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Regulation of primordial follicle assembly and development

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The assembly of the primordial follicles early in ovarian development and the subsequent development and transition of the primordial follicle to the primary follicle are critical processes in ovarian biology. These processes directly affect 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 occur. The inappropriate coordination of these processes contributes to ovarian pathologies such as premature ovarian failure (POF) and infertility. Primordial follicle assembly and development are coordinated by locally produced paracrine and autocrine growth factors. Endocrine factors such as progesterone have also been identified that influence follicular assembly. Locally produced factors that promote the primordial to primary follicle transition include growth factors such as kit ligand (KL), leukaemia inhibitory factor (LIF), bone morphogenic proteins (BMP's), keratinocyte growth factor (KGF) and basic fibroblast growth factor (bFGF). Factors mediating both precursor theca–granulosa cell interactions and granulosa–oocyte interactions have been identified. A factor produced by preantral and antral follicles, Müllerian inhibitory substance, can act to inhibit the primordial to primary follicle transition. Observations suggest that a complex network of cell–cell interactions is required to control the primordial to primary follicle transition. Elucidation of the molecular and cellular control of primordial follicle assembly and the primordial to primary follicle transition provides therapeutic targets to regulate ovarian function and treat ovarian disease.

Key words: granulosa/growth factors/ovary/primordial follicle/theca

Introduction

Primordial follicle assembly and development is a critical aspect of female reproduction, but a poorly understood process on a mechanistic level. The assembly of primordial follicles that occurs in the later stages of fetal development for a human and in the early postnatal period for the rodent is a distinct process from the induction of primordial follicle development involving the primordial to primary follicle transition. In mammals, the majority of literature suggests that the pool of primordial follicles a female is born with is the cohort of follicles that will develop throughout the female's reproductive life span. Abnormalities in primordial follicle development lead to a number of pathophysiologies [e.g. premature ovarian failure (POF)] and female infertility. The current manuscript reviews the molecular and cellular control of primordial follicle development. Several different cell types (e.g. oocyte, granulosa and precursor theca) participate in primordial follicle development. The focus of this review is on the cell–cell interactions and factors involved that control primordial follicle assembly and development.

The terms used in the current review are defined as described below. Primordial follicle development refers to both the assembly process and primordial to primary follicle transition process. Nests of germ cells (i.e. oogonia) develop in the embryonic period and have been also referred to as clusters or cysts. Owing to the space/fluid filled reference to cysts in the ovary, nests are more accurate and will be used in the current review. The assembly of primordial follicles, also referred to as primordial follicle formation, requires a transition from nests to primordial follicles. The follicle does not exist until the primordial follicle assembles. The primordial follicle pool is generally in a resting state and not developing. The subsequent development of the primordial follicle involves the primordial to primary follicle transition. This is the most accurate reference to this aspect of primordial follicle development. The term follicle growth is misleading for the primordial follicles do not proliferate or undergo mitosis, which is the general reference to the term growth. Although the oocyte does increase in size (i.e. grow), the term follicle growth will not be used so as to not suggest proliferation. Follicle activation is not precise and could refer to expression of a specific gene or cell growth. Therefore, the terms nests, primordial follicle assembly, primordial to primary follicle transition and for more global reference 'primordial follicle development' will be used.

The ability of somatic cells in the gonad to control and maintain the process of gametogenesis is an essential requirement for reproduction. The basic functional unit in the ovary is the ovarian follicle

that is composed of somatic cells and the developing oocyte. The two primary somatic cell types in the ovarian follicle are the theca cells and granulosa cells. These two somatic cell types are the site of action and synthesis of a number of hormones that promote a complex regulation of follicular development. The proliferation of these two cell types is in part responsible for the development of the antral ovarian follicle. The elucidation of factors that control ovarian somatic cell growth and development is critical to understand ovarian physiology.

Granulosa cells are the primary cell type in the ovary that provide the physical support and microenvironment required for the developing oocyte. Granulosa cells are an actively differentiating cell with several distinct populations. Alteration in cellular differentiation is required during folliculogenesis from a primordial stage of development through ovulation to a luteal stage of development. Regulation of granulosa cell cytodifferentiation requires the actions of a number of hormones and growth factors. Specific receptors have been demonstrated on granulosa cells for the gonadotropins FSH and LH (Richards and Midgley, 1976). In addition, receptors have been found for factors such as epidermal growth factor (EGF) (Vlodavsky *et al*., 1978; Wandji *et al*., 1992), insulin-like growth factor (IGF) (Adashi, 1998) and inhibiting substance [i.e. anti-Müllerian hormone (AMH)] (Peng *et al*., 1996). The actions of these hormones and growth factors on granulosa cells vary with the functional marker being examined and the stage of differentiation. The biosynthesis of two important ovarian steroids, estradiol (E_2) and progesterone, is a primary function of the granulosa cells in species such as the cow, human and rodent. As the follicle develops, granulosa cells differentiate and estrogen biosynthesis increases. FSH promotes this follicular development via the actions of cAMP. As the follicle reaches the developmental stages before ovulation, the granulosa cells develop an increased capacity to synthesize and secrete progestins under the control of LH. In contrast to secondary, preantral and antral follicles, the early primordial follicle stage granulosa are gonadotropin and steroid hormone independent and are non-steroidogenic (Richards and Midgley, 1976; Oktay *et al*., 1997).

Another important cell type in the ovary is the ovarian theca cell. These are differentiated stromal cells that surround the follicle and have also been termed theca interstitial cells (Erickson, 1983). The inner layer of cells, the theca interna, has a basement membrane separating it from the outermost layer of mural granulosa cells. One of the major functions of theca cells in species such as the cow, human and rodent is the secretion of androgens (Fortune and Armstrong, 1977). Theca cells respond to LH by increasing the production of androgens from cholesterol (Erickson and Ryan, 1976). Theca cells also produce progestins under gonadotropin control (McNatty *et al*., 1979; Channing, 1980; Evans *et al*., 1981; Haney and Schomberg, 1981; Braw-Tal and Roth, 2005). At the primordial stage, no theca cells are present; however, during transition to the primary stage, theca cells (i.e. precursor non-steroidogenic cells) are recruited to the follicle. Theca cell association with the primary follicle has been morphologically demonstrated in the rat, but has not been rigorously investigated in the mouse, cow or human. As with primordial follicle granulosa cells, theca cells at this stage of development are gonadotropin and steroid independent and non-steroidogenic (Braw-Tal and Roth, 2005). Previous studies discussed below suggest theca cell recruitment and association with the developing primordial follicles is critical in most mammalian species, including the human.

During embryonic development, oogonia proliferate and do not consistently associate with somatic cells (Pepling and Spradling, 2001). Later in fetal development in the human and cow or early postnatally in the rodent, primordial follicles assemble. Primordial follicles consist of a single oocyte and generally an incomplete layer of squamous (i.e. flattened) pre-granulosa cells (Figure 1). These primordial follicles exist in a stromal-interstitial cell environment with no apparent theca cell layers or organized mesenchymal tissue surrounding the follicles (Rajah *et al*., 1992). Females are

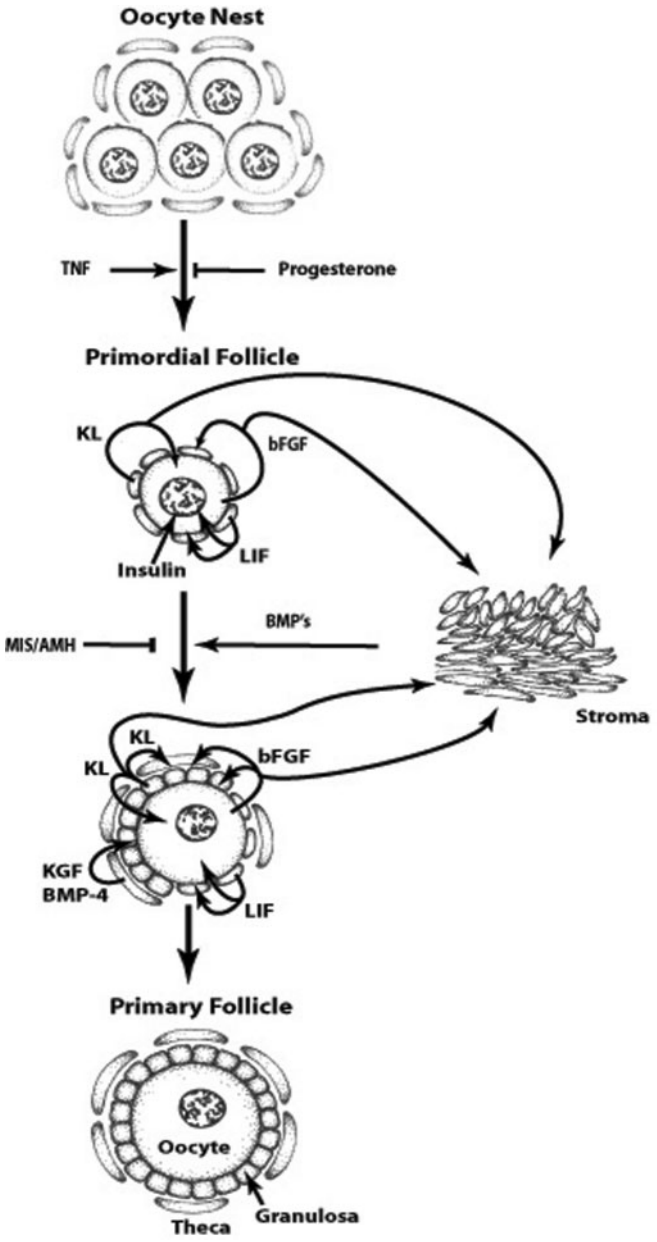

Figure 1. Schematic of the proposed cellular interactions in primordial follicle development. The follicle structures involving the oocyte, granulosa and theca cells are shown. Cell–cell interactions are mediated by tumour neucrosis factor alpha (TNFα), kit ligand (KL), basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), bone morphogenic protein-4 (BMP-4), keratinocyte growth factor (KGF), insulin and Müllerian inhibitory substance (MIS).

born with a pool of primordial follicles or oocytes. Literature suggests this pool represents the complete supply of oocytes that may potentially develop and ovulate. After primordial follicle development is initiated, the follicles are destined to ovulate or degenerate through atresia. When the supply of oocytes (i.e. primordial follicles) is diminished, menstrual cyclicity ends and humans undergo menopause.

A recent report suggested the potential presence of a female germ-line stem cell population in the ovarian surface epithelium covering the surface of the ovary (Johnson *et al*., 2004). This intriguing observation leads to the speculation that this stem cell population may provide a continual supply of primordial follicles, such that the primordial follicle pool may not be finite and regenerate. In contrast, the majority of literature in most mammalian species and a large number of transgenic mouse models have demonstrated the lack of a regenerative pool of primordial follicles (Foxcroft and Hunter, 1985; Adashi, 1994; Lass, 2001; Schlessinger and Van Zant, 2001; Durlinger *et al*., 2002; Fortune, 2003; van Zonneveld *et al*., 2003; Mayer *et al*., 2004; Rajkovic *et al*., 2004). No previous study has demonstrated the presence of a replenishing or regenerative pool of follicles (Guigon *et al*., 2003; Shirota *et al*., 2003), other than some shown in fish or bird models. The interesting potential presence of an adult female germ-line stem cell (Johnson *et al*., 2004) requires further studies to confirm the presence of this cell and its ability to contribute to the primordial follicle pool. Independent of the absence or presence of this potential stem cell population, the basic mechanisms involved in primordial follicle assembly and development will be functioning.

The majority of primordial follicles exist in a quiescent state with the oocyte arrested in prophase I of meiosis. In sexually mature animals, follicles leave the arrested pool and undergo the primordial to primary follicle transition (Faddy and Gosden, 1995, 1996; McGee and Hsueh, 2000; Fortune, 2003). One of the initial events in primordial follicle development (i.e. primordial to primary transition) is a change in granulosa cells from a squamous to cuboidal morphology (Lintern-Moore and Moore, 1979). The developing primordial follicles transition to primary follicles results in an oocyte with an increased diameter and a single layer of cuboidal granulosa cells (Lintern-Moore and Moore, 1979; Hirshfield, 1991; Rajah *et al*., 1992; Fortune, 1994, 2003) (Figure 1). Another early event in the process of folliculogenesis is the recruitment of theca cells from the stromal-interstitial cell population, and subsequent proliferation of both granulosa and theca cells. Factors produced by the granulosa cells that may be involved in theca cell recruitment and subsequent theca cell proliferation are reviewed below. A morphologically distinct theca cell in the early stage follicles has been reported in the rat, but is less defined in other models (Hirshfield, 1991). Epithelial cells do not develop or differentiate in the absence of an adjacent mesenchymal cell (Grobstein, 1967; Kratochwil, 1972). Therefore, the stromal/mesenchymal cells that associate with the primordial follicle in transition to the primary follicle are likely early stage precursor theca cells. Although both the granulosa and theca populations at this stage of development are not gonadotropin hormone responsive or steroidogenic, they are the less differentiated precursor populations of these cells. Because of the evolutionary conservation of this process in mammals it is proposed that this process of precursor theca cell recruitment and requirement for primordial follicle development is needed for all mammals, including human, independent of whether there is a morphologically distinct population of cells.

The initiation of primordial to primary follicle transition has been shown to be stimulated when ovarian tissue is cultured *in vitro* (Wandji *et al*., 1996; Hovatta *et al*., 1999; Parrott and Skinner, 1999, 2000; Nilsson *et al*., 2001, 2002; Durlinger *et al*., 2002; Kezele *et al*., 2002, 2005; Nilsson and Skinner, 2002, 2003, 2004; Kezele and Skinner, 2003;). This has been shown in several different species with a variety of procedures (Fortune *et al*., 1998; Hovatta *et al*., 1999; Miller *et al*., 1999; Faddy, 2000; Meredith *et al*., 2000; Oktay *et al*., 2000; Campbell *et al*., 2004; Silva *et al*., 2004). Primordial follicle development is hormone independent (Balla *et al*., 2003; Campbell *et al*., 2004; Silva *et al*., 2004; Braw-Tal and Roth, 2005), but the lack of developing follicles can influence primordial follicles (Balla *et al*., 2003; Fortune, 2003). The culture of primordial follicles has recently been proposed as a source of viable oocytes for assisted reproductive technologies (Hovatta, 2000; Picton and Gosden, 2000; Cortvrindt and Smitz, 2001; Liu *et al*., 2001). These cultures have also provided insight into the factors that regulate primordial follicle development (Murray and Spears, 2000; Picton and Gosden, 2000; Telfer *et al*., 2000; Campbell *et al*., 2004; Silva *et al*., 2004). Ovary cultures have been used to assess the importance of vascularization (Fortune *et al*., 2000), apoptosis (Reynaud and Driancourt, 2000; Flaws *et al*., 2001) and growth factors (De Felici, 2000; Derrar *et al*., 2000; Gougeon and Busso, 2000; Erickson, 2001; Nilsson and Skinner, 2001) to primordial follicle development (O'Brien *et al*., 2003). A number of these factors are discussed below with a focus on the factors shown to influence primordial to primary follicle transition. The actions of these factors on primordial follicles will be compared to their actions on primordial follicle assembly and later stage follicle development. This information is relevant to several physiological conditions including aspects of POF (Gosden and Faddy, 1998) and oocyte protection from radiotherapy and chemotherapy (Meirow and Nugent, 2001; Bath *et al*., 2003).

Primordial follicle assembly

The assembly or formation of the primordial follicles requires individual oocytes to segregate and associate with squamous (i.e. precursor) granulosa cells. Nests of associated oocytes undergo random apoptosis of individual oocytes to derive isolated oocytes that then associate with precursor squamous granulosa cells (Pepling and Spradling, 1998, 2001; Pepling *et al*., 1999; McNatty *et al*., 2000; Depalo *et al*., 2003; Vaskivuo and Tapanainen, 2003) (Figure 1). The factors controlling this primordial follicle assembly have not been thoroughly investigated (Lunenfeld *et al*., 1975), but neurotropins have been implicated (Dissen *et al*., 2001; Matzuk, 2001). The culture of 0-day rat ovaries has been utilized to investigate this follicle assembly. It was found that the total number of follicles/oocytes did not change in the cultures with different treatments (Kezele and Skinner, 2003). Therefore, the apoptosis rate as judged by TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) analysis did not change (Kezele and Skinner, 2003). This is an important criterion to examine to allow data interpretation of changes in follicle development versus survival. It is anticipated that different cell–cell interactions and factors will be involved in the assembly of

primordial follicles compared to those of the primordial to primary follicle transition.

An interesting observation from the *in vitro* culture of 0-day-old rat ovaries (Kezele and Skinner, 2003) was that the majority of primordial follicles that assembled continued to develop spontaneously into primary follicles. This is in contrast to 4-day-old rat ovary cultures that require an external stimulus [e.g. the kit ligand (KL)] to induce this degree of primordial to primary follicle transition (Parrott and Skinner, 1999). Therefore, primordial follicle assembly occurs *in vitro*; however, subsequent primordial to primary follicle transition dramatically increases compared to *in vivo* ovaries (Kezele and Skinner, 2003), or 4-day-old ovary cultures. An inhibitory mechanism appears to be established *in vivo* that was not established in the 0-day-old ovary cultures that allowed spontaneous primordial follicle development. To investigate what factors may influence or inhibit this development, the cultures were treated with several agents (Kezele and Skinner, 2003). Both estrogen at a 10^{-6} M concentration and progesterone at a 10^{-6} M concentration dramatically inhibited the primordial to primary follicle development (Kezele and Skinner, 2003). A preliminary experiment with KL showed no effect on primordial follicle development in the cultures from 0-day ovaries. Primordial follicle assembly was inhibited by progesterone to a greater degree than estrogen, but both steroids inhibited the assembly process (Kezele and Skinner, 2003). The effect of steroid hormones on the 0-day-old ovary cultures was unexpected and suggests that an alteration in ovarian steroid levels may be a factor in the control of primordial follicle assembly. A decline in ovarian steroid levels initially derived from maternal/placental sources may influence primordial follicle assembly. Previously, in the rodent it has been shown that primordial follicle assembly occurs by approximately day 2 and 3 postnatally. The serum concentrations of E_2 drop between day 0 and day 2 from 155 pg/mL to 5.6 pg/mL (Montano *et al*., 1995). In the cow embryonic ovary, steroid levels also drop dramatically at the later stage of fetal development, which coincides with primordial follicle assembly (Tanaka *et al*., 2001). Estrogen levels appear to regulate fetal ovarian maturation in the primate (Zachos *et al*., 2002). The previous studies have lead to the hypothesis that 'high levels of ovarian steroid hormones prevent primordial follicle assembly and that a decline in ovarian steroid levels in part initiates primordial follicle assembly through locally produced growth factors' (Kezele and Skinner, 2003) (Figure 1 and Table I).

This previous study also demonstrated that the ability of progesterone to inhibit the assembly of primordial follicles is in part mediated through a reduction in oocyte apoptosis (Kezele and Skinner, 2003). Previously it has been shown that oocytes exist unassembled in nests and that apoptosis of surrounding oocytes causes isolated oocytes to develop and assemble with precursor granulosa cells to form primordial follicles (McNatty *et al*., 2000). When the 0-day ovary cultures are incubated with progesterone, the apoptosis is inhibited and individual follicles do not form (Kezele and Skinner, 2003). The apoptosis of random oocytes in the oocyte nests is required for primordial follicle assembly (McNatty *et al*., 2000; Hussein, 2005) and tumour necrosis factor-alpha (TNFα) appears to be involved in this process (Marcinkiewicz *et al*., 2002). TNFα appears to promote this random oocyte apoptosis to allow primordial follicle assembly. The ability of progesterone to prevent apoptosis is speculated to be linked to a suppression of TNFα expression in the oocyte (Figure 1). Observations provide some of the first insights into primordial follicle assembly and require further investigation of the relationship of steroid hormone actions, local growth factors, oocyte apoptosis and follicle assembly.

A recent study utilized a micro-array analysis to investigate the changes in gene expression between unassembled oocytes, primordial follicles and primary follicles to correlate this with the growth factor mediated cell–cell interactions of interest (Kezele *et al*., 2005). The developmental time points compared were the 0-day ovary that is predominately unassembled oocytes, the day 4 freshly isolated ovary that is predominately primordial follicles and cultured 0-day ovary that is predominately primary follicles (Kezele and Skinner, 2003). The ovarian transcriptomes of these developmental stages were compared. An Affymetrix micro-array analysis was utilized to investigate the simultaneous expression of all the genes of interest [e.g. KL, basic fibroblast growth factor (bFGF), leukaemia inhibitory factor (LIF), keratinocyte growth factor (KGF) and bone morphogenic protein-4 (BMP-4)]. The micro-array data were compared between the developmental periods and follicle populations with a focus on the genes related to the growth factor and hormone actions of interest (Kezele *et al*., 2005). The micro-array data demonstrated a decline in KL, negligible change in bFGF and KGF, and an increase in LIF. Several control genes such as inhibin, 3 beta-hydroxysteroid dehydrogenase and Zona Pellucida 2 that were expected to increase were found to increase as the primordial follicle assembled. New growth factors were identified for further analysis in the regulation of primordial follicle assembly and development (Kezele *et al*., 2005). A similar analysis with a mouse micro-array has also identified potentially important genes for primordial follicle formation (Herrera *et al*., 2005). A human primordial follicle complementary DNA library has recently been generated as a resource to investigate human primordial follicle development (Serafica *et al*., 2005). Therefore, genomic procedures are being utilized to help elucidate this critical aspect of primordial follicle development.

Table I. Primordial follicle development regulatory factors

Regulatory factor	Cellular source	Cellular site of action
Tumour necrosis factor-alpha	Oocyte	Oocyte
Basic fibroblast growth factor	Oocyte	Granulosa, theca, stroma
Kit ligand	Granulosa	Oocyte, theca, stroma
Leukaemia inhibitory factor	Granulosa	Oocyte, granulosa
Keratinocyte growth factor	Theca	Granulosa
Bone morphogenic protien-4	Theca/stroma	Granulosa
Bone morphogenic protein-7	Stroma	Granulosa
Insulin	Endocrine	Oocyte
Progesterone	Endocrine	Oocyte
Müllerian inhibitory substance	Antral follicle	Primordial follicle

Primordial to primary follicle transition

Previous observations have demonstrated that theca cells produce transforming growth factor (TGF) α, KGF and hepatocyte growth factor (HGF) that can regulate granulosa cells, whereas granulosa cells produce KL that can influence theca cells and the oocyte. More recently, studies have demonstrated that KL, bFGF, LIF, KGF and BMP-4 can influence primordial follicle development

(Kezele *et al*., 2002b). The current review discusses the roles of some of these factors (KL, bFGF, LIF, KGF and BMP-4) in mediating interactions between precursor theca cells, granulosa cells and the oocyte with a focus on primordial follicle development.

Kit ligand

The KL (also termed stem cell factor, steel factor and multipotent growth factor) is a growth factor with both soluble and membrane bound forms that have a wide range of effects on various cell types (Besmer, 1991). KL was originally characterized because of its ability to influence stem cell growth and differentiation. The receptor for KL is the ckit tyrosine kinase receptor (Zsebo *et al*., 1990). Previous studies on mice with mutations at the white-spotting (W) and steel (Sl) locus have demonstrated deficient gametogenesis (Russell, 1979; Silvers, 1979). The W locus encodes ckit (Chabot *et al*., 1988) and the Sl locus the KL (Huang *et al*., 1993). Different alleles are available that vary in phenotype from a total lack of germ cells to normal fertility in both males and females (Russell, 1979; Silvers, 1979; Chabot *et al*., 1988). An interesting allele is the steel panda (Sl^{pan}/Sl^{pan}) that has a relatively normal complement of primordial follicles and developing follicles arrested at the one layer cuboidal granulosa stage (i.e. primary follicle) (Huang *et al*., 1993; Bedell *et al*., 1995). This mutation appears to influence the level of KL produced (Huang *et al*., 1993). The ability of this mutation to have a block at the primary stage of follicle development suggests that additional factors may compensate for the lack of KL expression during the primordial to primary follicle transition. *In situ* hybridization and immunocytochemistry for ckit with normal ovarian sections revealed high levels of expression in developing oocytes (Manova *et al*., 1990; Horie *et al*., 1991; Robinson *et al*., 2001). Analysis of KL expression revealed high levels of KL expression in granulosa cells (Huang *et al*., 1993; Manova *et al*., 1993). The genetic evidence and localization studies suggested an important interaction between granulosa cells and oocytes via KL. *In vitro* studies with neutralizing antibodies and KL also support the importance of KL in granulosa cell controlled oocyte development (Packer *et al*., 1994). The *in situ* analysis of ovary sections also revealed low levels of ckit in stromal-interstitial cells and theca cells (Manova *et al*., 1990; Kang *et al*., 2003). This observation led to the proposal that granulosa derived KL may also influence theca cell recruitment and proliferation. Observations demonstrate that granulosa derived KL has a role in the recruitment of theca cells from the stromal-interstitial cell population and subsequently regulates theca cell proliferation (Parrott and Skinner, 1999; Faddy, 2000; Kang *et al*., 2003).

KL was found to induce the primordial to primary follicle transition (Parrott and Skinner, 1999). In this analysis, multiple experiments were performed in replicate such that over 7000 follicles were counted. The morphological criteria for primordial follicle development are shown in Figure 1. The stage 0 primordial follicles have a single oocyte and an incomplete layer of squamous (i.e. flattened) pre-granulosa cells and no theca cells. As the follicles develop, the granulosa cells develop a cuboidal structure and surround the oocyte while theca cells (i.e. pre-theca) are recruited from the stromal cells and organize around the follicle (Nilsson *et al*., 2001) (Figure 1). KL produced by granulosa cells appears to act on the oocyte to stimulate it to enlarge and initiate development (Parrott and Skinner, 1999; Kezele and Skinner, 2003). KL was found to stimulate stromal cell and theca cell growth. KL stimulates theca cell androgen production, but has no effect on stromal cell steroidogenesis (Parrott and Skinner, 2000). KL also was found to stimulate KGF and HGF mRNA levels in theca cells. KL expression was stimulated by gonadotropins, KGF and HGF. Therefore, KL appears to be a critical factor in primordial to primary follicle transition (Figure 1 and Table I).

Basic fibroblast growth factor

bFGF has been localized to the oocytes of primordial and primary follicles of several species (van Wezel *et al*., 1995; Nilsson *et al*., 2001). The bFGF is localized to granulosa cells of developing preantral follicles, but not to granulosa cells of human primordial follicles (Yamamoto *et al*., 1997). Theca cells of developing follicles also stain positive for bFGF (van Wezel *et al*., 1995; Yamamoto *et al*., 1997). Receptors for bFGF have been reported in rat (Shikone *et al*., 1992) and cow (Wandji *et al*., 1992) antral granulosa cells. bFGF is important in regulating a wide range of ovarian functions including granulosa cell mitosis (Gospodarowicz *et al*., 1989; Lavranos *et al*., 1994), steroidogenesis (Vernon and Spicer, 1994), differentiation (Anderson and Lee, 1993) and apoptosis (Tilly *et al*., 1992). In addition, bovine granulosa cells have been shown to produce bFGF (Neufeld *et al*., 1987) in the preantral and antral follicle stages.

When 4-day-old rat ovary organ cultures were treated with bFGF there was a dramatic increase in primordial follicle development similar to that observed with KL (Nilsson *et al*., 2001). Organ cultures treated with EGF, insulin-like growth factor-I (IGF-I) or HGF did not alter follicle development (Parrott and Skinner, 1999; Nilsson *et al*., 2001). The effects of bFGF on the ovary cross-sections after 14 days of treatment was investigated with multiple experiments done in replicate (Nilsson *et al*., 2001). bFGF treatment dramatically decreased primordial follicle numbers and increased the number of developing follicles, similar to the actions of KL (Parrott and Skinner, 1999; Nilsson *et al*., 2001). Therefore, bFGF, like KL, appears to be a primordial follicle inducing factor. bFGF was found to be primarily localized to the oocyte of the primordial and early stage follicles. bFGF was found to stimulate both theca cell and stromal cell growth (Nilsson *et al*., 2001), as has previously been shown for granulosa cells (Gospodarowicz *et al*., 1989; Lavranos *et al*., 1994). Therefore, bFGF produced by the oocyte appears to act on the adjacent somatic cells to influence primordial follicle development (Figure 1 and Table I).

Leukemia inhibitory factor

LIF is a factor that acts at a specific GP130 transducing receptor involving the JAK-STAT pathway (Gadient and Patterson, 1999) to influence a number of developmental systems (Abe *et al*., 1991; Taupin *et al*., 1998). LIF has been shown to be a differentiation inducer and an influence stem cells in various tissues (Abe *et al*., 1991). An LIF knockout was shown to influence implantation (Stewart and Cullinan, 1997) and LIF has a role in pregnancy and uterine biology (Senturk and Arici, 1998; Vogiagis and Salamonsen, 1999; Kholkute *et al*., 2000). Detailed analysis of the ovaries of LIF knockout mice remains to be carried out, but they do have the potential to ovulate. LIF has been shown to be present in follicular

fluid and appears to be at higher levels as the follicle develops (Coskun *et al*., 1998; Ozornek *et al*., 1999). Like KL, LIF has been shown to influence primordial germ cells *in vitro* and *in vivo* (Morita *et al*., 1999), but its role in oocyte development remains to be elucidated (Shim and Anderson, 1998).

When 4-day-old rat ovary organ cultures were treated with LIF, there was an increase in primordial to primary follicle transition, (Nilsson *et al*., 2002). LIF treatment resulted in a dramatic decrease in primordial follicle numbers and corresponding increase in developing follicle numbers. A previous study suggests that insulin (Figure 1), but not IGF-I, can enhance primordial follicle development (Kezele *et al*., 2002a). Insulin was found to enhance LIF actions on the primordial follicles (Nilsson *et al*., 2002). The LIF neutralizing antibody also decreased slightly the spontaneous follicle development observed in control cultures, (Nilsson *et al*., 2002). Therefore, LIF can promote primordial follicle development (Figure 1), and LIF neutralizing antibody can partially inhibit spontaneous follicle development. These data are similar to that observed with KL neutralizing antibodies.

Keratinocyte growth factor

A mesenchymal-derived growth factor that mediates mesenchymal–epithelial interactions is KGF. KGF is a fibroblast growth factor (FGF7), -related molecule found to stimulate epithelial cell proliferation (Rubin *et al*., 1989). KGF is a 28 kDa protein that appears to primarily be produced by mesenchymal cells and is an epithelial cell mitogen (Finch *et al*., 1989). The receptor to KGF is an FGF receptor isoform, FGFR2 splice variant that specifically binds KGF (Miki *et al*., 1991). The KGF receptor appears to be primarily localized on epithelial cells (Miki *et al*., 1992). Previous studies have documented the production of KGF by theca cells from antral follicles and its potential to influence granulosa cell growth (Parrott and Skinner, 1998).

KGF has also been found to stimulate the primordial to primary follicle transition (Nilsson and Skinner, 2003; Kezele *et al*., submitted for publication). When 4-day-old rat ovaries were cultured in the presence of 50 ng/mL, KGF a significant increase in primordial follicle transition was observed with a corresponding decrease in primordial follicle numbers. KGF is localized to the newly recruited precursor theca cells in contact with the layer of developing granulosa cells (Kezele *et al*., submitted for publication). This suggests that the KGF is produced by the recruited precursor theca cells and acts on the adjacent granulosa cell. This is one of the first specific markers for this developing precursor theca cell population and supports the concept that precursor theca cells are an integral part of primordial follicle development (Figure 1 and Table I).

Bone morphogenic proteins

The BMP family of growth factors is in the TGFβ superfamily. The first BMP shown to be associated with primordial follicle development was BMP-15 (Dean, 2002). BMP-15 is produced by oocytes and appears to influence follicle development (Dube *et al*., 1998). The regulatory role of BMP-15 on primordial follicle development remains to be elucidated. Other BMPs have been found to influence antral follicle development (Shimasaki *et al*., 1999; Dobens and Raftery, 2000; Elvin *et al*., 2000). BMP-7 appears to have a role in early follicle development (Lee *et al*., 2001, 2004) and involves a stroma cell interaction with the primordial follicle (Figure 1 and Table I).

BMP-4 has been shown to promote the primordial to primary follicle transition and appears to be essential for oocyte survival (Nilsson and Skinner, 2003). BMP-4 has been shown to be primarily produced by mesenchymal cells and act on adjacent epithelial cells (Winnier *et al*., 1995; Lawson *et al*., 1999). A previous study demonstrated that the developing theca cells produce BMP-4 to act on granulosa cells to sustain oocyte survival. BMP-4 was found to significantly increase primordial to primary follicle transition (Nilsson and Skinner, 2003). Immunocytochemistry for BMP-4 detected BMP-4 in the theca cells associated with the primary follicles (Nilsson and Skinner, 2003), similar to KGF expression (Kezele *et al*., submitted for publication). In contrast, BMP-4 was also found in islands of stromal cells not associated with the follicles using immunocytochemistry. Interestingly, when a neutralizing antibody to BMP-4 was used in the organ culture, all the oocytes in the organs initiated apoptosis and were lost within 14 days of culture (Nilsson and Skinner, 2003). Observations suggest that BMP-4 is a required survival factor for the primordial follicle and oocyte (Figure 1 and Table I).

Müllerian inhibiting substance

Müllerian inhibiting substance (MIS) or Anti-Mullerian Hormone (AMH) has been shown to regulate primordial follicle development (Durlinger *et al*., 1999, 2001, 2002; Gruijters *et al*., 2003; Salmon *et al*., 2004; Weenen *et al*., 2004; Visser and Themmen, 2005). MIS/AMH is not expressed in the primordial follicle, but appears to have the capacity to block primordial follicle development and is derived from developing follicles (Durlinger *et al*., 2001, 2002; Gruijters *et al*., 2003; Salmon *et al*., 2004; Weenen *et al*., 2004; Visser and Themmen, 2005) (Figure 1 and Table I). MIS/AMH is produced in the early secondary follicles, the preantral follicles and antral follicles (Visser and Themmen, 2005). Similar expression patterns are seen in the human ovary (Weenen *et al*., 2004). Therefore, MIS/AMH produced by the developing follicles can inhibit primordial follicle development (Durlinger *et al*., 1999, 2001, 2002; Gruijters *et al*., 2003; Salmon *et al*., 2004; Weenen *et al*., 2004; Visser and Themmen, 2005) (Figure 1). Currently this is the only negative regulatory factor for primordial to primary follicle transition. The ability of developing follicles to feedback and regulate primordial follicle development provides an efficient communication between follicles.

Other factors

Several additional factors have been shown to be associated with primordial follicles or mutations in the factor (e.g. knockout) that alter primordial follicle development. Growth factors that are associated with primordial follicles include EGF, TGFα and EGF receptor (Singh and Armstrong, 1995; Singh *et al*., 1995; Qu *et al*., 2000; Silva *et al*., 2004), neurotropins (Dissen *et al*., 2002; Paredes *et al*., 2004) and activins (Martins da Silva *et al*., 2004). These factors have not been shown to directly regulate primordial to primary follicle transition, but require further investigation. Knockout mice have also suggested roles for factors in primordial follicle development (Galloway *et al*., 2000; Matzuk, 2000; Vitt *et al*., 2000; Mazerbourg and Hsueh, 2003). Several transcription

Table II. Primordial follicle associated transcription factors

Transcription factor	Family
Aryl hydrocarbon receptor	Basic helix-loop-helix
Figα	Basic helix-loop-helix
Fox12	Wing helix
NOBOX	Homeobox

factors have been shown to be essential for oocyte survival and primordial follicle development including a transcription factor (aryl hydrocarbon receptor) (Robles *et al*., 2000; Matzuk, 2001), basic-helix-loop-helix transcription factor Figα (Dean, 2002; Bayne *et al*., 2004), a winged-helix transcription factor Fox12 (Schmidt *et al*., 2004) and a homeobox NOBOX gene in oocytes (Rajkovic *et al*., 2004) (Table II). The specific roles of many of these factors in the regulation of primordial follicle development remain to be elucidated.

Another factor investigated as a regulatory agent in the induction of primordial follicles is growth differentiation factor-9 (GDF-9). GDF-9 is a member of the TGFβ family and is expressed by oocytes in the primary stage of follicle development through ovulation (Dong *et al*., 1996; Mazerbourg and Hsueh, 2003). A GDF-9 homologue GDF-9B was identified as BMP-15 (Aaltonen *et al*., 1999; Jaatinen *et al*., 1999). Synergistic actions of GDF-9 and BMP-15 have a role in oocyte–cumulus cell interactions in developing follicles (Su *et al*., 2004; McNatty *et al*., 2005), but not in primordial follicles (Juengel *et al*., 2004). GDF-9 knockout mice have a block in follicle development at the primary stage (Carabatsos *et al*., 1998; Elvin *et al*., 1999). This and localization data (Vitt and Hsueh, 2001) suggests that GDF-9 does not influence primordial follicles. A recent report suggests that GDF-9 may influence follicle growth factor expression such as KL (Joyce *et al*., 2000; Wang and Roy, 2004). How GDF-9 may influence primordial follicle development was investigated. Four-day-old rat ovary organ cultures were treated with GDF-9 for 14 days and then follicle development was examined. GDF-9 was found to have no influence on primordial to primary follicle transition, (Nilsson and Skinner, 2002). None of the stage 0–2 follicle numbers changed after an optimal dose (100 ng/mL) of GDF-9 (Nilsson and Skinner, 2002). GDF-9 was found to influence granulosa cell gene expression demonstrating its bioactivity (Nilsson and Skinner, 2002). GDF-9 is initially expressed at the primary stage 2 follicle (Matzuk, 2000). Observations suggest that GDF-9 may influence primary follicle progression, but does not appear to have a role in primordial follicle development (Nilsson and Skinner, 2002). As discussed above, factors found not to influence primordial follicle development are GDF-9, IGFI, EGF and HGF. Therefore, among the factors investigated KL, bFGF, LIF, KGF, MIS, insulin and BMP-4 are specific in their ability to influence primordial follicles (Figure 1 and Table I).

Summary

The previous studies demonstrated the importance of cell–cell interactions involved in primordial follicle development. The hypothesis and potential cell–cell interactions examined are schematically shown in Figure 1. The primordial to primary follicle transition is distinct from subsequent follicle development because of hormone independence and less differentiated cell populations. The role KL, bFGF, KGF, BMP-4 and LIF have in this process of primordial to primary follicle transition has been established. The control of primordial follicle assembly appears to be distinct from the control of primordial to primary follicle transition. The proposed current model is shown in Figure 1 regarding primordial follicle development.

Primordial follicle assembly and development are highly conserved processes in mammalian species. Because of this high degree of evolutionary conservation, the regulation of primordial follicle development is speculated to be similar among different species, including humans. Slight morphological differences and distinct developmental timing should not be interpreted as major differences in the regulation of the process. Because of the need to assure reproductive success, the regulatory mechanisms likely have a high degree of compensation such that multiple factors are involved. For this reason, null mutations in specific factors (e.g. bFGF or LIF) do not have major primordial follicle phenotypes, due to compensation by other factors. Further investigation into the regulatory mechanisms controlling primordial follicle development in different species is needed to confirm this speculation. The hypothesis has proposed that the regulatory mechanisms and cell–cell interaction summarized in Figure 1 are common among species including humans.

The cell–cell interactions required during follicle development are speculated to be mediated in part through the local production and actions of a variety of factors (Parrott and Skinner, 1999, 2000; Nilsson *et al*., 2001, 2002; Kezele *et al*., 2002b, 2005; Nilsson and Skinner, 2002, 2003, 2004; Kezele and Skinner, 2003). How these different factors interact and the sequence of cellular interactions required remain to be elucidated. Inappropriate expression and action of these growth factors may result in the abnormal ovarian physiology associated with specific forms of infertility and ovarian pathology (e.g. POF). Therefore, the studies reviewed provide insights into the specific factors that may regulate the onset and progression of primordial follicle development. This information is critical to the future design of diagnostic procedures and therapeutic treatments of ovarian pathologies associated with primordial follicle development.

Human health issues where a manipulation of the primordial follicle pool size and alterations in primordial follicle development could be important include (i) increasing the initial primordial follicle pool size to increase the longevity of female fertility; (ii) delay the development of primordial follicles to delay the decrease in the pool size and delay onset of menopause; (iii) manipulate primordial follicle pool size to regulate the onset and timing of menopause and (iv) increase primordial follicle development to increase fertility in subfertile women. The basic information reviewed identifies potential therapeutic targets to potentially manipulate primordial follicle development. Several human disease states also exist that could benefit from such a therapeutic approach. Subfertile women could potentially increase fertility by increasing primordial follicle development to obtain a normal cohort of follicles. The premature menopausal woman could prolong fertility and the onset of menopause by inhibiting primordial follicle development. The POF patient could be treated with a therapeutic agent to inhibit primordial follicle development and thus allow fertility at a later date.

Future studies also will need to involve the development of diagnostic assays to assess potential mutations in the genes for the growth factors and receptors found to be associated with primordial follicle development. This type of assay could include single nucleotide polymorphism or mutation arrays. Such an assay could potentially diagnose and elucidate the genetic causal factors in disease states such as POF in humans and be used to identify high-risk patients.

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