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Autophagy is a cell survival program for female germ cells in the murine ovary

Thomas R Gawriluk, Amber N Hale, Jodi A Flaws[1], Christopher P Dillon[2], Douglas R Green[2] and Edmund B Rucker III

[1]*Department of Biology, University of Kentucky, 313 Thomas Hunt Morgan, Lexington, KY 40506, USA,* [2]*Department of Veterinary Biosciences, University of Illinois, 2001 S. Lincoln Ave., Urbana, IL 61802, USA and* [3]*Department of Immunology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA*

Correspondence should be addressed to E B Rucker; Email: edmund.rucker@uky.edu

Abstract

It is estimated that infertility affects 15–20% of couples and can arise from female or male reproductive defects. Mouse models have ascribed roles to over 100 genes in the maintenance of female fertility. Although previous models have determined roles for apoptosis in male and female fertility, we find that compromised autophagy within the perinatal ovary, through the loss of *Becn1* or *Atg7*, results in the premature loss of female germ cells. *Becn1*$^{+/-}$ ovaries have a 56% reduction of germ cells compared with control ovaries at post-natal day 1, whereas *Atg7*$^{-/-}$ ovaries lack discernable germ cells at this stage. Thus autophagy appears to be a cell survival mechanism to maintain the endowment of female germ cells prior to establishing primordial follicle pools in the ovary.

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Introduction

Oocyte development can be classically split into six broad steps: 1) colonization of the indifferent gonad by primordial germ cells (PGCs), 2) formation of oogonia, 3) meiotic arrest at prophase I, 4) primordial follicle formation, 5) follicle maturation, and 6) ovulation (Edson *et al*. 2009). Early female germ cell development encompasses the first four stages and occurs during fetal and perinatal development. The fetal ovary is endowed with the greatest number of germ cells prior to entering meiotic arrest. After this stage, most female germ cell loss occurs either during the fetal/perinatal window or from post-natal follicular atresia. Ovarian follicular atresia is a cell death event that occurs in more than 99% of mature follicles (Byskov 1978), and excessive follicular atresia is associated with premature ovarian failure, one of the most common causes of infertility in women (Anasti 1998, Matzuk & Lamb 2008). The loss of germ cells has been attributed, in part, to genes devoted to programmed cell death (PCD).

PCD, an important physiological process required for development, is classified as type I (apoptosis), type II (autophagy), or type III (non-lysosomal vesiculate degradation). Autophagy is a conserved mechanism, from yeast to mammals, for bulk recycling of proteins and organelles (Klionsky & Emr 2000). This process is important for normal development, tissue/organ remodeling, and cell death/survival and can be triggered under stress conditions such as nutrient deprivation. A wide array of diseases manifest from altered autophagy including neurodegenerative disorders (e.g. Alzheimer's, Parkinson's, and Huntington's diseases), liver disease, myodegenerative disorders, heart disease, inflammatory diseases, and cancer (Levine & Kroemer 2008). Initially, the proteins/organelles are sequestered by lipids called 'isolation membranes', which then encapsulate the targets with a double-membrane structure called the autophagosome. These autophagosomes then fuse with liposomes to degrade the internal components. The yeast autophagy (Atg) proteins responsible for this process are classified into four groups: 1) induction of autophagy (ULK1 (ATG1) protein kinase complex), 2) vesicle nucleation (phosphatidylinositol 3-kinases class III (PI3KC3) lipid kinase complex), 3) vesicle expansion (ATG7–ATG12–ATG5 and GABARAPL2 (ATG8) pathways), and 4) the Atg protein retrieval system (Maiuri *et al*. 2007). Beclin 1 (BECN1), the mammalian counterpart of the yeast ATG6 protein, is integral in the vesicle nucleation phase for autophagosome formation. BECN1 forms a complex with u.v. radiation resistance-associated gene (UVRAG) and BAX-interacting factor-1 (BIF1) to regulate the PI3KC3 kinase complex and promote autophagosome formation (Liang *et al*. 2006, Takahashi *et al*. 2007). ATG7 is an E1-like protein that is required for the conjugation of ATG5–ATG12 and the addition of phosphatidylethanolamine to LC3/GABARAPL2 (microtubule-associated protein light chain 3).

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Previous genetic mouse models have revealed the importance of more than 20 genes that impact the germ cell populations or the establishment of primordial follicles in the murine ovary (Matzuk & Lamb 2002, 2008). Of these, three apoptosis-associated genes are known to impact germ cell numbers in the fetal–neonatal ovary: *Bcl2l1* (*Bcl-x$_L$*), *Bax*, and caspase-2 (Bergeron *et al.* 1998, Rucker *et al.* 2000, Greenfeld *et al.* 2007). BCL2L1 and BAX have been shown to govern PGC numbers after colonization of the fetal gonads, around E11.5–E13.5, when a wave of apoptosis reduces the gonocyte populations. In contrast, the developmental window of germ cell loss at the time of parturition may be dependent on mechanisms other than apoptosis. Ablation of BCL2L1 in the fetal ovaries after this initial wave of germ cell loss does not impact the primordial follicle pool in the murine ovary (Riedlinger *et al.* 2002). Several additional reports suggest that the ovary may rely on alternative pathways of cell survival and cell death. Analysis of the mouse ovary between E19.5 and P2 revealed a 44% reduction in the number of follicles during this window (Rodrigues *et al.* 2009). At this stage, few germ cells or somatic cells were found to be apoptotic, whereas lysosome amplification and increased lysosomal-associated membrane protein 1 (LAMP1) expression occurred, suggesting a role for autophagy. During the germ nest breakdown and establishment of the primordial follicle pool, the germ cells may need to maintain energy homeostasis through autophagy. Autophagy can promote cell survival or lead to cell death, depending on the context (Codogno & Meijer 2005). Therefore, we determine the effect of autophagy, through the genetic loss of *Becn1* or *Atg7*, on the endowment of the perinatal ovary as a pro-survival or pro-death mechanism.

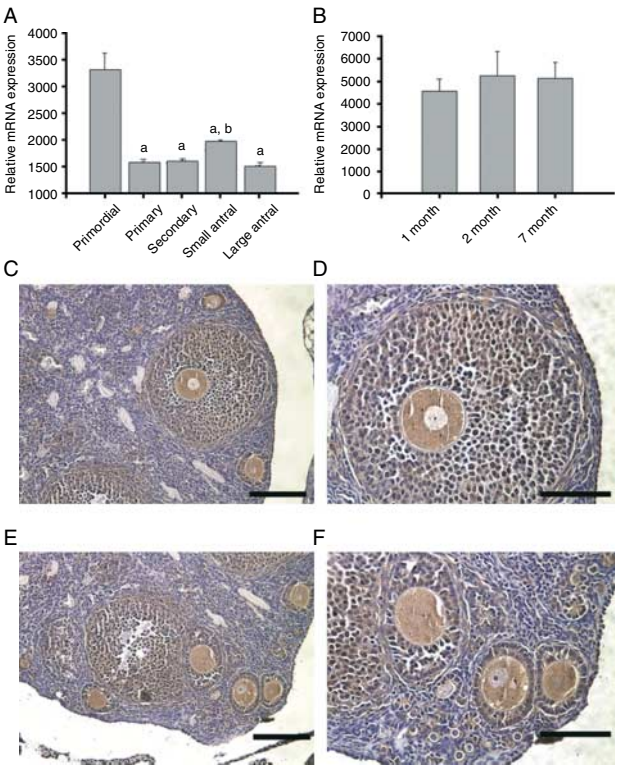

Figure 1 Expression profile of *Becn1* mRNA and protein in the murine ovary. (A) Oocyte *Becn1* mRNA expression in different stages of follicles ($n=3$, error bar; S.E.M.; $^{a}P<0.05$ compared with primordial follicles; $^{b}P<0.05$ compared with primary, secondary, and antral staged follicles). (B) Ovarian *Becn1* mRNA expression at different stages of development from diestrus mice ($n=2$; error bar; S.E.M.). Figure was generated from a re-analysis of microarray data originally published by Pan *et al.* (2005). BECN1 immunolabeling in 3-month-old Sv129 ovary is present in all follicles (theca and granulosa cells) and oocytes, however absent from ovary epithelium. (C and E) 20× Magnification showing several follicles. (D) 40× Magnification of (B). (F) 40× Magnification of (E) showing several primordial and primary follicles. Images were taken using Nikon E800, scale bar equals 100 μM.

Results

Characterization of mRNA from different oocyte stages was possible due to the efforts of Pan *et al.* (2005). They isolated oocytes from different follicle stages (primordial, primary, secondary, small antral, and large antral), extracted total RNA from each set, generated biotinylated cRNA, and hybridized probes to the 'Mouse Expression Set 430' MOE430A and MOE430B Affymetrix GeneChips (Pan *et al.* 2005). These arrays, which contain mouse maintenance genes as normalization controls, interrogate the expression levels of over 39 000 transcripts from the mouse transcriptome. From this data set, *Becn1* mRNA was found to be expressed at highest levels in the murine ovary within the primordial oocyte (Fig. 1). With respect to stage, the highest relative expression levels were found in the primordial oocyte population (3200 avg) compared with primary (1500 avg), secondary (1500 avg), small antral (2000 avg), and large antral oocytes (1500 avg). *Becn1* mRNA expression did not differ between 1-month ovaries (4500 avg) compared with 2-month (5000 avg) and 7-month ovaries (5000 avg). *Atg7* mRNA expression was present at all oocyte stages from the primordial to the large antral stage but did not statistically vary (not shown). Protein analysis by immunohistochemistry revealed that BECN1 is localized to the follicle (Fig. 1). BECN1 expression is not found in the cortex or medulla, but throughout the follicle (oocyte, granulosa cells, and theca cells). As homozygous *Becn1* null mice (−/−) are embryonic lethal at E9.5 (Qu *et al.* 2003, Yue *et al.* 2003), we decided to examine hemizygous *Becn1* ovaries (+/−) to compare them with *Atg7*-deficient ovaries at post-natal day 1 (P1). Analysis at this timepoint was chosen because of the perinatal lethality of ATG5- and ATG7-deficient neonates at P1 (Kuma *et al.* 2004, Komatsu *et al.* 2005). For analysis of *Becn1*, we generated a floxed beclin mouse model by gene targeting in embryonic stem cells,

Table 1 Germ cell counts in post-natal day 1 murine ovaries.

Genotype of oocyte	Counts	Mean (S.E.M.)
Becn1$^{+/+}$	10 090	8870 (962)
	6970	
	9550	
Becn1$^{+/-}$	4680	4065 (476)
	2190	
	3610	
	3830	
	4430	
	5650	

$P<0.001$ between *Becn1*$^{+/+}$ and *Becn1*$^{+/-}$.

which harbor loxP sites around exons 1 and 2. A null allele for *Becn1* was generated by crossing with the *MMTV-CreA* line, a Cre transgenic that is expressed in the ovary for germline-specific deletions (Wagner *et al.* 1997). Although this transgene is 'leaky', we previously used the *MMTV-CreA* model to perform an oocyte-specific deletion of a floxed *Bcl2l1* gene because of its extremely high efficiency of recombination (Riedlinger *et al.* 2002).

Compromised autophagy, through loss of ATG7 or a dose-dependent reduction of BECN1, reduces female germ cell populations in the post-natal day 1 (P1) ovaries. Morphometric analyses were performed to quantitate germ cells, similar to methods from our previous studies (Rucker *et al.* 2000, Borgeest *et al.* 2002, Riedlinger *et al.* 2002, Greenfeld *et al.* 2007). Quantitative analysis of germ cells in the P1 ovary revealed a BECN1-dependent reduction of germ cells (Table 1). Wild-type, control P1 ovaries had an average of 8870 germ cells (S.E.M.=962; $n=3$), compared with an average of 4065 germ cells (S.E.M.=476; $n=6$) in *Becn1*$^{+/-}$ hemizygous ovaries (*Becn1*$^{+/-}$, *MMTV-CreA*; *Becn1*$^{+/-}$, or *MMTV-CreA*, *Becn1*$^{fl/+}$). There was a high loss of fetuses with the *MMTV-CreA*, *Becn1*$^{fl/-}$ genotype associated with heart and brain defects at embryonic day 11.5–12.5. This was confirmed in subsequent breedings with the *EIIa-Cre* line as well, which have mosaic Cre expression beginning at the eight-cell stage of development. From over 100 collected neonates, only two survived to the P1 stage (expected 1:4 ratio). Histological examination of the mutant P1 ovaries showed an altered distribution and a reduction in primordial follicles compared with the control ovaries (Fig. 2). Wild-type ovaries had a more uniform distribution of follicles and were larger due to the presence of more germ cells and primordial follicles. To substantiate the role of BECN1 and autophagy in the maintenance of female germ cell pools, we also collected P1 ovaries from *Atg7*$^{-/-}$ neonates ($n=4$). ATG7-deficient ovaries had a loss of germ cells, and moreover, the altered appearance of the germ cells within the ovary made them impossible to quantitate (Fig. 3).

Discussion

In the context of autophagy, there are six major steps associated with the process: 1) regulation of induction, 2) isolation membrane recruitment and vesicle nucleation, 3) vesicle elongation, 4) protein retrieval, 5) docking and fusion of autophagosome and lysosome, and 6) vesicle breakdown and degradation (Maiuri *et al.* 2007). BECN1 is involved with both nucleation and autophagosome maturation, whereas ATG7 is needed for vesicle elongation. Although autophagy can proceed through either ATG7-dependent or ATG7-independent pathways, our results demonstrate that female germ cells utilize both ATG7 and BECN1 for induction of autophagy for cell survival. Determination of germ cell numbers in ATG7/BECN1-deficient ovaries would clarify whether alternative pathways are utilized. Our study demonstrates that autophagy may be an important regulator of germ cell survival prior to formation of the primordial follicular pool.

In addition to this early window of germ cell loss, evidence is mounting that the atresia within the pre-pubertal murine ovary is dependent upon autophagy. A non-apoptotic mechanism for loss of primordial follicle pools has been highlighted in two recent studies. Atretic oocytes from P1 to P28 wild-type rat ovaries were described as having apoptotic markers of active caspase-3 and positive staining for TUNEL as well as the autophagic markers of increased numbers of autophagosomes (Escobar *et al.* 2008*a*, 2008*b*). Of particular interest was that hallmark apoptotic characteristics, pyknotic nuclei and membrane blebbing were never observed. In the pre-pubertal mouse ovary, apoptotic characteristics (nuclear condensation, caspase 3 activation, PARP1 'poly (ADP–ribose) polymerase 1'

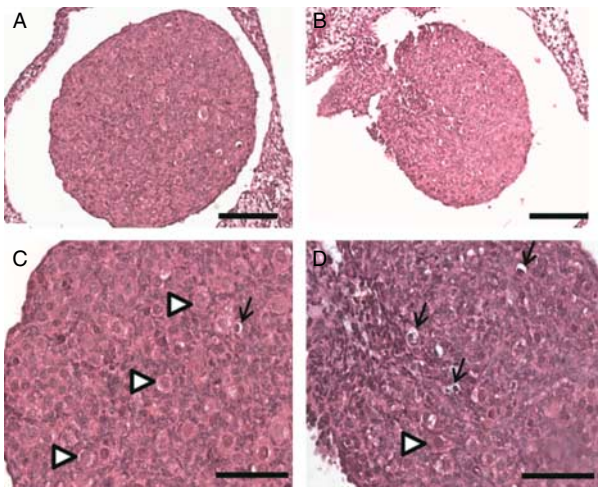

Figure 2 Reduction of germ cells in BECN1-deficient ovaries. Representative ovaries from *Becn1*$^{+/+}$ (a and c) and *Becn1*$^{+/-}$ females (b and d). Control ovary (a and c) shows a more uniform distribution of healthy germ cells than *Becn1*$^{+/-}$ ovary (b and d). Arrowheads: healthy germ cells; arrows: germ cells with 'pyknotic' nuclei. Images were taken using Nikon E800, scale bar equals 100 μM.

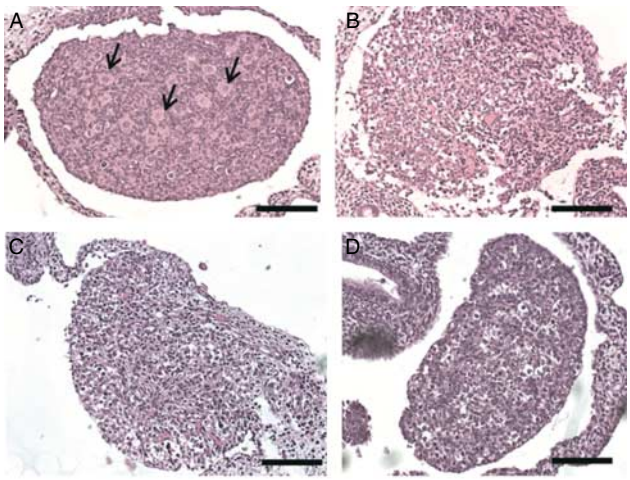

Figure 3 ATG7-deficient ovaries demonstrate loss of germ cells. (a) Postnatal day 1 (P1) control ovary contains healthy germ cells (marked by arrows). In contrast, P1 ovaries from three *Atg7*$^{-/-}$ females show loss of distinguishable germ cells (b–d). Images were taken using Nikon E800, scale bar equals 100 μM.

cleavage, and DNA fragmentation) were not correlated with primordial follicles that were lost from atresia (Tingen *et al*. 2009). *In vitro* analyses of murine prepubertal oocytes showed that both apoptotic and autophagic processes were involved in the cell death of the oocytes (Lobascio *et al*. 2007, Escobar *et al*. 2008*a*, 2008*b*). The importance of autophagy in follicular atresia has been demonstrated across multiple species including *Drosophila*, teleostei, caecilians, stingray, dove, quail, sheep, rats, and humans (Rosales-Torres *et al*. 2000, Velentzas *et al*. 2007, Gaytan *et al*. 2008, Santos *et al*. 2008, Zarnescu 2004).

Autophagy has been demonstrated to have a crucial function immediately after parturition within hours of birth, when neonates experience a starvation period. This is a crucial timepoint at which the neonate must utilize its energy reserves before the suckling response triggers milk ejection from the mother for sustenance. Knockout studies from *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ mice have shown a perinatal lethality (die within 1 day of birth) associated with a loss of autophagy during this early starvation period (Kuma *et al*. 2004, Komatsu *et al*. 2005). A burst of autophagy occurs upon parturition, peaks in tissues within 12 h, and then returns to basal levels within 1–2 days. *In utero*, the autophagy-deficient fetuses are able to obtain metabolites through the placenta; however, amino acid levels within tissues and plasma from the neonates are reduced compared with control siblings. Thus, autophagy appears to be a requisite mechanism to maintain energy homeostasis within the neonate.

In the context of the fetal and neonatal ovary, regulation of autophagy may occur through the KIT ligand/KIT signaling pathway. Naturally occurring mutations of KIT ligand, *Steel Panda* (*Sl*^{pan}) and *Steel Contrasted* (*Sl*^{con}), have been shown to result in reduced germ cell levels and fewer oocytes in the murine ovary (Huang *et al*. 1993, Bedell *et al*. 1995). Addition of anti-KIT antibody to fetal or neonatal oocytes *in vitro* caused a dramatic reduction in their growth (Packer *et al*. 1994). KIT has been localized by immunohistochemistry to female germ cells at 14 dpc (days post-coitum) and 16 dpc, and in the oocyte in the P2 and P7 murine ovary (Kang *et al*. 2003). KIT signaling is traditionally known to activate the PI3K–AKT–MTOR signaling pathway to promote cell survival, and suppression of this survival pathway leads to premature follicle activation and infertility as revealed in knockout studies (Reddy *et al*. 2008, 2009, Adhikari *et al*. 2009, 2010). As MTOR acts to suppress autophagy, the question then becomes how can cells concomitantly activate AKT and autophagy pathways? This apparent contradiction may be reconciled by the notion that signaling can occur through two distinct isoforms of the PI3K p110 catalytic subunit. For AKT activation, signaling primarily occurs through the PI3K p110α catalytic subunit; however, it has recently been demonstrated that the PI3K p110β catalytic subunit triggers autophagy through activation of the PI3K class III complex (Dou *et al*. 2011). A recently developed floxed PI3K p110β mouse model could be used to address the activation mechanism for autophagy in the developing ovary (Jia *et al*. 2008).

Regulation of autophagy may also occur through cross talk with apoptosis proteins, because BECN1 contains a BH3 region that serves as a BCL2 binding domain (Shimizu *et al*. 2004, Maiuri *et al*. 2007, Oberstein *et al*. 2007, Maiuri *et al*. 2010). *In vitro* abrogation of the BECN1–BCL2/BCL2L1 interaction with the pharmacological mimetic ABT737 triggers autophagy by reducing available BCL2/BCL2L1 (Maiuri *et al*. 2007). Similarly, *Bcl2* siRNA in MCF-7 cells induces autophagy, whereas overexpression of BCL2L1 in CHO cells suppressed both apoptosis and autophagy (Akar *et al*. 2008, Kim *et al*. 2009). However, BECN1 and BCL2 co-expression in HeLa cells did not demonstrate an effect of BECN1 on inhibiting the anti-apoptotic effects of BCL2 (Ciechomska *et al*. 2009). Although it is clear that BCL2 and BCL2L1 can regulate autophagy, there is some discrepancy as to the role BECN1 has on the integration of apoptotic signaling. Therefore, the susceptibility of cells to undergo apoptosis and autophagy can be linked to the free, unbound levels of the anti-apoptotic BCL2 family members. Within the developing ovary, this regulatory mechanism does not seem to be relevant. The reduction of BECN1 in the BECN1$^{+/-}$ ovary would result in an increase in the unbound BCL2/BCL2L1 fraction, leading to a surfeit of germ cells. Since the opposite is occurring with the loss of germ cells, this suggests that autophagy is not functioning in an autophagic cell death role, but rather in a pro-survival role in the ovary. This mislabeling of autophagy as a PCD pathway seems to be a prevailing view in the scientific

community. Currently, we are using different Cre lines to perform cell-specific deletions of *Becn1* in the murine ovary to determine its role in follicle maturation and atresia. In the future, *in vivo* and *in vitro* models of atresia and premature ovarian failure should take into consideration the combined effects of apoptosis and autophagy.

Materials and Methods

Gene targeting and generation of Becn1 *conditional knockout mouse*

For the targeting vector, a three loxP plasmid vector (ploxP3–NeoTK) with phosphoglycerol kinase (PGK)-neomycin and PGK–thymidine kinase cassettes for positive–negative selection was used. Genomic DNA isolated from 129SvEv mice was used to amplify three regions of homology for the targeting arms: a 2.7 kb *Becn1* arm #1 (*Becn1* promoter sequence), a 3.0 kb *Becn1* arm #2 (promoter, exon 1, intron 1, exon 2, and a portion of intron 2), and a 2.0 kb *Becn1* arm #3 (intron 2 sequence). For the amplification, the AccuPrime Pfx was used according to manufacturer's directions to generate blunt PCR fragments that were gel purified (Gel extraction kit, Qiagen) and cloned into pBlunt vector (Invitrogen). For the electroporations, 25 μg NotI-linearized DNA was resuspended in 25 μl electroporation buffer (Chemicon, Billerica, MA, USA) and electroporated into 1×10^7 129SvEv ES cells using the GenePulser II (250 V and 500 μFd; Bio-Rad) with 0.4 cm cuvettes. G418 selection (200 μg/ml) was started on day 1 after electroporation and continued thereafter; ganciclovir selection (2 μM) was performed on days 4–7 after electroporation. On day 12, 3× 96-well plates were picked and expanded for cell stocks and DNA isolation. Clones were initially screened by pooled PCRs using LaTaq (Takara) with a 5′-flanking reverse primer (5′-CCC TAG CTG GCC TGG AAC TCA GAA ATC T-3′) and neomycin-specific reverse primer (5′-TAC CGG TGG ATG TGG AAT GTG TGC GA-3′) set. The presence of the third loxP site was confirmed using flanking PCR primers (forward: 5′-CAG GAG AAG TGC CAT GGT GCA TCC TCT T-3′; reverse: 5′-CAA AGC CAA GGT TTC CAT GCT AAT GCC-3′). Individual clones were subjected to PCR confirmation from positive pools. Positive clones were confirmed by Southern blot diagnostics with an external 5′ probe. Targeted ES cells were expanded and used for blastocyst injections at the Transgenic Animal Core facility at Texas A&M University.

Generation of mice for ovary collections

Beclin 'floxed' mice were generated by gene targeting in 129SvEv ES cells and will be detailed elsewhere. *Becn1* null alleles were generated through heterozygous breedings of *Becn1*$^{fln/+}$ and *MMTV-CreA* mice. Mice carrying homozygous floxed alleles and neomycin cassettes were confirmed by PCR of tail-snipped DNA. True floxed alleles were generated by crossing *Becn1*$^{fln/+}$ and *Ella-CreA* mice. *CreA*$^{+/-}$ *Becn1*$^{+/-}$ transgenic mice were generated through breeding of homozygous *MMTV-CreA* (Jackson Labs, 003551; Bar Harbor, ME, USA) and heterozygous *Becn1*$^{+/-}$mice, or those heterozygous for a 'null' Cre-generated locus. *CreA* strain genotypes were confirmed using Jackson Labs Protocol for *Tg(MMTV-Cre)1Mam*. ATG7$^{-/-}$ ovaries were provided by Dr Doug Green. All animal work was conducted using protocols approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Histological follicle counts

To assess follicle numbers, whole ovaries including oviduct and ~1 cm uterus were collected from control ($n=3$), *Becn1*$^{+/-}$ ($n=6$), and *Atg7*$^{-/-}$ ($n=4$) ovaries and fixed in 4% (w/v) paraformaldehyde for at least 24 h. After fixation, tissues were dehydrated, embedded in Paraplast (VWR International, West Chester, PA, USA), serially sectioned (8 μm), mounted on glass slides, and stained with Weigert's hematoxylin–picric acid methylene blue. A stratified sample consisting of every tenth section was used to estimate total number of primordial and naked germ cells (germ cells not surrounded by somatic cells) per ovary. Only follicles with a visible nucleus were counted to avoid double counting. In addition, all follicles were counted without knowledge of the genotype of the animal.

Immunohistochemistry

Whole ovaries including oviduct and a small portion of the uterus were fixed in 4% (w/v) paraformaldehyde for at least 24 h. After fixation, tissues were cryo-protected by washing for 1 h in 10% sucrose, 1 h in 20% (w/v) sucrose, and at least 12 h in 30% (w/v) sucrose at 4 °C. Ovaries were then embedded in O.C.T. Compound (Sakura, Tokyo, Japan), frozen using a liquid nitrogen–isopentane bath, serially sectioned (15 μm) and mounted on glass slides. Slides were then washed for 5 min with PBS and stained using ImmPRESS Reagent Kit – Anti-Rabbit IgG (Vector Labs, MP-7401; Burlingame, CA, USA) in combination with primary antibody (anti-BECN1 at 1:80 dilution (Santa Cruz, sc-11427)) overnight at 4 °C. We confirmed the specificity of the antibody in control and BECN1-deficient mammary glands (WAP-Cre; *Becn1*$^{fl/-}$) at lactation day 1. All sections were counterstained with Hematoxylin QS (Vector Labs, H-3404) and visualized using a Nikon Eclipse E400. total rabbit-IgG 1:200 dilution (Santa Cruz, sc-2027) was used as a primary antibody-negative control.

Statistical analysis

Differences in germ cell numbers were evaluated by one-way ANOVA, with statistical significance assigned at $P<0.05$. When a significant P value was obtained, the Scheffe's test was used in the *post hoc* analysis. The SPSS (version 10; SPSS Inc., Somers, NY, USA) program was used to compile statistics from the obtained data. For microarray data, statistical analysis, ANOVA, and Holm–Sidak pairwise tests were completed with Sigma Plot (Systat, Chicago, IL, USA).

Microarray data

Becn1 mRNA expression data was obtained from microarray experiments of oocytes from primordial, primary, secondary,

small, and large antral follicles obtained from B6SJLF1 animals. Data represents four replicates and bars = S.E.M., $*P<0.05$ versus primordial, $**P<0.05$ versus secondary/large antral. Data is obtained from previously published microarray experiments found in NCBI GEO (GDS1266 and GDS1265) (Pan *et al*. 2005).

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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