Alkaline phosphatase-conjugated anti-rabbit, anti-goat or anti-mouse IgG were employed as secondary antibody as appropriate to the primary antibody. Anti-goat (Sigma) was employed as the secondary antibody for anti- β -actin. Visualization of alkaline phosphatase-labelled protein bands was performed using Enzyme linked Chemi-fluorescence Substrate (ECF) (GE Healthcare). The intensity of bands was quantified with ImageQuant version TL (GE Healthcare, Buckinghamshire, UK). The intensity of each band (for the protein of interest) is normalised with the intensity of the band for β -actin. In order to get the NS (Non Stimulated) value equal

to 1, the NS value that was extracted from the previous calculation (intensity of each band (for the protein of interest) is normalised with the intensity of the band for β -actin) was divided by itself. All the other values (which correspond to time points) are divided to the NS value.

2.6 MATERIALS

2.6.1 Chemical inhibitors

Chemical inhibitors that are used in this study are listed in **Table 2-8** below:

Inhibitors	Target	Working concentration	Reference/source
Gq inhibitor	Gq	100nM	M. Taniguchi, Astellas Pharma Inc (Takasaki et al., 2004)
Cetorelix	GnRH antagonist	1 μ M	Calbiochem
Wortmannin	PI3K	100nM	Calbiochem
LY294002	PI3K	5 μ M/30 μ M	Calbiochem
PP2	c-Src kinase	5 μ M	Calbiochem
AG1478	EGFR	100nM	Calbiochem
PD98059	MEK	20 μ M	Calbiochem
U73122	PLC	20 μ M	Calbiochem
ET-18-OCH3	PLC	20 μ M	Calbiochem
Ro-31-8220	PKC	100nM	Calbiochem
Go6983	PKC	21 μ M	Calbiochem
H89	PKA	10 μ M	Calbiochem
JKNII	JNK	5 μ M	Calbiochem
Akt1/2	AKT	1 μ M	Sigma
BMS-345541	IKK	1,5 μ M	Sigma-Aldrich

Table 2-8: Chemical inhibitors used in this study

2.7 CELL NUMBER ASSAY AND CELL COUNTING

2.7.1 Cell number assay

Cell seeding and treatment

SCL60 cells were seeded in 6-well plates at density of 0.2×10^6 cells per well in complete medium. Cells were treated with doses of D-Trp⁶-GnRH I (1×10^{11} , 1×10^{10} , 1×10^9 , 1×10^8 , 1×10^7 , 1×10^6 M) and were incubated for 5 days.

Cell fixation

Cells attached to the plastic substratum were fixed by gentle addition of 0.5ml of 25% (v/v) trichloroacetic acid (TCA) on top of the growth medium in each well. The cultures were incubated at 4°C for 1 hour and then washed five times with tap water to remove TCA, growth medium, low-molecular-weight metabolites, and serum protein. Plates were dried in an incubator at 37°C.

Sulforhodamine B (SRB) staining

TCA-fixed cells were stained for 30 minutes with 0.4% (w/v) SRB in 1% (v/v) acetic acid. At the end of the staining period, SRB was removed and cultures were quickly rinsed five times with 1% (v/v) acetic acid to remove unbound dye. Plates were dried in an incubator at 37°C.

Protein-bound dye extraction

Protein-bound dye was solubilised and extracted in 1ml of 10mM Tris (tris (hydroxymethyl) aminomethane, pH 10.5) per well.

Optical density measurement

The OD of these protein extracts was measured in 590nm using a spectrophotometer. This assay gives a direct indication of remaining number of live cells following GnRH treatment.

2.7.2 Cell counting

The growth rate of the SCL60 cells in response to D-Trp⁶-GnRH I was measured by cell counting using a haemocytometer. SCL60 cells were seeded in 6-well plates at a density of 0.2×10^6 cells per well in complete medium. The next day (day 1), cells were treated with D-Trp⁶-GnRH I. The cell number was counted in a similar manner every day thereafter (days 2, 3, 4, 5 and 6) after washing and trypsinisation.

2.8 PHARMACOLOGICAL CHARACTERISATION OF GnRH FUNCTION

2.8.1 GnRH binding assay

Cells were seeded in 12-well plates at a range of densities between 2×10^5 to 2×10^6 cells per well, and were incubated overnight. The next day ¹²⁵I-radiolabeled (His⁵,D-Tyr⁶)-GnRH I analogue) was added to HEPES/DMEM/0.1%BSA so that 0.5ml contained approximately 100,000cpm, and this mixture was kept on ice (labelled media). Unlabelled native GnRH I (cold ligand) was added to the mixture at a concentration of 10^{-6} M (labelled media plus cold ligand). Cells were washed twice with cold PBS (with Ca²⁺/Mg²⁺). 500µl of the mixture (labelled media plus cold ligand) was added to the wells in triplicate and cells were incubated for a minimum of 4 hours at 4°C. The radiolabelled mixture was aspirated and wells washed twice

with ice cold PBS. 500µl of 0.1M NaOH was added to each well and the plates were shaken for 15-20 minutes at room temperature in order to solubilise cells. Samples were transferred (from each well) into individual 12x75mm plastic tubes and radioactivity was counted on 1470 Automatic Gamma Counter (Perkin Elmer).

2.8.2 Inositol Phosphate (IP) assay

Cells were seeded at a density of 1×10^6 per well in 12-well plates and were incubated at 37°C overnight. The next day, cells were loaded with [³H] myo-inositol in pre-warmed inositol free SPECIAL DMEM at a concentration of 0.5µCi [³H] myo-inositol/0.5ml SPECIAL DMEM/well. The cells were then incubated overnight. Media was aspirated off and the cells were washed with 0.5ml HEPES-DMEM/0.1%BSA. 0.5ml HEPES-DMEM/0.1%BSA containing a final concentration of 10mM LiCl was added to each well and plates were incubated at 37°C for 30 minutes. This buffer was removed from the wells and 0.5ml HEPES-DMEM/10mM LiCl/0.1%BSA with or without GnRH I was added at appropriate dilutions. Cells were incubated at 37°C for 60 minutes. Media was aspirated from the wells and 1ml of 10mM formic acid was added to each well followed by incubation at 4°C for 30 minutes. 0.5ml of AG-1-X8 resin (Bio-Rad) was added into plastic tubes (12x75mm) and formic acid samples were transferred from the wells to the tubes containing AG-1-X8 resin and the mixture vortexed. Resin was allowed to settle and the supernatant was aspirated. 1 ml of water was added, the mixture was vortexed and resin was allowed to settle. Then supernatant was aspirated and 1ml of 60mM ammonium formate/5mM sodium tetraborate was added, vortexed and resin was allowed to settle. Finally, the supernatant was removed and 1ml of 1M ammonium

formate/0.1M formic acid was added vortexed and resin was allowed to settle. 0.8ml of the supernatant was removed into scintillation vials and 2.5ml of scintillation fluid (Optiphase Hisafe 3, Fisher Scientific) was added and ^3H was counted for 1 minute on a 1450 Microbeta Beta counter (TriLux; Perkin Elmer).

2.9 CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

L β T2 cells were seeded at a density of 2×10^6 in 60mm plates. 37% formaldehyde in DMEM was added directly to the cell culture and cross-linking was allowed to proceed for 10 min at room temperature. Cross-linking was stopped by the addition of glycine stop-fix solution (Active Motif). Cross-linked cells were scraped with cell scraping solution (Active Motif). Pellets were collected by micro-centrifugation, re-suspended and incubated in ice-cold lysis buffer supplemented with PIC (protease inhibitor) and PMSF (phenylmethanesulfonylfluoride) and lysed with an ice cold dounce homogenizer. Nuclei were again pelleted by centrifugation. These pellets were resuspended in Digestion Buffer (Active Motif) and incubated at 37°C for 5 minutes. Chromatin was sheared with the Enzymatic Shearing Cocktail (Active Motif). Sheared chromatin was collected by centrifugation for 10 minutes at 15.000rpm. 7 μg of sheared chromatin was added to the ChIP reaction. 1-3 μg of the appropriate antibody was added to the aliquots of chromatin and mixed gently on an end-to-end rotator for 16 hours at 4°C. Antibody against FOXO3a was purchased from Abcam and IgG (sc-2027) was purchased from Santa Cruz. Following extensive washing, bound DNA fragments were eluted and analyzed by qRT-PCR. Bound DNA fragments were analyzed by qRT-CR with TaqMan DNA Polymerase (Invitrogen) using primers and probes listed in the **Table 2-6** above.

2.10 CELL-CYCLE ANALYSIS

L β T2 and PWE-1-NB26-3 cells were plated at a density of 0.5×10^6 cells per dish in complete medium and in serum-free keratinocyte medium respectively. After treatment with 100nM D-Trp⁶-GnRH I for 96 hours, 10 μ g/ml Hoechst stain was added to the medium, and cells were incubated for 60 more minutes at 37°C. After trypsinisation and centrifugation, the cell pellets were resuspended in the appropriate complete medium. The cellular content of DNA was determined by flow cytometry analysis with a BD LSR Fortessa cell analyzer. Cells were gated according to physical characteristics and therefore subcellular debris and clumps were distinguished from single cells during the analysis. The data were analyzed with DIVA Software. (Data analysis (gating and computational analysis) was performed with the cooperation of Ms. Shonna Jonhston from University of Edinburgh, Centre for Inflammation Research, Flow Cytometry Facility).

2.11 IMMUNOHISTOCHEMISTRY

2.11.1 Tissues

Tissues that are used in this study are listed on **Table 2-9** below:

Tissue	Species	Fixation Method
Pituitary	Human	NBF
Pituitary	Mouse	NBF
Pituitary	Mouse	Bouins
Pituitary	Rabbit	NBF
Pituitary	Rabbit	Bouins

Table 2-9: Tissues that are used in this study

(NBF: neutral buffered formalin, is a general purpose fixative for all tissues for surgical tissues and necropsy specimens)

2.11.2 Immunohistochemistry procedure

Fixation and Tissue processing

The tissues that were used in this study had been fixed previously (the fixation methods are listed in the table above) and processed with paraffin prior to use.

Tissue sectioning

Sections were cut on a paraffin microtome (Leica RM2125RTF) in sections of 5µm. Sections were floated onto heated water at 45°C and were lifted onto superfrost plus microscope slides. Slides were then dried in an incubator overnight at 37°C.

Staining

Paraffin wax was removed from the slides in xylene, series dilutions of ethanol (100%, 95% and 70%) and distilled water. In selective cases antigen retrieval was performed using the pressure cooking method in pre-heated 0.01M sodium citrate buffer pH 6.0 followed by cooling for a further 20 minutes under running tap water (Tissue fixation alters the three dimensional structure of the tissue proteins which results in a modification of the antigen's epitope. The loss of an epitope results in an antigen's inability to react with the paratope of the antibody, and this can only be corrected by the restoration (antigen retrieval process) of the epitope). Next, slides were blocked in 300mls 3% hydrogen peroxidase in methanol, on a rocker at room temperature for 30 minutes, to block endogenous peroxide. Two washes with tris buffered saline (TBS) followed. Afterwards slides were incubated in normal goat serum (NGS) diluted in TBS (1:5 v/v) containing 5% (w/v) BSA for 30 minutes at room temperature. Slides were drained, and primary antibody diluted in NGS/TBS/BSA was added. Slides were incubated overnight at 4°C. Two staining methods were used to visualise the proteins of interest:

1. *DAB detection method*

After over-night incubation, slides were washed twice in TBS for 5 minutes and biotinylated goat anti-Rabbit at 1:500 in NGS/TBS/BSA was added and incubated for 30 minutes at room temperature. Slides were washed twice with TBS for 5 minutes and streptavidin HRP at 1:1000 in TBS was added and incubated for 30 minutes at room temperature. Slides were washed twice in TBS for 5 minutes and 3,3' Diaminobenzidine (DAB substrate) was added. The colour development was monitored microscopically (the usual time for DAB exposure was 5 minutes) and the colour development was stopped by washing in water. Next nuclear counterstain was performed using heamatoxylin; washed slides were rinsed in acid alcohol (ethanol/distilled water/hydrochloric acid) for 5 seconds. Then slides were washed in water and were added to Scott's tap water (sodium hydrogen carbonate/magnesium sulphate/distilled water) for blueing up the nucleus and nuclear membrane for 30 seconds. Next, slides were washed in water for 20 seconds and were dehydrated in 70% ethanol, 95% ethanol and absolute ethanol for 20 seconds and in xylene for 5 minutes. Finally, slides were cover-slipped using Pertex (). The immunostaining sections were visualized with an Olympus Provis microscope.

2. *Tyramide detection method-double staining*

After over-night incubation, slides were washed twice with TBS for 5 minutes and goat anti-Rabbit peroxidise Fab at 1:500 in NGS/TBS/BSA was added and incubated for 30 minutes at room temperature. Slides were washed twice in TBS for 5 minutes and Tyramide-Fluorescent Green (1/50) was added and incubated for 10 minutes at

room temperature. Slides were washed twice in TBS for 5 minutes and antigen retrieval process was performed (as described above). Afterwards slides were incubated in normal goat serum (NGS) diluted in TBS (1:5 v/v) containing 5% (w/v) BSA for 30 minutes at room temperature., slides were drained, and primary antibody diluted in NGS/TBS/BSA was added. Slides were incubated overnight at 4°C.

After over-night incubation, slides were washed twice in TBS for 5 minutes and goat anti-mouse peroxidise Fab at 1:500 in NGS/TBS/BSA was added and incubated for 30 minutes at room temperature. Slides were washed twice in TBS for 5 minutes and Tyramide-Fluorescent Red (1/50) was added and incubated for 10 minutes at room temperature. Slides were washed twice in TBS for 5 minutes. Nuclei were counterstained using DAPI (1:1000 in TBS) for 10 minutes. Finally slides were cover-slipped using permafluor ().

2.12 STATISTICAL ANALYSIS

All data from each experiment were combined and presented as means and standard error of the mean (\pm SEM). The numbers of independent experiments are denoted by the number (n) and are given in figures legends and text. Statistical analysis was performed using the student t-test with Graph Prism 5.00 software (Graph Prism Software Inc.). A p-value $P < 0.05$ was taken as statistical significance in robust data sets.

CHAPTER THREE

**REGULATION OF FOXO TRANSCRIPTIONAL
ACTIVITY BY GnRH**

3.0 ABSTRACT

In this Chapter, the regulation of FOXO transcriptional activity following GnRH-R activation is investigated, and a novel mechanism by which GnRH targets FOXO3a activity is described. Since the transcriptional activity of FOXO is determined by post-translational modifications and the action of co-factors, such as β -catenin, the role of these post-translational modifications and the involvement of β -catenin are characterised. Finally, the role of FOXOs in targeting the expression of several FOXO-target genes is assessed, highlighting a specific role for FOXO3a in mediating GADD45 and FasL expression in response to GnRH.

3.1 BACKGROUND AND OBJECTIVES

The mammalian FOXOs are emerging as an important family of proteins that are involved in a variety of cellular processes, including cell-cycle regulation, apoptosis, DNA damage repair and response to oxidative stress (Burgering, 2008). FOXOs modulate the expression of specific genes that mediate these processes, including the apoptotic *FasL* and the oxidative stress resistant *GADD45* genes. The most well-characterised regulatory mechanism of FOXO transcriptional activity is through their phosphorylation by the PI3K/Akt signalling pathway. Interestingly, the PI3K/Akt signalling pathway is also regulated by GnRH-R activation (Birkenkamp and Coffey, 2003). However, to date, there are no studies describing the regulation of FOXO transcriptional activity by GnRH. Thus, the potential regulation of FOXO activity by GnRH-R activation was investigated.

In this Chapter, the mechanisms and signalling pathways through which GnRH regulates FOXO transcriptional activity was examined. In particular, the main objectives of this Chapter were the following:

- to examine whether GnRH regulates FOXO transcriptional activity
- to investigate the mechanisms and signalling pathways through which GnRH regulates FOXO activity
- to examine whether GnRH targets the expression of FOXO-target genes, and whether FOXOs are involved in this expression.

In Chapters three, four and five cells were treated with GnRH I (referred to as GnRH), unless otherwise indicated. In Chapters three and four, all studies were conducted in the stable HEK293/ratGnRH-R expressing cell line (referred to as SCL60 cells), unless otherwise indicated.

3.2 RESULTS

3.2.1 FOXO1 and FOXO3a phosphorylation and sub-cellular localisation in response to GnRH treatment

3.2.1.1 *GnRH treatment targets phosphorylation of FOXO3a at Ser²⁵³ in SCL60 cells*

The primary pathway regulating FOXO function is the PI3K/Akt signalling pathway, in which FOXOs are phosphorylated at Akt phosphorylation sites, following PI3K activation by several proteins, including G-proteins (Van Der Heide *et al.*, 2004). A short time-course treatment of cells with 1µM GnRH, demonstrated that FOXO3a was only phosphorylated at Ser²⁵³ reaching maximal phosphorylation after 10

minutes (**Fig. 3-1A**). Whether GnRH-induced phosphorylation of FOXO3a at Ser²⁵³ was specifically mediated by GnRH binding and activation of the Type I GnRH-R, was also examined. For this purpose, a GnRH antagonist (Cetrorelix) was used. Co-treatment with Cetrorelix inhibited GnRH-targeted phosphorylation at Ser²⁵³ (**Fig. 3-1B**). This confirmed that phosphorylation of FOXO3a at Ser²⁵³ was mediated by GnRH-R activation.

A longer time-course treatment of SCL60 cells with 1 μ M GnRH over 24 hours resulted in decreased phosphorylation of FOXO3a at Ser²⁵³ of up to 35% (**Fig. 3-1C**). A GnRH dose-response treatment for FOXO3a phosphorylation at Ser²⁵³ site was also performed, producing an EC50 of 1.2nM, which is in agreement with activation of the Type I GnRH-R (**Fig. 3-1D**).

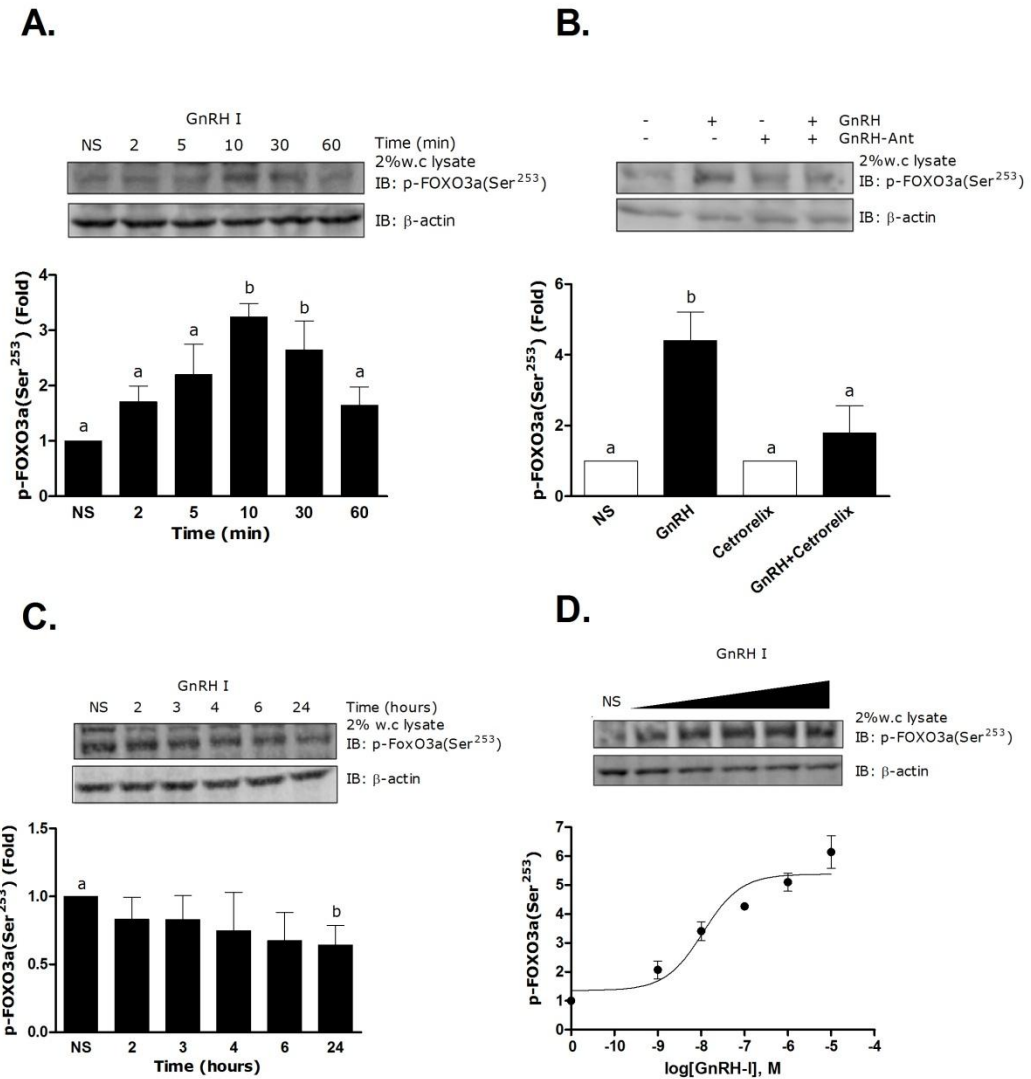


Figure 3-1: Phosphorylation of FOXO3a at Ser²⁵³ in response to GnRH.

(A) SCL60 cells were serum-starved 24 hours before being treated with 1 μ M GnRH for the indicated times, or left untreated (NS). (B) SCL60 cells were serum-starved 24 hours before being treated for 10 minutes with 1 μ M GnRH, 1 μ M Cetrorelix, or both or left untreated (NS). (C) SCL60 cells were treated with 1 μ M GnRH for the indicated times. (D) SCL60 cells were serum-starved 24 hours before being treated with 10 μ M, 1 μ M, 1nM, 100nM, 10nM, 1nM GnRH or left untreated (NS) for 10 minutes. Levels of p-FOXO3a(Ser²⁵³) were analysed using a p-FOXO3a(Ser²⁵³)-specific antibody. Quantification of the immunoblots were performed using β -actin as a loading control. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a; P<0.05)

Phosphorylation at other sites on FOXOs was also examined by Western Blot analysis, and FOXO3a was not phosphorylated at the Ser³¹⁸ or Ser³²¹ phosphorylation sites following GnRH treatment (**Supplementary Fig. 7-1A**). Additionally, GnRH did not target phosphorylation of FOXO1 at Ser²⁵⁶ or Ser³¹⁹ (**Supplementary Fig. 7-1B and C**), no did GnRH treatment lead to the phosphorylation of FOXO4 at the Ser¹⁹³ Akt-phosphorylation site (**Supplementary Fig. 7-1D**).

GnRH-targeted phosphorylation of FOXO3a at Ser²⁵³ in the LNCaP prostate cancer cell line was also examined. In contrast to SCL60 cells, GnRH did not mediate phosphorylation of FOXO3a at the Ser²⁵³ Akt phosphorylation site in LNCaP cells (**Supplementary Fig. 7-2**).

3.2.1.2 *p-FOXO3a(Ser²⁵³), but not p-FOXO1(Ser²⁵⁶), is redistributed between the nucleus and cytoplasm in response to GnRH in SCL60 cells*

In order to visualise GnRH-induced p-FOXO3a(Ser²⁵³) and p-FOXO1(Ser²⁵⁶) localisation, immunofluorescence confocal microscopy was performed. The presence of p-FOXO3a(Ser²⁵³) and p-FOXO1(Ser²⁵⁶) was low in non-stimulated cells. Following GnRH treatment, p-FOXO3a(Ser²⁵³) staining increased significantly in both the nucleus and cytoplasm. After 30 and 60 minutes of GnRH treatment p-FOXO3a(Ser²⁵³) appears to translocate from the nucleus to cytoplasm (**Fig. 3-2**), however since no quantification analysis has been performed, no robust conclusion can be extracted. These data are consistent with the Western Blot data showing increased p-FOXO3a(Ser²⁵³) in response to GnRH treatment. In contrast, p-FOXO1(Ser²⁵⁶) staining was only slightly increased following GnRH treatment. Localisation of p-FOXO1(Ser²⁵⁶) in the nucleus was also observed in response to

GnRH. Although these data appear to contradict the Western Blot data, it is possible that the antibody used was not specific and cross-reacted with other FOXOs, such as FOXO4 (Supplementary Fig. 7-3).

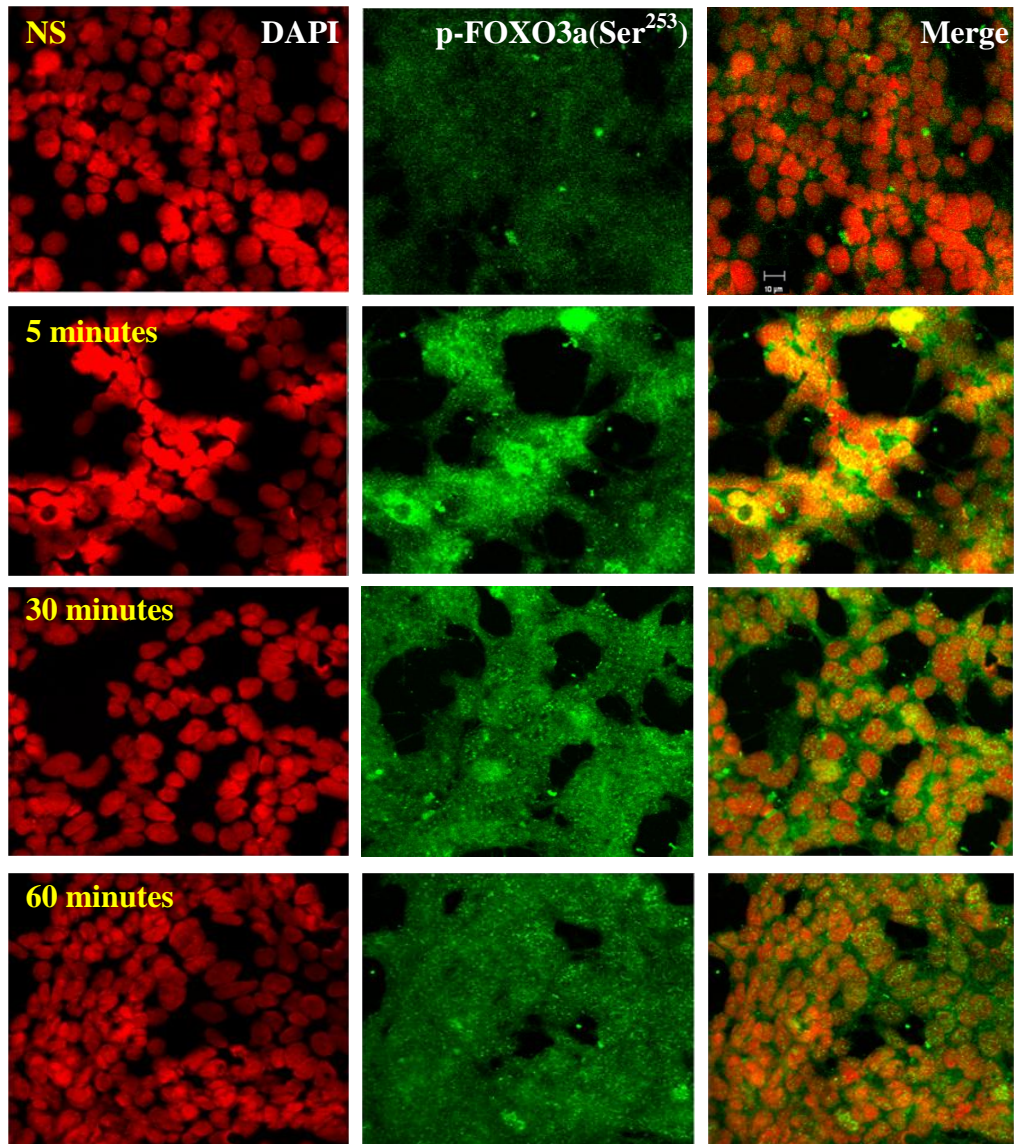


Figure 3-2: p-FOXO3a(Ser²⁵³) redistribution between nucleus and cytoplasm following GnRH stimulation in SCL60 cells.

Immunofluorescence confocal microscopy was performed to visualise redistribution of p-FOXO3a(Ser²⁵³) (green) between the nucleus and cytoplasm following 1μM GnRH treatment at the time intervals indicated. DAPI (red) is a marker for nucleus and co-localisation is indicated as yellow.

3.2.1.3 *Phosphorylation of FOXO3a at Ser²⁵³ in SCL60 cells is mediated by a G_q-coupled, PI3K/Akt-mediated signalling pathway*

Having demonstrated that GnRH-targeted phosphorylation of FOXO3a at Ser²⁵³, various pharmacological inhibitors were subsequently used in order to identify the signalling pathway that mediates this phosphorylation event. Cetrorelix and the G_q inhibitor YM254890 clearly inhibited phosphorylation of FOXO3a at Ser²⁵³ in response to GnRH (**Fig. 3-3** (upper panel)). In addition, the PI3K/Akt signalling pathway appears to be involved in this phosphorylation, as the PI3K inhibitors Wortmannin and LY294002, and the Akt inhibitor Akt1/2 reduced FOXO3a phosphorylation at Ser²⁵³ in response to GnRH (**Fig. 3-3** (lower panel)). Inhibitors of c-Src, EGFR, MEK, PLC, PKC and PKA were also tested. Results from these experiments indicated that none of these potential mediators appeared to be involved in phosphorylation of FOXO3a at Ser²⁵³ in response to GnRH (**Supplementary Fig. 7-4**).

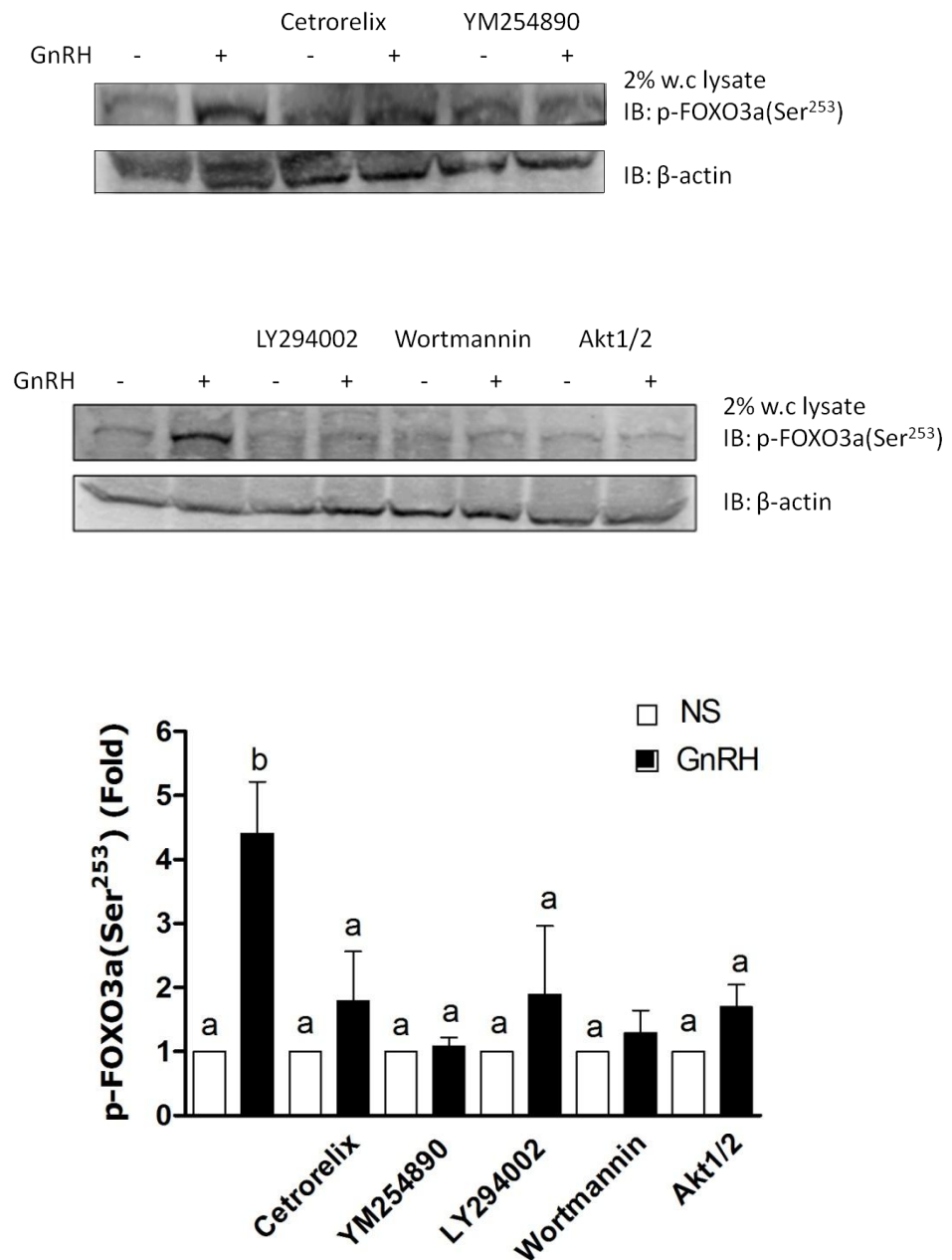


Figure 3-3: GnRH induces phosphorylation of FOXO3a at Ser²⁵³ via a G_q-coupled, PI3K/Akt-mediated signalling pathway.

SCL60 cells were serum-starved for 24 hours before cells were pre-treated for 30 minutes with: GnRH antagonist Cetorelix (1μM), G_q inhibitor YM254890 (100nM), PI3K inhibitors Wortmannin (100nM) and LY294002 (30μM), Akt inhibitor Akt1/2 (1μM), and were then treated for 10 minutes with 1μM GnRH. Quantification of the immunoblots were performed using β-actin as a loading control. Blots are representative of 3 independent experiments. Data are shown as mean fold change over NS±SEM from at least 3 independent experiments. (b is significantly different from a; P<0.05)

3.2.2 GnRH targets FOXO transcriptional activity

3.2.2.1 *GnRH targets GADD45 transcription in SCL60 cells by regulating FOXO3a transcriptional activity*

Phosphorylation is a known post-translational modification that regulates FOXO activity. Therefore, having demonstrated that GnRH stimulation mediates phosphorylation of FOXO3a, the involvement of GnRH in regulating FOXO transcriptional activity was examined. Three known FOXO-targeted promoter luciferase reporters were utilised; GADD45-, FHRE- and 6×DBE-promoter luciferase constructs, henceforth referred to as GADD45-, FHRE- and 6×DBE-reporters. FHRE (FH-Response Element) is the consensus binding site within the promoter of the FasL gene for FOXO transcription factors (Brunet *et al.*, 1999). DBE (Daf-16 family protein Binding Element) is a core binding sequence shared by all four FOXOs, TTGTTTAC.

Cells were treated with GnRH for 24 hours following pre-incubation in either complete or starving medium. Cells were pre-incubated in starving medium in order to examine the effect of GnRH exclusively, eliminating the effect of other potential stimulators of the pathway of interest. Alternatively, cells were pre-incubated in complete medium in order to examine whether the GnRH effect was dominant among the other factors that potentially affect FOXO activity. GADD45-, FHRE- and 6×DBE-reporter activities were increased following GnRH treatment (**Fig. 3-4**). To further examine whether FOXO transcriptional activity was induced by GnRH acting at the Type I GnRH-R, Cetrorelix was utilised. Co-treatment with Cetrorelix inhibited FHRE-reporter activity, previously observed in response to GnRH treatment (**Fig. 3-5**). However, treatment only with Cetrorelix decreases the FHRE-

reporter activity compared to non-stimulated control, which may suggest that Cetrorelix partially inhibits GnRH-independent signalling pathways, which also regulate FHRE-reporter activity. An explanation for this result could be related to the ligand-induced selective signalling (LISS) effect. Based on that, the GnRH receptor can assume a number of distinct active conformations that selectively target different intracellular signalling pathways, and therefore different GnRH analogues would impart signalling-selectivity by stabilising these distinct GnRH receptor active conformations.

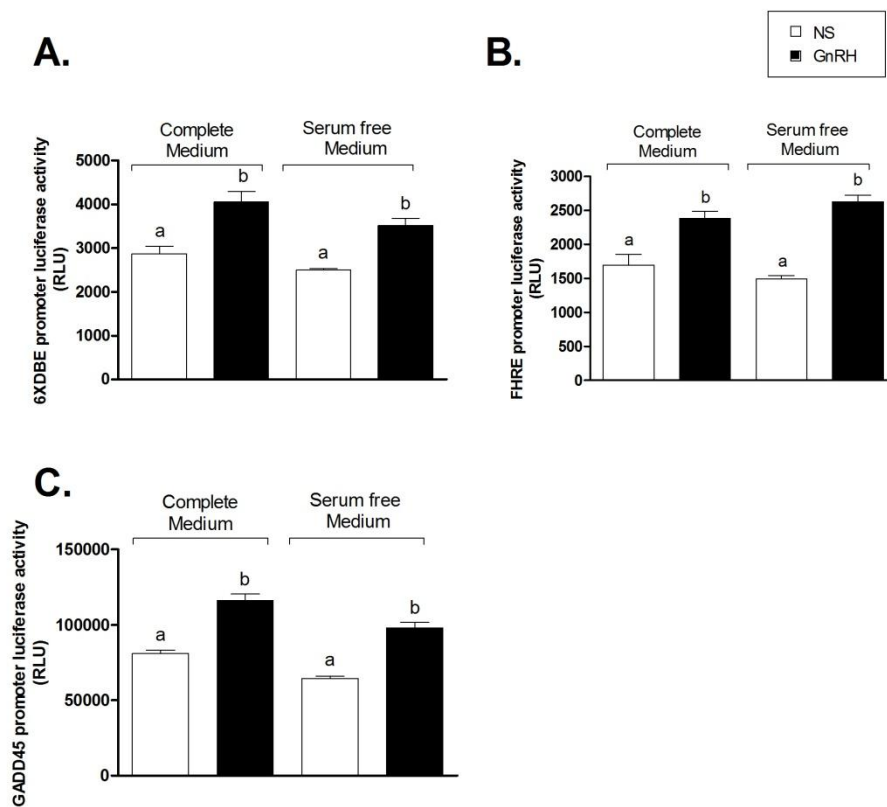


Figure 3-4: GnRH-induced GADD45-, FHRE- and 6xDBE-reporters.

SCL60 cells were transiently transfected with 10 μ g of (A) 6xDBE-, (B) FHRE- and (C) GADD45-reporter. Cells were treated with 1 μ M GnRH for 24 hours or were serum-starved 16 hours before treatment with 1 μ M GnRH for 24 hours. Samples were then assayed for luciferase activity. In all

panels open bars denote no stimulation and close bars denote GnRH stimulation. Data are shown as RLU, from at least 3 independent experiments. (b is significantly different from a; $P < 0.05$)

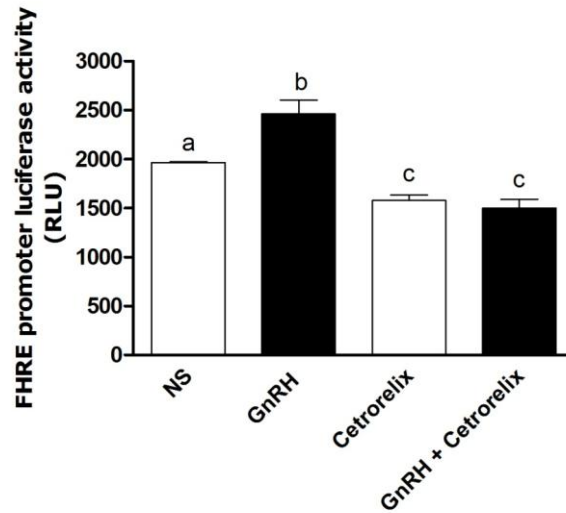


Figure 3-5: GnRH-induced FHRE-reporter activity.

SCL60 cells were transiently transfected with 10 μ g of FHRE-reporter. Cells were serum-starved for 16 hours and then treated with 1 μ M GnRH for 24 hours, or with 1 μ M Cetorelix or with 1 μ M GnRH plus 1 μ M Cetorelix. Samples were then assayed for luciferase activity. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a and c is significantly different from a and b; $P < 0.05$)

FOXO3a was subsequently over-expressed in order to investigate the transcriptional activity of FOXO3a above the endogenous FOXO1/3a/4 background. In particular, wild type FOXO3a (FOXO3a-WT) or a triple mutant form of FOXO3a (FOXO3a-TM) were used. FOXO3a-TM has mutations at its three Akt phosphorylation sites (Thr³²Ala, Ser³¹⁵Ala and Ser²⁵³Ala; TM denotes triple mutant) (Burgering and Kops, 2002). In contrast to FOXO3a-WT, FOXO3a-TM cannot be phosphorylated by Akt, resulting in its nuclear retention. FOXO3a-TM was therefore used in order to eliminate the effect of Akt signalling on the localisation/redistribution of FOXO3a.

SCL60 cells were co-transfected with GADD45-reporter together with increasing amounts of FOXO3a-TM, followed by treatment with 1 μ M GnRH. The basal levels of GADD45-reporter activity and GnRH-induced GADD45-reporter activity were increased with higher levels of over-expressed FOXO3a-TM (**Fig. 3-6**). A pGL3 vector backbone was also used as a control to determine background luciferase activity. When FOXO3a was over-expressed, the luciferase activity of pGL3 was not significantly increased compared to the FOXO-activated GADD45-promoter-dependent reporter in response to GnRH (**Fig. 3-7**).

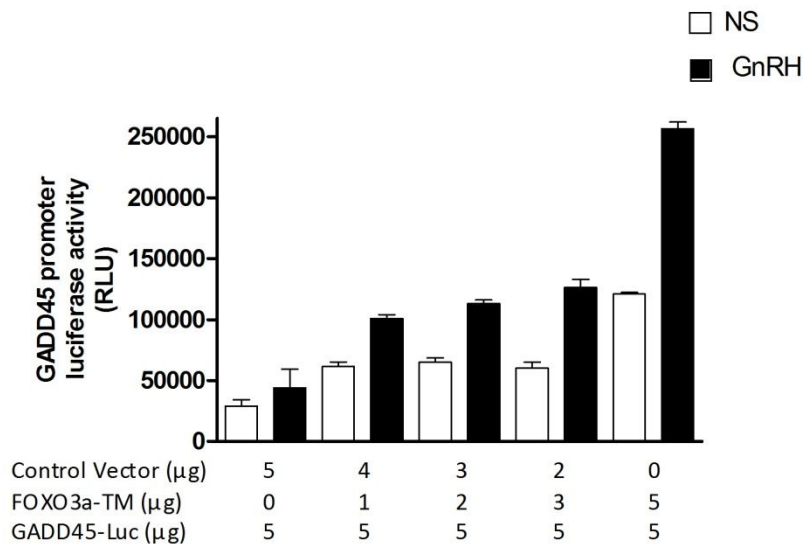


Figure 3-6: GnRH-induced GADD45-reporter activity in a time-course treatment.

SCL60 cells were transiently transfected with 5 μ g of GADD45-reporter and increasing amounts of FOXO3a-TM, and decreasing amounts of control vector (pcDNA3.1). Cells were treated with 1 μ M GnRH for the indicated time points or left untreated (NS). Samples were then assayed for luciferase activity. Data from two experiments are shown.

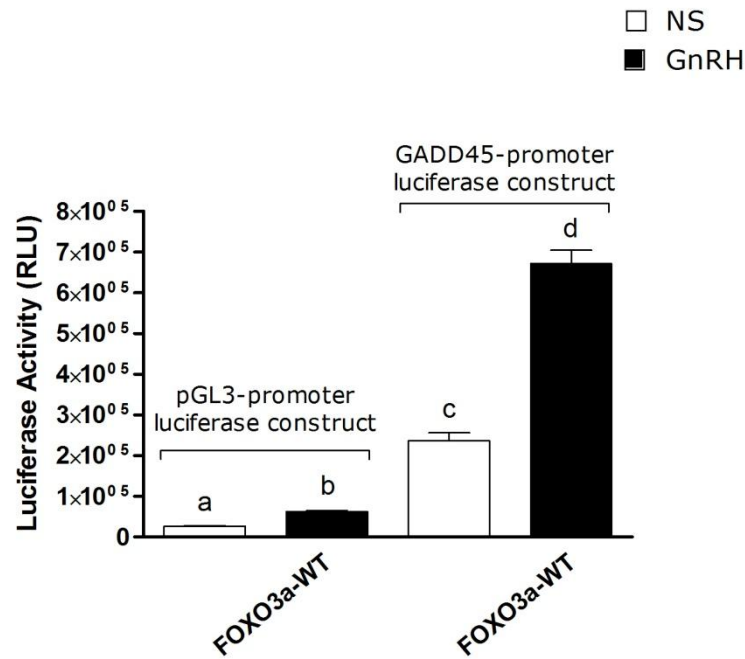


Figure 3-7: GnRH-induced pGL3- and GADD45-reporter activity.

SCL60 cells were transfected with 3.5 μ M of pGL3- or GADD45-reporter and 6.5 μ M of FOXO3a-WT. Cells were treated for 24 hours with 1 μ M GnRH in starving medium. Samples were assayed for luciferase activity. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a, c is significantly different from a and b, and d is different from a, b and c; P<0.05)

Next, SCL60 cells were co-transfected with the GADD45-reporter and pcDNA3.1 (henceforth referred to as vector control), FOXO3a-WT or FOXO3a-TM. Treatment with GnRH enhanced the transcriptional activity of both FOXO3a-WT and FOXO3a-TM by 2-fold compared to control over 24 hours of treatment (**Fig. 3-8A**). Of note is that GnRH treatment induced the same fold increase in FOXO3a transcriptional activity in cells that were co-transfected with either FOXO3a-WT or FOXO3a-TM. This is interesting with regards to previous results (see **Figure 3-1A** and **C**) that GnRH targets FOXO3a phosphorylation at an Akt phosphorylation site over short time-course treatment (5-60 minutes), while targeting FOXO3a de-

phosphorylation at the same phosphorylation site over 24 hours of treatment. The role of FOXO phosphorylation in regulating its transcriptional activity is well-established. Combining the above findings, that GnRH targets FOXO3a dephosphorylation over 24 hours of treatment, and that GnRH treatment enhanced the transcriptional activity of both FOXO3a-WT, and FOXO3a-TM that cannot be phosphorylated by Akt, by the same fold, suggests that targeting of FOXO3a phosphorylation by GnRH is not the dominant mechanism regulating its transcriptional activity.

While over-expression of FOXO3a-TM increased GnRH-induced GADD45-reporter activity compared to vector control, over-expression of FOXO3a-TM Δ DB, bearing mutations at its three Akt phosphorylation sites (Thr³²Ala, Ser³¹⁵Ala and Ser²⁵³Ala) in addition to a deletion of the DNA binding domain (denoted by Δ DB), reduced the GnRH-mediated GADD45-reporter activity compared to the effect of FOXO3a-TM (**Fig. 3-8B**). This suggests that FOXO3a binds directly to the GADD45 promoter and regulates the expression of the *GADD45* gene. A FOXO3a-TM Δ CT construct that has a deletion of the trans-activation domain, and cannot bind potential co-factors that may act to regulate FOXO3a transcriptional activity (denoted by Δ CT) was also tested. Over-expression of FOXO3a-TM Δ CT partially reduced the GnRH-mediated induction of GADD45-reporter activity compared with cells in which FOXO3a-TM was over-expressed (**Fig. 3-8B**). This indicates that FOXO3a acts in co-ordination with other co-factors in response to GnRH.

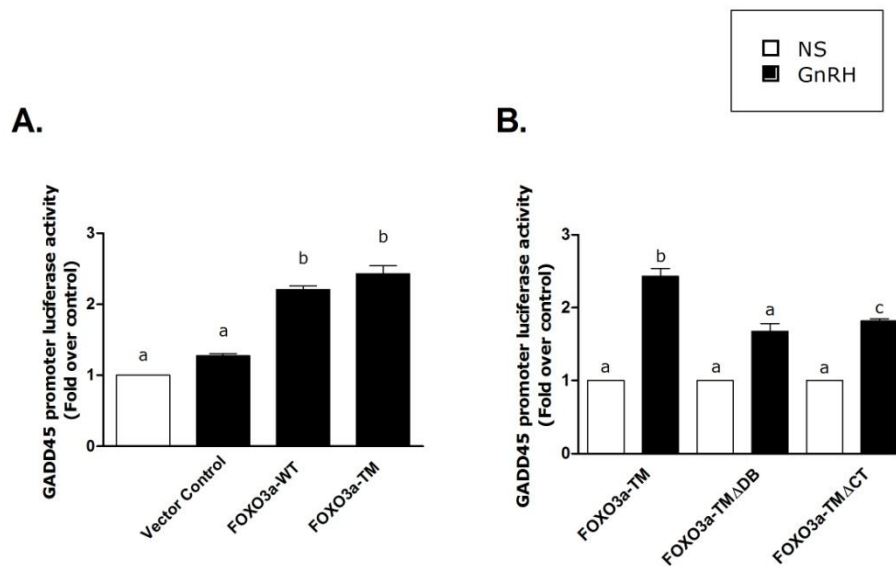


Figure 3-8: GnRH-induced GADD45-reporter activity by direct binding of FOXO3a to the GADD45 promoter.

SCL60 cells were transiently transfected with 1 μ g of GADD45-reporter and (A) 9 μ g of control vector (pcDNA3.1) or (A) FOXO3a-WT or (A and B) FOXO3a-TM or (B) FOXO3a-TM Δ DB or FOXO3a-TM Δ CT. Cells were serum-starved 16 hours before being treated with 1 μ M GnRH for 24 hours. Samples were assayed for luciferase activity. In all panels open bars denote no stimulation and close bars denote GnRH stimulation. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a, and c is significantly different from a and b; $P < 0.05$)

Although GnRH does not appear to target FOXO1 phosphorylation (see Section 3.2.1.1) a possible role for FOXO1 in GnRH-induced GADD45 expression was examined. For this purpose, cells were co-transfected with FOXO1-WT or FOXO1-TM constructs. Over-expression of FOXO1-TM increased GnRH-induced GADD45-reporter activity compared to vector control, however this was not significant (Supplementary Fig. 7-5).

3.2.2.2 *GnRH targets FOXO3a transcriptional activity independently of FOXO3a phosphorylation at any of its Akt phosphorylation sites*

Since a clear regulation of GADD45-reporter activity by FOXO3a following GnRH treatment was demonstrated (Section 3.2.2.1), the role of Akt phosphorylation in GnRH-mediated FOXO3a transcriptional activity was examined. When raw luciferase assay data was analysed, the transcriptional activity of FOXO3a-WT was lower than the activity of FOXO3a-TM in the absence of GnRH treatment (**Fig. 3-9A**). In this case raw data were used in order to be able to show the effect of the over-expression of FOXO3a-TM on GADD45-promoter luciferase activity. These results were expected since the triple mutant form of FOXO3a (FOXO3a-TM) cannot be phosphorylated by Akt, resulting its nuclear retention. When the corresponding normalised data was analysed, a similar fold-increase in GADD45-reporter activity was observed when either FOXO3a-WT or FOXO3a-TM was over-expressed (**Fig. 3-9B**). These data support the notion that phosphorylation of FOXO3a in response to GnRH treatment does not appear to play a role in its transcriptional activity. Thus, GnRH appears to target FOXO3a transcriptional activity independently of FOXO3a phosphorylation at any of its Akt phosphorylation sites.

To further investigate the role of any of the individual Akt phosphorylation sites in regulating FOXO3a transcriptional activity, cell were co-transfected with FOXO constructs bearing individual mutations at the three Akt phosphorylation sites (HA-FOXO3a Ser²⁵³Ala, HA-FOXO3a Ser³¹⁵Ala, HA-FOXO3a Thr³²Ala). The fold-change in luciferase activity following GnRH treatment was similar for the three mutants (**Fig. 3-9C**), suggesting that although GnRH targets phosphorylation of

FOXO3a at Ser²⁵³, this modification is not the main modulator of its transcriptional activity.

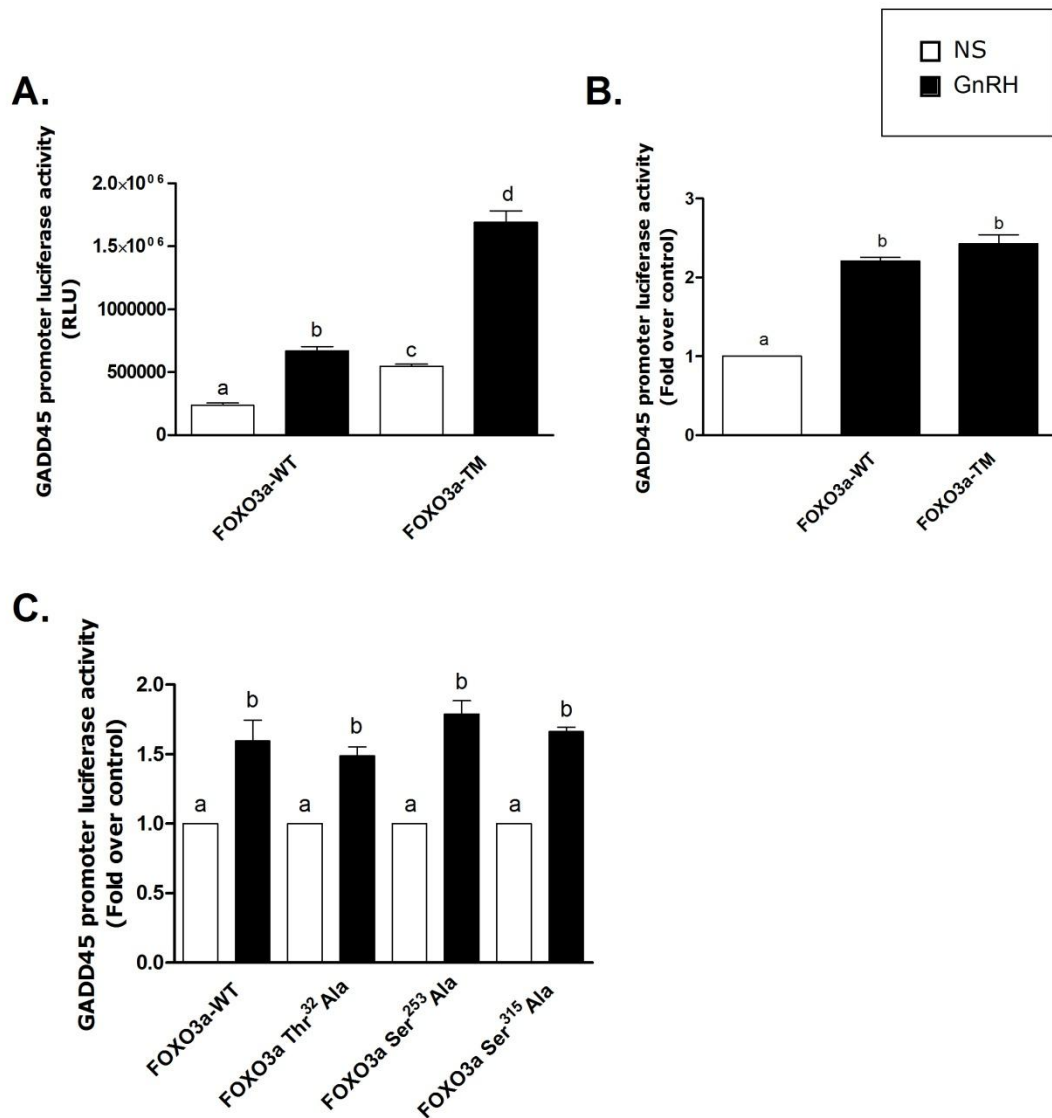


Figure 3-9: Effect of Akt-dependent FOXO3a phosphorylation on GnRH-induced GADD45-reporter activity.

(A and B) SCL60 cells were transiently transfected with 1 μ g of GADD45-reporter and 9 μ g of control vector (pcDNA3.1), or FOXO3a-WT or FOXO3a-TM. (C) Cells were transiently transfected with 5 μ g of GADD45-reporter and 5 μ g of control vector (pcDNA3.1), or FOXO3a-TM, FOXO3a Thr³²Ala or FOXO3a Ser²⁵³Ala or FOXO3a Ser³¹⁵Ala. Cells were serum-starved 16 hours before were they treated with 1 μ M GnRH for 24 hours. Samples were then assayed for luciferase activity. In all panels open bars denote no stimulation and close bars denote GnRH stimulation. Data are shown as mean fold

change over NS±SEM from at least 3 independent experiments. (b is significantly different from a, and c is significantly different from a and b, d is also significantly different from a, b and c; P<0.05)

3.2.2.3 *GnRH-induced FOXO3a transcriptional activity occurs via G_q, Akt- and IKK-mediated signalling pathways*

Based on the finding that the transcriptional activity of FOXO3a was enhanced following GnRH treatment, the signalling pathways through which this occurred was examined next.

GnRH induction of GADD45-reporter activity by FOXO3a was inhibited by co-treatment with the G_q inhibitor (YM254890) (**Fig. 3-10A**). Next, the role of Akt phosphorylation in GnRH-induced GADD45-reporter activity via FOXO3a was examined. A specific Akt inhibitor (Akt1/2) (**Fig. 3-10B**) and a dominant-negative Akt construct (DN-Akt) were utilised (**Fig. 3-10C**). Both Akt inhibitor and DN-Akt construct inhibited GnRH-induced GADD45-reporter activity.

FOXO3a can also be phosphorylated by that IκB kinase (IKK), which can inhibit FOXO3a transcriptional activity independently of Akt phosphorylation (Hu *et al.*, 2004). The role of IKK in GnRH-induced GADD45-reporter activity by FOXO3a was examined by utilising an IKK inhibitor (BMS-345541). Following 24 hour GnRH stimulation, GADD45-reporter activity was measured in SCL60 which had been transiently transfected FOXO3a-WT, in the presence of the IKK inhibitor. The IKK inhibitor inhibited GnRH-induced GADD45-reporter activity in cells with FOXO3a-TM over-expression (**Fig. 3-10D**). Subsequently, the role of IKK phosphorylation in GnRH-induced GADD45-reporter activity via FOXO3a was examined. Phosphorylation of FOXO3a at Ser⁶⁴⁴ has been reported to inhibit

FOXO3a transcriptional activity (Hu *et al.*, 2004). To investigate this, a mutant FOXO3a-TM-reporter was generated by replacing Ser⁶⁴⁴ with Alanine (FOXO3a-TM Ser⁶⁴⁴Ala). Over-expression of FOXO3a-TM Ser⁶⁴⁴Ala did not inhibit GnRH-induced GADD45-reporter activity compared to over-expression of FOXO3a-TM (**Fig. 3-10E**), suggesting that the role of IKK in regulating FOXO3a in response to GnRH, is not mediated by phosphorylation at the Ser⁶⁴⁴ site.

Having demonstrated that GnRH regulates FOXO3a transcriptional activity via Akt, the role of PDK1 phosphorylation at Thr³⁰⁸ and Ser⁴⁷³ in GnRH-induced GADD45-reporter activity via FOXO3a, was examined. PDK1 is involved in the PI3K/Akt signalling pathway since it phosphorylates Akt at Thr³⁰⁸ and Ser⁴⁷³, resulting in full Akt activation. A time-course of GnRH treatment revealed the phosphorylation of Akt at Thr³⁰⁸ over 60 minutes, reaching maximal phosphorylation after 5 and 10 minutes (**Fig. 3-11 (upper panel)**). No phosphorylation of Ser⁴⁷³ was detected in response to GnRH.

The involvement of three other potential mediators (PI3K inhibitor (Wortmannin), PKC inhibitor (Ro-31-8220) and PLC inhibitor (ET-18-OCH3)) was next examined by utilising the appropriate chemical inhibitors. None of them prevented GnRH-induced GADD45-reporter activity with FOXO3a over-expression (**Supplementary Fig. 7-6**).

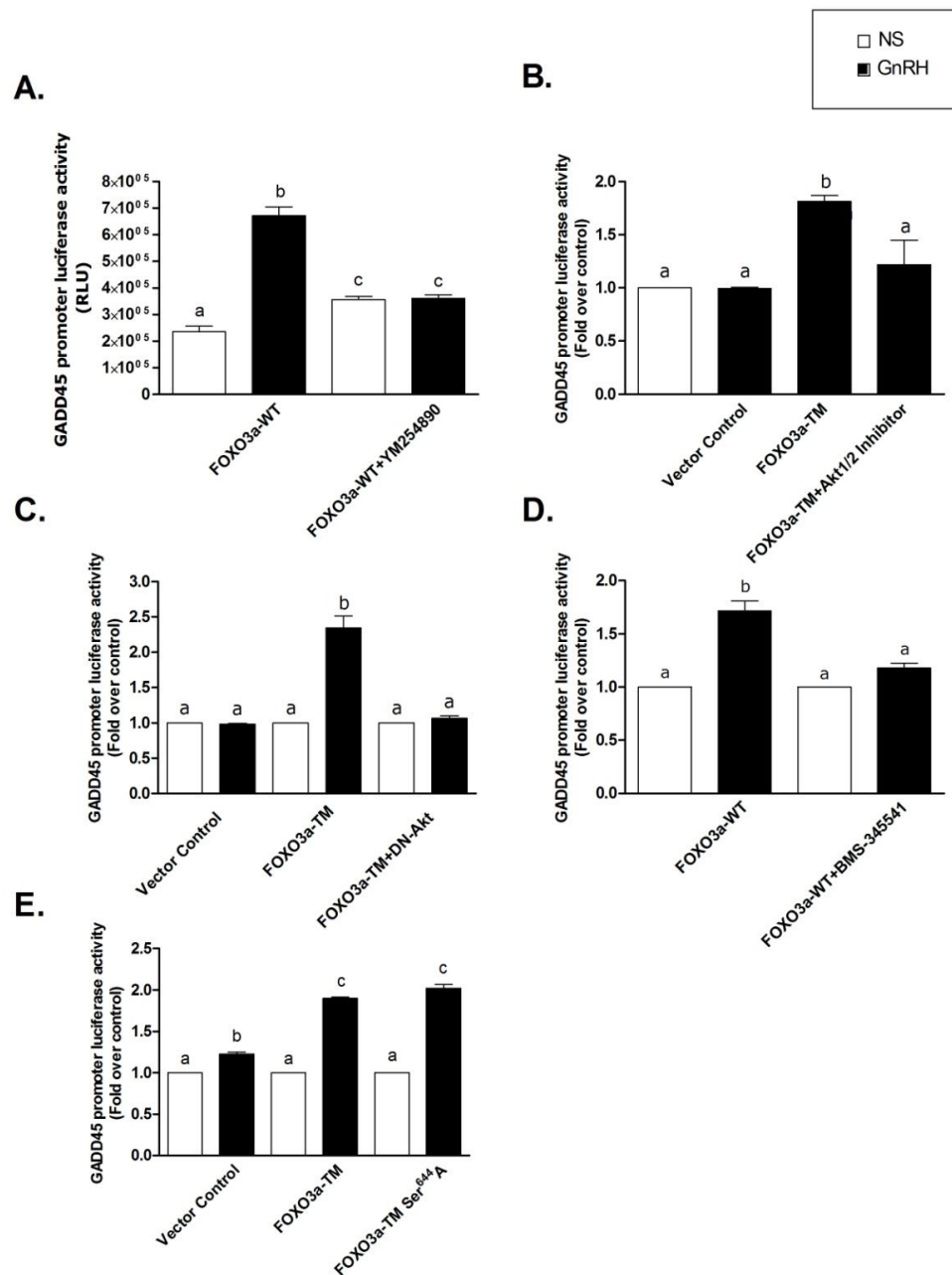


Figure 3-10: Role of Gq, Akt and IKK in the GnRH-induced GADD45-reporter activity.

(A, B, C, D and E) SCL60 cells were transiently transfected with 1 μ g of GADD45-reporter and (B, C and E) 9 μ g of control vector (pcDNA3.1) or (A and D) FOXO3a-WT or (B, C and E) FOXO3a-TM or (E) FOXO3a-TM Ser⁶⁴⁴A or (C) DN-Akt. Cells were serum-starved 16 hours before were pre-treated for 30 minutes with (D) 1,5 μ M BMS-345541 inhibitor or (B) 1 μ M Akt1/2 inhibitor or (A) 100nM YM254890 and thereafter were treated with 1 μ M GnRH for 24 hours. Samples were assayed for luciferase activity. In all panels open bars denote no stimulation and close bars denote GnRH stimulation. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a, and c is significantly different from a and b; $P < 0.05$)

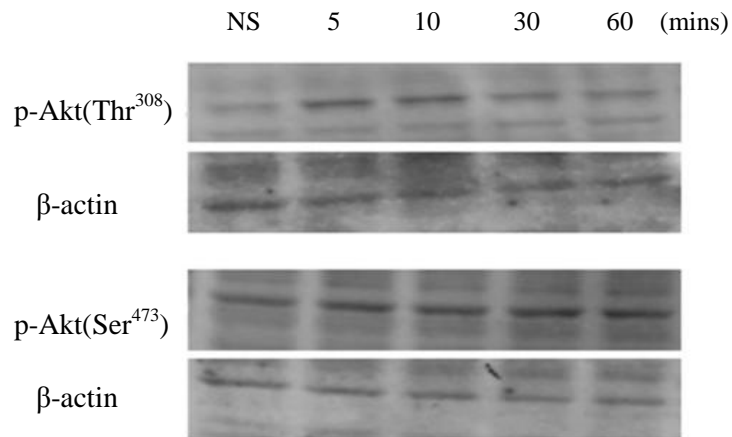


Figure 3-11: Immunoblots of GnRH-induced Akt phosphorylation.

SCL60 cells were serum-starved for 16 hours before treatment with 1 μ M of GnRH or left untreated (NS). Protein level were analysed by Western Blot using the appropriate antibody. Blots are representative of 3 independent experiments.

3.2.2.4 *The role of β -catenin in GnRH-induced GADD45-reporter activity*

An interaction between β -catenin and FOXO3a has previously been reported (Essers *et al.*, 2005). Thus, a role for β -catenin as co-factor to regulate the transcriptional activity of FOXO3a following GnRH treatment was examined. When nuclear β -catenin levels were increased by LiCl treatment, the transcriptional activity of FOXO3a increased in the absence of GnRH treatment (**Fig. 3-12A**). However, when cells were treated with both LiCl and GnRH GnRH-induced GADD45-reporter activity by FOXO3a-WT was decreased (**Fig. 3-12B**) compared with the increase observed with GnRH treatment alone (**Fig. 3-12C**). These data suggest the β -catenin-mediated effect of GnRH-induced FOXO3a transcriptional activity, is not a dominant regulatory mechanism of GnRH-induced FOXO3a activity.

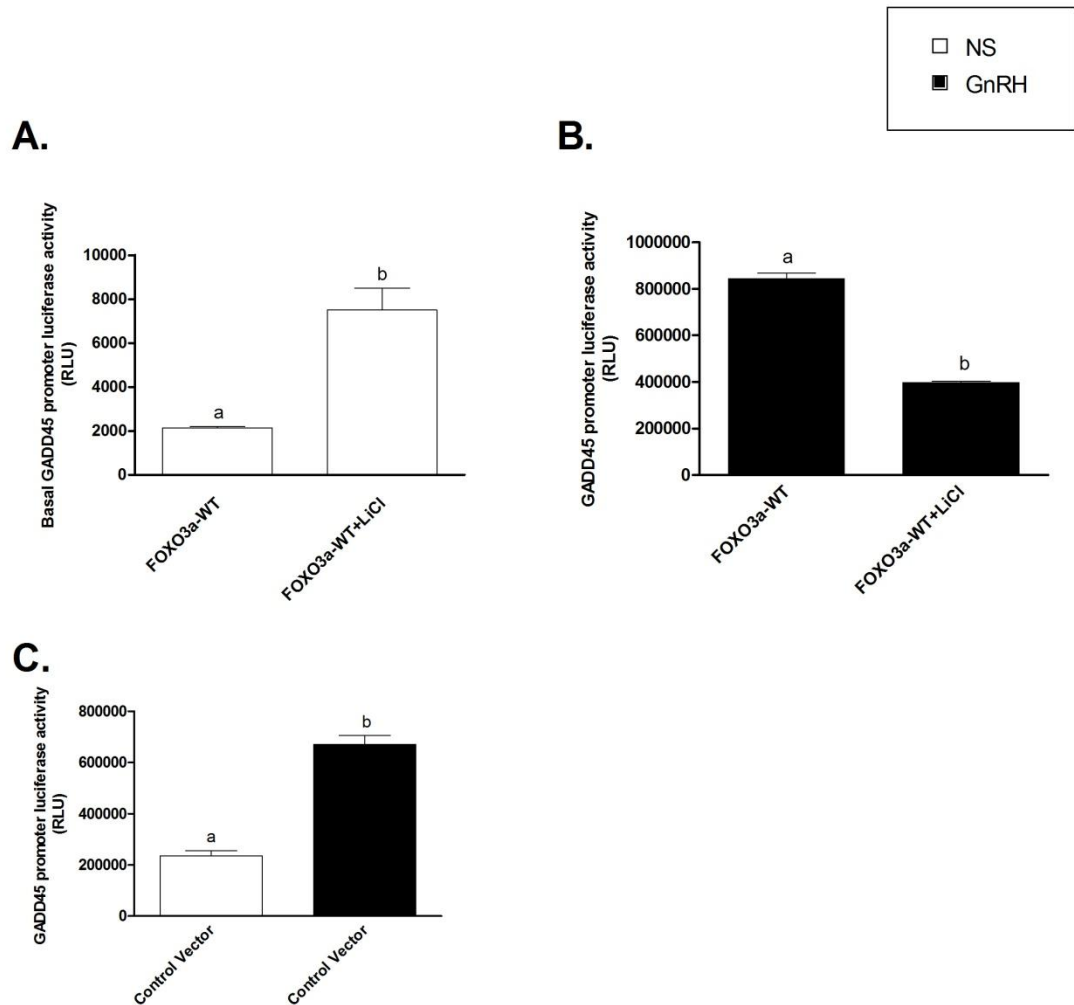


Figure 3-12: GnRH-induced transcriptional activity of FOXO3a is mediated by β -catenin.

SCL60 cells were transfected with 3.5 μ M of (A, B and C) GADD45-reporter or (A and B) 6.5 μ M of FOXO3a-WT or (C) control vector. Cells were serum-starved 16 hours before (B and C) cells were treated for 24 hours with 1 μ M GnRH and/or (A and B) 20 μ M of LiCl. Samples were then assayed for luciferase activity. In all panels open bars denote no stimulation and close bars denote GnRH stimulation. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a; $P < 0.05$)

3.2.2.5 *The role of acetylation in GnRH-induced GADD45-reporter activity*

Acetylation is another post-translational modification that regulates FOXO transcriptional activity. In order to examine whether GnRH targets FOXO3a transcriptional activity by regulating FOXO3a acetylation, the acetyltransferase

SIRT1 was utilised. SIRT1 is a de-acetylase which de-acetylates FOXO3a leading to its nuclear translocation (Motta *et al.*, 2004).

Over-expression of SIRT1 decreased basal GADD45-reporter activity by 2-fold (**Fig. 3-13A**). However, following a 24 hour treatment with GnRH, over-expression of SIRT1 increased GADD45-reporter activity compared with the vector control (**Fig. 3-13B**). In order to examine the effect of SIRT1 on the transcriptional activity of FOXO3a in response to GnRH, SCL60 cells were co-transfected with FOXO3a-WT, or with FOXO3a-WT and SIRT1. GADD45-reporter activity was increased approximately 3-fold in response to GnRH, whether SIRT1 was co-expressed or not (**Fig. 3-13C**). Finally, over-expression of SIRT1 decreased the basal level of GADD45-reporter activity when co-expressed with FOXO3a-WT (**Fig. 3-13D**). The data suggest FOXO3a acetylation is not a dominant regulatory mechanism of GnRH-induced FOXO3a transcriptional activity.

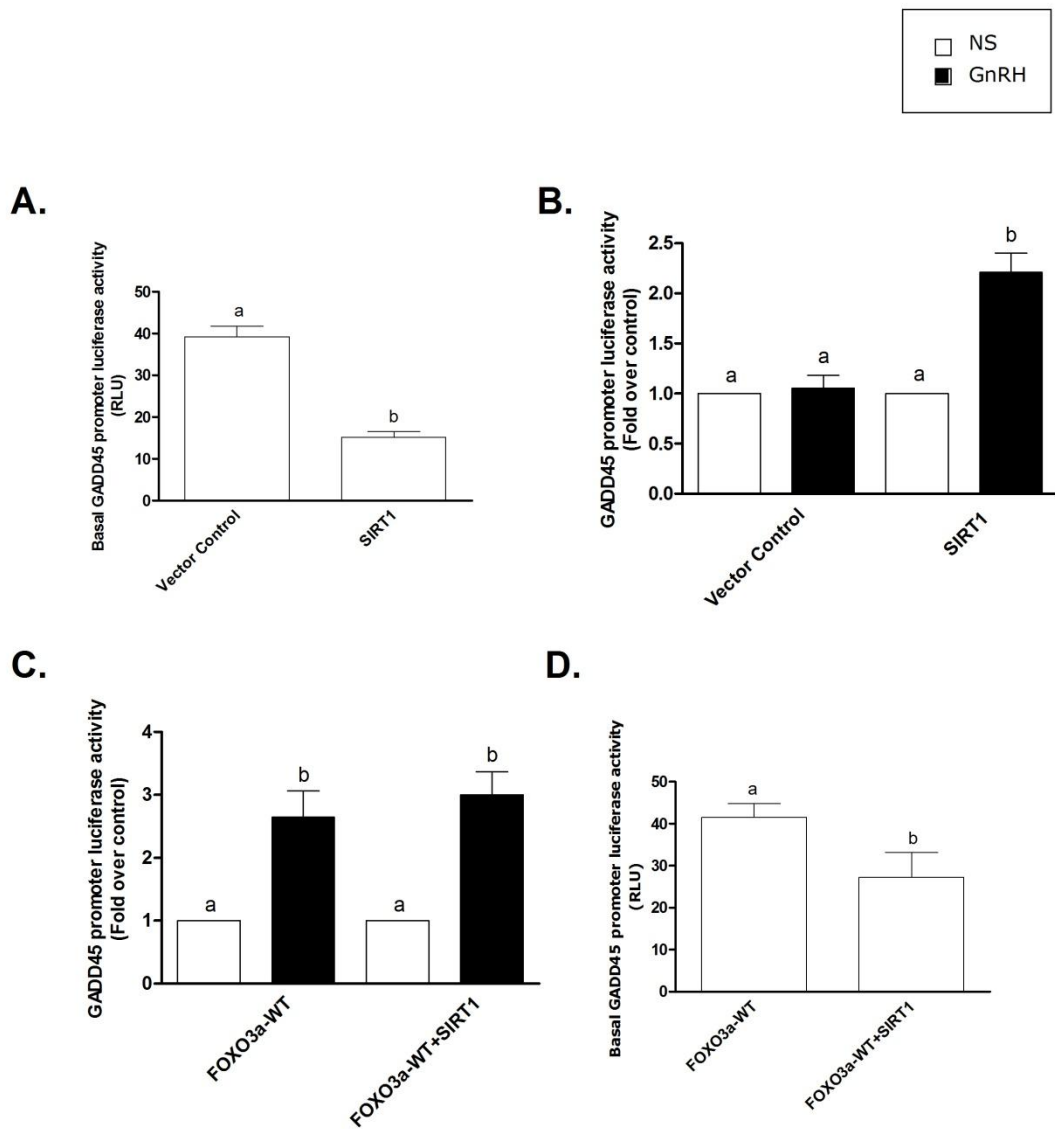


Figure 3-13: The role of acetylation in GnRH-induced transcriptional activity by FOXO3a.

SCL60 cells were transfected with 3.5 μ M of GADD45-reporter and 3.5 μ M of control vector (pcDNA3.1) or (C and D) 3.5 μ M of FOXO3a-WT or (A, B, C and D) 3.5 μ M of SIRT1. Cells were serum-starved 16 hours (B and C) before they were treated for 24 hours with 1 μ M GnRH. Samples were then assayed for luciferase activity. In all panels open bars denote no stimulation and close bars denote GnRH stimulation. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a; P<0.05)

3.2.3 GnRH targets the expression of known FOXO3a-target genes by regulating FOXO3a transcriptional activity

3.2.3.1 *GnRH regulation of FOXO3a-target gene expression over 24 hours and 4 days*

Having demonstrated that GnRH regulates GADD45-reporter activity by targeting FOXO3a, the mRNA expression levels of several other FOXO-target genes was assessed. The expression levels of *GADD45*, *FasL*, *p27kip1* and *p21Cip1* mRNAs in response to GnRH was measured by Taqman Quantitative RT-PCR analysis. Significant up-regulation of *GADD45* mRNA expression was observed over 24 hours of GnRH treatment (**Fig. 3-14A**), and 4 days of D-Trp⁶-GnRH I treatment (**Fig. 3-15A**). D-Trp⁶-GnRH I, a GnRH agonist, is a more stable analogue of GnRH, and was therefore used for treatment beyond 24 hours. The expression of *FasL* was reduced over the first 24 hours of treatment (**Fig. 3-14B**), but was up-regulated after 24 hours of treatment with GnRH (**Fig. 3-15B**). *p27Kip1* mRNA expression was reduced following treatment over 24 hours (**Fig. 3-14C**), or 4 days of treatment (**Fig. 3-15C**). *p21Cip1* mRNA expression was up-regulated after 4 and 6 hours (**Fig. 3-14D**), but there was no change over 4 days of treatment (**Fig. 3-15D**). There was no significant change in the *BIM* mRNA level in response to GnRH or D-Trp⁶-GnRH I treatment over 24 hours or 4 days (**Fig. 3-14E** and **3-15E**).

3.2.3.2 *GnRH regulates expression of GADD45 and FasL by FOXO3a*

Having identified that GnRH regulates the expression of the FOXO-target genes, *GADD45*, *FasL*, *p27Kip1*, *p21Cip1* and *BIM*, the role of FOXO3a in GnRH-regulation of the above genes was examined next. The involvement of FOXO3a in

GnRH-induced GADD45 expression has been already demonstrated utilising a GADD45-reporter in Section 3.2.2.1. The involvement of FOXO3a in GnRH-induced GADD45 expression was further examined here by utilising qRT-PCR.

Cells were transfected with control vector, FOXO3a-TM or FOXO3a-TM Δ DB. Following a time-course treatment with D-Trp⁶-GnRH I, the mRNA expression levels of *GADD45*, *FasL*, *BIM* and *p27Kip1* genes were measured. The pattern of mRNA expression for *GADD45* and *FasL* was similar in cells transfected with control vector or FOXO3a-TM Δ DB, (**Fig. 3-16A** and **B**). However, over-expression of FOXO3a-TM constructs followed by D-Trp⁶-GnRH I treatment, up-regulated the expression of GADD45 (**Fig. 3-16B**). This was consistent with previous results from luciferase assays (see Section 3.2.2.1). FOXO3a-TM over-expression down-regulated the expression of FasL compared to the control vector (**Fig. 3-16A**).

The expression of BIM, p21Cip1 and p27Kip1 was also measured in a time-course treatment with D-Trp⁶-GnRH I, in cells which had been transfected with control vector, FOXO3a-TM or FOXO3a-TM Δ DB. The expression pattern of each of these genes suggested that GnRH does not regulate BIM, p27Kip1 and p21Cip1 expression by FOXO3a (**Supplementary Fig. 7-7**). In particular, over-expression of FOXO3a-TM showed no difference in the expression patterns of any of these genes compared to vector control or FOXO3a-TM Δ DB over-expression.

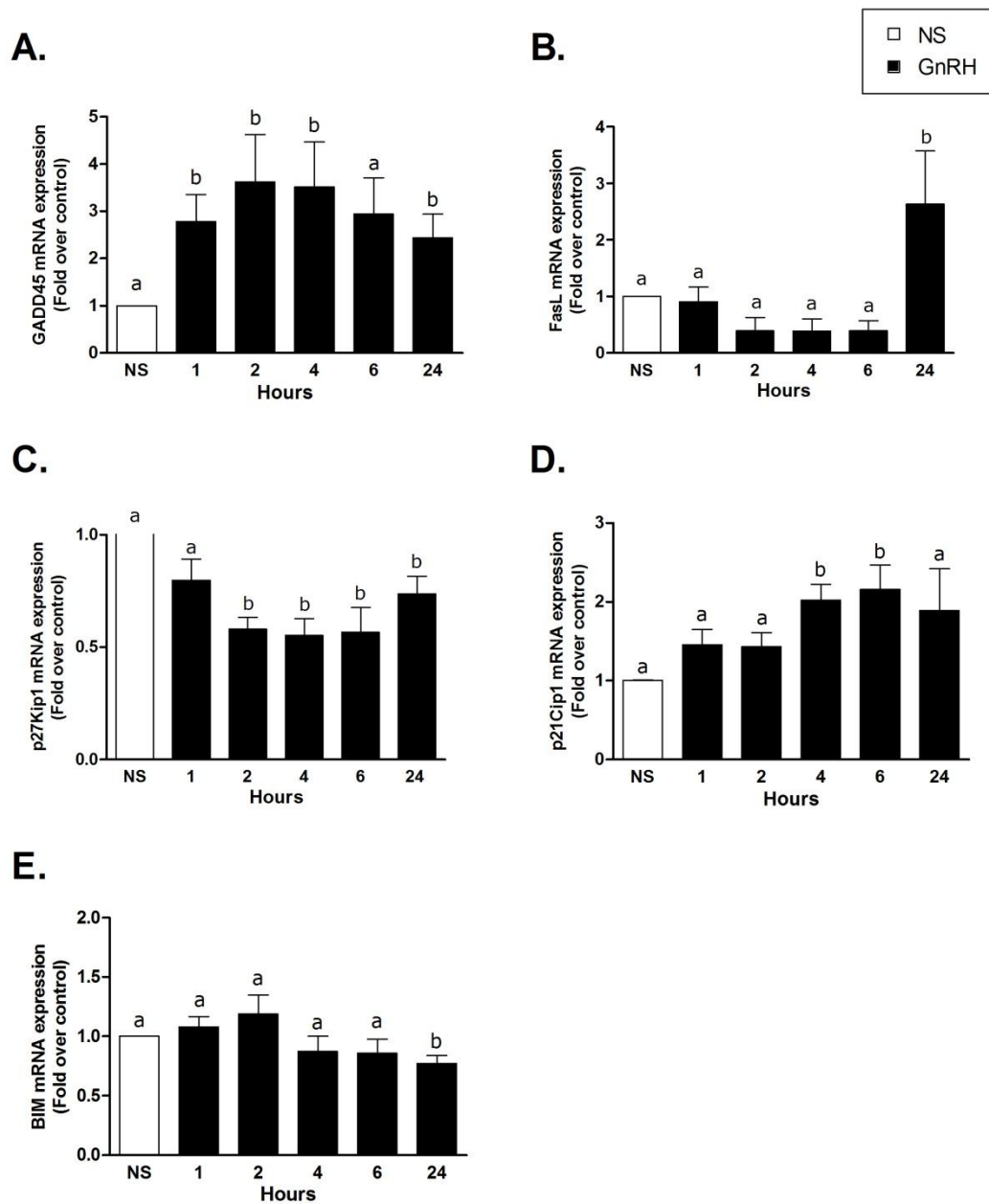


Figure 3-14: Effect of GnRH on FOXO-target genes mRNA expression in SCL60 cells over 24 hours.

Relative mRNA expression of (A) *GADD45*, (B) *FasL*, (C) *p27Kip1*, (D) *p21Cip1* and (E) *BIM* in SCL60 cells treated with 1 μ M GnRH over 24 hours or vehicle (NS), as determined by quantitative RT-PCR analysis. Data are shown as mean fold over NS \pm SEM over 24 hour treatment from at least 3 independent experiments. (b is significantly different from a; P<0.05)

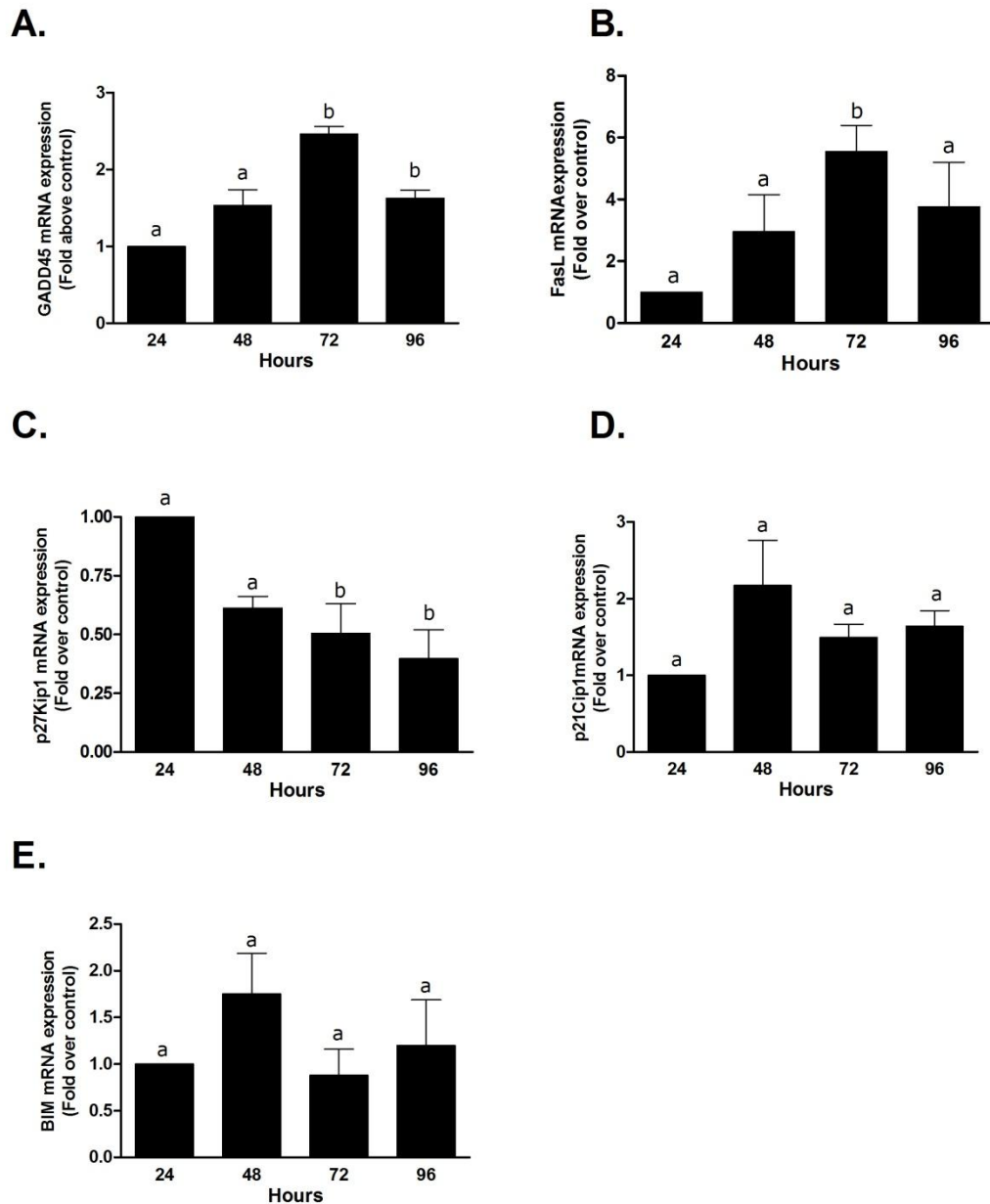


Figure 3-15: Effect of GnRH on FOXO-target genes mRNA expression in SCL60 cells over 4 days.

Relative mRNA expression of (A) *GADD45*, (B) *FasL*, (C) *p27Kip1*, (D) *p21Cip1* and (E) *BIM* in cells treated with $1\mu\text{M}$ D-Trp⁶-GnRH I or left untreated (each value represent the mRNA expression level for treated over untreated cells at each time point (e.g. 24h, 48h)) over 4 days, was determined by quantitative RT-PCR analysis. Data are shown as mean fold over the treated/untreated \pm SEM over 4 days from at least 3 independent experiments. (b is significantly different from a; $P < 0.05$)

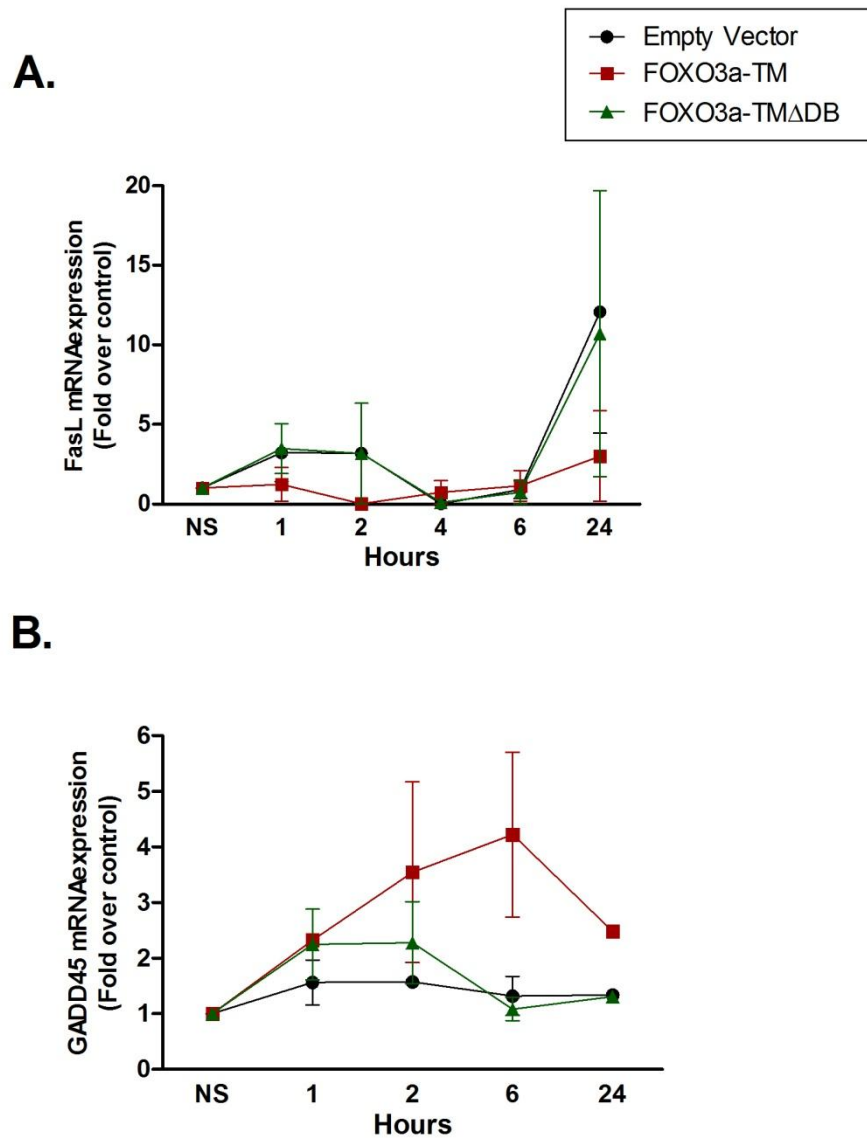


Figure 3-16: Role of FOXO3a in FOXO-target genes mRNA expression following GnRH treatment.

SCL60 cells were transfected with 10 μ g of FOXO3a-TM or FOXO3a-TM Δ DB. Relative mRNA expression of (A), *FasL* (B) and *GADD45* in cells treated with 1 μ M D-Trp⁶-GnRH over indicated time intervals or vehicle (NS) was determined by quantitative RT-PCR analysis. Data are shown as mean fold over NS \pm SEM over 24 hour treatment from at least 3 independent experiments.

3.2.3.3 *FOXO1 and FOXO3a auto-regulation*

A recent study has demonstrated that the FOXO1 promoter contains FOXO binding sites, suggesting that FOXOs can regulate their own expression (Essaghir *et al.*, 2009). In order to examine whether there is an auto-regulation of *FOXO* gene expression in response to GnRH, *FOXO1* and *FOXO3a* mRNA levels in SCL60 cells which have been transfected with a control vector, FOXO1-WT or FOXO3a-WT were assessed. As expected, over-expression of FOXO1-WT and FOXO3a-WT caused an increase in *FOXO1* (**Fig. 3-17A**) and *FOXO3a* mRNA levels (**Fig. 3-18A**) respectively, compared to vector control. In contrast, over-expression of FOXO3a-WT did not increase *FOXO1* mRNA levels (**Fig. 3-17A**), and over-expression of FOXO1-WT did not increase *FOXO3a* mRNA levels (**Fig. 3-18A**), compared to vector control.

To further investigate a possible FOXO auto-regulatory effect in response to GnRH, a time-course treatment with GnRH was performed and the mRNA level of *FOXO1* and *FOXO3a* was measured in cells which had been transfected with FOXO3a-WT and FOXO1-WT constructs, respectively. Over-expression of FOXO3a-WT did not appear to regulate *FOXO1* gene expression (**Fig. 3-17B**). Over-expression of FOXO1-WT displays a trend towards a reduction (this reduction did not reach statistical significance with n=3) for *FOXO3a* gene expression, in response to GnRH compared to the vector control (**Fig. 3-18B**).

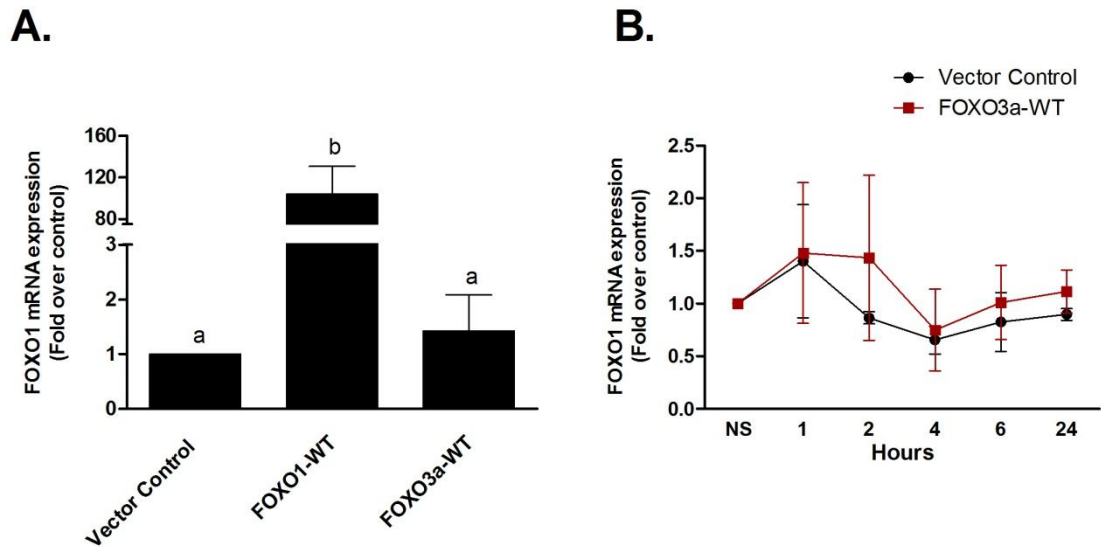


Figure 3-17: Effect of FOXO1 and FOXO3a on *FOXO1* mRNA expression in response to GnRH.

(A) SCL60 cells were transfected with 10 μ g of control vector (pcDNA3.1) or FOXO1-WT or FOXO3a-WT. Relative mRNA expression levels of *FOXO1* in cells that were treated with 1 μ M GnRH for 24 hours was determined by quantitative RT-PCR analysis. (B) SCL60 cells were transfected with 10 μ g of control vector (pcDNA3.1) or FOXO3a-WT. Relative mRNA expression levels of *FOXO1* in cells that were treated with 1 μ M GnRH over indicated time intervals or left untreated (NS), was determined by quantitative RT-PCR analysis. Data are shown as mean fold over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a; $P < 0.05$)

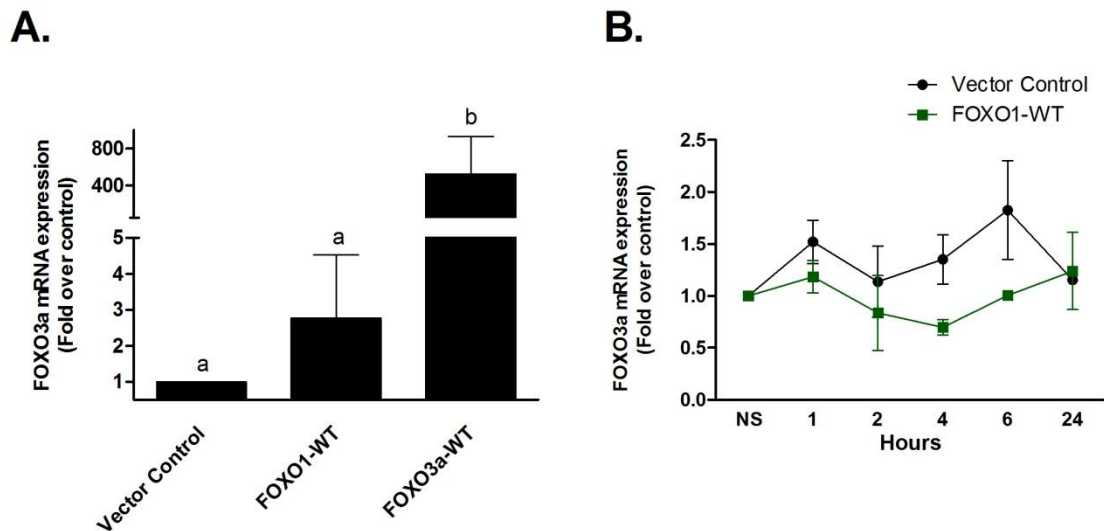


Figure 3-18: Effect of FOXO1 and FOXO3a on *FOXO3a* mRNA expression in response to GnRH.

(A) SCL60 cells were transfected with 10 μ g of control vector (pcDNA3.1) or FOXO1-WT or FOXO3a-WT. Relative mRNA expression levels of *FOXO3a* in cells that were treated with 1 μ M GnRH for 24 hours was determined by quantitative RT-PCR analysis. (B) SCL60 cells were transfected with 10 μ g of control vector (pcDNA3.1) or FOXO3a-WT. Relative mRNA expression levels of *FOXO3a* in cells that were treated with 1 μ M GnRH over indicated time intervals or left untreated (NS), was determined by quantitative RT-PCR analysis. Data are shown as mean fold over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a; P<0.05)

3.2.3.4 *GnRH does not regulate FOXO1 and FOXO3a gene expression and protein synthesis*

In the next set of experiments, the involvement of GnRH in regulating *FOXO* mRNA levels and protein expression was examined. Cells were treated with GnRH over 24 hours or with D-Trp⁶-GnRH I over 5 days, and mRNA levels and protein expression of FOXO1 and FOXO3a were assessed by qRT-PCR or Western Blot analysis, respectively. The results indicated that GnRH does not regulate *FOXO1* and *FOXO3a* gene expression (Fig. 3-19) or protein levels (Fig. 3-20). To further examine the effect of GnRH on FOXO protein levels, immunofluorescence confocal

microscopy was used to visualise FOXO1 and FOXO3a levels in cell following treatment with GnRH for 6 and 24 hours. FOXO3a was primarily located in the nucleus in untreated and treated cells, and GnRH treatment appears not to change the level of total FOXO3a (t-FOXO3a) (**Fig. 3-21**). FOXO1 appears to be localised both in the nucleus and cytoplasm in untreated and in 6-hour treated cells. 24 hour treatment with GnRH appears to cause FOXO1 translocation to the nucleus. (**Fig. 3-22**).

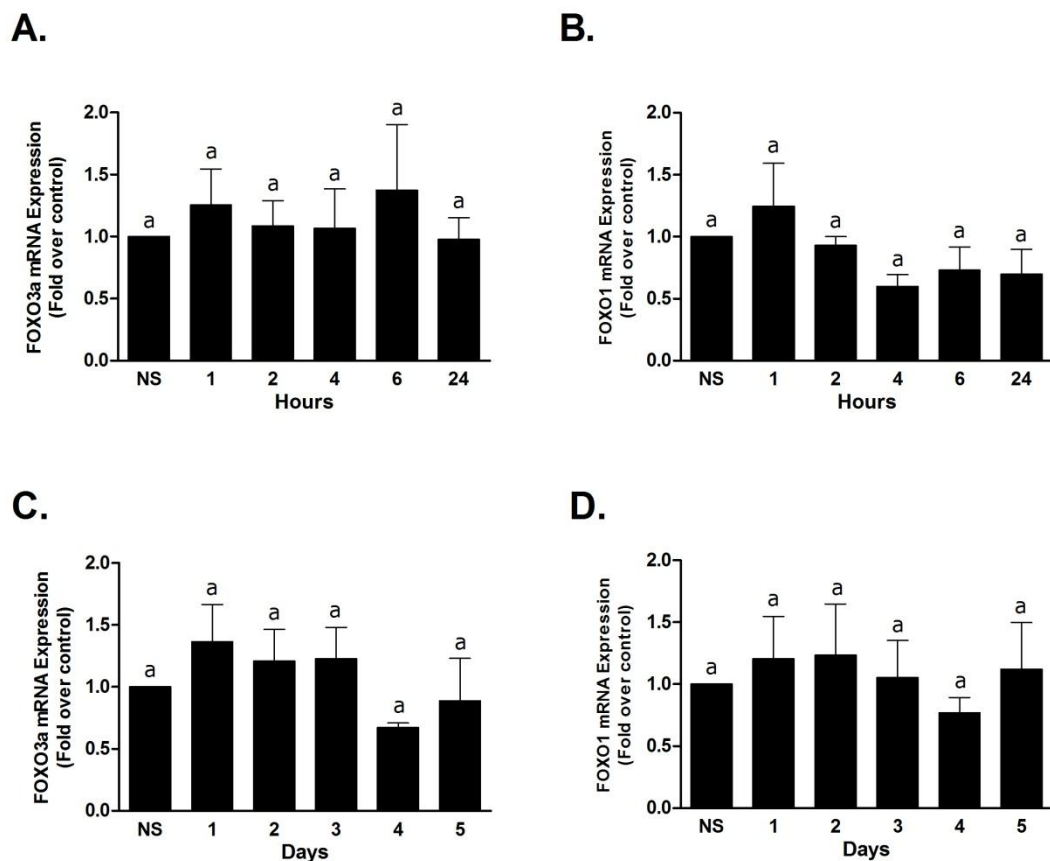


Figure 3-19: Effect of GnRH on FOXO1 and FOXO3a mRNA expression in SCL60 cells.

Relative mRNA expression of FOXO3a and FOXO1 in SCL60 that were treated (**A** and **B**) with 1 μ M GnRH for 24 hours (**C** and **D**) or with 1 μ M D-Trp⁶-GnRH I over 5 days, or vehicle (NS), was determined by qRT-PCR analysis. Data are shown as mean fold over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a; P<0.05)

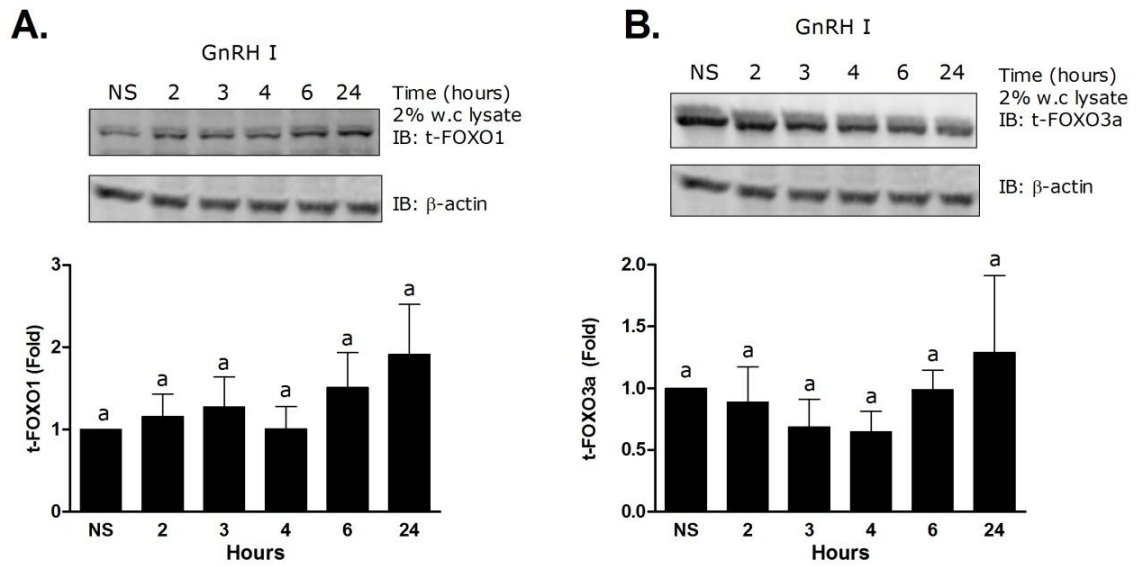


Figure 3-20: Effect on total FOXO1 (t-FOXO1) and total FOXO3a (t-FOXO3a) protein levels following GnRH treatment.

SCL60 cells were treated with 1 μ M of GnRH or left untreated (NS). Proteins level were analysed using the appropriate antibody. Quantification of the immunoblots was done by using β -actin as a loading control. Data are shown as mean fold over NS \pm SEM from at least 3 independent experiments.

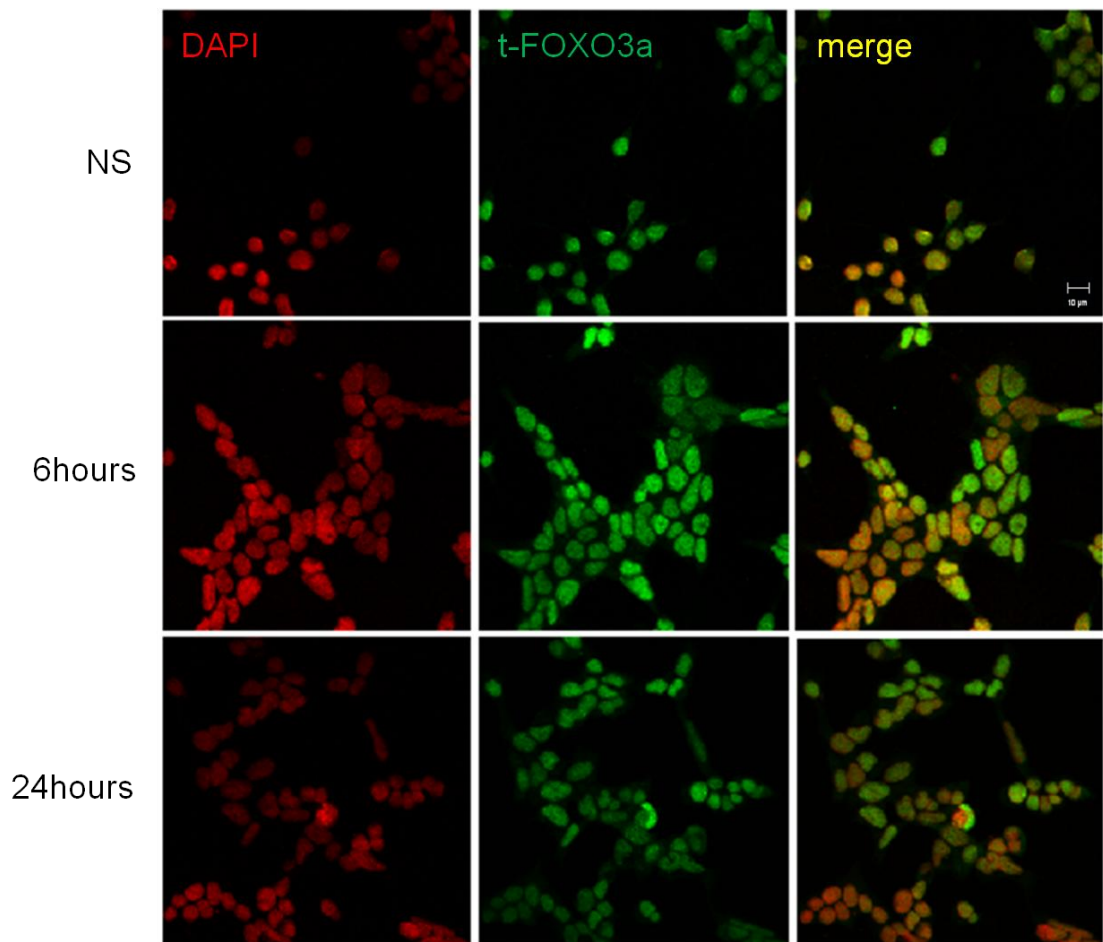


Figure 3-21: t-FOXO3a levels following GnRH stimulation in SCL60 cells.

Immunofluorescence confocal microscopy was used to visualise the change in t-FOXO3a levels (green) following 1 μ M GnRH treatment at the time intervals indicated. DAPI (red) is a marker for nucleus and co-localisation is indicated in yellow.

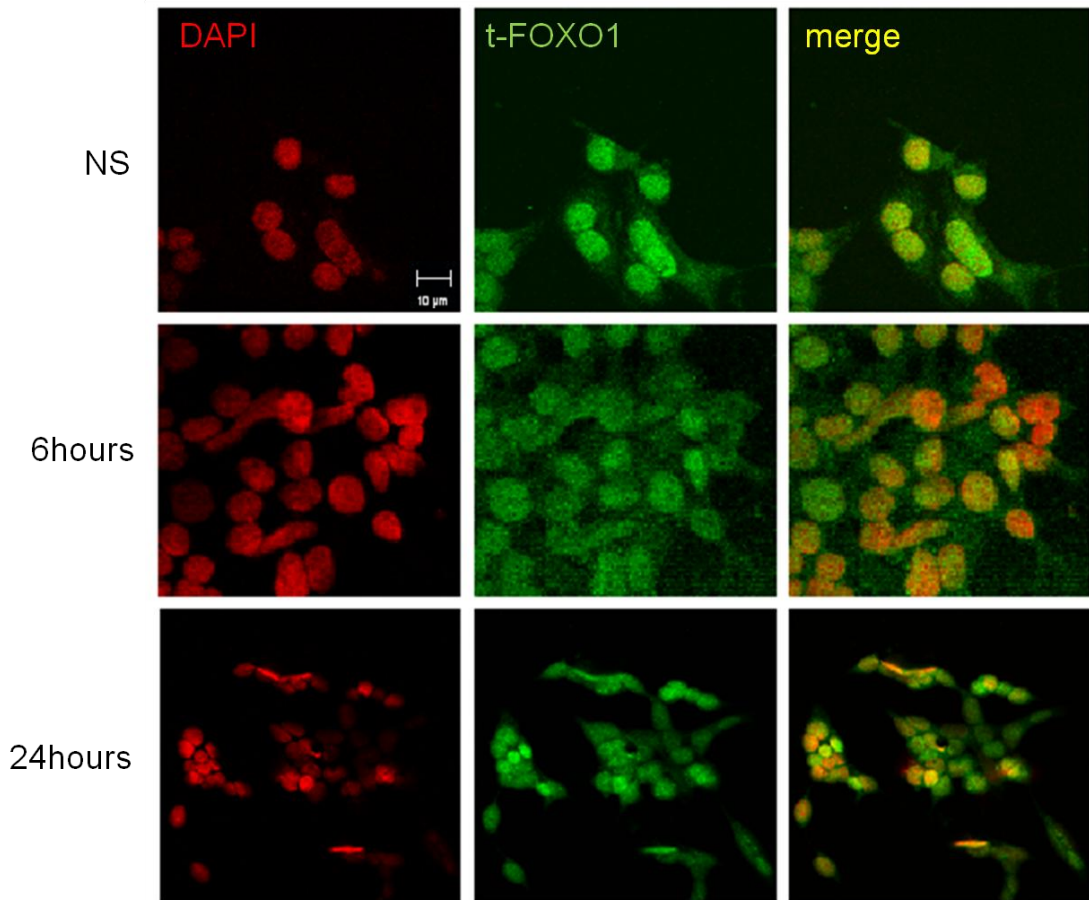


Figure 3-22: t-FOXO1 levels following GnRH stimulation in SCL60 cells.

Immunofluorescence confocal microscopy was used to visualise the change in t-FOXO1 levels (green) following 1μM GnRH treatment at the time intervals indicated. DAPI (red) is a marker for nucleus and co-localisation is indicated in yellow.

3.2.4 *Monitoring the role of FOXO3a in cell-cycle regulation in response to GnRH*

Up-regulation of any of the FOXO-target genes, mediate a specific cell-growth inhibitory effect. For example, up-regulation of FasL mediates apoptosis whereas up-regulation of GADD45 mediates blocking of the cell-cycle at G2 phase. Having shown that GnRH treatment induces the expression of these genes, cell-cycle analysis was performed in response to D-Trp⁶-GnRH I treatment. The reason for this

experiment was to examine whether the cell-cycle profile of SCL60 cells and PWE-1-NB26-3 prostate cancer cells followed the effect of the GnRH-induced genes, in response to D-Trp⁶-GnRH I treatment. The proportion of cells found in the sub-diploid population increased approximately by 3-fold in SCL60 cells, but did not increase in PWE-1-NB26-3. Furthermore, the proportion of SCL60 cells in G1 phase were decreased from 68.4% to 31.6%, while the proportion of cells in G2 phase were increased from 14.6% to 40.3%, (**Fig. 3-23B and D**). In contrast, the proportion of PWE-1-NB26-3 cells found in G2 phase was decreased from 77.9% to 66.6% and in G1 from 9% to 17.3% (**Fig. 3-23A and C**).

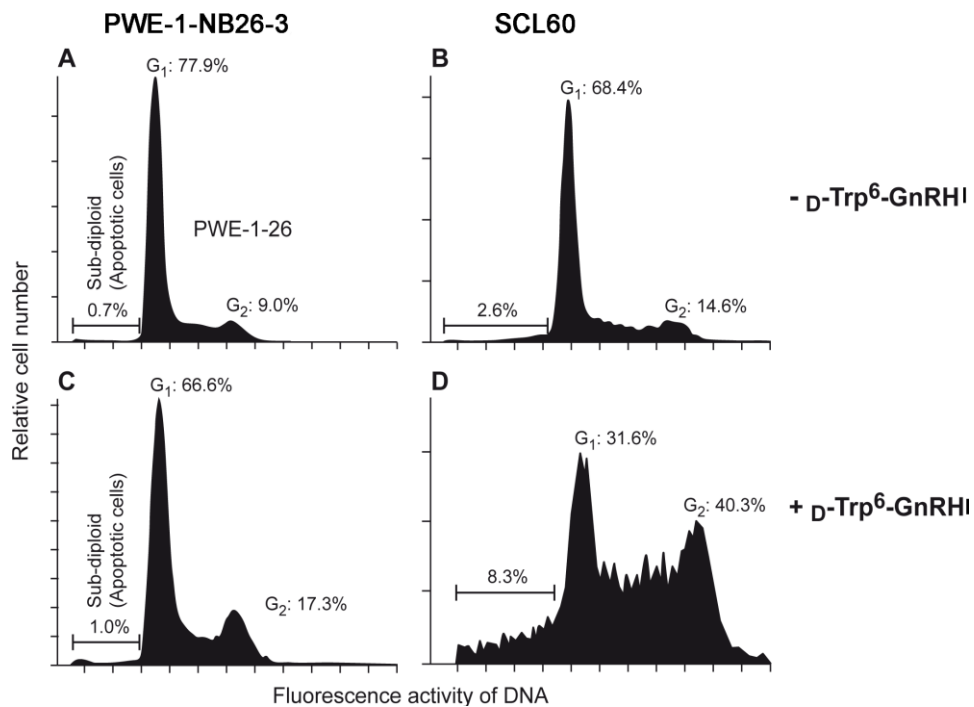


Figure 3-23: Flow cytometry analysis in PWE-1-NB26 and SCL60 cells and cells following D-Trp⁶-GnRH treatment.

Cells were treated with 100nM D-Trp⁶-GnRH I for 96 hours, fixed, permeabilised and stained with Hoechst for analysis by flow cytometry. The graphs represent a typical/single experiment. (Data analysis (gating and computational analysis) was performed with the cooperation of Ms. Shonna Jonhston from University of Edinburgh, Centre for Inflammation Research, Flow Cytometry Facility).

3.3 DISCUSSION

Scientific interest in FOXO transcription factors has increased markedly in the past eight years. Both *in vivo* and *in vitro* studies have been conducted in order to gain an understanding of the role of FOXOs in diverse cellular processes including cell proliferation, and the role of FOXO in diseases, including diabetes and cancer. However, to date, the mechanisms that regulate FOXO function are not well understood.

In the present study, the impact of GnRH on FOXO function was examined. Taken together, the results presented in this Chapter demonstrate that GnRH modulates FOXO3a function in a model HEK293 cell line (SCL60 cells). The specific mechanisms by which GnRH modulates the function of FOXOs were also examined. The dominant GnRH-initiated pathway that regulates the function of FOXOs was found to involve G_q-coupling and Akt-, IKK-dependent signalling (**Fig. 3-10**).

GnRH treatment of SCL60 cells for 24 hours resulted in increased FOXO transcriptional activity. The transcriptional activity initially measured was probably the combined transcriptional activity from all three FOXOs; FOXO1, FOXO3a and FOXO4. In order to begin to understand the mechanisms underlying GnRH-induced FOXO transcriptional activity, the effect of GnRH on FOXO1 and FOXO3a phosphorylation at their Akt phosphorylation sites was examined (**Fig. 3-1 & SFig. 7-1**). This was considered to be a reasonable approach since phosphorylation of FOXOs results in their translocation to the cytoplasm, and therefore the inhibition of their transcriptional activity (van der Horst and Burgering, 2007). Indeed, GnRH treatment initially (5-60 minutes) led to FOXO3a phosphorylation at Ser²⁵³, which is

an Akt-targeted phosphorylation site (**Fig. 3-24**). However, FOXO1 phosphorylation was not detected.

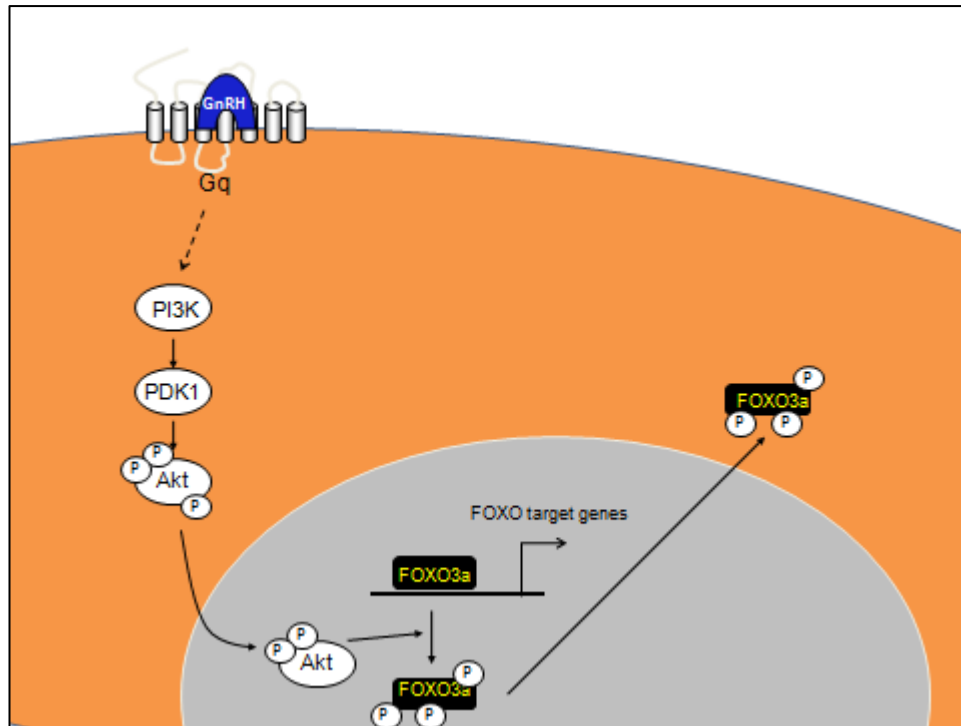


Figure 3-24: Regulation of FOXO3a by phosphorylation following GnRH treatment.

Treatment of SCL60 cells with GnRH leads to FOXO3a phosphorylation at Ser²⁵³ by a G_q-coupled, PI3K/Akt-dependent signalling pathway (direct activation of PI3K by GnRH via G_q has not been demonstrated in this study). Phosphorylation of FOXO3a by this pathway leads to its cytoplasmic localisation. Broken lines indicate an indirect regulation.

The targeting of FOXO3a phosphorylation at Ser²⁵³ by GnRH suggested that the change in phosphorylation status could result in its redistribution between the nucleus and cytoplasm. This redistribution could have an effect on FOXO3a transcriptional activity. Using confocal microscopy, the cellular redistribution of phosphorylated FOXO3a was visualised. Indeed, phosphorylation of FOXO3a at Ser²⁵³ following GnRH treatment for 5 to 60 minutes, resulted in p-FOXO3a(Ser²⁵³)

translocation to the cytoplasm (**Fig. 3-2**), in line with previous work, in which phosphorylation of FOXO3a, independent of GnRH treatment, led to its cytoplasmic translocation (van der Horst and Burgering, 2007). Phosphorylation at Ser²⁵³ promotes FOXO3a interaction with the 14-3-3 chaperone protein which sequesters FOXO3a in the cytoplasm inhibiting its transcriptional activity (Brunet *et al.*, 1999). Interestingly, the confocal microscopy images revealed punctate nuclear p-FOXO3a(Ser²⁵³) staining (**Fig. 3-2**). These puncta could correspond to a complex of p-FOXO3a(Ser²⁵³) either with chaperones such as 14-3-3 that shuttles FOXOs to the cytoplasm, or with other co-factors, such as β -catenin which act to regulate FOXO3a transcriptional activity.

Next, the role of FOXO3a phosphorylation in regulating its transcriptional activity following GnRH treatment was assessed. Surprisingly, activation of FOXO3a transcriptional activity appeared to be independent of its phosphorylation status (**Fig 3-8**). The finding that the transcriptional activity of FOXO3a-TM was higher than FOXO3a-WT was probably because FOXO3a-TM was retained in the nucleus because it could not be phosphorylated by Akt. Therefore the targeting of FOXO3a phosphorylation by GnRH does not appear to be the dominant mechanism regulating FOXO3a transcriptional activity.

Having shown that GnRH up-regulates GADD45-reporter activity via FOXO3a independently of FOXO3a phosphorylation at its Akt phosphorylation sites, other potential signalling pathways that modulate FOXO transcriptional activity were investigated (**Fig 3-10**). GnRH-induced transcriptional activity of FOXO was found to be mediated by a G_q-coupled, Akt- and IKK-dependent signalling pathway,

although it is not clear whether these three mediators belong to the same signalling pathway (**Fig. 3-25**). However, not all of these factors were involved in the phosphorylation of FOXO3a at Ser²⁵³. This finding appears to support the above suggestion that GnRH-targeted phosphorylation does not appear to be the dominant mechanism regulating FOXO3a transcriptional activity. Furthermore, while the targeting of FOXO3a phosphorylation was mediated by PI3K, a pharmacological inhibitor of PI3K did not inhibit GnRH-induced GADD45-reporter activity (**Fig 7-6**). It is possible that Akt and IKK participate in two different signalling pathways following GnRH treatment. Further investigation is required to determine this. It is important to note that in this thesis only the role of known signalling mediators that are recruited in pathways targeting the regulation of FOXO3a activity have been assessed. However, these signalling pathways may recruit a variety of additional mediators which may play a pivotal role in the regulation of FOXO3a activity, and which would be important to further dissect.

The Akt inhibitor, in contrast to the PI3K inhibitor (Wortmannin) (**Fig. 7-6**), inhibited the FOXO3a-mediated GADD45-reporter activity. This was an unexpected result, since the activation of Akt by PI3K, via the PDK1, is a well-established signalling pathway. PDK1 phosphorylates Akt at Thr³⁰⁸ and Ser⁴⁷³ phosphorylation sites, which is required for its full Akt activation (Liu *et al.*, 2009; Vanhaesebroeck and Alessi, 2000). The finding that GnRH targeted the phosphorylation of Akt at Thr³⁰⁸ suggested that Akt was activated by PDK1 (**Fig 3-11**). The inability of the PI3K inhibitor to inhibit the FOXO3a-mediated GADD45-reporter activity suggested that PDK1 was activated by a PI3K-independent pathway. The mechanism by which PDK1 was activated in response to GnRH remains to be determined.

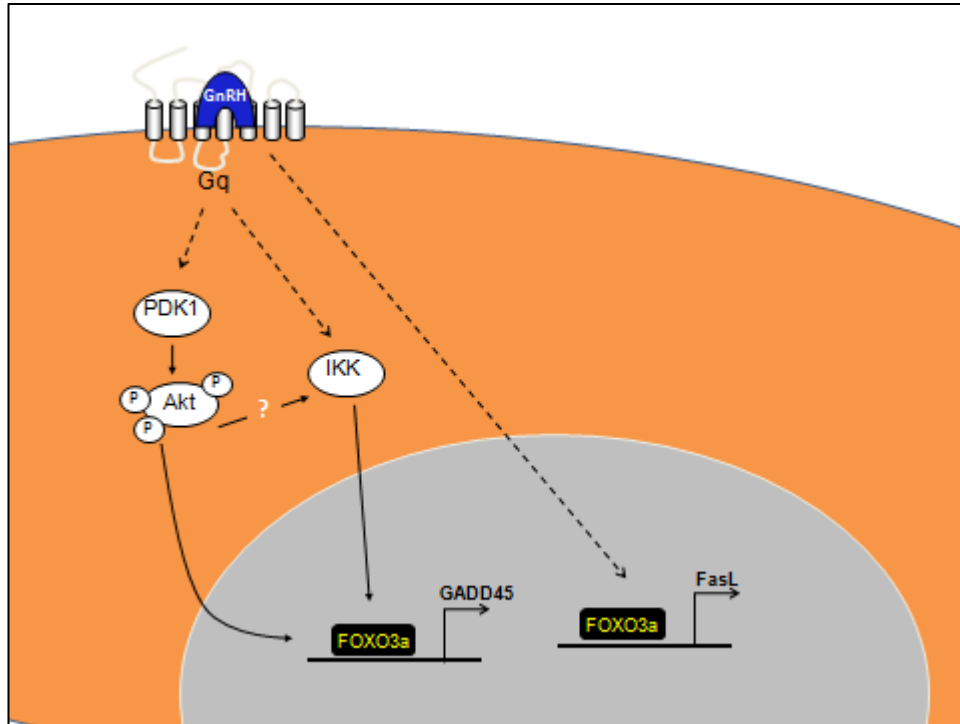


Figure 3-25: Regulation of *GADD45* and *FasL* gene expression by FOXO3a.

GnRH regulates *GADD45* and *FasL* gene expression by regulation of FOXO3a. In addition, GnRH mediates the regulation of FOXO3a transcriptional activity via a Gq-coupled, Akt- and IKK-dependent signalling pathway. Broken lines indicate an indirect regulation.

A further interesting result was the fact that IKK was found to be involved in the control of GADD45-reporter activity in response to GnRH. A role for IKK in the regulation of FOXO3a transcriptional activity has previously been demonstrated in a study conducted by Hu *et al.* (2004). In particular, IKK phosphorylated FOXO3a at Ser⁶⁴⁴ resulting in its translocation to the cytoplasm. However, co-transfection of a FOXO3a-TM Ser⁶⁴⁴Ala construct into SCL60 cells, did not inhibit the GnRH-induced GADD45-reporter activity (Hu *et al.*, 2004). This suggests that in response to GnRH, either IKK phosphorylates FOXO3a at another putative IKK recognition sequence, which could have a similar effect on FOXO3a transcriptional activity, or

that IKK does not mediate FOXO3a transcriptional activity directly, but instead as part of another signalling pathway.

In addition to phosphorylation, acetylation is also an important post-translational modification that is involved in regulating FOXO activity. Previous studies have demonstrated that FOXO de-acetylation by SIRT1 led to its cytoplasmic localisation and a reduction in FOXO transcriptional activity (Motta *et al.*, 2004). In contrast to these results, over-expression of SIRT1 increased GADD45-reporter activity compared to vector control following GnRH stimulation in SCL60 cells (**Fig. 3-13**). It is possible that SIRT1 de-acetylates another transcription factor, other than FOXO3a, which could bind to and regulate GnRH-induced GADD45-reporter activity. In order to test this possibility, SCL60 cells were co-transfected with FOXO3a and SIRT1, and GADD45-reporter activity measured. Co-expression of FOXO3a with SIRT1 reduced basal FOXO3a transcriptional activity compared with over-expression of FOXO3a alone, which is in line with previous work (Motta *et al.*, 2004). Following GnRH treatment, co-expression of FOXO3a and SIRT1 increased GADD45-reporter activity, but the increase was to the same level as observed in cells which had been transfected with FOXO3a alone. This suggested that, although SIRT1 de-acetylates FOXO3a, GnRH-R activation regulates FOXO3a transcriptional activity via an alternative pathway or post-translational modification, which is dominant compared to the pathway that led to FOXO3a acetylation.

The effect of β -catenin on the transcriptional activity of FOXO3a following GnRH stimulation was also examined. β -catenin has previously been shown to interact with FOXO3a (Essers *et al.*, 2005), and β -catenin accumulation in the nucleus following

GnRH stimulation has also been demonstrated (Gardner *et al.*, 2007). Based on these studies, a role of β -catenin in regulating FOXO3a transcriptional activity in response to GnRH was expected. In order to confirm this functional interaction, LiCl treatment was used in order to be able to further increase β -catenin accumulation in the nucleus, above GnRH-mediated β -catenin nuclear accumulation (**Fig. 3-12**). Treating with LiCl increased the transcriptional activity of FOXO3a. However, following treatment with both LiCl and GnRH treatment, the transcriptional activity of FOXO3a decreased. This suggests that GnRH possibly alters the way in which FOXO3a interacts with β -catenin, and that the binding of β -catenin to FOXO3a, following GnRH treatment, down-regulates its transcriptional activity. However, the transcriptional activity of FOXO3a increased following GnRH stimulation alone, even though β -catenin accumulates in the nucleus. Thus, the inhibitory effect of β -catenin on FOXO3a may not be the dominant mechanism for regulating FOXO3a activity in response to GnRH. This may account for the observed increase in FOXO3a transcriptional activity following GnRH stimulation alone in the absence of LiCl.

It is important to note that although LiCl treatment increased β -catenin nuclear accumulation (Rao *et al.*, 2005), it may also target other signalling pathways which could interfere with the GnRH-induced signalling pathways. Therefore, it would be important to further examine the role of β -catenin in regulating FOXO3a transcriptional activity by utilising an alternative regulator for β -catenin cellular levels (other than LiCl), for example Axin, which promotes degradation of cellular β -catenin.

Upon establishing that GnRH treatment targets FOXO3a transcriptional activity, a role for FOXO3a in GnRH-induced cell-growth inhibition and apoptosis was examined. qRT-PCR analysis demonstrated that the expression of apoptotic (*FasL*) and cell-cycle regulatory (*GADD45*, *p21Cip1* and *p27Kip1*) FOXO-target genes were regulated following 4 days of treatment with D-Trp⁶-GnRH I (**Fig. 3-15**). Additionally, the expression profiles of the FOXO-target genes over 4 days of D-Trp⁶-GnRH I treatment was analysed. Following D-Trp⁶-GnRH I stimulation, the expression of cell-cycle regulatory proteins GADD45 and p21Cip1 increased over 24 hours, reaching maximal expression after 6 hours. In contrast, expression of the pro-apoptotic protein FasL and the cell-cycle regulatory protein p27Kip1 decreased over 24 hours of treatment. Further treatment with D-Trp⁶-GnRH I over 3 days increased the expression of FasL and GADD45 proteins, whereas the expression of p27Kip1 protein was down-regulated.

Since GnRH has been shown to target cell-growth inhibition, (Kraus *et al.*, 2006), the above result suggest that D-Trp⁶-GnRH I treatment blocks cell-cycle progress prior to inducing an apoptotis. In particular, the up-regulation of *GADD45* gene expression possibly results in blockade of cell-cycle progress at G2 phase. In addition, the up-regulation of *FasL* gene expression possibly induces apoptosis. Finally, D-Trp⁶-GnRH I treatment could mediate a decrease of the cell population in G1 phase by the reduction of *p27Kip1* gene expression.

Thus, cell-cycle analysis of SCL60 cells was performed following 4 days of treatment with D-Trp⁶-GnRH I to examine the above speculation (**Fig. 3-23**). Indeed, the proportion of cells found in the G2 phase was increased by 2.5-fold in response

to D-Trp⁶-GnRH I, which may be a consequence of the induction of GADD45 expression. Following D-Trp⁶-GnRH I stimulation, the proportion of apoptotic cells (sub-diploid population) was increased by 4-fold, possibly as a result of FasL expression. The cell population that was found in G1 phase was reduced, possibly due to the decrease in p27Kip1 expression. The results from cell-cycle analysis were in line with a study conducted by Miles *et al.* (2004) (Miles *et al.*, 2004). However, cell-cycle analysis following a time-course treatment, would be required in order to determine how GnRH causes cell-cycle changes over time, and clarify whether these above-mentioned genes are the main mediators of this regulation.

Taken together, the research presented in this Chapter describes a novel regulatory mechanism initiated by GnRH-R activation. This involves the regulation of FOXO3a transcriptional activity, and the expression of FOXO-target genes by GnRH. In Chapters four and five, the impact of GnRH regulation of FOXO3a transcriptional activity on cell function will be assessed. Chapter four describes a role for FOXO3a in the well-documented GnRH-induced cell-growth inhibition and apoptosis. Chapter five describes a novel role for FOXO3a in GnRH-induced LH β -subunit expression in the gonadotropes.

CHAPTER FOUR

THE ROLE OF FOXO3a IN GnRH-INDUCED CELL- GROWTH INHIBITION

4.0 ABSTRACT

In this Chapter, the role of FOXO3a in the GnRH-induced cell-growth inhibition was examined. The results presented demonstrate that FOXO3a protects cells from the GnRH-induced cell-growth inhibition. In addition, GnRH recruits FOXO3a to regulate FasL expression to induce cell-growth inhibition. The role of FOXO3a in the GnRH-induced cell-growth inhibition by targeting GADD45 expression is also assessed.

4.1 BACKGROUND AND OBJECTIVES

Activation of the GnRH-R by GnRH has been shown to inhibit cell proliferation and induce apoptosis in cancer-delivered cell lines. The anti-proliferative and apoptotic effects of GnRH on tumour cells are also well-documented (Reviewed in Kraus *et al.* 2006). Several studies have reported that these events can occur as a result of changes in the expression profiles of specific cell-cycle regulatory and apoptotic genes (Imai *et al.*, 1998a; Wu *et al.*, 2009). Many of these are FOXO-target genes. FOXOs are involved in a number of biological processes, including cell-fate decision and tumour suppression in a variety of cancers (Fu and Tindall, 2008). Indeed, FOXO3a activation not only promotes apoptosis *in vitro*, but also restricts tumour growth *in vivo* in breast cancer cells (Yang and Hung, 2009), while constitutively active FOXO4 reduces tumour size (Greer and Brunet, 2005).

The regulation of FOXO3a transcriptional activity by GnRH-R activation has been demonstrated in Chapter three, and in this chapter the role of FOXO3a signalling pathway in the GnRH-induced cell-growth inhibition was examined. In particular the main objectives of this chapter were the following:

- to explore the potential role for FOXO3a activity in mediating the anti-proliferative and apoptotic effect of GnRH
- to examine the role of FOXO3a in the cell-cycle regulation following GnRH stimulation.

4.2 RESULTS

4.2.1 Role of FOXO3a in GnRH-induced cell-growth inhibition

4.2.1.1 *Generation of FOXO3a knock-down cell lines*

To elucidate the role of FOXO3a in GnRH-induced cell-growth inhibition, FOXO3a knock-down stable cell lines were generated using lentivirus-delivered short hairpin RNA (shRNA). Firstly, the conditions for optimal transduction efficiency were determined. SCL60 cells were transduced with GFP-lentivirus at ratios (cells to lentivirus) of 1:1, 1:2 and 1:5 in the presence or absence of polybrene (0.6µg/ml) over 1, 2, 3, 4 and 5 days (**Supplementary Fig. 7-8**). Polybrene is a positively charged molecule that binds to cell surfaces and neutralizes the surface charge of the cell, increasing the efficiency of infection (Aubin *et al.*, 1988). The optimal conditions for lentivirus transduction of SCL60 cells were the following: 3 days of transduction with lentivirus in the absence of polybrene at a ratio of 1:5.

Subsequently, cells were transduced with lentivirus-delivered shRNA to knock-down the expression of FOXO3a. Cells were also transduced with lentivirus-delivered scrambled shRNA to generate a control cell line. Western Blot analysis confirmed that transduction of cells with lentivirus-delivered shRNA against FOXO3a reduced the protein levels of FOXO3a, with the highest reduction obtained in a clone with a

double shRNA combination (shRNA codes: sh2978 and sh1687) (this clone will henceforth be referred to as **SCL840**). A control cell line was also generated (this clone will henceforth be referred to as **SCL839**) (**Fig. 4-1**).

The generation of a stable FOXO3a knock-down prostate cancer cell line (PWE-1-NB26-3) was also attempted in order to confirm the involvement of FOXO3a in cell-growth inhibition following GnRH treatment in another cell system. However, clones with a significant reduction of FOXO3a protein levels could not be generated.

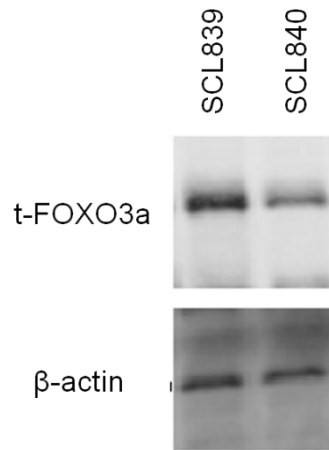


Figure 4-1: FOXO3a protein level in the control and FOXO3a knock-down stable cell lines.

SCL60 cells were transduced for 3 days with lentivirus-delivered short hairpin RNA (shRNA) against FOXO3a or lentivirus-delivered scrambled shRNA at a ratio of 1:5 (cells:lentivirus). A single clone of control cells (SCL839) and FOXO3a knock-down cells (SCL840) was isolated, and the reduction of total FOXO3a (t-FOXO3a) protein level was measured compared to the control cells by Western Blot analysis.

4.2.1.2 FOXO3a knock-down does not affect GnRH-R functionality

Variations of GnRH-R expression levels differentially affect the GnRH-induced apoptotic machinery in different cell lines and contribute to cell type-specific cell-growth inhibition in response to GnRH treatment (Morgan *et al.*, 2008). Before using

the SCL839 and SCL840 cell lines that had been generated, the effect of transduction with lentivirus on the level of GnRH-R expression, and the ability of the GnRH-R to activate intracellular signalling pathways following GnRH treatment was examined. [¹²⁵I]-GnRH receptor binding assays demonstrated that SCL840 expressed slightly lower numbers of receptors at the cell-surface compared to SCL839 cells (**Fig. 4-2A**). In addition, a [³H]-inositol phosphate assays were performed in order to assess the ability of the two cell lines to activate intracellular signalling. Treatment of SCL839 generated approximately an IC₅₀ of 1nM and treatment of SCL840 generated an IC₅₀ of 10nM (**Fig. 4-2B**). Nevertheless, the maximal responses were 6343 cpm for SCL839 and 2243 cpm for SCL840 (**Fig. 4-2C**). These results suggested that both SCL839 and SCL840 clones had the ability of functional coupling to PLC and initiating a signalling pathway to produce inositol phosphate were.

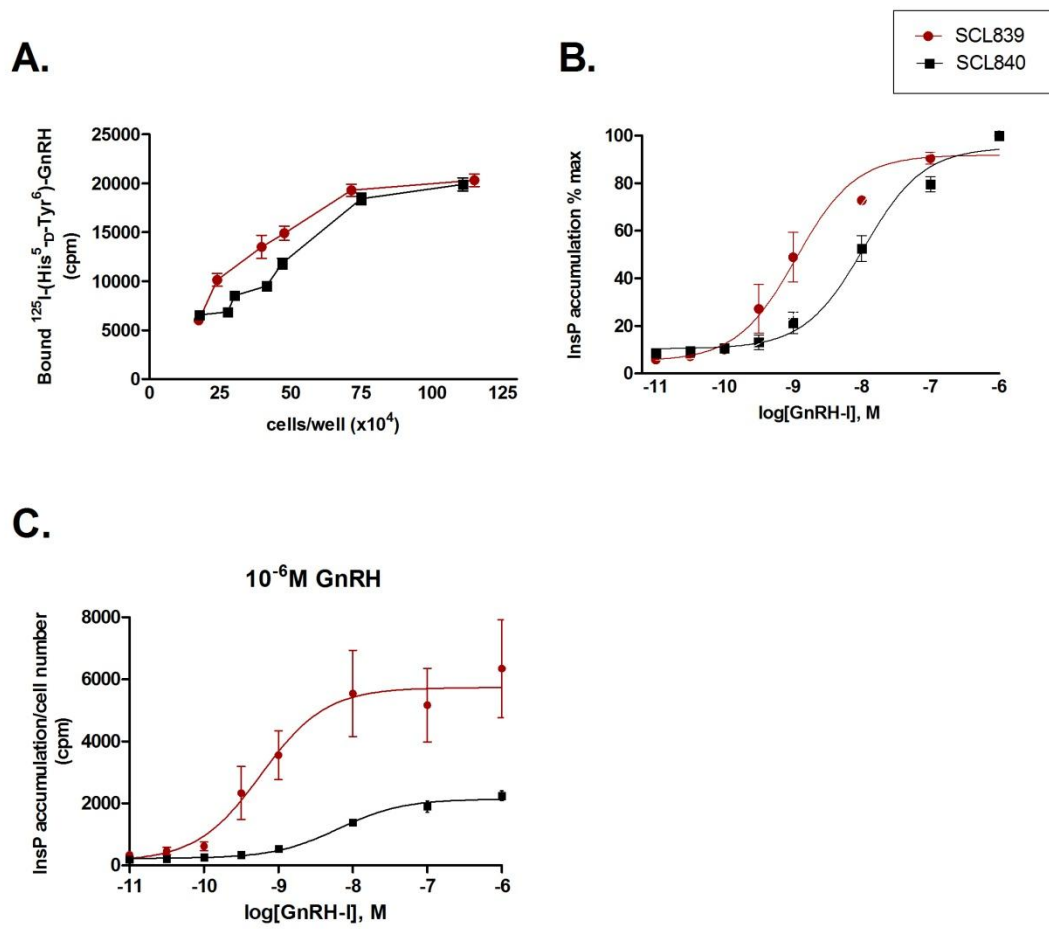


Figure 4-2: [^{125}I]-GnRH receptor binding and [^3H]-inositol phosphate assay performed in SCL839 and SCL840 cells.

(A) Competitive replacement of ^{125}I -[His⁵-D-Tyr⁶]-GnRH with 10^6M of GnRH in SCL840 (black squares) and SCL839 (red circles). (B) Inositol phosphate production in response to (10^{-6} - 10^{-10} M) GnRH in SCL840 (black squares) and SCL839 (red circles) expressed as percent of maximum response. (C) Maximal inositol phosphate production in response to (10^{-6} M) GnRH in SCL840 (black squares) and SCL839 (red circles). Data are shown as mean \pm SEM from at least 3 independent experiments performed in triplicate.

4.2.1.3 *FOXO3a* protects SCL60 cells from GnRH-induced cell-growth inhibition

The effect of GnRH treatment on the cell-growth rate of the SCL839 and SCL840 cell lines was compared next. Cells were treated for five days with different doses of D-Trp⁶-GnRH I (10^{-6} - 10^{-10} M). Doses from 0.5nM to $1\mu\text{M}$ caused approximately

35% higher cell-growth inhibition in the SCL840, in spite of having slightly lower receptor numbers, compared to SCL839 cells (**Fig. 4-3A**). To further confirm the differential effect of D-Trp⁶-GnRH I on cell-growth inhibition in these two cell lines, a cell-growth assay, in the presence or absence of 0.5nM D-Trp⁶-GnRH I treatment, was performed over 5 days. Cell-growth inhibition in response to D-Trp⁶-GnRH I was higher for the SCL840 cells (**Fig. 4-3B**) compared with SCL839 cells (**Fig. 4-3C**), which is consistent with the cell viability assay (see **Fig. 4-3A**) In addition, the cell-growth rate of SCL840 cells was reduced compared to the control SCL839 cells in absence (**Fig. 4-4A**) or in presence of 0.5nM D-Trp⁶-GnRH I (**Fig. 4-4B**).

When the data in figure 4-3 was further analysed, the cell-growth rate was found to be reduced by approximately 23.5% for control SCL839 cells, and by 73% for SCL840 cells, followed treatment with 0.5nM D-Trp⁶-GnRH I (analysis was performed with Graph Prism 5.00, growth rate was calculated by taking the derivative of the curve equation describing the number of cells over time) (**Fig. 4-5**). Cell-doubling times for these two cell lines, measured in the presence or absence of D-Trp⁶-GnRH I treatment respectively, were 1.8 and 1.4 hours for SCL839, and 7.8 and 2.3 SCL840 cells (**Table 4-1**).

The growth rate of SCL60 cells was also examined and compared to that of SCL839 and SCL840 cells. The growth rate of SCL60 cells was similar to that of SCL839 without D-Trp⁶-GnRH I treatment (**Fig. 4-4A**), while it was comparable to the growth rate of the SCL840 cells in the presence to 0.5nM D-Trp⁶-GnRH I (**Fig. 4-4B**).

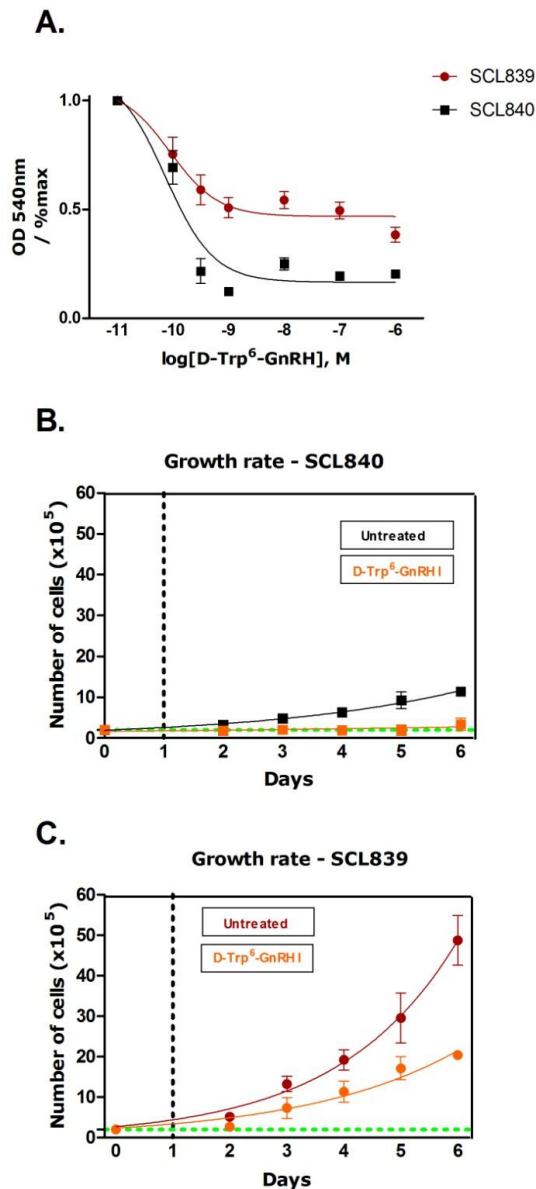


Figure 4-3: Effect of GnRH on cell-growth rate in SCL840 and control SCL839 cell lines.

(A) SCL839 and SCL840 cells in 12-well plates were treated on day 0 with the indicated doses of GnRH analogue (D-Trp⁶-GnRH). Cells were fixed, stained with sulforhodamine B, and quantified spectrophotometrically following cell-growth for 5 days. (B and C) SCL840 and SCL839 cells were plated in 100mm plates and treated at day 1 with 0.5nM D-Trp⁶-GnRH I or left untreated. Cell number was counted on subsequent days using a haemocytometer. (The vertical black line indicates that cells were treated in day 1, the horizontal green line indicates the number of cells in day 0). The data are from four independent experiments performed in triplicate. Data are shown as mean \pm SEM from at least 4 independent experiments performed in triplicate.

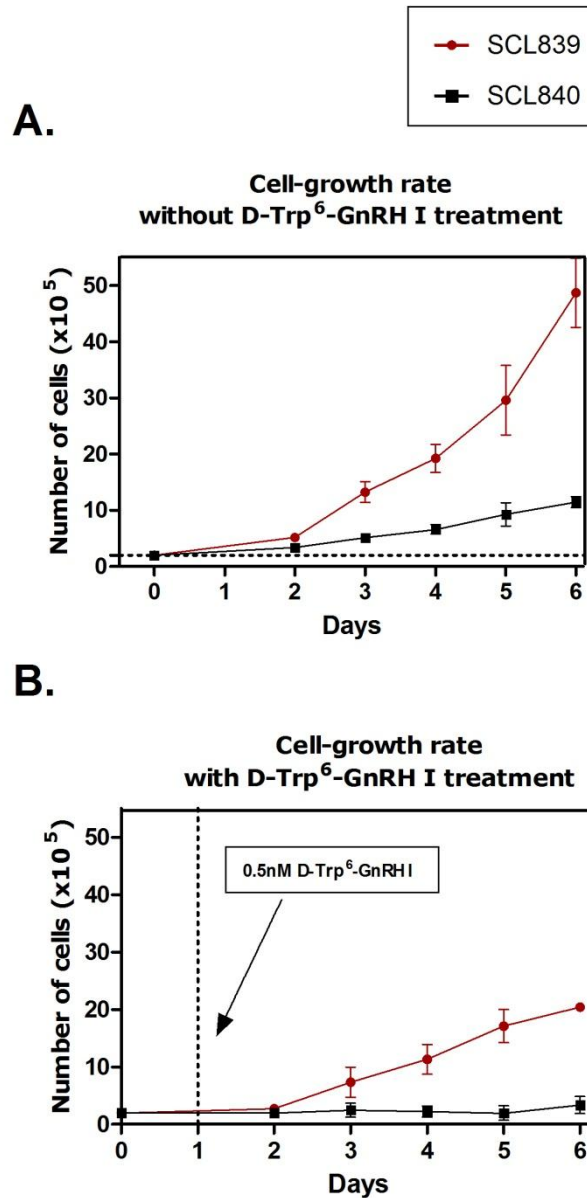


Figure 4-4: Effect of GnRH on cell growth rate.

(*A* and *B*) SCL839 and SCL840 cells were plated in 100mm plates. Thereafter, (*B*) cells were treated at day 1 with 0.5nM D-Trp⁶-GnRH I (the vertical black line indicates that cells were treated in day 1) or (*A*) left untreated (the horizontal green line indicates the number of cells in day 0). (*A* and *B*) Cell number was counted the following days using a haemocytometer. Data are shown as mean±SEM from at least four independent experiments performed in triplicate.

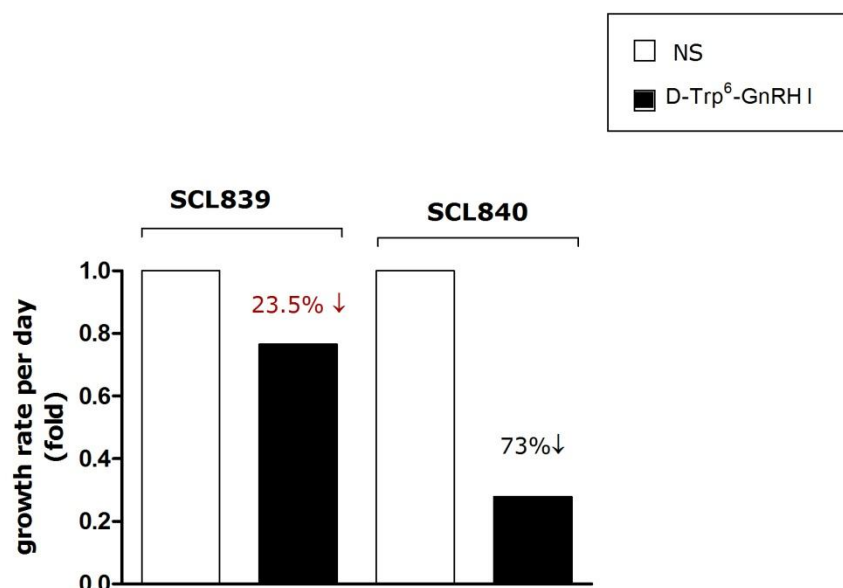


Figure 4-5: Growth rate of SCL839 and SCL840 cells in the presence or absence of 0.5nM D-Trp⁶-GnRH I.

These data are a re-analysis of the data presented in Figure 4-3.

Cell line	D-Trp ⁶ -GnRH I treatment	Doubling time (hours)
SCL839	No	1.44 (0.98-4.47)
	Yes	1.82 (0.96-3.75)
SCL840	No	2.32 (1.92-3.24)
	Yes	7.88 (2.69-high)

Table 4-1: SCL839 and SCL840 cell-doubling time in the presence or absence of 0.5nM D-Trp⁶-GnRH I.

4.2.1.4 *The effect of FOXO3a on FasL expression in response to D-Trp⁶-GnRH I treatment*

FOXO3a plays a role in GnRH-induced *FasL* expression as demonstrated in Section 3.2.3.2 in Chapter three. To confirm the involvement of FOXO3a in *FasL* gene expression in response to D-Trp⁶-GnRH I, SCL839 and SCL840 cells were treated with D-Trp⁶-GnRH I over 5 days, and *FasL* mRNA levels measured. *FasL* gene expression was higher in SCL840 cells compared to SCL839 cells at co-responding

intervals of D-Trp⁶-GnRH I treatment (**Fig. 4-6**). These data suggest that FOXO3a negatively regulates *FasL* expression following GnRH treatment.

FasL activates the Fas receptor to induce apoptosis through the activation of a signalling pathway which involves mediators including FADD, caspase 3 and caspase 8. To further confirm that FOXO3a mediates GnRH-induced FasL expression, Western Blot analysis was performed to examine whether the levels of FADD, caspase 3 and caspase 8 proteins were differentially regulated in SCL839 and SCL840 cells. However, no protein bands could be detected in the Western Blots performed, possibly due to poor expression levels.

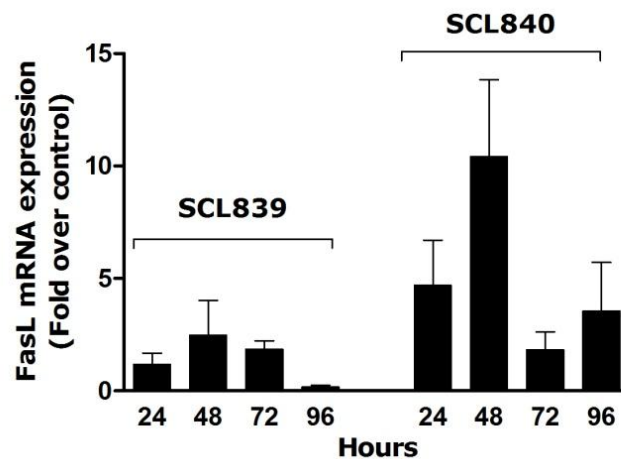


Figure 4-6: The effect of D-Trp⁶-GnRH I on *FasL* mRNA expression in SCL839 and SCL840 cells.

Relative mRNA expression of *FasL* in SCL839 cells and SCL840 cells that were treated with 0.5nM D-Trp⁶-GnRH I for the indicated times was determined by quantitative RT-PCR analysis. Data are shown as mean \pm SEM from two independent experiments performed in triplicate.

4.2.1.5 *Effect of Akt and IKK signalling on GnRH-induced cell-growth inhibition in SCL60, SCL839 and SCL840 cells*

GnRH-induced FOXO3a transcriptional activity occurs via Akt- and IKK-dependent signalling pathways as demonstrated in Section 3.2.2.3 in Chapter three. Furthermore, the involvement of FOXO3a in GnRH-induced cell-growth inhibition was also demonstrated in Section 3.2.4.3. Therefore, the role of IKK and Akt in GnRH-induced cell-growth inhibition was examined next. SCL60 cells were pre-treated with pharmacological inhibitors of IKK or Akt, and cell-growth was measured following 100nM D-Trp⁶-GnRH I treatment. Pre-treatment with IKK or Akt1/2 inhibitors reduced cell-growth rate over 5 days by approximately 20% and 30% respectively in the absence of D-Trp⁶-GnRH I treatment (**Fig. 4-7A**). However, Akt1/2 inhibitor shows a trend towards a reduction (19%) of GnRH-induced cell-growth inhibition, even though the reduction did not reach statistical significance (**Fig. 4-7B**).

Akt and IKK were found to inhibit GnRH-induced FOXO3a transcriptional activity in SCL60 cells (see Section 3.2.2.3). Furthermore, the Akt inhibitor partially rescues GnRH-induced cell-growth inhibition in SCL60 cells (**Fig. 4-7B**). Thus, it is possible that Akt (and possibly IKK) could be involved in the GnRH-FOXO3a inhibitory signalling pathway. If Akt and IKK are involved in this pathway, by regulating FOXO3a transcriptional activity, then Akt and IKK inhibitors would be expected to differentially effect GnRH-induced cell-growth inhibition in SCL839 and SCL840, because FOXO3a protein levels are lower in SCL840 compared to SCL839 cells. To test this hypothesis, SCL839 and SCL840 cells were pre-treated with Akt and IKK inhibitors, and cell-growth inhibition was measured following 0.5nM D-Trp⁶-GnRH

I treatment. While the IKK inhibitor had no significant effect on the cell-growth rate of both cell lines (**Fig. 4-8A** and **C**), the Akt inhibitor reduced the cell growth rate only in SCL840 cells (**Fig. 4-8A**). Even so, neither of these two inhibitors completely rescued GnRH-induced cell-growth inhibition (**Fig. 4-8B** and **D**). However, the Akt inhibitor reduced the level of GnRH-induced cell-growth inhibition of SCL840 to approximately the same level as for SCL839 following D-Trp⁶-GnRH I treatment alone (**Fig. 4-8D** compared to **Fig. 4-8B**, denoted by arrows). This result could implicate Akt involvement with FOXO3a in the GnRH-induced cell-growth inhibition signalling pathway.

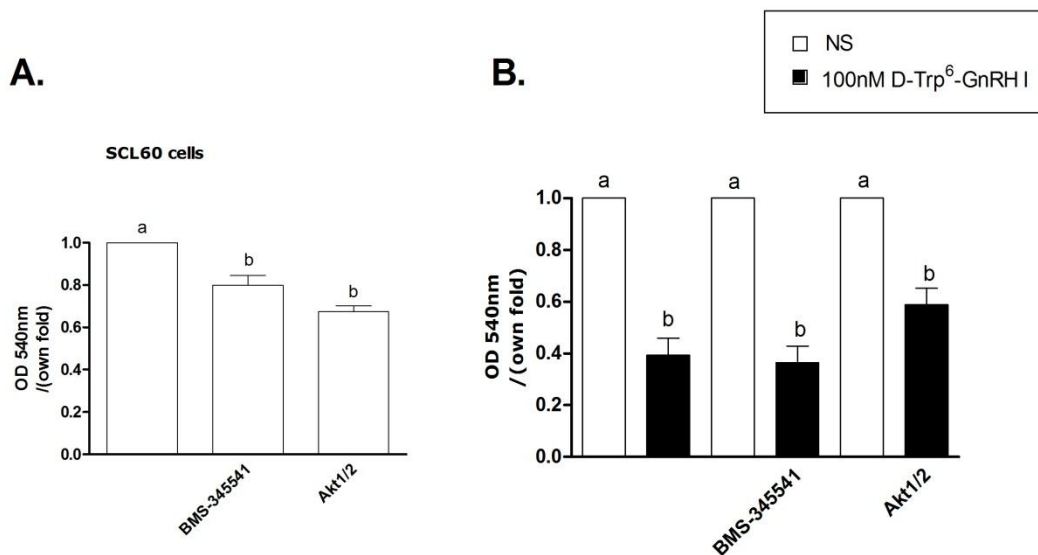


Figure 4-7: Effect of Akt and IKK on GnRH-induced cell-growth inhibition in SCL60 cells.

(**A** and **B**) SCL60 cells were treated with 1.5 μ M of IKK inhibitor (BMS-345541) or 1 μ M of Akt inhibitor (Akt1/2) and (**B**) 100nM of GnRH analogue (D-Trp⁶-GnRH I). Cells were fixed, stained with sulforhodamine B, and quantified spectrophotometrically following cell growth for 5 days. Data are shown as mean \pm SEM from at least four independent experiments performed in triplicate. (b is significantly different from a; P<0.05)

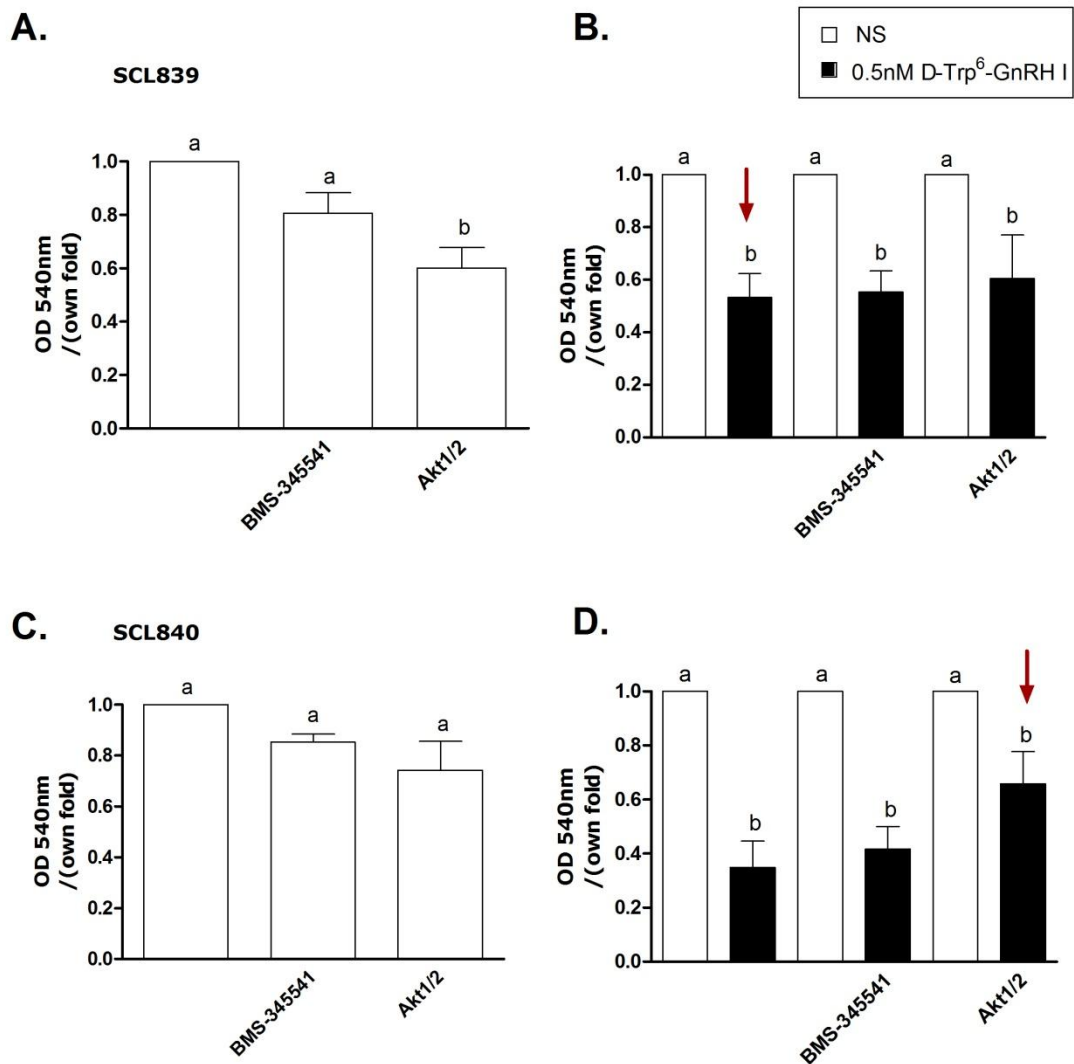


Figure 4-8: Effect of Akt and IKK on GnRH-induced cell-growth inhibition in SCL839 and SCL840 cells.

(A, B, C and D) SCL839 and SCL840 cells were treated with 1.5 μ M of IKK inhibitor (BMS-345541) or 1 μ M of Akt inhibitor (Akt1/2) and (B and D) 0.5nM of GnRH analogue (D-Trp⁶-GnRH I). Cells were fixed, stained with sulforhodamine B, and quantified spectrophotometrically following cell growth for 5 days. Data are shown as mean \pm SEM from at least three independent experiments performed in triplicate. (b is significantly different from a; $P < 0.05$)

4.2.1.6 *Monitoring the role of FOXO3a in cell-cycle regulation in response to GnRH*

The role of FOXO3a in GnRH-induced cell-growth inhibition was also confirmed by flow cytometry analysis. This is a simple method for detecting sub-diploid cell populations, an indication of apoptotic cells, by staining DNA. Cell-cycle analysis also allows the detection of the proportion of cells found at specific stages of the cell-cycle. In response to D-Trp⁶-GnRH I treatment, the sub-diploid population was increased approximately 3-fold in SCL839 cells (**Fig. 4-9A** and **C**), and approximately 7-fold in SCL840 cells (**Fig. 4-9B** and **D**). Thus, SCL840 cells are more sensitive to D-Trp⁶-GnRH I-induced cell-growth inhibition compared to the SCL839 cells, confirming the cell viability assay results presented earlier (see Section 4.2.3). The proportion of cells found in G1 and G2 were similar in both stable cell lines in the absence of D-Trp⁶-GnRH I treatment. The proportion of cells in G1 phase decreased from 60.1% to 46.4% in SCL839 cells following D-Trp⁶-GnRH I treatment (**Fig. 4-9A** and **C**), and from 60% to 36.5% in SCL840 cells (**Fig. 4-9B** and **D**), while no change in proportion of cells in G2 phase was observed in response to D-Trp⁶-GnRH I treatment in both stable cell lines.

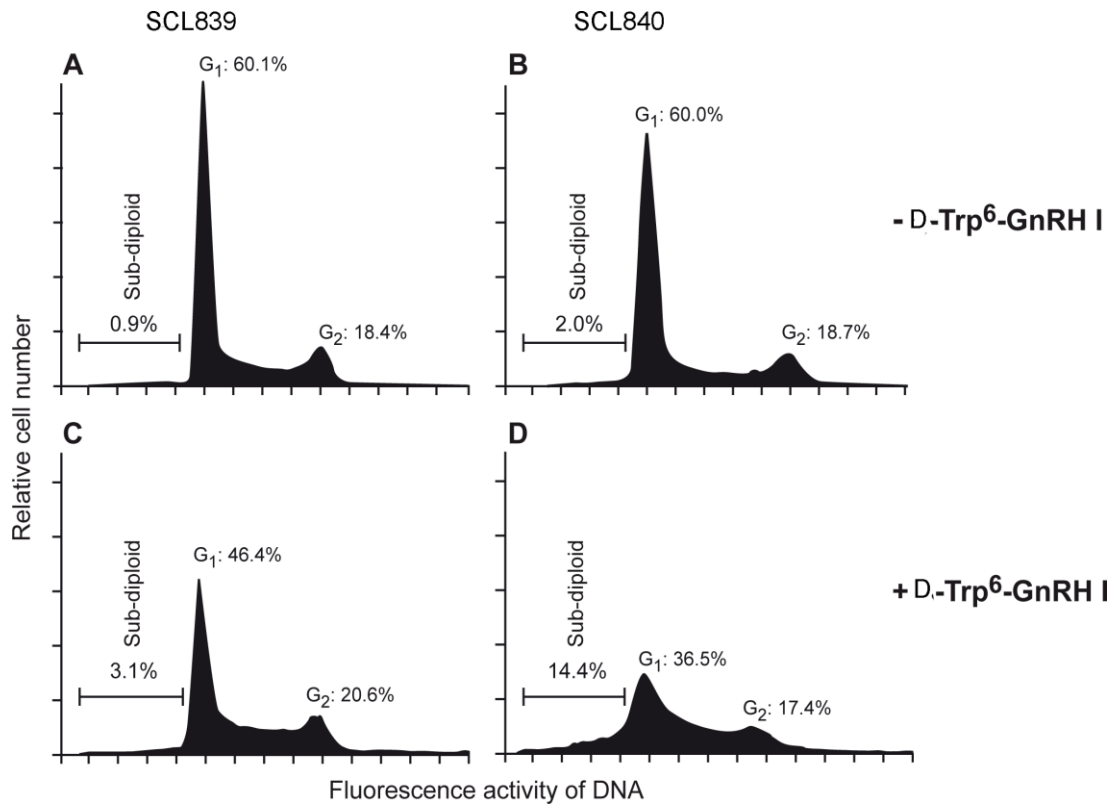


Figure 4-9: Flow cytometry analysis of SCL839 and SCL840 cells following D-Trp⁶-GnRH I treatment.

Cells were treated with 0.5nM D-Trp⁶-GnRH I for 96 hours, fixed, permeabilised and stained with Hoechst for analysis by flow cytometry.

4.3 DISCUSSION

Although the signalling pathways that mediate GnRH-induced cell-growth inhibition in different cell systems have been examined previously (Kraus *et al.*, 2006), there is no report implicating FOXO3a in these pathways. The results presented thus far indirectly implicate a role for FOXO3a in GnRH-induced cell-growth inhibition. GnRH has previously been demonstrated to induce the expression of the FOXO-target genes, *FasL* and *GADD45* (Imai *et al.*, 1998a; Wu *et al.*, 2009). The results presented in Chapter three demonstrated that GnRH regulates the *FasL* and *GADD45* expression by targeting FOXO3a activity.

In order to directly examine the role of FOXO3a, a stable FOXO3a knock-down cell line was generated (SCL840 cells, together with control SCL839 cells) (**Fig. 4-1**). Stable cell lines were generated instead of transiently knocking-down FOXO3a expression because stable knock-down cell lines ensure long-term reduction of FOXO3a protein levels, which are useful for experiments that run over days rather hours. However, by generating a stable cell line, other parameters should be taken into account. Transduction of cells with lentivirus-delivering shRNA may cause phenotypic changes in the generated stable lines. For example, in SCL60 cells, it may alter the function and/or the expression of the GnRH-R. However, GnRH-R binding assays showed no significant differences in the number of GnRH-R expressed at the cell surface of the two stable cell lines (control and knock-down). GnRH stimulation of Inositol phosphate was also similar in both cell lines, indicating that functional G_q coupling was comparable (**Fig. 4-2**). Nevertheless, it would be valuable to generate more than a single control and FOXO3a knock-down cell line.

Unfortunately, there was lack of significant reduction of FOXO3a protein levels in all other clones. Generation of a prostate cancer FOXO3a knock-down cell line expressing GnRH-R was also attempted, but again no clones with a significant reduction of FOXO3a expression were obtained.

In order to assess the role of FOXO3a in GnRH-induced cell growth inhibition, cells numbers were counted after treatment with D-Trp⁶-GnRH I (**Fig. 4-3**). The relative growth rate of SCL840 cells was slower compared to that of the SCL839 cells. A role for FOXO3a in cell proliferation, visualised by microscopy, was also confirmed in cell viability assays. Interestingly, reduction of FOXO3a levels in SCL840 cells, increased cell-growth inhibition following D-Trp⁶-GnRH I stimulation, which suggests that FOXO3a protects cells from GnRH-induced cell-growth inhibition. Cell-cycle analysis also confirmed the involvement of FOXO3a in protecting cells from GnRH-induced cell-growth inhibition via the monitoring of the sub-diploid population which represents the apoptotic cells. This result, is in contrast with most previous studies in other cell systems demonstrating that FOXO activation promoted cell-growth inhibition and apoptosis (Brunet *et al.*, 1999; Ramaswamy *et al.*, 2002; Roy *et al.*, 2010). However, Srinivasan *et al.* (2005) demonstrated that activation of FOXO3a was required for neuron survival (Srinivasan *et al.*, 2005), suggesting that the role of FOXO3a in cell-growth may be cell-type specific.

The role of FasL in FOXO3a-mediated GnRH-induced cell-growth inhibition was also examined (**Fig. 4-6**). Previous studies have also examined the role of FasL in the GnRH-induced cell-growth in different cell systems (Morgan *et al.*, 2008). In particular, the effect of GnRH on apoptotic markers which are involved in FasL

signalling apoptotic pathway was assessed. Small elevations of phosphorylated FADD, which is involved in the FasL apoptotic pathway, were detectable in SCL60 cells in response to GnRH (Morgan *et al.*, 2008). The involvement of FasL in the GnRH-induced cell-growth inhibition has been proposed in several other studies (Imai *et al.*, 1997; Imai *et al.*, 1998a). In contrast, Huang *et al.* (2002) demonstrated that GnRH did not up-regulate FasL expression, while it mediated cell-growth inhibition in leiomyoma cells (Huang *et al.*, 2002), again suggesting that the mechanism of GnRH-induced cell-growth inhibition is cell-type specific.

FOXO3a level		Gene	Effect on GnRH-induced cell-growth inhibition
Control	↑	<i>FasL</i> (Fig. 3-16A)	↑↑ (Fig. 4-4A and B , SCL60 cells)
Over-expressed (FOXO3a-TM)	↓	<i>FasL</i> (Fig. 3-17A)	↓ (not measured)
Knock-down	↑↑	<i>FasL</i> (Fig. 4-6)	↑↑↑ (Fig. 4-3A)
Control	↑	<i>GADD45</i> (Fig. 3-16A)	↑↑ (Fig. 4-4A and B , SCL60 cells)
Over-expressed (FOXO3a-TM)	↑↑	<i>GADD45</i> (Fig. 3-17B)	↑ (not measured)
Knock-down	(not measured)		↑↑↑ (Fig. 4-3A)

Table 4-2: This table summarises the effect of GnRH on the expression of FasL and GADD45, and the effect of GnRH on the cell-growth inhibition in the SCL60 model cell line, with regards to FOXO3a levels (Arrows denote relative increase or decrease in gene expression and cell-growth inhibition)

Based on the results presented in this Chapter, GnRH potentially recruits FOXO3a in order to regulate FasL expression to induce cell-growth inhibition. This reveals a novel role of FOXO3a in the GnRH-induced cell-growth inhibition process. A

number of other observations strengthen the above speculation. Cell-cycle analysis in SCL839 and SCL840 cells demonstrated that the sub-diploid (apoptotic) population of SCL840 cells was increased compared with that of the SCL839 cells in response to D-Trp⁶-GnRH I (**Fig 4-9**). This suggests that the different cell-growth inhibition effects of D-Trp⁶-GnRH I, as demonstrated in the cell-viability assays, between the two stable cell lines, was due to the induction of apoptosis and not cell-cycle blockade. This is a reasonable explanation which is further supported by the finding that GnRH-induced *FasL* expression was higher in SCL840 cells compared to SCL839 cells, as observed by qRT-PCR analysis (**Fig. 4-6**). This result was expected since over-expression of FOXO3a in SCL60 cells decreased *FasL* gene expression.

In summary, GnRH treatment increases FasL expression resulting in induction of apoptosis in SCL60 cells, while over-expression of FOXO3a reduced FasL expression and apoptosis in SCL60 cells. Furthermore, knocking-down FOXO3a further increased FasL expression and apoptosis in response to GnRH treatment in SCL840 cells, compared to control cells (see **Table 4-2**). These observations therefore appear to suggest that in response to GnRH treatment, FOXO3a acts to repress FasL expression. ChIP analysis will be helpful to further understand the activity of FOXO3a at the *FasL* promoter in response to GnRH stimulation.

Interestingly, IKK and Akt inhibitors, previously shown to inhibit GnRH-induced GADD45 activity, were unable to completely rescue SCL60, SCL839 and SCL840 cells from GnRH-mediated cell-growth inhibition. The Akt inhibitor did not completely rescue GnRH-mediated cell-growth inhibition in SCL60 cells, and it partially rescued GnRH-mediated growth inhibition, in SCL840 by about 20.4% and

in SCL839 cells by 4.2%. Moreover, growth inhibition for SCL840 cells was approximately 25% higher compared to inhibition of SCL839 cells. Interestingly, the Akt inhibitor reduced the GnRH-induced cell-growth inhibition of SCL840 cells by approximately the same level as for SCL839 cells after GnRH treatment alone. This suggests that, the Akt inhibitor showed a trend towards a reduction of the additive GnRH-induced cell-growth inhibition effect in SCL840 cells compared to SCL839 cells. This suggests that the Akt inhibitor may block a GnRH-induced signalling pathway that mediates the more profound anti-proliferative effect in SCL840 cells compared to SCL839 cells, and that FOXO3a is part of this pathway.

The involvement of FOXO3a and FasL, as part of the same signalling pathway, in the GnRH-induced cell-growth inhibition process has been already suggested in this Chapter. In addition, the role of GADD45, as downstream target of FOXO3a, in GnRH-induced cell-growth inhibition was also examined. However, whether GnRH induces cell-growth inhibition in SCL60 cells by activating GADD45 expression by FOXO3a, was not clear. Thus, while FOXO3a appears to be involved in the induction of apoptosis, by regulating FasL expression following GnRH treatment, the role of FOXO3a in the GnRH-induced cell-cycle blockade, by induction of GADD45 was not clear (see **Table 4-2**). The results from this Chapter demonstrated that Akt inhibitor partially rescues GnRH-induced cell-growth inhibition in SCL60 cells. As was mentioned above, GnRH possibly induces cell-growth inhibition by blocking cell-cycle progress at G2 phase, by up-regulating GADD45 expression. Since FOXO3a mediates GnRH-induction of GADD45 expression, a role for FOXO3a in mediating GADD45 expression in GnRH-induced cell-growth inhibition is strongly suggested. Cell-cycle analysis in SCL839 and SCL840 cells demonstrated an

increase in sub-diploid population in SCL840 cells compared to SCL839 cells, which can be explained by the increase of FasL expression. However, there is no difference in the proportion of the G2 population between the two cell lines. However, differences in GADD45 expression are expected because of different FOXO3a levels should lead to differences on the cell-cycle profile between the SCL839 and SCL840 cells. In addition, following D-Trp⁶-GnRH I treatment, the change in the proportion of SCL839 and SCL840 found in G2 phase was similar, although FOXO3a levels were lower in SCL840 cell line. This result suggests that GnRH treatment cause cell-growth inhibition by up-regulating GADD45 expression, but not exclusively by FOXO3a, and possibly involving another transcription factor.

In conclusion, a role of FOXO3a in GnRH-induced cell-growth inhibition is proposed in this Chapter. The involvement of FOXO3a regulated expression of FasL and GADD45 in response to GnRH is also suggested to play a role in this process. Although this research was conducted in a heterologous HEK293/ratGnRH-R expressing model cell line, it is likely that these findings apply to other cell systems to highlight additional novel roles for the targeting of FOXO3a activity by GnRH. Indeed, this is demonstrated in Chapter five, which describes a novel role for FOXO3a in the regulation of LH β -subunit expression in the pituitary gonadotropes.

CHAPTER FIVE

**THE ROLE OF FOXOs IN THE REGULATION OF LH β
EXPRESSION IN RESPONSE TO GnRH IN THE
GONADOTROPE**

5.0 ABSTRACT

In this Chapter, the role of FOXO1 and FOXO3a in GnRH-induced expression of LH β , FSH β and the GnRH-R in gonadotropes was examined. The data presented demonstrates that FOXO3a and FOXO1 are expressed in human pituitary gonadotropes, and a role for FOXO3a in the regulation of LH β expression in response to GnRH treatment is highlighted. In addition, the regulation of Egr1 expression via a FOXO3a-dependent signalling pathway was demonstrated. Chromatin immunoprecipitation (ChIP) assays were used to assess FOXO3a recruitment to Egr1 and LH β promoters. FOXO3a targets Egr1 expression to, at least in part, indirectly regulate LH β promoter activity. β -catenin is also involved in the regulation of the LH β expression in co-ordination with FOXO3a in response to GnRH. These findings suggest that GnRH regulates LH β -subunit expression through one or more FOXO3a-mediated mechanisms.

5.1 BACKGROUND AND OBJECTIVES

Gonadotropin-releasing hormone (GnRH) is an essential regulator of the reproductive process, and stimulates the synthesis of the gonadotropins (LH and FSH) in pituitary gonadotropes, thereby regulating gametogenesis and steroidogenesis. GnRH is synthesised and secreted by hypothalamic GnRH neurons, and delivered to the anterior pituitary gland where it binds to the GnRH-R expressed on the surface of the gonadotrope (Naor, 2009; Seeburg *et al.*, 1987; Vale *et al.*, 1977).

Diverse signalling pathways have been reported to regulate LH β -subunit expression in response to GnRH, including the ERK/p38MAPK/JNK cascades and factors such as Egr1, SF1 and β -catenin (Thackray *et al.*, 2010). The LH β promoter comprises of

two DNA binding sites for Egr1 and SF1, and one for Pitx1 which is flanked by the Egr1 and SF1 binding sites (Halvorson *et al.*, 1996; Keri and Nilson, 1996; Quirk *et al.*, 2001; Tremblay and Drouin, 1999). Although the presence of all three of these factors as a complex on the promoter of the *LH β* gene is required for optimal LH β expression (Dorn *et al.*, 1999; Jiang *et al.*, 2005; Tremblay and Drouin, 1999), only Egr1 levels are regulated by GnRH-R activation (Dorn *et al.*, 1999; Tremblay and Drouin, 1999). It therefore appears that GnRH up-regulates *LH β* mRNA expression levels via induction of Egr1 expression in gonadotrope cells.

Although the aforementioned mechanism has been extensively described, it is not yet been completely understood. Recent studies highlighting the co-operativity between β -catenin and FOXO3a in mediating gene expression (Essers *et al.*, 2005; Hoogeboom *et al.*, 2008; Kwon *et al.*, 2010), suggested a potential role for FOXOs in regulating gonadotrope function. FOXO3a is one of four members of the “O” subgroup of Forkhead box (FOX) transcription factors, and plays an important role in a variety of cellular processes such as tumour suppression, cell-cycle regulation and metabolism (Burgering, 2008). To date, there are no studies demonstrating a role for FOXOs in GnRH-induced gonadotropin expression. Therefore, the main objectives of this Chapter were the following:

- to examine whether FOXO1 and FOXO3a are expressed in gonadotropes
- to investigate whether GnRH regulates LH β , FSH β or GnRH-R expression via FOXO1 or FOXO3a
- to examine the involvement of FOXO3a in GnRH-induced LH β expression

5.2 RESULTS

5.2.1 The role of FOXOs in GnRH-induced regulation of LH β , FSH β and GnRH-R expression

Having identified the involvement of GnRH in FOXO3a transcriptional activity in Chapter three, the role of FOXOs in GnRH-induced regulation of LH β , FSH β and GnRH-R expression in gonadotropes was examined. To investigate this, L β T2 cells were used as model gonadotrope cell line. These cells were co-transfected with LH β -promoter, FSH β -promoter or GnRH-R-promoter reporters together with control vector, wild type FOXO1 (FOXO1-WT) or wild type FOXO3a (FOXO3a-WT). Over-expression of FOXO1 increased the basal GnRH-R- and FSH β -reporter activity, whereas has no affect on basal transcriptional activity of the LH β -reporter was observed compared to vector control. Over-expression of FOXO3a did not alter basal FSH β -reporter activity but slightly decreased basal LH β -reporter activity compared to vector control (**Fig. 5-1A, C and E**).

L β T2 cells were treated with 1 μ M GnRH, and the role of FOXO1 and FOXO3a in the GnRH-induced LH β , FSH β and GnRH-R expression was examined. GnRH treatment increased LH β -, FSH β - and GnRH-R-reporter activity in cells transfected with control vector (**Fig. 5-1B, D and E**). FOXO1-WT over-expression decreased GnRH-induced GnRH-R-reporter activity, but has no detectable effect on LH β - or FSH β -reporter activity compared to vector control. FOXO3a-WT over-expression increased GnRH-induced LH β -reporter activity by 3-fold compared to vector control but did not affect FSH β -reporter activity compared to vector control (**Fig. 5-1B, D and F**). Together, this set of data points to a role for FOXO3a in GnRH-induced

regulation of LH β but not in FSH β regulation. FOXO3a also appears to have a negative role in GnRH stimulation of GnRH-R expression; however this potential role for FOXO3a was not further examined in this thesis. It is also interesting that after over-expression of FOXO1 the FSH β expression was increased significantly, while the GnRH-induced FSH β expression was not increased. This possibly means that FOXO1 acts as a transcription factor for FSH β , however GnRH does not regulate its transcriptional activity. The next section of work aims to delineate the role of FOXO3a in GnRH-induced LH β expression.

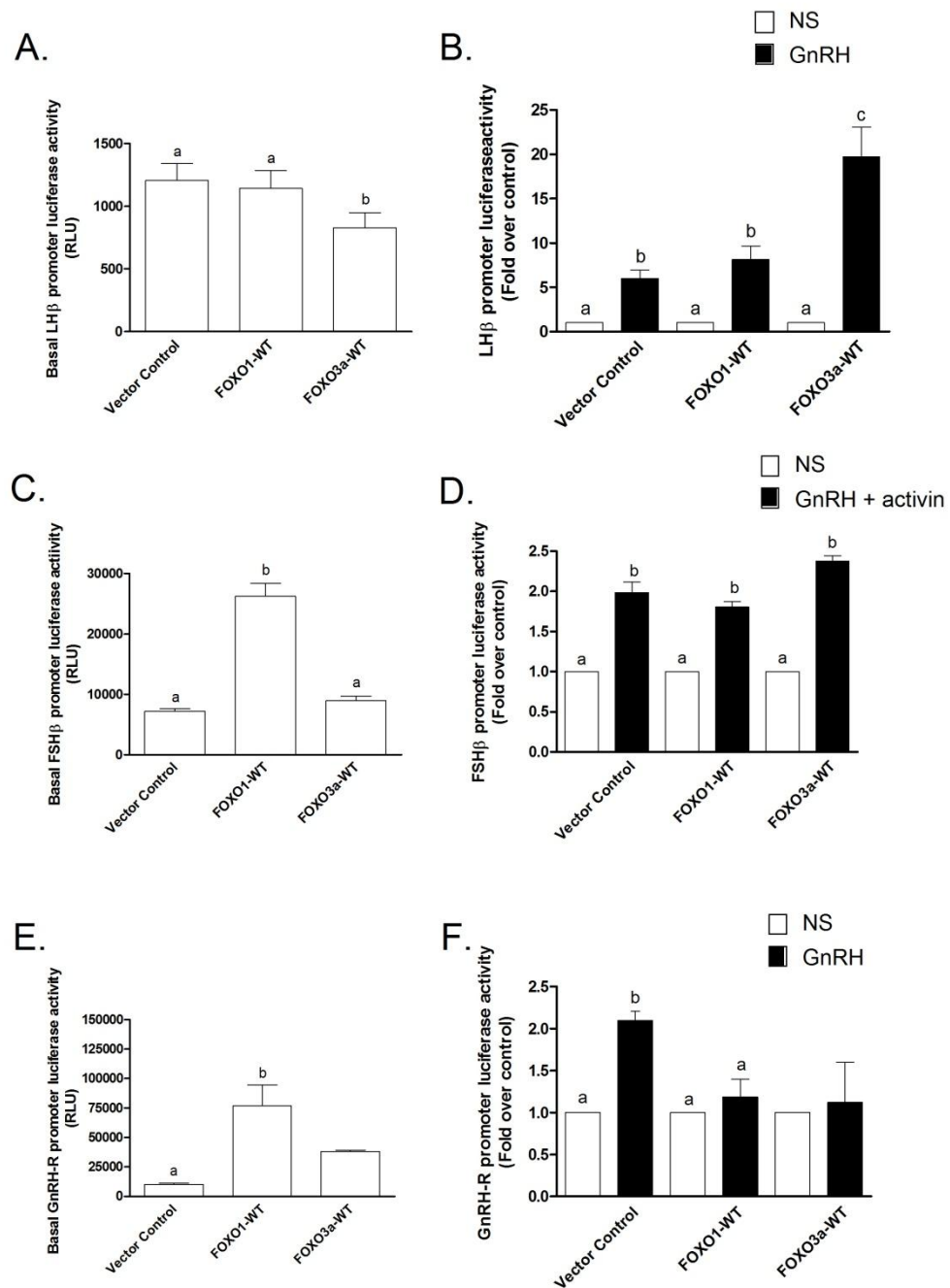


Figure 5-1: LH β , FSH β and GnRH-R-reporter activity levels in response to GnRH.

L β T2 cells were transiently transfected (*A* and *B*) with 5 μ g of LH β -, (*C* and *D*) FSH β - and (*E* and *F*) GnRH-R-reporter and 5 μ g of control vector (pcDNA3.1) or FOXO1-WT or FOXO3a-WT. Cells were treated (*B* and *F*) with 1 μ M GnRH for 6 hours (*D*) or with 1 μ M GnRH plus activin for 8 hours. Samples were then assayed for luciferase activity. In all panels open bars denote no stimulation and close bars denote GnRH stimulation. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments (in *E*) and (*F*), assay with over-expression with FOXO3a-WT is n=2). (b is significantly different from a, and c is significantly different from a and b; P<0.05)

5.2.2 GnRH regulates LH β -subunit expression via FOXO3a activity

To further investigate the role of FOXO3a in GnRH-induced LH β -reporter activity, L β T2 cells were co-transfected with LH β -reporter, together with FOXO3a-TM, bearing mutations at its three Akt phosphorylation sites (Thr³²Ala, Ser³¹⁵Ala and Ser²⁵³Ala; TM denotes triple mutant) (Burgering and Kops, 2002), or with FOXO3a-TM Δ DB with the same three Akt phosphorylation site mutations in addition to a deletion of the DNA binding domain (denoted by Δ DB). In contrast to FOXO3a-WT, FOXO3a-TM cannot be phosphorylated by Akt, resulting in its nuclear retention, thus eliminating the effect of Akt signalling on the localisation/redistribution of FOXO3a. While over-expression of FOXO3a-TM increased GnRH-induced LH β -reporter activity compared to vector control, over-expression of FOXO3a-TM Δ DB reduced GnRH-mediated LH β -reporter activity compared to the effect of FOXO3a-TM (**Fig. 5-2A**).

To further confirm the involvement of FOXO3a in GnRH-induced LH β expression, the effect of transiently knocking-down FOXO3a expression by lentivirus-delivered short hairpin RNA (shRNA) was examined in LH β -reporter activity experiments. Attempts to generate stable FOXO3a knock-down L β T2 cell lines using lentivirus-delivered shRNA were unsuccessful. The cells were not viable after 8-10 days of transduction with lentivirus-delivered shRNA with or without the addition of the selective antibiotic, implying that the level of FOXO3a protein may be an important for the viability of gonadotropes. Therefore, transiently transduced cells were used in further experiments. In order to achieve the highest transient reduction of FOXO3a protein levels, L β T2 cells were transduced using lentivirus-delivery of shRNA

targeted towards FOXO3a, and FOXO3a protein levels were measured after 1, 2, 3 and 5 days of transduction by Western Blot analysis. Maximum FOXO3a protein level reduction was achieved after 4 days of transduction with lentivirus-delivery shRNA1624 (**Supplementary Fig. 7-9**). Control cells were also generated by transduction with lentivirus-delivered scrambled shRNA (these cells will henceforth be referred to as control cells).

Basal LH β -reporter activity was reduced in FOXO3a knock-down cells compared to control cells (**Fig. 5-2B**), and GnRH induction of LH β expression was lower in FOXO3a knock-down L β T2 cells compared to control cell levels (**Fig. 5-2C**). Taken together, these data suggest that FOXO3a regulates LH β expression, either directly by acting at the *LH β* promoter, or indirectly by targeting the expression and/or co-opting the activity of one or more factors which in-turn play a role in regulating LH β expression.

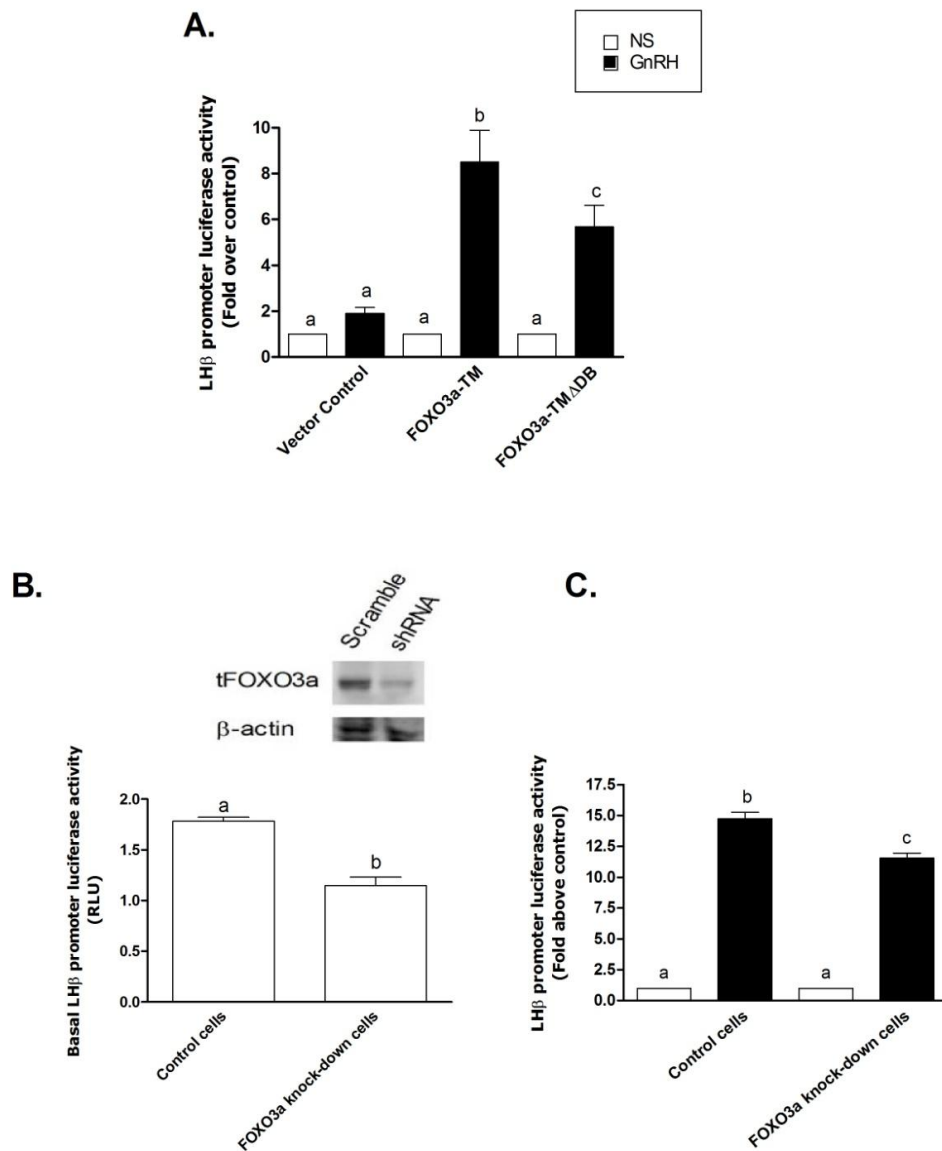


Figure 5-2: GnRH stimulates LH β -reporter activity by FOXO3a.

(A) L β T2 cells were co-transfected with 2 μ g of LH β -reporter and 8 μ g of control vector (pcDNA3.1), FOXO3a-TM or FOXO3a-TM Δ DM. Cells were serum-starved for 24 hours followed by treatment with 1 μ M GnRH for 6 hours. Samples were then assayed for luciferase activity. For knock-down cells, lentivirus-delivered shRNA against FOXO3a or scrambled shRNA (control cells) was added to L β T2 cells. The following day, virus was removed and cells incubated with fresh medium (**B** and **C**). Cells were subsequently co-transfected with LH β -reporter and serum-starved for 24 hours after transfection, (**C**) followed by stimulation with 1 μ M GnRH for 6 hours. In all panels open bars denote no stimulation and close bars denote GnRH stimulation. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a, and c is significantly different from a and b; $P < 0.05$)

5.2.3 GnRH regulation of Egr1 expression by FOXO3a

Egr1 is a well-known intermediate in the GnRH-induced LH β expression pathway (Kaiser *et al.*, 2000; Maudsley *et al.*, 2007; Weck *et al.*, 2000). Having identified the involvement of FOXO3a in GnRH-induced LH β expression, the role of FOXO3a in the GnRH-induced Egr1 expression, was examined next. Given that GnRH treatment causes a maximal increase in LH β -reporter activity after 6 hours of treatment, the effect of FOXO3a on GnRH-induced Egr1-reporter activity over 2, 3, 4 and 5 hours was examined, on the assumption that GnRH increases the Egr1 expression prior to LH β expression reaching its maximum level. L β T2 gonadotrope cells were co-transfected with an Egr1-reporter and either the FOXO3a-WT or FOXO3a-TM constructs, and treated with 1 μ M GnRH. A 5 hour time-course treatment with GnRH in vector controlled L β T2 cells showed that GnRH-induced Egr1 activity was increased reaching maximal expression after 5 hours. The same trend was observed in cells with FOXO3a-WT or FOXO3a-TM over-expression, in which over-expression of FOXO3a-WT or FOXO3a-TM increased the GnRH-induced Egr1-reporter activity compared to vector control, strongly implicating FOXO3a in GnRH-induced Egr1 expression (**Fig. 5-3**).

The effect of FOXO3a on GnRH-induced Egr1 expression in response to GnRH over 6 hours of treatment was examined in order to compare the effect of FOXO3a on Egr1 expression at the same time point that GnRH induced maximal LH β expression. L β T2 cells were co-transfected with Egr1-reporter and the FOXO3a-TM construct. Over-expression of FOXO3a-TM up-regulated Egr1-reporter activity in response to GnRH (**Fig. 5-4A**), but had no effect on basal Egr1-reporter activity (**Fig. 5-4B**).

Notably, over-expression of FOXO3a-TM Δ DB increased basal Egr1-reporter activity (Fig. 5-4B), possibly suggesting negative regulation of Egr1 expression by FOXO3a.

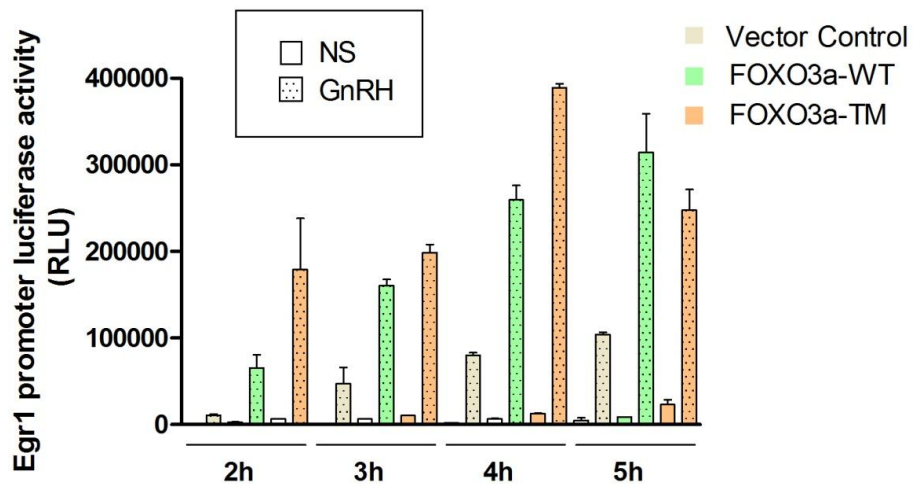


Figure 5-3: Time-course treatment with GnRH for Egr1-reporter activity.

L β T2 cells were co-transfected with 0.1 μ g Egr1-reporter and 9.9 μ g of control vector (pcDNA3.1), FOXO3a-WT or FOXO3a-TM. Cells were serum-starved 24 hours after transfection followed by stimulation with 1 μ M GnRH hours. Samples were then assayed for luciferase activity. Data are shown as mean fold change over NS \pm SEM from at least 2 independent experiments.

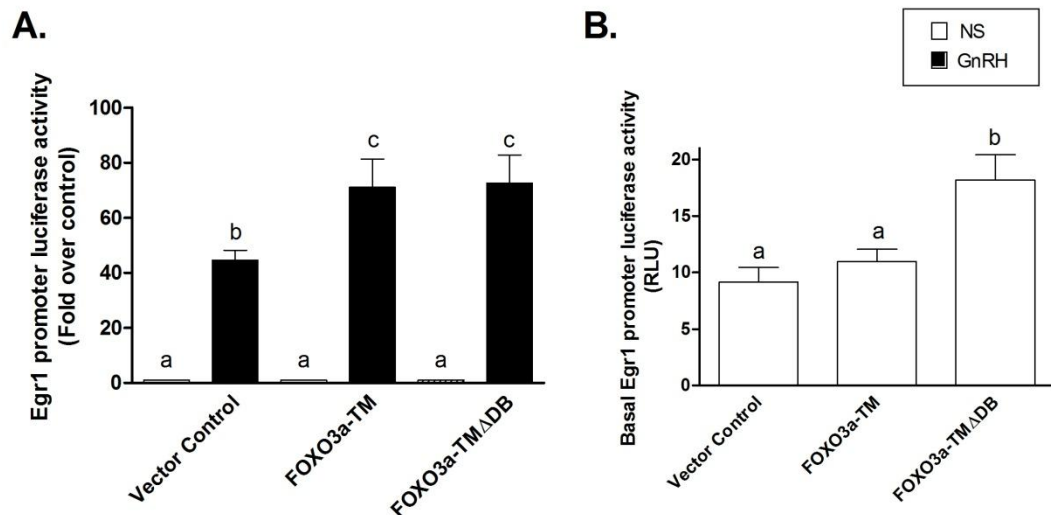


Figure 5-4: The role of FOXO3a in the GnRH-induced Egr1 expression.

L β T2 cells were co-transfected with 0.1 μ g Egr1-reporter and (A and B) 9.9 μ g of control vector (pcDNA3.1), FOXO3a-TM or (B) FOXO3A-TM Δ DB. (A and B) Cells were serum-starved 24 hours after transfection followed by stimulation (B) with 1 μ M GnRH for 6 hours. Samples were then assayed for luciferase activity. In all panels open bars denote no stimulation and close bars denote GnRH stimulation. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a, and c is significantly different from a and b; $P < 0.05$)

5.2.4 GnRH regulation of LH β -subunit expression by targeting Egr1 expression by FOXO3a

Despite the data showing that GnRH induced Egr1 and LH β expression via FOXO3a, it was not clear whether FOXO3a regulated GnRH-induced LH β expression via an Egr1-independent pathway. To test this possibility, the induction of LH β -reporter activity in L β T2 cells was examined by over-expression of FOXO3a-TM or Egr1, or Egr1 together with FOXO3a-TM. Egr1 over-expression alone increased basal LH β expression (Fig. 5-5A), but did not up-regulate LH β expression in response to GnRH (Fig. 5-5B). Although over-expression of FOXO3a-TM up-regulated LH β -reporter activity compared to vector control, co-expression of

FOXO3a-TM and Egr1 failed to increase LH β -reporter activity in response to GnRH (Fig. 5-5B).

To further investigate the role of Egr1 and FOXO3a in LH β expression in response to GnRH, L β T2 cells were transfected with either a -136 ovine LH β -promoter reporter fragment (dLH7-luc) or a -136 ovine LH β -promoter reporter fragment with a double deletion of the Egr1 binding sites (mutated distal and proximal mdpEgr1 (mdpLH7-luc construct)) (Fig. 5-5C). Over-expression of FOXO3a-TM up-regulated dLH7-reporter activity compared to vector control, but did not significantly up-regulate mdpLH7-reporter activity. Thus, FOXO3a was unable to up-regulate LH β expression in the absence of the Egr1 binding sites on the LH β promoter. Taken together, these data suggest a role for FOXO3a in GnRH-induced Egr1 expression, which is essential for maximal LH β expression.

5.2.5 GnRH regulation of LH β expression via FOXO3a requires β -catenin activity

In a previous study by Gardner *et al.* (2007), GnRH treatment promoted the stabilisation and nuclear accumulation of β -catenin in L β T2 cells (Gardner *et al.*, 2007). β -catenin acts as a co-activator of GnRH-induced LH β expression by forming a complex with Egr1 and SF1, which act together at the LH β promoter to drive maximal LH β expression (Salisbury *et al.*, 2007). Thus, the potential role of FOXO3a in the functioning of this complex in response to GnRH was assessed. In particular, whether FOXO3a acts together with, or independently of β -catenin to regulate GnRH-induced LH β expression in L β T2 cells was examined. L β T2 cells were co-transfected with the LH β -reporter together with FOXO3a-TM or Axin alone,

or with FOXO3a-TM together with Axin (**Fig. 5-6A**). Axin is known to promote the degradation of cellular β -catenin (Salisbury *et al.*, 2007). As expected, Axin over-expression suppressed LH β -reporter activity in response to GnRH compared to vector control. However, co-expression of Axin together with FOXO3a-TM also suppressed LH β -reporter activity compared to FOXO3a-TM over-expression alone (**Fig. 5-6A**), suggesting that FOXO3a and β -catenin co-operate to drive LH β -reporter activity.

Additionally, a possible involvement of β -catenin, as a co-factor with FOXO3a in Egr1 activity in response to GnRH was assessed. In particular, it was examined whether FOXO3a acts together with, or independently of β -catenin to regulate GnRH-induced Egr1 expression in L β T2 cells was examined. L β T2 cells were co-transfected with the Egr1-reporter together with FOXO3a-TM or Axin alone, or with FOXO3a-TM together with Axin (**Fig. 5-6B**). Although over-expression of FOXO3a-TM increased Egr1-reporter activity, Axin over-expression failed to suppress Egr1-reporter activity in response to GnRH compared to vector control. In addition, co-expression of Axin together with FOXO3a-TM did not change Egr1-reporter activity compared to FOXO3a-TM over-expression alone (**Fig. 5-6A**). Taken together these data suggest that β -catenin does not co-operate with FOXO3a to regulate Egr1 expression.

5.2.6 GnRH regulation of FOXO3a binding at *Egr1* and *LHβ* promoters

Since a potential role for FOXO3a in driving the expression of LHβ and *Egr1* in response to GnRH has been demonstrated, whether FOXO3a acts directly at the promoters of these two genes, and whether it acts as an activator or suppressor was examined next.

In order to assess the specific role of FOXO3a at these two promoters, ChIP assays were performed (calculations for RT-PCR analysis for the ChIP assay are described in **Supplementary Table 7-10**). The DNA that was immuno-precipitated with FOXO3a or IgG (control) antibody in response to GnRH was amplified by Taqman qRT-PCR using primers for the promoter regions or primers that anneal to distal upstream regions of the *LHβ* and *Egr1* genes (**Fig. 5-7**).

The results suggest that GnRH treatment increased the binding of FOXO3a to the *LHβ* promoter to regulate LHβ-subunit expression (**Fig. 5-8A**). In contrast, GnRH treatment appeared to result in the release of FOXO3a from the *Egr1* promoter at 6 hours, which may indicate that under basal conditions FOXO3a acts to repress *Egr1* expression (**Fig. 5-8B**), again suggesting that FOXO3a negatively regulates *Egr1* under basal conditions (see **Figure 5-4B**)

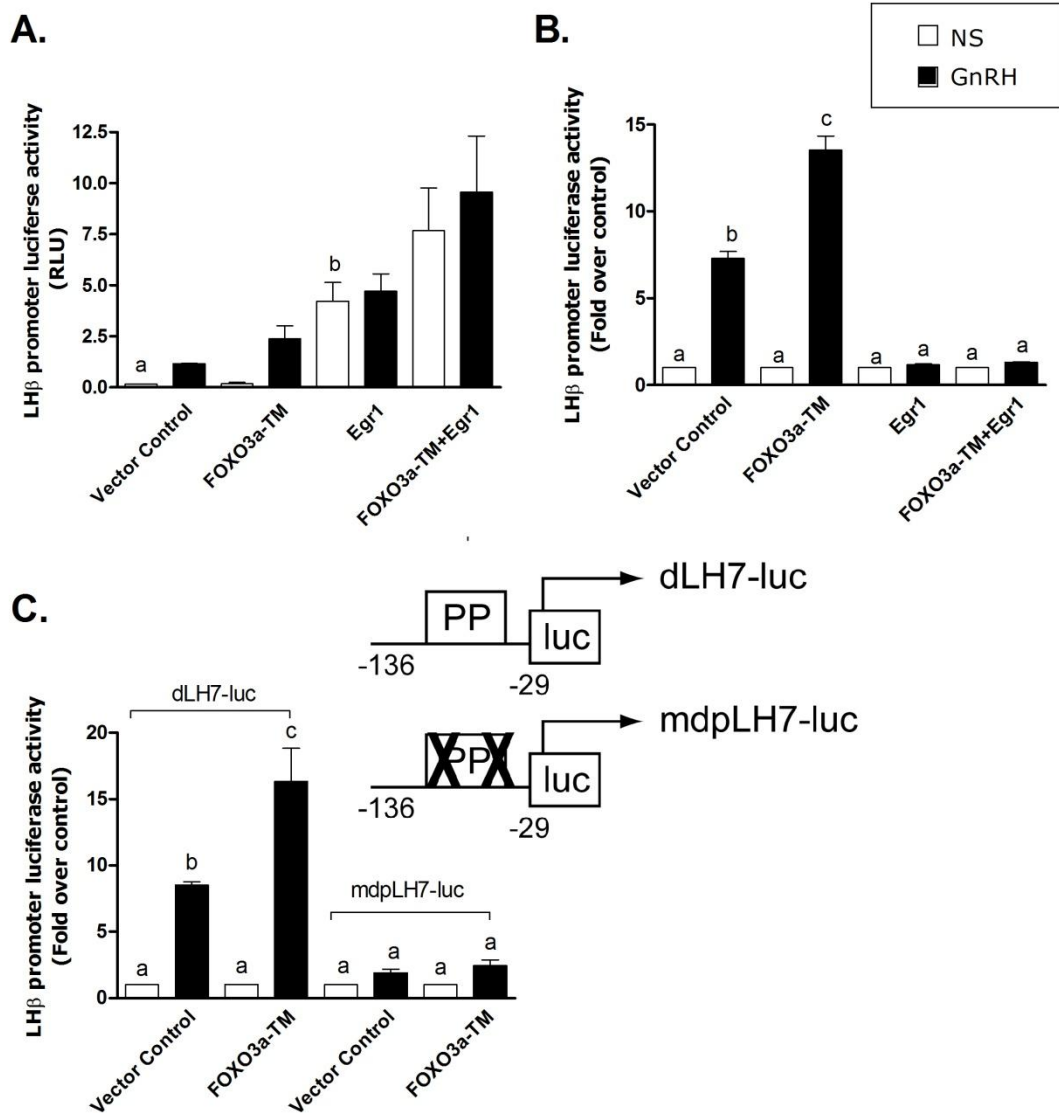


Figure 5-5: Egr1 and FOXO3a in the GnRH-induced LH β expression.

(A and B) L β T2 cells were co-transfected with 2 μ g LH β -reporter and 4 μ g of control vector (pcDNA3.1), FOXO3a-TM, Egr1 or FOXO3a-TM+Egr1 as indicated. (C) L β T2 cells were co-transfected with 2 μ g dLH7- or mdpLH7⁽¹⁾-reporter and 8 μ g of control vector (pcDNA3.1) or FOXO3a-TM. Cells were serum-starved 24 hours after transfection followed by stimulation with 1 μ M GnRH for 6 hours. Samples were then assayed for luciferase activity. In all panels open bars denote no stimulation and close bars denote GnRH stimulation. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a, and c is significantly different from a and b; P<0.05)

⁽¹⁾mdpLH7-luc: -136/-29 ovine LH β -reporter fragment with a double deletion of the two Egr1 binding sites on the proximal promoter (PP) region.

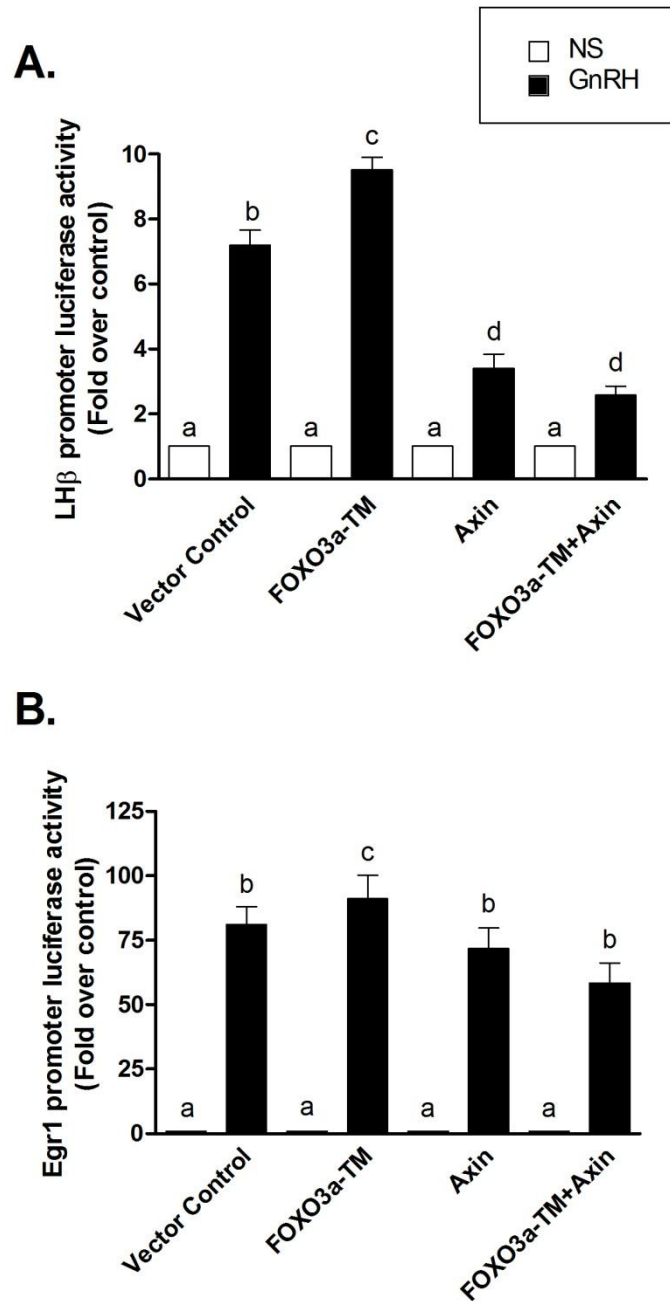


Figure 5-6: GnRH-induced LH β -reporter activity requires the presence of FOXO3a and β -catenin.

L β T2 cells were co-transfected with 2 μ g of (A) LH β - or (B) Egr1-reporter and 4 μ g of control vector (pcDNA3.1), FOXO3a-TM or Axin as indicated. Cells were serum-starved 24 hours after transfection followed by stimulation with 1 μ M GnRH for 6 hours. In all panels open bars denote no stimulation and close bars denote GnRH stimulation. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments. (b is significantly different from a, c is significantly different from a, and b and d are significantly different from a, b and c; $P < 0.05$).

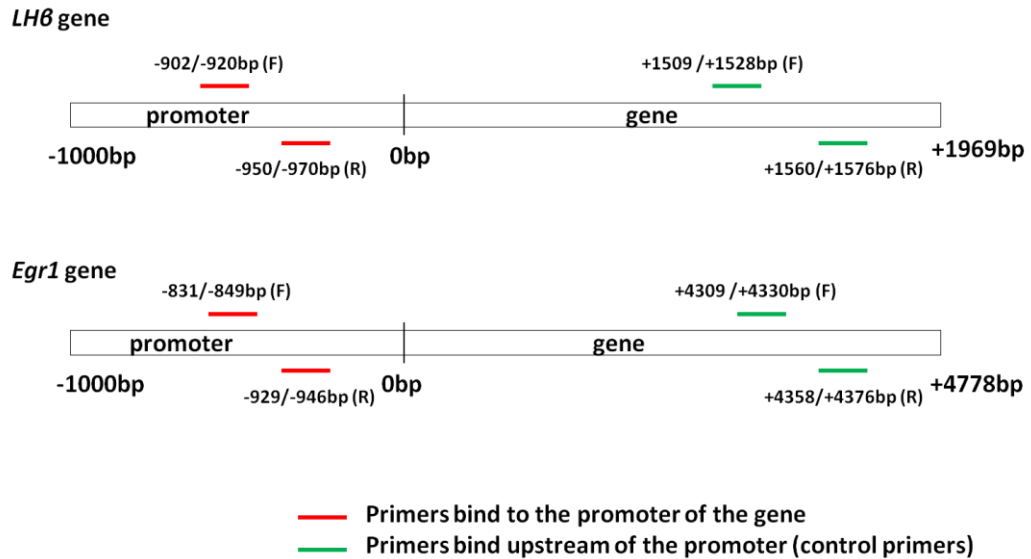


Figure 5-7: Schematic representation of the mouse *LH β* and *Egr1* genes, and the primers pairs that bind to the promoter of the genes, or upstream of the promoter (control primers).

Primers corresponding to *LH β* and *Egr1* are located in the promoter of these genes, and the control primers located at a region upstream of the promoter.

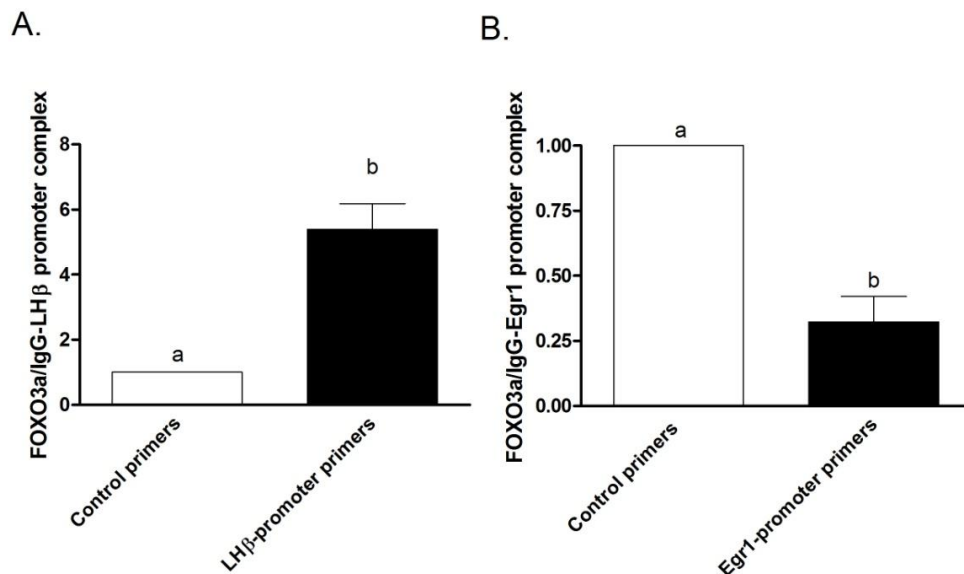


Figure 5-8: GnRH regulation of FOXO3a binding to *LH β* and *Egr1* promoter.

The regulation of FOXO3a binding to the promoter of *LH β* and *Egr1* in response to GnRH was measured by ChIP and quantitative RT-PCR using primers for the *LH β* and *Egr1* promoter and primers for distal upstream region of the *LH β* gene and *Egr1* gene. L β T2 cells were stimulated with 1 μ M GnRH for 6 hours. Data are shown as mean fold change over “control primers” \pm SEM from 3 independent experiments. (b is significantly different from a; $P < 0.05$).

5.2.7 Immunohistochemistry of FOXOs in pituitary tissues

5.2.7.1 *Optimisation of the staining for FOXO1, FOXO3a and p-FOXO3a(Ser²⁵³) antibodies*

Having confirmed the involvement of FOXO3a in the regulation of GnRH-induced LH β -reporter activity in the L β T2 cells, immunohistochemistry was utilised in order to examine whether FOXO1 and FOXO3a are expressed in the pituitary. Pituitary tissues from human, rat and mouse tissues were utilised. In order to examine whether there was expression of FOXOs in these tissues, optimisation of antibodies was required, since different dilutions will be optimal for each tissue depending on the FOXO expression level. In addition, immunohistochemistry with and without an antigen retrieval step for unmasking the epitopes through pressure cooking was performed.

Immunostaining of pituitary tissues from rat and mouse showed low staining for LH β , but no staining for FOXO1 and FOXO3a (FOXO1 and FOXO3a antibody with and without antigen retrieval used at the following dilutions: 1:20, 1:500, 1:1,000, 1:5,000 and 1:10,000).

Immunostaining of pituitary tissues from adult human was also performed. FOXO1, FOXO3a and p-FOXO3a(Ser²⁵³) antibodies in serial dilutions of 1:10, 1:50, 1:200, 1:1,000 were used showing very low staining or nonspecific background with antigen retrieval, while without antigen retrieval, specific and clear staining for all of the above serial antibody dilutions was not observed. However, immunostaining showed expression of FOXO1 and FOXO3a (and localisation of p-FOXO3a(Ser²⁵³)) with antibody dilutions 1:100, 1:20 and 1:100 respectively with antigen retrieval.

For the tyramide double-staining detection method, tissues were stained with a series of dilutions of 1:400, 1:600 and 1:800 for FOXO1, FOXO3a and p-FOXO3a(Ser²⁵³) antibodies. The 1:800 dilution was found to be the optimal dilution for all three antibodies. Next, the tissues were double-stained with the second primary antibody (LH β) at dilutions of 1:200, 1:500 and 1:1000. The optimal dilution for this antibody was 1:200 for double-staining with FOXO1 and p-FOXO3a(Ser²⁵³), and 1:500 for double-staining with FOXO3a. Only satisfactory staining images are presented in this Chapter with optimal antibody dilutions.

5.2.7.2 *Adult human gonadotropes express FOXO1 and FOXO3a*

Having confirmed the involvement of FOXO3a in the regulation of LH β promoter activity in response to GnRH in L β T2 cells, immunohistochemistry was used to examine the expression of FOXO1 and FOXO3a in the pituitary of adult human males. Immunohistochemical staining throughout the anterior pituitary revealed broad expression of FOXO1 and FOXO3a. FOXO1 was located predominantly in the cytoplasm, whereas FOXO3a was located exclusively in the nucleus (**Fig 5-9**). To localise FOXO1 and FOXO3a to gonadotropes, double immunofluorescent staining for LH and FOXO1 or FOXO3a was performed. In cells positive for LH β (i.e. gonadotropes), FOXO3a was found to be expressed in the nucleus (**Fig. 5-10A**) whereas FOXO1 was mainly expressed in the cytoplasm (**Fig. 5-10B**). The control sections showed no immuno-reactivity.

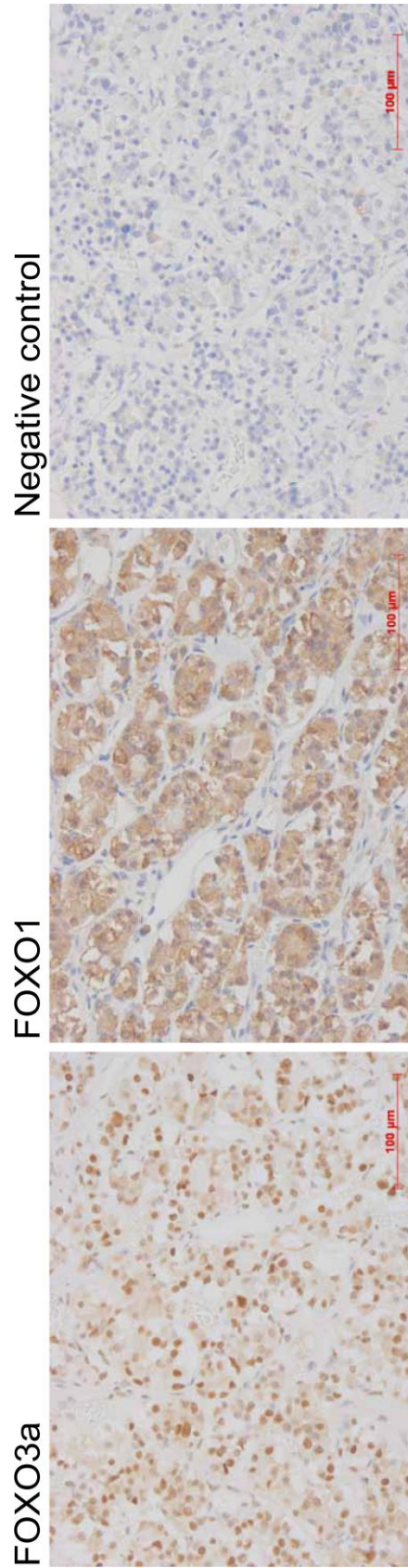


Figure 5-9: FOXO1 and FOXO3a expression and immuno-localisation in adult human pituitary tissue. Immunohistochemistry in human pituitary tissue. FOXO3a (brown) expressed mainly in the nucleus (purple), and FOXO1 (brown) is expressed mainly in the cytoplasm. Negative control is used to evaluate non-specific binding.

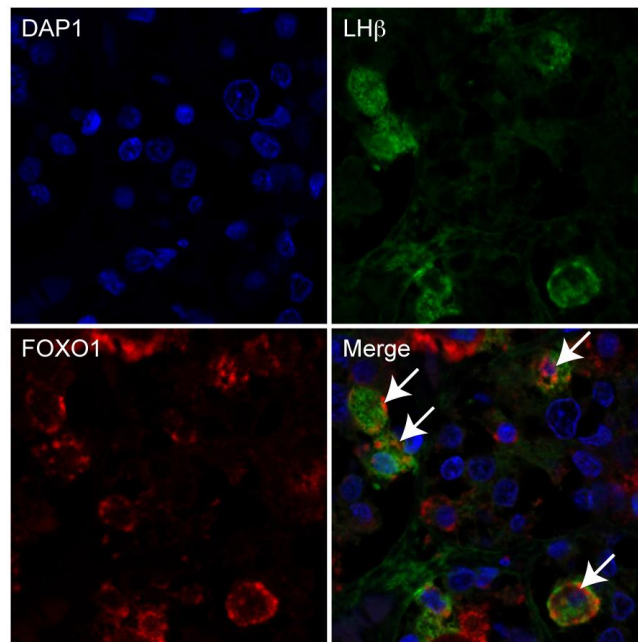
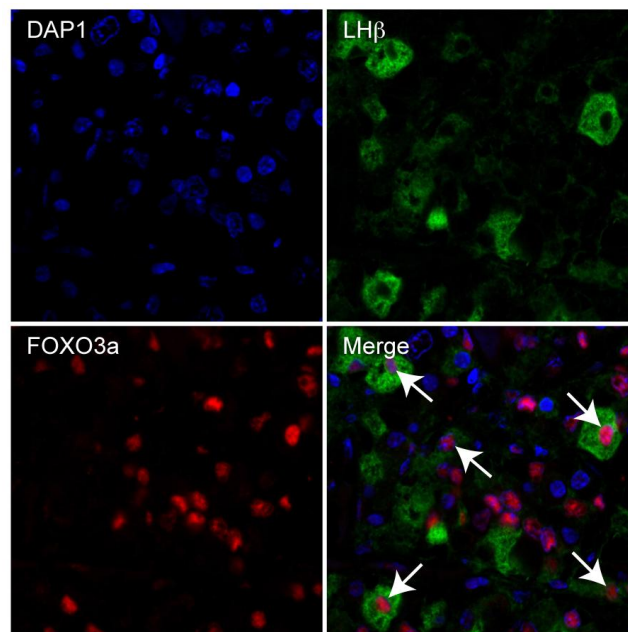
A.**B.**

Figure 5-10: FOXO1 and FOXO3a expression and immuno-localisation in adult human pituitary gonadotropes.

Confocal dual immunofluorescence images of adult human pituitary sections immunoreactive for (A) FOXO1 or (B) FOXO3a (Red fluorescence, TSA-Cy3) and LHβ (green fluorescence, TSA-fluorescence). Nuclei are stained in blue by DAPI. Arrows indicate the gonadotrope cells that also co-express (A) FOXO1 or (B) FOXO3a.

5.2.8 GnRH-induced FOXO3a phosphorylation

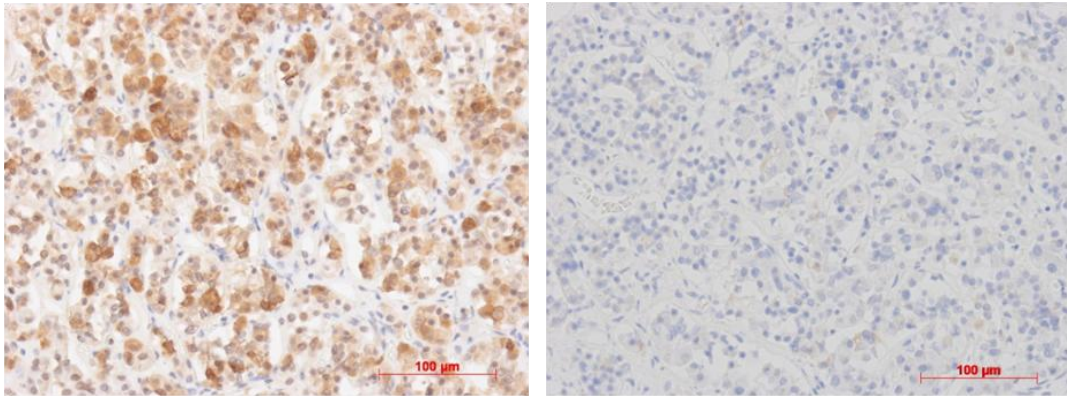
Having shown that FOXO3a is expressed in human pituitary tissues, phosphorylation of FOXO3a in response to GnRH was examined in L β T2 gonadotrope cells. Firstly, immunohistochemical staining revealed phosphorylated FOXO3a in human pituitary gonadotropes (**Fig 5-11A**). p-FOXO3a(Ser²⁵³) was localised both in the nucleus and in cytoplasm (**Fig. 5-11A**). In cells positive for LH β (i.e. gonadotropes) p-FOXO3a(Ser²⁵³) was found to be similarly expressed (**Fig. 5-11B**).

Next, immunofluorescence confocal microscopy was used in order to determine visually whether p-FOXO3a(Ser²⁵³) was redistributed between nucleus and cytoplasm in L β T2 gonadotrope cells in response to GnRH. p-FOXO3a(Ser²⁵³) was localised in both the nucleus and the cytoplasm, and no significant translocation was observed in response to GnRH. Phosphorylation of FOXO3a at Ser²⁵³ was increased following 5 minutes treatment with 1 μ M GnRH. However, after 10 and 60 minutes of treatment, levels of p-FOXO3a(Ser²⁵³) returned to non-stimulated levels (**Fig. 5-12A**). This was consistent with the results of a Western Blot analysis, where phosphorylation of FOXO3a at Ser²⁵³ was increased following 5 minutes treatment with 1 μ M GnRH. Western Blot analysis demonstrated that phosphorylation of FOXO3a at Ser²⁵³ was also increased following 30 minutes treatment with 1 μ M GnRH (**Fig. 5-12B**).

A.

p-FOXO3a(Ser²⁵³)

Negative control



B.

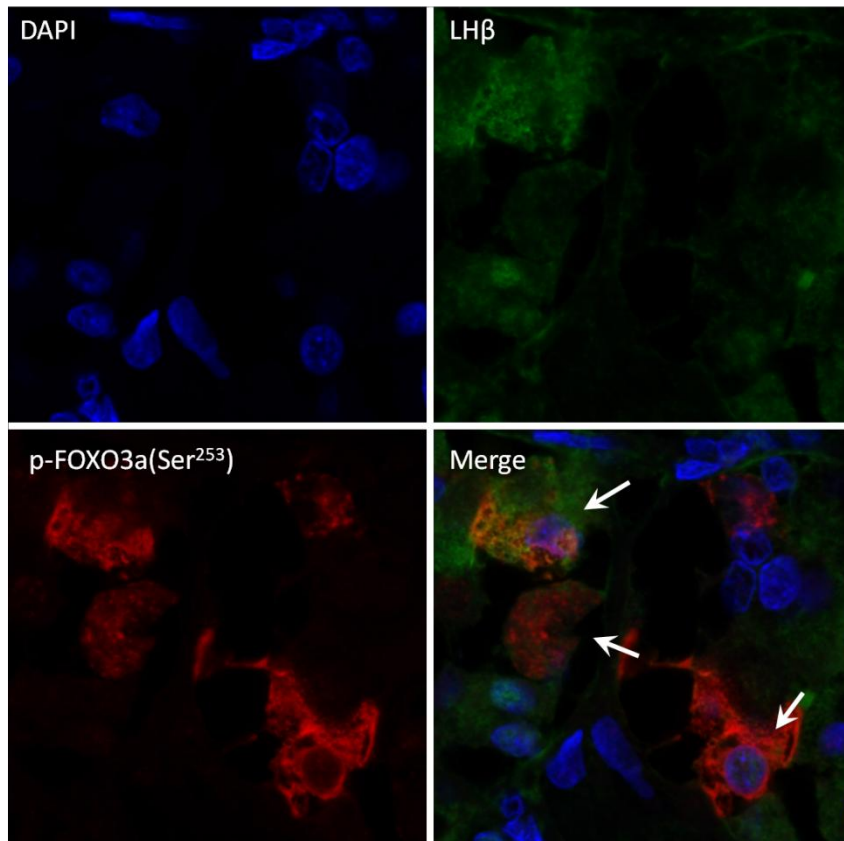


Figure 5-11: p-FOXO3a(Ser²⁵³) staining in gonadotropes.

(A) Immunohistochemistry in human anterior pituitary p-FOXO3a(Ser²⁵³) (brown) is expressed in the nucleus (purple) and in the cytoplasm. Negative control is used to evaluate non-specific binding. (B) Co-localisation of p-FOXO3a Ser²⁵³ in human anterior pituitary tissue. p-FOXO3a(Ser²⁵³) is indicated in red, LHβ is green and nuclei are stained in blue by DAPI. Arrows indicate gonadotropes that also express p-FOXO3a(Ser²⁵³).

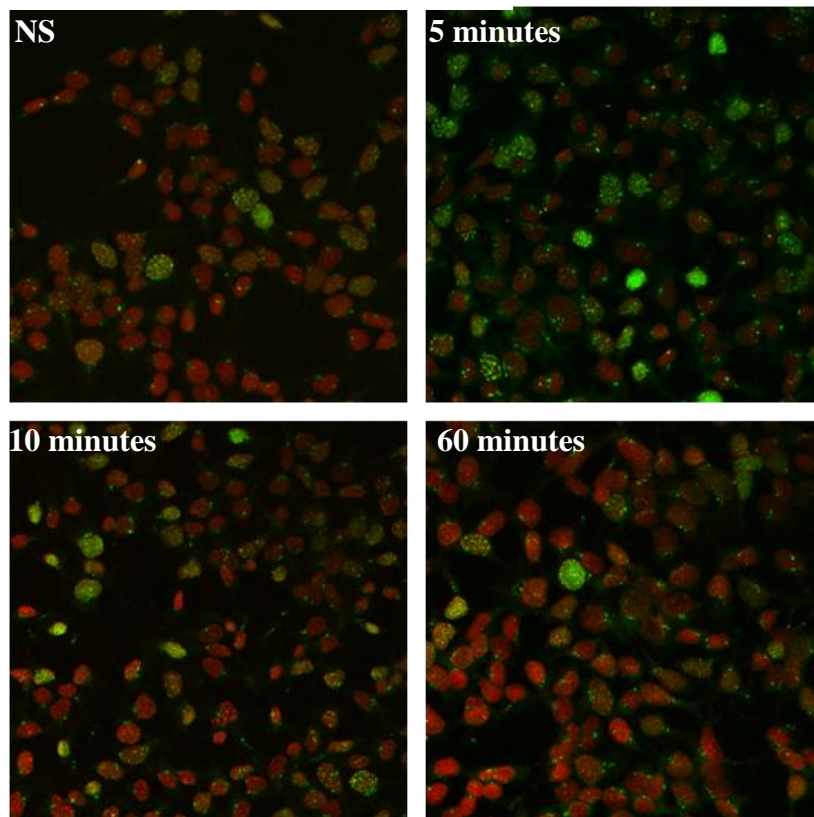
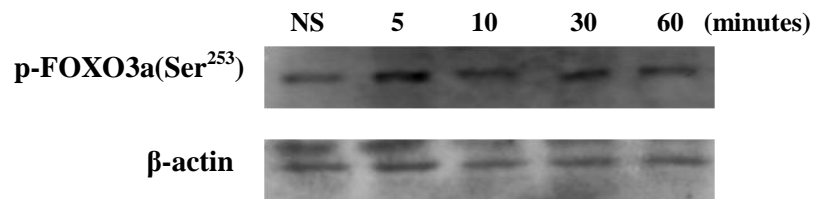
A.**B.**

Figure 5-12: FOXO3a phosphorylation in LβT2 cells.

(A) LβT2 cells were seeded into eight-well chamber slides and serum-starved (24h) before stimulation with 1μM GnRH for indicated times, or left untreated (NS). Cells were fixed and immunocytochemistry performed. Detection of the immuno-reactive proteins was achieved using Alexafluor (488nm, green) conjugate for p-FOXO3a(Ser²⁵³). The merged image also shows DAPI staining (red) and co-localisation as yellow. (B) Western Blot analysis for p-FOXO3a(Ser253), in LβT2, which were treated with 1μM GnRH for the indicated times, or left untreated (NS). Blot is representative of 3 independent experiments.

5.3 DISCUSSION

In the present study the expression of FOXO1 and FOXO3a in L β T2 cells and in human pituitary tissue was demonstrated. A role for FOXO3a in mediating GnRH-induced LH β and Egr1 expression was shown. ChIP assay results led to speculation that FOXO3a binds to the LH β promoter, but it is released from the Egr1 promoter, following GnRH treatment. Overall, the results from this Chapter suggested that GnRH targets FOXO3a activity to regulate LH β expression directly, through the binding of FOXO3a to the LH β promoter in complex with co-factors, and indirectly through the release of FOXO3a from the Egr1 promoter, on which it probably acts as a repressor.

The expression of FOXO1 and FOXO3a in the human pituitary, as well as expression of both transcription factors in human pituitary gonadotropes was demonstrated using double immunofluorescence confocal microscopy (**Fig 5-10**). Of particular interest was the differential localisation patterns of the two FOXOs in human pituitary gonadotropes; FOXO3a was found exclusively in the nucleus and FOXO1 in the cytoplasm. The co-localisation of FOXO3a to the nucleus was pertinent as this was the factor that influenced LH β and Egr1 expression, and was shown to bind to the promoters of these genes. In contrast, FOXO1 was localised to the cytoplasm and had no effect on LH β and Egr1 expression.

Several lines of evidence that GnRH-induced LH β expression is mediated by FOXO3a were provided. First, over-expression of FOXO3a up-regulated LH β expression (**Fig. 5-1**). Second, in FOXO3a knock-down L β T2 cells, the basal level of LH β expression was lower compared to control cell (**Fig 5-2B**). Third, GnRH

receptor activation in the FOXO3a knock-down L β T2 cells did not lead to an increase in LH β expression (**Fig 5-2C**). On the contrary, LH β expression was reduced compared to that of control cells. These findings support the proposal that FOXO3a is an important mediator for GnRH-induced LH β expression.

The involvement of FOXO3a in modulating physiological LH levels was revealed in a study by Castrillon *et al.* (2003). In particular, FOXO3a^{-/-} mice exhibited serum levels of LH which were higher compared to the control FOXO3a^{+/+} mice (Castrillon *et al.*, 2003). Since the FOXO3a knock-down was not specific to the pituitary, and would therefore be expected to affect the biology of multiple tissues including other reproductive tissues, there are two possible explanations for this observation. Either, FOXO3a normally represses LH production in the pituitary gonadotrope, or FOXO3a is involved in some form of feedback regulation from the ovary or testis. Although over-expression or knocking-down of FOXO3a in L β T2 cells in the present study resulted in a direct effect on LH β -subunit expression, it is unclear what the mechanistic role of FOXO3a is in regulating LH levels *in vivo*. Furthermore, FOXO3a regulation of LH β expression in L β T2 cells is restricted to the GnRH effect, while in the *in vivo* situation, this effect is also supplemented by a number of physiological factors such as steroid and peptide hormones affecting gonadotropes (Ooi *et al.*, 2004). It is known that gonadotrope cells only comprise 5-15% of the cells in anterior pituitary (Ooi *et al.*, 2004), and that there are four other types of secretory cell types present in the anterior pituitary which secrete factors that may effect gonadotrope cell function (Thackray *et al.*, 2010).

The study by Castrillon *et al.* (2003) also demonstrated that FOXO3a^{-/-} female mice were viable and showed age-dependent infertility without histological abnormalities of the pituitary tissue (Castrillon *et al.*, 2003). They further reported that FOXO3a is not required for gonadotropin responsiveness in ovarian follicles (Castrillon *et al.*, 2003). These data, in combination with the findings from this Chapter, that GnRH can regulate LH β expression via a FOXO3a-mediated mechanism, may suggest that the infertility in the FOXO3a^{-/-} mice could be due to the inability of GnRH, through FOXO3a regulation, to optimally increase LH β expression or the release of mature LH from the gonadotropes leading to reduced serum LH levels.

The present data demonstrated that GnRH treatment stimulates the transcriptional activity of FOXO3a, but not of FOXO1 to regulate LH β expression (**Fig. 5-1**). This is consistent with the results from Chapter three in which GnRH was shown to regulate the transcriptional activity of FOXO3a but not FOXO1 in the heterologous SCL60 cell system. Since both FOXO1 and FOXO3a share common DNA binding sites, one could argue that they could mediate expression from the same promoters (Burgering, 2008). However, GnRH-induced LH β -subunit expression is mediated by FOXO3a, and not by FOXO1, suggesting that GnRH may regulate these two transcription factors via distinct signalling pathways. Alternatively, the exclusive localisation of FOXO1 to the cytoplasm of gonadotrope cells may not allow FOXO1 mediation of GnRH-induced LH β expression.

Having demonstrated that GnRH regulates LH β expression by targeting FOXO3a activity, the mechanism by which this occurs was further investigated. Following the finding that GnRH-induced *Egr1* expression was regulated in part by FOXO3a, a

number of possible mechanisms related to the regulation of LH β expression were examined. Firstly, whether FOXO3a regulates the expression of LH β or *Egr1* or both by direct binding to their promoters was examined. Secondly, whether FOXO3a could regulate the *LH β* promoter independently of *Egr1* in response to GnRH was investigated, and finally, whether FOXO3a regulates *LH β* gene expression in cooperation with other co-factors was assessed. Results from the ChIP assay suggested that upon GnRH stimulation, FOXO3a binds to the *LH β* promoter, increasing its transcriptional activity (**Fig. 5-8**). In contrast, FOXO3a appears to be released from the *Egr1* promoter in response to GnRH. These findings suggested that FOXO3a acts as an enhancer at the *LH β* promoter and as a repressor at the *Egr1* promoter. The latter was further supported by over-expression of FOXO3a-TM Δ DB, which led to an increase in *Egr1*-reporter activity compared to control cells. This may also indicate that FOXO3a acts synergistically with other co-factors on the *Egr1* promoter. The reduction of *Egr1*-reporter activity by co-expression of FOXO3a-TM Δ DB may indicate that FOXO3a-TM Δ DB competes with endogenous FOXO3a for binding with its functional co-factors (**Fig 5-4**). Thus, FOXO3a-TM Δ DB release from the *LH β* promoter in response to GnRH would result in the sequestration of co-factors that are essential for maximal *Egr1* gene expression.

Having demonstrated in a time-course treatment with GnRH for *Egr1*-reporter activity, with over-expression of FOXO3a-WT and FOXO3a-TM, that *Egr1* expression increases over 4 hours but decreases thereafter (**Fig. 5-3**), another possible mechanism with regards to the role of FOXO3a on *Egr1* gene expression could be revealed. In particular, GnRH treatment up-regulates *Egr1* gene expression,

possibly by binding of FOXO3a to the *Egr1* promoter over 4 hours of treatment with GnRH. Thereafter, FOXO3a is released from the *Egr1* promoter, and *Egr1* protein binds to the *LHβ* promoter inducing *LHβ* expression.

FOXO3a regulates the GnRH-induced *LHβ* expression, however this regulation is partly dependent on GnRH-induced *Egr1* expression. To assess the role of *Egr1* in GnRH-induced *LHβ* expression via FOXO3a, two *LHβ*-reporters, the dLH7-reporter (-136 to -29bp) and the mpdLH7-reporter (-136 to -29bp) were utilised (**Fig. 5-5**). Although the length of the dLH7 promoter (-136/-29) was about 550bp shorter than the full length *LHβ* promoter (-692 to -29bp), over-expression of FOXO3a led to an increased GnRH-induced *LHβ* expression compared to control. This suggests that the effect of FOXO3a on the *LHβ* promoter is restricted to the proximal promoter (PP) region (mapped within -320bp of the transcription start site) of the promoter (Maudsley *et al.*, 2007). In addition, the result demonstrating that over-expression of FOXO3a does not significantly increase the mpdLH7-reporter activity compared with vector control confirms the previous results, that GnRH-induced *LHβ* expression is dependent on FOXO3a-regulated *Egr1* expression. This suggests that FOXO3a is released from the *Egr1* promoter in response to GnRH, up-regulating its expression level, since it acts as a repressor (**Fig. 5-13**). *Egr1* protein then binds to the *LHβ* promoter, possibly as a complex with β -catenin and FOXO3a for maximal *LHβ* expression (**Fig 5-13**). Absence of FOXO3a, which binds to *LHβ* promoter in response to GnRH in this complex prevents maximal activity of *LHβ* expression.

Maximal activity of *LHβ* expression requires the presence of SF1, *Egr1* and β -catenin on the *LHβ* promoter (Salisbury *et al.*, 2007). Therefore, the involvement of

FOXO3a in this regulation by both direct binding on the *LHβ* and *Egr1* promoters, and by interaction with other co-factors was suggested. A study using a yeast two-hybrid system by Essers *et al.* (2005) demonstrated that β-catenin interacts with FOXO3a (Essers *et al.*, 2005). Whether or not FOXO3a acts together with, or independently of β-catenin at the *LHβ* promoter was examined. Over-expression of Axin, which promotes degradation of cellular β-catenin levels, reduced GnRH-induced LHβ expression; however co-expression of Axin with FOXO3a did not restore the GnRH-induced LHβ-reporter activity that could be obtained by over-expression of FOXO3a alone.

Overall, results presented in this Chapter implicate FOXO3a in the regulation of GnRH-induced LHβ-subunit expression, in addition to the previously published roles of Egr1, SF1, PITX1 and β-catenin. In summary, GnRH treatment up-regulates *Egr1* gene expression, possibly by releasing FOXO3a from the Egr1 promoter. Thereafter, Egr1 protein binds to the *LHβ* promoter inducing LHβ expression. GnRH treatment also induces FOXO3a localisation to the LHβ promoter. FOXO3a and Egr1 act synergistically on the *LHβ* promoter for maximal *LHβ* gene expression. In addition, FOXO3a-mediated GnRH-induced LHβ maximal expression appears to require β-catenin co-operativity (**Fig. 5-13**).

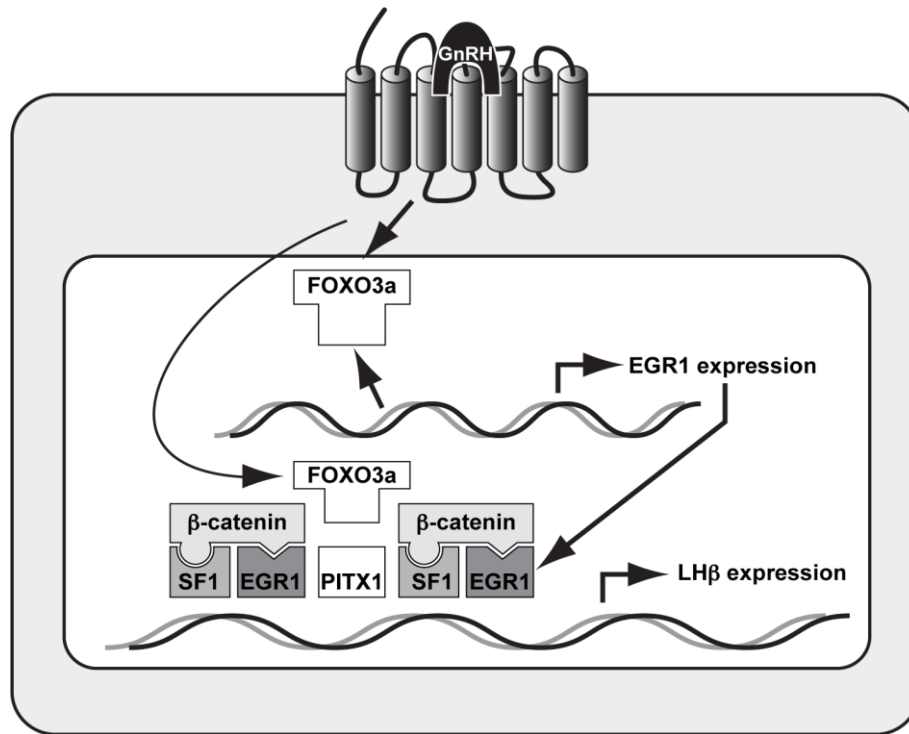


Figure 5-13: Model of *LHβ* and *Egr1* gene regulation by FOXO3a in response to GnRH.

The results from this chapter suggest that in the absence of GnRH treatment, FOXO3a represses *Egr1* expression. In response to GnRH treatment, FOXO3a appears to be released from the *Egr1* promoter, resulting in increased *Egr1* gene expression; *Egr1* then binds to the *LHβ* promoter to drive *LHβ* expression. A complex of FOXO3a, *Egr1* and β -catenin on the *LHβ* promoter is required for maximal *LHβ* gene expression.

CHAPTER SIX

CONCLUDING DISCUSSION

6.1 THESIS MOTIVATION

GnRH-Rs belong to the Rhodopsin-like family of the GPCRs and are activated following the binding of GnRH (Millar and Newton, 2010). GnRH-R activation targets a variety of signalling pathways that mediate important cellular processes. Among these are GnRH-induced cell-growth inhibition in cancer-derived cell lines (Cheng and Leung, 2005; Everest *et al.*, 2001; Franklin *et al.*, 2003; Kraus *et al.*, 2006; Limonta *et al.*, 2003; Maudsley *et al.*, 2004; Miles *et al.*, 2004; Morgan *et al.*, 2008), and GnRH-induced LH and FSH synthesis and secretion in pituitary gonadotropes (Naor, 2009). The importance of GnRH-induced cell-growth inhibition is easily understood by the fact that GnRH analogues are currently used for cancer treatment (Bifulco *et al.*, 2004; Carr *et al.*, 1993; Hofstra *et al.*, 1999; Klijn and de Jong, 1982; Mizutani *et al.*, 1998; Tolis *et al.*, 1982). In addition, because GnRH plays a central role in reproduction, the regulation of gonadotropin production by GnRH analogues is used for treatment of infertility (White *et al.*, 2008b).

Although both of the aforementioned cellular processes have been extensively described, the mechanisms and signalling pathways that mediate them are not yet completely understood. When the current study was initiated, a role for FOXO transcription factors in regulating GnRH-induced cell-growth inhibition and gonadotropin synthesis had not been proposed. Nevertheless, the important role of FOXOs in cell-cycle regulation and induction of apoptosis is well-documented (Fu and Tindall, 2008). The involvement of FOXOs in cell proliferation has been shown to be mediated by the PI3K/Akt signalling pathway, a known GnRH-targeted

signalling pathway. These facts provided the basis for pursuing the research described in this thesis.

6.2 THESIS OBJECTIVES

Since no previous studies had described the regulation of FOXOs by GnRH-R activation, the initial objective in **Chapter three** was to examine whether GnRH regulates FOXO activity. Thereafter, the mechanisms by which FOXO regulation is mediated following GnRH-R activation were investigated. The regulation of FOXO activity by post-translational modifications and the action of co-factors is well-established. Therefore, the role of such modifications (phosphorylation and acetylation), and the effect of β -catenin on FOXO activity in response to GnRH, were also examined in **Chapter three**. In addition, the regulation of FOXO-target genes following GnRH-R activation was assessed, and a cell-cycle analysis of GnRH-R-expressing cells was performed.

In complementary studies presented in **Chapter four**, a role for FOXO3a in mediating GnRH-induced cell-growth inhibition was assessed. GnRH-induced cell-growth inhibition pathways, and the diverse factors that mediate them have previously been examined by many research groups (Cheng and Leung, 2005; Everest *et al.*, 2001; Franklin *et al.*, 2003; Kraus *et al.*, 2006; Limonta *et al.*, 2003; Maudsley *et al.*, 2004; Miles *et al.*, 2004; Morgan *et al.*, 2008). By generating a FOXO3a knock-down cell line, the potential importance of FOXO3a in regulating cell-growth rate and GnRH-induced cell-growth inhibition was investigated.

Finally, the mechanisms that mediate the synthesis of LH β and FSH β in gonadotropes were further explored in **Chapter five**. The role of transcription factors and co-factors, such as Egr1, SF1, Sp1 and more recently β -catenin in regulating LH β promoter activity are now fairly well-characterised (Thackray *et al.*, 2010). Recent studies highlighting the co-operativity between β -catenin and FOXOs in mediating gene expression (Essers *et al.*, 2005; Hoogeboom *et al.*, 2008; Kwon *et al.*, 2010), suggested a potential role for FOXOs in regulating gonadotrope function. The expression of FOXOs in gonadotropes was assessed by double immunostaining in human pituitary tissues, and the involvement of FOXOs in regulating LH β , FSH β and GnRH-R promoter activity in response to GnRH was examined.

6.3 CONCLUSIONS

In **Chapter three**, the regulation of FOXO3a activity following GnRH treatment was demonstrated. This novel regulation is described for the first time in this thesis using the SCL60 model cell line. The mechanism by which GnRH regulates FOXO activity appears to link two known signalling pathways; GnRH-activated PI3K/Akt signalling and growth factor-activated PI3K/Akt-induced regulation of FOXO activity. Further exploration of the mechanism of this regulation demonstrated FOXO3a phosphorylation at an Akt phosphorylation site (Ser²⁵³), and its subsequent translocation from nucleus to the cytoplasm. Since GnRH treatment promoted the phosphorylation and activation of Akt (phosphorylation at Thr³⁰⁸), it remains unclear why GnRH does not target phosphorylation of FOXO3a at the other Akt phosphorylation sites (Ser³¹⁵ and Thr³²), or indeed phosphorylation of FOXO1 and FOXO4 Akt sites. This could be explained by the observation that GnRH was unable

to promote the phosphorylation of Akt at both sites (Thr³⁰⁸ and Ser⁴⁷³), which is apparently required for its full activation. By utilising a panel of pharmacological inhibitors and dominant-negative mutants, the pre-dominant signalling pathway by which GnRH regulates FOXO3a activity was identified. GnRH does not regulate FOXO transcriptional activity via a PI3K-dependent pathway, but instead via another Akt- and IKK-dependent pathway. It is not clear whether these mediators are part of the same signalling pathway, or how Akt is activated by a PI3K-independent pathway.

One of the unexpected findings in this chapter was that FOXO3a de-phosphorylation by GnRH over 24 hours of treatment was not the dominant mechanism regulating FOXO3a transcriptional activity. This arises from the observation that although GnRH targets FOXO3a de-phosphorylation over 24 hours of treatment, GnRH treatment increased FOXO3a transcriptional activity by the same fold in cells co-transfected with either FOXO3a-WT, or FOXO3a-TM that cannot be phosphorylated by Akt, clearly indicating that phosphorylation is not the dominant post-translational modification that regulates FOXO3a transcriptional activity following GnRH treatment. A further important finding presented in this chapter was the correlation of the expression profile of FOXO-target genes (*FasL*, *GADD45*, *p21Cip* and *p27Kip1*) in response to GnRH, with the cell-cycle profile of SCL60 cells, which was assessed by a cell-cycle analysis.

Having demonstrated that GnRH regulates FOXO3a transcriptional activity in **Chapter three**, a FOXO3a knock-down cell line was generated in order to examine the role FOXO3a in GnRH-induced cell-growth inhibition. The results in **Chapter**

three suggested that FOXO3a protects cells from GnRH-induced cell-growth inhibition. Cell-cycle analysis of FOXO3a knock-down cells revealed a negative role for FOXO3a in GnRH-induced cell-cycle arrest and apoptosis, in which FOXO3a mediates G2 phase arrest and apoptosis, probably by regulating *GADD45* and *FasL* gene expression respectively.

The impact of GnRH regulation on FOXO3a transcriptional activity in the gonadotropes was assessed in **Chapter five**. In particular, a role for FOXO3a in GnRH-induced LH β -subunit expression was investigated. Initially, expression of FOXO1 and FOXO3a was demonstrated in gonadotropes in human pituitary tissue. Although the role of FOXO1 in gonadotropes remains unclear, FOXO3a was found to regulate LH β expression. Interestingly, in this study, the role of FOXO3a as a transcription factor on the *Egr1* promoter was also demonstrated. Expression of the *Egr1* gene, and binding of Egr1 to the promoter of LH β , is required for maximal LH β expression. Taken together, the results presented in **Chapter five** demonstrate the involvement of FOXO3a in LH β expression, by both direct binding on the LH β and *Egr1* promoters, and by interaction with other co-factors such as β -catenin.

6.4 FUTURE RESEARCH AND PERSPECTIVES

Work presented in this thesis described for the first time the regulation of FOXO3a transcriptional activity by GnRH-R activation. In addition, this thesis assessed the biological significance of this regulation. By investigating the role of the FOXO3a in GnRH-induced cell-growth inhibition and GnRH-induced synthesis of the

gonadotropins, new opportunities arise for further investigation of the current findings, or for exploring novel applications of these findings.

The regulation of FOXO3a activity by GnRH was demonstrated in **Chapter three**. By utilising pharmacological inhibitors, the signalling pathway that mediates the GnRH-induced FOXO3a transcriptional activity was characterised. Akt and IKK have been shown to mediate this effect, whereas this regulation appears to be independent of PI3K. The results demonstrated that PDK1 targets phosphorylation and subsequent activation of Akt in response to GnRH. Although several of studies have previously demonstrated PI3K-independent activation of Akt via a variety of mechanisms (Vanhaesebroeck and Alessi, 2000), further investigation in order to determine alternative mechanisms for PDK1 or Akt activation in response to GnRH stimulation would be of great interest, and would highlight a novel signalling modality.

By generating a FOXO3a knock-down cell line, the role of FOXO3a in GnRH-induced cell-growth inhibition was examined *in vitro* in **Chapter four**. Generating other FOXO3a knock-down stable cell lines using cancer-derived cell lines from human tumours that endogenously express GnRH receptor would be important to confirm the role of FOXO3a in the GnRH-induced cell-growth inhibition. The main advantage by using cancer-derived cell lines in order to examine the role of FOXO3a in GnRH-induced cell-growth inhibition, is that these cell systems are more relevant to the pathophysiological situations that exist *in vivo*.

In addition to these proposed *in vitro* assays, tumour xenograft studies could be used to complement the *in vitro* analysis. The FOXO3a knock-down cell lines (either

SCL60 or other cancer-derived cell lines) could be implanted into nude mice, and the size of the tumour following GnRH treatment then measured to enable confirmation of the *in vitro* data. However, in an *in vivo* experiment other factors could impact directly or indirectly on the GnRH-induced antiproliferative effect. Nevertheless, the *in vivo* experiments would mimic the conditions that exist in an organism in contrast to an *in vitro* cell viability assay.

The role of FOXO3a in LH β , but not in FSH β , expression was demonstrated in **Chapter five**, by chronic treatment with GnRH. In contrast, FOXO1 had no effect on LH β and FSH β expression following chronic GnRH treatment. A potential issue that should be highlighted from this approach, is the fact that in this study gonadotropin expression is stimulated by continuous GnRH administration instead of pulsatile treatment. The frequency of GnRH release influences the differential regulation of LH β and FSH β expression in gonadotropes of the pituitary, since low GnRH pulses (>120-240min) favour the expression of FSH, while high pulses (<1hour) favour the expression of LH (Ferris and Shupnik, 2006). Thus, treatment with low or high GnRH pulses could reveal additional roles for FOXO1 and FOXO3a in gonadotropin synthesis. In addition, having proposed in **Chapter five** that FOXO3a binds to *LH β* promoter, but it is released from the *Egr1* promoter following continuous GnRH treatment, a possible role of FOXO3a in how different pulsatile GnRH treatments influence the expression of LH β and FSH β could be also examined by performing ChIP assays in time-course intervals with pulsatile GnRH treatment.

Finally, having demonstrated that GnRH-R activation regulates the transcriptional activity of FOXO3a, novel applications based on this regulation could be

investigated. Of interest are tissues and cell types that express both FOXOs and high levels of GnRH-Rs. Thus far, expression of both FOXOs and GnRH-R has been demonstrated in placenta (Lappas *et al.*, 2010), in gonadotropes in human pituitary (see Chapter five), in prostate cancer cells (unpublished data) and in the endometrium (Rudd *et al.*, 2007; Ward *et al.*, 2008). In the latter case, the regulation of the transcriptional activity of progesterone and estrogen receptors via FOXO was demonstrated (Rudd *et al.*, 2007). Thus, a possible impact of GnRH on the human endometrium by targeting FOXO activity, is interesting and worth further investigation. Taken together, studies detailed in this thesis may provide the basis for future identification of new FOXO targets and functions in different reproductive cell types.

APPENDICES

APPENDIX 7.1: Supplier Details

Abgene	Epson, UK
Active Motif	Rixensart, Belgium
ATCC	Teddington, UK, (http://www.atcc.org/)
BD Biosciences	New Jersey, USA
BMG Lab technology	Aylesbury, UK
BioRad Laboratories	Hertfordshire, UK
Carl Zeiss	New York, USA
Cell Signalling Technologies	Hertfordshire, UK
GE Healthcare	Buckinghamshire, UK
Graph Prism Software Inc.	San Diego, USA
Immunotech	Marseilles, France
Invitrogen	Paisley, UK
Labsystem	Victoria, Australia
Novo Laboratory	WA, USA
Perkin Elmer	Cambridgeshire, UK
Promega	Madison, USA
Qiagen	Crawley, UK
Roche	Welwyn, Garden City, UK
Santa Cruz Biotechnology	Heidelberg, Germany
Sigma	Dorset, UK

Table 7-1: List of Suppliers details

APPENDIX 7.2: Supplementary Data

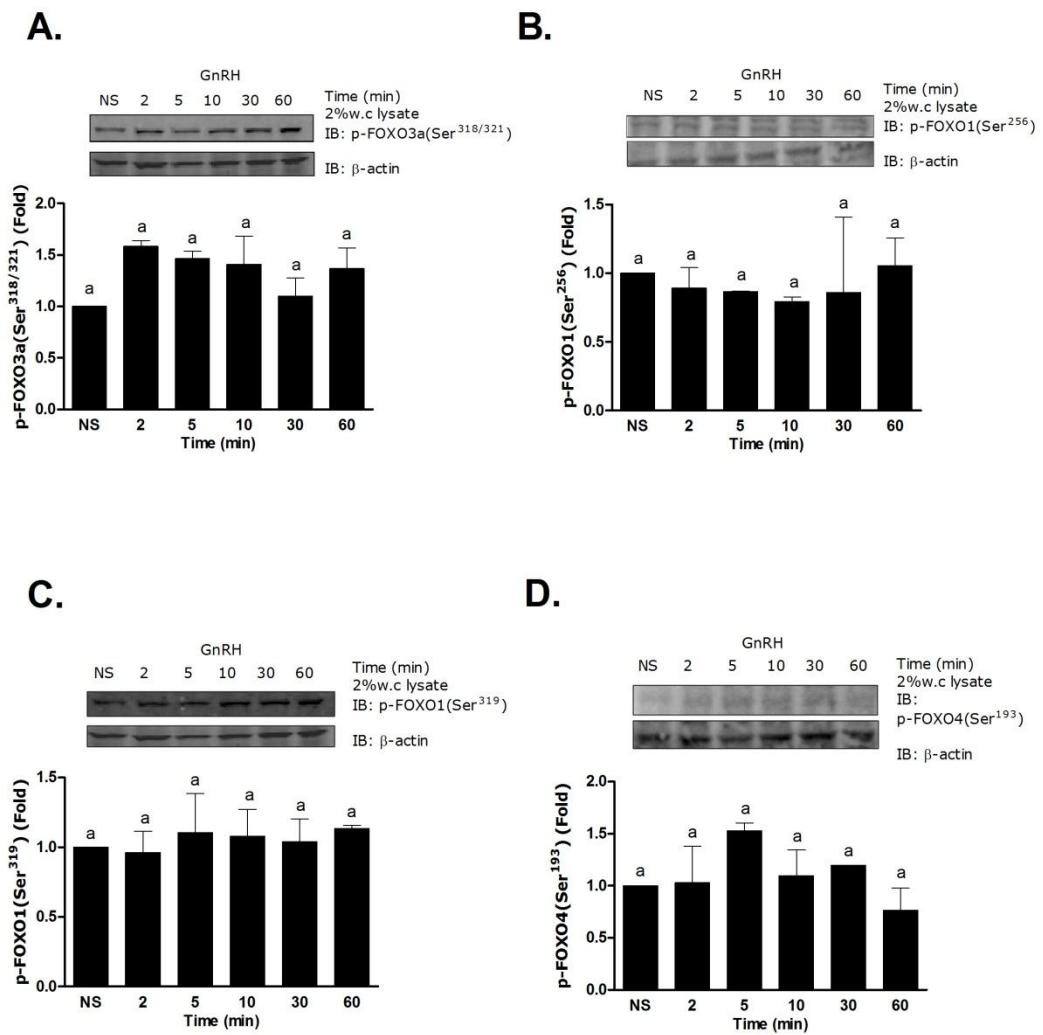


Figure 7-1: Immunoblots of GnRH-induced FOXO3a and FOXO1 phosphorylation in SCL60 cells.

SCL60 cells were serum-starved 24 hours before treatment with 1 μ M GnRH for the indicated times, or left untreated (NS). Levels of (A) p-FOXO3a(Ser^{318/321}), (B) p-FOXO1(Ser²⁵⁶), (C) p-FOXO1(Ser³¹⁹), (D) p-FOXO4(Ser¹⁹³), (D) p-FOXO1(Ser¹⁹⁶) were analysed using the appropriate antibodies. Data are shown as mean fold change over NS \pm SEM from at least 3 independent experiments.

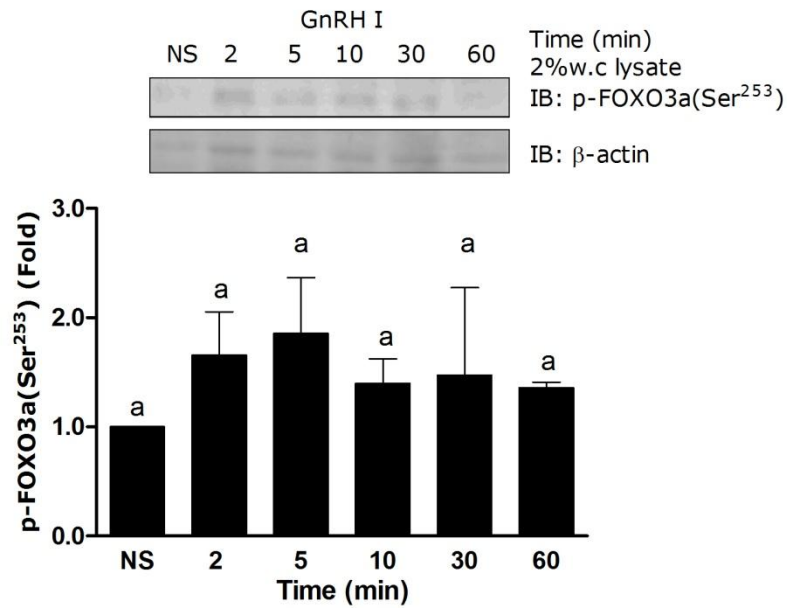


Figure 7-2: Immunoblots of GnRH-induced FOXO3a phosphorylation in LNCaP prostate cancer cells.

LNCaP cells were serum-starved 24 hours before treatment with 1μM GnRH for the indicated times, or left untreated (NS). Level of p-FOXO3a(Ser²⁵³) were analysed using the appropriate antibody. Data are shown as mean fold change over NS±SEM from at least 3 independent experiments. (b is significantly different from a; P<0.05)

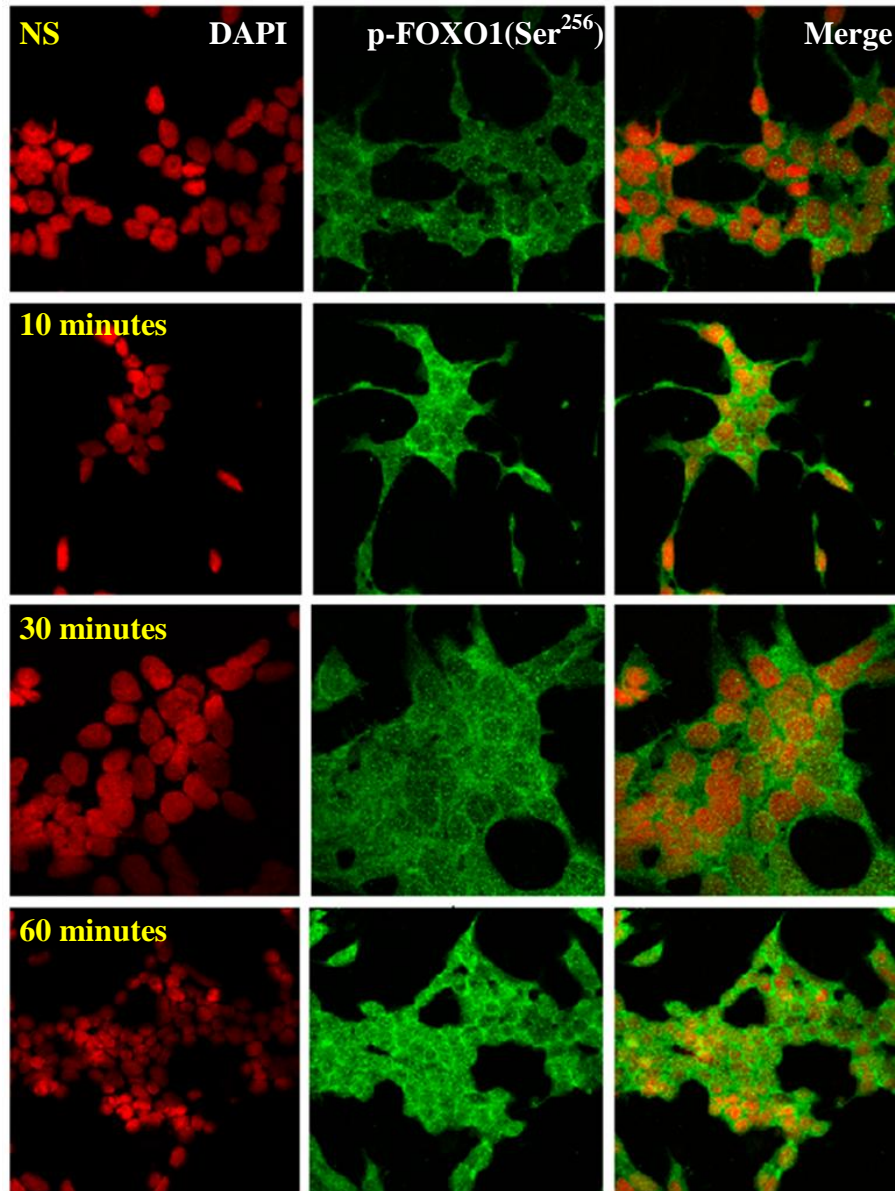


Figure 7-3: p-FOXO1(Ser²⁵⁶) distribution between nucleus and cytoplasm following GnRH stimulation in SCL60 cells.

Immunofluorescence confocal microscopy was performed to visualise the redistributed of p-FOXO1(Ser²⁵⁶) (green) between the nucleus and cytoplasm following 1 μ M GnRH treatment at the time intervals as indicated. DAPI (red) is a marker for nucleus and co-localisation is indicated as yellow.

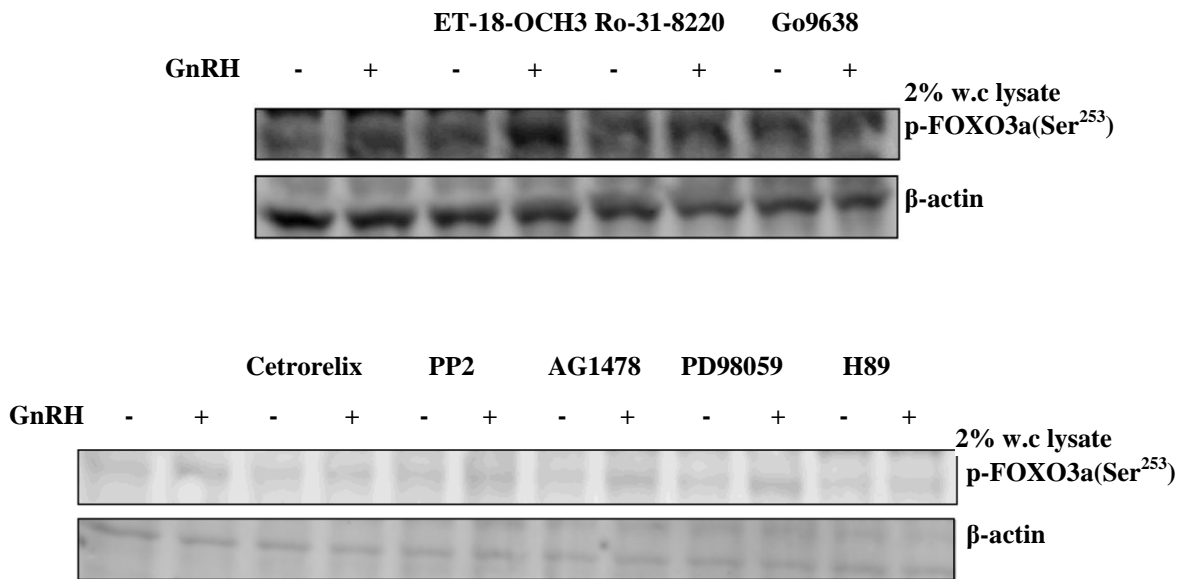


Figure 7-4: The effect of chemical inhibitors on GnRH-targeted FOXO3a phosphorylation at Ser²⁵³.

SCL60 cells were serum-starved 24 hours before cells were pre-treated for 30 minutes with: PLC inhibitor ET-18-OCH3 (20μM), PKC inhibitors Ro-31-8220 (100nM) and Go9638 (21μM), GnRH-I antagonist Cetrorelix (1μM), c-Src inhibitor PP2 (5μM), EGFR inhibitor AG1478 (100nM), MEK inhibitor PD98059 (20μM) and PKA inhibitor H89 (10μM), followed by treatment for 10 minutes with 1μM GnRH. Blots are representative of 3 independent experiments.

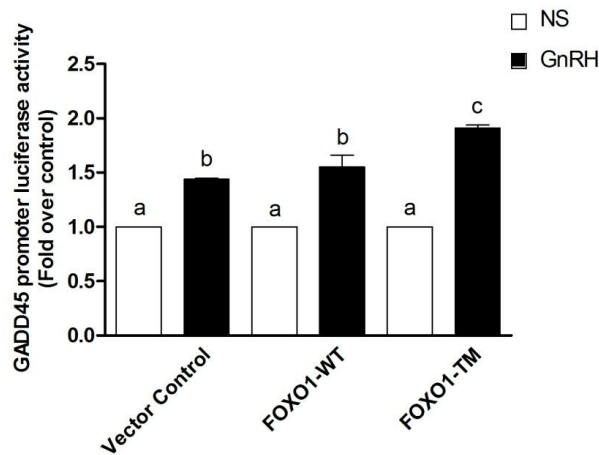


Figure 7-5: The role of FOXO1 in GnRH-induced GADD45-reporter activity.

SCL60 cells were transiently transfected with 1µg of GADD45-reporter and 9µg of control vector (pcDNA3.1) or FOXO1-WT or FOXO1-TM. Cells were treatment with 1µM GnRH for 24 hours. Samples were then assayed for luciferase activity. Data are shown as mean fold change over NS±SEM from at least 3 independent experiments. (b is significantly different from a, and c is significantly different from a and b; P<0.05)

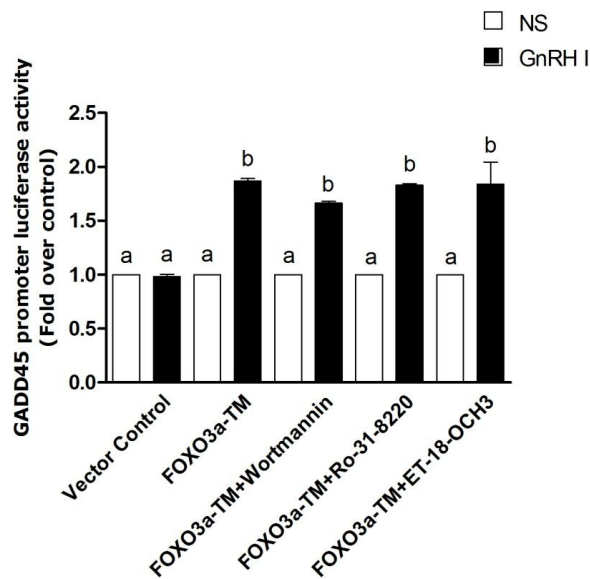


Figure 7-6: The role of PI3K, PKC and PLC in the GnRH-induced GADD45-reporter activity.

SCL60 cells were transiently transfected with 1µg of GADD45-reporter and 9µg of control vector (pcDNA3.1) or FOXO3a-TM. Cells were serum-starved 16 hours before they were pre-treated for 30 minutes with 100nM PI3K inhibitor (Wortmannin), 100nM PKC inhibitor (Ro-31-8220) or 20µM PLC inhibitor (ET-18-OCH3). Thereafter were treated with 1µM GnRH for 24 hours. Samples were then assayed for luciferase activity. Data are shown as mean fold change over NS ±SEM from at least 3 independent experiments. (b is significantly different from a; P<0.05)

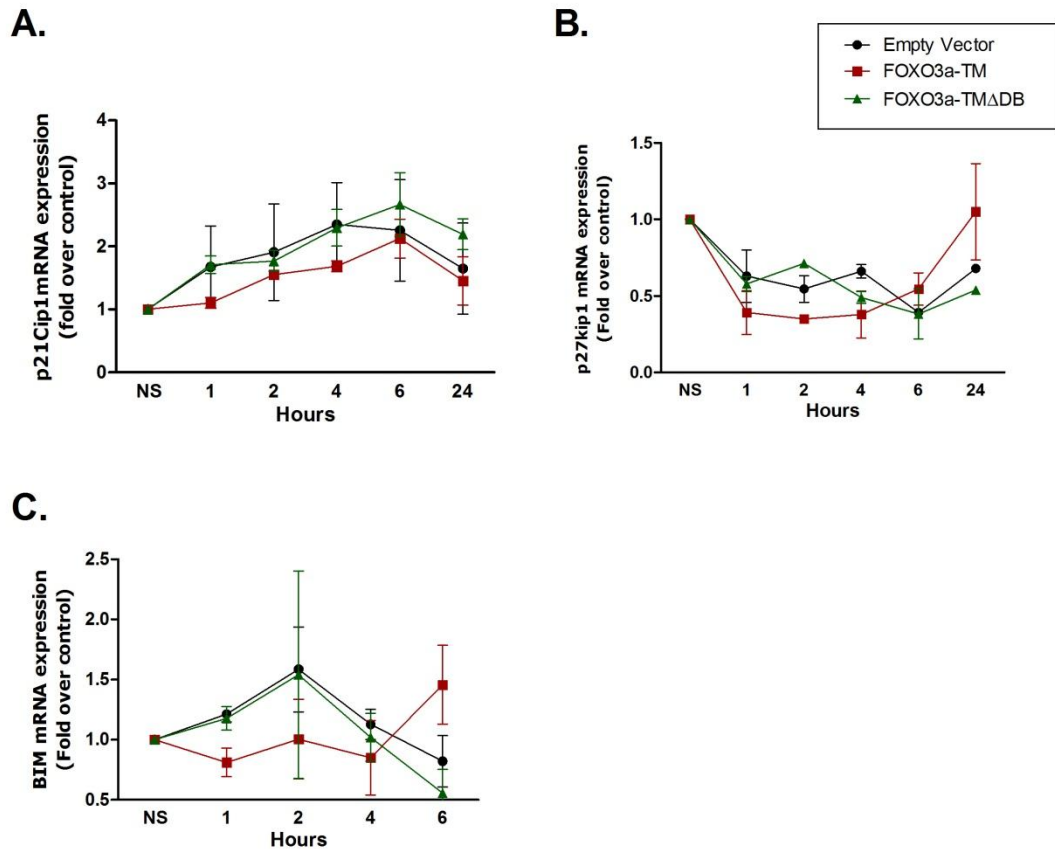
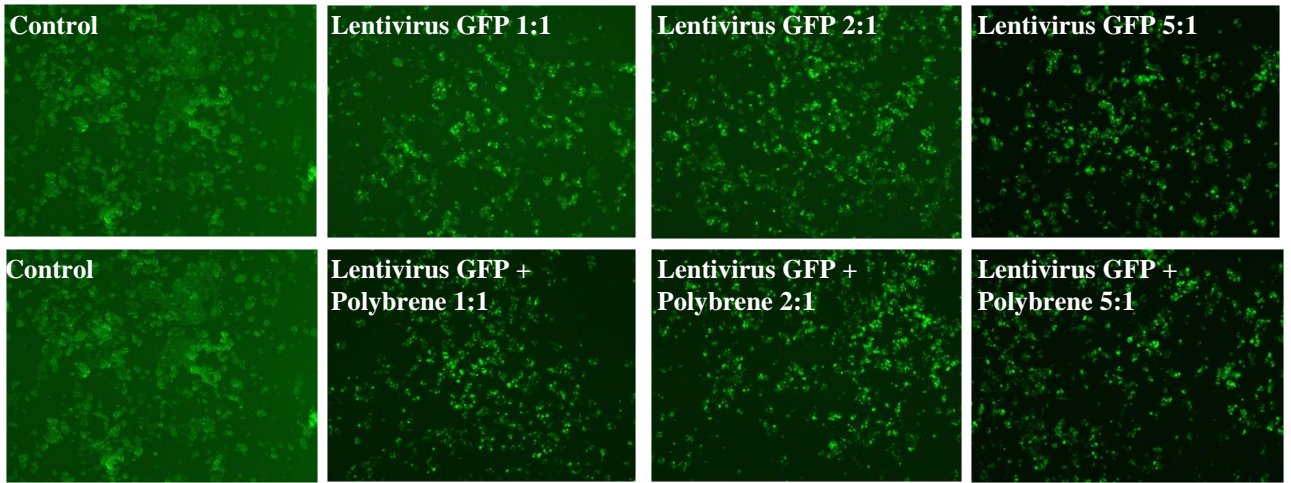


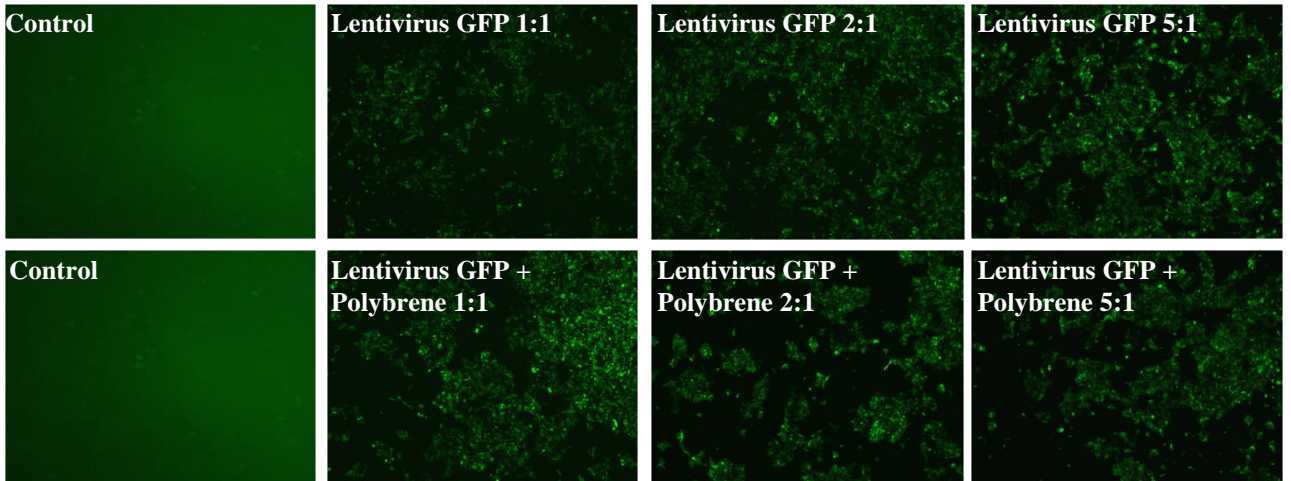
Figure 7-7: The role of FOXO3a in FOXO-target genes mRNA expression following GnRH treatment.

SCL60 cells were transfected with 10 μ M of FOXO3a-TM or FOXO3a-TM Δ DB. Relative mRNA expression of (A) *p21Cip1*, (B) *p27Kip1* and (C) *BIM* in cells treated with 1 μ M D-Trp⁶-GnRH over the indicated time intervals or vehicle (NS) was determined by quantitative RT-PCR analysis. Data are shown as mean fold over NS \pm SEM over 24 hour treatment from at least 3 independent experiments.

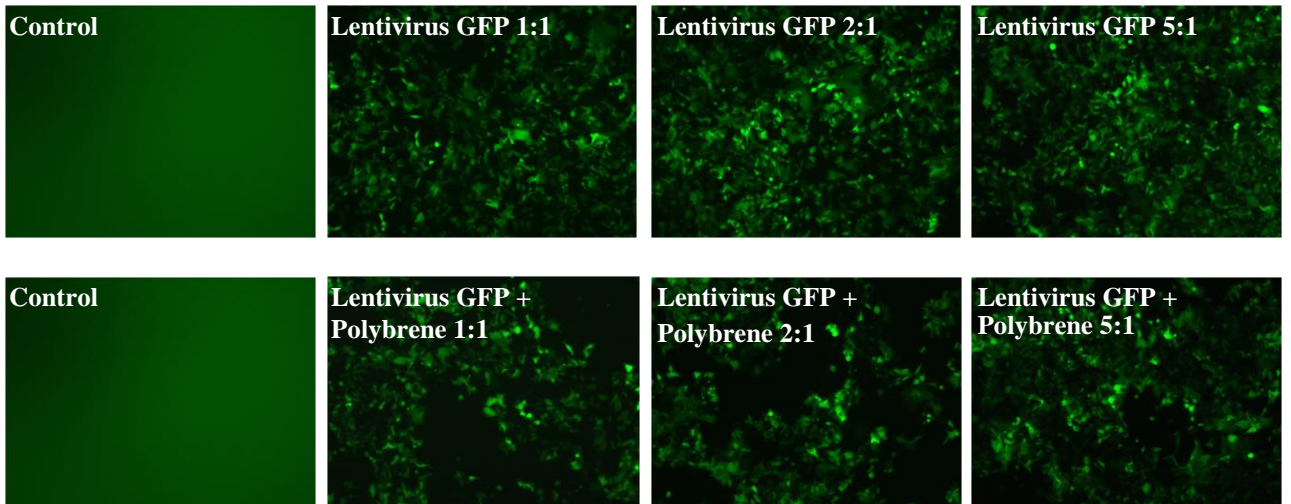
Day 1



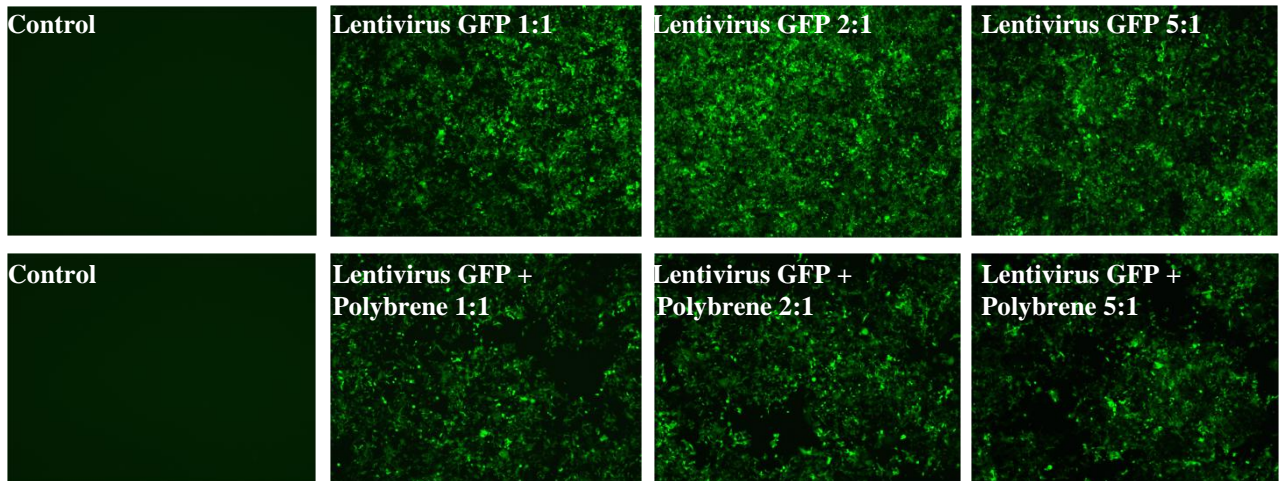
Day 2



Day 3



Day 4



Day 5

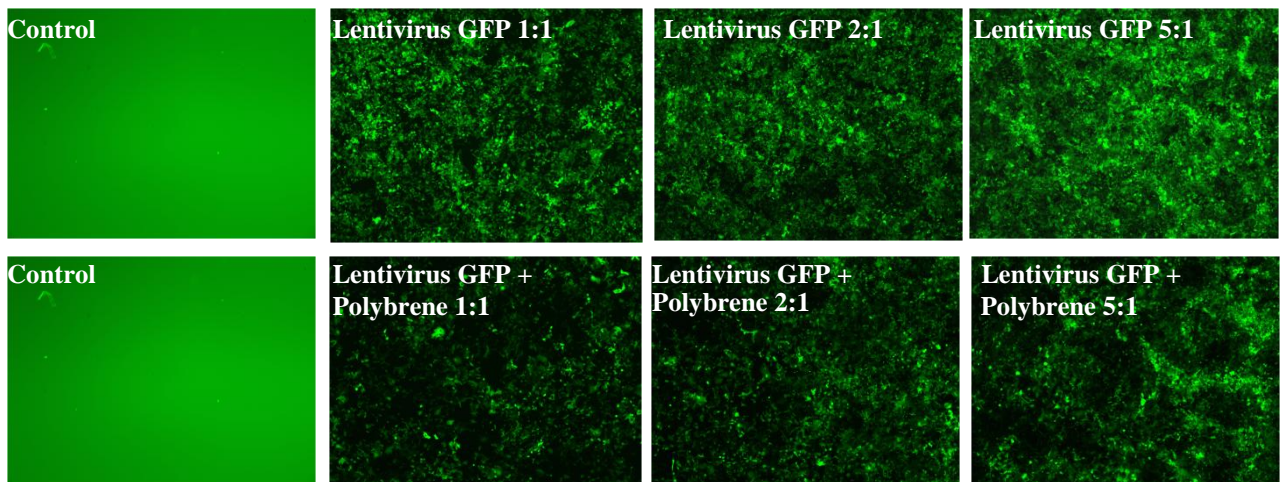


Figure 7-8: Transduction efficiency of lentivirus in SCL60 cells.

SCL60 cells were transduced with GFP-tagged lentivirus. Transduction was performed with different ratios of virus to cells (1:1, 2:1 and 5:1) and cells without transduction with lentivirus were used as control. Polybrene was also used.

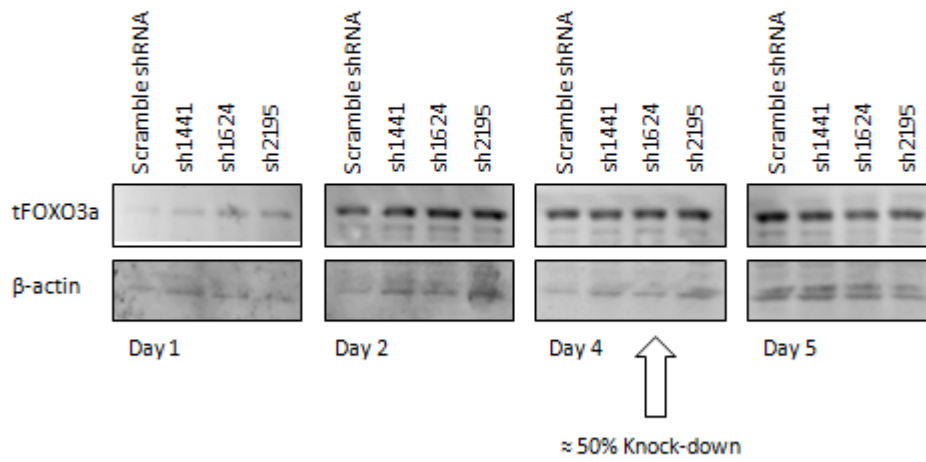


Figure 7-9: LβT2 transient FOXO3a knock-down cells.

LβT2 cells were transduced with lentivirus-delivering shRNA against FOXO3a (FOXO3a knock-down cells) or with lentivirus-delivering scrambled shRNA (control cells). The following day viruses were removed and cells were incubated with fresh complete medium. Total FOXO3a protein levels were measured 1, 2, 4 and 5 days after transduction by Western Blot analysis.

primers	CHIP Ab	GnRH	Ct	Avg Ct	DDCt	2-DDCt	Column 1	Column 2	Column 3	Column 4	
LH control	IgG	no	40	38.595953	1.405	0.377618	-0.696657	1.934966	2.608981	1	
		no	38.17845		-0.416552	1.334734					
		no	40		1.405	0.377618					
	foxo3a	no	37.60941		-0.98559	1.980123					
		no	39.09879		0.503785	0.705254					-1.348007
		no	38.15283		-0.442168	1.358644					
LH control	IgG	yes	40	39.835532	0.165	0.891929	-1.01316	5.048291	4.588283		
		yes	39.5066		-0.328404	1.255624					
		yes	40		0.165	0.891929					
	foxo3a	yes	36.64243		-3.19257	9.142381					
		yes	39.27408		-0.56092	1.47521				-5.114727	
		yes	37.5942		-2.2408	4.726591					
LH promoter	IgG	no	40	38.991854	1.009	0.496891	-1.678937	4.422108	11.97074		
		no	40		1.009	0.496891					
		no	36.97556		-2.015437	4.04303					
	foxo3a	no	35.78966		-3.20134	9.198126					
		no	36.06443		-2.926566	7.602985				-7.424441	
		no	36.53888		-2.452124	5.472212					
LH promoter	IgG	yes	40	40	0	1	1	28.99443	0.822035		
		yes	40		0	1					
		yes	40		0	1					
	foxo3a	yes	35.1423		-4.857704	28.99443				-28.99443	
		yes									
		yes									
Egr1 control	IgG	no	31.18275	31.594262	-0.411255	1.329842	-1.026467	1.218699	0.822035		
		no	31.99407		0.400072	0.75782					
		no	31.60597		0.011968	0.991739					
	foxo3a	no	31.15601		-0.43799	1.354716					
		no	31.39523		-0.198769	1.147719				-1.250955	
		no	31.27158		-0.322424	1.25043					
Egr1 control	IgG	yes	30.50733	30.799041	-0.29167	1.224056	-1.010564	1.001813	0.325635		
		yes	30.94446		0.145462	0.90409					
		yes	30.94533		0.146332	0.903545					
	foxo3a	yes	30.64985		-0.149151	1.108917					
		yes	30.78881		-0.01019	1.007088				-1.012396	
		yes	30.91744		0.118442	0.921182					
Egr1 promoter	IgG	no	40	40	0	1	1	11.07727	0.267683		
		no	40		0	1					
		no	40		0	1					
	foxo3a	no	36.53047		-3.46953	11.07727				-11.07727	
		no									
		no									
Egr1 promoter	IgG	yes	40	40	0	1	1	2.965197			
		yes	40		0	1					
		yes	40		0	1					
	foxo3a	yes	38.57386		-1.426144	2.687275				-2.965197	
		yes	40		0	1					
		yes	37.61918		-2.380817	5.208316					

Figure 7-10: qRT-PCR calculations for ChIP assay.

Column 1: Calculation was done as for the real-time PCR analysis. Column 2: Value that obtained from the division of the value obtained by using the IgG antibody to the value obtained by using the FOXO3a antibody (with or without GnRH treatment). In each case this value (obtained by using the FOXO3a antibody) is normalised with the value obtained by using the IgG antibody (IgG antibody is using as a control and its not affected by GnRH treatment). Column 3: Value that obtained from the division of the value obtained from treated cells with GnRH to the value obtained from the un-treated cells. Column 4: Value that obtained from the division of the value obtained by using primers against the LH or Egr1 promoter to the value obtained by using control primers.

APPENDIX 7.3: Antibodies

Antibody	Supplier
FoxO1 (76E10)	Cell Signaling
FoxO3a	Cell Signaling
Phospho-FoxO3a (Ser253)	Cell Signaling
Phospho-FoxO3a (Ser318/321)	Cell Signaling
Phospho-FoxO4 (Ser193)	Cell Signaling
FoxO4	Cell Signaling
Phospho-FoxO1 (Ser256)	Cell Signaling
Phospho-FoxO1 (Ser319)	Cell Signaling
Actin (I-19): sc-1616	Santa Cruz Biotechnology, Inc
Cleaved Caspase-9 (Asp330)	Cell Signaling
p-FADD (Ser191)	Cell Signaling
LH β	Novo Laboratories

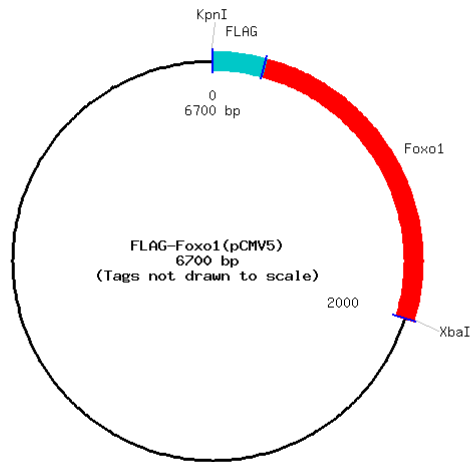
Table 7-2: List of antibodies

APPENDIX 7.4: Abbreviation of amino acids

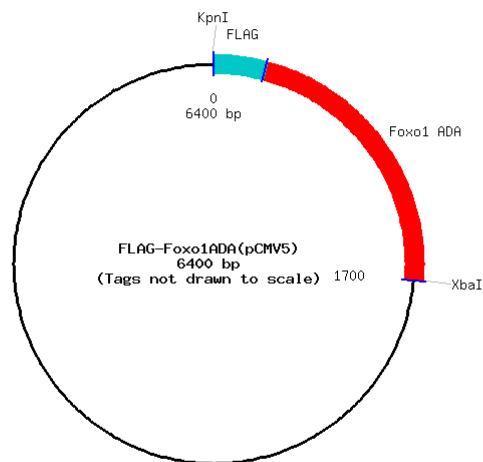
Amino acid	Abbreviation	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Try	W
Tyrosine	Tyr	Y
Valine	Val	V

Table 7-3: Amino acid abbreviations and one letter codes.

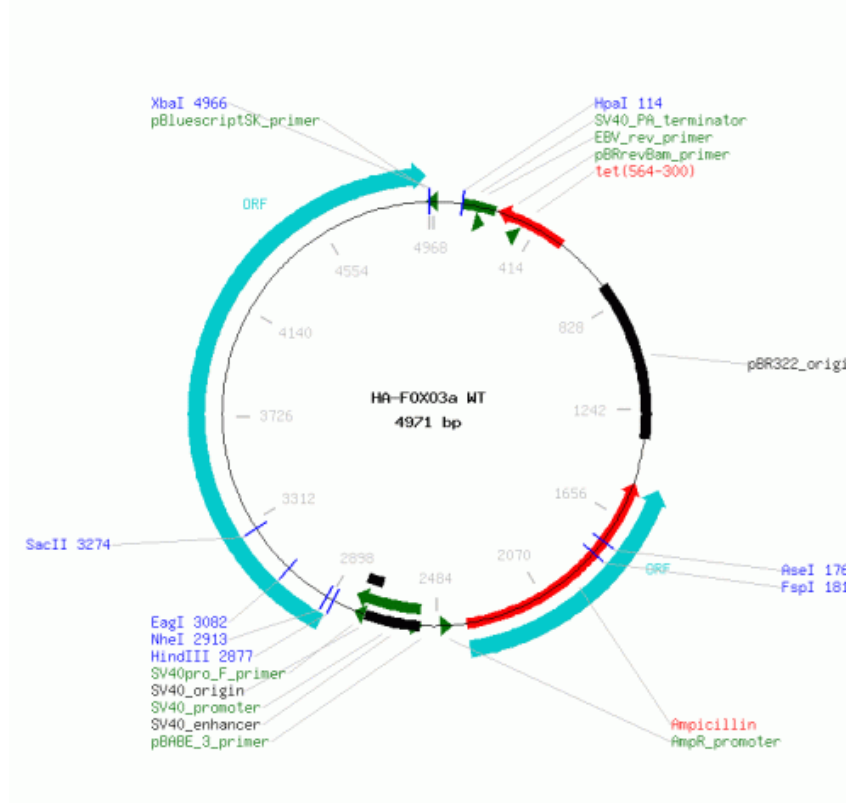
APPENDIX 7.5: Constructs



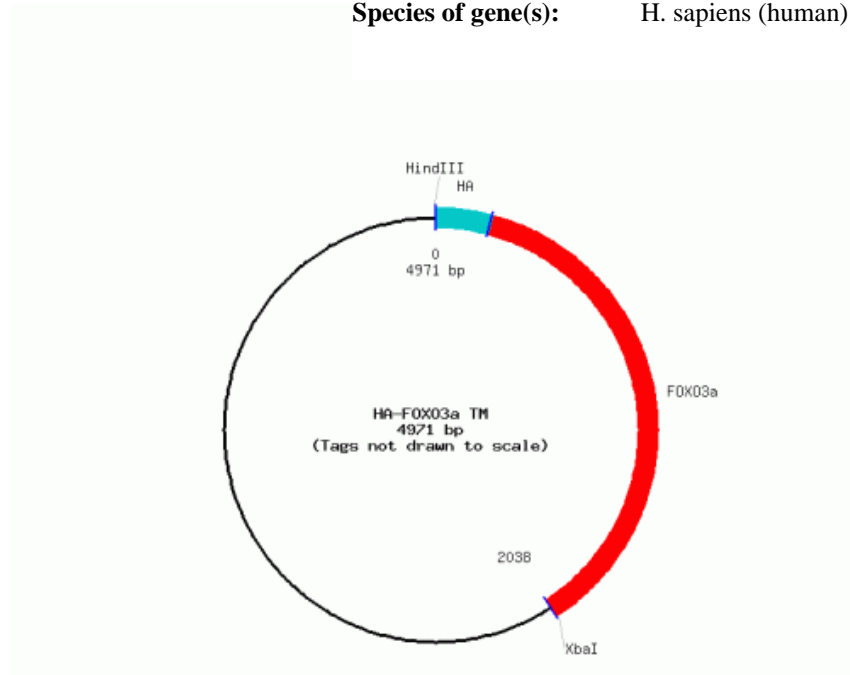
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Species of gene(s): *M. musculus* (mouse)



Insert size (bp): 1700
Species of gene(s): *M. musculus* (mouse)
Relevant mutations/deletions: Thr²⁴Ala, Ser²⁵³Ala, Ser³¹⁶Ala

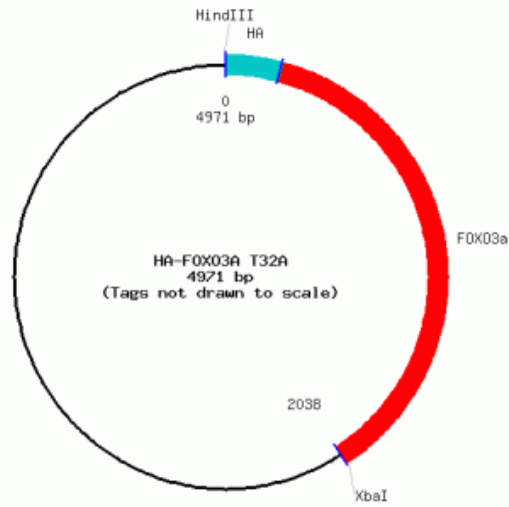


Species of gene(s): H. sapiens (human)

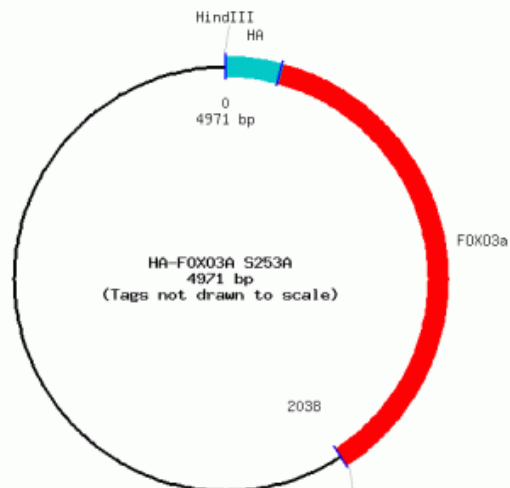


Species of gene(s): H. sapiens (human)

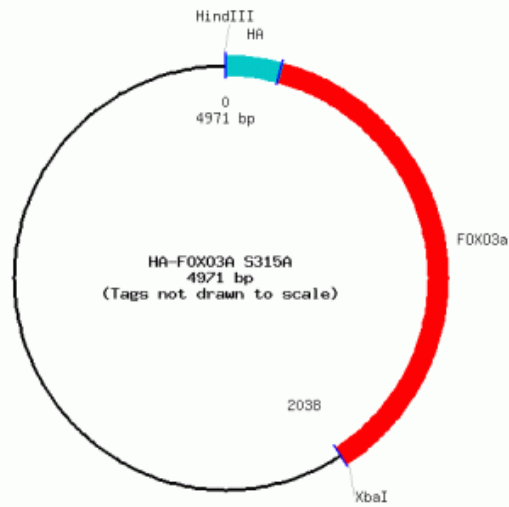
Relevant mutations/deletions: Thr³²Ala, Ser²⁵³Ala, Ser³¹⁵Ala



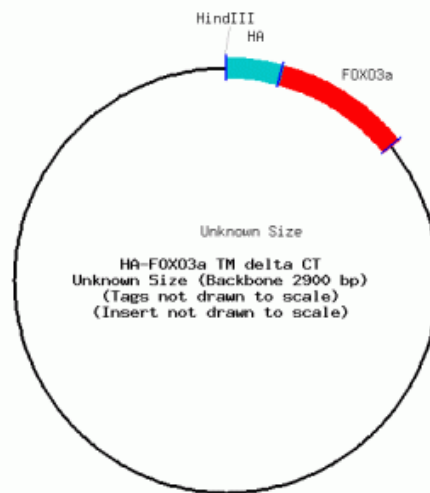
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Species of gene(s): H. sapiens (human)
Relevant mutations/deletions: Thr³²Ala



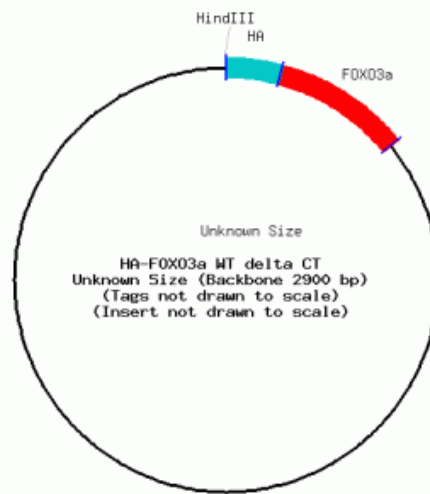
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Species of gene(s): H. sapiens (human)
Relevant mutations/deletions: Ser²⁵³Ala



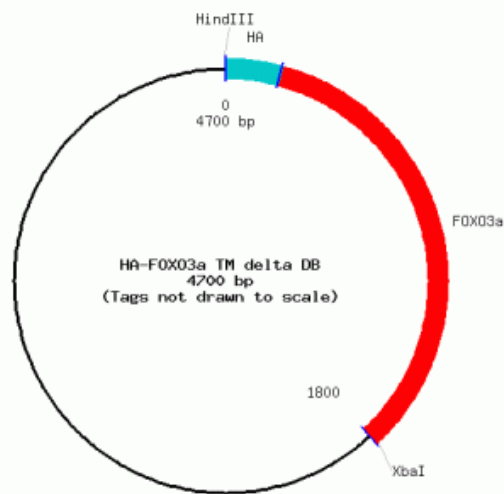
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Relevant mutations/deletions: Ser³¹⁵Ala



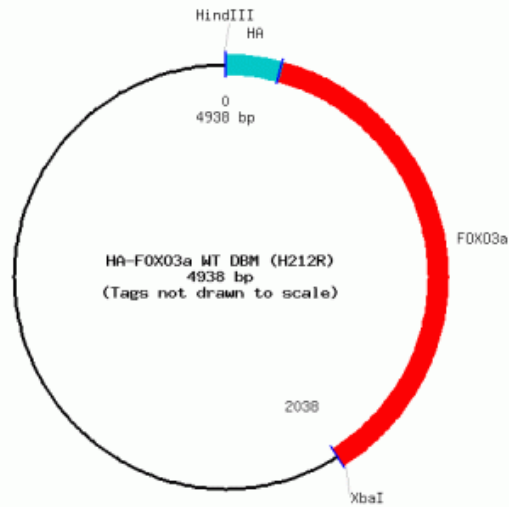
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Relevant mutations/deletions: Thr³²Ala, Ser²⁵³Ala, Ser³¹⁵Ala, delta CT



Insert size (bp): Unknown
Species of gene(s): H. sapiens (human)
Relevant mutations/deletions: delta CT



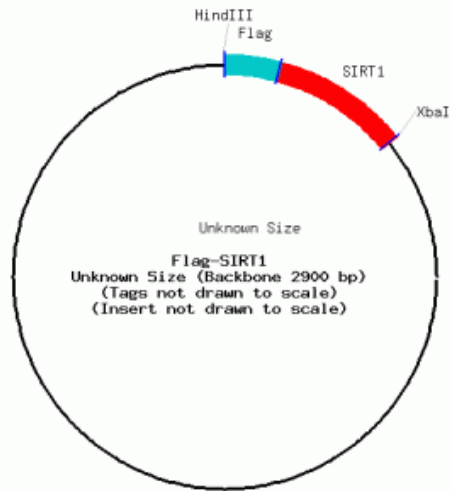
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Relevant mutations/deletions: Thr³²Ala, Ser²⁵³Ala, Ser³¹⁵Ala, delta DB



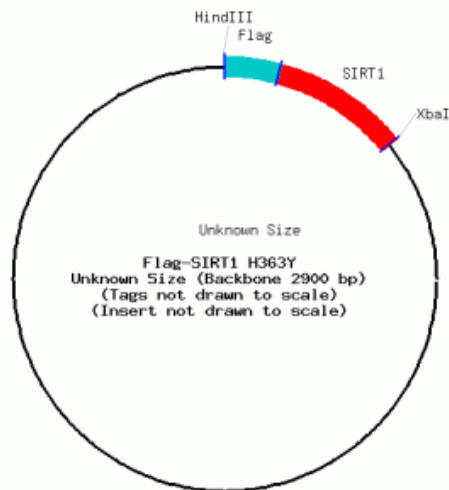
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Relevant mutations/deletions: His212R



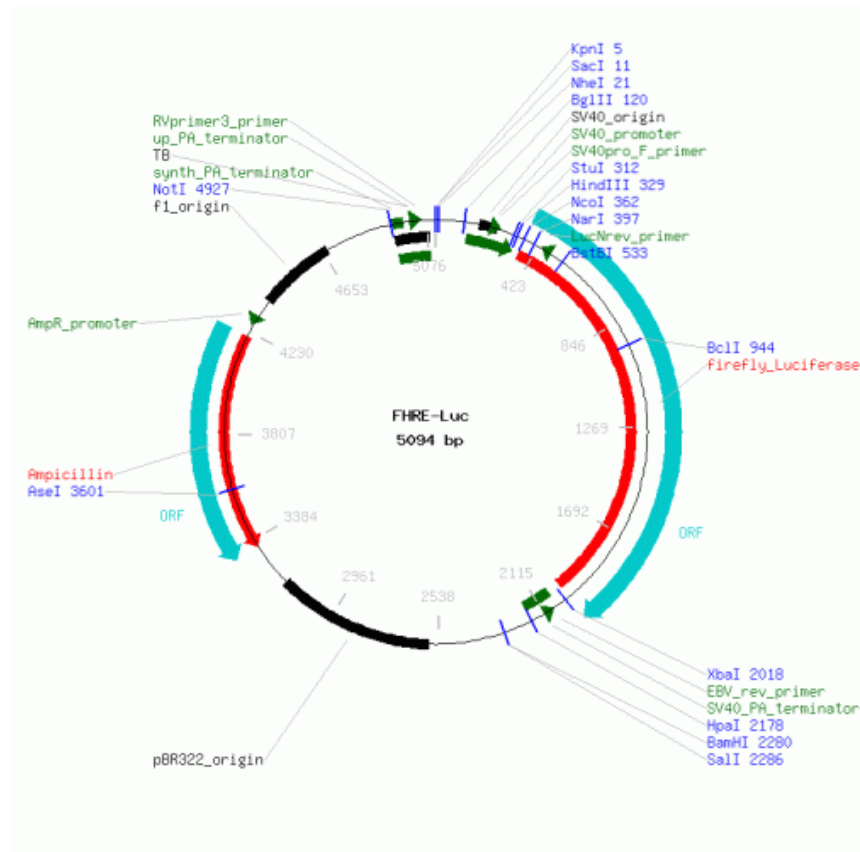
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Insert size (bp): Unknown
Species of gene(s): H. sapiens (human)



Insert size (bp): Unknown
Species of gene(s): H. sapiens (human)
Relevant mutations/deletions: His³⁶³Y, deacetylase domain mutation



Insert size (bp): 96
Species of gene(s): H. sapiens (human)
Relevant mutations/deletions: 3 copies of FHRE

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