THE UNIVERSITY of EDINBURGH

Title	Regulation of collagen pathway biology during follicle maturation in the rat ovary
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Qualification	PhD
Year	2007

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Regulation of collagen pathway biology during follicle maturation in the rat ovary

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Doctor of Philosophy

2007

Declaration

The studies in this thesis were the unaided work of the author, except where acknowledgement is made by reference. The work described in this thesis has not been previously accepted for, or is currently being submitted for, another degree or qualification.

Kirsty D. Shearer
January 2007

Acknowledgements

First I would like to thank my supervisors Prof. Steve Hillier and Dr Tony Bramley for their support and encouragement over the last three years.

My thanks also goes to Dr Mick Rae for all his wit and expert knowledge, and all those words for encouragement as well as all those stories; Dr Chris Harlow for the many hours of the day and night spent in the animal house injecting animals and in the tissue culture lab culturing rat cells; Dr Tom Van Agtmael for all those 'collagen chats' as well as collecting the ovaries from his raw mice and supplying me with basement membrane antibodies, 'it only takes a trip to America to meet someone from the same building!'; Deborah Price for imparting all her technical knowledge on the molecular aspects of this thesis; Dr Michelle O'Reilly and June Noble for showing me the art of *in situ* hybridisation; Eva Gay and the histology team for all their histology advise; the staff of the animal house for looking after my animals, especially Mark Fisken for setting up my breeding colony. Thanks also to Prof. John Mullins and Dr Linda Mullins for providing the ovarian tissue from the 11βHSD1$^{-/-}$ and CYP11B1$^{-/-}$ mice.

Many thanks to my tireless proof readers for spotting every spelling mistake and missing comma. I would like to thank my fellow third years, Michelle, Nicole, Sarah and Cynthia – anyone for pizza? - as well as Michelle, Scott and Anastasia for all the laughs, and all the other wonderful people I have met at in the CRB.

Finally, I would like to thank all my family and friends for all their love and encouragement, as well as Basil, Hagar and Tilly for keeping me sane!

Presentations

Shearer KD, Rae MT, Harlow CR, Hillier SG

Local regulation of ECM remodelling in rat the ovary.

Oral presentation at Society for Reproduction and Fertility Conference, Leeds, July 2006

Shearer KD, Rae MT, Harlow CR, Hillier SG

Expression of genes that regulate the ovarian extracellular matrix.

Oral presentation: Munro Kerr Society Meeting, Edinburgh, February 2006

Shearer KD, Rae MT, Hillier SG

Regulation of lysyl oxidase (LOX) and related genes in rat granulosa cells during follicular maturation.

Oral presentation: 196th Meeting of the Society for Endocrinology Conference, London, November 2005

Shearer KD, Rae MT, Hillier SG

Lysyl oxidase and related genes in rat granulosa cells in the maturing follicle.

Poster presentation: 14th Simpson Symposium, Edinburgh, August 2005

Shearer KD, Rae MT, Hillier SG

Regulation of synthetic tissue remodelling-associated genes in rat granulosa cells.

Poster presentation: GRC Collagen, Connecticut, July 2005

Publications

Harlow CR, Bradshaw AC, Rae MT, Shearer KD, Hillier SG (in press) **Oestrogen formation and connective tissue growth factor (CTGF) gene and protein expression in rat granulosa cells.** Journal of Endocrinology.

Shearer KD, Rae MT, Harlow CR, Hillier SG (2006). **Local regulation of ECM remodelling in the rat ovary.** Reproduction Abstract Series. 33:O22

Shearer KD, Rae MT, Hillier SG (2005). **Regulation of lysyl oxidase (LOX) and related genes in rat granulosa cells during follicular maturation.** Endocrine Abstracts. 10:OC1

Abstract

Ovulation is a natural, recurrent injury involving sustained breakdown and remodelling of extracellular matrix (ECM) proteins, as follicles grow, ovulate and luteinise during each reproductive cycle. It is generally accepted that ovulation-associated tissue injury is the end-product of an acute inflammatory cascade driven by gonadotrophins (follicle-stimulating hormone (FSH) and luteinising hormone (LH)) and inflammatory mediators that signal up-regulation of matrix metalloproteinase enzymes, which proteolytically digest the follicle wall to allow oocyte release. The principle ECM component of the basement membrane separating granulosa cells (GC) and theca cells (TC) in the follicle wall is collagen IV. The terminal stage in mature collagen formation is cross-linkage of collagen fibrils catalysed by lysyl oxidase (LOX). The pro-LOX protein encoded by LOX mRNA is hydrolysed extracellularly to active LOX by bone-morphogenetic protein-1 (BMP-1), which also hydrolyses procollagens to mature forms of collagen. BMP-1 action on pro-collagen is enhanced by procollagen C-proteinase enhancer (PCPE). This thesis examines where and how expression of these collagen pathway component genes are regulated at mRNA and protein levels throughout follicular development, asking in which cell types are they expressed; are they developmentally (i.e. gonadotrophin) regulated; and do local pro- and anti-inflammatory mechanisms influence their activity?

Quantitative RT-PCR analysis of total RNA from rat ovaries and *in situ* hybridisation studies revealed presence of LOX, BMP-1 and PCPE mRNA in both GC and TC of rat ovary. In immature follicles, LOX mRNA principally located to GC where its abundance declined during gonadotrophin-induced follicular development. Conversely, BMP-1 and PCPE mRNA were present at similar levels in GC and TC. No developmental regulation was determined in isolated cells but visualisation by in situ hybridisation on whole ovaries saw these genes associated with the theca layer declining as maturation occurred. LOX and BMP-1 protein were located in both GC and TC layers. These results suggest a two-cell mechanism of ECM formation involving GC LOX and TC BMP-1.

GC and TC isolated from rat ovarian follicles were cultured with development-associated regulatory cues: FSH (GC), LH (TC) and TGF-β for immature follicles; LH, TGF-β and IL-1α for preovulatory follicles, in presence and absence of anti-inflammatory steroids (progesterone or corticosterone), to determine how these factors affect collagen pathway gene expression *in vitro*. Results established that components of ECM remodelling varied

with the changing profile of ovarian regulators. In the immature follicle FSH in the GC, LH in the TC and TGF-β in both cell types caused an overall increase in up-regulation of collagen pathway activity to allow for follicle growth. Towards ovulation LH, progesterone, IL-1 and a probable lack of TGF-β caused a cessation of active remodelling. Then post-ovulation remodelling was resumed due to the positive influences of TGF-β and corticosterone.

The classic anti-inflammatory glucocorticoid corticosterone, acts to minimise or prevent fibrosis. Evidence from culture experiments showed corticosterone influenced collagen pathway gene expression. Within glucocorticoid target tissues corticosterone formation is amplified by 11β-hydroxysteroid dehydrogenase (11βHSD) type 1 and suppressed by 11βHSD type 2. Both 11βHSD isoforms are known to be regulated at the level of gene expression in the ovary. Immunolocalisation of 11βHSD proteins in normal rat ovary showed that 11βHSD1 increased as follicles developed, whereas 11βHSD2 declined, suggesting that active glucocorticoid becomes available to influence collagen deposition and remodelling after ovulation. However, ovaries from mice null for the 11βHSD1 gene, in which corticosterone cannot be locally activated, remained fertile and no changes were seen in protein expression of LOX or BMP-1. Therefore other mechanisms may rate-limit intra-ovarian glucocorticoid action during ovulation.

Finally, collagen pathway components are instrumental in forming stable collagen structures and are believed to influence collagen IV networks. In the ovary collagen IV is associated with the follicular basement membrane. But mice with partial disruptions of COL4α1 did not show gross abnormalities in fertility or collagen pathway gene location. This result suggests that the follicular basement membrane is a dynamic structure that can accommodate partial disturbances without becoming dysfunctional.

The conclusion of this thesis is that there are changes in intrafolliclular ECM remodelling driven by endocrine, paracrine and intracrine signals. Collagen pathway components are key to this process and present targets for future studies of tissue injury and repair in ovary and elsewhere in the body.

Abbreviations

11βHSD	11β-hydroxysteroid dehydrogenase
17βHSD	17β-hydroxysteroid dehydrogenase
3βHSD	3β-hydroxysteroid dehydrogenase
AAS	α-aminoadipic-δ-semialdehyde
ACP	alcohol condensation product
ACTH	adrenocorticotrophic hormone
ADAMTS	A disintegrin and metalloproteinase with thrombospondin 1 motifs
Asp	aspartic acid
BAPN	β-aminoproprionitrile
BM	basement membrane
BMP-1	bone morphogenetic protein-1
Bru	bruised
Ca^{+}	calcium
CBG	corticosteroid-binding globulin
CNBr	cyanogens bromide
CNS	central nervous system
COL	collagen
CORT	corticosterone
COX	cyclooxygenase
CTGF	connective tissue growth factor
Cu^{2+}	copper
CUB	C-terminal embryonic sea urchin protein Uegf BMP-1
CYP11A	cholesterol side-chain cleavage cytochrome P450
CYP11B1	11β-hydroxylase cytochrome P450
CYP17	17^{α}-hydrolase/$C_{17\text{-}20}$lyase cytochrome P450
CYP19	aromatase/cytochrome P450
CYP21	21-hydroxylase cytochrome P450
DAB	3,3'-Diaminobenzidine tetrahydrochloride

deLNL	anhydrolysinonorleucine
DEPC	diethylpyrocarbonate
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	ethylenediaminetetaacetic acid
EGF	epidermal growth factor
ECM	extracellular matrix
ENU	N-ethyl-N-nitrosourea
EtOH	ethanol
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
g	gram
GAG	glycosaminoglycan
GBM	glomerular basement membrane
GDF-9	growth differentiation factor-9
Gly	glycine
GM-CSF	granulocyte-macrophage colony-stimulating factor
GnRH	gonadotrophin releasing hormone
GR	glucocorticoid receptor
h	hour
HIF	hypoxia-inducible factor
HSC	hepatic stellate cells
IL-1	interleukin-1
IL-1RA	interleukin-1 receptor antagonist
kDa	kilo-daltons
l	litre
LB	Luria-Bertani
LH	luteinising hormone

LHR	luteinising hormone receptor
LOX	lysyl oxidase
LTQ	lysine tyrosylquinone
Lys	lysine
MeOH	methanol
min	minute
miRNA	microRNA
MMP	matrix metalloproteinase
MR	mineralocorticoid receptor
mRNA	messenger RNA
mth	month
mTLD	mammalian tolloid
mTLL	mammalian tolloid-like
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
NADPH	nicotinamide-adenine dinucleotide phosphate (reduced form)
NC1	noncollagenous-1 domain
NTR	netrin
OSE	ovarian surface epithelium
PA	plasminogen activation
PBS	phosphate buffered saline
PCOS	polycystic ovarian syndrome
PCP	procollagen C-proteinase
PCPE	procollagen C-proteinase enhancer
Raw	retinal arteriolar wiring
RNA	Ribonucleic acid
rrg	*ras* recision
sec	second
StAR	steroidogenic acute regulatory protein
Svc	small with vacuolar cataracts

TBST	Tris buffered saline with Tween-20
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of matrix metalloproteinase
TNF-α	tumour necrosis factor-alpha
Tyr	tyrosine

1. Review of the Literature

1.1. Ovulation as an inflammatory event

The process of ovulation is described as an inflammatory reaction (Espey 1980; Espey 1994). This model states that the surge of luteinising hormone (LH) prior to ovulation causes an inflammatory reaction within the preovulatory follicle that subsequently results in the rupture of the healthy follicle and the release of the oocyte. Before detailing ovulation as an inflammatory event, folliculogenesis will be briefly reviewed to give an outline of non-inflammatory events in the ovary prior to ovulation, because if these events do not occur ovulation cannot take place.

The ovary consists of follicles at different stages of development embedded in a connective tissue matrix known as the stroma, as shown in Figure 1.1A. The external surface of the ovary is covered by a simple cuboidal epithelial layer of cells known as the ovarian surface epithelium (OSE). Follicles are made up of granulosa cells and theca cells, which are separated by a basement membrane (Fig. 1.1B). The theca layer is vascular whereas the granulosa layer is avascular. The basement membrane prohibits the invasion of vessels into the granulosa layer but allows passage of nutritional and instructional substances between the two cell types and therefore ultimately to the oocyte.

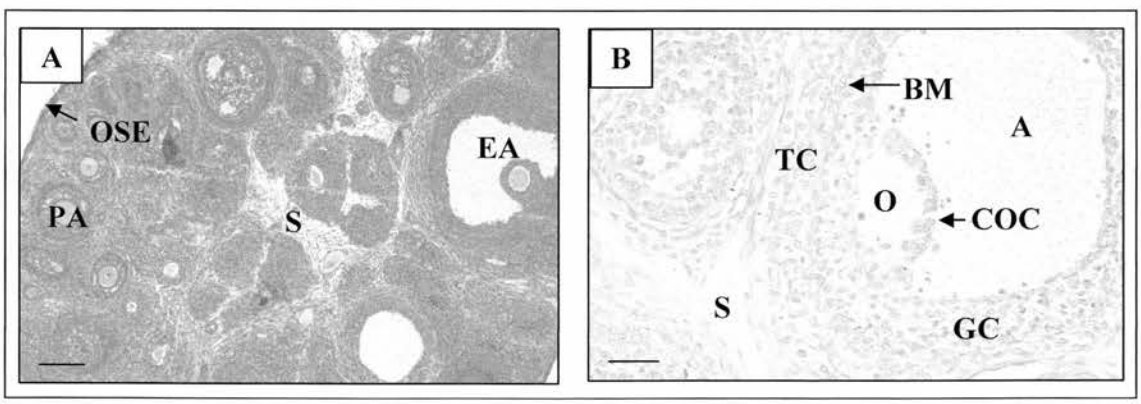

Figure 1.1. Collagen content and basement membrane location in the rat ovary. A, immature rat ovary stained with Masson's Trichrome illustrating a population of preantral and antral follicles within the stroma, scale bar 100μm. B, antral rat follicle showing the basement membrane separating the granulosa and theca cells within the follicle, scale bar 50μm. Ovarian surface epithelium (OSE), preantral follicle (PA), stroma (S), early antral follicle (EA), theca cells (TC), oocytes (O), basement membrane (BM), cumulus oophorus complex (COC), antrum (A), granulosa cells (GC).

The follicles in the ovary are laid down before birth as primordial follicles, each consisting of an oocyte (arrested at prophase I of meiosis) and a single layer of flattened precursor granulosa cells. Primordial follicles are recruited throughout reproductive life to grow and mature, either fully to result in ovulation and potential fertilisation or incompletely to become atretic and degenerate.

1.1.1. Folliculogenesis

The process of follicle growth is known as folliculogenesis and involves endocrine regulation from the hypothalamus and pituitary gland as well as paracrine signalling within the ovary itself. Follicles mature (Fig 1.2A) from primordial through primary (granulosa cells are cuboidal and the number of layers are increasing) and secondary (increasing layers of granulosa cells surrounded by thecal cells recruited from the stroma) to tertiary (when an antrum is formed) and onwards to become preovulatory (Rajkovic *et al.* 2006). However the fate of most follicles is not ovulation but degeneration. This maturation process of degeneration is known as atresia and is due to programmed cell-death of somatic follicular cells and oocytes. The life-cycle of a follicle is summarised in Fig. 1.2.

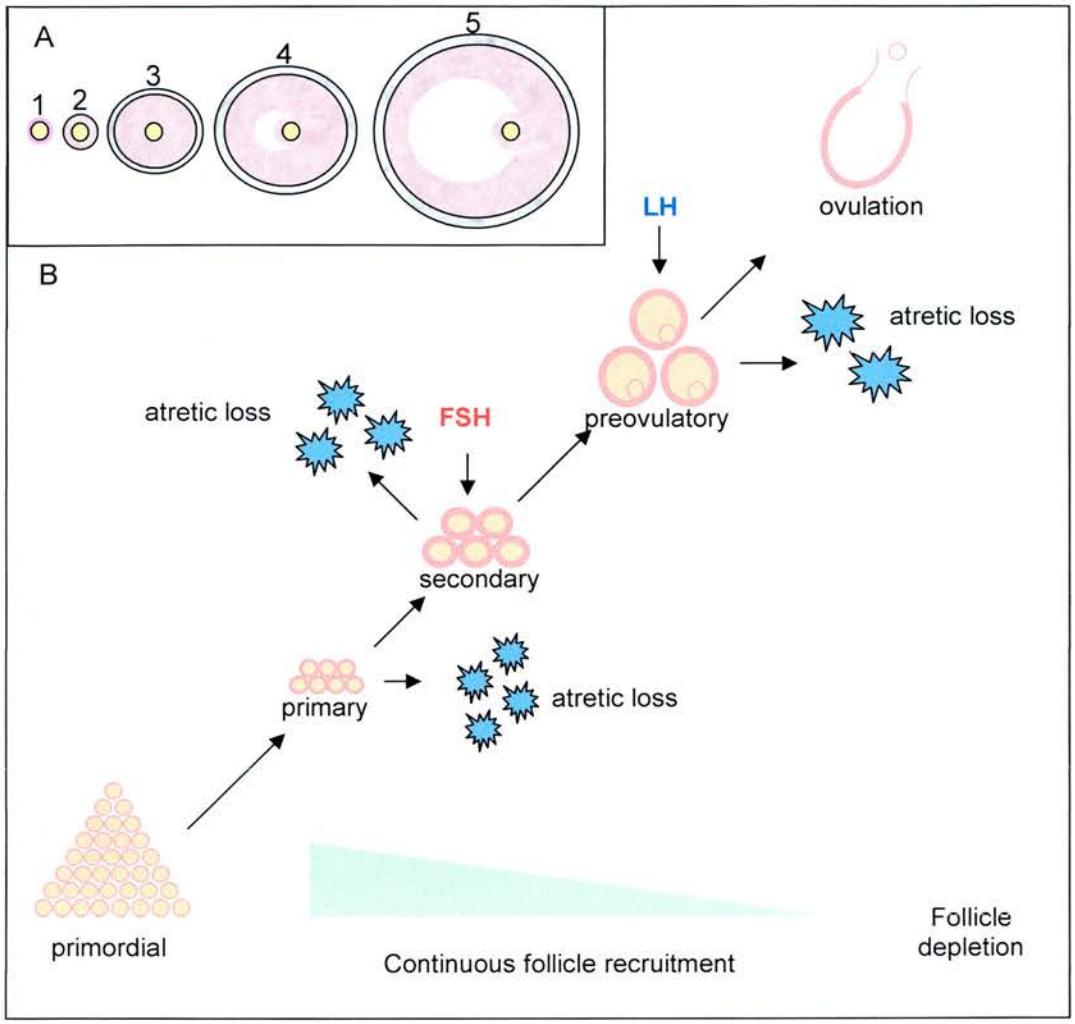

Figure 1.2. The life-cycle of an ovarian follicle. A, illustration depicting follicular maturation: 1, primordial follicle; 2, primary follicle; 3, secondary follicle; 4, tertiary follicle; 5, preovulatory follicle. B, graphical representation of the potential fates of a follicle during maturation emphasising that the number of primordial follicles declines throughout reproductive life until depletion. This conventional perception has recently been challenged by the work of Jonathan Tilly (Johnson *et al.* 2004) suggesting that there is primordial follicle renewal. Adapted from McGee and Hsueh (2000).

Folliculogenesis has two successive phases: gonadotrophin-independent (preantral) followed by gonadotrophin-dependent (antral).

1.1.1.1. Gonadotrophin-independent follicle growth

Primordial follicles are continuously recruited to grow as cohorts in the cortical stroma and are activated to become primary follicles. The signals that stimulate activation are not fully understood but possibly involve the removal of a local inhibitory factor (Suh *et al.* 2002). At the preantral stage, paracrine factors cause the growth and differentiation of oocytes, proliferation and cytodifferentiation of granulosa cells, and recruitment and development of theca cells. The development of a thecal layer in the preantral follicle is essential for any subsequent response of the follicle to LH (Magoffin 2005). Although not well characterised, various growth and differentiation factors are assumed to work in a series of paracrine feedback loops to orchestrate early follicle development and initiate stages of development that eventually require the presence of FSH (Suh *et al.* 2002). Mice null for the growth differentiation factor-9 (GDF-9) gene are unable to form a theca layer, indicating that this growth/differentiation factor is important in theca development (Dong *et al.* 1996). Whether such factors emanate from oocytes or granulosa cells is uncertain but it can be concluded that paracrine communication between both cells is an absolute requirement for normal folliculogenesis.

Once growth is initiated and 2-3 layers of granulosa cells have developed, the follicular antrum starts to form (Suh *et al.* 2002). This cavity fills with follicular fluid comprised of granulosa cell secretions and substances passing from plasma across the follicular basement membrane. At this stage further growth and development becomes critically dependent on stimulation by gonadotrophins.

1.1.1.2. Gonadotrophin-dependent follicle growth

Once antral development has started, follicle growth becomes gonadotrophin-dependent. This is revealed by the phenotype of the follicle-stimulating hormone-β (FSHβ)-null mouse where folliculogenesis does not progress beyond preantral stages, likewise in the luteinising hormone (LH) receptor null mouse, where follicles only reach early antral stages (Kumar *et al.* 1997; Zhang *et al.* 2001). Follicles not in receipt of adequate gonadotrophic support become atretic and degenerate.

The gonadotrophins are members of the glycoprotein hormone family that also includes thyroid stimulating hormone. Each of these heterodimeric hormones shares a common

α-subunit with a unique β-subunit that confers individual receptor specificity. It is gonadotrophin-releasing hormone (GnRH) produced by the hypothalamus that stimulates release of FSH and LH from the anterior pituitary gland (Rajkovic *et al.* 2006). Negative and positive feedback loops along the hypothalamo-pituitary-gonadal axis exist to regulate the release of these hormones.

FSH and LH act via their receptors (FSHR and LHR) on ovarian cells to promote follicle maturation. In particular, FSH causes further granulosa cell proliferation and differentiation. Preovulatory differentiation is signalled through increased aromatisation of androgen to oestrogens and expression of the granulosa cell LHR. LH mainly causes theca cells to produce androgens for aromatisation by granulosa cells and is eventually necessary for ovulation to take place (Richards 1994). As well as FSH and LH, the maturing follicle utilises locally-produced oestrogen to support granulosa cell proliferation. Oestrogen biosynthesis requires two cell-types (granulosa and theca) and two gonadotrophins (FSH and LH). This is described as the 'two-cell, two-gonadotrophin' mechanism (Fig. 1.3.).

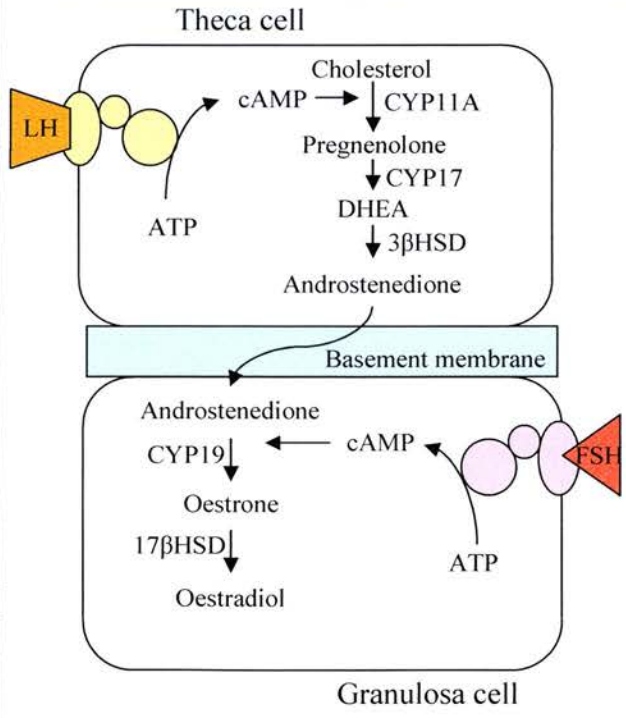

Figure 1.3. The 'two-cell, two-gonadotrophin' model of oestrogen synthesis adapted from Magoffin (2005). LH binds to its G protein-coupled receptor on the theca cell membrane and via cAMP signalling stimulates the expression of cholesterol side-chain cleavage cytochrome P450 (CYP11A), 17α-hydroxylase/C_{17-20} lyase cytochrome P450 (CYP17) and 3β-hydroxysteroid dehydrogenase (3βHSD). This cascade results in the synthesis of androstenedione which passes across the basement membrane to the granulosa cells. FSH binds to its G protein-coupled receptor and via cAMP causes the expression of aromatase cytochrome P450 (CYP19) and 17β-hydroxysteroid dehydrogenase (17βHSD). These enzymes metabolise androstenedione to oestradiol.

In this classic model the granulosa and theca cells work together to regulate oestrogen synthesis. The theca cells convert cholesterol to androgens following stimulation signalled by LH through the G protein-coupled LHR on their cell surface. However, theca cells cannot produce the aromatase enzyme cytochrome P450 (CYP19) needed to convert androgen to oestrogen (Magoffin 2005). On the other hand the granulosa cells cannot produce androgen due to the absence of 17α-hydroxylase/C_{17-20}lyase cytochrome P450 (CYP17) enzyme, which they can convert androgen to oestrogen since they express CYP19, following FSH stimulation via the FSHR (Magoffin 2005). Hence theca cells produce androgen in the form of androstenedione, which passes through the basement membrane to the granulosa cells to be metabolised into oestrogen. LH enhances the production of androgens both by increasing the number of LHR present on theca cells and by increasing the expression of steroidogenic acute regulatory protein (StAR) which aids cholesterol transport within the cell (Magoffin 2005). Oestrogen production is limited by the amount of androgen available. Therefore the increased amount of oestrogen secreted by the maturing follicle is coupled to the increased ability of its theca cells to produce androgens, due to endocrine stimulation by LH and paracrine stimulation by granulosa cell-derived inhibin (Hillier 1991). Oestrogens created in the ovary also drive the proliferation and differentiation of steroidogenic ovarian cells (Findlay *et al.* 2001)

As follicular oestrogen secretion continues to increase and plasma levels rise beyond a critical threshold level, oestrogen exerts positive feedback action on the hypothalamus and pituitary gland. This triggers the release of a surge of LH, which causes ovulation to occur with release of the oocyte. The LH surge also causes the luteinisation of the granulosa and theca cells of the preovulatory follicle (Richards 1994) to form the corpus luteum, which is a highly vascularised endocrine gland that primarily produces progesterone. The corpus luteum has one of two fates: it can either be maintained if pregnancy occurs or if not it will regress and a new ovarian cycle will begin.

1.1.2. Inflammation at ovulation

At ovulation the preovulatory follicles rupture to release oocytes for potential fertilisation. This occurs through the LH surge triggering the secretion of proteases to degrade the apical follicular wall. In addition to causing ovulation and luteinisation the LH surge also initiates the progression of oocyte meiosis to metaphase II (where it is arrested until fertilisation) and the down-regulation of CYP17 (Magoffin 2005)

What is the evidence that ovulation is an inflammatory event? Classic inflammation is characterised by: redness, heat, swelling, pain and organ dysfunction. Fig. 1.4 shows a human ovary at ovulation. It can clearly be seen that the site of ovulation is red and swollen.

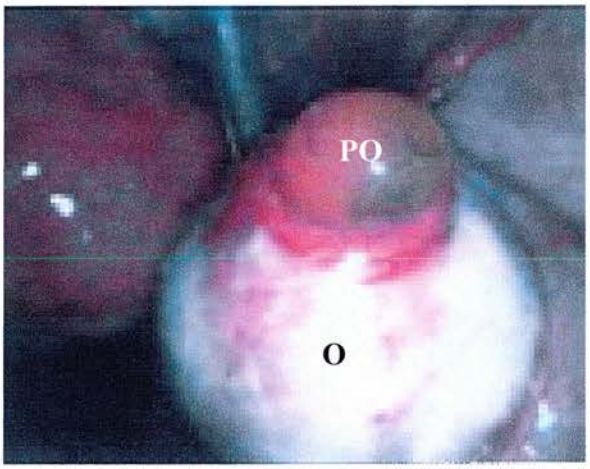

Figure 1.4. The human ovary at ovulation. Preovulatory follicle (PO), ovary (O). Source www.oakbrookfertility.com/files/fertility.html

If non-steroidal anti-inflammatories are given during the first 80% of ovulation the process will be stopped (Espey 1980). These drugs inhibit acute inflammation (unlike steroidal anti-inflammatories that work on chronic conditions and have no effect on inhibiting ovulation) (Espey 1994).

It is the LH surge that triggers these inflammatory changes around the preovulatory follicle. The LH surge starts several hours prior to the physical act of follicle rupture and oocyte release (the time span varies in different species). During this time physical changes are occurring to the cells of the follicle and the surrounding tissue. There are

five layers of cells that must be breached at ovulation; these are the OSE, the tunica albuginea, the theca externa, the theca interna and the granulosa layer (Espey 1994). These layers have associated collagenous ECM structures such as basement membranes that must be broken down for ovulation to occur. The inflammation of ovulation initiates protease activity that breaks down these tissue layers (Espey & Richards 2006). As ovulation is imminent, the follicle wall becomes very thin due to degradation and the follicle bulges from the surface of the ovary (Espey & Richards 2006). The LH surge causes the fibroblasts in the collagenous tissues to be activated and proliferate leading to the weakening of the follicle wall and eventual rupture of the follicle (Espey 1994).

Many genes involved in processes of inflammation in the body are evident in the process of ovulation. Interleukin-1 (IL-1) is a pro-inflammatory cytokine and will induce ovulation in the rat while IL-1 receptor antagonists (IL-1RA) will reduce the incidence of ovulation (Brannstrom *et al.* 1993; Peterson *et al.* 1993) Leukocytes are recruited to the follicle shortly after the LH surge and are regulated by IL-1 (Brannstrom & Enskog 2002). Ovarian perfusion *in vitro* with leukocytes taken from the peripheral circulation increases the ovulation rate 3-fold (Brannstrom & Enskog 2002). Other cytokines expressed at ovulation are IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor-alpha (TNF-α) (Brannstrom *et al.* 1994). Prostagladins are also key mediators in inflammation and their production is regulated by the LH-dependent cyclooxygenase-2 (COX-2) enzyme. Ovulation is prevented by inhibition of prostaglandin biosynthesis by non-steroidal anti-inflammatories and antisera to prostaglandin will also prevent follicle rupture (Sirois *et al.* 2004). Selective COX-2 inhibitors prevent ovulation in mice, showing inhibition of prostaglandin production is at the level of COX-2 (Sirois *et al.* 2004). The COX-2 null mouse fails to ovulate (Lim *et al.* 1997) but this phenotype is rescued by the administration of exogenous prostaglandin (Davis *et al.* 1999). All this evidence indicates that inflammation is a key feature of ovulation.

1.2. Anti-inflammatory mechanism of ovulation

Since ovulation is an inflammatory event, the ovary must be able to resolve this inflammation before each new ovarian cycle begins.

Work in human OSE cells has shown that the active glucocorticoid, cortisol, down-regulates pro-inflammatory associated genes and up-regulates anti-inflammatory regulators (Rae *et al.* 2004b). Progesterone also causes down regulation of pro-inflammatory induced genes in these cells but has no effect on anti-inflammatory regulators (Rae *et al.* 2004b). This suggests that glucocorticoids with the assistance of progesterone play a major role in inflammatory resolution.

1.2.1. Glucocorticoids

Glucocorticoids have many important functions emphasised by the fact that most cells in the body possess receptors for these hormones. Glucocorticoids stimulate gluconeogenesis which results in glucose formation from amino acids and lipids. They also induce enzymes that metabolise these substrates from tissues and are involved in glucose conservation. Active glucocorticoids are produced in acute periods of stress in response to the 'fight-or-flight' mechanism. This burst of 'stress hormone' causes a rapid increase in energy and immunity, heightened cognitive function, a lowering in pain threshold and maintains homeostasis in the body; all of which lead to an increased chance of survival in an adverse environment (Alberts *et al.* 1997). Glucocorticoids have anti-inflammatory actions that are mediated through interaction with the glucocorticoid receptor (GR) (Cato & Wade 1996). Anti-inflammatory glucocorticoids inhibit the synthesis of prostaglandins and pro-inflammatory cytokines in the ovary (Goppelt-Struebe 1997; Telleria *et al.* 1998). Glucocorticoids also have immunosuppressive properties and these two properties have led to the use of glucocorticoids in autoimmune and inflammatory diseases.

The fact that glucocorticoids have so many functions in the body highlights the importance of understanding how they are regulated and influence different biological processes.

1.2.1.1. Glucocorticoid synthesis and regulation

1.2.1.1.1. The synthesis of glucocorticoids

Glucocorticoids are synthesised in the adrenal cortex from precursor cholesterol (Brook & Marshall 2001). The synthesis and secretion of glucocorticoid by the adrenal gland is stimulated by adrenocorticotrophic hormone (ACTH) from the pituitary gland (Brook & Marshall 2001). ACTH causes an increase in the production of cholesterol side-chain cleavage (CYP11A1) that converts cholesterol to pregnenolone (Fig. 1.5) (Brook & Marshall 2001; Michael *et al.* 2003; Miller 1988). In the adrenal gland of most mammals, pregnenolone can either be converted to 17α-hydroxypregnenolone via 17α-hydroxylase (CYP17) or to progesterone via 3β-hydroxysteroid dehydrogenase (3βHSD) (Fig. 1.5) (Brook & Marshall 2001). The exception to this is in the rodent, in which CYP17 is not produced in the adrenals (Keeney *et al.* 1995). Therefore, in the rodent adrenal pregnenolone is only converted to progesterone. Glucocorticoids are produced from either progesterone or 17α-hydroxyprogesterone substrates via the enzymic activities of 21-hydroxylase (CYP21) (Brook & Marshall 2001) and 11β-hydroxylase (CYP11B1). Glucocorticoids principally circulate in human blood as 'active' cortisol and 'inactive' cortisone. As discussed below in the rodent, the active glucocorticoid is corticosterone and the inactive form deoxycorticosterone. The difference between human and rodent glucocorticoids is the absence of the hydroxyl groups at position C17 in the rodent, but the biological actions are the same (Michael *et al.* 2003).

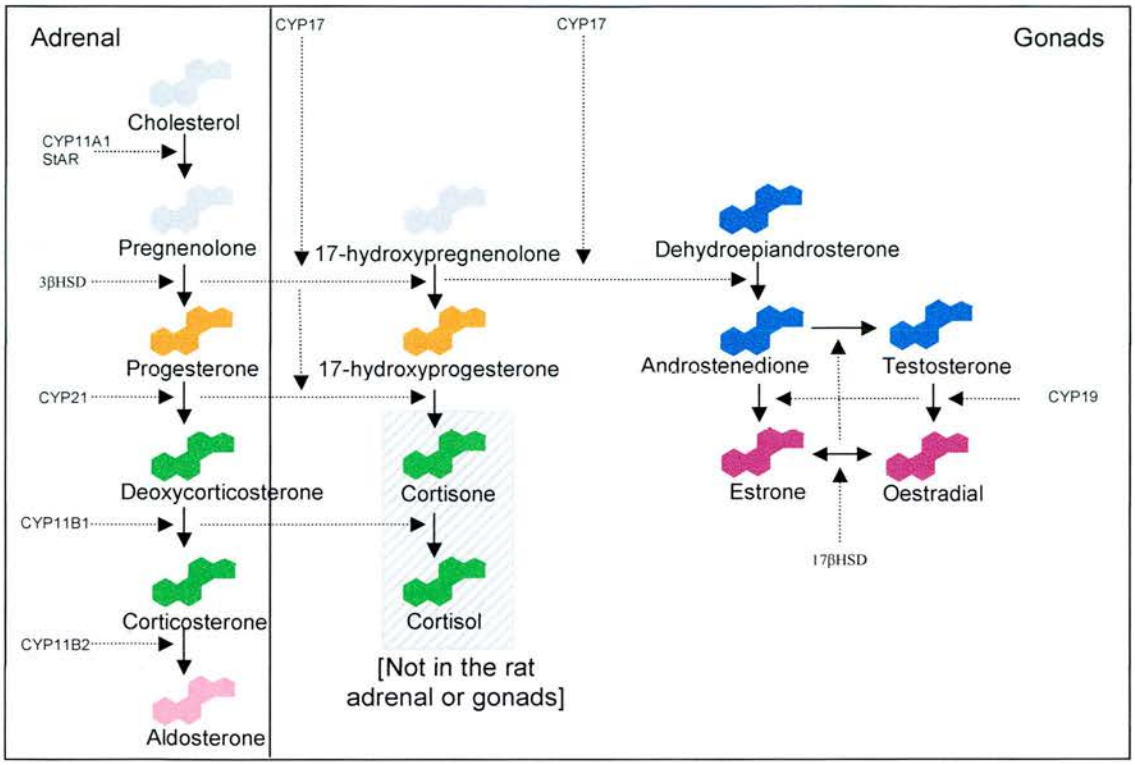

Figure 1.5. Principal pathways of steroid biosynthesis. The steroid metabolism conversion pathway. Precursor molecules are shown in grey, progestins in orange, androgens in blue, oestrogens in purple, glucocorticoids in green and mineralocorticoids in pink. Metabolising enzymes are shown against directional arrows. Adapted from source www.mwcpharmacy.com/Hormone_Pathway.htm

1.2.1.1.2. Regulation of glucocorticoid activity

A number of mechanisms regulate glucocorticoid formation and function. ACTH from the pituitary gland acts directly on the adrenal gland to maintain blood levels of glucocorticoids, which negatively feed back via the hypothalamic-pituitary-adrenal axis to regulate ACTH release. Cortisol and corticosterone in the plasma bind with high affinity to corticosteroid-binding globulin (CBG) and with low affinity to albumin, which together regulate the bioavailability of glucocorticoids as well as transporting these steroids around the body (Andersen 2002; Ho *et al.* 1999). In the human, CBG binds 80% or more of the circulating cortisol with albumin binding 14% and only 6% circulating free (Lewis *et al.* 2005). In rodents, corticosterone usually circulates bound to CBG whereas 11-dehydrocorticosterone circulates unbound (Kotelevtsev *et al.* 1997). CBG protects glucocorticoids from degradation in the circulation. CBG-bound glucocorticoid is not available to GR, thereby CBG helps determine the amount of glucocorticoid circulating for action on target tissues.

At the tissue level, pre-receptor metabolism via cellular 11β-hydroxysteroid dehydrogenases (11βHSD) isoforms further regulates the bioavailability of glucocorticoids to bind and activate GR. There are two isoforms of the 11βHSD enzyme produced by different genes; 11βHSD type 1 (11βHSD1) and 11βHSD type 2 (11βHSD2). They are biochemically different from one another and share only 30% homology at the amino acid level (Kotelevtsev *et al.* 1997). Both 11βHSDs are members of the alcohol dehydrogenase superfamily (Penning 1997) and the mechanism by which they regulate glucocorticoid activity is summarised in Figure 1.6. 11βHSD1 functions as either a dimer or a tetramer predominantly to produce active glucocorticoids by NADPH-dependent reduction of the 11-keto-steroid (cortisone/11-dehydrocorticosterone). 11βHSD1 is bidirectional and can also inactivate glucocorticoids, the direction of conversion being dependent on the NADP(H) state of the cell (Michael *et al.* 2003), and the low binding affinity of 11βHSD1 for cortisol or corticosterone (Stewart & Mason 1995). In intact cells the main direction of conversion catalysed by 11βHSD1 is from inactive to active. 11βHSD2 is a NAD+-dependent 11β-

dehydrogenase and works only in a unidirectional manner to inactivate glucocorticoids (Albiston *et al.* 1994). 11βHSD2 has a high binding affinity for cortisol or corticosterone (Stewart *et al.* 1994). Since 11βHSD1 and 11βHSD2 essentially catalyse glucocorticoid activation and inactivation, they constitute a 'steroid shuttle' as shown in Fig. 1.6.

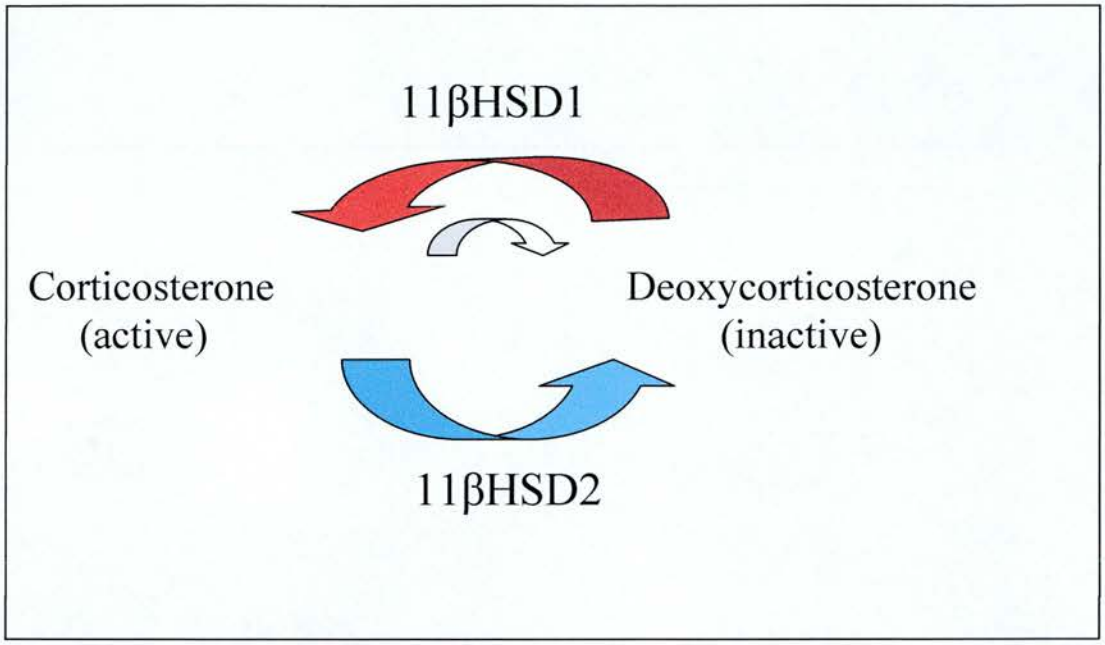

Figure 1.6. The glucocorticoid shuttle in the rodent. 11βHSD1 mainly converts the inactive glucocorticoid deoxycorticosterone to the active form corticosterone and 11βHSD2 inactivates corticosterone. Therefore relative presence determines availability of steroid to act in target cells via the GR.

Actions of adrenal glucocorticoids and mineralocorticoids (which are involved in regulating electrolytes and water balance) are transduced through intracellular receptors that are members of the nuclear receptor family of ligand-dependent transcription factors (Schaaf & Cidlowski 2003): GR and mineralocorticoid receptor (MR). GR mediates the many different cellular functions associated with glucocorticoid action and selectively binds glucocorticoids. Two isoforms of GR exist: -α and –β; of which GRα is the active receptor (Pujols *et al.* 2004). MR mediates the actions of the mineralocorticoid aldosterone, which is responsible for sodium resorption and electrolyte excretion. MR and GR share a high degree of homology and have a similar binding affinity for glucocorticoids and aldosterone. Therefore, a local level of regulation needs to be in place to protect MR being engaged by glucocorticoid. The 11βHSD isoforms serve this

role. Thus in glucocorticoid target organs such as the liver 11βHSD1 provides active glucocorticoids to stimulate the GR (Whorwood *et al.* 1992), whereas in mineralocorticoid target organs such as the kidney and placenta 11βHSD2 protects the MR from being stimulated by glucocorticoids (Shimojo *et al.* 1997; Stewart & Mason 1995). This highlights the critical role of pre-receptor metabolism in glucocorticoid action.

1.2.1.2. Glucocorticoids in the ovary

The ovary does not express 21-hydroxylase (CYP21) and therefore is unable to synthesise glucocorticoids *de novo* (Omura & Morohashi 1995). However, this tissue does possess a glucocorticoid shuttle. Ovary can be both glucocorticoid and mineralocorticoid target organ depending on the levels of follicular activity (Tetsuka *et al.* 1999b). Immature follicles are probably protected from the effects of circulating glucocorticoids by 11βHSD2 whilst ovulation-associated inflammation is thought to be resolved by anti-inflammatory glucocorticoids generated by 11βHSD1 (Tetsuka *et al.* 1999b).

Glucocorticoids can be inhibited in ovarian cells by the actions of gonadotrophins and steroids (Hsueh & Erickson 1978; Michael *et al.* 1993). The type of 11βHSD expression in granulosa cells (hence potential for glucocorticoid activation) is dependent on their degree of gonadotrophin-induced differentiation (Michael *et al.* 2003). Granulosa cells that have not been exposed to the LH surge express both 11βHSD2 and MR (Tetsuka *et al.* 1999b; Tetsuka *et al.* 1997). After the LH surge the expression of 11βHSD2 decreases and 11βHSD1 becomes the predominant isoform (Tetsuka *et al.* 1999b; Tetsuka *et al.* 1997; Yong *et al.* 2000). There is also a decrease in the expression of MR whereas GR expression being constitutive is relatively stable (Tetsuka *et al.* 1999b; Tetsuka *et al.* 1997). This all contributes to a net increase in activation of the glucocorticoids in the ovary as ovulation approaches. Yong *et al* (2000) showed that theca cells from human periovulatory follicles could not convert cortisone to cortisol but immature theca cells could convert cortisol to cortisone. 11βHSD2 protein has been localised to human theca cells with immature cells having lower levels than more mature

cells (Ricketts *et al.* 1997). Therefore these cells presumably require protection from glucocorticoid stimulation. By studying immature human granulosa cells *in vitro* it was shown that hFSH caused an increase in the activity of 11βHSD1 in these cells and hLH causes an increase in 11βHSD1 in periovulatory granulosa cells (Yong *et al.* 2000).

Free cortisol levels in follicular fluid increase once the LH surge has occurred and ovulation approaches, perhaps indicating disassociation from CBG (Harlow *et al.* 1997; Yong *et al.* 2002). This could occur due to the excessive levels of progesterone present in the follicular fluid at this time. Thus CBG has a higher affinity for progesterone than cortisol such that cortisol is displaced when excess amounts of progesterone are present (Andersen 2002). CBG may also be inactivated due to cleavage by proteolytic enzymes that are active in the preovulatory follicle, which would also increase the free cortisol level (Hammond *et al.* 1990).

Intrafollicular changes in 11βHSD isoform expression are not only regulated by gonadotrophins but also by pro-inflammatory cytokines such as IL-1, in an inflammatory cascade (Hillier 2003). Cultured rat granulosa cells show an increase in 11βHSD1 expression when stimulated with gonadotrophins (Tetsuka *et al.* 1999b). This is increased further by the addition of IL-1 to the culture and attenuated by the presence of IL-1RA (Tetsuka *et al.* 1999a). Also by studying human OSE cells, which are sites of inflammation-associated damage at ovulation, an increase in the expression of 11βHSD1 was observed in response to IL-1 (Rae *et al.* 2004a; Rae *et al.* 2004b; Yong *et al.* 2002). When cortisol was also added to these cultures an enhanced IL-1-induced increase in 11βHSD1 was seen, while cortisol alone had no effect (Rae *et al.* 2004b). This indicates that human OSE cells in an inflammatory environment (created by the presence of IL-1) will increase their own production of cortisol to resolve inflammation.

All of this points to a local role for anti-inflammatory glucocorticoids at the time of ovulation, principally due to up-regulate 11βHSD1.

1.3. Collagen Pathway Biology

Collagens are major components of connective tissues that are subject to inflammatory remodelling such as bone, tendon and ligament. However, collagens also have other important biological roles such as regulating cell adhesion, differentiation, growth and survival (Gelse *et al.* 2003). Through the receptor-like properties they possess, they also serve as reservoirs for substances the body requires during growth and development. Finally, the non-structural collagens can have roles in modulating tumorigenesis and angiogenesis (Gelse *et al.* 2003). Therefore, collagens are essential components of any functional tissue system, and it is important to understand how they are synthesised and processed to accomplish their biological actions. Moreover, an understanding of normal function should lead to a better appreciation of states of disease. However, since collagen biology covers such a diverse field only a brief overview of the topic can be given here. Key collagen pathway components relevant to this investigation are collagen type IV, lysyl oxidase (LOX), bone morphogenetic protein-1 (BMP-1) and procollagen C-proteinase enhancer protein (PCPE).

1.3.1. Collagen

Collagens are insoluble extracellular glycoproteins that are the most abundant proteins in the body. To date at least 27 vertebrate collagens have been identified (Myllyharju & Kivirikko 2004). Collagen polypeptide chains comprise Gly-Xaa-Yaa repeats in which the Xaa and Yaa are usually proline and hydroxyproline, which limit the amount of rotation of the collagen chains (Canty & Kadler 2005; Prockop & Kivirikko 1995). Collagen forms a left-handed helix which combines with other primary polypeptide units to form a right-handed elongated triple helix (Prockop & Kivirikko 1995). The member-chains can be repeats or products of different collagen genes.

Collagens fall into 6 different categories; fibril-forming, basement membrane, microfibrillar, anchoring, transmembrane and fibril-associated (Gelse *et al.* 2003). (Only fibril-forming collagens and basement membranes will be discussed in this review. For a review of the other types of collagen, refer to Gelse *et al* (2003). The

different forms of collagen vary in their diversity and complexity due to additions of non-collagen domains.

The most abundant collagens are the fibril-forming collagens that give bone and cartilage their tensile strength. The fibril-forming collagens are types I-III, V and XI, with type I being the most abundant and well-studied. Collagens have large helical domains which confer strength to biological structures. Type I is an abundant, ubiquitous collagen whereas the other fibrillar collagens are more tissue-specific (Prockop & Kivirikko 1995). The importance of collagen type I is highlighted in homozygous mice with deleted expression of COL1A1. These mice die *in utero*. Moreover even though heterozygous mice survive, their bone strength is subnormal and they have loss of hearing (Prockop & Kivirikko 1995).

The important collagen in basement membranes is type IV collagen, which forms the flexible meshwork of these structures. Basement membranes form divisions between cellular structures but also function to determine cell polarity and anchorage, forming barriers or filters and templates for cell migration (Alberts *et al.* 1997).

1.3.1.1. Processing of collagen

Investigations into how collagens are processed have mostly focused on collagen type I but similar mechanisms are believed to apply to other collagens, though one must appreciate that significant differences may occur. Collagen type I is used as an example in this text.

The box below summarises collagen biosynthesis, from transcription through to helix formation.

Box 1. Summary of key steps involved in collagen biosynthesis and maturation (adapted from Gelse *et al* (2003) and Trackman (2005)).

1. Transcription and translation
   - regulation of collagen genes
2. Post-translational modifications
   - hydroxylation (3- and 4-hydroxylases/lysyl hydroxylase) forms intramolecular hydrogen bonds; intermolecular cross-links between fibrils; create sites for carbohydrate attachment.
   - Glucosylation and galactosylation
   - Conformational folding (peptidyl-prolyl *cis-trans* isomerase/chaperones)
   - Disulphide bond formation (disulphide isomerase) forms inter- and intra-bonds in procollagen.
3. Secretion
   - Via secretory vesicles into the extracellular space
4. Cleavage
   - Procollagen domain cleavage (Procollagen C-proteinase/ Procollagen N-proteinase)
5. Fibril formation
   - Spontaneous aggregations form ('self assembly')
6. Stabilisation
   - Covalent cross-linkage (LOX) creates mechanical resilience.

From this summary, it can be seen that collagen protein is subject to many modifications before it can fulfil its roles in the body. The foci of this investigation are the extracellular modifications of collagen, in particular the proteins involved in its cleavage and stabilisation.

The triple helices of the fibril-collagens formed intracellularly are flanked by non-collagenous globular protein domains (Gelse *et al.* 2003), the C-propeptide and the N-propeptide (Fig. 1.7), which keep procollagens in a soluble form until they are cleaved after secretion from the cell. The propeptides are linked to the collagen helices by telopeptides, which may be involved in forming covalent cross-links once the procollagen has been processed or/and may link to other structures (Gelse *et al.* 2003). Collagen IV is not formed as a procollagen and therefore is not cleaved before forming helices.

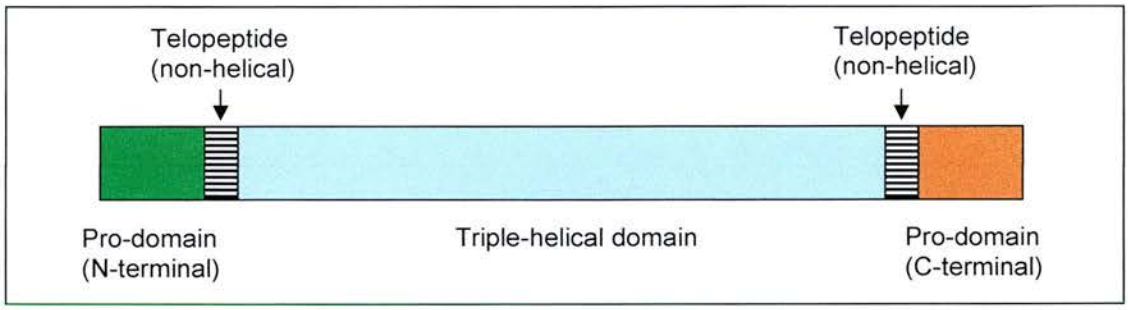

Figure 1.7 The molecular structure of fibrillar procollagen. Adapted from (Gelse *et al.* 2003).

If prodomains are not removed, collagen cannot assemble into helices (Prockop *et al.* 1998). Procollagen undergoes two different point cleavages. The cleavage at the N-terminus is accomplished by A Disintegrin and Metalloproteinase with Thrombospondin 1 Motifs (ADAMTS) family members, while the C-terminus is cleaved by BMP-1 (Prockop *et al.* 1998). Removal of the C-terminal is critical for collagen assembly and is therefore more efficient than N-terminal removal (Kadler *et al.* 1987).

1.3.1.1.1. Procollagen N-proteinase

The N-terminus of procollagen is cleaved by ADAMTS-2, -3, and -14. ADAMTS proteins have zinc metalloproteinase domains that are required to cleave procollagens and have a binding site for integrins (Colige *et al.* 1997). ADAMTS proteins are activated through cleavage by furin, then go on to cleave the proforms of collagens I, II, III and V (Colige *et al.* 2005). All three of the ADAMTS proteins act on procollagens I, II and III, whilst ADAMTS-2 also acts on procollagen V (Colige *et al.* 2005). Each of

these enzymes are expressed in a tissue-specific manner (Trackman 2005). Mice null for ADAMTS-2 have brittle skin due to a lack of collagen type I cleavage and fibrils not increasing in diameter, so tensile strength is lost (Porter *et al.* 2005). Presumably ADAMTS-3 and -14 are not present in the skin as they do not compensate for the lack of ADAMTS-2 (Li *et al.* 2001), confirming that different tissues require different members of these procollagen N-proteinases for cleaving procollagen. Interestingly, female ADAMTS-2 knockouts are fertile but males are sterile due to a lack of mature sperm (Li *et al.* 2001). This perhaps indicates that these proteins have roles other than procollagen cleavage or that fibrillar collagen is important in spermatogenesis. These proteinases appear only to cleave procollagens that are in their native folded conformations (Prockop *et al.* 1998).

1.3.1.2.2. Procollagen C-proteinase

Procollagen C-proteinase (PCP), also known as BMP-1, is fundamental for the assembly of the ECM (Kessler *et al.1990*). The importance of BMP-1 is highlighted by the fact that mice null for this gene die shortly after birth due to abnormal collagen fibrillogenesis (Suzuki *et al.* 1996).

BMP-1 differs structurally from other BMPs. It is unrelated to TGF-β, and is a member of the astacin family of the zinc-requiring metalloproteinase endopeptidases (Li *et al.* 1996). Astacin proteins are important in early developmental decisions that coordinate embryo development as well as embryonic hatching and dorsal-ventral patterning (Li *et al.* 1996).

BMP-1 processes collagens and noncollagenous molecules of the ECM (Medeck et al, 2003). As shown in Fig. 1.8, BMP-1 cleaves the C-terminus of procollagens I, II, III, V and XI. At the same time, the N-terminus is cleaved by procollagen N-proteinase thus forming active collagens (Medeck *et al.* 2003). By removing the C-terminus of procollagen, BMP-1 reduces the solubility of the peptide (Prockop & Kivirikko 1995), thus allowing the collagen helix to form into an ordered unit (Trackman 2005). When BMP-1 was inhibited in MC3T3-E1 cells collagen assembly was profoundly affected (Pischon *et al.* 2005). The collagen in these cells had retained C-terminal domains,

resulting in an increase in fibril diameter presumably due to improper alignment (Pischon *et al.* 2004). BMP-1 also processes probiglycan, the γ2 and α3 laminin chains, chordin and prolysyl oxidase. These are all involved in ECM formation. Trackman (2005) hypothesises that BMP-1 involvement in so many different aspects of ECM maturation affords an economically sound strategy for fundamental control, which also permits specific regulation of the individual processes to occur at a local level.

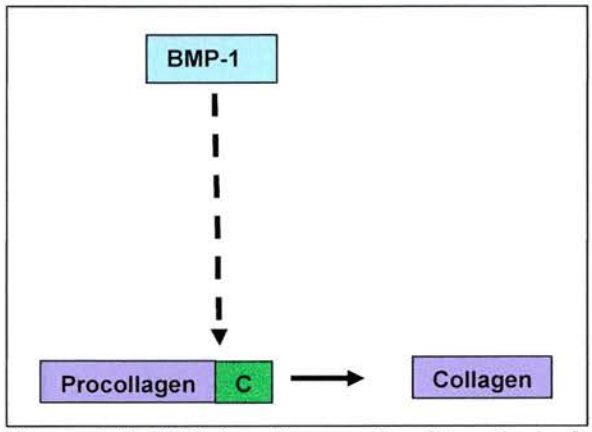

Figure 1.8. BMP-1 action on the C-terminal of procollagen, resulting in active collagen.

BMP-1 action on procollagens is enhanced by PCPE, a glycoprotein found in connective tissues (Moschcovich *et al.* 2001). Three other BMP-1 related proteinases, mammalian tolloid (mTLD), tolloid-like 1 (mTLL-1) and tolloid-like 2 (mTLL-2) also have procollagen C-proteinase activity with varying substrate specificity (Uzel *et al.* 2001). All members of the astacin family contain an N-terminal astacin-like metalloproteinase domain, an epidermal growth factor (EGF)-like motif and C-terminal embryonic sea urchin protein Uegf, BMP-1 (CUB) domains (Takahara *et al.* 1994). BMP-1 has three CUB domains, with CUB2 and CUB3 being separated by an EGF-like domain (Hartigan *et al.* 2003). Investigations into the structure of BMP-1 using domain-swapping techniques show that for BMP-1 to be secreted the CUB1 domain is needed, positioned next to the metalloproteinase domain. CUB3 and the EGF-like domain are not essential for activity but CUB2 is necessary for BMP-1 C-proteinase function (Hartigan *et al.* 2003).

1.3.1.2.2.1. Processing of BMP-1

BMP-1 is synthesised as an inactive proenzyme (Bond & Beynon 1995). Leighton and Kadler (2003) have demonstrated that the prodomain is rapidly cleaved by furin-like/paired basic proprotein convertases in the trans-Golgi network. This rapid conversion may allow active BMP-1 to work efficiently in the local ECM. Regulation of furin may be a control mechanism for assembly of the ECM (Leighton & Kadler 2003). By inhibiting furin, BMP-1 was secreted in an inactive form, as demonstrated by the lack of procollagen cleavage (Leighton & Kadler 2003). Therefore it is interesting that the prodomain does not have to be cleaved for BMP-1 to be secreted from the cell (Leighton & Kadler 2003). This may maintain the enzyme in an inactive form, so that it is only cleaved when activation is required.

1.3.1.3.1. Procollagen C-Proteinase Enhancer

PCPE enhances BMP-1 action on procollagen and two PCPE proteins have been identified: PCPE-1 and -2. Both are glycoproteins of the ECM that stimulate the processing of the C-terminus of procollagens (Steiglitz *et al.* 2002; Moschcovich *et al.* 2001; Kessler & Adar 1989). The two PCPEs are comparable structurally (43% homology) and have similar functions but it appears that PCPE-2 is not as widely expressed as PCPE-1 (Steiglitz *et al.* 2002; Trackman 2005). Unless otherwise stated, it is PCPE-1 that is referred to in the following text.

1.3.1.3.1.1. PCPE function on BMP-1

PCPE does not have inherent proteolytic activity (Kessler & Adar 1989; Moschcovich *et al.* 2001), but it binds to C-propeptide domains of procollagens where it enhances the proteolytic effect of BMP-1 (Fig 1.9) on procollagen up to 20-fold (Kessler & Adar 1989; Ricard-Blum *et al.* 2002). PCPE recognises not only the amino acid sequence of BMP-1 but also its three-dimensional structure (Moali *et al.* 2005).

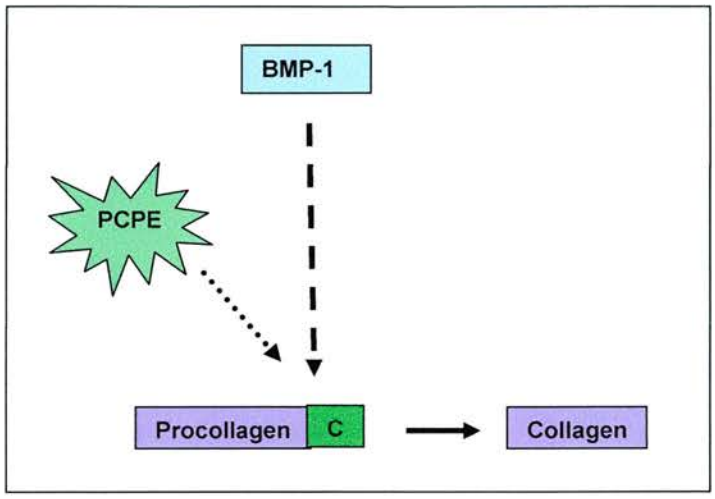

Figure 1.9. PCPE enhancement of the BMP-1 cleavage of the C-terminal of procollagen.

PCPE has two CUB domains and a netrin (NTR)-like module (Banyai & Patthy 1999; Takahara *et al.* 1994) arranged in a rod-like structure (Bernocco *et al.* 2003). To date, the exact mechanism by which PCPE enhances BMP-1 activity on procollagen is unclear. It is postulated that it might be due to the CUB domains, as these domains can mediate protein-protein interactions (Takahara *et al.* 1994). PCPE binds in a 1:1 ratio with BMP-1 at the N-terminus of the molecule, although PCPE is not thought to enhance BMP-1 activity directly (Moali *et al.* 2005; Ricard-Blum *et al.* 2002). Structural studies of PCPE show that it binds to both the BMP-1 and the procollagen molecule. It is also proposed that PCPE causes a structural change in the C-terminus of procollagen to allow BMP-1 to bind and cleave the pro-domain (Ricard-Blum *et al.* 2002) (Adar *et al.* 1986). Because PCPE stimulates C-terminal processing of several procollagens it probably shares a common recognition site on procollagens (Moali *et al.* 2005).

The NTR module may inhibit MMPs as it is homologous to tissue inhibitors of MMPs (TIMPs) (Banyai & Patthy 1999) and fragments of C-terminal PCPE (CT-PCPE) (16.5 kDa) isolated from human brain tumour had characteristics of TIMPs (Mott *et al.* 2000). The CT- PCPE fragments were shown to inhibit MMP-2 in solution with an IC_{50} value of 560nM, which probably indicates that this is not its principal target (Mott *et al.* 2000). Full-length PCPE did not have TIMP activity, nor did the 34kDa or 36kDa fragments

that are thought to enhance BMP-1 (Hulmes *et al.* 1997; Mott *et al.* 2000), suggesting that the C-terminus only has this function if cleaved from the rest of the protein. The possible TIMP-like activity of PCPE suggests that this molecule may have multiple roles in facilitating ECM maintenance and accumulation.

1.3.1.3.1.2. Other functions of PCPE

PCPE works in a 1:1 ratio with BMP-1 yet in fibroblasts it is expressed to a greater degree than BMP-1 (Takahara *et al.* 1994). Also, investigations into the temporal expression patterns of PCPE and BMP-1 in bone showed differences in their patterns of expression, suggesting additional roles for PCPE (Scott *et al.* 1999). Matsui *et al* (2002) postulated that PCPE may regulate protein synthesis in hepatic stellate cells (HSC), as the PCPE molecule has RNA-binding motifs in its sequence and reduced expression of PCPE appeared to suppress the levels of both collagenous and non-collagenous synthesised proteins. PCPE expression, elevated in Rat2 cells using a retroviral vector, resulted in changes in cell morphology, contact inhibition and anchorage-independent growth, indicating that PCPE may act as a tumour suppressor (Masuda *et al.* 1998; Trackman 2005).

1.3.2. Collagen stability

Collagen is synthesised, processed, formed into helical structures and incorporated into the ECM. It must then be stabilised and held in position so that it can serve its tensile role. As previously stated, this is accomplished by: hydroxylases, glycosyltransferases and an oxidase (Myllyharju & Kivirikko 2004).

The formation of stabilising covalent cross links between collagen fibrils is catalysed by LOX through oxidative deamination of lysine residues in collagen and elastin fibrils (Siegel 1976).

1.3.2.1. Lysyl oxidase

LOX is a copper-dependent amine oxidase that catalyses critical post-translational alteration needed to generate connective tissue matrices (Smith-Mungo & Kagan 1998). It is also linked with the following: cancer cells (Kirschmann *et al.* 1999), healing (Kagan *et al.* 1981), motility and migration of cells (Jo *et al.* 2004), and cell differentiation (Omori *et al.* 2002). This array of functions indicates that LOX is a multi-functional protein. The LOX gene is highly conserved between mouse, rat and human, showing that this gene has evolutionary importance (Chinoy *et al.* 2000).

1.3.2.1.1. Mechanism of LOX cross-linking

In the ECM, LOX catalyses the covalent cross-linking of elastin and collagen fibrils. LOX substrate specificity could be low as the sequence surrounding lysine residues present in collagen and elastin are very different. So too are their physical structures and it has been shown that LOX will oxidise lysine peptides in an array of basic globular proteins (Kagan *et al.* 1984), indicating that LOX has other potential roles other than to covalently cross-link collagens. Cross-linking is achieved by oxidative deamination of peptidyl lysine found in elastin and collagen to peptidyl α-aminoadipic-δ-semialdehyde (AAS) (Smith-Mungo & Kagan 1998). The stoichiometry of this reaction is shown in Fig. 1.10A.

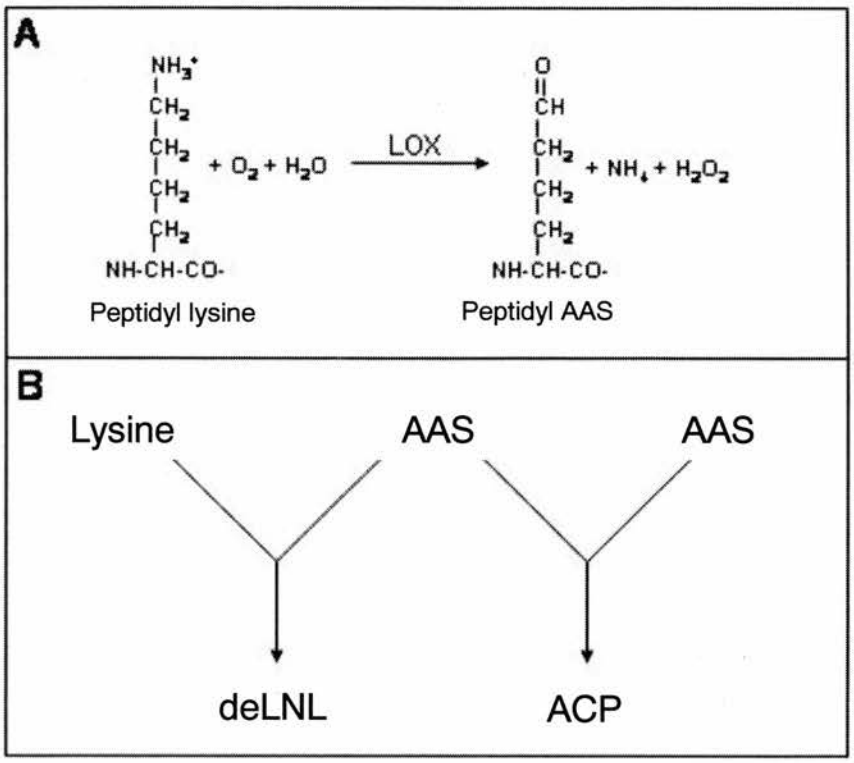

Figure 1.10. The peptide cross-linking achieved by LOX. A, The stoichiometry of the LOX catalysed reaction from a pepitidyl lysine to a peptidyl AAS. B, the spontaneous formation of cross-links. Adapted from Lucero and Kagan (2006).

This aldehyde spontaneously condenses with other amino groups or peptidyl aldehydes forming the covalent cross-linkages between fibrils (Kagan *et al.* 1986). This is achieved by condensation with an unmodified lysine forming the anhydrolysinonorleucine (deLNL) cross-link and two AAS residues that form an alcohol condensation product (ACP) (Fig. 1.10B). Therefore, LOX may be a controlling factor in ECM accretion and stability, as a cross-linked matrix is less vulnerable to proteolysis. This is supported by inhibiting LOX which retains collagen in its soluble and easily degradable form (Vater *et al.* 1979).

LOX requires two co-factors to function: copper (Cu^{2+}) and lysine tyrosylquinone (LTQ), which is derived from tyrosine and lysine residues (Lucero & Kagan 2006). If Cu^{2+} is not bound to LOX, the enzyme is inactive (Smith-Mungo & Kagan 1998). LTQ is formed within the nascent LOX enzyme and links Lys 314 to Tyr 349 via a quinine ring creating a transient electron sink in the catalytic mechanism of LOX (Lucero &

Kagan 2006). LOX is classified kinetically as a "ping-pong" catalyst, as it carries out amine oxidation in two kinetically distinct steps (Williamson & Kagan 1986). These general steps are summarised below:

- $RCH_2NH_2 + Enz_{Oxidised} \longrightarrow RCH{=}{=}O + Enz_{Reduced}$
- $Enz_{Reduced} + O_2 + H_2O \longrightarrow Enz_{Oxidised} + H_2O_2 + NH_3$

The mechanism of LOX action in relation to the ping-pong catalytic mechanism is summarised below in Box 2, to show how Cu^{2+} and LTQ are integral factors in producing covalent cross-links (Lucero & Kagan 2006).

Box 2. Key stages in LOX catalysed cross-linkage of collagen fibrils

1. A Schiff base is formed with LTQ, which undergoes a rate-limiting general base facilitated α-proton abstraction resulting in a reduction of LTQ.
2. Hydrolysis of the imine intermediate releases the reactive AAS product (to form spontaneous cross-links) from the reduced LTQ.
3. The reduced enzyme is re-oxidised with the aid of Cu^{2+} (electron transfer) forming and releasing H_2O_2.
4. The enzyme is then hydrolysed producing NH_3 and the cycle is completed as LTQ is regenerated.

It is thought that the oxygen-dependent reoxidation of this reaction could be rate limiting for LOX catalysis (Shah *et al.* 1993).

1.3.2.1.2. Biological functions of LOX

As LOX causes covalent cross-linking of collagen and elastin, it is obviously important in wound healing. In osteoblast cultures LOX increases dramatically just prior to increasing collagen deposition (Trackman 2005). Studies into wound healing in longitudinal injuries to the rat forebrain have shown that LOX is secreted spatiotemporally by cells in the injury locus (Gilad *et al.* 2001). When the central nervous system (CNS) is injured, scarring results from the deposition of collagen (Gilad *et al.* 2001). If the amount of collagen present at an injury site is reduced, less scarring occurs (Stichel & Muller 1998). Therefore as LOX causes collagen cross-linking it would be reasonable to presume that inhibition of LOX would also reduce scarring. This has been confirmed using β-aminoproprionitrile (BAPN) an irreversible inhibitor of LOX to inhibit LOX in a unilateral spinal cord injury. This resulted in an increase in healing and better functional recovery (Gilad & Gilad 2001). Also when atherosclerosis was induced in rabbits by continual feeding of an atherogenic diet, LOX activity measured in aortas increased detectably after 30d, and peaked at 90d (Kagan *et al.* 1981). Evidence that LOX activity is increased in the blood vessel walls and plaque-like structures of people with Alzheimer's disease suggests that LOX is associated with the ECM and may be instrumental in plaque formation (Gilad *et al.* 2005).

LOX has also been shown to be involved in fibrotic diseases (Smith-Mungo & Kagan 1998). LOX was greater in sclerodermatous (scaly) skin than in normal skin (Chanoki *et al.* 1995) and was increased in the inflammatory oedematous stage (when mononuclear cells are surrounding the collagen fibres of the fibrosis) rather than in the sclerotic stage (when collagen becomes tightly packed) (Chanoki *et al.* 1995). LOX mRNA and activity were also increased in synthetic liver fibrotic models compared to normal liver (Wakasaki & Ooshima 1990).

Even though LOX is instrumental in the cross-linking of collagen and elastin, recent research has shown that LOX may also have other functions, which are summarised below.

1.3.2.1.3. LOX and cancer

A role for LOX in cancer was first recognised when the murine LOX gene was shown to be identical to the murine *ras* recision gene (*rrg*), which is a recognised tumour suppressor (Contente *et al.* 1990). *Ras* induces DNA synthesis in the nuclei of inactive cells to cause proliferation (Malumbres & Pellicer 1998). *rrg* regulates the expression of *ras* by working through the *ras* signalling pathway (Contente *et al.* 1990). The mode of regulation appears to be by suppression of *ras*-induced proliferation to prevent a cancerous phenotype developing (Contente *et al.* 1990). Palamakumbura *et al* (2004) have shown that it is the released LOX propeptide that is responsible for the effects on *ras*, and not the active enzyme. BAPN does not have an inhibitory effect on these actions of LOX indicating that this property of LOX is completely removed from the enzyme acting on the ECM (Li *et al.* 2003). The *rrg*/LOX gene is expressed at lower than normal levels in human malignant cell lines (Contente *et al.* 1990; Hamalainen *et al.* 1995; Kenyon *et al.* 1991). In many cancers, the chromosome that LOX is located on is deleted at high frequencies and the LOX promoter can be heavily methylated, indicating two methods of down-regulating LOX in tumour cells (Kaneda *et al.* 2004; Wieland *et al.* 1996). Recent work on microRNAs (miRNA) has show that the LOX gene promoter contains a single miRNA binding site that is down-regulated in many cancers, indicating another element of LOX control by cancer cells (Dalmay & Edwards 2006). LOX also has anti-mitogenic effects on basic fibroblast growth factor (bFGF) by oxidising lysine residues, resulting in a loss of cell cycle progression and growth in fibroblasts and tumour cell lines (Li *et al.* 2003). Other identified intracellular substrates are histones. Therefore LOX could potentially influence histone-DNA interactions and ultimately gene transcription (Giampuzzi *et al.* 2003). LOX may act to stop abnormal growth of cells and could provide a possible anti-tumorigenic mechanism. Consequently, it appears to be a key target for down-regulation by tumorigenic cells.

Reports of LOX in different types of tumours do not follow a consistent expression pattern indicating that the stage/type of the cancer and/or the tissue of origin may influence LOX expression. In general LOX appears to be down-regulated in carcinomas but up-regulated in invasive metastasis (Bouez *et al.* 2006; Erler *et al.* 2006).

Expression and activity of LOX in malignant cell lines were down-regulated and LOX expression and protein location in breast and skin cancer tumours was observed only in the areas surrounding the tumour (Bouez *et al.* 2006; Hamalainen *et al.* 1995; Peyrol *et al.* 1997). In early tumour stages LOX expression may act as a defence mechanism to contain the tumour in a cross-linked matrix and the lack of LOX in later stages of tumour invasion may allow the tumour to metastasise (Peyrol *et al.* 1997).

LOX is up-regulated in metastatic breast cancer compared to primary cancer tissues (Jo *et al.* 2004). LOX has recently been associated with hypoxia-induced metastatic development from breast cancer tumours in mice, as the inhibition of LOX prevented the formation of metastasis (Erler *et al.* 2006). Interestingly, inhibition of LOX had no effect on the primary tumour. Erler *et al* (2006) also showed that LOX mRNA was up-regulated by hypoxia-inducible factor-1 (HIF-1). It is proposed that LOX could be a tumour-secreted factor needed to form a permissive niche for metastatic growth (Erler *et al.* 2006). LOX is required for focal adhesion kinase activation, which is involved in cell survival and proliferation, as well as fibronectin activation, a possible ligand that is instrumental in permissive niche formation (Kaplan *et al.* 2005), and of course, LOX is vital for ECM formation, which would provide a repository of substances needed for cell survival (Erler *et al.* 2006).

Evidence implies that LOX is regulated by tumours depending on their environment of demands, so established cancers down-regulate this gene in order to metastasise. Then the break-away cancer cells use LOX to form a favourable environmental niche where these cells can proliferate. It would be predicted that once metastatic cells become established at a new site, LOX is down-regulated.

1.3.2.1.4. Other functions of LOX

As LOX catalyses cross-linking, it produces hydrogen peroxide (H_2O_2) and ammonia (Li *et al.* 2000). LOX can also translocate to cells where it is proposed that it might act on an unknown substrate to form H_2O_2 (Li *et al.* 2000). *In vitro* vascular smooth muscle cells migrate towards the H_2O_2 produced by LOX (Li *et al.* 2000). Human monocytes also migrate towards LOX (Lazarus *et al.* 1995). This could help explain the role of

LOX in atherosclerosis, as smooth muscle cells and monocytes are seen to migrate across the endothelium of blood vessels to contribute to plaque formation (Lusis 2000). Atherosclerosis is viewed as a response to injury with a pro-inflammatory environment being produced and the resultant plaques are formed via production of ECM (Lusis 2000). LOX mRNA in other cell systems has been shown to increase in response to pro-inflammatory mediators, indicating how this enzyme might be controlled in atherosclerosis (Rae *et al.* 2004a; Trackman *et al.* 1998). So, LOX could be contributing to ECM stabilisation and cell migration in this disease.

When LOX is inhibited in highly invasive cancer cell lines, a decrease in cell motility and adhesion is observed (Jo *et al.* 2004). More detailed investigations have shown that LOX causes changes in the actin filament polymerisation of cells (Jo *et al.* 2004). This chemoattractant property of LOX could be important to other aspects of LOX function such as when substances need to be attracted to a particular tissue during wound healing.

1.3.2.1.5. Processing dynamics of LOX

LOX is synthesised as a 46kDa preproenzyme. This (precursor) preproenzyme then undergoes cleavage of a signal peptide and N-glycosylation to produce a proenyzme of 50kDa (Smith-Mungo & Kagan 1998). The proenzyme is cleaved to a 32 kDa active enzyme in the extracellular space by the metalloproteinase BMP-1 (Trackman *et al.* 1992). BMP-1 cleaves pro-LOX at the Gly-Asp bond in the N-terminus (Cronshaw *et al.* 1995). If BMP-1 is inhibited, LOX activity but not mRNA expression will be inhibited (Pischon *et al.* 2005). LOX processing is summarised in Figure 1.11.

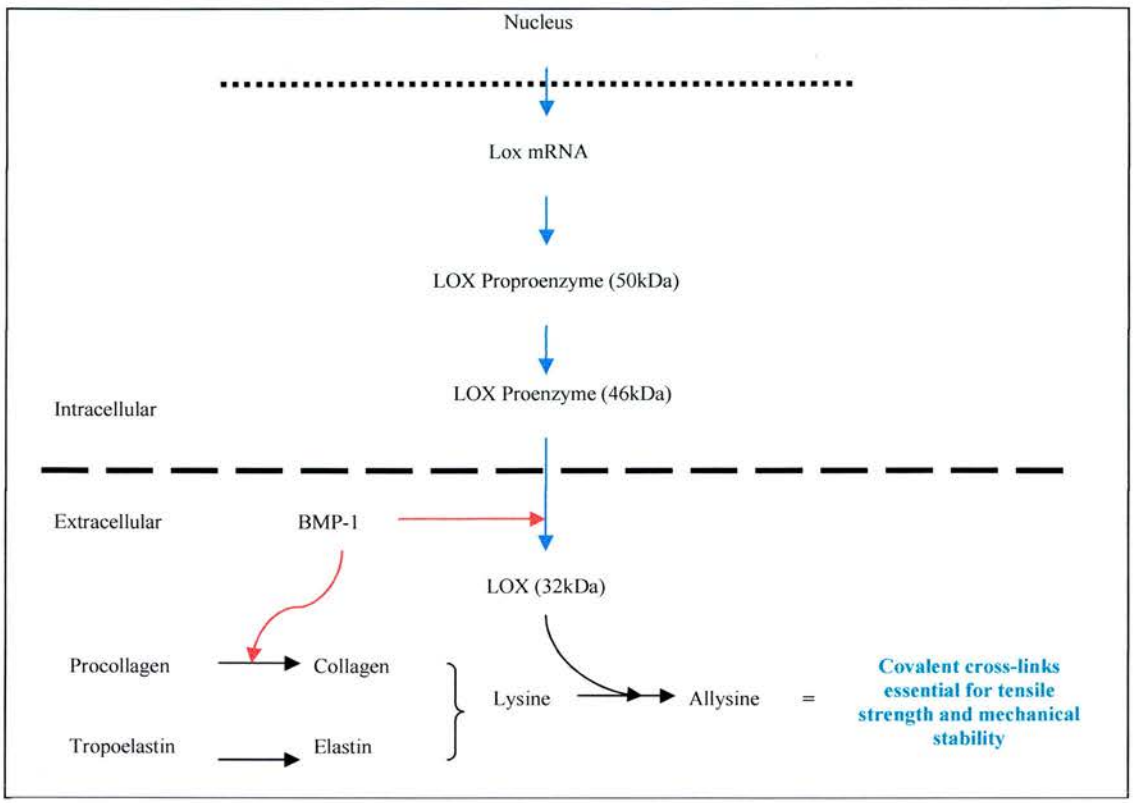

Figure 1.11. LOX processing summarised. LOX mRNA is translated into a preproenzyme that is processed intracellulary to proLOX, which moves into the extracellular environment where it is cleaved by BMP-1 to form active LOX. Active LOX causes the covalent cross-linking of collagen and elastin.

As previously described in section 1.3.1.2.2, fibril-collagens are cleaved by BMP-1. Subsequently these collagens can then be oxidised by LOX (Moschcovich *et al.* 2001; Siegel 1974; Smith-Mungo & Kagan 1998). Therefore, BMP-1 has a dual function in the formation of stable collagen through cleaving susceptible procollagen substrate and activating LOX (Smith-Mungo & Kagan 1998). This is shown schematically in Fig. 1.12.

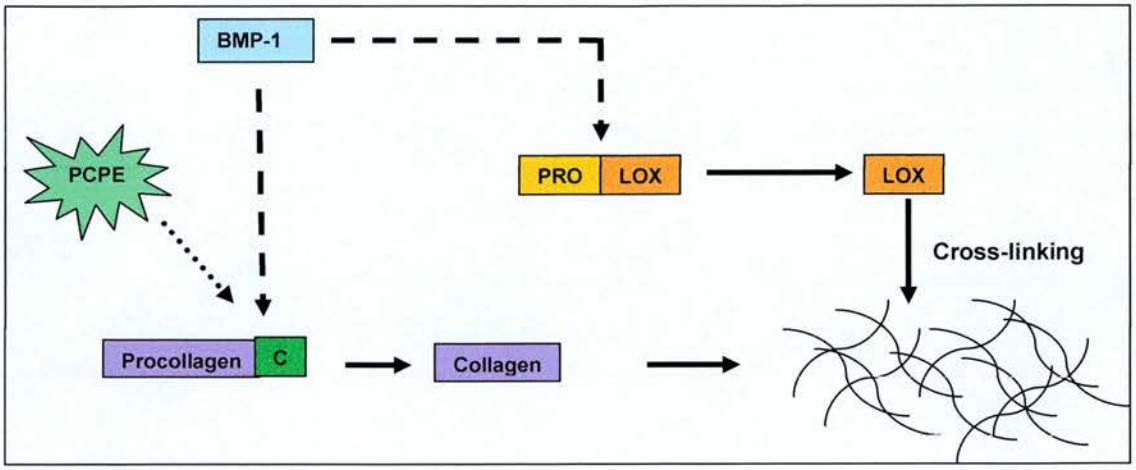

Figure 1.12. How BMP-1 and LOX process collagen, which results in covalent cross-linking of collagen in the ECM.

As previously stated, other proteinases similar to BMP-1 include mTLD, mTLL-1 and mTLL-2. These proteinases have been shown to exhibit procollagen C-proteinase activity and can process pro-LOX (Uzel *et al.* 2001). BMP-1 has been shown to be the most efficient processor of pro-LOX (Uzel *et al.* 2001). Culturing fibroblasts from mice null for either BMP-1 or mTLL-1 (the next most efficient proteinase after BMP-1) or null for both genes showed that active LOX was present in wild-type and single nulls but the double null had a 70% decline in LOX activity. This evidence suggests that these enzymes are responsible for the majority of LOX activation in fibroblasts (Uzel *et al.* 2001).

1.3.2.1.6. The existence of a LOX-like family

A series of genes highly related to LOX exists, suggesting that there is a LOX family (Smith-Mungo & Kagan 1998). These genes are LOX-like (LOXL), LOXL2, LOXL3 and LOXL4. LOX genes probably share a common ancestor as close homology exists between exon regions (Molnar *et al.* 2003; Saux *et al.* 1999). These proteins have domains characteristic of LOX in the carboxy terminus, including a copper-binding domain, lysyl-tyrosyl-quinoneresidues and a cytokine receptor-like domain (Maki *et al.* 2001; Saux *et al.* 1999){Asuncion, 2001 #223}(Kim *et al.* 1995). However, the N-terminal domains appear to differ, suggesting that they have different biological roles (Borel *et al.* 2001).

Null LOX mice develop to birth then die shortly after due to aortic aneurysms (Maki *et al.* 2002). Embryonic lungs from these mice are developmentally abnormal (Maki *et al.* 2005). Collagen and elastin are present in these mice but presumably not extensively cross-linked, since cardiovascular irregularities result (Maki *et al.* 2002). The fact that these mice survive to birth may indicate that other members of the LOX family contribute towards cross-linking but presumably this is not sufficient to ensure further survival. This indicates that LOX is the main instigator of biological cross-linking especially in the vasculature.

The different structures and patterns of expression of the members of the LOX family suggest that each member has specific biological roles that may help to regulate ECM remodelling. This is demonstrated by LOX and LOXL2 being expressed in different locations and at different times in fetal reproductive tissues (Saux *et al.* 1999). However, there is still much work remaining to determine the functions of these LOX-like proteins.

1.3.3. Extracellular Matrix

The ECM is a highly dynamic structure which constantly renews itself where tissue repair occurs and interactions among many different cell types take place (Bosman & Stamenkovic 2003b). The ECM has many roles including influencing differentiation, development, migration, cell behaviour, fluid dynamics and mechanical support of tissues. (Meredith *et al.* 1993; Ortega & Werb 2002; Irving-Rodgers *et al.* 2002 Rodgers *et al.* 2000; Rodgers *et al.* 2003). In addition, nutrients and hormones must traverse the ECM to reach target cells. Regulation of cellular mechanisms by the ECM is in part through its ability to bind growth factors (Taipale & Keski-Oja 1997). Bound growth factors are kept in a latent state in the ECM and are activated when disassociated from the matrix by proteolytic release, allowing for rapid availability of signalling messages (Lyons *et al.* 1988).

The composition and structure of the ECM is organ-specific and thus provides an organ-specific microenvironment (Brown *et al.* 2006; Rodgers *et al.* 2000). The ECM has two principal forms: basement membrane and interstitial matrix or connective tissue. The

basement membrane is a condensed layer of matrix adjacent to and providing support for parenchymal epithelial cells (Bosman & Stamenkovic 2003a). The interstitial matrix is a fibrillar network that surrounds cells, allowing their movement in discrete tissue zones as well as providing structural support (Schwarzbauer 1999). Both these matrix layers have a collagen scaffold but the two scaffolds differ in composition (as described below) (Bosman & Stamenkovic 2003a). The collagen network provides a backbone to the matrix, which also consists of adhesive glycoproteins and proteoglycans that interact with cells via matrix receptors such as integrins (Bosman & Stamenkovic 2003a). When the ECM undergoes remodelling new signals are produced between basement membrane and surrounding cells (Ortega & Werb 2002).

1.3.3.1. Components necessary for a functional ECM

1.3.3.1.1. Interstitial matrix

The interstitial matrix primarily supports cells and allows substrates required by these cells to pass among them. This matrix is composed of collagens, elastin, fibronectin, glycosaminoglycans (GAGs), proteoglycans and integrins. Collagens give tensile strength to the interstitial matrix and elastin gives the matrix flexibility (Alberts *et al.* 1997). The collagens of the interstitial matrix are mainly types I, II and III. Different matrices are composed of different types of collagen that confer the required properties on the specific matrices such as collagen type I and III in skin. Fibronectin, which is involved in matrix adhesion as well as cell migration and differentiation, has rigid and flexible domains and binds heparin sulphate, collagen, hyaluronic acid and fibronectin (Johansson & Hook 1980). GAGs are unbranched polysaccharides including hyaluronan and heparin sulphate (Alberts *et al.* 1997). Proteoglycans are highly glycosylated proteins that can influence the diffusion of small molecules through the matrix due to their size. Integrins are transmembrane glycoproteins that connect the matrix to the cytoskeleton (Hynes 1987). The diversity of components alludes to the importance of the interstitial matrix not only as a structural support but also in creating microenvironments and contributing to the regulation of biological processes.

1.3.3.1.2. Basement membranes

Basement membranes are specialised thin layers of ECM that are found in association with endothelial cells, epithelial cells, muscle fibres, adipocytes and nerves (Erickson & Couchman 2000; Ortega & Werb 2002). They are found throughout the body, usually between epithelial cells and mesenchyme, consequently compartmentalising tissues (Kalluri 2003).

The basement membrane contains members of several different protein families, the main components being collagen type IV, laminin, nidogen or entactin and heparin sulphate proteoglycans (the main one of which is perlecan). Basement membrane specificity is regulated by the composition and variety of matrix receptors (Gelse *et al.* 2003). It has been proposed that there are two networks of basement membrane, one composed of collagen IV and the other of laminin, and these two structures are linked by entactin-1 (Timpl & Brown 1996). Collagen IV is a major component of the basement membrane and is discussed in detail in the next section of this review.

Laminins form gel-like aggregates made up of three chains (α, β and γ), and each of these chain types comprises different sub-chains, which can form many different laminin molecules contributing to the diversity of the basement membrane and accounting for much of its complexity (Irving Rodgers & Rodgers 2005). Laminin is responsible for the adhesion of adjacent cells to the basement membrane, and is involved in cell migration and differentiation (Bosman & Stamenkovic 2003a). Mice null for the laminin gamma-1 subunit die *in utero* at day 6.5 due to disorganisation of the basement membrane resulting in an absence of gastrulation, showing that laminin is necessary for the development of the basement membrane (Smyth *et al.* 1999). Laminins signal to cells via integrins. They thereby act as mediators between the ECM and the cells. Nidogen links laminin to type IV collagen and perlecan, thereby maintaining the integrity of the basement membrane (Gelse *et al.* 2003; Timpl & Brown 1996).

1.3.3.1.2.1. The importance of collagen type IV

Collagen IV, the major collagen associated with basement membranes, is highly conserved between vertebrates and invertebrates and functions in cell migration and adhesion (Gelse *et al.* 2003; Gould *et al.* 2005). It also has roles in angiogenesis and

structural roles in the vasculature (Gould *et al.* 2005). Collagen IV appears to bind many basement membrane components and is therefore suggested to be an essential structural component (Brauer & Keller 1989). Each collagen IV monomer consists of a triple helix made from three collagen IV α-chains (Hudson *et al.* 1993). Collagen IV itself consists of 6 polypeptide chains (α1-α6), encoded by three pairs of genes: COL4A1 and COL4A2, COL4A3 and COL4A4, COL4A5 and COL4A6. Each gene-pair is arranged in close proximity, head-to-head, sharing a bidirectional promoter that coordinates their transcription via downstream transcription elements, as shown in Fig. 1.13. (Pollner *et al.* 1997).

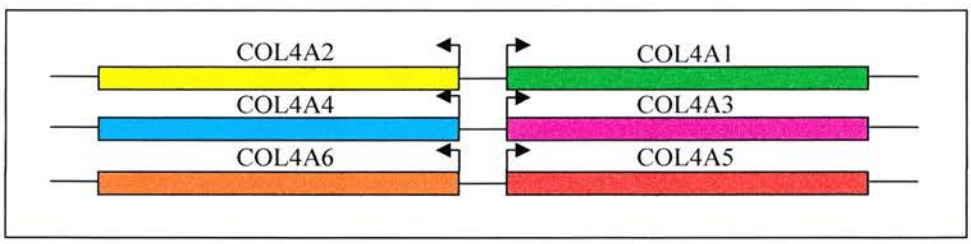

Figure 1.13. The arrangement of the collagen IV α-chain genes and their bidirectional promoters. Adapted from (Hudson *et al.* 1993).

The six α-chains assemble into three distinctive protomers α1α1α2(IV), α3α4α5(IV) and α5α5α6(IV) that form three separate networks: the α1/α2(IV) arrangement which is ubiquitous, α3α4α5(IV) and a structure formed from α1α1α2(IV)/ α5α5α6(IV) (Borza *et al.* 2002; Gelse *et al.* 2003). The two latter aggregations appear to occur later in development where they replace the α1/α2(IV) system in a tissue-specific manner (Gelse *et al.* 2003). In adults, α1(IV) and α2(IV) are found together in many different tissues whereas the other four α chains are expressed in a more precise temporal and spatial pattern (Fleischmajer *et al.* 1997; Miner & Sanes 1994). When mutations occur in α3(IV) and α5(IV) characteristic phenotypes are seen in the tissues where these chains are specifically expressed (Gelse *et al.* 2003).

The murine endodermal-derived M1536-B3 cells have been shown to make ECM *in vitro* even though they lack collagen IV (Brauer & Keller 1989). The resulting basement membrane is characteristically similar to collagen IV-containing basement membrane suggesting that matrix can form without collagen IV in culture or that collagen IV does

not provide the basic structural framework for basement membrane assemblage (Brauer & Keller 1989). The α1/α2(IV) aggregation is first detected at the 32-64 cell stage of development in the mouse embryo giving the impression that it must be important in early development. However, when the locus for COL4 α1/α2 is inactivated, embryos survive to E10.5 – 11.5 suggesting that while this network is not crucial to early basement membrane formation it may have other functional and structural roles (Gelse *et al.* 2003). Possibly, when these α1/α2(IV)-chains are absent other α-chains take over the roles of the missing α-chains to some extent. This has not been shown for α1/α2(IV) complexes but in male dogs mutant for α5(IV) where the α3α4α5(IV) complexes are not formed and humans that lack the α3α4α5(IV) complex, fetal expression of α1/α2(IV) continues (Harvey *et al.* 1998; Kalluri *et al.* 1997). Embryonic lethality (or premature death as seen in the α3(IV) null mouse (Cosgrove *et al.* 1996)) seen in COL4 α-chain homozygous mutant mice implies that collagen IV chains work together in complex ways and all are needed to function harmoniously. Certainly, all are needed as the structural integrity of the embryo becomes more complex. All this suggests that COL4 is essential for the maintenance of the structure of the basement membrane but not the formation.

1.3.3.1.2.2. Network formation

Each collagen IV α chain is made up of three distinct domains: a cysteine-rich N-terminal 7S domain, a triple-helical domain that is found at the centre of the helix and a highly conserved C-terminal non-collagenous globular domain (NC1) (Ortega & Werb 2002). The whole structure forms a flexible helix (Gelse *et al.* 2003). When NC1 are removed by enzymic digestion the folding ability of collagen IV is lost (Dolz *et al.* 1988)

The collagen IV network is formed by protomers interacting with each other to form a mesh. Various interactions that can occur between the protomers are shown in Fig. 1.14. These include NC1 domains interacting with one another to form dimers and then with 7S domains to form tetramers (Boutaud *et al.* 2000). Additionally, supercoiling and looping of protomers contributes to network formation (Hudson *et al.* 2003). The N-

terminus can form disulphide bonds within and between molecules, which establish the overall 'suprastructure' of the collagen IV network (Kalluri *et al.* 1997). The diverse molecular associations made by collagen IV allow for the formation of diverse types of basement membrane, consistent with their many tissue-specific roles throughout the body.

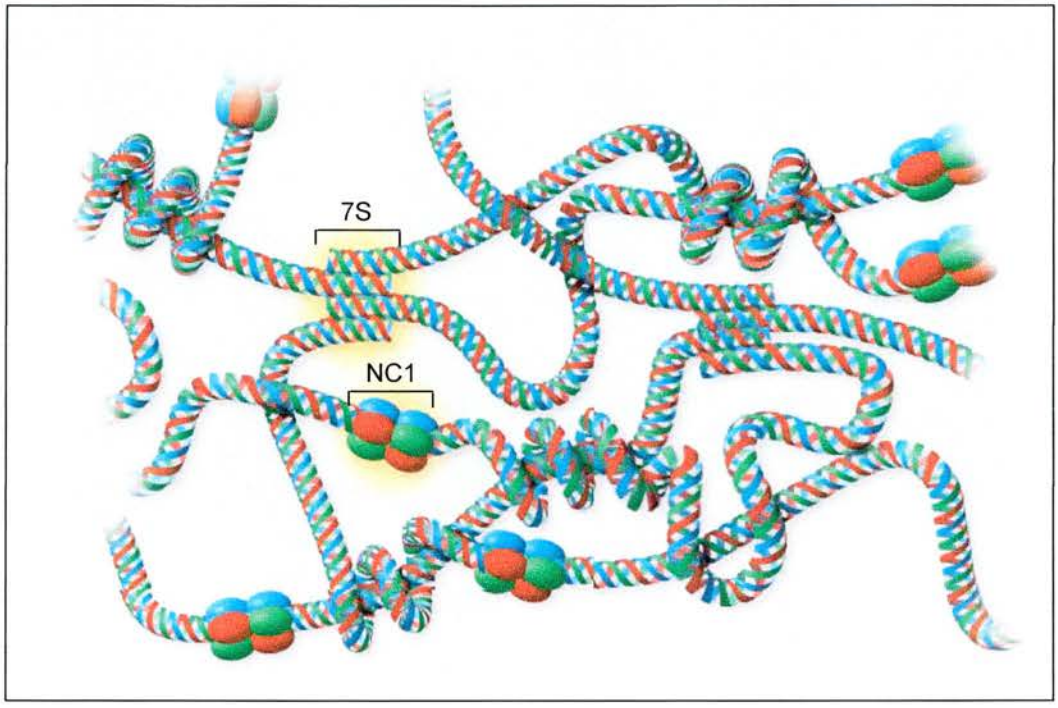

Figure 1.14. Schematic representation of collagen IV 'suprastructure' and network (taken from Hudson *et al* 2003). Each protomer helix consists of three α-chains, then NC1 domains link to form dimers and 7S domains link to form tetramers. Protomers also loop and coil to form the collagen IV network.

The existence of six α-chains capable of forming triple helices creates the theoretical potential for many different combinations of collagen IV helices to be formed into networks. Yet in nature only three different combinations actually form and the reason for this is unknown. Possibly, there is molecular specificity between the α-chains, such that only three combinations can form. It has been shown that α1(IV) and α2(IV) form helices through recognition at NC1 domains (Boutaud *et al.* 2000; Soder & Poschl 2004). Thus it is possible that the NC1 domains define and limit the formation of

2004). Thus it is possible that the NC1 domains define and limit the formation of particular collagen IV complexes. The NC1 domain may also include intraprotomer and interprotomer recognition sites necessary for helix and network specificity. Alternatively, the spatiotemporal expression of particular genes within tissues could dictate the composition of protomers that are formed. This could be because each type of chain confers a distinctive strength potential and different tissues require basement membranes with varying strengths. For example, in the COL4α5 null mouse the kidney glomerular basement membrane lacks the α3α4α5(IV) complex, meaning that although it still has the α1/α2(IV) protomer the membrane is unable to handle the strain of filtration, resulting in proteinuria (Hamano *et al.* 2002).

Collagen IV protomers are also stabilised by undetermined covalent cross-links at the C- and N- termini (Bailey *et al.* 1984). These cross-links may be lysine aldehydes, pointing towards the cross-links being similar to those in fibrillar collagens and hence possibly being formed by LOX (Bailey *et al.* 1984). This is important as it has not conclusively been proven that LOX is instrumental to collagen IV network formation. Collagen I, III and IV in LOX null mice are histologically normal, but electron microscopic analysis has revealed that the fibrillar collagens were dispersed and had shorter fibres compared to the tightly bundled wild-type phenotype (Maki *et al.* 2005). These authors did not comment on basement membrane structure or location and no electron microscope studies of basement membranes were undertaken. However, the lungs and cardiovascular system in LOX-null mice are morphologically abnormal and dysfunctional (Maki *et al.* 2002; Maki *et al.* 2005). Since, collagen IV is associated with these tissues this phenotype may indicate the involvement of LOX in stabilising collagen IV. However, this interpretation is potentially confounded by the fact that the fibrillar collagens are also associated with these structures.

A possible binding site for LOX, similar to that in fibrillar collagen, has been identified in collagen IV (Bailey *et al.* 1993). The cross-link is reducible by mercaptoethanol (Bailey *et al.* 1984) as are cross-links created by LOX (Harris *et al.* 1974). More recent investigations into the NC1 domain, which is essential for network formation as it forms hexamers between helices, suggest that this region is stabilised by a covalent cross-link

between methionine and lysine residues of opposing α1 and α2 chains. However, there is much debate as to the nature of this link with some evidence suggesting that the cross-link is possibly a S-hydroxylysylmethionine (Than *et al.* 2005; Than *et al.* 2002; Vanacore *et al.* 2005). Although deliberation on the nature of the cross-linkages of collagen IV continues, the balance of the evidence to date suggests that LOX may be involved. Certainly, no evidence has been provided to refute this possibility.

1.3.4. Intraovarian tissue remodelling

As discussed in section 1.1, the ovary is continually being subjected to a series of injury and repair/remodelling episodes during the course of follicle growth and ovulation. Therefore, collagen pathway biology must have a functional role in maintaining ovarian tissue integrity and associated ECM remodelling. Processes controlling ovarian tissue remodelling have been reviewed extensively by Ny *et al* (2002) and Curry & Osteen (2003). These reviews summarise the roles of MMPs and the plasminogen activation (PA) system in the ovary, which are beyond the scope of this literature survey. Instead, the focus here is on formation and accumulation of ECM.

1.3.4.1. Ovarian ECM

1.3.4.1.1. Stroma

The ovarian stroma is an understudied tissue. The stromal matrix supports the resting and growing follicle populations of the ovary as well as the ovarian vasculature, and consists of collagen type I and III (Palotie *et al.* 1984; Paranko 1987). Theca cells are recruited from unspecialised mesenchymal progenitor cells in the stroma, and once differentiated these cells remain associated with the stroma (Magoffin 2005). Therefore the stromal ECM may facilitate migration of theca precursors towards growing follicles. This need only be over a short distance as evidence suggests theca cell recruitment is controlled by granulosa cell secretions (Magoffin & Magarelli 1995).

1.3.4.1.2. Follicular basement membrane

In the ovarian follicle, granulosa cells are supported by the follicular basement membrane (also known as the basal lamina or lamina basalis) that separates them from the surrounding theca interna (Rodgers *et al.* 2000). This basement membrane influences granulosa cell proliferation and differentiation (Amsterdam *et al.* 1989). Basement membranes are also found in the subendothelial theca cell layer (Rodgers *et al.* 2000). The focus of the following text is the basement membrane that separates the granulosa and theca cells (Fig. 1.1).

In the healthy follicle, the basement membrane stops capillary invasion and entry of white blood cells and nerves into the granulosa layer until ovulation when the basement membrane is degraded (Rodgers *et al.* 2000). The basement membrane maintains the polarity and specialisation of the granulosa cells and most probably adjusts its composition in response to granulosa cell behaviour during follicle growth (Rodgers *et al.* 2000). The semi-permeable follicular basement membrane also excludes large molecular weight plasma proteins such as low density lipoproteins and molecules from the antrum of the follicle (Andersen *et al.* 1976). Therefore, large molecules that are synthesised by the granulosa cells and oocytes may be retained within the follicular fluid (Rodgers *et al.* 2000). The molecular weight cut-off for the follicular basement membrane is estimated to be 100–850 kDa (Andersen *et al.* 1976). Although it may change throughout follicular development (Rodgers & Irving Rodgers 2002), this cut-off is well beyond the size of most of the classic growth factors, which can almost certainly move freely between the theca and granulosa cell layers. The proportion of extracellular, large proteins in the follicular fluid increases as the follicle grows (Andersen *et al.* 1976). This indicates that the movement of protein into the antrum is governed by the follicular basement membrane. So, the basement membrane can act as a barrier between the two main somatic follicular cell layers. However, since ECM can also bind various growth factors it can act as a reservoir for instructional substances needed by the different cell types at particular stages of follicular development (Rodgers *et al.* 2000). For instance, the ECM binds and pools active TGF-β, which is effectively an ECM component (Gualandris *et al.* 2000).

1.3.4.1.2.1. Early development of the follicular basement membrane

Even at very early stages of ovarian development, basement membranes are in evidence as oogonial cords become separated from the ovarian stroma (Rodgers *et al.* 2003). Then, as cords develop into primordial follicles the pregranulosa cells are also surrounded by basement membrane (Irving Rodgers & Rodgers 2005). Eventually, as growth of these follicles is initiated, the basement membrane expands to accommodate later stages of follicle growth (Rodgers *et al.* 2003). Throughout, the basement membrane can influence follicle growth by regulating substances that pass among theca cells and granulosa cells, and beyond to the oocyte.

1.3.4.1.2.2. Basement membrane during follicular maturation

Cytoplasmic oocyte maturation occurs throughout follicular development and some of the early changes occur in close contact with the primordial follicular basement membrane (Oksjoki *et al.* 1999). Pre-ovulatory follicular development and post-ovulatory development of the corpus luteum also intimately involve ECM (Oksjoki *et al.* 1999). As each stage occurs, the ECM undergoes extensive destruction, repair and displacement, indicating that its role is instructional as well as structural (Woessner 1991). Studies have shown that the MMPs and their inhibitors, TIMPs, have developmental-associated patterns of expression throughout folliculogenesis, highlighting that formation and breakdown of ECM is precisely regulated (Duncan *et al.* 1998). In the mouse ovary during the oestrous cycle, different mRNA expression patterns have been shown for type III, IV and VI collagens as well as for several small proteoglycans (Oksjoki *et al.* 1999). This indicates that the destruction and construction of the follicular ECM are influenced by cyclic changes and are a feature of normal ovarian function.

Dormant primordial follicles contain a small, quiescent oocyte and a single layer of non-replicating granulosa cells enclosed by the basement membrane (Irving-Rodgers & Rodgers 2000). As previously stated (Section 1.1.1.1), follicular growth becomes gonadotrophin-dependent once the antral cavity begins to form, which fills with fluid composed in part from secretions of the granulosa cells (Hillier 1994). Extracellular matrices have been shown to influence fluid movements within tissues that set up

osmotic barriers can affect osmosis and filter soluble substances, both of which properties would be important in the accumulation of follicular fluid and the nutritional and instructional signals therein (Rodgers *et al.* 2003).

The granulosa cell layer expands as the follicle matures. As a general approximation, the number of granulosa cells is four times that of the follicular surface area (Rodgers *et al.* 1999a). In rat and cow ovary, the composition of the follicular basement membrane has been shown to change as development occurs (Frojdman *et al.* 1998; van Wezel *et al.* 1998). However, it has not yet been shown whether these changes are linked to changes in the granulosa cells or to the permeability of the ECM. At ovulation the basement membrane is degraded, allowing blood cells and inflammatory cells such as macrophages to enter the granulosa cell compartment (Irving-Rodgers *et al.* 2002).

1.3.4.1.2.3. Basement membrane influences on cell morphology

It is thought that the follicular basement membrane influences the shape and function of the granulosa cells (Huet *et al.* 2001). Differences have been observed between the phenotypes of granulosa cells that are in direct contact with the basement membrane and those that are situated in inner layers (Huet *et al.* 2001). Some follicles have basal cells that are columnar in form, middle layers composed of rounded cells and antral granulosa cells that are flattened, whilst other follicles have granulosa cells that are all rounded (Irving-Rodgers & Rodgers 2000). The cell shape may reflect the proliferation status of these cells as rounded basal granulosa cells have a greater rate of proliferation compared to antral cells (Irving-Rodgers & Rodgers 2000; Jablonka-Shariff *et al.* 1994). These differences within the follicle may be due to cellular location in relation to the basement membrane.

Ultrastructural studies of bovine follicles reveal two main phenotypes. In healthy preantral and antral follicles, the conventional single layer of basement membrane is seen (Irving-Rodgers & Rodgers 2000). At the preantral stage the basement membrane is enlarged and partially laminated. A loopy phenotype can also be observed in some preantral follicles and antral follicles $\leq$ 4mm in diameter (Irving-Rodgers & Rodgers 2000). However, is not seen in larger follicles (Irving-Rodgers & Rodgers 2000). When

observed in antral follicles, the loopy phenotype was associated with columnar granulosa cells, whereas normal basement membrane phenotype was observed in connection with rounded granulosa cells (Irving-Rodgers & Rodgers 2000). The basement membrane in preantral follicles was thicker than that of antral follicles, which may reflect differences in the permeability of this layer as maturation occurs (Irving-Rodgers & Rodgers 2000). The loopy phenotype suggests that cell shedding and, therefore, cell replacement is occurring (Irving-Rodgers & Rodgers 2000). When follicles with loopy and normal phenotypes were compared it was found that the normal follicles were of a better 'quality' but a cause-effect relationship has not been established (Irving Rodgers & Rodgers 2005).

A recent study of the bovine follicular matrix has highlighted a novel form of matrix within the granulosa cell layer of antral/preovulatory follicles. Irving-Rodgers *et al* (2004) term this the 'focimatrix' as it appears as aggregates of basement membrane in between granulosa cells but its function is unclear. The role of focimatrix is unknown but it is hypothesised to be important to the epithelio-mesenchymal transition of granulosa cells through dispersing any positional cues being provided by the basement membrane of the follicle (Irving Rodgers & Rodgers 2005).

1.3.4.1.2.4. Composition of the follicular basement membrane

The molecular components of the follicular basement membrane are believed to originate primarily from granulosa cells (van Wezel *et al.* 1999). However, theca cells also express several matrix components, which leaves the contribution of this issue open to debate (van Wezel *et al.* 1999). The expression pattern of basement membrane components in the ovary is summarised in Figure 1.15.

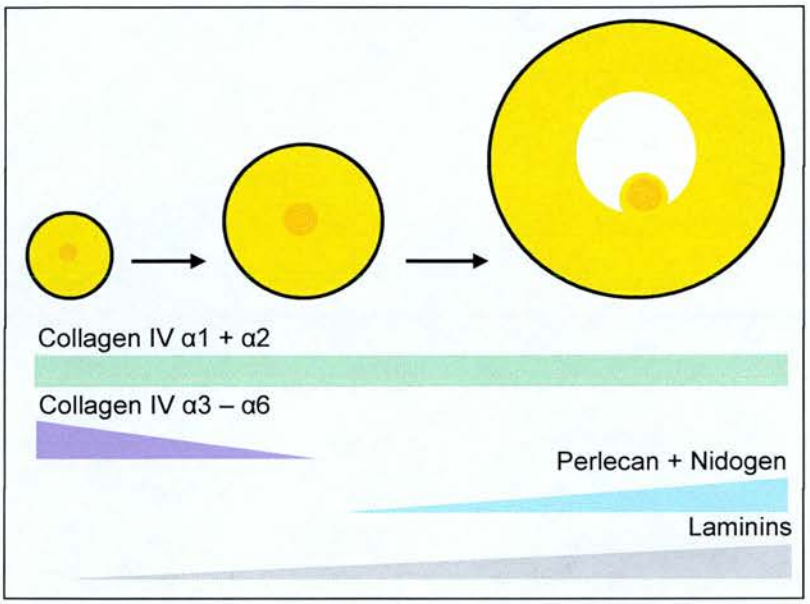

Figure 1.15. Schematic diagram of the expression pattern of basement membrane components in the ovary (adapted from Irving-Rodgers and Rodgers 2005).

Laminins are localised to the basement membrane of all healthy follicles and in the theca layers of antral follicles (van Wezel *et al.* 1998). All collagen IV α-chains are expressed in primordial follicles (Rodgers *et al.* 1998). Therefore there is potential for all three collagen IV helices (α1α1α2(IV)/α3α4α5(IV)/α5α5α6(IV)) to be formed although to date the complexes that actually exist have not been documented. As follicles increase in size only α1(IV) and α2(IV) become expressed (Rodgers *et al.* 1998). Expression of collagen IV α3 through α6 declines, and perlecan and nidogen expression begins (Irving-Rodgers *et al.* 2006). This switch occurs around the time of antrum formation, suggesting that the presence of α3(IV),α4(IV) and α5(IV) opposes antral development. Alternatively the loss of these α-chains may allow easier degradation of the basement membrane at ovulation, as α3α4α5(IV) networks have a greater degree of protection from proteases due to heavy cross-linking (Kalluri *et al.* 1997).

1.3.4.2. Collagen biology in the ovary

So how is the collagenous composition of the follicular basement membrane regulated in the ovary? Collagen synthesis and cross-linking catalysed by LOX are liable to be critical, as are the activities of BMP-1 and PCPE (that to date have not been reported in the ovary). This literature review concludes with an overview of what little data there are describing LOX and ovarian function.

1.3.4.2.1. The role of LOX in remodeling of the ovary

Investigations into LOX in the ovary are very limited even though tissue remodelling and repair are very important in this tissue due to follicular growth and ovulation. As follicles grow their basement membranes and the surrounding stroma must accommodate the increase in size. LOX mRNA is strongly expressed in preantral and early antral granulosa cells (Harlow & Hillier 2002) and *in situ* hybridisation showed LOX was most highly expressed in preantral/early antral follicles with much lower expression in large preovulatory follicles (Slee *et al.* 2001).

At ovulation, localised tissue breakdown is necessary to release oocytes for potential fertilisation. Each time ovulation occurs a natural wound develops that must be repaired before the next ovulation. In the rabbit ovary, LOX activity increases after hCG-induced ovulation and an increase in LOX mRNA occurs after progestin-induced ovulation in the perch (Himeno 1986; Langenau *et al.* 1999).

In preantral/early antral follicles the LOX gene is negatively regulated by FSH in a dose-dependent manner, as is connective tissue growth factor (CTGF) (Harlow *et al.* 2002; Harlow *et al.* 2003). The down-regulation of LOX by FSH is mediated through the elevation of cAMP which co-ordinates signals that up-regulate steriodogenic enzymes and LH receptors (Harlow *et al.* 2003). cAMP also suppresses basal and TGF-β-induced collagen and LOX mRNA levels in human lung fibroblasts (Choung *et al.* 1998) but has the opposite effect on LOX in vascular smooth muscle cells, suggesting a tissue specific response (Ravid *et al.* 1999).

The expression of CTGF mRNA is up-regulated at the early stages of antral follicle development at the same point that LOX gene expression increases (Harlow & Hillier 2002). This is also when intense connective tissue biosynthesis occurs and ECM is laid

down (Harlow & Hillier 2002), implying that the regulation of CTGF and LOX may be linked.

Interactions between androgens and FSH enhance differentiation processes such as sex steroid synthesis, induction of LH receptors and carbohydrate metabolism (Harlow *et al.* 2003). Androgens intensify the action of cAMP, and when FSH and the androgen dihydrotestosterone (DHT) were cultured with rat granulosa cells an enhanced down-regulation of both LOX mRNA and activity was seen (Harlow *et al.* 2003; Hillier & de Zwart 1982). DHT alone caused an increase in LOX expression (Harlow *et al.* 2003; Slee *et al.* 2001) showing that FSH strongly influences the timing and extent of LOX expression.

LOX has also been investigated in polycystic ovarian syndrome (PCOS) where ovaries have a thickened fibrotic tunica alguginea as well as cystic follicles. When PCOS was induced in a rat model using dehydroepiandrosterone (DHEA) an increase in LOX expression was observed whilst there was a reduction in MMP-2 expression. This was opposite to what was observed in normally cyclic controls (Henmi *et al.* 2001). These findings could help to explain the cystic, thickened ovarian phenotype of PCOS and also suggests how important LOX is to normal ovarian function.

LOX (as well as BMP-1 and PCPE) in follicular development remains poorly understood.

Therefore the overall scope and purpose of this thesis is to determine how collagen pathway biology alters in the developing ovarian follicle, asking specifically how LOX, BMP-1 and PCPE are involved; if these genes and their resultant proteins are developmentally and hormonally regulated; and whether they are subject to pro- and/or anti-inflammatory modulation.

1.4. Hypothesis

1. I propose the collagen pathway genes are localised in different ovarian compartments and function via paracrine signalling to create functional ECM remodelling during follicular development. I hypothesise that BMP-1 and PCPE will be localised to the theca cell compartment to regulate collagen processing in the stroma. BMP-1 will also act on pro-LOX to create active enzyme. LOX is known to be expressed by the granulosa cells implying that BMP-1 will cross the basement membrane to cleave pro-LOX. LOX would then move to collagenous areas of the ovary to stabilise collagens in these matrices. This hypothesis is summarised in figure 1.16

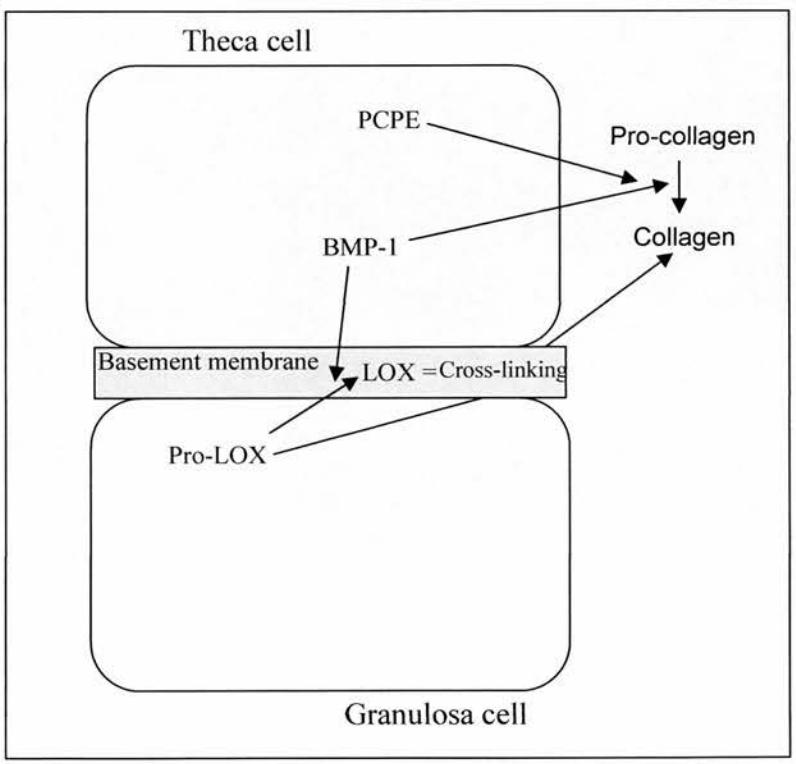

Figure 1.16. The proposed two-cell model for collagen pathway genes.

Therefore if the candidate collagen pathway genes are present in different compartments their expression and activation is most probably regulated by the gonadotrophins that regulate the development of ovarian follicles. Other influences

could be ovarian steroids, growth factors and cytokines. Ovarian steroids include glucocorticoids, the accumulation of which is regulated by the expression of 11βHSD isoforms. Glucocorticoids as anti-inflammatories, would be expected to have strong influences on collagen pathway genes.

2. As glucocorticoids are likely to be key post-ovulatory anti-inflammatories this raises the question what might occur if their formation was dysregulated? If glucocorticoids were not present in the biological system, or if glucocorticoids could not be activated normally at ovulation, ovulation-associated inflammation might not be adequately resolved. This would be predicted to involve measurable alteration in collagen pathway biology.
3. Collagen IV is possibly the most important collagen in the ovary as it is part of the basement membrane. Therefore if collagen IV is partially disrupted it is predicted that the basement membrane would not form normally. This could lead to abnormal communication between granulosa and theca cells, perhaps resulting in impaired localisation of collagen pathway biology and reduced fertility.

Thus, the principal aims of this thesis were to establish if:

1. Collagen pathway genes (PCPE, BMP-1 and LOX) are present in ovary.
2. Compartmentalisation of gene expression and translated proteins for collagen pathway genes and glucocorticoid signal regulators occurs in ovary.
3. Collagen pathway genes are regulated by gonadotrophins, growth factor, cytokine and steroids during follicular development.
4. Disruptions in the glucocorticoids regulatory mechanism affect ovarian function and collagen pathway biology.
5. Disruptions in collagen IV of the ovarian basement membrane affect ovarian function.

2. Materials and Methods

The commercial sources of all reagents/chemicals/plastics/antibodies used in these investigations are shown in Appendix 1.

2.1 Animals

2.1.1 Wistar rat model for follicular maturation

Immature 21-day old female Wistar rats were housed on a 12h light:12h dark lighting regime in a temperature-controlled environment. Rats were fed rat chow *ad libitum* and water was freely available. Rats were allocated to one of three experimental groups to simulate different stages of the oestrous cycle by giving hormone treatments *in vivo*. All hormones were made up in filtered Dulbecco's phosphate buffered saline (DPBS) supplemented with 1% bovine serum albumin (BSA).

- Group 1 received no *in vivo* hormone injections. The ovaries of these animals consisted of preantral and early antral follicles.
- Group 2 received 10IU/rat pregnant mare serum gonadotrophin (PMSG) 48 hours before sacrifice. The ovaries of these animals produced large numbers of preovulatory follicles.
- Group 3 received 10IU/rat PMSG 60h before sacrifice and 10IU/rat human chorionic gonadotrophin (hCG) 48h after the PMSG injection and 12h before sacrifice. These ovaries provided a population of largely periovulatory follicles.

All injections were given inter-peritoneally in accordance with Home Office regulations. Animals were killed by CO_2 asphyxiation followed by cervical dislocation. Ovaries were immediately resected and immersed in culture medium or placed in fixative.

2.1.2. 11βHSD1-null mice

11βHSD1 was mutated in the mouse genome using targeted gene disruption (Kotelevtsev *et al.* 1997). 11βHSD1-null mice were housed under a 12 hour light: 12 hour dark regime with free access to food and water. Female 11βHSD1-null mice aged 3 months and 12 months were allocated to one of three groups and received the same hormone treatments as the Wistar rat model (Section 2.1.1), to generate untreated

ovaries, PMSG-treated ovaries and PMSG/hCG treated ovaries. Mice were sacrificed and tissues collected as stated above (Section 2.1.1).

2.1.3. COL4α1-mutant mice

Missense mutations of the COL4α1 gene were generated as part of an N-ethyl-N-nitrosourea (ENU) mutagenesis project to generate mice with a dominant mutant eye phenotype (Thaung *et al.* 2002). Of the many phenotypes generated, three were accessible for these investigations through collaboration with Dr Tom Van Agtmael (The University of Edinburgh). The three groups of mutant mice were:

1. retinal arteriolar wiring (Raw)
2. small with vacuolar cataracts (Svc)
3. Bruised (Bru)

The reported phenotypes of these three mutants are shown in Table 2.1. Raw and Svc were created using a BALB/c mouse strain and Bru originated from a C3H/101 strain (Van Agtmael *et al.* 2005). Wild-type littermates were used as controls.

Table 2.1. Reported phenotypes of Raw, Svc and Bru mutant mice (Van Agtmael *et al* 2005; Thaung *et al* 2002).

Mutant	Phenotype
Raw	Retinal arterioles have a silvery appearance.
Svc	Silvering of the arteriolar, vacuolar cataracts, body size is small and pups are bruised at birth.
Bru	Eye defects (including opaque cornea, iris and corneal adhesions), bruising at birth, peripheral kidney glomerulopathy and small body size.

Mutant and wild-type mice were housed under a 12h light:12h dark lighting regime in a temperature-controlled environment. Mouse chow and water were available *ad libitum*. Female mice were culled by CO_2 asphyxiation at three months, six months and nine months and ovaries were resected and fixed. Post-fixation, excess tissue and fat were removed from the ovaries using forceps. The ovaries were weighed post-fixation.

A cohort of Raw and WT 21-day old female mice received 10IU PMSG/mouse 60h before sacrifice and 10IU hCG/mouse 48h after PMSG injection and 12h before

sacrifice. After sacrifice, the mice were individually weighed then ovaries were resected and weighed separately before being fixed.

2.1.4 CYP11B1 null mice

CYP11B1 was mutated in the mouse genome using targeted gene disruption. CYP11B1-deficient mice were accessible for these investigations through collaborations with Dr Linda Mullins, Dr Chris Kenyon and Dr Judy McNeilly (The University of Edinburgh). Animals were housed under a 12 hour light: 12 hour dark regime with free access to food and water. Females were sacrificed by CO_2 asphyxiation aged 3 months, when ovaries were removed and fixed as stated in Section 2.1.1.

2.2. Granulosa cell culture

2.2.1. Granulosa cell collection and preparation for cell culture

Granulosa cells were collected from the ovaries of female 21-day old Wistar rats that had received the hormone treatments previously described (Section 2.1.1.). Ovaries were resected and visualised under a dissecting microscope to enable the removal of extraneous tissue and fat. The ovaries were punctured using a 27 gauge needle and granulosa cells were expelled into culture medium (Medium 199 supplemented with 2mM L-glutamine, penicillin 50IU/ml, streptomycin 50μg/ml, 0.1% (w/v) BSA). Unless stated otherwise this supplemented medium is referred to as Medium 199 throughout the remainder of this text. Cells were gently pipetted through a 10ml pipette to break up cell clumps. The cell suspension was centrifuged at 120xg for 5min to form a pellet of cells, which was resuspended in a known volume of Medium 199. A 20μl volume of cell suspension was mixed with an equal volume of trypan blue to assess cell number and viability using a haemocytometer.

2.2.2. Granulosa cell culture

Cells were plated into 24-well tissue culture plates that had been pre-coated overnight with donor calf serum to provide a thin layer of ECM-related substances that encourage the cells to adhere to the plastic. Plates were washed twice with phosphate-buffered saline (PBS) before cells were plated out at 50,000 cells per well and cultured overnight in 500µl Medium 199 to allow cell attachment to the culture dishes. Cells then received different treatments (Table 2.2) and were incubated for 48 hours at 37°C in an atmosphere of 20% (v/v) O_2 and 5% (v/v) CO_2.

Table 2.2. Culture treatments given to granulosa cells and number of experimental repeats. Corticosterone (CORT), follicle-stimulating hormone (FSH), transforming growth factor-β (TGF), luteinising hormone (LH), interleukin-1 (IL-1), progesterone (PROG).

Untreated rat granulosa cells

Experimental treatments	Number of experimental repeats
Control/CORT/FSH/CORT+FSH	4
Control/CORT/TGF/CORT+TGF	4
CORT dose response (0/0.01/0.1/1µM)	3

PMSG-treated rat granulosa cells

Experimental treatments	Number of experimental repeats
Control/CORT/LH/CORT+LH	6
Control/CORT/TGF/CORT+TGF	3
Control/CORT/IL-1/CORT+IL-1	6
CORT dose response (0/0.01/0.1/1µM)	3
Control/PROG/LH/PROG+LH	3
Control/PROG/IL-1/PROG+IL-1	3

PMSG/hCG-treated rat granulosa cells

Experimental treatments	Number of experimental repeats
Control/CORT/LH/CORT+LH	3
Control/CORT/TGF/CORT+TGF	3
Control/CORT/IL-1/CORT+IL-1	3
CORT dose response (0/0.01/0.1/1µM)	3
Control/PROG/LH/PROG+LH	4
Control/PROG/IL-1/CORT+IL-1	4

2.2.4. Termination of culture

Spent medium was collected and stored at –20°C. Cells were removed for later RNA extraction by incubation with trypsin until they detached from the plastic surface. The resulting suspension was centrifuged at 300xg for 5min and the pellet was washed in 1ml DPBS. This suspension was centrifuged at 600xg for 3min before being resuspended in 350µl Qiagen lysis buffer containing 10µl/ml β-mercaptoethanol. Cells were homogenised by passing them through a fine-gauge needle several times. Alternatively cells were removed from culture by placing 350µl Qiagen lysis buffer containing 10µl/ml β-mercaptoethanol onto cells after medium had been removed, followed by a brief vortex. The cell homogenates were stored at –70°C. Both methods were in accordance with the Qiagen RNasy kit protocol used to extract RNA. No differences were observed between the two methods when granulosa cell RNA quality and quantity were compared.

2.3. Theca cell culture

2.3.1. Theca cell extraction

Theca cells were extracted from residual ovary tissue using a protocol adapted from Magoffin & Erickson (1982) and Smyth *et al* (1993). Once granulosa cells had been removed, the remains of the ovaries were rinsed in Medium 199, then incubated in 2ml digestion medium (0.1% collagenase, 0.01% DNase, Medium 199) for 30min with agitation. The suspension was dispersed to achieve a single-cell suspension by gentle pipetting every 15min. Cells were centrifuged at 800xg for 5min, followed by resuspension in 2ml Medium 199 containing 5% (v/v) donor calf serum. Cell number and viability were assessed with a haemocytometer.

2.3.2. Theca cell separation

Following the digestion step the theca cell suspension was enriched using discontinuous gradient centrifugation. This was achieved by creating two layers of Percoll® in a Rohen tube. 2ml of Percoll® solution adjusted to a specific gravity of 1.055 was placed into the tube. The specific gravity was calculated using the following formula:

$$V_o = V \left(\frac{d - 0.1\, d_x - 0.9}{d_o - 1} \right)$$

V_o = volume of Percoll® (ml)

d_o = density of Percoll® (1.130g/ml)

d_x = density of Medium 199 (1.058g/ml)

V = volume required for final working solution (ml)

d = density required for final working solution (g/ml)

1ml of 44% Percoll® was placed beneath the 1.055 specific-gravity layer using a glass pipette. The dispersed ovarian cells were layered over the Percoll® layers and the tube centrifuged at 400xg for 20 min. Theca cells were aspirated from between the two layers of Percoll®. Cells were washed in Medium 199, collected by centrifugation and resuspended in Medium 199 with 5% donor calf serum. Density and viability were checked with a haemocytometer, then cells were plated at 50, 000 per well in 24-well culture dishes in 500µl Medium 199 with 5% donor calf serum. After 12h the medium was changed to Medium 199 and cells were treated with specific treatments (Table 2.3).

Table 2.3 Culture treatments given to theca cells and number of experimental repeats. Corticosterone (CORT), follicle-stimulating hormone (FSH), transforming growth factor-β (TGF), luteinising hormone (LH), interleukin-1 (IL-1), progesterone (PROG).

Untreated rat theca cells

Experimental treatments	Number of experimental repeats
Control/CORT/LH/CORT+LH	3
Control/CORT/TGF/CORT+TGF	4
CORT dose response (0/0.01/0.1/1μM)	3

PMSG-treated rat theca cells

Experimental treatments	Number of experimental repeats
Control/CORT/LH/CORT+LH	3
Control/CORT/TGF/CORT+TGF	3
Control/CORT/IL-1/CORT+IL-1	4
CORT dose response (0/0.01/0.1/1μM)	5
Control/PROG/LH/PROG+LH	4
Control/PROG/IL-1/PROG+IL-1	4

PMSG/hCG-treated rat theca cells

Experimental treatments	Number of experimental repeats
Control/CORT/LH/CORT+LH	3
Control/CORT/TGF/CORT+TGF	3
Control/CORT/IL-1/CORT+IL-1	3
CORT dose response (0/0.01/0.1/1μM)	3
Control/PROG/LH/PROG+LH	3
Control/PROG/IL-1/CORT+IL-1	3

2.3.3. Termination of culture

Theca cells were removed from culture as described in Section 2.2.4 for granulosa cells.

2.4. Radioimmunoassays

2.4.1. Androstenedione

Coat-a-count® direct androstenedione kits were used to measure androstenedione quantity in cell culture medium from theca cells. Briefly, the coat-a-count® kit supplies polypropylene tubes coated with polyclonal androstenedione antibody, direct androstenedione calibrators and ^{125}I-androstenedione. Total counts and non-specific binding were measured for each assay. Calibration solutions of known androstenedione concentrations were used to create a standard curve. Culture cell medium was added to coated tubes in duplicate at 100µl/tube. Tubes were incubated at room temperature for 2h, then decanted and counted on a gamma counter (1261 Multigamma, LK3 Wallac) for 1 min. Androstenedione values were read from the standard curve using MultiCal analysis software (Wallac).

2.5. Extraction of RNA from cells

2.5.1. RNA Extraction

RNA was extracted from freshly isolated and cultured granulosa and theca cells, using Qiagen RNasy kits with a centrifugation protocol. All centrifugation steps were carried out at 900xg in a mini-centrifuge. Cell suspensions stored in lysis buffer supplemented with 10µl/ml β-mercaptoethanol were defrosted, diluted with an equal volume of 70% (v/v) EtOH and applied to the mini columns supplied with the kit. The columns were washed with the supplied wash buffer, and then treated with DNase for 15min, to remove any contaminating genomic DNA. DNase was inactivated and removed by washing with wash buffer. Columns were then washed twice with supplied elution buffer and RNA was eluted into 30µl RNase-free water.

2.6. RT-PCR

2.6.1. cDNA for PCR

Extracted RNA was quantified using the Agilent 2100 Bioanalyser in conjunction with a RNA_{6000} nano chip. RNA was used to synthesise cDNA for use in standard RT-PCR. First a Superscript III first strand was synthesised. For each different RNA sample a mix of the reagents shown in Table 2.4. was prepared in 0.2ml Eppendorfs tubes.

Table 2.4. Reagents required for Superscript III first-strand synthesis (x denotes variable quantity).

Reagent	Amount
Oligo dT	1µl
10mM dNTP mix	1µl
2µg total RNA	**X**µl
Nuclease free H_2O	to a total of 13µl

Eppendorf tubes were heated to 65°C for 5min, then placed on ice for 1 min and briefly centrifuged. The next step produces cDNA from the first strand synthesis. The reagents shown in Table 2.5. were added to each tube depending on whether cDNA synthesis was required (positive) or not (negative control).

Table 2.5. Reagents added to each first-strand reaction for cDNA synthesis.

Reagent	+ve	-ve
5x 1st strand buffer	4µl	4µl
0.1M DTT	1µl	1µl
RNase OUT	1µl	1µl
Superscript III	1µl	
Nuclease free H_2O		1µl

Reagents were mixed and heated to 50°C for 50min (transcription), followed by 70°C for 15min (final extension step). The resultant cDNA was stored at -20°C.

2.6.2. Reverse Transcriptase (RT)-PCR

cDNA was used for PCR, using primers generated specifically for this project or primers that had been constructed previously in the laboratory. Mastermix solutions shown in Table 2.6. were made fresh for each reaction from a double-strength Promega PCR stock.

Table 2.6. Mastermix for a 1 x reaction for RT-PCR.

Reagent	Volume
Promega mastermix	25µl
DNase/RNase free H_2O	19µl
5' primer (50pmol)	2µl
3' primer (50pmol)	2µl

The mastermix was vortexed and aliquotted into 0.2ml Eppendorf tubes. 2µl cDNA was added to each reaction, mixed by pipetting and then on a heat cycled TECHNE TC-312 PCR machine with the following PCR programme.

➢ Step 1	93°C	3min	1 cycle	initialisation
➢ Step 2	93°C	30 sec		denaturing
	Variable °C	30 sec	30-40 cycles	annealing
	72°C	1 min		extension
➢ Step 3	72°C	5 min	1 cycle	final extension

Hold at 4°C

The variable temperature (U) was calculated for each set of primers using the following formula:

$[2 \times (A + T) + 4 \times (C + G)] - 5 = U^{\circ}C$

2.6.3. Gel electrophoresis

PCR products were size-fractionated by electrophoresis in the presence of ethidium bromide for visualisation under UV light. PCR products were mixed with 5x loading dye in a 1:5 ratio and loaded onto a 1.5% (w/v) agarose gel (1.8g agarose, 120ml 1xTAE buffer, 2µl ethidium bromide) in 1xTAE buffer. A 100-base pair ladder was used as a reference size marker. Electrophoresis was done at 100 volts until the PCR products had run three-quarters the length of the gel. Bands were observed and photographed under UV light (254nm).

2.7. Quantitative real-time PCR

2.7.1. cDNA synthesis for quantitative real-time PCR

RNA extracted from ovarian cells was quantified and its quality assessed using an Agilent 2100 Bioanalyser in conjunction with a RNA_{6000} nano chip. RNA that displayed intact 18S and 28S peaks was reverse transcribed using the Applied Biosystems Taqman™ reverse transcriptase reagents kit. A mastermix of the reagents was prepared for the desired number of reactions as shown in Table 2.7.

Table 2.7. Reverse transcription regents required for a 1 x reaction mastermix.

Reagent	x 1 reaction
Nuclease-free water	1.85µl
10x Taqman buffer	1.0µl
$MgCl_2$	2.2µl
dNTPs	2.0µl
Random hexamers	0.5µl
RNase inhibitor	0.2µl

7.75µl of the mastermix was removed to be used as an RT-negative control. Multiscribe Reverse Transcriptase was added to the remaining mastermix using the calculation 0.25 x number of reactions required - 0.25. The mastermix was vortexed, and portions (8µl for a single volume reaction or 16µl for a double volume reaction), were transferred into 0.2ml Eppendorf tubes. 2µl (single volume) or 4µl (double volume) sample RNA was

added to each reaction tube (including the RT-negative reaction) and 2µl/4µl of nuclease-free water was added to a separate tube as an RT-H_2O control. All reaction tubes were subjected to the following temperature cycle: 25°C for 1h (incubation step), 48°C for 45min (reverse transcription step), 95°C for 15min (denaturing step).

2.7.2. qRT-PCR

Quantitative real-time RT-PCR was used to measure gene expression in freshly isolated and cultured ovarian cells. Quantitative real-time RT-PCR can be performed using fluorescence-labelled custom-made primer and probe sets (previously validated) or using Assay-On-Demand assays. Both were used in this project. An endogenous control of 18S was used in all quantitative real-time RT-PCR assays. Table 2.8. shows the custom-made primer/probe sets and Assay-On-Demand assays used in this project.

Table 2.8. Primer/probe sequences for the genes of interest used in this project

Gene	Forward primer sequence	Reverse primer sequence	Probe sequence
LOX	TGGCACCGGT TACTTCCAGTA	ACTACATCCA GGCATCCACGT	CCCGGACCTG GTACCCGATCCC
BMP-1	CCAATTACC CCGACGATT	CACGTGGAAA CCCTCAGACA	CTGGATCCGCCA GATGCAGACTTTG
PCPE	TTTCCAGCAC TCCCCCTACA	AAGGAATG GCCCCAGCA	AATGCTGCCTGC TGCCCTAACCTCC
11BHSD1	GAGGTCAACTTC CTCAGCTATGTG	TGGAGGAGAT GATGGCAATG	CCTTGCCCATGCT GAAACAGAGCAAT
11βHSD2	TGAACTTCTTT GGTGCACTTGAG	AATACGTCCCC TTGAGTGACGTA	TGACCAAGGGC CTCCTGCCACTC
MMP-2	Assay-On-Demand assays – sequences are not published		
MMP-9			

Working reagent solutions were prepared for either custom-made primer/probe sets or for Assay-On-Demand assays. Typical mastermix components for a 1 x reaction for primer/probe sets and Assay-On-Demand assays are shown in Table 2.9.

Table 2.9. Mastermix for primer/probe sets and Assay-On-Demand assays for quantitative real-time PCR

Custom primer/probe			Assay-on-demand	
Reagent	1 x mastermix		Reagent	1 x mastermix
Taqman Universal mastermix	12.5µl		Taqman mastermix	12.5µl
Nuclease-free water	8.525µl		Nuclease-free water	8.875µl
18s primer/probe	0.375µl		18s primer/probe	0.375µl
Forward primer	0.3µl		Assay-on-demand primer/probe	1.25µl
Reverse primer	0.3µl			
Gene probe	1.0µl			

The reagent mix was vortexed and aliquotted into 0.2ml Eppendorf tubes at a volume of 23µl/tube. Custom-made primer/probe sets were run in triplicate and Assay-On-Demand assays in duplicate. cDNA was added to the mastermix aliquots at a volume of 2µl/tube. A PCR water-control was set up by adding nuclease-free water in place of cDNA. After vortexing the Eppendorf tubes, 23µl portions of their contents were transferred to the chambers of 96-well optical reaction plates. The plates were sealed with optical caps or film and then processed on the ABI PRISM® 7900 heat-cycler system. The PCR cycle was: stage 1, 50°C for 2min; stage 2, 95°C for 10min; stage 3, 95°C for 15sec; and stage 4, 59°C for 1min; stage 3 was repeated for 40 cycles.

2.7.3. Data analysis

Data were analysed using ABI 7900 software. Three important parameters were considered when analysing such data. These were the 'baseline', 'threshold' and 'threshold cycle' (C_T) values. The baseline value refers to baseline fluorescence in the reaction plate. The threshold is the numerical value reflecting the point above the calculated baseline that represents statistical significance. The threshold was set in the exponential region of the amplification plot (i.e. not in the plateau phase or baseline region). C_T reflects the cycle number taken for the fluorescence generated in a reaction to cross the threshold. Therefore, the C_T value for each sample indicates when

amplification has significantly exceeded the baseline value. Data was exported to a Microsoft Excel spreadsheet for further analysis.

The 'comparative C_T method' was used to further analyse raw data. The comparative C_T method uses arithmetic formulae to calculate relative quantitation. This is summarised below:

Step 1: calculate ΔC_T for each well in the optical plate

$$\Delta C_T = C_{T\ \text{target gene}} - C_{T\ \text{reference gene}}$$

Step 2: calculate the average of duplicates/triplicates

Step 3: calculate $\Delta\Delta C_T$

$$\Delta\Delta C_T = \Delta C_{T\ \text{target gene}} - \Delta C_{T\ \text{reference gene}}$$

Step 4: calculate the arithmetic formula

$$2^{-\Delta\Delta CT}$$

Values were expressed as fold-difference relative to the untreated controls. Repeated measures ANOVA with the Tukey post-hoc test were used to assess differences between gene expression levels, with significance set at $P<0.05$.

2.8. Lysyl oxidase enzyme assay

LOX oxidatively deaminates peptidyl lysine to peptidyl α-aminoadipic-δ-semialdehyde to cause collagen and elastin cross-linking. Tropoelastin can be used as a substrate to measure LOX activity as the rate of peptidyl lysine oxidation. Two principal assay methods have been described for measuring LOX activity. Both were used here:

1. Tritium vacuum distillation (Bedell-Hogan *et al.* 1993). This method uses tritium-labelled tropoelastin as substrate and measures the amount of tritiated water formed when samples are incubated with the tracer (as LOX activity is a direct measurement of released tritium).
2. Fluorometric LOX activity assay (Palamakumbura & Trackman 2002). This assay utilises 1, 5-diaminopentane as a substrate. The reaction releases hydrogen peroxide, which is detected by N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) in horseradish peroxidase-coupled reactions. Unfortunately, this method proved too insensitive for the present purpose, so all measurements of LOX

activity described in this thesis were carried out by the tritium vacuum distillation method as described in Section 2.8.1.

2.8.1. Lysyl oxidase tritium distillation enzyme assay

2.8.1.1. Tropoelastin biosynthesis

The procedure was adapted from Bedell-Hogan *et al* (1993). An E.*coli* clone for recombinant tropoelastin (pAS-tElastin) was obtained from Dr Joel Rosenbloom (University of Philadelphia, School of Dental Medicine, Philadelphia, PA.). Clones were grown overnight at 37°C on Luria-Bertani (LB) plates supplemented with 50µl/ml ampicillin. A single colony was picked and grown overnight in 100ml LB broth supplemented with 50µl of 100mg/ml ampicillin. 25ml culture was transferred into two 1l Erlenmeyer flasks containing 500ml of fresh LB media supplemented immediately before use with ampicillin. The culture was immediately agitated at 37°C until the OD_{620nm} of the suspension reached 0.3-0.4. This was then centrifuged at 4000xg for 10min at 4°C. Cell pellets were washed three times in lysine-free medium and transferred into a sterile 1l Erlenmeyer flask. 500ml of lysine-free medium was added and the culture was agitated for 10min at 37°C to reach an OD_{620nm} of 0.6. To induce protein expression, 2.0ml fresh nalidixic acid (dissolved in 0.1N NaOH) was added to the culture, and agitated for a further 2h at 37°C. 2 mCi [^{3}H]-(4,5)-lysine was added to the culture and incubated for 3h at 37°C with shaking. Cells were centrifuged at 4000xg and washed twice with PBS. The cell pellet was resuspended in 20ml cold buffer A (50mM Tris pH 8.0, 2mM EDTA, 1mM dithiothreitol containing 15% (v/v) glycerol) and stored overnight at -80°C.

After defrosting the cells on ice, 1/100 volume of fresh phenylmethylsulphonyl fluoride (PMSF) was added to inhibit proteases. The cells were then centrifuged at 4000xg for 10min and resuspended in cold buffer A containing 5% (v/v) glycerol. To break down the polysaccharide cell wall of the bacteria, 200µl of lysozyme was added and incubated on ice for 30min. Cells were centrifuged at 15000xg for 20min and resuspended in 10ml of cold buffer A containing 5% glycerol. 1/100 volume PMSF and sodium deoxycholate (0.05% w/v) were added to further break down cell membranes and cells were

homogenised. The resultant suspension was centrifuged at 15000xg for 30min and resuspended in 6.3ml of 70% (v/v) formic acid. 1g cyanogen bromide (CNBr) was added and stirred overnight under nitrogen at room temperature in a fume hood. CNBr cleaves all peptide bonds at methionine residues. Tropoelastin is one of the few proteins that does not possess this bond and is therefore not cleaved.

To neutralise CNBr, 2ml water was added to the solution. After 4-5h, the homogenate was centrifuged at 15000xg for 15min. Radioactivity of a 10µl sample was measured by β-scintillation counting and the supernatant was dialysed against 4l 0.1% (v/v) acetic acid to remove unwanted solutes. Dialysis solution was replaced every 12h. The dialysed solution was centrifuged and its radioactivity content measured. Finally, the solution was distributed into microfuge tubes (1 x 10^6 cpm/tube), freeze-dried and stored at -80°C.

2.8.1.2. Sample incubation

Culture media were assayed for LOX enzyme activity using the vacuum distillation assay. For each sample, two positive and one negative assay tubes were set up. The positive assay tubes contained 750µl test sample, 650µl borate buffer (0.1M sodium borate, 0.15M NaCl, pH 8.0) and 100,000cpm of [^{3}H]-tropoelastin tracer. The negative control tubes contained 750µl test sample, 450µl borate buffer, 100,000cpm [^{3}H] tropoelastin tracer and 50µM β-aminopropionitrile (BAPN), an irreversible inhibitor of LOX activity. All assay tubes were incubated at 55°C for 4h, and then frozen at -20°C in preparation for distillation.

2.8.1.3. Vacuum distillation

The reaction product, tritiated water, was collected by vacuum distillation in a fume cupboard. A rolled up sheet of Kleenex tissue was inserted into the top of each assay tube (Fig. 2.1 insert) to prevent excessive rapid evaporation. The assay tubes were placed in a heated (70°C) water bath and collection tubes were placed in a cooled (-70°C) dry ice/industrial methylated spirit bath (Fig. 2.1). The two tubes were connected by a glass swan-neck and were connected to a vacuum pump by tubing and valve taps. The distillation apparatus contained a bleed-valve to control the distillation rate.

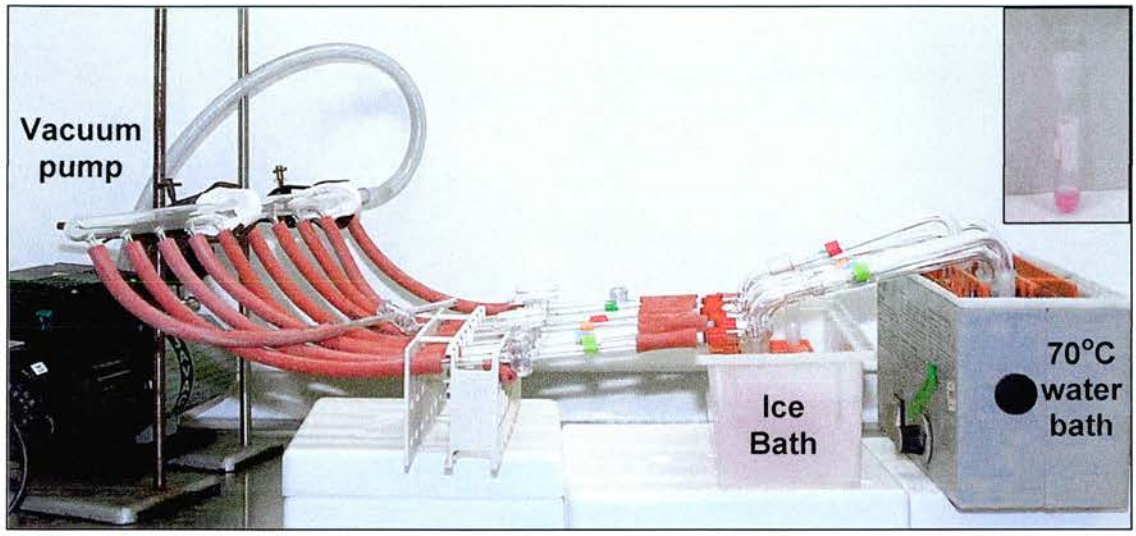

Figure 2.1. Vacuum distillation equipment used for measurement of LOX enzyme activity. When the vacuum pump is switched on samples in the 70°C water bath boil under vacuum and pass along the swan-neck tubes to condense in the ice bath. Insert shows assay tube with assay medium below a Kleenex tissue inserted to prevent rapid evaporation.

When the system was ready for use the vacuum pump was turned on and the bleed-valve shut. This caused the assay samples in the 70°C water bath to boil (the water vapour then transfers along the swan necks and condenses in the tubes immersed in the ice bath). The procedure was run for 10min, whereupon the vacuum pump was switched off and the bleed-valve opened to release the vacuum. The collection tubes were disconnected and 1ml distillate was mixed with scintillation fluid and counted on a Tri-Carb 2100R β-scintillation counter (Packard).

2.9. Histological staining

2.9.1. Tissue processing

Animal tissue from hormone-treated Wistar rats, raw COL4α1-mutant mice, CYP11B1$^{-/-}$ mice and 11βHSD1$^{-/-}$ mice that had been fixed in Bouin's for 6h were submerged in 70% EtOH and processed through a series of EtOH washes before being embedded in paraffin wax blocks. Tissue blocks were sectioned (5μm) using a Microm microtome, and sections floated onto water (50°C) and placed on Superfrost slides. Tissue was heat-fixed to slides overnight at 40°C.

2.9.2. Haematoxylin and Eosin staining

Slides were placed in xylene (2x5min) to remove paraffin wax and then re-hydrated through successive EtOH solutions [100%, 95% (v/v), 70% (v/v), 50% (v/v)]. The nuclei of the cells in the fixed tissue were stained with haematoxylin, and sections were washed in running tap water. The intensity of staining was checked microscopically and if satisfactory was "blued up" in Scott's tap water, followed by a further wash in tap water. The slides were then placed in eosin for 20-30sec to stain cytoplasm and washed in tap water. When the required staining had been achieved, slides were dehydrated quickly through increasing solutions of EtOH concentrations [50%(v/v), 70% (v/v), 95% (v/v), 100%, 100%] followed by xylene (2x5min). Coverslips were mounted using DPX mounting medium and left to dry before being examined and photographed under brightfield microscopy (Provis AX70, Olympus Corp. London, UK, fitted with a Canon DS6031 camera).

2.9.3. Picro-sirius red staining

The Picro-sirius red staining protocol stains collagens. Slides were dewaxed in xylene (2x5min) and rehydrated through decreasing EtOH concentrations [100%, 100%, 95% (v/v), 85%(v/v), 70% (v/v)]. Following a 30min wash in tap water, tissue sections were stained with Picro-sirius red stain (sirius red 0.5g; aqueous picric acid 500ml) for 60min. Two changes of acidified water (5ml glacial acetic acid in 1l water) then followed. Excess water was shaken from the slides and they were dehydrated in 100% EtOH, cleared in xylene and mounted with DPX mounting medium. When viewed under polarised light, stained collagens appeared as bright areas against the dark non-collagenous areas, due to the birefringence of the collagen fibrils (Junqueira *et al.* 1981). The birefringent properties of collagens are due to the parallel orientation of fibrils (Montes 1996). Photographs were taken under brightfield and polarising microscopy (Leitz DMRB, Leica, Milton Keynes, UK, connected to a JVC KY-F55B camera).

2.9.4. Jones methenamine silver staining

The Jones methenamine silver staining protocol stains basement membranes. Following dewaxing in xylene (2x5 min) and rehydration through decreasing EtOH solutions [100%, 100%, 95% (v/v), 85% (v/v), 70% (v/v)], slides were placed in distilled water. The sections were oxidized with 1% periodic acid for 15min, then washed in distilled water. Slides were immersed in methenamine silver working solution (1.25ml 5% silver nitrate; 25ml 3% methenamine; 10ml 5% borax; 2ml distilled H_2O) and heated to 58°C for 30-60min until basement membranes started to turn black. After washing in distilled water, 0.2% gold chloride was used to emphasise intensity of staining (1min). Excess gold chloride was removed by washing in distilled water, followed by fixation for 2min in 3% sodium thiosulphate and further washing with distilled water. A light haematoxylin nuclear counterstain was used before slides were dehydrated through a series of ethanol concentrations [50% (v/v), 70% (v/v), 95% (v/v), 100%, 100%] and mounted with DPX mounting medium. Basement membranes were stained black. Photographs were taken through a brightfield microscope as stated in Section 2.9.2.

2.9.5. Immunohistochemistry

2.9.5.1. Dewaxing and rehydration

Slides were dewaxed in xylene (2x5 min) and rehydrated through decreasing alcohol concentrations [100%, 100%, 95% (v/v), 85% (v/v), 70% (v/v)] followed by a wash in deionised water for 5min. Three washes in 0.01M PBS buffer with agitation for 3min followed.

2.9.5.2 Antigen retrieval

Antigen retrieval allows the exposure of epitopes to allow antibodies to bind, whilst preserving the morphology of the tissue. This was performed by pressure cooking sections in 0.01M Na citrate (pH 6.0) buffer for 5min and leaving the slides to rest for a further 20min. The slides were washed (3x3min) in 0.01M PBS buffer with agitation.

2.9.5.3. Blocking

Endogenous peroxidase blocking prevents unrelated peroxidases from being visualised. An endogenous peroxidase block (3% H_2O_2 in MeOH) was performed for 10min at room temperature. Sections were washed in 0.01M PBS buffer (3x3min) with agitation. An avidin/biotin block was used to prevent non-specific binding. This consisted of incubations with both blocking agents for 15min, each separated by a 2min wash with 0.01M PBS. Serum blocking with non-immunised animal serum prevents binding of unrelated antibody, helping to reduce background staining. A non-immunised block was carried out using a 1 in 5 dilution of goat serum (rabbit serum for the 11βHSD2 antibody) in 0.01M PBS plus 5% BSA (this is referred to as goat serum in the following text) for 60min.

2.9.5.4. Primary antibodies

Primary antibodies diluted in goat serum were added (50µl per slide), covered with parafilm and incubated at 4°C overnight. Table 2.10. shows the primary antibodies and dilutions used. Negative controls were performed with each immunohistochemistry performed; these controls were correctly matched IgG antibodies. Fig. 2.2 shows an example of two negative control slides, all negative controls were negative for staining but not all are shown in the following experimental chapters.

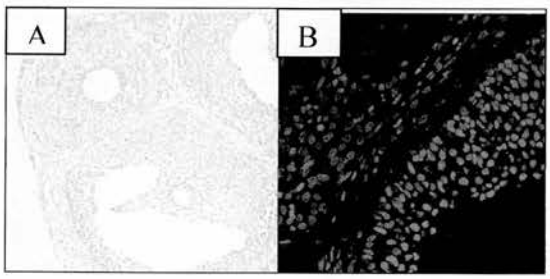

Figure 2.2. Examples of negative controls for DAB (A) and fluorescence (B) immunohistochemistry.

Table 2.10. Primary antibodies used on tissue.

Primary antibody	Dilution	Host species	Secondary antibody	Source
11βHSD1	1:100	Rabbit	Goat anti-rabbit	Cayman
11βHSD2	1:600	Sheep	Rabbit anti-sheep	Gift[1]
BMP-1	1:100	Rabbit	Goat anti-rabbit	Sigma
GR	1:1000	Rabbit	Goat anti-rabbit	ABR
Laminin	1:100	Rabbit	Goat anti-rabbit	Abcam
LOX	1:100	Rabbit	Goat anti-rabbit	Gift[2]
MMP-2	1:200	Rabbit	Goat anti-rabbit	Nova Castra
Mouse COL4α1	1:100	Rat	Rat anti-mouse	Gift[3]
Mouse COL4α2	1:100	Rat	Rat anti-mouse	Gift[3]
Mouse COL4α3	1:100	Rat	Rat anti-mouse	Gift[3]
Mouse COL4α4	1:100	Rat	Rat anti-mouse	Gift[3]
Mouse COL4α5	1:100	Rat	Rat anti-mouse	Gift[3]
Mouse COL4α6	1:100	Rat	Rat anti-mouse	Gift[3]
Nidogen 1	1:500	Rabbit	Goat anti-rabbit	Gift[4]
Nidogen 2	1:500	Rabbit	Goat anti-rabbit	Gift[4]
Perlcan	1:500	Rabbit	Goat anti-rabbit	Gift[4]

Gift[1] a kind gift from Prof. Ian Mason, The University of Edinburgh.

Gift[2] a kind gift from Dr Dawn Kirschmann, University of Iowa

Gift[3] a kind gift from Dr Tom Van Agtmael, The University of Edinburgh (originally sourced from Dr Yoshikazu Sado, Shigei Medical Research Institute, Japan).

Gift[4] a kind gift from Dr Tom Van Agtmael, The University of Edinburgh (originally sourced from Prof. Rupert Timpl, The Max Planck Institute of Biochemistry, Germany).

2.9.5.5. Secondary antibodies

Positive and negative slides were kept separate through all the wash stages. All washes on the second day were performed in PBST (8g NaCl, 100µl Tween-20/litre 0.01M PBS). Tween-20 is a detergent and helps to remove non-specifically bound antibody. Overnight parafilm covers were removed and slides were washed (3x3min). Biotinylated secondary antibodies were diluted 1:500 using goat serum and incubated on slides at room temperature for 60min. The slides were washed (3x3min). The ABC method was used to facilitate visualisation of primary-antibody staining by using the affinity of avidin for biotin attached to the secondary antibody. ABC Elite was prepared by adding 2 drops of solution A and 2 drops of solution B to 5ml 0.01M PBS buffer. ABC Elite was prepared 30min prior to use to allow cross links to form between the two

solutions. ABC Elite was then added to each slide. Tissue sections were covered and incubated at room temperature for 60min. This was followed by 3x3min washes.

2.9.5.6. Antigen detection

Localisation of bound antibodies was determined using 3,3'-Diaminobenzidine tetrahydrochloride (DAB) to visualise biotin peroxidase attached to the primary antibody (ABC complex). One drop of DAB solution was mixed with 2ml of DAB buffer. The bound antibody complex was visualised following 30sec incubation with DAB, and the reaction was stopped by washing with distilled water. Slides were counterstained in haematoxylin and dehydrated through increasing alcohol concentrations [70% (v/v), 85% (v/v), 95% (v/v), 100%, 100%] followed by xylene (2x5min) and mounted with DPX mounting medium. Photographs were taken using brightfield microscopy as stated in Section 2.9.2.

2.9.5.7. Fluorescence visualisation

Preparation for fluorescent visualisation is as detailed above up to the addition of the primary antibody. After these steps positive and negative slides were kept separate through all the wash stages. All washes were performed in PBST for 5min followed by 0.01M PBS for 5min.

Slides were washed and the secondary antibody was diluted (1:200 in goat serum) and incubated at room temperature for 30min. Streptavidin 463 (red) or streptavidin 488 (green), which bind to the antibody complex and fluoresce, was diluted 1:200 in 0.01M PBS and incubated on tissue sections for 1h. Visualisation of the 11βHSD1 antibody was enhanced using a tyramide visualisation system (TSA™-Plus Tiramide Cy3nine 3 system). This kit is used in the place of streptavidin and involves a 10min incubation of tyramide diluted 1:50 in the kit diluent. The slides were then washed and counterstained with TOPRO for 3min or Sytox green for 5min, to visualise cell nuclei. Slides were washed with 0.01M PBS alone and mounted with Permafluor. Slides were stored at 4°C in dark conditions. Staining was visualised using confocal microscopy (Zeiss LSM 510 Meta Axiovert 100M confocal microscope; Carl Zeiss Ltd., Welwyn Garden City, UK).

2.10. Western Blotting

2.10.1. Sample preparation and electrophoresis

Animal tissues were homogenised in a protein lysis buffer supplemented with a protease cocktail, using a Qiagen TissueLyser. Homogenised tissues were mixed with 4µl of 5 x protein loading buffer (50mM Tris-HCl, 2% SDS, 0.1% Bromophenol Blue, 10% Glycerol, 100mM β-mecaptoethanol), denatured by heating to 100°C for 5min, briefly centrifuged and then loaded on to a polyacrylamide gel in a tank containing 1 x running buffer (25mM Tris-base, 250mM Glycine (pH 8.3), 0.1% SDS). A voltage of 100 volts was applied to the gel until the samples had run three-quarters of the way down the gel.

2.10.2. Blotting

The gel was then removed and placed into transfer buffer (192mM glycine, 25mM Tris, 20% MeOH). A transfer cassette was prepared consisting of: a fibre pad soaked in transfer buffer placed against the black side of the cassette, and overlaid with a sheet of filter paper. The gel was then placed onto the filter paper surface. A sheet of Immobilon-P transfer membrane was placed in 100% MeOH for 1min, rinsed in water then in transfer buffer, and then placed over the gel. A second sheet of filter paper and a fibre pad were placed on top of the membrane. The cassette was closed and placed in the transfer gel tank with an ice-cooling unit. Transfer buffer was added to the gel tank and 100 volts were applied for one hour. The membrane was then removed and washed in 1xTBST (10xTBST – 0.5M Tris, 9% NaCl, 0.5% Tween 20, pH 7.4, diluted 1:10 for working concentration).

2.10.3. Blocking and primary antibody incubation

The membrane was placed into a blocking solution (5% milk in 1xTBST) for one hour. The primary antibody was diluted 1:1000 in blocking solution and the membrane was placed in this solution overnight at 4°C.

2.10.4. Secondary antibody incubation

The membrane was washed in 1xTBST (3x5min) at room temperature. The secondary antibody was added to the membrane at a dilution of 1:5000 in 1xTBST and incubated at room temperature for one hour, followed by further washes in 1xTBST (3x5min).

2.10.5. Detection

Chemiluminescence was used to detect antibody binding using ECL Plus™ Western Blotting Detection Reagents. ECL Plus™ was brought to room temperature according to the protocol. Solutions A and B were mixed in a 1:40 ratio and applied to the membrane for 1min. Excess ECL Plus was drained from the membrane which was then wrapped in Saran Wrap™ and exposed to Kodak BioMax XAR film in the darkroom for different exposure times and developed using a Compact x4 automatic developer (Xograph, Imaging System Ltd).

2.11. ^{35}S *in situ* hybridisation

2.11.1. *In situ* probe preparation

All probes were designed previously and inserted into plasmid vectors. Plasmids were amplified using RT-PCR with primers to T3 (AATTAACCCTCACTAAAGGG), T7 (GTAATACGACTCACTATA) or SP6 (AGCTATTTAGGTGACACTATAGA). Table 2.11. shows the plasmids, the inserted sequences and the primers used to amplify these sequences.

Table 2.11. Plasmids into which probe sequences were inserted and primers used to amplify these sequences.

Probe sequence	Plasmid	Primers
LOX	pBluescript SK	T7 (antisense), T3 (sense)
BMP-1	pGEM T easy	T7 (antisense), SP6 (sense)
PCPE	pGEM T easy	T7 (antisense), SP6 (sense)
11βHSD1	pBluescript KS	T3 (antisense), T7 (sense)
11βHSD2	pGEM T	T7 (antisense), SP6 (sense)

11βHSD1 and 11βHSD2 were kind gifts from the Endocrinology Unit, The University of Edinburgh.

Amplification creates a cDNA template for generating the *in situ* labelled probe. cDNA templates were run on low melting-point agarose electrophoresis gels and visualised under UV light. Bands of the correct size were excised with a clean scalpel blade, then purified using a QIAquick gel extraction kit. An aliquot of the extracted DNA bands was run on an agarose electrophoresis gel to confirm recovery.

2.11.2. Radiolabelling of RNA

cDNA template at a concentration of 0.5-1μg/μl was radiolabelled using a transcription reaction containing 1mM ATP, CTP and GPT, 4μl ^{35}S-UTP (1.48 MBq/μl), 10mM dithiothreitol (DTT), 0.4μl RNase inhibitor and 1μl of the appropriate RNA polymerase (T3, T7 or SP6) to a total volume of 10μl in 1 x transcription buffer. After incubation at either 37°C (T3 and T7) or 40°C (SP6) for 90min, 1μl of RNase-free DNase was added and incubated for a further 15min to degrade the template. Probes were then incubated on ice for 5min before purification using NICK columns. Columns were washed with 3ml TE buffer (10mM Tris HCl, 1mM EDTA, pH 8.0) before application of the probe. The column was washed with 400μl TE buffer then the probe was eluted in 400μl TE buffer. Total activity of each probe was determined by scintillation counting using a β-counter. Probes were frozen at -20°C and used within 4 weeks.

2.11.3. Tissue sectioning

Paraffin embedded tissues were cut under RNase-free conditions using a Microm microtome cleaned with RNAzap. Sections were floated on DEPC-H_2O at 50°C, and placed onto RNase-free polysine-coated slides. Slides were placed in baked slide racks and tissue was heat-fixed overnight at 40°C.

2.11.4. De-waxing and fixation

All glassware was baked to remove RNases and DEPC-H_2O was used to make up solutions. Slides were dewaxed (2x10min) in xylene then rehydrated through decreasing EtOH solutions each for 2min (3x100% EtOH, 95% EtOH, 85% EtOH, 70% EtOH, 50% EtOH and 30% EtOH). EtOH was removed by washing in 0.9% NaCl for 5min. Tissue sections were then permeabilised in 0.3% Triton X-100 in 1xPBS for 15min, followed by 2x5min washes in 1xPBS. Proteinase K digestion was performed on paraffin-

embedded tissue to facilitate penetration of the probe. Proteinase K (1μg/ml in 100mM Tris-HCl, pH 8.0; 50mM EDTA) was incubated for 30 min at 37°C. Slides were washed in 0.2% glycine to stop the proteinase K digestion.

Sections were immersed in cold 4% (w/v) paraformaldehyde in 0.1M phosphate buffer (20mM NaH_2PO_4, 80mM $NaHPO_4$) for 10min to fix tissue, then washed (2x5min) in 1xPBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_2). Tissue sections were acetylated with 0.25% (v/v) acetic anhydride in 0.1M triethanolamine to reduce non-specific binding of the probe to positively-charged amino groups in the tissue. Slides were washed in 1xPBS for 5 min then dehydrated through increasing EtOH each for 2min (70% EtOH, 80% EtOH and 95% EtOH). Slides were left to dry, then either pre-hybridised or stored at -20°C for 1-2 days (if stored slides need to be brought to room temperature before prehybridisation).

2.11.5. Pre-hybridisation

Double strength pre-hybridisation buffer was prepared as follows; DEPC-H_20 (5.88ml), 5M NaCl (2.4ml), 1M Tris, pH 7.5 (200μl), 50 X Denhardt's (400μl), 250mM EDTA, pH 8.0 (80μl), 10mg/ml Salmon Sperm DNA (1ml), 50mg/ml Yeast tRNA (40μl). The buffer was mixed well, aliquotted into RNase-free Eppendorf tubes and stored at -20°C.

'Box' buffer was prepared by mixing solutions in the following ratio (v/v): 20% 20 X SSC (3M NaCl, 0.3M trisodium citrate in distilled-H_2O, pH 7.0 and autoclaved), 30% DEPC-H_20, 50% deionised formamide (150ml formamide mixed with 15g mixed bed for 1h at RT, then filtered twice and stored in a light-proof bottle). Whatman 3MM chromatography paper was cut to fit hybridisation boxes. Two pieces of paper were placed in each box and 20ml 'box' buffer was added to create a humid atmosphere.

A pre-hybridisation mix was prepared by diluting 2 x pre-hybridisation buffer 1:1 in molecular-grade deionised formamide. Each slide required even dispersion of 200μl of pre-hybridisation mix and was then placed in the hybridisation box. Slides were incubated at 50°C for 2-3h.

2.11.6. Hybridisation

Double strength hybridisation buffer was prepared as follows; DEPC-H_20 (6.68ml), 5M NaCl (2.4ml), 1M Tris, pH 7.5 (200µl), 50 X Denhardt's (400µl), 250mM EDTA, pH 8.0 (80µl), 10mg/ml Salmon Sperm DNA (200µl), Dextran sulphate (2.0g), 50mg/ml Yeast tRNA (40µl). The buffer was mixed well, aliquoted into RNase-free Eppendorf tubes and stored at -20°C.

The hybridisation step was carried out in a dedicated radioactive area. Probes were removed from the -20°C freezer and allowed to thaw behind a Perspex screen. A hybridisation mix was prepared by first calculating the required volume of mix. Each slide required 200µl of hybridisation mix and an antisense and a sense mix was prepared for each probe. For each hybridisation mix, 10 million counts were required per ml. This was calculated from the cpm measured when the probes were generated. For each ml of hybridisation mix, 500µl molecular grade deionised formamide was added, plus the calculated amount of probe. The remaining volume was made up with 2 x hybridisation buffer. The hybridisation mix was vortexed and incubated at 75°C for 10min to denature the probe. The hybridisation mix was cooled on ice, then 10µl/ml 1M dithiothreitol (DTT) was added to reduce cross-linking of sulphur residues.

The prehybridisation mix was drained off the slides and excess solution was carefully wiped off with a lens tissue. The hybridisation mix was then added to the slides, before being returned to the hybridisation box and incubated at 50°C for 16h.

2.11.7. RNase treatments and washes

At this stage equipment no longer needs to be RNase-free, and distilled-H_20 can be used. Hybridisation mix was drained from the slides before placing the slides into racks. Slides were washed (3x5min) in 2xSSC, and then treated with RNase to remove any probe that had not hybridised. RNase 'box' buffer was prepared as follows for each box: 5M NaCl (1ml), 1M Tris (100µl), 250mM EDTA (40µl) made up to 10ml with distilled-H_20. The 'box' buffer was added to Whatman 3MM chromatography paper cut to fit the RNase boxes. 200µl of the same buffer was added to each slide, then RNase (30µg/ml) was added (at 3µl/ml) to the RNase buffer. 2xSSC was drained from the

slides and dried using lens tissue. Finally, the RNase mix was added and slides were incubated at 37°C for 1h.

Slides were then subjected to three stringent washes (2xSSC at room temperature for 1h, 0.1xSSC at 60°C for 1h followed by 0.1xSSC at 60°C). The latter solution was removed from the heat source when slides were placed into it allowing the temperature to fall for 1h. Slides were then dehydrated through 50% (v/v) EtOH, 70% (v/v) EtOH and 90% (v/v) EtOH (all containing 0.3M ammonium acetate), and allowed to dry overnight in a fumehood.

2.11.8. Autoradiography

After the slides had dried the signal was visualised by exposure to Kodak BioMax MR film for 1-7 days. Slides were placed into an autoradiography cassette in darkroom conditions. Kodak BioMax MR film was positioned (matt side towards the slides) and the cassette was closed. Films were developed using a Compact x4 automatic developer (Xograph, Imaging System Ltd).

2.11.9. Nuclear emulsion

Nuclear emulsion was used to visualise radioactive grains where the target mRNA had bound. Ilford G.5 emulsion was diluted 1:1 with distilled water and heated to 42°C. Slides were dipped into the emulsion, and then left to dry overnight. Dry slides were stored in light-proof boxes at 4°C for varying lengths of time (1-7 weeks) before being developed.

2.11.10. Developing

All solutions were cooled on ice to 15°C and the developing protocol was conducted in darkroom conditions. Slides were taken through the following solutions: Kodak D-19 developer (diluted 1:2 with tap water) for 4min, tap water (10sec), Kodak GBH fixer (diluted 1:5 with tap water) for 10min, followed by a final 5min wash in tap water. Slides were allowed to air dry for 1-2h.

2.11.11. Staining and mounting

Slides were counterstained with haematoxylin (10sec), washed in tap water, and followed by 20sec in Scott's tap water to "blue up" the haematoxylin stain. After a further wash in tap water, slides were dehydrated through a series of alcohols, and placed in Xylene (2x5min). Coverslips were mounted with DPX mounting medium. Photographs were taken under darkfield microscopy (Provis AX70; Olympus Corp. London, UK, fitted with a Canon DS6031 camera).

3. Spatiotemporal expression of collagen pathway biology genes and glucocorticoid signalling components in the rat ovary: isolated cell studies.

3.1. Introduction

The ovarian ECM is subject to remodelling and injury during each oestrous cycle as follicles grow and approach ovulation. The inflammatory mechanisms underpinning tissue degradation leading to ovulation are well characterised and known to involve proteases such as MMPs and their inhibitors (TIMPs) (Curry & Osteen 2003; Ny *et al.* 2002). However, the mechanisms involved in ECM reorganisation/remodelling during follicular development have not been extensively investigated. One such mechanism is likely to involve processing and cross-linking of collagen. Important collagen biology pathway genes are BMP-1, LOX and PCPE. BMP-1 cleaves the C-terminal of procollagen to create active collagen; a prerequisite for collagen incorporation into the ECM. BMP-1 also cleaves pro-LOX to form active LOX and it is this latter enzyme that leads to the covalent cross-linking of collagen that gives it its tensile strength (Panchenko *et al.* 1996; Smith-Mungo & Kagan 1998). PCPE is important for the action of BMP-1 on procollagen as it enhances the cleavage of the collagen precursor (Hulmes *et al.* 1997). LOX has previously been shown to be present in the ovary of the mouse (Hayashi *et al.* 2004), rat (Slee *et al.* 2001), rabbit (Himeno 1986), perch (Langenau *et al.* 1999) and human (Yong *et al.* 2002). Slee *et al* (2001) have shown that LOX expression dramatically decreases in rat granulosa cells as the ovarian follicle matures. Within the ovary LOX has only been studied in granulosa cells and OSE. It is not yet known whether this gene is expressed in theca cells. BMP-1 and PCPE have not been studied to date in the ovary. However, their presence would be predicted as collagen processing occurs in this tissue. Also since LOX is known to be active in the ovary (Harlow *et al.* 2003) BMP-1 is an obvious candidate for causing this activation.

Glucocorticoids are potential regulators of collagen pathway biology components in the ovary because of their involvement in anti-inflammatory signalling. The ovary does not actively synthesise glucocorticoids *de novo*, due to absent or immeasurably low levels of

CYP21 and CYP11B1 expression (Omura & Morohashi 1995). However, pre-receptor regulation of glucocorticoid action presumably occurs due to differential expression of 11βHSD isoforms that interconvert active and inactive circulating adrenal glucocorticoids (Mercer & Krozowski 1992). 11βHSD1, the enzyme that activates glucocorticoids (Section 1.2.1.2) increases as the follicle matures being most strongly expressed at ovulation where it is proposed to be instrumental in the local regulation of corticosterone that contributes to resolution of ovulation-associated inflammation (Tetsuka *et al.* 1999a; Tetsuka *et al.* 1999b). Conversely, 11βHSD2 is responsible for inactivating glucocorticoids and decreases with follicle maturation (Tetsuka *et al.* 1999b). Since ovarian GR seems to be expressed constitutively throughout follicular maturation (Tetsuka *et al.* 1999b), the differential expression of 11βHSD1 and 11βHSD2 is likely to be the major determinant of glucocorticoid signalling.

Evidence for the importance of glucocorticoid signalling in ovarian tissue remodelling includes that the MMP and urokinase PA degradation pathways in granulosa cells are suppressed by the synthetic glucocorticoid dexamethasone (Canipari *et al.* 1987; Harlow *et al.* 1987; Pross *et al.* 2002). Additionally, dexamethasone has been shown to enhance LOX expression in fetal lung cell cultures (Chinoy *et al.* 2000). Taken together this evidence suggests that different aspects of tissue remodelling can be negatively regulated by glucocorticoids. Therefore if both collagen pathway genes and glucocorticoid regulatory genes are expressed in the ovary it is possible that glucocorticoid signalling during follicular development could influence collagen pathway genes.

This chapter aims to determine whether individual collagen pathway components are expressed in particular ovarian cell types and whether their pattern of expression changes as follicles mature. It also aims to confirm the presence of integral modulators of the glucocorticoid signal.

3.2. Materials and methods

3.2.1. Animals

Female rats treated with hormones to stimulate different stages of follicle development as described in Section 2.1.1.

3.2.2. Cell isolation

Granulosa and theca cells were freshly isolated from rat ovaries as described in Sections 2.2.1 and 2.3.1.

3.2.3. RT-PCR

Qualitative RT-PCR was performed as described in Section 2.6, using the primers shown in Table 3.1. that were designed specifically for rat tissue.

Table 3.1. Primer sequence and annealing temperature used for RT-PCR

Gene	Forward primer sequence	Reverse primer sequence	Annealing temperature
LOX	CACTGGCAGTCTATGTCTGC	TACTTCCAGTACGGTCTCC	55°C
BMP-1	AGAGGTAGATGAGTGTTCAAGG	GTCAGACTTGAACTCCACACGC	61°C
PCPE	GCATCTGGACAATAACGGTGCC	CAGTATGTGTCAGGCTCC	55°C
GR	CTCTGCCTGGTGTGCTCCGATGAA	CCTTAGGAACTGAGGAGAGAAGCA	65°C
11βHSD1	GTCCTGGTGCTCTGCCTGGGT	ATGAAGCCGAGGACACAGAGA	65°C
11βHSD2	GATGGACCTGACCAAGCCAGAGGATA	CACATTAGTTCACTGCCTCTGTCTT	65°C
Actin	CATTGAACACGGCATTGTCACC	GGATGCCACAGGATTCCATACC	62°C

3.2.4. qRT-PCR

qRT-PCR was performed as described in Section 2.7 using custom designed primer/probes for LOX, BMP-1 and PCPE rat mRNAs. Custom made primer/probe sets for 11βHSD1 and 11βHSD2 rat mRNAs were also tested but proved ineffective. Rat MMP-2 mRNA was quantified using an Assay-On-Demand™ assay (#Rn01538172_g1, Applied Biosystems). Quantification of rat MMP-9 using the corresponding Assay-On-Demand™ (#Rn00675894_g1, Applied Biosystems) was also attempted but failed due to technical problems.

3.2.5. Statistical Analysis

Statistical analysis was performed on qRT-PCR data using ANOVA with Tukey post-hoc testing or Student's paired t-test, depending on number of treatments. Statistical difference was assigned at $P<0.05$. For each experimental set of data treatment groups

are compared to the untreated control group which was defined as a value of 1 and therefore is shown graphically without variation about the mean. However statistical analysis was performed on raw data (CT values) and this does account for variation around the mean.

3.3. Results

3.3.1. Expression of collagen pathway genes in relation to follicular maturation.

Qualitative RT-PCR revealed that mRNA encoding the collagen pathway genes LOX, BMP-1 and PCPE were all expressed in granulosa and theca cells irrespective of stage of follicular maturation (Fig. 3.1).

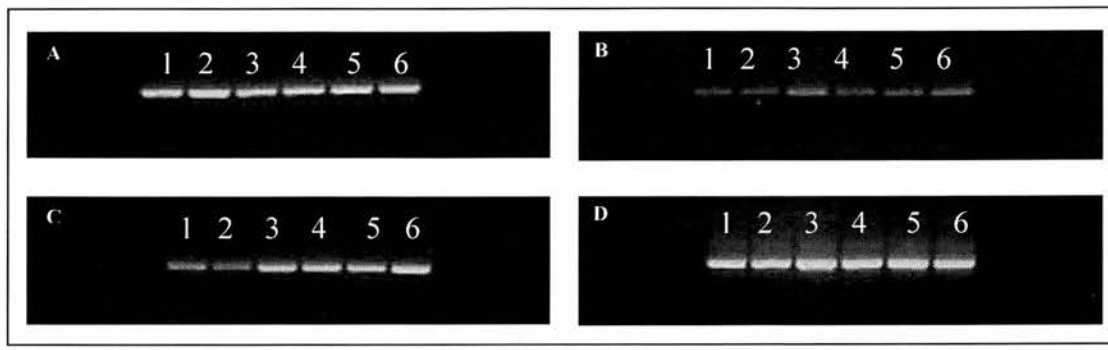

Figure 3.1. Expression of collagen pathway genes during follicular maturation. RT-PCR was performed as described in Section 2.6 on freshly isolated granulosa and theca cells, from 21-day old female rats treated *in vivo* with or without gonadotrophins. Electrophoresis was used to separate and visualise PCR products to show qualitative gene expression of the collagen pathway genes: A, LOX; B, BMP-1; C, PCPE; D, β-Actin control. Loading for each gel is as follows: lane 1, untreated granulosa cells; lane 2, untreated theca cells; lane 3, PMSG granulosa cells; lane 4, PMSG theca cells; lane 5, PMSG/hCG granulosa cells; lane 6, PMSG/hCG theca cells.

3.3.2. Expression of glucocorticoid signalling genes in relation to follicular maturation.

Qualitative RT-PCR showed the presence of mRNAs encoding gene products necessary for the production and activation of glucocorticoid metabolism and action throughout follicular development (Fig. 3.2) i.e. both isoforms of 11βHSD and GR.

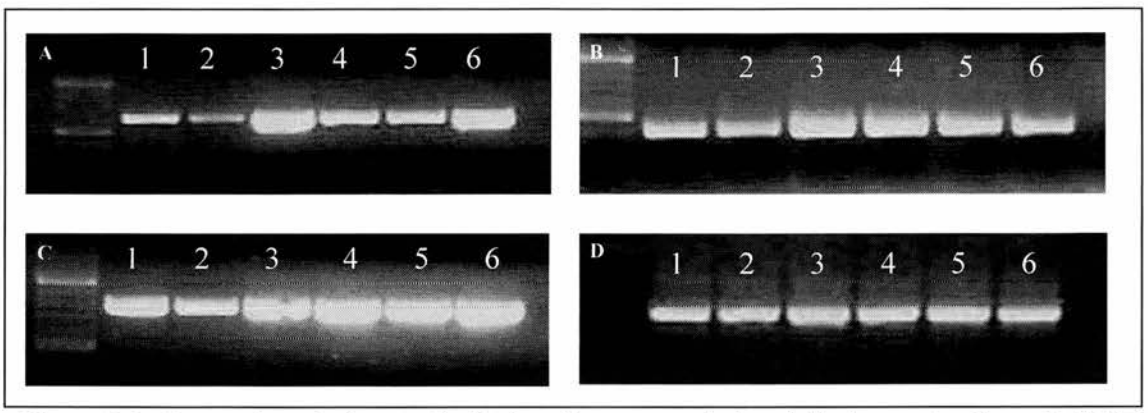

Figure 3.2. Expression of glucocorticoid signalling genes during follicular maturation. RT-PCR was performed as described in Section 2.6 on freshly isolated granulosa and theca cells, from 21-day old female rats treated *in vivo* with or without gonadotrophins. Electrophoresis was used to separate and visualise PCR products to show qualitative gene expression PCR gels showing qualitative gene expression of the mechanisms that regulate the anti-inflammatory glucocorticoids: A, 11βHSD1; B, 11βHSD2; C, GR; D, β-Actin control. Loading for each gel is as follows: lane 1, untreated granulosa cells; lane 2, PMSG granulosa cells; lane 3, PMSG/hCG granulosa cells; lane 4, untreated theca cells; lane 5, PMSG theca cells; lane 6, PMSG/hCG theca cells.

3.3.3. Quantitative assessment of collagen pathway genes in freshly isolated rat granulosa cells in relation to follicle development.

Freshly isolated granulosa cells from follicles at various stages of development were used for quantitative assessment of target mRNAs for LOX, BMP-1, PCPE and MMP-2. The mRNA expression of these genes is shown in Figure 3.3. LOX mRNA progressively declined with increasing follicular maturity. Conversely, MMP-2 mRNA significantly increased during follicle maturation. BMP-1 and PCPE mRNAs did not alter significantly as maturation progressed, although there was a trend for expression to increase during maturation.

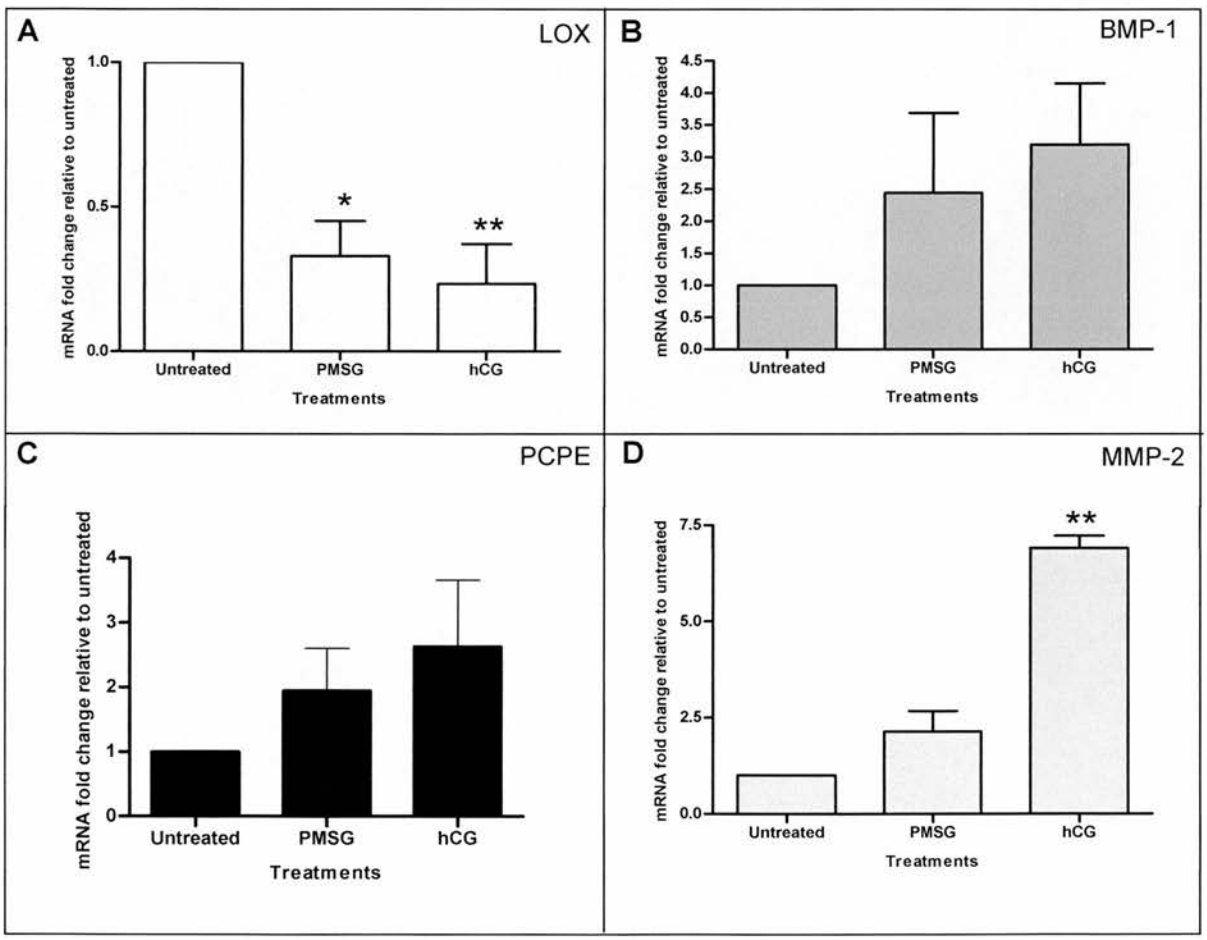

Figure 3.3. Quantitative measurement of collagen pathway gene expression in freshly isolated granulosa cells from 21-day old female rats treated *in vivo* with and without gonadotrophins. qRT-PCR, as described in Section 2.7, was used to measure mRNA expression of LOX (A), BMP-1 (B), PCPE (C) and MMP-2 (D). * = $P<0.05$, ** = $P<0.01$ (compared to untreated).

3.3.4. Quantitative expression of collagen pathway genes in freshly isolated rat theca cells in relation to follicle development.

The levels of LOX, BMP-1, PCPE and MMP-2 mRNAs in freshly isolated rat theca cells were determined by qRT-PCR (Fig. 3.4). No significant effects of gonadotrophin treatment were observed for BMP-1 or PCPE expression, although trends similar to those observed for granulosa cells were detected. LOX mRNA decreased and MMP-2 expression increased with preovulatory follicle maturation, both significantly so in response to PMSG/hCG.

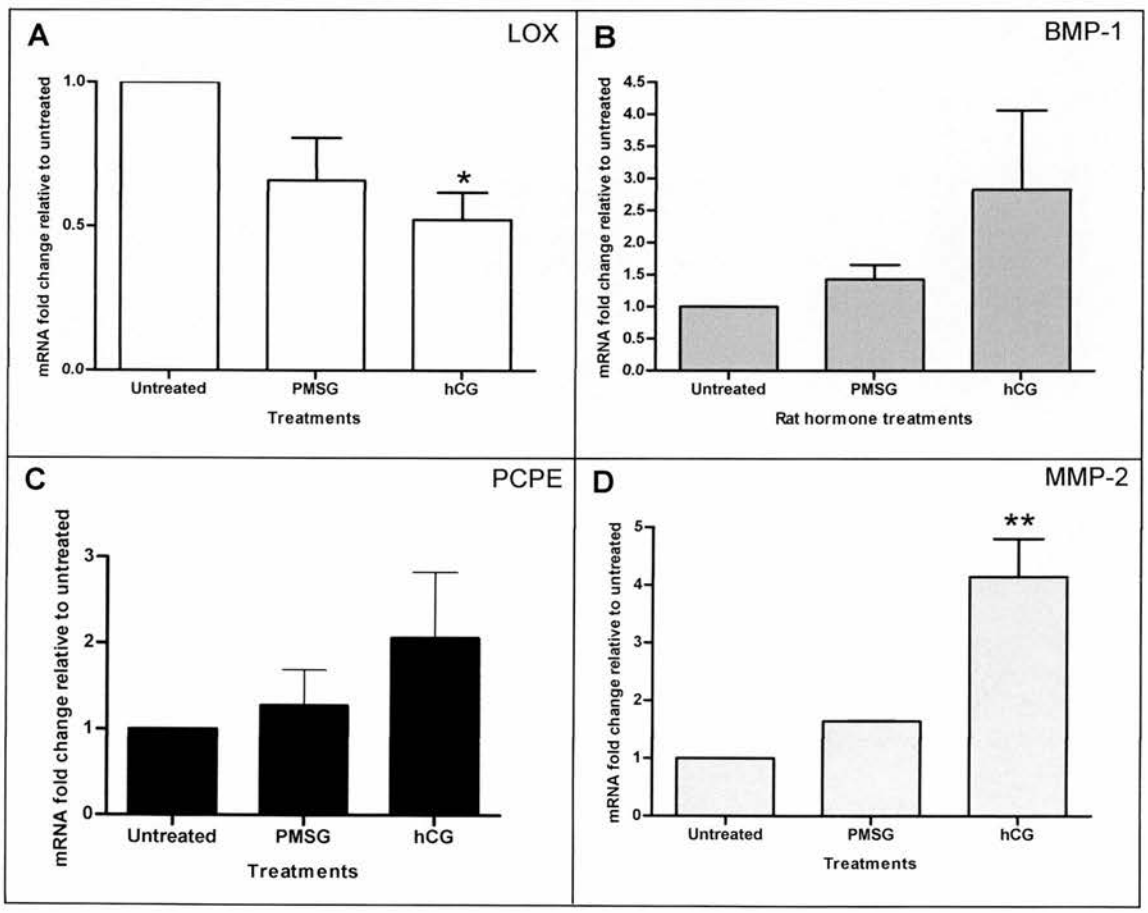

Figure 3.4. Quantitative measurement of collagen pathway gene expression in freshly isolated theca cells from 21-day old female rats treated *in vivo* with or without gonadotrophins. qRT-PCR, as described in Section 2.7, was used to measure mRNA expression of LOX (A), BMP-1 (B), PCPE (C) and MMP-2 (D). * = $P<0.05$, ** = $P<0.01$.

3.3.5. Granulosa versus theca expression of collagen pathway genes.

Total mRNA from freshly isolated granulosa and theca cells was compared with respect to quantitative gene expression of LOX, BMP-1, PCPE and MMP-2 (Fig. 3.5). There was a significant difference between the expression of LOX mRNA in these two cell types, with the greater expression in granulosa cells. No significant differences were observed between the two cell types for BMP-1 or PCPE mRNAs. MMP-2 mRNA was significantly higher in theca cells than in granulosa cells at all stages of follicle development.

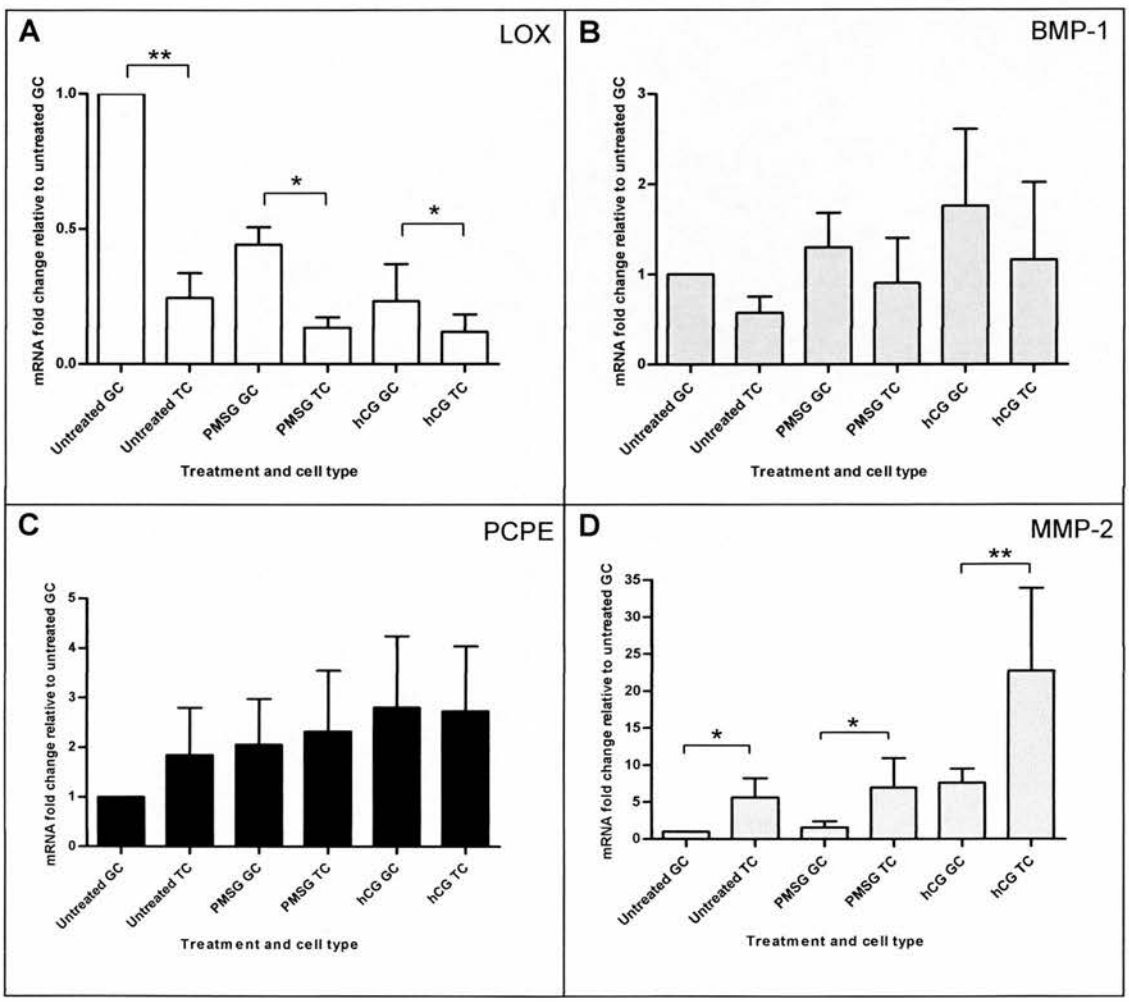

Figure 3.5. Comparison of collagen pathway mRNA from granulosa and theca cells freshly isolated from gonadotrophin treated or untreated 21-day old female rats. LOX, BMP-1, PCPE and MMP-2 mRNA levels were compared using qRT-PCR as described in Section 2.7. Granulosa cells (GC), theca cells (TC). * = P<0.05, ** = P<0.01.

3.4. Discussion

These data show that genes involved in collagen deposition and remodelling are present in the rat ovary, along with those genes required for glucocorticoid action and pre-receptor glucocorticoid metabolism. Furthermore, these genes are regulated as follicles mature and are expressed at different levels in granulosa and theca cells.

Most notably, LOX expression decreased as the follicle matured. The granulosa cell located LOX decrease has previously been shown (Slee *et al.* 2001) but the LOX data for theca cells are novel. Moreover, the results show that the LOX mRNA content of theca cells is significantly lower than that of granulosa cells. This suggests that the main follicular site of LOX mRNA expression is granulosa cells. LOX expression in theca cell preparations could be due to granulosa cell contamination as the cell population studied was essentially an enriched thecal cell population unavoidably contaminated with granulosa cells.

Since the granulosa cell layer does not contain collagen the question arises as to why LOX is expressed there. Presumably LOX that is involved in collagen cross-linking may move to the basement membrane separating the granulosa and theca cells where it could be instrumental in cross-linking collagen IV. However, it should be noted that it has not been conclusively demonstrated that LOX causes cross-linking in the collagen IV network, as discussed in Section 1.3.3.1.2.2. Interestingly, OSE cells also synthesise and secrete large amounts of LOX (Rae *et al* 2007 in preparation). OSE cells also sit on a basement membrane, further suggesting that LOX may have a role in cross-linking of collagen IV. Collagens I and III are present in the stromal matrix (Palotie *et al.* 1984), and LOX may cross the basement membrane to cross-link these collagens too.

Increased MMP-2, which is known to hydrolyse collagen substrate and basement membrane components (Johnson *et al.* 1998), was observed in granulosa cells as follicles matured. Thus the pattern of MMP-2 expression was inverse to that of LOX. This might be expected given the opposing functions of these two remodelling enzymes. Overall the data suggest that LOX is likely to be instrumental in matrix remodelling as follicles mature. Thus as follicles grow, LOX action could allow for the expansion and remodelling of the basement membrane and stromal collagens. As ovulation

approaches, the follicle ceases to grow and the surrounding tissues must be broken down to allow ovulation to occur. Hence the expression of MMPs such as MMP-1, -2, -9 and -13 (Curry & Osteen 2003) increases, leading to degradation of the follicle wall to allow oocyte release. The expression of these genes must therefore be tightly regulated to sustain an environment conducive to development of the oocyte (within the growing follicle) and its release at the appropriate time (ovulation). *In vivo* gonadotrophin treatment in rats has shown that MMP-2 mRNA and activity were up-regulated as ovulation approached (Curry *et al.* 2001). Therefore, there could be a direct influence of gonadotrophins on MMP-2 expression and/or an indirect (autocrine or paracrine) effect following cell stimulation by gonadotrophin. It is possible that the positive regulator of MMP-2 may have an opposing effect on LOX. These questions are yet to be answered.

The data shown here confirm the prediction that BMP-1 and PCPE are present in both granulosa and theca cells. Unlike LOX there is no significant regulation of these two genes as follicles mature, suggesting that gonadotrophins may not regulate BMP-1 and PCPE with increasing maturation. However, there was an increasing trend in the expression of BMP-1 and PCPE. This could result in the synthesis of collagen necessary for cross-linkage after ovulation, a time when LOX is up-regulated as shown in rabbit and perch ovaries (Himeno 1986; Langenau *et al.* 1999). Fibrillar collagens are mainly seen in corpus luteum (Luck & Zhao 1993). Thus collagen types I and III are both increased in luteinised granulosa cells and are expressed in the early corpus luteum of the cow and mouse (Oksjoki *et al.* 1999; Zhao & Luck 1995; Zhao & Luck 1996). This would create a depot of substrate for LOX to cross-link during the development of the corpus luteum, and during its subsequent remodelling throughout gestation if pregnancy occurs.

When BMP-1 and PCPE expression were compared in granulosa and theca cells, no differences were observed in relation to follicular maturation. This could be due to cross-contamination of the cell populations by each other. More likely, these genes are expressed in both cell types. As the thecal cells appear to express both BMP-1 and PCPE, this would suggest that the area in which they are found outside the basement membrane is rich in collagen. Their expression by granulosa cells may help boost that

of the theca cells to cope with the high demand needed at times of intense tissue reorganisation. This implies that, similar to LOX, both BMP-1 and PCPE have to cross the basement membrane from the granulosa cell compartment to process procollagen. This is plausible, as the molecular weight cut-off for the basement membrane is estimated to be 100-850 kDa (Andersen *et al.* 1976) and the largest of these proteins is BMP-1 which has an Mr of 70 kDa. Collagen I is expressed in the theca layer of the follicle, but some collagen III is produced in the granulosa cell layer (Zhao & Luck 1995) suggesting that some procollagen processing may occur in the granulosa cell compartment. Mature collagen could then pass across the basement membrane where it could be incorporated into stromal ECM.

MMP-2 is a gelatinase that breaks down collagen. This enzyme has been reported previously to be primarily expressed in theca/interstitial cells. The results of the comparison of mRNA expression in this investigation confirm that there is a higher level of expression in the theca cell population. This implies that MMP-2 may have an important role in the degradation of collagens surrounding the follicle, especially at ovulation when it was significantly up-regulated. Interestingly, compartmentalisation of MMP-2 and LOX mRNA places them either side of the follicular basement membrane where they exist in a distinctly inverse relationship. Perhaps paracrine interactions between granulosa and theca cells control the expression of these genes, or possibly unknown regulators of these genes act on both cell types. As yet, these questions cannot be answered.

Proteolytic elements of tissue remodelling have been shown to be regulated by glucocorticoids. In the ovary, it has been shown that the urokinase-PA system, (which converts plasminogen to plasmin triggering a proteolytic cascade), is suppressed by glucocorticoids (Canipari *et al.* 1987; Harlow *et al.* 1987; Ny *et al.* 2002). Potential regulation of ovarian collagen pathway genes is via the (in)activation of glucocorticoids by 11βHSD enzymes, the presence of which, along with GR, were confirmed here at the mRNA level. Although technical problems prevented quantitation of 11βHSD mRNA levels during this work, reference data published elsewhere are informative (Tetsuka *et al.* 1999a; Tetsuka *et al.* 1999b; Tetsuka *et al.* 1997). Thus the preovulatory rise in

11βHSD1 and corresponding fall in 11βHSD2 activities cause a net increase in the intra-follicular availability of active glucocorticoid (Harlow *et al.* 1997; Tetsuka *et al.* 1997). At the same time LOX gene expression is depressed. So the potentially increased availability of active glucocorticoid could suppress collagen remodelling. However, whether the increase in 11βHSD1 (i.e. increased glucocorticoid availability) and decrease in LOX are causally related remains to be determined.

In summary, collagen pathway genes and glucocorticoid regulatory genes are expressed and regulated as the rat ovarian follicle matures. Therefore, there is potential for glucocorticoid regulation of these collagen pathway genes. Whether and how such regulation might occur is further explored in the following chapters.

4. Spatiotemporal expression of collagen pathway genes and glucocorticoid signalling components in the rat ovary: histological studies.

4.1. Introduction

In the previous chapter the expression of collagen pathway genes and glucocorticoid signalling regulators in freshly isolated rat ovarian cell types were explored and it was concluded that expression changed during follicular development. Once expressed, genes are then translated into protein, which then communicates, via signalling, to alter the function of target cells. Therefore, it is important to assess the localisation of these proteins as well as gene expression within these tissue compartments. This will give valuable information on the sites of protein action and can shed light on relationships between sites of synthesis and action in the different cellular compartments. The classic ovarian example of this is the two-cell, two-gonadotrophin model of oestrogen synthesis described in Section 1.1.1.2.

As discussed in Sections 1.1 and 1.3.3, granulosa and theca cells are separated by a basement membrane. This basement membrane has been shown to contain collagen IV, laminin, nidogen and perlecan proteins (McArthur *et al.* 2000; Rodgers *et al.* 1998; van Wezel *et al.* 1998). Focimatrix, a novel ECM, is located between granulosa cells of antral follicles (Irving Rodgers *et al.* 2004). Much of the work on the follicular basement membrane has been conducted on bovine tissue. Therefore in some instances, cross-species conclusions as to the nature of the basement membranes have been drawn. Collagen pathway biology is important in the follicle, as LOX cross-links collagen and BMP-1 processes the ECM components probiglycan, the γ2 and α3 laminin chains and chordin, as well as pro-lysyl oxidase. Outside the follicle, BMP-1 is essential in processing fibril procollagen, a process enhanced by PCPE, and the resultant collagen fibrils are stabilised by LOX.

Protein localisation studies for BMP-1 and PCPE expression have not been conducted in ovarian tissue. Investigations in rat bone types have revealed that BMP-1 protein is present in the chondrocytes of growth plates as well as in osteoblasts and their progenitor fibroblasts of the metaphyseal, metaphysic and epiphysis regions (Anderson *et al.* 2000). These are areas of intense ECM remodelling during development, where BMP-1 would be needed to cleave procollagens and pro-LOX, suggesting that BMP-1 is required in areas of concentrated remodelling. BMP-1 and PCPE mRNA have been localised in identical areas of mouse embryos using *in situ* hybridisation (Scott *et al.* 1999). This would be expected, as PCPE augments BMP-1 action on procollagens. Therefore, given that growth of ovarian follicles is a period of intense remodelling, it is likely that BMP-1 and PCPE proteins will be co-localised in follicles.

Studies of LOX protein expression in different mouse tissues show this protein is found in most, if not all, regions of the body (Hayashi *et al.* 2004), as would be predicted, given the role of LOX. LOX and LOXL proteins have been localised to the granulosa cells of primary follicles of the mouse ovary (Hayashi *et al.* 2004). However, detailed studies of LOX protein distribution have not been conducted during follicle growth. *In situ* hybridisation showed LOX was confined to the granulosa cell layer of follicles of the rat ovary, suggesting that LOX processing of the ECM stems from this cell layer (Slee *et al.* 2001).

If collagen pathway components are regulated by glucocorticoids, the location of the glucocorticoid signal regulators in the ovary will determine whether glucocorticoids could affect expression of these genes. As the ovary does not produce glucocorticoids *de novo* it is important to look at glucocorticoid signal regulators (11βHSD1, 11βHSD2 and GR). Studies of localisation of these regulators in the ovary are limited. Ricketts *et al* (1997) showed that 11βHSD1 protein was present in human liver, adrenal, ovary, decidua and adipose tissue. 11βHSD1 protein was observed in the oocyte and luteinised granulosa cells of the corpus luteum of human ovarian tissue (Ricketts *et al.* 1997), reflecting the expression of 11βHSD1 following ovulation (Tetsuka *et al.* 1997). 11βHSD2 protein localisation in the ovary has not been studied, but as gene expression of this protein decreases as follicles mature it may be predicted that the protein will

mirror this pattern (Tetsuka *et al.* 1997). Previous *in situ* hybridisation analysis of rat ovaries has shown 11βHSD2 to be expressed in late luteal stages, indicating that this tissue autoregulates glucocorticoid availability (Roland & Funder 1996). 11βHSD isoforms regulate the availability of active glucocorticoid, which then signals via GR. GR protein has not been localised in the ovary. Immunolocalisation of GR protein expression in human uterus is unchanged across the menstrual cycle, in line with mRNA expression in this tissue (McDonald *et al.* 2006). Hence it would be predicted that GR protein will be present throughout follicle development in the rat ovary, mirroring the expression pattern of GR mRNA (Tetsuka *et al.* 1999b).

This chapter aims to establish the spatiotemporal pattern of collagen pathway and glucocorticoid signalling genes at mRNA and protein levels during rat follicular development as the basis for mechanistic studies that will be described in Chapter 5.

4.2 Materials and methods

4.2.1 Animals

Immature female rats were given *in vivo* hormone treatments to stimulate different stages of follicular development as described in Section 2.1.1.

4.2.2 Tissue

Whole ovaries were fixed as described in Section 2.9.1.

4.2.3 Histological staining

The following histological stains were performed on the tissue: haematoxylin and eosin as described in Section 2.9.2, Jones' methenamine silver basement membrane stain as described in Section 2.9.4, and the picro-sirius red collagen stain as described in Section 2.9.3.

4.2.4 Western blotting

Ovarian homogenates were blotted for LOX and BMP-1 as described in Section 2.10, to verify that these antibodies identified proteins of the correct size. PCPE was not studied, as an antibody for this protein was not available. Other antibodies were verified previously, either by other workers in the laboratory or by original sources.

4.2.5 Immunohistochemistry

Immunohistochemistry was performed on tissue sections as described in Section 2.9.5. Table 4.1. shows the antibodies used, the type of detection method and the concentrations at which they were applied to the tissue sections.

Table 4.1. Antibodies used, their dilutions and method of detection used for visualisation.

Antibody	Dilutions	Detection method
BMP-1	1:100	Fluorescence
Collagen type IV	1:25	DAB and fluorescence
Laminin	1:100	DAB
LOX	1:100	Fluorescence
MMP-2	1:400	DAB
11βHSD1	1:100	Fluorescence
11βHSD2	1:600	Fluorescence

4.2.6 *In situ* hybridisation

In situ hybridisation was performed for BMP-1, LOX, PCPE, 11βHSD1 and 11βHSD2 as described in Section 2.11. *In situ* hybridisation for 11βHSD1 was unsuccessful.

4.3 Results

4.3.1. Morphology of rat ovary following gonadotrophin treatment *in vivo*.

The oestrous cycle is associated with follicular maturation and is driven by gonadotrophins. Figure 4.1 shows the morphology of ovaries from pre-pubertal rats that have not been exposed to exogenous gonadotrophins. Follicles are situated within the cortical interstitial tissue (Fig. 4.1A). Such follicles are recruited from primordial cohorts, members of which have a single layer of flattened granulosa cells and no distinguishable theca cell layer (Fig. 4.1B insert): through primary preantral follicles (Fig. 4.1B) with one layer of granulosa cells, to secondary preantral follicles (Fig. 4.1B) with multiple granulosa cell layers. In preantral follicles, the oocyte is in close contact with the granulosa cells, allowing easy signalling between these cells (Fig. 4.1C). As follicle growth progresses a cavity known as the antrum forms and the oocyte is surrounded by cumulus cells to form the cumulus oocyte complex (Fig. 4.1D). Within the cohort of follicles, which will be at different stages of development, larger follicles begin to emerge (Fig. 4.1E). These are preovulatory follicles, which have multiple layers of granulosa cells and are surrounded by several layers of theca cells (Fig. 4.1E). The increase in follicle size is due to increased FSH stimulation, which was simulated by giving PMSG *in vivo*. The final stage of follicle development is caused by a surge of LH (mimicked by administration of hCG *in vivo*) to produce follicles on the verge of ovulation (Fig. 4.1G/H).

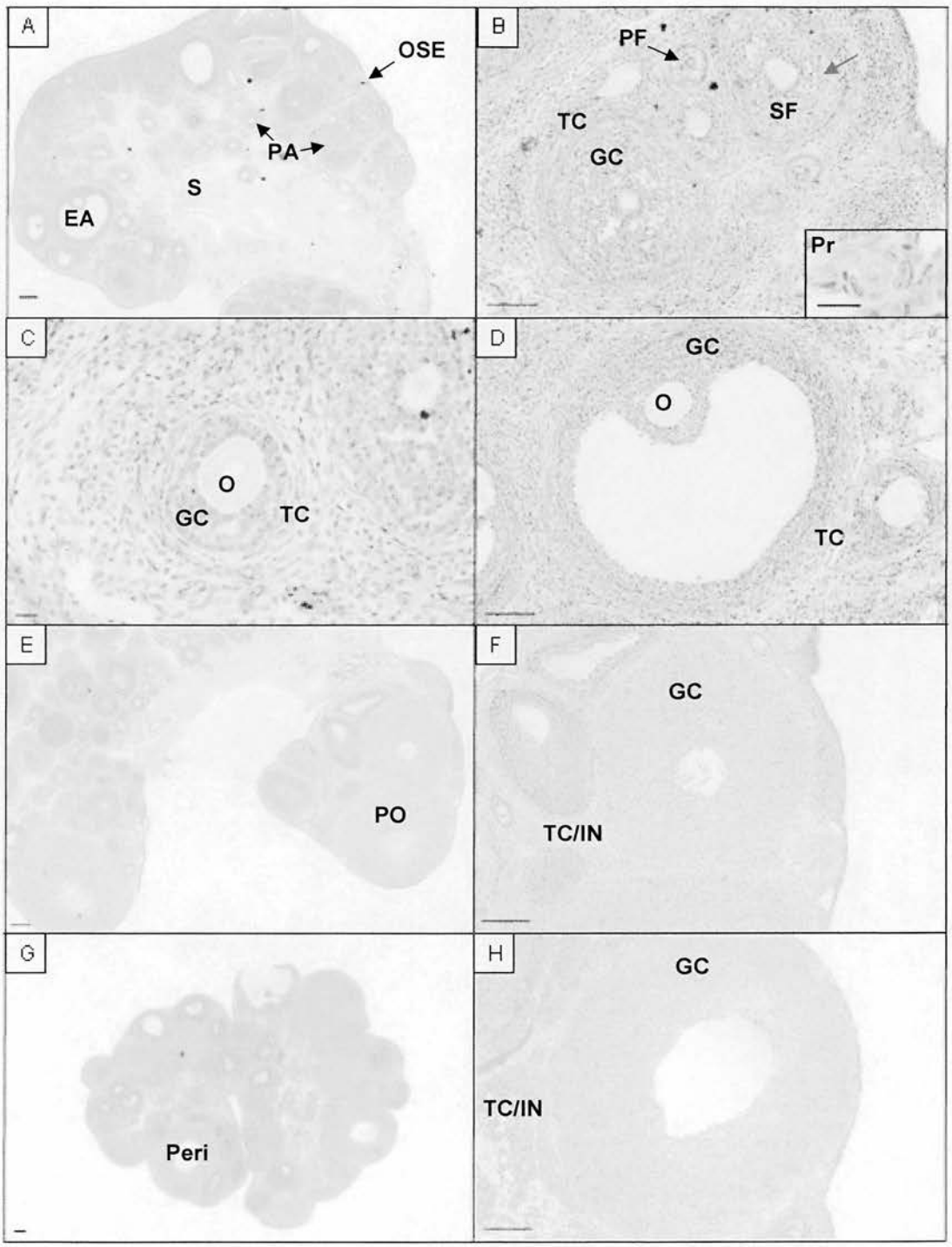

Figure 4.1. Ovarian morphology of *in vivo* gonadotrophin-treated rat ovaries. Fixed ovaries were stained with haematoxylin and eosin as described in Section 2.9. Immature ovaries (A-D), pre-ovulatory ovaries (E-F), peri-ovulatory ovaries (G-H). A, immature rat ovary, scale bar 100μm; B, preantral follicles, scale bar 50μm, black arrow, primary follicle, green arrow, secondary follicle, the insert shows a primordial follicle, scale bar 20μm; C, preantral follicles, scale bar 20μm; D, antral follicle, scale bar 50μm; E, pre-ovulatory ovary, scale bar 100μm; F, preovulatory follicle, scale bar 100μm; G, peri-ovulatory ovary, scale bar 100μm; H, peri-ovulatory follicle, scale bar 100μm. Primordial follicle (Pr), ovarian surface epithelium (OSE), preantral follicle (PA), early antral follicle (EA), primary follicle (PF), secondary follicle (SF), antral cavity (A), granulosa cells (GC), theca cells/interstitial tissue (TC/IN), oocyte (O), stroma (S).

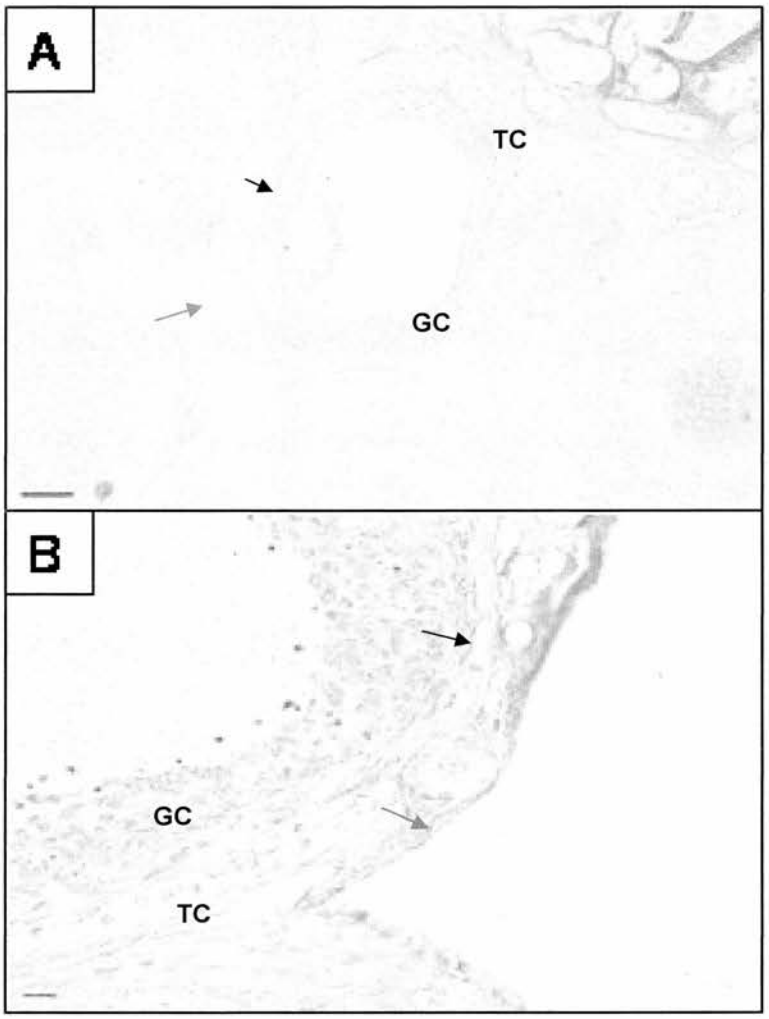

Figure 4.2. Histological localisation of basement membranes in the rat ovary. Fixed rat ovaries were sectioned and stained for basement membrane using Jones' methenamine silver basement membrane stain, as described in Section 2.9.4. A, preantral and antral follicles in untreated rat ovary, scale bar 100μm, black arrow shows basement membrane between granulosa and theca cells, purple arrow shows basement membrane enclosing thecal layer; B, preovulatory follicle in PMSG-treated ovary, scale bar 50μm. Black arrow shows basement membrane between granulosa and theca cells; red arrow shows the basement membrane on which the OSE are situated. Granulosa cells (GC), theca cells (TC).

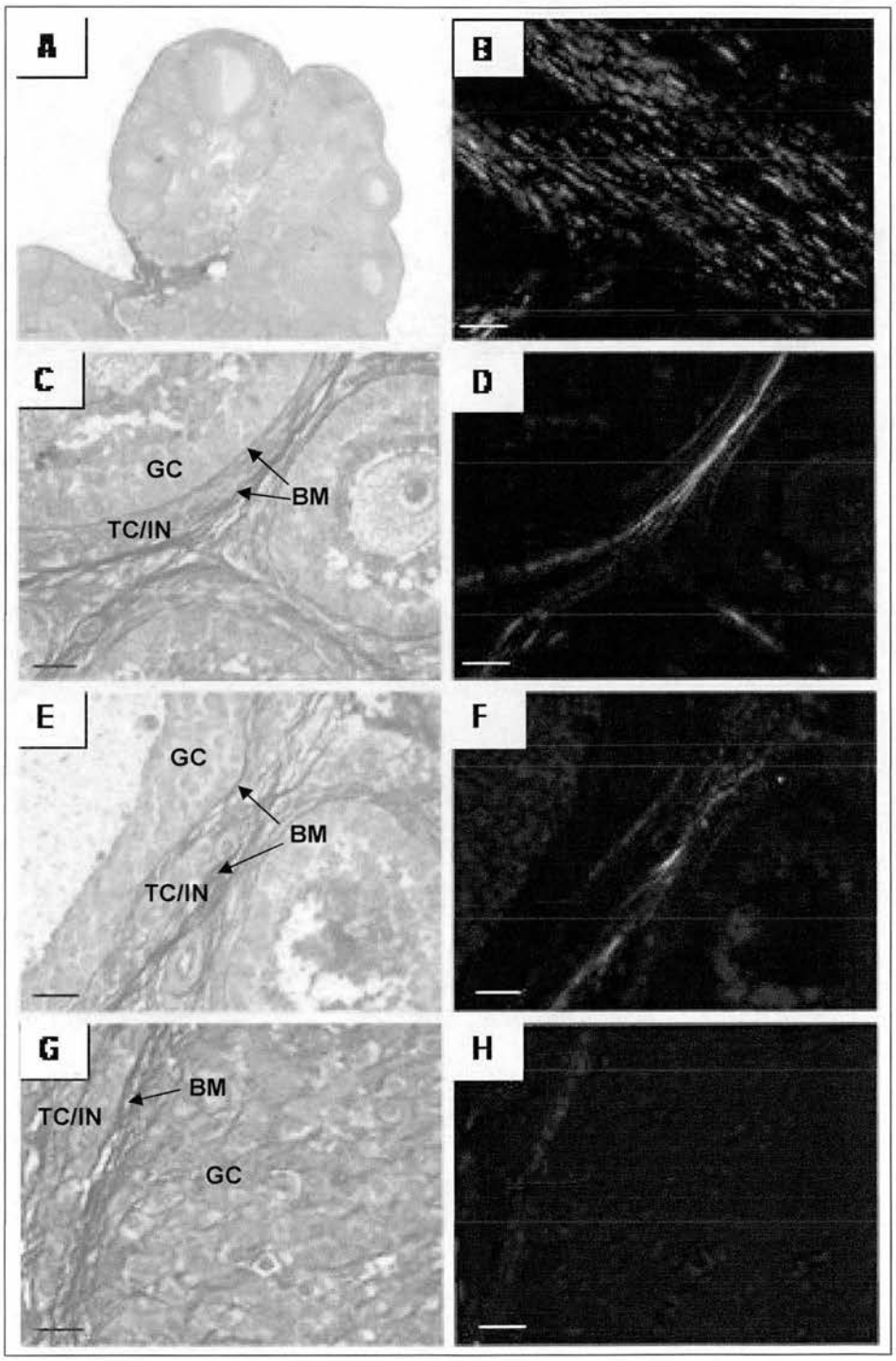

Figure 4.3. Histological localisation of ovarian basement membranes during rat follicular development. Ovaries from gonadotrophin treated rats were stained with picro-siruis red as described in Section 2.9.3. A, picro sirius red staining in the rat ovary viewed under light microscopy, scale bar 100µm; B, ovarian stroma viewed under polarising light microscopy, scale bar 50µm; C, untreated rat ovary viewed under light microscopy, scale bar 50µm; D, untreated rat ovary (seen in C) viewed under polarising light microscopy, scale bar 50µm; E, preovulatory follicle wall viewed under light microscopy, scale bar 50µm; F, follicle wall (from E) viewed under polarising light microscopy, scale bar 50µm; G, periovulatory follicle wall viewed under light microscopy, scale bar 50µm; H, follicle wall from G viewed under polarising light, scale bar 50µm. Granulosa cells (GC), theca cells/interstitial tissue (TC/IN), basement membrane (BM).

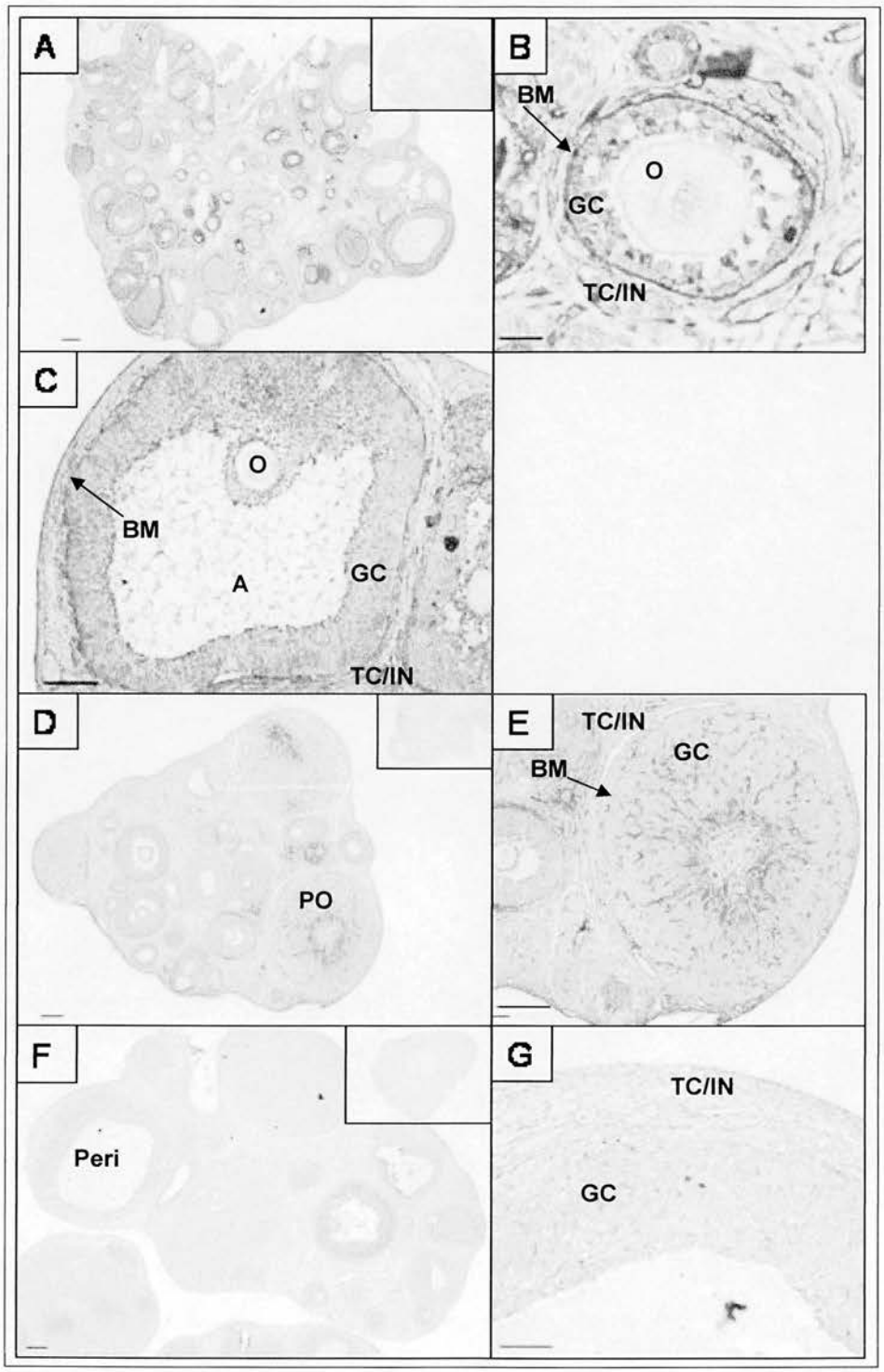

Figure 4.4. Localisation of laminin protein during follicular development. Ovaries from gonadotrophin-treated 21-day old rats were fixed and stained with laminin antibody as described in Section 2.9.5. A, immature ovary, insert shows negative control, scale bar 100μm; B, preantral follicle, scale bar 20μm; C, early antral follicle, scale bar 50μm; D, pre-ovulatory ovary, insert negative control, scale bar 100μm; E, preovulatory follicle, scale bar 50μm; F, peri-ovulatory rat ovary, insert negative control, scale bar 100μm; G, follicle wall of periovulatory follicle, scale bar 50μm. Basement membrane (BM), – preovulatory follicle (PO), periovulatory follicle (Peri), antral cavity (A), granulosa cells (GC), theca cells/interstitial tissue (TC/IN), oocyte (O).

4.3.3. Visualisation of collagen pathway gene and protein expression in the ovary

In situ visualisation of the location of mRNA expression was performed using radio-labelled probes for candidate collagen pathway genes (BMP-1, LOX and PCPE). Protein localisation was performed for BMP-1, LOX and MMP-2. Western blotting confirmed that the antibodies for BMP-1 and LOX detected proteins of the required size (Fig. 4.5).

PCPE mRNA in rat ovarian tissue (Fig. 4.6) showed mRNA present around follicles, with more intense hybridisation in the thecal region of the immature ovary. Expression declined as follicle maturation progressed, with PCPE being faint or absent in pre-ovulatory and peri-ovulatory follicles.

BMP-1 mRNA labelling in the maturing rat ovary was most intense in the immature ovary, and mainly localised to the stromal/theca area around follicles (Fig. 4.7). Follicular maturation was associated with a decrease in BMP-1 mRNA, although periovulatory follicles had some thecal/stromal hybridisation, with discrete patches being observed in the granulosa cells (Fig. 4.7). BMP-1 protein was not observed around primordial follicles nor at the outer surface of the immature ovary (Fig. 4.8A). However, protein staining was strong in the granulosa cells of preantral and early antral follicles (Fig. 4.8B/C). In these follicles there was some nuclear staining of the granulosa cells. As follicles matured, BMP-1 was also detected in the theca layers (Fig. 4.8D/E/F). BMP-1 protein was localised in oocytes (Fig. 4.8A/C), oocytes are recognised as a sticky tissue therefore caution should be used when drawing conclusions as to the specificity of the location of protein in this tissue.

Preantral and early antral follicles of untreated rat ovaries had the highest expression of LOX mRNA, and expression appeared to decline as follicles matured (Fig. 4.9). LOX mRNA in these follicles was confined to the granulosa layer. There was some LOX expression in pre-ovulatory ovaries, at the periphery of the pre-ovulatory follicles. Lower levels of LOX were present in peri-ovulatory ovaries. In the immature ovary, LOX protein was not associated with primordial follicles (Fig. 4.10A) but was seen in the granulosa cell layer and thecal region of preantral and early antral follicles (Fig.

4.10B/C). Small amounts of LOX protein were seen in the OSE basement membrane (Fig. 4.10A). LOX protein continued to be associated with granulosa and theca cells as follicles matured (Fig. 4.10D/E/F).

Expression patterns for the collagen pathway genes are compared in Figure 4.12.

In situ hybridisation was not attempted for MMP-2 but protein was localised to the interstitial tissue of the immature ovary (Fig. 4.11A/B). However, as ovulation approached some MMP-2 protein was observed in the granulosa cell compartment, although the majority was outside this layer (Fig. 4.11C/D). This trend continued up to ovulation (Fig. 4.11E/F).

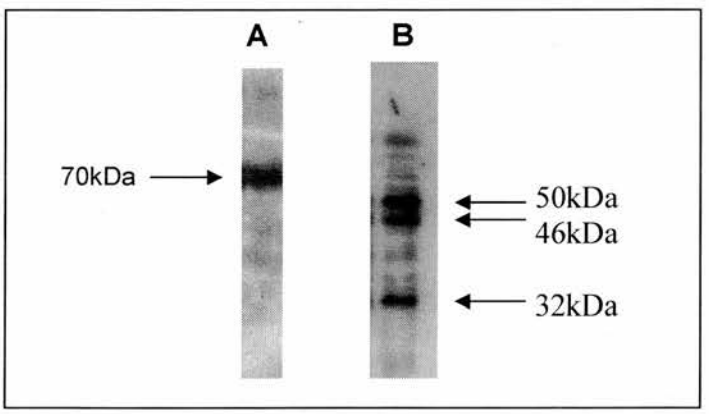

Figure 4.5. Western blotting for BMP-1 and LOX in rat ovarian tissue. Antibodies for BMP-1 and LOX were used to detect protein in rat ovary culture medium or tissue homogenate as described in Section 2.10. Samples were run against a commercial size marker. A, BMP-1 in granulosa cell culture medium; B, LOX in ovary homogenates.

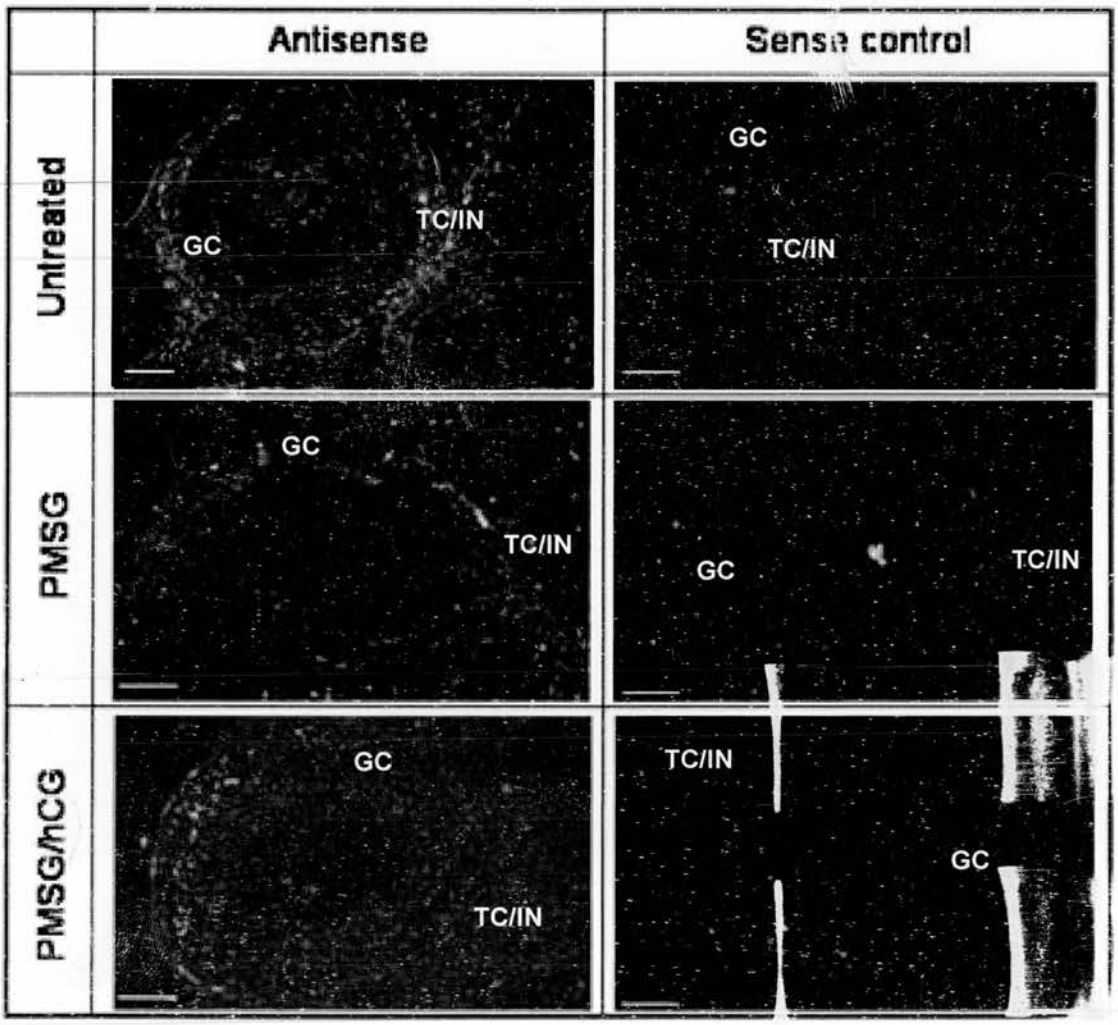

Figure 4.6. PCPE mRNA localisation during follicular development. *In situ* hybridisation as described in Section 2.11 was performed on fixed ovarian tissue from untreated or gonadotrophin-treated 21-day old female rats. Anti-sense positively hybridised tissue is shown on the left and sense controls on the right for each of the three rat treatment groups. Granulosa cells (GC), theca layers/interstitial tissue (TC/IN). Scale bars untreated antisense 50 µm, all other scale bars 100µm.

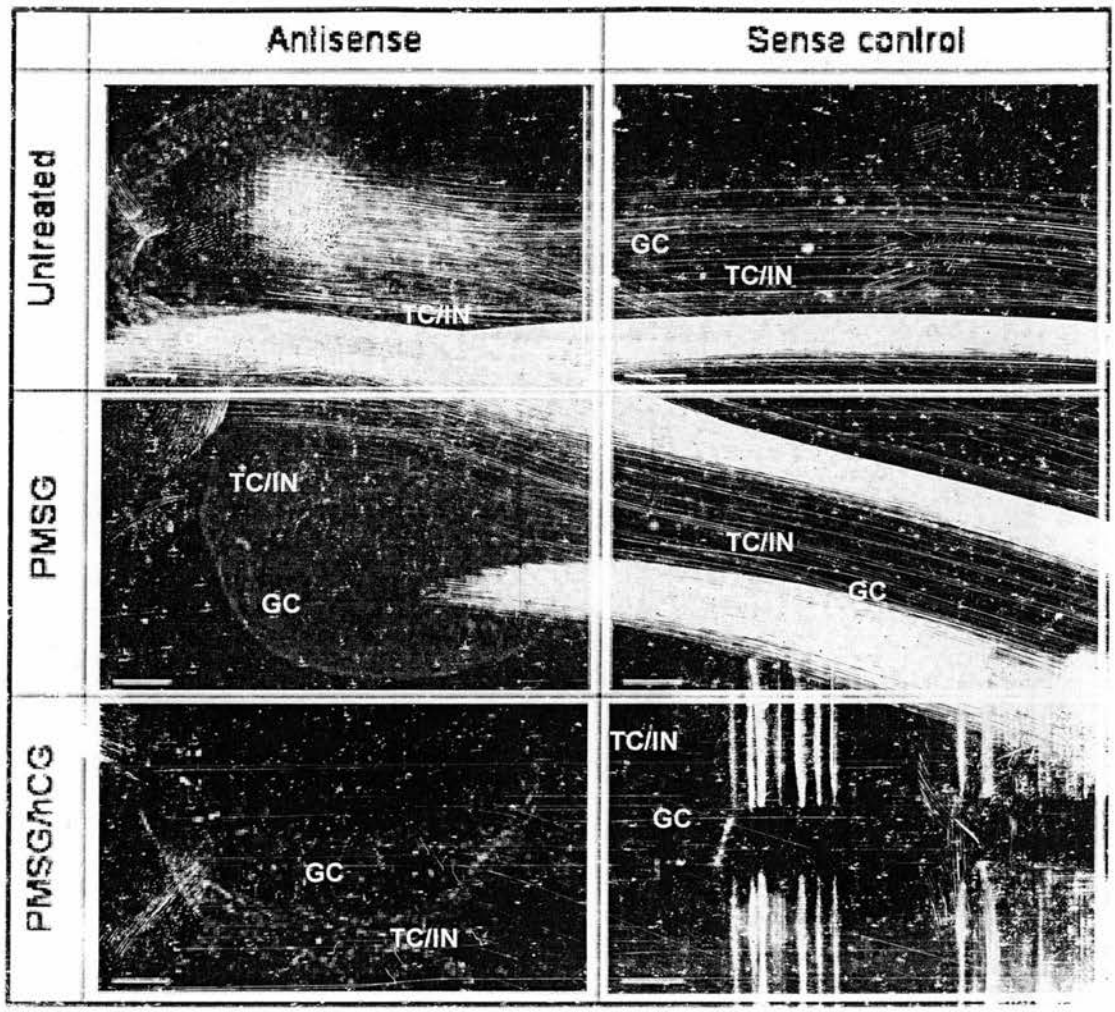

Figure 4.7. BMP-1 mRNA localisation during follicular development. *In situ* hybridisation as described in Section 2.11. was performed on fixed ovarian tissue from untreated or gonadotrophin-treated 21-day old female rats. Anti-sense positively hybridised tissue is shown on the left and sense controls on the right for each of the three rat treatment groups. Granulosa cells (GC), theca layers/interstitial tissue (TC/IN). Scale bars untreated antisense 50 µm, all other scale bars 100µm.

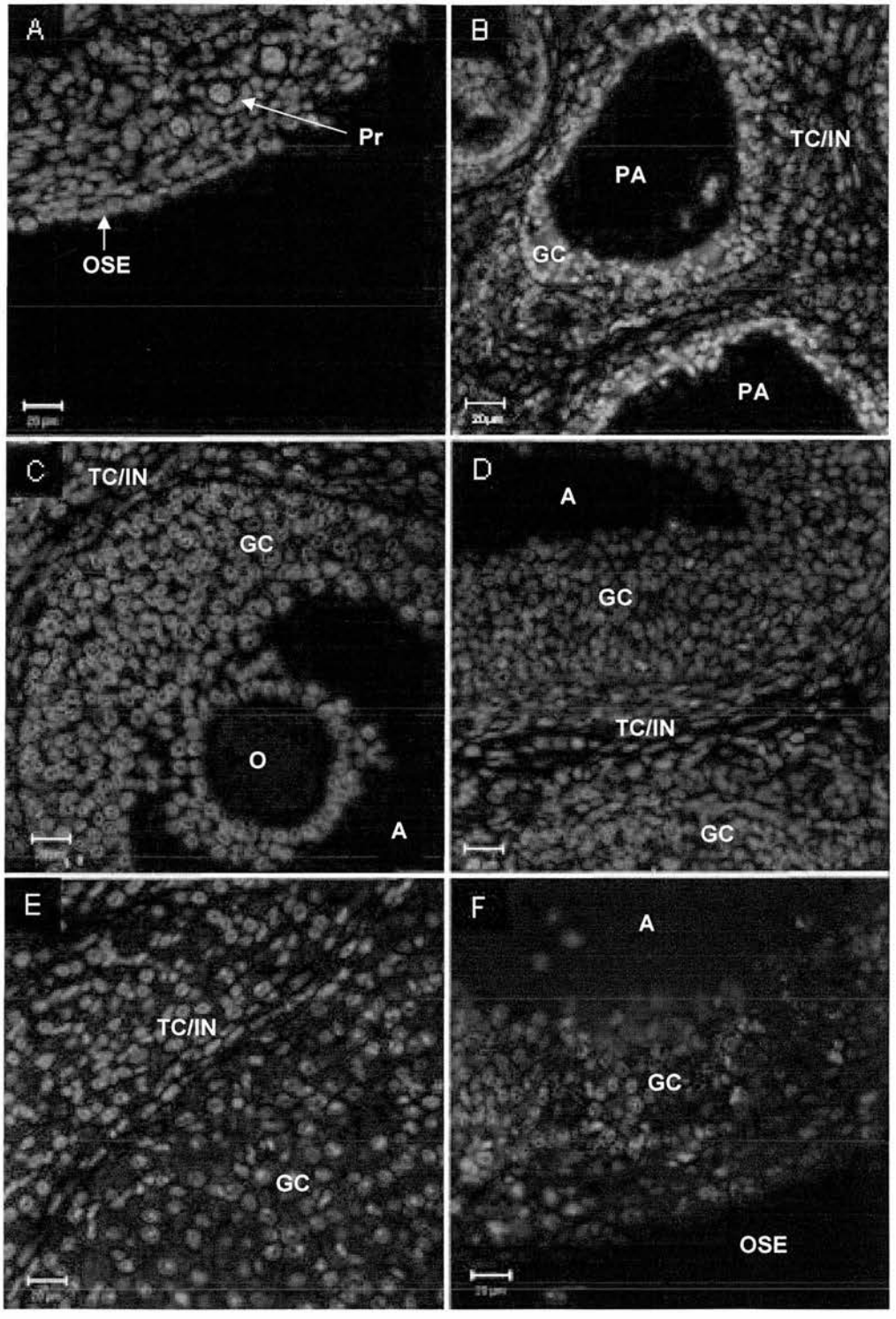

Figure 4.8. Localisation of BMP-1 protein during follicular development. Ovaries from untreated or gonadotrophin-treated 21-day old female rats were fixed and stained with BMP-1 antibody as described in Section 2.2.9.5. Red = BMP-1, Green = nuclear counter stain. A, Immature ovary with primordial follicles and OSE; B, immature ovary with preantral follicles; C, antral follicle from immature ovary; D, follicle wall of a preovulatory follicle (PMSG treated); E, follicle wall of a periovulatory follicle (inner section); F, follicle wall of a periovulatory follicle (outer section) with OSE edge. Primordial follicle (Pr), ovarian surface epithelium (OSE), preantral follicle (PA), antral cavity (A), granulosa cells (GC), theca cells/interstitial tissue (TC/IN), oocyte (O). Scale bars 20μm.

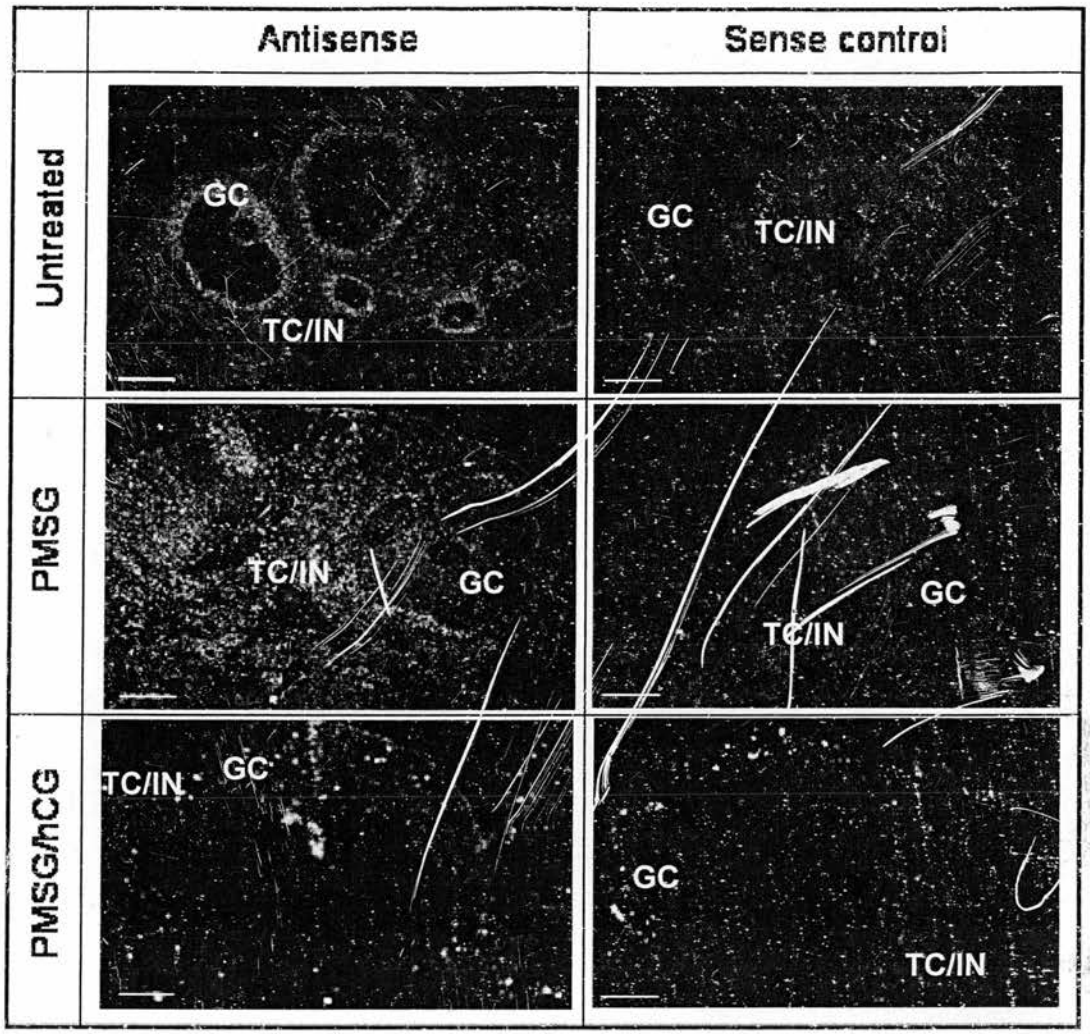

Figure 4.9. LOX mRNA localisation during follicular development. *In situ* hybridisation as described in Section 2.11. was performed on fixed ovarian tissue from untreated or gonadotrophin-treated 21-day old female rats. Anti-sense positively hybridised tissue is shown on the left and sense controls on the right for each of the three rat treatment groups. Granulosa cells (GC), theca layers/interstitial tissue (TC/IN). Scale bars 100μm.

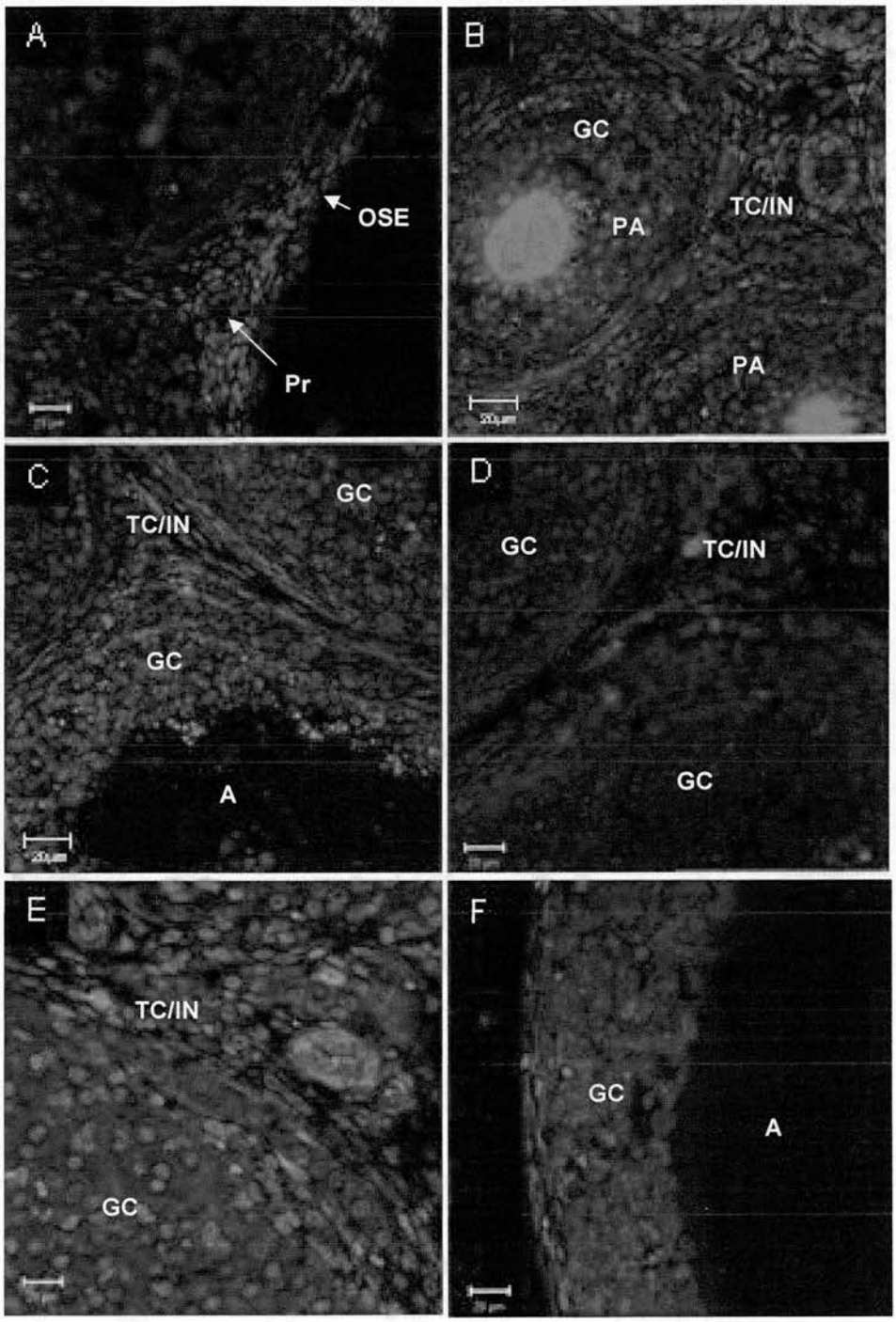

Figure 4.10. Localisation of LOX protein during follicular development. Ovaries from untreated or gonadotrophin-treated 21-day old female rats were fixed and stained with LOX antibody as described in Section 2.2.9.5. Red = LOX, green = nuclear counterstain. A, immature ovary showing OSE and primordial follicles; B, preantral follicle from immature ovary; C, early antral follicle from immature ovary; D, preovulatory follicle; E, inner wall of periovulatory follicle; F, external wall of periovulatory follicle. Antral cavity (A), granulosa cells (GC), theca cells/interstitial tissue (TC/IN), ovarian surface epithelium (OSE), primordial follicle (Pr), preantral follicle (PA). Scale bars 20μm.

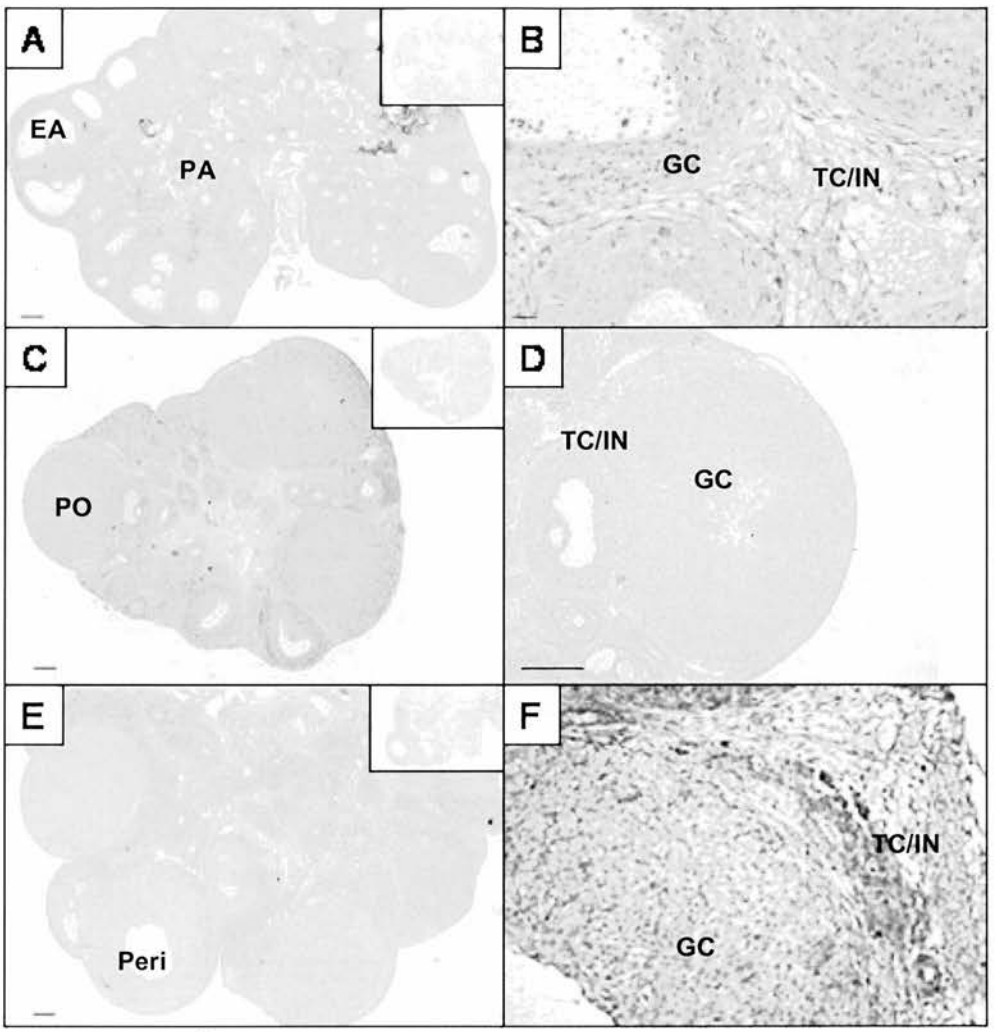

Figure 4.11. Localisation of MMP-2 protein during follicular development. Ovaries from untreated or gonadotrophin-treated 21-day old female rats were fixed and stained with MMP-2 antibody as described in Section 2.2.9.5. Rat ovaries stained with MMP-2 antibody. A, immature ovary, scale bar 100μm, insert negative control; B, interstitial area between immature follicles, scale bar 20μm; C, preovulatory rat ovary, scale bar 100μm, insert negative control; D, preovulatory follicle, scale bar 100μm. E, periovulatory ovary, scale bar 100μm, insert negative control; F, periovulatory follicle, scale bar 20μm. Preantral follicle (PA), early antral (EA), antral cavity (A), granulosa cells (GC), theca cells/interstitial tissue (TC/IN), preovulatory follicle (PO), periovulatory follicle (Peri).

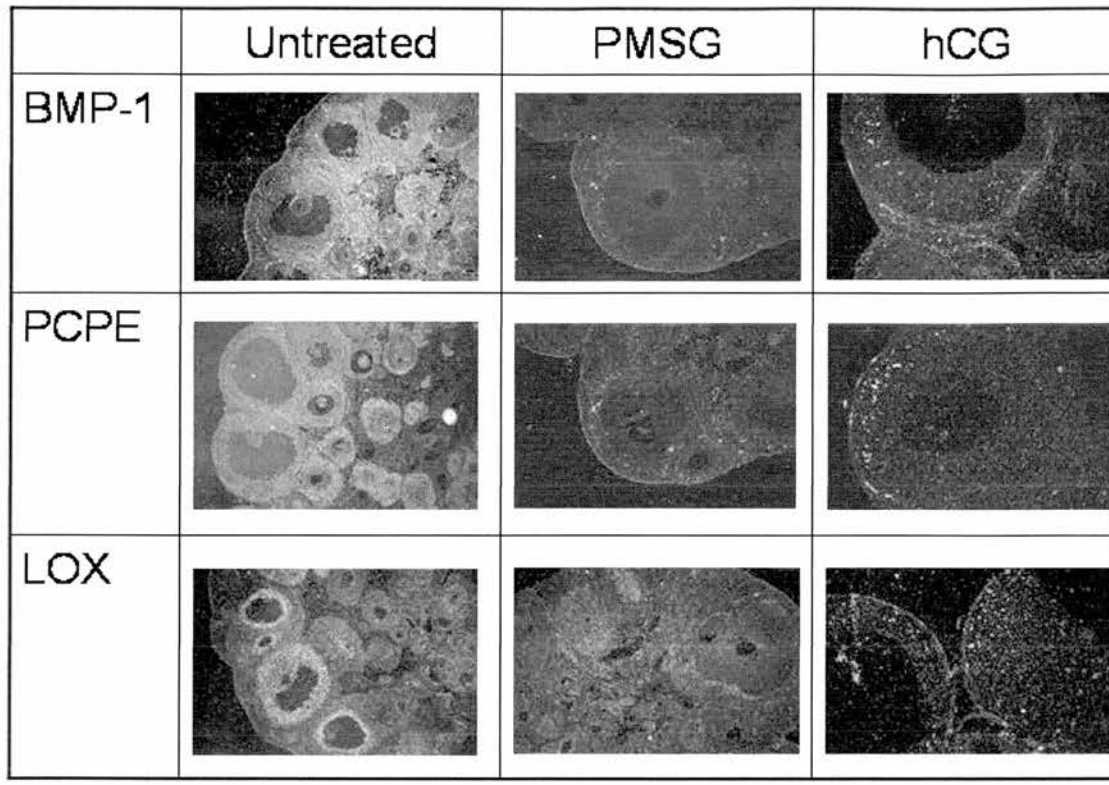

Figure 4.12. The spatiotemporal expression patterns of the collagen pathway genes during follicular development. Here comparisons between the expression patterns of PCPE, BMP-11 and LOX can be made at the different simulated stages of follicular maturation shown in the figures above using *in situ* hybridisation as described in Section 2.11. on fixed ovarian tissue from untreated or gonadotrophin-treated 21 day old female rats.

4.3.4. Localisation of glucocorticoid regulation mechanisms during follicular maturation.

Glucocorticoid signalling components were localised by *in situ* hybridisation and/or immunohistochemistry.

The *in situ* hybridisation analysis of 11βHSD1 (Fig. 4.13) generally showed a very low level of specific hybridisation signal with a possible increase in peri-ovulatory ovaries. The overall impression of the 11βHSD1 expression by *in situ* hybridisations would suggest that the analysis failed for unknown technical reasons. 11βHSD1 protein was, however, clearly located around granulosa and theca cells of preantral and antral follicles from immature ovaries (Fig. 4.14A/B). As follicles matured 11βHSD1 protein was mostly associated with granulosa cells (Fig. 4.14C/D).

The 11βHSD2 *in situ* hybridisation analysis was successful and showed 11βHSD2 mRNA expression in rat ovaries (Figure 4.15). The expression of this gene was greatest in the granulosa region of follicles from immature rat ovaries and decreased as follicles grew. This was reflected in the protein localisation, where 11βHSD2 was strongly associated with preantral and early antral granulosa cells and decreased as follicles matured (Fig. 4.16).

GR *in situ* hybridisation was not attempted. GR protein was mainly localised to follicles at all stages of development and was present in both the granulosa and theca layers (Fig. 4.17). Stromal tissue did not contain much GR.

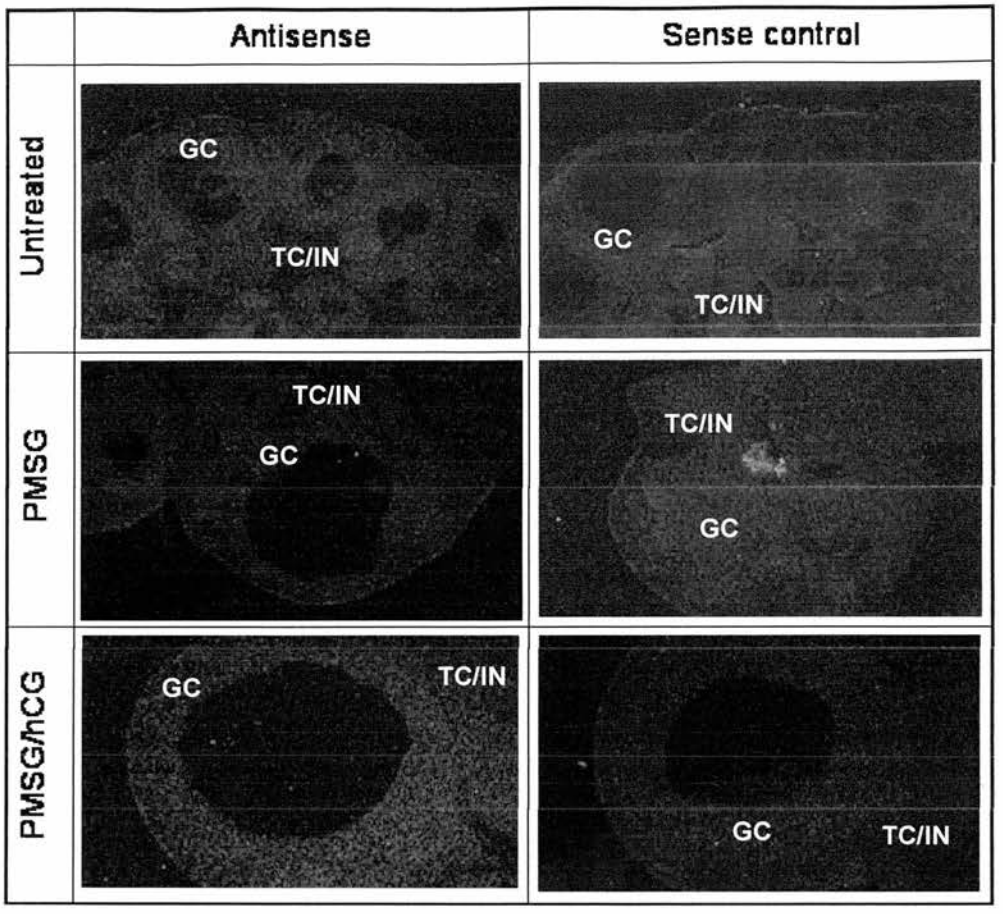

Figure 4.13. 11βHSD1 mRNA localisation during follicular development. *In situ* hybridisation as described in Section 2.11. was performed on fixed ovarian tissue from untreated or gonadotrophin-treated 21-day old female rats. Anti-sense positively hybridised tissue is shown on the left and sense controls on the right for each of the three rat treatment groups. Granulosa cells (GC), theca layers/interstitial tissue (TC/IN). Scale bars 100μm.

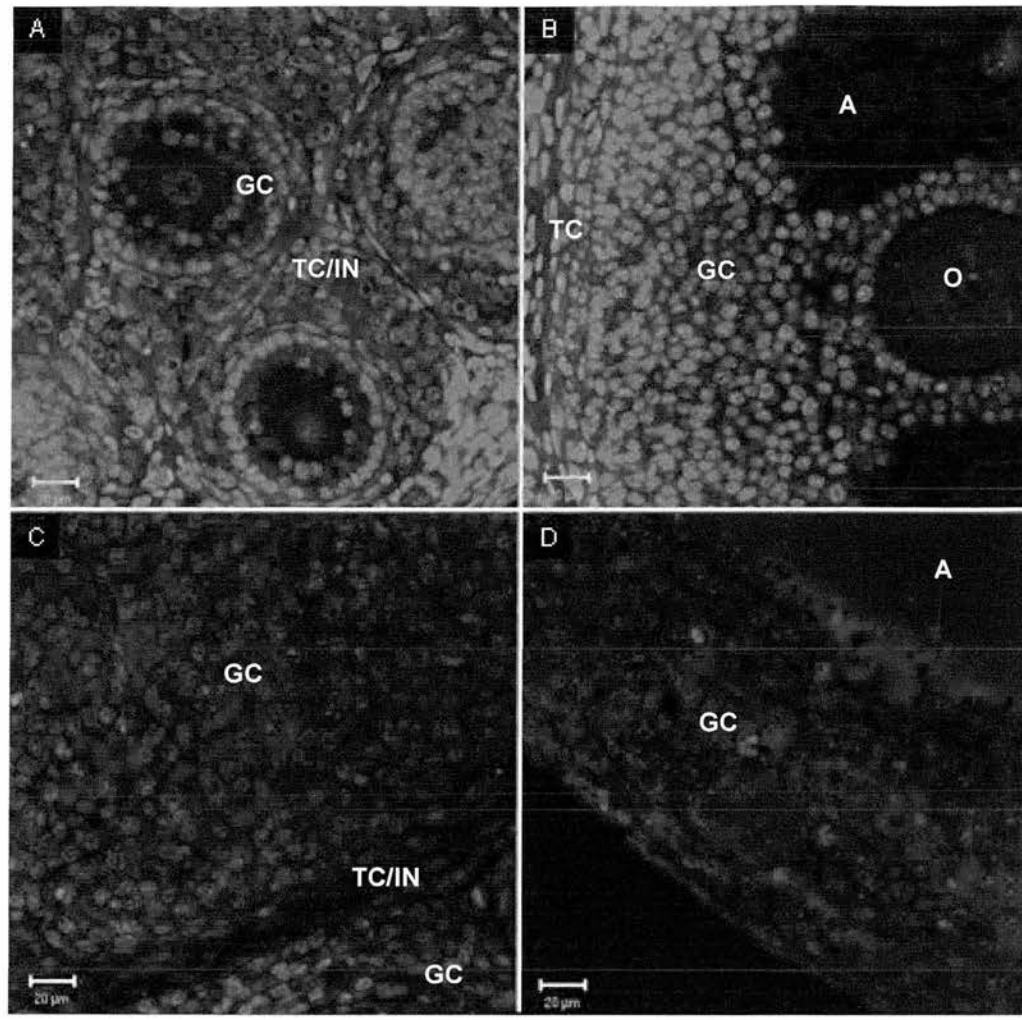

Figure 4.14. Localisation of 11βHSD1 protein during follicular development. Ovaries from untreated or gonadotrophin-treated 21-day old rats were fixed and stained with 11βHSD1 antibody as described in Section 2.2.9.5. Red = 11βHSD1, green = nuclear counter stain. A, preantral follicles from immature ovaries; B, early antral follicle from immature ovary; C, wall of preovulatory follicle; D, wall of a periovulatory follicle. PA, preantral follicle. Antral cavity (A), granulosa cells (GC), theca cells/interstitial tissue (TC/IN), oocyte (O). Scale bars 20µm.

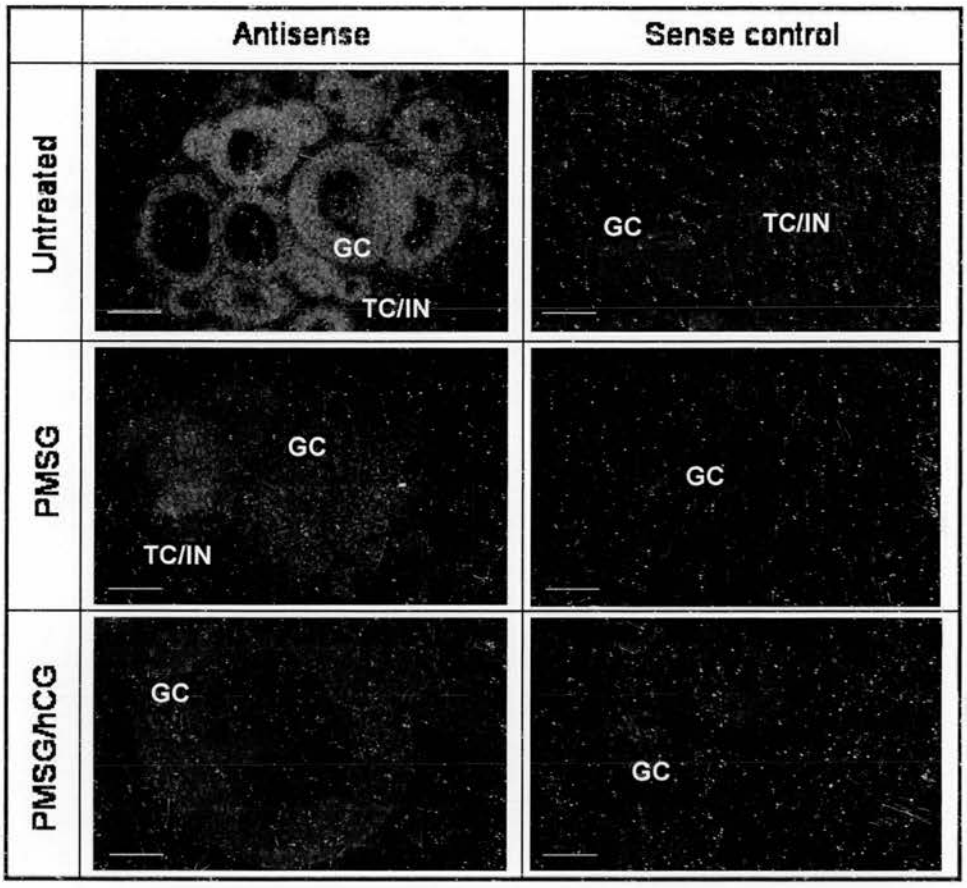

Figure 4.15. 11βHSD2 mRNA localisation during follicular development. *In situ* hybridisation as described in Section 2.11. was performed on fixed ovarian tissue from untreated or gonadotrophin-treated 21-day old rats. Anti-sense positively hybridised tissue is shown on the left and sense controls on the right for each of the three rat treatment groups. Granulosa cells (GC), theca layers/interstitial tissue (TC/IN). Scale bars 100μm.

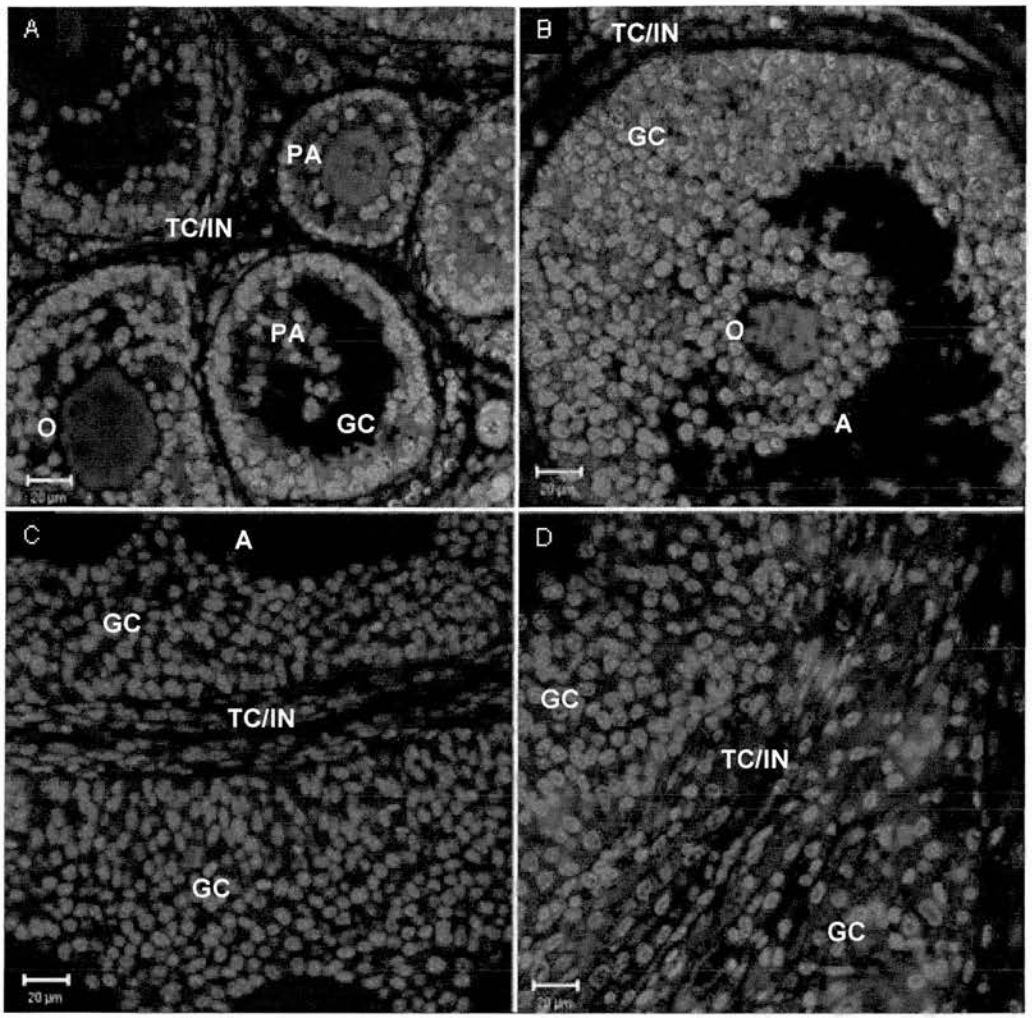

Figure 4.16. Localisation of 11βHSD2 protein during follicular development. Ovaries from untreated or gonadotrophin-treated 21-day old rats were fixed and stained with 11βHSD2 antibody as described in Section 2.2.9.5. Red = 11βHSD2, green = nuclear counter stain. A, preantral follicles from immature ovaries; B, early antral follicle from immature ovary; C, follicle wall of preovulatory follicles from PMSG treated rats; D, periovulatory follicle wall. Preantral follicle (PA), antral cavity (A), granulosa cells (GC), theca cells/interstitial tissue (TC/IN), oocyte (O). Scale bars 20µm.

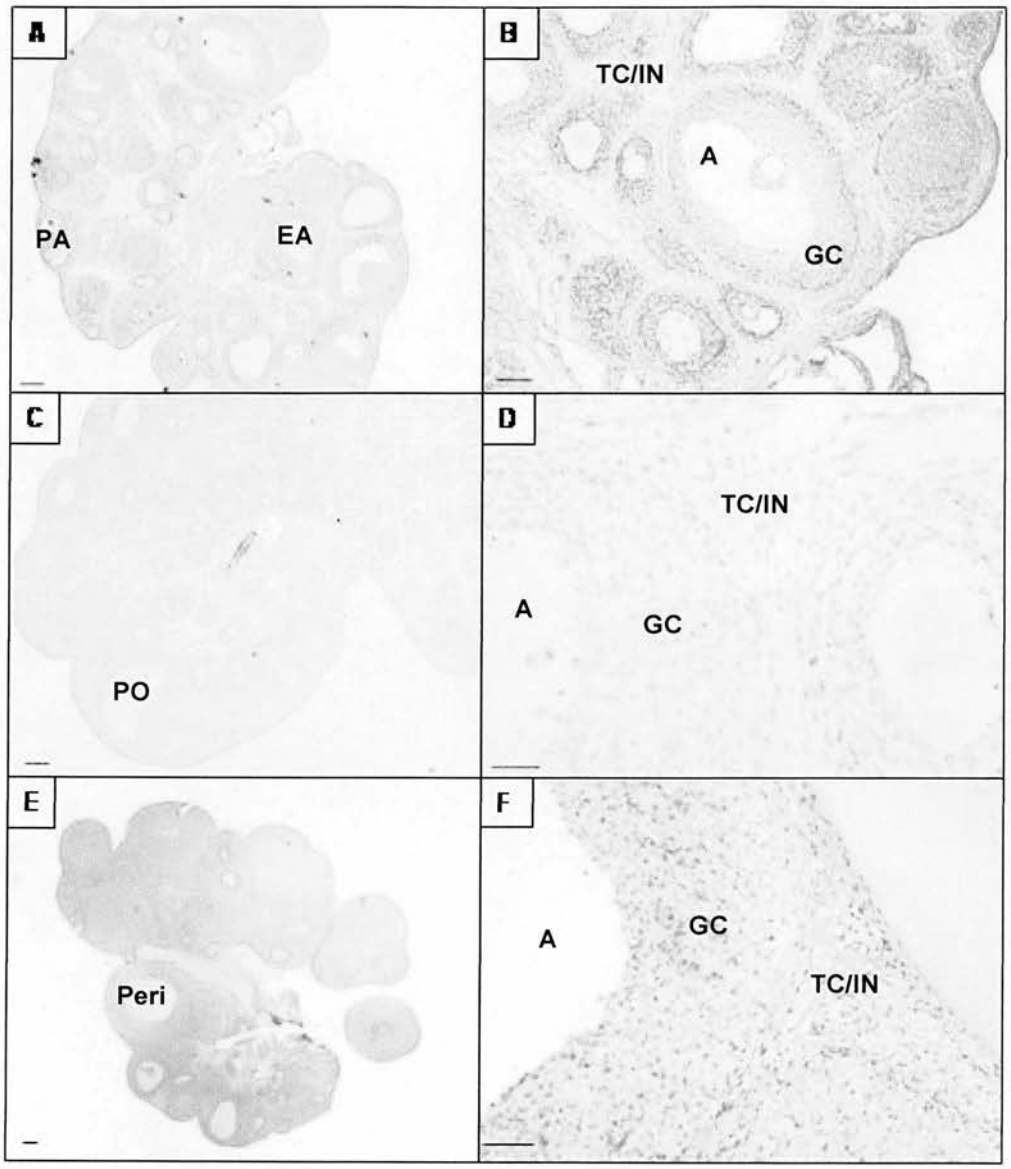

Figure 4.17. Localisation of GR protein during follicular development. Ovaries from untreated or gonadotrophin-treated 21-day old rats were fixed and stained with GR antibody as described in Section 2.2.9.5. A, immature ovary, scale bar 100μm; B, preantral and antral follicles, 50μm; C, pre-ovulatory ovary, scale bar 100μm, D, pre-ovulatory follicle wall, scale bar 20μm; E, periovulatory ovary, scale bar 100μm; F, wall of a periovulatory follicle, scale bar 50μm. Preantral follicle (PA), early antral (EA), Antral cavity (A), granulosa cells (GC), theca cells/interstitial tissue (TC/IN), oocyte (O), periovulatory follicle (Peri).

4.4 Discussion

As seen in the previous chapter, mRNA levels for collagen synthesis and degradation genes change in response to *in vivo* gonadotrophin treatment of immature female rats. By further using gonadotrophin-treated rats, ovaries were obtained at staged points during follicular development (Fig. 4.1). These developmentally staged ovaries were used to establish spatiotemporal distribution of genes and their transcribed proteins from the collagen pathway (BMP-1, LOX, PCPE and MMP-2) and from glucocorticoid signalling (11βHSD1, 11βHSD2 and GR).

The major findings from these investigations were that LOX mRNA was prominently localised to the granulosa cells of immature follicles, whereas the translated protein was observed in all ovarian compartments. BMP-1 mRNA was mostly located in the theca and interstitial regions with protein mainly detected in the granulosa layers. The two 11βHSD isoforms were seen to have an inverse relationship; with 11βHSD2 being predominant in early follicle development and 11βHSD1 prevails as follicles matured.

The rat ovary is compartmentalised by the basement membrane between granulosa and theca cells, as well as basement membranes that enclose the thecal layer, and the external circumference of the ovary on which the OSE reside (Fig 4.2). Rat ovaries showed fibrillar-collagens I and III in the stroma, and collagen IV in the basement membrane of the follicles (Fig. 4.3). Although faint it could be seen that this basement membrane appeared to lose its integrity as follicles approached ovulation. Fragmentation of the basement membrane has been reported previously in luteinising and atretic rat follicles (Bagavandoss *et al.* 1983; Palotie *et al.* 1984). This is a consequence of the actions of degrading proteases which have been shown to destroy the follicular basement membrane and which are up-regulated approaching ovulation (Espey 1994; Ohnishi *et al.* 2005).

To confirm the position of basement membranes in the rat ovary, laminin was used as a marker as this protein is one of the major components, forming a network that interacts with the collagen IV network (Sasaki *et al.* 2004). Immunohistochemical localisation showed that laminin protein in maturing ovaries was localised to the basement membrane, as previously shown by Leardkamolkarn & Abrahamson (1992). As follicles

matured, laminin staining became less discrete at the basement membrane, further highlighting the effects of degradation initiated by the LH surge that breaks down this structure in preparation for ovulation (Espey 1994; Ohnishi *et al.* 2005).

Laminin protein detected within the granulosa cell layer of antral follicles could mark what has been termed the 'focimatrix', a novel matrix between granulosa cells of antral bovine follicles that is believed to effect cell polarity (Rodgers *et al.* 2004). ECM components, in particular laminin, appear to promote cell survival and proliferation of granulosa cells (Le Bellego *et al.* 2002; Taipale & Keski-Oja 1997). Given that laminin was more abundant in growing follicles compared to follicles on the verge of ovulation, its distribution mirrors that of cell proliferation that occurs through development.

The previous chapter showed that collagen pathway components were present in the rat ovary. Here these elements were visualised within this tissue. *In situ* hybridisation for PCPE and BMP-1 showed similar patterns of expression (Fig. 4.12). These two genes have been shown to be similarly distributed in other tissues (Scott *et al.* 1999). PCPE and BMP-1 were both expressed in preantral/early antral follicles, with expression being most intense in the theca layer. However, there was also some expression observed within the granulosa cell layer. Presence of PCPE mRNA in the immature follicles implies that the translated protein would serve to enhance the actions of BMP-1 in cleaving procollagen to produce the substrate for LOX to form mature collagen in the stroma. Collagen I and III have been observed in the connective tissue of the theca layer during follicle maturation (Palotie *et al.* 1984). As follicles developed, expression of PCPE and BMP-1 declined. This would result in a decrease in the cleavage of procollagen, reflecting a decline in the need for mature collagen. Prior to ovulation, PCPE and BMP-1 mRNA were expressed in the theca layer and in discrete regions of the granulosa layer of the periovulatory follicle, indicating a resumption of collagen processing. After ovulation, early luteal tissue contains collagen fibrils (Luck & Zhao 1993). Perhaps the expression of BMP-1 and PCPE in follicles on the verge of ovulation produces collagen substrate for the remodelling necessary to form the corpus luteum. Given the speed at which this structure forms, a pre-synthesised source of collagen may aid this remodelling event.

The presence of PCPE and BMP-1 denotes the production of mature collagen fibrils, therefore the localisation of LOX will give information as to where these collagen fibrils are stabilised into rigid structures by cross-linking in the ovary. The localisation of LOX gene expression using *in situ* hybridisation showed that the main site and stage of LOX mRNA expression was in preantral/early antral granulosa cells. This discrete mRNA expression pattern of LOX could create a tight window of expression in preantral/early antral follicles that might produce enough pro-LOX to be used during follicular remodelling. This could in turn be regulated by BMP-1 in areas where remodelling needs to occur. Evidence for this comes from studies in skin wounding, that show that there can be an extensive period of time between mRNA expression and activation of LOX protein (Fushida-Takemura *et al.* 1996; Siegel 1979).

LOX protein was observed at all stages of follicular growth, apart from primordial follicles (which are quiescent) and protein was localised to both granulosa and theca cell compartments in growing follicles. Other possible sources of LOX are ovarian fibroblasts though they may not have been visualised on the *in situ* hybridisation. Myofibroblasts cells surrounding human breast tumours and fibroblasts associated with the skin have been shown to produce LOX (Chanoki *et al.* 1995; Peyrol *et al.* 1997). In the ovary, fibroblasts are thought to reside found in the stroma and thecal regions, potentially providing a source of LOX. As these cells were not observed by *in situ* hybridisation, it may be assumed that their contribution would be small.

In the previous chapter, it was shown that the theca cell population expressed significantly less LOX mRNA than the granulosa cell population. These mRNA levels were measured by quantitative real-time PCR, which is a more sensitive technique than *in situ* hybridisation, suggesting that the theca population may in part contribute to ovarian LOX. Another ovarian source of LOX are the OSE cells (Rae *et al.* 2004a; Rae *et al* 2007 in preparation). As LOX gene expression was predominantly localised to the granulosa cell layer of preantral/early antral follicles, and protein was seen throughout the ovary, it is possible that the protein moves from its site of origin. This would involve traversing the basement membrane, a region where LOX may act to stabilise collagen IV by cross-linking.

Interestingly, not every preantral follicle expressed LOX mRNA. Is the lack of expression a factor determining the fate of a follicle, or a consequence of its fate? TGF-β is low or undetectable in atretic follicles (Chegini & Flanders 1992) and TGF-β stimulates LOX expression and activation (Harlow *et al.* 2003; Hong *et al.* 1999; Shanley *et al.* 1997). This suggests that in follicles that do not express TGF-β, LOX will not be stimulated and therefore LOX will not be present to stabilise collagen remodelling around the follicle leading to atresia.

The antibody used in these investigations was shown to detect pro-forms of LOX (Fig. 4.5) as well as the active form. Hence, LOX staining may not necessarily parallel biochemical enzyme activity. It is not known how much of the protein that was visualised was active. Even if not all the protein is active, this staining represents a potential pool from which active protein can be formed. This phenomenon is seen in wounding studies, where mRNA levels peak at 3 days post-wounding but activity is measured only 8-10 days after injury (Fushida-Takemura *et al.* 1996; Siegel 1979).

The focus of these studies was the function of LOX in ECM remodelling and LOX protein was either localised to the cytoplasm of cells or was extracellular; it was not localised to the nuclei of cells. However, this enzyme has also been shown to have intracellular functions and other systems see LOX associated with the nuclei, at nuclear pores and in association with condensed chromatin in the nucleus, indicating involvement in the cell cycle (Kagan & Li 2003; Molnar *et al.* 2005). Work by Palamakumbura *et al* (2004) has shown that the cleaved pro-peptide region of LOX will associate with the nucleus of cells. This region has been shown to have tumour-suppressor properties by stopping over-proliferation via the *ras* pathway (Contente *et al.* 1990; Palamakumbura *et al.* 2004). The probable intracellular targets are histones, which can be oxidised by LOX and subsequently influence gene transcription (Giampuzzi *et al.* 2003). LOX (and LOXL) have been localised in proliferating cells such as spermatocytes, chondocytes, cornea epithelia as well as granulosa cells (Hayashi *et al.* 2004). The significance of this localisation to proliferating cells has not been determined but may relate to the nuclear functions of LOX. The antibody used here

does not detect this 18kDa pro-region. Hence LOX could have nuclear actions on the cell cycle of granulosa cells.

The distribution of BMP-1 may provide clues as to where LOX is active in the ovary, as BMP-1 must cleave pro-LOX to form the active protein (Pappano *et al.* 2003; Uzel *et al.* 2001). Hence, areas that contain both BMP-1 and LOX protein are most likely to be areas where LOX is active. BMP-1 protein was localised in the granulosa cells of preantral follicles, which are sites of intense LOX mRNA expression. As follicles matured, BMP-1 was observed more extensively throughout the ovary in areas where LOX protein was also observed. This implies that LOX has the potential to be activated in these areas during development where it is likely to function locally in remodelling.

By *in situ* hybridisation, BMP-1 gene expression was predominantly thecal. Therefore the BMP-1 protein presumably passes across the basement membrane to the granulosa cells of immature follicles in order to activate LOX. This can be likened to thecal androgen passing to granulosa cells to provide substrate for aromatisation (Ryan & Short 1965), reinforcing the concept that cell-to-cell communication is integral to follicular maturation. BMP-1 also cleaves probiglycan, the γ2 and α3 laminin chains and chordin. There are no specific reports of these ECM components in the rat follicle. However, *in situ* hybridisation in embryonic mouse tissue showed that BMP-1 and chordin were expressed in similar patterns in the hindlimb (Scott *et al.* 1999). Therefore, BMP-1 may transverse the basement membrane to cleave other proteins besides pro-LOX. The presence of BMP-1 would permit the activation of several ECM elements, highlighting again the importance of protein components moving across the basement membrane of the follicle to act in different cellular compartments.

Gene expression of MMP-2 was shown in Chapter 3 to have an inverse relationship with that of LOX during follicle maturation, and was strongly expressed in theca cells. Here MMP-2 protein was mainly localised to the thecal/interstitial area of all stages of follicular development in the rat (Fig. 4. 11). MMP-2 is a gelatinase that breaks down collagen, and as ovulation approaches this protein and other proteases start to degrade the follicle wall of preovulatory follicles (Ny *et al.* 2002). Consequently, MMP-2 protein is seen in granulosa cells as well as theca cells. MMP-2 mRNA has previously

been localised to the thecal/interstitial area of the ovary by *in situ* hybridisation during follicular development (Curry *et al.* 2001; Liu *et al.* 1998). Thus initially, both MMP-2 mRNA and protein are confined to the theca/interstitial area but as the LH surge initiates the process of ovulation, proteases are activated and MMP-2 protein can move to the granulosa cell layer as the basement membranes degraded. In chapter 3 the levels of MMP-2 mRNA were seen to increase as ovulation approached, yet the immunohistochemistry does not appear to show an increase in protein levels. This could be due to qRT-PCR being a more sensitive technique than immunohistochemistry and therefore able to detect discreet changes in expression, changes in mRNA may not be reflected in protein translation or may be seen after a timelag and immunohistochemistry relies on the sensitivity of the antibody used.

Previous investigations have studied the mRNA levels of the signal regulators of glucocorticoids in cells isolated from the rat ovary but there are only limited reports about their localisation within this tissue.

The ovary does not produce glucocorticoid *de novo* but does contain the enzymatic machinery to regulate the availability of active glucocorticoid (Tetsuka *et al.* 1997). This regulation occurs through the expression of 11βHSD isoforms (Lakshmi & Monder 1988; Mercer & Krozowski 1992). The expression profile of 11βHSD2, as shown by *in situ* hybridisation, was greatest in the granulosa cells of immature follicles, but absent in more mature follicles, confirming previous reports by Tetsuka *et al* (1999b). 11βHSD2 protein was localised to the same areas as the mRNA.

Gene localisation studies for 11βHSD1 gave inconclusive results in this study. Therefore published data alluding to 11βHSD1 mRNA localisation in the granulosa cells has to be relied upon (Tetsuka *et al.* 1999b). When the protein localisation of 11βHSD1 was examined, this enzyme appeared to be most strongly expressed in more mature follicles, where the protein was present in both granulosa and theca cells. In the present work, staining of 11βHSD1 was not as intense as expected. This may be explained by the time-lag of only 12h between the *in vivo* treatment of hCG and the time of animal sacrifice. This time-delay is accounted for by the need for 11βHSD1 mRNA translation to protein as well as subsequent glucocorticoid conversion. At the stage at which the

ovaries in the present investigation were removed, mRNA would have been increasing but protein levels may have only just have started increase, accounting for the low intensity of the staining observed.

The data for the 11βHSD isoforms shows their inverse relationship in the ovary, allowing for tightly-controlled regulation of glucocorticoid action in this tissue. Another possible level of control is GR, as receptor availability ultimately determines whether cells can receive the available signal. GR mRNA is expressed continually throughout the rat oestrous cycle (Tetsuka *et al.* 1999b), and here GR protein was observed in both the granulosa and theca layers of the follicle at all stages studied. Therefore, glucocorticoid signalling during follicle development is not limited by receptor availability as GR is readily available throughout follicle development. This highlights the likely importance of the inverse expression pattern of 11βHSD isoforms in regulating glucocorticoid activity.

In summary, these investigations into mRNA and protein localisation have shown that cellular sites of collagen pathway gene expression are reasonably compartmentalised and change during follicular growth, whereas translated proteins are not so discretely confined and are observed in most areas of the ovary during follicular development. The genes encoding 11βHSD isoforms involved in glucocorticoid pre-receptor regulation are differentially expressed and developmentally regulated during follicular development. Therefore, spatiotemporal expression of 11βHSD genes has the potential to influence collagen pathway biology within the ovary. The implications of this are examined in the next chapter by using *in vitro* cultures of rat granulosa and theca cells to investigate the actions not only of glucocorticoids but also of other known effectors of follicular maturation on collagen pathway biology.

5. Spatiotemporal expression of collagen pathway genes in the rat ovary: *in vitro* studies.

5.1. Introduction

As seen in the previous chapters, collagen pathway genes and proteins are regulated during the process of follicle development in the female rat. The factors responsible for these changes can be scrutinised by removing granulosa and theca cells from the rat model and studying cause-effect relationships.

As LOX seems to be active in many different situations, its expression is presumably regulated by specific factors such as cytokines and growth factors (Smith-Mungo & Kagan 1998). Some of these factors have been described, with TGF-β being the most widely studied. TGF-β has been shown to increase LOX mRNA and activity time- and dose-dependently, as well as up-regulating the LOX substrate, collagen type I (Feres-Filho *et al.* 1995). In neonatal rat aorta smooth muscle cells, TGF-β increased LOX mRNA expression but decreased cell proliferation (Gacheru *et al.* 1997). It is thought that the increase in LOX by TGF-β is due to stabilisation of LOX mRNA (Gacheru *et al.* 1997).

LOX is activated by BMP-1, therefore regulators of this protein will also influence the availability of LOX. BMP-1 was first isolated in a complex with two other proteins from human bone (Wozney *et al.* 1988). The two other proteins were BMP-2 and -3, which are part of the TGF-β superfamily, and suggested that BMP-1 may be involved in activation of TGF-β family members (Bond & Beynon 1995). TGF-β is initially secreted in a latent form that can be processed by BMP-1 causing a positive feed-forward regulation of matrix deposition (Ge & Greenspan 2006; Lee *et al.* 1997).

BMP-1 is up-regulated by TGF-β in fibrogenic cells and keratinocytes (Lee *et al.* 1997). This positive regulation may involve TGF-β up-regulation of furin, the protease that activates BMP-1 by cleaving its pro-domain (Leighton & Kadler 2003). Procollagen N-proteinase is also up-regulated by TGF-β in fibrogenic cells (Wang *et al.* 2003) and demonstrates that collagen cleavage can be regulated by TGF-β. Evidence for BMP-1/TGF-β interactions also comes from the Drosophila *tolloid* (*tld*) gene. This has 41%

homology to human BMP-1 and displays interactions during embryogenesis with *decapentaplegic* (*dpp*), the Drosophila form of BMP-2, a TGF-β superfamily member (Shimell *et al.* 1991; Wozney *et al.* 1988). Null mutants for *dpp* and *tld* show similar phenotypes indicating a possible link between these two genes (Finelli *et al.* 1994; Shimell *et al.* 1991).

BMP-1 cleaves procollagen to active collagen, a process that is enhanced by PCPE. Therefore PCPE regulation will influence the effect that BMP-1 has on procollagen cleavage. Collagen production shows a similar pattern to PCPE expression in bovine smooth muscle cells (SMC) with respect to the stage of cell proliferation (Kindy *et al.* 1988). Presumably PCPE expression is stimulated or inhibited by cytokines expressed by proliferating cells. Possible candidates also include TGF-β, as it causes the expression of PCPE in SMC (Kanaki *et al.* 2000) and the up-regulation of PCPE and procollagen I in rat liver stellate cell lines (Ogata *et al.* 1997).

TGF-β is an important regulator of ECM accumulation (Feres-Filho *et al.* 1995; Lee *et al.* 1997; Massague 1990). In the rat ovary all three members of the TGF superfamily, TGFβ-1, -2 and -3, are differentially expressed in developing oocytes, granulosa cells and theca cells (Chegini & Flanders 1992; Schmidt *et al.* 1992; Teerds & Dorrington 1992). Most studies of TGF-β influencing collagen pathway biology use TGF-β1. This member of the TGF family is first expressed in preantral oocytes and granulosa cells then at the time of antrum development appears in theca cells (Teerds & Dorrington 1992). The expression of TGF-β1 increases in both cell types with follicle maturation. Receptors for TGF-β family proteins are present in oocytes and follicular cells, as are signal pathway components, Smad-2 and -3 (Juengel & McNatty 2005). In rodents, TGF-β causes granulosa cell proliferation (although in other systems TGF-βs can be inhibitory) (Dorrington *et al.* 1988). TGF-β enhances the production of FSH-stimulated oestrogen production in granulosa cells (Adashi *et al.* 1989). However, in theca cells, TGF-β has inhibitory effects on LH-stimulated androgen production, but promotes differentiation (Magoffin *et al.* 1989).

To date there are no investigations of the regulation of BMP-1 and PCPE in the ovary, but it would be predicted that these genes would be influenced by regulators of follicle development.

Thus the available data suggest that TGF-β and related growth and differentiation factors are likely to be of importance in mediating developmental-related changes in collagen pathway biology gene expression during follicular development. The likelihood that these genes are also subject to anti-inflammatory (glucocorticoid) modulation effects of corticosterone may also be important. Here I describe interactions between gonadotrophins/TGF-β/corticosterone on collagen pathway genes in isolated follicular cells from immature rat follicles. In more mature follicles, where LH is the major gonadotrophin drive, ovulation-associated cytokines such as IL-1 are also likely to be important. Therefore the effect of this pro-inflammatory cytokine was also examined on cells from preovulatory follicles.

5.2. Materials and methods

5.2.1. Animals

Female Wistar rats were given *in vivo* hormone injections as described in Section 2.1.

5.2.2. Cell culture

Granulosa and theca cells were isolated and cultured as described in Sections 2.2 and 2.3.

5.2.3. RIA

Androstenedione was measured in culture medium used for theca cell cultures, as described in Section 2.4.

5.2.4. RT-PCR

Freshly isolated granulosa and theca cells were assessed for the presence or absence of aromatase (CYP19) gene expression as described in Section 2.6. The primer sequences are shown in Table 5.1.

Table 5.1. Primer sequence and annealing temperature used for RT-PCR

Gene	Forward primer sequence	Reverse primer sequence	Annealing temperature
Aromatase	GTATGAGAGATCCGTCAAGGAC	TTGATCATCACATGGCGATGTAC	60°C

5.2.5. Quantitative real-time PCR

The mRNA expression for LOX, BMP-1 and PCPE was measured using quantitative real-time PCR as described in Section 2.7.

5.2.6. LOX activity assay

LOX activity was measured in culture samples using the vacuum distillation method described in Section 2.8. Many problems were encountered using this method so activity data is not complete for all culture experiments.

5.2.7 Statistical analysis

All data (average dCT valves for qRT-PCR) were statistically tested with either a paired t-test or repeated measures ANOVA according to the number of treatment groups being assessed. The lowest level of accepted significance was $P<0.05$. For each experimental

set of data treatment groups are compared to the untreated control group which was defined as a value of 1 and therefore is shown graphically without variation about the mean. However statistical analysis was performed on raw data (CT values) and therefore does account for variation around the mean.

5.3. Results

5.3.1. Cell morphology

Granulosa cells cultured from rats that have received *in vivo* hormone treatments showed phenotypic differences when cultured (Fig 5.1). Granulosa cells (highlighted with black arrows) from untreated rats (Fig. 5.1A) and rats that received PMSG (Fig. 5.1B) are relatively small in size, whereas luteinised cells from animals treated with PMSG and hCG (Fig. 5.1C) are larger. Theca cell populations (shown by blue arrows) from the three groups of female rats showed similar differences in morphology (Fig. 5.1). Stromal fibroblasts were also present in theca cell populations (highlighted by white arrows).

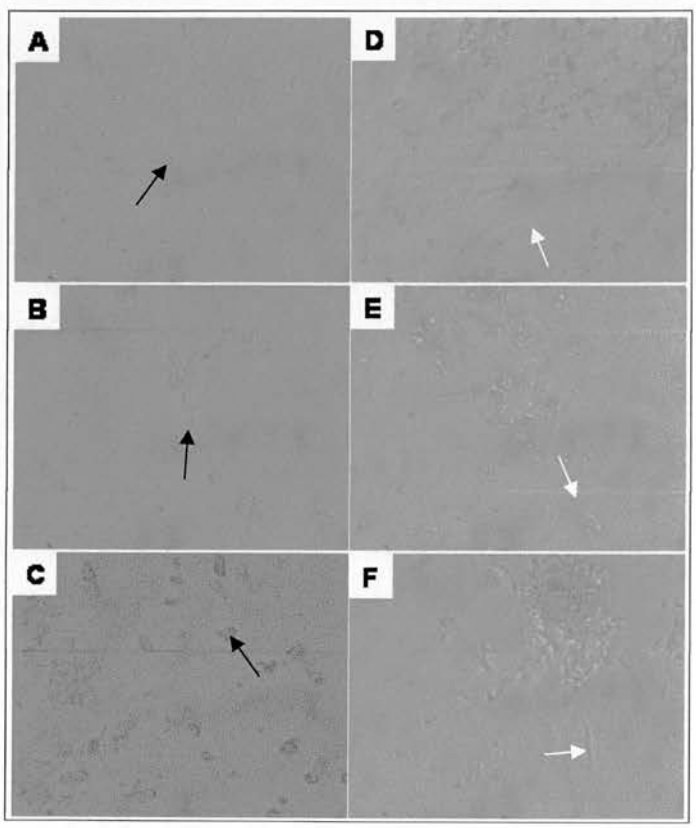

Figure 5.1. Cultured ovarian cell morphology. Cells were isolated as described in Sections 2.2 (granulosa cells) and 2.3 (theca cells) and cultured for 48h. A, untreated rat granulosa cells; B, PMSG treated rat granulosa cells; C, PMSG- plus hCG-treated rat granulosa cells; D, untreated rat theca cells; E, PMSG treated rat theca cells; F, PMSG- plus hCG-treated rat theca cells. Arrows highlight representative cells, black – granulosa cells, blue – theca cells, white – stromal fibroblasts.

5.3.2. Assessment of ovarian cell purity

The purity of the theca cell population studied was assessed by measuring androgen (androstenedione) production. As shown in Fig. 5.2., treatment with LH (0.01IU/ml) for 48h significantly increased androstenedione production, confirming the presence of functional thecal cells. Further evidence for the quality of the thecal cell preparations came from comparisons of CYP19 (aromatase) levels in separated theca and granulosa cells. As shown in Fig. 5.3., theca cells were devoid of aromatase mRNA, whereas it was readily detectable in granulosa cells from the same ovaries. This latter result, with evidence that aromatase mRNA expression by granulosa cells was enhanced by PMSG treatment *in vivo*, also served to validate the granulosa cell preparation.

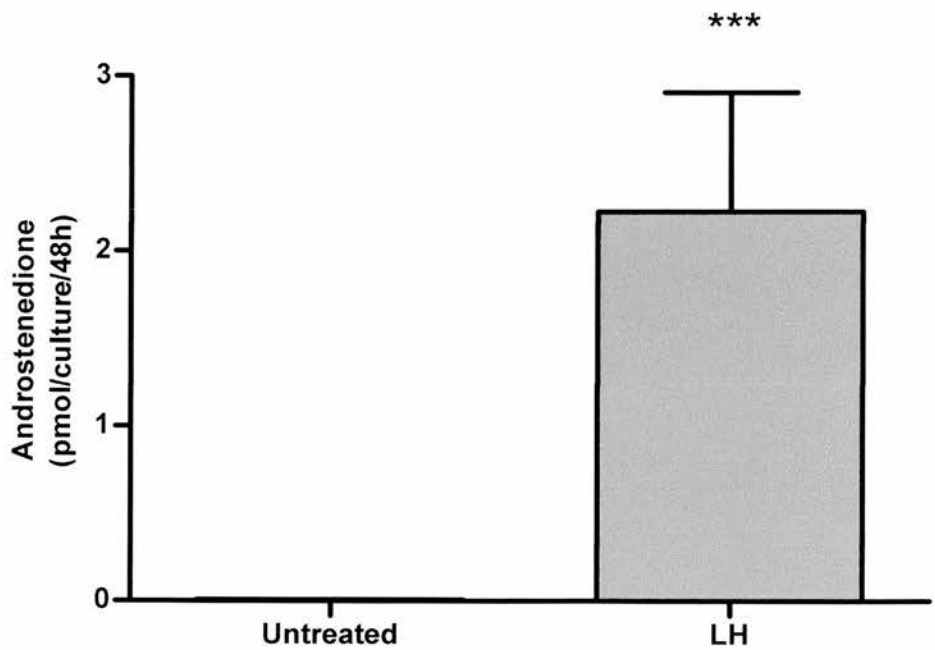

Figure 5.2. Theca cell population responsiveness to LH stimulus measured by production of androstenedione. Thecal cells were cultured as described in Section 2.4. with and without LH [0.01IU/ml], androstenedione production was measured in spent culture medium as described in Section 2.4. N=4. *** = P<0.001 (compared to untreated).

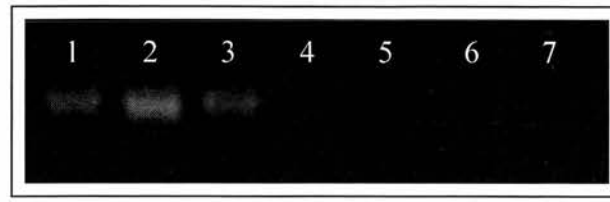

Figure 5.3. Qualitative gene expression of aromatase during follicular development. Cultured granulosa and theca cells were used for RT-PCR as described in Section 2.6. Loading for each gel is as follows: lane 1, untreated granulosa cells; lane 2, PMSG granulosa cells; lane 3, PMSG/hCG granulosa cells; lane 4, untreated theca cells; lane 5, PMSG theca cells; lane 6, PMSG/hCG theca cells; lane 7, negative control.

5.3.3. Immature ovarian cells

Immature ovarian cells respond to an array of different growth factors, until the antrum starts to form and the follicle becomes gonadotrophin-dependent. It is at this time that granulosa cells respond to the influences of FSH and theca cells respond to LH.

5.3.3.1. Granulosa cells

Collagen pathway gene expression was assessed in granulosa cells cultured for 48h with TGF-β (10ng/ml) or FSH (0.01IU/ml) alone and in combination with corticosterone (CORT) (1μM). Cells were also cultured with increasing concentrations of CORT (0μM, 0.01μM, 0.1μM, 1μM). Changes in mRNA levels for PCPE, BMP-1 and LOX were assessed as well as LOX enzyme activity.

No significant differences were found in mRNA levels for BMP-1 with any of the culture treatments. PCPE mRNA expression was significantly decreased in the presence of CORT. This was not consistently observed in additional experiments, therefore may not be a real effect (Fig. 5.4). CORT alone did not significantly change LOX gene expression or enzyme activity (Fig. 5.4).

When granulosa cells were cultured in the presence of TGF-β, a known enhancer of LOX, a significant increase in both mRNA and activity was observed (Fig. 5.5). CORT did not alter the TGF-β stimulated increase in LOX mRNA but may have reduced activity levels (Fig. 5.5).

Immature granulosa cells were also cultured with FSH, the gonadotrophin to which these cells would respond *in vivo*. LOX mRNA was not affected by FSH alone, but was significantly decreased when both CORT and FSH were present (Fig. 5.6). A significant difference was observed between cells treated with CORT with and without FSH, indicating that FSH depressed the expression of this gene. Interestingly, FSH significantly increased LOX enzyme activity data, and this effect was significantly suppressed by the addition of CORT (Fig. 5.6).

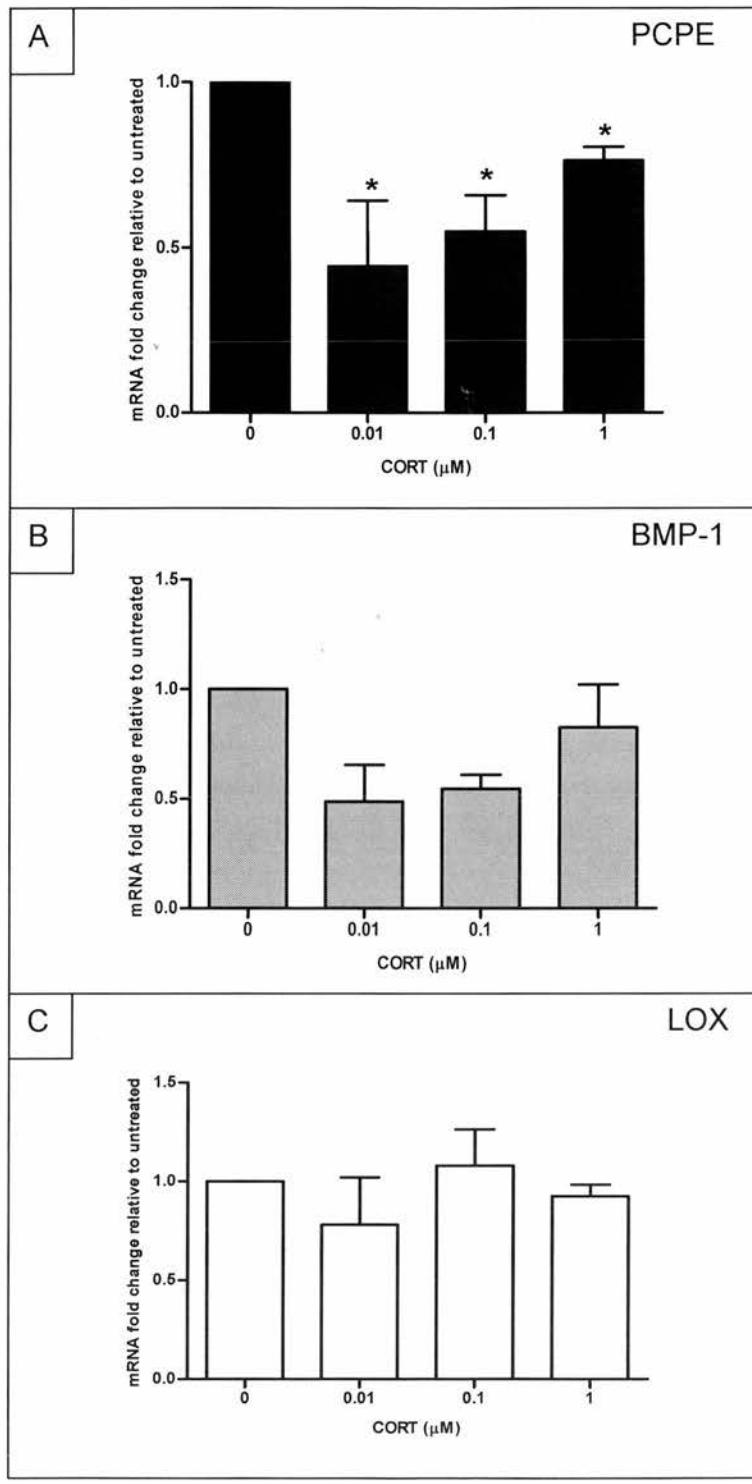

Figure 5.4. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured granulosa cells from untreated immature female rats. Granulosa cells were cultured with increasing concentrations of CORT for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3, * = $P<0.05$.

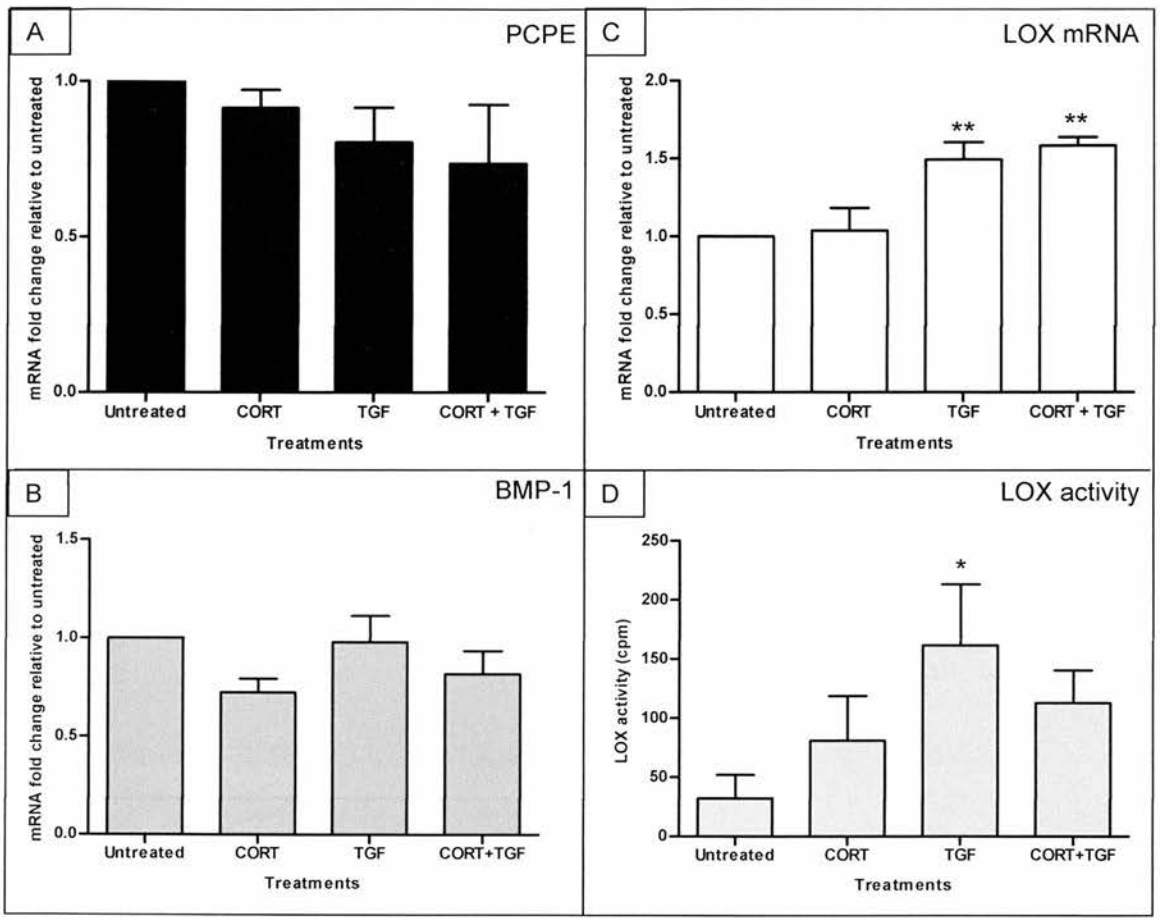

Figure 5.5 PCPE (A), BMP-1 (B), LOX (C) mRNA expression levels and LOX enzyme activity (D) levels in cultured granulosa cells from untreated immature female rats. Granulosa cells were cultured with CORT [1μM] and/or TGF-β [10ng/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for LOX mRNA levels using qRT-PCR as described in Section 2.7. Culture medium was assessed for LOX enzyme activity using the LOX tritium vacuum distillation enzyme assay as described in Section 2.8.1. N=4, * = $P<0.05$, ** = $P<0.01$.

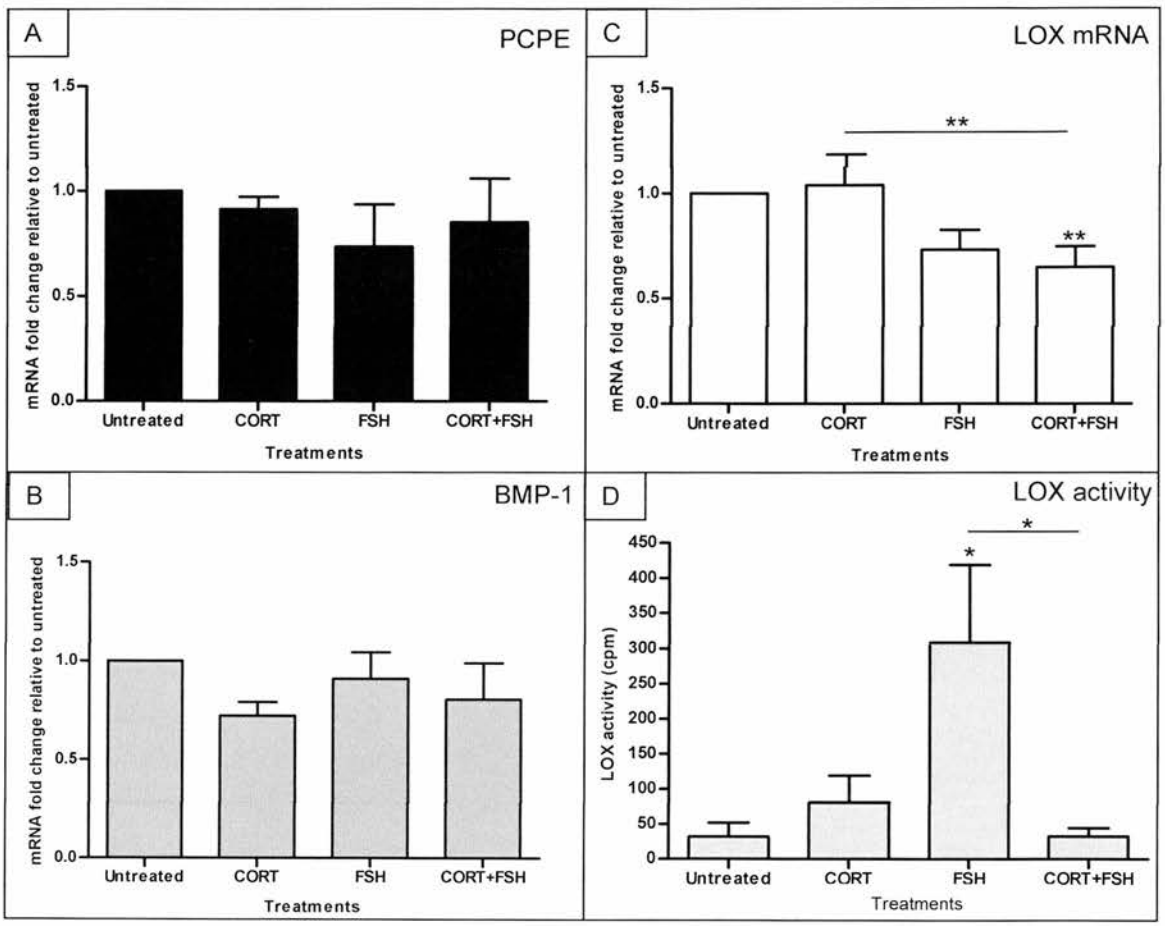

Figure 5.6 PCPE (A), BMP-1 (B), LOX (C) mRNA expression levels and LOX enzyme activity (D) levels in cultured granulosa cells from untreated immature female rats. Granulosa cells were cultured with CORT [1μM] and/or FSH [0.01IU/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for LOX mRNA levels using qRT-PCR as described in Section 2.7. Culture medium was assessed for LOX enzyme activity using the LOX tritium vacuum distillation enzyme assay as described in Section 2.8.1. N=4, * = P<0.05, ** = P<0.01.

5.3.3.2. Theca cells

Results for theca cells from immature follicles cultured with increasing concentrations of CORT (0μM, 0.01μM, 0.1μM, 1μM) or LH (0.01IU/ml) or TGF-β (10ng/ml) with and without CORT (1μM) are shown in Figures 5.7–5.9. LOX enzyme activity was not measured in these cells.

BMP-1 gene expression was not affected by any of the culture treatments. PCPE mRNA was not significantly affected by CORT or TGF-β, and LOX mRNA was not influenced by LH. LOX mRNA was significantly increased by CORT alone at 0.1μM and 1μM (Fig. 5.7). TGF-β was stimulating and the effects of TGF-β and CORT were additive (Fig.5.8). There was a significant difference between CORT with or without TGF-β.

Theca cells express LH receptors and therefore respond to LH. This gonadotrophin significantly increased PCPE mRNA levels but this was reduced when CORT was also present (Fig. 5.9).

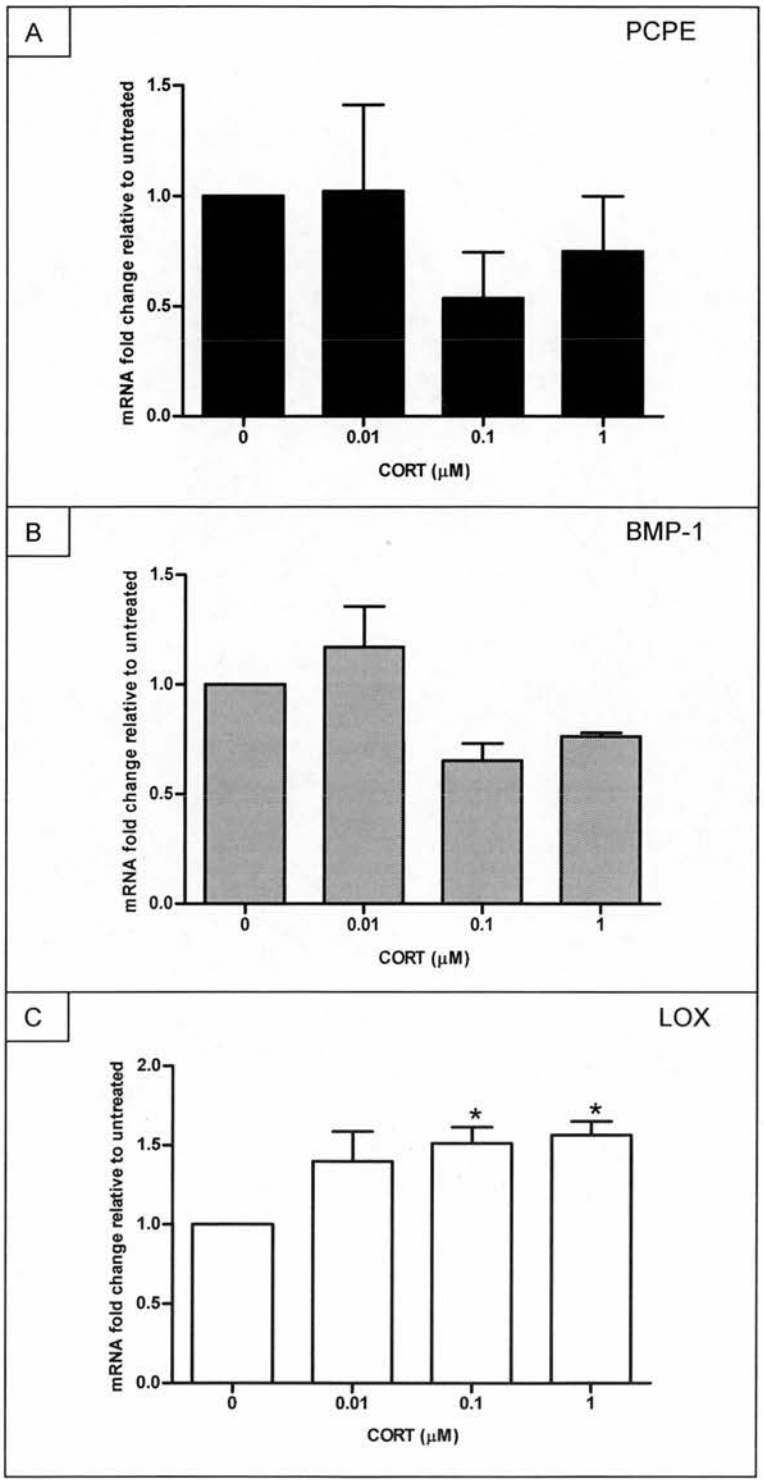

Figure 5.7. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from untreated immature female rats. Granulosa cells were cultured with increasing concentrations of CORT for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=4, * = $P<0.05$.

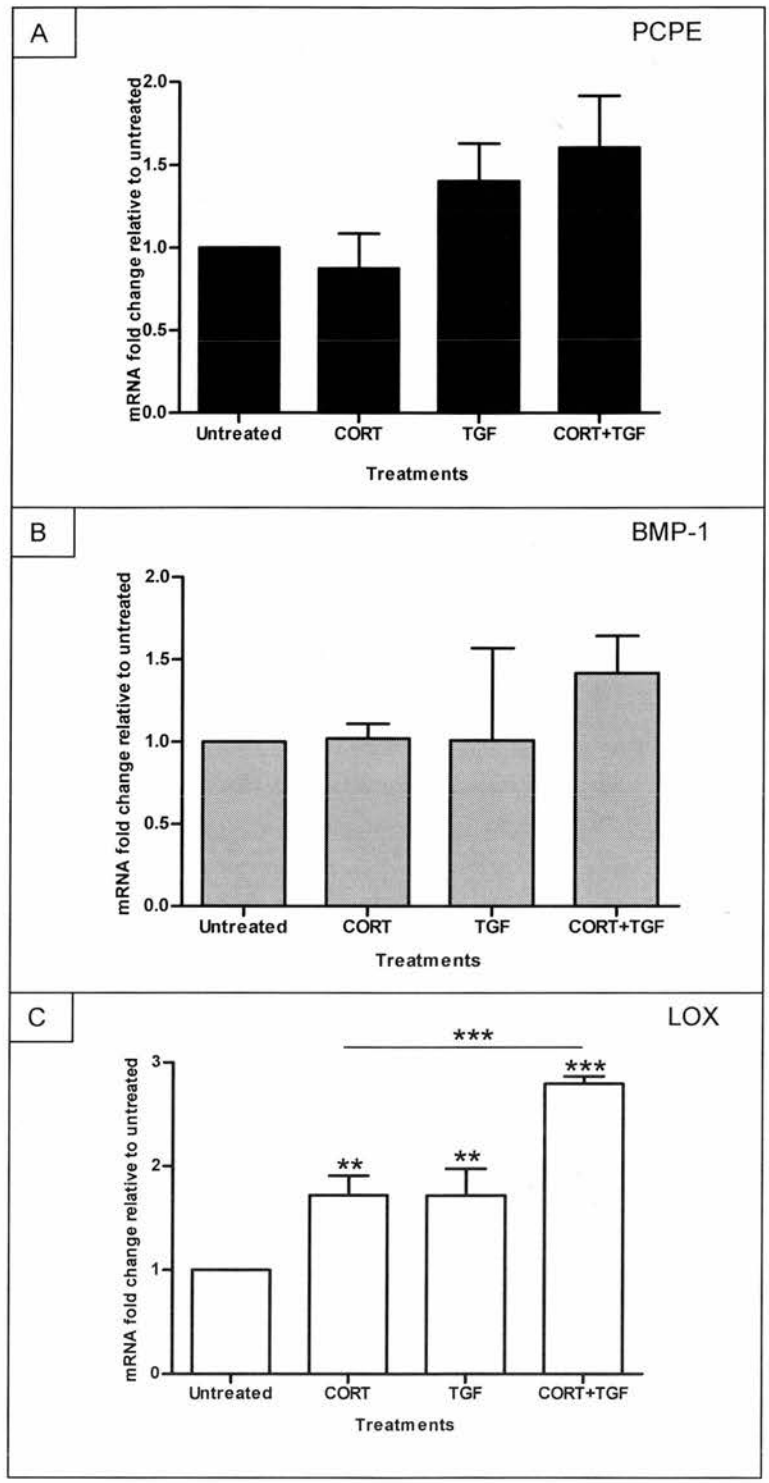

Figure 5.8. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from untreated immature female rats. Granulosa cells were cultured with CORT [1μM] and/or TGF-β [10ng/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3, * = P<0.05, ** = P<0.01, *** = P<0.001.

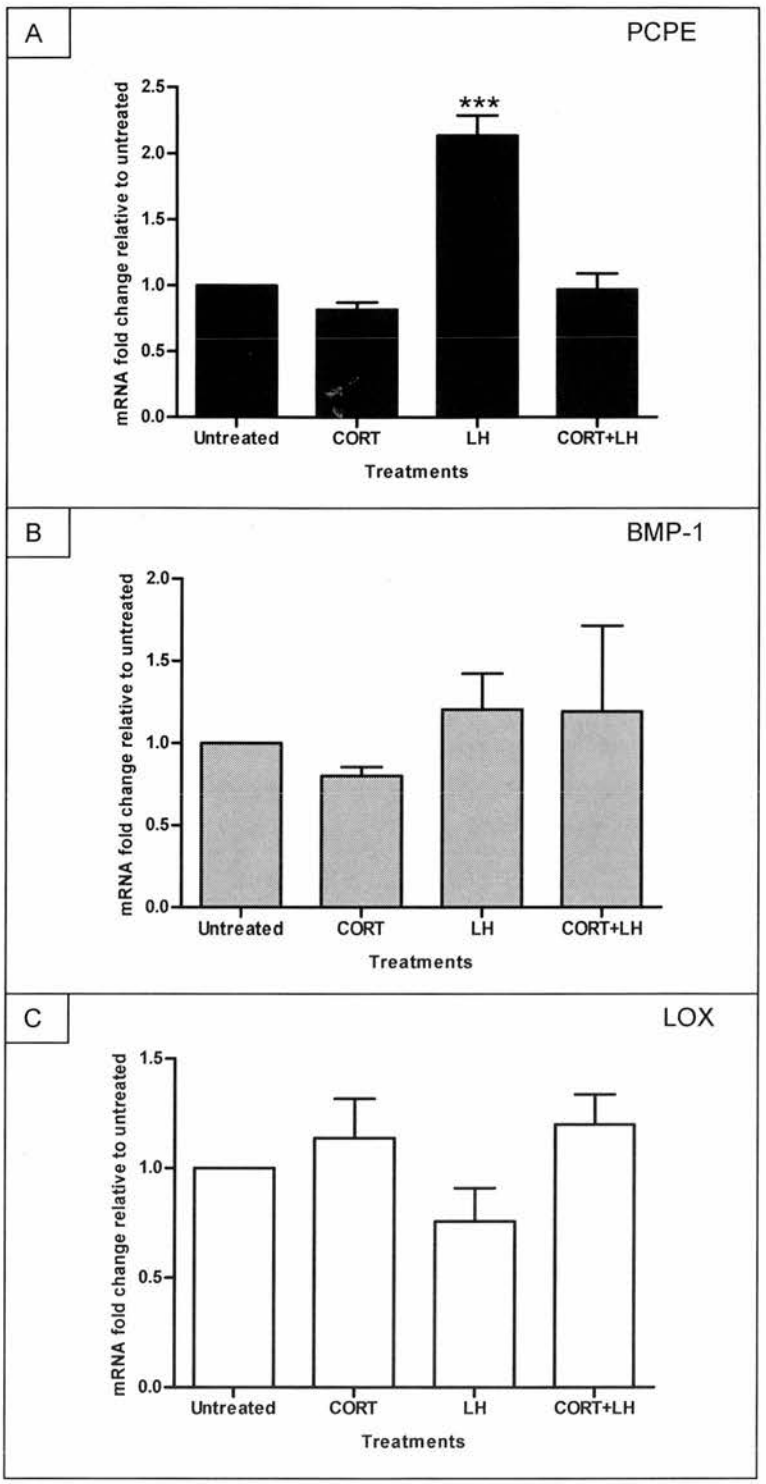

Figure 5.9. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from untreated immature female rats. Granulosa cells were cultured with CORT [1μM] and/or LH [0.01IU/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=4. *** = P<0.001.

5.3.4. PMSG-treated ovarian cells

In vivo treatment of PMSG in immature female rats simulates cells receiving FSH. This allows continued development of the antrum so the follicle reaches its preovulatory stage. Prior to ovulation granulosa cells begin to express LHR as a consequence of ovarian FSH (Burns *et al.* 2001). Therefore cells from these follicles respond to LH as well as growth factors. At this stage of development theca cells are responsive to progesterone as they have receptors for this steroid. Granulosa cells however do not acquire receptors until after the LH surge (Drummond 2006).

5.3.4.2. Granulosa cells

Granulosa cells from immature rats that had received PMSG to stimulate preovulatory follicular maturation were cultured with LH (0.01IU/ml), TGF-β (10ng/ml) or IL-1 (500pg/ml), with and without anti-inflammatory steroids: CORT or progesterone (PROG) (1μM). Readouts assessed were PCPE, BMP-1 and LOX mRNA, as well as LOX enzyme activity.

PCPE was not affected by CORT, BMP-1 (Fig. 5.10) was significantly decreased by CORT, while LOX gene expression and activity were significantly increased by CORT (1μM) (Fig. 5.10/11).

TGF-β did not alter BMP-1 and PCPE expression, but did significantly increased LOX mRNA expression with and without the addition of CORT (Fig. 5.11). LOX enzyme activity was increased by CORT plus TGF-β over the untreated control and over corticosterone alone (Fig. 5.11).

BMP-1 and LOX mRNA as well as LOX enzyme activity were unaffected by either LH or IL-1. However, LH given to granulosa cells with or without CORT significantly decreased PCPE gene expression (Fig. 5.12). The cytokine IL-1, used to simulate the inflammation of ovulation, significantly decreased PCPE mRNA expression (Fig. 5.13) when given alone or with corticosterone.

No significant differences were observed for any of the genes when PROG was given to cells (Fig. 5.14/15).

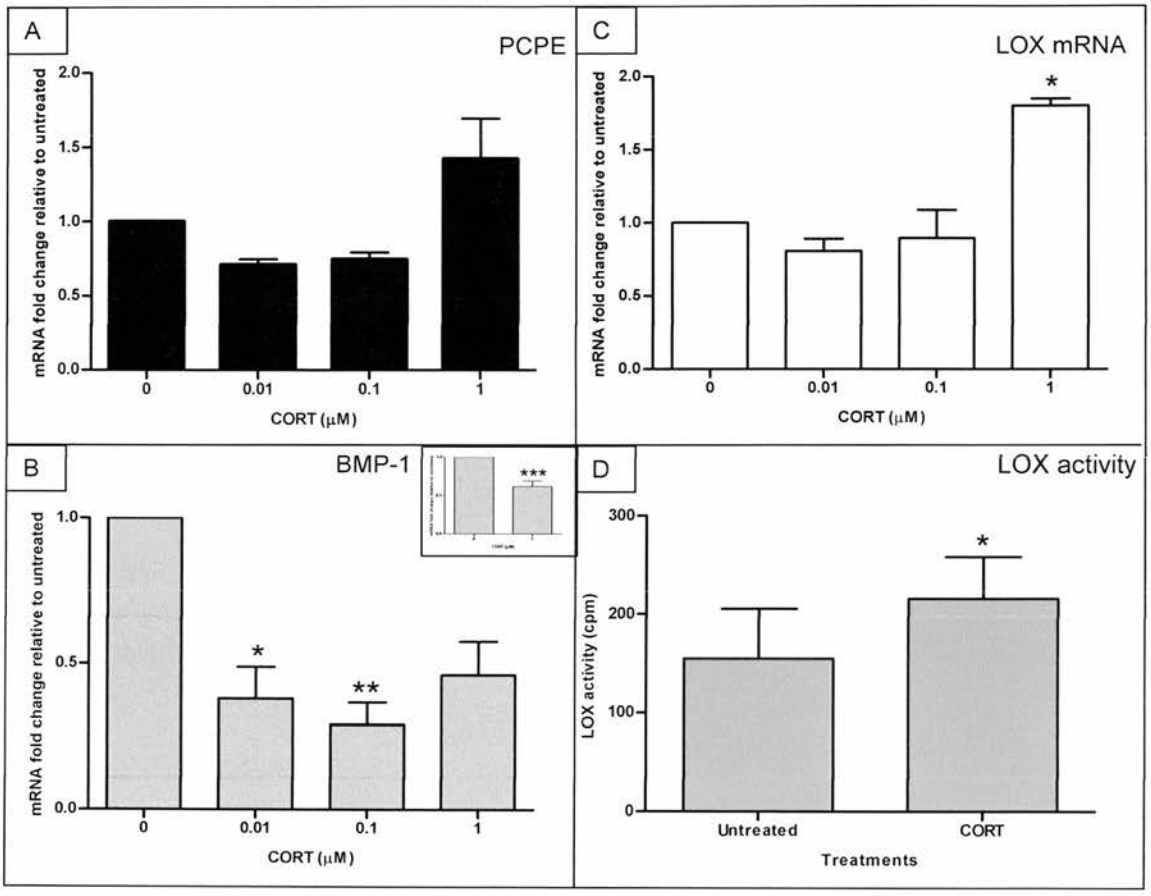

Figure 5.10. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured granulosa cells from PMSG-treated immature female rats. Granulosa cells were cultured with increasing concentrations of CORT [0, 0.01, 0.1, 1μM] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=4. Insert for BMP-1 shows CORT [0μM] compared to CORT [1μM] for N = 11. * = P = P<0.05, ** = P<0.01, *** = P<0.001.

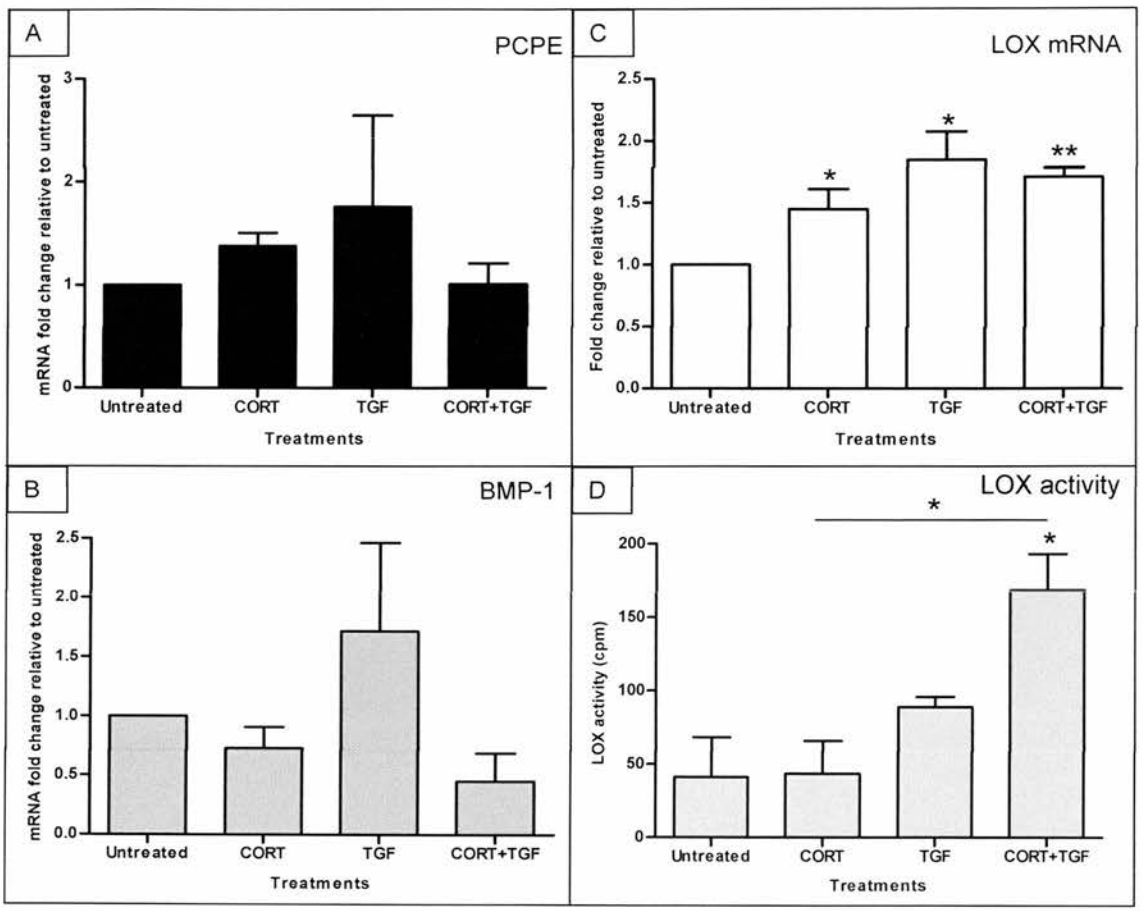

Figure 5.11 PCPE (A), BMP-1 (B), LOX (C) mRNA expression levels and LOX enzyme activity (D) levels in cultured granulosa cells from PMSG-treated immature female rats. Granulosa cells were cultured with CORT [1μM] and/or TGF-β [10ng/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for LOX mRNA levels using qRT-PCR as described in Section 2.7. Culture medium was assessed for LOX enzyme activity using the LOX tritium vacuum distillation enzyme assay as described in Section 2.8.1. N=3, * = $P<0.05$, ** = $P<0.01$.

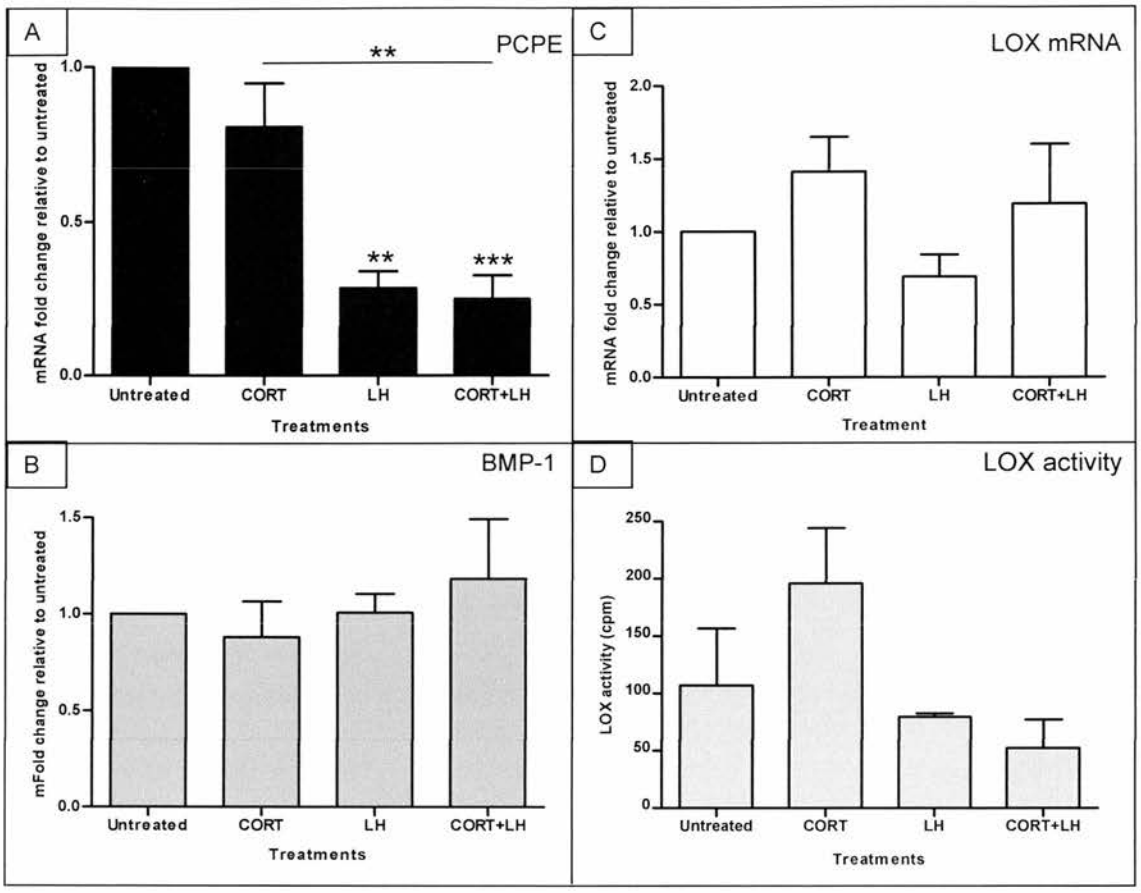

Figure 5.12. PCPE (A), BMP-1 (B), LOX (C) mRNA expression levels and LOX enzyme activity (D) levels in cultured granulosa cells from PMSG-treated immature female rats. Granulosa cells were cultured with CORT [1μM] and/or LH [0.01IU/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for LOX mRNA levels using qRT-PCR as described in Section 2.7. Culture medium was assessed for LOX enzyme activity using the LOX tritium vacuum distillation enzyme assay as described in Section 2.8.1. N=6. ** = P<0.01, *** = P<0.01.

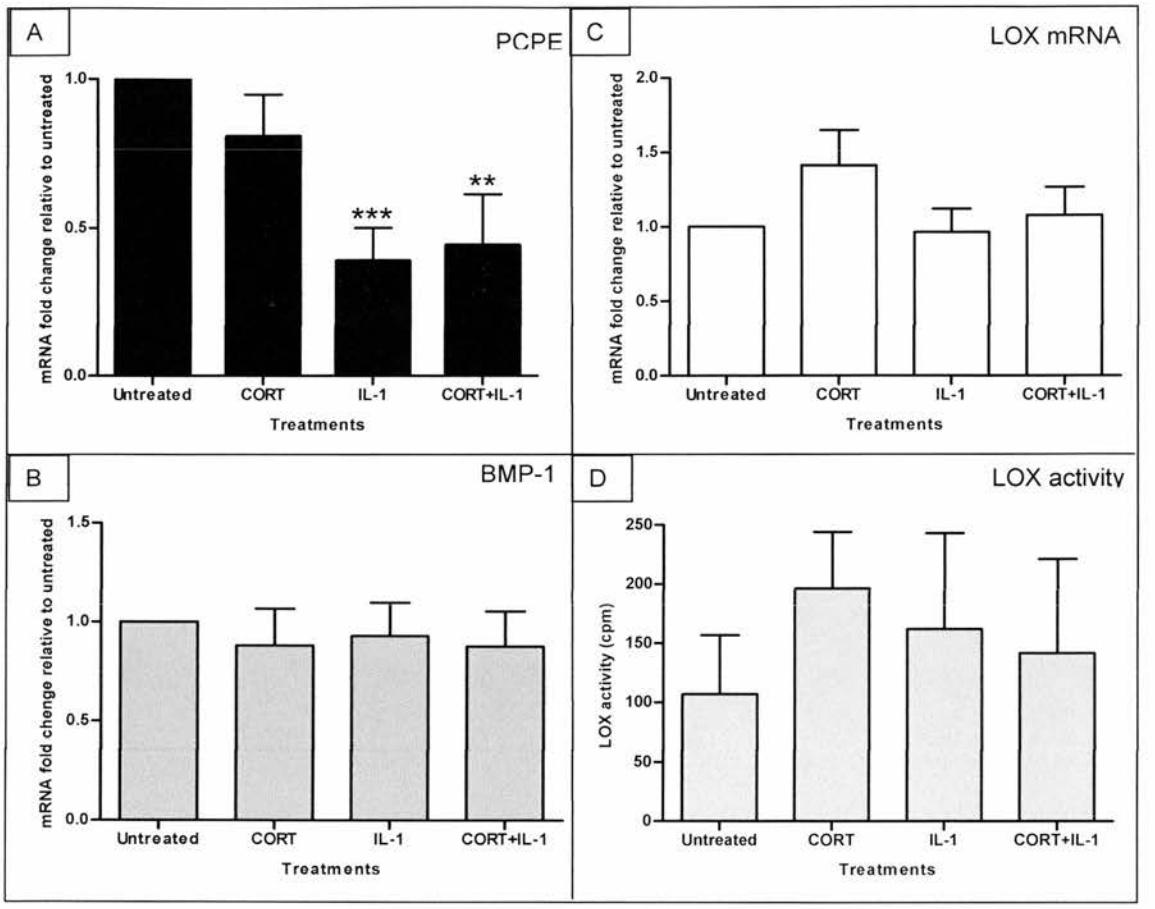

Figure 5.13. PCPE (A), BMP-1 (B), LOX (C) mRNA expression levels and LOX enzyme activity (D) levels in cultured granulosa cells from PMSG-treated immature female rats. Granulosa cells were cultured with CORT [1μM] and/or IL-1 [500pg/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for LOX mRNA levels using qRT-PCR as described in Section 2.7. Culture medium was assessed for LOX enzyme activity using the LOX tritium vacuum distillation enzyme assay as described in Section 2.8.1. N=6. ** = P<0.01, *** = P<0.01.

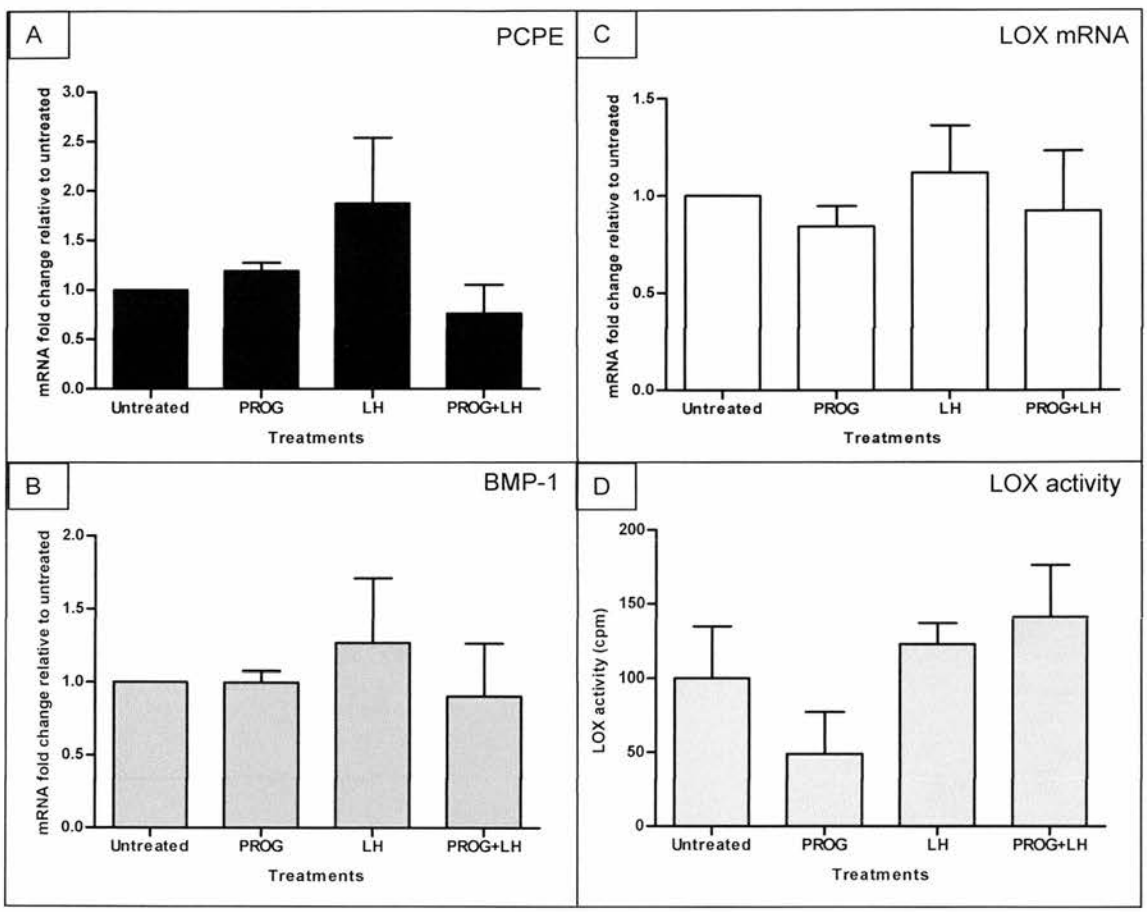

Figure 5.14. PCPE (A), BMP-1 (B), LOX (C) mRNA expression levels and LOX enzyme activity (D) levels in cultured granulosa cells from PMSG-treated immature female rats. Granulosa cells were cultured with PROG [10μM] and/or LH [0.01IU/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for LOX mRNA levels using qRT-PCR as described in Section 2.7. Culture medium was assessed for LOX enzyme activity using the LOX tritium vacuum distillation enzyme assay as described in Section 2.8.1. N=3.

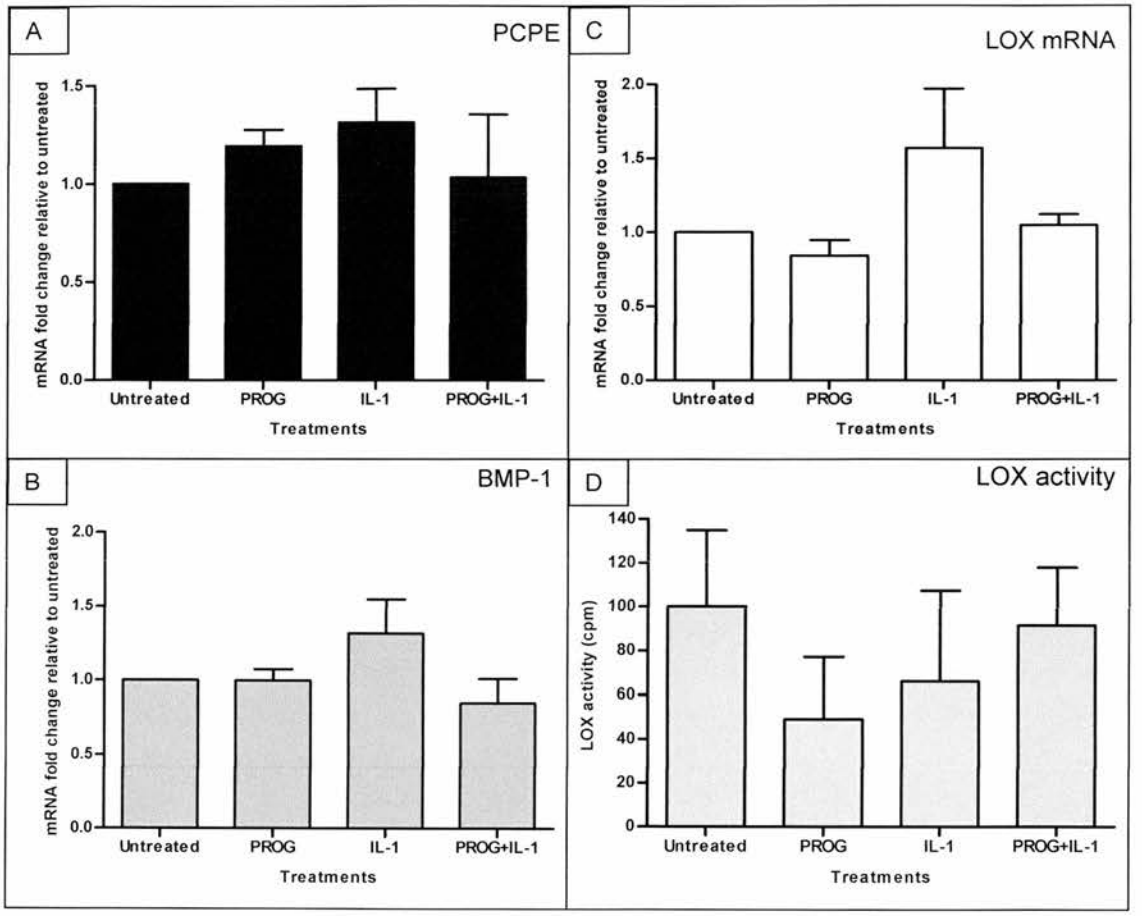

Figure 5.15. PCPE (A), BMP-1 (B), LOX (C) mRNA expression levels and LOX enzyme activity (D) levels in cultured granulosa cells from PMSG-treated immature female rats. Granulosa cells were cultured with PROG [10μM] and/or IL-1 [500pg/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for LOX mRNA levels using qRT-PCR as described in Section 2.7. Culture medium was assessed for LOX enzyme activity using the LOX tritium vacuum distillation enzyme assay as described in Section 2.8.1. N=3. ** = P<0.01, *** = P<0.01.

5.3.4.2. Theca cells

Theca cells from 21-day old female Wistar rats treated *in vivo* with PMSG were quantitatively assessed for mRNA levels of the collagen pathway biology genes. Theca cells were cultured with CORT (0μM, 0.01μM, 0.1μM, 1μM), TGF-β (10ng/ml), LH (0.01IU/ml), IL-1 (500pg/ml) or PROG (10μM).

CORT significantly down-regulated BMP-1 and PCPE mRNA, whilst up-regulating LOX mRNA (Fig. 5.16).

TGF-β did not alter the expression of BMP-1 or PCPE mRNAs. This growth factor did significantly up-regulate LOX mRNA expression (Fig. 5.17) with or without the addition of CORT. A significant difference was also observed between CORT and CORT plus TGF-β, and there was an additive effect of these two treatments.

LH did not affect BMP-1, but caused a suppression of PCPE mRNA expression (Fig. 5.18). Whilst LH alone did not influence LOX gene expression, the addition of CORT significantly up-regulated LOX mRNA (Fig. 5.18).

The pro-inflammatory IL-1 did not affect LOX or PCPE mRNA levels, however it did significantly down-regulate BMP-1 mRNA (Fig. 5.19).

PROG did induce a significant down-regulation of BMP-1 and PCPE (Fig. 5.20). PROG alone had no effect on LOX, but when cultured with a combination of PROG and IL-1, LOX expression was significantly increased over the expression with either treatment alone (Fig. 5.21).

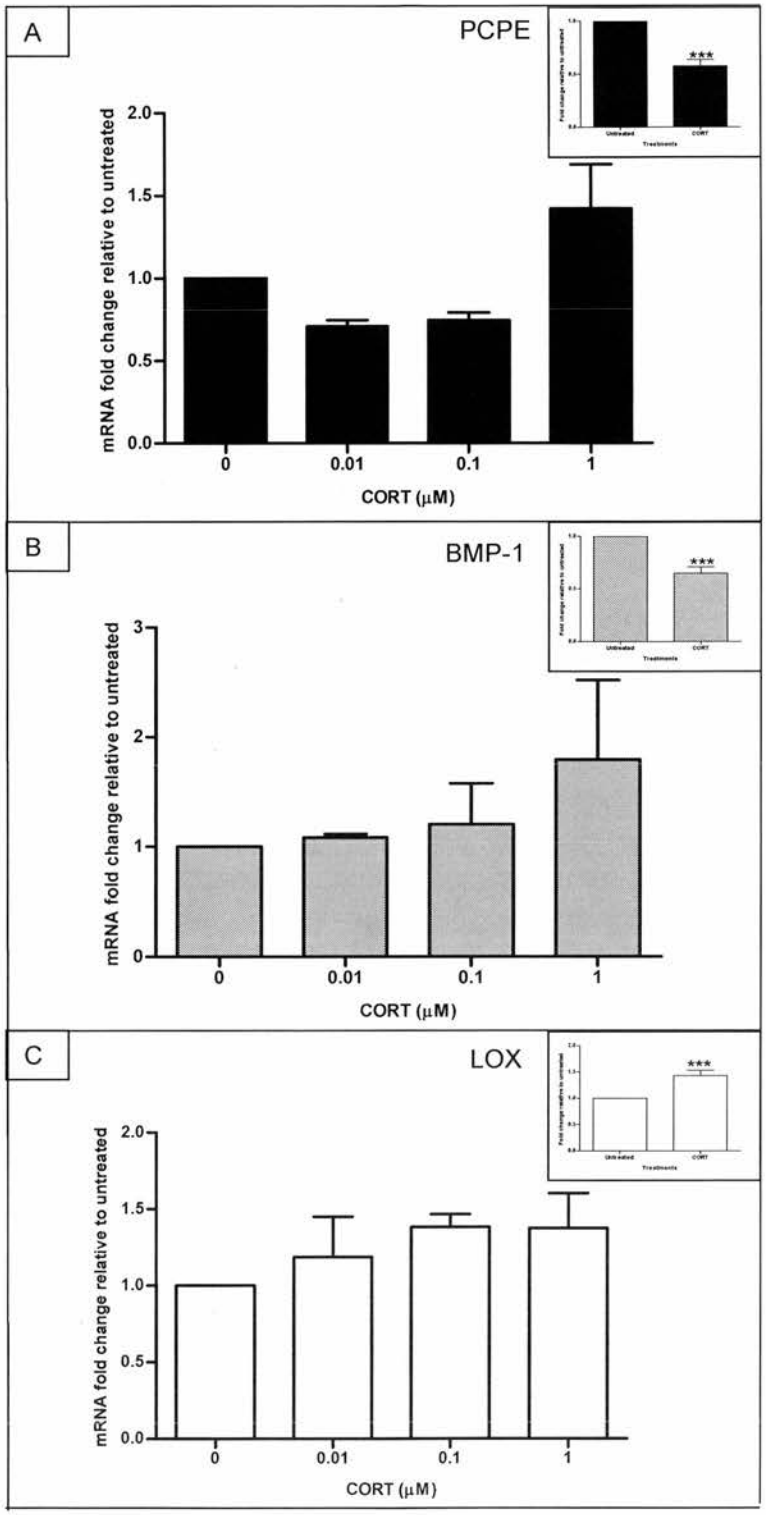

Figure 5.16. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from PMSG-treated immature female rats. Theca cells were cultured with increasing concentrations of CORT [0, 0.01, 0.1, 1μM] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=5. Inserts for all genes show CORT [0μM] compared to CORT [1μM] for N = 9. * = P<0.05, ** = P<0.01, *** = P<0.001.

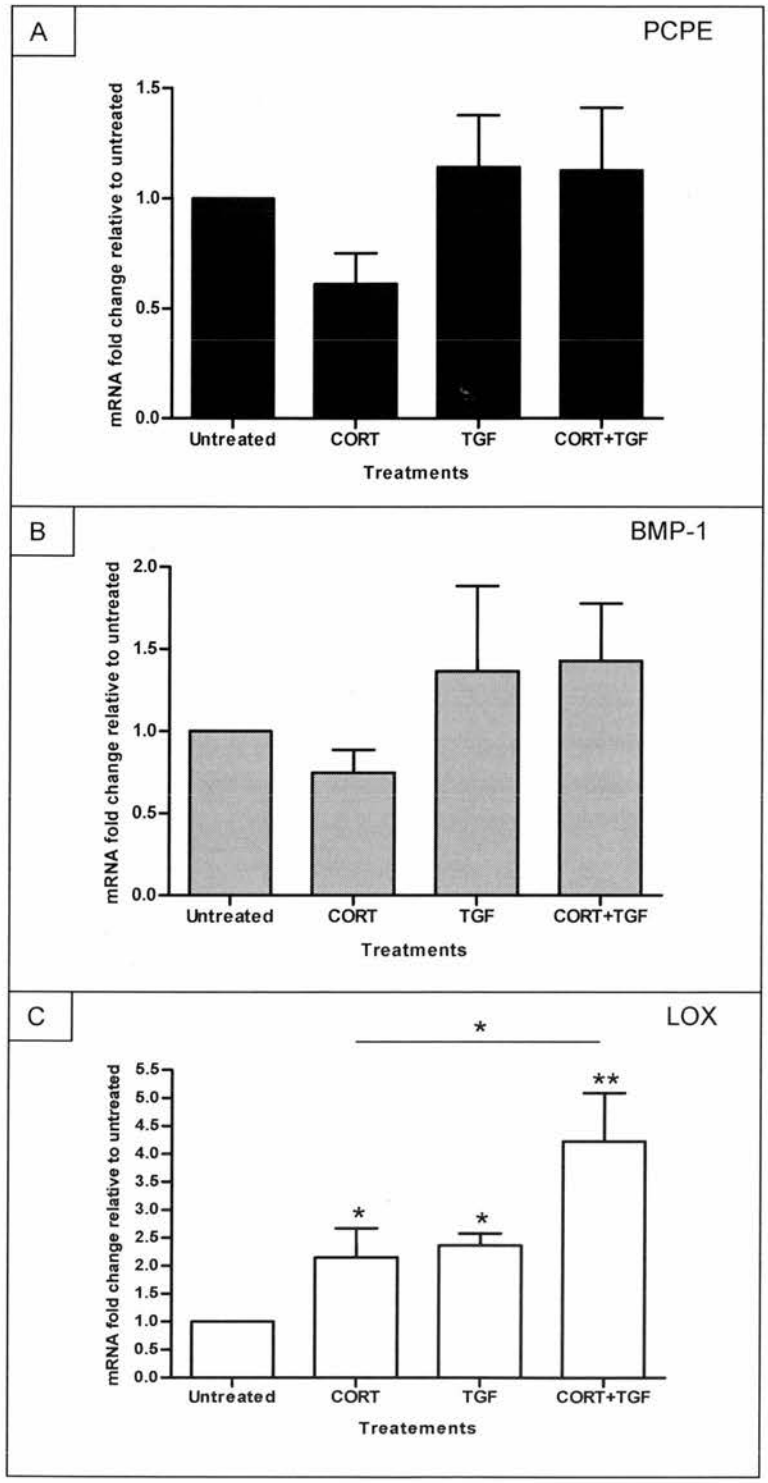

Figure 5.17. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from PMSG-treated immature female rats. Theca cells were cultured with CORT [1μM] and/or TGF-β [10ng/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3, * = P<0.05, ** = P<0.01, *** = P<0.001.

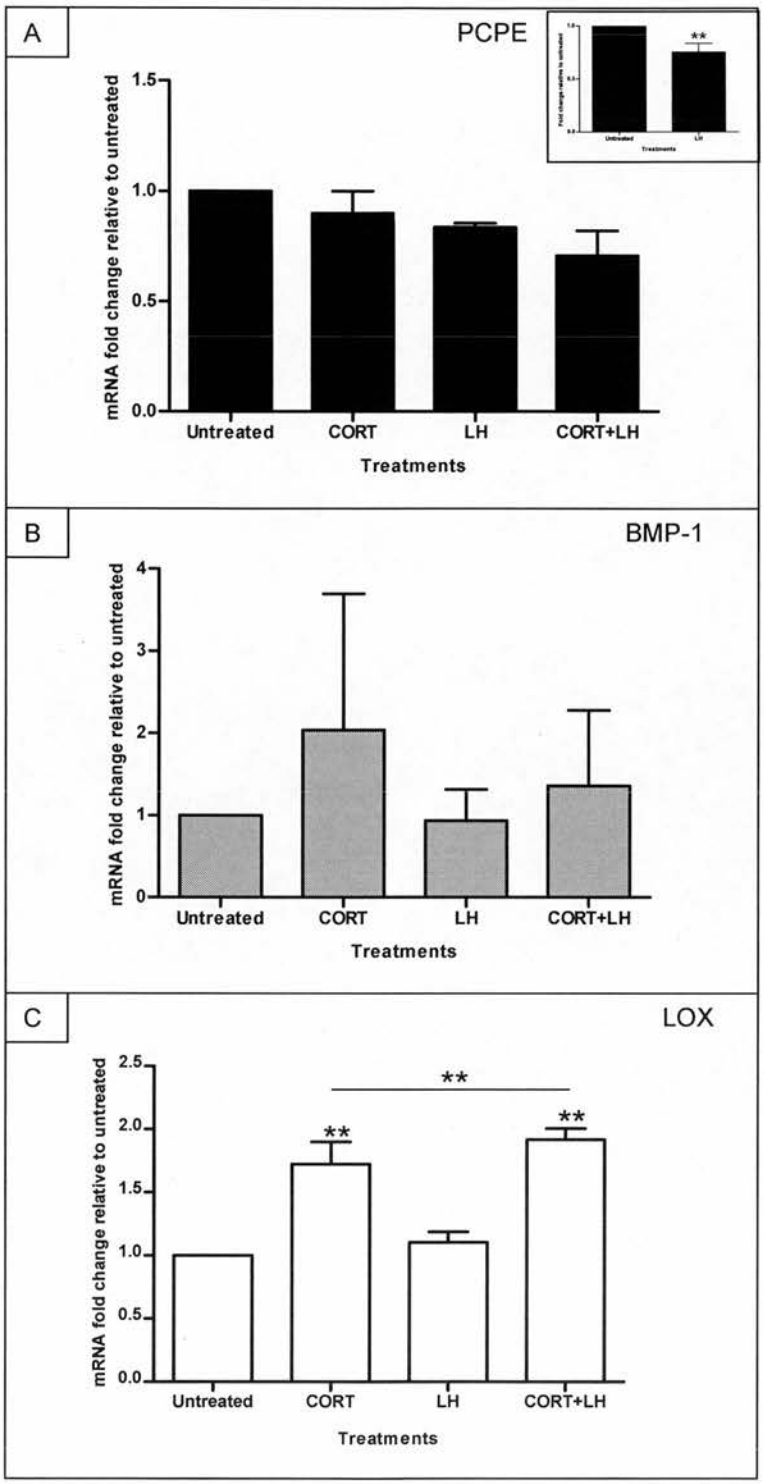

Figure 5.18. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from PMSG-treated immature female rats. Theca cells were cultured with CORT [1μM] and/or LH [0.01IU/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3. Insert for PCPE (A) shows untreated compared to LH [0.01IU/ml] alone for N = 4. * = P<0.05, ** = P<0.01, *** = P<0.001.

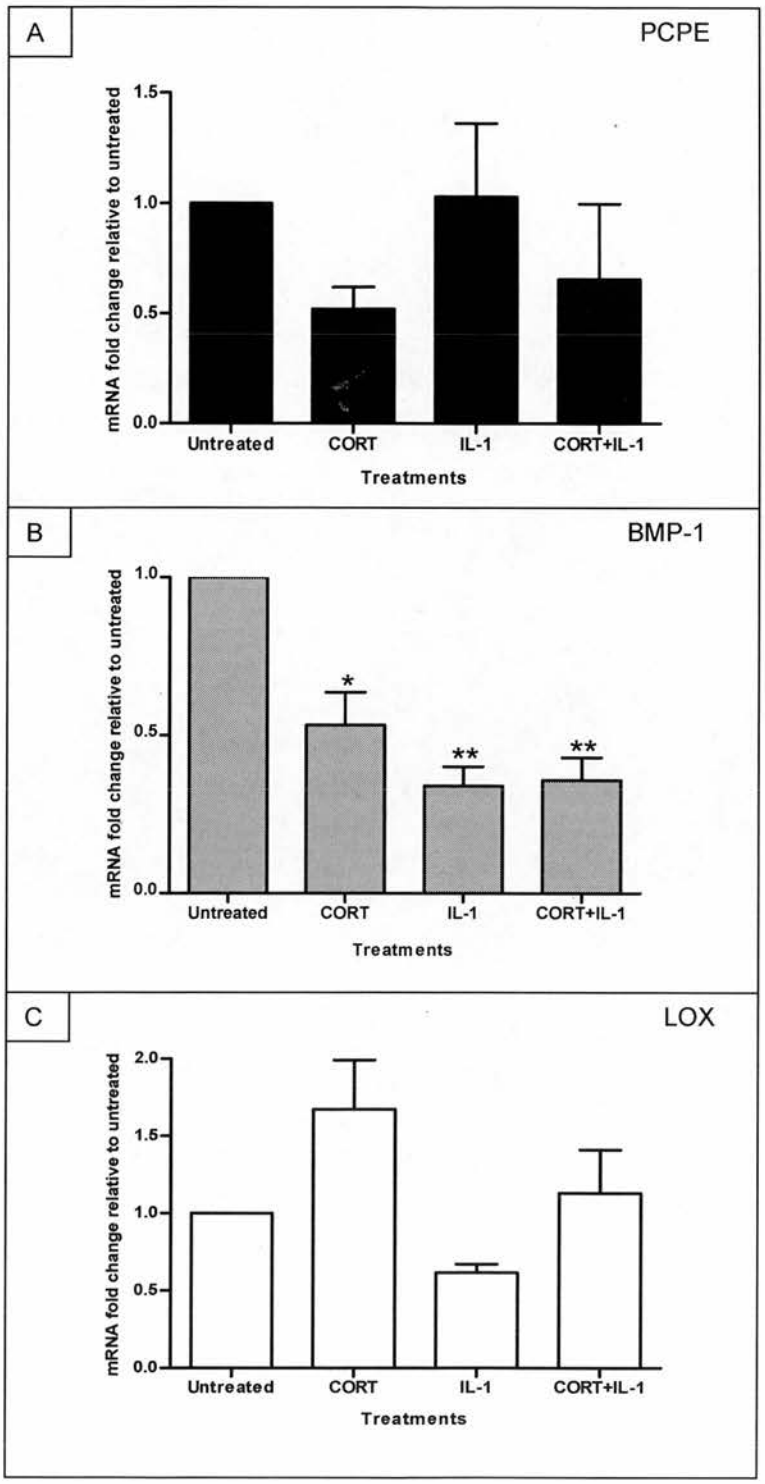

Figure 5.19. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from PMSG-treated immature female rats. Theca cells were cultured with CORT [1μM] and/or IL-1 [500pg/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=4. * = P<0.05, ** = P<0.01.

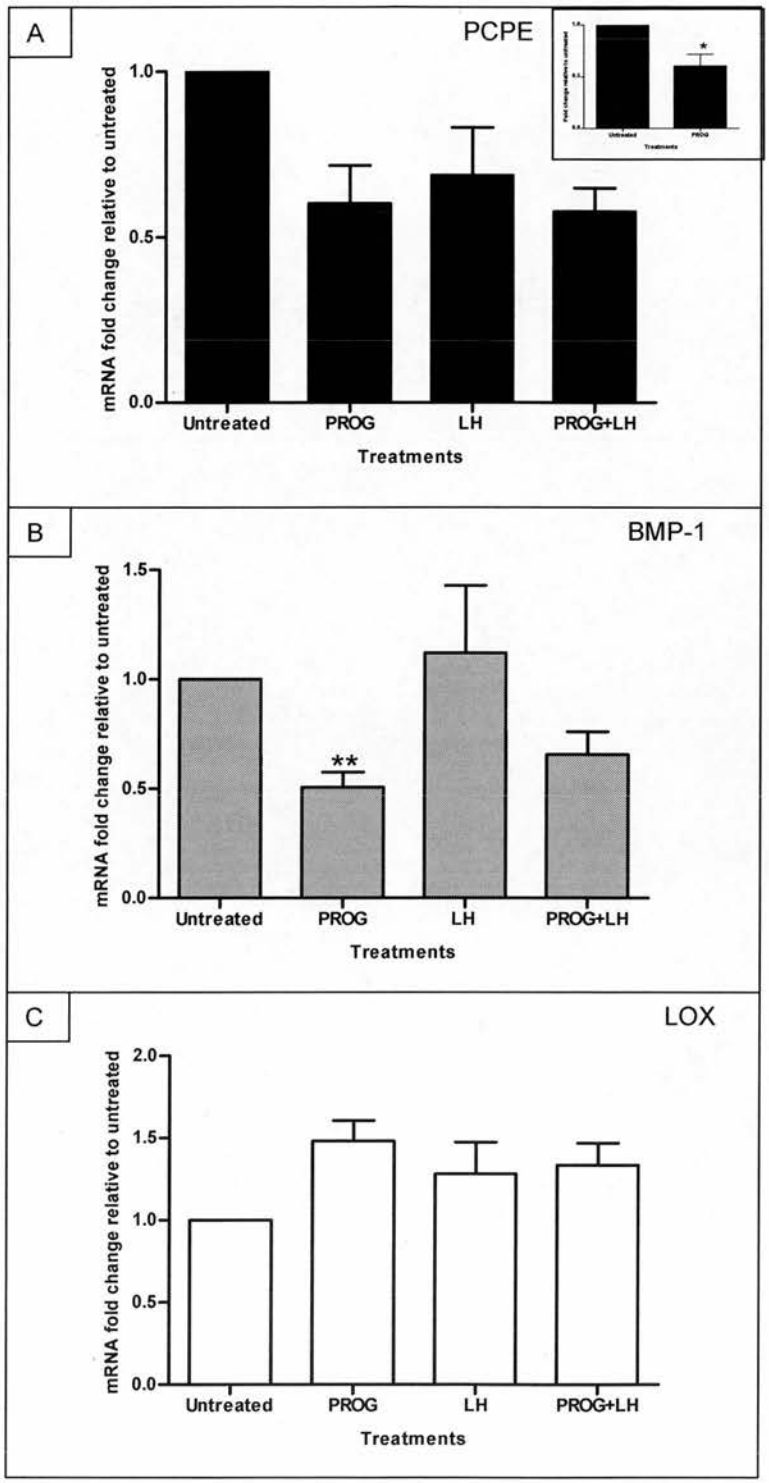

Figure 5.20. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from PMSG-treated immature female rats. Theca cells were cultured with PROG [10μM] and/or LH [0.01IU/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=4. Insert for PCPE (A) shows untreated compared to LH [0.01IU/ml] for N = 4. * = $P<0.05$, ** = $P<0.01$.

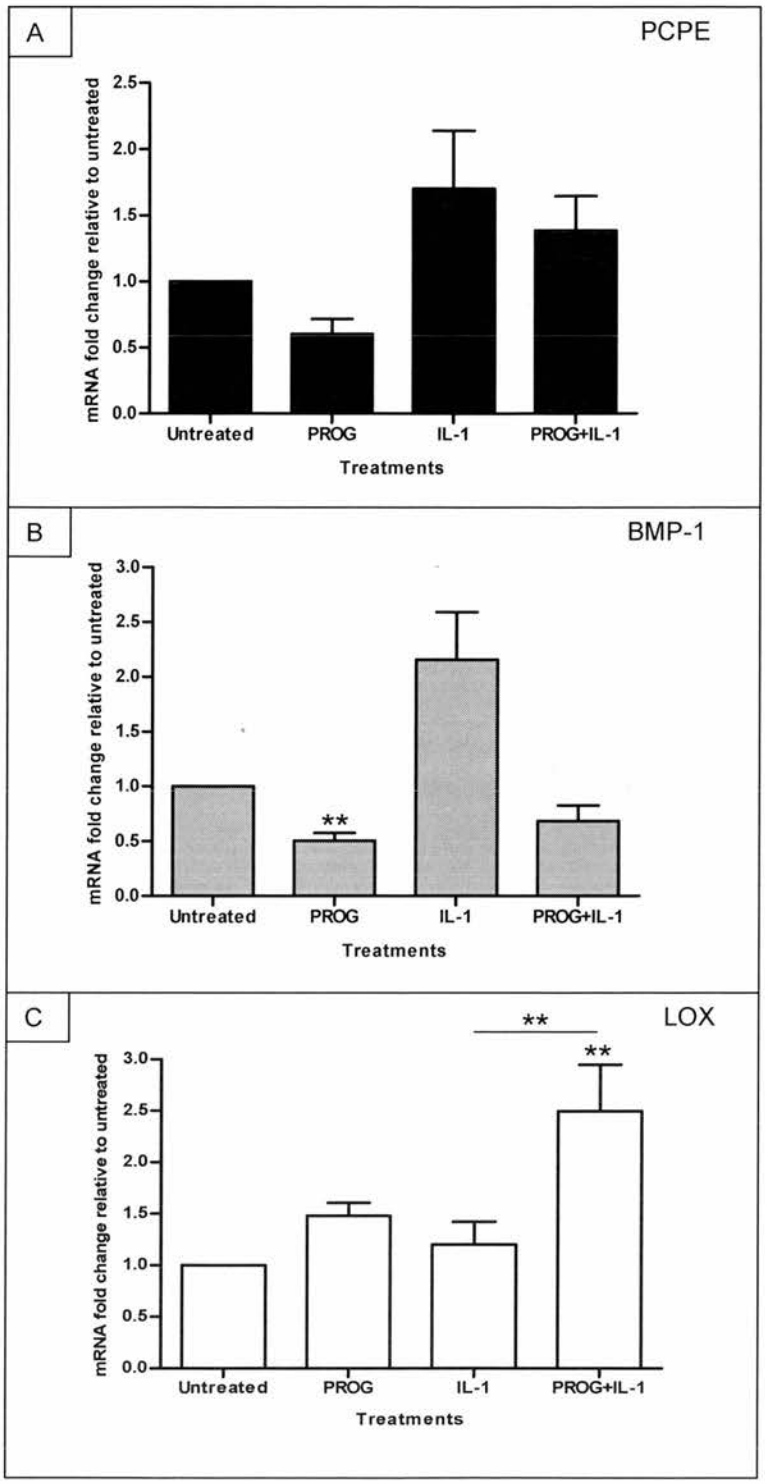

Figure 5.21. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured granulosa cells from PMSG-treated immature female rats. Granulosa cells were cultured with PROG [10μM] and/or IL-1 [500pg/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=4. ** = P<0.01.

5.3.5. Results for ovarian cells from rats treated with PMSG + hCG

PMSG replicates the actions of FSH, by also giving an *in vivo* treatment of hCG the action of the LH surge is simulated, producing a population of periovulatory follicles. The cells of these follicles have begun the process of luteinisation which commences with the LH surge. Thus expression of the LH receptor is being down-regulated so cells are less responsive to LH (Towns *et al.* 1999). Granulosa cells now respond to progesterone as the LH surge causes the expression of progesterone receptors (Drummond 2006).

5.3.5.1. Granulosa cells

Granulosa cells from 21-day old rats that had received PMSG and hCG were cultured with CORT (0, 0.01, 0.1, 1μM), TGF-β (10ng/ml), LH (0.01IU/ml), IL-1 (500pg/ml) and/or PROG (10μM), and gene expression levels measured for collagen pathway genes using real-time PCR. Measurements for LOX enzyme activity were unobtainable due to problems with the distillation assay.

PCPE gene expression was unchanged by any of the culture treatments. However, LOX mRNA expression was significantly increased in the presence of CORT (1μM) (Fig. 5.22). LOX mRNA in granulosa cells was significantly up-regulated by TGF-β, with or without CORT (Fig. 5.23). LH alone suppressed BMP-1 and LOX (Fig. 5.24), whilst LOX expression was also suppressed by IL-1 (Fig. 5.25). PROG alone caused a significant increase in LOX gene expression (Fig. 5.26). No combined effects were observed for the addition of progesterone with either LH or IL-1 (Fig. 5.26/27).

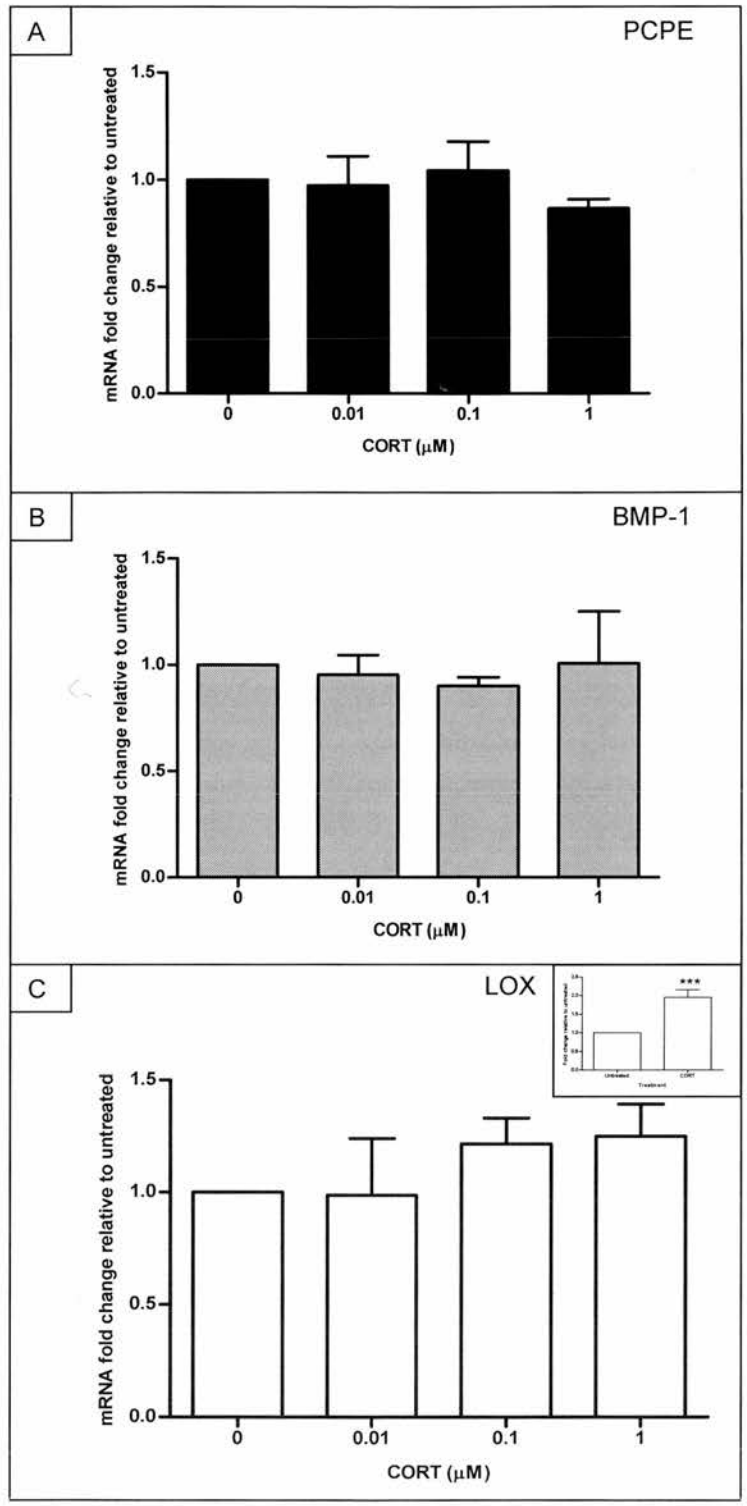

Figure 5.22. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured granulosa cells from PMSG-plus hCG-treated immature female rats. Granulosa cells were cultured with increasing concentrations of CORT [0, 0.01, 0.1, 1μM] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3. Insert for LOX (C) shows CORT [0μM] compared to CORT [1μM] for N = 6. *** =P<0.001.

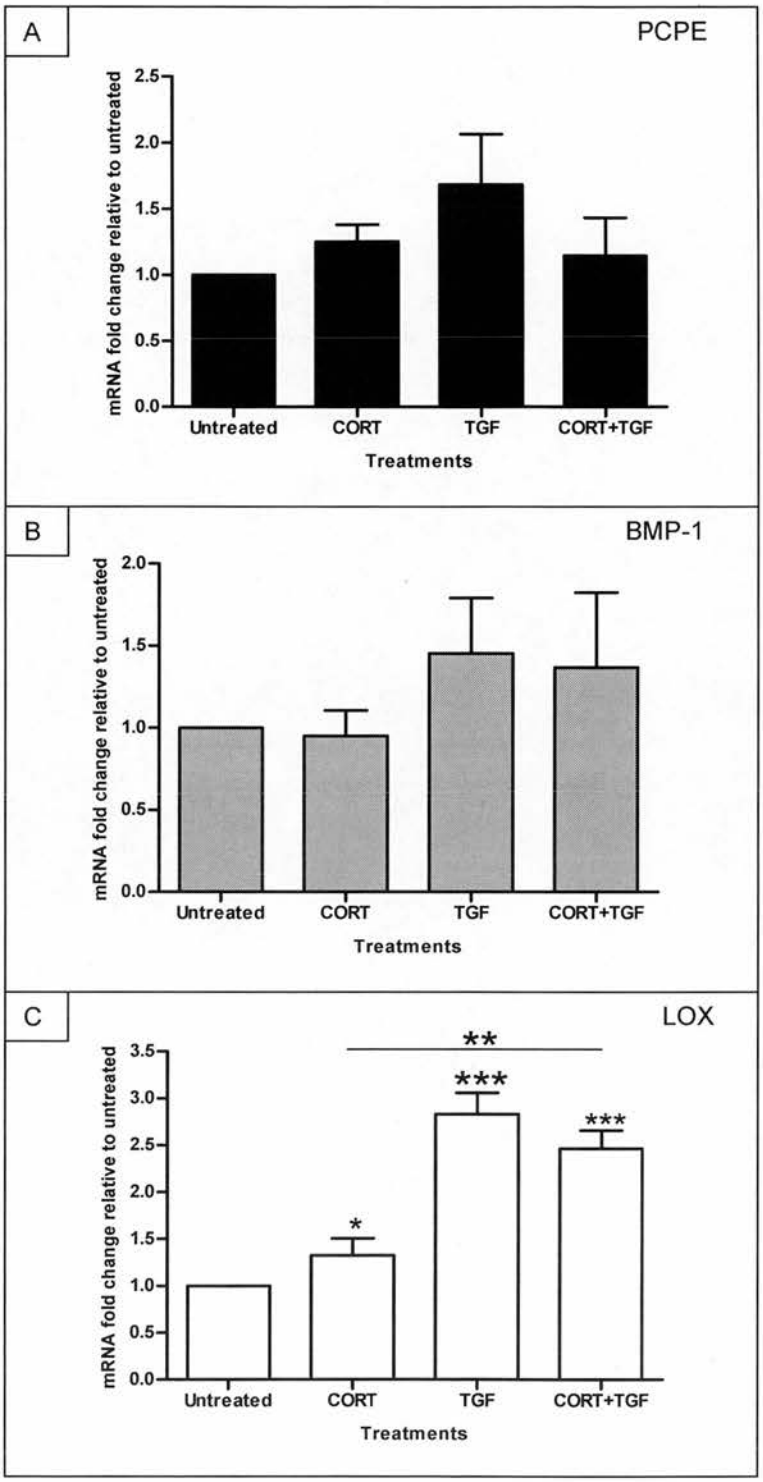

Figure 5.23. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured granulosa cells from PMSG- plus hCG-treated immature female rats. Granulosa cells were cultured with CORT [1μM] and/or TGF-β [10ng/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3. * = P<0.05, ** = P<0.01, *** = P<0.001.

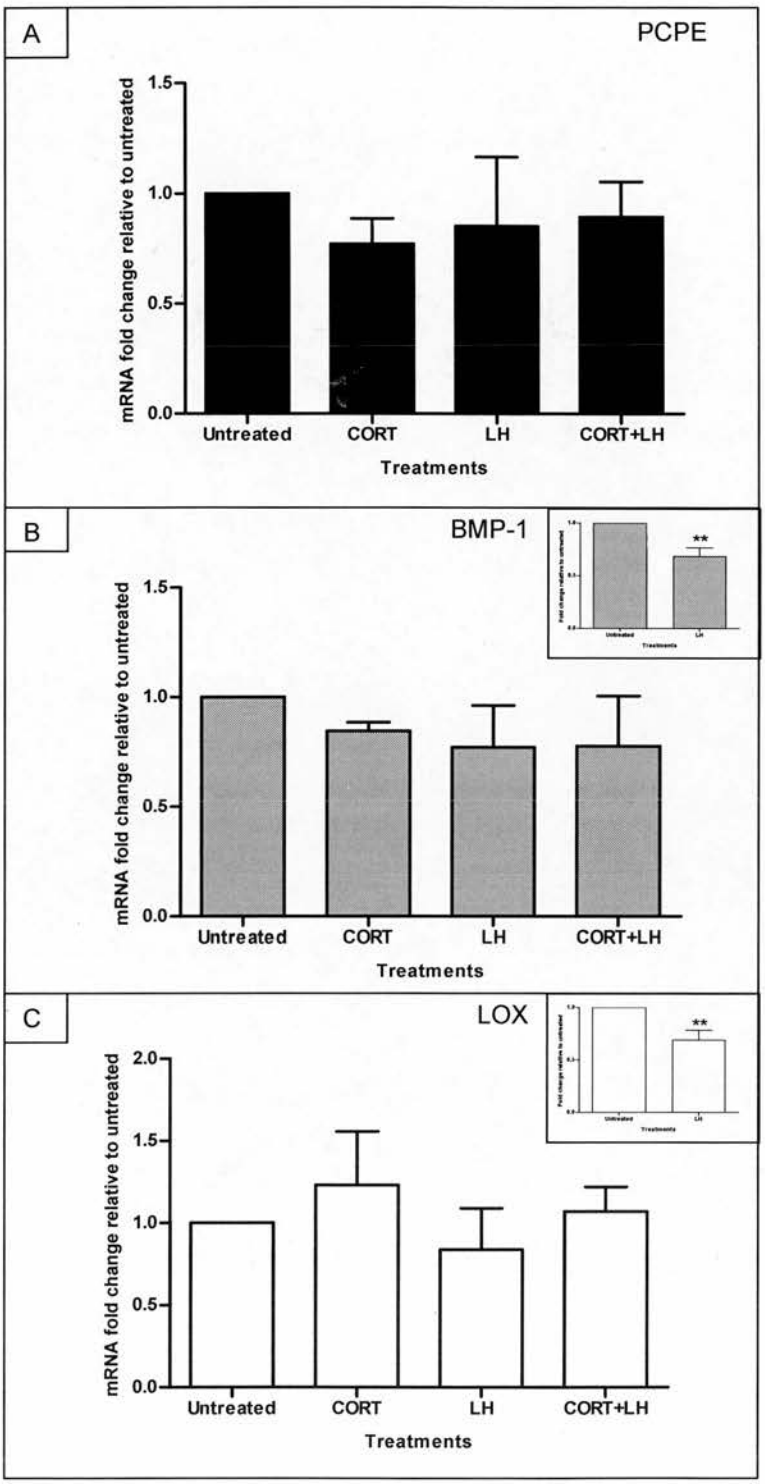

Figure 5.24. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured granulosa cells from PMSG- plus hCG-treated immature female rats. Granulosa cells were cultured with CORT [1μM] and/or IL-1 [500pg/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3. Inserts BMP-1 (B) and LOX (C) show untreated compared to LH [0.01IU/ml] for N = 5. ** = P<0.01.

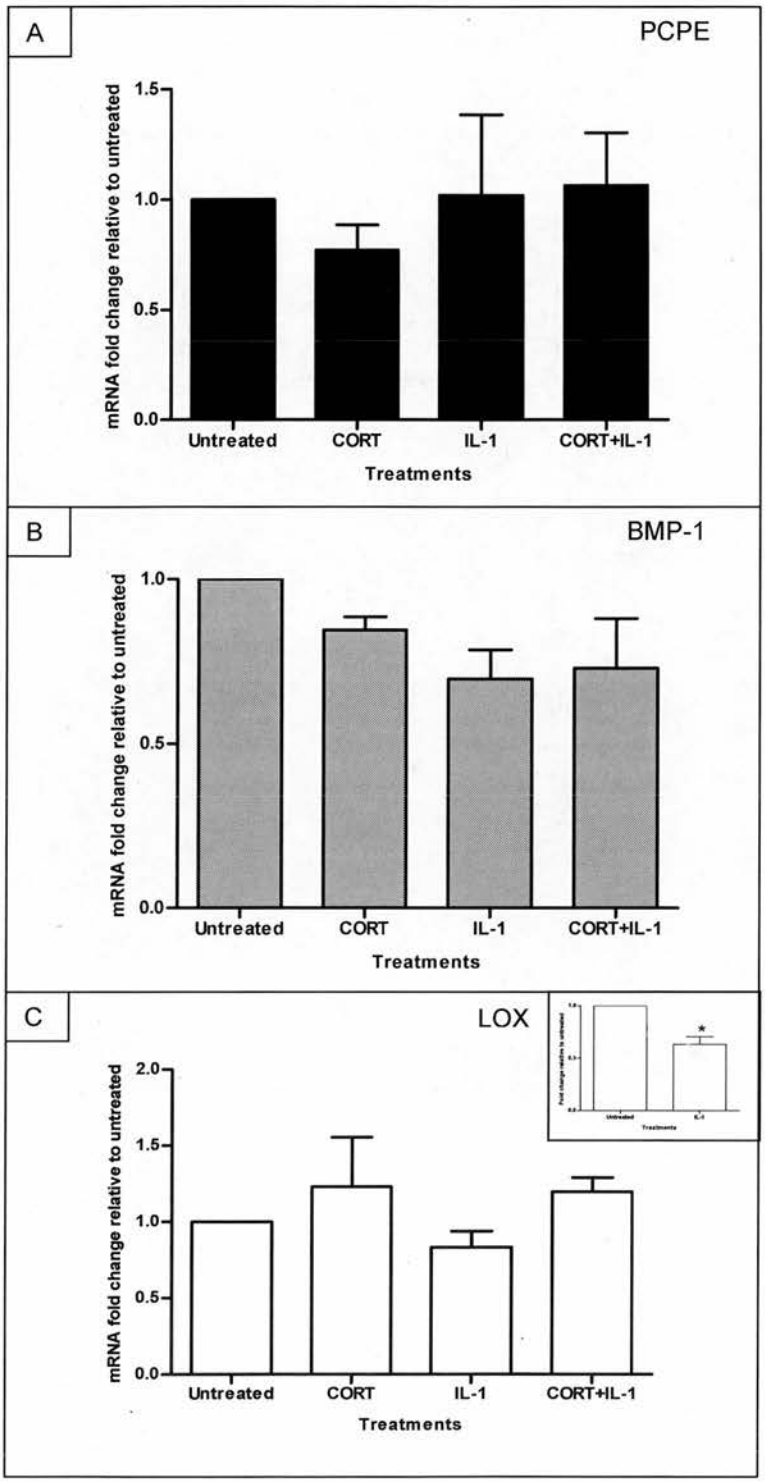

Figure 5.25. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured granulosa cells from PMSG- plus hCG-treated immature female rats. Granulosa cells were cultured with CORT [1μM] and/or IL-1 [500pg/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3. Insert BMP-1 (B) shows untreated compared to IL-1 [500pg/ml] for N = 7. * = $P<0.05$.

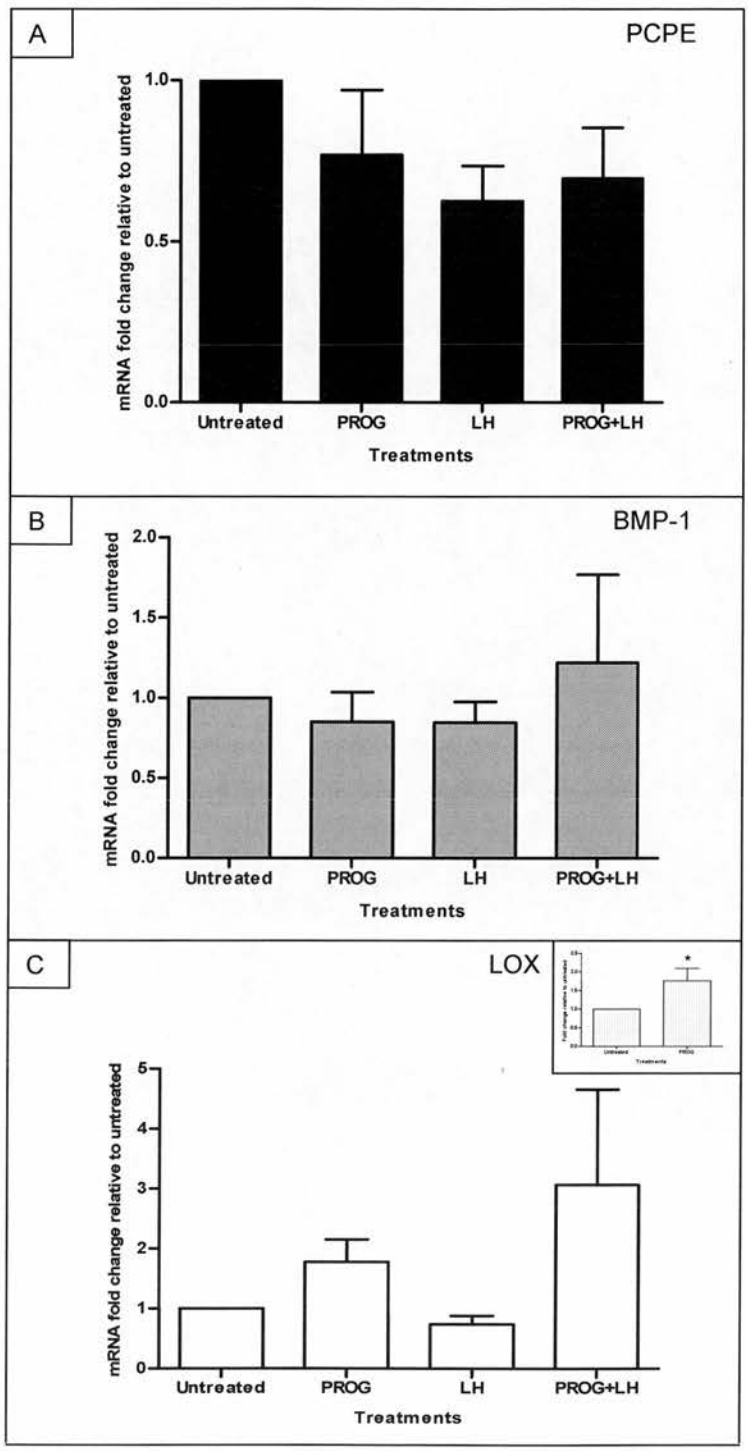

Figure 5.26. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured granulosa cells from PMSG- plus hCG-treated immature female rats. Granulosa cells were cultured with PROG [10μM] and/or LH [0.01IU/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=4. Insert LOX (C) shows Untreated compared to PROG [10μM] for N=7. * = $P<0.05$.

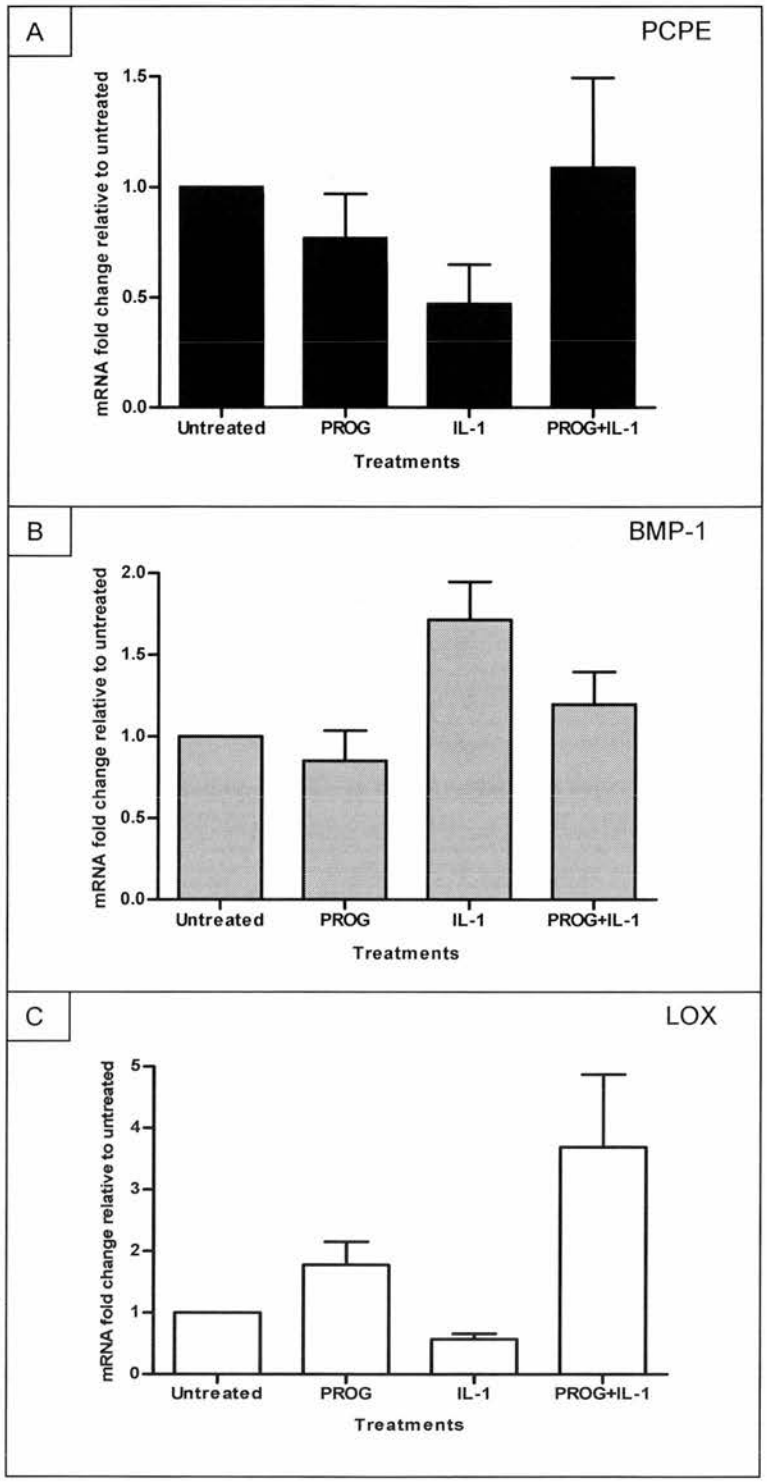

Figure 5.27. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from PMSG- plus hCG-treated immature female rats. Granulosa cells were cultured with PROG [10μM] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=4. * = P<0.05, ** = P<0.01, *** = P<0.001.

5.3.5.2. Theca cells

The expression of mRNA for LOX, BMP-1 and PCPE was measured in cultured theca cells from female rats that had been treated *in vivo* with PMSG followed by hCG. Cells were cultured with CORT (1μM), TGF-β (10ng/ml), LH (0.01IU/ml), IL-1 (500pg/ml) or PROG (10μM).

BMP-1 and PCPE were unaffected by CORT. LOX was significantly up-regulated by CORT (Fig. 5.28).

When theca cells were cultured with TGF-β, LOX expression was significantly increased with or without CORT (Fig. 5.29), but expression of both BMP-1 and PCPE was unaffected.

The presence of LH in culture had no effect on LOX gene expression but the addition of corticosterone significantly increased LOX expression over LH-treated cells (Fig. 5.30). LH caused an up-regulation of PCPE and a down-regulation of BMP-1 (Fig. 5.30).

The significant up-regulation of LOX by CORT was suppressed by the addition of IL-1 (Fig. 5.31), whilst not altering the expression of BMP-1 or PCPE.

PROG caused an up-regulation of LOX mRNA which just failed to reach significance (Fig. 5.32). PROG was also combined in culture treatments with LH and IL-1, but no differences were observed (Fig. 5.32/33).

Table 5.2. shows a summary of all the *in vitro* culture experiments described in this results section.

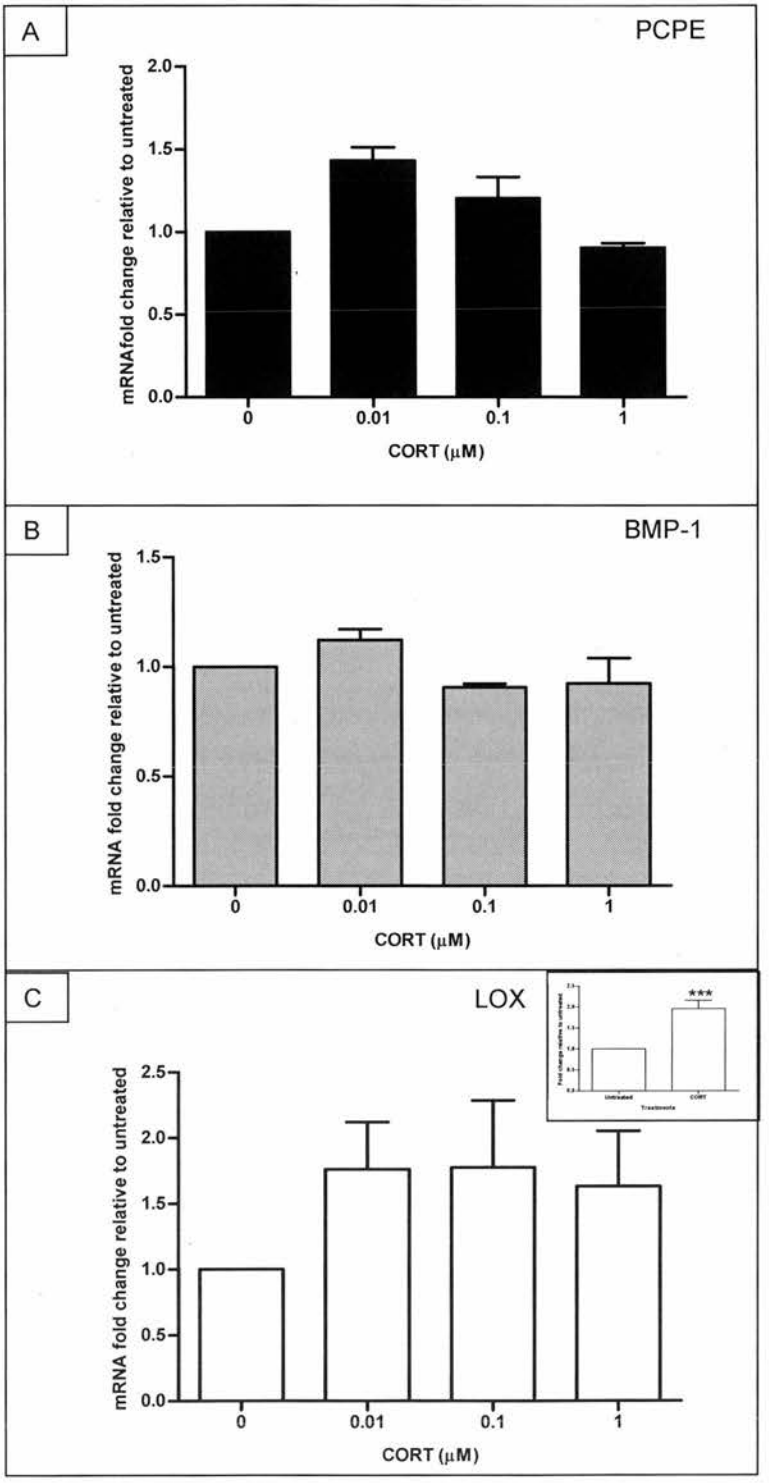

Figure 5.28. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from PMSG- plus hCG-treated immature female rats. Theca cells were cultured with increasing concentrations of CORT [0, 0.01, 0.1, 1μM] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3. Insert LOX (C) shows CORT [0μM] compared to CORT [1μM] for N = 13. * = P<0.001.

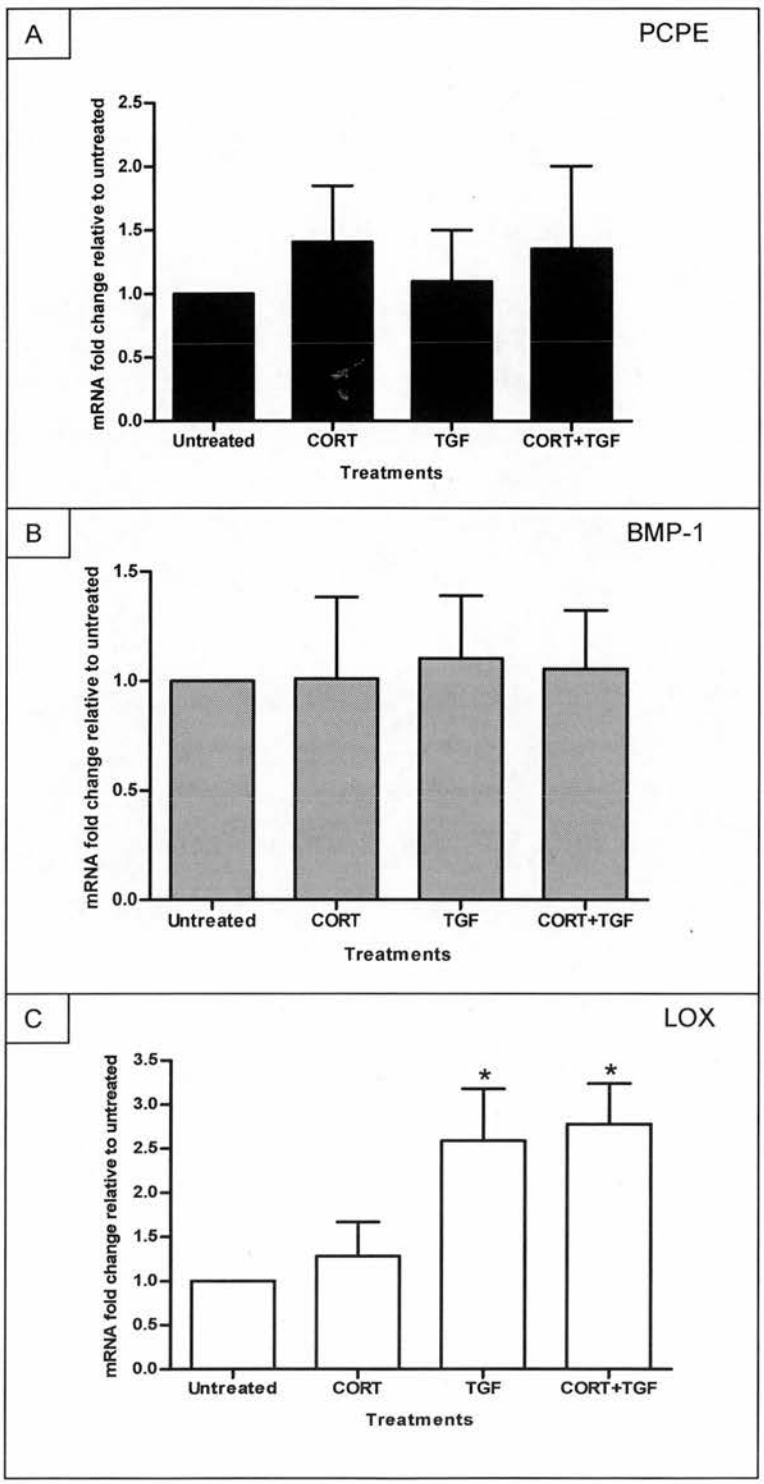

Figure 5.29. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from PMSG- plus hCG-treated immature female rats. Theca cells were cultured with CORT [1μM] and/or TGF-β [10n/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3. * =P<0.05.

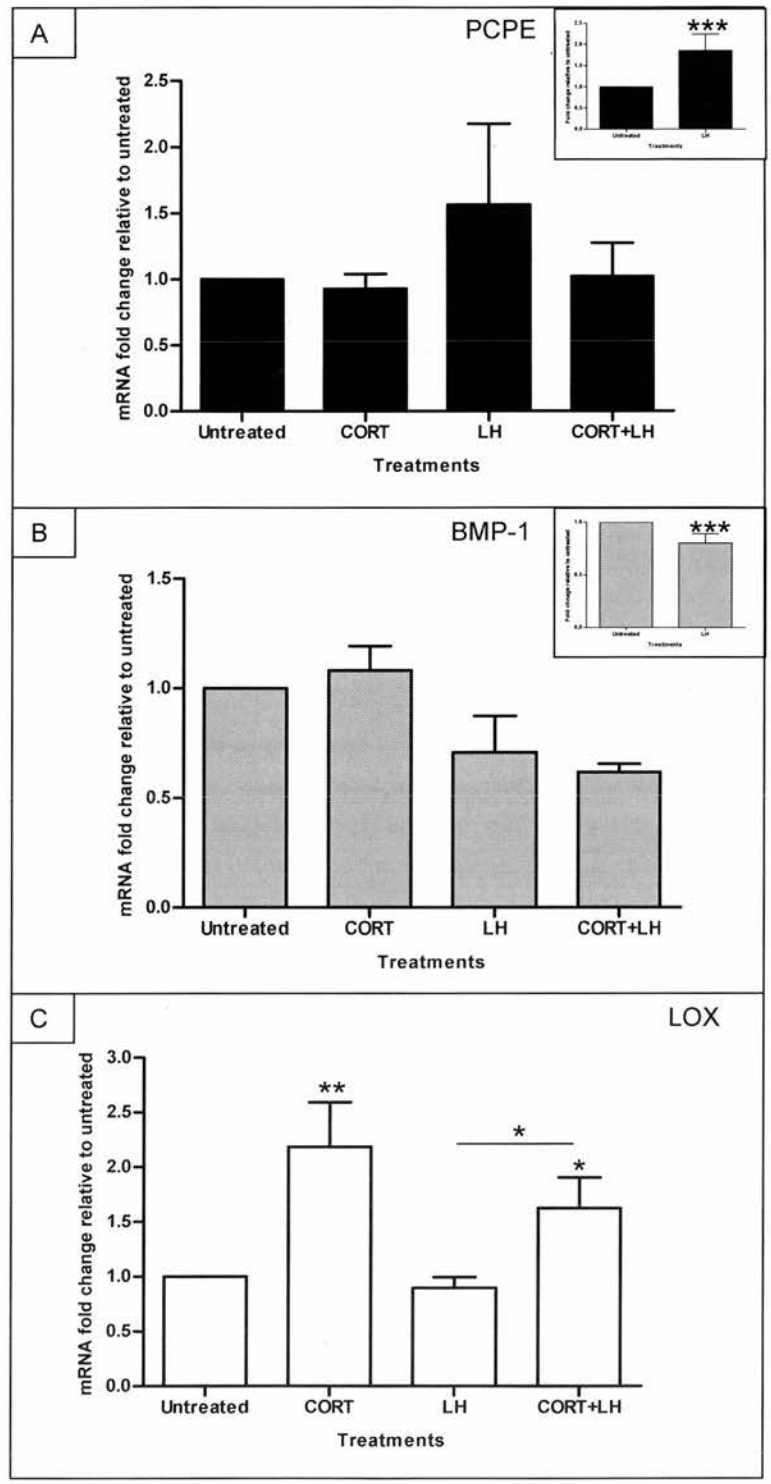

Figure 5.30. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from PMSG- plus hCG-treated immature female rats. Theca cells were cultured with CORT [1μM] and/or LH [0.01IU/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3. Inserts PCPE (A) and BMP-1 (B) show untreated compared to LH [0.01IU/ml] for N = 6. * = P<0.05, ** = P<0.01, *** =P<0.01.

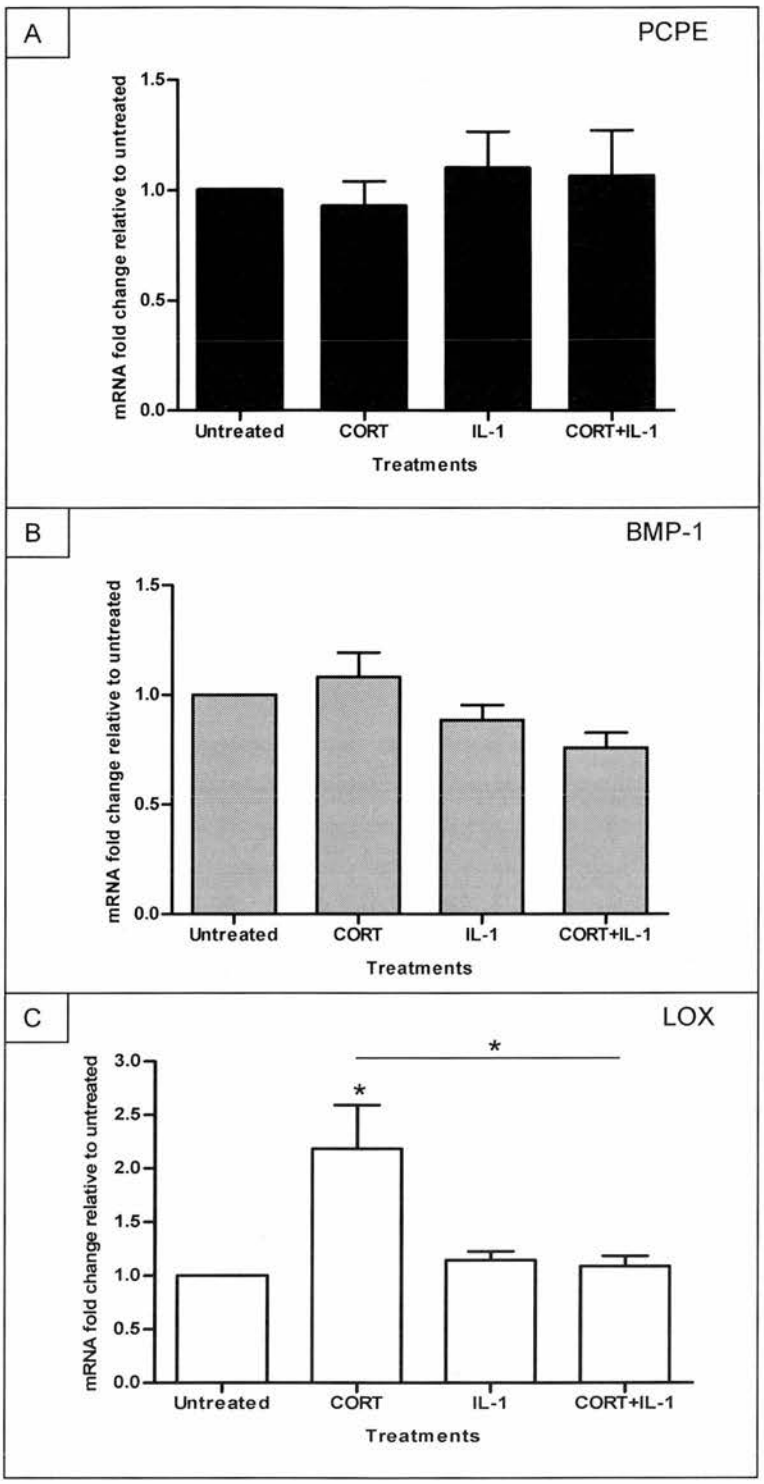

Figure 5.31. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from PMSG- plus hCG-treated immature female rats. Theca cells were cultured with CORT [1μM] and/or IL-1 [500pg/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3. * =P<0.05

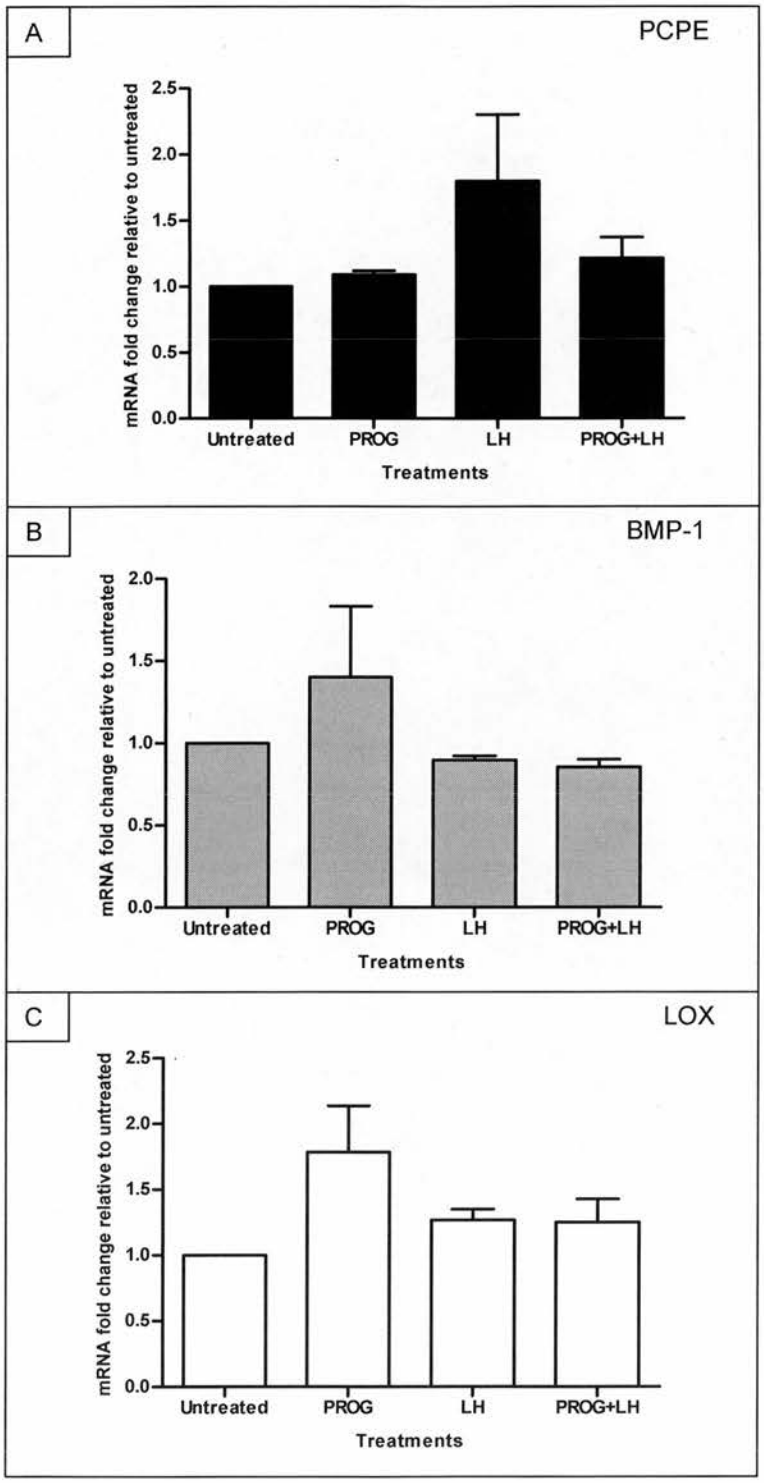

Figure 5.32. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from PMSG- plus hCG-treated immature female rats. Theca cells were cultured with PROG [10μM] and/or LH [0.01IU/ml] for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3.

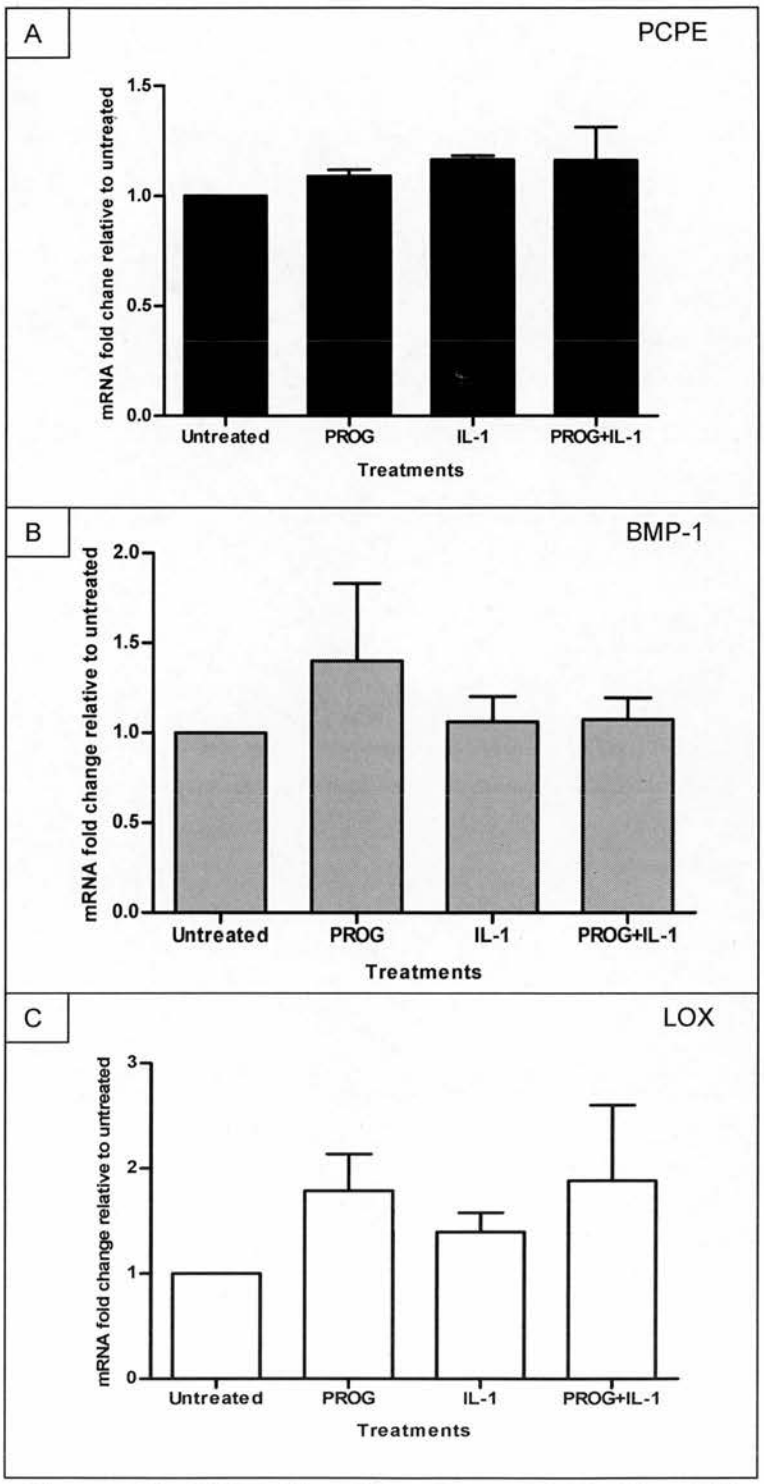

Figure 5.33. PCPE (A), BMP-1 (B) and LOX (C) mRNA expression levels in cultured theca cells from PMSG- plus hCG-treated immature female rats. Theca cells were cultured with increasing concentrations of CORT for 48h as described in Section 2.2. Resultant mRNA was measured for collagen pathway mRNA levels using qRT-PCR as described in Section 2.7. N=3.

Table 5.2. Results summary for the gene expression changes described above for the rat granulosa and theca cell culture experiments from animals that had either received no *in vivo* treatment or that had received in vivo treatment with gonadotrophins.

PCPE		**FSH**	**LH**	**TGF-β**	**CORT**	**PROG**	**IL-1**
Immature	Granulosa				↓		
	Theca		↑				
Preovulatory	Granulosa		↓				
	Theca		↓		↓	↓	
Periovulatory	Granulosa						
	Theca		↑				

BMP-1		**FSH**	**LH**	**TGF-β**	**CORT**	**PROG**	**IL-1**
Immature	Granulosa						
	Theca						
Preovulatory	Granulosa				↓		
	Theca				↓		↓
Periovulatory	Granulosa		↓				
	Theca		↓				

LOX		**FSH**	**LH**	**TGF-β**	**CORT**	**PROG**	**IL-1**
Immature	Granulosa			↑			
	Theca		↑	↑	↑		
Preovulatory	Granulosa			↑	↑		
	Theca			↑	↑		
Periovulatory	Granulosa		↓	↑	↑	↑	↓
	Theca			↑	↑		

5.4. Discussion

The previous chapter identified the localities of genes and their translated proteins from collagen pathway biology and glucocorticoid signalling regulator mechanisms, showing that gonadotrophins and potentially glucocorticoids can influence collagen pathway mRNA and protein levels in the rat ovary. To further analyse these interactions granulosa and theca cells from gonadotrophin-treated 21-day old female rats were cultured with specific hormones, growth factors, cytokines and steroids that would influence normal follicle development.

Major findings were that components of ECM remodelling were regulated by the changing profile of ovarian regulators. In the immature follicle, FSH in the granulosa cells, LH in the theca cells and TGF-β in both cell types causes an overall increase in up-regulation of collagen pathway biology to allow for follicle growth. Towards ovulation, LH, progesterone, IL-1 and a proposed lack of TGF-β (Derman *et al.* 1999; Ingman *et al.* 2006; Juneja *et al.* 1996) caused a cessation of active remodelling. Post-ovulation, remodelling was again active due to the positive influences by TGF-β and corticosterone. Ovaries from immature rats have not undergone puberty. Therefore, the follicles have not developed past the early antral stage of maturation as gonadotrophins are required to sustain follicular development. These ovaries provided a population of immature follicles to assess regulation of collagen pathway genes by substances known to influence follicles at this stage of development, such as growth factors. As the antrum begins to form, growth factors alone cannot maintain development and FSH causes the continued proliferation of granulosa cells and growth of follicles (Matzuk 2000), as well as inducing the expression of aromatase necessary for the production of oestrogen (discussed in section 1.1.1.2.). Receptors for FSH are present only on granulosa cells, hence only these cells can respond to this gonadotrophin (Simoni *et al.* 1997). The addition of FSH to granulosa cells only affected LOX mRNA expression by reducing its expression. Intriguingly, there was a significant increase in LOX enzyme activity contradictory to LOX gene expression. This indicates that there is a residual pool of LOX that is activated directly or indirectly by FSH. As FSH did not affect BMP-1

mRNA, LOX may be either activated by an unidentified factor, or latent BMP-1 is present and activated via the actions of FSH. Results for LOX activity are contrary to results obtained previously by Harlow *et al* (2003) using immature rat granulosa cells. This could be because this investigation used cells from untreated immature rats, whereas Harlow and workers treated their rats with diethylstilboestrol (DES) to increase the number of follicles present in the ovaries. DES is a synthetic oestrogen and may have effects on the expression of LOX in these cells. Harlow *et al* (2003) also used a higher dose of FSH (10ng/ml) compared to the dose used in this study (0.01IU/ml = 3.2ng/ml). This suggests that FSH at initially low levels could stimulate LOX activity when first acting on granulosa cells, but this effect becomes inhibitory at higher concentrations. These effects could be direct or indirect due to FSH induction of oestrogen production, suggesting that perhaps oestrogen has inhibitory effects on LOX expression.

Theca cells do not respond to FSH, but as the antrum begins to develop these cells begin to acquire the mechanisms required to produce androstenedione, which includes LHR. Consequently, theca cells respond to LH, and this gonadotrophin increased, PCPE mRNA levels 2-fold. PCPE increases BMP-1 action on procollagen 20-fold (Ricard-Blum *et al.* 2002), so small changes in PCPE would greatly enhance the cleavage of procollagen by BMP-1 even though BMP-1 expression itself was unchanged by LH. The limiting aspect of this cleavage would be the amount of BMP-1 present in the system, as PCPE works in a 1:1 ratio with BMP-1 (Ricard-Blum *et al.* 2002). Other possible roles indicated for PCPE are its TIMP-like activities (Mott *et al.* 2000). Therefore this increase in PCPE could be reducing ECM degradation of the ovarian ECM.

As discussed in Section 5.1, TGF-β has been shown to enhance the synthesis of ECM, including up-regulating the expression of LOX, BMP-1 and PCPE in different cell types whilst inhibiting the production of proteolytic enzymes (Border & Ruoslahti 1992; Centrella *et al.* 1992; Davidson *et al.* 1993; Massague *et al.* 1994; Okuda 1992; Verrecchia & Mauviel 2002). Hence it was unsurprising to find that TGF-β significantly increased LOX mRNA and activity in granulosa cells and LOX mRNA expression in

theca cells. Interestingly, BMP-1 and PCPE mRNA were not affected by TGF-β in these cells. Changes in granulosa cell expression *in vivo* could be regulated in a paracrine fashion, as TGF-β is produced in theca cells at this stage of development (Skinner *et al.* 1987). TFG-β also exists in a latent form which is bound to ECM, and which can be activated by BMP-1 combined with the action of MMPs (D'Angelo *et al.* 2001; Ge & Greenspan 2006; Yu & Stamenkovic 2000).

The rat glucocorticoid, corticosterone, at the high dose (1μM) did not affect BMP-1 or PCPE mRNA in granulosa and theca cells, but did significantly increase LOX mRNA in theca cells but not granulosa cells (mRNA and enzyme activity). Corticosterone would be present in preantral and early antral follicles at basal levels, with most glucocorticoid present being inactivated by the greater ratio of 11βHSD2 to 11βHSD1 (Tetsuka *et al.* 1997). As the granulosa cells show no response to corticosterone, perhaps it is these cells that contain the 11βHSD isoforms that regulate the availability of corticosterone as hypothesised by Tetsuka *et al* (1999b). Therefore, corticosterone added to granulosa cells would be inactivated, whereas corticosterone given to theca cells would remain active.

Corticosterone was given to ovarian cells in combination with TGF-β, FSH (granulosa cells) or LH (theca cells) to establish whether additive effects of ovarian regulators occurred, as *in vivo* follicles would receive a multitude of different cellular signals. No additive effects were observed in theca cells when corticosterone and LH were added. In theca, but not granulosa cells, the up-regulation of LOX expression by TGF-β was further increased by the addition of corticosterone showing a positive additive effect. Granulosa cells treated with corticosterone and FSH showed a decrease in LOX enzyme activity. Similar depressions in LOX activity, and mRNA expression have been observed when FSH was combined with DHT, TGF-β, GDF-9 or activin (Harlow *et al.* 2003).

The up-regulation of LOX in immature follicles, along with the unchanging expression pattern of BMP-1, suggests that a pool of pro-LOX may accumulate for use throughout follicle development and is activated as required. Delays in activation of pro-LOX have been shown in the bone cell line, MC3T3-E1. When these cells were given TGF-β, an

increase in LOX mRNA was observed, but activity only increased after 6h (Feres-Filho *et al.* 1995). Studies *in vivo* have also shown that pro-LOX can accumulate prior to collagen substrate production (Fushida-Takemura *et al.* 1996). This was most notable in studies of dermal wounding, where LOX was initially produced in the first few days but full activation was not reached until 8–10 days later (Fushida-Takemura *et al.* 1996; Siegel 1979). This indicates that post-translational processing of LOX is quite slow and could be a rate-limiting factor.

Overall, the developmental influences assessed here in immature follicles initiates active remodelling, allowing ovarian ECM to accommodate the increasing size of the follicle, processes regulated by the same mechanisms (gonadotrophins and growth factors).

In vivo treatment of immature female rats with PMSG simulates FSH stimulation, and progresses follicle development allowing continued development of the antrum, and progression of the follicle to the preovulatory stage. The preovulatory follicle is not yet ready to undergo ovulation, as a surge of LH is required for the final development and initiation of the ovulatory process. Ovarian cells taken from 21-day old female rats treated with PMSG were cultured with hormones, steroids and cytokines that mimic the changes that occur *in vivo*.

Prior to ovulation, granulosa cells begin to express LHR as a consequence of FSH stimulation (Burns *et al.* 2001). Therefore cells from these follicles respond to LH as well as growth factors. LH down-regulated PCPE mRNA in both granulosa and theca cells. The decrease in PCPE expression would limit the amount of procollagen cleavage by BMP-1, which would in turn reduce the substrate for LOX to cross-link, resulting in a net decrease in ECM accumulation. As follicles approach ovulation there would be a reduction in accumulation of ECM, and a shift towards ECM remodelling and degradation in preparation for oocyte release.

At this stage of development theca cells are responsive to progesterone as they express receptors for this steroid (Gava *et al.* 2004). Theca cells cultured with progesterone showed lower expression of BMP-1 and PCPE mRNA. Similar to the effects of LH, this would result in a decrease in ECM accretion. If granulosa cells from PMSG-treated rats (which now express LHR) experience an LH surge progesterone receptor expression will

be induced (Drummond 2006). Granulosa cells given LH and progesterone show no effects on gene expression.

Interleukins (IL-1) are pro-inflammatory cytokines that induce cells to act as though they are in an inflammatory environment. In the ovary, IL-1s are one cytokine family of many (Gerard *et al.* 2004). IL-1 is principally involved in the ovulatory process, and is greatly up-regulated at this time (Simon *et al.* 1994). The only effect of IL-1 observed in cultured granulosa cells was a decrease in PCPE expression, and IL-1 decreased BMP-1 mRNA expression in theca cells. LOX expression was unchanged by IL-1, though it has been shown to be decreased by other pro-inflammatory cytokines, such as TNFα in osteoblasts (Pischon *et al.* 2004). As ovulation is an inflammation-associated process, the inhibition of genes involved in ECM build-up by pro-inflammatory mechanisms is important in suppressing production of the ECM.

Ovarian cells treated with PMSG are approaching ovulation, the follicle will still be increasing in size and remodelling of the ECM is actively continuing, though there is a fine balance between proteolysis and ECM accumulation. Addition of TGF-β to granulosa cells caused an up-regulation in LOX mRNA and enzyme activity and mRNA in theca cells. Therefore, TGF-β could be involved in regulating ECM remodelling as ovulation approaches. TGF-β has been shown to increase LOX mRNA and activity time- and dose-dependently as well as up-regulating collagen type I (Feres-Filho *et al.* 1995). Therefore the amount of TGF-β available should influence the expression of LOX and the amount of available substrate. A reduction in TGF-β at ovulation would decrease active ECM remodelling. There is no direct evidence indicating the involvement of TGF-β in ovulation, rather evidence suggesting that the presence of TGF-β is inhibitory to ovulation, but it may be involved in triggering the processes that lead to a successful ovulation (Derman *et al.* 1999; Ingman *et al.* 2006; Juneja *et al.* 1996). Even though the precise mechanism of TGF-β down-regulation in ovulation has not been reported, the evidence that this growth factor can up-regulate LOX would suggest that TGF-β effects would be suppressed at this stage of the follicular cycle.

As ovulation approaches, 11βHSD1 mRNA increases (Tetsuka *et al.* 1997), and corticosterone is made available, which could have important effects on these follicles.

Corticosterone increased LOX mRNA, but not its activity, when increasing doses were given to granulosa cells. BMP-1 mRNA in both granulosa and theca cells, was down-regulated by corticosterone and PCPE expression was down-regulated in theca cells. Therefore, corticosterone could induce an accumulation of pro-LOX due to decreased BMP-1 levels, so slowing the activation of LOX. Furthermore, decreasing PCPE (and BMP-1) would also limit the accumulation of LOX substrate (collagen). Hence a pool of pro-LOX may be created ready for activation post-ovulation, when repair of the injury of ovulation is required, as seen in dermal wounding repair (Fushida-Takemura *et al.* 1996; Siegel 1979).

When corticosterone in combination with TGF-β was given to granulosa and theca cells, LOX mRNA and activity were further up-regulated. However, when corticosterone was combined with LH, only PCPE expression was decreased in granulosa cells, whereas LOX was up-regulated in theca cells. The change in granulosa cell PCPE mRNA appeared to be due to the effects of LH, whereas in the theca cells, corticosterone was acting on the cells. The combination of corticosterone and IL-1 reflected the decrease in PCPE mRNA achieved with IL-1 alone in granulosa cells and for BMP-1 in theca cells. In conclusion, several mechanisms in follicles approaching ovulation lead to a reduction in ECM accumulation. This is vital for ovulation to occur.

PMSG replicates the actions of FSH and by combining PMSG treatment with a bolus of hCG *in vivo*, the action of the LH surge is simulated, producing a cohort of periovulatory follicles on the verge of ovulation. The cells of these periovulatory follicles luteinise commencing with the LH surge. The main steroid secreted in the luteal phase is progesterone produced by luteinised granulosa and theca cells. These cells also possess progesterone receptors, and can respond to this steroid (Nelson *et al.* 1992). Once ovulation has occurred these cells form the corpus luteum. This is a period of intense ovarian remodelling as the injury of ovulation is repaired and the corpus luteum created. Progesterone increased LOX mRNA expression in luteinised granulosa cells, but had no effect on the BMP-1 and PCPE expression and did not alter expression of these genes in theca cells.

Both luteinised rat granulosa and theca cells express LHR and respond to LH by producing steroids (Nelson *et al.* 1992). Pulsatile bursts of LH maintain progesterone secretion (Filicori *et al.* 1984; Wuttke *et al.* 1998). In granulosa cells, LH down-regulated LOX and BMP-1 mRNA, but did not affect PCPE, whereas in theca cells, LH down-regulated BMP-1 but up-regulated PCPE and did not alter LOX expression. Although complex, these expression patterns would result in decreased ECM accumulation.

Components of the IL-1 system are present in luteinised follicle cells and have been shown to affect the function of these cells (Gerard *et al.* 2004), but the addition of IL-1 to cell cultures did not affect collagen pathway genes, and would therefore not affect ECM remodelling.

TGF-β is expressed by rodent luteal cells (Ghiglieri *et al.* 1995; Matsuyama & Takahashi 1995). Therefore, if collagen pathway genes are influencing remodelling in luteal tissue, this known regulator of ECM deposition may be involved. TGF-β increased the expression of LOX in both cell types. TGF-β present in luteal cells peaks at the mid-luteal phase and decreases in the late luteal phase (Chegini & Flanders 1992). Hence TGF-β would be available to regulate collagen pathway genes in these cells, and could therefore regulate ECM remodelling in the luteal phase.

Corticosterone is the dominant glucocorticoid in the ovary immediately after ovulation, where it is involved in resolving the inflammation of ovulation (Hillier & Tetsuka 1998). GR is present in the rat corpus luteum (Towns *et al.* 1999), allowing any corticosterone present to act on these cells. Corticosterone had no effects on granulosa or theca cell BMP-1 or PCPE, but did increase the expression of LOX. Corticosterone levels in early luteal cells may reduce fairly rapidly and the aged corpus luteum of the rat has been shown to have up-regulation of 11βHSD2 mRNA and activity but 11βHSD1 mRNA was undetectable (Hillier & Tetsuka 1998; Tetsuka *et al.* 2003; Waddell *et al.* 1996). This results in a decrease in corticosterone as the luteal phase progresses. Therefore if corticosterone does regulate collagen pathway genes it has more potential to act early in the luteal phase: a time when tissue remodelling would be most active.

Taken as a whole these results suggest that the collagen pathway is activated in these luteinised ovarian cells to resolve the injury of ovulation and to remodel the follicle to create the corpus luteum. A possible mechanism for post-ovulation remodelling, in regard to the regulators assessed in these studies, would suggest an initial up-regulation by corticosterone then, as this declines in the system, other regulators such as TGF-β continue this process.

In summary, conclusions that can be drawn about collagen pathway biology across follicle development in the rat from these investigations are that immature follicles show changes in the expression pattern of collagen pathway genes and enzyme activities to produce a net up-regulation of the factors that are actively involved in tissue remodelling. As follicles develop and ovulation approaches, the remodelling priorities of the follicle change as it prepares for release of the oocyte. Therefore a switch towards ECM degradation emerges, and active ECM formation is suppressed. During ovulation, follicle cells begin to luteinise. At this time, the injury of ovulation must be repaired. Hence active remodelling of the follicle ECM is once again required. These conclusions can be illustrated by proposing a model for the changes to collagen pathway biology during follicular development in relation to the ovarian regulators studied (Fig. 5.34).

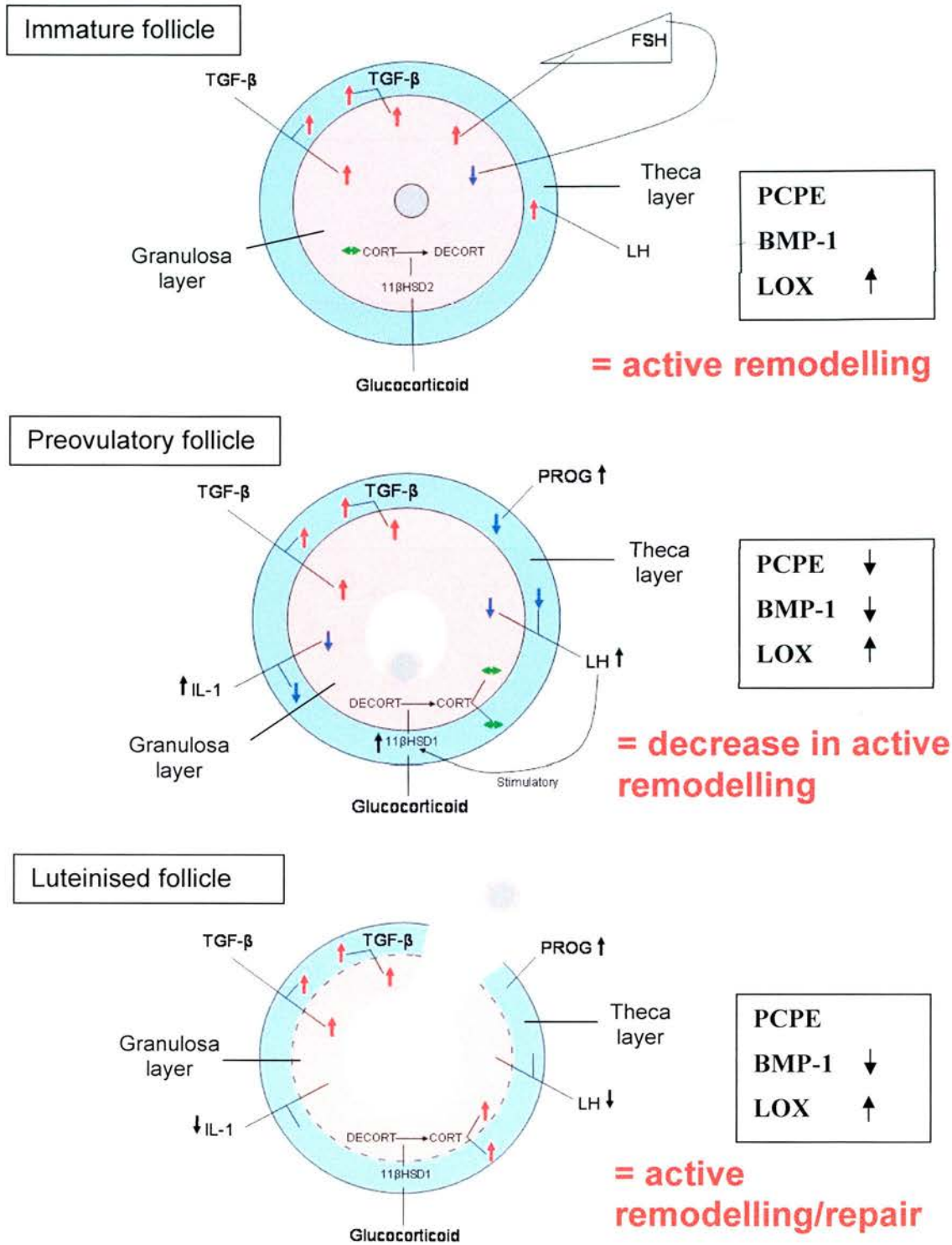

Figure 5.34. Proposed model of collagen pathway biology in the developing ovarian follicle based on current knowledge from these investigations. Red arrows indicate overall activation of remodelling; blue arrows indicate overall non-activation of remodelling; green arrows indicate accumulation of in-activated substrates of the collagen pathway. Boxes indicate changes observed in the collagen pathway genes.

In summary, active ovarian remodelling is promoted in the immature follicle by the positive influences of TGF-β and low granulosa levels of FSH as well as LH acting on the thecal cells. In the preovulatory follicle as ovulation approaches, active remodelling decreases due to negative influences of progesterone, LH and IL-1, although if TGF-β is present at this time it will drive active remodelling. Post ovulation the collagen remodelling in the luteinised follicle is positively driven by TGF-β and corticosterone.

6. Collagen pathway biology and glucocorticoid signalling in the ovary: transgenic models studies

6.1. Introduction

The previous chapters have shown that collagen pathway biology is an integral part of ovarian development, affecting the stability of collagens present in the ovary. This process is regulated during ovarian development by an array of endocrine and paracrine factors. During this project the opportunity arose to study three different transgenic mouse models, allowing insight into the regulation and function of the collagen pathway genes in these altered environments. Animal models with novel genotypes allow biological systems to be manipulated to scrutinise specific gene functions. Individual genes can be manipulated in such mouse models allowing investigation of (abnormal) function and phenotype of that biological system. This yields information about disease states which can lead to an increased understanding and the development of treatments. The three model systems available were the raw mouse (which has a mutation in its collagen IV α1 chain), the CYP11B1$^{-/-}$ mouse (which does not produce adrenal corticosterone), and the 11βHSD1$^{-/-}$ mouse (which does not enhance the activation of deoxycorticosterone to corticosterone). Unfortunately the 11βHSD2$^{-/-}$ mice could not be obtained.

Raw mice have a point mutation known as a missense mutation that renders the resulting protein non-functional. This mutation was located in exon 34 of the COL4a1 gene and has been shown to substitute a positively charged lysine residue at the Yaa position of the Gly-Xaa-Yaa repeat with a negatively charged glutamic acid (Van Agtmael *et al.* 2005). Homozygous animals are dominant negative with adverse effects on normal protein synthesis, secretion and function, whereas heterozygous animals show a semi-dominant phenotype where adverse effects are not so severe (Van Agtmael *et al.* 2005). This allows investigation of the effects of collagen substrate and enzyme movement across the partially disrupted basement membrane. Raw mice were created from an ENU mutagenesis project to generate phenotypes with eye defects (Thaung *et al.* 2002).

Two other similar, but more severe phenotypes (Bruised and Small with vacuolar cataracts) were also generated and available to study. However, due to severe reductions in male and female fertility, it was not feasible to generate sufficient numbers of animals to study. Homozygous raw mice are not viable, and do not develop past embryonic day E9.5 (Van Agtmael *et al.* 2005). This is probably due to failure of basement membrane integrity, as this is also seen in mutants that are null for the COL4α1/α2 locus (Poschl *et al.* 2004). The semi-dominant phenotype of the raw mouse shows that the basement membrane of the eye was thinned, partially disintegrated, ruptured and detached from the underlying cells, resulting in arteriolar silvering of the eye (Van Agtmael *et al.* 2005). To date, other basement-membrane related phenotypes have emerged in the kidneys and aortas of these mice (personal communication, Dr Tom Van Agtmael). Such mice are fertile but fertility levels and ovarian phenotype have not been investigated. Collagen IV is an important component of the basement membrane of ovarian follicles. Therefore, if basement membranes are disrupted, as observed in other body systems, it would be predicted that follicle development might be affected. Furthermore, as collagen pathway components appear to traverse the follicular basement membrane, disruption may influence the movement of these proteins.

The literature has shown extensively that glucocorticoids are important in the resolution of the inflammation-associated with ovulation (Section 1.2). CYP11B1 is the enzyme required to convert deoxycorticosterone to the active glucocorticoid, corticosterone. CYP11B1$^{-/-}$ mice are viable even though they have lower circulating corticosterone compared to wild-types (personal communication, Dr Chris Kenyon). The adrenals and pituitaries of these mice are enlarged as they attempt to compensate for the lack of corticosterone by trying to over-produce this steroid. The gonads of CYP11B1$^{-/-}$ male mice are phenotypically normal and these animals are fertile, but females are infertile (personal communication, Dr Chris Kenyon). This would be predicted to be due to an impaired anti-inflammatory repair system due to a lack of corticosterone produced by the adrenal gland.

The local activation of glucocorticoids is regulated by the two isoforms of 11βHSD. Tissue-specific regulation of the 11βHSD isoforms determines whether or not active

glucocorticoid is available in a specific tissue. The 11βHSD1$^{-/-}$ mouse disrupts the balance between these two isoforms. 11βHSD1$^{-/-}$ mice cannot enhance the conversion of deoxycorticosterone to corticosterone and as a result there is a compensatory hyperplasia in the adrenals as more corticosterone is being secreted in response to ACTH (Kotelevtsev *et al.* 1997). Subsequently, these mice are not affected by hyperglycemia caused by obesity or stress (Kotelevtsev *et al.* 1997). Moreover, the lack of 11βHSD1 in these knockout mice has shown to be important, for cognition with age, improved glucose tolerance and a resistance to obesity (Morton *et al.* 2001; Morton *et al.* 2004; Yau *et al.* 2001). It might be predicted that these mice (similar to the CYP11B1$^{-/-}$ mouse) would be infertile due to a lack of resolution of inflammation-associated with ovulation. However, 11βHSD1$^{-/-}$ mice are fertile (Kotelevtsev *et al.* 1997).

Corticosterone was shown in Chapter 5 to affect collagen pathway biology in ovarian cells, implying that disruption in glucocorticoid signalling in both the CYP11B1$^{-/-}$ and the 11βHSD1$^{-/-}$ mice might have ECM-related phenotypes. Therefore, as glucocorticoids are believed to be important in the ovary, investigations of these two mouse models may lead to a better understanding of the actions of these steroids and their regulation in this tissue.

6.2 Materials and methods

6.2.1 Animals

Transgenic animals were generated as described in Section 2.1. Female wild-type and CYP11B1$^{-/-}$ mice were culled at 3 months as described in Section 2.1.2. Female wild-type and 11βHSD1$^{-/-}$ mice aged 3 months and 12 months were given hormone injections *in vivo* and culled as described in Section 2.1.3. Raw mutant mice and wild-type litter mates were culled at 3 months and 9 months old. Also a cohort of wild-type and raw mice were injected with PMSG and hCG as described in Section 2.1.4.

6.2.2 Tissue

Whole ovaries were collected and fixed as described in Section 2.9.1.

6.2.3 Histological staining

Haematoxylin and eosin staining was performed on ovarian sections as described in Section 2.9.2.

6.2.4 Immunohistochemistry

Immunohistochemistry was performed on tissue sections as described in Section 2.9.5. Table 6.1 shows the antibodies used on these tissues.

Table 6.1 Antibodies, dilutions and methods of detection used in this study.

Antibody	Dilution	Detection method
11βHSD1	1:100	Fluorescence
11βHSD2	1:600	Fluorescence
BMP-1	1:100	Fluorescence
COL4α1	1:100	Fluorescence
COL4α2	1:100	Fluorescence
COL4α3	1:100	Fluorescence
COL4α4	1:100	Fluorescence
COL4α5	1:100	Fluorescence
COL4α6	1:100	Fluorescence
Laminin	1:100	DAB
LOX	1:100	Fluorescence
Nidogen 1	1:500	Fluorescence
Nidogen 2	1:500	Fluorescence
Perlecan	*1:500*	Fluorescence

6.3 Results

6.3.1 Raw mice

No histological differences were observed between wild-types and raw mutants (Fig. 6.1) and there were no differences among age groups (data not shown). There was no significant difference between ovary:body weight ratios between wild-type and raw mice (Fig. 6.2). *In vivo* horomone treatments with PMSG and hCG showed no differences between wild-type and Raw animals (data not shown).

Staining for collagen IV (COL4α-1, -2 and -5 chains) showed less continuous staining at the follicular basement membranes in raw mice (Fig. 6.3). Collagen pathway proteins LOX and BMP-1 showed no differences in immuno-localisation between wild-types and mutants (Fig. 6.4).

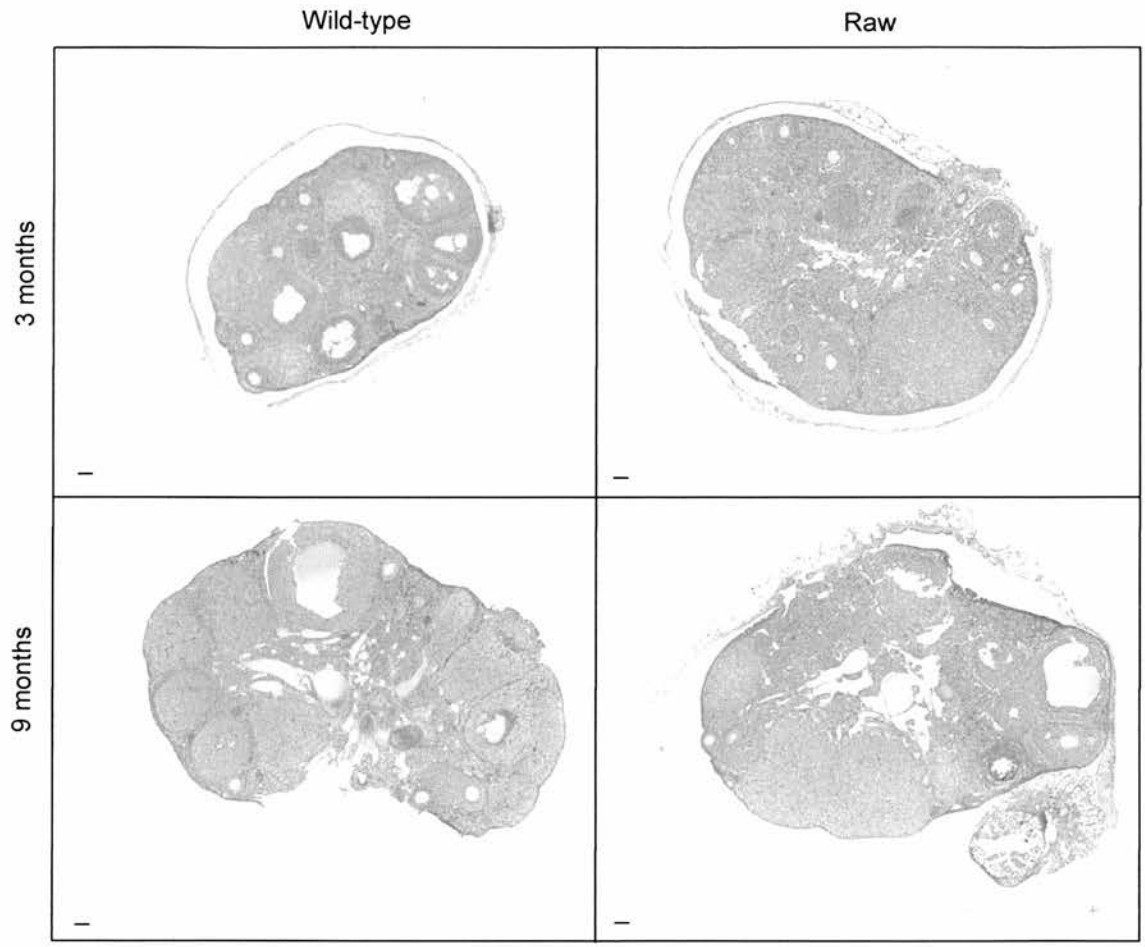

Figure 6.1 Haematoxylin and eosin staining of ovaries from wild-type and raw mice aged 3 months and 9 months. Tissue was fixed and stained as described in Section 2.9. All scale bars 100μm.

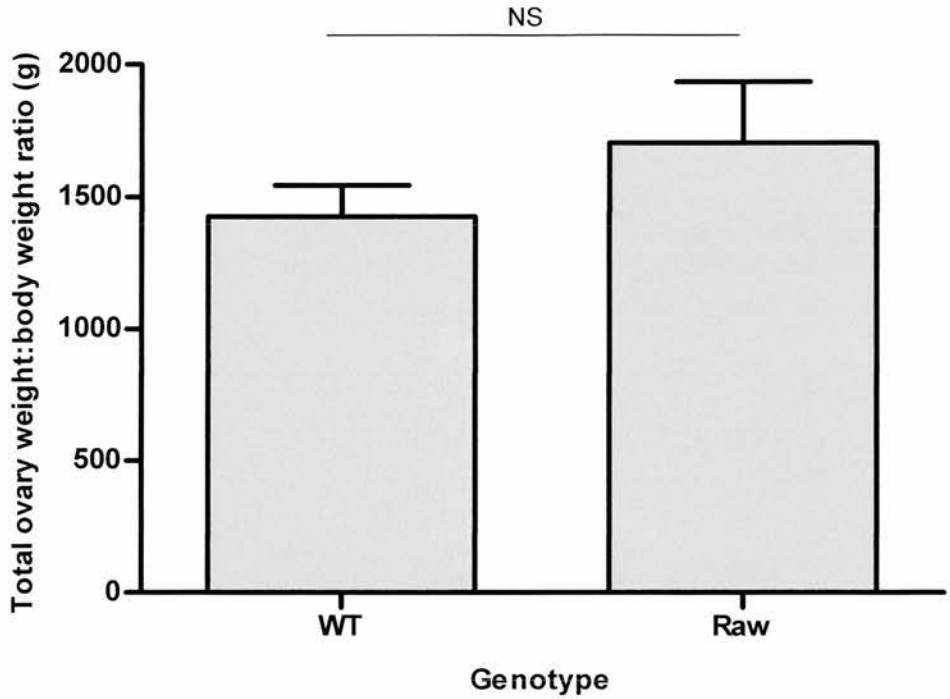

Figure 6.2. Comparison of ovary:body weight ratio for wild-type and raw mice. A cohort of wild-type and Raw mice were bred and given *in vivo* injections of PMSG and hCG as described in Section 2.1.4. No significant difference in ovarian weight was observed between the wild-type and Raw mice, which was validated with the observation of no difference between body weight of the two animal groups. Wild-type (N=7), Raw (N=6).

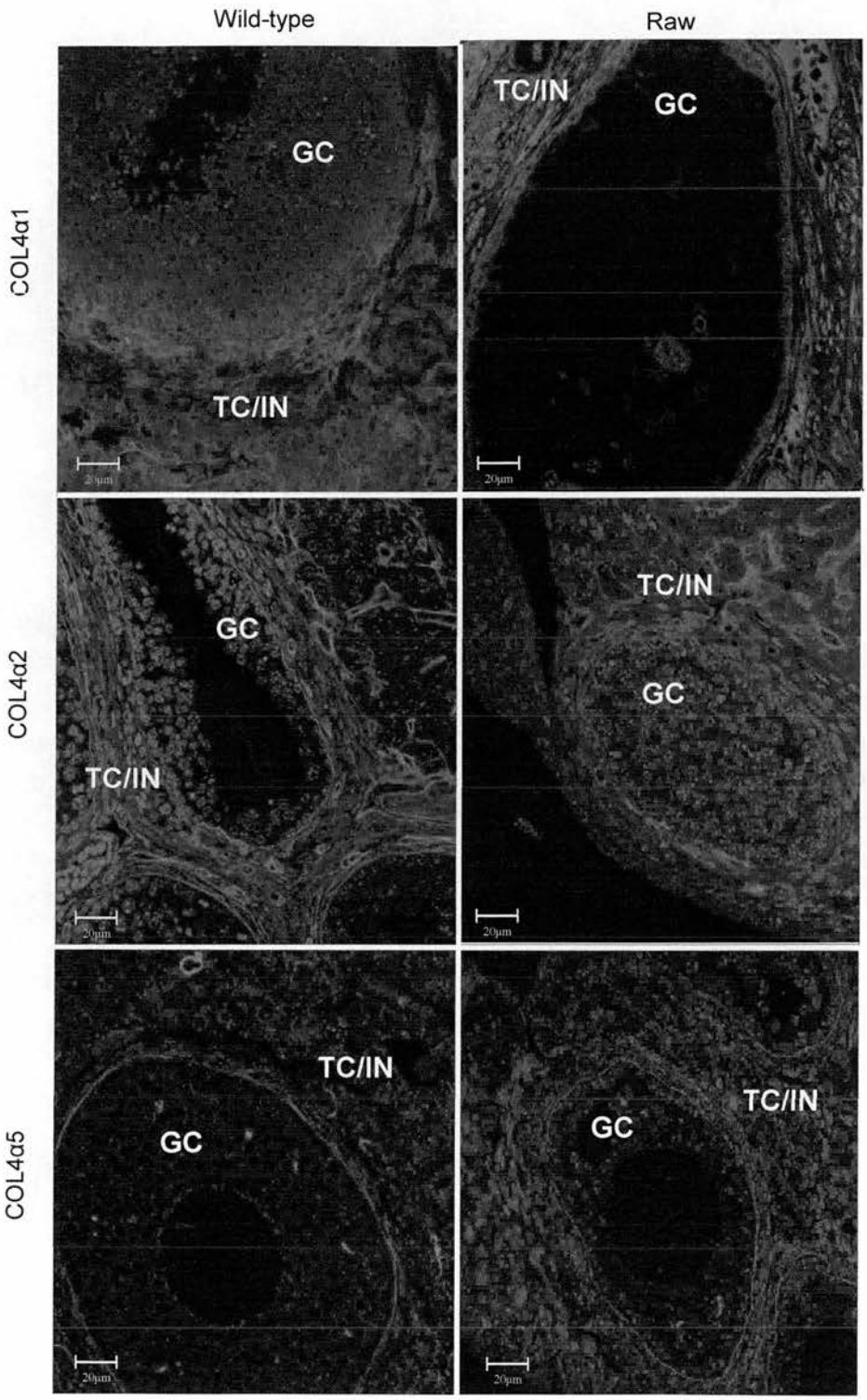

Figure 6.3. Immunohistological localisation of collagen IV α-chain proteins in wild-type and raw ovaries. Ovaries were fixed and stained with antibodies raised against collagen IV α-1, -2 and -5 as described in Section 2.9.5. Red, collagen α-chains; blue, nuclear stain. Granulosa cells (GC), Theca cells/interstitial tissue (TC/IN). All scale bars 20μm.

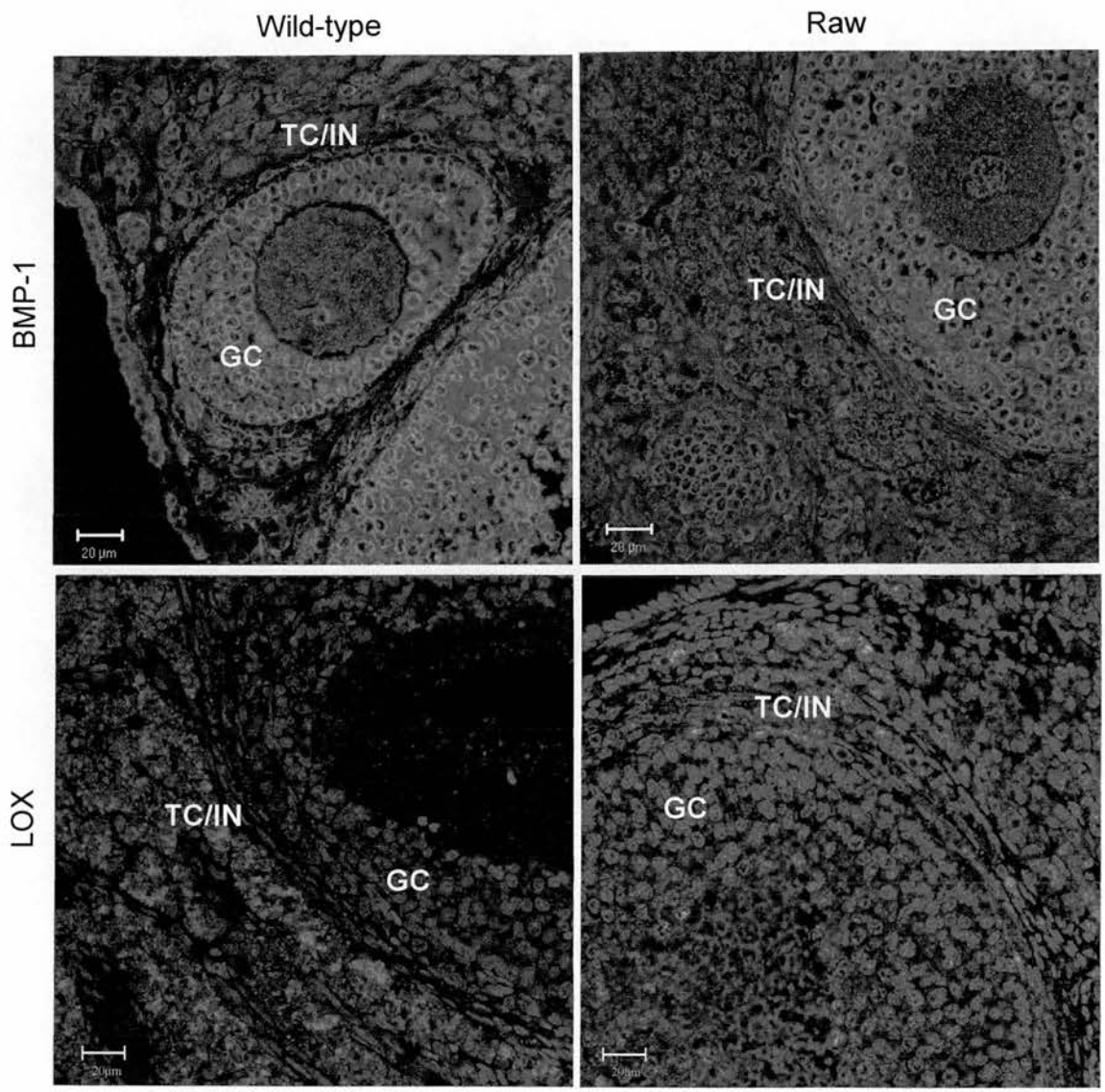

Figure 6.4. Immunohistological localisation of BMP-1 and LOX proteins in wild-type and Raw ovaries. Ovaries were fixed and stained with antibodies raised against BMP-1 and LOX as described in Section 2.9.5. Red, BMP-1 or LOX; green, nuclear stain. Granulosa cells (GC), Theca cells/interstitial tissue (TC/IN). All scale bars 20µm.

6.3.2 CYP11B1$^{-/-}$ mice

The ovaries from wild-type and CYP11B1$^{-/-}$ mice showed phenotypic differences. Wild-type ovaries showed normal follicular development, and contained follicles of increasing size as well as corpora lutea that were discretely arranged within the stromal tissue (Fig. 6.5). The ovaries from CYP11B1$^{-/-}$ mice show antral follicle development (Fig. 6.5), but entrapped oocytes have been observed (personal communication, Dr Judy McNeilly). These ovaries also appear to contain luteinised granulosa cells similar to those normally associated with corpora luteal structures. However, unlike wild-type ovaries, these cells in the CYP11B1 ovaries (termed luteinised-like cells) were not located within obvious corpora lutea. Instead they were present throughout the ovarian interstitum.

Laminin and collagen IVα2, the major components of the basement membrane which are linked by perlecan, nidogen-1 and -2, were clearly detected in CYP11B1$^{-/-}$ ovarian follicles, similar to wild-types (Fig. 6.6/6.7). In the luteinised-like cells of the CYP11B1$^{-/-}$ ovaries, these basement membrane proteins showed less intense staining and were more disorganised than in wild-type ovaries (Fig. 6.6/6.7).

Collagen pathway proteins appeared less abundant in the CYP11B1$^{-/-}$ ovaries and were not associated with particular ovarian structures (Fig. 6.8).

The 11βHSD isoforms were localised in follicles and corpora lutea of wild-type ovaries (Fig. 6.9). Whereas, 11βHSD1 was hardly detected in the CYP11B1$^{-/-}$ ovary, 11βHSD2 was widespread and did not appear to be localised to any particular structure in CYP11B1$^{-/-}$ ovaries (Fig. 6.9).

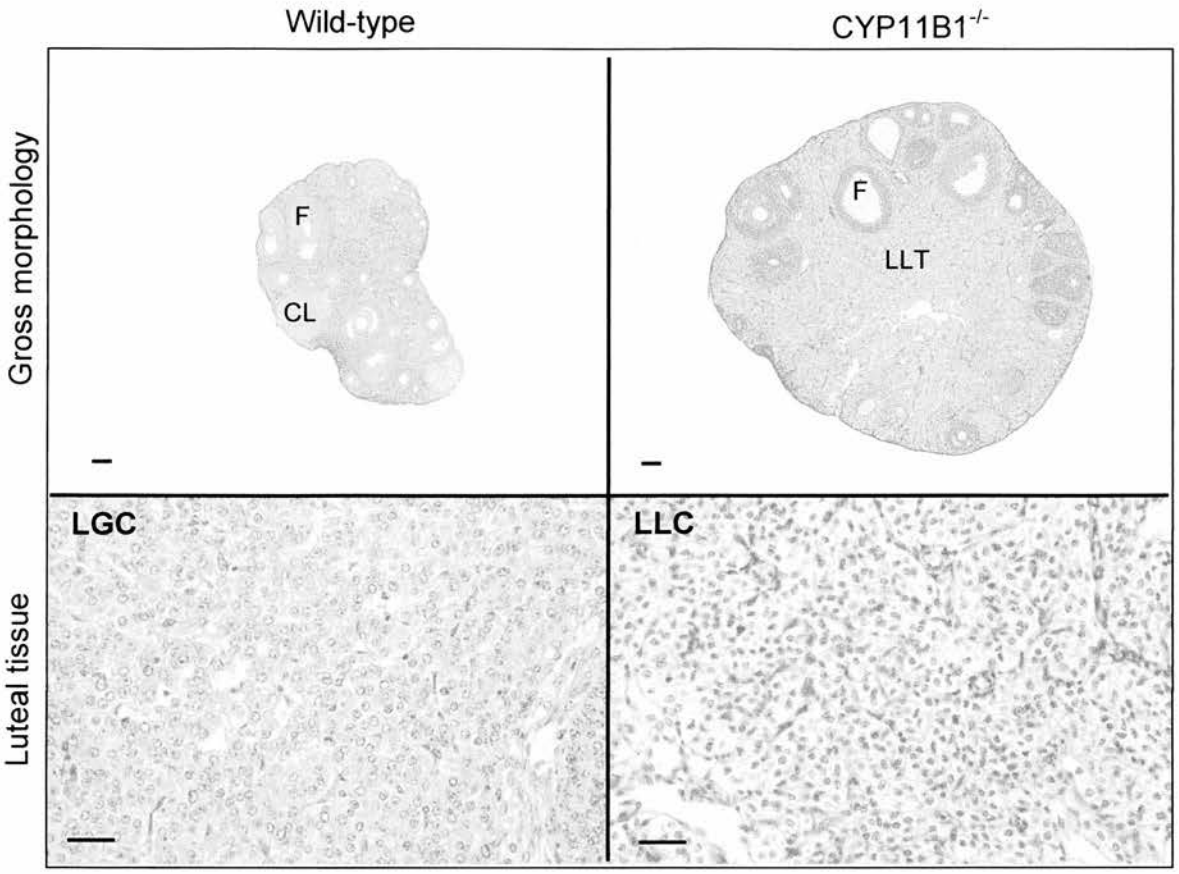

Figure 6.5. Haematoxylin and eosin staining of wild-type and CYP11B1-/- ovaries. Ovaries from wild-type and CYP11B1-/- mice were fixed and stained with haematoxylin and eosin as described in Section 2.9.5. Follicles (F), corpus luteum (CL), luteinised-like tissue (LLT), luteinised granulosa cells (LGC), luteinised-like cells (LLC). Upper panels scale bars 100μm; lower panels scale bars 20μm.

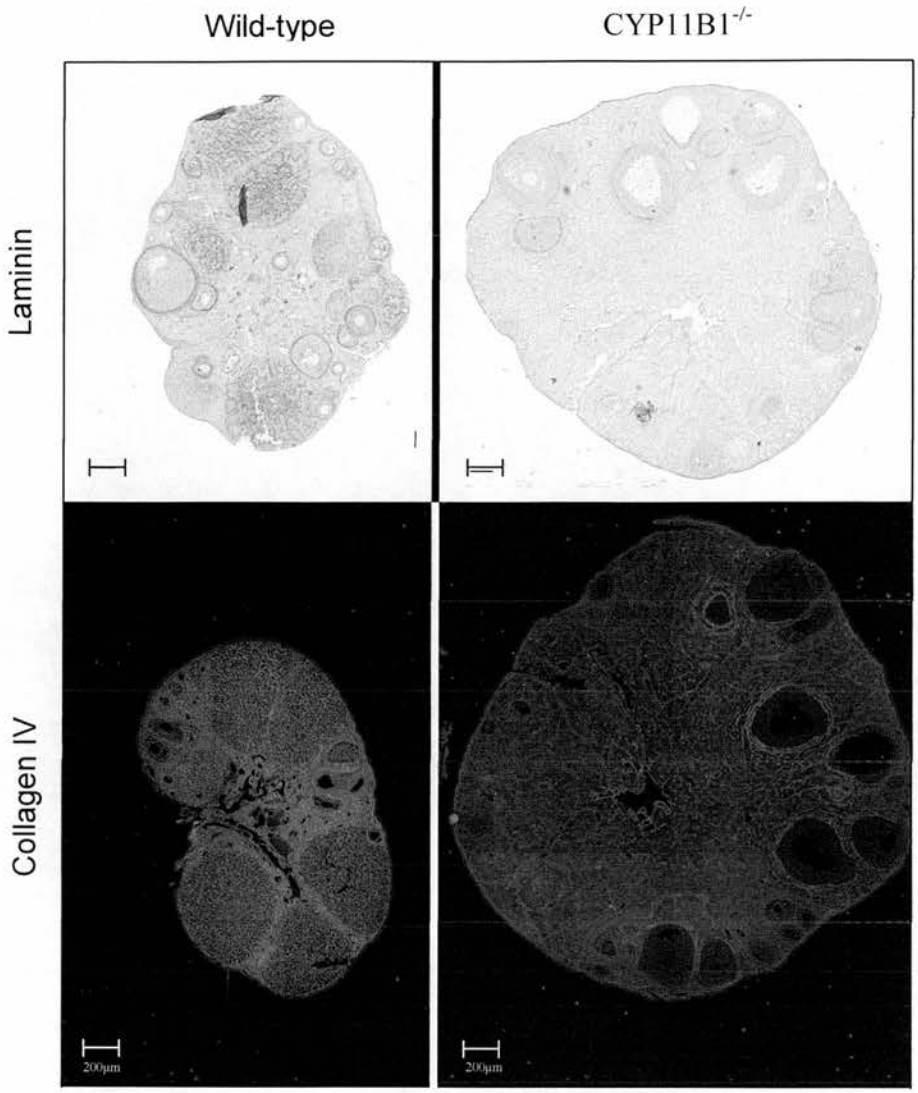

Figure 6.6. Immunohistochemical localisation of laminin and collagen IVα2 proteins in wild-type and CYP11B1$^{-/-}$ ovaries. Ovaries were fixed and stained with antibodies raised against laminin and collagen IVα2 as described in Section 2.9.5. Red, collagen IVα2; green, nuclear staining. The nuclear staining of the CYP11B1$^{-/-}$ ovaries was not optimal in the fluorescence photographs. No apparent reasons for this could be determined as immunohistochemistry was performed on wild-type and CYP11B1$^{-/-}$ tissue in parallel using the same reagents. Time and tissue availability did not allow for further investigations. All scale bars 200µm.

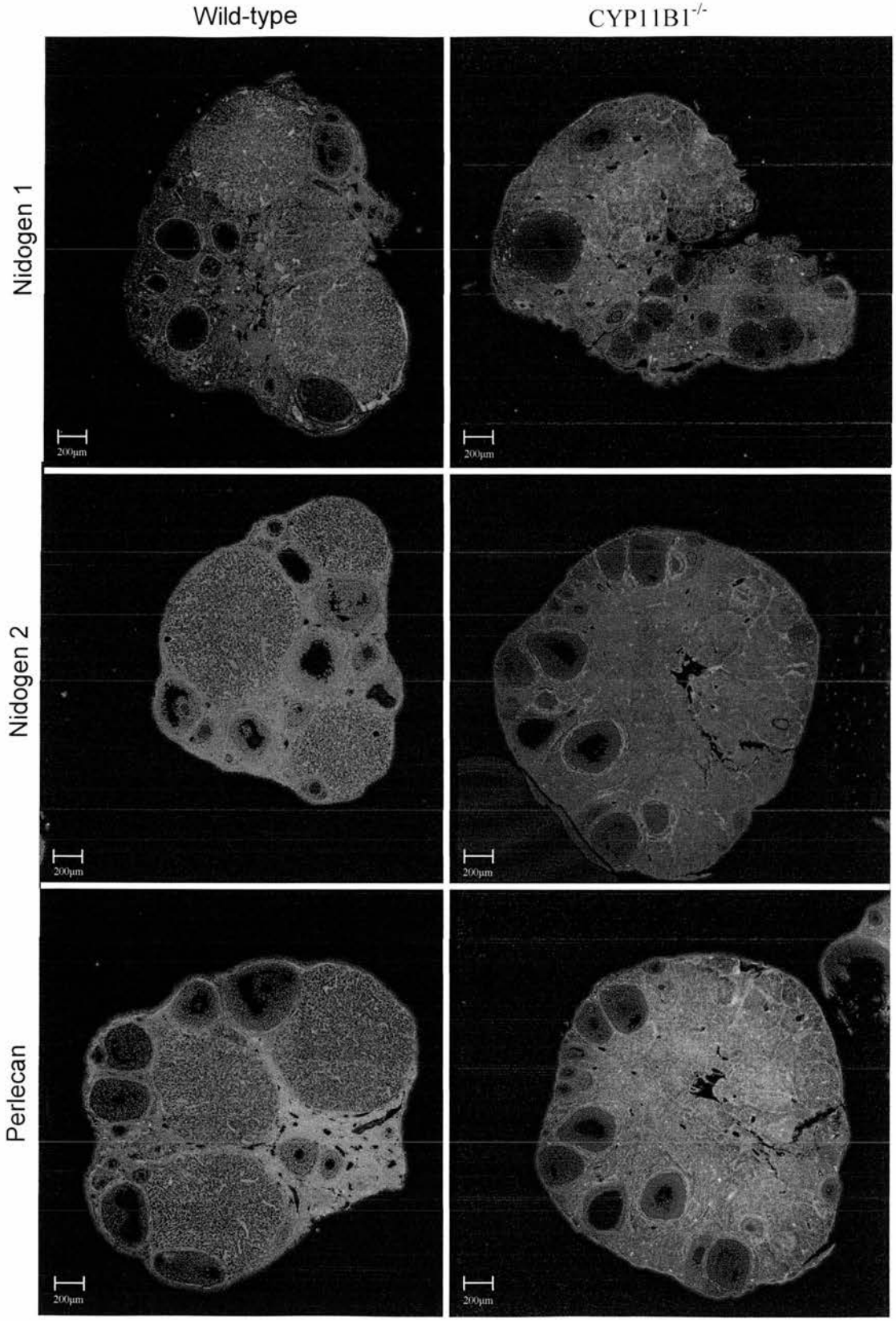

Figure 6.7. Immunohistochemical localisation of nidogen-1, -2 and perlecan proteins in wild-type and CYP11B1$^{-/-}$ ovaries. Ovaries were fixed and stained with antibodies raised against nidogen-1, -2 and perlecan as described in Section 2.9.5. Red, nidogen-1,-2 or perlecan; green, nuclear staining. The nuclear staining of the CYP11B1$^{-/-}$ ovaries was not optimal in the fluorescence photographs. No apparent reasons for this could be determined as immunohistochemistry was performed on wild-type and CYP11B1$^{-/-}$ tissue in parallel using the same reagents. Time and tissue availability did not allow for further investigations. All scale bars 200µm.

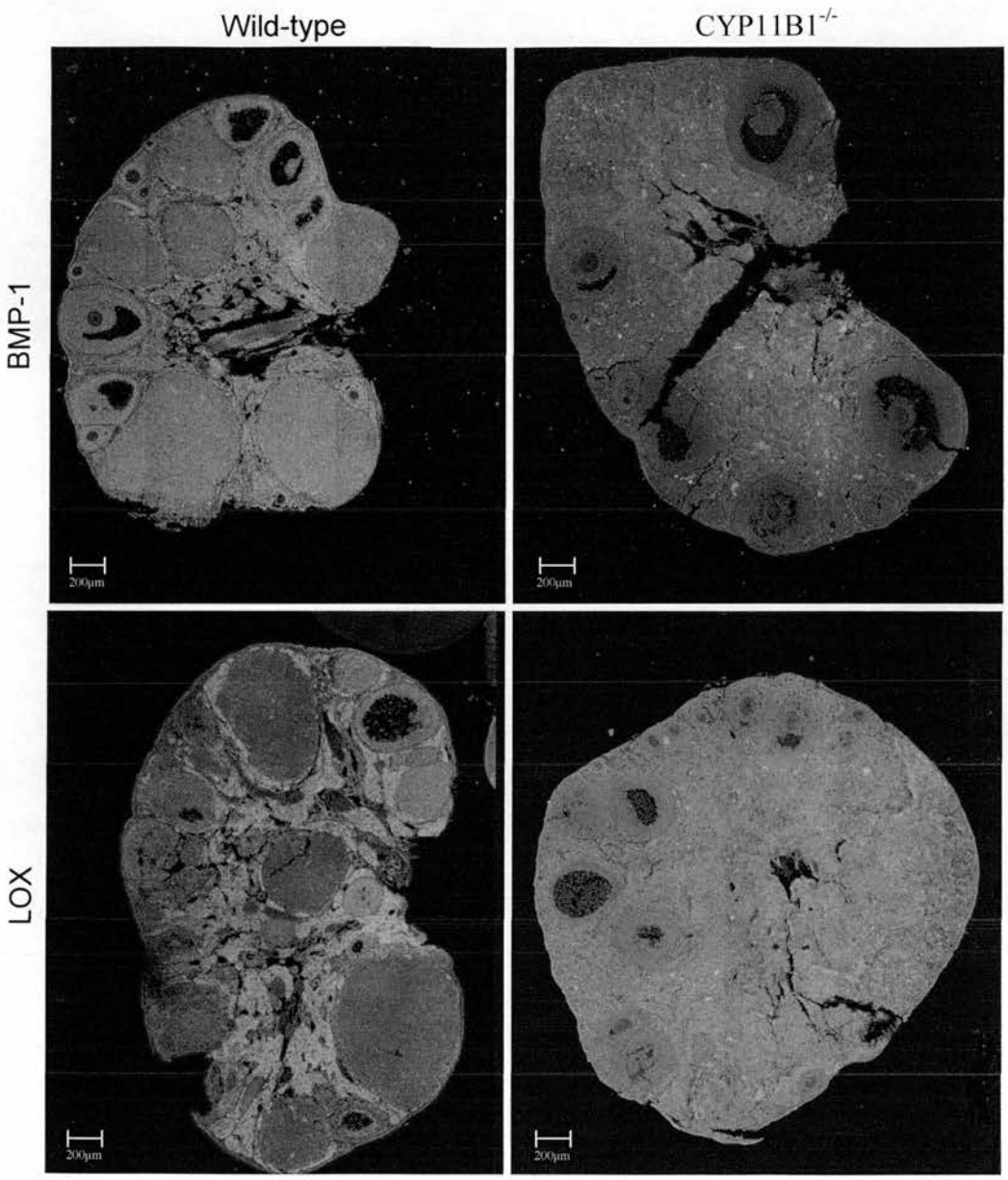

Figure 6.8. Immunohistochemical localisation of BMP-1 and LOX proteins in wild-type and CYP11B1$^{-/-}$ ovaries. Ovaries were fixed and stained with antibodies raised against BMP-1 and LOX as described in Section 2.9.5. Red, BMP-1 or LOX; green, nuclear staining. The nuclear staining of the CYP11B1$^{-/-}$ ovaries was not optimal in the fluorescence photographs. No apparent reasons for this could be determined as immunohistochemistry was performed on wild-type and CYP11B1$^{-/-}$ tissue in parallel using the same reagents. Time and tissue availability did not allow for further investigations. All scale bars 200µm.

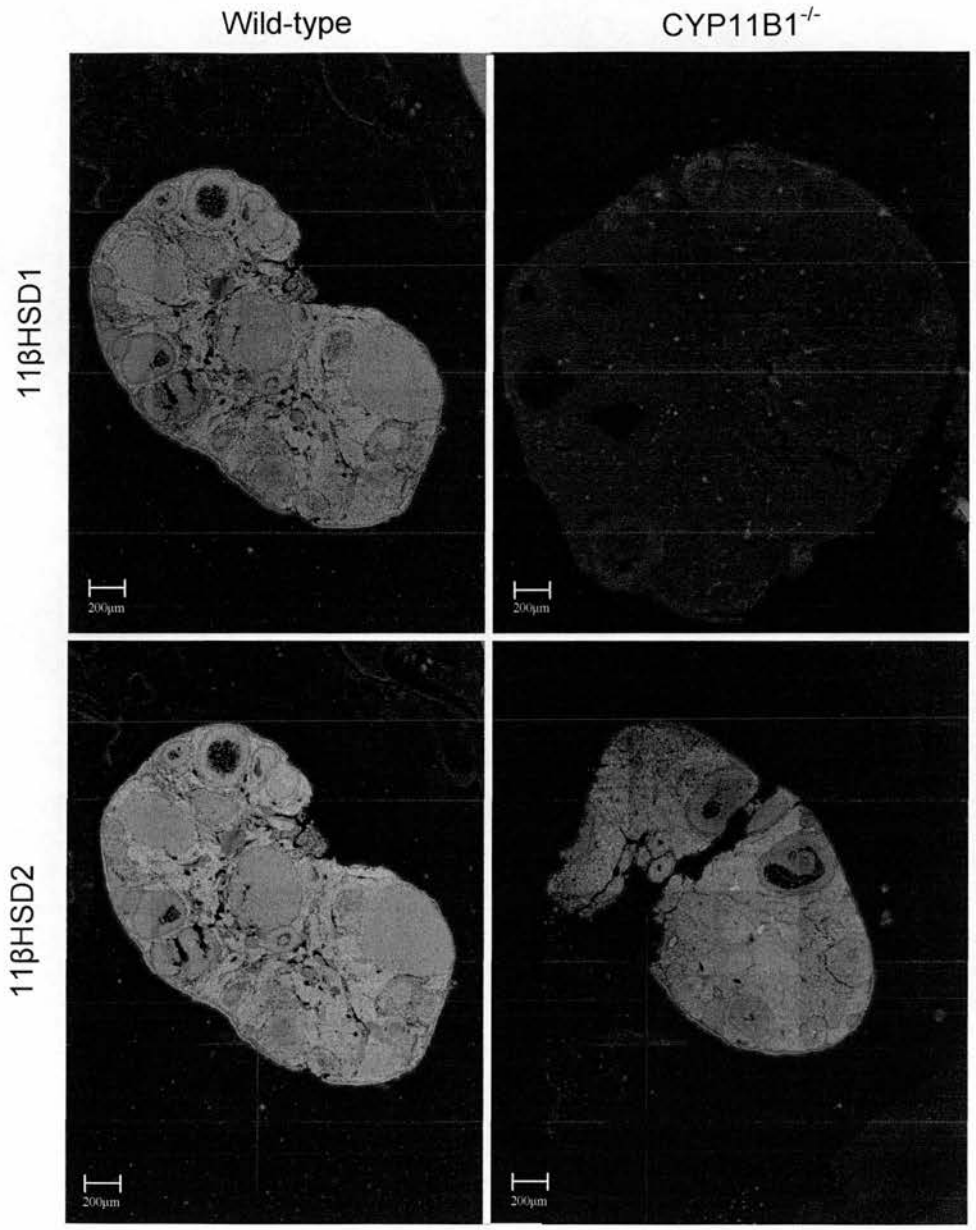

Figure 6.9 Immunohistochemical localisation of 11βHSD1 and 11βHSD2 proteins in wild-type and CYP11B1$^{-/-}$ ovaries. Ovaries were fixed and stained with antibodies raised against 11βHSD1 and 11βHSD2 as described in Section 2.9.5. Red, 11βHSD1 or 11βHSD2; green, nuclear staining. The nuclear staining of the CYP11B1$^{-/-}$ ovaries was not optimal in the fluorescence photographs. No apparent reasons for this could be determined as immunohistochemistry was performed on wild-type and CYP11B1$^{-/-}$ tissue in parallel used the same reagents. Time and tissue availability did not allow for further investigations. All scale bars 200μm.

6.3.3 11βHSD1$^{-/-}$ mice

Haematoxylin and eosin staining of ovaries from mice aged 3 months and 12 months that had received *in vivo* treatment to simulate gonadotrophin-driven follicle maturation showed no differences between wild-type and 11βHSD1$^{-/-}$ mice for any treatment groups (Fig. 6.10/6.11).

Ovaries were immuno-stained for BMP-1 and LOX (Fig. 6.12/6.13). The only major difference observed was an increased localisation of BMP-1 protein in untreated 11βHSD1$^{-/-}$ compared to wild-type tissue. There were no major differences between wild-type and 11βHSD1$^{-/-}$ mice with either antibody. Subtle differences observed were an apparent lack of LOX staining in the granulosa cells of the PMSG-treated 11βHSD1$^{-/-}$ mice compared to wild-types, and preantral follicles of 11βHSD1$^{-/-}$ appeared to have more BMP-1 localised to the granulosa layer.

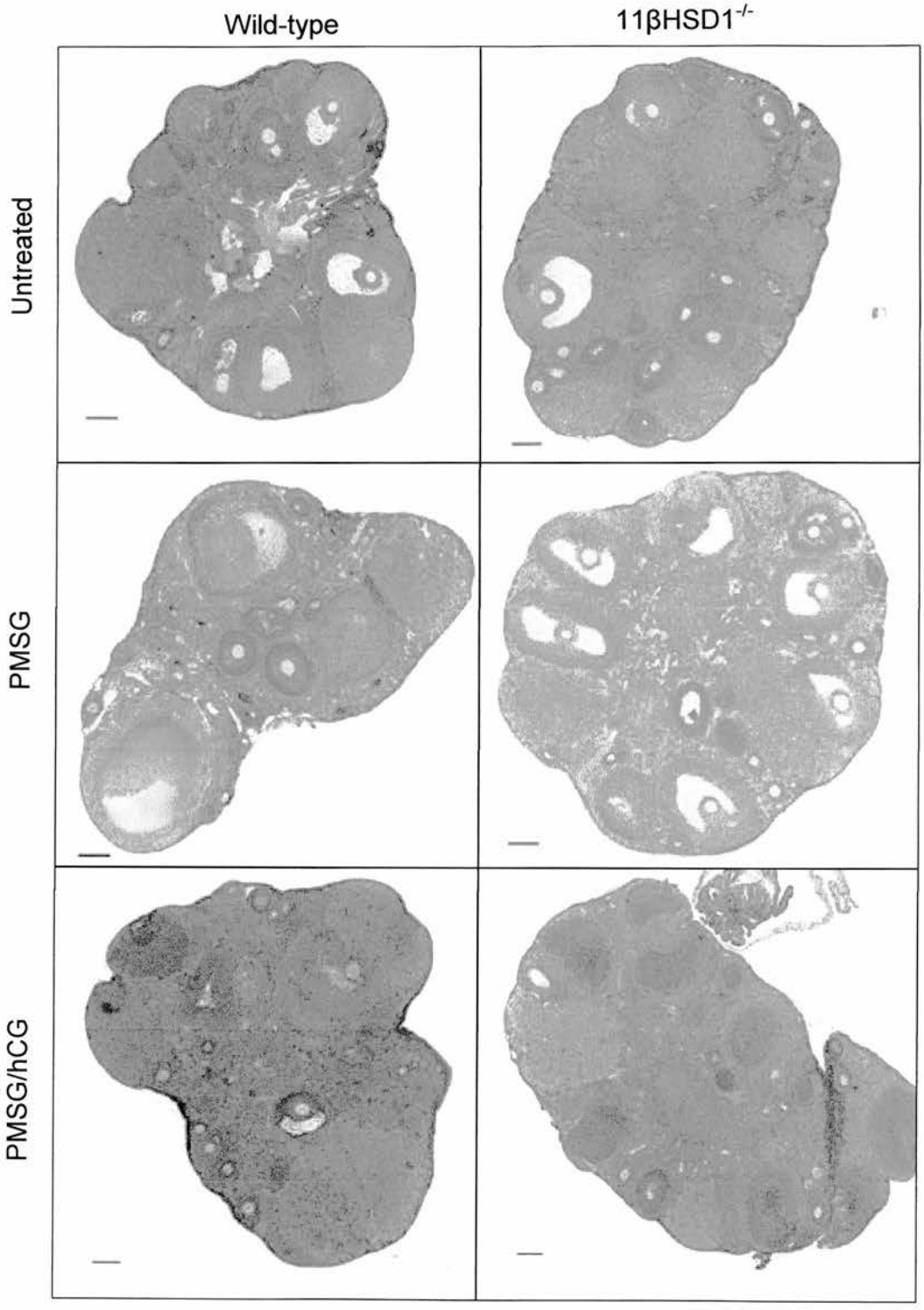

Figure 6.10. Haematoxylin and eosin staining of ovaries from wild-type and 11βHSD1$^{-/-}$ mice aged 3 months. These mice had been treated *in vivo* with either no treatment, PMSG or PMSG/hCG as described in Section 2.1. All scale bars 100μm.

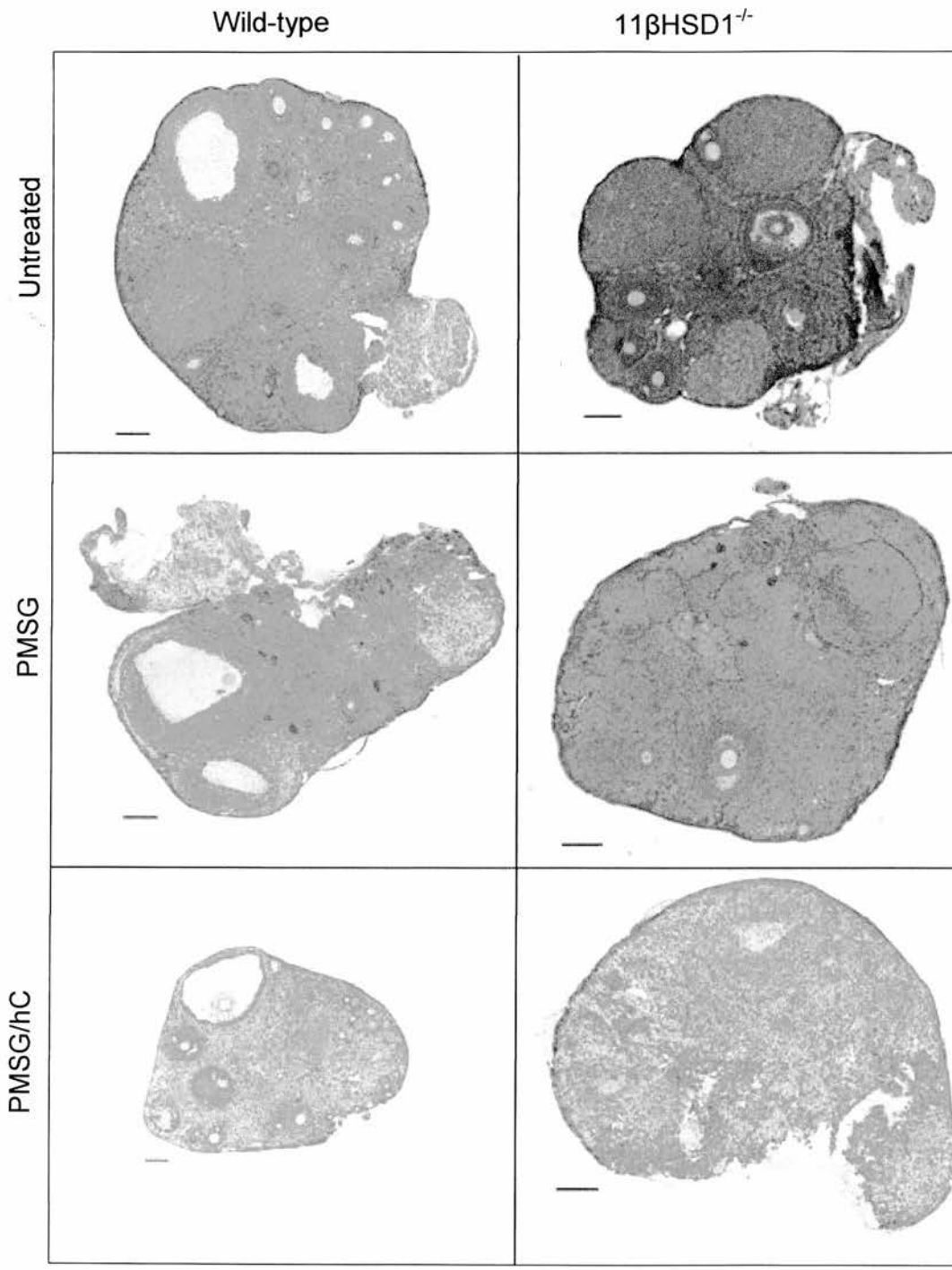

Figure 6.11. Haematoxylin and eosin staining of ovaries from wild-type and 11βHSD1$^{-/-}$ mice aged 12 months. These mice had been treated *in vivo* with either no treatment, PMSG or PMSG/hCG as described in Section 2.1. All scale bars 100μm.

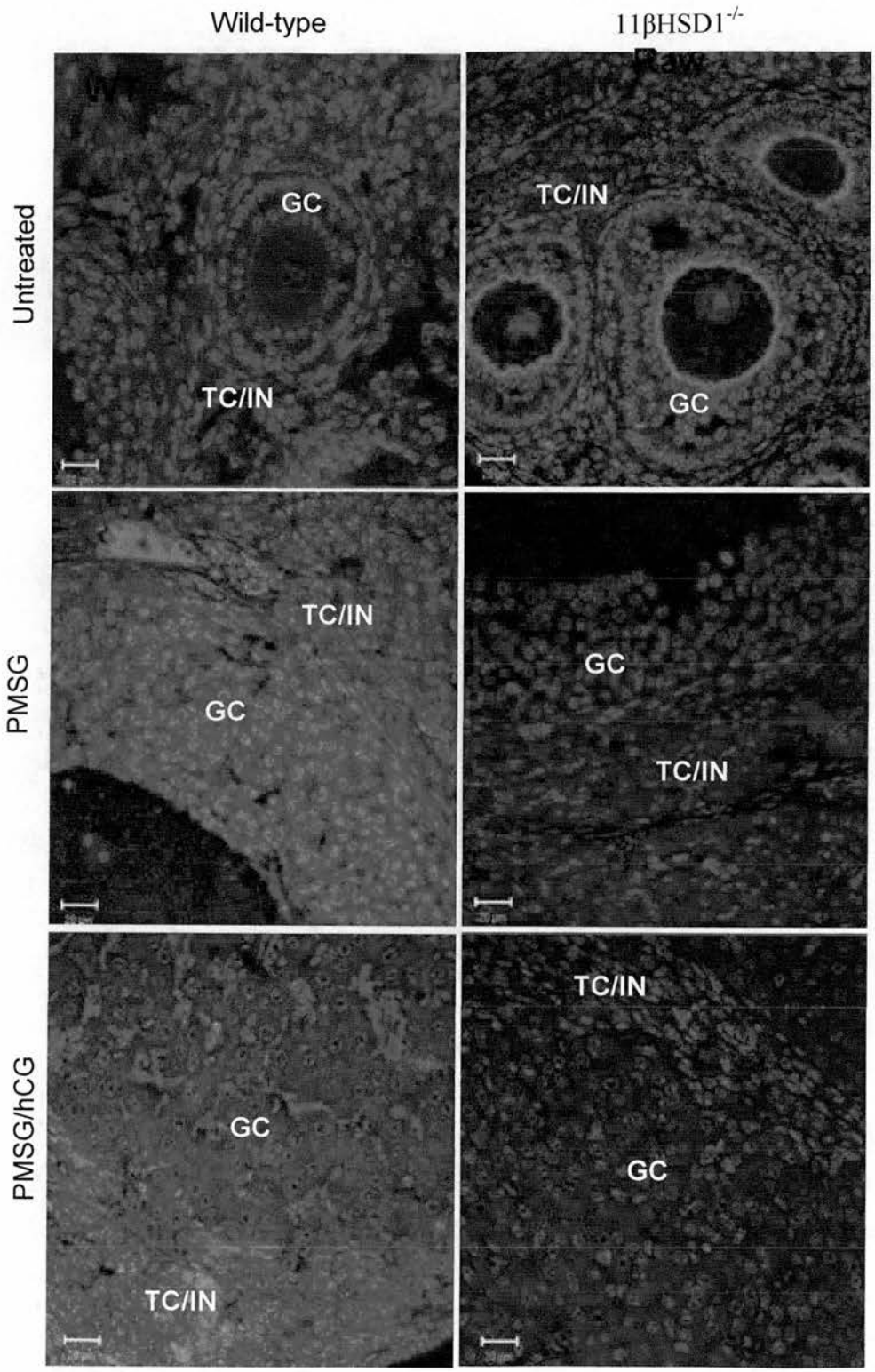

Figure 6.12. Immunohistochemical localisation of BMP-1 protein in wild-type and 11βHSD1$^{-/-}$ ovaries. Mice had been treated *in vivo* with either no treatment, PMSG or PMSG/hCG as described in Section 2.1. Ovaries were fixed and stained with an antibody raised against BMP-1 as described in Section 2.9.5. Red, BMP-1; green, nuclear stain. Granulosa cells (GC, theca cells/interstitial tissue (TC/IN). All scale bars 20μm.

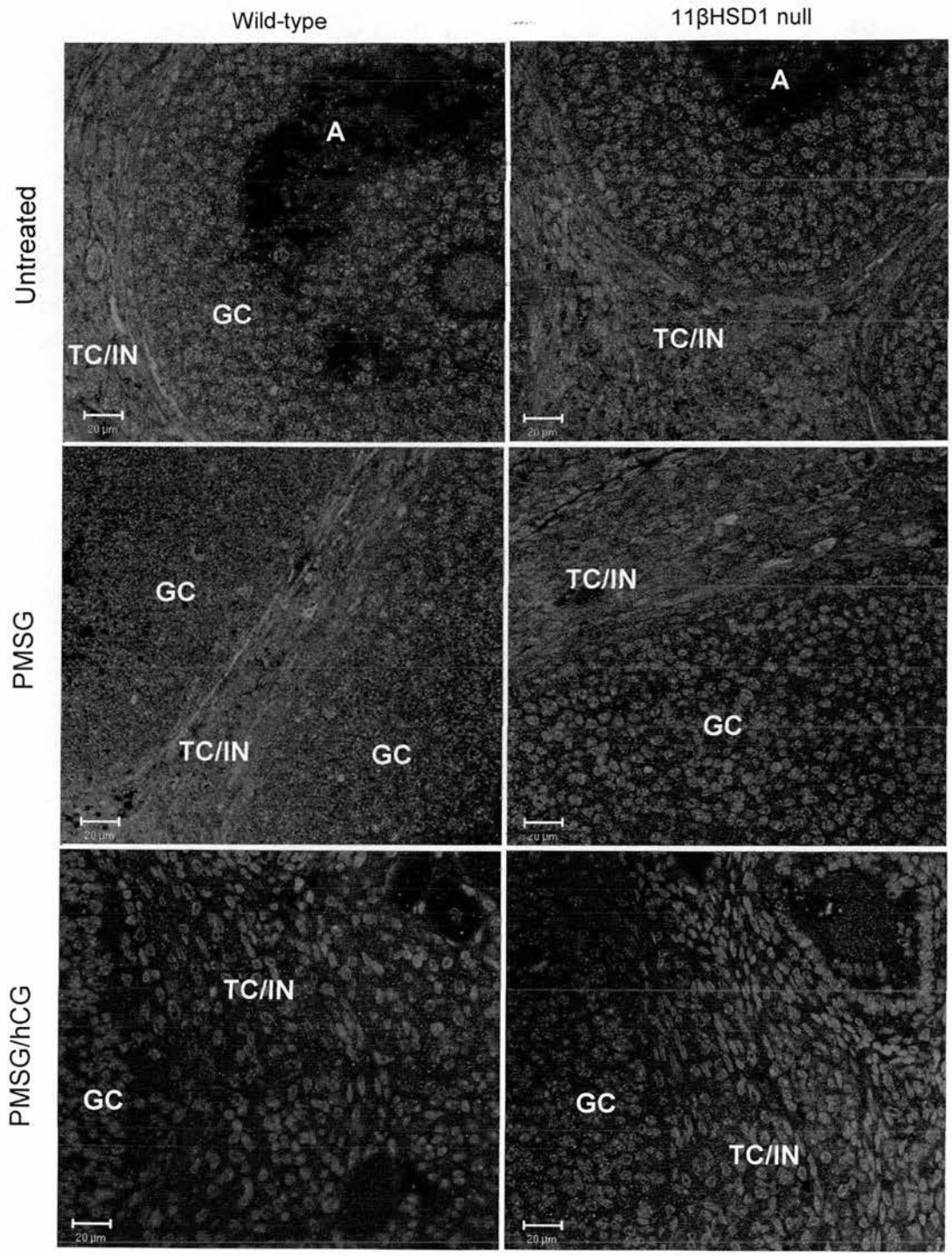

Figure 6.13. Immunohistochemical localisation of LOX protein in wild-type and 11βHSD1$^{-/-}$ ovaries. Mice had been treated *in vivo* with either no treatment, PMSG or PMSG/hCG as described in Section 2.1. Ovaries were fixed and stained with an antibody raised against LOX as described in Section 2.9.5. Red, LOX; green, nuclear stain. Antrum (A), Granulosa cells (GC), theca cells/interstitial tissue (TC/IN). All scale bars 20µm.

6.4 Discussion

Previous chapters have explored the localisation and regulation of collagen pathway genes and proteins in the rat ovary. Here these proteins were investigated in transgenic mice that were deficient in either basement membrane components (raw mice that have partially disrupted COL4A1) or glucocorticoid regulation (CYP11B1$^{-/-}$ mice and 11βHSD1$^{-/-}$ mice). Investigations using these model systems may expand understanding of the role of collagen pathway biology in the ovary.

Raw mice were heterozygous for mutations in the collagen IV α1-chain, and therefore had partial disruption of their basement membranes. The follicular basement membrane has been shown to contain collagen IV α1 throughout development (Rodgers *et al.* 1998). Hence, it was predicted that follicular development might have been disrupted in some way in these animals. However, these mice were fertile, and although some individual cases of infertility were observed and there was only a slight reduction in average litter size (personal communication, Dr Tom Van Agtmael).

No obvious ovarian phenotype was observed at the level of gross morphology. The distribution of collagen IV α1 and α2 appeared uneven in the ovaries from the raw animals, but collagen IV α5 was unaffected. These disruptions did not appear to be associated with abnormal ovarian function in the raw animals. Furthermore, no differences were observed in the distribution of BMP-1 or LOX proteins either. These data suggest that the function of the follicular basement membrane was unaltered in these mice. However, basement membranes of other organs of these animals such as the eye and kidney, showed prominent abnormal phenotypes that progressively worsened with age (personal communication, Dr Tom Van Agtmael). Possible reasons for this are that the basement membranes of the eye and the kidney are established structures that function continuously throughout the life of the animal. Basement membranes are highly stable and, once established, are not continually remodelled (Hudson *et al.* 1993). Exceptions to this are developmental situations where structures are forming, and the basement membrane of the ovarian follicle, which begins to develop as follicles are recruited to grow throughout reproductive life (Rodgers *et al.* 2003). Once growth commences the ovarian follicle in the mouse develops in only 4 days and then is

destroyed at ovulation (Mandl 1951). Therefore, the period that the follicular basement membrane is functional in the ovary is brief, when compared to other basement membranes. The follicular basement membrane is also subjected to continuous remodelling, and this may not allow time for gross abnormalities to develop. This is supported by the report that raw mice show similar phenotypic alterations to the human disease, Hereditary Angiopathy with Nephropathy, Aneurysm and Cramps (HANAC) syndrome, an inherited disease that has no reported ovarian dysfunction (Plaisier *et al* 2007, in preparation).

The ovary does not produce glucocorticoids *de novo*. Therefore levels of circulating corticosterone and local regulation by 11βHSD isoforms determine the amount of exposure of ovarian cells to this steroid. CYP11B1$^{-/-}$ mice do not produce circulating corticosterone. The females are infertile, and the ovaries of these animals contain antral follicles, but are devoid of structurally normal corpora lutea. The areas that are normally stroma appeared to be full of weakly luteinised cells that were positive for 3βHSD (the steroidogenic marker of luteinised ovarian tissue) (personal communication, Dr Judy McNeilly). When observed under high power microscopy, these cells did not have the appearance of healthy luteinised granulosa cells, but were negative for CYP17, indicating that they were not thecal cells (personal communication, Dr Judy McNeilly). This, together with the observation of entrapped oocytes, implies that that ovulation may not occur normally, and that follicle cells do not luteinise to form a functional corpus luteum. In the normal corpus luteum, basement membrane components surround the luteinised cells. However, the corpus luteum-like cells of the CYP11B1$^{-/-}$ mouse had a reduced amount of the major structural proteins (laminin and collagen IV) and basement membrane cross-linkers (perlecan, nidogen-1 and -2).

The localisation of BMP-1 and LOX was irregular throughout the CYP11B1$^{-/-}$ ovary. The basement membrane and collagen pathway protein localisations observed were probably due to the abnormal morphology rather than a cause implying that functional ovulation and steroidogenesis are needed for normal expression. Interestingly, the developing follicles do not appear to bulge at the ovarian surface as seen in wild-type ovaries, perhaps indicating an abnormality in follicle development or that a physical

restriction is occurring that may account for the failure of ovulation. There were no differences between CYP11B1$^{-/-}$ and wild-type in collagen IV localisation at the surface of the ovary. Perhaps another collagen may be deposited in this area. The deficiency of corticosterone in the CYP11B1$^{-/-}$ mice appeared to dramatically reduce 11βHSD1 protein in the ovary, but did not affect 11βHSD2 expression. Cortisol has previously been shown to enhance the expression of 11βHSD1 in human OSE cells (Rae *et al.* 2004b).

A problem that arose with the CYP11B1$^{-/-}$ ovaries was the poor quality of the fluorescent nuclear stain. The staining was repeated at different times on different tissue sections with the same results. Reasons for the staining may include the way the tissue has been fixed, this was performed by a third party therefore fixation may not have been optimal. If the fixation was performed correctly the difference seen in nuclear staining between wild-type and CYP11B1-/- may reflect an effect caused by the genotype of these mutant animals. This difference requires further investigation, which was beyond the scope of this thesis.

The 11βHSD1$^{-/-}$ mouse, in which circulating deoxycorticosterone is not converted to corticosterone in target tissues, showed no impairment in ovarian function. Ovaries were phenotypically normal compared to wild-type controls. The only difference observed in collagen pathway proteins in these ovaries was an increased localisation of BMP-1 protein in the 11βHSD1$^{-/-}$ untreated ovaries. Reasons for this difference are likely to be the lack of corticosterone enhancement in this tissue implying that corticosterone maybe involved in regulating BMP-1. It was predicted that the ovaries of the 11βHSD1$^{-/-}$ mice would be similar to the CYP11B1$^{-/-}$ mice as there should be a deficiency in corticosterone exposure post-ovulation. However, any impairment of ovarian repair caused by 11βHSD1 absence appeared to be rescued. These mice may have an increased level of corticotrophic releasing hormone, which would lead to raised ACTH levels and ultimately increased circulating corticosterone (Harris *et al.* 2001). Presumably an elevation in circulating corticosterone could compensate for the lack of 11βHSD1 and could be sufficient to resolve the inflammation of ovulation. However, progesterone has also been shown to have anti-inflammatory effects on reproductive

tissue (Kelly *et al.* 2001; Rae *et al.* 2004a), and progesterone is elevated in the corpus luteum and suppresses the IL-1 stimulated inflammation response in OSE cells (Rae *et al.* 2004a). Therefore, the actions of progesterone may be instrumental in resolving the ovulation-associated inflammation in 11βHSD$^{-/-}$ mice. To date, circulating progesterone levels have not been measured in these mice. Even though a deficiency in 11βHSD1 did not present an ovarian phenotype, there could be other effects on reproduction in these mice, as they have been shown to have reduced litter sizes without a reduction in ovulation rate (personal communication, Dr Chris Harlow), implying an impairment in reproduction at some point after ovulation. 11βHSD1 has been shown to be present in placenta, stromal decidua and endometrium, making these tissues targets for further investigations (McDonald *et al.* 2006; Thompson *et al.* 2002).

These investigations have shed light on possible mechanisms involved in ovarian function as well as collagen pathway biology. Further investigations will allow broader conclusions to be drawn on all aspects identified in these initial observations.

7. General Discussion

Early reports on the structure of the follicle during development indicated that the basement membrane structure was sensitive to the effects of hormones (Baccarini 1971). This has since been demonstrated in many species, suggesting that the mechanisms involved in remodelling the basement membrane are influenced by ovarian hormones (Palotie *et al.* 1984; Rodgers *et al.* 1999b). The follicular basement membrane is degraded by proteases that are up-regulated by the LH surge, an effect that can be inhibited *in vivo* by anti-sera to the protease, MMP-2 (Espey 1994; Gottsch *et al.* 2002). Components of the basement membrane have also been shown to enhance the survival and proliferation of granulosa cells during the period of follicle growth (Le Bellego *et al.* 2002). The destruction of the basement membrane ultimately allows for ovulation. However, this degradation process may also remove growth factors such as TGF-β that are required for proliferation, but are not necessary for ovulation. The collagen pathway is one aspect of this remodelling process that had not been extensively investigated in the rat ovary before the studies described here. Information gained in relation to this can be combined with known concepts of MMP and PA regulation to expand our current knowledge on the dynamics of tissue remodelling in the ovary.

Data showed that the collagen pathway genes, PCPE, BMP-1 and LOX, were all expressed most intensely in the immature follicle and that as follicles matured expression of these genes declined. Protein expression of BMP-1 and LOX was also seen in follicles at all stages, implying that depots of inactive enzymes synthesised when follicle growth is initiated are available for use throughout the period of follicle growth. Perhaps this could provide a finite amount of collagen pathway enzymes for follicle remodelling, confined to the preovulatory phase of the oestrous cycle, but that did not hinder ovulation. Visualisation of the compartmentalisation of collagen pathway genes revealed that BMP-1 mRNA was mostly thecal-derived, whereas LOX mRNA was located in the granulosa cells. However, protein location was not confined to the sites of gene expression. This implies that the encoded proteins must move across the basement membrane to other target sites, and highlights the need for co-operation between the two principal somatic ovarian cell types, a concept seen in the two-cell pathway of oestrogen production (Ryan

& Short 1965). Figure 7.1 refines the model for interactions of the collagen pathway genes in the follicle, proposed in Section 1.4.

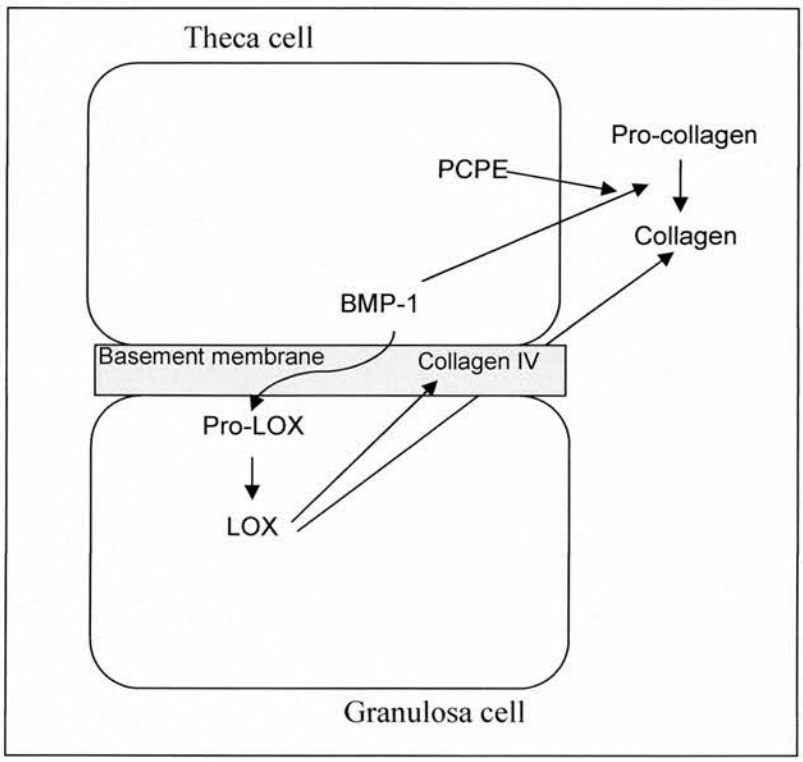

Figure 7.1. Model of proposed movements of collagen pathway components in the ovary.

The pattern of expression of the collagen pathway components during the oestrous cycle may be orchestrated by ovarian regulators such as glycoprotein hormones, growth factors and steroids. Glucocorticoids are known anti-inflammatory agents that have been shown to aid the resolution of ovulation-associated inflammation (Hillier & Tetsuka 1998; Rae & Hillier 2005). Therefore it is predicted that they may influence collagen pathway components involved in tissue remodelling post-ovulation. Localisation studies showed that 11βHSD2 was predominantly associated with immature follicles, indicating that active glucocorticoid does not affect these follicles at a stage when the collagen pathway components were most intensely expressed, and consequently implying that a lack of glucocorticoid allows active remodelling. The initiation of the LH surge sees a change in the expression of the 11βHSD isoforms, resulting in the predominant expression of 11βHSD1, which makes more active glucocorticoidd available in the ovary. When rat

ovarian cells were cultured with corticosterone, LOX mRNA was increased but BMP-1 and PCPE mRNAs were decreased, resulting in pro-LOX accumulation without activation and a lack of substrate formation. Similar to the inverse relationship of the 11βHSD isoforms, degrading proteases increase in expression as genes involved in collagen synthesis decrease in the preparation for ovulation. The common feature in all these changes in expression is the action of the gonadotrophic hormones. As these are the co-ordinating factors in functional follicle development, it is the direct or indirect affects of these hormones that drive the changes in expression patterns. Although this does not rule out interactions between the different cells, it highlights the complexity involved in the process of ovulation. This is summarised in Figure 7.2.

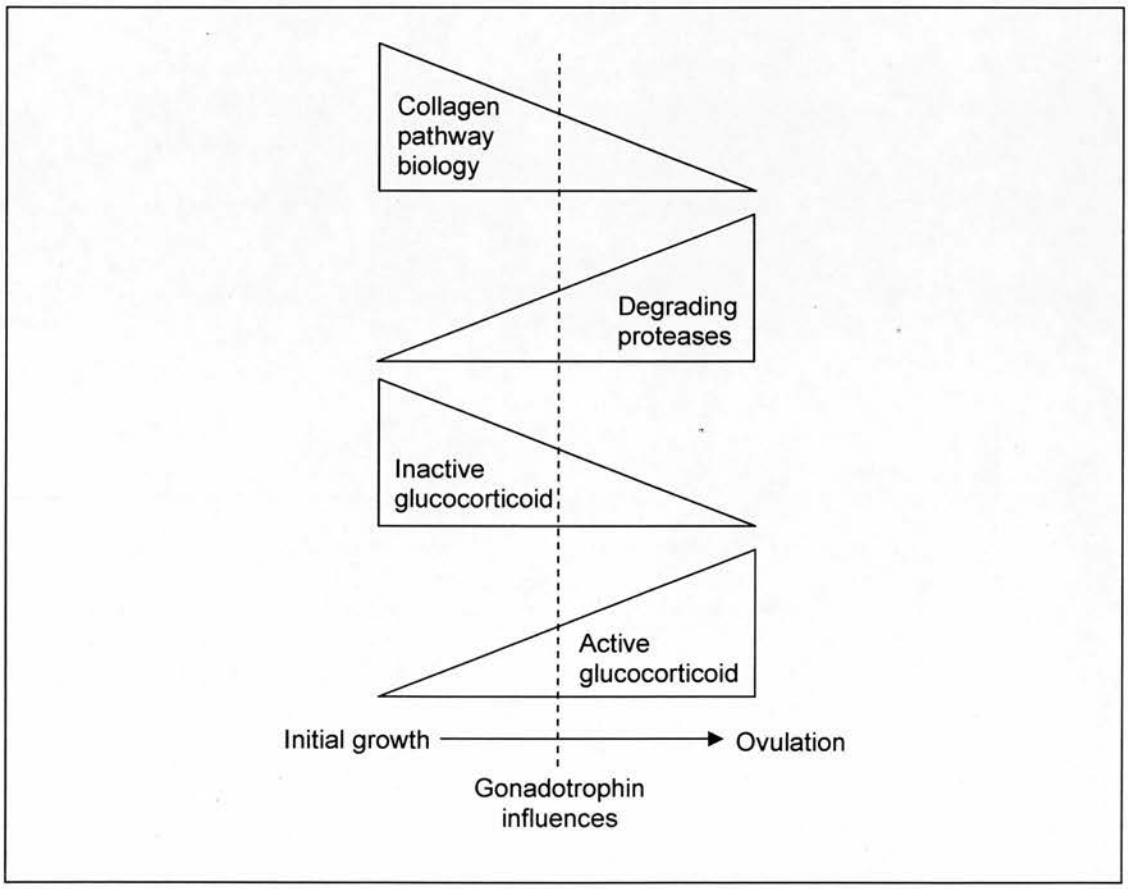

Figure 7.2. Summary of changing profiles of remodelling pathways (collagen synthesis and degrading proteases) and glucocorticoid activity in the ovary.

TGF-β up-regulates all members of the collagen pathway in many other body systems (Gacheru *et al.* 1997; Kanaki *et al.* 2000; Lee *et al.* 1997). However, in the ovary, TGF-β only appears to up-regulate LOX. In MG-63 human osteosarcoma cells TGF-β up-

regulates BMP-1 at 12h and peaks at 24h (Lee *et al.* 1997). In a variety of cell types induction was delayed when compared to TGF-β induction of other ECM proteins (at 3-5h) (Lee *et al.* 1997; Massague 1990). Ovarian cell cultures in these studies showed an upwards trend for BMP-1 and PCPE mRNA expression in the presence of TGF-β at 48h, suggesting that accumulation of pro-LOX was followed by activation (BMP-1) and substrate cleavage (BMP-1/PCPE). This suggests that the time-scale of TGF-β induction of collagen pathway genes in the ovary is shifted relative to other cell types studied, or that cells require a period of adjustment to the culture environment.

It is well documented that TGF-β improves wound healing by exerting effects on cell proliferation, differentiation and migration through regulating deposition of ECM constituents. To date, about 60 ECM-related genes have been identified as downstream targets of TGF-β (Verrecchia *et al.* 2001). Figure 7.3 indicates the effects TGF-β may have in the ovarian follicles on a small number of these downstream targets, and suggests that the effects of the LH surge possibly target TGF-β to reduce ECM accumulation and remodelling at ovulation. TGF-β itself has not been demonstrated to effect the expression of 11βHSD isoforms, but activin, a TGF-β superfamily member, has been shown to up-regulate 11βHSD2 in luteinised granulosa cells (Myers *et al* 2007, in preparation). This may suggest a role for other TGF-β superfamily members such as TGF-β itself in 11βHSD isoform regulation.

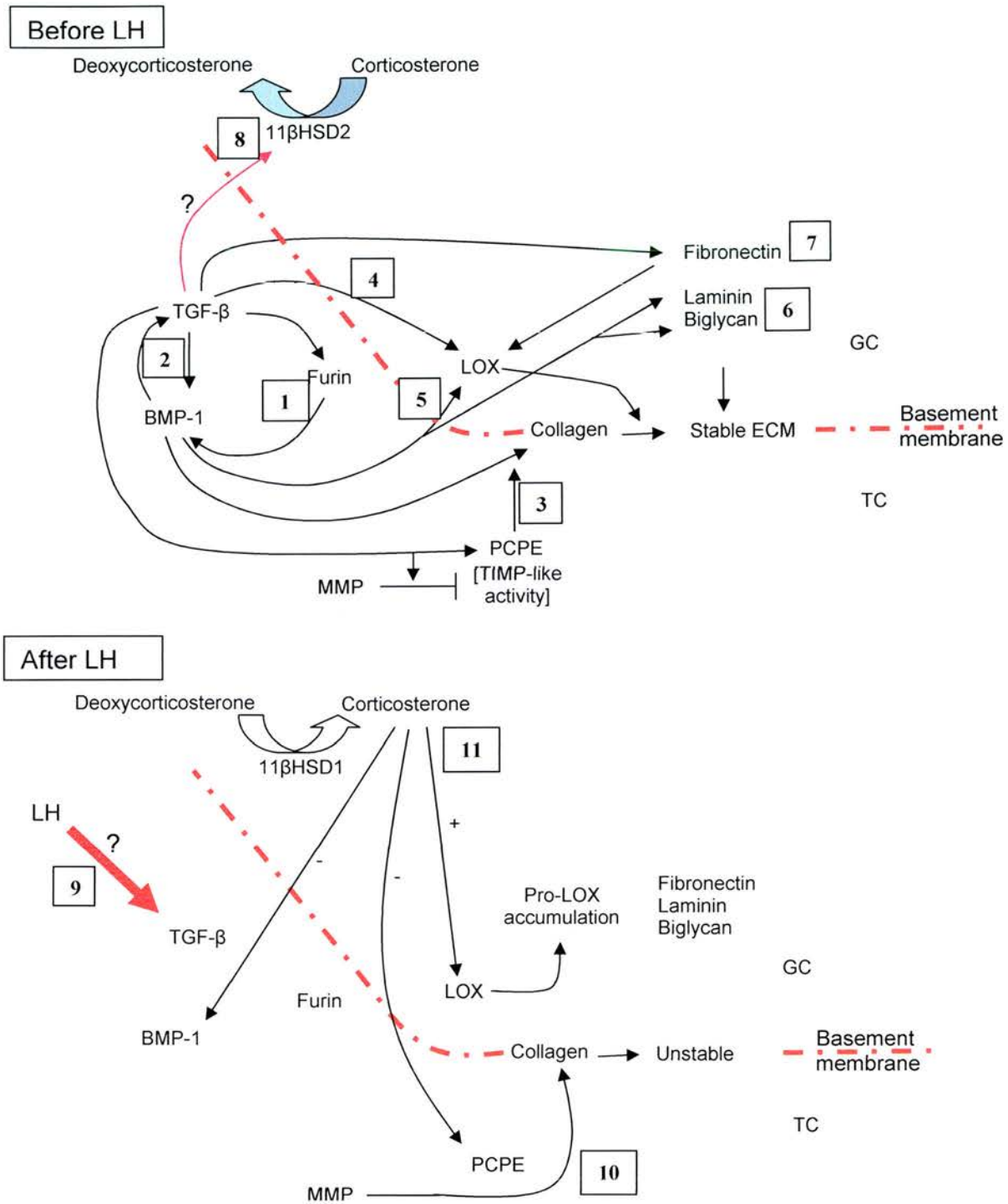

Figure 7.3. Proposed influences of TGF-β in the ovarian follicle before and after the LH surge. Before the LH surge, TGFβ is central to the accumulation and stabilistaion of the ECM and basement membrane. In the ovary TGFβ is produced by the theca cells where it will increase the production of furin, which will activate BMP-1 (1). BMP-1 will increase the cleavage of latent TGFβ, therefore the actions of TGFβs actually increase it own activation (2). TGFβ increases PCPE production which enhances BMP-1 effects on procollagen cleavage (3), therefore producing more collagen. TGFβ increases LOX expression (4) and BMP-1 activates LOX by cleaving it propeptide (5). BMP-1 also cleaves the proforms of other ECM components such as laminin and biglycan (6). Other acions of TGFβ on ECM include up-regulation of fibronectin which has been shown to increase LOX activation. At this time no direct evidence exists demonstrating TGFβ action on glucocorticoid activation. However unpublished data from Myers et al shows that the TGFβ superfamily member activin up-regulates 11βHSD2, indicating that TGFβ may have a similar effect and will therefore inactivate glucocorticoids subsequently allowing active remodelling. It is predicted that the LH surge initiates a decrease in ovarian TGFβ (9). This decline in the central driving force of ECM accumulation will subsequently cease and existing ECM structures such as basement membrane are degraded by MMPs (10). 11βHSD1 is now the predominant regulator of ovarian glucocorticoids driving the production of corticosterone which down-regulates BMP-1 and PCPE and up-regulates LOX that will accrue as a pool of pro-LOX as BMP-1 is lacking in the system (11).

pathway biology which have not yet been identified. Recent work by Forelgren *et al* (2005) suggests that fibronectin could provide a specific microenvironment that would regulate LOX activity. Using solid-phase binding assays, it was shown that fibronectin binds LOX, with cellular fibronectin having a higher affinity than plasma fibronectin (Fogelgren *et al.* 2005). Fibronectin also binds collagen (Johansson & Hook 1980) thereby localising LOX substrate to areas where LOX is present. In the fibronectin-null mouse, active LOX was decreased by 90% and pro-LOX increased (Fogelgren *et al.* 2005). There were also marked decreases in measurable LOX activity but BMP-1 levels were unchanged (Fogelgren *et al.* 2005), indicating that fibronectin is needed for the activation of LOX enzyme and that LOX enzyme activity can be regulated by its microenvironment. Studies in the ovary have shown that fibronectin in preantral/early antral follicles is localised to granulosa cells, but in larger follicles it is more strongly associated with the theca/stroma (Berkholtz *et al.* 2006). In the bovine corpus luteum, fibronectin is initially abundant but decreases as the corpus luteum is formed and the same pattern of expression has been shown for collagen type I (Silvester & Luck 1999). Hence, fibronectin seems to be available in the ovary at times when pools of pro-LOX are formed.

In conclusion, these studies have shown that the collagen pathway components are regulated throughout rat ovarian follicle development and ECM remodelling changes as the follicle matures, influenced by endocrine, paracrine and intracrine signalling.

Future work

Tissue remodelling during follicle development and post-ovulation is essential for normal function of the ovary. This work has established a basis for further detailed investigations into collagen pathway biology in the ovary.

Even though LOX was observed to be present throughout the ovary, other LOX family members should be considered. Immunohistochemistry and *in situ* hybridisation for LOX, LOXL and LOXL2 in human fetal membranes and placentae show that LOXL and LOXL-2 have both overlapping and distinct areas of protein expression compared to LOX (Hein *et al.* 2001; Saux *et al.* 1999). LOX and LOXL have been co-localised to skin, aorta, heart, lung, liver and cartilage but are seen in different localities in kidney,

stomach, small intestine, colon, retina, ovary, testis and brain (Hayashi *et al.* 2004). In the mouse ovary, LOX protein was principally seen in the theca cells of primary follicles and LOXL protein was expressed in the granulosa cells of these follicles (Hayashi *et al.* 2004). Therefore these proteins may have different functions in different ovarian cell types and would be prime candidates for future investigations.

Similar to LOX, BMP-1 has other family-associated members with similar proteolytic actions. These include mTLD, mTLL-1 and mTLL-2, which are all astacin family members (Uzel *et al.* 2001). *In situ* hybridisation of embryonic mouse hind limb shows that mTLL-1 and mTLL-2 are expressed more discretely than BMP-1 (Scott *et al.* 1999). Perhaps these BMP-1 related proteins provide a fine level of control within tissues. To date, these astacin family members have not been reported in the ovary, so it is not known whether they contribute to the actions of BMP-1 in this tissue.

It has been shown herein that the growth factor TGF-β regulates collagen pathway genes during follicle maturation. As the primary development of the ovarian follicle is dependant on growth factors, these may also influence collagen pathway gene expression. Possible regulators include other members of the TGF-β superfamily. It has been shown previously that GDF-9 and activin A, both members of the TGF-β superfamily, increase LOX mRNA and activity in immature rat granulosa cells. Contrary to positive effects on collagen synthesis during early follicle development, many pro-inflammatory cytokines are up-regulated at ovulation and may negatively regulate collagen pathway gene expression. Such effects have been shown in lung fibroblast cultures, where TGF-β effects on LOX are lost in the presence of the pro-inflammatory prostanoid, PGE2 (Roy *et al.* 1996).

The studies described in this thesis have begun to determine the locations and activities of collagen pathway components. However, due to technical difficulties with the LOX enzyme activity assay and the lack of a PCPE antibody, complete profiles for all the involved proteins have still to be established. Potential use of the LOX western could be utilised in future investigations to quantify the relative amounts of inactive and active LOX in isolated cell types. Similary western analysis could be performed for BMP-1 protein to determine activity of this protein and therefore the potential for activating LOX.

Comparisons between the qRT-PCR results from freshly isolated follicle cells (Chapter 3) and follicle cells cultured for 48h (Chapter 5) can begin to provide evidence for the regulators that influence collagen pathway biology at difference stages of follicle growth. The results from chapter 3 show the overall state of gene expression in maturing follicles. Whereas the results from chapter 5 show the influences of individual regulators, therefore influences that reflect the gene expression in freshly isolated cells may be more prominent in collagen pathway gene regulation at these points in follicle development. However, it should be remember that many hormones, growth factors and cytokines interact and regulate gene expression *in vivo*. This comparison has highlighted that LOX can be influenced by TGFβ at all stages of follicle development, but is probably most important in immature follicles when LOX expression is highest. The gene expression BMP-1 and PCPE in cultured cell experiments showed general down-regulation or no change implying that other factors up-regulate the expression of these genes during follicle maturation. These studies provide a basis for the regulation of collagen pathway biology in the ovary, and there are many other potential regulators that justify further investigation for example the influences of other mediators of ovarian inflammation could be examined such as TNFα, which in other tissue has been shown to influence LOX. Further investigations into the transgenic animal models studied in this thesis are required to build upon preliminarily investigations, additional studies will be insightful with respect to the animal model, as well as to the genes of interest being examined. Of particular interest in the models of interrupted glucocorticoid regulation (CYP11B1$^{-/-}$ and 11βHSD1$^{-/-}$) would be the examination of inflammatory markers to determine the degree of inflammation to be resolved in these models.

A further interesting observation between results chapters showed that qRT-PCR studies for BMP-1 and PCPE mRNA expression in isolated follicle cells (Chapter 3) showed an increasing trend as follicles matured, whereas the *in situ* hybridization studies (Chapter 4) showed a qualative decline in mRNA as follicles matured. Possible reasons for these differences include the sensitivies of these two techniques. qRT-PCR directly measures the amount of gene expression quantitatively from RNA extracted from cells and provides a highly sensitive method for measuring gene expression. *In situ* hybridization is performed on fixed tissue sections which require permeablisation and protein digestion

to allow cDNA probes to penetrate tissue to bind to sites of gene expression. All these steps can be optimised but can also limit the sensitivity of this technique. When *in situ* hybridsation was performed for these studies it was decided to use the same conditions for all tissues so comparisons could be drawn between stages of follicle maturation. If these studies were to be repeated it could be considered to look at the differently staged ovary tissue separately as they may require different optimal *in situ* hybridisation conditions to allow a clearer view of mRNA expression within these tissue.

In addition to further investigations into ovarian LOX function, it will be important to establish the relationship between LOX and collagen IV cross-linking, as the literature is still unclear as to whether LOX does cause cross-linking of the collagen network (Section 1.3.2.2.2). The importance of LOX in collagen cross-linking was observed in animals feed lathyrongenic agents such as legume plant family members or BAPN, these animals had weakened bones, skin, tendon and ligament attachments as well as impaired wound healing (Levene & Gross 1959). Subsequently it was shown that BAPN inhibited the activity of LOX extracted from chick cartilage (Siegel & Martin 1970). Collagen extracts from chick bone were further used to demonstrate that the addition of LOX stabilised collagen (reduced amounts of soluble collagen were recovered from cultures) and this effect decreased in the presents of BAPN (Siegel & Martin 1970). By re-visiting these original studies but substituting fibrillar collagens for collagen IV could confirm or refute the role of LOX in stabilising collagen IV structure.

Finally, as patterns of normal regulation and localisation are established, the role of LOX in ovarian pathology can be investigated. LOX has been shown to have a role in cancer (Section 1.3.2.1.3.). The incidence of ovarian cancer is ranked the fourth most common type of cancer and cause of death in women (NHS, Cancer in Scotland Statistics, 2003). Ovarian cancer arises from the OSE cells, which have been shown to produce LOX (Rae *et al* 2007, in preparation). Therefore, the role of LOX in these cells may be a fruitful avenue of investigation in ongoing investigations into ovarian cancer. Another ovarian disorder is polycystic ovary syndrome (PCOS). This is the most common endocrinopathy in women of reproductive age (Diamanti-Kandarakis & Economou 2006). The ovaries of these women are characterised by a thickened and fibrotic interstitial tissue containing cystic follicles in various phases of growth and atresia

(Henmi *et al.* 2001). Previous work using a rat model has shown that LOX is up-regulated and MMP-2 down-regulated in PCOS ovaries (Henmi *et al.* 2001). This indicates that LOX, and other collagen pathway components could be targets for endocrine inducers of this syndrome, perhaps offering markers of disease status and novel treatment strategies.

Finally, the ovary provides a model of scar-free remodelling and repair that can be used more widely as a model for wound repair mechanisms where scarring occurs and disrupts normal function elsewhere in the body.

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Appendix

Reagent	Source
β-mercapethanol	Sigma-Aldrich (Poole, Dorset, UK)
1,5-diaminopentane	Sigma-Aldrich (Poole, Dorset, UK)
100 bp ladder	Promega (Chilworth, Southampton, UK)
18s primer/probe mix	Applied Biosystems (Warrington, UK)
2 mCi [^{3}H] (4,5) lysine	Amersham (Chalfont St. Giles, Bucks UK)
3,3'-diaminobenzidine tetrahydrochloride (DAB)	DAKO (Ely, Cambs. UK)
35S-UTP (1.48 MBq/µl)	Amersham (Chalfont St. Giles, Bucks UK).
ABC Elite	Vector Laboratories (Orton Southgate, Peterborough UK)
Acetic anhydride	Sigma-Aldrich (Poole, Dorset, UK)
Adivin/biotin block	Vector Laboratories (Orton Southgate, Peterborough UK)
Agarose	Sigma-Aldrich (Poole, Dorset, UK)
Agilent 2100 Bioanalyser	Agilent Technologies (Wokingham, UK)
Amplex red	Sigma-Aldrich (Poole, Dorset, UK)
Aqueous picric acid	Sigma-Aldrich (Poole, Dorset, UK)
Assay-On-Demand assays	Applied Biosystems (Warrington, UK)
ATP, CTP and GPT	Ambion (Huntingdon, Cambs, UK)
BAPN	Sigma-Aldrich (Poole, Dorset, UK)
Borax	Sigma-Aldrich (Poole, Dorset, UK)
Bovine serum albumin	Sigma-Aldrich (Poole, Dorset, UK)
Bromophenol blue	Sigma-Aldrich (Poole, Dorset, UK)
CNBr	Sigma-Aldrich (Poole, Dorset, UK)
Coat-a-count direct androstenedione kits	DPC (Llanberis, Gwynedd, UK)
Collagenase	Sigma-Aldrich (Poole, Dorset, UK)
Denhardt's solution	Sigma-Aldrich (Poole, Dorset, UK)
DEPC	Sigma-Aldrich (Poole, Dorset, UK)
Dextran sulphate	Sigma-Aldrich (Poole, Dorset, UK)
Dithionthreitol	Sigma-Aldrich (Poole, Dorset, UK)
DNase I	Sigma-Aldrich (Poole, Dorset, UK)
Donor calf serum	Invitrogen (Paisley, UK)
dPBS	Invitrogen (Paisley, UK)
DPX mounting medium	VWR (Lutterworth, Leics, UK)

Duolite	VWR (Lutterworth, Leics, UK)
ECL Plus™ Western Blotting Detection Reagents	Amersham (Chalfont St. Giles, Bucks UK)
EDTA	Sigma-Aldrich (Poole, Dorset, UK)
Eosin	Sigma-Aldrich (Poole, Dorset, UK)
Ethidium bromide	Sigma-Aldrich (Poole, Dorset, UK)
EtOH	Fisher Scientific Ltd (Loughbough, Leics, UK)
First strand superscript III synthesis reagents	Invitrogen (Paisley, UK)
Formamide	Sigma-Aldrich (Poole, Dorset, UK)
Formic Acid	Sigma-Aldrich (Poole, Dorset, UK)
G.5 Nuclear emulsion	Ilford Imaging Ltd (Mobberley, Cheshire. UK)
Glacial acetic acid	Sigma-Aldrich (Poole, Dorset, UK)
Glycerol	VWR (Lutterworth, Leics, UK)
Glycine	Sigma-Aldrich (Poole, Dorset, UK)
Goat serum	Diagnostics Scotland (Glenrothes, UK)
Gold chloride	Sigma-Aldrich (Poole, Dorset, UK)
Haematoxylin	Sigma-Aldrich (Poole, Dorset, UK)
hCG	Sigma-Aldrich (Poole, Dorset, UK)
Horseradish peroxidase	Sigma-Aldrich (Poole, Dorset, UK)
Hydrogen peroxide	Sigma-Aldrich (Poole, Dorset, UK)
Immobilon-P transfer membrane	Fisher Scientific Ltd (Loughbough, Leics, UK)
Industrial methylated spirit	Fisher Scientific Ltd (Loughbough, Leics, UK)
Kodak BioMax MR	VWR (Lutterworth, Leics, UK)
Kodak BioMax XAR	Sigma-Aldrich (Poole, Dorset, UK)
Kodak D-19 developer	Calumet Photographic (Milton Keynes. UK)
Kodak GBH fixer	Sigma-Aldrich (Poole, Dorset, UK)
L-Glutamine	Sigma-Aldrich (Poole, Dorset, UK)
Lysine free medium	Promo Cell, SickingstraBe, Heidelberg, DE
Lysozyme	Sigma-Aldrich (Poole, Dorset, UK)
Medium 199	Invitrogen (Paisley, UK)
MeOH	Fisher Scientific Ltd (Loughbough, Leics, UK)
Methenamine	VWR (Lutterworth, Leics, UK)
Milk powder	Tesco (Dundee, UK)
Na citrate	Sigma-Aldrich (Poole, Dorset, UK)
NaCl	Sigma-Aldrich (Poole, Dorset, UK)

NaCl	Sigma-Aldrich (Poole, Dorset, UK)
Nalidixic acid	Sigma-Aldrich (Poole, Dorset, UK)
NICK columns	Amersham (Chalfont St. Giles, Bucks UK)
Optical reaction plates	Applied Biosystems (Warrington, UK)
Paraformaldehyde	Sigma-Aldrich (Poole, Dorset, UK)
PBS tablets	Sigma-Aldrich (Poole, Dorset, UK)
Penicillin	Sigma-Aldrich (Poole, Dorset, UK)
Percoll	Sigma-Aldrich (Poole, Dorset, UK)
Periodic acid	Sigma-Aldrich (Poole, Dorset, UK)
Permafluor	Beckman Coulter (High Wycombe, UK)
Phenylmethylsulfonyl fluoride	Sigma-Aldrich (Poole, Dorset, UK)
Pregant Mare Serum Gonadotrophin	Sigma-Aldrich (Poole, Dorset, UK)
Promega mastermix	Promega (Chilworth, Southampton, UK)
Protease cocktail	Sigma-Aldrich (Poole, Dorset, UK)
Proteinase K	Sigma-Aldrich (Poole, Dorset, UK)
QIAquick gel extraction kit	Qiagen (Crawley, UK)
Rabbit serum	Diagnostics Scotland (Glenrothes, UK)
RLT buffer	Qiagen (Crawley, UK)
RNA polyermase (T3, T7 or SP6)	Promega (Chilworth, Southampton, UK)
RNase free water	Sigma-Aldrich (Poole, Dorset, UK)
RNase inhibitor	Promega (Chilworth, Southampton, UK)
RNase inhibitor	Promega (Chilworth, Southampton, UK)
RNasy Kit	Qiagen (Crawley, UK)
RNAzap (Ambion	Ambion (Huntingdon, Cambs, UK)
S35 UTP	Amersham (Chalfont St. Giles, Bucks UK)
Salmon Sperm DNA	Sigma-Aldrich (Poole, Dorset, UK)
Saran Wrap™	VWR (Lutterworth, Leics, UK)
Scintillation fluid	VWR (Lutterworth, Leics, UK)
Scott's tap water	Sigma-Aldrich (Poole, Dorset, UK)
SDS	Sigma-Aldrich (Poole, Dorset, UK)
Silver nitrate	Sigma-Aldrich (Poole, Dorset, UK)
Sirius red	Sigma-Aldrich (Poole, Dorset, UK)
Sodium borate	Sigma-Aldrich (Poole, Dorset, UK)
Sodium deoxycholate	Sigma-Aldrich (Poole, Dorset, UK)
Sodium thiosulphate	VWR (Lutterworth, Leics, UK)
Streptadivin 463	Molecular Probes (Paisley, UK)
Streptomycin	Sigma-Aldrich (Poole, Dorset, UK)

Sytox green	Molecular Probes (Paisley, UK)
Taqman reversre transcriptase regeants	Applied Biosystems (Warrington, UK)
Taqman Universal mastermix	Applied Biosystems (Warrington, UK)
Topro	Molecular Probes (Paisley, UK)
Triethanolamine	Sigma-Aldrich (Poole, Dorset, UK)
Tris-base	Sigma-Aldrich (Poole, Dorset, UK)
Tris-HCl	Sigma-Aldrich (Poole, Dorset, UK)
Trisodium citrate	Sigma-Aldrich (Poole, Dorset, UK)
Trypan Blue	Sigma-Aldrich (Poole, Dorset, UK)
Trypsin	Sigma-Aldrich (Poole, Dorset, UK)
TSA™ Tiramide Cy3nine 3 system	Perkin Elmer (Beaconsfield, Bucks, UK)
Tween-20	VWR (Lutterworth, Leics, UK)
Urea	Sigma-Aldrich (Poole, Dorset, UK)
Xylene	Fisher Scientific Ltd (Loughbough, Leics, UK)
Yeast tRNA	Invitrogen (Paisley, UK)

Antibodies	**Source**
11βHSD1	Cayman/IDS Ltd (Bolton, UK)
Collagen IV	Chemicon (Chandlers Ford, Hamps, UK)
Goat anti-rabbit	Vector Laboratories (Orton Southgate, Peterborough UK)
GR	ABR (Golden, CO, USA)
Laminin	Abcam (Cambridge, UK)
MMP-2	Nova-castra (Newcastle, UK)
Rabbit anti-sheep	Vector Laboratories (Orton Southgate, Peterborough UK)
Rat anti-mouse	Vector Laboratories (Orton Southgate, Peterborough UK)

Product	**Source**
24-well culture plates (tissue culture grade)	Fisher Scientific Ltd (Loughbough, Leics, UK)
Eppendorfs	Fisher Scientific Ltd (Loughbough, Leics, UK)
Syringe needles	Fisher Scientific Ltd (Loughbough, Leics, UK)
Syringes	Fisher Scientific Ltd (Loughbough,

	Leics, UK)
Hybridisation boxes	Fisher Scientific Ltd (Loughbough, Leics, UK)
Rohen tubes	Sarstedt, Germany
Whatman 3MM chromatography paper	VWR (Lutterworth, Leics, UK)
Falcon tubes	Fisher Scientific Ltd (Loughbough, Leics, UK)
Glassware	Fisher Scientific Ltd (Loughbough, Leics, UK)
Plasticware	Fisher Scientific Ltd (Loughbough, Leics, UK)
Superfrost microscope slides	VWR (Lutterworth, Leics, UK)