

**The Control and Manipulation of Angiogenesis
in the Primate Ovarian Follicle**

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Declaration

I hereby declare that this thesis has been composed by myself and is my own work. Any contribution of others has been fully acknowledged. The work described in this thesis has not been submitted in full or in part for any other degree, diploma or professional qualification.

Paul Taylor

May 2005

Dedication

For my family and my friends

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Abbreviations

3 β HSD	3 β -hydroxysteroid dehydrogenase
11 β HSD1	11 β -hydroxysteroid dehydrogenase type 1
11 β HSD2	11 β -hydroxysteroid dehydrogenase type 2
ACE	adrenal-cortex-derived capillary endothelial
ADAMTS-1	A disintegrin and metalloproteinase with thrombospondin-like motifs
aFGF	acidic fibroblast growth factor
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
ANOVA	analysis of variance
ANSA	8-anilino-1-naphthalenesulphonic-acid
AMH	anti-müllerian hormone
APAAP	alkaline phosphatase-anti-alkaline-phosphatase
AS-MX	3-hydroxy-2-naphthoic acid 2,4-Dimethylanilide
bFGF	basic fibroblast growth factor
BMP-7	bone morphogenic protein-7
bp	base pair
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
CAM	chick chorioallantoic membrane
cAMP	cyclic adenosine monophosphate
CD31	cluster differentiation factor 31
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CL	corpus luteum
COX-2	cyclooxygenase-2
cpm	counts per minute
CRE	cAMP response element
DAB	diaminobenzadine
DARS	donkey anti-rabbit serum

DAGS	donkey anti-goat serum
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EGF	epidermal growth factor
EG-VEGF	endocrine gland vascular endothelial growth factor
FDA	Food and Drug Administration
Flt	<i>fms</i> -like tyrosine kinase
FSH	follicle stimulating hormone
GDF-9	growth differentiation factor-9
GnRH	gonadotrophin releasing hormone
GPCR	G protein-coupled receptor
H & E	haematoxylin and eosin
hCG	human chorionic gonadotrophin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HGF	hepatocyte growth factor
ICD	intercapillary distances
IGF	insulin-like growth factor
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl- β -D-thiogalactopyranoside
ISNT	<i>in situ</i> nick translation
i.v.	intravenous
IVF	<i>in vitro</i> fertilisation
KDR	kinase insert domain containing region
KGF	keratinocyte growth factor
LH	luteinising hormone
LIF	leukaemia inhibitory factor
LSD	least significant difference
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging

mRNA	messenger ribonucleic acid
MVD	microvessel density
NBF	neutral buffered formalin
NBT	nitro blue tetrazolium
NGS	normal goat serum
NRS	normal rabbit serum
NSB	non-specific binding
OHSS	ovarian hyper-stimulation syndrome
P450 _{arom}	cytochrome P450, aromatase
P450 _{c17}	cytochrome P450, 17 α hydroxylase
P450 _{scc}	cytochrome P450, side chain cleavage
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PG	prostaglandin
PGF _{2α}	prostaglandin F _{2α}
PK-1	prokineticin-1
PKA	protein kinase A
PKI	protein kinase inhibitors
PIGF	placental growth factor
QC	quality control
rATP	riboadenosine triphosphate
rCTP	ribocytosine triphosphate
rGTP	riboguanine triphosphate
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcription
rUTP	ribouridine triphosphate
SEM	standard error of the mean
SSC	salt sodium chloride

StAR	steroidogenic acute regulatory protein
TBS	Tris buffered saline
TEA	triethanolamine
TEM	tumour endothelial marker
TGF β	transforming growth factor β
Tie	tyrosine kinase with immunoglobulin and epidermal growth factor homology domains
TNF α	tumour necrosis factor- α
tRNA	transfer ribonucleic acid
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP nick end labelling
UTP	uridine triphosphate
UV	ultra violet
VEGF	vascular endothelial growth factor
VEGFR1	vascular endothelial growth factor receptor 1
VEGFR2	vascular endothelial growth factor receptor 2
VPF	vascular permability factor
VPRA	venom protein A
X-gal	5-bromo-4-chloro-3-indolo- β -D-galactopyraniside

Publications and Presentations Relating to this Thesis

Publications in Peer Reviewed Journals

Taylor PD, Hillier SG, Fraser HM (2004) Effects of GnRH antagonist treatment on follicular development and angiogenesis in the primate ovary. *J Endocrinol* 183(1):1-17

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Oral Presentation

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Poster Presentations

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Abstract of Thesis

The ovary is one of the most plastic tissues in the body undergoing constant serial remodelling throughout its reproductive lifetime, during both folliculogenesis and the formation and regression of the corpus luteum. The process of follicle growth and selection is intimately associated with the *de novo* establishment of vasculature supporting the developing follicles. Blood vessels are recruited from the ovarian stroma to form vascular sheaths surrounding each developing follicle supplying steroid hormone precursors, oxygen and nutrients to the expanding follicle. During folliculogenesis only the theca of the developing follicle becomes vascularised, the granulosa cells remaining avascular until ovulation at which point the basement membrane that has been separating the granulosa and theca breaks down. After ovulation the granulosa cells become heavily vascularised during the process of luteinisation and the formation of the corpus luteum. Angiogenesis, the growth of new blood vessels from the pre-existing vasculature, requires the degradation of the established vessels followed by endothelial cell proliferation and finally stabilisation of the new vessels. Recently, techniques to quantify angiogenesis, identify putative molecular regulators, and inhibit them *in vivo* have become available. The work in this thesis applies these advances to the following questions:

1) What is the effect of the inhibition of the gonadotrophins, using a gonadotrophin releasing hormone (GnRH) antagonist, on follicular angiogenesis? The hypothesis being tested was that follicular angiogenesis would be dependent on follicle stimulating hormone (FSH) / luteinising hormone (LH) and be severely inhibited by GnRH antagonist treatment. *In vivo* follicular angiogenesis was assessed by quantitative immunocytochemistry of bromodeoxyuridine and the endothelial cell marker CD31. The effect of treatment on follicular development and angiogenesis at the molecular level was assessed by *in situ* hybridisation of mRNA for vascular endothelial growth factor (VEGF) and aromatase. The results suggest that while VEGF expression in the preovulatory follicle is under gonadotrophic control, it is not dependent on normal gonadotrophin secretion in tertiary follicles, indicating that there are other paracrine factors regulating VEGF expression in the

developing ovarian follicle. The second chapter extends the findings by determining granulosa cell response to FSH stimulation with respect to induction of the VEGF and aromatase genes.

2) What is the effect of inhibition of vascular endothelial growth factor, using the antagonist, VEGF trap R1R2, on follicular angiogenesis, follicular development, ovulation and the establishment of the corpus luteum (CL)? The hypothesis being tested was that VEGF is essential for increasing permeability and the growth of the selected follicles. The immunocytochemical techniques used in the first study were again employed. The effect of treatment on the molecular regulation of angiogenesis was assessed by *in situ* hybridisation of mRNA for VEGF and its two receptors. *In vivo* inhibition of VEGF caused dramatic reductions in angiogenesis and in VEGF receptor expression but did not reliably prevent dominant follicle growth or ovulation once dominant follicle selection had occurred.

3) Is a novel factor, endocrine gland vascular endothelial growth factor (EG-VEGF) expressed in our animal model? The hypothesis being tested was that EG-VEGF is an additional angiogenic factor that is expressed in the marmoset and the human ovary. This was assessed by *in situ* hybridisation in various marmoset tissues as well as in the human corpus luteum. Findings demonstrated that EG-VEGF is expressed in the granulosa lutein cells in the human corpus luteum while the marmoset ovary does not appear to express EG-VEGF.

This thesis has improved our understanding of the gonadotrophic control of follicular angiogenesis and the role VEGF plays in the latter stages of folliculogenesis.

Chapter 1 Introduction

1.1 Overview

Angiogenesis is the growth of new blood vessels from the pre-existing vasculature, and requires the degradation of these vessels, followed by endothelial cell proliferation and finally stabilisation of these new vessels. Physiological angiogenesis is rare in the healthy adult apart from in the female reproductive system, with both the ovaries and uterus undergoing serial angiogenesis. In the ovary, cyclic angiogenesis occurs during folliculogenesis and the formation of the corpus luteum. It is the regulation of ovarian follicular angiogenesis that is the subject of this thesis.

The function of the ovarian follicle is to produce a fertilisable oocyte at ovulation and to prepare the reproductive tract for pregnancy. Many follicles initiate development during the female reproductive lifetime; however, through the process of dominant follicle selection only one (human), or two to three (marmoset monkey) preovulatory follicle(s) develops per ovarian cycle.

The process of folliculogenesis and dominant follicle selection is intimately associated with angiogenesis. Blood vessels are recruited from the ovarian stroma to form vascular sheaths surrounding each developing follicle. The new vasculature supplies steroid hormone precursors, oxygen and nutrients to the developing follicle. The extent of follicle vascularisation has also been hypothesised to play an important role in the process of selection of the dominant follicle. During folliculogenesis, only the theca of the developing follicle becomes vascularised; the granulosa cells remain avascular until ovulation, where the basement membrane that has been separating the granulosa and theca breaks down. After ovulation, the granulosa cells become heavily vascularised during the process of luteinisation and the formation of the corpus luteum. Vascular endothelial growth factor (VEGF) is known to be one of the key regulators of ovarian follicular angiogenesis.

This chapter reviews the current understanding of the factors regulating ovarian follicle development and the angiogenic process. It focuses on experimental evidence from studies in women and non-human primates. However, where there is a lack of available evidence, extrapolation from findings in other species is also used. First the process of folliculogenesis and ovulation is discussed, followed by the

control of angiogenesis, including the history, molecular regulation, and therapeutic relevance of inhibiting angiogenesis. This chapter serves as an introduction to the following experimental chapters that report studies on the regulation and inhibition of angiogenesis in the primate ovarian follicle.

1.2 Pituitary-Ovarian axis

Ovarian function is controlled by the brain through the secretion of messages from the hypothalamus that act on the ovary via the gonadotrophins: follicle stimulating hormone (FSH); and luteinising hormone (LH). The signal from the hypothalamus to the pituitary is gonadotrophin releasing hormone (GnRH). GnRH is a decapeptide secreted in a pulsatile manner by the hypothalamus into the hypophysial portal blood system that connects the hypothalamus and the pituitary gland. GnRH is perceived by GnRH receptors at the anterior pituitary, and activation of these receptors stimulates the production and release of both FSH and LH from the anterior pituitary. FSH and LH are both heterodimeric polypeptides with a common α -subunit and a structurally related, but unique, β -subunit that gives them their biological specificity, this is reviewed by Clarke (1996).

1.3 Folliculogenesis

The structures we now recognise as ovarian follicles were first noted in 1672 by Graaf. Whilst examining human ovaries he mistook the developing follicles for what he thought to be ova (Jocelyn and Setchell 1972).

Folliculogenesis is the growth of ovarian follicles from a primordial or dormant state to the preovulatory stage of development. For many years it has been thought that primordial follicle formation only occurred during foetal life, and that all primordial follicles were formed during the formation of the ovary itself (Pearl and Schoppe 1921, Baker 1963). However, recent work has challenged this pillar of female reproductive biology, with new evidence of germline stem cells present in the adult mouse ovary (Johnson *et al.* 2004). In the human, there are thought to be between 266,000 and 472,000 primordial follicles present in each ovary at birth (Block 1953, Forabosco *et al.* 1991, Gougeon *et al.* 1994). However many primordial follicles there are in the ovary and whether or not they are replenished

during adult life, only approximately 400 will develop into a preovulatory follicle and ovulate (Hillier 1994). The rest will be cleared from the ovary by atresia, a topic discussed later in this chapter.

1.3.1 Follicle recruitment

The signal or cause for a resting primordial follicle to begin to develop is not completely understood. Current research has uncovered a number of factors involved in the regulation of the initiation of growth of primordial follicles (initial recruitment). Basic fibroblast growth factor (bFGF) (Nilsson *et al.* 2001, Nilsson and Skinner 2004), leukaemia inhibitory factor (LIF) (Nilsson *et al.* 2002), kit-ligand (Motro and Bernstein 1993, Parrott and Skinner 1999, Nilsson and Skinner 2004), the neurotrophins (Dissen *et al.* 2001, Paredes *et al.* 2004), and bone morphogenic protein-7 (BMP-7) (Lee *et al.* 2001) have all been implicated in the initiation of primordial follicle development. It is known that both kit-ligand and bFGF must be active in order to optimally promote the changes that occur in the oocyte, granulosa cells and stromal cells when a primordial follicle initiates development in the rat (Nilsson and Skinner 2004), but the precise mechanism is still unclear.

Strong correlations exist between the number of primordial follicles initiating development at any one time and the size of the primordial follicle pool at that time: the more follicles in the resting pool, the more follicles start to develop (Gougeon *et al.* 1994). However, as the number of resting primordial follicles decreases through both ovulation and atresia, the proportion of the remaining follicles which develop increases (Krarup *et al.* 1969, Gougeon and Chainy 1987). These observations on follicular dynamics indicate that there is an inhibitory signal from the developing follicles that prevents other primordial follicles from initiating development. A prime candidate for this signalling molecule is now thought to be anti-müllerian hormone (AMH). AMH is a dimeric glycoprotein related to the transforming growth factor β (TGF β) family (Cate *et al.* 1986) and is primarily thought of as being involved in male sexual differentiation, being essential for the regression of the Müllerian ducts in the male foetus, the initial step of organogenesis of the male genital tract (Josso *et al.* 1977, Josso *et al.* 1998). However, AMH is also produced by the granulosa cells of developing follicles in the foetal ovaries from about week

36 of gestation through to the menopause (Lee *et al.* 1996, Rajpert-De Meyts *et al.* 1999) and is implicated in the inhibition of early folliculogenesis through mice knock-out studies (Ueno *et al.* 1989, Baarends *et al.* 1995, Durlinger *et al.* 2001, Durlinger *et al.* 2002a).

A primordial follicle consists of an oocyte arrested in prophase of the first meiotic division, surrounded by a single layer of flattened epithelial cells (Block 1951, Koering 1969, Dekel and Beers 1978). When primordial follicles leave the resting pool, the flattened epithelial like granulosa cells become cuboidal and begin to show signs of proliferation, although the rate of proliferation is slow, with follicles developing at this level for approximately 65 days in the human (Hirshfield 1985, Gougeon 1996, Wandji *et al.* 1996, Wandji *et al.* 1997). During the early stages of folliculogenesis, cells close to the developing follicles in the ovarian stroma are recruited to form the theca surrounding the granulosa cells. A distinct thecal layer is evident at different stages of folliculogenesis in different species. In rodents, it can be seen as early as the late primary/early secondary stage of follicle development (Fortune and Eppig 1979), but cannot be seen in the primate, bovine or ovine follicle until the mid or late secondary stage of follicular development (Gougeon 1996, Braw-Tal and Yossefi 1997, Lundy *et al.* 1999). The mechanism by which the theca is recruited is poorly understood and requires much further research.

1.3.2 Tertiary follicle development

By the time follicles reach the late secondary stage of development, small fluid filled pockets between the granulosa cells start to appear. These pockets are caused by the production of glycosaminoglycans by the granulosa cells under FSH stimulation. Glycosaminoglycans are a major component of the follicular fluid that fills the antral cavity (Hillier 1991a) and, as these pockets expand and connect, the continuous antral cavity is formed. Tertiary, FSH-sensitive, follicles containing an antral cavity can be observed at all stages of the human ovarian cycle, but it is only the 20 or so small tertiary follicles present in the late luteal phase of the cycle that will be selected for further growth and potentially go on to ovulate (Gougeon and Lefevre 1983, McNatty *et al.* 1983). This is because, during the luteal phase of the ovarian cycle, the circulating levels of oestradiol, progesterone and inhibin in the blood are

high and have been shown to inhibit gonadotrophin secretions in the rhesus monkey (Karsch *et al.* 1973, Channing *et al.* 1981, Bassett and Zeleznik 1990). Both oestradiol and inhibin directly inhibit FSH β subunit messenger ribonucleic acid (mRNA) production (McNeilly *et al.* 2003), thus the circulating level of FSH is not sufficient to stimulate selection of a dominant follicle. However, as the corpus luteum undergoes luteolysis, the circulating levels of oestradiol, progesterone and inhibin decrease and, in turn, the circulating level of FSH increases so that the process of dominant follicle selection can begin (Roseff *et al.* 1989).

The ovarian follicle has two main functions to fulfil: one is to produce a fertilisable oocyte at ovulation; the other is to prepare the reproductive tract for pregnancy. Once FSH-dependent folliculogenesis has started, these goals are achieved by a tightly regulated system controlled by the gonadotrophins. Unlike the uncertain role of the gonadotrophins in the start of follicular development, they are essential for the maintenance of follicular endocrine functions in the developing tertiary follicles. LH stimulation of theca cells leads to the production of androstenedione (androgen) in the theca cells (Erickson *et al.* 1985). Stimulation of FSH receptors on the cell surface of developing granulosa cells leads to the activation of adenylyl cyclase and cyclic adenosine monophosphate (cAMP)-dependent protein kinase(s) (Adashi and Resnick 1986). This activation leads in turn to the activation and expression of a range of genes required for granulosa cell differentiation including: cytochrome P450 aromatase (P450_{arom}), which is essential for converting theca produced androgen to oestrogen in the granulosa cells (Falck 1959, Steinkampf *et al.* 1987, Simpson *et al.* 1994); cytochrome P450 side chain cleavage (P450_{ssc}), the cholesterol side chain cleavage enzyme that is essential for granulosa cell progesterone production (Richards 1994); the LH receptor (Segaloff and Ascoli 1993); and a range of other genes necessary for ovarian follicle maturation.

1.3.3 Selection of dominant follicle(s)

By the mid follicular phase of the ovarian cycle, dominant follicle(s) can already be identified as the largest healthy follicle(s) in the ovaries (Gougeon and Lefevre 1983). As the tertiary follicles continue to grow under the influence of FSH, their

granulosa cells become steroidogenically active. As discussed above, oestrogen production has an inhibitory effect on FSH secretion, and it is this negative feedback via oestrogen that will go on to regulate the acquisition of follicular dominance.

As follicles undergo stimulation by FSH and mature to the preovulatory stage of development, they become more sensitive to FSH and also begin to express LH receptors on their granulosa cells (Zelevnik *et al.* 1974, Segaloff and Ascoli 1993). These processes serve to protect the selected follicle(s) from the imminent decline in FSH concentrations that occurs due to the follicular oestrogen production. It is at this point that the dominant follicle(s) becomes increasingly dependent on LH relative to FSH (Yong *et al.* 1992a). As the circulating concentration of FSH decreases, the FSH-induced expression of the LH receptor on the granulosa cells provides a protective effect for the dominant follicle(s). The dominant follicle(s) also starts to produce increasing amounts of androgen and oestrogen in response to LH stimulation (McNatty *et al.* 1976), which continues to augment the decrease in FSH production, and hence the maintenance of follicle dominance. It is probably the follicle(s) that is most sensitive to FSH during follicle recruitment that will begin to produce oestradiol before the others, and hence gain dominance (Zelevnik and Kubik 1986). During the preovulatory follicular period in rats, the density of FSH receptors on the granulosa cells increases to confer dominance to the developing follicle(s) as the concentration of FSH falls (LaPolt *et al.* 1992). Less mature follicles have fewer FSH receptors and may not be as sensitive to FSH stimulation, or express as many LH receptors, which therefore makes them more susceptible to the decline in FSH concentrations than are the dominant follicles, and more likely to die.

Of the follicles initially selected to begin preovulatory development, only one (human), or two to three (marmoset monkey) will eventually go on to secrete enough oestrogen and become the dominant follicle(s) in that cycle (Goodman *et al.* 1977, Zelevnik 1981).

1.4 Ovulation

Ovulation marks the completion of the follicular phase of the ovarian cycle, culminating in the rupture of the dominant follicle(s) and the onset of luteinisation. It is triggered by the LH surge, a large discharge of LH from the anterior pituitary (Yen *et al.* 1975).

1.4.1 The LH surge

Sustained high levels of circulating oestradiol produced by the dominant follicle(s) trigger the LH surge. How oestrogen exerts this positive feedback mechanism remains to be fully understood, but it is thought to involve an effect on both the hypothalamus and pituitary. Oestrogen increases the sensitivity of the gonadotrope cells in the pituitary to the effects of GnRH, resulting in progressively more and more LH release throughout the follicular phase, reviewed by Baird (1991). The LH surge is likely to be due to a pulsatile GnRH release from the hypothalamus acting on a hypersensitive pituitary, resulting in a large release of LH. Progesterone also plays an important role in triggering the LH surge. In macaques the LH surge is caused by an increase in GnRH release that is induced by progesterone (Woller and Terasawa 1994). In both women (Liu *et al.* 1987, Ho 2001) and monkeys (Collins and Hodgen 1986, Wolf *et al.* 1989), treatment with progesterone antagonists has been shown to prevent the late follicular LH surge.

The LH surge induces sequential transcription of a number of genes involved in ovulation (Espey and Richards 2002), which can be broken down into three main processes: reactivation of oocyte maturation; follicle rupture; and luteinisation of the granulosa cells (Tsafriri and Dekel 1994).

1.4.2 Oocyte reactivation

The oocyte does not express LH receptors, so LH responsive cells must therefore mediate the trigger for reactivation of the oocyte. The oocyte nucleus, otherwise known as the germinal vesicle, remains arrested in prophase of the first meiotic division until oocyte maturation commences (Dekel and Beers 1978). It is believed that intra-oocyte cAMP is critical to the control of meiosis and that it is the oocytes

intimate contact with the granulosa cell that prevents it from spontaneously maturing before ovulation. An oocyte will mature spontaneously when removed from its cumulus granulosa cells (Espey and Lipner 1994). However, this can be prevented by addition of cAMP to the oocyte as it is thought that the cumulus granulosa cells supply cAMP to prevent the oocyte from maturing prematurely (Tsafriri and Dekel 1994). Purines present in the follicular fluid are also thought to contribute to the accumulation of cAMP within the oocyte (Eppig *et al.* 1985). The LH surge causes the cytoplasmic oocyte-cumulus granulosa cell connections to break down, thus lowering oocyte cAMP levels and releasing the oocyte to mature prior to ovulation. However, the role for a positive stimulus for oocyte maturation has been suggested from observations that in mouse cumulus-enclosed oocytes meiotic resumption *in vivo* (Su *et al.* 2002), and *in vitro* (Downs *et al.* 1988, Su *et al.* 2002, Su *et al.* 2003) under conditions which maintain continued meiotic arrest, can be induced by an overriding signal originating in the cumulus cells as a direct effect of gonadotrophin action. However, the chain of events leading to oocyte maturation remains to be delineated.

1.4.3 Follicle rupture

Approximately 36 hours after the LH surge the follicle wall ruptures, allowing the oocyte to be extruded from the follicle (Baird and Fraser 1975). Many theories have been advanced as to the nature of the process of follicle rupture at ovulation, such as those based on smooth muscle contraction and intrafollicular pressure, reviewed by Espey and Lipner (1994). However, nowadays ovulation is thought to be similar to an acute inflammatory response triggered by the LH surge.

Many cell layers that do not express the LH receptor are affected during the process of follicle rupture. There are therefore many interactions occurring to prepare the preovulatory follicle for rupture. These interactions are initiated by the LH surge stimulating steroidogenic cells to induce an inflammatory response (Espey 1980). The inflammatory response leads to the activation of a range of genes including the expression of cyclooxygenase-2 (COX-2) and epiregulin, both of which are involved in prostanoid synthesis in the preovulatory follicle (Espey and Richards 2002). Prostaglandins, including prostaglandin F_{2α}, are produced by the

granulosa cells in response to the LH surge (Evans *et al.* 1983). These prostaglandins are primarily involved in the increase in vascular permeability in the preovulatory follicle, and help to maintain the positive intrafollicular pressure during follicular fluid leakage.

The LH surge also stimulates 11β -hydroxysteroid dehydrogenase type 1 (11β HSD1) and simultaneously down-regulates 11β HSD2 expression in the granulosa cells of preovulatory follicles (Tetsuka *et al.* 1997, Tetsuka *et al.* 1999). Since 11β HSD1 converts cortisone to cortisol, whereas 11β HSD2 inactivates cortisol to cortisone (Stewart 1996), the changes induced by LH favour local accumulation of anti-inflammatory cortisol at a time when rapid healing of the ruptured surface will be required to quickly restore normal ovarian function (Hillier and Tetsuka 1998). This is the mechanism by which the balance between inflammation and wound repair is maintained at ovulation.

As ovulation approaches, an area of the preovulatory follicle wall becomes weaker due to proteolytic disintegration and a follicle apex or stigma will form. This stigma is characterized by a reduction in vascular density and will go on to become the point of ovulation. A range of specific collagenases mediates these proteolytic processes. In the mouse model, proteolytic enzymes such as Cathepsin-L and a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS-1) are both selectively induced in granulosa cells of preovulatory follicles by the LH surge (Robker *et al.* 2000). However, other common proteases such as matrix metalloproteinase-2 (MMP-2) and MMP-9 are both thought to be redundant during the process of ovulation, as mice that are null for MMP-9 nevertheless ovulate, and the LH surge selectively decreases the activation of MMP-2 in follicles (Robker *et al.* 2000).

At ovulation, the apex of the follicle ruptures and the cumulus-oocyte complex flows out of the follicle and onto the ovarian surface, where it will pass into the oviduct. During this process, the granulosa cells in the preovulatory follicles are also undergoing luteinisation.

1.4.4 Granulosa cell luteinisation

Luteinisation of the granulosa cells from the preovulatory follicle is vital for the

formation of the corpus luteum. During luteinisation, there is a dramatic change in the steroidogenic activity of granulosa cells. During folliculogenesis, the main steroidogenic process in the granulosa cells is the aromatisation of theca-derived androgen to oestrogen (Hillier 1991b). In the preovulatory follicle, oestrogen synthesis declines as a result of the inhibition of thecal cytochrome P450 17 α hydroxylase (P450_{17 α}), and the subsequent lack of androgen as a substrate for aromatisation in the granulosa cells. The LH surge causes an increase in the expression of P450_{scc}, 3 β -hydroxysteroid dehydrogenase (3 β -HSD), and steroidogenic acute regulatory protein (StAR) in the granulosa cells of the preovulatory follicle. These enzymes are essential for the conversion of cholesterol to progesterone (Miller 1988, Yong *et al.* 1992a, Stocco 2001). Thus, as ovulation approaches, the ovary increasingly produces progesterone while oestrogen production falls (Hillier 1985). The induction of these enzymes causes follicular fluid progesterone levels to rise, and it is thought that this preovulatory increase is essential to follicular rupture. *In vitro* culture of follicle wall sections in progesterone has been shown to accelerate the net loss of collagen fibres from the follicular wall (Tjugum *et al.* 1984), caused by the up-regulation of plasminogen activator synthesis that in turn activates collagenases, facilitating follicular wall breakdown, reviewed by Zeleznik and Hillier (1996). **Figure 1.1** illustrates the different stages of follicular development during the ovarian cycle.

1.5 Ovarian follicular steroidogenesis

The production of ovarian steroids is critical in regulating ovarian function. The ovarian steroids play a crucial role in feedback regulation of gonadotrophin secretion from the pituitary. They also act in both paracrine and autocrine capacities in the ovarian follicle itself (Billiar 1993).

1.5.1 Androgen

Androgens are produced in theca cells through the oxidation of the 17 α -OH androgen

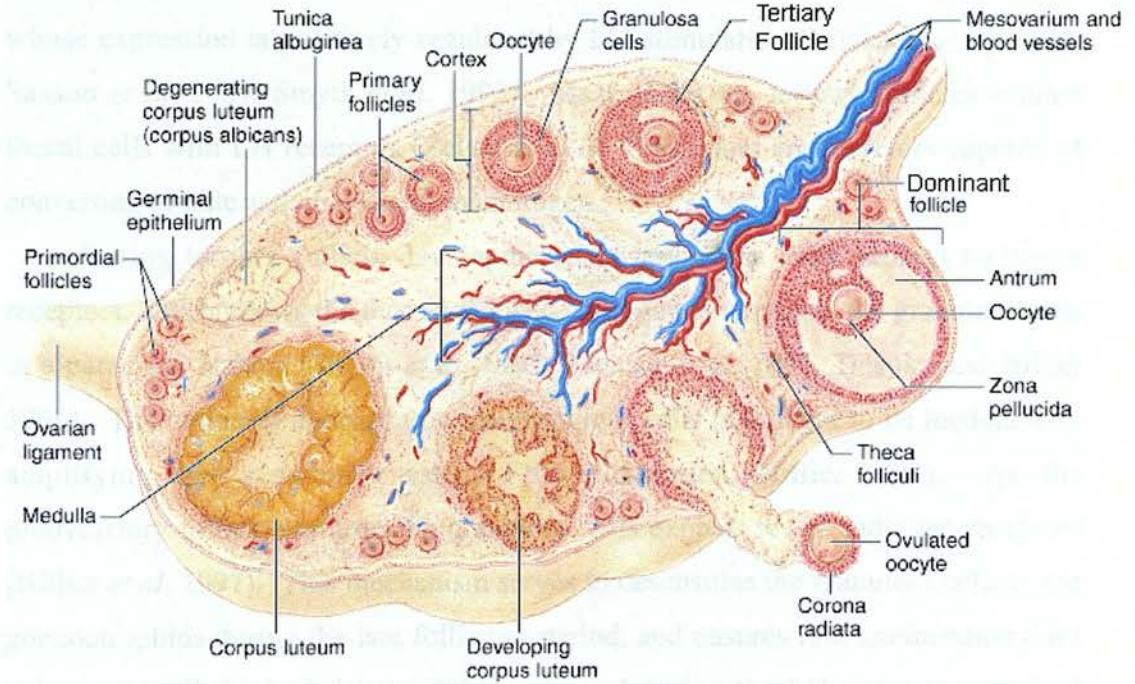


Figure 1.1 Diagram of the ovary.

The various stages of follicular development, ovulation and luteinisation encountered throughout the mammalian ovarian cycle are depicted in the diagram. Adapted from ©2001 Benjamin Cummings, an imprint of Addison Wesley Longman, Inc.

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1.5.1 Androgen

Androgens are produced in theca cells through the expression of the P450_{17 α} enzyme, whose expression is positively regulated by LH stimulation (Erickson *et al.* 1985, Sasano *et al.* 1989, Smyth *et al.* 1993). Most, if not all, tertiary follicles contain thecal cells with LH receptors (Zelevnik *et al.* 1981), and are therefore capable of converting acetate and cholesterol to androgen.

During tertiary follicle development, the granulosa cells express androgen receptors, which allows the theca-produced androgen to stimulate the granulosa cells in a paracrine fashion (Smyth *et al.* 1995, Tetsuka *et al.* 1995, Tetsuka and Hillier 1996). The action of androgen on the granulosa cells is thought to be mediated by amplifying their responsiveness to FSH stimulation (Hillier 2001). As the preovulatory follicle matures, the granulosa cells express fewer androgen receptors (Hillier *et al.* 1997). This mechanism serves to desensitise the granulosa cells to the gonadotrophins during the late follicular period, and ensures that luteinisation does not occur until the high levels of LH achieved during the LH surge is perceived (Hillier and Tetsuka 1997).

1.5.2 Oestrogen

Granulosa cells are the predominant, if not exclusive, site of oestrogen synthesis in the ovarian follicle. Immunocytochemical and *in situ* hybridisation studies have localised cytochrome P450_{arom} protein (Tamura *et al.* 1992) and mRNA (Fitzpatrick and Richards 1991, Whitelaw *et al.* 1992) to the granulosa cells. Isolated cell cultures of both granulosa and theca cell have also demonstrated that more than 99% of a follicle's capacity to aromatise androgens lies in the granulosa cells (Hillier *et al.* 1981). However, the granulosa cells are unable to produce oestrogen without

theca-produced androgen as a substrate because they do not express the P450_{17 α} enzyme necessary for androgen production. Conversely, theca cells are not able to synthesise oestrogen directly as they do not contain the P450_{arom} enzyme. As both cell types are required for oestrogen production, this has become known as the two-cell theory of steroidogenesis, illustrated in **Figure 1.2** and reviewed by Hillier *et al* (1994).

High oestrogen production in a follicle reflects its ability to respond to FSH stimulation, as FSH regulates expression of the P450_{arom} enzyme that is required to convert androgen to oestrogen. Oestrogen production is considered the hallmark of preovulatory follicular development (Hillier 2001). The granulosa cells also express oestrogen receptors that can mediate autocrine oestrogen action within the granulosa cell population, again serving to augment the action of FSH on the granulosa cells (Richards 1994).

1.5.3 Progesterone

As the preovulatory follicle matures, it increasingly produces progesterone. Progesterone is produced in the granulosa cells under the regulation of P450_{ssc} that catalyses the rate-limiting conversion of cholesterol to pregnenolone (Richards *et al.* 1998). Progesterone is not thought to play either an autocrine or a paracrine role in the developing follicle, as progesterone receptors are not expressed in the developing follicle until the LH surge (Natraj and Richards 1993, Park-Sarge and Mayo 1994). However it is essential for the process of ovulation, particularly follicle rupture, as discussed earlier in this chapter.

1.8 Follicular paracrine signalling

Folliculogenesis is not solely regulated by the gonadotrophins. Locally produced factors also act in either an autocrine or paracrine manner to modulate a follicle's responsiveness to FSH and LH. In the human ovary many locally produced factors have been shown to modify FSH action *in vitro* and thus serve to increase a follicle's sensitivity to the gonadotrophins (Holler 1991b). The best characterised ovarian growth factors to date are insulin-like growth factors I and II (IGF-I and IGF-II) (Adashi 1990); and members of the transforming growth factor β (TGF β) superfamily (Gaddy-Kurten *et al.* 1995) such as inhibin and activin (Holler 2010). However, other factors including TGF- α and TGF- β (Maguiness 1990), fibroblast growth factor VEGF and FGF. However, the most important factor derived from a non-endocrine source including human, cynomolgus and rhesus monkey ovaries, which appears to

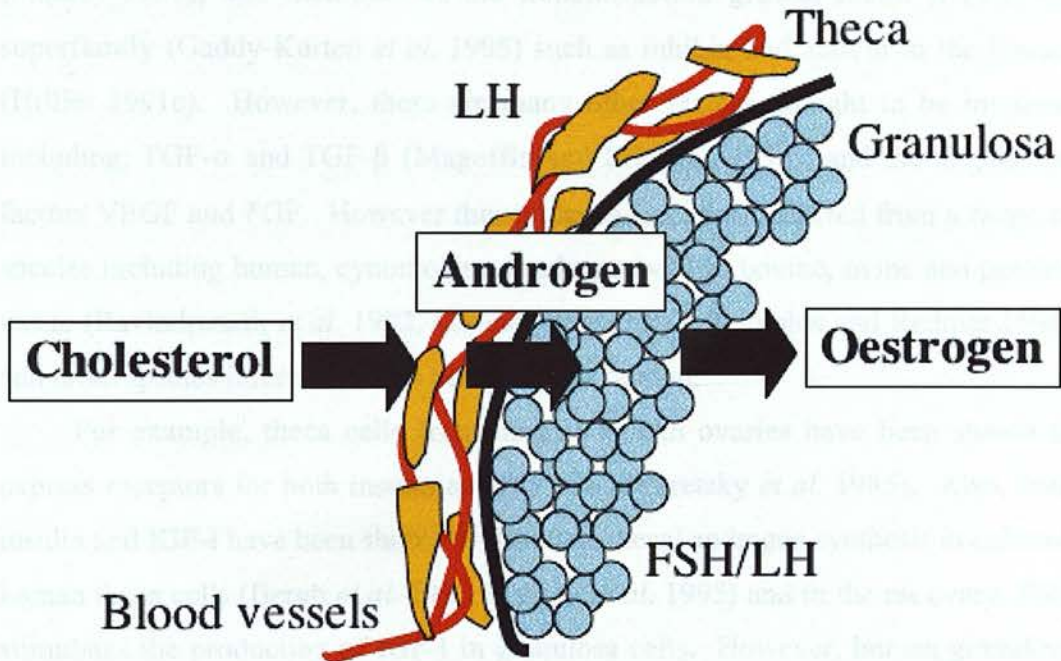


Figure 1.2 The two-cell theory of steroidogenesis.

Cholesterol is converted into androgen in theca cells. The androgen is then transported to the granulosa cells where it is converted into oestrogen. Based on Armstrong and Dorrington (1977).

1.6 Follicular paracrine signalling

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For example, theca cells from normal human ovaries have been shown to express receptors for both insulin and the IGFs (Poretsky *et al.* 1985). Also, both insulin and IGF-I have been shown to stimulate thecal androgen synthesis in cultured human theca cells (Bergh *et al.* 1993, Nahum *et al.* 1995) and in the rat ovary, FSH stimulates the production of IGF-I in granulosa cells. However, human granulosa cells do not produce IGF-I, although they do produce IGF-II, which suggests a paracrine role for IGF-II produced in the granulosa cells acting on the theca to stimulate thecal androgen synthesis (Hernandez *et al.* 1988, Nahum *et al.* 1995).

Activins and inhibins produced in the granulosa cells also have potent modulatory effects on thecal androgen production. In the developing human follicle activin is inhibitory, and inhibin stimulates thecal androgen production (Hsueh *et al.* 1987, Hillier *et al.* 1991a, Hillier *et al.* 1991b). During tertiary follicle development in the rat, activin predominates over inhibin and promotes FSH stimulated granulosa cell proliferation and steroidogenic differentiation (Miro and Hillier 1996). As the dominant follicle matures, both inhibin and the activin-binding follistatin are produced in relatively larger amounts compared to activin (Nakatani *et al.* 1991).

This leads to the stimulatory action of inhibin on the thecal androgen synthesis increasing as the follicle develops and starts to produce increasing amounts of oestradiol in the process of follicle selection and dominance.

1.7 Follicular atresia

Follicular atresia is evident at every stage of folliculogenesis, as the majority of follicles in the ovary will become atretic and never reach ovulation (Gougeon 1986). In the ovary there are a number of specific regulators affecting apoptosis, reviewed by Markstrom *et al* (2002). Once the 'decision' to enter follicular atresia has been made, the execution of apoptotic cell death requires the coordinated activation of several cellular sub-programmes (Hengartner 2000). Two main regulatory families of genes required for apoptosis are the caspases and the Bcl-2 family (Hengartner 2000). Follicular atresia is a stage-dependent process, with different factors effecting a follicle's decision to die at different stages of follicular development (Markstrom *et al.* 2002). The main physiological regulators of ovarian follicle survival are the gonadotrophins (Markstrom *et al.* 2002), but there are many other factors regulating follicular atresia, summarised in **Figure 1.3**.

1.7.1 Primordial follicle atresia

Follicular apoptosis in primordial follicles is most probably oocyte regulated (Markstrom *et al.* 2002). Oocyte apoptosis in rodent species has been reviewed by (Morita and Tilly 1999) and (Reynaud and Driancourt 2000). The importance of kit-ligand interactions in the survival of primordial follicles in the mouse model has been highlighted (Driancourt *et al.* 2000), and growth differentiation factor 9 (GDF-9) has also been shown to play a major role in primordial follicle survival, as mice lacking GDF-9 do not develop follicles beyond the primary or early secondary stage of development (Dong *et al.* 1996)

1.1.3 Preantral follicle atresia

Little is known about the factors involved in the early stages of development. FSH stimulation of cultured rat preantral follicles does not extend beyond three antral stages, although follicles are sensitive to FSH in vivo (Dyball *et al.* 1974, Kauskij *et al.* 1993), and both FSH and LH play major roles in preantral follicle development (Mörner *et al.* 1997b).

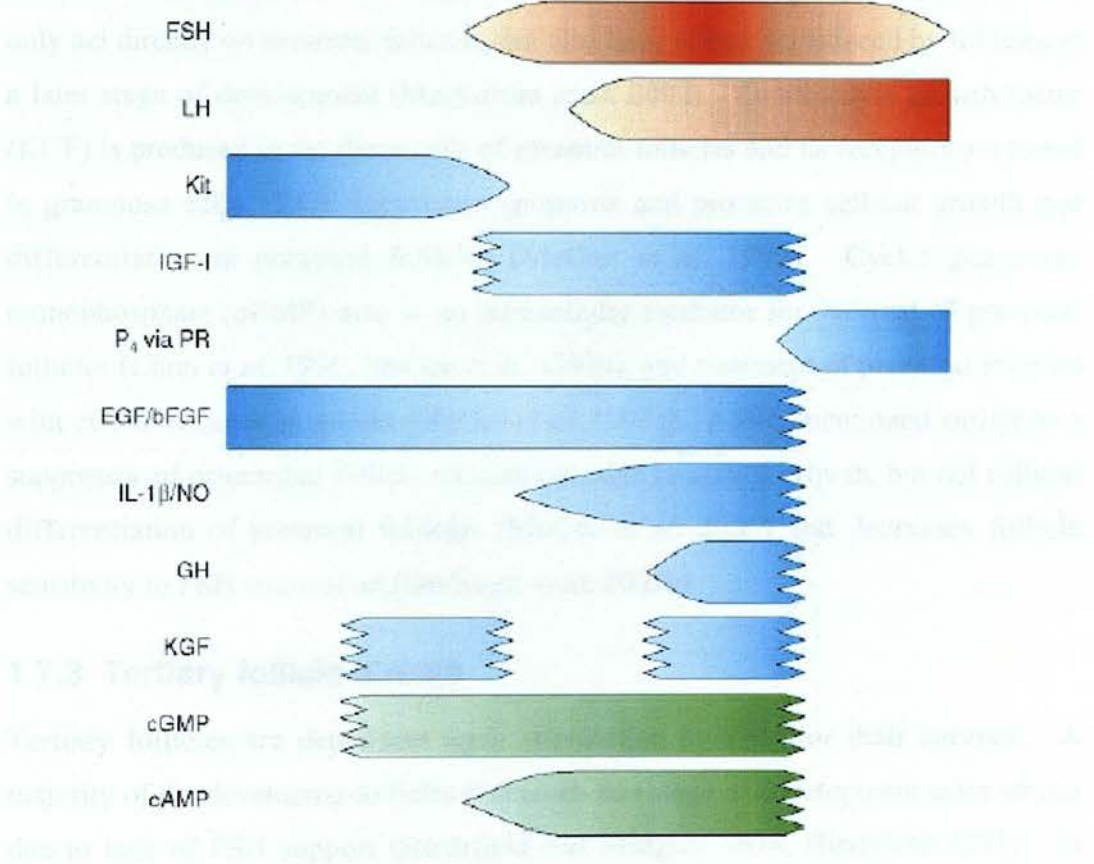


Figure 1.3 Factors regulating stage dependent survival in the ovarian follicle.

Gonadotrophins (Red), locally produced survival factors (Blue) and intracellular mediators (Green). The data is an amalgamation from studies in a number of species. Jagged ends indicate studies have not been published regarding earlier or later stages of folliculogenesis. BFGF: basic fibroblast growth factor; EGF: epidermal growth factor; GH: growth hormone; IGF-1: insulin-like growth factor; IL-1 β : interleukin 1 β ; KGF: keratinocyte growth factor; NO: nitric oxide; P₄: progesterone; PR: progesterone receptor. Adapted from Markstrom *et al.* (2002).

1.7.2 Preantral follicle atresia

Little is known about follicle survival at this stage of development. FSH stimulation of cultured rat preantral follicles does not effect rescue from apoptosis, although follicles are sensitive to FSH at this stage (Dunkel *et al.* 1994, Rannikki *et al.* 1995), and both FSH and LH play important roles in preantral follicle development (McGee *et al.* 1997b). From these findings it is suggested that the gonadotrophins do not only act directly on preantral follicles, but also have effects transduced by follicles at a later stage of development (Markstrom *et al.* 2002). Keratinocyte growth factor (KGF) is produced in the theca cells of preantral follicles and its receptors are found in granulosa cells. KGF suppresses apoptosis and promotes cellular growth and differentiation in preantral follicles (McGee *et al.* 1999). Cyclic guanosine monophosphate (cGMP) acts as an intracellular mediator for survival of preantral follicles (Chun *et al.* 1996, McGee *et al.* 1997a), and treatment of preantral follicles with cGMP reduces apoptosis (McGee *et al.* 1997a). AMH, mentioned earlier as a suppressor of primordial follicle recruitment, also promotes growth, but not cellular differentiation of preantral follicles (McGee *et al.* 2001) and decreases follicle sensitivity to FSH stimulation (Durlinger *et al.* 2002b).

1.7.3 Tertiary follicle atresia

Tertiary follicles are dependent upon stimulation by FSH for their survival. A majority of the developing follicles that reach this stage of development enter atresia due to lack of FSH support (Hirshfield and Midgley 1978, Hirshfield 1991). In isolated rat tertiary follicles, FSH is the most crucial follicle survival factor (Chun *et al.* 1996), suppressing apoptosis by up to 60%. Locally produced factors such as IGF-I (Chun *et al.* 1994, Chun *et al.* 1996), epidermal growth factor (EGF), and bFGF (Tilly *et al.* 1992a, Chun *et al.* 1996) have all been implicated in their ability to rescue tertiary follicles from apoptosis to varying extents.

1.7.4 Preovulatory follicle atresia

By the preovulatory stage of follicular development, both FSH and LH play a critical role in suppressing apoptosis in isolated preovulatory rat follicles (Braw and Tsafiri 1980, Chun *et al.* 1994). Interestingly, both EGF and bFGF have been shown to be

equally as effective as the gonadotrophins in suppressing apoptosis in cultured granulosa cells from preovulatory follicle (Tilly *et al.* 1992a). IGF-I has been shown to be a survival factor for cultured preovulatory rat follicles (Chun *et al.* 1994), exerting its effect on the granulosa cells through paracrine signalling from the theca (Tilly *et al.* 1992b).

1.7.5 Perioovulatory follicle atresia

Follicles at this stage of development are dependent upon the LH surge. Inhibition of the LH surge at this stage causes such follicles to degenerate (Talbert *et al.* 1951, Braw and Tsafiriri 1980). After the LH surge, perioovulatory follicles are less susceptible to atresia (Svensson *et al.* 2000). Expression of the nuclear progesterone receptor is induced shortly after the LH surge in both rat and human granulosa cells. Progesterone has been demonstrated to play an important role in the perioovulatory follicle, as mice lacking progesterone receptors are anovulatory (Lydon *et al.* 1995, Lydon *et al.* 1996, Svensson *et al.* 1999, Makrigiannakis *et al.* 2000) and treatment of women with anti-progestins such as RU486 block ovulation (Ledger *et al.* 1992).

1.8 Follicular vascularisation

The smallest early growing follicles do not have an independent blood supply and are essentially devoid of a capillary network. Once a number of granulosa cell layers have formed and the thecal layer has been established, the follicle will start to recruit a vascular supply of its own that will continue to develop throughout follicular growth (Reynolds *et al.* 1992, Suzuki *et al.* 1998). The follicular vasculature is thought to be required for the delivery of steroid hormone precursors, oxygen, and nutrients to the developing follicle, and it has been hypothesised that adequate vasculature is a key factor influencing the selection of the dominant follicle that will continue developing and eventually ovulate (Zelevnik *et al.* 1981, Augustin 2000). Follicular angiogenesis is discussed in more detail later in this chapter.

Vascular cells in different organs acquire specialised characteristics that allow these cells to optimally perform specific functions in each organ (Ruoslahti and Rajotte 2000). For example, endothelial cells in the brain are tightly linked to each other, and are surrounded by numerous pericytes that form a barrier to protect the brain cells from potentially toxic blood-derived molecules. In contrast, vessels in endocrine glands, i.e. the ovarian follicle, are leaky and their endothelial cells have fenestrations, allowing hormone trafficking (Carmeliet 2004).

1.9 Angiogenesis

1.9.1 History of angiogenesis

Blood vessels were recognised thousands of years ago by the Egyptians. Their physicians recognised that ‘there were vessels in him for every part of the body, which were hollow, having a mouth which opens to absorb medications and eliminate waste elements’ (Carmeliet 2004). More recently, in 1896, J.G. Clark started to study ‘ovarian circulation’, he drew detailed illustrations of vasculature development in ovaries from newborn to post menopausal women (Clark 1900) and with that began the field of ovarian vascular biology. By the 1930s, vascular biology had advanced to observing blood vessels at sites of surgical insult. E.R. and E.L. Clark would insert glass-windowed chambers into the ears of rabbits and make exceptionally detailed drawings of the branched blood vessel networks they observed at the wound site (Clark and Clark 1932). Newly formed blood vessel infiltration was also observed in rabbit tumours using similar techniques (Ide *et al.* 1939). The first demonstration that tumours actively attract new blood vessels was carried out in mouse tumours, when blood vessels were observed migrating toward tumours using wound chambers adapted from the rabbit for use in the mouse (Algire 1943a, Algire 1943b, Algire *et al.* 1945).

The process of vascular development takes place by angiogenesis, which is defined as the growth of new blood vessels by proliferation and migration of pre-existing vessels. Angiogenic activity was first described by studies on retinopathic vessel growth (Michelson 1948). An unknown factor was thought to mediate abnormal blood vessel growth in the eye. It was not until 20 years later that it was demonstrated that tumours release diffusible factors that attract new blood vessels to the tumour site (Ehrmann and Knoth 1968, Greenblatt and Shubi 1968).

In 1971, Judah Folkman and collaborators presented an experimental protocol to isolate a fraction of ascites fluid that stimulated blood vessel growth (Folkman *et al.* 1971). This discovery was pivotal in the field of cancer research. Later that year, Folkman went on to write the first review of tumour angiogenesis, which literally defined the birth of this field of scientific research. It was the first suggestion that

angiogenic inhibitors could be used to treat cancer and other angiogenic dependent diseases such as diabetic retinopathy (Folkman 1971). Although viewed with some disbelief and scepticism at the time, Folkman's early work is now viewed as a seminal step in the field of angiogenesis.

In 1972, the first paper to go beyond circumstantial evidence and show that the progressive growth of a tumour can indeed be completely dependent on angiogenesis was published. Experiments using small clusters of tumour cells suspended in different parts of the eye demonstrated that tumours only grow when they are able to attract vessels from pre-existing vasculature (Gimbrone *et al.* 1972).

1.9.2 Physiological angiogenesis

The maxim "blood vessels grow where they are needed" still holds true today, as the molecular regulation of angiogenesis is increasingly understood (Augustin 2000). Vasculogenesis and angiogenesis occur primarily during embryonic and foetal development. The development of a vascular network is essential for organ development and differentiation during embryogenesis and neonatal growth. In the healthy adult, angiogenesis is primarily down-regulated as there is very little physiological growth and tissue remodelling, apart from during wound healing and during the female reproductive cycle in both the ovaries and uterus.

Angiogenesis is a pivotal event in the endochondral ossification (bone formation) of the longitudinal bones during vertebrate development (Poole 1991). The long bones of the limbs are laid down by cartilaginous elements, which then become ossified. This process requires the precise coupling of cartilage production and bone formation. Cartilage is usually an avascular tissue, apart from when capillaries invade the growth plate to convert cartilage into bone. Blood vessels grow into the hypertrophic cartilage and erode it to produce a scaffold on which osteoblasts settle to produce woven bone (Alini *et al.* 1996). Alini *et al.* discovered that hypertrophic cartilage secretes an angiogenic factor (Alini *et al.* 1996). Other angiogenic factors have also been localised to the cartilage growth plate, including members of the FGF family (Baron *et al.* 1994), and members of the TGF- β family (Jingushi *et al.* 1995). VEGF, produced by the hypertrophic chondrocytes in the cartilage, has also been shown to be required for this process, as inactivation of

VEGF during this process leads to a complete disruption of the normal vascular pattern of the growth plate (Gerber *et al.* 1999b).

VEGF-mediated angiogenesis is also a critical rate limiting step in determining organ size and function. Inhibition of VEGF in neonatal mice causes abnormal liver development, severe renal failure and tissue hypoxia (Gerber *et al.* 1999a). These effects of VEGF inhibition are only evident during neonatal life, and not in the fully developed adult. Angiogenesis is a rare event in the healthy adult, apart from pathological angiogenesis observed during wound healing. However, the female reproductive system undergoes serial angiogenesis and vessel regression during the reproductive cycle. In the ovary, angiogenesis occurs during both follicular development and the establishment of the corpus luteum. This review focuses on follicular vascularisation.

1.9.3 Follicular angiogenesis

Dormant primordial, small primary and early secondary follicles do not have a vascular supply of their own and have to rely on vessels in the surrounding stroma for their supply of nutrients and oxygen. As the recruited follicles develop, they start to acquire their own vascular network. This network consists of two concentric vascular sheaths, located in the theca interna and theca externa of the follicle. The arterioles and venules of the outer vascular network send tiny branches into the theca interna to form the inner capillary plexus, which consists of a single layer of endothelial cells. These endothelial cells are immediately outside of the membrana propria (basement membrane) that separates the granulosa and theca cells. This vascular network continues to undergo angiogenesis throughout folliculogenesis, but does not penetrate the granulosa cells of the follicle until after ovulation (Findlay 1986, Reynolds *et al.* 1992, Gordon *et al.* 1995, Goede *et al.* 1998, Suzuki *et al.* 1998, Augustin 2000). This vasculature is not evenly distributed between the different classes of ovarian follicle (Hazzard and Stouffer 2000). Colour Doppler studies have shown that there is a positive correlation between the development of ovarian follicles and their blood flow (Campbell *et al.* 1993, Balakier and Stronell 1994). By the time a follicle reaches the preovulatory stage of development it will have become highly vascularised with blood vessels approximately 2 times larger

than in other follicles (DiZerega *et al.* 1980, Zeleznik *et al.* 1981). It has also been hypothesised that it is the establishment of an adequate vasculature that is a key factor influencing the selection of a dominant follicle, which will continue developing and eventually ovulate (Zeleznik *et al.* 1981, Augustin 2000).

The principal angiogenic factor with an established role in follicular angiogenesis is VEGF. VEGF mRNA and protein have been localised (in the ovary) to the granulosa cells of the developing follicles, and in preovulatory follicles to the theca (Phillips *et al.* 1990, Ravindranath *et al.* 1992, Shweiki *et al.* 1993). Inhibition of VEGF *in vivo* has been shown to severely suppress both thecal vascularisation and follicle development in the marmoset (Wulff *et al.* 2001b, Wulff *et al.* 2002), and to inhibit the follicular phase of the cycle in macaques (Zimmermann *et al.* 2001b, Zimmermann *et al.* 2002). VEGF is expressed in the granulosa and theca cells of follicles as they mature, and is concurrent with the acquisition of steroidogenic activity, suggesting that the expression is hormonally regulated (Shweiki *et al.* 1993). It has been demonstrated that VEGF expression in granulosa cells from preovulatory follicles is up regulated by FSH stimulation *in vitro* (Christenson and Stouffer 1997, Hazzard *et al.* 1999). LH also induces VEGF mRNA expression in both cultured bovine and primate granulosa cells (Garrido *et al.* 1993, Hazzard *et al.* 1999). Forskolin, a potent activator of adenylate cyclase, also induces VEGF mRNA expression in cultured bovine granulosa cells (Garrido *et al.* 1993).

1.9.4 Pathological angiogenesis

As already mentioned, angiogenesis is a relatively rare event in the healthy adult. However, excessive capillary growth is associated with various pathological conditions including: tumour growth; psoriasis; retinopathies; and rheumatoid arthritis (Reynolds *et al.* 1992, Moretti *et al.* 1999, Folkman 2001, Kerbel and Folkman 2002). The female reproductive organs are one of the only sites in the adult to undergo serial angiogenesis followed by vessel regression and degradation, and it is therefore not surprising that there are also many pathological conditions associated with the miss-regulation of angiogenesis in the female reproductive tract. These include: polycystic ovary syndrome (PCOS); ovarian hyperstimulation syndrome (OHSS); benign ovarian neoplasms; and menorrhagia (Smith 1998, Geva and Jaffe

2000, Livingstone and Fraser 2002).

PCOS affects approximately 5% of women of reproductive age (Franks 1995, Knochenhauer *et al.* 1998, Legro and Strauss 2003, Azziz *et al.* 2004). In women with PCOS, increased serum concentrations of VEGF are found during *in vitro* fertilisation treatment, and Doppler blood flow velocities within the ovarian stromal vessels are also increased in parallel with the rising levels of VEGF (Agrawal *et al.* 1998a, Agrawal *et al.* 1998b). OHSS can be a life-threatening complication of ovulation induction using gonadotrophins and ovarian steroid hormones. Because certain steroids are known to stimulate angiogenesis, i.e. stimulation of VEGF expression (Folkman and Ingber 1987), administration of hCG can lead to excessive vascular permeability via stimulation of VEGF production in the hCG responsive granulosa cells (Neulen *et al.* 1995). These techniques of ovulation induction can lead to high levels of VEGF expression in follicles that result in vascular leakage. This leakage can, in turn, lead to extravasation of fluid out of the intravascular space, accounting for virtually all of the manifestations of OHSS (Golan *et al.* 1989). Immunohistochemical studies on ovaries from postmenopausal women have revealed VEGF staining in epithelial inclusion cysts and in serous cystadenomas. The VEGF protein has been observed in the epithelial layers of the cysts (Gordon *et al.* 1996), suggesting that VEGF in benign serous neoplasms may be responsible for fluid accumulation. Moreover, if epithelial ovarian cancer originates within epithelial inclusion cysts (Perez *et al.* 1991), VEGF within these cysts may represent a means by which a developing neoplasm is able to induce the formation of an increased vascular supply (Gordon *et al.* 1996).

1.10 The angiogenic process

Angiogenesis *in vivo* is a complex process involving the degradation of the capillary basement membrane, the migration and proliferation of endothelial cells, and tube formation. Mature blood vessels comprise lining endothelial cells and one or more enveloping layers of supporting cells. In arteries and veins, these cells are smooth muscle cells; in capillaries and venules they are termed pericytes (Rhodin 1968). Angiogenesis is the remodelling of the existing vasculature through both pruning and vessel enlargement and can be divided into at least three steps (Folkman and Klagsbrun 1987).

- 1) Breakdown of the basement membrane of the existing blood vessel
- 2) Migration of the endothelial cells toward an angiogenic stimulus
- 3) Establishment and stabilisation of blood vessels

Angiogenesis initiates with vasodilatation. Vascular permeability increases in response to VEGF and is mediated by the formation of fenestrations, vesiculo-vacuolar organelles and the redistribution of platelet endothelial cell adhesion molecules (PECAM-1/CD31), and vascular endothelial (VE)-cadherin (Eliceiri *et al.* 1999). This dilation of the blood vessels may serve to loosen inter-endothelial cell contacts and render them more responsive to growth factors. As pericytes are thought to limit the rate of endothelial cell proliferation in both vascular pruning and remodelling (Orlidge and D'Amore 1987, Benjamin *et al.* 1998, Sundberg *et al.* 2001), endothelial cell migration must involve dissociation of the endothelial cells from their periendothelial cell support. However, pericytes also appear to aid the earliest stages of capillary sprouting. Pericytes are found lying at and in front of the advancing tips of endothelial sprouts and, at many sites, pericytes can be observed bridging the gap between the leading edges of opposing endothelial sprouts, which are apparently preparing to merge. This suggests that pericytes may serve as guiding structures aiding the outgrowth of endothelial cells (Nehls *et al.* 1992).

Extracellular matrix components also play an important role in angiogenesis by serving as a reservoir of growth factors (Hood and Cheresch 2002, Hynes 2002).

Proteinases of the plasminogen activator, MMP, chymase and heparinase families aid angiogenesis by breaking down the extracellular matrix and, in doing so, activate or liberate many angiogenic growth factors that are sequestered within the extracellular matrix (Coussens *et al.* 1999, Carmeliet 2000). This proteolytic degradation of the extracellular matrix must, however, be tightly controlled, as excessive breakdown can remove critical support and guidance cues for migrating endothelial cells and can have a deleterious effect on angiogenesis (Carmeliet 2004). Once the extracellular matrix has been degraded, endothelial cell proliferation and migration can begin.

Endothelial cells of resting, established, vasculature are generally quiescent. However, endothelial cell proliferation forms the major component of capillary growth. Proliferating endothelial cells respond to a mitogenic stimulus by migration, during which time their morphology is more elongated than usual (Ingber and Folkman 1989). The majority of proliferation occurs in the endothelial cells behind the tip of the growing capillary, the tip usually remaining quiescent (Klagsbrun and D'Amore 1991). VEGF and its receptor VEGF receptor 2 (VEGFR2) are thought to mediate much of the endothelial cell proliferation and migration during angiogenesis, as VEGF binding to VEGFR2 results in a chemotactic and pro-survival signal for the endothelial cells on which it is expressed (Ferrara *et al.* 2003b).

The generation of new blood vessels is completed by their formation into capillaries. Endothelial cells stop dividing and form tubes through curvature as they start to adhere to each other. The recruitment of pericytes — from either pre-existing microvessels or newly derived pericytes from fibroblasts — to cover the outer walls of the newly formed vessels and thus to stabilise the new vasculature, is thought to be controlled by the endothelial cells themselves through secretion of diffusible factors (Crocker *et al.* 1970, Hirschi and D'Amore 1996, Goede *et al.* 1998, Hirschi *et al.* 1999, Redmer *et al.* 2001). Among the diffusible factors secreted by the endothelial cells are platelet derived growth factor (PDGF) and bFGF, both of which are thought to act in a paracrine fashion, stimulating mural cell proliferation and migration towards the new capillaries (Montesano *et al.* 1986, Westermarck *et al.* 1990). The process of pericyte recruitment is also thought to be relatively rapid, as pericytes are evident around new blood vessels in the forming corpus luteum (Goede *et al.* 1998, Wulff *et al.* 2001a). Blood flow in these new vessels begins slowly, as

the new vessels remain highly permeable until a basement membrane surrounding the endothelial cells is laid down and pericyte recruitment and stabilisation is complete (Findlay 1986).

1.10.1 Measurement of angiogenesis

There is a long history of measuring tumour vascularity to provide prognostic information and to study the effect of treatment on a tumour (West *et al.* 2001). Intercapillary distances (ICD) have been used as an index of tumour oxygenation and hence vascularisation, although these have recently been shown to only provide a weak measure of the hypoxic state of the tissue (West *et al.* 2001). A more commonly used histological assessment of tumor angiogenesis is measurement of microvessel density (MVD) (Vermeulen *et al.* 1996), where the density of the blood vessels in areas of neovascularisation is analysed.

Less invasive methods of measuring the extent of vascularisation and blood flow in an organ are also available. Colour Doppler ultrasonography is a non-invasive technique for monitoring vascular function and degree of vascularisation of an organ. The technique involves scanning the organ of interest with an ultrasound scanner enabling the calculation of organ volume and rate of blood flow. This technique has been used to study blood flow in both the preovulatory follicle (Brannstrom *et al.* 1998, Acosta *et al.* 2003) and the corpus luteum (Miyazaki *et al.* 1998, Acosta *et al.* 2002). Recent advances in magnetic resonance imaging (MRI) can now also be applied to the *in vivo* measurement of blood flow, blood volume, vessel permeability and extracellular leakage (Padhani and Dzik-Jurasz 2004).

The quantification of vascularisation and angiogenesis in tissue sections using immunocytochemical markers for the endothelium such as CD31, has greatly enhanced the study of angiogenesis. Expression of CD31, a 130-kDa glycoprotein belonging to the immunoglobulin (Ig) superfamily of cell adhesion molecules (Newman 1994), is restricted to cells of the vascular system platelets, monocytes, neutrophils, selected T cells, and endothelial cells (Newman 1997), and is commonly used as an endothelial specific marker (Ilan *et al.* 2000). It is the extracellular Ig domain of CD31 that contains specialised sites that mediate trans-homophilic interactions between CD31 molecules on adjacent cells (Sun *et al.* 1996). CD31

knockout mice have been noted to suffer from prolonged bleeding times after surgical insult, which is at least in part due to disrupted endothelial-platelet interactions (Mahooti *et al.* 2000). In addition to CD31's role in endothelial cell adhesion, there is growing evidence that CD31 is able to transduce signals that suppress endothelial cell apoptosis (Noble *et al.* 1999, Newman and Newman 2003). Co-localisation of CD31 and a proliferating cell marker, such as BrdU or Ki67, has also allowed for the quantification of active endothelial cell proliferation in a fixed tissue (Wulff *et al.* 2001b, Wulff *et al.* 2002).

There are also several bioassays that have been developed to measure angiogenesis. The most common ones are endothelial cell migration and proliferation *in vitro* (Folkman *et al.* 1979, Glaser *et al.* 1980), and capillary growth in the developing chick chorioallantoic membrane (CAM) or cornea *in vivo* (Ausprunk *et al.* 1974, Gimbrone *et al.* 1974). The CAM is an extra-embryonic membrane that serves as a gas exchange surface with its respiratory function provided by an extensive capillary network (Ausprunk *et al.* 1974). Because of its extensive vascularisation the CAM has been used as an *in vivo* model for the evaluation of both angiogenic and anti-angiogenic molecules (Ribatti *et al.* 1996, Ribatti *et al.* 2000, Ribatti *et al.* 2001), **Figure 1.4**.

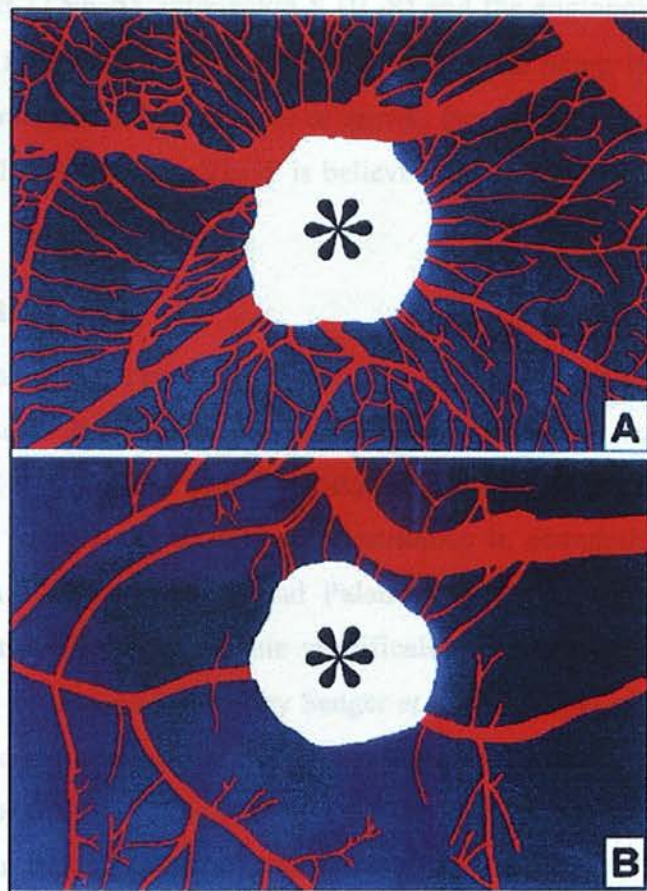


Figure 1.4 Image analysis of CAM vascularisation.

Vascularisation of CAM after incubation with (A) a pro-angiogenic agent, fibroblast growth factor-2 (FGF-2) and (B) an angiostatic molecule, TNP 470. Numerous vessels develop radially towards the gelatin sponge (*) soaked with FGF-2 in a spoked-wheel pattern. Whereas very few vessels are recognizable around the sponge (*) treated with TNP-470. Adapted from (Ribatti *et al.* 2001)

1.10.2 Angiogenic promoters

A large number of growth factors have been implicated in the positive regulation of angiogenesis, aFGF, bFGF, TGF- α , TGF- β , hepatocyte growth factor (HGF), tumour necrosis factor- α (TNF- α), interleukin-8 (IL-8) and the angiopoietins (Ang-1 and Ang-2) have all been shown to be involved (Folkman and Shing 1992, Yancopoulos *et al.* 2000). However, of the multitude of growth factors that regulate physiological and pathological angiogenesis, VEGF is believed to be the most important (Ferrara 2004).

1.10.2.1 VEGF

VEGF is a pro-angiogenic molecule that has been implicated in various steps in the angiogenic process. It is known also as vascular permeability factor (VPF) on the basis of its ability to induce vascular leakage (Senger *et al.* 1983, Dvorak *et al.* 1995), and has been found to induce fenestrations in endothelial cells of small venules and capillaries (Roberts and Palade 1995). VEGF is also the only angiogenic peptide that is mitogenic specifically for vascular endothelial cells. VEGF was first discovered in 1983 by Senger *et al.* (Senger *et al.* 1983) when they described the partial purification of a protein from the conditioned medium of a guinea-pig tumor cell line. The protein was able to induce vascular leakage in the skin and was thus named vascular permeability factor. However, in 1989 a separate group reported the isolation of a diffusible endothelial-cell specific mitogen from medium conditioned by bovine pituitary follicular cells, which they named vascular endothelial growth factor, to reflect the target cell specificity of this molecule (Ferrara and Henzel 1989). Sequencing of purified VEGF proved that this protein was distinct from the known endothelial cell mitogens such as aFGF or bFGF (Ferrara and Henzel 1989) and cloning of VEGF (Leung *et al.* 1989) and VPF (Keck *et al.* 1989) cDNAs demonstrated that VEGF and VPF were in fact the same molecule. The finding that VEGF is potent, diffusible, and specific for vascular endothelial cells led to the hypothesis that this molecule might play a role in the regulation of physiological and pathological growth of blood vessels (Ferrara and Henzel 1989, Leung *et al.* 1989).

VEGF is a member of the heparin-binding growth factor family (Leung *et al.* 1989). The VEGF family of molecules consists of six growth factors (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor). VEGF-A (from here on referred to as VEGF) has six isoforms generated as a result of alternative splicing of the 8 exons of the VEGF gene: VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₃, VEGF₁₈₉ and VEGF₂₀₆ (Stimpfl *et al.* 2002). These splice variants are illustrated in **Figure 1.5**. The domain encoded by exons 1–5 contains information required for the recognition of the known VEGF receptors (Keyt *et al.* 1996), and is present in all of the VEGF isoforms. The mRNA encoding VEGF₁₆₅ and VEGF₁₂₁ are dominant in normal human ovaries (Fujimoto *et al.* 1998, Otani *et al.* 1999). In contrast, VEGF₂₀₆ is a rare isoform, and has been described only in the human foetal liver (Ferrara and Davis-Smyth 1997). VEGF-C and VEGF-D are involved in the regulation of lymphatic angiogenesis (Karkkainen *et al.* 2002, Stacker *et al.* 2002).

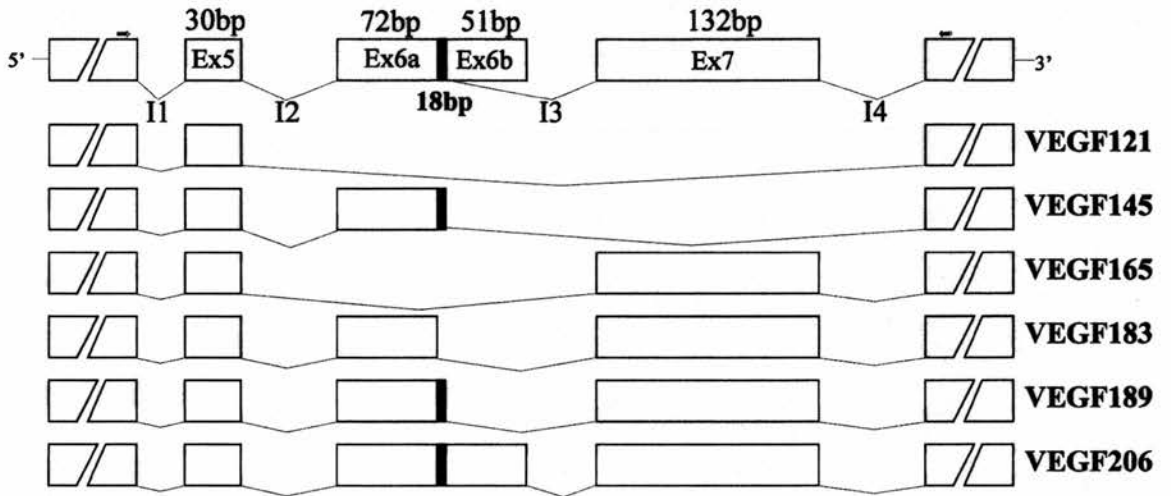


Figure 1.5 The VEGF isoforms.

Model for alternative splicing of VEGF mRNA generating six VEGF variants. Exons are represented by rectangles, introns by solid lines. Adapted from (Stimpfl *et al.* 2002)

VEGF mRNA expression and protein secretion is up regulated by a variety of cytokines and growth factors. IGF-I induces VEGF mRNA and protein production by both an increase in the transcriptional rate of the VEGF gene, and the stability of mRNA (Warren *et al.* 1996). EGF, TGF- α and TGF- β , bFGF, interleukin (IL)-1 and IL-6 all up-regulate VEGF expression (Ferrara and Davis-Smyth 1997). VEGF mRNA is also highly expressed through induction by hypoxia *in vitro* and *in vivo* via hypoxia-inducing factor (HIF)-1 signalling (Shweiki *et al.* 1992, Popovici *et al.* 1999), via elevated cyclic adenosine monophosphate (cAMP) concentration, increased cAMP levels increasing VEGF mRNA expression (Takagi *et al.* 1996). Both of the gonadotrophins FSH and LH primarily signal through cAMP post receptor mechanisms, allowing them to stimulate VEGF expression (Garrido *et al.* 1993).

All members of the VEGF family bind to cell-surface receptors (VEGFRs) to initiate a cellular response via activation of an intracellular tyrosine kinase domain. The biological effects of VEGF are mediated by two main tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1), although other receptors such as neuropilin-1 and -2 can also bind VEGF. A further tyrosine kinase receptor, VEGFR3 (flt-4) also exists, but binds VEGF-C and VEGF-D and is more important in the development of lymphatic vessels (Larrivee and Karsan 2000).

The signalling and biological properties of VEGFR1 and VEGFR2 are strikingly different. They both have seven immunoglobulin (Ig)-like regions in the extracellular domain, a single transmembrane region, and a consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain (Ferrara and Davis-Smyth 1997). Both VEGFR1 and VEGFR2 bind VEGF with high affinity, although VEGFR2 has a somewhat lower affinity than VEGFR1 (de Vries *et al.* 1992). Their signal transduction properties, however, are very different. In transfected cells expressing the VEGFR2 receptor, chemotaxis and mitogenic activity have been found in response to VEGF. In contrast, transfected cells expressing VEGFR1 lack such responses. VEGFR2 undergoes strong ligand-dependent tyrosine phosphorylation in intact cells, while phosphorylation of VEGFR1 is weak (Waltenberger *et al.* 1994). Therefore, interaction with VEGFR2 is a critical requirement to induce the full spectrum of VEGF biological responses. Hence,

VEGFR2 is thought to be the key receptor that triggers angiogenesis and vascular permeability (Gille *et al.* 2001), with the role of VEGFR1 being less clear, but possibly mediating cell migration and vascular permeability (Stacker *et al.* 1999, Kanno *et al.* 2000) and also acting as a decoy receptor when in soluble form, preventing VEGF binding to VEGFR2 (Park *et al.* 1994). Park *et al.* were the first to propose that VEGFR1 may not be the primary VEGF receptor transmitting the mitogenic signal, but rather a decoy receptor, able to regulate VEGF activity on the endothelium in a negative way by sequestering VEGF and rendering it less available to VEGFR2 (Park *et al.* 1994).

Much of our understanding of how VEGF and its receptors operate comes from studies in the mouse. Mice with a homozygous mutation in the VEGFR1 locus die *in utero* between days e8.5 and e9.5 (Fong *et al.* 1995). Further work on VEGFR1 knockout mice revealed a disorganised phenotype in the embryos, due to an increase in the number of endothelial cell progenitors by altered cell fate determination (Fong *et al.* 1999). Work on VEGF mutant mice showed that inactivation of one VEGF allele is embryonic lethal between days e11 and e12 (Carmeliet *et al.* 1996, Ferrara *et al.* 1996). Blood island formation in these VEGF^{+/-} embryos was impaired and they also displayed defects in formation of the nervous system. This VEGF^{+/-} phenotype is due to gene dosage and not maternal imprinting, emphasising the critical need for the precise regulation of VEGF expression during vasculogenesis and angiogenesis (Carmeliet *et al.* 1996).

1.10.2.2 Angiopoietins

The angiopoietins and their receptors constitute another significant signalling system involved in blood vessel maintenance, growth and stabilisation. The inter-relationship between VEGF and the Ang family of angiogenic regulators plays an important role in angiogenesis. The angiopoietins consist of approximately 500 amino acids, and are ligands for only one of the Tie tyrosine kinase receptors, Tie-2. Both Tie-1 and Tie-2 are selectively expressed within the vascular endothelium. Tie-1, however, is an orphan tyrosine kinase receptor (Partanen *et al.* 1992), which shares little identity, in its extracellular domains, with Tie-2 and thus does not bind any of the angiopoietins (Kontos *et al.* 2002). There are four known angiopoietins,

Ang1-4, all of which bind to Tie-2 (Valenzuela *et al.* 1999). Northern blot analyses from adult human tissues has localised expression of the angiopoietins. Ang-1 is widely expressed, although only in small amounts in the heart and liver. However, Ang-2 expression is only readily detectable in ovary, placenta and uterus, the three predominant sites of vascular remodelling in the healthy adult (Maisonpierre *et al.* 1997).

Ang-1 is known to both promote and inhibit angiogenesis, depending upon the tissue in which it is expressed. It is primarily involved in the stabilisation of vessel walls, tightening blood vessels via an effect on PECAM-1/CD31, VE-cadherin and occludin (Chen *et al.* 1996, Lawson and Weinstein 2002), and acts as an angiogenic inhibitor in this sense. However, it stimulates vessel growth in skin, ischaemic limbs, gastric ulcers and some tumours (Shyu *et al.* 1998, Suri *et al.* 1998, Chae *et al.* 2000, Shim *et al.* 2002), presumably because of its role as an endothelial cell survival factor. When acting in conjunction with VEGF, Ang-2 stimulates angiogenesis by loosening endothelial cell interactions with their pericytic cells, and aids the degradation of the extracellular matrix by up-regulating proteinase expression, thus allowing the endothelial cells to migrate. However, without an angiogenic signal acting on the destabilised endothelial cell, the action of Ang-2 causes vessel regression and endothelial cell death (Maisonpierre *et al.* 1997). Hence, the balance between the expression of Ang-1 and Ang-2 competitively binding Tie-2 needs to be finely tuned during angiogenesis. Mice over-expressing Ang-2 in their blood vessels have shown that over-expression disrupts blood vessel formation in the embryo. The embryos have a collapsed endocardium, vascular abnormalities in and around the heart, and lack a regular dendritic capillary plexus in the head, reminiscent of embryos lacking Tie-2 or Ang-1. Ang-2 is highly expressed in the theca of the preovulatory follicle, and during formation of the corpus luteum, a time at which high levels of VEGF are also expressed, which promotes angiogenesis. During luteal regression in both the human (Wulff *et al.* 2000) and in the macaque (Hazzard *et al.* 2000) Ang-2 is again highly expressed where as VEGF is not, leading to vessel destabilisation and endothelial cell death. In the rat, however, different mechanisms appear to be involved in luteal vessel destabilisation and degradation as Ang-2 mRNA is no longer detectable in the regressing CL (Wiegand *et al.* 2000).

The relative roles of VEGF, the Ang family and their receptors is summarised in **Figure 1.6**.

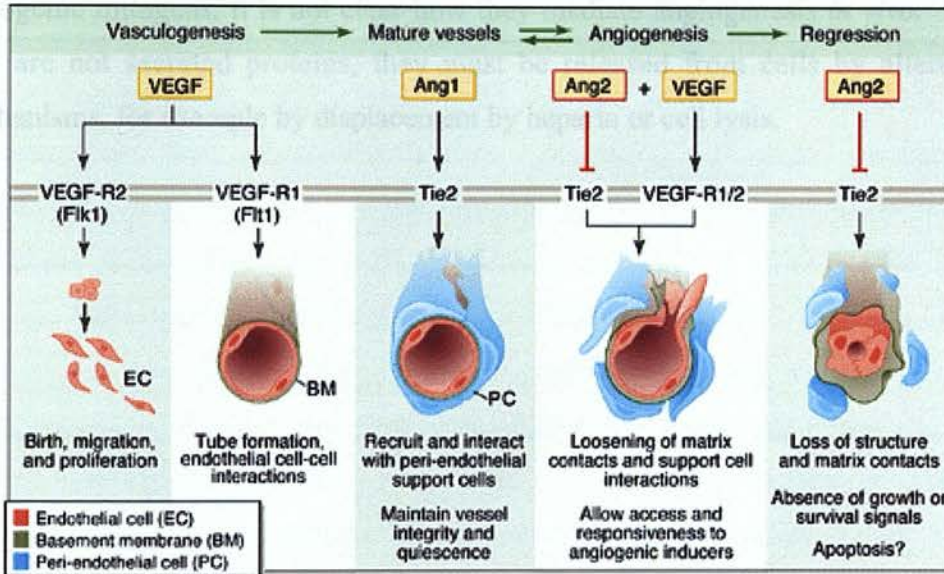


Figure 1.6 Factors influencing the angiogenic process.

Regulation of vascular morphogenesis, maintenance, and remodelling by the receptor tyrosine kinases and their ligands. Adapted from Hanahan (1997).

1.10.2.3 Fibroblast growth factors

One of the most potent of the known angiogenic factors is bFGF (Klagsbrun and D'Amore 1991), which was first purified by heparin-affinity chromatography in 1985 (Esch *et al.* 1985). FGF *in vitro* is mitogenic and chemotactic for endothelial cells, stimulating them to produce collagenases and plasminogen activator proteases capable of degrading the basement membrane. FGF also induces capillary endothelial cells to migrate into three-dimensional collagen matrices and to form capillary like tubes (Klagsbrun and D'Amore 1991). Both aFGF and bFGF are angiogenic factors in the CAM and cornea assays (Klagsbrun and D'Amore 1991). However, they are not only endothelial growth factors, but are also mitogenic for vascular smooth muscle, fibroblasts, and for some epithelial cells. Endothelial cells

synthesise large amounts of bFGF (Schweigerer *et al.* 1987), but bFGF is also synthesised by many other cell types. Neither bFGF nor aFGF contains a consensus signal peptide in the open reading frame, and are therefore cellular and not secreted (Abraham *et al.* 1986, Vlodavsky *et al.* 1987). Although aFGF and bFGF are both angiogenic mitogens, it is not clear how they mediate angiogenesis *in vivo*. Since they are not secreted proteins, they must be released from cells by alternative mechanisms, for example by displacement by heparin or cell lysis.

1.11 Manipulation of angiogenesis

1.11.1 Development of anti-angiogenic agents

The application of angiogenesis inhibitors as anticancer agents is still in its early stages, but over 25 drugs are currently under development in various stages of trials (Kerbel and Folkman 2002). These drugs fall into several functional categories, including inhibitors of: growth factors; endothelial cell signal transduction; endothelial cell proliferation; matrix metalloproteinases; endothelial cell survival; and bone marrow precursor cells (Hagedorn and Bikfalvi 2000, Kerbel 2001). Whereas some agents, such as antibodies to VEGF and VEGFR2, continue to show promise, a few drugs have proved disappointing. For example, matrix metalloprotease inhibitors with anti-angiogenic activity have poor efficacy and also exhibit side effects (Coussens *et al.* 2002). Naturally occurring inhibitors of angiogenesis that are encrypted within larger proteins devoid of angiogenic activity have been identified. Angiostatin, an internal fragment of plasminogen, is a potent inhibitor of angiogenesis and selectively inhibits endothelial cell proliferation. Endostatin, a 20 kDa C-terminal fragment of collagen XVIII, specifically inhibits endothelial proliferation and showed potential to inhibit angiogenesis and tumor growth (O'Reilly 1997, O'Reilly *et al.* 1997). Despite generating much publicity at the time, the potent anti-tumour activity of some of these proteins, such as endostatin and angiostatin, has proved difficult to reproduce (Marshall 2002). Nevertheless, there is still intense interest in angiogenesis inhibitors as future clinical tools and much work is in progress (Kerbel and Folkman 2002, Novak 2002).

Ferrara and colleagues were the first to show that antibodies to VEGF slowed tumour growth in the mouse model (Kim *et al.* 1993), and a variety of therapeutic strategies aimed at blocking VEGF or its receptor signalling system are currently being developed for the treatment of neoplastic diseases. VEGF/VEGFR blockade by monoclonal antibodies and inhibition of receptor signalling by tyrosine kinase inhibitors are to date the most studied approaches. VEGFR1 ribozymes, VEGF toxin conjugates, and soluble VEGF receptors are also being investigated. Of these, bevacizumab (rhuMab VEGF, AvastinTM, Genentech Inc, South San Francisco, CA,

USA), a humanised monoclonal antibody directed at VEGF, is the most advanced in clinical development, and has shown promising results in clinical trials (Ferrara 2004). In February of this year, the U.S. Food and Drug Administration (FDA) approved AvastinTM to be used in combination with chemotherapy as a treatment for patients with first-line or previously untreated metastatic cancer of the colon or rectum. Although in August, the FDA and Genentech issued an important warning that there is evidence of an increased risk of serious arterial thromboembolic events, including cerebrovascular accident, myocardial infarctions, transient ischemic attacks, and angina related to AvastinTM use.

1.11.2 Vascular targeting

The concept of vascular targeting is related to anti-angiogenesis but involves a different approach. Rather than inhibiting the formation of new vessels, drugs are aimed at destroying the existing vasculature, with consequent tumour regression. A fundamental principle of this approach is that tumour vasculature is different from that in normal tissues, and much evidence exists to support this (Ruoslahti 2002). For example, the vasculature is exposed to inflammatory cytokines released by tumour cells and also hypoxic conditions (Helmlinger *et al.* 1997). Targeting tumour vasculature with toxic drugs as an effective anti-tumour strategy was proved when an anti-major histocompatibility complex class II antibody, coupled to ricin toxin, was used to destroy tumour vasculature in experimental mice models (Burrows and Thorpe 1993). They showed that this approach could be used to specifically eradicate large solid tumours *in vivo*. The largest stumbling block to further progress in this field has been the failure to identify candidate targets in human tumour endothelium. However, two recent advances have led to a resurgence of interest in vascular targeting: the development of novel, low-molecular-weight drugs that are selectively toxic to tumour vasculature; and the advances in bioinformatics that have enabled the analysis and identification of novel targets. For example, the discovery of three novel tumour endothelial marker (TEM1, 5, 8) genes that display elevated expression during tumor angiogenesis (Carson-Walter *et al.* 2001), and magic roundabout (robo4) (Huminiacki *et al.* 2002), an endothelial-specific gene in tumours, are both of considerable interest. Magic roundabout is present on the cell

surface and is therefore ideal for targeting. Moreover, it appears to be a developmental gene because it is not expressed in adult tissue and, as such, its targeting should have no deleterious effects in the adult. Another such gene is Delta4, which is also endothelial specific, and is only found on tumour endothelium in the adult (Mailhos *et al.* 2001). The identification of a tumour-homing peptide that specifically targets tumour lymphatic vessels (Laakkonen *et al.* 2002) could also target therapies to tumour lymphatics. It has been shown recently that active immunisation against tumour endothelial markers (e.g. the VEGF receptors) (Li *et al.* 2002b, Niederman *et al.* 2002) can inhibit tumour growth and metastasis. It has long been realised that, at the molecular level, angiogenesis is likely to be a complex process that involves many players. Nevertheless, targeting key molecules, such as VEGF and its receptor VEGFR2, and the rapid increase in endothelial-specific markers in tumours that represent targets, confirms that research into angiogenesis and the tumour vasculature is at an exciting stage (Bikfalvi and Bicknell 2002).

1.12 Clinical relevance of follicular angiogenesis research

As discussed above, there is a great deal of research being conducted into the regulation of tumour angiogenesis and techniques to either inhibit blood vessel growth or indeed destroy specific vasculature altogether. The lessons learned from these studies on the regulation of angiogenesis should be applied to the physiological angiogenesis that occurs in the developing ovarian follicle. It is a tightly regulated process that, if upset by a disturbance in the balance of angiogenic factors regulating it, can lead to severe reproductive dysfunction. The parallel systems, such as VEGF and its receptors, associated with both physiological and pathological angiogenesis have long been appreciated. However, the endocrine mechanisms regulating angiogenesis and in the ovarian follicle have received much less attention in the literature. Understanding the specific mechanisms regulating follicular angiogenesis may allow formulation of treatments for such reproductive disorders as PCOS and OHSS.

1.13 Aims of this thesis

At the beginning of this project little was known about the effect of the inhibition of the gonadotrophins or VEGF on angiogenesis in the primate ovarian follicle. The angiogenic activity associated with follicular development in the marmoset and methods for its quantification had already been established in earlier studies (Wulff *et al.* 2001b, Wulff *et al.* 2002). The aims of this thesis were to increase the understanding of control of primate follicular angiogenesis by comparing the effect of inhibiting either the gonadotrophins or VEGF directly, and assessing the effect of these inhibitions on follicular development and vascularisation in a primate model. To assess the endocrine control of follicular angiogenesis, a GnRH antagonist was used. This was done to analyse the effect of the inhibition of the gonadotrophins on follicular angiogenesis. The hypothesis being that follicular angiogenesis would be dependent upon both FSH and LH, and be severely inhibited by GnRH antagonist treatment. To extend this investigation, the availability of primary marmoset granulosa cell cultures allowed the study of the sensitivity of both angiogenic and steroidogenic gene expression in response to FSH stimulation *in vitro*. This was done to investigate whether a different level of FSH stimulation is required for activation of genes involved in regulating both follicular angiogenesis and follicular steroid hormone synthesis. Secondly, the effect of inhibition of angiogenesis directly was studied by using VEGF trap, a soluble form of the VEGF receptors that is able to sequester VEGF protein and block its action *in vivo*. The hypothesis being that VEGF is essential for increasing permeability and growth of the selected follicles and that inhibition of VEGF would have serious effects on follicular angiogenesis, follicular development, ovulation and the establishment of the corpus luteum. Finally, during this project, the discovery of a novel endocrine gland specific angiogenic mitogen stimulated the search for this gene in both the marmoset monkey and the human. The hypothesis being that endocrine gland vascular endothelial growth factor (EG-VEGF) is an additional angiogenic factor regulating blood vessel development and maintenance in the primate ovary.

Chapter 2 General Materials and Methods

2.1 Source of reagents

Unless otherwise stated all reagents were supplied by Sigma-Aldrich Company Ltd (Poole, Dorset, UK).

2.2 Experimental model

The marmoset monkey (*Callithrix jacchus*) was chosen for this *in vivo* exploration of follicular angiogenesis. No one primate model is ideal for investigations pertaining to the human, but marmosets offer several distinct advantages over other non-primate models (Fraser and Lunn 1999). Some of the key advantages of the common marmoset for use in reproductive research are: ease of handling; high fecundity; absence of post-partum acyclicity related to lactation; and quick sexual maturation (Torii *et al.* 1996). Also, the ability to induce luteolysis and synchronise the ovarian cycle precisely by prostaglandin injection makes the marmoset a very powerful model in which to study precise stages in the ovarian cycle (Summers *et al.* 1985, Duncan *et al.* 1998, Gilchrist *et al.* 2001).

2.2.1 Marmoset husbandry

All marmosets were housed in the R. V. Short Building, Bush Estate, Edinburgh. Staff at the centre were responsible for the care and maintenance of the animals. Common marmoset monkeys were housed in rooms kept at between 20°C and 25°C, which were artificially lit between the hours of 07:00 and 19:00. The animals were housed in cages measuring 1.15m x 1.1m x 0.6m. The cages contained larch branches and a nest box, and the floor was filled with wood chippings to allow foraging.

The animals were fed daily with a selection of fruit and SDS Mazuri (E) primate diet pellets. In addition, they were given high protein porridge with multivitamin supplements three times per week. Water was continuously available. Adult females with a body weight of approximately 350g, and who had regular ovarian cycles, were housed together with a younger sister or prepubertal female, unless otherwise stated. Blood samples were collected three times per week by femoral venepuncture without anaesthesia, while the animals were held in a

restraining device (Hearn *et al.* 1978). Syringes were sealed and centrifuged for 20 mins at 1000 x g; plasma was then removed and stored at -20°C until required.

2.2.2 The Marmoset ovarian cycle

In marmosets the ovarian cycle starts at approximately 14 to 18 months of age (McAnulty 1994). The ovarian cycle lasts for approximately 28 days, typically with two or three ovulations per cycle (Harding *et al.* 1982, Hearn 1983). The follicular phase lasts for 8 or 9 days with a luteal phase of 20 days (Harding *et al.* 1982). In most cycles, 2 or 3 dominant follicles grow at similar rates with ovulation occurring at up to 12-hour intervals (Torii *et al.* 1996).

2.2.3 Synchronisation of follicular development

Induction of luteolysis by administration of $\text{PGF}_{2\alpha}$ was used to synchronise follicular development in the marmoset (Gilchrist *et al.* 2001). To synchronise the stage of the ovarian cycle, all control and experimental animals were given $1\mu\text{g}$ $\text{PGF}_{2\alpha}$ analogue (cloprostenol, Planate, Coopers Animal Health Ltd., Crewe, UK) intramuscularly between days 12-16 of the luteal phase of the pre-treatment cycle to induce luteolysis: this marks day zero of the follicular phase. It has been demonstrated that after $\text{PGF}_{2\alpha}$ induced luteolysis, an average of 10.7 days is required for growth of the dominant follicle to the preovulatory stage (Summers *et al.* 1985). $\text{PGF}_{2\alpha}$ administration is also a commonly-used tool in the investigation of marmoset luteal regression (Summers *et al.* 1985, Webley *et al.* 1989, Michael and Webley 1993, Fraser *et al.* 1995a, Duncan *et al.* 1998, Fraser *et al.* 1999b).

2.2.4 Treatment regimes

Ovaries were collected throughout the follicular phase and into the early luteal phase of the cycle. The basic principle of the experiments was to prevent follicular angiogenesis by administering either a GnRH antagonist, Antarelix [$\text{N-Ac-D-Nal}^1, \text{D-pCl-Phe}^2, \text{D-Pal}^3, \text{D-(Hci)}^6, \text{Lys(iPr)}^8, \text{D-Ala}^{10}$]GnRH (Europeptides, Argenteuil, France) (Deghenghi *et al.* 1993), or the VEGF trap R1R2 (Regeneron Pharmaceuticals, Tarrytown, NY, USA) (Holash *et al.* 2002) during either the early- or the mid-follicular phase of the treatment cycle. An explanation of the nature of

the VEGF trap can be found in Chapter 5. Details of dose, timing and duration of treatment can be found in the relevant experimental chapters. Staff at the primate centre undertook administration of treatments. All experiments were carried out in accordance with the Animals Scientific Procedures Act 1986, and were approved by the local ethical review committee.

2.2.5 Classification of follicle stage

Stages of follicular development were classified as follows: primordial (oocyte surrounded by a single flat layer of follicle epithelial cells); primary (single layer of cuboidal granulosa cells surrounding an oocyte); early secondary (two to four granulosa cell layers surrounding an oocyte); late secondary (more than four granulosa cell layers surrounding an oocyte, no antrum formation); tertiary (follicles containing an antrum, less than 2000 μm in diameter); and dominant (follicles with a diameter greater than 2000 μm). Healthy follicles were classified as having a normally shaped oocyte and no sign of pycnosis in the granulosa cells. Atretic follicles were classified as having pycnotic granulosa cells, the first morphological sign of follicular atresia (Durlinger *et al.* 2000).

2.2.6 Criteria for follicle analysis

To allow for analysis of follicle populations in the ovaries certain criteria was established for counting follicles. Every 40th ovarian section was stained with haematoxylin and eosin (2.6) for analysis. Only follicles with a normal shaped oocyte surrounded by granulosa cells that were regularly opposed on an intact basement membrane and did not show any signs of pycnosis were classified as health and included in analysis. Only follicles with a visible oocyte where included in the analysis to insure that the same follicle was not included in the analysis twice.

2.2.7 Classification of cycle stage

For a number of cycles before the animals were used, marmoset plasma was collected three times per week to measure the concentration of plasma progesterone. Such routine blood sampling has been shown not to disturb ovarian cyclicity or the hypothalamic-pituitary-adrenal axis (Saltzman *et al.* 1994, Saltzman *et al.* 1997a,

Saltzman *et al.* 1997b). Plasma was separated and frozen at -20°C . A plasma progesterone reading of $<30\text{nmol/L}$ was taken to indicate the follicular phase; when the plasma progesterone rose above 30nmol/L and continued to rise, the luteal phase was deemed to have begun.

2.3 Plasma progesterone plate assay

Ninety-six well plates were coated in DARS coating antibody, Rivanol purified DARS (Donkey anti-rabbit serum, pH 9.6, SAPU, Scottish Antibody Production unit, Carlisle, Lanarkshire, UK) and left overnight at 4°C . Plates were then washed 5 times with washing solution (302g Tris, 450g NaCl and 25ml Tween 20 up to 2L with H_2O , pH 7.5) diluted 1:25 in water before use. Marmoset plasma samples were thawed, vortexed and diluted. Plasma ($2.5\ \mu\text{l}$) was added to $97.5\ \mu\text{l}$ of assay buffer (17.85g Na_2HPO_4 , 7.75g citric acid, 1g gelatin, 0.1g thiomersalate in 1L deionised H_2O , pH 6). In each assay, a low, medium and high progesterone concentration quality control (QC) was used. Samples and QCs were prepared in a similar way. A standard curve was prepared from a serial dilution of progesterone standards diluted in assay buffer to 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and $1000\text{pg}/50\ \mu\text{l}$. Fifty micro litres of each standard plus $2.5\ \mu\text{l}$ of charcoal stripped male marmoset plasma was added to $47.5\ \mu\text{l}$ assay buffer. To determine maximum binding (B_0), $2.5\ \mu\text{l}$ of charcoal stripped male marmoset plasma was diluted in $97.5\ \mu\text{l}$ of buffer. All samples, standard curve, B_0 and QC's were prepared in duplicate. Fifty micro litres of primary antibody, rabbit anti-progesterone (SAPU), diluted 1:50,000 in assay buffer were added to each well. In addition, to assess non-specific binding, charcoal stripped male marmoset plasma — which does not contain progesterone — was diluted in $147.5\ \mu\text{l}$ of assay buffer, and primary antibody omitted. Plates were covered and left overnight at room temperature. After incubation overnight, $50\ \mu\text{l}$ per well of labelled ligand (progesterone-11-glucuronide-biotin complex) diluted 1:20,000 in assay buffer was added with 2mg/ml ANSA (8-anilino-1 naphthalene sulphonic acid). Plates were then covered and left at room temperature for 3 h. After the incubate was removed, the plates were washed 10 times before $100\ \mu\text{l}$ of Streptavidin-Horseradish peroxidase diluted 1:2000 with normal phosphate buffer

(1% casein, pH7.4) was added to each well. After 1 h, the plates were again washed 10 times before 200 μ l per well of substrate OPD (5mM O-Phenylenediamine/0.03% H₂O₂), dissolved in substrate buffer, (10.3g citric acid, 17.79g Na₂HPO₄ dihydrate up to 1L with deionised H₂O, pH 5), was added. After 10-20 mins, the reaction was stopped by adding 50 μ l of stopping solution (2N sulphuric acid) to each reaction. Plates were read at 490nm on a spectrophotometer plate counter (VICTOR 1420 Multilabel Counter, Wallac, Turku, Finland). Inter assay coefficients of variation for low, medium and high level quality controls were 11.6%, 7.0% and 12.6% respectively. The detection limit of the assay was 4.6 nmol/L. Our in house assay lab staff conducted this assay.

2.4 Plasma oestradiol radioimmuno assay

To extract the steroid hormones from the plasma samples and the QCs, plasma samples and QCs were first thawed and vortexed. Tubes (10mm x 75mm) were set up with 10 μ l of sample or QC (high, medium or low) as well as 40 μ l of distilled water. Tubes were then vortexed and 2ml of Diethyl Ether was added to each tube. Tubes were then vortexed for 10 mins on a multitube clamp vortex, after which they were snap frozen in dry ice and methanol. The unfrozen fraction was then decanted off and was left to evaporate in a fume hood overnight. After all of the Diethyl Ether had evaporated, 200 μ l of 0.1M PGBS (pH 7.4, 11.46g Na₂HPO₄, 2.61g NaH₂PO₄.2H₂O, 9.0g NaCl, 1.0g Gelatin, 0.1g thiomersalate to 1L deionised water) was added to each tube, tubes were then vortexed and allowed to solvate for 30 mins. A standard curve was prepared from oestradiol standards diluted in 0.1M PGBS to 0.06, 0.12, 0.24, 0.49, 0.98, 1.95, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250 and 500pg/100 μ l. One hundred microlitres of each standard and 100 μ l of 0.1M PGBS was added to fresh tubes. To determine non-specific binding (NSB) 300 μ l of 0.1M PGBS was added to fresh tubes. To determine maximum binding (B₀), 200 μ l of 0.1M PGBS was added to fresh tubes. To all tubes, except NSB's, 100 μ l of oestradiol MAIA kit antibody (Adaltis Italia Casalecchio di Reno. Italy), diluted 1:10 from kit stock was added. 100 μ l of MAIA tracer, diluted to 10-12,000cpm, was then added to all tubes tube and incubated at room temperature for 3-4 h. After incubation, 100 μ l of diluted secondary antibody (1:1 ratio of 1:200 Normal Rabbit

Serum, SAPU and 1:60 GARG, Valeant Pharmaceuticals International, Costa Mesa, C.A., U.S.A.) was added to each tube and incubated overnight at 4°C. One millilitre of 4%PEG/0.2%Triton in 0.9% Saline was then added and all tubes centrifuged at 1600 x g for 30min at 4°C. The supernatant was then poured off and the tubes left to dry for one hour before being counted on a gamma counter (1261 multigamma, Wallac). All samples, standards, B0 and QC's were prepared in duplicate. The detection limit for the assay was 3.7 pmol/l. Inter assay coefficients of variation for low, medium and high quality controls were 15.5%, 6.5% and 6.4% respectively. Our in house assay lab staff conducted this assay. There is a caveat associated with this assay in that the antibody used can cross react with oestrone and thus at low levels there may be some cross reaction with oestrone which may distort the results at low levels.

2.5 Collection and processing of tissue

2.5.1 Tissue collection

Animals were sedated with 200µl ketamine hydrochloride (Park-Davis Veterinary, Pontypool, UK) intramuscularly, euthanised with a 400µl intravenous injection of Euthetal (sodium pentobarbitone, Rhone Merieux, Harlow, Essex, UK) and exsanguinated with a needle and syringe. Ovaries were removed immediately, weighed, and fixed in 4% NBF for 24 h before paraffin wax embedding.

2.5.2 Tissue fixation and processing.

Tissue fixation was needed to stop autolysis, putrefaction, bacterial attack and other undesirable cellular changes. It also keeps the tissue in as close a state as possible to its *in vivo* form. Ovaries were fixed in 4% neutral buffered formalin (NBF) for 24 h and then transferred to a 70% alcohol (C₂H₅OH) solution until processed into paraffin wax. Processing and paraffin wax embedding was carried out by the Unit histology laboratory staff. Tissue was dehydrated through a series of graded alcohols before saturation in paraffin wax. This process was carried out using a 17.5 h automated cycle on a Leica TP-1050 processor (Leica UK Limited, Milton Keynes,

UK). The tissue was then embedded onto plastic cassettes for sectioning.

2.5.3 Sectioning of paraffin blocks

All ovaries were serial sectioned at a thickness of 5 μ m on to RNase free, electrostatically charged slides (BDH, Merck Co, Inc, Poole, UK). Slides had to be RNase free for *in situ* hybridisation and the electrostatic charging improved the binding of the tissue to the slides. Sections were cut using a hand-operated microtome (Leica) with disposable blades. Sections were floated onto diethyl pyrocarbonate (DEPC) treated water (1ml per litre ultra pure water, autoclaved three times) at 50°C, then transferred onto slides and dried overnight.

2.5.4 Dewaxing and rehydrating

Sections were dewaxed in two 5 min washes of xylene before being taken through graded (100%, 90%, 70%) alcohols and finally taken into water.

2.6 Haematoxylin and eosin staining

Haematoxylin and Eosin (H&E) staining is used to visualise the tissue for morphometric analysis. Haematoxylin is a purple nuclear stain, Eosin is a red cytoplasmic stain. These stains were prepared by Histology staff. Harris's haematoxylin is prepared by dissolving 2.5g of haematoxylin (BDH) in absolute alcohol, which is added to alum previously dissolved in 500ml warm deionised water. This solution was then boiled and either mercuric oxide (1.25g) or sodium iodate (0.5g) carefully added. The stain was then rapidly cooled by plunging the flask into a sink of cold water. Once cold, 20ml of glacial acetic acid was added. Eosin Y was prepared by making a 1% solution in distilled water and adding 0.5% volume for volume of acetic acid (Bancroft and Stevens 1996). Once dewaxed, slides were stained in haematoxylin for 5 mins, washed in water, washed in acid alcohol for 20 seconds, washed in water, washed in Scott's tap water for 30 seconds, washed in water, stained in eosin for 20 seconds and finally washed in water.

2.7 Immunocytochemistry

Immunocytochemistry is a method used to localise a specific antigen within a cell or

on the surface of a cell using an antibody raised to an epitope of the protein of interest. This is a powerful technique, as it allows an antigen to be localised to a specific cell type and often a specific cellular location. The immunocytochemical process involves building up layers of antibodies to increase the available binding sites for the antibody and detection system applied. Quantification of immunocytochemical staining is possible and is generally based on parameters such as: number of positive cells in a population; the area of staining in a given tissue or structure (in this case the ovarian follicle); or a combination of the two. The advent of image analysis systems and software has increased the efficiency and accuracy of quantification of such immuno-staining.

2.7.1 Antigens detected

2.7.1.1 BrdU (proliferating cells)

5-bromo-2-deoxyuridine also known as BrdU is a base analogue of thymidine, one of the bases of the nucleic acid found in deoxyribonucleic acid (DNA). Since the 1970s BrdU incorporation has been used as a tool for measuring DNA synthesis in cells and tissues and is a common chemical used in the detection of proliferating cells (Dolbeare 1995). BrdU injected into animals one hour prior to euthanasia will incorporate into the DNA of cells replicating during the interval before euthanasia. Newly synthesized DNA will (partly) incorporate BrdU instead of thymidine. The incorporated BrdU can then be visualised by immunocytochemistry. Other endogenous markers of cellular proliferation are available, such as proliferating cell nuclear antigen (PCNA) and Ki67, although these are often thought of as 'soft' markers, as their expression is rarely restricted to a specific cell cycle phase, owing to time delays in synthesis and degradation (Rew and Wilson 2000).

2.7.1.2 CD31 (endothelial cells)

CD31 is a 130-kDa glycoprotein belonging to the Ig superfamily of cell adhesion molecules and is commonly used as an endothelial specific marker (Ilan *et al.* 2000). CD31 expression begins early in development at the stage of hemangioblast formation and continues throughout adult life (Newman 1994, Newman 1997,

Newman 1999). CD31 expression is restricted to cells of the vascular system platelets, monocytes, neutrophils, selected T cells, and endothelial cells (Newman 1997, Newman 1999). CD31 knockout mice have been noted to suffer from prolonged bleeding times after surgical insult. Which is at least in part due to disrupted endothelial-platelet interactions (Mahooti *et al.* 2000). In addition to the role of CD31 in endothelial cell adhesion, there is growing evidence that CD31 is able to transduce signals that suppress endothelial cell apoptosis (Noble *et al.* 1999, Newman and Newman 2003).

2.7.1.3 Caspase-3 (apoptotic cells)

Some of the other and more commonly used histological methods for the detection of apoptosis is the detection of DNA fragments through terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) or *in situ* nick translation (ISNT) techniques (Barrett *et al.* 2001). Both of these techniques, although useful, involve issues of specificity and interpretation (Allen *et al.* 1997). The development of antibodies specific to more generally distributed apoptotic markers such as the cleaved form of caspase-3 are more applicable to the detection of apoptosis (Barrett *et al.* 2001). The caspase family of proteins are regulators of the apoptotic process, for review see (Thornberry and Lazebnik 1998). Caspase-3 is one of the predominant caspases activated in apoptotic cells (Faleiro *et al.* 1997). A diagram of caspase-3's position in the apoptotic cascade in ovarian granulosa cells is provided in **Figure 2.1**.

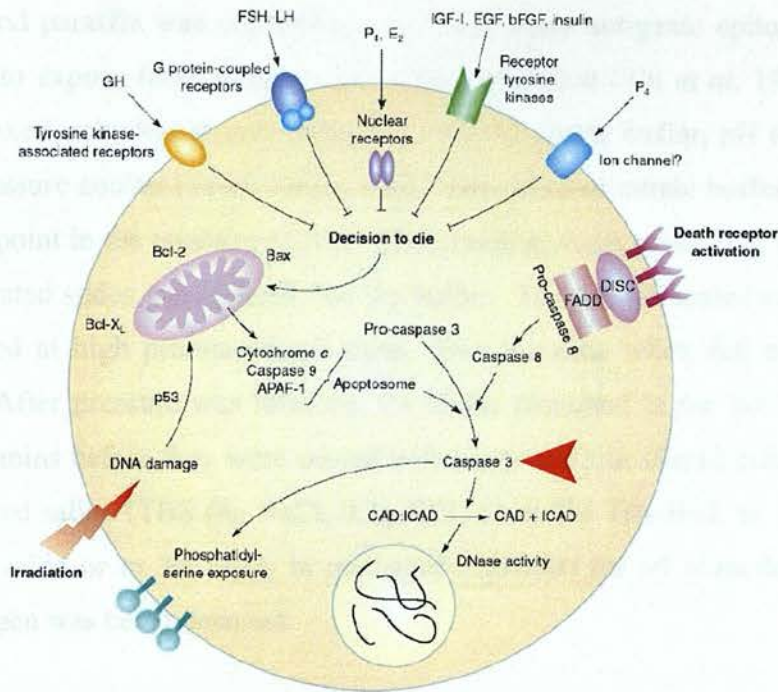


Figure 2.1 Overview of the apoptotic process in follicular granulosa cells.

A range of hormones and locally produced factors regulate the ‘decision to die’ via their receptors. Execution of the apoptotic process converges in the mitochondria, which in turn releases pro-apoptotic molecules that subsequently activate downstream caspases, such as caspase-3. Downstream of caspase-3, the apoptotic programme culminates in orchestrated DNA fragmentation. Apoptosis can also be triggered by DNA damage through radiation and subsequent mitochondrial activation. The red arrow indicates the position of Caspase-3. Figure adapted from (Markstrom *et al.* 2002).

2.7.2 Antigen retrieval

Fixation and paraffin wax embedding can mask some antigenic epitopes. It was necessary to expose these antigens using heat treatment (Shi *et al.* 1991). Slides were dewaxed, rehydrated, and exposed to 0.01M citrate buffer, pH 6, in a Tefal Clypso pressure cooker (Tefal, Essex, UK). Two litres of citrate buffer was heated to boiling point in the pressure cooker. Once boiling, racks containing the dewaxed and rehydrated slides were placed into the buffer. The lid was sealed and the slides were heated at high pressure for 7 mins, from the time when full pressure was reached. After pressure was released, the slides remained in the hot buffer for a further 20 mins before they were cooled with water and transferred either to 0.05M Tris buffered saline (TBS (8g NaCl, 0.2g KCl, 10ml 1M Tris-HCL to 1L H₂O) pH 7.4) for 5 mins or to 3% H₂O₂ in methanol (CH₃OH) for 30 mins depending on which antigen was being detected.

2.7.3 Blocking non-specific binding

The main cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections (Kraehenbuhl and Jamieson 1974). It is usually the first immune serum that gives rise to the highest background staining. Blocking the sites that show a non-specific affinity for the antibodies can reduce this. This is achieved by incubating the sections in normal whole serum from the species in which the secondary antibody was raised. For example, when mouse BrdU was localised using rabbit anti-mouse as a secondary antibody, blocking was performed using normal rabbit serum. All slides were treated with the relevant blocking serum diluted 1:5 in TBS with 20% bovine serum albumin (BSA) for 30 mins at room temperature. Details for each specific antigen are given in **Table 2.1**.

2.7.4 Immunocytochemical procedure

An automated incubation method, the Sequenza System (Shandon Scientific, Runcorn, Cheshire, UK), was used for all incubations apart from the peroxidase block. A Sequenza works by capillary action and reduces the volumes of reagents required. Each section was retained within its own Sequenza cassette, which

prevents cross-contamination and antibody-antigen complexes are prevented from precipitating onto sections, which would give rise to unwanted background staining. Unbound antibody was washed from each section before the proceeding step in each protocol. Detection systems vary according to antibody, details are given in **Table 2.1**. After the appropriate block had been applied and allowed to incubate for 30 mins, sections were given three 5 min washes with TBS. The primary antibody, diluted in the appropriate blocking serum used for blocking non-specific binding, was then added to the sections and incubated overnight at 4°C. After overnight incubation and three 5 min washes with TBS, the secondary antibody, diluted accordingly in the appropriate blocking serum was added and incubated at room temperature for 40 mins. Detection systems vary somewhat from this stage of the protocol and therefore will be dealt with separately.

Primary Antibody	Species raised in	Block	Secondary antibody	Tertiary antibody	Detection	Counter stain
BrdU	Mouse monoclonal	Normal rabbit serum	Rabbit anti mouse	Mouse APAAP	NBT	Yes
CD31	Mouse monoclonal	Normal rabbit serum	Rabbit anti mouse	Mouse APAAP	NBT	No
CD31/BrdU	Mouse monoclonal	Normal rabbit serum	Rabbit anti mouse	Mouse APAAP	Fast red	Yes
	Sheep polyclonal		Rabbit anti sheep	Mouse APAAP	NBT	
Caspase-3	Rabbit polyclonal	Normal goat serum	Goat anti mouse	Goat anti rabbit peroxidase labelled polymer	DAB	Yes

Table 2.1 Antibodies and reagents used in immunocytochemistry protocols.

Sheep BrdU (Fitzgerald Industries international, Concord, MA, USA), Mouse BrdU (Boehringer Mannheim, Germany), CD31 (Dako Corp, Carpinteria, CA, USA) and Cleaved Caspase-3 (Cell Signalling Technology, Beverly, MA, USA).

2.7.5 Specific immunocytochemistry techniques

2.7.5.1 BrdU and CD31

Slides incubated with non-biotinylated secondary antibodies were subsequently incubated with a tertiary alkaline phosphatase-anti-alkaline-phosphatase (APAAP) antibody (Cordell *et al.* 1984). The secondary antibody was applied in excess so that one of its two identical binding sites would bind to the primary antibody, the other to the APAAP complex, illustrated in **Figure 2.2**. The APAAP was added, again diluted in the appropriate serum block for 40 mins, before slides were washed in and then transferred to TBS. The APAAP detection method can be used with a variety of substrates giving rise to a variety of colours. The two used in this thesis were nitroblue tetrazolium (NBT), which forms a deep blue/black stain (McGadey 1970), and fast red used with naphthol AS-MX phosphate sodium, which gives a bright red stain.

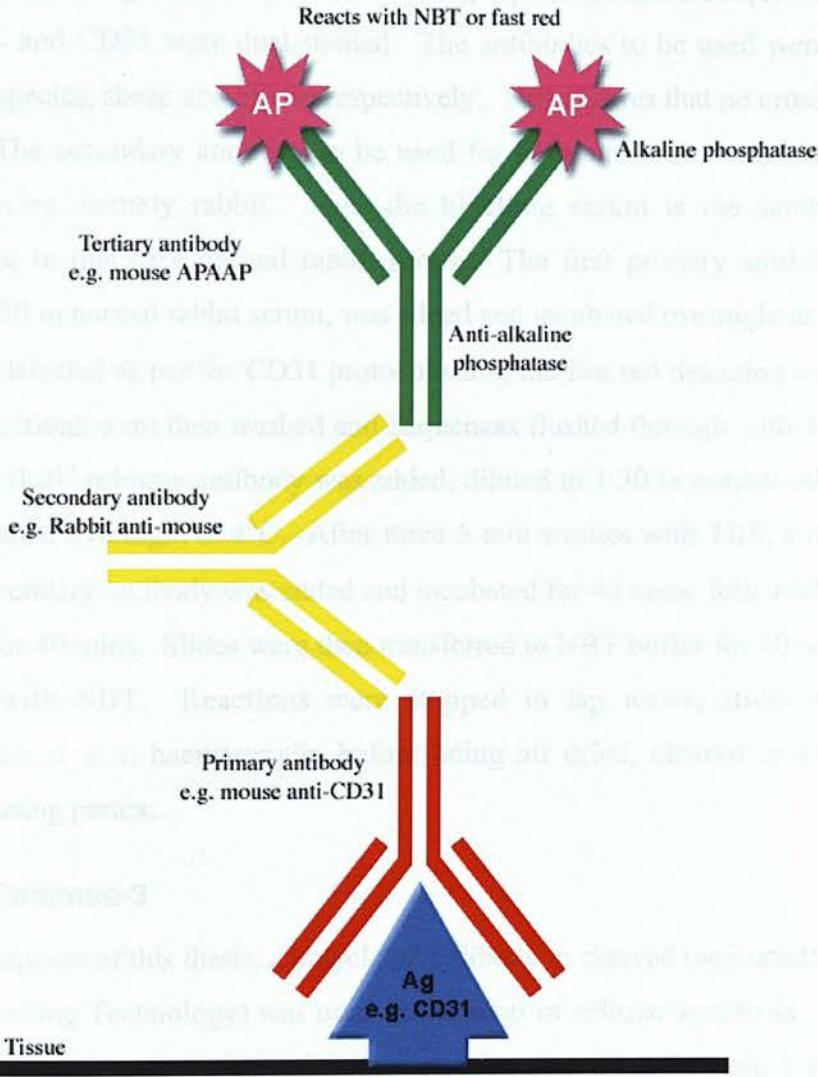
Where NBT was used, slides were transferred to NBT buffer for 10 mins at room temperature (40ml 0.5M MgCl + 40ml 1MTris/1MNaCl + 320ml distilled water). The area of the slide surrounding the tissue was then dried, and NBT solution added, 10ml NBT buffer + 45 μ l NBT substrate (Boehringer Mannheim) + 35 μ l Xphosphate (Boehringer Mannheim) +10 μ l levamisole. Slides were left for colour to develop over approximately 10 mins.

Where fast red was to be used for detection, slides were removed from TBS and the area around tissue sections wiped dried. Fast red was added in solution at 1mg/ml in fast red buffer (20mg naphol AS-MX phosphate, 2ml dimethyl formamide to 98ml 0.1M Tris, pH 8.2) and left for approximately 20-30 mins until a red colour developed. Reactions were stopped in tap water.

Where appropriate, sections were counterstained in haematoxylin for approximately 20 seconds before being dehydrated. If stained with NBT, slides were dehydrated in graded alcohols, cleared in xylene and mounted in pertex (Cell Path, Hemel Hempstead, UK). If stained with fast red, slides were air dried before being cleared and mounted.

2.2.5.2 Co-localisation

In order that two antigens could be detected and differentiated on the same slide, two separate detection systems were used. For the purposes of this thesis, it was required that DAPI and CD31 were identified. The antibodies to be used were raised in different species, sheep and mouse, respectively, so that no cross reactivity occurs. The secondary antibody can be used for both species, namely rabbit anti-mouse and rabbit anti-sheep. The first primary antibody, CD31, diluted 1:20 in normal rabbit serum, was applied to the slide overnight at 4°C. This was then detected as per the CD31 protocol. The second primary antibody, mouse anti-CD31, was then washed and incubated with rabbit anti-mouse IgG diluted to 1:30 in normal rabbit serum for 40 minutes. Slides were transferred to NBT buffer for 10 minutes staining with NBT. Reactions were stopped in tap water, slides were then mounted and air dried, cleared in xylene, and mounted using permount.



2.2.5.3 Caspase-3

For the purposes of this thesis, caspase-3 (Casp-3) (Santa Cruz Technology) was used for the detection of apoptosis. The primary antibody was diluted in PBS containing 0.1% Triton X-100 and 0.1% BSA. The secondary antibody was diluted in PBS containing 0.1% Triton X-100 and 0.1% BSA. The primary antibody was applied to the slide for 1 hour at 37°C. The secondary antibody was applied to the slide for 1 hour at 37°C. The slide was then washed and incubated with NBT buffer for 10 minutes staining with NBT. Reactions were stopped in tap water, slides were then mounted and air dried, cleared in xylene, and mounted using permount.

Figure 2.2 Illustration of APAAP detection system.

A primary anti-body binds to the specific antigen. The secondary antibody binds to the primary and also to the APAAP complex. Either NBT or fast red is then used for detection of stain.

2.7.5.2 Co-localisation

In order that two antigens could be detected and differentiated on the same slide, two separate detection systems were used. For the purposes of this thesis, it was required that BrdU and CD31 were dual-stained. The antibodies to be used were raised in different species, sheep and mouse respectively. This ensures that no cross reactivity occurs. The secondary antibody to be used for both antibodies was raised in the same species, namely rabbit. Thus the blocking serum is the same for both antibodies, in this case normal rabbit serum. The first primary antibody, CD31 diluted 1:20 in normal rabbit serum, was added and incubated overnight at 4°C. This was then detected as per the CD31 protocol using the fast red detection as described above. Sections were then washed and Sequenzas flushed through with TBS before the sheep BrdU primary antibody was added, diluted to 1:30 in normal rabbit serum and incubated overnight at 4°C. After three 5 min washes with TBS, a rabbit anti-mouse secondary antibody was added and incubated for 40 mins, followed by mouse APAAP for 40 mins. Slides were then transferred to NBT buffer for 10 mins before staining with NBT. Reactions were stopped in tap water, slides were then counterstained with haematoxylin before being air dried, cleared in xylene, and mounted using pertex.

2.7.5.3 Caspase-3

For the purposes of this thesis, a polyclonal antibody to cleaved (activated) caspase-3 (Cell Signalling Technology) was used as a marker of cellular apoptosis. Instead of NBT or fast red used for other immunocytochemical visualisation, a peroxidase labelled polymer was used with 3,3' diaminobenzadine tetrahydrochloride (DAB) as a detection chromagen. DAB yields a crisp, insoluble, dark brown reaction product (Graham and Karnovsky 1966), a diagram illustrating this technique is provided in **Figure 2.3**. Some endogenous peroxidases in the animal tissue can react with the DAB, and cause a blurring of the resulting stain. Treatment with saturating amounts of hydrogen peroxide results in the irreversible inactivation of these endogenous peroxidases. Therefore, immediately after the pressure cooking step of the protocol, slides were incubated in absolute methanol containing 3% hydrogen peroxide for 30

mins, without being transferred to TBS first (Streefkerk 1972). After methanol incubation, slides were transferred to TBS, sections were then washed three times for 5 mins with TBS then incubated for 30 mins at room temperature with a goat anti-rabbit secondary antibody conjugated to an EnVision peroxidase labelled polymer complex. Slides were washed and transferred to TBS before the tissue sections were dried around and DAB substrate added. Staining was produced after approximately 1 min. Reactions were stopped in tap water and sections were counterstained in haematoxylin for approximately 20 seconds before being dehydrated through graded alcohols, cleared and mounted using pertex.

2.7.6 Negative controls

For immunocytochemical detection using a specific antibody for the first time on a specific tissue, negative control experiments were carried out to assess the potential of false positive results. The primary antibody was replaced with IgG from the same species the primary was raised in and at the same concentration. No sign of false negative results were observed for any of the antibodies used.

2.8 Quantification of immunocytochemistry

BrdU and CD31 immunostaining were quantified using Image Pro Plus 3.0 software (Media Cybernetics, Silver Spring, Maryland, USA). Images were captured at x200 magnification and converted to grey scale. The area (CD31) or number of dark objects (BrdU) was measured per area of interest, theca and granulosa. In all cases, the threshold for recognition of a “dark” object was set so that the captured image mirrored the live image as closely as possible. Dual-stained follicles were analysed manually at x400 magnification using a tally counter. These analysis techniques are commonly used to quantify immunocytochemistry (Wulff *et al.* 2001b, Wulff *et al.* 2001c, Rowe *et al.* 2002, Wulff *et al.* 2002). The pairs of ovaries removed from each animal was treated as n=1 for analysis. Not all immunohistochemical stains were quantified: caspase-3 was performed purely for the purpose of confirming follicle characterisation and health.

2.8.1 Bromodeoxyuridine

Sections were analysed under x200 magnification. The image analysis package was set up to analyse the number of BrdU positive cells and the total number of cells in the outlined cellular compartment of interest, theca and granulosa. The proliferation index is expressed as the percentage of cells proliferating from the total number of cells in that compartment. Calculated for the granulosa and thecal compartments separately, the proliferation index is expressed as a mean value for each follicular stage per animal assessed.

2.8.2 CD31

The endothelial cell area was measured at x200 magnification. Capturing and thresholding a grey scale image into a binary image measured CD-31 positive cells. The whole area of the thecal compartment and the CD31 positive area were measured, and the CD31 positive area was calculated per unit area of the thecal compartment. It is expressed as a mean value for each follicular stage per animal assessed.

2.8.3 BrdU/CD31 co-localisation

The number of dual-stained cells (both BrdU and CD31 positive cells) and the total number of proliferating cells (BrdU positive cells) were counted in the thecal compartment. The proportion of dual-stained cells of proliferating endothelial cells (dual-stained cells) is expressed as a percentage of the total number of proliferating cells. The mean value was calculated for the number of follicles assessed within each follicular class and per animal. Non-endothelial cell proliferation was calculated by multiplying the total thecal proliferation index (BrdU index) by the percentage of proliferation that was not endothelial (not dual stained, BrdU positive cells on a dual stained slide), per follicle class per animal.

2.8.4 Caspase-3

Although not quantified, caspase-3 staining was used to assess that morphological characterisation of follicular atresia, for example pyknotic granulosa cells, was correct. **Figure 2.4.**

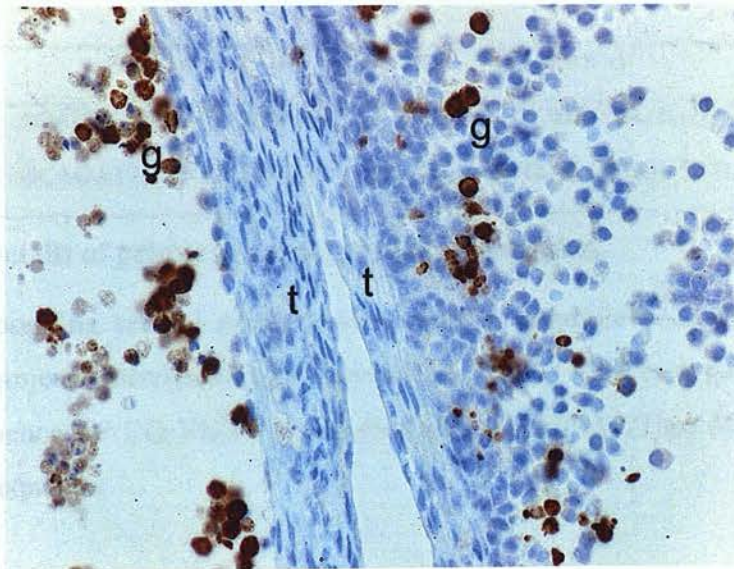


Figure 2.4 Caspase-3 staining of atretic follicles

Two atretic dominant follicles from a GnRH antagonist days 5-10 treated animal stained for cleaved caspase-3. The staining is isolated to the degenerating pyknotic granulosa cells. g = granulosa, t = theca.

2.9 Construction of riboprobes for *in situ* hybridisation

In situ hybridisation was used for the detection of specific mRNAs in tissue sections by hybridisation to complementary radio labelled riboprobes, and subsequent detection using photographic emulsion. This is again a very powerful technique allowing the localisation and quantification of specific mRNAs to tissues and cells of interest.

2.9.1 Design of riboprobes

Riboprobes were designed and constructed for human endocrine gland vascular endothelial growth factor (EG-VEGF) and marmoset aromatase mRNAs, the accession numbers for the sequences are: NM_032414 and AY034779 respectively. Specific primers were designed for each gene sequence using primer 3 software, available at: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Details of the primers are provided in **Table 2.2**.

	Forward 5'-3'	Reverse 5'-3'
EG-VEGF	AGAGGCATCTAAGCAGGC	AGGTATGTCTGCCTGTGTGC
Aromatase	GCAGATTCCTGTGGATGG	GAAGAGCGTGTTAGAGGTGTCC

Table 2.2 Details of primers used to generate riboprobes.

The corresponding sets of primers were used to generate complimentary DNA (cDNA) fragments corresponding to nucleotides 10-533 (523 base pairs (bp)) of the human sequence for EG-VEGF and nucleotides 188-609 (421bp) of the marmoset aromatase sequence.

2.9.2 Reverse transcription of RNA

Total RNA extracted from the human corpus luteum was used to construct the EG-VEGF probe. Total RNA extracted from marmoset ovaries was used to construct the aromatase probe. RNA was reverse transcribed using Oligo DT priming from a taqman reverse transcription (RT) kit (Roche Applied Bioscience, Lewes, Sussex, UK). The following RT protocol was used:

Nuclease free H ₂ O	96µl
RNA (2µg/µl)	10µl
x10 RT Buffer	20µl
dNTPs (10mM, 2.5mM of each)	20µl
Rnase inhibitor (20U/µl)	10µl
Oligo(dT)s (50µM)	10µl
Reverse transcriptase (50U/µl)	10µl
MgCl ₂ (25mM)	24µl
Final Volume	200µl

Reaction mix was split in to 20µl reactions to allow efficient heating to occur. The following programme was used:

23°C	10 mins
42°C	1 h
95°C	10 mins

2.9.3 PCR of specific cDNA insert

Using the single stranded DNA synthesised in the RT reaction, specific cDNA fragments were generated by Polymerase Chain Reaction (PCR) using the primers designed to generate each probe (**Table 2.2**). Firstly a PCR was carried out to determine the optimal temperature for primer annealing. PCR reagents were from Abgene, Epsom, Surrey, UK. A standard PCR mix used was:

Nuclease free H ₂ O	47.2μl
10x ThermoStart buffer	8μl
dNTP mix (5mM mixture, 5mM each of A, C, G and T)	8μl
Forward and reverse primers (5μM)	8μl
Taq DNA polymerase (5U/μl)	0.8μl
cDNA (1μg)	8μl
MgCl ₂ (25mM)	6μl
Total volume	80μl

The ThermoStart enzyme requires a heat activation step at the beginning of the PCR.

A standard thermoStart PCR protocol used was:

95°C	10min	Enzyme activation	
95°C	30secs	Denature] x30 cycles
Gradient 51.2°C-63.9°C	30secs	Primer annealing	
72°C	90secs	Extension	
72°C	10min	Final extension	

The optimal temperature for both primer pairs was determined by eye as the intensity of the PCR product when run out on a 1% agarose gel (0.5g agarose dissolved in 50ml TBE buffer: 1M Tris, 1M boric acid and 0.02M EDTA in distilled water, 2μl ethidium bromide, then set in a gel tray and wells made with a comb). The gel was run at 120V for 30 mins and the PCR products visualised and photographed under ultra violet (UV) light. Once the optimal annealing temperatures had been determined, 80μl PCRs were performed at the optimum temperature to obtain large quantities of the specific cDNAs required for purification and ligation into vectors.

2.9.4 PCR product purification

The PCR products were then purified using a Boehringer High Pure PCR Product purification kit (Boehringer Mannheim) as per the manufacturer's instructions. Simply, the PCR product is bound to the purification column while the PCR primers

and other contaminants are washed through with a series of washing buffers. The purified product is then eluted off the column using nuclease free water. Concentrations of PCR products were measured on a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech, Cambridge Science Park, Cambridge, UK). The products were run on a 1% agarose gel as before, to verify the correct products had been purified, as the length of the expected PCR product sequences was known (**Table 2.3**). The purified product was also sequenced for extra confirmation.

Probe	Vector	Host Bacterium	Length of cDNA insert (bp)	RNA polymerase initiation sites (AS/S)
VEGF	pBluescript	Epicurian Coli XL1 - blue	579	T7/T3
VEGFR1 (Flt)	pBluescript	Epicurian Coli XL1 - blue	273	T7/T3
VEGFR2 (KDR)	pBluescript	Epicurian Coli XL1 - blue	275	T3/T7
EG-VEGF	pGEM-TEasy	JM109	523	SP6/T7
Aromatase	pGEM-TEasy	JM109	421	T7/SP6

Table 2.3 Riboprobe plasmids, orientations and cDNA insert lengths.

Epicurian Coli XL1 - blue (Stratagene, La Jolla, CA, US)

2.9.5 Ligation of purified PCR products

The purified PCR products were ligated into vectors using a TA bidirectional cloning system. The pGEM-T Easy vector system (Promega Corporation, Madison, WI, USA) was used as per the manufactures instruction, with in insert to vector ratio of 3:1. A map of the vector is provided in **Figure 2.5**.

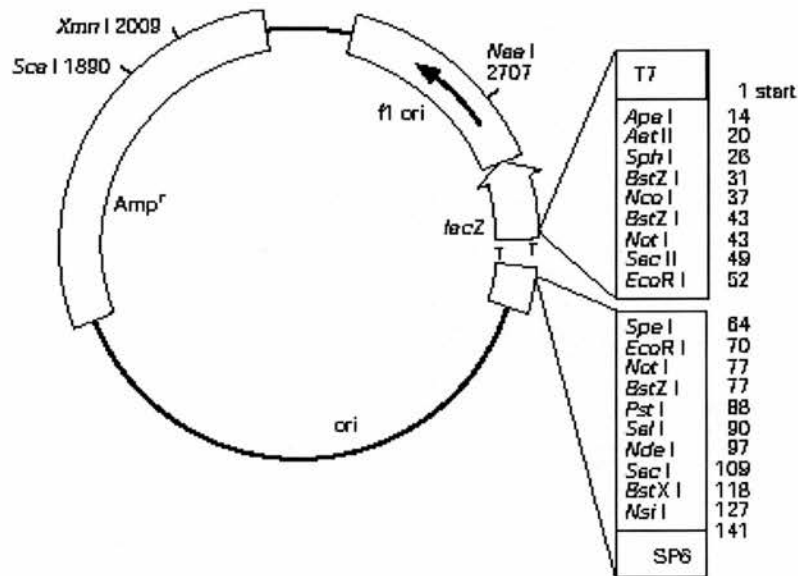


Figure 2.5 Circle map of the pGEM-T Easy vector.

Restriction enzyme cleavage sites are present on either side of the insertion site in the vector that interrupts the *lacZ* gene. The numbers corresponding to the restriction sites are measured from the T7 transcription initiation site. Adapted from pGEM-T and pGEM-T Easy Vector Systems Technical manual No. 042 (Promega Corp).

2.9.6 Transformation of plasmid constructs

Once the ligation reactions were complete the vector-insert constructs (plasmids) were transformed into JM109 competent cells (Promega Corp.) as per the manufactures instructions. LB Broth agar plates containing ampicillin at 100µg/ml (Gibco BRL, Life Technologies Ltd., Paisley, UK) were poured and once set, each plate had 100µl of 100mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega) and 20µl of 50mg/ml X-Gal (Promega) spread over their surface and allowed to absorb for 30 mins at 37°C. A blue/white selection screen was set up on these plates to select colonies that contained the plasmid constructs. As the plasmids containing the insert will have their LacZ gene broken (**Figure 2.5**) they will appear white because they are unable to metabolise the X-Gal on the plates. Clonal expansion of single white colonies was used to ensure that only single colonies were selected with the insert in only one orientation. After clonal expansion, selected cells were grown up overnight in 10ml LB Broth (Anachem, Luton Beds, UK) containing ampicillin at 100µg/ml (Gibco) on a shaking platform at 37°C. A control tube containing broth and ampicillin was also prepared. After overnight growth, plasmids were purified from their host cells as detailed below, if the control solution remained clear, i.e. if no contamination was present in the LB Broth. Glycerol stocks of the cells containing the plasmid constructs were also taken at this stage, 700µl of cells in LB and 300µl of 50% glycerol was vortexed and stored at -20°C for future use.

2.9.7 Purification of plasmids

Plasmids were purified from their host bacterium using a QIAprep Spin miniprep kit (QIAGEN Ltd, Crawley, West Sussex, UK) as per the manufacturers instructions. DNA concentration was determined using the GeneQuant Pro spectrophotometer (Amersham). Purified plasmids were then sequenced (See **2.13**) and the results aligned to the genes they had been constructed to verify specificity.

2.9.8 Validation of probes

To validate that the constructed probes only detected the mRNA they were designed to hybridise to, Northern analysis was conducted. An agarose gel containing 2.0g

agarose dissolved in 20ml x10 MOPS (41.86g of 3[n-morpholino]propane sulphonic acid, 6.805g of sodium acetate and 3.722g of EDTA to 1 litre of deionised water), 34 ml formaldehyde and 146 ml of deionised water was prepared. Ten micrograms of each RNA sample (marmoset testicular and ovarian RNA, human luteal and placental RNA) was loaded into the gel along with 2µl of a 0.2-10 Kb RNA ladder. Samples were run out on the gel at 80V for 3 h and then photographed and the position of both the 18S and 28S RNA measured. The separated RNA was then transferred to a nylon membrane (Amersham) by capillary action in 20x salt sodium chloride (SSC) [20xSSC: 175.3g NaCl, 88.2g Citric acid (Tri Sodium salt) to 1L H₂O, pH 7.0] overnight and baked for 2 h at 80°C the following day to permanently bond the RNA to the membrane. The dry blot could then be stored in a plastic bag at 4°C for future use.

Blots were prepared for hybridisation by placing them in hybridisation tubes, adding 16ml of Ultrahyb (Ambion) and incubating at 42°C for 1 h whilst rotating. The Probes were labelled by adding 25ng of purified probe plasmid in 45µl of water and 5µl of [α -³²P] dCTP (Amersham) to Rediprime II DNA Labelling System tubes (Amersham) and incubated as per the manufacturers instructions. The probes were then fractionated to 400µl volumes using NICK sephadex G-50 DNA grade purification columns (Amersham), with 4µl of each fraction being collected and counted in 3.5ml of scintillant on a β counter (Microbeta Trilux 1450, Wallac) to check the probe had labelled properly. The second and third fractions (the fractions containing labelled probe) were then heated at 99°C for 5 mins and then quenched in ice to linearise the probes. Probes were then added to the hybridisation tubes containing the blots and Ultrahyb and incubated overnight at 42°C whilst rotating. The following day two 5 min 2xSSC/0.1%SDS, followed by two 15 min 0.1xSSC/0.1%SDS stringency washes, were used to remove un-hybridised probe from the blots. All washes were carried out 42°C. Blots were then removed from the hybridisation tubes, wrapped in Saran film and analysed on an autoradiograph (instant imager, Canberra Packard, Pangbourne, Berkshire, UK) and exposed to photographic film.

2.10 *In situ* hybridisation technique

2.10.1 Preparation of plasmids

Riboprobes were generated from plasmids containing the specific cDNA insert of interest and RNA polymerase initiation sites. Probes for VEGF, VEGFR1 and VEGFR2 were gifts from Dr Stephen Charnock-Jones (Department of Obstetrics and Gynaecology, University of Cambridge) and were received pre-inserted into their host plasmid. The vector, host bacterium, cDNA insert length and orientation in the vector for each probe are detailed in **Table 2.3**. The complete nucleotide sequence and probe binding region are given in **Appendix A**.

Plasmids were grown overnight on a shaking platform at 37°C by taking 10µl of glycerol stock and inoculating 10ml LB Broth (Anachem) containing 100µg/ml ampicillin (Gibco), a control tube containing broth and ampicillin was again prepared to check the LB Broth was not contaminated. After overnight growth, plasmids were purified from their host cells as detailed in **2.9.7**.

2.10.2 PCR of plasmid

In order to amplify the sequence of interest within the plasmid, specific forward and reverse PCR primers were designed to the RNA polymerase initiation sites contained within the plasmid. The sequences of the primers were (5' - 3'):

-T7	TAATACGACTCACTATAGGGCGA
-T3	AATTAACCCTCACTAAAGGGAAC
-SP6	ATTTAGGTGACACTATAGAATAC

PCRs were performed using the standard reaction mix and protocol as detailed in **2.9.3**. The only differences in standard PCR protocol between probes were the annealing temperatures, which were: 52°C (VEGF, VEGFR1, VEGFR2); and 59°C (EG-VEGF and aromatase). PCR products were then purified using a Boehringer High Pure PCR Product purification kit (Boehringer Mannheim) as detailed in **2.9.4**. The concentration of DNA was measured on the GeneQuant Pro spectrophotometer

(Amersham) and the products run on a 1% agarose gel as before, to verify that the correct product had been amplified.

2.10.3 Synthesis of riboprobes (cold labelling)

Riboprobes were synthesised from the purified the PCR products. First it was necessary to check that the PCR product could be transcribed into sense and antisense RNA probes by cold labelling. Sense and antisense riboprobes were synthesised from the purified PCR products using T7, T3 or SP6 RNA polymerases. A MAXIscript *in vitro* transcription kit (Ambion Inc., Austin, Texas, USA) was used for riboprobe synthesis. The following protocol was used:

Nuclease free H ₂ O	X μ l
300ng DNA in H ₂ O	Y μ l (X + Y = 13 μ l)
10 x transcription buffer	2 μ l
rCTP, rGTP ,rATP rUTP (10mM)	1 μ l of each
RNA polymerase	1 μ l
Final Volume	20 μ l

This mixture was incubated at 37°C for 1 h. After 1 h, 1U DNase (deoxyribonuclease) was added and the reaction was incubated for a further 15 mins at 37°C. The product was run on a 1% agarose gel to check that transcription had been successful.

2.10.4 Synthesis of riboprobes (hot labelling)

This is essentially the same as the cold labelling detailed above with the addition of radiolabelled UTP. The following protocol was used.

Nuclease free H ₂ O	X μ l
300ng DNA in H ₂ O	Y μ l (X + Y = 11.5 μ l)
RNA polymerase	1 μ l
10 x transcription buffer	2.5 μ l
rCTP, rGTP ,rATP (10mM)	1 μ l of each
rUTP (0.1mM)	1 μ l
³⁵ S labelled UTP (2.3MBq)	5 μ l
Dithiothreitol (100mM)	1 μ l
Final Volume	25 μ l

A 1:100 dilution of unlabelled UPT is included in the reaction mix because ³⁵S labelled UTP (NEN life science products, Boston, MA, USA) is a large molecule and if every UTP in the probe were to be radio labelled stereo inhibition may occur. By adding a low concentration of unlabelled UTP, stereo inhibition is prevented as not all UTP will be radiolabelled ³⁵S UTP.

Reaction mixtures were incubated at 37°C for 45 mins and then for a further 45 mins after the addition of another 10U enzyme. Finally, 1U of DNase was added and incubated for 15 mins at 37°C. After riboprobe synthesis, the percentage of incorporated ³⁵S UTP was measured by counting the total radioactivity (counts per min, cpm) of 1 μ l of the final reaction mixture; then spinning the remaining solution through a ChromaSpin column (Clonetech Laboratories Inc., Palo Alto, CA, USA), as per manufacturer's instructions, to retain any free radioactivity before counting 1 μ l of the eluate. The incorporated count was expressed as a percentage of total counts per min (cpm).

2.10.5 *In situ* hybridisation procedure

In situ hybridisation experiments were conducted for VEGF, VEGFR1, VEGFR2,

aromatase and EG-VEGF. For each tissue of interest two serial sections were used, one was treated with the sense probe and the other with the antisense probe. It was essential that solutions and all equipment used for the experiment were ribonuclease (RNase) free. To ensure this was the case, sterile plastics were utilised and glassware and metal racks were baked at 180°C. All solutions used on the first day of the *in situ* hybridisation were made up with DEPC treated water. After hybridisation, subsequent solutions were made up in ultra pure water. Additionally, chemicals were only retained for *in situ* use if they had been used exclusively with sterile equipment and gloves. Fresh xylene and alcohols were prepared before each experiment. Tissue sections were dewaxed, rehydrated, incubated in 0.2N HCL (to denature proteins) for 20 mins, followed by two 5 min DEPC water washes. Proteolytic digestion of tissues was performed using 5µg/ml proteinase K in 1M Tris, 0.5M EDTA, for 30 mins at 37°C. Next, the proteinase K digestion was inhibited by transferring the slides to a 0.2% glycine solution at 4°C for 10 mins. Slides were then transferred to 0.1M triethanolamine buffer (TEA, pH 8 with 5M NaOH) for 5 mins before incubation in TEA buffer/0.25% acetic anhydride for 10 mins. Slides were then washed in 4x SSC for 5 mins.

Tissue sections were dried around, laid in sterile humid boxes and incubated for 2 h at 55°C. Each tissue section was covered by 100µl of prehybridisation buffer containing: 50% deionised formamide, 4x SSC, 1xDenhardtts, 125µg/ml salmon testes DNA, 125µg/ml yeast transfer ribonucleic acid (tRNA), and 10mM dithiothreitol (DTT). Prehybridisation buffer was drained off and replaced with 50µl per slide of hybridisation buffer (containing the same as the prehybridisation buffer plus 10% dextran sulphate, to increase the rate of reassociation of nucleic acids, and approximately 0.5×10^6 cpm of probe per 50µl (VEGF, aromatase) and 1×10^6 cpm per 50µl for all other probes. Before the addition of hybridisation buffer to the slides, the probe (in buffer) was denatured immediately before use by heating to 95°C for 10 mins. Sections were coverslipped (Gel Bond) and incubated overnight at 55°C in a humid box.

The following day, RNase free conditions were no longer necessary. After removal of coverslips and a subsequent 10 min wash in fresh 4xSSC, slides

underwent RNase-A treatment (20 μ g/ml RNase-A in 1M Tris, 0.5M EDTA, 0.5M NaCl buffer, pH 8) at 37°C for 30 mins. This was followed by a wash in RNase buffer containing no RNase-A for 30 mins. Slides were then subjected to repeated 30 min, stringency washes to remove unhybridised probe (2xSSC, 1 x SSC at 65°C and 0.1 x SSC).

Finally, slides were dehydrated in 50% (v/v) ethanol/0.3M ammonium acetate, 85% ethanol/0.3M ammonium acetate and 94% ethanol/0.3M ammonium acetate for 2 mins each, before being allowed to air dry for 3-4 h. Slides were dipped in G5 emulsion (Illford, H + A West, Edinburgh, UK), diluted 1:1 in distilled water, and heated at 45°C in a dark room until liquefied.

Slides were then dried overnight in a lightproof humid box at 4°C, before desiccant was added and the emulsion exposed at 4°C for a varying number of weeks, depending on probe. VEGF *in situ* were exposed for 4 weeks, VEGFR1 and VEGFR2 for 9 weeks and EG-VEGF and aromatase 3 weeks. Exposure times were determined by including trial slides in each *in situ* hybridisation experiment for each probe and tissue type developed at intervals until such time as optimum hybridisation had occurred. All experimental slides within one run were developed at the same time. Slides were developed in filtered Kodak D19 developer (Edinburgh Cameras, Edinburgh, UK) for 4 mins at 14°C, rinsed in distilled water, then fixed in Kodak GBS Fixer diluted 1:5 in distilled water, for 10 mins at room temperature. Slides were again rinsed in distilled water, counterstained with haematoxylin for 20 seconds, dehydrated in graded alcohols, cleared in xylene and mounted in pertex.

2.10.6 Negative controls

In all *in situ* hybridisation experiments negative control were included. These negative controls were the sense transcription of the specific probe used, as opposed to the antisense that binds to the mRNA to give specific binding. All sense slides were devoid of specific hybridisation and only displayed background levels of hybridisation.

2.10.7 Quantification of *in situ* hybridisation

Slides were analysed qualitatively under light field and quantitatively under dark field illumination. Specific hybridisation to follicular compartments of interest was quantified by grain density measurements. Grain density was either determined at x400 magnification using image analysis software and is given as number of grains per μm^2 , or scoring by eye.

Scoring by eye was carried out by Paul Taylor and Hamish Fraser with the following classification: - (0) No detectable specific signal above background levels; + (1) low grain density clearly above background levels; ++ (2) moderate grain density; +++ (3) high grain density. Scoring of tissues was blind and random by each observer.

2.11 Statistical analysis of data

Data was first tested for normality using a Shapiro-Wilk test. Where more than two variables were being compared analysis of variance (ANOVA) with a least significant difference (LSD) post-hoc test was used if the data was parametric. Non-parametric data was tested using a Kruskal-Wallis test with Dunn's post-hoc test. Where two variables were being compared a two-tailed unpaired t-test was used for normally distributed data. A Mann-Whitney test was used for data that was not normally distributed. The level of significance for all tests was $P < 0.05$. Tests were performed using SPSS 11 for Macintosh OSX (SPSS, Inc, Chicago, IL, USA) or Prism 2.0 for Macintosh OS 9 (GraphPad Software, Inc. San Diego, CA, USA).

2.12 Digital photomicroscopy

Tissue sections were photographed using a digital camera, Kodak DCS420 (Eastman Kodak, Rochester, NY, USA) mounted on an Olympus Provis microscope (Olympus Optical, London, UK). Figures were compiled using Adobe Photoshop 7.0 for Macintosh OSX (Adobe Systems Inc., Mountain View, CA, USA).

2.13 DNA sequencing

All DNA sequencing was carried out by either the Medical Research Council Human Reproductive Sciences Unit genomics and proteomics service or the Medical

Research Council Human Genetics Unit sequencing service.

**Chapter 3 Effect of GnRH Antagonist Treatment on
Follicular Development and Angiogenesis**

3.1 Introduction

The ovary is one of few organs in the body to undergo serial angiogenesis which is intimately involved in ovarian follicle development (Fraser and Wulff 2001, Wulff *et al.* 2001b, Zimmermann *et al.* 2001b, Wulff *et al.* 2002, Zimmermann *et al.* 2003). Angiogenesis in the ovarian follicle is restricted to the thecal compartment, the resulting capillaries forming a vascular sheath around the growing follicle that continues to develop during folliculogenesis (Wulff *et al.* 2001b). The degree of vascularisation achieved has been hypothesised to be involved in the establishment of follicle dominance (Zeleznik *et al.* 1981), facilitating the delivery of gonadotrophins, steroid precursors, oxygen and nutrients from the blood stream, but whether an abundant vasculature is a cause or consequence of follicle dominance remains to be determined.

The principal angiogenic factor with an established role in follicular angiogenesis is VEGF. VEGF mRNA and protein have been localised in the ovary to the granulosa cells of the developing follicles and the theca of preovulatory follicles (Phillips *et al.* 1990, Ravindranath *et al.* 1992, Shweiki *et al.* 1993). Inhibition of VEGF *in vivo* in the marmoset monkey has been shown to severely suppress both thecal vascularisation and follicle development (Wulff *et al.* 2001b, Wulff *et al.* 2002) and to inhibit the follicular phase of the cycle in macaques (Zimmermann *et al.* 2001b, Zimmermann *et al.* 2002). As VEGF is expressed in the granulosa and thecal cells of follicles as they mature it is concurrent with the acquisition of steroidogenic activity, suggesting that the expression is hormonally regulated (Shweiki *et al.* 1993). It has also been demonstrated that VEGF expression in granulosa cells from preovulatory follicles is up regulated by FSH stimulation *in vitro* (Christenson and Stouffer 1997, Hazzard *et al.* 1999).

In this study, the role of the gonadotrophins was assessed by administration of a GnRH antagonist *in vivo* during follicular development in the marmoset, a species in which the development of the follicular vasculature has been described in detail (Wulff *et al.* 2001b, Wulff *et al.* 2002). Since it is established that GnRH antagonist treatment suppresses follicular development by inhibiting both FSH and LH secretion from the pituitary (Fluker *et al.* 1991, Hall *et al.* 1991, Fraser *et al.* 1999a),

the aim of this study was to test the hypothesis that this suppression resulted in a reduced production of follicular VEGF mRNA with resultant inhibition of angiogenesis. The effects of the antagonist on follicular angiogenesis were examined using BrdU immunocytochemistry as a proliferation marker, CD31 as an endothelial specific cell marker and dual staining to distinguish proliferating endothelial cells. Effects of treatment on expression of VEGF and aromatase mRNA was determined by *in situ* hybridisation.

3.2 Specific methods

Tissue collection, immunocytochemistry and *in situ* hybridisation in this chapter were conducted as described in Chapter 2.

3.2.1 GnRH antagonist treatment

We employed the GnRH antagonist, Antarelix, a gift from Dr R. Deghenghi (Europeptides, Argenteuil, France) (Deghenghi *et al.* 1993), dissolved in water to concentration of 10 mg/ml. To provide a slow-release depot, Antarelix was administered at a dose of 12mg/kg, injected subcutaneously on follicular day 0 (n=4), treatment schedule 1 or follicular day 5 (n=6), treatment schedule 2 (**Figure 3.1**). Timing of ovarian cyclicity was synchronised by PG injection and ovaries from the treated animals were collected 10 days after PG administration, corresponding to the peri-ovulatory period in control animals. Control marmosets were studied on day 0 (n=3), day 5 (n=4) and day 10 (n=6) relative to PG administration. After PG administration, blood samples continued to be collected from the animals 3 times a week to monitor progesterone levels. All animals were injected i.v. with 20 mg BrdU (Roche) in saline 1 h before being sedated.

To confirm the potency of the GnRH antagonist treatment, 3 marmosets with regular ovarian cycles were given a single 12mg/kg dose of Antarelix and allowed to recover normal cycles as determined by measuring plasma progesterone in blood samples collected 3 times per week. Ovulation was suppressed in all three animals and a progesterone rise was not observed until between 80-110 days post treatment. The results from one treated animal are shown in **Figure 3.2**.

3.2.2 *In situ* hybridisation quantification

In situ slides were analysed blind by two investigators, separately. A scoring system of 0 = no hybridisation above background, + = detectable but low grain density clearly above background levels, ++ = moderate grain density, +++ = high grain density was employed to denote the level of hybridisation observed in each follicle and the results compared for agreement.

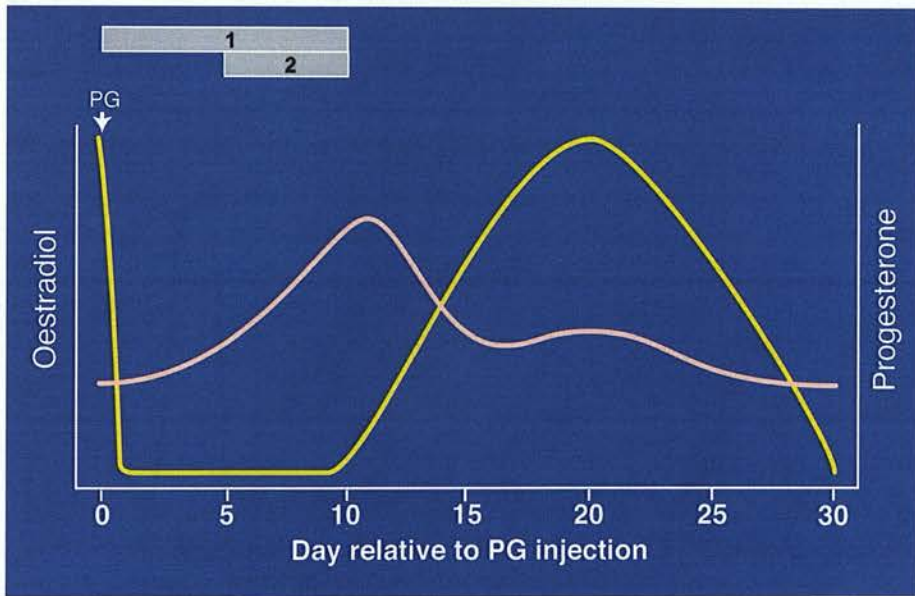


Figure 3.1 GnRH antagonist treatment schedule.

Two treatment regimes were employed in this study. Regime 1 consisted of a single GnRH antagonist injection on the day of PG injection. Regime 2 consisted of a single GnRH antagonist injection 5 days after PG injection. Ovaries from treated animals were collected on follicular day 10. The two grey boxes denote the two treatment regimes. Note the sharp decline in progesterone associated with induction of luteolysis, the ovulatory progesterone rise and the follicular phase increase in oestradiol that would be observed in control animals.

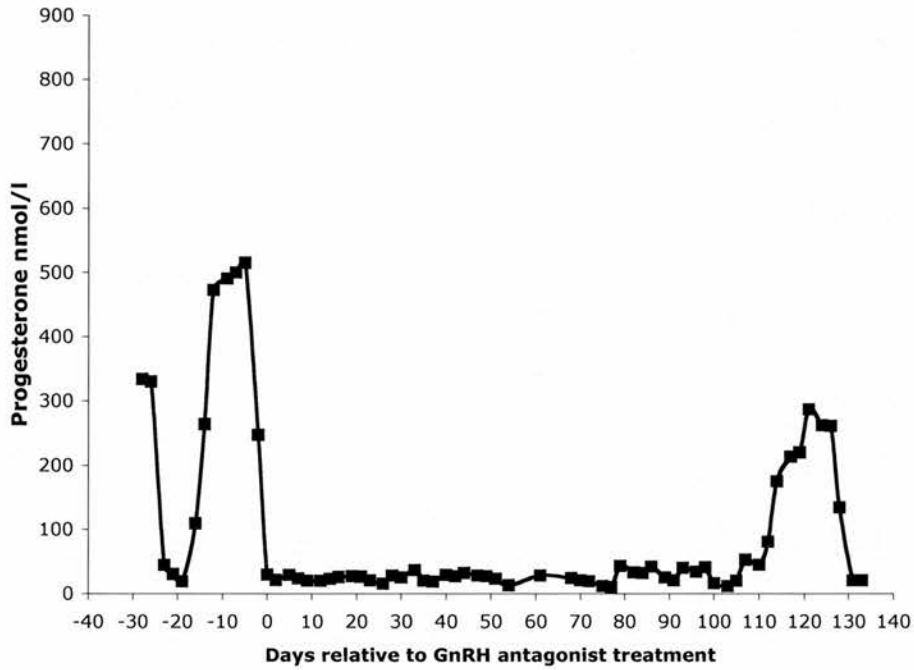


Figure 3.2 Circulating progesterone after GnRH antagonist treatment.

Circulating progesterone after a single injection of GnRH antagonist (12mg/kg). A sharp decline in progesterone is observed and an ovulatory rise in progesterone is not observed until 110 days post treatment in this case.

3.3 Results

3.3.1 Hormonal changes

All marmosets responded to PG injection with a rapid fall in plasma progesterone concentration that remained at follicular phase levels for the duration of the study. Treatment also suppressed serum oestradiol-17 β levels, consistent with inhibition of gonadotrophin release, significant reductions being observed between late follicular controls (1096 ± 332 pmol/L) and both treatment groups days 0-10 (72 ± 10 pmol/L, $p=0.022$) and days 5-10 (127 ± 41 pmol/L, $p=0.027$) (**Figure 3.3**).

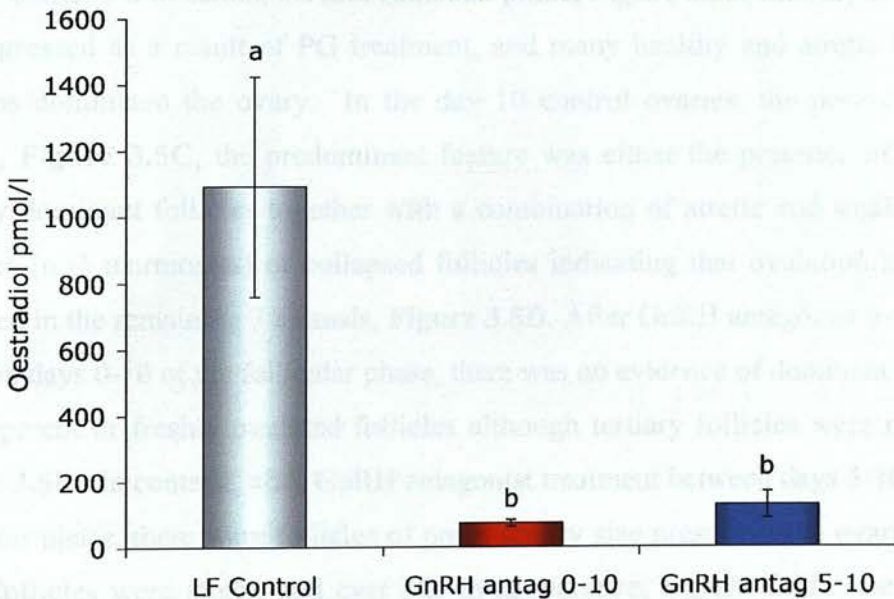


Figure 3.3 Terminal plasma oestradiol concentrations.

Significant reductions in terminal plasma oestradiol were observed after both GnRH antagonist treatments (days 0-10, 72 ± 10 pmol/L, $p=0.022$ and days 5-10, 127 ± 41 pmol/L, $p=0.027$) compared to late follicular controls (1096 ± 332 pmol/L). Values are means \pm sem. Superscripts a and b denotes statistical significance. Statistics used: one way ANOVA with an LSD post hoc test.

3.3.2 Ovarian Morphology

Both of the GnRH antagonist treatment schedules significantly reduced paired ovary weight, days 0-10 (102.5 ± 20.9 mg, $p=0.026$), days 5-10 (109.2 ± 8.0 mg, $p=0.029$) compared to pre-ovulatory weight of late follicular control ovaries (165.0 ± 22.2 mg), **Figure 3.4**.

In cross-section through a typical control ovary at day 12-16 of the luteal phase, the time of PG injection, **Figure 3.5A**, the corpus luteum was the dominant tissue in the organ, accompanied by numerous small and medium sized tertiary follicles. The late luteal control tissue was collected specifically for these experiments by monitoring progesterone and collecting the ovaries at the time prostaglandin would be injected in both follicular phase control and treated animals. By day 5 after PG injection, the mid-follicular phase, **Figure 3.5B**, the corpus luteum had regressed as a result of PG treatment, and many healthy and atretic tertiary follicles dominated the ovary. In the day 10 control ovaries, the periovulatory period, **Figure 3.5C**, the predominant feature was either the presence of 1 to 3 healthy dominant follicles together with a combination of atretic and small antral follicles ($n=3$ marmosets) or collapsed follicles indicating that ovulation had just occurred in the remaining 3 animals, **Figure 3.5D**. After GnRH antagonist treatment between days 0-10 of the follicular phase, there was no evidence of dominant follicle development or freshly ovulated follicles although tertiary follicles were present, **Figure 3.5E**. In contrast, after GnRH antagonist treatment between days 5-10 of the follicular phase, there were follicles of preovulatory size present in the ovaries, but these follicles were atretic and cyst like in appearance, **Figure 3.5F**. Details of specific animals are provided in **Appendix D**.

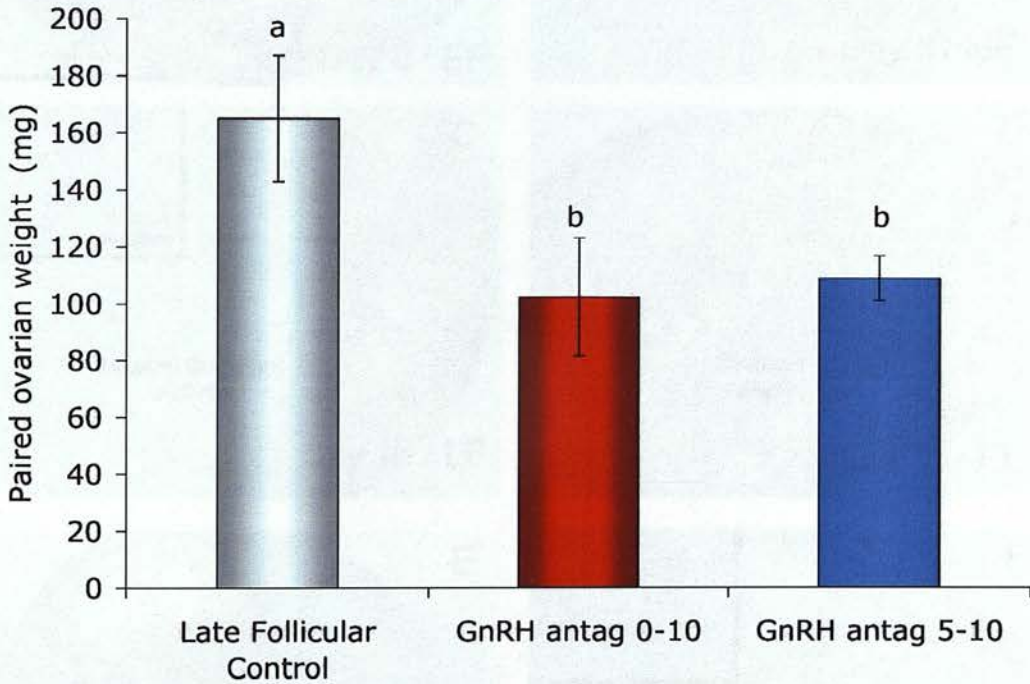


Figure 3.4 Paired ovarian weight.

Both days 0-10 (102.5 ± 20.9 mg, $p=0.026$) and days 5-10 (109.2 ± 8.0 mg, $p=0.029$) GnRH antagonist regimes significantly reduced the mean paired ovary weight compared to preovulatory late follicular controls (165.0 ± 22.2 mg). Values are means \pm sem. Superscripts a and b denote statistical significance. Statistics used: one way ANOVA with an LSD post hoc test.

3.3.3 Effect on tertiary follicle development

The accuracy measurements of follicle populations are shown in Figure 3.6. Healthy

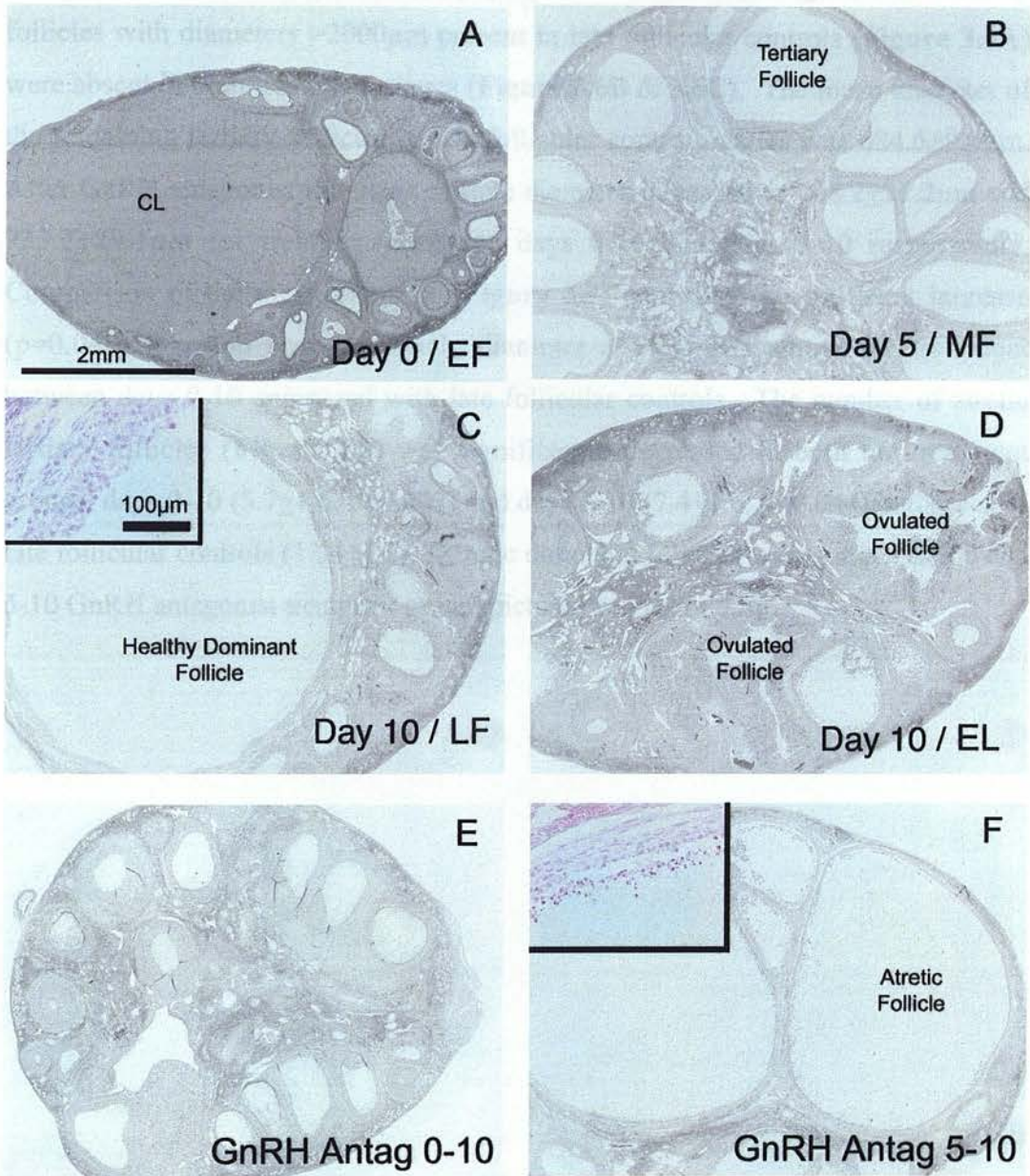


Figure 3.5 Haematoxylin and eosin stained ovary sections.

Sections of control day 0 early follicular phase (A), day 5 mid follicular phase with tertiary follicles (B), day 10 late follicular phase with healthy dominant follicles containing intact granulosa cells (inset) (C) or early luteal phase (D), GnRH antagonist treatment days 0-10 (E) and GnRH antagonist treatment days 5-10 showing atretic granulosa cells (inset) (F). Note the lack of healthy dominant follicles in both of the treated groups.

3.3.3 Effect on tertiary follicle development

Frequency measurements of follicle populations are shown in **Figure 3.6**. Healthy follicles with diameters $>2000\mu\text{m}$ present in late follicular controls (**Figure 3.6A**) were absent in both treatment groups (**Figure 3.6B & 3.6C**). The mean diameter of the remaining tertiary follicles in late follicular control ovaries was $684.6\pm 9.0\mu\text{m}$. After GnRH antagonist the mean follicle diameter increased to $768.2\pm 27.2\mu\text{m}$ and $733.2\pm 29.1\mu\text{m}$ for treatment between days 0-10 and days 5-10 respectively. Comparison of the mean diameter (**Figure 3.7**) confirmed a significant increase ($p=0.048$) in mean tertiary follicle diameter after GnRH antagonist treatment between days 0-10 compared with late follicular controls. The number of atretic tertiary follicles (**Figure 3.8**) was significantly decreased in both the treatment groups, days 0-10 (5.7 ± 1.6 , $p=0.007$) and days 5-10 (7.4 ± 1.6 , $p=0.044$) compared to late follicular controls (12.4 ± 1.5). Atretic dominant follicles were unique to the day 5-10 GnRH antagonist treatment group, pictured in **Figure 3.5F**.

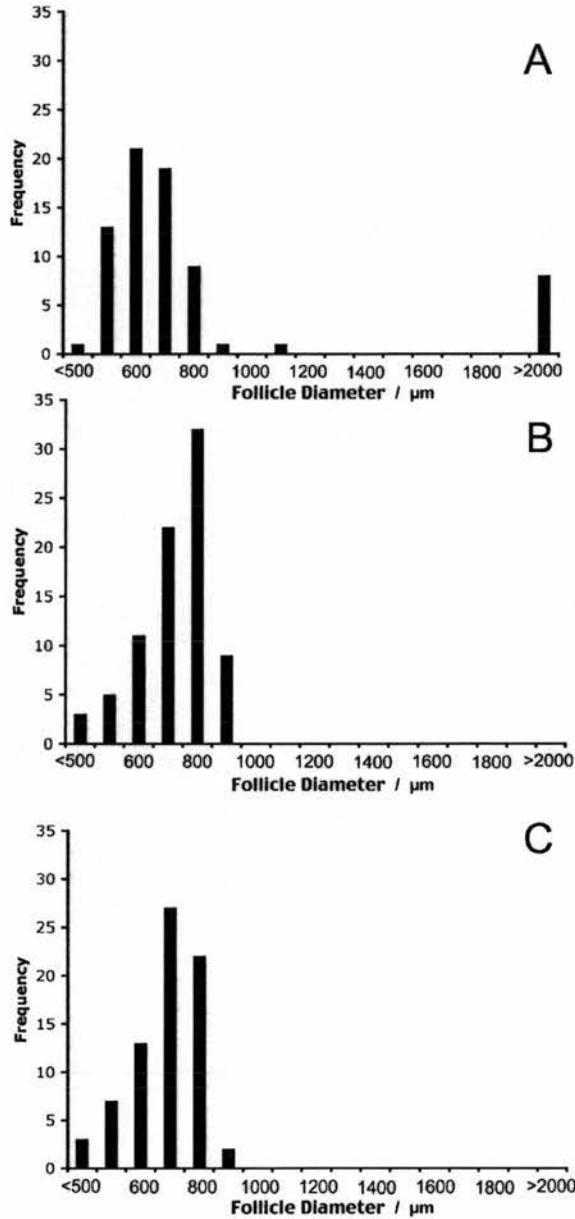


Figure 3.6 Tertiary follicle frequency.

Histograms showing distribution of tertiary follicle size in late follicular control animals (A), day 0-10 GnRH antagonist treated animals (B) and day 5-10 GnRH treated animals (C). The effect of both the treatments was to positively skew the frequency of antral follicles present in the ovaries compared to controls. Measurements are in microns.

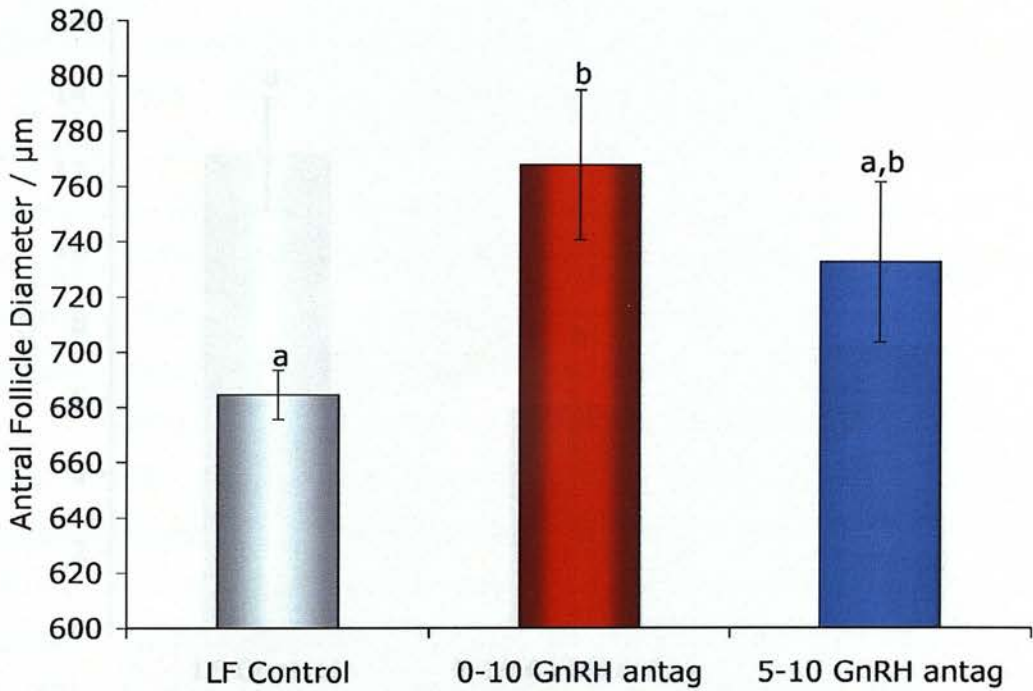


Figure 3.7 Mean tertiary follicle diameter.

After both GnRH antagonist treatment for 0-10 days ($768.2 \pm 27.2 \mu\text{m}$) and 5-10 days ($733.2 \pm 29.1 \mu\text{m}$) the mean tertiary follicle diameter increased compared to late follicular controls ($684.6 \pm 9.0 \mu\text{m}$). A significant increase was observed between late follicular controls and GnRH antagonist treatment between days 0-10 ($p=0.048$). Values are means \pm sem. Superscripts a and b denote statistical significance. Statistics used: one way ANOVA with an LSD post hoc test.

3.3.4 Effect on cellular proliferation

A typical tertiary follicle from a late follicular-control ovary is shown in Figure 3.9A. The total number of proliferating cells were obtained as a percentage of the total number of cells present in the follicle compartment in question. Analysis of granulosa cell proliferation in different classes of follicles from late follicular-control ovaries revealed a similar daily significant increase in proliferation between early

secondary ($6.5\% \pm 1.5$) and late secondary follicles ($12.4\% \pm 1.5$, $p=0.004$) and a significant decrease in proliferation between late secondary ($12.4\% \pm 1.5$) and dominant follicles ($8.0\% \pm 0.4$, $p=0.009$) (Figure 3.10A). Similarly, significant

increases in the number of proliferating cells were observed between early secondary ($0.7\% \pm 0.2$) and late secondary follicles ($8.0\% \pm 0.4$, $p=0.001$) and late secondary ($8.0\% \pm 0.4$) and dominant follicles ($11.3\% \pm 1.2$, $p=0.041$) (Figure 3.10B).

Significant increases in proliferation were also observed between early secondary ($0.7\% \pm 0.2$) and late secondary follicles ($8.0\% \pm 0.4$, $p=0.001$) and late secondary ($8.0\% \pm 0.4$) and dominant follicles ($11.3\% \pm 1.2$, $p=0.041$) (Figure 3.10C).

Significant increases in proliferation were also observed between early secondary ($0.7\% \pm 0.2$) and late secondary follicles ($8.0\% \pm 0.4$, $p=0.001$) and late secondary ($8.0\% \pm 0.4$) and dominant follicles ($11.3\% \pm 1.2$, $p=0.041$) (Figure 3.10D).

Figure 3.8 Atretic tertiary follicles per ovary.

Analysis showed a significant decrease in the number of atretic follicles between controls (12.4 ± 1.5) and either treatment group, day 0-10 (5.7 ± 1.2 , $p=0.007$) and day 5-10 (7.4 ± 1.6 , $p=0.044$). Values are means \pm sem. Superscripts a and b denote statistical significance. Statistics used: one way ANOVA with an LSD post hoc test.

compared to late follicular-control ovaries.

3.3.4 Effect on cellular proliferation

A typical tertiary follicle from a late follicular control ovary is shown in **Figure 3.9A**. The total numbers of proliferating cells were counted as a percentage of the total number of cells present in the follicle compartment in question. Analysis of granulosa cell proliferation in different classes of follicles from late follicular control ovaries revealed a statistically significant increase in proliferation between early secondary ($6.3\% \pm 0.9$) and late secondary follicles ($12.4\% \pm 1.6$, $p=0.004$) and a significant decrease in proliferation between late secondary ($12.4\% \pm 1.6$) and dominant follicles ($5.8\% \pm 0.6$, $p=0.008$) (**Figure 3.10A**). Similarly, significant increases in the rate of theca cell proliferation were observed between early secondary ($0.7\% \pm 0.3$) and late secondary follicles ($8.0\% \pm 0.8$, $p=0.001$), and late secondary ($8.0\% \pm 0.8$) and tertiary follicles ($12.5\% \pm 1.8$, $p=0.041$). A significant decrease in the rate of proliferation was observed between tertiary ($12.5\% \pm 1.8$) and dominant follicles ($4.1\% \pm 0.2$, $p=0.002$) (**Figure 3.11B**).

Comparison of typical tertiary follicles from a late follicular control (**Figure 3.9A**), a GnRH antagonist treated ovary from day 0-10 (**Figure 3.9B**) and a GnRH antagonist treated ovary from day 5-10 (**Figure 3.9C**) showed a decrease in both granulosa and thecal proliferation after both treatment schedules. Quantitative analyses confirmed significantly decreased cell proliferation in the granulosa, day 0-10 ($4.6\% \pm 1.5$, $p=0.013$) and day 5-10 ($6.5\% \pm 0.4$, $p=0.009$) (**Figure 3.10B**) and theca day 0-10 ($5.55\% \pm 2.0$, $p=0.034$) day 5-10 ($7.1\% \pm 1.3$, $p=0.029$) (**Figure 3.11B**) of tertiary follicles after treatment compared to late follicular controls (granulosa $9.0\% \pm 0.6$, theca $12.5\% \pm 1.8$). In the dominant follicles, a significant reduction in cellular proliferation in both the granulosa ($5.8\% \pm 0.6$ to $1.4\% \pm 0.7$, $p=0.044$) (**Figure 3.10B**) and theca ($4.1\% \pm 0.2$ to $1.1\% \pm 0.5$, $p=0.01$) (**Figure 3.11B**) compartments was also seen after GnRH antagonist treatment between days 5-10 of the follicular phase compared to late follicular controls.

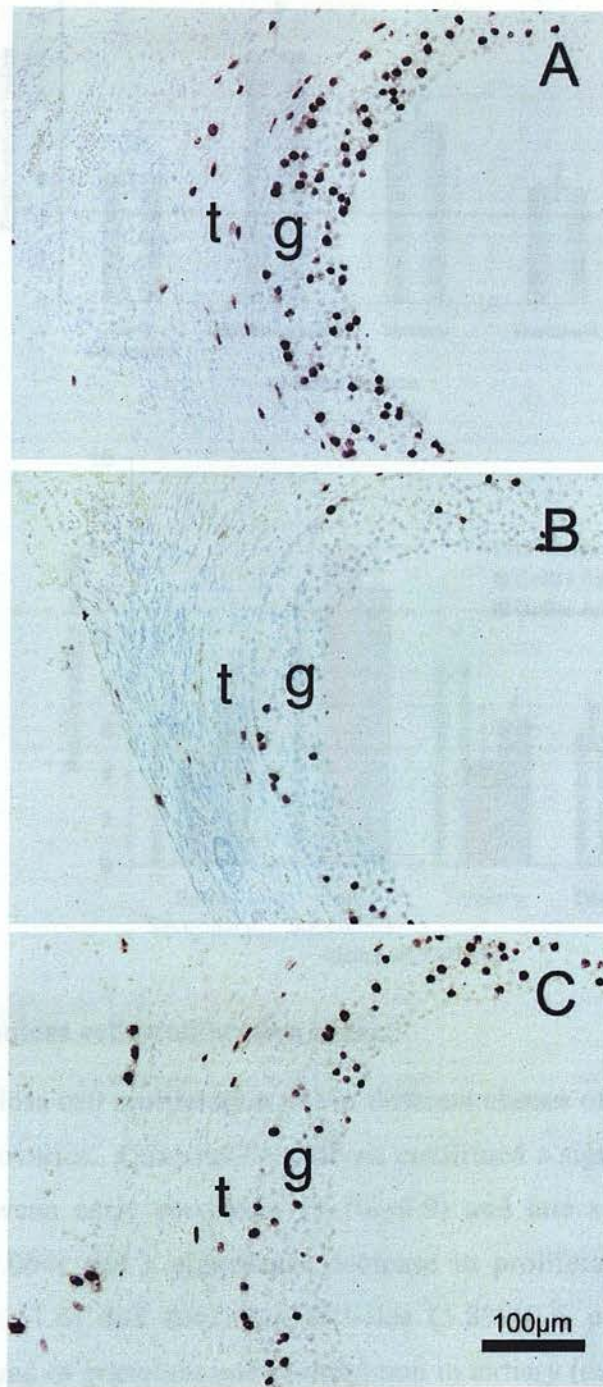


Figure 3.9 BrdU immunocytochemistry in tertiary follicles.

Tertiary follicles of (A) late follicular control, (B) day 0-10 GnRH antagonist treated and (C) day 5-10 GnRH antagonist treated ovaries are shown. Note the decrease in both granulosa and theca proliferation after both treatment schedules. Proliferation cells have a black nuclear stain. g=granulosa, t=theca.

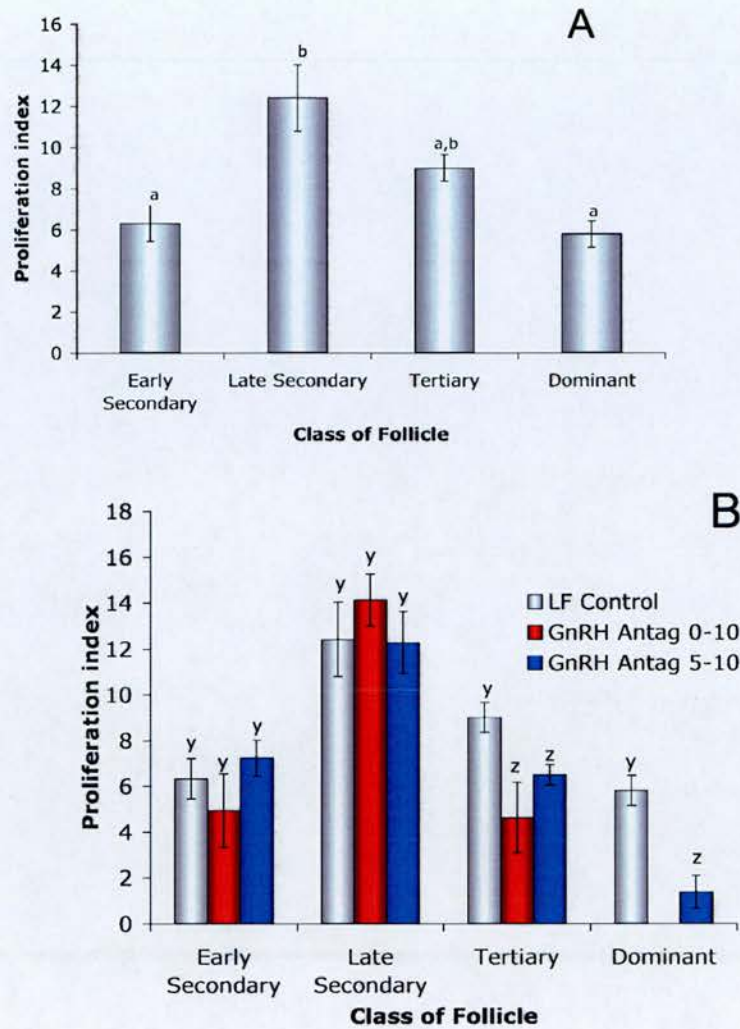


Figure 3.10 Granulosa cell proliferation index.

Analysis of granulosa cell proliferation (A) in different classes of follicles from late follicular control ovaries. Quantitative analysis confirmed a significant increase in proliferation between early secondary ($6.3\% \pm 0.9$) and late secondary follicles ($12.4\% \pm 1.6$, $p=0.004$) and a significant decrease in proliferation between late secondary ($12.4\% \pm 1.6$) and dominant follicles ($5.8\% \pm 0.6$, $p=0.008$). (B) A significant decreased in granulosa cell proliferation in tertiary (day 0-10, $4.6\% \pm 1.5$, $p=0.013$ and day 5-10, $6.5\% \pm 0.4$, $p=0.009$) and dominant follicles (day 5-10 $1.4\% \pm 0.7$, $p=0.044$) was also confirmed compared to late follicular controls (tertiary $9.0\% \pm 0.6$ and dominant $5.8\% \pm 0.6$). Values are means \pm sem. Superscripts a, b, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

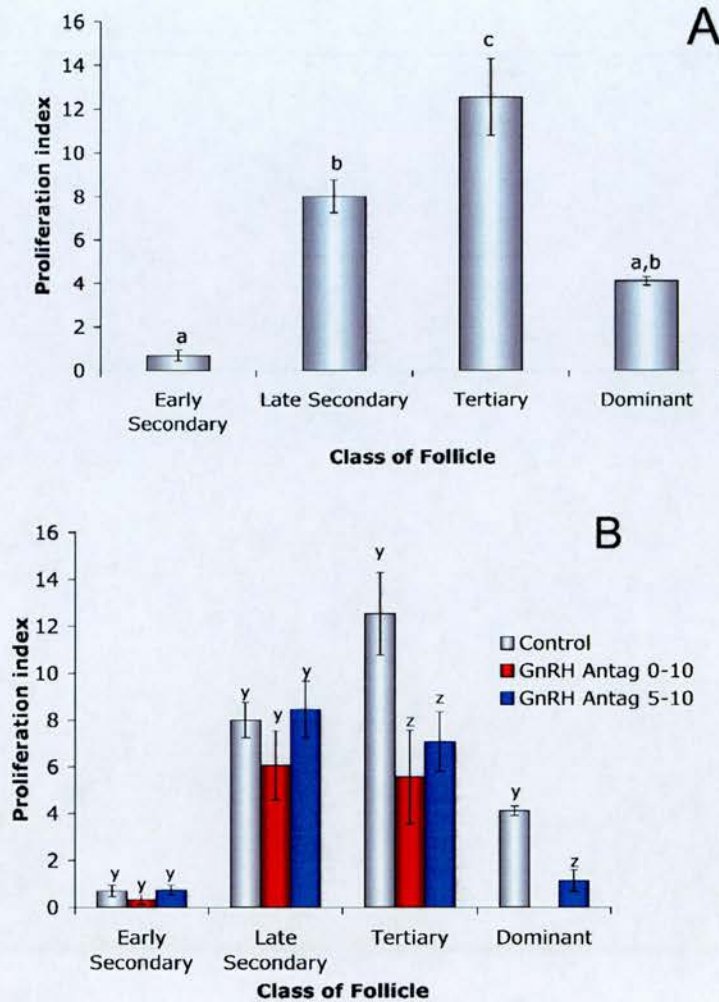


Figure 3.11 Thecal proliferation index.

Analysis of theca cell proliferation in (A) different classes of follicles from late follicular control ovaries. Quantitative analysis confirmed significant increases in proliferation between early secondary ($0.7\% \pm 0.3$) and late secondary follicles ($8.0\% \pm 0.8$, $p=0.001$), late secondary ($8.0\% \pm 0.8$) and tertiary follicles ($12.5\% \pm 1.8$, $p=0.041$) and a significant decrease in proliferation between tertiary ($12.5\% \pm 1.8$) and dominant follicles ($4.1\% \pm 0.2$, $p=0.002$). (B) Significant decreases in theca cell proliferation in tertiary (day 0-10, $7.1\% \pm 1.3$, $p=0.034$ and day 5-10, $5.5\% \pm 2.0$, $p=0.029$) and dominant follicles (day 5-10, $1.1\% \pm 0.5$, $p=0.01$) compared to late follicular controls was also confirmed (tertiary= $12.5\% \pm 1.8$, dominant= $4.1\% \pm 0.2$). Values are means \pm sem. Superscripts a, b, c, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

3.3.5 Endothelial cell proliferation

An index of the number of proliferating cells in the thecal compartment that were endothelial cells by co-localisation of CD31 and BrdU was used to determine the extent to which the treatment had impaired endothelial cell division and hence ongoing angiogenesis. In late follicular control ovaries (**Figure 3.12A**), dual-labelled cells were readily apparent. Quantitative analysis of follicles from late follicular control ovaries showed no rise in endothelial cell proliferation between late secondary and tertiary stages of follicle development (**Figure 3.13A**). A significant increase in the proportion of proliferating cells that were endothelial was observed between tertiary ($16.8\% \pm 3.1$) and dominant follicles ($51.0\% \pm 1.8$, $p < 0.001$) (**Figure 3.13A**). In late secondary follicles from ovaries from GnRH antagonist treatment between days 0-10 (**Figure 3.12B**) dual labelled cells were reduced significantly ($7.2\% \pm 1.5$, $p = 0.014$) compared to late follicular controls ($19.7\% \pm 2.6$) (**Figure 3.13B**). In tertiary follicles from ovaries from GnRH antagonist treatment between days 0-10 dual labelled cells were reduced significantly ($16.8\% \pm 3.1$ to $7.2\% \pm 2.0$, $p = 0.043$) (**Figure 3.13B**). After treatment between days 5-10 (**Figure 3.13C**) there was no reduction in dual labelled cells in tertiary follicles. Endothelial cell proliferation was also significantly reduced between dominant but atretic follicles from the day 5-10 GnRH antagonist treatment group ($20.7\% \pm 9.90$) and dominant follicles from late follicular controls ($51.0\% \pm 1.8$, $p = 0.036$) (**Figure 3.13B**).

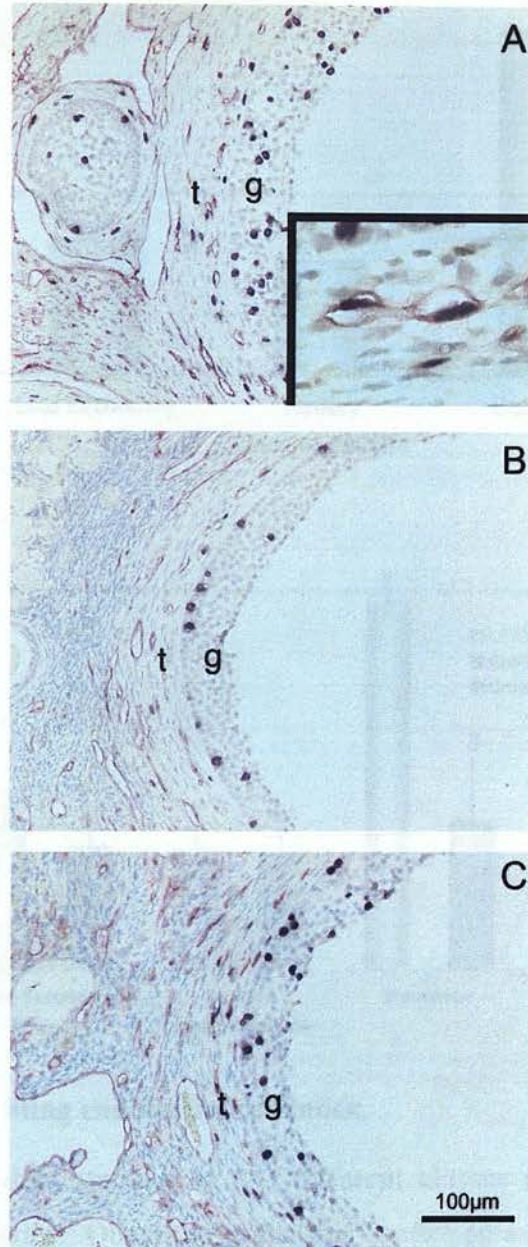


Figure 3.12 BrdU and CD31 dual staining immunocytochemistry in tertiary follicles.

Tertiary follicles of (A) late follicular control, (B) day 0-10 GnRH antagonist treated and (C) day 5-10 GnRH antagonist treated ovary are shown. Note the decrease in the number of co-localised BrdU and CD31 cells. Proliferating cells have a black nuclear stain, endothelial cells have a red stain, co-localised cells have both. g=granulosa, t=theca.

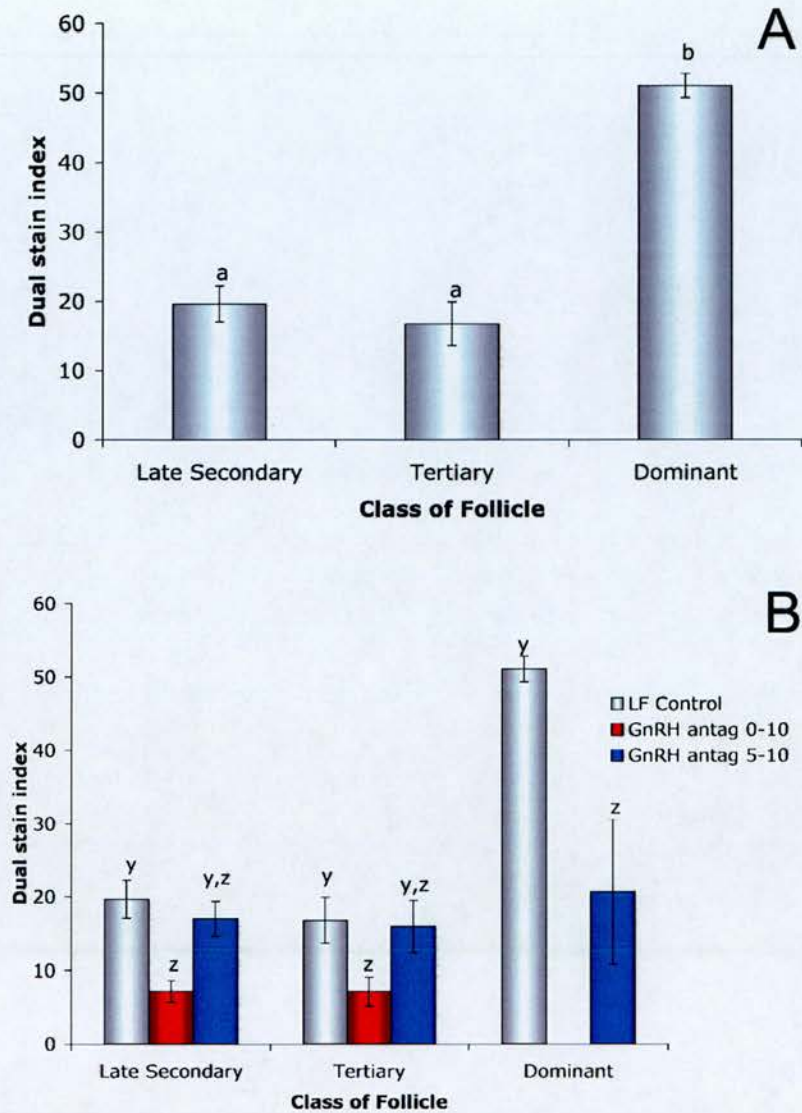


Figure 3.13 Proliferating endothelial cell index.

Analysis of thecal dual staining in (A) different classes of follicles from late follicular control ovaries. Quantitative analysis confirmed a significant increase in dual staining cells proliferation between tertiary ($16.8\% \pm 3.1$) and dominant follicles ($51.0\% \pm 1.8$, $p < 0.001$). (B) Significant decreases in dual cell proliferation in late secondary (day 0-10, $7.2\% \pm 1.5$ compared to $19.7\% \pm 2.6$, $p = 0.014$), tertiary (day 0-10, $7.2\% \pm 2.0$ compared to $16.8\% \pm 3.1$, $p = 0.043$) and dominant follicles (day 5-10, $20.7\% \pm 9.9$ compared to $51.0\% \pm 1.8$, $p = 0.036$) compared to late follicular controls was also confirmed. Values are means \pm sem. Superscripts a, b, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

3.3.6 Non-endothelial cell proliferation

Analysis of non-endothelial, presumably predominantly thecal, cell proliferation in the theca showed a significant increase between late secondary ($6.5\% \pm 0.7$) and tertiary follicles ($10.7\% \pm 1.5$, $p=0.041$) (**Figure 3.14A**), a significant decrease was observed between tertiary ($10.7\% \pm 1.5$) and dominant follicles ($1.9\% \pm 0.2$, $p=0.001$) (**Figure 3.14A**).

After GnRH antagonist treatment the amount of non-endothelial cell proliferation in the theca was also examined. GnRH antagonist treatment between days 0-10 resulted in a significant ($5.0\% \pm 1.8$) reduction in the number of non-endothelial cells proliferating in tertiary follicles compared to late follicular controls ($10.7\% \pm 1.5$, $p=0.029$) (**Figure 3.14B**). GnRH antagonist treatment from days 5-10 resulted in a non-significant ($6.1\% \pm 1.3$) reduction in non-endothelial cell proliferation in tertiary follicles compared to late follicular controls ($10.7\% \pm 1.5$, $p=0.051$) and a significant reduction in non-endothelial cell proliferation in dominant but atretic follicles ($0.8\% \pm 0.3$) compared with late follicular controls ($1.9\% \pm 0.2$, $p=0.018$) (**Figure 3.14B**).

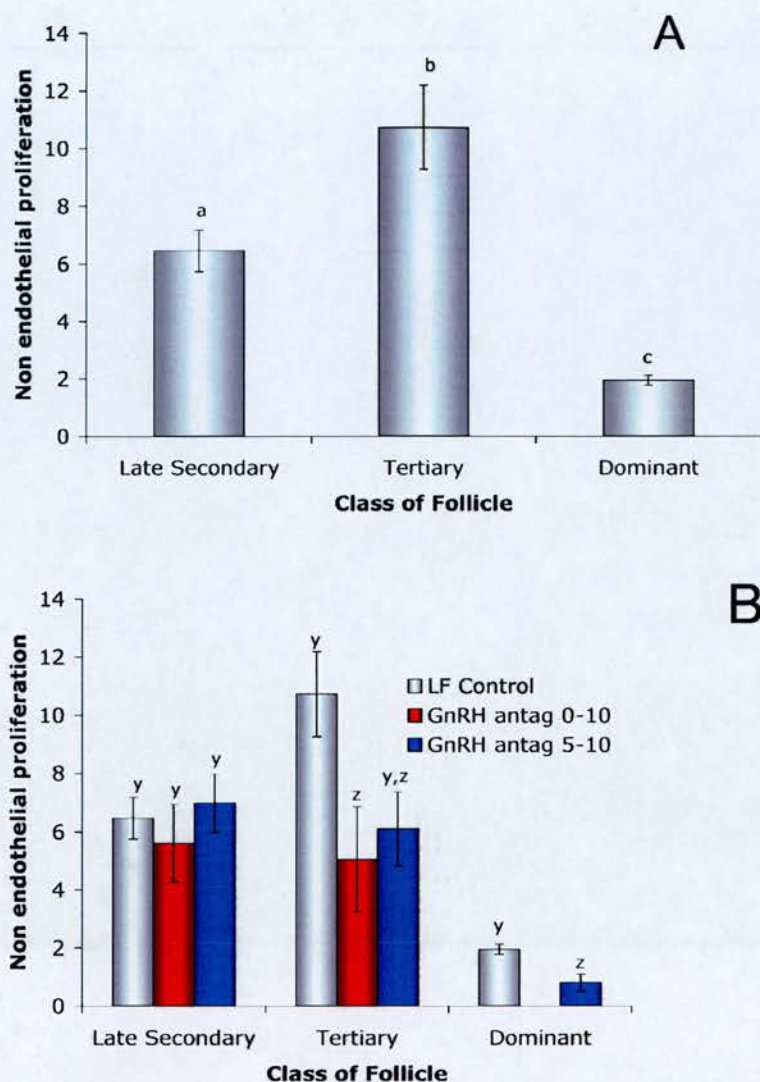


Figure 3.14 Non endothelial cell proliferation.

Analysis of non-endothelial cell proliferation in the theca showed (A) a significant increase between late secondary ($6.5\% \pm 0.7$) and tertiary follicles ($10.7\% \pm 1.5$, $p=0.041$) and a significant decrease between tertiary ($10.7\% \pm 1.5$) and dominant follicles ($1.9\% \pm 0.2$, $p=0.001$). (B) GnRH antagonist treatment between days 0-10 resulted in a significant ($5.0\% \pm 1.8$ compared to $10.7\% \pm 1.5$, $p=0.029$) reduction in non-endothelial cell proliferation in tertiary follicles and a significant ($0.8\% \pm 0.3$ compared to $1.9\% \pm 0.2$, days 5-10 $p=0.018$) reduction in non-endothelial cell proliferation in dominant but atretic follicles compared with late follicular controls. Values are means \pm sem. Superscripts a, b, c, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

3.3.7 Effect on vasculature

Localisation of blood vessels using an endothelial cell marker in tertiary follicles is shown in **Figure 3.15**. Large vessels were located in the outer theca while smaller vessels were observed within the vicinity of the lamina basalis. Extensive vascularisation was observed in both controls (**Figure 3.15A**) and after GnRH antagonist treatment between days 0-10 (**Figure 3.15B**) and days 5-10 (**Figure 3.15C**) of the follicular phase. Quantitative analysis of staining of follicles from late follicular control ovaries showed a significant increase in the extent of vascularisation between tertiary ($5.8\% \pm 0.3$) and dominant follicles ($12.3\% \pm 0.8$, $p < 0.001$) (**Figure 3.16A**). Analysis indicated that there was no difference in the extent of thecal vascularisation between the groups treated and control groups (**Figure 3.16B**).

However, as follicles develop the theca becomes thicker. Significant increases in thecal diameter were observed between late secondary ($24.9 \pm 2.0 \mu\text{m}$) and tertiary ($57.3 \pm 2.7 \mu\text{m}$, $p < 0.001$) and between tertiary ($57.3 \pm 2.7 \mu\text{m}$) and dominant follicles ($74.9 \pm 4.1 \mu\text{m}$, $p = 0.002$) from late follicular control ovaries, **Figure 3.17A**. A thinning of the theca was observed between follicles from late follicular controls and both treatment groups, with a significant decrease in the mean thecal thickness between dominant follicles from GnRH antagonist treatment between days 5-10 ($52.21 \pm 4.5 \mu\text{m}$) of the follicular phase and dominant follicles from late follicular controls ($74.9 \pm 4.1 \mu\text{m}$, $p = 0.006$) (**Figure 3.17B**). To adjust for the effect of the thinner theca, a conversion factor was used to quantify an absolute value of CD31 positive staining in each class of follicle, based on a technique applied by Wulff *et al* (Wulff *et al.* 2001a). The conversion factors were calculated as a ratio of mean thecal thickness against the late follicular control mean for each class of follicle, giving the late follicular control conversion a value of one. After adjustment, no increase in thecal vascularisation was observed between late secondary and tertiary follicles from late follicular control ovaries. Although a significant increase in thecal vascularisation was observed between tertiary ($5.8\% \pm 0.3$) and dominant follicles from late follicular control ovaries ($12.3\% \pm 0.9$, $p < 0.001$) (**Figure 3.18A**). Also, after GnRH antagonist treatment a significant reduction in thecal vascularisation was

demonstrated between tertiary follicles from late follicular controls ($5.8\% \pm 0.3$) and day 0-10 GnRH antagonist treatment ($4.0\% \pm 0.6$, $p=0.04$) (**Figure 3.18B**). A significant reduction was also observed between dominant follicles after day 5-10 GnRH antagonist treatment ($8.1\% \pm 0.4$) and late follicular controls ($12.3\% \pm 0.9$, $p=0.014$) after adjustment for thecal thickness (**Figure 3.18B**).

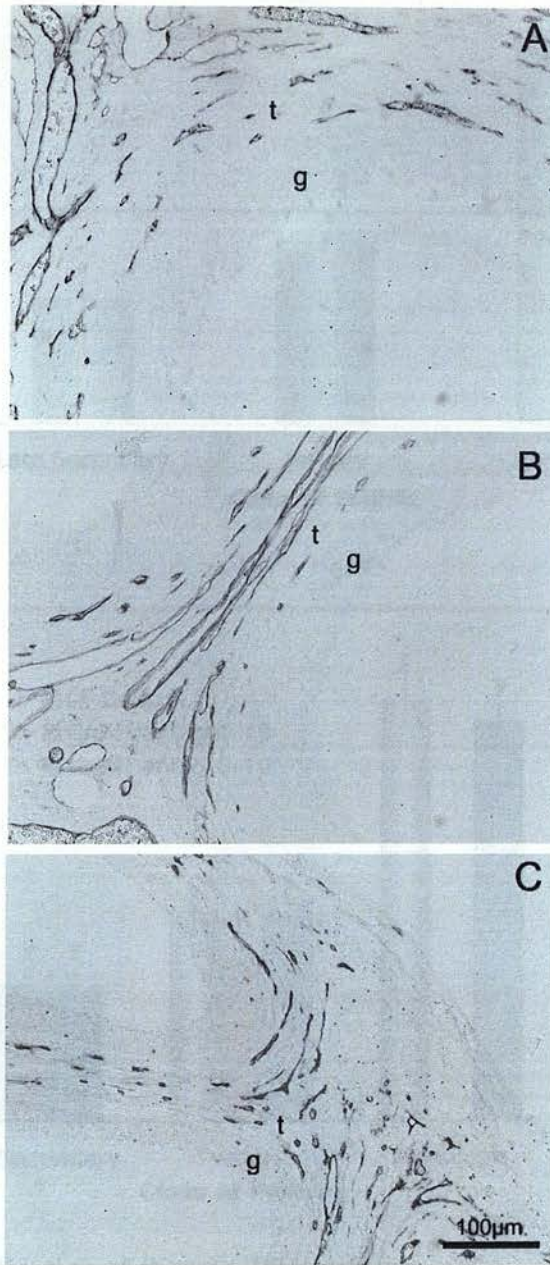


Figure 3.15 CD31 immunocytochemistry in tertiary follicles.

Tertiary follicles of (A) late follicular control, (B) day 0-10 GnRH antagonist treated and (C) day 5-10 GnRH antagonist treated ovary are shown. Note the uniform positioning of the blood vessels in ovaries from both treated and control animals. g=granulosa, t=theca

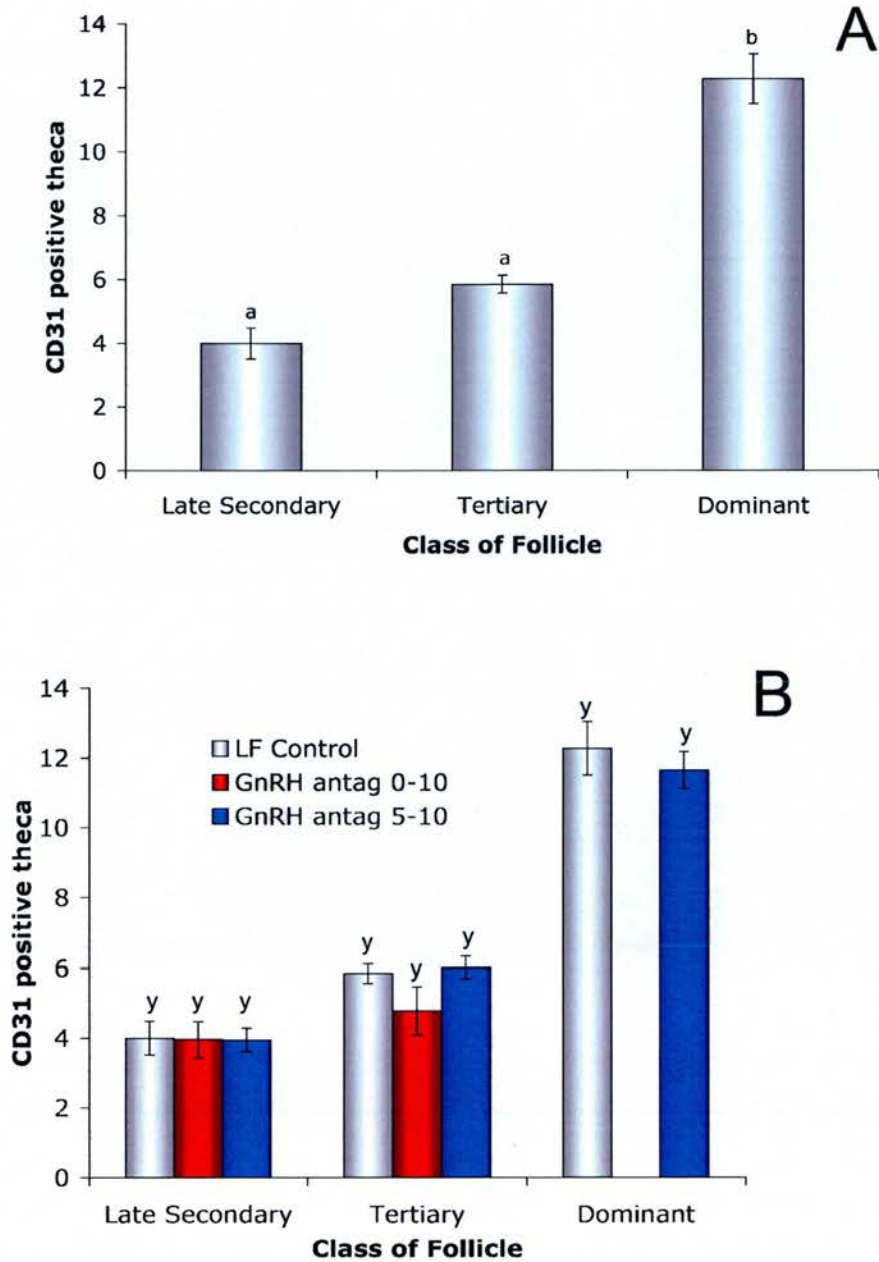


Figure 3.16 Quantification of thecal CD31 staining.

Quantitative analysis of the CD31 staining in the theca showed (A) a significant increase in the extent of follicular vascularisation between tertiary ($5.8\% \pm 0.3$) and dominant follicles in late follicular control ovaries ($12.3\% \pm 0.8$, $p < 0.001$). (B) No difference in the extent of vascularisation after either treatment. Values are means \pm sem. Superscripts a, b and y denote statistical significance. Statistics used: one way ANOVA with an LSD post hoc test.

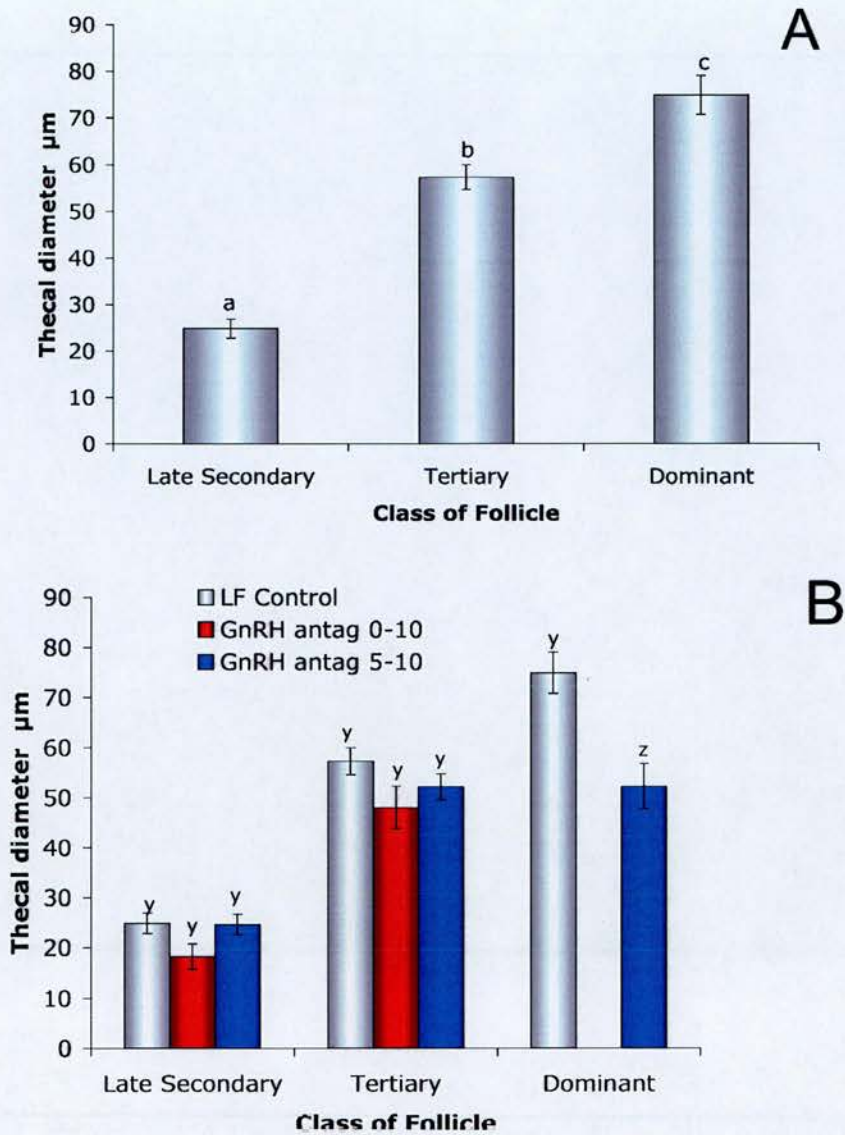


Figure 3.17 Quantification of thecal thickness.

(A) Significant increases in thecal diameter were observed between late secondary ($24.9 \pm 2.0 \mu\text{m}$) and tertiary ($57.3 \pm 2.7 \mu\text{m}$, $p < 0.001$) and between tertiary ($57.3 \pm 2.7 \mu\text{m}$) and dominant follicles from late follicular control ovaries ($74.9 \pm 4.1 \mu\text{m}$, $p = 0.002$). (B) A significant decrease in the mean thecal thickness between dominant follicles from GnRH antagonist treatment between days 5-10 of the follicular phase ($52.2 \pm 4.5 \mu\text{m}$) and dominant follicles from late follicular controls ($74.9 \pm 4.1 \mu\text{m}$, $p = 0.006$) was also observed. Values are means \pm sem. Superscripts a, b, c, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

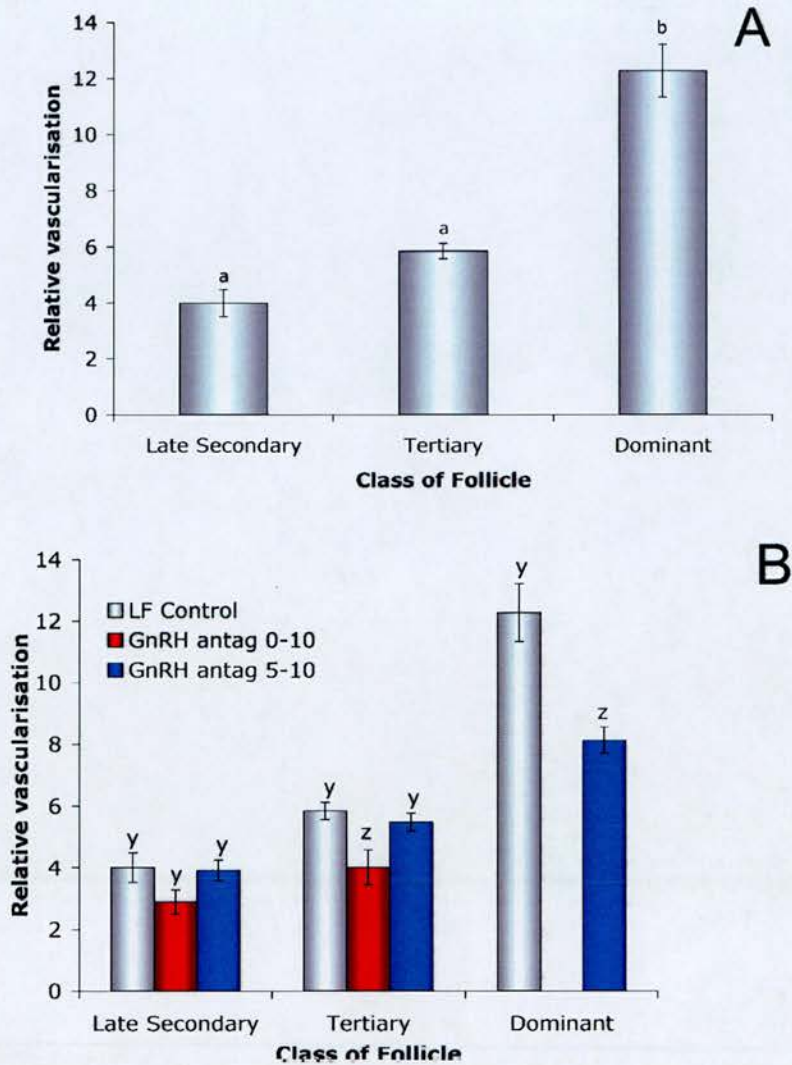


Figure 3.18 Relative vascularisation of theca

After adjustment a significant increase in thecal vascularisation was observed (A) between tertiary ($5.8\% \pm 0.3$) and dominant follicles ($12.3\% \pm 0.9$, $p < 0.001$) from late follicular control ovaries. (B) After GnRH antagonist treatment a significant reduction in thecal vascularisation was demonstrated between tertiary follicles from late follicular controls and day 0-10 GnRH antagonist treatment ($4.0\% \pm 0.6$ compared to $5.8\% \pm 0.3$ $p = 0.04$) and between dominant follicles after day 5-10 GnRH antagonist treatment and late follicular controls ($8.1\% \pm 0.4$ compared to $12.3\% \pm 0.9$, $p = 0.014$). Values are means \pm sem. Superscripts a, b, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

3.3.8 Localisation of VEGF mRNA

In situ hybridisation revealed VEGF mRNA in both control and treated groups was absent in primordial, primary and early secondary follicles. VEGF mRNA was expressed in the granulosa of late secondary and tertiary follicles from late follicular controls (**Figure 3.19A&B**), after GnRH antagonist treatment between days 0-10 (**Figure 3.19E&F**), days 5-10 (**Figure 3.19G&H**)(**Figure 3.21B**) and in mid follicular controls (**Figure 3.19I&J**). VEGF mRNA was also expressed in the theca of atretic follicles in the late follicular controls (**Figure 3.19C&D**), with a significant increase in the level of expression between tertiary (1.3 ± 0.2) and atretic follicles (2.4 ± 0.2 , $p=0.019$) from late follicular control ovaries (**Figure 3.19A-D**)(**Figure 3.22A**). However, thecal expression was absent in the theca of atretic follicles from both the days 0-10 and days 5-10 GnRH antagonist treatment groups (0.2 ± 0.15 , $p=0.024$ and 0.4 ± 0.1 , $p=0.004$ respectively)(**Figure 3.22B**), and the mid follicular controls (**Figure 3.19E-J**).

In the dominant follicles of late follicular controls, VEGF mRNA continued to be expressed in the granulosa cells (**Figure 3.20A&B**). However, in follicles that appeared to be closer to the time of ovulation, VEGF mRNA expression in the granulosa cells was reduced and an intense punctate expression pattern in cells in the thecal layer on the border of the granulosa cells was observed (2.4 ± 0.3), being significantly increased compared to tertiary follicles (1.3 ± 0.2 , $p=0.024$) (**Figure 3.20C&D**, **3.22A**). This punctate pattern was also observed in postovulatory follicles (**Figure 3.20E&F**) at the edge of the newly forming corpus luteum. In contrast, VEGF mRNA expression was absent from both granulosa and theca in all the dominant follicles from the day 5-10 GnRH antagonist treatment group (**Figure 3.20G&H**). Quantitative analysis confirmed these observations (1.7 ± 0.3 compared to 0.25 ± 0.25 , $p=0.016$ and 2.4 ± 0.3 compared to 0.4 ± 0.2 , $p<0.001$ respectively) (**Figure 3.21B**, **3.22B**).

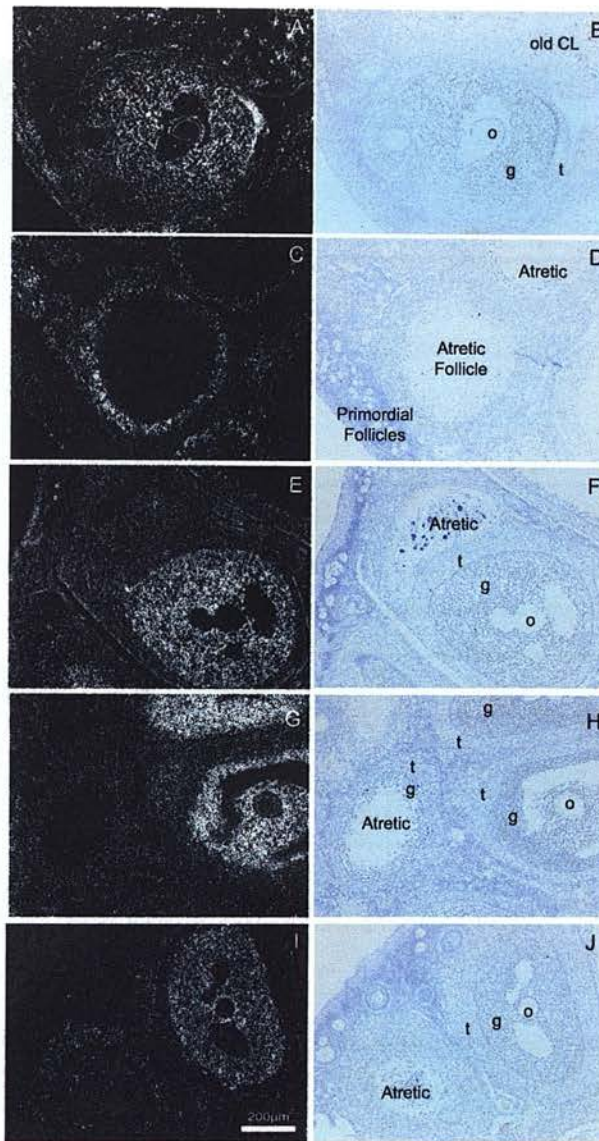


Figure 3.19 Dark and light field photomicrographs showing *in situ* hybridisation for VEGF mRNA.

(A&B) VEGF mRNA localised in the granulosa of late follicular phase tertiary follicles. The same expression pattern was found in both treatment groups days 0-10 (E&F), days 5-10 (G&H) and in mid follicular controls (I&J). VEGF mRNA was also expressed in the theca of atretic follicles in the late follicular controls (C&D) but was absent in atretic follicles in both treatment groups and the mid follicular controls (E&J). Note frequent occurrence of high expression in cumulus cells surrounding the oocyte. g=granulosa, t=theca, o=oocyte.

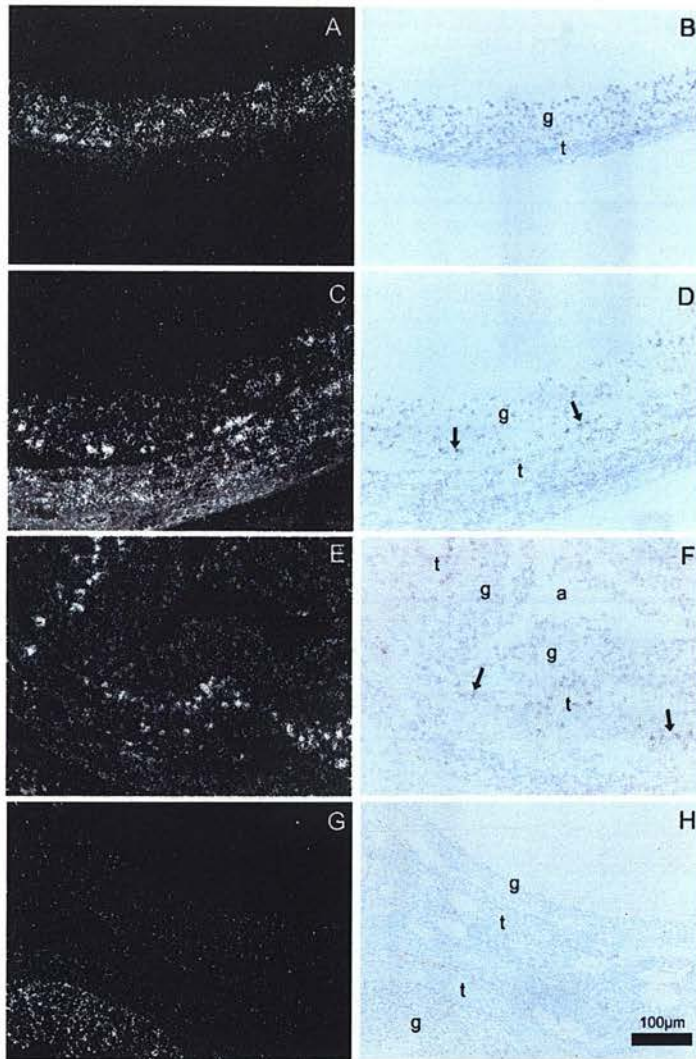


Figure 3.20 Dark and light field photomicrographs showing *in situ* hybridisation for VEGF mRNA.

(A&B) VEGF mRNA localised in the dominant follicles of late follicular controls VEGF mRNA is expressed in the granulosa cells. (C&D) Before ovulation, VEGF mRNA expression in the granulosa cells is reduced while a punctate expression pattern in the theca cells on the border of the granulosa cells is observed. (E&F) This punctate pattern is also observed in postovulatory follicles at the edge of the newly forming CL. (G&H) This expression is absent in the dominant follicles from the 5-10 day GnRH antagonist treatment group while expression is maintained in the granulosa of tertiary follicles. g=granulosa, t=theca, a=antrum

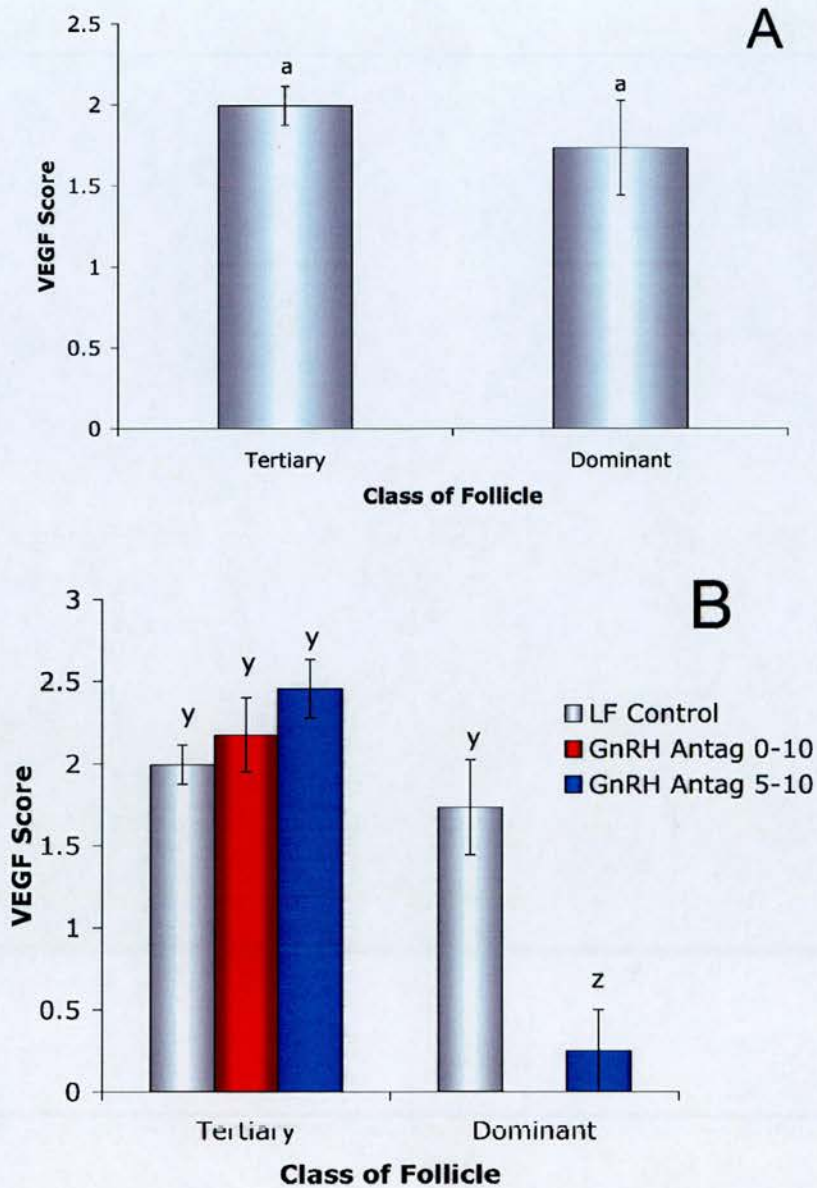


Figure 3.21 Quantification of VEGF mRNA expression in granulosa cells.

Quantitative analysis of VEGF mRNA expression in granulosa cells from: (A) Different follicle classes from late follicular control ovaries showed no significant difference in VEGF mRNA expression; (B) GnRH antagonist treatment for days 5-10 significantly reduced VEGF mRNA expression in the granulosa cells of dominant but atretic follicles (0.25 ± 0.25) compared to late follicular controls (1.7 ± 0.3 , $p=0.016$). Values are means \pm sem. Superscripts a, b, y and z denote statistical significance. Statistics used: two tailed unpaired t-test in panel A and one way ANOVA with LSD post hoc tests in panel B.

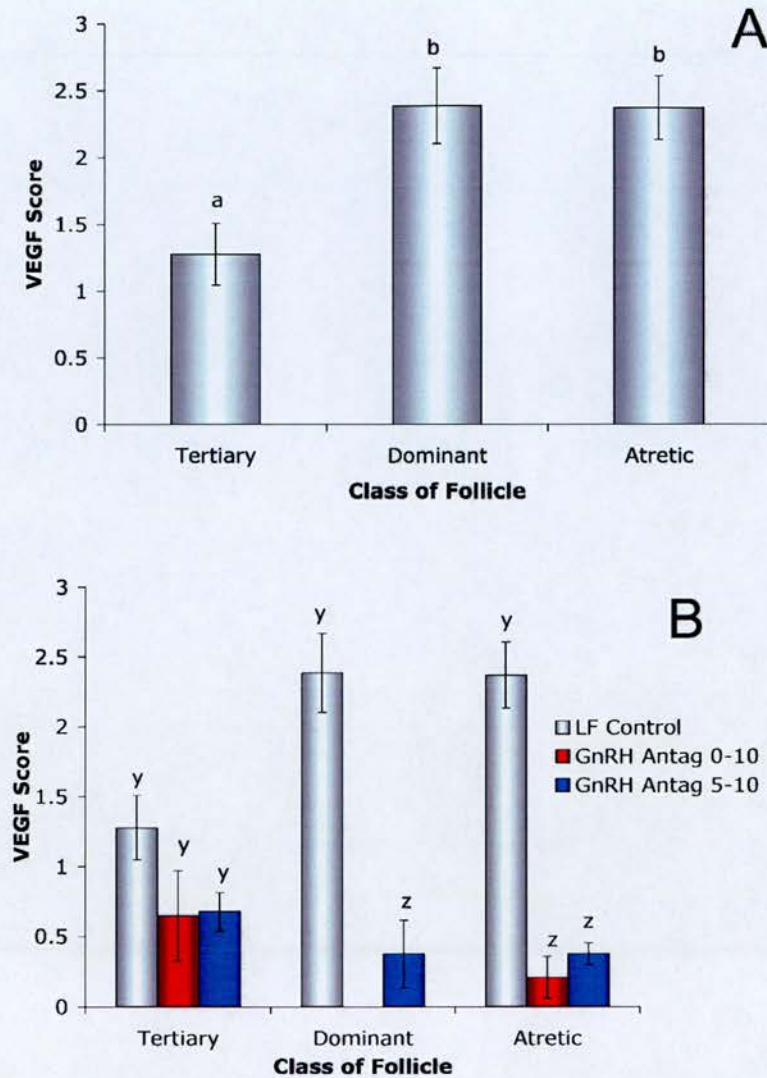


Figure 3.22 Quantification of VEGF mRNA expression in the theca.

Quantitative analysis of VEGF mRNA expression in the theca showed: (A) a significant increase between tertiary (1.3 ± 0.2) and dominant follicles (2.4 ± 0.3 , $p=0.024$) and between tertiary (1.3 ± 0.2) and atretic follicles (2.4 ± 0.2 , $p=0.019$) in late follicular control ovaries; (B) After GnRH antagonist treatment for days 0-10 a significant reduction in VEGF expression was seen in atretic follicle (0.2 ± 0.15 compared to 2.4 ± 0.2 , $p=0.024$) and after treatment for days 5-10 significant reduction in both dominant and atretic follicles (0.4 ± 0.2 compared to 2.4 ± 0.3 , $p<0.001$ and 0.4 ± 0.1 compared to 2.4 ± 0.2 , $p=0.004$ respectively). Values are means \pm sem. Superscripts a, b, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

3.3.9 Aromatase mRNA

Aromatase mRNA expression was measured to establish the level of differentiation of the granulosa cells. In control preovulatory follicles, aromatase mRNA was expressed in the mural granulosa cells closest to the membrana propria (**Figure 3.23A&B**), along with some expression in old luteal tissue and newly forming extra-luteal tissue. After both the GnRH antagonist treatment regimes there was an absence of aromatase mRNA expression (day 5-10 shown **Figure 3.23C&D**). Some expression in old luteal tissue was maintained. Quantitative analysis confirmed this observation showing a significant reduction in aromatase expression ($p=0.016$) (**Figure 3.24**).

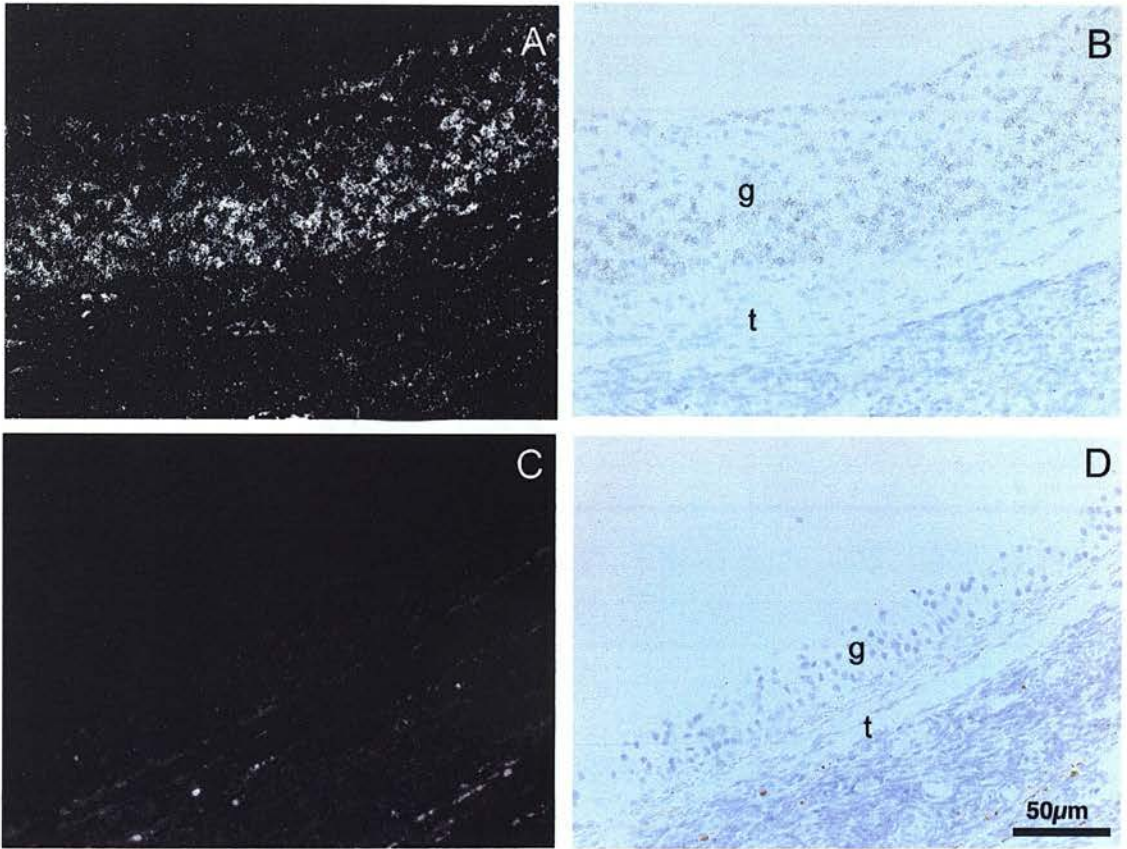


Figure 3.23 Photomicrograph of *in situ* hybridisation for aromatase mRNA.

(A&B) Aromatase *in situ* in a preovulatory follicle and Day 5-10 GnRH antagonist treated (C&D). Note the absence of aromatase mRNA expression in the treated ovary. g=granulosa, t=theca.

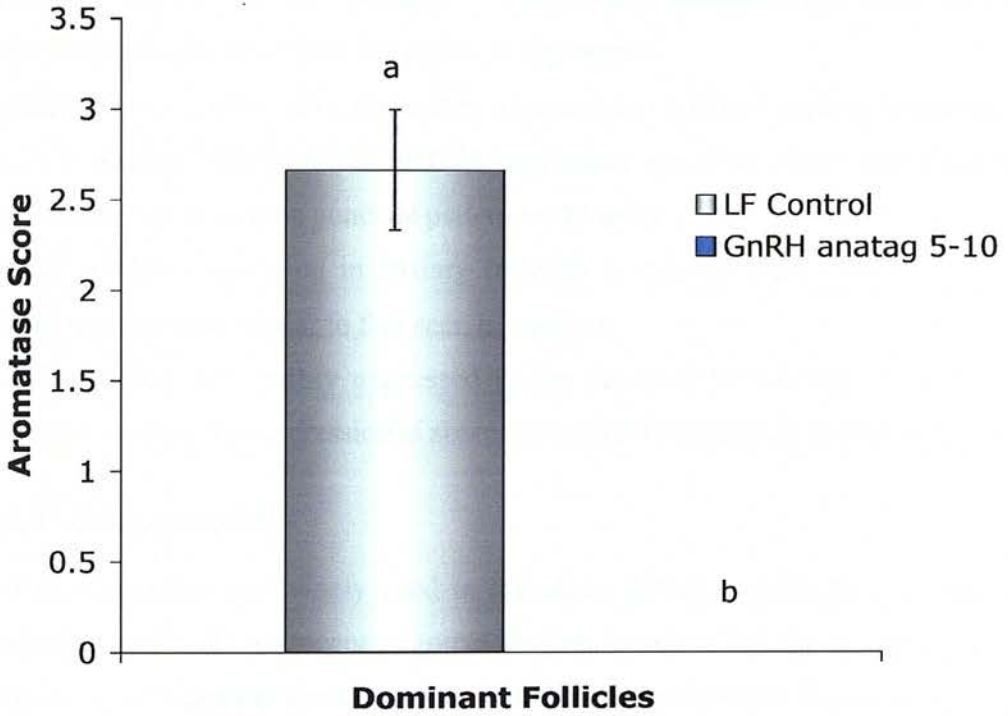


Figure 3.24 Quantification of aromatase mRNA expression.

Quantification of aromatase mRNA expression showed a significant reduction in expression in dominant follicle after GnRH antagonist treatment for days 5-10 compared to late follicular controls ($p=0.016$). Values are means \pm sem. Superscripts a and b denote statistical significance. Statistics used: Rank Sum of independent samples (Mann-Whitney test).

3.4 Discussion

3.4.1 Key results

- The development of dominant follicles was completely inhibited by the 0-10 day GnRH antagonist treatment.
- Dominant follicles did develop after 5-10 day GnRH antagonist treatment, although these follicles were abnormal and cystic in appearance.
- Follicular angiogenesis was moderately inhibited by GnRH antagonist treatment.
- In preovulatory follicles VEGF mRNA expression was absent from granulosa cells but activated in an intense punctate pattern in the theca.
- VEGF mRNA expression in tertiary follicles in ovaries from GnRH antagonist treated animals was similar to that seen in controls.
- VEGF mRNA was highly expressed in the theca of atretic follicles from late follicular ovaries, this expression is absent after GnRH antagonist treatment.

3.4.2 Background

GnRH antagonists are widely used in the clinic during ovarian hyperstimulation programmes to block endogenous gonadotrophin secretion and the pre-ovulatory LH surge while exogenous gonadotrophins are being administered (Evans *et al.* 2002, Huirne *et al.* 2004). Many studies have examined the effects of GnRH antagonist treatment on pituitary-ovarian function throughout the follicular phase in non-human primates and in women and have demonstrated a suppression in LH and FSH secretion associated with a fall in serum oestradiol production (Ditkoff *et al.* 1991, Fluker *et al.* 1991, Hall *et al.* 1991, Karnitis *et al.* 1994, Nelson *et al.* 1995, Fraser *et al.* 1999a). However, the effect upon vascularisation of the follicles during GnRH antagonist treatment has not been determined. Previous studies have shown that GnRH antagonist treatment of marmosets during the early luteal phase had a marked inhibitory effect upon the intense angiogenesis normally observed in the corpus luteum at this time (Dickson and Fraser 2000) and that this was associated with luteal cellular degeneration (Dickson and Fraser 2000) and a marked reduction in expression of VEGF as demonstrated by in situ hybridisation (H.M. Fraser,

unpublished observations).

Angiogenesis is intimately involved in ovarian follicle development (Fraser and Wulff 2001, Zimmermann *et al.* 2001b, Zimmermann *et al.* 2003) and is necessary for normal follicular growth (Wulff *et al.* 2001b, Wulff *et al.* 2002). The expression pattern of VEGF mRNA in the ovarian follicle suggests it is regulated by gonadotrophic stimulation (Ravindranath *et al.* 1992, Shweiki *et al.* 1993). Hence the effect of GnRH antagonist treatment on follicular development and vascularisation in the marmoset ovary might be expected to result in a suppression of VEGF expression, inhibition of angiogenesis and follicular development.

3.4.3 Analysis

The most expected findings were observed in the day 0-10 GnRH antagonist treated animals in which development of large dominant follicles was completely inhibited and oestradiol secretion was markedly suppressed. In the remaining healthy tertiary follicles, granulosa and theca cell proliferation was reduced. Angiogenesis was also lower in these follicles and this resulted in a reduction in endothelial cell area after the reduction in thecal thickness was taken into account. However, these effects were much less than after inhibition of VEGF by a specific antagonist (Wulff *et al.* 2002) and Chapter 5. Surprisingly, VEGF mRNA expression was similar to controls in these tertiary follicles. Similarly, in the day 5-10 treated group, VEGF mRNA continued to be highly expressed in the granulosa cells of tertiary follicles. This suggests that at this stage of development, follicular VEGF expression is not tightly controlled by the gonadotrophins.

3.4.3.1 Gonadotrophin inhibition

It is reasonable to suggest that follicular development and angiogenesis may be maintained by residual gonadotrophin secretion. Unfortunately LH and FSH could not be measured in the present study because these assays are problematic in the marmoset and there are restrictions in the volume of plasma that can be collected. However, a degree of residual gonadotrophin secretion would be expected to persist after GnRH antagonist treatment with FSH being less suppressed than LH. Evidence from a series of clinical studies using GnRH antagonists in humans would suggest

the concentration of FSH to be suppressed by approximately 33%, with LH concentrations suppressed by approximately 70%, although the concentrations of antagonist used in these studies was in some cases over one hundred times less than those used in this study (Ditkoff *et al.* 1991, Fluker *et al.* 1991, Hall *et al.* 1991, Nelson *et al.* 1995, Oberye *et al.* 1999). While this suppression is sufficient to reduce the ability of mature follicles to produce oestradiol, low levels of LH and FSH remaining may be sufficient to maintain the activity of tertiary follicles. In addition, the possibility of stimulation of VEGF expression by hypoxia, as seen in solid tumours (Shweiki *et al.* 1992) and the endometrium (Sharkey *et al.* 2000), occurring in granulosa cells in tertiary follicles deprived of gonadotrophic support requires to be investigated. However, this phenomenon does not appear to occur in follicular granulosa cells from pre-ovulatory follicles (Martinez-Chequer *et al.* 2003). Paracrine mechanisms could also be acting in these tertiary follicles that may still function during the withdrawal of the gonadotrophins to help maintain VEGF mRNA expression. Recent evidence also suggests that locally produced factors and cytokines such as insulin-like growth factors -1 and -2 play a role in regulating ovarian VEGF mRNA expression in pre-ovulatory follicles (Martinez-Chequer *et al.* 2003). Such mechanisms may also explain the continued VEGF mRNA expression in tertiary follicles in the current study. The fact that there was a decrease in endothelial cell proliferation in these follicles after 10 days GnRH antagonist treatment does suggest that a deleterious effect on the VEGF pathway was occurring, perhaps at the level of translation of VEGF mRNA into biologically active protein or by disruption of the process of transfer of the VEGF protein from the granulosa cells to the thecal layer.

One further point that should be considered is that there is evidence that GnRH can act directly on the ovary through specific, high-affinity receptors in a number of species, including the rat (Knecht *et al.* 1981), pig (Maruo *et al.* 1985) and rabbit (Koos and LeMaire 1985). Some of the effects of GnRH antagonist treatment may therefore be mediated by the direct inhibition of GnRH and not via the gonadotrophins. Studies in the marmoset do suggest that GnRH can directly affect ovarian function although not through the high affinity receptors found in other species (Wickings *et al.* 1990, Fraser *et al.* 1996).

3.4.3.2 Follicle dynamics

Although pre-ovulatory follicles developed during treatment with GnRH antagonist between days 5-10, these follicles were abnormal and cystic in appearance. This indicates a stage of development after which normal levels of gonadotrophic support for follicle expansion is not required, presumably because the dominant follicles have already been selected by the mid follicular phase, as suggested by Gilchrist *et al* (2001). Angiogenesis was decreased in these cyst-like follicles and VEGF mRNA was absent from both the theca and the granulosa cell layers. The observation that follicles grew to pre-ovulatory size but undergo atresia and fail to produce oestradiol indicates a differential level of gonadotrophic stimulation required for proliferation (growth) and cellular differentiation (steroid synthesis) in the dominant follicle. In addition, the marked reduction in aromatase mRNA expression in these dominant but atretic follicles gives further support to the hypothesis that a differential level of gonadotrophic support is required for dominant follicle expansion and differentiation. GnRH antagonist treatment in cynomolgus monkeys has shown that FSH alone is capable of stimulating follicle expansion as determined by ultrasound monitoring, whilst not stimulating oestradiol production due to lack of LH stimulated androgen production (Karnitis *et al.* 1994) although the health of these large follicles was not assessed.

It might have been expected that the rate of follicular atresia would have increased after GnRH antagonist treatment. However, there was a reduction in the number of atretic follicles and an increase in the mean tertiary follicle diameter after both GnRH antagonist treatment schedules. This may be explained by the fact that tertiary follicles not having sufficient FSH stimulation to continue developing appear to be held in an arrested state of development and fail to be cleared from the ovary by apoptosis (Tsafirri and Braw 1984) as they would be in a normal follicular phase ovary approaching ovulation. Also, the reduction in thecal thickness observed in the dominant follicle from the day 5-10 treatment group is probably due to a deficiency in gonadotrophic support for both the theca directly and the granulosa cells stimulating thecal development in a paracrine manner.

3.4.3.3 VEGF mRNA expression

In follicles close to the point of ovulation in control ovaries, a shift in the VEGF mRNA expression pattern was observed, disappearing from the granulosa cells but switching on in an intense punctate pattern in the theca in cells close to the blood vessels. In healthy pre-ovulatory follicles the reduction in VEGF mRNA expression is consistent with the finding that VEGF production is switched off in the granulosa cells of gilts after treatment with human chorionic gonadotrophin (hCG) (Barboni *et al.* 2000). The punctate VEGF mRNA expression observed in the thecal compartment of dominant follicles on follicular day 10 in the marmoset appears to be similar to that first noted in the rhesus monkey ovary (Ravindranath *et al.* 1992). It has also been suggested from observations in the sheep ovary that pericytes in the thecal layer play a key role in the transition from preovulatory follicle to the new corpus luteum (Redmer *et al.* 2001). The VEGF mRNA positive cells observed in the marmoset theca are possibly vascular pericytes. However, these conclusions do not appear to concur with other studies on expression of VEGF mRNA and protein in the rhesus monkey (Christenson and Stouffer 1997, Hazzard *et al.* 1999), where cultures of aspirated granulosa cells exposed to regimes of FSH and LH *in vivo* showed that VEGF expression is stimulated by the gonadotrophins.

The absence of VEGF mRNA expression in the theca of atretic dominant follicles from the day 5-10 GnRH antagonist treatment group indicates that the expression of VEGF in the thecal cells directly prior to ovulation is dependent upon the LH surge and not hypoxia as suggested by Neeman *et al.* (1997). The LH surge is absent in both GnRH antagonist treated groups, while the morphologically intact theca of these follicles would still be responsive to LH. The absence in VEGF mRNA expression in the dominant atretic follicles from the day 5-10 GnRH antagonist treated group also suggests that normal physiological levels of VEGF are not required for dominant follicle expansion between the mid to late follicular period, even though VEGF is thought to play a role as a permeability factor in follicle development (Koos 1995), allowing the movement of fluid into the follicle to expand the antral cavity. Surprisingly, VEGF mRNA expression was also detected in the theca of atretic tertiary follicles in the late follicular control animals. This expression was again absent in GnRH antagonist treated animals and was not present

in atretic follicles from mid-follicular phase ovaries. This suggests that the LH surge triggers the strong expression of VEGF mRNA in the theca of atretic follicles at the time of ovulation, which may play a role in the establishment of extra luteal tissue from the atretic tertiary follicles present at ovulation, a common feature in the marmoset (McAnulty 1994).

3.4.4 Conclusion

In conclusion, the use of a potent GnRH antagonist to block gonadotrophin release did not result in complete inhibition of follicular angiogenesis as had been predicted from the results of their use during the luteal phase of the cycle. This raises the possibility of other factors and paracrine mechanisms regulating VEGF and angiogenesis in the primate ovarian follicle. Also, the dynamic expression pattern of VEGF mRNA evident as the dominant follicles develop towards ovulation poses interesting question as to VEGF's regulation during the periovulatory period.

3.4.5 The next step

To further investigate and extend the findings of this chapter, the next chapter assesses the level of FSH stimulation required to stimulate follicular angiogenesis and follicular steroid hormone synthesis as evidence from this chapter suggests that angiogenic activity should be stimulated at a lower threshold of FSH stimulation than steroid hormone production. This evidence being that whilst angiogenesis was moderately inhibited by GnRH antagonist treatment ovarian hormone production was severely suppressed. Use of primary granulosa cell cultures and measurement of angiogenic (VEGF) and steroidogenic (aromatase) gene induction under graded FSH stimulation will be used to test this hypothesis.

**Chapter 4 The Effect of FSH Stimulation on VEGF
and Aromatase Gene Expression *in vitro***

4.1 Introduction

Data presented in Chapter 3 suggested that there could be a differential level of FSH stimulation required for follicular angiogenesis (VEGF expression) and granulosa cell differentiation (aromatase expression), with follicles becoming vascularised but not undergoing differentiation or producing steroid hormones. This chapter aims to further investigate the level of FSH stimulation required for VEGF and aromatase gene expression in granulosa cells from the marmoset ovarian follicle. The hypothesis being that VEGF production will be stimulated at a lower concentration of FSH than aromatase, i.e. angiogenesis is stimulated in follicles before steroid production is.

In order to investigate this issue, it is necessary to appreciate the mechanisms that regulate FSH stimulated gene expression in the granulosa cells. FSH signals through the FSH receptor, a single 75kDa polypeptide with a 348 residue extracellular domain containing three N-linked glycosylation sites, which is identical in its structural design to the LH receptor (Sprenkel *et al.* 1990). Furthermore, both receptors display 50% sequence similarity in their large extracellular domains and 80% identity across the seven transmembrane segments (Sprenkel *et al.* 1990). The first direct evidence that granulosa cells express the FSH receptor was obtained by autoradiographic localisation of ¹²⁵I-labelled FSH binding to immature rat ovarian sections (Zeleznik *et al.* 1974). The post-receptor signalling systems that relay FSH stimulation to the granulosa cell nucleus rests mainly on adenylyl cyclase, cAMP production, and activation of protein kinase A (PKA) (Richards 1994). At the early stages of follicular growth FSH supports mitotic cell division in the granulosa cells (Hirshfield 1986), whereas during the final stages of preovulatory development, FSH induces the expression of specific genes, leading to major changes in granulosa cell differentiation (Richards 1980). Despite the presence of similar numbers of FSH receptors per cell, granulosa cells of small antral follicles produce less cAMP in response to FSH than do granulosa cells of preovulatory follicles (Richards 1980). This highlights the possibility of different transducers to which the FSH receptor can couple or that the activity of the adenylyl cyclase enzyme is modified in some way between different classes of follicle.

In order to better understand the level of FSH stimulation required for follicular angiogenesis versus differentiation, primary granulosa cell cultures were employed. VEGF mRNA was used as a marker of angiogenesis, VEGF being expressed in the granulosa cell *in vivo*, signalling to the endothelial cells in the theca to promote angiogenesis. Aromatase mRNA was used as a marker of granulosa cell differentiation and maturation. The granulosa cells sensitivity to FSH stimulation with respect to the expression of these two genes was measured after the cells were cultured in the presence of increasing concentrations of FSH *in vitro*.

4.2 Specific methods

4.2.1 Source of marmoset tissue

Subordinate adult female marmosets, in which follicular development and ovulation are known to be suppressed (Abbott *et al.* 1981) were euthanised as detailed in Chapter 2. The ovaries were immediately removed and transferred to serum free medium 199 (Gibco-Europe, Glasgow, UK) containing Earle's salts, 25mM HEPES, 2.68mM L-glutamine, penicillin (50 IU/ml), streptomycin (50µg/ml) and 0.1% BSA and transported back to the laboratory on ice.

4.2.2 Collection and separation of marmoset granulosa cells

Antral follicles, >500µm, were dissected from the ovaries under a stereomicroscope using watchmaker's forceps. Granulosa cells were harvested into fresh culture medium by puncturing individual follicles with a 12-gauge needle. Examples of dissected follicles both before and after granulosa cell removal are shown in **Figure 4.1**. Follicles that displayed gross signs of atresia (either degenerate or absent oocyte) or fragmentation of the granulosa cells were not studied and discarded. The granulosa cells were then pooled, centrifuged for 5 mins at 300g and re-suspended in fresh medium. At this point, cellular viability was assessed by trypan blue exclusion using a haemocytometer.

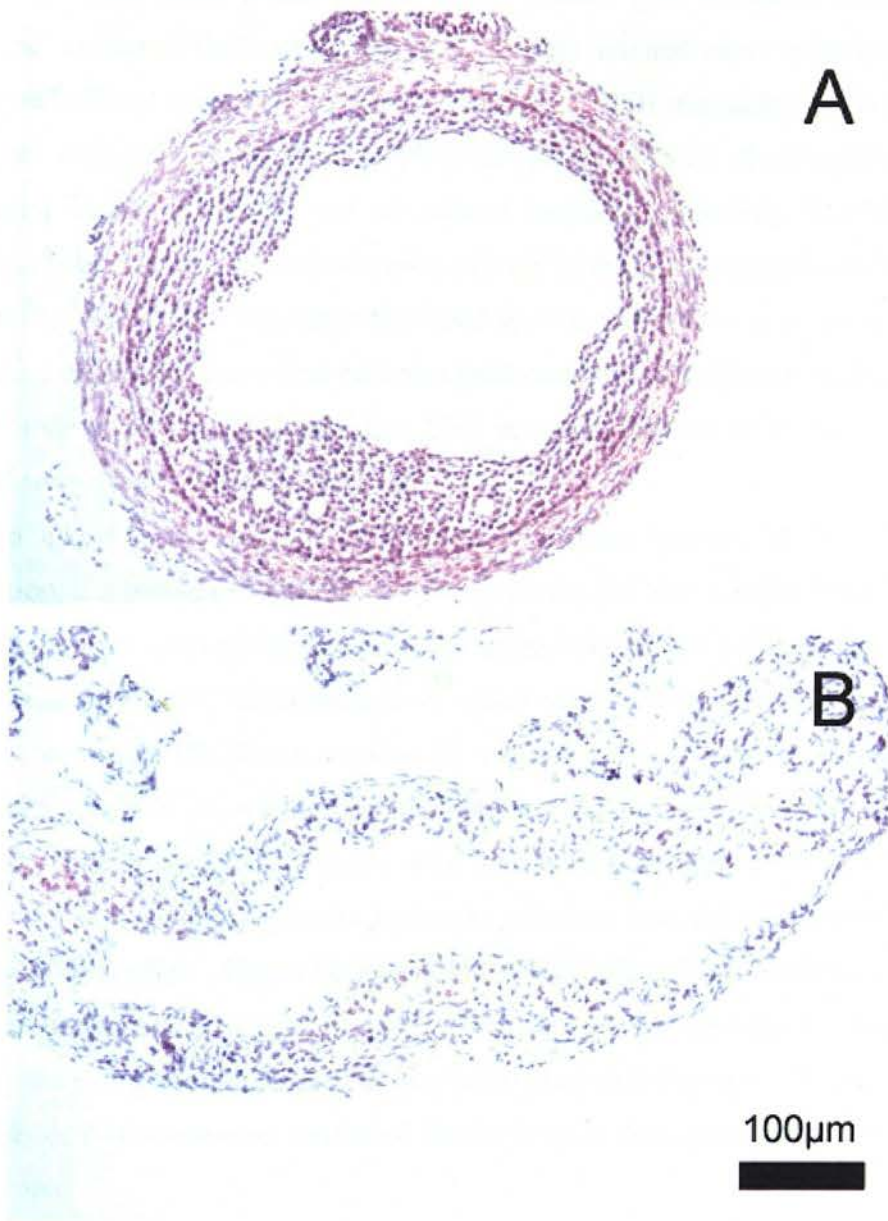


Figure 4.1 H&E of isolated follicles before and after granulosa cell removal.

(A) Example of a tertiary follicle isolated from a subordinate marmoset ovary before removal of the granulosa cells. (B) Example of a follicle once it had been punctured and had the granulosa cells removed.

4.2.3 Cell culture

Twentyfour wells plates (Nunc plastic dishes, Gibco) were precoated with aseptic donor calf serum (Gibco) overnight at 37°C then washed twice with Dulecco's phosphate buffered saline (DPBS, Gibco) before use. FSH was added to the plates at double the required concentration in 250µl of culture medium. Each well was then inoculated with a further 250µl of culture medium containing 70,000 viable granulosa cells, halving the concentration of FSH to the final required concentration in the wells. The dishes were then incubated at 37°C in 5% CO₂ in air for 48 h, in a humidified incubator. Each treatment was performed in quadruplicate. Recombinant FSH (Puregon, Organon, Cambridge, UK) at concentrations of 0, 0.1, 1 and 10 mIU/ml were used to stimulate the cells.

To quantify the amount of aromatase enzyme activity elicited by FSH stimulation, a separate cell culture was set up during the above experiments. These experiments were carried out as above, but using only 25,000 cells per well. After 45 h incubation the cell monolayers were rinsed twice with 1ml pre-warmed (37°C) DPBS containing 0.1% BSA to remove all traces of residual steroid. Five hundred micro litres of fresh pre-warmed culture medium containing (1µM) testosterone (3 wells) or control medium (1 well) was then added to each dish. The added testosterone acts as an exogenous androgen substrate that the aromatase enzyme converts to oestradiol. After a 3h incubation at 37°C the culture medium from each well was collected and stored at -20°C until assayed for oestradiol. Aromatase activity was expressed as the level of oestradiol produced during a 3 h incubation in the presence of testosterone, corrected for the level accumulated in the absence of testosterone.

4.2.4 Total RNA extraction from cells

Total RNA was extracted from cultured cells after 48 h incubation using a Qiagen RNA extraction kit as per the manufacturers instructions (Qiagen, Crawley, West Sussex, UK). After removal and storage of culture medium from wells, the cells were washed in 37°C DPBS to remove any dead, non-adherent cells, (live cells remained adherent to the well surface). The remaining cells were then trypsinised at 37°C for 10mins and pipetted repeatedly to dislodge them from the base of the wells.

Cells from all four replicate wells were pooled into a microfuge tube and centrifuged for 5 mins at 300g. The resulting cell pellet was then washed in PBS. After re-sedimenting, PBS was removed and 350µl of RLT extraction buffer (containing guanidine thiocyanate) added. The cells were then passed through a 12-gauge needle 10 times to aid lysis. Threehundred and fifty microlitres of 70% ethanol was then added and the mix loaded on a Qiagen RNA silica gel column. Washes and elution of RNA was then performed as per the manufacturer instructions, and stored at -70°C until required. One micro litre of the harvested RNA was taken for analysis of quality and concentration using an Agilent 2100 Bioanalyzer (Agilent Technologies Ltd, South Queensferry, UK) in conjunction with RNA nano chips (Agilent)

4.2.5 Reverse transcription

In order that expression of specific genes of interest can be assessed by quantitative real time PCR, cDNA was produced from the extracted RNA. Reverse transcription (RT) was conducted using a Taqman reverse transcription kit (Applied Biosystems, Cheshire, UK), according to manufacturers instructions. A mixture of buffer, dNTPs, random hexamers, RT enzyme, MgCl_2 , RNase free water and RNA (200ng per 20µl reaction mixture) was incubated at room temperature for 10 min before heating to 42°C for 1 h followed by a 10 min incubation at 95°C . For every sample an RT-negative was included. This contained all reagents apart from reverse transcriptase.

4.2.6 Quantitative real time PCR

The LightCycler (Roche, East Sussex, UK) was used to amplify the cDNA in a cyclic process of DNA denaturation, specific primer annealing and primer extension to exponentially increase the primer specified cDNA fragment.

Quantification of real time PCR using this method is based on calculations of amount of PCR produced per cycle, taking into account both the specific gene of interest (VEGF or aromatase) and a control (house keeping) gene (β actin in this case).

The fluorescent dye SYBR Green I, used in these reactions, binds to the minor groove of the DNA double helix. The unbound dye exhibits very little fluorescence,

however, fluorescence is greatly enhanced upon DNA-binding. As the PCR reaction continues, the amount of double stranded DNA the SYBR Green can bind to increases, therefore fluorescence emitted by the SYBR Green also increases. The LightCycler's optics are set to detect the wavelengths of excitation and emission by SYBR Green. A measurement of fluorescence is taken after each individual cycle, therefore the total amount of PCR product in the reaction is measured per cycle. This principle is outlined in **Figure 4.2**.

The fluorescence of each individual reaction is measured at a specific temperature after each cycle. This is to make sure that no non-specific double stranded DNA is detected, which would disturb the results. The measuring temperature for all of these reactions was 82°C. **Figure 4.3** illustrates the melt peaks for both the VEGF and β actin PCR products, both just above 82°C. The melt peaks are the points at which the PCR products dissociate and are no longer detectable. This analysis was also used to determine if more than one PCR product was being produced due to genomic contamination, as different PCR products have different melt peaks. Genomic contamination would produce larger PCR products, as all primers were designed over intron-exon boundaries, with different melt points and thus different melt peaks.

Relative quantification of the specific PCR products is achieved by normalisation to known concentrations of serial diluted standards. For these experiments serially diluted whole ovarian marmoset cDNA was used, and was reverse transcribed in the same reverse transcription experiment as the experimental samples to eliminate any variation in RT efficiency. A standard curve was then generated from the concentration of the standards. The standard curve for each run was constructed using second derivative maximum software incorporated into the LightCycler programme. An example of a standard curve is shown in **Figure 4.4**. The concentration of the PCR products can then be calculated using the standard curves from both the gene of interest and the control gene. All samples were run in duplicate.

4.2.7 Statistical analysis

Data was first tested for normality using a Shapiro-Wilk test. Where more than two

variables were being compared analysis of variance (ANOVA) with a least significant difference (LSD) post-hoc test was used if the data was parametric. Non-parametric data was tested using a Kruskal-Wallis test with Dunn's post-hoc test.

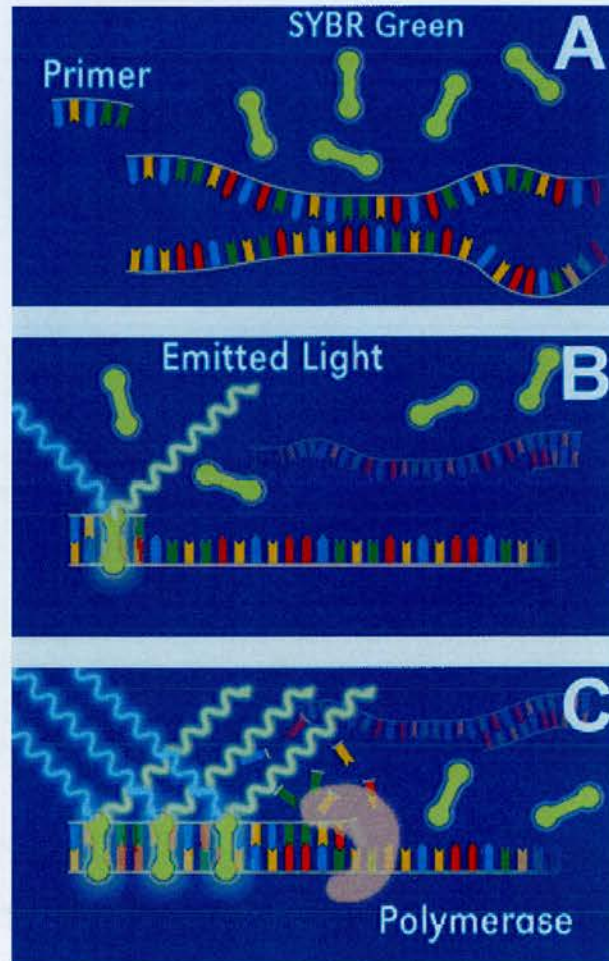


Figure 4.2 The principal behind SYBR Green DNA detection.

(A) At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and the dye. The unbound dye molecules weakly fluoresce. (B) After annealing of the primers, a few dye molecules can bind to the double strand. DNA binding results in a dramatic increase in the amount of fluorescence from the SYBR Green. (C) During elongation, more and more SYBR Green binds to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real-time. Figure adapted from pictures at <http://www.roche-applied-science.com/lightcycler-online/>.

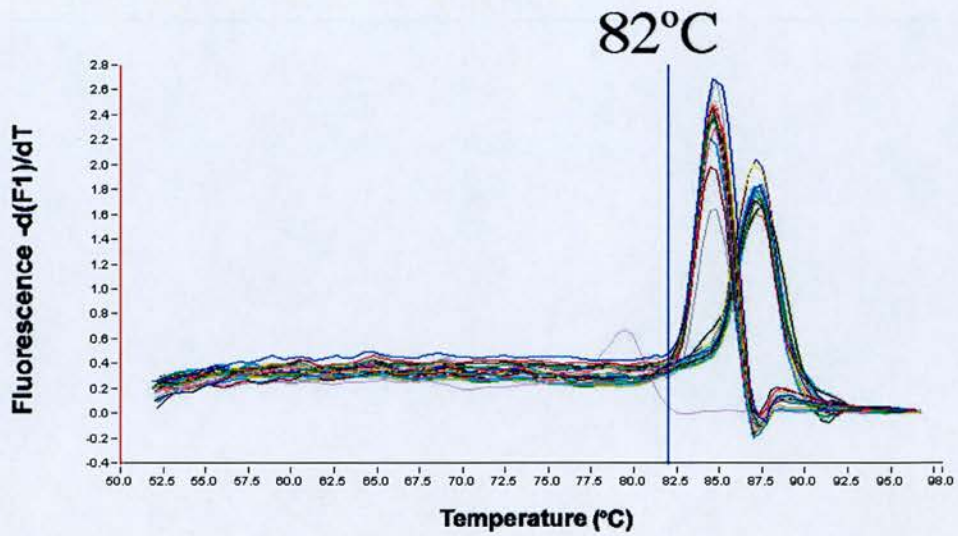


Figure 4.3 Melt peak analysis

Each coloured line on the chart identifies one PCR reaction tube. Two melt peaks are evident in the analysis, one for VEGF and the other for β actin, both of which lie just above 82°C, the optimised measuring temperature for these LightCycler reactions.

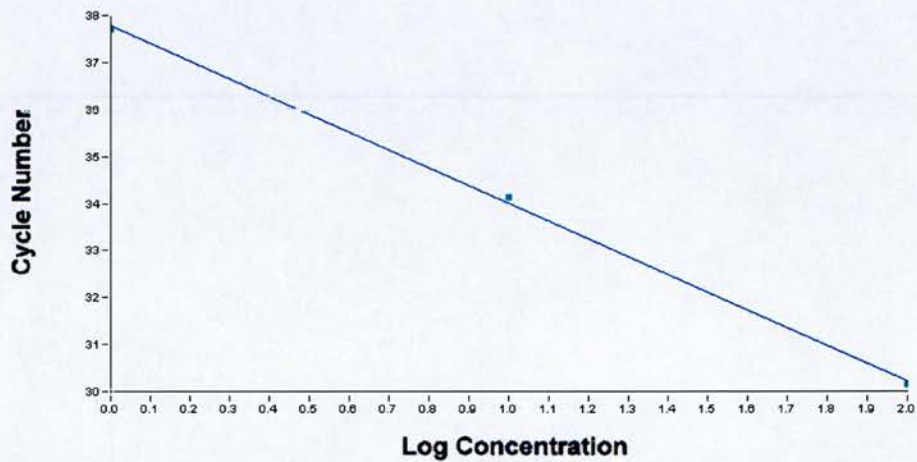


Figure 4.4 Standard curve

This standard curve was constructed using the second derivative maximum software to apply a line of best fit through the points generated by the standards in the reaction.

4.2.8 Probe design and optimisation

For quantification purposes on the LightCycler, the PCR primers designed for use must give a short product, no bigger than ≈ 200 bp. **Figure 4.5** shows the three PCR products generated from the three sets of primer pairs designed for use in these experiments. Primers should also be designed so that they span an intron of the gene of interest. This is to insure that genomic DNA contamination in a sample can be detected due to the fact that it would produce a larger PCR product because of the inclusion of the intron in the PCR product and therefore show up as a different product during melt point analysis. Complementary DNA sequences for target genes were acquired from GenBank and primers designed using Primer 3 software; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Details of the primers and their positions in the genes of interest and the housekeeping gene are given in **Appendix B**. All PCR conditions were optimised on the LightCycler, details of the reaction conditions are given below:

	VEGF	Aromatase
LightCycler SYBR Green (enzyme)	1 μ l	1 μ l
Nuclease free H ₂ O	5.7 μ l	6 μ l
MgCl ₂	1.3 μ l	1 μ l
Primer pairs (5 μ m)	1 μ l	1 μ l
cDNA	1 μ l	1 μ l
Annealing Temperature ($^{\circ}$ C)	59	51
Measure Temperature ($^{\circ}$ C)	82	82
Number of Cycles	45	45

The housekeeping gene (β actin) operated optimally in both reaction conditions.

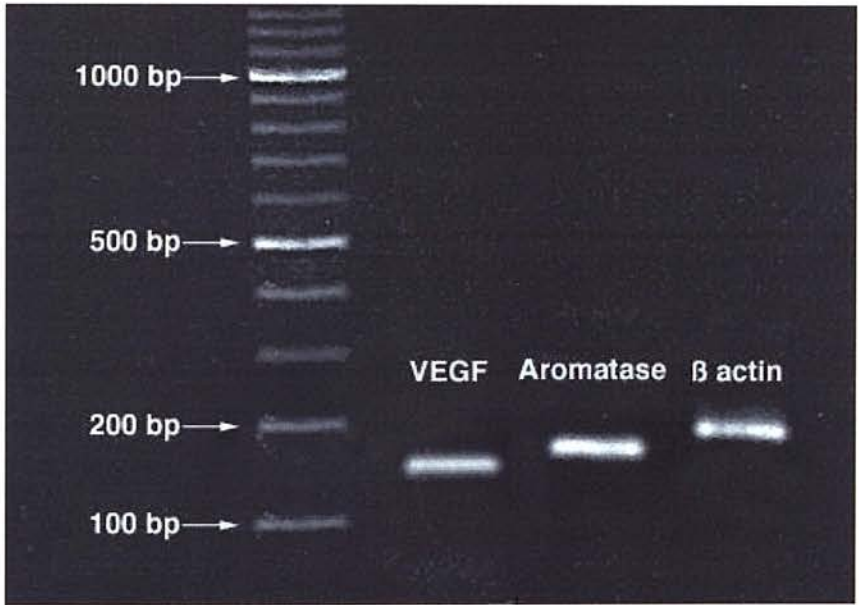


Figure 4.5 PCR products generated using LightCycler primers

The three PCR products are all of between 155 and 202 bp in length. Note the single bands in all three lanes indicating the generation of a single PCR product, i.e. no genomic contamination.

4.2.9 Steroid hormone assay on culture medium

4.2.9.1 Progesterone

Ninety-six well plates were coated in DARS coating antibody and left overnight at room temperature. Plates were then washed 5 times with washing solution (302g Tris, 450g NaCl and 25ml Tween 20, to 2L pure H₂O pH 7.5), diluted 1:25 in pure water before use. Culture medium samples were then diluted 1:50 in assay buffer (17.85g Na₂HPO₄, 7.75g citric acid, 1g gelatin, 0.1g thiomersalate in 1L pure H₂O, pH 6) and 50µl of diluted medium was then added to the plates. In each assay, a low, medium and high progesterone concentration QC was used. A standard curve was prepared from a progesterone standard serially diluted in assay buffer to 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000pg/50µl. Fifty micro litres of each standard in assay buffer was added to the plate. To determine maximum binding (B₀), 100µl of assay buffer was added separately. To determine none-specific binding 150µl of assay buffer was added separately. All samples, standard curve, B₀, NSB and QC's were prepared in duplicate. Fifty micro litres of primary antibody, rabbit anti-progesterone (SAPU), diluted 1:50000 in assay buffer were added to each well (except NSBs). Plates were covered and left overnight at room temperature. After incubation overnight, 50µl per well of labelled ligand (progesterone-11-glucuronide-biotin complex) diluted 1:7000 in assay buffer was added with 2mg/ml ANSA. Plates were then covered and left at room temperature for 3 h. After the incubate was removed, the plates were washed 5 times before 100µl of Streptavidin-Horseradish peroxidase diluted 1:2000 with normal phosphate buffer (1% casein, pH7.4) was added to each well. After 1 h, the plates were again washed 5 times before 200µl per well of substrate OPD (5mM O-Phenylenediamine/0.03% H₂O₂), dissolved in substrate buffer,(10.3g citric acid, 17.79g Na₂HPO₄ dihydrate up to 1L with deionised H₂O, pH 5), was added. After 10-20 mins, the reaction was stopped by adding 50µl of stopping solution (2N sulphuric acid) to each reaction. Plates were read at 490nm on a spectrophotometer plate counter. Inter assay coefficients of variation for low-, medium- and high-level quality controls were 20.5%, 15.6% and 19.8% respectively.

4.2.9.2 Oestradiol

The method for the oestradiol assay was similar to that of the progesterone assay, however the specifics of the assay were: Coating antibody DAGS (Donkey anti-goat serum) not DARS; primary antibody is Sheep anti-oestradiol, diluted 1:750,000 in assay buffer; assay buffer is 11.46g Na₂HPO₄, 2.61g Na₂HPO₄.2H₂O, 9.0g NaCl, 1g gelatin, 0.1g Thiomersalate in 1L deionised H₂O, pH 7.4; oestradiol-biotin complex is used instead of progesterone complex and it is added along with the primary antibody at the end of day two and not at the start of day three; the standard curve was prepared from an oestradiol standard serially diluted in assay buffer to 0.06, 0.12, 0.24, 0.49, 0.98, 1.95, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250 and 500pg/100µl; no medium-level QC used; and the culture medium was added undiluted. Inter assay coefficients of variation for low- and high-level quality controls were 13.3% and 16.9% respectively.

4.3 Results

4.3.1 mRNA

The level of VEGF mRNA increased with increasing levels of FSH stimulation. There was a significant increase in the expression of VEGF mRNA with 1 mIU/ml FSH (0.27 ± 0.04) compared to no stimulation controls (0.09 ± 0.04 , $p=0.016$) (**Figure 4.6**). Aromatase mRNA showed similar trends in expression although there were no statistically significant results (**Figure 4.7**). It is interesting to note that after 10 mIU/ml FSH stimulation there appears to be a trend for the level of mRNA expression of both genes to decrease.

4.3.2 Progesterone assay

Measurement of the amount of progesterone present in the culture medium after the 48 h FSH treatments displayed an increase in the level of progesterone produced as the level of FSH stimulation increased. Statistically significant increases were observed after 1 (145.5 ± 56.4 nmol, $p=0.049$) and 10 (220.5 ± 106.4 nmol, $p=0.014$) FSH mIU/ml stimulation compared to controls (24.0 ± 5.7 nmol) (**Figure 4.8**).

4.3.3 Oestradiol assay

Measurement of the amount of oestradiol produced over a 3 h period, after the granulosa cells had been cultured for 45 h under increasing concentrations of FSH stimulation. FSH stimulation elicited dose dependent increases in the amount of oestradiol produced by the granulosa cells. Stimulation with 1 (167.5 ± 63.4 pmol, $p=0.016$) and 10 (655.2 ± 81.8 pmol, $p<0.001$) mIU FSH caused statistically significant increases in the amount of oestradiol produced compared to controls (13.2 ± 3.1 pmol) (**Figure 4.9**).

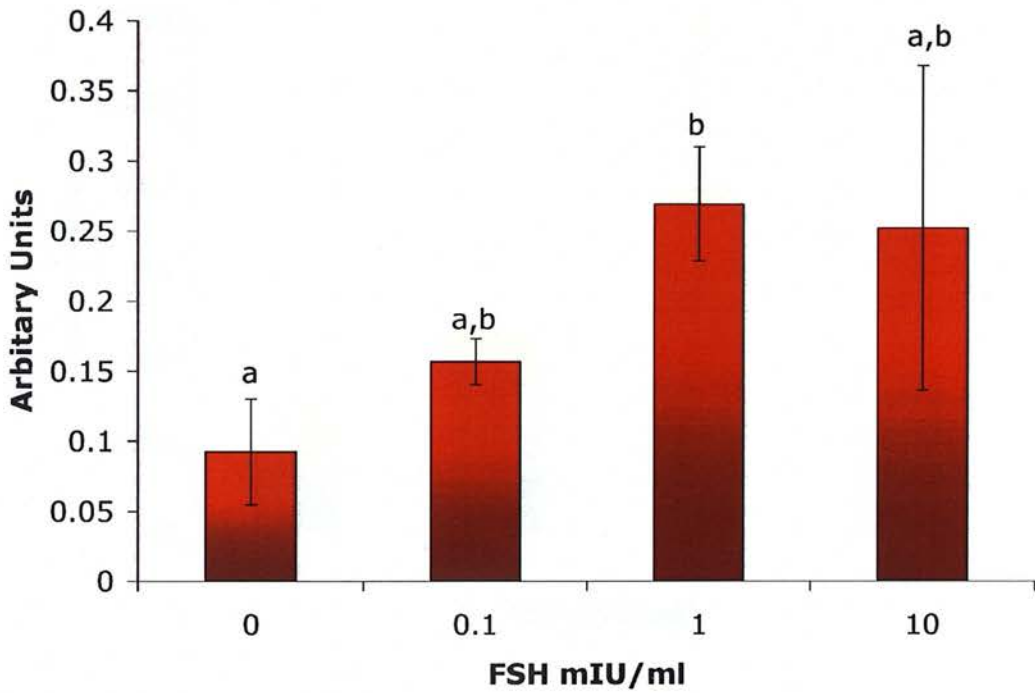


Figure 4.6 VEGF mRNA.

VEGF mRNA expression in granulosa cells stimulated for 48 h by graded concentrations of FSH. A statistically significant increase was observed after 1 mIU/ml (0.27 ± 0.04) FSH stimulation compared to controls (0.09 ± 0.04 , $p=0.016$). Superscripts a and b denote statistical significance. Statistics used: Kruskal-Wallis test with Dunn's post hoc test.

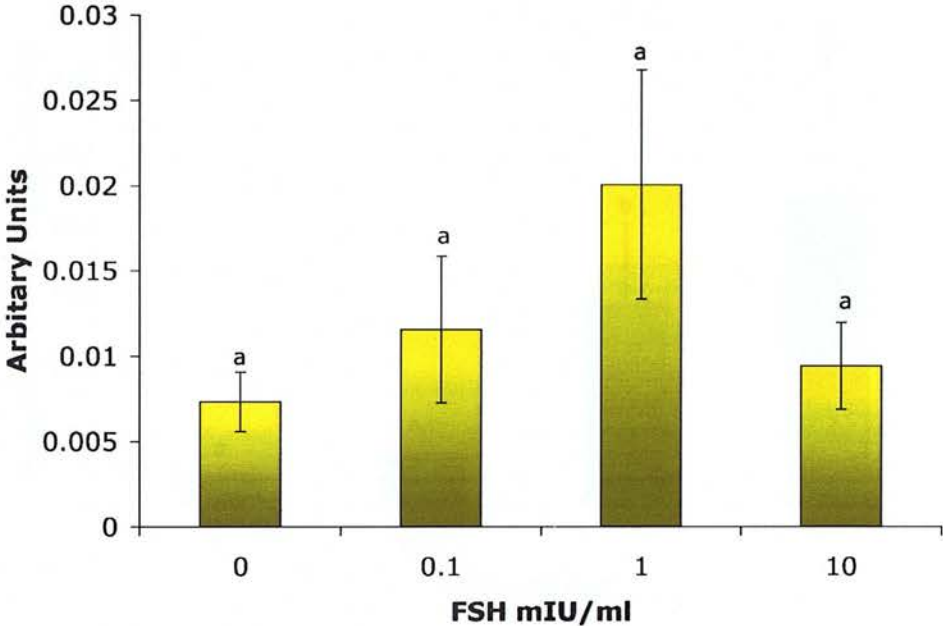


Figure 4.7 Aromatase mRNA.

Aromatase mRNA expression in granulosa cells stimulated for 48 h by graded concentrations of FSH. Superscript a denotes statistical significance. Statistics used: Kruskal-Wallis test with Dunn’s post hoc test.

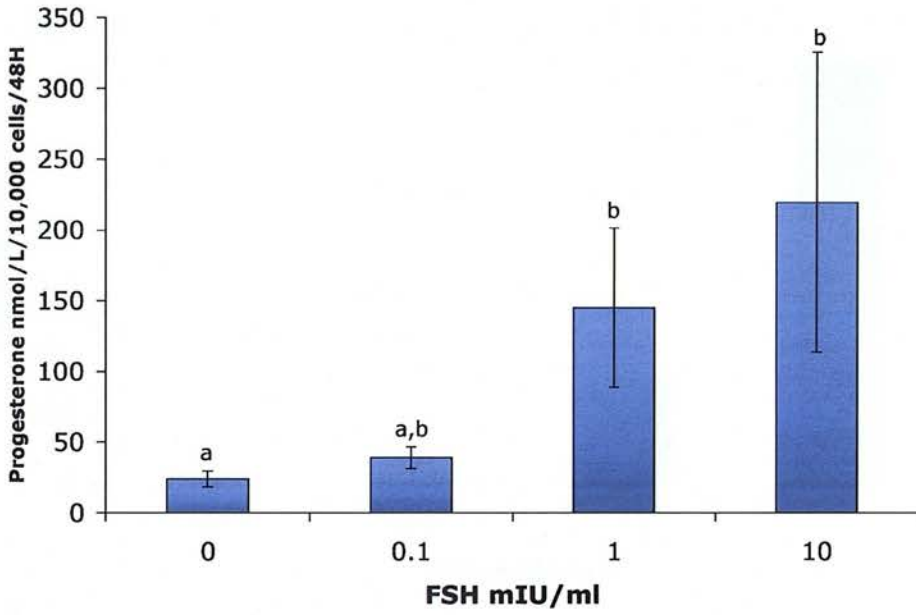


Figure 4.8 Progesterone concentration in culture medium.

Progesterone accumulation in culture medium from 48 h FSH culture experiments. Progesterone concentration showed a dose dependent increase in response to FSH stimulation. Statistically significant increases were observed after both 1 (145.5±56.4nmol, p=0.049) and 10 (220.5±106.4nmol, p=0.014) FSH mIU/ml compared to control (24.0±5.7nmol). Superscripts a and b denote statistical significance. Statistics used: one way ANOVA with an LSD post hoc test.

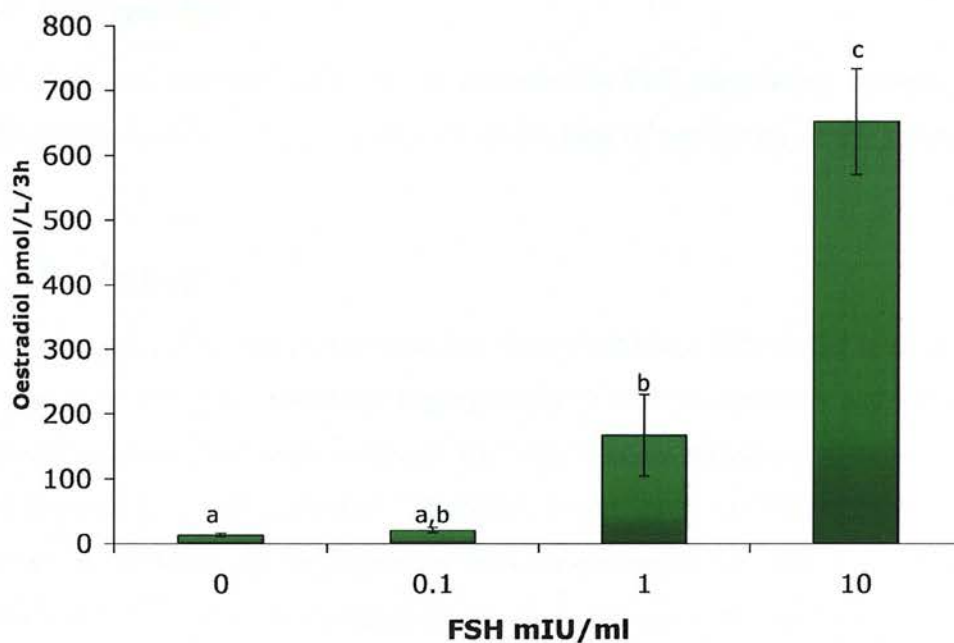


Figure 4.9 Granulosa cell oestradiol production.

The amount of oestradiol produced by the granulosa cells over a 3 h period after 45 h of culture. Oestradiol production elicited a dose dependent response following FSH stimulation. Statistically significant increases were observed after both 1 (167.5 ± 63.4 pmol, $p=0.016$) and 10 (655.2 ± 81.8 pmol, $p<0.001$) mIU/ml FSH stimulation compared to controls (13.2 ± 3.1 pmol). Superscripts a, b and c denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

4.4 Discussion

4.4.1 Key results

-Both VEGF and aromatase mRNA was increased by FSH stimulation, however little can be drawn from these results because of the lack of sensitivity of the techniques used.

4.4.2 Background

Data presented in Chapter 3 suggested that there could be a differential level of FSH stimulation required for follicular angiogenesis (VEGF expression) and granulosa cell differentiation (aromatase expression), with follicles becoming vascularised but producing low levels of oestradiol. Therefore, lower levels of FSH stimulation may be required for follicular angiogenesis than are necessary for follicular oestradiol production. To this end, experiments in this chapter were designed to further investigate the level of FSH stimulation required for follicular angiogenesis and follicular oestradiol production. Granulosa cells isolated from subordinate female marmoset ovaries were cultured in graded concentrations of FSH and the activation of the VEGF and aromatase genes measured.

4.4.3 Analysis

Although the mRNA results presented in this chapter may suggest that there is a differential level of FSH stimulation required for the expression of VEGF mRNA than there is for aromatase mRNA in cultured granulosa cells, much further work is required to confirm this hypothesis. Stimulation with 1 mIU/ml of FSH caused a significant increase in VEGF mRNA compared to no FSH stimulation controls, whereas there was no significant rise in aromatase mRNA expression over the same range of stimulation. Although there were very similar trends in stimulation between the two genes, the aromatase results were not statistically significant. Also, there was a significant increase in the level of aromatase enzyme activity after 1 mIU/ml FSH stimulation compared to controls. The measurement of the amount of oestradiol produced by the cells shows that the cells were stimulated sufficiently to elicit aromatase enzyme activity and therefore activation of the gene. Further

quantification of the amount of VEGF protein secreted by the cells in culture would allow for quantification of the actual amounts of functional gene translation elicited by FSH stimulation and not only the mRNA expression.

4.4.3.1 Granulosa cell sensitivity to FSH

The mechanism by which granulosa cell sensitivity to FSH stimulation is regulated is as yet unclear. One possible explanation is to look at what is known about the promoter regions of the genes. The granulosa cells response to FSH stimulation may involve changes in expression or activation of either *cis*- or *trans*-acting factors that regulate the expression of specific genes. Analysis of the VEGF gene promoter region reveals a single major transcription start that lies near a cluster of potential Sp1 factor-binding sites along with several potential binding sites for the *cis* acting transcription factors AP-1 (a transcription factor formed from a heterodimer of the products of the proto-oncogenes *fos* and *jun*, also known as *c-jun*) and AP-2 (activating enhancer binding protein 2 α), thought to be involved in cAMP regulated gene expression (Tischer *et al.* 1991). VEGF expression has been shown to be regulated by elevated cyclic cAMP concentration, as cAMP levels increase, VEGF mRNA expression also increases (Takagi *et al.* 1996). Forskolin (a naturally occurring diterpene that directly activates adenylyl cyclase) can mimic the effects of FSH stimulation on granulosa cells and studies using bovine granulosa cells stimulated with forskolin have shown that activation of adenylyl cyclase can regulate VEGF gene expression in a time-dependent fashion (Garrido *et al.* 1993). The aromatase promoter contains a *cis*-acting DNA element known to respond to cAMP/A-kinase. This cAMP response element (CRE) is found in the promoter regions of many genes induced by cAMP (Kurten and Richards 1989, Habener 1990, Fitzpatrick and Richards 1993, Fitzpatrick and Richards 1994). Functional studies of the aromatase gene promoter have identified two regions involved in regulating cAMP inducibility in granulosa cells (Fitzpatrick and Richards 1993, Fitzpatrick and Richards 1994).

With respect to this it is of interest to discuss previous work regarding the different effect of FSH and LH on cultured granulosa cells. FSH and LH are both presumed to act predominantly, if not exclusively, via cAMP mediated post-receptor

signalling (Yong *et al.* 1992a, Richards 1994). FSH positively regulates granulosa cell growth and at the same time initiates steroid synthesis, whereas LH has a negative effect on cell growth but causes maximal steroid production (Yong *et al.* 1992b). This is thought to be possible because LH stimulates greater levels of endogenous cAMP than FSH in cultured human granulosa cells (Yong *et al.* 1992a). It is therefore possible that the level of receptor activation may mediate the differential gene expression. There is evidence that individual cAMP responsive genes show varying sensitivity to cAMP (Karin 1989). These responses could be mediated through the multiple isoforms of A-kinase within the ovary (Richards 1994), one of which (RII β) has been shown to be hormonally regulated during folliculogenesis (Kurten *et al.* 1992). Also, inhibitors of A-kinase subunit activity, known as protein kinase inhibitors (PKI) may play a role in this regulation. PKIs have been shown to be regulated by FSH in the testis (Tash *et al.* 1981) and PKI α has been shown to be expressed in the ovary (Van Patten *et al.* 1992).

4.4.3.2 Intra-follicular regulators of FSH sensitivity

In vivo, there are also many other factors that may mediate FSH induced gene expression in the granulosa cells. It is known that intra-follicular androgens and oestrogens can act to enhance the responsiveness of the granulosa cells to FSH (Richards 1980, Hsueh *et al.* 1984, Richards *et al.* 1987) and androgens have been shown to augment oestradiol production in cultured granulosa cells (Harlow *et al.* 1986). This begs the question as to a potential role for the steroid hormones in modulating VEGF gene expression *in vivo*? In addition to the gonadotrophins and the steroids influencing granulosa cell gene expression, granulosa cell development is also modulated by oocyte factors capable of stimulating granulosa cell proliferation but suppressing their differentiation. The nature of these oocyte factors is unclear (Vitt *et al.* 2000), but factors like GDF-9 have been shown to regulate the proliferation and differentiation of rat granulosa cells *in vitro* (Vitt *et al.* 2000). GDF-9 has been shown to stimulate granulosa cell proliferation in both tertiary and dominant follicles but also suppress FSH-induced differentiation of the same cells (Vitt *et al.* 2000). The inhibitory effects of GDF-9 on FSH-induced granulosa cell differentiation is also accompanied by decreases in the FSH-induced cAMP

production, the major route through which FSH signals. Thus, other factors, such as oocyte-derived factors, the granulosa cells would be exposed to *in vivo*, should not be overlooked when examining the regulation of granulosa cell gene expression.

There is also a possibility that VEGF gene expression in granulosa cells is up regulated by hypoxia as is it in other cell and tissue types (Shweiki *et al.* 1992, Sharkey *et al.* 2000). However, the mechanism(s) responsible for hypoxic induction of VEGF has not been clearly delineated and it appears that the same hypoxic response seen in both tumour biology and in the uterus do not appear to operate in differentiated granulosa cells from preovulatory follicles (Martinez-Chequer *et al.* 2003). Thus, it remains unclear whether regulators of VEGF production in these other cell types and tissues, such as hypoxia (Shweiki *et al.* 1992, Sharkey *et al.* 2000) local cytokines and growth factors (Robinson and Stringer 2001) influence the expression of VEGF in the developing ovarian follicle. Notably IGF-I, which plays a key local role in ovarian biology (Poretsky *et al.* 1999), induces VEGF mRNA and protein production by both an increase in the transcriptional rate of the VEGF gene, and the stability of mRNA (Warren *et al.* 1996). IGF-I has also been shown to influence VEGF production in granulosa cells isolated from preovulatory follicles (Martinez-Chequer *et al.* 2003) and other tissues such as the retina (Bermont *et al.* 2001, Hellstrom *et al.* 2001). To further characterise the regulation of gene expression by FSH stimulation, promoter-enhancer regions of genes of interest will be required. Looking at which FSH responsive genes are modulated by cAMP versus cAMP-independent pathways would also be very interesting.

4.4.3.3 Further experiments

In addition to the granulosa cell cultures conducted in this chapter it would also have been interesting to harvest and culture the theca cells collected during these experiments. Stimulation of the theca cells with either graded concentrations of VEGF or possibly the medium the granulosa cells were cultured in would allow for the analysis of thecal cell response to the indirect FSH stimulation they would be subjected to *in vivo*. Although VEGF receptors are not localised to the theca cells by *in situ* hybridisation there is the possibility that unknown mechanisms may transduce VEGF signalling to the theca directly, as evidenced by the large reduction in theca

cells proliferation when blocking VEGF action (Wulff *et al.* 2002). Although the theca cell response would have to be separated from the endothelial cell response as there would undoubtedly be endothelial cells associated with the theca in follicles at this stage of development. Stimulation of the theca cells with the medium the granulosa cells were cultured in would also be very interesting, although FSH would probably still be present in the medium that could possible mediate an effect on its own biasing the results. Human umbilical vein endothelial cell response to the culture medium would also be interesting to measure extent of angiogenesis elicited by the culture medium from the graded FSH stimulate culture medium, functioning as a bio assay.

4.4.3.4 Limitations of study

The mRNA results may be in agreement with the hypothesis that the induction of VEGF gene expression is more sensitive to FSH stimulation than the aromatase gene. However, the differences between the aromatase mRNA and the aromatase activity results suggest that there are caveats associated with the results. Steroid hormone assays being generally more robust than mRNA data, the discrepancy in results probably resides in the mRNA data. The stability of the mRNAs of the respective genes may influence the data, one mRNA species may be more stable than the other, thus skewing the results. The inclusion of a time-course experiment to determine the point at which maximal mRNA production was elicited in the cells would also identify if there was indeed a period before 48h at which mRNA levels reached their maximal expression and if they had declined by 48h. Forty eight hours was chosen for these experiments based on previous work in primary cell cultures of both marmoset (Harlow *et al.* 1986, Harlow *et al.* 1988) and rat (Harlow *et al.* 2002) granulosa cells. Furthermore, the technique used to measure the abundance of the two genes may not be sensitive enough to quantify changes over the scale that the genes were expressed at in these experiments.

The amount of progesterone produced by the cells in these experiments was measured to assess if the cells were healthy and that the doses of FSH they were exposed to were physiologically relevant. The progesterone results indicate that the range of FSH concentrations used was within physiological parameters, as a dose

dependent response to FSH stimulation would be expected. However, when comparing the oestradiol and the progesterone results it must be remembered that there was no progesterone precursor (low density lipoprotein) added to the culture medium as there was for oestradiol (testosterone) and this may bias the results in favour of oestradiol production.

4.4.4 Conclusion

In conclusion it would appear that there are very complex mechanisms regulating FSH induced gene expression in the developing granulosa cells that require a great deal more investigation in order that the exact mechanisms by which angiogenesis is regulated in the developing ovarian follicle are delineated. This preliminary experiment suggests that VEGF may be more sensitive to FSH stimulation than aromatase.

4.4.5 The next step

This chapter has looked at the level of FSH stimulation required induce both angiogenesis and steroid hormone production in the ovarian follicle. The next chapter moves away from the regulation of the VEGF gene and hence angiogenesis by the gonadotrophins to the direct inhibition of VEGF, using specific antagonist designed to block VEGF action *in vivo*. The next chapter has been designed to assess the effects of inhibition of angiogenesis through the direct blocking of VEGF action on follicle selection, development, ovulation and establishment of the CL.

**Chapter 5 Effect of VEGF Trap R1R2 Treatment on
Follicular Angiogenesis and Development**

5.1 Introduction

As discussed in Chapter 1, the ovary is one of few organs in the body to undergo serial angiogenesis, which is intimately involved in ovarian follicle development (Fraser and Wulff 2001, Wulff *et al.* 2001b, Zimmermann *et al.* 2001b, Wulff *et al.* 2002, Zimmermann *et al.* 2003). The principal angiogenic factor with an established role in follicular angiogenesis is VEGF (Wulff *et al.* 2001b, Wulff *et al.* 2002). VEGF mRNA and protein have been localised in the ovary to the granulosa cells of the developing follicles and the theca of preovulatory follicles in a number of species (Phillips *et al.* 1990, Ravindranath *et al.* 1992, Shweiki *et al.* 1993), including the marmoset as shown in Chapter 3 (Taylor *et al.* 2004).

It is well established that angiogenesis plays a key role in normal luteal function, with inhibition of VEGF severely disrupting luteal angiogenesis and the maintenance of the established corpus luteum (Ferrara *et al.* 1998, Fraser *et al.* 2000, Dickson *et al.* 2001, Wulff *et al.* 2001c). Previous studies have also established the importance of angiogenesis in follicular development (Wulff *et al.* 2001b, Wulff *et al.* 2002). Inhibition of VEGF *in vivo* in the marmoset monkey has been shown to severely suppress both thecal vascularisation and follicular development (Wulff *et al.* 2001b, Wulff *et al.* 2002) and to inhibit the follicular phase of the cycle in both macaques (Zimmermann *et al.* 2001b) and mice (Zimmermann *et al.* 2003). This study aims to extend previous experiments using the VEGF trap and assess the effects of inhibition of VEGF action, and hence angiogenesis, on follicular development from both the time of follicle selection and dominant follicle maturation. The hypothesis being tested was that inhibition of VEGF would result in inhibition of dominant follicle maturation and ovulation and that VEGF is essential for increasing permeability and the growth of the selected follicles. The VEGF trap has been designed to inhibit VEGF by preventing it from binding to its receptors. It has been previously used to inhibit follicular development in the marmoset (Wulff *et al.* 2002) and has also been used to inhibit the ovarian cycle in macaques (Fraser *et al.* 2005b).

The effects of VEGF trap treatment on follicular angiogenesis were examined using BrdU immunocytochemistry as a proliferation marker, CD31 as an endothelial

specific cell marker and dual staining to distinguish proliferating endothelial cells. Effects of treatment on expression of VEGF and its receptors mRNA was determined by *in situ* hybridisation.

5.2 Specific methods

Tissue collection, immunocytochemistry and *in situ* hybridisation in this chapter were conducted as described in Chapter 2.

5.2.1 The VEGF trap R1R2

The VEGF trap R1R2 used in this study is a recombinant chimeric protein comprising the second Ig domain of VEGFR1 and the third Ig domain of VEGFR2 expressed in sequence with the Fc portion of human IgG. The incorporation of the Fc domain results in homodimerisation of the recombinant protein, creating a high affinity VEGF trap (Holash *et al.* 2002, Wulff *et al.* 2002). The VEGF trap was expressed in Chinese hamster ovary cells and was purified by protein-A affinity chromatography followed by size-exclusion chromatography. The VEGF trap binds all isoforms of both VEGF-A, VEGF-B and placental growth factor (PlGF) (S. J. Wiegand, personal communication). The specificity of VEGF binding to the VEGF trap was determined by Biacore (Uppsala, Sweden) (Wulff *et al.* 2002). The VEGF trap was a gift from Dr Stanley. J. Wiegand (Regeneron Pharmaceuticals). A schematic of the VEGF trap is provided in **Figure 5.1**.

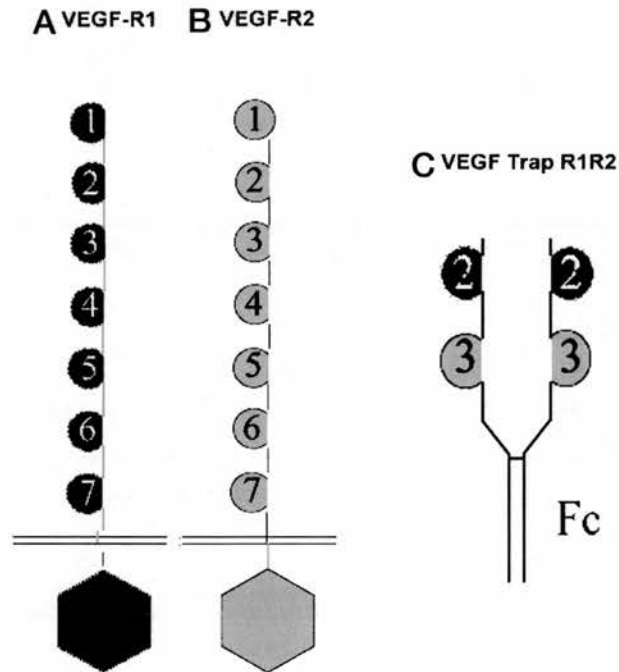


Figure 5.1 Structure of VEGF receptors and the VEGF trap.

(A) VEGFR1 and (B) VEGFR2 both contain seven extracellular domains (Ferrara and Davis-Smyth 1997), which differ between the two receptors. Four of these extracellular domains make up the VEGF binding portion of VEGF trap (C). Figure adapted from Wulff *et al* (2002).

5.2.2 Animals and treatment

Both animals receiving the VEGF trap and control animals were given 1µg of prostaglandin F_{2α} analogue intramuscularly on days 12-15 of the 21 day luteal phase of the pre-treatment cycle to induce luteolysis marking follicular day 0. Using this technique to induce luteolysis creates a 10-day follicular phase with control animals ovulating on follicular day 10. Early follicular (n=3), midfollicular (n=4) and late follicular controls (n=7) were used for these experiments. Three treatment regimes were used for these experiments. Each regime consisted of one 25mg/kg dose of the VEGF trap administered subcutaneously at the start of each treatment regime. Treatment regime 1 was for 10 days starting from follicular day 0 (n=3). Treatment regime 2 was for 5 days from follicular day 5 (n=5). Treatment regime 3 was for 10 days starting from follicular day 5 (n=7). The treatment regimes are illustrated in **Figure 5.2**. At the end of each treatment schedule ovaries were collected and fixed as detailed in Chapter 2.

The dose of 25mg/kg was chosen as previous studies by Wulff *et al* (2002) had used 25mg/kg every other day throughout the follicular phase to severely suppress angiogenesis. Further to this, recent work by Fraser *et al* (2005b) using the VEGF trap in the Macaque has shown that ovarian function is suppressed for more than 40 days with a single 12.5mg/kg dose and a dose of 0.25mg/kg in the mid follicular phase of the cycle, one hundredth that used in this study, suppresses a rise in progesterone for approximately 20 days. Thus, the 25mg/kg dose used in this study was designed to severely and permanently suppress angiogenesis and ovarian function throughout the short study period, a maximum of 10 days.

5.2.3 *In situ* hybridisation

In situ hybridisation for VEGF, VEGFR1, VEGFR2 and aromatase was conducted using the probes and protocols detailed in Chapter 2. *In situ* hybridisation experiments were quantified using image analysis software at x400 magnification.

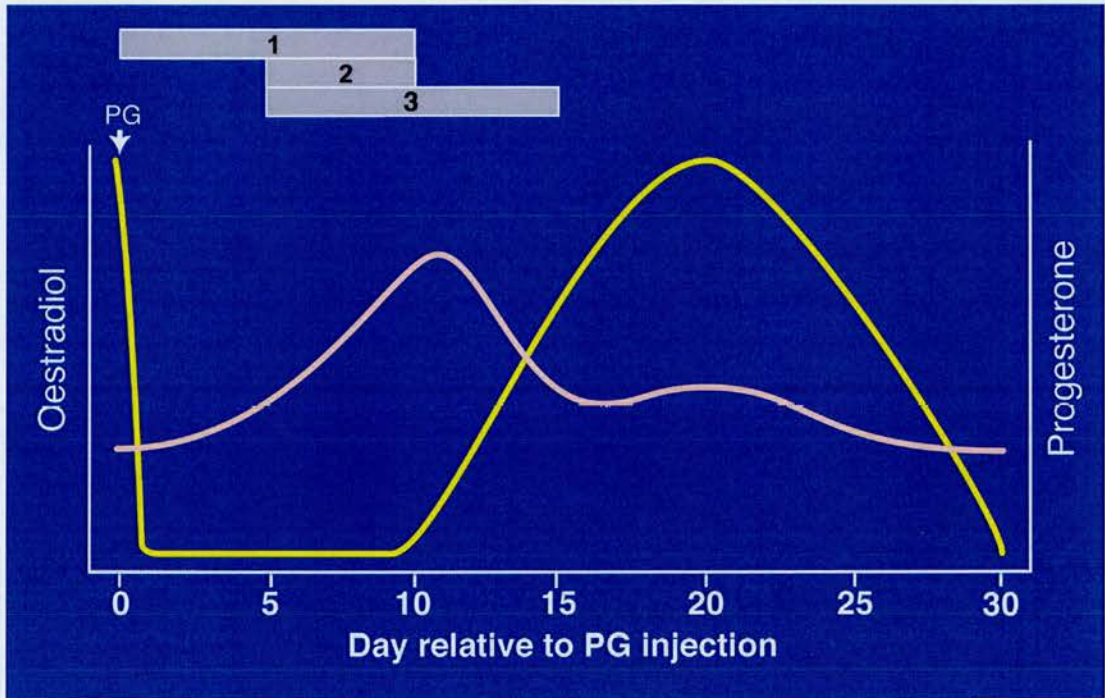


Figure 5.2 Treatment schedules for VEGF trap experiments.

Prostaglandin (PG) was administered in the late luteal phase of the pre-treatment cycle to induce luteolysis, marking follicular day 0. Treatment regime 1 was for 10 days starting from follicular day 0. Treatment regime 2 was for 5 days from follicular day 5. Treatment regime 3 was for 10 days starting from follicular day 5. Treatment regimes are denoted as grey bars in the figure. Note the sharp decline in progesterone associated with induction of luteolysis, the ovulatory progesterone rise and the follicular phase increase in oestradiol that would be observed in control animals.

5.3 Results

5.3.1 Hormonal changes

All marmosets responded to PG with a rapid fall in plasma progesterone concentration due to the induction of luteolysis. Terminal plasma progesterone concentrations for regimes 1 and 2 are shown in **Figure 5.3A**. Significant inhibition of plasma progesterone is observed after both days 0-10 ($20.8 \pm 2.3 \text{ nmol}$, $p=0.024$) and 5-10 ($21.9 \pm 1.4 \text{ nmol}$, $p=0.042$) VEGF trap treatment compared to late follicular controls ($38.4 \pm 6.2 \text{ nmol}$). Treatment with VEGF trap for days (5-15 $21.2 \pm 3.1 \text{ nmol}$, $p=0.002$) caused a significant reduction in plasma progesterone levels compared to early luteal control animals ($136 \pm 20.5 \text{ nmol}$), **Figure 5.3B**.

Terminal plasma oestradiol concentrations were also significantly suppressed by both VEGF trap treatment regimens. Days 0-10 (252 ± 21.3 , $p=0.010$) and days 5-10 (614 ± 300.0 , $p=0.024$) compared to late follicular controls (1871.3 ± 392.4), **Figure 5.4**.

5.3.2 Effect of treatment on ovary weight

Treatment with VEGF trap for follicular days 0-10 significantly reduced paired ovary weight ($84.3 \pm 2.7 \text{ mg}$) compared to late follicular controls ($165.0 \pm 22.2 \text{ mg}$, $p=0.032$). A non-significant reduction was observed after treatment with the trap for 5-10 days ($124.8 \pm 18.8 \text{ mg}$), **Figure 5.5A**. Treatment with the VEGF trap for days 5-15 ($91.9 \pm 10.4 \text{ mg}$) caused a significant reduction in paired ovary weight compared to early luteal controls ($225.3 \pm 25.9 \text{ mg}$, $p=0.001$), **Figure 5.5B**.

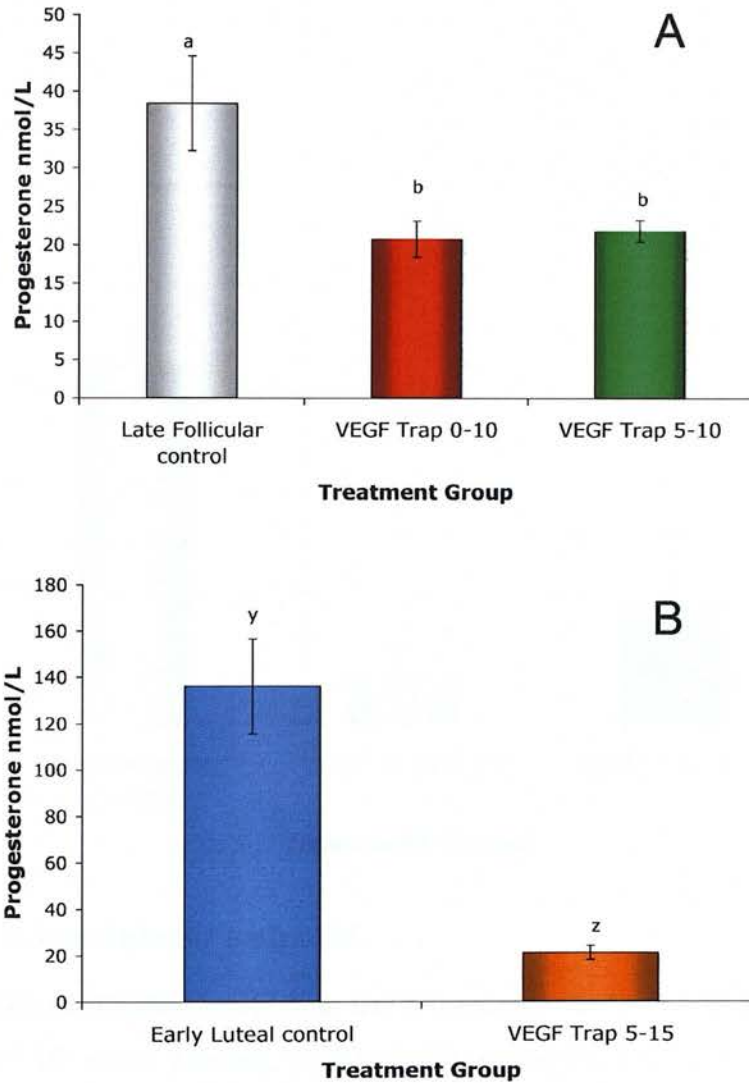


Figure 5.3 Terminal plasma progesterone.

Terminal plasma progesterone levels from (A) late follicular control (38.4 ± 6.2 nmol) and VEGF trap days 0-10 (20.8 ± 2.3 nmol, $p=0.024$) and days 5-10 (21.9 ± 1.4 nmol, $p=0.042$) treated animals and (B) Early luteal control (136.0 ± 20.5 nmol) and VEGF trap day 5-15 (21.2 ± 3.1 nmol) treated animals that had ovulated ($p=0.002$). Values are means \pm sem. Superscripts a, b, y and z denote statistical significance. Statistics used: ANOVA with an LSD post hoc test in panel A and two-tailed unpaired t test in panel B. Statistics used: one way ANOVA with an LSD post hoc test in panel A and a two-tailed unpaired t test in panel B.

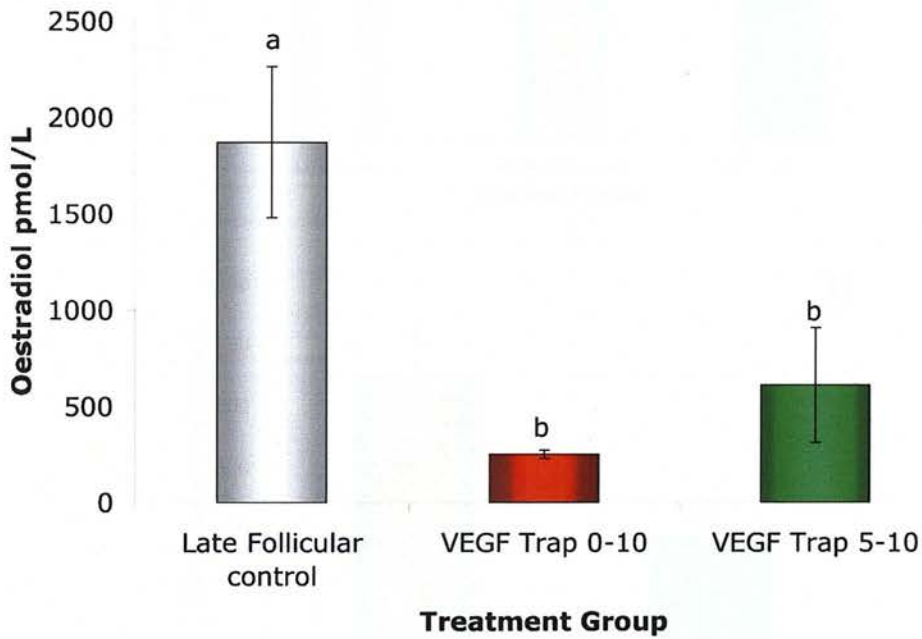


Figure 5.4 Terminal plasma oestradiol.

Terminal plasma oestradiol levels from late follicular control and VEGF trap days 0-10 and days 5-10 treated animals. Significant reduction were seen after both VEGF trap treatments days 0-10 (252.3 ± 21.3 pmol/L, $p=0.010$) and days 5-10 (614.0 ± 300.0 pmol/L, $p=0.024$) compared to late follicular control values (1871.3 ± 392.4 pmol/L). Statistics used: ANOVA with an LSD post hoc test.

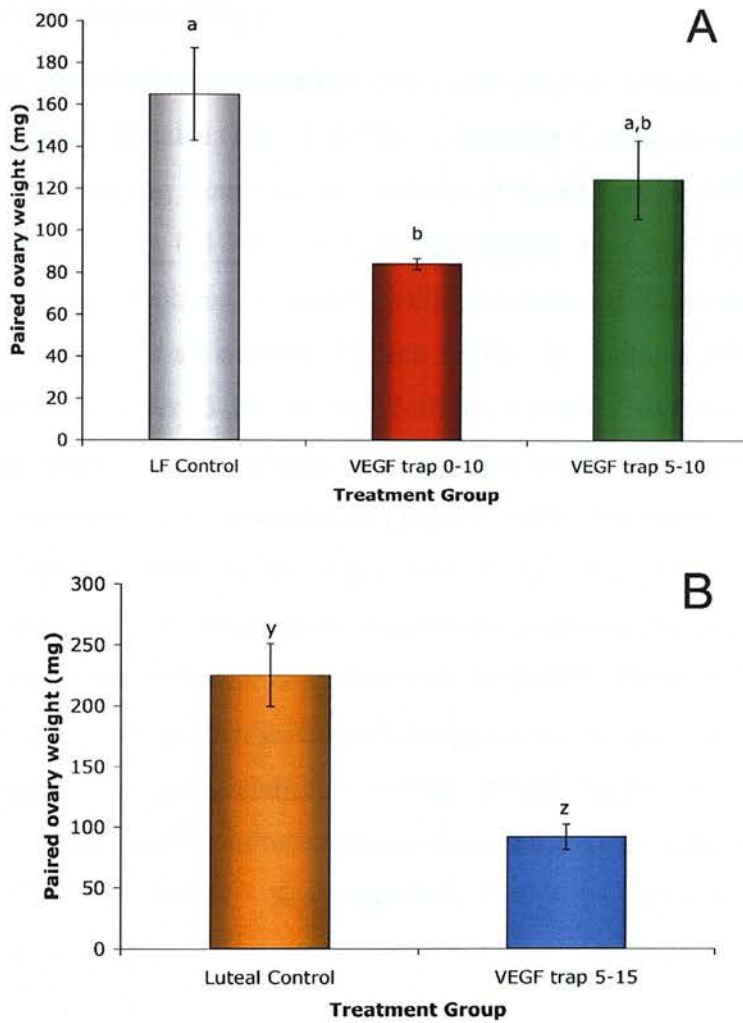


Figure 5.5 Paired ovary weights.

(A) Mean paired ovary weight of late follicular control ovaries and ovaries from animals treated with VEGF trap for either follicular days 0-10 or 5-10. A significant reduction in weight was observed after treatment for days 0-10 (84.3 ± 2.7 mg, $p=0.032$) compared to late follicular controls (165.0 ± 22.2 mg). (B) Mean paired ovary weight of early luteal control and days 5-15 VEGF trap treated animals. A significant reduction was observed after days 5-15 VEGF trap treatment (91.9 ± 10.4 mg, $p=0.001$) compared to luteal controls (225.3 ± 25.9 mg). Superscripts a, b, y and z denote statistical significance. Statistics used: one way ANOVA with an LSD post hoc test in panel A and a two-tailed unpaired t test in panel B.

5.3.3 Ovarian morphology

Late follicular control ovaries contained either preovulatory follicles (**Figure 5.6A**) or recently ovulated follicles (**Figure 5.6B**). Luteal day 5 control ovaries contained new luteal tissue forming from ovulated follicles (**Figure 5.6C**). After VEGF trap treatment between days 0-10 of the follicular phase, there was no evidence of dominant follicle development or freshly ovulated follicles although healthy tertiary follicles were present in all ovaries (**Figure 5.6D**). In contrast, after VEGF trap treatment between days 5-10 of the follicular phase, ovaries displayed a heterogeneous response to treatment ranging from healthy preovulatory follicles (**Figure 5.6E**) to fresh points of ovulation (**Figure 5.6F**). The mean diameter of the dominant follicles present in the days 5-10 VEGF trap treated ovaries was significantly reduced ($2367.80\mu\text{m}$, $n=4$) compared to dominant follicles ($3232.90\mu\text{m}$, $n=4$) present in the late follicular control ovaries ($p=0.017$), **Figure 5.7**. VEGF trap treatment for days 5-15 also resulted in heterogeneous morphology with varying degrees of luteinisation and ovulation occurring. A new “micro CL” (**Figure 5.6G**) and a healthy non-ovulated dominant follicle (**Figure 5.6H**) from the days 5-15 trap treatment groups are shown. See **Appendix D** for descriptions of the gross morphology of the ovaries.

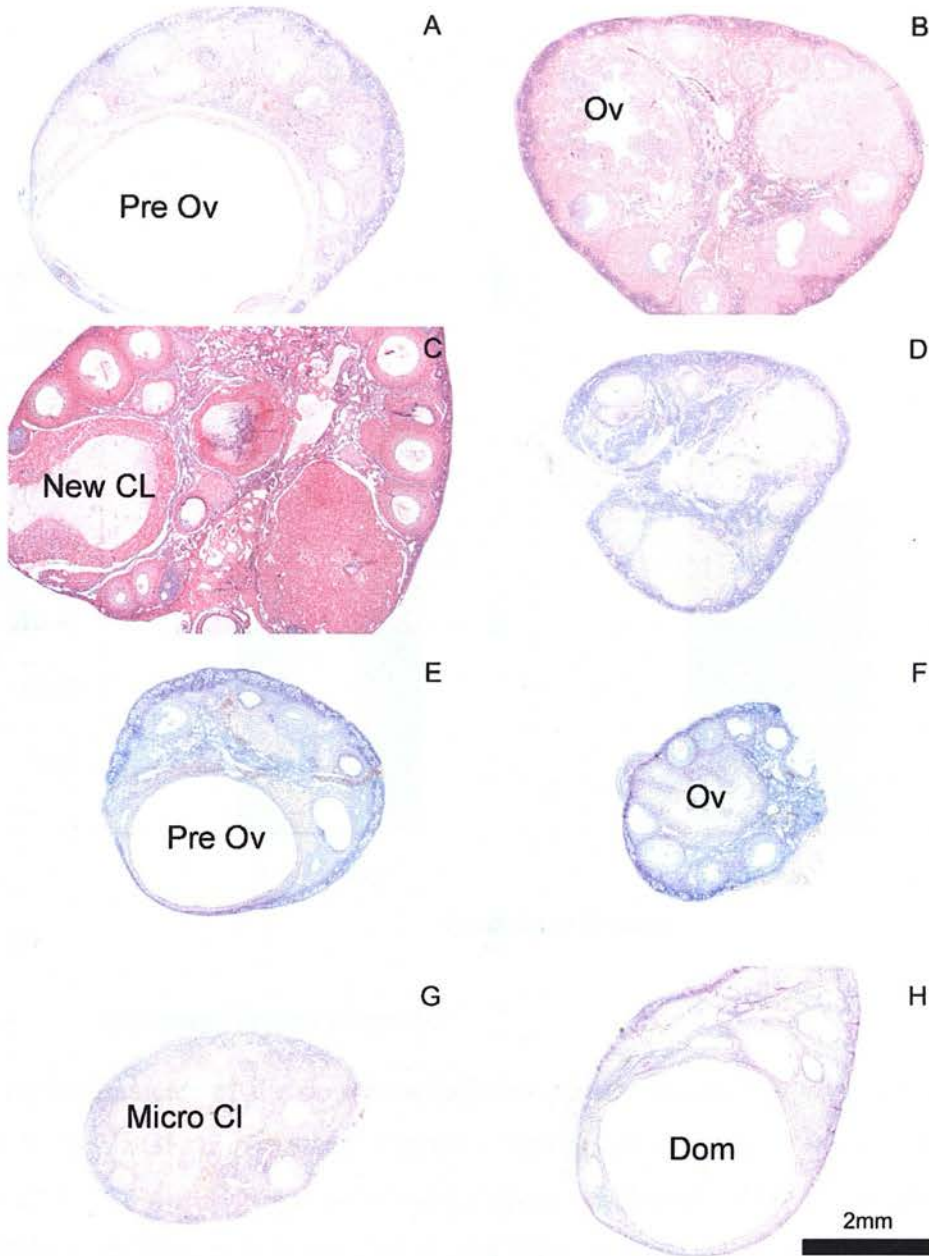


Figure 5.6 Ovarian morphology.

Haematoxylin and eosin stained sections of control late follicular phase (A&B), early luteal control (C), VEGF trap days 0-10 (D), days 5-10 (E&F) and days 5-15 (G&H) treated ovaries. Note the reduction in size of the ovaries after all of the treatment regimes. Pre Ov = Preovulatory follicle, Ov = Ovulated follicle, New CL = New corpus luteum, Micro CL = small, avascular formed after ovulation in VEGF trap treated animals, Dom = dominant but unovulated follicle found in VEGF trap treated animals.

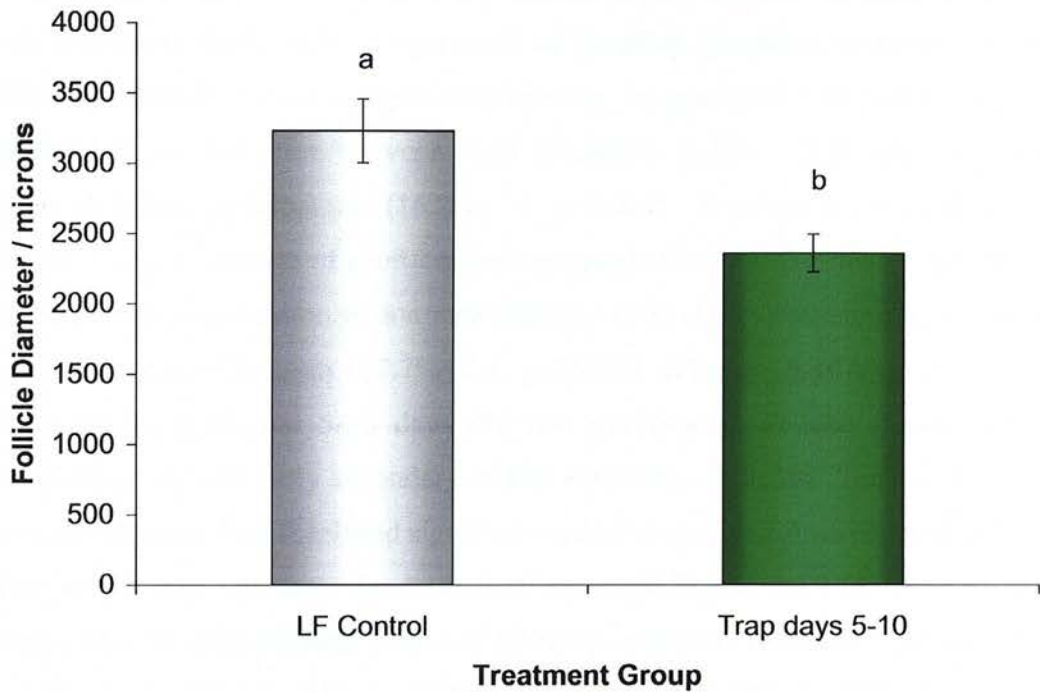


Figure 5.7 Dominant follicle diameter

The mean diameter of the dominant follicles present in late follicular control and days 5-10 VEGF trap treated ovaries. The mean dominant follicle diameter ($3232.90 \pm 226.4 \mu\text{m}$ $n=4$) was significantly reduced after trap treatment ($2367.80 \pm 135.3 \mu\text{m}$ $n=4$, $p=0.017$). Eight follicles from 4 animals were assessed from both control and treated ovaries. Values shown are means \pm sem. Superscripts a and b denote statistical significance. Statistics used: two-tailed unpaired t test.

5.3.4 Effect of VEGF trap treatment on cellular proliferation

Tertiary and dominant follicles from ovaries from late follicular control and VEGF trap treated animals are shown in (Figure 5.8, 5.9) respectively. The total number of proliferating (BrdU positive) cells was counted as a percentage of the total number of cells present in the follicle compartment in question, granulosa or theca. In late follicular control ovaries a significant increase in granulosa cell proliferation is observed in the transition between early secondary ($6.6\% \pm 1.5$) to late secondary stages of follicle development ($16.8\% \pm 1.8$, $p=0.007$). Between late secondary and tertiary follicles the rate of proliferation remained relatively constant. The rate of granulosa cell proliferation in dominant follicles ($7.8\% \pm 2.2$) decreased significantly from tertiary proliferation ($17.7\% \pm 2.4$, $p=0.029$), (Figure 5.10A). VEGF trap treatment for 0-10 and 5-10 days did not significantly reduce granulosa cell proliferation in both early secondary or late secondary follicles. Tertiary follicles, shown in (Figure 5.8), displayed significant reductions in granulosa cell proliferation after VEGF trap treatment for both 0-10 ($2.1\% \pm 0.2$) and 5-10 ($4.3\% \pm 0.4$) days compared to tertiary follicles from late follicular controls ($17.7\% \pm 2.4$, $p<0.001$ and $p=0.001$ respectively), (Figure 5.10B). Sections of the follicle wall of dominant follicles from late follicular control and VEGF trap 5-10 day treated ovaries is shown in (Figure 5.9). VEGF trap treatment between days 5-10 resulted in no significant reduction in granulosa cell proliferation in dominant follicles. In late follicular control ovaries, theca cell proliferation showed a significant increase in the rate of proliferation from early secondary ($0.7\% \pm 0.3$) to late secondary stages of follicle development ($8.1\% \pm 0.7$, $p<0.001$). A further increase was observed between late secondary ($8.1\% \pm 0.7$) and tertiary follicles ($12.5\% \pm 1.8$, $p=0.044$) and a decrease in the rate of theca cell proliferation was observed between tertiary ($12.5\% \pm 1.8$) to dominant follicles ($5.0\% \pm 1.0$, $p=0.002$), (Figure 5.11A). Both VEGF trap treatment for 0-10 and 5-10 days had marked inhibitory effects on the rate of thecal proliferation in all follicles from the late secondary stage of development onwards. Late secondary follicles showed significant reductions in cellular proliferation after VEGF trap treatment between days 0-10 ($8.1\% \pm 0.7$ to $1.9\% \pm 0.2$, $p=0.001$) and 5-10 ($8.1\% \pm 0.7$ to $2.1\% \pm 0.8$, $p<0.001$). Tertiary follicles showed the same pattern of

suppression after 0-10 ($12.5\% \pm 1.8$ to $0.1\% \pm 0.02$, $p < 0.001$) and 5-10 days ($12.5\% \pm 1.8$ to $0.5\% \pm 0.3$, $p < 0.001$). This pattern continued in the dominant preovulatory follicles ($5.0\% \pm 1.0$) seen in the late follicular control ovaries and the ovaries from marmosets treated for days 5-10 ($0.2\% \pm 0.1$, $p = 0.002$) (**Figure 5.11B**).

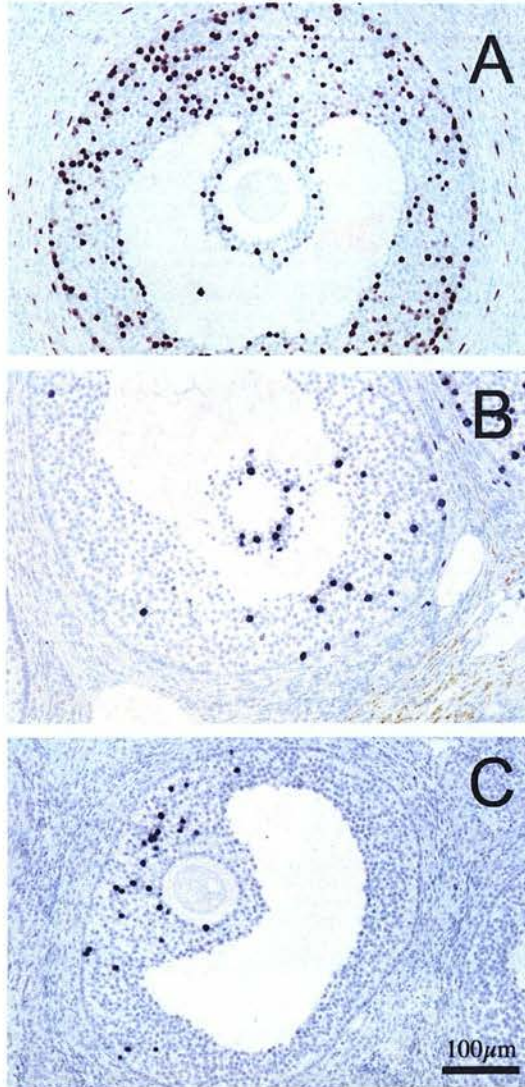


Figure 5.8 The effect of VEGF trap on cellular proliferation in tertiary follicles.

BrdU stained tertiary follicles from (A) late follicular control ovaries, (B) 0-10 day VEGF trap treated ovaries and (C) 5-10 day treated ovaries. Note the reduction in granulosa cell proliferation and the severe reduction of proliferation in the thecal layer in both treatment groups. Also note the only remaining proliferating granulosa cells are located close to the oocyte.

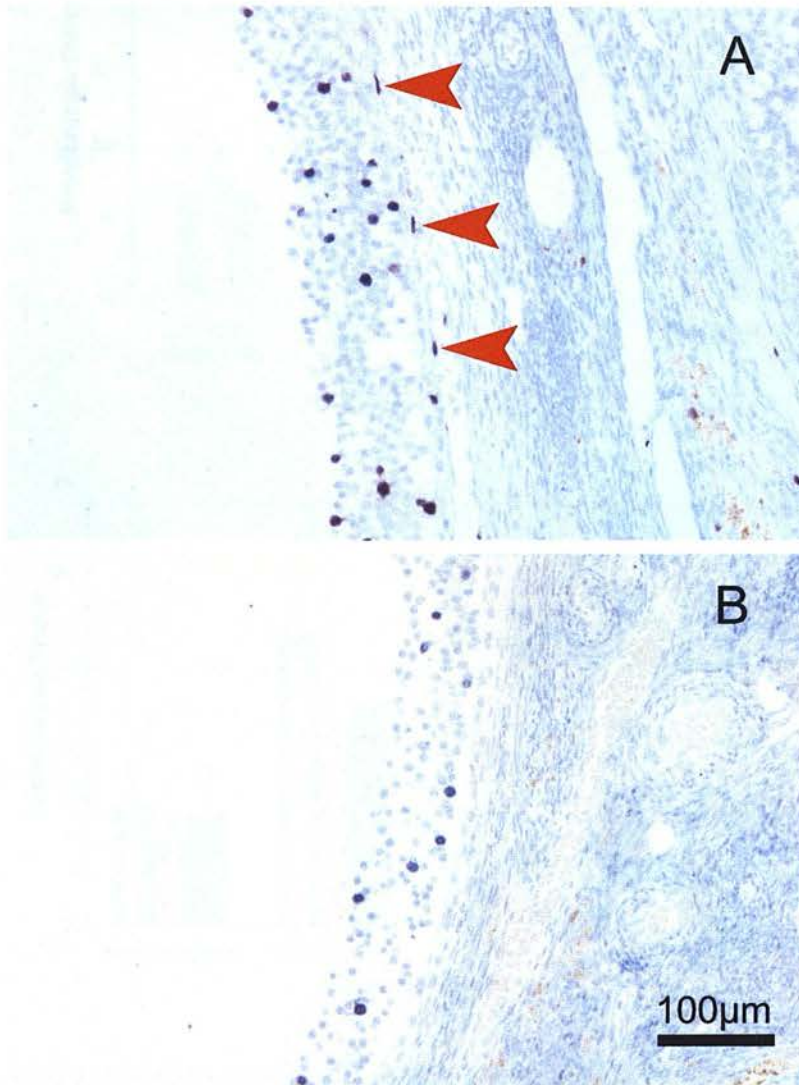


Figure 5.9 The effect of VEGF trap treatment on cellular proliferation in dominant follicles.

BrdU stained dominant follicles from (A) late follicular control ovaries and (B) 5-10 day VEGF trap treated ovaries. Note the reduction in granulosa cell proliferation and the absence of proliferation in the thecal layer in treated ovaries. Red arrows highlight proliferating thecal cells.

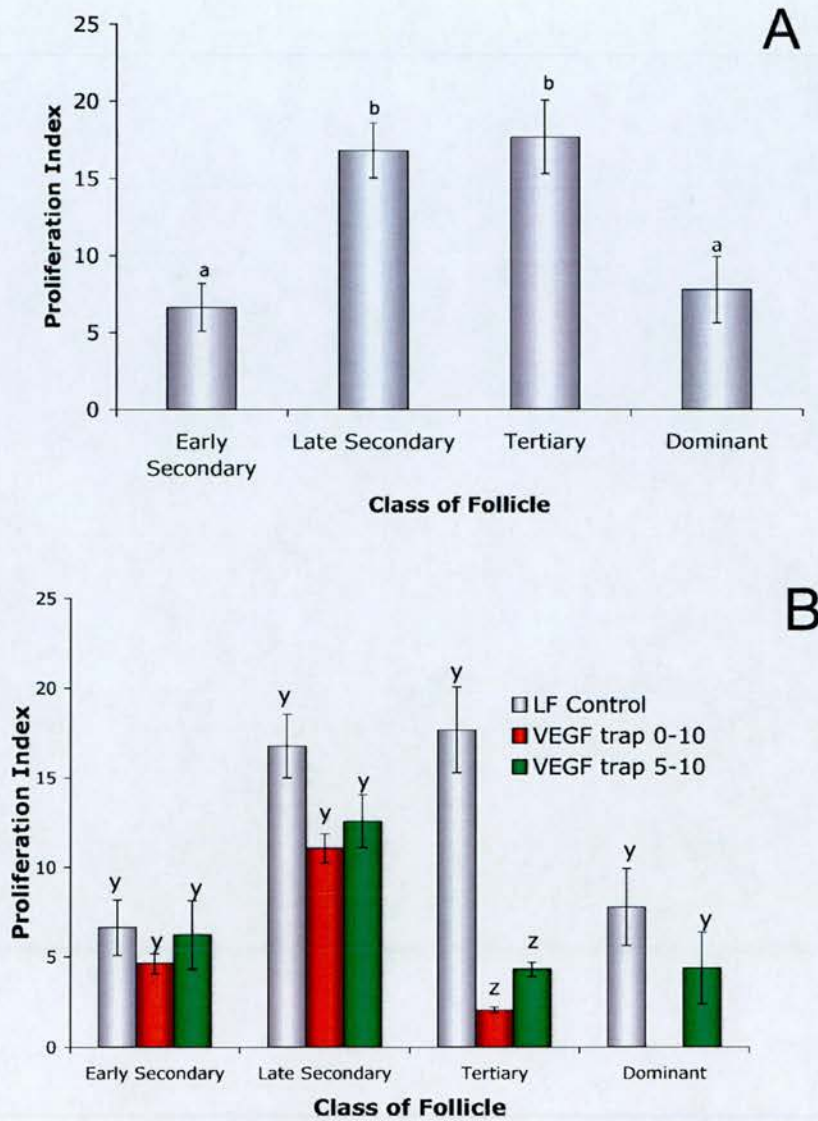


Figure 5.10 Quantitative analysis of granulosa cell proliferation.

(A) Analysis of granulosa cell proliferation from developing follicles from late follicular control ovaries. A significant increase between early secondary ($6.6 \pm 1.5\%$) and late secondary follicles ($16.8 \pm 1.8\%$, $p=0.007$) and a significant decrease between tertiary ($17.7 \pm 2.4\%$) and dominant follicles ($7.8 \pm 2.2\%$, $p=0.029$) was observed. (B) VEGF trap treatment for both 0-10 and 5-10 days significantly reduced granulosa cells proliferation in tertiary follicles ($2.1 \pm 0.2\%$, $p<0.001$ and $4.3 \pm 0.4\%$, $p=0.001$ respectively when compared to the control value of $17.7 \pm 2.4\%$). Values shown are means \pm sem. Superscripts a, b, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

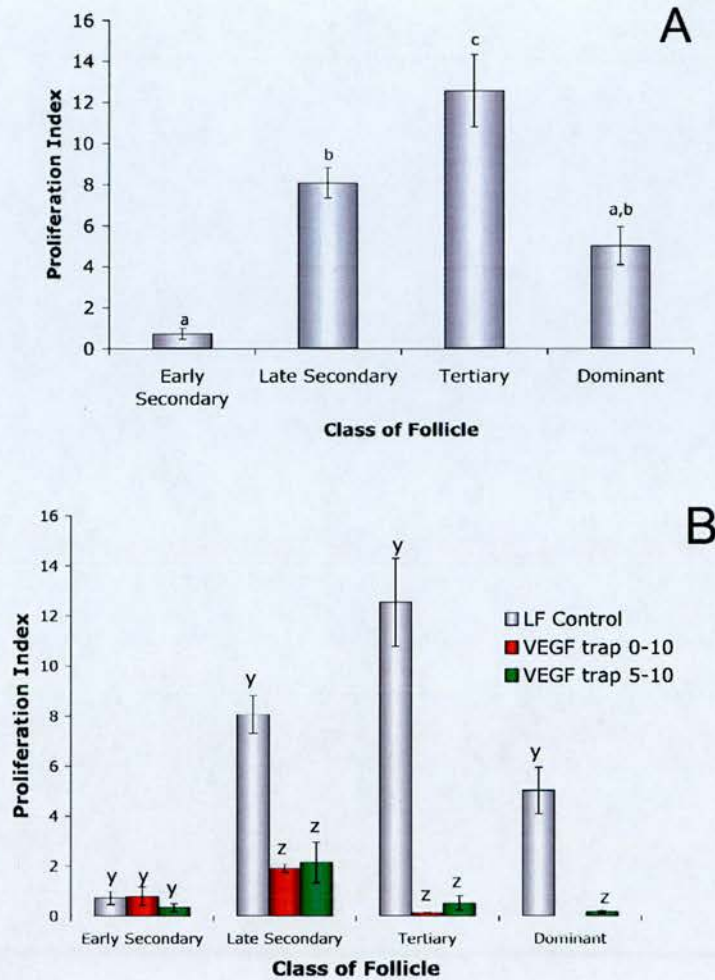


Figure 5.11 Quantitative analysis of theca cell proliferation.

(A) Analysis of theca cell proliferation from developing follicles from late follicular control ovaries. Significant increases between early secondary ($0.7 \pm 0.3\%$) and late secondary ($8.1 \pm 0.7\%$, $p < 0.001$), late secondary ($8.1 \pm 0.7\%$) and tertiary ($12.5 \pm 1.8\%$, $p = 0.044$) and a significant decrease between tertiary ($12.5 \pm 1.8\%$) and dominant follicles ($5.0 \pm 1.0\%$, $p = 0.002$) were observed. (B) VEGF trap treatment severely inhibited cellular proliferation in the theca. Significant decreases after both treatment for both days 0-10 and 5-10 in late secondary ($1.9 \pm 0.2\%$, $p = 0.001$ and $2.1 \pm 0.8\%$, $p < 0.001$ respectively), tertiary ($0.1 \pm 0.02\%$, $p < 0.001$ and $0.5 \pm 0.3\%$, $p < 0.001$ respectively) and dominant follicles from days 5-10 trap treatment ($0.2 \pm 0.1\%$, $p = 0.002$) compared to late follicular controls. Values are mean \pm sem. Superscripts a, b, c, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

5.3.5 Endothelial cell proliferation

The effect of VEGF trap treatment on endothelial cell proliferation was assessed by quantification of co-localisation of BrdU and CD31 in the theca of developing follicles. Tertiary follicles from late follicular control ovaries and both days 0-10 and 5-10 VEGF trap treatment groups are shown in (**Figure 5.12**), dominant follicles from late follicular controls and days 5-10 VEGF trap are shown in (**Figure 5.13**). Quantitative analysis of follicles from late follicular control ovaries showed no rise in the proportion of endothelial cell proliferation between late secondary and tertiary stages of follicle development, (**Figure 5.14A**). A significant increase in the proportion of proliferating cells that were endothelial was observed between tertiary ($18.7\% \pm 2.3$) and dominant follicles ($56.0\% \pm 5.9$, $p < 0.001$), (**Figure 5.14A**). Both the 0-10 day and the 5-10 day VEGF trap treatment regimes almost completely ablated endothelial cell proliferation in all classes of follicle. Quantitative analysis revealed significant reductions in endothelial cell proliferation in late secondary follicles from both treatment groups ($0.8\% \pm 0.8$, $p < 0.001$ and $0.0\% \pm 0.0$, $p < 0.001$ respectively compared to controls $19.8\% \pm 2.6$). This trend continued in the tertiary follicles ($0.0\% \pm 0.0$, $p < 0.001$ and $0.8\% \pm 0.5$, $p < 0.001$ respectively compared to late follicular controls $18.7\% \pm 2.3$) and the dominant follicles from the days 5-10 VEGF trap treatment group ($0.0\% \pm 0.0$, $p < 0.001$ compared to late follicular controls $56.0\% \pm 5.9$), (**Figure 5.14B**).

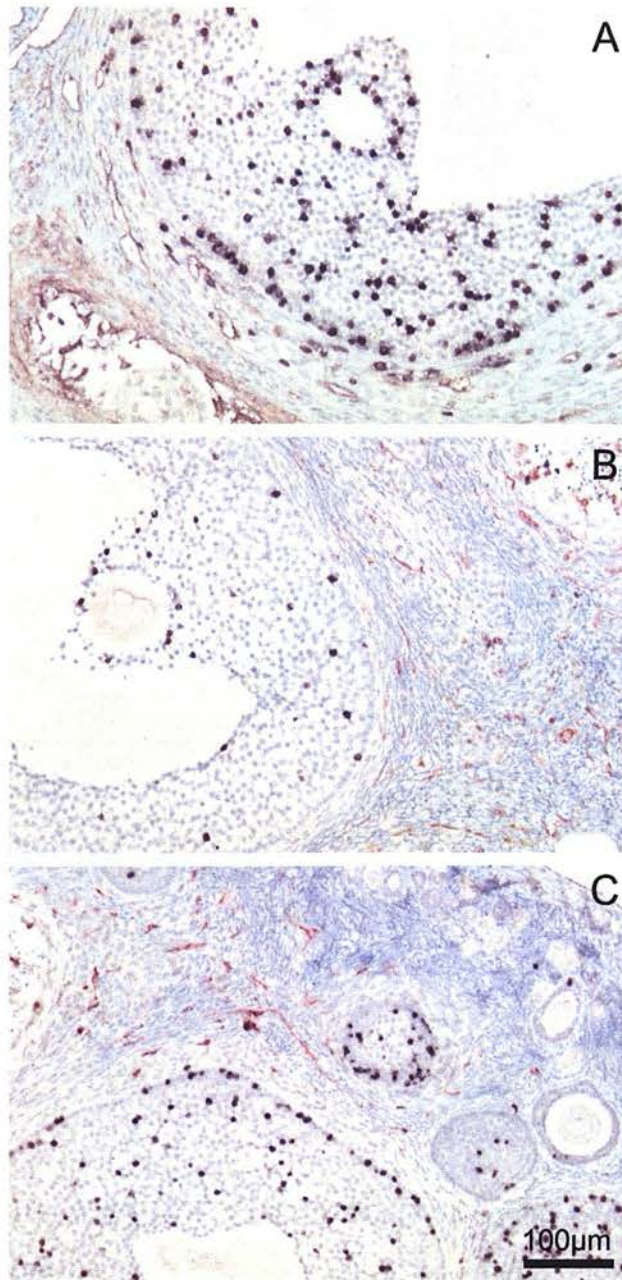


Figure 5.12 The effect of VEGF trap treatment on endothelial cell proliferation in tertiary follicles.

Dual (BrdU/CD31) stained tertiary follicles from (A) late follicular control ovaries, (B) VEGF trap treatment for days 0-10 and (C) 5-10 of the follicular phase ovaries. Note the reduction in co-localisation of BrdU (black nuclear) and CD31 (red endothelial) in the theca of both treatment groups.

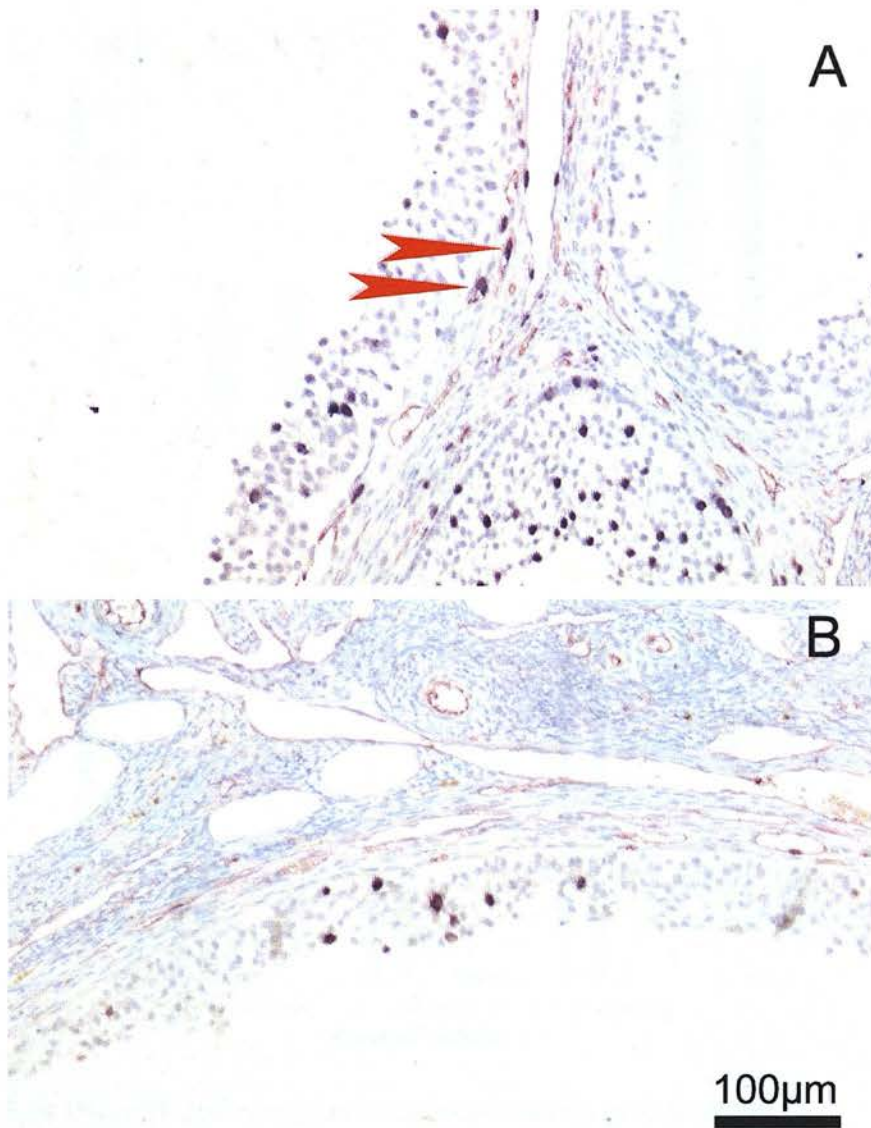


Figure 5.13 The effect of VEGF trap treatment on endothelial cell proliferation in dominant follicles.

Dual (BrdU/CD31) stained dominant follicles from (A) late follicular control ovaries and (B) days 5-10 trap treated ovaries. Note the reduction in co-localisation of BrdU (black nuclear) and CD31 (red endothelial) in the theca of follicles from treated ovaries. Red arrows indicate dual stained cells.

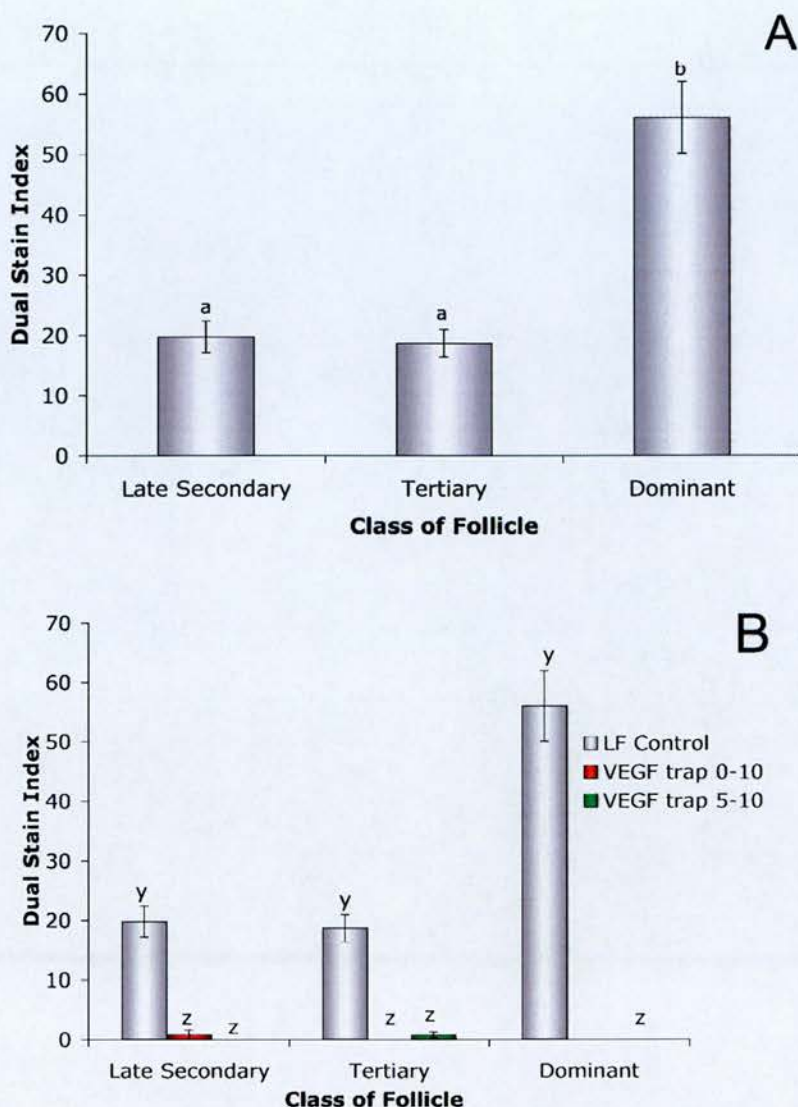


Figure 5.14 Quantitative analysis of endothelial cell proliferation.

(A) Analysis of endothelial cell proliferation in the theca of developing follicles from late follicular control ovaries. Significant increase between tertiary ($18.7 \pm 2.3\%$) and dominant follicles ($56.0 \pm 5.9\%$, $p < 0.001$). (B) VEGF trap treatment for both days 0-10 and 5-10 severely inhibited endothelial cell proliferation in late secondary ($0.8 \pm 0.8\%$, $p < 0.001$ and $0.0 \pm 0.0\%$, $p < 0.001$ respectively), tertiary ($0.0 \pm 0.0\%$, $p < 0.001$ & $0.8 \pm 0.5\%$, $p < 0.001$ respectively) and dominant follicles ($0.0 \pm 0.0\%$, $p < 0.001$) in trap treated days 5-10 ovaries. Values are means \pm sem. Superscripts a, b, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

5.3.6 Endothelial cell area

Typical tertiary and dominant follicles from ovaries from late follicular control and VEGF trap treated animals are shown in (**Figure 5.15, 5.16**) respectively. The effect of VEGF trap treatment on vascular abundance in the theca layer was determined by measurement of endothelial cell area in the theca. Quantitative analysis of CD31 staining / endothelial cell area in follicles from late follicular control ovaries showed a progressive significant increase between late secondary ($3.8\% \pm 0.4$) and tertiary follicles ($6.1\% \pm 0.2$, $p=0.002$) and between tertiary ($6.1\% \pm 0.2$) and dominant follicles ($12.9\% \pm 0.7$, $p<0.001$), (**Figure 5.17A**). Treatment with VEGF trap between days 0-10 and 5-10 of the follicular phase significantly reduced the CD31 positive area in the theca of late secondary follicles ($1.9\% \pm 0.3$, $p=0.014$ and $2.2\% \pm 0.2$, $p=0.014$ respectively) and tertiary follicles ($2.0\% \pm 0.3$, $p<0.001$ and $4.0\% \pm 0.4$, $p=0.001$ respectively). Typical examples of tertiary follicles from late follicular control, and both VEGF trap treatment for days 0-10 and 5-10 are shown in (**Figure 5.15**). Follicles at the tertiary stage of development in late follicular control ovaries had a vascular networking that reached the membrana propria that separates the granulosa cells from the theca cells (**Figure 5.15A**). Both VEGF trap treatment for days 0-10 and 5-10 prevented this development, (**Figure 5.15B&C**). Sections of dominant follicle walls from late follicular control and days 5-10 VEGF trap treated ovaries are shown in (**Figure 5.16**). A fully developed follicular vascular was evident in dominant follicles from both late follicular controls and days 5-10 VEGF trap treated ovaries. Quantitative analysis of endothelial cell area in dominant follicles showed no reduction after VEGF trap treatment for follicular days 5-10, (**Figure 5.17B**).

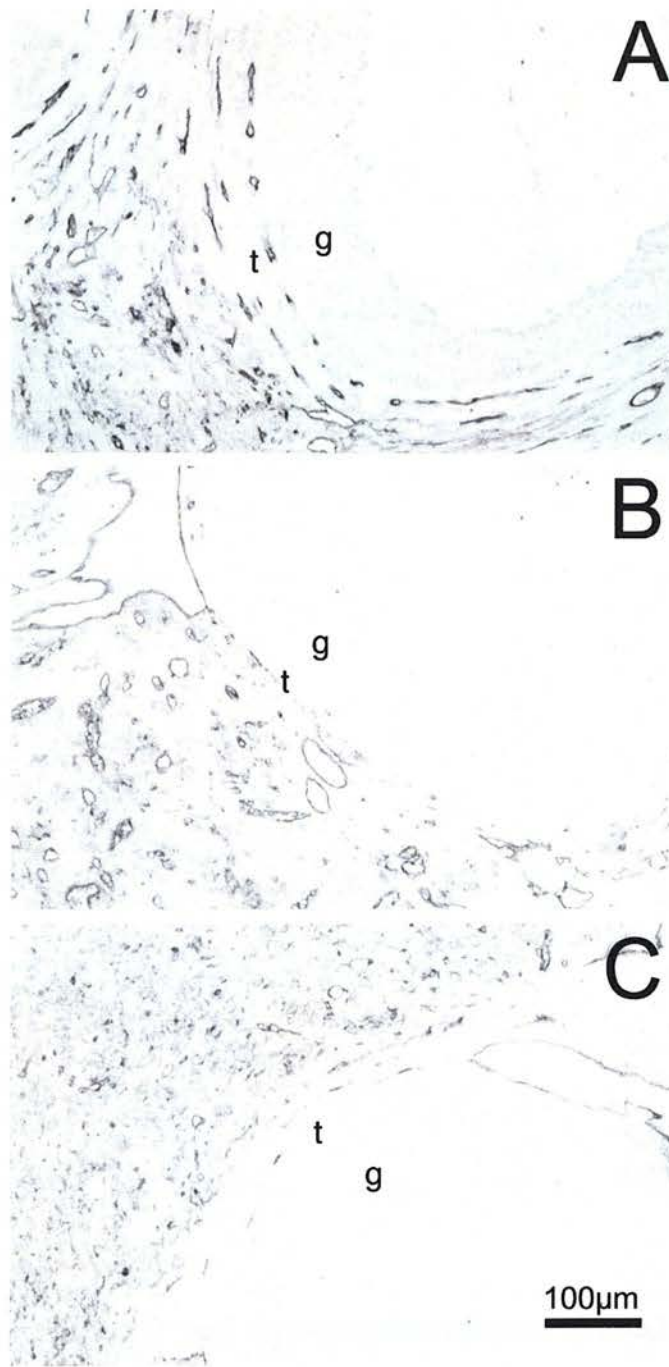


Figure 5.15 Effect of VEGF trap on endothelial cell area in tertiary follicles.

CD31 stained tertiary follicles from (A) late follicular control ovaries, (B) days 0-10 VEGF trap treated ovaries and (C) days 5-10 VEGF trap treated ovaries. Note the absence of vascular growth in the vicinity of the membrana propria separating the granulosa and theca cells after both treatments. g=granulosa, t=theca.

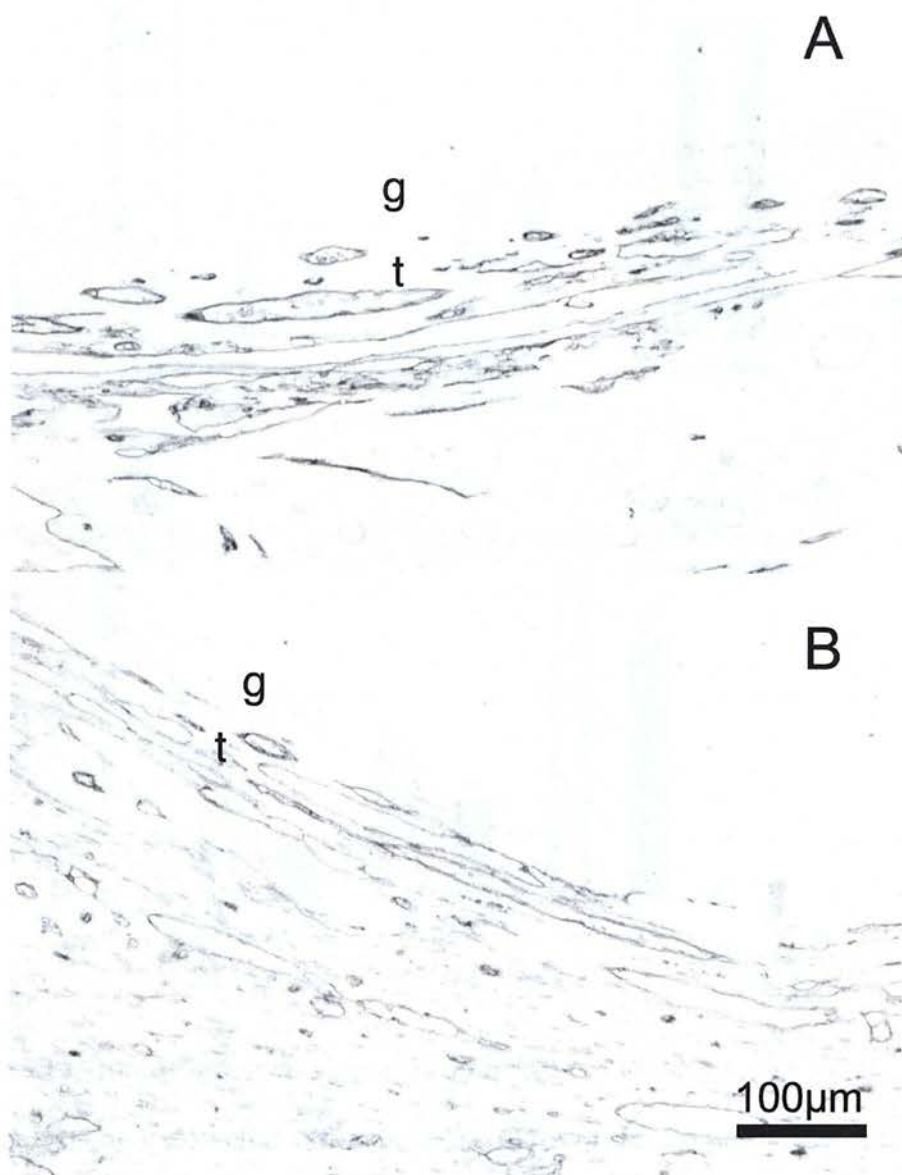


Figure 5.16 Effect of VEGF trap on endothelial cell area in dominant follicles.

CD31 stained dominant follicles from (A) late follicular control ovaries and (B) days 5-10 VEGF trap treated ovaries. Note the uniform pattern of endothelial cells throughout the theca of both control and treated follicles. g=granulosa, t=theca.

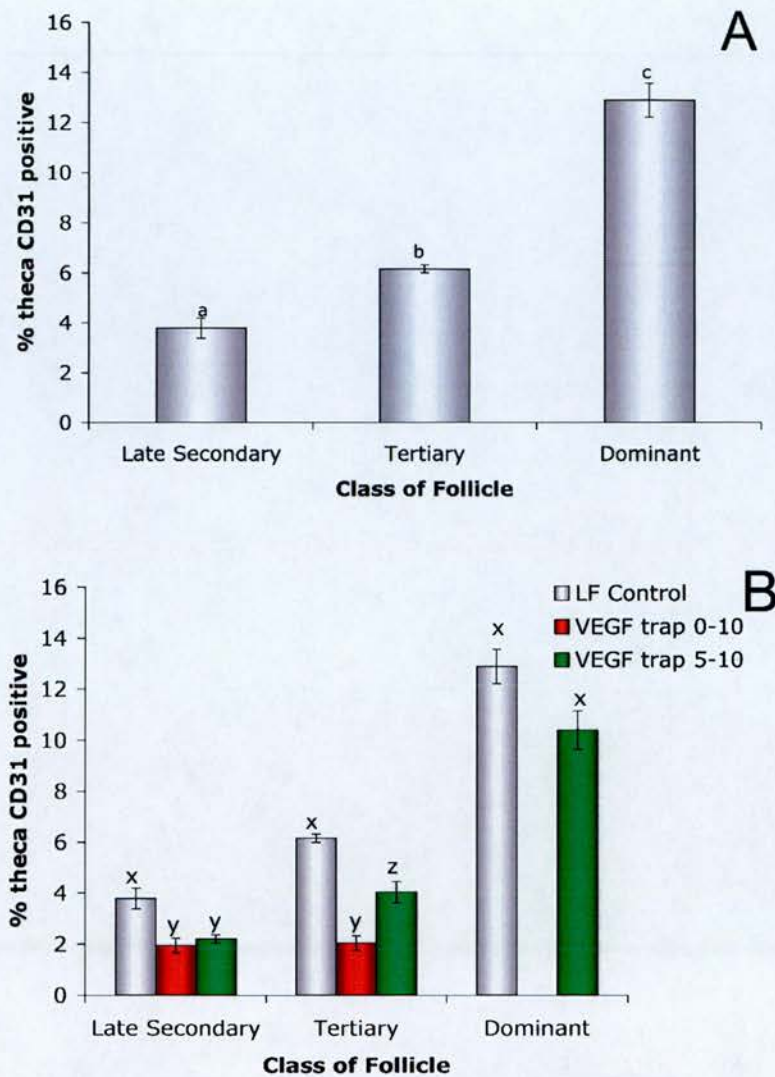


Figure 5.17 Quantitative analysis of endothelial cell area.

(A) Analysis of endothelial cell area in the theca of developing follicles from late follicular control ovaries. Significant increases in endothelial cell area were observed between late secondary ($3.8 \pm 0.4\%$) and tertiary ($6.1 \pm 0.2\%$, $p=0.002$) and tertiary ($6.1 \pm 0.2\%$) and dominant follicles ($12.9 \pm 0.7\%$, $p<0.001$). (B) VEGF trap treatment for both days 0-10 and 5-10 reduced endothelial cells area in late secondary ($1.9 \pm 0.3\%$, $p=0.014$ and $2.2 \pm 0.2\%$, $p=0.014$ respectively) and tertiary follicles ($2.0 \pm 0.3\%$, $p<0.001$ and $4.0 \pm 0.4\%$, $p=0.001$ respectively). Values are mean \pm sem. Superscripts a, b, c, x, y and z denote statistical significance. Statistics used: one way ANOVA and LSD post hoc tests.

Quantification of thecal thickness in follicles from late follicular control ovaries revealed significant increases in thecal thickness as the follicles developed from late secondary ($27.0 \pm 2.3 \mu\text{m}$) to tertiary ($57.0 \pm 3.5 \mu\text{m}$, $p < 0.001$) and from tertiary ($57.0 \pm 3.5 \mu\text{m}$) to dominant ($74.9 \pm 4.1 \mu\text{m}$, $p = 0.009$), (**Figure 5.18A**). VEGF trap treatment between days 0-10 ($17.7 \pm 0.6 \mu\text{m}$, $p = 0.015$) or days 5-10 ($18.6 \pm 0.9 \mu\text{m}$, significantly reduced thecal thickness in late secondary and tertiary ($29.6 \pm 3.1 \mu\text{m}$, < 0.001 and $32.8 \pm 2.0 \mu\text{m}$, $p < 0.001$ respectively) and dominant follicles ($44.7 \pm 5.8 \mu\text{m}$, $p = 0.003$) compared to late follicular controls, (**Figure 5.18B**). Taking the diameter of the theca into account allows the quantification of the total 'amount' of vasculature associated with the follicles. After thecal diameter is taken into account analysis again showed significant increases in thecal vascularisation as follicles developed: Late secondary ($3.8\% \pm 0.4$) to tertiary ($6.1\% \pm 0.2$, $p = 0.002$); tertiary ($6.1\% \pm 0.2$) to dominant follicles (12.9 ± 0.7 , $p < 0.001$), (**Figure 5.19A**). This analysis revealed that there was a significant reduction in the amount of blood vessels in every class of follicle after both of the VEGF trap treatment regimes: VEGF trap treatment significantly reduced the thecal vascularisation in late secondary ($1.3\% \pm 0.2$, $p = 0.001$ and $1.5\% \pm 0.1$, $p = 0.001$ respectively), tertiary ($1.1\% \pm 0.1$, $p < 0.001$ and $2.3\% \pm 0.2$, $p < 0.001$) and dominant follicles ($6.2\% \pm 0.4$, $p = 0.001$), (**Figure 5.19B**).

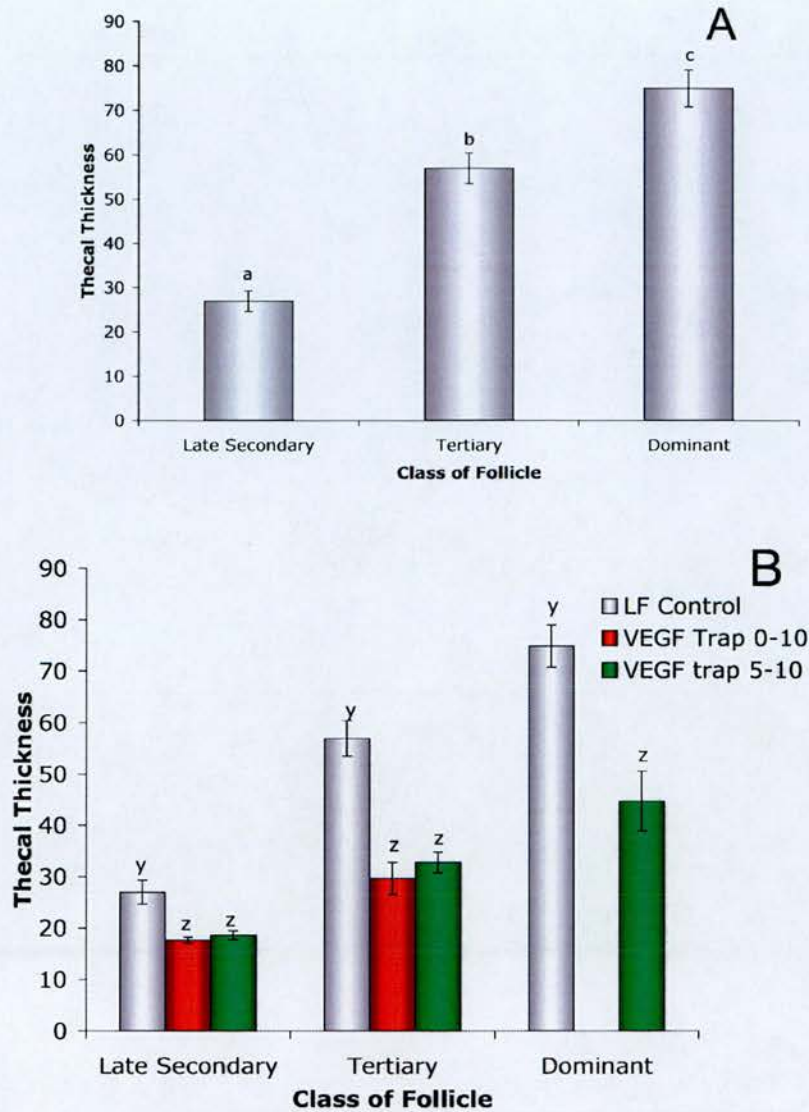


Figure 5.18 Analysis of thecal diameter.

(A) Analysis of theca diameter of follicles from late follicular control ovaries. Significant increases in thecal diameter were observed between late secondary ($27.0 \pm 2.3 \mu\text{m}$) and tertiary follicles ($57.0 \pm 3.5 \mu\text{m}$, $p < 0.001$) and between tertiary ($57.0 \pm 3.5 \mu\text{m}$) and dominant follicles ($74.9 \pm 4.1 \mu\text{m}$, $p = 0.009$). (B) VEGF trap treatment for both days 0-10 and 5-10 reduced thecal diameter in late secondary ($17.7 \pm 0.6 \mu\text{m}$, $p = 0.015$ and $18.6 \pm 0.9 \mu\text{m}$, $p = 0.012$ respectively), tertiary ($27.0 \pm 3.1 \mu\text{m}$, $p < 0.001$ and $32.8 \pm 2.0 \mu\text{m}$, $p < 0.001$ respectively) and dominant follicles in the days 5-10 trap treated group ($44.7 \pm 5.8 \mu\text{m}$, $p = 0.003$). Values are means \pm sem. Superscripts a, b, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

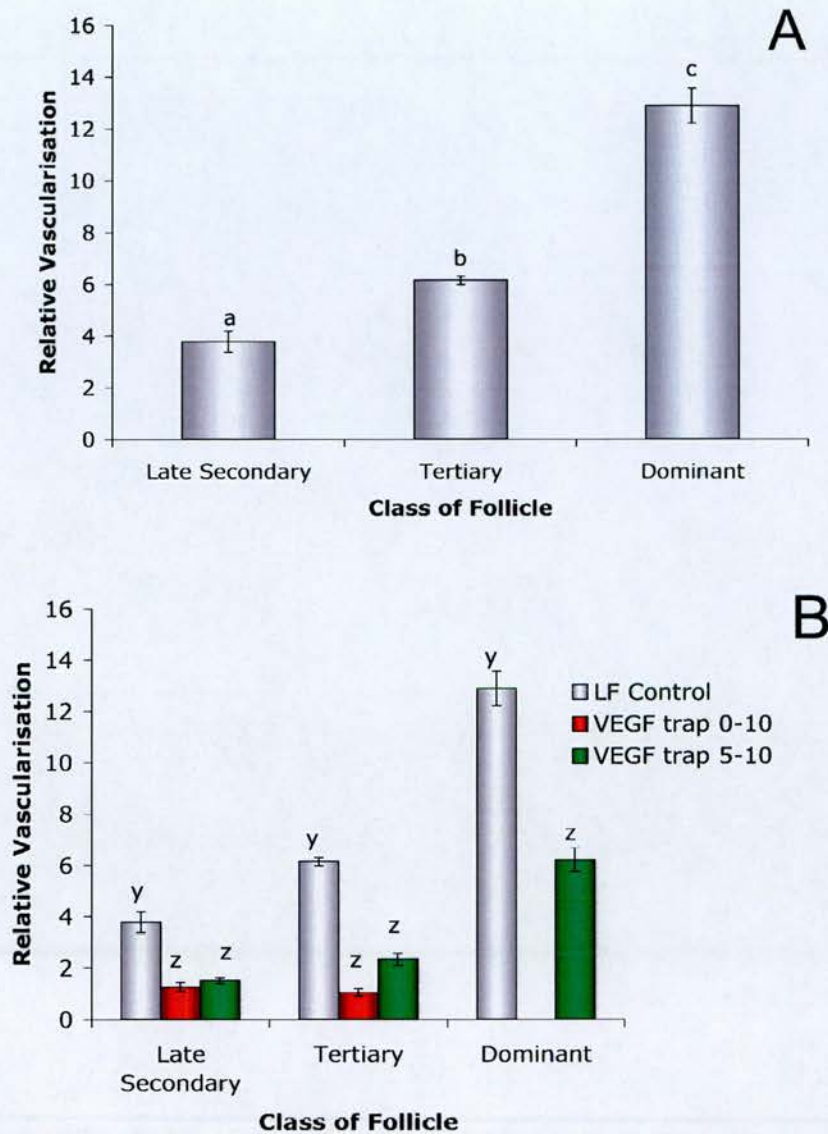


Figure 5.19 Analysis of total thecal vasculature.

Accounting for thecal diameter, the total thecal vasculature of (A) follicles from late follicular control ovaries showed significant increases between late secondary ($3.8 \pm 0.4\%$) and tertiary ($6.1 \pm 0.2\%$, $p=0.002$) and tertiary ($6.1 \pm 0.2\%$) and dominant follicles ($12.9 \pm 0.7\%$, $p<0.001$). (B) VEGF trap treatment for both days 0-10 and 5-10 reduced the total vasculature in late secondary ($1.3 \pm 0.2\%$, $p=0.001$ and $1.5 \pm 0.1\%$, $p=0.001$ respectively), tertiary ($1.1 \pm 0.1\%$, $p<0.001$ and $2.3 \pm 0.2\%$, $p<0.001$ respectively) and dominant follicles present in the days 5-10 trap treatment group ($6.2 \pm 0.4\%$ $p=0.001$). Values are means \pm sem. Superscripts a, b, c, y and z denote statistical significance. Statistics used: one way ANOVA and LSD post hoc tests.

5.3.7 VEGF mRNA expression

VEGF mRNA expression was localised to the granulosa and theca cells in developing follicles from late follicular control ovaries (**Figure 5.20A&B, 5.21A&B**). Quantitative analysis of the level of VEGF mRNA expression in granulosa cells of follicles from late follicular control ovaries showed a significant increase in expression between late secondary (0.011 ± 0.0005) and tertiary follicles (0.024 ± 0.0016 , $p < 0.001$), (**Figure 5.22A**). Examples of late follicular and days 0-10 and 5-10 VEGF trap treated tertiary follicles showing VEGF mRNA expression are shown in (**Figure 5.20**). Similarly, sections of dominant follicles from late follicular controls and VEGF trap treated days 5-10 ovaries are shown (**Figure 5.21**). A significant decrease in VEGF mRNA expression in the granulosa cells was observed between tertiary (0.024 ± 0.0016) and dominant follicles (0.015 ± 0.0021 , $p = 0.010$), (**Figure 5.22A**). Both VEGF trap treatment for days 0-10 and 5-10 caused non-significant increases in the level of VEGF mRNA expressed in the granulosa cells of each class of follicle after either treatment, (**Figure 5.22B**).

VEGF mRNA expression in the theca of follicles from late follicular control ovaries showed a significant increase in VEGF gene expression between tertiary (0.009 ± 0.002) and atretic follicles (0.024 ± 0.004 , $p = 0.033$). There was also a non-significant increase in VEGF expression between tertiary and dominant follicles, (**Figure 5.23A**). Neither the 0-10 or the 5-10 days VEGF trap treatment regimes caused significant increases in VEGF mRNA expression in the theca, (**Figure 5.23B**).

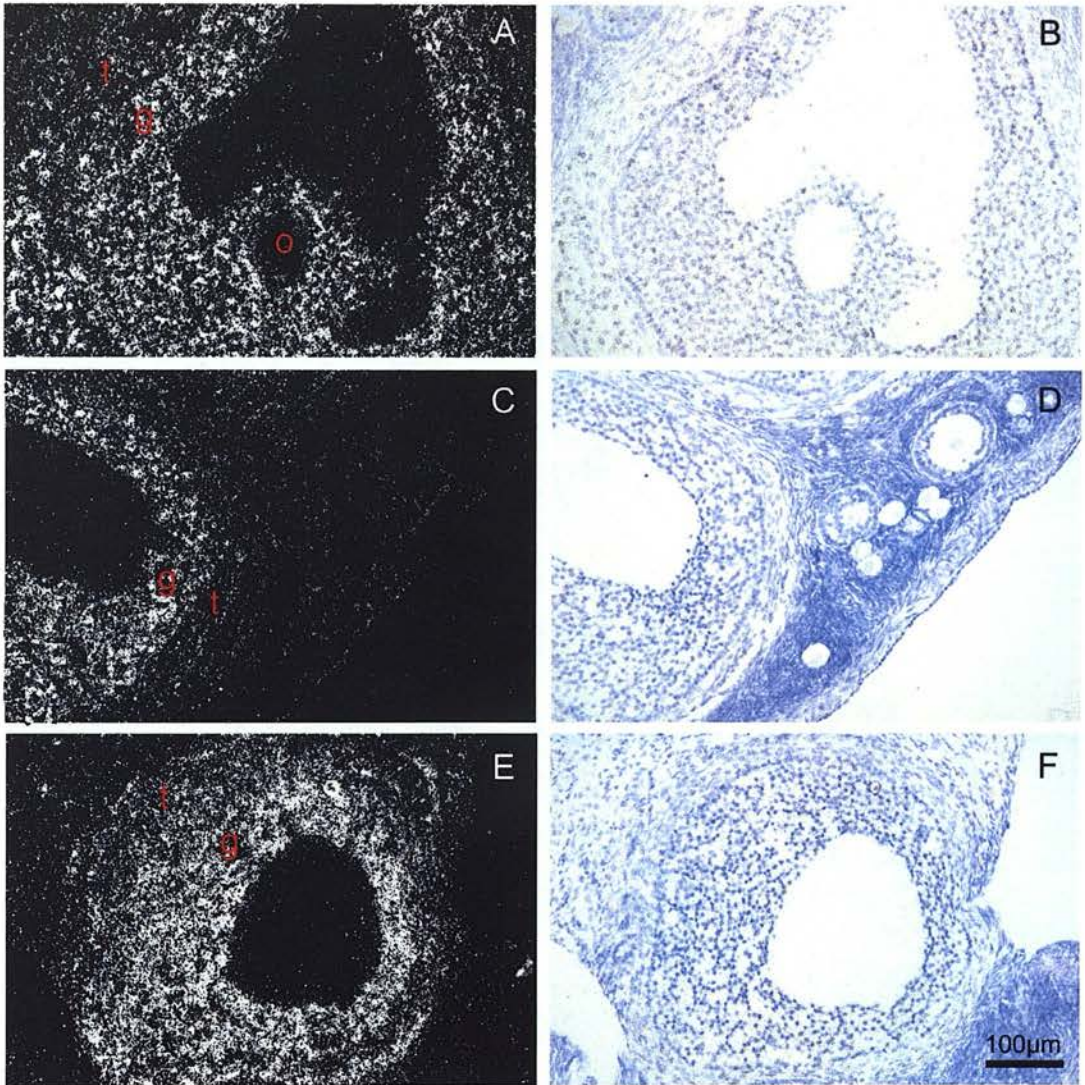


Figure 5.20 *In situ* hybridisation of VEGF mRNA in tertiary follicles.

VEGF mRNA expression in tertiary follicles from (A&B) late follicular control, (C&D) days 0-10 VEGF trap treated and (E&F) days 5-10 VEGF trap treated ovaries. g=granulosa, t=theca, o=oocyte.

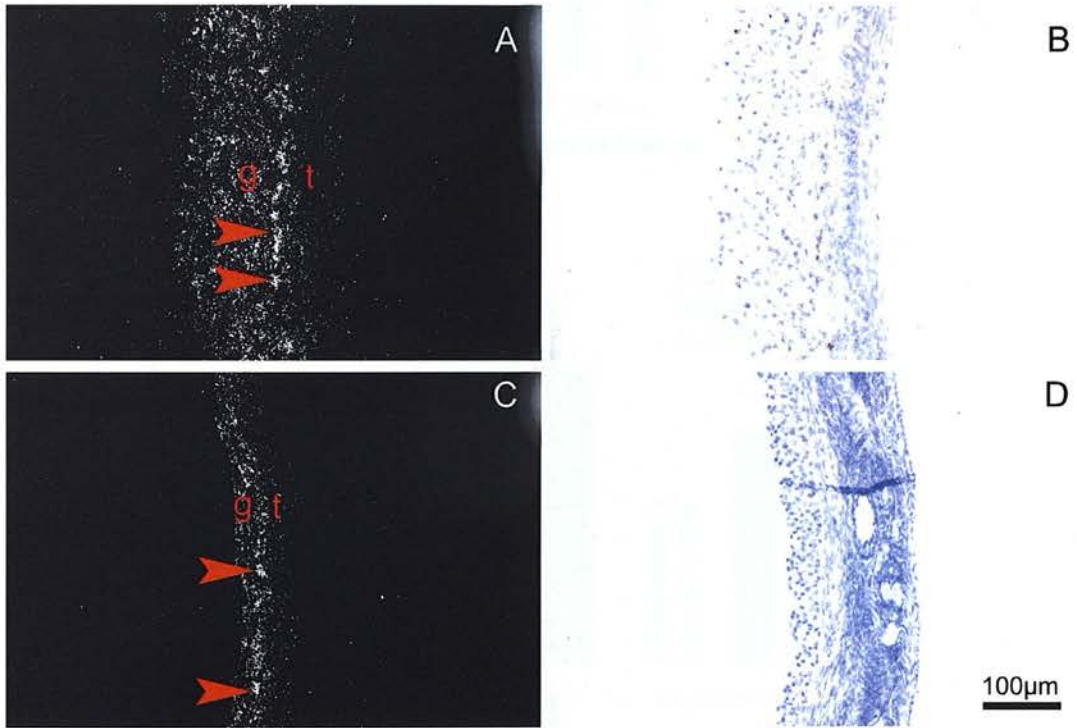


Figure 5.21 *In situ* hybridisation of VEGF mRNA in dominant follicles.

VEGF mRNA expression in dominant follicles from (A&B) late follicular control and (C&D) day 5-10 VEGF trap treated ovaries. Red arrows indicate punctate VEGF mRNA observed bordering the granulosa and theca cells in preovulatory follicles in dominant follicles from both follicular control and VEGF trap 5-10 days treatment groups. g=granulosa, t=theca.

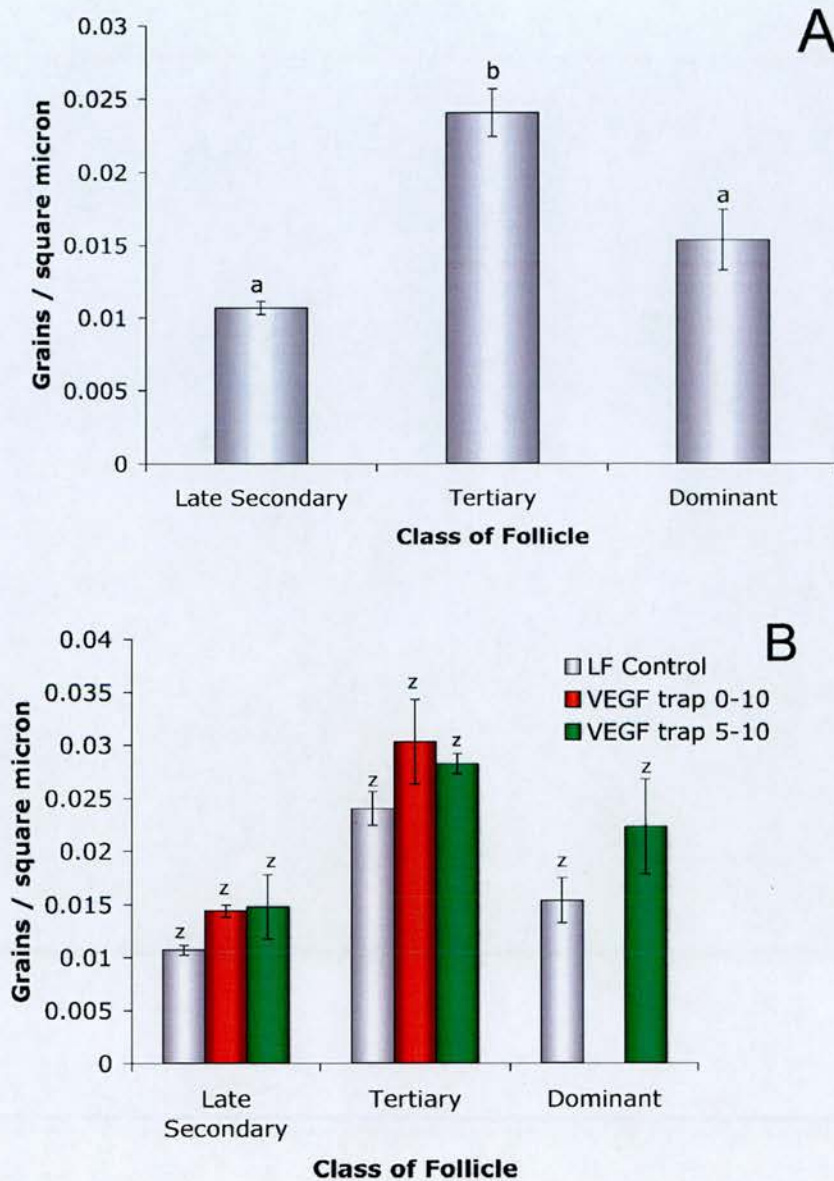


Figure 5.22 Quantitative analysis of granulosa cell VEGF mRNA expression.

(A) VEGF mRNA expression in follicles from late follicular control ovaries. A significant increase between late secondary (0.011 ± 0.0005) and tertiary follicles (0.024 ± 0.0016 , $p < 0.001$) and a significant decrease between tertiary (0.024 ± 0.0016) and dominant follicles (0.015 ± 0.0021 , $p = 0.010$) was observed. (B) VEGF trap treatment for both 0-10 and 5-10 did not cause any significant differences in VEGF mRNA expression in the granulosa. Values are means \pm sem. Superscript a, b and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

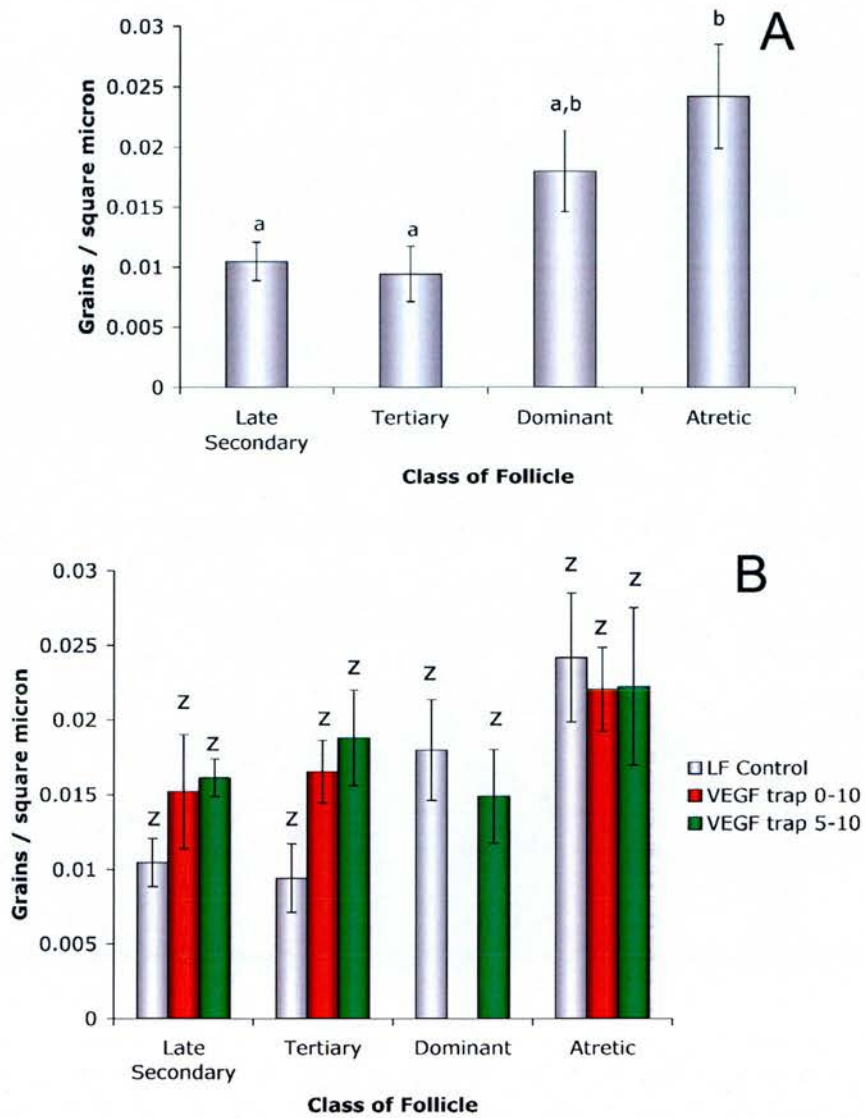


Figure 5.23 Quantitative analysis of thecal VEGF mRNA expression.

(A) VEGF mRNA expression in follicles from late follicular control ovaries. A significant increase between tertiary (0.009 ± 0.002) and atretic (0.024 ± 0.004 , $p=0.033$) follicle VEGF mRNA expression was observed. (B) VEGF trap treatment for both days 0-10 or 5-10 did not cause any significant changes in expression. Values are means \pm sem. Superscripts a, b and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

5.3.8 VEGFR1 mRNA expression

VEGFR1 mRNA was localised to the endothelial cells in the theca of both developing and atretic follicles. VEGFR1 mRNA expression in tertiary follicles from late follicular control ovaries and both VEGF trap treatment for days 0-10 and 5-10 are shown in (**Figure 5.24**). Similarly VEGF mRNA expression in dominant follicles from late follicular and days 5-10 treated ovaries is shown in (**Figure 5.25**). VEGFR1 mRNA expression in the theca of follicles from late follicular control ovaries showed no significant variation apart from a significant decrease between tertiary (0.010 ± 0.002) and atretic follicles (0.004 ± 0.001 , $p=0.028$), (**Figure 5.26A**). VEGF trap treatment for both days 0-10 or days 5-10 of the follicular phase significantly reduced VEGFR1 mRNA expression in all follicular classes: Late secondary (0.002 ± 0.0006 , $p=0.018$ and 0.001 ± 0.0003 , $p=0.006$); tertiary (0.0014 ± 0.0002 , $p<0.001$ and 0.0018 ± 0.0003 , $p<0.001$); dominant (0.002 ± 0.000 , $p<0.001$); and atretic (0.0016 ± 0.0001 , $p=0.009$ and 0.0019 ± 0.0003 , $p=0.012$), (**Figure 5.26B**).

5.3.9 VEGFR2 mRNA expression

VEGFR2 mRNA expression was localised to the endothelial cells in the theca of both developing and atretic follicles. VEGFR2 mRNA expression in tertiary follicles from both late follicular control and VEGF trap treated ovaries is shown in (**Figure 5.27**). Similarly VEGFR2 mRNA expression in dominant follicles from late follicular control and 5-10 day VEGF trap treated ovaries is shown in (**Figure 5.28**). Quantitative analysis of VEGF mRNA expression in follicles from late follicular control ovaries showed no significant variation across follicle class in late follicular control ovaries, (**Figure 5.29A**). VEGF trap treatment between days 0-10 and days 5-10 of the follicular phase significantly reduced VEGF mRNA expression in all classes of follicle observed: late secondary (0.0026 ± 0.0007 , $p=0.001$ and 0.0026 ± 0.0003 , $p=0.001$ respectively); tertiary (0.003 ± 0.001 , $p=0.001$ and 0.002 ± 0.0002 , $p<0.001$ respectively); dominant (0.0016 ± 0.0003 , $p=0.005$); and atretic (0.0019 ± 0.0003 , $p=0.002$ and 0.0024 ± 0.0008 , $p=0.003$ respectively), (**Figure 5.29B**).

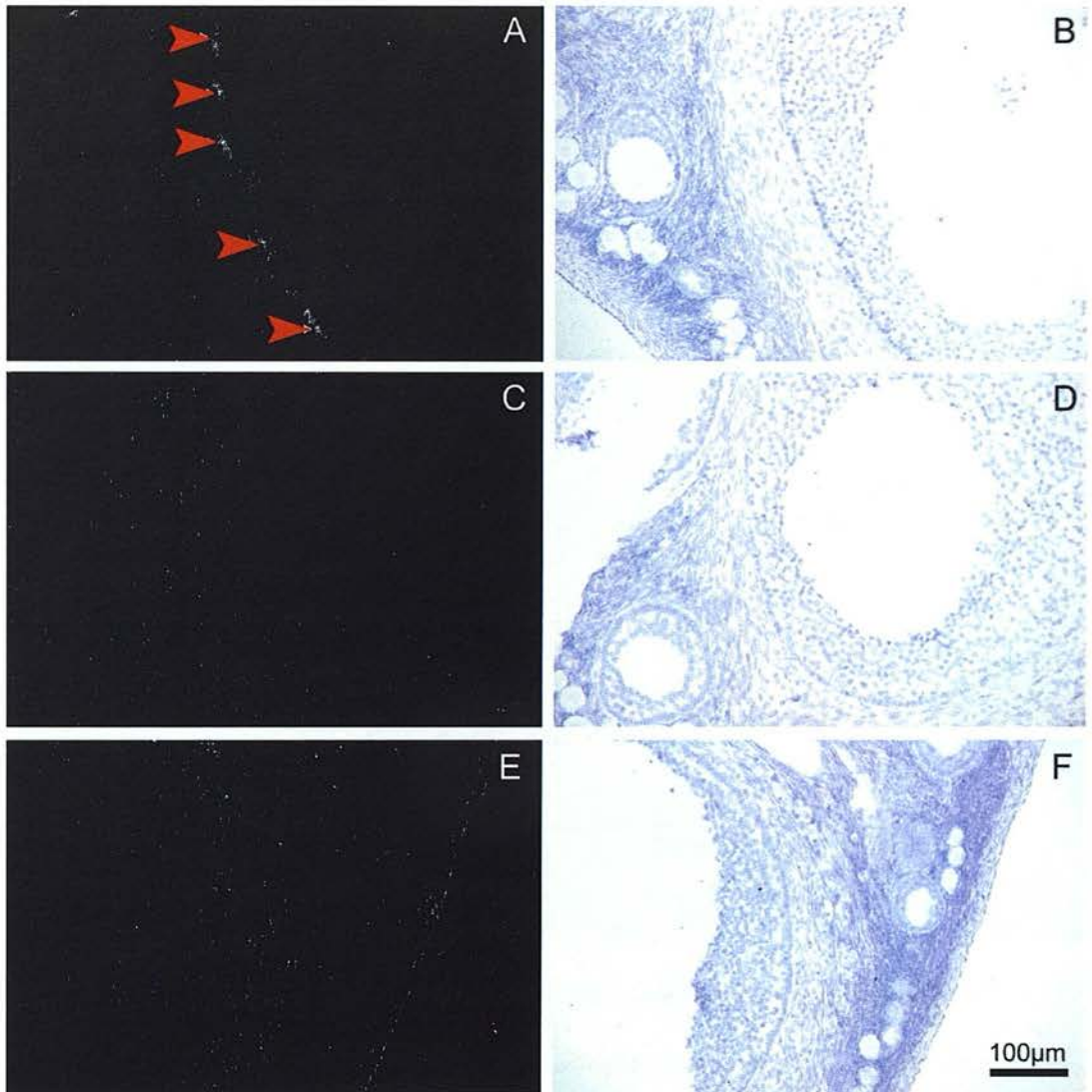


Figure 5.24 *In situ* hybridisation of VEGFR1 mRNA in tertiary follicles.

VEGFR1 mRNA expression in tertiary follicles from (A&B) late follicular control, (C&D) 0-10 day VEGF trap treated and (E&F) VEGF trap 5-10 day treated ovaries. Note the absence of VEGFR1 mRNA expression after both treatment regimes. Red arrow highlight expression.

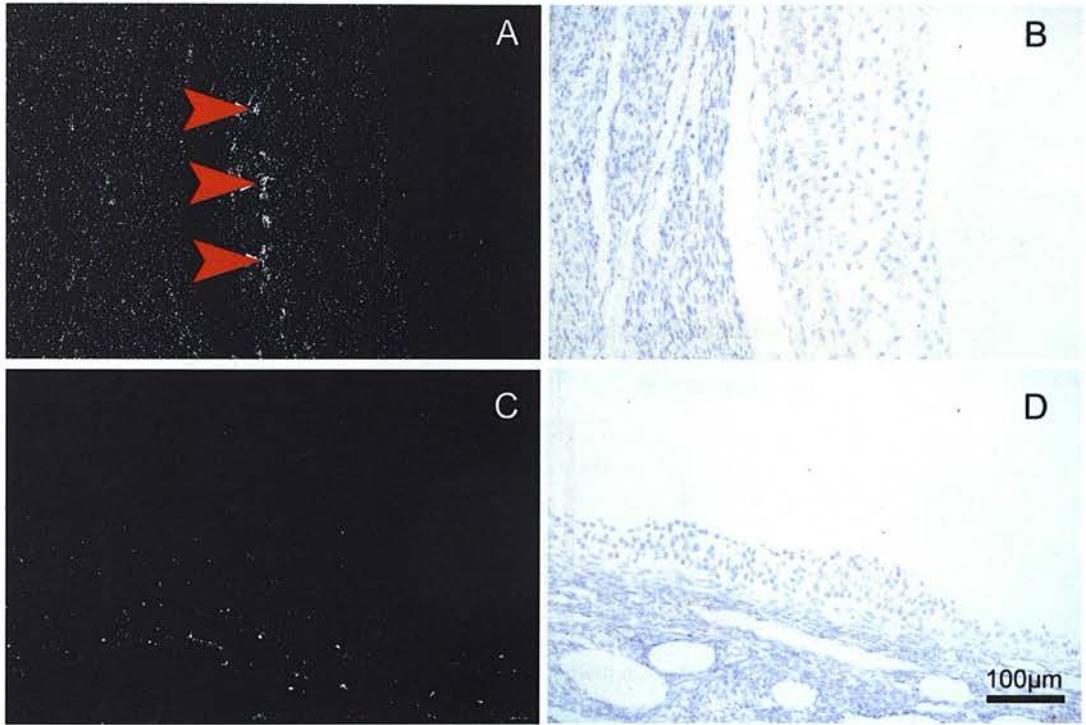


Figure 5.25 *In situ* hybridisation of VEGFR1 mRNA in dominant follicles.

VEGFR1 mRNA expression in dominant follicles from (A&B) late follicular control and (C&D) 5-10 day VEGF trap treated ovaries. Note absence of VEGFR1 mRNA expression after treatment. Red arrows highlight expression.

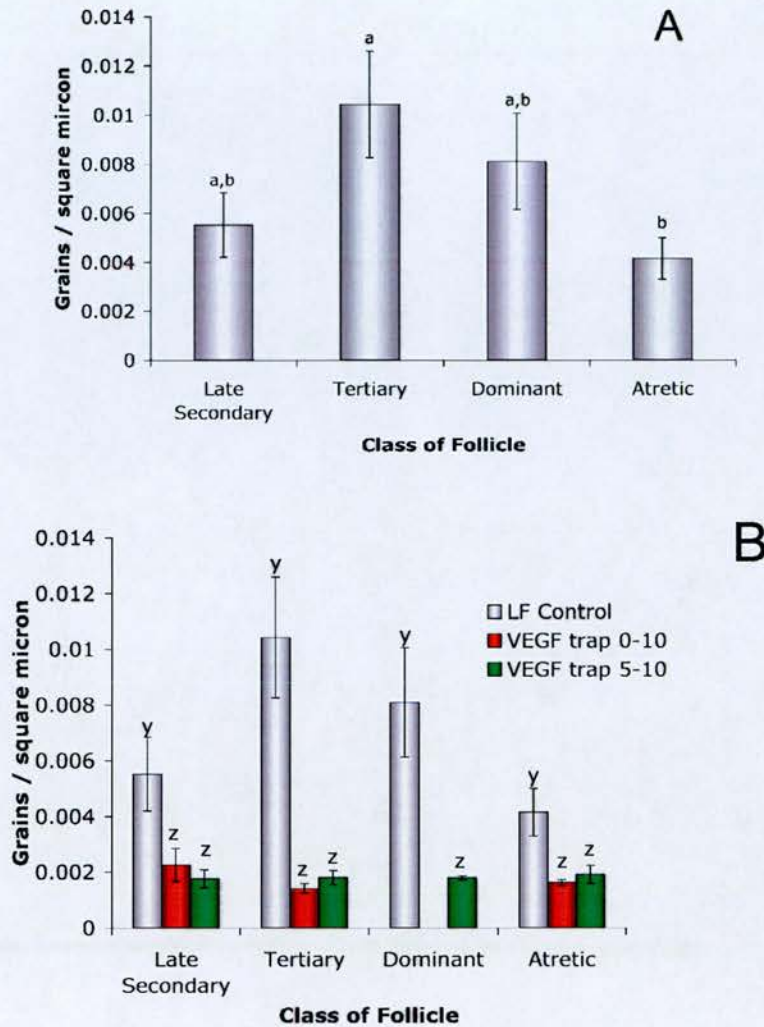


Figure 5.26 Quantitative analysis of thecal VEGFR1 mRNA expression.

(A) VEGFR1 mRNA expression in follicles from late follicular control ovaries. A significant decrease in expression was observed between tertiary (0.01 ± 0.002) and atretic follicles (0.004 ± 0.001 , $p=0.028$). (B) VEGF trap treatment for both 0-10 and 5-10 days caused significant reductions in expression in late secondary (0.002 ± 0.0006 , $p=0.018$ and 0.001 ± 0.0003 , $p=0.006$ respectively), tertiary (0.004 ± 0.0002 , $p<0.001$ and 0.0018 ± 0.0003 , $p<0.001$ respectively), atretic (0.01 ± 0.002 $p=0.009$ & $p=0.012$ respectively) and dominant follicle present in the days 5-10 treatment group (0.002 ± 0.0001 $p<0.001$) compared to late follicular controls (0.005 ± 0.0013 , 0.010 ± 0.0022 , 0.008 ± 0.0020 and 0.004 ± 0.0009 respectively). Values are means \pm sem. Superscripts a, b, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

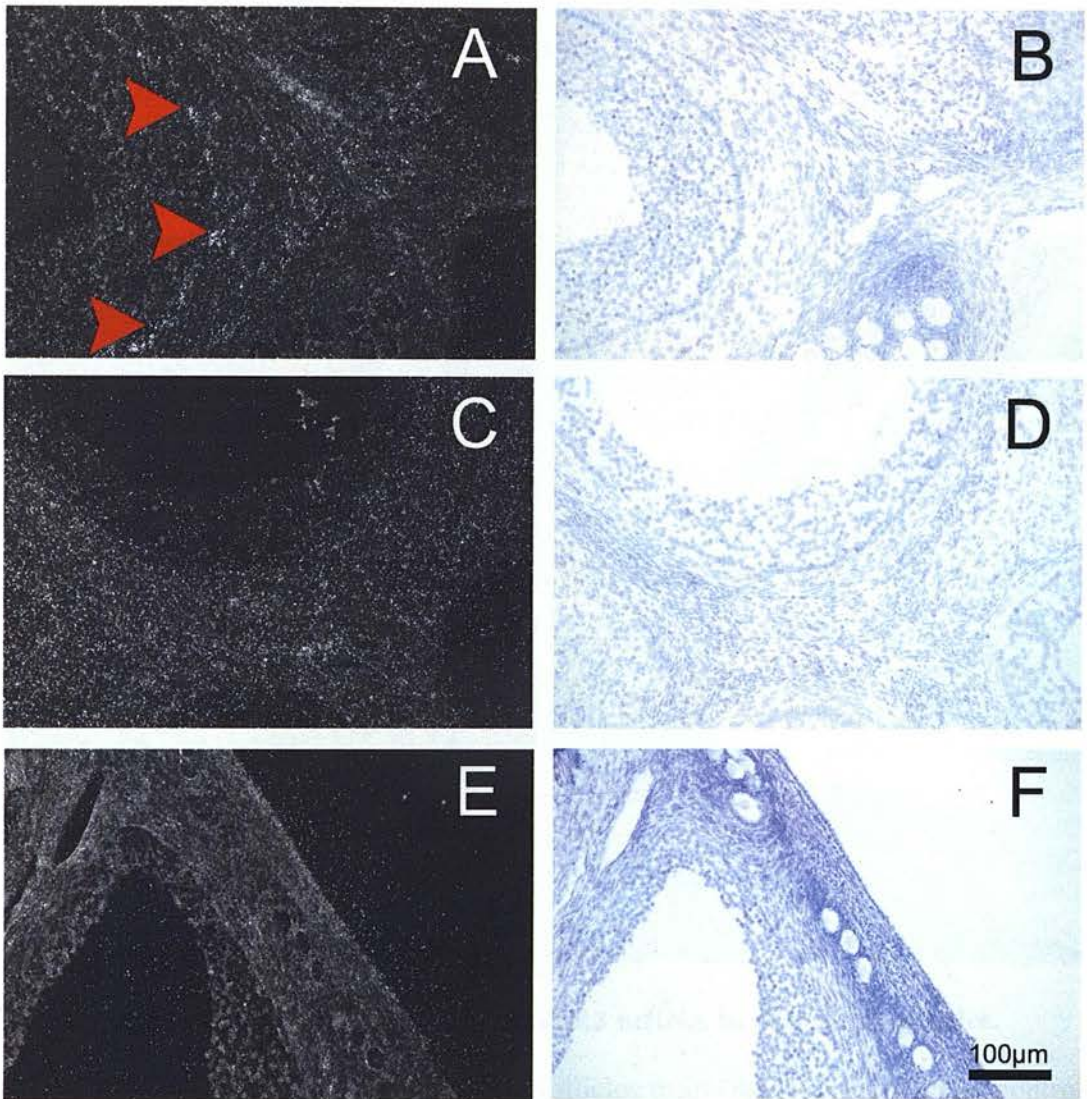


Figure 5.27 *In situ* hybridisation of VEGFR2 mRNA in tertiary follicles.

VEGFR2 mRNA expression in tertiary follicles from (A&B) late follicular control, (C&D) 0-10 day VEGF trap treated and (E&F) VEGF trap 5-10 day treated ovaries. Note the absence of expression after trap treatment. Red arrows highlight expression.

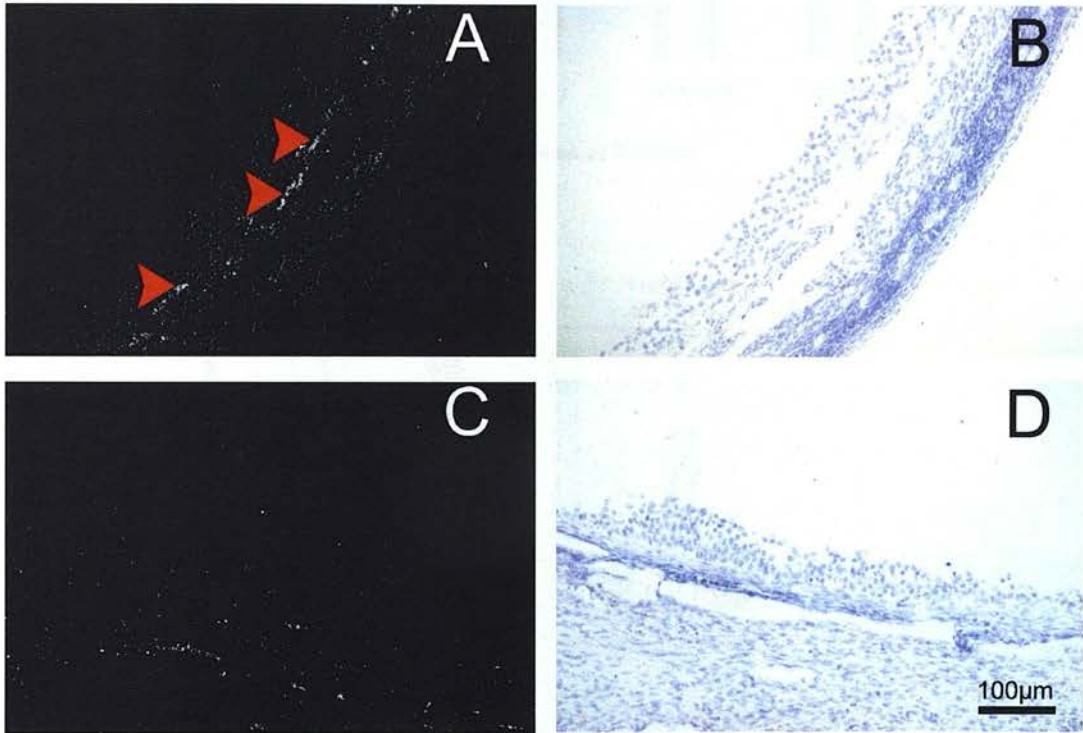


Figure 5.28 *In situ* hybridisation of VEGFR2 mRNA in dominant follicles.

VEGFR2 mRNA expression in dominant follicles from (A&B) late follicular control and (C&D) 5-10 day VEGF trap treated ovaries. Note the lack of expression after VEGF trap treatment. Red arrows highlight expression.

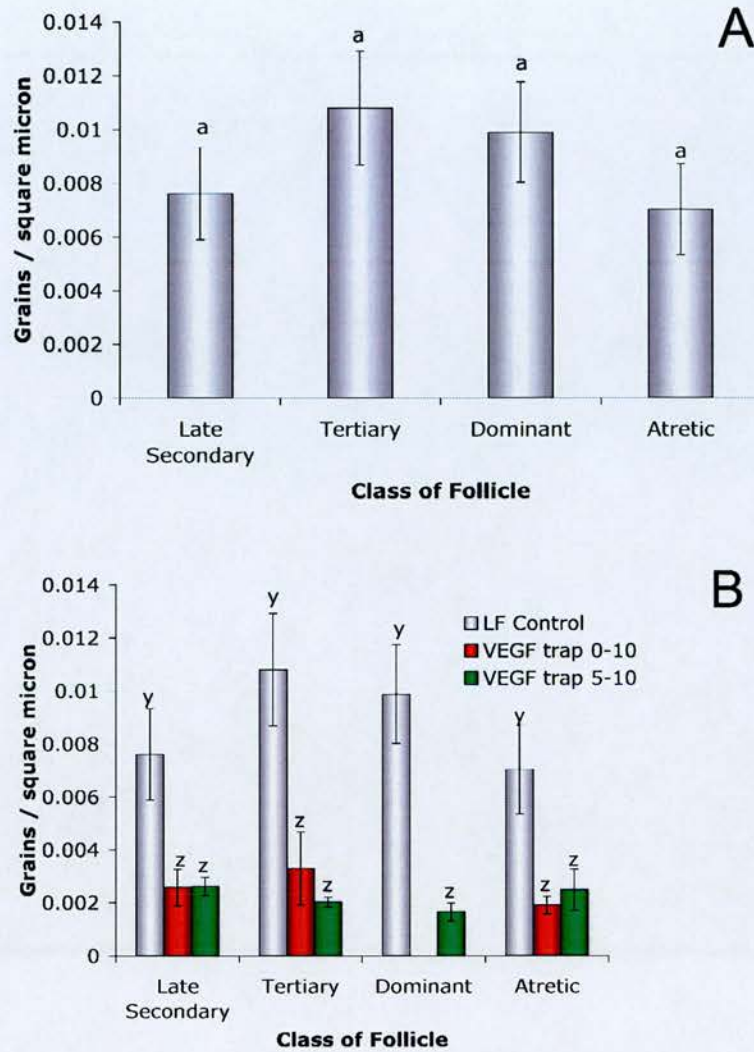


Figure 5.29 Quantitative analysis of thecal VEGFR2 mRNA expression.

(A) VEGFR2 mRNA expression in follicles from late follicular control ovaries. No significant variation was observed between different classes of follicle. (B) VEGF trap treatment for between days 0-10 and 5-10 days both significantly reduced expression in late secondary (0.0026 ± 0.0007 , $p=0.001$ and 0.0026 ± 0.0003 , $p=0.001$ respectively), tertiary (0.003 ± 0.001 , $p=0.001$ and 0.002 ± 0.0002 , $p<0.001$ respectively), atretic (0.0019 ± 0.0003 , $p=0.002$ and 0.0024 ± 0.0008 , $p=0.003$ respectively) and dominant follicles (0.0016 ± 0.0003 , $p=0.005$) present in the days 5-10 trap treatment group compared to late follicular controls (0.008 ± 0.0017 , 0.011 ± 0.0021 , 0.010 ± 0.0019 and 0.007 ± 0.0017 respectively). Values are means \pm sem. Superscripts a, b, y and z denote statistical significance. Statistics used: one way ANOVA with LSD post hoc tests.

5.3.10 Characteristics of the 'micro CL'

The follicles that did ovulate in the VEGF trap days 5-15 VEGF treated group formed small 'micro CL' after ovulation, these CLs were much smaller than those found in early luteal control ovaries. Haematoxylin and eosin stained sections of early luteal control and 'micro CL' are shown in **Figures 5.30A&B** respectively. Although not quantified the rate of cellular proliferation in the 'micro CLs' also appeared to be drastically reduced compared to early luteal controls (**Figures 5.30C** control, **5.30D** micro CL). The extent of vascularisation achieved in these micro CL also appeared to be severely reduced compared to early luteal controls (**Figures 5.30E** control, **5.30F** micro CL). The rate of angiogenesis in the micro CL also appeared to be severely inhibited compared to early luteal controls (**Figures 5.30G** control, **5.30H** micro CL).

Also, however not quantified, the expression of both VEGF and aromatase mRNA appeared to remain at levels similar to those found in early luteal controls. The punctate VEGF mRNA observed in the large steroid producing cells in the early luteal control tissue (**Figure 5.31A&B**) was also evident in the luteal cells in the micro CLs (**Figure 5.31E&F**). Similarly expression of aromatase mRNA observed in early luteal controls (**Figure 5.31C&D**) was also expressed in the micro CLs (**Figure 5.31G&H**).

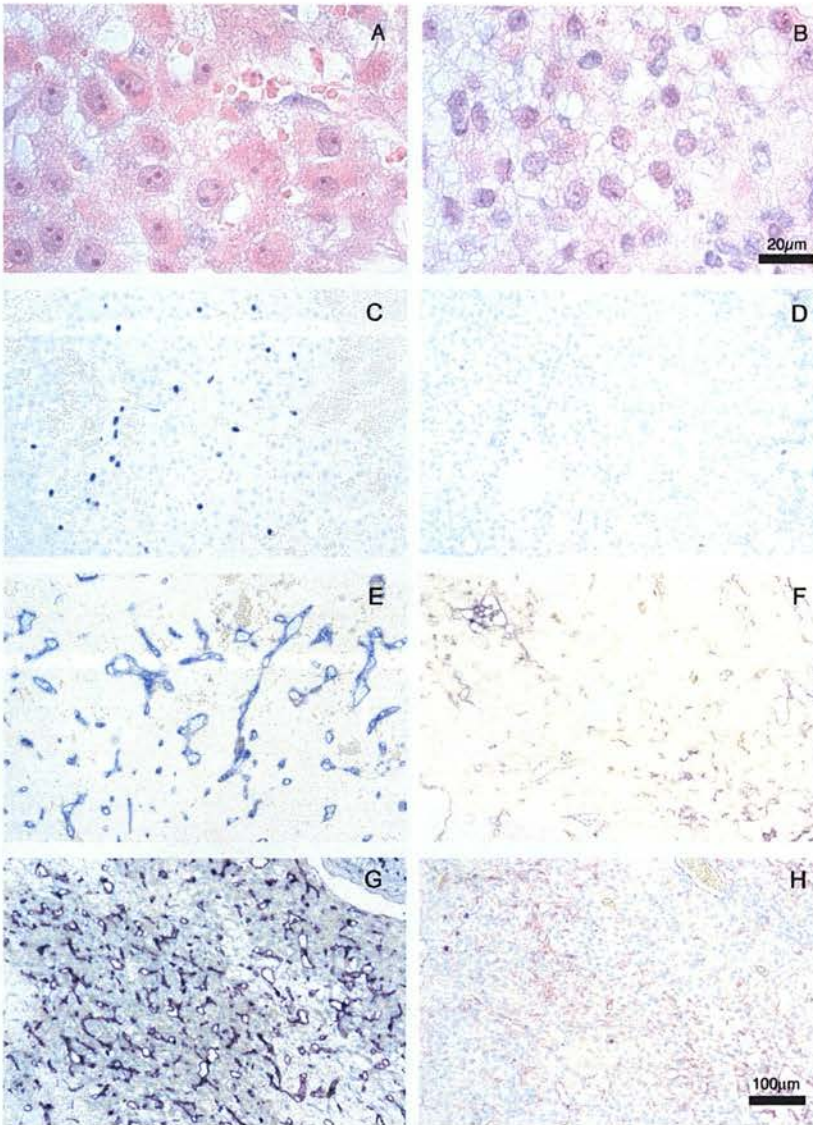


Figure 5.30 Characteristics of CL from early luteal control and VEGF trap treated micro CL ovaries.

Haematoxylin and eosin stained CL from early luteal control (A) and day 5-15 VEGF trap treated ovaries (B). BrdU stained CL from early luteal control (C) and day 5-15 VEGF trap treated ovaries (D). CD31 stained CL from early luteal control (E) and day 5-15 VEGF trap treated ovaries (F). Dual stained (black nuclear stain proliferating cells and red stained vasculature, co-localised staining indicates proliferating endothelial cells) CL from early luteal control (G) and day 5-15 VEGF trap treated ovaries (H).

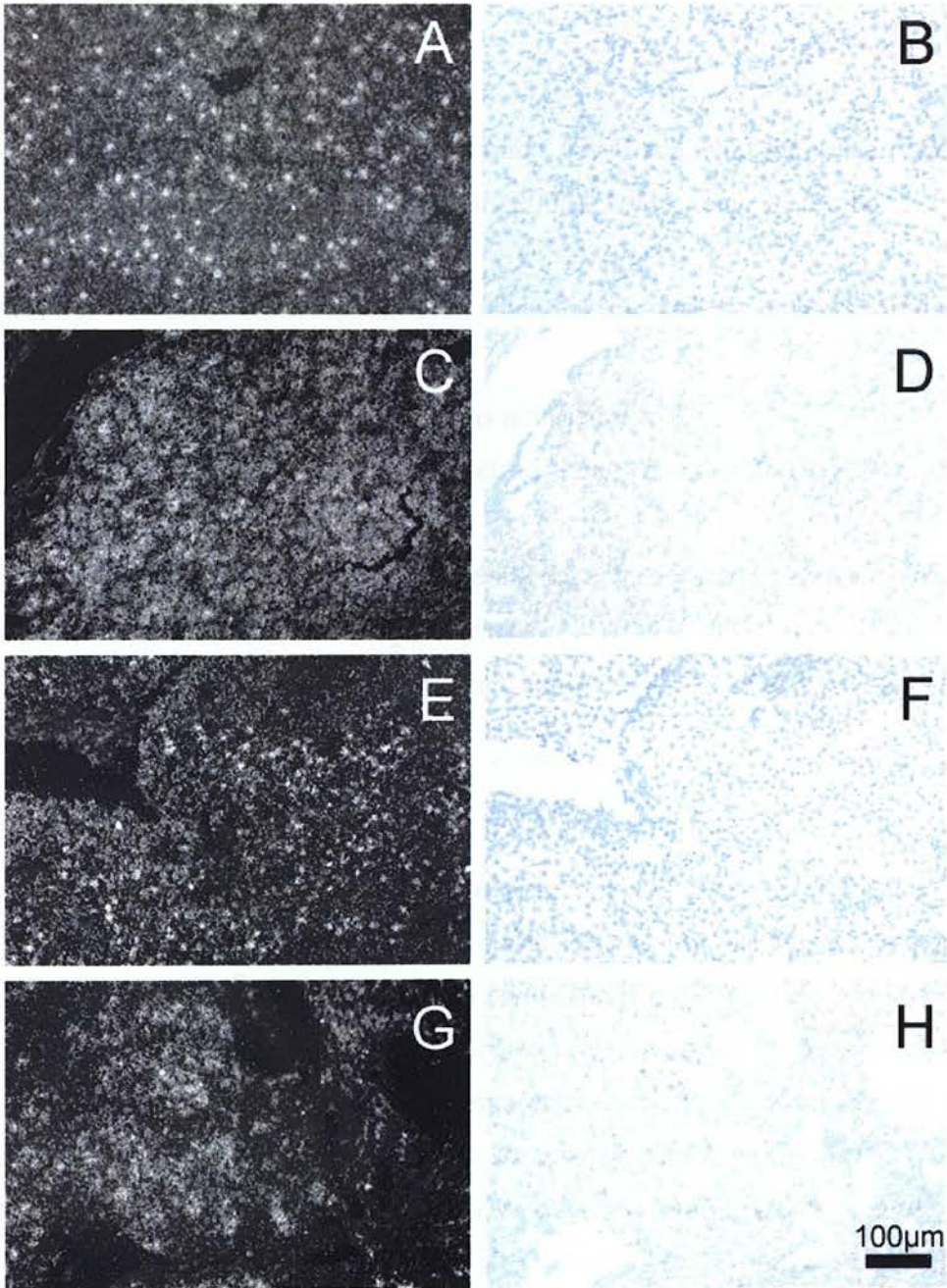


Figure 5.31 *In situ* hybridisation of VEGF and aromatase mRNA.

VEGF mRNA expression in early luteal control CL (A&B). Aromatase mRNA expression in early luteal control (C&D). VEGF mRNA expression in micro CL formed in day 5-15 VEGF trap treated animals (E&F). Aromatase mRNA expression in micro CL formed in day 5-15 VEGF trap treated animals (G&H).

5.4 Discussion

5.4.1 Key results

-A single injection of VEGF trap on follicular day 0 achieved the same results as those previously observed by Wulff *et al* (2002), when using multiple injections throughout the follicular phase.

-Ovulation was not definitively inhibited when the trap was administered in the mid follicular phase of the cycle.

-If ovulation did occur, a functional CL was not established.

-Both angiogenesis and thecal proliferation severely inhibited by VEGF trap treatment.

-In all follicles assessed, the volume of endothelial cells associated with each class of follicle was reduced after VEGF trap treatment.

-VEGF mRNA expression was maintained whilst VEGFR1 and VEGFR2 ligand dependant expression was inhibited.

5.4.2 Background

It is established that thecal development precedes follicular angiogenesis, with marmoset follicles developing a vascular supply during the late secondary stage of development, after the theca has begun to form (Wulff *et al.* 2001b). Angiogenesis and VEGF have been shown to be intimately involved in ovarian follicle development (Fraser and Wulff 2001, Zimmermann *et al.* 2001b, Zimmermann *et al.* 2003) and are necessary for normal follicular growth (Wulff *et al.* 2001b, Wulff *et al.* 2002). VEGF is also known as a potent vascular permeability factor on the basis of its ability to induce vascular leakage (Senger *et al.* 1983, Dvorak *et al.* 1995), and is thought to play a role as a permeability factor in the final stages of follicle development (Koos 1995) allowing the movement of fluid into the follicle and has been shown to be the mediator of hCG induced vascular permeability associated with OHSS (Gomez *et al.* 2002). Hence, the effect of VEGF trap treatment on follicular development and expansion in the marmoset ovary would be expected to result in suppression of dominant follicle expansion and ovulation.

Previous studies have examined the effects of inhibition of VEGF during

folliculogenesis and ovulation using either the VEGF trap (Wulff *et al.* 2002), earlier prototypes of the trap (Wulff *et al.* 2001b, Hazzard *et al.* 2002) and antibodies against VEGF (Zimmermann *et al.* 2001b) or VEGFR2 (Zimmermann *et al.* 2002, Zimmermann *et al.* 2003). Through the inhibition of VEGF, these studies established the requirement for VEGF during follicle vascularisation, antrum formation and ovulation. The requirement for angiogenesis and VEGF during formation and maintenance of a functional corpus luteum has also already been demonstrated (Ferrara *et al.* 1998, Fraser *et al.* 2000, Dickson *et al.* 2001, Wulff *et al.* 2001c). The most recently published work using the VEGF trap in the marmoset monkey involved a series of VEGF trap (25 mg/kg) injections, one injection every other day, throughout the follicular phase to prevent follicular vascularisation, antrum development and ovulation (Wulff *et al.* 2002).

5.4.3 Analysis

The use of a single injection of VEGF trap on follicular day 0 achieved the same results as those previously observed by Wulff *et al.* (2002), when using multiple injections. Also, in this current study the dissection of the follicular phase and assessment of the requirement for VEGF during both dominant follicle selection and the expansion of the dominant follicles through to ovulation has extended those previous findings. The results in this study reveal that inhibition of VEGF and thus both angiogenesis and vascular permeability have dramatic effects on both dominant follicle maturation and ovulation.

5.4.3.1 Cellular proliferation

The effects of VEGF trap treatment on thecal and endothelial cell proliferation were as expected from experience in previous studies, where thecal angiogenesis and thecal steroid producing cell proliferation in all stages of follicle development assessed, were almost completely suppressed (Wulff *et al.* 2002). The effect on both endothelial and theca proliferation was thought to be due to the effect of the lack of paracrine VEGF signalling from the granulosa cells acting on the endothelium in the theca. VEGF inhibition has an obvious role in reducing the rate of endothelial cell proliferation, however VEGF trap treatment also having a major effect on the rate of

theca cell proliferation may also possibly be a consequence of a reduction in the level of perceived LH stimulation due to reduced vessel permeability. The observed reduction in the rate of granulosa cell proliferation is thought to be a secondary effect of the reduction in thecal proliferation and vessel permeability as insufficient FSH stimulation would slow granulosa proliferation, although it is also possible that VEGF plays an autocrine role in promoting granulosa cell proliferation as it has recently been shown to protect granulosa cells from atresia (Greenaway *et al.* 2004). Although, *in situ* hybridisation results in this chapter do not localise VEGFR1 or VEGFR2 mRNA to the granulosa cells of any class of follicle. It is also very interesting to examine the localisation of the few proliferating granulosa cells after VEGF trap treatment, they were all located close to the oocyte. This observation is almost certainly due to oocyte secreted factors stimulating granulosa cell proliferation locally, candidate molecules for this role are GDF-9 and BMP-15, although interspecies variation of the effects elicited by these molecules has recently been reported (McNatty *et al.* 2005).

5.4.3.2 Vascularisation

In all follicles assessed, the volume of endothelial cells associated with each class of follicle was reduced after VEGF trap treatment. Again, this was as predicted from previous work using the VEGF trap (Wulff *et al.* 2002). However, the dominant follicles that developed in the VEGF trap days 5-10 and 5-15 treatment groups had a well developed vascular network penetrating into the theca up to basement membrane separating the theca from the granulosa cells. This is as far as the vascular network would be expected to penetrate until the basement membrane breaks down during ovulation. It is therefore proposed that even though the angiogenesis usually associated with dominant follicle maturation was severely inhibited, the dominant follicles already selected by follicular day 5 had a sufficient vascular supply to continue developing up to and past the point of ovulation. Although the necessity for angiogenesis again became apparent after ovulation as follicles that had ovulated did not form functional CLs with, although not quantified in this chapter, a severely reduced vascular supply. Thus, the crux of whether these dominant follicles have the potential to ovulated or not is dependent upon the state of

the follicle vasculature at the start of VEGF treatment.

5.4.3.3 mRNA

It was predicted that the inhibition of VEGF using the VEGF trap would result in the increased expression of VEGF mRNA, however no significant increase in VEGF mRNA expression was observed in these studies. Increases in VEGF mRNA expression have been reported after VEGF trap treatment, although these increases were not quantified in those previous studies (Wulff *et al.* 2002). The decrease in mRNA expression for the VEGF receptors due to lack of the positive feed back mechanisms that stimulate VEGFR1 and VEGFR2 by VEGF was also as previously reported (Barleon *et al.* 1997, Shen *et al.* 1998, Sato *et al.* 2000, Wulff *et al.* 2002).

5.4.3.4 Steroid hormone production

The VEGF traps effects on progesterone secretion observed after each of the VEGF treatments were as predicted. Only small differences would be expected between the late follicular controls and treatment with the VEGF trap for both days 0-10 or 5-10, as follicular phase (low) progesterone levels would be expected in control animals as well as those treated with the VEGF trap. The suppression of progesterone secretion in the animals that ovulated from the VEGF trap days 5-15 treatment group were also as would be expected as luteal angiogenesis and therefore establishment of a functional corpus luteum was suppressed by VEGF trap treatment, this also concurs with findings administering the VEGF trap in the macaque (Fraser *et al.* 2005b). The suppression of oestradiol production after VEGF trap treatment for both days 0-10 and 5-10 is concurrent with the inhibition of follicular development, particularly in the days 0-10 group. In the 5-10 day VEGF trap treatment group oestradiol was suppressed but residual production remained. This moderate production from the selected follicles again highlights the persistence of the selected follicles to continue their development, all be it at a reduced level.

5.4.3.5 Morphology

The morphological appearance and the major structures in the ovaries after VEGF trap treatment are of great interest. The results from the days 0-10 VEGF trap treatment group were as would have been predicted, with a complete absence of

either dominant follicle development or newly ovulated follicles due to the inhibition of angiogenesis in developing follicles that is essential for follicle selection (Wulff *et al.* 2002). VEGF trap treatment for days 0-10 also caused significant reductions in paired ovary weights, presumably through the inhibition of follicle development and suppression of vessel permeability. VEGF trap treatment for days 5-10 resulted in ovaries displaying a range of phenotypes similar to those observed in late follicular control ovaries. Preovulatory and recently ovulated follicles could be observed in the ovaries, although the size of the preovulatory follicles was reduced compared to those in late follicular control ovaries. Other phenotypes included follicles in which the granulosa cells appeared to be luteinising but have not ruptured, although this can also be observed during ovulation in late follicular control ovaries.

The 5-15 days VEGF trap treatment resulted in a range of outcomes different from those seen in early luteal control ovaries. These ranged from the presence of healthy follicles of large preovulatory size to the presence of new 'micro CL' in the ovary. The most likely explanation for this heterogeneity in results is the level in gonadotrophin the developing follicles were exposed during treatment. As already discussed, these follicles have an established vascular supply, however vessel permeability must also be considered.

5.4.3.6 Vessel permeability

Vascular cells in different organs acquire specialized characteristics that allow them to perform organ specific functions (Ruoslahti and Rajotte 2000). For example, endothelial cells in the brain are tightly linked to each other and are surrounded by numerous pericytes that form a barrier to protect the brain cells from potentially toxic blood-derived molecules. In contrast, vessels in endocrine glands, i.e. the ovarian follicle, are 'leaky' and their endothelial cells have fenestrations, which allows hormone trafficking to occur (Carmeliet 2004). VEGF is also known as VPF on basis of its ability to induce vascular leakage (Senger *et al.* 1983, Dvorak *et al.* 1995) has been found to induce these fenestrations in endothelial cells of small venules and capillaries (Roberts and Palade 1995). Specific inhibition of VEGFR2 is known to inhibit vascular permeability (Gomez *et al.* 2002). Therefore, inhibition of VEGF using the VEGF trap causes the endothelial cells in the developing follicles

and the 'micro CL' to behave more like the vasculature observed in the brain and hence minimizes that amount of hormone trafficking, thus inhibiting proper endocrine function. This restriction on vascular permeability has to be considered when assessing the results from the ovaries in both the VEGF trap days 5-10 and 5-15 treatment groups.

5.4.3.7 Removal of negative feedback...

Unfortunately the levels of both LH and FSH could not be measured in this study because these assays are problematic in the marmoset and there are restrictions in the volume of plasma that can be collected. However, extrapolation from other studies using the VEGF trap in the macaque may be able to explain these observation. With angiogenesis inhibited and vascular permeability reduced, the amount of gonadotrophin perceivable by the developing dominant follicles in the day 5-10 and 5-15 treatment groups would be severely reduced. However, after VEGF trap treatment during the follicular phase in the macaque, both FSH and LH become extremely elevated. FSH levels rise to well above those seen at any stage of the normal cycle and LH rises to levels equivalent to those observed during the LH surge (Fraser *et al.* 2005b). The gonadotrophins remain at these elevated levels until the circulating levels of the VEGF trap have subsided to very low levels compared to doses given in this study. Similar results have also been demonstrated in the macaque using anti-VEGFR2 antibody during the early follicular period (Zimmermann *et al.* 2001b). This hypergonadotrophism is thought to arise in response to the removal of negative feed back from the developing follicles as it is associated with sharp decreases in both oestradiol (Fraser *et al.* 2005b) and inhibin B (H.M. Fraser, unpublished results). This is concurrent with the significant inhibition of oestradiol observed in the chapter.

It is interesting to note that the punctate VEGF mRNA expression observed in Chapter 3, hypothesised to be stimulated by the LH surge, was also evident in the dominant follicles present in the VEGF trap days 5-10 treatment group suggesting that high levels of LH were being perceived in the theca of these follicles. It is also interesting to comment upon the morphology of the luteal cells present in follicles with varying degrees of luteinisation and in the micro CLs. The luteal cells appeared

healthy with few morphological changes and little sign of atresia. This is in contrast with observations on the characteristic effect of withdrawal of LH during the early luteal phase where cellular degradation is prevalent (Dickson and Fraser 2000), suggesting that significant levels of LH were being perceived by the cells in this study. This also concurs with the observations from ovaries in the VEGF trap treated days 5-15 group that ovulated that appear to have steroidogenically active luteal cells expressing both aromatase and VEGF mRNA in the 'micro CL' formed after ovulation. The steroidogenic luteal cells would be either starved of steroid hormone precursors or unable to secrete the hormones that they produce due to both the lack of vasculature and the reduced permeability of the available blood vessels. The failure of ovulated follicles to form functioning CLs has been shown to be primarily due to the lack of vascularisation of the luteal tissue (Wulff *et al.* 2001c). However the remaining hormone producing cells that would be trying to drive the establishment of the CL via VEGF production are still expressing both VEGF and aromatase mRNA and show little sign of luteolytic change as would be expected after removal of LH stimulation (Fraser *et al.* 1999b, Dickson and Fraser 2000).

The results from the VEGF trap days 5-15 treatment group are similar to those of Zimmermann (Zimmermann *et al.* 2001a), when administering an anti-VEGFR2 antibody immediately prior to induction of ovulation in the mouse. Blocking VEGFR2 function results in a reduced number of corpora lutea present in the ovaries and a major reduction in vasculature in the corpora lutea that were present. They also found that treatment had reduced secretion of progesterone, but not by the level to which they had expected (Zimmermann *et al.* 2001a). Again this is similar to the results reported in this chapter where there is both morphological and *in situ* hybridisation evidence that luteinisation had occurred. Similar results have also been demonstrated in the rat (S. J. Wiegand, personal communication). Combining the changes in vascular permeability and the vastly elevated level of gonadotrophins observed in other species, it is clear that the VEGF trap treatment drastically alters the ovarian environment the developing follicles were exposed to.

5.4.4 Conclusion

In conclusion, this study has demonstrated that inhibition of VEGF and hence

angiogenesis is able to prevent dominant follicle selection and ovulation when administered during follicle selection. Administration after dominant follicle selection has taken place, although having drastic effect on angiogenesis, vessel permeability and follicle expansion, does not inhibit ovulation to the extent of which may have been predicted from previous studies. VEGF trap treatment drastically alters the ovarian environment and follicle development. This study also confirms that VEGF mediate angiogenesis is essential for establishment of the corpus luteum.

5.4.5 The next step

VEGF, as studied in this chapter, is known as one of the most pivotal mediators of the angiogenic process. However, it is not the only angiogenic mitogen thought to be functioning in the ovary. In 2001 a novel endothelial cell mitogen, endocrine gland vascular endothelial growth factor (EG-VEGF), was described (Li *et al* 2001, LeCouter *et al.* 2001). Unrelated to the VEGF family, or any of the other known families of endothelial cell mitogens (LeCouter and Ferrara 2003), EG-VEGF posed a very exciting prospect as it appeared that a pro-angiogenic, specific to the endocrine glands had been discovered. The next chapter looks at the expression of this new angiogenic factor in both the human and the marmoset.

**Chapter 6 EG-VEGF mRNA Expression in the
Marmoset and the Human**

6.1 Introduction

EG-VEGF is a novel endothelial cell mitogen discovered in 2001 (Li *et al.* 2001, LeCouter *et al.* 2001) unrelated to the VEGF family, or any of the other known families of endothelial cell mitogens (LeCouter and Ferrara 2003). In the human ovary EG-VEGF is thought to play a role in both early folliculogenesis and corpus luteum development (Ferrara *et al.* 2003a) and has also been implicated as having a potential function in PCOS (Ferrara *et al.* 2003a). EG-VEGF has recently also been implicated in testicular development and function (Samson *et al.* 2004) and the expression pattern of EG-VEGF in the endometrium is consistent with its regulation by the steroid hormones (Battersby *et al.* 2004). Given the tissue specific expression of EG-VEGF to the endocrine glands, it is potentially as a very exciting new target for tissue specific modulation and inhibition of angiogenesis in the reproductive organs. The aim of this study was to investigate whether EG-VEGF is expressed in the marmoset and if so, which cell types its expression localised to. This was assessed by *in situ* hybridisation in various marmoset tissues.

6.1.1 Discovery of EG-VEGF

EG-VEGF or Prokineticin-1 (PK-1), as it was originally termed, was first identified in human gastrointestinal smooth muscle in 2001 (Li *et al.* 2001). It was discovered as one of a pair of human homologues to either the mamba intestinal toxin (PK-1) (Schweitz *et al.* 1999) or the yellow bellied toad secreted protein Bv8 (PK-2) (Joubert and Strydom 1980). Later that year, in a separate study, EG-VEGF was identified from a library of purified human secreted proteins for its ability to induce proliferation in primary bovine adrenal-cortex-derived capillary endothelial (ACE) cells and termed endocrine-gland-derived vascular endothelial growth factor (LeCouter *et al.* 2001). EG-VEGF promotes proliferation, survival and chemotaxis of ACE cells but does not stimulate the same reactions in other endothelial cell types such as human umbilical vein endothelial cells (LeCouter *et al.* 2001).

6.1.2 Expression of EG-VEGF

Northern blot analysis of RNAs from a range of human tissues has revealed EG-

VEGF expression in ovary, testis, adrenal gland, placenta (LeCouter *et al.* 2001) and stomach (Li *et al.* 2001). EG-VEGF mRNA has been localised to the human CL, where its expression is not detectable until the early- to mid-stage of luteal development, persisting at least throughout the mid-luteal phase, a point at which VEGF mRNA expression is much reduced (Ferrara *et al.* 2003a), presumably giving EG-VEGF an alternate role to VEGF in the corpus luteum. Ferrara and colleagues also localised EG-VEGF mRNA to the granulosa cells of small follicles that are initiating folliculogenesis and also in the stroma and theca of PCOS ovaries (Ferrara *et al.* 2003a) giving EG-VEGF a complimentary pattern of expression to that of VEGF (Ferrara *et al.* 2003a). EG-VEGF has also been identified in the macaque corpus luteum (R. L. Stouffer, personal communication). Also, more recently, EG-VEGF has been implicated in playing a role in both early testis development and adult testis function in the human (Samson *et al.* 2004).

6.1.3 Structure of EG-VEGF

EG-VEGF does not belong to the VEGF family, or any of the other known families of endothelial cell mitogens (LeCouter and Ferrara 2003). It is a member of a structurally related class of peptides including the digestive enzyme colipase; the *Xenopus* head organiser gene, dickkopf (Glinka *et al.* 1998); mamba intestinal toxin 1 (MIT1) (Schweitz *et al.* 1999) also known as venom protein A (VPRA) (Joubert and Strydom 1980), a non-toxic protein purified from the venom of the black mamba snake; and the secreted yellow bellied toad protein Bv8 (Joubert and Strydom 1980). Both VPRA (Schweitz *et al.* 1999) and Bv8 (Mollay *et al.* 1999) have been shown to induce gastrointestinal motility. The common structural motif between these proteins is 10 cysteine residues that form five disulfide bridges within a conserved span (Aravind and Koonin 1998). G proteins are thought to be involved in EG-VEGF signalling (Li *et al.* 2001) and EG-VEGF has been identified as the ligand for two, until recently, orphan G protein-coupled receptors (GPCRs) of the neuropeptide Y receptor class (Lin *et al.* 2002, Masuda *et al.* 2002).

6.2 Materials and Methods

6.2.1 Human luteal tissue

Laboratory stocks of human luteal tissue collected by the Obstetrics and Gynaecology department in the New Royal Infirmary of Edinburgh were used in this study. Briefly, corpora lutea were enucleated from healthy woman aged 32-45 with regular menstrual cycles, who were undergoing hysterectomy and who had not received hormonal treatment during the previous three months. Informed consent was obtained from all patients before tissue collection. Once collected, portions of the corpus luteum were fixed in NBF for 24 hours as described for marmoset tissue in Chapter 2. Five-micron sections were then taken from the tissue blocks as and when required.

6.2.2 Marmoset tissue

Laboratory stocks of marmoset organs obtained from control animals were used for this study. Ovaries at different stages of the cycle were used along with uterine tissue and portions of small intestine.

6.2.3 *In situ* hybridisation

In situ hybridisation was performed as described in Chapter 2. EG-VEGF slides were left exposed for 3 weeks.

6.3 Results

In situ hybridisation revealed EG-VEGF mRNA expression in the granulosa lutein cells of the mid stage corpus luteum. No hybridisation was evident in any of the marmoset tissues (**Figure 6.1**).

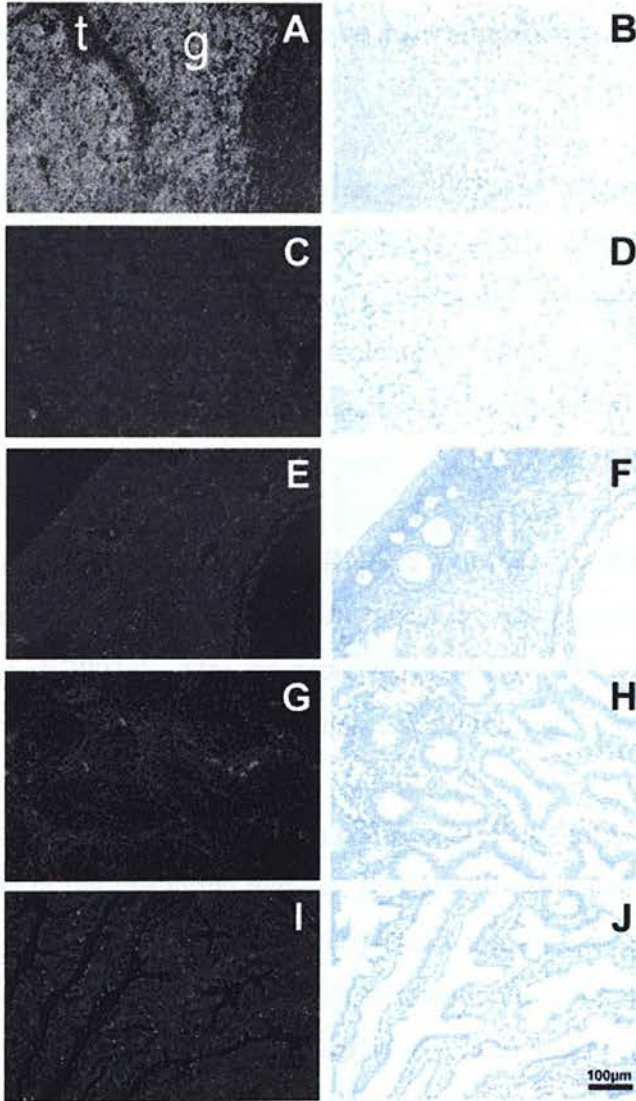


Figure 6.1 EG-VEGF mRNA expression.

All figures shown are corresponding dark and light field images. EG-VEGF mRNA expression in: The mid-stage human corpus luteum (A&B); the mid-stage marmoset corpus luteum (C&D); the marmoset ovarian follicle (E&F); the marmoset placenta (G&H); and the marmoset small intestine (I&J). The only detectable expression was observed in the human CL. g=granulosa, t=theca.

6.4 Discussion

6.4.1 Key results

-EG-VEGF mRNA is expressed in the granulosa lutein cells of the human corpus luteum.

-No EG-VEGF mRNA was detected in any of the marmoset tissue analysed.

6.4.2 Background

EG-VEGF was first discovered in 2001 (Li *et al.* 2001, LeCouter *et al.* 2001) and to this day poses intriguing questions regarding organ specific regulation of angiogenesis and offers a very exciting opportunity to modulate and inhibit angiogenesis in reproductive tissues specifically, which will lead to a better understanding of, or even treatments for, angiogenic related reproductive disorders.

6.4.3 Analysis

This chapter identifies that EG-VEGF mRNA is expressed in the granulosa lutein cells of the human corpus luteum and not the thecal lutein cells as suggested by other studies (Ferrara *et al.* 2003a). Also, no expression of EG-VEGF was observed in any marmoset tissue, suggesting that the marmoset does not make use of the EG-VEGF system for endocrine gland angiogenesis.

In the human, the expression pattern of EG-VEGF suggests that it is regulated by specific factors in the endocrine glands. To this end, consensus-binding sites for the NR5A1 orphan nuclear receptor are present within the human EG-VEGF promoter (LeCouter *et al.* 2003). NR5A1 is considered to be a key regulator of endocrine development and function (Parker *et al.* 2002) and the NR5A1 protein is strongly expressed in both theca and granulosa cells of antral follicles (Takayama *et al.* 1995). Recent data from the human endometrium suggests that EG-VEGF expression may be regulated by progesterone as its expression is elevated during the secretory phase of the cycle (Battersby *et al.* 2004). This may occur by a direct effect of progesterone on EG-VEGF gene transcription, as analysis of the promoter region of the human EG-VEGF gene also identifies two putative progesterone-receptor binding sites (Battersby *et al.* 2004).

To address the question of why no EG-VEGF mRNA was detected in any of the marmoset tissue, it is interesting to look at what is known about cross species expression of EG-VEGF. For example there is a strong possibility that the lack of EG-VEGF expression in the marmoset results from changes in the gene promoter sequence. Examples of divergent regulation of the EG-VEGF gene is found between different species. For example EG-VEGF has also been identified in the mouse. The mouse EG-VEGF gene encodes a peptide 88% identical and 93% homologous to the human protein (LeCouter *et al.* 2003). Mouse EG-VEGF also maps to a region of chromosome 3 that is syntenic with human chromosome 1p13.1, the locus for human EG-VEGF (LeCouter *et al.* 2003). However, the expression pattern of mouse EG-VEGF mRNA is very different from that of human. The predominant sites of mouse EG-VEGF mRNA expression are hepatocytes in the foetal liver and the epithelial tubule cells in the adult kidney (LeCouter *et al.* 2003). The NR5A1 nuclear receptor present within the human EG-VEGF promoter is absent in the mouse promoter (LeCouter *et al.* 2003), giving EG-VEGF a very different role between these species. Therefore, although the EG-VEGF gene may be well conserved between humans and mice, and show the same pattern of expression in old world primates (humans and macaques), the marmoset being a new world primate may have evolved either mutations in the EG-VEGF gene or different mechanisms of regulation of the EG-VEGF gene, leading to lack of expression in reproductive tissue. To assess this hypothesis, the sequence of the marmoset EG-VEGF gene would need to be analysed, looking for either mutations in the promoter sequence arresting its transcription or other regulatory elements in the promoter giving it a different pattern of expression from other primates.

The findings in this chapter that contradict Ferrara and colleagues previous work on localisation of EG-VEGF in the human CL (Ferrara *et al.* 2003a) are likely to be due to the misidentification of the cell type the mRNA was observed in. This chapter clearly identifies the granulosa lutein cells as the cells expressing EG-VEGF mRNA. Further work by H. M. Fraser and colleagues has conclusively demonstrated EG-VEGF mRNA expression in granulosa lutein cells by immunocytochemistry of 3β -HSD, a granulosa lutein cell specific enzyme and 17α hydroxylase, to localise theca derived lutein cells (Fraser *et al.* 2005a). They have also demonstrated that

EG-VEGF expression increases as the CL matures (Fraser *et al.* 2005a). EG-VEGF has also been found to be expressed in immortalized human granulosa lutein cells (Kisliouk *et al.* 2003). It is interesting to note that once the marmoset CL has formed there is no distinguishable differences between the granulosa and theca lutein cells, this difference in cell lineage is obvious in both the human and the macaque CL. This difference in CL structure may have implications in the differential expression in EG-VEGF mRNA between human and marmoset observed in this chapter.

Another possibility that should not be overlooked is that the marmoset EG-VEGF mRNA sequence may have diverged so far from human sequence, against which the probe was designed, that the probe was unable to hybridise to marmoset EG-VEGF mRNA. However, considering the conservation between the human and the mouse sequence, this is unlikely. Further work on the sequencing of the marmoset EG-VEGF gene will help to reveal both its function and pattern of expression in the marmoset.

6.4.4 Conclusion

In conclusion, the localisation of EG-VEGF to the granulosa lutein cells of the human CL provides support for the role of EG-VEGF in the corpus luteum with respect to the regulation of luteal angiogenesis. The lack of EG-VEGF expression in the marmoset tissues requires further investigation, but indicates variation between species that raises the question of how important EG-VEGF is when compared to other ubiquitously expressed angiogenic factors such as VEGF in the regulation of luteal angiogenesis and vessel stabilisation.

Chapter 7 General Discussion

7.1 Introduction

This thesis has presented the results of a series of investigations into the control of ovarian follicular angiogenesis in the primate and its *in vivo* manipulation in the marmoset monkey. The results from each experimental chapter have been discussed in the relevant chapters. This section therefore provides an opportunity to summarise the main findings of the thesis, discuss the relevant merits of the model used for inhibition of follicular angiogenesis, clinical implications of the thesis findings in both reproductive medicine and other angiogenesis related disorders and to suggest areas for further study.

7.2 The findings of this thesis

Angiogenesis has long been hypothesised to play an essential role in follicular development and the establishment of follicle dominance (Zelevnik *et al.* 1981). Subsequently, it has been demonstrated by direct *in vivo* manipulation that VEGF is one of the principal angiogenic factors involved in follicular angiogenesis (Wulff *et al.* 2001b, Zimmermann *et al.* 2001b, Wulff *et al.* 2002, Zimmermann *et al.* 2002). VEGF mRNA and protein have been localised in the ovary to the granulosa cells of the developing follicles, concurrent with the acquisition of steroidogenic activity, and the theca of preovulatory follicles (Phillips *et al.* 1990, Ravindranath *et al.* 1992, Shweiki *et al.* 1993, Taylor *et al.* 2004). It has also been demonstrated that VEGF expression in granulosa cells of preovulatory follicles is up regulated by FSH stimulation *in vitro* (Christenson and Stouffer 1997, Hazzard *et al.* 1999). This evidence suggested that the expression is hormonally regulated. Therefore, the rationale behind the first hypothesis was to use the marmoset monkey, a species in which the development of the follicular vasculature has been described in great detail (Wulff *et al.* 2001b, Wulff *et al.* 2002), as a model for *in vivo* inhibition of the gonadotrophins during the follicular phase. It was postulated that their suppression would inhibit follicular angiogenesis, as had been demonstrated for the CL using a similar approach in the luteal phase in the marmoset (Dickson and Fraser 2000). The finding that blocking gonadotrophin release did not result in complete inhibition of follicular angiogenesis as had been predicted raises the possibility that there are other

factors and paracrine mechanisms regulating VEGF and angiogenesis in the primate ovarian follicle. For example the oocyte-granulosa interactions that coordinate follicle development (Eppig 2001, Eppig *et al.* 2002) could play an important role in VEGF regulation in the granulosa cells. It follows that the reason GnRH antagonist treatment is more effective in suppressing early luteal angiogenesis (Dickson and Fraser 2000) is likely to be because the corpus luteum is heavily dependent upon LH (Fraser *et al.* 1986). This dependence would include the local compensatory mechanisms that are relatively gonadotrophin independent in the developing follicle with the coordinate stimulation of both the gonadotrophins and the oocyte both playing their part.

The *in vivo* inhibition of VEGF has been shown to severely suppress thecal vascularisation, follicle development and inhibit the ovarian cycle (Wulff *et al.* 2001b, Zimmermann *et al.* 2001b, Wulff *et al.* 2002, Zimmermann *et al.* 2002). The second hypothesis to be tested in this thesis was based on these previous studies and was that VEGF is essential for the increasing permeability and the growth of the follicles selected for dominance by the mid-follicular point in the cycle. *In vivo* inhibition of VEGF caused dramatic reductions in angiogenesis and in VEGF receptor expression but did not reliably prevent dominant follicle growth or ovulation once dominant follicle selection had occurred. As mentioned in the previous paragraph the role of the oocyte was again highlighted in this study where granulosa proliferation was marginally maintained, presumably by stimulation from oocyte secreted factors. Comparing these results to the direct inhibition of angiogenesis throughout the follicular phase with the VEGF trap (Wulff *et al.* 2002) has demonstrated that as the developing follicle grows it becomes less dependant upon angiogenesis. Also, comparing these results to the direct inhibition of VEGF in the CL (Ferrara *et al.* 1998, Fraser *et al.* 2000, Wulff *et al.* 2001c) indicates that luteal function is more dependant upon VEGF than the developing dominant follicle.

At this point it is of interest to compare the results of inhibiting the gonadotrophins with a GnRH antagonist and the effects of the specific targeting of VEGF using the VEGF trap, on follicular angiogenesis and development. A much higher degree of inhibition of follicular angiogenesis was observed when directly inhibiting VEGF rather than inhibiting the gonadotrophins (**Figure 7.1**). In the case

of the GnRH antagonist treatment, angiogenesis continued in the follicles, although at a reduced level, as the VEGF system was not severely inhibited by the treatment. The GnRH antagonist inhibition of gonadotrophin secretion may have had little effect on the ability of hypoxia and local paracrine mechanisms to mediate or modulate VEGF synthesis and action in the ovarian follicle. These compensatory mechanisms may have been responsible for continued angiogenesis. In contrast, the VEGF trap had a much more dramatic effect in follicular angiogenesis and it is suggested that no matter what compensatory mechanisms came into operation to restore VEGF action, the VEGF trap negated them.

The effect of both GnRH antagonist or VEGF trap treatment from the early follicular period was as had been predicted, with both treatments inhibiting the selection and development of a dominant follicle. When the GnRH antagonist or VEGF trap were given from the mid-follicular phase the selected dominant follicles were severely effected in terms of their 'health', some becoming anovulatory and displaying signs of atresia, however neither treatment managed to consistently inhibit expansion of the selected follicles to a preovulatory size. This suggests that neither the gonadotrophic support nor the normal physiological vessel permeability and angiogenesis associated with the development of the dominant follicle play major roles in its mechanical expansion. There is evidence that reductions in the frequency of the GnRH pulse has severe inhibitory effects on the quality of follicle development in the rhesus monkey (Pohl *et al* 1983), although these studies determine follicle development and quality in terms of the gonadotrophins and circulating progesterone and oestradiol concentrations, and do not look directly at the mechanical expansion of the follicle, where as evidence from this thesis suggests that there is a dissociation between mechanical expansion and follicular steroid production as a follicle matures.

Finally, the identification of the novel angiogenic molecule EG-VEGF in the granulosa lutein cells of the human CL is a very exciting and intriguing result, which has fuelled further research into its regulation and pattern of expression throughout the luteal phase.

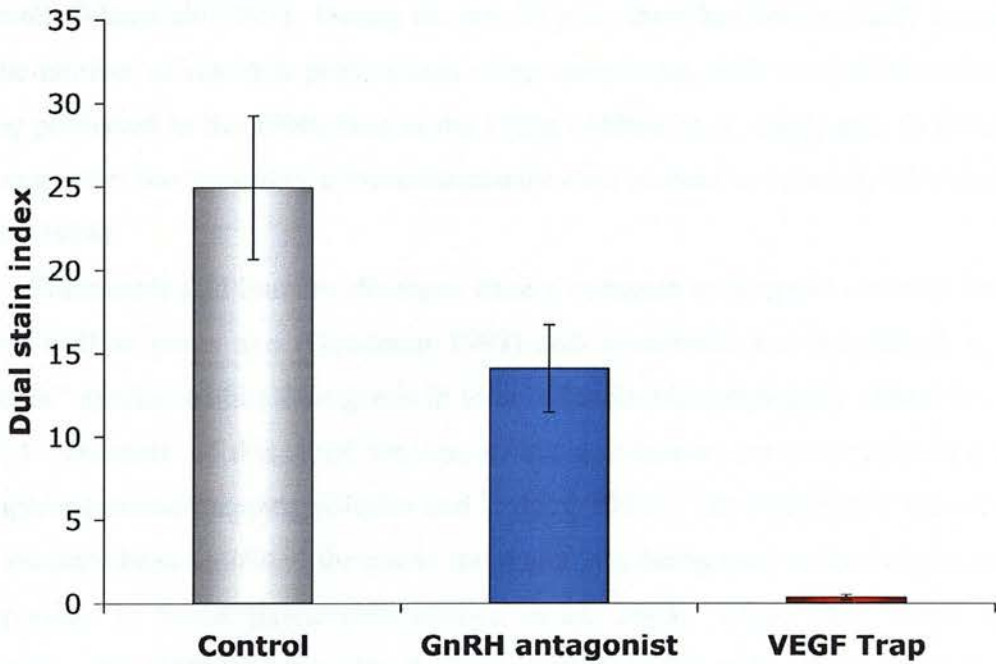


Figure 7.1 Comparison of the inhibition of follicular endothelial proliferation.

The effect of the administration of either a GnRH antagonist or the VEGF trap on follicular endothelial cell proliferation. Note the difference in suppression between the GnRH antagonist (14.1 ± 2.6) and the VEGF trap (0.3 ± 0.2) compared to controls (25.0 ± 4.3). Sources are Chapter 3 and Chapter 5. Values are the combined means of late secondary, tertiary and dominant follicles \pm sem.

7.3 The marmoset model

7.3.1 Use in biomedical research

The common marmoset is used extensively in biomedical research and has typically been used in reproductive biology, neuroscience, infectious disease and behavioural research (Mansfield 2003). During the last 40 years there has been a steady increase in the number of research publications using marmosets, with over 40 times more being published in the 1990s than in the 1960s (Abbott *et al.* 2003) and, in Europe, the marmoset has become the most commonly used primate in research laboratories (Scott 1994).

Marmosets and humans diverged from a common anthropoid ancestor about 35-40 million years ago (Goodman 1999) and marmosets are considered as an “anchor” species within their genus in terms of molecular phylogeny (Abbott *et al.* 2003). Because of this, they are one of six non-human primates proposed for complete genome mapping (Eichler and DeJong 2002). The Affymetrix GeneChip has recently been validated for use in the marmoset, being used to investigate gene expression in brain tissue (Marvanova *et al.* 2003). Also, their small size (approximately 350g) compared to the macaque (12-15 kg) makes them ideal for use when only small amounts of highly valuable recombinant or synthetically produced antagonist are available for *in vivo* studies.

As far as female reproductive biology is concerned, the marmoset has an ovarian cycle of approximately 28 days with ovulation occurring on day 10 when synchronised using PG injection (Gilchrist *et al.* 2001) and shows similar gonadotrophin and steroid hormone profiles to the human throughout the cycle (Harlow *et al.* 1984). Their use in reproductive biology has facilitated the development of human embryonic stem cell biology (Thomson *et al.* 1996, Thomson *et al.* 1998) and they are an oft used model in the study of both luteal angiogenesis (Dickson *et al.* 2001, Fraser and Lunn 2001, Wulff *et al.* 2001a, Wulff *et al.* 2001c, Rowe *et al.* 2002) and luteal regression (Fraser *et al.* 1995a, Fraser *et al.* 1995b, Duncan *et al.* 1998, Fraser *et al.* 1999b).

7.3.2 The marmoset as a model for follicular angiogenesis

The marmoset is a commonly used primate for the study of female reproductive medicine (Fraser and Lunn 1999, Mansfield 2003). The availability of healthy, cyclic marmosets, through our in-house breeding programme permitted their use in these terminal studies, where it is necessary to collect tissue for cellular and molecular analysis.

The studies in this thesis utilised the marmoset monkey as an experimental model in which to investigate factors regulating ovarian follicular angiogenesis, a species in which the development of the follicular vasculature has been described in great detail (Wulff *et al.* 2001b, Wulff *et al.* 2002). Using prostaglandin injection, the ability to synchronise the timing of the ovarian cycle in such a precise fashion makes the marmoset an extremely useful and practical model with which to study primate reproductive biology. Previous work in our laboratory has used the marmoset model to great effect, dissecting the necessity for both the gonadotrophins and VEGF during precise time points in the luteal phase of the cycle (Fraser *et al.* 2000, Dickson *et al.* 2001). The studies in this thesis applied those timing techniques to study the effects of treatment at specific points in the follicular phase of the cycle.

7.3.3 Limitations of the model

The marmoset model does have its limitations when extrapolating data to the human. The marmoset is not a mono-ovulatory species, it does not menstruate as humans do, and there is debate over the existence of an age related decline in the rate of ovulation (Tardif and Jaquish 1997, Gilchrist *et al.* 2001). The reproductive cycles of old world primates are more similar to the human, but the invasiveness of the techniques used in these studies dictated that higher primates were not used. Also, as Chapter 6 of this thesis highlights, the recent identification of a novel angiogenic molecule that the marmoset does not appear to express draws scrutiny as to the relationship between the molecular mechanisms functioning during ovarian angiogenesis between the marmoset and the human.

It would also be valuable to be able to evaluate the changes in both gonadotrophin and steroid hormone concentrations after treatment. However the marmoset is not a good model in which to measure the gonadotrophins, because

suitable assays are not yet available. An FSH assay is available but has only been reported to measure urinary FSH successfully (Gilchrist *et al.* 2001). The marmosets in these studies could not be housed in urinary collection cages, as ethical approval was not obtained. It may have been possible to harvest urine from the bladder of the animals after they had been euthanised, however this was not done and the bladder was disposed of after other organs had been removed. An LH assay based upon an antibody to bovine LH, as described by Saltzman *et al.* (1998), has been tested in our laboratory, but it was found to lack the sensitivity to monitor changes in the cycle successfully. This may possibly be due to the recent finding that marmosets have a missing exon in the LH receptor gene (Zhang *et al.* 1997) and the hypothesised redundancy of LH in the marmoset, with the absence of LH expression, the role of LH being replaced by chorionic gonadotrophin produced by gonadotropes in the pituitary (Gromoll *et al.* 2003, Muller *et al.* 2003, Muller *et al.* 2004). A new oestradiol assay has been used in this thesis, but is still temperamental and remains problematic. In addition, because of the small volume of blood taken from the marmoset at each sampling interval, assay of multiple hormones is restricted.

7.4 Clinical implications of the thesis findings

The vascularisation of the developing ovarian follicles is a tightly regulated process. A disturbance in the balance of angiogenic factors regulating it may play a role in severe reproductive dysfunction, specifically, PCOS and OHSS. PCOS is probably the most common but least understood reproductive endocrine disorder, affecting approximately 5% of women in the developed world (Franks 1995, Knochenhauer *et al.* 1998, Legro and Strauss 2003, Azziz *et al.* 2004) and there is growing evidence that suggests familial inheritance of this disorder (Franks *et al.* 1997, Legro *et al.* 1998), although there are inherit problems associated with establishment of the maternal phenotype (Legro and Strauss 2003). PCOS is generally characterised by hyperandrogenism and ovulatory dysfunction (Zawadzki and Dunaif 1992). The ovarian dysfunction is manifest by chronic anovulation, hypervascularisation of the theca and increases in stromal blood flow (Zawadzki and Dunaif 1992, Ehrmann *et al.* 1995, Franks 1995, Pan *et al.* 2002). An increase in VEGF is associated with the hyperthecotic ovarian stroma of these patients (Kamat *et al.* 1995) and it has been

suggested that VEGF may play an important role in this pathogenesis.

OHSS is a severe and potentially life-threatening complication that can arise through treatment with gonadotrophins given during *in vitro* fertilisation (IVF) procedures (Pellicer *et al.* 1999). It is associated with enlargement of the ovaries, ascites, an increase in vascular permeability and is associated with increased expression in VEGF via hCG stimulation (Wang *et al.* 2002). An increased concentration of VEGF in the follicular fluid is also associated with the condition (Pellicer *et al.* 1999, Albert *et al.* 2002) and although having many years of clinical experience there are no precise methods or protocols to completely prevent severe OHSS (Orvieto 2005), apart from withholding the ovulation inducing hCG trigger and hence aborting the procedure, costing not only valuable clinical time and money but also causing devastating disappointment for couples struggling to conceive. Since both PCOS and OHSS are associated with increases in both angiogenesis and vascular permeability, both studies on the mechanisms regulating ovarian follicular angiogenesis and its inhibition may allow formulation of treatments for these major cause of infertility, and the complications that can be associated with IVF treatment. Until this study, the endocrine mechanisms regulating angiogenesis in the ovarian follicle had received little attention in the literature. This study has made inroads into unravelling the complicated process of ovarian angiogenesis.

Also, the discovery that EG-VEGF is expressed in the granulosa lutein cells of the human corpus luteum (Chapter 6) and the endometrium (Battersby *et al.* 2004) provides another putative target for manipulation of luteal function and perhaps inhibition of blastocyst implantation as an emergency contraceptive, as the ability to specifically modulate and inhibit angiogenesis in the endocrine glands potentially offers a much more selective treatment for angiogenic disorders than inhibiting angiogenesis *per se*, with more generally targeted antagonists.

7.5 Relevance to other systems

Forming new blood vessels is a complex and delicate process. If insufficient vasculature forms, tissue can become ischaemic and die. If over-vascularisation occurs, both tumours and chronic inflammatory disorders can become aggravated (Carmeliet 2004). Novel insights into the regulation of angiogenesis are therefore

relevant for the future treatment of many angiogenic disorders, the most prevalent being: cancer; abnormal uterine bleeding; arthritis; psoriasis; and retinopathies (Folkman 2001, Kerbel and Folkman 2002, Carmeliet 2004). But, even a high fat diet has been shown to lead to the activation of angiogenic gene expression in adipose tissue (Li *et al.* 2002a) and studies using the angiogenic inhibitor TNP-470 have shown that inhibition of angiogenesis can be used to control the deposit of adipose tissue (Rupnick *et al.* 2002).

Conversely there are also a number of disorders associated with under-vascularisation of tissues. Studies in women have shown that elevated levels of VEGFR1 are associated with the incidence of preeclampsia (Levine *et al.* 2004) and the progressive loss of the microvasculature underlies many age related diseases (Carmeliet 2004). Age-related bone loss is associated with a decrease in the osteoblastic secretion of VEGF (Martinez *et al.* 2002), angiogenesis is positively correlated with the survival rate in stroke patients (Krupinski *et al.* 1994, Manoonkitiwongsa *et al.* 2001) and insufficient VEGF levels cause can cause motor neurone degradation (Oosthuysen *et al.* 2001). Therefore, with an increasing understanding of the mechanisms that regulate angiogenesis can come treatments for a large number of angiogenic-related disorders.

There are a large number of anti-angiogenic molecules currently undergoing various stages of clinical trials. Of these, AvastinTM (bevacizumab), a humanised monoclonal antibody directed at VEGF, is the most advanced, and has showed promising results in clinical trials (Ferrara 2004). In February 2004, the FDA approved AvastinTM to be used in combination with chemotherapy as a treatment for patients with first-line or previously untreated metastatic cancer of the colon or rectum. However, six months later, the FDA and the manufactures of AvastinTM issued an important warning that there is evidence of an increased risk of serious arterial thromboembolic events related to AvastinTM use. All of these anti-angiogenic agents can generally be divided into two distinct classes, direct inhibitors and indirect inhibitors. The direct inhibitors prevent endothelial cells from proliferating, migrating or avoiding apoptosis in response to a spectrum of pro-angiogenic proteins, including VEGF. Indirect inhibitors prevent the expression or block the activity of proteins that activate angiogenesis or block the expression of

their receptors on endothelial cells (Kerbel and Folkman 2002). A summary of some of these molecules is given in **Table 7.1**.

Action	Drug	Target
Direct	Arresten	Believed to bind integrin- $\alpha_1\beta_1$ to inhibit endothelial-cell proliferation, migration, tube formation and neovascularisation
Direct	Avastin™	Recombinant humanised monoclonal antibody against VEGF
Direct	Canstatin	Believed to bind integrin- $\alpha_v\beta_3$ to inhibit endothelial-cell proliferation, migration and tube formation
Direct	NM-3	An isocoumarin small-molecule inhibitor of VEGF. Is shown to selectively inhibit endothelial-cell proliferation, sprouting and tube formation <i>in vitro</i>
Direct	2-methoxyoestradiol	Inhibits microtubule function in proliferating endothelial cells, resulting in endothelial-cell apoptosis
Direct	VEGF Trap R1R2	Chimeric protein of VEGFR1 and VEGFR1, inhibits endothelial cell proliferation and migration
Direct	Vitaxin	A humanised monoclonal antibody against integrin $\alpha_v\beta_3$
Action	Drug	Production of Proteins effected
Indirect	Herceptin	Inhibits VEGF, angiopoietin-1, TGF- β , PAI1; up regulates thrombospondin-1
Indirect	IFN- α	Inhibits expression of bFGF by cancer cells
Indirect	PTK787; ZD6474; 166,168 SU6668; SU11248	Inhibits VEGF receptor expression on endothelium
Indirect	PTK787; SU11248	Inhibits PDGF receptor expression

Table 7.1 Novel anti-angiogenic agents

Novel angiogenesis inhibitors in various stages of development. Table adapted from Kerbel and Folkman (2002).

7.6 Suggestions for further study

It is established that during the process of ovulation, blood vessel permeability in the ovulating follicle increases (Cavender and Murdoch 1988, Koos 1995). These changes in vascular permeability are likely to be very important in the function of the ovarian follicle and the treatment of ovarian reproductive disorders. There are few studies to date that assess the molecular regulation of vascular permeability in the primate. The results reported in this thesis, specifically Chapter 5, demonstrate that inhibition of VEGF in the marmoset does not reliably inhibit follicle expansion and subsequent ovulation. Further experiments should be conducted to assess follicular blood vessel permeability in ovaries from control and both GnRH antagonist and VEGF trap treated animals. This is possible through either the use of Doppler flow studies or the injection of dyes prior to euthanasia, to allow for the immunocytochemical analysis of vessel permeability.

The establishment of a model in which to study both PCOS and OHSS would also be invaluable to the progression of the development of treatment for both the disorders. Exposure to androgen excess during early gestation has been shown to cause LH hypersecretion (Dumesic *et al.* 1997), impaired insulin secretion (Eisner *et al.* 2000), hyperandrogenism, anovulation (Eisner *et al.* 2002) and increased adiposity (Eisner *et al.* 2003) in adult female rhesus monkeys. All of these consequences of foetal androgenisation bare a striking resemblance to manifestations of PCOS (Ehrmann *et al.* 1995, Franks 1995, Dunaif 1997). Establishment of a model in which terminal studies could be conducted, i.e. the marmoset, could lead to advances in the treatment of PCOS. The marmosets short time to sexual maturation and ovarian cyclicity would expedite the establishment of a primate model of PCOS in which terminal studies could be conducted on the effects of inhibition of angiogenic factors.

In 2001, just before work on this thesis commenced, a novel endocrine gland specific vascular endothelial growth factor was described. Chapter 6 describes the localisation of EG-VEGF to the granulosa lutein cells in the human corpus luteum, but fails to identify EG-VEGF in any of the marmoset tissues examined. To progress the identification of EG-VEGF in the marmoset, analysis of the promoter region of

the gene should be conducted. The human and mouse promoters have both been sequenced and have been found to be relatively divergent from one another (LeCouter *et al.* 2003). Promoter analysis of the marmoset sequence may shed further light on EG-VEGF expression in the marmoset model.

7.7 Summary

Overall, this thesis has demonstrated that the developing ovarian follicle, though susceptible to insult by either deprivation of gonadotrophic support or the inhibition of angiogenesis, is a much more robust structure than the corpus luteum after dominant follicle selection has occurred. Suppressing gonadotrophic support, angiogenesis and vessel permeability has a much less of an effect on follicle development than had been hypothesised from previous studies. The dissociation of the mechanical functioning of the ovary, i.e. follicle expansion and ovulation and the steroid hormone profile throughout the follicular phase of the cycle observed in this thesis, specifically chapters 3 and 5, raises the possibility of alternative interpretation of results previously published in the literature that directly infer suppression of follicle expansion and ovulation on purely hormonal evidence, highlighting the importance of extrapolating data to far.

Also, the localisation of the novel angiogenic molecule EG-VEGF to human granulosa lutein cells has contradicted findings from other research groups and has helped initiate the elucidation of the function of EG-VEGF in the human CL.

In a broader context, this thesis forms part of the ongoing research into reproductive angiogenesis by Prof Hamish Fraser's team. There is still plenty of exciting work to be done to develop a full understanding of the regulation of blood vessel development and regression in the female reproductive system and how this process can be manipulated, inhibited and stimulated to improve reproductive health.

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Appendices

Appendix A: Sequences for *in situ* hybridisation riboprobes

Nucleotide sequence of the gene is in black. The region of the mRNA that the riboprobe recognises is in red.

VEGF

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1 tcgcggaggc ttggggcagc cgggtagctc ggaggtcgtg gcgctggggg ctagcaccag
 61 cgctctgtcg ggaggcgcag cgggttaggtg gaccggtcag cggactcacc ggccagggcg
121 ctcgggtcgtg gaatttgata ttcattgato cgggttttat ccctcttctt tttcttataa
181 cttttttttt taaaactgta ttgtttctcg ttttaattta tttttgcttg ccattcccca
241 cttgaatcgg gccgacggct tggggagatt gctctacttc cccaatcac tgtggatttt
301 ggaaccacg agaaagagga aagaggtagc aagagctcca gagagaagc gaggaagaga
361 gagacggggt cagagagagc gcgcggggcg gcgagcagcg aaagcgacag ggcacaaagtg
421 agtgacctgc ttttgggggt gaccgccgga gcgcggcgtg agccctcccc cttgggatcc
481 cgagctgac cagtcgcgct gacggacaga cagacagaca ccgccccag cccagctac
541 cacctcctcc cggccggcg gcggacagtg gacgcggcgg cgagccgcgg gcaggggccc
601 gagcccgcg ccggaggcgg ggtggagggg gtcggggctc gggcgctcgc actgaaactt
661 ttcgtccaac ttctgggctg ttctcgcttc ggaggagccg tggctcgcgc gggggaagcc
721 gagccgagcg gagccgcgag aagtgtctag tcgggccggg aggagccgca gccggaggag
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841 aagccgggct catggacggg tgaggcggcg gtgtgcgacg acagtgtctc agccgcgcgc
901 gctccccagc ccctggccc ggcctcgggg cggggaggaa gagtgtctcg ccgagggccc
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1021 ggtcgggctt ccgaaacctt gaactttctg ctgtcttggg tgcattggag ccttgccttg
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1141 cagaatcatc acgaagtggg gaagtctatg gatgtctatc agcgcagcta ctgccatcca
1201 atcgagacc tgggtggacat cttccaggag taccctgatg agatcgagta catcttaag
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1381 cagcacatag gagagatgag cttctctacg cacaacaaat gtgaatcgag accaaagaaa
1441 gatagagcaa gacaagaaaa atgtgacaag ccgaggcggg gagccgggca ggaggaagga
1501 gccctcccca gggtttcggg aaccagatct ctcaccagga aagactgata cagaacgatc
1561 gatacagaaa ccacgctgcc gccaccacac catcaccatc gacagaacag tccttaatcc
1621 agaaacctga aatgaagaaa gaggagactc tgcgcagagc actttgggtc cggagggcga
1681 gactccggcg gaagcattcc cgggcccgtg acccagcagc gtcctctctg gaattggatt
1741 cgccatttta tttttcttgc tgctaaatca ccgagcccgg aagattagag agttttat
1801 ctgggattcc tgtagacaca cccaccaca tacatacatt tatatatata tatattatat
1861 atatataaat taa

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VEGFR1

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1 gcggacactc ctctcggctc ctccccggca gcggcggcgg ctcgagcggg gctccggggc
 61 tcgggtgcag cggccagcgg gcctggcggc gaggattacc cggggaagtg gttgtctcct
121 ggtctggagcc gcgagacggg cgctcagggc gcggggcccg cggcggcgaa cgagagagcg
181 gactctggcg gccgggtcgt tggccggggg agcgcgggca ccggcgagc agccgcgctc
241 gcgctcaccg tggtcacgta ctgggacacc ggggtcctgc tgtgcgctgt gctcagctgt
301 ctgcttctca caggatctag ttcaggttca aaattaaaag atcctgaact gatttataaa
361 ggcaccagc acatcatgca agcaggccag acactgcac tcctaatgca gggggaagca
421 gccataaat ggtctttgcc tgaatggtg agtaaggaaa gcgaaaggct gacataact
481 aaatctgctt gtggaagaaa tggcaacaa ttctgcagta ctttaacctt gaacacagct
541 caagcaaac acactggctt ctacagctgc aaatatctag ctgtacctac tcaaaagaag
601 aaggaaacag aactgcaat ctatatattt attagtgata caggtagacc ttcgtagag
661 atgtacagt aaatccccga aattatacac atgactgaag gaaggagct cgtcattccc
721 tgccgggtta cgtcacttaa catcactgtt actttaaaaa agtttccact tgacactttg
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841 acgtacaag aaatagggtt tctgacctgt gaagcaacag tcaatgggca ttgtataag
901 acaactatc tcacacatcg acaaccaat acaatcatag atgtccaaat aagcacacca
961 cgcccagtc aattacttag agccatact cttgtcctca attgtactgc taccactccc
1021 ttgaacacga gattcfaat gacctggagt taccctgatg aaaaaataa gagagctcc
1081 gtaaggcgag gaattgacca aagcaattcc catgccaaca tattctacag tttcttact
1141 attgacaaaa tgcagaacaa agacaaagga ctttactt gtcgtgtaag gattggacca
1201 tcattcaaat ctgttaacac ctcagtgcat atatatgata aagcattcat cactgtgaaa
1261 catcgaaaac agcaggtgct tgaaccgta gctggcaagc ggtctaccg gctctctatg
1321 aaagtgaagg catttccctc gccggaagtt gtatggtaa aagatgggtt acctgagct
1381 gagaaatctg ctgcctattt aactcgtggc tactcgttaa ttatcaagga cgtaaactgaa
1441 gaggatgcag ggaattatac aactctgtg agcataaac agtcaaatgt gtttaaacac
1501 ctactgcca ctctaattgt caatgtgaaa cccagattt acgaaaggc cgtgtcatcg

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1561 ttccagacc cgctctcta cccactgggc agcagacaaa tctgacttg taccgcatat
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1681 gcaaggtgtg acctttgttc caataatgaa gagtccctta tctctggatgc tgacagcaac
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2221 caggaagcac catacctcct gcgaaacctc agtgatcaca cagtggccat cagcagttcc
2281 accactttag actgtcatgc taatggtgtc cccgagcctc agatcacttg gtttaaaac
2341 aaccacaaa tacaacaaga gcctggaatt attttaggac caggaagcac cagcgtgtt
2401 attgaaagc tcaagaaga ggatgaaggt gtctatcact gcaagccac caaccagaag
2461 ggctctgtgg aaagtccagc atacctcact gttcaaggaa cctcggacaa gtctaactg
2521 gag

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VEGFR2

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1 atggagagca aggtgctgct ggccgtgcc ctgtggctct gcgtggagac cggggccgc
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2581 acttgacgga cagttagcag caaaatggtg caaagaaggag caacacagc tgagctcga
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3361 gaaggaacta gaatgagggc ccctgattat actacaccag aaatgtacca gaccatgctg
3421 gactgctggc acggggagcc cagtcagaga cccacgtttt cagagtgggt ggaacatttg
3481 ggaatctct tgcagactaa tgctcagcag gatggcaaa actacattgt tcttccgata
3541 tcagagactt tgagcatgga agaggattct ggactctctc tgccctacctc acctgtttcc
3601 tgtatggagg aggaggaagt atgtgacccc aaattccatt atgacaacac agcaggaatc
3661 agtcagtatc tgcagaacag taagcgaaa agccggcctg tgagtgtaaa aacatttgaa
3721 gatatcccgt tagaagaacc agaagtaaaa gtaatcccag atgacaacca gacggacagt
3781 ggtatggttc ttgcctcaga agagctgaaa actttggaag acagaaccaa attatctcca
3841 tcttttggtg gctaccagtc cagcaaaaagc agggagtctg tggcatctga aggtcaaac
3901 cagacaagcg gctaccagtc cggatatcac tccgatgaca cagacaccac cgtgtactcc
3961 agtgagggaag cagaactttt aaagctgata gagattggag tgcaaacccg tagcacagcc
4021 cagattctcc agcctgactc ggggaccaca ctgagctctc ctctgttta a

```

EG-VEGF

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1 ggggaagcga gaggcattca agcagggcagt gttttgcctt caccccaagt gaccatgaga
  61 ggtgccacgc gagtctcaat catgctctc ctagtaactg tgtctgactg tgcgtgcatc
 121 acaggggctt gtgagcggga tgtccagtgt ggggcaggca cctgctgtgc catcagcctg
 181 tggtctcgag ggctcgggat gtgcaccccg ctggggcggg aaggcgagga gtgccacccc
 241 ggcagccaca aggtcccctt cttcaggaaa cgcaagcacc acacctgtcc ttgcttgccc
 301 aacctgctgt cgtccaggtt cccggacggc aggtaccgct gctccatgga cttgaagaac
 361 atcaattttt aggcgcttgc ctggtctcag gataccacc atccttttct gagcacagcc
 421 tggattttta tttctgccat gaaacccagc tcccattgact ctcccagtc ctaccatgac
 481 taccctgact tctctgtct agtacgcaca tatgcacaca ggcagacata cctccatca
 541 tgacatggtc cccagcttgg cctgaggatg tcacagcttg aggctgtggt gtgaaagggtg
 601 gccagcctgg ttctcttccc tgcctcaggct gccagagagg tggtaaatgg cagaaggac
 661 attccccctc ccctcccagc gtgacctgct ctctttctg ggccctgccc ctctcccac
 721 atgtatccct cggcttgaat tagacattcc tgggcacagg ctcttggtg cattgtctcag
 781 agtcccaggt cotggcctga ccctcaggcc cttcacgtga ggtctgtgag gaccaatttg
 841 tgggtagttc atcttcccctc gattggttaa ctcttagtt tcagaccaca gactcaagat
 901 tggctctccc cagagggcag cacacagtc cccaaggca ggtgtaggga gccoagggag
 961 gccaatcagc cccctgaaga ctctggtccc agtcagctg tggctgtggy cctgtgacct
1021 gtgacctctc gccagaattg tcatgctctc gaggcccctc cttaccacac tttaccagtt
1081 aaccactgaa gcccccaatt cccacagctt ttccattaaa atgcaaatgg tgggtggtca
1141 atctaactcg atattgacat attagaagc aattagggtg tttccttaa caactcctt
1201 ccaaggatca gccctgagag caggttggtg actttgagga gggcagtcct ctgtccagat
1261 tggggtggga gcaagggaca gggagcagg caggggctga aaggggcact gattcagacc
1321 agggaggcaa ctacacacca acctgctggc tttagaataa aagcaccaac tgaactg

```

Aromatase

```

1 atggtcttgg aatgctgaa ccgatgcat tataacatca ccagcatggt gcttgaagcc
  61 atgcctgctg ccaccatgcc aatcctgctg ctcaactggc ttttctcct ggttggaat
 121 tatgagggca catcctcaat accaggtccc ggctactgca tgggaattgg acccctcatc
 181 tcccacggca gatctctgtg gatggggatc ggcaatgcct gcaactacta caaccggatg
 241 tatggagaat tcattgagat ctggatctct ggagaggaaa cactcattat cagcaagtcc
 301 tcaagtatgt tccatgtaat gaagcacaat cattatagct ctcgattcgg cagcaactt
 361 gggctgcagt gcattggtat gcatgagaaa ggcattcatat ttaacaacaa tccagatctc
 421 tggaaaaaaa ctcgccccct ctttatgaaa gctctgtcag gccctggctt tgttcgcagt
 481 gtcacagtct gtgctgaatc cctcaacacg catctggaca ggttggagga ggtgaccaat
 541 gagtccaggt tcatcgacgt gttgaccctc ctgctgtgca tcatgctgga cacctctaac
 601 acgctcttct tgaggatccc tttggacgaa aatgccatcg tgtttaaaat ccaaggttat
 661 tttgatgcat ggcaagctct cctcatcaa ccagacatct tcttaagat tcttggtcta
 721 tacaaaaagt atgagaagtc tgtcaaggat ttgaaagatg ccatagaagt tctgatggca
 781 gaaaaaagac gcaggatttc cgcagaagag aaactggaag aacatataga ctttgccact
 841 gatttgattt tagccgagaa acgtgggtgac ctgacaagag agaactgtgaa ccagtgcagt
 901 ttggaaatgc tgattgcagc tcctgacacc atgtctgtct ctctgttctt catgctattc
 961 ctgattgcaa agcaccctaa tgtggaagag gcaataatga aggaaatcca gactgttgtt
1021 ggtgagagag acgtaaaagt tgatgatata caaaaattaa aagtgggtgaa aaacttcatt
1081 tatgagagca tggcgtacca gctgtttgtg gacttggctc tgcgcaaagc cttagaagat
1141 gatgtgatcg atggctaccc agtgaaaaag gggacgaaca ttatctgaa tattggaagg
1201 atgcacagac togagttttt ccccaaaccc aatgaattta ctcttgaaaa ttttgcaag
1261 aatgttctct acaggtactt tcagccattt ggttttgggc cccgtagctg tgcaggaaag
1321 tacatcgcca tggttgatg gaaatccatt ctcgttacgc tctgagaag attccacgtg
1381 aagacattgg ggggagagt cgttgagagc ctacagaaga caaacgactt ggccttgcac
1441 ccagatcaca ctaaaagcat gctggaaatg atttttacc caagaaactc agcgtggtgc
1501 ctggaacact aa

```

Appendix B: LightCycler primer and probe sequences

Primers are highlighted in red in the sequence.

VEGF

Forward 5' - caccatggcagaaggag

Reverse 5' - cacacaggatggcttgaaga

PRODUCT SIZE: 155

```

1  tgggccaggctgcaccatggcagaaggaggagggcagaatcatcacgaagtgggtaag
61  ttcattgatgtctatcagcgcagctactgccatccaatcgagaccctggggacatcttc
121 caggagtaccctgatgagatcgagtacatcttcaagccatcctgtgtgcccctgatgcga
181 tgcgggggctgctccaatgacgagggcctggagtggtgtgccactgaggagtccaacatc
241 accatgcag

```

Aromatase

Forward 5'- cactggcctttttctcttgg

Reverse 5'- agagatccagactcgcgatgaa

PRODUCT SIZE: 178

```

1  atggttttgaaatgctgaaccgatacattataacatcaccagcatcgtgcctgaagcc
61  atgcctgctgccaccatgccagtcctgctcctactggcctttttctcttggtggtggaat
121 tatgagggcacatcctcaataaccaggtcctggctactgcatgggaattggaccctcatc
181 tcccacggcagattcctgtggatggggatcggcagtgctgcaactactacaaccgggta
241 tatggagaatttcatgcgagctctggatctctggagaggaaacactcattatcagcaagt

```

 β actin

Forward 5' - ggcacctcaccctgaagta

Reverse 5' - ggggtggtgaaggtctcaaa

PRODUCT SIZE: 202

```

1  cagggcgtgatggtgggcatgggtcagaaggattcctatgtggggcgacgaggccagagc
61  aagagaggcatcctcaccctgaagtaccccatcgagcagggcatcgtcaccaactgggac
121 gacatggagaaaaatctggcaccacaccttctacaatgagctgcgtgtggctcccaggag
181 caccocgtgctgctgaccgagggccccctgaacccaaggccaaccgcgagaagatgcc
241 agatcatgtttgagaccttcaacaccccagccatgtacgttgctatccaggctgtgctat
301 ccctgtacgcctctggcctgactgacatcgtgatggactccggtgacggggtcacc
361 aactgtgccatctacgaggggatgcccctccccatgccatcctgcgtctggacctgg
421 ctggccgggacctgactgactacctcatgaagatcctcaccgagcgcggctacagctca
481 ccaccacggccgagcgggaaatcgtgcgtgacattaaggagaagctgtgctacgtcgcc
541 tggacttcgagcaagagatggccaacggctgcttccagctcctccctggagaagagctacg
601 agctgcctgacggccaggtcatcaccattggcaatgagcgggtcccgctgcctgaggcac
661 tcttcagccttctctctg

```

Appendix C: Number of follicles assessed.

The number of follicles from each follicular class and each treatment group measured for each immunocytochemical and *in situ* analysis. ES = early secondary, LS = late secondary, T = tertiary, Dom = dominant, A = atretic.

	BrdU				Dual			CD31			VEGF mRNA				Arom mRNA
	ES	LS	T	Dom	LS	T	Dom	LS	T	Dom	LS	T	Dom	A	Dom
LF Control	50	105	33	9	29	24	9	23	27	9	10	18	9	28	7
GnRH antag 0-10	22	24	17	-	25	25	-	21	20	-	5	16	-	7	-
GnRH antag 5-10	41	55	34	8	27	23	8	27	21	8	14	29	7	14	7
MF Control	-	-	-	-	-	-	-	-	-	-	-	23	-	14	-

	BrdU				Dual			CD31		
	ES	LS	T	Dom	LS	T	Dom	LS	T	Dom
LF Control	42	79	29	9	29	14	8	31	36	7
VEGF trap 0-10	14	14	14	-	17	17	-	13	15	-
VEGF trap 5-10	14	19	19	7	42	52	8	28	25	6

	VEGF mRNA (Gran)			VEGF mRNA (Theca)				VEGFR1 mRNA				VEGFR2 mRNA			
	LS	T	Dom	LS	T	Dom	A	LS	T	Dom	A	LS	T	Dom	A
LF Control	10	12	4	10	12	4	12	9	10	6	7	10	13	6	8
VEGF trap 0-10	10	9	-	10	9	-	12	11	8	-	10	10	14	-	10
VEGF trap 5-10	11	11	8	11	11	8	7	13	13	7	14	15	8	8	10

Appendix D: Animals used in studies

Animal number	Treatment Group	Gross morphology
995	Early follicular control	Majority luteal tissue
902	Early follicular control	Majority luteal tissue
932	Early follicular control	Majority luteal tissue
829	Mid follicular control	Many tertiary follicles
955	Mid follicular control	Many tertiary follicles
960	Mid follicular control	Many tertiary follicles
990	Mid follicular control	Many tertiary follicles
895	Late follicular control	3 recent ovulations
940	Late follicular control	2 recent ovulations
944	Late follicular control	2 preovulatory follicles
958	Late follicular control	2 recent ovulations
966	Late follicular control	2 preovulatory follicles
983	Late follicular control	2 preovulatory follicles
997	Late follicular control	3 preovulatory follicles
972	GnRH antagonist 0-10	No dominant follicles
996	GnRH antagonist 0-10	No dominant follicles
2Y	GnRH antagonist 0-10	No dominant follicles
15Y	GnRH antagonist 0-10	No dominant follicles
922	GnRH antagonist 5-10	2 dominant atretic follicles
946	GnRH antagonist 5-10	2 dominant atretic follicles
952	GnRH antagonist 5-10	2 dominant atretic follicles
973	GnRH antagonist 5-10	1 dominant atretic follicle
982	GnRH antagonist 5-10	1 dominant atretic follicle
985	GnRH antagonist 5-10	2 dominant atretic follicle
855	R1R2 0-10	No dominant follicles
49Y	R1R2 0-10	No dominant follicles
77Y	R1R2 0-10	No dominant follicles
45Y	R1R2 5-10	2 recent ovulations
54Y	R1R2 5-10	3 dominant follicles, all partially luteinised
57Y	R1R2 5-10	2 dominant follicles, 1 healthy, 1 partially atretic
67Y	R1R2 5-10	2 dominant follicles, both heavily luteinised
97Y	R1R2 5-10	1 dominant follicle, partially luteinised
63Y	R1R2 5-15	3 micro CL
65Y	R1R2 5-15	4 dominant follicles, little sign of atresia in any
76Y	R1R2 5-15	2 dominant follicles, some atresia, 2 micro CLs
93Y	R1R2 5-15	1 recent ovulation
99Y	R1R2 5-15	2 micro CL, 1 luteinising follicle
105Y	R1R2 5-15	2 dominant follicles, some sign of atresia in both
110Y	R1R2 5-15	3 healthy dominant follicles

Appendix E: Published papers

Effects of GnRH antagonist treatment on follicular development and angiogenesis in the primate ovary

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Abstract

Angiogenesis is required for normal follicular development but the role of gonadotrophins in the control of follicular angiogenesis remains to be elucidated. This study investigated the effects of treatment with GnRH antagonist *in vivo* on follicular development and angiogenesis in the marmoset. GnRH antagonist was administered on either follicular day 0 or day 5 of the 10-day follicular phase with ovaries collected on day 10. Ovaries from control marmosets were studied at day 5 (mid follicular phase) and day 10 (perioovulatory period). Ovaries were fixed, serial sectioned and subjected to morphological analysis and immunocytochemistry to determine cell proliferation and follicular endothelial cell area and *in situ* hybridization to assess changes in expression of vascular endothelial growth factor (VEGF). Treatment with GnRH antagonist from day

0–10 resulted in an absence of dominant preovulatory follicles seen in controls. In the remaining tertiary follicles granulosa, theca and endothelial cell proliferation was reduced, resulting in a minor reduction in vascular density. However, VEGF mRNA expression was unaffected by treatment. Treatment from day 5–10 did not prevent development of ovulatory size follicles, but they were atretic and lacked VEGF mRNA. These results suggest that while VEGF expression in the preovulatory follicle is under gonadotrophic control it is not dependent on normal gonadotrophin secretion in tertiary follicles, indicating that there are other paracrine factors regulating VEGF expression in the developing ovarian follicle.

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Introduction

The ovary is one of few organs in the body to undergo serial angiogenesis which is intimately involved in ovarian follicle development (Fraser & Wulff 2001, Wulff *et al.* 2001b, 2002, Zimmermann *et al.* 2001, 2003). Angiogenesis in the ovarian follicle is restricted to the thecal compartment, the resulting capillaries forming a vascular sheath around the growing follicle that continues to develop during folliculogenesis (Wulff *et al.* 2001b). The new blood vessels associated with dominant follicles do not penetrate the membrana propria, leaving the granulosa cells of the follicles avascular until the point of ovulation at which time the basement membrane breaks down. The degree of vascularization achieved has been hypothesized to be involved in the establishment of follicle dominance (Zelevnik *et al.* 1981), facilitating the delivery of gonadotrophins, steroid precursors, oxygen and nutrients from the blood stream, but whether an abundant vasculature is a cause or consequence of follicle dominance remains to be determined.

The principal angiogenic factor with an established role in follicular angiogenesis is vascular endothelial growth

factor (VEGF). VEGF mRNA and protein have been localized in the ovary to the granulosa cells of the developing follicles and the theca of preovulatory follicles (Phillips *et al.* 1990, Ravindranath *et al.* 1992, Shweiki *et al.* 1993). Inhibition of VEGF *in vivo* in the marmoset monkey has been shown to severely suppress both thecal vascularization and follicle development (Wulff *et al.* 2001b, 2002) and to inhibit the follicular phase of the cycle in macaques (Zimmermann *et al.* 2001, 2002).

VEGF is expressed in the granulosa and thecal cells of follicles as they mature and is concurrent with the acquisition of steroidogenic activity, suggesting that the expression is hormonally regulated (Shweiki *et al.* 1993). It has also been demonstrated that VEGF expression in granulosa cells is up-regulated by follicle-stimulating hormone (FSH) stimulation *in vitro* (Christenson & Stouffer 1997, Hazzard *et al.* 1999). In the current study, the role of the gonadotrophins was assessed by administration of a gonadotrophin-releasing hormone (GnRH) antagonist *in vivo* during follicular development in the marmoset, a species in which we have described the development of the follicular vasculature in detail (Wulff *et al.* 2001b,

2002). Since it is established that GnRH antagonist treatment suppresses follicular development by inhibiting both FSH and luteinizing hormone (LH) secretion from the pituitary (Fluker *et al.* 1991, Hall *et al.* 1991, Fraser *et al.* 1999b), the aim of the present study was to test the hypothesis that this suppression resulted in a reduced production of follicular VEGF mRNA with resultant inhibition of angiogenesis. The effects of the antagonist on follicular angiogenesis were examined using bromodeoxyuridine (BrdU) immunocytochemistry as a proliferation marker, CD31 as an endothelial specific cell marker and dual staining to distinguish proliferating endothelial cells. Effects of treatment on expression of VEGF and aromatase mRNA were determined by *in situ* hybridization.

Materials and Methods

Animals

Adult female common marmoset monkeys (*Callithrix jacchus*), 2–3 years old with a body weight of approximately 350 g and regular (28-day) ovulatory cycles, as determined by plasma progesterone concentrations on blood samples collected three times per week (Wulff *et al.* 2001c), were housed together with a younger sister or prepubertal female, as described previously (Fraser *et al.* 1999a).

Treatment

Experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986, and were approved by the Local Ethical Review Process Committee. To synchronize the timing of ovulation, marmosets were treated with 1 µg prostaglandin (PG) F_{2α} analogue (cloprostenol, Planate; Coopers Animal Health Ltd, Crewe, Cheshire, UK), intramuscularly on days 12–15 of the 20-day luteal phase to induce luteolysis. The day of PG injection was designated follicular day 0. This regime was based upon that of Gilchrist *et al.* (2001), who have described detailed changes in follicular populations during the subsequent follicular phase. We employed the GnRH antagonist Antarelix (Deghenghi *et al.* 1993), [N-Ac-D-Nal¹, D-pCl-Phe², D-Pal³, D-(Hci)⁶, Lys(iPr)⁸, D-Ala¹⁰]GnRH (Europeptides, Argenteuil, France) dissolved in water to a concentration of 10 mg/ml. To provide a slow-release depot Antarelix was administered at a dose of 12 mg/kg, injected subcutaneously on follicular day 0 (*n*=4) or follicular day 5 (*n*=6). Ovaries from the treated animals were collected 10 days after PG administration, corresponding to the periovulatory period in control animals. Control marmosets were studied on day 0 (*n*=3), day 5 (*n*=4) and day 10 (*n*=6) relative to PG administration. After PG administration, blood samples continued to be collected from the animals three times

a week to monitor progesterone levels. All animals were injected i.v. with 20 mg BrdU (Roche Molecular Biochemicals, Essex, UK) in saline 1 h before being sedated using 100 µl ketamine hydrochloride (Parke-Davis Veterinary, Pontypool, Gwent, UK) and killed with an i.v. injection of 400 µl Euthetal (sodium pentobarbitone; Rhone Merieux, Harlow, Essex, UK). After cardiac exsanguination, ovaries were removed, weighed and immediately fixed in 4% neutral buffered formalin. After 24 h, the ovaries were transferred to 70% ethanol, dehydrated and embedded in paraffin according to standard procedures.

To confirm the potency of the GnRH antagonist treatment, three marmosets with regular cycles were given the treatment described above, but allowed to recover normal cycles as determined by measuring plasma progesterone in blood samples collected three times per week.

Steroid assays

Oestradiol concentrations in serum collected by exsanguination were determined by an ELISA assay adapted from Mann *et al.* (1995). The detection limit for the assay was 1 ng/l. Interassay coefficients of variation for low, medium and high quality controls were 15.5%, 6.5% and 6.4% respectively. All samples were run in a single assay. Plasma progesterone concentrations were determined by an ELISA assay, as previously described (Wulff *et al.* 2001c).

Morphological characterization of ovarian follicles

Stages of follicular development were defined as follows: primary (oocyte surrounded by one granulosa cell layer), early secondary (two to four granulosa cell layers, no antrum), late secondary (more than four granulosa cell layers, no antrum), tertiary (follicles containing an antrum) and ovulatory (large antral follicles >2 mm). Follicles were classified as healthy if they contained a normal-shaped oocyte surrounded by granulosa cells that were regularly apposed on an intact basement membrane with normal appearance of granulosa cell nuclei without signs of pycnosis. Follicles were classified as atretic when they showed signs of granulosa cell pycnosis, the first morphological sign of follicular atresia (Durlinger *et al.* 2000). Only follicles with a visible oocyte containing a nucleus were considered to ensure proper follicular classification. Follicles not fulfilling these criteria were classified as unsuitable for analyses.

Haematoxylin–eosin staining

The embedded ovaries were serially sectioned, and tissue sections (5 µm) were placed onto BDH SuperFrost

slides (BDH, Merck Co., Inc., Poole, Dorset, UK). For morphological and morphometric analyses every 40th section of the ovaries was used. Sections in between were subjected to immunocytochemistry and *in situ* hybridization. Sections were dewaxed in xylene, rehydrated in descending concentrations of ethanol, washed in distilled water and stained with haematoxylin (Richard-Allan, Richland, MI, USA) for 5 min, followed by a wash in water and acetic alcohol before staining with eosin (Richard-Allan) for 20 s. After dehydrating in ascending concentrations of ethanol and xylene, sections were mounted.

Immunocytochemistry

The effects of the treatment on the establishment of the thecal vascular network were studied by (1) quantifying the number of proliferating cells stained for BrdU, (2) identifying endothelial cells using CD31 staining and (3) distinguishing proliferating endothelial from non-endothelial cells by colocalization of BrdU and CD31.

For BrdU and CD31 immunostaining, antigen retrieval was performed by pressure cooking (Tefal Clynso pressure cooker; Tefal, Langley, Berks, UK) sections in 0.01 M citrate buffer, pH 6, for 6 min at high pressure setting 2. Slides were then left for 20 min in hot buffer and washed in Tris-buffered saline (TBS; 0.05 mol/l Tris and 9 g/l NaCl). To reduce non-specific binding, sections were blocked in normal rabbit serum (NRS; 1:5 diluted in TBS containing 5% bovine serum albumin) for 30 min. Primary antibodies CD31 (mouse anti-human CD31; DAKO Corp., Copenhagen, Denmark) or BrdU (mouse anti-BrdU; Roche Molecular Biochemicals) were diluted 1:20 or 1:30 in TBS respectively. Incubation was carried out overnight at 4 °C. Slides were washed three times in TBS. Incubation with the secondary antibody, rabbit anti-mouse Ig (1:60 diluted in NRS:TBS; DAKO Corp.), was performed for 40 min at room temperature, followed after two washes in TBS by incubation of the alkaline phosphatase-anti-alkaline phosphatase complex (1:100 dilution in TBS; DAKO Corp.) for 40 min at room temperature. Visualization was performed using 500 µl/slide nitro blue tetrazolium (NBT) solution containing 45 µl NBT substrate (Roche Molecular Biochemicals), 10 ml NBT buffer, 35 µl 5-bromo-3-chloro-3-indolyl-phosphate and 10 µl levamisole. Sections for BrdU were counterstained with haematoxylin, whereas sections for CD31 were not counterstained, so that quantitative image analysis could be performed. For dual labelling, slides were incubated first with CD31 and visualized with Fast Red (Sigma, Poole, Dorset, UK); 1 mg Fast Red in 1 ml Fast Red buffer (20 mg 3-hydroxy-2-naphthoic acid 2,4-dimethylamide phosphate, 2 ml dimethyl formamide and 98 ml 0.1 M Tris, pH 8.2). After staining for CD31, incubation with BrdU was performed. BrdU-stained cells were visualized with NBT as described above.

Quantification of immunocytochemistry

It was previously demonstrated that angiogenesis is initiated in follicles containing more than four granulosa cell layers (Wulff *et al.* 2001b). Thus, in this study, only follicles with four or more granulosa cell layers were analysed. Captured images were thresholded, and the thecal and granulosa cell compartments were outlined and analysed separately. Four sections per ovary were analysed under × 200 magnification. Cellular proliferation was measured using an image analysis system set up to measure the number of dark-stained nuclei (BrdU positive), and the number of dark- and light-stained nuclei (total number of cells) in the outlined compartment of interest. A proliferation index (BrdU-positive cells expressed as a percentage of the total number of cells) was calculated in the thecal and granulosa compartments for each follicle. The proliferation index is expressed as a mean value for the number of follicles assessed within each follicular stage and per animal.

The automated image analysis of BrdU in the granulosa of secondary follicles failed to reliably distinguish between single cells because granulosa cells have only a small cytoplasmic volume, so that the nuclei of different cells are in close vicinity. Thus, the granulosa cell proliferation index in these follicles was obtained by manual counting of the number of granulosa cells and the granulosa cell area from 50 secondary follicles, calculating a mean cell number per unit area and measuring granulosa cell area and converting the area to cell number.

The endothelial cell area (CD31-positive cells) was measured by thresholding a captured grey scale image and converting it to a binary image. The whole area of the thecal compartment and the CD31-positive area within the compartment were measured. The CD31-positive area was then calculated per unit area of the thecal compartment and expressed as a mean value for the number of follicles assessed within each follicular stage and per animal.

Proliferating endothelial cells (dual-stained cells) were counted manually at × 200. Only endothelial cells (red stain) that were also BrdU positive (black stain) were counted as proliferating endothelial cells. Non-endothelial cell proliferation was calculated by multiplying the total thecal proliferation index (BrdU count) by the percentage of proliferation that was not endothelial (not dual-stained, BrdU-positive cells on a dual-stained slide) per animal. Quantitative analysis was performed using an image analysis system linked to an Olympus Corporation camera, and the data were processed using Image-Pro Plus version 3.0 for Windows (Microsoft).

In situ hybridization

In situ hybridization was performed as described previously (Fraser *et al.* 2000, Wulff *et al.* 2001c). As the marmoset

Table 1 The number of follicles from each follicular class and each treatment group measured for each immunocytochemical and *in situ* analysis

	BrdU			CD31			Dual			VEGF mRNA				Aromatase mRNA
	LS	T	Dom	LS	T	Dom	LS	T	Dom	LS	T	Dom	A	Dom
LF control	105	33	9	23	27	9	29	24	9	10	18	9	28	7
GnRH antag 0–10	24	17		21	20		25	25		5	16		7	
GnRH antag 5–10	55	34	8	27	21	8	27	23	8	14	29	7	14	7
MF control										2	23		14	

LF, late follicular phase; MF, mid follicular phase; GnRH antag 0–10, GnRH antagonist treatment on day 0–10; GnRH antag 5–10, GnRH antagonist treatment on day 5–10.

shows very close homology of the known human gene sequence, a cRNA probe for human VEGF probe was used. For aromatase, a marmoset-specific cRNA probe was generated. Sense and antisense probes were prepared using an RNA transcription kit (Ambion, Inc. Austin, TX, USA) and were labelled with [³⁵S]uridine 5'-triphosphate (NEN Life Science Products, Boston, MA, USA). Deparaffinized sections were treated with 0.1 M HCl and then digested in proteinase K (5 µg/ml; Sigma) for 30 min at 37 °C. After prehybridization for 2 h at 55 °C subsequent hybridization was performed in a moist chamber overnight. High stringency post-hybridization washings and ribonuclease treatment were used to remove excess probe. Slides were then dehydrated, dried and dipped in Ilford G5 liquid emulsion (H A West, Edinburgh, UK) and exposed for 3 weeks. Slides were subsequently developed (D19 developer; Kodak, Rochester, NY, USA) and fixed (GBX fixer; Kodak). All slides were counterstained with haematoxylin (Richard-Allan), dehydrated and mounted.

In situ slides were analysed blind by two investigators separately. A scoring system of 0=no hybridization above background, +=detectable but low grain density clearly above background levels, ++=moderate grain density and +++=high grain density was employed to denote the level of hybridization observed in each follicle and the results were compared for agreement.

Statistical analysis

Data obtained for different cycle and follicular stages were tested for significant differences using ANOVA. Effects of the treatment compared with late follicular controls were determined using a two-tailed, unpaired *t*-test. *In situ* analysis was tested for significant differences using the Kruskal–Wallis test. Differences were considered to be significant at $P<0.05$. The tests were performed using SPSS version 11 for Macintosh (SPSS, Inc., Chicago, IL, USA). All values are given as the mean \pm S.E.M. Table 1 details the number of follicles analysed for each follicular class and stain.

Results

Hormonal changes

All marmosets responded to PG with a rapid fall in plasma progesterone concentration that remained at follicular phase levels for the duration of the study. Treatment suppressed serum oestradiol-17 β levels, consistent with inhibition of gonadotrophin release, significant reductions being observed between late follicular controls and both treatment groups (day 0–10 $P=0.022$, day 5–10 $P=0.027$) (Fig. 1). Additional evidence for potency of the GnRH antagonist treatment was demonstrated in animals left to recover after a single 12 mg/kg injection of Antarelix. Ovulation was suppressed and a progesterone rise was not observed until between 80 and 110 days after treatment (data not shown).

Ovarian morphology

In a cross-section through a typical control ovary at days 12–15 of the luteal phase, the time of PG injection

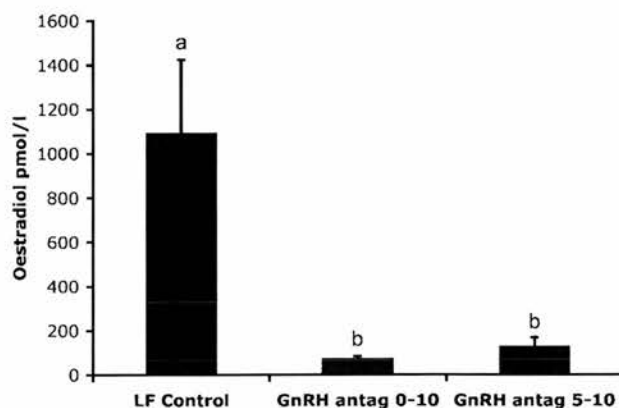


Figure 1 Plasma oestradiol levels in control marmosets at day 10 of the follicular phase (LF) and after treatment with GnRH antagonist between day 0–10 (GnRH antag 0–10) or 5–10 (GnRH antag 5–10). Oestradiol levels were significantly reduced by both of the treatments. Values are means \pm S.E.M. Different letters indicate statistically significant differences.

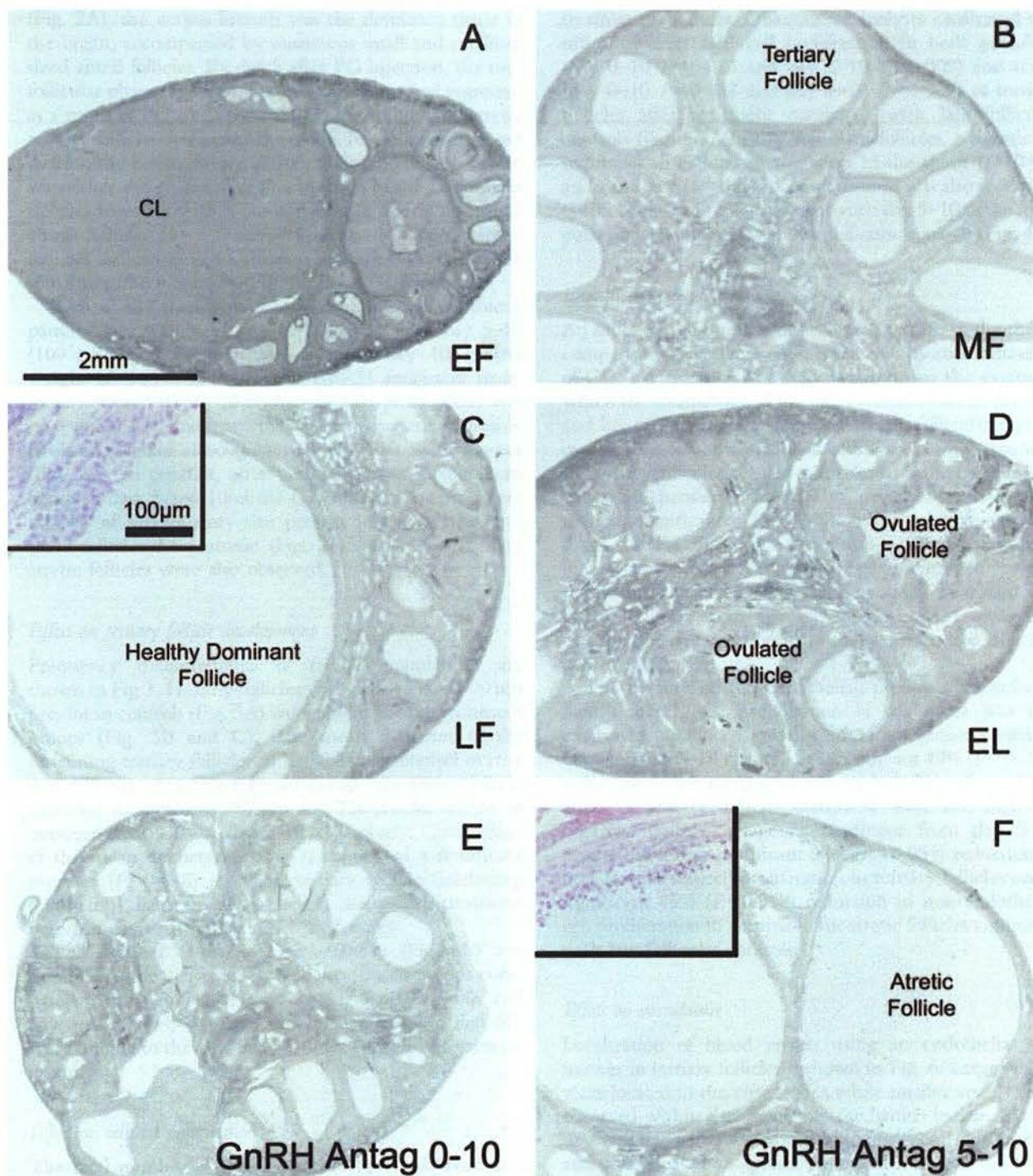


Figure 2 Haematoxylin and eosin-stained sections of (A) control early follicular phase (EF), representative of days 14–16 of the luteal phase (CL=corpus luteum), (B) mid follicular phase (MF) with tertiary follicles, (C) late follicular phase (LF) with healthy dominant follicles containing intact granulosa cells (inset), (D) early luteal phase (EL), (E) GnRH antagonist (Antag) treatment day 0–10 and (F) GnRH antagonist treatment day 5–10 showing atretic granulosa cells (inset). Note the lack of healthy dominant follicles in both of the treated groups.

(Fig. 2A), the corpus luteum was the dominant tissue in the organ, accompanied by numerous small and medium sized antral follicles. By day 5 after PG injection, the mid follicular phase (Fig. 2B), the corpus luteum had regressed as a result of PG treatment, and many healthy and atretic tertiary follicles dominated the ovary. In the day 10 control ovaries, the periovulatory period, the predominant feature was either the presence of one to three healthy dominant follicles together with a combination of atretic and small antral follicles ($n=3$ marmosets) (Fig. 2C) or collapsed follicles indicating that ovulation had just occurred in the remaining three animals (Fig. 2D).

Both of the treatment schedules significantly reduced paired ovary weight; day 0–10 (103 ± 21 mg), day 5–10 (109 ± 8 mg) compared with a mean day 10 control weight of (165 ± 22 mg). After GnRH antagonist treatment between day 0–10 of the follicular phase, there was no evidence of dominant follicle development or freshly ovulated follicles although tertiary follicles were present (Fig. 2E). In contrast, after GnRH antagonist treatment between days 5 and 10 of the follicular phase, there were follicles of preovulatory size present in the ovaries, but these follicles were atretic (Fig. 2F). Again tertiary and atretic follicles were also observed.

Effect on tertiary follicle development

Frequency measurements of follicle populations are shown in Fig 3. Healthy follicles with diameters >2000 μm present in controls (Fig. 3A) were absent in both treatment groups (Fig. 3B and C). The mean diameter of the remaining tertiary follicles in late follicular control ovaries was 680 μm . After GnRH antagonist, the mean follicle diameter increased to 767 μm and 736 μm for treatment between day 0–10 and day 5–10 respectively. Comparison of the mean diameter (Fig. 3D) confirmed a significant increase ($P=0.048$) in mean tertiary follicle (excluding dominant) diameter after GnRH antagonist treatment between day 0–10 compared with controls.

The number of atretic antral follicles (Fig. 3E) was significantly decreased in both the treatment groups compared with late follicular controls (day 0–10 $P=0.007$ and day 5–10 $P=0.044$). Atretic dominant follicles (Fig. 3F) were unique to the days 5–10 GnRH antagonist treatment group.

Effect on cellular proliferation

The total number of proliferating cells was counted as a percentage of the total number of cells present in the follicle compartment in question. Comparison of a section from the wall of a typical tertiary follicle from a late follicular control (Fig. 4A), a GnRH antagonist-treated ovary from day 0–10 (Fig. 4B) and a GnRH antagonist-treated ovary from day 5–10 (Fig. 4C) revealed a decrease in both granulosa and thecal proliferation after both

treatment schedules. Quantitative analyses confirmed significantly decreased cell proliferation in both granulosa (day 0–10 $P=0.013$ and day 5–10 $P=0.009$) and theca (day 0–10 $P=0.034$ and day 5–10 $P=0.029$) of tertiary follicles after treatment compared with late follicular controls (Fig. 4D). In the dominant follicles, a significant reduction in cellular proliferation in the theca ($P=0.01$) and granulosa ($P=0.044$) compartments was also seen after GnRH antagonist treatment between day 5–10 of the follicular phase compared with late follicular controls (Fig. 4E).

Endothelial cell proliferation

An index of the number of proliferating cells in the thecal compartment that were endothelial cells by co-localization of CD31 and BrdU was used to determine the extent to which the treatment had impaired endothelial cell division and hence ongoing angiogenesis. In late follicular control ovaries (Fig. 5A), dual-labelled cells were readily apparent. In tertiary follicles from ovaries from GnRH antagonist treatment between day 0–10 dual-labelled cell were reduced significantly ($P=0.043$). After treatment between day 5–10 (Fig. 5C), there was a non-significant reduction in dual-labelled cells. Endothelial cell proliferation was also significantly reduced ($P=0.036$) between dominant but atretic follicles from the day 5–10 GnRH antagonist treatment group and dominant follicles from late follicular controls (Fig. 5D).

The amount of non-endothelial, presumably predominantly thecal, cell proliferation in the theca was also examined (data not shown). GnRH antagonist treatment between day 0–10 resulted in a significant 49% ($P=0.029$) reduction in the number of non-endothelial cells proliferating in tertiary follicles compared with late follicular controls. GnRH antagonist treatment from day 5–10 resulted in a non-significant 57% ($P=0.051$) reduction in non-endothelial cell proliferation in tertiary follicles and a significant 45% ($P=0.018$) reduction in non-endothelial cell proliferation in dominant but atretic follicles compared with late follicular controls.

Effect on vasculature

Localization of blood vessels using an endothelial cell marker in tertiary follicles is shown in Fig. 6. Large vessels were located in the outer theca while smaller vessels were observed within the vicinity of the lamina basalis. Extensive vascularization was observed in both controls (Fig. 6A) and after GnRH antagonist treatment between day 0–10 (Fig. 6B) and day 5–10 (Fig. 6C) of the follicular phase. Quantitative analysis of staining (Fig. 6D) indicated that there was no difference in the extent of thecal vascularization between the groups.

There was, however, a thinning of the theca between follicles from late follicular controls and both treatment groups, with a significant ($P=0.006$) decrease in the mean

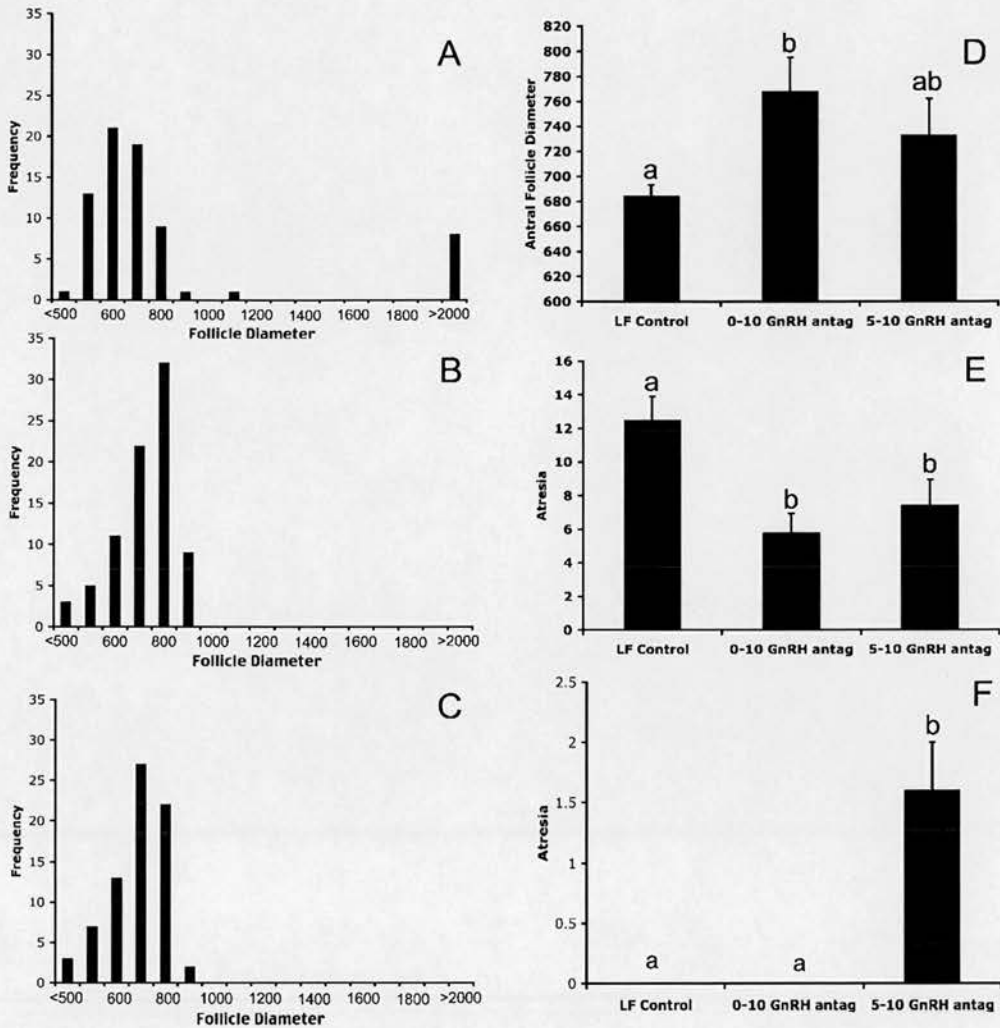


Figure 3 Histograms showing distribution of tertiary follicle size in (A) late follicular control animals, (B) days 0–10 GnRH antagonist-treated animals and (C) day 5–10 GnRH-treated animals. The effect of both the treatments was to positively skew the frequency of antral follicles present in the ovaries compared with controls, with (D) a significant increase in the mean tertiary follicle diameter between day 0–10-treated animals and controls and (E) a significant decrease in the number of atretic follicles between controls and either treatment group. (F) Number of atretic dominant follicles in controls and treated groups. Values are means \pm S.E.M. Different letters indicate statistically significant differences.

thecal thickness between dominant follicles from GnRH antagonist treatment between day 5–10 of the follicular phase and dominant follicles from late follicular controls (Fig. 6E). To adjust for the effect of the thinner theca, a conversion factor was used to quantify an absolute value of CD31-positive staining in each class of follicle, based on a technique applied by Wulff *et al.* (2001a). The conversion factors were calculated as a ratio of mean thecal thickness against the late follicular control mean for each class of follicle, giving the late follicular control conversion a value of one. After adjustment for thecal thickness a significant ($P=0.04$) reduction in thecal vascularization was demonstrated between tertiary follicles from late follicular con-

trols and day 0–10 GnRH antagonist treatment (Fig. 6F). A significant reduction ($P=0.014$) was also observed between dominant follicles after day 5–10 GnRH antagonist treatment and late follicular controls after adjustment for thecal thickness (Fig. 6F).

Localization of VEGF mRNA

In situ hybridization revealed that VEGF mRNA in both control and treated groups was absent in primordial, primary and early secondary follicles. VEGF mRNA was expressed in the granulosa of late secondary and tertiary follicles from late follicular controls (Fig. 7A and B), after

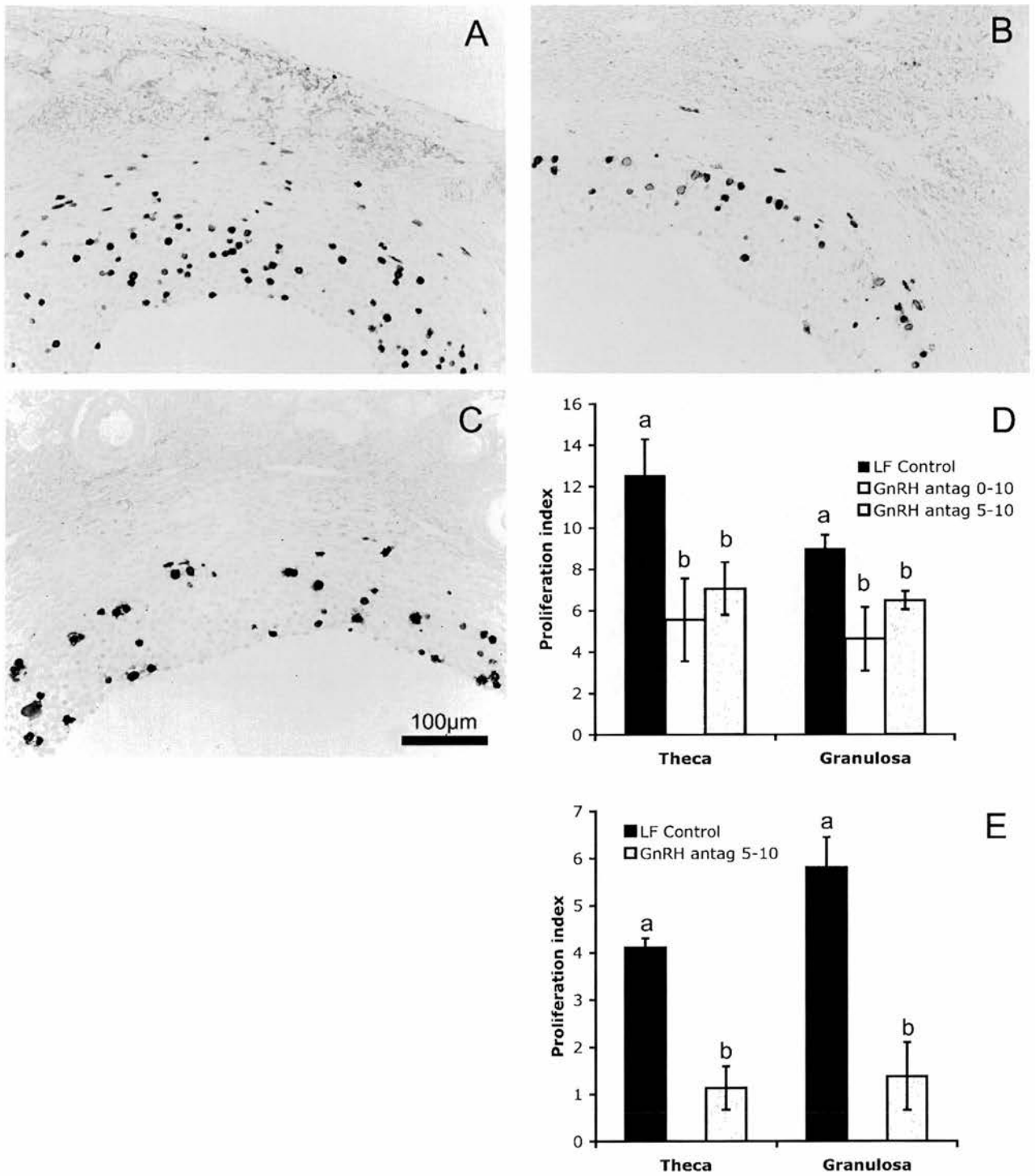


Figure 4 Effects of the treatment on granulosa and thecal cell proliferation as indicated by BrdU immunocytochemistry. (A, B and C) Tertiary follicles of a control, day 0–10- and day 5–10-treated ovary are shown respectively. Note the decrease in both granulosa and thecal proliferation after both treatment schedules. Quantitative analyses confirms the observation of decreased cell proliferation in (D) tertiary follicles and (E) dominant follicles after either treatment. Values are means \pm S.E.M. Different letters indicate statistically significant differences.

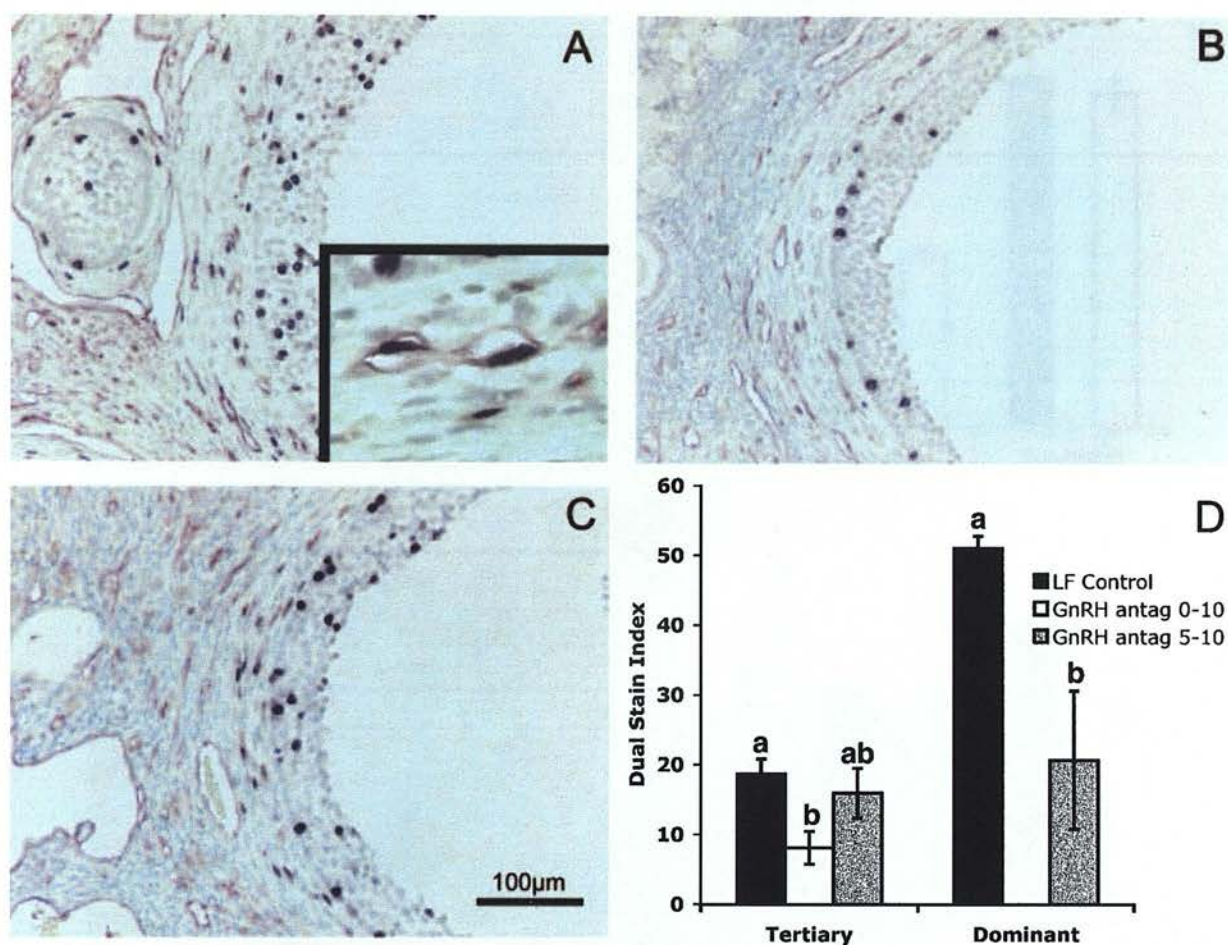


Figure 5 Photomicrographs showing dual staining of proliferating endothelial cells (black nuclei and red cell surface). (A) Control (dual stain shown in inset), (B) days 0–10 and (C) day 5–10 GnRH antagonist treatment. (D) Quantification showing that there was a significant reduction in the number of proliferating endothelial cells between controls and GnRH antagonist treatment between day 0–10. Different letters indicate statistically significant differences.

GnRH antagonist treatment between day 0–10 (Fig. 7E and F) and day 5–10 (Fig. 7G and H) and in mid follicular controls (Fig. 7I and J). VEGF mRNA was also expressed in the theca of atretic follicles in the late follicular controls (Fig. 7C and D). However, thecal expression was absent in the theca of atretic follicles from either of the treatment groups and the mid follicular controls (Fig. 7E–J).

In the dominant follicles of late follicular controls, VEGF mRNA continued to be expressed in the granulosa cells (Fig. 8A and B). However, in follicles that appeared to be closer to the time of ovulation, VEGF mRNA expression in the granulosa cells was reduced and an intense punctate expression pattern in cells in the thecal layer on the border of the granulosa cells was observed (Fig. 8C and D). This punctate pattern was also observed in postovulatory follicles (Fig. 8E and F) at the edge of the newly forming corpus luteum.

In contrast, VEGF mRNA expression was absent from both granulosa and theca in all the dominant follicles from the day 5–10 GnRH antagonist treatment group (Fig. 8G and H). Quantitative analysis confirmed these observations (Fig. 9A and B).

Aromatase mRNA

Aromatase mRNA expression was measured to establish the level of differentiation of the granulosa cells. In control preovulatory follicles, aromatase mRNA was expressed in the mural granulosa cells closest to the membrana propria (Fig. 10A and B), along with some expression in old luteal tissue and newly forming extra-luteal tissue. After both the GnRH antagonist treatment regimes there was an absence of aromatase mRNA expression (day 5–10 shown

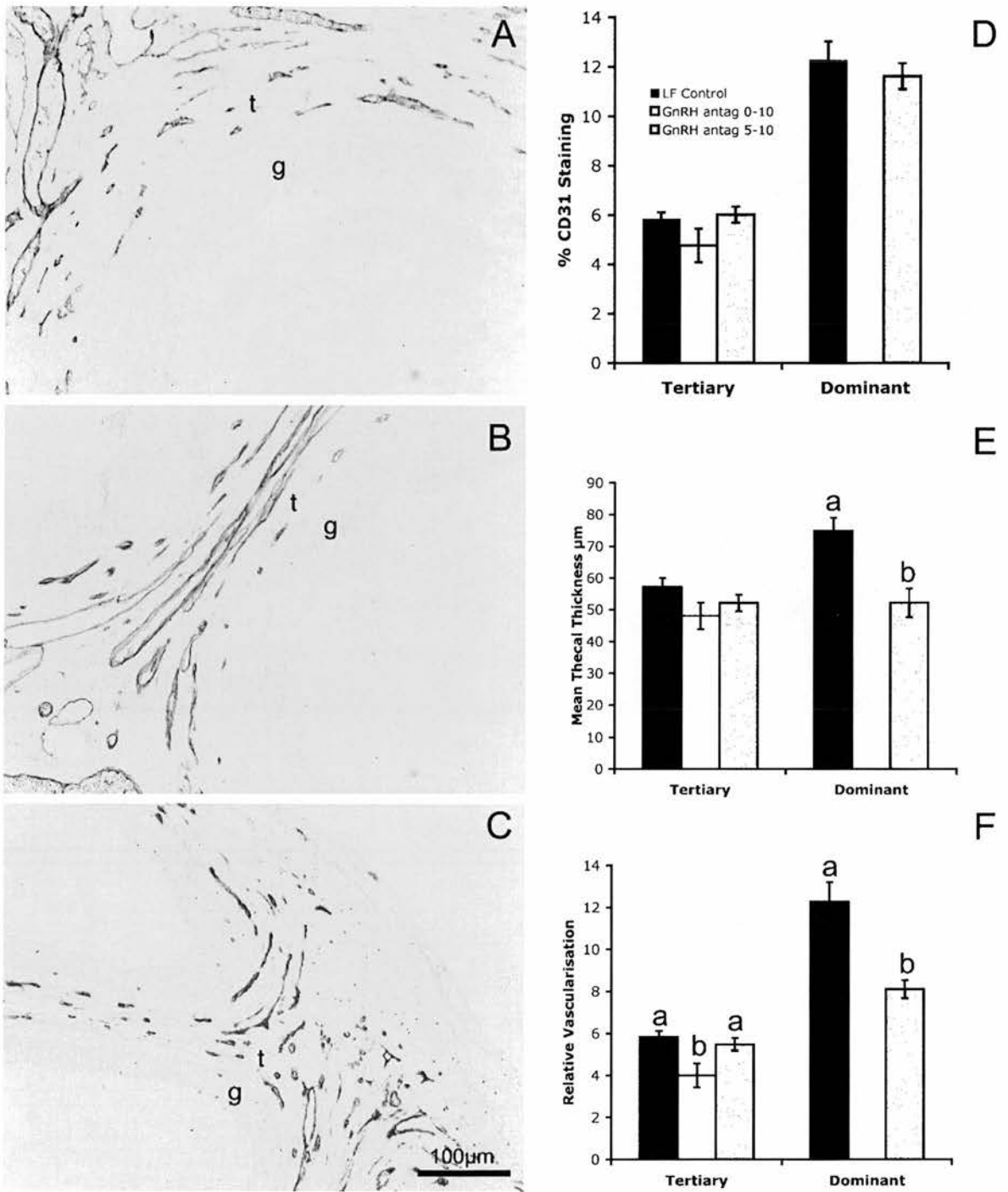


Figure 6 Photomicrographs of tertiary follicles stained for CD31 to visualize endothelial cells. (A) A tertiary follicle from control, (B) day 0–10 and (C) day 5–10 GnRH antagonist-treated ovaries. Note the uniformity between the control and treatment groups. (D) Quantitative analyses confirmed the observation of uniform vascularization between the control and treated groups. Measurement is the percentage CD31-positive staining divided by the total area of the thecal compartment. (E) Measurement of mean thecal thickness between control and treated groups revealed a significant reduction between day 0–10-treated and control ovaries. t, theca; g, granulosa. (F) Quantification of endothelial area between control and treated groups after conversion by the ratio of mean thecal thickness.

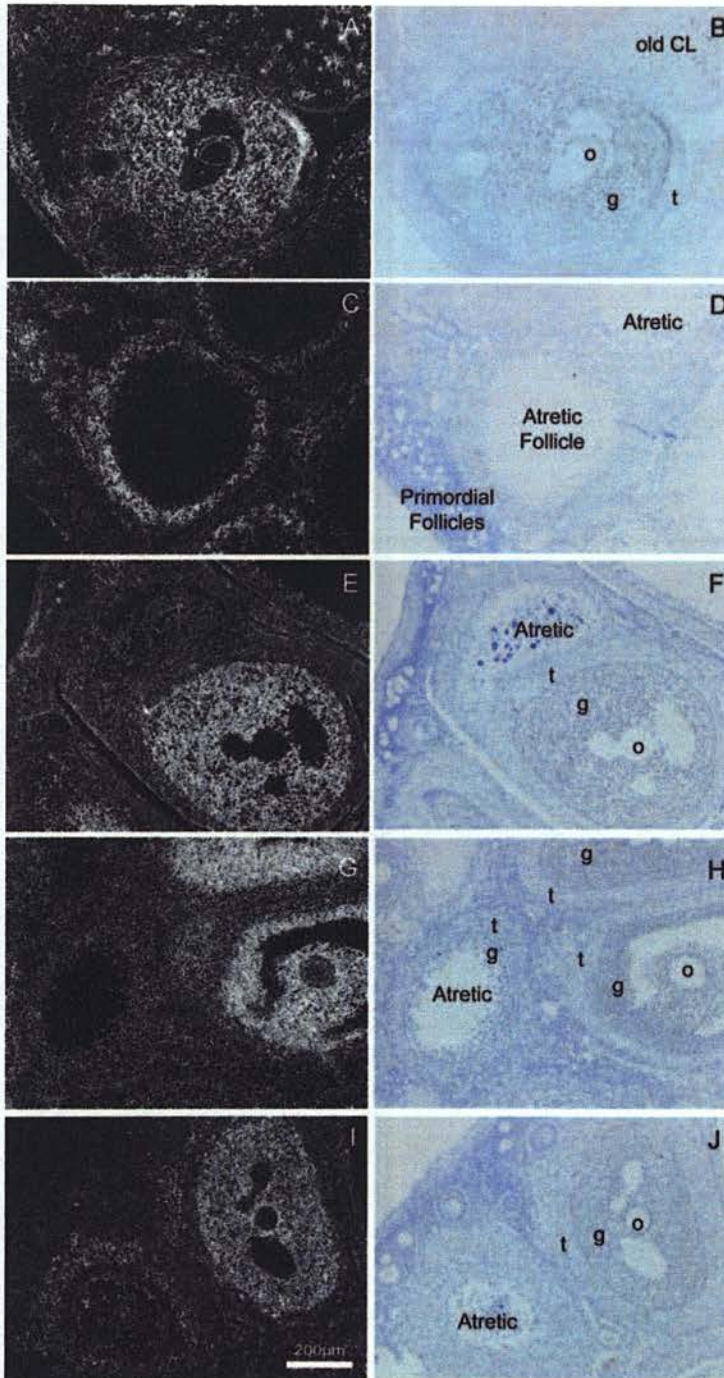


Figure 7 Dark and light field photomicrographs showing *in situ* hybridization for VEGF mRNA. (A and B) VEGF mRNA localized in the granulosa (g) of late follicular phase tertiary follicles. The same expression pattern was found in both treatment groups, (E and F) day 0–10, (G and H) day 5–10 and (I and J) in mid follicular controls. VEGF mRNA was also expressed in the theca (t) of atretic follicles in the late follicular controls (C and D) but was absent in atretic follicles in both of the treatment groups and the mid follicular controls (E–J). Note the frequent occurrence of high expression in cumulus cells surrounding oocyte (o).

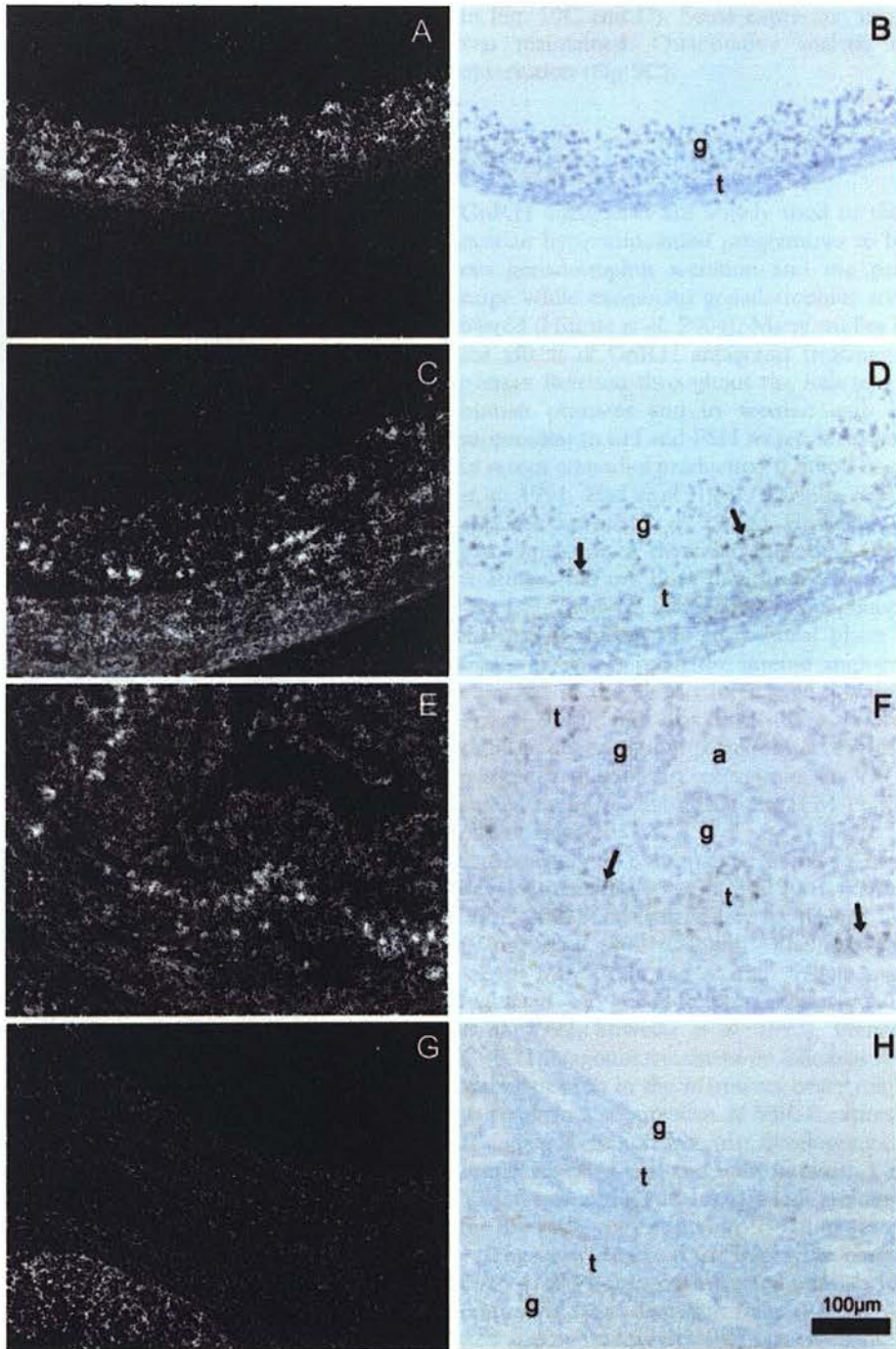
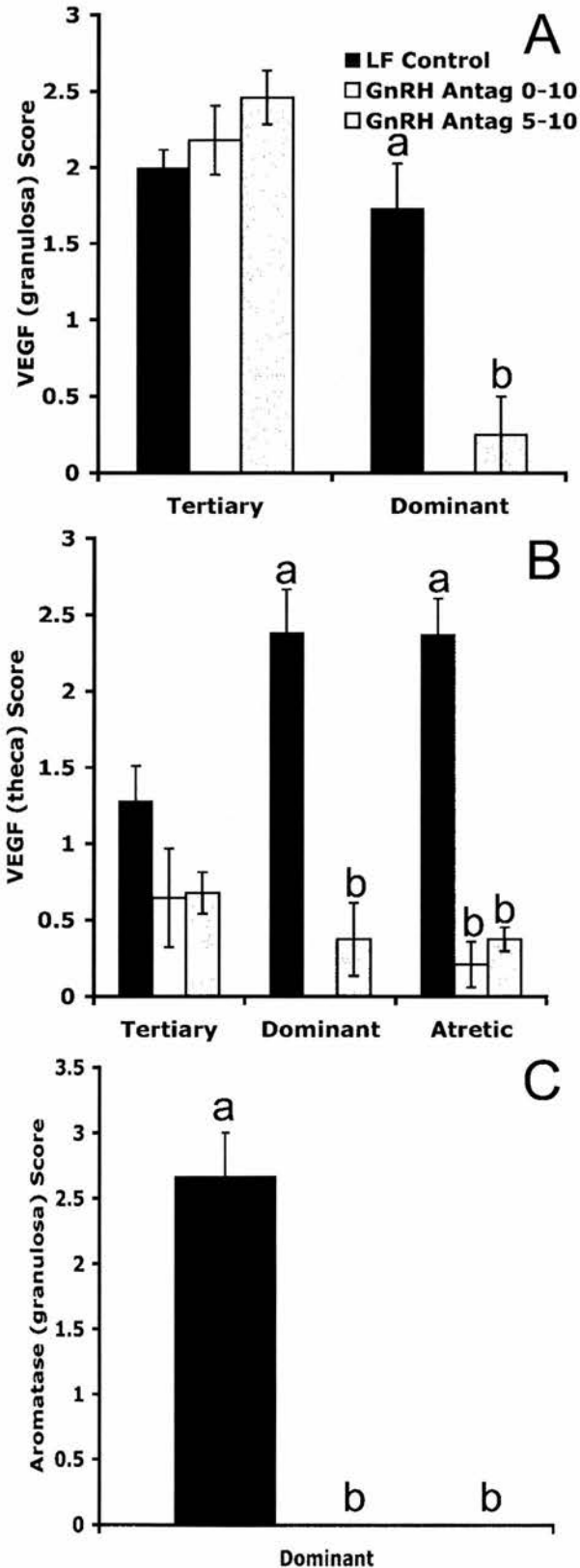


Figure 8 Dark and light field photomicrographs showing *in situ* hybridization for VEGF mRNA. (A and B) VEGF mRNA localized in the dominant follicles of late follicular controls. VEGF mRNA is expressed in the granulosa cells (g). (C and D) Before ovulation, VEGF mRNA expression in the granulosa cells is reduced while a punctate expression pattern in the theca cells (t) on the border of the granulosa cells is observed (arrows). (E and F) This punctate pattern is also observed in postovulatory follicles at the edge of the newly forming corpus luteum (arrows). (G and H) This expression is absent in the dominant follicles from the day 5–10 GnRH antagonist treatment group while expression is maintained in the granulosa of tertiary follicles.



in Fig. 10C and D). Some expression in old luteal tissue was maintained. Quantitative analysis confirmed this observation (Fig 9C).

Discussion

GnRH antagonists are widely used in the clinic during ovarian hyperstimulation programmes to block endogenous gonadotrophin secretion and the preovulatory LH surge while exogenous gonadotrophins are being administered (Huirne *et al.* 2004). Many studies have examined the effects of GnRH antagonist treatment on pituitary-ovarian function throughout the follicular phase in non-human primates and in women and demonstrated a suppression in LH and FSH secretion associated with a fall in serum oestradiol production (Ditkoff *et al.* 1991, Fluker *et al.* 1991, Hall *et al.* 1991, Karnitis *et al.* 1994, Nelson *et al.* 1995, Fraser *et al.* 1999b). However, the effect upon vascularization of the follicles during GnRH antagonist treatment has not been determined. In a previous study, we have shown that GnRH antagonist treatment of marmosets during the early luteal phase had a marked inhibitory effect upon the intense angiogenesis normally observed in the corpus luteum at this time (Dickson & Fraser 2000) and that this was associated with luteal cellular degeneration (Dickson & Fraser 2000) and a marked reduction in expression of VEGF as demonstrated by *in situ* hybridization (H M Fraser, unpublished observations).

Angiogenesis is intimately involved in ovarian follicle development (Fraser & Wulff 2001, Zimmermann *et al.* 2001, 2003) and is necessary for normal follicular growth (Wulff *et al.* 2001b, 2002). The expression pattern of VEGF mRNA in the ovarian follicle suggests that it is regulated by gonadotrophic stimulation (Ravindranath *et al.* 1992, Shweiki *et al.* 1993). Hence the effect of GnRH antagonist treatment on follicular development and vascularization in the marmoset ovary might be expected to result in a suppression of VEGF expression, inhibition of angiogenesis and follicular development. However, the results revealed that the links between GnRH, follicular VEGF expression and angiogenesis are more tenuous than for the early corpus luteum.

The most expected findings were observed in the day 0–10 GnRH antagonist-treated animals in which development of large dominant follicles was completely inhibited and oestradiol secretion was markedly suppressed. In the remaining healthy tertiary follicles, granulosa and theca

Figure 9 Quantification of *in situ* hybridization. (A) VEGF mRNA in granulosa cells of tertiary and dominant follicles. (B) VEGF mRNA in thecal compartment of tertiary, dominant and atretic follicles. (C) Aromatase mRNA expression in the granulosa cells of dominant follicles. Values are means \pm S.E.M. Different letters indicate statistically significant differences.

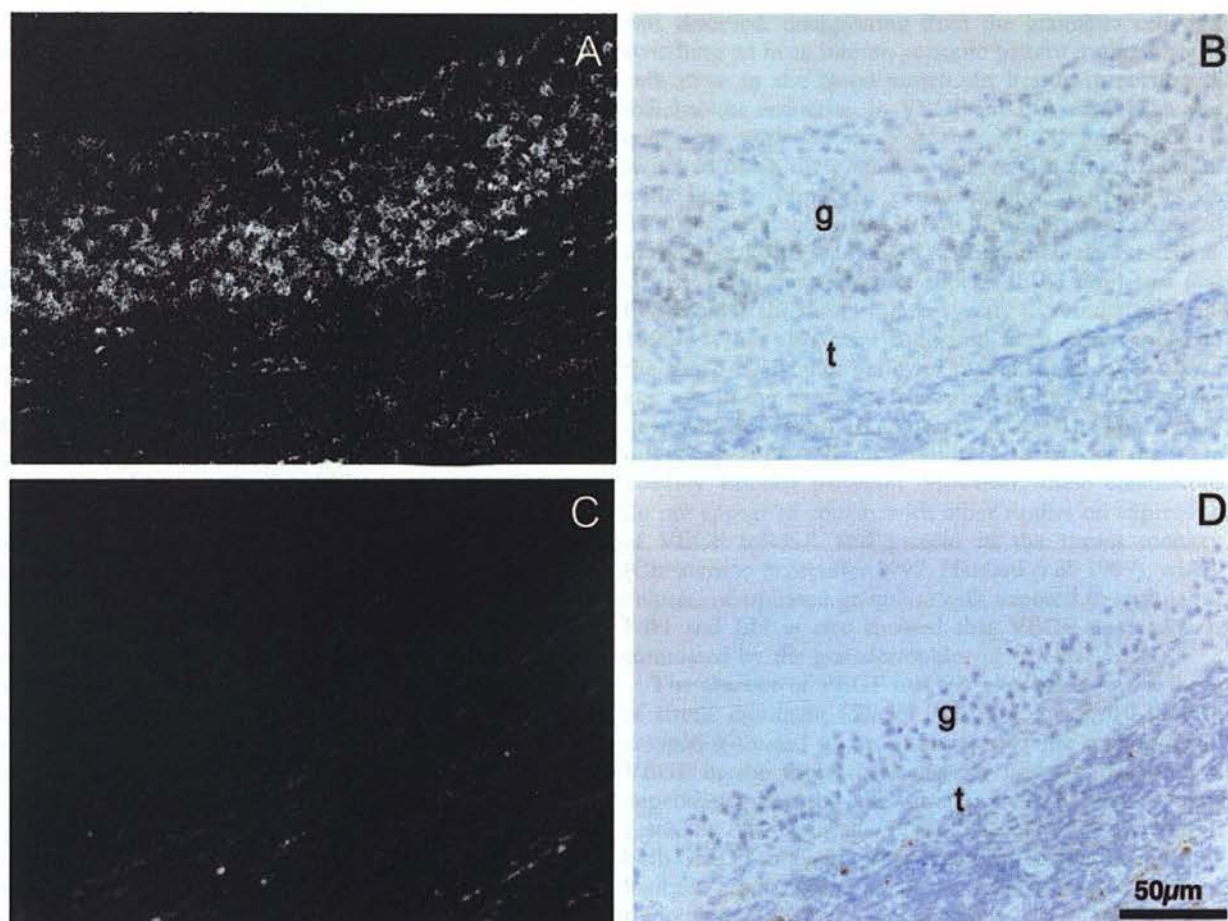


Figure 10 Photomicrograph of *in situ* hybridization for aromatase mRNA. (A and B) Aromatase *in situ* in a preovulatory follicle and (C and D) day 5–10 GnRH antagonist treated. Note the absence of aromatase mRNA expression in the treated ovary. t, theca; g, granulosa.

cell proliferation was reduced. Angiogenesis was also lower in these follicles and this resulted in a reduction in endothelial cell area after the reduction in thecal thickness was taken into account. However, these effects were much less than after inhibition of VEGF by a specific antagonist (Wulff *et al.* 2002). Surprisingly, VEGF mRNA expression was similar to controls in these tertiary follicles. Similarly, in the days 5–10-treated group, VEGF mRNA continued to be highly expressed in the granulosa cells of tertiary follicles. This suggested that, at this stage of development, follicular VEGF expression is not tightly controlled by the gonadotrophins. It is reasonable to suggest that expression may be maintained by residual gonadotrophin secretion. Unfortunately LH and FSH could not be measured in the present study because these assays are problematic in the marmoset and there are restrictions in the volume of plasma that can be collected. However, a degree of residual level of gonadotrophin secretion would be expected to persist after GnRH

antagonist treatment, with FSH being less suppressed than LH (Ditkoff *et al.* 1991, Fluker *et al.* 1991, Hall *et al.* 1991, Nelson *et al.* 1995, Oberye *et al.* 1999). While this suppression is sufficient to reduce the ability of mature follicles to produce oestradiol, the low levels of LH and FSH remaining may be sufficient to maintain the activity of tertiary follicles. In addition, the possibility of stimulation of VEGF expression by hypoxia, as seen in solid tumours (Shweiki *et al.* 1992) and the endometrium (Sharkey *et al.* 2000), occurring in granulosa cells in tertiary follicles deprived of gonadotrophic support requires to be investigated. However, this phenomenon does not appear to occur in follicular granulosa cells from preovulatory follicles (Martinez-Chequer *et al.* 2003). Paracrine mechanisms could also be acting in these tertiary follicles that may still function during the withdrawal of the gonadotrophins to help maintain VEGF mRNA expression. Recent evidence also suggests that locally produced factors and cytokines such as insulin-like growth

factors-I and -II play a role in regulating ovarian VEGF mRNA expression in preovulatory follicles (Martinez-Chequer *et al.* 2003). Such mechanisms may also explain the continued VEGF mRNA expression in tertiary follicles in the current study. The fact that there was a decrease in endothelial cell proliferation in these follicles after 10 days of GnRH antagonist treatment does suggest that a deleterious effect on the VEGF pathway was occurring, perhaps at the level of translation of VEGF mRNA into biologically active protein or by disruption of the process of transfer of the VEGF protein from the granulosa cells to the thecal layer.

Although preovulatory follicles developed during treatment with GnRH antagonist between day 5–10, these follicles were abnormal and cystic in appearance. This indicates a stage of development after which normal levels of gonadotrophic support for follicle expansion is not required, presumably because the dominant follicles have already been selected by the mid follicular phase, as suggested by Gilchrist *et al.* (2001). Angiogenesis was decreased in these cyst-like follicles and VEGF mRNA was absent from both the theca and the granulosa cell layers. The observation that follicles grew to preovulatory size but undergo atresia and fail to produce oestradiol indicates a differential level of gonadotrophic stimulation required for proliferation (growth) and cellular differentiation (steroid synthesis) in the dominant follicle. In addition, the marked reduction in aromatase mRNA expression in these dominant but atretic follicles gives further support to the hypothesis that a differential level of gonadotrophic support is required for dominant follicle expansion and differentiation. GnRH antagonist treatment in cynomolgus monkeys has shown that FSH alone is capable of stimulating follicle expansion as determined by ultrasound monitoring, whilst not stimulating oestradiol production due to lack of LH-stimulated androgen production (Karnitis *et al.* 1994), although the health of these large follicles was not assessed.

It might have been expected that the rate of follicular atresia would have increased after GnRH antagonist treatment. However, there was a reduction in the number of atretic follicles and an increase in the mean tertiary follicle diameter after both GnRH antagonist treatment schedules. This may be explained by the fact that tertiary follicles not having sufficient FSH stimulation to continue developing appear to be held in an arrested state of development and fail to be cleared from the ovary by apoptosis (Tsafiri & Braw 1984) as they would be in a normal follicular phase ovary approaching ovulation. Also, the reduction in thecal thickness observed in the dominant follicle from the day 5–10 treatment group is probably due to a deficiency in gonadotrophic support for both the theca directly and the granulosa cells stimulating thecal development in a paracrine manner.

In follicles close to the point of ovulation in control ovaries, a shift in the VEGF mRNA expression pattern

was observed, disappearing from the granulosa cells but switching on in an intense punctate pattern in the theca in cells close to the blood vessels. In healthy preovulatory follicles, the reduction in VEGF mRNA expression was consistent with the finding that VEGF production is switched off in the granulosa cells of gilts after treatment with human chorionic gonadotrophin (Barboni *et al.* 2000). The punctate VEGF mRNA expression observed in the thecal compartment of dominant follicles on follicular day 10 in the marmoset appears to be similar to that first noted in the rhesus monkey ovary (Ravindranath *et al.* 1992). It has also been suggested from observations in the sheep ovary that pericytes in the thecal layer play a key role in the transition from preovulatory follicle to the new corpus luteum (Redmer *et al.* 2001). The VEGF mRNA-positive cells observed in the marmoset theca are possibly vascular pericytes. However, these conclusions do not appear to concur with other studies on expression of VEGF mRNA and protein in the rhesus monkey (Christenson & Stouffer 1997, Hazzard *et al.* 1999), where cultures of aspirated granulosa cells exposed to regimes of FSH and LH *in vivo* showed that VEGF expression is stimulated by the gonadotrophins.

The absence of VEGF mRNA expression in the theca of atretic dominant follicles from the days 5–10 GnRH antagonist-treated group indicates that the expression of VEGF in the thecal cells directly prior to ovulation is dependent upon the LH surge and not hypoxia as suggested by Neeman *et al.* (1997). The LH surge is absent in both GnRH antagonist-treated groups, while the morphologically intact theca of these follicles would still be responsive to LH. The absence of VEGF mRNA expression in the dominant atretic follicles from the day 5–10 GnRH antagonist-treated group also suggests that normal physiological levels of VEGF are not required for dominant follicle expansion between the mid to late follicular period, even though VEGF is thought to play a role as a permeability factor in follicle development (Koos 1995), allowing the movement of fluid into the follicle to expand the antral cavity.

Surprisingly, VEGF mRNA expression was also detected in the theca of atretic tertiary follicles in the late follicular control animals. This expression was again absent in GnRH antagonist-treated animals and was not present in atretic follicles from mid follicular phase ovaries. This suggests that the LH surge triggers the strong expression of VEGF mRNA in the theca of atretic follicles at the time of ovulation, which may play a role in the establishment of extra luteal tissue from the atretic tertiary follicles present at ovulation, a common feature in the marmoset (McAnulty 1994).

It is of interest to compare the current results with the effects of specific targeting of VEGF on follicular angiogenesis and development in the marmoset. A much higher degree of inhibition of angiogenesis and follicular development was observed when directly inhibiting VEGF

with a potent, receptor-based antagonist VEGF Trap R1R2 (Wulff *et al.* 2002). This antagonist binds VEGF with high affinity and prevents it reaching its receptors. Thus, no matter what compensatory mechanisms come into operation to restore VEGF, they are negated by the VEGF inhibitor. In contrast, while treatment with GnRH antagonist inhibits gonadotrophin secretion this may have little effect on the ability of hypoxia and local paracrine mechanisms to mediate or modulate VEGF action in the ovarian follicle, as these compensatory mechanisms may be allowed to function. It follows that the reason that GnRH antagonist treatment is more effective in suppressing early luteal angiogenesis (Dickson & Fraser 2000) is likely to be because the corpus luteum is heavily dependent upon LH (Fraser *et al.* 1986). This dependence would include the local compensatory mechanisms that are relatively gonadotrophin independent in the developing follicle.

In conclusion, the use of a GnRH antagonist to block gonadotrophin release did not result in complete inhibition of follicular angiogenesis and vascularization and raises the possibility of other factors and paracrine mechanisms regulating VEGF and angiogenesis in the primate ovarian follicle.

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Localization and Quantification of Cyclic Changes in the Expression of Endocrine Gland Vascular Endothelial Growth Factor in the Human Corpus Luteum

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Angiogenesis is essential for normal growth and function of the corpus luteum. The roles of various angiogenic factors in these events are being elucidated. Endocrine gland vascular endothelial growth factor (EG-VEGF) has recently been described in the human ovary. To define the localization of EG-VEGF mRNA in the corpus luteum and determine changes in its expression, dated human corpora lutea were studied at the early, mid-, and late luteal phases. Quantitative RT-PCR was employed to determine changes in EG-VEGF mRNA and compare expression to its related factor prokineticin-2 and the established angiogenic factor, VEGF. *In situ* hybridization was used to localize sites of production of EG-VEGF. To investigate whether expression of EG-VEGF was under the influence of LH or progesterone, luteinized granulosa cells were stimulated with human chorionic gonadotropin in the pres-

ence or absence of a progesterone synthesis inhibitor. EG-VEGF mRNA increased throughout the luteal phase, whereas there was no change in VEGF mRNA. The relative abundance of RNAs based upon PCR signal intensity showed that VEGF and EG-VEGF were highly expressed, whereas expression of prokineticin-2 was low. EG-VEGF mRNA was localized predominantly to granulosa-derived cells of the corpus luteum. Human chorionic gonadotropin stimulated both VEGF and EG-VEGF mRNA *in vitro*, but the level of expression was not influenced by progesterone. These results establish that in the human corpus luteum EG-VEGF is principally derived from granulosa lutein cells and that its synthesis is highest during the mid- to late luteal phase. (*J Clin Endocrinol Metab* 90: 427–434, 2005)

DURING THE TRANSFORMATION of the ovulatory follicle to a fully functional corpus luteum, the vascular endothelial cells undergo an intense period of proliferation followed by recruitment of perivascular cells. Whereas endothelial cells in most tissues live for several years, those in the corpus luteum of a nonfertile cycle die in the space of a few weeks during the process of luteolysis (1). Understanding the molecular mechanisms that regulate these divergent mechanisms is a major challenge in reproductive biology with implications for the understanding of pathological angiogenesis. Factors that regulate luteal vasculature play a major role in the regulation of luteal function, and several studies have described the localization of mRNA and protein for vascular endothelial growth factor (VEGF) in the human corpus luteum (2, 3). Furthermore, inhibition of VEGF *in vivo* during the luteal phase in the nonhuman primate prevents luteal angiogenesis and suppresses progesterone secretion (4, 5).

A novel angiogenic factor with a degree of specificity for the ovary and endocrine glands, endocrine gland vascular

endothelial growth factor (EG-VEGF), has recently been described in the human ovary (6–8). The protein was designated EG-VEGF because it was also found in the testis, adrenal, and placenta (6), and this has been confirmed by others (9). However, EG-VEGF is also present at much lower levels in other tissues such as the small intestine (10), where it was discovered independently and named prokineticin (PK)-1 (10). EG-VEGF/PK-1 acts via G protein-coupled receptors, termed PK-R1 and PK-R2 (11). EG-VEGF is a member of a class of proteins that includes Bv8, otherwise known as PK-2, which has an 83% homology and appears to be preferentially expressed in the testis (12). Structurally, EG-VEGF/PK-1 is distinct from VEGF but exhibits functional similarities.

The aim of the current study was to define the localization of EG-VEGF mRNA and determine changes in its expression in dated human corpora lutea from the early, mid-, and late luteal phase using *in situ* hybridization and quantitative RT-PCR analysis. In addition, to gain more information on the factors regulating EG-VEGF expression, a primary culture system for luteinized granulosa cells was employed in which the cells were stimulated with human chorionic gonadotropin (hCG) in the presence or absence of an inhibitor of progesterone synthesis.

Subjects and Methods

Tissue

The study was approved by the Lothian Pediatrics and Reproductive Medicine Ethics Committee, and informed consent was obtained from

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Abbreviations: EG-VEGF, Endocrine gland VEGF; hCG, human chorionic gonadotropin; 3β HSD, 3β hydroxysteroid dehydrogenase; LDL, low-density lipoprotein; PK, prokineticin; RT, reverse transcription; SSC, saline sodium citrate; TBS, Tris-buffered saline; and VEGF, vascular endothelial growth factor.

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all patients before tissue collection. Corpora lutea were enucleated at the time of hysterectomy as described previously (13). All women were healthy and aged 32–45 yr and with benign gynecological conditions, typically uterine fibroids. Only women with regular menstrual cycles who had not received any form of hormonal treatment during the previous 3 months took part in the study. The date of the preovulatory LH surge was determined by measuring LH concentration in serial early-morning urine samples collected before operation (13). On this basis, eight corpora lutea were classified as early luteal (LH + 1 to + 5 d), 12 as mid-luteal (LH + 6 to + 10 d), and six as late luteal (LH + 11 to + 14 d). There were no differences in the average age between the groups. Serum was collected at the time of surgery for progesterone determination (13).

Corpora lutea were enucleated from the ovary by blunt dissection. The tissue was immediately divided into radial blocks to ensure standard representation of all regions of the corpus luteum, and portions were either frozen on dry ice and stored at -70°C until RNA extraction or fixed in 4% neutral buffered formalin for paraffin embedding. In each case, an endometrial biopsy was obtained, and dating of the luteal-phase endometrium (14) confirmed the luteal-phase classification.

Preparation of cDNA

A potential disadvantage of the RT-PCR approach using whole tissue containing heterogeneous cell types is that specimens have variable degrees of stromal or extracellular matrix component. To reduce this potential problem in the present study, a large proportion of the corpus luteum (between 30 and 50%) was extracted. Thus, total RNA was extracted from 0.2- to 0.5-g frozen corpora lutea samples using Tri Reagent (Sigma-Aldrich Co. Ltd., Gillingham, UK) according to the manufacturer's instructions. The integrity of the RNA was confirmed from the absorbance 260/280-nm ratio and by ethidium bromide gel electrophoresis. RNA concentration was calculated by absorbance at 260 nm, measured on a Gene Quant-Pro spectrophotometer (Amersham Biosciences, Little Chalfont, UK).

To eliminate genomic DNA contamination, 10 μg RNA was DNase treated (RQ-1 RNase-free DNase, Promega, Southampton, UK) by incubation at 37°C for 30 min and the reaction stopped by the addition of Stop Solution (20 mM EGTA, Promega) and heating to 70°C for 10 min. Reverse transcription (RT) was performed under carefully controlled conditions, using a Taqman Reverse Transcription kit with random hexamers (Applied Biosystems, Warrington, UK). All samples were treated simultaneously from the same reagent mix containing 2 μl RT buffer; 2 μl deoxynucleotide triphosphates (10 mM); 1 μl each of RNase inhibitor (20 U/ μl), random hexamers (50 μM), and Multiscribe RT enzyme (50 U/ μl); 2.4 μl MgCl_2 (25 mM); and 0.2 μg of DNase-treated RNA in a final sample volume of 20 μl . RT was performed at room temperature for 10 min and at 42°C for 60 min and was stopped by incubation at 95°C for 10 min. The resulting cDNA was stored at -20°C until use. An equal amount of human placental RNA (Cambridge Bioscience, Cambridge, UK, 1 mg/ml) was concomitantly reverse transcribed to provide cDNA for the generation of standard calibration curves for each gene examined.

Primer design

Oligonucleotide PCR primers for each gene investigated were designed using Primer3 software (available at [http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi](http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)) from DNA sequences obtained from GenBank (<http://www.ncbi.nlm.nih.gov>). Primers were synthesized by MWG (MWG-AG Biotech, Milton Keynes, UK). For VEGF-A (GenBank accession no. E15157), primers were 5'-TACCTC-CACCATGCCAAGTG-3' and 5'-TAGCTGCGCTGATAGACATCCA-3', giving a 103-bp product. Primers for EG-VEGF (GenBank accession no. AF333024) were 5'-GCAAGCGCCTAAAAATTGAT-3' and 5'-CCT-TCTTCAGGAAACGCAAG-3' and gave a 124-bp product, and for PK-2 (GenBank accession no. AF333025), the primers were 5'-CCCACTCCT-GCTCCTCTTG-3' and 5'-CCAGATACTGACAGCACAGCA-3', and the product was 133 bp.

Optimization of PCRs

Each assay was optimized with regard to anneal temperature and magnesium concentration. Firstly, PCR amplification of human placen-

tal cDNA was performed with ThermoStart Taq Polymerase (Advanced Biotechnologies, Epsom, UK) at anneal temperatures between 51 and 65°C , using the gradient feature of a DNA Engine gradient cycler (MJ Research Inc., Watertown, MA). All products were examined by gel electrophoresis to confirm the presence of a single band at the correct size and to determine the optimum anneal temperature for each primer set. The identity of each PCR product was further confirmed by sequence analysis.

Successful reactions were then transferred to the LightCycler, and the magnesium concentration was titrated in the range 3–5 mM, using the LightCycler Fast Start DNA Master SYBR Green 1 kit (Roche, Lewes, UK), according to the manufacturer's instructions. The PCR cycling conditions were denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec, annealing at the temperature defined above for 5 sec, and extension at 72°C for 15 sec. A melting curve analysis following the amplification schedule allowed optimization of the temperature used to quantify the level of gene expression by minimizing background fluorescence from nonspecifically amplified DNA. All PCR assays exhibited a single DNA melting curve peak. The optimized PCR conditions were as follows: VEGF-A annealed at 65°C using 3 mM MgCl_2 , and fluorescence was measured at 83°C ; EG-VEGF annealed at 61°C using 5 mM MgCl_2 , and fluorescence was measured at 84°C ; and PK-2 annealed at 66°C using 3 mM MgCl_2 , and fluorescence was measured at 83°C . The VEGF-A product melted at 85°C , the EG-VEGF product at 87°C , and the PK-2 product at 90°C .

Quantitative real-time PCR

Quantitative real-time PCR was performed in duplicate 10- μl reaction volumes on the LightCycler using the Mastermix supplied with the LightCycler Fast Start DNA Master SYBR Green 1 kit (Roche), at the optimized conditions described above. In each case a standard curve was generated using serial dilutions of human placental cDNA.

Using the second derivative maximum method provided in the LightCycler software (version 3.3), a standard curve was generated by plotting the external standard concentration against threshold cycle. This analysis method ensured interrun consistency. The software automatically calculated PCR product concentration for each tissue sample. All samples were analyzed in duplicate, and assay variation was typically within 10%. Data were normalized according to the expression level of glucose-6-phosphate-dehydrogenase determined in duplicate by reference to a serial dilution calibration curve as above. The generation of calibration curves for both the gene of interest and the normalizing gene allows for any difference in amplification efficiencies between the genes. PCR products were extracted from the LightCycler capillaries and analyzed on 2% agarose gels to confirm product size and reaction specificity.

In situ hybridization

To investigate possible changes in expression patterns of the mRNA for EG-VEGF in the corpus luteum, *in situ* hybridization was performed. Forward 5'-AGAGGCATCTAAGCAGGC and reverse 5'-AGGTAT-GTCTGCTGTGTGC primers were used to generate a cDNA fragment corresponding to nucleotides 10–533 of the human sequence (GenBank accession no. NM032414). This fragment was cloned into a pGEM-T easy vector (Promega Corp., Madison, WI) containing both T7 and SP6 transcription sites. Sense and antisense probes were prepared using a RNA transcription kit (Ambion, Inc., Austin, TX) and were labeled with [^{35}S]UTP (NEN Life Science Products, Boston, MA). The synthesized probes were purified from free bases using Chroma Spin-100 columns (Clontech Laboratories, Inc., Palo Alto, CA).

Paraffin sections (5 μm) were mounted onto SuperFrost Plus glass slides (BDH, Dorset, UK). Sections were deparaffinized in xylene and hydrated through descending concentrations of ethanol. Sections were treated with 0.1 N HCl and then digested in proteinase K (5 $\mu\text{g}/\text{ml}$, Sigma, St. Louis, MO) for 30 min at 37°C . The digestion was stopped by treating the slides with 0.2% glycine for 10 min at 4°C . After acetylation with 0.2% acetic anhydride in triethanolamine buffer (Sigma), the slides were washed in $4\times$ SSC (saline sodium citrate). A prehybridization step was carried out by incubation in prehybridization buffer (50 $\mu\text{l}/\text{slide}$, containing 50% formamide, $4\times$ SSC, $1\times$ Denhardt's solution, 125 $\mu\text{g}/\text{ml}$ salmon testes DNA, 125 $\mu\text{g}/\text{ml}$ yeast transfer RNA, and 10 mmol/liter

dithiothreitol at 55 C in a moist chamber for 2 h. Hybridization was performed in a moist chamber overnight at 55 C. The hybridization buffer was similar to the prehybridization buffer, but contained 10% dextran sulfate additionally. Two sections per slide were exposed to the antisense or the sense sequences. After hybridization, slides were rinsed in 4× SSC and treated with ribonuclease A (20 μg/ml; Sigma) for 30 min at 37 C to remove all excess probe; desalted in descending concentrations of SSC (2× SSC for 30 min at room temperature, 1× SSC at 65 C, and 0.1× SSC at room temperature); and dehydrated for 2 min each in 50, 84, and 94% ethanol containing 0.3 mol/liter ammonium acetate. Dry slides were dipped in Ilford G5 liquid emulsion (Ilford Imaging, Cheshire, UK), exposed for 5 wk at 4 C, and subsequently developed (Kodak D19 developer, Eastman Kodak Co., Rochester, NY) and fixed (Kodak GBS). All slides were counterstained with hematoxylin, dehydrated, and mounted.

The slides were analyzed under both light- and dark-field conditions. The intensity of the hybridization signal was scored for the various compartments (granulosa-derived lutein cells, theca-derived lutein cells, stroma, and endothelium) as follows: 0, no expression above tissue background; 1, detectable grains above background; 2, low expression; 3, moderate expression; 4, high expression; and 5, intense expression with grain coalescence. Two observers scored the sections “blind.” Comparison of the results between the observers showed excellent agreement.

Immunohistochemistry

To determine the origin of the steroid-producing luteal cells, 5-μm paraffin-embedded sections were immunostained for 3β hydroxysteroid dehydrogenase (3βHSD), which stains granulosa-derived cells strongly and theca-derived cells weakly, and 17α-hydroxylase, which stains theca-derived cells only. Paraffin-embedded tissue sections were dewaxed in xylene and rehydrated through decreasing grades of alcohol. Endogenous peroxidase activity was blocked by 30-min incubation in 3% hydrogen peroxide in methanol. For the 3βHSD immunostain, nonspecific binding was reduced by blocking with normal goat serum and diluted 1:5 in Tris-buffered saline (TBS) containing 5% BSA. Sections were incubated overnight at 4 C in rabbit polyclonal 3βHSD antibody (kindly donated by Prof. J. I. Mason, University of Edinburgh, Edinburgh, UK) diluted 1:1000 in normal goat serum block. Slides were washed three times in TBS. Incubation with the secondary antibody biotinylated goat antirabbit IgG (Dako Ltd, Cambridgeshire, UK), diluted 1:500, was performed for 40 min at room temperature. For the 17α-hydroxylase immunostain, a normal porcine serum block was used to reduce nonspecific staining as above. The primary antibody, rabbit anti-pig 17α-hydroxylase (CYP17, kindly donated by Prof. J. I. Mason), was applied at a dilution of 1:750 and incubated overnight at 4 C. A biotinylated swine antirabbit secondary antibody (Dako Ltd.) was used in a 40-min incubation as above. Detection in each case involved, after washing in TBS, a 30-min incubation in avidin-biotin horseradish peroxidase complex (Dako Ltd.) followed by incubation in diaminobenzidine (Dako Ltd.) to give a brown color. Sections were lightly counterstained with hematoxylin, dehydrated, and mounted in Pertex (CellPath, Powys, UK). In each case negative controls, with omission of the primary antibody, were included.

Collection of human luteinized granulosa cells

The Reproductive Medicine Subcommittee of the Lothian Medical Ethics Committee approved the collection of luteinized granulosa cells from patients undergoing assisted conception. Follicular fluid was collected from women undergoing transvaginal oocyte retrieval for IVF following ovarian stimulation using a standard procedure. Briefly, a long protocol-stimulated cycle was followed, using nasal nafarelin (Pharmacia, Milton Keynes, Bucks, UK) for down-regulation and daily purified gonadotrophins (Menopur, Ferring Pharmaceuticals, Langley, Berks, UK) for ovarian stimulation. When at least three follicles reached 18 mm in diameter, 10,000 IU of hCG was administered. Transvaginal oocyte collection was performed under sonographic guidance 35 h later.

Granulosa cells were obtained from the follicular aspirates after the removal of the oocytes. Individual follicles were not distinguished, and all follicular fluid from the same individual was pooled and centrifuged at 1500 rpm for 10 min. The cells were resuspended in culture medium

(DMEM/F12 Ham mixture, Life Technologies, Inc., Gaithersburg, MD), layered over a 45% Percoll/culture medium mixture, and centrifuged at 1200 rpm for 30 min to pellet the blood cells. Luteinized granulosa cells, visible in the interface, were collected by pipette and washed three times in PBS. The cells were resuspended in culture medium, and viable cells were counted using a Trypan blue exclusion test. Eighty thousand viable cells were plated onto each well of 24-well plates precoated with Matrigel (15, 16) and cultured using 1 ml culture medium at 37 C in 5% CO₂ in air. Cells were cultured for 7–8 days using culture medium supplemented with glutamine (2 mmol/liter), insulin (6.25 μg/liter), transferrin (6.25 mg/liter), selenious acid (6.25 μg/liter), amphotericin (2.5 mg/liter), penicillin (50 mg/liter), and streptomycin (60 mg/liter) as described previously (16). Media were changed every 2–3 d over the course of the culture period.

After 7–8 d in culture, fresh serum-free medium was added containing the following treatments: 1) low-density lipoprotein (LDL; 50 mg/liter) (Sigma), 2) hCG (10 ng/ml) (Serono) and LDL (50 mg/liter), 3) hCG (100 ng/ml) and 4) hCG (100 ng/ml) and aminoglutethamide (100 μM) (Sigma). This treatment regime was designed to manipulate progesterone concentrations in the presence of hCG. After 24 h, medium was collected for progesterone measurement, and cells were collected for mRNA extraction. The treatment regimens and doses used had previously been optimized, and the experiments were repeated in triplicate on three different occasions.

Preparation of cDNA from cultured cells

After removal and storage of culture medium, cells were rinsed in PBS and Tri Reagent was added. The resulting solution was stored at –70 C until batch extraction of RNA was carried out. RNA was extracted following the manufacturer's instructions. To remove contaminating genomic DNA, RNA was treated with DNase I at a concentration of 1 U per μg RNA for 30 min at 37 C. After stopping the reaction with Stop Solution, the samples were heated to 70 C for 10 min. Using random hexamers, 200 μg RNA was reverse transcribed in a solution containing 5.5 mM MgCl₂, 2.5 μM random hexamers, 500 μM each deoxynucleotide triphosphates, 0.4 U/μl RNase inhibitor, and 1.25 U/μl Multiscribe reverse transcriptase (PE Applied Biosystems, Warrington, Cheshire, UK). Samples were incubated at room temperature for 10 min, followed by 42 C for 60 min and 95 C for 10 min. Two controls were used; one omitted the Multiscribe enzyme and the other the template RNA. The resulting cDNA was used for PCR.

Measurement of progesterone

Progesterone concentrations in serum and in culture media collected were measured using a plate modification of a standard in-house progesterone RIA (13). This assay has a detection limit of 0.1 nmol/liter and intra- and interassay coefficient of variation of less than 4% and less than 11%, respectively.

Statistical analysis

Differences between groups with respect to level of mRNA expression after *in situ* hybridization were analyzed using the Kruskal-Wallis test, significance being ascribed at a level of $P < 0.05$. To determine changes in serum progesterone and differences in expression between stages of the luteal phase after RT-PCR and after cell culture, data were exported into Microsoft Excel, and statistical analyses (ANOVA) with the Bonferroni test for pairwise comparisons were performed with SPSS (Statistical Package for Social Sciences, SPSS Inc, Chicago, IL), version 10.7 for Macintosh, with significance being indicated by $P < 0.05$.

Results

Serum progesterone

The serum concentrations of progesterone at the time of surgery were 24.7 ± 6.3 nmol/liter (mean \pm SEM) for the early luteal phase, rising to 56.0 ± 8.1 nmol/liter at mid-luteal phase before declining significantly ($P < 0.05$) to 10.3 ± 4.5 nmol/liter in the late luteal phase group.

Quantitative RT-PCR

The amplification efficiencies of each primer set were similar as detected by generation of standard curves by the LightCycler software. Quantitative RT-PCR showed there was no significant change in the expression of VEGF mRNA during the luteal phase (Fig. 1). EG-VEGF mRNA progressively increased throughout the luteal phase, showing a significant increase in expression ($P < 0.05$) between early and late luteal phase (Fig. 1). Expression of PK-2 showed a non-significant trend to increase through the luteal phase, in a similar pattern to EG-VEGF mRNA (Fig. 1).

Comparison of the levels of VEGF, EG-VEGF, and PK-2 based on crossing point data relative to glucose-6-phosphate-dehydrogenase showed that VEGF and EG-VEGF were highly expressed, whereas PK-2 expression was extremely low.

In situ hybridization and immunohistochemistry

In situ hybridization showed that localized concentrations of silver grains representing EG-VEGF mRNA were present in all 26 specimens studied. Typical examples from the early, mid-, and late luteal phases are shown in Fig. 2. Early luteal

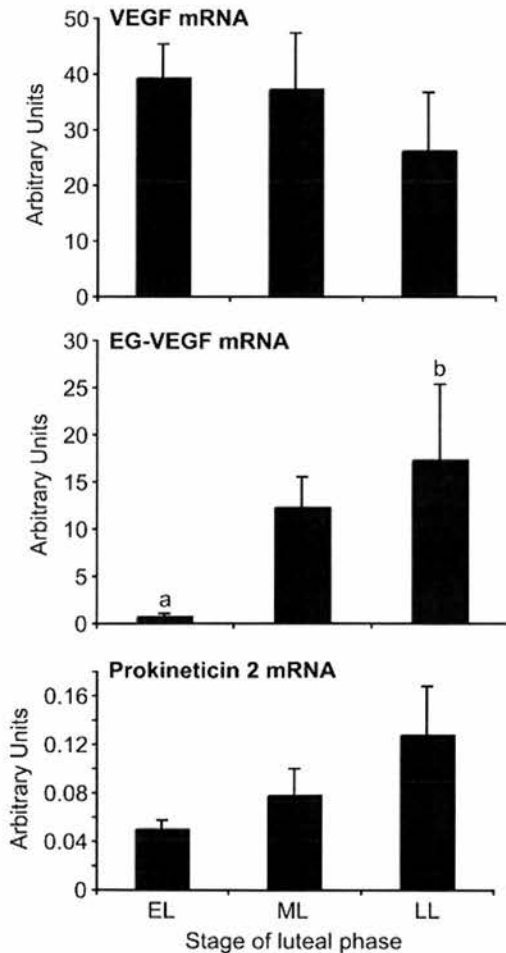


FIG. 1. Changes in expression of VEGF, EG-VEGF, and PK-2 mRNA in the early, mid-, and late luteal phase as determined by RT-PCR. EL, ML, and LL indicate early, mid-, and late luteal phases, respectively. Different letters show where differences are significant.

phase specimens had relatively low-intensity expression, whereas those of the mid- and late luteal phase showed EG-VEGF mRNA to be moderately to intensely expressed. Grains were clearly predominantly localized to the granulosa-lutein cells over which they were uniformly distributed, and this was confirmed by comparison with sections stained with 3 β HSD and 17 α -hydroxylase, which localized to hormone-producing and theca-derived cells, respectively (Fig. 2). The sense probe showed absence of tissue localization above background (Fig. 2J). Control sections for 3 β HSD and 17 α -hydroxylase immunostaining were negative (Fig. 2, K and L).

Light-field examination of the *in situ* slides showed that in early luteal phase specimens, granulosa-lutein cells expressed in the weak to moderate range, whereas expression was also observed in the theca-derived cells, generally at a lower level. In the mid-luteal specimens, expression was extensive in the granulosa lutein cells, whereas theca lutein cells were negative. The late luteal specimens also confirmed the granulosa-derived cells as the site of highest EG-VEGF expression, whereas the theca-derived cells were negative or demonstrated expression in the occasional cell (Fig. 3). Endothelium showed no hybridization, whereas stromal expression was weak or absent and was unaffected by stage (Fig. 3).

Scoring showed that intensity of grains in granulosa lutein cells was significantly increased ($P = 0.0008$) from the early to mid- and late luteal phases, whereas in the theca-derived cells intensity of grains was lower ($P = 0.02$) than in the other two stages at the mid-luteal phase (Fig. 4). There were no stage-specific significant differences in the low levels of stromal grain intensity.

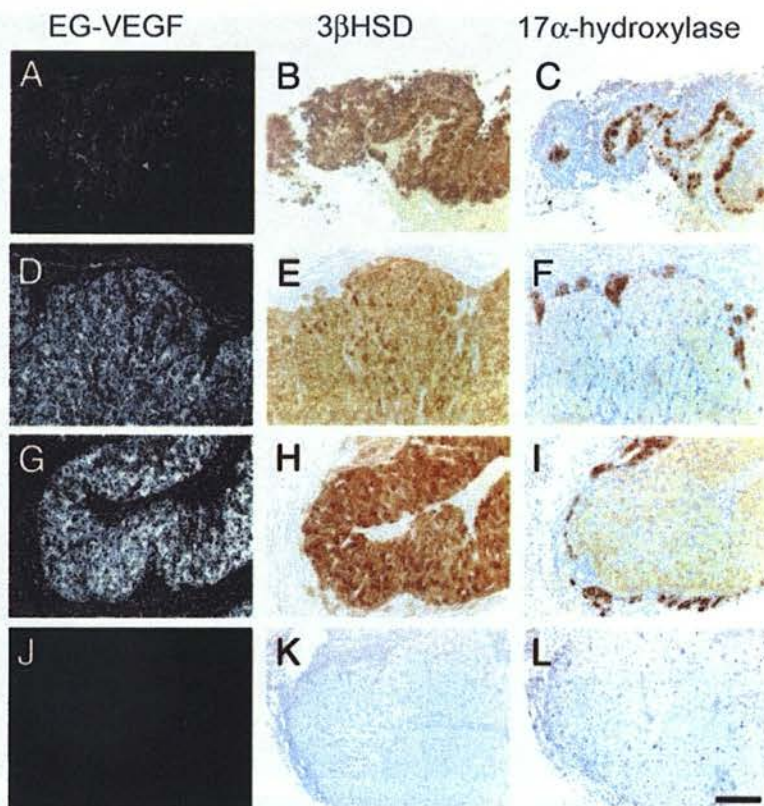
Luteinized granulosa cells

Incubation of luteinized granulosa cells with hCG significantly increased ($P < 0.05$) progesterone production and significantly ($P < 0.05$) increased expression of both VEGF and EG-VEGF mRNA (Fig. 5). However, stimulation of progesterone production further by addition of LDL failed to induce a further rise in either VEGF or EG-VEGF mRNA. In addition, reduction of hCG and LDL-stimulated progesterone synthesis by aminoglutethamide also failed to alter synthesis of either factor.

Discussion

In this study, the complementary approaches of real-time PCR and *in situ* hybridization have been used to define the changes in expression of EG-VEGF mRNA in the human corpus luteum throughout the luteal phase. Both techniques showed that expression increased as the corpus luteum matured. Comparison of expression with the established luteal endothelial mitogen, VEGF, showed that by RT-PCR in contrast to EG-VEGF, VEGF mRNA was already being expressed at maximal levels during the early luteal phase and was not significantly altered during the luteal phase, in agreement with our previous *in situ* hybridization findings (17). VEGF and EG-VEGF mRNA are largely similar in their localization predominantly to the granulosa lutein cells. The relative abundance of RNAs based upon PCR signal intensity

FIG. 2. Expression of EG-VEGF mRNA (silver grains) in the human CL as revealed by *in situ* hybridization and staining of the hormone-producing cells by 3 β HSD and theca-derived cells by 17 α -hydroxylase antibodies (brown stain) from the same corpora lutea from the early (A–C), mid- (D–F), and late (G–I) luteal phases. Dark-field *in situ* photographs show grain density increasing from the early to the mid- and late luteal phases. Immunocytochemical identification of the luteal cell types shows that expression of EG-VEGF mRNA is localized predominantly to granulosa-derived luteal cells. Control slide for the late luteal section hybridized with sense probe is shown in panel J, whereas the slides shown in panels K and L were incubated in the absence of antibodies for 3 β HSD and 17 α -hydroxylase, respectively. Scale bar, 100 μ m.



showed that VEGF and EG-VEGF/PK-1 were highly expressed, whereas expression of the related factor PK-2 was low. These findings strongly suggest that EG-VEGF, in addition to VEGF, has a physiological role in regulation of the luteal vasculature. In contrast, PK-2 is unlikely to have a significant role in the human corpus luteum as has been suggested from studies in other species (18).

The observation of independent changes in levels of expression between VEGF and EG-VEGF within the corpus luteum also occurs in the human follicle (19) where EG-VEGF, but not VEGF mRNA is found in granulosa cells of primordial and primary follicles. As follicles mature, VEGF expression increases, whereas that of EG-VEGF mRNA declines in the granulosa and increases in the theca (19). In addition, different expression rates between the two factors have also been reported in a human granulosa-lutein cell line (20). The study on the human follicle (19) also described the localization of both mRNAs in the human corpus luteum. Although results on localization of VEGF mRNA to the granulosa lutein cells agree with our results using *in situ* hybridization (17), they differ in that the theca-lutein cells appeared to be the predominant site of EG-VEGF production (19). In our study, the site of major expression of EG-VEGF was identified as the granulosa-derived cells by visual inspection of hematoxylin-stained slides. To confirm the localization of EG-VEGF mRNA, sections of all corpora lutea were stained with 3 β HSD to localize steroidogenic cells and 17 α -hydroxylase to localize theca-derived cells specifically. These results established that the predominant site of EG-VEGF expression was the granulosa-lutein cells with some expression in theca-lutein cells in the early luteal specimens and the oc-

casional positive theca-derived cell in the late luteal phase. The previous study on human ovaries (19) was based upon the ovaries of 13 patients collected at random stages of the cycle (assuming six to seven in luteal phase) and staged by histological criteria. This contrasts with the 26 corpora lutea described herein that were staged according to hormonal analysis. Staging from histological criteria alone may result in inaccurate conclusions in some cases, especially with what appears to be the late luteal phase. Both studies agree in that the early corpus luteum exhibits relatively low expression of EG-VEGF. Both studies agree that in the mid-luteal phase, EG-VEGF is highly expressed, but our results provide stronger evidence of the granulosa-lutein cells, rather than the theca-lutein, being the predominant source of EG-VEGF. However, in an ovary estimated to be at d 8 postovulation, a major decrease in VEGF signal with EG-VEGF confined to apparent theca-derived cells was reported (19). At this stage of the luteal phase, VEGF should be highly expressed (2, 17). In the absence of fundamental assessment by progesterone or 3 β HSD immunostaining, it is uncertain whether this sample was correctly timed. In our study, this stage was characterized by moderate to high EG-VEGF in all 12 specimens. Finally, in the other study of a regressing corpus luteum, both VEGF and EG-VEGF expression were virtually absent (19), contrasting markedly with our results, which showed that in all six late luteal specimens EG-VEGF mRNA was either moderately or highly expressed, including in specimens with apoptosis. It may be that for the late luteal phase ovary described (19), the age may have been underestimated, especially as the luteal cells were highly vacuolated and several large antral follicles were present (19).

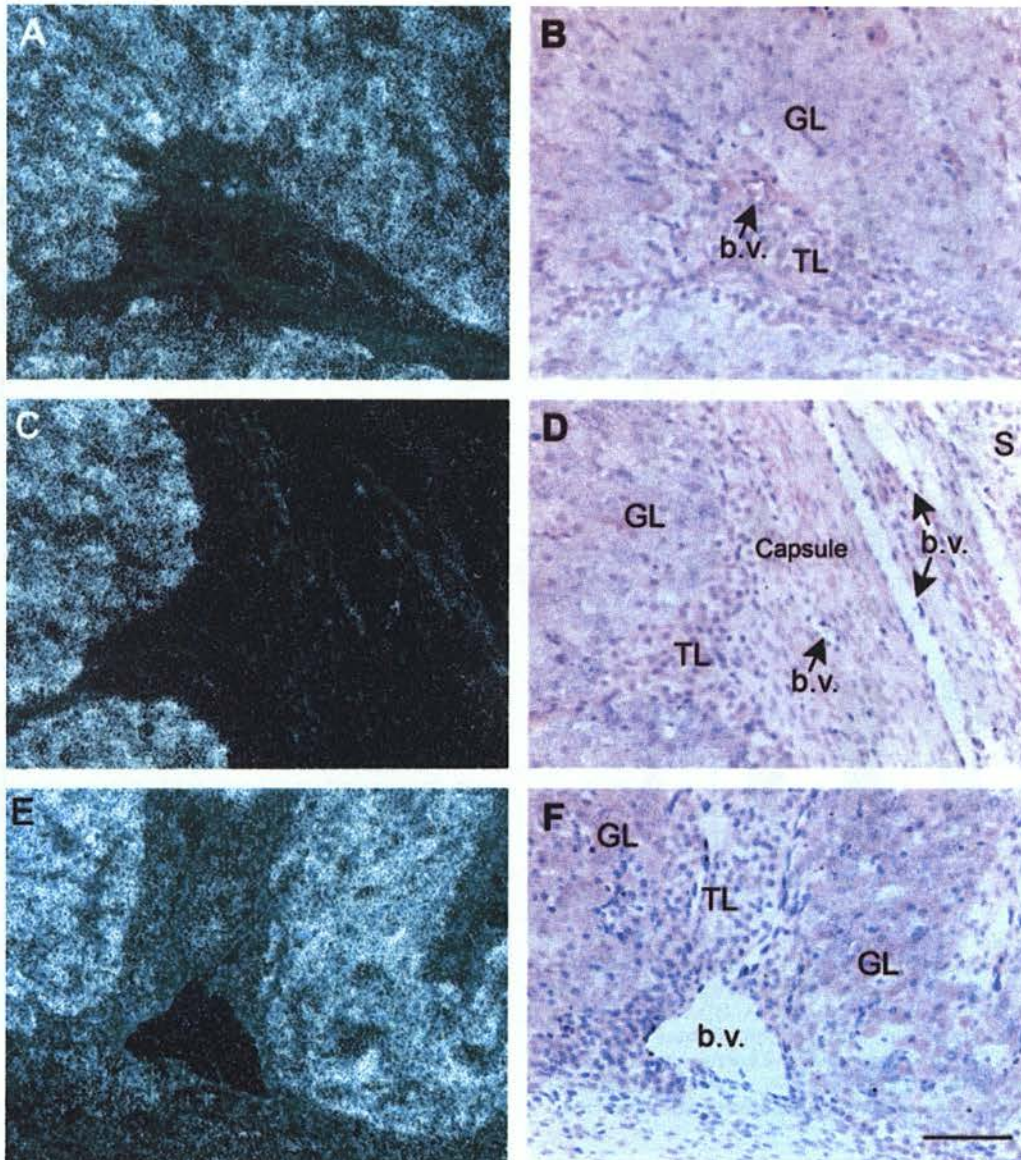


FIG. 3. Dark-field and corresponding light-field high-power photographs of *in situ* hybridization of EG-VEGF mRNA in human corpus luteum to show sites of expression in detail. Mid-luteal phase corpus luteum (A–D) showing expression is localized predominantly to the granulosa lutein cells (GL); virtually absent from theca lutein cells (TL); and absent from endothelial cells of blood vessels (b.v.), the luteal capsule, and stroma (S). In a late-stage corpus luteum (E and F), a similar pattern of expression is observed, although low expression in some theca lutein cells can also be observed. Scale bar, 50 μ m.

With respect to the physiological roles of VEGF and EG-VEGF in the corpus luteum, it has been shown that VEGF is essential for normal luteal angiogenesis and function in the nonhuman primate (4, 5). The high level of VEGF mRNA expression during the early luteal phase (2) is likely to be responsible for the intense angiogenesis seen at this time (17). The maintenance of VEGF expression throughout the functional life span of the corpus luteum may serve as a survival factor for endothelial cells and as an ovarian permeability factor (21). Immunoneutralization of VEGF during the mid-luteal phase in the marmoset resulted in suppression of plasma progesterone levels even though the luteal microvascular tree is largely complete by this stage (21), suggesting a continued requirement for VEGF for normal luteal function.

The role of EG-VEGF has been proposed as a regulator of vascular function exhibiting a selectivity for the endocrine glands (8, 22). The current findings support the view that EG-VEGF could have an important role in the functioning of the human corpus luteum. Establishing a physiological role for EG-VEGF must await *in vivo* studies in which pharmacological inhibitors can be employed to selectively prevent its action or block its receptors as has been carried out for VEGF (5, 23). From the current information, what is of greatest interest is that EG-VEGF mRNA is most highly expressed during the late luteal phase. By this period, the intense angiogenesis associated with the establishment of the luteal microvascular tree has subsided (3). This supports the established role for VEGF in the intense angiogenesis that takes

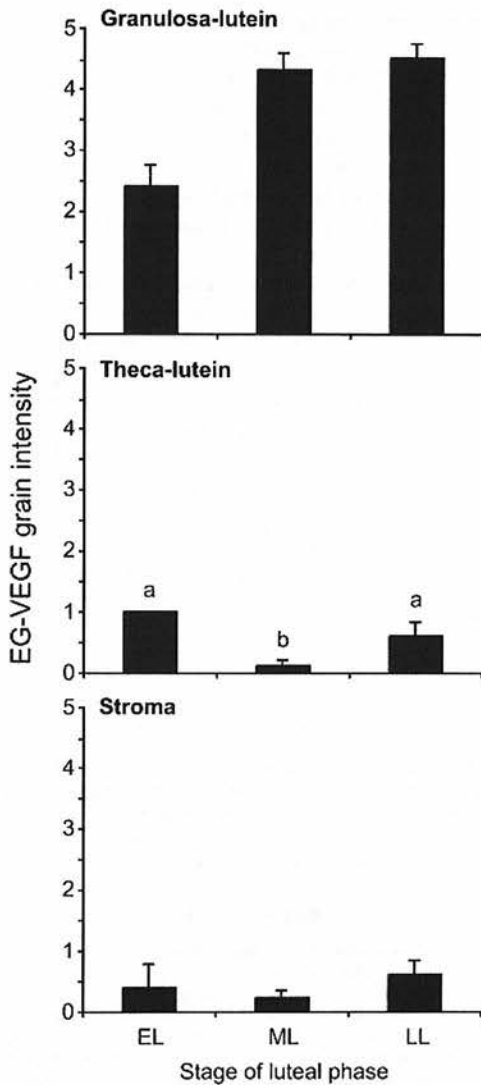


FIG. 4. Scoring of sections after *in situ* hybridization for EG-VEGF mRNA at different stages of the luteal phase in the various compartments of the tissue: granulosa-derived cells, theca-derived cells, and stroma. Silver grain intensity was significantly higher in the mid- and late luteal specimens than during the early luteal phase in the granulosa-derived cells, whereas theca-derived cells had the highest expression in the early and late luteal phases. EL, ML, and LL indicate early, mid-, and late luteal phases, respectively. Different letters represent significant differences.

place during the early luteal phase. It has been demonstrated that EG-VEGF enhances the development of fenestrae that allow the passage of molecules across the vascular endothelium. The most likely role for EG-VEGF at the mid- to late luteal phase is the regulation of vascular permeability to enhance transport of LDLs into the luteal cells and secretion of progesterone and other luteal products into the bloodstream (6). This may be of particular importance in allowing the corpus luteum to respond to hCG in early pregnancy.

With respect to the factors regulating the synthesis of EG-VEGF within the corpus luteum, the two most likely candidates are LH and progesterone. LH is an essential trophic factor for luteal function (24), whereas incubation of human luteinized granulosa cells by hCG stimulates VEGF

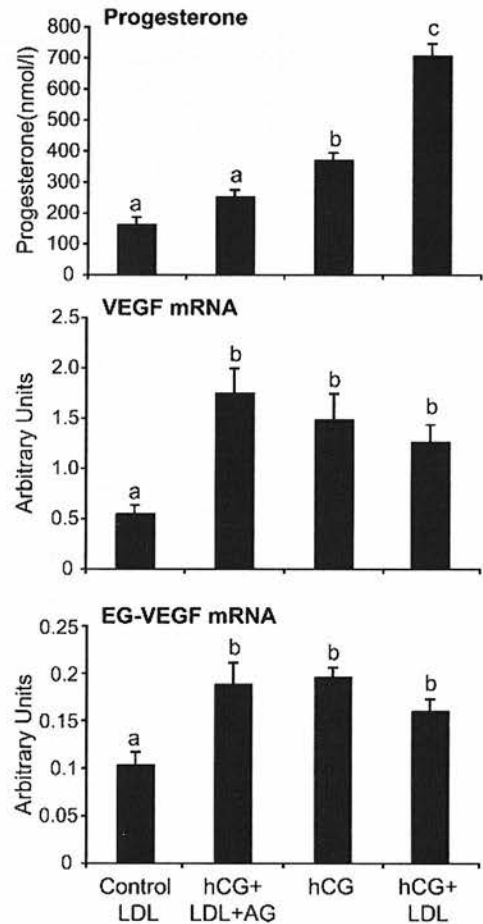


FIG. 5. Progesterone secretion into culture medium and VEGF and EG-VEGF expression in cultures of luteinized granulosa cells. Treatment groups were designed to expose the cells to hCG or increased progesterone: hCG + LDL gave high progesterone concentrations, hCG alone medium progesterone concentrations, and hCG + aminoglutethamide control levels of progesterone. The effect on VEGF and EG-VEGF expression was the same regardless of progesterone concentrations. Different lowercase letters represent significant differences.

expression (25, 26). Our observation that hCG stimulates the synthesis of both VEGF and EG-VEGF in human luteinized granulosa cells provides evidence for a role of LH in the regulation of this novel factor. Because the distinguishing feature between patterns of VEGF and EG-VEGF synthesis in the corpus luteum was the increased production of EG-VEGF in the mature tissue, it was tempting to believe that synthesis of EG-VEGF could be stimulated as a result of progesterone stimulation. We therefore manipulated progesterone concentrations in the presence of hCG. The addition of LDL significantly increased progesterone concentrations but failed to produce a further rise in either factor. In addition, the reduction of progesterone to basal levels by the addition of aminoglutethamide for 24 h also failed to alter gene expression. Although a role for basal progesterone secretion cannot be excluded, these data suggest that a direct stimulation by progesterone is not a major factor in the regulation of EG-VEGF mRNA in the human corpus luteum. Additional factors regulating ovarian EG-VEGF synthesis such as hyp-

oxia (6, 20), thrombin (20), or local growth factors such as insulin-like growth factors (27) require further investigation.

A clearer picture of the role of EG-VEGF in the ovary may become apparent when the expression patterns of its receptors, PK-R1 and PK-R2, are determined. In our laboratory, we have been unable to detect these receptors on the luteal sections by *in situ* hybridization, probably as a result of low levels of expression (H.W. and H.M.F., unpublished observations).

In conclusion, a profile of the expression of EG-VEGF within the corpus luteum has been provided by the complementary approach of *in situ* hybridization and RT-PCR. The results provide support for a role for EG-VEGF within the corpus luteum with respect to the regulation of the luteal vasculature, which is vital for normal function.

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