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Basal lamina and other extracellular matrix produced by bovine granulosa cells in anchorage-independent culture

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Abstract. Bovine granulosa cells from 3–7 mm follicles were cultured without anchorage in soft agar/methylcellulose solution for 14 days, with or without 50 ng/ml basic fibroblast growth factor. The granulosa cells divided to form colonies of cells. These were analysed by light and electron microscopy, immunohistochemistry and Western immunoblotting. In approximately 20% of the colonies extracellular matrix was clearly visible at the light-microscope level. Ultrastructurally the matrix resembled a basal lamina 30–100 nm thick and was composed of tangled fibres or cords. Unidentified spherical structures of less than 50 nm diameter were sometimes present and attached to this basal lamina. The basal lamina of follicles had similar features, except that the basal lamina produced in vitro was a large aggregate of many convoluted layers. The cells produced collagen type IV and the cellular form of fibronectin. Intercellular areas not associated with basal lamina were identified. Ruthenium red staining revealed these areas to be rich in proteoglycan granules. Free granules were clustered near the cell surface, and the lumina of these areas were rich in fibres decorated with ruthenium red. This material did not resemble follicular fluid of antral follicles. Thus, granulosa cells in anchorage-independent cultures have a follicular cell morphology and secrete two distinct extracellular matrices, one similar to the follicular basal lamina.

Key words: Basal lamina – Basement membrane – Ovary – Follicle – Granulosa cell – Follicular fluid – Collagen type IV – Fibronectin – Proteoglycan – Ruthenium red – Bovine

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Introduction

In mammals the ovarian follicular basal lamina resembles that of other basal laminae (Bjersing and Cajander 1974; Anderson et al. 1978) and components of basal laminae, such as collagen type IV, laminin, fibronectin and heparan sulphate proteoglycans, have been localized to the follicular basal lamina (Bagavandos et al. 1983; Wordinger et al. 1983; Palotie et al. 1984; Leardkamolkarn and Adamson 1992; Luck 1994; Zaho and Luck 1995; Luck et al. 1995). However, we know very little of this particular basal lamina. It has not been isolated, and studies of its composition have been hampered by the presence of the endothelial basal laminae that are often adjacent to the follicular basal lamina. We do not know what stimulates its production during follicular growth and there is uncertainty as to which cells produce it and we do not know what its role is, other than it probably acts as a selective filter of plasma proteins during follicular fluid accumulation (Shalgi et al. 1973; Andersen et al. 1976; Chang et al. 1976; Simpson et al. 1980).

There is also a pressing need to know which growth factors can bind to the follicular basal lamina. Already there has been much research on the production of growth factors within follicles, either by granulosa cells that in turn act on theca cells found just outside of the basal lamina, or vice versa. These factors must, of course, traverse the follicular basal lamina in order to act on the adjoining compartment. The major factors studied, including transforming growth factor β, activin, insulin-like growth factor (IGF) I and II, and basic fibroblast growth factor (bFGF), either directly or via their binding proteins, bind to extracellular matrix components that can form part of a basal lamina (see Ruoslahti 1989; Massague 1992; Butzow et al. 1993; Sugino et al. 1993; Jones et al. 1993). Thus, there is a great need to study the follicular basal lamina, if only to test the hypotheses concerning the movement of growth factors from one compartment of the follicle to the other.

The other major extracellular matrices of the ovarian follicle are the intrafollicular 'fluids'. Fluid is present in

small pockets or gaps between cells, long before a large fluid-filled antrum appears. The content of this former extracellular matrix has not yet been determined, however, follicular fluid that fills the antrum has been extensively analysed. It is similar to plasma but with the additions of secretions of granulosa cells and without the larger components of plasma, particularly low-density lipoprotein (Chang et al. 1976; Simpson et al. 1980) and α2-macroglobulin. Proteins of intermediate size, such as fibronectin, are partially excluded (Shalgi et al. 1973; Andersen et al. 1976). Heparan sulphate and chrondroitin/dermatan sulphate proteoglycans have been identified in follicular fluid and in culture medium of granulosa cells (Yanagishita and Hascall 1979, 1983, 1984), and the production of hyaluronic acid by the cumulus cells has been extensively studied (Eppig 1979; Salustri et al. 1990). These latter cells make up a small proportion of the total number of cells enclosed by the follicular basal lamina.

Since the follicular basal lamina aligns one side of the membrana granulosa and follicular fluid the other, it is likely that the granulosa cells are important in the production of both of these extracellular matrices. However, the majority of in vitro studies on granulosa cells has been conducted on granulosa cells cultured in monolayer. In these anchorage-dependent culture systems cells attach to the bottom of the dish, coated or uncoated with extracellular matrix components. Under these conditions granulosa cells spontaneously differentiate (Luck et al. 1990; Meidan et al. 1990), thereby taking on the characteristics of luteal cells, which in vivo are not associated with either a basal lamina or a fluid similar to follicular fluid. This phenomenon makes it difficult to study the characteristics of follicular granulosa cells and their extracellular matrix production. Recently, the anchorage-independent technique of soft agar culture was adapted for culturing bovine granulosa cells (Lavranos et al. 1994). This was chosen because we realized that granulosa cells divide in vivo without contact inhibition, a property of stem or tumour cells which also have the ability to divide in vitro without anchorage. It was found that granulosa cells cultured in an anchorage-independent culture system under control conditions or more so in the presence of bFGF (Lavranos et al. 1994) or IGF I (Lavranos and Rodgers 1994), continued to divide rather than differentiate. The cells also retained the ultrastructural features of follicular granulosa cells seen in vivo and were observed to secrete an extracellular matrix (Lavranos et al. 1994). Basic FGF was used in these studies because it has been localized to the oocytes of primordial follicles and hypothesized to be an early growth factor in the reactivation of follicle growth (van Wezel et al., unpublished observations).

Therefore we have examined the extracellular matrix produced by bovine granulosa cells cultured without anchorage under control conditions or in the presence of bFGF, and for comparison, we examined the follicular basal lamina and follicular fluid.

Materials and methods

Cell culture

Granulosa cells were isolated from bovine ovaries using a modification of the method of Lavranos et al. (1994). Briefly, bovine ovaries were collected into Hepes-buffered Earle's balanced salt solution without calcium or magnesium from nonpregnant cycling animals killed at a local abattoir. In the laboratory, granulosa cells were gently scraped from small- and medium-sized follicles (3- to 7-mm diameter) into prewarmed (39° C) α-modification minimal essential medium (MEM; Code 50-112-PA; Biosciences Pty. Ltd., Castle Hill, NSW, Australia) containing 5 μg/ml deoxyribonuclease I (DNAase; Code DN-25; Sigma Chemical Co., St. Louis, Mo., USA). The cells were rinsed four times, and then resuspended in MEM containing 5 μg/ml deoxyribonuclease I, then slowly syringed 10 times through a 27G needle to gently disperse larger clumps of cells before filtering. Cells were then rinsed three times in MEM, and the proportion of viable cells and yields were determined by counting cells in a haemocytometer in 0.2% trypan blue. Using the method of Lavranos et al. (1994), 2.5×10^4 viable cells (as single cells or clumps of 2–10 cells) were aliquoted onto 1 ml of 0.5% gelled agar (Difco Laboratories, Detroit, Mich. USA) in MEM with 20% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia) and antibiotics (100 μg/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone; Commonwealth Serum Laboratories) in 35 mm dishes. The cells were overlayed with 1 ml 1% methylcellulose (Methocell; Dow Chemical Co., Midland, Mich., USA) in the same medium as the agar base and then cultured under these conditions (control) or with the addition of 50 ng/ml bFGF (Boehringer Mannheim GmbH, Mannheim, Germany). Cells were cultured for 14 days (some examined at 7 days) at 37° C in a humidified atmosphere of 7% O_2 /10% CO_2/83% N_2. A sample of granulosa cells was also taken at day 0 and stored at −20° C until required for Western blotting or fixed for microscopic examination. At the end of each culture period the methylcellulose solution overlay from each dish was collected into a microfuge tube by rinsing with 0.5 ml MEM to recover the colonies of cells quantitatively, and the recovered cells were washed twice in MEM. The colonies of cells were either stored at −20° C for Western immunoblotting of fibronectin or processed for immunohistochemistry or light and electron-microscopic examination.

Light and electron-microscopic examination

Pellets of cells collected prior to culture and colonies of cells cultured for 14 days were fixed in a solution of 2.5% glutaraldehyde with or without 100 μg/ml ruthenium red (Sigma Chemical Co.) in either 0.1 M MOPS (4-morpholinepropanesulphonic acid) buffer (pH 7.3) or 0.1 M sodium cacodylate buffer (pH 7.3) (n=18). The colonies of cultured cells were then resuspended in 100 μl 2% sodium alginate in MOPS buffer. Colonies of cells in this suspension were centrifuged to the bottom of the alginate solution and then a solution of 50 mM $CaCl_2$ in MOPS buffer layered on top of the alginate solution, causing the alginate to gel (Tamponnet et al. 1988) and act as a support for the cells during further processing. The cells were postfixed in 2% osmium tetroxide, dehydrated in acetone, and embedded in epoxy resin. One-micrometer-thick sections were cut and stained in 1% methylene blue solution for light-microscopy examination, and 100 nm sections were stained with uranyl acetate and lead citrate and examined in a JOEL CS1200 electron microscope.

In order to process antral follicles for electron microscopy reproductive tracts were removed from cows killed at an abattoir within 20 min of death. The ovarian artery was cannulated and the ovary perfused with Dulbecco's modified Eagle's medium solution (30 ml) delivered from a syringe using gentle manual pressure. Each ovary was then perfused with 25 ml 2.5% glutaralde-

hyde with or without 100 µg/ml ruthenium red in MOPS buffer. The ovary was cut near the side of the antral follicle and the follicle (n=6) placed in fixative. Later when the follicular fluid had gelled due to fixation (approximately 2 h) the follicle was cut into smaller segments with the follicular fluid attached to the membrana granulosa and these pieces of antral follicle further fixed. Where ruthenium red was used in the fixative for both cells and tissues, ruthenium red was included in all buffers up to postfixation of the cells and tissues.

Immunohistochemistry

Colonies of cells (n=3) were mixed with 200 µl O.C.T. embedding compound (Miles Inc., Elkhart, Ind., USA) in a microfuge tube, centrifuged at 5000 rpm for 3 min, and frozen on dry ice. Following removal of the frozen pellet from the tube, 10-µm-thick sections were cut, mounted on Vectabond-coated (Vecta Laboratories, Bulingeine, Calif., USA) slides and then air dried. Following fixation in formalin (4% formaldehyde) the sections were rinsed in phosphate-buffered saline and incubated with blocking solution of 5% serum (normal goat serum or nomal donkey serum) for 1 h at room temperature. The sections were incubated in primary antiserum for 3 h at room temperature, then rinsed (3×5 min) with phosphate-buffered saline before being incubated with secondary antiserum for 1 h. The sections were further rinsed, then incubated with biotinylated secondary antisera (1:100 for polyclonal primary antiserum and 1:300 for monoclonal antiserum), rinsed again and then with peroxidase-labelled avidin-biotin complex (Vectastain ABC kit from Vecta Laboratories) for 1 h, and subsequently rinsed again. Development of peroxidase activity (10 min) used the chromogen 3′3′-diaminobenzidine tetrahydrochloride (0.7 mg/ml) and hydrogen peroxide (0.7 mg/ml) in 0.06 M TRIS buffer ('Sigma FAST' from Sigma Chemial Co.). The sections were further rinsed, dehydrated in ethanol and mounted. The primary antisera used were rabbit polyclonal antiserum specific to human fibronectin (F-3648; Sigma Chemical Co.), rabbit polyclonal antiserum to collagen type IV from human placenta (PCO; EuroDiagnostics, BV, Apeldoom, The Netherlands) and culture supernatant containing mouse monoclonal antibodies to the NC1 domain of the α_1 chain of collagen type IV from bovine kidney (677/7C1; MonoCarb, AB, Lund, Sweden), and all were used at a 1:50 dilution. Normal rabbit serum, mouse serum or a mouse monoclonal antiserum to the La antigen, all at 1:50 dilution, were used to replace the specific antisera in control incubations. Secondary antisera were goat anti rabbit IgG (1:300 dilution) and horse anti mouse IgG (1:100 dilution) from Vecta Laboratories.

Western immunoblotting of fibronectin

Immunoblotting for fibronectin (n=5) was carried out as reported in detail for bovine (Rodgers et al. 1986b) and ovine (Rodgers et al. 1988) samples. Briefly, the cultured cells pooled from five 35-mm dishes from each treatment, and cells collected prior to culture were all each homogenized in 200 µl homogenisation buffer [58.4 mM TRIS, pH 6.8, 2% sodium dodecyl sulphate (SDS), 2% 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride and 6 M urea] in a hand-held homogenizer. Homogenates were centrifuged to remove any particulate matter and the supernatant subjected to electrophoresis on SDS/polyacrylamide gels under reducing conditions (Laemmli 1970). The proteins were electroblotted onto nitrocellulose-coated nylon membrane (Hybond-C; Amersham Australia Pty. Ltd., Castle Hill, NSW, Australia) and incubated with rabbit polyclonal antiserum specific to human fibronectin (F-3648; Sigma Chemical Co.), using a method similar to that of Towbin et al. (1979) and reported in detail previously (Rodgers et al. 1986b, 1988). The secondary goat anti-rabbit IgG (R-4880; Sigma Chemical Co.) was labelled with ^{125}I (Thorell and Johnsson 1971) and autoradiography of the blots was carried out with Kodak XAR film. Bovine serum fibronectin (F-1141) used in the fibronectin blots was obtained from Sigma Chemical Co. Molecular weight markers were obtained from Pharmacia (Australia) Pty. Ltd. (North Rhyde, NSW, Australia). The components (laminin A chain, laminin B chains and nidogen/entactin) of Matrigel (Collaborative Research Inc., Two Oak Park, Bedford, Mass., USA) were also used as molecular weight markers.

Results

Morphology of the colonies and cells

The cells from 18 different experiments were examined. The control and bFGF-treated granulosa cells divided in a contact uninhibited manner giving rise to visible colonies of varying size up to 300-µm. In cross-section the individual cells in the colonies appeared elongated, close together and arranged in the colonies with their long axes positioned circumferentially (Fig. 1). In about a fifth of the colonies, particularly the larger colonies, an extracellular matrix was visible (Fig. 1). The bFGF-treated colonies were similar to control colonies, only larger and more frequently containing more extracellular matrix.

Both the control and bFGF-treated cells had similar ultrastructural morphological appearances (not shown) with similarities to that of follicular granulosa cells, as described previously (Rodgers et al. 1986a), and to that of the cells collected prior to culture. They had a low cytoplasmic/nuclear ratio, low to moderate volume densities of mitochondria and endoplasmic reticulum, both smooth and rough. Golgi apparatus were prominent and the surfaces of adjacent cells were in close apposition, not folded and occasionally contacted by gap junctions. The shape of the cultured cells was less cuboidal than is found in vivo and lipid droplets were present in higher concentrations. Some cisternae of rough endoplasmic reticulum were very dilated and contained visible flocculent-like material (not shown), unlike those of uncultured cells and those in vivo (Rodgers et al. 1986a).

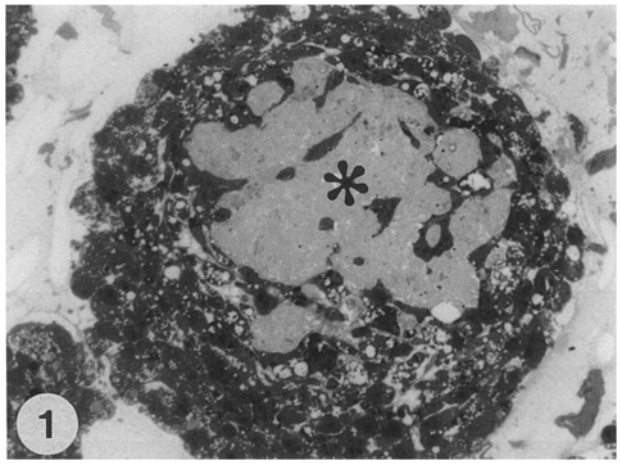


Fig. 1. Light micrograph of a cross-section of a colony of granulosa cells cultured for 7 days in the presence of bFGF (50 ng/ml). The colony was embedded in epoxy resin and the section was stained with methylene blue. The lighter staining material (*) is an extracellular matrix. ×325

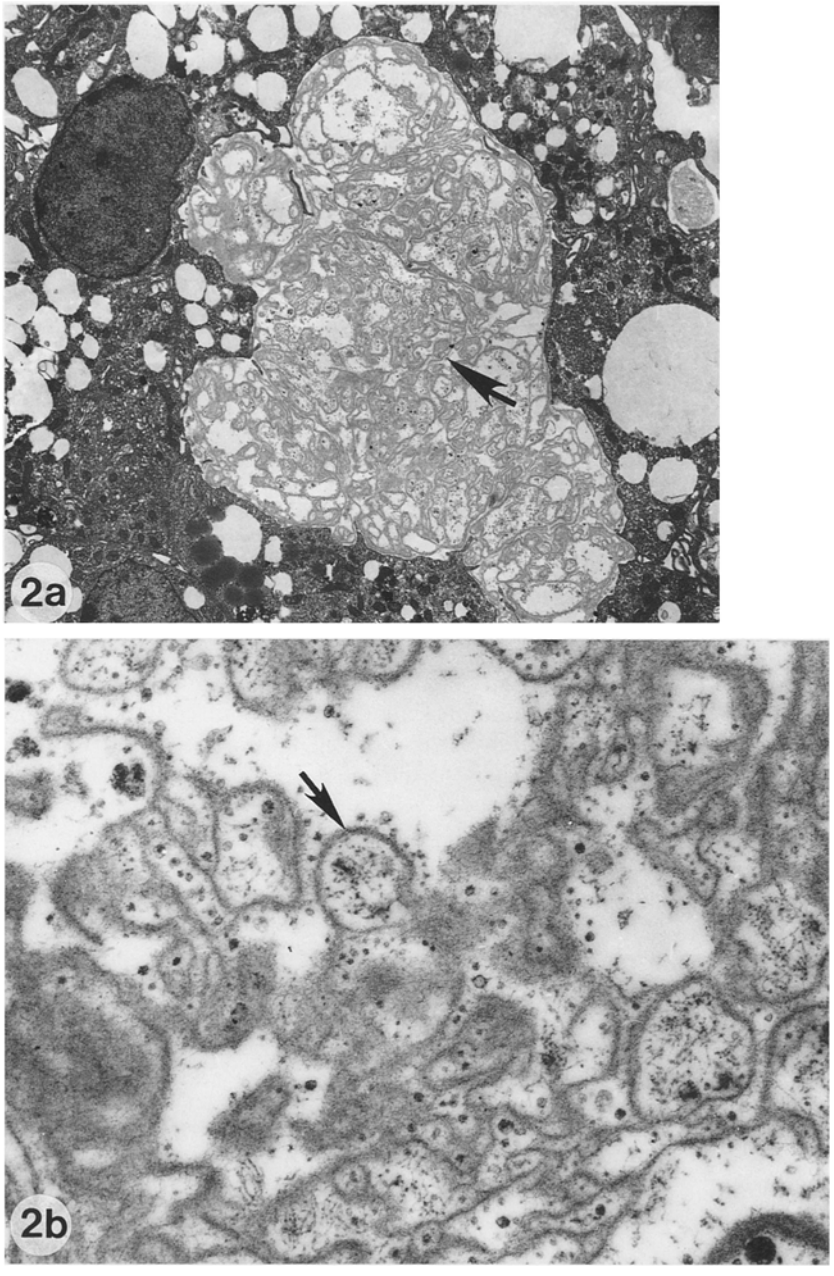


Fig. 2a, b. Electron micrographs of extracellular matrix produced by granulosa cells cultured for 14 days in the presence of bFGF (50 ng/ml). The extracellular matrix was assembled into a basal lamina (*arrows*) of convoluted layers and was not a single layer aligning a cell surface. **a** ×3500, **b** ×30000

Basal lamina

In many instances the extracellular matrix observed at the light-microscopic level appeared in the electron microscope as a large aggregate of basal lamina of moderately electron-dense material (Fig. 2a), convoluted and therefore often cut tangentially (Fig. 2b) and measuring 30 to 100 nm across. At higher magnification the material was composed of tangled fibres or strands (Fig. 3a). In other areas of the colonies a more amorphous matrix that probably represented non-assembled basal lamina was visible (not shown). These areas were often continuous with areas that contained assembled basal lamina. Small unidentified spherically shaped structures (less than 50 nm diameter) were sometimes present and adjacent to the basal lamina (Fig. 3a). The follicular basal lamina in vivo was 30 to 100 nm thick and was composed to tangled fibres or cords. It was found to be uniformly aligned to the surface of the granulosa cells, although sometimes it was more than one layer and these were often joined together forming a branching network. Spherical structures similar in appearance to those observed in vitro were sometimes seen in association with the basal lamina in vivo (Fig. 3b).

To gain further evidence that the extracellular matrix secreted in vitro represented a true basal lamina, bFGF-treated colonies were immunostained at the light-microscopic level for the presence of collagen IV (Fig. 4a) and fibronectin (Fig. 4b), and the molecular weight of fibronectin was determined by Western immunoblotting (Fig. 5). Collagen IV was identified by the two antisera used and fibronectin was also identified; no staining was ob-

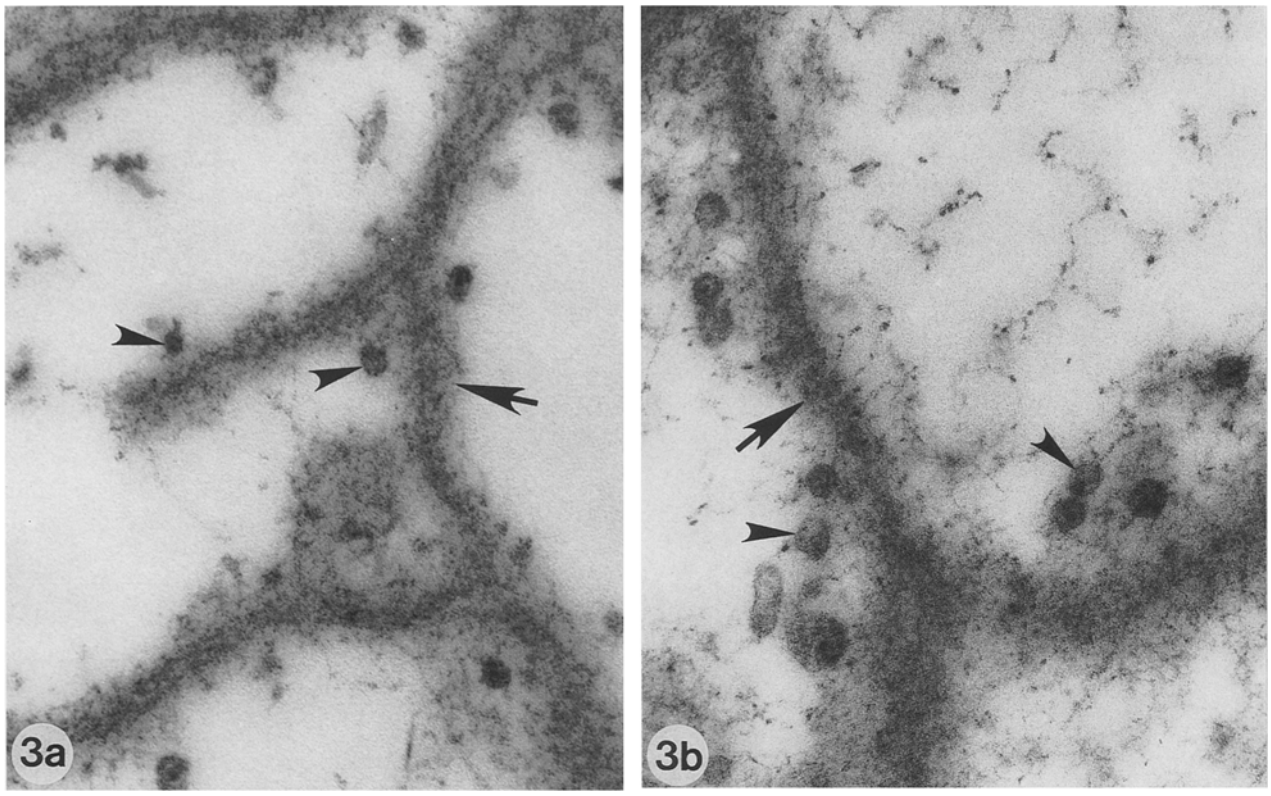


Fig. 3a, b. Electron micrographs of (**a**) basal lamina (*arrows*) produced by granulosa cells cultured for 14 days in the presence of bFGF (50 ng/ml) and (**b**) a follicular basal lamina (*arrows*) from a 3-mm diameter antral follicle. Note the presence of spherical material (*arrowheads*) associated with both types of basal lamina. **a** ×100000, **b** ×100000

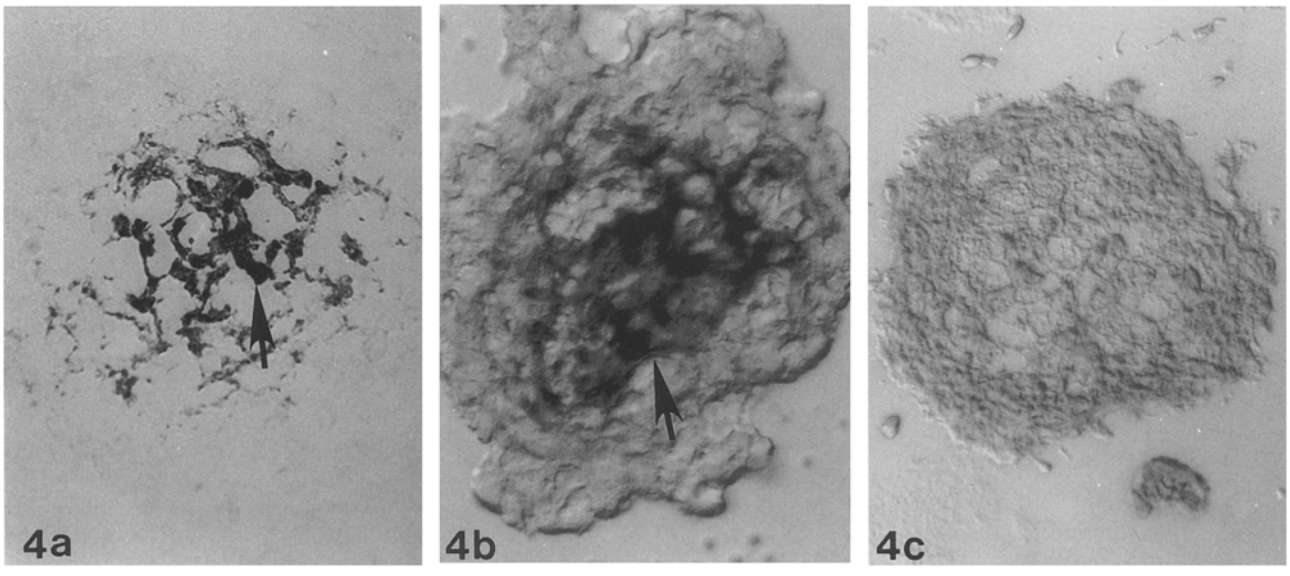


Fig. 4a–c. Photomicrographs of colonies of granulosa cells cultured for 14 days in the presence of bFGF (50 ng/ml) and immunostained with (**a**) monoclonal antiserum to collagen type IV, (**b**) rabbit polyclonal antiserum to fibronectin or (**c**) rabbit non-immune serum. *Arrows* depict areas of positive immunostaining. **a** ×325, **b** and **c** ×430; **b** and **c** were photographed using differential interference contrast lenses

served when rabbit non-immune serum (Fig. 4c) or mouse non-immune serum were used as the primary antisera. Only about a fifth of the colonies stained positively for collagen type IV or fibronectin, approximately the proportion of colonies that had identifiable extracellular matrix (Rodgers et al., unpublished observations). Fibronectin was produced by cells cultured in the presence or absence of bFGF, and detected by Western immunoblotting as a diffuse band of molecular weight greater than 200 kDa (Fig. 5). This molecular weight was greater

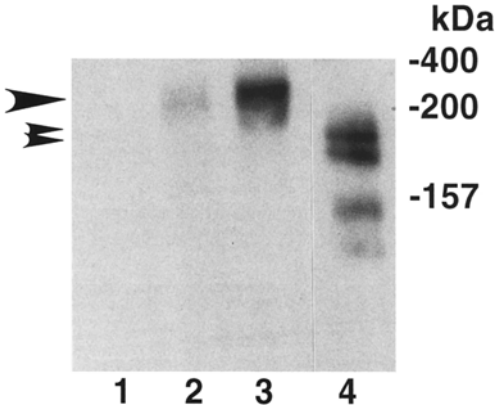


Fig. 5. Western immunoblot of fibronectin from granulosa cells prior to culture (*lane 1*) or following 14 days of culture under either control conditions (*lane 2*) or in the presence of 50 ng/ml bFGF (*lane 3*); 50 ng of bovine plasma fibronectin was loaded in *lane 4*. The *large arrow* indicates the fibronectin secreted from the granulosa cells and the *smaller arrows* indicate the position of the lower molecular weight species of fibronectin from bovine plasma

than that of fibronectin from bovine serum, suggesting that it was a cellular form (Vartio et al. 1987) and that it was derived from a splice variant (Oyama et al. 1989; Hershberger and Culp 1990) different to that encoding the serum form (Fig. 5). Since it had a greater molecular weight than that from serum we can also be certain that the fibronectin was synthesized by the granulosa cells and not merely concentrated from the bovine fetal serum present in the culture medium.

Proteoglycans

Colonies were fixed and processed with ruthenium red to identify and localize proteoglycans. Ruthenium red forms an electron-dense granule by specifically interacting with the negatively charged glycosaminoglycan side-chains of proteoglycans. Each ruthenium red granule is believed to represent an individual proteoglycan monomer (Hascall 1980). The size and number of glycosaminoglycan side-chains and the size of the core protein determine the size of the ruthenium red granule (Iozzo et al. 1982). Thus, the size of the granules, their association with fibres and the distances between them were observed. In the colonies small areas between cells, not associated with either the assembled or unassembled basal lamina as described above, were observed and found to be rich in proteoglycan granules (Fig. 6a). Free proteoglycan granules, up to 50 nm diameter, were observed to be very common near the surface of the cells facing onto these areas, and tightly packed in the small intercellular spaces between cells, giving the impression that these granules were being secreted and directed into these areas (Fig. 6b). Further to the middle of these areas were well defined fibres up to 500 nm in length and decorated with rutheniium red granules (Fig. 6c). The fibres were irregular and did not seem to form a complete network. The fibres were 10 nm thick with much thinner branches. The ruthenium red granules were located on the thick portion of the fibres and were themselves variable in diameter, 15 to 50 nm thick, and were spaced at irregular intervals on the fibres.

Ovarian antral follicles were fixed and processed with ruthenium red to visualize and localize their proteoglycans. In the antral follicles no free proteoglycan granules were observed at the surface of the granulosa cells facing the follicular fluid, nor were there any free granules in the intercellular spaces facing the fluid, as observed in the colonies of granulosa cells in vitro. The antral follicular fluid was composed entirely and uniformly of a course network of aggregated material decorated with ruthenium red granules (Fig. 6d). The granules were 25 nm diameter and the minimum distance apart was approximately 100 to 150 nm. Many of the granules were associated with one or two fine filaments radiating from each granule and often seen to be connecting with another granule (Fig. 6d). Associated with this network was electron-dense flocculent material (Fig. 6d).

Discussion

For the first time in any species we have found that granulosa cells cultured under anchorage-independent conditions in the absence of a luteotrophin retain their follicular phenotype and as such secrete extracellular components such as fibronectin and collagen type IV. They secrete and assemble a basal lamina similar to the follicular basal lamina and secrete another extracellular matrix rich in proteoglycans. This latter matrix, whilst composed of fibres containing proteoglycans as does follicular fluid, does not resemble the follicular fluid extracellular matrix of antral follicles. Thus, these findings are and will be extremely valuable for the study of ovarian follicular formation and function.

The evidence that the granulosa cells secreted a basal lamina in vitro was partially based upon the presence of collagen type IV in a proportion of colonies. These colonies grew from the dispersed cells during culture and thus we can be sure that the collagen type IV was synthesised during culture. Ultrastructurally, large amounts of extracellular matrix were observed in about a fifth of the colonies. These observations support the conclusion that granulosa cells of themselves are capable of synthesizing basal lamina and that they may be the source of the follicular basal lamina. However, recent investigations have suggested that the structural components of the follicular basal lamina could originate from the thecal layer (Zhao and Luck 1995; Luck et al. 1995) outside of the basal lamina. These authors acknowledge that the observed synthesis of basal lamina components in the thecal layer may in fact be destined for the capillaries of the thecal layer. If the thecal layer does contribute to the follicular basal lamina, which theca cells would produce it? The cells of the thecal layer adjoining the basal lamina vary from one area of the follicle to another; it could be a steroidogenic theca cell, an endothelial cell (surrounded by its own basal lamina), a fibroblast or a white blood cell, and in primordial follicles fibroblasts appear

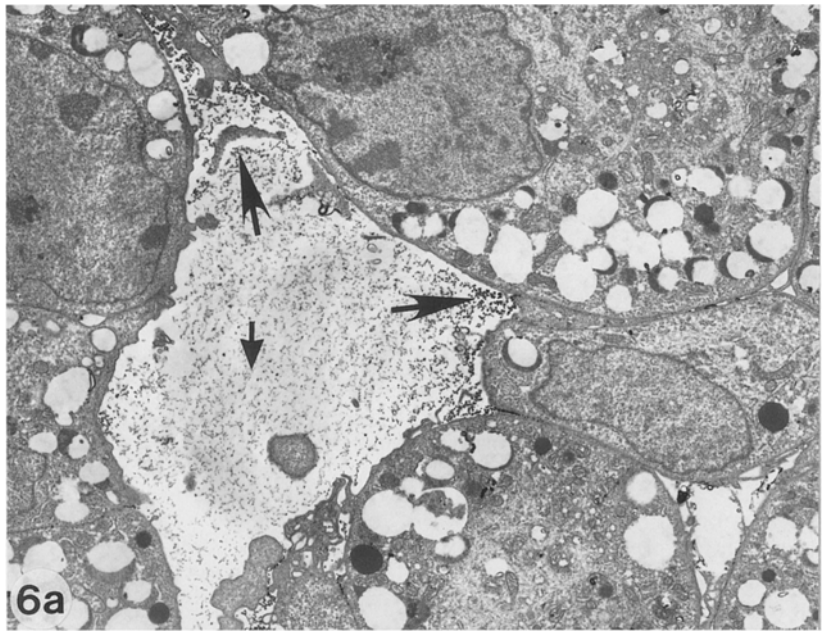
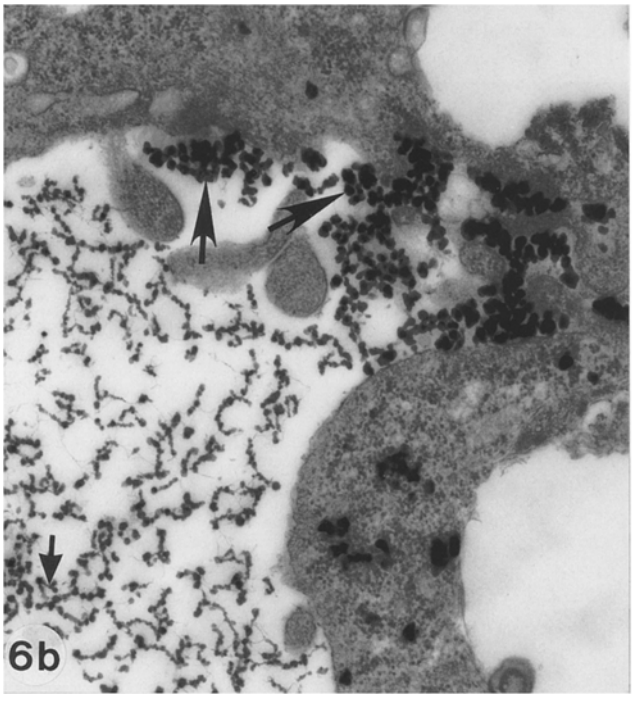
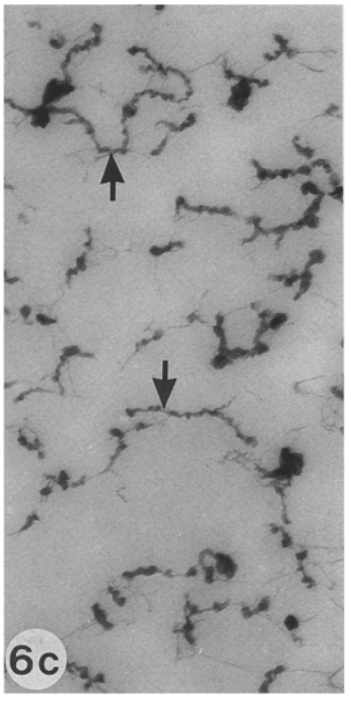
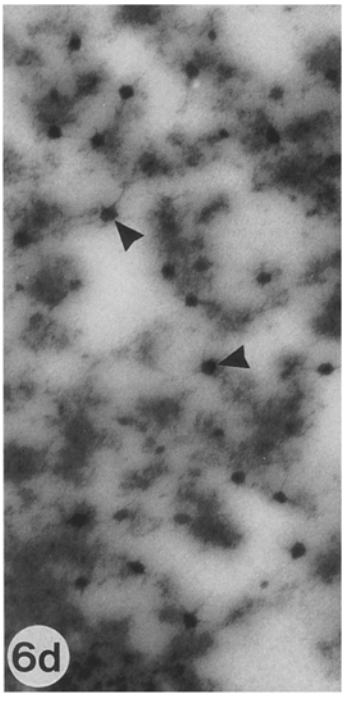


Fig. 6a–d. Electron micrographs of extracellular matrix produced by granulosa cells cultured for 14 days in the presence of 50 ng bFGF/ml (**a**, **b**, **c**) and follicular fluid of an antral follicle (**d**), all stained and fixed in the presence of ruthenium red (100 μg/ml). *Large arrows* denote ruthenium red proteoglycan granules near the surface of the cells and not associated with fibres (**a**, **b**), and *small arrows* denote ruthenium red granules associated with fibres (**b**, **c**). *Arrowheads* depict the ruthenium red granules associated with radiating fine filaments and a coarse network of aggregated material in follicular fluid (**d**). **a** ×4000, **b** ×30000, **c** ×65000, **d** ×65000







to be most common. This variability in the cell type aligning the basal lamina on the thecal side and our current observations strongly support the hypothesis that the granulosa cells are the source of the follicular basal lamina. In other situations epithelial cells are responsible for the synthesis of the basal lamina (see Timpl and Dziadek 1986) but it is clear also that in some instances the stromal layer secretes the structural components needed for basal lamina production (Simon-Assmann et al. 1988; Weiser et al. 1990). Clearly, further work needs to be performed to resolve this issue for the follicular basal lamina.

Fibronectin is a component of basal lamina although not specific to it. There are many forms arising from the one gene by differential splicing of the encoding RNA (Oyama et al. 1989; Hershberger and Culp 1990). A proportion of colonies contained fibronectin that was greater than 200 kDa and thus larger than the plasma form. Thus, not only was evidence obtained that the fibronectin was synthesised by the granulosa cells but also that this fibronectin was of a size to be a cellular form, reported to be approximately 220 kDa. Others have also shown that fibronectin is produced by granulosa cells (Savion and Gospodarowicz 1980; Skinner and Dorrington, 1984; Skinner et al. 1985; Carnegie 1990).

Many of the roles of proteoglycans in follicular fluid and those secreted by granulosa cells have still to be determined. Hyaluronic acid secreted by the cumulus cells

surrounding the oocyte causes the cumulus to expand (Eppig 1979; Salustri et al. 1990), and a role for the proteoglycans in the formation of follicular fluid has been postulated (Yanagishita et al. 1979). The secretion of the proteoglycans observed here in vitro was very directed. All cells aligning a cavity of fibres decorated with ruthenium red granules, had proteoglycan granules at their surfaces and between the tight spaces between them, thus appearing to direct the secretion of proteoglycans towards these areas. If directed secretion of proteoglycans larger than 500 kDa towards the centre of the follicle occurs in vivo it would exert an osmotic pressure to attract fluid to accumulate in this area and form follicular fluid (Yanagishita et al. 1979). However, experiments to test this theory have still to be carried out.

The structure of extracellular matrix containing proteoglycans as seen by ruthenium red staining and electron microscopy was substantially different between granulosa cells in vitro and that seen in the follicular fluid of antral follicles. Small fibres decorated with ruthenium red granules associated with large aggregates of material were present in the follicular fluid. This aggregated material could have been proteins derived from the plasma. In antral follicles no free proteoglycans were observed near the surface or between the granulosa cells as seen in vitro. However, in vitro, fibres decorated with ruthenium red were observed. The sizes of these fibres and of the ruthenium red granules associated with the fibres were different to those observed in antral follicular fluid. Thus, the proteoglycans produced in vitro were of a different size (Iozzo et al. 1982) and possibly a different type to those observed in antral follicular fluid.

Granulosa cells are often loosely referred to as if they represent one cell type. Clearly there are subtypes as has been recognized by differences in morphology and content of steroidogenic enzymes (see Amsterdam and Rotmensch 1987). From the results presented here it is clear that only a proportion of the colonies produce collagen type IV or fibronectin. As well within colonies not all cells secreted proteoglycans, suggesting that there are subtypes based upon their ability to produce extracellular matrix. In bovine ovaries all these cell types are, as are the specialized cumulus cells that surround the oocyte in antral follicles, derived from the 24 cells found in bovine primordial follicles (van Wezel and Rodgers; unpublished observations). Whether these cells of primordial follicles are pluripotent or are a mixture of committed cells has still to be determined.

In summary, the findings of the present study are important for they have shown that granulosa cells are capable of producing and assembling the components of a basal lamina. They have also shown that granulosa cells in anchorage-independent culture produce another extracellular matrix rich in proteoglycans. These findings thus provide us with the opportunity to discover the components, properties, and regulation of the follicular basal lamina and extracellular matrix of ovarian follicles.

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