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Extracellular Matrix Functions in Follicle Maturation

Courtney B. Berkholtz, B.A.[1,2], **Lonnie D. Shea, Ph.D.**[2,3], and **Teresa K. Woodruff, Ph.D.**[2,4,5]

1*Interdepartmental Biological Sciences Program, Northwestern University, Evanston, Illinois*

2*Center for Reproductive Research, Northwestern University, Evanston, Illinois*

3*Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois*

4*Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois*

5*Department of Medicine, Northwestern University, Evanston, Illinois.*

Abstract

The extracellular matrix (ECM) promotes and/or inhibits many cellular processes, including but not limited to proliferation, differentiation, and survival, which must occur for follicle growth and oocyte maturation. The ECM regulation of cellular processes in ovarian cells is being investigated in many animal models, including avian, rat, bovine, porcine, rabbit, sheep, human, and mouse. Granulosa cells are more frequently employed; however, the culture of intact follicles and ovaries has been developed and enables ECM functions in folliculogenesis to be studied. ECMcomponents that have been examined are used individually (collagen, laminin, fibronectin) or collectively (Matrigel, isolated basal lamina, and ECM produced by cell lines) in both two- and three-dimensional model systems. In granulosa cell cultures, ECM affects morphology, aggregation and communication, survival, proliferation, and steroidogenesis; whereas follicle and ovary cultures demonstrate a regulation of folliculogenesis. This article describes the ECM functionality on ovarian cells throughout development, and highlights the potential of developing technologies to identify structure–function relationships in follicle maturation.

Keywords

Extracellular matrix; granulosa cells; morphology; proliferation; survival

Ovarian follicle development is regulated by endocrine, paracrine, and autocrine factors in a spatially and temporally regulated manner, and is characterized by dramatic changes throughout maturation. The ovary contains a large reserve of inactive primordial follicles arrested in prophase I, with a small cohort activated daily. Prior to activation, these immature follicles contain nongrowing oocytes and nondividing, flattened (squamous) pregranulosa cells surrounded by a basal lamina. Following activation, follicle growth begins with the transformation of squamous pregranulosa cells into a single layer of cuboidal granulosa cells surrounding a growing oocyte. Granulosa cells proliferate to form multiple layers, requiring expansion of the basal lamina, and leading to two-layered and multilayered follicle stages. At this time, an outer layer of cells, termed the theca layer, is recruited (or differentiated from existing stroma[1]) and surrounds the basal lamina. A fluid-filled antral cavity, or antrum, forms in the granulosa cell compartment of the follicle later in folliculogenesis. Small antral follicles develop into mature preovulatory follicles in the presence of follicle-stimulating hormone

Address for correspondence and reprint requests: Teresa K. Woodruff, Department of Neurobiology and Physiology, Northwestern University, 2205 Tech Dr, Hogan 4-150, Evanston, IL 60208-3520. E-mail: tkw@northwestern.edu.
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(FSH). The oocyte from a mature follicle, characterized by a large antrum and the ability to respond to the luteinizing hormone (LH) surge, is ovulated. Developmental competence of an oocyte is correlated with follicular size, with larger oocytes being more developmentally competent. The basal lamina is degraded upon ovulation and the remaining granulosa cells differentiate into luteal cells and form the corpus luteum. Follicles unable to mature or ovulate undergo atresia (follicle death).

Tissue remodeling and turnover occurs during the folliculogenesis, suggesting that ECM is an important factor in folliculogenesis. Autocrine and paracrine signaling by secreted extracellular matrix (ECM) molecules can regulate follicle fate, yet these mechanisms have been studied less than the traditional growth factor and hormone regulation of folliculogenesis. ECM plays various roles in cellular development in many systems.[2,3] The role of ECM in granulosa cell function has been studied since the early 1980s[4]; however, ECM regulation of follicle and ovarian culture have developed more recently, which has been possible due to advancements with in vitro culture systems. In ovarian cells, ECM influences a variety of cellular processes, such as cell morphology, aggregation and communication, proliferation, survival, and steroidogenesis. This review describes the functions of ECM in the ovary and identifies new frontiers for investigation.

EXTRACELLULAR MATRIX

The ECM is known to participate in the regulation of a variety of cellular functions for many tissues, such as the ovary. In vivo, ECM is the natural substrate supporting cellular processes such as adhesion, migration, survival, and proliferation, which results from a combination of mechanical and chemical signals. The structure of the ECM provides mechanical support to the tissue, whereas the biochemical composition can interact directly with cells through specific receptors or can bind growth factors that are released upon matrix degradation.[5] In addition, the ECM can act as a barrier that restricts cellular access, thereby defining specific cellular compartments and specialization necessary for proper function.[6] Overall, the ECM is a significant contributor to the microenvironment by providing the signals to support differentiated cell function.

The ECM is composed of a variety of molecules, which can include collagens, laminin, fibronectin (reviewed elsewhere in this issue), proteoglycans, and polysaccharides. These components are being used individually or in combinations, such as in Matrigel and ECMs produced by different cell lines, for in vitro culture systems. For example, Matrigel is composed of solubilized basement membrane from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. This tumor is rich in ECM proteins including laminin, type IV collagen, heparan sulfate proteoglycans, entactin, and nidogen.[7] Fibronectin, laminin, and certain collagens have been localized in the ovaries of many species and their effects on follicle development have been examined (Huet et al,[8] Bortolussi et al,[9] Luck,[10] van Wezel et al[11]; and Berkholtz et al, unpublished data, 2006). Type IV collagen and laminin are localized specifically to the basal lamina of follicles, a specialized ECM found between the epithelioid granulosa cells and the mesenchymal theca cells. Those cells located adjacent to the basal lamina have different characteristics than those cells further away, suggesting that this interaction influences function.[6,12,13]

An alternative to whole-ECM proteins or combinations involves using known sequences of matrix peptides, such as RGD (Arg-Gly-Asp). Whole ECM molecules are large and can be difficult to engineer or manipulate. In contrast, small peptide sequences can be readily manipulated, yet may not recapitulate the entire functionality of the intact ECM molecule. Peptide sequences can provide a simplified system in which to identify the function of specific regions. For example, the RGD motif is common to many ECM proteins, whereas other

sequences are more specific, such as the laminin-derived IKVAV and the tenascin-C derived VFDNFVLK. These short peptides can be adsorbed directly to surfaces, or covalently attached to synthetic matrices that would not normally mediate cellular interactions. [14,15] Granulosa cells have been shown capable of interaction with the RGD sequence on a two-dimensional (2D) surface. This interaction affects attachment, spreading, and steroid production of granulosa cells.[16] In a three-dimensional (3D) system, RGD was also able to effect two-layer secondary follicle growth but not multi-layered follicle growth compared with other ECM molecules. Oocyte competence (from multilayered follicles) was increased in the presence of RGD compared with other ECM molecules.[17]

Granulosa cell adhesion and spreading on ECM-modified surfaces indicates their ability to interact specifically with ECM molecules, which has been linked directly to integrin ligation. Integrins are transmembrane receptors composed of α:β heterodimers, which are necessary for development and function of all tissues. There are 24 members of the human integrin family, 18 α- and eight β-subunits, and the various combinations of these proteins can provide specificity for interacting with different ECM molecules.[18] Integrins provide the mechanism for interacting with the matrix, and transduce signals that ultimately influence cellular behaviors. [15,19] Upon binding, integrins initiate signaling cascades involved in cell migration, endocytosis, and proliferation.[20] The expression and localization of integrins in wild-type and knockout mouse ovaries have been determined, providing insight into the function and regulation of cell-matrix interactions. Integrins recognize specific motifs in matrix proteins, including the RGD motif.

REGULATION OF OVARIAN CELL MORPHOLOGY

Cell morphology describes many cellular characteristics including shape, attachment, and cytoskeletal arrangement. The ECM plays a substantial role in structural organization and function, affecting shape and the reorganization of the cytoskeleton.[21] The experimental systems to investigate ECM function in cellular responses generally can be categorized into three categories: (1) cells cultured on ECM proteins adsorbed to tissue culture plastic or other substrate, (2) cells cultured on a hydrogel (2D culture) containing ECM proteins, and (3) cells encapsulated within a hydrogel (3D) containing ECM proteins. The cellular responses in these three systems are dependent upon the identity and density of the ECM, the structure of the ECM (2D versus 3D), and the mechanical integrity of the gels.

Cell Shape and Attachment

Cellular shape affects processes such as metabolism, attachment, and migration,[22–25] and employing the different in vitro culture systems can investigate the structure–function relationships to help clinicians understand cellular function in vivo.[26] Cell adhesion is perhaps the first indicator of interaction with the ECM, which subsequently leads to influences on cell shape. Porcine granulosa cells have increased attachment after plating on type I or IV collagen, fibronectin, and laminin compared with tissue culture plastic. Note that cells cultured on laminin and type IV collagen surfaces have a round morphology.[27] Rat and human granulosa cells plated on bovine corneal epithelium ECM or type I collagen gel attached significantly faster compared with cells plated on tissue culture plastic (all cells were observed as round/epithelioid in shape).[28–30] The interaction between the granulosa cell and ECM is highly regulated and specific, given that ovine granulosa cell binding to laminin could be reduced with antibodies specific to anti- α_6 integrin antibody.[31] In addition to the ECM identity, the density of ECM molecules can regulate granulosa cell adhesion.[16]

The normal granulosa cell morphology in vivo is round, and this round shape is not always maintained in vitro. Granulosa cells seeded onto uncoated tissue culture plastic flatten in a monolayer and spread with little interaction between neighboring cells,[21,29,30,32–36] similar

to fibroblast cells. This phenotype is also observed when granulosa cells are cultured on some ECM-coated surfaces. Mouse, rabbit, and ovine granulosa cells plated on laminin, fibronectin, poly-lysine, and RGD-modified surfaces spread and are flat.[16,37,38] However, granulosa cells maintain their spherical, epithelioid shape when plated on type I collagen gels, isolated basal lamina, and Matrigel.[21,32,33,38,39] Chicken granulosa cells plated on basal lamina or plated with solubilized basal lamina isolated from large preovulatory chicken follicles remain round in comparison to the cells plated on tissue culture plastic, which flatten within 60 minutes.[32] Similarly, granulosa cells from human, rat, ovine, porcine, and rabbit plated on ECM (type I collagen, EHS, or ECM produced by bovine corneal epithelial cells of mouseHR9 cells) also become spherical.[21,27–29,33,34,37–39] Whole bovine follicles cultured on type I collagen, fibronectin and Matrigel attached maintaining their spherical shape, whereas follicles on tissue culture plastic or laminin did not remain in the culture well after agitation.[40]

The development of novel surfaces for granulosa cell culture have been developed by attaching adhesive peptides to otherwise nonadhesive materials, which can correlate specific sequences to granulosa cell behavior. One such material, alginate, is a polysaccharide isolated from algae, and when cultured alone with mammalian cells promotes minimal cell adhesion.[14] Mouse granulosa cells plated on RGD-modified alginate attached to the surface, whereas there was little attachment to unmodified alginate.[16] Attachment to the ECM by granulosa cells is indicative of a specific binding reaction and is supported by this alginate-RGD system. Steroid production by adhered granulosa cells was density dependent, and this system could be used to investigate a variety of other peptide sequences, or combinations in granulosa cell function.

Regulation of the Cytoskeleton

The morphology of the granulosa cell is maintained by an intricate cytoskeleton arrangement. Rat granulosa cells plated on ECM from bovine corneal epithelium have fewer actin cables, maintain their epithelioid shape, and have a diffuse actin pattern compared with those cells on tissue culture plastic, which have numerous stress fibers. Gene expression of viniculin, α-actininin, and actin reveal that those cells on ECM had lower expression compared with cells on tissue culture plastic. [21] Actin cytoskeleton development occurred in a similar system on both bovine corneal epithelium and laminin.[41]

REGULATION OF CELL AGGREGATION AND COMMUNICATION

Cellular communication in the follicle is maintained through a highly organized network of connections between the granulosa cells and the oocyte and among the granulosa cells themselves.[42–44] Gap junctions between the granulosa cells are necessary for passage of small metabolites, ions, and second messengers.[45] Aggregation of granulosa cells in culture mimics the in vivo environment in which granulosa cells are in constant contact with one another within the follicle. This aggregation is either maintained or stimulated for culture in Matrigel.[46] Human[36,47] and rat[21,28] granulosa cells formed multilayered aggregates on the Matrigel surface as well as on ECM from bovine corneal epithelium. Cell-to-cell communication can be disrupted in the flat architecture represented in 2D systems, unlike those cells that aggregate on particular ECM surfaces or within 3D hydrogels. Those cells maintaining a spherical shape in vitro have increased cytoplasmic processes and gap junctions[21,29,39,47] for proper communication between neighboring cells.

SURVIVAL

The ECM composition can influence survival of granulosa cells, which generally do not survive beyond a few days in culture.[48,49] In bovine, the ECM composition changes when a follicle becomes atretic.[9,50,51] Rat, ovine, and human granulosa cells plated on tissue culture plastic either become apoptotic or undergo rapid cell death, whereas granulosa cells plated on ECM

survive many days in culture.[36,37,41] Rat granulosa cells survive on HR9 mouse endodermal ECM and laminin, whereas human granulosa cells survive on Matrigel.[36,41] Ovine granulosa cells plated on fibronectin and laminin have significantly increased survival compared with those cells plated on type I collagen-coated plates, RGD-modified tissue culture plastic, type I collagen gels, tissue culture plastic alone, poly(2-hydroxyethyl methacrylate) (pHEMA)-coated plates, and heparin-coated plates.[37] Human ovarian tissue slices cultured on Matrigel have an increased number of viable follicles 2 weeks after the beginning of culture.[52]

PROLIFERATION

Granulosa cell proliferation continues throughout folliculogenesis, providing not only a specialized microenvironment for oocyte growth, but also nutrients for oocyte growth. Bovine granulosa cells plated on bovine corneal epithelium ECM proliferate more rapidly than cells plated on tissue culture plastic, which require the presence of basic fibroblast growth factor (bFGF) to mimic the proliferation observed on ECM.[4] In a bovine granulosa cell line, the ED-1 domain of fibronectin (as a recombinant peptide in culture) promotes thymidine incorporation in a dose-dependent manner.[53] Fibronectin and laminin increase proliferation of ovine granulosa cells.[37] Laminin's effect on the promotion of proliferation in ovine granulosa cells is associated with its interaction with the integrin α6β1.[31] In a 3D culture system for intact follicles, type I collagen mixed with alginate and RGD-modified alginate promoted an increase in size of two-layer follicles yet had no significant effect on multilayered preantral follicles. Laminin and fibronectin mixed with the alginate actually inhibited the proliferation of granulosa cells in multilayered follicles.[17] For follicles plated on either type IV collagen or laminin, 5-day-old mouse ovaries have increased follicle densities and increased follicle growth initiation, compared with ovaries plated on poly-lysine. Addition of activin A to these cultures increased multilayer follicle development (an increase in the ratio of multilayered follicles to the total number of follicles) when ovaries were cultured on laminin. When ovaries were cultured on type IV collagen, multilayered follicle growth was suppressed in the presence of activin A.[54] The examination of the effect ECM has on proliferation in the above studies indicates that the stage of the follicle, the absence or presence of particular growth factors, and the ECM present all contribute to the regulation of cell behavior.

STEROIDOGENESIS

Regulation of Progesterone

Progesterone plays a central role in ovulation and subsequently establishing and maintaining pregnancy. The progesterone receptor knockout mouse models of infertility and subfertility indicate the importance of progesterone in the late stages of folliculogenesis,[55] and ECM can influence progesterone production. Mouse granulosa cells were stimulated to produce progesterone when plated on RGD-modified alginate compared with alginate alone.[16] In rat and human granulosa cells, progesterone levels have been shown to be increased in the presence of bovine corneal endothelial ECM, HR9 mouse endodermal ECM, fibronectin, laminin, and type I collagen, but decreased with Matrigel.[21,28,29,34,39,41,46] In contrast, human granulosa cells plated on laminin have decreased progesterone levels compared with uncoated plates.[56] Type I collagen coating, type I collagen gels, poly-hema modified plates, and heparin-coated plates affect the production of progesterone negatively in ovine granulosa cells isolated from large follicles (4 to 7 mm).[37] Ovine granulosa cells plated on laminin, in the presence of anti- α_6 antibody, have decreased levels of progesterone compared with those cells that did not come in contact with the antibody.[31] Again, this finding indicates the importance of the interaction between the ECM and integrins. Chicken granulosa cells plated on isolated basal lamina or with solubilized basal lamina from preovulatory chicken follicles resulted in different progesterone production.

Immature and mature chicken granulosa cells plated with the solubilized basal lamina all showed increased progesterone production compared with cells plated on tissue culture plastic. However, when plated on solid basal lamina, chicken granulosa cells from mature follicles had decreased progesterone production, whereas the immature granulosa cells mimic the effect seen in liquid basal lamina.[57] This finding suggests that not only is the stage at which granulosa cells are isolated important, but also the form of the ECM employed.

Porcine granulosa cells showed increased progesterone production when plated on type I collagen, fibronectin, and laminin,[27] but when type I collagen gel was overlaid on the cells plated on type I collagen, progesterone production was decreased.[58] This finding that adding another dimension affects steroid production will be important in examining results in 2D and 3D systems. As with the examination of proliferation, progesterone production is affected by many variables including the maturity of the granulosa cell, the type of ECM, the method by which the ECM is presented (liquid versus solid), and the dimensionality of the culture (2D versus 3D).

Regulation of Estradiol

Estradiol is involved in overall follicle growth through FSH stimulation, LH receptor expression, antrum formation, gap junction development, and prevention of atresia.[59] Human granulosa cells on serum-coated tissue culture plastic did not produce estradiol without the addition of androstenedione to the media. In the presence of this steroid, however, estradiol and testosterone were secreted.[33] Yet, human granulosa cells on type I collagen gel have been shown to secrete estradiol without the addition of steroid.[29] Mouse granulosa cells secreted estradiol when plated on RGD-modified alginate but not alginate alone.[16] Ovine granulosa cells isolated from large follicles had increased estradiol secretion on type I collagen-coated tissue culture plastic, type I collagen gels, pHEMA, and heparin-modified tissue culture plastic, but decreased secretion on fibronectin and laminin surfaces.[37] In the presence of the anti- α_6 antibody, ovine granulosa cells on laminin had increased estradiol production,[31] again indicating a specific interaction between ECM and granulosa cells. These results are similar to those for progesterone in terms of how the ECM type and the dimension affect the ability of granulosa cells to produce estradiol.

Regulation of Steroidogenic Enzymes

Steroidogenesis is a vital part of folliculogenesis as demonstrated by the knockout mouse models of steroid receptors.[60–62] The alterations in progesterone and estradiol secretion have also been investigated for alterations in the steroidogenic enzymes. Expression of cholesterol side-chain cleavage cytochrome P450 ($P450_{scc}$) and the steroid acute regulatory protein was examined in rat granulosa cells plated on HR9 mouse endodermal ECM modified with bFGF or plated on laminin alone. Only those cells on the bFGF-modified ECM maintained expression, whereas those on laminin did not.[41] Porcine granulosa cells plated between type I collagen gels had reduced $P450_{scc}$ gene expression when compared with those cells without the overlaid gel.[58] Yet, bovine granulosa cells cultured in an anchorage-independent system had higher levels of $P450_{scc}$ and 3β-hydroxysteroid dehydrogenase in response to cyclic adenosine monophosphate.[63]

DIFFERENTIATION

At the time of ovulation, granulosa cells differentiate into luteal cells, which would produce the hormones required to sustain pregnancy until placental development. Human granulosa cells are isolated when oocytes are removed for in vitro fertilization (IVF), making these cells mature and ready to undergo differentiation. Characteristics of these differentiating cells are mitochondria clustering, increased number of lipid droplets, microvilli, and clustering

granulosa cells forming lumen-like structures. [64] Human granulosa cells cultured on Matrigel contain numerous lipid droplets, microvilli, and lumen-like structures.[36] Human granulosa cells cultured on bovine corneal epithelium ECM contain lipid droplets, [30] mitochondria clusters, and lysosomes,[39] indicating that Matrigel and bovine corneal epithelium ECM affect cell differentiation. This cellular behavior is not repeated on tissue culture plastic. Similar to human granulosa cells isolated at the time of IVF, rat granulosa cells isolated from pregnant mare serum gonadotropin-primed preovulatory follicles plated on laminin become enlarged and are referred to as steroidogenic cells.[46] The ECM is not only involved in the development and maturation of the follicle but also the differentiation of granulosa cells after ovulation.

ADDITIONAL EFFECTS OF ECM

Some studies have indicated the ECM is also involved in protein expression, cellular currents, and oocyte maturation. Rabbit granulosa cells have different levels of zona pellucida proteins depending on the culture surface— poly-D-lysine or EHS.[38] Bovine granulosa cells cultured in an anchorage-independent system produced their own matrix consisting of type IV collagen and fibronectin. [63,65] Granulosa cells isolated from chicken follicles plated on basal lamina or in the presence of solubilized basal lamina from preovulatory chicken follicles had a slower Ca^{2+}-dependent Cl^{-} current than those granulosa cells plated on tissue culture plastic.[66] The presence of ECM not only affects the granulosa cells but the oocyte. In the 3D mouse follicle culture system, fibronectin-, laminin- and RGD-modified alginate increase the rate at which oocytes from multilayered follicles proceeded to MII and polar body extrusion compared with unmodified alginate alone.[17] The ECM is a dynamic regulator involved in numerous cellular processes in all follicular cell types.

FUTURE DIRECTIONS

ECM regulation of cellular processes and tissue development has been documented in virtually all organs, and the basic techniques have also been applied to studies in the ovary.[49] Recently, the introduction of tissue engineering principles to investigate structure–function relationships have advanced the understanding of follicle development and provided a versatile tool set for additional studies.[17,67,68] These systems have been adapted for the 3D culture and maturation of ovarian follicles in vitro, given that these systems more faithfully mimic the in vivo follicle environment. The ability to present ECM molecules to the follicle in 3D, rather than just the granulosa cells in 2D, is critical to identifying the structure function relationships for the ECM. As mentioned, the dimensionality of a system affects multiple processes, such as proliferation, steroidogenesis, and morphology. These and similar systems have the potential to advance our understanding of the signals that regulate folliculogenesis, given that these systems provide the 3D context in which both soluble and insoluble signals can be manipulated to identify signals that promote or inhibit folliculogenesis.

CONCLUSION

Many studies of folliculogenesis examine growth factors and hormones in follicle development, ignoring the structure and composition of the ECM. Type I and IV collagen, fibronectin, laminin, Matrigel, and other ECM proteins or combinations affect the morphology, survival, proliferation, and steroidogenesis of granulosa cells, follicles, and whole ovaries in culture. The ECM is a significant contributor to the follicle microenvironment, and the role of growth factors and hormones and their respective signaling pathways must be considered within the context in which they are presented. The adaptation of synthetic matrices for the culture of granulosa cells, follicles, and ovaries provides a tool with which to investigate the contributions of each component.

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