The roles of the ovarian extracellular matrix in fertility

RJ Rodgers and HF Irving-Rodgers

Robinson Institute, Research Centre for Reproductive Health, Discipline of Obstetrics and Gynaecology, University of Adelaide, Adelaide, South Australia 5005, Australia

In the mammalian ovary there is considerable and continuous remodelling of tissue during both fetal and adult life, necessitating changes in extracellular matrix. Matrix is a diverse group of molecules varying in its composition and roles, which include regulation of growth factor activity and cell behaviour. Here we discuss four topical aspects of matrices in ovaries. (1) Our current state of knowledge of latent TGFβ binding proteins that can bind the extracellular matrix fibrillins. Fibrillins and latent TGFβ binding proteins may be very important given the genetic linkage data implicating a role for fibrillin 3 in polycystic ovarian syndrome. They will almost certainly be important in the stromal compartments of the ovary by regulating TGFβ bioactivity. (2) Follicles which have an unusual ultrastructural follicular basal lamina and poor quality oocytes. The results suggest that the use of oocytes from these follicles should be avoided in assisted reproductive technologies. (3) Evidence that expression of components of focimatrix correlates with expression of aromatase and cholesterol side-chain cleavage in granulosa cells. Focimatrix is a novel type of basal lamina associated with granulosa cells with expression beginning before deviation and continuing until ovulation. It may be involved in maturation of granulosa cells and selection of the dominant follicle. (4) Evidence is presented in support of a hypothesis that follicular fluid accumulates in follicles due to the osmotic potential of hyaluronan and versican, which are matrices produced by granulosa cells and too large to traverse the follicular antrum. These examples illustrate the diversity of matrix and foreshadow potential important discoveries involving extracellular matrix in ovaries.

Introduction

In the mammalian ovary there is considerable ongoing remodelling of tissue during both fetal and adult life. This includes the formation, activation and growth of follicles which culminates in atresia or ovulation. There is also formation and regression of corpora lutea. These processes are regulated by hormones and growth factors in particular, which have all been well studied. Extracellular matrix also regulates cells and tissues, but its study is relatively limited in the ovary. However it is probably no less important as nearly all cells interact with matrix during development.

Corresponding author E-mail: ray.rodgers@adelaide.edu.au

Extracellular matrix is a very diverse range of molecules that evolved with multicellular organisms and then expanded by gene duplication during the evolution of vertebrates (Huxley-Jones *et al.* 2007). Matrix molecules can be grouped according to which tissue compartment they are located in. The first compartment is that of epithelia, which in the ovary includes surface epithelium and the membrana granulosa, and endothelia as well as some specialized cells such as adipocytes or neurons. Cells in these compartments are surrounded by basal lamina either individually or as groups of cells, providing unique compartments within which these cells reside. In epithelia and endothelia, basal laminas initiate cell polarity, they can control cell proliferation and differentiation and act as barriers to the migration of cells and the passage of molecules across them. They are composed of a lattice-type network of collagen type IV intertwined with a network of laminin, stabilised by the binding of entactin/nidogen-1 or –2 (Schymeinsky *et al.* 2002) to the collagen and laminin. The cells outside these basal laminas are broadly classified as stromal. They contain amongst other cell types fibroblasts and the structural collagens which maintain the integrity of tissues compartments. The non-epithelial or stromal compartments of the ovary include the tunica albuginea, cortical stroma and the specialised theca externa and interna.

Extracellular matrix molecules have many different functions and during evolution matrix also acquired motifs to bind growth factors or their binding proteins. This enabled growth factors to act locally and not as hormones which are broadly dispersed throughout the body upon secretion. We have studied the changing nature of matrix within different compartments of the ovary in several species including bovine, mouse, and human. Here we consider different types of matrix in the bovine ovary and their bearing upon fertility.

Stromal matrix in the ovary

Whilst the theca interna has been well studied the theca externa, tunica albuginea and cortical stroma have received less attention. This is unfortunate as these compartments are altered in human ovaries in the polycystic ovary syndrome (PCOS) which affects up to 5-7% of women in western societies. Women with PCOS often have an increased risk of infertility due to chronic anovulation caused by hyperandrogemia (Balen *et al.* 1995). Up to 20% of women have phenotypically polycystic ovaries, characterized by a multitude of small antral follicles and enlarged theca interna which contributes to the elevated levels of circulating androgens in PCOS women. Interestingly theca cells maintained and passaged in long-term cultures continue to have elevated basal and cAMP-stimulated progesterone, DHEA, 17-hydroxyprogesterone, androstenedione and testosterone production on a per cell basis (Nelson *et al.* 1999), due to elevated expression of steroidogenic enzymes HSD3B and CYP17 (Nelson *et al.* 2001). One conclusion to draw from these studies is that the thecal cells of PCOS ovaries are permanently altered. Another feature of PCOS ovaries is an extremely thickened ovarian capsule or tunica albuginea, and an increased amount of ovarian cortex (Hughesdon 1982). It has been observed that the amount of tunica was increased by 50 per cent in cross sectional area and contained more collagen, while the cortical stromal thickness was increased by one third, and the subcortical stroma, whether deep cortical or medullary, by five fold (Hughesdon 1982). Animal models, including ruminants also exhibit some ovarian features of PCOS (Padmanabhan *et al.* 2006).

It has not been shown if changes to the tunica or or cortical stroma and their collagen content in either women or animal models of PCOS affect their fertility per se, but it is of at least of academic interest to understand their origins and functions. In other normal and fibrotic tissues, Transforming Growth Factor β (TGFβ) stimulates fibroblast function and production and deposition of collagen. During fibrosis of many organs TGFβ activity is also enhanced (Bottinger 2007; Kisseleva & Brenner 2007). TGFβs are part of a superfamily that include activins, bone morphogenic proteins (BMPs), anti-mullerian hormone (AMH or MIS), and growth/differentiation

factors (GDF). Members of all of these are expressed in ovaries (Knight & Glister 2006). GDF9 and BMP15 are expressed by oocytes and AMH and activins are expressed by granulosa cells. Studies on expression TGFβ1, 2 and 3 vary somewhat across species. The specialized stromal theca layers of follicles express or contain TGFβ1 (human and sheep), 2 (human, cow and sheep) or 3 (cow) (Chegini & Flanders 1992; Nilsson *et al.* 2003; Juengel *et al.* 2004). The epithelial granulosa cells at different stages of development as well as stroma can express different TGFβ molecules.

Fibrillins are extracellular matrix glycoproteins. Fibrillins form connective tissue microfibrils associated with elastin fibres or extracellular microfilaments (Ramirez & Pereira 1999; Kielty *et al.* 2002). Studies of fibrillin 1 and 2 show that they have a second role, namely they interact with some of the latent TGFß binding proteins (LTBP) (discussed below). The fibrillins and LTBPs can therefore limit local bioavailability and action of TGFβ in a tissue. Direct evidence of this relationship between fibrillins and TGFβs is seen in Marfan's Syndrome and other related syndromes which can be caused by mutations either in fibrillin 1 or in the TGFβ type 2 receptor. Fibrillin 3 is expressed in brain and was only discovered in 2001 and hence much of its activity or roles can only be inferred from our knowledge of fibrillins 1 and 2.

There are four LTBPs. LTBP 1, 3 and 4 can form covalent disulfide complexes with some of the propeptide TGFßs during processing within cells and these are secreted as large latent TGFß complexes (Fig. 1). LTBP 2 does not form such complexes. LTBP 1 and 3 can bind any of the TGFß 1, 2 or 3 propeptides. LTBP 4 can bind only TGFß1 and then less efficiently than LTBP 1 and 3. Secreted LTBPs with or without associated propeptide TGFßs can associate extracellularly with fibrillins at the N terminals. LTBP 2 can dissociate LTBP 1 from fibrillin 1 (Hirani *et al.* 2007) and so can additionally be involved in the regulation of the TGFß bioactivity.

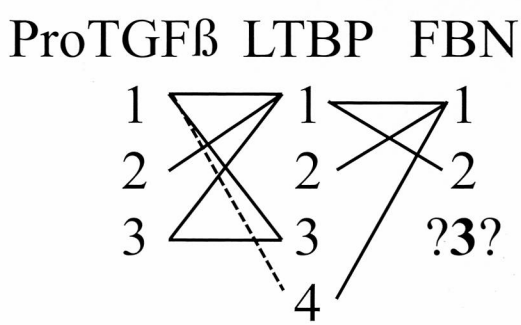

Fig. 1. Interactions between proTGFβ, LTBP and fibrillin (FBN) family members. Lines connect molecules known to interact, and a dashed line indicates weaker association. Associations with fibrillin 3 have not been investigated.

Fibrillin 1, 2 and 3 and LTBP 1 and 2 are expressed in human and bovine (Figs 2 and 3) ovaries (Prodoehl *et al.* 2009a; Prodoehl *et al.* 2009b). However, the expression of fibrillin 3 is very low in both normal and PCOS ovaries and in bovine ovaries. LTBP 3 is also expressed (Penttinen *et al.* 2002) and LTBP 4 has not been examined. The fibillins and LTBPs in bovine ovaries are localized to cells in theca, stroma and tunica albuginea (Prodoehl *et al.* 2009b). The expression patterns appear to be unique with some overlapping co-localization of these proteins while the staining pattern is mostly fibrillar (Figs 2 and 3) (Prodoehl *et al.* 2009b). The expression levels between a number of these genes is highly correlated in ovaries (Prodoehl *et al.* 2009a; Prodoehl *et al.* 2009b), as has been observed in other tissues. Their roles and regulation have not been precisely determined in ovaries but based upon studies in other tissues they are likely to participate in tissue remodelling of stromal compartments during ovarian development and during follicular and luteal development and regression.

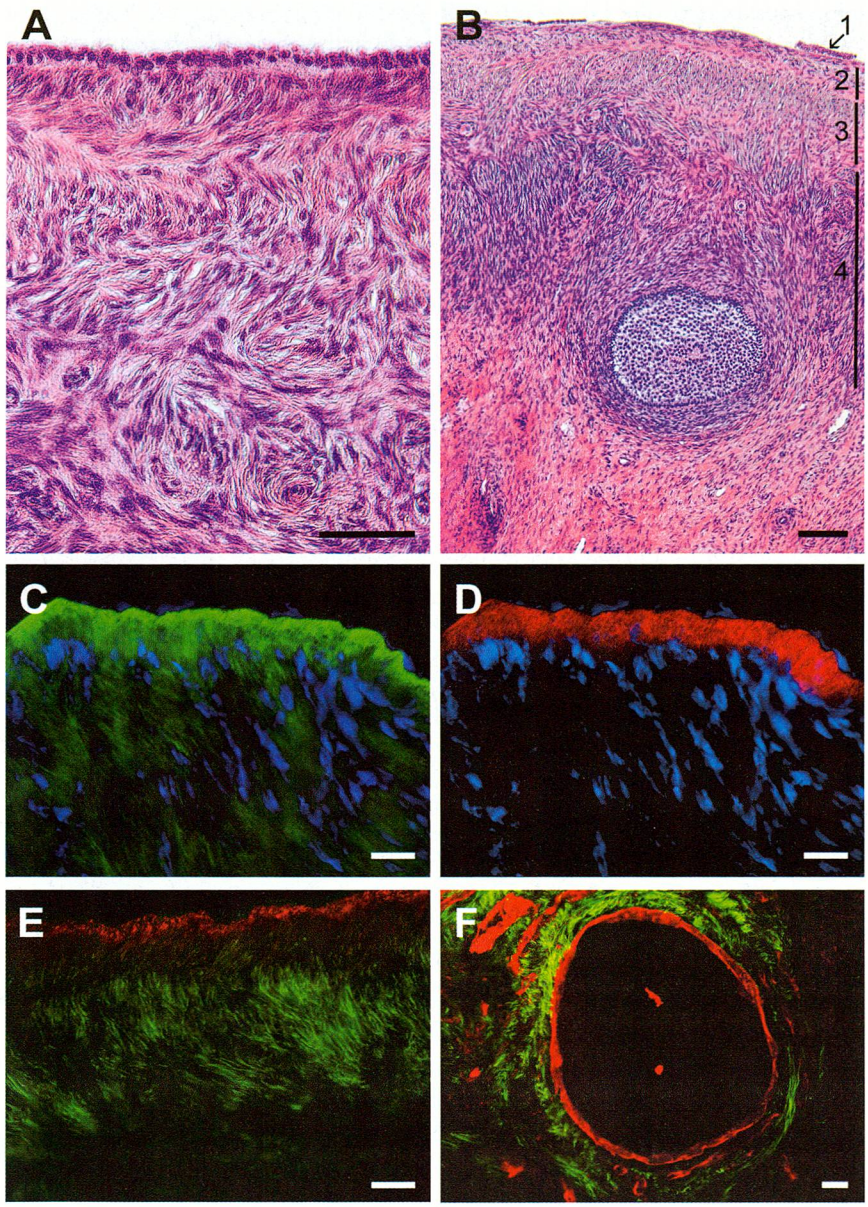

Fig. 2. Localisation of fibrillin 1 and LTBP 1 and 2 in stromal areas of the bovine ovary. Ovarian sections stained with haematoxylin and eosin (A, B) showing surface epithelium (1), the outer (2) and inner (3) regions of the tunica albuginea and the cortical stroma (4). Note the variations in the cellular density and arrangements within the ovary. Fibrillin 1 is most concentrated in the tunica albuginea and less concentrated throughout the cortical stroma (C, green). LTBP2 is present in the tunica albuginea (D and E, red) where it co-localises with fibrillin 1 (C and D are the same section). LTBP 1 is present in the cortical stroma (E, green) and surrounding preantal follicles (F, green; laminin 111 staining in red localizes the follicular basal lamina and sub-endothelial basal laminas). Nuclei are counterstained with DAPI (C, D, blue). Scale bars equal 50 μm (A), 100 μm (B), 20 μm (C, D, F) and 30 μm (E). Parts of this Figure were reprinted with permission from (Prodoehl *et al.* 2009b).

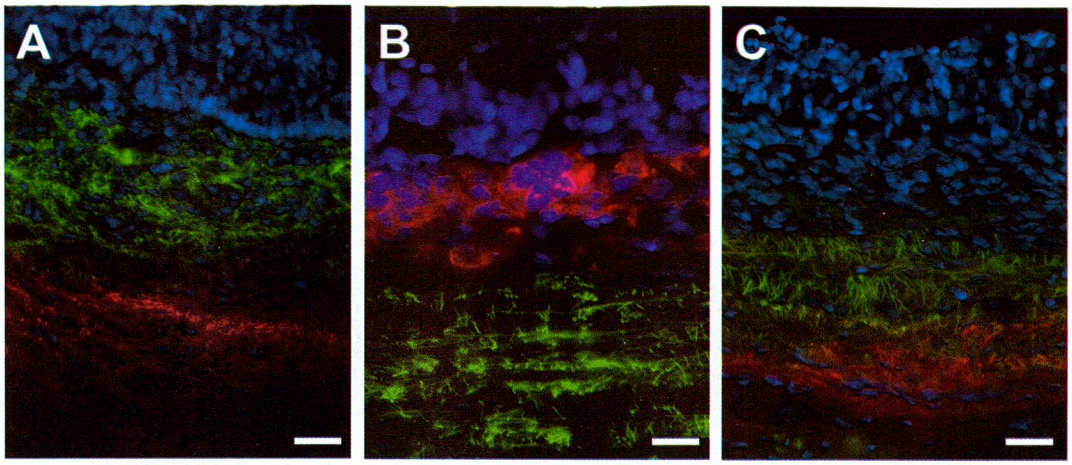

Fig. 3. Localisation of fibrillin 1 and LTBP 1 and 2 in bovine antral follicles. Fibrillin 1 is present in theca interna (A, green). LTBP2 is present in the theca externa (A and C, red). LTBP 1 (B and C, green) present in basal regions of the theca interna and in adjoining theca externa. B, red is CYP17 in theca interna. Nuclei are counterstained with DAPI (blue). Scale bars equal 30 μm (A) and 20 μm (B, C). This Figure was reprinted with permission from (Prodoehl *et al.* 2009b).

Basal laminas and oocyte quality

Generally basal laminas constitute a single layer of lamina densa aligned to the cell surface. However, a number of physiological and pathological conditions lead to different morphological appearances of basal laminas. In patients suffering from diabetes mellitus additional layers of basal lamina are present in kidney, microvasculature, neuronal tissues and the retina (Abrahamson 1986). These expanded multi-layered basal laminas may contribute to the secondary pathology of these organs. In patients with pili annulati, multi layers of basal lamina have been observed in the abnormal hair shafts (Giehl *et al.* 2004). In tadpoles undergoing metamorphosis into frogs, folding of the small intestinal basal lamina has been observed (Murata *et al.* 1994). These changes in morphology of basal laminas presumably reflect or cause changes in basal lamina function.

The follicular basal lamina underlies the membrana granulosa at all stages of development from primordial (van Wezel & Rodgers 1996; Irving-Rodgers *et al.* 2009b), preantral (Irving-Rodgers & Rodgers 2000b; Irving-Rodgers *et al.* 2009b) and antral follicles (Irving-Rodgers & Rodgers 2006) until ovulation when the basal lamina is degraded (Irving-Rodgers *et al.* 2006a). The follicular basal lamina changes in composition during follicular development in bovine (Rodgers *et al.* 2003) and mouse (Irving-Rodgers *et al.* 2010). Extracellular matrix and specifically the follicular basal lamina can influence granulosa cell proliferation and differentiation (Amsterdam *et al.* 1989; Richardson *et al.* 1992; Irving-Rodgers & Rodgers 2006). Additionally, in healthy but not in atretic follicles (Irving-Rodgers *et al.* 2002) the follicular basal lamina excludes capillaries, blood cells and nerve processes from the membrana granulosa.

Different ultrastructural phenotypes of follicular basal lamina have also been observed in follicles in both cattle (Irving-Rodgers & Rodgers 2000a; Irving-Rodgers *et al.* 2002) and humans (Irving-Rodgers *et al.* 2009b). At all follicular stages many follicles have a conventional follicular basal lamina of a single layer which is aligned to the basal surface of the adjacent

granulosa cells. At the preantral stage these conventional basal laminas become substantially thicker or even partially laminated in both human and bovine (Irving-Rodgers & Rodgers 2000a; Irving-Rodgers *et al.* 2009b). On atresia the basal lamina is not completely degraded even if macrophages, endothelial cells, and fibroblasts breach the follicular basal lamina as they migrate from the thecal layer into the degrading membrana granulosa. However, on atresia as cells of the membrana granulosa die and the follicle shrinks the follicular basal lamina becomes concertinaed in appearance (McArthur *et al.* 2000; Irving-Rodgers *et al.* 2002).

In addition to changes in the follicular basal lamina during growth and atresia, substantial differences in the ultrastructure of follicular basal laminas have also been observed in follicles of similar developmental stages in both bovine (Irving-Rodgers & Rodgers 2000b) and human (Irving-Rodgers *et al.* 2009b) ovaries. This phenotype occurs in approximately 50% of bovine preantral and antral follicles up to 4 mm diameter (Irving-Rodgers & Rodgers 2000a). The follicular basal lamina of these follicles has additional layers and are referred to as 'loopy' because they have the appearance of having additional 'loops' of basal lamina when observed in cross section by electron microscopy. Unlike in diabetic patients where the pathological changes in basal laminas occur in many organs of the body, these different follicular basal lamina phenotypes exist only in a proportion of ovarian follicles. In diabetics the follicular basal lamina abnormalities are proposed to be due to advanced glycation end-products (Gardiner *et al.* 2003; Goldin *et al.* 2006) or to the action of reactive oxygen species (Meyer zum Gottesberge & Felix 2005), which are likely to affect all organs in the body. If such mechanisms were the cause of the 'loopy' follicular basal laminas then this must be a localised insult, as only a proportion of follicles have a 'loopy' basal lamina.

We have speculated that follicles with a 'loopy' basal lamina have a slowly expanding antrum, and thus produce excess basal lamina that is subsequently shed (Rodgers *et al.* 2001). This implies that when comparing two follicles of the same size the one with the a 'loopy' basal lamina has been growing for longer (Rodgers *et al.* 2001). We subsequently hypothesised that these follicles would contain oocytes of poorer quality, and examined the ability of such oocytes to undergo in vitro maturation, fertilization and development to blastocyst (Irving-Rodgers *et al.* 2009b). Bovine oocytes from follicles (less than 5 mm diameter) with an aligned basal lamina had nearly twice the capacity to develop to blastocyst with a 65% success rate compared to those from a follicle with a 'loopy' basal lamina. How and why such follicles exist is still unknown. It could be that their oocytes are defective in stimulating follicle growth and thus producing the 'loopy' follicular basal lamina. Irrespective of their cause, avoiding these follicles in assisted reproductive technologies should improve success rates.

In these studies we also examined the oocyte quality of atretic follicles. Most of the atretic follicles had a 'loopy' basal lamina which we considered was due to the collapse of the follicle during atresia and therefore was not analogous to the 'loopy' basal laminas of healthy follicles. The small atretic antral follicles had oocytes with a greater developmental capacity than healthy follicles of an equivalent size as has been observed by others previously (Blondin & Sirard 1995; Nicholas *et al.* 2005; Feng *et al.* 2007). Atresia of antral follicles is characterized by death of granulosa cells and it is not until a very advanced stage that the culumus cells (Yang & Rajamahendran 2000) or oocytes (Rajakoski 1960) die, during which time the oocyte could presumably mature, giving them greater capacity to successfully undergo maturation in vitro. Furthermore it has been hypothesised that in cattle atresia mimics preovulatory maturation (Sirard *et al.* 1999). Attempts to utilise this knowledge of the association between atresia and oocyte quality by induction of apoptosis in cumulus cells did not significantly enhance maturation of oocytes (Rubio Pomar *et al.* 2004).

Focimatrix, steroidogenesis and selection of the dominant follicle.

In cows there are two or three waves of development of large antral follicles during an oestrous cycle when a dominant follicle continues to grow as the smaller subordinate follicles in the cohort subsequently regress (Fortune *et al.* 1991; Ginther *et al.* 1996). Dominant follicle development culminates in ovulation, or if the follicular wave is in the luteal phase, atresia ensues. By comparing follicles that are smaller or larger than at which deviation in the growth rate of individual follicles occurs, it has been shown that the expression in granulosa cells of FSH receptor is static or declining, whilst expression of the LH receptor, CYP11A1 (cholesterol side-chain cleavage), 3ß-hydroxysteroid dehydrogenase and CYP19A1 (aromatase) increases [reviewed in (Knight & Glister 2003; Beg & Ginther 2006)]. In follicular fluid there is an increase in oestradiol and progesterone [reviewed in (Knight & Glister 2003; Beg & Ginther 2006)] and free IGF-I and decreased IGFBP-4 and -5 (Mihm *et al.* 2000; Rivera & Fortune 2003; Beg & Ginther 2006) in dominant compared to subordinate follicles.

More recently it has been shown in bovine follicles that a specialised basal lamina-type matrix, focal intra-epithelial matrix (focimatrix) appears before deviation (> 5 mm diameter) and increases in abundance as follicles enlarge to preovulatory sizes (Irving-Rodgers *et al.* 2004; Irving-Rodgers *et al.* 2006b). Focimatrix is deposited as plaques or aggregates of basal lamina material between the epithelial granulosa cells and exists in cattle (Irving-Rodgers *et al.* 2004), sheep (Huet *et al.* 1997), humans (Yamada *et al.* 1999; Alexopoulos *et al.* 2000) and mice (Nakano *et al.* 2007; Irving-Rodgers *et al.* 2010). This matrix is a novel basal lamina because instead of being a sheet enclosing a cell or a group of cells (epithelia or endothelia) and thus creating compartments within tissues, focimatrix exists as aggregates between cells and therefore cannot perform many known functions of basal lamina. Focimatrix in bovine is composed of basal lamina components type IV collagen α1 (COL4A1) and α2, laminin chains α1, β2 (LAMB2) and γ1, perlecan (HSPG2), and nidogen 1 and 2. Like the follicular basal lamina, focimatrix is degraded at ovulation (Irving-Rodgers *et al.* 2006b) hence it is not found in corpora lutea of humans, cattle or mice (Irving-Rodgers *et al.* 2004; Irving-Rodgers *et al.* 2006c; Nakano *et al.* 2007). Thus focimatrix is developmentally regulated and is present before and after follicles deviate in size, suggesting that it may play a role in follicular development including during the period of follicular dominance.

What initiates or controls dominance of one follicle over the others in a cohort has been difficult to determine. Ideally, biopsies would be taken from individual follicles before deviation and then growth monitored until after deviation thus identifying the future dominant and subordinate follicles. Whilst follicular fluids have been sampled in vivo and follicles are apparently unaltered and continue to grow following this procedure (Ginther *et al.* 1997; Mihm *et al.* 2000), taking a biopsy of the wall is likely to ablate the follicle. Therefore most studies have compared dominant and subordinate follicles after deviation, or identified changes that occur during follicle growth at the sizes when follicles deviate.

To investigate if focimatrix is involved in the selection and dominance of ovarian follicles and/or in the maturation of granulosa cells, we quantified focimatrix components in the membrana granulosa and examined the expression of markers of granulosa cells (Irving-Rodgers *et al.* 2009a). Many changes were observed as predicted, due to the size differences between dominant and subordinate follicles. However after statistically removing the effect of follicle size, the volume density of focimatrix components (immunostaining with antibodies to laminin 111) was still significantly elevated in dominant follicles as was the expression of *CYP11A1*. *CYP11A1* mRNA levels were very highly correlated with the genes for focimatrix components and we suggested that focimatrix and *CYP11A1* have a role in follicles gaining dominance (Irving-Rodgers *et al.* 2009a).

To investigate these hypotheses further we examined bovine follicles of equal size before deviation and measured expression of a number of genes as well as the steroid hormone concentrations in follicular fluids (Matti *et al.* 2010). We then sorted the follicles within each animal into two groups for each of three parameters hypothesised to be important in follicular dominance – follicular fluid oestradiol, and TGFß1 (*TGFB1*) and *CYP11A1* expression. One group consisted of follicles with the highest oestradiol concentration or the highest *CYP11A1* expression, which are up regulated during growth, or the lowest *TGFB1*, which is conversely down regulated, and for each parameter the group was compared with a group formed from the remaining follicles. For example, considering the oestradiol groups, for the 14 animals the high group had 14 follicles by taking the follicle with the highest oestradiol level from each animal, and the other group was composed of all the other follicles (n = 21). Similarly for the *CYP11A1* and *TGFB1* comparisons. Before emergence of a dominant follicle, sorting follicles either on *TGFB1* (Fig. 4) or oestradiol levels did not identify a pattern of gene regulation (Matti *et al.* 2010) that would indicate that these parameters were involved in dominance. However, by sorting follicles into two groups on the basis of the levels of *CYP11A1* (Fig. 5) it was clear that one of the follicles in the wave had significantly elevated levels of *CYP11A1, CYP19A1, COL4A1, LAMB2* and *HSPG2* in granulosa cells (Fig. 5) suggesting that the up-regulation of focimatrix and steroidogenic enzymes could be involved in a follicle attaining dominance (Matti *et al.* 2010). Since focimatrix expression precedes deviation and the up-regulation of *CYP11A1* and *CYP19A1*, our current working hypothesis is that focimatrix induces the expression of these steroidogenic enzymes.

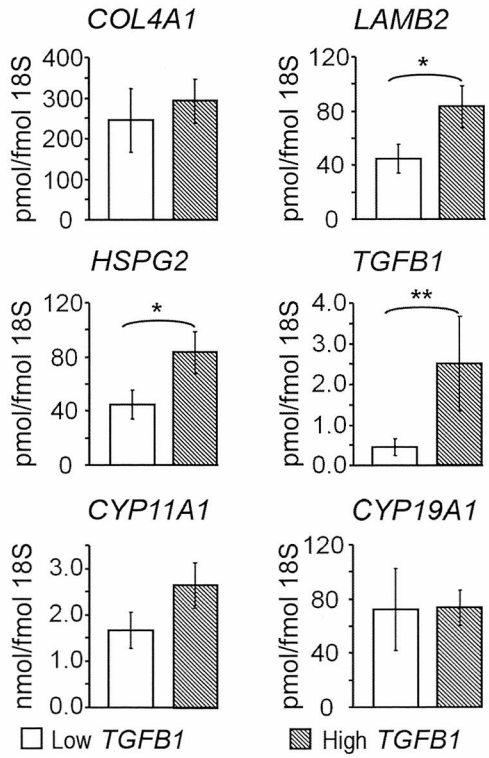

Fig. 4. Expression levels in granulosa cells of predeviated follicles sorted into lowest and higher levels of *TGFB1*. Mean ± SEM levels of all measured parameters when follicles were sorted into groups with the lowest (n = 14) and higher levels (n = 21) of *TFGB1*. $*P < 0.05$; $**P < 0.01$ significantly different between groups. This Figure was modified and reprinted with permission from (Matti *et al.* 2010).

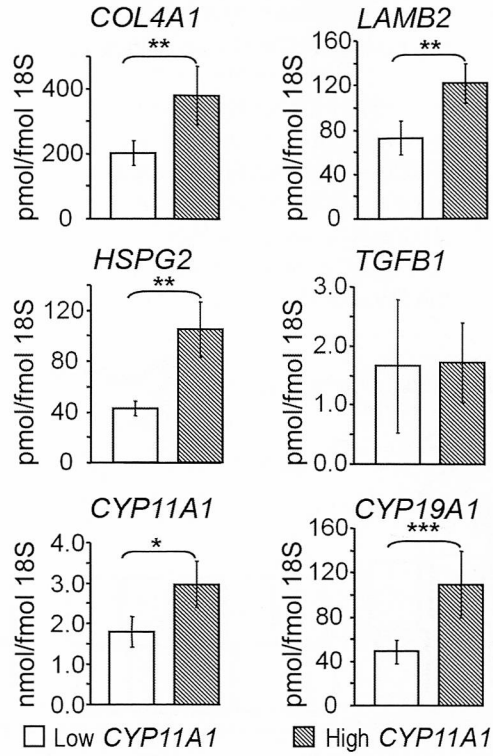

Fig. 5. Expression levels in granulosa cells of predeviated follicles sorted into highest and lower levels of *CYP11A1*. Mean ± SEM levels of all measured parameters when the follicles were sorted into groups with the highest (n = 14) and lower levels (n = 21) of *CYP11A1*. $*P<0.05$; $**P<0.01$; $***P<0.001$ significantly different between groups. This Figure was modified and reprinted with permission from (Matti *et al.* 2010).

Formation of follicular fluid

Formation of follicular fluid and expansion of the follicular antrum are important processes in follicle growth, especially as only large follicles can ovulate. The follicular fluid is probably derived from plasma flowing through thecal capillaries. Capillaries are sparse around primordial follicles (van Wezel & Rodgers 1996; Herrmann & Spanel-Borowski 1998) and generally the stroma surrounding follicles becomes vascularised when the theca and antrum begin to develop. In species with small follicles the thecal capillaries form only a single-layered network, but in species with larger antra the network is multilayered, especially as the follicle increases in size (Yamada *et al.* 1995; Jiang *et al.* 2003). Thus the degree of vascularization of follicles is related to the amount of fluid formed but it is unlikely that vascularisation of theca is a rate limiting step for the formation of follicular fluid since the volume of blood flowing into the thecal capillaries would be considerably greater than the volume needed to fill the antrum. However, there are differences in blood flow or vascularization between dominant and subordinate follicles (Berisha & Schams 2005; Acosta 2007), between follicles with different quality oocytes (Huey *et al.* 1999) and between healthy and atretic follicles (Jiang *et al.* 2003; Clark *et al.* 2004).

Increases in permeability of the thecal capillaries leads to oedema of the thecal tissue, as observed following the LH surge (Espey 1980; Cavender & Murdoch 1988); but transport of

fluid from the theca into the follicular antrum requires it to cross the endothelium and sub-endothelial basal lamina before traversing the thecal interstitium, the follicular basal lamina and the membrana granulosa (Fig. 6). In earlier literature both a sodium pump and cleavage of glycoaminoglycans were considered as mechanisms to raise osmotic pressure in the preovulatory follicle and recruit fluid into the centre of the follicle [reviewed by (Gosden *et al.* 1988)]. However, since granulosa cells lack a network of tight junctions an osmotic gradient across the membrana granulosa could not be established with small molecules like sodium. However, it would be possible to establish one with large molecules as there is a nominal 'blood follicle barrier' operating at sizes above 100 kDa (Shalgi *et al.* 1973; Andersen *et al.* 1976). This barrier probably exists largely at the level of the follicular basal lamina.

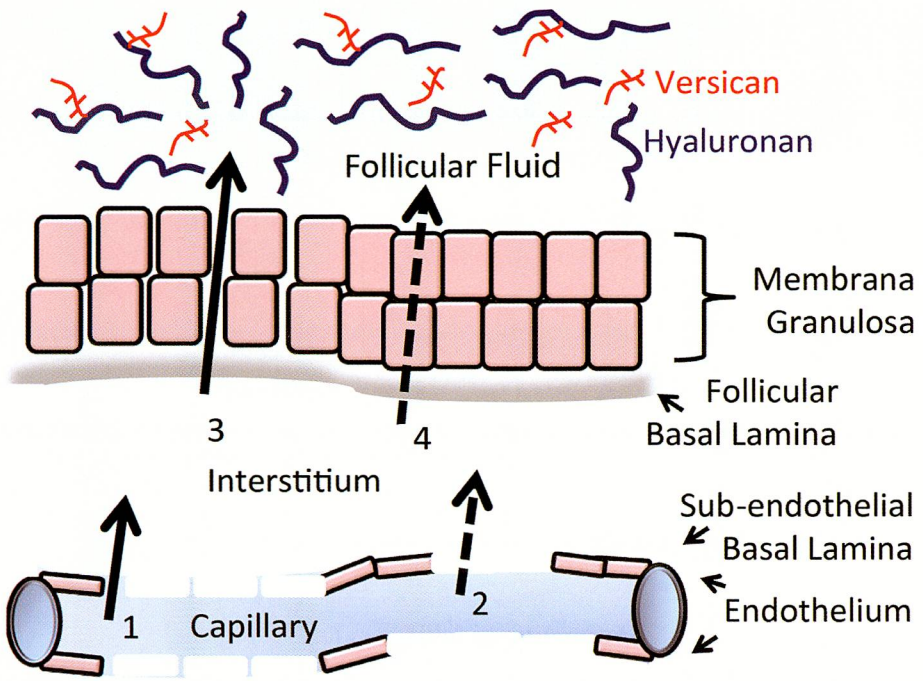

Fig. 6. Drawing illustrating the routes fluid can take from the thecal capillary to the follicular fluid and the potential barriers of the endothelium, sub-endothelial basal lamina, interstitium, follicular basal lamina and membrana granulosa. Routes 1 and 3 show movement of fluid between the cells (solid arrows), and routes 2 and 4 show potential transcellular routes (hatched arrows) which either involve aquaporins or transcytosis. Reprinted with permission from (Rodgers & Irving-Rodgers 2010).

The central hypothesis on how fluid is recruited to the follicular antrum suggests that granulosa cells produce osmotic molecules too large to escape the follicular antrum enabling their osmotic potential to recruit fluid from the thecal vasculature (Fig. 6) (Clarke *et al.* 2006; Rodgers & Irving-Rodgers 2010). In a previous study we found that degradation and removal of the glycosaminoglycans, hyaluronan and chondroitin sulphate/dermatan sulphate, and DNA from follicular fluid resulted in a reduction in osmotic pressure, suggesting that these molecules could contribute to the osmotic potential of the follicular fluid (Clarke *et al.* 2006). The hyaluronan in bovine follicular fluid was found to be up to 2×10^6 in molecular weight (Clarke

et al. 2006), too large to escape from the antral cavity. The chondroitin sulphate proteoglycans identified in our bovine studies were versican (V1 and V0 splice forms) and inter-α-trypsin inhibitor (bikunin with heavy chains 1, 2 and 3), pre-α-trypsin inhibitor (bikunin with heavy chain 3), and inter-α-like trypsin inhibitor (bikunin with heavy chain 2) (Clarke *et al.* 2006). Versican is a large chondriotin sulphate proteoglycan hyalectan expressed in many tissues. It has been shown to be present in extracts of bovine follicles (McArthur *et al.* 2000), follicular fluid of non-ovulating (Clarke *et al.* 2006) and ovulating follicles (Eriksen *et al.* 1999), and in the follicular membrana granulosa (McArthur *et al.* 2000; Irving-Rodgers *et al.* 2004) and is expressed by granulosa cells (Russell *et al.* 2003). Thus versican may directly contribute to the osmotic potential of follicular fluid by virtue of the high sulphation status of chondroitin sulphate side chains. However, versican may also contribute by cross-linking other components like hyaluronan (Zimmermann & Dours-Zimmermann 2008) to form larger molecular weight components, ensuring that both remained trapped in the follicular antrum.

At this stage it is not clear if inter-α-trypsin inhibitor members contribute to the formation of follicular fluid. There are four genes involved in the synthesis of these inter-α-trypsin inhibitor members. The liver expresses these and secretes members of the inter-α-trypsin inhibitor family which are found abundantly in serum. Serum is the source of components of the inter-α-trypsin inhibitor family found in mouse follicular fluid at ovulation (Chen *et al.* 1992). However inter-α-trypsin inhibitor, pre-α-trypsin inhibitor, and inter-α-like trypsin inhibitor exist in bovine (Clarke *et al.* 2006) and porcine (Nagyova *et al.* 2004) follicular fluids well before the LH surge and in follicles that are smaller than pre-ovulatory size. If these molecules are derived from serum they would not contribute to a net gain of fluid by contributing to the osmotic pressure within the antrum, unless actively transported there. However, they may contribute by cross linking to hyaluronan (Rugg *et al.* 2005) ensuring they remain in the follicular antrum. Cross linking of the heavy chains of inter-α-trypsin inhibitor to hyaluronan is catalyzed by tumour necrosis factor-stimulated gene 6 (TNAIFP6) (Milner *et al.* 2007) which is only up regulated after the LH surge at ovulation in pigs (Nagyova *et al.* 2004) and mice (Yoshioka *et al.* 2000). Thus it is unlikely that inter-α-trypsin inhibitor members contribute to the formation of follicular fluid, even if present. Therefore our central hypothesis on follicular fluid formation suggests that production of hyaluronan and versican by granulosa cells generates an osmotic gradient which functions to recruit fluid from the thecal vasculature. Additional studies are required to validate this hypothesis.

Summary

Here we have discussed matrix in ovarian stroma, the follicular basal lamina, focimatrix and follicular fluid and where or how these may impinge upon fertility. That such diverse cellular processes all involve extracellular matrix attests to the complexity and multitude of roles of extracellular matrix. The examples presented also foreshadow many other potential important discoveries to be made involving extracellular matrix in ovaries.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The authors' laboratory is funded by the National Health and Medical Research Council of Australia.

References

Abrahamson DR 1986 Recent studies on the structure and pathology of basement membranes. *J Pathol* **149** 257-78.

Acosta TJ 2007 Studies of follicular vascularity associated with follicle selection and ovulation in cattle. *J Reprod Dev* **53** 39-44.

Alexopoulos E, Shahid J, Ongley HZ & Richardson MC 2000 Luteinized human granulosa cells are associated with endogenous basement membrane-like components in culture. *Mol Hum Reprod* **6** 324-30.

Amsterdam A, Rotmensch S, Furman A, Venter EA & Vlodavsky I 1989 Synergistic effect of human chorionic gonadotropin and extracellular matrix on in vitro differentiation of human granulosa cells: progesterone production and gap junction formation. *Endocrinology* **124** 1956-64.

Andersen MM, Kroll J, Byskov AG & Faber M 1976 Protein composition in the fluid of individual bovine follicles. *J Reprod Fertil* **48** 109-18.

Balen AH, Conway GS, Kaltsas G, Techatrasak K, Manning PJ, West C & Jacobs HS 1995 Polycystic ovary syndrome: the spectrum of the disorder in 1741 patients. *Hum Reprod* **10** 2107-11.

Beg MA & Ginther OJ 2006 Follicle selection in cattle and horses: role of intrafollicular factors. *Reproduction* **132** 365-77.

Berisha B & Schams D 2005 Ovarian function in ruminants. *Domest Anim Endocrinol* **29** 305-17.

Blondin P & Sirard MA 1995 Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol Reprod Dev* **41** 54-62.

Bottinger EP 2007 TGF-beta in renal injury and disease. *Semin Nephrol* **27** 309-20.

Cavender JL & Murdoch WJ 1988 Morphological studies of the microcirculatory system of periovulatory ovine follicles. *Biol Reprod* **39** 989-97.

Chegini N & Flanders KC 1992 Presence of transforming growth factor-beta and their selective cellular localization in human ovarian tissue of various reproductive stages. *Endocrinology* **130** 1707-15.

Chen L, Mao SJ & Larsen WJ 1992 Identification of a factor in fetal bovine serum that stabilizes the cumulus extracellular matrix. A role for a member of the inter-alpha-trypsin inhibitor family. *J Biol Chem* **267** 12380-6.

Clark LJ, Irving-Rodgers HF, Dharmarajan AM & Rodgers RJ 2004 Theca interna: the other side of bovine follicular atresia. *Biol Reprod* **71** 1071-8.

Clarke HG, Hope SA, Byers S & Rodgers RJ 2006 Formation of ovarian follicular fluid may be due to the osmotic potential of large glycosaminoglycans and proteoglycans. *Reproduction* **132** 119-31.

Eriksen GV, Carlstedt I, Morgelin M, Uldbjerg N & Malmstrom A 1999 Isolation and characterization of proteoglycans from human follicular fluid. *Biochem J* **340** 613-20.

Espey LL 1980 Ovulation as an inflammatory reaction--a hypothesis. *Biol Reprod* **22** 73-106.

Feng WG, Sui HS, Han ZB, Chang ZL, Zhou P, Liu DJ, Bao S & Tan JH 2007 Effects of follicular atresia and size on the developmental competence of bovine oocytes: a study using the well-in-drop culture system. *Theriogenology* **67** 1339-50.

Fortune JE, Sirois J, Turzillo AM & Lavoir M 1991 Follicle selection in domestic ruminants. *J Reprod Fertil Suppl* **43** 187-98.

Gardiner TA, Anderson HR & Stitt AW 2003 Inhibition of advanced glycation end-products protects against retinal capillary basement membrane expansion during long-term diabetes. *J Pathol* **201** 328-33.

Giehl KA, Ferguson DJ, Dean D, Chuang YH, Allen J, Berker DA, Tosti A, Dawber RP & Wojnarowska F 2004 Alterations in the basement membrane zone in pili annulati hair follicles as demonstrated by electron microscopy and immunohistochemistry. *Br J Dermatol* **150** 722-7.

Ginther OJ, Kot K, Kulick LJ & Wiltbank MC 1997 Sampling follicular fluid without altering follicular status in cattle: oestradiol concentrations early in a follicular wave. *J Reprod Fertil* **109** 181-6.

Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR & Kot K 1996 Selection of the dominant follicle in cattle. *Biol Reprod* **55** 1187-94.

Goldin A, Beckman JA, Schmidt AM & Creager MA 2006 Advanced glycation end products: sparking the development of diabetic vascular injury. *Circulation* **114** 597-605.

Gosden RG, Hunter RH, Telfer E, Torrance C & Brown N 1988 Physiological factors underlying the formation of ovarian follicular fluid. *J Reprod Fertil* **82** 813-25.

Herrmann G & Spanel-Borowski K 1998 A sparsely vascularised zone in the cortex of the bovine ovary. *Anat Histol Embryol* **27** 143-6.

Hirani R, Hanssen E & Gibson MA 2007 LTBP-2 specifically interacts with the amino-terminal region of fibrillin-1 and competes with LTBP-1 for binding to this microfibrillar protein. *Matrix Biol* **26** 213-23.

Huet C, Monget P, Pisselet C & Monniaux D 1997 Changes in extracellular matrix components and steroidogenic enzymes during growth and atresia of antral ovarian follicles in the sheep. *Biol Reprod* **56** 1025-34.

Huey S, Abuhamad A, Barroso G, Hsu MI, Kolm P, Mayer

J & Oehninger S 1999 Perifollicular blood flow Doppler indices, but not follicular pO2, pCO2, or pH, predict oocyte developmental competence in in vitro fertilization. *Fertil Steril* **72** 707-12.

Hughesdon PE 1982 Morphology and morphogenesis of the Stein-Leventhal ovary and of so-called "hyperthecosis". *Obstet Gynecol Surv* **37** 59-77.

Huxley-Jones J, Robertson DL & Boot-Handford RP 2007 On the origins of the extracellular matrix in vertebrates. *Matrix Biol* **26** 2-11.

Irving-Rodgers HF, Catanzariti KD, Aspden WJ, D'Occhio MJ & Rodgers RJ 2006a Remodeling of extracellular matrix at ovulation of the bovine ovarian follicle. *Mol Reprod Dev* **73** 1292-302.

Irving-Rodgers HF, Catanzariti KD, Aspden WJ, D'Occhio MJ & Rodgers RJ 2006b Remodeling of extracellular matrix at ovulation of the bovine ovarian follicle. *Mol Reprod Dev* **73** 1292-1302.

Irving-Rodgers HF, Friden BE, Morris SE, Mason HD, Brannstrom M, Sekiguchi K, Sanzen N, Sorokin LM, Sado Y, Ninomiya Y & Rodgers RJ 2006c Extracellular matrix of the human cyclic corpus luteum. *Mol Hum Reprod* **12** 525-34.

Irving-Rodgers HF, Harland ML & Rodgers RJ 2004 A novel basal lamina matrix of the stratified epithelium of the ovarian follicle. *Matrix Biol* **23** 207-17.

Irving-Rodgers HF, Harland ML, Sullivan TR & Rodgers RJ 2009a Studies of granulosa cell maturation in dominant and subordinate bovine follicles: novel extracellular matrix focimatrix is co-ordinately regulated with cholesterol side-chain cleavage CYP11A1. *Reproduction* **137** 825-34.

Irving-Rodgers HF, Hummitzsch K, Murdiyarso LS, Bonner WM, Sado Y, Ninomiya Y, Couchman JR, Sorokin LM & Rodgers RJ 2010 Dynamics of extracellular matrix in ovarian follicles and corpora lutea of mice. *Cell Tissue Res* **339** 613-24.

Irving-Rodgers HF, Morris S, Collett RA, Peura TT, Davy M, Thompson JG, Mason HD & Rodgers RJ 2009b Phenotypes of the ovarian follicular basal lamina predict developmental competence of oocytes. *Hum Reprod* **24** 936-44.

Irving-Rodgers HF, Mussard ML, Kinder JE & Rodgers RJ 2002 Composition and morphology of the follicular basal lamina during atresia of bovine antral follicles. *Reproduction* **123** 97-106.

Irving-Rodgers HF & Rodgers RJ 2000a Ultrastructure of the basal lamina of bovine ovarian follicles and its relationship to the membrana granulosa. *J Reprod Fertil* **118** 221-8.

Irving-Rodgers HF & Rodgers RJ 2000b Ultrastructure of the basal lamina of bovine ovarian follicles and its relationship to the membrana granulosa. *J Reprod Fertil* **118** 221-8.

Irving-Rodgers HF & Rodgers RJ 2006 Extracellular matrix of the developing ovarian follicle. *Semin Reprod Med* **24** 195-203.

Jiang JY, Macchiarelli G, Tsang BK & Sato E 2003 Capillary angiogenesis and degeneration in bovine ovarian antral follicles. *Reproduction* **125** 211-23.

Juengel JL, Bibby AH, Reader KL, Lun S, Quirke LD, Haydon LJ & McNatty KP 2004 The role of transforming growth factor-beta (TGF-beta) during ovarian follicular development in sheep. *Reprod Biol Endocrinol* **2** 78.

Kielty CM, Sherratt MJ & Shuttleworth CA 2002 Elastic fibres. *J Cell Sci* **115** 2817-28.

Kisseleva T & Brenner DA 2007 Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis. *J Gastroenterol Hepatol* **22 Suppl 1** S73-8.

Knight PG & Glister C 2003 Local roles of TGF-beta superfamily members in the control of ovarian follicle development. *Anim Reprod Sci* **78** 165-83.

Knight PG & Glister C 2006 TGF-beta superfamily members and ovarian follicle development. *Reproduction* **132** 191-206.

Matti N, Irving-Rodgers HF, Hatzirodos N, Sullivan TR & Rodgers RJ 2010 Differential expression of focimatrix and steroidogenic enzymes before size deviation during waves of follicular development in bovine ovarian follicles. *Mol Cell Endocrinol* **321** 207-14.

McArthur ME, Irving-Rodgers HF, Byers S & Rodgers RJ 2000 Identification and immunolocalization of decorin, versican, perlecan, nidogen, and chondroitin sulfate proteoglycans in bovine small-antral ovarian follicles. *Biol Reprod* **63** 913-24.

Meyer zum Gottesberge AM & Felix H 2005 Abnormal basement membrane in the inner ear and the kidney of the Mpv17-/- mouse strain: ultrastructural and immunohistochemical investigations. *Histochem Cell Biol* **124** 507-16.

Mihm M, Austin EJ, Good TE, Ireland JL, Knight PG, Roche JF & Ireland JJ 2000 Identification of potential intrafollicular factors involved in selection of dominant follicles in heifers. *Biol Reprod* **63** 811-9.

Milner CM, Tongsoongnoen W, Rugg MS & Day AJ 2007 The molecular basis of inter-alpha-inhibitor heavy chain transfer on to hyaluronan. *Biochem Soc Trans* **35** 672-6.

Murata E, Akita M, Kaneko K & Merker HJ 1994 Changes associated with the basal lamina during metamorphosis of Xenopus laevis. *Acta Anat (Basel)* **150** 178-85.

Nagyova E, Camaioni A, Prochazka R & Salustri A 2004 Covalent transfer of heavy chains of inter-alpha-trypsin inhibitor family proteins to hyaluronan in in vivo and in vitro expanded porcine oocyte-cumulus complexes. *Biol Reprod* **71** 1838-43.

Nakano K, Naito I, Momota R, Sado Y, Hasegawa H, Ninomiya Y & Ohtsuka A 2007 The distribution of type IV collagen alpha chains in the mouse ovary and its correlation with follicular development. *Arch Histol Cytol* **70** 243-53.

Nelson VL, Legro RS, Strauss JF, 3rd & McAllister JM 1999 Augmented androgen production is a stable steroidogenic phenotype of propagated theca cells from polycystic ovaries. *Mol Endocrinol* **13** 946-57.

Nelson VL, Qin KN, Rosenfield RL, Wood JR, Penning TM, Legro RS, Strauss JF, 3rd & McAllister JM 2001 The biochemical basis for increased testosterone production in theca cells propagated from patients

with polycystic ovary syndrome. *J Clin Endocrinol Metab* **86** 5925-33.

Nicholas B, Alberio R, Fouladi-Nashta AA & Webb R 2005 Relationship between low-molecular-weight insulin-like growth factor-binding proteins, caspase-3 activity, and oocyte quality. *Biol Reprod* **72** 796-804.

Nilsson EE, Doraiswamy V & Skinner MK 2003 Transforming growth factor-beta isoform expression during bovine ovarian antral follicle development. *Mol Reprod Dev* **66** 237-46.

Padmanabhan V, Manikkam M, Recabarren S & Foster D 2006 Prenatal testosterone excess programs reproductive and metabolic dysfunction in the female. *Mol Cell Endocrinol* **246** 165-74.

Penttinen C, Saharinen J, Weikkolainen K, Hyytiainen M & Keski-Oja J 2002 Secretion of human latent TGF-beta-binding protein-3 (LTBP-3) is dependent on co-expression of TGF-beta. *J Cell Sci* **115** 3457-68.

Prodoehl MJ, Hatzirodos N, Irving-Rodgers HF, Zhao ZZ, Painter JN, Hickey TE, Gibson MA, Rainey WE, Carr BR, Mason HD, Norman RJ, Montgomery GW & Rodgers RJ 2009a Genetic and gene expression analyses of the polycystic ovary syndrome candidate gene fibrillin-3 and other fibrillin family members in human ovaries. *Mol Hum Reprod* **15** 829-41.

Prodoehl MJ, Irving-Rodgers HF, Bonner W, Sullivan TM, Micke GC, Gibson MA, Perry VE & Rodgers RJ 2009b Fibrillins and latent TGF□ binding proteins in bovine ovaries of offspring following high or low protein diets during pregnancy of dams. *Molecular and Cellular Endocrinology* **307** 133-144.

Rajakoski E 1960 The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical, end left-right variations. *Acta Endocrinol Suppl (Copenh)* **34(Suppl 52)** 1-68.

Ramirez F & Pereira L 1999 The fibrillins. *Int J Biochem Cell Biol* **31** 255-9.

Richardson MC, Davies DW, Watson RH, Dunsford ML, Inman CB & Masson GM 1992 Cultured human granulosa cells as a model for corpus luteum function: relative roles of gonadotrophin and low density lipoprotein studied under defined culture conditions. *Hum Reprod* **7** 12-8.

Rivera GM & Fortune JE 2003 Selection of the dominant follicle and insulin-like growth factor (IGF)-binding proteins: evidence that pregnancy-associated plasma protein A contributes to proteolysis of IGF-binding protein 5 in bovine follicular fluid. *Endocrinology* **144** 437-46.

Rodgers RJ & Irving-Rodgers HF 2010 Formation of the ovarian follicular antrum and follicular fluid. *Biol Reprod* **82** 1021-9.

Rodgers RJ, Irving-Rodgers HF & Russell DL 2003 Extracellular matrix of the developing ovarian follicle. *Reproduction* **126** 415-24.

Rodgers RJ, Irving-Rodgers HF, van Wezel IL, Krupa M & Lavranos TC 2001 Dynamics of the membrana granulosa during expansion of the ovarian follicular antrum. *Mol Cell Endocrinol* **171** 41-8.

Rubio Pomar FJ, Roelen BA, Slot KA, van Tol HT, Colenbrander B & Teerds KJ 2004 Role of Fas-mediated apoptosis and follicle-stimulating hormone on the developmental capacity of bovine cumulus oocyte complexes in vitro. *Biol Reprod* **71** 790-6.

Rugg MS, Willis AC, Mukhopadhyay D, Hascall VC, Fries E, Fulop C, Milner CM & Day AJ 2005 Characterization of complexes formed between TSG-6 and inter-alpha-inhibitor that act as intermediates in the covalent transfer of heavy chains onto hyaluronan. *J Biol Chem* **280** 25674-86.

Russell DL, Ochsner SA, Hsieh M, Mulders S & Richards JS 2003 Hormone-regulated expression and localization of versican in the rodent ovary. *Endocrinology* **144** 1020-31.

Schymeinsky J, Nedbal S, Miosge N, Poschl E, Rao C, Beier DR, Skarnes WC, Timpl R & Bader BL 2002 Gene structure and functional analysis of the mouse nidogen-2 gene: nidogen-2 is not essential for basement membrane formation in mice. *Mol Cell Biol* **22** 6820-30.

Shalgi R, Kraicer P, Rimon A, Pinto M & Soferman N 1973 Proteins of human follicular fluid: the blood-follicle barrier. *Fertil Steril* **24** 429-34.

Sirard MA, Picard L, Dery M, Coenen K & Blondin P 1999 The time interval between FSH administration and ovarian aspiration influences the development of cattle oocytes. *Theriogenology* **51** 699-708.

van Wezel IL & Rodgers RJ 1996 Morphological characterization of bovine primordial follicles and their environment in vivo. *Biol Reprod* **55** 1003-11.

Yamada O, Abe M, Takehana K, Hiraga T, Iwasa K & Hiratsuka T 1995 Microvascular changes during the development of follicles in bovine ovaries: a study of corrosion casts by scanning electron microscopy. *Arch Histol Cytol* **58** 567-74.

Yamada S, Fujiwara H, Honda T, Higuchi T, Nakayama T, Inoue T, Maeda M & Fujii S 1999 Human granulosa cells express integrin alpha2 and collagen type IV: possible involvement of collagen type IV in granulosa cell luteinization. *Mol Hum Reprod* **5** 607-17.

Yang MY & Rajamahendran R 2000 Morphological and biochemical identification of apoptosis in small, medium, and large bovine follicles and the effects of follicle-stimulating hormone and insulin-like growth factor-I on spontaneous apoptosis in cultured bovine granulosa cells. *Biol Reprod* **62** 1209-17.

Yoshioka S, Ochsner S, Russell DL, Ujioka T, Fujii S, Richards JS & Espey LL 2000 Expression of tumor necrosis factor-stimulated gene-6 in the rat ovary in response to an ovulatory dose of gonadotropin. *Endocrinology* **141** 4114-9.

Zimmermann DR & Dours-Zimmermann MT 2008 Extracellular matrix of the central nervous system: from neglect to challenge. *Histochem Cell Biol* **130** 635-53.