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Extracellular matrix associated with human luteinizing granulosa cells

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ABSTRACT

FACULTY OF MEDICINE
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Doctor of Medicine

Extracellular matrix associated with human luteinizing granulosa cells

by Evangelos Demetrios Alexopoulos

Human granulosa cells (GC), prepared from follicular aspirates using a non-enzymic method, were maintained on chamber slides in a defined medium without additional attachment factors. In this system, GC clustered to a limited extend and attached only loosely to the substratum necessitating medium replacement through repeated partial changes to avoid cell loss. Cell size and progesterone production increased consistently with continuing luteinization. These processes were associated with maintenance and deposition of the endogenous extracellular matrix components heparan sulphate proteoglycan and laminin ($\alpha 2$, $\beta 1$, $\gamma 1$ subunits) as shown by both Western blotting and immunohistochemistry. Collagen IV seemed to be present between freshly prepared GC and was maintained in culture. The addition of either epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) into the culture medium did not reveal any marked effect on deposition of laminin components during culture. Release of cell clusters, particularly in the absence of gonadotrophin, was noted during the later stages of culture, constituting the so-called 'lift off' phenomenon. This has been correlated with an increasing concentration of matrix metalloproteinases (MMP-2) in the culture medium during the culture period. To conclude, luteinization and maintenance of the GC-derived layer of the corpus luteum is likely to involve deposition and conservation of pericellular extracellular matrix components, actively synthesized by the GC themselves and controlled by the presence of gonadotrophin.

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ABBREVIATIONS

bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cAMP	cyclic adenosine mono-phosphate
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
FSH	follicle stimulating hormone
GC	granulosa cells
GnRH	gonadotrophin releasing hormone
hCG	human chorionic gonadotrophin
HSPG	heparan sulphate proteoglycan
IGF	insulin-like growth factor
IU	international unit
IVF	in vitro fertilization
LDL	low density lipoproteins
LH	luteinizing hormone
mg	milligram
ml	millilitre
MMP	matrix metalloproteinase
ng	nanogram
PBS	phosphate buffered saline
PBS-A	PBS with bovine serum albumin
PG $F_{2\alpha}$	prostaglandin $F_{2\alpha}$
$P450_{arom}$	cytochrome P450 aromatase
$P450_{scc}$	cytochrome P450 cholesterol side chain cleavage
SDS	sodium dodecyl sulphate
StAR	steroidogenic acute regulatory protein
TBST	tris-buffered saline with tween
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
TRIS	tris (hydroxy methyl) methylamine
VEGF	vascular endothelial growth factor
3β-HSD	3β-hydroxysteroid dehydrogenase

LIST OF PUBLICATIONS

Luteinized human granulosa cells are associated with endogenous basement membrane-like components in culture.
E Alexopoulos, J Shahid, HZ Ongley, & MC Richardson
Molecular Human Reproduction (2000) vol. 6, no.4 pp324-330.

Basement membrane-like components associated with human granulosa cells luteinised in culture.
J Shahid, E Alexopoulos, HZ Ongley and MC Richardson
Journal of Reproduction and Fertility, Abstract Series 23, page 29, 1999.
Poster presented at the Society for the Study of Fertility Annual Conference, Aberystwyth, U.K.

Control of extracellular matrix associated with luteinising granulosa cells: the present state of play and recent results describing endogenous matrix associated with granulosa cells in culture.
MC Richardson, HZ Ongley, J Shahid and E Alexopoulos.
Presented at the National Ovarian Workshop (UK) 1999, Southampton, U.K.

DEDICATION

This thesis is dedicated to my family for their constant support and encouragement during the 6 years of my training in the United Kingdom.

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Chapter 1

INTRODUCTION

Contents

1.1 Overview

The corpus luteum is an endocrine gland of the ovary with a sophisticated function and a vital role in human reproduction. It is formed from the remnants of the dominant follicle after ovulation has occurred as a response to the midcycle surge of luteinizing hormone (LH). During its short life span it plays a fundamental role in hormonal synthesis and secretion, as it is the most active gland in the human body (Zeleznik and Fairchild Benyo, 1994).

In the 14 days of its life, the corpus luteum produces estradiol and progesterone and it is largely under the control of LH. The progesterone, a C21 steroid, provides the necessary changes in the intrauterine environment for implantation of the blastocyst and early embryonic development (Stouffer, 1988). The secretion of the human chorionic gonadotrophin (hCG) by the trophoblast of a successfully implanted embryo maintains the integrity and secretory capacity of the corpus luteum beyond the time of the missed menstrual period (Baird, 1985). The importance of hCG to luteal rescue has been proved as hCG injections given to women extended the life cycle of the corpus luteum (Hanson et al., 1971). Progesterone levels normally rise sharply after ovulation, reaching a peak approximately 8 days after the LH surge (Figure 1.1). The corpus luteum can then be recognized as a two centimeter, orangy-red structure on the surface of the ovary reflecting its high vascularity and lipid content.

Assuming that implantation has not occurred, the functional and structural integrity of the corpus luteum is lost (luteolysis) and progesterone production falls. This causes destabilization of the endometrium, which through an inflammatory-like process leads to endometrial shedding, resulting in menstruation (Salamonsen and Wooley, 1996). At the time of menstruation the corpus luteum converts into an avascular fibrous remnant, which can be identified on the surface of the ovary.

It is therefore evident that the corpus luteum constitutes the primary interface between menstruation and maternal recognition of pregnancy. Despite its key role in reproduction, cellular mechanisms underlying luteal regression and rescue remain poorly understood, particularly in primates and represent a major unsolved puzzle in biology. Recent work suggests a central role for extracellular matrix located around the luteal cells, the stability of which is likely to be regulated by a balance between the degrading activity of matrix metalloproteinases (MMPs) and the inhibitory action of tissue inhibitors of matrix metalloproteinases (TIMPs).

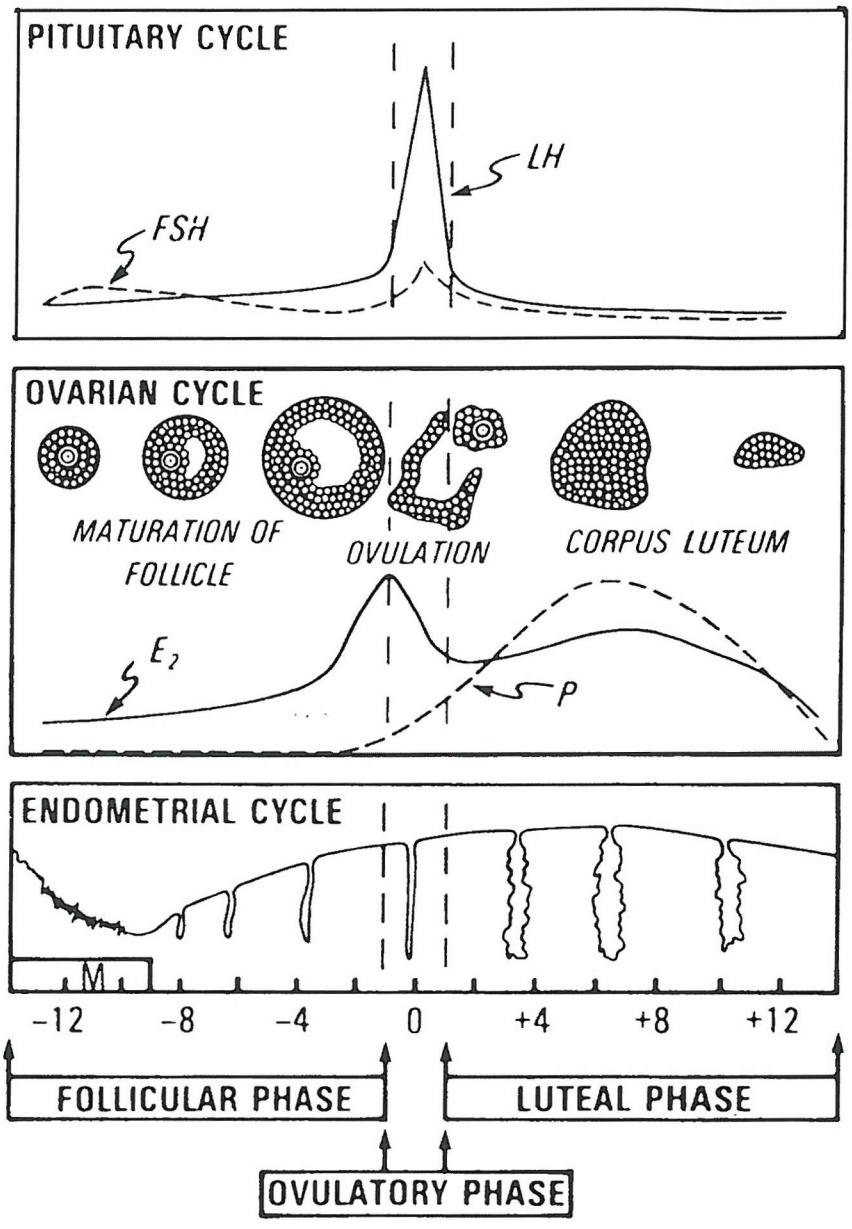


Figure 1.1 The human menstrual cycle. Diagrammatic representation of the changes in serum concentrations of the sex steroids 17β-estradiol (E_2) and progesterone (P) and the gonadotrophins luteinizing hormone (LH) and follicle stimulating hormone (FSH), throughout the menstrual cycle. Endometrial and follicular changes in correlation with the menstrual cycle are also indicated.

The corpus luteum remains one of the great enigmas of modern reproductive endocrinology and this thesis will attempt to increase our understanding of the processes involved in its formation, maintenance and regression. The overall guiding hypothesis postulates that a predominance of MMP activity will lead to extracellular matrix degradation, loss of cell function and viability resulting in luteal regression, whereas a predominance of TIMP activity will encourage matrix stability, continued cell function and luteal rescue. As part of a broader plan to examine this hypothesis, the thesis will be mainly concerned with looking at the nature of the extracellular matrix around luteal cells.

1.2 Follicular growth in the human ovary

The structure, function and control of the corpus luteum all have their origin in the preovulatory follicle. Therefore it is fundamental prior to any research on this enigmatic gland to ensure a good understanding of general ovarian physiology and particularly of follicular growth and development. Normal folliculogenesis constitutes a prerequisite for the normal function of the corpus luteum.

Small follicles developing from the pool established in the fetus do not need gonadotrophin support. Larger follicles follow time-dependent growth patterns and require follicle stimulating hormone (FSH) and LH support. The pool of follicles in the human fetus expands rapidly to 5 X 10^6 oocytes at 4-5 months of gestation, then declines as many follicles degenerate in successive waves of atresia. Some follicles begin to differentiate as soon as they are formed, so that folliculogenesis begins in fetal life and is then continuous throughout a woman's reproductive life. At birth approximately 2 million oocytes remain and this pool is then depleted as follicles migrate away until very few or none are left at the menopausal transition.

Human ovaries have a pool of primordial follicles consisting of an oocyte arrested in prophase I of meiosis, surrounded by flattened granulosa cells (Zeleznik and Fairchild Benyo, 1994). Enlargement and proliferation of the

granulosa cells with an increase of the oocyte's size leads to the formation of the primary follicle (Gougeon, 1996). A basement membrane supports the outermost layer of the granulosa cells while a non-cellular layer, the zona pellucida, separates the innermost granulosa cells from the oocyte. Some stromal cells near the basal lamina become aligned parallel to each other, changing into epithelioid-like cells and stratify into the theca cell layer (Figure 1.2).

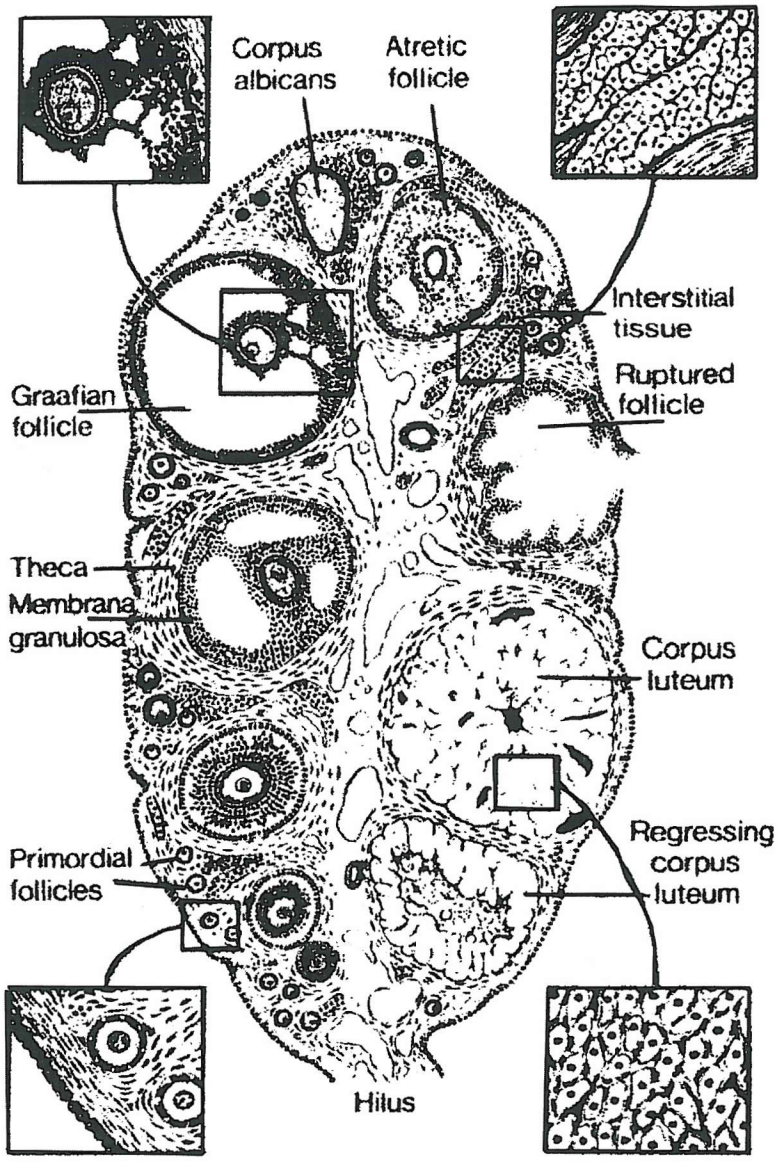


Figure 1.2 The mammalian ovary. Idealised drawing of the structure of the mammalian ovary showing follicles at various stages of their development and the formation and regression of corpora lutea.

Fluid is secreted within the granulosa cell layer causing the development of small fluid-filled spaces, gradually coalescing to form the follicular antrum (Weiss, 1983). A specific group of granulosa cells surrounds the oocyte and forms the cumulus oophorus. The theca cells are differentiated into a highly vascular layer of columnar cells, the theca interna and the outer fusiform cells, the theca externa. The oocyte is pushed to one side of the follicle in an eccentric position forming the secondary follicle. This non gonadotrophin-dependent stage of follicular growth lasts about 85 days.

Tertiary follicular growth occurs only in 1 or 2 of the secondary follicles, between cycle days 5 and 12. The dominant follicle expands rapidly by mitosis and fluid accumulation from 6.9±0.5mm in the early follicular phase to 18.8±0.5 mm in the late follicular phase (Pache et al., 1990). Good blood flow is essential during these stages of growth. The preovulatory follicle becomes highly vascular via an endothelial cell proliferation process within the theca cell layers (McClure et al., 1994). The granulosa cell layer remains avascular so that nutrients are obtained by diffusion.

1.3 Ovulation

As the graafian follicle grows, it gradually reaches the surface of the ovary and ultimately protrudes above it. Necrosis of the overlying tissue rather than pressure within the follicle is the principal factor resulting in follicular rupture. LH, acting on specific receptors, induces ovulation by influencing factors which act on the outer layers of the follicle (Espey and Lipner, 1994). Eicosanoids and steroids have been implicated to stimulate and augment an inflammatory-like reaction at the stigma on the follicular wall. The cumulus-oocyte complex is released within a wave of follicular fluid. The collapsing follicle with its luteinising granulosa cells becomes the corpus luteum.

1.4 The corpus luteum

Following the LH surge, the basement membrane between the theca interna and the granulosa cells breaks down. The extended granulosa layer undergoes pronounced folding whilst the theca engorges with blood becoming oedematous. The theca cells, placed at the periphery of the corpus luteum, invaginate into the granulosa cell layer becoming hypertrophic and forming the theca-lutein cells. The granulosa cells hypertrophy also and are termed granulosa lutein cells. The theca derived cells are easily distinguished from the granulosa ones in the human corpus luteum (Rodger et al., 1995) and their complex is surrounded by a dense fibrous stroma containing blood vessels, fibroblasts, immune cells and extracellular matrix (Behrman et al., 1993).

The corpus luteum has an extremely rich blood and lymph supply and it is the most vascular tissue in the body, with a blood supply per unit mass eight times higher than that of the kidney (Ford et al., 1982). The newly formed blood vessels invade the granulosa layer towards the centre as cords of endothelial cells, which represent over fifty percent of the corpus luteum cellular tissue (Reynolds et al., 1992; Zheng et al., 1994). Blood vessel formation involves two distinct phases (Reynolds et al., 1992). The first phase involves the dissolution of the endothelial cell basement membrane under the action of proteolytic enzymes. The next stage is the proliferation and migration of the endothelial cells. Such angiogenic activity is stimulated by a variety of growth factors, cytokines and interleukins. Epidermal Growth Factor (Huang et al., 1995), Insulin-like Growth Factor-1 (Igf-1) (Jones and Clemmons, 1995), Transforming Growth Factor-α, Transforming Growth Factor-β (Tamura et al., 1995), Tumor Necrosis Factor-α (TNFα), Interleukin-1 and Interleukin-6 (Bagavandoss and Wilks, 1991) are all thought to influence luteal angiogenesis. It has been postulated that basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are the principle luteal endothelial mitogens (Redmer and Reynolds, 1996; Redmer et al., 1996, Ferrara et al., 1998).

Macrophages, T-lymphocytes, neutrophil polymorphonuclear leukocytes, eosinophils and monocytes are immune cells found in the corpora lutea, which seem to have an important role in the regulation of luteal function (Brannstrom et al., 1994; Best et al., 1996). Macrophages are the prominent immune cells in the corpora lutea with an increasing number as the luteal phase progresses (Best et al., 1996).

The extracellular matrix holds together the various constituents that make up the corpus luteum. It has a structural role and consists mainly of collagen, proteoglycans, laminin and fibronectin. Additionally, there is a dynamic function of the extracellular matrix involving its interactions with the steroidogenic and endothelial cells (Behrman et al., 1993) and plays a vital role during the growth, development and atrophy of the corpus luteum. The structural components of the ECM of the human corpus luteum as well as their relationship with the function of the corpus luteum constitute a fundamental basis of this thesis, and are discussed separately.

1.5 The corpus luteum function

The main physiological role of the corpus luteum is the secretion of hormones that play a prime role in the establishment and maintenance of pregnancy. Luteal products include the steroid hormones estrogens, progesterone and androgens and also peptides such as relaxin, inhibin and oxytocin (Behrman et al., 1993). Progesterone secretion is the primary function of the corpus luteum. The human corpus luteum secretes up to 25 mg of progesterone per day.

Luteinisation is the process of formation of the corpus luteum from the ruptured dominant follicle and includes both structural and functional changes. The predominantly FSH-stimulated estrogen production of the follicle changes at mid-cycle under the effect of pituitary LH so that progesterone production now becomes dominant. During this process the granulosa cells become larger, develop intracellular granules and become

rich in mitochondria, smooth and rough endoplasmic reticulum (Zeleznik and Fairchild Benyo, 1994). They can synthesize and secrete progesterone as well as oestrogens by aromatisation from the thecal androgens.

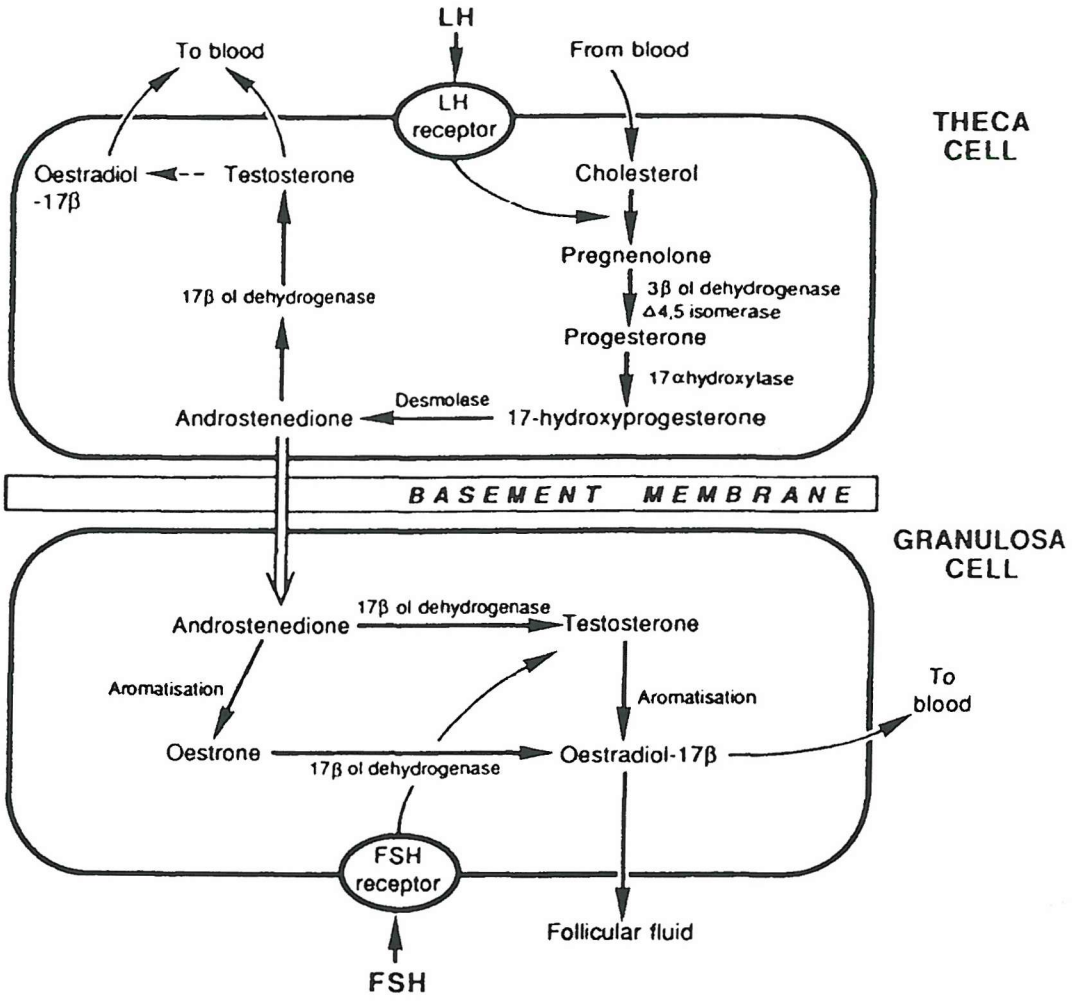


Figure 1.3 Steroid production.

The currently accepted model for oestrogen synthesis (the two-cell, two-gonadotrophin hypothesis). Luteinizing hormone (LH) acts on the theca cells to stimulate androgen synthesis. The granulosa cells are stimulated by follicle-stimulating hormone (FSH) to aromatise these androgens to oestrogens.

1.5.1 Steroidogenesis

The sex steroid synthetic pathway is well defined. Luteal cholesterol is derived directly from the plasma and the mobilization of intracellular stores (Strauss et al., 1981). It is transported to the ovary mainly in the form of low-density lipoproteins (LDL) where it is internalized and incorporated into luteal lysosomes (Figures 1.3 & 1.4). The initial conversion of cholesterol to pregnenolone within mitochondria is considered to be the rate-limiting step in steroidogenesis (Hall, 1985). This is carried out by the P450 cholesterol side-chain cleavage enzyme complex (P450scc), which is located on the inner mitochondrial membrane (Stocco and Clark, 1996). Cyclic AMP (cAMP), released within the cell, causes an increase in the translocation of cholesterol across the mitochondrial membrane. It is the LH receptor activation of cAMP production that makes this possible and so demonstrates the method by which gonadotrophins stimulate steroidogenesis (Hall, 1985). The transfer of the hydrophobic cholesterol to the inner mitochondrial membranes is achieved via the steroidogenic acute regulatory protein (StAR), which seems to be required for regulated steroidogenesis (King et al., 1995; Waterman, 1995). In the cytosol, pregnenolone is converted into progesterone by the action of 3β-hydroxysteroid dehydrogenase (3β-HSD). Progesterone can be converted into androstenedione, which ultimately converts to 17β-estradiol through the action of P450 aromatase (P450arom) (Strauss and Miller, 1991). Human theca lutein cells produce both progesterone and androgens, while granulosa lutein cells produce mainly progesterone and some oestradiol.

In addition to steroid hormones the corpus luteum also synthesizes and releases protein hormones such as relaxin, oxytocin, inhibin and activin.

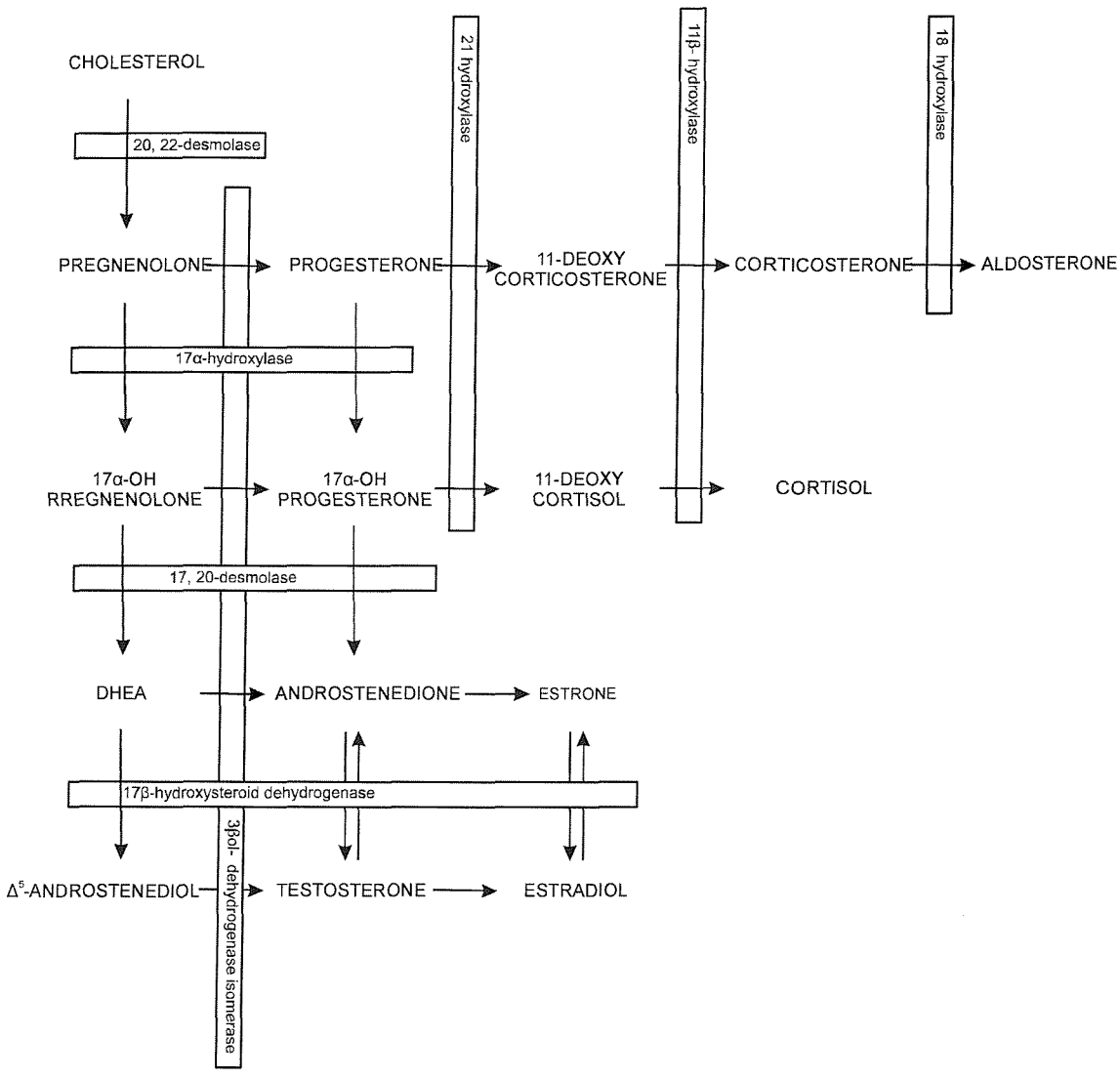


Figure 1.4 The synthetic pathway for sex steroid hormones. The synthetic pathway and the enzymes involved in the production of sex steroids.

1.5.2 Regulators of steroidogenesis

The steroidogenic cells of the corpus luteum express specific receptors to LH (Ravindranath et al., 1992), which is the principal regulator of luteal function. Removal of the pituitary surgically results in a rapid loss of luteal function (Fraser et al., 1986), whilst addition of LH to isolated luteal cells and luteal tissue can augment progesterone production.

Binding of LH with its receptors stimulates the enzyme adenyl cyclase to produce cyclic adenosine mono-phosphate (cAMP), which is a messenger molecule responsible for the stimulation of steroidogenesis. However various molecules acting in autocrine and paracrine pathways can also modify progesterone synthesis. These molecules include proteins, growth factors, cytokines, eicosanoids and steroids. IGF stimulates progesterone production from isolated luteal cells and this effect seems to be mediated by specific IGF receptors (Devoto et al., 1995). In contrast, there is now evidence of increased activin concentration during luteolysis (Muttukrishna et al., 1996) suggesting an inhibitory role in luteal steroidogenesis. Although it has been postulated that there is a role for progesterone in the local control of luteal function (Duffy and Stouffer, 1995), specific physiological roles have not yet been clearly identified. An inhibitory action of androgens in progesterone synthesis and LH receptor expression on luteinized granulosa cells has been demonstrated. (Polan et al., 1986) In addition, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Abayasekara et al., 1993), arachidonic acid itself, leukotrienes (Ciereszko et al., 1995) and TNFα (Wang et al., 1992) seem to have an antisteroidogenic effect.

1.6 Luteolysis

Corpus luteum regression (luteolysis) will occur in the absence of conception. Historically, this process has been divided into two distinct stages: functional and structural luteolysis. Functional luteolysis refers to the suppression of progesterone synthesis and secretion (Zeleznik and

Fairchild Benyo, 1994) whereas structural luteal regression which follows involves dissolution and absorption of the gland (Behrman et al., 1993). These historical definitions were based on studies in rats (Malven, 1969) where these processes occur as separate events. Functional and structural luteolysis may be separate events in the human, although the interval between the loss of function and beginning of structural involution is unclear.

It is known that the corpus luteum is very dependent on circulating LH during the luteal phase. For example, administration of GnRH antagonists results in the rapid loss of corpus luteum integrity (Fraser et al., 1986). However, physiological luteolysis does not seem to be due to a drop in circulating LH as the end of the luteal phase approaches. Thus it has been concluded that changes in pulse frequency of LH during the luteal phase are not sufficient to cause luteolysis (Ellingwood et al., 1984). It therefore appears that there is a change in responsiveness of the corpus luteum to LH in the late luteal phase (reviewed by Behrman et al., 1993).

The role of the LH receptors of the corpus luteum and their ability to bind to LH has also been studied in non-human primates (Cameron and Stouffer, 1982). It appears that falling progesterone production in spontaneous luteolysis is not preceded by a change in the number or affinity of the LH receptors. We are left to conclude that there are post-receptor events associated with loss of responsiveness in the late luteal tissue.

The role of an alteration in the steroidogenic enzymes P450scc, 3β-HSD (Sanders and Stouffer, 1997) and StAR (Kiriakidou et al., 1996) during the lifespan of the corpus luteum has been examined. These fall as a result of the change in LH-responsiveness. Although there is clear evidence of a luteolytic action of $PGF_{2\alpha}$ in the rat corpus luteum (Riley et al., 1991), in the human the role of $PGF_{2\alpha}$ is less certain.

Regarding structural luteolysis, the loss of luteal cells and the consequent change in luteal size and structure are via a physiological cell death

process known as apoptosis (Arends and Wyllie, 1991). During apoptosis the cells shrink and become denser with pyknotic nuclei. Small membrane-bound packages of nucleus and cytoplasm, the apoptotic bodies, are phagocytosed by macrophages. These morphological changes of apoptosis can be seen in tissues by light microscopy (Wyllie, 1994).

Induction of luteolysis in the primate increases the rate of apoptosis (Young et al., 1997) whilst there is a reduction in the early stages of pregnancy (Shikone et al., 1996). It is therefore possible that luteal regression involves apoptotic cell death and luteal rescue inhibits it.

Histological features of structural luteolysis in the rat demonstrate a reduction in the corpus luteum diameter with an increase of the connective tissue content, which consists mainly of fibroblasts. Finally, scarring tissue remains with fibroblasts surrounding islands of luteal cells.

Biochemically, apoptosis is associated with a characteristic pattern of deoxyribonucleic acid (DNA) degradation. It is regulated by a variety of proteins, genes and proto-oncogenes (Hale et al., 1996).

Abundant evidence has demonstrated the importance of the extracellular matrix in the modulation of cell function and differentiation.

1.7 Extracellular matrix in the corpus luteum.

It will become apparent that there is an important role for extracellular matrix in the control of ovarian function both at the follicular and luteal stages. Although there may be a role for non-basement membrane extracellular matrix, the thesis will concentrate on the identities and functions of 'basement membrane components' which appear to be particularly important in ovarian function. As a start, it is worth considering some of the general features of the molecular architecture of basement membranes as follows.

The general structure of basement membranes has been reviewed by Yurchenco and Schittny (1990). To summarize, basement membranes provide cellular support, cell regulatory and sieving functions within various tissues. They are formed through complex and specific molecular interactions between various macromolecules (see Figure 1.5). Firstly, collagen IV chains, using terminal and lateral associations, form a covalently stabilized polygonal framework. We can regard this as a sort of 'trellis' on which the other macromolecules arrange themselves. Thus laminin, a four-armed glycoprotein, assembles to form a second polymer network associated with the collagen framework. A small linker molecule (entactin or nidogen) helps with this association. Finally, large heparan sulphate proteoglycan molecules are firmly anchored in the membrane, binding both laminin and collagen IV through the glycosaminoglycan chains of the proteoglycan. There is some heterogeneity of structure between tissues and potentially changes in composition as tissues develop or undergo cyclical changes. This heterogeneity may sometimes be brought about through the inclusion of different laminin isoforms. Also, each collagen IV molecule is composed of three alpha chains, selected from at least six different types of alpha chain discovered to date.

Figure 1.5 A current model of the molecular structure of a basal lamina. Reproduced from: 'Molecular Biology of the Cell' by Alberts et al.(1994).

In order to understand the potential role of basement membrane in the corpus luteum, we need to begin with an appreciation of the disposition of basement membrane in the follicle. Put simply, there is a clear, well-characterized basement membrane which separates the granulosa and thecal layers. It is estimated that the surface area of the bovine follicle doubles nineteen times during development implying very active synthesis of basement membrane during this time (see Van Wezel et al., 1998). Also, there is some evidence for changing laminin isoform composition during follicular development (Van Wezel et al., 1998).

The basement membrane of the follicle has a number of potential functions. Apart from a purely structural function, it has an important 'sieving' action reducing the entry of large molecules (such as low density lipoprotein) into the granulosa compartment and follicular fluid (see Van Wezel et al, 1998). It will also have a regulatory action on the granulosa cells which may be particularly important for those cells directly in contact with it. It may also be involved in preventing vascularization of the granulosa layer. The question as to which cells produce the follicular basement membrane remains controversial. Although there is evidence that bovine granulosa cells alone are capable of producing basement membrane components in culture (Rodgers et al., 1996), it is possible that the follicular basement membrane in vivo requires a contribution from both the granulosa cells and the stroma or thecal cells (discussed in Van Wezel et al., 1998). In pre-ovulatory follicles of women it has been shown that collagen IV may be detected patchily, but clearly among the granulosa cells (Yamada et al., 1999). Perhaps this is part of some mechanism whereby collagen IV is synthesised throughout the granulosa cell layer and then 'passed down' to be assembled into the basement membrane.

At ovulation, the basement membrane in the follicle breaks down, presumably under the influence of the mid-cycle peak of LH. What happens to the breakdown products at this stage is not known. The cellular

re-organisation that now occurs involves granulosa, endothelial and thecal cells.

1.7.1 Granulosa cells

These enlarge and increase their level of progesterone production. It has been shown in rats that this process of luteinisation may be dependent on cell-matrix interactions through integrins (Aten et al., 1995). Clues as to the nature of this matrix (which may be associated with the granulosa cells in the developing corpus luteum) have been provided by several studies using immunohistochemistry to examine matrix disposition in the corpus luteum. Thus, Wordinger et al. (1983) showed the presence of laminin around individual luteal cells in the mouse suggesting the presence of basement membrane elements. Also, in the rat it has been shown that after 6 hours following ovulation, dot-pattern positive reactions for collagen IV and laminin can be detected in the granulosa layer consistent with the acquisition of thin basement membrane-like structures by the individual granulosa cells (Matsushima et al., 1996). Could it be that the basement membrane producing capacity of pre-ovulatory granulosa cells is continued into the luteal phase?

1.7.2 Endothelial cells

Although not a major concern of this thesis, it is worth bearing in mind that in vivo, the breakdown of the basement membrane at mid-cycle preludes the invasion of the previously avascular granulosa cells by capillaries. This is a major example of physiological angiogenesis which is probably governed by release of VEGF by the granulosa cells (reviewed by Fraser and Lunn, 2000). As the endothelial cells form new capillaries during the invasion of the granulosa layer, some basement membrane material will be laid down in association with the endothelial cells. Examination of electron micrographs of human corpus luteum suggests that this basement membrane of the endothelial cells is quite separate from that in association with the granulosa cells (unpublished results from our laboratory).

1.7.3 Thecal cells

The thecal cells remain in largely segregated areas on the periphery of the corpus luteum in the human. Their role in matrix re-modelling in the developing corpus luteum is not known.

Extracellular matrices are not inert. They modulate cell function actively through cell surface receptors for extracellular matrix components. Integrins, a family of transmembrane heterodimeric glycoproteins, are one class of cell surface receptors. They can therefore interfere in the regulation of many fundamental cellular processes including proliferation, migration and survival as well as differentiation and tissue morphogenesis. Their presence has been identified in both animal and human ovaries playing a regulatory role in granulosa cells function. It has been shown that antiserum against integrin β1-subunit prevents the progestin secretory activity of cultured granulosa cells from preovulatory follicles in rats (Aten et al., 1995). In humans integrin α6β1 is expressed on the cell surface of luteinizing granulosa cells in the early luteal phase, and its ligand, laminin, is also detected around the granulosa cells (Fujiwara et al., 1997). Integrin α2 is a differentiation-related molecule of human granulose and theca interna cells and its ligand collagen type IV, is rapidly produced around luteinizing granulosa cells during ovulation and interacts with the cell surface of granulosa cells (Yamada et al., 1999).

Integrins are composed of α and β subunits, the pairing of which confers on the integrins their ligant specificity for extracellular matrix factors. There are 14 α subunits and 8 β subunits that associate in different combinations to form at least 20 distinct integrins which differ in ligant binding.

Interactions between integrins and extracellular matrix ligands activate a variety of intracellular signaling molecules including tyrosine, serine-threonine and lipid kinases and phospholipases. In addition, binding of integrins to their respective ligands modulates the generation of second messengers by growth factors, cytokines and hormones.

1.8 Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) in the corpus luteum.

The degradation of extracellular matrix is largely under the control of MMPs and their corresponding inhibitors – TIMPs. It is likely that the balance between MMP and TIMP activity in a tissue is important in determining the extent of matrix breakdown. Changes in this balance between degradation and inhibition of degradation may govern how the corpus luteum either survives in early pregnancy (under the influence of hCG), or undergoes luteolysis in a non-conception cycle (O'Sullivan et al., 1997). Evidence for the involvement of MMPs and TIMPs in luteal function will now be examined separately.

1.8.1 MMPs in corpus luteum.

The role of MMPs in reproductive function and their major role in the manipulation of matrix in tissue remodelling has been reviewed by Hulboy et al. (1997). The MMP family is a group of structurally related proteins that degrade extracellular matrix in a zinc-dependent manner. They are produced as proenzymes and secreted into the extracellular matrix where they are activated by proteolytic cleavage in areas of active matrix remodelling. In addition to the 'pro' domain (removed at activation), most MMPs have a 'catalytic' domain involved in holding the zinc ion, and a haemopexin-like domain which has been shown to be involved in their association with various extracellular components. Hulboy et al. (1997) describe 17 different MMPs, some of which (the membrane-type (MT)-

MMPs) are membrane associated. The various MMPs have a range of substrate specificities.

The role of MMPs in ovarian function has been reviewed by McIntush and Smith (1998). Very little is known about the function of MMPs in the corpus luteum. Most studies have emphasised the importance of MMP-2 and MMP-9 in luteal function. Thus these are present in rat corpora and notably the increased production and activation of MMP-2 is associated with structural luteolysis (Endo et al., 1993). Work on cultured, luteinised human granulosa cells (Stamouli et al., 1996) and extracts of human corpora lutea (Duncan et al., 1998) also focused on the two gelatinases, MMP-2 and MMP-9. It is likely that MMP-2 is made mainly by the theca-derived cells in the corpus luteum (Duncan et al., 1998), trace amounts in human granulosa cell preparations (Stamouli et al., 1996) being due to contamination with small numbers of theca cells. MMP-9 was shown to be made by scattered cells within the granulosa layer of the corpus luteum. It is possible that these are white cells and that these are present in granulosa cell cultures as used by Stamouli et al., 1996. Luteal rescue may be associated with a reduction in MMP activity (compared with luteolytic tissue) brought about by the action of hCG. This has been shown both in the culture studies (Stamouli et al., 1996) and the work on extracts of corpora lutea (Duncan et al., 1998). In particular, this latter study noted a reduction in MMP-2 activity in the 'rescued' corpora lutea indicating an important role for this MMP in the rescue process.

1.8.2 TIMPs in corpus luteum.

A survey of the expression of TIMP-1 mRNA in various murine tissues (Nomura et al., 1989) showed that this mRNA was very highly expressed in the corpus luteum. This has now been confirmed in a range of other species (discussed in O'Sullivan et al., 1997) including the human (Duncan et al., 1996). It would appear that the production of TIMP-1 is mainly confined to the granulosa-derived cells in the corpus luteum (Duncan et al.,

1996) so that when these are cultured, TIMP-1 protein is secreted into the medium (Morgan et al., 1994; O'Sullivan et al., 1997).

How does TIMP-1 production change during the life of the corpus luteum? Post-ovulation, the development of the corpus luteum (under the control of gonadotrophin in many species) involves the up-regulation of TIMP-1 production. This is implied by the high level of TIMP-1 expression in corpus luteum compared with pre-ovulatory tissues (Nomura et al., 1989), and confirmed by in vitro studies showing that TIMP production by rat granulosa cells is responsive to LH (Mann et al., 1991). Whether luteal rescue with hCG in women involves an upregulation or maintenance of TIMP-1 production compared with tissue undergoing luteolysis, remains controversial. In vivo studies of Duncan et al. (1996) in women suggested that this was not the case, the amounts of TIMP-1 in corpora lutea appearing to be unresponsive to administered hCG. However, work on luteinized , cultured human granulosa cells showed a clear stimulation of TIMP-1 production by hCG implying that luteal rescue could be associated with increased TIMP-1 production (O'Sullivan et al., 1997).

1.9 Overall working hypothesis for extracellular matrix involvement in luteal function.

Bringing together the evidence relating to matrix involvement in the corpus luteum, we are able to piece together a working hypothesis which is depicted in diagram form in Figure 1.6. A number of features of this model are envisaged:

a) As part of corpus luteum development we envisage that each granulosa-derived cell will become associated (and possibly synthesize) a pericellular extracellular matrix.
b) Luteal rescue with hCG in women will involve maintenance or up-regulation of TIMP-1 production and suppression of MMP action. The resulting shift in balance towards inhibition of MMP action will lead to a stabilisation of extracellular matrix and continued progesterone production.

c) Luteal regression will involve the opposite scenario whereby lack of LH action within the granulosa cells (not necessarily associated with falling circulating LH) leads to less TIMP-1 production and more MMP production. This leads to a switch towards greater MMP action, degraded extracellular matrix and decreasing progesterone production and luteal regression.

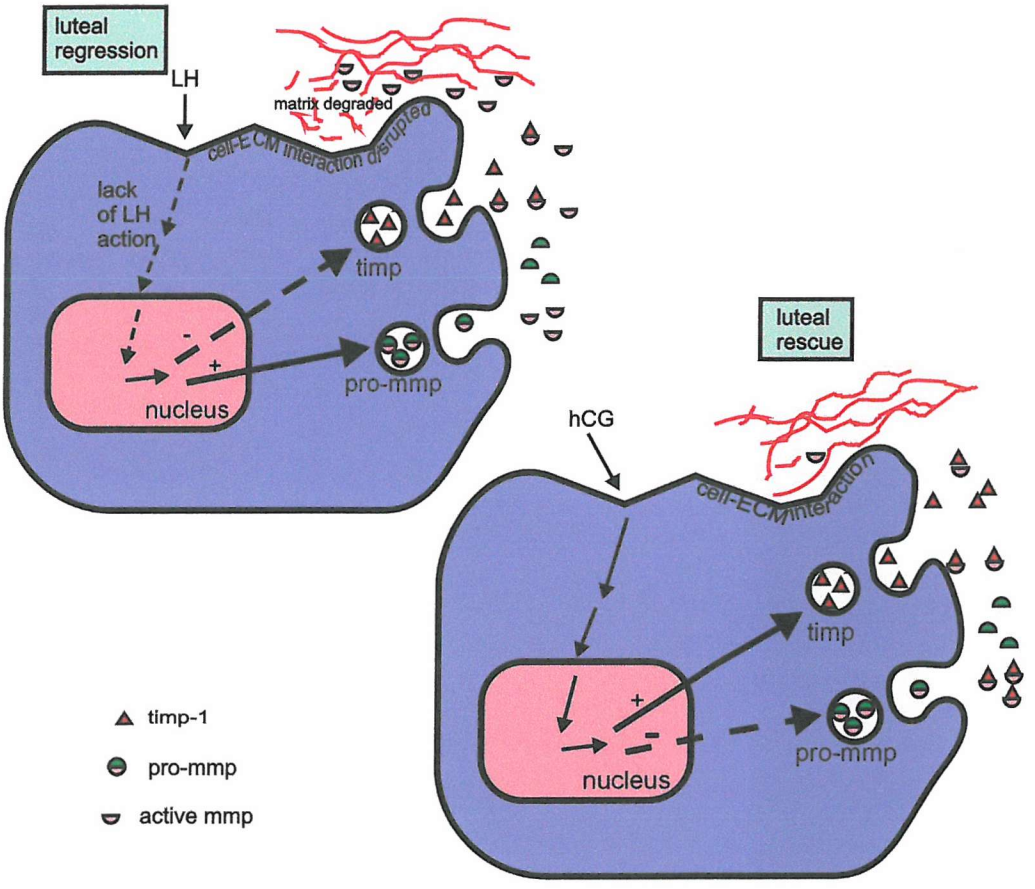


Figure 1.6 Luteal regression and luteal rescue. Diagrammatic representation occurring at the cellular level in the corpus luteum during luteal regression and rescue.

To tackle all the issues raised by this overall hypothesis would be very ambitious and impractical considering the time available. The thesis will therefore mainly concern itself with examining the first of

these proposals. Thus the more restricted hypothesis examined by this thesis is as follows:

We hypothesize that pre-ovulatory granulosa cells acquire a peri-cellular extracellular matrix as they luteinize. They may actively synthesize this matrix which may contain elements of basement membrane. We further hypothesize that matrix production may be controlled by gonadotrophin or growth factors.

The following chapters will describe how this hypothesis was examined using cultures of luteinizing granulosa cells obtained from IVF aspirates.

Chapter 2

MATERIALS AND METHODS

Contents:

2.1 Patients

Follicular aspirates and washes were obtained at ovum collection for in vitro fertilization (IVF) according to a procedure approved by our local ethical committee. The treatment protocol, as previously described (Jenkins et al., 1991) involved down-regulation of pituitary function with gonadotrophin releasing hormone analogue (Nafarelin: 400μg intranasally twice daily) started in the luteal phase (21st day of cycle) of the preceding cycle. From day 4 of the IVF cycle, a dose of 150-600 IU FSH was administered daily. A constant dose of FSH was used daily, the level determined by patient age and previous response to gonadotrophins. Also pretreatment levels of FSH were taken into account i.e. higher levels of FSH were administered to patients with high pretreatment FSH levels. In practice, it was found that the 60 patients utilised for the study could be largely

restricted to those with 'tubal' problems, those where a 'male factor' contributed to subfertility, and women acting as 'egg donors'.

When the leading two follicles had a diameter of >18mm and serum oestradiol concentration was >300pmol/L for each follicle which was over 14 mm in diameter, 10,000 IU hCG was administered. Oocytes were collected 34 hours later under transvaginal ultrasound guidance. All follicles with a diameter >15mm were aspirated. Each follicle was aspirated in turn including two further washes with flushing medium (phosphate buffered saline (PBS) containing 60mg/l Penicillin, 50mg/l Streptomycin and 10,000i.u. /l heparin).

2.2. Preparation and culture of granulosa cells

Initial follicular aspirates and washes were combined for each patient and brought to the laboratory. The method used for preparation was adapted from a method previously described (Richardson et al., 1992). The details are as follows:

The medium used throughout the preparation and culture was a mixture (50:50) of Ham's F12 and Dulbecco's modified Eagle's medium supplemented with glutamine (2 mmol/l), penicillin (100,000 IU/l), streptomycin (100mg/l), amphotericin (0.25mg/l), insulin (6.25mg/l), transferrin (6.25mg/l), selenious acid (6.25µg/l), bovine serum albumin (BSA; 1.25g/l) and linoleic acid (5.35mg/l).

The granulosa cells were separated from the follicular fluid and wash medium by centrifugation at 1200rpm (200g) for 10 minutes. The supernatant was discarded and the pellets of cells resuspended in culture medium. A mixture of 9ml of Percoll (Pharmacia, St. Albans, UK), 1ml of 10 x Ham's F12 and 10 ml of culture medium was prepared giving a solution of 45% Percoll. The cell mixture was then carefully layered on the surface of

the Percoll solution prior to centrifugation for 30 min at 1200 rpm. The red blood cells derived from contaminating blood in the aspirates were pelleted at the bottom of the tube while the granulosa cells stayed at the interface between the two layers. The granulosa cells were aspirated using a pastette and transferred to a separate tube where they were diluted with culture medium. The granulosa cells were washed twice by centrifugation (1200rpm for 10 min) and resuspension to give a final cell suspension. This was strained through a 70μm strainer to remove clusters or tissue pieces. The number of cells was then estimated using a haemocytometer and diluted to 5×10^5 cells/ml using culture medium. Viability was estimated to be >90% using a trypan blue exclusion test (see O'Sullivan et al., 1997).

Aliquots of cell suspension (0.45 ml/well) were added to 8-well Labtek-II chamber slides (Nunc brand, obtained through Merck, Poole, UK), which provided a pre-washed glass surface for cell attachment. Additions of medium, hCG (to give 100ng/ml) or growth factors were included to give a total incubate volume of 0.5ml. The concentration of hCG used was chosen to give a maximal progesterone response (Richardson et al., 1992).

Cells were cultured in a humidified incubator in the presence of 5% CO_2 in air and medium changed regularly using a method designed to avoid cell loss. Complete removal and replacement of medium was found to dislodge cells from the culture surface. Therefore two methods were developed for changing the media.

Firstly, for time-course experiments, medium was changed carefully every 48hours by a multi-step procedure involving four cycles of partial removal and replacement of medium. By this method we calculated that a 96% reduction of media constituents was achieved, assuming perfect mixing occurred (Table 2.1). Cells remained undisturbed by this procedure.

Secondly, for other experiments, a simpler one-cycle procedure was adopted involving removal and replacement of 50% of the medium. For

time-course experiments, different sets of cultures were established which were intended for termination at the different time-points. At the allotted time, all the medium was removed for storage at -20^0 C, and the cells remaining taken for DNA assay or extracted for Western analysis.

Table 2.1 Multi-step medium changes during time-course experiments

Step	Control wells	hCG wells
1	+0.3 ml medium	+0.3 ml medium
2	- 0.4 ml medium	-0.4 ml medium
3	+0.4 ml medium	+0.4 ml medium
4	- 0.4 ml medium	-0.4 ml medium
5	+0.4 ml medium	+0.4 ml medium
6	- 0.4 ml medium	-0.4 ml medium
7	+0.4 ml medium	+0.4 ml medium
8	- 0.3 ml medium	-0.35 ml medium
9		+0.05 ml hCG

2.3 DNA assay

This method is based on the observation that the amount of DNA per cell is constant. Therefore a DNA assay provides a relatively simple method to obtain a measurement, which accurately reflects the number of cells in each well.

A DNA assay buffer was made up by mixing two stock solutions: "A" and "B".

"A": 0.05M Na_2HPO_4 and 2M NaCl

"B": 0.05M NaH_2PO_4 and 2M NaCl

Solution "B" was gradually added into solution "A" and pH monitored to achieve a pH of 7.4.

After aspiration of the culture medium, the adherent cells in each well were collected by adding 300µl assay buffer to the well and scraping thoroughly the cell layer from the base of the well with the use of a 1ml (blue) pipette tip. To ensure good recovery the procedure was repeated once more to give a total of 600µl. Tubes containing these "scrapings" in DNA buffer were stored frozen at -20^0 C until assay.

When the DNA assay was carried out, a standard curve was constructed using a stock DNA standard (calf thymus DNA: Sigma, Poole, Dorset), which contained 1mg of DNA in 25 ml of DNA assay buffer. Sequential dilutions of the above standard were prepared in 3ml UV cuvettes using assay buffer to give 4, 2, 1, 0.5 and 0.25µg DNA per cuvette. Using the method of Labarca and Paigen (1980) 1ml of bis-benzamide solution (2µg/ml; H33258; Sigma, Poole) was added to each cuvette prior to measurement of fluorescence using a fluorimeter (model TKO 100: Hoefer Scientific Instruments, San Francisco, USA).

The unknown sample solutions were briefly homogenized. They were then prepared by mixing 0.3ml of the cell sample with 0.7 ml of assay buffer and 1 ml of the bis-benzimide solution in a 3ml UV cuvette. Following fluorimetry the amount of DNA in each well could be estimated precisely by using the standard curve. An equation for each standard curve was calculated assuming linearity and values for the unknowns calculated.

2.4 Progesterone assay

Progesterone was assayed in culture medium by our Chemical Pathology Department using an automated, solid-phase, chemiluminescent enzyme immunoassay (Immunolite Analyser System; Diagnostic Products Corporation, Llanberis, UK). Over a working range of 20-120 nmol/l; the intra- and inter-assay variations were <7% and 12% respectively. Samples

from one experiment were always run as one batch. Progesterone production by cells was corrected for DNA measured for the time and culture condition shown.

2.5 Immunocytochemistry

This method of qualitative analysis of the cultured cells was adopted in order to identify the potential extracellular matrix components around the granulosa cells. It was hoped that this information would complement the findings obtained through Western analysis of extracts. The immunofluorescence technique relied upon the use of primary antibodies to extracellular matrix components followed by a second antibody, which is attached to a fluorescent molecule. This was then visualized using laser scanning confocal microscopy.

Cultured or freshly prepared cells (allowed to settle) were fixed with 4% (w/v) paraformaldehyde for 15 minutes. The supernatant was removed with a pastette and PBS added. This was then replaced with 10% (v/v) normal goat serum in PBS and the chambers left for 30 minutes. Cells were then exposed to primary antibodies diluted 1:40 in the 10% goat serum and left overnight at 4° C. The chamber slides were covered with "clingfilm" to avoid evaporation. Control chambers without primary antibody were run in parallel.

Primary antibodies used were: monoclonal antibodies against human collagen IV (Novocastra, Newcastle-upon-Tyne, UK), heparan sulphate proteoglycan (HSP; 10E4 epitope; AMS Biotechnology, Witney, UK), and a polyclonal antibody raised in rabbits against human placental laminin (Autogen Bioclear, Calne, UK).

After incubation with primary antibody, the chambers were drained and rinsed in PBS-A (PBS containing 0.2% w/v bovine serum albumin) for 4 x 5

minutes. An appropriate rhodamine-labelled F(ab')2 fragment secondary antibody (Stratech) at a 1:100 dilution was then added and left for 1 hour at room temperature. Following two final washes with PBS-A for 10 minutes, the upper sections of the chamber slides were removed and the cultures mounted in Citifluor mountant (Citifluor Products, Canterbury, UK).

Preparations were examined using a Leica TCS 4D confocal microscope, with a crypton/argon laser with excitation light source arranged for epifluorescence. In this way, 3-dimensional images were generated which allowed positive identification of the antigen concerned. Images were composed of a selection of these 'confocal slices' which often enabled pericellular location of extracellular matrix to be clearly visualized. Where comparisons were made between images, scanning parameters were standardised.

2.6 Western analysis

This method, based on sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, allows a qualitative analysis of the potential extracellular matrix components around the granulosa cells. Electrophoresis allows estimation of protein size by differential migration through the small pores of gel matrix. SDS binds to the proteins, disrupts their shape and imposes net charge densities on the proteins. Therefore, the main variable determining migration is size. Determining the molecular weight of the unknown is achieved by comparison with a coloured, standard molecular weight protein marker (Kaleidoscope marker from Bio-Rad, Hemel Hempstead, UK). Specific proteins can then be identified immunochemically by Western blotting. Proteins in samples are transferred to a membrane to which the proteins bind tightly, retaining their electrophoretic pattern. The use of specific antibodies for the protein of interest allows the identification of a single protein among a mass of other proteins. The details of the method used are as follows (Table 2.2):

Table 2.2 Antibodies used in immunocytochemistry

Extracellular matrix component	Primary antibody	Secondary antibodies – all from Stratech, Luton, UK.
Collagen IV	Anti-collagen IV. Mouse monoclonal raised against human glomerular collagen IV, specificity detailed by Gusterson et al., 1984. Obtained through Novocastra Labs. Newcastle, UK	Goat anti-mouse Rhodamine-labelled $F(ab')_2$ fragment
Heparan sulphate proteoglycan	Anti-heparan sulphate proteoglycan (HSPG)-10E4 epitope – mouse monoclonal. Raised against HSPG from human fetal lung fibroblasts. Prepared by Seikagaku Corp. and supplied by AMS, Witney, UK	Goat anti-mouse Rhodamine-labelled $F(ab')_2$ fragment
Laminin	Anti-human laminin. Polyclonal raised in rabbits against purified human laminin. Supplied by Autogen Bioclear, Calne, UK	Goat anti-rabbit Rhodamine-labelled $F(ab')_2$ fragment

50 µl of sample buffer [3% (w/v) SDS, 10%(v/v) glycerol, 1mg/ml bromophenol blue, 0.0625 mol/l TRIS-HCl (adjusted to pH 6.8)] was added to each well after being pre-heated at ~ 60^0 C. Cells were then scraped off the culture surface with a pipette tip and extracts were combined and stored at -80^0 C. Equivalent wells were scraped and the cells stored for DNA assay. Freshly prepared cells were centrifuged, medium removed and hot sample buffer (50µl for each 0.45ml of cell suspension) was added, prior to vigorous mixing and storage as above.

Before electrophoresis, samples were taken out of the freezer to allow thawing. Aliquots of extracts (~30 µl) were then reduced with 100mmol/l dithiothreitol during heating at 95^0 C for 10 minutes. After vigorous mixing, a further 10-minute heating period at 95^0 followed. Volumes were then standardized for the cell content on the basis of DNA assay so that the same "cell-equivalent" was run in each lane. The samples were subjected to SDS-polyacrylamide gel electrophoresis using 6% resolving gels. Also run was the coloured kaleidoscope molecular weight marker, a reduced extract of Matrigel (Stratech, Luton, UK) providing subunits of mouse laminin and a reduced extract of human placental laminin (Life Technologies, Paisley, UK). The proteins on the gel were blotted on to polyvinylidene difluoride membranes using the Mini Trans-blot Cell (Bio-Rad). The membranes were blocked for 1 hour in TRIS-buffered saline with Tween (TBST; 0.05 mol/l TRIS-HCl, 0.15 mol/l NaCl, 0.1% (v/v) Tween 20, pH 7.5) containing 10 % dried milk powder. Blots were then exposed at room temperature for 2 hours to primary antibodies (listed in Table 2.3) dissolved in TBST with 10% milk powder. After several washes with TBST, membranes were incubated for 1 hour with the peroxidase conjugate of the appropriate secondary antibody (listed in Table 2.3). Dilutions in TBST were those recommended by the supplier. Controls were carried out under identical conditions, but without primary antibody.

Finally the blots were developed using the ECL-plus system (Amersham Pharmacia Biotech, Little Chalfont, UK). This is a non-radioactive, chemiluminescent detection system for use with peroxidase-labeled antibodies. The system is based on the enzymatic generation of an acridinium ester, which produces a signal. The combined peroxidase and peroxide-catalyzed oxidation of Lumigen PS-3 acridan substrate generates thousands of acridinium ester intermediates per minute. These intermediates react with peroxide under slight alkaline conditions to produce a sustained, high intensity chemiluminescence with maximum emission at a wavelength of 430nm.

The chemiluminescence emitted on the blot was recorded using overlaid photographic film, positive bands showing as dark bands on the photographic film. Control blots where primary antibody had been omitted proved largely negative.

2.7 Zymography

Medium removed from the wells where granulosa cells were cultured were assessed with zymography for gelatinase activity. The technique used was originally described by Herron et al. (1986). It involves separation of the proteins present in the sample according to their molecular weight. The gel contains a gelatin substrate which is degraded by the two gelatinases, matrix metalloproteinase 2 and 9 respectively, at the position where they come to rest following electrophoresis. SDS-PAGE was used with the inclusion of 0.8 mg gelatin/ml in the 7.5% resolving gel.

The samples were electrophoresed at 40V for the first 10 min and then at 80V for a further 90 min in buffer (25mmol/l TRIS, 0.4 mol/l glycine and 1% (w/v) SDS). Subsequently, the gels were washed in 2.5% (v/v) Triton-X100 for 30min and incubated overnight in buffer (50mmol/l TRIS-HCl, 5 mmol/l $CaCl_2$, pH 8.0).

Table 2.3 Antibodies used in Western analysis

Laminin sub-unit	Primary antibody	Secondary antibody
Alpha-1	Mouse monoclonal MAB 1924 Chemicon	Anti-mouse IgG (whole molecule) Peroxidase conjugate
Alpha-2	Mouse monoclonal anti-human merosin Reacts with 80 kDa fragment of α2 chain. Life Technologies	Anti-mouse IgG (whole molecule) Peroxidase conjugate
Beta-1	Anti-β1 monoclonal antibody (MAB 1928) Obtained from Chemicon, Harrow, UK	Anti-rat IgG (whole molecule) Peroxidase conjugate
Beta-2	Mouse monoclonal Anti-β2 laminin antibody Donated by Dr J. Aplin Dept Obst&Gyn St Mary's Hospital Manchester	Anti-mouse IgG (whole molecule) Peroxidase conjugate
Gamma-1	Anti-γ1 monoclonal antibody (MAB 1914) Obtained from Chemicon	Anti-rat IgG (whole molecule) Peroxidase conjugate
Complete laminin	Anti-human laminin. Polyclonal raised in rabbits against purified human laminin. Supplied by Autogen Bioclear, Calne, UK	Anti-rabbit IgG (whole molecule) Peroxidase conjugate

The gels were stained the following day with Coomassie blue for 1 hour and destained in 30% methanol: 10% acetic acid in water. Gelatinase activity was visualized as clear bands against a blue background.

2.8 Buffers & Reagents

2.8.1 Western blotting

Electrophoresis buffer:

6g TRIS	(to give 25mM)	diluted in 2l distilled H_2O
28.8g glycine	(to give 192mM)	
2g SDS	(to give 0.1%)	

Transfer buffer:

6.06g TRIS	(to give 25mM)	made up to 2l with distilled H_2O
28.8g glycine	(to give192mM)	
400ml methanol	(to give 20%)	

Blotting Buffer (TBST):

6.05g TRIS	(to give 50mM)	made up to 1l with distilled H_2O, adjusted pH to 7.5
8.76g NaCl	(to give 150mM)	
800ml	distilled H2O	
1ml	Tween 20	

Sample buffer for SDS-PAGE (Western blotting):

6.25ml 0.5M TRIS	(to give 0.0625M)	made up to 50 ml in distilled H_2O, adjusted pH to 6.8
5ml glycerol	(to give 10%)	
15ml 10%SDS	(to give 3%)	
50mg bromophenol blue		

2.8.2 Zymography

Electrophoresis buffer (see Western blotting)

Sample buffer:

1.514g TRIS	(to give 0.25M)	diluted in 50ml distilled H_2O adjusted pH to 6.8
5g SDS	(to give 10%)	
2.5g sucrose	(to give 5%)	
50mg bromophenol blue		

Incubation buffer:

12g TRIS	(to give 0.05M)	diluted in 2l distilled H_2O adjusted pH to 8
1.11g $CaCl_2$	(to give 5mM)	
0.4g sodium azide	(to give 0.02%)	

Coomasie Blue:

250ml methanol	(to give 50%)	made up to 500 ml in distilled H_2O
35ml acetic acid	(to give 17%)	
1.25g Coomasie R280	(to give 0.25%)	

Destain:

150ml methanol	(to give~30%)
35ml acetic acid	(to give~7%)
300ml distilled H_2O	

Chapter 3

CELL CULTURE

Contents:

3.1 Introduction

Following ovulation, granulosa cells undergo a process of luteinization which involves cell enlargement and increased progesterone production. These luteinized granulosa cells eventually form the dominant progesterone-producing layer of the corpus luteum (see review by Behrman et al., 1993). In order to study the changes that occur in the granulosa cells during the process of luteinization different culture models have been suggested. Thus, culture systems which required exogenous matrix (e.g. Richardson et al., 1992) or the provision of attachment factors (usually in the form of serum in the medium or as a pre-coating) have been used in the past. The added attachment factors in those culture systems (e.g. fibronectin present in serum, see Tapanainen et al., 1987) allow cells to spread out on the culture surface giving them a flattened morphology with radiating processes.

Because the aim of this thesis was to investigate the potential synthesis of new extracellular matrix by human cultured granulosa cells derived from

follicular aspirates, we could not adopt the above described culture systems which use the addition of exogenous matrix or other attachment factors. Therefore, we developed a novel method for culturing human granulosa cells on specially washed glass surfaces of Lab-Tek II chamber slides (Nunc brand, obtained through Merck, Poole, U.K.), avoiding the use of exogenous additions for cell attachment. On this system the granulosa cells utilized cell-matrix interactions endogenously generated within the culture, settled on to the glass surface and retained a rounded morphology. The details of this method, the morphology of the cells and characteristics of progesterone production are now described.

3.2 Methods

In outline, granulosa cells were prepared from follicular aspirates from IVF patients using a Percoll step to remove red blood cells. The details of the cell preparation are described in Chapter 2.

For the particular experiments shown in Figures 3.4 & 3.5, aliquots of cell suspension (5 x 10^5 cells/ml) were added to the 8-well chamber slides at 0.45 ml per well. Additions of medium or hCG (to give 100ng/ml; activity approximately 14,000 IU/mg; Sigma, Poole, UK) for the 'hCG treated' wells, were included to give a total incubate volume of 0.5 ml. The cell preparations were then cultured at 37° C in 5% (v/v) CO_2 in air using a humidified incubator. Separate wells were set up for cultures intended for different culture times. In other words, 'Day 2' wells, 'Day 4' wells, 'Day 6' wells etc, were set up for each experiment.

Because it was found that complete removal and replacement of medium led to loss of loosely attached cells from the culture surface, a method of repeated partial changes was evolved and this is detailed in Chapter 2. Using this method, cell loss was kept to a minimum even for loosely attached cells. In fact, using our method, cells seemed to need several days to become more firmly attached, possibly because attachment involved cell-generated extracellular matrix that was slow to form.

When particular cells reached the end of their incubation period (e.g. 'Day 2' wells or 'Day 4', etc) all the medium was removed gently (and this did not disturb the attachment of the cells) and then stored at –20° C for later steroid assay. DNA assay buffer was then added to the wells, the attached cells scraped free from the culture surface, and the resulting mixture stored at –20° C for later assay of DNA to give an assessment of cell number (see Chapter 2).

3.2.1 Calculation of progesterone production per µg DNA.

Following assay of progesterone in the medium and DNA in the 'scraped' cells, progesterone production was calculated in terms of progesterone/µg DNA. This progesterone will have been produced over the preceding 2 days of culture in each case. The changing pattern of progesterone production/µg DNA is illustrated in Figures 3.1 & 3.2.

3.2.2 Cell morphology.

Cells were photographed under the inverted microscope on the particular days shown in Figures 3.4 & 3.5.

3.3 Results

Figure 3.3 shows how the amount of DNA per well, which reflected the number of attached cells, changed during the culture period. There was some loss of cellular material during the culture both in the presence and absence of hCG. However, the amount of cell loss without hCG was greater.

3.3.1 Progesterone production

The changes in progesterone production/µg DNA over the culture period are shown in Fig. 3.1 for a typical experiment. A combined graph for three

experiments is shown in Figure 3.2. The cells cultured without hCG showed an initial increase in progesterone production followed by a later reduction. This is particularly clear in Figure 3.1 where progesterone production increases to a peak on 'Day 4' and then decreases progressively towards the end of the culture. Granulosa cells cultured in the presence of hCG (100ng/ml) again showed an increase in progesterone production over the initial period of culture similar to that seen in the absence of hCG. However, in the presence of hCG, progesterone production was then further increased above that seen in the control cultures and was maintained until the end of the culture period. Thus, by the end of the culture, the rates of progesterone production per cell were substantially elevated in the presence of hCG compared with the equivalent control culture.

3.3.2 Cell morphology

On day 2 of culture, both control and hCG-treated cells appeared rounded and the outline of each cell was clearly defined. From the scale bar we can estimate roughly that the size of each cell was of the order of 20μm. The sizes of the control and hCG treated cells were similar. Even by Day 2, the cells appeared as clusters with an appearance similar to a 'bunch of grapes'.

As the culture progressed, the outlines of the individual 'control' cells became less well defined. This occurred progressively over the period of the experiment (compare morphology of 'Day 8' cells with that shown for 'Day 2' cells). In the presence of hCG, the loss of cell definition that was apparent under control conditions did not occur so that the cell clusters retained their 'bunch of grapes' morphology. Clusters of 'control' cells had lost this morphology and appeared rounded.

3.4 Discussion

Our culture system clearly showed that there was an increased level of progesterone production per cell as the culture progressed and that this

occurred initially, whether or not hCG was present. We speculate that this may be due to the continued effectiveness of hCG stimulation which was initiated in vivo during final maturation of the follicles with hCG (i.e. during the 34-36 hours following injection of 10,000IU of hCG). This initial increase in progesterone production occurred at a time when the cell diameters were increasing. Thus the original, freshly prepared cells are approximately 12-13μm in diameter (see Figure 4.1, Chapter 4 and previously published work of Webley et al, 1991). These cell diameters seem to increase during the first few days of culture so that even by Day 2 diameters of approximately 20μm are apparent (see Figure 3.4). We speculate that both the increase in progesterone production and cell size indicate that the process of luteinization, initiated in vivo by hCG, is continuing in culture.

In the absence of hCG it appears that the process of luteinization is not maintained so that progesterone production falls. This was associated with the loss of the 'bunches of grapes' appearance. Thus it would appear that the effectiveness of the 'in vivo' administration of hCG is only short-lived.

In the presence of hCG in culture, the initial increase in progesterone production (along with increased cell size) is maintained as the 'in vitro' stimulus takes over from the original 'in vivo' stimulus. Consequently progesterone production is maintained and moreover goes on increasing to a plateau reached after about 'Day 6'. We would like to believe that these cells are now well luteinized and, in a sense, may be equivalent to granulosa cells 'rescued' by hCG in early pregnancy.

To conclude, the freshly prepared cells (equivalent to the pre-ovulatory stage) may be relatively unluteinized in that they are still small compared with mature luteal cells. They are able to greatly increase their level of progesterone production further in culture particularly in the presence of hCG. The granulosa cells are therefore undergoing a process in vitro associated with developing luteinization.

Although at this stage of the project, we had no direct evidence for or against matrix deposition in our system, several observations provided circumstantial evidence suggesting the possibility of matrix production:

i) Published evidence for rat granulosa cells has shown that cell-matrix interaction is important for luteinization (Aten et al., 1995). The observation that our cells were luteinizing in culture therefore raised the possibility that endogenous matrix may be present.

ii) The development of clusters of granulosa cells seems to be associated with the provision of exogenous matrix (Richardson et al., 2000). Thus granulosa cells on Matrigel show intense clustering. The observation that some cell clustering was occurring in our system was again not inconsistent with the presence of some endogenous matrix.

iii) The general features of steroid production of the present culture system in the presence or absence of hCG, are similar to features of progesterone production shown by granulosa cells cultured on Matrigel (Richardson et al., 1992).

The observation in Figure 3.3 that there is some gradual cell loss over the culture period and that this is partially prevented by hCG, is open to a range of possible explanations:

i) It has been shown that hCG is capable of reducing MMP expression by granulosa cells (Stamouli et al., 1996). It is possible that hCG is inhibiting breakdown of endogenous matrix and that this is encouraging the retention of cells on the culture surface.

ii) It is possible that there is some cell death in the cell population and that this is partially offset by hCG as suggested for rabbit corpus luteum (Dharmarajan et al., 1994)

Without further studies, the exact explanation for this cell loss will remain speculative.

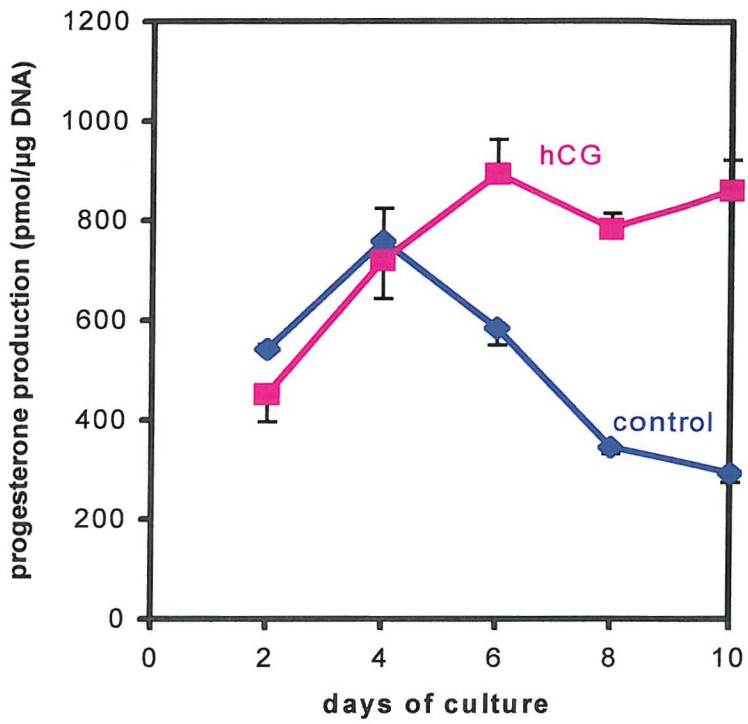


Figure 3.1 Progesterone production by granulosa cells cultured for 10 days with either no additions (control) or with hCG (100ng/ml). Error bars represent S.E.M. calculated to reflect variation between replicate cultures.

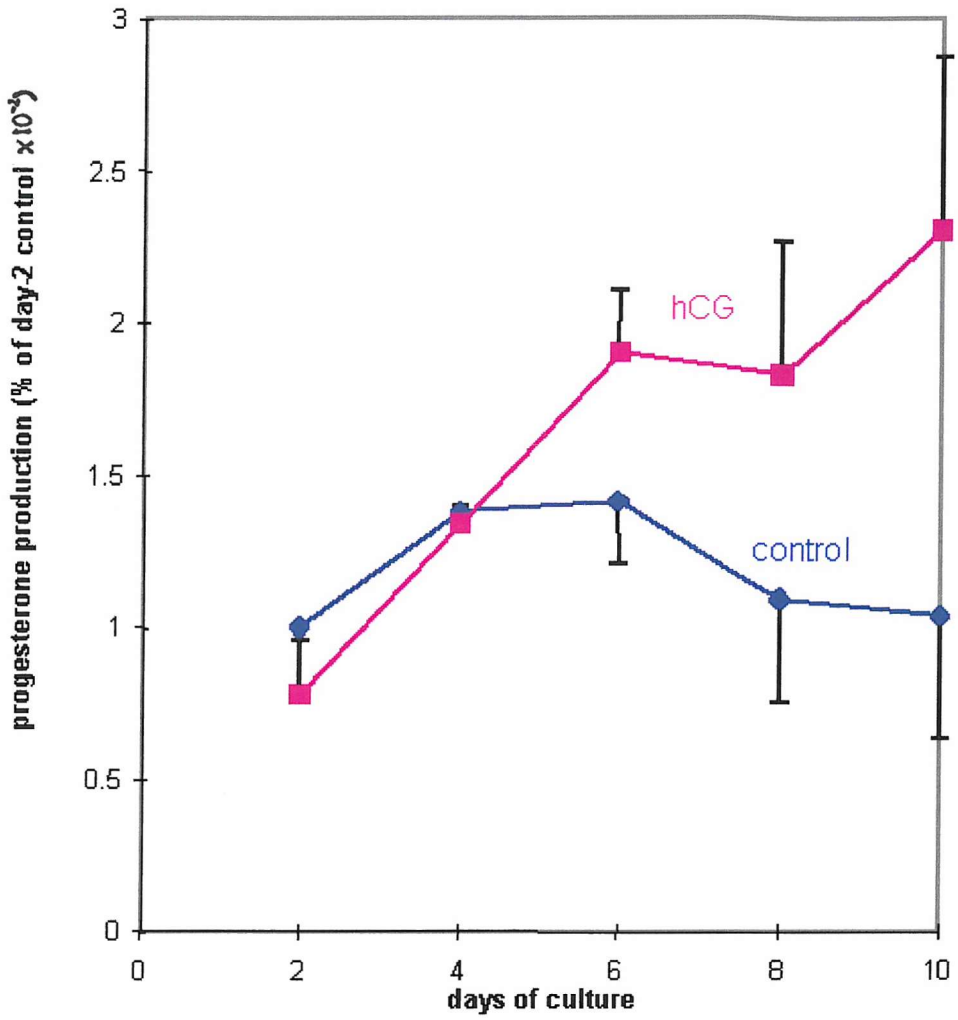


Figure 3.2 Progesterone production by granulosa cells under control conditions or in the presence of hCG. Data shown represent a combination of 3 experiments and error-bars (giving S.E.M.) represent variation between patients.

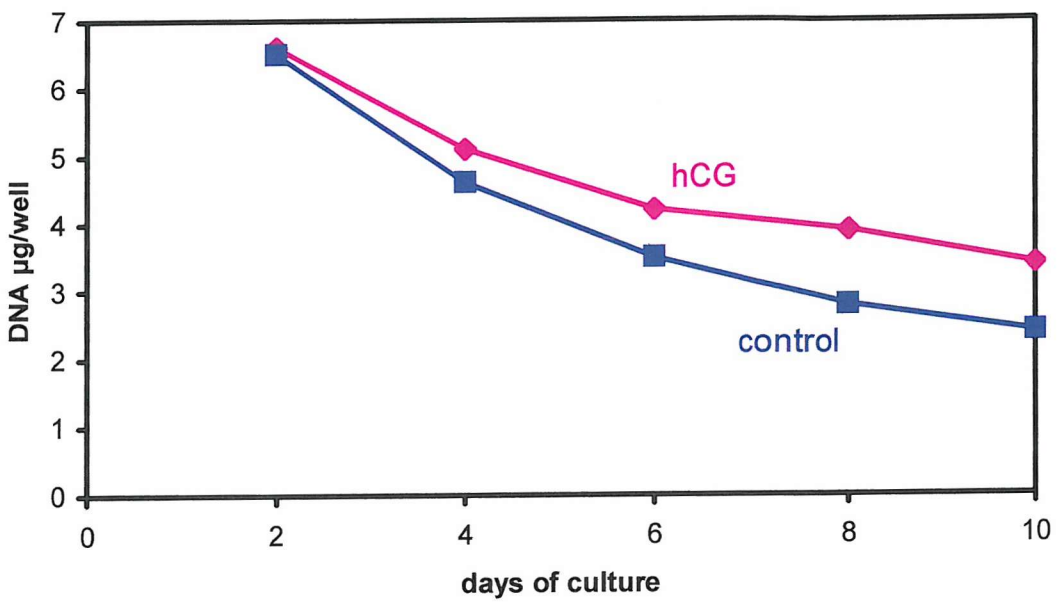
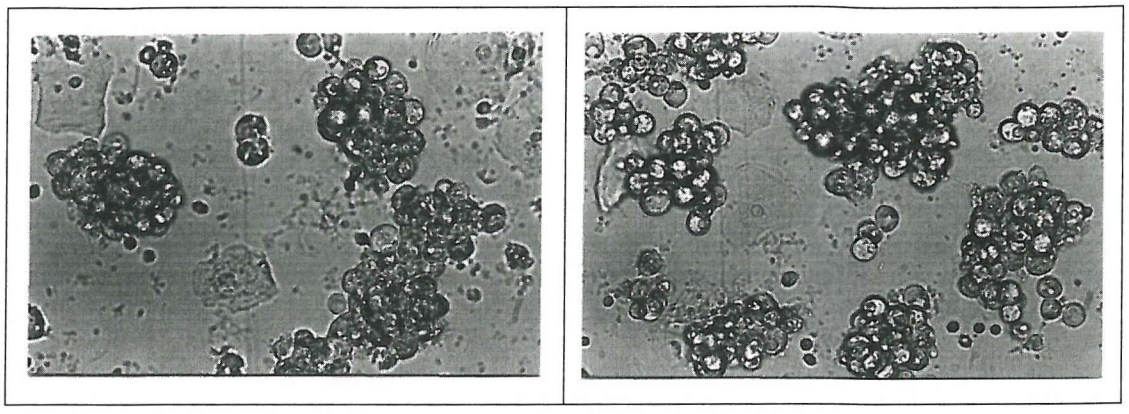
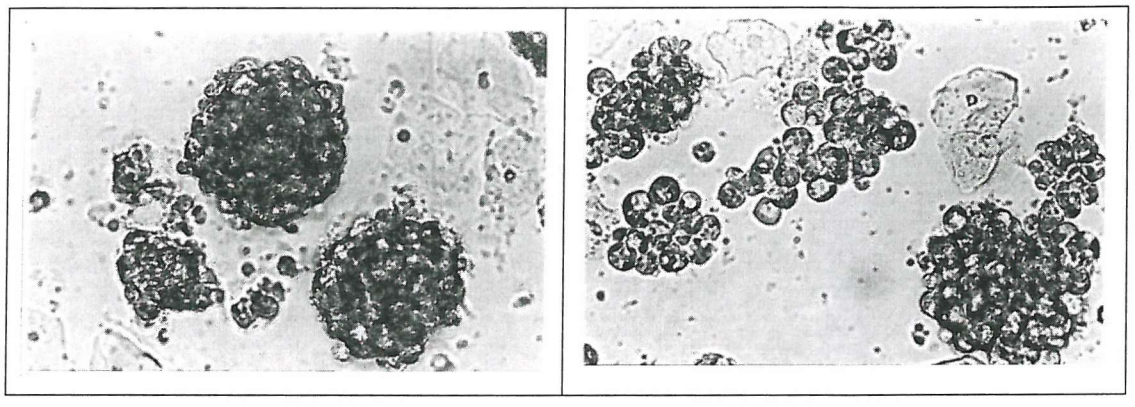


Figure 3.3: Adherent granulosa cells in culture as measured by DNA-assay, in the presence or absence of hCG (100ng/ml).



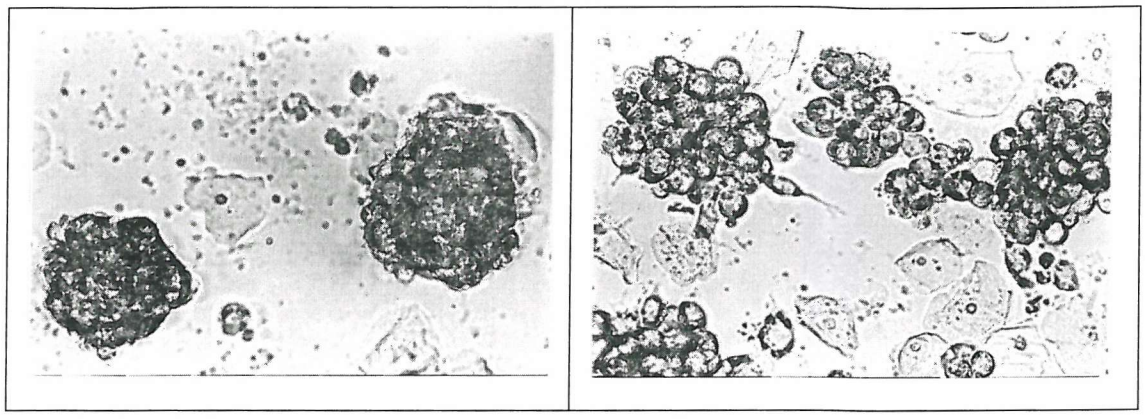
Day 2 – control Day 2 – hCG



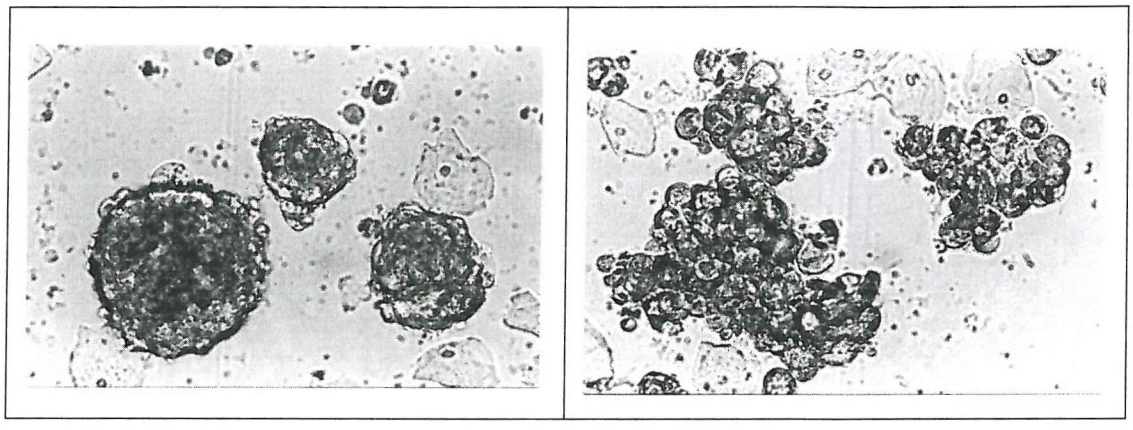
Day 4 – control Day 4 - hCG

100 microns

Figure 3.4 Light micrographs of cells cultured for different periods with and without hCG (100ng/ml).



Day 6 – control Day 6 –hCG



Day 8 – control Day 8 - hCG

100 microns

Figure 3.5 Light micrographs of cells cultured for different periods with and without hCG (100ng/ml).

Chapter 4

EXTRACELLULAR MATRIX COMPONENTS

Contents

4.1 Introduction

The development of the corpus luteum in the mammalian ovary is characterized by functional and structural changes. Functionally, the cells of the granulosa layer and theca interna differentiate into progesterone-secreting cells. Structurally, both the granulosa and theca cells differentiate into luteal cells after the degradation of the basement membrane, which originally separated the two layers in the follicle.

The significance of the extracellular matrix of the luteal cells has attracted the interest of many studies. Apart from its structural role as the supporting "glue", it also seems to have a pivotal functionality for the survival and maintenance of the epithelial derived granulosa cells, which secrete progesterone, the main pregnancy-supporting hormone. Animal tissues have been used as models in order to identify the separate extracellular matrix components. Early studies revealed immunocytochemical evidence of the presence of laminin, a noncollagenous glycoprotein, at the periphery of individual corpora luteal cells in mice (Wordinger et al., 1983). More specifically, dot-pattern positive reactions for type IV collagen and laminin were detected in the granulosa layer, and thin basement membrane structures appeared in association with the luteinizing granulosa cells in rats (Matsushima et al., 1996). There is also good evidence that bovine

pre-ovulatory granulosa cells produce basement membrane-like material in culture (Rodgers et al., 1996). There is an intriguing possibility that the pre-ovulatory potential of granulosa cells for producing basement membrane elements (Rodgers et al., 1996), is carried over into the early luteal phase in order to establish a new extracellular matrix now important for developing corpus luteum function.

The IVF programme at the Princess Anne Hospital, with the associated ethical permission for harvesting granulosa cells from follicular aspirates, enabled the establishment of a model system for looking at deposition of extracellular matrix components by granulosa cells in the corpus luteum of the primate. The new model constitutes, to the best of our knowledge, the first attempt to identify the endogenous extracellular matrix components produced by human granulosa cells in vitro in the absence of any other exogenous matrix or attach ment factors.

4.2 Methods

Detailed methods described in Chapter 2 are now summarized: Follicular aspirates were obtained at ovum collection of patients who had undergone ovulation induction with gonadotrophins. A boosting injection of hCG (10,000i.u.) was given in patients, 34 hours prior to egg recovery, mimicking the LH surge. The harvested granulosa cells were then prepared and separated from the rest of the aspirates and washes by a method adapted to avoid the use of enzymic dispersion. Granulosa cells were placed on chamber slides, which provided a pre-washed glass surface without the addition of any substrate. Granulosa cells were cultured for various times, in the presence or absence of hCG.

Freshly prepared or cultured cells were exposed, after fixation, to specific primary antibodies against potential components of the extracellular matrix. The primary antibodies used were: monoclonal antibodies against human collagen IV (Novocastra, Newcastle-upon-Tyne, UK), heparan sulphate proteoglycan (HSP; 10E4 epitope; AMS Biotechnology, Witney, UK), and a

polyclonal antibody raised in rabbits against human placental laminin (Autogen Bioclear, Calne, UK). An appropriate fluorescently labelled secondary antibody was then used in each case.

The preparations were examined by the use of confocal microscopy.

4.3 Results

Immunocytochemistry for the main constituents of the basement membrane is shown in Figures 4.1, 4.2 & 4.3 both in the freshly prepared granulosa cells (Figure 4.1A, 4.2A, 4.3A) and in the enlarged cells evident after culture (Figure 4.1B, 4.2B&C, 4.3B&C). Results for the extracellular matrix components were consistent over a total of five experiments.

Immunofluorescent staining for collagen IV revealed that it was apparently present between both small fresh cells (Figure 4.1A) and enlarged cultured cells (Figure 4.1B). Its appearance was consistent with small, irregular patches between the cells, revealing an irregular pattern. Although immunocytochemistry is a semi-quantitative technique, within its limits, there was no apparent difference in the levels of collagen IV staining between hCG-treated and control cells (only hCG-treated cells are shown in Figure 4.1B). Therefore the addition of hCG in the medium of cultured cells seemed to have had no effect on the distribution of collagen IV, which maintained the same immunocytochemical staining during culture (Table 4.1)

Table 4.1. Collagen IV in association with human granulosa cells before culture, and after culture with and without human chorionic gonadotrophin (hCG).

Matrix component	Immunocytochemical staining[a] before and after culture[b]		
	Before culture	After culture (without hCG)	After culture (with hCG)
Collagen IV	+	+	+

[a]Staining scored as +.

[b]Culture for 7 days with or without hCG (100ng/ml).

Immunostaining for heparan sulphate proteoglycan was only faintly visible in the freshly prepared granulosa cells having a pericellular distribution (Figure 4.2A). However, after culture, the amount of heparan sulphate proteoglycan increased significantly around the enlarged granulosa cells (Figure 4.2B&C). The amount seemed to be particularly elevated around the enlarged cultured cells which had been exposed to hCG (Figure 4.2C), where a continuous, very intense, pericellular coating was apparent suggesting enhanced deposition. When heparitinase was added to the treated cells, the marked pericellular staining was removed, confirming the specificity of the antibodies used (Figure 4.4, kindly donated by Miss Sian Llewellyn). A summary of the results for heparan sulphate proteoglycan in the extracellular matrix of granulosa cells is shown in table 4.2.

Table 4.2. Heparan sulphate proteoglycan (HSPG) in association with human granulosa cells before culture, and after culture with and without human chorionic gonadotropin (hCG).

Matrix component	Immunocytochemical staining[a] before and after culture[b]		
	Before culture	After culture (without hCG)	After culture (with hCG)
HSPG	+	++	+++

[a]Staining scored as +, ++, +++.

[b]Culture for 7 days with or without hCG (100ng/ml).

Immunostaining using the polyclonal antibody against laminin showed highly variable levels of laminin around the smaller, freshly prepared cells, with many showing negligible immunoreactivity (Figure 4.3A). After culture, the surviving, large granulosa cells in clusters showed a thin but more consistent and uniform pericellular layer of laminin which is shown well in Figure 4.3C. It should be emphasized that this layer was spread over a much larger surface area of the cultured cells, suggesting there had been net deposition of laminin.

Although the particular cell cluster shown in Figure 4.3B shows a less organized deposition of laminin in the control cultures, the overall level of laminin staining in the control versus hCG-treated cells in our experiments did not enable us to make a firm judgement as to whether hCG had stimulated laminin deposition. It should be noted that Western analysis of extracellular matrix reported in Chapter 5 will show a lack of effect of hCG on laminin deposition by cultured granulosa cells. Taken as a whole, our immunocytochemical results for laminin were not inconsistent with the Western data.

4.4 Discussion

It has already been mentioned in the Introduction that the extracellular matrix may play an important role in the tissue re-modeling that occurs as the corpus luteum develops. It has been suggested that its multiple role may involve such functions as holding the cellular components together, inducing cell differentiation, influencing cell survival and providing specific signals for cell migration.

Although its precise composition varies from tissue to tissue and even from region to region in the same lamina, most mature basal laminae contain type IV collagen, heparan sulphate proteoglycan, laminin, fibronectin and entactin. In this study, we used immunocytochemistry in order to identify the presence of the first three constituents in the extracellular matrix of the

granulosa cells, as these molecules represent the major components (see Introduction).

Collagen IV was immunostained in a 'patchy' pattern amongst freshly prepared human granulosa cells (Figure 4.1A) and this finding is consistent with a report by Yamada et al. (1999). In their experiments collagen IV was demonstrated using flow cytometry and immunocytochemistry among granulosa cells in the pre-ovulatory follicles but not in growing follicles. They also showed staining for the integrin α_2 subunit, part of a heterodimeric integrin molecule on the cell surface, which mediates the interaction between the granulosa cells and the extracellular matrices. Our work now shows at a higher magnification using confocal microscopy how these small patches of collagen IV are positioned around and between the granulosa cells. In both studies, it should be taken into account, that freshly prepared cells have already been exposed to hCG in vivo. This may have started the process of luteinization in the granulosa cells, although they have not expanded at this stage (i.e. at egg recovery) and progesterone production has not reached a maximum (Figure 3.1 & 3.2). Therefore, we assume that collagen IV deposition in the extracellular matrix of freshly prepared granulosa cells represents either a process which is occurring pre-luteinization or a process which is associated with the early stages of luteinization before cell expansion.

The physiological significance of collagen IV remains unclear. Its formation could be an aspect of the process whereby basement membrane components are synthesized for the formation of the basement membrane between granulosa cells and theca cells in the follicle (Rodgers et al., 1996; Zhao and Luck, 1996). Its absence in the growing follicles however, in combination with its expression intensity among luteinizing granulosa cells, could imply that it is involved in early luteinization.

Our finding that the deposits of collagen IV are, at least, maintained in culture is consistent with the results of Yamada et al. (1999). In that study, the expression of collagen IV with immunocytochemical staining around the

granulosa cells was obviously augmented during a 4-day culture. This was not substantiated in our study, as any potential differences between uncultured and cultured cells were not of sufficient magnitude to show up clearly using immunocytochemistry. Also Yamada et al. (1999) showed that the concentration of collagen IV in culture media was enhanced by hCG, implying a potential up-regulation by circulating LH in vivo. Our study did not measure output of collagen IV into the medium. Despite these differences between the present study and that of Yamada et al. (1999), the two studies agree on the conclusion that if collagen IV is part of the luteinization process, this process is well underway in women before ovulation, at least in patients undergoing IVF treatment.

Although there is no previous work published regarding the presence of heparan sulphate proteoglycan around human granulosa cells, our findings are consistent with previous studies in animal models. These studies have shown the synthesis of heparan sulphate proteoglycan by rat granulosa cells (Yanagishita and Hascall, 1983) and its presence around rat luteal cells (Asakai et al., 1993). In humans, there is evidence of heparan sulphate proteoglycan's presence in the follicular fluid (Eriksen et al., 1997). Although this may be indicative of an important role in primates, there appears to be little related published information on its cellular production and disposition. The much stronger signal for heparan sulphate proteoglycan after culture of granulosa cells (compared with the freshly prepared cells) suggests that significant extra deposition of this extracellular matrix constituent may occur as part of luteinization. The newly formed heparan sulphate proteoglycan would have the potential to bind a range of growth factors e.g. basic fibroblast growth factor. This growth factor has been implicated in the development and differentiation of the corpus luteum (Asakai et al., 1993, Aharoni et al., 1997). Clearly the role of HSPG in luteinization is an important area for further study.

The amount of laminin bound to freshly prepared granulosa cells was very variable. This finding is consistent with previously published work (Fujiwara et al., 1997) where both flow cytometry and immunohistochemistry revealed

the presence of laminin in varied amounts on the cell surface region of granulosa cells derived from patients undergoing IVF treatment. It is possible that this variable deposition of laminin reflects variability in the extent of early luteinization due to hCG exposure of cells in vivo, in both studies.

A clear, dense pericellular staining was revealed around the enlarged granulosa cells after their culture (Figure 4.3C). Taking into account the considerable cell enlargement after culture and the clearer peripheral staining around cells, we concluded that the amount of laminin had increased in cultured cells. This was confirmed by Western analyses of cell extracts, presented in the following chapter. These showed a clear and progressive accumulation in culture of laminin detected using the polyclonal antibody to laminin (Figure 5.6). Thus, our observations on cultured human cells in vitro, using this novel method for studying the endogenous production of laminin, appear to reproduce the findings of previous studies examining luteinization in vivo, in animals. Such studies showed that corpora lutea exhibited positive laminin localization around the periphery of individual corpora luteal cells in mice (Wordinger et al., 1983). As the presence of laminin was not demonstrated around follicle cells prior to ovulation, it was suggested by this study that the processes of ovulation and corpora lutea formation are associated with laminin deposition around individual luteal cells. Similarly, thin basement membrane structures appeared in association with the luteinizing granulosa cells in rats 6 hours after ovulation. These basement membrane-like structures showed positive immunostaining for laminin (Matsushima et al., 1996). It is also possible that the increased synthetic capacity for laminin noted in bovine early corpus luteum (Zhao and Luck, 1995) can now be attributed, at least in part, to the granulosa cell fraction.

As a conclusion to the above findings, the luteinization process appears to involve deposition of considerable amounts of heparan sulphate proteoglycan and laminin around the human granulosa cells. More work would be necessary to clarify if this process of ECM deposition is an

obligatory part of luteinization. From a clinical standpoint, it is interesting to speculate that defective formation of ECM may potentially contribute to sub-optimal corpus luteum function and associated luteal insufficiency.

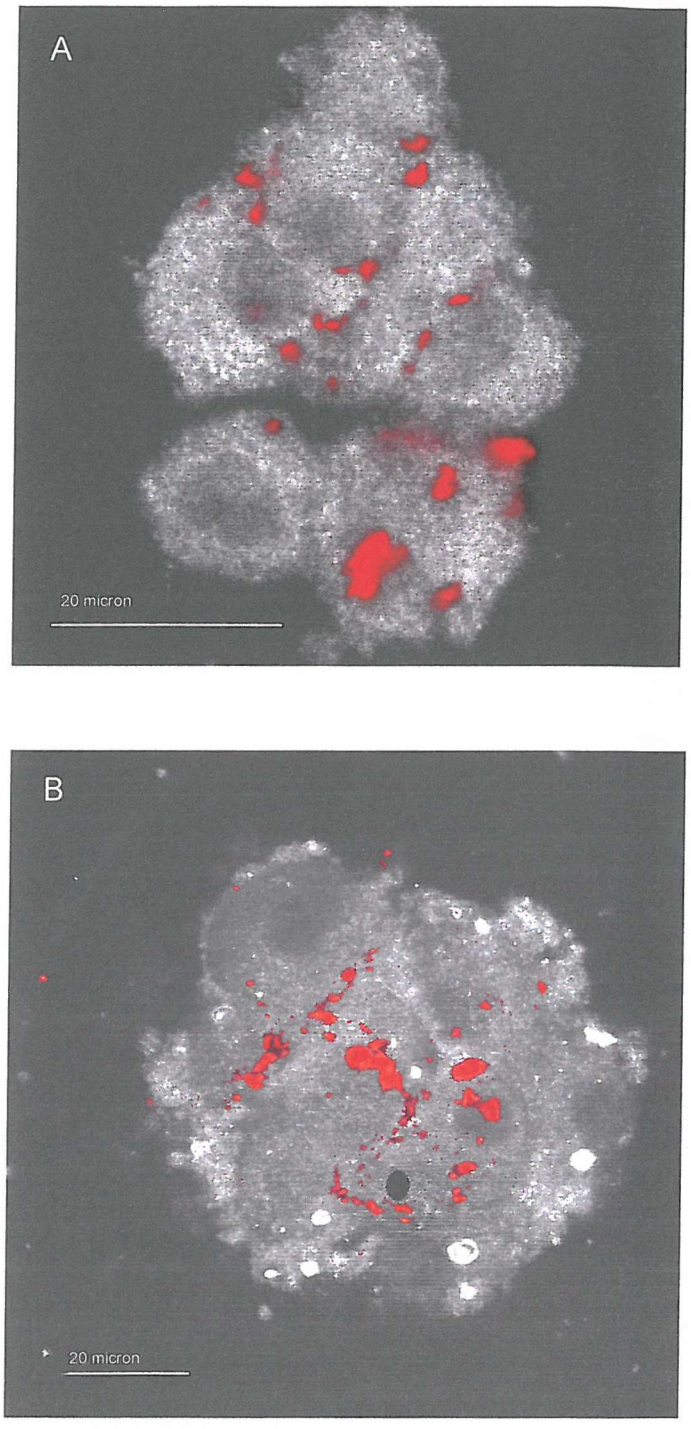


Figure 4.1 Immunocytochemistry of collagen IV

A: freshly prepared granulosa cells
B: granulosa cells cultured for 7 days with hCG (100ng/ml) for 7 days

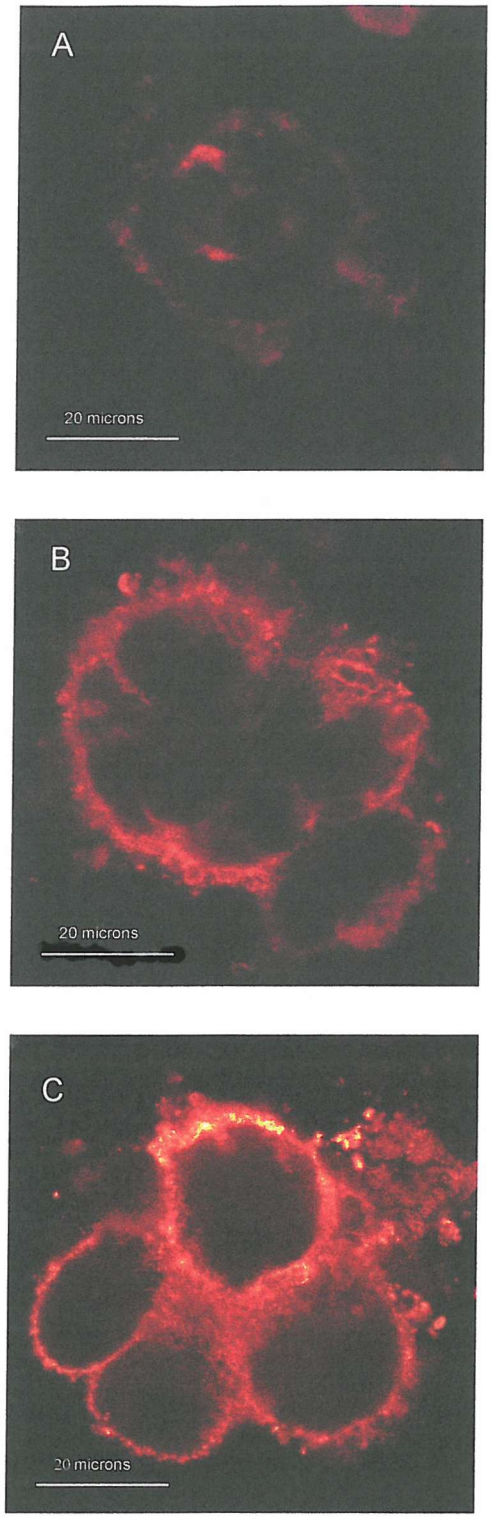


Figure 4.2 Immunocytochemistry of heparan sulphate proteoglycan

A: freshly prepared granulosa cells
B: granulosa cells cultured without gonadotrophins for 7 days
C: granulosa cells cultured with hCG (100ng/ml) for 7 days

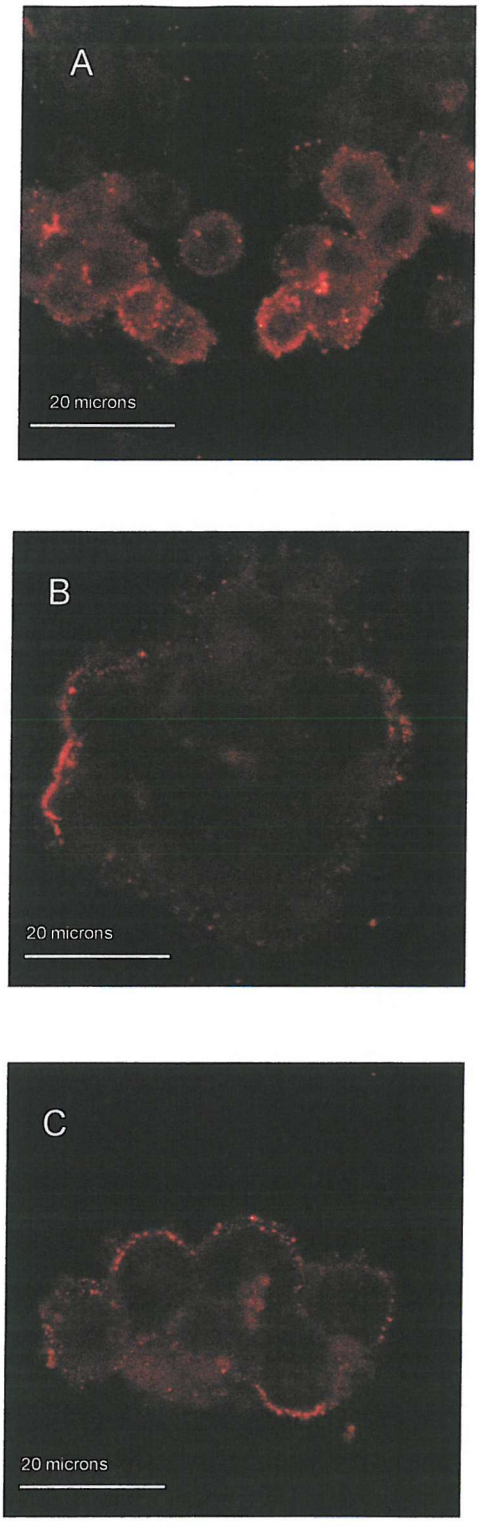


Figure 4.3 Immunocytochemistry for laminin using a polyclonal antibody raised against purified human laminin.

A. freshly prepared granulosa cells
B. granulosa cells cultured without gonadotrophins for 7 days
C. granulosa cells cultured with hCG (100ng/ml) for 7 days

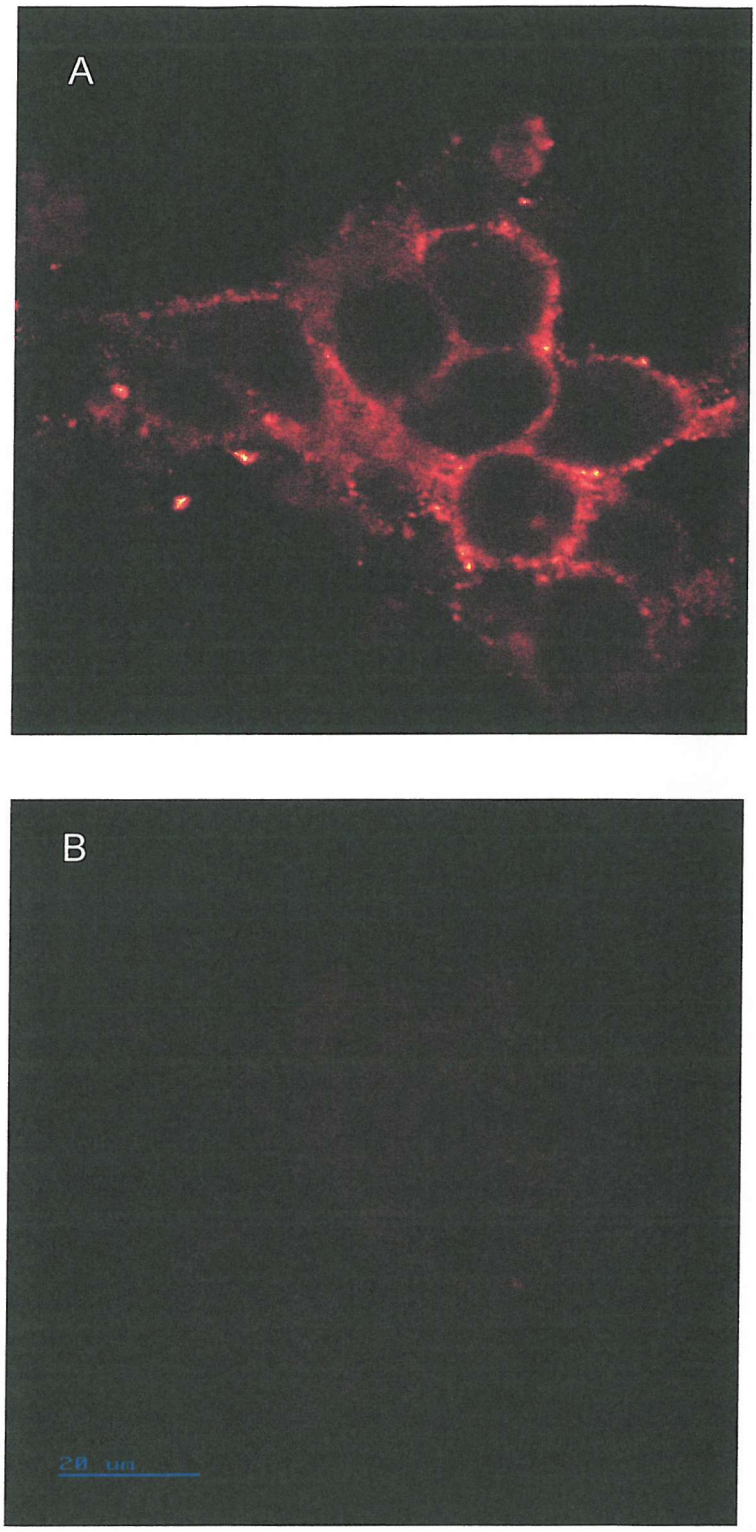


Figure 4.4 (kindly donated by Miss Sian Llewellyn) Immunohistochemistry for heparan sulphate proteoglycan in granulosa cells

(A) Before and
(B) After treatment with heparitinase

Chapter 5

LAMININ SUBUNITS

Contents

5.1 Introduction

5.1.1 The laminin family

Laminins are a family of extracellular matrix glycoproteins. It has been advocated that there is a close correlation between cellular phenotype and specialized extracellular matrix formation. Cells synthesize an extracellular matrix according to their state of maturation and function. Matrix components are able to modulate fundamental cellular properties such as proliferation and differentiation. Laminin seems to be related to advanced cellular differentiation. In various cell lineages, laminin is a prerequisite for terminal differentiation and the realization of specialized cell functions.

5.1.2 Laminin isoforms

Laminin is a heterotrimeric cross-shaped molecular complex of 850,000 Da. The laminin molecule consists of a large α chain and two different smaller chains, the β and the γ chains, connected by disulfide bridges in a

cruciform-shaped molecule (figure 5.1). The long arm of the cross comprises a linear assembly of α-helical domains associated in a triple helix. Laminin is bound to other extracellular matrix components i.e. heparan sulphate proteoglycan, type IV collagen, entactin and to other receptor proteins. The assembly of various possible heterotrimeric combinations has been described, giving rise to a family of eleven different laminin isoforms (Table 5.1).

Table 5.1 Current and previous nomenclature of the laminin family constituents and their heterotrimeric composition (Burgeson et al., 1994; Timpl, 1996)

Name composition	Previous name	Chain
Laminin 1	EHS Laminin	α1β1γ1
Laminin 2	Merosin	α2β1γ1
Laminin 3	S-Laminin	α1β2γ1
Laminin 4	S-Merosin	α2β2γ1
Laminin 5	Kalinin/Nicein	α3β3γ2
Laminin 6	K-Laminin	α3β1γ1
Laminin 7	K-S-Laminin	α3β2γ1
Laminin 8		α4β1γ1
Laminin 9		α4β2γ1
Laminin 10		α5β1γ1
Laminin 11		α5β2γ1

The prototype laminin (laminin 1) was originally described in the matrix of the Engelbreth-Holm-Swarm (EHS) mouse tumor and on isolation was found to be a large heterotrimer (Timpl et al., 1979). The three subunits α1 (molecular weight ~400kDa), β1 (~220kDa) and γ1 (~200kDa) assemble to yield its molecule with one long and three short arms.

Laminin 2 (merosin) was originally isolated from human placenta and mouse heart and found to contain a novel α chain homologue along with a

β1 and γ1 subunit (Paulsson et al., 1991). The α2 subunit is unique in having two non-covalently associated chains of ~300 and 80kDa; these are post-translational proteolytic products derived from one gene product (LAMA2). Laminin 3 (s-laminin) contains a β chain homologue (β2~190kDa) associated with an α1 and a γ1 chain.

Laminin 4 (s-merosin) is a combination of the α2 and β2 subunits along with a γ1 chain.

The laminin isoforms can be identified in situ by chain-specific antibodies or by mRNA in situ hybridization with probes applied to the individual chains (Kosmehl et al., 1996).

Laminin binding to the cell surface can be mediated by several members of the integrin family (Mercurio, 1995). These interactions have in some cases been localized to discrete domains of the laminin molecule.

There is limited research described in the literature regarding the different laminin subunits that constitute the continuously remodeling ovarian basal laminae. In one study, the α1, β2 and γ1 chains have been immunolocalized to the basal lamina in bovine antral follicles whilst α2 and β1 were rarely present (van Wezel et al., 1998). Laminin γ1 was detected in bovine granulosa and basement membrane/theca fractions of maturing follicles and from corpora lutea of the early, middle and late luteal phase. Laminin β1 was expressed in all the above tissues except the granulosa (Zhao and Luck, 1995). There is evidence from animal studies that the composition of the follicular basal lamina, at least in terms of laminin, changes during follicular development and atresia (van Wezel et al., 1998).

5.1.3 Histopathological aspects of laminin isoforms

Laminin 1, the most widely studied member, is the first extracellular matrix component to be detected in the developing embryo. Its α1 subunit is

expressed in the mouse at the 16-cell stage. The β1 and γ1 chains are detected even earlier. In this context, laminin may play a role both in the organization of early matrix deposition and the polarization of the trophectodermal epithelium. Laminin-cell interactions are important in mediating polarization, adhesion and migration in a wide variety of cell types (Mercurio, 1995). Laminin 1 has also been shown to modulate other aspects of cell phenotype. It can stimulate the production of matrix metalloproteinases and invasive behavior in malignant cells as well as promoting angiogenesis.

Abnormal expression of the α2 chain of laminin-2 (merosin) is associated with congenital muscular dystrophies, a heterogenous group of autosomal recessive skeletal muscle disease mutations in the LAMA2 gene (Helbling-Leclerc et al., 1995). Laminin 3 in muscles is concentrated at the sites of synapses. After injury, it can mediate the regeneration of motor axons to these sites.

In dermatoses characterized by altered adhesion (blistering) in the epithelium-stroma interface of skin and mucous membranes, mutations in the genes of laminin-5 can be found (Pulkkinen et al., 1994).

Laminin can trigger secretory differentiation in mammary epithelial cells. Laminin is highly antigenic and autoantibodies are associated with a number of pathologies including pre-eclampsia and systemic lupus erythematosus (Garcia Lerna et al., 1995). Administration of laminin antibodies to pregnant mice has been shown to cause spontaneous abortions (Foidart et al., 1983). Its presence in the endometrium plays a role in controlling the adhesion, migration, and differentiation of invading trophoblast cells and therefore in the successful implantation of the blastocyst.

5.2 Methods

The five different monoclonal primary antibodies used had been developed in animals against the specific laminin subunits α1, α2, β1, β2 and γ1 (Table 2.3 2nd Chapter). An additional polyclonal antibody raised in rabbits against purified human whole laminin was used in order to provide a good 'general' impression of the presence of laminin.

The method of Western analysis is based on SDS polyacrylamide gel electrophoresis which allows estimation of protein size by differential migration through the small pores of gel matrix. The molecular weight of the unknown proteins was estimated by the use of a standard molecular weight protein marker. The proteins were transferred to a membrane by Western blotting retaining their electrophoretic pattern. Identification of the individual protein was achieved via the use of the specific antibody for the protein of interest, as described in detail in Chapter 2.

5.3 Results

Western analysis of cell extracts was carried out in order to obtain additional evidence about laminin synthesis in culture. It was hoped that these results would serve to complement the immunocytochemical studies already described. The results are shown in Figures 5.2, 5.3, 5.4, 5.5 & 5.6 both for the freshly prepared granulosa cells and for the cultured cells in the absence or presence of the hCG (100ng/ml). Results for the three subunits and the polyclonal antibody to human laminin were consistent over a total of five experiments.

Western analysis using monoclonal antibodies against specific laminin subunits is shown in Figures 5.2, 5.3, 5.4 &5.5.

The use of the antibody specific to the α_1-subunit was not clear-cut and did not reveal or exclude the presence of the α1-subunit in the extracts of the granulosa cells. This was mainly due to its high molecular weight (~400

kDa), which made clear separation difficult using the SDS-PAGE system employed. Our results for the α1-subunit are therefore not shown.

The antibody to the α2-subunit is known to be specific for an 80kDa fragment which is generated by post-translational proteolysis, non-covalently associated with intact laminin and released during the extraction process. Strong bands of immunoreactivity at ~80kDa in extracts from the cultured granulosa cells (and human placental laminin standard) are therefore consistent with the presence of the α2-laminin (Figure 5.2). In order to confirm the specificity of the primary antibody that was used and to identify the presence of the non-specific immunostained bands the experiment was performed with and without the addition of the primary antibody (Figure 5.3a & 5.3b). This revealed, quite clearly, that the addition of the primary antibody gives a strong immunostained signal at ~80kDa, compared to the control without primary antibody.

Similarly, blots developed with the antibody against β1-laminin (Figure 5.4), showing strong immunoreactivity at about 200kDa in extracts of cultured cells and Matrigel (containing mouse subunits), are consistent with the presence of β1-laminin. It is worth noticing that the antibody used, reacts with both the human and mouse β1-laminin and therefore detects the human-derived and Matrigel-derived subunits. This experiment was duplicated with loaded amounts, which were either 'adjusted' or 'non-adjusted' for cell number (Figures 5.4a & 5.4b). No difference in the immunostaining was noticed, within the limits of this semi-quantitative method, when 'adjusted' and 'non-adjusted' loadings were compared. Higher levels of laminin subunits were found in the extracts from cultured cells compared to freshly prepared cells.

Blots developed with the use of the antibody against β2-laminin (results not shown) did not reveal the presence of the specific subunit either in the extracts from the freshly prepared cells or from the cultured cells.

There was evidence for the presence of the γ1-subunit of laminin (Figure 5.5). Like the results obtained with the α2- and β1-subunit, much lower levels of laminin subunits were found in the extracts from freshly prepared cells irrespective of whether a loading adjustment was made for cell number. Also, levels of the three subunits were not noticeably altered after hCG exposure in culture.

An anti-human polyclonal antibody was also used for Western analysis in extracts from freshly prepared and cultured cells, revealing a major immunoreactive band at ~200kDa. This is probably reflecting the level of β- and/or γ-subunits of laminin and is likely to provide a good "general" impression of the presence of laminin. The levels of this "200kDa laminin" were higher in the cultured cells than in the freshly prepared cells (Figure 5.5). The lower level of laminin in the original cells was a reproducible observation apparent in a total of five preparations examined and was evident whether or not the amount loaded onto the gels was adjusted for cell content on the basis of DNA. The experiments performed did not detect an increased level of laminin deposition following hCG stimulation.

Further experiments were performed in order to examine the time course of laminin deposition in the cultured cells. The polyclonal antibody to laminin and the monoclonal antibodies were used in a series of experiments. Results from the use of the antibody to the α2-subunit and the polyclonal antibody are shown in Figures 5.7 & 5.8. The evidence for a progressive deposition of laminin in culture was particularly clear when the polyclonal antibody was used (Figure 5.8). Results for the α2-subunit were less clear-cut as deposition was well underway by day 2 of culture. The rates of which the various subunits are deposited thus seem to be subunit specific.

5.4 Discussion

Tissue remodeling and cell differentiation are a characteristic feature of the ovarian cycle. The transition from mature follicle to corpus luteum involves

marked changes in tissue structure and alterations of the endocrine cell phenotype. There is increasing evidence that the extracellular matrix proteins are affecting the cell phenotype in reproductive tissues during development, remodeling and repair. The composition of a basal lamina seems to affect the fate of adjacent epithelial cells. Therefore it differs in composition with tissue development, and between different tissues in the body.

The role of these proteins in the ovarian cycle is unclear, largely because their identity and locations within the ovary are poorly described. Laminin is one of its components and its detection at the different stages of corpus luteum formation constitutes one of the aims of this study. Laminins consist of one α, one β and one γ chain, of which there are at least five, three and two isoforms, respectively. We have adopted the new nomenclature used for the laminin subunits (see Burgeson et al, 1994). Although there have been previous studies which tried to immunolocalize a range of individual laminin chains in ovarian follicles in animals (van Wezel et al, 1998), this is the first systematic attempt in humans.

Previous work in the bovine ovary, revealed a changing pattern in laminin isoforms during follicular development (van Wezel et al, 1998). The α2 and β1 subunits were rarely present in the follicular basal laminae of healthy antral follicles and this is consistent with our finding that they are absent from the extracellular matrix of the freshly prepared granulosa cells. In extracts from bovine follicles and corpora lutea it has been shown that there is expression of laminin β1 in corpora lutea but it is not expressed in granulosa cells (Zhao and Luck, 1995). In the same study, there was also an increased expression of the γ1 subunit during the developmental phase of the corpus luteum. Both the above findings from animal experiments seem consistent with changes detected in the human granulosa cells, and suggest increased laminin synthesis as the corpus luteum is established.

The present study looks at the laminin which may be deposited around luteinizing granulosa cells in women and clearly shows the presence of the

α2-, β1- and γ1- subunits although with some diversity in the intensity of staining. Laminin-2 (merosin) is known to contain these subunits and is present in muscle, peripheral nerve and trophoblast (reviewed by Church et al., 1997). As abnormal expression of this specific laminin is associated with congenital muscular dystrophies in humans, further studies in this subpopulation, could provide useful information regarding the corpus luteum formation and functionality. Our findings may also have implications for the development of cell culture techniques for granulosa cells. If we are to provide extracellular matrix in vitro we may be obliged to supply the cells with the physiological laminin isoform for luteinized granulosa cells, rather than the mouse laminin-1 used in previous studies (e.g. Fujiwara et al., 1997). This could be the explanation of the enigmatic finding that laminin suppresses progesterone production by human luteinizing granulosa cells, as it has been advocated from the above study, while it is also synthesized by the granulosa cells as part of their luteinization.

It should be noted that a larger study would need to be carried out in order to exclude the possibility of the presence of other laminins such as laminin-4 (α2β2γ1), and in establishing how the subunit composition of laminin may change as function changes during the menstrual cycle and more importantly in luteinization. Our work on the existence of the laminin subunits in luteinizing granulosa cells could lead on to future studies which might look at the physiological or pathological consequences of alteration in matrix deposition after ovulation. For example, it is possible that reduced or 'faulty' laminin formation in luteinization could have negative consequences for luteal function with reduced progesterone production in the luteal phase. The ability of hCG to 'rescue' the corpus luteum in early pregnancy could also be compromised.

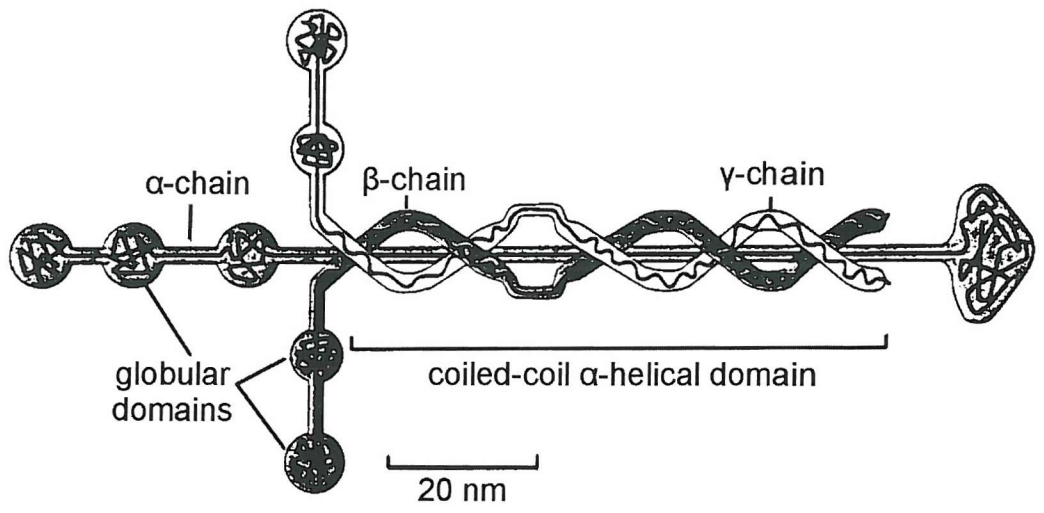


Figure 5.1 The structure of laminin. This multidomain glycoprotein is composed of three polypeptides (α-, β- & γ- subunits) that are disulfide bonded into an asymmetric cross like structure. Each of the polypeptide chains is more than 1500 amino acid residues long. Adapted from 'Molecular Biology of the Cell' by Alberts et al. (Third edition: 1994).

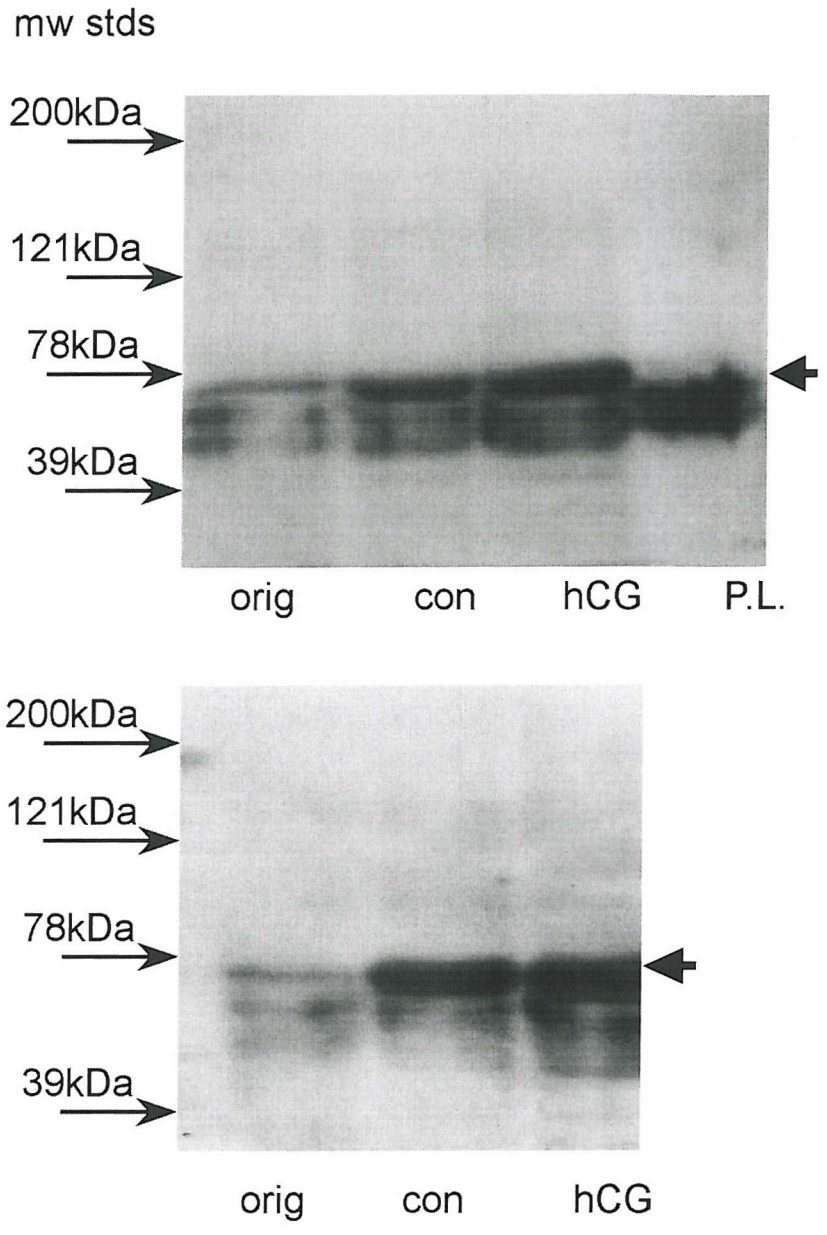


Figure 5.2 Detection of laminin-α2-subunit by Western blotting in extracts of cultured human granulosa cells in the absence or presence of hCG (100ng/ml) for 7 days. Two separate experiments are shown.

orig: cells freshly prepared
con: cells cultured without gonadotrophins
hCG: cells cultured with hCG (100ng/ml)
P.L.: placental laminin, 10 micrograms
mw stds: molecular weight standards
➡ : position of α2-subunit

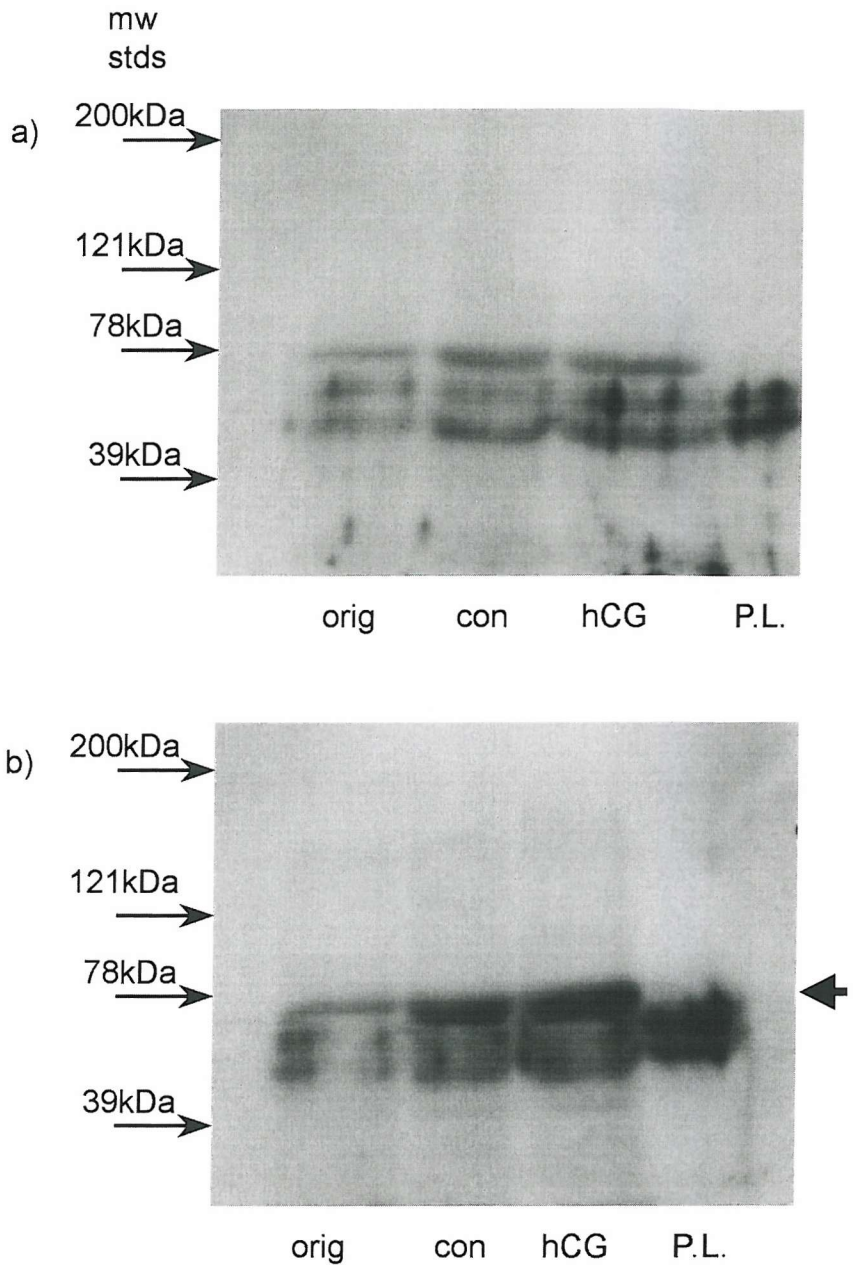


Figure 5.3 Detection of laminin-α2-subunit by Western blotting in extracts of cultured human granulosa cells. Cultures were maintained either in the absence or presence of hCG (100ng/ml) for 7 days. Results are shown from Western blots prepared either without (a) or with (b) the addition of the primary antibody.

orig: cells freshly prepared
con: cells cultured without gonadotrophins
hCG: cells cultured with hCG (100ng/ml)
mw stds: molecular weight standards
➡ : position of α2-subunit

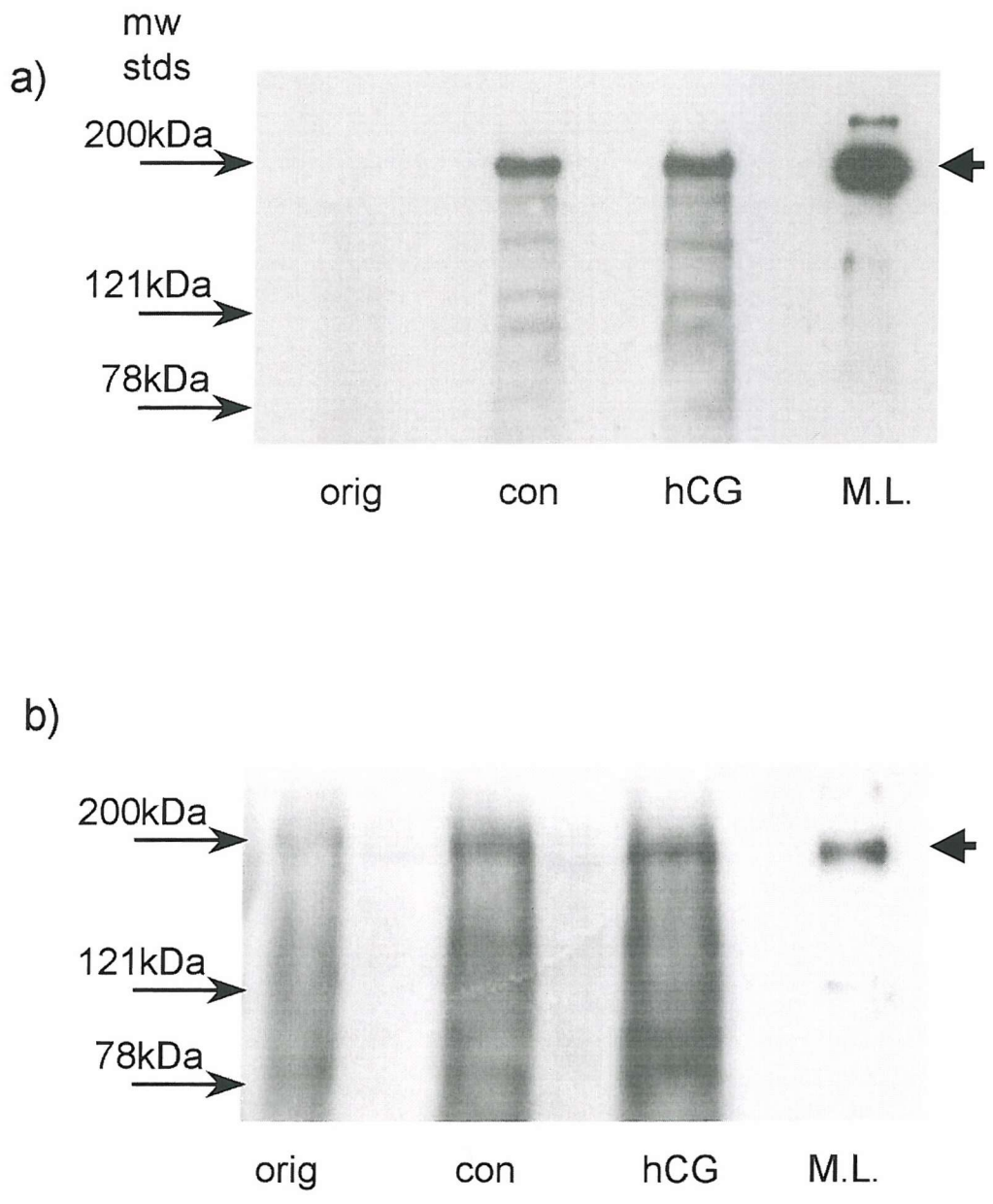


Figure 5.4 Detection of laminin-β1-subunit by Western blotting in extracts of cultured human granulosa cells. Cultures were maintained either in the absence or presence of hCG (100ng/ml) for 7 days. Amounts loaded were either adjusted (b) or not adjusted (a) for cell number.

orig: cells freshly prepared
con: cells cultured without gonadotrophin
hCG: cells cultured with hCG (100ng/ml)
M.L.: mouse laminin in the form of Matrigel
mw stds: molecular weight standards
➔ : position of β1-subunit

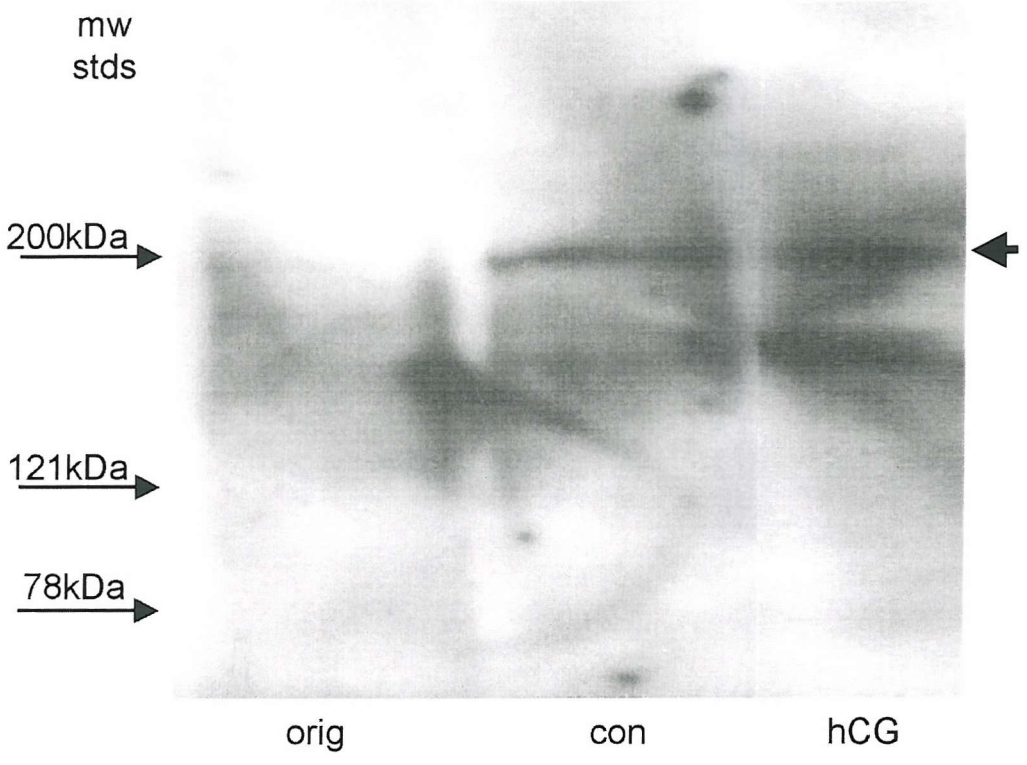


Figure 5.5 Detection of laminin-γ1-subunit by Western blotting in extracts of cultured human granulosa cells. Cultures were maintained either in the absence or presence of hCG (100ng/ml) for 7 days. Amounts loaded were either adjusted (b) or not adjusted (a) for cell number.

orig: cells freshly prepared
con: cells cultured without gonadotrophin
hCG: cells cultured with hCG (100ng/ml)
mw stds: molecular weight standards
→ : position of γ1-subunit

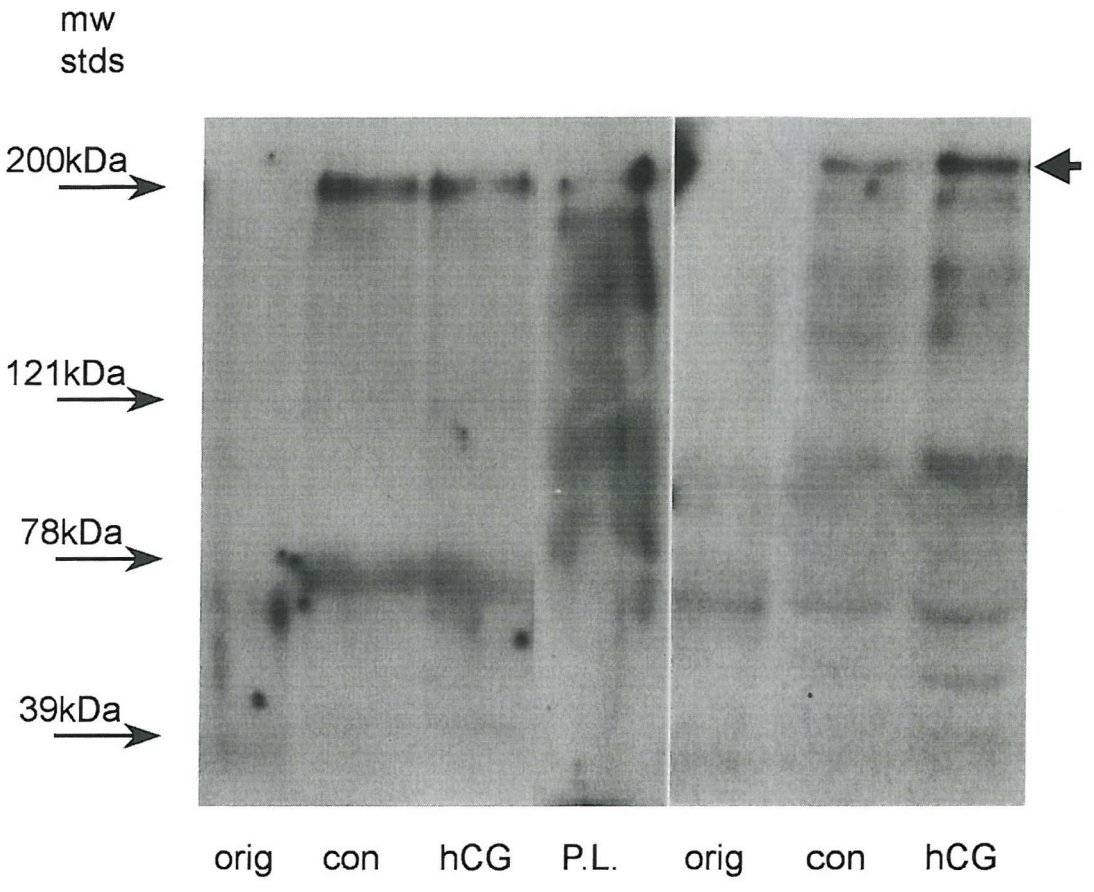


Figure 5.6: Detection of laminin subunits by Western blotting in extracts of cultured human granulosa cells. A polyclonal primary antibody raised against purified human laminin was used. Cultures were maintained either in the absence or presence of hCG (100ng/ml) for 7 days.

orig: cells freshly prepared
con: cells cultured without gonadotrophins
hCG: cells cultured with hCG (100 ng/ml)
P.L.: placental laminin
mw stds: molecular weight standards
➡ : position of positive bands of interest

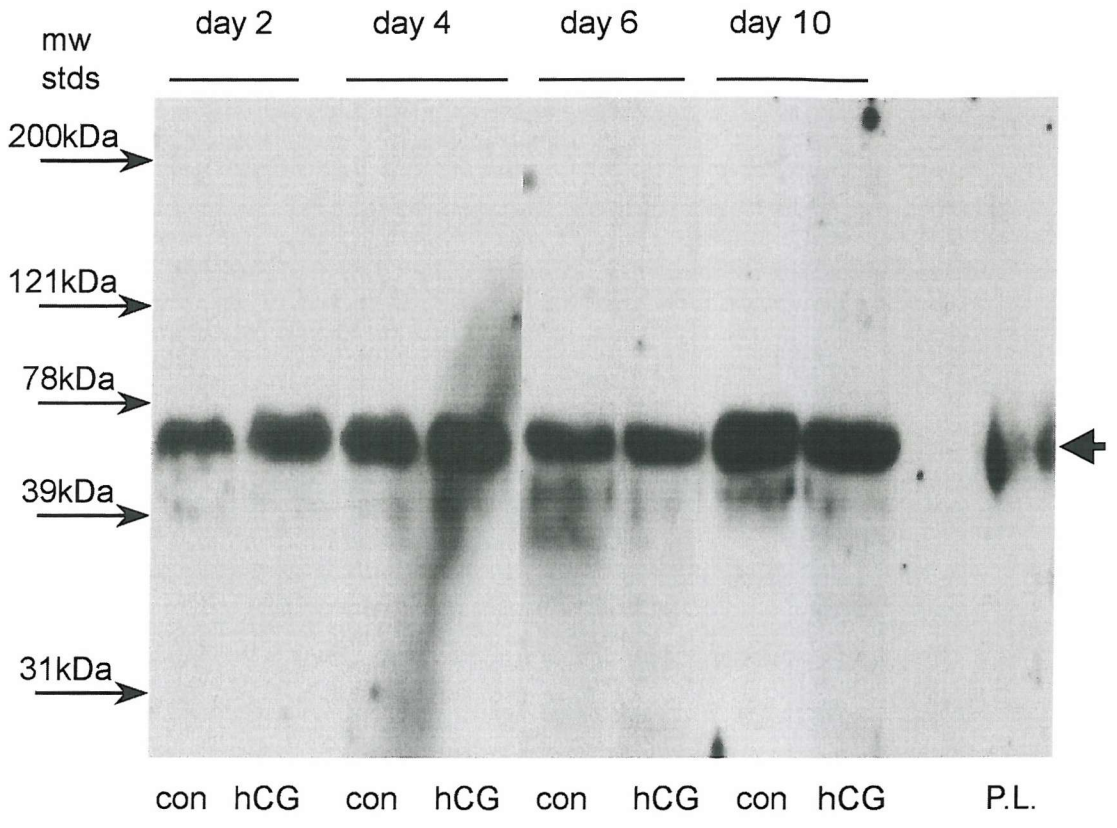


Figure 5.7. Time course of production of laminin-α2-subunit by cultured granulosa cells as measured by Western analysis using a monoclonal atibody. Amount loaded into each well was corrected for cell number.

con: cells cultured without gonadotrophin
hCG: cells cultured with hCG (100ng/ml)
P.L.: placental laminin
mw stds: molecular weight standards
➡ : position of α2-subunit

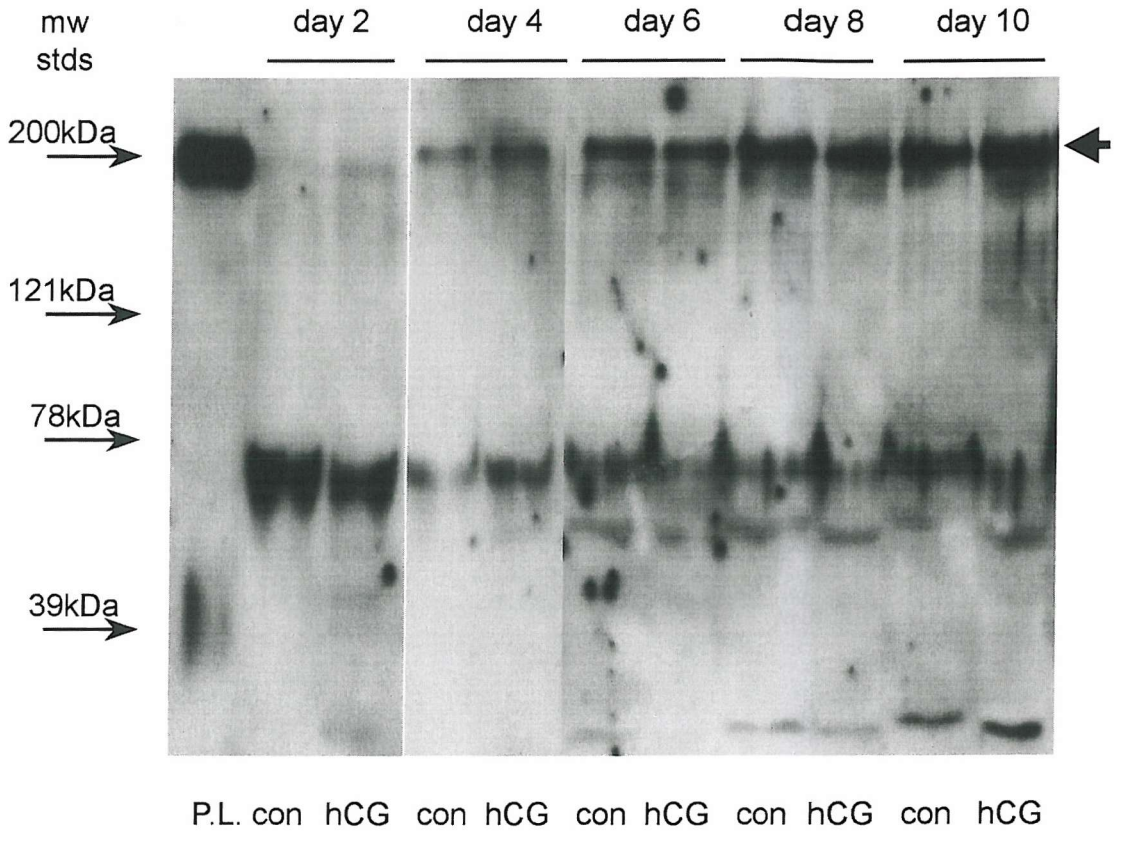


Figure 5.8 Time course of production of laminin by cultured granulosa cells as measured by Western analysis using a polyclonal antibody. Amount loaded into each well was corrected for cell number.

PL: placental laminin, 10 micrograms.
con: cells cultured without gonadotrophin
hCG: cells cultured with hCG (100ng/ml)
mw stds: molecular weight standards
➡ : position of positive bands of interest

Chapter 6

GROWTH FACTORS

Contents

6.1 Introduction

Evidence has been presented that the granulosa cells in the corpus luteum are in contact with a specific extracellular matrix that develops as luteinization occurs. As growth factors may affect matrix deposition by cells (see below), we speculated that luteal extracellular matrix might be influenced by granulosa cells when subjected to modulation by growth factors as well as gonadotrophins. The novel culture system we developed, without the addition of exogenous matrix, enabled us to measure endogenous matrix production and how this may be influenced by the addition of epidermal growth factor and fibroblast growth factor.

6.1.1 Fibroblast growth factor (FGF)

Although a range of growth factors have now been recognised in the FGF family, discussion here is restricted to basic FGF (abbreviated to bFGF).

A compound with angiogenic activity, found in extracts of corpus luteum, was investigated by Gospodarowicz et al. (1985) and identified as bFGF. It was later found that bovine granulosa cells produce bFGF (Neufeld et al.,

1987). Later work (Stirling et al., 1991) looked at the expression of mRNA encoding bFGF in bovine corpora lutea and cultured luteal cells. Also, Watson et al. (1992) measured the expression of mRNA for bFGF in human granulosa and cumulus cells using reverse transcription and nested polymerase chain reaction. This locally produced bFGF may have a number of effects in the corpus luteum:

1. The bFGF probably acts in conjunction with other growth factors (notably VEGF) to cause the angiogenesis that occurs as the corpus luteum is established. This was suggested by Gospodarowicz et al. (1985).

2. The bFGF may have an effect on cell survival in the corpus luteum. Thus Tilly et al. (1992) showed that bFGF (with EGF) suppresses the spontaneous onset of apoptosis in cultured rat ovarian granulosa cells and follicles. More recently, it has been shown that the observed positive effect of progesterone on rat large granulosa cell viability may be mediated by bFGF synthesised by small granulosa cells (Peluso and Pappalardo, 1999).

3. Aharoni et al. (1997) suggested that bFGF has a positive effect on granulosa cell differentiation (i.e. maintaining steroidogenic enzymes).

4. Rodgers et al. (1996) looked at the production of extracellular matrix by granulosa cell colonies in vitro. They showed that these colonies produced basement membrane-like material. The sizes of the colonies were increased in the presence on bFGF (presumably an effect on cell replication) and also the amount of matrix deposition was enhanced. Production of fibronectin (considered to be a component of extracellular matrix) was greatly increased in the presence of bFGF. However, whether the overall production of matrix per cell was increased in the presence of bFGF was not made entirely clear in this paper.

Therefore, in view of these studies, it was thought that it was possible that bFGF would stimulate the production of matrix by human granulosa cells. Obviously one potential problem is that there could be endogenous production of bFGF by the cultures which could mask effects of exogenously added material.

6.1.2 Epidermal growth factor

It has been known for some time that there are receptors to EGF on granulosa and luteal cells (Vlodavsky et al., 1978). This applies to a range of species including the human where specific EGF receptors have been demonstrated in corpora lutea of menstrual cycles and pregnancy (Khandawood et al., 1995). There appears to be a stimulatory effect of EGF on adenylate cyclase in luteal cell membranes (Budnik and Mukhopadhyay, 1991). This leads to a number of modulatory actions within the cell:

1. In rat granulosa cells, EGF has the ability to stimulate the production of progestins (Jones et al., 1982). This effect appears to be via induction of the cholesterol side-chain cleavage enzyme complex in these cells (Trzeciak et al., 1987). Stimulation of progesterone production (the main product of the corpus luteum in primates) by EGF certainly applies to human granulosa cells (Richardson et al., 1989).

2. EGF clearly stimulates the production of IGFBP-1 by human granulosa cells (Angervo et al., 1992; Yap et al., 1998)

3. EGF (along with bFGF) has a marked anti-apoptotic effect on granulosa cells (Tilly et al., 1992).

Overall, it appears that EGF (or an EGF-like substance, such as transforming growth factor-alpha) is likely to be involved in luteinization (discussed by Murray et al., 1993). As we are postulating that production of

matrix may be part of the luteinization process, we hypothesise that EGF (or an EGF-like substance) may be involved in regulating matrix production at this stage during formation of the corpus luteum. Thus, along with FGF, the effect of exogenous EGF was investigated in our model system.

6.2 Methods

Follicular aspirates were obtained from patients who had undergone IVF treatment. The harvested granulosa cells were then prepared and separated from the rest of the aspirates according to a method previously reported (Richardson et al., 1992) and described in detail in Chapter 2. Granulosa cells were cultured for 7 days on chamber slides, which provided a pre-washed glass surface without the addition of any substrate. The culture medium was replaced regularly using the simple one-cycle replacement procedure described in Chapter 2. The granulosa cells were cultured in the presence or absence of hCG (100ng/ml), EGF (25ng/ml) and FGF (25ng/ml).

The method of Western analysis was then used for the identification of laminin synthesis in culture. The method is based on SDS polyacrylamide gel electrophoresis, which separates proteins on the basis of size by differential migration through gel matrix. The proteins were transferred to a membrane by blotting retaining their electrophoretic pattern. Specific antibodies for the laminin subunits (α2 and β1), already identified in the extracellular matrix of human granulosa cells (Chapter 5), and an antibody for the complete laminin molecule were used for the detection of the laminin components from the extracts of the treated cells (see Table 2.3).

6.3 Results

Western analysis of cell extracts was carried out in order to obtain evidence about laminin synthesis in culture in the absence or presence of the growth factors. The results are shown in Figures 6.1, 6.2, 6.3 & 6.4 both for the freshly prepared granulosa cells and for the cultured cells in the absence or

presence of hCG (100ng/ml) with or without EGF (25ng/ml) or FGF (25ng/ml). Results for the two subunits (α2 and β1) and the polyclonal antibody to human laminin were consistent over a total of five experiments. Results from the use of the γ1 subunit are not shown here.

Western analysis using the polyclonal antibody to human laminin with and without the addition of EGF in the culture medium is shown in Figure 6.1a. Very little immunoreactive material was found in uncultured cells. However, after culture there was clear evidence of the presence of laminin components (detected by the polyclonal antibody) in all cultured samples with or without the addition of hCG, EGF or the combination of both of them. Similar findings were revealed by the use of monoclonal antibodies against specific laminin subunits (α2 and β1) as shown in Figures 6.1b and 6.2. Within the limits of the semi-quantitative method used the levels of the three components were not noticeably altered after EGF exposure in culture.

The results of further Western analysis on the effects of hCG and bFGF in culture are shown in Figures 6.3 & 6.4. Using the same antibodies as above for the analysis, no clear effect of the growth factor on the production of laminin components was observed although there was some evidence that bFGF alone may have caused an increase in laminin β1 production (Figure 6.4). The results were equivocal, however, and much more work would need to be carried out to substantiate this effect.

6.4 Discussion

It was discussed earlier how growth factors influence a range of functions of granulosa cells. These included effects on steroid production by EGF (Jones et al., 1982; Trzeciak et al., 1987) and bFGF (Aharoni et al., 1997). Of particular interest was the observation by Rodgers et al. (1996) that the production of extracellular matrix by bovine granulosa cells was directly stimulated by the presence of bFGF. Effects of growth factors on granulosa cell survival (Tilly et al., 1992) have also been reported.

As our results suggest that synthesis of extracellular matrix components may be part of the routine function of granulosa cells, it was reasonable to suppose that growth factors could influence this aspect of their function. Our observations (Figures 6.1, 6.2, 6.3 and 6.4) revealed no marked effect of either EGF or bFGF on the deposition of laminin components in our system although there was some evidence that bFGF may have influenced the production of the β1-subunit. It would appear, therefore, that the major influence of bFGF on the synthesis of extracellular matrix shown by Rodgers et al. (1996) was not apparent in our culture system. There are many differences between that work and our own which could explain the different results. Firstly, the work of Rodgers et al. involved pre-ovulatory cells. It is possible that the ability to respond to bFGF in terms of matrix synthesis is influenced by luteinization which is occurring in our system. Also, the effect of the bFGF was examined partially in relation to fibronectin synthesis which was not investigated in the present study. It would be informative, therefore, to extend the present work to the influence of the growth factors on other extracellular matrix components.

It is possible that the lack of any marked effect of growth factors in our system could have been related to the presence of endogenous growth factor material in the cultures. Thus Watson et al. (1992) in this laboratory showed that human granulosa cells have the capability to produce bFGF in culture. It is feasible that the effect of additional bFGF provided exogenously in our experiments was masked by pre-existing endogenous bFGF in the cultures.

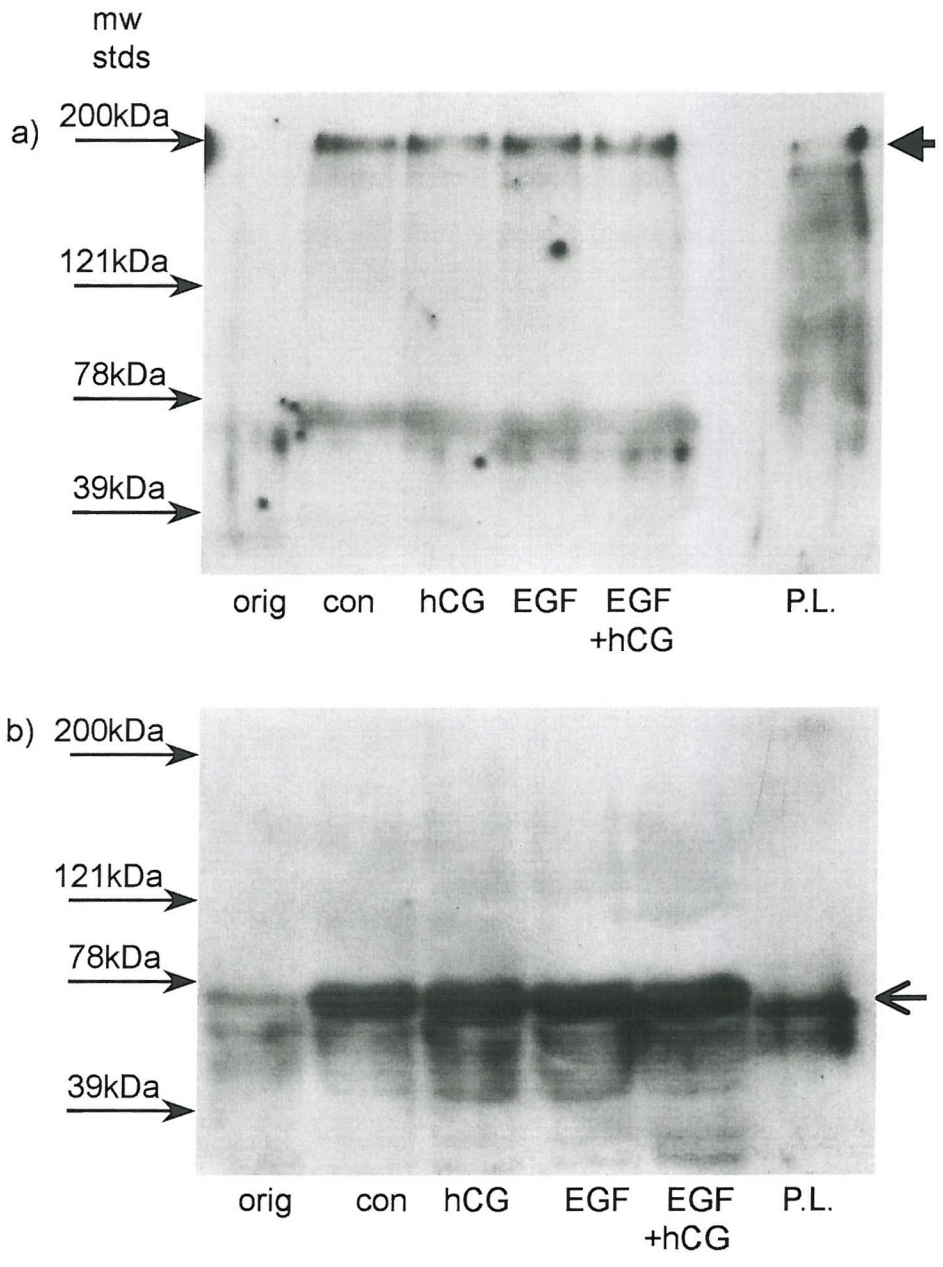


Figure 6.1 Detection of laminin subunits by Western blotting in extracts of cultured human granulosa cells in the presence or absence of hCG (100ng/ml) and EGF (25ng/ml) for 7 days. A polyclonal primary antibody (a) and a monoclonal one specific for the laminin-α2-subunit (b) were used.

orig: cells freshly prepared
con: cells cultured without gonadotrophin
hCG: cells cultured with hCG (100ng/ml)
EGF: cells cultured with EGF (25ng/ml)
P.L.: placental laminin, 10 micrograms
mw stds: molecular weight standards
→ : position of α2-subunit
➔ : position of positive bands of interest

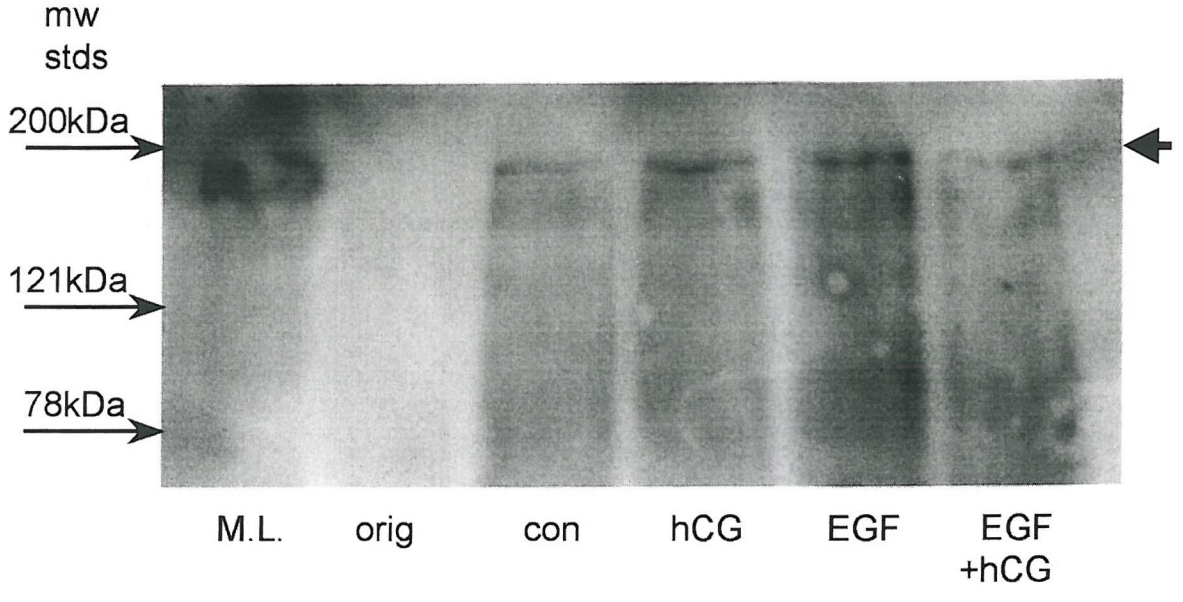


Figure 6.2 Detection of laminin subunits by Western blotting in extracts of cultured human granulosa cells in the presence or absence of hCG (100ng/ml) and EGF (25ng/ml) for 7 days. A specific monoclonal antibody for the laminin β1-subunit was used.

orig: cells freshly prepared
con: cells cultured without gonadotrophin
hCG: cells cultured with hCG (100ng/ml)
EGF: cells cultured with EGF (25ng/ml)
P.L.: placental laminin, 10 micrograms
mw stds: molecular weight standards
➡ : position of β1-subunit

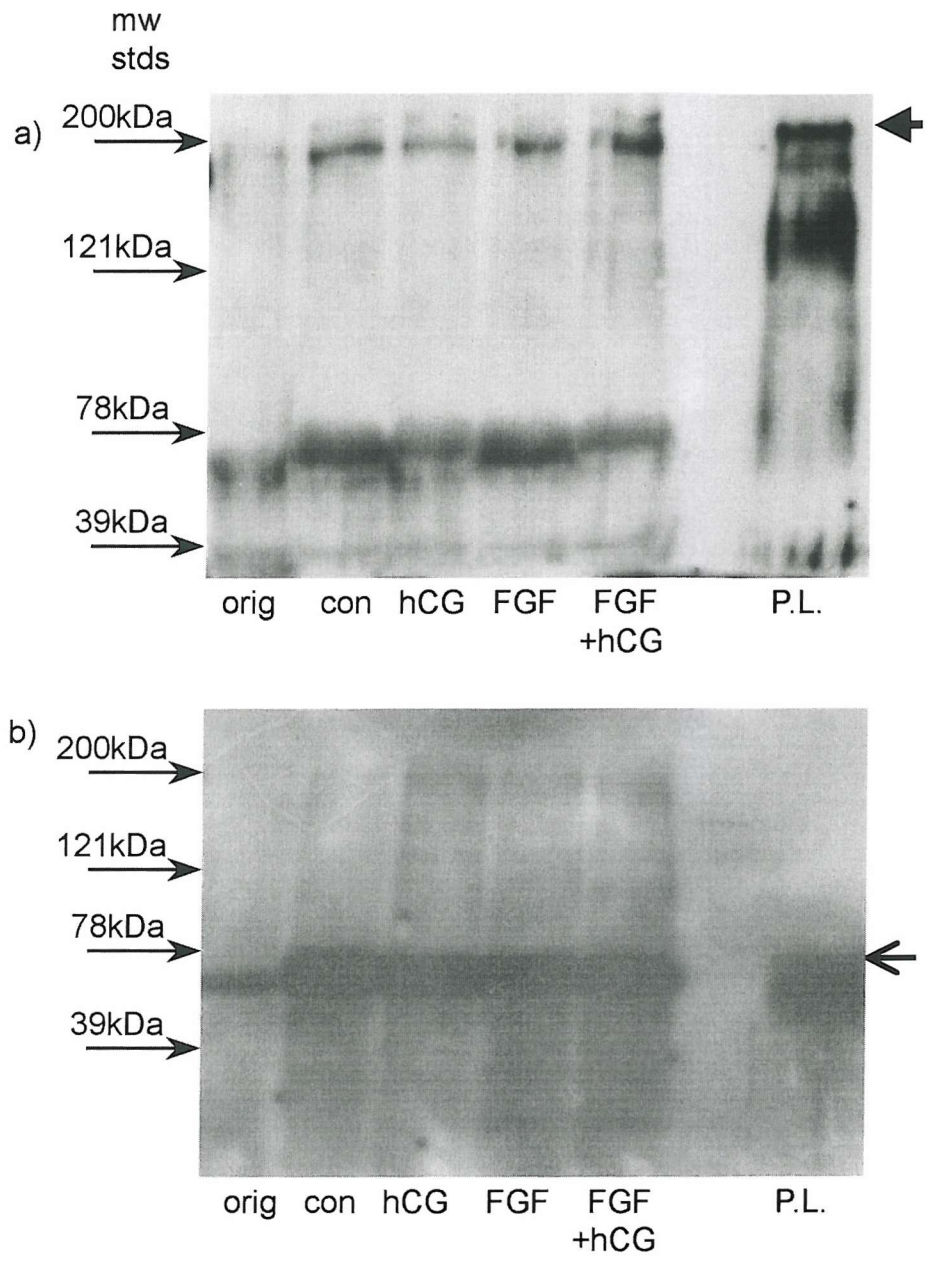


Figure 6.3 Detection of laminin subunits by Western blotting in extracts of cultured human granulosa cells in the presence or absence of hCG (100ng/ml) and FGF (25ng/ml) for 7 days. A polyclonal primary antibody (a) and a monoclonal one specific for the laminin-α2-subunit (b) were used.

orig: cells freshly prepared
con: cells cultured without gonadotrophin
hCG: cells cultured with hCG (100ng/ml)
FGF: cells cultured with FGF (25ng/ml)
P.L.: placental laminin, 10 micrograms
mw stds: molecular weight standards
→ : position of α2-subunit
➔ : position of positive bands of interest

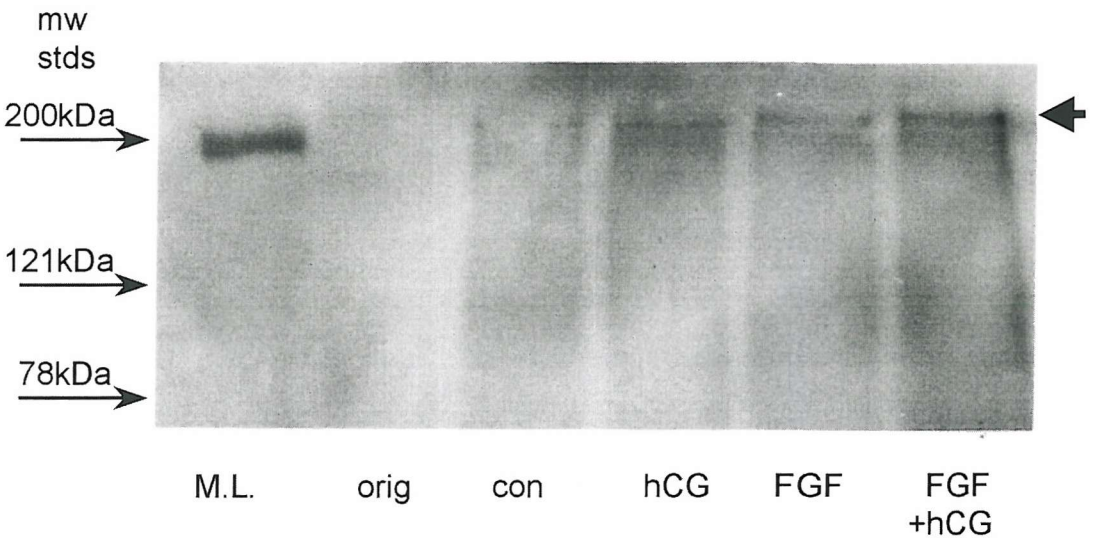


Figure 6.4 Detection of laminin subunits by Western blotting in extracts of cultured human granulosa cells in the presence or absence of hCG (100ng/ml) and FGF (25ng/ml) for 7 days. A specific monoclonal antibody for the laminin-β1-subunit was used.

orig: cells freshly prepared
con: cells cultured without gonadotrophin
hCG: cells cultured with hCG (100ng/ml)
FGF: cells cultured with FGF (25ng/ml)
P.L.: placental laminin, 10 micrograms
mw stds: molecular weight standards
➡ : position of β1-subunit

Chapter 7

'LIFT OFF' PHENOMENON AND METALLOPROTEINASES

Contents

7.1 Introduction

The general importance of metalloproteinase action in the function of the corpus luteum is well recognised and has been reviewed recently (Hulboy et al., 1997; McIntush & Smith, 1998). In Chapter 1 it was described how the balance between metalloproteinase activity and their inhibitors (TIMPs) may be important in the control of extracellular matrix degradation and how changes in this balance may occur as the corpus luteum develops or regresses.

Several metalloproteinases have been examined in relation to luteal function. In the rat, active MMP-2 is associated with structural luteolysis (Endo et al., 1993). In the human corpus luteum, both MMP-2 and MMP-9 have been investigated for their role in luteal regression (Duncan et al., 1998).

The possibility of using cultured granulosa cells for the study of metalloproteinase control in the corpus luteum was raised by the study of Stamouli et al. (1996) which showed that both MMP-2 and MMP-9 are produced in these cultures – the same metalloproteinases detectable in extracts of whole corpus luteum (Duncan et al., 1998). Moreover, the

observation that hCG was able in vitro to cause the suppression of metalloproteinase action suggested that control mechanisms pertaining in vivo might be operative within the culture model system.

Evidence has been presented that changes in metalloproteinase action in the granulosa cultures can lead to changes in the retention of the cells to the culture surface (Aston et al., 1996a; Aston et al., 1996b). A loss of cells from the culture surface in the absence of gonadotrophic support (the so-called 'lift off' phenomenon) may have been due to degradation of extracellular matrix by elevated levels of metalloproteinases present in these cultures. These former studies utilised exogenous matrix in the form of Matrigel.

It could be argued that the results of Aston et al. (1996a and b) were influenced by the inclusion of the exogenous matrix, Matrigel. It has already been shown in this thesis that the endogenous matrix surrounding granulosa cells may be quite different from Matrigel which contains mainly laminin-1. It was therefore important to extend the earlier work of Aston et al. to the new culture system discussed here that relies exclusively on the presence of endogenous matrix.

The studies to follow were carried out to establish whether the 'lift off' phenomenon would occur in the new culture system and whether associated changes in metalloproteinases were also apparent.

7.2 Methods

Granulosa cells were cultured using methods described in Chapter 3. The medium was changed every 2 days using the 'repeated partial change' method which left the cells relatively undisturbed on the culture surface.

Despite this method for medium renewal, eventually in the cultures without hCG, the cell clusters loosened from the culture surface. When this was

apparent, all the culture medium was removed gently from both the control and hCG-treated wells. The supernatants were centrifuged and the cell pellets were estimated for DNA. Cells remaining on the culture surface were also treated with DNA assay buffer and included in the analysis. In this way, a quantitative reflection of the 'lift off' phenomenon was obtained (Figure 7.1).

In order to collect media, which were used for the time-course experiment (Figure 7.2), the following method was adopted. Separate wells were set up which were intended for termination at specific days of culture (so that 'day 2' wells finished on day 2 of culture, 'day 4' wells finished on day 4 of culture, etc). When particular wells reached the end of their culture period, the entire medium was gently removed for storage, and the cells remaining in the wells were scraped and stored for later DNA assay. Zymography was performed on the media collected and bands of activity compared with bands resulting from standards of MMP-2 and MMP-9 obtained commercially (Chemicon, Harrow, UK). Amounts loaded on the gels were adjusted for cell number using the DNA measurements (i.e., volume of medium used for day 2 was adjusted for DNA value obtained for that day of culture). Zymograms for selected days of culture enabling replication in triplicate lanes were run in a similar way and are shown in Figure 7.3.

Experiments were performed to study the influence of 'medium changing' on the 'lift off phenomenon'. Cultures were maintained under our standard conditions (with or without hCG) where medium was changed every 2 days using the 'repeated partial change' method. Extra wells were included where medium was not replaced in the normal way. After the addition of 0.3ml of culture medium in the first step (see table 2.1), medium was removed from the cultures and the same medium re-added so that only the physical effect of medium changing was experienced by the cells – this was called 'sham' changing. As the cultures progressed they were examined under the inverted microscope and photographs taken on day 10 (Figure 7.4).

7.3 Results

As shown in Figure 7.1 the number of cells that remained attached to the culture surface was higher in those wells where hCG was added to the culture medium. Consequently, less cells were detected in the culture medium in the wells where hCG had been added compared to the control. Thus in this experiment between day 6 of culture (when a standard medium change was applied) and day 8 (when the measurements of cell release were made), there was a marked and substantial release of cells from the culture surface.

The time course experiment shown in Figure 7.2 shows the metalloproteinases in media collected on days 2, 4, 6, 8 and 10 of culture when cells were incubated in the presence or absence of hCG. The metalloproteinases in the culture medium were identified as MMP-2 and MMP-9 by comparison with the commercially available standards for these metalloproteinases. The release of MMP-2 into the medium tended to be higher as the culture progressed (Figure 7.2). Although zymography constitutes a semi-quantitative method, within its limitations, a higher MMP-2 concentration was detected in the medium from control cells compared to that from hCG-treated cells. This was more apparent during the last days of the experiment. Regarding MMP-9 production, the results obtained did not show any clear trend during the culture period.

In order to examine in more detail the changes occurring later in culture, triplicate samples obtained on days 7, 8 and 10 were examined by zymography (Figure 7.3). These results show a very clear elevation of MMP-2 levels in the culture medium of control cells compared with medium derived from cells cultured with hCG.

Using the inverted microscope, cell cluster formation was visualised under all conditions and was not dependent on the presence of gonadotrophin (Figure 7.4). Using our standard method for changing medium every two

days, eventually the cell clusters under control conditions lifted from the culture surface and this is apparent in Figure 7.4A. When examining the cells under the microscope, detachment was very clear and could be confirmed by gentle tapping of the microscope stage. In cultures which were maintained in the presence of hCG (Figures 7.4C and D), clusters remained attached to the culture surface. Also, where 'sham medium changes' were adopted (i.e. where medium was removed and the same medium replaced) the phenomenon of cell detachment was not detected (Figure 7.4B).

7.4 Discussion

The observation in Figure 7.1 establishes that in the absence of gonadotrophin a substantial release of cell clusters can occur during the later stages of culture. This so-called 'lift-off' phenomenon had been previously observed (Aston et al., 1996a) where granulosa cells had been maintained on Matrigel providing an artificial extracellular matrix. The present study now shows for the first time that this 'lift-off' can occur in the absence of exogenous matrix. The original work of Aston et al. (1996a and b) suggested that the 'lift-off' phenomenon resulted from degradation of the exogenous extracellular matrix and that this was associated with greater levels of MMP-2 in culture media. The finding that the 'lift-off' can occur in the absence of exogenous matrix may suggest that in our experiments where endogenous matrix is present in the culture system (see Chapters 4 and 5), that this too may be degraded causing release of the cells. As with the original work by Aston et al., this may be associated with changes in metalloproteinase concentrations in the culture system.

As shown previously by Aston et al., hCG prevented the detachment of cell clusters from the culture surface (Figure 7.1). Again, as suggested by Aston et al., the possibility arises that the action of hCG on the cultured cells results in the prevention of degradation of matrix within the cultures. A

range of possible mechanisms could be considered including changes in metalloproteinases and their inhibitors.

As a way of investigating the role of metalloproteinases in the culture system, levels of MMP-2 and MMP-9 in culture media were examined (Figures 7.2 and 7.3). The identity of these metalloproteinases shown by comparison with standards in our study, confirms more detailed identification through Western analysis of MMP-2 and MMP-9 in granulosa cell media demonstrated by Stamouli et al. (1996). MMP-2 and MMP-9 were also considered to be the predominant metalloproteinases in extracts of human corpus luteum (Duncan et al., 1998).

Our finding that levels of MMP-2 tended to increase during the culture period, particularly in the absence of gonadotrophin, is consistent with the previously published work of Stamouli et al. (1996). In that study, however, the amount of medium loaded on to the gels for zymography was not adjusted for cell number. The adoption of this adjustment in the present study is therefore novel and may have led to a clearer demonstration of changes in metalloproteinases in different culture conditions. Thus, results shown in Figure 7.3 show a very marked difference in MMP-2 levels in media from control versus hCG-treated cells. Elevation of MMP-2 clearly shown under control conditions (days 7, 8 and 10 of culture) is remarkable when compared to the lower levels in media from hCG-treated cells. These results are in agreement with previous work of Duncan et al. (1998) which showed that hCG in vivo was able to suppress MMP-2 levels measured later in extracts of corpora lutea.

Our finding that MMP-9 levels did not appear to be modulated in culture with hCG (Figure 7.2 and 7.3) is not consistent with earlier work of Stamouli et al. (1996) which showed some suppression of this metalloproteinase in hCG-treated cultures. Our work may have been influenced by the volumes of media loaded on to the gels used for zymography. Further work using a range of dilutions of media would be necessary to establish whether MMP-9

production is effected by the presence of gonadotrophins in the culture medium.

The photographs shown in Figure 7.4 provide an excellent visual record of the 'lift-off' phenomenon. Virtually all the clusters seen in Figure 7.4A are detached from the culture surface while those seen in the other micrographs (Figures 7.4B, C and D) remain attached. This cluster release (also shown quantitatively in Figure 7.1) can happen relatively suddenly after some period of culture and occurred leading up to day 10 in the experiment shown. The exact timing of the more rapid release of clusters from the control cultures varied between experiments. The suppression of cluster release by hCG (Figure 7.4B) confirms the quantitative results already shown in Figure 7.1.

The observation that the retention of clusters under control conditions was influenced by the removal of constituents during medium changing is a novel finding. Thus the lack of detachment of clusters when culture medium was removed and then the same medium re-added (Figure 7.4B), indicates that cluster release is inhibited by factors which build up in the medium. The identities of these factors are presently unknown but could be substances such as TIMP or progesterone. These same agents could be mediating the inhibition of cluster release by hCG. Further work would be necessary to clarify the mechanisms involved.

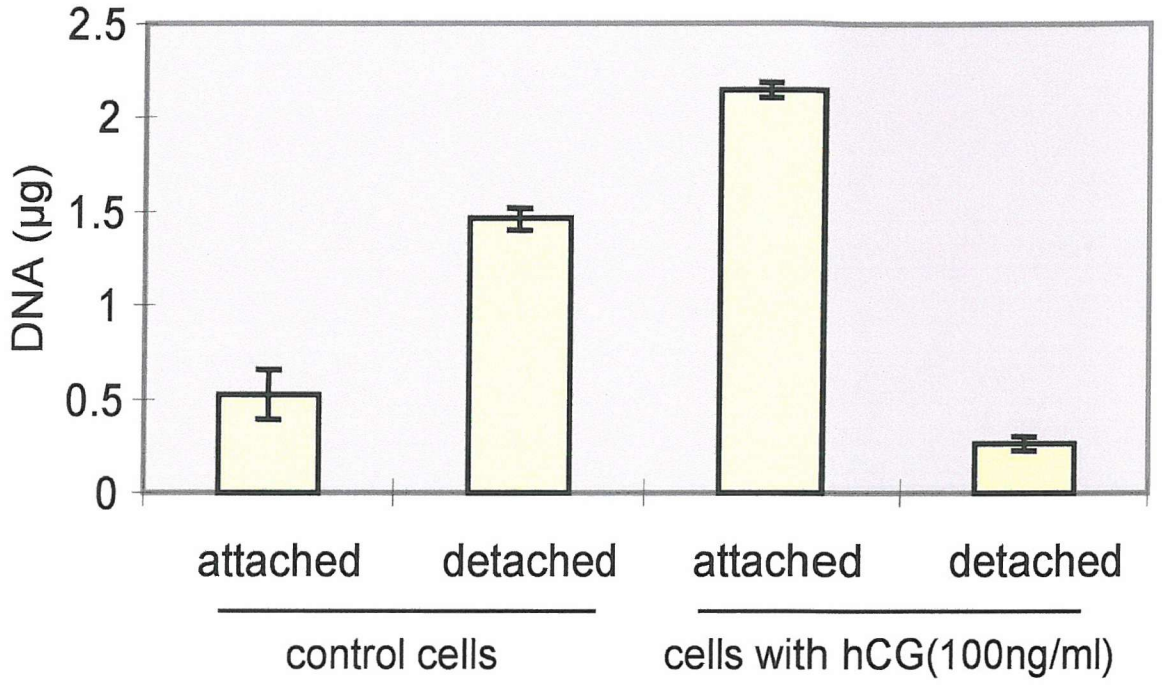


Fig 7.1 Release of granulosa cells from the culture surface under control and hCG stimulated conditions measured on day 8 of culture. DNA values relate to cells contained in one well. Bars represent the range of duplicate estimates.

attached: cells remaining attached to the culture surface
detached: cells (normally as clusters) released from the culture surface into the medium

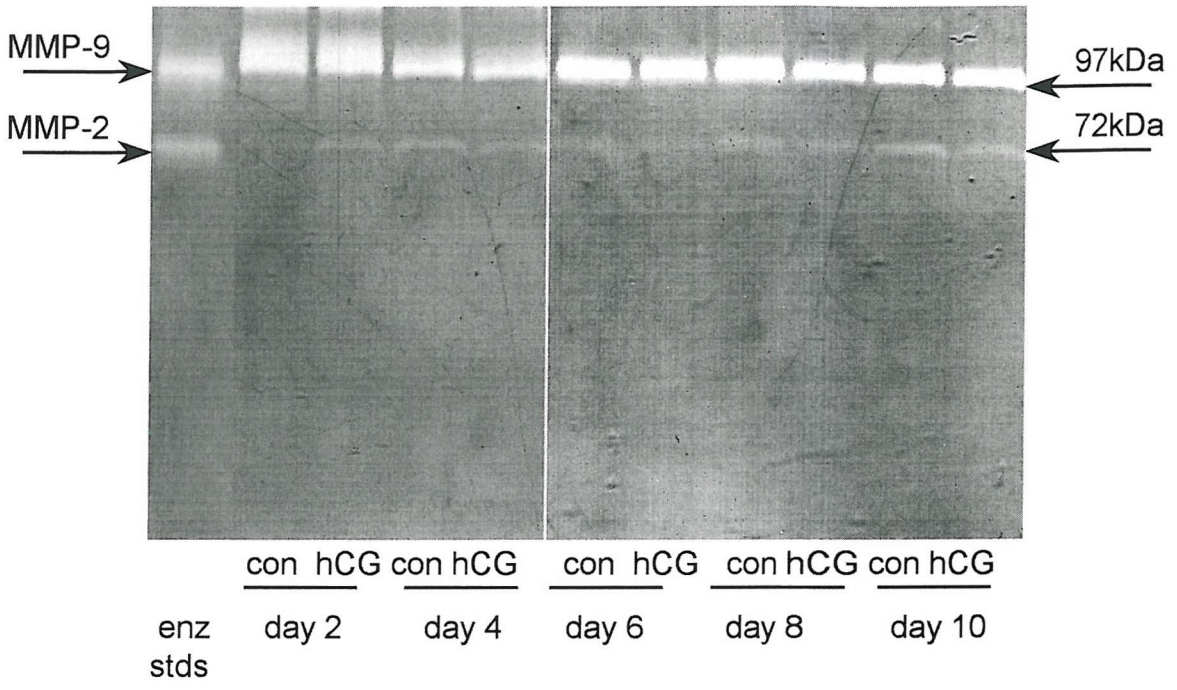


Figure 7.2 Time course of production of MMP-2 and MMP-9 by cultured granulosa cells as measured by zymography. Cultures were maintained in the presence or absence of hCG (100ng/ml) for the time shown. The media was changed every 48 hours. Amount loaded into each well was corrected for cell number.

con: cells cultured without gonadotrophin
hCG: cells cultured with hCG (100ng/ml)
enz stds: commercial preparation containing MMP-2 and MMP-9

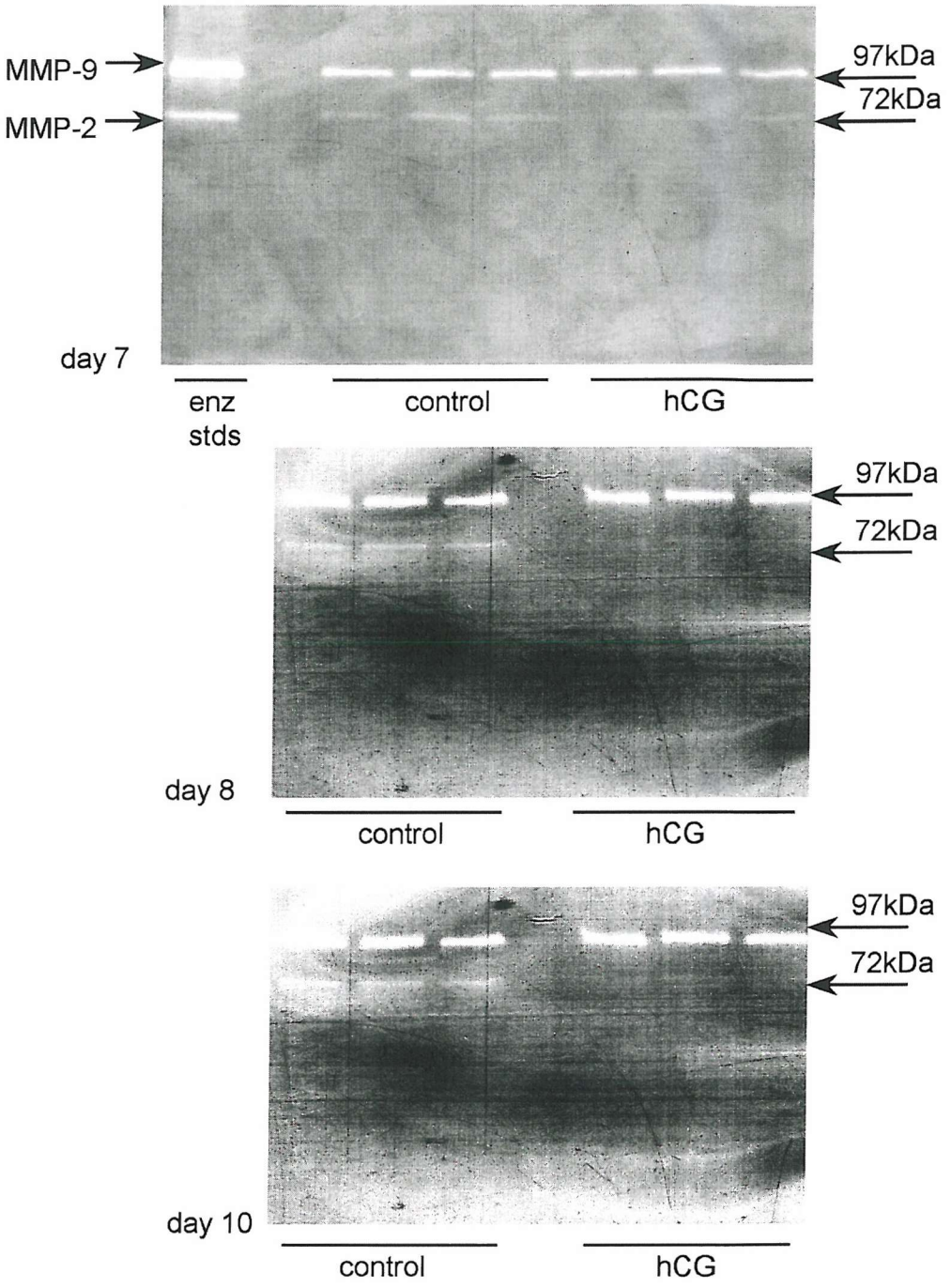
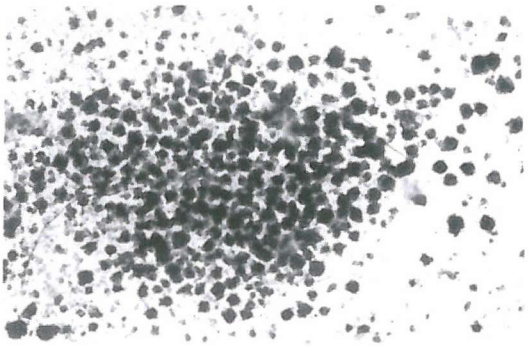


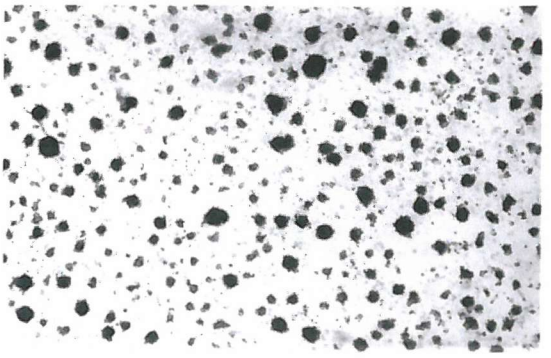
Figure 7.3 Zymography of media from granulosa cell cultures showing presence of MMP-9 and MMP-2. Cultures were maintained in the presence or absence of hCG (100ng/ml) for the time shown. Elevated levels of MMP-2 are evident in cultures without hCG.

control: cells cultured in absence of gonadotrophin
hCG: cells cultured with hCG
enz stds: commercial preparation containing MMP-2 and MMP-9

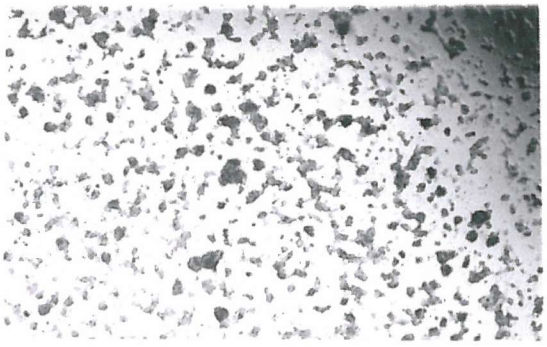
a: most clusters detached



b: clusters attached



c: clusters attached



d: clusters attached

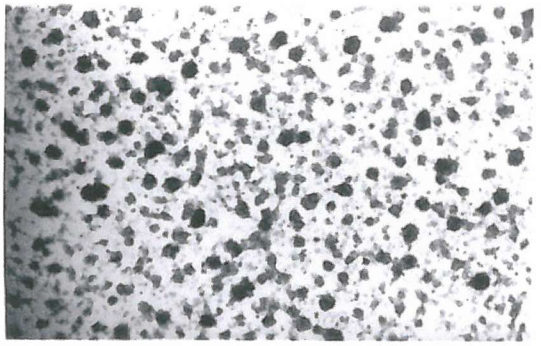


Figure 7.4 Photomicrographs of granulosa cells at day-10 of incubation.

a. cells incubated without hCG; medium changed by repeated partial changes. When the microscope stage was tapped it was evident that the clusters were free floating.

b. cells incubated without hCG; same medium aspirated and replaced to give 'sham' change

c. cells incubated with hCG; medium changed by repeated partial changes

d. cells incubated with hCG; same medium aspirated and replaced to give 'sham' change

Chapter 8

CONCLUSION

Contents

8.1 Introduction

In order to bring together the various findings and conclusions of the preceding chapters, it is worth re-considering the hypotheses originally outlined in Chapter 1. Although the hypotheses examined were part of a larger set of ideas on extracellular matrix control in corpus luteum development, rescue and regression, the central concepts investigated were as follows:

Do pre-ovulatory granulosa cells acquire a peri-cellular extracellular matrix as part of the luteinization process? Do they synthesise this matrix and is this based on elements of basement membrane? Is matrix production and degradation controlled by gonadotrophin or growth factors?

This concluding chapter will be largely concerned with establishing whether these hypotheses have been substantiated. Implications of our findings will be discussed and thoughts on future work elaborated.

8.2 Development of the novel cell culture system.

In order to investigate the luteinization process of granulosa cells in relation to matrix deposition, a cell culture system was required which did not involve the use of exogenous matrix components or additional attachment factors as had been used in previous studies. The presence of any newly synthesised matrix would have been masked by pre-existing exogenous matrix material. A new system was therefore developed using chamber slides which provided a pre-washed glass surface enabling cells to settle and to loosely adhere in the absence of exogenous matrix or attachment factors.

The use of a special method for the routine changing of medium was also adopted. This involved repeated, partial changes of culture medium leaving cells relatively undisturbed on the culture surface. We had found that the usual method involving complete removal and replacement of the culture medium resulted in the severe disturbance of the cultured cells. The new development of the repeated, partial change method was therefore very important in establishing a culture system suitable for our studies.

The combined use of the glass chamber slides and the special method for changing medium proved satisfactory for our requirements and enabled easy progression to immunohistochemistry and removal of cells for extraction prior to Western analysis.

Measurement of progesterone production and examination of the cells under the inverted microscope enabled us to establish that the granulosa cells, in the presence of hCG, were able to enlarge and develop increased

and sustained levels of progesterone production consistent with luteinization. These various features of the new culture model developed, provided reassurance that our in vitro model included luteinized granulosa cells which may have been equivalent to luteinized granulosa-derived cells of the mature corpus luteum.

8.3 Extracellular matrix components associated with the cultured granulosa cells.

The new methods developed enabled the examination of matrix components associated with the luteinizing granulosa cells. To our knowledge, this is the first systematic attempt to identify the matrix components associated with human granulosa cells during the luteinization process.

8.3.1 Heparan Sulphate Proteoglycan (HSPG).

Clear evidence was presented that HSPG was increased around the enlarged granulosa cells during the luteinization process and this was particularly evident after hCG exposure. This observation is consistent with our original hypothesis that the luteinization process of human granulosa cells involves deposition of matrix.

The HSPG around the granulosa cells may have importance for the binding of a range of growth factors which could have key roles in cellular differentiation. This is fully explained in Chapter 4 and clearly is an area for future study.

8.3.2 Collagen IV

Evidence is presented that freshly prepared human granulosa cells are associated with small, irregular patches of collagen IV and that this is consistent with the work of Yamada et al. (1999). In our hands the amount of collagen IV deposited between granulosa cells did not increase during culture either in the presence or absence of hCG. The study by Yamada et

al. (1999) indicated that collagen IV may be released into the medium during culture and that this release is stimulated by hCG. In the present study collagen IV was examined in adherent granulosa cultures and not in culture medium.

Although the present work and that of Yamada et al. (1999) disagree about continued synthesis of collagen IV in culture, the two studies are in agreement that if collagen IV deposition is part of the luteinization process, this is underway before ovulation, at least in women undergoing IVF.

8.3.3 Laminin

The present study revealed the presence of laminin in variable amounts around the freshly prepared granulosa cells. Its deposition was enhanced during the culture period either in the presence or absence of hCG, a finding consistent with our initial hypothesis that the luteinization process involves matrix deposition. The two methods used, immunohistochemistry and Western blotting, gave consistent results.

The observation that hCG-treated cells did not show enhanced deposition of laminin compared to the control cells may be explained by the fact that the control cells had already been exposed to hCG for 34 hours in vivo. Although the freshly prepared granulosa cells are still relatively small at this stage, the effectiveness of the in vivo hCG stimulus may still be operative during the first stages of in vitro culture and, indeed, this may lead to the temporary rise in progesterone production seen in our experiments under control conditions. In order to clarify the effect of gonadotrophin matrix deposition, further work on 'natural-cycle' pre-ovulatory granulosa cells may be necessary.

Further investigation with Western blotting allowed the identification of the presence of the three laminin subunits: α2, β1, γ1 as constituents of the luteinized granulosa cells' extracellular matrix. It is therefore possible that laminin-1 (present in Matrigel) which has been used in the past as the

substratum for granulosa cell culture does not constitute the ideal extracellular matrix for culture. The observed presence of the α2 subunit in the formation of the laminin molecule associated with the granulosa cells is of interest. The pathogenesis of several muscular dystrophies has been closely related with the defective formation of the α2-laminin subunit. In this group of patients, it would be informative to examine the implications of the defective formation of the α2-laminin for corpus luteum functionality and for women's fertility in general.

The presence of the three laminin subunits identified in this study cannot exclude the possibility that other subunits may be involved in the extracellular matrix formation of the luteinizing granulosa cells. Larger studies may be necessary in order to eliminate such a possibility.

8.3.4 Other extracellular matrix components

It is accepted that other components, including fibronectin and entactin, contribute to basement membrane formation (see Chapter 1). Investigations on the role of these other components could represent further avenues for research.

Taken together, our results (particularly those comparing freshly prepared and cultured cells) substantiate the hypothesis that extracellular matrix is deposited around granulosa cells as they luteinise in culture. The patchy location of the collagen IV component, which contrasted with the more peri-cellular disposition of the HSPG and laminin, may suggest that we are not seeing the co-ordinated deposition of a complete basement membrane structure in vitro. More work would be required to clarify the comparative disposition of the various matrix components.

8.4 Control of synthesis and degradation of extracellular matrix

8.4.1 Synthesis of matrix.

It is well established that LH plays a dominant role in the control of luteinization of granulosa cells in vivo (see Chapter 1). As hCG acts on the same receptor population as LH, it would be expected that addition of hCG to pre-ovulatory cultured granulosa cells in vitro would induce luteinization and associated matrix deposition. However in our experiments such stimulation by hCG of extracellular matrix deposition was not clearly manifested. Similar amounts of extracellular matrix were detected in both the control and hCG-treated cells. This could be explained by the fact that the granulosa cells had already been exposed to hCG for 34 hours in vivo, thus initiating the luteinization process. This process was probably continued to some degree during the initial phase of culture of the non-hCG treated cells resulting in extracellular matrix deposition. In order to demonstrate clearly whether gonadotrophin stimulates matrix deposition in granulosa cells it may be necessary to carry out experiments on cells obtained from follicles prior to their exposure to ovulatory stimulation with hCG.

Apart from the gonadotrophins, growth factors-mainly bFGF and EGF- have also been suggested to influence the luteinization process of granulosa cells in a variety of functions including progesterone production and suppression of apoptosis (see Chapter 6). In particular, the work of Rodgers et al. (1996) on bovine pre-ovulatory granulosa cells suggested that bFGF may influence matrix deposition.

It was therefore important for us to examine the effect of growth factors on matrix deposition in relation to the luteinization process of human granulosa cells. Our observations on the effects of growth factors were not conclusive regarding deposition of several laminin subunits. It would be useful to carry out work on other matrix components during luteinization in pre-ovulatory

granulosa cells to examine further the effect of growth factors in this process.

8.4.2 Degradation of matrix

It is generally recognised that extracellular matrix degradation and remodelling depends upon the ratio of MMPs and their inhibitors, TIMPs (reviewed by McIntush and Smith, 1998 and discussed in Chapter 1). A ratio in favour of MMP activity results in extracellular matrix degradation, whereas a ratio favouring TIMPs inhibits extracellular matrix degradation and is permissive for its deposition.

It has been shown that both MMPs and TIMPs are present within cultures of human granulosa cells. Studies on a previously used culture system involving exogenous extracellular matrix in this laboratory have shown that MMP-9, MMP-2 and TIMP-1 are produced by cultures of human granulosa cells (Stamouli et al., 1996; O'Sullivan et al., 1997). Clearly, changing ratios of MMP to TIMP activity in the cultures under various conditions are a possibility and these changes may lead to alterations in the extracellular matrix.

It was an important step forward to establish that our new culture system for granulosa cells enabled the production of MMP-2 and MMP-9 (see Chapter 7). Moreover, the suppressive effect of hCG on MMP production (previously noted by Stamouli et al., 1996) was also apparent and, indeed, was shown very clearly for MMP-2 in the new culture system. The fact that a similar suppression of MMP-2 activity by hCG has been shown for the corpus luteum in vivo (Duncan et al., 1998), confirms that the system developed may represent a good in vitro model for studying the mechanisms involved in luteal 'rescue'. Certainly, the clear suppression of MMP-2 activity by hCG both in vivo and in our model system is remarkable and may enable the in vivo mechanisms involved to be investigated in the laboratory.

A further advance was achieved in confirming that the 'lift-off' phenomenon, previously noted in our laboratory using a different culture method involving exogenous extracellular matrix, applied to the new culture model. Thus it was shown that cells cultured without gonadotrophin eventually were released from the culture surface and that this could progress quite rapidly once underway. The work performed previously by Aston et al. (1996a) suggested that the 'lift-off' phenomenon may have been associated with a change in the ratio of MMP to TIMP activity. It is possible, therefore, that the increase in MMP-2 activity which occurs in the absence of gonadotrophin may be related to the 'lift-off' phenomenon.

Our model system may thus provide an excellent opportunity to investigate the complex changes that occur in culture as a result of a 'switch' in the MMP to TIMP ratio favouring MMP action. These complex changes could involve increased degradation of matrix by MMP-2 and this could be instrumental in causing release of clusters from the culture surface. The methods of analysis for extracellular matrix components evolved during the course of the present study could now be applied to investigations focusing on the potential changes in endogenous matrix when the 'lift-off' phenomenon occurs. This work may shed light on changes in extracellular matrix associated with luteal regression in vivo.

8.5 Future work

1. More work needs to be carried out to establish which cells in the granulosa cell preparations are producing the metalloproteinases identified. Duncan et al. (1998) have shown that in the corpus luteum, it appears to be only a minority of cells in the granulosa-derived layer that is producing MMP-9. It is possible that these cells are macrophages or other white cells and that these may be present in our cultures. Also, the MMP-2 present in the cultures may be derived from contaminating theca cells. By using

immunohistochemistry for MMPs in conjunction with detection of white cell or theca cell markers, the exact cellular sites of production of the metalloproteinases could be established.

2. Although a start has been made in the identification of matrix components that are associated with luteinising granulosa cells, more work needs to be done on components other than those examined in this thesis. As antibodies become available other laminin subunits could be examined. Also fibronectin and entactin in association with the granulosa cells could be studied.

3. There is a strong probability that the HSPG on the surface of granulosa cells could act as an important reservoir for a range of bioactive molecules. As well as the growth factors (such as FGF and the longer forms of VEGF) it is possible that certain IGF-binding proteins are also bound by the HSPG (see Hwa et al., 1999). The role of HSPG in the binding of these various molecules would be a subject of further study.

4. It is well accepted that the quality of granulosa cells could vary widely between patients. For example, the extent of apoptosis in granulosa cells varies between patients and this is related to success in IVF cycles (Oosterhuis et al., 1998). Whether the matrix associated with granulosa cells, or their ability to produce matrix, varies between patients is unknown. If such variation does occur, the ability of the granulosa cells to luteinize and to form a corpus luteum which is 'rescuable' by hCG, could be affected. In other words, poor matrix formation at luteinization may lead to a problem with luteal rescue and therefore difficulty with establishment of early pregnancy. Future work could begin to look at this aspect examining variation in the quality of matrix production between patients and correlating this with clinical indicators such as pregnancy outcome.

5. The functional significance to the granulosa cell of the various extracellular matrix components could be studied. Specific removal of particular components could be carried out and effects on morphology and steroidogenesis monitored. For example, enzymes specific for the degradation of HSPG and collagen IV are available and could be used in these studies.

6. Work could be extended to examine matrix deposition by human pre-ovulatory granulosa cells unexposed to either mid-cycle LH or exogenous gonadotrophin, overcoming problems in the present study concerning prior exposure of granulosa cells to hCG in vivo.

8.6 Concluding remark

The thesis has probably broken some new ground on the role of extracellular matrix in the life of the post-ovulatory granulosa cell. Avenues for further studies have arisen from this research and should be undertaken in order to enlarge our knowledge on this enigmatic area. Correlations of cellular variations in in vitro experiments with clinical manifestations in vivo, regarding human fertility, might constitute the future challenge for research in this area.

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