The role of Hypoxia Inducible Factors in regulating ovarian function

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Abbreviations

ARNT Aryl hydrocarbon receptor nuclear translocator

bHLH Basic helix-loop-helix

BSA Bovine serum albumin

CAD Carboxyl-terminal trans-activation domain

CC Cumulus cell

CL Corpora lutea

COC Cumulus-oocyte-complex

$CoCl_2$ Cobalt chloride

DAPI Diamindino phenylindole

DFO Desferrioxamine

DMEM Dulbecco's modified Eagle's medium

DP 2,2'-dipiridyl

eCG Equine chorionic gonadotropin

EGFP Enhanced green fluorescent protein

EPAS-1 Endothelial PAS domain 1

EPO Erythropoietin

FSH Follicle stimulating hormone

GDF9 Growth differentiating factor-9

hCG Human chorionic gonadotropin

HIF Hypoxia inducible factor

HLF HIF-1α-like factor

HRE Hypoxia response element

IPAS Inhibitory PAS protein

IVM *In vitro* maturation

LH Luteinizing hormone

MEF Mouse embryonic fibroblast

MGC Mural granulosa cell

N-TAD N-terminus transactivation domain

ODD Oxygen dependent domain

PAS Per-arnt-sim

PBS Phosphate buffered saline

PCOS Polycystic ovarian syndrome

PCR Polymerase chain reaction

PHD Prolyl hydroxylase domain enzyme

ROS Reactive oxygen species

SEM Standard error of the mean

TGFβ1 Transforming growth factor beta-1

TH Theca

VEGF Vascular endothelial growth factor

Abstract

Hypoxia inducible factors (HIFs) are heterodimeric bHLH-PAS domain transcriptional factors that mediate physiological responses to low oxygen. There are 3 known isoforms. In normoxic conditions, HIFs are rapidly degraded via the ubiquitin-proteasome pathway. In hypoxic conditions, HIFs are stabilised and translocate to the nucleus, where they activate the Hypoxia Response Elements (HRE) upstream of numerous target genes involved in angiogenesis and glycolysis. Hypoxia has been established as the major inducer of HIFs, but other factors, such as transition metals and hormones, can also induce HIF target gene expression by increasing HIF protein stability and/or expression.

In the ovary, oocytes grow in follicle structures surrounded by a basement membrane that excludes the vasculature. Follicular development occurs under the influence of signals from both the oocyte and the systemic environment mediated by the ovarian stroma. During follicular development, the follicle develops an antrum. In the antral follicle, an oxygen gradient is thought to exist across the follicle, potentially limiting the oocyte's ability for oxidative phosphorylation. In contrast, following the ovulatory signal, blood vessels cross the basement membrane between theca and granulosa layers and continue a rapid growth to sustain corpus luteum development and function.

This study hypothesised that these transitional events in follicular growth, especially the formation of an antrum and the formation of the corpus luteum involve HIF-signalling.

To assist with unravelling the role of HIFs in the ovary, this study utilised a transgenic C57BL/6-Tg(HRE(4)-SV40-EGFP) HIF reporter mouse line, designed to produce EGFP following HIF binding to the HRE. Examination of ovaries collected from cycling and pregnant mice revealed that EGFP was absent from all primordial, primary and preantral follicles. EGFP was, however, variably detected within the theca of antral follicles of all sizes. Furthermore, FSH was unable to induce HIF 1α protein or increased expression of HIF activated genes in cultured mouse granulosa cells, despite readily doing so in the presence of hypoxic

mimetics (e.g. $CoCl_2$). In contrast, I observed a stimulatory effect of hCG on HIF 1α protein levels within granulosa cells. Temporal analyses following eCG/hCG treatment *in vivo* revealed a time-dependent increase in EGFP localisation within granulosa cells post hCG, in synchrony with immunoreactive HIF 1α protein levels in granulosa cells *in vivo*, with maximal levels around the time of ovulation. Corpora lutea (CL) also expressed EGFP, suggesting that HIFs play a role during follicle differentiation and luteinisation as a response to ovulatory signals.

As there was little evidence of HIF activity in developing follicles *in vivo*, I assessed whether cumulus cells displayed oxygen sensitive-gene expression. Previous studies in our laboratory used microarrays to examine cumulus cell gene expression following *in vitro* maturation (IVM) at varying oxygen concentrations (2, 5, 10 & 20%) and demonstrated up-regulation of known HIF-regulated genes. I confirmed using quantitative Real Time RT-PCR that expression of these known HIF-regulated genes in cumulus cells was regulated by oxygen concentration *in vitro*. Surprisingly, I was unable to stabilise HIF 1α protein in cumulus cells, despite investigating a variety of incubation conditions. This contrast with results for mural granulosa cells, where HIF 1α is readily detectable and levels can be altered by oxygen concentration, hypoxia mimetics, proteasome inhibition and the oocyte-specific TGFβ family member, GDF-9. These results demonstrate a clear distinction between HIF activity within cumulus cells and mural granulosa cells and may lead to a new understanding of how follicular development, especially antrum formation and growth, can occur within an avascular environment.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Kimberley Kai Yen Tam and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

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Publications arising from these and related studies

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Hormonally regulated follicle differentiation and luteinization in the mouse is associated with hypoxia inducible factor activity.

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Abstracts arising from these and related studies

Kimberley Tam, Kelly Banwell, Darryl Russell, David Froiland, Karen Kind, Jeremy Thompson. (2009) "Oxygen regulated gene expression in cumulus cells during in vivo maturation." Proceedings of the Fortieth Annual Conference of The Society for Reproductive Biology, Adelaide, Australia.

Kimberley Tam, Darryl Russell, Karen Kind, Jeremy Thompson. (2008) " Follicle differentiation and luteinisation in the mouse is associated with hypoxia inducible factor activity." Proceedings of the Thirty-Ninth Annual Conference of The Society for Reproductive Biology, Melbourne, Australia.

Kimberley Tam, Darryl Russell, Karen Kind, Jeremy Thompson. (2008) "Post hCG follicle differentiation in the mouse is associated with an increase in hypoxia inducible factor activity." Forty-First Annual Meeting for the Study for the Study of Reproduction, Kailua-Kona, Hawaii, USA.

CHAPTER 1 Literature review

1.1 Folliculogenesis

The primary function of the ovary is to release a mature oocyte from a follicle, ready for fertilization (McGee & Hsueh, 2000). However, folliculogenesis begins from the primordial follicle. The number of primordial oocytes present at birth varies according to species. There are approximately 10,000 primordial oocytes in mice and 2 - 7 million in humans and in domestic species such as sheep and cow (Gosden & Telfer, 1987). Primordial follicles are characterized by a single layer of flattened granulosa cells surrounding an inactive oocyte (Figure 1.1a) and primary follicles differ by having a single layer of cuboidal granulosa cells (Figure 1.1b). A primary follicle becomes a preantral follicle (Figure 1.1c) as its granulosa cells proliferate to form multiple layers; in addition, it acquires an outer layer of thecal cells, from interstitial stroma cells, which lies anterior on the basement membrane. The initial vascular supply to the follicle begins at this stage and consists of only one or two arterioles terminating in an increasingly complex wreath-like network as the follicle develops (Findlay, 1986). At this preantral stage, steroidogenic thecal cells express luteinizing hormone (LH) receptors and granulosa cells express follicle stimulating hormone (FSH) receptors. Prior to this stage, follicular growth appears to be gonadotropin independent. FSH is an anterior pituitary hormone that promotes antrum formation and angiogenesis.

The next stage is antral follicle formation accompanied by the formation of a single antral cavity known as an antrum. Antrum formation may be a mechanism through which the follicle can continue to grow yet avoid becoming hypoxic (Gosden & Byatt-Smith, 1986; Redding *et al.*, 2007). In antral follicles, the oocyte is surrounded by closely associated and specialized granulosa cells, also known as cumulus cells, forming a cumulus-oocyte-complex (COC) connected with mural granulosa cells that surround the follicular antrum.

At the antral stage, 99.9% of follicles undergo atresia (follicle death, Figure 1.1e) (Barnett *et al.*, 2006). It is well established that the remaining follicles, under the influence of FSH and estrogen, continue to proliferate and mature to a pre-ovulatory follicle (Figure 1.1f) (Goldenberg *et al.*, 1972; Gore-Langton & Daniel, 1990;

Hirshfield, 1991; Gougeon, 1996; Vegetti & Alagna, 2006). FSH effects on follicle growth and maturation can be mimicked by the exogenous administration of equine chorionic gonadotropin (eCG). The LH surge causes final maturation of the oocyte and induces follicular ovulation and the formation of the corpus luteum (Figure 1.1g) and can be mimicked by exogenous administration of human chorionic gonadotropin (hCG).

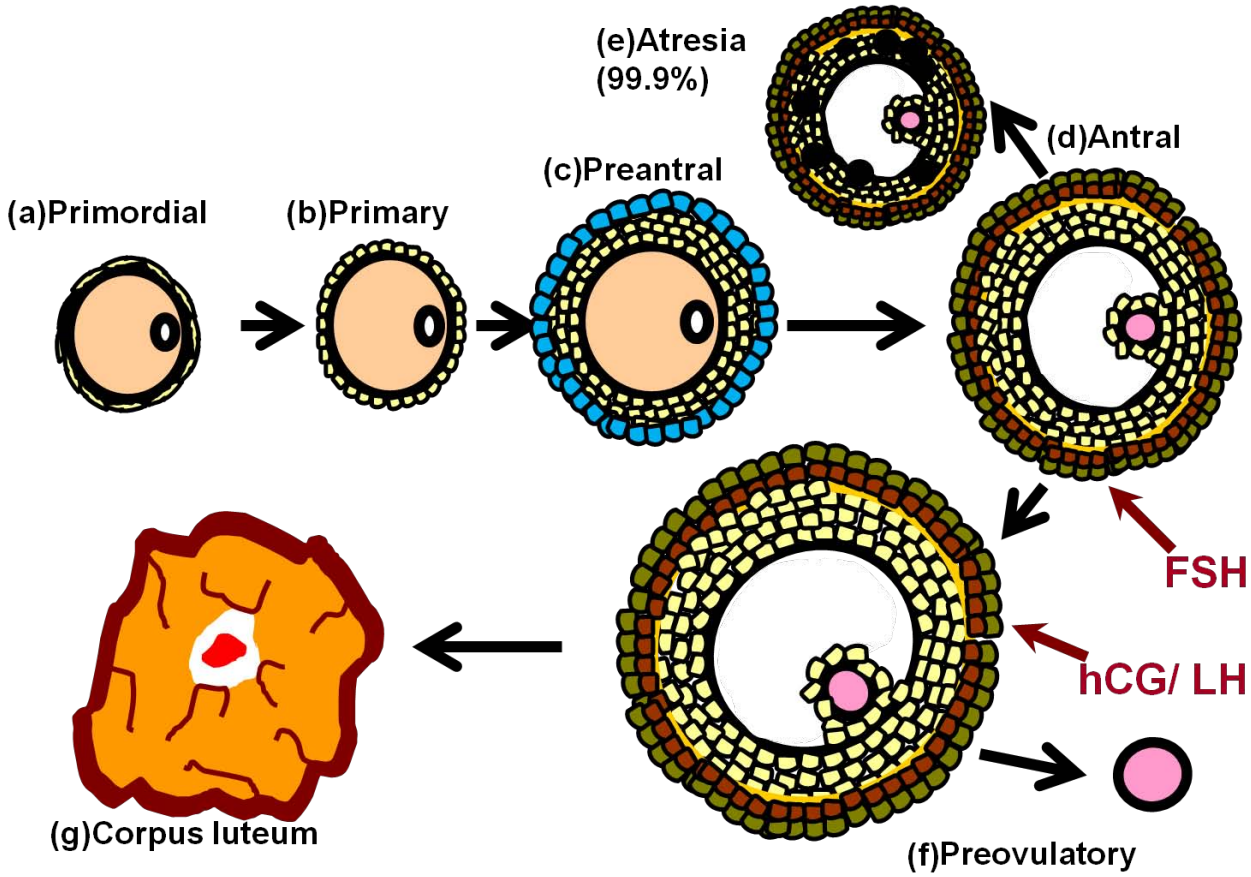

Figure 1.1 A schematic of folliculogenesis in the mammalian ovary.
Folliculogenesis begins with the establishment of a finite pool of primordial follicles containing an inactive oocyte (a); Primary follicles (b) are characterized by a cuboidal shaped granulosa cell layer; (c) the preantral stage with proliferating granulosa cells (cream coloured) and steroidogenic theca layer (blue); Antral stage (d) fluid fluid cavity (white) called an antrum with defined thecal layers [theca interna (brown) and theca externa (olive)]. Follicles at this stage either follow 2 pathways (e) Atresia or, under the influence of FSH, continue to proliferate and mature to a preovulatory follicle (f). After ovulation, the remaining granulosa and thecal cells differentiate into an endocrine structure known as the corpus luteum (g).

1.2 Oxygen regulation in follicular development

Oxygen regulation has been implicated as a crucial aspect of ovarian physiology. During early stages of follicle development prior to antrum formation, oxygenation occurs by the process of passive diffusion. As the follicle develops, the granulosa cells proliferate and a zona pellucida forms and oxygen diffusion is hindered towards the oocyte (Hirshfield, 1991; Neeman *et al.*, 1997). Oocytes grow in follicle structures surrounded by a basement membrane that excludes vasculature; however, it has been demonstrated by mathematical modelling that cumulus cells do not consume much oxygen, allowing sufficient oxygenation of the oocyte (Clark *et al.*, 2006). It has also been demonstrated that pyruvate and oxygen consumption is increased in oocytes from primary follicles and declines at the early antral stage of development (Billig & Magnusson, 1985). The requirement for oxygen consumption by the oocyte is increased at ovulation, suggesting the Krebs cycle activity together with oxidative phosphorylation both increase for ATP production, as it is also well known that oocytes have relatively low glycolytic activity (Sutton-McDowall *et al.*, 2006; Thompson *et al.*, 2007; Harris *et al.*, 2009). During follicular development, oxygen concentration forms a decreasing gradient towards the oocyte and therefore may be limiting the capacity of oocyte oxidative phosphorylation as granulosa cell numbers proliferate (Gosden & Byatt-Smith, 1986; Neeman *et al.*, 1997). There are several studies that have attempted to measure *in vivo* follicular oxygen concentrations following follicle aspiration. It has been proposed that the dissolved oxygen concentration ranges from approximately 1.3 - 5.5% in humans (Van Blerkom *et al.*, 1997; Huey *et al.*, 1999), 7% in porcine (Knudsen *et al.*, 1978) and 3 - 7% in the cattle (Redding *et al.*, 2006). There is no evidence for the dissolved oxygen concentration in murine follicles, however, researchers have been using 5% or 20% [O_2] for *in vitro* maturation (IVM) studies of oocytes. A study in the early 70s showed oocyte developmental competence during *in vitro* maturation of mouse oocytes decreased as oxygen tension increased (Haidri *et al.*, 1971). Subsequently, it was shown that 5% oxygen concentration was optimal for oocyte maturation in hamsters and oocytes cultured under 20% oxygen displayed rapid necrosis (Gwatkin & Haidri, 1974). Epigg and

Wigglesworth (1995) also demonstrated that there was an increase in the percentage of oocytes that were able to cleave to the two-cell stage when matured under 5% [O_2] compared to oocytes matured under 20% [O_2]. This contrasts with a study performed in primates where IVM oocytes had lower developmental competence when cultured in 5% [O_2] compared to 20% [O_2] (Yeoman *et al.*, 1999). However, a recent study demonstrates that varying the oxygen concentration during mouse oocyte IVM did not influence the rate of embryo implantation and development but there was an impact on subsequent fetal development, as fetal weight was reduced following oocyte maturation at 5% [O_2] compared to 20% or 2% [O_2] (Banwell *et al.*, 2007). These studies support the notion that a low oxygen environment in which the oocyte lies within may be a determinant factor of oocyte quality for oocyte maturation.

Additionally, inadequate oxygen supply has also been related to follicle atresia (Hirshfield, 1991). A study performed on more than 1000 follicular fluid aspirates from human follicles showed that oocytes from severely hypoxic (approximately 1% O_2 or less) and poorly vascularised follicles had higher a frequency of abnormalities in chromosome number, spindle organization and cytoplasmic structure (Van Blerkom *et al.*, 1997). Another study utilised Doppler ultrasonography to grade follicular vascularity (Grade 1 and 2 = poor flow) and (Grade 3 and 4 = good flow) and demonstrated that Grade 3/4 follicular vascularity is associated with increased pregnancy rate (Chui *et al.*, 1997). These studies provide evidence that poor follicular vascularity and therefore low oxygen concentration are associated with poor developmental competence of the oocyte and/or IVF outcome (Chui *et al.*, 1997; Van Blerkom *et al.*, 1997).

1.3 Hypoxia inducible factors

Oxygen regulation is crucial in mammals to maintain tissue homeostasis. During hypoxia, a physiological response occurs that enables adaptation to the low oxygen conditions. Hypoxia describes a deficiency in normal oxygen delivery to cells. It was originally described that cells are able to quickly overcome this oxygen deficiency by regulation of the gene encoding the peptide hormone erythropoietin (EPO) which

stimulates the production of red blood cells through erythropoiesis (Goldberg *et al.*, 1988), and by longer term solutions such as increasing angiogenesis. Hypoxia inducible factors (HIFs) were initially found when they were shown to regulate EPO production under hypoxia. The HIFs were found to bind to hypoxia response elements (HREs) found in the region of the EPO gene that corresponded to the 3' un-translated portion of its mRNA (Semenza *et al.*, 1991).

HIF 1α is an 826-amino acid (120 kDa) protein (Figure 1.2). There are other known members of the HIF family which includes HIF 1α, HIF 2α/EPAS-1, HIF 3α/IPAS. HIF 1α and HIF 2α are members of the bHLH and PAS domain protein family. In general, 31% of all HIF 1α amino acid residues are proline (P), glutamic acid (E), serine (S) or threonine (T) (PEST) residues, which are common to many proteins targeted for rapid intracellular degradation (Rogers *et al.*, 1986). The N-terminus transactivation domain (N-TAD) region contains the basic-Helix-Loop-Helix (bHLH)-Per-ARNT-Sim (bHLH-PAS) domain required for dimerisation and DNA binding (Wang & Semenza, 1993a). The N-terminal and C-terminal TADs are localised in the C-terminal half of HIF 1α (aa 531-575 and 786-826, respectively). The NTAD overlaps the oxygen dependent domain (ODD) domain (aa 401-603) which is responsible for degradation of HIF 1α under normoxic conditions (Huang *et al.*, 1998).

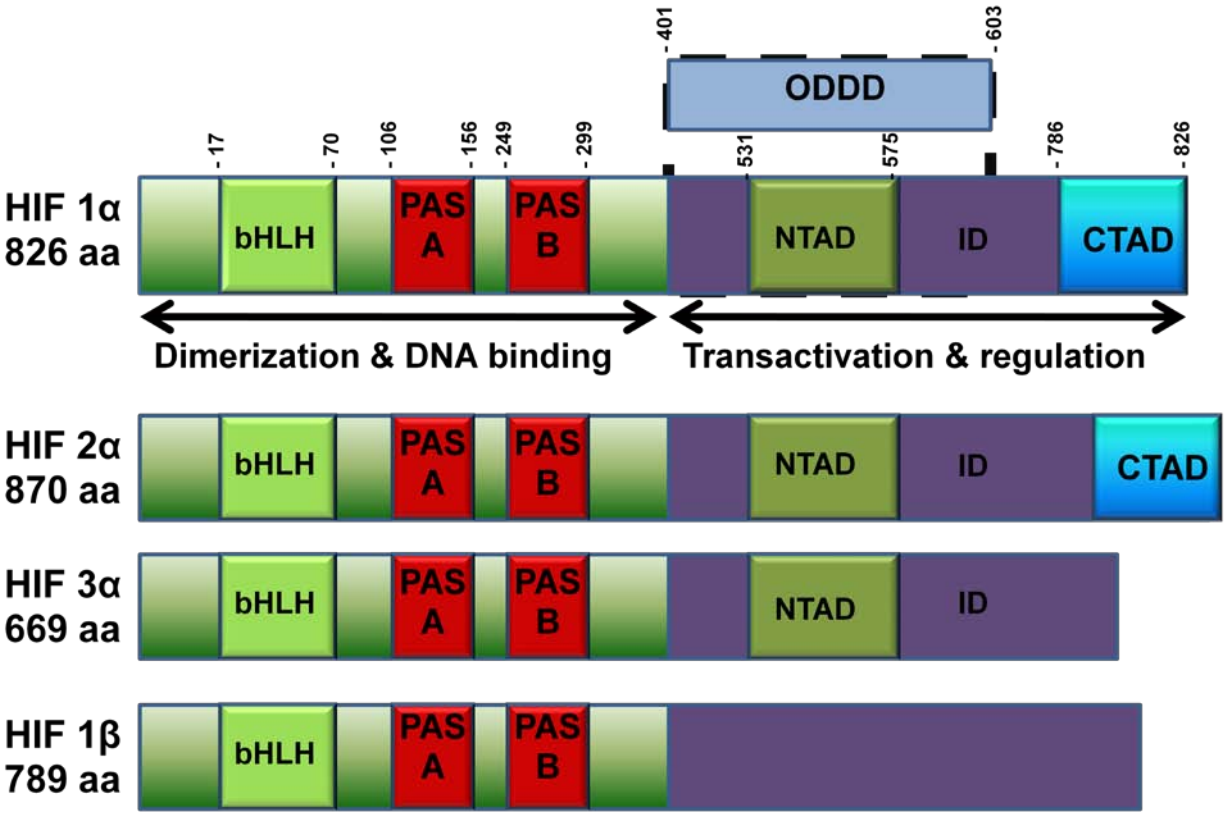

Figure 1.2 Structures of HIF proteins.

All members of the HIF family are bHLH/PER-ARNT-Sim homology (bHLH/PAS) transcription factors, grouped by conserved amino-terminal bHLH and PAS domains which are necessary for protein dimerisation and DNA binding. The regulatory regions of HIF 1α and HIF 2α are comprised of 2 tranactivation domain (N-TAD and C-TAD) within the C-terminus, separated by an inhibitory domain (ID). PHDs hydroxylate Pro 402 and Pro 564 on the O_2-dependent degradation domain (ODDD). The constitutive HIF 1β lacks these regulatory sequences.

Under normoxic conditions, HIF 1α protein is constitutively expressed but is rapidly degraded via the ubiquitin-proteasome pathway due to post translational hydroxylation (Huang *et al.*, 1998). A system of energy (ATP)-consuming protein degradation that involves linking of ubiquitins to specific proteins and the subsequent targeting of these polyubiquitinylated proteins to 26S proteasome (a multicatalytic process) and HIF 1α has a short half life ($t_{1/2}$~five minutes), however its half life is increased to approximately thirty minutes when cells are exposed to hypoxia (i.e., 1% [O_2]) (Huang *et al.*, 1998).

When a cell is exposed to a hypoxic environment, the HIF 1α protein is stabilised. A heme protein and/or the generation of mitochondrial derived reactive oxygen species (ROS) may be necessary for the hypoxia induction of HIF 1α protein levels (Agani *et al.*, 2000). HIF 1α forms a DNA binding heterodimer with another bHLH-PAS protein called the aryl hydrocarbon receptor nuclear translocator (ARNT), also termed HIF 1β, of approximately 90 - 94 kDa, critical during transcriptional response to hypoxia (Wang *et al.*, 1995; Gradin *et al.*, 1996; Maltepe *et al.*, 1997; Tian *et al.*, 1997). Basic HLH-PAS proteins contain an N-terminal bHLH domain and two PAS domains. The bHLH domain is required for dimerisation and DNA binding, whereas the PAS regions appear primarily to be involved in binding to the hypoxia response element (HRE) region (Figure 1.2). The HIF 1α-HIF 1β heterodimer then binds to the HRE and activates the transcription of downstream target genes (Figure 1.3). The HRE contains the core 5'-(A/G)CGTG-3' element to the regulated promoter in hypoxic-regulated genes (Semenza *et al.*, 1991; Wang & Semenza, 1993a). The transcription co activator CBP/p300 is involved in this transcription activation event (Arany *et al.*, 1996; Schofield & Ratcliffe, 2004). It is well documented that HIF target genes play key roles in angiogenesis, vascular angiogenesis, glucose and energy metabolism, erythropoiesis and cell proliferation (Table 1.2). HIF 1α also is involved in the stabilisation of p53 protein and may play a role in hypoxic-induced apoptosis (An *et al.*, 1998).

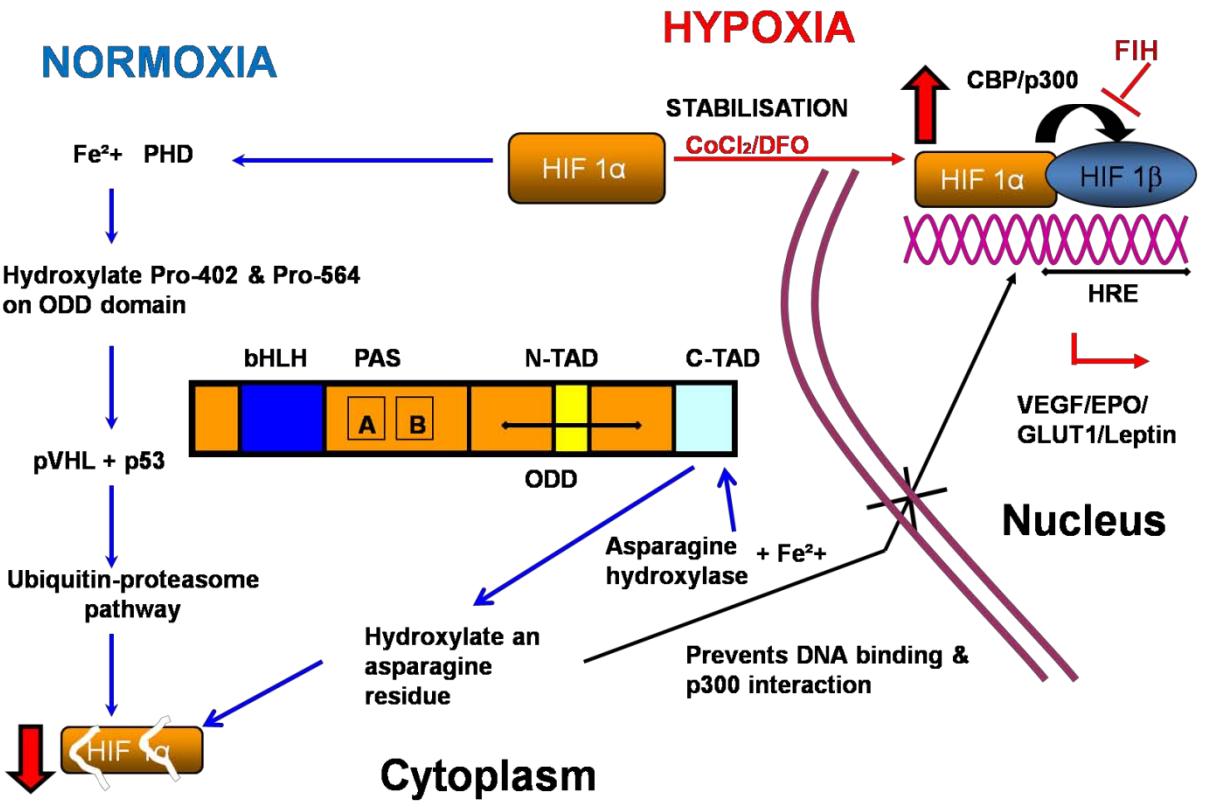

Figure 1.3 The HIF regulation of transcription pathway.

Hypoxic conditions occur when cells are exposed to concentrations below normal physiological conditions. This can lead to several cellular and molecular changes, many of which are affected through the basic-helix-loop-helix factor HIF 1α. Under normoxic conditions, the HIF 1α protein is rapidly ubiquitinated and degraded. Under hypoxic conditions, the protein is stabilised, heterodimerises with ARNT (aryl hydrocarbon nuclear translocator), and translocates to the nucleus where it activates transcription from a number of hypoxia-responsive genes, including *Vegfa*, *Epo* and *Slc2a1*.

Many published reports show that the levels of HIF 1α and HIF 2α protein levels increase in response to hypoxia, proteasomal inhibitors, the transition metals (Co^{2+}, Mn^{2+} and Ni^{2+}), iron chelators [hydrophilic desferrioxamine (DFO) and the lipophilic 2, 2'-dipiridyl (DP)] and other stress responses, whereas the mRNA levels remains stable (Gradin *et al.*, 1996; Wiesener *et al.*, 1998; Linden *et al.*, 2003). It has been shown that iron chelators and transition metals inhibit the interaction between iron-mediated hydroxylation of HIF α and pVHL binding respectively and also directly inhibit the hydroxylation of a key proline residue within the ODD domain of HIF 1α (Wiesener *et al.*, 1998). Further studies were able to show that iron chelators DP and DFO were able to induce optimal up regulation of HIF-1-dependent reporter gene expression under normoxic conditions and this up-regulation did not further increase when exposed to hypoxia (Linden *et al.*, 2003).

1.4 Functionality and distribution of HIFs

Although HIF 1β displays a ubiquitous distribution, distinct patterns have been described for the HIF α isoforms. Stroka *et al*., (2001), using their own novel chicken (IgY) anti-HIF-1α polyclonal antibody were able to localize HIF 1α protein in mouse brain, kidney, liver, heart and skeletal muscle. HIF 2α and HIF 3α are two other members of the bHLH-PAS superfamily which have also been described. HIF 2α, also referred to as endothelial PAS domain protein 1 (EPAS-1) or HIF-1α-like-factor (HLF) bears functional similarity to HIF 1α in regards to hypoxic stabilisation and ARNT binding. HIF 2α shares 48% amino acid sequence identity with HIF 1α and 83% identity in the bHLH domains (Tian *et al.*, 1997), and may be differentially regulated depending on the duration and severity of hypoxia exposure (Holmquist-Mengelbier *et al.*, 2006). Clearly, however, investigators have found similarities with HIF 1α and HIF 2α, but there are important differences (Wiesener *et al.*, 2003). Wiesener and colleagues were able to show in liver, kidney, brain, heart, lung and duodenum of the rat using immunoblotting that HIF 2α protein expression was not present in normoxic conditions and when studied under hypoxia, the expression of HIF 2α was sustained for

more than 6 h, which contrasts with HIF 1α (Wiesener *et al.*, 2003). These authors were also able to induce *Hif2α* mRNA in rat lung whereas previous reports were unable to show any HIF 1α expression there (Yu *et al.*, 1998; Compernolle *et al.*, 2002; Wiesener *et al.*, 2003; Uchida *et al.*, 2004). Similarly, HIF 2α protein was not detectable in MEF isolates (Bracken *et al.*, 2005; Hu *et al.*, 2006) and hypoxia-induced gene expression in MEFs occurred solely through the action of HIF 1α, while endogenous HIF 2α remained inactive due to cytoplasmic trapping (Park *et al.*, 2003). However, it was found in mouse embryonic stem cells that targeted replacement of HIF 1α by a HIF 2α "knock in" allele promoted tumour growth, increased microvessel density and specific HIF 2α target genes such as Oct4, a transcription factor essential for maintaining stem cell pluripotency, survival and maintenance (Covello *et al.*, 2006). HIF 2α is also involved in vascular remodelling and angiogenesis (Ema *et al.*, 1997; Tian *et al.*, 1997; Peng *et al.*, 2000; Brusselmans *et al.*, 2001). These studies provide strong evidence for tight regulation of HIF 2α in ES cells and early embryonic development.

Although HIF 2α has been referred to as endothelial PAS protein 1 (EPAS-1), HIF 2α is not endothelial specific and is expressed in other cell types including kidney fibroblasts, hepatocytes, pancreatic interstitial cells, heart myocytes and lung type II pneumocytes (Compernolle *et al.*, 2002; Wiesener *et al.*, 2003; Haase, 2006). Furthermore, in tissues where both HIF 1α and HIF 2α are co-expressed, each is localised to a distinct cell population. For example, in the brain, HIF 1α is specific to neuronal cells, and HIF 2α is limited to non-parenchyma such as glia cells (Wiesener *et al.*, 2003).

In contrast to the other HIFs, less is known about HIF 3α. As seen in Figure 1.2, the HIF 3α polypeptide is much shorter. At least six alternatively spliced isoforms of HIF 3α are thought to exist and sequencing of mouse inhibitory PAS protein (IPAS) genomic structure revealed IPAS is a splicing variant of the HIF 3α locus (Makino *et al.*, 2002). The N-terminal bHLH-PAS domain of HIF 3α shares amino acid (aa) sequence identity with that of HIF 1α and HIF 2α (57% and 53% identity, respectively). IPAS is a 307-aa composed of

bHLH and PAS domains, but lacks nearly all of the C-terminal regulatory sequences needed for transactivation (Makino *et al.*, 2001). This splicing variant of the HIF 3α locus may act as the dominant regulator by heterodimerising with other HIFs and forming a transcriptionally inactive complex (Makino *et al.*, 2002).

HIF 3α has been shown to be expressed in adult thymus, lung, brain, heart and kidney (Gu *et al.*, 1998). In mice, it was also observed to be predominantly expressed in Purkinje cells of the cerebellum and in corneal epithelium of the eye (Makino *et al.*, 2002). IPAS is important for the avascular phenotype of the cornea (Makino *et al.*, 2002). A recent study also suggests that HIF 3α acts as a suppressive regulator of HIF 1α (Forristal *et al.,* 2009). Silencing HIF 1α in human embryonic stem (hES) cells was shown to promote the up-regulation of HIF 3α expression and vice versa (Forristal *et al.,* 2009). Although much has been described about the roles of HIF 1α and HIF 2α in activating the transcription of hypoxia-responsive genes, the mechanism by which HIF 3α levels within cells are regulated by hypoxia is still poorly understood.

1.5 Knock out models of HIFs

Studies have shown that mice with HIF 1α, HIF 1β and HIF 2α mutations are lethal. Mice with HIF 1α or HIF 1β mutations die at midgestation with vascular defects primarily with HIF 1α also exhibiting morphological developmental differences. Between embryonic days 8 and 8.5, there is a complete lack of cephalic vascularisation, a reduction in the number of somites, abnormal neural fold formation (Ryan *et al.*, 1998), with HIF 1β null mutants dying at day 8.5 from deficiencies in the extra embryonic circulation including: defective yolk sac and brachial arch vascularisation; stunted development and embryo wasting (Maltepe *et al.*, 1997). On the contrary, HIF 2α null mutants have a variable phenotype. In one report, embryos died at day 13.5, due to specific defect in catecholamine production (Tian *et al.*, 1997) with no obvious vascular defects indicating that that HIF 2α expression in vascular endothelium is sufficient for normal development. However, in another report, HIF 2α -/- neonatal mice died of cardiac failure and had problems with VEGF-mediated lung maturation resulting in perinatal death (Compernolle *et al.*, 2002). Recent studies have also shown that HIF 2α -/- mice exhibit marked retinopathy consistent with the complete loss of vision by 1 month of age due to the thinning of the retina and abnormal retinal vasculature (Ding *et al.*, 2005). Interestingly, a brain specific knock out model of HIF 1α reduces, rather than increases, hypoxic ischemic damage, consistent with a proapoptotic role of HIF 1α, suggesting that a loss of HIF 1α function may serve as a protective mechanism in some tissues (Helton *et al.*, 2005).

1.6 HIF prolyl hydroxylases and asparagine hydroxylases

Extensive studies have demonstrated that prolyl hydroxylase domain enzymes (PHDs) and asparagine hydroxylases act as cellular oxygen sensors regulating the activity of HIFs in response to the changing oxygen levels. These two hydroxylating pathways in the presence of iron are catalysed by a member of the 2-oxoglutarate-dependent-oxygenase superfamily (Epstein *et al.*, 2001; Lando *et al.*, 2002).

The di oxygen-dependent HIF r egulatory pat hway i s r egulated by 3 PHDs w hich m ediate t rans-4-hydroxylation at two conserved proline residues (Pro-402 and Pro-564 in human HIF 1α; Pro-405 and Pro-531 in human HIF 2α) under normoxia, sending signals to the E3 ubiquitin ligase complex containing the von Hippel- Lindau tumour suppressor protein (pVHL), which targets the HIF α subunits for degradation via the u biquitin-proteasome p athway. T he r egulation of VHL bi nding by proline hydroxylation r epresents a mechanism by which HIF protein levels are controlled. However, it has been proposed that inactivation of PHD 2 a lone is s ufficient to up-regulate HIF 1α protein levels and HIF target genes in oxygenated cells indicating that P HD 2 i s the more important enzyme in the regulation of HIF α in normoxia (Berra *et al.*, 2003). Recent genetic studies suggest that PHD3 is mainly responsible for HIF 2α hydroxylation (Bishop *et al.*, 2008).

The other important pathway in the regulation of HIF activity requires a asparagine hydroxylase, originally identified as a protein that binds HIF and named factor inhibiting HIF (FIH). In normoxia, FIH hydroxylates a conserved asparagine residue in the carboxyl-terminal transactivation domain in HIF α proteins (Mahon *et al.*, 20 01). T his pa thway prevents D NA binding an d t he r ecruitment of p300/CBP t ranscriptional c o activators leading to transcriptional repression (Lando *et al.*, 2002; Schofield & Ratcliffe, 2004; Soilleux *et al.*, 2005). Under hypoxic conditions, these oxygen- dependent hydroxylases are inactive, which allows the HIF α subunit to escape the pVHL-mediated destruction thus recruiting p300/CBP co activators leading to the up-regulation of target genes (Schofield & Ratcliffe, 2004).

1.7 Non-**hypoxic induction of HIF 1α**

Hypoxia-independent nuclear induction of HIFs can also be achieved *in vitro* by chemical mimetics, certain growth factors, cytokines and gonadotropins (Table 1.1).

Growth factors, angiotensin II (Ang II), thrombin, platelet-derived growth factor (PDGF) and other hormones can also increase HIF 1α protein in vascular smooth muscle cells (VSMC) to levels that are substantially more elevated than hypoxia treatment alone (Richard *et al.*, 2000). Similarly, transforming growth factor beta-1 (TGFβ1), a growth factor which is highly expressed in tumour cells, induces HIF 1α accumulation through specific regulation of PHD2 levels (McMahon *et al.*, 2006). Although TGFβ1 alone is able to induce HIF 1α protein expression and HIF-modulated *Vegf* transcriptional activity, this magnitude of amplification is further enhanced when cells are exposed to synergistic cooperation of hypoxia and TGFβ1 (Sanchez-Elsner *et al.*, 2001).

There is emerging evidence for effects of gonadotropins such as FSH and hCG in stimulating HIF activity (Alam *et al.*, 2004; Herr *et al.*, 2004; Alam *et al.*, 2008; van den Driesche *et al.*, 2008). The effects of gonadotropin-induced HIF activity are important to this study and will be discussed further below.

1.8 Targets of HIFs

It is estimated that 1 - 2% of all human genes are regulated by hypoxia (Mazure *et al.*, 2004). HIF 1α and HIF 2α form heterodimers that bind to DNA through core consensus sequence (A/G)CGTG in the HRE regulatory region of target genes (Table 1.2). However, some of these genes such as tumour associated carbonic anhydrase IX (*Ca9*) and *Vegf* require additional binding sites such as Sp1, implying cooperative effects with HIF transcription factors and other binding sites (Kaluz *et al.*, 2003). Although HIF 1α and HIF 2α have structurally similar subunits in their DNA-binding and dimerisation domains, they differ in their transactivation domains implying they may regulate unique target genes and require distinct transcriptional co factors. For example, the hypoxic regulation of glycolytic enzymes is regulated by HIF 1α but not HIF 2α (Semenza *et al.*, 1996; Hu *et al.*, 2003). However, many genes such as *Vegf* and *Slc2a1* are activated by both HIF 1α and HIF 2α (Chen *et al.,* 2001; Covello *et al*., 2006; Duncan *et al.,* 2008). Recent reports indicate that *Oct4* and *TGF-α* appear to be preferentially induced by HIF 2α (Covello *et al.*, 2005; Covello *et al.*, 2006).

Table 1.1: Factors known to stabilise HIF under normoxic conditions

Factor	Reference
Angiotensin 2 (AngII)	(Richard *et al.*, 2000)
Estrogen	(Kazi & Koos, 2007)
Endothelin 1	(Spinella *et al.*, 2002)
Epidermal growth factor (EGF)	(Zhong *et al.*, 2000)
Follicle stimulating hormone (FSH)	(Alam *et al.*, 2004)
Heat shock protein 90 (HSP90)	(Ibrahim *et al.*, 2005)
Hepatocyte growth factor (HGF)	(Tacchini *et al.*, 2004)
Human chorionic gonadotropin (hCG)	(Herr *et al.*, 2004; van den Driesche *et al.*, 2008)
Insulin-like growth factor 1 (IGF-1)	(Fukuda *et al.*, 2002)
Insulin-like growth factor 2 (IGF2)	(Feldser *et al.*, 1999)
Insulin	(Zelzer *et al.*, 1998; Treins *et al.*, 2002)
Interleukin 1(IL1)	(Thornton *et al.*, 2000)
Interferon-alpha	(Gerber & Pober, 2008)
Nitric oxide (NO)	(Sandau *et al.*, 2000)
Platelet-derived growth factor	(Richard *et al.*, 2000)
P53 tumour suppressor (p53)	(An *et al.*, 1998)
Prostaglandin E_2 (PGE_2)	(Fukuda *et al.*, 2003)
Tumour necrosis factor alpha (TNFα)	(Haddad & Land, 2001)
Thrombin	(Richard *et al.*, 2000)
Transforming growth factor beta 1 (TGFβ1)	(McMahon *et al.*, 2006)

Table 1.2: Examples of HIF induced genes

Gene	Involved	Reference
Aldo	Glycolysis	(Semenza *et al.*, 1996)
Bnip3	Apoptosis	(Raval *et al.*, 2005)
Ca9	Tumour marker	(Kaluz *et al.*, 2003; Salnikow *et al.*, 2008)
Ccng2	Proliferation & survival	(Wykoff *et al.*, 2000)
Col5a1	Extracellular matrix	(Wykoff *et al.*, 2000)
Edn2	Follicular ovulation	(Na *et al.*, 2008)
Eno1	Glycolysis	(Semenza *et al.*, 1994; Semenza *et al.*, 1996)
Epo	Erythropoiesis	(Wang & Semenza, 1993b; Semenza *et al.*, 1994)
Ets1	Transcription factor	(Oikawa *et al.*, 2001; Salnikow *et al.*, 2008)
Gapdh	Glycolysis	(Semenza *et al.*, 1996)
Ldha	Glycolysis	(Firth *et al.*, 1994; Semenza *et al.*, 1996)
Leptin	Energy homeostasis	(Ambrosini *et al.*, 2002)
Ndrg1	Cellular proliferation	(Salnikow *et al.*, 2008)
*Oct4	Embryonic development	(Covello *et al.*, 2006)
Pgk1	Glycolysis	(Firth *et al.*, 1994; Semenza *et al.*, 1996)
Scd1	Fatty acid metabolism	(Li *et al.*, 2006)
Slc2a1	Glucose transport	(Chen *et al.*, 2001)
Vegf	Angiogenesis	(Forsythe *et al.*, 1996; Duncan *et al.*, 2008)

***denotes specificity to HIF 2α regulation**

1.9 The role of HIFs within tumours

The partial pressure of oxygen within solid tumours varies, ranging from 5% O_2 in well vascularised regions to near anoxic conditions in regions often surrounding areas of necrosis (Brown & Wilson, 2004). Insufficient blood supply to developing tumours results in expression of hypoxia inducible genes (Semenza, 2003). There are many studies implicating roles for HIF 1α and HIF 2α in tumour promotion and progression (Semenza, 2002, 2003). HIF 1α have been reported to be over expressed in 13 of 19 tumour types compared with their respective normal tissues, including colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate and renal carcinomas (Zhong *et al.*, 1999; Jiang & Feng, 2006). On the other hand, HIF 2α has been shown to play a critical role in tumour formation by pVHL-deficient renal clear cell carcinoma (RCC) by regulating HIF 2α specific target genes such as *OCT4* (Hu *et al.*, 2003; Raval *et al.*, 2005). Although HIFs are frequently co expressed in human tumours (Talks *et al.*, 2000), the pattern of expression and distributions differ between HIFs as it has been implicated that HIF 1α primarily mediates fast (acute) responses and HIF 2α is continuously accumulated during chronic hypoxia (Holmquist-Mengelbier *et al.*, 2006). It has been implicated that HIFs play a part in the pathophysiology of many major human diseases such as cancer, myocardial infarction, ischaemia and preeclampsia (Semenza, 2001).

1.10 Angiogenesis during follicular development

Angiogenesis (blood vessel formation) is an important physiological component in follicular growth and formation of corpora lutea (Findlay, 1986; Tamanini & De Ambrogi, 2004; Fraser, 2006). In the ovary, there is an intensive network of blood vessel formation and increased permeability of blood vessels during follicular development, ovulation and subsequent development of the corpus luteum. There are extensive studies of the vascular development in the ovary (Findlay, 1986). The outgrowth of new blood capillaries from pre-existing blood vessels is essential to supply nutrients, to remove carbon dioxide and other metabolic by-products and to transfer hormones from endocrine glands to target cells, promoting follicular growth and corpus luteum formation in the ovary.

Primordial, primary and preantral follicles receive oxygen and nutrients by passive diffusion from stromal blood vessels. The expanding follicle continues its growth via the development of an individual capillary network and continued angiogenesis. As the follicle acquires an antrum, a vascular sheath develops in the thecal layer confined to outside the basement membrane of the follicle (Hazzard & Stouffer, 2000). The capillaries do not penetrate the basement membrane nor enter the membrana granulosa of unruptured follicles (Findlay, 1986; Fraser, 2006). The granulosa layer remains avascular until the breakdown of the basement membrane at ovulation (Fischer *et al.*, 1992).

It is well established that the ovary is an organ where the vasculature undergoes development and degeneration on a periodic basis (Hazzard & Stouffer, 2000). Interestingly, granulosa (mural and cumulus) cells and the oocyte are excluded from follicular vasculature yet, as the follicle develops, the COC is still able to maintain developmental competency. The mechanism by which the COC is able to remain viable within an avascular environment is a question inadequately resolved.

Vascular endothelial growth factor (VEGF) is a secreted endothelial cell-specific mitogen and plays a major role in angiogenesis (Shweiki *et al.*, 1993). VEGFs are controlled by endocrine, paracrine and autocrine

regulation, by growth factors, cytokines and a variety of environmental influences such as hypoxia. VEGF mRNA was shown to be expressed in cells surrounding the developing vasculature in the ovary and was predominantly produced in regions of the ovary that acquire new capillary networks such as the theca layers and endometrial stroma. On the other hand, no VEGF was found in atretic or degenerating corpus luteum (Gordon *et al.*, 1996). The involvement of VEGF in follicular development is further supported by experimental data demonstrating a positive correlation between VEGF production by granulosa cells, as measured in follicular fluid, and follicle size (Geva & Jaffe, 2000). A study performed in Rhesus monkeys showed that blocking VEGF receptors led to the inability for growth of recruited antral follicles and subsequent dominant follicle selection, illustrating that the final follicular growth stages are VEGF dependent (Zimmermann *et al.*, 2001).

Angiogenesis is associated with follicular development, excessive follicular angiogenesis is likely to be associated with some types of infertility. In polycystic ovarian syndrome (PCOS) there is excessive angiogenesis and ovarian hyperstimulation syndrome (OHHS) has been associated with increased capillary permeability. Thus maintaining a proper balance of follicular angiogenesis is important as an over expression of VEGF can lead to infertility; whilst down-regulation of angiogenesis can lead to follicular hypoxia or follicle death.

1.11 Relevance of the HIF pathway in the ovary

Hypoxia-inducible factors (HIFs) are key transcriptional factors regulating the hypoxic response in both adult and embryonic development (Maltepe *et al.*, 1997; Ryan *et al.*, 1998). Many recent studies have suggested a role for HIFs in the ovary (Alam *et al.*, 2004; Boonyaprakob *et al.*, 2005; Alam *et al.*, 2008; Duncan *et al.*, 2008; Na *et al.*, 2008; Kim *et al.*, 2009). As discussed earlier, mammalian oocytes are dependent on oxidative phosphorylation for the generation of ATP and have a low capacity for glycolytic activity (Sutton-McDowall *et al.*, 2006; Thompson *et al.*, 2007). During follicular development, the declining oxygen gradient towards the oocyte may be the limiting factor for oocyte oxidative phosphorylation as granulosa cell numbers proliferate (Gosden & Byatt-Smith, 1986; Neeman *et al.*, 1997). Mathematical modelling in sheep demonstrates that the formation of an antrum may be a mechanism by which the follicle overcomes a mounting degree of hypoxia (Redding *et al.*, 2007). Gonadotropins such as FSH and hCG (a hormone which mimics the LH surge) have been shown to up-regulate HIF activity. There is evidence that FSH treatment can stimulate HIF 1α activity in cultured rat granulosa cells and this is necessary for up-regulation of FSH target genes (Alam *et al.*, 2004) and may require multiple signalling pathways such as PI3-Kinase/AKT and ERK (Alam *et al.*, 2008). In addition, hCG can up regulate *HIF1α* mRNA (van den Driesche *et al.*, 2008) and *HIF2α* mRNA (Herr *et al.*, 2004) in human granulosa lutein cells *in vitro*. VEGF is highly expressed in granulosa lutein cells, and is up-regulated by hCG *in vitro* and *in vivo* (Koos, 1995; Zimmermann *et al.*, 2001). A recent publication has demonstrated that progesterone receptor (PR), a mediator of the ovulation cascade (Robker *et al.*, 2000), is necessary for the expression of HIF and known HIF target genes such as *Vegfa* and *Edn2* (Kim *et al.*, 2009). Finally, there are suggestions that corpora lutea and tumours display similar characteristics, such as a hypoxic core, both requiring rapid angiogenesis (Neeman *et al.*, 1997; Semenza, 2002). HIF 1α is an important mediator of the response to hypoxia during follicular differentiation and tumour progression, however, the mechanism of its regulation in the ovary is poorly understood.

1.12 THESIS HYPOTHESES & AIMS

The oxygen environment may vary during follicular development and luteinisation as the avascular primordial follicle develops, matures to a preovulatory follicle and finally undergoes luteinisation which involves highly regulated angiogenesis. HIFs mediate oxygen and gonadotropin dependent gene expression changes of a number of genes thought to be important for this process within the ovary, however, the expression and localisation of HIFs within murine ovaries is yet to be determined. HIFs may also provide a mechanism by which COCs respond to surrounding oxygen concentration, which then determines oocyte developmental competence.

With these points in mind the following hypotheses were proposed:

- Hypoxia as well as non-hypoxic stimuli co-ordinately regulate both granulosa cell function and luteal formation through HIFs;
- Hypoxia as well as non-hypoxic stimuli can promote and regulate hypoxia inducible factors in a transgenic reporter mouse expressing the Enhanced Green Fluorescent Protein (EGFP) under the control of a promoter region containing hypoxia response elements;
- Oxygen regulates gene expression in cumulus cells via HIF activation.

The following aims were established:

- To investigate HIF 1α protein stabilisation and target gene activation in FSH stimulated granulosa cell cultures and hCG-treated granulosa lutein cell cultures, in the presence and absence of low oxygen and/or with a hypoxic mimetic.
- To assess *in vivo* the role of HIF 1α during granulosa cell luteinisation.

- To determine function and localisation of HIF activity in the ovary utilising a transgenic reporter mouse expressing EGFP under the control of a promoter region containing hypoxia response elements.
- To assess HIF 1α protein stabilisation and gene expression changes in murine cumulus cells exposed to varying oxygen concentrations during *in vitro* maturation.

CHAPTER 2 MATERIALS AND METHODS

2.1 Chemicals and solutions

All chemicals were of analytical grade and were purchased from Sigma Chemical Company (St. Louis, MI, USA), unless otherwise stated. All buffers, media and solutions were prepared using Milli Q water (Millipore Corporation) and stored in Nunc Brand Products (Nunc Interenational, Roskilde, Denmark). All media were purchased from MP Biomedicals (Australia) and antibiotics were purchased from Sigma-Aldrich (St. Louis, MI, USA) unless indicated. All media used for culture was equilibrated overnight prior to use under the appropriate gas atmosphere at 37.5 °C. Antibody sources and dilutions are described in Table 2.2. Recipes, sterilization and storage for solution, buffer concentrations are described in Appendix 7.1. Specific techniques are outlined in appropriate chapters.

2.2 Animals

All procedures were approved by The University of Adelaide Animal Ethics Committee. Female C57BL/6 mice were obtained from the Central Animal House at The University of Adelaide. Female hybrid CBAB6F1 were obtained from either the Central Animal House at The University of Adelaide or Animal Resources Centre (Western Australia, Australia). The animals were housed under a 12:12 h light-dark regimen, and were fed a standard pellet diet ad libitum with free access to water.

2.2.1 HRE-EGFP transgenic reporter mouse

A licence from the Office of the Gene Technology Regulator was obtained for the production of transgenic mice (NLRD 229/2002). A previously described (Ema *et al.*, 1997) pSV40 promoter-EpoHRE-Luc plasmid was used to generate the HRE-EGFP construct. The plasmid contained four HREs of the *Epo* gene (coding strand, 5'-GATCGCCCTACGTGCTGTCTCA-3') inserted in tandem into the *Bgl*II site of pGL3 promoter plasmid (Promega) (Ema *et al.*, 1997). An Xba I/Hind III fragment from the pEGFP-N1 vector (Clontech), containing the coding sequence for EGFP, was inserted into the plasmid. A lentiviral vector containing the

HRE-SV40-EGFP sequence was then generated by Ozgene Pty Ltd, and transgenic mice were produced by lentiviral incorporation of the vector (Ozgene, Bentley, WA, Australia). Tail clippings of transgenic animals were obtained at 3 weeks of age for extraction of genomic DNA, and genotyping was performed by PCR by a laboratory technician. Liver and kidney were used as additional verification that the mice used in these studies were positive for a functioning construct (Figure 3.1).

2.3 Ovarian stimulation procedure

All experiments were conducted according to the National Health and Medical Research Council of Australia guidelines for the use of animals following approval from The University of Adelaide Animal Ethics Committee. For all cell culture experiments, granulosa cells were isolated from transgenic or non transgenic mice (21 days old) that received 5 - 7.5 IU equine chorionic gonadotropin (eCG) (Folligon serum gonadotropin; Internet, Boxmeer, Holland) injected intraperitoneally 46 - 48 h prior to granulosa cell extraction. All hormones for injection were prepared in 0.9% saline. Hormonal stimulation protocol used in this study is the gold standard mouse ovarian stimulation protocol used by the laboratory (Banwell *et al.*, 2007; Pringle *et al.*, 2007).

2.4 Granulosa cell collection and cultures

Gonadotropin-stimulated ovaries were collected into HEPES buffered 199, pH 7.4, supplemented with 3% bovine serum albumin (BSA). Granulosa cells were collected by follicular puncture by puncturing visible antral follicles present on the ovary surface with a 30 gauge needle and were cultured in DMEM/HAMS F12 media supplemented with 1 % fetal calf serum, 1% L-glutamine and 2% penicillin/streptomycin in an incubator atmosphere of 20% O_2, 5% CO_2 at 37.5 ºC overnight.

2.5 Localisation of EGFP in tissue sections

Ovaries from C57BL/6-Tg(HRE(4)-SV40-EGFP) mice collected for fluorescent microscopy were fixed in 4% (w/v) paraformaldehyde in PBS overnight, followed by washes in PBS for 24 h and 18% sucrose for 24 h. Ovaries were mounted in Tissue-Tek® O.C.T. Compound (Miles Inc., Elkhart, IN, USA) and stored at -20 °C until sectioned. Serial sections (8 µm) were prepared using a CM 1850 Leica cryostat (Leica Microsystems Pty Ltd) were mounted onto SuperFrost slides and fixed in 100% ethanol for 5 min at room temperature followed by washing in PBS for 5 min twice. All sections were counterstained with the nuclear stain 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (3 µM; Molecular Probes, Eugene, OR, USA) for 30 min at room temperature and mounted in Dako fluorescent mounting media (Dako Corporation, Carpinteria, CA, USA) prior to fluorescent imaging (Nikon Eclipse, TE2000-E, Nikon Corporation, Tokyo, Japan). Parameters of filters used for fluorescent analyses are listed in Table 2.1.

The images were processed using the CoolSNAP-ES CCD digital camera and IPLab 3.6 software (Scanalytic, Inc., Fairfax, VA, USA). Subsequent sections were stained with Haematoxylin and Eosin (H&E), dehydrated, and coverslipped using DPX mountant (BDH Laboratory Supplies, Poole, U.K.) for general morphology. (Appendix section 7.1.5 for H&E protocol).

Table 2.1: Filter parameters used for fluorescence imaging

Filter	Excitation Wavelength (nm)	Barrier Wavelength (nm)
UV (Blue)	330 – 380	420
GFP (Green)	460 – 500	510
Cy3 (Red)	540 – 565	605

2.6 Collection and processing of samples for Western blot analyses

Cultured granulosa cells were washed twice with cold PBS, and total cell extracts were collected by scraping cells and lysing in RIPA buffer (50 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) in the presence of protease inhibitors (Protease Inhibitor Mixture, Sigma). Protein concentrations were determined by the Bradford method (Bradford, 1976), using protein standard assay 2 (Bio-Rad, Hercules, CA) as a standard. For Western blot analyses, concentrations of 20 - 50 µg of protein extracts were used for granulosa cells and luteinised granulosa cells; 100 µg of protein extract was used for residual ovarian stroma; 50 µg were used for ovaries post - copulation days 1, 4, 8 and 12.

2.6.1 Concentrating the protein sample with Microcon Centrifugal Filter (YM-10)

Due to the limited sample volume that can be loaded within a well on the SDS-PAGE gel; certain cell samples were processed by Microcon centrifugal filter device, YM-10 (Millipore). Microcon centrifugal filter devices simply and efficiently concentrate and desalt macromolecular solutions to a concentrated sample of approximately 25 - 40 µl. YM-10 has a nominal molecular weight limit of 10 kDa; the ability to retain molecules above a specified molecular weight. The protein sample was pipetted into the supplied Microcon sample reservoir in vial. The vial containing the Microcon sample reservoir was spun at 14,000 r.c.f for 30 min at room temperature. Following this, the sample reservoir was placed upside down and spun at 1,000 r.c.f for 2 min at room temperature to transfer the concentrated protein sample into a new supplied vial. Samples were stored at -80 ºC to prevent protein breakdown.

2.7 Western blotting analyses

Proteins were loaded onto the gel with the inclusion of 5X loading buffer. Proteins were resolved by SDS-PAGE on 4% - 10% gradient gels and transferred onto PVDF membranes. All gels for Western blotting included pre- stained protein molecular weight markers (Bio-Rad). Membranes were blocked for 1 h with 5% (w/v) skim milk, 1x TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0. 05% Tween 20]. Detection of target proteins was accomplished by using primary antibodies (Table 2.2). Membranes were washed and incubated 1 h at RT with HRP conjugated secondary antibodies (Table 2.2). All antibodies were diluted in 5% (w/v) skim milk, 1x TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.05% Tween 20]. After several TBST washes, bands were detected by enhanced chemiluminescence detection as per the manufacturer's instructions (Amersham, GE healthcare Life Sciences, Rydalmere, NSW, Australia). The membranes were stripped and re-probed with an antibody to β-actin (Sigma). Film images were captured using Image Quant ECL (Bio-Rad) and the images were quantified using Image Quant 1D gel analyses (Bio-Rad).

Table 2.2: Antibodies and conditions used for Western blotting

Antigen	Predicted band size (kDa)	Host	Primary Antibody Dilution (Source)	HRP conjugated Secondary Antibody Dilution (Source)
HIF 1α	~ 120	Rabbit Polyclonal	1:1000 (NB100-449)	Goat anti-Rabbit-HRP (SC-2004) 1:5000
HIF 1α	Blocking Peptide	Specific for NB100-449	200 fold excess of NB100-449	Goat anti-Rabbit-HRP (SC-2004) 1:5000
HIF 2α	~118	Rabbit Polyclonal	1:1000 (NB100-122)	Goat anti-Rabbit-HRP (SC-2004) 1:5000
HIF 3α	~ 75	Rabbit Polyclonal	1:500 (NB100-2287)	Goat anti-Rabbit-HRP (SC-2004) 1:5000
β-actin	~42	Mouse Monoclonal	1:25000 (A 3854; SI)	Goat anti-Mouse-HRP (AB 6789) 1:5000

Company abbreviations: NB = Novus Biologicals, Littleton, CO, USA; SC = Santa Cruz Biotechnologies, Santa Cruz, CA, USA; SI = Sigma, St Louis, MO, USA; AB = Abcam Inc, Cambridge, MA, USA.

2.8 General protocols for RNAse-free conditions

Ribonuclease (RNAse)-free conditions were maintained for materials, chemicals and solutions used in the generation and handling of all materials used during RNA isolation and analyses. This included the use of disposable plastic pipette tips and tubes where possible, and disposable latex gloves were regularly replaced during experimental procedures and handling of samples.

2.9 Quantitative Real Time RT- PCR

Total RNA from granulosa cells and granulosa lutein cells was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with the inclusion of 7.5 µg Blue glycogen (Ambion Inc., Austin, TX) during precipitation. Whole ovary was homogenized in TRIzol using Precellys 24 (Geneworks, Hindmarsh, SA, Australia). RNA concentrations were quantified using the NanoDrop ND-100 spectrophometer (NanoDrop Technologies, Wilmington, DE). Total RNA was treated with 1 U of DNase as per the manufacturer's instructions (Ambion Inc). First strand complimentary DNA (cDNA) was synthesized from total RNA (400 ng for granulosa cells and lutein cells; 1 µg for whole ovary) using random primers (Geneworks) and Superscript III reverse transcriptase (Invitrogen Australia Pty. Ltd).

Specific gene primers for quantitative Real Time RT-PCR were designed against published mRNA sequences using Primer Express software (PE Applied Biosystems, Foster City, CA) and obtained from Geneworks. Primer sequences for gene expression studies are listed in Table 2.3. For *eGfp*, sequence data for the pEGFP-N1 vector (Clontech) was used for primer design. Real Time RT-PCR was carried out on an ABI-PRISM 5700® sequence detection system (Applied Biosystems, Foster city, CA, USA). For *Vegfa*, *Slc2a1, Star* and *Hif1α*, 4 µl of cDNA (equivalent to 20 ng of total RNA), 1 pmol of each forward and reverse primer and 10 µl of SYBR Green Master Mix (Applied Biosystems) were added, and H_2O was added to a

final volume of 20 µl. For *eGfp*, 1 µl of cDNA (equivalent to 5 ng of total RNA), 5 pmol of each forward and reverse primer and 10 µl of SYBR Green master mix were added, and H_2O was added to a final volume of 20 µl. PCR cycling conditions were 2 min at 50 ºC then 10 min at 95 ºC followed by 40 cycles of 15 sec at 95 ºC and 60 sec at 60 ºC. Each assay included duplicates of each sample, an ovary standard and a non template control. The ovarian standard was generated by whole ovary extraction of RNA from a pool of 3 random naturally cycling C57BL/6-Tg(HRE(4)-SV40-EGFP) mice. Relative mRNA expression for each gene of interest was calculated using the standard curves produced from serial dilutions of the whole ovary standard cDNA. The expression of *18S* rRNA was used to normalise samples for the amount of cDNA used per reaction and results are presented as the relative expression of each gene after normalisation against *18S* rRNA. Dissociation curve analyses was performed to confirm the amplification of a single product.

2.10 Statistical Analyses

Statistical significance was determined using SPSS software for windows, version 14.0 (SPSS, Chicago, IL). For *in vitro* experiments, each treatment group was repeated in triplicate and collected on 3 separate occasions. For *in vivo* experiments, each time course experiment was repeated at least 3 times. Band densities from Western blot analyses conducted following cell culture experiments were analysed by analyses of variance and comparisons made by least-significant difference (LSD). After plotting the mean ± S.E.M data, an analysis of variance fitting a polynomial model was used to assess the relationship of band densities from Western analyses over time after hCG treatment for both the *in vivo* residual ovaries and granulosa cells. Gene expression results were analysed using one-way analyses of variance and least-significant differences unless the significance of Levene's test was ≤ 0.05. In those cases, a non-parametric Kruskal-Wallis test followed by post-hoc analyses (Mann-Whitney) was used. The data are presented as the mean ± S.E.M. *P* values of ≤ 0.05 compared to appropriate control were regarded as statistically significant.

Table 2.3: Primers used for quantitative Real Time RT-PCR analyses

Gene	GenBank accession no.		Primers	Amplicon length (bp)
eGfp		Forward:	GTCCAGGAGCGCACCATCT	
		Reverse:	CGATGCCCTTCAGCTCGAT	
Ldha	NM_010699	Forward:	GGACAGTGCCTACGAGGTGATC	107
		Reverse:	GCACCCGCCTAAGGTTCTTC	
Slc2a1	M23384	Forward:	CCAGCTGGGAATCGTCGTT	76
		Reverse:	CAAGTCTGCATTGCCCATGAT	
Vegfa	NM_009505	Forward:	CTGCTGTGCTGTAGGAAGCTCAT	75
		Reverse:	CCACGTCAGAGAGCAACATCA	
Hif1α	NM_010431	Forward:	TCAGAGGAAGCGAAAAATGGA	77
		Reverse:	AGTCACCTGGTTGCTGCAATAAT	
Star	NM_011485	Forward:	TAACTCACTTGGCTGCTCAGTATTG	101
		Reverse:	GGTGGTTGGCGAACTCTATCTG	
Lhcgr	NM_013582	Forward:	GGATAGAAGCTAATGCCTTTGACA A	149
		Reverse:	GGTCCGGATGCCTGTGTTAC	

Table 2.3 (Continued)

Gene	GenBank accession no.		Primers	Amplicon length (bp)
Acss2	BC051432	Forward:	AAAAGATTGGCCCCATTGC	101
		Reverse:	AATCTTCCGGAGAACTCGCC	
Bnip3	BC046603	Forward:	GGTTTTCCTTCCATCTCTGTTACTG	74
		Reverse:	TCAGACGCCTTCCAATGTAGATC	
Elovl6	BC051041	Forward:	AGCAGTTCAACGAGAACGAAGC	101
		Reverse:	CCGACCACCAAAGATAAAGGC	
Eno1	BC003891	Forward:	CAAAGTGAACCAGATCGGCTC	108
		Reverse:	TCCTCAGTTTCCCCAGATCG	
Inpp5e	AF226683	Forward:	TCTGGAGATGGGAAGGTAGCA	135
		Reverse:	CTCATCAAACCGGGTAGTGACA	
Mecr	BC003864	Forward:	CCCGAGACAAAAACCATCTTCA	90
		Reverse:	CCGGAGCAGCTCTGTAGAACTC	
Ndrg1	BC015282	Forward:	AGTACTTTGTGCAGGGCATGG	94
		Reverse:	AGGGATGTGACACTGGAGCC	
Pgk1	BC083355	Forward:	TTGATGAGAATGCCAAGACTGG	131
		Reverse:	AACAATCTGCTTAGCTCGACCC	

Table 2.3 (Continued)

Gene	GenBank accession no.		Primers	Amplicon length (bp)
Scd1	AF509567	Forward:	CGCATCTCTATGGATATCGCC	104
		Reverse:	GTGGTGGTAGTTGTGGAAGCC	
18S	AF176811	Forward:	AGAAACGGCTACCACATCCAA	91
		Reverse:	CCTGTATTGTTATTTTTCGTCACTACCT	
Rpl19	NM_009078	Forward:	TTCCCGAGTACAGCACCTTTGAC	103
		Reverse:	CACGGCTTTGGCTTCATTTTAAC	

CHAPTER 3 The role of hypoxia inducible factors during folliculogenesis

3.1 Introduction

The main function of the ovary is to release a mature oocyte ready for fertilization (McGee & Hsueh, 2000). At birth, there are approximately 10,000 primordial oocytes in mice and 2 - 7 million in humans. During folliculogenesis, follicles develop through primordial, primary and preantral stages before the acquisition of a follicular antrum, the latter is defined as an antral follicle. At this stage, approximately 99.9% of antral follicles undergo atresia, whereas the survivors (under the influence of gonadotropin stimulation) continue to develop by increasing differentiation of granulosa and theca cells and by increasing steroidogenic activity, to reach the preovulatory stage (Hirshfield, 1991; Gougeon, 1996). It is well established that follicle stimulating hormone (FSH) is necessary for follicular growth and maturation (Gore-Langton & Daniel, 1990) and can be mimicked by the exogenous administration of equine chorionic gonadotropin (eCG). As the follicle acquires an antrum, a vascular sheath develops in the thecal layer confined outside the basement membrane of the follicle (Hazzard & Stouffer, 2000) (Chapter 1, Figure 1.1). Folliculogenesis concludes when a mature cumulus oocyte complex is ovulated from an antral follicle following the LH surge and the remaining follicle cells remodel to an endocrine structure known as the corpus luteum, a progesterone secreting organ important for the maintenance of pregnancy.

During follicular development, a declining oxygen gradient towards the oocyte may limit the capacity of oocyte oxidative phosphorylation, as granulosa cell numbers proliferate (Gosden & Byatt-Smith, 1986; Neeman *et al.*, 1997). The definition of whether a tissue is hypoxic is described as a deficiency in O_2 whereby its demand exceeds supply. It is suggested that the preovulatory follicle has similar characteristics to a tumour structure whereby they both contain a hypoxic core (Neeman *et al.*, 1997). It has been further hypothesized that this declining gradient of oxygen concentration is the stimulus for the formation of the follicular antrum to alleviate hypoxia in the centre of the growing follicle (Gosden & Byatt-Smith, 1986; Hirshfield, 1991). Mathematical modelling has been used to support this hypothesis, whereby oxygen

transport is facilitated by the presence of an antrum (Gosden & Byatt-Smith, 1986; Redding *et al.*, 2007). As previously described, the follicular microenvironment is crucial for proper follicular development and therefore one aspect that this study focuses on is oxygen regulation, especially low oxygen (hypoxia), as it has been demonstrated that this inadequacy of oxygen supply has also been associated with follicle atresia (Hirshfield, 1991). The peri-follicular blood flow has been related to follicular oxygenation and can differ between follicles within an ovary (Van Blerkom *et al.*, 1997). In addition, ATP content of the oocyte and dissolved oxygen content within follicular fluid has been related to oocyte/embryo development (Van Blerkom *et al.*, 1995; Van Blerkom *et al.*, 1997). Furthermore, poor follicular vascularity and low oxygen concentration have been linked to poor developmental competence of the oocyte and/or IVF outcome (Chui *et al.*, 1997; Van Blerkom *et al.*, 1997; Borini *et al.*, 2004). These reports provide evidence for supporting the hypothesis that oxygen sensing and molecular mechanisms allowing responsiveness to oxygen concentration are fundamental requirements for the development of the follicle.

A role for the hypoxia inducible factors in follicular development has been implicated (Kim *et al.*, 2009) There is evidence that HIF 1α activity can be stimulated in cultured rat granulosa cells by FSH treatment and this is necessary for the up-regulation of FSH-responsive target genes (Alam *et al.*, 2004). FSH also stimulates VEGF production (a well characterized HIF 1 target) in ovarian cancer cells (Huang *et al.*, 2008). A study performed in primates demonstrated that the granulosa cells from large antral follicles synthesise and secrete VEGF in response to FSH (Christenson & Stouffer, 1997). Recently, immunostaining in ovarian sections during the normal follicular phase of primates also showed HIF 1α in the cytoplasm of cells of the developing follicle, including the oocyte, theca and granulosa cells. Although in this report there was no nuclear HIF 1α localisation, HIF 1α was observed in preantral, antral or preovulatory follicles and a clear up-regulation of HIF 1α in granulosa cells was seen when VEGF was inhibited (Duncan *et al.*, 2008). These studies suggest that upon FSH stimulation, HIF may play a role in up-regulating genes necessary for follicle development as a mechanism to overcome hypoxia.

As previously mentioned, angiogenesis is essential for antral follicle development (Tamanini & De Ambrogi, 2004) and it has been previously shown in primates that the inhibition of VEGF during folliculogenesis resulted in small, poorly vascularised antral follicles and prevented further follicular development (Fraser & Wulff, 2001; Wulff *et al.*, 2002). Similarly, the inhibition of VEGF signalling has been reported to prevent antrum formation and thecal angiogenesis in eCG stimulated mice (Zimmermann *et al.*, 2003).

I hypothesised that hypoxia inducible factors play a role during folliculogenesis, especially around the time of antrum formation. To test this hypotheses, our laboratory has developed a transgenic reporter mouse expressing EGFP under the control of a promoter region containing hypoxia response elements derived from the erythropoietin gene sequence (Ema *et al.,* 1997), to assess HIF activity. Therefore, the aim of this study was to characterise the ovaries from the HRE-EGFP transgenic reporter mouse to assess *in vivo* expression of HIF activity within follicles and to determine whether components of the HIF pathway are present in granulosa cells in normal/EGFP mouse. In addition, experiments assessed whether HIF 1α protein stabilisation and target gene activation is inducible in granulosa cells following FSH stimulation *in vitro*.

3.2 Material and Methods specific to this chapter

Mouse ovarian stimulation and granulosa cell cultures were performed as outlined in Chapter 2.

3.2.1 Functional positive C57BL/6-Tg(HRE(4)-SV40-EGFP) mouse identification

Tail clippings of transgenic animals were obtained at 3 weeks of age for genomic DNA and genotyping was performed by PCR by a laboratory technician. To identify functional positive C57BL/6-Tg(HRE(4)-SV40-EGFP) mice, tissue samples of kidney and liver of PCR-positive mice were collected when killed and processed for microscopic fluorescent localisation of positive EGFP protein as previously described in Chapter 2.

3.2.2 Morphological classification of follicles with HIF activation in the ovary of functional C57BL/6-Tg(HRE(4)-SV40-EGFP) mice

Utilising mice positive for the functional HRE-EGFP reporter, direct fluorescent visualisation of EGFP was performed on the ovaries of naturally cycling mice (n = 4). Follicles at all stages of folliculogenesis were assessed from serial sections. Mice were between stages of diestrus - proestrus at the time of killed as determined by vaginal smearing (Appendix section 7.1.6 for vaginal smearing protocol). Haematoxylin and Eosin staining was performed on subsequent sections for general morphology and to classify follicles as primordial, primary, preantral and antral follicle stages. Follicles were classified as primordial if they contained an oocyte surrounded by a layer of squamous granulosa cells. Primary follicles showed a single layer of cuboidal granulosa cells. Preantral follicles displayed more than 1 layer of granulosa cells with no visible antrum. Many previous investigations classify mouse follicles according to the classification scheme of Pedersen & Peters (1968), which determines class based on granulosa cell number rather than follicle and granulosa cell appearance. Although such classification is biased by the section thickness, the classification used in this study to quantify the range from primordial to preantral follicles approximates to types 1 to 5b (Pedersen & Peters, 1968). For the purpose of classification in this study, and in accordance

with Pedersen and Peters, type 6 - 8 follicles that have a follicular antrum were grouped as antral follicles. Atretic follicles were assessed based on morphology of membrana granulosa and the presence of apoptotic cells and were not included in analyses.

3.2.3 Immunohistochemistry

Ovaries from C57BL/6-Tg(HRE(4)-SV40-EGFP) mice were collected as previously described in Chapter 2. Serial sections (8 µm) were used for immunohistochemical localisation of F4/80 (a macrophage marker), DDX4/MVH (a primordial germ cell marker) and HIF 1α. All primary and secondary antibodies, sources, dilutions and blocking agents used in this study are listed in Table 3.1. All sections were counterstained with the nuclear stain 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (3 µM; Molecular Probes, Eugene, OR, USA) for 30 min at room temperature and mounted in Dako fluorescent mounting media (Dako Corporation, Carpinteria, CA, USA).

For DDX4/MVH and HIF 1α, ovarian serial sections (8 µm) from functional HRE-EGFP reporter mice were removed from -20 °C and dried under vacuum for 5 min before fixation in either 100 % ethanol or left unfixed. All sections were incubated in 10% normal goat serum in antibody diluents containing 0.55 M sodium chloride and 10 mM sodium phosphate for 1 h at room temperature to block non-specific binding. Sections were then incubated overnight with the primary antibody in a humidified chamber at room temperature. After three washes in PBS, the sections were incubated for 1 h or 24 h with their relevant biotinylated secondary antibody, followed by Cy3-conjugated-streptavidin (1:100; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in antibody diluent.

For F4/80 immunohistochemistry, the slides were thawed to room temperature and then placed in 1% (w/v) BSA for 2 min. Excess BSA was removed and slides were incubated with antibody overnight at 4 ºC overnight in a humidified chamber. Antibody was used as undiluted hybridoma supernatant with 10% mouse serum. After three washes in PBS for 5 min, sections were placed in 1% (w/v) BSA for 2 min and then for 2

h at 4 ºC w ith bi otinylated r abbit a nti-rat I gG [1:300; D akoCytomation Polyclonal R abbit A nti-Rat IgG/Biotinylated (Code E 0468)] in a humidified chamber. Sections were then washed and incubated for 1 h with Cy3-conjugated-streptavidin (1:100, Jackson ImmunoReasearch Laboratories, Inc., West Grove, PA, USA). The primary and s econdary ant ibodies w ere di luted i n s olution c ontaining 1% B SA, 10% nor mal mouse serum and PBS.

Negative controls used either antibody diluents or irrelevant IgG in place of the primary antibodies.

3.2.4 Granulosa cell culture and Western Blotting

Granulosa cells were collected as described in Chapter 2. In the first instance, granulosa cells were treated with or without the hypoxic mimetics, cobalt chloride ($CoCl_2$, 150 µM or 250 µM) or 2'2-Dipyridyl (DP, 150 µM or 250 µM) for 4 h to assess if HIF 1α was detectable by Western blot. Antibodies used for Western blot analyses are listed in Chapter 2 (Table 2.2). Commercially available COS-7 $CoCl_2$-treated and untreated nuclear extracts (NB800-PC26, Novus Biologicals, Littleton, CO, USA) were used as HIF 1α positive control.

Secondly, granulosa cells were treated with recombinant human follicle stimulating hormone (rhFSH) (Puregon; Organon, Sydney, NSW, Australia) 50 mIU overnight, during which time cells plated down. The cells were then cultured for a further 4 h, with or without $CoCl_2$ treatment, or were exposed to low oxygen conditions for 4 h in a modular incubator chamber (Billups-Rothenburg, Del Mar, USA) flushed with 2% O_2, 5% C O_2 balanced w ith N_2 at 37 .5 °C. Additional granulosa c ells w ere also c ultured overnight i n t he presence of 50 mIU rhFSH and treated with 150 µM DP for 4 h.

At the completion of the culture period the cells were collected for Western blot analyses as outlined in Section 2.7.

Granulosa cells were collected and pooled on 4 separate occasions, from ovaries of a total of 31 C57BL/6-Tg(HRE(4)-SV40-EGFP) mice for quantitative Real Time RT-PCR analyses.

3.2.5 RNA extraction and Reverse Transcription

RNA extraction and reverse transcription were performed as outlined in section 2.9.

3.2.6 Quantitative Real Time RT-PCR

Quantitative Real Time RT-PCR was performed using the ABI PRISM 5700® sequence detection system (Applied Biosystems, Foster City, CA, USA) as outlined in Chapter 2. Primer sequences for *Vegfa, Slc2a1, Ldha, Lhcgr*, *eGfp* and *18S* are listed in Table 2.3.

3.2.7 Statistical Analyses

All data are presented as mean ± S.E.M. The gene expression results for *Vegfa*, *Slc2a1*, *Ldha* and *Lhcgr* mRNA were measured using granulosa cells from C57BL/6 and was conducted on 3 separate occasions in triplicate *in vitro* (n = 9 per treatment group). Gene expression results for *eGfp* mRNA using pooled granulosa cells from ovaries of 31 EGFP positive C57BL/6-Tg(HRE(4)-SV40-EGFP) mice was performed on 4 separate occasions (n = 4 per treatment group). A non-parametric Kruskal-Wallis test followed by post-hoc analyses (Mann-Whitney) was used following Levene's test ≤ 0.05. *P* values of ≤ 0.05 compared to appropriate control were regarded as statistically significant. Statistical analyses were carried out using SPSS software for Windows, version 14.0 (Chicago, IL, USA).

Table 3.1: Antibodies (Primary and Secondary), sources, dilutions used in these studies

Antigen agent	Primary antibody	Source	Dilutions used/tested	Secondary antibody	Dilution	Blocking
DDX4/MVH serum	Rabbit Polyclonal	Abcam (AB13840)	1:50; 1:100	Biotin-goat-anti-Rabbit-IgG	1:500	10% Goat
HIF1α serum	Rabbit Polyclonal	Novus Biologicals (NB100-449)	1:100	Biotin-goat-anti-Rabbit-IgG	1:500	10% Goat
F4/80 serum	Anti-IgG2b	Gift[1]	1:20	Biotin-rabbit-anti-Rat-IgG	1:400	10% Mouse

[1] Kind gift from P.Kenny, Flinders University, South Australia, Australia.

3.3 Results

3.3.1 Characterising HIF activation in the ovary of functional C57BL/6-Tg(HRE(4)-SV40-EGFP).

To determine functional positive C57BL/6-Tg(HRE(4)-SV40-EGFP) mice, both kidney and liver were analysed for EGFP and were consistent with another investigator (Stroke *et al*., 2001) which has shown using immunohistochemical techniques that kidney and liver are known to express HIF 1α under normoxic conditions (Figure 3.1).

To identify EGFP positive follicles at different stages of folliculogenesis, ovaries were collected from functional positive C57BL/6-Tg(HRE(4)-SV40-EGFP) mice as described in Section 3.2.2. Fluorescent localisation of EGFP expression was examined in every 5th section of ovaries from naturally cycling C57BL/6-Tg(HRE(4)-SV40-EGFP) mice and H&E was performed on the subsequent section for confirmation of follicle classification (Figure 3.2). Naturally cycling transgenic mice exhibited no follicular EGFP in primordial, primary and preantral follicles (Figure 3.2) (Table 3.2). However, upon antrum formation, intermittent EGFP, indicating HIF activated expression, within the theca layer was observed (Figure 3.3 C - H). EGFP was also readily observed in CL (Figure 3.3, A, B, G and H) (Table 3.2). Interestingly, EGFP was not observed in mural granulosa cells or within COCs at any stage of folliculogenesis.

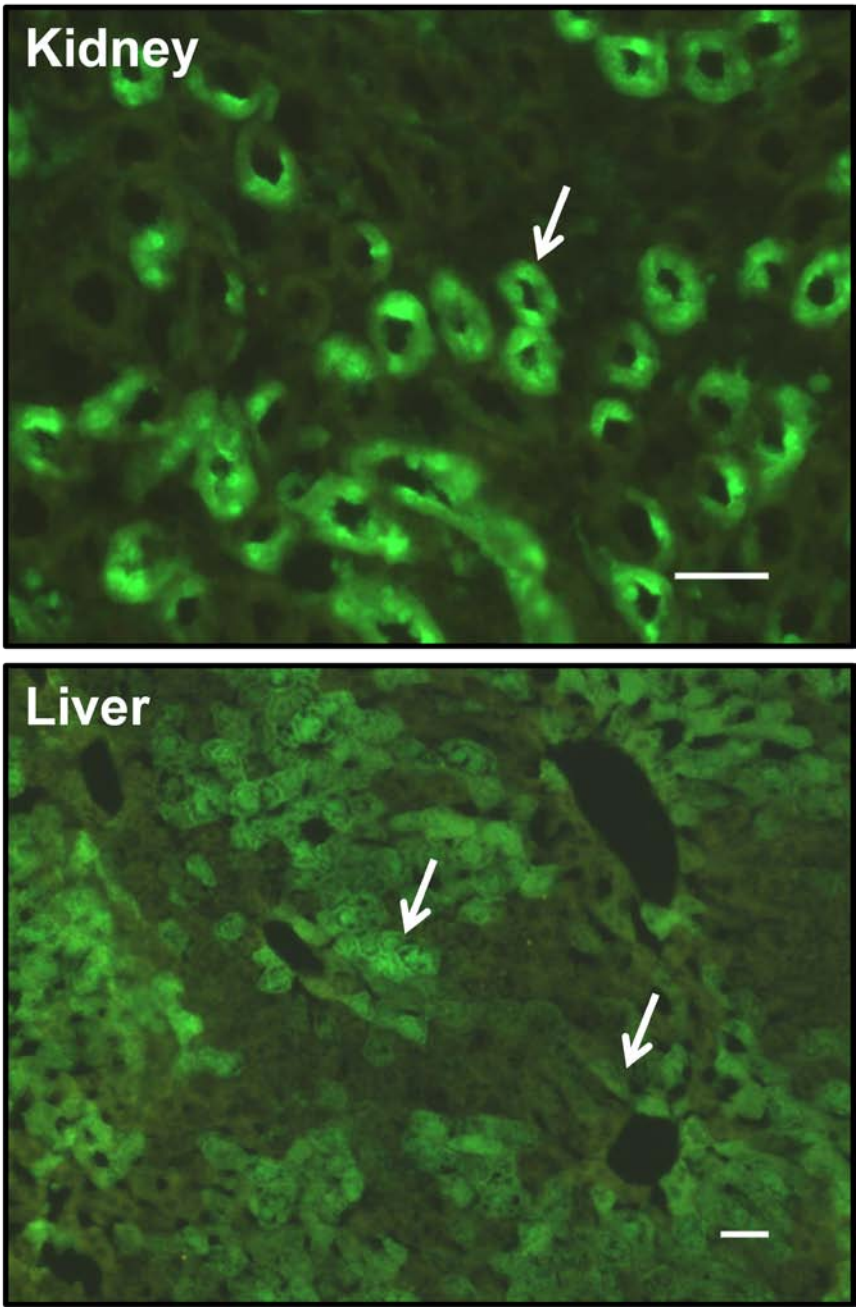

Figure 3.1 Cross section of EGFP protein localisation in kidney and liver of C57BL/6-Tg(HRE(4)-SV40-EGFP) mice.
Kidney - 200x magnification, Liver – 100x magnification. Arrows = EGFP positive cell.

Scale bar = 50 µm.

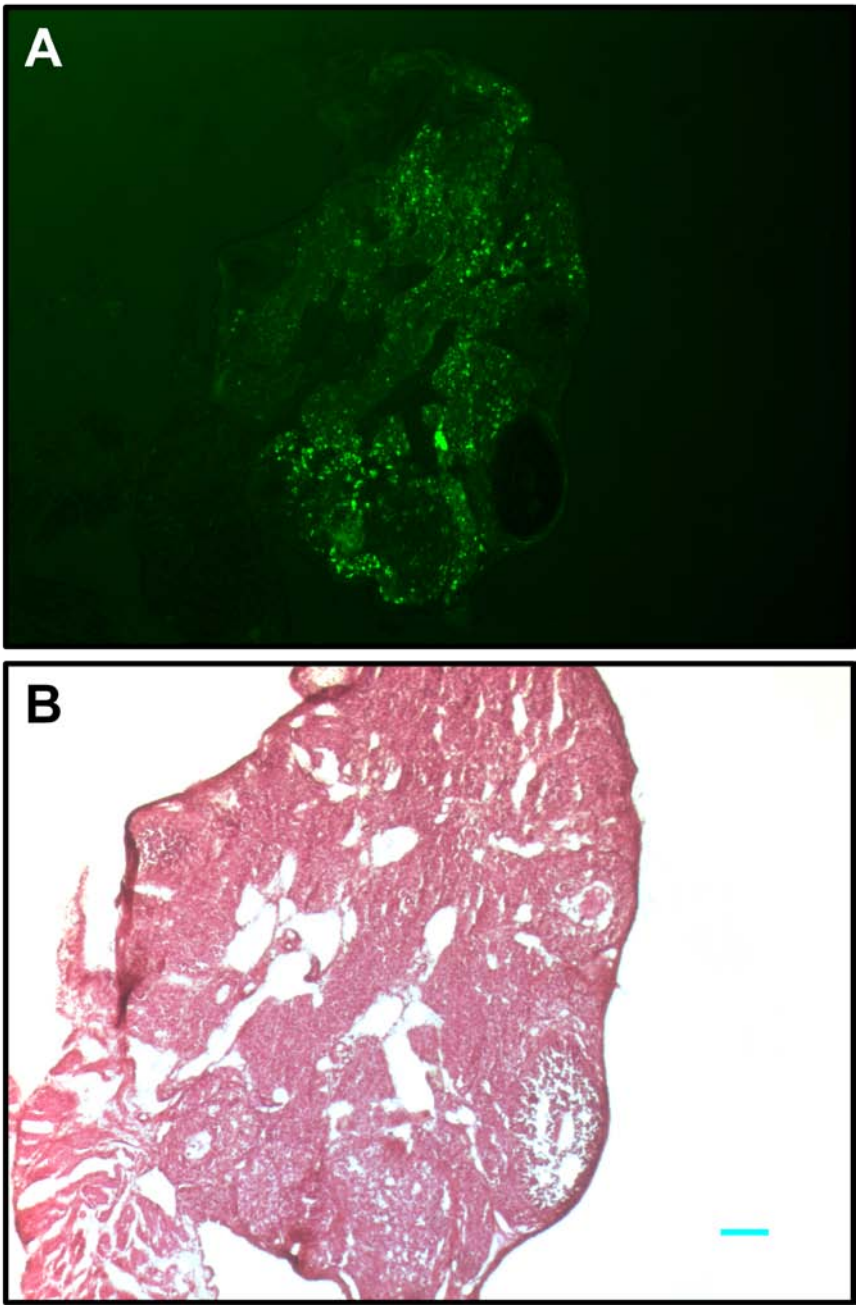

Figure 3.2 Cross section of EGFP protein localisation in ovary of naturally cycling C57BL/6-Tg(HRE(4)-SV40-EGFP).

Panel A – Fluorescent localisation of EGFP in an ovarian cross section. 40x magnification.

Panel B – Haematoxylin and Eosin stain of ovarian cross section. 100x magnification; Scale bar = 50 µm.

Table 3.2: Classification and quantification of EGFP positive follicles and corpora lutea in naturally cycling C57BL/6-Tg(HRE(4)-SV40-EGFP) mice (n = 4)

Stage	EGFP positive	Total follicle count
Primordial	0	55
Primary	0	30
Preantral	0	15
Antral	14	26
Corpus luteum	8	21

3.3.2 **Immunohistochemical localisation of DDX4/MVH, HIF 1α and F4/80.**

In order to aid identify and quantify primordial follicles, DDX4/MVH, a primordial germ cell marker was used. To identify cells in the ovary that were producing HIF 1α, immunohistochemical analysis of HIF 1α was also attempted. Immunohistochemical analyses were tested using various dilution factors, incubation times and different conditions, however, optimisation and co-localisation of antibodies to their respective targets was not achieved in this study.

On the other hand, macrophages have been shown to be localised within theca of antral follicles and their numbers increase at ovulation and are believed to play a role in facilitating follicle rupture (Hume *et al.*, 1984; Brannstrom *et al.*, 1993). Furthermore, macrophages have been shown to infiltrate newly developing CL following follicle rupture and degenerated CL of diestrus staged ovaries collected from mice but was not associated with further infiltration of CL during pregnancy (Hume *et al.*, 1984). Therefore, F4/80 (which recognises a cell surface glycoprotein of macrophages) (Austyn & Gordon, 1981) was used, however, F4/80 did not co-localise to the same cells that were producing EGFP within sections visualised (data not shown).

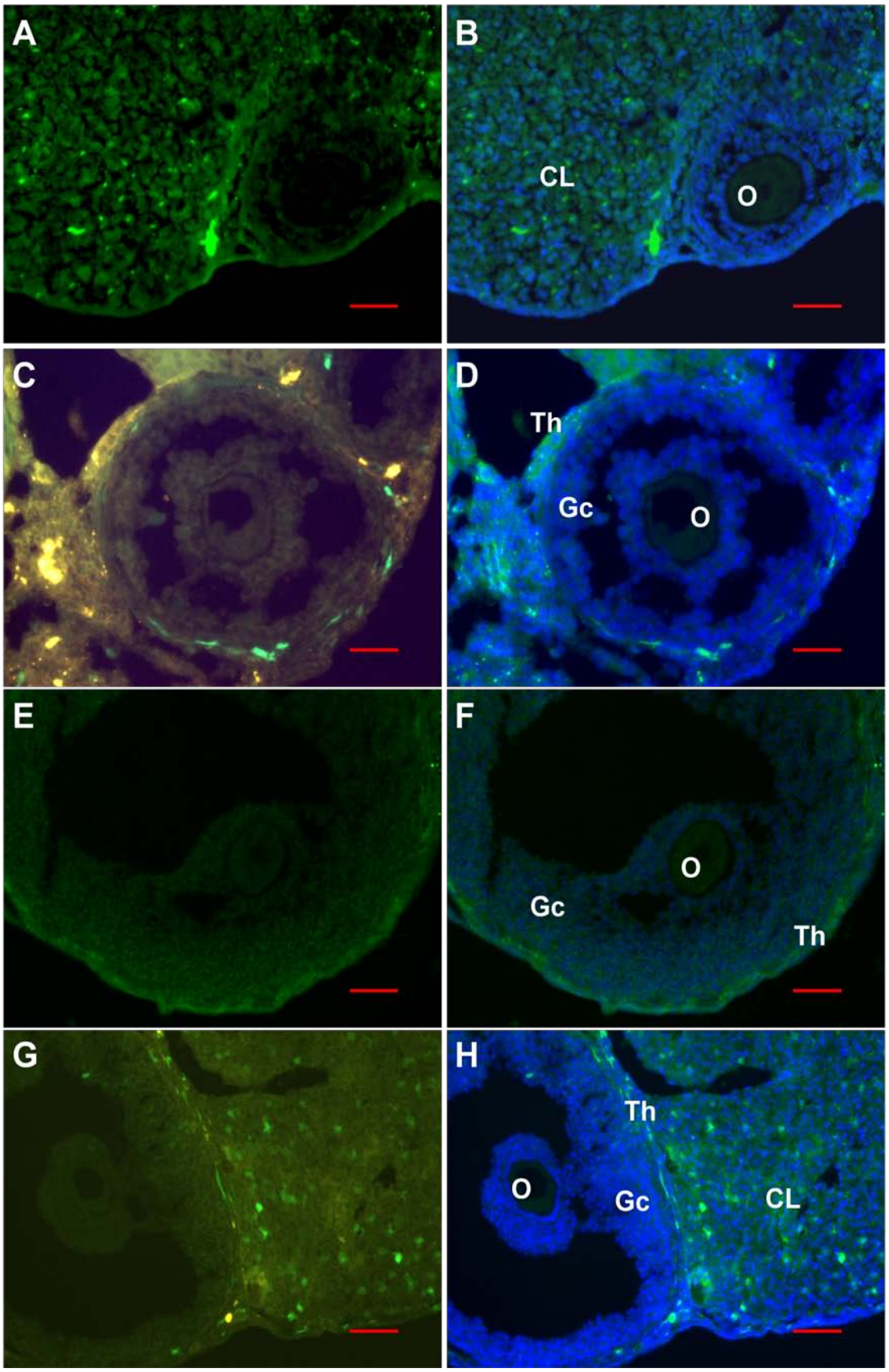
A
B
CL
O
C
D
Th
Gc
O
E
F
O
Gc
Th
G
H
Th
O
Gc
CL

Figure 3.3 EGFP fluorescent localisation in naturally cycling C57BL/6-Tg(HRE(4)-SV40-EGFP) adult mice.

Ovarian sections consisting of various stages of antral follicle development and CLs were prepared as described in Section 3.2.3. Panels A, C, E and G are images taken under direct EGFP fluorescence. Panels B, D, F and H are images acquired using the same parameters at 200x magnification. All panels represent the magnified images after brightness and contrast adjustment to visualise the localisation of EGFP protein.Gc, granulosa cell layer; Th, theca cell layer; O, oocyte; CL, corpus luteum. EGFP (green) and DAPI (nuclear staining- blue). Scale bar = 50 µm.

3.3.3 Establishing Western blotting **for the detection of HIF 1α** and the effect of hypoxic mimetics **on HIF 1α stabilisation in granulosa cell cultures.**

To determine if HIF 1α protein can be stabilised in granulosa cells *in vitro*, cells were cultured in the presence or absence of a hypoxic mimetic and compared to commercially available HIF 1α positive control as outlined in Chapter 2 and in Section 3.2.4. There was a significant accumulation of HIF 1α protein at 120 kDa when cultured granulosa cells were treated with DP compared to control (Figure 3.4; Lanes 1 and 2). Interestingly, HIF 1α protein was readily detected in the commercially available untreated COS-7 lysate, and as expected, there was a greater accumulation of HIF 1α in $CoCl_2$-treated COS-7 cells (Figure 3.4; Lanes 3 and 4). The hypoxic mimetic, $CoCl_2$ (150 or 250 μM) and low oxygen was also able to stabilise HIF 1α protein at 120 kDa (Figure 3.5A and 3.5C).

3.3.4 **The effect of rhFSH on HIF 1α protein stabilisation in cultured granulosa cells** *in vitro*.

To determine the effect of rhFSH on HIF 1α protein stabilisation in cultured granulosa cells *in vitro*, cells were cultured in the presence or absence of 50 mIU rhFSH as described in Section 3.2.4. Western blotting demonstrates that HIF 1α (120 kDa) can be stabilised upon addition of hypoxic mimetic, $CoCl_2$ (Figure 3.5A). HIF 1α protein (~120 kDa) was also accumulated in total cell extracts of mouse granulosa cells that had been exposed for 4 hr to 2% oxygen or 150 μM DP (Figure 3.5C). However, there was no effect of 50 mIU FSH on HIF 1α protein levels in mouse granulosa cells in the presence or absence of the hypoxia mimetic (Figure 3.5B). These results demonstrate that HIF 1α protein abundance in cultured granulosa cells was increased by low oxygen and $CoCl_2$ but not by FSH.

3.3.5 **The effect of rhFSH on HIF 2α protein stabilisation in cultured granulosa cells *in vitro*.**

HepG2 cells that were treated with low oxygen, DP and untreated protein lysates were kindly provided by Dr. Daniel Peet, The University of Adelaide, South Australia, Australia. As demonstrated in Figure 3.6, HIF 2α (MW ~118 kDa) was induced in HepG2 cells treated with low oxygen and under hypoxic mimetic, however, HIF 2α in the presence or absence of 50 mIU FSH, with and without the addition of $CoCl_2$ could not be detected.

3.3.6 Gene expression analyses in granulosa cells *in vitro*.

To determine the effect of FSH on gene expression in cultured granulosa cells, quantitative Real Time RT-PCR was performed on RNA collected from these cells to determine expression of *Vegfa*, *Slc2a1* and *Ldha*. Biological activity of rhFSH was investigated by gene expression analyses of *Lhcgr*. *eGfp* mRNA expression was also measured in RNA collected from cultured C57BL/6-Tg(HRE(4)-SV40-EGFP) mice. Results demonstrate that the addition of FSH alone did not alter expression of HIF target genes *Vegfa, Slc2a1* and *Ldha* in cultured granulosa cells ($P > 0.05$). Expression of *Vegfa*, *Slc2a1* and *Ldha* were markedly increased in cultured granulosa cells by $CoCl_2$ (Figure 3.7, A – C; $P \leq 0.005$). There was no further induction of gene expression in the presence of both $CoCl_2$ and FSH (Figure 3.2; $P > 0.05$). Biological activity of the rhFSH used was confirmed by assessing luteinizing hormone receptor (*Lhcgr*) mRNA. A 9.6 fold up regulation of *Lhcgr* mRNA was observed when 50 mIU rhFSH was added to granulosa cell cultures (Figure 3.7D; $P \leq 0.004$) and when used in combination with $CoCl_2$ (Figure 3.7D; $P \leq 0.006$). However, *eGfp* mRNA expression in granulosa cell cultures from C57BL/6-Tg(HRE(4)-SV40-EGFP) reporter mouse was unaltered across all treatments (Figure 3.7E; $P > 0.05$).

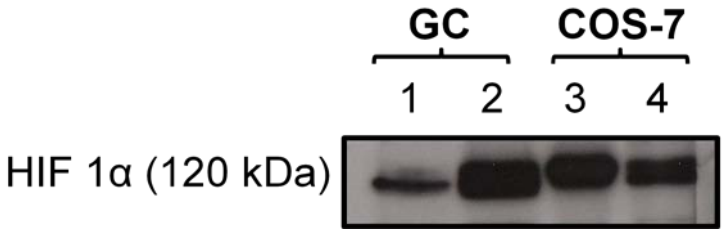

Figure 3.4 **Detection of HIF 1α protein by Western blot compared to commercially available HIF 1α positive and** negative control.

Granulosa cells (GC) prepared as described in Chapter 2 were cultured overnight and exposed with or without 250 µM 2,2'-Dipyridyl for 4 h. Protein whole cell lysates were collected and 50 µg of protein lysates were loaded for Western Blot analyses. HIF 1α protein stabilisation in granulosa cells was compared to commercially available $CoCl_2$-treated and untreated COS-7 cells (NB800-PC26, Novus Biologicals, Littleton, CO, USA).

Lane 1: granulosa cells control;

Lane 2: DP-treated granulosa cells;

Lane 3: $CoCl_2$-treated COS-7 cells;

Lane 4: untreated-COS-7 cells.

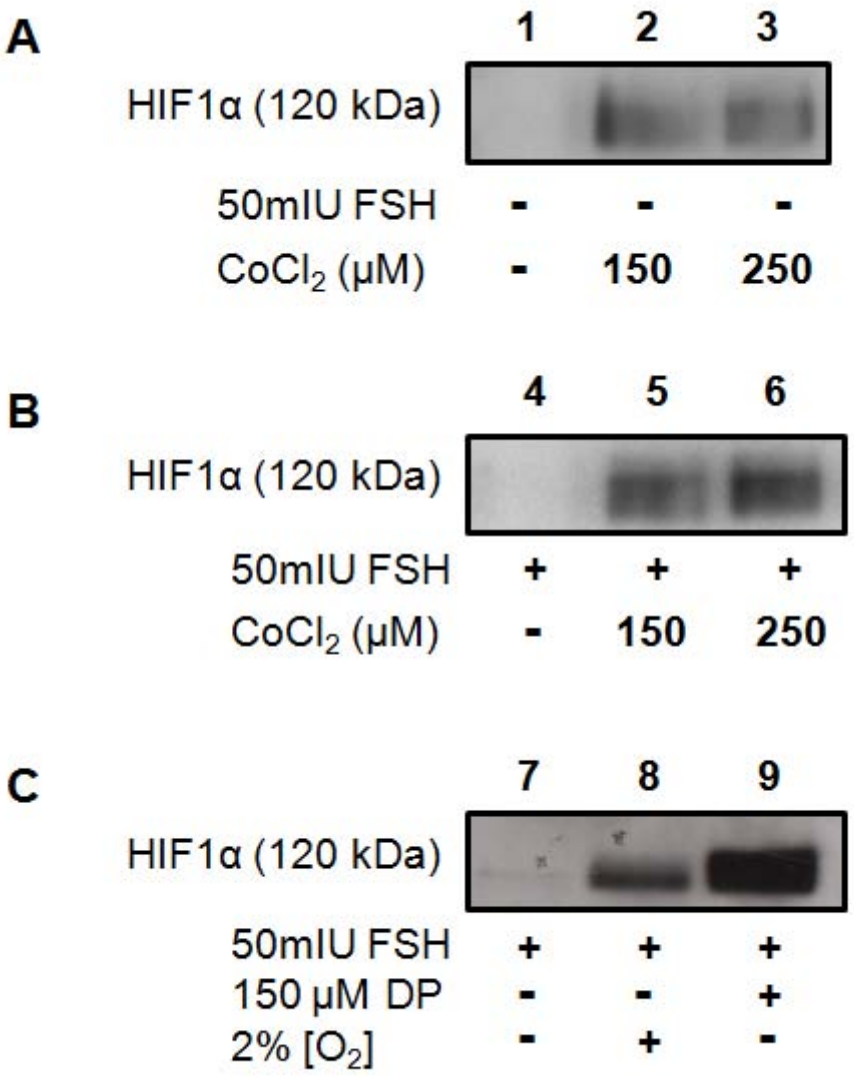

Figure 3.5 Effect of FSH, low O_2 and hypoxic mimetic on HIF 1α protein in cultured granulosa cells.

Granulosa cells were cultured with and without 50 mIU FSH and incubated in the presence of hypoxic mimetic $CoCl_2$ (150 μM or 250 μM) at 37.5 ºC, 20% O_2 5% CO_2 or low oxygen (2% oxygen) for 4 h as indicated. Western blots of total cell extracts were probed with an antibody to HIF 1α as described in Section 3.2.4.

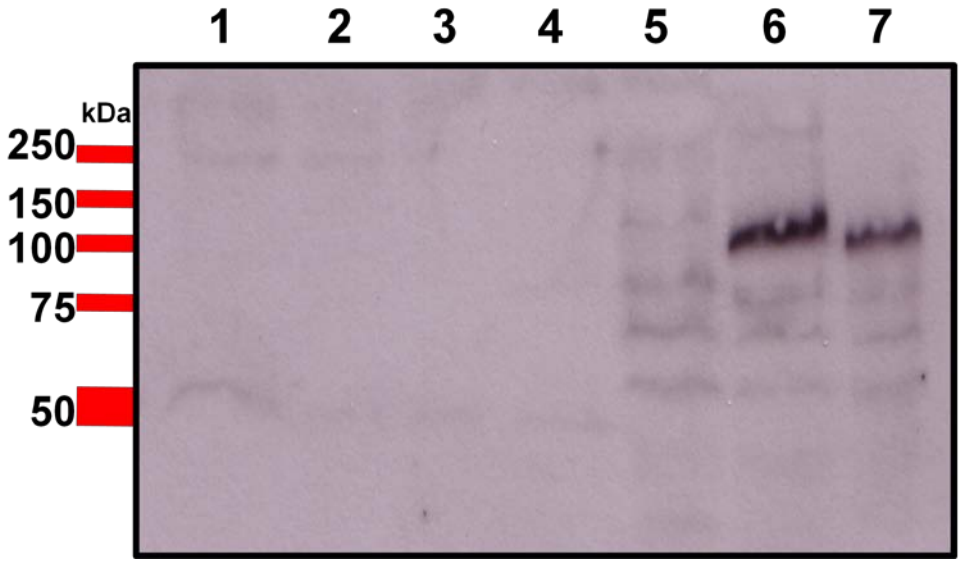

Figure 3.6 **HIF 2α Western blot** analyses on cultured mouse granulosa cells.

Western blot probed with an antibody to HIF 2α on granulosa cells cultured in the presence or absence of FSH and/ without the addition of hypoxic mimetic as previously described, using HepG2 cells as positive control.

Lane 1: granulosa cells with 50 mIU FSH

Lane 2: granulosa cells with 50 mIU FSH and 250 μM $CoCl_2$

Lane 3: granulosa cells control

Lane 4: granulosa cells with 250 μM $CoCl_2$

Lane 5: HepG2 (normoxia)

Lane 6: HepG2 (1% oxygen)

Lane 7: HepG2 with 100 μM 2'2-Dipyridyl

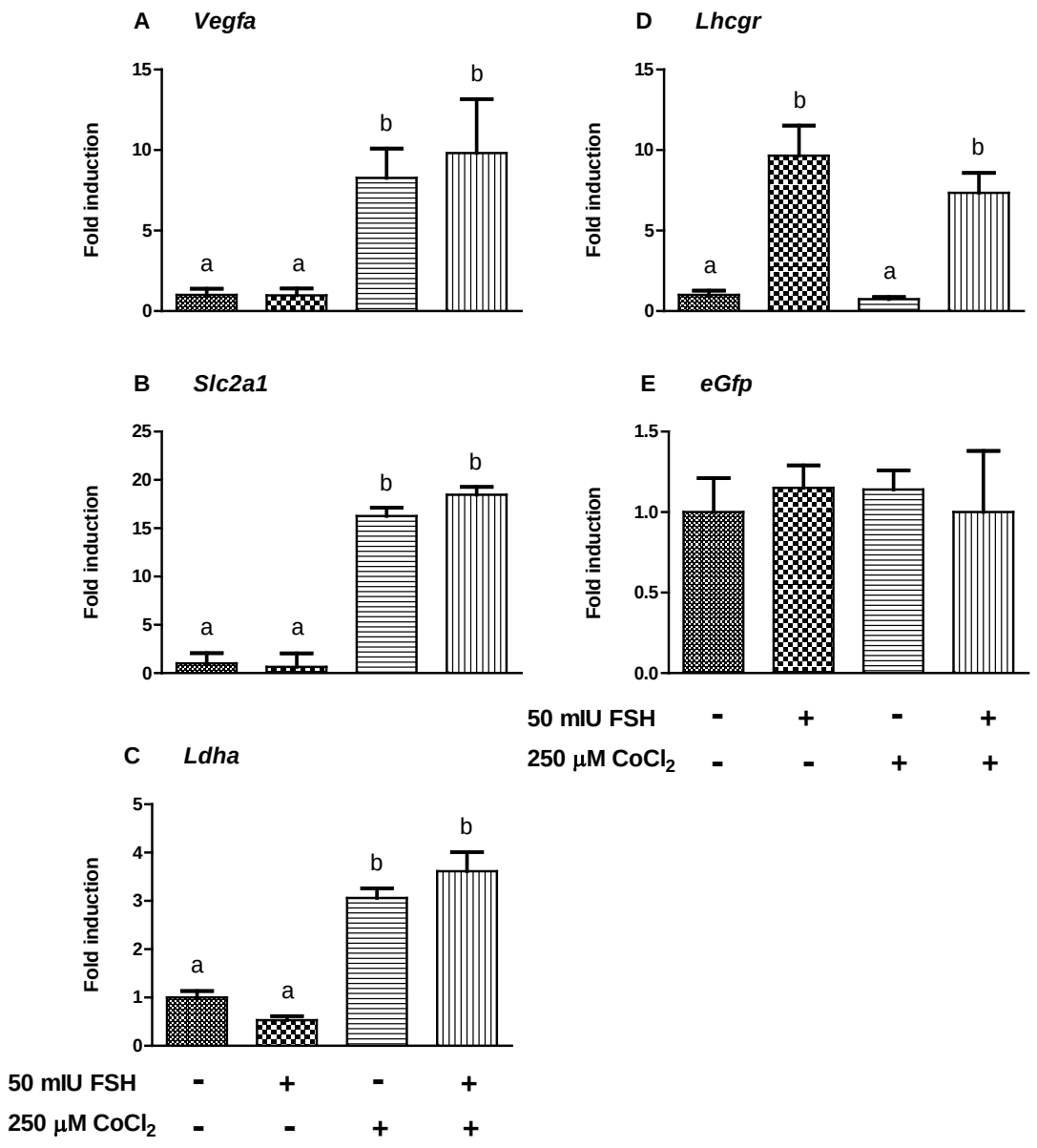

Figure 3.7 Effect of FSH and hypoxic mimetic on expression of HIF target genes in cultured granulosa cells.

In panel A – E, granulosa cells were cultured with and without 50 mIU FSH and incubated in the presence or absence of 250 μM $CoCl_2$ for 4 h. Quantitative Real Time RT-PCR was performed to determine expression of (A), *Vegfa* mRNA, (B) *Slc2a1* mRNA, (C) *Ldha* mRNA and (D) *Lhcgr* mRNA. The data for *Vegfa*, *Slc2a1*, *Ldha* and *Lhcgr* mRNA are presented as fold induction relative to control and represent the means from 3 independent experiments (mean ± S.E.M). Each experiment was replicated in triplicate on 3 separate occasions. Additionally, granulosa cells from gonadotropin-primed C57BL/6-Tg(HRE(4)-SV40-EGFP) mice were used to determine expression of (E) *eGfp* mRNA, which was conducted as individual experimental replicates on 4 separate occasions (n = 4 per treatment group). Non-parametric Kruskal-Wallis test followed by Mann-Whitney post-hoc test was used to compare values; Superscripts denotes significance $P \leq 0.05$. All gene expression results were normalised against *18S* rRNA.

3.4 Discussion

Reported here is the first characterisation of ovarian classification in the C57BL/6-Tg(HRE(4)-SV40-EGFP) mouse, designed to produce EGFP following HIF binding to the HRE. EGFP cells in this study are indicative of HIF activation. Examination of ovaries collected from naturally cycling transgenic mice revealed that EGFP was absent from all follicle classes prior to antrum formation (Table 3.2 and Figure 3.2). However, upon antrum formation, EGFP was detected within the theca cell layer of antral follicles of all sizes (Figure 3.3). This led us to investigate if EGFP positive cells were F4/80 positive (macrophage marker) as macrophages are known to have receptors for VEGF and are present within theca of antral follicles and their numbers increase at ovulation (Brannstrom *et al.*, 1993). Observations in this study demonstrated that F4/80 did not co-localise with EGFP cells in C57BL/6-Tg(HRE(4)-SV40-EGFP) mouse ovarian sections (data not shown), however a negative Immunohistochemical assessment is not conclusive and further analysis is required.

It is well known that hypoxic mimetics such as cobalt chloride and iron chelators stabilise HIF 1α at the protein level under acute exposures and conditions (Semenza & Wang, 1992; Wang & Semenza, 1993a). Results in the present study indicate that granulosa cells can respond to low oxygen and hypoxic mimetics ($CoCl_2$ and DP) by stabilising the 120 kDa HIF 1α protein (Figure 3.4 and 3.5).

Knowing that the ovarian follicular vasculature is restricted to the thecal compartment suggests that the granulosa cell layer and oocyte develop in an avascular environment and that the controlled O_2 and nutrient supply may be hindered within the developing follicle. Granulosa cells of follicles at any stage of folliculogenesis in the ovaries from the C57BL/6-Tg(HRE(4)-SV40-EGFP) mouse had no active EGFP present. HIF 1α protein could not be detected in granulosa cells by immunohistochemistry. However, studies have reported that FSH promotes HIF 1α abundance to mediate hypoxia responsive genes in cultured rat granulosa cells during follicular development (Alam *et al.*, 2004), and this stimulates *Vegfa*

expression via the PI3K/Akt pathway (Alam *et al.*, 2008). This study investigated the roles of low oxygen, hypoxic mimetics ($CoCl_2$ and DP) and FSH on HIF stabilisation and HIF mediated gene expression using an *in vitro* murine granulosa cell system. The results demonstrate that HIF 1α protein levels in cultured murine granulosa cells under hyperoxic conditions (20% O_2) are undetectable, even in the presence of FSH, suggesting that the hyperoxic conditions completely degrade HIF 1α protein and FSH does not increase either protein production or stabilisation. This contradicts earlier work utilising rat granulosa cells (Alam *et al.*, 2004). On the other hand, HIF 1α protein is readily detectable in mouse granulosa cells under low oxygen conditions or in the presence of hypoxic mimetic (DP or $CoCl_2$), but there was no further interactive effect of FSH with these treatments on HIF 1α induction (Figure 3.5).

In the present study, another member of the HIF superfamily, HIF 2α, was examined to determine if it may be involved in folliculogenesis, as it has been shown that HIF 1α and HIF 2α can function as indistinguishable transcription factors known to regulate many of the same target genes (Tian *et al.*, 1997). However, HIF 2α protein could not be detected by Western blot analyses in granulosa cells, even under HIF stabilising conditions (Figure 3.6). It must be taken into consideration that although HIF 2α was detected in HepG2 cells under low oxygen and DP, a suitable positive control to confirm that the antibody would detect mouse HIF 2α was not available. Studies have shown that fibroblast-like cells treated with hypoxic mimetics can induce HIF 2α (Haase, 2006) and HIF 2α is primarily localised in the cytoplasm of mouse embryonic fetal fibroblasts (MEFs) regardless of oxygenation (Park *et al.*, 2003). It has been suggested that utilising liver nuclear extracts from anaemic mice would be a suitable candidate for a positive HIF 2α mouse control (Volker Hasse, personal communication). However, the inability to stabilise HIF 2α is not surprising as studies have shown that HIF 2α expression is up-regulated during chronic exposure, whereas acute exposure to hypoxia induces HIF 1α (Holmquist-Mengelbier *et al.*, 2006; Forristal *et al.*, 2009). Further studies are required to optimise Western blots of HIF 2α in mouse tissues to confirm the presence or absence of HIF 2α from cultured granulosa cells.

As expected, there were alterations in the expression of HIF target genes in cultured granulosa cells following an acute exposure to $CoCl_2$. Quantification of mRNA expression in granulosa cells revealed that *Vegfa*, *Slc2a1* and *Ldha* mRNA abundances significantly increased following exposure to the hypoxic mimetic (Figure 3.7,A - C; $P < 0.005$), compared with granulosa cells cultured in the absence or presence of rhFSH. $CoCl_2$ stimulated expression of *Vegfa*, *Slc2a1* and *Ldha* mRNA abundance in rhFSH stimulated cells compared to those treated with rhFSH alone. There was no further interactive effects on gene expression when granulosa cells were cultured in the presence of both rhFSH and $CoCl_2$ when compared to cells treated only with $CoCl_2$ (Figure 3.7, A - C). The gene expression data presented is consistent in that hypoxic mimetics can up regulate HIF responsive genes but on the other hand contradicts the study by Alam and colleagues who demonstrated that FSH can up regulate hypoxia responsive genes in cultured rat granulosa cells (Alam *et al.*, 2004).

Because results demonstrated no observable effect of HIF 1α protein from rhFSH treatment, it was necessary to determine if rhFSH used in this study was biologically active. Real time RT-PCR analyses of *Lhcgr* mRNA abundance demonstrated FSH-responsiveness by granulosa cells (Figure 3.3.7D; $P \leq 0.006$). Interestingly, *eGfp* mRNA abundance was not altered across all treatments, even in the presence of $CoCl_2$ (Figure 3.3.7E; $P > 0.05$). This result was surprising, and suggests that the EGFP construct is less sensitive to acute exposures, or less responsive to hypoxia-mediated gene induction in the absence of further co-factors.

Nevertheless, results presented in this study contradict work by Alam *et al.,* (2004), who demonstrated FSH was able up regulate HIF target genes such as *Vegfa* in cultured granulosa cells. However, HIF 1α protein stabilisation observed in their rat FSH-treated granulosa cell culture were cultured in combination with a hypoxic mimetic or a HIF degradation inhibitor and therefore may not reflect accurately the conditions *in vivo*.

In conclusion, the results demonstrate that within granulosa cells, HIF 1α protein can be stabilised by hypoxia and hypoxic mimetics, but there was no effect of FSH on HIF induction nor its target genes. Furthermore, and most surprisingly, there appears little evidence of significant HIF activity during folliculogenesis in adult mice. This contradicts the body of evidence which suggests strongly that follicle development should involve oxygen-regulated signalling, which are best characterised by the HIF transcription family. In contrast, EGFP was readily observed in corpora lutea in naturally cycling mice. This observation led me to subsequently investigate the role of HIFs, especially HIF 1α, during follicle differentiation to corpus luteum (CL) formation.

CHAPTER 4 The role of hypoxia inducible factors in follicle differentiation and luteinisation.

4.1 Introduction

In the previous chapter, it was demonstrated that HIF 1α protein abundance in cultured granulosa cells increased under low oxygen or when exposed to a hypoxic mimetic but FSH had no effect on either HIF 1α protein abundance or HIF 1α target genes. Furthermore, histological examinations of fluorescent EGFP expression in C57BL/6-Tg(HRE(4)-SV40-EGFP) mice demonstrated that EGFP was absent in follicles prior to antrum formation. However, upon antrum formation, EGFP localisation was present amongst cells within the thecal layer, indicating that HIF activated gene expression is restricted to theca during folliculogenesis. In contrast, EGFP was readily observed in many corpora lutea of naturally cycling transgenic mice. Nevertheless, and interestingly, EGFP was absent in some CLs. The evidence presented in Chapter 3 suggests that HIF may not participate, or plays only a minor role during the early stages of folliculogenesis, however, may have a role during follicle differentiation and luteinisation.

As previously mentioned in Chapter 1, pituitary-secreted gonadotropin hormones such as FSH and LH control the ovarian follicular cycle. Follicle differentiation and ovulation occurs post LH surge. After ovulation, the follicle differentiates into an endocrine structure known as the corpus luteum which is the primary source for progesterone production. It is well established that the LH surge causes final maturation of the oocyte and induces follicular ovulation and luteinisation (CL formation) and can be mimicked within responsive follicles by exogenous administration of human chorionic gonadotropin (hCG) (Pierce & Parsons, 1981).

After ovulation, angiogenesis becomes more extensive, in association with the process of CL development (Redmer & Reynolds, 1996; Fraser & Duncan, 2009). Earlier studies demonstrated little change in blood flow in CLs during early pregnancy; however, the CL undergoes a major growth phase over day 10 – 16 of gestation in rats and ovarian blood flow increases at mid-pregnancy (Meyer & Bruce, 1979; Bruce *et al.*, 1984). Other studies performed in marmoset monkeys have established that VEGF is essential during luteal

angiogenesis. A VEGF immunoneutralisation technique resulted in a significant decrease in endothelial cell proliferation and the same study also showed that anti-VEGF treatment caused a significant reduction in plasma progesterone which is an indicator of reduced luteal function (Dickson *et al.*, 2001). The integrity of the CL is dependent on VEGF and its receptors (Zimmermann *et al.*, 2001) and VEGF protein is only down-regulated after initiation of luteolysis (Dickson *et al.*, 2001).

There are suggestions of similarities between a 'hypoxic' periovulatory follicle and subsequent CL formation to tumours, in that they may have a hypoxic core and require rapid angiogenesis (Neeman *et al.*, 1997; Semenza, 2002). The oxygen pressure within solid tumours varies from 5% O_2 in well vascularised regions to hypoxia and anoxia in regions often surrounding areas of necrosis (Brown & Wilson, 2004) and it has been well described that it is this insufficiency of O_2 supply within tumours which regulates HIFs (Semenza, 2003; Hopfl *et al.*, 2004). Furthermore, it has been well established that under low oxygen environments, HIF-induced transcription factors are the main regulators in up-regulating the angiogenic factor, VEGF.

A most recent publication has demonstrated that progesterone receptor (PR), a mediator of the ovulation cascade (Robker *et al.*, 2000), is necessary for the expression of HIF and known HIF target genes such as *Edn2* and *Vegfa* in granulosa cells of the preovulatory follicle (Boonyaprakob *et al.*, 2005; Duncan *et al.*, 2008; Na *et al.*, 2008; Kim *et al.*, 2009). In addition, earlier work demonstrated that hCG can up-regulate *HIF1α* mRNA (van den Driesche *et al.*, 2008) and *HIF2α* mRNA (Herr *et al.*, 2004) in human luteinised granulosa cells *in vitro*. In addition, a further study has demonstrated that inhibition of VEGF can lead to the up-regulation of HIF 1α protein in primate granulosa cells and corpora lutea (Duncan *et al.*, 2008). Taken together, I hypothesise that low oxygen conditions as well as non-hypoxic stimuli regulate HIF during follicle differentiation and luteinisation. To test my hypotheses regarding the role of HIF in follicle differentiation and luteinisation, I have investigated the effects of hCG on granulosa cell cultures, in the presence and absence of low oxygen, on HIF 1α protein stabilisation and target gene activation. In addition, I assessed *in vivo*

expression of HIF 1α protein in ovarian follicles of hCG-stimulated mice, and used the C57BL/6-Tg(HRE(4)-SV40-EGFP) transgenic reporter mouse to assess HIF activity during follicle differentiation and corpus luteum formation. Lastly, as I previously mentioned that EGFP is absent in some CLs, I assessed the ratio of EGFP positive CLs to the total number of CLs and investigated the role of HIF 1α during CL development at early to mid stages of pregnancy.

4.2 Materials and methods specific to this chapter

Mouse ovarian stimulation, granulosa cell collection, cell cultures and western blotting procedures were performed as outlined in Chapter 2.

4.2.1 Animals

This study was approved by the Animal Ethics Committee of The University of Adelaide. C57BL/6-Tg(HRE(4)-SV40-EGFP) males and females were maintained at The University of Adelaide Medical School Animal House under PC2 conditions. Transgenic females were housed with transgenic males overnight and checked for the presence of vaginal copulatory plugs the following morning. Transgenic animals were approximately 4 - 9 months old. The morning that the vaginal plug was observed was designated as day 1 of pregnancy. Additional 6 week old non-transgenic C57BL/6 female mice were obtained on days 1, 4, 8 and 12 following mating (n = 3 per time point) and were also housed at The University of Adelaide Medical School Animal House. Non-transgenic mice were housed and cared for at The University of Adelaide Medical School Animal House, as outlined in Chapter 2.

4.2.2 Luteinised granulosa cell cultures

Ovaries were collected from eCG treated mice and granulosa cells were isolated by follicular puncture, as described in Section 2.4. Cells were washed once in HEPES 199 buffer, pH 7.5, supplemented with 3% bovine serum albumin (BSA), followed by 2 washes with 50% Dulbecco Modified Eagle medium (DMEM) and 50% Hams F12 with 29.2 mg/ml L-glutamine, 5 mg/ml penicillin/streptomycin, 10 ng/ml testosterone and 50 mIU rhFSH. Cells were incubated on serum coated 24-well plates at a density of approximately 5 x 10^5 cells per well at 37.5 ºC, 20% O_2 5% CO_2 in a humidified atmosphere. Twenty-four hours later, cells were treated with or without 10 IU hCG (Pregnyl, Organon, Oss, The Netherlands). The following day, granulosa lutein cells were incubated at either 20% O_2, 5% CO_2 and 75% N_2 or 2% O_2, 5% CO_2 and 83%

N_2 in modulator incubators for 4 h. Granulosa lutein cells were treated in the presence or absence of 250 µM $CoCl_2$ at 20% O_2, 5% CO_2 and 75% N_2. Cells were collected for Western blot analyses or for analyses of gene expression as described in Section 2.7 and 2.9 respectively.

4.2.3 Radio Immuno Assay (RIA) for progesterone hormone

Culture media were collected prior to harvesting granulosa lutein cells cultured for 48 h with or without hCG. Progesterone concentrations were determined using commercially available RIA kits (Cat # DSL 3400; Diagnostic Systems Laboratories Inc, Webster, TX, USA) following the manufacturers' protocol. Samples were grouped as with or without 10 IU hCG-treated granulosa cells as all samples contained FSH (n = 7). The sensitivity of the assay and the intra-assay CV were 0.10 ng/mL and 5.5% respectively.

4.2.4 Immunofluorescence & histochemistry

Histological analyses of C57BL/6-Tg(HRE(4)-SV40-EGFP) ovaries at each day of days 1 to 12 post copulation, obtained from 4 - 5 different mice per time point, were performed. Mice were killed by cervical dislocation. One ovary per mouse was collected and fixed in 4% (w/v) paraformaldehyde in PBS overnight, transferred to fresh PBS the following day for 24 h and finally transferred to 18% sucrose overnight prior to mounting in Tissue-Tek® O.C.T. Compound (Miles Inc., Elkhart, IN, USA) and stored at -20 ºC until sectioned. Ovaries were cut in 8 µm serial sections and fixed in 100% ethanol for 5 min at room temperature, followed by washing in PBS for 5 minutes twice prior to quantification analyses. The following section was stained with haematoxylin and eosin (H&E) for general morphology and to quantify total number of corpora lutea present. This method of quantification removes possible bias against fluorescence quantification.

4.2.5 *In vivo* ovarian analyses

C57BL/6 mice or transgenic C57BL/6-Tg(HRE(4)-SV40-EGFP) mice were injected with 5 IU eCG at 21-25 days of age. Forty-four hours post eCG, mice undergoing hCG stimulation received 5 IU hCG (Pregnyl; Organon, Oss, The Netherlands). Ovaries from C57BL/6 mice were collected for protein extraction at 44 h post eCG and at 4, 8, 12, 16 and 24 h post hCG. Granulosa cells isolated by follicle puncture and the residual ovaries were collected for Western blot analyses (n = 3 animals per time point). Whole ovaries from C57BL/6-Tg(HRE(4)-SV40-EGFP) collected at times 44 h post eCG, 12 and 24 h post hCG were snap-frozen for RNA extraction (n ≤ 4 - 5 animals per time point). Additional ovaries from prepubertal transgenic mice were collected for fluorescence localisation at times 44 h post eCG, 4, 8, 12, 16 and 24 h post hCG (n ≤ 4 - 5 animals per time point).

4.2.6 Protein extraction from whole ovary or residual ovary

Residual or whole ovaries were homogenized using ReadyPrep™ Mini Grinders (Cat #163-2146; Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. In brief, the supplied grinding tubes containing grinding resin in solution (the grinding resin is a neutral abrasive material made of a high-tensile microparticle and does not bind with protein or damage high molecular weight proteins) were centrifuged at 20,000 rpm for 20 sec, and the supernatant removed with a pipette. Either residual (following granulosa cells collection) or whole ovary was added to the tube containing the grinding resin. Approximately 150 – 250 µl of RIPA buffer (Sigma) and Protease Inhibitor cocktail (1:100; Sigma) were added to the tube. Samples were homogenised within the tube with the supplied pestle. Tubes containing the homogenate were centrifuged at 20 000 rpm for 30 min at room temperature to pellet the resin and cellular debris. The supernatant was then transferred to a new Eppendorf tube without disturbing the pellet. The protein concentration was determined by the Bradford method (Bradford, 1976).

4.2.7 Quantitative Real Time RT- PCR

RNA extraction and reverse transcription were performed as outlined in Section 2.9. Quantitative Real Time RT-PCR was performed using the ABI PRISM 5700® sequence detection system (Applied Biosystems, Foster City, CA, USA) as outlined in Section 2.9. Primer sequences for *Star*, *Vegfa*, *Slc2a1*, *Hif1α*, *eGfp* are listed in Table 2.3. All gene expression analyses were performed using the relative standard curve method and results were normalised against *18S* rRNA housekeeping gene.

4.2.8 Statistical Analyses

All data are presented as mean ± S.E.M, except for corpora lutea quantification where data were presented as a percentage of positive EGFP CL against total number of CL. To analyse progesterone production from cultured granulosa cells, a Student's t-test was performed. For *in vitro* experiments, each treatment group was performed in triplicates and repeated on 3 occasions (n = 9 per treatment). For *in vivo* experiments, each time course experiment was repeated at least 3 times. Band densities from Western blot analyses conducted following the cell culture experiments were analysed by Kruskal-Wallis test followed by least-significant difference (LSD) pairwise comparison as a post-hoc test. After plotting the mean ± SEM data, an analysis of variance fitting a polynomial model was used to assess the relationship of band densities from Western blot analyses over time after hCG treatment for both the *in vivo* residual ovaries and granulosa cells. Gene expression results were analysed by one-way analysis of variance and LSD unless the significance of Levene's test ≤ 0.05. In these cases, a non-parametric Kruskal-Wallis analyses followed by post-hoc Mann-Whitney test was used. The data are presented as the mean ± S.E.M. *P* values of ≤ 0.05 compared to appropriate control were regarded as statistically significant. Statistical analyses were carried using SPSS software for Windows, version 14.0 (Chicago, IL, USA).

4.3 Results

4.3.1 Effect of hCG on granulosa cell cultures.

To determine the effect of hCG on granulosa cell cultures, cells were cultured in the presence or absence of hCG after 24 h of incubation. Images were taken for validation of hCG effects on granulosa cell morphology and progesterone concentrations were measured for validation of the luteinisation transformation within granulosa cells. After 24 h, granulosa cells without the addition of hCG had a typical plated granulosa cell phenotype, which consists of long spindle like morphology (Figure 4.1A). Not surprisingly, granulosa cells cultured in the presence of hCG after 46 h altered the granulosa cell phenotype to a luteinised granulosa cell which displays a morphologically more flattened, fibroblast-like phenotype (Figure 4.1B). After the addition of 250 μM $CoCl_2$ for 4 h, luteinised granulosa cells were not morphologically altered in appearance (Figure 4.1, C and D). Progesterone concentration in culture media of luteinised granulosa cells (n = 7) increased following exposure to hCG when compared to control (Figure 4.2; $P \leq 0.001$).

4.3.2 **HIF 1α protein in** luteinised granulosa cells *in vitro*.

To determine if HIF 1α protein was present in luteinised granulosa cells *in vitro*, cells (n = 3 replicates) were lysed for Western blot analyses. hCG alone did not promote HIF 1α protein accumulation in cultured luteinised granulosa cells (Figure 4.3). When cells were exposed to 250 μM $CoCl_2$, an increase in HIF 1α protein was observed (Figure 4.3, B and D). In contrast, exposure to 2% oxygen did not induce an increase in HIF 1α protein accumulation in granulosa lutein cells (Figure 4.3, A and C). However, HIF 1α protein increased when hCG was added in either the presence of 2% oxygen (Figure 4.3, A and C; $P \leq 0.05$) or 250 μM $CoCl_2$ (Figure 4.3, B and D; $P \leq 0.05$).

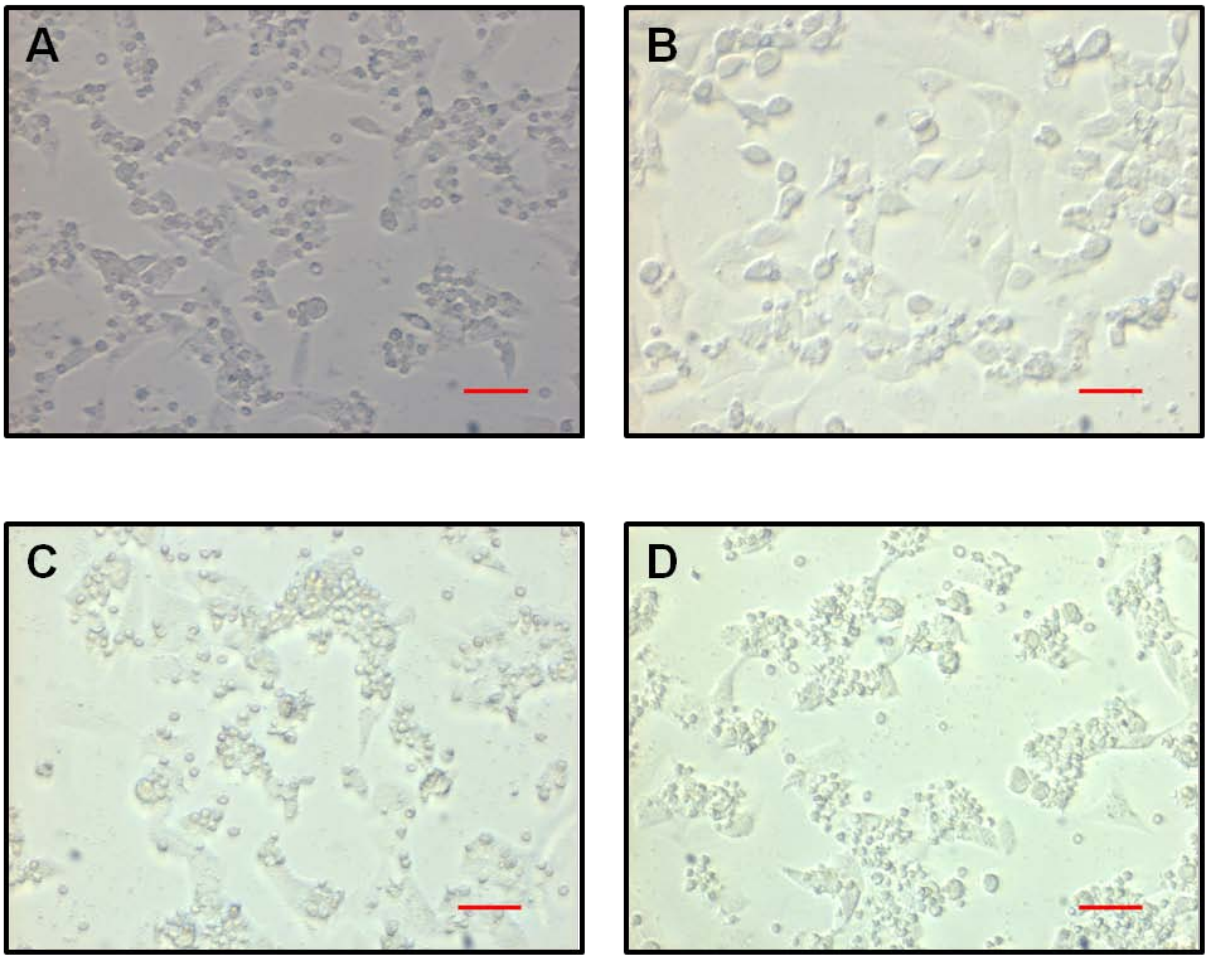

Figure 4.1 Luteinisation of granulosa cells *in vitro*.

Panels A – D, 21-23 day old C57BL6 mice were treated with 7.5 IU eCG and 46-48 h later, granulosa cells were collected and cultured to lutein cells as described in Section 4.2.2. Magnification – 200x, Scale bar = 50 µm.

Panel A: granulosa cells 24 h after collection exhibited a long spindle like morphology.

Panel B: granulosa cells were incubated in the presence of 10 IU hCG and 46 h later, exhibited a more flattened, fibroblast-like morphology, phenotypic of a granulosa lutein cell.

Panel C: granulosa cells were incubated for 50 h without hCG (control).

Panel D: granulosa lutein cells following 46 h hCG treatment + 4 h 250 µM $CoCl_2$.

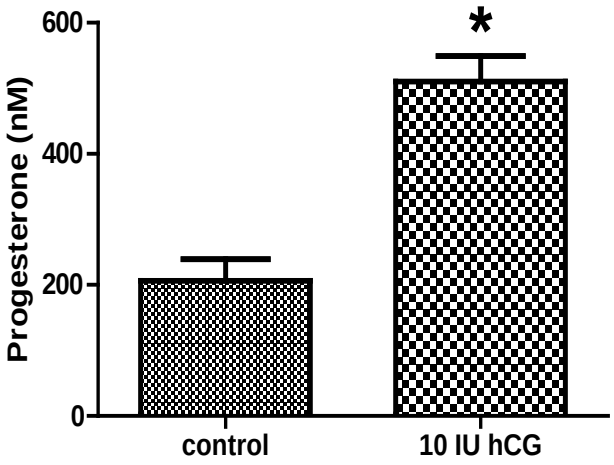

Figure 4.2 Progesterone production from cultured granulosa cells.

Progesterone concentrations in culture media from granulosa cells treated with or without 10 IU hCG. After 48 h of culture, hCG-induced luteinisation of granulosa cells *in vitro* induced a significant increase of progesterone production above control levels ($P \leq 0.001$). The data is shown as mean ± S.E.M (n = 7 per treatment).

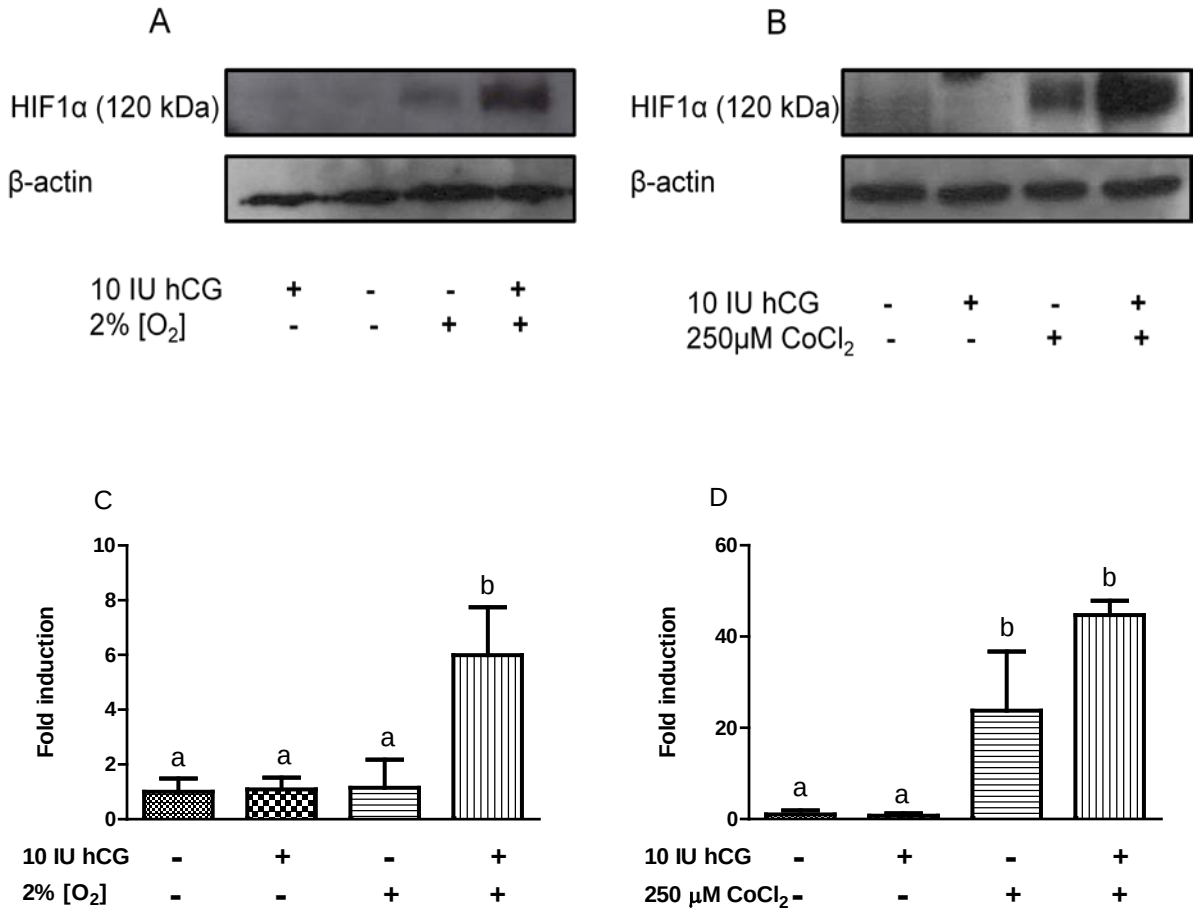

Figure 4.3 HIF 1α protein expression in granulosa lutein cells *in vitro.*

Panel A, Western blot of HIF 1α (molecular weight of ~ 120 kDa) protein expression in granulosa lutein cells as described in Section 4.2.2. Cells were incubated in the absence or presence of 10 IU hCG overnight and exposed to 20% oxygen or 2% oxygen for 4 h (n = 3). In panel B, Western blot of HIF 1α (molecular weight of ~ 120 kDa) protein expression in granulosa lutein cells cultured with and without 10 IU hCG overnight and incubated in the presence or absence of 250 μM $CoCl_2$ for 4 h (n = 3). Figure 4.3; C and D are representative of total quantitative densitometric analyses of 3 replicates of Figure 4.3; A and B, respectively. Data are presented as fold induction relative to control and results were normalised against β-actin. Results were analysed using Kruskal-Wallis followed by Mann-Whitney post-hoc test ($P \leq 0.05$ denoted by different superscripts).

4.3.3 Gene expression analyses in luteinised granulosa cells *in vitro*.

To determine the effect of low oxygen or 250 µM $CoCl_2$ on expression of well characterised HIF 1α target genes, *Vegfa* and *Slc2a1,* in cultured luteinised granulosa cells, quantitative Real Time RT-PCR was performed on mRNA collected from these cells. Samples were collected in triplicates on 3 separate occasions and results were normalised against *18S* rRNA. *Star* mRNA was analysed as another indicator of luteinisation in granulosa cells following stimulation with 10 IU hCG. *Hif1α* and *eGfp* mRNA were both also measured in luteinised granulosa cells cultured with and without 10 IU hCG and incubated in the presence or absence of 250 µM $CoCl_2$. There was no effect of low oxygen (2%) or $CoCl_2$ on *Star* expression in luteinised granulosa cells (Figure 4.4A and 4.5A). hCG increased the expression of *Vegfa* (Figure 4.5B; $P \leq 0.05$), although this effect was not consistent across experiments (Figure 4.4B). hCG alone also had no effect on *Slc2a1* mRNA expression (Figure 4.4C and 4.5C). Exposure of luteinised granulosa cells to low oxygen (2%) or to $CoCl_2$ increased *Vegfa* expression (~3-fold and 6-fold, respectively when compared to control) (Figure 4.4B and 4.5B; $P \leq 0.05$). Exposing luteinised granulosa cells to low oxygen or to $CoCl_2$ also increased *Slc2a1* expression (~4- and 19-fold when compared to control) (Figure 4.4C and 4.5C; $P \leq 0.05$). However, no further increase was observed in *Vegfa* or *Slc2a1* mRNA when hCG was used in synergy with low oxygen or $CoCl_2$.

hCG treatment increased the abundance of *Hif1α* mRNA in granulosa lutein cells (Figure 4.5D; $P \leq 0.05$). In contrast, $CoCl_2$ treatment did not alter *Hif1α* mRNA. However, addition of $CoCl_2$ to hCG-treated granulosa lutein cells reduced *Hif1α* levels, when compared to cells treated with hCG alone (Figure 4.5D; $P \leq 0.05$). Interestingly, hCG alone, or addition of $CoCl_2$, did not alter *eGfp* mRNA in granulosa lutein cells when compared to control. However, there was an increase in *eGfp* mRNA abundance observed when hCG was used in synergy with $CoCl_2$ when compared to control (Figure 4.5E; $P \leq 0.05$).

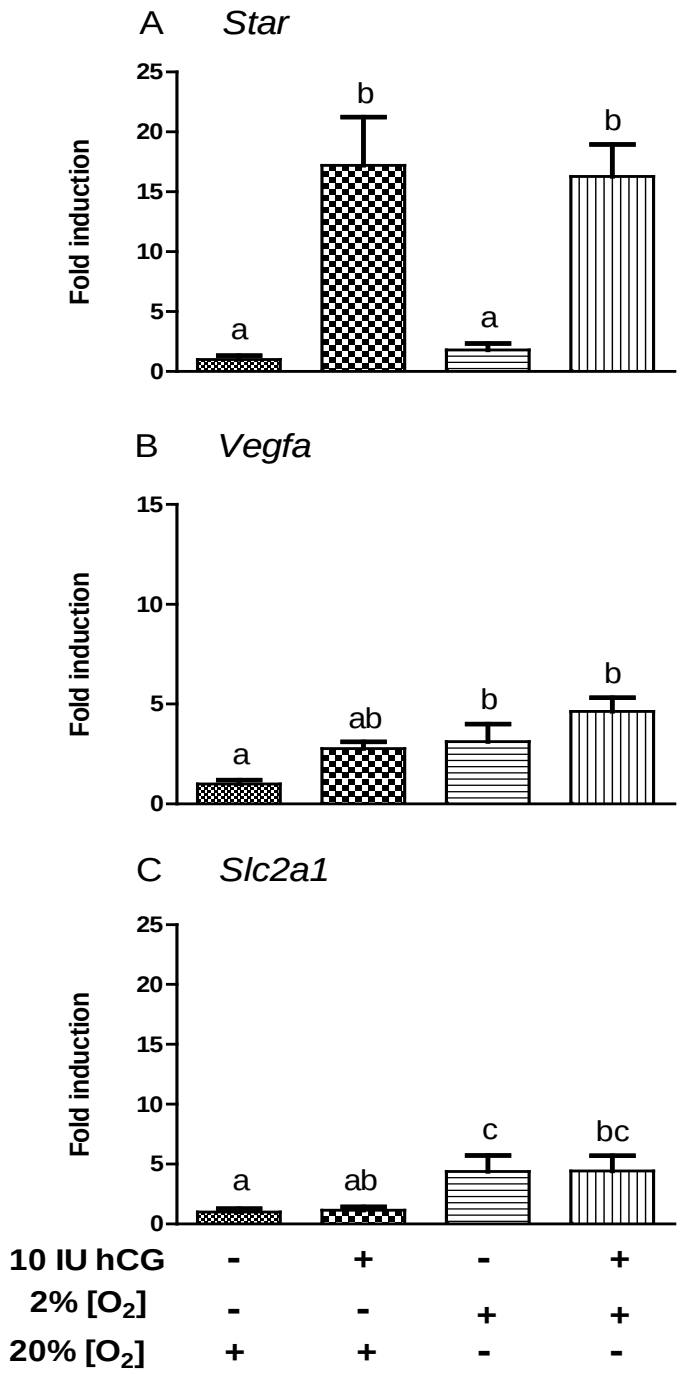

Figure 4.4 Effect of low oxygen on induction of HIF target genes in hCG induced granulosa lutein cells *in vitro*.

In panels A, B and C, luteinised granulosa cells were cultured overnight in the absence or presence of 10 IU hCG and exposed to 20% oxygen or 2% oxygen for 4 h. Quantitative Real Time RT-PCR was performed to determine expression of *Star* mRNA, *Vegfa* mRNA, *Slc2a1* mRNA and results were normalised against *18S* rRNA. Data are presented as fold induction relative to control. Data is expressed as mean ± S.E.M of triplicate measures and is representative of 3 separate experiments. Results were analysed using Kruskal-Wallis followed by Mann-Whitney post-hoc test ($P \leq 0.05$ denoted by different superscripts).

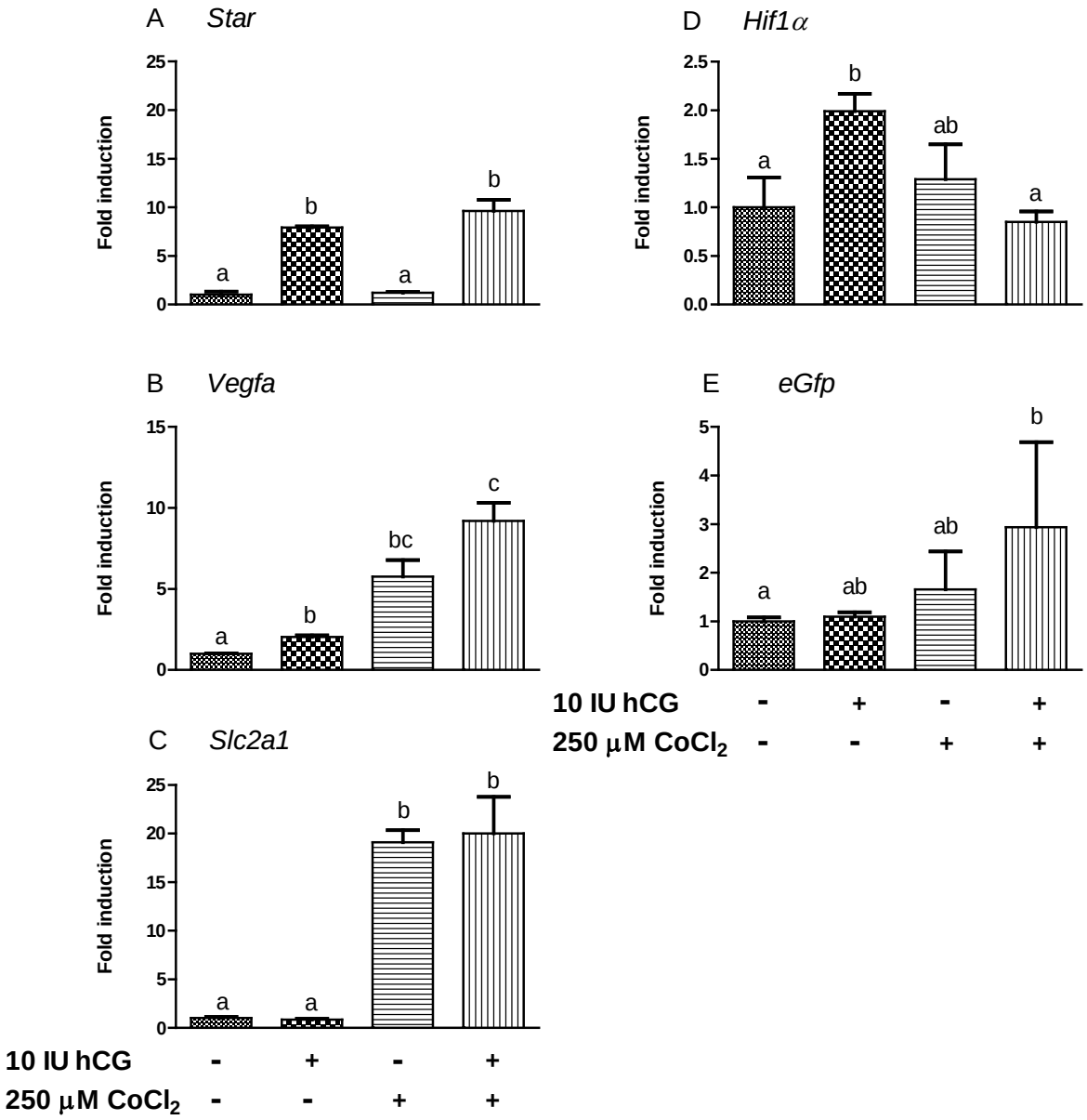

Figure 4.5 Effect of hypoxic mimetic – cobalt chloride on induction of HIF target genes in hCG induced granulosa lutein cells *in vitro*.

In panel A - E, luteinised granulosa cells were cultured with and without 10 IU hCG overnight and incubated in the presence or absence of 250 μM $CoCl_2$ for 4 h. Real Time RT-PCR was performed to determine expression of *Star* mRNA, *Vegfa* mRNA, *Slc2a1* mRNA and *Hif1α* mRNA. Luteinised granulosa cells from gonadotropin-primed prepubertal C57BL/6-Tg(HRE(4)-SV40-EGFP) mice were used to determine the expression of *eGfp* mRNA. All gene expression results were normalised against *18S* rRNA. Data are presented as fold induction relative to control. Data is expressed as mean ± S.E.M of triplicate measures and is representative of 3 separate experiments. Results were analysed using Kruskal-Wallis followed by Mann-Whitney post-hoc test ($P \leq 0.05$ denoted by different superscripts).

4.3.4 **HIF 1α protein** in granulosa cells and residual ovary following hCG stimulation *in vivo*.

Next, to determine the effect of hCG stimulation in granulosa cells and residual ovary *in vivo*, prepubertal C57BL6 mice (n = 3 per time point) were treated with 5 IU hCG 46 h post eCG. Protein was collected from granulosa cells and residual ovary at various time points at 44 h post eCG, 4, 8, 12, 16 and 24 h post hCG. HIF 1α protein in granulosa cells decreased 4 h post hCG. HIF 1α then increased with a maximal stabilisation at 12 h, approximately when ovulation occurs (Figure 4.6C; $P \leq 0.02$). Although, HIF 1α appears to be increasing in residual ovary post hCG (Figure 4.6B), densitometric analyses of residual ovary HIF 1α did not show a significant difference over time (Figure 4.6D; $P = 0.345$).

4.3.5 HRE-EGFP localisation post hCG in ovarian sections of C57BL/6-Tg(HRE(4)-SV40-EGFP).

As the above results demonstrated that HIF 1α can be stabilised post hCG, further work was performed to determine if hCG stimulation in transgenic mice would increase EGFP *in vivo*. Fluorescent localisation of EGFP expression was examined in sections of ovary from gonadotropin stimulated prepubertal C57BL/6-Tg(HRE(4)-SV40-EGFP) mice. Prepubertal transgenic mice were injected with eCG to stimulate follicle development, and 46 h later injected with hCG to induce ovulation. EGFP was restricted to thecal cells at 44 h post eCG (Figure 4.7A). However, 4-12 h post hCG, EGFP fluorescence was primarily localised in granulosa layers of luteinising ovarian follicles (Figure 4.7; B - D), with maximal EGFP fluorescent intensity at 12 h post hCG (Figure 4.7D). Interestingly, EGFP localisation within the granulosa cell layer decreased at 16 h post hCG (Figure 4.7E) however, 24 h post hCG, EGFP localisation was evident in the early corpus luteum (Figure 4.7F).

4.3.6 Gene expression in HRE-EGFP ovaries post hCG.

To determine whether hCG stimulated HIF 1α induced transcriptional activity *in vivo*, ovaries were collected from C57BL/6-Tg(HRE(4)-SV40-EGFP) at 44 h post eCG, and 12 h and 24 h post hCG and the expression of *eGfp*, *Vegfa* and *Slc2a1* was measured. *eGfp* mRNA abundance in the ovary was increased by 5.8 fold, 12 h post hCG stimulation when compared to pre-hCG treated mice ($P \leq 0.05$). *eGfp* mRNA levels returned to baseline at 24 h post hCG (Figure 4.8A; $P \leq 0.05$). There was an increase in *Vegfa* mRNA levels 12 h post hCG (Figure 4.8B; $P \leq 0.05$). However, *Slc2a1* expression in the ovary was not altered following hCG treatment *in vivo* (Figure 4.8C).

4.3.7 EGFP expression in corpora lutea post - copulation *in vivo*.

As previously described in Chapter 3, EGFP is readily expressed in corpora lutea but this expression was not evident in every CL (Figure 4.9). The numbers of corpora lutea that were positive for EGFP were quantified during various stages of pregnancy, days 1, 4, 8 and 12 post - copulation (Table 4.1). Interestingly, there was an increase in the percentage of CLs that were positive for EGFP from post - copulated day 4 'early pregnancy' CLs compared to post - copulated day 12 'mid-pregnancy' CLs (Table 4.1; $P \leq 0.05$).

4.3.8 **HIF 1α protein in whole ovary post** - copulation.

To determine whether HIF 1α protein increased in the ovary at days 1, 4, 8 and 12 post - copulation, ovaries were collected (n = 3 per time point) and HIF 1α protein was measured by Western blot. HIF 1α protein abundance in the whole ovary was maintained across the different stages of pregnancy (Figure 4.10).

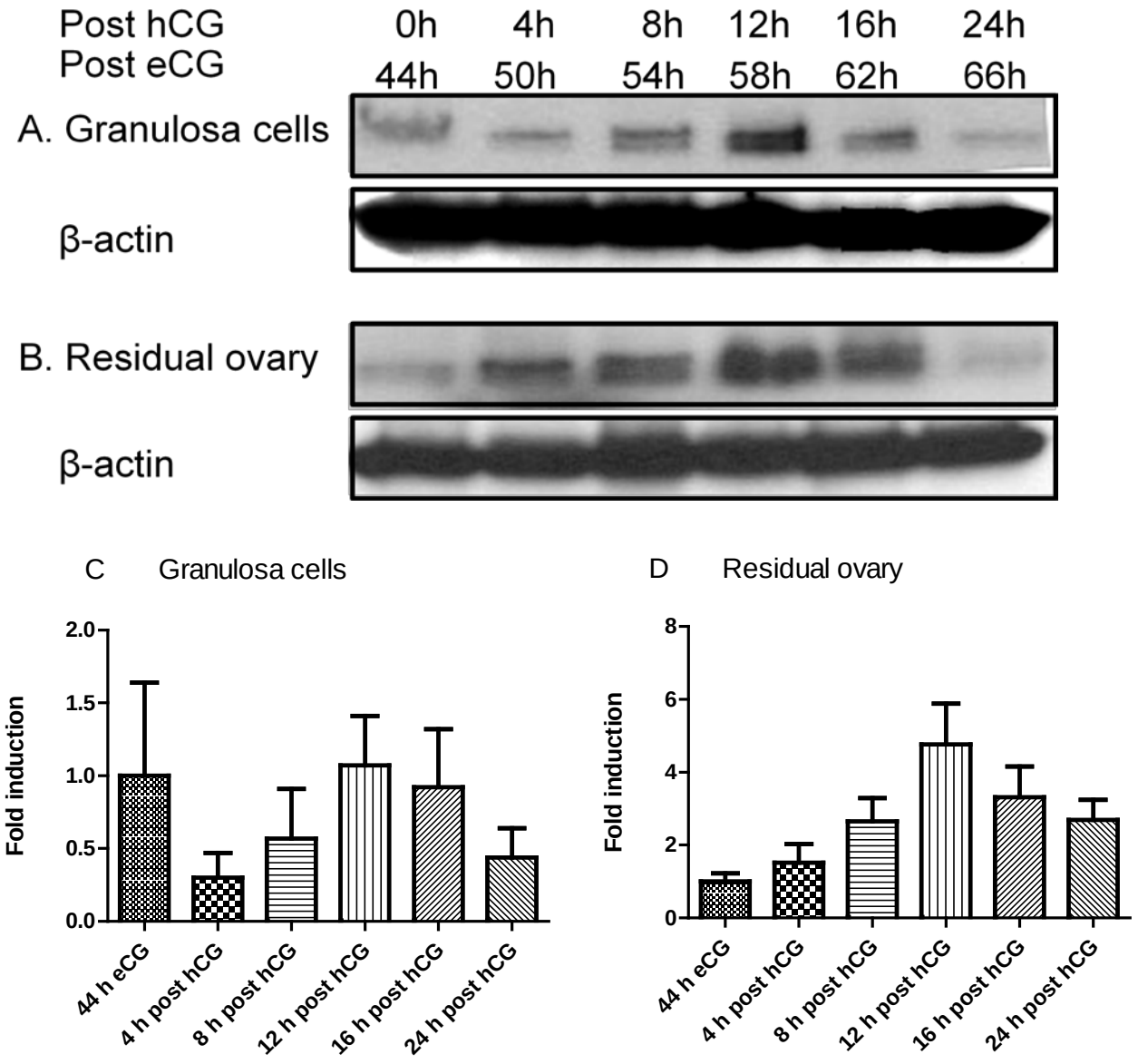

Figure 4.6 **Western blot of HIF1α protein in granulosa cells and residual ovary following hCG** stimulation *in vivo*.

Prepubertal C57BL/6 mice (n = 3 animals per time point) were treated with 5 IU hCG 46 h post eCG. Ovaries and granulosa cells were collected at times indicated, 50 µg of granulosa whole cell (Figure 4.6A) protein extracts and 100 µg of residual ovarian (Figure 4.6B) protein total cell extracts were separated by SDS-PAGE and blotted onto nitrocellulose, and membranes were incubated with an antibody to HIF 1α as described in Section 2.7. β-actin was used as a loading control. Figure 4.6, C and D are representative of densitometric analyses (mean ± SEM) of Western blotting in granulosa cells and residual ovarian stroma respectively. One representative experiment of 3 independent replicates. Results were analysed using univariate quadratic effect over 4 h post hCG. All samples were normalised to 44 h eCG (P = 0.015 for granulosa cells and P = 0.345 for residual ovarian stroma).

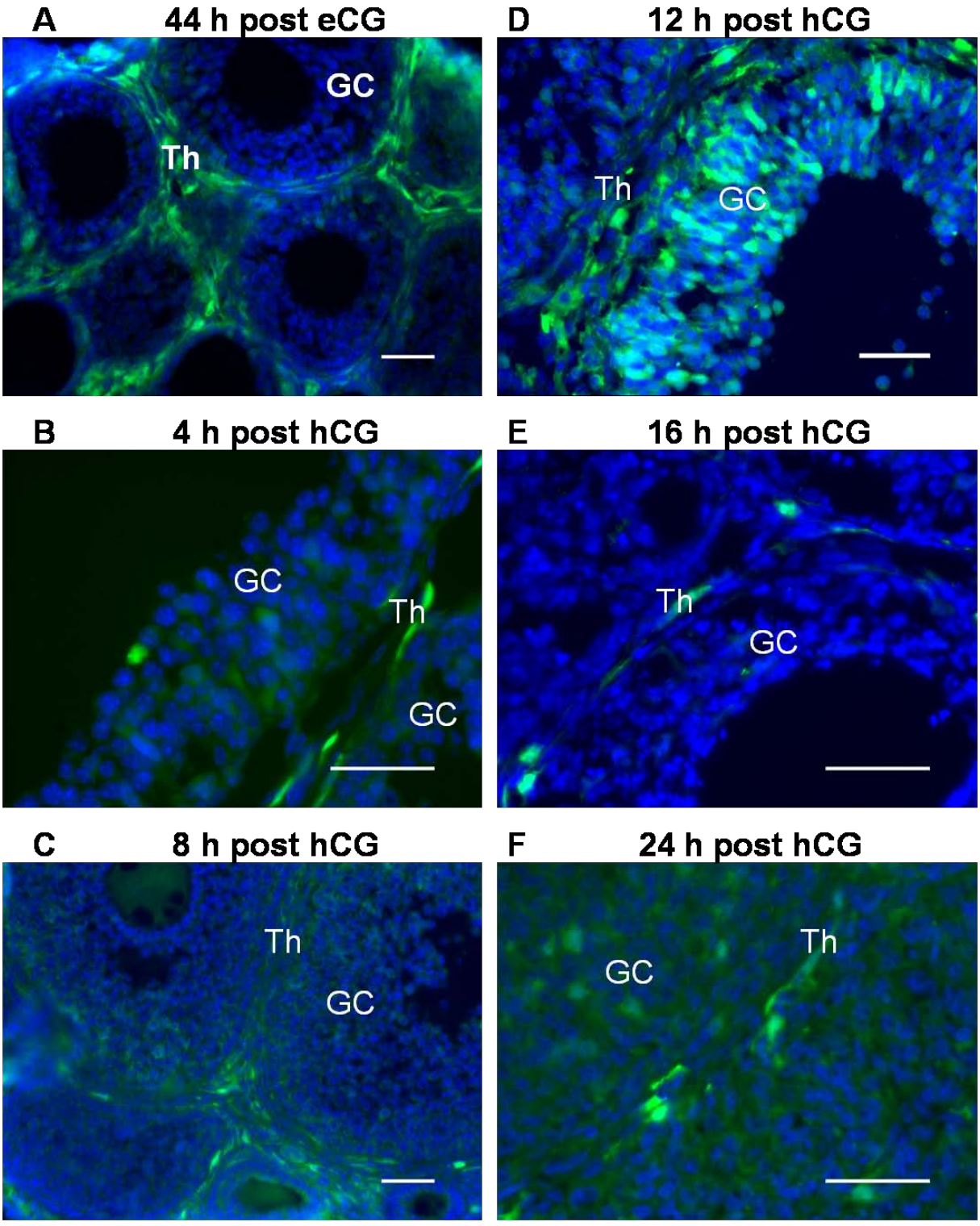

Figure 4.7 EGFP protein localisation during the periovulatory period in ovaries from gonadotropin-primed prepubertal C57BL/6-Tg(HRE(4)-SV40-EGFP) mice.

GC, granulosa cell layer; Th, Theca cell layer. EGFP (green) and DAPI (nuclear staining-blue). Panel A, C and D – 200x magnification, B, E and F – 400x magnification. Scale bar = 50µm.

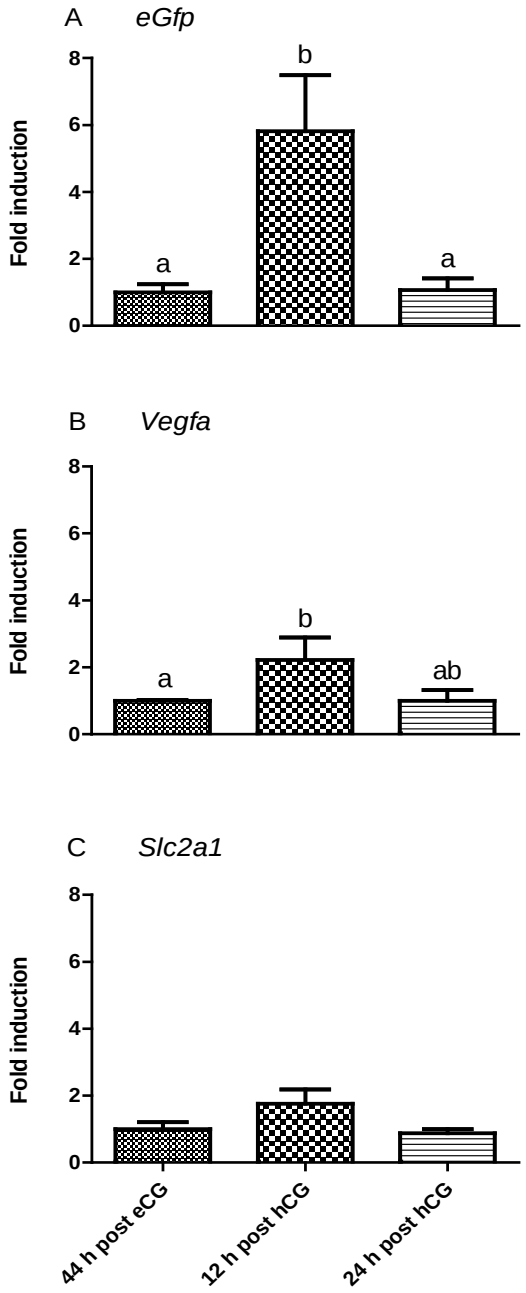

Figure 4.8 hCG treatment increases *eGfp* mRNA.

In panels A, B and C, prepubertal C57BL/6-Tg(HRE(4)-SV40-EGFP) mice were treated with 5 IU hCG at indicated times after the initial administration of 5 IU eCG. Ovaries were collected as described in Section 4.2.5. Quantitative Real Time RT-PCR was performed to determine expression of *eGfp* mRNA, *Vegfa* mRNA and *Slc2a1* mRNA. Results are normalised against *18S* rRNA, and expressed as a fold change relative to the 44h post eCG time point. Data represent mean ± S.E.M of n = 4 animals per time point. Results were analysed using one-way analyses of variance and comparisons were made by LSD pairwise comparison ($P \leq 0.05$ denoted with different superscripts denotes significance).

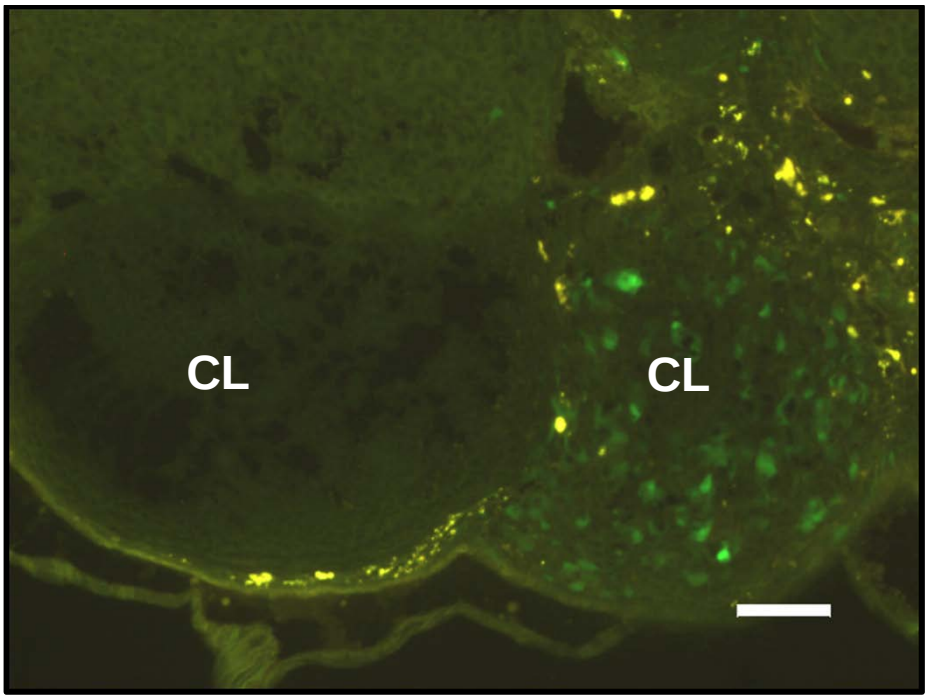

Figure 4.9 EGFP localisation in corpus luteum (CL) of C57BL/6-Tg(HRE(4)-SV-40-EGFP) mice. Ovaries were observed to contain CLs both positive and negative for EGFP, as illustrated in this ovary collected on days 4 post-copulation. 200x magnification; scale bar = 50 µm.

Table 4.1: Corpora lutea quantification in naturally mated C57BL/6-Tg(HRE(4)-SV40-EGFP) mice.

Days post – copulation	Total CL	Total positive CL	% EGFP positive	Animals (n)
1	33	16	43	5
4	32	11	34[a]	5
8	17	9	53	4
12	32	20	67[b]	5

Ovaries were collected from naturally mated transgenic mice. Table represents histological quantification of total number of corpora lutea that were positive or negative for EGFP.

[a,b] Values with different superscripts are significantly different ($P \leq 0.05$).

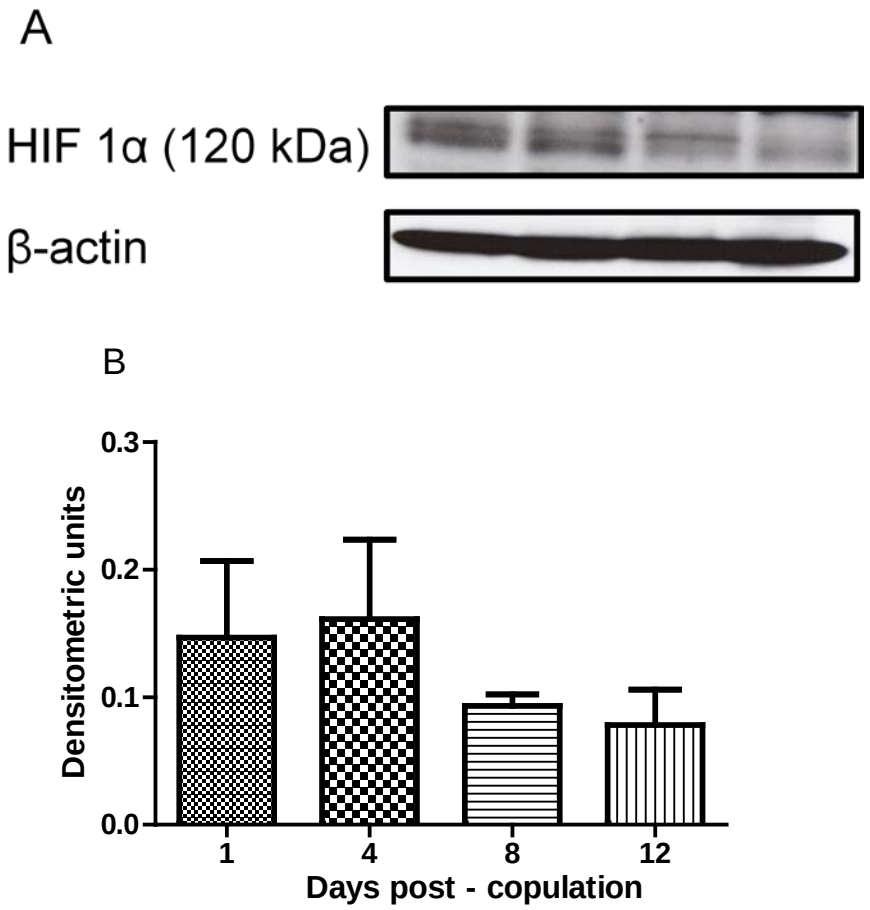

Figure 4.10 **Western blot of HIF1α protein** in whole ovaries collected at days 1, 4, 8 and 12 post – copulation.

6 – 8 week old C57BL/6 mice (n = 3 animals per time point) were mated with fertile males and ovaries were collected at times indicated, 50 µg of ovarian whole cell protein extracts were separated by SDS-PAGE and blotted onto nitrocellulose, and membranes were incubated with an antibody to HIF 1α as described in Section 2.7. β-actin was used as a loading control. Figure 4.10B is representative of densitometric analyses of Western blotting. One representative experiment of 3 independent replicates. Results were analysed using non-parametric Kruskal-Wallis test followed by Mann-Whitney post-hoc test for significance.

4.4 Discussion

The ovarian follicular vasculature is restricted to the thecal compartment, thus the granulosa cell layer and oocyte develop in an avascular environment, suggesting that the controlled O_2 and nutrient supply may be important within the developing follicle. After ovulation, the basement membrane breaks down prior to the rupture of the follicular wall and the theca and remaining granulosa cells reorganise to form the corpus luteum of pregnancy.

Results reported within Chapter 3 were unable to demonstrate a role for HIF, in particular HIF 1α, during the early stages of folliculogenesis; however, it has been recently reported that hCG can up-regulate *Hif1α* mRNA abundance to mediate hypoxia responsive genes during ovulation (Kim *et al.*, 2009). This study investigated the effects of low oxygen and hCG on HIF stabilisation and HIF mediated gene expression in the ovary using *in vitro* and *in vivo* systems, which included a novel HIF-reporter mouse to examine HIF-activity during follicular differentiation through to subsequent CL development. Although hypoxia is the best characterised activator of HIF activity, recent evidence has shown that HIF expression can also be hormonally regulated (Alam *et al.*, 2004; Duncan *et al.*, 2008; van den Driesche *et al.*, 2008). The present study indicates that hCG stimulation is an important regulator of HIF 1α protein abundance in the ovary. The data also support and extends recent work that demonstrates a role for HIF activity at the time of ovulation and CL formation (Duncan *et al.*, 2008; van den Driesche *et al.*, 2008; Kim *et al.*, 2009).

As previously mentioned, gonadotropins, such as FSH and LH, are important in regulating follicle differentiation and ovulation, and LH is also important for granulosa-lutein transformation (Fraser *et al.*, 1987). A study suggested that ovulatory signalling stimulates a HIF-mediated up-regulation of VEGF (Duncan *et al.*, 2008). Furthermore, hCG has been shown to increase *HIF1α* mRNA and *HIF2α* mRNA in human cultured luteinised granulosa cells (Herr *et al.*, 2004; van den Driesche *et al.*, 2008). Here, these

results show that hCG stimulated HIF 1α protein levels in luteinised granulosa cells, but only when stabilised in the presence of low oxygen or with a hypoxic mimetic.

HIF 1α protein accumulated in the residual ovary post hCG treatment, however, granulosa cells displayed a different pattern of induction, whereby HIF 1α actually showed a decrease in protein accumulation immediately after hCG treatment but then increased over time and stabilised around the time of ovulation (i.e. 12 h post-hCG). One possible explanation is that immediately prior to ovulation induction, the ovulatory follicle is highly hypoxic due to its size and as such, granulosa cell HIF 1α is stabilised. However, on hCG administration, a transient increase in blood flow to the follicle occurs (by approximately 150% within the first 2-4 h in humans but flow rate returns to normal by 8 h), thereby resolving this hypoxic state (Fischer *et al.*, 1992). Therefore, it is feasible that HIF 1α protein levels initially fall upon hCG stimulation. Another study demonstrated that hCG promotes HIF 1α protein accumulation, but this was in whole ovarian cell extracts (Kim *et al.*, 2009) and agree with my data on HIF 1α accumulation in residual ovarian tissue after granulosa cell retrieval. An increase in *Hif1α* mRNA was also observed following hCG stimulation of luteinised granulosa cells in the current study and these results are consistent with the studies which demonstrate that *HIF1α* mRNA can be up-regulated upon gonadotropin stimulation (Alam *et al.*, 2004; Herr *et al.*, 2004; van den Driesche *et al.*, 2008; Kim *et al.*, 2009). Regulation of HIF 1α at the level of transcription has been reported for other non hypoxic stimuli (Bilton & Booker, 2003). Increased HIF 1α protein levels following exposure to $CoCl_2$ occurred in the absence of any change in *Hif1α* mRNA, consistent with inhibition of proteasomal degradation being the primary mechanism of HIF regulation by the hypoxia mimetic. However, in luteinised granulosa cells exposed to both hCG and the hypoxia mimetic, *Hif1α* mRNA levels were not altered when compared to control. This suggests that the kinetics of HIF 1α protein activation by hCG differs under conditions where HIF 1α protein stabilisation is promoted, with a synergism between hCG and hypoxia operating at a post-transcriptional level.

HCG-treated granulosa cells from the transgenic reporter mouse were cultured in the presence of the hypoxia mimetic and were unable to visualise an increase in EGFP fluorescence in cultured luteinised granulosa cells. However, the expression of *eGfp* mRNA abundance increased when compared to control. This increase suggests that the process of luteinisation stabilises HIF 1α protein and the following *eGfp* expression occurs post hCG in combination with low oxygen. Next, using the transgenic mouse as a tool for visualisation of direct activation of HRE, EGFP fluorescence was observed in the ovary following the HIF 1α protein stabilisation pattern post-hCG. Furthermore, up-regulation of *eGfp* and *Vegfa* mRNA was observed by 12 h post-hCG, but had returned to pre-hCG levels by 24 h. This supports studies performed in primates where VEGF secretion into follicular fluid was up-regulated 6 - fold by hCG administration after 12 h (Hazzard *et al.*, 1999) indicating the ability for the up-regulation of HIF gene targets following the ovulatory cascade signal. The results are also consistent with studies that have reported hCG induced up-regulation of *Hif1α* mRNA in luteinised granulosa cells accompanied by a marked increase in *VEGF* mRNA (Laitinen *et al.*, 1997; van den Driesche *et al.*, 2008). It has been suggested that glucose transporters play a role in successful ovulation (Kol *et al.*, 1997; Zhou *et al.*, 2000). The present results demonstrated that *Slc2a1* mRNA expression did not increase following hCG stimulation either *in vitro* or *in vivo*. Others have also reported a statistically insignificant 2 - fold increase in *Slc2a1* mRNA in the rat ovary following hCG stimulation and the same study demonstrated that *Slc2a3* mRNA (another member of the glucose transporter family that is HIF-regulated) expression was found to increase in granulosa cells of gonadotropin-stimulated, periovulatory follicles which is suggestive that Slc2a3 may be the major glucose transporter during the ovulatory period (Kol *et al.*, 1997). Further studies are required to determine the effect of hCG stimulation and low oxygen on *Slc2a3* gene expression in granulosa cells. Nevertheless, results demonstrated in this study indicate several distinct mechanisms for HIF responsive gene regulation in ovarian cells, including the *Vegfa* pattern of induction by hCG and hypoxia, and *Slc2a1*, which was found to be induced *in vitro* only by low oxygen, with no overt requirement for hormone. Such differences between

gene expression patterns for such well characterised HIF-responsive genes suggests that hormone-mediated attenuation of expression is selective to certain genes within specific tissues.

As previously mentioned, Kim *et al.,* (2009) demonstrated that the progesterone receptor (PR) is necessary for the expression of HIF and known HIF target genes such as *Edn2* and *Vegfa* (Kim *et al.*, 2009). Echinomycin, a factor known to inhibit HIF DNA binding and transcriptional activity was also found to inhibit genes that regulate ovulation (Kim *et al.*, 2009). However, another study showed that echinomycin is not specific in blocking HIF activity and revealed activity of this drug against Sp1 binding elements (Vlaminck *et al.*, 2007), another transcription factor that is able to bind onto the VEGF promoter (Curry *et al.*, 2008). Furthermore, the PR/HIF responsive induction of *Edn2* demonstrated by Kim *et al.*, (2009) differs from another study displaying maximal expression of *Edn2* mRNA, 12 h post hCG in cultured granulosa cells (Na *et al.*, 2008). However, in the latter study, *Edn2* mRNA levels were not affected when treated with a PR antagonist (Na *et al.*, 2008). Therefore, although PR may indeed regulate HIF 1α downstream of hCG during the ovulatory cascade, these results suggest that low oxygen remains important for HIF accumulation and HIF mediated gene expression in addition to hormone-mediated induction.

Post ovulation, the formation of the CL has been associated with extensive angiogenesis and by the mid luteal phase, the CL is fully functional and has the greatest blood flow amongst any tissue in the body. In this study, EGFP positive CLs were found at all stages of pregnancy examined, with the percentage of positive EGFP-CLs increasing from early CL formation to mid-pregnancy (day 12). Earlier studies performed in rats have demonstrated little change in blood flow measured in CLs during early pregnancy but the CL undergoes a major growth phase and increase in blood flow by mid pregnancy (Meyer & Bruce, 1979). This suggests that the CL during early pregnancy may recruit active HIF for intensive neovascularisation to occur. Interestingly, HIF 1α protein accumulation in the ovary was maintained during any stage of pregnancy investigated in this study although densitometric analyses suggested that HIF 1α decreases as

the CL develops which is consistent with studies that have demonstrated that *HIF1α* mRNA expression tended to decrease as the CL matured (Boonyaprakob *et al.*, 2005; van den Driesche *et al.*, 2008). However, it must be taken into consideration that mice used in the post-copulation study were naturally cycling adults, and CLs from previous cycles may affect results as it has been shown previously that CLs from the previous cycles have increased blood circulation and can still develop (Bruce *et al.*, 1984). The current results demonstrated that HIF 1α activity is maintained during early pregnancy to mid pregnancy. However, more work is needed to understand what occurs after the mid luteal stage, particularly during luteolysis, as HIF 1α immunostaining was detected in marmoset ovaries within CLs undergoing luteolysis (Duncan *et al.*, 2008). Furthermore, VEGF is only down-regulated after the initiation of luteolysis (Dickson *et al.*, 2001), which possibly explains the lowered level of vascularity in the regressing CL.

These results indicate that HIF is present in granulosa cells, thecal cells and luteinised cells of the corpora lutea, however, the localisation of HIF in the cumulus cells or the oocyte has yet to be explored. In conclusion, the results reported here are the first to report that HIF 1α protein is maintained during the development and maintenance of CLs during pregnancy.

CHAPTER 5 The effects of different oxygen concentrations during oocyte maturation *in vitro* – the role of hypoxia inducible factors.

5.1 Introduction

In previous Chapters, it was demonstrated that oxygen regulation during folliculogenesis is essential for follicular differentiation and ovulation. In Chapter 3, using the transgenic HRE-GFP mouse as a tool, it was demonstrated that HIF 1α activity was not present in follicles prior to antrum formation. Upon antrum formation, follicles expressed EGFP in cells within the thecal layer. HIF 1α protein was present in granulosa cells (GCs) upon stimulation with hypoxia or a hypoxia mimetic but was not detected in FSH-treated cultured granulosa cells. In Chapter 4, HIF 1α was found to be highly associated with follicular differentiation and was induced in granulosa cells and residual ovary around the time of ovulation. EGFP was also expressed in corpora lutea post copulation and increased as luteinisation progressed. These studies indicate that HIF is present in granulosa cells, thecal cells and luteinised cells of the corpora lutea. The localisation of HIF in the cumulus cells or the oocyte has yet to be explored.

The preantral to antral follicle transition is an important step during follicular development, where preantral granulosa cells form two subpopulations of GCs. In large antral follicles mural granulosa cells (MGCs) attach to the basement membrane enclosing the follicle and display steroidogenic properties and differentiation towards luteal cell differentiation. Cumulus cells (CCs) are coupled by gap junctions to both the oocyte and mural granulosa cells.

The transdifferentiation from GCs to form CCs is tightly regulated by paracrine signalling from the oocyte (Gilchrist *et al.*, 2006). CCs are closely associated with the oocyte forming the cumulus-oocyte complex (COC). CCs are required to facilitate oocyte developmental competence (Diaz *et al.*, 2007; Gilchrist *et al.*, 2008). In response to the preovulatory LH surge, a defining feature of CCs is their ability to promote oocyte competence and to undergo cumulus expansion after the preovulatory LH surge (Diaz *et al.*, 2006). MGCs do not undergo expansion *in vivo*. Studies have shown that under the influence of FSH, GC differentiation is geared towards MGC (Eppig *et al.*, 1997) and elimination of oocyte paracrine signalling by physically

removing the oocyte by oocytectomy (OOX) (Eppig *et al.*, 1997) or by inactivating SMAD2/3 signalling (an oocyte paracrine signalling pathway) (Dragovic *et al.*, 2007) causes CCs to lose their distinctive phenotype and GC tend to display typical characteristics of MGCs. However, treating OOX with oocyte secreted factors fully restores CC characteristics demonstrating that oocytes suppress FSH induced GC differentiation toward luteinisation (Eppig *et al.*, 1997). Alternatively, an early study has demonstrated that the differentiation of granulosa cells to functional cumulus cells as well as the process of cumulus cell expansion is highly regulated by the oocyte or 'cumulus expansion enabling factor' (Vanderhyden *et al.*, 1990). Therefore, treating MGC with denuded oocytes and/or oocyte secreted factors such as growth-differentiation factor-9 (GDF-9) and bone morphogenic protein-15 (BMP-15) promotes proliferation of MGC as a functional CC phenotype (Gilchrist *et al.*, 2001; Gilchrist *et al.*, 2006).

It is well established that *in vitro* matured oocytes are inferior in their developmental competence compared with their *in vivo* matured counterparts (Eppig & O'Brien, 1998; Banwell *et al.*, 2007). Several studies have shown that cumulus cells are required during maturation for the oocyte to acquire full cytoplasmic maturation, as removal of cumulus cells at the beginning of IVM adversely affects oocyte developmental competence in several species including cattle (Fukui & Sakuma, 1980), pigs (Maedomari *et al.*, 2007), rabbit (Lu *et al.*, 2009) and in mice (Gilchrist *et al.*, 2008).

As previously mentioned, despite the oocyte being localised within the avascular follicular antrum, it utilies oxidative phosphorylation; this may limit the oocyte's ability to generate ATP (Gosden & Byatt-Smith, 1986; Neeman *et al.*, 1997; Thompson *et al.*, 2007). Studies have attempted to measure intrafollicular oxygen concentration *in vivo* following follicle aspiration. These investigators demonstrated that the dissolved oxygen concentration is low and can vary between species (Knudsen *et al.*, 1978; Van Blerkom *et al.*, 1997; Huey *et al.*, 1999; Redding *et al.*, 2006). Furthermore, it has been demonstrated that cumulus cells consume relatively little oxygen, sparing it for the oocyte (Clark *et al.*, 2006). Several groups have examined

the effects of lower oxygen tensions on *in vitro* oocyte growth and development and it was found that maturation of mouse oocytes under 5% oxygen compared to 20% oxygen increases oocyte developmental competence (Eppig & Wigglesworth, 1995; Hu *et al.*, 2001). However, reduced availability of oxygen in the follicle has been associated with chromosomal abnormalities and reduced pregnancy rates in human (Chui *et al.*, 1997; Van Blerkom *et al.*, 1997). A recent study demonstrated that varying the oxygen concentration during mouse oocyte IVM perturbs subsequent fetal and placental outcomes but not in a dose dependent relationship (Banwell *et al.*, 2007). Yet, the effect of oxygen concentration on HIF protein abundance and activity in cumulus cells and/or the oocyte during IVM has yet to be fully established.

Microarray technology was previously used to determine the impact of altering oxygen concentration (20%, 10%, 5%, 2% O_2) during murine IVM on cumulus cell gene expression (Banwell, 2008). The analyses revealed 218 differentially expressed probes, of which 34 were up-regulated in cumulus cells with decreasing oxygen levels. The great majority of these were classified as HIF-regulated genes. Microarray analyses also showed that genes involved in fatty acid synthesis, transcripts including *Mecr, Scd1, Elovl6, Inpp5e* and *Acss2,* were significantly down-regulated upon exposure to hypoxia during IVM (Banwell, 2008). As previously mentioned, HIF 1α is a well established oxygen dependent transcription factor known to up-regulate a number of genes involved in angiogenesis, glucose metabolism and proliferation (Semenza *et al.*, 1994; Forsythe *et al.*, 1996; Semenza *et al.*, 1996; Salnikow *et al.*, 2008). A study suggests that HIF 1α-dependent activation of fatty acid metabolism in cancer cells is necessary (Menendez *et al.*, 2005). However, a recent publication utilising inactivated HIF 1α or HIF 2α in the livers of pVHL-deficient mice and microarray technology suggested that glycolysis and pyruvate metabolism were mainly HIF 1α-dependent whereas, HIF 2α played a central role in lipogenesis and fatty acid synthesis (Rankin *et al.*, 2009).

Knowing that the majority of genes up-regulated in cumulus cells during IVM of murine COCs under low oxygen conditions were downstream of the HIF-dependent pathway, I hypothesized that oxygen regulates

gene expression in cumulus cells via HIF activation. Thus, the aim of this study was to analyse gene expression changes and HIF 1α protein stabilisation in murine cumulus cells and cumulus-oocyte complexes exposed to varying oxygen concentrations during *in vitro* maturation.

5.2 Materials and methods specific to this Chapter

5.2.1 Animals and gonadotropin stimulation

All experiments were conducted according to the National Health and Medical Research Council guidelines and approved by The University of Adelaide Animal Ethics Committee. Prepubertal 21 day old hybrid CBAB6F1 hybrid mice were obtained from Animal Resources Centre (Western Australia, Australia). The animals were housed at The University of Adelaide Medical School Animal House under a 12:12 h light-dark regimen and fed a standard pellet diet ad libitum with free access to water. Cumulus-oocyte-complexes (COC) were isolated from mice that received 7.5 IU eCG (Folligon serum gonadotropin; Intervet, Boxmeer, Holland) injected intraperitoneally 46 h prior to COC retrieval.

5.2.2 *In Vitro* Maturation (IVM) of cumulus oocyte complexes

COCs were collected into HEPES-buffered αMEM media supplemented with 50 μg/ml streptomycin sulphate, 75 μg/ml penicillin G and 5% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) by gently puncturing visible antral follicles present on the ovary surface with a 30 x ½ gauge needle. Germinal vesicle stage oocytes with healthy layers of cumulus cells were collected and pooled from a minimum of 10 - 12 animals.

Groups of 10 COCs were matured per 100 μl droplets of bicarbonate buffered αMEM supplemented with 50 μg/ml Streptomycin, 75 μg/ml Penicillin G, 5% FBS and 50 mIU/ml recombinant human follicle stimulating hormone (rhFSH) (Puregon; Organon, Sydney, Australia) under oil in 35-mm Falcon 1008 culture dishes (Becton-Dickinson Labware, Franklin Lakes, USA). Maturation of COCs under different oxygen concentrations was performed for 17 h at 37.5 °C in modular incubation chambers (Billups-Rothenburg, Del Mar, USA) filled with test gas mixtures. The gas mixtures used were 2, 5 or 20% oxygen

(6% carbon dioxide and balance of nitrogen). Culture dishes were prepared a day ahead and were allowed to equilibrate in the modulators at 37.5 °C overnight.

5.2.3 Cumulus cell collection

Following IVM, COCs from each treatment group were treated with 50 U/ml ovine hyaluronidase and all cumulus cells were detached with the aid of gentle pipetting. All denuded oocytes were removed and media containing cumulus cells were collected into sterile Eppendorf tubes. Cumulus cells were washed in fresh HEPES buffered αMEM twice and spun down at 13,200 rpm for 2 min. The supernatant was removed and cells were snap frozen and stored at -80 °C. To minimize any change in oxygen concentration, care was taken to ensure that the procedure was conducted in minimal handling time.

5.2.4 Cumulus cell mRNA extraction

Total RNA was isolated from cumulus cell samples using the RNeasy Micro Kit (Qiagen, Doncaster, USA). All 15 samples were extracted at one time to prevent variation in extraction conditions. After each centrifugation step, the flow through was discarded and the tube retained unless otherwise stated. All centrifugation steps were performed at room temperature. 100 µl of Buffer RLT containing 1µl/ml β-mercaptoethanol was added to each sample together with 5 µl of carrier RNA working solution (310 µg supplied with kit, made up to 310 µg/ml in 1 ml RNase free water, stored at -20 °C, diluted to 4 ng/µl working solution). Samples were vortexed for 1 min. One hundred and five µl of 70% ethanol made with molecular grade 99.9% ethanol and RNase free water was added to each homogenized lysate and mixed by pipetting. The sample was then applied to RNeasy MinElute Spin Column in a 2 ml collection tube. The tube was closed gently and centrifuged at ≥ 10,000 rpm for 15 sec. The flow through was discarded and the collection tube was reused. To wash the column, 350 µl of Buffer RW1 was added onto the membrane surface and the column centrifuged at≥ 10,000 rpm for 15 sec. Eighty µl of DNase incubation mix (1:7

working ratio of DNase and Buffer RDD) was carefully pipetted directly onto the spin column silica gel membrane and incubated at room temperature for 15 min. To wash, 350 µl of RW1 buffer was added to column followed by centrifugation at ≥ 10,000 rpm for 15 sec. A new collection tube was fitted to the column and 500 µl of RPE buffer was added, the cap closed gently and the column centrifuged at ≥ 10,000 rpm for 15 sec. To the surface of each column was added 500 µl of 80% ethanol made with molecular grade 99.9% ethanol and RNase-free water. The lid was closed and the column centrifuged at 10,000 rpm for 2 min. Following this, the column was carefully transferred to a new 2 ml collection tube and centrifuged at maximum speed for 5 min to dry the column completely. The column was transferred to the final 1.5 ml collection tube and 14 µl of RNase-free water was pipetted directly onto the centre of the column membrane and, with the lid closed, the column was centrifuged at maximum speed for 1 min. RNA solution flow through was collected into a 0.6 ml Eppendorf and stored at -80 °C.

5.2.5 Reverse Transcription

Refer to materials and methods in Chapter 2 (Section 2.9).

5.2.6 Cumulus cell gene expression analyses.

The effect of IVM oxygen concentration on expression of selected genes of interest in cumulus cells was analysed by quantitative Real Time RT-PCR. Cumulus cells were collected following IVM at 2, 5 and 20% oxygen concentration and RNA from these cells was extracted and processed for RT-PCR. Primers for each gene of interest (Table 2.3) were designed using Primer Express version 2.0 software (PE Applied Biosystems, Foster City, CA, USA) based on the corresponding sequence found in GenBank. Quantitative analyses of mRNA samples were performed using an ABI PRISM® 5700 sequence detection system (Applied Biosystems, Foster City, CA, USA).

A minimum of 5 replicates were collected for each treatment with each replicate representing an equal proportion of RNA from cumulus cells pooled from a minimum of 80 COCs. The cDNA synthesized from all cumulus cells groups was subjected to a Real Time RT-PCR using the *Rpl19* or *18S* primer to test that there were no significant differences in the relative abundance of housekeeping genes between treatments. Final quantitative analyses was performed using the relative standard curve method and results are reported as the fold difference after normalisation of the transcript amount relative to *Rpl19* and *18S* independently as both housekeeping genes were analysed separately.

5.2.7 Western blotting

5.2.7.1 'Normal' processing of cumulus cells.

Following IVM, COCs from each treatment group were treated with 50 U/ml ovine hyaluronidase and all cumulus cells were detached with the aid of gentle pipetting. All denuded oocytes were removed and media containing cumulus cells were collected into sterile Eppendorf tubes. Cumulus cells were washed in fresh HEPES buffered αMEM twice and spun down at 13,200 rpm for 2 min. The supernatant was removed and cells were lysed in 15 µl of RIPA buffer in the presence of 10 µl/ml protease inhibitor cocktail.

5.2.7.2 'Rapid' processing of cumulus cells, oocytes and Cumulus oocyte complexes.

Following IVM, COCs from each treatment group were treated with 25 U/ml ovine hyaluronidase and all cumulus cells were detached with the aid of gentle pipetting. The concentration of hyaluronidase was reduced from here on to prevent salt accumulation during concentration of protein step (Section 2.6.1) as samples processed using 50 U/ml ovine hyaluronidase caused proteins to resolve inefficiently within the resolving gel. All denuded oocytes were removed into Eppendorf tubes containing 350 µl RIPA buffer containing 10 µl/ml protease inhibitor cocktail and media containing cumulus cells were collected into sterile

Eppendorf tubes. COCs were collected using pulled glass pipettes directly into Eppendorf tubes containing RIPA buffer and 10 µl/ml protease inhibitors (Sigma).

5.2.8 **The effect of different hypoxic mimetics on HIF 1α induction** in cumulus cells, denuded oocytes and granulosa cells *in vitro*.

COCs were collected as previously described. COCs were then matured *in vitro* for 13 h in maturation media previously described and transferred to either 250 µM $CoCl_2$ or 100 µM dexsoferramine (DFO) treated maturation media, as well as a control for a further 4 h. (All 3 treatment groups were cultured for a total of 17 h). Cumulus cells were detached with the aid of gentle pipetting in the presence of 25 U/ml ovine hyaluronidase. All denuded oocytes were removed and media containing cumulus cells were collected into sterile Eppendorf tubes containing RIPA buffer and protease inhibitors. Mural granulosa cells from the same group of mice were also collected and plated in 4-well dishes for 24 h. Mural granulosa cells were exposed to either 250 µM $CoCl_2$ or 100 µM dexsoferramine (DFO) treated granulosa cell maturation media, as well as a control for a further 4 h. Granulosa cell lysates were collected as previously described in Chapter 2 and Section 3.2.4.

5.2.9 Addition of MG 132, a potent and cell-permeable proteasome inhibitor.

COCs were collected as stated under '*in vitro* maturation' and groups of 10 COCs were matured per 100 µl droplet of bicarbonate buffered αMEM supplemented with 50 µg/ml Streptomycin, 75 µg/ml Penicillin G, 5% FBS and 50 mIU/ml recombinant human follicle stimulating hormone (rhFSH) (Puregon; Organon, Sydney, Australia) with or without the addition of 10 µM of MG 132 (Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) (Calbiochem, San Diego, CA, USA) under oil in 35-mm Falcon 1008 culture dishes (Becton-Dickinson Labware, Franklin Lakes, USA). Maturation of COCs was performed for 13 h at 37.5 °C in 20% O_2, 5 % CO_2 and balance of N_2. COCs were treated for a further 4 h with or without 250 µM $CoCl_2$. Collection of cumulus cells and denuded oocytes was performed via 'rapid processing' (Section 5.2.7.2).

5.2.10 Transdifferentiation of granulosa cells into mural granulosa cells or cumulus cells.

Forty-six hours following 7.5 IU eCG stimulation, granulosa cells and COCs were collected via follicular puncture in HEPES-TCM 199 buffer. Cumulus cells were dissociated by vortexing COCs for 5 min and all oocytes were removed with the aid of glass pipetting. Granulosa cells and cumulus cells were cultured in 24-well plates (Falcon, Franklin Lakes, NJ, USA) containing DMEM/HAMS F12 media supplemented with 1% fetal calf serum, 1% L-glutamine and 2% penicillin/streptomycin in an incubator of 20% O_2, 5% CO_2 and balance of N_2 at 37.5 °C for 24 h. Depending on the individual experiments, reagents and media were added to wells giving a final volume of 1 ml. A 2 X 2 factorial design was used for the addition of 50 ng/ml GDF-9 (R&D Systems Inc, Minneapolis, MN, USA) to granulosa cells and cumulus cells. All cells were incubated with 250 µM $CoCl_2$ after 24 h incubation for a further 4 h. For collection of protein lysates, media was removed, plated granulosa cells or cumulus cells were washed with 250 µM $CoCl_2$-treated PBS and lysed with 100 µl of RIPA and 10 µl/ml protease inhibitors using a sterile cell scraper.

All protein samples collected were processed and concentrated using the Microcon Centrifugal Device (YM-10), as described in Chapter 2 (Section 2.6.1).

For Western blot analyses, concentrations of 50 - 100 µg of protein extracts were used. Proteins were resolved by SDS-PAGE on 4% - 10% gradient gels and transferred onto PVDF membranes. All gels for Western blotting included pre-stained protein molecular weight markers (Bio-Rad). Membranes were blocked for 1 h with 5% (w/v) skim milk, 1x TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.05% Tween 20]. Detection of target proteins was accomplished by using a rabbit polyclonal antibody specific for HIF1α (1:1000, NB100-449, Novus Biologicals, Littleton, CO, USA) followed by a goat anti-rabbit secondary antibody conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:5000 dilution in 5% (w/v) non-fat milk, TBST and 0.05% Tween 20 for 1 h and detected by enhanced chemiluminescence detection as per the manufacturer's instructions (Amersham, GE healthcare Life Sciences, Rydalmere,

NSW, Australia). The membranes were stripped and re-probed with an antibody to β-actin (Sigma) at dilution of 1:25 000.

5.2.11 Peptide competition assay (PCA) of HIF 1α.

For PCA, concentrations of ~ 40 µg of 250 µM $CoCl_2$-treated granulosa cells, 'rapid' processed 20% [O_2] treated COCs and PyMT- tumour protein extracts were used. Two identical test samples were resolved by SDS-PAGE on 4% - 10% gradient gels and transferred onto PVDF membranes. All gels for Western blotting included pre- stained protein molecular weight markers (Bio-Rad). Membranes were blocked for 1 h with 5% (w/v) skim milk, 1 x TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.05% Tween 20]. On one test sample, detection of target proteins was accomplished by using a rabbit polyclonal antibody specific for HIF 1α (1:1000, NB100-449, Novus Biologicals, Littleton, CO, USA). For the second test sample (PCA), peptide blocking antibody (200-fold molar excess of peptide, NB100-449PEP, Novus Biologicals, Littleton, CO, USA) together with HIF 1α antibody (NB100-449) were incubated together for 2 h. Both test samples were followed by a goat anti-rabbit secondary antibody conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:5000 dilution in 5% (w/v) non-fat milk, TBST and 0.05% Tween 20 for 1 h and detected by enhanced chemiluminescence detection as per the manufacturer's instructions (Amersham, GE healthcare Life Sciences, Rydalmere, NSW, Australia).

5.2.12 Statistical Analyses

Statistical significance was determined using SPSS software for Windows, version 14.0 (SPSS, Chicago, IL, USA). Cumulus cell gene expression results were analysed using one-way analysis of variance followed by least-significant difference (LSD) post-hoc test, when the significance of Levene's test ≤ 0.05; non-parametric Kruskal-Wallis analysis followed by Mann-Whitney post-hoc test was used. The data are

presented as the mean ± S.E.M. Superscripts denotes significance with *P* values of ≤ 0.05 compared to appropriate control were regarded as statistically significant.

5.3 Results

5.3.1 Genes involved in proliferation, apoptosis and glucose metabolism are up-regulated in cumulus cells cultured under low oxygen environments.

Chosen genes of interest were previously identified by microarray studies to be altered in cumulus cells following *in vitro* maturation under varying oxygen conditions.

Slc2a1, *Ldha*, *Eno1* and *Pgk1* mRNA levels in cumulus cells were up-regulated following *in vitro* maturation at 2% or 5% when compared to 20% [O_2]. A further significant increase in *Slc2a1* mRNA was seen when cumulus cells were cultured under 2% [O_2] when compared to 5% [O_2] (Figure 5.1 and 5.2; $P < 0.01$).

Bnip3 mRNA abundance was increased in cumulus cells exposed to 5% [O_2] when compared to 20% [O_2]. A further up regulation was observed when cumulus cells cultured in 2% [O_2] were compared to those cultured at 5% [O_2] when *Rpl19* housekeeper was used (Figure 5.1; $P < 0.01$). There was no change in *Bnip3* mRNA when cumulus cells were exposed from 5% [O_2] to 2 % [O_2] when *Bnip3* mRNA was normalised to *18S* housekeeping gene (Figure 5.2).

Ndrg1 mRNA levels in cumulus cells were up-regulated when cultured in 5% [O_2] compared to 20% [O_2]. A further significant ~ 65-fold and 46-fold increase was seen when cumulus cells were cultured under 2% [O_2] compared to 20% [O_2] relative to *Rpl19* and *18S* respectively (Figure 5.1 and 5.2; $P \leq 0.03$).

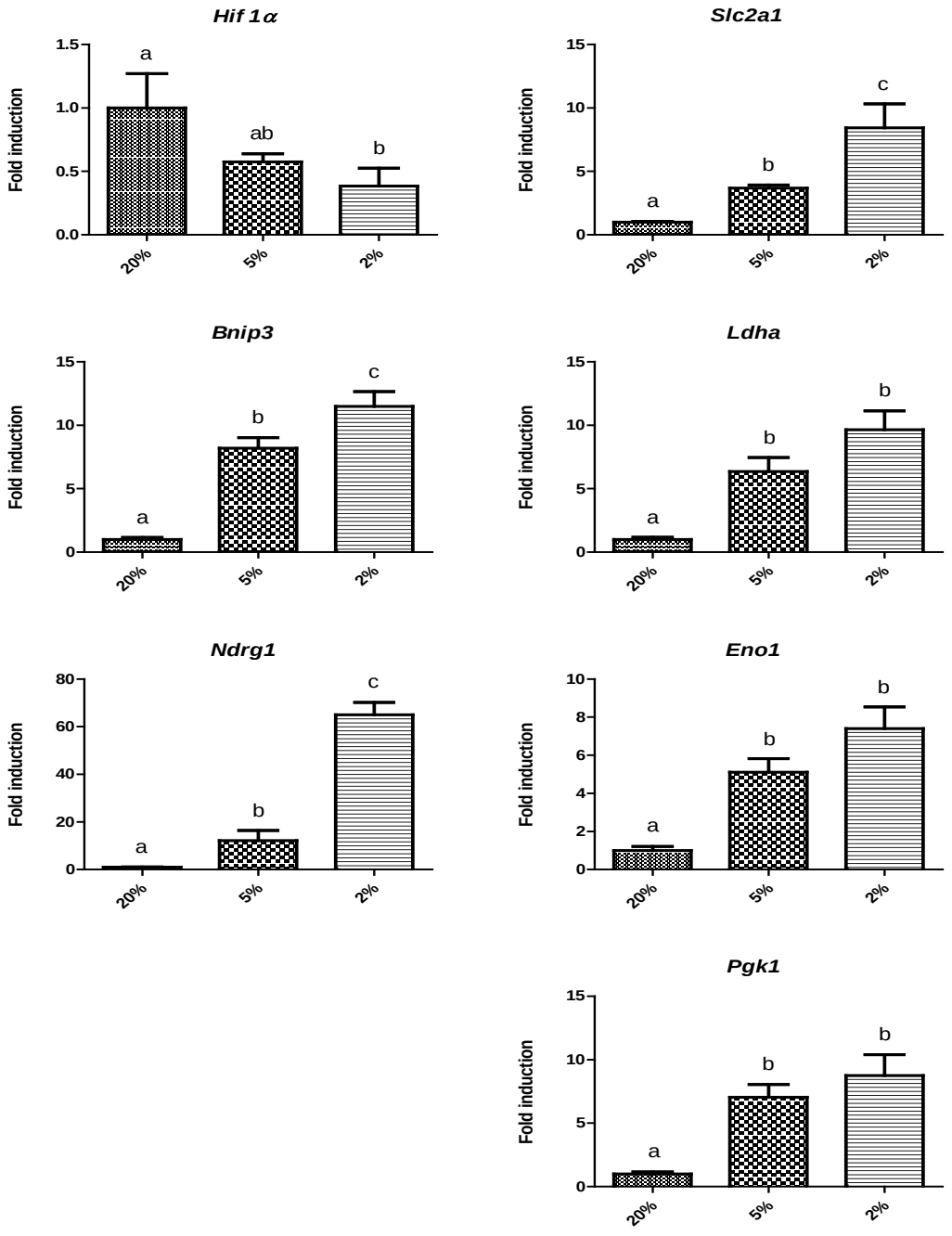

Figure 5.1 The effect of oxygen concentration during *in vitro* maturation on cumulus cell gene expression normalised against housekeeper *Rpl19.*

Quantitative Real Time RT-PCR was performed to determine the expression of *Hif1α* mRNA, *Bnip3* mRNA, *Ndrg1* mRNA, *Slc2a1* mRNA, *Ldha* mRNA, *Eno1* mRNA and *Pgk1* mRNA normalised against housekeeper *Rpl19*. The data are presented as fold induction compared to 20% [O_2] and represents the mean from 5 independent experiments (mean ± S.E.M) (n = 5 per treatment group). Cumulus cell gene expression results were analysed using one-way ANOVA and LSD post-hoc test. When significance of Levene's test ≤ 0.05; non-parametric Kruskal-Wallis followed by Mann-Whitney post-hoc test was used. Superscripts denote significance P ≤ 0.05.

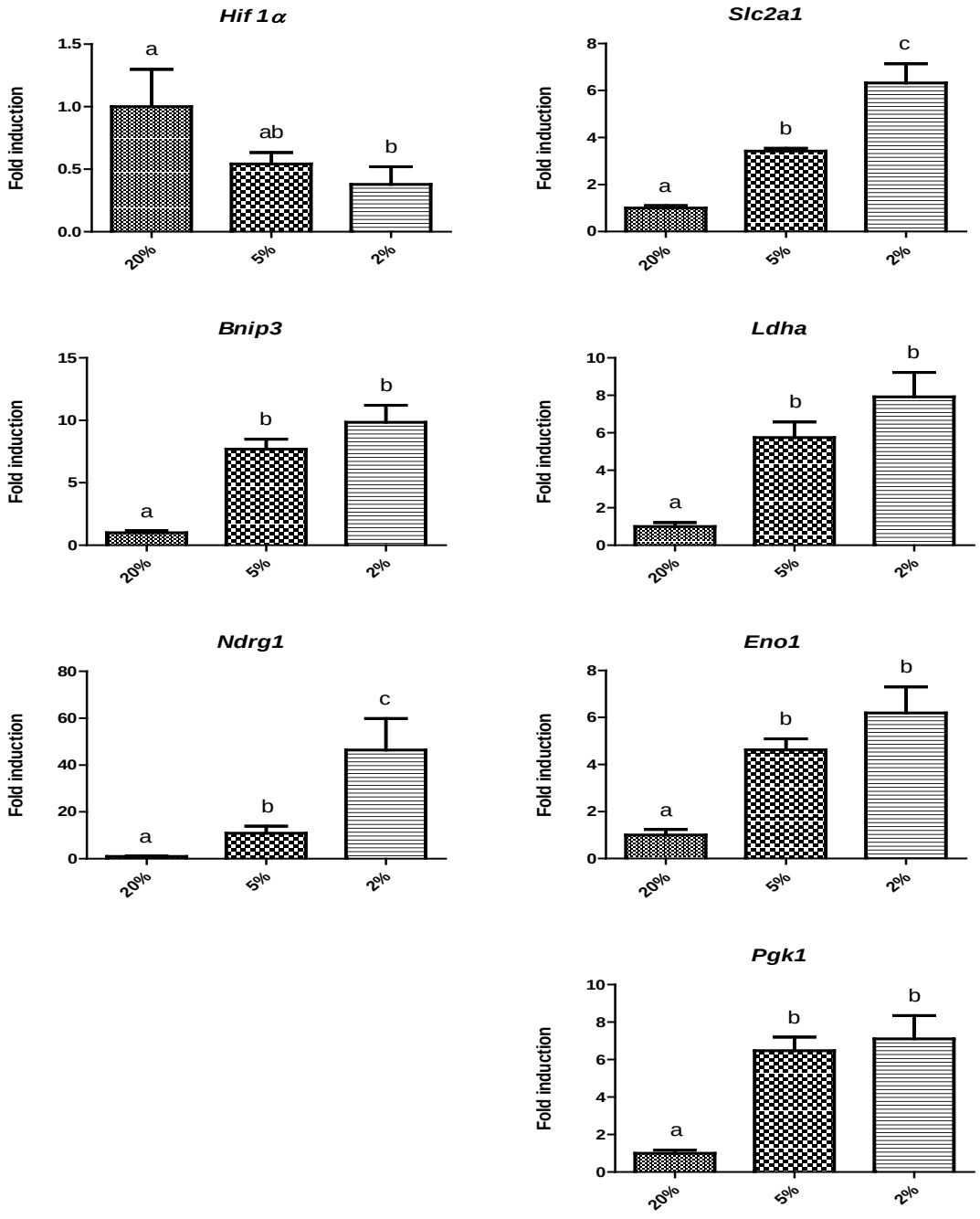

Figure 5.2 The effect of oxygen concentration during *in vitro* maturation on cumulus cell gene expression normalised against housekeeper *18S.*

Quantitative Real Time RT-PCR was performed to determine the expression of *Hif1α* mRNA, *Bnip3* mRNA, *Ndrg1* mRNA, *Slc2a1* mRNA, *Ldha* mRNA, *Eno1* mRNA and *Pgk1* mRNA normalised against housekeeper *18S.* The data are presented as fold induction compared to 20% [O_2] and represents the mean from 5 independent experiments (mean ± S.E.M) (n = 5 per treatment group). Cumulus cell gene expression results were analysed using one-way ANOVA and LSD post-hoc test. When significance of Levene's test ≤ 0.05; non-parametric Kruskal-Wallis followed by Mann-Whitney post-hoc test was used. Superscripts denote significance P ≤ 0.05.

5.3.2 Fatty acid synthesis genes, *Elovl6* mRNA and *Scd1* mRNA were down-regulated under low oxygen environments.

I went on to compare the mRNA expression of *Mecr, Scd1, Elovl6, Inpp5e* and *Acss2* to understand the effects of different oxygen concentrations on cumulus cell gene expression specific to fatty acid synthesis (Figure 5.3 and 5.4), as suggested from microarray results. There were no changes in mRNA expression of *Mecr, Inpp5e* and *Acss2* under different oxygen concentrations in cumulus cells. However, there was a significant down-regulation of *Elvol6* mRNA in cumulus cells developed at 2% [O_2] compared to 20% [O_2] or 5% [O_2] (Figure 5.3 and 5.4; $P \leq 0.02$). *Scd1* mRNA in cumulus cells was down-regulated following IVM in 2% [O_2] when compared to 20% [O_2] (Figure 5.3 and 5.4; $P < 0.01$). There were no differences in statistical analyses between different housekeepers used.

5.3.3 **HIF 1α protein (MW ~ 120 kDa) was not detectable in cumulus cells, however, a HIF 1α** immunoreactive polypeptide (MW~ 75 kDa) was identified in COCs.

To investigate the effects of different oxygen environments on HIF 1α protein stabilisation in cumulus cells during *in vitro* maturation Western blotting analyses were performed on cumulus cells and COCs following maturation under varying oxygen concentrations for 17 h.

There was no HIF 1α protein (120 kDa) or any other bands detected in cumulus cells at varying oxygen concentrations (Figure 5.5; Lanes 1 - 3). However, a HIF 1α immunoreactive polypeptide was detected at around 75 kDa in cell lysates of IVM- cultured COCs treated in both 2% [O_2] or 20% [O_2] (Figure 5.5; Lane 4 and 5). A stronger 75 kDa HIF 1α polypeptide was seen in the 2% [O_2] COC (Figure 5.5; Lane 4). PyMT-tumour cells were used as a positive control for mouse HIF 1α, and showed a stronger 120 kDa band, as well as non-specific binding.

Given the unexpected findings, I repeated the same IVM experiment but this time, I collected the granulosa cells from the CBAB6F1 mice and cultured the granulosa cells with or without a hypoxic mimetic – cobalt

chloride ($CoCl_2$). Similarly to the results observed in Chapter 3 for HIF 1α protein induction in granulosa cells from C57BL/6 mice, we were able to induce the 120 kDa HIF 1α protein in granulosa cells under 250 µM $CoCl_2$. I also collected COCs and exposed them to 4 h of 250 µM $CoCl_2$. As expected, I was able to induce 120 kDa HIF 1α protein in 250 µM $CoCl_2$ –treated granulosa cells (Figure 5.6; Lanes 1 - 2). A 75 kDa HIF 1α immunoreactive polypeptide was induced in 2% [O_2] - IVM treated COCs (Figure 5.6; Lane 3). Interestingly, there was no HIF 1α protein present when COCs were treated with 250 µM $CoCl_2$ for 4 h.

5.3.4 **Peptide competition assay for HIF 1α immunoreactivity**.

To test cross reactivity of our HIF 1α antibody (NB100-449, Novus Biologicals, Littleton, USA), especially to determine the 75 kDa band, we employed a blocking peptide (NB100-449PEP, Novus Biologicals, Littleton, USA) specific to NB100-449. In Figure 5.7, test samples (1A, 2A and 3A) were probed with an antibody to HIF 1α and the same corresponding test samples (1B, 2B and 3B) were probed with the HIF 1α antibody in the presence of the blocking peptide as described in Section 5.2.11. Western blot analyses demonstrated that the 120 kDa and 75 kDa seen in test samples 1A and 2A were indeed HIF 1α immunoreactive as the blocking peptide blocked the signal in test samples 1B and 2B.

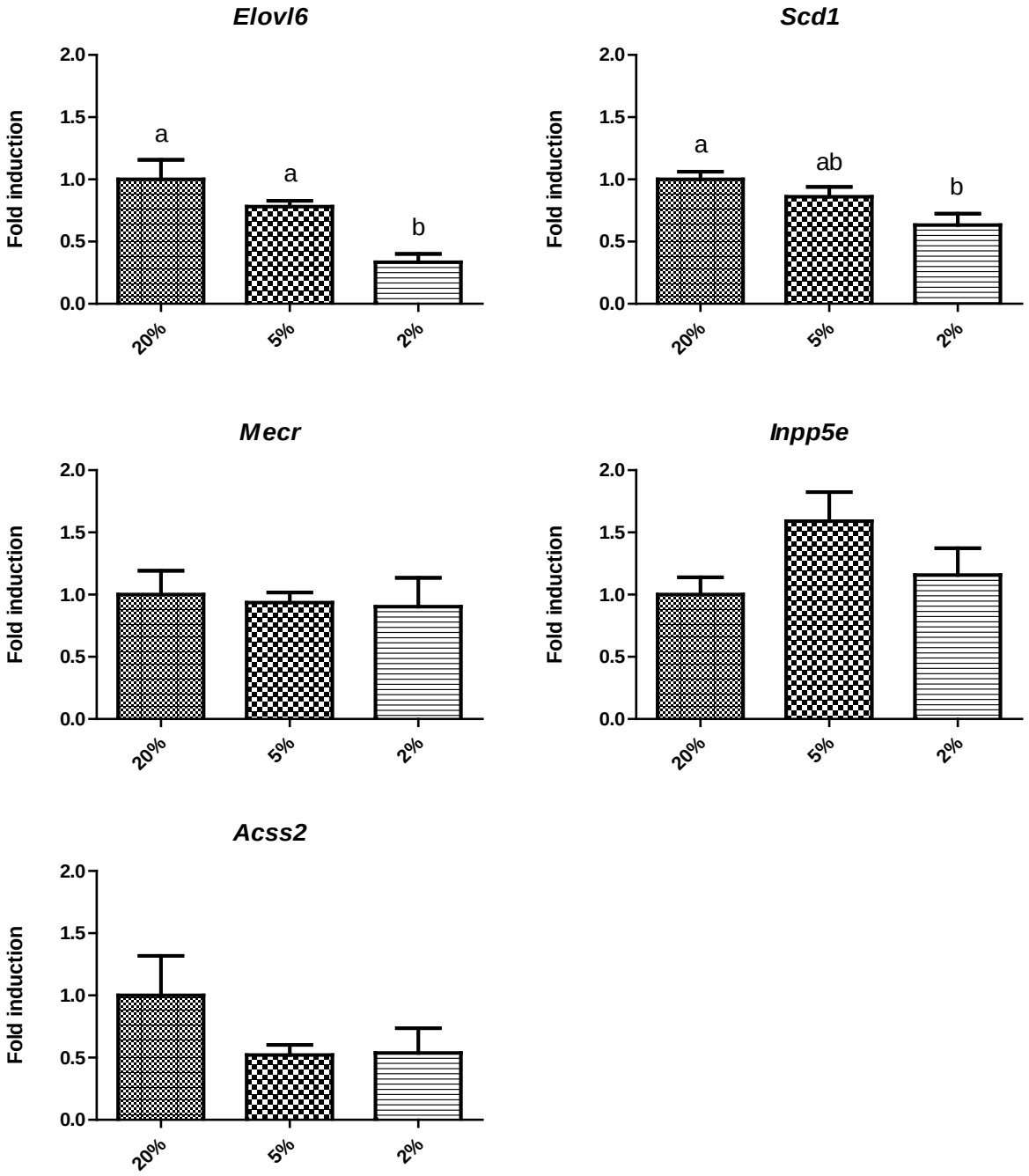

Figure 5.3 The effect of oxygen concentration during *in vitro* maturation on cumulus cell gene expression normalised against housekeeper *Rpl19.*

Quantitative Real Time RT-PCR was performed to determine the expression of *Elovl6* mRNA, *Mecr* mRNA, *Acss2* mRNA, *Scd1* mRNA and *Inpp5e* mRNA normalised against housekeeper *Rpl19*. The data are presented as fold induction compared to 20% [O_2] and represents the mean from 5 independent experiments (mean ± S.E.M) (n = 5 per treatment group). Cumulus cell gene expression results were analysed using one-way ANOVA and LSD post-hoc test. When significance of Levene's test≤ 0.05; non-parametric Kruskal-Wallis followed by Mann-Whitney post-hoc test was used. Superscripts denote significance P ≤ 0.05.

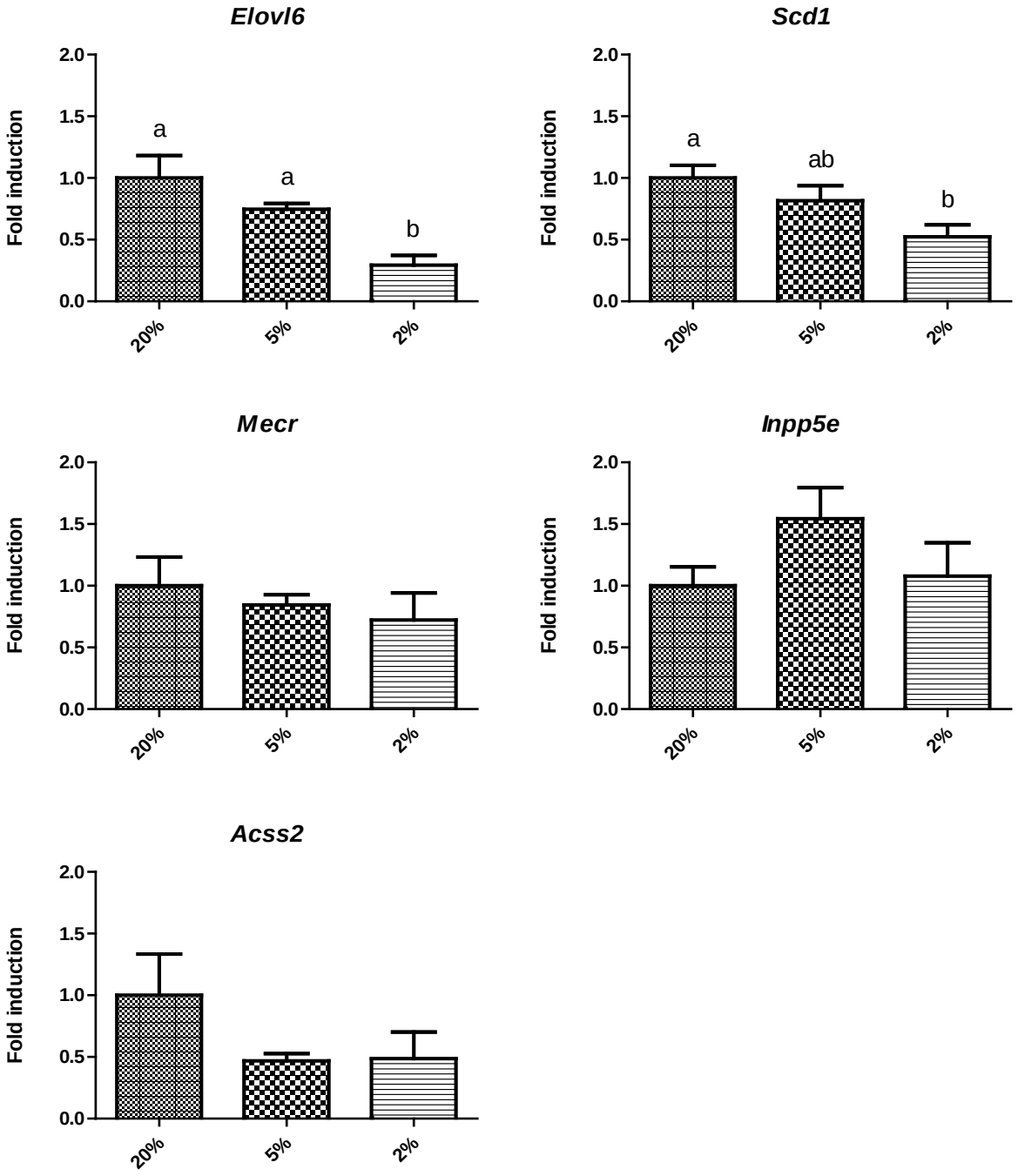

Figure 5.4 The effect of oxygen concentration during *in vitro* maturation on cumulus cell gene expression normalised against housekeeper 18S.

Quantitative Real Time RT-PCR was performed to determine the expression of *Elovl6* mRNA, *Mecr* mRNA, *Acss2* mRNA, *Scd1* mRNA and *Inpp5e* mRNA normalised against housekeeper *18S*. The data are presented as fold induction compared to 20% [O_2] and represents the mean from 5 independent experiments (mean ± S.E.M) (n = 5 per treatment group). Cumulus cell gene expression results were analysed using one-way ANOVA and LSD post-hoc test. When significance of Levene's test≤ 0.05; non-parametric Kruskal-Wallis followed by Mann-Whitney post-hoc test was used. Superscripts denote significance P ≤ 0.05.

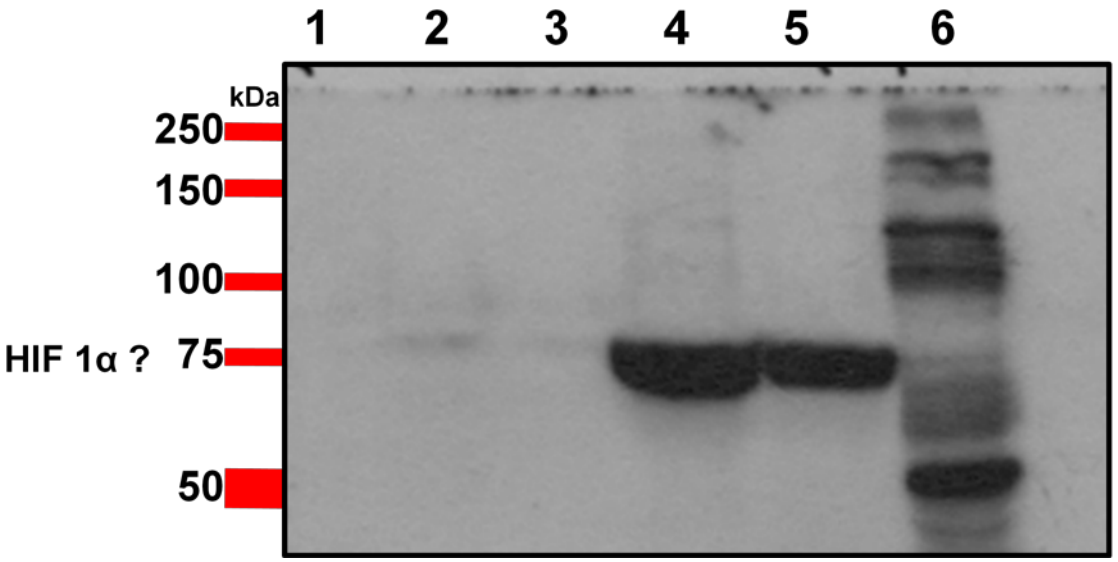

Figure 5.5 The effects of varying oxygen concentrations during *in vitro* maturation on **HIF 1α** protein in cumulus cells and COCs.

Western blot with an antibody to HIF 1α was performed to determine the effects of oxygen concentrations during IVM (17 h). Lanes 1 - 3: 5%, 2% and 20% [O_2]-treated cumulus cells following IVM for 17 h; Lanes 4 - 5: 2% and 20% [O_2]-treated COC; Lane 6: PyMT-tumour control.

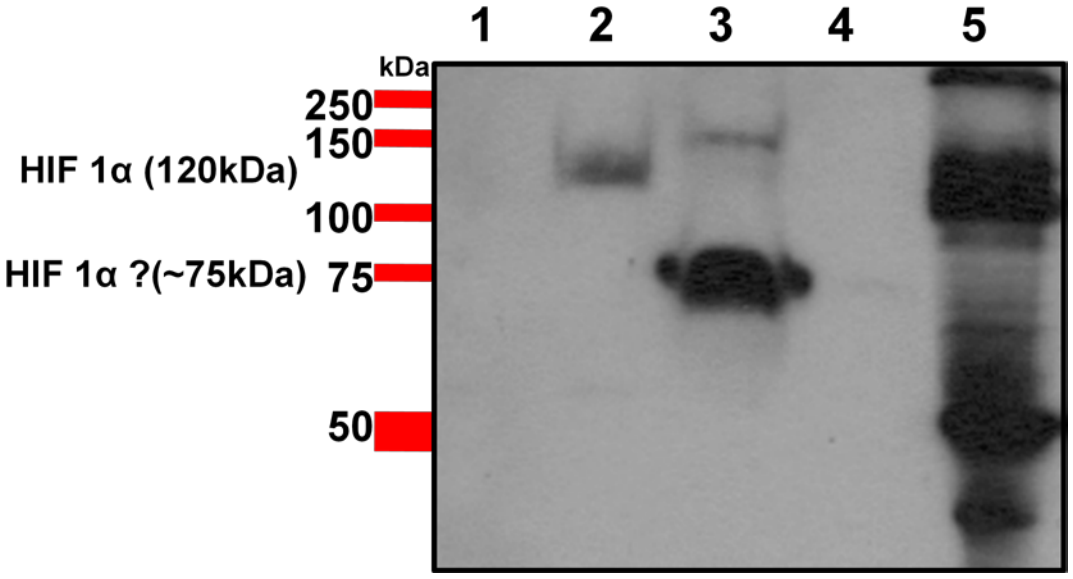

Figure 5.6 The effects of $CoCl_2$ on **HIF 1α** protein in granulosa cells and COCs.

Western blot analyses was performed with an antibody to HIF 1α to determine the effect of $CoCl_2$ (4 h) on granulosa cells and COCs. Lane 1: granulosa cell control; Lane 2: granulosa cells treated with 250µM $CoCl_2$; Lane 3: 2% [O_2]-treated COC; Lane 4: COC treated with 250µM $CoCl_2$; Lane 5: PyMT tumour.

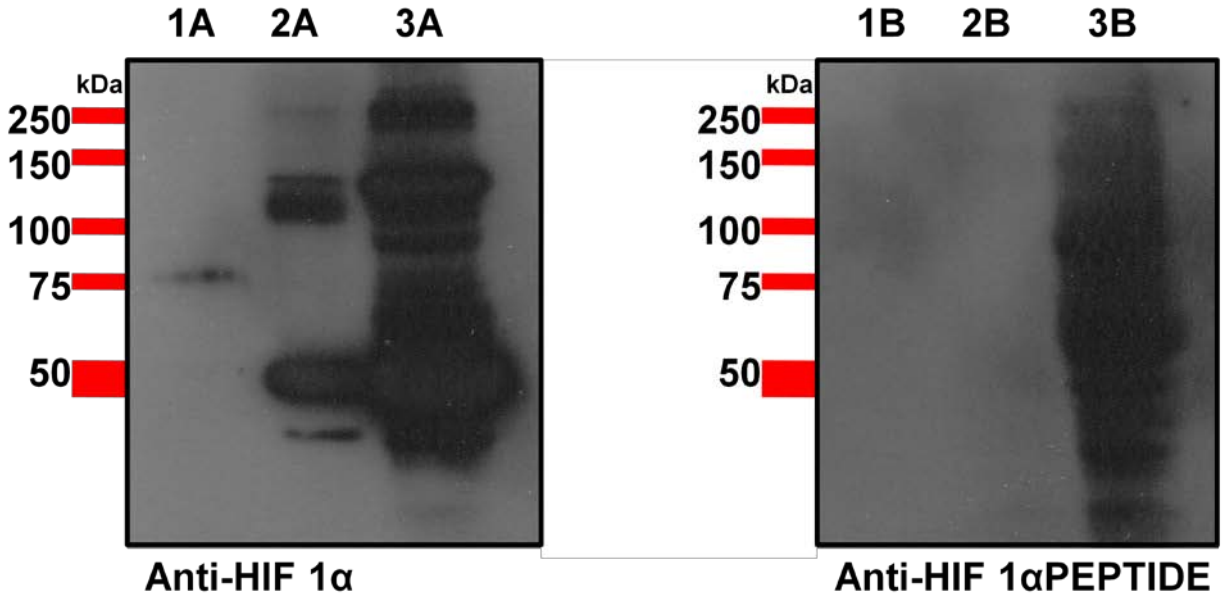

Figure 5.7 Western blot analyses **of Peptide competition assay against HIF 1α.**
Two identical test samples (1A and 1B: 20% [O_2] treated COC; 2A and 2B: granulosa cells treated with 250μM $CoCl_2$; 3A and 3B: PyMT-tumour). Samples 1A, 2A and 3A were probed with an antibody to HIF 1α and the corresponding test samples 1B, 2B and 3B were probed with the HIF 1α antibody (NB100-449) in the presence of the blocking peptide (NB100-449PEP) as described in Section 5.2.11.

5.3.5 **Effect of cobalt chloride or dexsoferramine on HIF 1α protein** stabilisation in cumulus cells, denuded oocytes and granulosa cells.

The effect of different hypoxic mimetics on HIF 1α protein stabilisation in cumulus cells, denuded oocytes and granulosa cells was assessed. Protein lysates were collected as described in Section 5.2.7.2. There was no HIF 1α protein stabilisation in cumulus cells that were treated with different hypoxic mimetics ($CoCl_2$ or DFO), however, 120 kDa HIF 1α accumulation was detected in denuded oocytes treated with hypoxic mimetics (Figure 5.8A). As expected, HIF 1α protein was detected at 120 kDa in granulosa cells treated with DFO, however, the signal intensity was higher in granulosa cells treated with $CoCl_2$ (Figure 5.8B).

5.3.6 Effect of MG 132 on **HIF 1α protein stabilisation in** cumulus cells, denuded oocytes and granulosa cells.

To determine the effect of MG 132, a potent and cell-permeable proteasome inhibitor, on HIF 1α protein stabilisation, protein lysates from cumulus cells, denuded oocytes and granulosa cells were collected as described in Section 5.2.9. HIF 1α protein (120 kDa) was not present in either the cumulus cells or denuded oocytes (Figure 5.9A), however, HIF 1α protein (120 kDa) was present in granulosa cells (Figure 5.9B). A 75 kDa HIF 1α immunoreactive polypeptide band was present in MG 132 treated denuded oocytes, however the band intensity was higher in $CoCl_2$ treated oocytes when compared to MG 132 treatment alone. Interestingly, MG 132 treated cumulus cells were not able to stabilise HIF 1α at any immunoreactive size, but cumulus cells, exposed to $CoCl_2$ in the presence or absence of MG 132 accumulated the 75 kDa immunoreactive HIF 1α (Figure 5.9A).

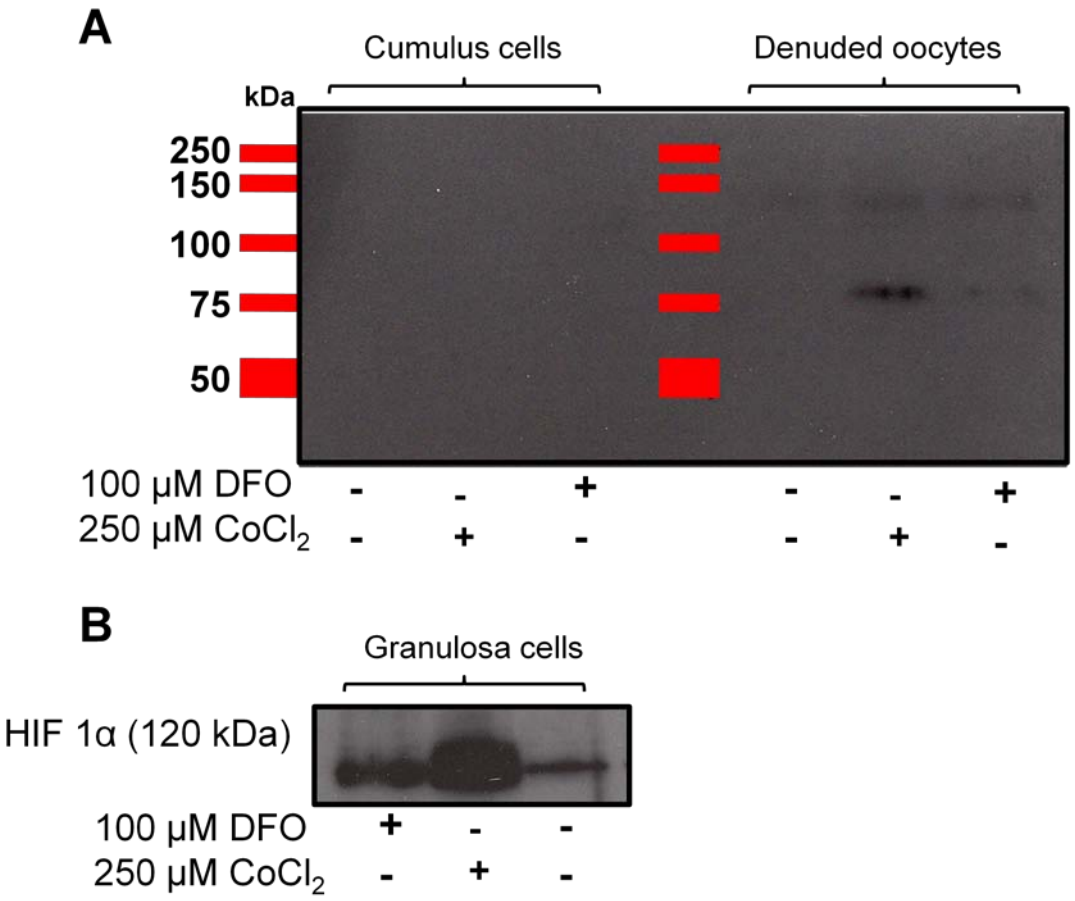

Figure 5.8 Western blot analyses **of HIF 1α** in cumulus cells, denuded oocytes and granulosa cells under different hypoxic mimetics.

In Western analyses panels A and B, cumulus cells, denuded oocytes and granulosa cells as described in Section 5.2.8.

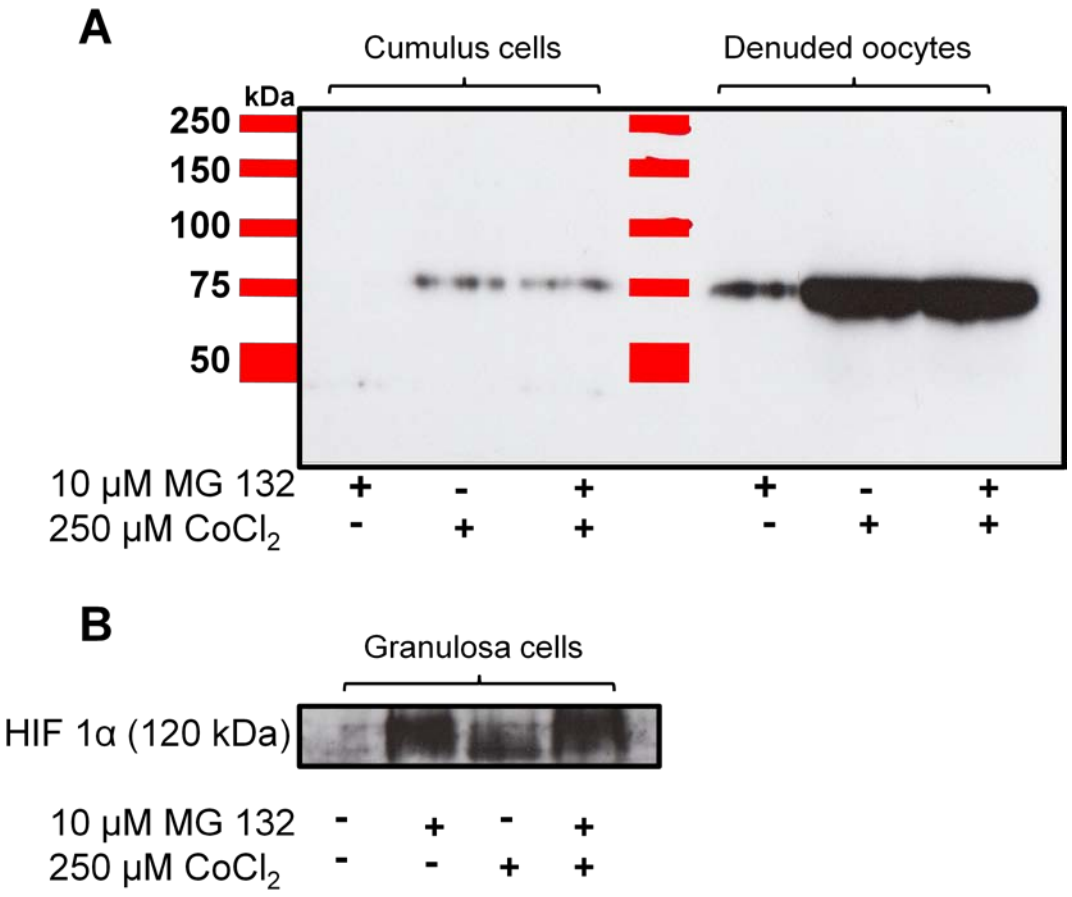

Figure 5.9 Western blot analyses of the effect of MG 132 on **HIF 1α** stabilisation in cumulus cells, denuded oocytes and granulosa cells.

In Western analyses panels A and B, cumulus cells, denuded oocytes and granulosa cells as described in Section 5.2.9.

5.3.7 Effect of GDF-9 on cumulus or mural granulosa cells.

As previously mentioned, the transdifferentiation of granulosa cells to form cumulus cells is tightly regulated by oocyte secreted factors, such as GDF-9. The aim of this experiment was to examine if transdifferentiating cumulus cells into granulosa cells would stabilise HIF 1α at 120 kDa and if mural granulosa cells under the influence of GDF-9 would differentiate cells into cumulus cells, therefore, suppressing HIF 1α at 120 kDa.

To determine if the addition of GDF-9 to mural granulosa cells can transdifferentiate mural granulosa cells into a cumulus cell phenotype, respective cells were cultured in the presence or absence of 50 ng/ml of GDF-9 (R&D Systems Inc, Minneapolis, MN, USA) *in vitro* as described in Section 5.2.10. Indeed, after 28 h, cumulus cells under the influence of GDF-9 were able to continue to differentiate into a plated cumulus cell phenotype whose morphology includes extensions branching out from cells to form connections with a neighbouring cell (Figure 5.10B) when compared to control (Figure 5.10A). On the other hand, mural granulosa cells, which were cultured in the presence of GDF-9 (Figure 5.10D) had a slightly more 'cumulus cell' phenotype compared to control at 28 h (Figure 5.10C). However, taking into consideration the cell density difference between cultured cumulus cells compared to granulosa cells, another subset of mural granulosa cells were plated down for a further 24 h to determine if longer incubation periods will promote transdifferentiation of granulosa cells into a more definite cumulus cell phenotype. Interestingly, mural granulosa cells cultured under the influence of GDF-9 for 48 h were morphologically no different to control (Figure 5.10; E and F).

5.3.8 The effect of GDF-**9 on HIF 1α pr**otein expression *in vitro*.

Lastly, to examine the effect of transdifferentiation of granulosa cells to cumulus cells under the influence of GDF-9 on HIF 1α, whole cell protein lysates were collected as described above. Western analyses showed a faint HIF 1α immunoreactive band at 50 kDa in cumulus cells treated with $CoCl_2$ in the absence of GDF-9.

However, mural granulosa cells under the influence of GDF-9 displayed a strong accumulation of the typical 120 kDa HIF 1α protein when compared to mural granulosa cells cultured only in the presence of $CoCl_2$ (Figure 5.11A). Interestingly, mural granulosa cells that were cultured for a total of 48 h under the influence of GDF-9 showed a higher accumulation of HIF 1α protein compared to 24 h (Figure 5.11B). These are the first results to demonstrate that GDF-9 promotes HIF 1α protein stabilisation in cultured mural granulosa cells.

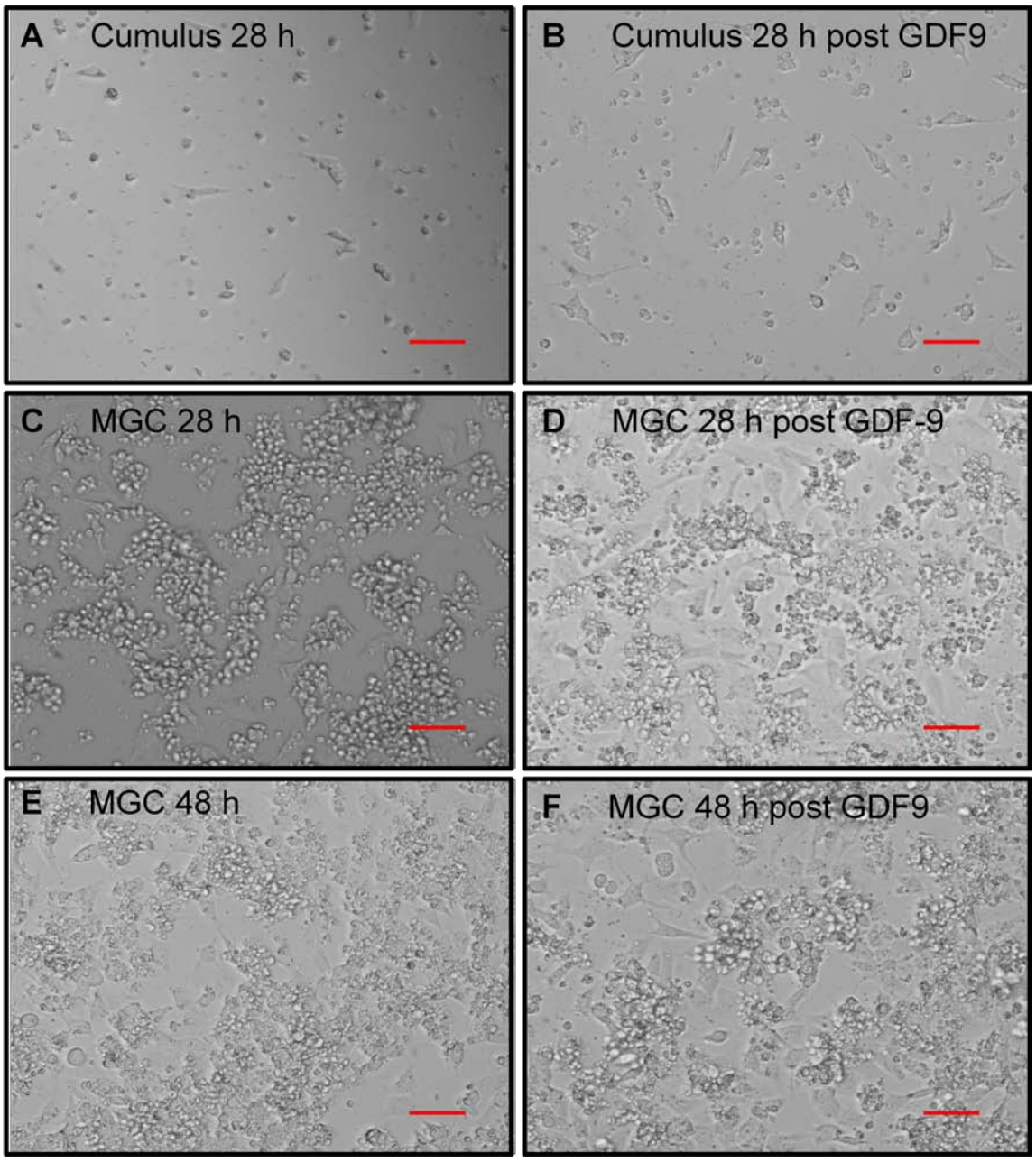

Figure 5.10 Effect of GDF-9 on cumulus and mural granulosa cells *in vitro*.

In panels A - F, 21 day old CBAB6F1 hybrid mice were treated with 7.5 IU eCG, 46 h later, mural granulosa cells and cumulus cells were collected and cultured as described in Section 5.2.10. All cultures were exposed to 250 µM $CoCl_2$ in the last 4 h.

Panel A, cumulus cells cultured for 28 h.

Panel B, cumulus cells cultured in the presence of GDF-9 for 28 h.

Panels C and E, mural granulosa cells cultured for 28 h and 48 h respectively.

Panels D and F, mural granulosa cells cultured in the presence of GDF-9 for 28 h and 48 h respectively.

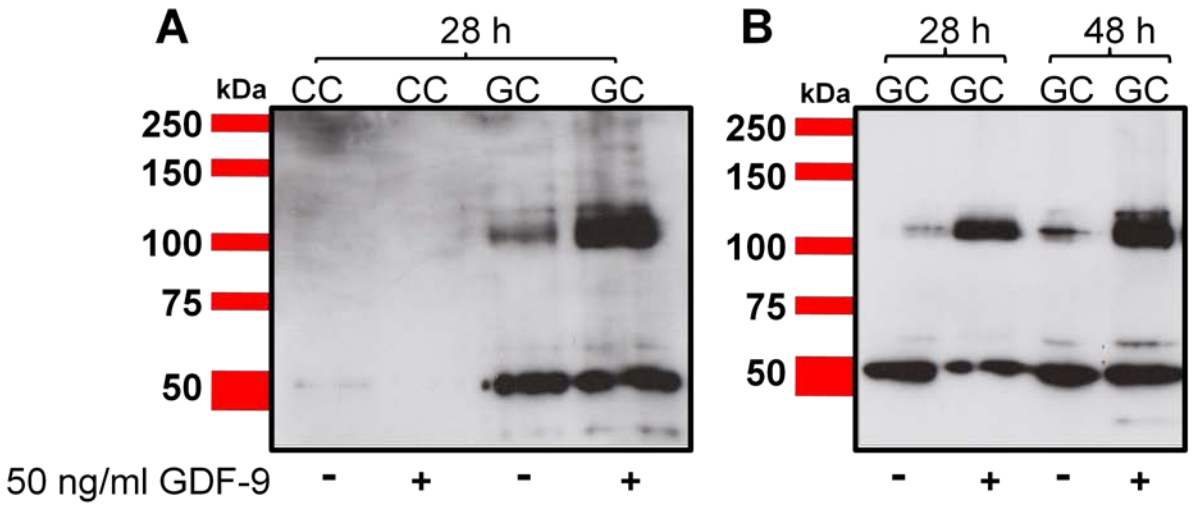

Figure 5.11 Western blot analyses **of HIF 1α** in cumulus cells and granulosa cells with the addition of GDF-9.

In panels A and B, 21 day old CBAB6F1 hybrid mice were treated with 7.5 IU eCG, 46 h later, granulosa cells (GC) and cumulus cells (CC) were collected and cultured in the presence or absence of 50 ng/ml GDF-9 as described in Section 5.2.10. HIF 1α was examined by Western blot analyses. All cultures were exposed to 250 μM $CoCl_2$ in the last 4 h and protein lysates were collected at times indicated.

5.4 Discussion

The environment of the COC during maturation is an important factor in determining oocyte developmental competence. Cumulus cells that surround the oocyte are important in maintaining metabolic activity (Eppig, 2005) and mediate the interaction of the oocyte within the ovarian follicular microenvironment through complex bidirectional interactions (Hussein *et al.*, 2006; Gilchrist *et al.*, 2008). Cumulus cells and oocytes share a close relationship and this relationship may be a potential mechanism affecting oocyte quality and subsequent development. Studies in several species have shown that removal of cumulus cells before *in vitro* maturation is detrimental to oocyte maturation (Vanderhyden & Armstrong, 1989; Zhang *et al.*, 1995; Lu *et al.*, 2009). Defining predictive markers in cumulus cells for oocyte health is the aim for much current oocyte biology research, as proposed in various species (Assidi *et al.*, 2008; Anderson *et al.*, 2009; Tesfaye *et al.*, 2009).

In the ovary, the COC lies within the avascular follicular environment, yet the cumulus cells have the ability to promote oocyte competence and display proliferative properties for cumulus expansion. This suggests that the regulatory mechanisms involved in COC maturation may be regulated by the oxygen concentration in which the COC is located. Therefore, the study of oxygen environment during *in vitro* maturation is important and it is also important to consider the impact on the cumulus cells. The present study aimed to analyse gene expression changes and HIF 1α protein stabilisation in cumulus cells exposed to varying oxygen concentrations during *in vitro* maturation.

The gene expression of nine of the twelve genes selected from the microarray varied in cumulus cells matured under varying oxygen concentrations. Low oxygen had a pronounced effect on most genes under investigations, especially in genes classified as HIF-regulated genes. HIFs are transcription factors that mediate a number of genes involved in physiological responses to low oxygen environments (Semenza, 1998; Semenza, 2000). The HIF family of genes contains hypoxia response elements (HREs) upstream of

target genes and induce transcription. It has been estimated that 1 - 2% of all human genes are regulated by hypoxia (Mazure *et al.*, 2004). There are other known members of the HIF superfamily which includes HIF 1α, HIF 2α/EPAS-1 and HIF 3α/IPAS. HIF 1α and HIF 2α share 48% amino acid sequence identity and 83% identity in the bHLH domains (Tian *et al.*, 1997), however, they may be differentially regulated depending on the duration and severity of hypoxic exposure (Wiesener *et al.*, 1998; Wiesener *et al.*, 2003; Holmquist-Mengelbier *et al.*, 2006).

The HIF responsive genes *Slc2a1, Ldha, Eno1, Pgk1, Bnip3* and *Ndrg1* were all up-regulated in cumulus cells following IVM at 2% and 5% compared to 20% oxygen. *Slc2a1, Ldha, Eno1* and *Pgk1* are involved in glucose metabolism suggesting these cells were activating the HIF response to low levels of oxygen (Figure 5.1 and 5.2). It is well established that genes involved in glycolysis are up-regulated during hypoxia (Semenza *et al.*, 1996; Rankin *et al.*, 2009). *Bnip3* was also up-regulated, increasingly so as oxygen decreased (at 2% and 5% there was ~ 9.6 and 7.6 fold change respectively compared to 20% oxygen). This gene belongs to the Nip3 protein family, is a member of the Bcl-2 family, and appears to be ubiquitously expressed (Chen *et al.*, 1997). Bnip3 is a well known proapoptotic factor and has been shown to accumulate dramatically in response to hypoxia in many cell lines (Bruick, 2000; Greijer & van der Wall, 2004). N-myc downstream regulated gene (*Ndrg1*) was also significantly increased (at 2% and 5% there was ~ 45.0 and 10.6 fold change compared to 20% oxygen) (Figure 5.1 and 5.2). Ndrg1 is an intracellular protein and a member of the NDRG gene family involved in cellular differentiation, proliferation, DNA damage response and tumour progression (Ellen *et al.*, 2008 ; Salnikow *et al.*, 2008) . Another study compared tumours (WHO grade 4 compared to WHO grade 2) and demonstrated that NDRG1 was over expressed (Said *et al.*, 2009). The up-regulation observed in our present study therefore suggests that the cumulus cells at 2% and 5% oxygen are under stress and activate protective physiological mechanisms to overcome these hypoxic stresses.

There was no change in *Hif1α* mRNA in cumulus cells following 17 h of IVM at 5%, compared to 20% oxygen; however, *Hif1α* mRNA was down-regulated in cumulus cells subjected to 2% oxygen compared to 20% oxygen. These results are similar to the study which demonstrates that prolonged and chronic exposure of mice to hypoxia decreases *Hif1α* mRNA but expression of HIF regulated genes such as *Aldo* mRNA increases (Wenger *et al.*, 1998). There are other studies that suggest that *Hif1α* mRNA appears to be down-regulated under prolonged and chronic hypoxia (Gradin *et al.*, 1996; Gassmann *et al.*, 1997) and acute hypoxia typically does not alter *Hif1α* mRNA expression (Gassmann *et al.*, 1997; Wenger *et al.*, 1997) but HIF 1α protein levels are stabilised under acute exposure to hypoxia (Wang *et al.*, 1995). This study further suggests that the kinetics of *Hif1α* mRNA expression is dependent on severity and duration of hypoxic treatment.

From the microarray analyses, there were a number of genes down-regulated in cumulus cells exposed to low oxygen conditions, compared to those exposed to 20% oxygen. From these genes, a common gene function emerged. Following IVM at 2% and 5% oxygen, cumulus cells were found to have a number of genes that play a role in fatty acid synthesis and metabolism down-regulated (Banwell, 2008). In this study, we analysed 5 of the genes involved in fatty acid synthesis that were identified by microarray as most highly down-regulated in cumulus cells exposed to low oxygen conditions. Real Time RT-PCR analyses determined that cumulus cell expression of only 2 of the genes (*Elovl6* and *Scd1*) was significantly down-regulated and the remaining 3 genes showed no significant differences in expression across the varying oxygen treatments during IVM.

Elongation of long-chain fatty acids family member 6 (*Elovl6*) belongs to a highly conserved family of endoplasmic reticulum enzymes as its name suggests, and is involved in the formation of long-chain fatty acids (Jakobsson *et al.*, 2006). Stearoyl-CoA desaturase 1 (*Scd1*) is also crucial in energy metabolism, insulin sensitivity and downstream pathway of lipid biosynthesis (Flowers *et al.*, 2006; Li *et al.*, 2006). SCD

catalyses the conversion of stearate, the end product of Elovl6, to oleate, the final product of endogenous lipogenesis (Matsuzaka & Shimano, 2009). To date, there is little evidence to prove the causative link between hypoxia and these genes involved in fatty acid synthesis, however, a study performed in mice that were exposed to intermittent hypoxia showed an up-regulation of SCD1 (Li *et al.*, 2006), it is important to note that this up-regulation was demonstrated in different cell type, conditions and the length of hypoxia. Recent evidence using microarray technology in HIF 1α and/or 2α mutant mouse livers demonstrated that the process of lipid metabolism and fatty acid synthesis were predominately regulated via HIF 2α (Rankin *et al.*, 2009). In this study, it is possible that the decrease in *Scd1* mRNA expression could partly contribute from the down-regulation of *Elovl6*. The RT-PCR performed in this study of *Elovl6, Scd1, Mecr, Inpp5e* and *Acss2* suggest that fatty acid synthesis and metabolism may not be affected by hypoxia during maturation *in vitro*.

As previously mentioned, cumulus cells are a derivative of granulosa cells that surround the oocyte. It was shown in earlier Chapters that HIF 1α protein can be stabilised upon stimulation with low oxygen environments and/or hypoxic mimetics in mural granulosa cells and in luteinised granulosa cells. This study was also able to demonstrate that HIF 1α protein was stabilised upon non-hypoxic stimulation (hCG) and was maximally induced around the time of ovulation yet I was unable to stabilise HIF 1α protein in cumulus cells during *in vitro* maturation. Experiments were performed where cumulus cells were collected and fully processed under 2% oxygen to ensure that the collection procedure did not result in HIF 1α degradation. Furthermore, we collected cumulus cells following exposure to 2 types of hypoxic mimetics (cobalt chloride and desferrioxamine) known to induce HIF 1α protein. HIF 1α at 120 kDa could not be stabilised in cumulus cells under any of these conditions. Proteasomal degradation inhibitor MG 132, which has been studied extensively and is known to prevent HIF 1α degradation was also used (Hagg & Wennstrom, 2005; Moroz *et al.*, 2009), however, only a 75 kDa HIF 1α polypeptide was evident when cumulus cells were cultured in the presence of both MG 132 and cobalt chloride.

GDF-9, an oocyte secreted factor, a member of the TGFβ superfamily known to promote granulosa cell differentiation into cumulus cells and prevent luteinisation (Diaz *et al.*, 2007; Gilchrist *et al.*, 2008; Su *et al.*, 2009) was shown to attenuate HIF 1α protein stabilisation in mural granulosa cells. This is not surprising, as there are several studies which have shown that TGFβ1 promotes HIF 1α stabilisation (Sanchez-Elsner *et al.*, 2001; McMahon *et al.*, 2006). Morphological analyses of GDF-9 induced differentiation of granulosa cells did not demonstrate phenotypic differences between control and granulosa cells exposed to GDF-9 after 48 h. Further experiments are necessary to determine successful transdifferentiation of mural granulosa cells under the influence of GDF-9, or other oocyte secreted factors to cumulus cells. Further analyses, such as to measure progesterone production (an indicator of luteinisation) in culture media or to analyse gene expression of *Slc38a3* mRNA (amino acid transporter) (Eppig, 2005) or (hyaluronan synthase 2) *Has2* mRNA (cumulus expansion endpoint markers) (Elvin *et al.*, 1999) should be pursued.

In addition, a recent review has demonstrated that oocytes play a role in secreting oocyte secreted factors (OSFs) via bidirectional communication to control metabolic activities such as glycolysis in cumulus cells (Su *et al.*, 2009). Results in this study demonstrated that glycolytic genes such as *Slc2a1*, *Ldha*, *Eno1* and *Pgk1* were up-regulated in cumulus cells from COCs matured *in vitro*, yet, HIF 1α protein was undetectable in cumulus cells. This paradox remains to be answered. One possibility is that HIF signalling is somehow suppressed in cumulus cells and that other oocyte-derived signalling is regulating cumulus cell function via paracrine signalling produced by the oocyte.

Lastly, my IVM protocol presents a chronic exposure to hypoxia, while studies have shown that HIF 1α tends to prefer acute exposure to hypoxia and the regulation is dependent on the duration and severity (Holmquist-Mengelbier *et al.*, 2006). However, HIF 1α could not be stabilised in cumulus cells even after acute exposure to the hypoxic mimetic. It is therefore possible that there may be other active isoforms of HIF present in cumulus cells. Future work such as utilising siRNA to down regulate HIF activity within

cumulus cells could be used to investigate if HIF target genes are unaffected under low oxygen conditions. The use of prolyl hydroxylases (PHD) inhibitors could also be used to determine if inhibiting the PHD pathway could lead to the up-regulation and stabilisation of HIF proteins in cumulus cells.

These results demonstrate a clear distinction between HIF activity within cumulus cells and mural granulosa cells. The current findings may lead to a new understanding of how follicular development, especially how granulosa cells differentiate into two subpopulations, may activate regulatory mechanisms very differently.

CHAPTER 6 Final discussion

6.1 Introduction

Folliculogenesis is the process by which the ovary produces a mature oocyte ready for fertilisation. This thesis explored the hypothesis that oxygen concentration is a significant regulator of folliculogenesis. This hypothesis has been proposed by others, suggesting that significant differentiation steps involved during the transitional processes of folliculogenesis are regulated by oxygen (Hirshfield, 1991; Redding *et al.*, 2007). Over the years, researchers have been trying to determine the *in vivo* oxygen concentration within follicles and have suggested that the oxygen levels are considerably lower than atmosphere (Van Blerkom *et al.*, 1997; Huey *et al.*, 1999). Further declining levels have been reported within the oviduct and uterus, and when this is mimicked *in vitro*, during embryo culture, it has been shown to improve subsequent outcomes in several species (Thompson *et al.*, 1990). It is well established that the Hypoxia Inducible Factor (HIF) family of transcription factors regulate oxygen-sensitive genes (see Chapter 1). Therefore, this study investigated the role of HIF signalling during folliculogenesis. Recent publications have advanced the knowledge concerning a role for HIF signalling during ovulation (Na *et al.*, 2008; Kim *et al.*, 2009) or during CL formation (Duncan *et al.*, 2008; van den Driesche *et al.*, 2008). However, this is the first study which utilises a HRE-EGFP transgenic mouse as a tool for visualisation of HIF activation and demonstrates a difference in HIF activation within follicle cells in the mouse ovary.

6.2 HIF activity during early folliculogenesis

The results in Chapter 3 demonstrated that EGFP, an indicator of HIF activation in the HRE-reporter mouse, was not detectable in primordial, primary and preantral follicles *in vivo* (Table 3.2). As granulosa cells proliferate forming multiple layers surrounding the oocyte, the avascular granulosa cell layer of the follicle is thought to inhibit O_2 diffusion and it has been proposed that the purpose of the antrum is to alleviate hypoxia in the growing follicle (Hirshfield, 1991). Mathematical modelling has been used to support this model, whereby the oxygen transportation is facilitated by an antrum (Gosden & Byatt-Smith, 1986;

Redding *et al.*, 2007). Interestingly during folliculogenesis, EGFP was only ever present in the theca, when follicles start developing a follicular antrum (Figure 3.3). In a sense, this supports my hypothesis, but with the caveat that I was expecting to observe EGFP within the granulosa cell layers and in cumulus cells surrounding the oocyte rather than the theca.

Studies have shown that FSH can stimulate HIF 1α protein in rat cultured granulosa cells via ERK/PI3K/Akt pathways (Alam *et al.*, 2004; Alam *et al.*, 2008). In contrast, there was no effect of FSH on HIF 1α protein induction nor its target genes within mouse granulosa cells in this study, however HIF 1α can be stabilised by low oxygen or hypoxic mimetics. These results suggest that either HIF signalling is not required prior to antrum formation or other members of the HIF family may be playing a role in preventing HIF 1α up-regulation. The Western blot of HIF 2α showed no stabilisation of HIF 2α protein in cultured granulosa cells (Figure 3.6). Although this suggests no participation by HIF 2α, the lack of a suitable mouse positive control must be taken into consideration. Another possible limitation in my study might be the moderately low oxygen concentration of 2% O_2 used. Nevertheless, it has been suggested that 2% is low enough to induce HIF 1α activity and target genes but is high enough to support oxidative phosphorylation in other cell types (Jiang *et al.*, 1996). In contrast, O_2 concentrations below 0.5% has been shown as limiting for oxidative phosphorylation (Chandel *et al.*, 2 000). A recent study performed in human embryonic stem cells demonstrated that a knockdown of HIF 3α resulted in the reappearance of HIF 1α protein (Forristal *et al.*, 2009). Previous studies have also demonstrated that IPAS, a splice variant of HIF 3α, acts as a dominant regulator of HIF 1α (Makino *et al.*, 2001; Makino *et al.*, 2002). Therefore, there are possibilities that HIF 3α, a dominant negative regulator of HIF 1α, may play a yet to be determined role during folliculogenesis prior to antrum formation. Thereby, more work is needed to unravel the influence of the other members of the HIF family, as it is possible that HIF 2α up-regulation or a down-regulation of HIF 3α could occur during early folliculogenesis. These unassessed HIFs may be the regulatory mechanism determining which follicles are destined to continue follicular growth and dominance.

Lastly, my data does suggest that the process of follicular development during early stages of folliculogenesis prior to antrum formation is not a oxygen-sensitive driven mechanism.

6.3 hCG induced HIF activity

In contrast, the present study identifies hCG as an important stimulator of HIF protein abundance *in vivo,* which is in agreement with the recent publication indicating that HIF signalling is a requirement for the process of ovulation (Kim *et al.*, 2009). Furthermore, hCG stimulated HIF 1α protein levels in luteinised granulosa cells, but only when in the presence of low oxygen or with a hypoxic mimetic. These results are in agreement with studies that have suggested the periovulatory follicle is highly hypoxic, which is resolved only after hCG administration, via a transient increase in blood flow (Fischer *et al.*, 1992).

During ovulation, the expression of both *eGfp* and *Vegfa* mRNA were up-regulated in response to hCG stimulation, which demonstrates the ability for up-regulation of HIF target genes following the ovulatory cascade signal. Interestingly, another chosen and well-characterised HIF 1α target gene, *Slc2a1* (Chen *et al.*, 2001) was unaltered in response to hCG. This observation suggests that not all HIF1-sensitive genes are up-regulated under the influence of hCG. This hormonally stimulated HIF activity may be quite gene selective. Interestingly, in support, *eGfp* mRNA abundance was unaltered in granulosa cells *in vitro* when cultured in the presence or absence of FSH and/or hypoxic mimetic (Figure 3.7E) although gene expression data of *Vegfa*, *Slc2a1* and *Ldha* were significantly up-regulated when granulosa cells were cultured under hypoxic mimetic alone or in combination with FSH (Figure 3.7, A - C). This specificity of the promoter construct within C57BL/6-Tg(HRE(4)-SV40-EGFP) mouse indicates that the promoter construct is not simply hypoxia regulated (although hypoxia is a well known inducer of HIF). Other non-hypoxic stimuli have been well documented to regulate HIF (Bilton & Booker, 2003). Thus, the transgenic mouse used here is definitely a valuable tool for identifying cells that may be HIF regulated, but further investigations are required to understand how hormones in other cell types interact with the reporter construct.

Additional observations in the transgenic mouse showed that EGFP was readily present in CLs of naturally cycling mice and the percentage of positive EGFP CL increased between early to mid pregnancy. However, HIF 1α protein levels were tending downwards during early to mid stages of pregnancy (Figure 4.10), although, more work is needed to investigate HIF signalling during CL regression in pregnancy. Studies in marmoset monkeys have shown cytoplasmic staining of HIF 1α during natural CL regression (Duncan *et al.*, 2008). Maturation of CL has also been associated with a decrease in *HIF1α* mRNA levels (Boonyaprakob *et al.*, 2005; van den Driesche *et al.*, 2008). These studies do suggest that a down-regulation of HIF 1α at both the transcriptional and translational level occurs during CL regression and possibly explains the withdrawal of vascularity in the regressing CL.

6.4 HIF activity in cumulus cells & oocytes

Previous work by other laboratory members had demonstrated through microarray that the expression of HIF target genes was altered in cumulus cells following *in vitro* maturation (IVM) at varying oxygen concentrations 2, 5, 10 and 20% (Banwell, 2008). To assess whether these genes were HIF regulated, firstly this study established that low oxygen had a pronounced effect on cumulus cell gene expression during *in vitro* maturation (IVM) (Figure 5.1 and 5.2) and this provided further support for a role for HIFs in the regulation of cumulus cell function. Surprisingly, HIF 1α protein stabilisation was never evident in cumulus cells at 120 kDa. However, a polypeptide at 75 kDa was faintly detectable occasionally (Figure 5.5). This is despite manipulating a variety of incubation conditions, as described in Chapter 5. In contrast, within oocytes, there was some evidence for the 120 kDa band, but this too was variable and the predominant band was at 75 kDa. There is little obvious explanation as to why mural granulosa cells have a clear 120 kDa band and cumulus cells have virtually no band at all. In an attempt to view any HIF 1α expression, I tried various treatments, including hypoxia mimetics (Figure 5. 8B), proteasome inhibition (Figure 5.9B) and GDF-9 (a member of the oocyte specific TGFβ superfamily) (Figure 5.11). Furthermore, a

different culture medium was used for lutein granulosa cells cultures and was still able to stabilise the 120 kDa HIF 1α under low oxygen or under a hypoxic mimetic (Figure 4.3). Therefore these data collectively indicates strongly that there are differences in HIF 1α polypeptides present in different follicle cells and different cell types may therefore vary with respect to their HIF 1α polypeptide variation. This is made even more complicated by the lack of EGFP in our transgenic mouse model in either granulosa cells or COCs (when observed).

Western blot revealed that HIF 1α at 120 kDa was never evident in cumulus cells and neither in COCs (Figure 5. 5). However, when cumulus cells were removed prior to protein lysis, the resultant denuded oocyte stabilised HIF 1α at 120 kDa (Figure 5.8A) and suggests mechanisms in CC which could suppress regulation of HIF 1α at the expected molecular mass of ~120 kDa. This could be either a failure for translation of HIF 1α or the presence of a dominant regulator, such as HIF 3α.

Although not conducted here, our laboratory has evidence that HIF 1α protein is found in cattle cumulus cells, and that HIF 1α can be detected in cumulus cells by immunohistochemistry although its function has not been assessed (Harvey, 2003). Another common observation throughout my study was the presence of a 50 kDa polypeptide in granulosa cells and in luteinised granulosa cells which was initially thought of as a degradation product of HIF 1α, but this peptide band was never present in denuded oocytes, COCs or in cumulus cells, with the exception of one experimental replicate of cumulus cells cultured for a period of 28 h with the addition of hypoxic mimetic in the last 4 h of culture (Figure 5.11). However, this observation of a 50 kDa peptide in cumulus cells requires replication and was not carried out due to time constraints. The question still remains as to whether the 75 kDa and 50 kDa peptides observed are isoforms or variants of HIF 1α, or simply degradation products. Future characterisation, such as the utilisation of proteomic technology to identify the sequence of the HIF 1α polypeptide variants within the different follicle cells would

help elucidate their role(s) and further our understanding of how HIF 1α translational mechanisms work and ultimately HIF 1α regulation within different cell types.

Additionally, gene expression results from cumulus cells collected after IVM under various O_2 analysed demonstrated that some of the genes involved in fatty acid metabolism were not altered and a recent publication suggested that HIF 2α played a central role in lipogenesis and fatty acid synthesis (Rankin *et al.*, 2009). This may suggest that HIF 2α rather than HIF 1α may be present in cumulus cells as it has also been shown that regulation of HIFs are highly dependent on the duration and severity to hypoxia (Holmquist-Mengelbier *et al.*, 2006). Therefore which HIF is active, remains unanswered and it would therefore be essential to optimise HIF 2α Western blot techniques in order to answer these questions.

6.5 A hypothesis: PHD activity regulating HIF target genes during prolonged hypoxia?

Is there a possible mechanism by which HIF protein is not present in cumulus cells, yet HIF sensitive genes remain up-regulated? As previously described in Chapter 1, it is well established that Prolyl-4-hydroxylase domain (PHDs) proteins are oxygen dependent enzymes that hydroxylates HIF-α subunits, leading to their subsequent ubiquitination and degradation in normoxic conditions. Studies have demonstrated that cells exposed to acute hypoxia inhibit PHD activity, leading to HIF stabilisation. In contrast, prolonged hypoxia can lead to diminished HIF 1α and HIF 2α protein levels but HIF mRNA level remains unaltered (Stiehl *et al.*, 2006; Ginouves *et al.*, 2008). HIF 1α target genes such as *Slc2a1* (Chen *et al.*, 2001) and *Ca9* (Kaluz *et al., 2003*) mRNA levels remain elevated during prolonged hypoxia and the hypoxic induction of PHD2 and PHD3 proteins is accompanied by decreased HIF 1α protein levels (Stiehl *et al.*, 2006). A HIF desensitisation mechanism is required to protect cells against necrotic cell death, a protective mechanism during prolonged hypoxia (Ginouves *et al.*, 2008). I propose that this mechanism accounts for the absence of cumulus cell HIF, despite significant HIF-sensitive gene expression. Further work could involve the addition of the hydroxylase inhibitor, dimethyloxallyl glycine (DMOG), to determine if the inhibition of these

PHD pathways could lead to the up-regulation of HIF proteins in cumulus cells maintained under low oxygen conditions.

6.6 Another hypothesis: Does the oocyte act as a "defacto" HIF regulator?

Is there another possible mechanism by which the oocyte acts as a defacto for the suppression of HIF signalling in cumulus cells? Oocytes are deficient in their ability in carrying out glycolysis and require cooperation from cumulus cells in up regulating glycolytic genes necessary for developmental competence (Eppig, 2005; Sugiura *et al.*, 2005). Numerous studies and a recent review have demonstrated that oocytes play a role in secreting oocyte secreted factors (OSFs) via bidirectional communication to control metabolic activities in cumulus cells (Su *et al.*, 2009). My results demonstrated that glycolytic genes such as *Slc2a1*, *Ldha*, *Eno1* and *Pgk1* were significantly up-regulated in cumulus cells from COCs matured *in vitro* (Figure 5.1 and 5.2). Furthermore, cumulus cells in the absence of GDF-9 displayed a faint HIF 1α signal at 50 kDa (Figure 5.11), which was previously undetectable in any other cumulus cell Western analyses, yet the HIF 1α at 120 kDa were detectable in denuded oocytes under different hypoxic mimetics (Figure 5.8A). Therefore, these data suggest a possible mechanism in which HIF signalling within the cumulus cells is suppressed via the oocyte, possibly by oocyte secreted factors. The oocyte, through its own endogenous HIF, senses O_2 concentration and responds by the secretion of paracrine factors that mediate a "defacto" HIF response in cumulus cells. Although highly speculative, this hypothesis has some appeal as it promotes the concept that the oocyte reacts to environmental cues and responds to alter cumulus cell behaviour. Further work could involve oocytectomy (OOX), which is the process by which oocytes are microsurgically removed from their complexes to determine if OOX prior to culture in a low oxygen environment could lead to an activation of HIF activity in cumulus cells.

6.7 Future directions and therapeutic benefits

A number of recent studies have demonstrated that macrophages respond to hypoxia by up-regulating HIF 1α and HIF 2α protein (Elbarghati *et al.*, 2008; Fang *et al.*, 2009) and it is well documented that macrophages are rapidly recruited to areas of hypoxic regions within tumours (Murdoch *et al.*, 2004). In antral follicles, macrophages are localised within the theca and their numbers increase at ovulation and are believed to play a role in facilitating follicle rupture (Brannstrom *et al.*, 1993). Although preliminary observations in this study indicated that the F4/80 antibody (a well-characterised macrophage marker) (Austyn & Gordon, 1981) did not co-localise with EGFP cells within ovarian sections of naturally cycling transgenic mice, the identity of the EGFP cells in transgenic ovarian sections post hCG remains to be elucidated. It is still possible that these cells are macrophages.

As previously mentioned in Chapter 5, it has been suggested that treating OOX with oocyte secreted factors (OSFs) fully restores CC characteristics demonstrating that oocytes suppress FSH induced GC differentiation towards luteinisation (Eppig *et al.*, 1997). Therefore, treating MGC with GDF-9 promotes a functional cumulus cell phenotype (Gilchrist *et al.*, 2001; Gilchrist *et al.*, 2006). However, preliminary results of the effect of GDF-9 on cumulus cells and mural granulosa cells *in vitro*, suggests that granulosa cells under the influence of 50 ng/ml of GDF-9 did not promote a transition towards a cumulus cell phenotype (Figure 5.10). In fact, the morphology of mural granulosa cells after 48 h of culture with GDF-9 suggested that GDF-9 did not prevent luteinisation. Future investigation is required, such as gene expression analyses of anti-Mullerian hormone (a cumulus cell specific marker) (Salmon *et al.*, 2004) or Luteinising hormone receptor and progesterone receptor (specific for mural granulosa cells) (Eppig *et al.*, 1997; Robker *et al.*, 2000) or even measuring the production of progesterone to identify the phenotype of treated and untreated cells. The induction of 120 kDa HIF 1α protein was more abundant in mural granulosa cells cultured under the influence of GDF-9 for 28 h, and this was further accentuated by 48 h culture (Figure 5.11). This is the first evidence that oocyte secreted factors may regulate HIFs in follicular cells and warrants further

examination. Could there be a role for HIF activity in polycystic ovary syndrome (PCOS) which affects 10% of women during their reproductive years? Polycystic ovaries (PCO) have been defined by the presence of ≥ 12 or more follicles (Jonard *et al.*, 2003). In PCO, several follicles are recruited but the growth of the follicles is restricted and ultimately the dominant follicle cannot proceed because of follicular arrest which has been associated with low levels of blood supply (Jarvela *et al.*, 2004). Results in Chapter 3 and 4 demonstrated that HIF 1α activity is important in regulating *Vegfa* during ovulation and CL development, and HIF is present in the theca of antral follicles. This may suggest HIF signalling is defective in patients with PCOS and therefore an opportunity to promote follicular blood vascularity and ultimately promote ovulation and subsequent CL growth may lie in stimulating HIF activity *in vivo*. In contrast, preventing HIF activity, by suppressing HIF 1/2 or by stimulating HIF 3α, during ovulation and CL formation may decrease fertility and provide a novel contraceptive therapy.

Although such therapeutic value is highly speculative, recently an integrated EU Framework project 2004-2009, termed EUROXY was established which involved numerous collaborators whom are experts in the HIF field. They demonstrated several pathways involved in transcription and translation control of hypoxic cell phenotype (Ebbesen *et al.*, 2009) and a recent study demonstrated that the anti-HIF 1α siRNA was effective in controlling tumour growth in mice by silencing the expression of HIF 1α protein. They utilised a method of delivering siRNA nanoparticles with tumour specific peptides which specifically bound to receptors that are over expressed in various cancers, including prostate, breast, lung and colon, ovarian and pancreatic cancers and malignant gliomas (Wang *et al.*, 2009). Such collaborative ventures may well open the door for the use of HIF to regulate ovarian events and reiterates the importance of studying the role of HIFs in ovarian physiology.

6.8 Conclusions

This study has established that prior to antrum formation, HIF 1α signalling within follicles is not detectable and established that the transitional stages, especially during the initiation of antrum formation, follicular differentiation and luteinisation involves HIF activity. These data have demonstrated that HIF 1α signalling is highly active during the ovulatory cascade and also in the formation and maintenance of the CL. Although gene expression results provided implications of HIF activity within cumulus cells, the study has yet to determine the role of HIF stabilisation in cumulus cells. I have proposed novel hypotheses to further explore this enigma. Nevertheless, the present study demonstrates a clear distinction in the regulation of HIF activity and stabilisation between different follicular cell types within antral follicles. Although there is much more to understand, my studies have contributed a foundation of new knowledge, which was previously unknown, regarding the role of HIFs and oxygen sensitivity in the function of the mouse ovary.

7.1 APPENDIX

MEDIA AND SOLUTIONS

All chemicals were obtained from Sigma unless otherwise stated. All media and solutions were filtered via a 0.2µm filter and stored at 4 ºC unless otherwise stated.

7.1.1 Stock solutions for granulosa cells and granulosa lutein cell culture

HEPES-TCM199

Dissolve 1 sachet of TCM-199 (MP Biomedicals; Catalogue: 1020120) in 1 L MQ water, add 50 mg Kanamycin Sulphate, 1.78 g of Hepes (free acid, 7.5 mM), 1.95 g Hepes (Na salt, 7.5 mM) and 420 mg $NaHCO_3$. Measure and adjust pH to 7.4.

Dulbecco's Modified Eagles Medium (DMEM)

Dissolve 1 sachet of DMEM (MP Biomedicals Catalogue: 1033120) in 1 L of MQ water, add 3.7 g $NaHCO_3$. Measure and adjust pH to 7.42.

HAMS F12

Dissolve 1 sachet of HAMS F12 (MP Biomedicals Catalogue: 1042120) in 1 L of MQ water, add 1.17 g $NaHCO_3$Sodium Bicarbonate. Measure and adjust pH to 7.42.

Media for granulosa cell culture

Granulosa cell handling media

To beaker with 100 ml HEPES-TCM199, add 300 mg Bovine Serum Albumin (BSA), place in 37.5 ºC incubator, once BSA has dissolved, filter and store.

Granulosa cell plating media

24 ml DMEM

24 ml HAMS F12

1 ml Penicillin-Streptomycin (CLS Biosciences, Catalogue: 05081901)

500 µl L-Glutamine (ICN Biomedicals inc; Costa Mesa, CA, USA, Catalogue: 16-801-49)

500 µl Fetal Calf Serum (FCS)

Filter and store at 37.5 ºC.

Media for granulosa lutein cell culture

Granulosa lutein handling media

To beaker with 100 ml HEPES-TCM199, add 300 mg Bovine Serum Albumin (BSA), place in 37.5 ºC incubator, once BSA has dissolved, filter and store at 37 ºC.

Granulosa lutein plating media (without FSH)

24 ml DMEM

24 ml HAMS F12

1 ml Penicillin-Streptomycin

500 µl Testosterone (10 ng)

500 µl L-Glutamine

Filter and store at 37.5 ºC.

Granulosa lutein plating media (with FSH)

Same media composition as granulosa lutein plating media with the addition of 50 mIU rhFSH.

7.1.2 Stock solutions for *In Vitro* Maturation (IVM) of murine cumulus oocyte complexes

2X αMEM (makes Bicarbonate buffered and HEPES) stock

Makes 2 x 250 ml of stock solution at 2X concentration.

Dissolve 1 sachet of αMEM powder (Gibco, Catalogue: 12000-014) in 200 ml Milli Q water. Divide equally between two 250 ml volumetric flasks.

For bicarbonate buffered (Maturation media)

To one flask add 1100 mg $NaHCO_3$, 25 mg Streptomycin Sulphate and 37.5 mg Penicillin G dissolved into a small amount of water (approximately 20 ml).

For HEPES (Handling media)

To the flask add 252 mg $NaHCO_3$, 2380 mg HEPES, 25 mg Streptomycin Sulphate and 37.5 mg Penicillin G dissolved into a small amount of water (approximately 20 ml).

Fill up both flasks to 250 ml with MQ water, filter and store for a maximum of 4 weeks at 4 ºC.

Media for IVM of murine cumulus oocyte complexes

Following media were made up, filtered one day prior to use.

For bicarbonate buffered (Maturation culture media)

Makes 40 ml.

To 20 ml of 2X αMEM bicarbonate buffered stock, add 17.8 ml of MQ water, 2 ml of non-heat inactivated FCS and 200 µl of (50 mIU) rhFSH(Puregon; Organon, Sydney Australia).

For HEPES (Handling media)

Makes 40 ml.

To 20 ml of 2X αMEM HEPES stock, add 18 ml MQ water and 2 ml of non-heat inactivated FCS.

7.1.3 Stock solutions for Western blotting

All solutions for Western blotting are stored at room temperature unless indicated.

5M NaCl

Dissolve 292.2 g of NaCl in 1 L of MQ water.

1M Tris-HCl pH 7.5

Dissolve 121.1 g Tris in 800 ml MQ water, measure and adjust to pH 7.5.

1.5 M Tris-HCl pH 8.8

Dissolve 27.23 g of Tris in 80 ml MQ water, measure and adjust to pH 8.8.

10X Running Buffer

30.3 g Tris

144 g Glycine

10 g SDS

Bring to 1 L with MQ water.

Solutions for Western Blotting

TBS + 0.05%Tween 20 (TBST)

30 ml 5 M NaCl

20 ml 1 M Tris-HCl pH 7.5

500 µl Tween 20

950 ml MQ water

5% Skim milk/ TBST

Dissolve 5 g of Skim milk in 100 ml TBST.

Phosphate buffered saline with 0.05% Tween20 pH 7.4 (PBST)

Dissolve 1 PBST tablet (Fluka, Biochemika, Catalogue: 08057) in 500 ml MQ water.

Running buffer

To 950 ml of MQ water, add 50 ml of 10X Running Buffer stock.

Transfer buffer

30 g of molecular grade Glycine

15 g of Ultrapure Tris

Dissolve in 2 L MQ water

Basic 1X Laemmli Sample Buffer

2% SDS

10% glycerol

0.002% bromphenol blue

0.063 M Tris HCl

5% β-mercaptoethanol

The solution has a pH of approximately 6.8

5X solution

10% SDS

50% glycerol

0.01% bromphenol blue-

0.313 M Tris HCl

Add β- mercaptoethanol prior to use

7.1.4 Solutions for immunofluorescence

Phosphate Buffered Saline

Dissolve 1 PBS tablet (Fluka, Biochemika, Catalogue: 23707208) in 200 ml MQ water.

18 % sucrose

180 g of Sucrose in 1L MQ water.

4 % Paraformaldehyde

4 g Paraformaldehyde

100 ml PBS

3 - 5 drops of 1M NaOH

Heat solution to 60 ºC on rotary shaker, filter in fume hood and store in a falcon flask at 4 ºC.

7.1.5 Haematoxylin and Eosin (H&E for frozen sections)

1. Thaw slide at room temperature.
2. Place slide in 2 changes of 100% ethanol for 2 min each.
3. 90% ethanol for 2 min and 70% ethanol for 2 min.
4. Wash briefly in MQ H_2O.
5. Stain in haematoxylin solution for approximately 3 - 4 min.
6. Rinse in running tap water for 5 min.
7. Rinse in MQ H_2O .
8. Counterstain in eosin solution for 1 min.
9. Dehydrate through 70% ethanol, 2 changes of 100% ethanol, 2 min each.
10. Mount in DPX mountant and coverslipped.

7.1.6 Vaginal smearing morphology

Females were checked daily between 0700 – 0900 h for external vaginal changes. Vaginal smears were collected daily using a pipette containing 20 µl of saline inserted gently into the vagina. It was taken into consideration that mechanical stimulation can induce a pseudopregnancy. The saline containing the vaginal fluid was then transferred onto a glass slide to determine cytological constituents of smear. Proestrus smears contained mostly nucleated epithelial cells and small numbers of cornified epithelial cells. Vaginal estrus smears comprised of only cornified cells that were irregularly shaped and maximally scattered. Metestrus smears contained scattered and clumped cornified epithelial cells and string mucus, and sometimes leukocytes. Diestrus smears consisted of non-cornified epithelial cells, string mucus and large numbers of leukocytes.

8.1 Bibliography

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