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.

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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 phosphorylated protein kinase A, and 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 Stee 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 mice raised without 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. Stee/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, granulosa cell division decreased. 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) or the following morning. The presence of a vaginal plug was denoted at 19.5 dpc (PND 1) and pups were euthanized at PND 1 and 3 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 chew 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, goat 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 (Y705) 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.

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 at 19.5 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.
culture. To determine the importance of KIT signaling at birth, ovaries \((n=8)\) were grown in culture for 5 days either in control media alone, or in control media at 17.5 dpc then switched to media supplemented with ACK2 after 2 days in culture. For cell death and proliferation studies, ovaries were cultured in control or ACK2 supplemented media for 3 days \((n=8\) ovaries per group). At the conclusion of culture, all ovaries were prepared for whole-mount immunohistochemistry.

To determine pathways regulated by KIT signaling, 17.5 dpc ovaries were grown in culture for 3 days in either control media or media supplemented with SCF \((n=8)\). At the conclusion of culture, ovaries were prepared for Western blotting.

**Fig. 1.** Expression of KIT in the fetal and neonatal ovary. Confocal sections from 16.5 dpc \((A-C)\), 17.5 dpc \((D-F)\), 18.5 dpc \((G-I)\), PND 1 \((J-L)\), and PND 3 \((M-O)\) ovaries labeled for KIT \((green)\) \((A, D, G, J\) and \(M)\) and the nuclear marker, propidium iodide \((red)\) \((B, E, H, K\) and \(N)\) with overlay shown in \(C, F, I, L\) and \(O)\. Inset in \(L)\ is an enlarged image of the area within the white circle and arrows indicate oocytes without KIT labeling while arrowheads denote oocytes with KIT labeling. Arrows in \(G-I\) indicate oocytes and arrowheads indicate somatic cells. \((P-R)\) KIT labels a subset of oocytes at PND 1. Confocal section of a PND 1 ovary labeled with \((P)\) the germ cell marker, VASA \((green)\) antibody, \((Q)\) KIT \((red)\) antibody and \((R)\) overlay. In \(P-R\), arrows indicate oocytes without KIT labeling and arrowheads denote oocytes with KIT labeling. Scale bar, 20 \(\mu m\).
**Immunohistochemistry**

Ovaries were fixed in 5% EM-grade paraformaldehyde (Electron Microscopy Sciences) overnight at 4 °C and stained as previously described ([Pepling and Spradling, 1998](#)). Whole ovaries were labeled with primary antibody overnight, then with anti-rabbit or anti-goat secondary antibody. Nuclei were labeled with TOTO-3 or propidium iodide (Invitrogen). A Zeiss LSM 710 Confocal Microscope was used to image ovaries. As a control, some ovaries were labeled with only anti-rabbit secondary antibody.

**Analysis of oocyte cyst breakdown and follicle development**

At the conclusion of cultures, ovaries prepared for immunohistochemistry were labeled with STAT3 antibody, a known oocyte marker and imaged with confocal microscopy ([Murphy et al., 2005](#)). In each ovary, two randomly selected cores were visualized and counted. A core is a region 135 × 135 µm² made up of optical sections at 4 separate depths in the ovary 15–20 µm apart, with the first section beginning in the outer cortex and the last section ending within the medullar region of the ovary so that ovarian development is assessed in all regions. For each ovary, 2 cores with 4 optical sections were analyzed for a total of 8 sections examined. The number of oocytes found in cysts relative to the total number of oocytes was determined for each ovary by analyzing each section and reported as percent single oocytes. In order to determine whether oocytes were in cysts or not, for each of the 4 optical sections in a core, a z-stack of images each 1 µm apart was obtained with 5 images above the section and 5 images below the section being analyzed. This allowed us to determine whether an oocyte was part of a germ cell cyst above or below the plane of focus. Follicle development was determined by counting the

![Fig. 2](image-url)
number of primordial, primary and secondary follicles present in relation to the total number of follicles found and reported as percent primordial, primary or secondary follicles.

**Analysis of oocyte numbers**

To determine oocyte numbers, we counted the number of oocytes found within the 8 optical sections used for analyzing cyst breakdown and follicle development. The numbers were averaged and reported as number of oocytes per section. Only ovaries that appeared to be of similar size and depth were used for counting oocyte numbers because the number of oocytes per section is an estimate of the total number of oocytes within an ovary.

**Analysis of cell death**

To analyze dying cells, ovaries were first labeled with the TUNEL labeling kit (Intergen) according to the manufacturer’s recommendations and then with the oocyte marker antibody, VASA. Ovaries were imaged with confocal microscopy. In each ovary, 4 randomly selected optical sections were visualized and analyzed by counting the number of TUNEL positive cells relative to the total number of cells present. This number was reported as % TUNEL positive cells per ovary.

**Western blot hybridization**

17.5 dpc ovaries were grown in culture for 3 days and then homogenized in 10 μl sample buffer (2% SDS, 10% Glycerol, 0.005% Bromophenol Blue, 0.0625 M Tris pH 6.8, and 2.5% 2-mercaptoethanol) plus mini-complete protease inhibitors (Roche) per ovary. Additional 2-mercaptoethanol was added to extracts at 1/10 of the volume of extract. Samples were then heated to 95°C and centrifuged at 6000 rpm for 1 min, separated on a 10% SDS-polyacrylamide gel (BioRad) and then electroblotted onto either Immobilon P PVDF (Millipore) or nitrocellulose (BioRad) membranes. Membranes were blocked in either Phosphate Buffered Saline plus 0.05% Tween (PBST) containing 5% nonfat milk for AKT, phospho-AKT, MAPK p44/42 and STAT3 antibodies or Tris Buffered Saline plus 0.05% Tween (TBST) with 5% BSA for phospho-p44/42 and phospho-STAT3 antibodies for 1 h at room temperature. Blots were then incubated with primary antibody diluted in the appropriate blocking solution for 1 h at room temperature. Membranes were washed 3–6 times in either PBST or TBST then incubated with secondary antibody diluted in 5% milk blocking solution for 1 h at room temperature. goat anti-rabbit horseradish peroxidase conjugated secondary antibody (ThermoScientific) for the AKT, pAKT, MAPK p44/42 and STAT3 hybridized blots was used at a dilution of 1:10,000 and at a dilution of 1:15,000 for phospho-p44/42 MAPK and phospho-STAT3 hybridized blots. Again, blots were washed 3–6 times in PBST or TBST and bands were visualized.

**Fig. 3.** Expression of KIT and cleaved PARP at PND 1. Confocal sections of a PND 1 ovary labeled with (A) and (D) the cell death marker, cleaved PARP (green) antibody, (B) and (E) KIT (red) antibody and (C) and (F) overlay. Arrowheads indicate oocytes with KIT labeling and arrows indicate oocytes without KIT labeling. (G) Percent of oocytes positive for both KIT and cleaved PARP proteins or negative for KIT but positive for cleaved PARP protein. Data are presented as the mean ± SEM.
visualized using the Supersignal kit (Pierce) on films. Blots were reprobed with anti-mouse GAPDH (1:5000; Santa Cruz Biotechnology) as a control.

**Statistical analysis**

A Student's t-test was used to analyze cell death and studies analyzing the effect of blocking KIT in the ovary at different time points. Effects of ACK2 and recombinant SCF on ovaries cultured for five days beginning on 17.5 dpc were analyzed with a one-way ANOVA using SPSS statistical software. For all results, p values less than 0.05 were considered significant.

**Results**

**Expression of KIT and KITL in the ovary**

We first examined the expression of both KIT and KITL in the fetal and neonatal ovary to determine if KIT signaling could be involved in oocyte survival and follicle formation at the time of birth. To evaluate expression, we harvested ovaries from 16.5 dpc, 17.5 dpc, 18.5 dpc, PND 1 and PND 3 mice, fixed and labeled them with an antibody to either KIT or KITL. Fig. 1 shows the expression of KIT weakly in the cytoplasm of oocytes at 16.5 (A–C) and 17.5 (D–F) dpc, and expression at the cell membrane appears in a few oocytes at 17.5 dpc (only one oocyte with expression at the membrane in the panel shown). At 18.5 dpc (Fig. 1G–I) KIT is expressed in a subset of the oocytes in either the cytoplasm or cell membrane but becomes pronounced at the cell membrane of oocytes by PND 1 (Fig. 1J–L). Interestingly, at PND 1 a small subset of cells within a cyst do not express KIT (inset Fig. 1L). KIT protein appears to be similarly expressed at PND 3 although it may be less pronounced at the membrane (Fig. 1M–O). To demonstrate that KIT labels oocytes, we used immunohistochemistry on PND 1 ovaries and labeled them with an antibody to KIT and to the germ cell marker, VASA. Fig. 1 Q–R shows that KIT labels only oocytes at PND 1 and that some oocytes within a cyst do not express KIT.

We also tested for the presence of KITL in fetal and neonatal ovaries. Fig. 2A–C shows weak expression of KITL in the cytoplasm of germ and somatic cells at 16.5 dpc. By 17.5 dpc, KITL appears to accumulate in small areas of some oocytes, which may represent expression of the membrane bound isoform of KITL (Fig. 2D–F). From 18.5 dpc onward, KITL expression remains in the cytoplasm of oocytes and somatic cells (Fig. 2G–O) and expression appears to increase from PND 1 to 3.

Based on previous work showing that KIT signaling was responsible for oocyte survival, we hypothesized that the oocytes within a cyst not expressing KIT at PND 1 would be marked for apoptosis. To test this, we double labeled PND 1 ovaries with an antibody to the apoptosis marker, cleaved PARP, and the germ cell marker, VASA. Fig. 3A–F shows representative confocal sections demonstrating that some oocytes expressing KIT and some oocytes not expressing KIT are

**Fig. 4.** Ovarian follicle formation is affected by KIT signaling. (A) Timeline showing the 5 day culture period (orange bar) and duration of ACK2 or KITL treatment (blue bar). (B) Number of oocytes, (C) percent single oocytes and (D) percent of follicles found at the primordial, primary or secondary stage per confocal section in ovaries cultured for 5 days either in control media alone or media supplemented with IgG at 1:100, ACK2 at 1:100, or 100 ng/mL KITL. Data are presented as the mean ± SEM. Asterisk (*) indicates a significant difference between groups (ANOVA, p < 0.05). (E) Confocal section of an ovary cultured for 5 days in control media or (F) with ACK2 antibody labeled for STAT3 (green) to visualize oocytes and propidium iodide (red) to visualize nuclei. Scale bar, 20 μm.
both positive for cleaved PARP. To quantify that data, we calculated the percentage of oocytes in each confocal section labeling with both the KIT and PARP antibodies and those that do not label with KIT but are positive for PARP (Fig. 3G). There was not a statistically significant difference between these groups. Thus there does not appear to be a correlation between KIT signaling and oocyte survival.

**KIT signaling regulates oocyte numbers, primordial follicle formation and cyst breakdown**

We first hypothesized that KIT signaling might regulate perinatal germ cell numbers by increasing oocyte survival for those expressing KIT. To test this, we harvested 17.5 dpc ovaries and placed them in organ culture for 5 days in either control media alone, media supplemented with a 1:100 dilution of the KIT function blocking antibody, ACK2, media supplemented with 100 ng/ml of recombinant KITL or media supplemented with 1:100 dilution of IgG as a negative control (Fig. 4A). Surprisingly, we found an increase in the number of oocytes present in the ACK2 inhibited ovaries (14.5 oocytes) and a reduction in the number of oocytes in KITL treated ovaries (5.8 oocytes) when compared with both control and IgG treated ovaries (8.7 oocytes and 8.1 oocytes; Fig. 4B). In addition, we found that when KIT signaling was blocked with ACK2, a large reduction in cyst breakdown occurred with only 47% single oocytes found, while an increase in cyst breakdown was present in KITL treated ovaries which contained 84% single oocytes (Fig. 4C). These were both statistically different from the control and IgG groups which had 71.1% single oocytes and 77.4% single oocytes, respectively (Fig. 4C). Finally, both blocking and activating the KIT signaling pathway resulted in changes in follicle development. The ACK2 inhibited ovaries showed a complete block on follicle progression.
with 100% of follicles found in the primordial stage and 0% found in the primary or secondary stages (Fig. 4D). Follicle development was facilitated by the addition of KITL to culture and resulted in an average of 87% primordial follicles, 13% primary follicles and 0% secondary follicles (Fig. 4D). Normal follicle development, as demonstrated by the control ovaries, showed an average of 87% primordial follicles, 13% primary follicles and 0% secondary follicles (Fig. 4D). Fig. 4E and F are representative confocal sections of both control and ACK2 treated ovaries demonstrating the increase in oocytes and reduction in cyst breakdown found in KIT inhibited ovaries.

**Kit signaling contributes to follicle formation before and after birth**

Yoshida and colleagues injected female neonatal mice with ACK2 every other day beginning on PND1 and found no effect on oocyte numbers prior to PND 5 (Yoshida et al., 1997). Additionally, they did not evaluate oocyte cyst breakdown and follicle formation, although they found normal numbers of primordial follicles in the ovary at PND 5. We wondered if the discrepancy between our results and those findings was a result of the timing of the ACK2 treatment, as cyst breakdown has been shown to begin prior to PND 1 (Pepling et al., 2010). Therefore, we wanted to test the effects of inhibiting KIT signaling on cyst breakdown and oocyte number specifically before birth to determine if the signal was important for follicle formation at that time. We began by culturing 17.5 dpc ovaries for 5 days either in media alone, or in media supplemented with ACK2 antibody for the first two days of culture and then in media alone for the remaining 3 days of culture to test the effects of inhibiting KIT signaling on oocyte number and cyst breakdown when ovaries were cultured with ACK2 only for the first two days of culture. There was, however, a significant decrease in follicle development when KIT signaling was inhibited only for the first two days of culture, with ACK2 treated ovaries containing 96% primordial and 4% primary follicles compared to 87% primordial and 13% primary in control ovaries (Fig. 5D). These data suggest that KIT signaling has either no effect on cyst breakdown or oocyte numbers before birth, or that both were affected but rescued by removing the KIT antibody from culture and allowing treated ovaries to grow in plain media for the remaining 3 days of culture.

To better clarify the effect of blocking KIT signaling before birth, we decided to place 17.5 dpc ovaries in culture and treat with ACK2 antibody again for the first two days, but this time cultured them only an additional day in plain media (Fig. 5E). A length of three days of culture was chosen because the final day would correspond to PND 2, the time when germ cell death peaks and cyst breakdown is well underway (Pepling and Spradling, 2001). As shown in Fig. 5F, there was no significant difference in the number of oocytes present in treated ovaries. However, cyst breakdown was greatly reduced with ACK2 treated ovaries containing only 49% single oocytes while control ovaries had 69% single oocytes (Fig. 5G). Finally, follicle development was inhibited when KIT signaling was inhibited before birth. Fig. 5H shows that ACK2 treated ovaries had 99% primordial and 1% primary follicles and control ovaries had 94% primordial and 6% primary follicles.

**Kit signaling is important for follicle formation after birth**

Since cyst breakdown is a continuous process lasting from approximately 17.5 dpc to PND 5, we wanted to determine whether KIT signaling was still important for follicle formation after birth (Pepling et al., 2010). To test this, we cultured 17.5 dpc ovaries for 5 days either in control media alone, or control media supplemented with ACK2 at 1:100 for the first two days of culture and then in ACK2 supplemented

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**Fig. 6.** KIT signaling is important for follicle formation in ovaries from PND 1–4. (A) Timeline showing the 5 day culture period (orange bar) and timing of ACK2 treatment (blue bar). (B) Number of oocytes, (C) percent single oocytes and (D) percent of follicles found at the primordial, primary or secondary stage per confocal section in ovaries cultured for 5 days either in control media alone (days 1–5) or control media (days 1 and 2) and subsequently in media supplemented with ACK2 at 1:100 (days 3–5). Data are presented as the mean ± SEM. * indicates a significant difference between groups (t-test, p < 0.05).
media for the remaining 3 days (Fig. 6A). While there was no significant difference in the number of oocytes in the ACK2 treated ovaries (9.8 oocytes) when compared with controls (8.2 oocytes; Fig. 6B), cyst breakdown was significantly reduced with ACK2 treatment (Fig. 6C) with inhibited ovaries having 59.1% single oocytes and control ovaries containing 77% single oocytes. Finally, follicle development was reduced with ACK2 treated ovaries containing significantly fewer primary follicles than control ovaries (Fig. 6D).

**Cell death is modulated by KIT inhibition**

Our results showed that KIT signaling is important for regulating cyst breakdown and oocyte numbers. Upon KIT inhibition in the ovary, we found the number of oocytes present in the ACK2 treated ovaries (9.8 oocytes) when compared with controls (8.2 oocytes; Fig. 6B), cyst breakdown was significantly reduced with ACK2 treatment (Fig. 6C) with inhibited ovaries having 59.1% single oocytes and control ovaries containing 77% single oocytes. Finally, follicle development was reduced with ACK2 treated ovaries containing significantly fewer primary follicles than control ovaries (Fig. 6D).

**KitL activates the MAP Kinase pathway**

Kit signaling can activate several different pathways downstream of the receptor, and we wondered which of these pathways might be involved in cyst breakdown. To evaluate the downstream signaling pathway activated by KIT signaling during cyst breakdown, ovaries were cultured for 3 days in either control media alone, or in media supplemented with recombinant KitL to activate KIT signaling (Fig. 8A). At the conclusion of culture, ovaries were prepared for Western blotting and extracts were probed for total AKT, MAPK and STAT3 as well as the phosphorylated forms of these proteins. Fig. 8 B–G shows that both control and KitL treated ovaries contain similar levels of total AKT, MAPK and STAT3 proteins. Phosphorylated AKT and STAT3 levels were also comparable between control and KitL ovaries. KitL treatment did, however, increase phosphorylation of MAPK, indicating that KitL activates the MAPK pathway downstream of KIT.

**Discussion**

Although many studies have shown a role for KIT signaling in ovarian development, there is sparse evidence examining its importance in oocyte cyst breakdown and primordial follicle formation. Several labs have examined mice mutant for either the Steel or W loci, and have found dramatic defects in PGC migration and proliferation that result in a complete loss of oocytes by birth (Huang et al., 1993; Reith et al., 1990). These mice are not optimal for observing the effects of KIT signaling on cyst breakdown, as cysts do not appear to form in these mutant ovaries and there are few oocytes remaining. Other Steel mutants show a decreased number of germ cells, but normal primordial follicle formation (Bedell et al., 1995). However, these mice continue to secrete low amounts of KitL which may allow for normal cyst breakdown and follicle formation. The present study examines the role of KIT signaling in cyst breakdown and primordial follicle assembly using an organ culture system which permits the study of KIT signaling specifically at the time these processes are occurring. Using this in vitro system, we have elucidated a role for KIT signaling in the fetal and neonatal mouse ovary.
We found KIT to be expressed at the oocyte membrane from 17.5 dpc to PND 3. This supports the findings of Manova et al., where Kit mRNA was localized to the oocyte beginning in just a few cells at 17.5 dpc then increasingly throughout the life of the oocyte (Manova et al., 1990). Interestingly, we found that at PND 1 a subset of oocytes within some cysts did not express the KIT. This may be due to expression of Activin in those cells. Using immunohistochemistry, Coutts and colleagues found human fetal ovaries expressed KIT incongruously in oocyte cysts and those that lacked expression stained positively for Activin (Coutts et al., 2008). We examined the expression of Activin in neonatal mouse ovaries and found that it was expressed in somatic cells and not in oocytes (data not shown). Thus, the expression pattern of Activin does not appear to be conserved between mice and humans. Finally, we found that KITL was expressed in the mouse ovary from 17.5 dpc through PND 3 in both oocytes and somatic cells, however the signal for KITL was strongest in oocytes at 17.5, but then becomes more evenly distributed between germ cells and granulosa cells by PND 3. It is possible that there is a switch from the KITL membrane bound isoform at 17.5 dpc in the oocyte to the soluble form by PND 1, and this switch may take part in regulation of cyst breakdown. This could indicate either an autocrine or paracrine signaling mechanism for the KIT/KITL system in the developing mouse ovary particularly during follicle formation as expression of both components was detected during this time period.

Given the positive expression of both KIT and KITL in the mouse ovary, we hypothesized that this system would play a role in cyst breakdown and primordial follicle formation. To test this, we used in vitro ovary organ culture to both inhibit and activate the KIT signaling pathway. When exposed to the KIT inhibiting antibody ACK2, cyst breakdown was greatly reduced, and upon activation with recombinant KITL, cyst breakdown was accelerated. Our data supports that of Wang and Roy in hamster ovary, where addition of KITL to culture promoted the formation of primordial follicles (Wang and Roy, 2004). Alternatively, our results contradict earlier findings that suggest that KIT signaling has no effect on follicle formation in mouse ovary (Yoshida et al., 1997). Using injections of ACK2 every other day beginning on the day of birth, Yoshida and colleagues failed to find that KIT played any role in primordial follicle formation, but instead blocked the primordial to primary follicle transition. This study utilized newborn mice at PND 1 and, based on work from our lab showing that cyst breakdown begins as early as 17.5 dpc, injecting at PND 1 may have been too late a time to affect cyst breakdown and follicle formation (Pepling et al., 2010). Additionally, the every other day injection schedule of ACK2 may have allowed a rescue of cyst breakdown, such that on the days when antibody was not given, normal signaling was permitted and primordial follicle formation occurred.

In an effort to clarify the differences in our results and those of Yoshida et al. 1997, we first wanted to test the effect of blocking KIT prior to PND 1 to determine its importance in inhibiting cyst breakdown before birth. To accomplish this, we cultured 17.5 dpc ovaries in the presence of ACK2 for 2 days and then allowed the ovaries to continue in culture in control media for the remaining 3 days. To our surprise, ovaries that were treated only on the first two days had only a slight decrease in cyst breakdown, but a delay in follicle development remained, suggesting a possible rescue of cyst breakdown and follicle formation on the 3 days ovaries were not in the presence of the antibody. To test the idea that follicle formation had been rescued, we again cultured 17.5 dpc ovaries in the presence of ACK2 for 2 days but this time only allowed them to be cultured for 1 more day before examining them. When removed from culture at the earlier time point, ovaries that were treated with ACK2 demonstrated a significant decrease in cyst breakdown and primordial follicle formation. This suggests that follicle formation can be rescued when ovaries are allowed to resume normal KIT signaling and in addition, the KIT signal is important before birth in cyst breakdown.

We also postulated that the KIT signal would continue to be important for cyst breakdown at birth, as this process continues
from 17.5 dpc to PND 5. To examine this, we cultured 17.5 dpc ovaries first in control media for 2 days and then in ACK2 supplemented media for the remaining 3 days. In support of our hypothesis, we found that cyst breakdown was again inhibited, though not as severely as when inhibition was started at 17.5 dpc. The KIT signal appears to be important for cyst breakdown on or after PND 1 in the neonatal ovary.

In our initial experiment, inhibiting KIT signaling in culture for 5 days from 17.5 dpc onward also resulted in an increase in the number of oocytes present in the ovary. We suspected that this increase might be due to a decrease in cell death. Using a TUNEL analysis, we were able to quantify the percentage of dying cells in both control and ACK2 treated ovaries and found that the inhibited ovaries contained a lower percentage of dying cells. Although we used VASA antibody to label oocytes, we cannot say for sure that the dying cells are actually oocytes because the germ cell marker antibody may lose reactivity in dying cells, therefore dying oocytes may no longer be positive for VASA (Pepling and Spradling, 2001). However, since KIT is expressed only at the oocyte membrane and we see an increase in oocyte numbers, it is likely that the decreased TUNEL labeling upon KIT inhibition is due to a reduction in oocyte specific death.

Much of the work in the ovary has focused on the activation of the PI3K pathway downstream of KIT in the oocyte. When KIT is activated by KITL, KIT is autophosphorylated at tyrosine 719 which is the primary binding site for the p85 subunit of PI3K leading to its subsequent activation. Mice with a point mutation in KIT at Y719 are not able to bind p85 and therefore cannot activate PI3K downstream of KIT (John et al., 2009). These mice undergo normal primordial follicle formation upon activation. Mice with a point mutation in KIT at Y719 are not able to bind p85 and therefore cannot activate PI3K downstream of KIT (John et al., 2009). However, since KIT is expressed only at the oocyte membrane and we see an increase in oocyte numbers, it is likely that the decreased TUNEL labeling upon KIT inhibition is due to a reduction in oocyte specific death.

In summary, we have found a role for KIT in the formation of primordial follicles using an in vitro ovary culture system to block KIT signaling specifically at the time when cyst breakdown and primordial follicle formation begin. While the in vitro system is a reliable method for testing the effects of hormones and signaling molecules on ovarian development, it may not accurately reflect what is happening in vivo. Developing and evaluating a tissue specific knockout of KIT would shed light on the importance of this pathway during cyst breakdown in vivo. Additionally, studying the genes regulated downstream of KIT is essential to understanding its role in primordial follicle formation and further, in its effect on somatic cell division. Trombly et al., found that blocking Notch signaling in vitro resulted in a defect in primordial follicle formation in the mouse ovary (Trombly et al., 2009). Interestingly, the Notch receptor (NOTCH2) is expressed on the granulosa cells and its ligand, Jagged 1 (JAG1), is present in oocytes. This may be a pathway involved in an oocyte granulosa cell feedback system with the KITL/KIT system, where activation of KIT in the oocyte might upregulate JAG1 expression in the oocyte, resulting in communication with NOTCH2 at the granulosa cells. Testing the expression of both NOTCH2 and JAG1 by quantitative RT-PCR or Western blot following organ culture with exogenous KITL would be a valuable follow up study. Likewise, culture with recombinant KITL could be followed by a microarray analysis to determine the global genes being regulated by KIT signaling in the ovary during cyst breakdown. Finally, it would be interesting to determine if KIT signaling might act synergistically or redundantly with other pathways that have been identified as regulators of cyst breakdown and primordial follicle formation. Studies examining Connective Tissue Growth Factor (CTGF) and Nerve Growth Factor (NGF) have demonstrated a role for both of these signaling pathways in primordial follicle assembly and cyst breakdown, so experiments that explore the effects of inhibiting and activating multiple signaling pathways in conjunction with KIT could prove worthwhile (Dissen et al., 2001; Schindler et al., 2010).

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References