[Frontiers in Bioscience 7, d1990-1996, September 1, 2002]

CELL-CELL INTERACTIONS IN PRIMORDIAL FOLLICLE ASSEMBLY AND DEVELOPMENT

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1. ABSTRACT

Two critical processes in ovarian biology are the assembly of the primordial follicles early in development and then the subsequent development and transition of the primordial follicle to the primary follicle. These processes directly effect the number of oocytes available to a female throughout her reproductive life. Once the pool of primordial follicles is depleted a series of physiological changes known as menopause begins in humans. The inappropriate coordination of these processes contributes to ovarian pathologies such as premature ovarian failure. Studies demonstrate primordial follicle assembly and development are coordinated by locally produced paracrine and autocrine factors. Factors have been identified that influence follicular assembly such as neurotropins. Several local factors that promote the primordial to primary follicle transition have also recently been identified. These include growth factors such as kit-ligand, leukemia inhibitory factor and basic fibroblast growth factor. Interestingly, recent studies demonstrate Müllerian inhibitory substance appears to inhibit the primordial to primary follicle transition. Therefore, observations suggest a mechanism for both positive and negative control of the primordial to primary follicle transition. The studies reviewed regarding the control of primordial follicle assembly and the primordial to primary follicle transition help elucidate these poorly understood aspects of ovarian biology.

2. INTRODUCTION

The assembly of the developmentally arrested primordial ovarian follicle and the subsequent transition of the primordial follicle to the primary follicle is a poorly understood process critical for female reproduction. The primordial follicle consists of an oocyte arrested at the

diplotene stage of prophase one of meiosis and is surrounded by squamous epithelial or pre-granulosa cells. The primordial follicles assemble directly after birth in rodents and by the third trimester in humans and cattle. Follicles assemble from clumps of newly proliferated germ cells known as ovarian nests. In the process of assembly the oocytes separate from each other and become surrounded by the pre-granulosa that are speculated to come from the ovarian stroma or the ceolomic epithelium. The primordial follicles remain developmentally arrested until individual follicles leave the resting pool and change into growing primary follicles. This is termed the primordial to primary follicle transition. A primary follicle displays a larger oocyte and a layer of proliferating cuboidal granulosa cells. Once a follicle has made the primordial to primary follicle transition it continues development unless it is destroyed by follicular atresia or it ruptures at ovulation.

The coordination of primordial follicle assembly and the primordial to primary follicle transition are of great importance in determining female reproductive competence. Primordial follicles do not proliferate after birth and are the sole source of gametes for a female's reproductive life. This primordial follicle source is termed the primordial follicle pool. When primordial follicles are exhausted a series of physiological changes associated with menopause begins in humans. Therefore, the proper control of the size of the primordial follicle pool is critical to maintain female fertility. Inappropriate control of follicular assembly or inappropriate activation of primordial follicle development appears to be a cause of certain reproductive diseases. The most notable of these is a set of conditions known collectively as premature ovarian failure. In these conditions the pool of primordial follicles is lost and the individual enters a precocious menopause. The investigation of the factors that coordinate primordial follicle assembly and the primordial to primary follicle transition is critical to develop potential therapeutic treatments for these conditions.

Primordial follicle assembly and the primordial to primary follicle transition appear to be regulated by locally produced ovarian factors. Follicles in ovaries and ovary fragments grown in serum free culture are fully competent to assemble and develop into primary follicles. A number of studies have investigated the role of locally produced factors in primordial follicle development. Observations reviewed below start to elucidate the control mechanism in assembly and the primordial to primary follicle transition.

2.1. Ovarian development

A useful description of ovarian development can be found in Hirshfield's review "Development of Follicles in the Mammalian Ovary" published in 1991 in the *International Review of Cytology* (1). The ovary develops early in embryonic life from the indifferent gonad. The indifferent gonad is formed from mesenchymal cells derived from the genital ridge. The structure is covered by a layer of coelomic epithelium (2). The germ cells that form the oocytes have an extra-ovarian origin. The germ cells migrate from the yolk sac starting at about embryonic day 8 in the mouse. They arrive at the genital ridge and enter the indifferent gonad around embryonic day 11-12 (E11-E12) (3). After the germ cells enter the gonad they organize into structures called ovarian germ cell nests. These consist of a pocket of germ cells surrounded by a layer of stromal cells. The origin of the stroma surrounding the germ cell nests is unclear (1, 4). Some evidence points to an invasion of the coelomic epithelium surrounding the ovary (4). Most of the available evidence points to cells originating from the ovarian rete (1, 5, 6). This distinction is important since the stromal layer appears to be the likely source for the pre-granulosa surrounding the primordial follicle.

After germ cell colonization a period of rapid germ cell proliferation begins. In rodents, this occurs between E11 and E12. In the mouse the number of germ cells increase from 25,000 to 83,000. The maximum number of germ cells in the rat has been determined at 75,000 (7). When the period of germ cell proliferation ends the cells enter meiosis (8). The germ cells are considered to be 'oocytes' when they enter meiosis. The oocytes enter meiotic arrest early at the diplotene phase of prophase 1 (9). The oocyte will not finish meiosis 1 until ovulation. Meiotic oocytes are first seen in the mouse at E14 when proliferation ends (10). While meiotic oocytes are first seen in humans at the second month of pregnancy the process is a very heterogeneous one. In humans the last germ cells enter meiosis at the seventh month of pregnancy (11). After the oocytes enter meiotic arrest a short but significant period of oocyte attrition begins. This results in a large decrease in the total number of oocytes and is contiguous with the time of follicular assembly. The number of oocytes in the rat plummets from 75,000 to 27,000 between E18.5 and postnatal day 2 (P2) (7). The apoptotic factor tumor necrosis factor appears to stimulate this oocyte atresia *in vivo* (12). A recent hypothesis is that this apoptosis isolates oocytes to facilitate the assembly of the primordial follicle (4).

2.2. Primordial Follicle Assembly

Follicular assembly is the process by which the primordial follicle is formed. Previous studies suggest they form by the fragmentation of the ovarian "nests" of oocytes (13, 14). A recent model suggests the oocyte attrition occurring at the same time drives follicular assembly leaving only a few oocytes in each cord to be covered by the surrounding stroma (15). The timing of follicular assembly depends on the species. In rodents it is a very regular process. All the remaining oocytes have formed follicles within three days post partum (16). The process is more heterogeneous in large monovulators such as the primate and the cow. These species have developing follicles and unassembled oocytes in the same fetal ovary (17).

The coordination of primordial follicle assembly seems to occur through locally produced factors. Of particular interest is the lack of importance of gonadotropins in this process. It has been demonstrated that mouse oocytes are fully competent to assemble into follicles in culture in the absence of hormones (18). It has

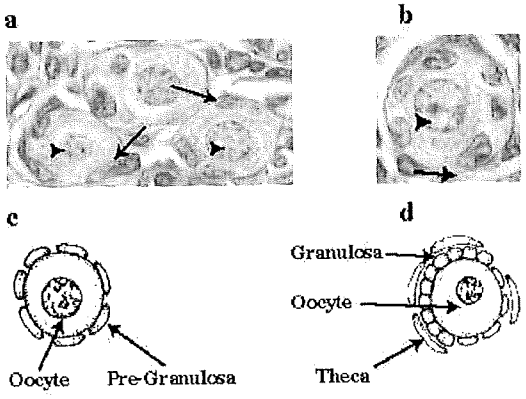

Figure 1. Primordial and Primary Follicle Morphology. a) Primordial follicles, an oocyte(arrowhead) encapsulated by squamous granusloa (arrow), 200x magnification. b) Primary follicles, an oocyte(arrowhead) encapsulated by cuboidial granulosa (arrow), 200x magnification c) Primordial follicle schematic and d) transitional follicle schematic with some cuboidal and squamous granulosa.

also been noted that hamster oocytes are able to form follicles in culture (19). Null mutant mouse studies have also been useful in determining the gonadotropin independence of follicle assembly. It has been observed that luteinizing hormone receptor knockout mice have normal folliculogenesis until the pre-antral stage of development (20). Follicle stimulating hormone receptor knockout mice also have normal folliculogenesis until the pre-antral stage (21). In contrast, the mice born to mothers given anti-FSH antibodies showed a delay in normal follicle assembly (22). Key locally produced factors appear to be derived in part from the oocyte. Busulfan-sterilized rats lacking germ cells have structures resembling ovarian "nests" but at no time will follicle like structures form (23). Ectopic germ cells that have inappropriate migration to the adrenal gland will form structures resembling primordial follicles (24). Locally produced neurotropins have been found to be significant in the process of follicle assembly. Null mutation studies have found that either the neurotropin-4 (NT-4) or brain derived nerve factor (BDNF) are needed for properly timed follicular assembly (25). Both BDNF and trkB (the BDNF and NT-4 high affinity receptor) mRNA levels increase at the time of follicular assembly then decrease when the process is over. The null mutant mouse for the neurotropin nerve growth factor (NGF) displays a higher number of oocytes unassembled into follicles than the wild type controls (26). Levels of mRNA for trkA (the NGF high affinity receptor) have been found to be higher at the time of follicular assembly. Other factors involved in primordial follicle assembly remain to be identified. A transcription factor has been found to be required for primordial assembly. Null mutant mice born without the basic helix-loop-helix transcription factor Fig α have normal appearing ovarian germ cell nests (27). However, histological examination of these mice found they do not undergo follicle assembly (27).

2.3. Primordial to Primary Follicle Transition

The control of the primordial to primary follicle transition is more defined than for primordial follicle assembly. When primordial follicles initiate development the squamous granulosa cells develop a cuboidal epithelial morphology (Figure 1). The granulosa cells also begin proliferating. Since all cells in the primordial follicle except the oocyte proliferate, a proliferation marker such as the proliferating cell nuclear antigen (PCNA) can be used to detect the primordial to primary follicle transition (28). Unfortunately, this stain cannot be used to detect the transition in primordial follicles of neo-natal ovaries due to the level of cell growth at this period (29).

The primordial to primary follicle transition appears to be coordinated by locally produced factors. Primordial follicles in culture of rodent ovaries are fully competent to make the primordial to primary follicle transition (18, 19, 30). As discussed above, animals with null-mutations in the gonadotropin receptors display normal folliculogenesis until the pre-antral stage (20, 21). The one endocrine factor that seems to effect the primordial to primary follicle transition is insulin. Insulin has been found to promote the primordial to primary follicle transition in cultured hamster ovaries (19) and rat ovaries (31). Insulin appears to act on the oocyte to maintain cell viability and developmental competence (31).

Several paracrine factors produced in the ovary have been found that stimulate the primordial to primary follicle transition *in vivo* or *in vitro*. Various mouse strains that have mutations for the growth factor kit-ligand/stem cell factor display a normal primordial follicle pool but retarded folliculogenesis (32, 33, 34). Kit-ligand (KL) has also been shown to sequester theca cells from the surrounding stroma (35). Neonatal rat ovaries cultured in the presence of kit-ligand display a higher rate of primordial to primary follicle transition (30). The kit ligand seems to be produced in the granulosa of the primary follicles (36, 37). The receptor for this factor is located in the oocyte and theca of developing follicles (36, 37, 38, 39). Therefore, KL is produced by the developing granulosa cells of the primordial follicle to act on the adjacent stromal cells to recruit theca cells (35), and on the oocyte to initiate the primordial to primary follicle transition (30), Figure 2.

Basic fibroblast growth factor (bFGF) is another factor implicated in the primordial to primary follicle transition. The oocytes of primordial and primary follicles seem to be the site of bFGF production (40), and the bFGF receptors are in the granulosa cells (41, 42). bFGF is important in a variety of follicular processes such as granulosa cell mitosis (43, 44, 45, 46) and differentiation (47). Neonatal rat ovaries cultured in the presence of bFGF display a higher rate of primordial to primary follicle transition (48). Therefore, bFGF is produced by the oocyte of developing primordial follicles to act on the adjacent granulosa and developing theca to initiate the primordial to primary follicle transition (48), Figure 2.

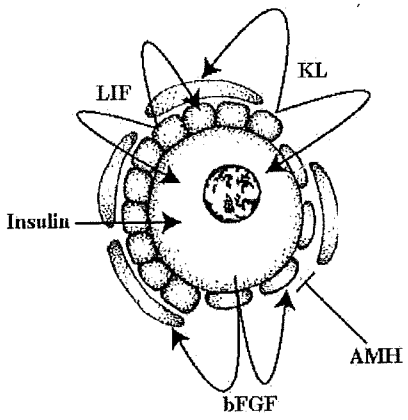

Figure 2. Proposed cell-cell interactions in the primordial to primary follicle transition. Kit ligand (KL) and Leukemia inhibitory factor (LIF) are produced by the granulosa and act on the oocyte and KL acts to promote theca cell recruitement. Basic fibroblast growth factor (bFGF) is produced by the oocyte and acts on the somatic cells. Insulin is an endocrine hormone that acts on the oocyte. Anti-Müllerian hormone (AMH) is derived from more advanced follicle stages and inhibits primordial to primary follicle transition.

Leukemia inhibitory factor (LIF) is another factor shown to promote primordial to primary follicle transition. LIF has been found in the follicular fluid of large follicles and its production can be altered by human chorionic gonadotropin (49, 50). Cultured granulosa cells have been shown to produce LIF (49, 50). LIF has been shown to be important in early germ cell survival (51). Neonatal rat ovaries cultured in the presence of leukemia inhibitory factor (LIF) display a higher rate of primordial to primary follicle transition (52). Immunolocalization studies found that LIF was produced in the granulosa, and appears to act on the oocyte and granulosa (52). Therefore, LIF is produced by the granulosa cell of the developing primordial follicle to act on the oocyte and granulosa to initiate the primordial to primary follicle transition (52), Figure 2.

GDF-9 is another candidate for a factor involved in primordial follicle development. Although GDF-9 is not expressed in the primordial follicle of rodents, it is expressed in the primary follicle. GDF-9 knockout mice display folliculogenesis arrested at the primary stage of development (53). Rats injected with GDF-9 displayed a higher rate of primordial to primary follicle transition *in vivo* and displayed a higher serum concentration of the theca cell marker CYP-17 (54). In contrast, neonatal rat ovaries cultured in the presence of GDF-9 do not display a higher degree of primordial to primary follicle transition (55). GDF-9 treated organs do display a higher degree of primary to secondary follicle transition (55). Therefore, GDF-9 does not appear to have a role in the primordial to primary follicle transition, but does act on the primary follicles. Potential interactions between the developing follicles and primordial follicles *in vivo* needs to be considered in assessing GDF-9 actions.

Culture of rodent ovaries or bovine ovary fragments containing primordial follicles both have some spontaneous primordial to primary follicle transition. A difference between the rodent and bovine model gives clues to the primordial to primary follicle transition. Small neonatal rodent ovaries can be cultured in their entirety (30). These ovaries contain approximately 20% developing pre-antral stage follicles in an ovarian cross section. Bovine ovaries are too large to be cultured so generally a cortical fragment enriched in primordial follicles that contain few larger follicles is used (17). It has been noted that the rate of primordial to primary follicle transition is much higher in the bovine cultures than the rodent ovary cultures (17). This has led to the hypothesis that the larger follicles may repress the primordial to primary follicle transition. Interesting co-culture experiments have tested this hypothesis. Bovine ovarian cortical segments cultured with a chick chorioallantoic membrane reduced the level of spontaneous primordial to primary follicle transition, while culturing cortical segments with actual large bovine follicles did not change the rate of primordial to primary follicle transition (17).

A factor that appears to inhibit the primordial to primary follicle transition is Anti-Müllerian Hormone (AMH). AMH is produced by the granulosa cells of developing pre-antral and antral follicles. The AMH null-mutant mouse displays a much higher level of primordial to primary follicle transition than wild type mice (56). Culturing mouse ovaries in the presence of AMH represses the primordial to primary follicle transition (57). It has been found that treating neonatal rats with high levels of estrogen stimulates AMH production in rat ovaries (58). These studies suggest that AMH is a negative regulator of the primordial to primary follicle transition, Figure 2, and its action might be linked to the actions of estrogen.

3. DISCUSSION

The previous studies suggest an initial model for primordial follicle assembly. Follicular assembly takes place by the breakdown of ovarian germ cell nests possibly through a process of germ cell apoptosis. The granulosa cells most likely originate from stroma surrounding the germ cell nests. Through a combination of pro-apoptotic factors such as TNF and cell survival growth factors, such as the neurotropins, primordial follicle assembly is controlled. The network of factors that control primordial follicle assembly remain to be more fully elucidated.

A model for the primordial to primary follicle transition is shown in Figure 2. This process likely involves both positive and negative regulators. The negative regulator(s) appear to come from larger more developed follicles. AMH from the granulosa cell is a promising candidate. AMH production is stimulated by estrogen. This might explain why culture of bovine cortical fragments with larger bovine follicles did not influence the rate of spontaneous primordial to primary follicle transition. The model suggested is that the larger developing follicles that are gonadotropin receptive produce AMH that inhibits the primordial to primary

follicle transition. It is likely that other factors will be identified that mediate interactions between developing follicles and primordial follicles.

The positive regulatory factors appear to be locally produced growth factors that initiate the primordial to primary follicle transition. These growth factors include KL, bFGF, and LIF (30, 48, 52). These factors act by coordinating the cell-cell interactions between the granulosa, theca, and oocyte (Figure 2). Once the primordial to primary follicle transition is initiated theca cells are recruited from the surrounding stoma. The production of KL by the developing granulosa cells and bFGF by the oocyte are postulated to influence the recruitment of these theca cells (35). The production of both KL and LIF by the developing granulosa cells to subsequently act on the oocyte to influence oocyte maturation associated with the primordial to primary follicle transition also appears to be critical. Production of bFGF by the oocyte and actions on granulosa cells may influence granulosa cell development and the expression of factors such as KL and LIF. This network of cell-cell interactions involved in primordial follicle development will likely expand with the identification of additional factors and elucidation of how the factors interact.

Insulin also seems to be an important endocrine factor for the primordial to primary follicle transition. The actions of insulin on the oocyte is likely to maintain oocyte viability and developmental competence. The female infertility associated with specific types of diabetes may in part be due to deficient insulin levels and inability to maintain primordial follicle development.

Both the primordial to primary follicle transition and follicular assembly set the size and rate of depletion of the primordial follicle pool. Once the primordial follicle pool is exhausted ovarian steroidogenesis stops and menopause begins. The inappropriate coordination of these processes can cause a premature loss of primordial follicles and the condition known as premature ovarian failure. This area of investigation will provide a greater understanding of the physiology behind ovarian pathologies such as premature ovarian failure and the potential development of new therapeutic targets. While several individual factors have been identified that influence primordial follicle assembly and the primordial to primary follicle transition, further research is required to elucidate the complex regulatory mechanisms of this aspect of ovarian biology.

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Key Words: Ovary, Primordial Follicle, Cell-Cell Interactions, Growth Factors, Review

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