

Angiogenesis in the Reproductive
Tract During Early Pregnancy

Amanda Rowe

BSc (Hons)

Doctor of Philosophy
University of Edinburgh

June 2004



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.

Amanda Rowe

June 2004

Dedication

For Mum and Dad,

Thanks for your unconditional love and support.

Table of Contents

Declaration	ii
Dedication	iii
Contents	iv
Figures and Tables	ix
Abbreviations	xiv
Publications and presentations related to this thesis	xviii
Acknowledgements	xix
Abstract of thesis	xx
1 INTRODUCTION	2
1.1 Overview	2
1.2 Experimental models	4
1.3 The marmoset ovulatory cycle	5
1.4 Folliculogenesis	7
1.4.1 Pre-antral development	9
1.4.2 Antral development	10
1.4.3 Selection of the dominant follicle	10
1.4.4 Gonadotrophin control of pre-ovulatory follicular development	11
1.4.5 Putative Intraovarian regulators	13
1.4.6 Steroidogenesis- Mechanisms of follicular oestrogen synthesis	15
1.4.7 Termination of follicular oestrogen synthesis	17
1.4.8 Atresia	17
1.5 Ovulation	17
1.5.1 The LH Surge	17
1.5.2 Follicle rupture	19
1.6 The corpus luteum	20
1.6.1 Corpus luteum formation and luteinisation	20
1.6.2 Neovascularisation	20
1.6.3 Luteal steroidogenesis	21
1.6.4 Cell types in the corpus luteum	25
1.6.5 Trophic regulation of the corpus luteum	28
1.6.6 Autocrine/Paracrine regulation of human luteal function and development	30
1.6.7 Cellular interactions	32
1.6.8 Humoral cellular interactions	33

1.6.9	Gap junctional (contact-dependent) cellular interactions	34
1.7	Luteolysis	35
1.7.1	Functional luteolysis	35
1.7.2	Structural luteolysis.....	39
1.8	Luteal rescue.....	41
1.9	The endometrium.....	43
1.9.1	The role of steroids throughout the menstrual cycle.....	43
1.9.2	Menstruation	46
1.10	Implantation	49
1.10.1	Adaptations of the uterus for implantation.....	49
1.11	Morphology of implantation in primates.....	54
1.11.1	Steroid receptors during pregnancy	60
1.12	Vascular growth	61
1.12.1	Uterine and placental tissues.....	61
1.12.2	The ovary	62
1.13	Angiogenesis	64
1.13.1	Pathological angiogenesis	64
1.13.2	Physiological angiogenesis	65
1.13.3	The angiogenic process	66
1.13.4	Endothelial cell proliferation	66
1.13.5	Capillary stabilisation and pericytes	68
1.14	Angiogenic growth factors.....	70
1.14.1	Angiogenic promoters.....	70
1.14.2	Receptors involved in angiogenesis	75
1.14.3	Angiogenic inhibitors.....	78
1.15	Manipulation of angiogenesis.....	78
1.15.1	Development of antiangiogenic agents	78
1.16	Hypothesis and objectives.....	84
2	MATERIALS AND METHODS	86
2.1	Sources of reagents, enzymes and antibodies	86
2.2	Marmoset husbandry.....	86
2.2.1	PGF _{2α} administration and induction of luteolysis.....	86
2.2.2	Collection of tissue.....	87
2.2.3	Details of animals used in each experiment.....	89
2.2.4	Progesterone ELISA plate assay	89
2.2.5	Assay for serum chorionic gonadotropin	92

2.2.6	Classification of the stage of the ovulatory cycle	92
2.3	Tissue fixation, processing and sectioning of paraffin blocks.....	92
2.4	Haematoxylin and eosin staining.....	94
2.5	Immunocytochemistry	94
2.5.1	Antigen retrieval.....	95
2.5.2	Blocking Endogenous enzymes	95
2.5.3	Blocking non-specific binding	96
2.5.4	Immunocytochemical protocol.....	97
2.5.5	Detection	97
2.5.6	Dual staining	99
2.5.7	Negative controls	103
2.6	Image analysis	103
2.6.1	Digital photomicroscopy.....	103
2.6.2	Quantification of immunocytochemical staining.....	103
2.7	In situ hybridisation	104
2.7.1	Plasmid Preparation	104
2.7.2	Polymerase Chain Reaction (PCR) of plasmids for riboprobes.....	105
2.7.3	Synthesis of riboprobe (cold labelling).....	106
2.7.4	35S labelling	107
2.7.5	<i>In situ</i> hybridisation procedure.....	108
2.7.6	Quantification of <i>in situ</i> hybridisation	110
2.8	Statistical analysis of data	110
3	ANGIOGENESIS IN THE CORPUS LUTEUM OF EARLY PREGNANCY	113
3.1	Introduction.....	113
3.1.1	Specific Methods.....	114
3.2	Results	115
3.2.1	Hormone profile.....	115
3.2.2	Lutein cell area.....	117
3.2.3	Cell Proliferation.....	119
3.2.4	Endothelial cell area.....	121
3.2.5	Pericyte cell area	123
3.2.6	VEGF	125
3.2.7	KDR	127
3.2.8	Flt	129
3.2.9	Ang-1, Ang-2 and Tie-2.....	131
3.3	Discussion.....	131

4	THE EFFECT OF PROGESTERONE ON VEGF EXPRESSION IN HUMAN LUTEAL STEROIDOGENIC CELLS <i>IN VITRO</i>.....	137
4.1	Introduction.....	137
4.2	Specific methods.....	139
4.2.1	Details of animals and samples used.....	139
4.2.2	Source of marmoset and human tissues	140
4.2.3	Progesterone receptor immunocytochemistry on fixed tissues.....	140
4.2.4	Collection and separation of human granulosa cells.....	141
4.2.5	Cell culture.....	141
4.2.6	Cell fixation and processing for immunocytochemistry	142
4.2.7	Progesterone receptor and 3 β HSD immunocytochemistry dual staining on fixed cells.	143
4.2.8	Cell culture treatments	143
4.2.9	Treatment regimes.....	144
4.2.10	Time course experiment.....	145
4.2.11	Progesterone assay on culture medium	145
4.2.12	Total RNA extraction and quantification of specific mRNA.....	146
4.3	Results	154
4.3.1	Progesterone receptor immunohistochemistry on marmoset and human tissues	154
4.3.2	Progesterone receptor immunocytochemistry of human luteinised granulosa cells.....	155
4.3.3	The effect of hCG on luteal steroidogenic cells <i>in vitro</i>	156
4.3.4	The effect of progesterone on luteal steroidogenic cell VEGF expression	158
4.3.5	The effect of duration of hCG treatment on luteal steroidogenic cells <i>in vitro</i>	160
4.4	Discussion.....	162
5	CELLULAR AND MORPHOLOGICAL CHANGES ASSOCIATED WITH EARLY PREGNANCY IN THE MARMOSET.....	169
5.1	Introduction.....	169
5.2	Specific methods.....	170
5.2.1	Details of animals used for the experiment.....	170
5.2.2	Dual immunostaining of CD31 and BrdU by Confocal microscopy ...	171
5.2.3	Quantification of dual immunostaining	172
5.3	Results	174
5.3.1	Gross morphology and patterns of cell proliferation in marmoset endometrium throughout the cycle.....	174
5.3.2	Description of the foetal-maternal interface	181
5.3.3	Gross morphology and patterns of cell proliferation at the time of implantation and in early pregnancy in marmoset endometrium.....	184

5.3.4	Uterine weight.....	190
5.3.5	Volume fraction analysis.....	192
5.4	Discussion.....	200
6	MOLECULAR REGULATION OF ANGIOGENESIS IN IMPLANTATION AND EARLY PREGNANCY	208
6.1	Introduction.....	208
6.2	Methods.....	209
6.2.1	Details of animals used in the experiment	209
6.2.2	<i>In situ</i> hybridisation	209
6.2.3	Quantification of <i>In situ</i> hybridisation	210
6.3	Results	211
6.3.1	Vascular Endothelial Growth Factor.....	211
6.3.2	KDR and Flt (VEGFR-1 and VEGFR-2).....	213
6.3.3	Angiopoietin-1	216
6.3.4	Angiopoietin-2	218
6.3.5	Tie-2	219
6.4	Discussion.....	224
7	GENERAL DISCUSSION.....	231
7.1	The findings of the thesis	231
7.2	The marmoset as a model for early pregnancy	241
7.3	Clinical implications of the thesis findings	241
7.4	Future speculation.....	242
7.5	Conclusions	244
	References	245
	Appendices	287

Figures and Tables

	Page
Chapter 1	
Figure 1.1 Overview of folliculogenesis	8
Figure 1.2 Gonadotrophin levels throughout the ovulatory cycle	12
Figure 1.3 Two-cell, two-gonadotrophin theory of steroidogenesis	16
Figure 1.4. Pathway for progesterone biosynthesis in a generic luteal cell	22
Figure 1.5. Development of the corpus luteum from a follicle	27
Figure 1.6 Cellular interactions in the corpus luteum	32
Figure 1.7 Changes in human endometrium during the menstrual cycle	44
Figure 1.8 Menstrual Induction hypothesis	49
Table 1.1 Relative time sequence of some developmental sites in the peri-implantation period	55
Figure 1.9 Expansion of the marmoset early implantation site	60
Figure 1.10 Expression of Ang-1 and Ang-2 mRNA in adult human tissues	73
Figure 1.11 Lessons from gene knockout mice	76
Figure 1.12 Regulation of vascular morphogenesis, maintenance and remodelling by Receptor Tyrosine Kinase's and their ligands	77
Chapter 2	
Figure 2.1 Schematic of study groups	88
Table 2.1 Details of animals used for the thesis	89
Figure 2.2 Schematic of ELISA for progesterone	91
Figure 2.3 APAAP detection of immunocytochemistry	100
Figure 2.4 ABC-HRP detection of immunocytochemistry	101
Table 2.2 Antibodies, reagents and summary protocol used for immunocytochemistry	102
Table 2.3 Specific plasmids used for generation of riboprobes for <i>in situ</i> hybridisation	104

Chapter 3

Table 3.1	Summary of animals used in this experiment	114
Figure 3.1	Mean plasma chorionic gonadotrophin (ng/ml) levels from animals at the different stages studied	115
Figure 3.2	Plasma progesterone concentrations from ovulation to day 21 in pregnant and non-pregnant animals.	116
Figure 3.3	Haematoxylin and eosin stained sections of marmoset corpora lutea	118
Figure 3.4	Cross-sectional area of lutein cells in corpora lutea of animals from the different study groups.	119
Figure 3.5	Cell Proliferation in the marmoset corpus luteum	120
Figure 3.6	Mean proliferation index in corpora lutea of animals from the different study groups	121
Figure 3.7	Immunocytochemical localisation of endothelial cell marker CD31	122
Figure 3.8	Mean endothelial cell area in corpora lutea of animals from the different study groups	123
Figure 3.9	Immunocytochemical localisation of α -smooth muscle actin	124
Figure 3.10	Mean pericyte area in corpora lutea of animals from the different study groups	125
Figure 3.11	Photomicrographs illustrating localisation of VEGF mRNA to steroidogenic cells (SC) by <i>in situ</i> hybridisation	126
Figure 3.12	Mean grain density/ μm^2 and mean grain area (μm^2) of VEGF hybridisation in nonpregnant and pregnant CL	127
Figure 3.13	Photomicrographs illustrating localisation of KDR mRNA to endothelial cells (EC) by <i>in situ</i> hybridisation	128
Figure 3.14	Mean grain density/ μm^2 and mean grain area (μm^2) of KDR hybridisation in nonpregnant and pregnant CL	129
Figure 3.15	Photomicrographs illustrating localisation of KDR mRNA to endothelial cells (EC) by <i>in situ</i> hybridisation	130
Figure 3.16	Mean grain density/ μm^2 and mean grain area (μm^2) of Flt hybridisation in nonpregnant and pregnant CL	131

Chapter 4

Table 4.1	Details of animals and samples used in this experiment	139
Figure 4.1	Monitoring PCR with SYBR Green 1 dye	149
Figure 4.2.	Real time amplification of fluorescence signal	150
Figure 4.3	Melt curve analysis	152
Figure 4.4	Standard curve	153
Figure 4.5	Progesterone receptor localisation by immunocytochemistry in human and marmoset reproductive tract	155
Figure 4.6	Dual immunostaining of progesterone receptor and 3 β HSD in human granulosa cells	156
Figure 4.7	Dose response graph to illustrate the effect of varying hCG concentrations (ng/ml) on progesterone secretion (nmol/l) from luteinised human granulosa cells <i>in vitro</i>	157
Figure 4.8	Dose response graph to illustrate the effect of varying hCG concentrations (ng/ml) on relative VEGF mRNA expression in luteinised human granulosa cells <i>in vitro</i>	158
Figure 4.9	Graph to illustrate the effect of different treatments on Progesterone secretion (nmol/l) from human luteinised granulosa cells <i>in vitro</i>	159
Figure 4.10	Graph to illustrate the effect of different treatments on relative VEGF expression in human luteinised granulosa cells <i>in vitro</i>	160
Figure 4.11	Time course data illustrating the effect of varying the duration of hCG treatment on progesterone secretion (nmol/l) from luteinised human granulosa cells <i>in vitro</i>	161
Figure 4.12	Time course data illustrating the effect of varying the duration of hCG treatment on relative VEGF mRNA expression in luteinised human granulosa cells <i>in vitro</i>	162

Chapter 5

Table 5.1	Details of animals used for this experiment	170
Figure 5.1	Example of the grid system used to obtain volume fraction data	173
Figure 5.2	Schematic illustrating grid placement	174
Figure 5.3	Uterine morphology in the Mid proliferative phase	176

Figure 5.4	Uterine morphology in the Late proliferative phase	177
Figure 5.5	Uterine morphology in the Early secretory phase	178
Figure 5.6	Uterine morphology in the Mid secretory phase	179
Figure 5.7	Uterine morphology in the late secretory phase	180
Figure 5.8	Cytokeratin immunocytochemistry for differentiation of trophoblast from maternal tissue in early pregnancy	182
Figure 5.9	Diagrammatic representation of the marmoset implantation site	183
Figure 5.10	Uterine morphology at 2 weeks of pregnancy	186
Figure 5.11	Uterine morphology at 3 weeks of pregnancy	187
Figure 5.12	Uterine morphology at 4 weeks of pregnancy	188
Figure 5.13	Confocal microscopy illustrating dual staining for CD31 and BrdU	189
Figure 5.14	Graphs to illustrate mean uterine weight before fixation from marmosets in the different study groups	191
Figure 5.15	Volume fraction of cellular compartments of the marmoset Uterus	193
Figure 5.16	Volume fraction of cellular compartments of the marmoset uterus (compartmentalised)	194
Figure 5.17	Mean volume fraction of proliferating luminal epithelium as a percentage of total luminal epithelium	196
Figure 5.18	Mean volume fraction of proliferating glandular epithelium as a percentage of total glandular epithelium	197
Figure 5.19	Mean volume fraction of proliferating glandular epithelium as a percentage of total glandular epithelium	198
Figure 5.20	Mean volume fraction of proliferating endothelial cells as a percentage of total endothelial cells	199
Figure 5.21	Mean volume fraction of proliferating endothelial cells as a percentage of total endothelial cells	200
Chapter 6		
Table 6.1	Details of animals used for this experiment	209
Figure 6.1	<i>In situ</i> hybridisation of VEGF mRNA in the marmoset Endometrium	212

Figure 6.2	<i>In situ</i> hybridisation of VEGF receptor	215
Figure 6.3	<i>In situ</i> hybridisation of Ang-1 mRNA in marmoset Endometrium	217
Figure 6.4	<i>In situ</i> hybridisation of Ang-2 mRNA in pregnant endometrium at day 28	218
Figure 6.5	<i>In situ</i> hybridisation of Tie-2 mRNA in marmoset endometrium	220
Table 6.2	Quantification of <i>in situ</i> hybridisation using relative grain density within different uterine compartments throughout the stages studied for VEGF, VEGF R1 and R2, Ang-1, Ang-2 and Tie-2	222
Figure 6.6	Diagrammatic representations of angiogenic factor expression in the marmoset endometrium	223
Figure 6.7	Flow diagram to illustrate putative roles of the angiogenic factors during implantation and early pregnancy	228

Abbreviations

α -SMA	α -smooth muscle actin
3 β HSD	3 β -hydroxysteroid dehydrogenase
ABC	avidin biotin complex
aFGF	acidic fibroblast growth factor
Ang-1	angiopoietin-1
Ang-2	angiopoietin-2
ANOVA	analysis of variance
ANSA	8-anilino-1-naphthalenesulfonic-acid
APAAP	alkaline phosphatase-anti-alkaline-phosphatase
bFGF	basic fibroblast growth factor
bp	base pair
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CAM	chorio-allantoic membrane
cAMP	cyclic adenosine monophosphate
CD31	cluster differentiation factor 31
cDNA	complementary deoxyribonucleic acid
CG	chorionic gonadotrophin
CL	corpus luteum
cpm	counts per minute
DAB	diaminobenzadine
DARS	donkey anti-rabbit serum
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
DTT	dithiothreitol
ECM	extracellular matrix
EGF	epidermal growth factor
ER	estrogen receptor
Flt-1	<i>fms</i> -like tyrosine kinase
FSH	follicle stimulating hormone
GMG	granulated metrial gland
GnRH	gonadotrophin releasing hormone
G6PDH	Glucose-6-phosphate dehydrogenase
H & E	haematoxylin and eosin
hCG	human chorionic gonadotrophin
HDL	high density lipoprotein
HRP	horse radish peroxidase
i.m.	intramuscular
i.v.	intravenous
IFN	interferon
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IgG	immunoglobulin G
IL	interleukin
kDa	kilodalton
KDR	kinase insert domain containing region
LDL	low density lipoprotein
LH	luteinising hormone
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NBF	neutral buffered formalin

NBT	nitro blue tetrazolium
NGS	normal goat serum
NK	natural killer
NRS	normal rabbit serum
P450 _{arom}	cytochrome P450, aromatase
P450 _{c17}	cytochrome P450, 17 α hydroxylase/C17-20 lyase
P450 _{scc}	cytochrome P450, side chain cleavage
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PFA	paraformaldehyde
PG	prostaglandin
PGE ₂	prostaglandin E ₂
PGF _{2α}	prostaglandin F _{2α}
PGI ₂	prostacyclin
PI	proliferation index
PIGF	placental growth factor
PR	progesterone receptor
QC	quality control
rATP	riboadenosine triphosphate
rCTP	ribocytosine triphosphate
rGTP	riboguanine triphosphate
RTK	receptor tyrosine kinase
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
rNTP	ribonucleotide triphosphate
RT	reverse transcription

SEM	standard error of the mean
SSC	salt sodium chloride
StAR	steroidogenic acute regulatory protein
TBS	tris buffered saline
TEA	triethanolamine
TGF β	transforming growth factor β
Tie	tyrosine kinase with immunoglobulin and epidermal growth factor homology domains
TIMP	tissue inhibitor of metalloproteinases
TNF	tumour necrosis factor
UTP	uridine triphosphate
VEGF	vascular endothelial growth factor
VEGFR-1	vascular endothelial growth factor receptor-1
VEGFR-2	vascular endothelial growth factor receptor-2

Publications and Presentations Relating to this Thesis

Publications in peer reviewed journals

Rowe AJ, Morris KD, Bicknell R and Fraser HM (2002) Angiogenesis in the corpus luteum of early pregnancy in the marmoset and the effects of vascular endothelial growth factor immunoneutralization on establishment of pregnancy *Biology of Reproduction* **67**:1180-8

Rowe AJ, Wulff C and Fraser HM (2003) Localisation of mRNA for vascular endothelial growth factor (VEGF), angiopoietins and their receptors during the peri-implantation period and early pregnancy in marmosets (*Callithrix jacchus*) *Reproduction* **126**:227-238

Oral presentations

Rowe AJ and Fraser HM (2002) Angiogenesis in the corpus luteum of early pregnancy in the marmoset and the effects of vascular endothelial growth factor immunoneutralization. *35th Annual Meeting for the Society for the Study of Reproduction*, Baltimore Marriott Waterfront Hotel, Baltimore, Maryland, USA

Poster presentations

Rowe AJ and Fraser HM (2003) Localisation of vascular endothelial growth factor and angiopoietins during the peri-implantation period and early pregnancy in the marmoset. *3rd Joint Meeting of the British Andrology Society, the British Fertility Society and the Society for Reproduction and Fertility*, The University of Aberdeen Scotland, UK

Acknowledgements

First of all I would like to acknowledge Dr Hamish Fraser. He warrants a special thank you for all his help and encouragement over the last three years. Without his expertise and continual input of ideas this thesis would not have been possible. Other members of our group have also been invaluable. Helen Wilson, who taught me all the laboratory techniques used in this thesis and to Julie Bell for her expert help with the light cycler. Not to mention Paul Taylor, the other PhD student in our group and of course Dr Christine Wulff for her unending ability to look on the bright side and for her intellectual contributions to this work. These people have made working at the MRC Human Reproductive Sciences Unit a pleasure and I thank them not only on a professional level but also for their friendship throughout.

Keith Morris and all the staff at the Primate Centre have also made this work possible, for the general care of the marmoset colony and collection of tissue, without them the project could not have taken place. I also thank Ian Swanston and Fiona Pitt in the assay lab for the accurate dating of tissue and monitoring of marmoset ovulatory cycles. A special thank you also goes to Dr Colin Duncan for his intellectual involvement throughout, also to Eva Gay and Dr Ismail for all their help with the cell culture work.

The histology department also warrant a special acknowledgement for undertaking all tissue processing and for their assistance with immunocytochemistry and image analysis. I also thank Ted Pinner for the preparation of posters and slides; always of the highest quality and prepared in the shortest time.

More personally, I would like to thank everyone at the unit for making the last three years an enjoyable and wonderful experience, particularly all the other PhD students- you know who you are! Thanks for everything.

Finally, a very special mention goes to my parents, without whose continual love, support and encouragement, even when things looked black, I could not have done it. Thank you both so much.

Abstract of thesis

Angiogenesis (formation of new blood vessels) is essential for tissue growth and development. The adult vasculature is generally quiescent, except in the ovaries and uterus, which undergo cyclical angiogenesis to ensure adequate provision of nutrients and hormone precursors essential for establishment and maintenance of pregnancy. These tissues provide excellent models in which to study angiogenesis. Clinically, inadequate luteal vascularisation may cause reduced luteal function and insufficient progesterone production, resulting in early embryo loss or failure of the blastocyst to implant directly resulting from an inadequately prepared endometrium. This thesis investigated angiogenesis in the primate corpus luteum and endometrium of early pregnancy.

The corpus luteum is a transient endocrine gland responsible for progesterone production to induce changes in uterine endometrium necessary for implantation of the developing blastocyst and establishment of pregnancy. In the absence of conception, progesterone declines resulting in endometrial destabilisation. Should conception occur, the corpus luteum is rescued by chorionic gonadotrophin synthesised by the developing trophoblast, and retains its functional capacity to synthesise and secrete progesterone until such time as this role is transferred to the placenta.

The first experiment investigated whether there exists a further burst of angiogenesis in the corpus luteum of early pregnancy at the time of luteal rescue which may be involved in prolonging its lifespan. Ovaries were collected from non-pregnant animals in the late luteal phase at day 21 after ovulation and from pregnant animals at day 21 and day 28 post ovulation. *In vivo* luteal angiogenesis was assessed by quantitative immunocytochemistry of 1) Bromodeoxyuridine (BrdU) incorporation, 2) Assessment of the microvascular network by CD31 endothelial cell marker and 3) Pericyte recruitment by smooth muscle α -actin staining. Molecular regulation of angiogenesis was assessed by *in situ* hybridisation of mRNA for Vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEGFR-2, followed by image analysis of grain density and grain area. No increase in any

cellular or molecular angiogenic parameter was observed in the marmoset corpus luteum during luteal rescue. It was concluded that the vasculature already established by the late luteal phase is all that is required to maintain early pregnancy.

At the uterine level, early pregnancy is associated with changes that allow implantation and give rise to an environment capable of supporting early embryonic development.

The metabolic demands of a newly implanted blastocyst are met by diffusion from the endometrial vasculature, as such, a stable and well developed maternal vascular network is an essential prerequisite for successful implantation and early pregnancy. The second experiment aimed to elucidate the angiogenic events occurring during the peri-implantation period and in early pregnancy. Uteri were studied at day 14, 21 and 28 of pregnancy, together with non-pregnant uteri from day 14 and day 21 post-ovulation. Cellular parameters were studied by volume fraction analyses of uterine sections dual stained with BrdU and CD31. Increased angiogenesis was observed in the endometrium at this time. VEGF, VEGFR-1, VEGFR-2, Angiopoietin-1, Angiopoietin-2 and Tie-2 mRNA's were localised by *in situ* hybridisation to describe the spatial and temporal expression of these factors and to indicate putative mechanisms by which the observed changes in the vasculature occur. Messenger RNA for VEGF and its receptors increased from the time of implantation throughout pregnancy, as did Angiopoietin-1. Particularly high levels of expression were observed at a focal point immediately adjacent to the implantation site. This work provides evidence that angiogenesis, regulated by VEGF and the angiopoietins, may be involved in the preparation of endometrium for implantation, remodelling of the maternal vasculature during the peri-implantation period and trophoblast invasion.

This thesis has improved our understanding of the angiogenic events occurring in the corpus luteum and endometrium of early pregnancy and its molecular regulation.

Chapter 1

Introduction

1 Introduction

1.1 Overview

Angiogenesis refers to formation of new blood vessels, or neovascularisation and is an essential component of tissue growth and development. Apart from wound healing, the adult vasculature is generally quiescent, with vascular endothelium representing an extremely stable population of cells with low mitotic rate. Intense or persistent capillary growth is associated with several pathological conditions including tumour growth, retinopathies, hemangiomas, fibroses and rheumatoid arthritis. Notable exceptions of physiological angiogenesis occur in the female reproductive tract, ovaries, uterus and placenta. These tissues undergo cyclical angiogenesis to ensure adequate provision of nutrients and hormone precursors essential for establishment and maintenance of pregnancy. As such the female reproductive tissues provide excellent models in which to study angiogenesis. It is the regulation of angiogenesis in the primate corpus luteum and endometrium of early pregnancy which form the subject of this thesis.

The corpus luteum is a transient endocrine gland principally responsible for progesterone production. One function of progesterone is to induce changes in uterine endometrium necessary for implantation of the developing blastocyst and establishment of pregnancy. The corpus luteum is formed from the dominant follicle after ovulation and persists throughout the luteal phase until such time as it undergoes functional and structural regression. Luteal regression results in declining progesterone production causing endometrial destabilisation and menstruation in the human.

The human luteal phase or functional lifespan of the corpus luteum is 14 days and 21 days in the marmoset. Should conception occur, the corpus luteum is rescued by chorionic gonadotrophin (humans and non-human primates) synthesised by the developing trophoblast, and retains its functional capacity to synthesise and secrete progesterone until such time as this role is transferred to the placenta.

During the follicular phase the follicular granulosa layer remains avascular, separated from the vascularised theca by a basement membrane. After ovulation, the

granulosa layer becomes vascularised transforming the newly formed corpus luteum into a highly active endocrine gland. Extensive angiogenesis occurs in the early luteal phase gradually decreasing throughout the lifespan of the corpus luteum. In a non-fertile cycle the corpus luteum undergoes functional and structural regression. Functional luteolysis is associated with a decline in progesterone production after which structural luteolysis occurs, associated with vessel atrophy, and formation of a relatively avascular structure, visible within the ovary in subsequent cycles.

In a fertile cycle, the maintenance or rescue of the corpus luteum ensures continued presence of progesterone, maintaining the intrauterine environment and preventing menstruation.

Progesterone acts on the endometrium as a differentiation factor. Oestrogens induce endometrial proliferation during the follicular phase, a process inhibited by progesterone in the luteal phase. Progesterone induces stromal differentiation, stimulates glandular secretions and changes the pattern of proteins secreted by endometrial cells. All these events prepare the uterus for implantation and give rise to an environment capable of supporting early embryonic development. Progesterone also inhibits myometrial contractility.

Many factors thought to be involved in the regulation of angiogenesis, with the exception of VEGF and bFGF, have been determined largely from gene knockout studies investigating embryonic vascularisation in transgenic mice, *in vitro* studies and from analysis of tumour angiogenesis. Luteal angiogenesis has been widely studied throughout the luteal phase in human and primates but the role of angiogenesis in luteal rescue *in vivo* in the primate has yet to be elucidated. The corpus luteum, being one of the exceptional adult tissues to undergo cyclic angiogenesis forms a unique model for studying the dynamic processes involved in such a rapid onset and tightly controlled process.

The clinical implications of angiogenesis research are paramount. Inadequate luteal vascularisation may be a cause of reduced luteal function and insufficient progesterone production. This could result in early embryo loss or indeed failure of the blastocyst to implant directly resulting from an inadequately prepared endometrium. There are many pathological conditions associated with unregulated

angiogenesis such as retinopathies, rheumatoid arthritis, tumour growth and psoriasis, therefore clinical research into this field remains important. Also, manipulation of luteal angiogenesis in both pregnant and non-pregnant cycles could lead to novel strategies in post-ovulatory or post-coital fertility control.

This chapter reviews the current understanding of 1) The formation and control of the corpus luteum in fertile and non-fertile cycles with regard to angiogenesis and 2) Cyclic changes in the endometrium and those associated with early pregnancy. Firstly, the ovarian cycle will be discussed: Folliculogenesis culminating in ovulation, subsequent luteal development, luteolysis, the maternal recognition of pregnancy and luteal rescue with emphasis on functional relevance of ovarian vascularisation. Molecular control of angiogenesis will then be discussed with reference to current understanding of its role in early pregnancy. Cyclic changes in endometrium, menstruation, implantation and placentation will also be addressed again with emphasis on the angiogenic process. Finally the clinical relevance of the study of *in vivo* angiogenesis in early pregnancy in the corpus luteum and endometrium will be evaluated with details of current understanding of manipulation of angiogenesis.

This chapter serves to introduce the field of clinical angiogenesis research and to evaluate current knowledge in order that rationale for the succeeding experimental chapters be appreciated.

In each section I have tried to give an overall review referring to human, non-human primates, rodents and domestic species. However, it should not be assumed that mechanisms occurring in one species will be the same in another and so unless experiments have been conducted to confirm species similarities or differences, I have not extrapolated from one to another.

1.2 Experimental models

With the exception of data presented in Chapter 4, all results presented in this thesis were obtained using the marmoset monkey (*Callithrix jacchus*) as an experimental model for this *in vivo* investigation into angiogenesis and tissue remodelling at the time of implantation and in subsequent early pregnancy in corpus luteum and uterus. Using a primate model allows the identification and manipulation

of angiogenic factors that may be important in the human therefore forming a bridge between basic research in the laboratory and clinical application. Mechanisms by which luteal function is regulated are markedly different in rodents compared to primates (Fraser and Lunn, 2001). In the non-fertile cycle in the rodent, the corpus luteum is active for less than a day making it difficult to discern discrete phases of vessel maturation and vessel regression that follow the angiogenic phase of the corpus luteum. In contrast, the marmoset corpus luteum remains active for 2-3 weeks allowing the stages of the luteal phase to be accurately dissected out and studied (Augustin, 2000). Another advantage is that the marmoset ovulatory cycle can be reliably synchronised by $\text{PGF}_{2\alpha}$ administration, any time after luteal day 8, causing luteal regression and subsequent ovulation 10 days later (Summers, et al., 1985). Marmosets also have a high fertility rate, short gestation (Fraser et al., 1994) and unlike the human and bovine are not a monovulatory species, ovulating two to three ova per cycle. Rodents, however, are multiple ovulators and are therefore arguably more dissimilar to the human than the marmoset (Augustin, 2000).

1.3 The marmoset ovulatory cycle

The marmoset ovulatory cycle lasts approximately 28 days and there are usually two to three ova shed at ovulation. The follicular phase lasts about 8 days followed by a luteal phase of about 20 days (Harding, 1982). At the end of the luteal phase, structural and functional regression of the corpus luteum occurs in non-pregnant cycles while in pregnancy, the corpus luteum is rescued by the presence of chorionic gonadotrophin.

Implantation in the marmoset occurs on day 11 after ovulation and the blastocyst remains in loose attachment to the surface epithelium without epithelial fusion. Penetration of trophoblast into maternal vessels occurs at around day 45 to 60 of gestation (Enders, 1993).

For the purposes of this thesis, pregnant marmosets were studied at day 14 (around the peri-implantation period), day 21 and day 28 of the luteal phase, together with non-pregnant animals from day 14 and day 21.

As a model for the human, the marmoset presents some differences. It is not a mono-ovulatory species, it has a luteal phase length of approximately 21 days, as

opposed to 14 days in the human, and the structure of its corpus luteum differs to that of the human. It displays subtly different implantation and placentation and does not menstruate. However, it has allowed for the first time, the study of physiological pregnancy as opposed to pharmacological pregnancy, by hCG treatment, in a primate model. Such invasive investigations of endometrial angiogenesis and structure of the early implantation site could not be carried out in higher primates. Its high fecundity, greater than 70% fertility rate, offers a considerable advantage over other commonly studied non-human primates, where fertility rate is in the region of 30%. The marmoset has an ovulatory cycle more analogous to the human than any rodent species, and so is an appropriate animal model.

In order to investigate angiogenesis in the marmoset and to compare this to the human, it is necessary to understand what is happening at this time in both species. Progesterone is a key player in early pregnancy. Maintenance of pregnancy in mammals requires progesterone production by the ovarian corpus luteum and in most species, the functional lifespan of the CL is extended to maintain this production (Zelevnik and Benyo, 1994). In humans, the CL remains the primary source of progesterone for 4-5 weeks after implantation until placental production becomes sufficient to maintain pregnancy. The mechanisms of luteal rescue vary among species. In rodents, coitus initiates rescue whereas in human and primates, rescue is initiated by the conceptus through its production of chorionic gonadotrophin.

The general pattern of progesterone production during the first trimester of pregnancy has been described in the human. Luteal rescue by hCG is associated with increased luteal progesterone secretion both in normal (Lenton and Woodward, 1988) and simulated early pregnancy (Mendizabal et al., 1984, Tay and Lenton, 2000). This observation forms the first discrepancy between these two species, for in the marmoset, progesterone levels are maintained in early pregnancy at levels already attained in the luteal phase but are not further augmented during luteal rescue (See chapter 3).

1.4 Folliculogenesis

The corpus luteum is pivotal to the control of the menstrual cycle and is formed from ovulation of the pre-ovulatory cycle; Once formed the corpus luteum secretes progesterone throughout the luteal phase and its regression signifies the beginning of the follicular phase of the subsequent cycle. Maturation changes occurring during the follicular phase are essential for adequate luteal function therefore it is important to firstly discuss events prior to ovulation in order to fully appreciate the role of this endocrine gland. The involvement of pituitary gonadotrophin secretion on folliculogenesis will also be considered as gonadotrophins play a fundamental and essential role in reproductive function at all levels.

During the follicular phase of the human menstrual cycle, the follicle destined to ovulate increases in size from a diameter of 2-5mm to over 20mm and becomes the major ovarian source of secreted oestrogen. This oestrogen synthesising capacity accompanies a programmed sequence of cell growth and differentiation in the follicle which terminates in ovulation and formation of the corpus luteum (Hillier, 1991). The sequence of events which transpire during folliculogenesis will be discussed in more detail in the subsequent section but **Figure 1.1** highlights the main stages.

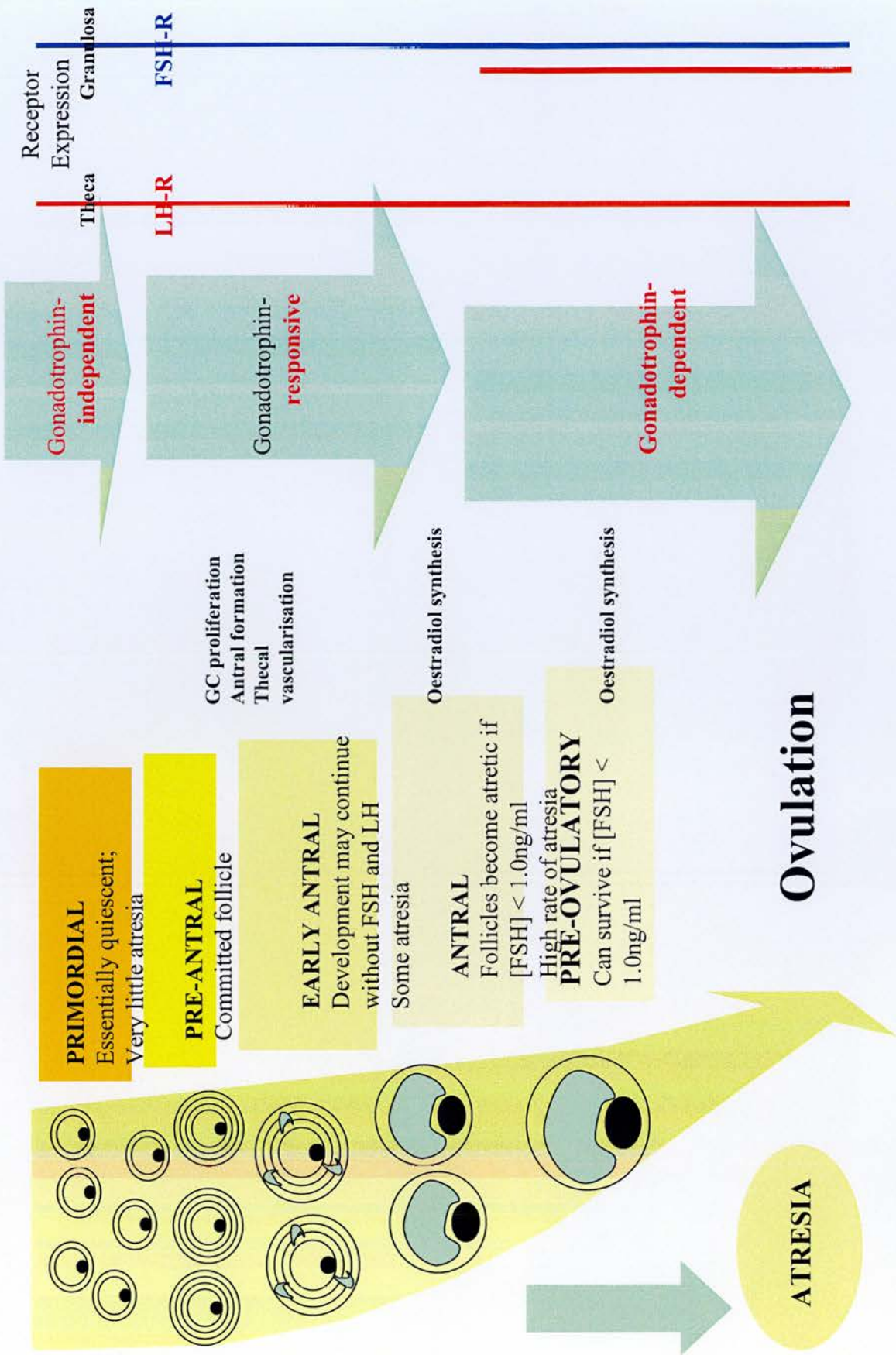


Figure 1.1
Overview of folliculogenesis

1.4.1 Pre-antral development

Individual follicles within the primordial follicle reserve are stimulated to commence development in a process known as folliculogenesis. The primordial follicle consists of an oocyte surrounded by a single layer of flattened epithelial cells and is essentially devoid of a capillary network. At this stage there is very little evidence of atresia and cell turnover rates are low (Hirshfield, 1991). Primordial follicles are recruited to undergo follicular maturation in an ordered sequence thought to commence with those from the innermost regions of the ovarian cortex (Smith et al., 1993). On leaving the primordial pool, follicles are irreversibly committed to growth and can no longer return to a quiescent state. Several theories have been suggested to explain initiation of follicle recruitment, but the stimulus for follicle growth still remains unclear (Baird, 1991). However, evidence from studies of hypophysectomised sheep indicate that pituitary hormones are not required (McNatty et al., 1992).

The first detectable events in the transformation from a primordial follicle to a committed follicle include RNA synthesis by the oocyte, formation of the zona pellucida, a change in shape of the granulosa cells from flat to cuboidal and granulosa proliferation which continues in concentric circles around the oocyte. When two or three layers of granulosa cells are formed, thecal cells differentiate from the surrounding stroma with a concentric alignment to the basement membrane (Scaramuzzi et al., 1993). Fluid subsequently accumulates in spaces between the epithelial cells and the follicle becomes vesicular. Once the granulosa layer has become two, three and then four layered, blood capillaries invade the fibrous layer of the theca externa and thus the theca becomes vascularised. These capillaries remain the only source of nutrients and hormone precursors to both the granulosa layer and oocyte by diffusion until ovulation and concurrent collapse of the basement membrane separating the two layers occurs (Baker, 1972).

During each reproductive cycle, under the influence of pituitary FSH, a pool of growing follicles are stimulated to undergo further growth and maturation. This pituitary dependent phase of follicular growth involves further multiplication in the number of granulosa cells and also passage of fluid into the spaces between them

(liquor folliculi). As the quantity of fluid increases so do the cavities that it occupies and they become confluent to form the antrum. The follicle is now of the Graafian type.

1.4.2 Antral development

By the time an antrum is formed (in women at a follicular diameter of 0.2mm), the gonadotrophins, follicle stimulating hormone (FSH) and luteinising hormone (LH) are required although relative amounts vary depending on stage of development. This concept will be further discussed below (Baird, 1991). Follicles entering the last 14 days of pre-ovulatory development, corresponding to the first half of the menstrual cycle, are between 2 and 4mm in diameter (Baird, 1991).

1.4.3 Selection of the dominant follicle

From the pool of small antral follicles (2-5mm diameter) a single follicle is selected for further development in the human and the rest become atretic. The trigger for this is still not fully understood. It may be that all these follicles are at subtly different stages of development despite being a similar size, not all receptive to the gonadotrophin milieu of high LH and FSH, following luteal regression (McNatty, 1982). In multiple ovulators for example the pig and marmoset, it is assumed that development of such follicles is synchronous so that several can concurrently be selected to undergo further maturation (Baird, 1987).

FSH is known to play a crucial role in selection. Large healthy follicles in women all contain easily detectable levels of FSH while those with lower FSH levels have much lower steroidogenic output (Zelevnik and Hillier, 1996). It is thought that local paracrine factors may play a role in selecting the dominant follicle while locally produced steroidal and non-steroidal factors probably modulate the responsiveness of the ovarian cells to gonadotrophins.

Once selected, it is the chosen follicle that ensures suppression of development of other follicles. It is thought that this is mediated by suppression of FSH due to the rise in oestradiol levels secreted by this dominant follicle. Inhibin and oestradiol both have the potential to negatively regulate pituitary FSH secretion (Groome et al., 1995), but there is evidence that it is the oestradiol that is the primary

regulator of FSH secretion during the follicular phase of the human and non-human primate ovarian cycles (Zelevnik and Kubik, 1986). As a result of this negative feedback, FSH levels fall too low to sustain the development of other follicles within the cohort and they become non-ovulatory and undergo atresia (Zelevnik and Hillier, 1996). The dominant follicle is thought to be protected from atresia as it is more sensitive to FSH than other less favoured follicles and thus can survive in FSH levels below the 'threshold' necessary to recruit further follicles. The presence of LH receptors on granulosa cells of the maturing follicle could also contribute to its protection from declining FSH levels (Yong et al., 1992). In addition to changes in the density of gonadotrophin receptors, vascularisation of the dominant follicle could also contribute to its survival, where gonadotrophins are preferentially delivered to it. In rhesus monkeys, the dominant follicle has a capillary supply three times more dense than subordinate follicles (Zelevnik et al., 1981). The dominant follicle may also secrete angiogenic mitogens causing its selective vascularisation. Vascular endothelial growth factor (VEGF) for example, is apparently selectively expressed by the maturing follicle (Ravindranath et al., 1992). There also exist a number of paracrine and autocrine regulators of FSH action which could serve a role in providing increased sensitivity of the dominant follicle to FSH, however details of these will not be provided at this point (Zelevnik and Hillier, 1996).

The follicle which will eventually ovulate is now approaching a diameter of about 10mm and is beginning to secrete increasing amounts of oestradiol (Baird, 1991). Over 95% of the oestradiol produced in the follicular phase of the cycle comes from the dominant follicle, thus by the mid-follicular phase, the selected follicle can dictate to the hypothalamo-pituitary unit, to secrete an optimal gonadotrophin cocktail, for its further development.

1.4.4 Gonadotrophin control of pre-ovulatory follicular development

Ovarian cyclicity is controlled by a feedback system involving the hypothalamus, anterior pituitary and the ovaries. FSH and LH are secreted by gonadotrophs in the anterior pituitary in response to gonadotrophin-releasing

hormone (GnRH) that interacts with specific receptors on the surface of the gonadotroph. Serum levels of LH and FSH are shown in **Figure 1.2**.

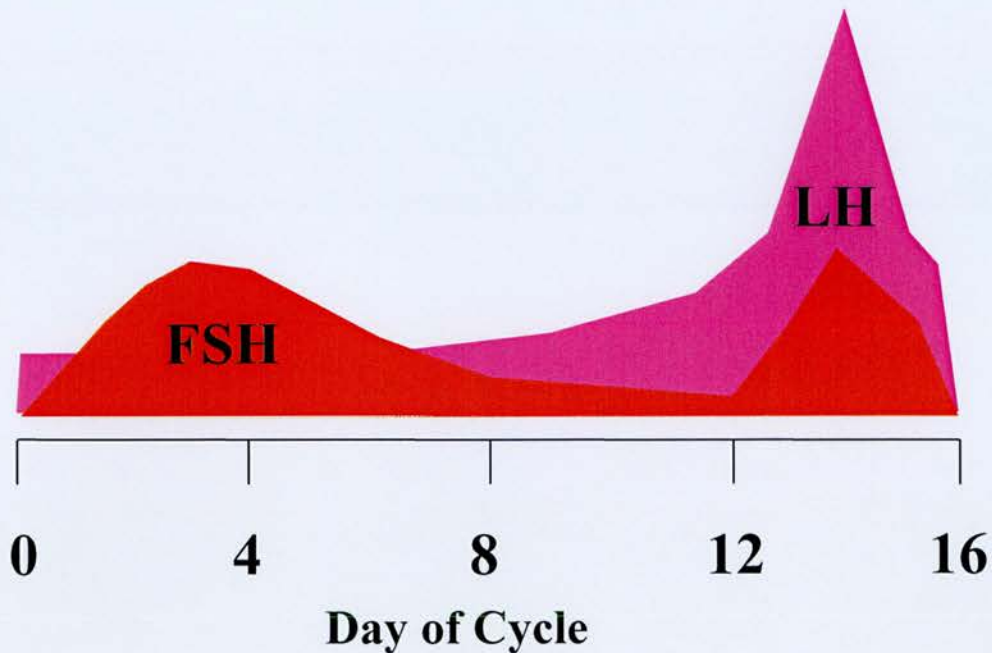


Figure 1.2 Gonadotrophin levels throughout the human menstrual cycle (Clarke,1996)

1.4.4.1 FSH

As the corpus luteum regresses, steroid and inhibin mediated negative feedback to the pituitary are removed and consequent increased pituitary FSH secretion occurs at the beginning of a subsequent cycle. This increased FSH production by the pituitary constitutes the primary signal for pre-ovulatory follicular development to commence (Hsueh et al., 1989). A 5mm antral follicle will require 10-12 days of sustained stimulation by FSH to attain a full pre-ovulatory diameter of approximately 20mm. During which time the number of granulosa cells increase to reach over 50 million (McNatty, 1981). It is FSH which stimulates granulosa cell proliferation directly and also induces LH-responsive mechanisms on these cells previously limited to the thecal layer (Channing and Tsafriiri, 1977). FSH stimulation

to granulosa cells also causes increased synthesis of steroids (progesterone and oestrogen) (McNatty, 1981) and overall increased responsiveness to FSH and LH (Hillier, 1985, Hsueh et al., 1984, Richards, 1980).

There exist several FSH inducible genes. These include the LH receptor itself (Segaloff et al., 1990) and some major steroidogenic enzymes including aromatase (P450arom) crucial in conversion of androstenedione and testosterone to oestradiol in granulosa cells of the small antral follicle (Mendelson et al., 1988). Proteases such as plasminogen activator and regulatory peptides, such as inhibin and follistatin may also be induced by FSH (Hillier, 1991).

1.4.4.2 LH

Steroidogenesis in the antral follicle is critically dependent on LH, as is the corpus luteum. In the early follicular phase of the cycle the concentration of LH rises as the suppressive effect of progesterone is removed following luteal regression. Follicular oestrogen synthesis occurs in the second half of the follicular phase when frequency of pituitary LH pulses increases and FSH declines. This occurs until mid-cycle where oestrogen secretion is suppressed by the ovulatory LH surge.

Granulosa LH receptors develop in response to FSH stimulation. LH receptors are also located on thecal cells and androgen synthesis in these cells is under direct LH control mediated through adenylyl cyclase signalling (Golos et al., 1987, Magoffin, 1989, Marsh, 1976).

1.4.5 Putative Intraovarian regulators

Despite the widely accepted role of gonadotrophins in folliculogenesis, the continued variable fate of follicles exposed to the same hormone milieu suggests the existence of other intraovarian modulatory systems (Adashi and Rohan, 1994).

It is thought that most intraovarian communication is autocrine or paracrine in nature. Paracrine communication involves local diffusion of regulators from their site of production to their designated target cells within the same organ. The adequate endocrine function of the follicle in terms of oestradiol synthesis depends upon the ability of the theca to provide the granulosa cells with androgenic substrate (Adashi and Rohan, 1994). The granulosa therefore may produce regulators which

could alter the function of the neighbouring theca. It has been suggested that granulosa-theca cell interactions are paramount in maintenance of follicular structure and function. Theca cells secrete inhibitory factors to suppress differentiation of immature granulosa cells while promoting that of mature cells during co-culture of bovine theca and granulosa cells; granulosa cells were also shown to secrete factors promoting both growth and differentiation of thecal cells (Yada et al., 1999).

Autocrine communication is based upon a cell modulating its activity by detecting its own message at surface receptors. In the ovary, the granulosa cells produce local autocrine factors which may alter granulosa cell function. Steroids may exert intracrine effects (regulation within the cell of origin), but there is little evidence for juxtacrine (contact-dependent regulation between immediately adjacent cells) mediators in the ovary (Adashi and Rohan, 1992).

Most studies of intraovarian regulation of folliculogenesis have been in rats or from *in vitro* experiments. From which several putative peptidergic intraovarian regulators have been discovered, some of which will be discussed briefly below.

Insulin-like growth factor-I for example, is produced in the rat granulosa cell and its receptors are located on both granulosa and theca cells. This signal molecule has been indicated to have many roles within the ovary including amplification of gonadotrophin action (Adashi and Rohan, 1994). Depletion of IGF action may be necessary for the onset of atresia and thus IGF may confer follicular survival. Conversely, transforming growth factor α has been put forward as a candidate attenuator of gonadotrophin action. Other peptidergic regulators of gonadotrophins include activin, inhibin and the interleukin-1 system, details of which will not be given here.

Steroids can also act as paracrine regulators of ovarian function. Locally produced androgens act via granulosa cell androgen receptors to modulate follicular responsiveness to gonadotrophins and thus serve as paracrine regulators of ovarian function (Hillier et al., 1997). Androgen receptor is down regulated during FSH-induced pre-ovulatory follicular development in the rat (Tetsuka and Hillier, 1996) and marmoset (Hillier, et al., 1997), suggesting that paracrine androgen action is influenced by development-related changes in granulosa cell androgen receptor expression.

1.4.6 Steroidogenesis- Mechanisms of follicular oestrogen synthesis

Both theca and granulosa cells are required for oestrogen synthesis by the antral follicle. Androstenedione and testosterone are produced by the theca interna under positive regulation by LH (Erickson et al., 1985). They are produced from cholesterol transported to the follicle by the vascularised thecal layer in a reaction catalysed by P450c17. This enzyme is expressed exclusively by thecal/interstitial cells and theca lutein cells and has both 17-hydroxylase and C-17,20-lyase activities (Richards et al., 1987, Sasano et al., 1989). These androgens act as precursors for oestrogen synthesis in the granulosa cells which are thought to be the exclusive source of oestradiol synthesis in the follicle (Dorrington and Armstrong, 1979). It has been calculated that over 99% of aromatase activity lies in this layer (Hillier et al., 1981). It is worth noting that despite these very specific roles of individual cell types, oestradiol production depends both on FSH and LH. FSH does indeed stimulate granulosa cell aromatase activity in antral follicles, but without the androgen precursors produced by the theca upon stimulation by LH this would not be possible. Follicular steroidogenesis is in fact a complex and tightly controlled event involving both cell types of small antral follicles. In this way, the granulosa cells are activated to utilize efficiently the increased amounts of androgen produced by the theca in response to the LH rise following luteal regression (Baird, 1991).

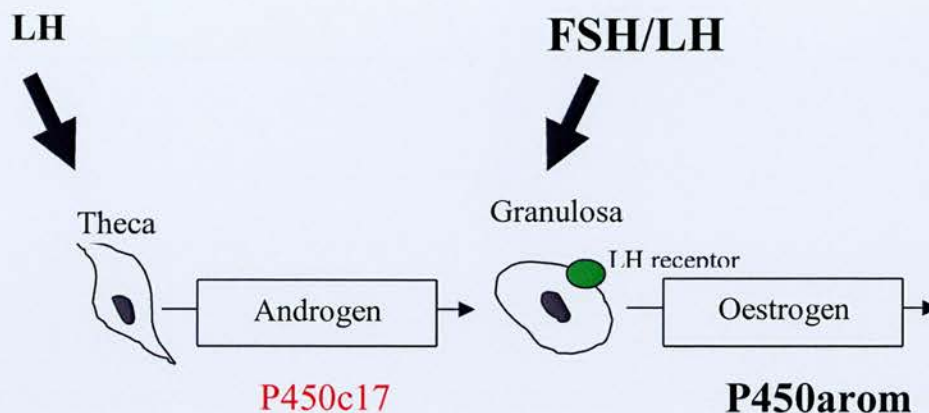


Figure 1.3 Two-cell, two-gonadotrophin theory of steroidogenesis, adapted from (Zeleznik and Hillier, 1996). Androgen synthesis occurs in the theca interna regulated by LH. This is the rate limiting step where C_{21} substrates are hydrolysed to C_{19} androgens catalysed by 17-hydroxylase/ C_{17-20} -lyase (P450c17). Aromatase (P450arom) is the granulosa cell enzyme that converts C_{19} androgens (androstenedione and testosterone) to C_{18} oestrogens (estrone and oestradiol)-induced by FSH. FSH also induces granulosa LH receptors which are coupled to aromatase. Thus androgen is synthesised in thecal cells and aromatised to oestrogen in granulosa cells.

Progesterone synthesis is only a minor granulosa cell function before the onset of the LH surge. However, LH responsive steroidogenic enzymes crucial to progesterone synthesis in the corpus luteum are induced by FSH during pre-ovulatory granulosa cell development, notably P450scc (Funkenstein et al., 1984, Richards et al., 1987). FSH-responsive aromatase activity and progesterone synthesis in granulosa cells increase markedly during preovulatory follicular development in rat (Hillier et al., 1978, Zeleznik et al., 1977), human (Hillier, et al., 1981) and non-human primate ovaries (Harlow et al., 1988). This is thought to be mediated by amplification of cAMP intracellular signalling and could further contribute to the dominant follicle's ability to continue to synthesise and secrete oestrogen in the face of declining levels of FSH.

1.4.7 Termination of follicular oestrogen synthesis

Several hours after the onset of the ovulatory LH surge, thecal androgen synthesis is suppressed resulting in reduced oestradiol output. The exact mechanism for this discontinuation of androgen synthesis is not fully understood but is thought to be a consequence of thecal desensitisation to LH brought about by the rapid and extreme increase in circulating LH levels characteristic of this stage of the ovulatory cycle. Desensitisation appears to involve impaired coupling of the LH receptor to the guanine nucleotide binding regulatory protein G_s (Ekstrom and Hunzicker-Dunn, 1989).

Once the LH surge subsides and ovulation has occurred, thecal androgen synthesis is reinitiated in the CL (Hillier, 1991). The LH surge does not suppress granulosa cell aromatase activity, it may in fact even enhance it. Progesterone synthesis in the granulosa layer is also enhanced by the LH surge in preparation for the progesterone secreting role of the corpus luteum (Hillier, 1985).

1.4.8 Atresia

Folliculogenesis occurs continually throughout life until the stock of oocytes is exhausted (Gougeon, 1982). The majority of follicles become atretic at the antral stage of development but the number attaining ovulatory status is more or less fixed for a given species by the levels of circulating gonadotrophins. A follicle is described as being functionally atretic when it has sustained irreversible degeneration preventing it from further development and ovulation (Scaramuzzi, et al., 1993).

1.5 Ovulation

Ovulation marks the pivotal point in the ovarian cycle. It marks the terminal step in the growth and differentiation of the follicle and an essential prerequisite for corpus luteum formation.

1.5.1 The LH Surge

Ovulation occurs in response to a massive discharge of LH from the anterior pituitary. It is the action of oestrogen on the hypothalamo-pituitary unit that

stimulates this pre-ovulatory LH discharge via a GnRH surge. How oestrogen exerts this positive feedback mechanism remains to be fully understood but it is thought to exert an effect at both the hypothalamus and pituitary.

Oestrogen sensitises the gonadotrophs to the effects of GnRH resulting in progressively more and more LH released throughout the follicular phase following GnRH injection, reviewed by Baird, 1991. Restoration of ovarian cyclicity and subsequent ability to ovulate can be restored to hypogonadotrophic rhesus monkeys by hourly pulses of GnRH (Wildt et al., 1981). The LH surge is likely to be due to pulsatile GnRH release from the hypothalamus, acting on a hypersensitive pituitary. Ovulation can also be induced in FSH primed pre-ovulatory follicles by administration of exogenous HCG as an LH substitute (Zeleznik and Hillier, 1996).

Circulating levels of progesterone rise prior to ovulation at about the same time as the onset of the LH surge. This is caused by cessation of follicular growth and increased expression of P450 scc, the major steroidogenic enzyme required for progesterone synthesis, in granulosa cells (Yong, et al., 1992). Oestrogen synthesis declines as a result of inhibition of thecal P450c17 and subsequent lack of androgen as a substrate for aromatase in the granulosa cells. Thus as ovulation approaches, the ovary increasingly produces progesterone while oestrogen levels transiently decline (Hillier, 1985). In the rat, progesterone facilitates the ability of oestrogen to provoke a discharge of LH (Fink, 1979). In rabbit (Kanayama et al., 1996), monkey (Collins and Hodgen, 1986, Wolf et al., 1989) and women (Ho, 2001, Liu et al., 1987), treatment with progesterone antagonist prevents the pre-ovulatory LH surge. Progesterone is also thought to further enhance the positive feedback effects of oestradiol at this time in women (Liu and Yen, 1983).

Progesterone levels continue to rise in follicular fluid following the LH surge and it is thought that this preovulatory increase is essential to follicular rupture, by accelerating the net loss of collagen fibres in the follicular wall as demonstrated *in vitro* in human (Tjugum et al., 1984). This loss of collagen fibres is caused by the upregulation of plasminogen activator synthesis by progesterone which in turn activates collagenase thereby facilitating follicular wall breakdown, reviewed by (Zeleznik and Hillier, 1996). There is further evidence for a specific action of progesterone in ovulation in rats and pigs where LH/hCG has been demonstrated to

stimulate transient expression of progesterone receptor mRNA in granulosa cells of preovulatory follicles (Park-Sarge and Mayo, 1994). Progesterone receptors have also been localised in granulosa cells of women (Chaffkin et al., 1993) and rhesus monkey (Duffy et al., 1994).

1.5.2 Follicle rupture

The LH surge provokes a proteolytic cascade within the preovulatory follicle resulting in its rupture approximately 36 hours later (Zeleznik and Hillier, 1996), earlier in sheep (Scaramuzzi, et al., 1993). During the final stages of folliculogenesis, a small area of the wall of the Graafian follicle becomes weaker, usually mediated by reduced blood flow to the apex or stigma, and increased proteolytic enzyme activity induced by second messenger systems which become activated in the thecal and granulosa cells. This area becomes the ovulation point. Similarly, disintegration of the follicle wall, basement membrane and theca also occurs, eventually leaving a single layer of granulosa cells separating the follicular fluid from the peri-ovarian space. In pigs and other species this breakdown is brought about by the action of prostaglandin F₂ α from granulosa cells produced in response to the LH surge (Evans et al., 1983). It is these locally produced prostaglandins which are primarily responsible for the increase in vascular permeability, that sustains positive intrafollicular pressure with the onset of follicular fluid leakage. VEGF could also be involved in this increase in permeability, but this will be discussed later. Just prior to ovulation the stigma becomes raised and resembles a fluid filled blister on the ovarian surface. With the onset of ovulation, follicular fluid and vascular transudate flow out of the ruptured follicle carrying the cumulus-enclosed oocyte onto the ovarian surface from which point it passes into the oviduct (Johnson and Everitt, 1995). The collapsed follicle now transforms into a progesterone secreting corpus luteum.

1.6 The corpus luteum

1.6.1 Corpus luteum formation and luteinisation

The luteal phase of the ovulatory cycle commences in response to the mid-cycle gonadotrophin surge resulting in follicular rupture and transformation of the follicular granulosa cells into luteal cells in a process known as luteinisation (Zelevnik, 1991). The corpus luteum is usually formed from the preovulatory follicle but ovulation is not a necessary prerequisite for luteinisation as corpora lutea can develop from non-ovulated follicles (Behrman et al., 1993). In antral follicles, the theca and granulosa cell layers are separated by a basement membrane consisting of collagen IV and laminin (Paulsson, 1992, Yurchenco and Schittny, 1990). This excludes capillaries, white blood cells and nerve processes from the granulosa layer until ovulation at which time this basal lamina is degraded. As such, degradation of the follicular membrane can be used as an early marker of luteinisation (Rodgers et al., 1998). In an *in vitro* study of cultured marmoset large antral non-luteinised follicles, Wehrenberg *et al*, using dissociation of the basement membrane, decrease of the gap junction protein connexin-43, expression of a luteal specific member of the integrin family and the expression of TGF- β receptor type II as markers of luteinisation, demonstrated that antral follicles can spontaneously luteinise without necessarily having undergone ovulation (Wehrenberg and Rune, 2000). These spontaneously luteinised follicles are termed accessory corpora lutea.

1.6.2 Neovascularisation

An adequate vascular supply is paramount in efficient steroid synthesis and secretion. Before such mechanisms of steroid biosynthesis are further discussed, the dramatic events associated with neovascularisation of the newly formed corpus luteum will first be addressed. The detailed mechanism of angiogenesis will follow in section 1.11. This is purely an introduction.

After follicle rupture at ovulation, newly formed blood vessels invade the avascular granulosa and within about 5 days, the CL becomes one of the most highly vascularised organs on a tissue-weight basis (Zelevnik and Benyo, 1994). New vessels originate as sprouts from small capillaries, the basement membrane of the endothelium is broken down and endothelial cells migrate into the granulosa layer as

a cord. This cord then forms a lumen, mitosis begins and growth occurs just behind the tip of the advancing vessel. Formation of the vessel is completed by the laying down of a basement membrane and migration of pericytes along the length of the vessel (Folkman, 1992). The CL has one of the highest blood flows per unit mass of any gland in the body (Baird, 1991, Bruce, 1976, Reynolds and Redmer, 1999). It is not surprising therefore that endothelial cells make a substantial contribution to total cells in the mature human corpus luteum, around 50% in fact. The capillary network of the CL is so extensive that in the mature corpus luteum, every steroid producing cell is in contact with a capillary. Endothelial cells increase in number from the early to mid luteal phase. Cellular proliferation is greatest in the early corpus luteum, resulting in a mitotic rate comparable to that of rapidly growing tumours (Jablonka-Shariff et al., 1993). This rate of proliferation gradually decreases throughout the luteal phase (Dickson and Fraser, 2000). Greater than 80% of these proliferating cells have been identified as endothelial in sheep (Jablonka-Shariff, et al., 1993), rat (Gaede et al., 1985), pig (Ricke et al., 1999), cow (Zheng et al., 1994), macaque (Christenson and Stouffer, 1996) and marmoset (Dickson and Fraser, 2000).

The mature corpus luteum receives most of the ovarian blood flow which is highly correlated with progesterone secretion (Redmer and Reynolds, 1996). An inadequate blood supply to the corpus luteum is associated with decreased luteal function. CL expected to be short lived have reduced LH receptor number and reduced luteal vascularity (Smith et al., 1996). Reduced progesterone secretion resulting from inadequate luteal development results in inadequate development of the endometrium for secure implantation of fertilised ova. This is clinically significant in infertility (Andrews, 1979, Crosignani, 1988).

1.6.3 Luteal steroidogenesis

The corpus luteum is fundamental to the control of the menstrual cycle and once formed, it has a functional lifespan of 14 to 16 days in women. During this time its major secretory products are progesterone and oestradiol.

During the follicular phase, granulosa and theca cells of the follicle co-ordinately produce oestrogens. Luteinisation of the follicle alters the steroidogenic pathway so that progesterone is the primary steroid hormone produced. Synthesis of

progesterone occurs in both theca-lutein cells and granulosa-lutein cells, although predominantly in the latter, and is the least complex biosynthetic pathway in the ovary. See **Figure 1.4**.

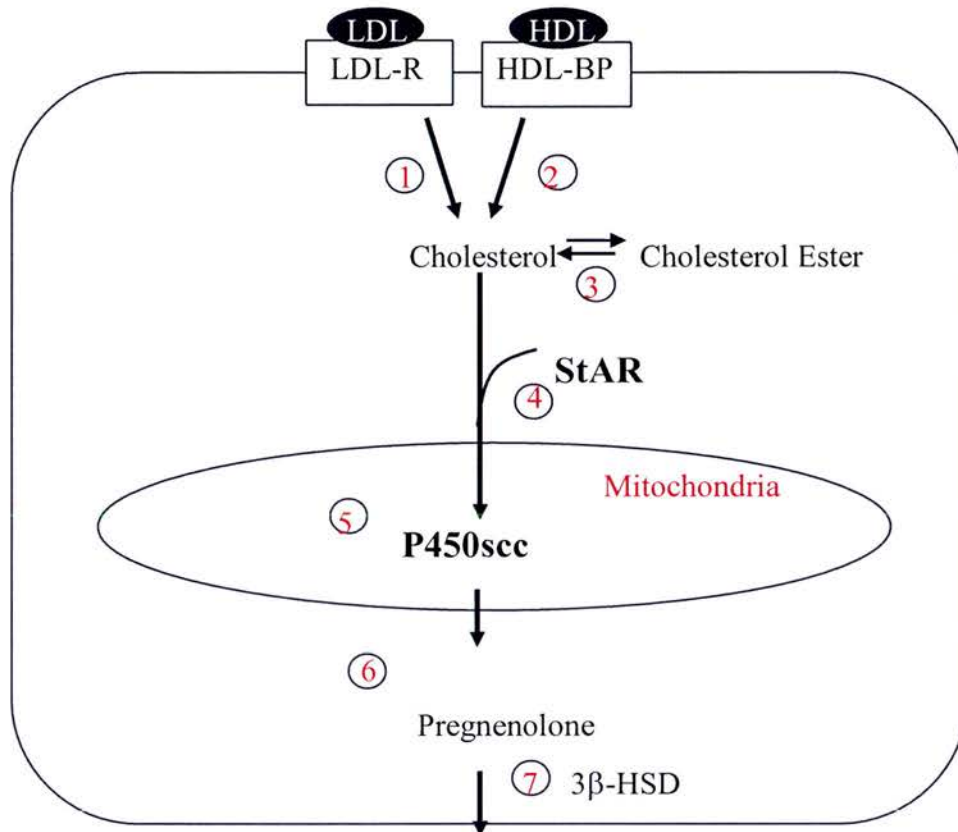


Figure 1.4. Pathway for progesterone biosynthesis in a generic luteal cell (Niswender et al., 2000). Three sources of cholesterol can be utilised for substrate: **1)** Low-density lipoprotein (LDL) **2)** High-density lipoprotein (HDL), or **3)** hydrolysis of stored cholesterol esters by cholesterol esterase. Free cholesterol is transported to mitochondria, thought to involve StAR **4)**. Cholesterol is converted to pregnenolone by cytochrome P-450 side-chain cleavage enzyme (P-450scc); **5)**. Pregnenolone is then transported out of mitochondria and converted to progesterone by 3 β -hydroxysteroid dehydrogenase (3 β -HSD) **6)**, present in smooth endoplasmic reticulum. **7)** Progesterone diffuses from luteal cell.

The substrate for steroidogenesis is cholesterol which predominantly comes from low density- and high density- lipoproteins (LDL and HDL respectively) in the blood stream. Either source can be utilised for steroid biosynthesis by most species but for simplicity only LDL will be considered further here. The uptake of LDL by luteal cells occurs by receptor mediated endocytosis (Brown and Goldstein, 1986). Once internalised, the endosomes combine with lysosomes where LDL dissociates from the receptor and is degraded making free cholesterol available to the cell. This free cholesterol in the cell is now transported to mitochondria where the cholesterol side-chain cleavage enzyme, P-450 complex (P450_{scc}) cleaves the side chain from cholesterol to form pregnenolone. This is the rate limiting step in this process and appears to be the primary site of acute positive and negative regulation of steroidogenesis by second messenger systems (Niswender, et al., 2000).

Stimulation of the steroidogenic pathway by trophic hormones requires the synthesis of a short-lived protein which when phosphorylated, upregulates steroid secretion. This protein is termed steroidogenic acute regulatory protein (StAR) and is thought to aid internalisation of cholesterol at the mitochondrial membrane. Once formed, pregnenolone is transported to the endoplasmic reticulum where it is converted to progesterone by 3 β -hydroxysteroid dehydrogenase Δ^5, Δ^4 isomerase (3 β -HSD). Progesterone is then thought to diffuse from the cell (Niswender, et al., 2000).

In the uterus, progesterone acts on the endometrium as a differentiation factor (Cummings and Yochim, 1984). Oestrogens induce endometrial cell proliferation during the follicular phase which is inhibited by progesterone in the luteal phase (Padykula et al., 1989). Progesterone induces stromal differentiation, stimulates glandular secretions and changes the pattern of proteins secreted by endometrial cells. This process is known as decidualisation. These uterine proteins provide an environment that supports early embryonic development (Niswender, et al., 2000). Progesterone also inhibits myometrial contractility.

Other products of this newly formed endocrine gland include androgens and peptides such as relaxin and oxytocin. Inhibin-A, eicosanoids, cytokines, growth factors and oxygen radicals have also been identified as luteal products but their function has yet to be elucidated (Behrman, et al., 1993). It should be noted that in

the primate, pig and rat, the corpus luteum retains the ability to secrete oestrogen in the luteal phase in addition to progesterone.

Progesterone, oestradiol and inhibin-A, secreted by the corpus luteum collectively exert negative feedback regulation on pituitary FSH secretion thereby preventing preovulatory follicular development (Zelevnik and Hillier, 1996). It has been demonstrated that the health of the dominant follicle from a subsequent cycle is superior when it develops in the contralateral ovary where no ovulation took place in the preceding cycle rather than in the ovary where the preceding ovulation took place (Fukuda et al., 1996). Further to this work it was proposed that the corpus luteum may exert a local negative effect on follicular growth during the luteal phase by secreting local ovarian factors which negatively affect follicular health of the responsive cohort of follicles of the next menstrual cycle (Fukuda et al., 1997). Due to the various levels of negative feedback exerted by the corpus luteum, both locally and at the level of the pituitary, its regression becomes a pivotal event in order that a new menstrual cycle and subsequent follicular development may occur (Zelevnik and Hillier, 1996).

As the corpus luteum matures and plasma progesterone levels rise, the capacity for luteal cells to biosynthesise progesterone declines, illustrated by **Figure 1.5** (Zelevnik and Hillier, 1996). Cellular levels of 3β -HSD and P450_{scc} mRNA's are also highest shortly after ovulation, declining progressively throughout the luteal phase. This declining rate of steroid production and biosynthetic potential may occur due to the fact that the newly formed CL is incompletely vascularised such that access to blood-borne cholesterol in the form of low density lipoprotein as a progesterone precursor is limiting. However, by the mid-luteal phase when the luteal vasculature is fully developed, this is no longer true so progesterone secretion rate progressively increases during the early to mid-luteal phase despite a decline in steroidogenic potential at the cellular level (Zelevnik, 1991).

Increased vascularisation also benefits oestradiol synthesis. It is thought that the two cell model for oestrogen synthesis is preserved in the primate corpus luteum with small theca-derived luteal cells producing androgens that serve as a substrate for the large granulosa-derived cells producing oestrogens (Sanders and Stouffer, 1997). Luteal oestrogen levels mimic those of progesterone, reaching a maximum in the

mid-luteal phase despite declining activity of aromatase. This can be explained by the fact that granulosa-lutein cells require precursor androgen from the theca-lutein cells in order that it may be aromatised to oestradiol. Therefore this process can also only benefit from increased vascularity (Zelevnik, 1991).

1.6.4 Cell types in the corpus luteum

Luteinisation includes several structural events and endocrinological components that shift a predominantly FSH regulated, oestrogen secreting organ to an organ dominated by LH whose function is predominantly progesterone secretion. (Behrman, et al., 1993). Over the last few years it has been extensively reported that the CL of several species, including the human is composed of several cell subpopulations which are either steroidogenic or non-steroidogenic (Vega and Devoto, 1997). It is widely accepted that steroidogenic cells are derived from both the granulosa and theca layers of the follicle. In women, these are easily recognised, with the theca lutein cells, also termed small lutein cells, at the periphery and cortical infoldings of the gland and the granulosa lutein cells, or large lutein cells, internal to this area (Behrman, et al., 1993).

Large luteal cells contain numerous mitochondria, well developed endoplasmic reticulum and secretory granules, all characteristic of a secretory cell. It is here that most of the progesterone is synthesised and on these cells there exist prostaglandin $F_{2\alpha}$ receptors. The small lutein cells however, have reduced mitochondrial numbers and a well developed endoplasmic reticulum lacking secretory granules (Vega and Devoto, 1997). Small lutein cells are the site of action of LH. Both theca and granulosa lutein cells are capable of progesterone, androgen and oestrogen production, but the regulatory mechanisms involved differ between the two cell types. In several domestic species and human, the majority of luteal LH and hCG receptors exist on small lutein cells (Niswender et al., 1985, Retamales et al., 1994). Large lutein cells are the sites where most steroidogenesis occurs even though they show poor response to several regulatory factors. Thus it is most likely that the regulation of the steroidogenic capacity of the human CL is by response of small lutein cells to different endocrine, paracrine and autocrine regulators (Vega and Devoto, 1997). In addition to steroidogenic cells, the corpus luteum contains non-

steroidogenic cell types including endothelial cells, fibroblasts, pericytes and immune cells. Despite serving as an endocrine organ principally responsible for progesterone secretion in the luteal phase and early pregnancy, the human corpus luteum contains more non luteal cells than luteal cells, also evident in bovine species (Lei, 1991). In most non-primate animals, follicular tissue is extensively reorganised during migration of luteal cells during development of the corpus luteum (Niswender and Nett, 1994), where large and small luteal cells, fibroblasts and endothelial cells are in close proximity (Niswender, et al., 2000). **Figure 1.5** illustrates development of the corpus luteum and its extensive cellular reorganisation (Niswender, et al., 2000).

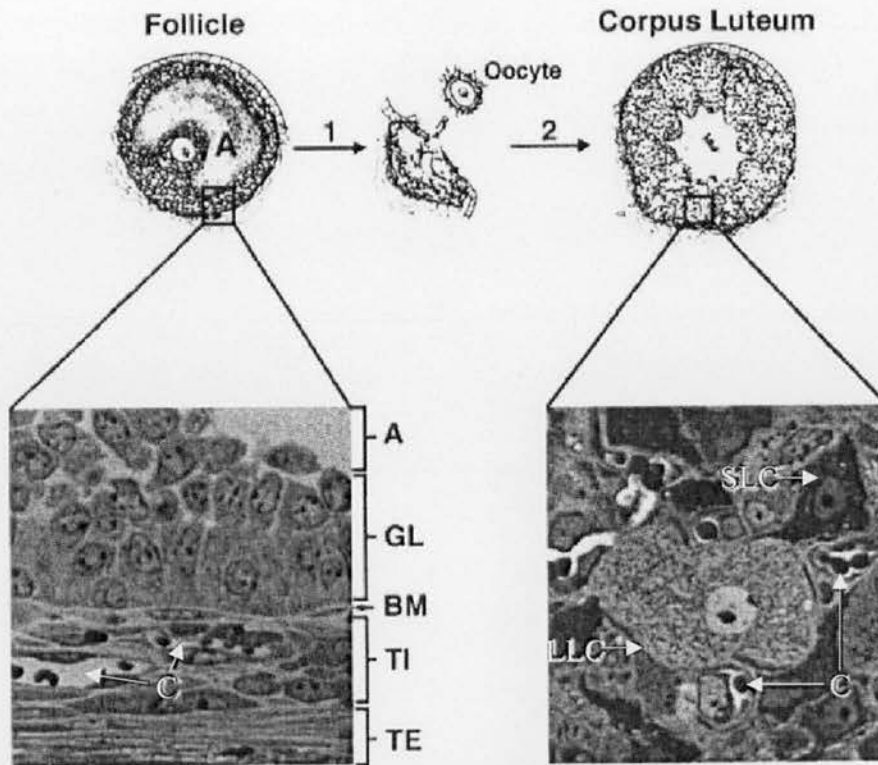


Figure 1.5. Development of the corpus luteum from a follicle (Niswender, et al., 2000). The follicle is organised into distinct layers, The Antrum (A), is a fluid filled lumen surrounded by a granulosa layer (GL). This layer and the oocyte are separated from the rest of the follicle by a basement membrane (BM). Outside the BM are the theca interna (TI) and theca externa (TE) layers of the follicle. Capillaries (C) are present in the thecal layer but not in the granulosa layer. LH causes breakdown of the follicular wall and release of the oocyte at ovulation (1). After ovulation the granulosa layer becomes vascularised as the basement membrane degrades and the follicular tissue develops into the corpus luteum (2). The Corpus luteum contains a heterogeneous population of cells including large and small luteal cells (LLC and SLC respectively). There are many capillaries indicative of the high degree of vascularisation of the CL.

Of the non-steroidogenic cells, leukocytes, particularly macrophages are of fundamental importance because of their capacity for secretion of growth factors, cytokines and reactive oxygen species increasingly recognised as intraluteal regulators of luteal function (Brannstrom and Friden, 1997). Macrophage numbers are highest at the end of the mid luteal phase, having tripled from initial numbers. These cells represent a greater proportion of total cells than fibroblasts which also increase in number from the mid to late luteal phase. (Lei, 1991). Besides their involvement in regressing corpora lutea (Gaytán et al., 1998), macrophages are

putative cells involved in luteal angiogenesis and progesterone synthesis (Vega and Devoto, 1997).

1.6.5 Trophic regulation of the corpus luteum

1.6.5.1 LH

LH is historically recognised as playing a fundamental and critical role in formation and maintenance of the CL (Niswender, et al., 2000) by its action at LH receptors. The number of LH receptors increases during the early to midluteal phase and declines progressively in the late luteal phase becoming undetectable after menstruation. Therefore, regulation of this receptor may in itself be an important mechanism controlling the function of the CL throughout the menstrual cycle (Nakano, 1997). From studies conducted on stump-tailed macaque, it was found that the corpus luteum is relatively unresponsive to high serum LH concentrations in the early luteal phase and that this responsiveness increases as the luteal phase progresses. However, the corpus luteum is sensitive to LH withdrawal regardless of stage of luteal phase (Fraser et al., 1986). It is possible that other luteotrophic factors such as prolactin may be involved in maintaining the morphological integrity of the CL but this is unlikely as inhibition of prolactin secretion does not result in reduced luteal function, reviewed by (Baird, 1991).

The overwhelming evidence would suggest that primates and women depend absolutely upon LH throughout the entire luteal phase to maintain secretion of progesterone by the corpus luteum (Zelevnik, 1991). GnRH antagonist administration has been shown to prevent normal luteal development and reduce progesterone synthesis (Hutchison and Zelevnik, 1984, Ravindranath et al., 1992, Webley et al., 1991). In hypophysectomised women, injection of HCG has been demonstrated to maintain the secretory activity of the corpus luteum for its normal lifespan of 14-16 days (Gemzell, 1965, Vande Wiele et al., 1970).

Dependence on LH for adequate luteal function has also been addressed in other species but the findings are somewhat less uniform. Ewes and pregnant swine treated with LH antisera have lower luteal weights and reduced luteal progesterone production (Fuller and Hansel, 1970, Reimers and Niswender, 1975, Snook et al., 1969, Spies and Niswender, 1971). Pulsatile release of LH is necessary for luteal

development in cattle (Peters et al., 1994), but once fully developed, the bovine CL is no longer LH dependent. Sheep do not appear to be critically dependent on LH at all as treatment with GnRH antagonist had no effect on luteal function in either the developing or mature CL (McNeilly et al., 1992).

It would appear therefore that LH support is critical in primates for normal luteal function and in development of the CL in cows. However, it appears not to be important for maintained progesterone secretion in the cow and not at all critical in formation or maintained CL function in ewes. Thus requirement for gonadotrophin support in the luteal phase is variable between species.

A close temporal association exists between bursts of LH and the subsequent secretion of progesterone by the corpus luteum (Ellinwood et al., 1984, Filicori et al., 1984, Mais et al., 1986). The speed of response in progesterone secretion following each pulse of LH implies an acute effect of LH on precursor cholesterol metabolism through existing steroidogenic pathways rather than upregulation of steroidogenic enzyme production, exactly how this is mediated is still unknown (Zelevnik and Hillier, 1996). It is thought to include the cyclic AMP-dependent mobilisation of cholesterol to the inner mitochondrial membrane for conversion into pregnenolone by P450_{scc} (Strauss, 1991). A longer term effect of LH on steroidogenesis is exerted through maintaining cellular levels of 3 β -HSD and P450_{scc} mRNA's (Bassett et al., 1991, Ravindranath, et al., 1992).

1.6.5.2 FSH

Luteal function itself is not directly regulated by FSH but does depend on development of the follicle. FSH induces appearance of LH receptors on granulosa cells but does not appear to stimulate progesterone secretion. The fact that exogenous LH and hCG can maintain luteal function and steroidogenic capacity in hypophysectomised women indicate that FSH does not play an obligatory role in regulation of the primate corpus luteum (Gemzell, 1965).

1.6.6 Autocrine/Paracrine regulation of human luteal function and development

Human corpora lutea undergo an extremely rapid period of growth, development and regression during the course of non-fertile cycles. Having discussed the dependence on pituitary-derived LH for steroidogenesis in women and other primates, there also exists intra-luteal regulation of steroid synthesis. Steroidogenic luteal cells and non-steroidogenic cells interact via endocrine and paracrine pathways, and by contact-dependent pathways (gap junctions). Thus, hormones and locally produced factors including steroids, growth factors, cytokines, reactive oxygen species and nitric oxide may modulate luteal lifespan and as such will be discussed briefly below (Devoto et al., 2000).

1.6.6.1 Oestradiol

The primate CL synthesises oestradiol where it has been implicated as an intraovarian regulator of luteal regression (Stouffer, 1996). In some species such as the rat, rabbit and pig, oestrogens are luteotrophic, however the dependence of the corpus luteum on oestradiol for support varies in these species. In rabbit and rat, where oestradiol is the major luteotrophic hormone, removing it causes cessation of progesterone synthesis (Niswender, et al., 2000). It is stimulation by oestrogen in these species that causes luteal cell hypertrophy during pregnancy (Holt, 1989). In the pig the luteotrophic role of oestrogen is ill-defined however it does inhibit PGF₂ α , thus preventing luteolysis (Ford et al., 1982). Oestrogen receptor mRNA was found to be absent from rhesus monkey CL suggesting an apparent absence of a role for oestradiol in this species (Chandrasekher et al., 1994) despite localisation of specific androgen and progesterone receptors (Vega and Devoto, 1997). Administration of large amounts of oestradiol suppresses plasma LH levels and results in premature luteal regression in human. Also, following administration of antioestrogens such as clomiphene or tamoxifen there is an increase in frequency, though not amplitude of LH pulses suggesting that oestradiol enhances the suppressive effect of progesterone on GnRH pulse frequency during the luteal phase. Thus, during the luteal phase under the combined effects of oestradiol and

progesterone the concentration of LH is suppressed to the lowest levels throughout the cycle (Baird, 1991).

1.6.6.2 Progesterone

Progesterone has been implicated as a universal luteotrophin for the mammalian CL and has been demonstrated as a potent regulator of its own secretion in rat (Rothchild, 1981). Besides important roles in periovulatory events, progesterone promotes synthesis of both LH receptor and luteotrophic prostaglandins in the bovine (Vega and Devoto, 1997). In the rhesus monkey, appearance of the progesterone receptor is driven by the LH peak. Luteinising granulosa cells from patients undergoing *in vitro* fertilisation undergo differentiation in response to progestins which in turn upregulate progesterone production (Rothchild, 1981). Furthermore, the observed rise in progesterone is maintained by progesterone itself and as such may be considered as a broader regulator of the functional lifespan of the primate CL (Vega and Devoto, 1997).

1.6.6.3 Prostaglandins

The role of prostaglandins as luteal regulators has long been postulated. The highest secretion of prostaglandins has been reported in the early luteal phase decreasing by the late luteal phase and as such their putative role in luteal function has been the subject of much research (Niswender, et al., 2000).

Production of both luteotrophic and luteolytic prostaglandins has been documented in the non-primate and primate CL over the years. Prostacyclin (PGI₂) is a major product of endothelial cells and has an implicated role in vasodilation. It is thought to be lutetrophic as its inhibition during luteal development has been shown to decrease progesterone production and bovine luteal lifespan (Milvae and Hansel, 1985). In women and non-human primates, stimulation of progesterone secretion by PGE₂ has been demonstrated *in vitro* and *in vivo* (Stouffer et al., 1979, Zelinski-Wooten et al., 1990, Zelinski-Wooten and Stouffer, 1990). In contrast, PGF_{2α} has been shown to reduce concentrations of progesterone *in vivo* (Auletta and Kelm, 1994, Sargent et al., 1988). In the marmoset, *in vivo* administration of cloprostenol, a synthetic PGF_{2α} analogue induces luteolysis during the mid-late luteal phases

(Summers et al., 1985). Treatment with PGE₂ can prevent PGF_{2α} induced premature functional luteolysis in the rhesus monkey but not spontaneous luteal regression (Zelinski-Wooten and Stouffer, 1990).

As such luteal function is thought to be regulated by complex interactions of luteotrophic and luteolytic prostaglandins (Fehrenbach et al., 1999). However the role of PGF_{2α} in luteolysis will be further discussed in a subsequent section.

1.6.7 Cellular interactions

Cellular interactions in the CL are mediated by several mechanisms including humoral (endocrine and paracrine) and contact-dependent (gap-junctional) pathways and involve heterogeneous cell populations including steroidogenic and non-steroidogenic cells. The existing interactions between cells in the corpus luteum are summarised in **Figure 1.6**

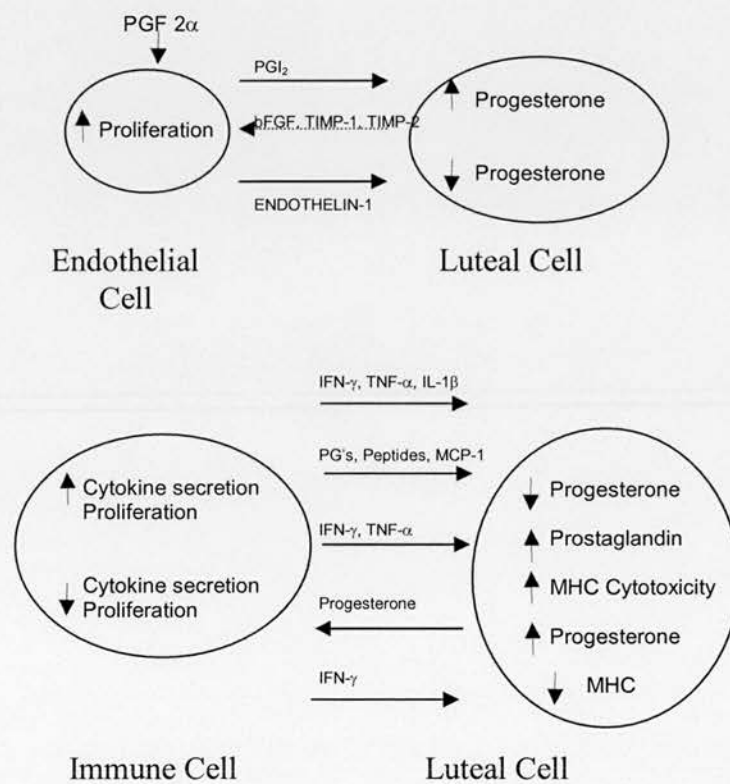


Figure 1.6 Cellular interactions in the corpus luteum adapted from (Pate, 1996).

1.6.8 Humoral cellular interactions

1.6.8.1 Cellular interactions among small and large steroidogenic luteal cells

Data from several studies suggest that cellular interactions are involved in the production of progesterone and luteolysis in the CL of several species, reviewed by (Grazul-Bilska et al., 1997). Numerous hormones and growth factors are produced by luteal cells that may mediate cellular interactions in a paracrine manner (Del-Vecchio, 1997). Steroidogenic luteal cells produce oxytocin, relaxin, inhibin and prostaglandins E_2 , $F_{2\alpha}$ and I_2 in several species (Ackland et al., 1992, Auletta and Flint, 1988, Einspanier et al., 1997, Einspanier et al., 1999, Fields, 1991, Hansel et al., 1991, Hansel, 1986, Khan-Dawood et al., 1997, Nappi et al., 1994, Stouffer and Brannian, 1993, Yamamoto et al., 1997). Large luteal cells contain the majority of $PGF_{2\alpha}$ receptors and as such may respond to $PGF_{2\alpha}$ produced by small luteal cells resulting in reduced LH-induced progesterone secretion. Oxtocin is also produced by large luteal cells and has an effect on progesterone secretion. In small concentrations it appears to be stimulatory becoming inhibitory with higher doses. It has also been implicated in luteinisation in the marmoset (Einspanier, et al., 1997) and has been shown to induce luteolysis by either stimulation of uterine $PGF_{2\alpha}$ or by acting directly on the CL as a paracrine down-regulator of steroidogenesis in sheep (Grazul-Bilska, et al., 1997). Oxytocin also stimulates oestradiol synthesis, which may effect expression of the gap-junction protein connexin-43 allowing interaction between large and small luteal cells (Khan-Dawood, 1997).

1.6.8.2 Cellular interactions among steroidogenic and endothelial cells

Interactions between steroidogenic and endothelial cells may be important for growth, differentiation and regression of luteal tissues. After the process of ovulation endothelial cells of the vascularised theca layer invade the previously avascular granulosa layer. Proliferation and migration of these endothelial cells are critical components of the angiogenic process. Under the influence of LH, steroidogenic tissues produce angiogenic factors, principally VEGF which target receptors on endothelial cells causing their migration towards this angiogenic stimulus (Grazul-

bilska et al., 1995, Redmer et al., 1988, Redmer et al., 1996, Redmer and Reynolds, 1996, Smith et al., 1994). The angiogenic process will be discussed more thoroughly in later sections. Steroidogenic cells also produce tissue inhibitors of matrix metalloproteinases which may regulate endothelial cell migration during this process. Just as luteal cells appear to regulate endothelial cells, the reverse is also true. Endothelial cells produce vasoactive compounds which have profound and unique effects on the steroidogenic functions of the CL for example endothelin (Meidan and Girsh, 1997).

Interactions between steroidogenic cells and endothelial cells may not only be humoral, but also contact dependent. *In vitro* these two cell types develop contact dependent communication which can be regulated by LH and $\text{PGF}_{2\alpha}$ (Grazul-Bilska, et al., 1997).

1.6.8.3 Cellular interactions among steroidogenic and immune cells

Cytokines secreted by immune cells eg. Interferons, tumor necrosis factor or interleukins exert effects on luteal cells. Additionally cytokines enhance expression of major histocompatibility complex molecules on luteal cells and may be cytotoxic in certain conditions (Pate, 1996).

1.6.9 Gap junctional (contact-dependent) cellular interactions

The mammalian gap junction is a junction of communication or electrical coupling between adjacent cells, which when open, allows exchange of nutrients, ions and regulatory molecules (Grazul-Bilska, et al., 1997). Gap junctions have been demonstrated in corpora lutea of numerous species by electron microscopy and more recently by detection of gap junctional proteins or connexins (Beyer et al., 1990).

The structure and function of gap junctions are thought to be regulated by numerous factors including hormones, growth factors and intracellular regulators (Grazul-Bilska, et al., 1997). The stage of luteal development affects gap junctional intercellular communication. The rate of gap junctional intercellular communication between small and large luteal cells is significantly greater in the early and mid-luteal phases in cows, when the CL is undergoing rapid growth and differentiation compared to the late luteal phase, when the CL is regressing (Grazul-Bilska et al.,

1996). The rate of gap junctional exchange between bovine or ovine luteal cells is increased by LH, indicating that the luteotrophic effects of LH extend to control of luteal interactions (Grazul-Bilska et al., 1996, Grazul-Bilska et al., 1997, Grazul-Bilska, 1996). In ewes and cows, $\text{PGF}_{2\alpha}$ affects basal and LH stimulated gap junctional intercellular communication so not only does it contribute to functional and structural luteolysis, but also modulates cell to cell communication among luteal cell types, reviewed by (Grazul-Bilska, et al., 1997). Current knowledge of the function of gap junctions in luteal function however, is limited.

1.7 Luteolysis

In a non-fertile cycle, 10-12 days after its formation, obligatory demise of the corpus luteum occurs in order that the negative effects of progesterone, oestrogen and inhibin on FSH secretion are removed. The removal of these inhibitors allows continued maturation of subordinate follicles culminating in follicular rupture in the subsequent cycle.

Corpus luteum regression or luteolysis is defined as lysis or structural demise of the corpus luteum. During normal luteolysis, two closely related events occur. Firstly, there is a loss of the capacity to synthesise and secrete progesterone, referred to as functional luteolysis followed by loss of the cells that comprise the corpus luteum, known as structural luteolysis (Niswender, et al., 2000). In rat and human, functional and structural luteolysis are separate events under separate endocrine control as corpora lutea of previous cycles are evident within the ovary. (Malven and Sawyer, 1966, Wang et al., 1993). The interval between loss of functional activity and the induction of structural involution is not known exactly in the human, but in rat, corpora lutea are known to persist for up to five oestrous cycles, reviewed by (Behrman, et al., 1993).

1.7.1 Functional luteolysis

This refers to suppression of progesterone synthesis and secretion, a process that precedes structural luteolysis. Several features characterise the decrease in progesterone secretion during functional luteolysis in the primate and other species. Functional luteolysis resembles the effects of LH withdrawal, such as those produced

by hypophysectomy or chronic treatment with gonadotrophin releasing hormone (GnRH) antagonist.

The mechanism of luteolysis remains unclear. LH is the principal luteotrophin and therefore has been implemented to play a role in luteolysis. However, it is not necessarily changes in LH secretion that appear to be responsible for luteal regression. The major change in LH secretion during the luteal phase is a progesterone-mediated reduction in the frequency of pulsatile LH secretion from the anterior pituitary. The number of pulses falls from 1 pulse per hour during the early luteal phase to as little as 1 pulse every 4-8 hours during the mid-late luteal phases (Ellinwood, et al., 1984, Filicori, et al., 1984). This cannot be the direct cause of luteal regression however, because administration of hourly LH pulses throughout the luteal phase does not prolong luteal lifespan (Knobil et al., 1980, Leyendecker et al., 1980) nor does reduction of the LH pulse frequency to 1 every 8 hours in the early luteal phase cause premature luteal regression (Hutchison et al., 1986). Complete withdrawal of LH however causes rapid cessation of luteal progesterone secretion and onset of premature menses, however, the regulatory action of LH on luteal progesterone secretion can be dissociated from its influence on luteal lifespan (Zeleznik, 1991).

The best explanation for induction of luteolysis in primates, is that the corpus luteum loses its sensitivity to LH with age, such that a progressively higher plasma level of LH is needed to sustain luteal function. It has been reported that the number of LH receptors decreases markedly in the late luteal phase of the cycle (Duncan et al., 1998, McNeilly et al., 1980). The corpus luteum therefore regresses due to its ever declining ability to perceive the low levels of plasma LH that prevail throughout the luteal phase (Zeleznik and Hillier, 1996). However, studies in non-primates have shown that the corpus luteum degenerates as a result of the action of various luteolytic factors including oestrogen, oxytocin, prostaglandins and various other polypeptides and growth factors (Braden et al., 1994). However, the luteolytic factor in primates remains unclear.

PGF_{2α}, produced by the uterus after priming with oestradiol and progesterone at the end of the cycle, serves as the main luteolytic factor in several mammals including sheep, pig, guinea pig and cow. Hysterectomy of heifers, ewes, pigs and

guinea pigs, results in delayed luteolysis (Baird, 1991). $\text{PGF}_{2\alpha}$ reaches the ovary by a local counter-current mechanism between the utero-ovarian vein and the ovarian artery. On reaching the ovary, it stimulates the secretion of oxytocin from the corpus luteum which acts on the uterus to provoke further release of oxytocin in a positive feedback fashion which ensures the demise of the corpus luteum (Flint and Sheldrick, 1982 nature ovarian secretion (Cao and Chan, 1993).

In women and monkeys however, the uterus does not appear to play a role in luteolysis as hysterectomy does not affect the luteal phase (Neill et al., 1969, Niswender, et al., 2000), nor do women with congenital absence of the uterus, have prolonged luteal lifespan (Baird, 1991). $\text{PGF}_{2\alpha}$ may still be a luteolytic factor in these species as a shortening of the luteal phase was observed in women given direct injections of $\text{PGF}_{2\alpha}$ into the CL (Korda et al., 1975). Locally produced $\text{PGF}_{2\alpha}$ acts via paracrine and/or autocrine mechanisms to induce luteolysis (Auletta and Flint, 1988, Bennegård et al., 1991). The corpus luteum is most sensitive to the luteolytic effects of $\text{PGF}_{2\alpha}$ in the late luteal phase (Messinis, 1997). $\text{PGF}_{2\alpha}$ decreases luteal progesterone synthesis in cows, ewes, sows, monkeys, humans and pseudopregnant/pregnant rats and rabbits, reviewed by (Niswender and Nett, 1994). There are several possible intracellular effects of $\text{PGF}_{2\alpha}$; 1) Down regulation of receptors for luteotrophic hormones, although this does not appear to be the case as it does reduce LH receptor levels in ewes but this occurs after progesterone levels have already declined (Diekman et al., 1978). It may however interfere with the ability of LH to activate PKA as adenylate cyclase activity decreases after $\text{PGF}_{2\alpha}$ administration (Niswender, et al., 2000). 2) Decreased cellular uptake of cholesterol, although this does not appear to be the case. 3) Decreased transport of cholesterol through the cell and/or across mitochondrial membranes. Treatment of ewes or cows with $\text{PGF}_{2\alpha}$ dramatically reduces concentrations of StAR mRNA, followed by a decline in StAR production, an essential component of cholesterol transport (Juengel et al., 1995, Pescador et al., 1996). 4) Decreased activity of the steroidogenic enzymes responsible for progesterone biosynthesis, P-450scc and 3β -HSD, but again this has been demonstrated not to be true, as doses of $\text{PGF}_{2\alpha}$ that reduce progesterone secretion were not seen to decrease levels of P-450scc mRNA or activity of the P450scc complex in several species, reviewed by (Niswender, et al., 2000).

Another potential luteolytic factor is oestrogen. Oestrogen is known to impair luteal function in the primate by suppression of LH secretion (Schoonmaker et al., 1982). On the nonhuman primate, oestrogen does not induce luteal regression when LH secretion is maintained by pulsatile GnRH administration (Hutchison et al., 1987). Thus oestrogen-induced luteal regression in the primate is thought to be due to suppression of LH secretion (Behrman, et al., 1993).

Evidence for many other putative intraovarian substances involved in luteolysis has been produced. Oxytocin is stimulated by $\text{PGF}_{2\alpha}$ and has been demonstrated to inhibit progesterone production in high doses in human luteal cell cultures (Tan et al., 1982). Progesterone itself may also play an autocrine role in luteolysis (Duffy, et al., 1994). Nitric oxide is a free radical gas and has been shown to participate in functional luteal regression by inhibition of steroid synthesis (Friden et al., 2000, Olson et al., 1996, Vega and Devoto, 1997). A characteristic feature of luteal regression is the infiltration of leucocytes that is documented in the human (Adams and Hertig, 1969) and other species including guinea pig (Paavola, 1979) and rabbit (Bagavandoss et al., 1990). These leucocytes are prominent generators of prostaglandins and cytokines as well as antioxidants and hydrogen peroxide. Thus infiltration and activation of leucocytes appears to be an essential process for the induction of luteolysis (Behrman, et al., 1993, Bukovsky et al., 1995). Cytokines that evoke antigonadotrophic or luteolytic actions include $\text{TNF}\alpha$, $\text{IFN}\gamma$ and IL's. $\text{IFN}\gamma$ activates macrophages and monocytes which in turn release $\text{TNF}\alpha$ and IL-1. These then evoke downstream activation of eosinophils and neutrophils to produce H_2O_2 , other radicals and cytotoxic peptides (Behrman, et al., 1993). These can cause matrix dissolution by induction of collagenase activity and disrupt the actin cytoskeleton. Control of collagenases is by tissue inhibitors of matrix metalloproteinases (TIMP's) released at the same time as collagenases to block activity. It is suggested that TIMP-1 may serve to prevent luteolysis (Behrman, et al., 1993, Duncan et al., 1996). In the rat a decrease in TIMP mRNA occurs at the time of functional luteolysis (Tanaka et al., 1992), and interestingly LH and hCG stimulate TIMP expression in follicular granulosa cells (Mann et al., 1991). In the rat, prolactin induces structural luteolysis (Malven and Sawyer, 1966).

In summary, immune cells and cytokines appear to play a role in luteolysis by regulating $\text{PGF}_{2\alpha}$ synthesis, steroidogenesis and phagocytosis.

1.7.2 Structural luteolysis

Structural luteolysis is defined as involution of the corpus luteum and is a distinct event occurring after functional regression. Several different patterns of structural luteolysis exist in the human (Morales, 2000). Luteal regression is generally associated with a dramatic decrease in luteal weight and cell size as well as with an increase in the number of dying cells (Plendl, 2000). The diameter of the corpus luteum decreases and connective tissue or stromal elements greatly increase. At the end of the luteal phase, in the human corpus luteum, granulosa cells are seen to have large vacuoles occupying almost all of the cytoplasm. Small densely stained pyknotic nuclei are evident and an occasional nucleus has degenerated into a dense irregular mass. This degeneration is even more advanced in the theca (Corner, 1956). Luteal cell degeneration is accompanied by a massive regression of blood vessels. Some blood vessels are retained and are thought to play an important role in resorption of the luteal tissue (Plendl, 2000). It has been reported in bovine that reduced ovarian blood flow plays a critical role in luteal regression and that endothelial cells are important in the luteolytic process (Girsh et al., 1995). In a study conducted on sheep, it was demonstrated that vascular changes involved both luminal contents and capillary walls. Endothelial cell degeneration was observed leading to ultimate degradation of vessels (O'Shea et al., 1977). $\text{PGF}_{2\alpha}$ has been implicated in this reduction in blood flow to the corpus luteum and may therefore cause luteolysis by depriving the gland of nutrients, steroidogenic substrates and luteotrophic support in bovine. It is thought in this species, that $\text{PGF}_{2\alpha}$ exerts its effects at endothelial cells as these possess $\text{PGF}_{2\alpha}$ receptors by causing endothelial cell degradation (Mamluk et al., 1998). Endothelial cell deletion during luteolysis has been widely reported in many species (Azmi and O'Shea, 1984, Gaytán et al., 1999, Hossain and O'Shea, 1981, O'Shea, et al., 1977, Zheng, et al., 1994) resulting in marked reduction in capillary density thereby reducing blood flow to the luteal parenchyma.. Recently, endothelin-1 has been implicated as a putative mediator of the effects of $\text{PGF}_{2\alpha}$ on luteal blood flow in bovine. $\text{PGF}_{2\alpha}$ stimulates endothelial

cells to produce endothelin-1 *in vitro* (Girsh et al., 1996) and *in vivo* (Ohtani et al., 1998). Endothelin-1 is a potent vasoconstrictor and inhibits the steroidogenic activity of luteal cells (Girsh, et al., 1996). It may also reduce blood flow during early luteolysis by causing arteriole constriction in this species (Ohtani, et al., 1998). The resultant hypoxic environment may cause additional endothelin-1 release thus generating a positive feedback response (Rakugi et al., 1990). Thus endothelial cells play an essential role in luteal steroidogenic activity by being involved in both luteotrophic and luteolytic processes (Meidan and Girsh, 1997).

PGF_{2α} elicits a series of morphological changes in rat, sheep, cow and marmoset. The proportion and size of luteal cells in the CL decreases within 24 hours of treatment with PGF_{2α} in ewes (Braden et al., 1988). However, these morphological changes in steroidogenic cells do not become evident until after the steroidogenic capacity of the cells is markedly reduced. PGF_{2α} acts by binding to specific receptors localised to large steroidogenic cells (Fitz et al., 1982). Upon binding to its receptor, PGF_{2α} induces activation of membrane bound phospholipase C which ultimately leads to increased cytosolic concentrations of IP₃. This in turn causes release of free calcium ions from the smooth endoplasmic reticulum into the cytoplasm and stimulation of protein kinase C. Activation of PKC is believed to mediate many of the antisteroidogenic actions of PGF_{2α} in large luteal cells and induces expression and activation of many proteins involved in apoptosis, reviewed by (Niswender, et al., 2000).

Apoptosis is thought to play a major role in luteolysis in many species, (Bacci et al., 1996, Fraser et al., 1995, McCormack et al., 1998, Shikone et al., 1996, Young et al., 1997, Young et al., 1998, Yuan and Giudice, 1997), although the process of structural regression in the primate is poorly understood and thought to consist of multiple cell death pathways (Fraser et al., 1999). Endothelial cells have been documented to be the first cell type to be eliminated during luteolysis with apoptosis taking place 24 to 48 hours after administration of cloprostenol in guinea pig (Azmi and O'Shea, 1984). Apoptosis is an active, energy dependent process by which nonessential populations of cells delete themselves from a tissue. Several biochemical and morphological changes are characteristic of apoptotic cells including nuclear fragmentation, appearance of membrane-bound vesicles of

cytoplasmic contents, ladderlike DNA fragmentation and changes in gene expression (Niswender, et al., 2000). The involution of many endocrine glands after removal of a trophic hormone occurs by apoptotic mechanisms. Human granulosa cells deprived of FSH undergo apoptosis during follicular atresia and epithelial cells of the human endometrium experience apoptosis when progesterone and oestrogen support declines (Hopwood and Levison, 1976)

The first morphological evidence that a cell is apoptotic is the appearance of nuclear fragments containing degenerate chromatin, cell shrinkage, and appearance of membrane-bound cytosolic fragments. These cell fragments or apoptotic bodies are targets for the phagocytic cells of the immune system. Macrophages augment the apoptotic process in populations of luteal cells by phagocytosing membrane-enclosed fragments of those cells (Gemmell et al., 1976).

1.8 Luteal rescue

If pregnancy is to become successfully established, the maintenance of the corpus luteum and continued secretion of progesterone is essential. Although mechanisms by which progesterone secretion is maintained vary between species, there exist a few general types of signal. Timing of this signal is crucial. In general, the conceptus secretes factors that either prevent the secretion or luteolytic actions of $\text{PGF}_{2\alpha}$ or that are directly luteotrophic. Around the start of implantation, about 7 days after ovulation in the human (Baird, 1991), and 11 days in the marmoset (Enders, 1993), the embryo secretes chorionic gonadotrophin (CG) which stimulates increased secretion of progesterone by the corpus luteum. The amounts of hCG secreted by the trophoblastic cells in the implanting blastocyst increase exponentially, reaching maximal levels in the first trimester. Structurally and functionally, the hCG molecule is similar to LH and both interact with the same cell-surface receptor. hCG very rapidly reaches levels that might be expected to down regulate LH receptor synthesis. However, LH receptors are maintained at luteal phase levels in the corpus luteum of early pregnancy in both the human and nonhuman primate unlike in the rat, although >90% of them are occupied by hCG rather than LH (Duncan et al., 1996). hCG increases luteal function when administered in the luteal phase and is used therapeutically for such purposes. hCG is

thought to not only act as a surrogate for LH in stimulating progesterone secretion, but also has a more generalised luteotrophic role by inhibiting processes involved in luteal regression (Baird, 1991). The fact that hCG and LH are so similar begs the question why can hCG unlike LH prevent luteal regression? It may be that hCG provides a more intense and sustained gonadotrophic stimulus to the ageing corpus luteum overriding its diminished responsiveness to LH. Also, the substantially longer half life of hCG compared to LH would serve to further enhance the strength of hCG as a luteotrophic signal (Monfort et al., 1989). In the rhesus monkey, hCG has been shown to completely abolish the luteolytic effect of $\text{PGF}_{2\alpha}$ (Auletta and Kelm, 1994), and cell degradation enzymes which are normally upregulated in luteolysis are down regulated, for example MMP's (Duncan et al., 1998). hCG has also been demonstrated to prevent the normal influx of macrophages into the CL in the late luteal phase (Duncan et al., 1998) and to prevent apoptotic luteal cell death (Shikone, et al., 1996).

It has been suggested that there may be a point of no return at the mid-luteal phase, beyond which, the corpus luteum can no longer be maintained by hCG (Baird et al., 1991). The cellular mechanisms responsible for the loss of responsiveness to LH and hCG that occur as the CL ages remain largely unknown. It is thought that this is by an impairment of the gonadotrophin receptor adenylyl cyclase system in luteal cells and a reduction in enzyme activity necessary for progesterone synthesis in rhesus monkeys (Stouffer et al., 1978).

In ruminants, trophoblast proteins are produced that belong to the $\text{IFN}\alpha$ family of proteins. These are thought to 'rescue' the corpus luteum in these species by blocking synthesis of $\text{PGF}_{2\alpha}$ (Imakawa et al., 1987). In rodents, stimulation of the cervix during copulation causes activation of a neuroendocrine reflex arc that results in a dramatic release of prolactin, FSH and LH on the morning of oestrous. The biphasic release of prolactin from the anterior pituitary continues for 8-10 days and extends the function of the corpus luteum for 10-12 days. This occurs in a pseudopregnant situation. In a true pregnancy where an embryo is present, biphasic release of hypophysial prolactin is also extended for 8-10 days (Niswender, et al., 2000).

Progesterone secretion by the corpus luteum is maintained in early pregnancy until such time as the role is assumed by the placenta. The luteo-placental shift occurs near the end of the third week of gestation in rhesus monkeys (Goodman and Hodgen, 1979) at five to six weeks in women (Stouffer, 1996), and around six weeks in the marmoset (Hodges et al., 1983).

1.9 The endometrium

For the purposes of this literature review, the following sections focus on the human and primate with little referral to the rodent or domestic species because of widespread species differences.

1.9.1 The role of steroids throughout the menstrual cycle

The uterus undergoes prominent steroid-dependent cyclic changes in structure and function. During each cycle, the uterus first prepares to receive and transport the spermatozoa from the cervix to the oviduct, and subsequently, it prepares to receive the conceptus from the oviduct and nourish it. The uterus consists of an outer peritoneal and serosal investiture, overlaying a thick myometrium of smooth muscle. Internally, the endometrium consists of a stromal matrix over which a simple, low columnar epithelium with glandular extensions penetrating into the stroma is found (Johnson and Everitt, 1995).

The dependence of the uterus on ovarian steroids is paramount to its function, and the cyclical regulation of the two are intimately regulated. The ovarian steroid profile throughout the human menstrual cycle is illustrated in **Figure 1.7** (Johnson and Everitt, 1995).

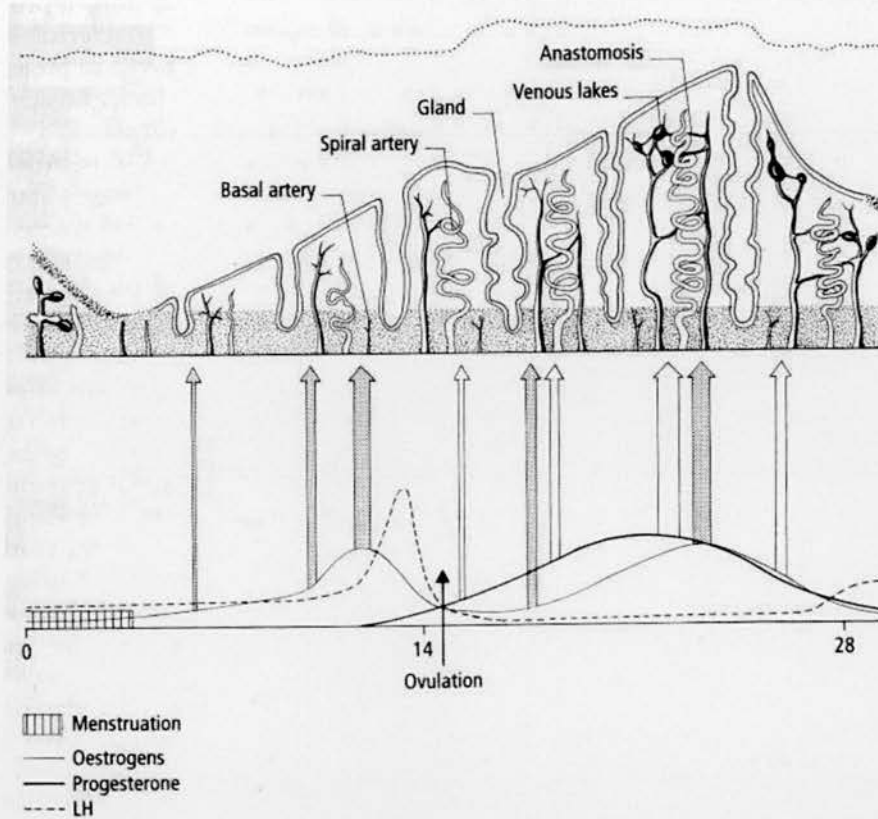


Figure 1.7 Changes in human endometrium during the menstrual cycle. Underlying steroid changes are indicated below and basal body temperature is indicated above. Thickness of arrows (oestrogens:stippled; progestagens:white) indicate strength of action (Johnson and Everitt, 1995).

After ovariectomy, and subsequent progesterone withdrawal, the uterus hypertrophies and its blood supply is reduced. Administration of oestrogen reverses this effect, with massive increases in RNA and protein synthesis, cellular division and growth. In human and non-human primates, the period during which oestrogen rises rapidly coincides with the latter half of the follicular phase of the menstrual cycle. In non-primates oestrogen rises are associated with the entire follicular phase. During this phase a similar uterotrophic effect of oestrogen is observed. Oestrogen causes the myometrium to increase its contractility and its excitability, accompanying increased spontaneous activity.

In the endometrium, stromal thickening occurs partly in response to stromal cell proliferation, hence the follicular phase, a term used in conjunction with the ovary, is called the proliferative phase when referring to the uterine cycle, and partly due to oedema.

Steroids interact with their target organs via specific nuclear receptors. The expression of endometrial sex steroid receptors, progesterone, oestrogen and androgen, varies both spatially and temporally across the menstrual cycle (Garcia et al., 1988, Lessey et al., 1988, Slayden et al., 2001, Snijders et al., 1992). Expression of both the oestrogen and progesterone receptors (ER and PR, respectively) are under the control of oestrogen and progesterone. Both are upregulated during the follicular phase by oestrogen and down regulated in the luteal phase by progesterone. The presence of a progesterone receptor is evidence that a functional oestrogen mediated pathway is in place. Administration of RU486, an anti-gestogen, in the early luteal phase blocks the progesterone-induced down regulation of both ER and PR in non-pregnant human endometrium (Cameron et al., 1996, Maentausta et al., 1993).

There are two distinct types of the human progesterone receptor (Clarke and Sutherland, 1990), that arise from a single gene and function as transcriptional regulators of progesterone responsive genes. The PRA is the shorter isoform, missing 164 amino acids at the N-terminus of the B subtype (Tung et al., 1993). PR expression declines significantly in the secretory phase compared to the proliferative phase, in the glands of the functionalis, (the region shed at menstruation). PR expression remains in the stroma, at particularly high levels cells in close proximity to the uterine vasculature. The basalis layer is differently regulated in that the glands of this layer continue to express PR throughout the cycle. These differences between the superficial and basal layers of the endometrium are likely to contribute functional importance when only the upper zones are shed at menstruation (Critchley et al., 2001).

The two forms of oestrogen receptor $ER\alpha$ and $ER\beta$ have also been described (Green et al., 1986, Kuiper et al., 1996). Both genes are encoded by eight exons with highest homology between the two present in the DNA and ligand binding domains (Enmark et al., 1997). The function of $ER\beta$ in the uterus is unknown. $ER\alpha$ expression increases in both glandular and stromal cells in the functionalis during the proliferative phase, and declines in the secretory phase as a consequence of increasing levels of progesterone which suppress it. In the basalis, $ER\alpha$ expression like PR remains unchanged throughout the cycle (Garcia, et al., 1988). In human and non-human primate, $ER\beta$ expression like $ER\alpha$, is expressed in the nuclei of

glandular epithelial and stromal cells, declining in the secretory phase in the functionalis. However, ER β , unlike ER α has been detected in endothelial cells, indicating that oestrogen may act directly on endometrial blood vessels and may thus be involved in angiogenesis and vascular permeability changes throughout the cycle (Critchley et al., 2001). Progesterone receptor is absent from the vascular endothelium (Critchley, et al., 2001) of the spiral arteries, suggesting that the effects of progesterone withdrawal on these vessels, which play a key role in menstrual induction, is directly mediated by the PR-positive peri-vascular stromal cells.

1.9.2 Menstruation

The endometria of humans and menstruating primates are subcompartmentalised (Rune et al., 1992). This compartmentalisation was described as follows: zone 1, luminal epithelium; zone 2, uppermost gland segments; zone 3, middle gland segments; and zone 4, basal gland segments. This definition was thought to provide a key to deciphering the mechanisms that underlie repetitive cyclicity (Rune, et al., 1992). The uterine experiments conducted for this thesis were performed using marmosets. Marmosets do not menstruate, and thus, according to (Padykula et al., 1984), are not expected to show endometrial zonation or to be suitable as primate models for the study of uterine cyclicity. This group were able to distinguish 4 zones in the rhesus monkey. In marmoset only 3 zones are detectable, a basal area, bordering on the myometrium, an adluminal region and the luminal zone based on morphological parameters. In a cross section through the endometrium, the glands of the basal region appear long and thin. Glands in the adluminal and luminal zone appear more rounded. The stroma of these zones appears more compact compared to the basal area. It would seem therefore that the subdivision of the basal area into 2 zones, seen in the human and rhesus endometrium seems to be absent in the marmoset (Rune, et al., 1992). They concluded that the existence of zonation per se in the marmoset endometrium is an integral feature that is independent of hormonal influences, whereas the differential behaviours of these zones throughout the cycle is due to varying steroid levels. Consequently, zonation of the endometrium is not a unique feature of menstruating species and thus not directly related to menstruation. Therefore the marmoset has the same zonal compartmentalisation of

the endometrium as the human and so despite it being a non-menstruating species is still an appropriate model to study the endometrium.

Menstruation involves the shedding of the superficial two thirds of the endometrium (the superficialis). Endometrial repair usually begins by the second day after bleeding has started, but proliferation begins around the fifth day of the cycle under oestrogen influence from the developing follicles (Ludwig and Metzger, 1976).

Menstruation is induced by the fall in concentration of progesterone that follows luteal regression. The molecular mechanisms by which steroids induce these changes, involve interactions between the endocrine and immune systems (Critchley, et al., 2001). In addition to the epithelial, stromal and endothelial cells that participate in cyclic proliferation, differentiation and desquamation, there is a dynamic population of leucocytes within the endometrium that plays an important role in the menstrual breakdown process. Details of this leucocyte population in terms of cell types and cell numbers will not be provided for the purposes of this thesis.

There exists a complex dialogue between the sex steroid hormones and the leukocytic cells of the immune system, locally produced cytokines and growth factors. These leucocytes are likely to be controlled directly by paracrine mediators and not by sex steroids directly as nuclear steroid receptors are not detectable in any of the macrophages, neutrophils found within the uterus (King, 2000, Poropatich et al., 1987). However, ER β expression has been reported in uterine natural killer (NK) cells (Henderson et al., 2003). Progesterone withdrawal causes a unique cascade of events that include expression of uterine cytokines that attract leucocytes into the uterine environment and a complex set of interactions between these cells and their products that ultimately result in the tissue sloughing and bleeding, characteristic of the menstrual process. This leucocytic invasion and production of inflammatory cytokines has led to menstruation being described as an inflammatory event (Finn, 1986, Kelly, 1994).

Transplanting explants of endometrium to the anterior chamber of the eye of the rhesus macaque causes vasoconstriction of spiral arteries induced by progesterone withdrawal (Markee, 1940). This constriction was associated with the

regression of stromal tissue and onset of degradation of the extracellular matrix. This vasoconstriction causes anoxia in the zones closest to the uterine lumen. MMP's are secreted by stromal cells in the upper zones after 48h following progesterone withdrawal (Rudolph-Owen et al., 1998). There has been much speculation as to what these endometrial vasoconstrictors are. As well as prostaglandins, there are other locally produced agents that have a vasoconstrictor action in the endometrium including endothelins (O'Reilly et al., 1992) and angiotensin II (Hunt et al., 1995).

Another unexpected observation was that KDR, one of the receptors for VEGF was upregulated in the stromal cells of the superficial endometrial zones during the pre-menstrual phase of both human and macaque endometrium (Nayak et al., 2000). VEGF itself can be upregulated by hypoxia as it has a hypoxia inducible factor in its promoter (Goldberg and Schneider, 1994). The KDR promoter however, lacks this response element and so it is therefore unlikely that hypoxia will regulate expression of this receptor (Gerber et al., 1997). It may be possible that hypoxia is upregulating VEGF and that this is in turn upregulating KDR expression (Critchley, et al., 2001).

In summary, demise of the corpus luteum causes a decline in progesterone. As a consequence of progesterone withdrawal, in cells that contain progesterone receptor, inflammatory mediators and locally produced prostaglandins are elevated. Spiral arteries are constricted, causing hypoxia in the uppermost endometrial zones. This in turn induces VEGF which may induce its own receptor KDR within stromal cells of this compartment. VEGF interaction with its receptor can augment MMP expression by stromal cells, while the inflammatory mediators induce a large influx of leucocytes. Therefore progesterone withdrawal would appear to firstly effect cells with progesterone receptors. Early events in the menstrual process are vasoconstriction and cytokine upregulation. Activation of lytic events occurs later, and involves cells that may lack progesterone receptors eg uterine leucocytes and epithelial cells (Kelly et al., 2001). **Figure 1.8** illustrates the current understanding of menstrual induction (Critchley, et al., 2001).

Menstrual Induction Hypothesis

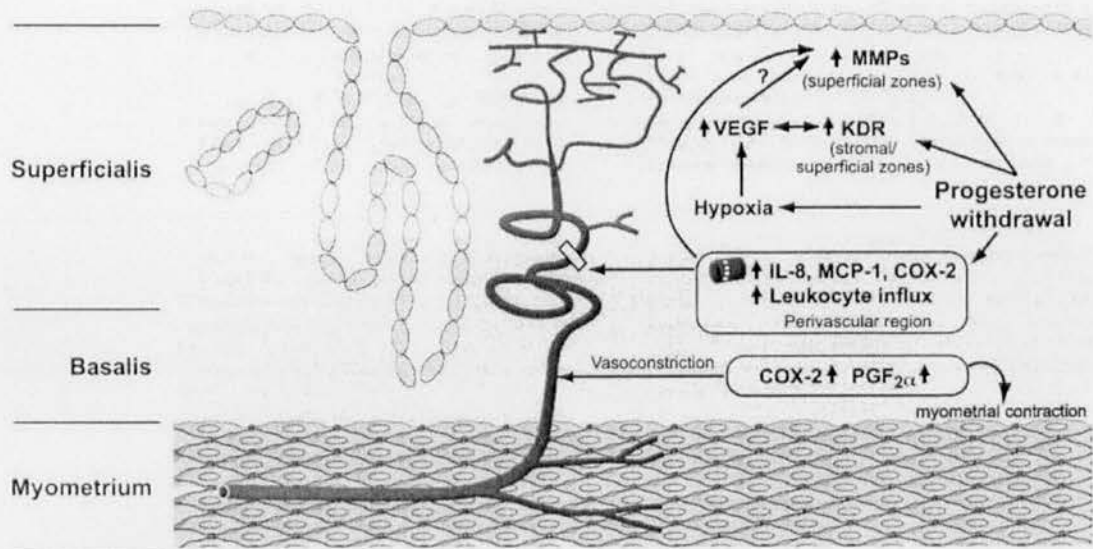


Figure 1.8 Menstrual Induction hypothesis (Critchley, et al., 2001)

1.10 Implantation

1.10.1 Adaptations of the uterus for implantation

The uterus, at the appropriate phase of the menstrual cycle, provides a hospitable and privileged environment for implantation of the blastocyst. This period of time is known as the “receptive window” and appears to be primarily regulated by ovarian steroids. Embryo implantation is the natural culmination of this period, and successful nidation requires the precise preparation of both the blastocyst and the endometrium. Enders and Schläpfe (Enders and Schläpfe, 1969) defined the morphological changes associated with implantation into three distinct stages: 1) apposition of the trophoblast and epithelium, 2) adhesion, involving the formation of junctional complexes between the two cell types, and 3) Epithelial penetration, which includes the spreading of trophoblast within the endometrium, regression of the uterine glands, and interruption of the integrity of the maternal vascular system.

1.10.1.1 Morphological adaptations

Detailed ultrastructural investigations into the morphology of the endometrium in response to pregnancy have been conducted (Cornillie et al., 1985, Wynn, 1974). Comparative morphological studies in the rhesus macaque and baboon (Fazleabas et al., 1995, Kraemer et al., 1977, MacLennan and Wynn, 1971) demonstrated that the non-human primate endometrium undergoes cyclic changes similar to that described for humans, with one notable exception. In humans, differentiation of periarteriolar stromal cells, referred to as the predecidual response, (Bell, 1983) begins during the final week of the menstrual cycle and spreads through the upper two thirds of the endometrium (Wynn, 1974). In contrast, stromal changes in the macaque (Brenner and Slayden, 1994) and baboon (Kraemer, et al., 1977) are minimal, and only those stromal cells that surround the spiral arteries enlarge substantially. Thus, it seems that only the human and chimpanzee show “predecidual” modifications of stromal fibroblasts in the absence of implantation and/or trauma (Enders, 1991, Graham, 1973).

1.10.1.2 Decidualisation

As discussed previously, the primate endometrium is a target tissue for oestrogen and progesterone. In response to these steroids and in the presence of a trophoblast, the endometrium is modified into the decidua of pregnancy. In a fertile cycle, blastocyst attachment to surface epithelium and its subsequent implantation is associated with local remodelling of the maternal stroma, smooth muscle and endothelium of the blood vessels by the trophoblast (Aplin, 1991). In addition, the glandular epithelium becomes highly secretory (Bell, 1988, Fazleabas et al., 1993), and the stromal cells differentiate into decidual cells, the major cell type of the gestational endometrium (Bell, 1983).

Ultrastructural studies on the human decidual cell indicate that they possess all the characteristics of a secretory cell (Wynn, 1974). While gap junctions are present on these cells, they do not join neighbouring cells, (Lawn et al., 1971) which may be advantageous for trophoblast invasion. It is thought the decidua has multiple functions during pregnancy, but its true function remains to be elucidated. It has been

suggested that the decidua may nourish the developing embryo, protect maternal tissue from excessive trophoblast invasion and/or protect the embryo against immunological rejections *in utero* (Beer and Boillingham, 1974).

Stromal cells of the rhesus monkey and baboon undergo cellular swelling during implantation (Ramsey et al., 1976), but the process is far more rapid in the human, where decidualisation is evident about 5 days following blastocyst implantation (Hertig et al., 1956). Also in non-human primates, the cells do not enlarge to the same extent as in the human, nor can decidualisation be induced by hormonal treatment, without the presence of a blastocyst (Fazleabas et al., 1993, Tarantino et al., 1992). In human, however, stromal cells obtained from non pregnant women will decidualise in the presence of steroids, relaxin and growth factors (Irwin et al., 1989, Irwin et al., 1991, Tseng et al., 1992). One characteristic of decidual cells is their ability to synthesise and secrete IGFBP-1, and while this can be induced *in vitro*, *in vivo* studies in the human suggest that the presence of the conceptus does enhance expression of IGFBP-1 and TGF- β in the decidualised endometrium (Selick et al., 1994). Therefore it would appear that despite the ability of human stromal cells to “predecidualise” *in vivo* and the fact that they can be induced to undergo decidualisation *in vivo*, decidual transformation in the human and non-human primate is far more efficiently regulated during pregnancy.

Despite the divergence in the timing of the onset of decidualisation between the human and non-human primate, in both, the starting cell, the stromal fibroblast, and the transformed secretory decidual cell are biochemically and morphologically similar (Fazleabas, et al., 1993). The regulation of this transformation could reflect the differences in the implantation process between these species (Enders, 1993, Ramsey, et al., 1976).

In the human, as soon as the blastocyst adheres to and begins to penetrate the uterine epithelium, the endometrium by definition is termed the decidua. The area underlying the blastocyst is called the decidua basalis, the endometrium overlying the blastocyst the decidua capsularis, and the rest of the endometrium becomes the decidua vera or the decidua parietalis.



1.10.1.3 Immune responses to implantation

It is not just stromal fibroblasts that respond to an implanting blastocyst by undergoing a decidual reaction, uterine epithelial cells, vascular endothelial cells and endometrial granular cells or large granular lymphocytes also respond.

The presence of a granulated mononuclear cells in the endometrium of the human and of the rhesus monkey was noted and associated originally with the premenstrual portion of the cycle and early pregnancy, reviewed by (Enders, 1991).

It is now clear that these cells, which are the predominant mononuclear cells of the basal plate in early pregnancy, belong to a subset of T-lymphocytes that constitute only a very small percentage of the peripheral blood large granular lymphocytes (King and Loke, 1990). These express markers of NK cells, and are generally CD3 and CD16 negative, and CD56 positive (King et al., 1989). These NK cells appear rounded with a kidney shaped nucleus, characterised by a cluster of large dense granules. The granules surround a cell centre composed of compact golgi apparatus and a pair of centrioles associated with the indented region of the nucleus (Cardell et al., 1969).

NK cells are in close association with the trophoblast cells which invade into the uterus and are often associated with decidual cells that surround small vessels (Enders, 1991). The function of these cells remains unclear. Many functions have been proposed, but there is no evidence to support any single proposal nor indeed whether these cells have single or multiple functions. They do appear to have a cytotoxic capability (Stewart, 1998). It has been postulated that these cells are involved in removal of labyrinthine cytotrophoblast which might otherwise lead to placental dysfunction in mouse (Stewart and Mukhtar, 1988).

In rodents, these NK cells are termed granulated metrial gland (GMG) cells and are by far the most remarkable population of cells in the decidualised uterine tissue. The observations that GMG cells are present in non-pregnant decidua where no foetus is present suggests that the origin of these cells is maternal (Zheng et al., 1991). They vary in number and maturation during pregnancy, suggesting that hormones, particularly progesterone and oestrogen, play a crucial role in controlling their differentiation (Sharma et al., 1986, Stewart, 1987). However, non-hormonal factors for example cytokines may also be important. Several cytokines, including

interleukin (IL)-2, IL-6, IL-7, IL-12 and IL-15 have been shown to be capable of inducing the differentiation and activation of NK cells, reviewed by (Liu and Young, 2001).

Human uterine NK cells are smaller than GMG cells of the rodent, but the proliferation and differentiation of these cells parallel those of the rodent. In human, these cells become prominent in the late secretory phase (Liu and Young, 2001), a status appropriate for implantation, provided conception occurs (King, et al., 1989). In the absence of conception, NK cells undergo apoptosis a few days prior to the onset of menstruation (King, et al., 1989). In a pregnant cycle, these cells persist and then increase remarkably in number in the decidua as gestation advances in the first trimester. At this stage uterine NK cells may account for up to 70% of the decidual leukocytes localised most densely at the implantation site where foetal trophoblasts infiltrate into maternal tissues (King et al., 1996, Starkey et al., 1988). These cells become less conspicuous by 20 weeks of pregnancy and virtually vanish by parturition. Unlike in the rodent, these cells in the human do not accumulate in specific regions of the pregnant uterus, rather they distribute throughout the decidual tissue but are more noticeable around the spiral arteries and near degenerative endometrial glands (Bulmer et al., 1983).

The exact role of uterine NK cells in pregnancy remains unclear. They may have a role in the implantation process and the subsequent orderly growth and development of the placenta (King et al., 1998). Additionally, they may modulate the growth, differentiation, breakdown and regeneration of the cycling uterine mucosa in a manner analogous to that proposed for regulation of the gut epithelium by resident lymphocytes (Boismenu and Havran, 1994). However, the presence of granulated lymphocytes at the maternal-foetal interface appears to be a common feature of many species during pregnancy (King, et al., 1998), suggesting that placental trophoblast cells are potential targets for uterine NK cells (King, et al., 1998). It has recently also been proposed that NK cells may play a role in endometrial angiogenesis after the finding that they produce VEGF-C and PlGF (Li et al., 2001).

1.10.1.4 Vascular endothelial cells

One of the more striking transformations that occurs during implantation in a number of non-human primates is the modification of the superficial capillaries and venules of the endometrium. The endothelial cells of these vessels become cuboidal, often with distinctive apically-situated nuclei. This transformation results in a series of anastomotic channels lined by tall endothelial cells (Enders, 1991). Endothelial cell hypertrophy is localised in vessels of the decidua basalis, and clearly involves all of the superficial vessels except the arterioles.

Arterioles directly beneath the placenta appear to be blocked by endovascular cells, the anastomotic complex of vessels lined by tall endothelial cells is probably the major pathway of blood both entering and leaving the intervillous spaces in the early stages of implantation and formation of the placenta (Enders, 1991).

1.11 Morphology of implantation in primates

Detailed descriptions of the morphological changes associated with implantation in human and non-human primates are available (Enders, 1993). While the same maternal and foetal tissues seem to be involved, the structure of the implantation site differs between species. A summary taken from (Enders, 1993) is given below in **Table.1**.

Day	Human	Baboon	Macaque	Marmoset
4	Morula to blastocyst			
5	Expanding blastocyst	Morula to blastocyst	Morula to blastocyst	
6	Expanded blastocyst	Blastocyst maturing	Blastocyst maturing	
7	Hatching blastocyst	Hatching blastocyst	Expanding blastocyst	
8	Attachment	Attachment	Hatching blastocyst	
9	Epithelial penetration	Penetration to basal lamina	Attachment, penetration to basal lamina	
10	Expanding site, amnion distinct	Penetration of maternal vessels	Trophoblastic plate	
11	Recruitment of parietal trophoblast	Trophoblast cleft and lacuna formation	Penetration of maternal vessels, secondary implantation	Attachment to uterus
12	Penetration of vessels, cleft formation	Expansion of lacunae	Expansion of lacunae	
13	Primary villi	Expansion of lacunae	Primary villi, primitive streak	Penetration to basal lamina
14	Primitive streak	Primary villi		
15	Secondary villi	Secondary villi	Secondary villi	
16	Notocordal process			Trophoblastic plate
18	Primitive pit			
19	Somites			Fusion of parietal trophoblast
21		Tertiary villi	Tertiary villi	
22	Heartbeat, tertiary villi			
45			Spread of trophoblast shell	Maternal vessels surround Tertiary villi
60				Penetration of maternal vessels

Table 1.1 Relative time sequence of some developmental sites in the peri-implantation period (Enders, 1993).

Apposition and adhesion

Implantation is not a single event, rather a series of events in the continued development and association of the blastocyst and the endometrium. Even if implantation is defined as starting when the blastocyst assumes a relatively fixed position within the uterus and a more intimate contact with the endometrium established, there is still a period of time between apposition of an adhesive blastocyst to the uterus and the time at which adhesion of the trophoblast to the uterine surface actually occurs. The lumen of the simplex uterus of primates is virtually closed at the time of implantation. This is thought to allow apposition of the endometrial epithelium to trophoblast of the blastocyst and is thought to be a result of the shape of the uterus, muscular activity, paucity of luminal fluid and endometrial oedema (Enders, 1993). Initial adhesion of the blastocyst to the uterine luminal epithelium occurs in the peri-inner cell mass region. In human, baboon, macaque and marmoset, the syncytial trophoblast developing in the peri-inner cell mass region invades the uterine epithelium (Enders et al., 1983). It would appear that the

adhesion stage prior to epithelial penetration is a short stage in primates. However, very little information regarding this stage of implantation is available.

Invasion of endometrial Epithelium

More information is available concerning invasion of the uterine epithelium by trophoblast. The initial invasion has been suggested to occur in the macaque and marmoset by intrusion of processes of syncytial trophoblast between epithelial cells (Enders, et al., 1983, Smith et al., 1987). This is followed by coalescence of the area of penetration to form a trophoblastic plate, which usually spreads in the plane of the epithelium (Enders, 1993). Epithelial invasion occurs within a day of adhesion in the baboon (Enders and King, 1991), 1-2 days in the macaque and a similar 1-2 days in the marmoset (Hearn et al., 1991). No information is available on the human yet, suggesting that epithelial penetration takes place over a very short space of time.

In the marmoset, the trophoblast intervenes between epithelial cells without apparent cellular damage. Syncytium is usually described as cytolytic implying its ability to erode the uterine epithelium and thereby penetrate to underlying vessels (Moore et al., 1985).

Trophoblast-Epithelial cell fusion

During the trophoblastic plate stage in the macaque and baboon, some fusion of syncytial trophoblast with maternal epithelial cells takes place. At the margin of the implantation site, cytoplasm that is confluent with syncytial trophoblast has apical vesicles similar to those in maternal epithelial cells. Second, in macaques, some masses of syncytium contain nuclei that have intranuclear channels similar to those seen in epithelial plaque cells. Third, near the basal lamina between nuclei, whorls and pockets of cell membrane suggest fusion of lateral cell membranes between cells (Enders, 1993).

Between days 13 and 15, the marmoset implantation site expands peripherally with areas of syncytial penetration between uterine luminal epithelial cells. Such penetrating masses bridge openings of endometrial glands, share junctional complexes with the uterine epithelial cells between which they are infiltrating and subsequently reach the basal lamina of the uterine luminal epithelium (Enders, 1993).

Stromal invasion and invasion of maternal vessels

With expansion of the trophoblastic plate, the epithelial basal lamina is penetrated as is the underlying stroma, followed by penetration of subepithelial maternal vessels. During these processes, variation between species increases. Trophoblast penetrates maternal blood vessels most readily in the baboon (Enders and King, 1991). Subepithelial stromal cells are dilated, and ectoplasmic processes from the syncytial trophoblast penetrate the vessels almost as soon as they have passed through the epithelial basal lamina (Enders, 1993). At the other extreme is the marmoset (Smith, et al., 1987), in which maternal vessels are slowly surrounded by trophoblast and penetration of trophoblast into vessels does not occur until 45 days to 60 days (Smith, et al., 1987) of gestation. In the macaque, penetration of vessels occurs almost as soon as epithelial basal lamina penetration, but a day or so later than in the baboon (Enders, 1993). It appears therefore that the trophoblast penetrates deeper into the stroma before breaching maternal vessels in marmoset (Enders and Lopata, 1999).

Trophoblast differentiation

While the trophoblast is tapping maternal vessels, profound changes are occurring in syncytial trophoblast in the macaque, baboon and human. The majority of this trophoblast converts from a multinucleate invasive tissue to a unilaminar microvillous absorptive tissue (Enders, 1989). This conversion occurs towards the end of the initial invasion of endometrium and during the early stages of formation of blood filled spaces within the trophoblast (Enders, 1993). The formation of an extensive series of clefts facilitates the subsequent expansion of these spaces into blood-filled intrasyncytial lacunae. In human, this conversion corresponds to the time of change in the rate of hCG production (Lenton and Woodward, 1988).

Expansion of the Implantation site

Once maternal vessels are tapped, there again exists variation between species (Enders, 1993). In the baboon, clefts in syncytial trophoblast develop rapidly into lacunae, and rapid growth of trophoblast lifts the placenta off the surface of the

endometrium (Tarara et al., 1987). During this time, most of the growth is within the placenta; little is within the endometrium (Enders, 1993).

In human, all of the trophoblast, including the abembryonic trophoblast, is converted into syncytial and cytotrophoblast with clefts and lacunae (Enders, 1989). This expansion of the lacunar area is largely contained within the endometrium (Enders, 1993).

In the macaque, abembryonic trophoblast forms a secondary implantation site on the opposite surface of the endometrium. As such, both placental discs lift from the surface of the endometrium. In the marmoset, the abembryonic trophoblast of twins and triplets fuses so that there comes to be a shared exocoelom (Smith, et al., 1987).

In summary, it would appear that the human previllous stage is most deeply embedded in the endometrium because the trophoblast grows within the stroma for a longer period of time before tapping vessels and because all of the trophoblast becomes involved (Enders, 1993).

The trophoblastic plate of the macaque and the baboon is a mixture of syncytium and cytotrophoblast. Vessels are initially penetrated by syncytial trophoblast, but as lacunae form, maternal blood comes into contact with both syncytium and cytotrophoblast. At this point, the border of trophoblast on the endometrium becomes smoother, and the cytotrophoblast cells become segregated largely to the embryonic side of the trophoblastic plate (Enders, 1993). In the macaque and baboon, cytotrophoblastic cells from the trophoblastic plate enter maternal vessels shortly after initial vessel penetration and become situated in the superficial arterioles that they at least partially plug (Enders and King, 1991).

At the end of the lacunar or previllous stage, proliferation of cytotrophoblast cells in clusters adjacent to the blastocyst cavity (human) or between lacunae (macaque and baboon) results in formation of the primary villi. Almost immediately, these become secondary villi once mesenchyme begins to grow into them. Despite vessels forming very quickly within these villi, no blood circulates in the vessels until an embryonic heartbeat is established (Enders, 1993). Thus, a period of placenta formation follows implantation for about 1 week. The reorganisation of trophoblast at the endometrial junction, results in establishment of more permanent structures

that persist for weeks or months rather than days. These structures are termed the anchoring villi. It is only at this stage (that of formation of the trophoblastic shell) that invasion of maternal arterioles occurs in the human (Enders, 1993).

Cytotrophoblast invasion into maternal stroma and blood vessels is a very dynamic process, associated with cell-cell and cell-matrix interactions (Tarara, et al., 1987). Several factors are secreted by decidual cells such as laminin, typeIV collagen, fibronectin, heparin and proteoglycans. These probably have a role in maintaining decidual integrity and enhancement of implantation (Charpin et al., 1985). It has been demonstrated *in vitro* that invading trophoblast cells require fibronectin to facilitate attachment, and that they adhere preferentially to laminin coated surfaces (Fazleabas, et al., 1995). The current evidence in the human (Aplin, 1991, Damsky et al., 1993) and the rhesus macaque (Blankenship and Enders, 1997) suggests that trophoblast invasion into the maternal endometrium and uterine vasculature is regulated by cell-cell and cell-matrix interactions.

As can be seen from **Table 1.** the marmoset embryo adheres to the uterine epithelium at a later gestational age than other primates. However, the maternal vascular bed beneath the surface epithelium of the endometrium is present throughout the uterus at the time of implantation. Sinuses enlarge in the vicinity of the embryo attachment as trophoblast extends along the uterine surface. The relatively slow development of the marmoset post-implantation embryo is therefore unlikely due to limitations of the initial placentation, but instead, probably an inherent developmental characteristic (Moore, et al., 1985). Marmoset implantation has been described as less invasive than that in the human and other primates because of the superficial position of the marmoset embryo in the uterine lumen (Hertig, et al., 1956).

Figure 1.9 is a diagram to illustrate expansion of the early implantation site in the marmoset. It illustrates differences in trophoblast-endometrial relationships during expansion of the site.

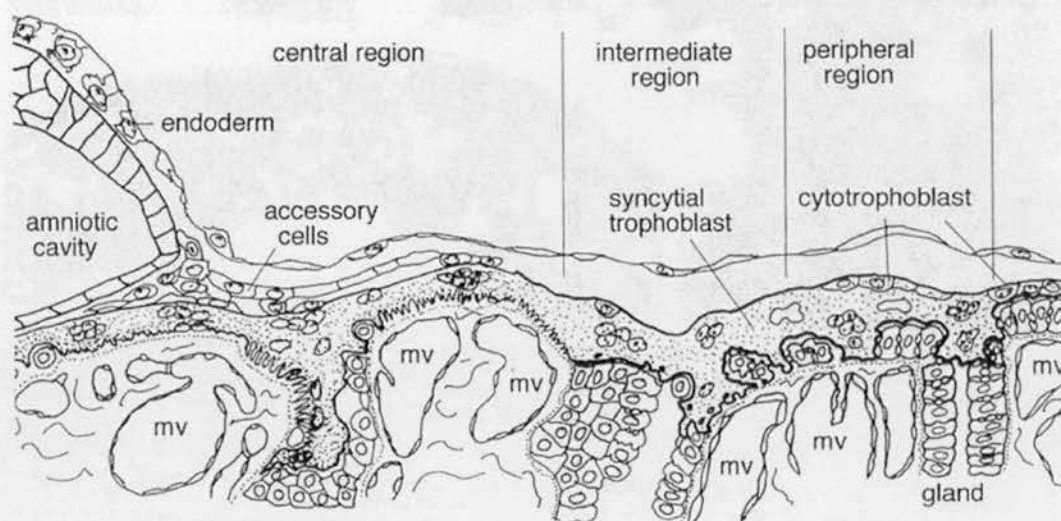


Figure 1.9 Expansion of the marmoset early implantation site (Enders and Lopata, 1999).

1.11.1 Steroid receptors during pregnancy

Regulation of ER during pregnancy is controlled by steroids. It is down regulated in glands and stroma as early as day 18 of pregnancy by progesterone as in the secretory phase of the non-pregnant cycle (Brenner et al., 1990). However, it does persist in spiral arteries throughout pregnancy where it may have a role in regulating blood flow, allowing the spiral arteries to adapt to trophoblast invasion and vessel occlusion (Enders, 1993) by increasing vasodilation to permit the unrestricted delivery of maternal blood to the developing embryo.

PR is also hormonally regulated during pregnancy but in a different way to that of the ER (Hild-Petito et al., 1992). In baboon, by day 18 of pregnancy, PR was absent from all glandular epithelium, but was in stroma surrounding the glands and spiral arteries. Stromal cells further away from the basal membrane of the glandular epithelium contained few PR positive cells, suggesting that the progesterone mediated glandular secretory activity may be regulated in a paracrine manner by adjacent stromal cells (Fazleabas, et al., 1993, Tarantino, et al., 1992). Furthermore, in baboon, PR expression in cells that do not contain ER suggests that PR maintenance in the primate endometrium during pregnancy is not via an ER-mediated mechanism. Insulin, IGF, EGF and cAMP have all been demonstrated to

increase PR expression in the absence of oestrogen *in vitro* (Aronica and Katzenellenbogen, 1991, Sumida et al., 1988, Sumida and Pasqualini, 1989). Following complete decidualisation, PR and IGFBP-1 are coexpressed in the same cells in the baboon uterus (Fazleabas et al., 1989, Hild-Petito, et al., 1992). It may be therefore, that IGF bound to IGFBP-1 can regulate PR expression in an autocrine manner in the pregnant baboon.

1.12 Vascular growth

1.12.1 Uterine and placental tissues

The mucosal, innermost layer of the uterus is termed the endometrium. In primates, growth of the endometrial vasculature begins during the proliferative (follicular) phase and continues throughout the secretory (luteal) phase of the menstrual cycle (Reynolds et al., 1992). Associated with endometrial capillary proliferation, is increased DNA synthesis of vascular endothelial cells (Ferenczy et al., 1979). Endometrial growth and development in response to systemic concentrations of ovarian steroids was demonstrated by classic experiments of Markee, 1932 and 1940 (as cited in (Reynolds and Redmer, 1992)). In these studies, endometrial explants of rabbits or monkeys were transplanted to the anterior chamber of the eye. Not only were these explants shown to quickly recruit a vascular supply, but they also underwent cyclic periods of growth and regression, which were associated with changes in systemic concentrations of steroids. Rapid vascularisation and cyclic growth of human endometrial explants that were transplanted to the hamster cheek pouch has been observed (Abel, 1985).

Rate of blood flow to uterine tissues also varies throughout the non pregnant cycle, being greatest at or just before ovulation when systemic oestrogen levels are greatest, and least during the luteal phase of the cycle when systemic levels of progesterone are highest. These regular patterns in uterine blood flow are temporarily associated with the ratio of oestrogen:progesterone in systemic blood (Reynolds, et al., 1992).

In eutherian mammals, the definitive placenta consists of maternal (endometrial) and fetal (chorioallantoic) tissues and is the site of physiological exchange between the maternal and foetal systems. Placental vascular growth begins

in early pregnancy and continues throughout gestation in association with a continual and dramatic increase in rates of uterine and umbilical blood flows (Ferrell, 1989). Increased blood flow to placental tissues satisfies the steadily increasing metabolic demands of foetal growth. The importance of placental vascular development means that any dysfunction may be a major contributor to embryonic wastage and reduced birth weights, two major socio-economic problems associated with pregnancy.

Angiogenesis affects numerous pathologic processes of the endometrium. These include, dysfunctional uterine bleeding and endometrial response to exogenous hormonal treatment (such as subdermal continuous released levonorgestrel or orally administered medroxyprogesterone acetate). Also, bleeding associated with an intrauterine device, uterine leiomyomata, endometriosis, complex endometrial hyperplasia and endometrial carcinoma in which greater depth of invasion and higher tumour grade are directly associated with increased angiogenic activity (Abulafia and Sherer, 1999).

1.12.2 The ovary

Before or soon after birth, the ovary of most mammals contains a stock pile of non-growing primordial follicles, each of which is composed of an oocyte surrounded by a layer of squamous pregranulosa cells (Greenwald and Terranova, 1988). In the postpubertal female, primordial follicles continually leave this non-growing pool. As they approach ovulation, the growing follicles begin to produce large quantities of oestrogens. A majority of these follicles undergo atresia, and regulation of growth and development of ovarian follicles therefore has long been recognised as a means to reduce or enhance fertility. After ovulation, the remaining cells of the ruptured follicle form a transient endocrine gland, the corpus luteum (CL). This is critical for successful maintenance of pregnancy in mammals because it is the primary source of the progestational hormone progesterone. In addition, the lifespan of the corpus luteum may be a primary determinant of the length of the non-fertile cycle. The extensive development of ovarian vascular beds has been well documented over the years, and it is recognised that capillary growth may be of primary importance in the growth and selection of ovulatory follicles as well as in the subsequent development and function of CL (Reynolds, et al., 1992). It has been

suggested that growth of the ovarian follicle and its corpus luteum may be governed by increased angiogenesis that occurs in the dominant follicle (Gospodarowicz and Thakral, 1978, Ravindranath, et al., 1992)

It has been demonstrated that reduced DNA synthesis of follicular endothelial cells, in association with reduced follicular vascularity, was one of the earliest signs of atresia (Greenwald, 1989), in addition, atretic follicles will regenerate when placed into culture. As such, decreased vascularity may limit access of atretic follicles to nutrients, substrates and trophic hormones, thus maintaining these follicles in an atretic state *in vivo* (Moor and Seamark, 1986). The extent to which angiogenesis governs follicular atresia and selection of the dominant follicle is of great interest. In some species a relationship has been demonstrated. The density of microvessels in the dominant, or selected follicle is at least twice that of other follicles in the rhesus ovary (Ravindranath, et al., 1992, Zeleznik, et al., 1981). This increased vessel density greatly increases the level of gonadotrophic hormone that is delivered to the dominant follicle *in vivo* suggesting that angiogenesis itself plays a role in selection of the preovulatory follicle. However, differences in follicle vasculature from one follicle to another are not obvious in the human ovary (Suzuki et al., 1998).

During the process of ovulation, the basement membrane loses its integrity and the thecal blood vessels access the granulosa cell layer. Subsequently, formation of the corpus luteum is associated with intense angiogenesis (Rodger et al., 1997). The formation and maintenance of the CL are essential for the support of early pregnancy, but the mechanisms that control these processes are unclear. In a fertile cycle in women, the corpus luteum persists in the presence of human chorionic gonadotrophin and secretes progesterone to maintain the developing conceptus. In a non-fertile cycle, the corpus luteum undergoes structural and functional regression. Approximately 50-70% of cells in the mature CL are endothelial cells and pericytes (Farin et al., 1989, Lei, 1991) resulting in the majority of steroidogenic cells being in contact with at least one capillary (Redmer and Reynolds, 1996, Reynolds, et al., 1992, Reynolds and Redmer, 1998). By the mid-luteal phase, when the CL is fully functional, blood flow is among the greatest of any tissue in the body, and ovarian blood flow is highly correlated with the rate of progesterone production (Niswender

et al., 1994). Proliferation of endothelial cells continues at a moderate rate decreasing towards the end of the cycle, so that, by the mid-luteal phase the microvascular tree is fully established. Pharmacological rescue of the corpus luteum of the CL in early pregnancy is associated with further angiogenesis in women (Wulff et al., 2001). Luteolysis in the sheep is associated with a rapid diminishment in luteal blood flow at a much faster rate than tissue weight, implying that luteal regression is preceded by changes in the vasculature (Bruce and Moor, 1976).

The newly ovulated follicle produces diffusible angiogenic substances that direct capillary proliferation into the newly formed corpus luteum (Gospodarowicz and Thakral, 1978). More recently, several angiogenic growth factors and their receptors have been shown to be present in the corpus luteum such as VEGF, KDR, Flt, Ang-1 and Ang-2 (Reynolds et al., 1994), (Goede et al., 1998, Hazzard et al., 1999, Maisonpierre et al., 1997, Redmer and Reynolds, 1996). More specific detail about expression of these factors and molecular regulation of angiogenesis will be included in the relevant experimental chapters.

1.13 Angiogenesis

Angiogenesis refers to formation of new blood vessels, or neovascularisation, and is an essential component of tissue growth and development (Folkman and Klagsbrun, 1987, Klagsbrun and D'Amore, 1991). It differs from vasculogenesis in which new embryonic microvessels arise *in situ* and endothelial cells are produced from progenitor cell types (Risau, 1991). It is regulated by a complex series of growth factor interactions, including stimulatory, modulatory and inhibitory regulators. This section will discuss physiological and pathological angiogenesis, the angiogenic process and its molecular regulation.

1.13.1 Pathological angiogenesis

Apart from during tumour growth and wound healing, the adult vascular endothelium is generally quiescent, representing an extremely stable population of cells with low mitotic rate. Rampant or persistent capillary growth is associated with numerous pathological conditions including tumour growth, retinopathies,

hemangiomas and rheumatoid arthritis (reviewed by Reynolds, et al., 1992). Conversely, insufficient capillary growth occurs in several disease states, including delayed wound healing, nonhealing fractures and chronic varicose ulcers. Due to the fact that regulation of the angiogenic process could become the primary method of treatment for these angiogenic disorders, and because capillary growth occurs infrequently in normal adult tissues, most work on angiogenesis and its regulation has focused on developmental or pathological processes (Klagsbrun and D'Amore, 1991).

1.13.2 Physiological angiogenesis

The notable exceptions of physiological angiogenesis are within the female reproductive tract; the ovaries uterus and placenta (Folkman, 1992). These tissues undergo cyclic changes in angiogenesis necessary to supply the nutrients and hormone precursors essential for the establishment and maintenance of pregnancy. During the menstrual cycle this physiological angiogenesis in the corpus luteum and endometrium is distinctive in that, in the absence of conception, it is followed by tissue regression and re-initiation of cyclicity. This tight physiological control contrasts to the situation in tumours, and raises the possibility that endogenous inhibitors of angiogenesis play an integral role in the reproductive tract.

Growth and regression of these tissues is extremely rapid. In the cow, for example, the ruptured ovulatory follicle, which represents less than 200mg of tissue, forms a mature corpus luteum weighing about 5-6g, in a period of about 10 days. Similar rapid growth is observed in uterine and placental tissues (Reynolds, et al., 1992). Rapid growth and regression of female reproductive tissues are accompanied by equally rapid changes in rates of blood flow. When fully functional, ovarian, uterine and placental tissues receive some of the greatest rates of blood flow, on a weight-specific basis, of any tissues in the body (Ford et al., 1982). Tissues of the female reproductive system are so dynamic that they provide a unique model for studying regulation of angiogenesis during the processes of growth, differentiation and regression in normal adult tissues. Understanding the mechanisms of control of angiogenesis in the reproductive system and the harnessing of major advances in the development of angiogenic inhibitors and stimulators may reveal new approaches to

regulation of reproductive function and treatment of pathological conditions of the female reproductive tract.

This section will discuss the current state of knowledge regarding the physiology of the angiogenic processes and their regulation in female reproductive tissues.

1.13.3 The angiogenic process

The angiogenic process begins with endothelial cell proliferation and culminates in the formation of a new microcirculatory bed composed of arterioles, capillaries and venules. The initial component of angiogenesis, capillary formation has been shown to consist of three processes:-

- 1) Fragmentation of the basal lamina of the existing vessel
- 2) Migration of endothelial cells from the existing vessel towards the angiogenic stimulus, and
- 3) Proliferation of endothelial cells (Reynolds, et al., 1992)

Neovascularisation is completed by formation of capillary lumina, by cessation of endothelial cell proliferation, curvature of the endothelial cell and adherence to each other to form the capillary lumen. The newly formed vessels differentiate into arterioles and venules, as pericytes emerge along the length of the sprout. Blood flow begins slowly. Newly formed vessels are highly permeable until the new basement membrane is formed and stabilisation by pericytes is complete (Findlay, 1986).

1.13.4 Endothelial cell proliferation

Endothelial cell proliferation forms the major component of capillary growth. Endothelial cells are generally quiescent and unresponsive to angiogenic stimuli. One important question in angiogenesis research is what maintains endothelial cells in this quiescent state and how are these barriers overcome during the initiation of neovascularisation? (Folkman, 1992). One of these barriers is cellular. Pericytes in the microvasculature appear to inhibit endothelial cell proliferation. In co-cultures of pericytes and endothelial cells, cell-cell contact results in production of latent TGF- β produced by both cell types (Antonelli-Orlidge et al., 1989). Interestingly, retinal

neovascularisation involves a process of “pericyte drop-out”, whereby the normal number of pericytes is reduced (Folkman, 1992).

Another barrier to neovascularisation is the endothelial cell itself. In culture, endothelial cells do not respond to growth factors when they are confluent. Increased sensitivity to bFGF, for example, only occurs by endothelial cell spreading or elongation (Ingber and Folkman, 1989). *In vivo*, small venules from which capillary sprouts arise under angiogenic stimulation appear to contain crowded endothelial cells analogous to a confluent cell culture (Folkman, 1992).

Vasodilation has been implicated as a possible first step in initiation of angiogenesis. This commonly occurs as a first step in angiogenesis before the emergence of the first capillary sprout (Dvorak et al., 1995). It could be that vasodilation serves to stretch endothelial cells to render them responsive to growth factors (Folkman, 1992).

A third barrier to neovascularisation is that access to vascular endothelium by potent angiogenic regulators may be prevented by their being sequestered by the basement membrane.

A fourth barrier to neovascularisation consists of endogenous inhibitors of endothelial growth that are present in the circulation and in tissues. These inhibitors will be discussed in more detail in a later section. Under mitogenic stimulation, endothelial cells leave the G₀ resting phase of growth and re-enter the cell cycle. Proliferation of endothelial cells can be quantified by expression of specific proteins associated with the cell cycle for example Ki67, or by the incorporation of nucleotide analogues during the DNA synthesis phase of growth. Studies using these approaches have quantified luteal endothelial cell proliferation in the human (Gaytán, et al., 1998, Rodger, et al., 1997), rhesus macaque (Christenson and Stouffer, 1996), sheep (Jablonka-Shariff, et al., 1993) and cow (Zheng, et al., 1994). All demonstrate peak endothelial cell proliferation in the early luteal phase associated with formation of the corpus luteum and decreased proliferation associated with luteal regression.

As mechanisms of initiating angiogenesis are revealed in the female reproductive tract, they may also be considered as therapeutic targets to control dysfunctional bleeding as well as targets of novel methods of contraception.

1.13.5 Capillary stabilisation and pericytes

Mature capillaries and postcapillary venules are comprised of two cell types, endothelial cells and pericytes. Pericytes are found in intimate association with endothelial cells, and this arrangement, unique to small vessels, is characterised by frequent sites of contact between the two (Orlidge and D'Amore, 1987). The position of the pericyte in the capillary and their close proximity to endothelial cells have led to several theories regarding their function. These include, regulation of permeability (De Oliveira, 1966) mediation of contractility (Tilton et al., 1979), maintenance of integrity (Rhodin, 1968) and control of proliferation (Crocker et al., 1970). The absence of pericytes has been correlated with the onset of neovascularisation. Before the onset of new vessel growth associated with diabetes mellitus, there is a loss of pericytes, termed “pericyte drop-out” (De Oliveira, 1966). Morphogenetic studies of vessel development demonstrate that newly forming capillaries are characterised by active endothelial cell proliferation and migration, and the absence of pericytes (Ausprunk and Folkman, 1977), whereas there is a decline in endothelial cell proliferation and migration associated with pericyte appearance (Ausprunk and Folkman, 1977). Such observations suggest a role for pericytes in the regulation of endothelial cell growth. *In vitro*, pericytes have been found to inhibit capillary endothelial cell proliferation, (Antonelli-Orlidge, et al., 1989, Orlidge and D'Amore, 1987). This inhibitory effect of pericytes appeared to be mediated by TGF- β . Activation of this factor requires formation of cell-cell contacts between pericytes and endothelial cells. Furthermore, the number of cell-cell contacts between pericytes and endothelial cells has been shown to increase with ongoing maturation of newly formed capillaries (Wakui et al., 1989). Capillary sprouting includes coordinated growth of both pericytes and endothelial cells rather than mere elongation of endothelial tubes, which subsequently become occupied by pericytes. Therefore, it may be that pericytes have a role in guiding capillary sprouts to find each other allowing their fusion to continuous capillary loops (Nehls et al., 1992). This has also been suggested in the sheep CL (Redmer et al., 2001). In view of these findings, stimulation and inhibition of endothelial cell proliferation during angiogenesis is a finely balanced process in which pericytes by an active involvement in capillary sprouting, are likely to play a key role.

Endothelial cells and pericytes communicate via bi-directional extracellular exchange of soluble mediators. Endothelial cells are a source of diffusible factors, regulating mural cell and possibly mural cell precursor proliferation and migration (Hirschi and D'Amore, 1996). Several diffusible soluble factors are synthesised and secreted by endothelial cells, among which are a variety of polypeptide growth factors, including platelet-derived growth factor (PDGF) (Westermarck et al., 1990) and bFGF (Montesano et al., 1986), which function in a paracrine fashion to promote mural cell proliferation/migration. Microcapillary endothelial cells coexpress PDGF-B and PDGF-B receptor genes, suggesting that endothelial cell proliferation may be stimulated in part by autocrine mediation by this molecule. Endothelial cells of larger developing vessels however, retain high expression of PDGF-B but have no detectable levels of PDGF-B receptor mRNA (Holmgren et al., 1991). The receptor is detectable in surrounding fibroblast-like cells and smooth muscle cells. These data suggest that endothelial cells can effect mural cell precursor migration and proliferation during vessel development by paracrine stimulation by PDGF-B. *In vitro* co-culture of endothelial cells and smooth muscle cell precursors has demonstrated that endothelial cells are capable of inducing migration of smooth muscle cells via PDGF-B. These mural precursors differentiated into smooth muscle cells in the presence of endothelial cells. If cultured alone their differentiation is driven by TGF- β (Hirschi et al., 1998). Basic fibroblast growth factor is angiogenic, but also mitogenic (D'Amore and Smith, 1993) and chemotactic (Sato et al., 1991) for smooth muscle cells. Although its mechanism of release remains unclear, it is another candidate for involvement in smooth muscle cell or pericyte-precursor recruitment by endothelial cells (Hirschi and D'Amore, 1996).

Pericytes appear to have a varied yet essential role in angiogenesis. Elevated awareness of these cells and increasing information obtained by further investigation will lead to better understanding of their role/s in microvascular function.

1.14 Angiogenic growth factors

1.14.1 Angiogenic promoters

Given the physiological and pathological importance of angiogenesis, a great deal of research has been devoted to the isolation, characterisation and purification of factors that can either stimulate or inhibit angiogenesis. Several bioassays have been developed to measure angiogenesis. The most common ones are endothelial cell migration (Glaser et al., 1980) and proliferation (Folkman et al., 1979) *in vitro*, and capillary growth *in vivo* in the developing chick chorioallantoic membrane (CAM) (Ausprunk et al., 1974) and the cornea (Gimbrone et al., 1974). Angiogenesis is regulated in the reproductive tract and elsewhere by at least twenty angiogenic growth factors, which have been identified to date. A number of stimulators of angiogenesis have been purified and characterised. One important promoter of angiogenesis appears to be vascular endothelial growth factor (VEGF), and it is the only angiogenic peptide that is mitogenic specifically for vascular endothelial cells. Other angiogenic promoters include acidic and basic fibroblast growth factors, platelet derived growth factor, which is structurally similar to VEGF, transforming growth factor- α , transforming growth factor- β , folliculostellate-derived growth factor, angiotropin, angiogenin (Abulafia and Sherer, 1999), and tumour necrosis factor- α (Okuda et al., 1999). Finally insulin-like growth factor II has been identified as an angiogenic promoter, in that it stimulates migration and morphological differentiation of endothelial cells (Lee et al., 2000). This is supported by earlier work by Fraser *et al* (Fraser et al., 1998), where it was found that insulin-like growth factor binding protein-3 mRNA was expressed in endothelial cells of the primate corpus luteum. This has more recently been implicated to play a role in luteal rescue. The regulated expression of IGFBP-3 may play a role in controlling angiogenesis and cell responses in the CL by autocrine/paracrine mechanisms (Fraser et al., 2000).

1.14.1.1 The Fibroblast Growth Factors

An angiogenic factor was first isolated from a tumour in 1971 (Folkman et al., 1971). This polypeptide was purified by heparin-affinity chromatography and when subsequently sequenced, it was found to be bFGF (Esch et al., 1985). Basic fibroblast growth factor is one of the most potent of the known angiogenic factors (Klagsbrun

and D'Amore, 1991). It is not only an endothelial growth factor, but also a mitogen for vascular smooth muscle, fibroblasts and for some epithelial cells. It is produced by virtually all vascular endothelial cells and by many other cell types. Therefore it may act as both an autocrine and paracrine growth factor. There also exists an acidic fibroblast growth factor (aFGF) Both bFGF and aFGF are structurally related, with a 53% absolute sequence homology (Esch et al., 1985).

FGF is one of the most widely distributed growth factors in the body, and it is synthesised by many cells in culture. It has been demonstrated to be a cellular rather than a secreted protein (Vlodavsky et al., 1987). Both bFGF and aFGF do not contain a consensus signal peptide in the open reading frame and are thus not secreted. (Abraham et al., 1986). Endothelial cells synthesise large amounts of bFGF (Schweigerer et al., 1987). 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. FGF *in vitro* is mitogenic and chemotactic for endothelial cells. It stimulates their production of collagenase and plasminogen activator proteases capable of degrading basement membrane, and it induces migration of capillary endothelial cells to migrate into three-dimensional collagen matrices to form capillary like tubes (reviewed by (Klagsbrun and D'Amore, 1991)). Both aFGF and bFGF are angiogenic factors in the CAM and cornea bioassays at levels as low as 10-100ng (reviewed by (Klagsbrun and D'Amore, 1991)).

In summary, both 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.

1.14.1.2 Vascular Endothelial Growth factors

By far the most widely studied of these angiogenic regulators in terms of regulation of angiogenesis is VEGF, also known as vascular permeability factor (Ferrara and Davis-Smyth, 1997). It was identified in 1983 by its ability to elicit vascular permeability (Senger et al., 1983), and has subsequently been shown to be a potent inducer of angiogenesis (Ferrara and Davis-Smyth, 1997). VEGF is a potent mitogen for micro and macrovascular endothelial cells but is devoid of appreciable

mitogenic activity for any other cell types (Keck et al., 1989, Leung et al., 1989). It is also known as a vascular permeability factor based on its ability to induce vascular leakage in the guinea pig skin (Senger, et al., 1983). More recent studies have also suggested that VEGF may be a factor that induces fenestrations in endothelial cells (Roberts and Palade, 1995)

VEGF is a member of the platelet derived growth factor family, and is a heparin-binding, secreted, dimeric glycoprotein. It exists as 5 different homodimeric isoforms generated by alternative splicing from a single gene. The secreted forms are composed of 121 and 165 amino acids respectively and act upon endothelial cells in which its receptor tyrosine kinases, VEGFR-1 and VEGFR-2 (Flt-1, KDR/Flk-1 respectively) are selectively expressed (Abulafia and Sherer, 2000). The most common secreted molecular species is VEGF₁₆₅. VEGF is an inducer of capillary tube formation and a vascular permeability-inducing agent, stimulated by hypoxia (Namiki et al., 1995), cytokines and hormones (Shweiki et al., 1992). VEGF is expressed in the ovary in a regulated manner, and its importance in ovarian angiogenesis has been established following studies in which its action has been inhibited during the ovulatory cycle. Targeted inactivation of one VEGF allele in embryonic stem cells results in abnormal formation of embryonic blood vessels (Carmeliet et al., 1996). Blood vessel formation is further impaired in homozygous VEGF allele deficient embryos, resulting in death during gestation. It appears that VEGF deficiency impairs most early steps of vascular development including in situ differentiation of blood islands (vasculogenesis), sprouting from pre-existing vessels (angiogenesis), lumen formation, formation of large vessels, establishment of interconnections, and spatial organisation of intra- and extra-embryonic vessels (Carmeliet, et al., 1996). Abnormal organogenesis can also occur due to inhibition of angiogenesis. This was demonstrated after exposure to thalidomide (associated with severe limb malformations, stunted limb growth and dysmelia) even at low concentrations in early human embryos (D'Amato et al., 1994).

1.14.1.3 Angiopoietins

VEGF has been the focus of a great deal of research in recent years. Particular attention has been given to the inter-relationship of VEGF with a novel

family of angiogenic regulators, the angiopoietins -1 and -2. These consist of about 500 amino acids and act via another endothelial cell receptor tyrosine kinase, Tie-2. Northern blot analyses of RNA from adult human tissues localised expression of these regulators. Angiopoietin-1 (Ang-1) was widely expressed, although in small amounts in the heart and liver. Angiopoietin-2 (Ang-2) expression was readily detectable only in ovary, placenta and uterus, three predominant sites of vascular remodelling in the normal adult (Maisonpierre, et al., 1997).

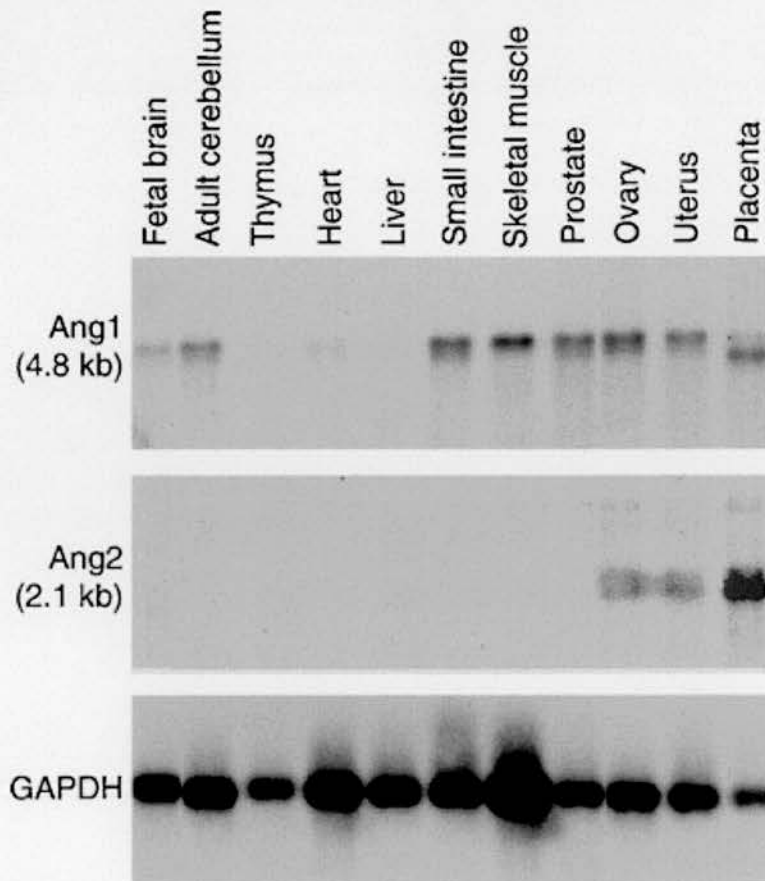


Figure 1.10 Expression of Ang-1 and Ang-2 mRNA in adult human tissues (Maisonpierre, et al., 1997).

It can be seen that the angiopoietins are relatively highly expressed in the ovary, uterus and placenta. Since they appear to play a major role in stabilisation of blood vessels on one hand and endothelial cell death on the other, the angiopoietins are attractive candidates as regulators of these divergent processes as they occur in the female reproductive tract. When angiogenesis is occurring in the presence of VEGF, Ang-2 acts in concert to enhance angiogenesis, while Ang-1 is involved in

the process of maturation and stabilisation of new blood vessels (Maisonpierre, et al., 1997). Paradoxically, in circumstances of reduced VEGF expression, Ang-2 may act to destabilise blood vessels and induce vascular regression by competitive inhibition of Ang-1. This was tested to determine if Ang-2 negatively regulates Tie-2 function *in vivo* (Maisonpierre, et al., 1997).

Transgenic mice specifically overexpressing Ang-2 in their blood vessels were generated by the use of an expression vector containing Tie-2 transcriptional regulatory elements, which direct expression of introduced genes to virtually all vascular structures in a developing mouse embryo (Maisonpierre, et al., 1997). Using this method they demonstrated that transgenic overexpression of Ang-2 disrupts blood vessel formation in the mouse embryo. This causes the embryo to be smaller than that of the wild type. The transgenic embryo had a collapsed heart endocardium, various vascular abnormalities in and around the heart and lacked a regular dendritic capillary plexus in the head, reminiscent of embryos lacking Tie-2 or Ang-1.

Ang-2 therefore has been demonstrated to be a natural antagonist for Tie-2 that disrupts *in vivo* angiogenesis. Natural antagonists for vertebrate receptor tyrosine kinases such as this are atypical, thus, the discovery of a negative regulator acting on Tie-2 emphasises the need for exquisite regulation of this angiogenic receptor system.

The temporal changes in the pattern of expression of VEGF and angiopoietin mRNA in the rat ovary have shown that VEGF is expressed in the pre-ovulatory follicle and in the hormone producing cells of the corpus luteum. Ang-2 is present in the theca of the preovulatory follicle and in the endothelial cell sprouts of the developing corpus luteum, suggesting an angiogenic role in concert with VEGF at this time. Interestingly, Ang-2 is highly expressed in regressing rat CL, when VEGF is declining, implying its involvement in the luteolytic process via destabilisation of blood vessels (Hazzard et al., 2000). Similarly, quantitative PCR in the bovine revealed that the Ang-2:Ang-1 ratio increased during luteolysis (Goede, et al., 1998). While luteolysis in the normal cycle is associated with endothelial cell loss, stabilisation and maturation of blood vessels may be crucial to the maintenance of the corpus luteum in early pregnancy. The mechanisms involved in these divergent processes are not fully understood. If luteolysis is associated with an increase in

Ang-2 expression and blood vessel destabilisation perhaps rescue of the corpus luteum is associated with an increase in recruitment of pericyte cells which stabilise the blood vessels? This has been demonstrated to be the case in CL of women treated with hCG to pharmacologically rescue their CL (Wulff, et al., 2001). However, this does not appear to be the case in all species. In the marmoset for example, this phenomenon was not observed (See Chapter 3).

1.14.2 Receptors involved in angiogenesis

Tie-2 knockout mice die somewhat later in embryogenesis (E9-E10.5), but the phenotype is distinct from that of VEGF receptor knockouts. Endothelial cells are present in normal numbers and are assembled into tubes, but the vessels are immature, lacking branching networks and proper organisation into large and small vessels. There is also an absence of the angiogenesis that vascularises the neuroectoderm by capillary sprouting from the primitive vascular network. Notably, the vessels that do form, lack an intimate encapsulation by periendothelial support cells. Thus the Tie-2 tyrosine kinase appears to control the capability of endothelial cells to recruit stromal cells to encase the endothelial tubes so as to stabilise the structure and modulate the function of blood vessels (Hanahan, 1997).

The fourth endothelial cell-selective RTK, Tie-1 is very similar in structure to Tie-2 and appears to control another aspect of vascular integrity. Knockout mice lacking Tie-1 die over a variable period of oedema and haemorrhage, implicating the Tie-1 signal in control of fluid exchange across capillaries in haemodynamic stress resistance, (the ability of a capillary to withstand changes in blood pressure) (Hanahan, 1997).

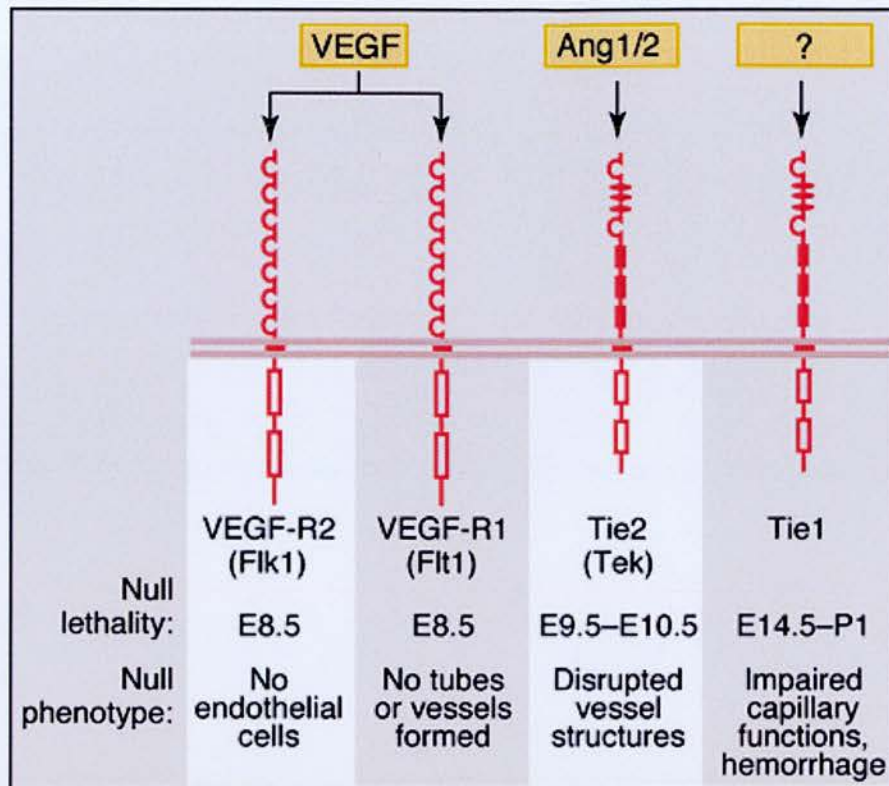


Figure 1.11 Lessons from gene knockout mice. The endothelial cell-selective RTK's have all been ablated in gene-knockout mice. Each RTK knockout produced embryonic lethality with vascular defects. However, their distinctive phenotypes indicate that each of these tyrosine kinases controls a specific, complementary function in endothelial cells that collectively account for a significant part of endothelial cell morphogenesis into functional vessels (Hanahan, 1997).

Summary of these interactions

- 1) Ang-1 is widely expressed in a variety of tissue types of adult and embryo. Ang-2 however, although expressed widely in the embryo, is expressed only in ovary, uterus and placenta of adult, the three tissues subject to physiological angiogenesis.
- 2) In early follicles, the vasculature is quiescent, and Ang-1 is expressed with little or no VEGF or Ang-2 expression
- 3) In late or preovulatory follicles and in the CL, where angiogenesis is ongoing, both VEGF and Ang-2 are upregulated while Ang-1 expression persists.
- 4) Finally in non-productive follicles which show vascular regression, Ang-2 is expressed at uniformly high levels, in the absence of VEGF expression.

These patterns of expression, collectively suggest a model for control of vasculogenesis, vessel maturation and maintenance, angiogenesis and regression (Hanahan, 1997).

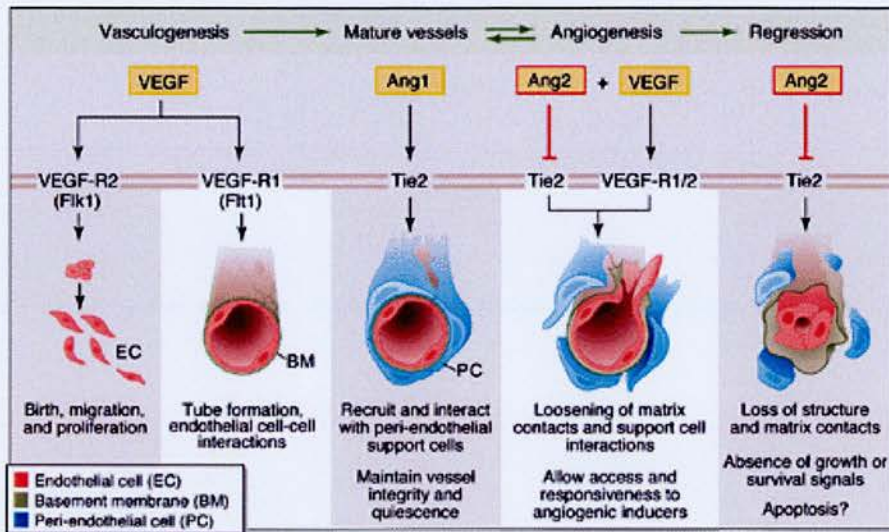


Figure 1.12 Regulation of vascular morphogenesis, maintenance and remodelling by RTKs and their ligands (Hanahan, 1997).

All three ligands (VEGF, Ang-1 and Ang-2) bind to RTK's that have very similar cytoplasmic signalling domains. Yet, their downstream signals elicit distinctive cellular responses. Only VEGF binding to VEGFR-2 sends a classical proliferative signal. When first activated in embryogenesis, this interaction induces the birth and proliferation of endothelial cells. In contrast, VEGF binding to VEGFR-1 elicits endothelial cell-cell interactions and capillary tube formation, a process that follows closely proliferation and migration of endothelial cells.

Ang-1 binding to the Tie-2 RTK recruits and likely maintains association of peri-endothelial support cells (pericytes, smooth muscle cells, myocardiocytes), thus solidifying and stabilising a newly formed blood vessel. Ang-2 does not activate the Tie-2 receptor, rather, it binds and blocks kinase activation in endothelial cells, thus it is a competitive antagonist of Ang-1. The Ang-2 negative signal causes vessel structures to become loosened, reducing endothelial cell contacts with matrix and disassociating peri-endothelial support cells. This loosening appears to render the endothelial cells more accessible and responsive to the angiogenic inducer VEGF. Finally, Ang-2 is expressed at uniformly high levels in vascular regression, in non-productive ovarian follicles, the lack of VEGF coexpression suggests that loosening

of cell-matrix interactions in the absence of a growth or survival signal elicits endothelial cell death, likely by apoptosis.

Although it will not be discussed in detail for the purposes of this literature review, changes in the extracellular matrix (ECM) are an essential part of the angiogenic process and are regulated by the metalloproteinases and their inhibitors. Cell adhesion to the ECM is primarily through integrins, a family of heterodimeric transmembrane proteins (Bazzoni et al., 1999). These mediate cell adhesion to the ECM leading to intracellular signalling events that regulate cell survival, proliferation and migration. During angiogenesis, it is thought that a number of integrins expressed on the surface of activated endothelial cells regulate critical adhesive interactions with a variety of ECM proteins (Eliceiri and Cheresh, 1999).

A complete understanding of these complex regulatory mechanisms within the ovary and uterus will allow systemic targeting in pathologies which have a vascular component.

1.14.3 Angiogenic inhibitors

An increasing number of angiogenesis inhibitors have been described including angiostatic steroids, interferons and platelet factor 4, which are non-specific, TNF- α , thrombospondin, TGF- β and protamine, reviewed by (Klagsbrun and D'Amore, 1991). Angiostatin, an internal fragment of plasminogen, and endostatin, a fragment of collagen XVIII derived from a human tumour cell line, are two examples of specific angiogenic inhibitors (O'Reilly et al., 1996, O'Reilly et al., 1997). No specific detail about angiogenesis inhibitors will be given for the purposes of this review.

1.15 Manipulation of angiogenesis

1.15.1 Development of antiangiogenic agents

Over 300 endogenous, natural or synthetic anti-angiogenic agents have been described (Thompson et al., 1999). The initial development involved screening for antiangiogenic activity in compounds developed for other pathologies. A second group required the design of specific antagonists to known positive angiogenic

factors and their receptors, while a third class have been identified from tumour sources as having negative effects on angiogenesis. The table below summarises this and is adapted from (Thompson, et al., 1999).

Agent	Mechanism
First Generation	
Suramin	Growth factor antagonist
Fumagillin derivative (TNP-470)	Unknown
Thalidomide	Unknown
Medroxyprogesterone	-
Anti-oestrogens	-

Inhibition of positive regulators

VEGF inhibitors:-

- VEGF immunoneutralising antibodies
- Truncated soluble receptor
- Tyrosine kinase receptor antagonists eg. Sugen 5416
- VEGF receptor immunoneutralising antibodies
- VEGF diphtheria toxin
- Ribozymes

Angiopoietin/Tie-2 receptor antagonists

Metalloproteinase inhibitors eg. Marimastat

Integrin inhibitors eg. Vitaxin

Administration of negative regulators

Angiogenin

Angiostatin

Thrombospondin

Endostatin

Numerous studies have shown that inhibition both by broad-spectrum or systemic targeting of the angiogenic pathway can have profound suppressive effects

upon tumour growth in animal models. Endostatin has been shown to specifically inhibit endothelial cell proliferation in tumours (O'Reilly, et al., 1997)

Other experiments include the effects of neutralisation of VEGF in an ovarian cancer model, with implications for a clinical application of this knowledge in inhibiting malignant ascites formation in ovarian cancer (Mesiano et al., 1998). Most of the clinical trials on angiogenesis have employed first generation compounds such as the fumagillin analogue, TNP-470, but as with many of these compounds, its mechanism of action is multi-factorial and is still under investigation.

Specific angiogenic peptide or receptor antagonists are being developed for clinical application. A humanised monoclonal antibody to VEGF is currently on a clinical trial (Presta et al., 1997) and the VEGF receptor is also being targeted using a soluble truncated Flt-1 receptor or tyrosine kinase inhibitors, the latter having the advantage of oral activity. Combination treatments such as VEGF inhibitor followed by Ang-2 may prove to be more effective in preventing angiogenesis (Hanahan, 1997).

Initial studies in experimental tumour models using negative regulators of angiogenesis, such as recombinant angiostatin are encouraging in that they demonstrate the successful treatment of tumours without generating resistance. Angiostatin has been shown to induce and sustain dormancy of human primary tumours in mice (O'Reilly, et al., 1996). Endostatin has also been shown to be an endogenous inhibitor of angiogenesis and tumour growth (O'Reilly, et al., 1997).

As increasing numbers of specific antagonists are developed, it should become possible to systemically target key steps in the angiogenic pathway at selected periods of the reproductive cycle using suitable animal models and their effects upon angiogenesis in the ovary, uterus and placenta determined. The potential for inhibition of physiological angiogenesis is currently being explored. Critical components of the reproductive pathway have been demonstrated to be suppressed after mice were treated with the angiogenesis inhibitor AGM-1470 (Klauber et al., 1997).

The importance of angiogenesis in the immature rat ovary has been shown in studies in which VEGF was blocked using a truncated Flt receptor. Follicular development was induced by gonadotrophin treatment and resulted in multiple

ovulations and elevated serum progesterone in control rats, whereas those treated with antagonist starting 4-6h prior to gonadotrophin administration showed small antral follicles and only few corpora lutea. The latter were relatively avascular and often demonstrated central ischaemic necrosis. While the uteri of the gonadotrophin-treated rats demonstrated morphological evidence of steroid stimulation, such changes were largely blocked in antagonist treated animals, presumably secondary to the comparative lack of steroid stimulation, but also in part as a result of inhibition of uterine VEGF (Ferrara et al., 1998). In marmoset, after treatment with a soluble truncated form of the Flt-1 receptor, vascular endothelial growth factor trap (A40) (VEGF trap) intense luteal endothelial proliferation was suppressed, a concomitant decrease in endothelial cell area confirmed the inhibition of vascular development, and a marked fall in plasma progesterone levels confirmed that luteal function was compromised (Wulff et al., 2001). In the follicle, treatment with the same compound resulted in an 87% decrease of proliferation in the theca of secondary and tertiary follicles, a reduction in endothelial cell area and a marked decline in Flt-1 mRNA expression. Granulosa cell proliferation also decreased. These results demonstrate that onset and establishment of the follicle vasculature takes place early during follicular development. Furthermore, the ability of VEGF trap treatment to severely restrict follicular angiogenesis establishes that VEGF is the major regulator of this process in the primate ovary (Wulff et al., 2001). Treatment with a VEGF neutralising antibody, commencing on day 7 after ovulation, for 3 days markedly suppressed luteal function as judged by a 50% reduction in plasma progesterone concentration (Dickson et al., 2001). It was concluded that ongoing angiogenesis in the mid-luteal phase is primarily driven by VEGF, that a proportion of endothelial cells of the mid-luteal phase vasculature are dependent on VEGF support and that the treatment may also be an anti-permeability agent, which would explain the decline in progesterone.

The mechanisms which regulate luteal function in rodents and primates are markedly different. For example, when macaques or marmosets were treated with TNP-470 there were no apparent effects upon angiogenesis or luteal function (Fraser et al., 1999) in contrast to the results in the mouse. However, luteal angiogenesis in the primate can be suppressed by neutralisation of VEGF (Fraser et al., 2000).

Less data is available on the effects of inhibition of VEGF on uterine function. It would appear that VEGF plays a fundamental role in implantation as an angiogenic factor but also as a potent inducer of permeability and edema. Its inhibition prevented implantation in rats (Rabbani and Rogers, 2001, Rockwell et al., 2002). In mice VEGF antagonist was seen to inhibit oestrogen induced uterine edema but had no effect on angiogenesis (Hastings et al., 2003). In marmoset, treatment with a neutralising antibody was seen to significantly suppress progesterone but had no significant effect on preventing establishment of early pregnancy. This result is not included in this thesis, but forms part of a publication included in the appendix (Rowe, et al., 2002). In this experiment, 10 marmosets were given a VEGF neutralizing antibody. Progesterone levels and pregnancy rates were compared to 6 control animals. Five out of six control animals became pregnant, whereas five out of ten treated animals became pregnant. This result was not significantly different despite there being significantly lower levels of progesterone in treated animals determined by 2-way ANOVA.

Angiogenesis is a very complex and tightly controlled process. Our understanding of its regulation in physiological circumstances and pathology is widening all the time. Targeted inhibition of angiogenesis and its manipulation are allowing this system to be dissected and no doubt further interventions will continue to improve our understanding of this vast subject.

1.15.1.1 Clinical relevance of angiogenesis research

Angiogenesis a very tightly regulated process. This review has highlighted its involvement in selection of the dominant follicle, subsequent formation of the corpus luteum and its role in uterus and placenta.

The corpus luteum is critical for successful maintenance of pregnancy as it is the primary source of the progestational hormone progesterone. Angiogenesis is intense during the early luteal phase where the previously avascular granulosa compartment of the ovulatory follicle becomes invaded by the thecal vasculature until such time as by the mid-luteal phase, every steroidogenic cell is in contact with at least one capillary. This is important in order that the necessary nutrients, oxygen and hormone precursors can be received by the gland to allow its efficient synthesis

of progesterone, then its export to receptive organs. In non-fertile cycles, the corpus luteum undergoes structural and functional regression accompanied by a demise of the vasculature. Luteal angiogenesis is clearly a tightly regulated process unlike that seen in tumours where prolonged and often unabated angiogenesis takes place.

There exist a number of endogenous inhibitors of angiogenesis that play as much of a role in its control as angiogenic promoters therefore this tightly regulated system represents an excellent model in which to study angiogenesis.

Inadequate progesterone production by the corpus luteum due to insufficient vascularisation could lead to early embryo loss, therefore promoters of angiogenesis may be of use in the follicle particularly, to help treat infertility. However, inhibition of luteal angiogenesis by an antagonist could prevent formation of the corpus luteum and thus act as a post-ovulatory contraceptive. Administration of the angiogenic inhibitor TNP-470, to mice resulted in inhibition of corpus luteum formation and endometrial maturation (Klauber, et al., 1997). TNP-470, however, has been shown to have no effect on luteal establishment and function in the primate (Fraser, et al., 1999). Therefore clearly not all treatments work the same way in different species and care must be taken when extrapolating from one to another.

The endometrium also undergoes cyclical angiogenesis. A poorly developed endometrial vasculature could have implications for successful implantation, establishment of early pregnancy, and placentation. Manipulation of angiogenesis here for example could have clinical implications in terms of treating women experiencing frequent miscarriage as a result of a poorly developed vasculature with pro-angiogenic agents or indeed conversely preventing implantation by anti-angiogenesis inhibitors. Studies addressing this issue are to date are limited to the rodent but form a basis for our understanding of this phenomenon (Rabbani and Rogers, 2001, Rockwell, et al., 2002).

Growth of tumours is dependent on an ever increasing vascular supply for provision of nutrients, oxygen and growth factors (Folkman, 1995). The study of angiogenesis *in vivo* using the female reproductive tract allows a unique opportunity to target factors involved therein that may have direct clinical relevance into several clinical conditions, both reproductive pathologies but also in other diseases.

1.16 Hypothesis and objectives

This thesis aims to elucidate the sites and mechanisms of angiogenesis in the marmoset during early pregnancy. In order to do this several hypotheses will be investigated:-

- 1) Based on existing evidence known about the human CL in early pregnancy where a further burst of angiogenesis is observed in early pregnancy (Wulff *et al.*, 2001), it was hypothesised that this phenomenon would also exist in the marmoset CL at this time.
- 2) This experiment was conducted to test the hypothesis that the upregulation in pregnant human CL, (Wulff *et al.*, 2001), is a progesterone mediated event and that it does not occur in the marmoset because progesterone levels are not significantly different in early pregnancy compared to the late luteal phase, (Rowe *et al.*, 2002)
- 3) This Chapter aimed to test the hypothesis that there would be an upregulation of angiogenesis in the marmoset endometrium in early pregnancy.
- 4) The final hypothesis was that if an upregulation in angiogenesis was observed in the marmoset endometrium in early pregnancy, this would be as a result of changes in local expression of the angiogenic factors. To investigate this, the localisation of these factors and their expression throughout the cycle in this tissue was examined.

Chapter 2

General Methods

2 Materials and Methods

2.1 Sources of reagents, enzymes and antibodies

Unless otherwise stated, all reagents and nuclease free water were obtained from Sigma (Poole, Dorset, UK) and all antibodies were from Dako Ltd, (High Wycome, Bucks, UK).

2.2 Marmoset husbandry

Animals were housed in a primate centre, the R.V.Short Building, Bush Estate, Edinburgh. Animals and the unit itself were maintained by primate centre staff. Marmosets were housed in cages in rooms maintained between 20°C and 25°C and artificially lit between 07.00h and 19.00h. The animals were fed daily with a selection of fruit, SDS Mazuri (E) primate dried pellets and high protein porridge with multivitamin supplements three times per week. Water was continuously available. Regularly cycling adult females with no previous breeding experience, aged between 2 and 2.5 years, weighing approximately 350g were housed together with a prepubertal female in cages measuring 1.15m in height, 1.1m in depth and 0.6m wide. The floor of each cage was covered with wood chippings to allow foraging and the cages contained large branches and a nest box. 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 minutes at 1000g, plasma was subsequently removed and stored at -20°C until assayed for progesterone to determine the date of ovulation and stage of the cycle and chorionic gonadotrophin (CG) to confirm pregnancy.

2.2.1 PGF_{2α} administration and induction of luteolysis

Administration of treatments was carried out by staff at the primate centre. All experiments were carried out in accordance with the Animals (Scientific Procedures) Act, 1986. In order that the timing of ovulation could be synchronised and subsequent day of cycle determined, all animals received 1µg PGF_{2α} analogue

(chloprostenol, Planate, Coopers Animal Health Ltd., Crewe, UK) intramuscularly (i.m.) during the mid-late luteal phase of the previous cycle to induce luteolysis and subsequent ovulation 10 days later (Summers, et al., 1985). At the time of prostaglandin administration, a fertile male was introduced. Two days prior to expected ovulation, vaginal lavages were obtained from female marmosets to determine whether or not they had mated. Blood samples continued to be taken until such time as tissues were collected. Terminal blood samples were assayed for chorionic gonadotrophin (CG) as a pregnancy test.

One parameter used to determine angiogenesis in this study was proliferation of endothelial cells. In order that this could be visualised and documented, bromodeoxyuridine (BrdU; Boehringer Mannheim, Sussex, UK) (twenty milligrams dissolved in 500 μ l physiological saline), was administered intravenously (i.v) 1h prior to tissue collection. BrdU is a thymidine derivative which is incorporated into the DNA of proliferating cells during the S-phase (DNA synthesis phase) of the cell cycle. This can then be visualised in tissue sections by immunocytochemistry. This will be discussed later.

2.2.2 Collection of tissue

Tissue was collected in the mid- and late- luteal phases from non-pregnant animals at days 14 (2 weeks) and 21 (3 weeks) from ovulation. Tissues from pregnant animals were also collected at 2 and 3 weeks post-ovulation. Additionally a final group of pregnant animals were studied at day 28 (4 weeks).

Animals were sedated using 100 μ l ketamine hydrochloride (Parke-Davis Veterinary, Pontypool, UK), i.m., and killed with an i.v. injection of 400ml Euthetal (sodium pentobarbitone, Rhone Merieux, Harlow, Essex, UK). Ovaries and uteri were removed immediately and weighed. Ovaries were halved at random across the widest point and fixed in either 4% paraformaldehyde (PFA) in phosphate-buffered saline (0.01M PBS; pH 7.4, containing 2.7mM KCl, 0.137M NaCl) or 4% neutral buffered formalin (NBF) in PBS for paraffin wax-embedding. All uteri were fixed in PFA.

These tissues were used for all results presented in chapters 3,5, 6 and 7. For Chapter 7, additional archived tissues were used from the early, mid and late follicular phase and the early and mid luteal phase.

Collection of human granulosa cells for Chapter 4 will be described later.

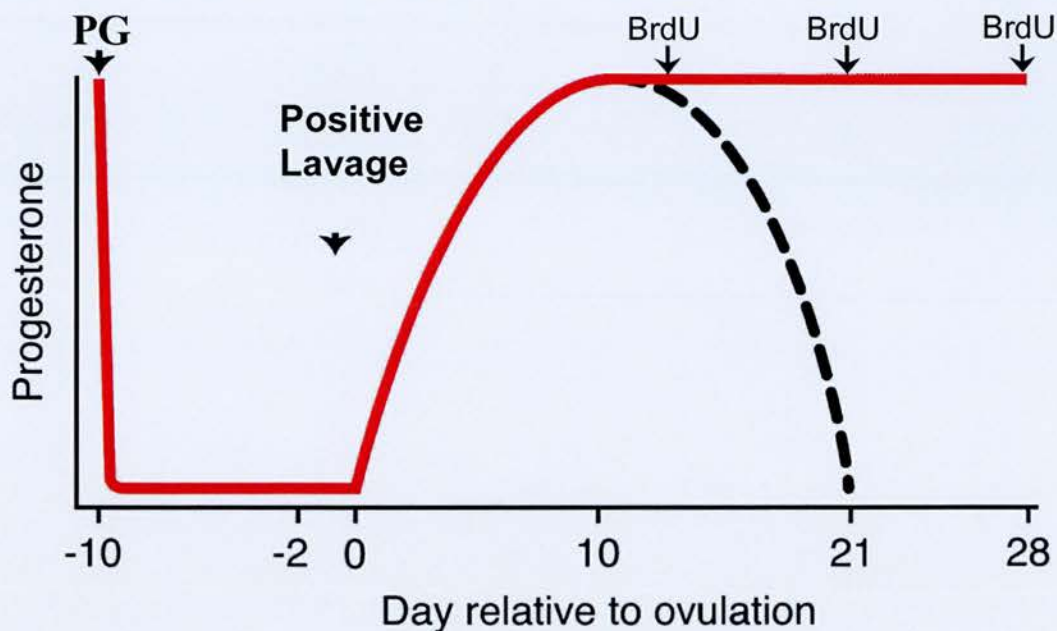


Figure 2.1 Schematic of study groups

Prostaglandin is administered in the mid to late luteal phase of the previous cycle to induce luteolysis (progesterone declines) and synchronise the timing of subsequent ovulation 10 days later as seen by the rise in progesterone concentration. Levels greater than 30nmol/l signify that ovulation has occurred. Pregnant and non-pregnant animals were studied at day 14 (progesterone remains elevated in both) and day 21 post-ovulation (in pregnant animals, where the corpus luteum is rescued and maintained by foetal CG, progesterone remains high, while in non-pregnant animals in the absence of CG, the corpus luteum regresses and progesterone levels decline). An additional group of pregnant animals were studied at day 28.

2.2.3 Details of animals used in each experiment

Study	Groups	Number of animals
Angiogenesis in the CL (Chapter 3)	Structurally intact late luteal	5
	Structurally regressed late luteal	3
	Pregnant Day 21	6
	Pregnant Day 28	6
Angiogenesis in the endometrium (Chapter 5)	Mid-luteal Day 14	5
	Late luteal (Day 21)	5 (all animals had structurally intact CL)
	Pregnant (Day14)	5
	Pregnant (Day21)	5
Gene expression in the endometrium (Chapter 6)	Pregnant (Day 28)	5
	Late luteal (Day 21)	5 (all animals had structurally intact CL)
	Pregnant (Day14)	5
	Pregnant (Day 21)	5
	Pregnant (Day 28)	5

Table 2.1. Details of animals used for the thesis

2.2.4 Progesterone ELISA plate assay

Marmoset ovulatory cycles were monitored by measuring plasma progesterone concentrations. Three blood samples were taken per week, plasma separated and frozen at -20°C.

Ninety-six well plates were coated in a 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 washed 5 times with washing solution diluted 1 in 25 before use (2.5M tris, 7M NaCl and 1.25% Tween, pH 7.5). Marmoset plasma samples were thawed, vortexed and diluted. 2.5µl of the plasma was added to 97.5µl of progesterone assay buffer, pH 6 (0.12M phosphate, 0.04M citrate, 0.1% gelatin and 0.01% thiomersalate in deionised water).

In each assay, a low, medium and high progesterone concentration quality control (QC) was used. These were prepared in the same way as samples.

A standard curve was prepared from a progesterone standard diluted in assay buffer to 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000pg/50µl. Fifty microlitres of each standard plus 2.5µl of charcoal stripped male marmoset plasma was added to 47.5µl assay buffer. Finally, to determine maximum binding (B0), 2.5µl of charcoal stripped male marmoset plasma was diluted in 97.5µl of buffer. All samples, standard curve, B0 and QC's were prepared in duplicate. To all wells, 50µl of primary antibody (rabbit anti-progesterone, SAPU, Carluke, Lanarkshire, UK) diluted 1:50,000 in assay buffer was added. In addition, to assess non-specific binding, charcoal stripped male marmoset plasma, which does not contain progesterone, was diluted in 147.5µl of assay buffer, primary antibody was omitted. The total volume in all wells was 150µl. Plates were covered and left overnight at room temperature.

After incubation overnight, 50µl per well of labelled ligand (progesterone-11-glucuronide-biotin complex) diluted 1:20,000 in assay buffer was added with 2mg/ml ANSA (8-anilino-1 naphthalene sulphonic acid). Plates were left at room temperature for 3h. After incubate was removed, plates were washed 10 times before 100µl per well of Streptavidin-Horseradish peroxidase diluted 1:2000 with normal phosphate buffer, (pH7.4, 1% casein). After 1 hour, plates were again washed 10 times before 200µl per well of substrate OPD, (5mM O-Phenylenediamine/0.03% H₂O₂) dissolved in substrate buffer, pH 5, (10.3g citric acid, 17.79g Na₂HPO₄ dihydrate up tp 1L with deionised water) was added. After approximately 15 minutes, once a sufficiently dark enough colouration had occurred, the reaction was stopped by adding 50µl per well of stopping solution (2N sulphuric acid). Plates were read at 490nm on a "Victor" spectrophotometer plate reader. Inter- and intra- assay coefficients of variation were 15% and 4% at the centre of the standard curve, becoming more varied at the extremes.

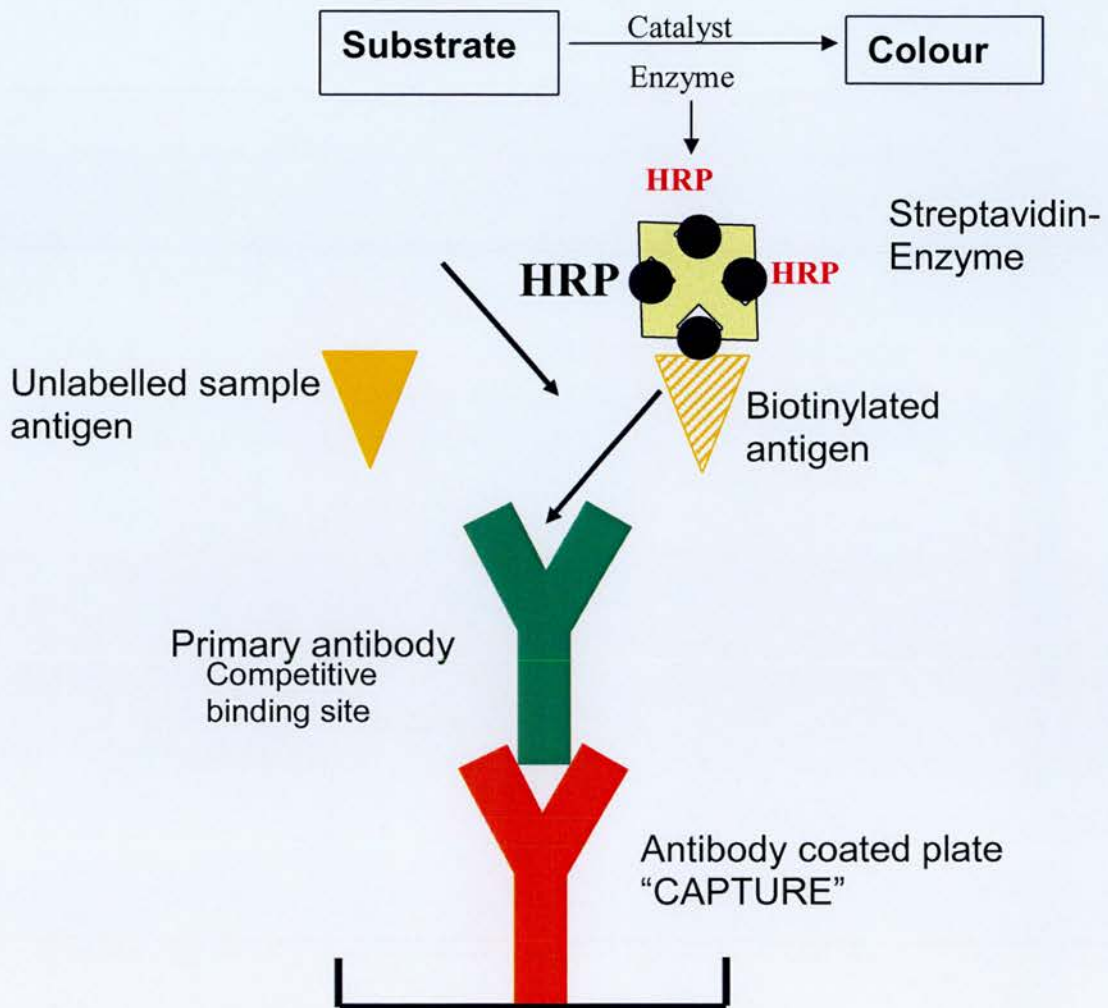


Figure 2.2 Schematic of ELISA for progesterone

The rabbit primary antibody binds to progesterone in the sample. This complex can then bind to the capture antibody (DARS) which is coating the plate. The biotin labelled progesterone is essentially exactly the same as the unlabelled sample antigen and binds to the primary antibody binding site with equal affinity. This labelled antigen can in turn bind the streptavidin- hydrogen peroxidase which is the enzyme responsible for the detectable substrate colour change. When in solution together, the labelled and unlabelled progesterone compete for the same binding site. There are a limited number of these binding sites but a proportionate amount of binding occurs depending upon the concentration of unlabelled progesterone in the sample. Therefore the more progesterone in the sample, the less labelled antigen can bind and the lower the colour intensity. Hence the colouration is inversely proportional to the amount of progesterone in the sample.

2.2.5 Assay for serum chorionic gonadotropin

This was conducted by Ian Swanston, using the method described by (Saltzman et al., 1998). The assay measures LH, which in marmoset has the same structure as CG apart from at the CHO terminus (Muller *et al*, 2004). The assay was a double-antibody RIA using the monoclonal antiserum 518B7 raised in mouse against bovine LH. The standard reference preparation was hCG CR-127 with a range of 0.1-5.0ng/tube. To validate the assay, three different marmoset plasma pools were assayed for parallelism. Each pool gave a displacement curve parallel to the standard curve. Assay sensitivity was 0.1ng/tube and inter and intra-assay CVs of a quality control pool were 6.88 and 5.96% respectively.

2.2.6 Classification of the stage of the ovulatory cycle

The classification of the stage of the marmoset ovulatory cycle was determined by plasma progesterone concentrations based on sampling three times per week, Monday, Wednesday and Friday. The follicular phase was defined as the period when plasma progesterone concentration was <30nmol/L. The luteal phase was classified as the stage when plasma progesterone concentration initially rose above 30nmol/L and was followed by a sustained increase. The first day when plasma progesterone rose above 30nmol/L being day 0, (the day of ovulation). Pregnancy was confirmed by the presence of trophoblast in serial sections of the uterus and plasma levels of CG greater than 20ng/ml.

2.3 Tissue fixation, processing and sectioning of paraffin blocks

Tissue fixation was necessary in order to prevent autolysis and bacterial degradation. Also fixation limits shape or volume changes during subsequent procedures, and allows tissues to be maintained as closely as possible to their *in vivo* state without significant loss or rearrangement of tissue morphology and RNA quality. All uteri and one half of the bisected ovaries were fixed in 4% paraformaldehyde (PFA). The other half of the bisected ovaries were fixed in 4%

neutral buffered formalin (NBF). The reason for this bisection and differing fixation of both halves was to examine any differences observed in results obtained from the tissues according to which fixative had been used. PFA had always been traditionally used in our laboratory but requires being made up on the day of use. NBF however, is more convenient as it can be stored at the primate centre ready for tissue collection at any time. Both of these fixatives work in the same way, by forming cross-links between proteins and aldehydes, resulting in a stable structure, without loss or damage to enzymes and antigenic sites; clearly important in immunocytochemistry. DNA and RNA do not react significantly with formaldehyde in their native states, but are thought to be physically trapped by the protein matrix formed in protein fixation.

Uteri obtained were fixed in 4% PFA for 24h then transferred to 70% EtOH until processing into paraffin wax. Ovaries were first bisected through the maximal part of the largest corpus luteum to ensure that the widest cross section through the gland could be obtained then fixed as above, one half in PFA, the other in NBF. Uteri were bisected after being fixed and transferred to EtOH. Bisection was performed longitudinally through the luminal cavity in order that sections would contain a full cross section of all compartments, myometrium, endometrium, luminal epithelium and uterine lumen. Processing and paraffin wax embedding were carried out by the MRC HRSU Histology Department. Tissue was dehydrated through a series of graded alcohols before saturation with paraffin wax. This process was conducted using a 17.5h automated cycle on a Leica TP-1050 processor (Leica UK Limited, Milton Keynes, UK). The tissue was then embedded with the cut surface facing upwards so that upon sectioning, maximal cross sections could be obtained. Five micron sections were cut on a hand operated microtome (Jung RM2035; Leica) with disposable blades. Super-frost plus slides, electrostatically charged for improved binding, were used for immunocytochemistry and *in situ* hybridisation (BDH, Merck Ltd., Poole, Dorset, UK). Sections were cut and floated onto a heated water bath (approximately 50°C containing either tap water for immunocytochemistry or RNase free water for *in situ* hybridisation). Sections were transferred onto slides and dried overnight before use. Uteri from females housed with a fertile male were serially sectioned in order to determine the presence of a trophoblast.

2.4 Haematoxylin and eosin staining

Haematoxylin and Eosin (H&E) stains were routinely prepared in the Histology department. Harris's haematoxylin was prepared by dissolving 2.5g of haematoxylin (BDH) in absolute alcohol. This is then added to alum that has been dissolved in 500ml of warmed distilled water in a 2L flask. The mixture was rapidly brought to the boil and the mercuric oxide (1.25g) or sodium iodate (0.5g) slowly added. The flask was then rapidly cooled by plunging into ice cold water. Once cold, 20ml glacial acetic acid was added (Stevens and Wilson, 1996). Eosin Y as a cytoplasmic stain was prepared as a 1% solution in distilled water with 0.5ml per litre of acetic acid.

Tissue sections for haematoxylin and eosin (H & E) staining were dewaxed in xylene and rehydrated through a series of graded alcohols to tap water. They were placed into Harris's haematoxylin for 5 minutes, then washed with tap water before being differentiated in 1% acid alcohol (1% HCl in 70% alcohol) for 5-10 seconds. Sections were again washed in tap water then placed into Scott's tap water until a 'blue' colour had developed (approximately 20 seconds). Sections were then washed again in tap water and placed into eosin for 20 seconds. After a final tap water wash, sections were dehydrated through a series of graded alcohols and placed into xylene for approximately 5 minutes before mounting in pertex (Cell Path, Hemel Hempstead, UK).

After serial sectioning of the uteri, every 10th slide was stained with H & E to determine the presence of trophoblast or foetal membranes.

2.5 Immunocytochemistry

Immunocytochemistry is a technique for identifying specific cellular or tissue constituents (antigens, usually proteins) by means of antibody-antigen interactions. The antigen is recognised within a cell by using a specific antibody raised to an epitope (monoclonal) or epitopes (polyclonal) of the protein in question. This technique allows specific localisation of proteins to individual cells and when quantified, provides a powerful tool for assessing protein expression. Quantification

is usually based upon parameters such as the number of positive cells within a given cell population, area of staining within a given field size and /or intensity of staining.

The power of immunocytochemistry is in the magnification of a signal produced by a bound antibody to a specific antigen by a series of secondary and tertiary antibodies to increase the available sites for the detection system. In this thesis, the majority of immunocytochemical techniques use either alkaline phosphatase-anti-alkaline phosphatase (APAAP) or Horseradish peroxidase (HRP). However, details of these specific detection systems will be provided separately.

2.5.1 Antigen retrieval

Fixation and embedding in paraffin wax to facilitate section cutting can mask or even destroy some antigenic epitopes. As such, after tissues had been dewaxed and rehydrated in a series of graded alcohols to water, an antigen retrieval step was used to reveal masked antigens. All antibodies used for the purposes of this thesis required an antigen retrieval step unless otherwise stated. Heat treatment was found to be the best antigen retrieval method for the antibodies used here after a series of optimisation experiments were carried out in order to ensure that no tissue damage or loss of morphology occurred.

Slides were subjected to pressure cooking in 0.01M citrate buffer, pH 6, in a Tefal Clypso pressure cooker (Tefal, Essex, UK). Two litres of citrate buffer were heated to boiling point in the pressure cooker. Once boiling, the dewaxed and rehydrated slides in a metal rack were lowered in. The lid was sealed and the slides were heated for 6 minutes at high pressure from when full pressure was reached (pressure 2). After pressure was released the slides remained in hot buffer for a further 20 minutes before cooling and either transferred to 0.01M phosphate buffered saline, pH 7.4, or 0.05M Tris buffered saline, pH 7.4, for 5 minutes.

2.5.2 Blocking Endogenous enzymes

If enzymes similar to those used in the tracer system are present in the tissue they may react with the substrate used to localise the tracer and cause problems with interpretation of results. For example, endogenous peroxidases may react with the DAB chromagen (3,3'-diaminobenzidine tetrahydrochloride) in the same way as the

exogenous horseradish peroxidase and give rise to non-specific staining. All antibodies which were detected using hydrogen peroxidase were incubated in absolute methanol containing 0.5% hydrogen peroxide for 20 minutes immediately after pressure cooking, without being transferred to TBS or PBS first (Streefkerk, 1972). After incubation, slides were transferred to TBS or PBS (details of which will be given in **Table 2.2**)

There are many types of alkaline phosphatase within tissues and most endogenous activity can be blocked using a 1mM concentration of levamisole in the final incubating medium. The alkaline phosphatase used here is calf intestinal alkaline phosphatase which is not inhibited by levamisole at this concentration so the specific signal is not quenched.

2.5.3 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). Generally, it is the first immune sera which gives rise to the highest background staining. This can be reduced by blocking those sites which show a non-specific affinity for immunoglobulins by incubating the section in normal whole serum (SAPU), from the species in which the secondary antibody was raised. For example, when mouse CD31 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 minutes at room temperature. This regime was used if no biotinylated secondary antibody would be subsequently used for detection. (See **Table 2.2** for details of respective blocking sera)

Marmoset ovaries tend to contain high levels of endogenous biotin. Thus a degree of non-specificity is incurred when using a biotinylated secondary antibody. Endogenous biotin was blocked in all cases where biotinylated secondary antibodies were used, by addition of exogenous avidin and biotin to the blocking sera. Using this blocking regime, exogenous avidin blocks the endogenous biotin, and all biotin binding sites on the avidin molecule were saturated with addition of excess biotin.

Avidin (Vector Laboratories Inc., Burlingame, CA, USA) was added at a concentration of 8 drops per ml of normal blocking serum for 30 minutes (as per kit instructions) then washed off with TBS or PBS. Followed by a 30 minute incubation with biotin in normal blocking serum (Vector, 8 drops per ml, as per kit instructions).

2.5.4 Immunocytochemical protocol

An automated incubation method, the Sequenza System (Shandon Scientific, Runcorn, Cheshire, UK) was used for all incubations apart from the peroxidase block. The Sequenza works by capillary action and reduces volumes of reagents required. Unbound antibody is washed from the section before addition of the next incubate. Each section is retained within its own sequenza coverslip preventing cross contamination and antibody-antigen complexes are prevented from precipitating onto sections giving rise to unwanted background staining.

The method detailed below is a general protocol. Detection systems vary according to antibody, as will the block used before addition of primary antibody and indeed whether or not TBS or PBS was used. Exact details are given in **Table 2.2**.

After the appropriate block had been applied and allowed to incubate for 30 minutes, sections were washed 3 times for 5 minutes each with either TBS or PBS. The primary antibody, diluted in the appropriate blocking serum used for blocking non-specific binding was added to the sections and incubated overnight at 4°C.

2.5.5 Detection

After 3 five minute washes with TBS or PBS, the secondary antibody, diluted accordingly in the appropriate blocking serum was added and incubated at room temperature for 40 minutes. At this point the standard protocol is somewhat divergent. Thus each detection system will be briefly discussed in turn.

APAAP (Alkaline phosphatase-Anti-alkaline phosphatase)

Slides incubated with non-biotinylated secondary antibodies were subsequently incubated with a tertiary APAAP antibody (Cordell et al., 1984). The secondary antibody is applied in excess so that one of its two identical binding sites binds to the primary antibody and the other to the APAAP complex (**Figure 2.3**).

The APAAP was added, again diluted in the appropriate serum block for 40 minutes before slides were washed and transferred to TBS or PBS.

The APAAP method can be used with a variety of substrates giving rise to a variety of colours. The two used for the purposes of this thesis were nitroblue tetrazolium (NBT) which forms a Deep blue/black immunostain (McGadey, 1970) and fast red TR used with naphthol AS-MX phosphate sodium which gives rise to a bright red end product.

Where NBT was used, slides were transferred to NBT buffer for 10 minutes 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 + 45µl NBT substrate (Boehringer Mannheim, Germany) + 35µl Xphosphate (Boehringer Mannheim) +10µl levamisole (Sigma). Slides were left for colour to develop over approximately 10 minutes.

Where Fast red was to be used for detection, slides were removed from TBS and tissues dried around. Fast red solution was added 1mg/ml fast red buffer and left for approximately 20-30 minutes until red colour developed. Reactions were stopped in tap water. Sections were counterstained in haematoxylin for approximately 20 seconds before being dehydrated in graded alcohols cleared and mounted in pertex (Cell Path) if stained with NBT or air dried before mounting if stained with fast red.

HRP

The favoured substrate for HRP was 3,3' diaminobenzidine tetrahydrochloride (DAB) which yields a crisp, insoluble, stable, dark brown reaction end product (Graham and Karnovsky, 1966). Where HRP was to be used as the detection enzyme, biotinylated secondary antibodies were used. After incubation with the secondary antibody, sections were washed three times for 5 minutes with PBS or TBS then incubated for 30 minutes at room temperature with ABC-HRP complex (one drop of solution 'A' + 1 drop of solution 'B' to 5ml 0.05M tris buffer with no salt, Dako). Slides were washed and transferred to either TBS or PBS before sections dried around and DAB substrate added (Dako). Staining was produced almost immediately. Reactions were stopped in tap water and sections were

counterstained in haematoxylin for approximately 20 seconds before being dehydrated, cleared and mounted in pertex (Cell Path).

Avidin-Biotin techniques rely on the marked affinity of the glycoprotein avidin for biotin. Avidin is composed of four subunits which form a tertiary structure possessing four biotin-binding hydrophobic pockets (Miller, 1996). The ABC-HRP complex works because 3 of the biotin binding sites on the avidin molecule are occupied by biotin molecules complexed to HRP while one site remains empty and can therefore bind to the biotinylated secondary antibody (**Figure 2.4**).

2.5.6 Dual staining

In order that two antigens can be detected and differentiated on the same slide, two separate detection systems must be 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 which is important in order 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, normal rabbit serum diluted 1 in 5 with TBS + 0.25g BSA.

Sections were dewaxed and rehydrated, then an avidin and biotin block was applied as described in 2.7.3. The first primary antibody, CD31 diluted accordingly in normal rabbit serum was added and incubated overnight at 4°C. This was then detected the following day by addition of a rabbit anti-mouse secondary antibody, followed by mouse APAAP and finally fast red as described above. Sections were then washed and sequenzas flushed through with TBS before the second primary antibody was added again diluted accordingly in normal rabbit serum overnight at 4°C. After post incubation washes with TBS, a biotinylated rabbit anti-mouse secondary antibody was added, followed by ABC-AP (Dako). The principle of this is the same as for ABC-HRP except that it uses NBT as a substrate instead of DAB. After incubation with the ABC-AP complex, slides were transferred to NBT buffer for 10 minutes before staining with NBT. Reactions were stopped in tap water, then slides were counterstained with haematoxylin before being dehydrated in ascending concentrations of alcohol, cleared in xylene and mounted in pertex (Cell Path).

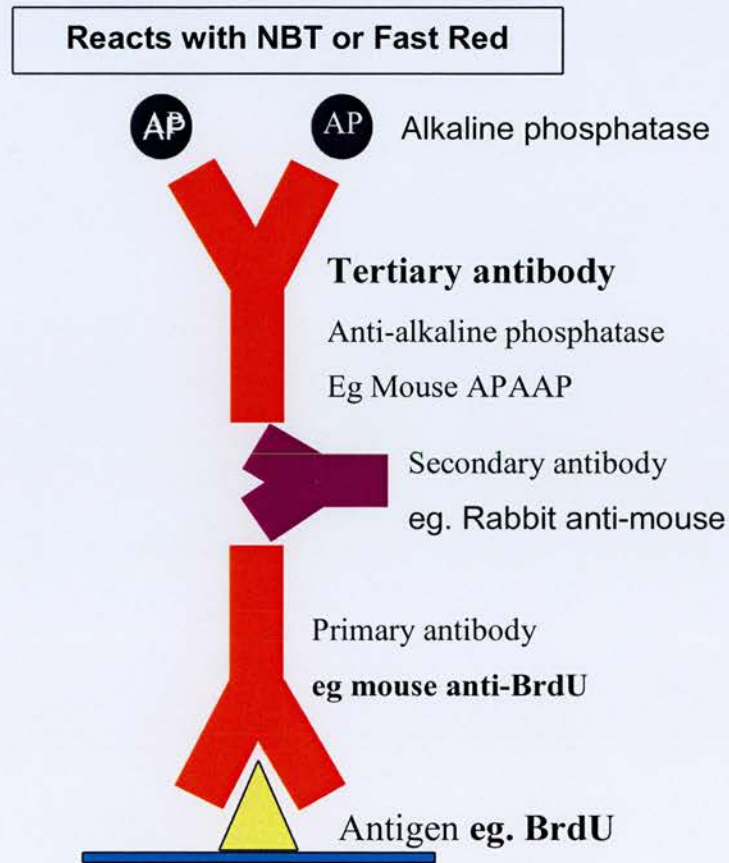


Figure 2.3. APAAP detection of immunocytochemistry

The primary antibody binds to its specific antigen. The secondary antibody is applied in excess so that one of its two identical binding sites binds to the primary antibody and the other to the APAAP complex. A coloured substrate is then used for visualisation

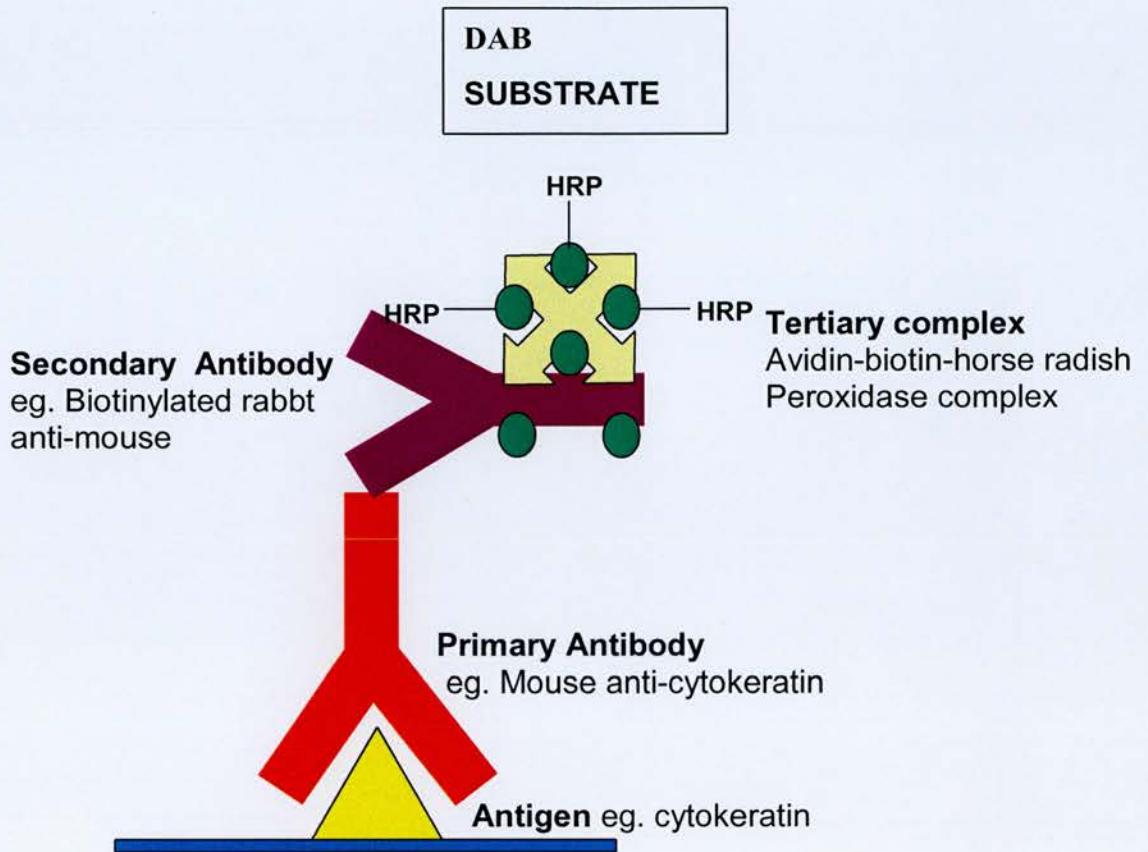


Figure 2.4. ABC-HRP detection of immunocytochemistry

The secondary antibody is biotinylated which permits binding of the tertiary complex, A coloured substrate, most commonly DAB is used for visualisation.

Antibody	Species raised in	Block	Secondary antibody	Tertiary antibody/complex	Detection	Washes	Counter-stain
CD31	Mouse Monoclonal 1:20	Normal rabbit serum	Rabbit anti-mouse 1:60	Mouse APAAP 1:100	NBT	TBS	No
BrdU	Mouse Monoclonal 1:30	Normal rabbit serum	Rabbit anti-mouse 1:60	Mouse APAAP 1:100	NBT	TBS	Yes
BrdU/CD31	Sheep/Poly 1:5000 Mouse/Mono 1:20	Normal rabbit serum	Rabbit anti-sheep biotinylated 1:500 Rabbit anti mouse 1:60	ABC-AP Mouse APAAP 1:100	NBT Fast Red	TBS	Yes
Cytokeratin	Rabbit Polyclonal 1:5000	Normal swine serum	Swine anti-rabbit 1:200	ABC-HRP	DAB	PBS	No
Pericytes	Mouse Monoclonal 1:20	Normal rabbit serum	Rabbit anti-mouse 1:60	Mouse APAAP 1:100	NBT	TBS	No

Table 2.2 Antibodies and reagents used for immunocytochemistry and protocol. All antibodies were used routinely in our laboratory and had been validated by the group previously. See Wulff C, Dickson SE, Duncan WC and Fraser HM (2001) for Pericytes, Fraser *et al*, 1999 for BrdU and Wulff C, Wiegand SJ, Saunders PT, Scobie GA and Fraser HM (2001) for CD31. Cytokeratin was an antibody borrowed from Dr. Simon Riley for a single morphological staining. Its specificity was tested on Human foetal membranes and marmoset endometrium as positive controls. Negative controls were conducted using rabbit IgG.

All antibodies are from DAKO apart from the sheep BrdU (Fitzgerald Industries international, Concord, MA, USA) and the mouse BrdU (Boehringer Mannheim). All detection reagents are from Sigma. See Appendix for details of antibodies and specificity.

2.5.7 Negative controls

For each antibody, negative controls were used in initial trials to determine the specificity of the antibody. The primary antibody was replaced with immunoglobulin G from the same species at the same concentration as the primary antibody.

2.6 Image analysis

2.6.1 Digital photomicroscopy

Tissue sections were examined by Digital Photomicroscopy using an Olympus Provis microscope (Olympus Optical, London, UK) and photographed using a digital camera, Kodak DCS420 (Eastman Kodak, Rochester, NY, USA). Figures were compiled using Adobe Photoshop 6.0 for Macintosh (Adobe Systems Inc., Mountain View, CA, USA) before being printed on a colour laser jet printer.

2.6.2 Quantification of immunocytochemical staining

Not all immunostains were quantified, some were purely performed for the purposes of localisation. In the corpus luteum, BrdU, CD31 and pericyte immunostaining were quantified using Image Pro Plus 3.0 software (Media Cybernetics, Silver Spring, Maryland, USA). Quantification was carried out across the complete ovarian cross section. An image is captured at x20 objective magnification and converted to grey scale. The captured image was examined to ensure that it mirrored the live image as closely as possible by eye. The area or number of dark objects on a white background per field of view ($3.2 \times 10^5 \mu\text{m}^2$) are measured using a specifically designed and pre-recorded macro. 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. The total cross section of the corpus luteum was analysed in this way.

In the uterus the dual staining of CD31 and BrdU were quantified using a grid system to record volume fraction measurements. Specific details of quantification protocols will be given in the relevant experimental chapters.

2.7 *In situ* hybridisation

This technique was used for the detection of specific mRNA in tissue sections by hybridisation to complementary radiolabelled riboprobes and subsequent detection using photographic emulsion.

I would like to thank Regeneron Pharmaceuticals, Inc., for the gift of Angiopoietin-1, -2 and Tie-2 cDNA riboprobes supplied to our laboratory preinserted into specific plasmids. Helen Wilson and Dr. Christine Wulff were responsible for transfection of these plasmids into *Epicurian coli* (XL-1 Blue; Stratagene, La Jolla, CA, USA) and production of glycerol stocks. I would also like to thank Dr. S. Charnock-Jones (University of Cambridge, Department of Obstetrics and Gynaecology) for the gifts of VEGF, VEGFR-1(Flt) and VEGFR-2 (KDR) cDNA probes. (All inserts are human sequences)

2.7.1 Plasmid Preparation

Riboprobes were generated from plasmids containing the specific cDNA insert of interest and RNA polymerase initiation sites. As stated above, these plasmids were received by myself already transfected into *Epicurian coli* as glycerol stocks. Details of specific plasmids used for each probe are given in **Table 2.3**.

Probe	Plasmid	Length of specific cDNA sequence (bp)	RNA polymerase initiation sites (AS/S)
VEGF-A	pBluescript	383	T7/T3
VEGFR-1 (Flt)	pBluescript	272	T7/T3
VEGFR-2 (KDR)	pBluescript	412	T3/T7
Ang-1	pKS+	570	T3/T7
Ang-2	pKS+	640	T3/T7
Tie-2	pKS+	1000	T3/T7

Table 2.3. Specific plasmids used for each cDNA insert. See appendix for specific sequences and homology to marmoset.

Plasmids were amplified by growth overnight on a shaking platform at 37°C of 10µl of glycerol stock inoculated into 10ml LB Broth (Anachem, Luton Beds, UK; 25 capsules per litre distilled water) containing 10µl Ampicillin (50m/ml, Gibco BRL, Life Technologies Ltd., Paisley, UK). A control tube containing broth and ampicillin was also prepared. After overnight growth, plasmids were purified from their host bacterium if the control solution remained clear.

Purification was performed using a QIAprep Spin miniprep kit (QIAGEN Ltd., Crawley, West Sussex, UK) according to the manufacturers instructions. Cells were centrifuged, resuspended then lysed by treatment with an alkaline solution. After addition of a neutralising solution and centrifugation, the supernatant was added to a QIAprep spin column. DNA becomes adsorbed to the silica in the presence of high salt, and is then eluted off the column. A GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech, Cambridge Science Park, Cambridge, UK) was used to determine the concentration and purity of the DNA. Additionally, the quality was checked using agarose gel electrophoresis. A 1% agarose gel was prepared by dissolving 0.5g agarose in 50ml TBE buffer (1M tris, 1M boric acid and 0.02M EDTA in distilled water). Once dissolved, 1µl ethidium bromide was added to the solution. The gel was cast and wells made with a comb. The purified plasmid was loaded (1µl) with 2µl loading buffer and 7µl nuclease free water. The gel was run at 100V for 45 minutes and the DNA visualised and photographed under UV light. If PCR (polymerase chain reaction) of riboprobes was not to commence immediately, purified plasmids were stored at -20°C.

2.7.2 Polymerase Chain Reaction (PCR) of plasmids for riboprobes

In order to amplify the sequence of interest within the plasmid. Specific forward and reverse primers were designed to the RNA polymerase initiation sites contained within the plasmid (See **Table 2.3**). The sequence of primers for T7 and T3 were:-

TAATACGACTCACTATAGGGCGA (T7)

AATTAACCCTCACTAAAGGGAAC (T3)

A standard PCR was performed using the following reaction mix:-

10µl 10x Thermostart standard buffer (Abgene, Epsom, Surrey, UK)

6 μ l MgCL₂ (1.5mM)

2 μ l dNTP mix (20mM mixture, 5mM each of A, C, G and T)

2 μ l each of forward and reverse primers (5 μ M each)

0.5 μ l Taq DNA polymerase (Abgene)

75.5 μ l nuclease free water

Total volume 100 μ l

The ThermoStart enzyme requires a heat activation step at the beginning of the PCR. Annealing temperature for VEGF, Flt, KDR, Ang-1, Ang-2 and Tie-2 was 52°C. The following PCR protocol was used:-

95°C	10min	Enzyme activation	
95°C	30secs	Denature	} 30 cycles
52°C	30secs	Primer annealing	
72°C	1min 30secs	Extension	
72°C	10mins	Final extension	

PCR products were then purified using a Boehringer High Pure PCR Product purification kit (Boehringer Mannheim) according to manufacturers instructions. As with the plasmid purification column, the riboprobe is bound to the column while plasmid template and other contaminants are washed through with a series of washing buffers. The purified product is then eluted off the column. Concentration of DNA was measured on a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech) and the product run on an agarose gel as before. The length of the expected cDNA sequence was known (**Table 2.3**) and so it was possible to verify that the correct product had been amplified by reference to the gel.

2.7.3 Synthesis of riboprobe (cold labelling)

Having purified the PCR product, it was necessary to determine whether or not the cDNA could be transcribed into a functional sense and antisense riboprobe. In order for this to be tested, a cold labelling was performed. Sense and antisense

riboprobes (generated from the same purified PCR product), were made using T7 and T3 RNA polymerases (See **Table 2.3**) Riboprobes were synthesised using a MAXIscript *in vitro* transcription kit (Ambion Inc., Austin, Texas, USA). 300ng purified PCR product was added to a reaction mixture containing 1 X transcription buffer (Ambion), 0.5mM of each rNTP (ribonucleotide triphosphate; riboadenosine triphosphate, rATP, ribocytosine triphosphate, rCTP, riboguanine triphosphate, rGTP and ribouridine triphosphate, rUTP), 10U RNA polymerase and nuclease free water to 20 μ l. This mixture was incubated at 37 °C for 1h. After which 1U DNase (deoxyribonuclease) was added for a further 15 minute incubation at 37 °C. The product was run on a gel to check that transcription had been successful.

2.7.4 ³⁵S labelling

This is essentially the same as the cold labelling. 300ng DNA are added to the following reaction mixture, 10U RNA polymerase, 1x transcription buffer 0.4mM rCTP, rGTP and rATP, (rUTP was added at 0.04mM concentration). This is to allow for some incorporation of cold UTP and some incorporation of ³⁵S labelled UTP. ³⁵S labelled UTP is a large molecule and so if incorporated in close proximity to another ³⁵S labelled UTP, it may be stereoinhibited. By adding a low concentration of cold UTP, this incorporates at random to limit this.

In addition to this standard reaction mixture 2.3MBq ³⁵S labelled UTP was added (NEN life science products, Boston, MA, USA) and 0.4mM Dithiothreitol (DTT), to prevent formation of disulphide bridges, up to a final volume of 25 μ l with nuclease free water. Reaction mixtures were incubated at 37 °C for 45 minutes and then for a further 45 minutes after addition of another 10U enzyme. Finally 1U of DNase was added and incubated for 15 minutes at 37 °C.

After riboprobe synthesis, incorporation of ³⁵S UTP was measured by counting the total radioactivity (counts per minute, 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), according to manufacturers 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 minute.

2.7.5 *In situ* hybridisation procedure

In situ hybridisation experiments were conducted for VEGF-A, Flt, KDR, Ang-1, Ang-2 and Tie-2. For each tissue of interest, two serial sections were cut, one was treated with the sense probe and the other with the antisense probe. Only tissues from the same *in situ* experiment were compared for analysis. 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. Tissue sections were cut wearing disposable gloves onto sterile slides. All solutions used on the first day of the *in situ* hybridisation were made up with Diethyl pyrocarbonate (DEPC, Sigma) treated water (1ml per litre ultra pure water), which had been autoclaved three times. After hybridisation subsequent solutions were made up in ultra pure water. Additionally, chemicals were retained for *in situ* use only with no usage without sterile equipment and gloves.

Fresh xylene and alcohols were prepared before each experiment. Tissue sections were dewaxed and rehydrated. Then placed in 0.2N HCL (to denature proteins) for 20 minutes followed by two 5 minute DEPC water washes. Proteolytic digestion of tissues was performed using 5µg/ml proteinase K in 1M Tris, 0.5M EDTA, for 30 minutes at 37°C. (Optimised for all probes). The proteinase K digestion was stopped by transfer of slides to a 0.2% glycine solution at 4°C for 10 minutes. Slides were then transferred to 0.1M triethanolamine buffer (TEA, pH 8 with 5M NaOH) for 5 minutes before incubation in TEA buffer/0.25% acetic anhydride for 10 minutes. Sections were then washed in 4X SSC (salt sodium chloride containing 0.6M NaCl, 0.06M Na citrate, pH 7) for 5 minutes.

Hybridisation

Sections were dried round, laid in sterile humid boxes and incubated for 2h at 55°C in 100µl prehybridisation buffer containing; 50% deionised formamide, 4x SSC, 1xDenhardtts, 125µg/ml salmon testes DNA, 125µg/ml yeast tRNA and 10mM 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 for VEGF-A and 1×10^6 cpm per

50µl for all other probes). Before addition of hybridisation buffer to slides, the probe (in buffer) was denatured immediately before use by heating to 95°C for 10 minutes. Sections were coverslipped (Gel Bond) and incubated overnight at 55°C in a humid box.

The following day, RNase free conditions were no longer required. After removal of coverslips and a subsequent 10 minute wash in fresh 4xSSC, slides underwent RNase-A treatment (20µg/ml Rnase A in 1M Tris, 0.5M EDTA, 0.5M NaCl buffer, pH 8) at 37°C for 30 minutes. This was followed by a wash in RNase buffer containing no RNase-A for 30 minutes. Slides were then subjected to repeated 30 minute stringent post hybridisation washes, at room temperature unless otherwise stated (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 minutes each, before being allowed to air dry for 3-4h.

Slides were dipped in G5 emulsion (Illford, H + A West, Edinburgh, UK), diluted 1:1 in distilled water and heated until liquefied at 45°C, in a dark room. Slides were then dried overnight in a light proof humid box at 4°C, before dessicant was added and emulsion exposed at 4°C for a certain number of weeks depending on probe. VEGF, Flt and KDR were exposed for 5, 9 and 9 weeks respectively for corpus luteum and 12 weeks for uterus. Ang-1, Ang-2 and Tie-2 were exposed for 12 weeks in uterus. Exposure times were determined by inclusion of trial slides in each *in situ* hybridisation experiment for each probe and tissue type that were 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 minutes at 14°C, rinsed in distilled water then fixed in Kodak GBS Fixer (Sigma), diluted 1:5 in distilled water, for 10 minutes at room temperature. Slides were again rinsed in distilled water, counterstained with haematoxylin for 20 seconds, cleared in xylene and mounted in pertex as previously described for immunocytochemistry.

2.7.6 Quantification of *in situ* hybridisation

Specific hybridisation to tissues was quantified by grain density measurements. This was either carried out using an image analysis macro for corpora lutea where the cross section of cells forms a homologous picture without zonation, or by eye in the uterus where tissue zonation was apparent. It would be impossible to use this image analysis macro on the uterine sections as it assumes homology of the tissue between fields as occurs in the corpus luteum, but clearly does not occur in the uterus where some fields may contain glands, others stroma etc. Grain area was also measured in ovaries using an image analysis macro. The reason that grain area and density were both used is that grain area gives an indication as to how many cells are expressing a gene, whereas grain density gives an indication as to how highly an individual cell is expressing a given gene. The image analysis macro was designed to distinguish silver grains in dark field at x20 objective magnification against the black background of the tissue. Where scoring was carried out by eye, in the case of the uterus, two independent observers used the following classification: - (0) No detectable specific signal above background levels, + (1) detectable but low grain density clearly above background levels, ++ (2) Moderate grain density, +++ (3) high grain density without grain coalescence and ++++ (4) grain coalescence. Scoring of tissues was blind and random by each observer. Statistical analyses were performed for the data obtained. If grain counts were conducted using a pre-designed macro, ANOVAs were used. However, if scoring was by eye, significant differences between groups were determined using a Kruskal-Wallis non-parametric test followed by Dunn's multiple comparisons test.

2.8 Statistical analysis of data

Specific details of statistical analyses will be given in the relevant chapters. Where more than two variables were being compared, an analysis of variance was used (ANOVA) followed by a Bonferroni's post hoc test if the data was parametric. In this thesis all data was normally distributed and hence an ANOVA was applied. One exception was in the scoring analysis of *in situ* hybridisation in the marmoset endometrium, where data was skewed towards the upper end of the distribution across the groups studies. In this circumstance, where data was non-parametric, a

Kruskal-Wallis, followed by Dunn's multiple comparisons test was used. If only two data sets were to be compared two-tailed unpaired t-tests were used. In all cases, the level of significance was $P < 0.05$. All tests were performed using SPSS 10 for the macintosh.

Chapter 3

Angiogenesis in the corpus luteum of early pregnancy

3 Angiogenesis in the Corpus Luteum of Early Pregnancy

3.1 Introduction

Angiogenesis has been shown to be intense during the first few days of the lifespan of the corpus luteum (CL) in a large number of species studied, including sheep, marmoset, macaque and women (Christenson and Stouffer, 1996, Dickson and Fraser, 2000, Rodger, et al., 1997, Zheng, et al., 1994) and is stimulated principally by vascular endothelial cell growth factor (VEGF) (Dickson, et al., 2001, Fraser, et al., 2000).

In the corpus luteum of early pregnancy, the changes in angiogenesis and the role of the luteal vasculature in the maintenance of progesterone secretion remain to be elucidated. When pharmacological 'rescue' of the CL by human chorionic gonadotrophin (hCG) was carried out in rhesus monkeys, no stimulation of angiogenesis, as monitored by Ki-67 incorporation, was observed despite maintaining plasma progesterone concentrations (Christenson and Stouffer, 1996). In apparent contrast, in the CL from women given hCG to mimic luteal rescue of early pregnancy, a second wave of angiogenesis, accompanied by vascular stabilisation by pericytes was associated with luteal rescue (Wulff, et al., 2001). This increase, documented by analysis of luteal sections, was dependent upon an increase in luteal cell volume being taken into account. In the study using rhesus monkeys luteal cell volume was not considered. It was also found that mRNA for VEGF increased in the CL after hCG rescue (Wulff et al., 2000). These findings suggest that luteal angiogenesis could be a component of luteal rescue which is important for prolonging the lifespan of the corpus luteum and the establishment of early pregnancy.

Since failure of the CL due to a malfunction of the vasculature could possibly lead to miscarriage or infertility, it was considered important to establish a non-human primate model of early pregnancy in which this issue could be addressed. The marmoset monkey offers several potential advantages for such studies. Firstly,

it has an exceptionally high rate of fertility (>70%) (Fraser, et al., 1994) when compared to other primates, so physiological pregnancy, rather than pharmacological hCG rescue may be studied. Secondly, the cellular and molecular regulation of angiogenesis during the normal cycle has been described previously (Dickson and Fraser, 2000). Angiogenesis was determined by comparing BrdU incorporation into corpora lutea of the late luteal phase versus early pregnancy. Vascular stability was monitored by determining pericyte area. Localisation of expression of VEGF mRNA and its receptors, KDR and Flt were determined and changes associated with pregnancy quantified using *in situ* hybridisation.

3.1.1 Specific Methods

3.1.1.1 Details of animals used for the experiment

Table 3.1. Summary of animals used in this experiment

Group	Mean age (years)	Mean Body mass (g)	Number of animals
LL Structurally intact	2.40	379	5
LL Structurally Regressed	3.12	325	3
P,21	2.19	341	6
P,28	2.68	356	6

3.1.1.2 Analysis of corpora lutea

In marmosets after ovulation, the follicle or follicles from which the oocytes were ovulated become luteinised and are termed primary corpora lutea. Often after ovulation, other follicles within the ovary also become luteinised. They do not differ in morphology to the primary CL except for the fact that they are smaller. While detailed descriptions of the analyses conducted in this experiment are given in Chapter 2, it should be noted that all primary and accessory corpora lutea were analysed in all cases. A comparison was conducted between the two groups for all parameters. In all cases, no significant difference between primary and accessory CL were found ($P < 0.05$ using 2 way ANOVA) hence results were combined.

3.2 Results

3.2.1 Hormone profile

Pregnancy was confirmed by the presence of a trophoblast and CG levels greater than 20ng/ml, while non-pregnant animals had a uterus compatible with an infertile cycle and non-detectable levels of CG (**Figure 3.1**).

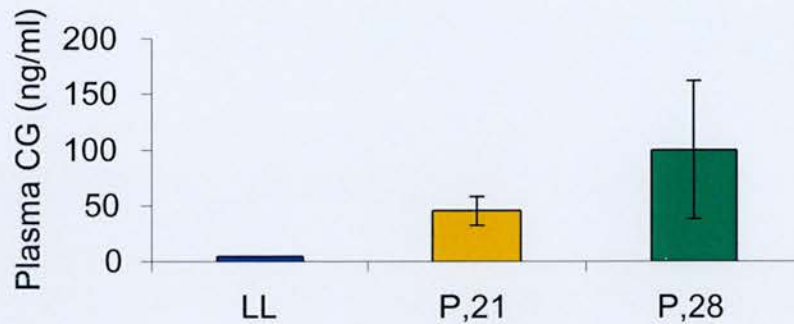


Figure 3.1 Mean plasma chorionic gonadotrophin (ng/ml) from animals at the different stages studied. Pregnancy was confirmed by CG levels greater than 20ng/ml. Values are means \pm SEM. (For LL n=8, and n=6 for P,21 and P,28)

Plasma progesterone profiles of pregnant and non-pregnant marmosets from ovulation (day 0) to day 21 of the cycle are illustrated in **Figure 3.2**. The late luteal non-pregnant animals have been subdivided into two groups, according to whether functional and structural regression had taken place by day 21. The corpora lutea of 5 animals in this group remained structurally intact despite being anticipated to shortly undergo structural regression. One of these intact CL was from an animal which was housed in the absence of a male. Thus the non-regressed state of the CL, was not dependent on a male being present. There was no observable difference in plasma progesterone levels between these non-pregnant control animals and the pregnant animals from day 0 to 21 of the cycle. Three of the late luteal control animals had undergone functional and structural regression. In these animals, there was a

significant difference in plasma progesterone as determined by a 2-way ANOVA compared to both the pregnant and control animals. In all four groups there was an initial rise in progesterone indicative of ovulation. Progesterone levels then remain elevated ($>150\text{nmol/L}$) until day 10-11 which corresponds to the time of implantation in marmosets (Enders, 1993). At day 10-11, the regressed late luteal control animals showed declining progesterone for the remainder of the recorded cycle, whereas a continued elevation of progesterone was observed in both groups of pregnant animals and the structurally intact control animals. Statistical analysis (2-way ANOVA) revealed that progesterone levels in the regressed control group were significantly different to the structurally intact controls and 3 (day 21) and 4 (day 28) week pregnant animals ($P<0.05$) from day 16. Progesterone levels remained elevated until tissue collection in day 28 pregnant animals although **Figure 3.2** only illustrates the progesterone profile until day 21. Data up to day 28 was not included in order that the graph be consistent across all groups studied. Progesterone levels remained at the same level between day 21 and 28 in day 28 pregnant animals. Progesterone levels were not compared against the number of corpora lutea per ovary.

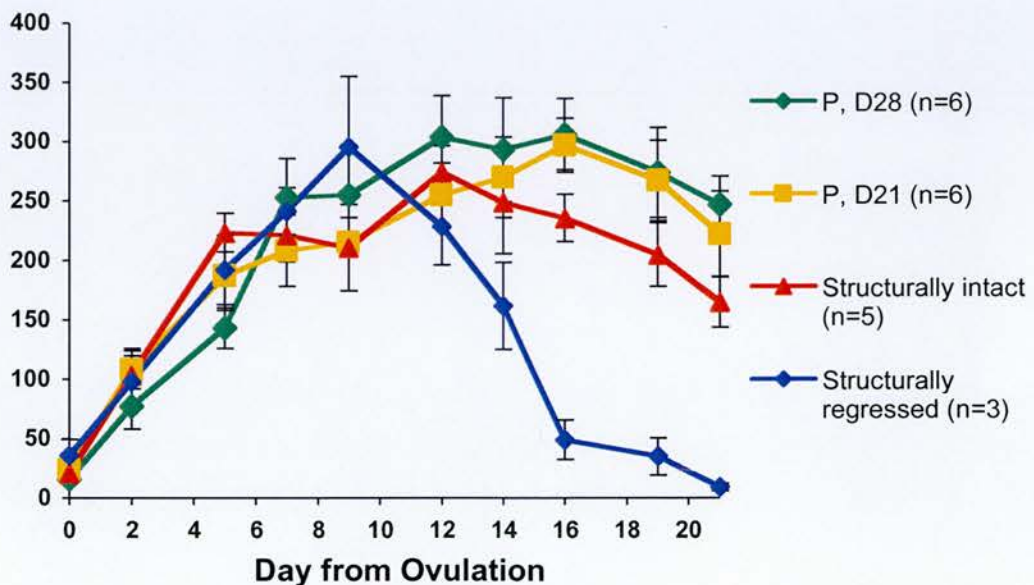


Figure 3.2 Plasma progesterone concentrations from ovulation to day 21 in pregnant

and non-pregnant animals. Values are means \pm SEM. (n=6, day 21 and 28 of pregnancy; n=5, day 21 structurally intact; and n=3 in day 21 regressed). In the five non pregnant animals with structurally intact CL, elevated progesterone levels continued up to day 21 despite anticipated luteal regression. Progesterone levels from three non pregnant animals with structurally regressed CL, however, had declined to follicular phase levels by Day 21.

3.2.2 Lutein cell area

3.2.2.1 Method of quantification

The marmoset is a multiovular species, therefore the ovary contains several corpora lutea. 10 fields of view were randomly chosen across the largest corpus luteum present in an ovarian cross section. Within each of these fields 10 cells were randomly selected and cross sectional area measured by free hand drawing around the circumference of the cell using image analysis equipment. This area was then filled in on the screen and the area of the blackened section measured.

3.2.2.2 Results

Cross-sectional area of individual lutein cells was not significantly different using a 1-way ANOVA ($P>0.05$) between structurally intact non-pregnant CL and the pregnant animals at day 21 and 28 (492 ± 26 , 530 ± 21 and 584 ± 34 , respectively. Values are means \pm SEM). The structural integrity of the regressed corpora lutea was such that individual lutein cells could not be identified and thus not measured (**Figure 3.3 and Figure 3.4**)

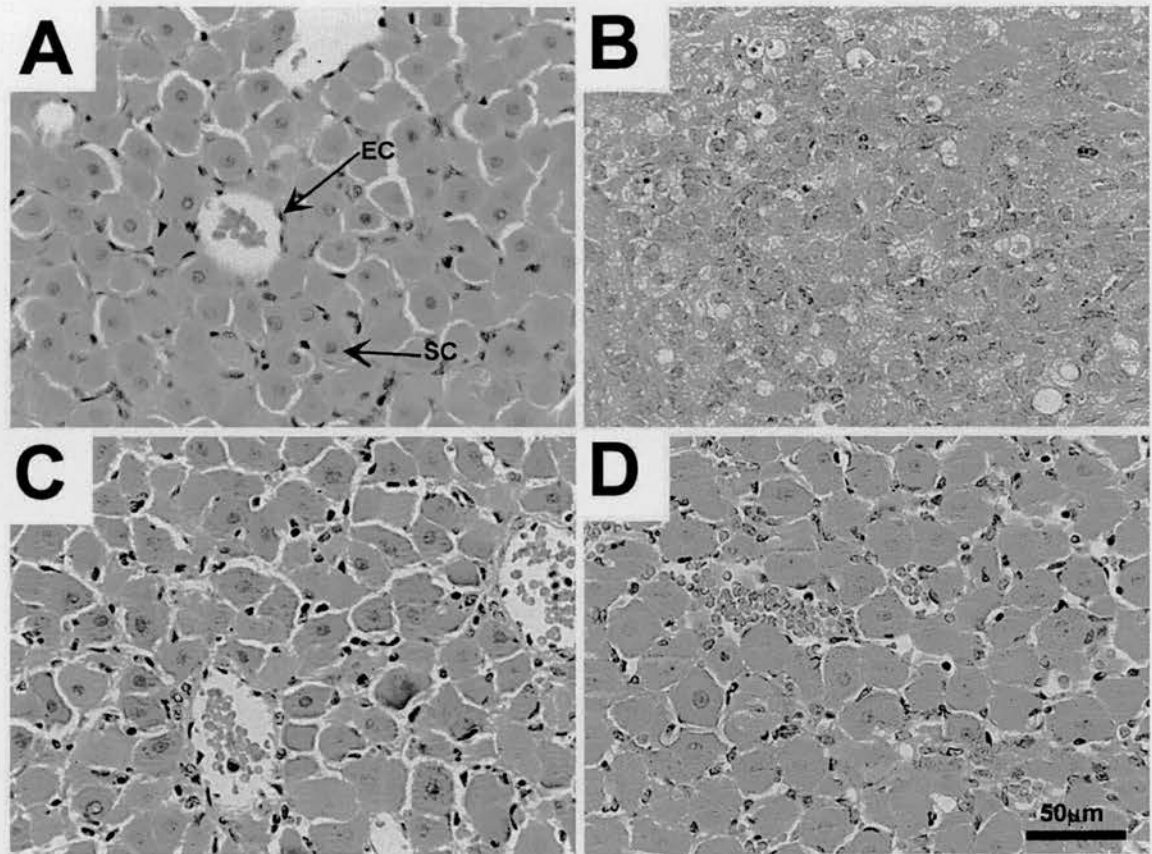


Figure 3.3 Haematoxylin and eosin stained sections of marmoset corpora lutea. (A) Structurally intact non-pregnant late luteal CL, (B) Structurally regressed non-pregnant late luteal CL, note absence of structural integrity, (C) CL at day 21 of pregnancy and (D) CL from day 28 of pregnancy. (EC, Endothelial cell, SC, Steroidogenic cell).

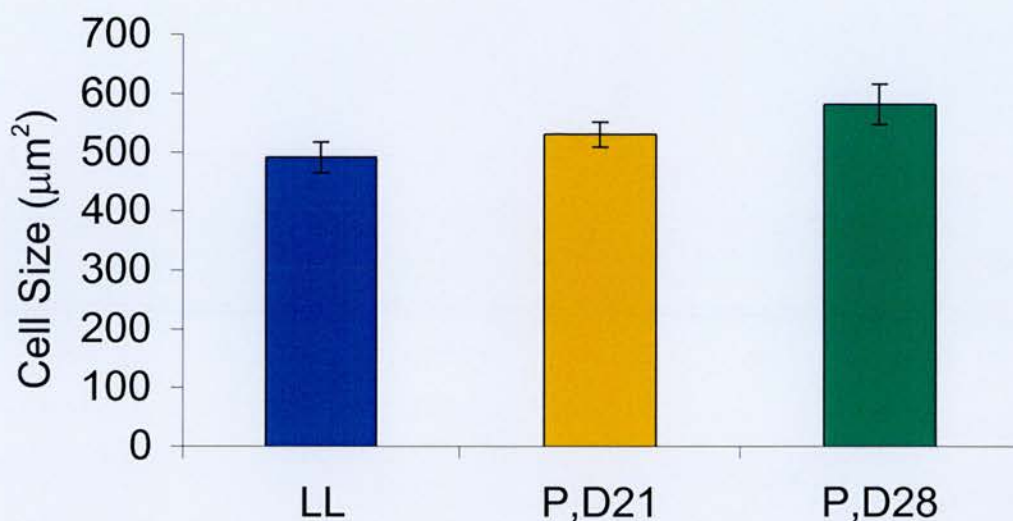


Figure 3.4 Cross-sectional area of lutein cells in corpora lutea of animals from the different study groups. Values are means \pm SEM. There were no significant differences between study groups

3.2.3 Cell Proliferation

BrdU immunostaining indicating proliferating cells was readily detected in all corpora lutea. When quantified, by 1-way ANOVA, no significant difference ($P > 0.05$) was observed between structurally intact late luteal and pregnant CL, however, proliferation indices from structurally regressed CL were significantly higher ($P < 0.05$) than for all other groups apart from day 14 pregnant animals (**Figure 3.5 and 3.6**). Previously published data (Dickson and Fraser, 2000), from the early and mid and late-luteal phase are included in **Figure 3.6** to show the changes in endothelial cell proliferation throughout the luteal phase of the cycle. An intense burst of angiogenesis with high endothelial cell proliferation is observed in the early luteal phase, decreasing in the mid-luteal phase and further still towards the end of the luteal phase. Data from 2 week (day 14) pregnant animals were included to investigate whether or not an intense burst of angiogenesis occurred earlier than 3 weeks (day 21). Day 14 corresponds to the peri-implantation period. However, proliferation indices were not found to be significantly different from other groups at this stage.

The insert in **Figure 3.5** illustrates the co-localisation of CD31 and BrdU immunostaining, ie. proliferating endothelial cells. Quantification showed that greater than 90 % of proliferating cells were endothelial cells.

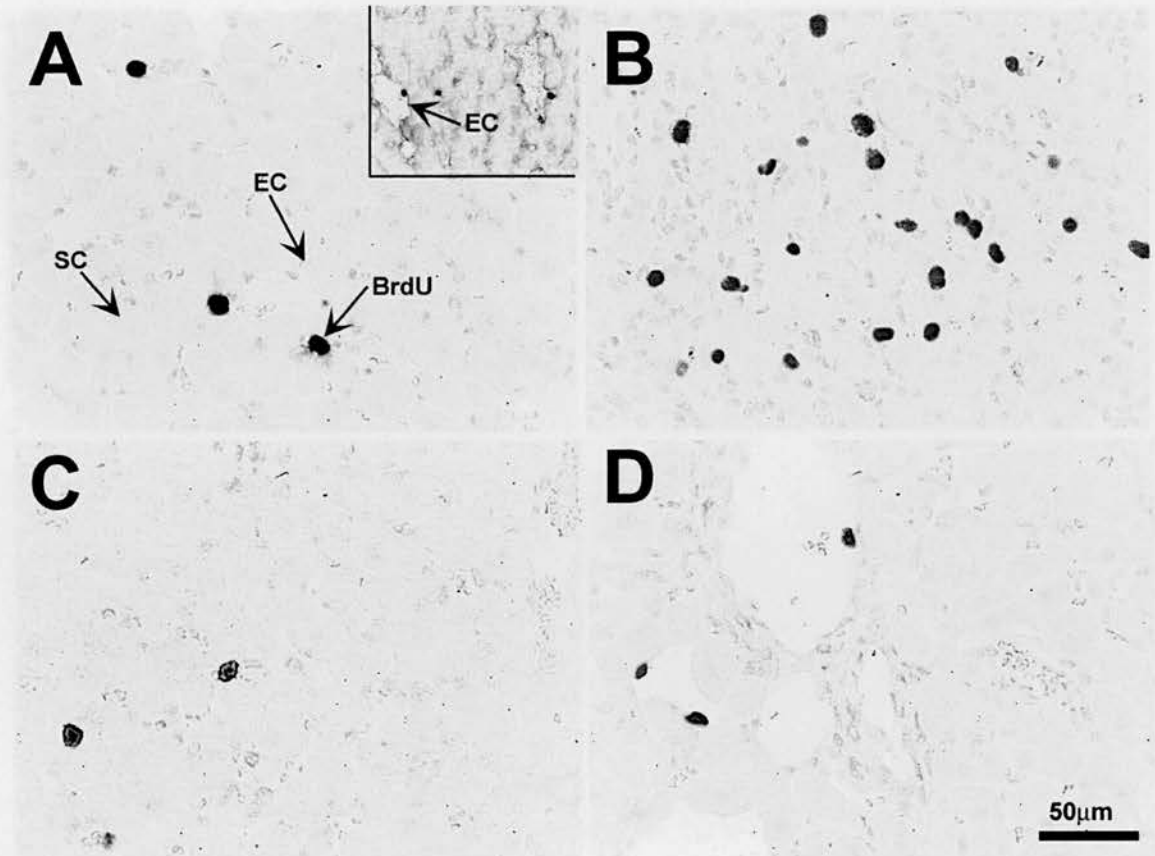


Figure 3.5 Cell Proliferation in the marmoset corpus luteum (x40 objective); (A) structurally intact late luteal CL, (B) structurally regressed late luteal, (C) Pregnant, day 21, (D) Pregnant, day 28. Insert in (A) shows colocalisation of CD31 and BrdU.

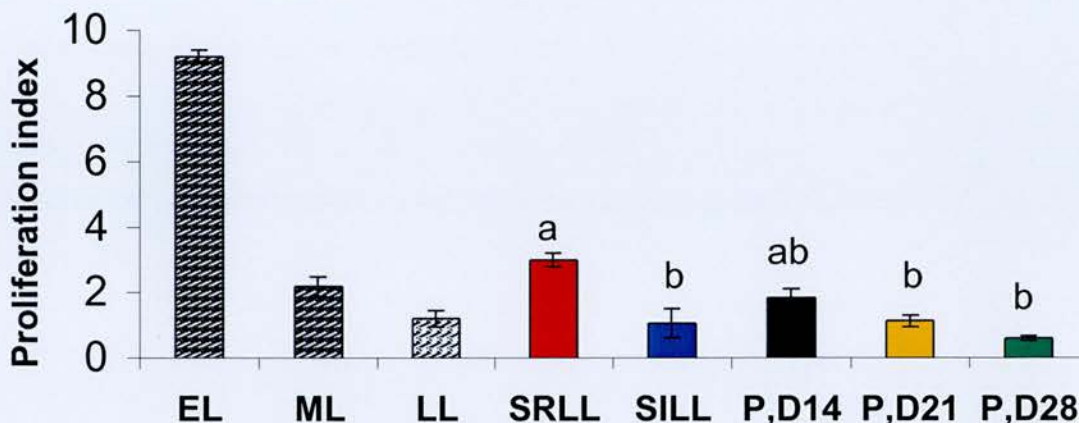


Figure 3.6 Mean proliferation index in corpora lutea of animals from the different study groups, early luteal (EL, day 4), Mid-luteal (ML, day 10) and late luteal (LL, day 21) from earlier studies, (Dickson *et al.*, 2000); Structurally regressed late luteal (SRLL), Structurally intact late luteal (SILL), Pregnant, day 14, 21 and 28 (P, D14, P,D21 and P,D28, respectively). Values are means \pm SEM. Mean proliferation index from structurally regressed CL was significantly higher than all other groups, apart from 2 week pregnant animals ($P < 0.05$), from 2-way ANOVA. Different letters denote significant differences.

3.2.4 Endothelial cell area

Measurement of CD31 was used to quantify endothelial cell area. There was no significant difference between groups ($P > 0.05$), using a 1-way ANOVA. (**Figure 3.7 and Figure 3.8**). By the late luteal phase, the corpus luteum is well vascularised and from the photomicrograph (**Figure 3.7**) it can be seen that every lutein cell is in contact with at least one endothelial cell. Structurally regressed CL were not included in the quantitative analysis because of poor structural integrity leading to diffuse staining, as can be seen in **Figure 3.7B**.

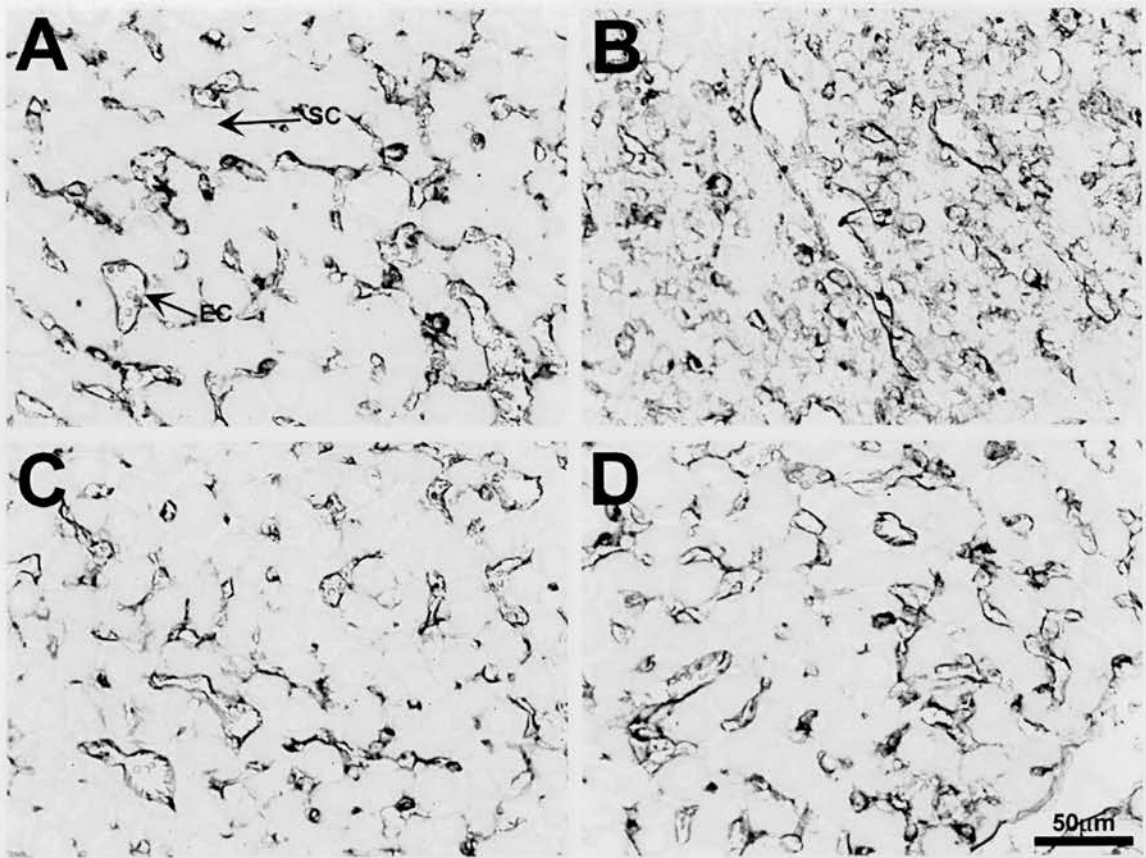


Figure 3.7 Immunocytochemical localisation of endothelial cell marker CD31. Structurally intact late luteal (A), structurally regressed late luteal (B), pregnant, day 21 (C) and pregnant, day 28 (D) corpora lutea are illustrated here. (SC) steroidogenic cell, (EC) endothelial cell. No significant difference in area of CD31 staining was observed between study groups.

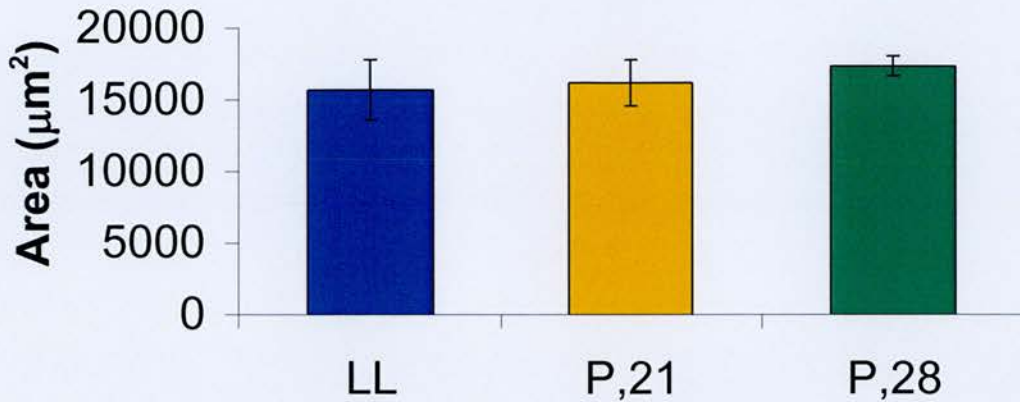


Figure 3.8 Mean endothelial cell area in corpora lutea of animals from the different study groups. Values are means \pm SEM. No significant differences were observed between groups.

3.2.5 Pericyte cell area

Immunostaining for α -smooth muscle actin was evident in all stages observed, predominantly in large luminal vessels, but also to a lesser extent in smaller vessels and capillaries (**Figure 3.9 and 3.10**). No significant difference in endothelium coverage by pericytes ($P > 0.05$) using 1-way ANOVA, was seen between study groups. Again structurally regressed CL were not quantified because of diffuse staining.

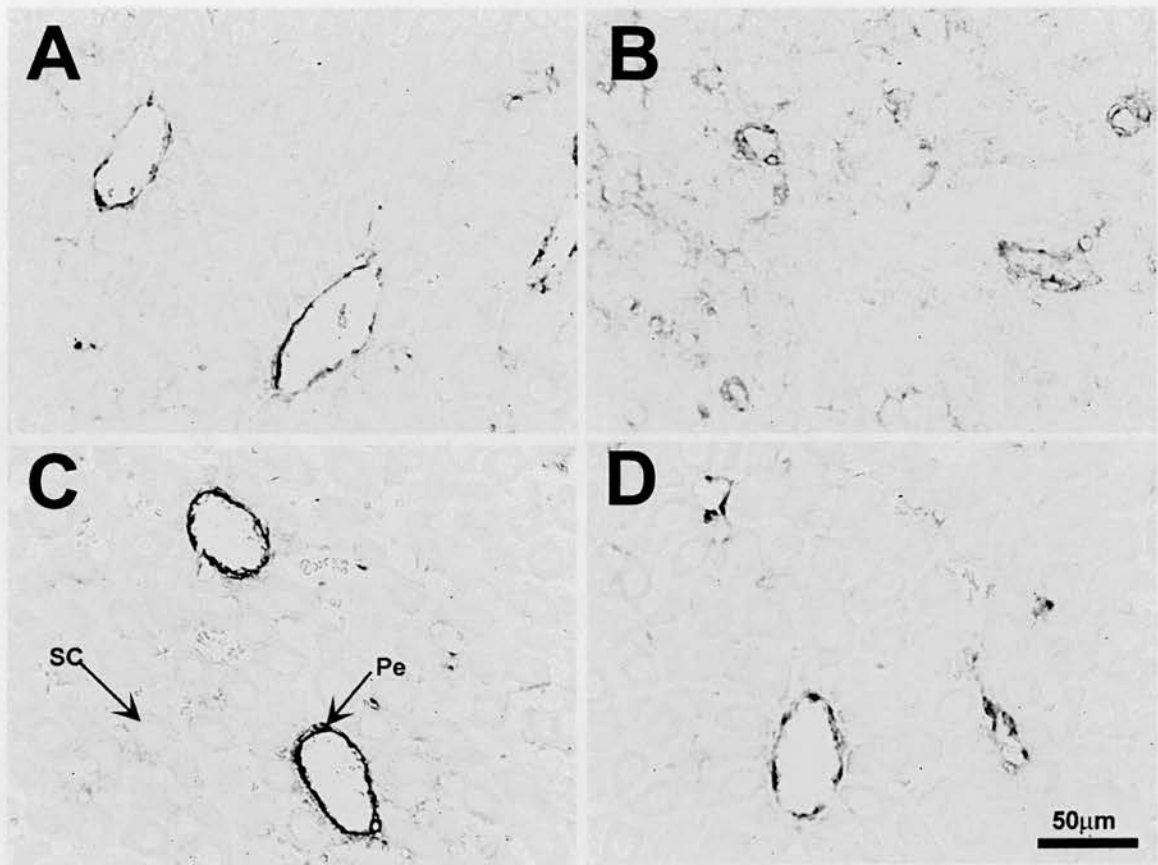


Figure 3.9 Immunocytochemical localisation of α -smooth muscle actin. Structurally intact late luteal (A), structurally regressed late luteal (B), pregnant, day 21 (C) and pregnant, day 28 (D) corpora lutea are illustrated here. (SC) steroidogenic cell, (Pe) Pericyte. No significant difference in area of α -smooth muscle actin staining was observed between study groups.

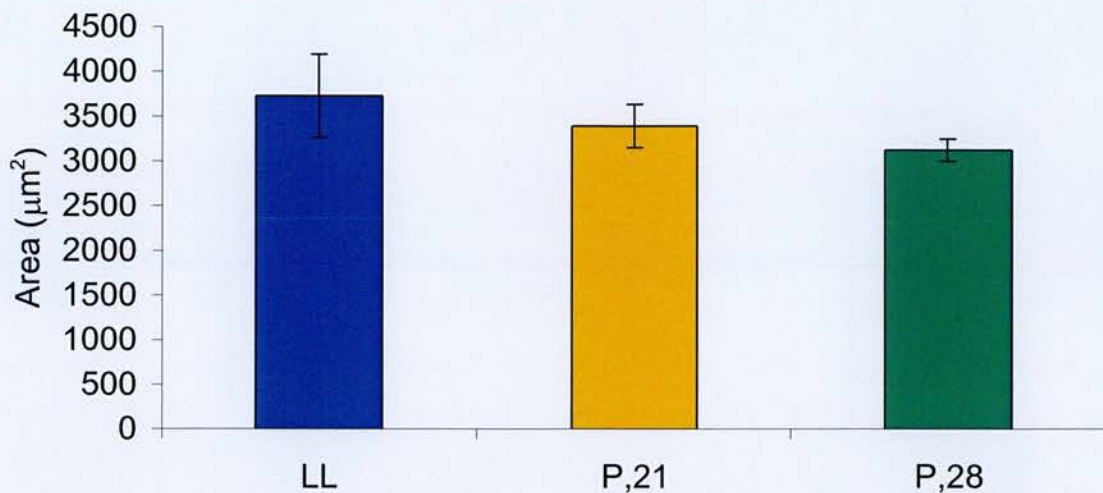


Figure 3.10 Mean pericyte area in corpora lutea of animals from the different study groups. Values are means \pm SEM. No significant differences were observed between groups.

3.2.6 VEGF

Both grain area and grain density were measured using an image analysis macro across the whole cross section of the corpus luteum. Intense hybridisation to VEGF mRNA was localised to lutein cells, with absence of hybridisation to endothelial cells. Distinct, punctate hybridisation in lutein cells was clearly seen in structurally intact control and pregnant CL (**Figure 3.11**). The exact cellular localisation of the grains was done by morphology of the sections under high power bright field microscopy. There was no significant difference using 1-way ANOVA ($P > 0.05$) in either mean grain area or mean grain density between these groups (**Figure 3.12**). In the structurally regressed CL, most of the lutein cells were devoid of punctate VEGF expression and there was a marked reduction in grain area and density (**Figure 3.11C and D**).

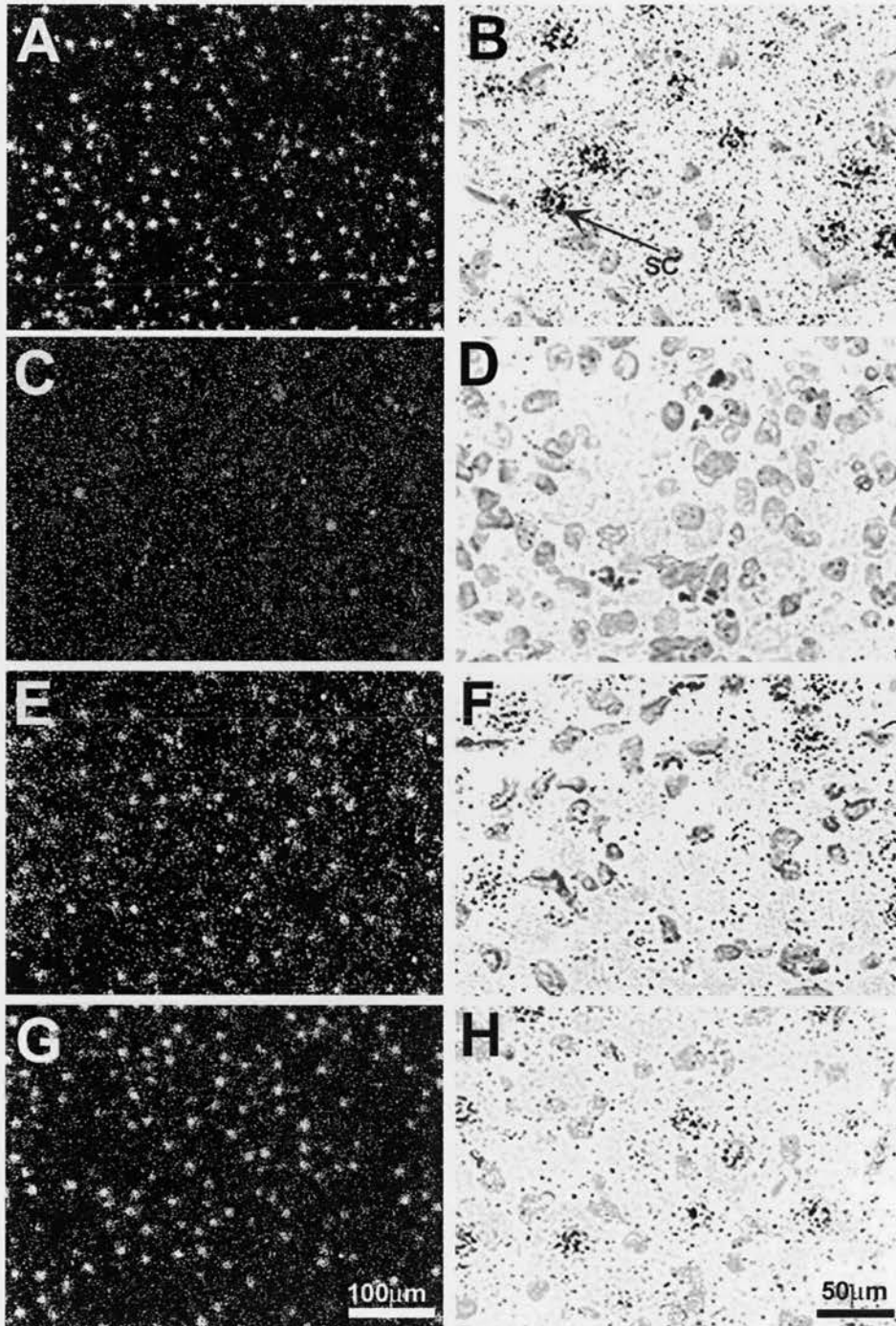


Figure 3.11 Photomicrographs illustrating localisation of VEGF mRNA to steroidogenic cells (SC) by *in situ* hybridisation. (A, C, E and G are dark field images of structurally intact, structurally regressed, pregnant, day 21 and pregnant, day 28 corpora lutea, while B, D, F and H are corresponding light field images.

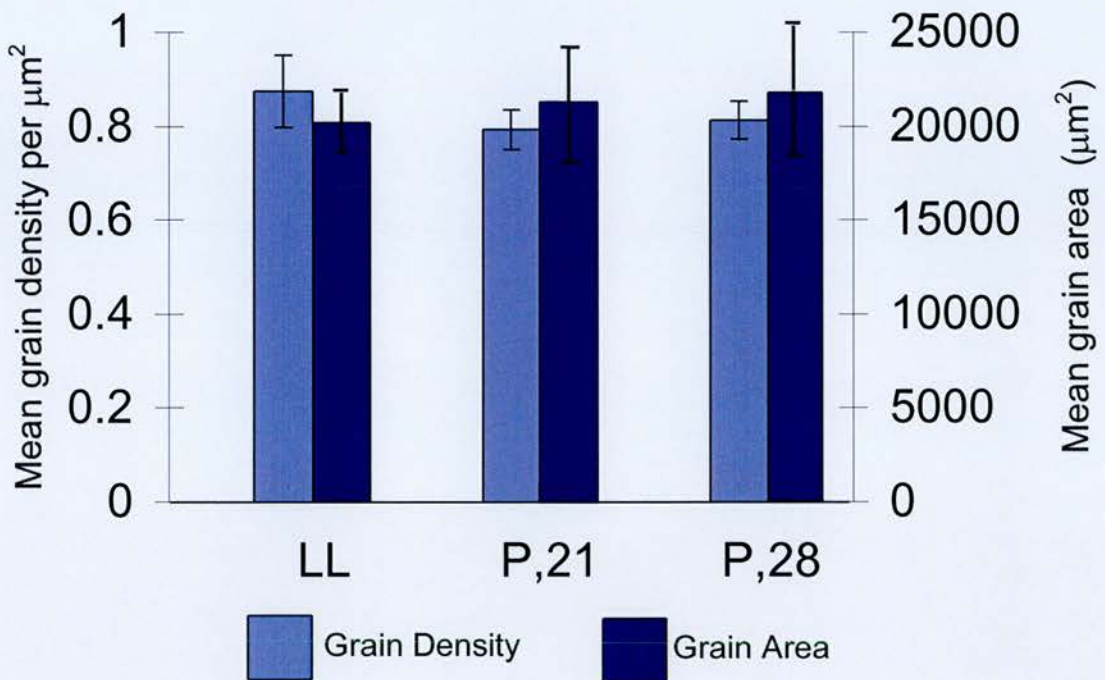


Figure 3.12 VEGF expression in marmoset CL. Mean grain density/ μm^2 and mean grain area (μm^2) in nonpregnant and pregnant CL. No significant differences were observed between these groups by 1-way ANOVA. Values are means \pm SEM.

3.2.7 KDR

Both grain area and grain density were measured using an image analysis macro across the whole cross section of the corpus luteum. Hybridisation to KDR mRNA was localised to endothelial cells (**Figure 3.13**). No significant difference in grain area or density was observed between study groups ($P > 0.05$) using 1-way ANOVA as illustrated by **Figure 3.14**. Structurally intact and regressed non-pregnant CL were analysed independently but, in contrast to VEGF, KDR expression was maintained in the regressed CL. Since grain area and density were the same in each group, the results were combined.

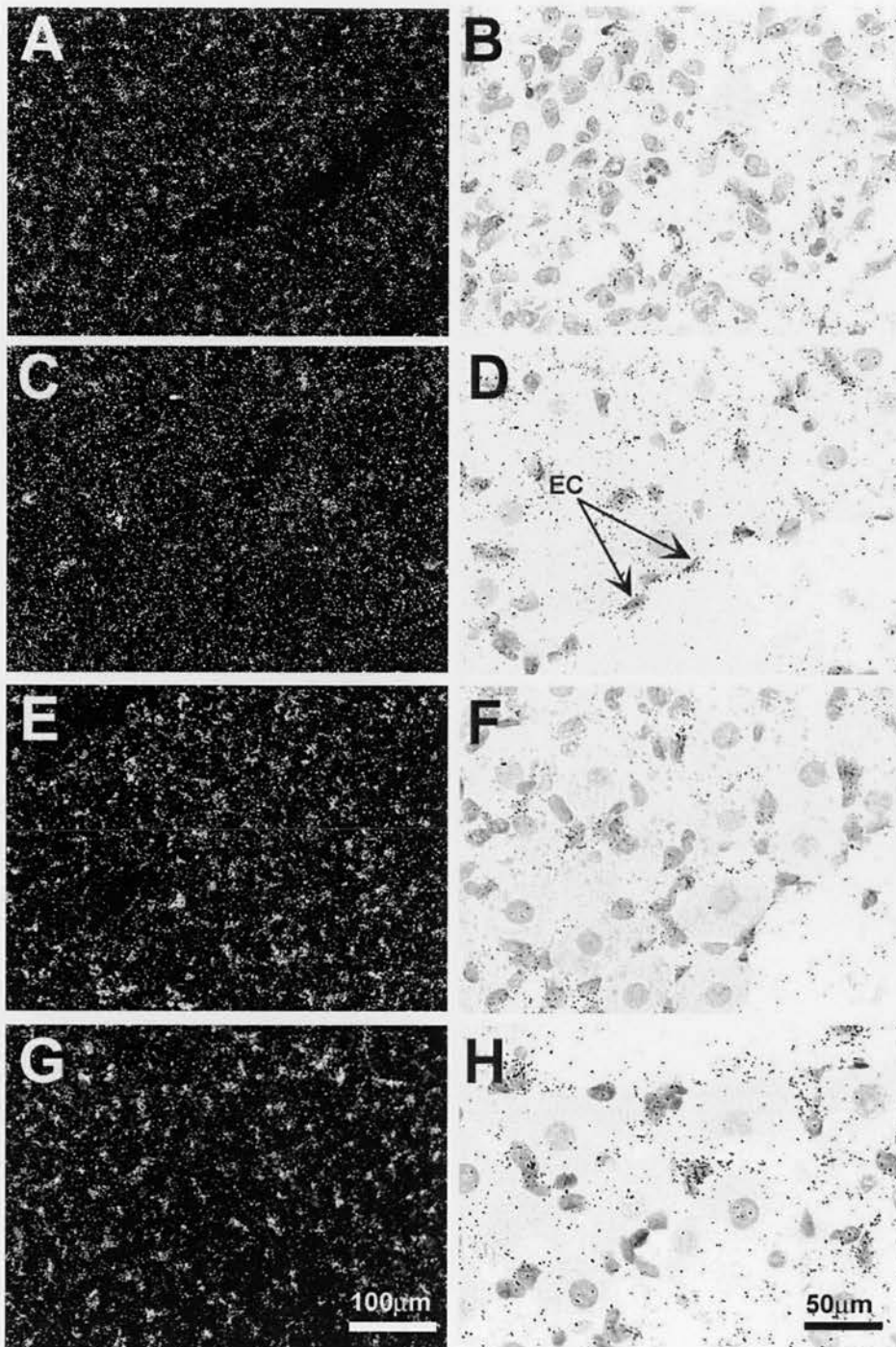


Figure 3.13 Photomicrographs illustrating localisation of KDR mRNA to endothelial cells (EC) by *in situ* hybridisation. (A, C, E and G are dark field images of structurally intact, structurally regressed, pregnant, day21 and pregnant, day 28 corpora lutea, while B, D, F and H are corresponding light field images.

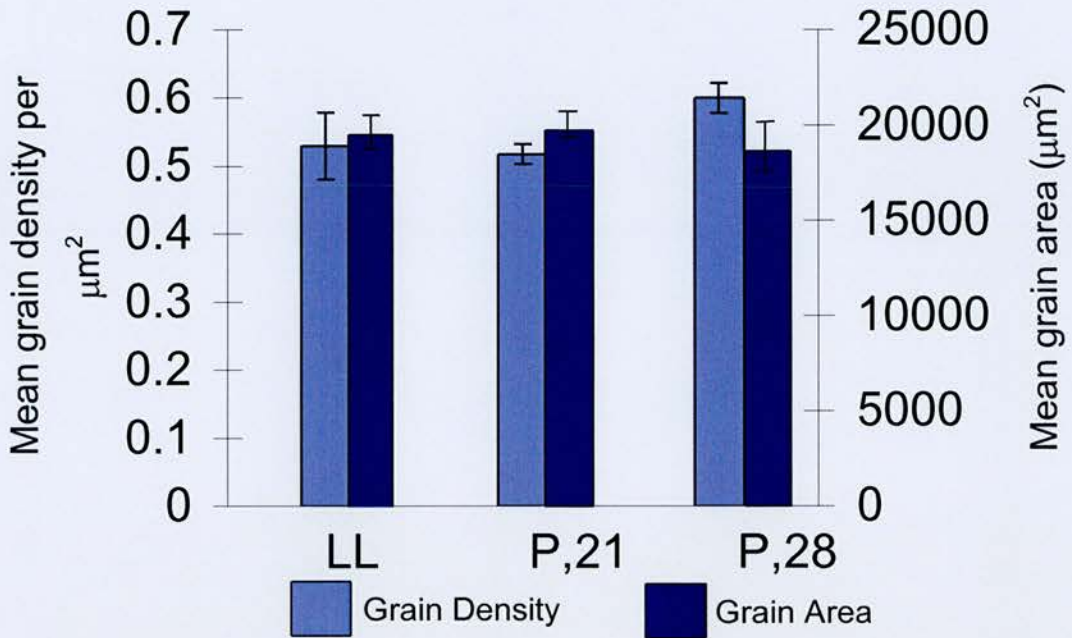


Figure 3.14 KDR expression in marmoset CL. Mean grain density/ μm^2 and mean grain area (μm^2) in nonpregnant and pregnant CL. No significant differences were observed between these groups by 1-way ANOVA. Values are means \pm SEM.

3.2.8 Flt

Both grain area and grain density were measured using an image analysis macro across the whole cross section of the corpus luteum. Hybridisation to Flt mRNA was localised to endothelial cells of the CL **Figure 3.15**. A significantly lower mean grain area was observed in day 28 pregnant corpora lutea than in late luteal animals ($P < 0.05$) by 1-way ANOVA, as illustrated by **Figure 3.16**. However, no significant difference in grain area was seen between late luteal and pregnant day 21 animals, nor was there any observable difference in grain density between groups. As above, late luteal regressed and intact controls were combined as there was no difference in Flt expression between them.

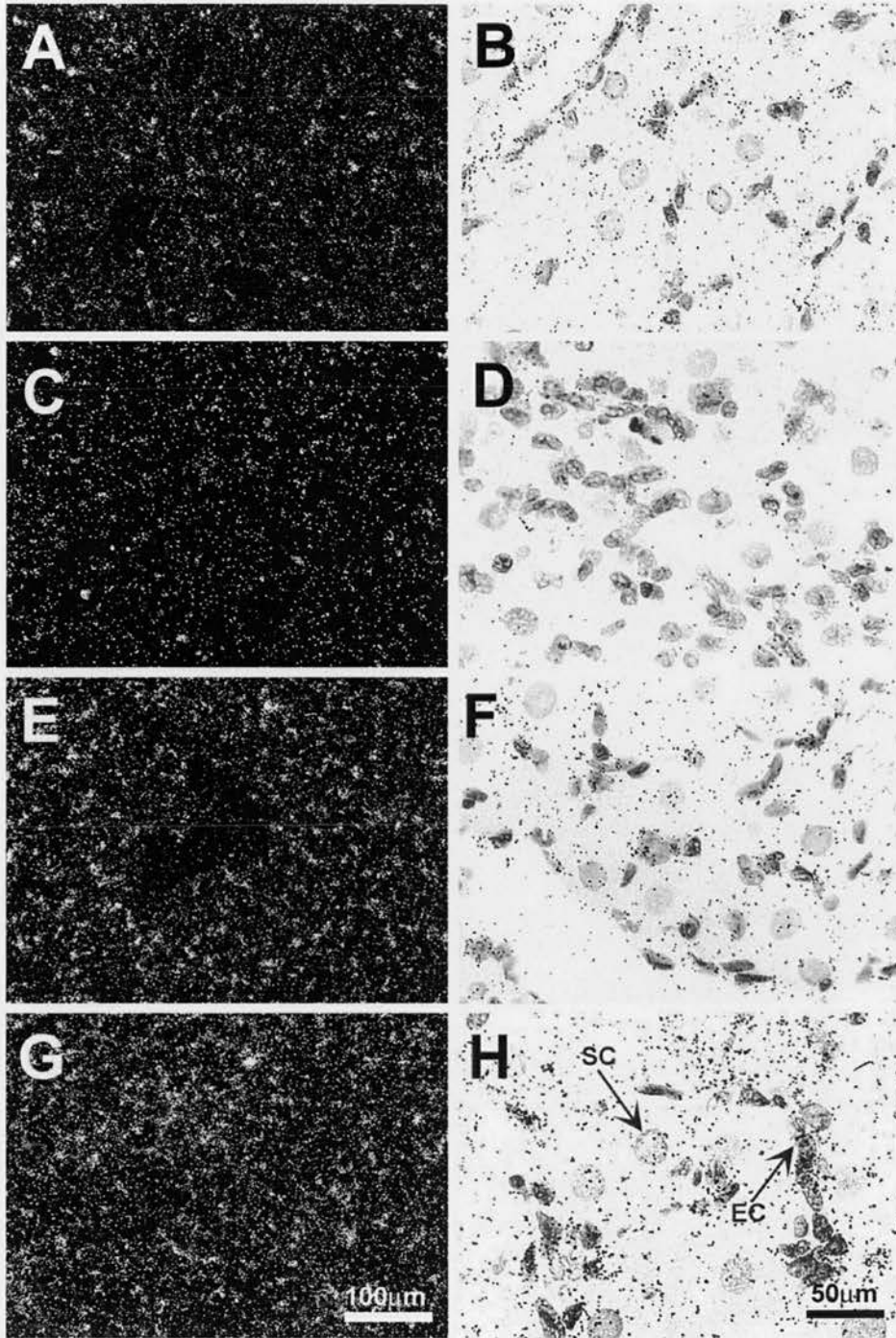


Figure 3.15 Photomicrographs illustrating localisation of Flt mRNA to endothelial cells (EC) by *in situ* hybridisation. (A, C,E and G are dark field images of structurally intact, structurally regressed, pregnant, day21 and pregnant, day 28 corpora lutea, while B, D, F and H are corresponding light field images.

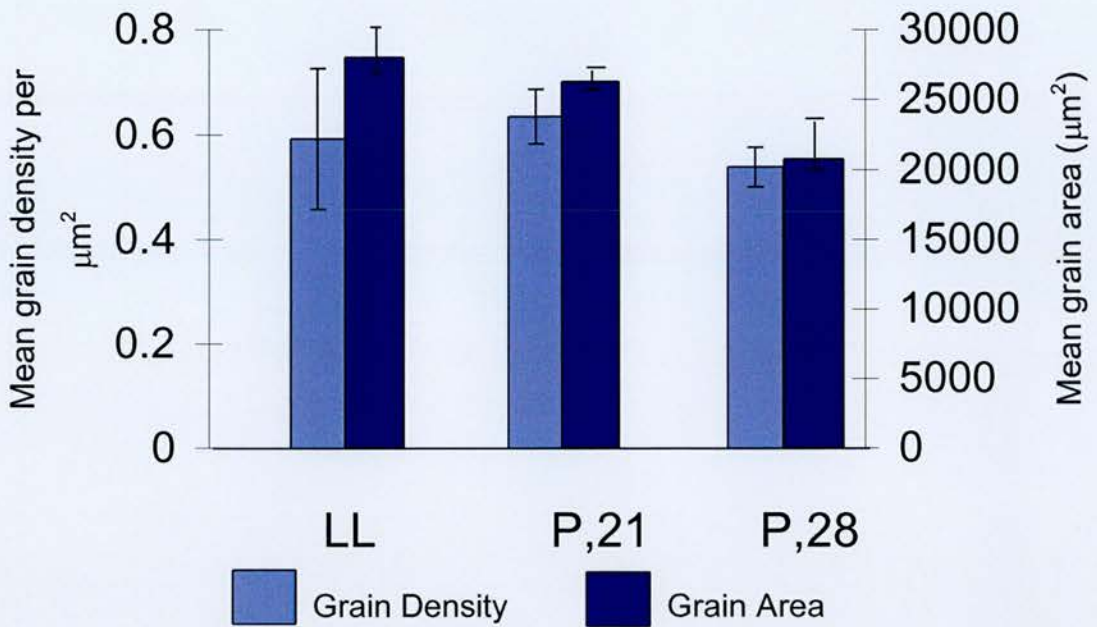


Figure 3.16 FIt expression in marmoset CL. Mean grain density/ μm^2 and mean grain area (μm^2) in nonpregnant and pregnant CL. A significantly lower mean grain area was observed in day 28 pregnant corpora lutea compared to late luteal animals ($P < 0.05$) by 1-way ANOVA. No other significant differences were observed. Values are means \pm SEM.

3.2.9 Ang-1, Ang-2 and Tie-2

No photographic or data are included for Ang-1, Ang-2 and Tie-2. However it should be noted that no expression above background levels of any of these genes was observed in the marmoset corpora lutea studied. Marmoset endometrium was used as a positive control in all experimental runs.

3.3 Discussion

This experimental chapter has described the cellular and morphological changes in the corpus luteum associated with early pregnancy in the marmoset. Neither luteal cell area, endothelial cell area, pericyte area or endothelial cell proliferation differed in early pregnancy from the late luteal CL of the non-pregnant

cycle. However, the proliferation index in structurally regressed CL was significantly greater than that recorded from CL of all other groups. It may be that these rapidly proliferating cells are white cells however, due to the disorganisation of the tissue at this time, immunocytochemical detection of white cells not carried out. We had no reason to believe that this was non-specific BrdU incorporation as the BrdU staining was specific in all other sections. In addition, levels of VEGF, KDR and Flt mRNA were largely unaltered. Thus, a further burst of angiogenesis does not occur in early pregnancy in the marmoset. It would appear that the late, pre-regressed luteal vasculature is equipped with the molecular and cellular components to perform the functions of the vasculature of the CL of early pregnancy.

In the human corpus luteum, endothelial cell proliferation mirrors that of the marmoset in the early, mid- and late luteal phase, where maximal proliferation occurs in the early luteal phase and declines significantly in the mid- and late luteal phase (Gaytán, et al., 1998, Rodger, et al., 1997). This is also true in the rhesus monkey, (Christenson and Stouffer, 1996), sheep (Jablonka-Shariff, et al., 1993, Jablonka-Shariff et al., 1997) and in cattle (Zheng, et al., 1994). During pharmacologically-induced luteal rescue in women by hCG, an increase in luteal cell volume, endothelial cell proliferation, pericyte recruitment and VEGF expression is observed, indicating an increased rate in angiogenesis accompanied by vessel stabilisation (Wulff, et al., 2001). This appears to mirror the situation observed in rat where endothelial cell proliferation is also seen to increase in early pregnancy (Meyer and McGeachie, 1988, Tamura and Greenwald, 1987). In contrast, there does not appear to be an increase in endothelial cell proliferation in early pregnancy in the marmoset, as in sheep (Jablonka-Shariff, et al., 1997) or in hCG-induced rescue of the CL in the rhesus monkey (Christenson and Stouffer, 1996).

The rescue of the corpus luteum of pregnancy allows it to persist functionally and structurally for longer than in the non-pregnant cycle. Presumably, this survival requires a stable vasculature, with recruitment of pericytes and prolonged endothelial cell survival, in addition to prolongation of the lifespan of the luteal cells. Since endothelial cell proliferation did not increase after administration of hCG in the rhesus monkey (Christenson and Stouffer, 1996), it was suggested that early pregnancy may be associated with an increase in vessel stability, and there is recent

evidence for hCG-induced increase in pericyte number in the human CL (Wulff, et al., 2001). However, no evidence for this was found here.

The increase in pericyte number in the human was dependent upon taking account of the increase in lutein cell volume after hCG administration (Wulff, et al., 2001). However, in the marmoset, no such increase was observed, consistent with Webley *et al.*, (Webley et al., 1990), who examined cell size distribution of luteal cells from pregnant and non-pregnant marmosets. It was concluded that there is a significant increase in mean cell diameter between day 6 and day 14 in both pregnant and non-pregnant animals but no further increase on day 20.

While in the structurally regressed CL, VEGF expression was markedly reduced, there was no apparent difference in VEGF expression between structurally intact late luteal controls and pregnant corpora lutea in the marmoset. VEGF expression is not significantly altered throughout the functional lifespan of the human corpus luteum, which is consistent with the high expression seen in the marmoset in the structurally intact CL (Berisha et al., 2000, Otani et al., 1999, Wulff, et al., 2000). A significant increase in VEGF expression, in terms of both grain area and density, compared to the late luteal phase was found in hCG rescued human CL, using the same method as the present study (Wulff, et al., 2001). Cell culture studies using luteinised granulosa cells, have also revealed that hCG treatment leads to increased VEGF mRNA levels (Neulen et al., 1998). This reported up-regulation of VEGF by hCG rescue supports the view that in the human, pregnancy is associated with further angiogenesis. In the marmoset however, no evidence for up-regulation of VEGF mRNA was found, consistent with observations in the early pregnant CL of the cow (Berisha, et al., 2000). It is also of interest that there is no evidence for a decline in VEGF mRNA preceding luteolysis, rather the decrease of VEGF mRNA was only observed at the time of structural regression.

Levels of KDR mRNA also did not change in the pregnant animals. Flt mRNA was unchanged in the day 21 pregnant group, but significantly decreased in the day 28 animals compared to the non-pregnant controls. It is interesting that only grain area was significantly reduced, not grain density. This suggests that the number of cells expressing Flt is reduced at this time, but that the intensity of expression within these cells is no less than in other groups. This could be explained by the fact

that Flt is associated with proliferating endothelial cells of which there are more in the earlier luteal phases of the cycle. Therefore by 28 days post ovulation, the endothelial cells from these earlier phases may be beyond the stage of high Flt expression. In the human CL, RT-PCR demonstrated that there was no increase in KDR or Flt mRNA levels in the CL of pregnancy compared to the non-pregnant cycle (Sugino et al., 2000). Thus, luteal rescue is not associated with an increase in VEGF receptors. These results lead us to propose that the late luteal CL of the marmoset retains its molecular (at least with regard to VEGF mRNA and its receptors), morphological and structural integrity and is similar to the rescued CL until such time as it does undergo structural regression.

In view of these results, it was considered that a period of intense angiogenesis may be occurring earlier than day 21, for example at around day 14 of early pregnancy, soon after implantation of the blastocyst on day 11. However, analysis of endothelial cell proliferation in the CL at day 14 of pregnancy, failed to demonstrate a significantly higher intensity of angiogenesis compared to non-pregnant structurally intact late luteal controls. While I have focussed on VEGF and its receptors in this study, it is possible that other angiogenic factors may be involved in regulation of the vasculature of early pregnancy. Since the angiopoietins are thought to participate in stabilisation of blood vessels, I examined mRNA levels for Angiopoietin-1 and -2 and their receptor Tie-2 using the same method and riboprobes employed by Wulff *et al.* (Wulff, et al., 2001), and marmoset endometrium as a positive control. However, no detectable specific signal above background grain density was observed in ovaries of any groups, thus the angiopoietins appear not to play a role in luteal rescue in the marmoset.

The length of the luteal phase in the non-pregnant marmosets varied greatly. This was advantageous here, as it allowed for the study of both structurally intact non-pregnant and structurally regressed CL, but the cause of this variation however, is still unclear. Some animals have functionally and structurally regressed CL by day 21 whereas other non-pregnant animals are still functionally and structurally active although anticipated to shortly undergo regression. Recent estimations from our colony have shown luteal length to vary between 16 and 24 days which is consistent

with previous reports on variability in luteal phase length (Harding, 1982, Harlow et al., 1984, Summers, et al., 1985).

It is well established that progesterone levels are maintained due to the continued responsiveness of luteal cells to gonadotrophins via CG in pregnancy (Stouffer, et al., 1978). It would appear that in the marmoset, despite the absence of CG, the late luteal CL of a non-pregnant cycle is maintained until it undergoes a rapid regression and thus decline in progesterone production. Luteal regression in the marmoset must therefore be a very rapidly triggered event, for up until that point the CL of a non-fertile cycle retains its morphology and functional capacity to the same extent as in a pregnant cycle.

These results have demonstrated that by the late luteal phase in the marmoset, the corpus luteum has already established a mature vascular system and the molecular capacity to synthesize VEGF and its receptors which are not further enhanced in early pregnancy. A pregnancy induced spurt of angiogenesis does not take place, rather a maintenance of the existing vasculature is all that is required for establishment of pregnancy in the marmoset. Therefore, it has to be concluded that the changing morphological and cellular events thought to be associated with early pregnancy in the human after hCG rescue do not occur in the marmoset.

Chapter 4

Comparison between marmoset and human luteal steroidogenic cells and the effect of progesterone on VEGF expression in human luteal steroidogenic cells *in vitro*

4 The effect of progesterone on VEGF expression in human luteal steroidogenic cells *in vitro*

4.1 Introduction

Chapter 3 demonstrated that there is no increase in angiogenesis associated with early pregnancy in the marmoset corpus luteum (Rowe, et al., 2002) in apparent contrast to the effects of hCG induced rescue of the CL in the human (Wulff, et al., 2001). One parameter used to assess angiogenesis in both of these studies was the level of expression of VEGF mRNA. This was unaltered in the marmoset in early pregnancy but was seen to increase in the human after hCG rescue. This chapter aims to investigate further this divergence between these species and to provide some preliminary data as to potential mechanisms for this discrepancy.

In order to investigate this issue, it is necessary to understand what is happening at this time in both species. Progesterone is a key player in early pregnancy. Maintenance of pregnancy in mammals requires progesterone production by the ovarian corpus luteum and in most species, the functional lifespan of the CL is extended to maintain this production (Zelevnik and Benyo, 1994). In humans, the CL remains the primary source of progesterone for 4-5 weeks after implantation until placental production becomes sufficient to maintain pregnancy. The mechanisms of luteal rescue vary among species. In rodents, coitus initiates rescue whereas in human and primates, rescue is initiated by the conceptus through its production of chorionic gonadotrophin.

The general pattern of progesterone production during the first trimester of pregnancy has been described in the human. Luteal rescue by hCG is associated with increased luteal progesterone secretion both in normal (Lenton and Woodward, 1988) and simulated early pregnancy (Mendizabal et al., 1984, Tay and Lenton, 2000). This observation forms the first discrepancy between these two species, for in

the marmoset, progesterone levels are maintained in early pregnancy at levels already attained in the luteal phase but are not further augmented during luteal rescue (Rowe, et al., 2002). This therefore gives rise to the possibility that progesterone could be responsible for the increase in VEGF seen in the human that is not apparent in the marmoset CL in early pregnancy. I hypothesise that the increase in VEGF mRNA observed in hCG rescue of the human CL is a progesterone mediated effect. This could be possible as the VEGF gene has been documented to contain a progesterone response element (Hyder and Stancel, 2000).

In order to understand this and to address this hypothesis, reception of progesterone in the CL will be investigated using immunocytochemistry for progesterone receptor in the marmoset and human. In order to fully dissect the system, an *in vitro* model of human luteal steroidogenic cells will be employed to allow this system to be studied by addition and withdrawal of progesterone to determine its role in promoting increased VEGF expression in the hCG rescued human corpus luteum.

4.2 Specific methods

4.2.1 Details of animals and samples used

Experiment	Group	Number of animals/culture samples
Progesterone receptor immunocytochemistry	Mid-luteal phase marmoset corpora lutea	3
	Human endometrium late secretory phase (positive control)	1
	Marmoset endometrium late secretory phase (positive control)	1 (animal had a structurally intact CL)
	Human corpora lutea. Mid-luteal phase	3
3 β HSD and progesterone receptor immunocytochemistry dual staining	human luteinised granulosa cells	3 culture samples from different women
The effect of hCG on human luteinised granulosa cells	Varying doses of hCG (0, 0.001, 0.01, 0.1, 1, 10 and 100ng/ml hCG)	5 culture samples from different women at each dose
The effect of progesterone on human luteinised granulosa cell VEGF expression	Control, hCG, Progesterone, hCG + Aminoglutethamide and hCG + aminoglutethamide + progesterone	8 culture samples from different women per treatment
The effect of duration of treatment of hCG on human luteal steroidogenic cells	12hours 24 hours 24 hours (control)	5 culture samples from different women per treatment

Table 4.1 Details of animals and samples used in the experiment

4.2.2 Source of marmoset and human tissues

Marmoset corpora lutea used in this experiment were collected from normally cycling marmosets in the mid-luteal phase (n=3). Details of marmoset husbandry, collection and processing of tissues are to be found in Chapter 2, sections 2.4 and 2.5. The marmoset endometrium used for the positive control was from the late secretory phase (n=1). Again uteri were collected and processed according to sections 2.4 and 2.5.

Human corpora lutea (n=3) were obtained from normally cycling women undergoing hysterectomy in the mid-luteal phase and were fixed and processed according to section 2.5. Human endometrium used for a positive control was collected in the early secretory phase from endometrial biopsy (n=1) and processed according to sections 2.4 and 2.5.

4.2.3 Progesterone receptor immunocytochemistry on fixed tissues

Tissues were dewaxed and rehydrated, as described in section 2.5 before being subjected to antigen retrieval. This was performed using pressure cooking in citrate buffer before blocking of endogenous peroxidase and endogenous biotin were performed as described in section 2.7. The normal serum used in this case was Normal horse serum (Vectastain ABC kit-Horse anti mouse, 3 drops of horse serum to 10ml PBS). Progesterone receptor antibody (mouse, monoclonal, Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK. This is raised against human PR. It detects both the A and B forms of the receptor, and does not detect non-genomic receptors), diluted 1:20 in normal horse serum block was added to slides and incubated overnight at 4 °C. A negative control was included and incubated with mouse IgG (SAPU) at a similar concentration (in this case 1:6000) overnight at 4 °C.

After overnight incubation with primary antibody, slides were washed twice in PBS before the secondary antibody was added, 1 drop of anti-mouse serum to 10ml PBS (Vectastain ABC kit- horse anti mouse). This incubate was left on for 1h at room temperature. After 2x PBS washes, previously prepared ABC-HRP solution (2 drops of solution A and 2 drops of solution B to 10ml PBS, made up at least 20 minutes in advance, Vectastain ABC kit- horse anti mouse) was added for 1h at room

temperature before detection by DAB immunostaining, counterstaining with haematoxylin, dehydration and mounting (see chapter 2).

4.2.4 Collection and separation of human granulosa cells

Follicular fluid was obtained from patients undergoing *in vitro* fertilisation treatment after transvaginal follicular aspiration. Typically these patients followed a long protocol down regulation with GnRH analogues for 2-3 weeks followed by daily injection of recombinant FSH with ultrasound monitoring. When three or more follicles were 18mm or greater, 10,000IU of urinary derived hCG was administered and follicular aspiration for oocyte collection was organised 35-36 hours later. Recruitment of patients and subsequent surgical procedures were performed by either Dr. W.C Duncan or Dr. W. Ismail. This study was approved by the Reproductive medicine subcommittee of the South East Scotland Research Ethics committee.

4.2.5 Cell culture

Follicular fluid, containing cells from within the follicle, was received after removal of oocytes by hospital staff. It was centrifuged at 1200rpm for 10 minutes and supernatant discarded. The gelatinous precipitate was resuspended to 5ml volume with warmed (37°C) medium. Dulbecco's modified Eagle's medium nutrient mixture F-12 ham, was used, supplemented with the following; To 480ml medium, 5ml each of 2mM L-glutamine, Penicillin-Streptomycin solution (100,000 units/l penicillin, 100mg/l streptomycin), 2.5mg/l amphotericin B and insulin-transferrin-sodium selenite media supplement (10mg/l insulin, 5.5mg/l transferrin, 5µg/l sodium selenite, 0.5g/l bovine serum albumin and 4.7µg/l linoleic acid).

Granulosa cells were separated over 45% percoll in culture medium. The cell suspension was layered over the percoll (Amersham Pharmacia Biotech, Little Chalfort, Bucks, UK) mixture and centrifuged at 252g for 30 minutes. After centrifugation the clearly visible granulosa layer was placed in a clean test tube and subjected to repeated washes in sterile phosphate buffered saline (PBS, 37°C) to remove red blood cell contamination. After the cells had been resuspended in PBS they were spun at 252g for 1 minute. Supernatant was discarded and cells resuspended in fresh PBS. These washes were repeated up to 5 times depending on how clean the samples were after each wash. Cells were ultimately resuspended in

1ml culture medium. Twenty microlitres of cell suspension were mixed 1:1 with trypan blue vital dye and mixed vigorously to break up cell clumps. Half of this mixture was placed in a haemocytometer and cells counted in each of four corner squares from the two chambers (8 squares in total). Total cells per ml were calculated;

Average count per square x dilution factor x 10^4 .

Cells were plated out in culture medium onto matrigel coated 24 well plates at a density of 75,000 cells per well. Incubation overnight at 37°C in 5% CO₂ allowed cells to adhere to plates before specific treatments were added, details of which will be discussed below. Matrigel (Beckton Dickinson UK, Ltd, Cowley, Oxford, UK), was thawed overnight on ice then mixed 1:1 with cold culture medium. Plates were cooled on an ice block before 50µl of the matrigel mixture was added and spread across the base of each well. This was allowed to dry for 45 minutes before 500µl culture medium was added per well. Plates coated in matrigel can be used for up to 14 days.

4.2.6 Cell fixation and processing for immunocytochemistry

Human luteinised granulosa cells were plated out onto matrigel coated plates as described in section 4.2.4 at a density of 75,000 cells per well and left to adhere for 24 hours.

After removal of culture medium, cells were washed with PBS and removed from the well with trypsin-EDTA solution (0.5g Trypsin, 0.2g EDTA made up to 100ml) for 2 minutes at 37°C. This puts the cells under stress and so medium containing 10% foetal calf serum was added as this enhanced cell survival and limited cell damage. After 900µl of culture medium containing 10% foetal calf serum was added, cells were gently scraped off the bottom of each well and placed into an eppendorf tube. Cells were centrifuged at 252g for 3 minutes before being fixed in 200µl 10% neutral buffered formalin. The cell pellet was then resuspended by vortexing and centrifuged 252g for 3 minutes.

After the supernatant was discarded, cells were resuspended in 5 to 10µl PBS, before being added to a liquefied 2% agarose pellet. Once the pellet had solidified it was stored in 70% ethanol until processing (as described in Chapter 2). Five micron

sections were cut onto BDH superfrost slides ready for haematoxylin and eosin staining as described in chapter 2, or immunocytochemistry as described below.

4.2.7 Progesterone receptor and 3 β HSD immunocytochemistry dual staining on fixed cells.

Immunostaining for progesterone receptor was performed exactly as described above except that 2 drops of nickel were added to the DAB substrate solution before detection so that the resultant nuclear immunostain would be black and thus clearly distinguishable from the cytoplasmic brown 3 β HSD immunostain to follow.

After detection of progesterone receptor, slides were incubated with normal goat serum (3 drops of goat serum to 10ml PBS, Vectastain ABC kit – Goat anti-rabbit), before 3 β HSD antibody, (rabbit polyclonal, kindly donated by Professor Van Luu-The (CHUL Research Centre, Quebec, Canada) diluted 1:1000 in normal goat serum was added and incubated overnight at 4°C (Duncan et al., 1999). After overnight incubation the secondary antibody, goat anti-rabbit was added for 1h at room temperature (1 drop biotinylated anti-rabbit serum to 10ml PBS, Vectastain ABC kit – Goat anti-rabbit). Detection was as above (ABC-HRP, 1h, room temperature and subsequent staining with DAB substrate).

Sections were dehydrated and mounted as described in chapter 2, without counterstaining.

4.2.8 Cell culture treatments

As stated in Section 4.2.4, human luteinised granulosa cells were plated out onto 24 well, matrigel coated plates. These were then left at 37°C in 5% CO₂ for 24 hours to allow the living cells to adhere to the base of the well. After 24h, culture medium was removed before fresh medium containing specific treatments was added (1ml medium per well). In all experiments, a lipid supplement of LDL cholesterol (Sigma) was included in the culture medium at a concentration of 50 μ g/ml, all treatments were given in duplicate and application of treatments was for 24 hours. Under no circumstances were cells from different women mixed. If there were not

enough cells from a sample to provide duplicate wells of at least 75,000 cells per well, for each treatment, then the sample was discarded. The number of individual experiments conducted for each part of this chapter ("n" number) refers to the number of separate samples utilised.

After 24 hours treatment duration, culture medium was removed and stored for analysis of progesterone while cells were treated with Tri reagent for RNA extraction and real time quantitative PCR for VEGF, as described below. At this stage, Tri reagent from duplicate wells was combined.

4.2.9 Treatment regimes

In order to determine the dose response of cultured human luteinised granulosa cells to hCG in terms of progesterone secretion and VEGF expression and to elucidate optimal concentrations to use for future experiments, urinary derived hCG was added to cells at concentrations of 0ng/ml, 0.001ng/ml, 0.01ng/ml, 0.1ng/ml, 1ng/ml, 10ng/ml and 100ng/ml.

To manipulate progesterone within this cell culture system, and to dissect out the effects of progesterone from hCG, several different strategies were used. All treatments were applied to cells in culture medium for 24h; (1) A control was included that received only culture medium containing 50µg/ml low density lipoprotein (LDL) as a substrate for progesterone synthesis. In order to increase progesterone secretion, both hCG and LDL need to be added. This control confirms that this is an effect of hCG rather than the LDL; (2) In order to assess the response of human luteal steroidogenic cells to hCG alone, 10ng/ml hCG + 50µg/ml LDL was added. This concentration of hCG was chosen because in dose response experiments it was demonstrated to evoke progesterone secretion from these cells towards the maximal range achieved, where the graph of dose response begins to flatten at saturated levels of progesterone secretion and VEGF expression; (3) To determine the effect of progesterone alone without addition of hCG, cells were treated with 10µM progesterone + 50µg/ml LDL; (4) In order to assess the involvement of progesterone in the observed upregulation of VEGF expression from human luteal steroidogenic cells, it was necessary to remove it from the system and to determine whether or not the previously seen upregulation of VEGF was maintained in the

presence of hCG treatment alone. To test this, a steroidogenesis inhibitor, aminoglutethamide, was used at appropriate concentrations previously reported (Fowkes et al., 2001). The treatment regime employed was 10ng/ml hCG + 100 μ M aminoglutethamide + 50 μ g/ml LDL. At this point, I wish to address the issue as to why RU486 was not chosen as a progesterone inhibitor. If steroidogenesis is blocked, no progesterone can be synthesised in response to hCG treatment, whereas if progesterone action alone is blocked, and its synthesis remains unabated, it cannot be proven in this system that RU486 is penetrating cells adequately and destroying any intracellular progesterone effects that may remain; (5) Finally, if progesterone is responsible for the increase in VEGF expression observed, reintroducing it exogenously to a system where its endogenous synthesis has been blocked should not only reinstate this effect, but evoke a further increase in VEGF expression. In order to test this, 10ng/ml hCG + 100 μ M Aminoglutethamide +10 μ M progesterone + 50 μ g/ml LDL were added.

4.2.10 Time course experiment

If the observed upregulation in VEGF expression was a progesterone mediated effect, it would be expected that increased progesterone synthesis would precede increased VEGF expression after hCG treatment. In order to test this hypothesis, human luteal steroidogenic cells were treated with 10ng/ml hCG for a duration of 12 and 24 hours before medium collection and RNA extraction as described in section 4.3. A control was also included which received only culture medium without hCG for 24 hours.

4.2.11 Progesterone assay on culture medium

This was essentially exactly the same as the protocol used for serum progesterone assays (Chapter 2), except that instead of 2.5 μ l of charcoal stripped male marmoset plasma being added to the standards, 2.5 μ l of culture medium was added.

4.2.12 Total RNA extraction and quantification of specific mRNA

4.2.12.1 RNA extraction from cells

Total RNA was extracted from human granulosa cells using Tri-Reagent, a mixture of guanidine thiocyanate and phenol in a mono-phase solution. This is a quick and convenient reagent for use in the simultaneous isolation of RNA, DNA and protein. This method is one of the most effective ways to isolate total intact RNA with little or no contaminating DNA or protein. The resulting RNA can be used for Northern blots, mRNA isolation, *in vitro* translation, RNase protection assay, cloning and PCR.

After removal and storage of culture medium from wells, the cells were washed in PBS to remove any dead, non-adherent cells, (live cells remained adherent to the well surface). Subsequently, 0.5ml Tri-reagent was added to each well, pipetted up and down to remove adherent cells and then left for approximately 5 minutes. All cell culture treatments (described in Chapter 4), were performed in duplicate. At this stage the tri reagent from each of the duplicate wells was combined before transfer to a clean eppendorf tube on ice. Then 200µl chloroform was added and mixed vigorously. The tube was then centrifuged at 19064 g at 4°C for 20 minutes. Centrifugation separates the mixture into three phases, a red organic phase containing protein, a white interphase containing DNA and a colourless upper aqueous phase containing RNA. This upper aqueous phase was transferred to a clean tube before 500µl isopropanol and 1µl glycogen were added (to aid visibility of the pellet). This was gently mixed by inversion before being left for at least one hour on ice at 4°C. After a further 15 minute centrifugation at 19064g, 4°C, a pellet was formed (not necessarily visible at this stage). The isopropanol was removed and 500µl 70% ethanol (made with DEPC water) added. After a gentle mix, the tube was once again spun for 10 minutes at 19064g, 4°C. The majority of the ethanol was removed by pipetting, while the remainder was allowed to evaporate off on ice. The pellet was resuspended in 20µl DEPC water. Concentration (A_{260}) and quality ($A_{260/280}$) of RNA was measured on a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech) and concentration adjusted to 1µg/µl. Additionally the quality of randomly selected RNA samples were assessed by heating to 70°C for 15 minutes

followed by immediate transfer to ice and 0.7% agarose gel electrophoresis and visualisation under UV light.

4.2.12.2 DNase treatment

In order that any contaminating genomic DNA from the extraction was removed, the RNA was DNase treated (Promega). The following reaction mixture was incubated at 37°C for 30 minutes:-

70µl DEPC water

10µl DNase buffer

10µl DNase 1 enzyme

1µl RNase inhibitor (Promega)

10µl RNA (10µg)

After incubation, 10µl of “stop solution” (supplied with the DNase) was added and the mixture incubated for a further 10 minutes at 70°C. RNA was either used immediately for reverse transcription or frozen at -70°C.

4.2.12.3 Reverse transcription (RT)

In order that expression of specific genes of interest can be assessed by quantitative real time PCR, complementary DNA (cDNA) must be produced from the extracted RNA. Reverse transcription was conducted using a Taqman reverse transcription kit (Applied Biosystems, Cheshire, UK), according to manufacturers instructions. A mixture of buffer, dNTP's, random hexamers, RT enzyme, MgCl₂, RNase free water and RNA (200ng per 20µl reaction mixture) was incubated at room temperature for 10 min before heating to 42°C for 1h followed by a 10 min incubation at 95°C. For every sample an RT-negative was included. This contained everything but the reverse transcriptase.

Once the cDNA had been made, it was amplified by PCR for Glucose-6-Phosphate Dehydrogenase (G6PDH) together with its negative control to ensure that there was no genomic contamination. G6PDH is not regulated in the human reproductive cycle across the stages studied here. This was tested by Julie Bell,

another member of our group, who passed this information on to me by personal communication). The resultant PCR product was run on a gel and visualised under UV light. The band produced in the positive control was checked to ensure that it was the expected size for the primers used and where bands were present in the negative controls, the cDNA was not used for quantitative PCR.

4.2.12.4 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. Complementary DNA sequences for target genes were acquired from Genbank, on the internet. Primers were designed by Julie Bell for earlier experiments within our laboratory group, using Primer 3 software;

(http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)

Primers were designed against segments of the sequence that displayed low homology with other genes of the same family and where possible spanning an intron. This allows identification of genomic contamination. If genomic DNA remains in the sample even after DNase treatment, two peaks with different melt temperatures will be produced when using a melt curve analysis or two bands will be visible on an agarose gel. Primer sequences are given below.

VEGF (103bp)

5' TACCTCCACCATGCCAAGTG 3' (forward)

5' TAGCTGCGCTGATAGACATCCA 3' (reverse)

G6PDH(239bp)

5' CGGAAACGGTCGTACACTTC 3' (forward)

5' CCGACTGATGGAAGGCATC 3' (reverse)

Real time quantification of this process allows the cDNA synthesised after each PCR step to be visualised. Visualisation is by fluorescence of SYBR Green I which fluoresces when bound to double stranded DNA. The principles used in this

technique are further clarified in **Figure 4.1– Figure 4.3**(<http://www.lightcycler-online.com/>).

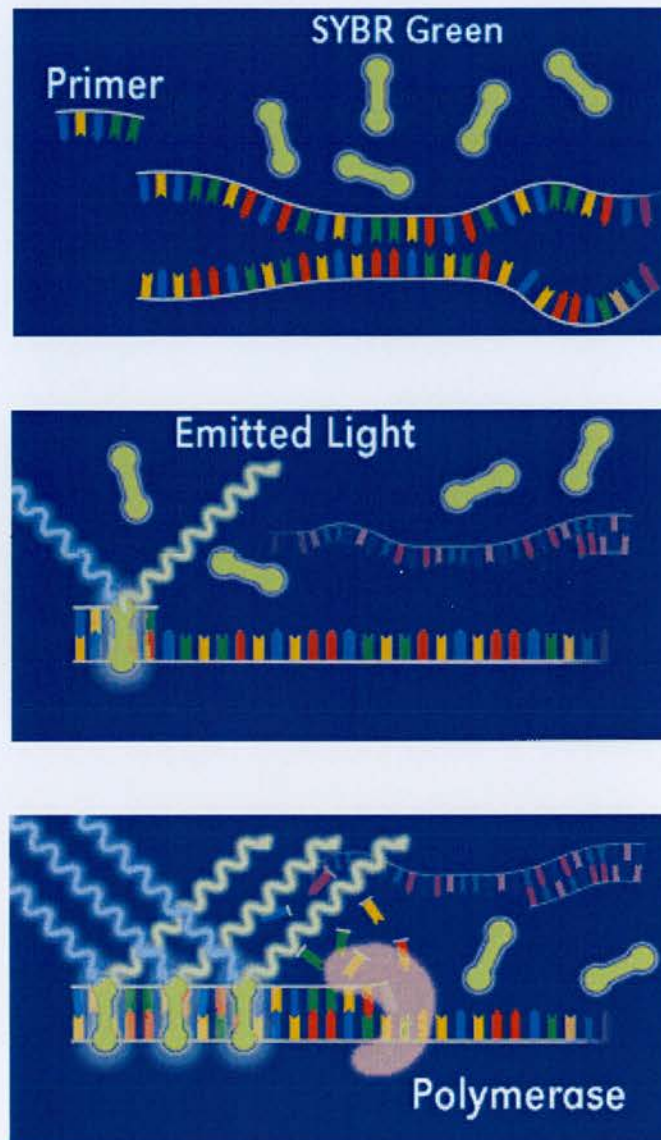


Figure 4.1 Monitoring PCR with SYBR Green 1 dye.

SYBR green I is a double stranded DNA binding dye. It binds to the minor groove of dsDNA which greatly enhances fluorescence. During the various stages of PCR, different intensities of fluorescence signals can be detected, depending on the amount of dsDNA that is present. (A) All DNA becomes single stranded after denaturation. At this stage the SYBR green I dye will not bind and the intensity of fluorescence signals is low. During annealing, the PCR primers hybridise to the target sequence

resulting in small parts of dsDNA to which SYBR green I can bind thereby increasing fluorescence intensity (B). In the elongation phase of the PCR, the PCR primers are extended, and more SYBR green I dye can bind (C). At the end of the elongation phase, all of the DNA has become double stranded and a maximum amount of dye is bound. The fluorescence (530nm) is recorded at the end of the elongation phase and increasing amounts of PCR product can be monitored from cycle to cycle. **Figure 4.2** illustrates the real time fluorescence readings in a typical light cycler run.

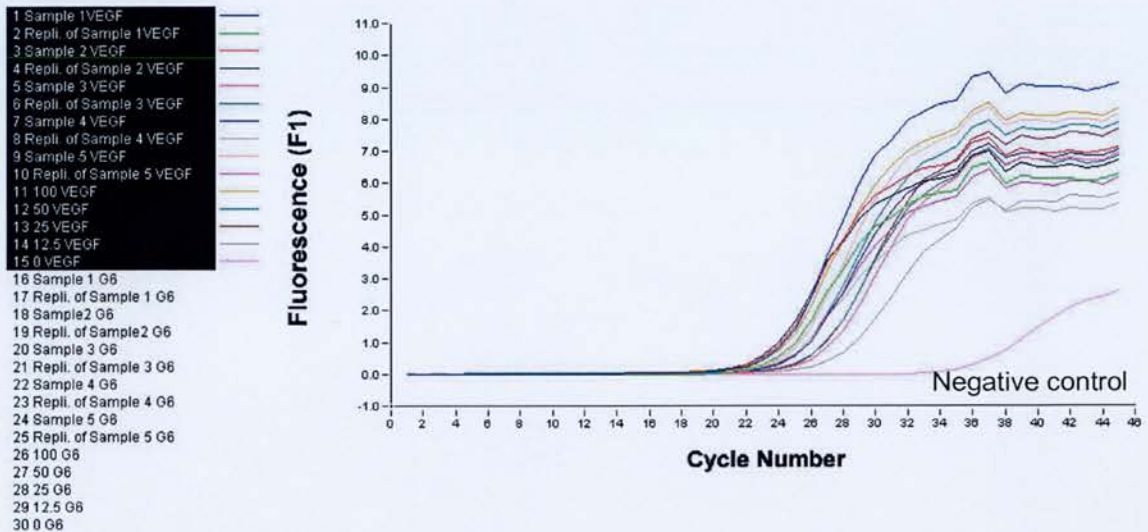


Figure 4.2. Real time amplification of fluorescence signal

There is some fluorescence detection from the negative control, but this only occurs at much higher cycle numbers than the samples.

Quantification of real time PCR is based on calculations taking into account a house keeping control gene (Glucose-6-phosphate dehydrogenase, G6PDH) in this case) and the gene of interest. The choice of control gene is critical. It has to be quantifiable over the same range and be amplified with the same or similar efficiency as the gene of interest. It must be constitutively expressed (equally within all cells, not changing according to the treatments the cells are given), and be optimally synthesised at the same primer annealing temperature with the same magnesium concentration as the gene of interest. VEGF and G6PDH reaction conditions were optimised to ensure that this was the case. The reagents used per capillary tube are listed below together with the PCR conditions (capillary tubes and reagents all supplied by Roche):

1µl Light cycler Fast start DNA Master SYBR Green (enzyme)
6.2µl nuclease free water
0.8µl magnesium chloride (3mM)
1µl (5µM) primer pairs
1µl cDNA

Reaction conditions

Heat activation	95°C	10 minutes
Denature	95°C	10 seconds
Primer annealing	65°C	5 seconds
Extension	72°C	15 seconds
Take measurement	83°C	1 second

Fluorescence measurements were taken at a temperature (optimised specifically for the gene of interest). This temperature was chosen to ensure that only fluorescence measurements of the specific product were taken while measurements of non-specific products such as primer-dimers were avoided. Primer dimers that may have been formed during the PCR will have been denatured at this temperature and are thus no longer binding SYBR green.

Measurement temperature was determined by producing “melt curve” data. A melt curve analysis is performed at the end of every run. The samples are heated to ensure that all double stranded DNA is denatured. The samples are then cooled and reheated slowly, while fluorescence measurements are constantly taken. At a specific temperature, melting of the DNA of interest occurs characterised by a sharp drop in fluorescence. Any non-specific product, for example primer dimers will have melted at a lower temperature. The temperature at which measurements are taken is between melting of non-specific products and melting of specific product. Therefore only the specific DNA of interest remains double stranded, and hence binding SYBR green I. By checking the melt curve produced at the end of each run, any genomic contaminants can be identified as these will produce melting peaks at different temperatures. Only a single melt peak should be produced from each sample (**Figure**

4.3). Note that the two melt curves have been combined for the purposes of this diagram but PCR for VEGF and G6PDH were carried out in different capillaries, therefore for each capillary only one melting peak would be produced, either VEGF or G6PDH.

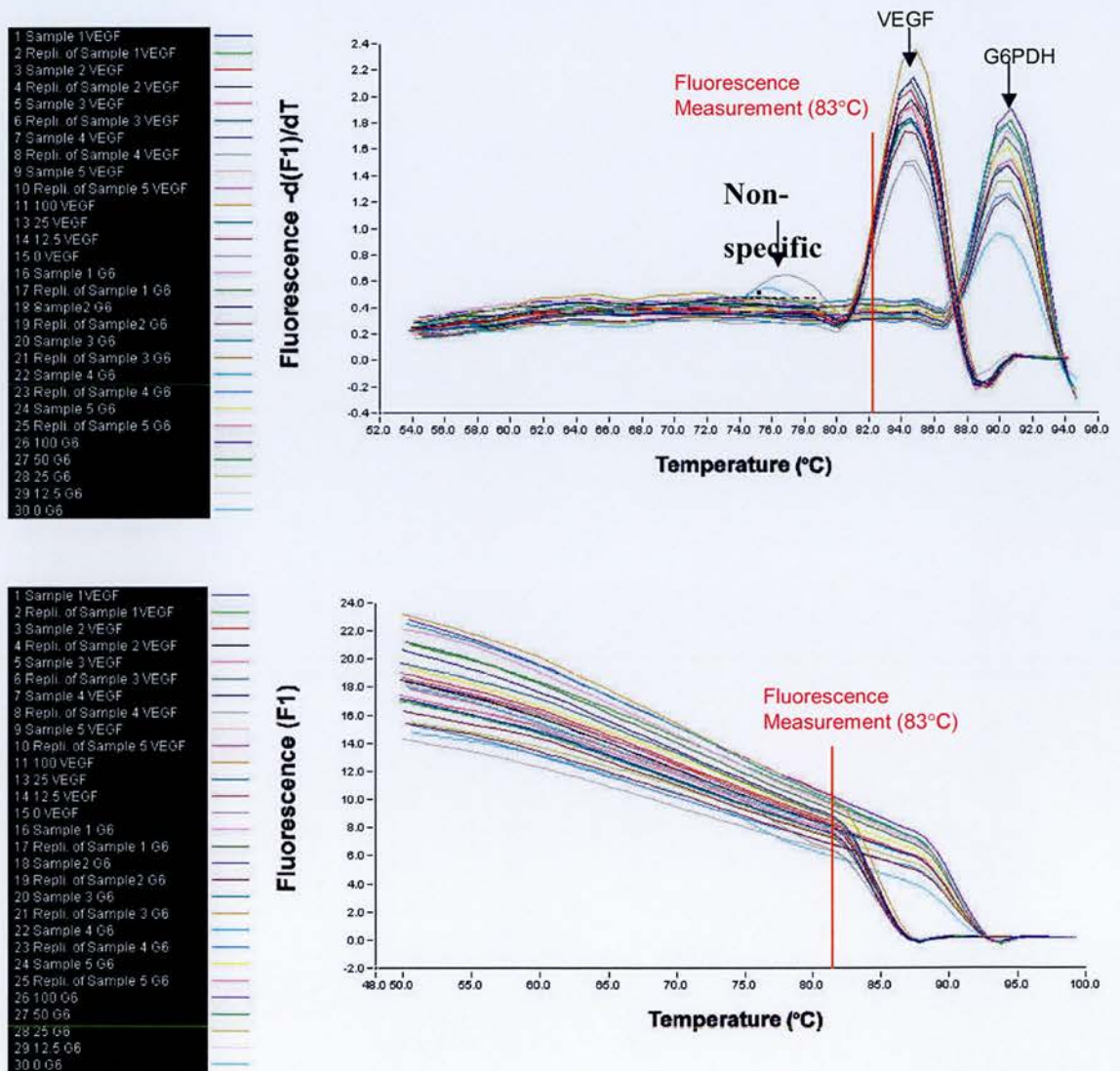


Figure 4.3 Melt curve analysis

A specific melting peak is produced for each product made, therefore any genomic contaminants producing non-specific products would be detectable. Primer dimer formation gives rise to melt peaks at a lower temperature than specific products (top diagram). The lower diagram illustrates the fluorescence profile as the temperature is slowly increased. The red line indicates the temperature at which fluorescence was measured at the end of each PCR cycle. This temperature is above that at which primer dimers remain double stranded so only specific product is measured. This temperature is the same for the specific gene of interest and housekeeper, therefore when choosing a housekeeping gene, it is essential to ensure that it melts in the same region as the gene of interest.

Relative quantification of specific cDNA is performed by normalisation to known concentrations of serially diluted standards (human placental cDNA reverse transcribed in the same reverse transcription experiment as the samples to be measured). This eliminates any variation in RT efficiency.

A standard curve with a gradient of -3.3 signified an efficient PCR. An efficient PCR was designed and optimised before any measurements were taken in order that separate runs of the Lightcycler could be accurately compared (**Figure 4.4**). The standard curve for each run was constructed using second derivative maximum software, supplied by Roche for the light cycler to apply a line of best fit through the standard points. This was considered to be the best option of standard curve fitting as it removes any operator influence.

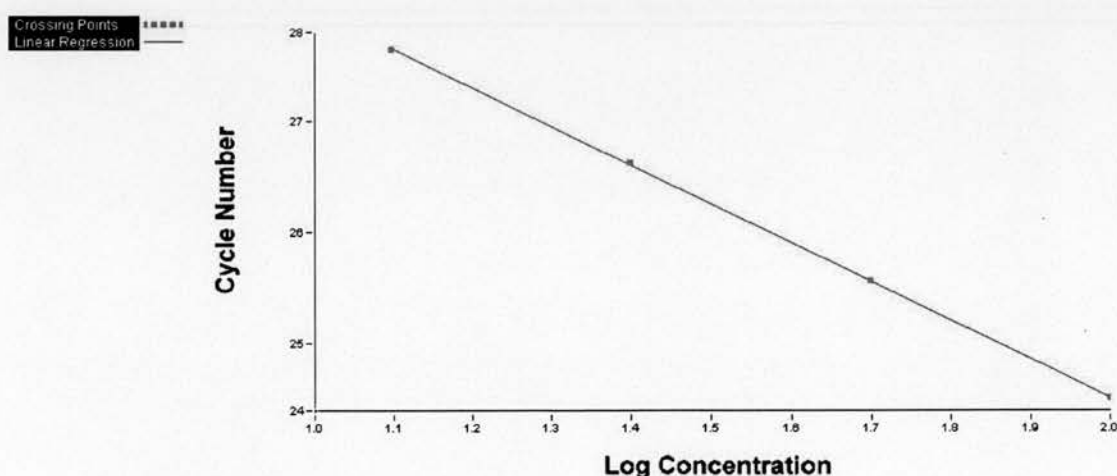


Figure 4.4. Standard curve

Constructed using second derivative maximum software to apply a line of best fit. The gradient of this standard curve was -3.5 from G6PDH standards.

Where results were to be directly compared, the same master mix was used and samples were run in duplicate for both VEGF and the housekeeping gene, G6PDH. Using SYBR green I and specific primers, without the need for additional specific hybridisation probes, requires that measurement of the gene of interest and G6PDH is from separate capillaries. The Lightcycler carousel can hold a maximum of 32 capillaries. This allows 5 separate samples to be included in each run of the Lightcycler:-

5 samples in duplicate =10 for VEGF

5 samples in duplicate =10 for G6PDH

4 standards (100%, 50%, 25% and 12.5%) for VEGF and 1 negative control =5

4 standards (100%, 50%, 25% and 12.5%) for G6PDH and 1 negative control =5

Total number of capillaries per run =30

4.3 Results

4.3.1 Progesterone receptor immunohistochemistry on marmoset and human tissues

Genomic progesterone receptors were localised by immunohistochemistry in marmoset and human tissues (**Figure 4.5**). The endometrium, a major target tissue for progesterone in both species, was used as a positive control in all runs of the experiment. Specific nuclear staining for progesterone receptors was seen in both stromal and glandular cells of the endometrium, as expected from previous studies (Critchley, et al., 2001). This confirmed the ability of the anti-human progesterone receptor antibody to detect genomic progesterone receptors in marmoset tissues. Marmoset and human endometrial sections are illustrated in (**Figure 4.5A and B**, respectively). Insert in (A) is a negative control.

As previously described, human granulosa lutein cells expressed progesterone receptor (Sasano and Suzuki, 1997), (**Figure 4.5C**) However, PR could not be immunolocalised in the steroidogenic luteal cells of the marmoset corpus luteum in any of the ovaries examined. Interestingly, PR was detectable in the granulosa and theca cells of the marmoset follicle, and in the ovarian stroma (**Figure 4.5D**).

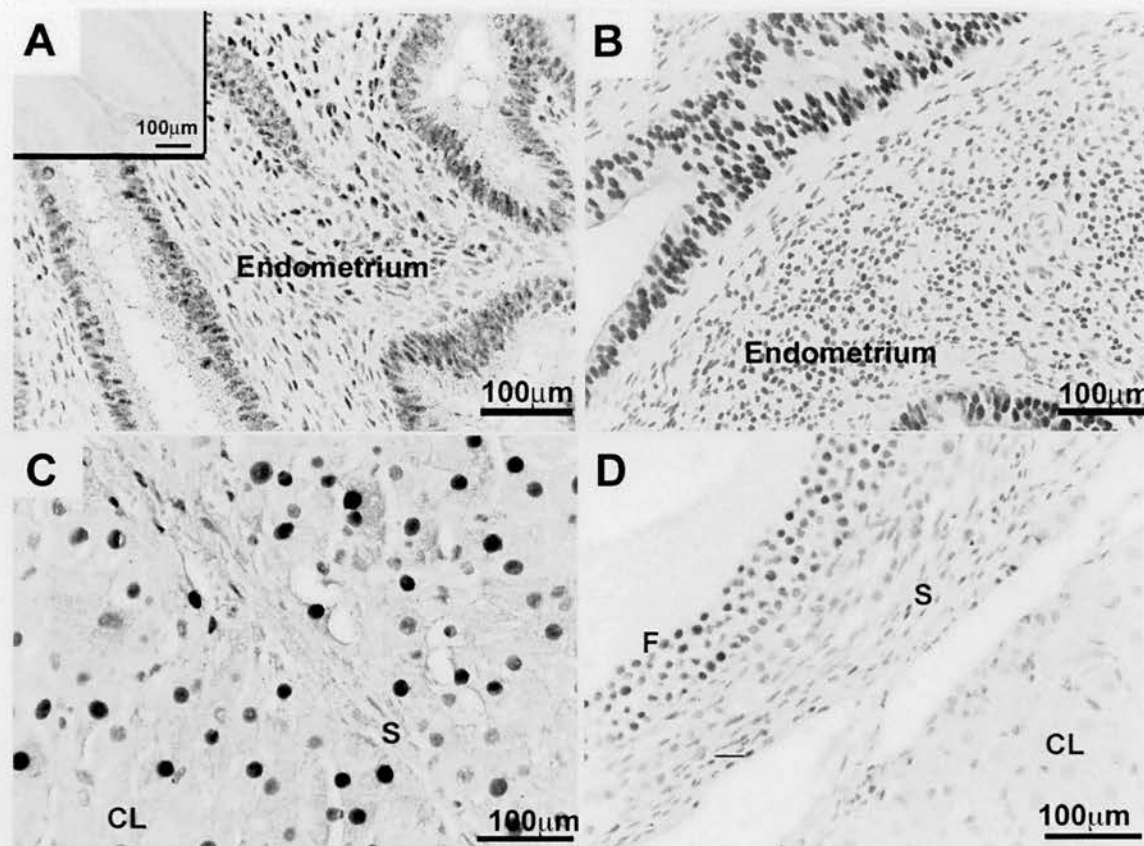


Figure 4.5 Progesterone receptor localisation by immunocytochemistry in human and marmoset reproductive tract. Positive staining is brown. (A) Marmoset endometrium, (B) Human endometrium, (C) human CL, note intense immunostaining in steroidogenic cells of the CL, and (D), marmoset ovary, note absence of staining in the CL, but positive staining in the follicle (F) and stroma (S). Insert in (A) represents a negative control section of marmoset endometrium.

4.3.2 Progesterone receptor immunocytochemistry of human luteinised granulosa cells

In order to confirm that the cells cultured were in fact luteal steroidogenic cells, immunocytochemistry for progesterone receptor and 3β HSD was performed. **Figure 4.6** illustrates dual staining of progesterone receptor, black, localised to cell nuclei with 3β HSD expression, brown, localised to cytoplasm. The function of luteal steroidogenic cells is to synthesise and secrete progesterone (Duncan, et al., 1999). In order to do this these cells must contain 3β HSD, the final enzyme in the progesterone steroidogenic pathway responsible for the conversion of pregnenolone to progesterone (Strauss, 1991). The immunostaining observed confirms that the

isolated cells are luteal steroidogenic cells and that the cell separation technique is appropriate, and that these cells continue to express genomic progesterone receptors in culture. It should be noted that the background staining is of the matrigel matrix used in the cell culture system and is not extracellular matrix non-specifically staining. The negative control is a section containing steroidogenic cells from the same culture without the addition of primary antibody.

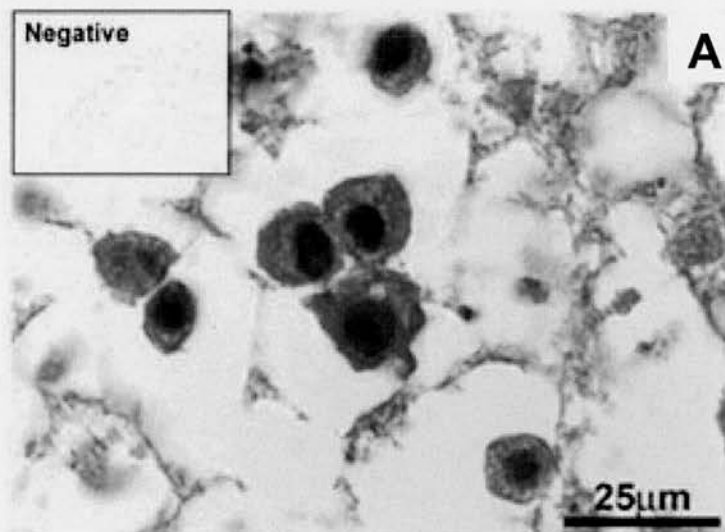


Figure 4.6 Dual staining of progesterone receptor (black, nuclear) and 3β HSD (brown, cytoplasmic) on human granulosa cells that have been fixed and paraffin embedded. Insert is the negative control with absence of specific staining above background levels.

4.3.3 The effect of hCG on luteal steroidogenic cells *in vitro*.

4.3.3.1 Effect of hCG on progesterone secretion

Figure 4.7 illustrates the response of luteal steroidogenic cells to increasing doses of hCG in terms of progesterone secretion into culture medium. No difference in the concentration of progesterone secreted was observed at doses of hCG, below 0.1ng/ml hCG. Doses of hCG between 0.1ng/ml and 100ng/ml gave rise to a tendency to increase levels of progesterone secretion, maximal responses occurring when cells were treated with 100ng/ml hCG.

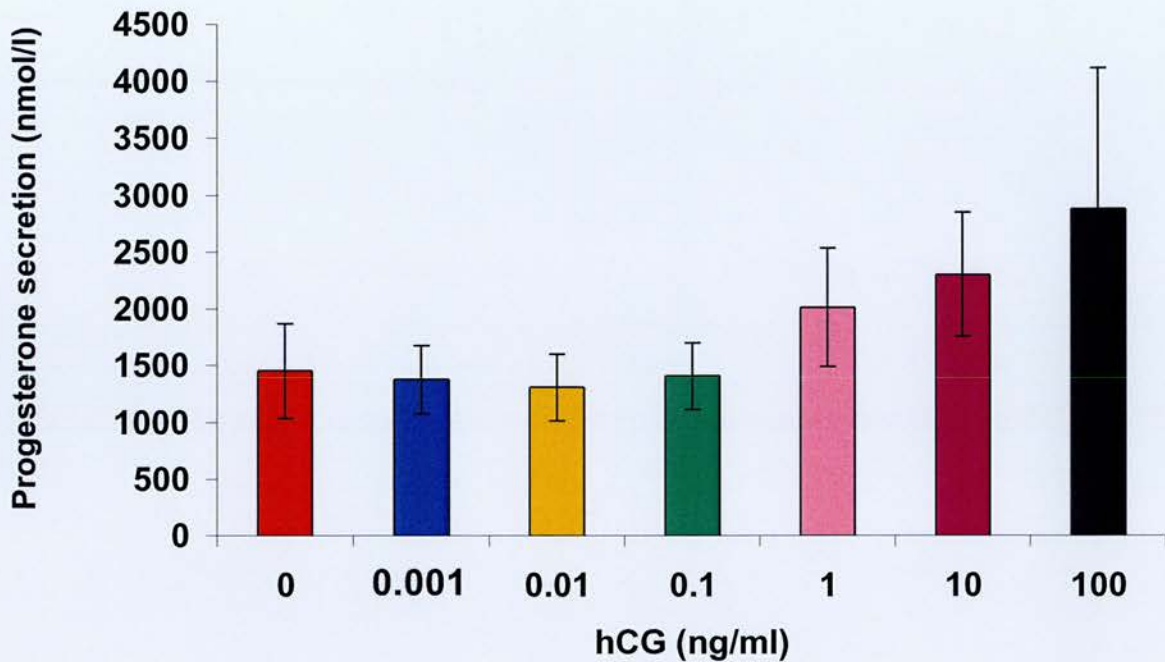


Figure 4.7 Dose response graph to illustrate the effect of varying hCG concentrations (ng/ml) on progesterone secretion (nmol/l) from luteinised human granulosa cells *in vitro*. Values are means \pm SEM (n=5 cell cultures from individual patients per hCG dose)

4.3.3.2 Effect of hCG on VEGF expression

I have established that these cells respond to hCG between 0.1ng/ml and 100ng/ml by increased secretion of progesterone. As such, **Figure 4.8** depicts levels of VEGF expression in cells treated with these concentrations of hCG. It appears that VEGF expression follows the same pattern as progesterone secretion in response to increasing levels of hCG. Using Pearson's correlation test, the two data sets were positively correlated with a correlation coefficient of 0.995, significant at the 0.01 level. From this initial dose response experiment, it was decided that 10ng/ml would be the concentration at which hCG would be used for the actual experiment. This is not the dose where a maximal response was observed, but it evokes a response in the upper part of the curve, before it plateaus ie. before saturation.

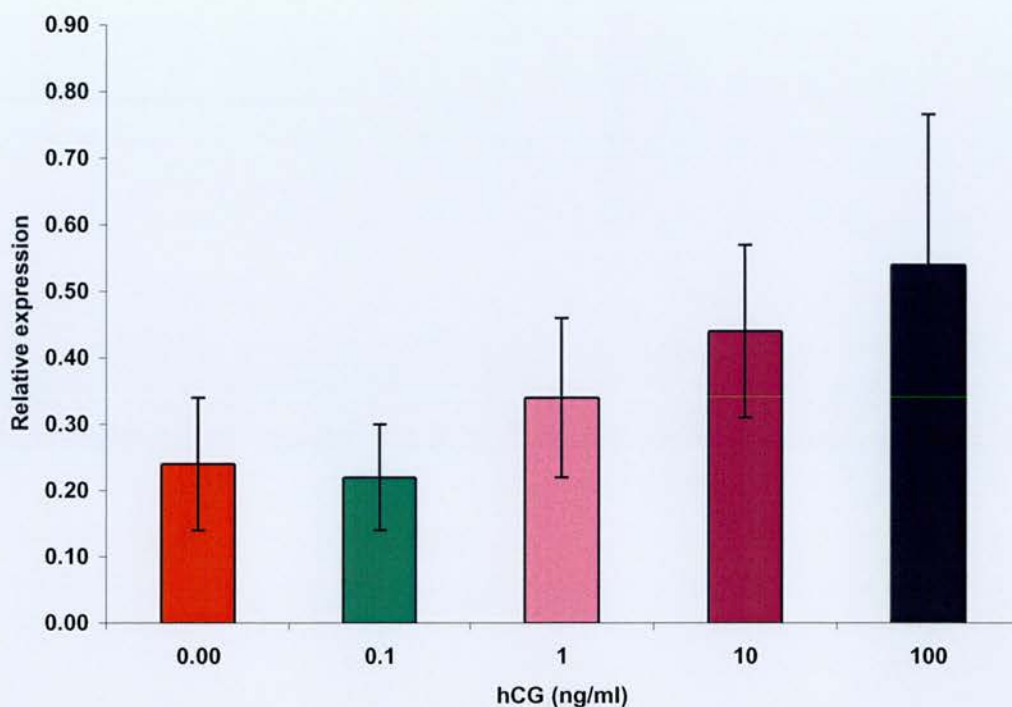


Figure 4.8 Dose response graph to illustrate the effect of varying hCG concentrations (ng/ml) on relative VEGF mRNA expression in luteinised human granulosa cells *in vitro*. Values are means \pm SEM (n=5 cell cultures from individual patients per hCG dose).

4.3.4 The effect of progesterone on luteal steroidogenic cell VEGF expression

From the previous experiment, it is clear that addition of hCG increased both progesterone secretion and VEGF expression in luteinised granulosa cells. The following experiments were designed to dissect the effect of hCG from that of progesterone. The strategy involved manipulating progesterone independently from hCG. To increase progesterone secretion from cells, either progesterone or hCG were added. As can be seen from **Figure 4.9**, both of these treatments significantly increased the progesterone concentration in cell medium as determined by 1-way ANOVA ($P < 0.05$). This effect was abolished when a steroidogenesis inhibitor, aminoglutethamide was added. This inhibits enzymes involved in the early, common steps in steroidogenesis. Where progesterone was added to culture medium,

obviously the progesterone assay is measuring what has been added as well as that produced. This confirms that the assay is working well.

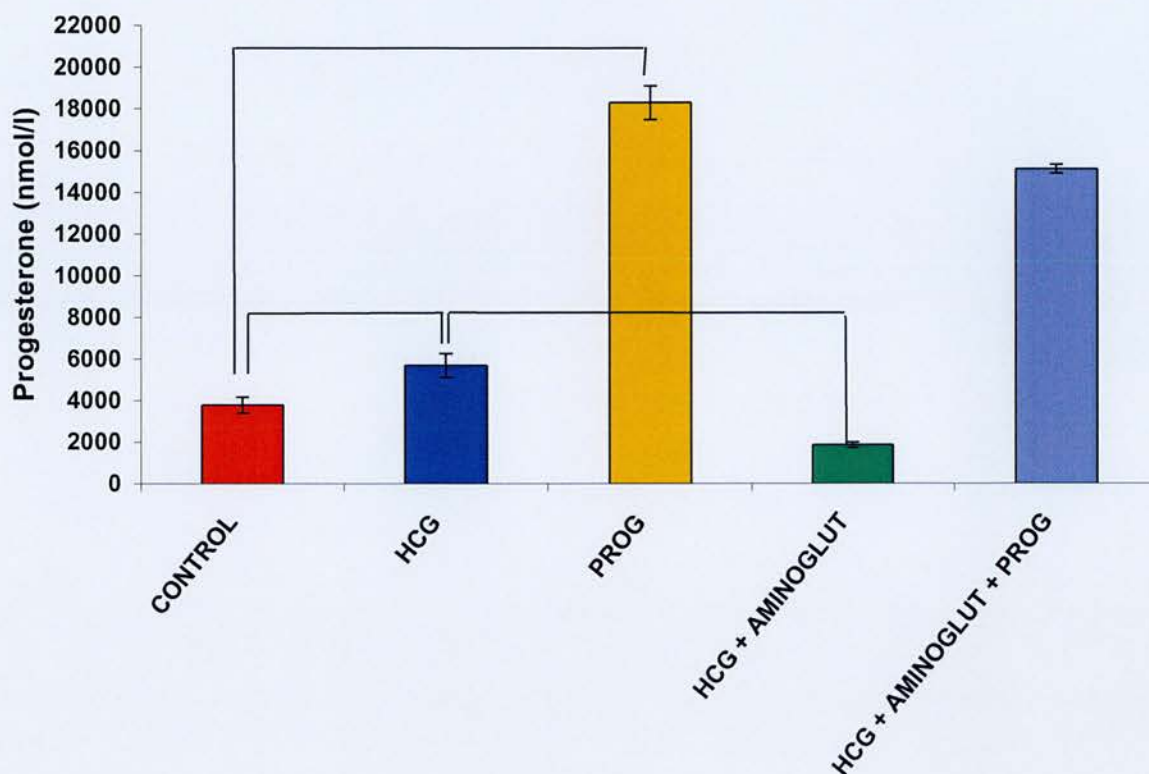


Figure 4.9 Graph to illustrate the effect of different treatments on progesterone secretion (nmol/l) from human luteinised granulosa cells *in vitro*. All treatments were applied to cells for 24 hours. Significant differences as determined by 1-way ANOVA ($P < 0.05$) are shown by connecting bars. Values are means \pm SEM ($n = 8$ cell cultures from individual patients per treatment).

There were no significant differences with regard to VEGF expression as determined by 1-way ANOVA (**Figure 4.10**) however, there was a tendency to higher expression with hCG treatment. This preliminary data provides valuable information eluding to the absence of a role for progesterone in upregulating luteal VEGF in human pregnancy. Treatment with progesterone failed to induce an upregulation of VEGF nor did inhibition of steroidogenesis by aminoglutethamide treatment cause a down regulation of VEGF expression, indicating that increased VEGF expression in the human CL of pregnancy is not a progesterone mediated effect. These data will be further examined in the discussion.

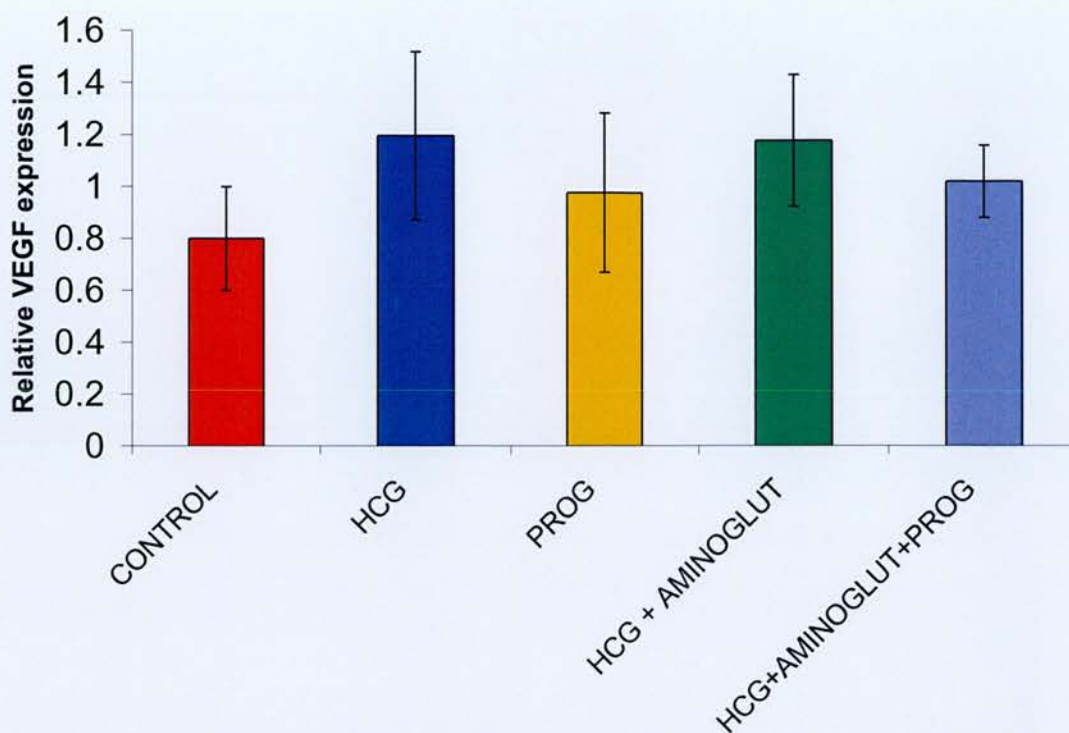


Figure 4.10 Graph to illustrate the effect of different treatments on relative VEGF expression normalised to G6PDH expression in human luteinised granulosa cells *in vitro*. All treatments were applied to cells for 24 hours. Values are means \pm SEM (n=8 cell cultures from individual patients per treatment)

4.3.5 The effect of duration of hCG treatment on luteal steroidogenic cells *in vitro*

4.3.5.1 The effect of duration of hCG treatment on progesterone

As illustrated above, these data do not yet support the hypothesis that progesterone has a direct effect on VEGF expression in luteinised granulosa cells. If progesterone were having an effect, it would be expected that the increase in VEGF would follow the increase in progesterone induced by hCG treatment.

Figure 4.11 and 4.12 depict responses of cells to treatment with 10ng/ml hCG for 12 and 24 hours in terms of progesterone secretion and VEGF expression, respectively. It would appear that the rise in progesterone observed after hCG treatment occurs after 24h, whereas the rise in VEGF expression occurs earlier and is

detectable at 12 hours after treatment. Therefore, hCG treatment appears to induce a rise in VEGF expression that precedes the rise in progesterone secretion.

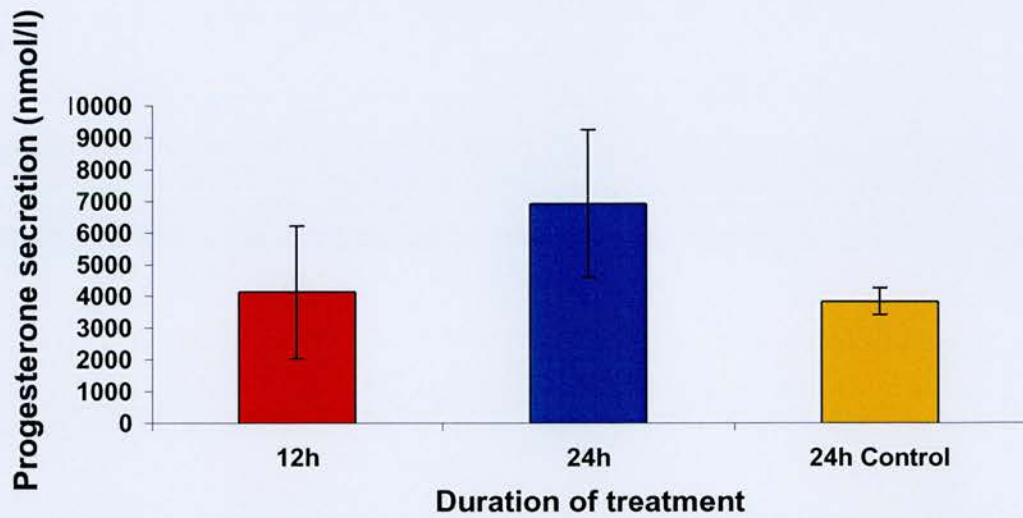


Figure 4.11 Time course data illustrating the effect of varying the duration of hCG treatment on progesterone secretion (nmol/l) from luteinised human granulosa cells *in vitro*. All cells received 10ng/ml hCG. Values are means \pm SEM (n=5 cell cultures from individual patients per treatment).

4.3.5.2 The effect of duration of hCG treatment on VEGF expression

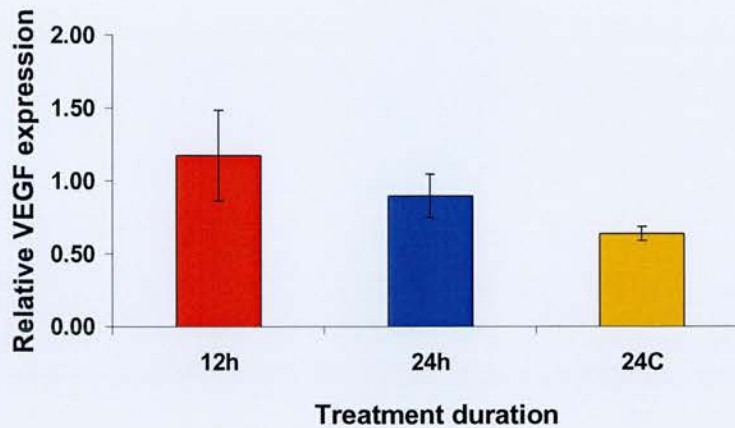


Figure 4.12 Time course data illustrating the effect of varying the duration of hCG treatment on relative VEGF mRNA expression in luteinised human granulosa cells *in vitro*. All cells received 10ng/ml hCG. Values are means \pm SEM (n=5 cell cultures from individual patients per treatment).

4.4 Discussion

Luteal rescue and maintenance of the corpus luteum is of profound importance for the success of early pregnancy. Until such time as the placenta takes over progesterone production, the corpus luteum is the sole provider of progesterone, without which pregnancy is abolished.

An earlier study conducted in women treated with hCG to induce pharmacological rescue of the corpus luteum has demonstrated increased luteal angiogenesis accompanied by an upregulation of VEGF mRNA as demonstrated by *in situ* hybridisation, associated with luteal rescue (Wulff, et al., 2001). I utilised the marmoset as an animal model where physiological early pregnancy could be studied as opposed to pharmacological rescue of the CL. It was surprising that a similar increase in angiogenesis did not occur in luteal rescue as in women, documented in Chapter 3. It was concluded that in the marmoset, maintenance of the already established late luteal vasculature is all that is required for establishment of early

pregnancy without the necessity for increased angiogenesis as observed in the human (Rowe, et al., 2002).

There does not appear to be an upregulation in VEGF mRNA during early pregnancy in the marmoset (Rowe, et al., 2002), however, like the human, the marmoset corpus luteum is “rescued” by a chorionic gonadotrophin molecule secreted by the trophoblast. This CG acts through LH receptors on the surface of the steroidogenic cells. If CG were directly responsible for increased VEGF expression, it is surprising that VEGF is not upregulated in the marmoset. One clear difference however, between early pregnancy in the marmoset and human is the effect of CG on progesterone. Progesterone levels in the marmoset in early pregnancy are maintained without further increase, at levels already attained in the late luteal phase. In the human, progesterone levels rise in early pregnancy compared to peak mid-luteal phase levels. This disparity between species brought forward the hypothesis that the observed upregulation of VEGF mRNA expression in the human could be a progesterone mediated effect.

Not only do marmosets not display increased levels of progesterone in luteal rescue, immunocytochemistry revealed that the marmoset CL does not have progesterone receptors in contrast to the human CL. Therefore, it could be that the increase in angiogenesis observed in the human CL is attributed to its ability to detect this rise in progesterone in response to early pregnancy. The marmoset, not having progesterone receptors in the CL cannot perceive a progesterone stimulus and so therefore does not display increased angiogenesis at this time. This may also explain why marmosets do not have elevated levels of plasma progesterone in early pregnancy compared to the mid luteal phase, as the corpus luteum is unable to respond to this stimulus anyway.

In order to answer the question as to whether or not increased VEGF expression in human CL is progesterone mediated, an *in vitro* luteal steroidogenic cell system was utilised. This system allows the precise addition or removal of various stimuli in order to investigate this hypothesis.

I was able to demonstrate that human luteal steroidogenic cells in culture respond to hCG by increased synthesis and secretion of progesterone accompanied by upregulation of VEGF expression. If a steroidogenesis inhibitor is added together

with hCG, progesterone secretion is reduced but the level of VEGF expression remains at levels similar to those observed with hCG treatment alone. Also, if exogenous progesterone is added to the system, no upregulation in VEGF expression was observed.

Although many of these results were trends rather than specific differences, this preliminary experiment suggests that the increase in VEGF mRNA observed in CL of women treated with hCG, is not a progesterone mediated effect as this increase is not blocked when progesterone synthesis is inhibited, nor is it enhanced by addition of exogenous progesterone to the system. Furthermore, time course experiments revealed that the increase in VEGF expression apparently precedes the increase in progesterone. Therefore, increased VEGF at this time cannot be a sequential effect of increased progesterone.

While progesterone and VEGF expression both increased with increasing doses of hCG, the levels reached were not as markedly different from control levels as anticipated. It could be that the cells should be left longer in culture before application of treatments. Comparing this system to the physiological situation, cells were collected upon aspiration of follicles, so in real time this represents the day of ovulation. Cells were then cultured for 24 hours before treatments were started so therefore cells were treated in the early luteal phase. Luteal rescue would not occur until the late luteal phase and so it may be that the cells should be left in culture for longer before addition of treatments in order that they become fully acclimatised and responsive to hCG at a more physiological time point. In the macaque, the rescue of the corpus luteum in early pregnancy by CG is transient, despite a continued rise in circulating CG. Therefore, age of the primate corpus luteum may be an important factor in determining the nature of the luteal response to CG (Wilks and Noble, 1983). CG administration to macaques in the mid-luteal phase has been demonstrated to cause a transient stimulation of luteal progesterone production similar to that observed in early pregnancy. CG administration in the early luteal phase resulted in persistent, rather than transient stimulation of luteal progesterone production. Therefore in the macaque, the mechanisms involved in the transitory response of the primate CL to CG during pregnancy appear to be inoperative during the early luteal phase of the menstrual cycle (Ottobre and Stouffer, 1984). This has also been

suggested in the marmoset where the CL is thought to gain responsiveness to CG between 6 and 14 days after ovulation (Webley et al., 1989). However, other *in vitro* experiments on human granulosa cells have reported responsiveness of granulosa cells to hCG in terms of upregulation of VEGF expression in the equivalent time period as the early luteal phase. This phenomenon was absent from control cells, not treated with hCG, suggesting that these cells are responsive to hCG at this time (Neulen, et al., 1998). In the current experiment, divergent responses in progesterone secretion and VEGF expression were observed between cells treated with hCG and control cells, suggesting that in these circumstances, the cells were responsive to hCG. Whether the response would be more marked, were the cells cultured for longer before the onset of treatment remains to be elucidated.

If the increase in VEGF expression observed in hCG rescued human CL is not a progesterone mediated effect, then what other factors could be involved to cause this disparity between the marmoset and human? Plasma progesterone levels in the marmoset are extremely high compared to those in women. As such it is possible that progesterone receptors in the marmoset corpus luteum are down regulated by hyperstimulation and this is why I was unable to detect them by immunocytochemistry. It may also be that progesterone can exert effects in the marmoset CL without acting at classic nuclear progesterone receptors. In rat, for example, progesterone receptors are not present in the corpus luteum of pregnancy despite being present in the endometrium and theca and granulosa cells of growing follicles as observed here. This group concluded that progesterone receptor is not required for progesterone action in the rat corpus luteum of pregnancy (Telleria et al., 1999).

It is also possible that hCG may not be the only important signal maintaining CL function at this time. This experiment has demonstrated that an upregulation in progesterone alone does not cause increased VEGF expression, therefore, in human it could be that a rise in progesterone in luteal rescue only when accompanied by upregulation of other local factors contributes to increased VEGF expression and angiogenesis at this time. The system that I have used here isolates luteal steroidogenic cells from the surrounding corpus luteum. It may be that endothelial cells, fibroblasts or immune cells, for example contribute to increased angiogenesis.

Other factors known to stimulate progesterone production by the CL in experimental studies include prostaglandins (Hamberger et al., 1991). Also the human CL increases production of substances other than progesterone during very early pregnancy, including, oestrogen, inhibin and relaxin, however these hormones do not all increase at the same time (Sunder and Lenton, 2000). It is probably hCG which is the first of several signals to the CL which causes up regulation of many hormones and growth factors for example VEGF. Also in the study by Wulff *et al*, hCG was the only signal introduced to the system to induce pharmacological rescue of the corpus luteum. While this probably is the first of several stimuli to be received by the CL in physiological pregnancy, without the presence of a trophoblast and resultant uterine reactions, it is impossible to determine the exact mechanisms occurring in the CL in physiological pregnancy as opposed to pharmacological rescue.

Luteal progesterone production is dependent on luteinizing hormone (LH) from the pituitary gland, despite continuing LH secretion, the human corpus luteum undergoes functional luteolysis unless it is "rescued" by hCG. LH and hCG act through a common receptor which is not down regulated during the maternal recognition of pregnancy in human (Duncan, et al., 1996). In women there is an increase in progesterone production by hCG stimulation, therefore despite LH and hCG acting at the same receptor, differential effects occur in response to each. It would appear that LH maintains the CL during its functional luteal lifespan but in order that the corpus luteum is maintained, beyond the normal time of luteolysis, an hCG stimulus is necessary evoking a divergent effect and increased progesterone synthesis and secretion. In the marmoset however, the corpus luteum of pregnancy did not appear to differ from that in the late luteal phase of non-pregnant cycles. It seems that the marmoset prepares for pregnancy every cycle. The corpus luteum is equipped to produce adequate levels of progesterone to maintain early pregnancy throughout the luteal phase so there is no need for an upregulation of progesterone synthesis nor angiogenesis upon detection of a CG stimulus. It may be that marmoset LH acts more like hCG and so the marmoset enters a "conception" luteal phase every cycle regardless of whether or not a blastocyst is present until such time as it undergoes structural and functional regression probably as a consequence of down regulation of LH receptors (Duncan, et al., 1998). One very interesting observation is

the nature of the marmoset LHR, in that it lacks exon 10 (Zhang et al., 1997). Recent data shows that the human LH receptor lacking exon 10 responds to hCG but not LH (Muller et al., 2003). It is possible therefore that marmoset LH is more like CG in its action. It is also of interest that normally fertile humans only have 22% chance of conception per cycle, while marmosets have greater than 80%.

It would appear that this is a very complex system which requires a great deal more investigation in order that the exact mechanisms by which increased angiogenesis occur in hCG rescue of the human corpus luteum occur. This preliminary experiment suggests that increased VEGF expression in luteal rescue is not a progesterone mediated effect.

Chapter 5

Cellular and morphological changes associated with early pregnancy in the marmoset endometrium

5 Cellular and morphological changes associated with early pregnancy in the marmoset

5.1 Introduction

Angiogenesis is a fundamental process of generating new blood vessels. In the female reproductive tract, angiogenesis has been suggested to be involved in such disorders as luteal phase defects, endometriosis, pregnancy loss, pre-eclampsia and cancer (Gordon et al., 1995). Embryo implantation requires a viable blastocyst and receptive stage endometrium. Angiogenesis in the human uterus is of paramount importance to support the reconstruction of endometrium after menstruation and to provide a vascularised, receptive endometrium for implantation and placentation three weeks later (Torry et al., 1996). This part of the project aims to describe the cellular and morphological events occurring in the marmoset endometrium throughout the cycle and in early pregnancy and to address whether there is increased angiogenesis associated therein.

Implantation in the marmoset occurs at day 12 after ovulation (Enders and Lopata, 1999), yet the maternal vasculature is not invaded until much later, at around day 60 (Smith, et al., 1987). The newly implanted blastocyst must therefore receive the nutrients and oxygen that it requires to meet its increasing metabolic demands by diffusion. In order that this transfer be efficient, it follows that a well developed uterine vasculature is of critical importance. The endometrium is controlled by ovarian steroids, under oestrogen dominance in the proliferative phase and progesterone in the secretory phase. This study describes the proliferative events occurring in the marmoset endometrium in response to ovarian steroids *in vivo* and begins to dissect the pre-implantation events occurring and whether they contribute to successful establishment of pregnancy. I have previously documented in chapter 3, no increase in angiogenesis in the marmoset corpus luteum in early pregnancy

compared to structurally intact non-pregnant controls (Rowe, et al., 2002), where the hormone milieu is the same except for the presence of chorionic gonadotrophin in the systemic circulation of pregnant animals. The ovarian and uterine systems are so intimately linked the question of “will there be any differences in angiogenesis in the endometrium?” begs to be answered.

Animals from the mid-proliferative, late proliferative, early luteal, mid luteal and late luteal stages were studied for morphological comparisons. Animals from the mid-luteal and late-luteal stages were compared to pregnant animals at day 14, 21 and 28 post ovulation. Qualitative studies were conducted on all groups but morphometric analyses of angiogenesis were only performed on non-pregnant animals from the mid (day14) and late (day 21) secretory phase and on all pregnant animals. The early implantation site and foetal maternal interface will be described but only briefly. The marmoset implantation site at the stages of pregnancy studied here have been previously described by others using light (Moore, et al., 1985) and electron microscopy (Smith, et al., 1987). This experiment aims to compliment these observations and to link morphological observations with quantitative analysis of angiogenic events occurring within the endometrium at this time.

5.2 Specific methods

5.2.1 Details of animals used for the experiment

Experiment	Groups	Number of animals
Angiogenesis in the endometrium	Mid-luteal Day 14	5
	Late luteal (Day 21)	5 (all animals had structurally intact CL)
	Pregnant (Day14)	5
	Pregnant (Day21)	5
	Pregnant (Day 28)	5

Table 5.1 Details of animals used for the experiments

5.2.2 Dual immunostaining of CD31 and BrdU by Confocal microscopy

This is a similar method to that employed in all other immunocytochemical techniques described previously except that a fluorescent detection system is employed and 0.05M TBS /1% tween is used for all washes and to make the normal serum block. Slides were dewaxed and rehydrated before undergoing antigen retrieval by pressure cooking in 0.01M citrate buffer as described in Chapter 2. This was followed by a standard peroxidase block and avidin/biotin block in NRS diluted in TBS/tween, (Chapter 2). Two primary antibodies, Anti-BrdU (sheep) and CD31 (mouse) were used, see Table 2.1, diluted together in normal rabbit serum at 1:300 and 1:20 respectively before application to slides overnight in a sequenza at 4°C.

After 3, 5 minute TBS/Tween washes, biotinylated rabbit anti-sheep was added (1:500) with rabbit anti-mouse peroxidase (1:500) in normal rabbit serum block for 1h at room temperature. Slides then received 3 TBS/tween washes followed by 2 0.01MPBS/1% tween washes and a final wash with PBS alone. In order to detect the CD31 antibody, a TSA-plus cyanine system was used (Perkin Elmer Life Sciences), diluted 1:50 in supplied buffer. This was applied for 10 minutes at room temperature before slides received 2 PBS/tween washes followed by a PBS wash. Slides were then incubated with Streptavidin Alexafluor 488, diluted 1:200 with PBS for 2-3h at room temp before being washed with PBS overnight.

Finally, propidium iodide was added (10µg/ml in PBS/tween) for 20 minutes at room temperature before slides were washed in PBS/tween for at least 2 hours. Slides were mounted straight from PBS/tween in permafluor aqueous mounting medium (Beckman Coulter, Luton, Beds, UK) and examined under a confocal microscope (Carl Zeiss laser scanning microscope LSM510, Axiovert 100M, version 3.2).

5.2.3 Quantification of dual immunostaining

5.2.3.1 Volume fraction measurements

In terms of quantitative analysis of proliferation, a grid system was devised according to Hastings *et al*, (Hastings, et al., 2003). This allows for the accurate volume fraction analysis of each of these different compartments across a uterine section and subsequent proliferation indices to be calculated. The uterus is an organ subject to oedematous changes and therefore analyses of immunocytochemical staining in the same way as employed for the CL were not appropriate. The methods employed take into account any volume changes and so therefore correct for this phenomenon and allow comparison between groups where changes in oedema may not occur to the same degree.

Volume fraction analyses were performed in three directions, from luminal epithelium to myometrium, each perpendicular to the luminal epithelium. The first, at the fundic end of the uterus. The second and third on the right and left hand side of the cross section respectively (See **Figure 5.2**) In each direction, every field of view was analysed, ensuring no overlap occurred between each. This gave rise to between 30 and 40 grids per animal, an example of which is given in **Figure 5.1**. The test grid, superimposed on the sections, consisted of 588 points. The number of test points falling on glands (including lumen), proliferating glandular epithelium, uterine lumen, stroma, proliferating stromal cells, myometrium, luminal epithelium, proliferating luminal epithelial cells, endothelial cells and proliferating endothelial cells (characterised by colocalised BrdU and CD31 immunostaining), were counted. The volume fraction occupied by each component was then calculated by expressing the number of points hitting that component as a percentage of the total number of test points applied. If, for example, the volume fraction of proliferating endothelial cells was required, it was expressed as volume fraction of proliferating endothelial cells as a percentage of volume fraction of total endothelial cells.

Analysis of randomly selected sections was conducted by two independent scorers. To validate this method the two data sets gained from each observer were compared using a T-test. No significant differences in results were seen signifying that this technique was repeatable and accurate.

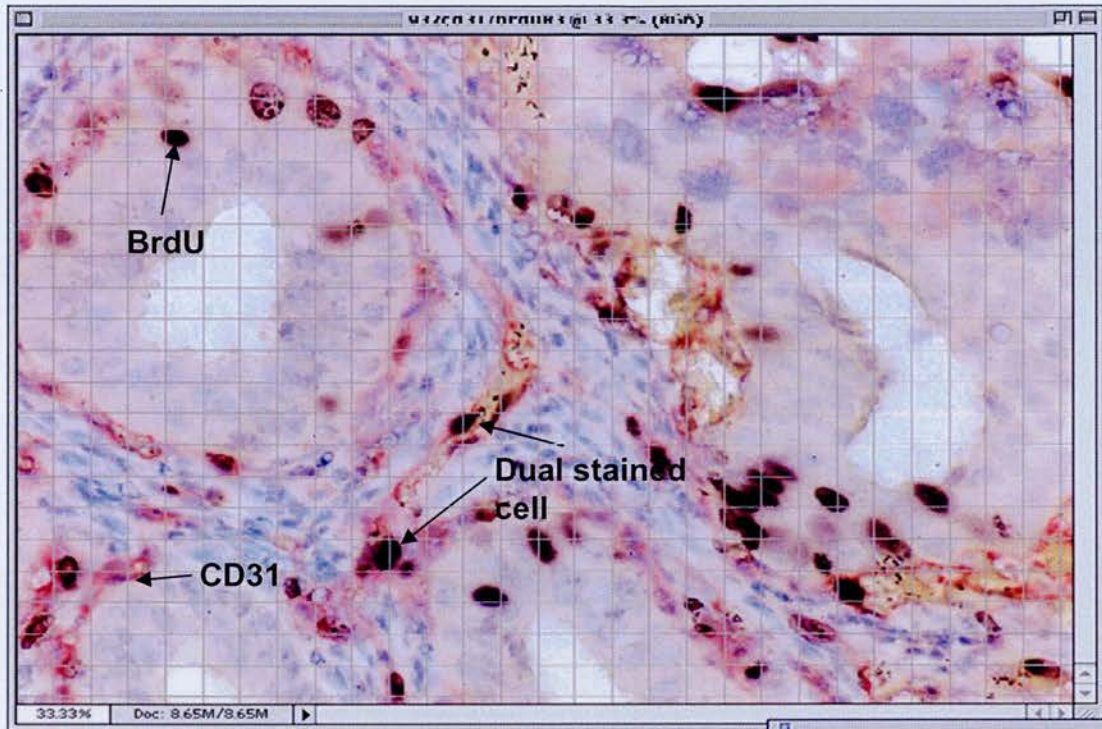


Figure 5.1 Example of the grid system used to obtain volume fraction data. The grid consists of 588 points (21 x 28). This grid size was chosen as it allows differentiation of individual cells with no cell being counted by more than one point. At each point on the grid, the cell type was recorded and expressed as a volume fraction of the total 588 points. The number of proliferating cells were expressed as a volume fraction of the total volume fraction of that cell type. For example the number of points overlaying proliferating endothelial cells was divided by the total volume fraction of endothelial cells to gain a volume fraction of proliferating endothelial cells.

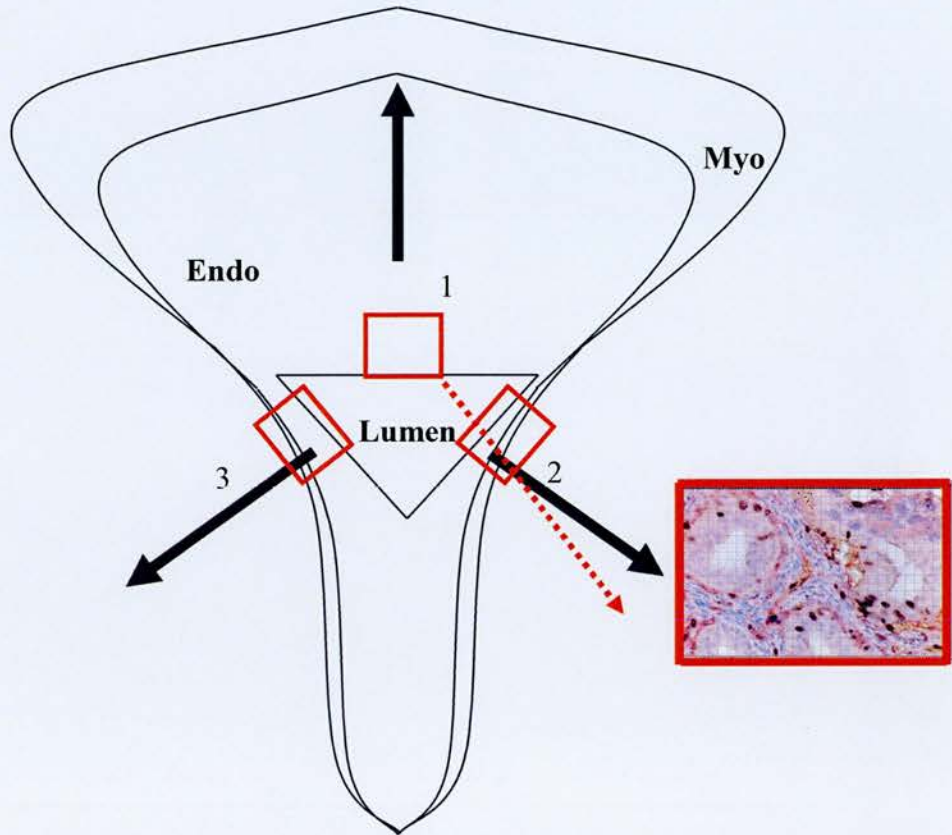


Figure 5.2 Schematic illustrating grid placement. Volume fraction analyses were performed in three directions, from luminal epithelium to myometrium, each perpendicular to the luminal epithelium. The first, at the fundal end of the uterus (1). The second and third on the right and left hand side of the cross section respectively, (2) and (3). In each direction, every field of view was analysed at x40 objective magnification, ensuring no overlap occurred between each. This gave rise to between 30 and 40 grids per animal.

5.3 Results

5.3.1 Gross morphology and patterns of cell proliferation in marmoset endometrium throughout the cycle

Figures 5.3 – 5.7 document uterine morphology and the patterns of cell proliferation throughout the ovulatory cycle of non-pregnant marmoset endometrium. **Figure 5.3A and B** are photomicrographs of H & E stained sections of full thickness endometrium from the mid-proliferative phase highlighting the luminal epithelium (LE), glandular epithelium (GE) and stroma (S). **(C and D)** depict BrdU incorporation into proliferating cells. In the mid-proliferative phase, the luminal epithelium and glandular epithelium of the functionalis are intensely proliferating, while glandular epithelium of the basalis region does not appear to be undergoing mitosis. No positively stained stromal cells were detected in the endometrium at this stage.

By the late proliferative phase, glandular epithelial proliferation has progressed throughout the full thickness endometrium and BrdU staining remains high in the luminal epithelium (**Figure 5.4C and D**). Low levels of BrdU immunostaining were present in the stroma at this stage.

Figure 5.5 documents uterine morphology (**A and B**) and BrdU incorporation (**C and D**) in the early secretory phase. At this stage of the cycle, there still exists a high level of cellular proliferation in the luminal epithelium and glandular epithelium. Stromal proliferation is apparent throughout the full thickness endometrium, particularly in the functionalis region. By the mid-secretory phase, no luminal epithelial or glandular epithelial proliferation was detectable in the functionalis (**Figure 5.6 D and F**) however, glandular proliferation is maintained in the basalis (**Figure 5.6B**). Also note accumulation of large maternal vessels (MV) beneath the luminal epithelial surface (**A**).

In the late secretory phase, (**Figure 5.7**), no glandular nor luminal epithelial proliferation was detectable in any zone of the endometrium. Stromal proliferation remained high in the functionalis and the large maternal vessels (MV) first observed in the mid-secretory phase are still apparent in the late luteal phase (**B**).

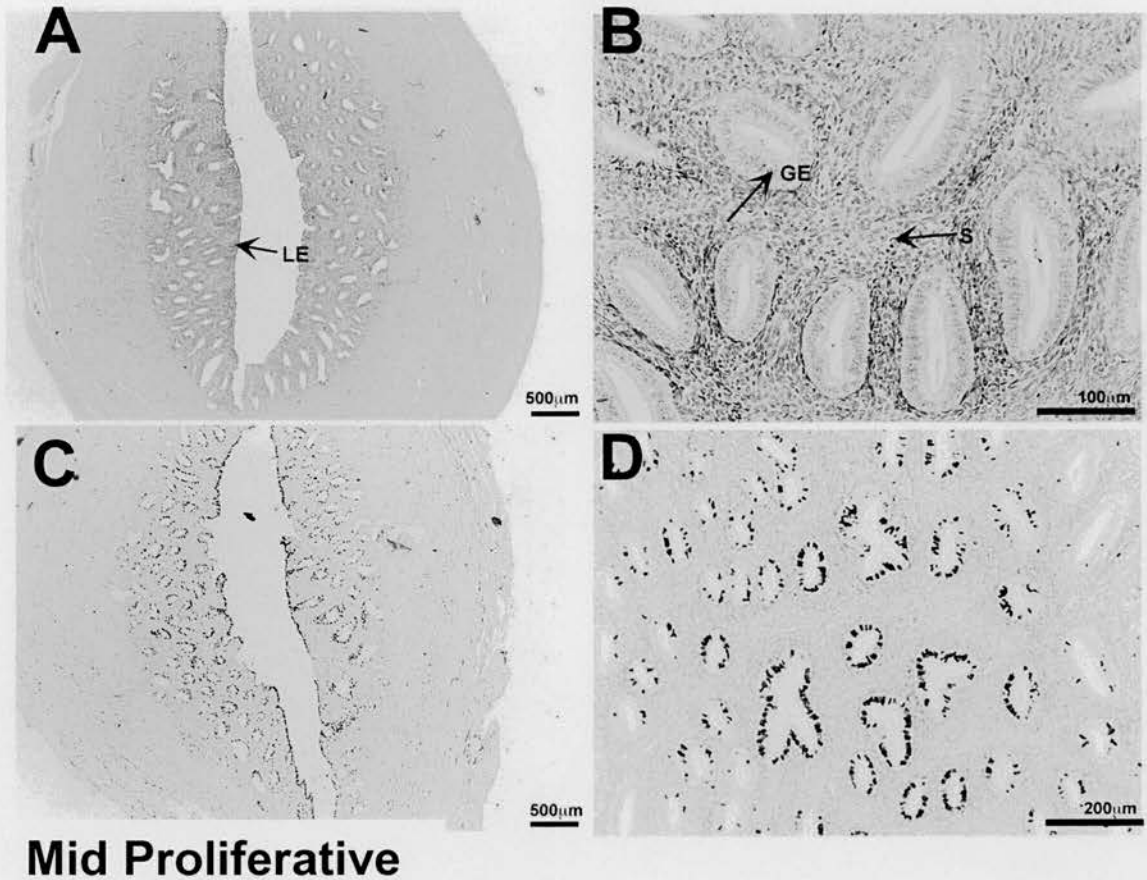


Figure 5.3 Uterine morphology in the mid-proliferative phase. (A) and (B) are H & E stained sections highlighting the luminal epithelium (LE), glandular epithelium (GE) and stromal (S) compartments of the uterus. (C) and (D) illustrate cell proliferation by immunocytochemical detection of BrdU. Note high level of glandular proliferation and absence of staining in the stroma.

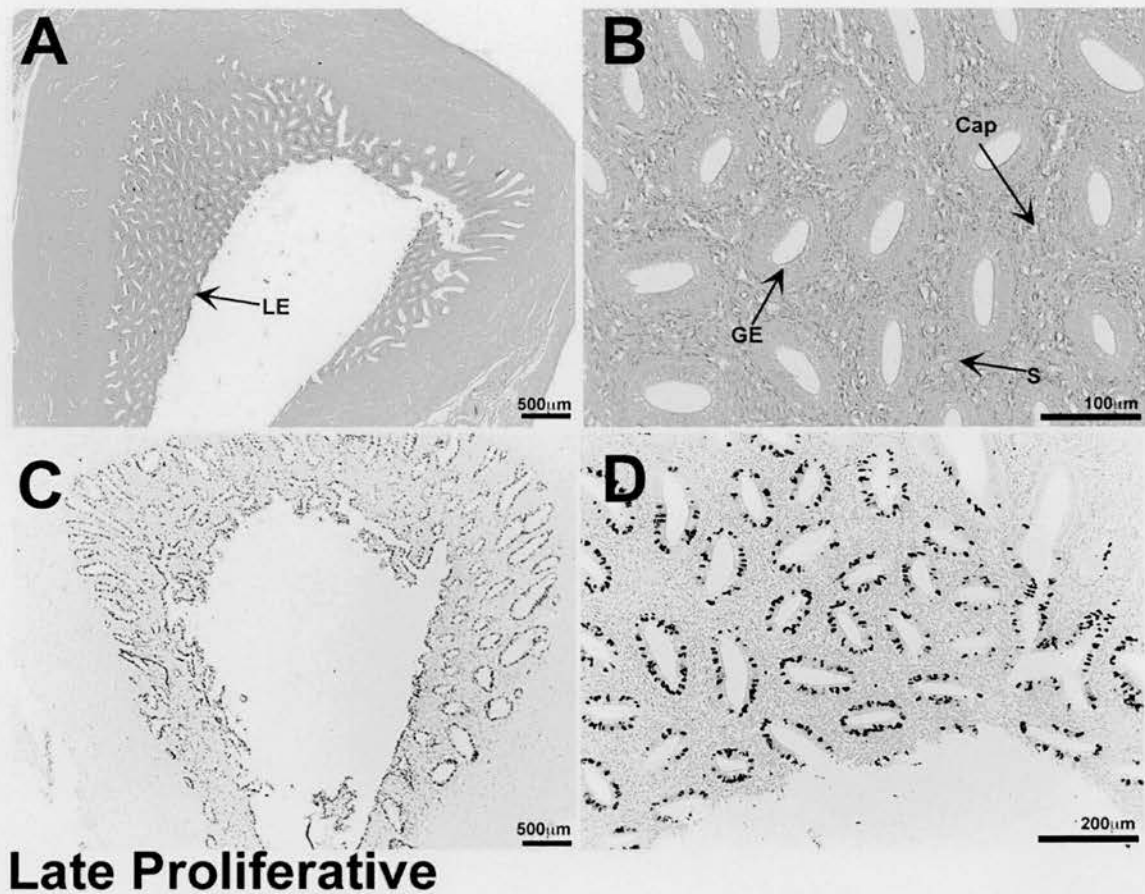
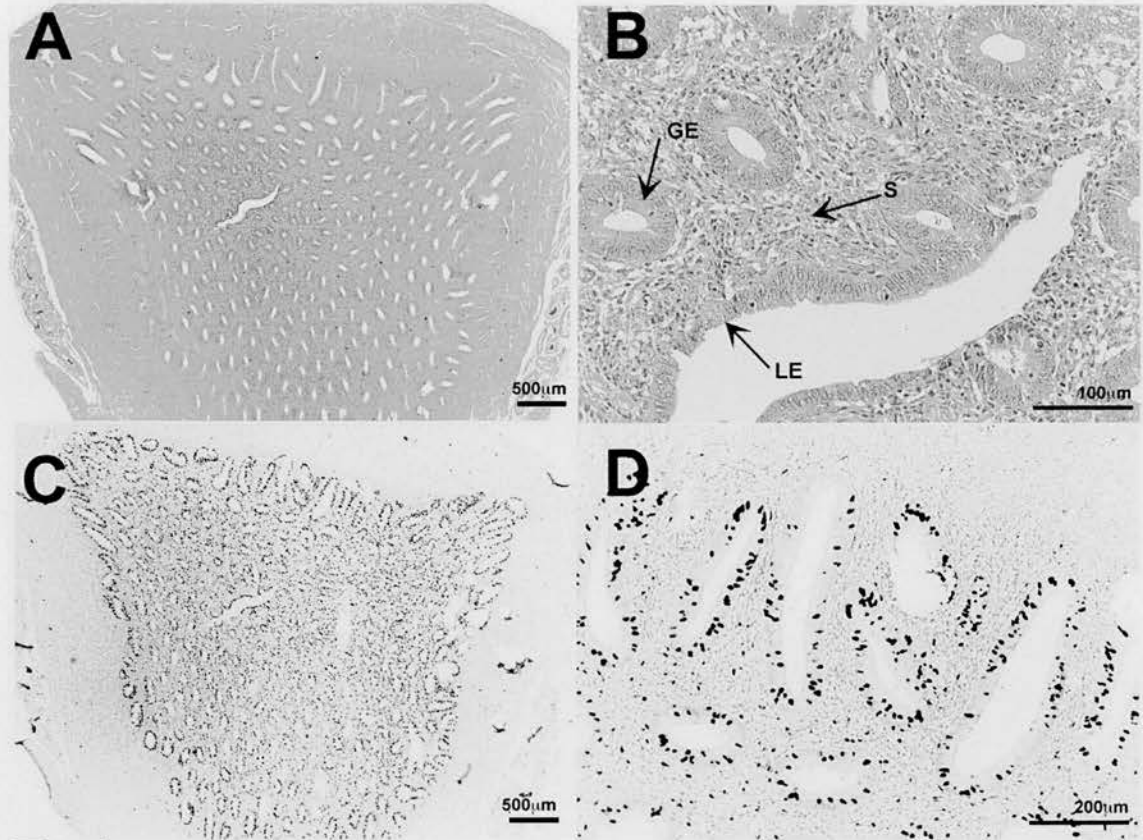
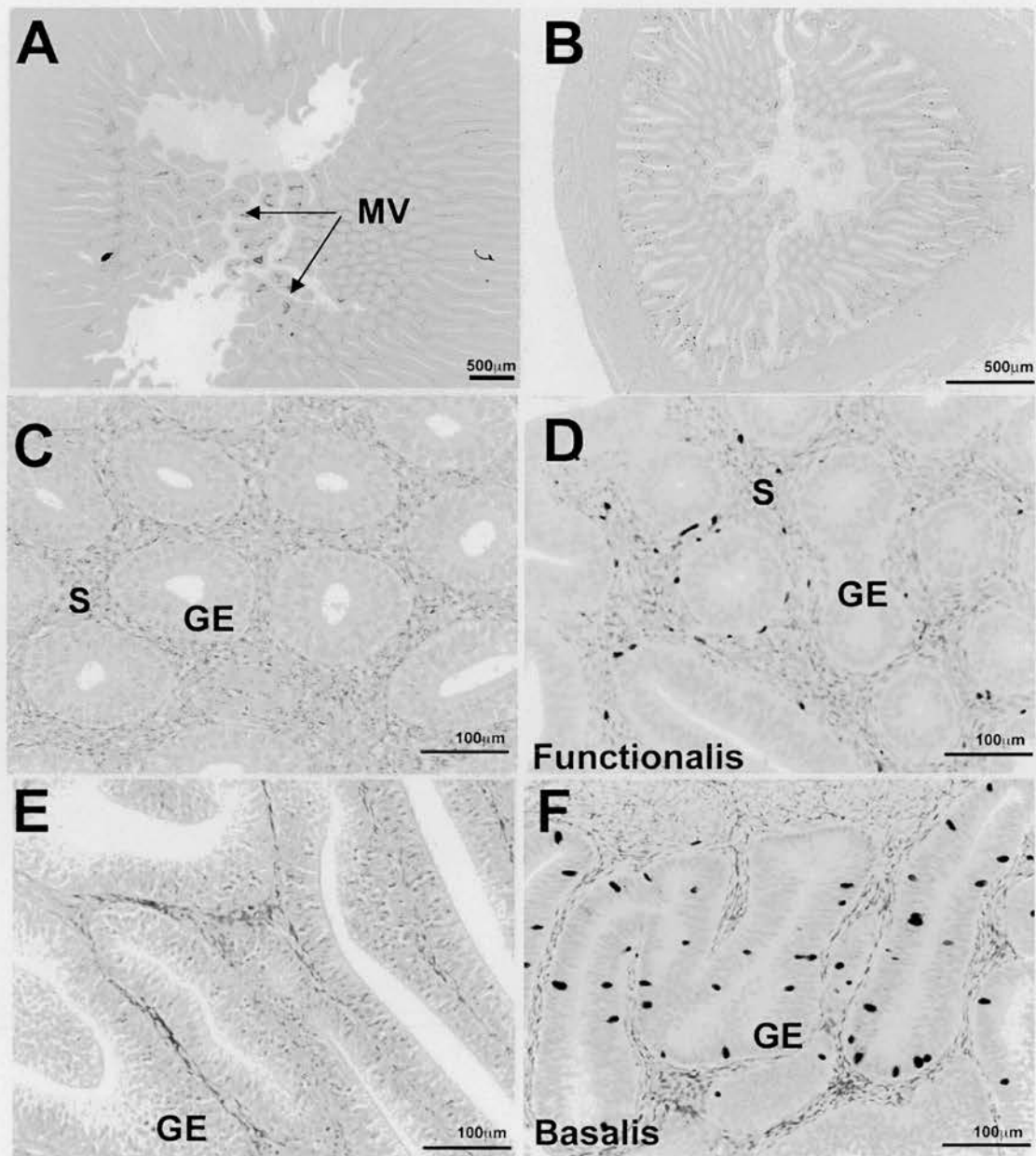


Figure 5.4 Uterine morphology in the late-proliferative phase. (A) and (B) are H & E stained sections highlighting the luminal epithelium (LE), glandular epithelium (GE) and stromal (S) compartments of the uterus. (C) and (D) illustrate cell proliferation by immunocytochemical detection of BrdU. Note high level of glandular proliferation and few immunopositive nuclei in the stroma.



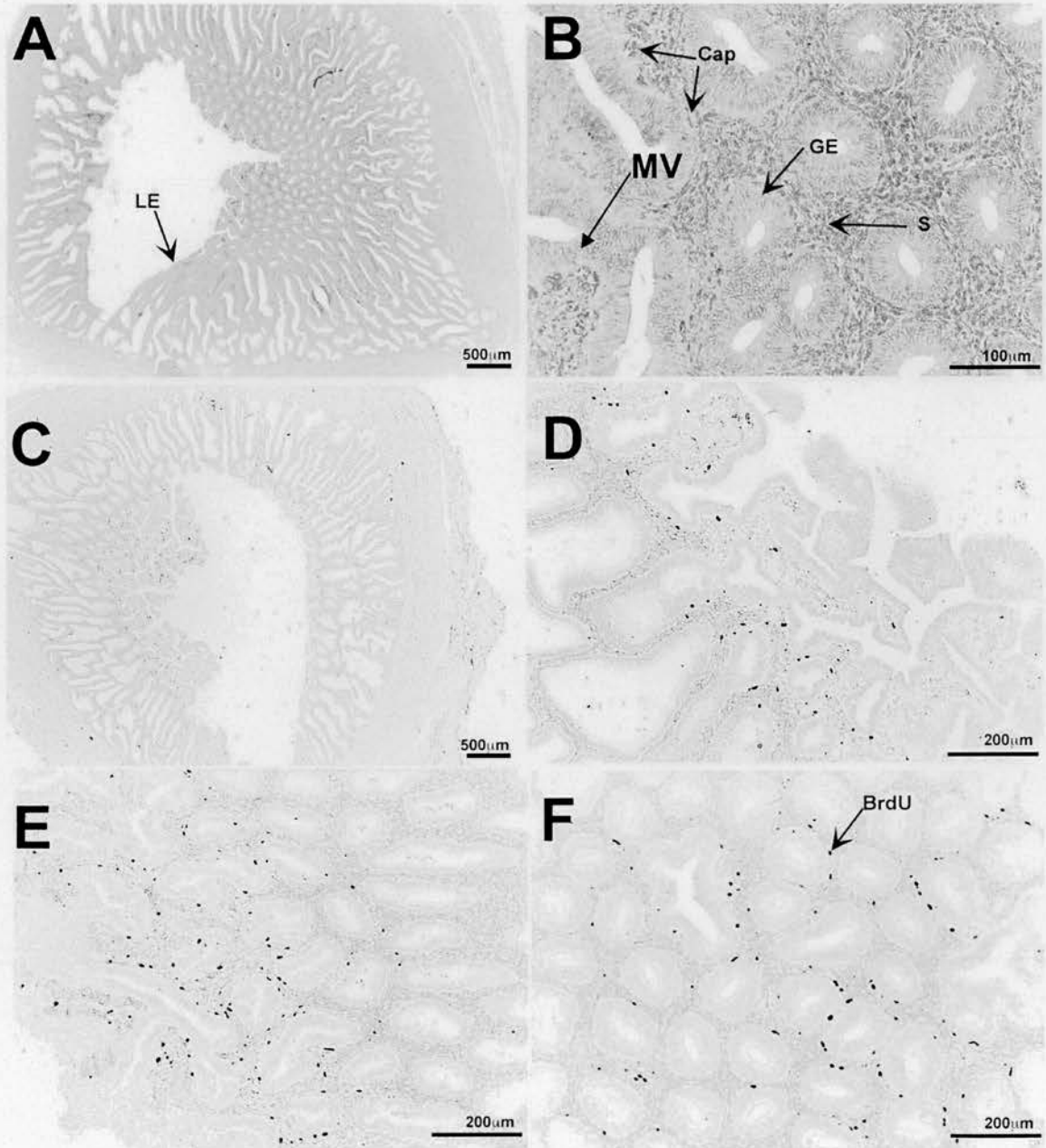
Early secretory

Figure 5.5 Uterine morphology in the early-secretory phase. (A) and (B) are H & E stained sections highlighting the luminal epithelium (LE), glandular epithelium (GE) and stromal (S) compartments of the uterus. (C) and (D) illustrate cell proliferation by immunocytochemical detection of BrdU. Note high level of glandular and stromal proliferation.



Mid-secretory

Figure 5.6 Uterine morphology in the mid-secretory phase. (A, C, E) are H & E stained sections. (B) illustrates cell proliferation across the entire endometrial section by immunocytochemical detection of BrdU. (D and F) illustrate BrdU incorporation in the functionalis and basalis layer, respectively. Note presence of glandular BrdU in the basalis (F), and absence of glandular proliferation in the functionalis (D). The large maternal vessels appearing just beneath the luminal epithelium have been highlighted (MV).



Non-pregnant late secretory

Figure 5.7 Uterine morphology in the late-secretory phase. (A) and (B) are H & E stained sections highlighting the luminal epithelium (LE), glandular epithelium (GE), stroma (S) and large capillaries near the lumen of the uterus (Cap). (C, D, E and F) illustrate cell proliferation by immunocytochemical detection of BrdU. Note absence of proliferation in the glandular epithelium, and high level of stromal proliferation particularly around the luminal epithelium.

5.3.2 Description of the foetal-maternal interface

Figure 5.8 illustrates cyokeratin immunocytochemistry at day 28 of pregnancy in order to distinguish the foetal maternal interface. Cytokeratin is a trophoblast marker. It was used to distinguish foetal and maternal tissues. Note the superficial attachment of the trophoblast to the surface epithelium of the endometrium without invasion of luminal epithelium. Maternal vessels (MV) depicted in (D) are not invaded by trophoblast at this stage. A diagrammatic representation of the marmoset implantation site is shown in **Figure 5.9**, depicting the main cell types and structures present at the implantation site. Foetal vessels have been included for completeness, although the trophoblast remains avascular during the stages of pregnancy studied for the purposes of this thesis and does not become vascularised until much later in pregnancy.

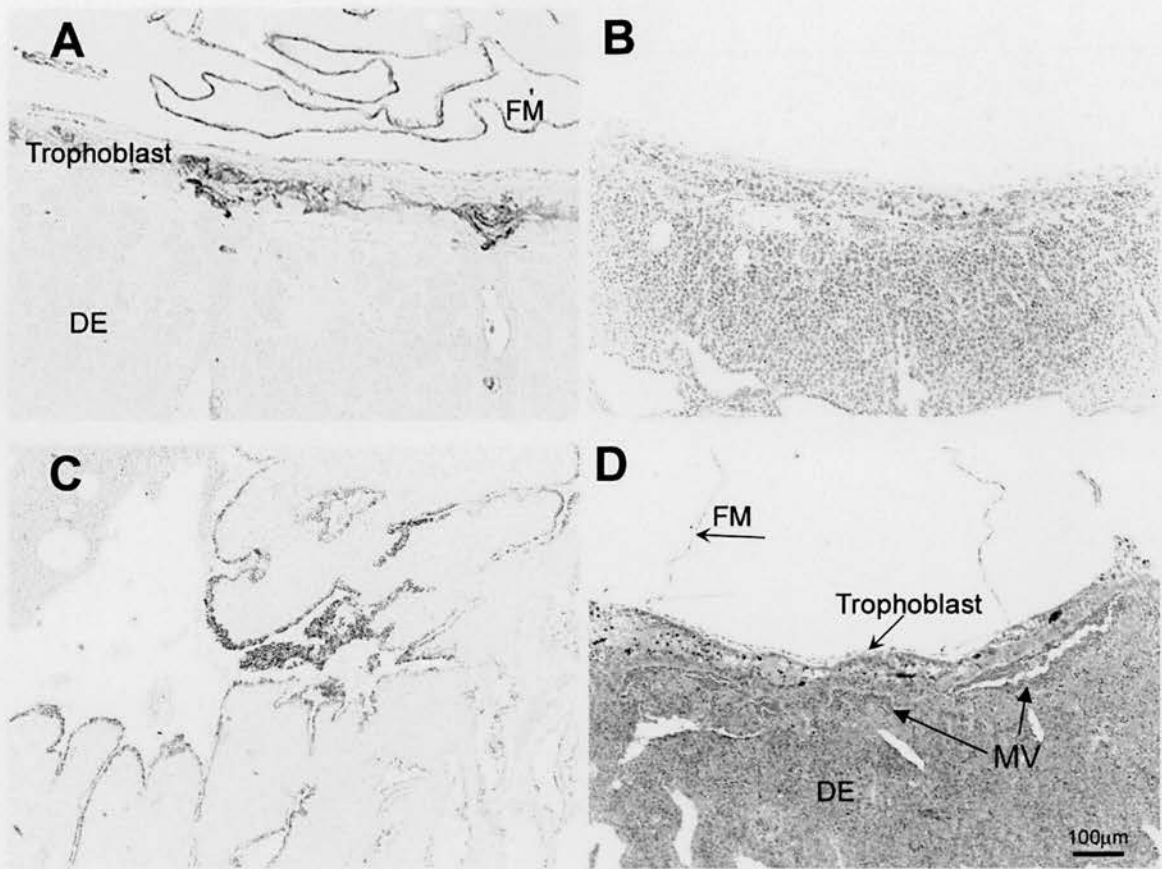


Figure 5.8 Cytokeratin immunocytochemistry for differentiation of trophoblast from maternal tissue in early pregnancy. (A) Note high level of immunostaining in the trophoblast and foetal membranes (FM) with absence of staining in the maternal decidua endometrium (DE). (B and C) are negative and positive controls respectively. (D) H & E stained section to illustrate uterine morphology at this time.

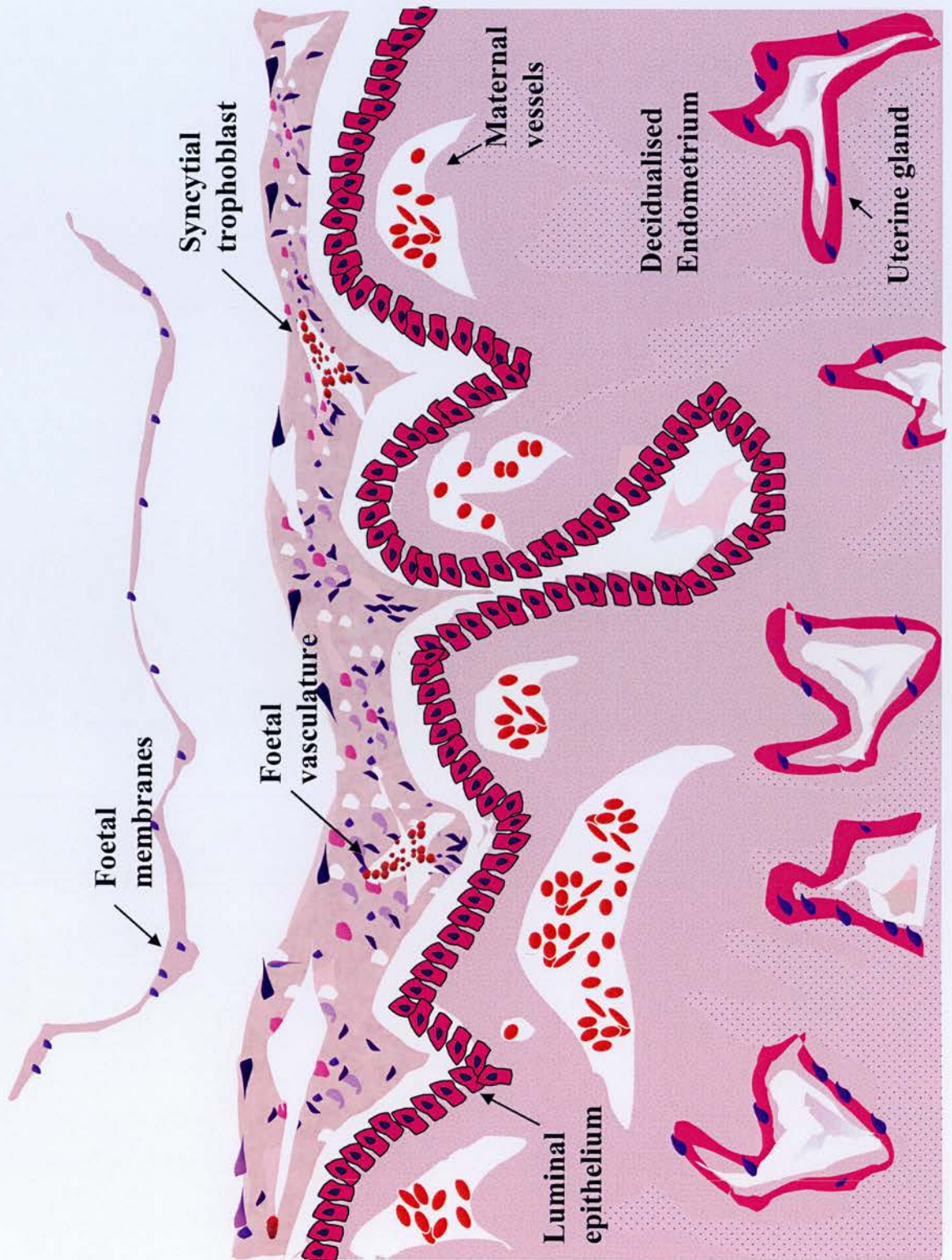


Figure 5.9 Diagrammatic representation of the marmoset implantation site

5.3.3 Gross morphology and patterns of cell proliferation at the time of implantation and in early pregnancy in marmoset endometrium

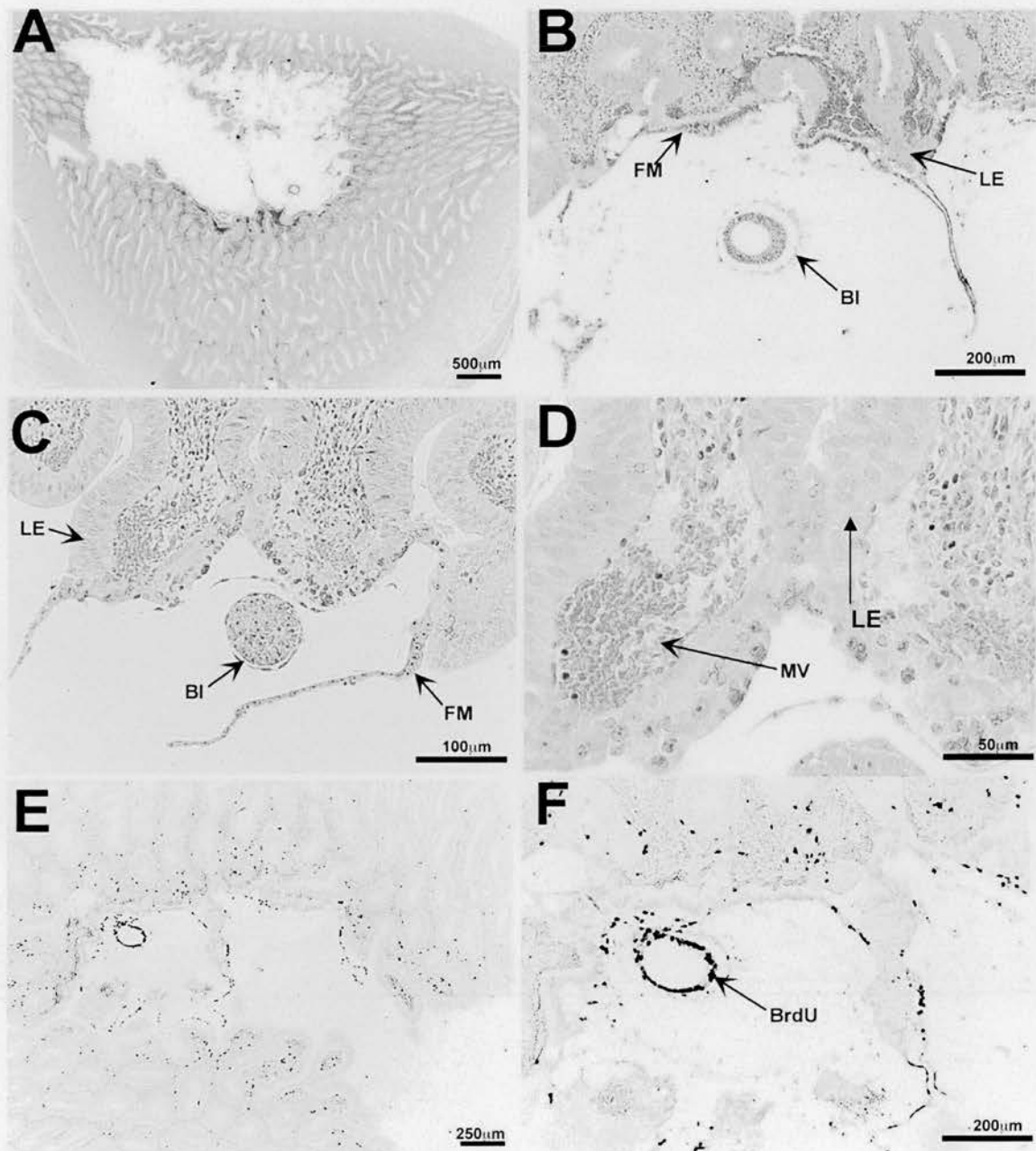
Figures 5.10 – 5.12 illustrate uterine morphology and cell proliferation during early pregnancy. **Figure 5.10** depicts morphology of the marmoset implantation site. Note the superficial attachment of the blastocyst (Bl) to the uterine epithelium (LE) best shown in (**Figure 5.10C**). The luminal epithelium appears to remain intact with minimal cellular damage by the newly implanted conceptus (**D**). Also highlighted in (**D**) are the large, maternal vessels immediately beneath the luminal surface. These appear larger than those in secretory phase non-pregnant endometrium, particularly those immediately beneath the implantation site, best depicted in (**A** and **B**). The pattern of BrdU incorporation is illustrated in (**E** and **F**). No glandular epithelial proliferation was observed in any region of the endometrium. Intense stromal proliferation was apparent in the functionalis, particularly immediately surrounding the glands of this region. The inner cell mass of the conceptus is also intensely proliferating at this time (**F**).

Figure 5.11 depicts the marmoset implantation site one week later at day 21. The blastocyst remains in superficial attachment to the endometrium with minimal invasion of luminal epithelium (**D**). Decidualisation of stromal cells is occurring, beginning in the functionalis around the entire luminal surface of the endometrium irrespective of the implantation site and progressing to the basalis (**A** and **E**). Stromal proliferation in the functionalis is very intense at this time particularly notable in decidua (**F**).

By week 4 of pregnancy (**Figure 5.12**), the stroma has become decidualised to a greater depth towards the basalis. While the trophoblast still remains in superficial attachment to the surface endometrium, penetration of syncytial trophoblast beneath the luminal epithelium is evident (**D**). Large maternal vessels (**MV**) rest beneath the luminal epithelium around the entire uterine lumen and there appears to be no penetration of these vessels by invading trophoblast. They clearly

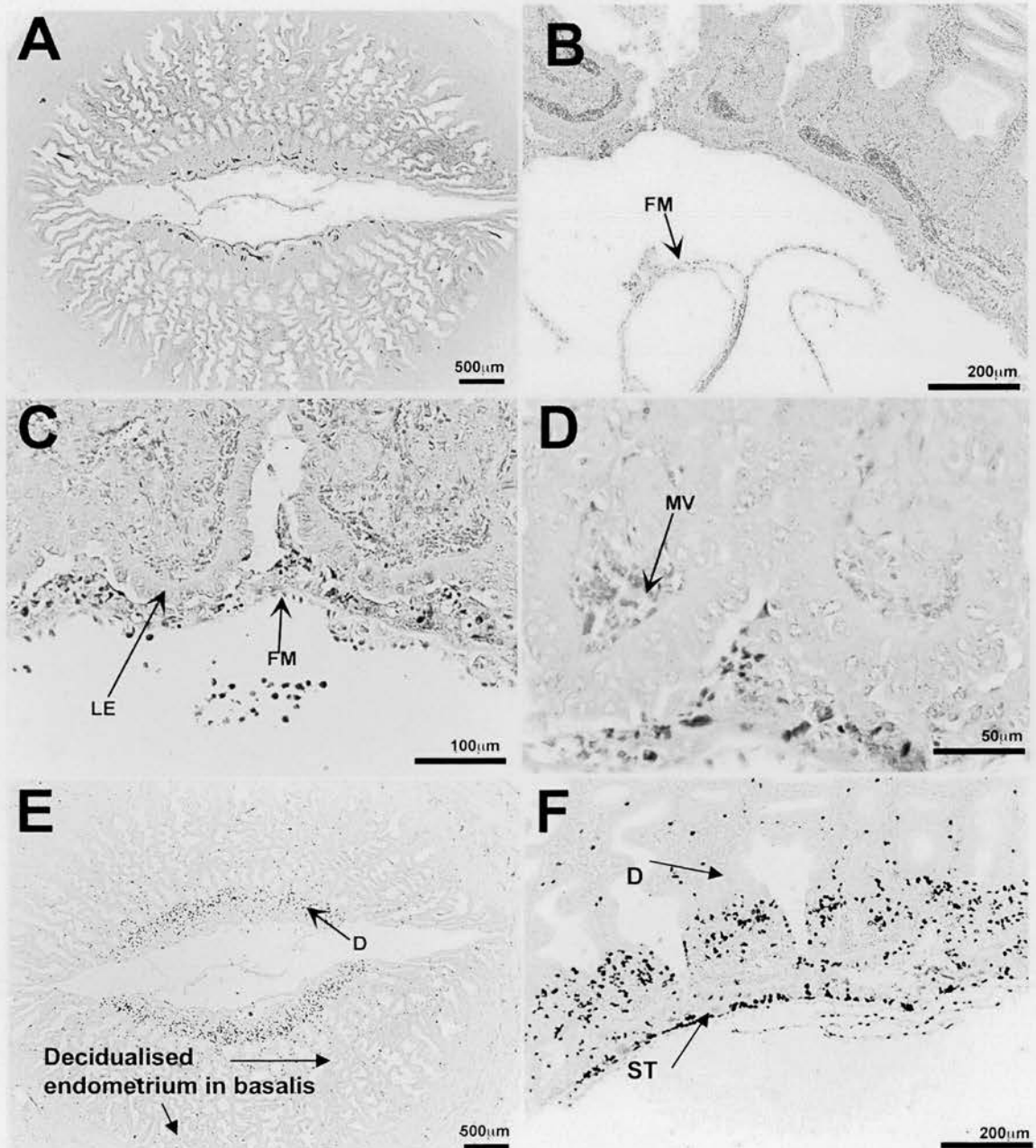
retain a continuous endothelium which is undisrupted. Note the high level of glandular epithelial proliferation in the basalis region at this stage of pregnancy (**E**), and the intensity of proliferation of decidualised stromal cells throughout the entire endometrium, most intensely in the functionalis (**F**).

Figure 5.13 illustrates confocal microscopy images of sections dual stained with CD31 (blue) and BrdU (green). This was done to determine whether or not the intensely proliferating decidual stromal cells depicted in **Figure 5.12F** are endothelial cells. A positive control from the marmoset corpus luteum is included (**C**). This illustrates a dual stained proliferating endothelial cell which appears yellow. In (**A** and **B**) all BrdU stained cells colocalise with CD31 giving rise to a yellow cell. However, in (**D**), the BrdU and CD31 do not colocalise and retain their individual green and blue colours respectively, therefore, these are not co-localising and so the proliferating decidual stromal cells in early pregnancy in the marmoset are clearly not endothelial cells. In (**A**), the foetal membranes are immunopositive for CD31 yet are avascular throughout pregnancy. It may be that the antibody is cross reacting here or that these cells may be endothelial precursors for later development into endothelial cells.



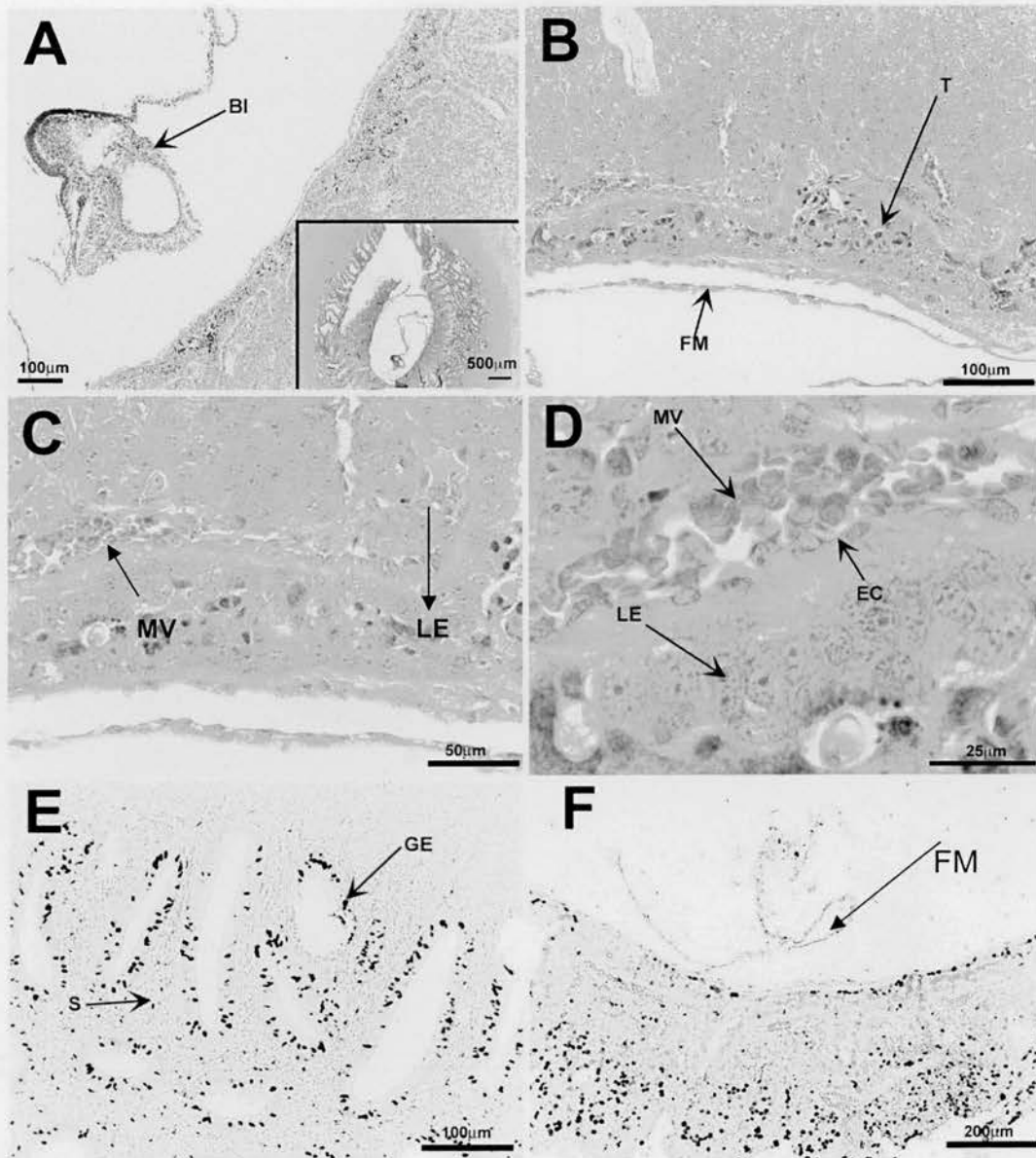
Pregnant, Day 14

Figure 5.10 Uterine morphology at 2 weeks of pregnancy. (A, B, C and D) are H & E stained sections highlighting the luminal epithelium (LE), blastocyst (BI) foetal membranes (FM) and large maternal vessels (MV). (E and F) illustrate immunocytochemical detection of BrdU. Note high level of proliferation in blastocyst and stroma nearest the luminal epithelium, while no BrdU was detected in the glandular epithelium.



Pregnant, Day 21

Figure 5.11 Uterine morphology at 3 weeks of pregnancy. (A, B, C and D) are H & E stained sections highlighting the luminal epithelium (LE), foetal membranes (FM) and large maternal vessels (MV). (E and F) illustrate immunocytochemical detection of BrdU. Note high level of proliferation in decidualised stroma nearest the luminal epithelium (F), while little BrdU was detected in the glandular epithelium. Also note syncytial trophoblast invasion of the uterine lumen at this stage (ST), (D) Decidua.



Pregnant, Day 28

Figure 5.12 Uterine morphology at 4 weeks of pregnancy. (A, B, C and D) are H & E stained sections highlighting the luminal epithelium (LE), foetal membranes (FM), blastocyst (BI), trophoblast (T), endothelial cells (EC) and large maternal vessels (MV). (E and F) illustrate immunocytochemical detection of BrdU. Note high level of proliferation in decidualised stroma nearest the luminal epithelium, and BrdU immunostaining in the glandular epithelium.

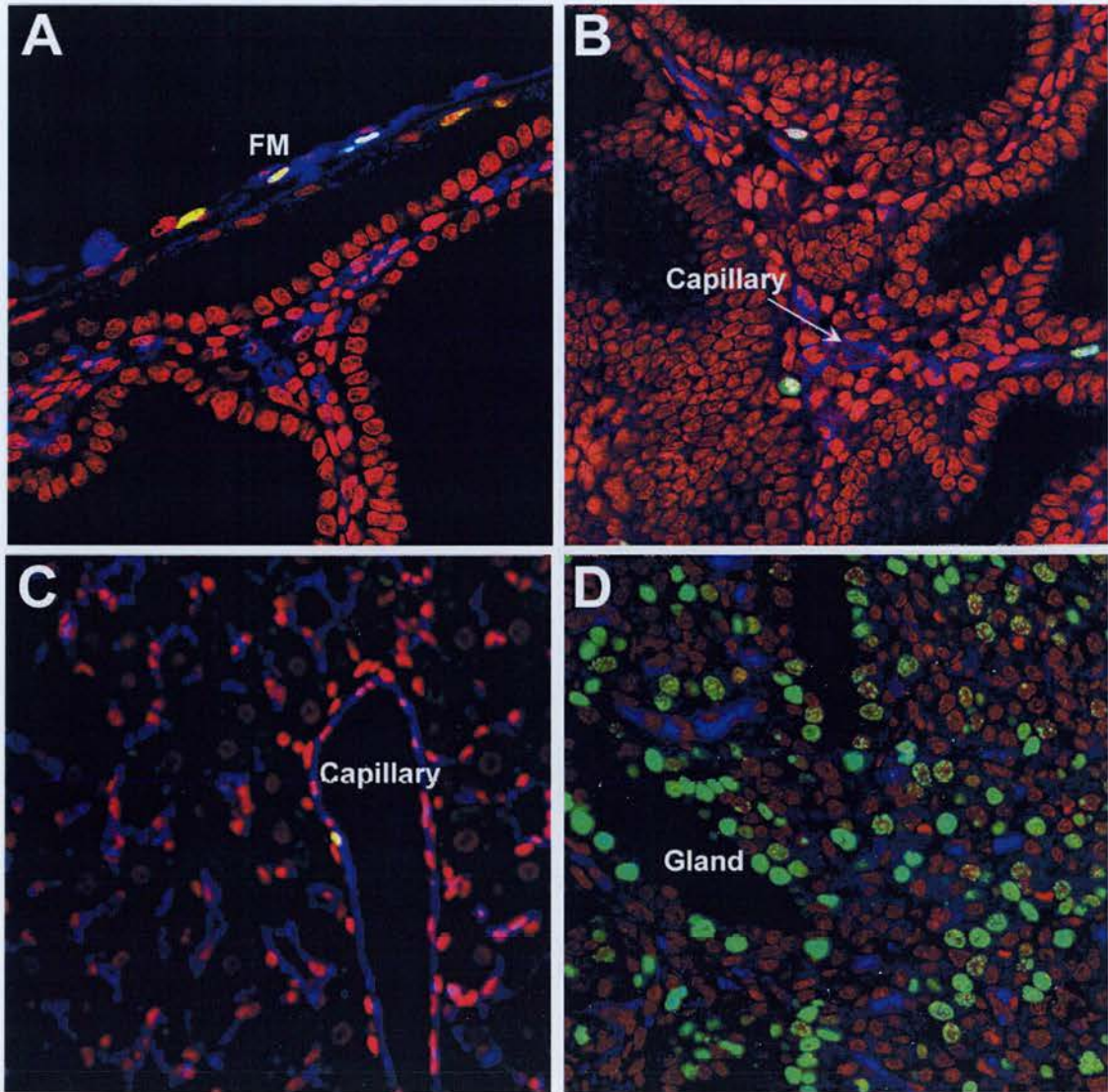


Figure 5.13 Confocal microscopy illustrating dual staining for CD31 (blue) and BrdU (green). (A) foetal membranes (FM) and upper zone endometrium at day 14 of pregnancy, (B) Non-pregnant late secretory phase endometrium at day 21. (C) Positive control from marmoset corpus luteum and (D) decidualised upper zone endometrium from day 28 of pregnancy. Where endothelial cells (blue) are colocalising with BrdU (green), a resulting yellow colour is seen as marked in (C). This depicts a proliferating endothelial cell.

5.3.4 Uterine weight

Figure 5.14 A and B illustrate mean uterine weights before tissue fixation. If precise uterine weights are plotted against stage of the cycle as in (A), there is a significant increase in uterine weight at day 28 of pregnancy compared to late luteal controls. However, this does not take into account animal body weight. If uterine weight is expressed as a relative proportion of body weight, it can be seen that uteri at pregnancy day 28 are heavier than both groups of non-pregnant controls.

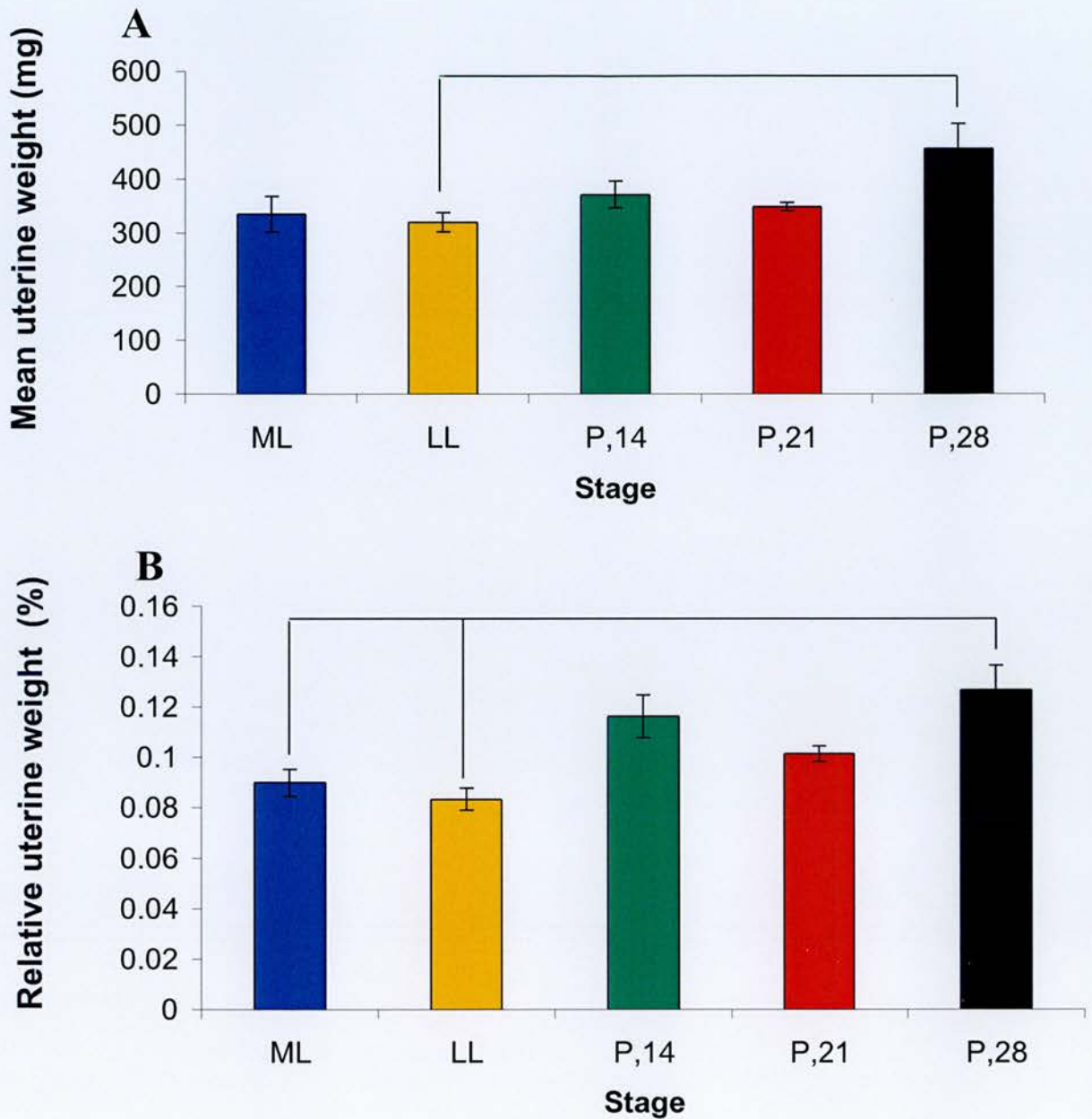


Figure 5.14 Graphs to show mean uterine weight before fixation from marmosets in the different study groups. (A) Uterine weight without correction for body weight. (B) Relative uterine weight as a percentage of body weight. Significant differences ($P < 0.05$) as determined by 1 way-ANOVA are denoted by adjoining lines. Values are means \pm SEM. ($n=5$ per group)

5.3.5 Volume fraction analysis

5.3.5.1 Cellular compartment analysis

Figure 5.15 (A and B) depict the differences in volume fraction occupied by glands (including lumen), luminal epithelium, endothelial cells and stroma throughout the stages of the cycle and early pregnancy studied. These figures illustrate total volume fraction across full thickness endometrium. There is no significant difference in volume fraction of endothelial cells between the stages studied here, nor does the volume fraction of stromal cells change between groups. Volume fraction of glands decreases as pregnancy progresses. At day 28 of pregnancy, there is a significantly lower volume fraction of glands compared to all other stages. The volume fraction of luminal epithelium is significantly reduced in the late luteal phase compared to the other study groups.

Figure 5.16 A and B illustrate the differences in volume fraction occupied by the different cellular compartments within the basalis and functionalis regions of the endometrium. The basalis was defined as the region where glands were long and thin in appearance on a cross section of the endometrium. The functionalis was defined as the region where glands were more rounded. This was an arbitrary assignment by eye. Similar trends are observed as in **Figure 5.15 A and B**. It is apparent that it is the volume fraction of glands in the functionalis that decreases as pregnancy progresses, because volume fraction of glands in the basalis are unaltered between the stages studied here. The statistical tests used was a 1-way ANOVA.

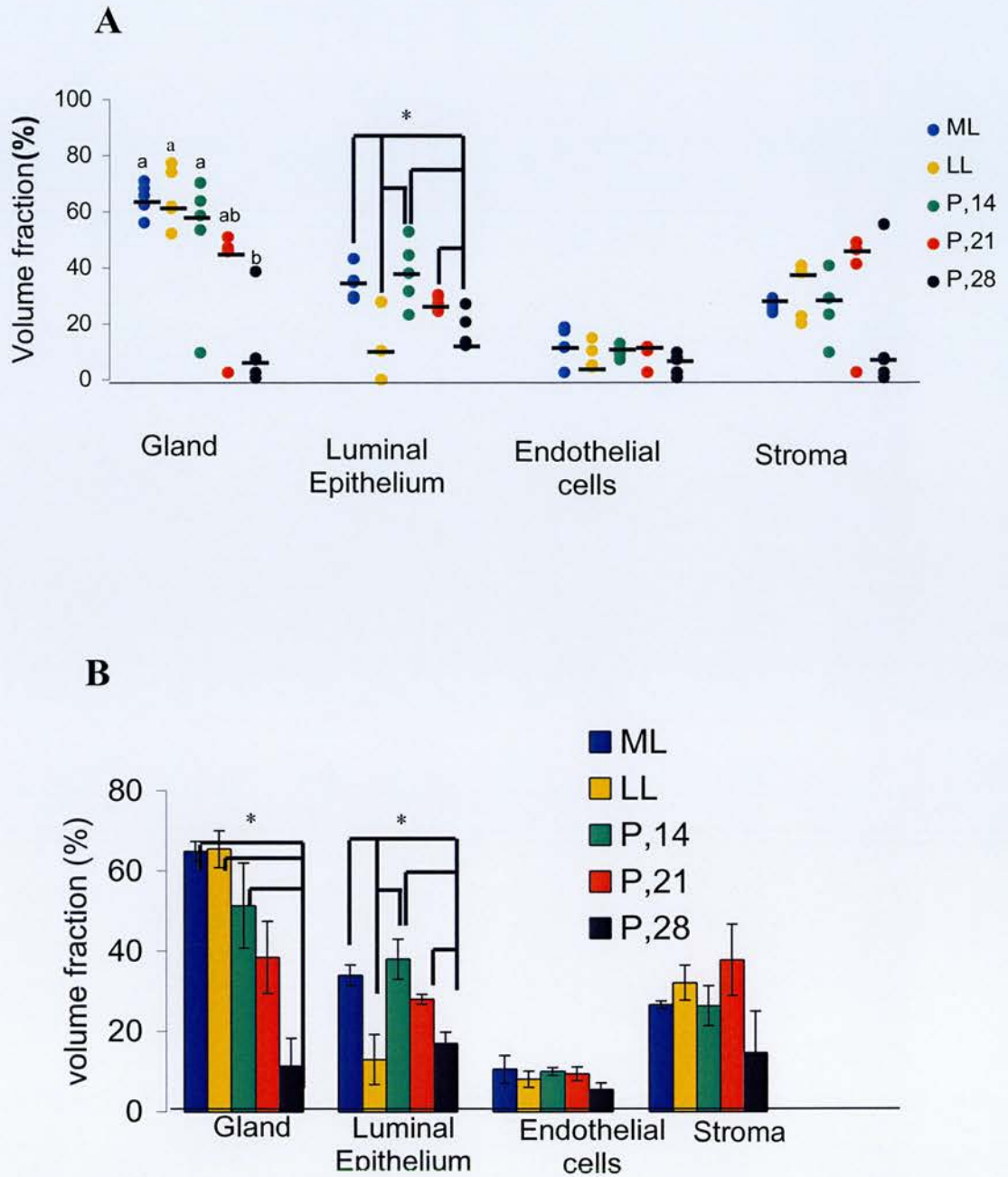


Figure 5.15 Scatter (A) and Mean (B) volume fraction of cellular compartments of the marmoset uterus. Volume fractions were measured using a test grid of 588 points and are represented as a percentage of the total volume. Significant differences ($P < 0.05$) as determined by 1-way ANOVA are denoted by different letters and/or bars. Values are means \pm SEM ($n=5$ in all groups).

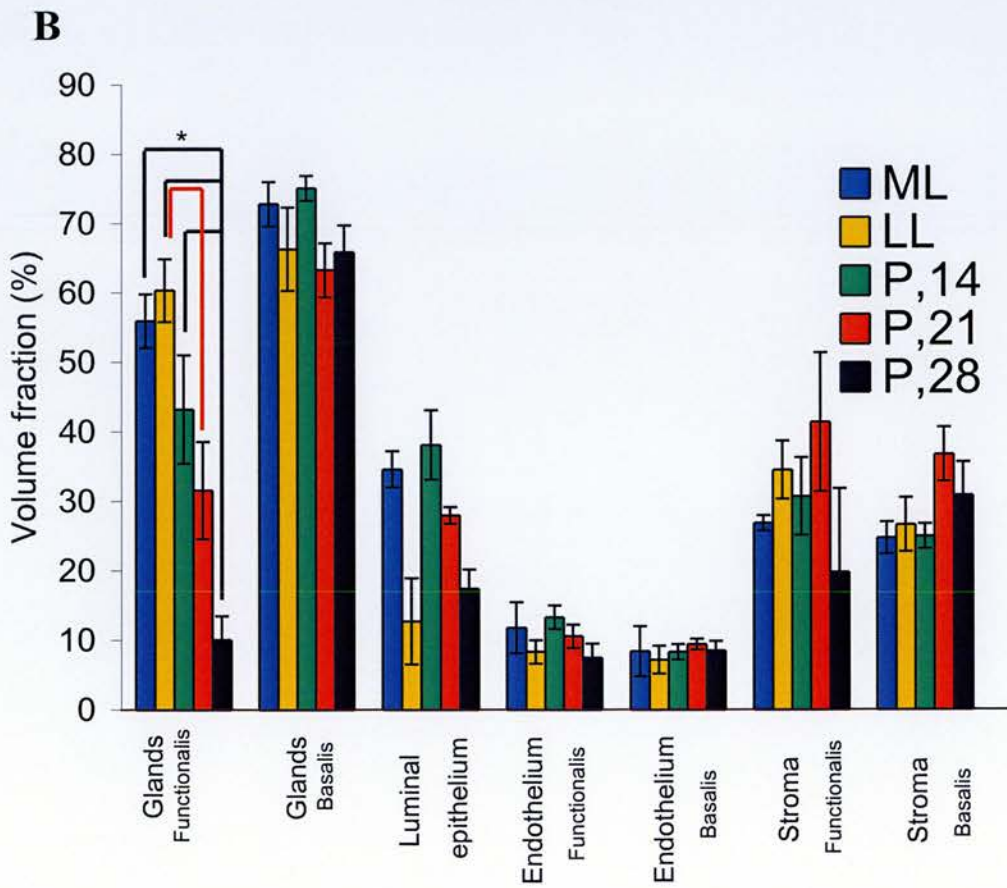
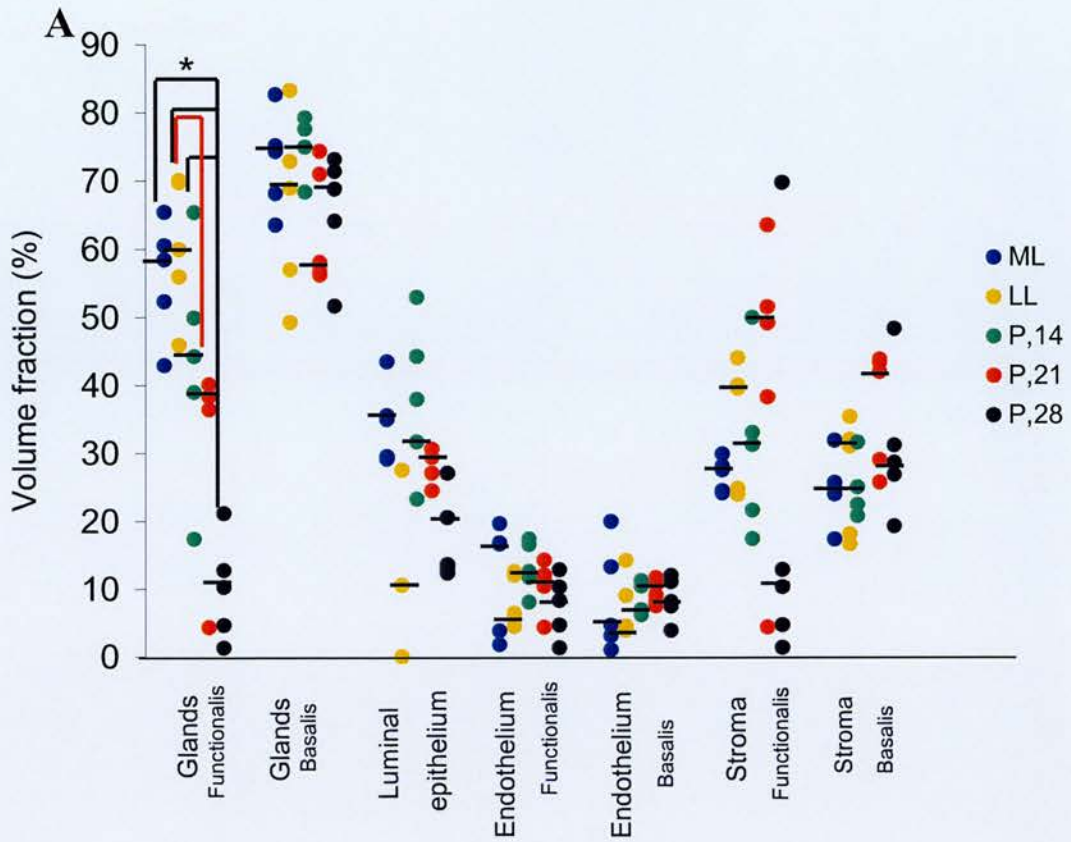


Figure 5.16 Scatter (A) and Mean (B) volume fraction of cellular compartments of the marmoset uterus. The endometrium has been subdivided into functionalis (upper) and basalis (lower) regions. Volume fractions were measured using a test grid of 588 points. Volume fraction is represented as a percentage of the total volume. Significant differences ($P < 0.05$) as determined by 1-way ANOVA between groups are denoted by connecting lines. Values are means \pm SEM ($n=5$ in all groups).

Luminal epithelial proliferation

Figure 5.17 illustrates luminal epithelial proliferation as a percentage of total luminal epithelium. There is a general increase in luminal epithelial proliferation as pregnancy progresses. Day 28 pregnant animals have significantly higher luminal epithelial proliferation compared to non-pregnant animals and pregnant animals at day 14 as determined by a 1-way ANOVA.

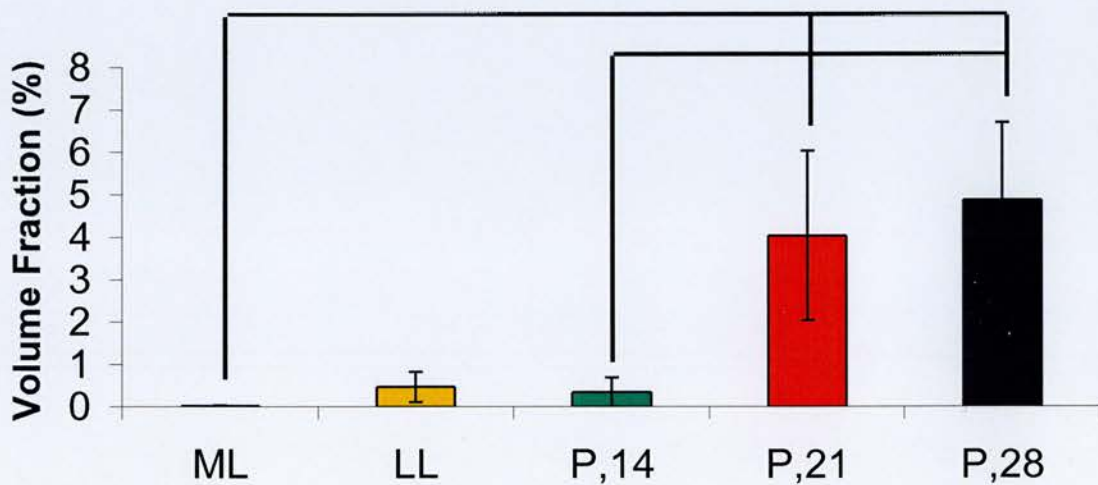


Figure 5.17 Mean volume fraction of proliferating luminal epithelium as a percentage of total luminal epithelium. Values are means \pm SEM. (n=5 in each group). Significant differences ($P < 0.05$) as determined by 1-way ANOVA are represented by adjoining bars.

Glandular epithelial proliferation

Figure 5.18 and 5.19 illustrate mean volume fraction of proliferating glandular epithelial cells as a percentage of total glandular epithelium. There is significantly higher glandular epithelial proliferation in the functionalis of day 21 and day 28 pregnant animals compared to the functionalis of other study groups. This level of proliferation in the functionalis is significantly higher than that recorded in the basalis region of these uteri.

In the basalis region of the mid luteal phase (day 14) uteri there is significantly higher glandular epithelial proliferation compared to the functionalis. This is also significantly higher than that recorded in the basalis from all other groups as determined by 1-way ANOVA.

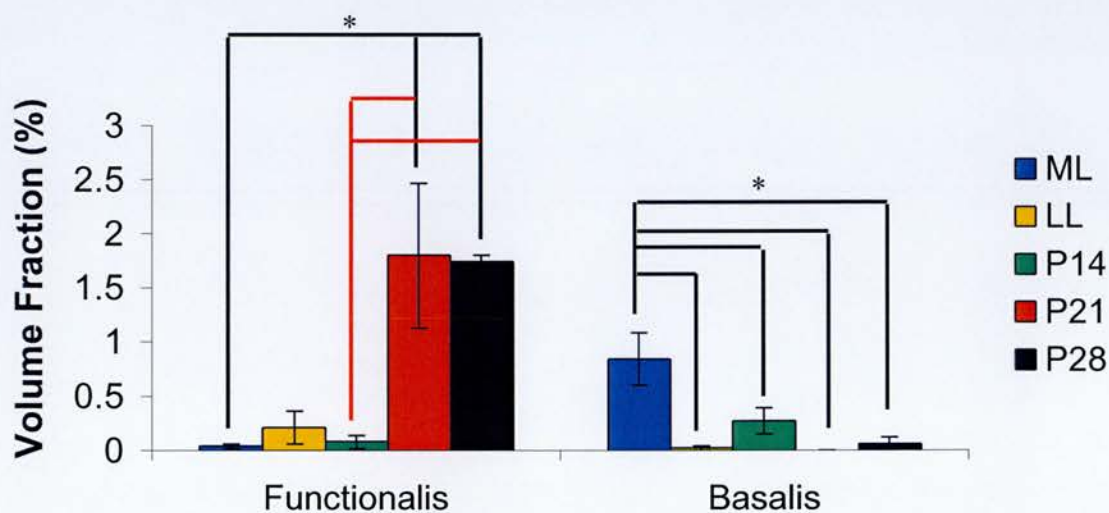


Figure 5.18 Mean volume fraction of proliferating glandular epithelium as a percentage of total glandular epithelium. This graph illustrates differences in glandular proliferation between groups within the functionalis layer and similarly within the basalis. It does not denote differences between the functionalis and

basalis. Significant differences ($P < 0.05$) as determined by 1-way ANOVA are denoted by adjoining bars. Values are means \pm SEM ($n=5$ in each group).

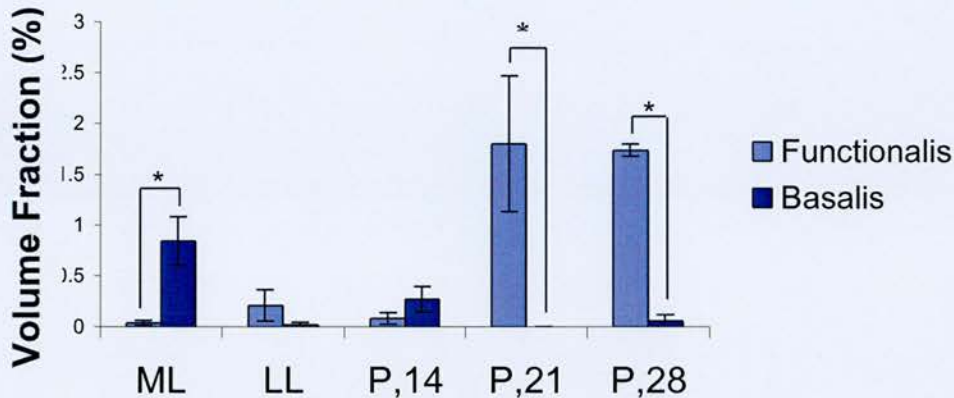


Figure 5.19 Mean volume fraction of proliferating glandular epithelium as a percentage of total glandular epithelium. This graph illustrates differences in glandular proliferation between the functionalis and basalis layer within each group. It does not denote differences between groups. Significant differences ($P < 0.05$) as determined by 1-way ANOVA are denoted by adjoining bars. Values are means \pm SEM ($n=5$ in each group).

Endothelial cell proliferation

Figures 5.20 and **5.21** depict differences in mean volume fraction of endothelial cell proliferation as a percentage of total endothelial cells. There is a significant increase in endothelial cell proliferation in the functionalis region of the endometrium at day 21 of pregnancy, compared to late secretory non-pregnant endometrium at day 21. There is no difference in the level of endothelial cell proliferation in the basalis region between the stages studied here as determined by 1-way ANOVA.

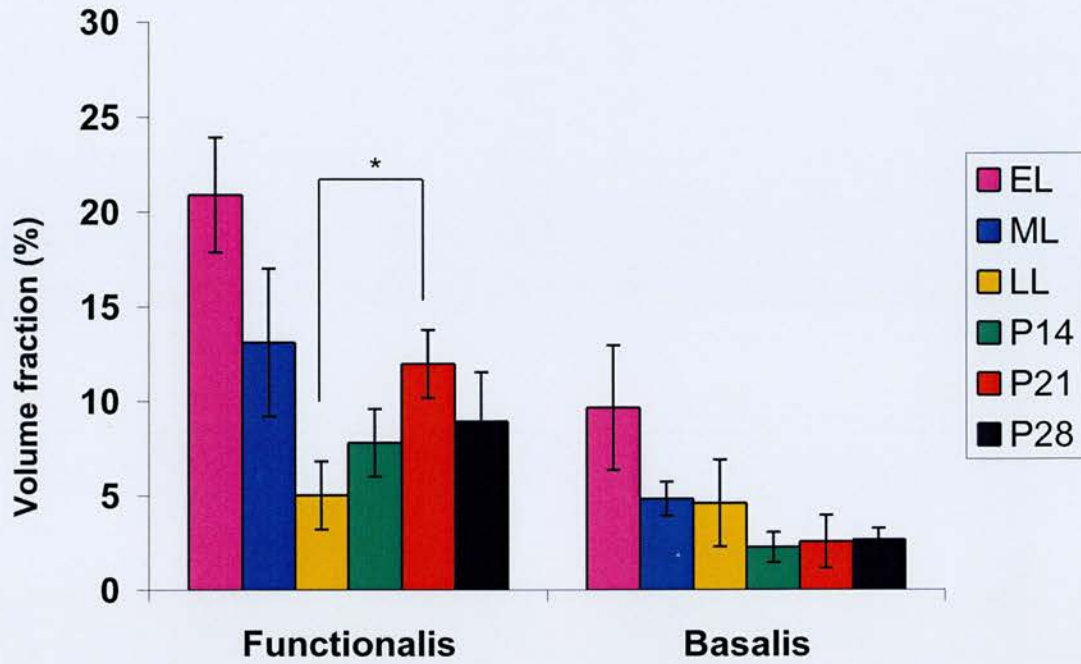


Figure 5.20 Mean volume fraction of proliferating endothelial cells as a percentage of total endothelial cells. This graph illustrates differences in endothelial proliferation between groups within the functionalis layer and similarly within the basalis. It does not denote differences between the functionalis and basalis. Values are means \pm SEM ($n=5$ in each group). Significant differences as determined by 1-way ANOVA are denoted by connecting bars. For this graph, animals from the early luteal phase were included to illustrate declining endothelial cell proliferation as the luteal phase progresses.

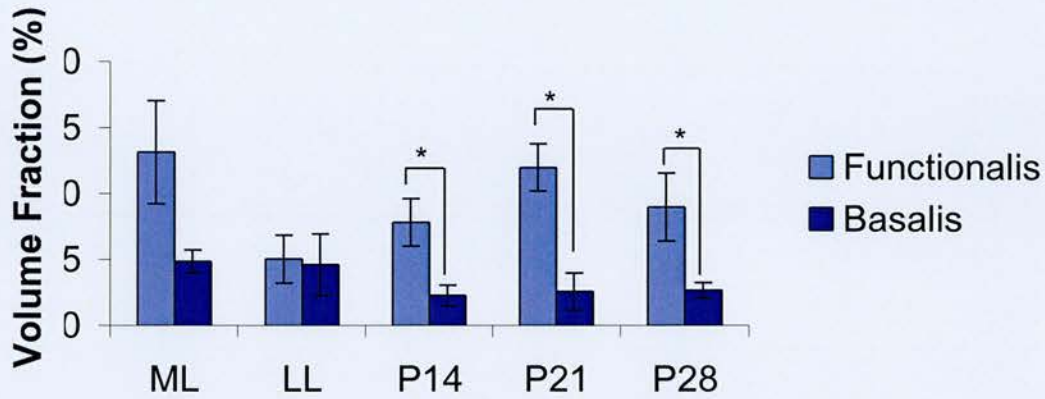


Figure 5.21 Mean volume fraction of proliferating endothelial cells as a percentage of total endothelial cells. This graph illustrates differences in endothelial proliferation between the functionalis and basalis layer within each group. It does not denote differences between groups. Values are means \pm SEM. Significant differences ($P < 0.05$) as determined by 1-way ANOVA are denoted by connecting bars. ($n=5$ in each group)

5.4 Discussion

Early miscarriage within the first few weeks after conception is a common phenomenon in women. Genetic and congenital abnormalities may account for some early pregnancy loss, but it is also possible that failure of the conceptus and endometrium to establish an effective implantation may be involved (Smith, et al., 1987). Marmoset implantation occurs at day 12 after ovulation (Enders, 1993) and in many ways displays a placentation similar to that in the human in that both develop a true hemochorial placenta. In human, the blastocyst implants superficially giving rise to an eccentric, discoid placenta, while the marmoset blastocyst implants superficially giving rise to a centric placenta (Smith, et al., 1987).

In women, baboon and macaque for example, subepithelial maternal vessels are penetrated early on in the process of implantation, at day 12, 10 and 11 of

gestation, respectively (Enders, 1993). The marmoset is at the other extreme, where maternal vessels are not penetrated by trophoblast until 45 to 60 days of gestation (Smith and Moore, 1990). It is therefore of paramount importance that the mechanisms required to meet the growing metabolic demands of the newly implanted blastocyst be in place throughout the early implantation stages. Increased angiogenesis at this time may be an important mechanism involved in ensuring that an adequate vasculature is available to support the conceptus. Nutrients and oxygen from the maternal circulation will be supplied to the trophoblast by diffusion, and so a well developed maternal vascular system is of paramount importance. A great deal of uterine remodelling occurs during early pregnancy. In ewes, vascularity of endometrial tissues, was elevated on Days 24 and 30 after mating. In addition, dramatic changes in uterine architecture (increased luminal diameter and decreased endometrial thickness) and in uterine microvascular development (increased abundance of large microvessels and development of a subepithelial capillary plexus) were observed by Day 24 after mating (Reynolds and Redmer, 1992). In pigs, blood flow dramatically increases to the uterine vascular bed in early pregnancy (Ford, et al., 1982). Characterization of the patterns of uterine growth and microvascular development will enable us to further define the role of previously reported uterine and conceptus-derived growth and angiogenic factors during early pregnancy (Reynolds and Redmer, 1992). Marmosets have a very high fertility rate compared to most other primates, and as such provide an excellent model for early pregnancy where the angiogenic events occurring therein can be addressed. This has allowed elucidation and speculation as to whether early embryo loss could be attributed to not only a poorly established implantation but also to an inadequately developed endometrial vasculature, by looking at successful implantation in a highly fecund species. I have examined the cellular and morphological events occurring in marmoset implantation at days 14, 21 and 28 of gestation and the angiogenic events associated therein.

Development of a receptive endometrium and its subsequent transformation into the decidua is a key aspect of mammalian implantation (Smith, 2000). The human endometrium undergoes angiogenesis to develop new capillaries, which then

undergo maturation and remodelling during each menstrual cycle. Abnormal vessel development may not only cause implantation failure but also lead to dysfunctional bleeding, endometriosis and menorrhagia (Albrecht and Pepe, 2003). The fate of the endometrium is cyclically dependent on ovarian steroids. Increased vascular permeability and angiogenesis are essential to successful implantation and placentation and several studies have provided evidence for the potential role for oestrogen and progesterone in these processes (Chakraborty et al., 1995, Halder et al., 2000, Hyder and Stancel, 1999). However, there appears to be conflicting evidence as to whether these hormones are pro- or anti-angiogenic. Although I will not detail mechanisms or sites of action of the steroid hormones in the marmoset endometrium, I have documented the cellular and proliferative changes throughout the cycle that I believe occur as a direct consequence of the hormone milieu at that time.

In the proliferative phase, under oestrogen dominance, a high level of luminal and glandular epithelial proliferation was observed, consistent with findings in the mouse (Martin and Finn, 1968). This group also demonstrated that progesterone inhibits oestrogen-mediated epithelial proliferation consistent with my observations where by the mid-secretory phase no epithelial proliferation remains in the functionalis region. However, in combination with oestrogen, progesterone has been reported to induce stromal cell proliferation (Martin and Finn, 1968). Under oestrogen dominance in the proliferative phase, very little stromal or endothelial proliferation was observed, thus it would appear that oestrogen may inhibit angiogenesis in the marmoset. In the secretory phase and in early pregnancy, I observed profound stromal and endothelial cell proliferation, probably directly attributed to progesterone being introduced to the system after ovulation. Not only does oestrogen not promote angiogenesis, in mouse, it has been shown to profoundly inhibit angiogenesis, but promoted uterine vascular permeability, whereas progesterone stimulates angiogenesis with little effect on permeability (Ma et al., 2001). This finding is somewhat inconsistent. Other groups have reported oestrogen to act as an inducer of rapid proliferation of endothelial cells, preceding endometrial tissue remodelling in mouse (Hastings, et al., 2003, Heryanto and Rogers, 2002,

Heryanto et al., 2003), raising the possibility that the ultimate mass of a tissue may be regulated in part by endothelial cell growth (Folkman, 1998). Progesterone has also been documented as an inhibitor of endothelial cell proliferation, in mouse, unless acting together with high dose oestrogen (Heryanto and Rogers, 2002).

Several immunohistochemical studies on endothelial cell proliferation indicate that classical sprouting angiogenesis does not occur in human endometrium, reviewed by (Gargett and Rogers, 2001). Most studies have failed to demonstrate oestrogen or progesterone receptors in human endometrial endothelium. However, oestrogen and progesterone receptors have been detected in human endometrial cells *in vitro* by immunocytochemistry and RT-PCR (Iruela-Arispe et al., 1999). ER β , specifically has also been reported in endothelial cells of macaque and women by immunocytochemistry (Critchley, et al., 2001).

Endothelial cell proliferation does vary widely throughout the menstrual cycle, but thought to show no relationship to the stage of the cycle, reviewed by (Gargett and Rogers, 2001). Early work using anti-proliferating cell nuclear antigen to identify proliferating cells and anti-CD34 to localize endothelial cells in human endometrium, demonstrated that proliferative activity of endothelial cells reached a peak in the mid to late proliferative phase of the cycle increasing again in the secretory phase (Goodger and Rogers, 1994). This is consistent with a more recent study in macaque endometrium, where a dramatic oestrogen dependent peak in endothelial cell proliferation during the mid-proliferative phase occurred (Nayak and Brenner, 2002). I did not perform quantitative analyses on proliferative phase sections, so I am unable to comment on whether a similar phenomenon exists in the marmoset. Stromal cell proliferation is apparent in the secretory phase and appears to increase as the secretory phase progresses.

Profound differences in endothelial cell proliferation were found between the superficial functionalis and basalis layers, where the functionalis always displayed more intense angiogenesis than the basalis, consistent with reports in women where proliferative activity was seen to remain fairly constant in the basalis throughout the cycle, but changing in the functionalis (Rogers, 1998).

In the face of the conflicting literature, from these results it would seem that in the marmoset in the proliferative phase, oestrogen acts to inhibit endothelial cell proliferation. This is concluded from the low level of BrdU incorporation observed in endometrial sections from the proliferative phase. However, this was not examined quantitatively. Oestrogen would appear to be promoting luminal and glandular epithelial proliferation at this time. Progesterone would appear to inhibit epithelial proliferation while stimulating endothelial proliferation.

Not surprisingly, the endometrium is a rich source of angiogenic growth factors. VEGF is thought to be regulated by progesterone and oestrogen, and contains progesterone and oestrogen response elements (Hyder and Stancel, 1999, Hyder and Stancel, 2000). VEGF expression and endothelial cell proliferation have been demonstrated to peak in the mid-proliferative phase in rhesus macaque endometrium, coinciding with a peak in oestrogen (Nayak and Brenner, 2002). This is a synchronised model system where animals were ovariectomised and then hormones replaced. This does not appear to be the case in human, where no correlation between VEGF production and endothelial cell proliferation was observed throughout the cycle (Gargett et al., 1999), however, this was not such an easy system to control as it was in the natural cycle rather than after ovariectomy and hormone replacement.

Expression of VEGF and its role in promoting vascular permeability and angiogenesis during early pregnancy and placentation have been frequently documented. I have described the specific localisation of VEGF, its receptors and the angiopoietins in marmoset endometrium during the peri-implantation period and in early pregnancy in Chapter 6 (Rowe et al., 2003). Increased expression of VEGF in stroma of the functionalis particularly adjacent to the implantation site during the peri-implantation period was observed, and continuing to increase up to day 28 of pregnancy. I also documented increased endothelial expression of KDR and Flt mRNA immediately surrounding the glands of the functionalis, once again most profoundly adjacent to the implantation site.

A significant increase in endothelial cell proliferation was observed in the functionalis layer of the endometrium in all pregnant groups compared to late luteal

non-pregnant controls. It is surprising that no significant differences in endothelial cell proliferation were observed between day 14 pregnant animals and non-pregnant marmosets. This is where the system becomes divergent in a fertile versus a non-fertile cycle, in that it is from this point onwards that the blastocyst is present in the pregnant uterus, chorionic gonadotrophin present in the system and where changes in angiogenesis may be expected to occur. It could be that it takes time for the endometrium to adapt to the newly implanted blastocyst before an upregulation in angiogenesis is observed. In a non-pregnant cycle, after functional regression of the CL, levels of VEGF decline (Rowe, et al., 2002). A similar phenomenon could be occurring here. It would appear that a divergence in endometrial angiogenesis occurs at the point at which the corpus luteum is either maintained or regresses. In a non-pregnant cycle, the corpus luteum undergoes functional luteal regression at around day 21, thus progesterone levels decline unlike in luteal rescue where they are maintained. Therefore it could be the continued perception of progesterone in the system at day 21 of pregnancy in the marmoset that induces this significant increase in angiogenesis, compared to late-secretory non-pregnant controls.

In the stages of pregnancy studied here, the trophoblast remains in relatively superficial attachment to the uterus with no development of its own vasculature. Maternal vessels are not invaded until much later in pregnancy (day 60) (Enders, 1993) and so foetal metabolic demands must be met by an alternative mechanism. This would appear to be by increased angiogenesis in the functionalis layer mediated by an increase in VEGF and its receptor mRNA. VEGF is also a potent permeability factor and no doubt has a role in inducing fenestration of maternal vessels to aid the diffusion process.

However, in the marmoset, unlike in the human, serum progesterone levels are not further increased in response to chorionic gonadotrophin, compared to late secretory phase levels, rather they are maintained at the level reached in the secretory phase (Rowe, et al., 2002). Therefore, if increased VEGF and consequently increased angiogenesis were purely attributable to progesterone alone, then this observed increase only evident in pregnant animals, would surely be apparent in the secretory phase regardless of conception? This is not the case and so increased angiogenesis at

this time is clearly not solely dependent on the stimulatory effect of progesterone on endothelial cells. Local factors possibly produced within the trophoblast itself and hypoxia are possible factors which could give rise to increased VEGF and increased angiogenesis in the functionalis. I observed increased VEGF mRNA throughout the whole of the functionalis layer but the most intense expression was observed immediately below the luminal epithelium adjacent to the implantation site providing evidence for focal upregulation in pregnancy (Rowe, et al., 2003).

In rats, embryo implantation is associated with increased endothelial cell proliferation (Macpherson and Rogers, 1993). They demonstrated that endothelial cell proliferation was increased from day 3 of pregnancy and increased throughout the entire endometrium up to day 5 of pregnancy. They implicated VEGF as being the main promoter of this observed increase in endothelial proliferation but found that although it appeared to be responsible for increased endometrial vascular permeability at implantation, it was not solely responsible for regulating endothelial cell proliferation at the site of implantation. Therefore other mechanisms may be involved in promoting this increase. Angiogenesis can be driven by multiple mechanisms with some considerable degree of redundancy between them.

This experiment also illustrated increased glandular and luminal epithelial proliferation as pregnancy progressed from the time of implantation. This could be due to the increased requirement for glandular secretions into the uterine lumen during early pregnancy. Also, the luminal epithelium is being invaded during the process of implantation and so may be undergoing rapid proliferation to replace damaged cells as a result of the epithelial reaction.

Chapter 6

Molecular regulation of angiogenesis in implantation and early pregnancy

6 Molecular regulation of angiogenesis in implantation and early pregnancy

6.1 Introduction

During the last few years, considerable progress has been made in understanding the molecular regulation of angiogenesis in the female reproductive tract. In particular, the role of vascular endothelial growth factor (VEGF) during follicular and luteal development in the marmoset model has been established by specific inhibition of this angiogenic factor *in vivo* (Fraser and Lunn, 2001). Since inhibitors of angiogenic factors are likely to be used clinically, it is important that their role during implantation and early pregnancy be elucidated. As a first step towards this goal, this experimental chapter aims to build on our knowledge of the regulation of angiogenesis in the reproductive tract of the female marmoset by investigating the expression patterns of angiogenic factors during this period.

Successful pregnancy is dependent on implantation of the blastocyst into the endometrium. Angiogenesis may have a key role in the implantation process, however, the mechanisms by which localised changes in vascular permeability and angiogenesis occur at the implantation site have yet to be elucidated.

VEGF and its receptors, Flt and KDR have been implicated as key players in vascular remodelling and placentation on the basis that their mRNA and proteins have been localised in endometrium of several species. These include human (Ahmed et al., 1995, Clark et al., 1996, Clark et al., 1998, Cooper et al., 1995, Geva et al., 2002, Goldman-Wohl et al., 2000, Helske et al., 2001, Sugino et al., 2002), sheep (Bogic et al., 2001), pig (Winther et al., 1999), marmoset (Wulff et al., 2002) mouse (Chakraborty, et al., 1995, Halder, et al., 2000), golden hamster (Yi et al., 1999) rat (Rabbani and Rogers, 2001), rabbit (Das et al., 1997) and rhesus monkey (Dhawan et al., 2000) during the first trimester of pregnancy. *In vivo* manipulation

experiments are limited to the rodent to date but have provided further evidence for the importance of VEGF in early pregnancy. In the mouse, inhibition of VEGF prevented estrogen-induced uterine oedema and implantation (Rockwell, et al., 2002) and in the rat, the number of implantation sites were reduced after administration of a VEGF blocking antibody (Rabbani and Rogers, 2001).

Angiopoietins also appear to have a major role in regulation of blood vessel growth, maturation and regression, but their importance at the time of implantation has yet to be studied. However their presence in placenta in later stages of pregnancy has been documented in human (Dunk et al., 2000, Goldman-Wohl, et al., 2000) and marmoset (Wulff, et al., 2002).

This experimental chapter aims to describe the localisation of mRNA for angiogenic factors and their receptors. In the marmoset, implantation occurs at day 11 after ovulation. This study was conducted on tissue collected at the peri-implantation period (2 weeks post ovulation), and 3 and 4 weeks of gestation. Angiogenic processes occurring at this early stage of pregnancy are still poorly understood in the human and with accumulating knowledge on effects of manipulation of angiogenesis in the marmoset, this species represents a potentially valuable model in which to study these events.

6.2 Methods

6.2.1 Details of animals used in the experiment

Study	Groups	Number of animals
Gene expression in the endometrium	Late luteal (Day 21)	5 (all animals had structurally intact CL)
	Pregnant (Day14)	5
	Pregnant (Day 21)	5
	Pregnant (Day 28)	5

Table 6.1. Details of animals used for the experiments

6.2.2 *In situ* hybridisation

Localisation of mRNA for VEGF, Flt, KDR, Ang-1, Ang-2 and Tie-2 was performed by *in situ* hybridisation as described in Chapter 2. H & E stained uterine sections were also photographed to confirm tissue morphology. In order to determine

whether or not endothelial cells expressed these angiogenic factors, CD31 immunocytochemistry was performed on serially cut sections to those used for the *in situ* hybridisation. It was not possible to co-localise CD31 surface antigen and mRNA for an angiogenic factor on the same slide as the stains used to detect CD31 interfered with dark field microscopy.

6.2.3 Quantification of *In situ* hybridisation

Uterine sections were scored and means calculated according to the grain density in the various different uterine compartments namely, luminal epithelium, upper zone stroma, (area beneath the luminal epithelium) upper zone glands, lower zone glands (area adjacent to myometrium), lower zone stroma and endothelial cells. Scoring was carried out by two independent observers, myself and Dr. H. Fraser, with the following classification: - (0) No detectable specific signal above background levels, + (1) detectable but low grain density clearly above background levels, ++ (2) Moderate grain density, +++ (3) high grain density without grain coalescence and ++++ (4) grain coalescence. Scoring of uteri was blind and random by each observer. For each compartment within a section, an overall score was assigned, resulting in the whole uterus being classified according to grain density. It should be noted that individual grain counts were not performed. Unlike the corpus luteum, the endometrium is not a uniform tissue. It is extremely heterogeneous with many different cellular compartments. As such, it was impossible to accurately count grains from a given field of view because inevitably each field of view contained different uterine compartments and thus could not be compared accurately to another equally sized area.

Significant differences between groups ($P < 0.05$) within a particular uterine compartment were determined using a Kruskal-Wallis non-parametric test followed by Dunn's multiple comparisons test.

6.3 Results

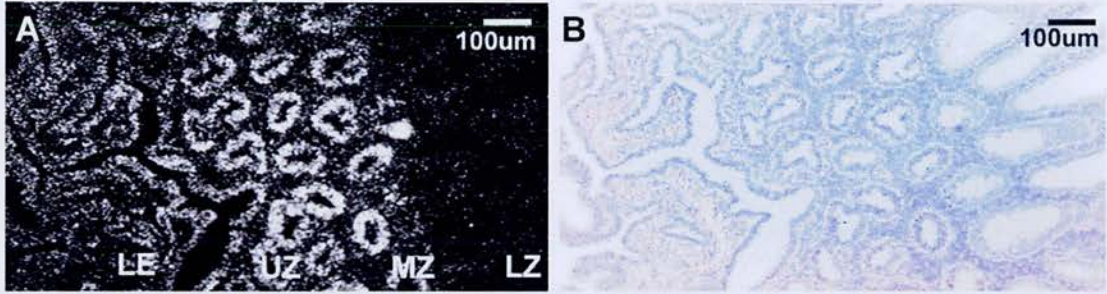
6.3.1 Vascular Endothelial Growth Factor

Photomicrographs of each *in situ* hybridisation are presented in this section (**Figures 6.1-6.5**) to illustrate the cellular localisation of each of the different angiogenic factors. Also, a table is included to summarise the scores assigned to each section in terms of mean grain density (**Table 6.1**).

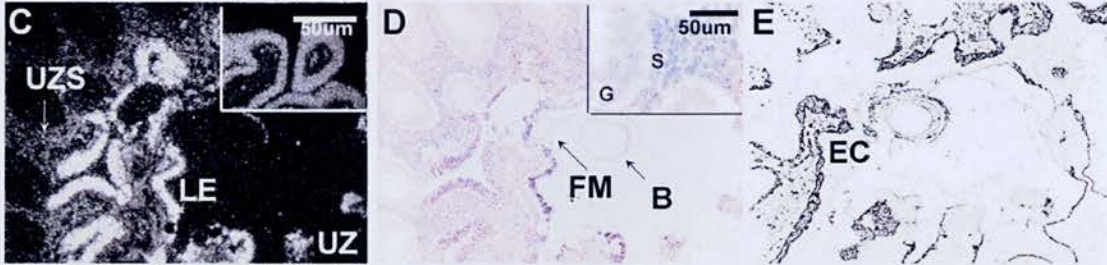
VEGF mRNA was localised to the glandular epithelium in the late secretory phase non-pregnant endometrium. Highest grain density was observed in the luminal epithelium and glands of the upper zones. Grain density was much reduced in the mid-zone glands, while grains were completely absent from the lower zone (**Figure 6.1A and B**). Exact cellular localisation of VEGF mRNA to glandular epithelium was confirmed at high power magnification (**Figure 6.1J**).

In early pregnant endometrium, 2 weeks post ovulation, a similar localisation was observed to that seen in the non-pregnant animals (**Figure 6.1C-E**). However, a low level of stromal VEGF mRNA expression was apparent just beneath the luminal epithelium (**Figure 6.1C and D**). At this very early stage of pregnancy, 3 days after implantation, no VEGF mRNA expression was observed in the blastocyst or foetal membranes (**Figure 6.1C and D**). By 3 weeks of pregnancy (day 21), VEGF mRNA levels remained high in the glandular epithelium and also appeared at a lower level in the lower zone glands (**Figure 6.1H and K**). At 3 weeks of pregnancy, VEGF expression was relatively high in luminal epithelium and in decidua where expression was punctate (**Figure 6.1F, G and I**). Also, by this stage of pregnancy the blastocyst and foetal membranes were expressing VEGF mRNA. Four week pregnant animals (day 28) had similar VEGF mRNA localisation to those animals at 3 weeks hence a representative figure at 4 weeks is shown. In all sense slides, no specific signal was detected above a low background (**Figure 6.1H insert**).

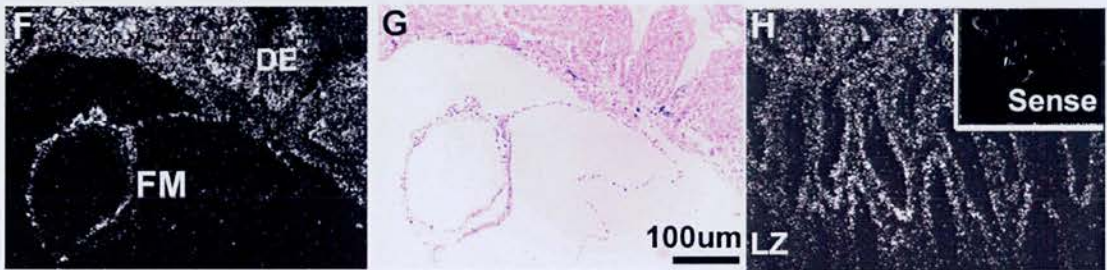
Late Secretory



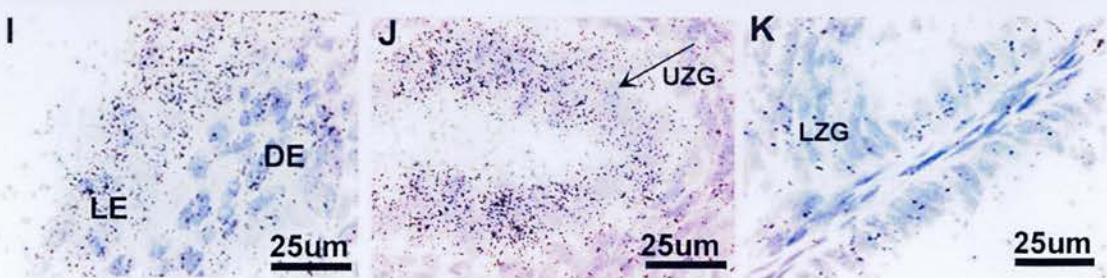
Pregnant Day 14



Pregnant Day 21/28



Cellular Localisation



VEGF

Figure 6.1 In each figure, a representative scale bar is. (A-K) *In situ* hybridisation of VEGF mRNA in the marmoset endometrium. Dark field (A, C and F) and corresponding light field images (B, D and G respectively). (A and B) Non-pregnant late secretory phase endometrium. Note high expression in upper zone glands (UZ) and luminal epithelium, declining in the mid-zone (MZ) and absent in the lower zone

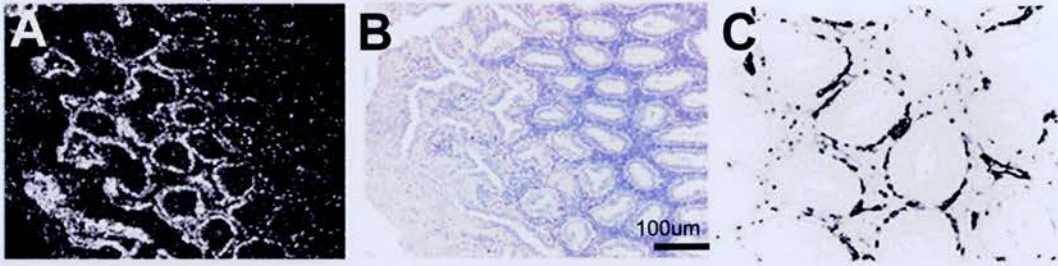
(LZ). (C, D, E) Pregnant marmoset endometrium at day 14 after ovulation. (C) Note high expression in luminal epithelium (LE) and upper zone (UZ) glands and absence of expression in the blastocyst (B) and foetal membranes (FM). A lower level of VEGF expression can be seen in the upper zone stroma (UZS). Insert in (C) is a higher power image of the luminal epithelium. Insert in (D) is a higher power bright field image of the upper zone stroma. (E) CD31 staining for endothelial cells (EC) to emphasise non-endothelial localisation of stromal VEGF. (F, G, H) Typical VEGF expression at day 21 and day 28. (F) Dark field image of marmoset early implantation site. Note high punctate expression in the decidualised endometrium (DE) and appearance of expression in the foetal membranes (FM). (G) Corresponding light field image. (H) Dark field image of lower zone endometrium where there is a low level of glandular VEGF expression. (I) is a higher power bright field image of the luminal epithelium (LE) of 3 week pregnant animals and also illustrates the hybridisation observed in decidualised stroma (DE). (J) is a high power image of the upper zone glands in the late secretory non-pregnant endometrium to confirm cellular localisation. (K) highlights glandular expression of VEGF in the lower zones at this time. Insert in (H) is a representative sense photograph.

6.3.2 KDR and Flt (VEGFR-1 and VEGFR-2)

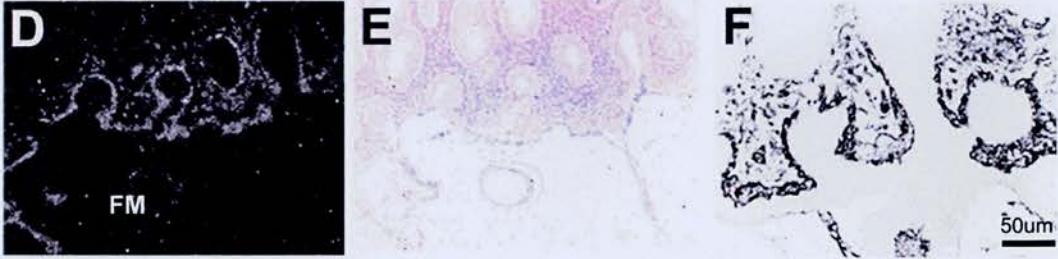
Figure 6.2 shows VEGFR-2 mRNA localisation. It should be noted that VEGFR-1 mRNA expression was consistently lower than that of VEGFR-2, but was found in the same cellular locations, thus mainly photographs of VEGFR-2 mRNA are represented here (**Figure 6.2 A-L**), however representative images are included of VEGFR-1 hybridisation at 2 weeks of pregnancy (**Figure 6.2M and N**). Each receptor localisation and grain density were scored separately and detailed in **Table 6.2**. In the late secretory non-pregnant endometrium, VEGFR-2 mRNA was found exclusively in endothelial cells surrounding the upper zone glands beneath the luminal epithelium (**Figure 6.2A, B and J**). This was confirmed by comparison to CD31 immunocytochemistry to identify endothelial cells in sequential sections (**Figure 6.2C**). In 2 week pregnant endometrium, VEGFR-2 mRNA was localised to the upper zone endothelium as in non-pregnant endometrium. Grains were absent from the blastocyst and foetal membranes (**Figure 6.2D and E**). At 3 and 4 weeks of pregnancy, VEGFR-2 mRNA expression remained in endothelial cells of the upper zone with highest grain density being seen around large maternal vessels beneath the luminal epithelium (**Figure 6.2G, H and K**), again confirmed by comparison to CD31 localisation (**Figure 6.2I**). VEGFR-2 mRNA was also observed to a lesser extent throughout the stromal compartment again localised to endothelial cells

(Figure 6.2L). In all sense slides, no specific signal was detected above a low background. This is shown in **(O)**, a x100 magnification bright field image of the corresponding sense slide to **(K)**.

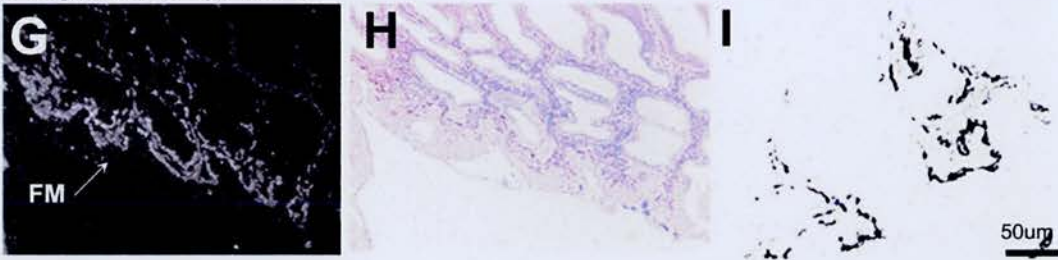
Late secretory



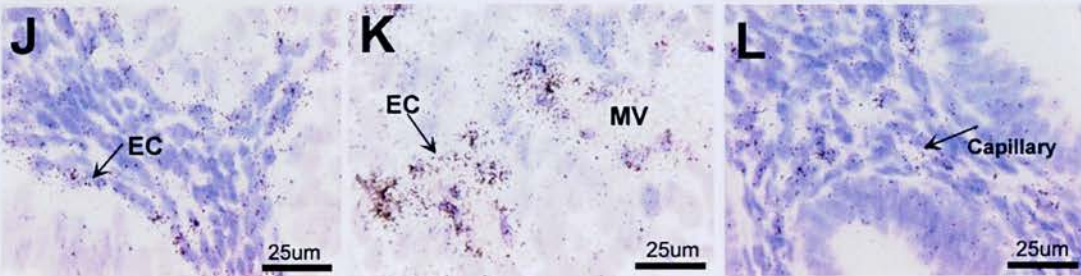
Pregnant, Day 14



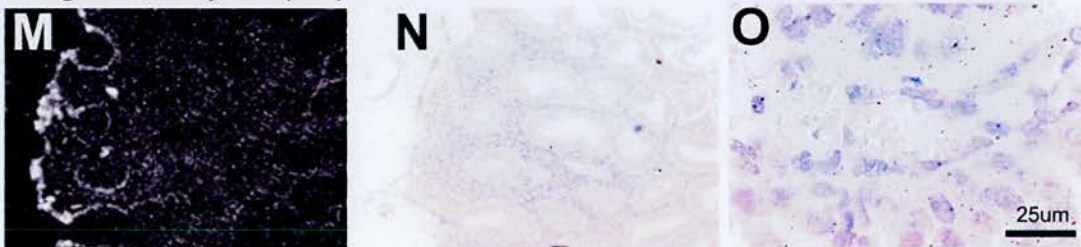
Pregnant, Day 21/28



Cellular localisation



Pregnant, Day 14 (R1)



KDR and FLT

Figure 6.2 *In situ* hybridisation of VEGF receptor R2 (A-L) and R1 (M,N) mRNA in marmoset endometrium. (A-C) Non-pregnant late secretory phase endometrium. (A) Dark field and (B) light field images of VEGF R2 expression in upper zone

endometrium. Note endothelial localisation immediately surrounding glands as demonstrated by CD31 immunocytochemistry (C) and high power bright field image (J). Also note punctate expression throughout the stroma in all stages studied illustrated by a representative high power bright field image (L). (D-F) Pregnant marmoset endometrium at day 14 after ovulation. (D) Dark field and (E) corresponding light field images showing localisation to large and small luminal blood vessels surrounding the upper most glands as indicated by CD31 immunocytochemistry for endothelial cells (F). Note absence of grains in blastocyst and foetal membranes (FM). (G) Dark field and (H) light field images of VEGF R2 expression. Note localisation in upper zone to luminal maternal vessels as demonstrated by CD31 immunocytochemistry (I), and high power bright field image (K). Also note absence of localisation to foetal membranes. (K) and punctate expression throughout the stroma of lower zones (L). A high power bright field sense image is given (O).

6.3.3 Angiopoietin-1

Ang-1 mRNA was localised to upper zone stroma and to a lesser extent the glandular epithelium of the upper zone in late secretory non-pregnant endometrium. In pregnant endometrium at day 14, Ang-1 mRNA is present at a high level in the lower zone glands (**Figure 6.3D, E and O**) but completely absent from adjacent compartments, the myometrium and mid-zone endometrium (see insert **Figure 6.3D**). In the upper zone, Ang-1 mRNA was expressed at a low level in the glandular epithelium but much more intensely in stroma (**Figure 6.3P**). Grains were absent from blastocyst and foetal membranes (**Figure 6.3F and G**). At three weeks of pregnancy, upper and lower zone glandular epithelial expression of Ang-1 mRNA was retained (**Figure 6.3H and J**). Grain density greatly increased in the stroma at this stage (**Figure 6.3H and I**). By Day 28, clear, punctate Ang-1 mRNA localisation was observed throughout the decidualised stroma (**Figure 6.3K, L and N**). In all sense slides, no specific signal was detected above a low background.

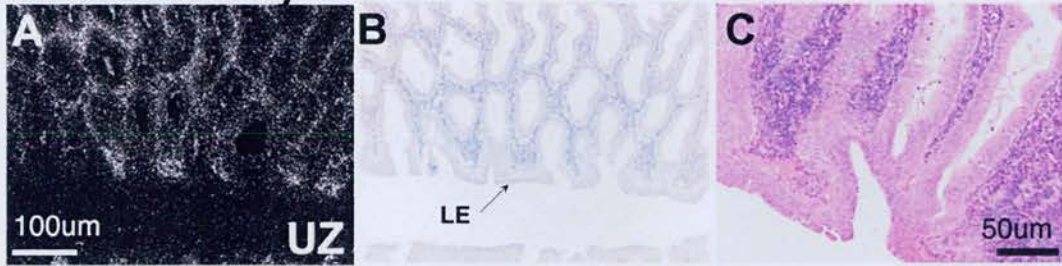
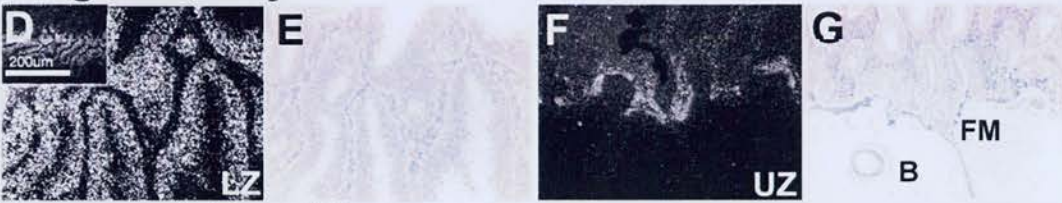
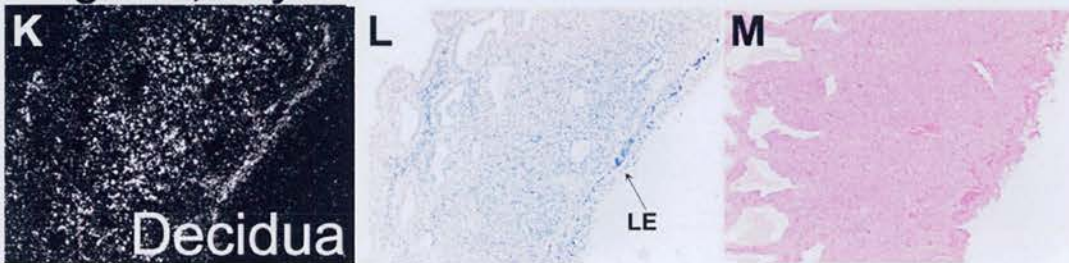
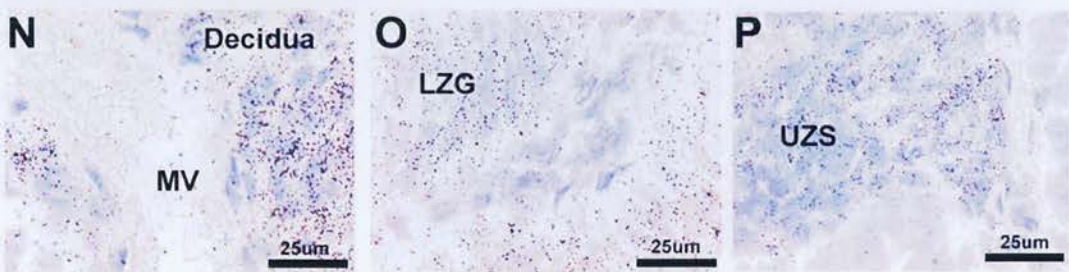
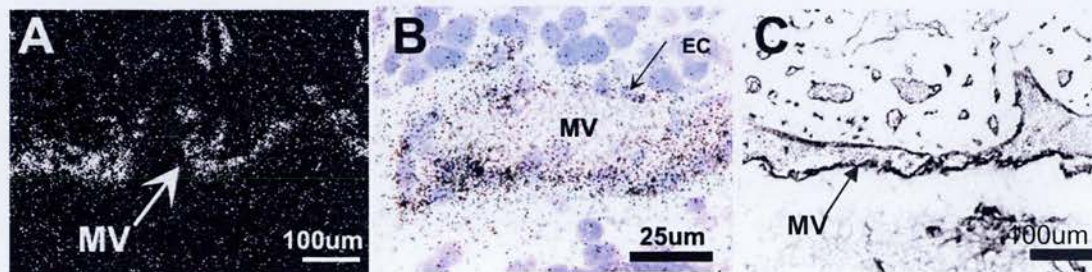
Late Secretory**Pregnant, Day 14****Pregnant, Day 21****Pregnant, Day 28****Cellular Localisation****Angiopoietin-1**

Figure 6.3 (A-P) *In situ* hybridisation of Ang-1 mRNA in marmoset endometrium. (A) dark field and (B) corresponding light field images of non-pregnant late

secretory phase endometrium. Note Ang-1 expression in stroma and a low level in glands of the upper zone endometrium. (C) Haematoxylin and eosin stained section of upper zone and luminal epithelium. (D-G) Day 14, pregnant marmoset endometrium. (D) Dark field and (E) corresponding light field images of Ang-1 expression at a high level in the glandular epithelium of the lower zone. Insert in (D) is a lower power image to demonstrate absence of grains in myometrium and mid-zone endometrium. (O) is a high power image to confirm cellular localisation to glands of the lower zone. (E) corresponding light field image of (D). (F) Dark field and (G) corresponding light field images of Ang-1 mRNA localisation in the upper zone. Note low glandular localisation together with stromal expression. Ang-1 is absent from the foetal membranes (FM) and blastocyst (B). (H-J) Ang-1 expression at day 21 (H) Dark field and (I) light field images of Ang-1 expression in the upper zone. Note expression in glandular epithelium and to a greater extent in stroma. This is also highlighted in (P), high power (x100) bright field image. (J) Dark field image of lower zone endometrium. Note glandular epithelial localisation of Ang-1. (K-M) Ang-1 expression at day 28 of pregnancy. (K) Dark field and (L) corresponding light field images of decidualised endometrium and luminal epithelium (LE). Note punctate expression of Ang-1 mRNA throughout decidualised stroma, not in endothelial cells, further illustrated in (N), a higher power bright field image. (M) Haematoxylin and eosin stained section to demonstrate decidual morphology.

6.3.4 Angiopoietin-2

Ang-2 mRNA was only detected in 4 week pregnant animals where it was localised exclusively in endothelial cells of maternal vessels (**Figure 6.4**). In all sense slides, no specific signal was detected above a low background.



Angiopoietin-2

Figure 6.4 (A-C) In situ hybridisation of Ang-2 mRNA in pregnant endometrium at day 28. (A) Dark field and (B) light field images of upper zone endometrium. Note Ang-2 expression in endothelial cells of large maternal vessels (MV), as demonstrated by CD31 immunocytochemistry in (C) and higher power image in (B). Ang-2 was absent from all other stages studied.

6.3.5 Tie-2

In non-pregnant late secretory endometrium Tie-2 mRNA was expressed predominantly in endothelial cells throughout the stroma of all zones (**Figure 6.5A,B and L**). At two weeks of pregnancy (day 14), the generalised stromal expression pattern seen in the non-pregnant animals is much reduced but still present at a low level. (**Figure 6.5D and E**). High grain density was observed in endothelium immediately surrounding the upper zone glands at this time (**Figure 6.5D, E and J**). By 3 and 4 weeks of pregnancy, luminal epithelial Tie-2 mRNA expression is lost but expression remains in upper zone decidualised stroma. (**Figure 6.5G**). By comparison to CD31 immunocytochemistry (**Figure 6.5I**) and high power haematoxylin and eosin stained sections (Insert **Figure 6.5H**), Tie-2 appeared to be expressed in blood vessels throughout the decidua. In sense slides, no specific signal was detected above a low background.

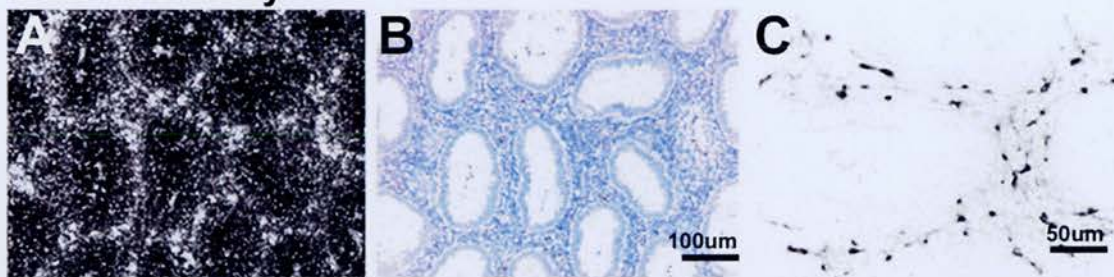
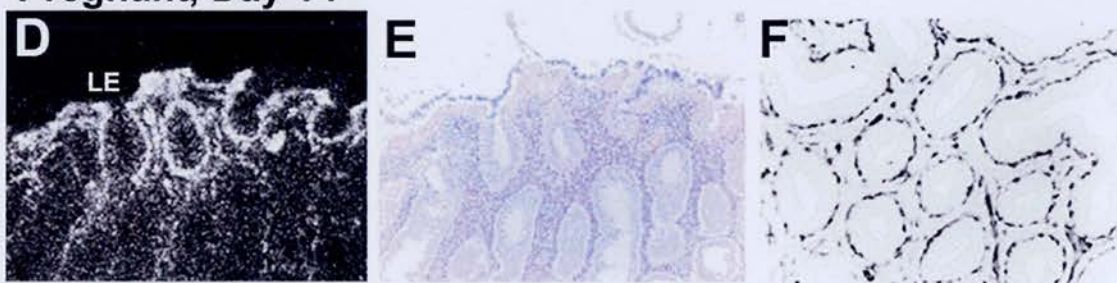
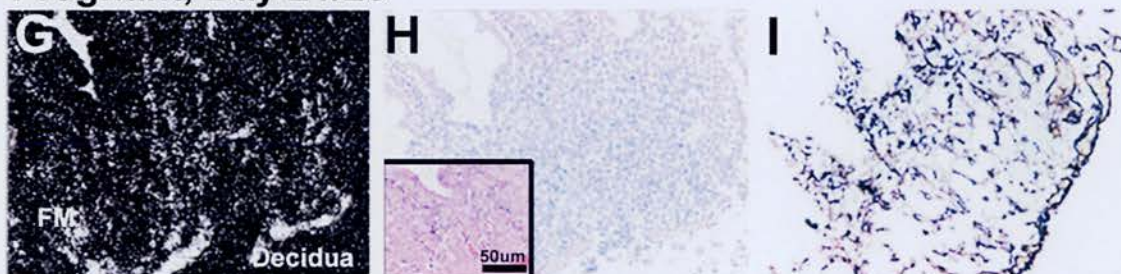
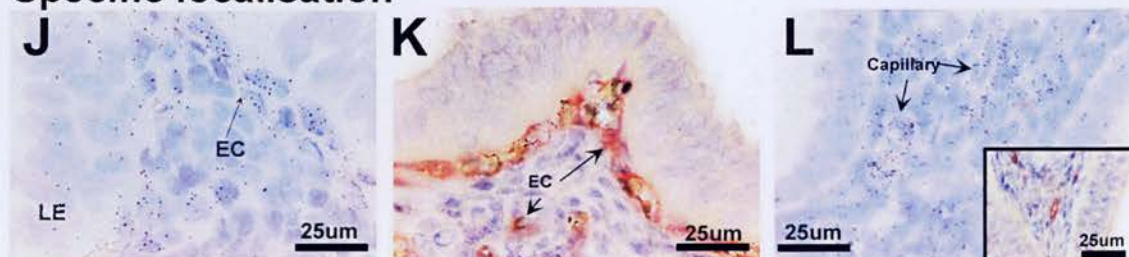
Late secretory**Pregnant, Day 14****Pregnant, Day 21/28****Specific localisation****Tie-2**

Figure 6.5 (A-L) In situ hybridisation of Tie-2 mRNA in marmoset endometrium. (A-C) Non-pregnant late secretory phase endometrium. (A) Dark field and (B) light field images of Tie 2 expression in stroma throughout all zones of the non-pregnant endometrium. Note expression localised to endothelial cells as demonstrated by CD31 immunocytochemistry (C) and (L). The insert in (L) illustrates CD31 immunocytochemistry, counterstained with haematoxylin. (D-F) Pregnant marmoset endometrium at day 14 after ovulation. (D) Dark field and (E) corresponding light field images of upper zone endometrium. Note localisation to endothelial cells immediately surrounding upper zone glands as demonstrated by (F) CD31 immunocytochemistry. (K) illustrates CD31 immunocytochemistry counterstained

with haematoxylin. (G-I) Typical Tie-2 expression at day 21 and day 28 of pregnancy. (G) Dark field and (H) light field images of upper zone decidualised endometrium. Note punctate expression of Tie-2 probably localised to endothelial cells as demonstrated by (I) CD31 immunocytochemistry. Insert shows a haematoxylin and eosin stained section. Note vessels similar to areas of grain localisation in (G).

Table 6.2 Quantification of *in situ* hybridisation using relative grain density within different uterine compartments throughout the stages studied for VEGF, VEGF R1 and R2, Ang-1, Ang-2 and Tie-2. This scoring was based on an overall impression of the region by two independent observers. Data was combined before statistical tests were applied. Values given are mean scores. Significant differences ($P < 0.05$) are marked with different letters. Where no differences occurred, no letters are given. (n=5 per group).

VEGF	LE	UZGE	UZS	LZGE	LZS	
LS	+++ ^a	+++	-	-	-	
P,D14	+++ ^{ab}	++	+	-	-	
P,D21	+ ^{ab}	++	++	+	-	
P,D28	- ^b	+	++	++	-	
R2 (R1)	LE	UZGE	UZS	Endo	LZGE	LZS
LS	-	-	-	+++ (+)	-	-
P,D14	-	-	-	++ (+)	-	-
P,D21	-	-	-	++ (+)	-	-
P,D28	-	-	-	++ (+)	-	-
Ang-1	LE	UZGE	UZS	LZGE	LZS	
LS	-	+	+	-	-	
P,D14	-	+	++	+++	-	
P,D21	-	+	+++	++	-	
P,D28	-	+	++	+	-	
Ang-2	LE	UZGE	UZS	Endo	LZGE	LZS
LS	-	-	-	- ^a	-	-
P,D14	-	-	-	- ^a	-	-
P,D21	-	-	-	- ^a	-	-
P,D28	-	-	-	+++ ^b	-	-
Tie-2	LE	UZGE	UZS	Endo	LZGE	LZS
LS	-	-	+	+++	-	+
P,D14	-	-	+	++	-	-
P,D21	-	-	+	++	-	+
P,D28	-	-	+	++	-	+

Row Headings (LE) Luminal Epithelium, (UZGE and LZGE) Upper and Lower Zone Glandular Epithelium, respectively, (UZS and LZS) Upper and Lower zone stroma, respectively, (UZEndo) Upper Zone Endothelium.

Column Headings Stage of cycle of endometrium (LS) Late Secretory non-pregnant, (P,D14) 2 weeks, (P,D21) 3 weeks and (P,28) 4 weeks of pregnancy. NOTE scores for VEGF R1 are given in brackets next to values for VEGF R2.

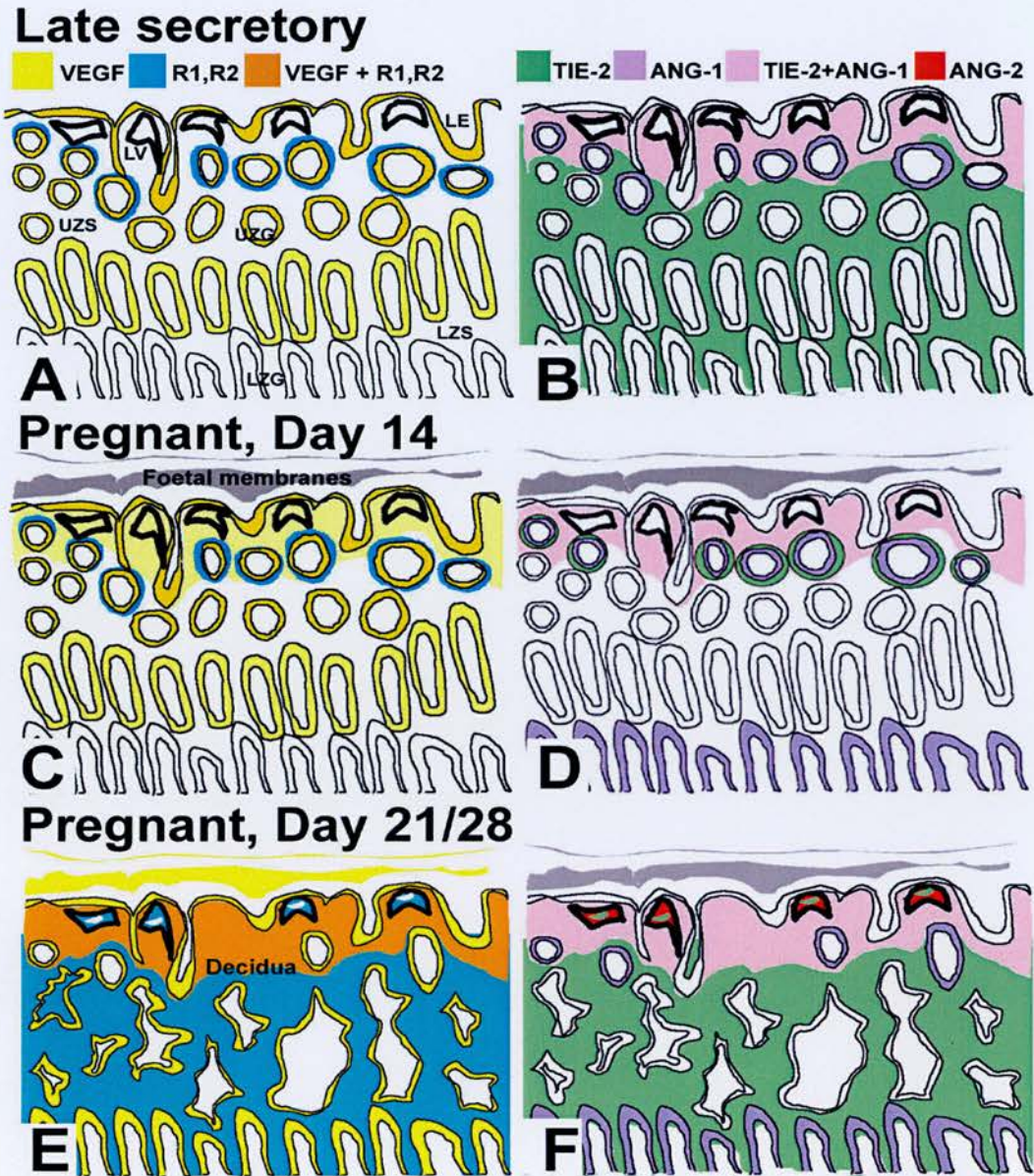


Figure 6.6 (A-F) Diagrammatic representations of angiogenic factor expression in the marmoset endometrium. Yellow represents VEGF, blue is VEGF receptors R1 and R2, orange is VEGF + R1R2 localised to the same areas. Green is Tie-2, Lilac represents Ang-1, pink is Ang-1 and Tie-2 in the same location and red is Ang-2. (A and B) Late secretory non-pregnant endometrium. (C and D) Pregnant endometrium, 14 days after ovulation. (E and F) Pregnant endometrium at 3 (21 days) and 4 (28 days) weeks of pregnancy. (LV) Luminal vessels, (UZS) Upper zone stroma, (UZG) Upper zone glands, (LE) Luminal epithelium, (LZS) Lower zone stroma and (LZG) Lower zone glands

6.4 Discussion

This experimental chapter has described the spatial and temporal expression patterns of mRNAs for VEGF-A, angiopoietins and their receptors in the marmoset uterus during the peri-implantation period, allowing us to begin to construct a working hypothesis as to their potential role in oxygen and nutrient exchange and maternal vascular remodelling. **Figure 6.6** is a summary diagram of the expression patterns detailed in the results sections and explained below as a system of angiogenesis highlighting the interplay between the different angiogenic factors.

Implantation occurs on day 11 after ovulation in the marmoset, three days later than in the human, and the blastocyst remains in loose attachment to the surface epithelium without epithelial fusion. Penetration of trophoblast into maternal vessels occurs quite late, days 45 to 60 of gestation in this species, compared to about day 12 in the human (Enders, 1993). Therefore, foetal demand for nutrients and oxygen during early pregnancy must be met by diffusion across the foeto-maternal interface, a process likely to be critically dependent on vascular permeability. Increased permeability is generally thought to accompany tissue growth and remodelling (Dvorak et al., 1999, Dvorak, 2000, Rabbani and Rogers, 2001). VEGF is a vascular permeability factor as well as a mitogen for endothelial cells (Keck, et al., 1989). Permeability and uterine oedema mediated by VEGF have been shown to be critical for successful implantation in the rodent where inhibition of VEGF prevented implantation (Rabbani and Rogers, 2001, Rockwell, et al., 2002). VEGF mRNA localisation to upper zone endometrium of all groups, increasing during the first few weeks of pregnancy as found here implies a role for VEGF as a permeability factor at this time.

The presence of VEGF mRNA in upper zone glandular epithelium and luminal epithelium of non-pregnant late secretory endometrium, with both VEGF R1 and VEGF R2 mRNA in endothelial cells of blood vessels immediately surrounding these glands suggests a local action of VEGF. This is also observed in 2 and 3 week pregnant endometrium where it may be involved in promoting fenestration of these vessels facilitating oxygen transfer to the avascular foetus. Although I observed

foetal membranes positive for CD31, they had no capillary formation. At 4 weeks of pregnancy, upregulation of stromal VEGF mRNA was observed. It is likely that the permeability role of VEGF is declining at this point, as glandular secretions are reduced and maternal sinusoids, although not yet invaded by trophoblast, have much more intimate contact with the more closely attached blastocyst thus facilitating diffusion. I also observed very strong expression of VEGFR-2 mRNA in endothelial cells of large maternal vessels immediately beneath the luminal epithelium where foetal VEGF could be acting to promote vessel migration in order that foetal metabolic demands can be adequately met. The pattern of mRNA localisation of VEGF and its receptors observed here is consistent with findings in human, where foetal VEGF expression in triprounuclear zygotes has been implicated to induce neoangiogenesis at the implantation site (Krussel et al., 2000). VEGF expression has been documented in human placenta and other species in later stages of placentation where it is reported to be expressed in maternal decidua, but unlike my findings, its receptor VEGFR-1 appeared to be expressed in invading syncytial trophoblast (Ahmed, et al., 1995, Clark, et al., 1996, Cooper, et al., 1995). It may be that foetal VEGF receptors are only present at later stages in the marmoset when a more intimate interplay between maternal and foetal tissues occurs (Wulff *et al.*, 2002). Soluble flt is produced by human trophoblast in pregnancy and is detectable in serum of pregnant women (Banks et al., 1998). It is possible that this also occurs in the marmoset, however no hybridisation of VEGFR-1 probe to trophoblast was observed in this study despite our probe detecting both the soluble and full length receptor. No serum analyses were conducted.

The roles of the angiopoietins and their receptor Tie-2 in early pregnancy are just beginning to be explored. In general, Ang-1 acts on its receptor Tie-2 to promote stabilisation of the endothelial cell-vascular smooth muscle structure (Suri et al., 1996), while Ang-2 acts as an antagonist by binding to the Tie-2 receptor without transmitting a signal (Lobov et al., 2002, Maisonpierre, et al., 1997). In the marmoset uterus, Ang-1 mRNA was the most abundantly expressed angiopoietin, localised to upper zone glands and stroma while Tie-2 mRNA was expressed in endothelial cells. In menstruating species following an infertile cycle, menstruation and shedding of the upper zones of the endometrium occur. The lower zone and

myometrium are comparatively more stable compartments from which a new vascular and new upper zone endometrium is supplied (Smith, 2001). Although the marmoset does not menstruate, in infertile cycles a degree of vascular remodelling may occur in the upper zones while the lower zones remain more stable, implicating a role for Ang-1 in this region as observed here. However, fertility rate is extremely high in marmosets (Rowe, et al., 2002), consequently this species may retain a stable upper zone endometrium without remodelling in response to progesterone withdrawal, associated with basal levels of expression of Ang-1 and Tie-2 in preparation for pregnancy. In fertile cycles, the endometrium is preparing to receive an implanting blastocyst and thus would require a stable maternal vasculature. Ang-1 mRNA may be expressed in the lower zones to maintain core vessel stability.

During the peri-implantation period, stromal Ang-1 and Tie-2 mRNA expression in the upper zones was greatly increased compared to non-pregnant endometrium, particularly adjacent to the implantation site. As gestation progressed into the third and fourth week, Ang-1 and Tie-2 mRNA expression in upper zone stroma and decidua was further increased. This corresponds with a continued requirement for a stable vasculature at the maternal-foetal interface and eventual placentation and development of a hemochorial placenta. This role appears to extend to later pregnancy in the marmoset, where Ang-1 mRNA is exclusively localised to syncytial trophoblast (Wulff, et al., 2002), suggesting that once the foetal vasculature begins to develop, there exists a need for stabilisation of newly formed foetal vessels.

The only site of Ang-2 mRNA localisation was in large maternal vessels immediately beneath the luminal epithelium of 4 week pregnant animals where it was co-localised with Tie-2 mRNA. This is similar to the situation observed in later stages of placentation in the marmoset (Wulff, et al., 2002). The presence of VEGF mRNA in the decidualised upper zone endometrium with its receptors in endothelial cells of this same region, including large maternal vessels, suggests a similar paracrine modulation of Ang-2 and VEGF. Ang-2, when expressed in conjunction with VEGF, is generally thought to be pro-angiogenic, promoting destabilisation of vessels and breakdown of basal lamina to allow migration of endothelial cells and subsequent angiogenesis. My findings suggest that at 4 weeks of pregnancy, VEGF

and Ang-2 could be working synergistically to promote migration of these vessels and establishment of an efficient interface for nutrient and oxygen exchange between the foetal and maternal circulation.

No angiopoietin or Tie-2 mRNA was detected in blastocyst or foetal membranes. Their presence in first trimester placenta has been reported in the human; Tie-2 has also been localised to trophoblast cells as well as endothelial cells (Dunk, et al., 2000). Tie-2 mRNA in trophoblast has been observed in later stages of pregnancy in the marmoset in our laboratory (Wulff, et al., 2002).

Ang-2 is expressed by uterine natural killer cells in human endometrium, particularly those situated beneath the luminal epithelium and around vascular smooth muscle cells (Smith, 2001). I found no evidence for this phenomenon in the marmoset where Ang-2 mRNA was found exclusively in endothelial cells of luminal maternal vessels as confirmed by CD31 immunocytochemistry.

In conclusion, this experimental chapter has described the molecular regulation of angiogenesis associated with implantation and vascular remodelling during the peri-implantation period in the marmoset. It provides evidence that VEGF/R1R2 and Ang-1,Ang-2/Tie-2 interactions may be involved in the preparation of endometrium for implantation, remodelling of the maternal vasculature during the peri-implantation period and trophoblast invasion.

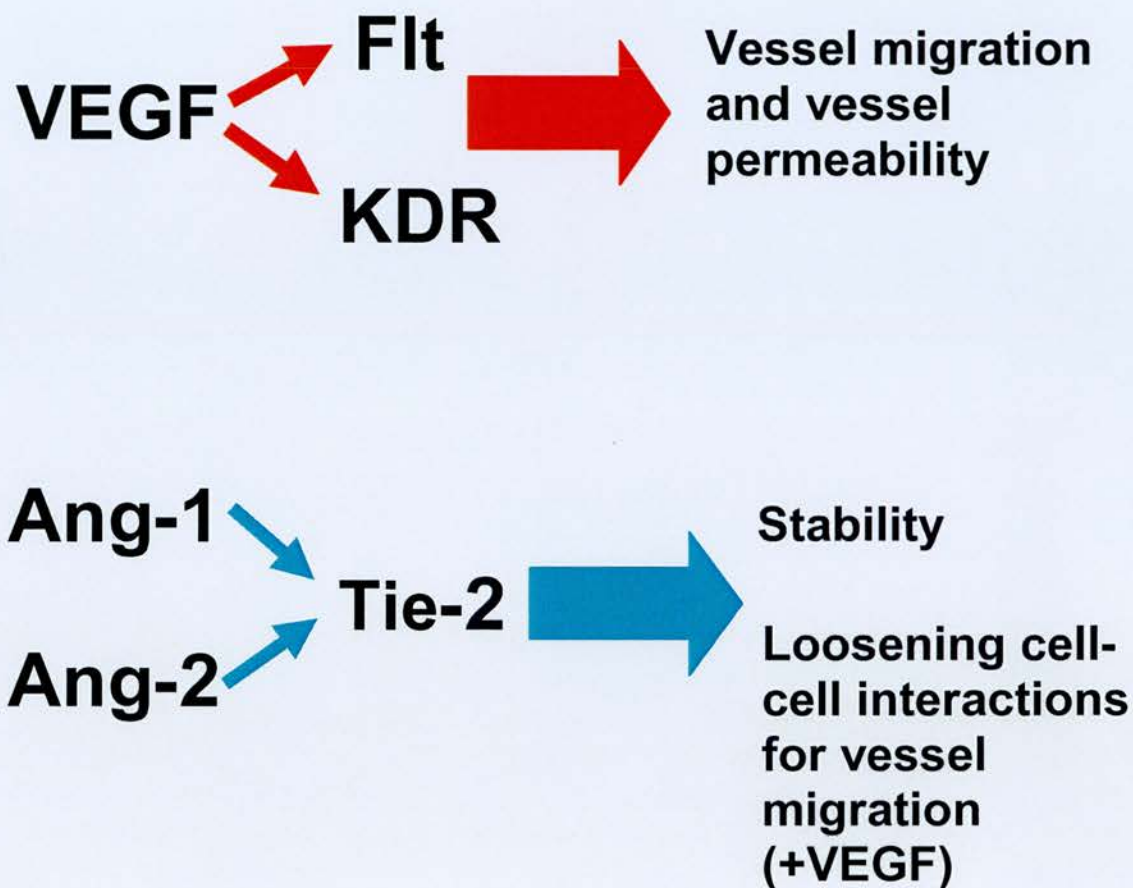


Figure 6.7 Flow diagram to illustrate putative roles of the angiogenic factors during implantation and early pregnancy

Figure 6.7 summarises the suggested roles for the angiogenic factors during the implantation process. From the *in situ* photographs it can be seen that with all probes, there appears to be an area of intense hybridisation immediately adjacent to the implantation site itself. This has caused a few apparent discrepancies between what is represented in Table 6.1 and what is presented as photographs. For the quantification, the uterus was compartmentalised into upper and lower zones, then further divided into cellular compartments. Therefore in terms of quantification, this area of intense hybridisation was not categorised separately, rather as part of the upper zone. This is apparent, for example with Tie-2 expression in the upper zone

endothelium. I refer to an increase in Tie-2 expression as pregnancy progresses. This however, does not appear to be the case in **Table 6.2** where the highest intensity of hybridisation was recorded in the non-pregnant late secretory phase. It is only in this isolated region of endometrium, immediately beneath the implantation site where Tie-2 expression increases as pregnancy progresses, as represented by the photographs.

I have proposed putative roles for the angiogenic factors studied here during this time point. I believe that VEGF is acting not only as an angiogenic mitogen to promote proliferation and migration of endothelial cells but also as a permeability factor. This would give rise to a well developed vasculature in the upper zone as pregnancy progresses and induce vessel fenestration; both greatly enhancing the diffusion process across the foetal-maternal interface to meet the growing metabolic demands of the newly implanted blastocyst.

I suggest that angiopoietin-1 is acting to stabilise these newly formed capillaries in order that placentation be successful with a well established maternal vasculature. Angiopoietin-2 was found to be highly expressed in upper zone large maternal vessels. Also at this cellular location, its receptor Tie-2 was found together with VEGF receptors KDR and Flt. Angiopoietin-2 is reported to have a destabilising role and involvement in vessel degradation. This could be true if maternal vessels were being invaded by syncytial trophoblast, however, in the marmoset this does not occur until later in pregnancy. I suggest that a complex synergism between VEGF and Angiopoietin-2 is in place by day 28 of pregnancy in order that the endothelial cells of these large vessels can proliferate and migrate to further increase the capillary density in this region, in preparation for true hemochorial placentation.

This experiment complements earlier work performed by our laboratory group documenting the molecular angiogenic events occurring from week 7 of pregnancy to term, and provides a comprehensive picture of implantation and placentation in this primate. Having proposed putative roles for these angiogenic regulators in this process, there exists potential for *in vivo* studies using specific antagonists to determine their physiological function.

Chapter 7

General Discussion

7 General Discussion

This thesis has illustrated the results of a series of investigations into the role of angiogenesis in the female reproductive tract in early pregnancy of the primate using the marmoset as an *in vivo* model.

The results from each experiment have been discussed in detail at the end of each chapter, and thus this general discussion provides an opportunity to dissect and summarise the main findings of the thesis, to discuss the appropriateness of the marmoset model and the clinical implications of this research.

The results presented in this thesis provide a comprehensive understanding of the role of angiogenesis in the corpus luteum and endometrium of early pregnancy, and allow for the opportunity to discuss possible future approaches to further investigate this complex process.

7.1 The findings of the thesis

The importance of angiogenesis in early pregnancy is a phenomenon that has been widely studied using pharmacological rescue of the corpus luteum, ie. treatment of women and experimental animals with hCG to mimic early pregnancy. While this gives valuable insight into the processes involved in rescue of the corpus luteum and preparation of the endometrium for early pregnancy, it is an isolated system where no conception has occurred and subsequently there is no implantation event. This is relevant because while hCG is recognised as being the primary stimulus for the maternal recognition of pregnancy, there may be other factors or indeed physical stimulation of an implantation event that trigger other mechanisms which would be lost in this model system. The marmoset allows us to overcome this problem as it is a highly fecund species and as such provides a primate model in which to study physiological pregnancy.

Angiogenesis has been shown to be intense during the first few days of the lifespan of the corpus luteum (CL) in a large number of species studied, including women (Christenson and Stouffer, 1996, Dickson and Fraser, 2000, Rodger, et al., 1997, Zheng, et al., 1994) and is stimulated principally by LH-driven vascular endothelial cell growth factor (VEGF) (Dickson, et al., 2001, Fraser, et al., 2000).

The rationale behind the initial hypothesis was to use the marmoset as a model for *in vivo* luteal angiogenesis in early pregnancy and to determine whether or not the same cellular and molecular events were taking place as had been reported in the human after hCG rescue of the corpus luteum. When pharmacological 'rescue' of the CL by hCG was carried out in women, a second wave of angiogenesis, accompanied by vascular stabilisation by pericytes was associated with luteal rescue (Wulff, et al., 2001). It was also found that mRNA for VEGF increased in the CL after hCG rescue (Wulff, et al., 2000). These findings suggested that luteal angiogenesis could be a component of luteal rescue which is important for prolonging the lifespan of the corpus luteum and the establishment of early pregnancy. Since failure of the CL due to a malfunction of the vasculature could possibly lead to miscarriage or infertility, it was considered important to establish a non-human primate model of early pregnancy in which this issue could be addressed experimentally.

The finding that the marmoset does not appear to undergo further angiogenesis during luteal rescue, gave rise to the second experiment presented in this thesis, whereby the effect of progesterone on VEGF expression *in vitro* was examined in human luteinised granulosa cells. This was done to determine whether or not the increase in VEGF expression observed in the human CL *in vivo* was a progesterone mediated effect.

The endometrium is cyclically dependent on ovarian steroids and as such was considered an ideal target tissue within which to explore possible angiogenic events that may be occurring throughout the non-pregnant cycle, during the peri-implantation period and in early pregnancy. The time points of early pregnancy studied here provide novel insights into the angiogenic process and its involvement during the peri-implantation period in a primate model.

A number of important findings have been described 1) I have demonstrated that by the late luteal phase in the marmoset, the corpus luteum has already established a mature vascular system and the molecular capacity to synthesize VEGF and its receptors which are not further enhanced in early pregnancy. A pregnancy induced spurt of angiogenesis does not take place, rather a maintenance of the existing vasculature is all that is required for establishment of pregnancy. Therefore,

it has to be concluded that the changing morphological and cellular events thought to be associated with early pregnancy in the human after hCG rescue do not occur in the marmoset. 2) From *in vitro* studies using human luteinised granulosa cells, it would appear that increased VEGF expression in luteal rescue is not a progesterone mediated effect. However, this is a complex system which requires further investigation in order that the exact mechanisms by which increased angiogenesis occurs in hCG rescue of the human corpus luteum are understood. 3) There exists an increase in angiogenesis associated with early pregnancy in the marmoset endometrium compared to late-secretory non-pregnant controls, and 4) I have described the molecular regulation of angiogenesis associated with implantation and vascular remodelling during the peri-implantation period in the marmoset. These data provide evidence that VEGF/R1R2 and Ang-1,Ang-2/Tie-2 interactions may be involved in the preparation of endometrium for implantation, remodelling of the maternal vasculature during the peri-implantation period and trophoblast invasion.

These results provide valuable insights into the importance of angiogenesis during early pregnancy in the marmoset female reproductive tract. Dissection of these systems will allow the exact roles of the angiogenic factors at this time to be elucidated by various manipulations including antagonist treatment.

The techniques employed in this thesis in order to assess the angiogenic process during early pregnancy are robust and reproducible. Incorporation of BrdU into proliferating cells during the S phase of the cycle has served as the basis for measuring vessel proliferation, the first step in the angiogenic process. In the corpus luteum, this method proved to be very accurate. The marmoset corpus luteum is a relatively homogeneous gland, where vascularisation is distributed evenly over the gland by the late luteal phase. As such, using image analysis to quantify proliferation, throughout the whole cross section of the CL allowed for the generation of extremely accurate results. This is true of all immunohistochemical techniques employed for the purposes of this thesis in the CL, equally true for *in situ* hybridisation, where, expression of VEGF, Flt and KDR is fairly consistent throughout the CL cross section. At this point I would like to comment on the fact that I was unable to localise the angiopoietins and Tie-2 in the marmoset CL by *in situ* hybridisation. I established that the human riboprobes used, do cross react in the

marmoset as demonstrated in Chapter 6 in the endometrium. I conclude therefore that the angiopoietins and their receptor Tie-2 mRNA are too low to be detected by *in situ* hybridisation in the marmoset CL. This is surprising however, because angiopoietin-1 is thought to have a role in vessel stabilisation and pericyte recruitment, so, having localised pericytes in the marmoset CL at this time, the mechanism by which these cells are recruited by the newly formed capillaries remains to be elucidated.

Another limitation of this experimental model was the difficulty in accurately assessing the processes involved in luteal regression. Those CL which had undergone structural regression by day 21 had extremely disrupted CL, with no clear morphology to the degree that steroidogenic cells could no longer be distinguished. As such, the methods employed to assess angiogenesis in the other experimental groups could not be used as these rely on the integrity of the tissue under analysis. Therefore it was impossible to determine the angiogenic response at this stage. I did observe that VEGF expression was completely obliterated at this time. VEGF could act as a survival factor for endothelial cells, so that its absence would cause vascular regression and consequently result in a hypoxic environment. However, it is difficult to make accurate conclusions about this, as it could be that due to the poor structural integrity of the tissue, that RNA degradation had occurred to such an extent that the riboprobes were unable to detect VEGF in these cells rather than it actually being "switched off". We have no evidence for a decline in VEGF prior to luteolysis.

Luteolysis in primates and the trigger for this remains unclear. The length of the luteal phase in marmosets is 21 days theoretically although I found this length to be variable, where some animals had undergone structural and functional regression by day 21, others still had intact and functionally active corpora lutea as late as day 25. As such, looking at triggers for luteolysis in this species is very difficult. It would appear that in the marmoset, the corpus luteum remains functionally and structurally active until such time as it undergoes luteolysis which seems to be a very rapidly triggered event.

In the endometrium, quantification of angiogenesis, both by immunocytochemistry and by *in situ* hybridisation proved more challenging. Unlike the CL, the uterus is not homogeneous. It is compartmentalised and not surprisingly these compartments showed varying levels of proliferation and expression of

angiogenic factors. In order that these problems could be overcome, the endometrium was separated into the various different compartments, namely, luminal epithelium, glandular epithelium, stroma and endothelium. In terms of quantitative analysis of proliferation, a grid system was devised according to Hastings *et al.*, (Hastings, et al., 2003). This allows for the accurate volume fraction analysis of each of these different compartments across a uterine section and subsequent proliferation indices to be calculated. The uterus is an organ subject to oedematous changes and therefore analyses of immunocytochemical staining in the same way as employed for the CL were not appropriate. The methods employed take into account any volume changes and so therefore correct for this phenomenon and allow comparison between groups where changes in oedema may not occur to the same degree.

In situ hybridisation was employed to localise and semi-quantify mRNAs for the angiogenic growth factors, but posed a higher degree of complexity. The level of proliferation of endothelial cells varied between endometrial compartments and between stages, and so therefore it was expected that the angiogenic factor distribution, presumably responsible for this would also vary. To address this issue, once again the endometrium was subdivided into cellular compartments and a scoring system employed. Grain counts per unit area, as used in the CL, would not have been an appropriate method of quantification here, due to this degree of variability.

Using the marmoset as a model for early pregnancy and taking into account the variability between cellular compartments and variations throughout the cycle, allows this important process and the angiogenic events associated therein to be elucidated. It was surprising that I did not observe an increase in angiogenesis in the CL associated with early pregnancy as had been reported in women. However, earlier experiments in hCG treated rhesus macaques had reported similar findings to the marmoset, where no increase in angiogenesis was associated with hCG rescue (Christenson and Stouffer, 1996). In the human corpus luteum, endothelial cell proliferation mirrors that of the marmoset in the early, mid- and late luteal phase, where maximal proliferation occurs in the early luteal phase and declines significantly in the mid- and late luteal phase (Gaytán, et al., 1998, Rodger, et al., 1997). This is also true in the rhesus monkey, (Christenson and Stouffer, 1996),

sheep (Jablonka-Shariff, et al., 1993, Jablonka-Shariff, et al., 1997) and in cattle (Zheng, et al., 1994).

The rescue of the corpus luteum of pregnancy allows it to persist functionally and structurally for longer than in the non-pregnant cycle. Presumably, this survival requires a stable vasculature, with recruitment of pericytes and prolonged endothelial cell survival, in addition to prolongation of the lifespan of the luteal cells. Since endothelial cell proliferation did not increase after administration of hCG in the rhesus monkey (Christenson and Stouffer, 1996), it was suggested that early pregnancy may be associated with an increase in vessel stability, and there is evidence from our group for hCG-induced increase in pericyte number in the human CL (Wulff, et al., 2001). However, no evidence for this was found here.

It is well established that progesterone levels are maintained due to the continued responsiveness of luteal cells to gonadotrophins via CG in pregnancy (Stouffer, et al., 1978). It would appear that in the marmoset, despite the absence of CG, the late luteal CL of a non-pregnant cycle is maintained until it undergoes a rapid regression and thus decline in progesterone production. Luteal regression in the marmoset must therefore be a very rapidly triggered event, for up until that point the CL of a non-fertile cycle retains its morphology and functional capacity to the same extent as in a pregnant cycle.

Having established this disparity between the marmoset and human, I addressed the issue of what could be responsible for the upregulation of VEGF seen in the human CL. Cell culture studies using luteinised granulosa cells, have revealed that hCG treatment leads to increased VEGF mRNA levels (Neulen, et al., 1998). This reported up-regulation of VEGF by hCG rescue supports the view that in the human, pregnancy is associated with further angiogenesis. The marmoset and human vary in terms of their progesterone profiles in early pregnancy. In the human, after hCG rescue, progesterone levels increase unlike in the marmoset where levels attained in the late luteal phase are maintained without further increase. This in itself provides compelling evidence for the disparity observed in the angiogenic response of these two species. The CL of pregnancy in the marmoset appears functionally and structurally the same as the structurally intact non-pregnant CL, as such, no upregulation of progesterone synthesis occurs. The second disparity is that marmoset

CL do not express progesterone receptors unlike the human. Therefore I proposed that progesterone produced by the human CL can be detected by its steroidogenic cells and that this stimulation causes a positive feedback response to further upregulate angiogenesis and progesterone synthesis. Marmosets, not having luteal progesterone receptors, could not undergo this phenomenon and therefore progesterone remains at levels attained in the late luteal phase.

A cell culture system was used to address the importance of progesterone in luteal rescue in the marmoset. Inhibition of steroidogenesis in these cells did not abolish VEGF upregulation upon hCG treatment. Nor did treatment with exogenous progesterone augment this effect. This leaves a very interesting question that remains to be answered. Clearly establishment and maintenance of pregnancy in the marmoset is a very efficient system, with fertility rates greater than 70% in our colony. How does this species establish early pregnancy without any apparent functional or structural changes in the CL at this time?

Angiogenic events occurring in the marmoset endometrium have not been addressed to date. The endometrium is very dynamic tissue undergoing profound cyclic changes in response to ovarian steroids. Also, angiogenic changes occurring during implantation and early pregnancy have not yet been elucidated. Marmoset implantation and placentation are much more superficial processes than in other primates. Maternal vessels are not invaded until around day 60 of gestation, as such the metabolic demands of the trophoblast must be met by diffusion. Consequently, a large degree of tissue remodelling would be expected as implantation is established but also, a degree of vascular remodelling in order that the nutrient and oxygen requirements of the newly implanting foetus be met.

VEGF and its receptors, Flt and KDR have been implicated as key players in vascular remodelling and placentation on the basis that their mRNA and proteins have been localised in endometrium of several species. These include human (Ahmed, et al., 1995, Clark, et al., 1996, Clark, et al., 1998, Cooper, et al., 1995, Geva, et al., 2002, Goldman-Wohl, et al., 2000, Helske, et al., 2001, Sugino, et al., 2002), sheep (Bogic, et al., 2001), pig (Winther, et al., 1999), marmoset (Wulff, et al., 2002) mouse (Chakraborty, et al., 1995, Halder, et al., 2000), golden hamster (Yi, et al., 1999) rat (Rabbani and Rogers, 2001), rabbit (Das, et al., 1997) and rhesus

monkey (Ghosh et al., 2000) during the first trimester of pregnancy. *In vivo* manipulation experiments are limited to the rodent to date but have provided further evidence for the importance of VEGF in early pregnancy. In the mouse, inhibition of VEGF prevented estrogen-induced uterine oedema and implantation (Rockwell, et al., 2002) and in the rat, the number of implantation sites were reduced after administration of a VEGF blocking antibody (Rabbani and Rogers, 2001).

Angiopoietins also appear to have a major role in regulation of blood vessel growth, maturation and regression, but their importance at the time of implantation has yet to be studied. However their presence in placenta in later stages of pregnancy has been documented in human (Dunk, et al., 2000, Goldman-Wohl, et al., 2000) and marmoset (Wulff, et al., 2002). Having established that there is an increase in angiogenesis in the functionalis of early pregnancy in the marmoset, compared to late-secretory non-pregnant controls, I wanted to determine putative molecular regulation of this event by describing the localisation of mRNA for angiogenic factors and their receptors. Angiogenic processes occurring at this early stage of pregnancy are still poorly understood in the human and with accumulating knowledge on effects of manipulation of angiogenesis in the marmoset, this species represents a potentially valuable model in which to study these events.

Not surprisingly, the endometrium is a rich source of angiogenic growth factors. VEGF expression and endothelial cell proliferation have been demonstrated to peak in the mid-proliferative phase in rhesus macaque endometrium, coinciding with a peak in oestrogen (Nayak and Brenner, 2002). Expression of VEGF and its role in promoting vascular permeability and angiogenesis during early pregnancy and placentation have been frequently documented. I have described the specific localisation of VEGF, its receptors and the angiopoietins in marmoset endometrium during the peri-implantation period and in early pregnancy in Chapter 6 (Rowe, et al., 2003)

Having described the distribution of VEGF, the angiopoietins and their receptors in the endometrium during the peri-implantation period and their upregulation in early pregnancy, it is clear that these factors must play a critical role in establishment of early pregnancy in this species. In order that their exact role be elucidated, antagonist experiments should be carried out to determine whether or not

they are critical for the establishment of early pregnancy. To date, most antagonist studies are limited to the rodent (Rabbani and Rogers, 2001, Rockwell, et al., 2002). However, we have treated marmosets with a VEGF antibody to examine the effects on progesterone secretion by the CL and establishment of pregnancy. While the antibody significantly suppressed progesterone secretion during the treatment period, it failed to significantly reduce pregnancy rate. The results were somewhat inconclusive, where in control animals, 5 of 6 became pregnant, while in treated animals, 5 of 10 became pregnant. Therefore, it appears that this treatment did prevent pregnancy in some animals and perhaps with a larger study group this may have been significant (Rowe, et al., 2002). It may also be that this antibody was not potent enough and that more potent VEGF inhibitors, such as VEGF traps, would prevent pregnancy in this species.

It is of great interest that despite significantly reduced levels of progesterone during the treatment period in this study, pregnancy could still be established. It would appear that the CL was able to recover and resume normal progesterone synthesis after cessation of treatment. This evokes questions about how important the normal quota of progesterone is in establishment of early pregnancy in the marmoset. It may be that in a normal cycle, levels of progesterone synthesised and secreted are well in excess of requirement for establishment of pregnancy, and that despite significant reduction in this level, enough progesterone remained in the system in order that pregnancy be established. In human, it is well known that treatment with progesterone antagonists can prevent implantation as well as terminating early and established pregnancy. The marmoset is a highly efficient reproductive species. It would appear from my results that the corpus luteum of every cycle resembles that of a pregnant cycle whereby adequate levels of progesterone and vascular development are established regardless of whether or not a conception event has occurred. The endometrium, does respond differently in a fertile cycle, after implantation where there is an upregulation of angiogenesis. It is at this point that the maternal recognition of pregnancy would be perceived. It would be feasible to assume that should no implantation event occur, thus no maternal recognition of pregnancy, that the corpus luteum would regress. The situation observed here, does not appear to be as simple as this. The CG stimulus "rescues" the CL, yet evokes no cellular or

morphological changes in terms of angiogenesis. Therefore it is as if the CL undergoes this response regardless of a conception event occurring as described before. "Rescue" of the CL cannot purely be a consequence of CG alone because timing of luteolysis is so variable. If it were, regression would be expected to occur at a set time in all animals should a CG stimulus not be detected. It may be that marmoset LH receptors undergo a refractory period when they are no longer sensitive to LH and that timing of this varies between animals. I propose that marmoset LH is not as dissimilar to CG as in other species and because they act through the same receptor on luteal steroidogenic cells, it could be that the marmoset detects the LH and hCG stimulus as the same thing and consequently prepares for early pregnancy in every cycle. It has been suggested that luteinizing hormone/chorionic gonadotropin bioactivity in the marmoset is due to a chorionic gonadotropin molecule with a structure intermediate between human chorionic gonadotropin and human luteinizing hormone (Simula et al., 1995).

Implantation in the marmoset occurs at day 12 after ovulation, in the mid-luteal phase. In a pregnant cycle, the blastocyst implants and angiogenesis in the endometrium is upregulated. The trigger for this upregulation is unclear. No angiogenic factors were detectable in the blastocyst until day 28, yet increased VEGF, angiopoietins and their receptors in the functionalis immediately surrounding the implantation site occur at the time of implantation, or by day 14 to use my time points. Therefore it is not the blastocyst per se that is stimulating an upregulation of these factors. It could be oestrogen. This is well known to cause uterine oedema by increased fenestration of vessels, possibly as a consequence of VEGF upregulation. It could also be a consequence of a hypoxic environment caused by the demands of the newly implanting blastocyst. It could also be a direct effect of CG produced by the invading trophoblast and acting locally on the endometrium, however, I am not aware of LH receptor expression in the marmoset uterus. It is not likely to be a direct effect of progesterone because levels of progesterone do not differ between pregnant and non-pregnant cycles. However, I have not looked at progesterone receptors at this time. It may well be that progesterone receptors are upregulated in marmoset endometrium in early pregnancy.

7.2 The marmoset as a model for early pregnancy

The studies in this thesis used the marmoset as a model for early pregnancy and to study the role of angiogenesis at this time in the CL and endometrium. As a model for the human, the marmoset presents some differences. It is not a mono-ovulatory species, it has a luteal phase length of approximately 21 days, as opposed to 14 days in the human, and the structure of its corpus luteum differs to that of the human. It displays subtly different implantation and placentation and does not menstruate. However, it has allowed for the first time, the study of physiological pregnancy as opposed to pharmacological pregnancy, by hCG treatment, in a primate model. Such invasive investigations of endometrial angiogenesis and structure of the early implantation site could not be carried out in higher primates. Its high fecundity, greater than 70% fertility rate, offers a considerable advantage over other commonly studied non-human primates, where fertility rate is in the region of 30%. The marmoset has an ovulatory cycle more analogous to the human than any rodent species, and so is an appropriate animal model.

The next step in our understanding of the role of angiogenesis in pregnancy would be to target it using specific antagonists, for which the marmoset should provide an invaluable model system.

7.3 Clinical implications of the thesis findings

The major function of the corpus luteum is progesterone production, resulting in provision of a receptive endometrium and quiescent myometrium for establishment and maintenance of early pregnancy. Since failure of the CL due to a malfunction of the vasculature could possibly lead to miscarriage or infertility, it was considered important to establish a non-human primate model of early pregnancy in which this issue could be addressed. We have demonstrated that the marmoset can become pregnant in the face of reduced progesterone synthesis, therefore further research is necessary to investigate the required level of progesterone suppression to prevent pregnancy. If progesterone levels could be suppressed to a level at which pregnancy could not be established, this could provide a model for post-ovulatory fertility control.

The demonstration that there is an upregulation of angiogenesis in the marmoset endometrium associated with early pregnancy provides the first step in our understanding of the role of angiogenesis in primate implantation. Targeting specific angiogenic factors could result in failure of implantation to occur, evoking possibilities for post-ovulatory or post-coital fertility control. It may be that if ongoing angiogenesis is targeted and inhibited during a specific window after implantation in the primate that an abortive agent could be developed.

Angiogenesis research in early pregnancy not only has implications in contraceptive development, it is also of relevance in understanding infertility. It may be that women suffering recurrent miscarriage may have inadequate luteal progesterone production, but also could have defects in the ability to establish an adequate endometrial vasculature in order to allow provision of nutrients and oxygen to the newly implanted foetus. This gives rise to opportunities for administration of pro-angiogenic agents to enhance this process. Pre-eclampsia is also a common illness associated with early pregnancy which has angiogenic implications.

Tumour growth is dependent on stimulation of new blood vessel growth from the pre-existing vasculature (Folkman and Klagsbrun, 1987), and therefore has been long proposed as a therapeutic target. Using the marmoset CL and endometrium as models for angiogenesis could result in development of new anti-angiogenic therapies not just for use in reproductive pathologies but for use in cancer treatment. These factors are also likely to have a role in other angiogenic pathologies such as rheumatoid arthritis, psoriasis and retinal neovascularisation in which uncontrolled angiogenesis occur. Pro-angiogenic factors are also likely to have great clinical importance in treating diseases where inadequate vascularisation is symptomatic, for example, in limb and heart ischaemia.

7.4 Future speculation

This thesis has provided a basis for studying angiogenesis in early pregnancy in the marmoset. Next steps into further dissecting this tightly regulated process include understanding the intricate development and progression of the vasculature in the embryo throughout the early stages of pregnancy. This thesis has focussed

predominantly on maternal tissues, but the capacity for investigating the relationship between the endometrium and foetus is unending.

Chapter 3 has described the angiogenic events associated with early pregnancy in the marmoset corpus luteum. In order to further understand this process other techniques could be employed such as Doppler imaging and perfusion fixing of tissue. This would allow for a more accurate determination of capillary volume without the effect of fixation after tissues are removed from the body. The process of exsanguination can cause small capillaries to collapse and therefore may interfere with the accuracy of the techniques employed in this thesis to examine capillary density. However, it was assumed that any degree of collapse would be occurring in all groups studied and therefore they were comparable.

Chapter 4 has allowed for the basic understanding into the role of progesterone in early pregnancy in rescue of the marmoset corpus luteum. It would be useful to look at different culture timings whereby the cell environment, length of culture time and hormone milieu more accurately represent the *in vivo* situation.

Chapter 5 has described the morphological and vascular status of the endometrium throughout the stages of pregnancy studied. This has provided a valuable insight into this field, but leaves much scope for future experiments. For example, it would be useful to look at the gene expression of ER and PR in the stages studies and to compare this with systemic levels of oestrogen and progesterone. I would also suggest that different groups of animals be studied with smaller windows in development between each.

Finally Chapter 6 has enabled for the first time the angiogenic factors to be localised and characterised in the marmoset uterus during early pregnancy. The real future benefit of this work will be to determine how critical these factors are in maintenance of early pregnancy. I feel that the most exciting future work arising from this thesis will be to look at potential antagonists to be administered during early pregnancy and to investigate the effect on the CL, uterus and embryo.

7.5 Conclusions

This thesis has provided powerful insights into the role of angiogenesis in early pregnancy in the primate. Using the marmoset as a model, studies reported in this thesis provide comprehensive evidence that the marmoset corpus luteum and maternal recognition of pregnancy differ somewhat to that of the human, and that angiogenesis is an important process in establishment of early pregnancy, particularly in the endometrium. Having established putative roles for angiogenic factors at the time of implantation, the potential to inhibit angiogenesis in the female reproductive tract should have important implications for clinical practice, be it in fertility control or pathology. This research provides a basis for our understanding of this process and opens doors for a plethora of future investigation.

References

- Abel MH** (1985) Prostanoids and menstruation. In: Baird DT, Michel EA (eds) *Mechanisms of menstrual bleeding*. Raven Press, New York, 139-156
- Abraham JA, Mergia A, Whang JL, Tumolo A, Friedman J, Hjerrild KA, Gospodarowicz D and Fiddes JC** (1986) Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor *Science* **233**:545-8
- Abulafia O and Sherer DM** (1999) Angiogenesis of the endometrium *Obstetrics and Gynecology* **94**:148-53
- Abulafia O and Sherer DM** (2000) Angiogenesis of the ovary *American Journal of Obstetrics and Gynecology* **182**:240-6
- Ackland JF, Schwartz NB, Mayo KE and Dodson RE** (1992) Nonsteroidal signals originating in the gonads *Physiological Reviews* **72**:731-87
- Adams EC and Hertig AT** (1969) Studies on the human corpus luteum I. Observations on the ultrastructure of development and regression of the luteal cells during the menstrual cycle *Journal of Cell Biology* **41**:696-715
- Adashi EY and Rohan RM** (1992) Intraovarian regulation: peptidergic signaling systems *Trends in Endocrinology and Metabolism* **3**:243-248
- Adashi EY and Rohan RM** (1994) Novel aspects of ovarian functions: Regulation of differentiation, ovulation and regression *Developmental Endocrinology* **6**:133-140
- Ahmed A, Li XF, Dunk C, Whittle MJ, Rushton DI and Rollason T** (1995) Colocalisation of vascular endothelial growth factor and its flt-1 receptor in human placenta *Growth Factors*. **12**:235-243
- Albrecht ED and Pepe GJ** (2003) Steroid hormone regulation of angiogenesis in the primate endometrium *Frontiers in Bioscience* **8**:416-429
- Andrews WC** (1979) Luteal phase defects. *Fertility and Sterility* **32**:501-9
- Antonelli-Orlidge A, Smith SR and D-Amore PA** (1989) Influence of pericytes on capillary endothelial cell growth *American Review of Respiratory Disease* **140**:1129-31
- Aplin JD** (1991) Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro *Journal of Cell Science* **99**:681-92
- Aronica SM and Katzenellenbogen BS** (1991) Progesterone receptor regulation in uterine cells: stimulation by estrogen, cyclic adenosine 3',5'-monophosphate,

- and insulin-like growth factor I and suppression by antiestrogens and protein kinase inhibitors *Endocrinology* **128**:2045-52
- Augustin HG** (2000) Vascular morphogenesis in the ovary *Bailliere's best practice & research. Clinical obstetrics & gynaecology* **14**:867-82
- Auletta FJ and Flint AP** (1988) Mechanisms controlling corpus luteum function in sheep, cows, nonhuman primates, and women especially in relation to the time of luteolysis *Endocrine Reviews* **9**:88-105
- Auletta FJ and Kelm LB** (1994) Mechanisms controlling corpus luteum function in the rhesus monkey (*Macaca mulatta*): Inhibitory action of hCG on luteolysis induced by PGF(2 alpha) *Journal of Reproduction and Fertility* **102**:215-220
- Ausprunk DH, Knighton DR and Folkman J** (1974) Differentiation of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study *Developmental Biology* **38**:237-48
- Ausprunk DH and Folkman J** (1977) Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis *Microvascular Research* **14**:53-65
- Azmi TI and O'Shea JD** (1984) Mechanism of deletion of endothelial cells during regression of the corpus luteum *Laboratory Investigation* **51**:206-217
- Bacci ML, Barazzoni AM, Forni M and Costerbosa GL** (1996) In situ detection of apoptosis in regressing corpus luteum of pregnant sow: evidence of an early presence of DNA fragmentation *Domestic Animal Endocrinology* **13**:361-72
- Bagavandoss P, Wiggins RC, Kunkel SL, Remick DG and Keyes PL** (1990) Tumor necrosis factor production and accumulation of inflammatory cells in the corpus luteum of pseudopregnancy and pregnancy in rabbits *Biology of Reproduction* **42**:367-76
- Baird DD, Weinberg CR, Wilcox AJ, McConnaughey DR, Musey PI and Collins DC** (1991) Hormonal profiles of natural conception cycles ending in early, unrecognized pregnancy loss *Journal of Clinical Endocrinology and Metabolism* **72**:793-800
- Baird DT** (1987) A model for follicular selection and ovulation: lessons from superovulation *Journal of Steroid Biochemistry* **27**:15-23
- Baird DT** (1991) The Ovarian Cycle. In: Hillier SG (ed) *Ovarian Endocrinology*. Blackwell Scientific, Oxford, 1-24

- Baker TG** (1972) Oogenesis and ovulation. In: Austin CR, Short RV (eds) *Germ Cells and Fertilisation* 2nd edition. Cambridge University Press, Cambridge, 17-45
- Banks RE, Forbes MA, Searles J, Pappin D, Canas B, Rahman D, Kaufmann S, Walters CE, Jackson A, Eves P, Linton G, Keen J, Walker JJ and Selby PJ** (1998) Evidence for the existence of a novel pregnancy-associated soluble variant of the vascular endothelial growth factor receptor, Flt-1 *Molecular Human Reproduction* **4**:377-386
- Bassett SG, Little-Ihrig LL, Mason JI and Zeleznik AJ** (1991) Expression of messenger ribonucleic acids that encode for 3 beta-hydroxysteroid dehydrogenase and cholesterol side-chain cleavage enzyme throughout the luteal phase of the macaque menstrual cycle *Journal of Clinical Endocrinology and Metabolism* **72**:362-6
- Bazzoni G, Dejana E and Lampugnani G** (1999) Endothelial adhesion molecules in the development of the vascular tree: the garden of forking paths *Current Opinion in Cell Biology* **11**:573-581
- Beer AE and Boillingham RE** (1974) Host responses to intrauterine tissue, cellular, and fetal allografts *Journal of Reproduction and Fertility (suppl)* **21**, 92
- Behrman HR, Endo T, Aten RF and Musicki B** (1993) Corpus luteum function and regression *Reproductive Medicine Review* **2**:153-180
- Bell SC** (1983) Decidualization and associated cell types: implications for the role of the placental bed in the materno-fetal immunological relationship *Journal of Reproductive Immunology* **5**:185-94
- Bell SC** (1983) Decidualization: Regional differentiation and associated function *Oxford reviews of reproductive biology* **5**:220-271
- Bell SC** (1988) Secretory endometrial/decidual proteins and their function in early pregnancy *Journal of Reproduction and Fertility (Suppl)* **36**:109-25
- Bennegård B, Hahlin M, Wennberg E and Norén H** (1991) Local luteolytic effect of prostaglandin F_{2α} in the human corpus luteum *Fertility and Sterility* **56**:1070-1076
- Berisha B, Schams D, Kosmann M, Amselgruber W and Einspanier R** (2000) Expression and tissue concentration of vascular endothelial growth factor, its receptors, and localization in the bovine corpus luteum during estrous cycle and pregnancy *Biol Reprod* **63**:1106-14
- Beyer EC, Paul DL and Goodenough DA** (1990) Connexin family of gap junction proteins *Journal of Membrane Biology* **116**:187-94

- Blankenship TN and Enders AC** (1997) Expression of platelet-endothelial cell adhesion molecule-1 (PECAM) by macaque trophoblast cells during invasion of the spiral arteries *The Anatomical Record* **247**:413-9
- Bogic LV, Brace RA and Cheung CY** (2001) Developmental expression of vascular endothelial growth factor (VEGF) receptors and VEGF binding in ovine placenta and fetal membranes *Placenta* **22**:265-75
- Boismenu R and Havran WL** (1994) Modulation of epithelial cell growth by intraepithelial gamma delta T cells *Science* **266**:1253-5
- Braden TD, Gamboni F and Niswender GD** (1988) Effects of prostaglandin F2 alpha-induced luteolysis on the populations of cells in the ovine corpus luteum *Biology of Reproduction* **39**:245-53
- Braden TD, Belfiore CJ and Niswender GD** (1994) Hormonal Control of Luteal Function *Molecular Biology of the Female*:259-287
- Brannstrom M and Friden B** (1997) Immune regulation of corpus luteum function *Seminars in Reproductive Endocrinology* **15**:363-70
- Brenner RM, West NB and McClellan MC** (1990) Estrogen and progesterin receptors in the reproductive tract of male and female primates *Biology of Reproduction* **42**:11-9
- Brenner RM and Slayden OD** (1994) Cyclic Changes in the Primate Oviduct and Endometrium *Physiology of Reproduction* **2**:541-569
- Brown MS and Goldstein JL** (1986) A receptor-mediated pathway for cholesterol homeostasis *Science* **232**:34-47
- Bruce NW** (1976) The distribution of blood flow to the reproductive organs of rats near term *Journal of Reproduction and Fertility* **46**:359-62
- Bruce NW and Moor RM** (1976) Capillary blood flow to ovarian follicles, stroma and corpora lutea of anaesthetized sheep *Journal of Reproduction and Fertility* **46**:299-304
- Bukovsky A, Caudle MR, Keenan JA, Wimalasena J, Upadhyaya NB and Vanmeter SE** (1995) Is corpus luteum regression an immune-mediated event? Localization of immune system components and luteinizing hormone receptor in human corpora lutea *Biology of Reproduction* **53**:1373-1384
- Bulmer D, Stewart I and Peel S** (1983) Endometrial granulocytes of the pregnant hamster *Journal of Anatomy* **136**:329-37

- Cameron ST, Critchley HO, Buckley CH, Chard T, Kelly RW and Baird DT** (1996) The effects of post-ovulatory administration of onapristone on the development of a secretory endometrium *Human Reproduction* **11**:40-9
- Cao L and Chan WY** (1993) Effects of Oxytocin and Uterine and Luteal Prostaglandins on the Functional Regression of the Corpus Luteum in Pseudopregnant Rats *Journal of Reproduction and Fertility* **99**:181-186
- Cardell RR, Jr., Hisaw FL and Dawson AB** (1969) The fine structure of granular cells in the uterine endometrium of the rhesus monkey (*Macaca mulatta*) with a discussion of the possible function of these cells in relaxin secretion *American Journal of Anatomy* **124**:307-39
- Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertszenstein M, Fahrig M, Vandenhoek A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W and Nagy A** (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele *Nature* **380**:435-9
- Chaffkin LM, Luciano AA and Peluso JJ** (1993) The role of progesterone in regulating human granulosa cell proliferation and differentiation in vitro *Journal of Clinical Endocrinology and Metabolism* **76**:696-700
- Chakraborty I, Das SK and Dey SK** (1995) Differential expression of vascular endothelial growth factor and its receptor mRNAs in the mouse uterus around the time of implantation *Journal of Endocrinology* **147**:339-352
- Chandrasekher YA, Melner MH, Nagalla SR and Stouffer RL** (1994) Progesterone receptor, but not estradiol receptor, messenger ribonucleic acid is expressed in luteinizing granulosa cells and the corpus luteum in rhesus monkeys *Endocrinology* **135**:307-314
- Channing CP and Tsafiriri A** (1977) Mechanism of action of luteinizing hormone and follicle-stimulating hormone on the ovary in vitro *Metabolism* **26**:413-68
- Charpin C, Kopp F, Pourreau-Schneider N, Lissitzky JC, Lavaut MN, Martin PM and Toga M** (1985) Laminin distribution in human decidua and immature placenta. An immunoelectron microscopic study (avidin-biotin-peroxidase complex method) *American Journal of Obstetrics and Gynecology* **151**:822-6
- Christenson LK and Stouffer RL** (1996) Proliferation of microvascular endothelial cells in the primate corpus luteum during the menstrual cycle and simulated early pregnancy *Endocrinology* **137**:367-74
- Clark DE, Smith SK, Sharkey AM and Charnockjones DS** (1996) Localization of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy *Human Reproduction* **11**:1090-1098

- Clark DE, Smith SK, Licence D, Evans AL and Charnock-Jones DW** (1998) Comparison of expression patterns for placenta growth factor, vascular endothelial growth factor (VEGF), VEGF-B and VEGF-C in human placenta throughout gestation *Journal of Endocrinology* **159**:459-467
- Clarke CL and Sutherland RL** (1990) Progesterin regulation of cellular proliferation *Endocrine Reviews* **11**:266-301
- Clarke IJ** (1996) The Hypothalamo-Pituitary Axis. In: Hillier SG, Kitchener HC, Neilson JP (eds) *Scientific Essentials of Reproductive Medicine*. W.B. Saunders Company, Ltd., London, 120-131
- Collins RL and Hodgen GD** (1986) Blockade of the spontaneous midcycle gonadotropin surge in monkeys by RU 486: a progesterone antagonist or agonist? *Journal of Clinical Endocrinology and Metabolism* **63**:1270-6
- Cooper JC, Sharkey AM, McLaren J, Charnock-jones DS and Smith SK** (1995) Localization of vascular endothelial growth factor and its receptor, flt, in human placenta and decidua by immunohistochemistry *Journal of Reproduction and Fertility* **105**:205-213
- Cordell JL, Falini B, Erber WN, Ghosh AK, Abdulaziz Z, MacDonald S, Pulford KA, Stein H and Mason DY** (1984) Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes) *Journal of Histochemical Cytochemistry* **32**:219-29
- Corner GWJ** (1956) The histological dating of the human corpus luteum of menstruation *American Journal of Anatomy* **98**:377-401
- Cornillie FJ, Lauweryns JM and Brosens IA** (1985) Normal human endometrium. An ultrastructural survey *Gynecologic and Obstetric Investigation* **20**:113-29
- Critchley HO, Brenner RM, Henderson TA, Williams K, Nayak NR, Slayden OD, Millar MR and Saunders PT** (2001) Estrogen receptor beta, but not estrogen receptor alpha, is present in the vascular endothelium of the human and nonhuman primate endometrium *Journal of Clinical Endocrinology and Metabolism* **86**:1370-8
- Critchley HO, Kelly RW, Brenner RM and Baird DT** (2001) The endocrinology of menstruation--a role for the immune system *Clinical Endocrinology (Oxf)* **55**:701-10
- Crocker DJ, Murad TM and Geer JC** (1970) Role of the pericyte in wound healing. An ultrastructural study *Experimental Molecular Pathology* **13**:51-65
- Crosignani PG** (1988) The defective luteal phase *Human Reproduction* **3**:157-60

- Cummings AM and Yochim JM** (1984) Differentiation of the uterus in preparation for gestation: a model for the action of progesterone *Journal of Theoretical Biology* **106**:353-74
- D'Amato RJ, Loughnan MS, Flynn E and Folkman J** (1994) Thalidomide is an inhibitor of angiogenesis *Proceedings of the National Academy of Sciences of the United States of America* **91**:4082-5
- D'Amore PA and Smith SR** (1993) Growth factor effects on cells of the vascular wall: a survey *Growth Factors* **8**:61-75
- Damsky C, Sutherland A and Fisher S** (1993) Extracellular matrix 5: adhesive interactions in early mammalian embryogenesis, implantation, and placentation *The FASEB Journal* **7**:1320-9
- Das SK, Chakraborty I, Wang J, Dey SK and Hoffman LH** (1997) Expression of vascular endothelial growth factor (VEGF) and VEGF- receptor messenger ribonucleic acids in the peri-implantation rabbit uterus *Biology of Reproduction* **56**:1390-9
- De Oliveira F** (1966) Pericytes in diabetic retinopathy *British Journal of Ophthalmology* **50**:134-43
- Del-Vecchio RP** (1997) The role of steroidogenic and nonsteroidogenic luteal cell interactions in regulating progesterone production. *Seminars in Reproductive Endocrinology* **15**:409-20
- Devoto L, Vega M, Kohen P, Castro A, Castro O, Christenson LK, Carvallo P and Strauss JF** (2000) Endocrine and paracrine-autocrine regulation of the human corpus luteum during the mid-luteal phase *Journal of Reproduction and Fertility (Suppl)* **55**:13-20
- Dhawan L, Ghosh D, Lalitkumar PG, Sharma DN, Lasley BL, Overstreet JW and Sengupta J** (2000) Anti-nidatory effect of vaginally administered (Ala8,13, 18)-magainin II amide in the rhesus monkey *Contraception* **62**:39-43
- Dickson SE and Fraser HM** (2000) Inhibition of early luteal angiogenesis by gonadotropin-releasing hormone antagonist treatment in the primate *Journal of Clinical Endocrinology and Metabolism* **85**:2339-44
- Dickson SE, Bicknell R and Fraser HM** (2001) Mid-luteal angiogenesis and function in the primate is dependent on vascular endothelial growth factor *Journal of Endocrinology* **168**:409-16

- Diekman MA, O'Callaghan PO, Nett TM and Niswender GD** (1978) Effect of prostaglandin F₂alpha on the number of LH receptors in ovine corpora lutea *Biology of Reproduction* **19**:1010-3
- Dorrington JH and Armstrong DT** (1979) Effects of FSH on gonadal functions *Recent Progress in Hormone Research* **35**:301-42
- Duffy DM, Hess DL and Stouffer RL** (1994) Acute administration of a 3 beta-hydroxysteroid dehydrogenase inhibitor to rhesus monkeys at the midluteal phase of the menstrual cycle: evidence for possible autocrine regulation of the primate corpus luteum by progesterone *Journal of Clinical Endocrinology and Metabolism* **79**:1587-94
- Duncan WC, Illingworth PJ and Fraser HM** (1996) Expression of tissue inhibitor of metalloproteinases-1 in the primate ovary during induced luteal regression *Journal of Endocrinology* **151**:203-13
- Duncan WC, McNeilly AS, Fraser HM and Illingworth PJ** (1996) Luteinizing hormone receptor in the human corpus luteum: Lack of down-regulation during maternal recognition of pregnancy *Human Reproduction* **11**:2291-2297
- Duncan WC, Illingworth PJ, Young FM and Fraser HM** (1998) Induced luteolysis in the primate: rapid loss of luteinizing hormone receptors *Human Reproduction* **13**:2532-40
- Duncan WC, McNeilly AS and Illingworth PJ** (1998) The effect of luteal 'rescue' on the expression and localization of matrix metalloproteinases and their tissue inhibitors in the human corpus luteum *Journal of Clinical Endocrinology and Metabolism* **83**:2470-2478
- Duncan WC, Rodger FE and Illingworth PJ** (1998) The human corpus luteum: reduction in macrophages during simulated maternal recognition of pregnancy *Human Reproduction* **13**:2435-2442
- Duncan WC, Cowen GM and Illingworth PJ** (1999) Steroidogenic enzyme expression in human corpora lutea in the presence and absence of exogenous human chorionic gonadotrophin (HCG) *Molecular Human Reproduction* **5**:291-8
- Dunk C, Shams M, Nijjar S, Rhaman M, Qiu Y, Bussolati B and Ahmed A** (2000) Angiopoietin-1 and angiopoietin-2 activate trophoblast Tie-2 to promote growth and migration during placental development *American Journal of Pathology* **156**:2185-99
- Dvorak HF, Brown LF, Detmar M and Dvorak AM** (1995) Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis *American Journal of Pathology* **146**:1029-1039

- Dvorak HF, Nagy JA, Feng D, Brown LF and Dvorak AM** (1999) Vascular Permeability Factor/Vascular Endothelial Growth Factor and the significance of Microvascular Hyperpermeability in Angiogenesis. In: Claesson-Welsh L (ed) *Current Topics in Microbiology and Immunology: Vascular Growth Factors and Angiogenesis*. Springer-Verlag Berlin Heidelberg, Germany, 97-132
- Dvorak HF** (2000) VPF/VEGF and the angiogenic response *Seminars in Perinatology* **24**:75-8
- Einspanier A, Jurdzinski A and Hodges JK** (1997) A local oxytocin system is part of the luteinization process in the preovulatory follicle of the marmoset monkey (*Callithrix jacchus*) *Biology of Reproduction* **57**:16-26
- Einspanier A, Nubbemeyer R, Schlote S, Schumacher M, Ivell R, Fuhrmann K and Marten A** (1999) Relaxin in the marmoset monkey: secretion pattern in the ovarian cycle and early pregnancy *Biology of Reproduction* **61**:512-20
- Ekstrom RC and Hunzicker-Dunn M** (1989) Homologous desensitization of ovarian luteinizing hormone/human chorionic gonadotropin-responsive adenylyl cyclase is dependent upon GTP *Endocrinology* **124**:956-63
- Eliceiri BP and Cheresh DA** (1999) The role of $\alpha_5\beta_1$ integrins during angiogenesis: insights into potential mechanisms of action and clinical development *Journal of Clinical Investigation* **103**:1227-1230
- Ellinwood WE, Norman RL and Spies HG** (1984) Changing frequency of pulsatile luteinizing hormone and progesterone secretion during the luteal phase of the menstrual cycle of rhesus monkeys *Biology of Reproduction* **31**:714-22
- Enders A** (1993) Overview of the Morphology of Implantation in Primates. In: Wolf D, Stouffer R, Brenner R (eds) *In vitro fertilisation and Embryo transfer in primates*. Springer-Verlag, New York, 145-157
- Enders AC and Schlafke S** (1969) Cytological aspects of trophoblast-uterine interaction in early implantation *American Journal of Anatomy* **125**:1-29
- Enders AC, Hendrickx AG and Schlafke S** (1983) Implantation in the rhesus monkey: initial penetration of endometrium *American Journal of Anatomy* **167**:275-98
- Enders AC** (1989) Trophoblast differentiation during the transition from trophoblastic plate to lacunar stage of implantation in the rhesus monkey and human *American Journal of Anatomy* **186**:85-98
- Enders AC** (1991) Current topic: structural responses of the primate endometrium to implantation *Placenta* **12**:309-25

- Enders AC and King BF** (1991) Early stages of trophoblastic invasion of the maternal vascular system during implantation in the macaque and baboon *American Journal of Anatomy* **192**:329-46
- Enders AC and Lopata A** (1999) Implantation in the marmoset monkey: expansion of the early implantation site *Anatomical Record* **256**:279-99
- Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M and Gustafsson JA** (1997) Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern *Journal of Clinical Endocrinology and Metabolism* **82**:4258-65
- Erickson GF, Magoffin DA, Dyer CA and Hofeditz C** (1985) The ovarian androgen producing cells: a review of structure/function relationships *Endocrine Reviews* **6**:371-99
- Esch F, Baird A, Ling N, Ueno N, Hill F, Denoroy L, Klepper R, Gospodarowicz D, Bohlen P and Guillemin R** (1985) Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF *Proceedings of the National Academy of Sciences of the United States of America* **82**:6507-11
- Esch F, Ueno N, Baird A, Hill F, Denoroy L, Ling N, Gospodarowicz D and Guillemin R** (1985) Primary structure of bovine brain acidic fibroblast growth factor (FGF) *Biochemical and Biophysical Research Communications* **133**:554-62
- Evans G, Dobias M, King GJ and Armstrong DT** (1983) Production of prostaglandins by porcine preovulatory follicular tissues and their roles in intrafollicular function *Biology of Reproduction* **28**:322-8
- Farin CE, Sawyer HR and Niswender GD** (1989) Analysis of cell types in the corpus luteum of the sheep *Journal of Reproduction and Fertility (Suppl)* **37**:181-7
- Fazleabas AT, Verhage HG, Waites G and Bell SC** (1989) Characterization of an insulin-like growth factor binding protein, analogous to human pregnancy-associated secreted endometrial alpha 1-globulin, in decidua of the baboon (*Papio anubis*) placenta *Biology of Reproduction* **40**:873-85
- Fazleabas AT, Donnelly KM, Mavrogianis PA and Verhage HG** (1993) Secretory and morphological changes in the baboon (*Papio anubis*) uterus and placenta during early pregnancy *Biology of Reproduction* **49**:695-704
- Fazleabas AT, Hild-Petito S and Donnelly KM** (1993) Overview of the Morphology of Implantation in Primates. In: Wolf D, Stouffer R, Brenner R

- (eds) *In vitro* fertilisation and Embryo transfer in primates. Springer-Verlag, New York, 169-181
- Fazleabas AT, Hild-Petito S and Verhage HG** (1995) The primate endometrium: morphological and secretory changes during early pregnancy - implications of the insulin-like growth factor axis *Seminars in Reproductive Endocrinology* **13**:120-132
- Fehrenbach A, Hodges JK and Einspanier A** (1999) Direct effects of the prostaglandins E2 and F2alpha on progesterone release by the corpus luteum of the marmoset monkey (*Callithrix jacchus*) studied by *in vitro* microdialysis *Journal of Endocrinology* **161**:433-43
- Ferenczy A, Bertrand G and Gelfand MM** (1979) Proliferation kinetics of human endometrium during the normal menstrual cycle *American Journal of Obstetrics and Gynecology* **133**:859-67
- Ferrara N and Davis-Smyth T** (1997) The biology of vascular endothelial growth factor *Endocrine Reviews* **18**:4-25
- Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, Chisholm V, Hillan KJ and Schwall RH** (1998) Vascular endothelial growth factor is essential for corpus luteum angiogenesis *Nature Medicine* **4**:336-40
- Ferrell CL** (1989) placental regulation of foetal growth. In: Champion DR, Hausman GJ, Martin RJ (eds) *Animal Growth Regulation*. Plenum, New York, 1-19
- Fields PA** (1991) Relaxin and other luteal secretory peptides: Cell localisation localisation and function in the ovary. In: Fammiliari G, Makabe S, Motta PM (eds) *Ultrastructure of the ovary*. Kluwer Academic Publishers, Boston, 177-198
- Filicori M, Butler JP and Crowley WF, Jr.** (1984) Neuroendocrine regulation of the corpus luteum in the human. Evidence for pulsatile progesterone secretion *Journal of Clinical Investigation* **73**:1638-47
- Findlay JK** (1986) Angiogenesis in reproductive tissues *Journal of Endocrinology* **111**:357-366
- Fink G** (1979) Neuroendocrine control of gonadotrophin secretion *British Medical Bulletin* **35**:155-60
- Finn CA** (1986) Implantation, menstruation and inflammation *Biological Reviews of the Cambridge Philosophical Society* **61**:313-28
- Fitz TA, Mayan MH, Sawyer HR and Niswender GD** (1982) Characterization of two steroidogenic cell types in the ovine corpus luteum *Biology of Reproduction* **27**:703-711

- Folkman J, Merler E, Abernathy C and Williams G** (1971) Isolation of a tumor factor responsible for angiogenesis *Journal of Experimental Medicine* **133**:275-88
- Folkman J, Haudenschild CC and Zetter BR** (1979) Long-term culture of capillary endothelial cells *Proceedings of the National Academy of Sciences of the United States of America* **76**:5217-21
- Folkman J and Klagsbrun M** (1987) Angiogenic factors *Science* **235**:442-7
- Folkman J** (1992) Angiogenesis in female reproductive organs. In: Alexander NJ, D'Arcangues C (eds) *Steroid Hormones and Uterine Bleeding*. AAAS Press, Washington DC, 143-158
- Folkman J** (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease *Nature Medicine* **1**:27-31
- Folkman J** (1998) Editorial: Is tissue mass regulated by vascular endothelial cells? Prostate as the first evidence *Endocrinology* **139**:441-442
- Ford SP, Magness RR, Farley DB and Van Orden DE** (1982) Local and systemic effects of intrauterine estradiol-17 beta on luteal function of nonpregnant sows *Journal of Animal Science* **55**:657-64
- Ford SP, Reynolds LP and Magness RR** (1982) Blood flow to the uterine and ovarian vascular beds of gilts during the estrous cycle or early pregnancy *Biology of Reproduction* **27**:878-85
- Fowkes RC, Chandras C, Chin EC, Okolo S, Abayasekara DR and Michael AE** (2001) Relationship between the production of prostaglandins and progesterone by luteinizing human granulosa cells *Journal of Endocrinology* **171**:455-62
- Fraser HM, Abbott M, Laird NC, McNeilly AS, Nestor JJ and Vickery BH** (1986) Effects of an LH-releasing hormone antagonist on the secretion of LH, FSH, prolactin and ovarian steroids at different stages of the luteal phase in the stumptailed macaque (*Macaca arctoides*) *Journal of Endocrinology* **111**:83-90
- Fraser HM, Recio R, Conn PM and Lunn SF** (1994) Gonadotropin-releasing hormone antagonist for postpartum contraception: outcome for the mother and male offspring in the marmoset *Journal of Clinical Endocrinology and Metabolism* **78**:121-125
- Fraser HM, Lunn SF, Cowen GM and Illingworth PJ** (1995) Induced luteal regression in the primate: evidence for apoptosis and changes in c-myc protein *Journal of Endocrinology* **147**:131-7

- Fraser HM, Lunn SF, Kim H and Erickson GF** (1998) Insulin-like growth factor binding protein-3 mRNA expression in endothelial cells of the primate corpus luteum *Human Reproduction* **13**:2180-5
- Fraser HM, Dickson SE, Morris KD, Erickson GF and Lunn SF** (1999) The effect of the angiogenesis inhibitor TNP-470 on luteal establishment and function in the primate *Human Reproduction* **14**:2054-60
- Fraser HM, Lunn SF, Harrison DJ and Kerr JB** (1999) Luteal regression in the primate: different forms of cell death during natural and gonadotropin-releasing hormone antagonist or prostaglandin analogue-induced luteolysis *Biology of Reproduction* **61**:1468-79
- Fraser HM, Dickson SE, Lunn SF, Wulff C, Morris KD, Carroll VA and Bicknell R** (2000) Suppression of luteal angiogenesis in the primate after neutralization of vascular endothelial growth factor *Endocrinology* **141**:995-1000
- Fraser HM, Lunn SF, Kim H, Duncan WC, Rodger FE, Illingworth PJ and Erickson GF** (2000) Changes in insulin-like growth factor-binding protein-3 messenger ribonucleic acid in endothelial cells of the human corpus luteum: a possible role in luteal development and rescue *Journal of Clinical Endocrinology and Metabolism* **85**:1672-7
- Fraser HM and Lunn SF** (2001) Regulation and manipulation of angiogenesis in the primate corpus luteum *Reproduction* **121**:355-62
- Friden BE, Runesson E, Hahlin M and Brannstrom M** (2000) Evidence for nitric oxide acting as a luteolytic factor in the human corpus luteum. *Molecular Human Reproduction* **6**:397-403
- Fukuda M, Fukuda K, Andersen CY and Byskov AG** (1996) Contralateral selection of dominant follicle favours pre-embryo development *Human Reproduction* **11**:1958-62
- Fukuda M, Fukuda K, Andersen CY and Byskov AG** (1997) Does corpus luteum locally affect follicular growth negatively? *Human Reproduction* **12(5)**:1024-1027
- Fuller GB and Hansel W** (1970) Regression of sheep corpora lutea after treatment with antibovine luteinizing hormone *Journal of Animal Science* **31**:99-103
- Funkenstein B, Waterman MR and Simpson ER** (1984) Induction of synthesis of cholesterol side chain cleavage cytochrome P-450 and adrenodoxin by follicle-stimulating hormone, 8-bromo-cyclic AMP, and low density lipoprotein in cultured bovine granulosa cells *Journal of Biological Chemistry* **259**:8572-7

- Gaede SD, Sholley MM and Quattropani SL** (1985) Endothelial mitosis during the initial stages of corpus luteum neovascularization in the cycling adult rat *American Journal of Anatomy* **172**:173-80
- Garcia E, Bouchard P, De Brux J, Berdah J, Frydman R, Schaison G, Milgrom E and Perrot-Appianat M** (1988) Use of immunocytochemistry of progesterone and estrogen receptors for endometrial dating *Journal of Clinical Endocrinology and Metabolism* **67**:80-7
- Gargett CE, Lederman FL, Lau TM, Taylor NH and Rogers PA** (1999) Lack of correlation between vascular endothelial growth factor production and endothelial cell proliferation in the human endometrium *Human Reproduction* **14**:2080-8
- Gargett CE and Rogers PA** (2001) Human endometrial angiogenesis *Reproduction* **121**:181-6
- Gaytán F, Morales C, García-Pardo L, Reymundo C, Bellido C and Sánchez-Criado JE** (1998) Macrophages, cell proliferation, and cell death in the human menstrual corpus luteum *Biology of Reproduction* **59**:417-425
- Gaytán F, Morales C, García-Pardo L, Reymundo C, Bellido C and Sánchez-Criado JE** (1999) A quantitative study of changes in the human corpus luteum microvasculature during the menstrual cycle *Biology of Reproduction* **60**:914-919
- Gemmell RT, Stacy BD and Thorburn GD** (1976) Morphology of the regressing corpus luteum in the ewe *Biology of Reproduction* **14**:270-279
- Gemzell C** (1965) Induction of ovulation with human gonadotrophins *Recent progress in hormone research* **21**:179-204
- Gerber HP, Condorelli F, Park J and Ferrara N** (1997) Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia *Journal of Biological Chemistry* **272**:23659-67
- Geva E, Ginzinger DG, Zaloudek CJ, Moore DH, Byrne A and Jaffe RB** (2002) Human placental vascular development: vasculogenic and angiogenic (branching and nonbranching) transformation is regulated by vascular endothelial growth factor-A, angiopoietin-1, and angiopoietin-2 *Journal of Clinical Endocrinology and Metabolism* **87**:4213-24
- Ghosh D, Sharkey AM, Charnock-Jones DS, Dhawan L, Dhara S, Smith SK and Sengupta J** (2000) Expression of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in conceptus and endometrium

- during implantation in the rhesus monkey *Molecular Human Reproduction* **6**:935-41
- Gimbrone MAJ, Cotran RS, Leapman SB and Folkman J** (1974) Tumor growth and neovascularization: an experimental model using the rabbit cornea *Journal of the National Cancer Institute* **52**:413-27
- Girsh E, Greber Y and Meidan R** (1995) Luteotrophic and luteolytic interactions between bovine small and large luteal-like cells and endothelial cells *Biology of Reproduction* **52**:954-62
- Girsh E, Milvae RA, Wang W and Meidan R** (1996) Effect of endothelin-1 on bovine luteal cell function: role in prostaglandin F₂alpha-induced antisteroidogenic action *Endocrinology* **137**:1306-12
- Glaser BM, D'Amore PA, Seppa H, Seppa S and Schiffmann E** (1980) Adult tissues contain chemoattractants for vascular endothelial cells *Nature* **288**:483-4
- Goede V, Schmidt T, Kimmina S, Koziar D and Augustin HG** (1998) Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis *Laboratory Investigation* **78**:1385-94
- Goldberg MA and Schneider TJ** (1994) Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin *Journal of Biological Chemistry* **269**:4355-9
- Goldman-Wohl DS, Ariel I, Greenfield C, Lavy Y and Yagel S** (2000) Tie-2 and angiopoietin-2 expression at the fetal-maternal interface: a receptor ligand model for vascular remodelling *Molecular Human Reproduction* **6**:81-7
- Golos TG, Strauss JF, 3rd and Miller WL** (1987) Regulation of low density lipoprotein receptor and cytochrome P-450scc mRNA levels in human granulosa cells *Journal of Steroid Biochemistry* **27**:767-73
- Goodger AM and Rogers PA** (1994) Endometrial endothelial cell proliferation during the menstrual cycle *Human Reproduction* **9**:399-405
- Goodman AL and Hodgen GD** (1979) Corpus luteum--conceptus--follicle relationships during the fertile cycle in rhesus monkeys: pregnancy maintenance despite early luteal removal *Journal of Clinical Endocrinology and Metabolism* **49**:469-71
- Gordon JD, Shifren JL, Foulk RA, Taylor RN and Jaffe RB** (1995) Angiogenesis in the human female reproductive tract *Obstet Gynecol Surv* **50**:688-97
- Gospodarowicz D and Thakral KK** (1978) Production of a corpus luteum angiogenic factor responsible for proliferation of capillaries and

- neovascularization of the corpus luteum *Proceedings of the National Academy of Sciences of the United States of America* **75**:847-51
- Gougeon A** (1982) Rate of follicular growth in the human ovary. In: Rolland R VH, E.V., Hillier, S.G., McNatty, K.P., & Schoemaker, J (ed) Follicular maturation and Ovulation. Excerpta Medica, Amsterdam, 155-63
- Graham CE** (1973) Chimpanzee endometrium and sexual swelling during menstrual cycle or hormone administration *Folia Primatologica (Basel)* **19**:458-68
- Graham RC, Jr. and Karnovsky MJ** (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique *Journal of Histochemical Cytochemistry* **14**:291-302
- Grazul-Bilska AT, Redmer DA, Zheng J, Killilea SD and Reynolds LP** (1995) Initial characterization of mitogenic activity of ovine corpora lutea from early pregnancy *Growth Factors* **12**:131-144
- Grazul-Bilska AT, Redmer DA, Johnson ML, Jablonka-Shariff A, Bilski JJ and Reynolds LP** (1996) Gap junctional protein connexin 43 in bovine corpora lutea throughout the estrous cycle *Biology of Reproduction* **54**:1279-87
- Grazul-Bilska AT, Reynolds LP, Kirsch JD and Redmer DA** (1996) Gap junctional intercellular communication of bovine luteal cells from several stages of the estrous cycle: effects of cyclic adenosine 3',5'-monophosphate *Biology of Reproduction* **54**:538-45
- Grazul-Bilska AT, Redmer DA and Reynolds LP** (1997) Cellular interactions in the corpus luteum *Seminars in Reproductive Endocrinology* **15**:383-93
- Grazul-Bilska AT, Reynolds LP and Redmer DA** (1997) Gap junctions in the ovaries *Biology of Reproduction* **57**:947-57
- Grazul-Bilska AT, Redmer, D.A. & Reynolds, L.P.** (1996) Effects of luteinising hormone and prostaglandin F₂-alpha on gap junctional intercellular communication of ovine luteal cells throughout the estrous cycle. *Endocrine* **5**:225-233
- Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P and Chambon P** (1986) Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A *Nature* **320**:134-9
- Greenwald GD and Terranova PR** (1988) Follicular selection and its control. In: Knobil E, Neill J (eds) *The Physiology of Reproduction*. Raven, New York, 387-445

- Greenwald GS** (1989) Temporal and topographic changes in DNA synthesis after induced follicular atresia *Biology of Reproduction* **41**:175-81
- Groome NP, O'Brien M, Illingworth P, Pai R, Mather J, Priddle J and McNeilly AS** Inhibin B is a major circulating form of inhibin in men and women. Abstracts of the 77th Annual Meeting of the Endocrine Society, Washington DC, 1995, p 103
- Halder JB, Zhao X, Soker S, Paria BC, Klagsbrun M, Das SK and Dey SK** (2000) Differential expression of VEGF isoforms and VEGF(164)-specific receptor neuropilin-1 in the mouse uterus suggests a role for VEGF(164) in vascular permeability and angiogenesis during implantation *Genesis* **26**:213-24
- Hamberger L, Hahlin M, Bennegard B and Sjoblom P** (1991) Human luteal function during implantation and early pregnancy *Annals of the New York Academy of Sciences* **626**:189-200
- Hanahan D** (1997) Signaling vascular morphogenesis and maintenance *Science* **277**:48-50
- Hansel W, Alila HW, Dowd JP and Milvae RA** (1991) Differential origin and control mechanisms in small and large bovine luteal cells *Journal of Reproduction and Fertility (suppl)* **43**:77-89
- Hansel W, Dowd, J.P.** (1986) New concepts of the control of corpus luteum function *Journal of Reproduction and Fertility* **78**:755-768
- Harding R, Hulme, MJ, Lunn, SF, Henderson, S & Aitken, RJ** (1982) Plasma Progesterone levels throughout the ovarian cycle of the common marmoset (*Callithrix jacchus*) *Journal of Medical Primatology* **11**:43-51
- Harlow CR, Hearn JP and Hodges JK** (1984) Ovulation in the marmoset monkey: endocrinology, prediction and detection *Journal of Endocrinology* **103**:17-24
- Harlow CR, Shaw HJ, Hillier SG and Hodges JK** (1988) Factors influencing follicle-stimulating hormone-responsive steroidogenesis in marmoset granulosa cells: effects of androgens and the stage of follicular maturity *Endocrinology* **122**:2780-7
- Hastings JM, Licence DR, Burton GJ, Charnock-Jones DS and Smith SK** (2003) Soluble vascular endothelial growth factor receptor 1 inhibits edema and epithelial proliferation induced by 17 beta estradiol in the mouse uterus *Endocrinology* **144**:316-344
- Hazzard TM, Molskness TA, Chaffin CL and Stouffer RL** (1999) Vascular endothelial growth factor (VEGF) and angiopoietin regulation by

- gonadotrophin and steroids in macaque granulosa cells during the peri-ovulatory interval *Molecular Human Reproduction* **5**:1115-21
- Hazzard TM, Christenson LK and Stouffer RL** (2000) Changes in expression of vascular endothelial growth factor and angiopoietin-1 and -2 in the macaque corpus luteum during the menstrual cycle *Molecular Human Reproduction* **6**:993-8
- Hearn JP, Abbott DH, Chambers PC, Hodges JK and Lunn SF** (1978) Use of the common marmoset, *Callithrix jacchus*, in reproductive research *Primate Medicine* **10**:40-9
- Hearn JP, Webley GE and Gidley-Baird AA** (1991) Chorionic gonadotrophin and embryo-maternal recognition during the peri-implantation period in primates *Journal of Reproduction and Fertility* **92**:497-509
- Helske S, Vuorela P, Carpen O, Hornig C, Weich H and Halmesmaki E** (2001) Expression of vascular endothelial growth factor receptors 1, 2 and 3 in placentas from normal and complicated pregnancies *Molecular Human Reproduction* **7**:205-10
- Henderson TA, Saunders PT, Moffett-King A, Groome NP and Critchley HO** (2003) Steroid receptor expression in uterine natural killer cells *Journal of Clinical Endocrinology and Metabolism* **88**:440-9
- Hertig AT, Rock J and Adams J** (1956) A description of 34 human ova within the first 17 days of development *American Journal of Anatomy* **98**:485-494
- Heryanto B and Rogers PA** (2002) Regulation of endometrial endothelial cell proliferation by oestrogen and progesterone in the ovariectomized mouse *Reproduction* **123**:107-13
- Heryanto B, Lipson KE and Rogers PAW** (2003) Effect of angiogenesis inhibitors on oestrogen-mediated endometrial endothelial cell proliferation in the ovariectomised mouse *Reproduction* **125**:337-346
- Hild-Petito S, Verhage HG and Fazleabas AT** (1992) Immunocytochemical localization of estrogen and progestin receptors in the baboon (*Papio anubis*) uterus during implantation and pregnancy *Endocrinology* **130**:2343-53
- Hillier SG, Zeleznik AJ and Ross GT** (1978) Independence of steroidogenic capacity and luteinizing hormone receptor induction in developing granulosa cells *Endocrinology* **102**:937-46
- Hillier SG, Reichert LE, Jr. and Van Hall EV** (1981) Control of preovulatory follicular estrogen biosynthesis in the human ovary *Journal of Clinical Endocrinology and Metabolism* **52**:847-56

- Hillier SG** (1985) Sex steroid metabolism and follicular development in the ovary *Oxford reviews of reproductive biology* **7**:168-222
- Hillier SG** (1991) Cellular Basis of Follicular Endocrine Function. In: Hillier SG (ed) Ovarian Endocrinology. Blackwell Scientific, Oxford, 73-106
- Hillier SG, Tetsuka M and Fraser HM** (1997) Location and developmental regulation of androgen receptor in primate ovary *Human Reproduction* **12**:107-11
- Hillier SGW** (1985) Cellular aspects of corpus luteum function. In: Jeffcoate SL (ed) The Luteal Phase. Wiley, London, 1-23
- Hirschi KK and D'Amore PA** (1996) Pericytes in the microvasculature *Cardiovascular Research* **32**:687-98
- Hirschi KK, Rohovsky SA and D'Amore PA** (1998) PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate *Journal of Cell Biology* **141**:805-14
- Hirshfield AN** (1991) Development of follicles in the mammalian ovary *International Reviews of Cytology* **124**:43-101
- Ho PC** (2001) Mifepristone: a potential postcoital contraceptive *Expert Opinion in Pharmacotherapy* **2**:1383-8
- Hodges JK, Henderson C and Hearn JP** (1983) Relationship between ovarian and placental steroid production during early pregnancy in the marmoset monkey (*Callithrix jacchus*) *Journal of Reproduction and Fertility* **69**:613-21
- Holmgren L, Glaser A, Pfeifer-Ohlsson S and Ohlsson R** (1991) Angiogenesis during human extraembryonic development involves the spatiotemporal control of PDGF ligand and receptor gene expression *Development* **113**:749-54
- Holt JA** (1989) Regulation of progesterone production in the rabbit corpus luteum *Biology of Reproduction* **40**:201-8
- Hopwood D and Levison DA** (1976) Atrophy and apoptosis in the cyclical human endometrium *Journal of Pathology* **119**:159-66
- Hossain MI and O'Shea JD** (1981) Vascular changes during regression of the corpus luteum of the guinea pig *Australian Journal of Biological Sciences* **34**:649-60

- Hsueh AJ, Adashi EY, Jones PB and Welsh TH, Jr.** (1984) Hormonal regulation of the differentiation of cultured ovarian granulosa cells *Endocrine Reviews* **5**:76-127
- Hsueh AJ, Bicsak TA, Jia XC, Dahl KD, Fauser BC, Galway AB, Czekala N, Pavlou SN, Papkoff H, Keene J and et al.** (1989) Granulosa cells as hormone targets: the role of biologically active follicle-stimulating hormone in reproduction *Recent progress in hormone research* **45**:209-73; discussion 273-7
- Hunt RA, Ciuffo GM, Saavedra JM and Tucker DC** (1995) Quantification and localisation of angiotensin II receptors and angiotensin converting enzyme in the developing rat heart *Cardiovascular Research* **29**:834-40
- Hutchison JS and Zeleznik AJ** (1984) The rhesus monkey corpus luteum is dependent on pituitary gonadotropin secretion throughout the luteal phase of the menstrual cycle *Endocrinology* **115**:1780-6
- Hutchison JS, Nelson PB and Zeleznik AJ** (1986) Effects of different gonadotropin pulse frequencies on corpus luteum function during the menstrual cycle of rhesus monkeys *Endocrinology* **119**:1964-71
- Hutchison JS, Kubik CJ, Nelson PB and Zeleznik AJ** (1987) Estrogen induces premature luteal regression in rhesus monkeys during spontaneous menstrual cycles, but not in cycles driven by exogenous gonadotropin-releasing hormone *Endocrinology* **121**:466-74
- Hyder SM and Stancel GM** (1999) Regulation of angiogenic growth factors in the female reproductive tract by estrogens and progestins *Molecular Endocrinology* **13**:806-11
- Hyder SM and Stancel GM** (2000) Regulation of VEGF in the reproductive tract by sex-steroid hormones *Histology and Histopathology* **15**:325-34
- Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites HG and Roberts RM** (1987) Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm *Nature* **330**:377-9
- Ingber D and Folkman J** (1989) Mechanochemical switching between growth and differentiation during fibroblastic growth factor-stimulated angiogenesis in vitro: Role of extracellular matrix *Journal of Cell Biology* **109**:317-330
- Iruela-Arispe ML, Rodriguez-Manzaneque JC and Abu-Jawdeh G** (1999) Endometrial endothelial cells express estrogen and progesterone receptors and exhibit a tissue specific response to angiogenic growth factors *Microcirculation* **6**:127-40

- Irwin JC, Kirk D, King RJ, Quigley MM and Gwatkin RB** (1989) Hormonal regulation of human endometrial stromal cells in culture: an in vitro model for decidualization *Fertility and Sterility* **52**:761-8
- Irwin JC, Utian WH and Eckert RL** (1991) Sex steroids and growth factors differentially regulate the growth and differentiation of cultured human endometrial stromal cells *Endocrinology* **129**:2385-92
- Jablonka-Shariff A, Grazul-Bilska AT, Redmer DA and Reynolds LP** (1993) Growth and cellular proliferation of ovine corpora lutea throughout the estrous cycle *Endocrinology* **133**:1871-9
- Jablonka-Shariff A, Grazul-Bilska AT, Redmer DA and Reynolds LP** (1997) Cellular proliferation and fibroblast growth factors in the corpus luteum during early pregnancy in ewes. *Growth Factors* **14**:15-23
- Johnson MH and Everitt BJ** (1995) Ovarian Function. In: Johnson MH, Everitt BJ (eds) *Essential Reproduction*. Blackwell Science, Oxford, 60-78
- Johnson MH and Everitt BJ** (1995) Actions of Steroid Hormones in the Adult. In: Johnson MH, Everitt BJ (eds) *Essential Reproduction*. Blackwell Science, Oxford, 122-142
- Juengel JL, Meberg BM, Turzillo AM, Nett TM and Niswender GD** (1995) Hormonal regulation of messenger ribonucleic acid encoding steroidogenic acute regulatory protein in ovine corpora lutea *Endocrinology* **136**:5423-9
- Kanayama K, Sankai T, Nariai K and Endo T** (1996) Blockade of ovulation in rabbits by RU-486, a competitive progesterone antagonist *Journal of Veterinary Medical Science* **58**:275-6
- Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J and Connolly DT** (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF *Science* **246**:1309-12
- Kelly RW** (1994) Pregnancy maintenance and parturition: the role of prostaglandin in manipulating the immune and inflammatory response *Endocrine Reviews* **15**:684-706
- Kelly RW, King AE and Critchley HO** (2001) Cytokine control in human endometrium *Reproduction* **121**:3-19
- Khan-Dawood FS, Yang J and Dawood MY** (1997) Immunohistological localization and expression of alpha-actin in the baboon (*Papio anubis*) corpus luteum. *Journal of Histochemistry and Cytochemistry* **45**:71-7
- Khan-Dawood FS** (1997) Oxytocin in intercellular communication in the corpus luteum *Seminars in Reproductive Endocrinology* **15**:395-407

- King A, Wellings V, Gardner L and Loke YW** (1989) Immunocytochemical characterization of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle *Human Immunology* **24**:195-205
- King A and Loke YW** (1990) Uterine large granular lymphocytes: a possible role in embryonic implantation? *American Journal of Obstetrics and Gynecology* **162**:308-10
- King A, Burrows T and Loke YW** (1996) Human uterine natural killer cells *Nature Immunology* **15**:41-52
- King A, Burrows T, Verma S, Hiby S and Loke YW** (1998) Human uterine lymphocytes *Human Reproduction Update* **4**:480-5
- King A** (2000) Uterine leukocytes and decidualization *Human Reproduction Update* **6**:28-36
- Klagsbrun M and D'Amore PA** (1991) Regulators of angiogenesis *Annual Review of Physiology* **53**:217-39
- Klauber N, Rohan RM, Flynn E and D'Amato RJ** (1997) Critical components of the female reproductive pathway are suppressed by the angiogenesis inhibitor AGM-1470 *Nature Medicine* **3**:443-6
- Knobil E, Plant TM, Wildt L, Belchetz PE and Marshall G** (1980) Control of the rhesus monkey menstrual cycle: permissive role of hypothalamic gonadotropin-releasing hormone *Science* **207**:1371-3
- Korda AR, Shutt DA, Smith ID, Shearman RP and Lyneham RC** (1975) Assessment of possible luteolytic effect on intra-ovarian injection of prostaglandin F-2ALPHA in the human *Prostaglandins* **9**:443-9
- Kraehenbuhl JP and Jamieson JD** (1974) Localisation of intracellular antigens by immunoelectron microscopy *International Review of Experimental Pathology* **12**:1-53
- Kraemer DC, Maqueo M, Hendrickx AG and Vera Cruz NC** (1977) Histology of the baboon endometrium during the menstrual cycle and pregnancy *Fertility and Sterility* **28**:482-7
- Krussel J, Behr B, Hirchenhain J, Wen Y, Milki AA, Cupisti S, Bielfeld P and Polan ML** (2000) Expression of vascular endothelial growth factor mRNA in human preimplantation embryos derived from tripronuclear zygotes *Fertility and Sterility* **74**:1220-6

- Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S and Gustafsson JA** (1996) Cloning of a novel receptor expressed in rat prostate and ovary *Proceedings of the National Academy of Sciences of the United States of America* **93**:5925-30
- Lawn AM, Wilson EW and Finn CA** (1971) The ultrastructure of human decidual and predecidual cells *Journal of Reproduction and Fertility* **26**:85-90
- Lee OH, Bae SK, Bae MH, Lee YM, Moon EJ, Cha HJ, Kwon YG and Kim KW** (2000) Identification of angiogenic properties of insulin-like growth factor II in in vitro angiogenesis models *British Journal of Cancer* **82**:385-91
- Lei ZM, Chegini, N. & Rao, Ch.V** (1991) Quantitative cell composition of human and bovine corpora lutea from various reproductive states *Biology of Reproduction* **44**:1148-1156
- Lenton EA and Woodward AJ** (1988) The endocrinology of conception cycles and implantation in women *Journal of Reproduction and Fertility (suppl)* **36**:1-15
- Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL and McCarty KS, Jr.** (1988) Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle *Journal of Clinical Endocrinology and Metabolism* **67**:334-40
- Leung DW, Cachianes G, Kuang WJ, Goeddel DV and Ferrara N** (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* **246**:1306-9
- Leyendecker G, Wildt L and Hansmann M** (1980) Pregnancies following chronic intermittent (pulsatile) administration of Gn-RH by means of a portable pump ("Zyklomat")--a new approach to the treatment of infertility in hypothalamic amenorrhea *Journal of Clinical Endocrinology and Metabolism* **51**:1214-6
- Li XF, Charnock-Jones DS, Zhang E, Hiby S, Malik S, Day K, Licence D, Bowen JM, Gardner L, King A, Loke YW and Smith SK** (2001) Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells *Journal of Clinical Endocrinology and Metabolism* **86**:1823-34
- Liu CC and Young JD** (2001) Uterine natural killer cells in the pregnant uterus *Advances in Immunology* **79**:297-329
- Liu JH and Yen SS** (1983) Induction of midcycle gonadotropin surge by ovarian steroids in women: a critical evaluation *Journal of Clinical Endocrinology and Metabolism* **57**:797-802
- Liu JH, Garzo G, Morris S, Stuenkel C, Ulmann A and Yen SS** (1987) Disruption of follicular maturation and delay of ovulation after administration

- of the antiprogestone RU486 *Journal of Clinical Endocrinology and Metabolism* **65**:1135-40
- Lobov IB, Brooks PC and Lang RA** (2002) Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo *Proceedings of the National Academy of Sciences of the United States of America* **99**:11205-10
- Ludwig H and Metzger H** (1976) The re-epithelization of endometrium after menstrual desquamation *Arch Gynakol* **221**:51-60
- Ma W, Tan J, Matsumoto H, Robert B, Abrahamson DR, Das SK and Dey SK** (2001) Adult tissue angiogenesis: evidence for negative regulation by estrogen in the uterus *Molecular Endocrinology* **15**:1983-92
- MacLennan AH and Wynn RM** (1971) Menstrual cycle of the baboon. I. Clinical features, vaginal cytology and endometrial histology *Obstetrics and Gynecology* **38**:350-8
- Macpherson AM and Rogers PAW** (1993) Uterine endothelial cell proliferation before and after embryo implantation in rats *Journal of Reproduction and Fertility* **99**:451-457
- Maentausta O, Svalander P, Danielsson KG, Bygdeman M and Vihko R** (1993) The effects of an antiprogestin, mifepristone, and an antiestrogen, tamoxifen, on endometrial 17 beta-hydroxysteroid dehydrogenase and progestin and estrogen receptors during the luteal phase of the menstrual cycle: an immunohistochemical study *Journal of Clinical Endocrinology and Metabolism* **77**:913-8
- Magoffin DA** (1989) Evidence that luteinizing hormone-stimulated differentiation of purified ovarian thecal-interstitial cells is mediated by both type 1 and Type 2 adenosine 3',5' monophosphate dependent protein kinases *Endocrinology* **125**:1464-73
- Mais V, Kazer RR, Cetel NS, Rivier J, Vale W and Yen SS** (1986) The dependency of folliculogenesis and corpus luteum function on pulsatile gonadotropin secretion in cycling women using a gonadotropin-releasing hormone antagonist as a probe *Journal of Clinical Endocrinology and Metabolism* **62**:1250-5
- Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN and Yancopoulos GD** (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis *Science* **277**:55-60
- Malven PV and Sawyer CH** (1966) A luteolytic action of prolactin in hypophysectomized rats *Endocrinology* **79**:268-74

- Mamluk R, Chen D, Greber Y, Davis JS and Meidan R** (1998) Characterization of messenger ribonucleic acid expression for prostaglandin F2 alpha and luteinizing hormone receptors in various bovine luteal cell types *Biology of Reproduction* **58**:849-56
- Mann JS, Kindy MS, Edwards DR and Curry TE, Jr.** (1991) Hormonal regulation of matrix metalloproteinase inhibitors in rat granulosa cells and ovaries *Endocrinology* **128**:1825-32
- Markee JE** (1940) Menstruation in intraocular transplants in the rhesus monkey *contributions to Embryology of the Carnegie Institution* **177**:211-308
- Marsh JM** (1976) The role of cyclic AMP in gonadal steroidogenesis *Biology of Reproduction* **14**:30-53
- Martin L and Finn CA** (1968) Hormonal regulation of cell division in epithelial and connective tissues of the mouse uterus *Journal of Endocrinology* **41**:363-371
- McCormack JT, Friederichs MG, Limback SD and Greenwald GS** (1998) Apoptosis during spontaneous luteolysis in the cyclic golden hamster: biochemical and morphological evidence *Biology of Reproduction* **58**:255-60
- McGadey J** (1970) A tetrazolium method for non-specific alkaline phosphatase *Histochemie* **23**:180-4
- McNatty KP** (1981) Hormonal correlates of follicular development in the human ovary *Australian Journal of Biological Science* **34**:249-68
- McNatty KP** (1982) Ovarian follicular development from the onset of luteal regression in human and sheep. In: Rolland R, Van Hall EV, Hillier SG, McNatty KP, Schoemaker J (eds) *Follicular maturation and ovulation*. Excerpta Medica, Amsterdam, 1-18
- McNatty KP, Smith P, N.L. H, Heath DA, Lun S and O WS** (1992) Follicular Development and steroidogenesis. In: Sjoberg NO, Hamberger L (eds) *Local Regulation of Ovarian Function*. The Pathenon Publishing Group, Lancashire, UK
- McNeilly AS, Kerin J, Swanston IA, Bramley TA and Baird DT** (1980) Changes in the binding of human chorionic gonadotrophin/luteinizing hormone, follicle-stimulating hormone and prolactin to human corpora lutea during the menstrual cycle and pregnancy *Journal of Endocrinology* **87**:315-25
- McNeilly AS, Crow WJ and Fraser HM** (1992) Suppression of pulsatile luteinizing hormone secretion by gonadotrophin-releasing hormone antagonist does not affect episodic progesterone secretion or corpus luteum function in ewes *Journal of Reproduction and Fertility* **96**:865-74

- Meidan R and Girsh E** (1997) Role of endothelial cells in the steroidogenic activity of the bovine corpus luteum *Seminars in Reproductive Endocrinology* **15**:371-82
- Mendelson CR, Merrill JC, Steinkampf MP and Simpson ER** (1988) Regulation of the synthesis of aromatase cytochrome P-450 in human adipose, stromal and ovarian granulosa cells *Steroids* **50**:51-59
- Mendizabal AF, Quiroga S, Farinati Z, Lahoz M and Nagle C** (1984) Hormonal monitoring of early pregnancy by a direct radioimmunoassay of steroid glucuronides in first morning urine *Fertility and Sterility* **42**:737-40
- Mesiano S, Ferrara N and Jaffe RB** (1998) Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization *American Journal of Pathology* **153**:1249-56
- Messinis IE** (1997) Luteal function--luteolysis *Annals of the New York Academy of Sciences* **816**:151-7
- Meyer GT and McGeachie JK** (1988) Angiogenesis in the developing corpus luteum of pregnant rats: a stereologic and autoradiographic study *Anatomical Record* **222**:18-25
- Miller K** (1996) Immunocytochemical techniques. In: Bancroft JD, Stevens A (eds) *Theory and Practise of Histological Techniques*. Churchill Livingstone, New York
- Milvae RA and Hansel W** (1985) Inhibition of bovine luteal function by indomethacin *Journal of Animal Science* **60**:528-31
- Monfort SL, Hess DL, Hendrickx AG and Lasley BL** (1989) Absence of regular pulsatile gonadotropin secretion during implantation in the rhesus macaque *Endocrinology* **125**:1766-73
- Montesano R, Vassalli JD, Baird A, Guillemin R and Orci L** (1986) Basic fibroblast growth factor induces angiogenesis in vitro *Proceedings of the National Academy of Sciences of the United States of America* **83**:7297-301
- Moor RM and Seamark RF** (1986) Cell signaling, permeability, and microvasculatory changes during antral follicle development in mammals *Journal of Dairy Science* **69**:927-43
- Moore HD, Gems S and Hearn JP** (1985) Early implantation stages in the marmoset monkey (*Callithrix jacchus*) *American Journal of Anatomy* **172**:265-78

- Morales G**, (2000) Different patterns of structural luteolysis in the human corpus luteum of menstruation *Human Reproduction* **15**:2119-2128
- Muller T, Gromoll J and Simoni M** (2003) Absence of exon 10 of the human luteinizing hormone (LH) receptor impairs LH, but not human chorionic gonadotropin action *Journal of Clinical Endocrinology and Metabolism* **88**:2242-9
- Nakano R** (1997) Control of the luteal function in humans *Seminars in Reproductive Endocrinology* **15**:335-44
- Namiki A, Brogi E, Kearney M, Kim EA, Wu T, Couffinhal T, Varticovski L and Isner JM** (1995) Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells *Journal of Biological Chemistry* **270**:31189-95
- Nappi C, Gargiulo AR and Di Carlo C** (1994) The human luteal paracrine system: current concepts *Journal of Endocrinological Investigation* **17**:825-36
- Nayak NR, Critchley HO, Slayden OD, Menrad A, Chwalisz K, Baird DT and Brenner RM** (2000) Progesterone withdrawal up-regulates vascular endothelial growth factor receptor type 2 in the superficial zone stroma of the human and macaque endometrium: potential relevance to menstruation *Journal of Clinical Endocrinology and Metabolism* **85**:3442-52
- Nayak NR and Brenner RM** (2002) Vascular proliferation and vascular endothelial growth factor expression in the rhesus macaque endometrium *Journal of Clinical Endocrinology and Metabolism* **87**:1845-55
- Nehls V, Denzer K and Drenckhahn D** (1992) Pericyte involvement in capillary sprouting during angiogenesis in situ *Cell and Tissue Research* **270**:469-74
- Neill JD, Johansson ED and Knobil E** (1969) Failure of hysterectomy to influence the normal pattern of cyclic progesterone secretion in the rhesus monkey *Endocrinology* **84**:464-5
- Neulen J, Raczek S, Pogorzelski M, Grunwald K, Yeo TK, Dvorak HF, Weich HA and Breckwoldt M** (1998) Secretion of vascular endothelial growth factor/vascular permeability factor from human luteinized granulosa cells is human chorionic gonadotrophin dependent *Molecular Human Reproduction* **4**:203-6
- Niswender GD, Schwall RH, Fitz TA, Farin CE and Sawyer HR** (1985) Regulation of luteal function in domestic ruminants: new concepts *Recent progress in hormone research* **41**:101-51

- Niswender GD, Juengel JL, McGuire WJ, Belfiore CJ and Wiltbank MC** (1994) Luteal function: the estrous cycle and early pregnancy *Biology of Reproduction* **50**:239-47
- Niswender GD and Nett TM** (1994) Corpus luteum and its control in infraprimates species. In: Knobil E, Neill JD (eds) *The Physiology of Reproduction*. Raven Press, New York, 781-816
- Niswender GD, Juengel JL, Silva PJ, Rollyson MK and McIntush EW** (2000) Mechanisms controlling the function and life span of the corpus luteum *Physiological Reviews* **80**:1-29
- Ohtani M, Kobayashi S, Miyamoto A, Hayashi K and Fukui Y** (1998) Real-time relationships between intraluteal and plasma concentrations of endothelin, oxytocin, and progesterone during prostaglandin F₂α-induced luteolysis in the cow *Biology of Reproduction* **58**:103-8
- Okuda K, Sakumoto R, Uenoyama Y, Berisha B, Miyamoto A and Schams D** (1999) Tumor necrosis factor alpha receptors in microvascular endothelial cells from bovine corpus luteum *Biology of Reproduction* **61**:1017-22
- Olson LM, Jones-Burton CM and Jablonka-Shariff A** (1996) Nitric oxide decreases estradiol synthesis of rat luteinized ovarian cells: possible role for nitric oxide in functional luteal regression *Endocrinology* **137**:3531-9
- O'Reilly G, Charnock-Jones DS, Davenport AP, Cameron IT and Smith SK** (1992) Presence of messenger ribonucleic acid for endothelin-1, endothelin-2, and endothelin-3 in human endometrium and a change in the ratio of ETA and ETB receptor subtype across the menstrual cycle *Journal of Clinical Endocrinology and Metabolism* **75**:1545-9
- O'Reilly MS, Holmgren L, Chen C and Folkman J** (1996) Angiostatin induces and sustains dormancy of human primary tumors in mice *Nature Medicine* **2**:689-92
- O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR and Folkman J** (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth *Cell* **88**:277-85
- Orlidge A and D'Amore PA** (1987) Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells *Journal of Cell Biology* **105**:1455-62
- O'Shea JD, Nightingale MG and Chamley WA** (1977) Changes in small blood vessels during cyclical luteal regression in sheep *Biology of Reproduction* **17**:162-77
- Otani N, Minami S, Yamoto M, Shikone T, Otani H, Nishiyama R, Otani T and Nakano R** (1999) The vascular endothelial growth factor/fms-like tyrosine

- kinase system in human ovary during the menstrual cycle and early pregnancy *Journal of Clinical Endocrinology and Metabolism* **84**:3845-51
- Ottobre JS and Stouffer RL** (1984) Persistent versus transient stimulation of the macaque corpus luteum during prolonged exposure to human chorionic gonadotropin: a function of age of the corpus luteum *Endocrinology* **114**:2175-82
- Paavola LG** (1979) The corpus luteum of the guinea pig. IV. Fine structure of macrophages during pregnancy and postpartum luteolysis, and the phagocytosis of luteal cells *American Journal of Anatomy* **154**:337-64
- Padykula HA, Coles LG, McCracken JA, King NWJ, Longcope C and Kaiserman-Abramof IR** (1984) A zonal pattern of cell proliferation and differentiation in the rhesus endometrium during the estrogen surge *Biology of Reproduction* **31**:1103-18
- Padykula HA, Coles LG, Okulicz WC, Rapaport SI, McCracken JA, King NW, Jr., Longcope C and Kaiserman-Abramof IR** (1989) The basalis of the primate endometrium: a bifunctional germinal compartment *Biology of Reproduction* **40**:681-90
- Park-Sarge OK and Mayo KE** (1994) Regulation of the progesterone receptor gene by gonadotropins and cyclic adenosine 3',5'-monophosphate in rat granulosa cells *Endocrinology* **134**:709-18
- Pate JL** (1996) Intercellular communication in the bovine corpus luteum *Theriogenology*. **45**:1381-1397
- Paulsson M** (1992) Basement membrane proteins: structure, assembly, and cellular interactions *Critical Reviews in Biochemistry and Molecular Biology* **27**:93-127
- Pescador N, Soumano K, Stocco DM, Price CA and Murphy BD** (1996) Steroidogenic acute regulatory protein in bovine corpora lutea *Biology of Reproduction* **55**:485-91
- Peters KE, Bergfeld EG, Cupp AS, Kojima FN, Mariscal V, Sanchez T, Wehrman ME, Grotjan HE, Hamernik DL, Kittok RJ and et al.** (1994) Luteinizing hormone has a role in development of fully functional corpora lutea (CL) but is not required to maintain CL function in heifers *Biology of Reproduction* **51**:1248-54
- Plendl J** (2000) Angiogenesis and vascular regression in the ovary *Anatomia, Histologia, Embryologia* **29**:257-66

- Poropatich C, Rojas M and Silverberg SG** (1987) Polymorphonuclear leukocytes in the endometrium during the normal menstrual cycle *International Journal of Gynecological Pathology* **6**:230-4
- Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, Winkler M and Ferrara N** (1997) Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders *Cancer Research* **57**:4593-9
- Rabbani MM and Rogers PA** (2001) Role of vascular endothelial growth factor in endometrial vascular events before implantation in rats *Reproduction* **122**:85-90
- Rakugi H, Tabuchi Y, Nakamaru M, Nagano M, Higashimori K, Mikami H, Ogihara T and Suzuki N** (1990) Evidence for endothelin-1 release from resistance vessels of rats in response to hypoxia *Biochemical and Biophysical Research Communications* **169**:973-7
- Ramsey EM, Houston ML and Harris JW** (1976) Interactions of the trophoblast and maternal tissues in three closely related primate species *American Journal of Obstetrics and Gynecology* **124**:647-52
- Ravindranath N, Little-Ihrig L, Benyo DF and Zeleznik AJ** (1992) Role of luteinizing hormone in the expression of cholesterol side-chain cleavage cytochrome P450 and 3 beta-hydroxysteroid dehydrogenase, delta 5-4 isomerase messenger ribonucleic acids in the primate corpus luteum *Endocrinology* **131**:2065-70
- Ravindranath N, Little-Ihrig L, Phillips HS, Ferrara N and Zeleznik AJ** (1992) Vascular endothelial growth factor messenger ribonucleic acid expression in the primate ovary *Endocrinology* **131**:254-60
- Redmer DA, Grazul AT, Kirsch JD and Reynolds LP** (1988) Angiogenic activity of bovine corpora lutea at several stages of luteal development. *Journal of Reproduction and Fertility* **82**:627-34
- Redmer DA, Dai Y, Li J, Charnock-Jones DS, Smith SK, Reynolds LP and Moor RM** (1996) Characterization and expression of vascular endothelial growth factor (VEGF) in the ovine corpus luteum *Journal of Reproduction and Fertility* **108**:157-65
- Redmer DA and Reynolds LP** (1996) Angiogenesis in the ovary *Reviews of Reproduction* **1**:182-92
- Redmer DA, Doraiswamy V, Bortnem BJ, Fisher K, Jablonka-Shariff A, Grazul-Bilska AT and Reynolds LP** (2001) Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum *Biol Reprod* **65**:879-89

- Reimers TJ and Niswender GD** (1975) Effects of specific neutralisation of luteinising hormone and prolactin on ovarian blood flow and progesterone secretion. In: Nieschlag E (ed) *Immunization With Hormones in Reproductive Research*. Elsevier, New York, 95-105
- Retamales I, Carrasco I, Troncoso JL, Las Heras J, Devoto L and Vega M** (1994) Morpho-functional study of human luteal cell subpopulations *Human Reproduction* **9**:591-6
- Reynolds LP, Killilea SD and Redmer DA** (1992) Angiogenesis in the female reproductive system *The FASEB Journal* **6**:886-92
- Reynolds LP and Redmer DA** (1992) Growth and microvascular development of the uterus during early pregnancy in ewes *Biology of Reproduction* **47**:698-708
- Reynolds LP, Grazul-Bilska AT, Killilea SD and Redmer DA** (1994) Mitogenic factors of corpora lutea *Progress in Growth Factor Research* **5**:159-75
- Reynolds LP and Redmer DA** (1998) Expression of the angiogenic factors, basic fibroblast growth factor and vascular endothelial growth factor, in the ovary *Journal of Animal Science* **76**:1671-81
- Reynolds LP and Redmer DA** (1999) Growth and development of the corpus luteum *Journal of Reproduction and Fertility (Suppl)* **54**:181-91
- Rhodin JA** (1968) Ultrastructure of mammalian venous capillaries, venules, and small collecting veins *Journal of Ultrastructural Research* **25**:452-500
- Richards JS** (1980) Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation *Physiological Reviews* **60**:51-89
- Richards JS, Jahnsen T, Hedin L, Lifka J, Ratoosh S, Durica JM and Goldring NB** (1987) Ovarian follicular development: from physiology to molecular biology *Recent progress in hormone research* **43**:231-76
- Ricke WA, Redmer DA and Reynolds LP** (1999) Growth and cellular proliferation of pig corpora lutea throughout the oestrous cycle *Journal of Reproduction and Fertility* **117**:369-77
- Risau W** (1991) Embryonic angiogenesis factors *Pharmacological Theriogenology* **51**:371-6
- Roberts WG and Palade GE** (1995) Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor *Journal of Cell Science* **108** (6):2369-79

- Rockwell LC, Pillai S, Olson CE and Koos RD** (2002) Inhibition of vascular endothelial growth factor/vascular permeability factor action blocks estrogen-induced uterine edema and implantation in rodents *Biology of Reproduction* **67**:1804-10
- Rodger FE, Young FM, Fraser HM and Illingworth PJ** (1997) Endothelial cell proliferation follows the mid-cycle luteinizing hormone surge, but not human chorionic gonadotrophin rescue, in the human corpus luteum *Human Reproduction* **12**:1723-9
- Rodgers RJ, van Wezel IL and Rodgers HF** (1998) Developmental changes in cells and matrix during follicle growth. In: Lauri A, Gandolfi F (eds) *Gametes: Development and Function*. Tabloid S.L.R, Rome, 85-99
- Rogers PA** (1998) Endometrial microvascular growth in normal and dysfunctional states *Human Reproduction* **9**:399-405
- Rothchild I** (1981) The regulation of the mammalian corpus luteum *Recent progress in hormone research* **37**:183-298
- Rowe AJ, Morris KD, Bicknell R and Fraser HM** (2002) Angiogenesis in the corpus luteum of early pregnancy in the marmoset and the effects of vascular endothelial growth factor immunoneutralization on establishment of pregnancy *Biology of Reproduction* **67**:1180-8
- Rowe AJ, Wulff C and Fraser HM** (2003) Localisation of mRNA for vascular endothelial growth factor (VEGF), angiopoietins and their receptors during the peri-implantation period and early pregnancy in marmosets (*Callithrix jacchus*) *Reproduction* **126**:227-238
- Rudolph-Owen LA, Slayden OD, Matrisian LM and Brenner RM** (1998) Matrix metalloproteinase expression in *Macaca mulatta* endometrium: evidence for zone-specific regulatory tissue gradients *Biology of Reproduction* **59**:1349-59
- Rune GM, Leuchtenberg U, Schroter-Kermani C and Merker HJ** (1992) Zonal differentiation of the marmoset (*Callithrix jacchus*) endometrium *Journal of Anatomy* **181**:301-12
- Saltzman W, Schultz-Darken NJ, Wegner FH, Wittwer DJ and Abbott DH** (1998) Suppression of cortisol levels in subordinate female marmosets: reproductive and social contributions *Hormones and Behaviour* **33**:58-74
- Sanders SL and Stouffer RL** (1997) Localization of steroidogenic enzymes in macaque luteal tissue during the menstrual cycle and simulated early pregnancy: immunohistochemical evidence supporting the two-cell model for estrogen production in the primate corpus luteum *Biology of Reproduction* **56**:1077-87

- Sargent EL, Baughman WL, Novy MJ and Stouffer RL** (1988) Intraluteal infusion of a prostaglandin synthesis inhibitor, sodium meclofenamate, causes premature luteolysis in rhesus monkeys *Endocrinology* **123**:2261-9
- Sasano H, Okamoto M, Mason JI, Simpson ER, Mendelson CR, Sasano N and Silverberg SG** (1989) Immunolocalization of aromatase, 17 alpha-hydroxylase and side-chain-cleavage cytochromes P-450 in the human ovary *Journal of Reproduction and Fertility* **85**:163-9
- Sasano H and Suzuki T** (1997) Localization of steroidogenesis and steroid receptors in human corpus luteum. Classification of human corpus luteum (CL) into estrogen-producing degenerating CL, and nonsteroid-producing degenerating CL *Seminars in Reproductive Endocrinology* **15**:345-51
- Sato Y, Hamanaka R, Ono J, Kuwano M, Rifkin DB and Takaki R** (1991) The stimulatory effect of PDGF on vascular smooth muscle cell migration is mediated by the induction of endogenous basic FGF *Biochemical and Biophysical Research Communications* **174**:1260-6
- Scaramuzzi RJ, Adams NR, Baird DT, Campbell BK, Downing JA, Findlay JK, Henderson KM, Martin GB, McNatty KP, McNeilly AS and et al.** (1993) A model for follicle selection and the determination of ovulation rate in the ewe *Reproduction Fertility and Development* **5**:459-78
- Schoonmaker JN, Bergman KS, Steiner RA and Karsch FJ** (1982) Estradiol-induced luteal regression in the rhesus monkey: evidence for an extraovarian site of action *Endocrinology* **110**:1708-15
- Schweigerer L, Neufeld G, Friedman J, Abraham JA, Fiddes JC and Gospodarowicz D** (1987) Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth *Nature* **325**:257-9
- Segaloff DL, Wang H and Richards JS** Hormone specific regulation of LH/CG receptor mRNA expression in rat ovarian follicles and corpora lutea. Proceedings of the 72nd Annual Meeting of the Endocrine Society, 1990
- Selick CE, Horowitz GM, Gratch M, Scott RT, Jr., Navot D and Hofmann GE** (1994) Immunohistochemical localization of transforming growth factor-beta in human implantation sites *Journal of Clinical Endocrinology and Metabolism* **78**:592-6
- Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS and Dvorak HF** (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid *Science* **219**:983-5

- Sharma R, Bulmer D and Peel S** (1986) Effects of exogenous progesterone following ovariectomy on the metrial glands of pregnant mice *Journal of Anatomy* **144**:189-99
- Shikone T, Yamoto M, Kokawa K, Yamashita K, Nishimori K and Nakano R** (1996) Apoptosis of human corpora lutea during cyclic luteal regression and early pregnancy *Journal of Clinical Endocrinology and Metabolism* **81**:2376-80
- Shweiki D, Itin A, Soffer D and Keshet E** (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis *Nature* **359**:843-5
- Simula AP, Amato F, Faast R, Lopata A, Berka J and Norman RJ** (1995) Luteinizing hormone/chorionic gonadotropin bioactivity in the common marmoset (*Callithrix jacchus*) is due to a chorionic gonadotropin molecule with a structure intermediate between human chorionic gonadotropin and human luteinizing hormone *Biology of Reproduction* **53**:380-9
- Slayden OD, Nayak NR, Burton KA, Chwalisz K, Cameron ST, Critchley HO, Baird DT and Brenner RM** (2001) Progesterone antagonists increase androgen receptor expression in the rhesus macaque and human endometrium *Journal of Clinical Endocrinology and Metabolism* **86**:2668-79
- Smith CA, Moore HD and Hearn JP** (1987) The ultrastructure of early implantation in the marmoset monkey (*Callithrix jacchus*) *Anatomy and Embryology (Berl)* **175**:399-410
- Smith CA and Moore HD** (1990) An ultrastructural study of early chorionic villus formation in the marmoset monkey (*Callithrix jacchus*) *Anatomy and Embryology (Berl)* **181**:59-66
- Smith GD, Sawyer HR, Mirando MA, Griswold MD, Sadhu A and Reeves JJ** (1996) Steady-state luteinizing hormone receptor messenger ribonucleic acid levels and endothelial cell composition in bovine normal- and short-lived corpora lutea *Biology of Reproduction* **55**:902-9
- Smith MF, McIntush EW and Smith GW** (1994) Mechanisms associated with corpus luteum development *Journal of Animal Science* **72**:1857-72
- Smith P, O WS, Hudson NL, Shaw L, Heath DA, Condell L, Phillips DJ and McNatty KP** (1993) Effects of the Booroola gene (*FecB*) on body weight, ovarian development and hormone concentrations during fetal life *Journal of Reproduction and Fertility* **98**:41-54
- Smith SK** (2000) Angiogenesis and implantation *Human Reproduction* **15 Suppl** **6**:59-66

- Smith SK** (2001) Regulation of angiogenesis in the endometrium *Trends in Endocrinology and Metabolism* **12**:147-51
- Snijders MP, de Goeij AF, Debets-Te Baerts MJ, Rousch MJ, Koudstaal J and Bosman FT** (1992) Immunocytochemical analysis of oestrogen receptors and progesterone receptors in the human uterus throughout the menstrual cycle and after the menopause *Journal of Reproduction and Fertility* **94**:363-71
- Snook RB, Brunner MA, Saatman RR and Hansel W** (1969) The effect of antisera to bovine LH in hysterectomized and intact heifers *Biology of Reproduction* **1**:49-58
- Spies HG and Niswender GD** (1971) Levels of prolactin, LH and FSH in the serum of intact and pelvic-neurectomized rats *Endocrinology* **88**:937-43
- Starkey PM, Sargent IL and Redman CW** (1988) Cell populations in human early pregnancy decidua: characterization and isolation of large granular lymphocytes by flow cytometry *Immunology* **65**:129-34
- Stevens A and Wilson I** (1996) The Haematoxylin and eosin. In: Bancroft JD, Stevens AS (eds) *Theory and Practice of Histological techniques*. Churchill Livingstone, USA
- Stewart I** (1987) Differentiation of granulated metrial gland cells in ovariectomized mice given ovarian hormones *Journal of Endocrinology* **112**:23-6
- Stewart I and Mukhtar DD** (1988) The killing of mouse trophoblast cells by granulated metrial gland cells in vitro *Placenta* **9**:417-25
- Stewart IJ** (1998) Granulated metrial gland cells in 'minor' species *Journal of Reproductive immunology* **40**:129-46
- Stouffer R** (1996) Corpus luteum formation and demise. In: Adashi EY, Rock JA, Rosenwaks Z (eds) *Reproductive Endocrinology, Surgery, and Technology*. Lippincott-Raven Publishers, Philadelphia, 251-270
- Stouffer RL, Nixon WE and Hodgen GD** (1978) The refractory state of luteal cells isolated from rhesus monkeys after prolonged exposure to chorionic gonadotropin during early pregnancy. *Biology of Reproduction* **18**:858-64
- Stouffer RL, Nixon WE and Hodgen GD** (1979) Disparate effects of prostaglandins on basal and gonadotropin-stimulated progesterone production by luteal cells isolated from rhesus monkeys during the menstrual cycle and pregnancy *Biology of Reproduction* **20**:897-903
- Stouffer RL and Brannian JD** (1993) The function and Regulation of cell populations composing the corpus luteum of the ovarian cycle. In: Adashi EY, Leung PCK (eds) *The Ovary*. Raven Press, New York, 245-259

- Strauss JFM, W.L.** (1991) Molecular basis of ovarian steroid synthesis. In: Hillier SG (ed) *Ovarian Endocrinology*. Blackwell Scientific Publications, Oxford
- Streefkerk JG** (1972) Inhibition of erythrocyte pseudoperoxidase activity by treatment with hydrogen peroxide following methanol *Journal of Histochemical Cytochemistry* **20**:829-31
- Sugino N, Kashida S, Takiguchi S, Karube A and Kato H** (2000) Expression of vascular endothelial growth factor and its receptors in the human corpus luteum during the menstrual cycle and in early pregnancy *Journal of Clinical Endocrinology and Metabolism* **85**:3919-24
- Sugino N, Kashida S, Karube-Harada A, Takiguchi S and Kato H** (2002) Expression of vascular endothelial growth factor (VEGF) and its receptors in human endometrium throughout the menstrual cycle and in early pregnancy *Reproduction* **123**:379-87
- Sumida C, Lecerf F and Pasqualini JR** (1988) Control of progesterone receptors in fetal uterine cells in culture: effects of estradiol, progestins, antiestrogens, and growth factors *Endocrinology* **122**:3-11
- Sumida C and Pasqualini JR** (1989) Antiestrogens antagonize the stimulatory effect of epidermal growth factor on the induction of progesterone receptor in fetal uterine cells in culture *Endocrinology* **124**:591-7
- Summers PM, Wennink CJ and Hodges JK** (1985) Cloprostenol-induced luteolysis in the marmoset monkey (*Callithrix jacchus*) *Journal of Reproduction and Fertility* **73**:133-8
- Sunder S and Lenton EA** (2000) Endocrinology of the peri-implantation period *Bailliere's best practice & research. Clinical obstetrics & gynaecology* **14**:789-800
- Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN and Yancopoulos GD** (1996) Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis *Cell* **87**:1171-80
- Suzuki T, Sasano H, Takaya R, Fukaya T, Yajima A and Nagura H** (1998) Cyclic changes of vasculature and vascular phenotypes in normal human ovaries *Human Reproduction* **13**:953-9
- Tamura H and Greenwald GS** (1987) Angiogenesis and its hormonal control in the corpus luteum of the pregnant rat *Biology of Reproduction* **36**:1149-54
- Tan GJ, Tweedale R and Biggs JS** (1982) Oxytocin may play a role in the control of the human corpus luteum *Journal of Endocrinology* **95**:65-70

- Tanaka T, Andoh N, Takeya T and Sato E** (1992) Differential screening of ovarian cDNA libraries detected the expression of the porcine collagenase inhibitor gene in functional corpora lutea *Molecular Cell Endocrinology* **83**:65-71
- Tarantino S, Verhage HG and Fazleabas AT** (1992) Regulation of insulin-like growth factor-binding proteins in the baboon (*Papio anubis*) uterus during early pregnancy *Endocrinology* **130**:2354-62
- Tarara R, Enders AC, Hendrickx AG, Gulamhusein N, Hodges JK, Hearn JP, Eley RB and Else JG** (1987) Early implantation and embryonic development of the baboon: stages 5, 6 and 7 *Anatomy and Embryology (Berl)* **176**:267-75
- Tay PY and Lenton EA** (2000) Corpus luteum response to exogenous HCG during the mid-luteal phase of the menstrual cycle *Clinical Endocrinology (Oxf)* **53**:345-50
- Telleria CM, Stocco CO, Stati AO and Deis RP** (1999) Progesterone receptor is not required for progesterone action in the rat corpus luteum of pregnancy *Steroids* **64**:760-6
- Tetsuka M and Hillier SG** (1996) Androgen receptor gene expression in rat granulosa cells: the role of follicle-stimulating hormone and steroid hormones *Endocrinology* **137**:4392-7
- Thompson WD, Li WW and Maragoudakis M** (1999) The clinical manipulation of angiogenesis: pathology, side-effects, surprises, and opportunities with novel human therapies *Journal of Pathology* **187**:503-10
- Tilton RG, Kilo C, Williamson JR and Murch DW** (1979) Differences in pericyte contractile function in rat cardiac and skeletal muscle microvasculatures *Microvascular research* **18**:336-52
- Tjugum J, Dennefors B and Norstrom A** (1984) Influence of progesterone, androstenedione and oestradiol-17 beta on the incorporation of [³H]proline in the human follicular wall *Acta Endocrinol (Copenh)* **105**:552-7
- Torry DS, Holt VJ, Keenan JA, Harris G, Caudle MR and Torry RJ** (1996) Vascular endothelial growth factor expression in cycling human endometrium *Fertility and Sterility* **66**:72-80
- Tseng L, Gao JG, Chen R, Zhu HH, Mazella J and Powell DR** (1992) Effect of progestin, antiprogestin, and relaxin on the accumulation of prolactin and insulin-like growth factor-binding protein-1 messenger ribonucleic acid in human endometrial stromal cells *Biology of Reproduction* **47**:441-50
- Tung L, Mohamed MK, Hoeffler JP, Takimoto GS and Horwitz KB** (1993) Antagonist-occupied human progesterone B-receptors activate transcription

without binding to progesterone response elements and are dominantly inhibited by A-receptors *Molecular Endocrinology* **7**:1256-65

Vande Wiele RL, Bogumil J, Dyrenfurth I, Ferin M, Jewelewicz R, Warren M, Rizkallah T and Mikhail G (1970) Mechanisms regulating the menstrual cycle in women *Recent progress in hormone research* **26**:63-103

Vega M and Devoto L (1997) Autocrine/paracrine regulation of normal human corpus luteum development *Seminars in Reproductive Endocrinology* **15**:353-62

Vlodavsky I, Fridman R, Sullivan R, Sasse J and Klagsbrun M (1987) Aortic endothelial cells synthesize basic fibroblast growth factor which remains cell associated and platelet-derived growth factor-like protein which is secreted *Journal of Cell Physiology* **131**:402-8

Wakui S, Furusato M, Hasumura M, Hori M, Takahashi H, Kano Y and Ushigome S (1989) Two- and three-dimensional ultrastructure of endothelium and pericyte interdigitations in capillary of human granulation tissue *J Electron Microsc (Tokyo)* **38**:136-42

Wang F, Riley JC and Behrman HR (1993) Immunosuppressive levels of glucocorticoid block extrauterine luteolysins in the rat *Biology of Reproduction* **49**:66-73

Webley GE, Richardson MC, Summers PM, Given A and Hearn JP (1989) Changing responsiveness of luteal cells of the marmoset monkey (*Callithrix jacchus*) to luteotrophic and luteolytic agents during normal and conception cycles *Journal of Reproduction and Fertility* **87**:301-10

Webley GE, Richardson MC, Smith CA, Masson GM and Hearn JP (1990) Size distribution of luteal cells from pregnant and non-pregnant marmoset monkeys and a comparison of the morphology of marmoset luteal cells with those from the human corpus luteum *Journal of Reproduction and Fertility* **90**:427-37

Webley GE, Hodges JK, Given A and Hearn JP (1991) Comparison of the luteolytic action of gonadotrophin-releasing hormone antagonist and cloprostenol, and the ability of human chorionic gonadotrophin and melatonin to override their luteolytic effects in the marmoset monkey *Journal of Endocrinology* **128**:121-9

Wehrenberg U and Rune GM (2000) Spontaneous luteinization of antral marmoset follicles in vitro *Molecular Human Reproduction* **6**:504-9

Westermarck B, Siegbahn A, Heldin CH and Claesson-Welsh L (1990) B-type receptor for platelet-derived growth factor mediates a chemotactic response by means of ligand-induced activation of the receptor protein-tyrosine kinase

Proceedings of the National Academy of Sciences of the United States of America **87**:128-32

- Wildt L, Hausler A, Hutchison JS, Marshall G and Knobil E** (1981) Estradiol as a gonadotropin releasing hormone in the rhesus monkey *Endocrinology* **108**:2011-3
- Wilks JW and Noble AS** (1983) Steroidogenic responsiveness of the monkey corpus luteum to exogenous chorionic gonadotropin *Endocrinology* **112**:1256-66
- Winther H, Ahmed A and Dantzer V** (1999) Immunohistochemical localization of vascular endothelial growth factor (VEGF) and its two specific receptors, Flt-1 and KDR, in the porcine placenta and non-pregnant uterus *Placenta* **20**:35-43
- Wolf JP, Hsiu JG, Anderson TL, Ulmann A, Baulieu EE and Hodgen GD** (1989) Noncompetitive antiestrogenic effect of RU 486 in blocking the estrogen-stimulated luteinizing hormone surge and the proliferative action of estradiol on endometrium in castrate monkeys *Fertil Steril* **52**:1055-60
- Wulff C, Wilson H, Largue P, Duncan WC, Armstrong DG and Fraser HM** (2000) Angiogenesis in the human corpus luteum: localization and changes in angiopoietins, tie-2, and vascular endothelial growth factor messenger ribonucleic acid *Journal of Clinical Endocrinology and Metabolism* **85**:4302-9
- Wulff C, Dickson SE, Duncan WC and Fraser HM** (2001) Angiogenesis in the human corpus luteum: Simulated early pregnancy by hCG treatment is associated with both angiogenesis and vessel stabilisation *Journal of Clinical Endocrinology and Metabolism* **85**:4302-4309
- Wulff C, Wiegand SJ, Saunders PT, Scobie GA and Fraser HM** (2001) Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc (vascular endothelial growth factor Trap(A40) *Endocrinology* **142**:3244-54
- Wulff C, Wilson H, Rudge JS, Wiegand SJ, Lunn SF and Fraser HM** (2001) Luteal angiogenesis: prevention and intervention by treatment with vascular endothelial growth factor trap(a40). *Journal of Clinical Endocrinology and Metabolism* **86**:3377-86
- Wulff C, Wilson H, Dickson SE, Wiegand SJ and Fraser HM** (2002) Hemochorial placentation in the primate: expression of vascular endothelial growth factor, angiopoietins, and their receptors throughout pregnancy *Biology of Reproduction* **66**:802-12

- Wynn RM** (1974) Ultrastructural development of the human decidua *American Journal of Obstetrics and Gynecology* **118**:652-70
- Yada H, Hosokawa K, Tajima K, Hasegawa Y and Kotsuji F** (1999) Role of ovarian theca and granulosa cell interaction in hormone production and cell growth during the bovine follicular maturation process *Biology of Reproduction* **61**:1480-6
- Yamamoto S, Konishi I, Nanbu K, Komatsu T, Mandai M, Kuroda H, Matsushita K and Mori T** (1997) Immunohistochemical localization of basic fibroblast growth factor (bFGF) during folliculogenesis in the human ovary *Gynecological Endocrinology* **11**:223-30
- Yi XJ, Jiang HY, Lee KK, O WS, Tang PL and Chow PH** (1999) Expression of vascular endothelial growth factor (VEGF) and its receptors during embryonic implantation in the golden hamster (*Mesocricetus auratus*) *Cell and Tissue Research* **296**:339-49
- Yong EL, Baird DT and Hillier SG** (1992) Mediation of gonadotrophin-stimulated growth and differentiation of human granulosa cells by adenosine-3',5'-monophosphate: one molecule, two messages *Clinical Endocrinology (Oxf)* **37**:51-8
- Young FM, Illingworth PJ, Lunn SF, Harrison DJ and Fraser HM** (1997) Cell death during luteal regression in the marmoset monkey (*Callithrix jacchus*) *Journal of Reproduction and Fertility* **111**:109-19
- Young FM, Illingworth PJ and Fraser HM** (1998) Ubiquitin and apoptosis in the corpus luteum of the marmoset monkey (*Callithrix jacchus*) *Journal of Reproduction and Fertility* **114**:163-8
- Yuan W and Giudice LC** (1997) Programmed cell death in human ovary is a function of follicle and corpus luteum status *Journal of Clinical Endocrinology and Metabolism* **82**:3148-55
- Yurchenco PD and Schittny JC** (1990) Molecular architecture of basement membranes *The FASEB Journal* **4**:1577-90
- Zeleznik AJ, Keyes PL, Menon KM, Midgley AR, Jr. and Reichert LE, Jr.** (1977) Development-dependent responses of ovarian follicles to FSH and hCG *American Journal of Physiology* **233**:E229-34
- Zeleznik AJ, Schuler HM and Reichert LE** (1981) Gonadotropin-binding sites in the rhesus monkey ovary: role of the vasculature in the selective distribution of human chorionic gonadotropin to the preovulatory follicle *Endocrinology* **109**:356-62

- Zeleznik AJ and Kubik CJ** (1986) Ovarian responses in macaques to pulsatile infusion of follicle-stimulating hormone (FSH) and luteinizing hormone: increased sensitivity of the maturing follicle to FSH *Endocrinology* **119**:2025-32
- Zeleznik AJ** (1991) Control of Luteal Endocrine Function. In: Hillier SG (ed) *Ovarian Endocrinology*. Blackwell Scientific, Oxford, 167-189
- Zeleznik AJ and Benyo DF** (1994) Control of follicular development, Corpus luteum function and the recognition of pregnancy in higher primates. In: Knobil E, Neill JD (eds) *The Physiology of Reproduction*. Raven Press, New York
- Zeleznik AJ and Hillier SG** (1996) The Ovary: Endocrine Function. In: Hillier SG, Kitchener HC, Neilson JP (eds) *Scientific Essentials of Reproductive Medicine*. W.B.Saunders Company Ltd., London, 133-145
- Zelinski-Wooten MB, Sargent EL, Molskness TA and Stouffer RL** (1990) Disparate effects of the prostaglandin synthesis inhibitors, meclofenamate, and flurbiprofen on monkey luteal tissue in vitro *Endocrinology* **126**:1380-7
- Zelinski-Wooten MB and Stouffer RL** (1990) Intraluteal infusions of prostaglandins of the E, D, I, and A series prevent PGF₂ alpha-induced, but not spontaneous, luteal regression in rhesus monkeys *Biology of Reproduction* **43**:507-16
- Zhang FP, Rannikko AS, Manna PR, Fraser HM and Huhtaniemi IT** (1997) Cloning and functional expression of the luteinizing hormone receptor complementary deoxyribonucleic acid from the marmoset monkey testis: absence of sequences encoding exon 10 in other species *Endocrinology* **138**:2481-90
- Zheng J, Fricke PM, Reynolds LP and Redmer DA** (1994) Evaluation of growth, cell proliferation, and cell death in bovine corpora lutea throughout the estrous cycle *Biology of Reproduction* **51**:623-32
- Zheng LM, Joag SV, Parr MB, Parr EL and Young JD** (1991) Perforin-expressing granulated metrial gland cells in murine deciduoma *Journal of Experimental Medicine* **174**:1221-6

Appendices

Angiogenesis in the Corpus Luteum of Early Pregnancy in the Marmoset and the Effects of Vascular Endothelial Growth Factor Immunoneutralization on Establishment of Pregnancy

Amanda J. Rowe,² Keith D. Morris,² Roy Bicknell,³ and Hamish M. Fraser^{1,2}

Medical Research Council Human Reproductive Sciences Unit,² Centre for Reproductive Biology,
Edinburgh EH3 9ET, United Kingdom

Molecular Angiogenesis Laboratories,³ ICRF, Institute of Molecular Medicine, John Radcliffe Hospital,
Oxford OX3 9DS, United Kingdom

ABSTRACT

This study investigated vascular and molecular changes in the corpus luteum (CL) of early pregnancy in the marmoset. Ovaries were studied on Days 21 ($n = 6$) and 28 ($n = 6$) of pregnancy and compared with corpora lutea from Day 21 (late luteal) of the nonconception cycle ($n = 8$). Endothelial cell proliferation was measured by immunocytochemical detection of incorporated bromodeoxyuridine. Endothelial cell and pericyte area were assessed by quantitative immunocytochemistry for CD31 and α -smooth muscle actin, respectively. Vascular endothelial growth factor (VEGF) and its receptors, kinase insert domain-containing region (KDR) and *fms*-like tyrosine kinase (Flt) mRNA, were localized and quantified in situ hybridization. In addition, the effects of immunoneutralization of VEGF on establishment and maintenance of pregnancy were investigated by administering a VEGF neutralizing antibody on Days 0–10 of the luteal phase during potentially fertile cycles ($n = 10$) and compared with fertile controls ($n = 6$). No differences in the cellular or morphological parameters were found between pregnant and structurally intact nonpregnant corpora lutea. No major differences were found in expression of VEGF, Flt, or KDR in these CL. VEGF immunoneutralization markedly suppressed plasma progesterone secretion during treatment, but pregnancy rate was not significantly reduced. Thus, a role for VEGF in early pregnancy in the marmoset remains to be established. These results show that, by the late luteal phase in the marmoset, the corpus luteum has established a mature vascular system and the molecular capacity to synthesize VEGF and its receptors. A pregnancy-induced spurt of angiogenesis or gene expression does not appear to take place; rather, maintenance of the existing vasculature is all that is required for the establishment of pregnancy.

corpus luteum, corpus luteum function, ovary, ovulation, progesterone

INTRODUCTION

Angiogenesis, the formation of new blood vessels via endothelial replication, has been shown to be intense during the first few days of the lifespan of the corpus luteum (CL) in a large number of species studied, including women [1–4], and is principally dependent on vascular endothelial cell growth factor (VEGF) [5, 6].

¹Correspondence: H.M. Fraser, Medical Research Council Human Reproductive Sciences Unit, 49 Little France Crescent, Edinburgh EH16 4SB, U.K. FAX: 44 131 242 6231; e-mail: h.fraser@hrc.ac.uk

Received: 20 November 2001.

First decision: 26 December 2001.

Accepted: 6 May 2002.

© 2002 by the Society for the Study of Reproduction, Inc.

ISSN: 0006-3363. <http://www.biolreprod.org>

In the corpus luteum of early pregnancy, the changes in angiogenesis and the role of the luteal vasculature in the maintenance of progesterone secretion remain to be elucidated. When pharmacologic “rescue” of the CL by human chorionic gonadotrophin (hCG) treatment in vivo was carried out in rhesus monkeys, no stimulation of angiogenesis, as monitored by Ki-67 incorporation, was observed despite maintaining plasma progesterone concentrations [3]. In apparent contrast, in women similarly treated with hCG, a second wave of angiogenesis, accompanied by vascular stabilization by pericytes and increased VEGF expression, was associated with luteal rescue [7, 8]. These findings suggest that luteal angiogenesis could be a component of luteal rescue, which is important for prolonging the lifespan of the corpus luteum.

Because failure of the CL due to a malfunction of the vasculature might lead to miscarriage or infertility, it was considered important to establish a nonhuman primate model of early pregnancy in which this issue could be addressed. The marmoset monkey offers several potential advantages for such studies. First, it has an exceptionally high rate of fertility (>70%) when compared with other primates, so physiological pregnancy, rather than pharmacologic hCG rescue, may be studied [9]. Second, we have a detailed description of the cellular and molecular regulation of angiogenesis during the normal cycle [4]. Angiogenesis was therefore determined by comparing bromodeoxyuridine (BrdU) incorporation into corpora lutea of the late luteal phase versus early pregnancy. Vascular stability was monitored by determining pericyte area. Localization of expression of VEGF mRNA and its receptors, KDR and Flt, was determined and changes associated with pregnancy quantified using in situ hybridization. Finally, the effect of neutralization of VEGF on luteal function and pregnancy rate in mated marmosets was studied.

MATERIALS AND METHODS

Angiogenesis and Pericyte Accumulation in the Corpus Luteum of Early Pregnancy

Experiments were carried out under the Animals (Scientific Procedures) Act (1986) and approved by the Ethical Review Committee. Blood samples were collected from adult female marmosets three times per week and assayed for presence of ovulatory rises in plasma progesterone [10]. During the mid to late luteal phase of the second recorded cycle, prostaglandin (PG) analogue, 1 μ g PGF_{2 α} (cloprostenol, Planate, Coopers Animal Health Ltd., Crewe, UK) i.m., was administered to induce luteolysis so that timing of the subsequent ovulation could be identified accurately.

At the time of PG administration, a fertile male was introduced. Marmosets were humanely killed on Day 21 or 28 postovulation after receiving BrdU as described previously [5]. Of 17 animals mated, 12 were confirmed pregnant by the presence of a trophoblast in serial sections of the uterus and plasma levels of CG of >20 ng/ml. CG was measured using the assay described by Saltzman et al. [11] and had a detection limit of 12 ng/ml. According to these criteria, six animals on Day 21 and six on Day 28 were pregnant. The nonpregnant animals were studied on Day 21 together with three additional animals, recruited by carrying out the above procedure in the absence of a male. Day 21 is the mean time of luteolysis in the marmoset, but this is variable. Of the eight nonpregnant animals, three had structurally regressed CL and the remainder had structurally intact CL. Uteri were fixed in 4% paraformaldehyde (PFA). Ovaries were bisected and fixed, one half of each ovary in 4% paraformaldehyde and the other in 4% neutral buffered formalin (NBF). Experiments were initially carried out on both NBF- and PFA-fixed tissue, but on analysis, no significant difference was found between the two fixatives; thus, only results from NBF-fixed tissues are presented here.

Immunocytochemistry

Cellular responses were studied by 1) measuring cell size in sections stained with hematoxylin and eosin, 2) quantifying the number of mitotic cells stained for BrdU, 3) examining the establishment of the microvascular network using CD31 staining to identify endothelial cells, 4) studying the recruitment of pericytes using immunocytochemical staining of smooth muscle α -actin, and 5) dual labeling to record the incidence of colocalization of BrdU and CD31.

Tissue sections (5 μ m) were cut onto Super-frost plus slides (Sigma, Poole, Dorset, UK) for immunocytochemistry. Sections were dewaxed in xylene, rehydrated in descending concentrations of ethanol, and washed in distilled water. Antigen retrieval was performed by boiling sections in a Tefal Clypso pressure cooker (Tefal, Essex, UK) 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, pH 7.4, NaCl₂ 9 g/L). To reduce nonspecific binding, sections were blocked in normal rabbit serum (diluted 1:5 in TBS) for 30 min. The primary antibodies used were CD31 (monoclonal; Dako, Denmark; diluted 1:20 in TBS), BrdU (monoclonal; Boehringer, Mannheim, Germany; diluted 1:30 in TBS), and α -smooth muscle actin (monoclonal; Dako, Denmark; diluted 1:20 in TBS). Incubation was carried out overnight at 4°C. Slides were then washed three times in TBS. Incubation with the secondary antibody (rabbit anti-mouse Ig; Dako, 1:60 diluted in TBS) was for 40 min at room temperature. This was followed by two TBS washes and incubation of the APAAP complex (Dako; 1:100 dilution in normal rabbit serum and TBS) for 40 min at room temperature. Visualization was performed using nitroblue tetrazolium (NBT) solution containing 45 μ l NBT substrate (Boehringer Mannheim), 10 ml NBT buffer, 35 μ l Xphosphate (Boehringer Mannheim), and 10 μ l levamisole (Sigma). Sections for BrdU were counterstained with hematoxylin (Richard-Allan, Richland, MI), whereas sections for CD31 and smooth muscle actin were not counterstained so that quantitative image analysis could be performed. For dual labeling, slides were incubated first with CD31 and visualized with fast red (Sigma), followed by incubation with BrdU and visualized with NBT as described above. Fast red solution contained 1 mg fast red/ml buffer (20 mg naphthol AS-MX phosphate, 2 ml dimethyl formamide, 98 ml 0.1 M Tris, pH 8.2; Sigma).

Localization of VEGF, Flt, and KDR mRNA in the Corpus Luteum

To investigate possible changes in expression patterns of the mRNA for angiogenic factors and their receptors in the corpus luteum of pregnancy, in situ hybridization was performed as described previously using complementary RNA probes for human VEGF A, Flt, and KDR [8]. Sense and antisense probes were prepared using an RNA transcription kit (Ambion, Austin, TX) and labeled with 35S uridine 5'-triphosphate (NEN, Boston, MA). Deparaffinized sections were treated with 0.1 N HCl for 20 min and then digested in proteinase K (5 μ g/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 at 55°C. High stringency posthybridization washings and ribonuclease A treatment were used to remove excess probe. Slides were then dehydrated, dried, and dipped in Ilford G5 liquid emulsion (H.A. West, Edinburgh, UK). Exposure times for VEGF, Flt, and KDR were 5, 9 and 9 wk, respectively. Slides were subsequently developed (Kodak D19 developer, Kodak, Rochester, NY) and fixed (Ko-

dak GBS, Kodak). All slides were counterstained with hematoxylin, dehydrated, and mounted.

Quantification

Quantification of lutein cell area. Hematoxylin and eosin-stained sections were examined under a 40 \times objective lens, images captured, and lutein cells identified according to morphologic appearance. Cell perimeter was accurately determined and cross-sectional area measured. Ten lutein cells from each of 10 fields were randomly selected from ovarian cross-sections from each animal.

Quantification of BrdU staining. Sections were examined using a 20 \times objective lens. Image Pro-Plus was used to measure the number of dark-stained nuclei only (those with incorporated BrdU) and the number of total cells per field (dark- and light-stained nuclei). These parameters were measured across the whole cross-section of the CL. To obtain a proliferation index, the number of BrdU-positive cells was expressed as a percentage of total cell nuclei per field of view. Mean values per animal were recorded.

Quantification of CD31 and smooth muscle α -actin. Sections were examined using a 20 \times objective lens and the area of CD31 and smooth muscle α -actin staining recorded across the whole corpus luteum. Because immunostaining was localized either to the cytoplasm or plasma membrane, visualization of individual cells was difficult; thus, it was not possible to measure absolute numbers of endothelial cells or pericytes. The image analysis system was therefore set to measure the area of staining within a given field of view. Mean values were calculated per animal.

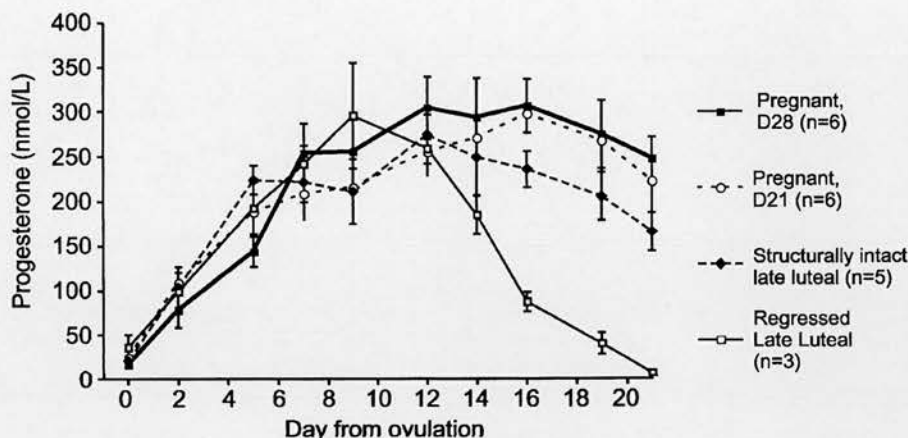
Quantification of dual labeling (BrdU and CD31). Dual labeling for BrdU and CD31 enabled the percentage of proliferating cells that were endothelial to be determined. Total incidences of dual staining were recorded and expressed as a percentage of total number of proliferating cells throughout the CL. Three animals from each group were assessed and a mean percentage calculated.

Quantification of VEGF, Flt, and KDR in situ. Quantification of VEGF, Flt, and KDR mRNA was carried out using an image-analysis macro designed to distinguish silver grains in dark field at 20 \times objective magnification against the black background of the tissue. Both grain area per field of view (μ m²), giving a relative estimate of number of cells per field ($3.2 \times 10^5 \mu$ m²) and grain density/ μ m², representative of amount of expression per cell, were recorded.

Effects of Immunoneutralization of VEGF on Establishment of Pregnancy

In a second experiment, we sought to investigate the role of luteal VEGF in the establishment and maintenance of early pregnancy by inhibiting its action with a neutralizing antibody in vivo. The experiment was designed so that effects could be monitored by measuring plasma progesterone concentrations during the first third of pregnancy and by recording number of live births. Females used in this experiment had all previously given birth to live young on at least two occasions. Each female had been continually housed with the same male for over a year in stable family groups consisting of the dominant female and male together with their offspring. In these conditions, the marmoset is a highly fecund species, commonly ovulating within 2 wk postpartum and becoming pregnant during the first postpartum cycle [9]. This was confirmed in the animals studied; in the pregnancy prior to treatment, 13 out of 16 (81%) had had an interbirth interval of 150–164 days (normal gestation length being 144 days). These animals therefore represented a potentially highly efficient group in which to study the effect of anti-VEGF treatment on incidence of pregnancy. Animals in which vaginal lavage had shown the presence of sperm on Day 7 or 9 postpartum were treated starting at Day 10 postpartum, i.e., around the time of anticipated ovulation. Ten marmosets received VEGF antibody in the regime described previously [5], i.e., 2 mg i.v. on Day 10, followed by 1 mg on Days 11, 12, 13, 15, 17, and 19, while six controls received the same dose of mouse gamma globulin (Sigma). Blood samples were collected daily from 2 days prior to onset of treatment (Day 0) to treatment Day 10, then three times per week until Day 40, and plasma assayed for progesterone concentration. This treatment had previously been shown to inhibit the development of the luteal microvascular tree and suppress plasma progesterone in normal cycles [5]. In the current study, suppression of progesterone was taken as an index of effectiveness of inhibition of luteal angiogenesis by VEGF antibody. Thus, the end point of the study was whether pregnancy rate was affected. The day of delivery, total number of offspring, and surviving offspring were recorded for each animal.

FIG. 1. Plasma progesterone concentrations from ovulation to Day 21 in nonpregnant and pregnant animals. Values are means \pm SEM ($n = 6$, Days 21 and 28 of pregnancy; $n = 5$, Day 21 structurally intact; and $n = 3$ in Day 21 regressed). In the five nonpregnant animals with structurally intact CL, elevated progesterone levels continued up to Day 21 despite anticipated luteal regression. Progesterone levels from three nonpregnant animals with structurally regressed CL, however, had declined to follicular phase levels by Day 21.



Statistical Analyses

Separate one-way analysis of variance (ANOVA) tests were conducted for each set of data and a Bonferroni post hoc test performed. $P < 0.05$ was taken as the level of significance for each test. Tests were performed using SPSS 10 for Macintosh. For determining differences in plasma progesterone between groups, repeated ANOVAs were used to compare study groups on each day of blood sampling.

To compare the number of successful pregnancies after anti-VEGF or mouse gamma globulin treatment, the Fisher exact test was used.

RESULTS

Plasma Progesterone and CG during Early Pregnancy

Pregnancy was confirmed by the presence of a trophoblast and CG levels greater than 20 ng/ml, while nonpregnant animals had a uterus compatible with an infertile cycle and nondetectable levels of CG. Plasma progesterone profiles of pregnant and nonpregnant marmosets from ovulation (Day 0) to Day 21 are shown in Figure 1. The late luteal nonpregnant animals were subdivided into two groups according to whether functional and structural regression had taken place by Day 21. In five animals, plasma progesterone levels were maintained and CL were structurally intact despite being anticipated to shortly undergo functional and structural regression. The nonregressed state of the CL was not dependent on a male being present, as one of this group included an animal that was housed in the absence of a male and we have commonly observed this phenomenon in other females in our colony. Three of the late luteal control animals had undergone functional and structural regression accompanied by low progesterone levels by Day 21. In these animals, there was a significant decline in plasma progesterone compared with both the pregnant and control animals.

Following the initial rise in progesterone indicative of ovulation, progesterone levels remained elevated (>150 nmol/L) in all four groups until Day 10–11 postovulation, which corresponds to the time of implantation in marmosets [12]. After Day 14, the regressed late luteal control animals showed declining progesterone for the remainder of the recorded cycle, whereas a continued elevation of progesterone was observed in both groups of pregnant animals and the structurally intact control animals. Progesterone remained elevated between Days 21 and 28 in the Day 28 pregnant marmosets (data not shown). Statistical analysis revealed that progesterone levels in the regressed control group were significantly different from the structurally intact controls and both groups of pregnant animals ($P < 0.05$) from Day 19.

Lutein Cell Area

Cross-sectional area of individual lutein cells was not significantly different ($P > 0.05$) between structurally intact nonpregnant CL and the pregnant animals at Days 21 and 28 postovulation (492 ± 26 , 530 ± 21 , and 584 ± 34 , respectively), while those in the regressed CL showed variable degrees of involution and degenerative change, which precluded meaningful measurements (Fig. 2).

BrdU Immunostaining

BrdU immunostaining indicating proliferating cells was readily detected in all corpora lutea. Previously published data [4] from the early, mid and late luteal phase are included in the graph to show the changes in endothelial cell proliferation throughout the luteal phase of the cycle (Fig. 3, A and B). An intense burst of angiogenesis with high endothelial cell proliferation is observed in the early luteal phase, decreasing in the mid luteal phase, and further still toward the end of the luteal phase. No significant difference ($P > 0.05$) was observed between structurally intact late luteal and pregnant CL. However, significantly higher ($P < 0.05$) proliferation indices were observed in regressed CL. The insert (Fig. 3a) illustrates the colocalization of CD31 and BrdU immunostaining, i.e., proliferating endothelial cells. Quantification of intact CL showed that more than 90% of proliferating cells were endothelial cells. In structurally regressed CL, it was not possible to determine whether or not these cells were endothelial due to the diffuse nature of the CD31 staining.

CD31 Immunostaining

Measurement of CD31 was used to quantify endothelial cell area. There was no significant difference between groups ($P > 0.05$) (Fig. 3, C and D). By the late luteal phase, the microvascular tree of the corpus luteum is extensive, and from the photomicrograph, it can be seen that every lutein cell is in contact with at least one endothelial cell. Structurally regressed CL were not included in the quantitative analysis because of poor structural integrity, leading to diffuse staining.

Pericyte Immunostaining

Immunostaining for α -smooth muscle actin was evident in all stages, predominantly in large luminal vessels but also to a lesser extent in smaller vessels and capillaries (Fig. 3, E and F). No significant difference in endothelium cov-

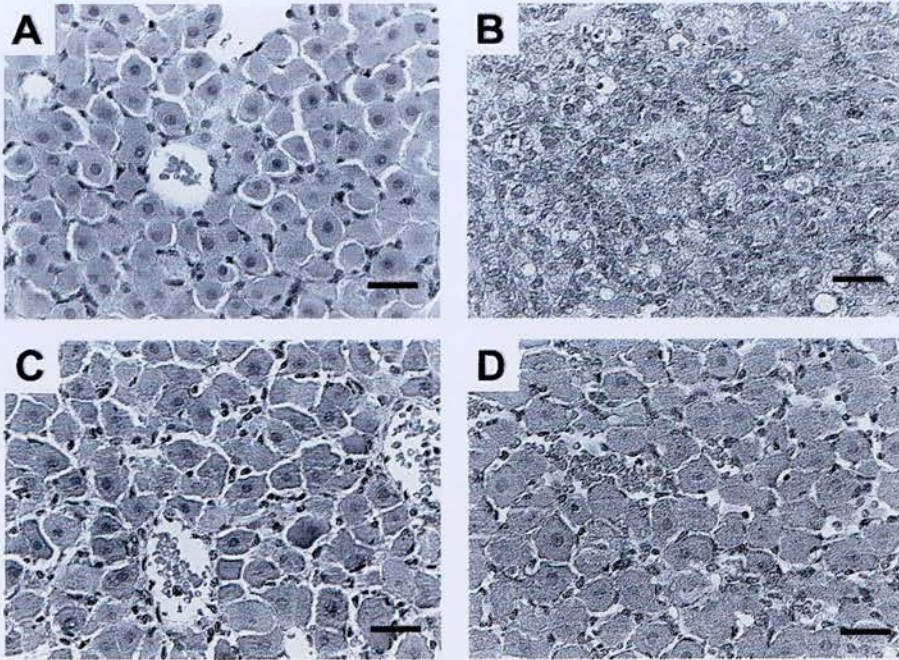


FIG. 2. Hematoxylin and eosin-stained sections from structurally intact late luteal CL (n = 5) (A), structurally regressed CL (n = 3) (B), Day 21 pregnant CL (n = 6) (C), and Day 28 pregnant CL (n = 6) (D). Bars = 50 μ m.

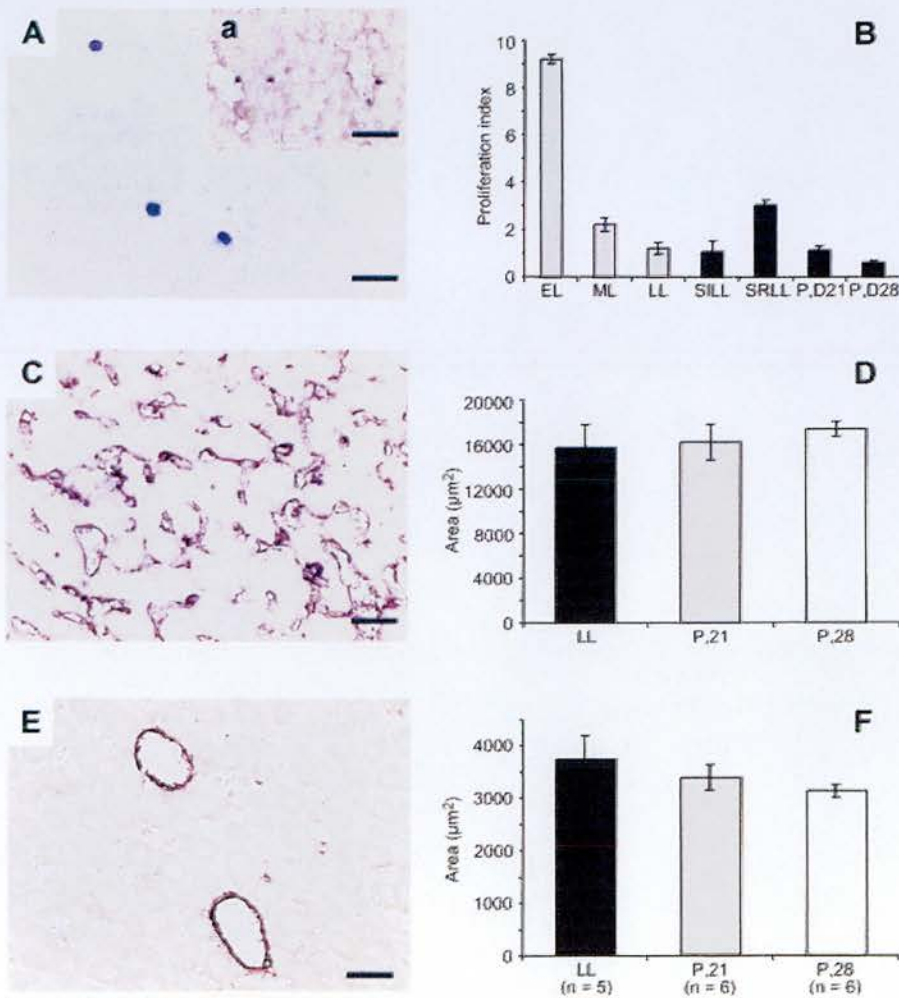
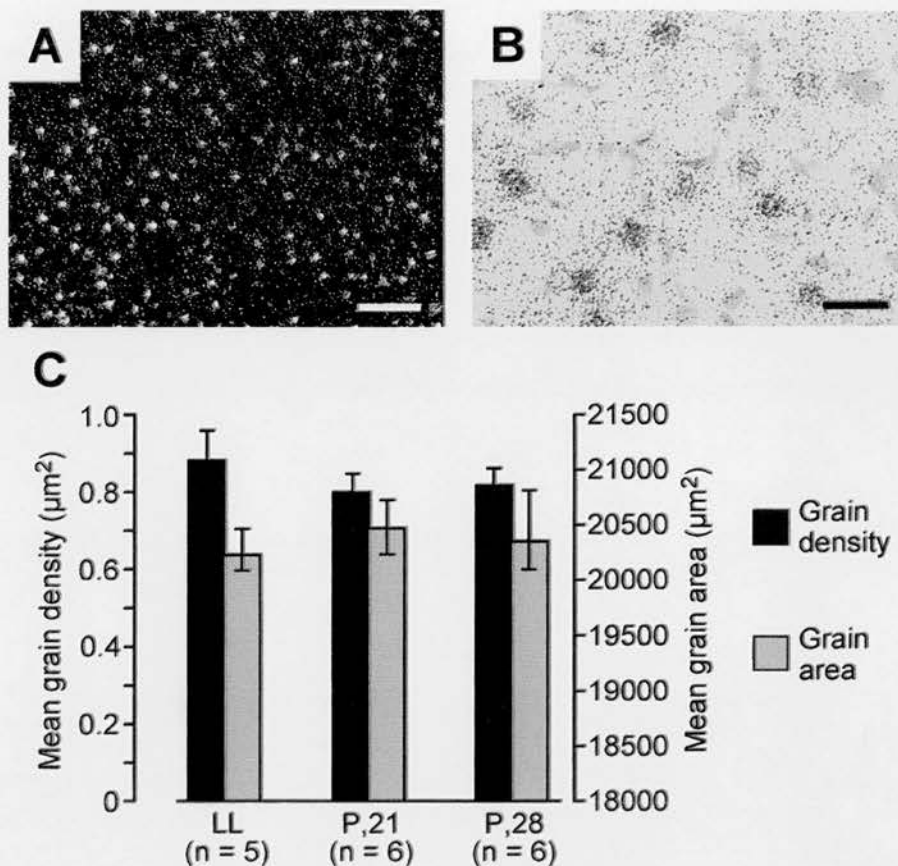


FIG. 3. A) Cell proliferation in the marmoset corpus luteum ($\times 40$ objective). Insert (a) shows colocalization of CD31 and BrdU ($\times 20$ objective). B) Mean proliferation index in corpora lutea of animals from the different study groups, structurally intact late luteal (SILL), structurally regressed late luteal (SRLL), Day 21 and Day 28 pregnant animals (P,D21 and P,D28, respectively). Previously published data from the early, mid, and late luteal phase are included in this graph. C) Immunocytochemical localization of endothelial cell marker CD31 ($\times 40$ objective). D) Mean endothelial cell area in corpora lutea of animals from the different study groups. E) Immunocytochemical localization of smooth muscle α -actin marker ($\times 40$ objective). F) Mean pericyte area in corpora lutea of animals from the different study groups. Bar = 100 μ m in a, 50 μ m in A, C, and E. Values are means \pm SEM. In all photographs and graphs, n = 5 for structurally intact CL, n = 6 for Day 21 pregnant, and n = 6 for Day 28 pregnant.

FIG. 4. Representative photomicrographs showing localization of VEGF mRNA to lutein cells by in situ hybridization in the CL (A, dark field; B, higher power light field). Structurally intact and pregnant CL were similar. Bars = 50 μm in A and 25 μm in B. C) Mean grain density/ μm^2 and mean grain area (μm^2) in nonpregnant and pregnant CL. No significant difference was seen between these groups. Values are means \pm SEM.



erage by pericytes ($P > 0.05$) was seen between study groups. Again, structurally regressed CL were not quantified because of diffuse staining.

Vascular Endothelial Growth Factor

Intense hybridization to VEGF mRNA was localized to lutein cells, with absence of hybridization to endothelial cells. Distinct, punctate hybridization in lutein cells was clearly seen in structurally intact control and pregnant CL (Fig. 4, A and B). There was no significant difference ($P > 0.05$) in either mean grain area or mean grain density between the groups (Fig. 4C). In the structurally regressed CL, most of the lutein cells were devoid of punctate VEGF expression and there was a marked reduction in grain area and density (Fig. 5, A and B).

Kinase Insert Domain-Containing Region and Flt

Hybridization to KDR mRNA was localized to endothelial cells (Fig. 6, A and B). No significant difference in grain area or density was observed between groups ($P > 0.05$) as illustrated by Figure 6E. Structurally intact and regressed nonpregnant CL were analyzed independently but, in contrast with VEGF, KDR expression was maintained in the regressed CL. Because grain area and density were the same in each group, the results were combined.

Hybridization to Flt mRNA was localized to endothelial cells (Fig. 6, D and E). A significantly lower mean grain area was observed in Day 28 pregnant corpora lutea than in Day 21 luteal animals ($P < 0.05$), as illustrated by Figure 6F. However, no significant difference in grain area was

seen between late luteal and pregnant Day 21 animals nor was there any observable difference in grain density between groups. As above, late luteal regressed and intact controls were combined because there was no difference in Flt expression between them.

Effects of Immunoneutralization of VEGF on Progesterone and Pregnancy Rate

Plasma progesterone profiles for pregnant control and anti-VEGF-treated marmosets from the time of postpartum ovulation are shown in Figure 7. In control marmosets, progesterone rises indicative of ovulation and establishment of early pregnancy were observed in five of the six animals (83%), with ovulation occurring, as expected, on Days 8–11 postpartum. In the treated animals, all 10 ovulated 9–11 days postpartum, as indicated by a rise in plasma progesterone, but during the treatment period, this level was significantly lower than in control animals ($P < 0.05$). In five marmosets in this group, the posttreatment period was associated with a variable degree of apparent partial recovery of progesterone, which was followed by functional luteolysis around Day 20–25 postovulation. These animals were classified as the delayed-pregnancy group (because they did not become pregnant during the treatment cycle but conception occurred during the subsequent cycle). In the remaining five treated animals, a more robust recovery of plasma progesterone was evident, followed by a sustained rise, reaching the same levels as attained in the pregnant control animals by Day 20–25. Because gestational length subsequently confirmed that these animals had become pregnant within the treatment cycle, they were thus

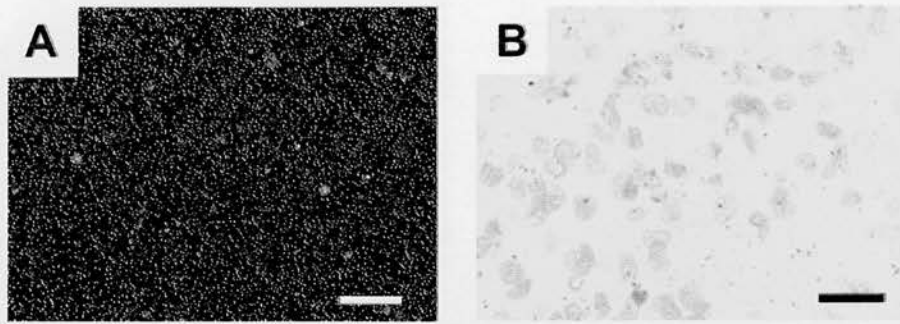


FIG. 5. Depiction of VEGF mRNA localization by in situ hybridization in structurally regressed nonpregnant CL (A, dark field; B, light field). Note low level of cellular localization. Bars = 50 μm in A and 25 μm in B.

classified as having no delayed pregnancy. A Fisher exact test to compare number of pregnancies achieved between the 6 control and 10 treated animals revealed no significant difference ($P > 0.05$). Plasma progesterone in the delayed pregnancy group continued to be significantly lower than controls until Day 27, when levels began to rise again, indicating ovulation, and all these animals became pregnant in the subsequent cycle.

Interbirth interval was recorded for all animals. Using the Student *t*-test, the 1-mo delay in conception observed in 50% of treated animals (188 days \pm 1.5 SEM) was significantly different from the interbirth interval seen in controls (157 days \pm 0.89 SEM) ($P < 0.05$). In the treated

group in which pregnancy was not delayed, the interbirth interval was not significantly different from controls (159 days \pm 1.8 SEM). The number of offspring was also compared for each group. The delayed pregnancy group had a significantly higher number of live births than the control group ($P < 0.05$) (Fig. 8). No abnormalities were observed in any of the offspring.

DISCUSSION

This study has described the cellular and morphological changes in the corpus luteum associated with early pregnancy in the marmoset, a commonly used species in repro-

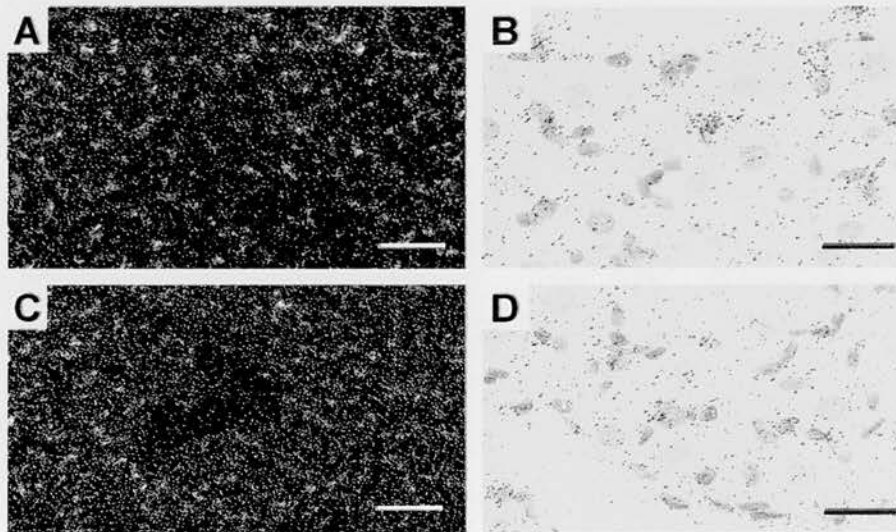


FIG. 6. KDR mRNA localization (A, dark field; B, light field). Dark field shows overall grain distribution (bar = 50 μm). Light field shows cellular localization to endothelial cells (bar = 25 μm). E) Mean grain density/ μm^2 and mean grain area (μm^2) for KDR in nonpregnant and pregnant CL. Depiction of Flt mRNA localization to endothelial cells (C, dark field; D, light field); mean grain density/ μm^2 and mean grain area (μm^2) for Flt in nonpregnant and pregnant CL (F). Values are means \pm SEM.

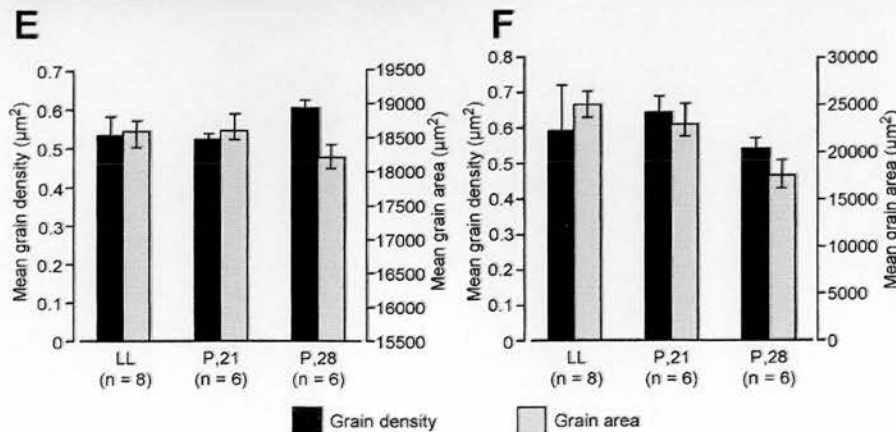
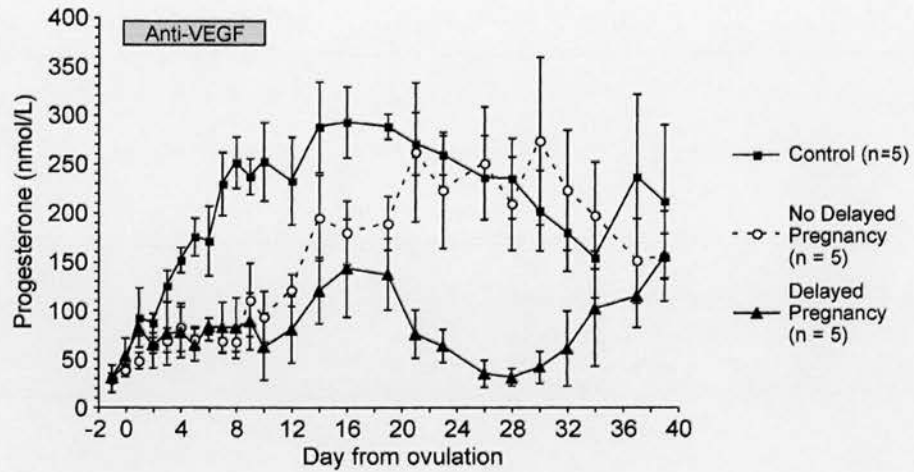


FIG. 7. Plasma progesterone concentrations in control (n = 5, ■—■) and marmosets treated with anti-VEGF on Days 0–10 of the luteal phase (n = 10). Values are means \pm SEM. Progesterone was significantly suppressed by treatment. After treatment, a degree of recovery in progesterone occurred. Five of the 10 treated animals became pregnant during the treatment cycle (○---○). In the remaining five animals (▲—▲), no recovery of progesterone was observed; thus, luteal development was blocked and they did not conceive until the subsequent cycle.



ductive research. Neither luteal cell area, endothelial cell area, pericyte area, or endothelial cell proliferation differed in early pregnancy from the late luteal structurally intact CL of the nonpregnant cycle. In addition, levels of VEGF, KDR, and Flt mRNA were largely unaltered. Thus, a further burst of angiogenesis does not occur in early pregnancy in the marmoset. It would appear that the late, pregressed luteal vasculature is equipped with the molecular and cellular components to perform the functions of the vasculature of the CL of early pregnancy.

In the human corpus luteum, the pattern of endothelial cell proliferation is similar to that of the marmoset in the early, mid, and late luteal phase, with maximal proliferation occurring in the early luteal phase and declining in the mid and late luteal phase [2, 13]. This is also true in the rhesus monkey [3], sheep [14, 15], and cattle [1]. During pharmacologically induced luteal rescue in women by hCG, an increase in endothelial cell proliferation was observed [7], accompanied by an increase in VEGF expression [7, 8]. In addition, when changes in luteal cell volume were taken into account, an increase in endothelial cell and pericyte area on luteal sections was found [7]. Taken together, these findings suggest that, in the human CL, hCG treatment results in increased angiogenesis accompanied by vessel stabilization [7]. This appears to mirror the situation observed in rats, where endothelial cell proliferation is also seen to increase in early pregnancy [16, 17]. In contrast, the ap-

parent absence of an increase in endothelial cell proliferation in early pregnancy in the marmoset is similar to findings in the sheep [14] and, after hCG-induced rescue of the CL, in the rhesus monkey [3].

The rescue of the corpus luteum of pregnancy allows it to persist functionally and structurally for longer than in the nonpregnant cycle. Presumably, this survival requires a stable vasculature, with increased recruitment of pericytes and prolonged endothelial cell survival in addition to prolongation of the lifespan of the luteal cells. Because endothelial cell proliferation did not increase after administration of hCG in the rhesus monkey [3], it was suggested that early pregnancy may be associated with an increase in vessel stability, and there is recent evidence for an hCG-induced increase in pericyte number in the human CL [7]. However, we found no evidence for this in the marmoset.

The increase in pericyte and endothelial cell area after hCG in the human was dependent on taking account of the increase in lutein cell volume after hCG administration [7]. However, in the pregnant marmoset, no such increase in lutein cell volume was observed. This is consistent with the findings of Webley et al. [18], who reported that size distribution of luteal cells from pregnant and nonpregnant marmosets revealed an increase in mean cell diameter from luteal Day 6 to 14 in both pregnant and nonpregnant animals but no further increase on Day 20 [18].

While in the structurally regressed CL, VEGF mRNA expression was markedly reduced, there was no apparent difference in VEGF expression between structurally intact late luteal controls and pregnant corpora lutea in the marmoset. VEGF expression is maintained in the CL of pregnancy in a number of species, implying a role in either angiogenesis or endothelial cell survival [8, 19, 20]. A significant increase in VEGF expression, compared with the late luteal phase, was found in hCG rescued human CL using the same method as in the present study [7]. Cell culture studies using human luteinized granulosa cells have also revealed that hCG treatment leads to increased VEGF mRNA levels [21]. This reported up-regulation of VEGF by hCG rescue supports the view that, in the human, pregnancy is associated with further angiogenesis. The absence of up-regulation of VEGF mRNA during early pregnancy in the marmoset is similar to observations in the early pregnant CL of the cow [20]. It is also of interest that there is no evidence for a decline in VEGF mRNA preceding an-

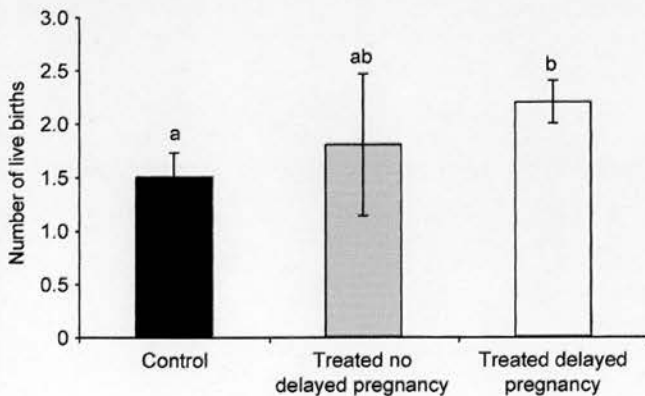


FIG. 8. Number of live births from the different study groups. Significant differences are represented by different letters.

anticipated luteolysis; rather, the decrease of VEGF mRNA was only observed at the time of structural regression.

Levels of KDR mRNA also did not change in the pregnant animals. Flt mRNA was unchanged in the Day 21 pregnant group but significantly decreased in the Day 28 animals compared with the nonpregnant controls. This could be explained by the fact that Flt expression is associated with proliferating endothelial cells, of which there are more in the earlier luteal phases of the cycle. Therefore, by 28 days postovulation, the endothelial cells from these earlier phases may have passed the stage of maximal Flt expression. In the human CL, RT-PCR demonstrated that there was no increase in KDR or Flt mRNA levels in the CL of pregnancy compared with the nonpregnant cycle [22]. Thus, luteal rescue is not associated with an increase in VEGF receptors. These results lead us to propose that the structurally intact late luteal CL of the marmoset retains its molecular integrity and is similar to the rescued CL with regard to VEGF mRNA and its receptors until such time as it does undergo structural regression.

In view of these results, it was considered that a period of intense angiogenesis may be occurring earlier than Day 21, e.g., at around Day 14 of early pregnancy, soon after implantation of the blastocyst on Day 11. However, a pilot study, examining endothelial cell proliferation in the CL from animals at Day 14 of pregnancy, failed to demonstrate increased angiogenesis at this time (unpublished data).

While we have focused on VEGF and its receptors in this study, it is possible that other angiogenic factors may be involved in regulation of the vasculature of early pregnancy. Because the angiopoietins are thought to participate in stabilization of blood vessels, we examined mRNA levels for Angiopoietin-1 and -2 and their receptor, Tie-2, as described for the human CL [7]. However, low expression of the angiopoietins was observed and, as with VEGF, there was no difference between rescued and regressing CL (unpublished data).

The length of the luteal phase in the nonpregnant marmosets varied. This was advantageous here because it allowed for the study of both structurally intact nonpregnant and structurally regressed CL, but the cause of this variation is unclear. Some animals have functionally and structurally regressed CL by Day 21, whereas other nonpregnant animals are still functionally and structurally active although anticipated to shortly undergo regression. Recent estimations from our colony have shown luteal length to vary between 16 and 24 days (unpublished data), which is consistent with previous reports on variability in luteal phase length [23–25]. It is well established that progesterone levels are maintained due to the continued responsiveness of luteal cells to gonadotropins via CG in pregnancy [26]. It would appear that, in the marmoset, despite the absence of CG, the late luteal CL of a nonpregnant cycle is maintained until it undergoes a rapid regression and thus decline in progesterone production. Luteal regression in the marmoset must therefore be a rapidly triggered event because, until that point, the CL of a nonfertile cycle retains its morphology and functional capacity to the same extent as in a pregnant cycle. As reported previously, increased BrdU incorporation was observed in the structurally regressed CL. It was not possible to determine whether or not these cells were endothelial due to the diffuse nature of the CD31 staining in these animals. It may be that these cells are fibroblasts, but this remains to be elucidated [27].

In view of the fact that the period of intense luteal angiogenesis in the fertile cycle of the marmoset is restricted

to the early luteal phase, the effects of inhibiting VEGF during this period were examined in animals in stable family groups containing a fertile male. A previous study in marmosets not exposed to pregnancy had shown that such treatment inhibited angiogenesis, prevented formation of the microvascular tree, and suppressed plasma progesterone by more than 60% [5]. The present results confirmed that the anti-VEGF treatment suppressed plasma progesterone in all animals. However, after cessation of treatment, a degree of recovery of progesterone secretion was observed. While in 5 of the 10 treated marmosets, luteal regression subsequently occurred, in the remainder, the corpus luteum function recovered and these animals went on to deliver offspring after a similar gestation period to the controls. The fact that pregnancy was successfully established in the face of a marked reduction in plasma progesterone concentrations suggests that, in the marmoset, the normal quota of luteal progesterone is in excess of requirement. Furthermore, the rise in plasma progesterone posttreatment in half of the animals implies that angiogenesis is capable of reinitiation after cessation of VEGF inhibition. It is of particular interest that, despite systemic administration of the antibody to VEGF, implantation must have taken place during the treatment cycle, gestation length was normal, and no abnormalities were observed in the offspring. Because VEGF is likely to be involved in implantation, it may be that the large molecular weight of the antibody prevented access to the fetal-placental unit.

Where delayed pregnancy occurred, the resulting number of live births in the subsequent cycle was significantly higher than in controls. This may be the result of an overcompensation in VEGF production during the treatment period, resulting in the development of more antral follicles and thus higher numbers of offspring per animal in the following cycle.

This study has demonstrated that, by the late luteal phase in the marmoset, the corpus luteum has already established a mature vascular system and the molecular capacity to synthesize VEGF and its receptors, which are not further enhanced in early pregnancy. A pregnancy-induced spurt of angiogenesis does not take place; rather, a maintenance of the existing vasculature is all that is required for establishment of pregnancy in the marmoset. Therefore, it has to be concluded that the changing morphological and cellular events thought to be associated with early pregnancy in the human after hCG rescue do not occur in the marmoset.

ACKNOWLEDGMENTS

We thank I. Swanston and F. Pitt for assays, Dr. W.C. Duncan for critical evaluation of the manuscript, Dr. S.J. Charnock-Jones for the gift of complementary DNA probes, and Dr. J. Roser, Dr. A.L. Parlow, and the National Hormone and Pituitary Program for reagents for the CG assay.

REFERENCES

1. Zheng J, Fricke PM, Reynolds LP, Redmer DA. Evaluation of growth, cell proliferation, and cell death in bovine corpora lutea throughout the estrous cycle. *Biol Reprod* 1994; 51:623–632.
2. Rodger FE, Young FM, Fraser HM, Illingworth PJ. Endothelial cell proliferation follows the mid-cycle luteinizing hormone surge, but not human chorionic gonadotrophin rescue, in the human corpus luteum. *Hum Reprod* 1997; 12:1723–1729.
3. Christenson LK, Stouffer RL. Proliferation of microvascular endothelial cells in the primate corpus luteum during the menstrual cycle and simulated early pregnancy. *Endocrinology* 1996; 137:367–374.
4. Dickson SE, Fraser HM. Inhibition of early luteal angiogenesis by gonadotropin-releasing hormone antagonist treatment in the primate. *J Clin Endocrinol Metab* 2000; 85:2339–2344.

5. Fraser HM, Dickson SE, Lunn SF, Wulff C, Morris KD, Carroll VA, Bicknell R. Suppression of luteal angiogenesis in the primate after neutralization of vascular endothelial growth factor. *Endocrinology* 2000; 141:995-1000.
6. Dickson SE, Bicknell R, Fraser HM. Mid-luteal angiogenesis and function in the primate is dependent on vascular endothelial growth factor. *J Endocrinol* 2001; 168:409-416.
7. Wulff C, Dickson SE, Duncan WC, Fraser HM. Angiogenesis in the human corpus luteum: simulated early pregnancy by hCG treatment is associated with both angiogenesis and vessel stabilization. *Hum Reprod* 2001; 16:2515-2524.
8. Wulff C, Wilson H, Lague P, Duncan WC, Armstrong DG, Fraser HM. Angiogenesis in the human corpus luteum: localization and changes in angiopoietins, tie-2, and vascular endothelial growth factor messenger ribonucleic acid. *J Clin Endocrinol Metab* 2000; 85:4302-4309.
9. Fraser HM, Recio R, Conn PM, Lunn SF. Gonadotropin-releasing hormone antagonist for postpartum contraception: outcome for the mother and male offspring in the marmoset. *J Clin Endocrinol Metab* 1994; 78:121-125.
10. Wulff C, Wilson H, Rudge JS, Wiegand SJ, Lunn SF, Fraser HM. Luteal angiogenesis: prevention and intervention by treatment with vascular endothelial growth factor trap(A40). *J Clin Endocrinol Metab* 2001; 86:3377-3386.
11. Saltzman W, Schultz-Darken NJ, Wegner FH, Wittwer DJ, Abbott DH. Suppression of cortisol levels in subordinate female marmosets: reproductive and social contributions. *Horm Behav* 1998; 33:58-74.
12. Enders A. Overview of the morphology of implantation in primates. In: Wolf DP, Stouffer RL, Brenner RM (eds.), *In Vitro Fertilisation and Embryo Transfer in Primates*. New York: Springer-Verlag; 1993: 145-157.
13. Gaytán F, Morales C, García-Pardo L, Reymundo C, Bellido C, Sánchez-Criado JE. Macrophages, cell proliferation, and cell death in the human menstrual corpus luteum. *Biol Reprod* 1998; 59:417-425.
14. Jablonka-Shariff A, Grazul-Bilska AT, Redmer DA, Reynolds LP. Cellular proliferation and fibroblast growth factors in the corpus luteum during early pregnancy in ewes. *Growth Factors* 1997; 14:15-23.
15. Jablonka-Shariff A, Grazul-Bilska AT, Redmer DA, Reynolds LP. Growth and cellular proliferation of ovine corpora lutea throughout the estrous cycle. *Endocrinology* 1993; 133:1871-1879.
16. Tamura H, Greenwald GS. Angiogenesis and its hormonal control in the corpus luteum of the pregnant rat. *Biol Reprod* 1987; 36:1149-1154.
17. Meyer GT, McGeachie JK. Angiogenesis in the developing corpus luteum of pregnant rats: a stereologic and autoradiographic study. *Anat Rec* 1988; 222:18-25.
18. Webley GE, Richardson MC, Smith CA, Masson GM, Hearn JP. Size distribution of luteal cells from pregnant and non-pregnant marmoset monkeys and a comparison of the morphology of marmoset luteal cells with those from the human corpus luteum. *J Reprod Fertil* 1990; 90:427-437.
19. Otani N, Minami S, Yamoto M, Shikone T, Otani H, Nishiyama R, Otani T, Nakano R. The vascular endothelial growth factor/fms-like tyrosine kinase system in human ovary during the menstrual cycle and early pregnancy. *J Clin Endocrinol Metab* 1999; 84:3845-3851.
20. Berisha B, Schams D, Kosmann M, Amselgruber W, Einspanier R. Expression and tissue concentration of vascular endothelial growth factor, its receptors, and localization in the bovine corpus luteum during estrous cycle and pregnancy. *Biol Reprod* 2000; 63:1106-1114.
21. Neulen J, Raczek S, Pogorzelski M, Grunwald K, Yeo TK, Dvorak HF, Weich HA, Breckwoldt M. Secretion of vascular endothelial growth factor/vascular permeability factor from human luteinized granulosa cells is human chorionic gonadotrophin dependent. *Mol Hum Reprod* 1998; 4:203-206.
22. Sugino N, Kashida S, Takiguchi S, Karube A, Kato H. Expression of vascular endothelial growth factor and its receptors in the human corpus luteum during the menstrual cycle and in early pregnancy. *J Clin Endocrinol Metab* 2000; 85:3919-3924.
23. Harding R, Hulme MJ, Lunn SF, Henderson S, Aitken RJ. Plasma progesterone levels throughout the ovarian cycle of the common marmoset (*Callithrix jacchus*). *J Med Primatol* 1982; 11:43-51.
24. Harlow C, Hearn JP, Hodges JK. Ovulation in the marmoset monkey: endocrinology, prediction and detection. *J Endocrinol* 1984; 103:17-24.
25. Summers PM, Wennink CJ, Hodges JK. Cloprostenol-induced luteolysis in the marmoset monkey (*Callithrix jacchus*). *J Reprod Fertil* 1985; 73:133-138.
26. Stouffer RL, Nixon WE, Hodgen GD. The refractory state of luteal cells isolated from rhesus monkeys after prolonged exposure to chorionic gonadotropin during early pregnancy. *Biol Reprod* 1978; 18: 858-864.
27. Young FM, Rodger FE, Illingworth PJ, Fraser HM. Cell proliferation and vascular morphology in the marmoset corpus luteum. *Hum Reprod* 2000; 15:557-566.

Localization of mRNA for vascular endothelial growth factor (VEGF), angiopoietins and their receptors during the peri-implantation period and early pregnancy in marmosets (*Callithrix jacchus*)

A. J. Rowe¹, C. Wulff² and H. M. Fraser^{1*}

¹Medical Research Council Human Reproductive Sciences Unit, Centre for Reproductive Biology, University of Edinburgh, Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, UK; and

²Department of Obstetrics and Gynaecology, University of Ulm, Prittwitzstrasse 43, 89075 Ulm, Germany

Implantation of a blastocyst into a receptive endometrium is a prerequisite for successful pregnancy. Angiogenesis is a key event in this process but the mechanisms by which localized changes in vascular permeability and angiogenesis occur have yet to be elucidated. Vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEGFR-2 have been implicated as key players in vascular remodelling and placentation. Angiopoietins also appear to have a significant role in regulation of blood vessel growth, maturation and regression. The aim of this study was to describe the molecular regulation of angiogenesis in the first month of pregnancy in marmosets and to address the putative physiological roles for these factors. Uteri were studied at weeks 2, 3 and 4 of pregnancy and compared with late secretory non-pregnant endometrium. Implantation in marmosets occurs at day 11 of pregnancy; hence, these time points were chosen so that the peri-implantation period and very early pregnancy could be studied. mRNAs for VEGF, VEGFR-1 and VEGFR-2, angiopoietin 1, angiopoietin 2 and their receptor Tie-2 were localized and quantified by *in situ* hybridization. Endothelial cells were

identified by CD31 immunocytochemistry. VEGF mRNA was present in all compartments except endothelial cells, and its expression generally increased throughout pregnancy except in upper zone glandular epithelium and luminal epithelium, where a decrease in expression was observed. VEGF receptor mRNAs were found in endothelial cells of the upper zones immediately surrounding glandular epithelium. Angiopoietin 1 mRNA was localized to glandular epithelium of the upper and lower zones throughout pregnancy, and increased in stroma at week 4. Expression of angiopoietin 2 mRNA was localized exclusively to endothelial cells of large luminal vessels and was higher in endometrium from marmosets at week 4 of pregnancy than in endometrium from all other stages. These data provide comprehensive evidence that VEGFR-1 and -2, and angiopoietin 1, angiopoietin 2 and Tie-2 interactions may be involved in the preparation of endometrium for implantation, remodelling of the maternal vasculature and trophoblast invasion during the peri-implantation period in this primate species.

Introduction

During the last few years, considerable progress has been made in understanding the molecular regulation of angiogenesis in the female reproductive tract. In particular, the role of vascular endothelial growth factor (VEGF) during follicular and luteal development in the marmoset (*Callithrix jacchus*) model has been established by specific inhibition of this angiogenic factor *in vivo* (Fraser and Lunn, 2001). As inhibitors of angiogenic factors are likely to be used clinically, it is important

that their role during implantation and early pregnancy is elucidated. The aim of the present study was to build on our knowledge of the regulation of angiogenesis in the reproductive tract of female marmosets by investigating the patterns of expression of angiogenic factors during this period.

Successful pregnancy is dependent on implantation of the blastocyst into the endometrium. Angiogenesis may have a key role in implantation; however, the mechanisms by which localized changes in vascular permeability and angiogenesis occur at the site of implantation have yet to be elucidated.

VEGF and its receptors, fms-like tyrosine kinase (Flt or VEGFR-1) and kinase insert domain-containing region

*Correspondence

Email: h.fraser@hrc.mrc.ac.uk

(KDR or VEGFR-2) have been implicated as key players in vascular remodelling and placentation in women (Ahmed *et al.*, 1995; Cooper *et al.*, 1995; Clark *et al.*, 1996, 1998; Goldman-Wohl *et al.*, 2000; Helske *et al.*, 2001; Geva *et al.*, 2002; Sugino *et al.*, 2002), sheep (Bogic *et al.*, 2001), pigs (Winther *et al.*, 1999) and marmosets (Wulff *et al.*, 2002) at later stages of pregnancy. VEGF has been localized in endometrium of mice (Chakraborty *et al.*, 1995; Halder *et al.*, 2000), golden hamsters (Yi *et al.*, 1999), rats (Rabbani and Rogers, 2001), rabbits (Das *et al.*, 1997) and Rhesus monkeys (Ghosh *et al.*, 2000) at this time. In mice, inhibition of VEGF prevents oestrogen-induced uterine oedema and implantation (Rockwell *et al.*, 2002), and in rats, the number of implantation sites was reduced after administration of a VEGF blocking antibody (Rabbani and Rogers, 2001).

Angiopoietins also appear to have a major role in regulation of blood vessel growth, maturation and regression, but their importance at the time of implantation has yet to be studied. However, their presence in placenta in later stages of pregnancy has been documented in women (Dunk *et al.*, 2000) and marmosets (Wulff *et al.*, 2002).

The aim of the present study was to describe the localization of mRNA for angiogenic factors and their receptors. Implantation occurs at day 11 after ovulation in marmosets. This study was conducted on tissue collected at the peri-implantation period (week 2 after ovulation) to week 4 of gestation. Angiogenic processes occurring at this early stage of pregnancy are still poorly understood in women and with accumulating knowledge on the effects of manipulation of angiogenesis in marmosets, this species represents a potentially valuable model in which to study these events.

Materials and Methods

Angiogenesis in marmoset endometrium

Experiments were carried out under the Animals (Scientific Procedures) Act (1986) and were approved by the local Ethical Review Committee. The tissues studied were collected from marmosets which were the subject of a previous report describing the changes in expression of angiogenic factors associated with early pregnancy in the corpus luteum (Rowe *et al.*, 2002). Blood samples were collected from adult female marmosets three times a week and were assayed for ovulatory increases in plasma progesterone concentration (Wulff *et al.*, 2001). During the mid- to late luteal phase of the second recorded cycle, prostaglandin analogue, 1 µg PGF_{2α} (cloprostenol; Planate, Coopers Animal Health Ltd, Crewe), was administered i.m. to induce luteolysis so that timing of the subsequent ovulation could be identified accurately. A fertile male was introduced at the time of prostaglandin administration. In anticipation of ovulation occurring 10 days after prostaglandin administration,

vaginal lavages were collected on days 7 and 9 of the follicular phase and examined for the presence of spermatozoa. Marmosets were killed humanely at week 2 ($n=4$), 3 ($n=6$) or 4 ($n=6$) after ovulation. A further six non-pregnant late secretory phase animals were also studied (Rowe *et al.*, 2002). Uteri were fixed in 4% (w/v) paraformaldehyde (Sigma, Poole).

Pregnancy was confirmed by examining serial sections of the uterus for the presence of a trophoblast and determining plasma concentrations of chorionic gonadotrophin. Chorionic gonadotrophin was measured according to the method of Saltzman *et al.* (1998). The detection limit for the assay was 12 ng ml⁻¹. All non-pregnant animals had concentrations of CG which were below the detection limit of the assay.

Haematoxylin–eosin staining

Tissue sections (5 µm thickness) were cut on to Super-frost plus slides (Sigma). Sections were dewaxed in xylene, rehydrated in decreasing concentrations of ethanol, and washed in distilled water before undergoing haematoxylin–eosin staining. The slides were dehydrated and mounted in pterex.

Localization of blood vessels by CD31 immunocytochemistry

Immunocytochemistry was performed to confirm suggested endothelial cell localization of VEGFR-1, VEGFR-2 and Tie-2 mRNA. Tissue sections (5 µm thickness) were cut on to Super-frost plus slides for immunocytochemistry. The sections were dewaxed in xylene, rehydrated in decreasing concentrations of ethanol and washed in distilled water. Antigen retrieval was performed by boiling sections in a Tefal Clypso pressure cooker in 0.01 mol citrate buffer l⁻¹, pH 6, for 6 min at high pressure setting 2. The slides were then left for 20 min in hot buffer and washed in Tris-buffered saline (TBS: 0.05 mol Tris l⁻¹, pH 7.4, 9 g NaCl l⁻¹). The sections were blocked in normal rabbit serum (diluted 1:5 in TBS) for 30 min to reduce non-specific binding. The primary antibody CD31 (monoclonal mouse anti-human endothelial cell CD31, Clone JC/70A, no. M0823; Dako, Glostrup) diluted 1:20 in TBS was used. Incubation was performed overnight at 4°C. The slides were washed three times in TBS. Incubation with the secondary antibody (rabbit anti-mouse Ig; Dako, 1:60 diluted in TBS) was performed for 40 min at room temperature (22°C). This was followed by two TBS washes and incubation of the APAAP complex (Dako; 1:100 dilution in normal rabbit serum and TBS) for 40 min at room temperature. Visualization was performed using nitroblue tetrazolium (NBT) solution containing 45 µl NBT substrate (Boehringer Mannheim, Mannheim), 10 ml NBT buffer, 35 µl Xphosphate (Boehringer Mannheim) and 10 µl levamisole (Sigma).

Localization of VEGF, VEGFR-1 VEGFR-2, angiopoietin 1, angiopoietin 2 and Tie-2 mRNA in the endometrium

In situ hybridization was performed as described previously using complementary RNA probes for human VEGF, VEGFR-1, VEGFR-2, angiopoietin 1, angiopoietin 2 and Tie-2 (Wulff *et al.*, 2000). The hVEGFR-1 probe was a 641 bp PCR fragment that hybridizes to base pairs 22–663 of the Flt mRNA. This comprises the region of the encoding extracellular domain of the Flt protein. Thus, the probe recognizes mRNA for both full-length and soluble Flt. Sense and anti-sense probes were prepared using an RNA transcription kit (Ambion, Austin, TX) and labelled with ^{35}S uridine 5[']-triphosphate (NEN, Boston, MA). Deparaffinized sections were treated with 0.1 mol HCl l^{-1} for 20 min and digested in proteinase K (5 $\mu\text{g ml}^{-1}$; Sigma) for 30 min at 37°C. After pre-hybridization for 2 h at 55°C, subsequent hybridization was performed in a moist chamber overnight at 55°C. High stringency post-hybridization washings and ribonuclease A treatment were used to remove excess probe. The slides were dehydrated, dried and dipped in Ilford G5 liquid emulsion (H. A. West, Edinburgh) and exposed for 12 weeks. The slides were subsequently developed (Kodak D19 developer; Kodak, Rochester, NY) and fixed (Kodak GBS, Kodak). All slides were counterstained with haematoxylin, dehydrated and mounted.

Quantification of *in situ* hybridization

Uterine sections were scored and means were calculated according to the grain density in the various different uterine compartments: namely, luminal epithelium, upper zone stroma (area beneath the luminal epithelium), upper zone glands, lower zone glands (area adjacent to myometrium), lower zone stroma and endothelial cells. Scoring was performed by two independent observers with the following classification: –: no detectable specific signal above background levels; + (1): detectable but low grain density clearly above background levels; ++ (2): moderate grain density; +++ (3): high grain density without grain coalescence; and ++++ (4): grain coalescence. Scoring of uteri was blind and random by each observer. An overall score was assigned for each compartment within a section. Significant differences between groups ($P < 0.05$) were determined using a Kruskal–Wallis non-parametric test followed by Dunn's multiple comparisons test. Representative photographs illustrate the localization of mRNAs. In general, these reflect the quantitative scoring but also show focal changes within a compartment not distinguished by the scoring.

Results

Vascular endothelial growth factor (VEGF)

VEGF mRNA was localized to the glandular epithelium in the late secretory phase non-pregnant endometrium.

Highest grain density was observed in the luminal epithelium and glands of the upper zones. Grain density was much reduced in the mid-zone glands, whereas grains were completely absent from the lower zone (Fig. 1a,b). Exact cellular localization of VEGF mRNA to glandular epithelium was confirmed under high power images (Fig. 1j).

In early pregnant endometrium, at 2 weeks after ovulation, a similar localization was observed to that in the non-pregnant animals (Fig. 1c–e). However, low expression of stromal VEGF mRNA was apparent just beneath the luminal epithelium (Fig. 1c,d). At this very early stage of pregnancy, 3 days after implantation, no expression of VEGF mRNA was observed in the blastocyst or fetal membranes (Fig. 1c,d). By week 3 of pregnancy (day 21), expression of VEGF mRNA remained high in the glandular epithelium. Lower expression was also observed in the lower zone glands (Fig. 1h,k). In decidua, VEGF expression was relatively high and punctate (Fig. 1f,g,i). In addition, by this stage of pregnancy the blastocyst and fetal membranes were expressing VEGF mRNA. Animals at week 4 of pregnancy (day 28) had similar VEGF mRNA localization to animals at week 3; hence, a representative figure at 3 weeks is shown (Fig. 1f (dark field) and Fig. 1g (bright field)). In all sense slides, no specific signal was detected above a low background (Fig. 1h insert).

KDR and Flt (VEGFR-1 and VEGFR-2)

VEGFR-2 mRNA localization is shown (Fig. 2). It should be noted that expression of VEGFR-1 mRNA was consistently lower than that of VEGFR-2, but it was found in the same cellular locations; thus, mainly photographs of VEGFR-2 mRNA are shown (Fig. 2a–l). However, representative images are included of VEGFR-1 hybridization at week 2 of pregnancy (Fig. 2m,n). Each receptor localization and grain density was scored separately (Table 1). In the late secretory non-pregnant endometrium, VEGFR-2 mRNA was found exclusively in endothelial cells surrounding the upper zone glands beneath the luminal epithelium (Fig. 2a,b,j). This observation was confirmed by comparison with CD31 immunocytochemistry to identify endothelial cells in sequential sections (Fig. 2c). In endometrium from week 2 of pregnancy, VEGFR-2 mRNA was localized to the upper zone endothelium as in non-pregnant endometrium. Grains were absent from the blastocyst and fetal membranes (Fig. 2d,e). At weeks 3 and 4 of pregnancy, expression of VEGFR-2 mRNA remained in endothelial cells of the upper zone with highest grain density observed around large maternal vessels beneath the luminal epithelium (Fig. 2g,h,k), which was again confirmed by comparison with CD31 localization (Fig. 2i). VEGFR-2 mRNA was also observed to a lesser extent throughout the stromal compartment, again localized to endothelial cells (Fig. 2l). In all sense slides, no specific

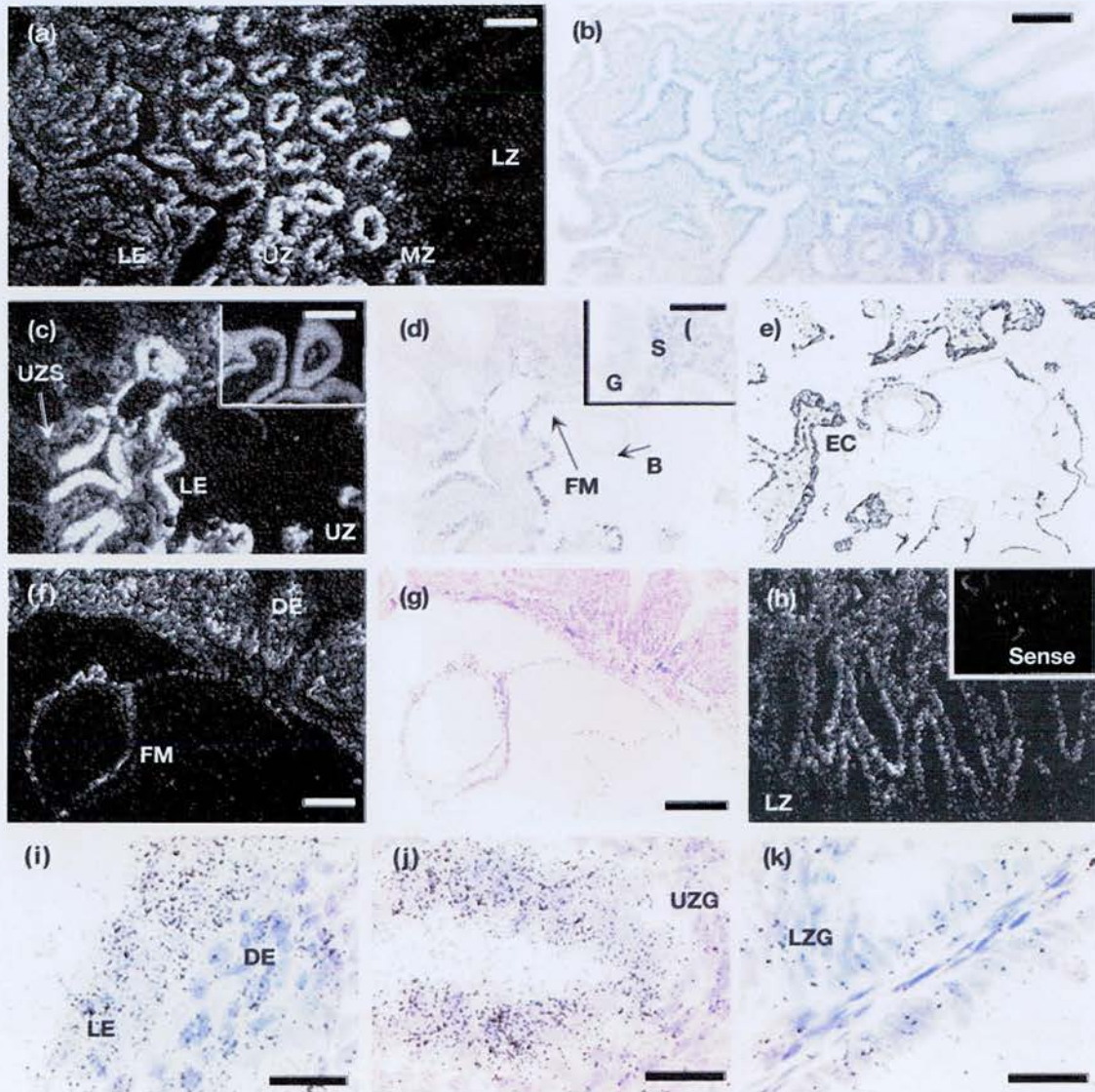


Fig. 1. (a–k) *In situ* hybridization of vascular endothelial growth factor (VEGF) mRNA in marmoset (*Callithrix jacchus*) endometrium. (a,c,f) Dark-field and (b,d,g) corresponding light-field images. (a,b) Non-pregnant late secretory phase endometrium. Note high expression in upper zone glands (UZ) and luminal epithelium (LE), which decreases in the mid-zone (MZ) and is absent in the lower zone (LZ). (c,d,e) Endometrium from pregnant marmoset at day 14 after ovulation. (c,d) Note high expression in luminal epithelium (LE) and upper zone (UZ) glands, and absence of expression in the blastocyst (B) and fetal membranes (FM). Lower VEGF expression can be seen in the upper zone stroma (UZS) in (c). Insert in (c) is a higher power image of the luminal epithelium ($\times 40$). Insert in (d) is a higher power bright-field image of the upper zone stroma ($\times 100$). (e) CD31 staining for endothelial cells (EC) to emphasize non-endothelial localization of stromal VEGF. (f–h) Typical VEGF expression at day 21 and day 28. (f) Dark-field image of marmoset early implantation site. Note high punctate expression in the decidualized endometrium (DE) and appearance of expression in the fetal membranes (FM). (g) Corresponding light-field image to (f). (h) Dark-field image of lower zone (LZ) endometrium where there is low glandular VEGF expression. Insert in (h) is a representative sense photograph. (i) Higher power ($\times 100$) bright-field image of the luminal epithelium (LE) of an animal at week 3 of pregnancy; this image also illustrates the hybridization observed in decidualized stroma (DE). (j) High power ($\times 100$) image of the upper zone glands (UZG) in late secretory non-pregnant endometrium to confirm cellular localization. (k) High power ($\times 100$) image to highlight expression of VEGF in the lower zone glands (LZG) at this time. G: gland; S: stroma. Scale bars represent (a,b,f,g) 100, (c,d inserts) 50 and (i–k) 25 μm .

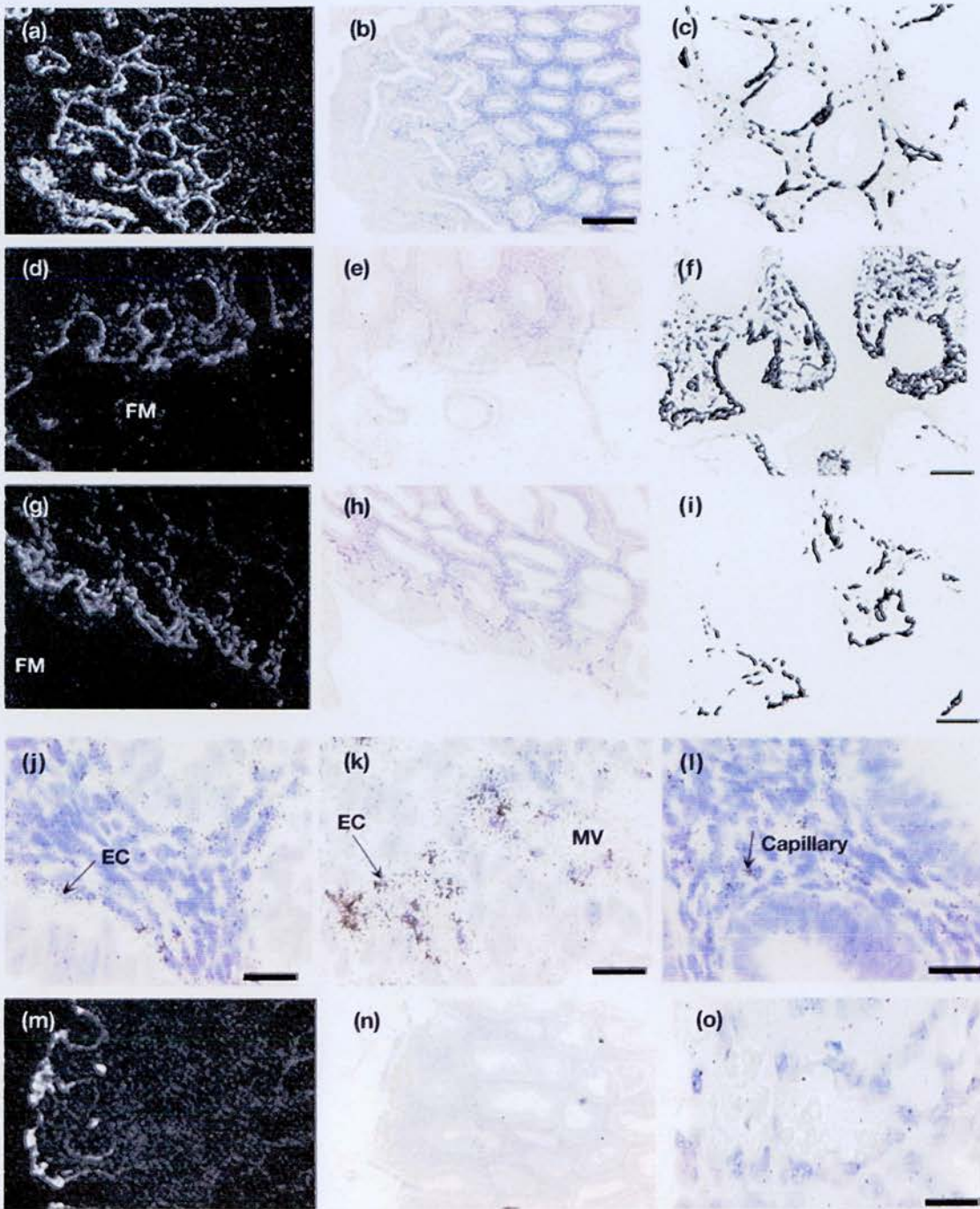


Fig. 2 *In situ* hybridization of vascular endothelial growth factor receptor (VEGFR) (a-l) -2 and (m,n) -1 mRNA in marmoset (*Callithrix jacchus*) endometrium. (a-c) Non-pregnant late secretory phase endometrium. (a) Dark-field and (b) light-field images of VEGFR-2 expression in upper zone endometrium. Note endothelial localization immediately surrounding glands as demonstrated by (c) CD31 immunocytochemistry and (j) high power ($\times 100$) bright-field image. Also note punctate expression in endothelial cells throughout the stroma in all stages studied illustrated by a representative high power bright-field image (l). (d-f) Pregnant marmoset endometrium at day 14 after ovulation. (d) Dark-field and (e) corresponding light-field images showing localization to large and small luminal blood vessels surrounding the uppermost glands as indicated by CD31 immunocytochemistry for endothelial cells (f). Note absence of grains in blastocyst and fetal membranes (FM). (g) Dark-field and (h) light-field images of VEGFR-2 expression at 3-4 weeks. Note localization in upper zone to luminal maternal vessels (MV) as demonstrated by CD31 immunocytochemistry (i), and high power bright-field image (k). In addition, note absence of localization to fetal membranes (g) and punctate throughout the stroma of lower zones (l). (o) A high power ($\times 100$) bright-field sense image. Scale bars represent (b) 100, (f,i) 50 and (j-l,o) 25 μm .

Table 1. Quantification of *in situ* hybridization using relative grain density within uterine compartments of marmosets (*Callithrix jacchus*) throughout the stages of pregnancy studied for vascular endothelial growth factor (VEGF), VEGFR-1 and -2, angiopoietin 1, angiopoietin 2 and Tie-2

Stage of cycle of endometrium	Luminal epithelium	Upper zone glandular epithelium	Upper zone stroma	Upper zone endothelium	Lower zone glandular epithelium	Lower zone stroma
VEGF						
Late secretory, non-pregnant	+++ ^a	+++	-	na	-	-
Week 2 of pregnancy	+++ ^{ab}	++	+	na	-	-
Week 3 of pregnancy	+ ^{ab}	++	++	na	+	-
Week 4 of pregnancy	- ^b	+	++	na	++	-
VEGFR-2 (-1)						
Late secretory, non-pregnant	-	-	-	+++ (+)	-	-
Week 2 of pregnancy	-	-	-	++ (+)	-	-
Week 3 of pregnancy	-	-	-	++ (+)	-	-
Week 4 of pregnancy	-	-	-	++ (+)	-	-
Angiopoietin 1						
Late secretory, non-pregnant	-	+	+	na	-	-
Week 2 of pregnancy	-	+	++	na	+++	-
Week 3 of pregnancy	-	+	+++	na	++	-
Week 4 of pregnancy	-	+	+++	na	+	-
Angiopoietin 2						
Late secretory, non-pregnant	-	-	-	- ^a	-	-
Week 2 of pregnancy	-	-	-	- ^a	-	-
Week 3 of pregnancy	-	-	-	- ^a	-	-
Week 4 of pregnancy	-	-	-	+++ ^b	-	-
Tie-2						
Late secretory, non-pregnant	-	-	+	+++	-	+
Week 2 of pregnancy	-	-	+	++	-	-
Week 3 of pregnancy	-	-	+	++	-	+
Week 4 of pregnancy	-	-	+	++	-	+

Analysis was performed by two independent observers. A Kruskal-Wallis test followed by a Dunn's multiple comparison test was used to determine significant differences within a particular uterine compartment between groups. Values given are mean scores. na: not assessed.

^{ab}Different letters denote significant differences ($P < 0.05$). No statistical comparisons were made between uterine compartments within the same stage of the cycle.

signal was detected above a low background. This is shown in (Fig. 2o), a $\times 100$ magnification bright-field image of the corresponding sense slide to Fig. 2k.

Angiopoietin 1

Angiopoietin 1 mRNA was localized to upper zone stroma and, to a lesser extent, the glandular epithelium of the upper zone in late secretory non-pregnant endometrium. In pregnant endometrium at day 14, there was high expression of angiopoietin 1 mRNA in the lower zone glands (Fig. 3d,e,o) but it was completely absent from adjacent compartments, the myometrium and mid-zone endometrium (see insert Fig. 3d). In the upper zone, expression of angiopoietin 1 mRNA was low in the

glandular epithelium but much more intense in stroma (Fig. 3p). Grains were absent from blastocyst and fetal membranes (Fig. 3f,g). At week 3 of pregnancy, upper and lower zone glandular epithelial expression of angiopoietin 1 mRNA was retained (Fig. 3h,j). Grain density increased in the stroma at this stage (Table 1; Fig. 3h,i). By day 28, clear, punctate angiopoietin 1 mRNA localization was observed throughout the decidualized stroma (Fig. 3k,l,n). In all sense slides, no specific signal was detected above a low background.

Angiopoietin 2

Angiopoietin 2 mRNA was detected only in animals at week 4 of pregnancy, and it was localized exclusively

in endothelial cells of maternal vessels (Table 1; Fig. 4). In all sense slides, no specific signal was detected above a low background.

Tie-2

In non-pregnant late secretory endometrium *Tie-2* mRNA was expressed predominantly in endothelial cells throughout the stroma of all zones (Fig. 5a,b,l). At week 2 of pregnancy (day 14), the generalized pattern of stromal expression observed in the non-pregnant animals was reduced but was still present (Table 1; Fig. 5d,e). High grain density was observed in endothelium immediately surrounding the upper zone glands at this time (Fig. 5d,e,j). By weeks 3 and 4 of pregnancy, luminal epithelial expression of *Tie-2* mRNA was not observed but expression was still observed in upper zone decidualized stroma (Fig. 5g). By comparison with CD31 immunocytochemistry (Fig. 5i) and high power haematoxylin–eosin-stained sections (insert Fig. 5h), *Tie-2* appeared to be expressed in blood vessels throughout the decidua. In all sense slides, no specific signal was detected above a low background.

Discussion

In the present study the spatial and temporal patterns of expression of mRNAs for VEGF, angiopoietins and their receptors in marmoset uterus were described during the peri-implantation period, allowing us to begin to construct a working hypothesis as to their potential role in oxygen and nutrient exchange and maternal vascular remodelling.

Implantation occurs on day 11 after ovulation in marmosets, 3 days later than in women, and the blastocyst remains in loose attachment to the surface epithelium without epithelial fusion. Penetration of trophoblast into maternal vessels occurs quite late, at days 45–60 of gestation, compared with about day 12 in women (Enders, 1993). Therefore, fetal demand for nutrients and oxygen during early pregnancy must be met by diffusion across the fetomaternal interface, a process likely to be critically dependent on vascular permeability. Increased permeability is generally thought to accompany tissue growth and remodelling (Dvorak *et al.*, 1999; Dvorak, 2000; Rabbani and Rogers, 2001). VEGF is a vascular permeability factor as well as a mitogen for endothelial cells (Keck *et al.*, 1989). Permeability and uterine oedema mediated by VEGF are critical for successful implantation in rodents, in which inhibition of VEGF prevented implantation (Dvorak *et al.*, 1999; Dvorak, 2000; Rabbani and Rogers, 2001). Our finding that VEGF mRNA was localized to upper zone endometrium of all groups, increasing during the first few weeks of

pregnancy, implies a role for VEGF as a permeability factor at this time.

The presence of VEGF mRNA in upper zone glandular epithelium and luminal epithelium of non-pregnant late secretory endometrium, with mRNA for both VEGFR-1 and -2 in endothelial cells of blood vessels immediately surrounding these glands, is indicative of a local action of VEGF. This is also observed in endometrium at weeks 2 and 3 of pregnancy where it may be involved in promoting fenestration of these vessels, thereby facilitating oxygen transfer to the avascular fetus. Although fetal membranes positive for CD31 were observed, they had no capillary formation. It may be that these cells are destined to become differentiated endothelial cells and thus already express CD31 surface antigens.

Upregulation of stromal VEGF mRNA was observed at week 4 of pregnancy. It is likely that the permeability role of VEGF is decreasing at this point, as glandular secretions are reduced and maternal sinusoids, although not yet invaded by trophoblast, have much more intimate contact with the more closely attached blastocyst, thus facilitating diffusion. Very strong expression of VEGFR-1 and -2 mRNA was also observed in endothelial cells of large maternal vessels immediately beneath the luminal epithelium where fetal VEGF could be acting to promote vessel migration such that fetal metabolic demands can be adequately met. The pattern of mRNA localization of VEGF and its receptors observed in the present study is consistent with findings in women, in whom fetal VEGF expression in tripronuclear zygotes has been implicated to induce neoangiogenesis at the site of implantation (Krussel *et al.*, 2000). VEGF expression has been documented in human placenta and in other species in the later stages of placentation where it is expressed in maternal decidua, but unlike our findings, its receptor VEGFR-1 appeared to be expressed in invading syncytial trophoblast (Ahmed *et al.*, 1995; Cooper *et al.*, 1995; Clark *et al.*, 1996). It may be that fetal VEGF receptors are present only at later stages in marmosets, when a more intimate interplay between maternal and fetal tissues occurs (Wulff *et al.*, 2002). Soluble Flt has been reported to be produced by human trophoblast in pregnancy and is detectable in the serum of pregnant women (Banks *et al.*, 1998). It is possible that this also occurs in marmosets; however, no hybridization of the VEGFR-1 probe to trophoblast was observed in the present study despite our probe detecting both the soluble and full-length receptor. No serum analyses were conducted.

The roles of the angiopoietins and their receptor *Tie-2* in early pregnancy are just beginning to be explored. In general, angiopoietin 1 acts on its receptor *Tie-2* to promote stabilization of the endothelial cell–vascular smooth muscle structure (Suri *et al.*, 1996), whereas angiopoietin 2 acts as an antagonist by binding to the *Tie-2* receptor without transmitting a signal (Maisonpierre *et al.*, 1997; Lobov *et al.*, 2002). In marmoset uterus,

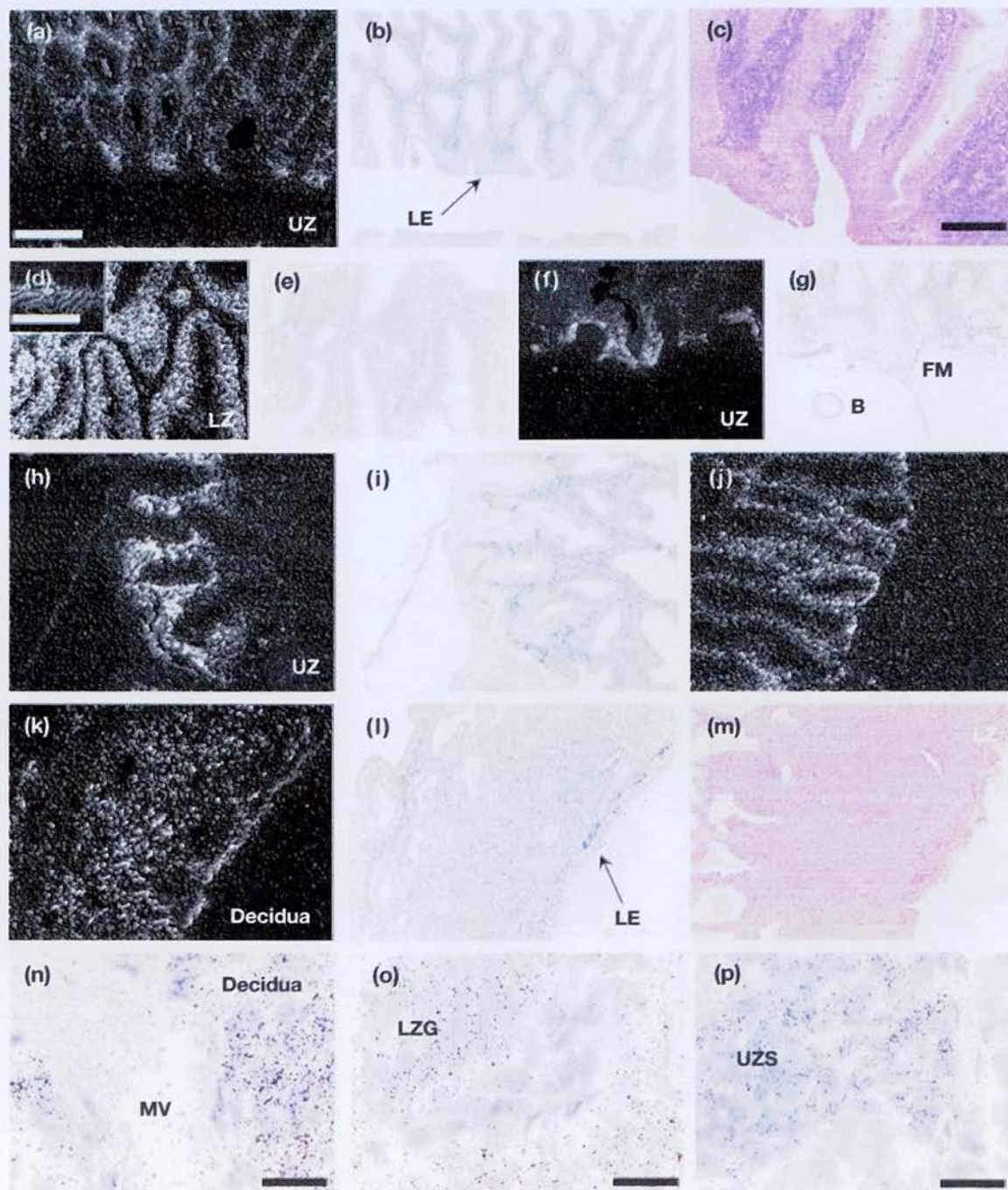


Fig. 3 (a–p) *In situ* hybridization of angiopoietin 1 mRNA in marmoset (*Callithrix jacchus*) endometrium. (a) Dark-field and (b) corresponding light-field images of non-pregnant late secretory phase endometrium. Note angiopoietin 1 expression in stroma and low expression in glands of the upper zone endometrium. LE: luminal epithelium. (c) Haematoxylin–eosinstained section of upper zone and luminal epithelium ($\times 40$). (d–g) Day 14 pregnant marmoset endometrium. (d) Dark-field and (e) corresponding light-field images of high expression of angiopoietin 1 mRNA in the glandular epithelium of the lower zone (LZ). Insert in (d) is a low power image ($\times 10$) to demonstrate absence of grains in myometrium and mid-zone endometrium. (e) High power image ($\times 100$) to confirm cellular localization to glands of the lower zone (LZG). (f) Dark-field and (g) corresponding light-field images of angiopoietin 1 localization in the upper zone (UZ). Note low glandular localization together with stromal expression. Angiopoietin 1 is absent from the fetal membranes (FM) and blastocyst (B). (h–j) Angiopoietin 1 expression at day 21 of pregnancy. (h) Dark-field and (i) light-field images of

angiopoietin 1 mRNA was the most abundantly expressed angiopoietin localized to upper zone glands and stroma, whereas Tie-2 mRNA was expressed in endothelial cells. In menstruating species after an infertile cycle, menstruation and shedding of the upper zones of the endometrium occur. The lower zone and myometrium are comparatively more stable compartments from which a new vascular and new upper zone endometrium is supplied (Smith, 2001). Although the marmoset does not menstruate, in infertile cycles a degree of vascular remodelling may occur in the upper zones, whereas the lower zones remain more stable, implicating a role for angiopoietin 1 in this region as was observed in the present study. However, the fertility rate is extremely high in marmosets (Rowe *et al.*, 2002) and hence this species may retain a stable upper zone endometrium without remodelling in response to progesterone withdrawal, associated with basal expression of angiopoietin 1 and Tie-2 in preparation for pregnancy. In fertile cycles, the endometrium is preparing to receive an implanting blastocyst and, thus, would require a stable maternal vasculature. It is possible that angiopoietin 1 mRNA may be expressed in the lower zones to maintain core vessel stability.

During the peri-implantation period, stromal angiopoietin 1 and Tie-2 mRNA expression in the upper zones adjacent to the site of implantation was increased compared with non-pregnant endometrium. As gestation progressed into weeks 3 and 4, expression of angiopoietin 1 and Tie-2 mRNA in upper zone stroma and decidua was further increased. This corresponds with a continued requirement for a stable vasculature at the maternal–fetal interface and eventual placentation and development of a haemochorial placenta. This role appears to extend to later pregnancy in marmosets, in which angiopoietin 1 mRNA is localized exclusively to syncytial trophoblast (Wulff *et al.*, 2002), indicating that once the fetal vasculature begins to develop, there may be a need for stabilization of newly formed fetal vessels.

The only site of localization of angiopoietin 2 mRNA was in large maternal vessels immediately beneath the luminal epithelium of animals at week 4 of pregnancy, where it was co-localized with Tie-2 mRNA. This is similar to the situation observed in later stages of placentation in marmosets (Wulff *et al.*, 2002). The presence of VEGF mRNA in the decidualized upper zone endometrium with its receptors in endothelial cells of this same region, including large maternal vessels, is indic-

ative of a similar paracrine modulation. It is thought that angiopoietin 2, when expressed in conjunction with VEGF, can be pro-angiogenic, promoting destabilization of vessels and breakdown of basal lamina to allow migration of endothelial cells and subsequent angiogenesis.

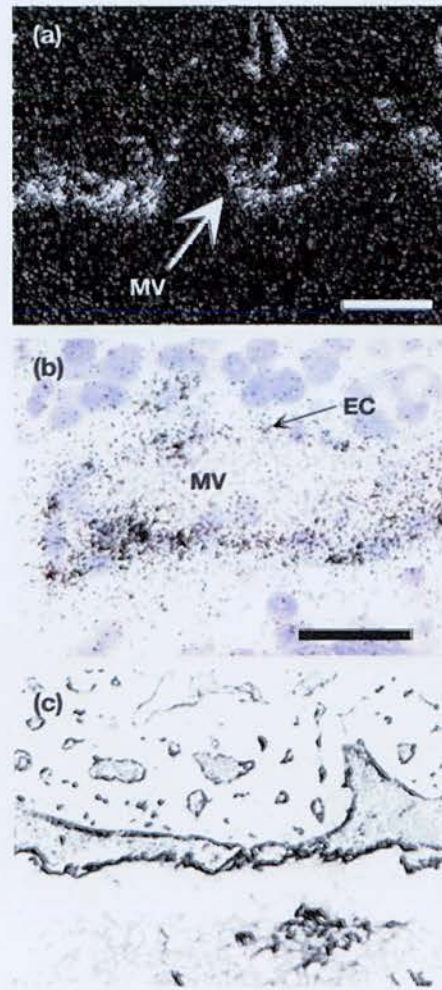


Fig. 4 (a–c) *In situ* hybridization of angiopoietin 2 mRNA in pregnant marmoset (*Callithrix jacchus*) endometrium at day 28. (a) Dark-field ($\times 20$) and (b) light-field ($\times 100$) images of upper zone endometrium. Note angiopoietin 2 expression in endothelial cells (EC) of large maternal vessels (MV), as demonstrated by CD31 immunocytochemistry in (c) and higher power image in (b). Angiopoietin 2 was absent from all other stages studied. Scale bars represent (a) 100 and (b) 25 μm .

angiopoietin 1 expression in the upper zone. Note expression in glandular epithelium and to a greater extent in stroma. This is also highlighted in (p), a high power ($\times 100$) bright-field image. UZS: upper zone stroma. (j) Dark-field image of lower zone (LZ) endometrium. Note glandular epithelial localization of angiopoietin 1. (k–m) Angiopoietin 1 expression at day 28 of pregnancy. (k) Dark-field and (l) corresponding light-field images of decidualized endometrium and luminal epithelium (LE). Note punctate expression of angiopoietin 1 mRNA throughout decidualized stroma, not in endothelial cells, which is further illustrated in (n), a higher power ($\times 100$) bright-field image. MV: maternal vessel. (m) Haematoxylin–eosin-stained section to demonstrate decidual morphology. Scale bars represent (a) 100, (c) 50, (d insert) 200 and (n–p) 25 μm .

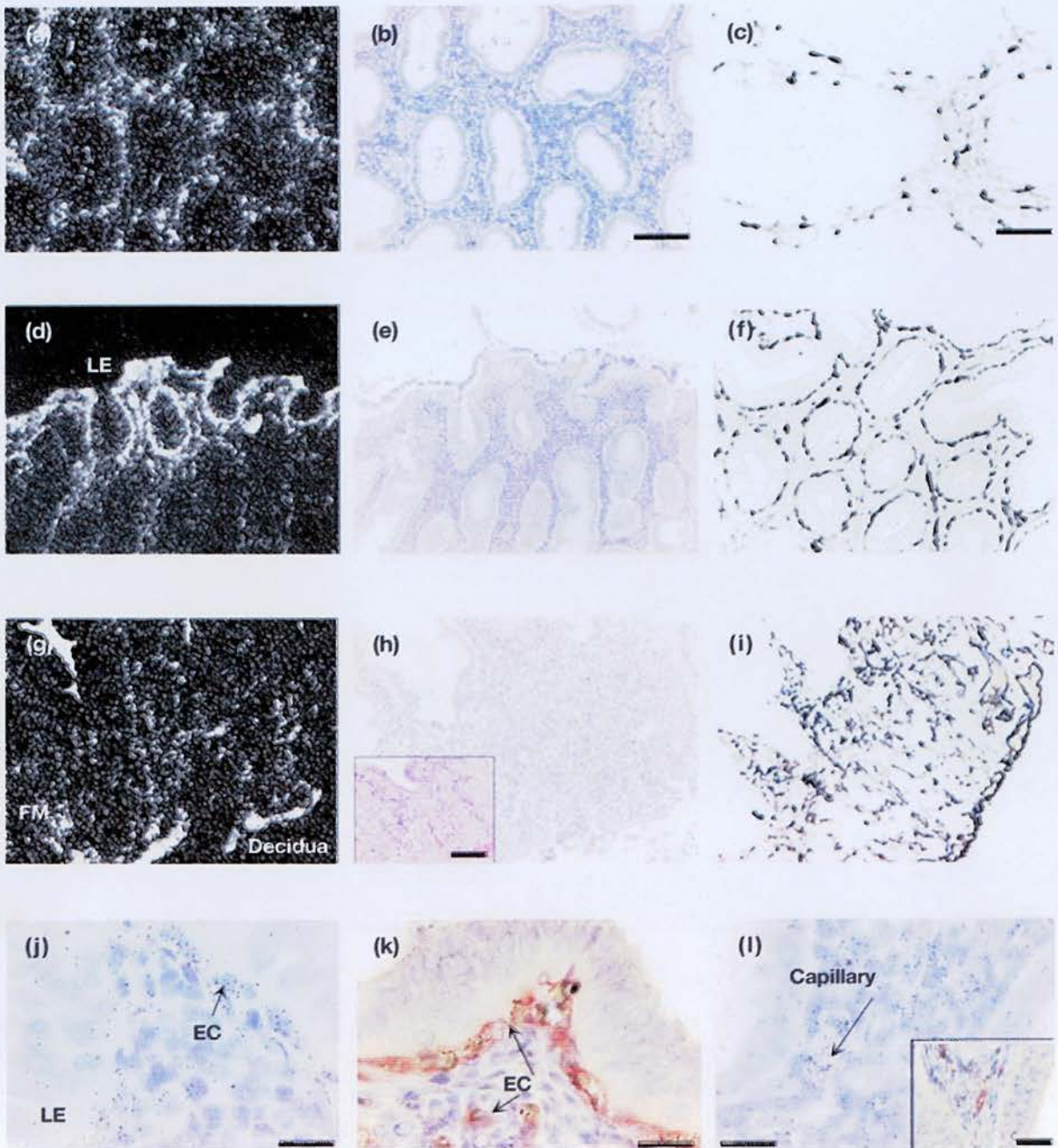


Fig. 5. (a–l) *In situ* hybridization of Tie-2 mRNA in marmoset (*Callithrix jacchus*) endometrium. (a–c) Non-pregnant late secretory phase endometrium. (a) Dark-field and (b) light-field images of Tie-2 expression in stroma throughout all zones of the non-pregnant endometrium. Note expression localized to endothelial cells as demonstrated by CD31 immunocytochemistry (c) taken at $\times 40$ objective and higher power ($\times 100$) bright-field images in (l). The insert in (l) illustrates CD31 immunocytochemistry, counterstained with haematoxylin. (d–f) Pregnant marmoset endometrium at day 14 after ovulation. (d) Dark-field and (e) corresponding light-field images of upper zone endometrium. Note localization to endothelial cells (EC) immediately surrounding upper zone glands as demonstrated by (f) CD31 immunocytochemistry and (j,k) higher power ($\times 100$) bright-field images. (k) CD31 immunocytochemistry counterstained with haematoxylin. LE: luminal epithelium. (g–i) Typical Tie-2 expression at day 21 and day 28 of pregnancy. (g) Dark-field and (h) light-field images of upper zone decidualized endometrium. FM: fetal membranes Note punctate expression of Tie-2 probably localized to endothelial cells as demonstrated by (i) CD31 immunocytochemistry. Insert shows a haematoxylin–eosin-stained section. Note vessels similar to areas of grain localization in (g). Scale bars represent (b) 100, (c,h insert) 50 and (j–l, l insert) 25 μm .

Our findings indicate that at week 4 of pregnancy, VEGF and angiopoietin 2 may be working synergistically to promote migration of these vessels and establishment of an efficient interface for nutrient and oxygen exchange between the fetal and maternal circulation.

Neither angiopoietin nor Tie-2 mRNA was detected in blastocyst or fetal membranes. Their presence in first trimester placenta has been reported in women; Tie-2 has also been localized to trophoblast cells, as well as endothelial cells (Dunk *et al.*, 2000). However, Tie-2 mRNA has been observed in trophoblast in later stages of pregnancy in marmosets (Wulff *et al.*, 2002).

It is also of interest that angiopoietin 2 is expressed by uterine natural killer cells in human endometrium, particularly those situated beneath the luminal epithelium and around vascular smooth muscle cells (Smith, 2001). No evidence for such expression was observed in marmosets, in which angiopoietin 2 mRNA was found exclusively in endothelial cells of luminal maternal vessels as confirmed by CD31 immunocytochemistry.

In conclusion, this study has described the molecular regulation of angiogenesis associated with implantation and vascular remodelling during the peri-implantation period in marmosets. It provides evidence that VEGFR-1 and -2 and angiopoietin 1, angiopoietin 2 and Tie-2 interactions may be involved in the preparation of endometrium for implantation, remodelling of the maternal vasculature during the peri-implantation period and trophoblast invasion. Taken together with our previous report on the molecular angiogenic events occurring from week 7 of pregnancy to term, a comprehensive picture of implantation and placentation in this primate species is provided. Having proposed putative roles for these angiogenic regulators in this process, there is potential for *in vivo* studies using specific antagonists to determine their physiological function.

The authors would like to thank Regeneron Pharmaceuticals, Inc., for the gift of angiopoietin and Tie-2 cDNA probes; S. Charnock-Jones for the gifts of VEGF, VEGFR-1 and VEGFR-2 cDNA probes; I. Swanston and F. Pitt for assays; and J. Roser, A. L. Parlow and NIDDK's National Hormone and pituitary Program for reagents for the CG assay.

References

- Ahmed A, Li XF, Dunk C, Whittle MJ, Rushton DI and Rollason T (1995) Colocalisation of vascular endothelial growth factor and its flt-1 receptor in human placenta *Growth Factors* **12** 235–243
- Banks RE, Forbes MA, Searles J *et al.* (1998) Evidence for the existence of a novel pregnancy-associated soluble variant of the vascular endothelial growth factor receptor, Flt-1 *Molecular Human Reproduction* **4** 377–386
- Bogic LV, Brace RA and Cheung CY (2001) Developmental expression of vascular endothelial growth factor (VEGF) receptors and VEGF binding in ovine placenta and fetal membranes *Placenta* **22** 265–275
- Chakraborty I, Das SK and Dey SK (1995) Differential expression of vascular endothelial growth factor and its receptor mRNAs in the mouse uterus around the time of implantation *Journal of Endocrinology* **147** 339–352
- Clark DE, Smith SK, Sharkey AM and Charnock-Jones DS (1996) Localization of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy *Human Reproduction* **11** 1090–1098
- Clark DE, Smith SK, Licence D, Evans AL and Charnock-Jones DW (1998) Comparison of expression patterns for placenta growth factor, vascular endothelial growth factor (VEGF), VEGF-B and VEGF-C in human placenta throughout gestation *Journal of Endocrinology* **159** 459–467
- Cooper JC, Sharkey AM, McLaren J, Charnock-Jones DS and Smith SK (1995) Localization of vascular endothelial growth factor and its receptor, flt, in human placenta and decidua by immunohistochemistry *Journal of Reproduction and Fertility* **105** 205–213
- Das SK, Chakraborty I, Wang J, Dey SK and Hoffman LH (1997) Expression of vascular endothelial growth factor (VEGF) and VEGF-receptor messenger ribonucleic acids in the peri-implantation rabbit uterus *Biology of Reproduction* **56** 1390–1399
- Dunk C, Shams M, Nijjar S, Rhaman M, Qiu Y, Bussolati B and Ahmed A (2000) Angiopoietin-1 and angiopoietin-2 activate trophoblast Tie-2 to promote growth and migration during placental development *American Journal of Pathology* **156** 2185–2199
- Dvorak HF (2000) VPF/VEGF and the angiogenic response *Seminars in Perinatology* **24** 75–78
- Dvorak HF, Nagy JA, Feng D, Brown LF and Dvorak AM (1999) Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. In *Current Topics in Microbiology and Immunology: Vascular Growth Factors and Angiogenesis* pp 97–132 Ed. I Claesson-Welsh. Springer-Verlag, Berlin, Heidelberg
- Enders A (1993) Overview of the morphology of implantation in primates. In *In Vitro Fertilisation and Embryo Transfer in Primates* pp 145–157 Eds D Wolf, RL Stouffer and RM Brenner. Springer-Verlag, New York
- Fraser HM and Lunn SF (2001) Regulation and manipulation of angiogenesis in the primate corpus luteum *Reproduction* **121** 355–362
- Geva E, Ginzinger DG, Zaloudek CJ, Moore DH, Byrne A and Jaffe RB (2002) Human placental vascular development: vasculogenic and angiogenic (branching and nonbranching) transformation is regulated by vascular endothelial growth factor-A, angiopoietin-1, and angiopoietin-2 *Journal of Clinical Endocrinology and Metabolism* **87** 4213–4224
- Ghosh D, Sharkey AM, Charnock-Jones DS, Dhawan L, Dhara S, Smith SK and Sengupta J (2000) Expression of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in conceptus and endometrium during implantation in the Rhesus monkey *Molecular Human Reproduction* **6** 935–941
- Goldman-Wohl DS, Ariel I, Greenfield C, Lavy Y and Yagel S (2000) Tie-2 and angiopoietin-2 expression at the fetal–maternal interface: a receptor–ligand model for vascular remodelling *Molecular Human Reproduction* **6** 81–87
- Halder JB, Zhao X, Soker S, Paria BC, Klagsbrun M, Das SK and Dey SK (2000) Differential expression of VEGF isoforms and VEGF(164)-specific receptor neuropilin-1 in the mouse uterus suggests a role for VEGF(164) in vascular permeability and angiogenesis during implantation *Genesis* **26** 213–224
- Helske S, Vuorela P, Carpen O, Hornig C, Weich H and Halmesmaki E (2001) Expression of vascular endothelial growth factor receptors 1, 2 and 3 in placentas from normal and complicated pregnancies *Molecular Human Reproduction* **7** 205–210
- Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J and Connolly DT (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF *Science* **246** 1309–1312
- Krussel J, Behr B, Hirchenhain J, Wen Y, Milki AA, Cupisti S, Biefeld P and Polan ML (2000) Expression of vascular endothelial growth factor mRNA in human preimplantation embryos derived from tripronuclear zygotes *Fertility and Sterility* **74** 1220–1226
- Lobov IB, Brooks PC and Lang RA (2002) Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival *in vivo*. *Proceedings National Academy of Sciences USA* **99** 11 205–210
- Maisonpierre PC, Suri C, Jones PF *et al.* (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis *Science* **277** 55–60

- Rabbani MM and Rogers PA** (2001) Role of vascular endothelial growth factor in endometrial vascular events before implantation in rats *Reproduction* **122** 85–90
- Rockwell LC, Pillai S, Olson CE and Koos RD** (2002) Inhibition of vascular endothelial growth factor/vascular permeability factor action blocks estrogen-induced uterine edema and implantation in rodents *Biology of Reproduction* **67** 1804–1810
- Rowe AJ, Morris KD, Bicknell R and Fraser HM** (2002) Angiogenesis in the corpus luteum of early pregnancy in the marmoset and the effects of vascular endothelial growth factor immunoneutralization on establishment of pregnancy *Biology of Reproduction* **67** 1180–1188
- Saltzman W, Schultz-Darken NJ, Wegner FH, Wittwer DJ and Abbott DH** (1998) Suppression of cortisol levels in subordinate female marmosets: reproductive and social contributions *Hormones and Behaviour* **33** 58–74
- Smith SK** (2001) Regulation of angiogenesis in the endometrium *Trends in Endocrinology and Metabolism* **12** 147–151
- Sugino N, Kashida S, Karube-Harada A, Takiguchi S and Kato H** (2002) Expression of vascular endothelial growth factor (VEGF) and its receptors in human endometrium throughout the menstrual cycle and in early pregnancy *Reproduction* **123** 379–387
- Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN and Yancopoulos GD** (1996) Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis *Cell* **87** 1171–1180
- Winther H, Ahmed A and Dantzer V** (1999) Immunohistochemical localization of vascular endothelial growth factor (VEGF) and its two specific receptors, Flt-1 and KDR, in the porcine placenta and non-pregnant uterus *Placenta* **20** 35–43
- Wulff C, Wilson H, Lague P, Duncan WC, Armstrong DG and Fraser HM** (2000) Angiogenesis in the human corpus luteum: localization and changes in angiopoietins, tie-2, and vascular endothelial growth factor messenger ribonucleic acid *Journal of Clinical Endocrinology and Metabolism* **85** 4302–4309
- Wulff C, Wilson H, Rudge JS, Wiegand SJ, Lunn SF and Fraser HM** (2001) Luteal angiogenesis: prevention and intervention by treatment with vascular endothelial growth factor trap(a40) *Journal of Clinical Endocrinology and Metabolism* **86** 3377–3386
- Wulff C, Wilson H, Dickson SE, Wiegand SJ and Fraser HM** (2002) Hemochorial placentation in the primate: expression of vascular endothelial growth factor, angiopoietins, and their receptors throughout pregnancy *Biology of Reproduction* **66** 802–812
- Yi XJ, Jiang HY, Lee KK, O WS, Tang PL and Chow PH** (1999) Expression of vascular endothelial growth factor (VEGF) and its receptors during embryonic implantation in the golden hamster (*Mesocricetus auratus*). *Cell and Tissue Research* **296** 339–349

Received 23 January 2003.

First decision 12 March 2003.

Revised manuscript received 4 April 2003.

Accepted 25 April 2003.

BrdU

The antibody reacts with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) and also with cellular DNA containing incorporated BrdU; it shows no crossreactivity with other cellular components. The antibody may be applied for the qualitative measurement of cell proliferation (BrdU incorporation) on a single cell level.

CD31

Clone JC70A

Code No./ Code/ Code-Nr. M 0823

Edition/ Ausgabe 06.02.03

Intended use For in vitro diagnostic use.

Monoclonal Mouse Anti-Human CD31, Endothelial Cell, Clone JC70A, is intended for use in immunocytochemistry. The antibody primarily labels endothelial cells, and is a useful tool for the identification of benign and malignant vascular disorders, including angiosarcomas. In addition, the antibody is valuable for the labelling of vessels when determining angiogenesis in several types of tumours.

Reagent provided Monoclonal mouse antibody provided in liquid form as cell culture supernatant dialysed against 0.05 mol/L Tris/HCl, pH 7.2, and containing

15 mmol/L NaN₃.

Clone: JC70A (1). Isotype: IgG1, kappa.

Mouse IgG concentration: see label on vial.

Immunogen Cell membrane preparation from the spleen of a patient with hairy cell leukaemia.

Specificity The antibody was clustered as anti-CD31 at the Fifth International Workshop and Conference on Human Leucocyte Differentiation

Antigens. The epitope recognized was found to be within the extracellular domain 1.

In Western blotting of membrane preparations from a spleen rich in the antigen or from normal platelets, the antibody labels bands of respectively 100 kDa and 130 kDa, the latter corresponding to classic CD31. The smaller band of 100 kDa observed with the splenic preparation may be due to proteolytic breakdown or to variations in glycosylation.

Specimen preparation Paraffin sections: The antibody can be used for labelling paraffin-embedded tissue sections fixed in formalin. Pre-treatment of tissues with heat-induced epitope retrieval is required.

Performance characteristics Cells labelled by the antibody predominantly display staining of the cell membrane, with weaker cytoplasmic staining.

Normal tissues: The antibody labels endothelial cells in a wide range of tissues, including endothelium in renal glomerular capillaries and the endothelium of vasa vasorum. In addition, the antibody labels megakaryocytes and occasional plasma cells in bone marrow. In frozen sections of human tonsil and spleen the antibody labels some non-endothelial cells, including some mantle zone B cells and T cells.

In blood smears, the antibody labels neutrophil polymorphs, 50% of the lymphocytes, all of the monocytes, and platelets.

Smooth Muscle Actin (Pericytes)

Clone 1A4

Code No./ Code/ Code-Nr. M 0851

Intended use For *in vitro* diagnostic use.

Monoclonal Mouse Anti-Human Smooth Muscle Actin, Clone 1A4, is intended for use in immunocytochemistry. The antibody labels smooth muscle cells, myofibroblasts and myoepithelial cells, and is a useful tool for the identification of leiomyomas, leiomyosarcomas and pleomorphic adenomas. Differential identification is aided by the results from a panel of antibodies. Interpretation must be made within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist.

Reagent provided Monoclonal mouse antibody provided in liquid form as cell culture supernatant dialysed against 0.05 mol/L Tris/HCl, pH 7.2, and containing 15 mmol/L NaN₃.

Clone: 1A4. The 1A4 clone is identical to the anti-asm-1. Isotype: IgG2a, kappa.

Mouse IgG concentration: see label on vial.

Immunogen N-terminal synthetic decapeptide of alpha-smooth muscle actin coupled to keyhole limpet haemocyanin (KLH).

Specificity In Western blotting and 2D-PAGE immunoblotting of the alpha-smooth muscle isoform of actin, the antibody labels a band corresponding to alpha-smooth muscle actin.

As demonstrated by Western blotting and/or immunocytochemistry, the antibody cross-reacts with the alpha-smooth muscle actin-equivalent protein in chicken, cow and rat.

Cytokeratin

Product No.	Description
C2931	Monoclonal Anti-Cytokeratin, pan antibody produced in mouse Clone C-11 Ascites fluid

Identifiers

Synonyms Monoclonal Anti-Pan Cytokeratin

Description

Immunogen keratin-enriched preparation from cultured human epidermoid carcinoma cell line A431.

Specificity The antibody recognizes human cytokeratins 4, 5, 6, 8, 10, 13, and 18. A broad spectrum antibody that reacts with a wide variety of normal, reactive, and neoplastic epithelial

tissues. This antibody may be used for staining cultured epithelial cell lines.

Properties

Application(s)	
Immunoblotting	suitable
Immunohistochemistry (formalin-fixed, paraffin-embedded sections)	suitable using protease-digested sections of human or animal tissues
Immunohistochemistry (frozen sections)	suitable
Indirect immunofluorescence	1:400 using protease-digested, formalin-fixed, paraffin-embedded sections of human or animal tissues
contains	15 mM sodium azide
Storage temp.	20°C
Shipped in	dry ice
Species reactivity	: human, bovine, frog, kangaroo rat, rat, mouse
Isotype	IgG1

References

Literature	Bartek, J., et al., <i>J. Pathol.</i> 164 , 215 (1991)
	Bartkova, J., et al., <i>Neoplasia</i> 38 , 439 (1991)
	Lane, E.B. and Alexander, C.M., <i>Semin. Cancer Biol.</i> 1 , 165 (1990)

Progesterone receptor

SOURCE

PR (C-19) is an affinity purified rabbit polyclonal antibody raised against a peptide mapping at the carboxy terminus of the progesterone receptor of human origin (identical to corresponding mouse sequence).

PRODUCT

Each vial contains 200 µg IgG in 1.0 ml of PBS contains 0.1% sodium azide and 0.2% gelatin.

Blocking peptide is available for competition studies (sc-538 P) (100 µg peptide in 0.5 ml PBS with 0.1% sodium azide and 100 µg BSA).

Also available as PR (C-19) X TransCruz antibody: sc-538 X for gel supershift studies; supplied as 200 µg IgG in 0.1 ml PBS contains 0.1% sodium azide.

SPECIFICITY

PR (C-19) is recommended for the detection of the progesterone

receptor (PR-A and PR-B) of mouse, rat and human origin by Western blotting, immunoprecipitation and immunohistochemistry (including paraffin-embedded sections).

PR (C-19) X TransCruz antibody is recommended for gel supershift studies.

Recommended dilution range for Western blot analysis:

1:200–1:1000. Recommended starting dilution: 1:200.

For research use only, not for use in diagnostic procedures.

BACKGROUND REFERENCES

1. Conneely, O.M., Mulac-Jericevic, B., DeMayo, F., Lydon, J.P., and O'Malley, B.W. 2002. Reproductive functions of progesterone receptors. *Recent. Prog. Horm. Res.* 57: 339-355

2. Giangrande, P.H., Pollio, G., and McDonnell, D.P. 1997. Mapping and characterization of the functional domains responsible for the differential activity of the A and B isoforms of the human progesterone receptor. *J. Biol. Chem.* 272: 32889-32900.

3. Giangrande, P.H. and McDonnell, D.P. 1999. The A and B isoforms of the human progesterone receptor: two functionally different transcription factors encoded by a single gene. *Recent Prog. Horm. Res.* 54: 291-313.

4. Giangrande, P.H., Kimbrel, E.A., Edwards, D.P., and McDonnell, D.P. 2000. The opposing transcriptional activities of the two isoforms of the human progesterone receptor are due to differential cofactor binding. *Mol. Cell. Biol.* 20: 3102-3115.

5. Clemm, D.L., Sherman, L., Boonyaratanakornkit, V., Schrader, W.T., Weigel, N.L., and Edwards, D.P. 2000. Differential hormone-dependent phosphorylation of progesterone receptor A and B forms revealed by a phosphoserine site-specific monoclonal antibody. *Mol. Endocrinol.* 14: 52-65.

6. Beck, C.A., Zhang, Y., Altmann, M., Weigel, N.L., and Edwards, D.P. 1996. Stoichiometry and site-specific phosphorylation of human progesterone receptor in native target cells and in the baculovirus expression system. *J. Biol. Chem.* 271: 19546-19555.

7. Zhang, Y., Beck, C.A., Poletti, A., Clement, J.P. IV, Prendergast, P., Yip, T.T., Hutchens, T.W., Edwards, D.P., and Weigel, N.L. 1997. Phosphorylation of human progesterone receptor by cyclin-dependent kinase 2 on three sites that are authentic basal phosphorylation sites *in vivo*. *Mol. Endocrinol.* 11: 823-832.

8. Law, M.L., Kao, F.T., Wei, Q., Hartz, J.A., Greene, G.L., Zarucki-Schulz, T., Conneely, O.M., Jones, C., Puck, T.T., O'Malley, B.W., *et al.* 1987. The progesterone receptor gene maps to human chromosome band 11q13, the site of the mammary oncogene int-2. *Proc. Natl. Acad. Sci. USA* 84: 2877-2881.

For product citations, please visit our website at www.scbt.com

DEFINITION Human VEGF mRNA.

LOCUS E15157 1873 bp RNA PAT 28-JUL-1999

SOURCE Homo sapiens.

ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;
Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 1873)

AUTHORS Uchida,K. .

TITLE PREPARATION OF ANTISENSE NUCLEIC ACID

JOURNAL Patent: JP 1998052285-A 24-FEB-1998;

BASE COUNT 422 a 506 c 608 g 337 t

ORIGIN

1 tcgctggaggc ttggggcagc cgggtagctc ggaggctcgt gcgctggggg ctgaccagc
61 cgctctgtcg ggaggcgcag cggtaggtg gaccggtcag cggactcacc ggccagggcg
121 ctgggtgctg gaatttgata ttcattgac cgggtttat ccctctctt ttttcttaa
181 cttttttt taaaactgta tgtttctcg ttttaatta ttttgcttg ccattcccca
241 ctgaatcgg gccgacggct tggggagatt gctctacttc cccaaatcac tgtgatttt
301 ggaaaccagc agaaagagga aagaggtagc aagagctcca gagagaagtc gaggaagaga
361 gagacggggg cagagagagc gcgctgggct gcgagcagcg aaagcgcagc gggcaaagt
421 agtgacctgc tttggggg gaccgccgga gcgctggcgt agccctccc ctgggatcc
481 cgcagctgac cagtcgcgct gacggacaga cagacagaca ccgccccag cccagctac
541 cacctctcc ccggccggcg gcggacagt gacgcggcgg cgagccgcgg gcagggggcg
601 gagcccgcc cggagggcg ggtggaggg gtcggggctc gcggcgtgc actgaaact
661 ttcgtccaac tctgggctg tctcgttc ggaggagccg tggcgcgc gggggaagc
721 gagccgagc gagccgcgag aagtgtagc tggggccgg aggagccga gccggaggag
781 ggggaggagg aagaagagaa ggaagaggag agggggccgc agtggcgact cggcgtcgg
841 aagccgggt catggacggg tgaggcggcg gtgtgcgcag acagtgtcc agccgcgcg
901 gctcccagg ccctggccc ggctcgggc cggggaggaa gagtagctc cggagggcc
961 gaggagagc ggccgcccc cagcccagc cggagagga gcgagagcc cgccggcccc
1021 ggtcggcct ccgaaacc **at gaactttctg ctgtctggg tgcattggg ccttgcttg**
1081 ctgcttacc tccaccatgc caagtgtcc caggctcac ccatggcaga aggaggagg
1141 cagaatcgc acgaagtggg gaagttcatg gatgtctatc agcgcagcta ctccatcca
1201 atcgagacc tgggtggacat ctccaggag taccctgatg agatcgagta catctcaag
1261 ccatactgtg tgcccctgat gcatgctggg gctgtctgca atgacgagg cctggagtgt
1321 gtgcccactg aggagtcaa catcaccatg cagattatgc ggaatcaacc taccaggc
1381 cagcacatag gagatgag ctctacag cacaacaaat gtgaatgcag accaaagaaa
1441 gatagagcaa gacaagaaaa atgtgacaag ccgagggcgt gagccgggca ggaggaagga
1501 gcctccctca gggtttcggg aaccagatct ctaccagga aagactgata cagaacgac
1561 gatacagaaa ccacgctgcc gccaccacac catcaccatc gacagaacag tcttaatcc
1621 agaaacctga aatgaaggaa gaggagactc tgcgagagc actttgggtc cggagggcga
1681 gactccggcg gaagcattcc cgggctgggtg acccagcacg gtccctctg gaattggatt
1741 cgccatttta ttttcttg tgctaaatca ccgagcccgg aagattagag agttttatt
1801 ctgggattcc ttagacaca cccaccaca tacatacatt tatatatata tatattat
1861 atatataat taa

DEFINITION Human mRNA for FLT.

LOCUS E14000 2523 bp DNA PAT 24-JUN-1998

SOURCE *Homo sapiens*.

ORGANISM *Homo sapiens*

Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria;
Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 2523)

AUTHORS Shibuya, M. .

TITLE POLYPEPTIDE CAPABLE OF BINDING TO VEGF

JOURNAL Patent: JP 1997255700-A 1 30-SEP-1997;

TOAGOSEI CO LTD

BASE COUNT 765 a 596 c 589 g 573 t

ORIGIN

```
1 gcggacactc ctctcgctc ctccccggca gcggcggcgg ctcgagcgg gctccggggc
61 tcgggtgcag cggccagcgg gcttggcggc gaggattacc cggggaagtg gttgtctct
121 ggctggagcc gcgagacggg cgctcagggc gcggggccgg cggcggcgaa cgagaggacg
181 gactctggcg gccgggtcgt tggccggggg agcgcgggca ccgggcgagc aggccgcgtc
241 gcgctacca tggcagcta ctggacacc ggggtcctgc tgtgcgcgt gctcagctgt
301 ctgcttca caggatctag ttcaggttca aaattaaaag atcctgaact gagttaaaa
361 ggcaccagc acatcatgca agcaggccag aactgcatc tccaatgcag gggggaagca
421 gccataaat ggtcttggc tgaaatggtg agtaaggaaa gcgaaaggct gagcataact
481 aaatcgcct gtggaagaaa tggcaaaaa ttctgcagta cttaacctt gaacacagct
541 caagcaaac aactggctt ctacagctgc aaatatctag ctgtacctac tcaagaag
601 aaggaaacag aatctgcaat ctatatatt attagtata caggtagacc ttcgtagag
661 atgtacagt aaatccccga aattatacac atgactgaag gaaggagct cgtattccc
721 tgccgggta cgtcacctaa catcactgtt acttaaaaa agtttccact tgacacttg
781 atccctgat gaaaacgcat aatctgggac agtagaaagg gcttcatcat atcaaatgca
841 acgtacaaag aaatagggtc tctgacctgt gaagcaacag tcaatgggca tttgataag
901 acaaactatc tcacacatcg acaaaccaat acaatcatag atgtccaaat aagcacacca
961 cgccagtc aattacttag aggcatact ctgtcctca attgtactgc taccactccc
1021 ttgaacacga gagtcaaat gacctggagt taccctgatg aaaaaataa gagagcttc
1081 gtaaggcgac gaattgacca aagcaattcc catgccaaca tattctacag tttcttact
1141 attgacaaaa tgcagaacaa agacaaagga cttatactt gtcgtgtaag gagtggacca
1201 cattcaaat ctgttaacac ctcagtgcat atatatgata aagcattcat cactgtgaaa
1261 catcgaaac agcagggtgt tgaaccgta gctggcaagc ggtctaccg gctctctatg
1321 aaagtgaagg catttccctc gccggaagtt gtatggtaa aagatgggtt acctgcgact
1381 gagaaatctg ctgctattt gactcgtgg tactcgttaa ttatcaagga cgtaactgaa
1441 gaggatgcag ggaattatac aatcttctg agcataaac agtcaaatgt gtttaaaaac
1501 ctactgcca ctctaattgt caatgtgaaa cccagattt acgaaaaggc cgtgtcatcg
1561 ttccagacc cggctctcta cccactgggc agcagacaaa tctgacttg taccgcatat
1621 ggatccctc aacctacaat caagtgggtc tggcaccct gtaaccataa tcattccgaa
1681 gcaagggtg actttgttc caataatgaa gactcctta tctggatgc tgacagcaac
1741 atgggaaaca gaattgagag catcactcag cgcatggcaa taatagaagg aaagaataag
1801 atggctagca ccttgggtgt ggctgactct agaatttctg gaatctacat ttgcatagct
1861 tccaataaag ttgggactgt gggaagaaac ataagcttt atatacaga tgtgccaat
1921 gggttcatg ttaactgga aaaaatgcc acggaaggag aggacctgaa actgtcttg
1981 acagtaaca agttctata cagagacgtt acttgattt tactgcggac agtaataac
2041 agaacaatgc actacagat tagcaagca aaaatggcca tcaactaagga gcaactccatc
2101 actcttaatc ttaccatcat gaatgttcc ctgcaagatt caggcaccta tgctgcaga
2161 gccaggaatg tatacacagg ggaagaaatc ctccagaaga aagaattac aatcagagat
2221 caggaagcac catacctct cgaaacctc agtgatcaca cagtggccat cagcagttcc
2281 accacttag actgtcatgc taatggtgtc cccagacctc agatcactg gtttaaaaac
```

2341 aaccacaaaa tacaacaaga gcttgaatt atttaggac caggaagcag cacgctgtt
2401 attgaaagag tcacagaaga ggatgaagggt gtctatcact gcaaagccac caaccagaag
2461 ggctctgtgg aaagttcagc atacctcact gtcaaggaa cctcggacaa gtctaactg
2521 gag

**DEFINITION Homo sapiens vascular endothelial growth factor receptor 2 (KDR)
mRNA, complete cds.**

LOCUS AF063658 4071 bp mRNA PRI 16-MAY-1998

SOURCE human.

ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;

Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 4071)

AUTHORS Yu,Y., Whitney,R.G. and Sato,J.D.

TITLE Coding region for human VEGF receptor KDR (VEGFR-2)

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 4071)

AUTHORS Yu,Y., Whitney,R.G. and Sato,J.D.

TITLE Direct Submission

JOURNAL Submitted (06-MAY-1998) Adirondack Biomedical Research Institute,
10 Old Barn Rd., Lake Placid, NY 12946, USA

BASE COUNT 1169 a 894 c 1025 g 983 t

ORIGIN

```
1 atggagagca aggtgctgct ggccgctgcc ctgtggctct gcgtaggagac ccgggcccgc
61 tctgtgggtt tgctagtgt ttctcttgat ctgcccaggc tcagcataca aaaagacata
121 ctacaatta aggctaatac aactctcaa attactgca ggggacagag ggactggac
181 tggctttgac ccaataatca gaggggcagt gagcaaaggg tggagggtgac tgagtgcagc
241 gatggcctct tctgtaagac actcacaatt ccaaaagtga tcggaaatga cactggagcc
301 tacaagtgt tctaccggga aactgacttg gctcgggtca tttatgtcta tgtcaagat
361 tacagatctc catttattgc ttctgttagt gaccaacatg gactcgtgta cattactgag
421 aacaaaaaca aaactgtggt gattccatgt ctgggtcca ttcaaactc caactgtca
481 ctttgtaa gataccaga aaagagattt gttctgatg gtaacagaat ttctgggac
541 agcaagaagg gcttactat tccagctac atgatcagct atgctggcat ggtctctgt
601 gaagcaaaaa ttaatgatga aagtaccag tctattatgt acatagttgt cgtgtaggg
661 tataggattt atgatgtgt tctgagtccg tctcatgaa tgaactatc tgttgagaa
721 aagctgtct taaattgtac agcaagaact gaactaaatg tggggattga ctcaactgg
781 gaataccctt ctgcaagca tcagcataag aaactgtaa accgagacct aaaaaccag
841 tctgggagtg agatgaagaa attttgagc accttaacta tagatgggtg aaccggagt
901 gaccaaggat tgtacacctg tgtagcatcc agtgggctga tgaccaagaa gaacagcaca
961 tttgcaggg tcatgaaaa acctttgtt gctttggaa gtggcatgga atctctggtg
1021 gaagccacgg tgggggagcg tgcagaatc cctgcgaagt acctgggta cccacccca
1081 gaaataaaat ggtataaaa tggaataccc ctgagtcca atcacacaat taaagcggg
1141 catgtactga cgattatgga agtgagtga agagacacag gaaattacac tgtatcctt
1201 accaatccca ttcaaagga gaagcagagc catgtggtct ctctggtgt gtatgtcca
1261 cccagattg gtgagaaatc ttaatctct cctgtggatt cctaccagta cggcaccat
1321 caaacgctga catgtacggt ctatgccatt cctccccgc atcacatcca ctggtattgg
1381 cagttggagg aagagtgcgc caacgagccc agccaagctg tctcagtac aaaccatac
1441 ccttgtaag aatggagaag tggaggagc ttccaggag gaaataaaat tgaagtaat
1501 aaaaatcaat ttgctctaat tgaaggaaaa acaaaaactg taagtaccct tgtatccaa
1561 gcgcaaatg tgcagctt gtacaaatgt gaagcggta acaaagtcgg gagaggagag
1621 aggggatct cctccacgt gaccaggggt cctgaaatta cttgcaacc tgacatgac
1681 cccactgagc aggagagcgt gctttgtgg tgcactgac acagatctac gttgagaac
1741 ctacatggt acaagcttg cccacagcct ctgccaatcc atgtgggaga gttgccaca
1801 cctgtttgca agaactgga tactcttgg aatgaatg ccacatgtt cttaatatg
1861 acaaatgaca tttgatcat ggagcttaag aatgatcct tcaggacca aggagactat
1921 gtctgcttg ctcaagacag gaagaccaag aaaagacatt gcgtggctcag gcagctcaca
1981 gtctagagc gttggcacc cacgatcaca gaaacctgg agaatcagac gacaagtatt
```

2041 ggggaaagca tcgaagtctc atgcacggca tctgggaatc cccctccaca gatcatgtgg
2101 tttaaagata atgagaccct tgtagaagac tcaggcattg tattgaagga tgggaaccgg
2161 aacctcacta tccgcagagt gaggaaggag gacgaaggcc tctacacctg ccaggcatgc
2221 agtgttcttg gctgtgcaaa agtggaggca ttttcataa tagaagggtc ccaggaaaag
2281 acgaacttgg aatcattat tctagtaggc acggcgggta ttgcatgtt ctctggcta
2341 ctctgtca tcatctacg gaccgtaag cgggccaatg gaggggaact gaagacaggc
2401 tactgtcca tcgtcatgga tccagatgaa ctcccattgg atgaacattg tgaacgactg
2461 cttatgatg ccagcaaatg ggaattcccc agagaccggc tgaagctagg taagcctctt
2521 ggccgtggg ccttgcca agtgattgaa gcagatgcct ttggaattga caagacagca
2581 actgcagga cagtagcagt caaaatgttg aaagaaggag caacacacag tgagcatcga
2641 gctctcatgt ctgaactcaa gatcctcatt catattgtc accatctcaa tgtggtaaac
2701 ctctaggtg cctgtaccaa gccaggaggg ccactcatgg tgatttgga attctgcaaa
2761 ttggaaacc tgtccactta cctgaggagc aagagaaatg aattgtccc ctacaagacc
2821 aaaggggac gattccgtca agggaaagac tacgttggag caatccctgt ggatcgaaa
2881 cggcgcttg acagcatcac cagtagccag agctcagcca gctctggatt tgtggaggag
2941 aagtcctca gtgatgtaga agaaggaggaa gctcctgaag atctgtataa ggactcctg
3001 acctggagc atctcatctg ttacagctc caagtggcta agggcatgga gttctggca
3061 tcgcgaaagt gtatccacag ggacctggcg gcacgaaata tcctctatc ggagaagaac
3121 gtggttaaaa tctgtgactt tggctggcc cgggatattt ataaagatcc agattatgc
3181 agaaaaggag atgctcgctt cctttgaaa tggatggccc cagaaacaat tttgacaga
3241 gtgtacacaa tccagagtga cgtctggctt tttgtgtt tgctgtgga aatatttcc
3301 ttaggtgctt ctccatatcc tgggtaaaag atgatgaag aattttag gcgattgaa
3361 gaaggaacta gaatgaggc cctgattat actacaccag aatgtacca gaccatgctg
3421 gactgctggc acggggagcc cagtcagaga cccacgtttt cagagttggt ggaacattg
3481 ggaaatctt tgcaagctaa tgctcagcag gatggcaaag actacattgt tctccgata
3541 tcagagactt tgagcatgga agaggattct ggactctctc tgcctacctc acctgttcc
3601 tgtatggagg aggaggaagt atgtgacccc aattccatt atgacaacac agcaggaatc
3661 agtcagtatc tgcagaacag taagcgaaag agccggcctg tgagtgtaaa aacattgaa
3721 gatatcccg tagaagaacc agaagtaaaa gtaatcccag atgacaacca gacggacagt
3781 ggtatgggtc ttgcctcaga agagctgaaa actttggaag acagaaccaa attatctcca
3841 tctttgtg gaatggtgcc cagcaaaagc agggagtctg tggcatctga aggctcaaac
3901 cagacaagcg gctaccagtc cggatatcac tccgatgaca cagacaccac cgtgtactcc
3961 agtgaggaag cagaactttt aaagctgata gagattggag tgcaaaccgg tagcacagcc
4021 cagattctcc agcctgactc ggggaccaca ctgagctctc ctctgttta a

DEFINITION Human angiopoietin-1 mRNA, complete cds.

LOCUS HSU83508 2149 bp mRNA linear PRI 26-MAR-1997
SOURCE Homo sapiens (human)
ORGANISM [Homo sapiens](#)

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 2149)

AUTHORS Davis,S., Aldrich,T.H., Jones,P.F., Acheson,A., Compton,D.L.,
Jain,V., Ryan,T.E., Bruno,J., Radziejewski,C., Maisonpierre,P.C.
and Yancopoulos,G.D.

TITLE Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by
secretion-trap expression cloning

JOURNAL Cell 87 (7), 1161-1169 (1996)

REFERENCE 2 (bases 1 to 2149)

AUTHORS Davis,S., Aldrich,T.H., Jones,P.F., Acheson,A., Compton,D.L.,
Jain,V., Ryan,T.E., Bruno,J., Radziejewski,C., Maisonpierre,P.C.
and Yancopoulos,G.D.

TITLE Direct Submission

JOURNAL Submitted (31-DEC-1996) Discovery, Regeneron Pharmaceuticals, 777
Old Saw Mill River Road, Tarrytown, NY 10591, USA

ORIGIN

```
1 cagctgactc aggcaggctc catgctgaac ggtcacacag agaggaaaca ataaatctca
61 gctactatgc aataaatatc tcaagttta acgaagaaaa acatcattgc agtgaataa
121 aaaattttaa aatttagaa caaagctaac aatggctag tttctatga ttctctca
181 aacgctttct ttgaggggga aagagtcaaa caacaagca gtttacctg aaataaaga
241 ctagtttag aggtcagaag aaaggagcaa gtttgcgag aggcacggaa ggagtgtgct
301 ggcagtacaa tgacagttt ctttcctt gcttctctg ctgccattct gactcacata
361 ggggtgcagca atcagcgccg aagtccagaa aacagtggga gaagatataa ccggattcaa
421 catgggcaat gtgcctacac ttcatctt ccagaacacg atggcaactg tctgagagt
481 acgacagacc agtacaacac aaacgctctg cagagagatg ctccacacgt ggaaccggat
541 ttctctccc agaaactca acatctggaa catgtgatg aaaattatac tcaatggctg
601 caaaaactg agaattacat tgtggaaaac atgaagtcgg agatggcca gatacagcag
661 aatgcagtc agaaccacac ggctaccatg ctggagatag gaaccagcct cctctctcag
721 actgcagagc agaccagaaa gctgacagat gttgagaccc aggtactaaa tcaaacttct
781 cgacttgaga tacagctgct ggagaattca ttatccacct acaagctaga gaagcaactt
841 ctcaacaga caaatgaaat ctgaagatc catgaaaaaa acagtttatt agaacataa
901 atcttagaaa tggaaggaaa acacaaggaa gagttggaca cctaaagga agagaaagag
961 aacctcaag gcttggtac tctcaaca tatataatcc aggagctgga aaagcaatta
1021 aacagagcta ccaccaaca cagtgtcctt cagaagcagc aactggagct gatggacaca
1081 gtccacaacc ttgtcaatct ttgactaaa gaaggtggtt tactaaaggg aggaaaaaga
1141 gaggaagaga aaccatttag agactgtgca gatgtatc aagctgggtt taataaaagt
1201 ggaatctaca ctattatat taataatg ccagaacca aaaaggtgtt ttgcaatg
1261 gatgtcaatg ggggaggtg gactgtaata caacatctg aagatggaag tctagattc
1321 caaagaggct ggaaggaata taaaatgggt ttggaaatc cctccggtga atattggctg
1381 gggaatgagt ttattttgc cattaccagt cagaggcagt acatgctaag aattgagta
1441 atggactggg aagggaaccg agcctattca cagtatgaca gattccacat aggaaatgaa
1501 aagcaaaact ataggttga ttaaaagggt cacactggga cagcaggaaa acagagcagc
1561 ctgatctac acgggtctga ttcagcact aaagatgctg ataatgaaa ctgtatgtc
1621 aaatgtgcc tcatgttaac aggaggatgg tggttgatg ctgtggccc ctccaatcta
1681 aatggaatgt tctatactgc gggacaaaac catggaaaac tgaatgggat aaagtggcac
1741 tactcaaag gcccagtta ctcttacgt tccacaacta tgatgattcg accttagat
1801 tttgaaagc gcaatgtcag aagcgattat gaaagcaaca aagaaatccg gagaagctgc
1861 caggtgagaa actgttgaa aactcagaa gcaacaata ttgtctcct tcagcaata
```

1921 agtggtagtt atgtgaagtc accaagggtc tfgaccgtga atctggagcc gtttgagtc
1981 acaagagtct ctactggggg tgacagtgct cacgtggctc gactatagaa aactccactg
2041 actgtcgggc tttaaaaagg gaagaaactg ctgagcttgc tgtgctcaa actactactg
2101 gacctattt tggaactatg gtagccagat gataaatatg gttaattc

DEFINITION Homo sapiens angiopoietin 2 (ANGPT2), mRNA.

LOCUS NM_001147 2269 bp mRNA linear PRI 20-DEC-2003

SOURCE Homo sapiens (human)

ORGANISM [Homo sapiens](#)

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 2269)

AUTHORS Zhou,Y., Bellingard,V., Feng,K.T., McMaster,M. and Fisher,S.J.

TITLE Human cytotrophoblasts promote endothelial survival and vascular remodeling through secretion of Ang2, PlGF, and VEGF-C

JOURNAL Dev. Biol. 263 (1), 114-125 (2003)

PUBMED [14568550](#)

ORIGIN

```
1 tgggttggtg tttatctcct cccagccttg agggagggaa caaactgta ggatctggg
61 agagaggaac aaaggaccgt gaaagctgct ctgtaaaagc tgacacagcc ctccaagt
121 agcaggactg ttctccac tgcaatctga cagttactg catgcctgga gagaacacag
181 cagtaaaaac caggtttgct actggaaaa gaggaagag aagacttca ttgacggacc
241 cagccatggc agcgtagcag ccctgcgtt cagacggcag cagctcgga ctctggacgt
301 gtgttgccc tcaagttgc taagctgctg gtttact gaagaaaga atgtggcagat
361 tgtttcttt actctgagct gtgatctgt ctggccgca gcctataaca actttcggaa
421 gagcatggac agcataggaa agaagcaata tcaggtccag catgggtcct gcagctacac
481 ttcctctc cagagatgg acaactgccg ctctctcc agcccctacg tgcctaatgc
541 tgtcagagg gacgcgccg tcgaatacga tgactcggtg cagaggctgc aagtctgga
601 gaacatcatg gaaaacaaca ctacgtggct aatgaagct gagaattata tccaggaaa
661 catgaagaaa gaaatgtag agatacagca gaatgcagta cagaaccaga cggctgtgat
721 gatagaaata gggacaaacc tgtgaacca aacagctgag caaacgcgga agttaactga
781 tgtggaagcc caagtata atcagaccac gagactgaa ctacagctc tgaacactc
841 cctctgaca acaaatgg aaaaacagat ttggaccag accagtga taaacaatt
901 gcaagataag aacagttcc tagaaaagaa ggtgctagct atggaagaca agcactcat
961 ccaactacag tcaataaag aagagaaaga tcagctacag gtgttagtat ccaagcaaaa
1021 ttcatcatt gaagaactag aaaaaaaaa atgactgcc acggtgaata attcagttc
1081 tcaaaagcag caacatgatc tcatggagac agttaataac ttactgacta tgaatccac
1141 atcaactca gtaaggacc cactgtgac taaagaagaa caatcagct tcagagactg
1201 tgctgaagta tcaaatcag gacacaccac aatggcacc tacacgtaa cattccctaa
1261 ttctacagaa gagatcaagg cctactgtga catggaagct ggaggaggcg ggtggacaat
1321 tattcagcga cgtgaggatg gcagcgtga tttcagagg acttgaaag aatataaagt
1381 gggatttgt aaccctcag gagaatattg gctgggaaat gagttgtt cgcaactgac
1441 taatcagcaa cgctatgtgc taaaataca cctaaagac tggaaggga atgaggctta
1501 ctcattgat gaacattct atctcaag tgaagaactc aattatagga ttcacctaa
1561 aggacttaca gggacagccg gcaaaataag cagcatcagc caaccaggaa atgatttag
1621 cacaaaggat ggagacaacg acaatgtat tgcaaatgt tcacaaatgc taacaggagg
1681 ctggtggtt gatgatgtg gtcctcca cttgaacgga atgtactatc cacagaggca
1741 gaacacaaat aagtcaacg gcattaatg gtactactgg aaaggctcag gctattcgt
1801 caaggccaca accatgatga tccgaccagc agattctaa acatcccagt ccacctgagg
1861 aactgtctc aactatttc aaagacttaa gccagtgca ctgaaagtca cggctgcgca
1921 ctgtgtcctc tccaccaca gagggcgtg gctcgggtg gacgggacc acatgctcca
1981 gattagagcc tgtaacttt atcactaaa ctgcatcac ttaacggacc aaagcaagac
2041 cctaaacatc cataattgtg attagacaga acacctatgc aaagatgaac cggaggctga
2101 gaatcagact gacagttac agacgtgct gtcacaacca agaatttat gtgcaagtt
2161 atcagtaaat aactggaaaa cagaacact atgtatata atacagatca tcttgaact
2221 gcattctct gagcactgt tatacactg gtaaatccc atatgtct
```

DEFINITION Homo sapiens TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal) (TEK), mRNA.

LOCUS NM_000459 4138 bp mRNA linear PRI 20-DEC-2003

SOURCE Homo sapiens (human)

ORGANISM [Homo sapiens](#)

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 4138)

AUTHORS Cascone,I., Audero,E., Giraud,E., Napione,L., Maniero,F., Philips,M.R., Collard,J.G., Serini,G. and Bussolino,F.

TITLE Tie-2-dependent activation of RhoA and Rac1 participates in endothelial cell motility triggered by angiopoietin-1

JOURNAL Blood 102 (7), 2482-2490 (2003)

PUBMED [12816861](#)

REMARK GeneRIF: Angiopoietin-1 is implicated in the maturation and remodeling of the vascular network during embryo development and in adult life through its tyrosine kinase receptor Tie-2 stimulates endothelial cells to migrate and change shape.

ORIGIN

```
1 cttctgtgct gttccttctt gcctctaact tgtaaacaag acgtactagg acgatgctaa
61 tggaaagtc acaaccgctg ggttttgaa aggatcctg ggacctcatg cacattgtg
121 gaaactggat ggagagattt ggggaagcat ggactctta gccagcttag ttctctgtg
181 agtcagctg ctcttctg gaactgtgga aggtgccatg gacttgatct tgatcaattc
241 cctacctt gtatctgatg ctgaaacatc tctcacctgc attgcctctg ggtggcgccc
301 ccatgagccc atcaccatag gaagggactt tgaagcctta atgaaccagc accaggatcc
361 gctggaagt actcaagatg tgaccagaga atgggctaaa aaagtgttt ggaagagaga
421 aaaggctagt aagatcaatg tgcttattt ctgtgaagg cgagttcgag gagaggcaat
481 caggatacga accatgaaga tgcgtcaaca agcttcttc ctaccagcta cttaactat
541 gactgtggac aaggagata acgtgaacat atcttcaaa aaggtattga taaagaaga
601 agatgcagtg attacaana atggctcct catccattca gtgccccggc atgaagtacc
661 tgatattcta gaagtacacc tgcctcatgc tcagccccag gatgctggag tgactcggc
721 caggtatata ggaggaaacc tctcacctc ggcctcacc aggctgatag tccggagatg
781 tgaagcccag aagtggggac ctgaatgcaa ccatctctgt actgcttga tgaacaatgg
841 tctctgcat gaagatactg gagaatgcat ttgccctct gggttatgg gaaggacgtg
901 tgagaaggct tgtgaactgc acacgtttgg cagaactgt aaagaaagg gacgtggaca
961 agagggatgc aagtcttatg tgttctgtct cctgacccc fatgggtgtt cctgtgccac
1021 aggctggaag ggtctgcagt gcaatgaagc atgccaccct ggttttacg ggccagattg
1081 taagcttagg tgcagctgca acaatgggga gatgtgat cgctccaag gatgtctctg
1141 ctctccagga tggcaggggc tccagtgtga gagagaaggc ataccgagga tgacccaaa
1201 gatagtggat ttgccagatc atatagaagt aacagtggt aaattaatc ccatttgcaa
1261 agcttctggc tggccgctac ctactaatga agaatgacc ctggtgaagc cggatgggac
1321 agtctccat ccaaaagact ttaaccatac ggatcattc tcagtagcca tattccat
1381 ccaccgatc ctccccctg actcaggagt ttgggtctgc agtgtgaaca cagtggctgg
1441 gatggtgaa aagccctca acatttctg taaagtctt ccaagcccc tgaatgccc
1501 aaacgtgatt gacactggac ataacttgc tgcatacaac atcagctctg agccttact
1561 tggggatgga ccaatcaaat ccaagaagct tctatacaaa cccgtaatc actatgaggc
1621 ttggcaacat attcaagtga caaatgagat tttacactc aactatttg aacctcggac
1681 agaatagaa ctctgtgac aactgtccg tcgtggagag ggtggggaag ggcacctgg
1741 acctgtgaga cgctcaca cagcttctat cggactccct cctccaagag gctaaatct
1801 cctgcctaaa agtcagacca ctctaaatt gacctggcaa ccaatattc caagctcgg
1861 agatgactt tatgtgaa tggagagaag gtctgtgcaa aaaagtgatc agcagaat
1921 taaagtcca ggcaactga ctcgggtgct acttaacaac ttacatcca gggagcagta
1981 cgtggtccga gctagagtca acaccaaggc ccaggggaa tggagtgaag atctcactgc
```

2041 ttggaccctt agtgacattc ttctcctca accagaaaac atcaagattt ccaacattac
2101 acactcctcg gctgtgattt ctggacaat attggatggc tattctattt ctctattac
2161 tatccgttac aagggtcaag gcaagaatga agaccagcac gttgatgtga agataaagaa
2221 tgccaccatc attcagtatc agctcaaggg cctagagcct gaaacagcat accaggtgga
2281 catthttgca gagaacaaca tagggtaag caaccagcc tttctcatg aactggtgac
2341 cctcccagaa tctcaagcac cagcggacct cggagggggg aagatgctgc ttatagccat
2401 ccttggctct gctggaatga cctgcctgac tgtgctgtg gccttctga tcatattgca
2461 attgaagagg gcaaatgtgc aaaggagaat ggccaagcc ttcaaaacg tgaggaaga
2521 accagtgtg cagtcaact caggactct ggcctaaac aggaaggta aaaacaacc
2581 agatctaca atttatccag tgctgactg gaatgacatc aaattcaag atgtattgg
2641 ggagggcaat ttggccaag ttctaaggc ggcacatcaag aaggatgggt tacggatgga
2701 tgctgcatc aaaagaatga aagaatgtc ctcaaagat gatcacaggg actttgcagg
2761 agaactgaa gttcttga aactggaca ccatcaaac atcatcaatc tctaggagc
2821 atgtaacat cgaggctact tgtacctggc cattgagtac gcgccccatg gaaacctct
2881 ggacttctt cgcaagagcc gtgtgctgga gacggacca gcattgcca ttgcaatag
2941 caccgctcc acactgtcct cccagcagct ccttacttc gctgccagc tggccccggg
3001 catggactac ttgagccaaa aacagttat ccacaggat ctggctgcca gaaacattt
3061 agttggtaa aactatgg caaaaatagc agatttggga tgtcccag gtcaagagg
3121 gtacgtgaaa aagacaatgg gaaggctccc agtgcgctgg atggccatc agtactgaa
3181 ttacagtgtg tacacaacca acagtatgt atgtctat ggtgtgtac tatggagat
3241 ttttagctta ggaggcacac cctactgagg gatgactgt gcagaactc acgagaagt
3301 gcccagggc tacagactgg agaagcccct gaactgtgat gatgagggt atgatcaat
3361 gagacaatgc tggcgggaga agcctatga gaggcatca ttgccaga tattgtgtc
3421 cttaaacaga atgttagagg agcgaagac ctactgaa accacgctt atgagaagt
3481 tacttatgca ggaattgact gttctgctga agaagcggcc taggacagaa catctgata
3541 ccctctgtt cccttact ggcatgggag accctgaca actgctgaga aaacatgct
3601 ctgcaaaagg atgtgatata taagtgtaca tatgtgctg aattcaaca agtcataggt
3661 taatattaa gacactgaaa aatctaagt atataatca gattctctc tctattta
3721 tccctacct gtacatgcc agtcccgtt cattagtca tgtgacct ctgtctgtg
3781 tttccacagc ctgcaagtc agtccaggat gctaacatc aaaaatagac taaatctca
3841 ttgcttaca gcctaagaat cttagagaa gtatacataa gtttaggata aaataatggg
3901 atttctttt ctthctctg gtaattatga ctgtatatt ttaagaaata acagaaagcc
3961 tgggtgacat ttgggagaca tgtgacatt atattgaa ttaatatcc tacatgtatt
4021 gcacattgta aaaagttta gtttgatga gttgtgagt tacctgtat actgtaggca
4081 cacttgcac tgatataca tgagtgaata aatgtctgc ctactcaaaa aaaaaaaa

Homology of probes with marmoset

VEGF

VEGF probe 578 bp

Marmoset sequence **ACCESSION** AJ278192 374bp

Matches very well from 120 -422 of our probe, bases 1-303 of the Marmoset sequence, with 6 mismatches, ie 98% homology.

The rest of the Marmoset 304 - 374 only gives 23% homology.

This corresponds to 1158 - 1531 of the published Human sequence **ACCESSION** E15157

FLT

Published Human sequence **ACCESSION** AJ278194

our probe 1507-1778

marmoset **ACCESSION** AJ278194

3592-3910 8 mismatches 98% homology

Our probe detects both sflt and Flt1

KDR

Published Human sequence **ACCESSION** AJ278193

our probe 1355-1767

marmoset sequence **ACCESSION** AJ278193

2491-2776 7 mismatches 97% homology

Ang-1, Ang-2 and Tie-2

No marmoset data available