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Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis

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Paracrine signalling between the oocyte and its surrounding somatic cells is fundamental to the processes of oogenesis and folliculogenesis in mammals. The study of animal models has revealed that the interaction of granulosa cell-derived kit ligand (KL) with oocyte and theca cell-derived c-Kit is important for multiple aspects of oocyte and follicle development, including the establishment of primordial germ cells within the ovary, primordial follicle activation, oocyte survival and growth, granulosa cell proliferation, theca cell recruitment and the maintenance of meiotic arrest. Though little is known about the specific roles of KL and c-Kit during human oogenesis, the expression profiles for KL and c-Kit within the human ovary suggest that they are also functionally relevant to female fertility. This review details our current understanding of the roles of KL and c-Kit within the mammalian ovary, with a particular focus on the functional diversity of this receptor–ligand interaction at different stages of oocyte and follicle development.

Key words: c-kit/folliculogenesis/kit ligand/ovary/stem cell factor

Introduction

The female gamete is stored in the ovary in the form of primordial follicles, which are comprised of small, non-growing functionally immature oocytes, surrounded by a single layer of squamous granulosa cells. Throughout the female's reproductive life span, a small number of primordial follicles are stimulated to grow, in a process referred to as follicle activation (review: Fair, 2003). Once growth is initiated, the follicle embarks on a pre-programmed course of maturation and development that is necessary for its successful ovulation and fertilization, or alternatively is lost from the follicle pool by atresia (review: Fair, 2003). During postnatal life, ovarian follicles continue to grow, mature and either ovulate or regress in a cyclic fashion. The continuous activation and apoptosis of primordial follicles eventually leads to exhaustion of the original pool, followed by reproductive senescence (Richardson *et al*., 1987). Consequently, ovarian endowment, primordial follicle activation and folliculogenesis are prerequisites for the development of mature oocytes, and are therefore basic determinants of female reproductive fitness in mammalian species.

Despite this importance, the factors that regulate many of the aspects of oogenesis and folliculogenesis remain a mystery. In particular, the specific mechanisms that maintain primordial follicle dormancy, as well as those that promote follicle activation, remain unknown. A deeper understanding of the mechanisms that regulate the primordial to primary follicle transition would be of both fundamental and clinical significance. For example, treatments for infertility associated with abnormal follicle recruitment and development may benefit from a more thorough understanding of the early stages of follicle growth. Additionally, this knowledge may be applied to generate mature oocytes from primordial follicles *in vitro* to propagate valuable domestic animals, and endangered wildlife species, using assisted reproductive technologies (review: Silva *et al*., 2004). Even those interested in the development of contraceptive agents to control fertility (Sacco *et al*., 1983), especially of pest animals (reviews: Epifano and Dean, 1994; Seamark, 2001), could gain from such research.

Kit ligand (KL) is a pleiotropic growth factor that exerts an influence on target cells through binding its cognate tyrosine kinase receptor, c-Kit. Both KL and c-Kit are actively expressed by a variety of developmentally distinct cell lineages during both embryogenesis and adult life. Roles for KL and c-Kit in gametogenesis, melanogenesis and haematopoiesis have been described (review: Fleischman, 1993).

Much of what is understood about the function of KL and c-Kit in the ovary has been garnered from the study of mouse mutants. KL and c-Kit are encoded by the *steel* and *white spotting* loci, respectively (Chabot *et al*., 1988; Copeland *et al*., 1990; Huang *et al*., 1990; Zsebo *et al*., 1990). There is considerable evidence from mice carrying mutations at these loci, to suggest a role for the receptor–ligand pair in primordial germ cell (PGC) survival, migration and proliferation, and also in follicle development (McCoshen and McCallion, 1975; Brannan *et al*., 1991; Flanagan *et al*., 1991; Brannan *et al*., 1992; Huang *et al*., 1993; Bedell *et al*., 1995; Bedell *et al*., 1996). For example, mice homozygous for the viable allele *steel dickie* (*Sld/Sld*) are sterile,

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anaemic, black-eyed and white-furred (Brannan *et al*., 1991; Flanagan *et al*., 1991). It is thought that infertility in these animals is due to the failure of PGCs to successfully migrate to the fetal genital ridge (Brannan *et al*., 1991; Flanagan *et al*., 1991). Follicles are present in the ovary of infertile mice carrying the *steel panda* (*Slpan*/*Slpan*) or *steel contrasted* (*Slcon/Slcon*) mutations, but they do not progress beyond the primary stage of development (Huang *et al*., 1993; Bedell *et al*., 1995). Less severe mutations that result in reduced expression of KL, such as *steel transfer* (*Sl/Slt*), permit follicle growth to the antral stage, though these animals ovulate sporadically and have limited fertility (Kuroda *et al*., 1988).

In humans, mutations in the c-Kit gene can result in piebaldism, a benign autosomal dominant disorder causing a pigmentation deficiency (Ezoe *et al*., 1995). Defects in the c-Kit gene have also been associated with human cancers including mastocytosis (Longley *et al*., 1996) and gastrointestinal tumours (Hirota *et al*., 1998). However, mutations in either c-Kit or KL genes have not yet been detected in cases of human female infertility.

Structure/function characteristics of KL

Kit ligand is classified as a 4-helix-bundle cytokine, the family members of which share little similarity at the amino acid level, though they can be aligned by their secondary structure (Rozwarski *et al*., 1994). Other members of this family include macrophage colony stimulating factor (M-CSF) (Pandit *et al*., 1992), platelet-derived growth factor (PDGF) (Oefner *et al*., 1992) and Flt3 ligand (Savvides *et al*., 2000). KL exists as both soluble and membrane-spanning proteins, which are synthesized from two alternatively spliced forms of the messenger RNA (mRNA) (Huang *et al*., 1992; Lu *et al*., 1992). In the mouse, KL-1, the 248-amino acid soluble form of KL, is initially membrane bound, but proteolysis at a cleavage site encoded by exon 6 releases 164–165 amino acids located in the extracellular portion of the protein (Figure 1) (Lu *et al*., 1992). In the 220-amino acid transmembrane form, KL-2, exon 6 is missing and so this protein remains primarily anchored to the membrane (Figure 1). Even though this protein lacks the major cleavage site, a soluble version of KL-2 has also been detected (Huang *et al*., 1992), suggesting that another proteolytic pathway exists.

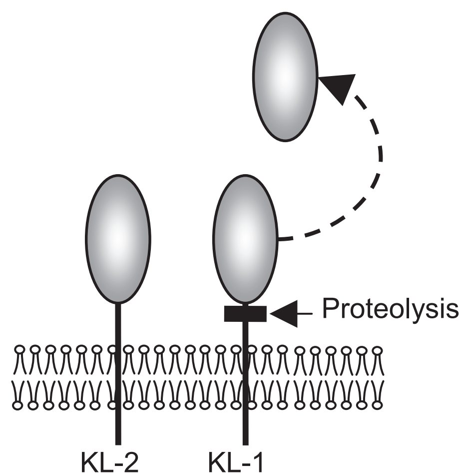

Figure 1. Structure of KL-1 and KL-2. KL-1 and KL-2 are comprised of an extracellular domain, hydrophobic transmembrane domain and a short cytoplasmic tail. Both forms of KL are initially anchored to the membrane. However, exon 6 of KL-2 encodes a proteolytic cleavage site that is absent in KL-1. Consequently, the KL-2 tends to remain membrane bound, whereas the KL-1 is usually found in soluble form.

Under physiological conditions, KL exists in monomeric form (Hsu *et al*., 1997). The crystal structure of KL predicts that two protomers interact head-to-head to form an elongated slightly bent dimer, which is stabilized by polar and non-polar interactions (Jiang *et al*., 2000; Zhang *et al*., 2000). Evidence suggests that the biological activity of both membrane-bound and soluble ligand is dependent on the formation of these non-covalent homodimers (Arakawa *et al*., 1991; Hsu *et al*., 1997). It has been hypothesized that dimerization regulates receptor activation and influences binding affinity (Lemmon *et al*., 1997; Zhang *et al*., 2000).

To identify those residues critical for biological activity, mutations, deletions and chimeric KL proteins have been made (Langley *et al*., 1994; Matous *et al*., 1996). These studies have shown that deletion of the first three residues from the amino terminus reduces the binding of KL to c-Kit by 50% (Langley *et al*., 1994). Similarly, deletion of the cysteine residue at position 4 inactivates the cytokine, while deletion of the cysteine residue at position 138 only reduces the activity (Langley *et al*., 1994). Therefore, the amino terminus and the integrity of the cysteine 4–cysteine 89 disulphide bond are essential for full biological function, whereas the second intramolecular disulphide bond at position cysteine 43–cysteine 138 is somewhat dispensable. Studies using KL/M-CSF chimeric proteins have shown that arginine 121, aspartic acid 124, lysine 127 and aspartic acid 128 are crucial for activity (Matous *et al*., 1996). Amino acids 61–65 and 91–95, which are located near the tail region of each molecule, are also essential (Matous *et al*., 1996). These data are in support of Langley *et al*. (1994), who proposed that KL contains a functional core comprising amino acids 1–141.

Amino acid residues important for the cell surface expression of KL have also been identified. Transport of KL to the plasma membrane is through the endoplasmic reticulum and requires the presence of a C-terminal valine residue located 19–36 amino acids from the border between the transmembrane and cytoplasmic domains (Paulhe *et al*., 2004). Interestingly, the basolateral expression of KL by Sertoli cells is important for spermatogonial survival (Brannan *et al*., 1992; Manova *et al*., 1993) and this polarized sub-cellular localization requires a leucine residue in the cytoplasmic tail of KL (Wehrle-Haller and Imhof, 2001).

To address the question of whether the cytoplasmic domain of KL has a role in intracellular signalling, *Sl17H* mice have been studied. *Sl17H* is a mutation in which the 36 amino acids comprising the cytoplasmic domain of KL are substituted for by 28 novel amino acids (Brannan *et al*., 1992; Tajima *et al*., 1998a; Kapur *et al*., 1999). Male, but not female mice carrying this mutation are infertile, though both sexes exhibit pigmentation deficiencies and anaemia. Defects in mice harbouring the *Sl17H* mutation have been shown to be the consequence of defective KL dimer formation, cellular processing and poor surface expression, suggesting that the cytoplasmic tail of KL itself does not have a direct role in signal transduction (Kapur *et al*., 1999; Tajima *et al*., 1998a).

Lastly, glycosylation appears to be important for the biological activity of KL. Natural and Chinese hamster ovary cell-derived recombinant KL is about 30% carbohydrate by weight. KL is heavily glycosylated, with both N-linked and O-linked sugars, on multiple asparagine, serine and threonine residues (Arakawa *et al*., 1991; Lu *et al*., 1991, 1992). Four putative asparagine glycosylation sites are found in the functional core: asparagine 65, asparagine 72, asparagine 93 and asparagine 120. Asparagine at position 72 is buried within the interface and is not glycosylated, asparagine 120 is always glycosylated, though its glycosylation status does not affect the binding of KL to c-Kit, and asparagine 65 and asparagine 93 are only occasionally glycosylated, with the glycosylation of these residues having an adverse affect on receptor binding (Lu *et al*., 1992).

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Structural characteristics of c-Kit

Kit ligand elicits its biological effects by binding and activating the type III tyrosine kinase receptor c-Kit (Lemmon *et al*., 1997). Other members of this family include PDGF receptors α and β and the M-CSF receptor (Qiu *et al*., 1988). These receptors are more similar at the primary amino acid level than their cognate ligands. All members of this family have five extracellular immunoglobulin-like loops, a hydrophobic transmembrane domain and a cytoplasmic protein kinase domain with an 80-amino acid insert (Figure 2) (review: Roskoski, 2005).

Evidence from mutagenesis studies, epitope mapping with site-specific c-Kit antibodies and crystal structure analyses indicate that KL binds to c-Kit at immunoglobulin loop-like domains 1, 2 and 3 (Figure 2) (Blechman *et al*., 1993; Zhang *et al*., 2000). Antibodies directed against the first two amino terminus immunoglobulin-like loop domains inhibit c-Kit activity (Blechman *et al*., 1993), and when the third immunoglobulin-like loop domain is deleted, the ability of KL to bind the receptor is significantly reduced (Blechman *et al*., 1993). Moreover Kit123, which contains only the first three immunoglobulin-like loops, binds KL in the same way as the complete receptor protein (Lemmon *et al*., 1997). KL-induced dimerization of the full-length membrane-associated c-Kit receptor requires immunoglobulin-like loop 4 (Figure 2) (Blechman *et al*., 1995). Dimerization is rapidly followed by phosphorylation, recruitment of signalling proteins, tyrosine phosphorylation of substrates and ultimately the activation of multiple signalling pathways (review: Roskoski, 2005).

A number of c-Kit isoforms have been described (Reith *et al*., 1991; Crosier *et al*., 1993; Wypych *et al*., 1995; Albanesi *et al*., 1996). Alternate splicing in both mice and humans results in isoforms characterized by the residues glycine–asparagine–asparagine–lysine (GNNK) in the juxta-membrane region of the extracellular domain (Crosier *et al*., 1993; Reith *et al*., 1991). Differences in the timing and level of c-Kit tyrosine kinase activity have been reported for the two variants (Voytyuk *et al*., 2003). Another splice variant identified in humans results in the presence, or absence, of a single serine residue in the interkinase region of the cytoplasmic domain of c-Kit. While these proteins can be co-expressed in humans, only the serine-containing variant is present in mice (Crosier *et al*., 1993). Truncated versions of c-Kit (tr-kit) lacking extracellular and transmembrane domains have been described in spermatids (Albanesi *et al*., 1996). Interestingly, microinjection of mouse oocytes with tr-Kit strongly activates the Src-family of kinases and induces resumption of meiosis in MII-arrested oocytes (Sette *et al*., 2002). Therefore, tr-kit may be involved in oocyte activation at fertilization. Finally, a soluble form of c-Kit, consisting of a part of the extracellular domain (KitS), has been detected in human serum (Wypych *et al*., 1995). KitS binds KL with high affinity and may therefore modulate the activity of KL *in vivo* by sequestering it and preventing its binding to membrane-associated c-Kit (Turner *et al*., 1995).

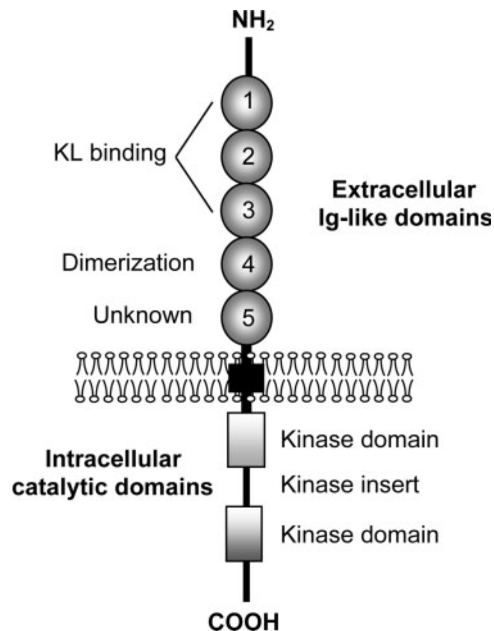

Figure 2. Structure of c-Kit. The extracellular domain of c-Kit is comprised of five immunoglobulin like (Ig-like) loops, of which the first three are believed to bind kit ligand (KL). The fourth Ig-like loop is important for receptor dimerization and the function of the fifth Ig-like loop is unknown. The intracellular domain of c-Kit is characterised by a kinase domain separated by a large kinase insert.

KL and c-Kit ovarian expression

The ovarian expression pattern of KL and c-Kit mRNA and protein has been previously studied in primates and humans (Horie *et al*., 1993; Gougeon and Busso, 2000; Hoyer *et al*., 2005; Stoop *et al*., 2005), sheep (Clark *et al*., 1996; Tisdall *et al*., 1997; Tisdall *et al*., 1999) and rodents (Manova *et al*., 1990; Horie *et al*., 1991; Keshet *et al*., 1991; Motro *et al*., 1991; Manova *et al*., 1993; Motro and Bernstein, 1993; Doneda *et al*., 2002). In general, PGCs, theca cells and oocytes express the c-Kit receptor, whereas granulosa cells and ovarian epithelial cells produce KL.

Use of the sensitive method of *in situ* RT-PCR has demonstrated the expression of both KL and c-Kit mRNAs in germ cells located in the outer regions of the fetal mouse ovary between 16.5 and 17.5 days post-coitus (Doneda *et al*., 2002). Histological analysis confirmed that the majority of co-expressing oocytes were in the zygotene/pachytene stage of meiotic prophase I, suggesting that this autocrine loop may have an important role during this stage of development (Doneda *et al*., 2002). By day 18.5 of embryonic development, KL expression is restricted to somatic cells in the medulla of the ovary (Doneda *et al*., 2002). At birth, KL mRNA and protein expression is high in the central cords of the mouse ovary, and thereafter KL expression becomes specifically associated with granulosa cells (Manova *et al*., 1993; Doneda *et al*., 2002).

In post-natal mouse ovaries, KL protein and mRNA expression is detected in the granulosa cells of follicles at all stages of development, though its expression is very low in primordial and primary follicles, and in the cumulus cells of antral follicles (Figure 3) (Manova *et al*., 1993; Motro and Bernstein, 1993). While only limited primordial follicle granulosa cell staining is detected in mice (Manova *et al*., 1993), more than 90% of primordial follicles in sheep consist of at least one granulosa cell expressing KL protein (Tisdall *et al*., 1997; Tisdall *et al*., 1999). Moreover, KL protein is detected in the oocytes of resting and growing murine follicles, presumably as a consequence of receptor-mediated endocytosis (Figure 3) (Manova *et al*., 1993; Jahn *et al*., 2002; Kang *et al*., 2003).

C-Kit mRNA and protein have been found in high levels in migrating and mitotic PGCs, but c-Kit mRNA is not present in germ cells undergoing the first stages of meiosis during fetal life in both mice and sheep (mice: Manova and Bachvarova, 1991; Manova *et al*., 1990; sheep: Clark *et al*., 1996). The expression of c-Kit mRNA then resumes in diplotene oocytes (mice: Manova *et al*., 1990; Horie *et al*., 1991; Doneda *et al*., 2002; sheep: Clark *et al*., 1996). The reason for this cessation in c-Kit expression is not yet understood. The oocytes of primordial and later stage follicles uniformly express c-Kit protein and mRNA, though expression decreases in antral follicles (Figures 3 and 4) (mice: Horie *et al*., 1991; sheep: Clark *et al*., 1996; Tisdall *et al*., 1999). Theca cells also express c-Kit protein and mRNA (Figure 3) (mice: Motro and Bernstein, 1993; Kang *et al*., 2003; cattle: Parrott and Skinner, 1997).

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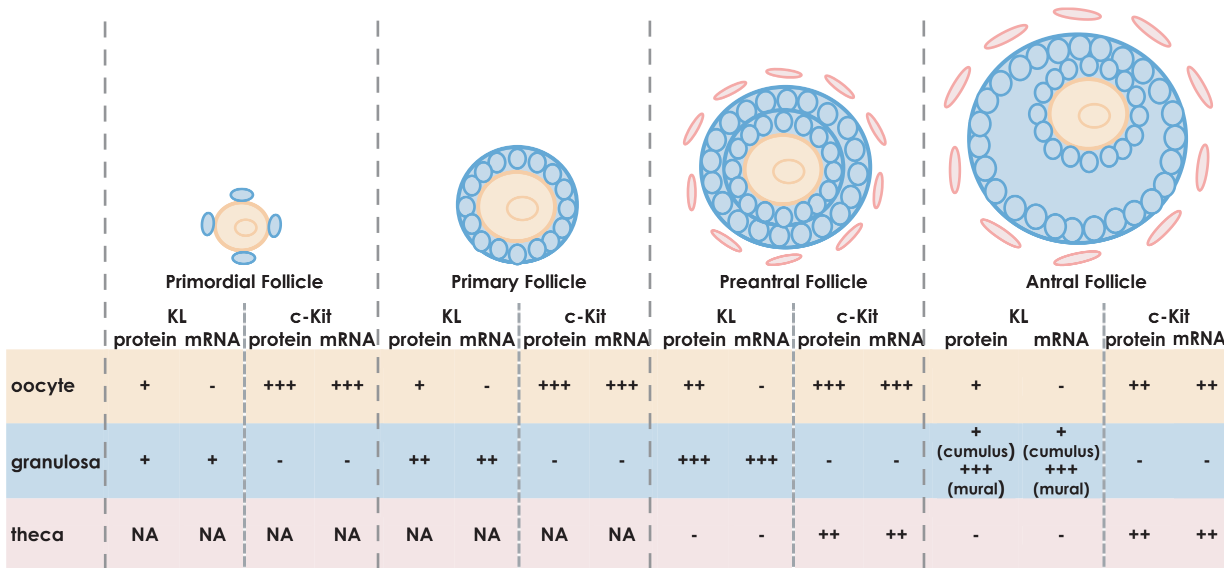

	Primordial Follicle				Primary Follicle				Preantral Follicle				Antral Follicle			
	KL		c-Kit		KL		c-Kit		KL		c-Kit		KL		c-Kit	
	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA	protein	mRNA
oocyte	+	-	+++	+++	+	-	+++	+++	++	-	+++	+++	+	-	++	++
granulosa	+	+	-	-	++	++	-	-	+++	+++	-	-	+ (cumulus) +++ (mural)	+ (cumulus) +++ (mural)	-	-
theca	NA	NA	NA	NA	NA	NA	NA	NA	-	-	++	++	-	-	++	++

Figure 3. Expression of kit ligand (KL) and c-Kit during folliculogenesis. In mice, KL protein and mRNA are expressed at low levels by granulosa cells in primordial follicles. Expression of KL by granulosa increases throughout the primary and pre-antral stage, but levels decrease in the cumulus cells of antral follicles, while the mural granulosa cells of these follicles continue to express high levels. KL protein, but not mRNA, can also be detected within oocytes at all stages of folliculogenesis. c-Kit protein and mRNA expression is high in the oocytes of primordial, primary and pre-antral follicles and then decreases in antral follicles. Theca cells also express moderate levels of c-Kit protein and mRNA. Relative expression levels: +, low; ++, moderate; +++, high. NA = Not applicable as no theca cells present at this stage of follicle development.

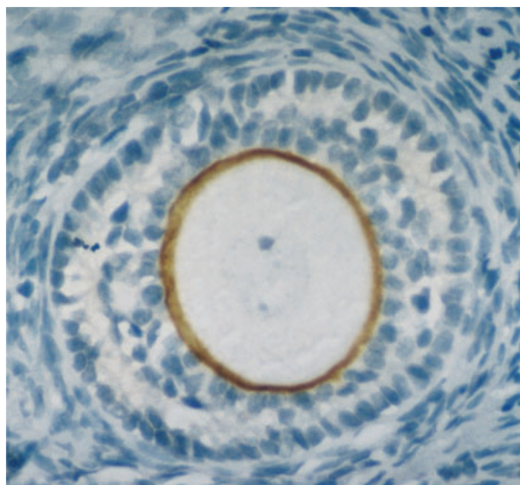

Figure 4. Immunolocalization of c-Kit within a pre-antral follicle. c-Kit is localized to the membrane of oocytes in preantral follicles (brown staining).

Given the limited availability of human samples, information regarding the temporal expression levels of KL and c-Kit during human oogenesis and folliculogenesis is less comprehensive. However, the few studies available are beginning to provide insight into the potential roles of KL and c-Kit in human female fertility. Similar to observations in mice, immunohistochemical studies have shown that in human fetal ovaries the oocytes of primordial follicles stain faintly for c-Kit (Robinson *et al*., 2001; Hoyer *et al*., 2005; Stoop *et al*., 2005). C-Kit expression initially intensifies in growing pre-antral follicles, only to decrease again with antrum formation (Hoyer *et al*., 2005; Stoop *et al*., 2005). Interestingly, there is some evidence to suggest that c-Kit protein is also expressed by the granulosa cells of newly formed human primordial follicles (Hoyer *et al*., 2005). KL protein has been immunolocalized to the granulosa cells of primordial, pre-antral and early antral follicles in human fetal ovaries (Hoyer *et al*., 2005). However, in another study, KL and c-Kit proteins were detected in the oocyte of follicles in both fetal and adult ovaries, but neither protein was detected in granulosa cells (Abir *et al*., 2004). Using RT-PCR and Western blotting, Tanikawa *et al*. (1998) reported that c-Kit mRNA and protein are expressed by both cumulus granulosa cells and metaphase II oocytes collected from women undergoing IVF. Collectively, these expression profiles support both autocrine and paracrine roles for KL and c-Kit during primordial follicle assembly and throughout folliculogenesis in humans.

Primordial germ cells

Oogenesis begins with the formation of PGCs during the early stages of embryonic development. Using a combination of passive transfer and amoeboid-like movement, proliferating PGCs migrate from the extra-embryonic mesoderm, through the hindgut, to eventually colonize the gonadal ridge (review: Molyneaux and Wylie, 2004). Once located in the developing ovary, PGCs lose their motility, cease to proliferate and enter the first stages of meiosis. The oocytes are enclosed in a layer of somatic cells and arrest in meiotic prophase I at the diplotene stage, becoming primordial follicles. Those oocytes not surrounded by granulosa cells are lost by apoptosis (De Pol *et al*., 1998; Pepling and Spradling, 2001). Roles for KL in PGC migration, proliferation and survival have been hypothesized (review: Molyneaux and Wylie, 2004).

In mouse embryos, KL protein can be detected in the somatic cells that line the path of PGC migration, and the level of KL expression progressively increases with proximity to the genital ridge (Matsui *et al*., 1990; Keshet *et al*., 1991). Given that PGCs express the c-Kit receptor, it is likely that the KL/c-Kit interaction facilitates their directed migration. This hypothesis is supported by the observation that in mouse embryos carrying the *steel* mutation *Sld/Sld*, germ cells migrate to ectopic sites (McCoshen and McCallion, 1975). Defects in migration are also a characteristic of some mutations at the *white spotting* locus. For example, in *We/We* embryos germ cells cluster in the hindgut and migrate more slowly than those in wild-type animals (Buehr *et al*., 1993). Furthermore, using a series of allelic mutations at

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the *steel* locus, Zama *et al*. (2005) demonstrated that PGCs have a differential requirement for KL before and after they start migrating, with KL being partially required for their migration from the hindgut, but not for their migration to the hindgut. This study suggested that KL is only partially required for the proliferation of germ cells in the hindgut, but is absolutely necessary for proliferation once they exit the hindgut *in vivo*. KL has also been shown to support germ cell proliferation during *in vitro* culture (Dolci *et al*., 1991; Matsui *et al*., 1991).

Kit ligand has also been identified as an important survival factor for PGCs both *in vivo* and *in vitro* and clues to its mode of action are now emerging (Dolci *et al*., 1991; Resnick *et al*., 1992; Pesce *et al*., 1993; Morita *et al*., 1999; Sakata *et al*., 2003). Sakata *et al*. (2003) demonstrated that activation of the c-Kit receptor negatively regulates Fas-mediated apoptosis in germ cells. Mice carrying a mutation in c-Kit (*Wv/Wv*) have ovaries devoid of oocytes, whereas the ovaries of mice mutated at both the c-Kit and Fas alleles (*Wv/Wv:Fas-/-*) contain many oocytes. Moreover, fibroblasts treated *in vitro* with exogenous KL down-regulate Fas Ligand expression (Sakata *et al*., 2003). These observations indicate that KL promotes germ cell survival by modulating the activity of the Fas ligand. These data also imply that germ cells fail to establish in the ovaries of *Wv/Wv* mutant mice due to excessive PGC death, rather than because they fail to migrate to their correct location in the gonadal ridge. A recent study has also suggested that KL, in synergy with IGF-1 and leukaemia inhibitory factor (LIF), promotes the entry of PGCs into meiosis in fetal mouse ovaries cultured *in vitro*, perhaps by improving germ cell survival (Lyrakou *et al*., 2002).

Primordial follicle activation and early follicle development

Given that the number of gametes stored within the ovary is finite, the size of the ovarian follicle reserve and the rate of primordial follicle activation, are critical determinants of a female's reproductive lifespan. For this reason, the study of factors responsible for follicle activation is an area of active research. However, the mechanisms permitting the continuous, but gradual exit of follicles from the resting pool remain largely a mystery, with KL being one of the few known factors with a demonstrated role in this process.

Evidence in support of a role for KL and c-Kit in early follicle development comes from the treatment of neonatal mice with a neutralizing antibody to c-Kit. Yoshida *et al*. (1997) injected mice with a function-blocking antibody to c-Kit (ACK-2) at selected time-points during the first 2 weeks of life. From this experiment they concluded that the KL/c-Kit interaction is important for primordial follicle activation, particularly during the first 5 days following birth. Neutralization of c-Kit caused disturbances in initial follicle recruitment, primary follicle growth, antrum formation and granulosa cell proliferation. With regard to the latter, the ability of KL to promote granulosa cell mitogenesis was later confirmed by the work of Reynaud *et al*. (2000) and Otsuka and Shimasaki (2002). Given that granulosa cells themselves do not express the c-Kit receptor, another unknown signal from the oocyte is necessary to mediate the proliferation of granulosa cells induced by KL. Among potential candidates are growth differentiation factor 9 (GDF-9) (Gilchrist *et al*., 2004) and bone morphogenetic factor 15 (BMP15) (Otsuka *et al*., 2000).

Parrott and Skinner (1999) reported that the activation of primordial follicles is promoted by KL during ovarian organ culture. Ovaries obtained from 4-day-old rats were cultured with and without recombinant KL (100 ng/ml) and/or neutralizing c-Kit antibody (ACK-2). The number and developmental stage of follicles in the cultured ovaries were compared with those in ovaries freshly isolated from 4-day-old rats. Sections from the freshly isolated ovaries contained 68% primordial follicles, with the remaining proportion of follicles in the growing pool. Following 14 days of *in vitro* culture, a number of follicles spontaneously activated such that 50% of follicles were primordial follicles and 50% were growing. Neutralizing antibody completely blocked the spontaneous activation of primordial follicles, suggesting that endogenous KL was responsible. Additionally, in those ovaries treated with KL, only 17% of follicles remained as primordial follicles, while 83% initiated growth, and this effect was also inhibited by neutralizing antibody. Based on these finding the authors hypothesized that KL was not only important for follicle growth but was in fact sufficient to induce primordial follicle activation. Despite this finding, it seems unlikely that KL acts alone to direct follicle activation *in vivo*. It is more plausible that multiple locally or distally produced factors are involved in regulating this highly controlled and fundamentally important process (see later section).

Kit ligand has also been reported to have a number of effects on isolated oocytes at early stages of development. These effects include the promotion of oocyte growth (Packer *et al*., 1994; Klinger and De Felici, 2002). Klinger and De Felici (2002) recovered naked oocytes approximately 10 μm in diameter from mouse embryos at 15.5–16.5 days post-coitus and cultured them under various conditions. Oocytes grew to 19 μm when cultured for 4 days in the presence of KL, which was significantly larger than oocytes cultured without KL. When these oocytes were seeded onto granulosa cells under conditions known to form gap junctions, oocyte diameter increased to 42 μm after an additional 3 days of culture. Subsequent reseeding of these oocytes onto fresh granulosa cells with no additional KL resulted in further oocyte growth. The authors concluded that oocyte growth in mice is characterized by three distinct phases: an initial stage of growth that can be promoted by KL but does not require gap junctions with granulosa cells, followed by a KL and gap junction-dependent growth phase, and then finally a KL-independent and gap junction-dependent growth phase. Recent work suggests that promotion of early follicle development by KL may be mediated via the protein kinase C and MEK pathway (Jin *et al*., 2005c).

Similar to the situation described for PGCs, KL may also be involved in promoting the survival of both primordial (Jin *et al*., 2005a) and pre-antral follicles (Yoshida *et al*., 1997; Reynaud *et al*., 2000). KL inhibited apoptosis in oocytes maintained in primordial follicles in whole mouse ovaries cultured *in vitro* (Jin *et al*., 2005a). In this study, KL up-regulated antiapoptotic proteins Bcl-2 and Bcl-cL, while down-regulating the expression of proapoptotic factor Bax. These antiapoptotic effects appeared to be mediated via the phosphoinositide-3 kinase pathway (Jin *et al*., 2005a). Others have demonstrated that inhibition of the KL/c-Kit interaction with antibody to c-Kit promotes the death of oocytes *in vitro* (Reynaud *et al*., 2000), though no effect on primordial follicle survival is observed when the c-Kit/KL interaction is inhibited *in vivo* (Yoshida *et al*., 1997). These discrepancies indicate that further studies are required before a role for KL in oocyte survival during the initial stages of folliculogenesis can be firmly established.

KL at later stages of folliculogenesis

In the small antral follicles of rats there is a gradient of KL mRNA expression, with cumulus granulosa cells exhibiting greater quantities of KL-1 and KL-2 transcripts than mural granulosa cells (Ismail *et al*., 1996; Ismail *et al*., 1997). In response to pregnant mare serum gonadotrophin treatment, the distribution of KL mRNA does not change, but there is a significant increase in the level of KL mRNA detected (Ismail *et al*., 1996). Human chorionic gonadotrophin (HCG)-induced meiosis not only results in a further increase in the quantity of KL mRNA, but also precipitates a dramatic redistribution of KL mRNA expression. KL mRNA drops to undetectable levels in cumulus cells and increases to high levels in mural cells (Ismail *et al*., 1996). Moreover,

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these changes are accompanied by a shift from the membrane form to the soluble form predominating. These findings are consistent with a role for KL in maintaining meiotic arrest. Ismail *et al*. (1997) demonstrated that when c-Kit antisense oligonucleotides were injected into meiotically arrested rat oocytes; the ability of oocytes to resume meiosis was increased. Additionally, meiosis was transiently blocked when oocytes were cultured in the presence of recombinant KL (Ismail *et al*., 1996). Therefore, *in vivo* the LH surge may cause a decrease in the production of KL by those granulosa cells adjacent to the oocyte, thereby allowing meiosis to resume. Fully grown oocytes themselves are believed to inhibit KL expression in cumulus granulosa cells, and this effect is possibly mediated by GDF-9 (Joyce *et al*., 1999; Joyce *et al*., 2000).

In cattle, KL together with keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) coordinates some of the granulosa cell–theca cell interactions that are important during the later stages of follicle development. Both KGF and HGF are expressed by theca cells, and together they influence granulosa cell function and growth, as well as KL expression (Parrott and Skinner, 1998). Granulosa cell-derived KL has also been shown to promote theca cell recruitment, and to up-regulate KGF and HGF expression (Parrott and Skinner, 1998; Parrott and Skinner, 2000). Evidence suggests that the growth-promoting effects of gonadotrophins on follicle development are mediated via KGF, HGF and KL (Parrott and Skinner, 1998). Testosterone and FSH have both been shown to up-regulate KL-1 and KL-2 expression in mice (Joyce *et al*., 1999), and KL also contributes to the regulation of androgen production by theca cells in antral follicles of pigs, cattle and mice (Parrott and Skinner, 1997; Reynaud *et al*., 2000; Brankin *et al*., 2003). KL has also been shown to up-regulate the mRNA and protein expression of SF-1, StAR and P450 aromatase in mice (Jin *et al*., 2005b).

Differential roles for KL-1 and KL-2

Studies have demonstrated that the levels of KL-1 and KL-2 differ between tissues and between different populations of granulosa cells (Ismail *et al*., 1996; Ismail *et al*., 1997). Indeed, differential roles for the membrane and soluble form of KL have been demonstrated in other cellular systems, with soluble KL mediating cellular migration and the membrane form contributing to cell survival and proliferation (Wehrle-Haller and Weston, 1995; Kunisada *et al*., 1998; Tajima *et al*., 1998b). In this regard, there is mounting evidence to indicate that the membrane and soluble forms of KL are differentially regulated within the ovary and have functions that vary in accordance with the phase of germ cell or follicular development.

Recent evidence indicates that the role of KL in PGC development may be dependent on a complex balance between the membrane and soluble forms of KL. In the absence of membrane-bound KL, soluble KL interacts with fibroblast growth factor (FGF) to promote PGC proliferation *in vitro* (Kawase *et al*., 2004). By contrast, in the presence of membrane-bound KL, soluble KL inhibited FGF-induced proliferation (Kawase *et al*., 2004). These findings indicate that the role of soluble KL in either promoting or inhibiting PGC proliferation depends on the presence or absence of membrane KL, which in turn may depend on the location of the PGCs on their migratory path towards the gonad. Interestingly, c-Kit promotes adhesion of PGCs to a variety of somatic cells *in vitro*, and this interaction appears to depend on the membrane-bound form of KL (Pesce *et al*., 1997). How this interaction modulates the activity of PGCs *in vivo* remains to be elucidated, though it has been hypothesized that it may promote their survival, proliferation and/or migration (Pesce *et al*., 1997). *In vitro* studies showing that the survival of PGCs is supported by membrane-bound KL, whereas the soluble form of KL has limited impact in this regard, suggests a specific functional role for membrane-bound KL in supporting cellular survival (Dolci *et al*., 1991).

Significant levels of both KL-1 and KL-2 transcripts are detected within the post-natal ovary (Manova *et al*., 1993; Joyce *et al*., 1999), and it is now becoming apparent that in addition to the total quantity of KL, the KL-1/KL-2 mRNA ratio is important for controlling oocyte growth. Evidence from the *in vitro* culture of pre-antral follicles suggests that FSH-stimulated oocyte growth is mediated by the KL–c-Kit interaction and this oocyte growth is dependent on a low KL-1/KL-2 mRNA ratio that is induced by low, but not high levels of FSH (Thomas *et al*., 2005). As described in an earlier section, the expression of both transcripts is selectively down-regulated in the cumulus cells of pre-ovulatory follicles, whereas the quantity of KL-1 produced by mural granulosa cells rises dramatically (Ismail *et al*., 1996; Ismail *et al*., 1997; Joyce *et al*., 1999).

In further support of a differential role for the two KL isoforms, Miyazawa *et al*. (1995) published evidence indicating that KL-1 and KL-2 have differential effects on c-Kit signalling. Whereas soluble KL down-regulates cell surface expression of c-Kit and promotes receptor proteolysis, the membrane-associated form of KL produces more sustained c-Kit signalling in a myeloid cell line (Miyazawa *et al*., 1995).

Synergism: modulation of KL by locally produced factors

It is becoming evident that multiple oocyte and granulosa cell-derived factors act both directly and synergistically to exert regulatory effects on follicular development. Of particular relevance to the current review are GDF-9, BMP15, FGF and LIF, as these factors have been shown to influence the expression and activity of KL. Molecular analysis of GDF-9-deficient mice revealed elevated levels of KL mRNA within the ovary, suggesting that GDF-9 negatively regulates KL expression (Elvin *et al*., 1999). This hypothesis was later corroborated by Joyce *et al*. (2000), who demonstrated that GDF-9 inhibits KL mRNA expression in cultured murine granulosa cells. In contrast to GDF-9, BMP15 stimulates KL expression, while KL down-regulates BMP15 expression, creating a paracrine-negative feed-back loop between the oocyte and granulosa cells (Figure 5) (Otsuka and Shimasaki, 2002; Thomas *et al*., 2005). Otsuka and Shimasaki (2002) demonstrated that

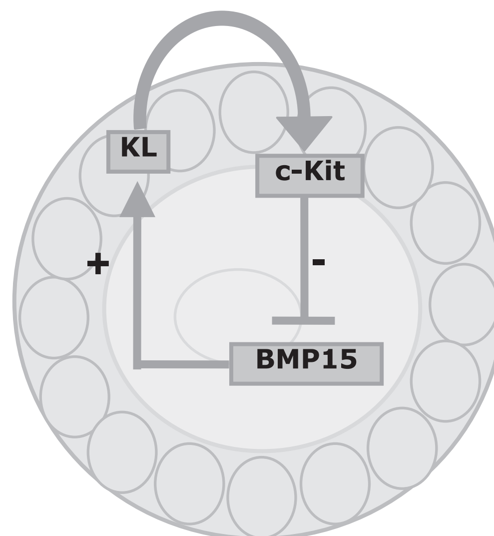

Figure 5. KL/BMP15 negative feedback loop. Oocyte derived BMP15 up-regulates the expression of KL by granulosa cells. In turn, KL acts through c-Kit located at the oocyte membrane to down-regulate the expression of BMP15.

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c-Kit signalling within the oocyte is important, though not necessary, for BMP15-mediated granulosa cell mitosis *in vitro*. Studies have also shown that the addition of partly grown oocytes to granulosa cell cultures increases KL expression, while fully grown oocytes suppress granulosa cell KL production (Joyce *et al*., 1999). This evidence led to the proposal that partly grown oocytes primarily secrete BMP15, promoting KL expression, while fully grown oocytes primarily secrete GDF-9, which inhibits KL expression (Joyce *et al*., 1999; Joyce *et al*., 2000). In synergy with KL, FGF promotes primordial follicle activation and early follicle growth *in vitro* (Nilsson and Skinner, 2004). Though KL does not regulate the expression of bFGF, bFGF up-regulates granulosa cell KL expression (Nilsson and Skinner, 2004). Therefore, bFGF may stimulate follicle activation by directly promoting granulosa cell mitosis, and it may also have an indirect influence by up-regulating granulosa cell KL expression. A recent study also suggests that KGF and KL act synergistically during early folliculogenesis to promote primordial follicle recruitment and KL produced by primordial follicle granulosa cells recruits precursor theca cells (Kezele *et al*., 2005). Finally, preliminary evidence suggests that LIF up-regulates KL expression in cultured granulosa cells (Nilsson *et al*., 2002).

Summary

Early observations that mice carrying mutations at the *steel* and *white spotting* loci exhibited severe reproductive phenotypes made it clear that KL and c-Kit are key players in female fertility. Subsequently, detailed localization and functional studies have begun to tease apart the exact nature of the influence of KL and c-Kit during oogenesis and folliculogenesis. Changes in the pattern and quantity of KL expression during follicle development emphasize the possibility that the requirement for KL is dynamic and that its function may vary dramatically at different stages of folliculogenesis. In this regard, KL and c-Kit play important roles in regulating PGC endowment, primordial follicle activation, theca cell recruitment, antrum formation and meiotic maturation. How the activity of KL is modulated to achieve these various functions in a timely and controlled manner, for the most part, remains to be elucidated.

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