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Regulation of ovarian folliculogenesis by Kit Ligand and the c-Kit system in mammals

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Abstract

The system comprised of Kit Ligand (KL) and its receptor c-Kit has proven to play a role in normal female reproduction and fertility in mammals. Gene expression studies have revealed that biological activities of ligands and receptors of the KL/c-Kit system are important in controlling apoptosis and cellular proliferation in reproductive tissues. Collectively, these studies have provided a better understanding of ovarian physiology and female fertility through the establishment of the concept that the KL/c-Kit system regulates the viability of primordial germ cells and follicles, initiation of primordial follicle growth, and further oocyte and follicular development through different signaling proteins. The purpose of this article is to review the importance of the KL/c-Kit system in ovarian follicular development, especially in the preantral phase of folliculogenesis.

Keywords: c-Kit system, follicle, Kit Ligand, ovary, signaling pathways.

Introduction

During the last decade, the role of growth factors in ovarian folliculogenesis has been extensively studied in several species, including rodents, domestic animals, and humans. In particular, Kit Ligand (KL), which was one of the first growth factors identified in the ovarian follicle, plays a key role in mammalian oogenesis and folliculogenesis (Thomas and Vanderhyden, 2006). Since its identification in 1990, *in vivo* and *in vitro* studies have shown that the functions of this system in the ovary include the establishment of primordial germ cells (PGCs), activation of primordial follicles, oocyte survival and growth, proliferation of granulosa cells, recruitment of theca cells, and maintenance of meiotic competence (Hutt *et al*., 2006b; Thomas *et al*., 2008), as shown in Fig. 1. This review will focus on the role of the KL/c-Kit system in ovarian follicle development, especially in the preantral phase of folliculogenesis.

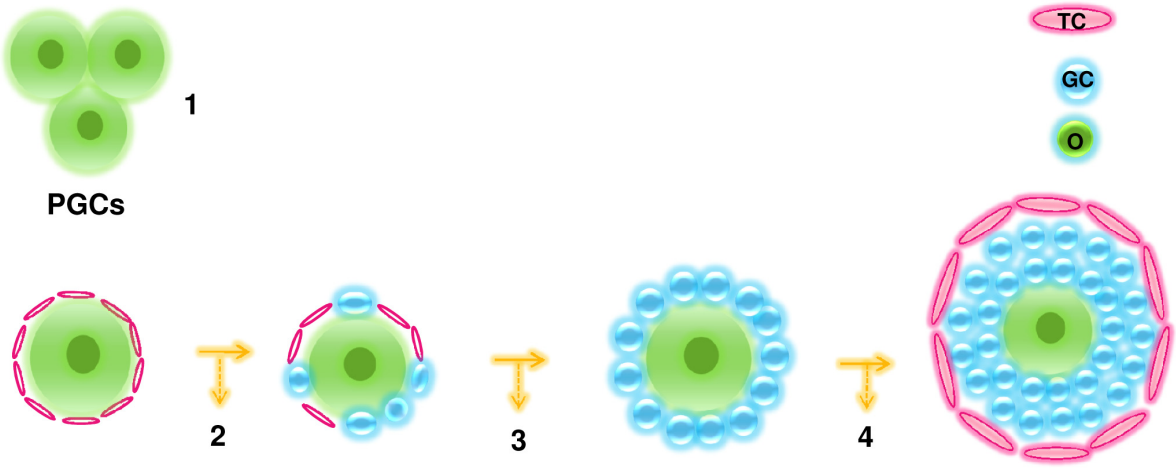

Figure 1. Several functions of this KL/c-Kit system in the ovary: 1) Establishment of primordial germ cells; 2) Activation of primordial follicles; 3) Oocyte survival and growth; 4) Proliferation of granulosa cells and recruitment of theca cells. PGCs: primordial germ cells; TC: theca cells; GC: granulosa cells; O: oocyte.

Expression of Kit Ligand and c-Kit in the ovary

Kit Ligand (KL), also known as Stem Cell Factor (SCF), Steel Factor (SF), and Mast Cell Growth Factor (MCGF), is a locally produced factor that has many roles in ovarian function from embryogenesis onward (Yoshida *et al*., 1997; Driancourt *et al*., 2000). In follicles, the expression of the mRNA for KL has been demonstrated in the granulosa cells of several species (rat: Ismail *et al*., 1996; ovine: Tisdall *et al*., 1999; mouse: Doneda *et al*., 2002; human: Hoyer *et al*., 2005 and caprine: Silva *et al*., 2006). Depending on how

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the mRNA is spliced, KL can be expressed as a soluble protein (KL-1) or as membrane-associated protein (KL-2; Huang *et al*., 1992). When translated, both transcripts yield membrane-associated products; however, KL-1 is efficiently cleaved and released as a soluble product due to a proteolytic cleavage site encoded by an 84-base pair exon. The other form, KL-2, lacks this cleavage site and therefore remains membrane-bound (Huang *et al*., 1992). KL-2 is the main isoform required for the growth and survival of oocytes (Thomas *et al*., 2008). Both isoforms are present in rodent (Ismail *et al*., 1997) and goat (Silva *et al*., 2006) ovaries. In goats, KL protein and mRNA are expressed in granulosa cells during all stages of follicular development as well as in corpus luteum, epithelium surface, and medullar tissue (Silva *et al*., 2006). KL affects target cells through binding to its receptor c-Kit, a member of the tyrosine kinase receptor family. During postnatal ovarian development, c-Kit mRNA and protein are found in oocytes of all stages of follicular development. In addition, c-Kit is expressed in interstitial and thecal cells of antral follicles (rodent: Motro and Bernstein, 1993; ovine: Clark *et al*., 1996; caprine: Silva *et al*., 2006). Fig. 2 illustrates action and expression of KL and its receptor c-Kit.

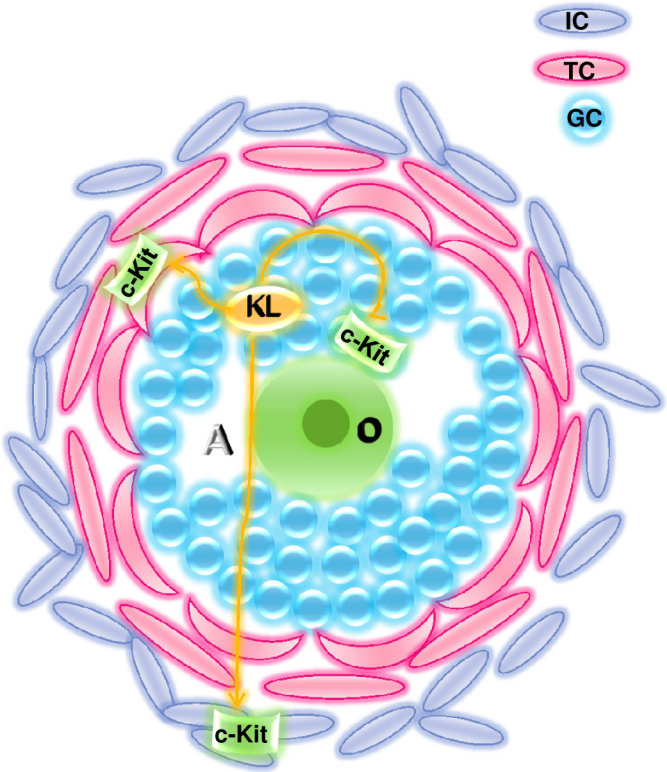

Figure 2. Autocrine action of Kit Ligand, expression in the granulosa cells and its role on the oocytes, interstitial and theca cells after binding to its receptor c-Kit, the tyrosine-kinase type. KL: Kit Ligand; c-Kit: receptor of Kit Ligand; A: antrum; O: oocyte; IC: interstitial cells; TC: theca cells; GC: granulosa cells.

Signaling pathways of the KL/c-Kit system in the regulation of oogenesis and folliculogenesis

The interaction between KL and its receptor is important for the development and differentiation of ovarian follicles in different species (Carlsson *et al*., 2006). KL produced by the granulosa cells in the oocyte acts by binding to c-Kit and may activate different signaling pathways. Currently, several studies have helped elucidate the pattern of signaling of KL/c-Kit via Phosphoinositide 3-Kinase (PI3K)-Akt-FKHRL1 and PTEN. The PI3K pathway is a fundamental signaling pathway for the regulation of cell proliferation, survival, migration, and metabolism, and it still plays an important role in regulating the activation of primordial follicles (Cantley, 2002). Primordial follicle activation (i.e., the transition from primordial to primary follicles) is a process that occurs very slowly *in vivo* (Fortune, 2003) and is influenced by positive and negative feedback loops. These loops are likely mediated by factors (John *et al*., 2008) that have not yet been definitively identified (Skinner, 2005). Some potential candidates include growth and differentiation factor-9 (GDF-9; Gilchrist *et al*., 2004) and bone morphogenetic protein-15 (BMP-15; Otsuka *et al*., 2000).

Studies by Reddy *et al*. (2005) using postnatal

mouse and rat ovaries revealed that the oocyte PI3K pathway is regulated by KL from granulosa cells, and this pathway is of great importance for early follicular development. These studies suggested that actions of KL on the primordial to primary follicle transition and subsequent follicle development may involve phosphorylation of the serine/threonine kinase Akt and the transcription factor FKHRL1, actions that most likely trigger Akt and inhibit FKHRL1 activities in oocytes. Akt is a signaling molecule that enhances cellular proliferation, survival, and glycogen and protein synthesis (Blume-Jensen and Hunter, 2001). FKHRL1(Foxo3a) is a member of the FOXO subfamily of forkhead transcription factors, which consists of Foxo3a, Foxo1 (FKHR), and Foxo4 (AFX); all of these are downstream effectors of the PTEN/PI3K/Akt pathway (Tran *et al*., 2003). Moreover, FKHRL1 is a substrate of Akt as well as a transcriptional factor that leads to apoptosis and cell cycle arrest. Therefore, it is suggested that Akt stimulates oocyte development, whereas FKHRL1 inhibits it.

Recently, John *et al*. (2008) also showed that the PI3K–Akt pathway has a key role in the initiation of oocyte growth (and hence in the maintenance of oocytes) and acts via Foxo3. Oocyte-specific ablation of the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) resulted in Akt hyperactivation, Foxo3 hyperphosphorylation, and Foxo3 nuclear export, culminating in global primordial follicle activation and premature ovarian failure. Surprisingly, oocyte-specific ablation of PTEN and Foxo3 resulted in virtually identical phenotypes of global primordial follicle activation, suggesting that Foxo3 is the primary if not sole effector of PI3K–Akt signaling in this physiologic context. Moreover, genetic evidence from mice lacking PTEN (a major negative regulator of PI3K) in oocytes demonstrates that the entire primordial follicle pool becomes activated. Subsequently, all primordial follicles become depleted in early adulthood, causing premature ovarian failure. This shows that the mammalian oocyte serves as the headquarters of follicle activation programming and that the oocyte PTEN-PI3K pathway governs follicle activation through control of the initiation of oocyte growth (Castrillon *et al*., 2003; Reddy *et al*., 2008). Figure 3 schematically illustrates the signaling pathway of KL/c-Kit via Phosphoinositide 3-Kinase (PI3K)-Akt-FKHRL1 and PTEN.

In addition to the PI3K–Akt pathway, several transcription factors are known to affect the regulation of oocyte-specific genes during early folliculogenesis. FIGLA (Factor in the Germline Alpha; Joshi *et al*., 2007), NOBOX (Newborn Ovary Homeobox gene; Choi *et al*., 2007), and Sohlh1 (Spermatogenesis and oogenesis helix-loop-helix 1; Pangas *et al*., 2006) are all critical to primordial follicle formation and maintenance. FIGLA is an oocyte-specific basic helix-loop-helix (bHLH) transcription factor that regulates the expression of many genes in the ovary, including zona pellucida genes (Soyal *et al*., 2000). NOBOX is a transcription factor necessary for the expression of several key oocyte-specific genes, including GDF-9. Sohlh1 is another germ cell-specific gene that lies upstream of Lhx8 (LIM-homeobox protein 8), a gene involved in oogenesis. Sohlh1 is preferentially expressed in primordial oocytes (Pangas *et al*., 2006). Manipulations that delete the Sohlh1 gene lead to disturbances in the formation of primary follicles from primordial follicles (Pangas *et al*., 2006); these findings are similar to those observed in tests blocking the c-Kit gene (Yoshida *et al*., 1997). More recently, the novel transcription factor Sohlh2, which is preferentially expressed in germ cells of the embryonic ovary and oocytes of primordial and primary follicles, was discovered (Ballow *et al*., 2006). Both factors (Sohlh1 and Sohlh2) have a crucial role in oogenesis. Choi *et al*. (2008b) demonstrated that Sohlh2-knockout adult female mice are infertile due to a lack of ovarian follicles. Further, Sohlh2-deficient ovaries can form primordial follicles and, despite limited oocyte growth, do not differentiate surrounding granulosa cells into cuboidal and multilayered structures. In addition, Sohlh2 deficiency causes infertility in female mice and affects the expression of numerous oocyte-specific genes (e.g., GDF-9 and c-Kit) in the ovary. Similar results were observed by Toyoda *et al*. (2009) after inhibition of the Sohlh2 gene in mice. These authors concluded that the Sohlh2 gene might be a key gene in the transcriptional cascade responsible for germ-cell differentiation through the acquisition of KIT expression.

Another critical factor for the maintenance and differentiation of oocytes during early oogenesis is Lhx8. It is a member of the LIM-homeobox transcription factor family and is preferentially expressed in germ cells and primordial, primary, and antral follicles within the mouse ovary (Pangas *et al*., 2006). Choi *et al*. (2008a) verified that Lhx8-deficient ($Lhx8^{-/-}$) ovaries are similar to newborn wild-type ovaries. After $Lhx8^{-/-}$ inhibition, a large oocyte loss in mice ovaries that led to female infertility was observed. $Lhx8^{-/-}$ ovaries fail to maintain the primordial follicles, and the transition from primordial to growing follicles does not occur. $Lhx8^{-/-}$ ovaries misexpress oocyte-specific genes, such as GDF-9 and NOBOX. In addition, $Lhx8^{-/-}$ ovaries demonstrated a decrease in the expression of Bax and caspases 2 and 3, without loss of Bcl2 gene expression. A drastic reduction in the KL and c-Kit genes was also observed, and this reduction may explain the loss in oocyte number. On the contrary, $NOBOX^{-/-}$ mice did not show decreased expression of KL, c-Kit, or apoptosis genes (Rajkovic *et al*., 2004).

The mechanism by which KL causes oocyte growth is unknown. Proteins involved in c-Kit signal transduction via PI3K, such as mitogen-activated protein kinase (MAPK) and Janus-Activated kinase 2 (JAK2), are possible candidates. It has been suggested that high levels of KL constantly activate PI3K signaling

in oocytes, increasing their growth (Reddy *et al*., 2005). KL appears to trigger oocyte growth, for example with the slow accumulation of factors (p34cd2, cyclin B1, MAPK, cdc25) required for meiosis resumption (Reddy *et al*., 2005). It is not clear whether these intracellular factors are activated by other cytokines and growth factors to balance the KL signaling for oocyte growth and follicular development under KL-deficient conditions (Moniruzzaman *et al*., 2007). Furthermore, the activation of MAPK is a key event for many cellular processes, including proliferation, differentiation, and apoptosis (Davis, 1993). There are three main classes of MAPK: extacellular-regulated kinases (Erks; Hunter, 1995), c-Jun NH2-terminal protein kinases (JNKs; Derijard *et al*., 1994), and p38-MAPKs (Lee *et al*., 1994; Goedert *et al*., 1997). Erks are mainly activated in response to growth factors and cytokines, whereas JNKs and p38-MAPKs are activated in response to different cell stresses. In a study by Jin *et al*. (2005), it was demonstrated that KL activated only Erk types 1 and 2.

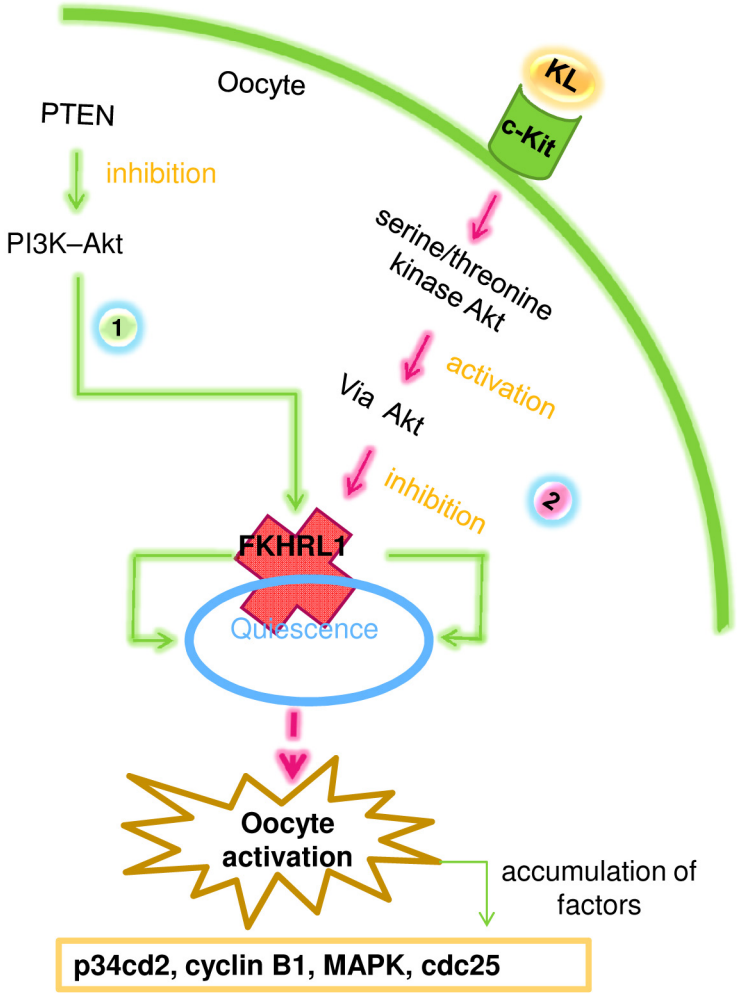

Figure 3. Pattern of signaling of KL/c-Kit system via Phosphoinositide 3-Kinase (PI3K)-Akt-FKHRL1 and PTEN. The oocyte PTEN-PI3K pathway governs follicle activation through control of initiation of oocyte growth, since it inhibits the PI3K-Akt pathway, which then allows the FKHRL1 to keep quiescent oocytes (1). The binding of KL to its receptor c-Kit phosphorylate serine/threonine kinase Akt group and activates Akt pathway, thus inhibiting the activity of FKHRL1 in oocytes allowing its activation (2). It is likely that KL starts oocyte growth, for instance, with the slow accumulation of factors required for meiosis resumption, such as p34cd2, cyclin B1, MAPK, cdc25. KL: Kit Ligand; c-Kit: receptor of Kit Ligand; Akt: signaling molecule; FKHRL1(Foxo3a): member of the FOXO subfamily and of forkhead transcription factors and is a substrate of Akt; PTEN: phosphatase and tensin homolog deleted on chromosome 10.

The cascade from granulosa cell KL to oocyte c-Kit PI3K-Akt-FKHRL1 is of great importance, not only to promote oocyte growth but also to promote the secretion of factors that influence granulosa cell proliferation and differentiation (Reddy *et al*., 2005). More recently, the action of glial-derived neurotrophic factor (GDNF) on the transition from primordial to primary follicles has been demonstrated (Dole *et al*.,

2008). GDNF signaling occurs via a protein complex. The receptor GDNF and the ligand-receptor complex activate the ubiquitous tyrosine kinase receptor RET (Amoresano *et al*., 2005; Carmillo *et al*., 2005; Pozas and Ibanez, 2005; Vargas-Leal *et al*., 2005). Activation of RET by this complex leads to the activation of intracellular signaling pathways involved in cell proliferation and differentiation (Naughton *et al*., 2006). GDNF is localized to the oocyte cytoplasm in follicles from all developmental stages as well as to cumulus, theca, and granulosa cells in rat antral follicles. Its receptor, α1 (GFRα1), was localized to the oocyte cytoplasm of primordial and primary follicles, and reduced levels were noted in the oocytes of antral follicles, theca cells, and epithelium (Dole *et al*., 2008). In observations from organ cultures, Dole *et al*. (2008) noted that ovaries treated with GDNF for 10 days contained a significant increase in developing follicles; this finding is similar to that observed with KL treatment, which was previously shown to promote follicle development. Moreover, it was verified that GDNF did not affect KL expression during primordial follicle development.

Effects of the KL/c-Kit system on PGC, oocyte, and follicle survival

It is known that KL is responsible for promoting the survival of different kinds of cells involved in the processes of hematopoiesis, melanogenesis, and gametogenesis, including PGCs, oogonia, and oocytes (Reynaud and Driancourt, 2000; Reynaud *et al*., 2001). *In vivo* studies have demonstrated that a mutation in the genes that encode KL and its receptor c-Kit induced sterility in mice, since PGCs did not survive during early oogenesis (Buehr *et al*., 1993). *In vitro* studies have demonstrated that the activation of the c-Kit receptor negatively regulates apoptosis of PGCs mediated by Fas antigen (a membrane-associated polypeptide that is a member of the tumoral necrosis factor receptor superfamily; Sakata *et al*., 2003). This inhibition occurs through PI3K-Akt signaling. Another study showed that the synergistic action of KL with insulin-like growth factor-1 (IGF-1) and leukemia inhibitory factor (LIF) improved the survival of PGCs of mice during *in vitro* culture (Lyrakou *et al*., 2002).

KL also seems to be involved in oocyte and preantral follicle survival (mice: Reynaud *et al*., 2000; rat: Jin *et al*., 2005). In these studies, *in vitro* culture of preantral follicles in a medium with KL either alone or with an anti-KL or anti-c-Kit antibody was performed. In mice, KL inhibited apoptosis of oocytes from primordial follicles after *in vitro* culture (Jin *et al*., 2005) through an increase in the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL and a reduction in the expression of the pro-apoptotic factor Bax. These anti-apoptotic effects seem to be mediated by PI3K signaling (Jin *et al*., 2005). Other authors have demonstrated that inhibition of the KL/c-Kit interaction via an anti/c-Kit antibody promoted oocyte death *in vitro* (mouse: Reynaud *et al*., 2000). When this interaction was inhibited *in vivo*, however, no effect on primordial follicle survival was observed (mouse: Yoshida *et al*., 1997). Moreover, an increase in the number of atretic follicles was verified after blockade of the c-Kit receptor in humans (Carlsson *et al*., 2006). In a mouse model, Doneda *et al*. (2002) showed that the addition of anti-KL or anti-c-Kit antibodies (in the absence of exogenous KL) to the culture medium leads to a significant increase in oocyte apoptosis. Furthermore, *in vitro* culture of fetal ovaries in the absence of KL causes a significant reduction (up to 99%) in the number of mouse oocytes after 72 h (Morita *et al*., 1999). According to Yan *et al*. (2000), KL production is much lower *in vitro* than *in vivo* due to the absence of FSH. Therefore, higher concentrations of this factor are necessary *in vitro*. In addition, some studies have reported that KL inhibits the expression of BMP-15, increasing the expression of FSH receptors (Thomas *et al*., 2005). This pathway is important for reducing follicular atresia in many species (human: Roy and Treacy, 1993; mouse: Baker and Spears, 1997; caprine: Matos *et al*., 2007).

The KL/c-Kit system regulates primordial follicle activation *in vitro*

To demonstrate the different signaling pathways involved in KL action, many studies have shown that KL promotes primordial follicle activation *in vitro*. Yoshida *et al*. (1997) demonstrated that blockade of c-Kit using a monoclonal antibody affects primordial follicle development. In addition, after *in vitro* culture of rat preantral follicles, Nilsson and Skinner (2004) have shown that KL (50 ng/ml) significantly increased the proportion of developing follicles in comparison to the control. This development did not occur after the addition of an anti-basic fibroblast growth factor antibody, which means that this antibody interferes with the ability of KL to promote follicular activation and development. An *in vitro* study showed that KL (100 ng/ml) increased the transition from rat primordial to primary follicles after 5 or 14 days of culture (Parrot and Skinner, 1999). According to these authors, low levels of KL may not be sufficient to promote follicular development. Therefore, it is necessary to use higher concentrations of this factor (Parrot and Skinner, 1997). Moreover, during culture of human preantral follicles, KL at 1, 10, or 100 ng/ml did not show any effect on the early stages of follicular development after 7 or 14 days (Carlsson *et al*., 2006). In a mouse model, however, 10 ng/ml of KL promoted the development of primordial follicles to the primary stage during *in vitro* culture for 9 days (Wang and Roy, 2004). Furthermore, after 8 days of culture, KL (50 or 150 ng/ml) led to the activation of mouse rather than rabbit primordial follicles (Hutt *et al*., 2006a). In

another study, follicular activation was observed when higher concentrations of KL (i.e., 100, 200, or 400 ng/ml) were used; in contrast, the lowest concentration (25 ng/ml) did not cause activation (Fernandez *et al*., 2008). According to the literature, there are many conflicting results. In spite of differences in culturing conditions (e.g., the culture medium used), these controversial results may suggest the existence of differences between species (Carlsson *et al*., 2006).

In vitro effects of the KL/c-Kit system on oocyte and follicle growth

The expression of both KL and c-Kit is consistent with the role of this system in early oocyte growth (Manova *et al*., 1993), which was already demonstrated *in vitro* (Packer *et al*., 1994; Klinger and De Felici, 2002). It is suggested that KL synthesized by granulosa cells binds to c-Kit present in the oocyte, thereby promoting oocyte growth. Klinger and De Felici (2002) working with mouse oocytes reported a two-fold increase in oocyte diameter compared to controls after 4 days of culture in the presence of 50 or 100 ng/ml of KL. These same authors reported that mouse oocyte growth is characterized by three distinct phases: the initial stage of growth can be promoted by KL and does not require gap junctions; followed by a growth phase which depends on KL and gap junctions; and finally, growth phase is dependent only on gap junctions and is independent of KL (Klinger and De Felici, 2002). Moreover, the use of 50 ng/ml of KL stimulated theca cell growth, as estimated by DNA synthesis and the increase in androstenedione production in the absence of gonadotropins (Parrot and Skinner, 1997). Furthermore, oocytes of prepubertal animals seemed to show the ability to increase mRNA for KL in granulosa cells (Packer *et al*., 1994); this finding was confirmed by Joyce *et al*. (1999). Therefore, it is suggested that the oocyte increases the expression of KL in the surrounding granulosa cells and this increase in KL stimulates oocyte growth (Driancourt *et al*., 2000). Nevertheless, Cecconi and Colonna (1996) did not observe any effect of KL on the growth of 12-day-old mouse oocytes, suggesting that KL may have different actions in each stage of oocyte development. In addition, after culture of whole mouse ovaries for 9 days, KL (100 ng/ml) did not promote oocyte growth (Wang and Roy, 2004). These differences in results within the same species may be attributed to the stage of oocyte development, different experimental techniques used for the evaluation of oocyte growth, or differences in culture conditions cited previously.

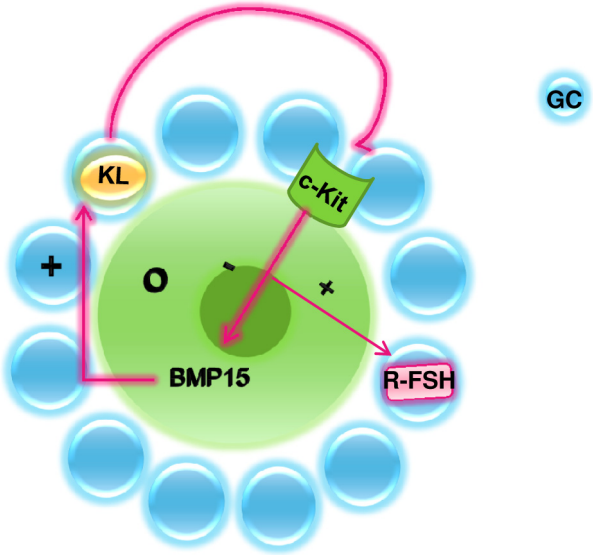

Figure 4. Kit Ligand/Bone Morphogenetic Protein negative feedback loop. BMP-15 produced by the oocyte increases KL expression by granulosa cells. Therefore, KL acts through c-Kit in the oocyte membrane to promote growth and negatively regulate BMP-15 expression, which produces a consequent increase in FSH receptors. KL: Kit Ligand; c-Kit: receptor of Kit Ligand; BMP-15: Bone Morphogenetic Protein-15; R-FSH: FSH receptor; O: oocyte; GC: granulosa cells. Adapted: Hutt *et al.* (2006b).

Finally, the complex interaction between oocyte and granulosa cells is influenced by KL/c-Kit, hormones, and growth factors. Some studies suggested that low concentrations of FSH are necessary for the appropriate regulation of the paracrine factors that trigger oocyte development. In this way, the correct concentration of FSH may be important for the proper modulation of KL and BMP-15, increasing oocyte growth (Thomas *et al*., 2005). BMP-15 produced by the oocyte increases KL expression by granulosa cells. Therefore, KL acts through c-Kit in the oocyte membrane to promote growth and negatively regulate

BMP-15 expression, which produces a consequent increase in FSH receptors (Hutt *et al*., 2006b; Thomas and Vanderhyden, 2006), as shown in Fig. 4. In humans, Carlsson *et al*. (2006) have demonstrated that different concentrations of KL (1, 10, or 100 ng/ml) did not increase follicular diameter compared to controls after 7 or 14 days of culture. Nevertheless, after 8 days of mouse and rabbit preantral follicle culture, the addition of 50 or 150 ng/ml of KL with FSH caused a significant increase in oocyte diameter relative to controls (Hutt *et al*., 2006a).

Collectively, these results underline the crucial role of the KL/c-Kit system in the control of mammalian reproduction, especially oogenesis and folliculogenesis. The KL/c-Kit system, through different signaling proteins, regulates PGCs and follicular viability, the initiation of primordial follicle growth, and oocyte and follicle development. Further research in this field will greatly advance our understanding of ovarian physiology, which may help clinicians and reproductive biologists to find a role for the KL/c-Kit system in the diagnosis and treatment of reproductive disorders affecting human and animal fertility.

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