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KIT signaling regulates primordial follicle formation in the neonatal mouse ovary

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ABSTRACT

The pool of primordial follicles determines the reproductive lifespan of the mammalian female, and its establishment is highly dependent upon proper oocyte cyst breakdown and regulation of germ cell numbers. The mechanisms controlling these processes remain a mystery. We hypothesized that KIT signaling might play a role in perinatal oocyte cyst breakdown, determination of oocyte numbers and the assembly of primordial follicles. We began by examining the expression of both KIT and KIT ligand in fetal and neonatal ovaries. KIT was expressed only in oocytes during cyst breakdown, but KIT ligand was present in both oocytes and somatic cells as primordial follicles formed. To test whether KIT signaling plays a role in cyst breakdown and primordial follicle formation, we used ovary organ culture to inhibit and activate KIT signaling during the time when these processes occur in the ovary. We found that when KIT was inhibited, there was a reduction in cyst breakdown and an increase in oocyte numbers. Subsequent studies using TUNEL analysis showed that when KIT was inhibited, cell death was reduced. Conversely, when KIT was activated, cyst breakdown was promoted and oocyte numbers decreased. Using Western blotting, we found increased levels of phosphorylated MAP Kinase when KIT ligand was added to culture. Taken together, these results demonstrate a role for KIT signaling in perinatal oocyte cyst breakdown that may be mediated by MAP Kinase downstream of KIT.

Introduction

Female fecundity is determined at the time of birth through the establishment of the primordial follicle pool and any aberration in the formation of primordial follicles can result in infertility. Follicular formation is tightly regulated and begins during fetal life in the mouse. After migration to the gonad, primordial germ cells (PGCs) divide rapidly but do not complete cytokinesis and are connected by intercellular bridges, forming germ cell cysts (Pepling and Spradling, 1998; Peters, 1970). As the germ cells enter meiosis, they cease mitotic division and remain connected until they begin to arrest in the diplotene stage of meiosis I. At that time, the germ cells, now called oocytes, begin to separate from each other and become surrounded by somatic granulosa cells, forming primordial follicles. The process of cyst breakdown and primordial follicle formation typically lasts from 17.5 days post coitum (dpc) to post natal day (PND) 5 (Borum, 1961; Menke et al., 2003). Concomitant with cyst breakdown is a large loss of oocytes, beginning at approximately 17.5 dpc and peaking between PND 2 and 3 of development (Pepling and Spradling, 2001). The mechanisms regulating both perinatal cyst breakdown and oocyte survival remain a mystery. One pathway of interest may be the KIT signaling pathway, as it has been previously shown to be important in germ cell survival and follicle progression at several stages of development.

KIT signaling is widely known for its ability to promote cell survival, proliferation and differentiation. The receptor, KIT, and its ligand, KIT ligand (KITL) also known as Stem cell factor (SCF), are encoded by the *White spotting* and *Steel* loci, respectively. Mutations at either locus have been studied extensively and result in an array of developmental defects in melanogenesis, hematopoiesis and gametogenesis (Roskoski, 2005). Signaling depends on the binding of KITL to KIT and when bound, KIT homodimerizes and autophosphorylates at tyrosine residues, attracting and binding downstream signaling molecules containing phosphotyrosine binding sites. KIT autophosphorylation is capable of activating several downstream cascades, including the Janus Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT), Phosphatidylinositol 3-kinase (PI3K), and Mitogen-activated Protein Kinase (MAPK) pathways (Schlessinger, 2000).

KITL exists in both a soluble isoform and a membrane bound isoform (Roskoski, 2005). The soluble form (KITL1) is a result of cleavage of the transmembrane portion of the protein, allowing the protein to dislodge from the cell membrane. The membrane

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bound isoform (KITL2) actually lacks exon 6 and therefore, the transmembrane cleavage site, forcing the protein to remain anchored at the cell membrane (Ashman, 1999). The exact function of each isoform is not well understood, however it has been reported that KITL2 binding results in more sustained KIT signaling (Miyazawa et al., 1995).

The KIT pathway has been shown to be important for many ovarian functions including germ cell survival and migration. Early studies examining *Steel* mutant mice demonstrated the importance of this signaling pathway in the proper migration of PGCs to the genital ridge. A decrease in KITL expression results in PGC migration to ectopic sites and a reduction in germ cell proliferation and survival (Huang et al., 1993; Huang et al., 1992; McCoshen and McCallion, 1975). Reynaud and colleagues demonstrated that KITL and KIT were also imperative to later follicular survival, protecting pre-antral follicles from apoptosis (Reynaud et al., 2001). Finally, ovaries of newborn rats exposed to recombinant KITL in culture had a lower percentage of dying oocytes than control ovaries (Jin et al., 2005).

Postnatal development of follicles is also dependent on the KIT ligand/KIT system. After birth, the flattened granulosa cells of primordial follicles become cuboidal and proliferate, forming the primary follicle. This change from primordial to primary follicle has been shown to be dependent on the KIT pathway in several studies. *Steel*^{panda} mutant mice, a KITL hypomorph which produces only a small amount of KITL, show a nearly complete block on the primordial to primary follicle transition, leaving a large pool of primordial follicles that cannot develop. These mice are sterile (Huang et al., 1993). In another experiment, when neonatal mice were injected with the KIT neutralizing antibody, ACK2, the transition from primordial to primary follicle was severely blocked (Yoshida et al., 1997). Conversely, when exogenous KITL was added to 4 day old rat ovaries in culture, there was a significant increase in developing primordial follicles (Parrott and Skinner, 1999). These experiments demonstrate the importance of KIT signaling in primordial follicle development, and studies have begun to describe an involvement of the KIT signaling pathway in an oocyte-granulosa cell feedback loop. During the primordial to primary transition, KIT activation at the oocyte regulates other molecules downstream that affect granulosa cell division. Otsuka and Shimasaki found that in the presence of KITL, granulosa cell mitosis increased, indicating an indirect regulation of granulosa cell division via the oocyte KIT signal (Otsuka and Shimasaki, 2002). In the same study, when a KIT neutralizing antibody was added, granulosa cell division decreased.

KIT signaling is also important in a subsequent stage of follicle development, the transition from preantral to antral follicle. As the granulosa cells of primary follicles divide to produce multiple layers, a secondary or preantral follicle is formed. These preantral follicles eventually gain a fluid filled space, and are then classified as antral follicles. Yoshida and colleagues were able to block the transition from the preantral to the antral follicle stage by ACK2 antibody injection (Yoshida et al., 1997). Similarly, when ACK2 was used to block KIT signaling in rat ovary culture, a block on gonadotropin induced preantral follicle development was seen (Parrott and Skinner, 1999).

Although much evidence exists for the role of KIT signaling in ovarian development, the contribution of KIT signaling to primordial follicle formation in later fetal and early neonatal development is unclear. In hamster, KIT signaling has been shown to promote cyst breakdown and primordial follicle formation in vitro (Wang and Roy, 2004). However, using an injection of a KIT neutralizing antibody, Yoshida et al., failed to find any role for KIT in primordial follicle formation in newborn mouse ovaries (Yoshida et al., 1997). Since KIT is directly involved in, and important to, early survival of oocytes and subsequent postnatal development of follicles, we hypothesized that this pathway might also regulate the formation of primordial follicles from oocyte cysts. In the current study we took advantage of an organ culture system to directly inhibit and activate KIT signaling in fetal ovaries, when cyst breakdown and follicle formation begin. Our results illustrate the importance of this pathway in the establishment of the primordial follicle pool.

Materials and methods

Animals

All C57BL/6 mice were purchased from Jackson Laboratories. Adult male and female mice were mated utilizing timed matings, and females were checked for the presence of a vaginal plug the following morning. The presence of a vaginal plug was denoted 0.5 dpc of pregnancy. Pregnant mice were euthanized on either 17.5 dpc for organ culture experiments or 16.5, 17.5 and 18.5 dpc for immunohistochemistry. Otherwise, pregnant dams gave birth at 19.5 dpc (PND 1) and pups were euthanized at PND 1 and 3 for immunohistochemistry.

All mice were housed under 12 h light/dark cycles, temperatures of 21–22 °C and had free access to chow and water. All animal protocols were approved by the Syracuse University Institutional Animal Care and Use Committee.

Antibodies

For immunohistochemistry, STAT3 (C-20) antibody (Santa Cruz Biotechnology) was used at a dilution of 1:500, KIT (C-19) and Stem Cell Factor (G-19) antibodies (Santa Cruz Biotechnology) were used at a dilution of 1:100, VASA antibody (Abcam) was used at a dilution of 1:250. The secondary antibodies, goat anti-rabbit Alexa 488, rabbit anti-goat Alexa 488 and goat anti-rabbit Alexa 568 (Invitrogen), were at a dilution of 1:200. A Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (Intergen) was used to visualize dying oocytes. For Western blotting, AKT 1/2/3 (H-136), phospho-AKT 1/2/3 (Ser473), STAT3 (C-20) and phospho-STAT3 (Tyr 705) antibodies (Santa Cruz Biotechnology) were used at a dilution of 1:1000. MAPK p44/42 ERK1/2 and Phospho-p44/42 (T202/Y204) antibodies (Cell Signaling) were used at a dilution of 1:1000 for Western blotting.

In vitro ovary organ culture

As previously described, ovaries were harvested and placed on 0.4 μm floating filters (Millicell-CM; Millipore Corp) in 4 well culture dishes (Nunc) with 0.4 ml culture media consisting of D-MEM/HAM'S F12 Media (Invitrogen), 0.1% Albumax (Invitrogen), 0.1% Fraction V BSA (Invitrogen), 5X ITS-X (Life Technologies), 0.05 mg/ml L-ascorbic acid (Sigma) and penicillin–streptomycin (Life Technologies) (Chen et al., 2007). Each floating filter held 2–3 ovaries, and a single drop of media was placed on each of the ovaries to keep them from drying out.

To assess the role of KIT signaling in the ovary, 17.5 dpc ovaries were cultured for 5 days in either media alone, media supplemented with a function blocking antibody to KIT, ACK2 (e-biosciences) at a 1:100 dilution, media supplemented with recombinant Stem Cell Factor (R&D Systems) at 100 ng/ml or media supplemented with IgG (Santa Cruz) at a 1:100 dilution ($n=8$ ovaries per group). To determine the role of KIT signaling before PND 1, ovaries ($n=8$ ovaries per group) were cultured for 3 days either in control media alone, or in media supplemented with ACK2 at 17.5 dpc then switched to control media after 2 days in