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Evolving tales of autophagy in early reproductive events

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ABSTRACT Cells learn to thrive under unfavorable conditions by various mechanisms, and autophagy, self-eating, is one such mechanism. Autophagy is always ongoing in cells at a basal level to turn over old proteins, provide building blocks for new proteins, and to dispose of unnecessary byproducts of metabolism, and normally it does not cause deleterious effects on other parts of basic cellular processes. Autophagy is often dubbed a "double-edged sword", as it is a necessary process for many cells, but its exaggeration may lead to cell death. Evidence is accumulating that autophagy is crucially involved in specific aspects of reproduction. Several recent studies have illustrated how the uniqueness of self-eating is manifested in germ cells and embryos. In this review, we attempt to portray where this relatively young field of autophagy research is heading in the context of reproductive biology research.

KEY WORDS: *autophagy, oocyte, sperm, embryo*

Introduction

Autophagy is an old term describing a cellular pathway by which cellular constituents are brought to lysosomes for degradation (Yang & Klionsky 2010). Christian de Duve, who was awarded a Nobel Prize for his work on the discovery of lysosomes, first employed the term when he observed intracellular vesicles encircling cytoplasmic materials (Deter & De Duve 1967; Yang & Klionsky 2010). This led to the realization that cells degrade their own macromolecules by delivering them to double-membrane vesicles, called autophagosomes, which then proceed to fuse with lysosomes. The fate of the degraded macromolecular constituents does not end in lysosomes; instead, the building blocks are salvaged and recycled. Thus, autophagy is considered a constructive cellular pathway necessary for the maintenance of cell functions. Three types of autophagy have been characterized to date: chaperone-mediated autophagy, macroautophagy, and microautophagy (Glick *et al.,* 2010). In this review, we focus on macroautophagy, which involves the initial sequestration of cytoplasmic materials into autophagosomes, as this process has been the focus of interest in physiology and disease research in recent years (Mizushima 2007; Yang & Klionsky 2010; Boya *et al.,* 2013).

Macroautophagy: a brief overview

Under a transmission electron microscope (TEM), the initiation of autophagy is distinguished by the formation of a phagophore, a horseshoe-shaped double-membranous structure incompletely encircling a target area within a cell (Klionsky *et al.,* 2012). A phagophore then expands by recruiting more lipid molecules and forms a complete vesicular structure, the autophagosome. When autophagosomes fuse with lysosomes, the lytic pathway ensues with the action of lysosomal enzymes. The inner membrane is also lysed during this process, and thus autophagolysosomes no longer exhibit the typical double-membrane morphology under TEM (Mizushima 2007; Klionsky *et al.,* 2012). Lipid molecules required for the formation of autophagosomes are provided mainly by the endoplasmic reticulum (ER), but other structures, such as the Golgi apparatus, mitochondria, and plasma membrane, also contribute (Tooze & Yoshimori 2010; Ravikumar *et al.,* 2010).

Many components of the autophagy pathway have names that begin with Atg, indicating autophagy (Boya *et al.,* 2013). Two main ubiquitin-like protein conjugation systems, Atg12 and Atg8, are involved in phagophore expansion and complete formation of autophagosomes (Geng & Klionsky 2008). Atg12 is covalently conjugated to Atg5 by a two-step process, which involves Atg7 and Atg10. The Atg12-Atg5 complex is then joined by Atg16, and this complex is required during the initial steps of phagophore expansion. Atg8, also called LC3, undergoes a reversible lipidation, rather than a covalent modification, with proteins. LC3-I is the cytosolic form of this protein prior to lipidation, and it is converted by the action

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Abbreviations used in this paper: ER, endoplasmic reticulum.

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of the Atg12-Atg5-Atg16 system to the phosphatidylethanolamine (PE)-conjugated form, LC3-II (Geng & Klionsky 2008). LC3-II proteins are incorporated into the autophagosomal membrane and remain there until they are cleaved by Atg4 and recycled (Geng & Klionsky 2008). In mammalian systems, the accumulation of LC3-II is widely used as a monitoring tool of autophagic activation (Klionsky *et al.,* 2012). Atg6, also known as Beclin1, is a subunit of the class III phosphoinositide 3-kinase (PI3K) complex, and it directly interacts with an anti-apoptotic protein, Bcl-2. Beclin1 is involved in induction of autophagy as a component of various PI3K complexes, and is also recognized as a seminal protein in bridging autophagy and apoptosis at the ER (Chang *et al.,* 2010; Wirawan *et al.,* 2012).

As for inducers of autophagy, "suboptimal environments" of a diverse nature are indicated (Yang & Klionsky 2010; Boya *et al.,* 2013). From the perspectives of individual cells and organisms, such inducers include, but are not limited to, starvation, lack of growth factors, adverse temperatures, toxic substances, reactive oxygen species (ROS), hypoxia, and crowding. The most well-established signaling regulator of autophagy is the mammalian target of rapamycin (mTOR) (Kapahi *et al.,* 2010). mTOR-containing signaling complexes work as integrators of cell growth by regulating protein translation, ER stress, metabolism, autophagy, and other processes (Kapahi *et al.,* 2010). Activation of mTOR signaling is achieved by many factors including amino acids, growth factors, and hormones, whereas its activation is inversely correlated with autophagy which is activated during suboptimal conditions of low nutrients and energy sources (Glick *et al.,* 2010; Kim *et al.,* 2011). Suppression of autophagy by mTOR involves phosphorylation of Atg1(Ulk) complex, a complex known to be required for the induction of autophagy (Kim *et al.,* 2011; Boya *et al.,* 2013). In contrast, AMP activated protein kinase (AMPK) promotes autophagy by phosphorylating Atg1 at a different site (Kim *et al.,* 2011).

Gene deficient mouse models: physiological roles first revealed

Yeast is the prototypical model organism in autophagy research, as there is high homology between the functional machinery of mammalian autophagy proteins with those of yeast (Boya *et al.,* 2013). The list of mammalian Atg homologs and associated genes of autophagy has been expanding, and the role of each Atg protein at a cellular level has been extensively investigated using mutant yeast and mammalian cell lines (Mizushima 2007). At the whole organism level, several gene-targeted mouse models have been produced and characterized (Yue *et al.,* 2003; Kuma *et al.,* 2004; Komatsu *et al.,* 2005; Marino *et al.,* 2007; Cann *et al.,* 2008; Marino *et al.,* 2010). As crucial upstream components of autophagic activation, Atg5 and Atg7 knockout animals were the first two models that demonstrated essential physiological roles for autophagy in the survival of neonates (Kuma *et al.,* 2004; Komatsu *et al.,* 2005). Atg5$^{-/-}$ pups failed to thrive after birth and died within several hours of birth (Kuma *et al.,* 2004). Atg7$^{-/-}$ mice were generated by conditional targeting of the floxed Atg7 gene with a Zp3-CRE transgenic mouse line, and they exhibit a very similar phenotype to Atg5$^{-/-}$ mice at birth (Komatsu *et al.,* 2005). These studies showed that autophagy is highly activated within several hours of birth in the muscles of neonates to provide immediate backup energy and nutrients to working muscles during a catastrophic change of environment at

birth (Schiaffino *et al.,* 2008). Autophagic activation is observed mainly in the liver and skeletal muscle in neonates, reflecting the fact that autophagy is required to cope with any sudden need for an independent energy source after being severed from placental circulation (Kuma *et al.,* 2004; Komatsu *et al.,* 2005; Schiaffino *et al.,* 2008). Beclin1-deficient embryos exhibit early lethality around E7.5, and this phenotype is associated with increased cell death (Yue *et al.,* 2003). Studies using Beclin1$^{-/-}$ and Atg5$^{-/-}$ embryonic stem (ES) cells further showed that these mutant ES cells fail to cavitate during embryoid body morphogenesis (Qu *et al.,* 2007). Thus, a role for autophagy in the clearance of apoptotic cells during cavitation was demonstrated. Other mouse models harboring tissue-specific deletion of Atg genes have been thoroughly described in recent review articles, along with their implications for broader disciplines (Cecconi & Levine 2008; Hale *et al.,* 2013).

Autophagy in reproduction: from germ cells to implantation

Death of germ cells in the prenatal ovary

In mammalian species, mechanisms of germ cell death function to maintain a finite follicular reserve in the ovary (Tam & Snow 1981). In the mouse ovary, the germ cell number steeply decreases after birth, and a gradual further reduction is noted in prepubertal and adult ovaries when atresia of primordial follicles is ongoing (Tilly 2001; Rodrigues *et al.,* 2009). In recent years, autophagy has been identified as one way of reducing the reserve of primordial follicles in addition to apoptosis in rodents (Lobascio *et al.,* 2007; Escobar *et al.,* 2008; Rodrigues *et al.,* 2009). When fetal mouse oocytes isolated at E15.5 were cultured *in vitro*, more than half showed signs of apoptosis within 3–4 days in culture (Lobascio *et al.,* 2007). Among these, a subset of oocytes contained membrane-bound cytoplasmic vesicles, indicating that autophagy was also activated. Most of the cultured fetal oocytes showed positive Beclin1 staining (Lobascio *et al.,* 2007). In the mouse ovary, increased autophagy along with acidification of lysosomes are implicated in the reduction of germ cell number after birth (Rodrigues *et al.,* 2009). A study using Atg gene-deficient mouse models presented evidence that the initial pool of primordial germ cells requires full-fledged autophagy for survival. On postnatal day 1, both Beclin1$^{+/-}$ and Atg7$^{-/-}$ mouse ovaries showed strikingly reduced numbers of germ cells (Gawriluk *et al.,* 2011). These studies collectively indicate that autophagy is involved in the survival of primordial germ cells in the prenatal period and also during the gradual reduction of the primordial follicle pool from birth through to adulthood in mice. The exact molecular mechanisms underlying these observations warrant further investigation.

Stress on the male reproductive system: temperature effect and beyond?

Whether autophagy is involved in the process of spermatogenesis is unknown. However, temperature has been identified as one factor that modulates autophagy in mammalian male germ cells (Gallardo Bolanos *et al.,* 2012; Zhang *et al.,* 2012). Heat stress causes male germ cell loss in the mouse testes, primarily via apoptosis (Yin *et al.,* 1997). A recent report has shown that heating of mouse testes is accompanied by evidence of autophagic activation, and autophagy seems to be implicated in enhancing the rate of apoptotic germ cell loss (Zhang *et al.,* 2012). Cold

temperatures are also recognized as an inducer of autophagy in mammalian sperm (Gallardo Bolanos *et al.,* 2012). Ethanol and formaldehyde have been shown to increase autophagy in rat Sertoli cells (Eid *et al.,* 2012; Han *et al.,* 2013). However, the potential roles that autophagy plays in developmental and pathological aspects of spermatogenesis remain to be identified and require further investigation.

Allophagy: breaking down paternally inherited organelles

Upon activation by the widely identified inducers of autophagy, such as oxidative stress, metabolic starvation, heat, and infection, specific subcellular organelles could succumb to autophagy (Bernales *et al.,* 2007; Kim *et al.,* 2007; Kraft *et al.,* 2008; Klionsky *et al.,* 2012). Terms to describe such selective types of autophagy include ER-phagy, ribophagy, and mitophagy (Klionsky *et al.,* 2012). Studies using *Caenorhabditis elegans* (*C. elegans*) revealed that mitophagy, in particular, is operative during fertilization when the paternal genome and mitochondria enter oocytes (Al Rawi *et al.,* 2011; Sato & Sato 2011). These studies showed that the selective degradation of paternal mitochondria by LC3-dependent autophagy, now termed allophagy, is an essential process for successful fertilization in *C. elegans* (Al Rawi *et al.,* 2011; Sato & Sato 2011). In mice, however, autophagy does not seem to participate in the removal of paternal mitochondria (Luo *et al.,* 2013).

Post-fertilization autophagy and embryonic development

Atg5$^{-/-}$ or Atg7$^{-/-}$ pups do not survive beyond a day after birth, and are thus not available to study the impact of the absence of autophagy in adult reproductive phenotypes (Kuma *et al.,* 2004; Komatsu *et al.,* 2005). Mice carrying the floxed Atg5 gene crossed with Zp3 promoter-drive CRE transgenic mice have been useful in examining the role of autophagy in early embryonic development (Tsukamoto *et al.,* 2008). Tsukamoto and colleagues first showed that autophagy is highly activated in oocytes just after fertilization, from 1- to 4-cell stages of development, by using GFP-LC3 transgenic mice (Tsukamoto *et al.,* 2008). GFP-LC3 transgenic mice are widely used as an *in vivo* model to visualize autophagy,

as this line expresses the GFP-LC3 fusion gene under a ubiquitously active strong promoter (Mizushima *et al.,* 2004). Crossing Atg5$^{flox/-}$;Zp3-CRE female mice with Atg5$^{+/-}$ male mice have never produced pups bearing a complete deletion of Atg5, which indicates that complete deletion of Atg5 during the preimplantation period led to their early demise *in utero* (Tsukamoto *et al.,* 2008). When examined at preimplantation stages of development, Atg5$^{flox/-}$;Zp3-CRE females bred with Atg5$^{+/-}$ males had a subset of embryos that were arrested from the 8-cell stage and never reached the blastocyst stage (Tsukamoto *et al.,* 2008). A copy of the Atg5 gene from Atg5^{+} sperm is expressed during zygotic gene activation at the 2-cell stage, and was able to partially rescue the phenotype of developmental arrest in Atg5-deficient oocytes after fertilization (Tsukamoto *et al.,* 2008). Autophagy-deficient preimplantation embryos showed a reduction in the rate of overall protein synthesis, and this observation suggested a role for autophagy in recycling building blocks from old proteins for new protein synthesis (Tsukamoto *et al.,* 2008). In porcine embryos, several maternal mRNAs were shown to remain at higher levels when an inhibitor of autophagy, 3-methyladenine (3-MA), was given to 2-cell- or 4-cell-stage embryos (Xu *et al.,* 2012). This result is also indicative of a role for autophagy in breaking down maternal macromolecules after fertilization. Further molecular mechanisms of autophagy governing the turnover of macromolecules in preimplantation embryos are yet to be investigated.

Environmental influence on embryonic autophagy

In the female reproductive tract, embryos are influenced by the surrounding environment and by the maternal health status. In this regard, elevated levels of glucose and endocannabinoids have been identified as inducers of autophagy in preimplantation embryos (Adastra *et al.,* 2011; Oh *et al.,* 2013). In the mouse uterus, the levels of endocannabinoids oscillate before and after implantation (Schmid *et al.,* 1997), and adequate levels of these lipid mediators are also important for the implantation competence of blastocysts (Wang *et al.,* 2003). When an endocannabinoid analog was given to pregnant mice prior to implantation in order to achieve a higher
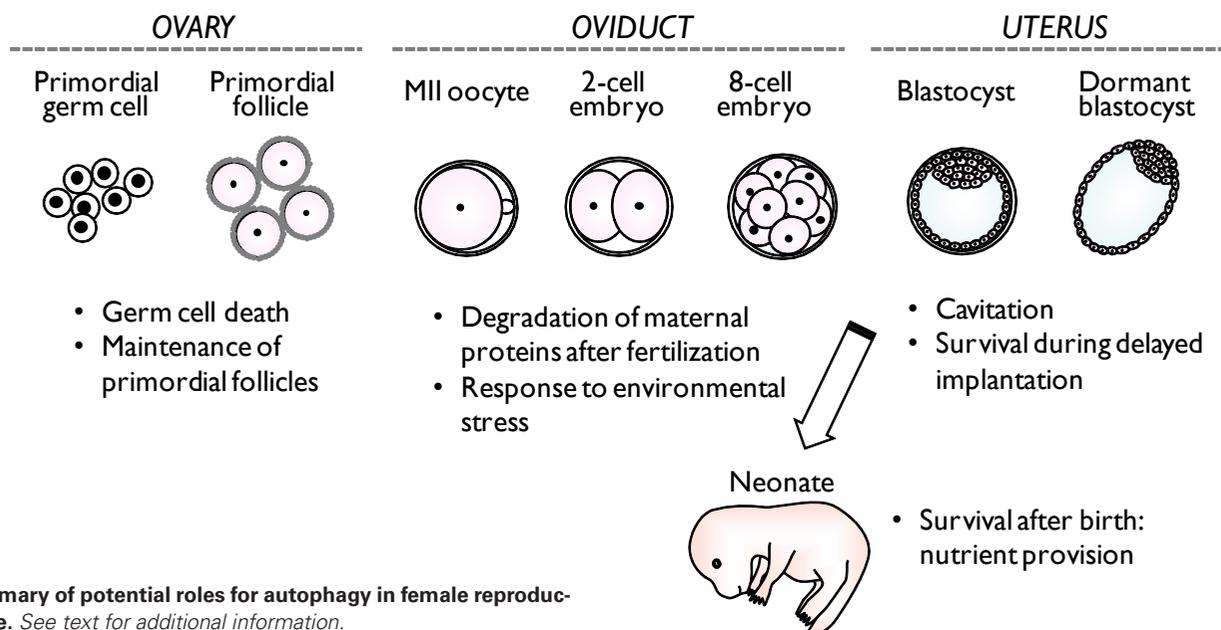

Fig. 1. Summary of potential roles for autophagy in female reproduction in mice. *See text for additional information.*

than normal level of endocannabinoids, it induced autophagic activation in blastocysts (Oh *et al.,* 2013). In a streptozotocin-treated diabetes model, oocytes and preimplantation embryos showed increased autophagic responses (Adastra *et al.,* 2011). Although the condition of hyperglycemia produces differential effects on autophagy in various tissues and organs (Fujitani *et al.,* 2009), embryos seem to respond to increased level of glucose *in vivo* and *in vitro* by activating autophagy (Adastra *et al.,* 2011). In the cases of both endocannabinoids and glucose, apoptosis is simultaneously upregulated, suggesting a link between these two death pathways (Adastra *et al.,* 2011; Oh *et al.,* 2013). Whether autophagy is further implicated as a response mechanism of mammalian oocytes and preimplantation embryos to other environmental insults still remains to be explored.

When implantation is delayed: survival or death by autophagy

Blastocysts implant when the uterus reaches the receptive phase under the regulation of ovarian progesterone and estrogen (Dey & Lim 2006). A small amount of estrogen is critical for activating the blastocyst and preparing the uterus for implantation (Dey & Lim 2006). When this estrogen is removed from pregnant mice prior to implantation, blastocysts cannot initiate implantation and enter "dormancy" (McLaren 1968). These dormant blastocysts are known to survive *in utero* for weeks in a free-floating state while the overall metabolic rate is reduced (Van Blerkom *et al.,* 1978; Weitlauf 1974). Delayed implantation in rodents naturally occurs during lactation, whereas in marsupials, mustelids, and several other species, this phenomenon is an obligate process during pregnancy (Renfree & Shaw 2000). Recently, autophagy has been suggested as a mechanism for survival in dormant blastocysts of experimentally induced delayed implanting mice (Lee *et al.,* 2011). Dormant blastocysts showed heightened levels of autophagy compared to blastocysts reactivated by estrogen for implantation (Lee *et al.,* 2011). As mentioned above, autophagy is considered a survival mechanism during stressful conditions, but its persistent activation could lead to cell death by further activating other death mechanisms (Shintani & Klionsky 2004). When the period of dormancy was maintained for a longer period of time, embryos became more vulnerable to death when autophagy was inhibited by the injection of 3-MA to the delayed implanting mice. 3-MA-exposed dormant blastocysts also showed higher rate of apoptosis than control embryos (Lee *et al.,* 2011). Thus, the involvement of autophagy in sustained survival of dormant blastocysts was evident, as well as a damaging effect of prolonged autophagy on embryonic survival (Lee *et al.,* 2011). It would be interesting to find out whether autophagic activation also occurs during obligate diapause in other species (Renfree & Shaw 2000) or in specific gene-targeted mice with a phenotype of deferred implantation (Song *et al.,* 2002).

Conclusion: conjecturing further implications of autophagy in reproduction

In summarizing the recent literature describing the potential roles for autophagy in various aspects of early pregnancy and development, it has become evident that many questions remain to be addressed. Where any kind of stress is implicated, autophagy may contribute either to the defense or offense in the maintenance of cell survival and homeostasis (Fig. 1). Whether the modulation of autophagy may be beneficial for improving the quality of embryos

in assisted reproductive technology is also in the pipeline for further investigation. Autophagy has also been identified as a mechanism utilized during dynamic reconstruction processes occurring within a cell population, such as embryonic cavitation and patterning (Qu *et al.,* 2007; Kawamura *et al.,* 2012). The impact of endocrine disruptors and other environmental toxins on autophagy of germ cells and embryos would also be an exciting topic of future research.

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