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Extracellular matrix of the human cyclic corpus luteum

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Extracellular matrix regulates many cellular processes likely to be important for development and regression of corpora lutea. Therefore, we identified the types and components of the extracellular matrix of the human corpus luteum at different stages of the menstrual cycle. Two different types of extracellular matrix were identified by electron microscopy; subendothelial basal laminas and an interstitial matrix located as aggregates at irregular intervals between the non-vascular cells. No basal laminas were associated with luteal cells. At all stages, collagen type IV α1 and laminins α5, β2 and γ1 were localized by immunohistochemistry to subendothelial basal laminas, and collagen type IV α1 and laminins α2, α5, β1 and β2 localized in the interstitial matrix. Laminin α4 and β1 chains occurred in the subendothelial basal lamina from mid-luteal stage to regression; at earlier stages, a punctate pattern of staining was observed. Therefore, human luteal subendothelial basal laminas potentially contain laminin 11 during early luteal development and, additionally, laminins 8, 9 and 10 at the mid-luteal phase. Laminin α1 and α3 chains were not detected in corpora lutea. Versican localized to the connective tissue extremities of the corpus luteum. Thus, during the formation of the human corpus luteum, remodelling of extracellular matrix does not result in basal laminas as present in the adrenal cortex or ovarian follicle. Instead, novel aggregates of interstitial matrix of collagen and laminin are deposited within the luteal parenchyma, and it remains to be seen whether this matrix is important for maintaining the luteal cell phenotype.

Key words: collagen type IV/corpus luteum/extracellular matrix/laminin/versican

Introduction

The temporary endocrine units within the adult ovary—follicles and corpora lutea—continually develop and regress, thus giving rise to the day-to-day variation in hormone secretion by the ovary. Their formation and regression require considerable tissue remodelling, cellular replication, specialization and death. Much effort has been expended in understanding the hormonal and growth factor regulation of these tissue and cellular events. In stark contrast, very little research has been conducted in the ovary on the roles of extracellular matrix (see reviews, Luck, 1994; Rodgers *et al*., 1998b, 1999, 2000, 2003), which also plays an important role in tissue remodelling in other organs.

Extracellular matrix has many different roles (Hay, 1991) including effects on cell adhesion, cell shape, migration, division, differentiation and cell death. All these cellular processes occur during development and regression of follicles and corpora lutea. Basal laminas are specialized sheets of extracellular matrix, which underlie the epithelial and endothelial cells and separate them from adjoining stroma (see reviews, Paulsson, 1992; Timpl and Brown, 1996; Miner and Yurchenco, 2004). They can also envelop individual cells such as muscle, nerve and fat cells and hence partition cells or groups of cells from the surrounding tissue. Basal laminas have been shown to influence epithelial cell proliferation and differentiation and orientate their polarity (Klein *et al*., 1988; Ekblom, 1989), and they can selectively retard the passage of soluble molecules. The basic structure of basal laminas, a collagen type IV lattice that is interconnected with a second network, is composed of laminins by the heparan sulphate proteoglycans and nidogens. Importantly, basal laminas in tissues differ in their ratios of these four major basal lamina components. Furthermore, each such 'component' often represents a family of different isoforms. For example, each molecule of collagen type IV comprises three α chains. However, there are six different chains of collagen type IV α (α1–α6, each encoded by a separate gene) (Hay, 1991). Because only three of

these α chains are required to make one collagen molecule, very many potential combinations of collagen type IV exist (e.g. α1α1α2 and α3α4α5), and each can be regarded as unique (Sado *et al*., 1995). Similarly, each laminin molecule is composed of three chains: one α, one β and one γ chain. There are five different α chains, three β chains and three γ chains (all encoded by separate genes and some with alternative splicing), giving rise to up to 15 identified isoforms (Miner and Yurchenco, 2004; Yurchenco *et al*., 2004). Thus, much complexity in the composition of basal laminas can be generated just by the different types of laminin and collagen type IV. It is now recognized that the unique composition of each basal lamina contributes to its specific functional properties.

In the ovary, the study of extracellular matrix has focused predominantly on follicles. Different types of extracellular matrix and different classes of molecules have been identified (reviewed Luck, 1994; Rodgers *et al*., 1998b, 1999, 2000, 2003). The follicular basal lamina, which underlies the membrana granulosa, changes considerably during follicle growth. In bovine, it commences at the primordial stage with expression of all the six collagen type IV α genes, then during growth, it loses the collagen type IV α1–4 chains, acquires nidogen 1 and perlecan and increases the expression of laminins α1, β2 and γ1, the components of the laminin 3 isoform. Upon approaching a preovulatory, size focimatrix develops within the membrana granulosa. Focimatrix is a novel, recently described, extracellular matrix appearing as aggregates of basal lamina-like material between granulosa cells and composed of the same components as the follicular basal lamina at the time it is produced (Irving-Rodgers *et al*., 2004). The other follicular layers also contain extracellular matrix. In the theca layers, the vasculature (endothelial and vascular smooth muscle) has its own associated basal laminas, and there is a generalized matrix in the theca interna referred to as the 'thecal matrix' (Rodgers *et al*., 1998a) composed of collagen type IV α1 and α2 and a laminin isoform containing the laminin γ1 chain. The origins or roles of the thecal matrix, if any, are not understood. In addition, the specialized extracellular matrix of the cumulus–oocyte complex is complex and dynamic.

At ovulation, the follicular basal lamina is degraded, and the epithelial nature of the membrana granulosa is lost, as the granulosa cells luteinize into granulosa lutein cells (large luteal cells) by enlarging considerably and increasing their capacity for progesterone synthesis. The process of luteinization is considered to be an epithelial–mesenchymal transition (Rodgers *et al*., 2001; Rodgers and Irving Rodgers, 2002). As the follicular fluid is released at ovulation, the follicle wall folds in on itself, and the inward protrusions from the theca layers are later represented in the mature corpus luteum by septa rich in larger blood vessels and connective tissue. Cells from the theca migrate into the area of the membrana granulosa. These include connective tissue elements and endothelial cells to vascularize the corpus luteum. Cells from the theca develop into a distinct population of small steroidogenic luteal cells. In ruminants, these are considerably interspersed between the granulosa-derived large luteal cells, but in humans, they more or less retain their location at the periphery of the corpus luteum (Adams and Hertig, 1969; O'Shea *et al*., 1989).

In view of the extensive tissue remodelling events that occur in the transition of the follicle into a corpus luteum and during regression of a corpus luteum, changes in the extracellular matrix are to be expected. However, to date, little or no information is available, and only the bovine corpus luteum has been recently examined (Irving-Rodgers *et al*., 2004). Here, we examine the extracellular matrix and its composition in the human corpus luteum during its formation through regression in the menstrual cycle. Note that a new nomenclature of laminins has been proposed (Aumailley *et al*., 2005). We use the traditional terminology here. Laminin 8 is now laminin 411, laminin 9 is now 421, laminin 10 is now laminin 511 and laminin 11 is now laminin 521.

Materials and methods

Tissues

Tissues used for immunolocalization studies were from women undergoing elective surgery at the Department of Gynecology at Sahlgrenska University Hospital, Goteborg, Sweden, for non-ovarian benign gynaecological conditions. All tissue was obtained from volunteers, and informed written consent was obtained in all cases. The study was approved by the ethical committee at Goteborg University, Sweden. Histories of the last three menstrual cycles were obtained, and only women with cycle lengths between 26 and 32 days were included. All women were free of any medication for the 3 months before surgery. At surgery, the corpus luteum was excised from the ovary in total as soon as possible. The morphology of the corpus luteum subsequently confirmed the stage within the ovarian cycle of the corpora lutea. The stages and number of corpora lutea examined in this study were early (0–4 days after ovulation, $n = 3$), mid (5–9 days after ovulation, $n = 5$), late (10–15 days after ovulation, $n = 6$) and regressing (excised during the follicular phase, $n = 1$).

Corpora lutea for light and electron microscopic analysis ($n = 8$) were collected from women undergoing elective hysterectomy and oophorectomy at the Queen Elizabeth Hospital, Adelaide, or the Royal Adelaide Hospital for conditions including fibrosis, dermoid or ovarian cysts and endometriosis. Human placentas for use as a positive control were obtained from women undergoing elective Caesarean section at the Women's and Children's Hospital, Adelaide, South Australia. Signed informed consent was obtained from each patient, and ethical approval was obtained from each of the relevant ethics committee in each hospital.

Light and electron microscopy

Tissues were fixed in 2% glutaraldehyde. Following several rinses with buffer to remove excess fixative, specimens were postfixed in 2% (v/v) aqueous osmium tetroxide for 1 h at 4°C, rinsed three times with distilled water (5 min each) and then dehydrated by successive washes on ice with acetone of increasing concentration to 100%. Following overnight infiltration with epoxy resin at room temperature, specimens were embedded in fresh resin and cured at 60°C overnight. For light microscopic examination, 1-μm thick epoxy sections were stained with 1% aqueous methylene.

Immunohistochemistry

Table I has a brief summary of the antibodies used for immunohistochemistry and the fixation conditions of the tissue for each antibody. Portions of corpora lutea embedded in optimal cutting temperature (OCT) compound (Sakura Finetechnical Co., Tokyo, Japan) were used for localization using an indirect immunofluorescence method. Methods for immunohistochemistry have been reported previously (Irving-Rodgers *et al*., 2002). Tissue sections (10 μm) were cut from each of the frozen ovaries using a CM1800 Leica cryostat (Adeal, Altona North, Victoria, Australia), collected on glass slides treated with 0.01% poly-L-ornithine hydrobromide (catalogue no. P-4638; Sigma Chemical Co., St. Louis, MO, USA) and stored at –20°C until used.

Sections were dried under vacuum for 5 min, followed either by fixation in 100% acetone, 100% ethanol or left unfixed. Following fixation, sections were then rinsed in 3 × 5 min changes of hypertonic phosphate–buffered saline (10 mM sodium/potassium phosphate with 0.274 M NaCl, 5 mM KCl; pH 7.2) before treatment with blocking solution [10% normal donkey serum (catalogue no. D–9663; Sigma Chemical Co.) in antibody diluent containing 0.55 M sodium chloride and 10 mM sodium phosphate (pH 7.1)] for 20 min at room temperature. Incubation with primary antibodies was carried out overnight at room temperature. For localization of molecules individually, the secondary antibodies used were biotin-SP-conjugated AffiniPure donkey anti-mouse immunoglobulin G (IgG) (1:100; catalogue no. 715-066-151), biotin-SP-conjugated AffiniPure donkey anti-rat IgG (1:100; catalogue no. 712-066-153) and biotin-SP-conjugated AffiniPure donkey anti–rabbit IgG (1:200; catalogue no. 711–066–152), followed by Cy3–conjugated streptavidin (1:100; catalogue no. 016–160–084), all from Jackson ImmunoResearch

Table I. Details of primary antibodies used for immunohistochemistry and the conditions of use

Antigen	Primary antibody (code or clone number)[a]	Source (reference)	Antibody dilution	Fixation
Type IV collagen	Rat anti-human purified NC1 domain of α1	Dr Sado (Sado *et al*., 1995)	1:100	100% Acetone
	Rabbit anti-EHS (Cat L-9393)	Sigma Chemical Co.	1:100	100% Ethanol
	Rabbit anti-mouse α1 (317)	Dr Sorokin (Durbeej *et al*., 1996)	1:100	100% Ethanol
	Mouse anti-human α2 (10E1)	Drs Sanzen and Sekiguchi (Sanzen and Sekiguchi, unpublished results)	1:50 ascites	Unfixed
Laminin	Mouse anti-human α3 (LAMININ5 2B10)	Dr Sekiguchi (Fujiwara *et al*., 2001; Hattori *et al*., 2003)	1:100 ascites	Unfixed
	Mouse anti-human α4 (3D12)	Dr Sorokin (Sorokin, unpublished results)	1:100	Unfixed
	Mouse anti-human α5 (5D6)	Dr Sekiguchi (Fujiwara *et al*., 2001; Hattori *et al*., 2003)	1:100 ascites	Unfixed
	Mouse anti-human β1 (clone 4E10)	Gibco BRL (Geberhiwot *et al*., 2000)	1:500 ascites	100% Acetone
	Mouse anti-bovine β2 (C4)	Hybridoma Bank Iowa (Sanes and Chiu, 1983)	1:100 2.9 mg/ml	100% Acetone
	Mouse anti-human γ1 (clone 2E8)	Gibco BRL (Geberhiwot *et al*., 2000)	1:500 ascites	100% Acetone
Versican	Mouse anti-human (12C5) recognizing isoforms V0–V3	Hybridoma Bank Iowa (Sztrolovics *et al*., 2002)	1:100 351 μg/ml	Formalin
von Willibrand factor	Rabbit anti-human (Cat F-3520)	Sigma Chemical Co.	1:50	Unfixed
PECAM-1 (CD31)	Goat anti-human (Cat sc-1505)	Santa Cruz. Santa Cruz, CA, USA.	1:50	100% Acetone

[a]Indicates polyclonal antisera for rabbit and goat. Mouse and rat are monoclonal antibodies.

Laboratories (West Grove, PA, USA). For dual localization with PECAM–1, sections were fixed in 100% acetone, and secondary antibodies used were biotin–SP–conjugated AffiniPure donkey anti–goat IgG (1:100; catalogue no. 712–066–147), followed by fluorescein isothiocyanate (FITC)–conjugated streptavidin (1:100; catalogue no. 016–090–084) in combination with Cy3–conjugated AffiniPure donkey anti–mouse IgG (1:100; catalogue no. 715–166–151) (laminins α5 and β1) or AffiniPure donkey anti–rat IgG (1:100; catalogue no. 712–165–153) (collagen type IV α1). Sections were mounted in mounting medium for fluorescence (catalogue no. S3023; Dako, Carpinteria, CA, USA). Controls without primary antibodies were conducted in each batch of immunostaining. No staining was observed with these controls. The validity of the staining was also supported by many different mouse or rabbit antibodies used in this study, each producing unique staining patterns.

Light and fluorescence microscopy

Sections of bovine ovary stained with methylene blue were examined using an Olympus BX50 microscope and Olympus DP12 digital camera (Olympus Australia, Mount Waverly, Australia). Sections processed for immunofluorescence staining were observed and photographed with an Olympus BX50 microscope with epifluorescence attachment and either Olympus DP12 digital camera or Spot RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Results

At the light microscope level, luteal cells and capillaries were evident, with fewer capillaries occurring in regressing corpora lutea (Figure 1). At the electron microscope level, several previously reported features of human corpora lutea (Lunn *et al*., 2002) were observed. Basal lamina underlying capillaries were observed throughout the corpus luteum (Figure 1), but there was no continuous basal lamina around individual luteal cells or around groups of luteal cells. However, occasionally, areas between luteal cells contained electron-dense extracellular matrix as illustrated in Figure 1D. These accumulations of matrix were irregularly distributed throughout the corpus luteum. The amount, location and frequency of occurrence of this matrix indicate that it is likely to be what we refer to below as interstitial matrix.

By immunostaining for von Willibrand factor, capillaries were clearly visible in corpora lutea at all stages of development (Figure 2). Immunostaining patterns of extracellular matrix molecules similar to that of von Willibrand factor were interpretable as representing the subendothelial basal laminas. In addition, punctate patterns of immunostaining were observed with some antibodies, which were interpreted as the luteal interstitial matrix observed above at the electron microscope level. Positive immunostaining for laminins and collagen type IV occurred in this extracellular matrix and, hence, would be predicted to be ultrastructurally similar to interstitial extracellular matrix illustrated in Figure 1D.

A summary of the staining patterns of laminins is summarized in Table II. Neither laminin α1 (Figure 3A) nor laminin α3 chains (data not shown) were detected in any corpora lutea, although they were readily detected in human placenta (Figure 3B). Laminin γ1 was localized exclusively in association with the vasculature (Figure 3C and D) in all the stages of corpora lutea examined. Laminin α4 was also localized in the subendothelial basal lamina but only in the mid and late stages and in regressing corpora lutea (Figure 4B). In early corpora lutea, punctate immunostaining for laminin α4 was observed throughout the luteal parenchyma (Figure 4A). Immunostaining for laminin β1 was similar to that of laminin α4 at all stages of corpus luteum development, but in addition, laminin β1 was localized to the interstitial matrix at all stages (Figure 4D–F). Laminin α5 (Figure 5A–D) and β2 chains (Figure 5E and F) localized to both the subendothelial basal lamina and the interstitial matrix at all the stages examined. Laminin α2 localized only to the interstitial matrix (Figure 5G and H) and did not change in intensity or distribution throughout development. Collagen type IV α1 was detected in the subendothelial basal lamina and in the interstitial matrix at all the stages of corpus luteum development (Figure 6A–D), with interstitial matrix staining being maximal at the mid and late stages (Figure 6B and C) as compared with the early stage (Figure 6A) and regressing corpora lutea (Figure 6D). Versican was localized to the fibrous extremities of the corpus luteum (Figure 6E and F).

Discussion

We describe here the extracellular matrix composition of the human corpus luteum at different stages of development during the menstrual cycle. In general, two different types of extracellular matrix were identified using electron microscopy: subendothelial basal laminas and an interstitial matrix located as aggregates at irregular intervals between the non-vascular cells. At all stages of the corpus luteum development, collagen type IV α1 and laminins α5, β2 and γ1 were localized to subendothelial basal laminas and collagen type IV α1 and laminins α2, α5, β1 and β2 localized to the interstitial matrix. Laminin α4 and β1 chains occurred in the subendothelial basal lamina only from mid-luteal to regressing stages. Laminin α1 and α3 chains were not detected in corpora lutea. Versican localized to the connective tissue capsule of the corpus luteum.

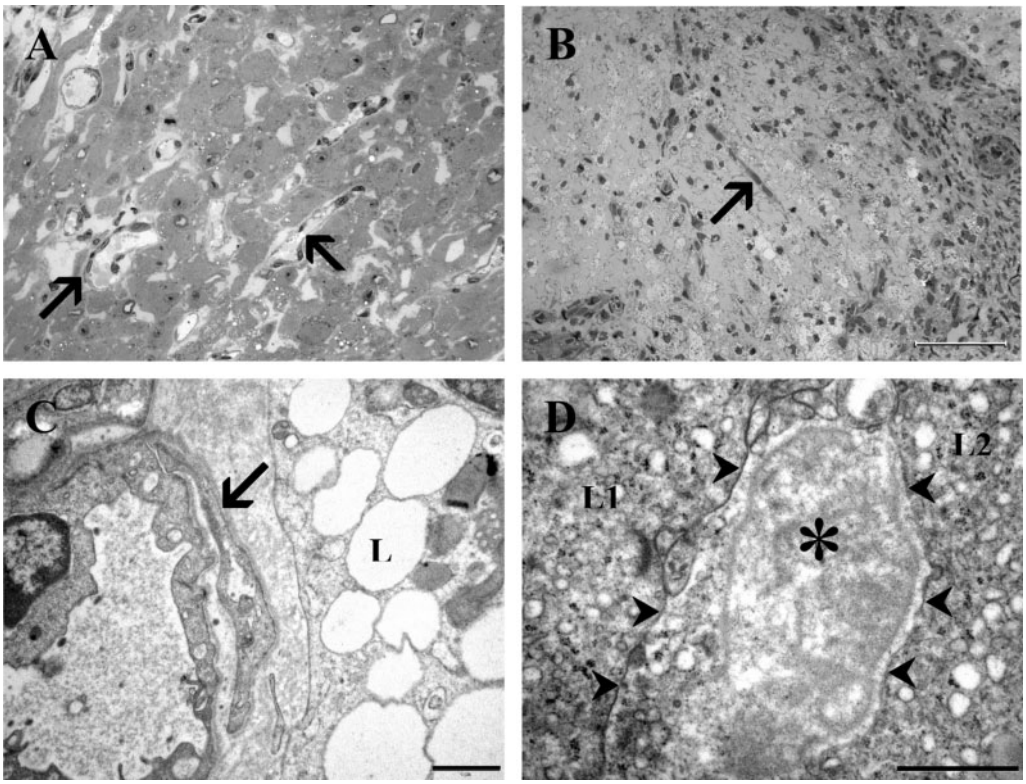

Figure 1. Light (**A** and **B**) and electron (**C** and **D**) micrographs of human corpora lutea. By light microscopy, blood vessels were clearly visible in mid (**A**) and regressing (**B**) corpora lutea (arrows) but less common during regression. (**C**) Endothelial cells of luteal capillaries are surrounded by a basal lamina (arrow). L, luteal cell. (**D**) Interstitial matrix (asterisk) between adjacent luteal cells (L1 and L2). Arrowheads indicate the plasma membranes of adjacent luteal cells. Scale bar = 50 μm (**A** and **B**); Scale bar =1 μm (**C** and **D**).

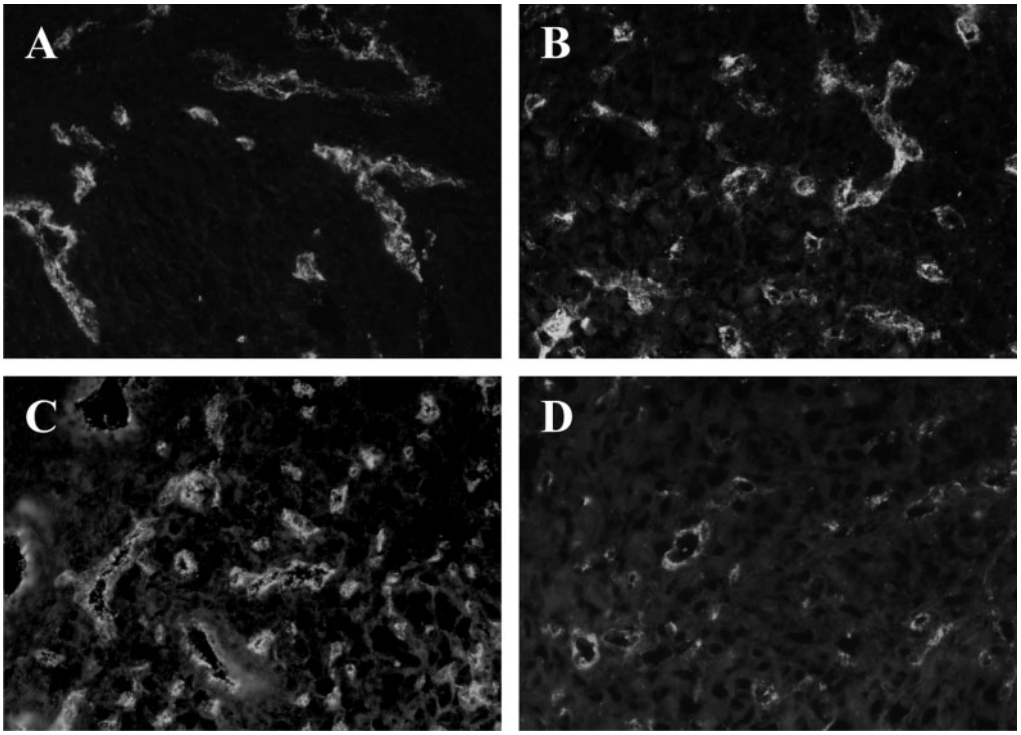

Figure 2. Localization of von Willibrand factor in early (**A**), mid (**B**), late (**C**) and regressing (**D**) human corpora lutea. The capillaries are clearly labelled with this antibody, showing higher density of capillaries in mid and late stages. Scale bar = 20 μm.

Table II. Summary of localization of laminin chains in human corpora lutea at different stages of development

Luteal development stage	Subendothelial basal lamina	Interstitial matrix
Early	α5, β2, γ1	α2, α4, α5, β1, β2
Mid	α4, α5, β1, β2, γ1	α2, α5, β1, β2
Late	α4, α5, β1, β2, γ1	α2, α5, β1, β2
Regressing	α4, α5, β1, β2, γ1	α2, α5, β1, β2

No staining was observed with either laminin α1 or laminin α3.

There has been speculation on the presence or absence of a basal lamina associated with luteal cells (Deane *et al*., 1966; Farin *et al*., 1986; O'Shea, 1987; Kenny *et al*., 1989). In an electron microscopic study of ovine corpus luteum, O'Shea *et al.* (1979) reported that 'Those areas of the surface of small luteal cells which did not make close contact with neighbouring cells ... basal laminae were occasionally seen on such areas'. When referring to large luteal cells, they reported that 'basal lamina formation on surfaces lacking cytoplasmic processes was more marked'. This would indicate that basal laminas do not completely envelope individual luteal cells in sheep. In rats, immunological electron microscopic studies identified 'laminin specifically within perisinusoidal areas (capillaries) and in basement membrane-like plaques between lutein cells' (Leardkamolkarn and Abrahamson, 1992). This suggests that basal laminas do not envelop or completely surround individual luteal cells of rats. Similarly, in bovine, a reticular network of fibres throughout the corpus luteum was observed by staining with antibodies to either laminin γ1 or components of laminin 1 (α1β1γ1; there was no laminin α1, α2, β1, β2 or collagen type IV α1, α2 or α3) (Irving-Rodgers *et al*., 2004). No basal lamina was observed surrounding bovine luteal cells. Light microscopic immunological studies described that laminin around luteal cells of mice (Wordinger *et al*., 1983) and collagen type IV (type undefined) has been localized in the human corpus luteum (Yamada *et al*., 1999). The pattern of immunostaining observed in the human is similar to that observed here for collagen type IV α1, wherein we claim that the staining pattern is due to the presence of collagen type IV α1 in both the subendothelial basal lamina and the interstitial matrix. Our claims are supported by electron microscopic examination and immunostaining of other matrix molecules and location of the endothelial cells by immunolocalizing von Willibrand factor and PECAM-1. Yamada *et al*. (1999) describe collagen type IV being 'highly detected around both large and small luteal cells'. Whilst this is not an inaccurate statement, it fails to convey correctly that there is not a basal lamina surrounding human luteal cells.

The basal lamina components of three steroidogenic tissues have now been comprehensively described: the adrenal cortex (Virtanen *et al*., 2003), the ovarian follicle (Huet *et al*., 1997; van Wezel *et al*., 1998; Rodgers *et al*., 1998a) and now the human corpus luteum. The composition and type of extracellular matrix in these different steroidogenic tissues could be related to the different steroid hormones produced by them. In the human adrenal cortex (Virtanen *et al*., 2003), the glandular basal lamina contains laminin β1 in the zona glomerulosa where aldosterone is produced, while laminin β2 is in the zona fasciculata and zona reticularis where cortisol and androgens are produced, suggesting that the difference in extracellular matrix composition may be related to the pattern of steroidogenic hormone production in the adrenal cortex. In ovarian follicles, as they mature, they first acquire the capacity to produce androgens by their specialized

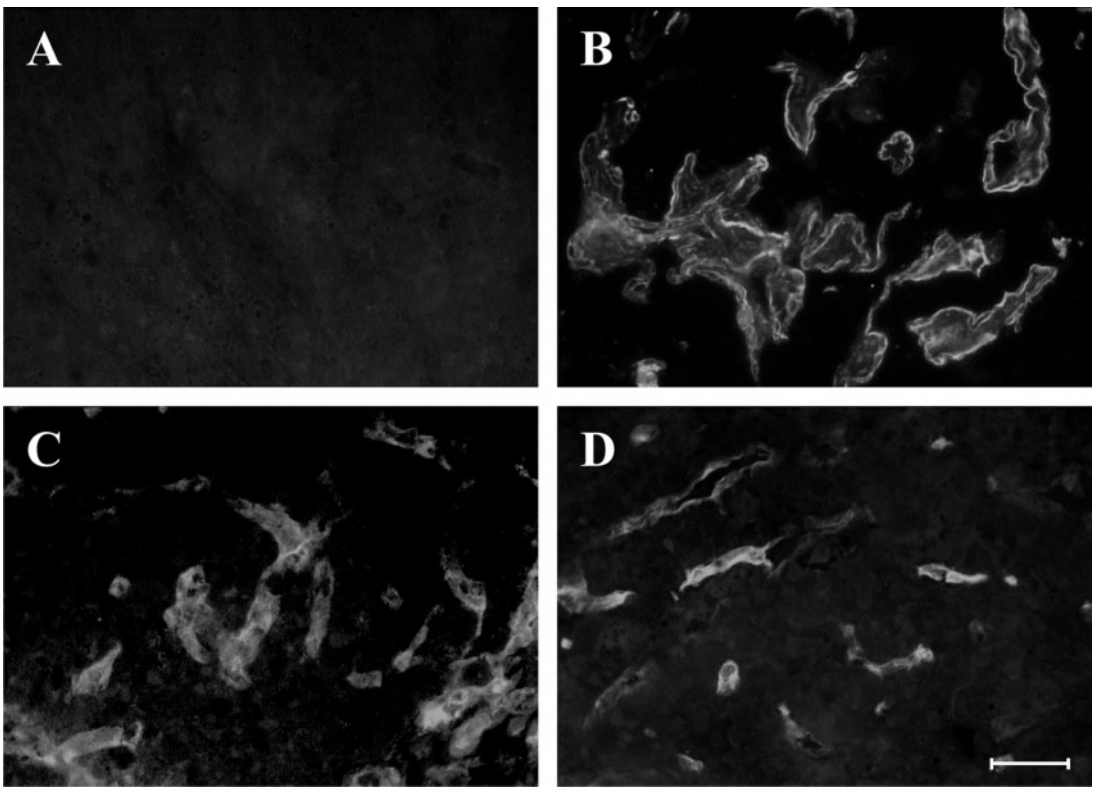

Figure 3. Localization of laminin α1 (**A** and **B**) and laminin γ1 (**C** and **D**) in human corpora lutea (**A**, **C** and **D**) and placenta (**B**). No staining of laminin α1 is present in corpora lutea (**A**, mid stage), even though it was readily detected with the same antibody in human placenta (**B**, positive control). Laminin γ1 was localized exclusively in association with the vasculature (arrows) at all stages of luteal development (**C**, early and **D**, regressing). Scale bar = 20 μm.

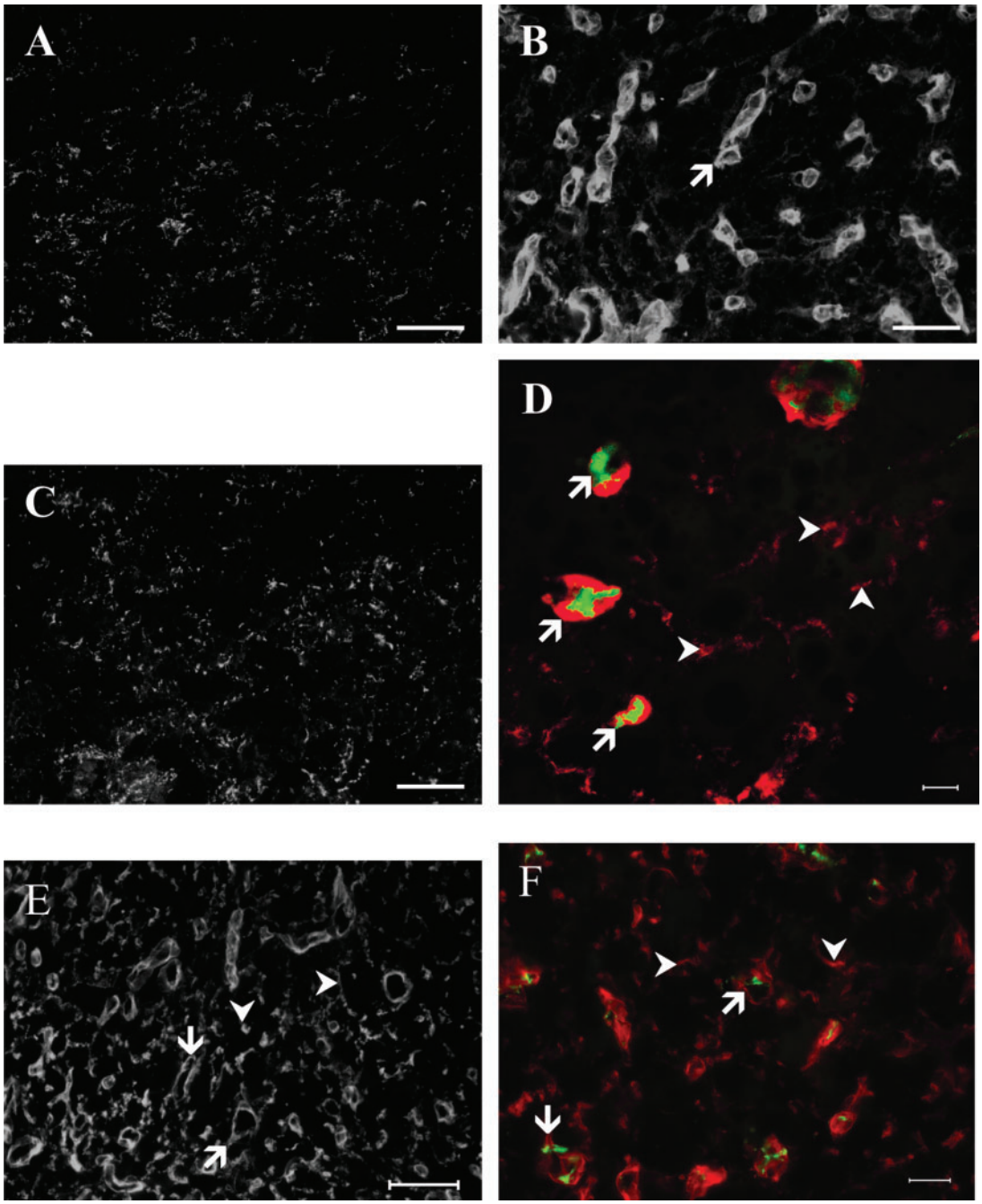

Figure 4. Localization of laminin α4 (**A** and **B**) and laminin β1 (**C**–**F**) in human corpora lutea. (**A**) In early corpora lutea, punctate staining was observed throughout the corpus luteum for laminin α4. However, at the later stages, laminin α4 was clearly localized to the vasculature (**B**, mid stage). Laminin β1 showed a similar staining pattern to laminin α4. In addition, laminin β1 was localized to the interstitial matrix (arrowheads) at all stages of luteal development. (**D** and **F**) Dual localization of laminin β1 (red) and PECAM-1 (CD31) (green). Arrows indicate blood vessels. (**C**) Early stage; (**D**) mid stage; (**E** and **F**) regressing stage. Scale bars = 20 μm.

stromal theca layer. Later, as the follicle enlarges approaching pre-ovulatory size, the granulosa cells acquire the capacity to produce progesterone and estradiol (E_2). However, in the bovine follicle, no changes have been observed in the follicular basal lamina during the transition of these stages, even though changes in composition have been observed much earlier in follicle development (van Wezel *et al*., 1998; Rodgers HF *et al*., 1998; McArthur *et al*., 2000). Instead, in the days leading to ovulation, a novel extracellular matrix composed of basal lamina-like material, called focimatrix and containing laminin α1, β2 and γ1 chains, nidogen 1, perlecan and collagens type IV α1 and α2, is produced (Irving-Rodgers *et al*., 2004) and deposited between the cells of the multilayered membrana granulosa in the bovine. This suggests that the production of focimatrix is related to the acquisition of steroidogenic capacity by the granulosa cells. Upon formation of the corpus luteum, there is a substantial increase in the capacity to produce progesterone, but the bovine corpus luteum has no equivalent of a follicular basal lamina nor focimatrix, instead it has a reticular fibre network. Of the limited number of matrix molecules examined in the bovine, this reticular fibre network in the bovine corpus luteum contains laminin γ1 but not laminins α1, α2,

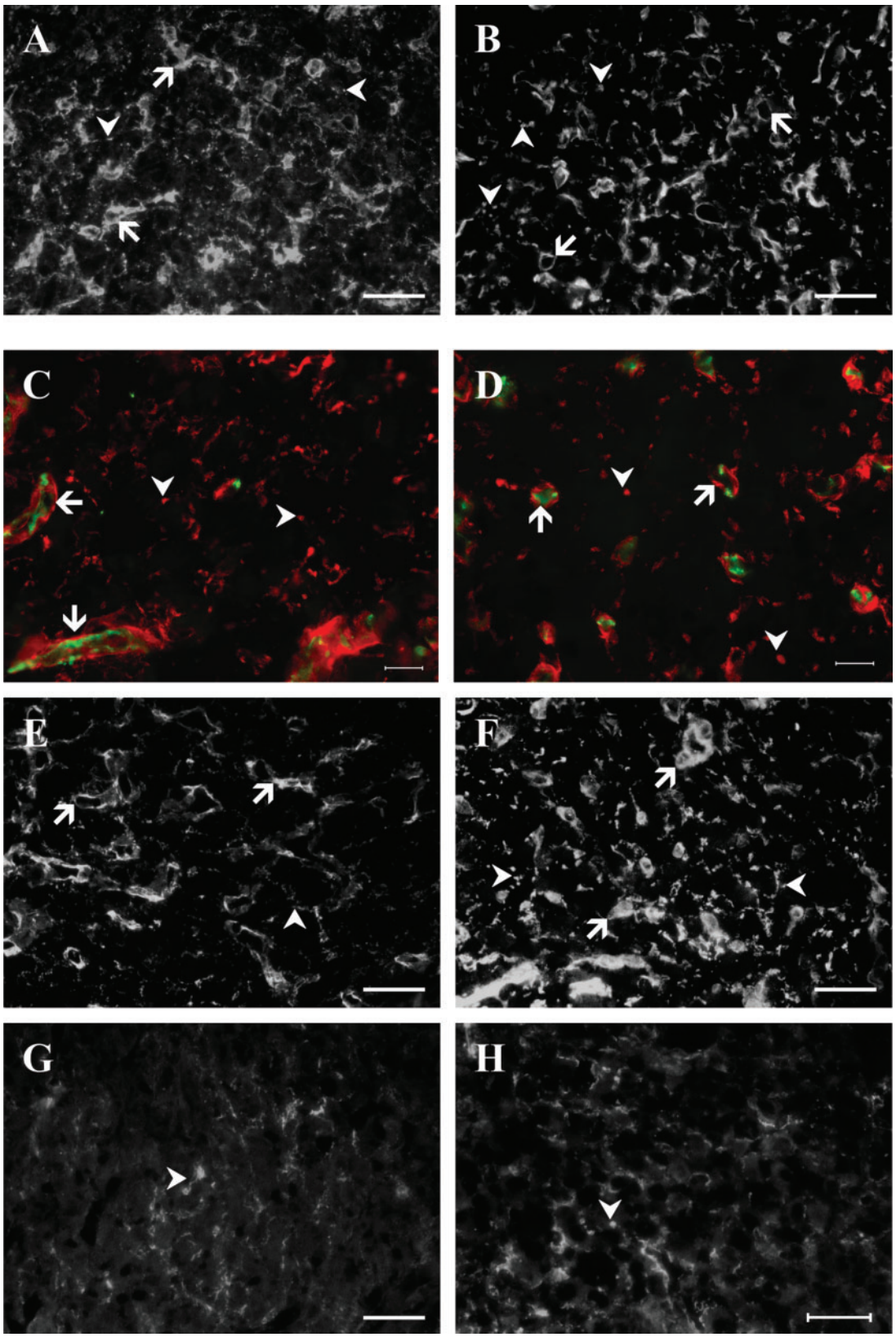

Figure 5. Localization of laminin α5 (**A**–**D**), β2 (**E** and **F**) and α2 (**G** and **H**) in human corpora lutea. Laminins α5 (**A**, early stage; **B** and **C**, mid stage; and **D** regressing) and β2 (**E**, early stage and **F**, regressing stage) localized to both the vasculature (arrows) and interstitial matrix (arrowheads) at all stages examined. (**C** and **D**) Dual localization of laminin α5 (red) and PECAM-1 (green). Laminin α2 localized only to the interstitial matrix (arrowheads) and did not change in intensity throughout development (**G**, early stage and **H**, mid stage). Scale bars = 20 μm.

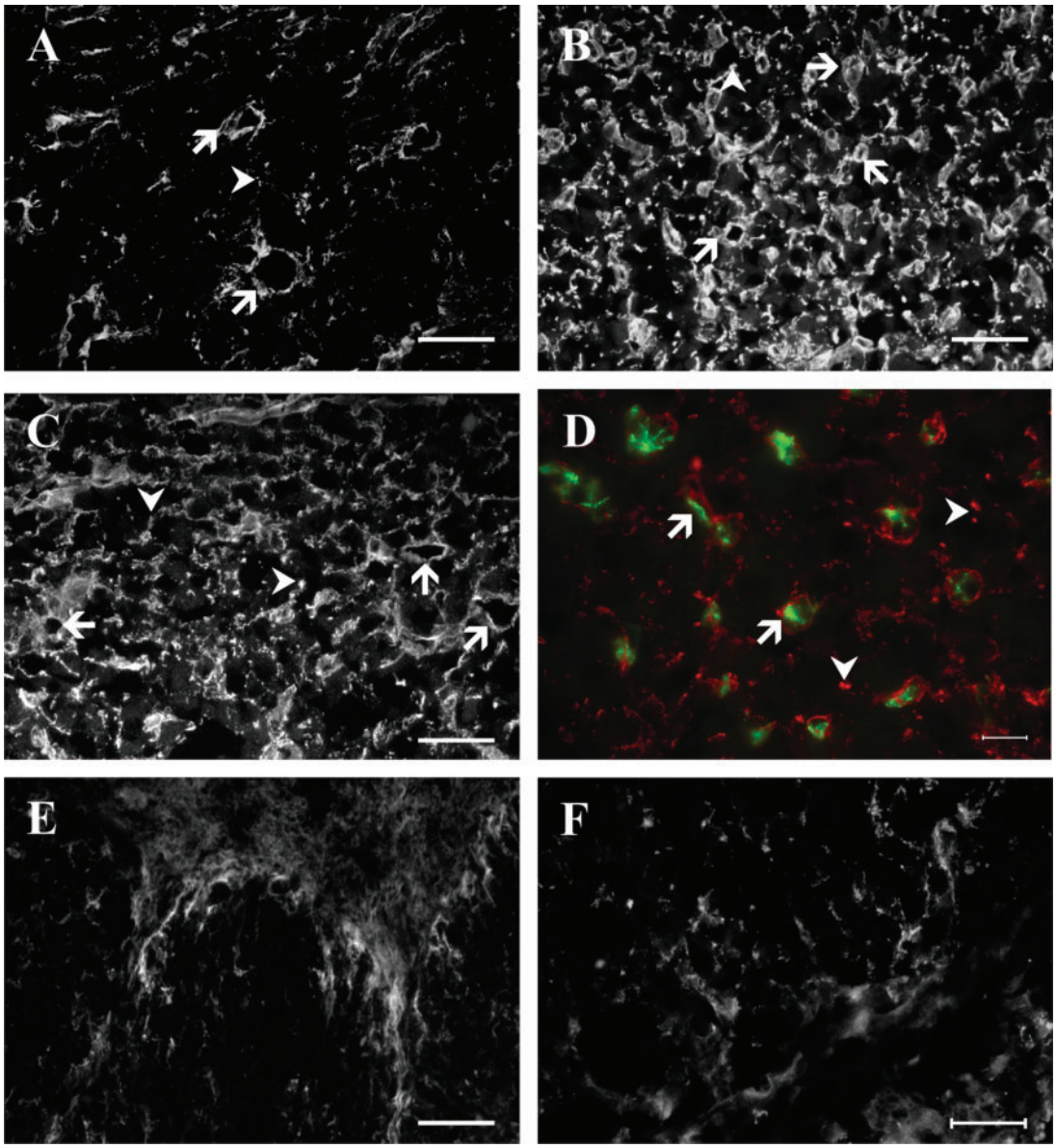

Figure 6. Localization of collagen type IV α1 (**A**–**D**) and versican (**E** and **F**) in human corpora lutea. Collagen type IV α1 was detected in the vasculature and interstitial matrix at all stages (**A**, early; **B**, mid; **C**, late; and **D**, regressing stages), with interstitial matrix staining being maximal at the mid and late stages (**B** and **C**). (**D**) Dual localization of collagen type IV α1 (red) and PECAM-1 (green). Versican was localized to the extremities of the corpus luteum (**E**, mid and **F**, late stages). Scale bars = 20 μm (**A**–**D** and **F**); Scale bar = 10 μm (**E**). Arrows, vasculature; Arrowheads, interstitial matrix.

β1 or β2 or collagen type IV α1, α2 or α3 (Irving-Rodgers *et al*., 2004). The human corpus luteum, which also has a very high capacity to produce progesterone and additionally E_2 (Sasano *et al*., 1989), has an interstitial matrix. It contains laminin α2, α5, β1 and β2 chains. It also contains collagen type IV α1. Thus, we can conclude that the different steroidogenic tissues have a variety of types of extracellular matrices and of differing composition, suggesting that they are not associated with production of any particular type of steroid hormone.

A more likely explanation for the type of matrix in steroidogenic tissues is that the matrices are related to the different cellular origins, formation and final structure of the tissues and glands themselves. The adrenal cortex has a glandular structure composed of nests of steroid-secreting cells surrounded by basal lamina, as indicated by the immunolocalization of laminins (Virtanen *et al*., 2003). Cells in the zona fasciculata and zona reticularis are interconnected by gap junctions (Davis *et al*., 2002). This is somewhat analogous to the ovarian follicle, in which the membrana granulosa cells are also connected by gap junctions (Fukushima, 1977) and surrounded by the follicular basal lamina (Rodgers *et al*., 2003). The corpus luteum is different in structure: the luteal cells are not epithelial but are mesenchymal or parenchymal. As shown here by immunohistochemistry and electron microscopy, there was no basal lamina around either groups or individual luteal cells but rather a diffuse and discontinuous interstitial matrix. In the human, this interstitial matrix appeared as roughly spherical aggregates that were randomly dispersed between but not associated with the vasculature. The extracellular matrix of the bovine corpus luteum has a reticular network of fibres. This difference between the human and bovine corpora lutea could be a reflection of the different level of penetration of the theca-derived luteal cells between the two species. In bovine, it is very extensive with many thecal derived cells interspersed between the granulosa-derived cells

(O'Shea *et al*., 1989), whilst in the human corpus luteum, the degree of penetration is much less with distinct discernable theca- and granulosa-derived luteal cell layers (Adams and Hertig, 1969; Gillim *et al*., 1969; Lunn *et al*., 2002). Thus, the differences between these steroidogenic tissues could reflect the different origins of the cells and the formation and the final structure of the tissues and glands themselves.

The interstitial matrix of the human corpus luteum and the reticular fibre network of the bovine appear to be newly synthesized during the development of the corpus luteum. Upon ovulation, the follicular basal lamina is degraded allowing migration of cells into the membrana granulosa. The newly described focimatrix, at least in bovine, is also degraded (Irving-Rodgers *et al*., 2006), and the present study found maximal levels of the interstitial collagen type IV α1 in the mature corpora lutea. All of these observations suggest that the luteal interstitial matrix is a newly synthesized matrix of the corpus luteum and not residual focimatrix nor follicular basal lamina derived from the ovulating follicle. Additional support comes from *in vitro* culture of human granulosa cells where, over time, there was an increase in the levels of laminins α2, β1 and γ1 (Alexopoulos *et al*., 2000) and collagen type IV (type undefined) (Yamada *et al*., 1999). However, in these cultures, the detection of laminin γ1, which we found only associated with subendothelial basal laminas may signal that these cultures also contain endothelial cells.

Laminin α4 and β1 chains had unusual distributions in developing corpora lutea, both showing a punctate distribution in early corpora lutea and subsequent association with subendothelial basal laminas at mid-luteal stages. This is unlike laminin α2, α5 and γ1 chains, which were associated with the vascular basal laminas from the early stages onwards. Therefore, human luteal subendothelial basal laminas potentially contain laminin 11 (α5β2γ1) early during luteal development and, additionally, laminin 8 (α4β1γ1), laminin 9 (α4β2γ1) and laminin 10 (α5β1γ1) when corpora lutea are fully formed by mid-luteal phase. Laminin α4 null mice are reported to have defects in neovascularization (Thyboll *et al*., 2002). Perhaps the distributional changes in laminin α4 reflect the neovascularization of the corpus luteum in the early stages reaching completion by the mid-luteal phase as observed previously by changes in the cross-sectional area of individual capillaries and their volume density (Suzuki *et al*., 1998; Gaytan *et al*., 1999).

The human follicle has not been extensively studied, but in the bovine follicle (van Wezel *et al*., 1998) and corpus luteum (Irving-Rodgers *et al*., 2004), only laminins α1 and α2 have been localized, but neither was detected in the subendothelial basal laminas. The subendothelial basal lamina in mature human corpora lutea contained laminins β1 and β2 as does the bovine corpus luteum (Irving-Rodgers *et al*., 2004) and the follicle (van Wezel *et al*., 1998), but neither has been described in regard to the vasculature in the adrenal cortex (Virtanen *et al*., 2003).

In ovaries, versican was originally identified in human follicular fluid aspirated from women undergoing oocyte retrieval (Eriksen *et al*., 1999) and in homogenates of bovine follicles (McArthur *et al*., 2000). Splicing produces four isoforms of versican—V0, the full-length transcript; V1, missing the GAGα domain; V2, missing the GAGβ domain and V3, missing both the GAGα and GAGβ domains (Dours-Zimmermann and Zimmermann, 1994). Using an antibody recognizing both the V0 and the V1 forms, versican was localized in both theca interna adjacent to the basal lamina and in the granulosa layers of bovine follicles (McArthur *et al*., 2000) but not in focimatrix (Irving-Rodgers *et al*., 2004). V0, V1 and V3 isoforms have been detected in mouse, rat and bovine ovaries, respectively (Russell *et al*., 2003a; Irving-Rodgers *et al*., 2006), and V2 is regarded as a brain-specific isoform (Schmalfeldt *et al*., 1998). At ovulation in the rat, it was recently suggested that versican produced by granulosa cells of ovulatory follicles is necessary for stabilization of the cumulus–oocyte matrix that develops in response to the LH surge (Russell *et al*., 2003a). Soon after, ADAMTS-1 is up-regulated in response to the LH surge, thus, proteolytically cleaving versican (Russell *et al*., 2003b) and initiating a rapid and continued degradation of extracellular versican as also observed in the bovine. Using an antibody recognizing all four isoforms of versican, versican was observed only at the periphery of the human corpus luteum where connective tissue elements and theca lutein cells are located. Versican isoforms have many different postulated roles, but the role of luteal versican is not known at this stage.

In summary, during the formation of the human corpus luteum, remodelling of extracellular matrix occurs producing aggregates of interstitial matrix but no basal lamina enveloping either individual or groups of luteal cells. The subendothelial basal lamina also changes composition during the formation of the corpus luteum. The structure of the extracellular matrix of the human corpus luteum is very different to that of the adrenal cortex and ovarian follicle. Whilst the extracellular matrix is dynamic in its composition and remodelled from the ovarian follicle, the precise roles played by the changing matrix have still to be identified.

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