

Minireview

Formation of the Ovarian Follicular Antrum and Follicular Fluid¹

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ABSTRACT

The formation of the follicular antrum and follicular fluid has received scant attention from researchers, yet both are important processes in follicular development. The central hypothesis on follicular fluid formation suggests that production by granulosa cells of hyaluronan and the chondroitin sulfate proteoglycan versican generates an osmotic gradient. This gradient draws in fluid derived from the thecal vasculature. Inter-alpha-trypsin inhibitor is also present in follicular fluid at least in species with large follicles, and inter-alpha-trypsin inhibitor and versican could additionally bind or cross-link with hyaluronan, resulting in the retention of these molecules within the follicular antrum. Barriers to the movement of fluid across the membrana granulosa are apparently minimal, as even relatively large serum proteins are present in follicular fluid. Despite the relative permeability of the follicular wall, aquaporins are present in granulosa cells and could be actively involved in the transport of water into the follicle. The formation of an antrum also requires movement of granulosa cells relative to each other to allow the fluid to accumulate. This presumably involves remodeling of cell-cell junctions and in species with small follicles may involve death of centrally located granulosa cells. Remodeling of the stroma and thecal layers also accompanies growth and expansion of the antrum and presumably involves similar processes that accompany growth of other glands.

follicular antrum, follicular fluid, hyaluronan, inter- α -trypsin inhibitor, proteoglycan, versican

INTRODUCTION

Growth of the follicle encompasses enlargement of the oocyte, replication of follicular cells, and formation and expansion of a central follicular antrum or cavity. Many in vitro studies of follicular growth have focused on the replication of granulosa cells, while in vivo studies using ultrasonography have focused on the expansion of the follicular antrum and its fluid. Replication of follicular cells and expansion of the follicular antrum are both important, and

both are probably stimulated by some of the same hormones and growth factors. They are, however, very distinct processes.

The rate at which the follicular antrum expands and follicular fluid accumulates differs between follicles, particularly between dominant and subordinate follicles [1, 2]. The proportion of a follicle that is follicular fluid at maximum size also varies from species to species. Generally, larger species such as ovine, equine, porcine, human, and bovine have larger follicles, with the fluid comprising a substantial proportion of the volume of the follicles at ovulation (estimated at >95% in bovine [3]). Smaller species such as rats and mice have smaller follicles with fractionally less follicular fluid.

With the advent of in vitro whole-follicle culture in hamsters and mice [4–6], which has been improved upon [7] and extended to other species, it is now potentially possible to study some aspects of follicular fluid formation in vitro. Hence, it is timely to review the state of our knowledge of follicular fluid formation. First, we briefly discuss fluid movement in other organs and tissues and then review our knowledge of the physiological and cellular events important for formation of the follicular antrum or cavity itself, as well as changes in the ovary that accommodate its formation and expansion. Then, we discuss the mechanism by which fluid could accumulate in the follicular antrum and the molecules involved in this process. Lastly, we address impediments and facilitators of fluid movement, to better understand how these processes are regulated.

HYDROSTATIC FORCES

Physical or hydrostatic pressures generated by contraction of cilia on individual cells (or groups of cells organized into contractile units such as in the heart or peristaltic organs like the intestine) can move large amounts of fluids efficiently and quickly. These organ systems usually move fluid within a sealed or closed system that is lined by endothelia or epithelia. Contraction of the heart generates hydrostatic pressure to move blood through the vasculature of many organs and tissues. As the heart pumps blood into arteries, the stretch and recoil of elastic fibers in arterial walls reduce the maximum pressure attained and raise the minimum pressure the blood falls to during each cycle of contraction and relaxation of the cardiac ventricles. Arteriolar smooth muscle tone can be regulated to restrict flow into any particular organ, and increased tone leads to reduced pressure within the capillaries and therefore reduced flow. Some capillary beds are extremely leaky (e.g., the fenestrated capillaries of the renal glomerulus). Other capillaries form an effective barrier to net movement of fluids, with both adherens and tight junctions between the endothelial cells effectively sealing the capillary lumen [8]. In this case, larger

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proteins find it difficult to transudate, and their osmotic pressure counterbalances the hydrostatic pressure. The osmotic pressure generated by the plasma proteins is called oncotic pressure and is not altered as a means of regulating capillary exudates, but its importance can be seen in pathological conditions or following severe hemorrhage. Capillary permeability can be altered dynamically, as the components of adherens and tight junctions are not static and can be altered to change the level of permeability [8]. Many factors have been shown to affect capillary permeability, including nitric oxide, prostacyclin, endothelin, vascular endothelial growth factor, insulin-like growth factor 1 (IGF1), substance P, and histamine.

Net fluid accumulated outside of the vasculature can be returned to the circulation via the lymphatic system. Lymphatic vessels are very permeable, allowing fluid and other molecules to enter, while the pumping mechanism to move fluid is achieved by movement of organs or limbs, effectively squeezing the vessels within them. The unidirectionality of lymphatic flow is achieved by way of valves in the larger lymphatic vessels and contraction of these vessels by their own smooth muscles. Evidence of the importance of lymphatics can be observed by the edema that occurs if the lymphatics are blocked by removal of the lymph nodes into which the lymphatic vessels drain. The lymphatic vessels eventually drain into the subclavian veins, thereby returning lymph to the circulation.

FLUID MOVEMENT ACROSS EPITHELIA

Within the body, groups of cells are able to form tight or adherens junctions between them, thus effectively creating a semipermeable cellular layer by sealing off all the pericellular spaces. This is common to both epithelia and endothelia. Thus, solutes or water crossing an endothelium or epithelium encounters either a cell membrane or the junctions between cells, ensuring that uncontrolled movement does not occur. The basal laminae that underlie endothelia and epithelia are also barriers to the passage of molecules, especially larger molecules, and potentially are also a barrier to growth factors that bind to basal lamina components such as the heparan sulfate proteoglycans.

Epithelia not only are barriers but also can be net absorptive (intestine), secretory (glands), or neither (epidermis), with absorption and secretion occurring to variable degrees in most epithelia. Absorptive epithelia function very differently than secretory epithelia as they work to move fluid against a hydrostatic gradient. Most secretory epithelia use directional secretion of solutes to achieve directional movement of fluid, with the exception of the glomerulus, where the hydrostatic pressure of blood in the adjacent capillaries is the driving force. For this arrangement to function in the glomerulus, there is lower pressure on the apical side, and the epithelium is relatively permeable. Many glands that secrete into larger cavities in the body or onto the external surfaces will have lower pressure on the apical side.

Some epithelia, however, face into a sealed filled cavity (choroid plexus, ovarian follicle, lens of the eye, or blocked glandular ducts); therefore, no draining of the fluid occurs, and net fluid accumulates as the cavity expands. With sealed cavities, it is possible to produce elevated turgidity within the cavity by increasing the levels of osmotic pressure of the fluid within it. The ovarian follicle is such a "sealed" cavity, and there are two important aspects to consider, namely, the formation of the antrum or cavity itself and the mechanism by which fluid moves into that cavity.

OSMOSIS AND DIRECTIONAL SECRETION

Osmosis depends upon a difference in solute concentrations between two areas, causing water to flow to the high solute concentration if the solute cannot move to the low concentration. The degree of inhibition of solute backflow depends upon a barrier to the migration of the solutes. In many of the equations for calculating osmotic pressure, a "semipermeable" membrane is invoked in which only water can flow and not the solutes. Biologically, only certain molecules are used to generate osmotic gradients, namely, those that can be directionally secreted or transported and those that can be prevented, even partially, from backflow. Epithelia, by their structure, can form barriers to movement of solutes across them; therefore, osmotic gradients are readily created across epithelia.

Directional secretion can occur apically or basally across an endothelium or epithelium because the cells are polarized. Additionally, material can be transported transcellularly in a basal to apical direction and vice versa by pinocytosis at one surface and exocytosis at the other surface of the cell. This sort of transcytosis does not generate osmotic gradients, as the material has the same composition on both sides of the cell, unless modification has taken place in transit. However, it is a mechanism by which fluid and solutes can be moved into or out of a lumen.

One of the best known osmotic gradients is that generated by sodium transport by Na,K-ATPase out of the ascending limb of the renal loop of Henle, creating a hyperosmotic concentration. The high concentration of sodium is the osmotic force for water to flow out of the collecting duct to concentrate the urine. Proteoglycans and their glycosaminoglycan side chains are also commonly used as osmotic solutes, resulting in fluid accumulation [9–14]. Proteoglycans are usually synthesized and directionally secreted rather than transported from one side of a cell to another. In addition, the movement of water can be either pericellularly or transcellularly facilitated by aquaporins. Aquaporins can allow the movement of water and other compounds such as glycerol [15], and if aquaporins exist on both basal and apical surfaces, water can flow into and out of a cell, thus allowing water to effectively transudate the cell.

OVARIAN FOLLICULAR FLUID FORMATION

Follicular fluid is probably derived from blood flowing through the thecal capillaries. Capillaries are rare in the region of the ovarian cortex containing primordial follicles [16, 17], and in most species they generally develop as a simple network around follicles at the early antral stages [18]. In species with small follicles, the thecal capillaries form a single-layered network, but in larger species the network is multilayered, especially as the follicle increases in size [18, 19]. Thecal networks are not uniform around the follicles, with fewer capillaries at the apex of the follicle compared with the lateral or basal regions [18], leading to corresponding differences in regional blood flow [20]. The capillary networks continue to develop and expand as the follicle grows, and it is generally accepted that blood vasculature or blood flow is usually not limiting. However, these could vary between dominant and subordinate follicles [21, 22], differ among follicles with oocytes of different quality [23], or decline upon follicular atresia [24, 25]. While the formation of follicular fluid commences when the theca is vascularized, the rate of fluid accumulation even during rapid growth is minor compared with the amount of blood flowing into the thecal capillaries. Hence, it is unlikely that the degree of thecal vascularization is a rate-limiting step for formation of follicular fluid, but

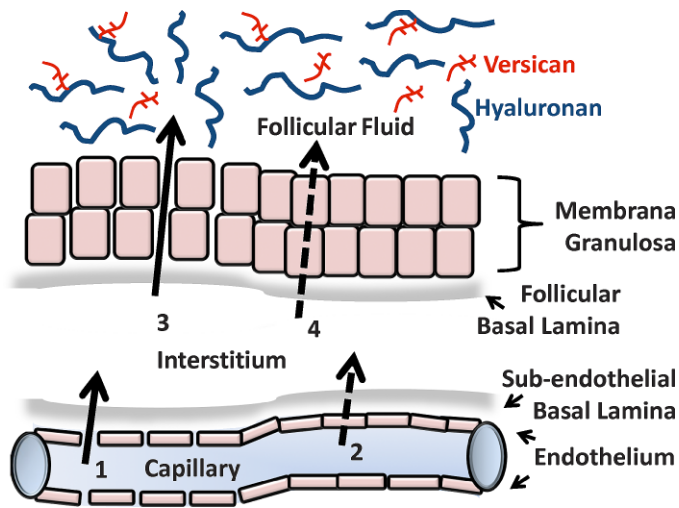


FIG. 1. Drawing illustrating the routes fluid can take from the thecal capillary to the follicular fluid and the potential barriers of the endothelium, subendothelial basal lamina, interstitium, follicular basal lamina, and membrana granulosa. Routes 1 and 3 show movement of fluid between the cells (solid arrows), and routes 2 and 4 show transcellular routes (hatched arrows) that either involve aquaporins or transcytosis.

alterations in thecal capillary blood pressure and flow could alter formation of fluid at critical times such as at ovulation.

For fluid from the theca to be transported into the follicular antrum, it obviously needs to cross the endothelium and subendothelial basal lamina before traversing the thecal interstitium, the follicular basal lamina, and the membrana granulosa (Fig. 1). Changes in permeability of the thecal capillaries will lead to edema of the thecal tissue, as observed following the luteinizing hormone (LH) surge [26, 27], but additional mechanisms are needed for fluid to accumulate in the follicular antrum. In earlier literature, both a sodium pump and cleavage of glycosaminoglycans to raise pressure in the preovulatory follicle were considered as such mechanisms [28]. Even if the cells of the membrana granulosa constitute a stratified epithelium and can directionally secrete osmotically active molecules toward the center of the follicle, because these cells lack a network of tight junctions, it would not be possible to establish an osmotic gradient across the membrana granulosa with small molecules like sodium. An early review found the evidence for a role for sodium to be “inconclusive” [29]. In fact, the composition of follicular fluid is similar to serum with respect to low-molecular-weight components, with most electrolytes being at the same concentrations in fluid and serum [29, 30]. However, for increasing sizes above 100 kDa, plasma proteins are found at progressively lower concentrations than in plasma [31–35]. This suggests that there is a nominal “blood-follicle barrier” at sizes above 100 kDa. This barrier probably exists at the level of the follicular basal lamina and additionally at the level of the thecal capillaries, especially for the larger molecules [36]. However, such a barrier may also exist in reverse in that large molecules produced by oocytes or granulosa cells cannot cross the membrana granulosa or follicular basal lamina, thereby establishing a potential osmotic gradient. This osmotic gradient could then be responsible for recruiting fluid to the center of the follicle.

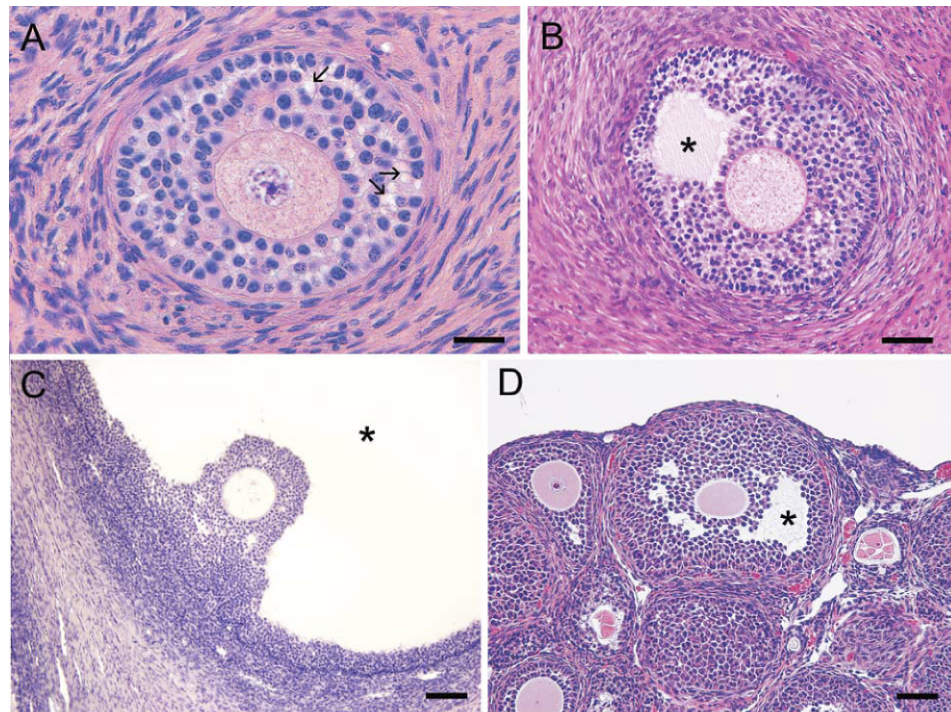
In a previous study [37], we sought evidence for the presence of large osmotically active molecules in ovarian follicular fluid. To determine which molecules contribute to the

osmotic potential, enzymes were used to degrade specific classes of molecules, followed by dialysis at molecular-weight cutoffs of 100 kDa and 300 kDa to remove digestion products. Removal of the glycosaminoglycans, hyaluronan and chondroitin sulfate/dermatan sulfate, 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. Hyaluronan, chondroitin sulfate, and dermatan sulfate are strongly hydrophilic and highly negatively charged and exert strong osmotic activity. The hyaluronan in bovine follicular fluid was found to be up to 2×10^6 in molecular weight [37], too large to escape from the antral cavity. Hyaluronan levels of human follicular fluid have been measured at 50 ng/ml, and hyaluronan has been previously localized adjacent to and between antral granulosa cells [38]. Granulosa cells in culture express hyaluronan synthase 2 (HAS2) and produce hyaluronan [39]. Therefore, hyaluronan clearly meets the necessary criteria to contribute to the osmotic potential of follicular fluid.

Chondroitin 4-sulfated proteoglycans were also identified as significant contributors to the osmotic potential of follicular fluid [37]. Earlier research in a number of species identified the glycosaminoglycans dermatan sulfate and chondroitin sulfate in follicular fluids, finding that glycosaminoglycans and proteoglycans are synthesized by the granulosa cells in vitro [40–43]. The chondroitin sulfate proteoglycans subsequently identified in our bovine investigations were versican (V1 and V0 splice forms) and inter- α -trypsin inhibitor (bikunin and 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) [37]. Versican is a chondroitin sulfate proteoglycan hyalactan with a broad tissue expression profile [44]. It has been shown to be present in extracts of bovine follicles [45], in follicular fluid of nonovulating [37] and ovulating follicles [46], and in the follicular membrana granulosa [45, 47]. It is also expressed by granulosa cells [48]. Many functional properties of versican are determined by two glycosaminoglycan attachment domains that are modified to allow for attachment of long chondroitin sulfate side chains. These chains are responsible for versican’s large molecular size and strong charge negativity. Versican may directly contribute to the osmotic potential of follicular fluid by virtue of the high sulfation status of chondroitin sulfate side chains attached to its core protein. However, versican may also contribute by cross-linking other components like hyaluronan [49, 50] to form larger-molecular-weight components, ensuring that they remained trapped in the follicular fluid.

Inter- α -trypsin inhibitor which consists of two heavy chains linked by a chondroitin sulfate chain to bikunin, is produced by the liver, and is found abundantly in serum. In mice, inter- α -trypsin inhibitor appears to be sequestered from the bloodstream, as it appears within the follicular fluid within minutes of the LH surge [51]. On entering the fluid following the LH surge, it associates with hyaluronan, being synthesized by the cumulus cells, liberating free bikunin, and producing a covalent bond between the heavy chains and hyaluronan [52]. However, in other studies, inter- α -trypsin inhibitor has been detected in mouse follicles before the LH surge by immunostaining with a polyclonal antibody [36]. This suggests that differing methodology used in these studies [36, 51] have lead to various conclusions as to when inter- α -trypsin inhibitor enters into mouse follicles. In other species however, inter- α -trypsin inhibitor, pre- α -trypsin inhibitor, and inter- α -like trypsin inhibitor exist in follicular fluids well before the LH surge and in follicle sizes smaller than preovulatory follicles (bovine

FIG. 2. Sections of bovine (A–C) and mouse (D) ovaries showing small preantral (A and D), early antral (B and D), and antral (C and D) follicles, with foci or pockets of follicular fluid (arrows) accumulating between the granulosa cells or in the antrum (asterisk). Tissues were fixed in Bouin solution and embedded in paraffin; the sections were stained with hematoxylin-eosin. Bars = 25, 50, 100, and 50 μ m in A, B, C, and D, respectively.



[37], porcine [53]). While inter- α -trypsin inhibitor-related molecules in follicular fluid are presumably not synthesized by granulosa cells and are derived from plasma, they could still contribute to osmotic potential of follicular fluid if their heavy chains cross-link to hyaluronan and they were retained in the follicular antrum. However, cross-linking of the heavy chains of inter- α -trypsin inhibitor to hyaluronan is catalyzed by tumor necrosis factor-stimulated gene 6 (*TNFAIP6*) [54], which is only upregulated after the LH surge at ovulation in pigs [53] and mice [55]. However, even a low level of expression of *TNFAIP6* in granulosa cells or transfer of heavy chains by means independent of *TNFAIP6* [54] could facilitate such cross-linking. Hyaluronan may also interact with proteoglycan link protein 1, which has been observed in follicular fluid of some species [56, 57]. Regardless of whether cross-linking to hyaluronan occurs, thus ensuring that hyaluronan remains trapped in the follicular fluid, the sizes of hyaluronan observed in bovine follicular fluid are far larger than the nominal blood-follicle barrier and thus unlikely to escape the follicular antrum anyway [37].

In our study [37] of osmotic potential in follicular fluids, we also discovered that DNA could exert osmotic potential. The DNA in follicular fluid is presumably derived from granulosa cells lining the follicular antrum. These granulosa cells do not appear to die by classic apoptotic mechanisms but rather by a process more common to cornification, releasing DNA of higher molecular weight than observed in apoptosis [58]. This DNA may be associated with larger molecules such as hyaluronan, as is suggested to occur in other tissues [59], and this may increase the osmotic effect of the hyaluronan. However, the DNA content in follicular fluid is probably not regulated and could easily be degraded by the release of cellular DNase. While contributing to the osmotic potential of follicular fluid, DNA is thus probably of less importance.

If granulosa cell production of large osmotically active molecules is the mechanism by which water is attracted into the follicular antrum, the water still has to move there. Evidence suggests that the granulosa cells can facilitate water transport

transcellularly via aquaporins. Aquaporins 7, 8, and/or 9 in rats [60] and aquaporins 1, 5, and 9 in pigs [61] have been detected in granulosa cells. By comparing the in vitro passive equilibration of water with that of inulin (with and without the addition of mercury chloride to block the aquaporins) in dissected large antral follicles from the rat (not specifically follicular fluid), it was demonstrated that 70% of the movement of water was transcellular and 30% pericellular. Herein lies a potential paradox. On the one hand, large plasma proteins up to 100 kDa in follicular fluid are in the same concentration as in plasma. If they gain access via a pericellular route, why do granulosa cells have or need aquaporins to facilitate transport of water, a molecule of very small size (molecular weight 18)? The aquaporins possibly have an additional role for transducing fluid at ovulation. Imaging studies of ovulating follicles show large volumes of fluid transudating the membrana granulosa and effectively flushing the follicle cavity (M. Brannstrom, personal communication). The aquaporins are perhaps present to transport other small-molecular-weight molecules or have other roles [15, 62]. Alternatively, the pericellular route for plasma proteins might not be as efficient as we assume. It could be that these proteins are transported by transcytosis involving vesicular trafficking from the basal to apical surfaces of the granulosa cells. Such a mechanism may not exclude large-molecular-weight molecules effectively. However, these could have already been proportionally excluded based on size by the follicular basal lamina. Caveolins are involved in vesicular trafficking and have been detected in bovine granulosa cells, and while they appear to be upregulated after the LH surge [63], transcytosis does not appear to be a major activity during follicular growth. Thus, the paradox remains unresolved.

OVARIAN FOLLICULAR ANTRUM FORMATION

Formation of a cavity or antrum in a follicle is more complex than for a simple epithelium. In a simple epithelium, cell-cell junctions exist only between the single layer of cells lining the basal lamina and not with cells on the opposite side of the cavity; because of this, a potential cavity already exists

between the two opposing layers of epithelial cells, even if not expanded. The ovarian follicle is different. It has multiple layers of cells before a cavity develops, and presumably cells within these layers have junctions with all neighboring cells. During growth of preantral follicles, multiple foci of fluid accumulate first, and as these expand and coalesce, a larger centrally located antrum develops (Fig. 2). These foci of fluid presumably accumulate in areas where there are fewer cell-cell contacts, or other specific events allow these foci to develop and then coalesce. Cavities or lumens can be formed by cell death, as occurs in blastocysts (where the inner ectodermal cells undergo apoptosis to create a cavity) [64, 65] and during in vitro "tube" formation [66] or in vivo lumen formation by endothelial cells [67]. It is possible that such events take place in preantral follicles, as dead granulosa cells are occasionally observed in apparently normal healthy follicles. If these cells were to die, fluid could accumulate in the space left, with DNA providing the osmotic force. While such a mechanism could be involved in the initial formation of foci of follicular fluid, it is unlikely that it would operate as the follicle enlarged to the antral stage. This would be particularly true in species with proportionally large follicular antral cavities, as substantially more death would need to occur than has been observed. However, such death may play a more substantive role in species with proportionally smaller follicular antral cavities such as mice or rats.

Whatever the mechanism by which small cavities develop within a follicle, osmotically active molecules would additionally need to be directionally secreted toward these foci in order for them to fill with fluid. In three-dimensional culture of bovine granulosa cells under anchorage-independent conditions, we previously observed directional secretion of glycosaminoglycans into small cavities within the colonies (Fig. 3) [68]. We noted that granulosa cells surrounding these small cavities "had proteoglycan granules at their surfaces and in the tight spaces between them, thus appearing to direct their secretion of proteoglycans towards these areas" [68]. Whether or not this is similar to the foci of follicular fluid formation is not known, but it does show that adjoining granulosa cells can coordinately directionally secrete proteoglycans. Additional evidence of directional secretion comes from the observation that hyaluronan and proteoglycans are located on the apical side of granulosa cells facing the follicular fluid [69; see *Note Added in Proof*].

As the follicle grows, the expansion of the follicular antrum clearly requires remodeling of the theca interna and externa, stroma, tunica albuginea, and surface epithelium. This remodeling presumably has much in common with growth of other glands that can grow and penetrate and even branch within stroma by replication of cells at their leading edges [70, 71]. Apart from the obvious need to remodel matrix and to expand the theca and its vasculature, the follicle eventually expands toward the surface of the ovary. An expansion would normally be in the direction of the area with the least resistance, but clearly the tunica is more collagenous than the stroma, so presumably remodeling of matrix in the tunica albuginea adjacent to the expanding follicle is an important process in follicular development. This process could be rate limiting in large antral stages; it may also be occurring to different degrees in dominant and subordinate follicles.

During regression and involution of follicles, the follicular fluid is resorbed. This could involve a number of mechanisms. In the bovine, the follicular basal lamina is not degraded during regression as it is at ovulation [72], so there cannot be a dramatic loss of fluid. However, cells from the theca, including

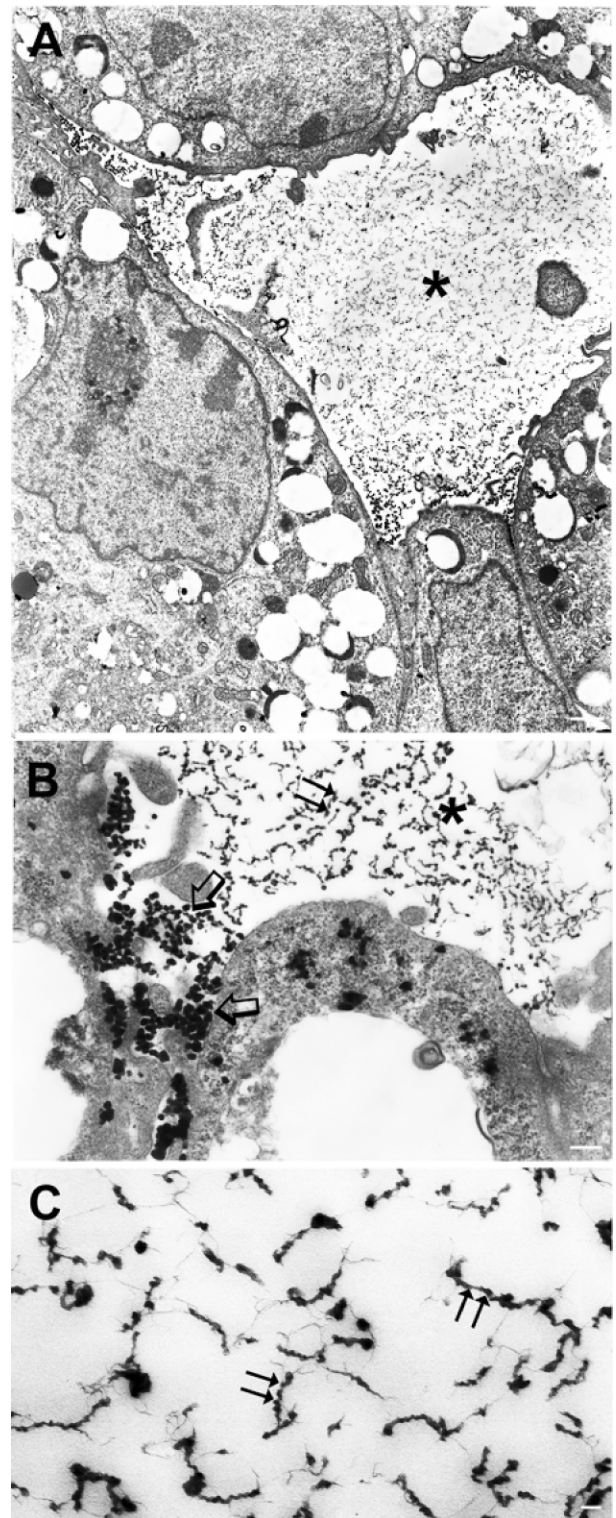


FIG. 3. Electron micrographs of extracellular matrix produced by a colony of granulosa cells cultured under anchorage-independent conditions and stained with ruthenium red. Ruthenium red forms an electron-dense granule by interacting with glycosaminoglycans, and each granule is believed to represent a proteoglycan. Asterisk denotes a cavity bounded by numerous granulosa cells. The large arrows denote ruthenium red proteoglycan granules near the surface of adjoining cells facing the cavity and not associated with fibers; small arrows denote fibers with associated ruthenium red granules in the cavity. Bars = 1 μ m (A), 200 nm (B), and 50 nm (C). (Reprinted from Rodgers et al. [68], with kind permission from Springer Science+Business Media.)

macrophages, endothelial cells, and fibroblasts, are able to penetrate through the follicular basal lamina [72]. This occurs when the basal lamina is no longer lined with granulosa cells [72], and it is possible that some fluid escapes via gaps made by traversing cells and is resorbed by lymphatic vessels. In addition, the osmotically active molecules within follicular fluid could be degraded by enzymes. We showed that removal of the glycosaminoglycans, hyaluronan and chondroitin sulfate/dermatan sulfate, and DNA from the follicular fluid of healthy follicles resulted in a reduction in osmotic pressure, but there was less effect following the removal of chondroitin sulfate/dermatan sulfate from the follicular fluid of atretic follicles, suggesting that these molecules may have already been degraded [37]. Many enzymes involved in degradation of glycosaminoglycans are located either on a cell surface or in lysosomes [73]; hence, cells need to be active and present in the follicular antrum to be able to degrade these molecules, unless only the protein component of proteoglycans is degraded, which could be accomplished by the release of proteases. It has been observed that hyaluronidases 1, 2, and 3 are upregulated in granulosa cells in atretic mouse follicles [74]. However, because in atresia the granulosa cells continue to die, their role in the metabolism of osmotically active molecules in later stages of regression and fluid resorption would be limited. Furthermore, our own investigations have demonstrated that, once follicular growth is arrested, granulosa cells show evidence of cell death [72]. Thus, we conclude that, while resorption of follicular fluid is important for the removal of the follicle from the ovary, it is probably not an early or initiating event in atresia.

REGULATION OF FOLLICULAR FLUID

In vivo, follicle-stimulating hormone (FSH) in particular stimulates follicular growth. The FSH leads to more follicles growing, while a reduction in FSH levels leads to fewer follicles growing, but does it actually stimulate expansion of the follicular antrum or is it merely stimulating overall follicle development? In vitro using follicle culture, a number of factors have now been shown to promote follicle growth, including antrum formation; again, the same question can be asked: are they stimulating follicular antrum expansion directly or indirectly by stimulating follicle growth? Transgenic knockout mice might be useful to identify regulatory molecules involved in follicular fluid formation. Mice null for *Gdf9* [75], *Fshb* [76], FSH receptor (*Fshr*) [77], and *Igf1* [78] have defects in follicular development at the preantral or early antral stages. Without further examination, it is not possible to know if these molecules are important for overall follicle development or are specifically involved in antrum formation. Mice null for estrogen receptor α (*Esr1*) [79] and aromatase (*Cyp19A1*) [80] have large cystic hemorrhagic follicles, and this phenotype may involve upregulation of molecules normally involved in follicular fluid accumulation.

Studies on the regulation of the synthesis of osmotically active molecules also shed light on the regulation of the follicular fluid formation. Early investigations of proteoglycan synthesis showed that in vitro rat granulosa cells synthesize a proteoglycan containing glycosaminoglycans sensitive to chondroitinase ABC [42]. An elution profile by gel filtration enabled us to infer that this molecule is probably versican, the synthesis of which was observed to be upregulated by gonadotropins, prostaglandins E1 and E2, and testosterone [42]. Later investigations of versican mRNA specifically found that forskolin, phorbol ester phorbol-12-myristate-13-acetate, and FSH plus testosterone could upregulate mRNA levels of

versican in rat granulosa cells [48]. HAS2 can also be induced by gonadotropins in bovine granulosa cells [39]. Thus, it would appear that synthesis of osmotically active molecules can be regulated by endocrine and autocrine mechanisms, and this might lead to regulation of formation of follicular fluid.

It was previously concluded that the rates of granulosa proliferation and maturation are not tightly or coordinately regulated with the timing or rate of antrum formation or expansion [3]. This conclusion was based primarily upon the variability of the numbers of layers of granulosa cells at different follicle sizes [81], which would be constant for any size of follicle if replication and antrum expansion were coordinately regulated. These observations suggest a lack of tight coordination between granulosa cell replication and antrum expansion. Additional data support this concept. Late in follicular development when the rate of fluid accumulation is increasing, the mitotic index of granulosa cells is declining [82, 83]. While there is a linear increase in the cross-sectional area of the membrana granulosa with increasing follicle diameter, the follicular antrum increases exponentially such that cells account for approximately 27% of a bovine follicle at 1 mm in diameter but represent only 6% at 4 mm [84]. This growth is accompanied by reduced thickness of the membrana granulosa and decreased granulosa cell density [85]. This thinner membrana granulosa results in the most apically situated granulosa cells being closer to the thecal vasculature.

Bovine follicles with different phenotypes of shape of basal granulosa cells and morphology of follicular basal lamina have been postulated to have different rates of antrum expansion [86] relative to granulosa cell replication, and these follicles have oocytes of different qualities [87]. Could it be that lack of oxygen or nutrients at the center of the follicle triggers centrally located granulosa/cumulus cells to secrete osmotically active molecules? Could the oocyte secrete factors to stimulate production of osmotically active molecules by granulosa cells and hence stimulate antrum formation? Such concepts are attractive, as they may explain the lack of a tight coordination between granulosa cell replication and antrum formation. The former could also explain the multiple foci of fluid accumulation that occur before a larger centrally located antrum begins to develop.

Follicular fluid formation may also be regulated by changes in the degree of functionality of impediments and facilitators of fluid movement. Despite the paradox of aquaporin expression in granulosa cells, as already discussed, if aquaporins are important for transport of fluid into the follicle, then their differential regulation could be a major determinant of the transition from preantral to antral stages and of the rate of follicular fluid accumulation thereafter. Additionally, the degree of vascularization, particularly at the early antral stages, could regulate follicular fluid accumulation. If caveolins are involved in movement of fluid across the membrana granulosa and if then the follicular basal lamina is the site of differential filtration of molecules, as already discussed, both the structure of the follicular basal lamina and level of caveolin activity could be important regulators of follicular fluid accumulation. It is relevant to note the follicular basal lamina changes in composition at around the preantral and primary stages, when collagens type IV $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\alpha 6$ are reduced in bovine [88] and murine [89, 90], while nidogen 1 [45], nidogen 2 [91], and perlecan [45] are upregulated in bovine at the preantral stages. These changes could alter the permeability of the follicular basal lamina. With the advent of follicle culture, it may be possible to identify the key regulated components involved in follicular fluid formation.

CUMULUS-OOCYTE COMPLEX MATRIX

From the forgoing, it can be seen that some of the components of follicular fluid are those found in the matrix of the cumulus-oocyte complex at the time of cumulus expansion following the LH surge in vivo [92–96]. These include hyaluronan, versican, and (in some species with large follicles) inter- α trypsin inhibitor [37, 45, 47, 53]. Yet, follicular fluid in many species is not a gel like the matrix associated with the cumulus cells. There are many differences that presumably can account for this. These could include differences in absolute concentrations of matrix molecules, and it should be noted that there is very little information on the concentrations of any of these molecules for a direct comparison. Versican processing could be different in the cumulus-oocyte complex compared with that in the membrana granulosa of growing follicles, noting that ADAMTS1 (a disintegrin and metalloprotease with thrombospondin motifs-1) processing of versican in the cumulus-oocyte complex is induced during ovulation in mouse [97]. In the cumulus-oocyte matrix, pentraxin-3 binds *TNFAIP6* and may form multimeric complexes with hyaluronan [98]. It is probably these components that allow the matrix of the cumulus-oocyte complex to form a gel. Expression of *TNFAIP6* and pentraxin-3 is comparatively low in mural granulosa cells before stimulation with LH or at ovulation [99, 100]. Additionally, the cumulus-oocyte complex at ovulation is in contact with the larger-molecular-weight molecules from serum, which only cross the membrana granulosa as the follicular basal lamina is degraded.

CONCLUSIONS

The main hypothesis on follicular fluid formation suggests that production by granulosa cells of hyaluronan and additionally versican generates an osmotic gradient to recruit fluid from the thecal vasculature. It is notable that these molecules are associated with formation of the matrix of the expanding cumulus-oocyte complex, which has additional molecules capable of cross-linking or interacting with hyaluronan. Many questions still remain unanswered such as what initiates or regulates formation of follicular fluid and what changes in cell-cell junctions occur to accommodate its formation. There is the paradox of aquaporins in granulosa cells through which water apparently flows, while large serum proteins easily gain access to the follicular antrum. With these questions in mind, the advent of follicular culture provides some unique opportunities to study and understand these important processes.

NOTE ADDED IN PROOF

Additional information regarding the follicular localization of hyaluronan can be found in an article by Rogers and Irving-Rogers, 2005 [101].

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