Multiple mechanisms of germ cell loss in the perinatal mouse ovary

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Abstract

In the perinatal ovary of most mammals, external and internal factors establish a primordial follicle reserve that specifies the duration of the reproductive lifespan of a given species. We analyzed the mechanism of follicle loss and survival in C57Bl/6 mice using static and dynamic assays of apoptosis, autophagy, and ovarian morphogenesis. We confirm an initial loss soon after birth, when about 44% of the germ cells detectable at the end of the fetal period abruptly disappear. The observations that (1) few germ or somatic cells were apoptotic in newborn ovaries, (2) vitally stained organ cultures exhibit active extrusion of non-apoptotic germ cells and (3) germ-cell lysosome amplification occurs at birth suggested that additional mechanisms are involved in perinatal germ cell loss. Newborn mouse ovaries cultured in the pH sensitive dye lysotracker red exhibit an increased incidence of acidified non-apoptotic germ cells when maintained in the absence but not in the presence of serum, implying a role for autophagy in germ cell attrition. Inhibitors of autophagy, but not apoptosis, reduce germ cell acidification induced by serum starvation in ovary organ cultures and protein mediators of both autophagy and apoptosis are expressed at birth. From these findings we suggest that multiple perinatal mechanisms establish the primordial follicle reserve in mice.

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Introduction

The transition from fetal to neonatal life is a critical phase of normal development in eutherian mammals during which organ systems adapt to post-parturition starvation. Many organ systems in newborns adjust progenitor cell density and function by engaging apoptotic and non-apoptotic mechanisms of programmed cell death (PCD; Edinger & Thompson 2004). There are three types of PCD. Type I, or apoptosis, is characterized morphologically by nuclear condensation and fragmentation, cell shrinkage and membrane blebbing (Tilly 2001, Edinger & Thompson 2004). Type II, or autophagy, is a lysosomal degradation pathway that involves formation of autophagic vacuoles during periods of massive cell elimination (Edinger & Thompson 2004, Qu *et al.* 2007). Type III, or necrosis, is characterized by plasma membrane breakdown causing an inflammatory reaction (Edinger & Thompson 2004). Apoptosis is coupled to autophagy as a means to maintain tissue viability and energy homeostasis in developing tissues during the demanding neonatal

phase in mammals (Kuma *et al.* 2004, Yu *et al.* 2004, Lum *et al.* 2005). This coupling occurs either via constitutive pathways, where lysosomes degrade the apoptotic bodies, or through an alternative pathway in which lysosomal proteases such as cathepsins, trigger apoptosis (Bursch 2001, Guicciardi *et al.* 2004). Amongst the organ systems that exhibit profound cell loss at birth is the ovary, where it has long been recognized that massive female germ cell attrition *post partum* precedes the establishment of a fixed follicle reserve that is progressively depleted during the reproductive lifespan (Reynaud & Driancourt 2000, Tilly 2001, Qu *et al.* 2007). Studies favoring a major role for germ cell apoptosis include those using ubiquitously targeted deletion of genes involved in Type I PCD. Accordingly, an increase or decrease in follicle numbers has been reported in postnatal mice bearing systemically targeted gene deletions for anti-apoptotic, or pro-apoptotic genes (Perez *et al.* 1999). Although apoptosis is believed to account for the bulk of germ cell loss in fetal mouse ovaries (Coucovanis *et al.*

1993, De Pol *et al.* 1997, Pepling & Spradling 2001), other processes such as germ cell extrusion (Wordinger *et al.* 1990) and autophagy also participate in pre- and postpartum adjustments of germ cell numbers (Wordinger *et al.* 1990, Lobascio *et al.* 2007). Both apoptosis (Edinger & Thompson 2004, Maiuri *et al.* 2007) and autophagy (Maiuri *et al.* 2007), lysosome-mediated PCD processes (Guicciardi *et al.* 2004), have been proposed to serve cooperatively during developmental transitions in other organs, but this prospect has yet to be studied during perinatal ovarian germ cell loss.

The present study addresses several questions related to morphogenesis of the mouse ovary revealing a previously unappreciated level of complexity in PCD that sets and maintains adequate numbers of follicles for reproductive function in mice without invoking replacement strategies. Our findings suggest that multiple mechanisms including autophagy mediate germ cell loss and are used in a coordinated and developmentally regulated fashion.

Results

Characterization of germ cell number during ovarian development

Given the wide range of variability reported for germ cells during ovarian development in mice, we adopted a technical modification for our morphometric analyses. Here, histological sections processed for detection of mouse vasa homologue (MVH), a specific germ cell marker (Fujiwara *et al.* 1994) combined with periodic acid Schiff's (PAS) staining so as to render extracellular matrix, including the zona pellucida, clearly discernible (Fig. 1). Sections of ovaries from C57Bl/6 females ranging in age from embryonic day (E) 15.5 through to postnatal day (P) 150 were morphometrically analyzed for total oocyte, follicle, and/or zona pellucida profiles

(Fig. 2 and Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental/). Examples of embryonic, postnatal, and prepubertal ovaries are shown in Fig. 1 to emphasize the striking histological transformation that takes place over these key developmental stages that include resolution of ovigerous cords into solitary follicles (Fig. 1A and B) and expansion of somatic cells including development of the vasculature (Fig. 1C).

In this study, we confirm the decrease in mean germ cell numbers per ovary at birth, with 5783 ± 475 (confidence intervals (CI): 4463–7103) total follicles observed prior to birth (E19.5) and 3252 ± 820 (CI: 976–5529) total follicles after birth (P2; Fig. 2 and Supplementary Table 1). Variations in mean oocyte number were also evident from immediately after birth through to the onset of reproductive cyclicity (data not shown). It should be emphasized that although germ cell number between P2 and P20 remains unchanged, considerable variability is observed between animals as expressed by the wide CI found for the mean numbers of total follicles at ages P2 (CI: 976–5529), 12 (CI: 264–4996) and 20 (CI: 947–5399; Fig. 2). In sharp contrast to the lack of stability in germ cell number observed pre-pubertally, the rate of decline in follicle number from the onset of cyclicity (P42, CI: 1830–3713 total follicles) through adulthood is consistent between time points and animals. This was further confirmed by performing similar analysis on animals in which stages of the estrous cycle were monitored (data not shown). Again, germ cell counts, corpora lutea and atretic follicles were similar at the age evaluated (P100, CI: 1419–2579 total follicles). Furthermore, estimates of the total number of zona pellucida remnants as a function of animal age also showed that their appearance coincided with the onset of reproductive cyclicity, from undetectable at P20 to 20.8 ± 3.2 at P42 and 34.9 ± 1.7 at P100 (mean number per ovary for $n=5$ animals for each time point). These

Figure 1 Germ cell disposition during ovarian histogenesis. MVH immunocytochemistry (brown reaction product) defines developmental variance in germ cell density and location. (A) Embryonic day 15.5 ovary, showing germ cell clusters and relative paucity of somatic tissue (inset). (B) Postnatal day 6 ovary, with cortical primordial follicles and medullary primary follicles; note apposition of peritoneal mesothelium to ovarian surface defining bursal cavity. (C) Postnatal day 16 ovary illustrating onset of antrum formation in centrally located follicles and cortically disposed preantral follicles with growing oocytes; inset shows 2 primordial follicles near surface and subtended by a primary follicle. Scale bars = 40 μ m for A, B and C, whereas for insets scale bars = 20 μ m.

Figure 2 Mean number of total germ cell number per ovary from embryonic day 15 (E15.5) through to adult day 150 (P150). Note marked decrease in mean germ cell number following birth ($P < 0.05$), and animal-to-animal variance in oocyte density between days P6 and P20 of age. Onset of cyclicity (P42) coincides with decrease in mean germ cell numbers that steadily declines up to P150. Data are presented as mean $\pm$ S.E.M. ($n = 5$ animals per age group). Arrow indicates day of birth (day 20 post coitum).

results are consistent with little if any net change in follicle number during prepubertal life and therefore prompted further enquiry into the nature of germ cell loss at perinatal developmental transitions.

Germ cell apoptosis during pre- and postnatal ovary remodeling

We first investigated the expression of various apoptosis markers prior to and following birth. Two common markers for early apoptosis, active caspase 3 and cleaved poly (ADP ribose) polymerase (PARP), were evaluated and the more traditional TUNEL assay for terminal stages of apoptosis. E15.5 and E19.5 samples exhibited limited staining for all markers in germ and somatic cells (Fig. 3A–C). This relative paucity of apoptotic germ cells was also evident in postnatal samples (Fig. 3D–F). As shown below, large clusters of germ cells were often seen at the surface of P2 ovaries, but even these clearly discarded oocytes were only rarely found to be positive for apoptotic markers. In contrast, ovaries from adult cycling females exhibited the apoptosis patterns well established for mammalian ovary (Fig. 3G–I), with granulosa cells (GCs), but not oocytes in atretic follicles positive for apoptosis markers. The rarity of apoptotic germ cells prior to and following birth is difficult to reconcile with the 40% reduction seen by us and others and prompted investigation of alternative cell death mechanisms.

Postnatal ovarian histogenesis and germ cell extrusion

We first evaluated germ cell extrusion or shedding, as a mechanism of germ cell loss. Oogonia and primordial follicles were scored based on their position relative to

the ovarian epithelium; these were classified as extra-ovarian if in the bursa cavity or intra-epithelial if positioned at the ovarian surface (Fig. 4A–D). As summarized in Fig. 4E, the mean number of extra-ovarian oogonia/oocytes observed in the bursa account for a minor fraction ($\sim 2\%$) of the total germ cells recorded at these time points. Intra-epithelial oogonia/oocytes represent a transient population of about 300 follicles at each stage examined up to P42, after which time they are not detected (Fig. 4E). Because of the difficulty in determining the flux rate of oocyte extrusion in fixed samples, we conducted live imaging studies in ovarian organ cultures. Intact E19.5, P0 (day of birth), and P1 ovaries were cultured for 4 or 18 h. Germ cells associated with the ovarian surface were large and spherical and rarely contained associated somatic cells (Supplementary Figure 1, which can be viewed online at www.reproduction-online.org/supplemental/) whereas those that accumulated on the bottom of the culture dish after 4 h in culture were found in clusters of 4–8 cells (Supplementary Figure 1). While active extrusion was observed at ages examined, attempts to calculate a rate of loss were unsuccessful. An estimated extrusion rate of 150 germ cells per ovary over an 18 h culture interval for P0 ovaries was obtained (Supplementary Figure 1) but in the absence of a way to follow single germ cells, we conclude that this mechanism of loss accounts for only a minor fraction of the loss observed until a more systematic analysis can be performed.

Lysosome compartment amplification and autophagy

Since lysosomes are involved in both autophagy and apoptosis (Guicciardi *et al.* 2004), we first analyzed their expression in somatic and germ cells of perinatal ovaries. Using LAMP1 antibody, which detects a membrane constituent of functional lysosomes, a striking increase in lysosome density was observed from E19.5 to post-natal ovaries (Fig. 5A and B). Figure 5C and D show further that the increase in LAMP1 staining in germ cells coincides with the onset of oocyte growth and follicle assembly. Quantification of LAMP1 by image analysis of stained ovarian sections further confirmed the lysosome amplification post-birth (Fig. 5E). Significant changes in the amplification of lysosomes were observed from E19.5 2.02 ± 0.21 (CI: 1.60–2.43) to P2 15.08 ± 0.95 (CI: 13.19–16.19) by measuring the mean LAMP1 staining per germ cell. The general pattern of increased lysosome expression after birth was also seen with the vital lysosome marker acridine orange (data not shown). Thus, lysosome amplification in all oocytes occurs upon birth and as in other postnatal tissues may represent an adaptive response to postnatal starvation. To directly address this question, an additional experimental strategy was used to ascertain the relative contributions of apoptosis and autophagy in the perinatal mouse ovary.

Figure 3 Pre- and postnatal apoptosis patterns. Comparison of apoptosis detection by cleaved-PARP (A, D, and G), active caspase 3 (B, E, and H) or TUNEL (C, F, and I) assays in E19.5 (A, B, and C), P2 *post partum* (D, E, and F), and P42 (G, H, and I) C57Bl/6 ovaries. (A) Confocal optical section of intact E19.5 ovary showing a solitary cleaved-PARP positive (green) germ cell amongst many negative germ cells (f-actin red, nuclei white). (D) Confocal optical section of intact ovary (P2) showing absence of staining in germ (one exception) or somatic cells for cleaved-PARP. Also note rare TUNEL positive cells in comparable ovaries (B, E). (G) (PARP red, GDF9 green), (H) (caspase red) and (I) (TUNEL brown) show that under identical processing conditions, apoptosis is readily demonstrable in granulosa cells of antral follicles in day P42 ovary. Scale bars=20 µm.

We adapted the methods of Zucker *et al.* (1998) to image intact ovaries that had been labeled with the probe LysoTracker Red (LTR) using high-resolution confocal microscopy to monitor cytoplasmic and organellar acidification in tissues in an unperturbed

state. In E19.5 ovaries, LTR reveals smaller apoptotic cells, macrophages identified by multiple lysosomes, and larger germ cells evidenced by acidified cytoplasm (Fig. 6A and B). Acidified LTR labeled germ cells were common at P2, but rare at later stages of ovarian

Figure 4 Developmental pattern of germ cell extrusion in mouse ovary. Germ cells were classified as extra-ovarian when within the bursal cavity (A–C) or intraepithelial when associated with or just beneath ovarian surface (D). (A–D) Bright field images of sections stained with MVH (brown), hematoxylin (blue) and PAS (pink). Bars=20 µm. (E) Comparison of extra-ovarian (filled bars) or intraepithelial (open bars) germ cells over developmental age range studied (E15.5–P150). Mean number primordial follicles ± s.e.m. per time point are plotted ($n=5$ ovaries/age).

Figure 5 LAMP1 expression in neonatal mouse ovaries. Prenatal ovaries (A and B) exhibit LAMP1 stained lysosomes (green) predominantly in germ cells (somatic and germ cell nuclei are white). After birth, large lysosomes are evident in oocytes that form primordial follicles (C–D, arrows) whereas smaller lysosomes within oocytes that are not fully enclosed by follicle cells (B, arrowheads). (E) Shows quantitation of LAMP1 staining in oocytes between E19.5 and P42; note the abrupt increase at P2 and stabilization of lysosome density from P16 on. Columns with different letters are significantly different from each other ($P < 0.05$). Scale bars = 20 μ m.

development (Fig. 6C and D). We next tested the effect of serum starvation on the loss of germ cells in an ovarian organ culture system.

To accomplish this, ovaries from P0 animals were cultured and exposed to LTR in the presence or absence of serum for 24 h. Complete Z stack projections were thresholded to demarcate acidified germ cells (Fig. 7A–C and Supplementary Figure 2, which can be viewed online at www.reproduction-online.org/supplemental/), which were counted and expressed as mean density volume per ovary. Direct comparison between P0 (56.93 ± 8.86 ; CI: 18.80–95.08) and P1 (mean of 20.99) for ovaries showed a 60% decrease at these time points with freshly isolated tissues. Comparison of

Figure 6 Analysis of LTR staining in intact mouse ovaries. E19.5 ovaries (A and B) just prior to birth containing both cytoplasmic acidification (arrows) and LTR foci (arrowheads) are seen within germ cell cluster (some circled for easy visualization). From P2 (C) to P6 (D), seem to have fewer cells with cytoplasmic acidification (arrows). Scale bars = 20 μ m.

serum treated (14.40 ± 3.64 ; CI: –15.41–44.22) vs serum starved (23.61 ± 5.08 ; CI: 1.77–45.45) organ cultures illustrates a higher proportion of acidified germ cells after serum removal (Fig. 7G). These findings indicate that maintaining newborn ovaries in the presence of serum lessens the incidence of autophagy. We next tested the effects of apoptosis or autophagy inhibitors on the density of LTR positive germ cells.

Autophagy as an effector of germ cell loss in the newborn ovary

3-Methyl adenine (3MA), a commonly used autophagy inhibitor (Seglen & Gordon 1982, Boya *et al.* 2005), and ZVAD, a commonly used pan-caspase inhibitor (Kim *et al.* 2001, Boya *et al.* 2005), were used to selectively impair autophagy or apoptosis in the organ culture system described above. Ovaries ($n=3$) were exposed to either inhibitor alone or a combination of both inhibitors for 24 h and processed for LTR staining. In the presence of 3MA, an apparent reduction in the density of acidified cells was evident. In contrast, ZVAD treatment had little discernible effect on the density of acidified cells compared to control ovaries (Fig. 7E and C respectively and Supplementary Figure 2). Interestingly, exposure to both 3MA and ZVAD (Fig. 7F) for 24 h reduced LTR-positive cell density to levels comparable with those observed in the 3MA treatment group (Fig. 7D and Supplementary Figure 2). These trends were confirmed by quantitative

Figure 7 Quantitation of acidified germ cell density volume (G) in perinatal mouse ovaries cultured with (filled bars) and without (open bars) serum in the presence or absence of autophagy (3MA) and/or apoptosis (ZVAD) inhibitors (open bars). Panels at the top represent complete confocal projections of intact ovaries after image thresholding to reveal germ cell density for each treatment group. Note decreased prevalence of LTR positive cells when ovaries are maintained in complete medium for 0 h (A) and 24 h (B) compared to serum deprivation for 24 h in culture (C). The autophagy inhibitor 3MA, either alone (D) or in combination with ZVAD (F) reduces the density of acidified germ cells under serum free conditions. Note that treatment with ZVAD alone (E) has little effect on germ cell density compared to controls (C). (G) Values presented as mean $\pm$ S.E.M. ($n=3$ ovaries except for combination of the 2 inhibitors, for which $n=2$ ovaries).

image analysis (Fig. 7G). Whereas the density of LTR-positive cells was similar in control (24 h, 23.61 ± 5.08 ; CI: 1.77–45.45) and ZVAD (28.77 ± 8.86 ; CI: –9.36–66.90) groups, 3MA alone (12.05 ± 3.45 ; CI: –2.79–26.90) or in combination with ZVAD (13.38 ± 1.28 ; CI: –2.89–29.85) resulted in cell densities comparable with those attained after culture in the presence of serum (14.41 ± 3.64 ; CI: –15.41–44.22). Collectively, these results indicate that inhibition of autophagy and not apoptosis influence germ cell loss in newborn ovaries. This was further supported by an analysis of protein expression in perinatal ovaries (Fig. 8). Mediators of apoptosis (caspase 3, cathepsin D) and autophagy (LC-3B and beclin 1) were probed by western blots of pooled ovary samples from animals of E19.5, P2, P6 and P20 ages. Figure 8 shows active

caspase 3 protein expression only on E19.5 (~ 17 kDa), and inactive caspase 3 expression (32 kDa) through all ages examined. Expression of the 16 kDa LC-3B, the autophagosomal membrane form of LC3B (Tanida *et al.* 2005), was evident between E19.5 and P6. The 36 kDa form of LC3B decreases at birth and remains relatively stable in expression at the later time points. Interestingly, cathepsin D gradually decreases until P20. Beclin levels show a slight decline at birth but also remain stable through to P20. Collectively, these data indicate that mediators of apoptosis and autophagy are differentially expressed while pre- and postnatal germ cell loss takes place.

Discussion

Most female eutherian mammals establish a stockpile of follicle-enclosed oocytes at or soon after birth that is gradually depleted through the organisms' reproductive lifespan. In such species, it has long been appreciated that the most profound period of germ cell loss occurs shortly after parturition (Faddy & Gosden 2007). Moreover, it is generally held that the primary determinant of germ cell survival involves parallel processes of germ cell enclosure in the primordial follicle and selective susceptibility of some oocytes to type I PCD, apoptosis (Coucovanis *et al.* 1993, De Pol *et al.* 1997, Reynaud & Driancourt 2000, Pepling & Spradling 2001, Tilly 2001). Since oocyte attrition is a fundamental tenet of reproductive aging and certain fertility disorders in women, there has been general interest in the mechanisms responsible for both the establishment and maintenance of the female germ cell supply (Gosden 2002, Matzuk 2005, Skinner 2005). Here, we used the commonly studied C57Bl/6 mouse strain to gain insight into the mechanisms of perinatal germ cell loss. While our results concur with previous studies indicating

Figure 8 Apoptotic and autophagic protein expression. Western blot analysis of caspase 3 and cathepsin D (apoptosis), LC-3B and beclin 1 (autophagy), and β -actin expression in E19.5, P2, P6 and P20 pooled ovaries.

that substantial germ cell attrition occurs at the onset of reproductive maturity and birth, they also question type I PCD as the sole cause for early germ cell loss. Instead, multiple perinatal mechanisms (apoptosis, germ cell extrusion, autophagy) interact to establish a finite follicle reserve for later use. The conclusion that different PCD mechanisms operate at discrete stages of ovarian development suggests a level of complexity in the regulation of ovarian germ cell and follicle survival that has only recently been appreciated (Lobascio *et al.* 2007).

Ovarian developmental transitions engage distinct PCD mechanisms

Unlike earlier histomorphometric analysis of germ cells and follicles in the mouse ovary, our methods using immunostaining of MVH for unequivocal germ cell identification provided an accurate counting method for analysis of all follicle stages from E15.5 to P150 (Fig. 2). This kind of analysis confirmed the sharp decline in oocyte number at birth noted by others (Baker 1966, Coucouvanis *et al.* 1993, Ratts *et al.* 1995, McClellan *et al.* 2003, Kerr *et al.* 2006) and further showed that over the postnatal period approaching puberty (P6–P42), little change in follicle numbers was detected. Our results are in agreement with those of Kerr *et al.* (2006) (Supplementary Table 1). Many studies have demonstrated the occurrence of germ cell loss through apoptosis in fetal mouse ovary (Coucouvanis *et al.* 1993, Pepling & Spradling 2001, De Felici *et al.* 2005) and in some cases have linked type I PCD to errors in meiotic cell cycle checkpoint control (Burgoyne & Baker 1985). It should be emphasized, however, that the frequency of TUNEL-positive oocytes seen in fetal mouse ovary is surprisingly small ranging from 0.5 to 5% (Pesce *et al.* 1997, Pepling & Spradling 2001). A recent re-examination of this stage of ovarian development by Lobascio *et al.* (2007) has concluded that previous studies failed to recognize the complexity of germ cell loss at fetal stages. Interestingly, these authors called attention to the fact that the mTOR inhibitor, rapamycin, increased TUNEL staining in germ cells presumably due to the negative effect of mTOR on autophagy. Moreover, calpain inhibitor 1 also induced apoptosis in germ cells after prolonged culture as evidenced by the appearance of caspase-negative and atypical TUNEL-positive oocytes (De Felici *et al.* 2007, Lobascio *et al.* 2007). The rare occurrence of apoptotic cells in perinatal ovaries described here further demonstrates that additional, PCD mechanisms, such as autophagy, are involved with germ cell attrition prior to and after birth in the mouse. These findings are immediately relevant to strategies seeking to prevent germ cell loss due to therapeutic or genetic causes and emphasize the need to clearly identify the PCD mechanisms involved.

Germ cell extrusion during ovarian morphogenesis

Oocytes have been noted to reside close to or within the ovarian epithelium in previous studies (Wordinger *et al.* 1990, Hirshfield 1992, Motta *et al.* 2003, Albertini & Barrett 2004) but the relative rarity of these and their disappearance with further postnatal development of the ovary have led most to conclude that this is a minor cause of germ cell loss (Byskov 1982, Hirshfield 1992, Motta *et al.* 2003). Estimates based on the present studies would tend to support this idea based upon analysis of static images at various time points. While this process could result in loss of a significant fraction of oocytes resident at the time of birth, a definitive assessment awaits development of technologies suitable for an analysis of this dynamic process.

Autophagy: an alternative mechanism for perinatal germ cell loss

The perinatal period of life in placental mammals marks an abrupt transition in energy homeostasis. Mounting evidence shows that up regulation of autophagy follows in the immediate hours of postnatal life in many tissues and organs (Kuma *et al.* 2004). It is now accepted that this burst of autophagy is an adaptive response to nutritional stress once the newborn organism is deprived of placental nutrients (Kuma *et al.* 2004). Coupled with studies on fetal ovaries cited above (De Felici *et al.* 2007, Lobascio *et al.* 2007), our results implicate autophagy in the establishment of the follicle reserve. That multiple PCD mechanisms would be involved in this process is not surprising since it has been shown in many other systems (Kuma *et al.* 2004). Moreover, ultrastructural studies of mammalian oocytes have repeatedly documented the presence of lysosomes and autophagosomes in many species (Hertig & Adams 1967, Wassarman & Albertini 1994) but the developmental time course of their appearance and its relationship to autophagy have not been established.

Lysosome amplification is a hallmark of autophagy (Bursch 2001). Using LAMP1 immunocytochemistry and vital markers for lysosomes, we document amplification of lysosomes in oocytes upon birth (Fig. 5). Moreover, accumulation of lysosomes was most apparent in oocytes enclosed within primordial follicles whereas these structures were less apparent in oocytes that were either extruded through the ovarian surface or undergoing PCD.

However, discriminating between germ cells actively undergoing autophagy or apoptosis required application of a vital staining method that would permit assessment of germ cell behavior at critical developmental stages. For this, we used the LTR method and confocal microscopy so that intact living ovaries could be evaluated by digital image analysis (Fig. 6). Fetal and neonatal ovaries exhibited cytoplasmic acidification of germ cells that were characterized by their size and disposition. These

germ cells were distinct from resident macrophages and smaller germ cells that exhibited fibrillar staining after LTR labeling. Quantitation of germ cells at (P0) and just after birth (P1) indicated a gradual decline in their density at this transition. Importantly, ovaries from newborn animals cultured in the presence of serum-enriched medium displayed fewer LTR positive germ cells than those maintained in a serum-free environment. This finding suggests that the removal of ovary from a growth factor-rich environment, rather than an endogenous signal, was able to elicit germ cell death at the time when major loss is occurring. Growth factors are known to prevent follicle loss in both *in vivo* and *in vitro* models of ovarian development (De Felici 2000, Nilsson & Skinner 2001, Skinner 2005, Bristol-Gould *et al.* 2006) and one likely candidate for maintaining germ cells is Kit ligand (Parrott & Skinner 1999, De Felici 2000, Hutt *et al.* 2006). While this experimental approach would be helpful in defining the physiological factors that regulate follicle formation and survival under dynamic conditions, some studies involving section analysis have supported the general notion that growth factor deprivation at birth is responsible for much of the germ loss seen at this time (Durlinger *et al.* 1999, Bristol-Gould *et al.* 2006). Whether the effects of serum on germ cell loss were mediated by apoptosis or autophagy was next investigated using selective inhibitors for each of these processes.

As shown above (Fig. 7 and Supplementary Figure 2), the selective autophagy inhibitor 3MA (Seglen & Gordon 1982, Boya *et al.* 2005) reduced the acidified germ cell density compared to controls whereas no effect was seen after treatment with ZVAD, a pan-caspase inhibitor. Moreover, use of both inhibitors reduced the density of acidified germ cells to the same extent as 3MA alone, suggesting further that autophagy rather than apoptosis is involved in germ cell loss after birth. That combinatorial mechanisms may mediate this process is further indicated by western blot analysis of components for either apoptosis or autophagy (Fig. 8).

Prominent perinatal expression of beclin 1 and LC-3B (16 kDa) in ovaries at E19.5, P2 and P6 directly implicate autophagy, but, as also shown, activated caspase 3 (~17 kDa) is present only in fetal ovaries. Since cathepsin D is also present over this time interval, it is tempting to suggest that apoptosis at low levels may occur both prior to and following birth whereas the activation of autophagy appears to be directly linked to parturition-induced starvation. Caspase 3 antibody also detects a 36 kDa component that most likely is the inactive, constitutively expressed form that requires cleavage for protease activation (Boone & Tsang 1998). Moreover, 16 kDa band corresponding to the LC3B II isoform is localized to autophagosomes and autolysosomes (Tanida *et al.* 2005). Together, these results support the existence of combinatorial mechanisms for germ cell loss and survival at birth. Further resolution of the interactions between both of these PCD pathways

will be facilitated by the use of conditional knockouts of genes involved in each of these processes.

In summary, these results suggest that multiple PCD mechanisms function in the perinatal mouse ovary to establish the primordial follicle reserve. The relative contributions of apoptosis and autophagy may vary at distinct stages of ovarian development. The present findings will be of relevance to emergent technologies seeking to optimize and preserve high quality oocytes for experimental or clinical use. Furthermore, they uncover a level of complexity in female germ line development in eutherian mammals not previously appreciated. Future studies seeking to rescue female germ cells in newborns, adolescents or adults will need to take into account the diverse mechanisms that are at play during different stages in the lifespan of the mammalian ovary.

Materials and Methods

Animals

Inbred C57Bl/6 mice (Charles River, or Taconic Farms Inc., Germantown, NY, USA) used for these experiments were housed in a 14 h light: 10 h dark environment at constant temperature. Food and water were provided *ad libitum*. All animals were killed by cervical dislocation. Mice were maintained and used in accordance with the policies of the University of Kansas Animal Care and Use Committee (protocol # 2007-1681).

Ovary collection and tissue preparation

Right and left ovaries from each animal were collected at embryonic (E15.5 and E19.5), prepubertal (P2, 6, 10, 12, 16, 20 days of age) and adult (P42, 100 and 150 days of age) stages. Five ovaries were collected at each stage from at least two separate litters. Intact bursa enclosed ovaries were fixed in either Bouin's fluid (Sigma; follicle counts) or 2% paraformaldehyde (PFA, Sigma; immunohistochemistry), for 4–6 h at room temperature and overnight at 4 °C respectively. Following fixation, ovaries were transferred to 70% ethanol and processed for paraffin embedding by standard techniques. For whole mount analysis, intact ovaries were fixed overnight in 2% formaldehyde microtubule stabilization buffer (MTSB-XF), (Messinger & Albertini 1991) at 4 °C and stored at 4 °C in a blocking buffer as previously described until use.

Immunocytochemistry

For follicle counts 5 µm sections were dewaxed and re-hydrated using conventional methods. Endogenous peroxidases were quenched in 0.3% hydrogen peroxide in methanol (5 min, room temperature). After three washes in Automation Buffer (Biomedex-Fisher, Foster City, CA, USA), slides were blocked for 30 min at room temperature in 9% goat serum (Zymed) containing 3% BSA (Sigma). Sections were labeled (overnight, 4 °C) with polyclonal rabbit

antibody against MVH, (a cytoplasmic germ cell specific protein, gift from Dr Noce) diluted 1:1200 in 1% BSA. Washed sections were then incubated with biotinylated goat anti-rabbit serum (Zymed; 1:200 in 1% BSA) for 30 min at room temperature, rinsed and incubated with HRP Avidin D (HRP; Vector, Burlingame, CA, USA; 1:500) for 10 min. Diaminobenzidine was used as a substrate for HRP. Sections were counterstained with PAS reagent and Harris hematoxylin (Protocol) containing 4% acetic acid. Tissues were dehydrated, cleared and coverslipped using Permount (Fisher, Pittsburgh, PA, USA). Control slides were processed identically but primary antibody was omitted.

Follicle morphology and classification

Follicles were classified according to Pedersen & Peters (1968) and Myers *et al.* (2004). Briefly, follicles were classified as primordial if the oocytes were partially or completely surrounded by squamous GCs, equivalent to Type 1–2 in the Pedersen & Peters (1968) classification. Primary follicles were those exhibiting one complete layer of cuboidal GCs, (Type 3–3b). In the transition from primordial to primary classification was made according to the predominant type of GCs present. Secondary follicles were classified as all follicles having more than one GC layer and no visible antrum (Type 4–5). Follicles with a small antrum were designated early antral (Type 6) and antral follicles (Type 7) when the follicle had a single large antral space.

Follicle counting

Counts were made using a 40× Nikon objective in an Alphaphot 2 SY2 Nikon microscope. Follicles with an intact oocyte nucleus containing a dark nucleolus were counted in every second section and repeat scoring was unlikely because ~12 µm germ cells in primordial and primary follicles would not be present in the third 8 µm section used for sampling frequency in this study. For these follicle classes, final totals were derived by doubling the follicles recorded in every second section. However, for secondary to pre-ovulatory follicles this adjustment was omitted in order to limit over-representation (Liu *et al.* 2002). The position of primordial follicles relative to the ovarian surface epithelium was also determined and these were scored as extra-ovarian (if in the bursal cavity) or intraepithelial (if associated with or subjacent to the epithelium).

Static and dynamic imaging of germ cell loss

PCD was studied using a combination of methods to discriminate between apoptosis and autophagy in histological samples or ovarian whole mounts.

Apoptosis detection

TUNEL was used to detect apoptosis following the manufacturer's protocol for the ApopTag Peroxidase *In Situ* apoptosis detection kit (Chemicon-Millipore, Billerica, MA, USA).

For active caspase 3 (1:100; R&D Systems, cat. # AF835), and poly (ADP ribose) polymerase (cleaved-PARP form; 1:50; AbCam cat. # ab32064) paraffin sections of PFA fixed ovaries were dewaxed and microwaved (high, 15 min) in sodium citrate buffer (0.01 M, pH 6.0) for antigen retrieval. After sections were washed and blocked they were incubated in primary antibody (see above). For fluorescent imaging, slides were exposed to Alexa-fluor goat anti-rabbit secondary antibodies (488 or 568; 1:800; Molecular Probes, Invitrogen; 37 °C; 1 h) rinsed (3×), incubated in Hoechst 33258 (1 µg/ml; Polysciences Inc., Warrington, PA, USA) for nuclear staining and mounted in Prolong Anti-fade Reagent (Molecular Probes, Invitrogen). Control slides were prepared as above by omitting primary antibody.

Whole mount preparations of intact or fragments of ovaries fixed in MTSB-XF (Messinger & Albertini 1991) were processed for confocal microscopy using the same primary and secondary antibodies noted above. These were diluted with wash solution and used with constant agitation for 24 h at 4 °C. Multiple wash steps (a total of 4 h, several changes; 37 °C) separated primary and secondary antibody incubations. Hoechst 33258 (nuclei) and Alexa 546 Phalloidin (f-actin; 1:100 dilution, Molecular Probes, Invitrogen) were used to define nuclear and cell boundaries. Ovaries were mounted on microscope slides in glycerol/PBS containing Hoechst 33258.

LTR is an aldehyde-fixable dye that concentrates in acidic membrane-bound intracellular compartments of living tissue, which has been adopted by Zucker *et al.* (1998) as an assay for PCD in rodent tissues. We modified the protocol as follows: intact ovaries were incubated in 5 µM LTR (Molecular Probes, Invitrogen) in MEM (Gibco, Invitrogen) for 1 to 2 h at 37 °C and 5% CO₂. After two washes in PBS the ovaries were fixed in a mixture of 4% PFA and 1% glutaraldehyde (Sigma) in PBS (2 h; 37 °C with shaking, followed by 4 °C, overnight), rinsed twice in PBS and dehydrated in a series of methanol/PBS solutions (50, 70, 95 and 100%-2×; 15 min each). Tissues were then cleared in a 1:2 mixture of benzyl alcohol to benzyl benzoate (BABB, Sigma). From absolute methanol, tissues were exchanged through 50% and 70% BABB-methanol and finally pure BABB solution (2 h each; at room temperature with constant agitation). For confocal microscopy, a single ovary was centrally positioned in a metal washer sealed by Permount (Fisher) to the center of a glass bottomed tissue culture dish (Delta T, Fisher). The depression was filled with BABB and a coverslip applied with Permount (Fisher).

Lysosome detection and autophagy

Both immunohistochemistry and live cell imaging were used to visualize the lysosomes and autophagic markers. Lysosome abundance and distribution in developing germ cells was determined using a rat antibody to LAMP1 (a lysosomal membrane glycoprotein; Developmental Studies Hybridoma Bank, University of Iowa, 1:100, cat. # 1D4B), and goat anti-rat Alexa Fluor 488 secondary antibody in both paraffin sections and whole mounts as described above. For analysis of acidic organelles in living tissues, intact ovaries (embryonic 19.5 and post-natal days 0 (day of birth), 1 and 2) were cultured for 4 and 18 h in Acridine Orange (1 µg/ml;

Molecular Probes, Invitrogen) or LysoSensor Yellow/Blue DND (10 µg/ml; Molecular Probes, Invitrogen). Individual ovaries were cultured in 100 µl drops of culture media (MEM with Earle's Salts and L-glutamine without phenol red (Gibco); 10% FCS (Hyclone, Logan, UT, USA), 100 ng/ml of leukemia inhibitory factor (Sigma), 100 ng/ml of stem cell factor (SCF/c-Kit, PreProtech), 50 ng/ml of IGF1 (Sigma), and 1% gentomycin (Sigma)), covered with mineral oil (Sigma) and incubated with at 37 °C with 5% CO₂. Glass bottom culture dishes allow live imaging of intact ovaries after 4 or 18 h of culture and Hoechst 33342 was used as a vital nuclear stain. LAMP1 was quantified using Integrated Morphometry Analysis (IMA) software (MetaMorph version 7.5 Universal Image Corporation, USA), to define individual germ cell boundaries (regions of interest) and measuring the area occupied by the LAMP1 signal. Values are represented as the percentage of LAMP1 per germ cell.

In vitro starvation of neonatal ovaries

To create *in vitro* conditions resembling the nutrient-deprived state of neonatal ovaries, P0 and P1 ovaries (3 per group from different litters) were incubated in 600 µl complete organ culture media (DMEM:Ham's F12, 1:1; 10% FBS, 1% ITS (Sigma), 1% penicillin/streptomycin (P/S, Sigma)), suspended in a 3 µm PCF insert Millicell (Millipore), and placed in a 24 well tissue culture plate. Following a stabilization period, ovaries were either incubated for an additional 21 h in complete media or starvation media (DMEM+1% P/S). At the end of the total 24 h incubation period, LTR (1%) was added to each well. For 0 h control ovaries, LTR was added for 3 h following the initial stabilization period. Hoechst 33342 was typically added as a nuclear counterstain. Ovaries were washed in PBS and fixed as for LTR in 4% PFA prior to confocal microscopy without BABB clearing.

Effect of apoptosis and autophagy inhibitors on starved neonatal ovaries

Using complete or starvation media (as above), E19.5 or P0 ovaries were incubated with inhibitors of PCD. For apoptosis the pan-caspase Inhibitor 1 ZVAD (OME)-FMK (50 µM; Calbiochem-EMD, Gibbstown, NJ, USA) was used; for autophagy, 3MA (10 mM; Sigma) was used. Drugs were added alone or in combination for 21 h and LTR and Hoechst 33342 were added for the final 3 h of incubation. Tissues were analyzed by confocal microscopy to determine the volume density of LTR labeled germ cells in Z-section composites of whole ovaries using IMA software as above. Briefly, LTR positive objects with a diameter greater than 80 µm² (determined to be the approximate diameter of germ cells) were thresholded and counted within 3-dimensional projections of a single ovary from each treatment group (*n*=3 per group). Approximately, 50% of the entire ovary volume was assayed for each replicate in each treatment group and the data are expressed as mean density volume per group.

Western blot analysis of apoptosis and autophagy proteins

Ovaries from fetal (E19.5), neonatal (P0, P1, P2, and P6), and pubertal (P20) mice were washed in cold PBS (4 °C), and lysed (20 mM Tris-HCl (pH 7.4; Sigma), 150 mM of sodium chloride (Sigma), 1 mM of EDTA (Sigma), 1 mM EGTA (Sigma), 1% Triton, 2.5 mM sodium pyrophosphate (Sigma), 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate (Sigma), 1 µg/ml leupetin (Sigma), 1 mM phenylmethanesulfonyl fluoride (Sigma)). Twenty micrograms of protein from pooled samples (E19.5, P2, 6, 16, and 20) was loaded on 12% SDS-Page gels and transferred to a PVDF membrane (Millipore). Membranes were incubated for 1 h in PBS-Tween 20 (0.05%) containing 5% nonfat milk and probed with primary antibodies to caspase 3 (Cell Signaling cat. # 9661), LC-3B (Cell Signaling cat. # 2775), cathepsin D (Santa Cruz, cat. # 10725) and beclin 1 (Sigma cat. # B6061) followed by incubation with the appropriate HRP-conjugated secondary antibody (Southern Biotechnologies Associates, Birmingham, AL, USA); bands were detected with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and an anti-actin antibody (Sigma cat. # A2066) was used to verify comparable loading between samples.

Image acquisition and analysis

Whole mount or sectioned ovary preparations were imaged using a LSM-510 Pascal confocal microscope (Zeiss) mounted on a Zeiss Axiovert 200M microscope equipped with excitation specific Diode (405 nm), Argon (458, 477, 488 and 514 nm) and Helium Neon lasers (594 nm). Single scans or Z series data sets were made using 20×, 40× and 63× (NA=1.25) objectives and data were archived and analyzed using LSM software. Bright field images were obtained using a Nikon Eclipse 8i microscope with 20× and 100× objectives and a Sony XWaveHAD color camera.

Statistical analysis

Data are presented as mean±S.E.M. and statistical analysis of follicle number counts was performed using Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). Data were analyzed by calculation of CI and non-parametric Kruskal-Wallis test of significance. Data were considered statistically significant in cases where *P*<0.05.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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