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Role of Autophagy in the Ovary Cell Death in Mammals

M.L. Escobar, O.M. Echeverría and G.H. Vázquez-Nin

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http://dx.doi.org/10.5772/54777

1. Introduction

The process of cell death is implicated in several other processes, such as tissue homeostasis, embrionary development, and the elimination of unwanted cells. Programmed cell death is classified first according to the morphological characteristics of the cells observed, and then by the molecular machinery involved in the process. To date, programmed cell death is known to involve apoptosis and autophagy, two processes with different morphological and molecular characteristics.

In mammals, germinal cells are contained in the follicles, specialized structures that develop through several phases of maturation. During follicular growth, cell proliferation and cell death are present simultaneously. During ovarian follicular development, the follicles not selected for the ovulation process are physiologically eliminated. Several studies have shown that in mammalian ovaries follicular atresia is governed by granulosa cell apoptosis (Manabe et al., 2004); however, recent evidence from studies of pre-pubertal (Ortíz et al., 2006; Escobar et al., 2008 and 2010) and adult rats (Escobar et al., 2012) shows that autophagy is an alternative route taken by some germinal cells to induce follicular atresia in the ovary. The emerging importance of autophagy in cellular elimination in the mammalian ovary is a very interesting development.

2. Autophagy as a cell death program

Autophagy is an evolutionary process that eliminates damaged cellular proteins and organelles (Ferraro and Cecconi, 2007). It also plays an important role in bioenergetic management during periods of starvation (Othman et al., 2009), and is the major pathway for the degradation and recycling of intracellular contents.

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The autophagy process occurs at a basal level in normal cells under certain adverse conditions, such as starvation, low oxygen levels, and growth factor withdrawal, among others. Under these conditions, autophagy functions as a cytoprotective program that helps maintain cellular homeostasis by recycling the cytoplasmic contents. Another function of autophagy is to eliminate damaged organelles so as to maintain correct cellular functions. Thus, all the features of autophagy in cells perform cytoprotective functions.

In eukaryotic cells, autophagy has been characterized according to the way in which it is carried out: microautophagy, chaperone-mediated autophagy, and macroautophagy (Klionsky, 2006; Massey et al., 2005). In microautophagy, the lysosomal surface directly engulfs the cytoplasm that is to be degraded (Figure 1A). In chaperone-mediated autophagy, the material to be degraded crosses the lysosomal membrane directly (Figure 1B), while macroautophagy, commonly referred to simply as autophagy, is characterized by a double-membrane vesicle (Figure 1C) that encloses (sequesters) organelles and portions of the cytosol (reviewed in Yang and Klionsky, 2009).
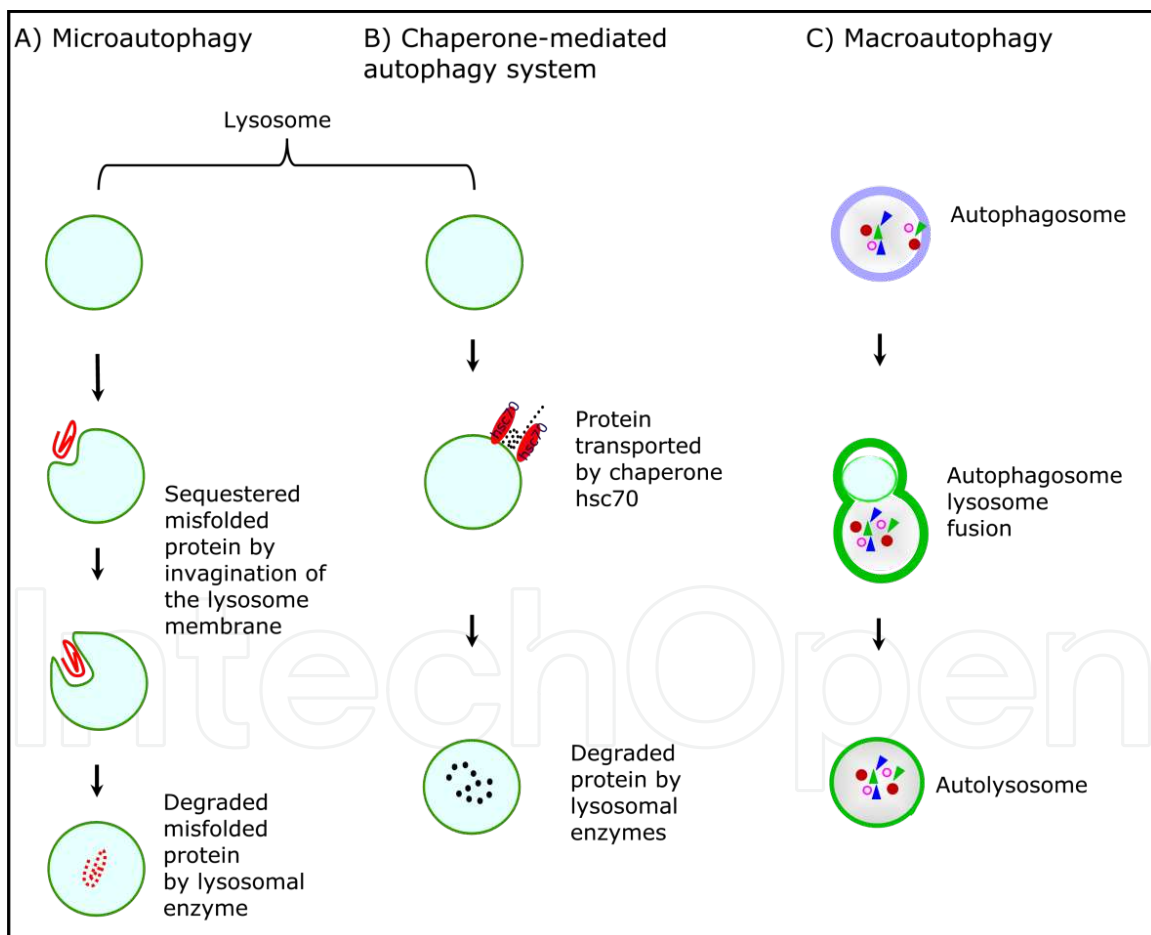

Figure 1. Schematic drawing of autophagic routes. A) Microautophagy: the cytoplasmic contents is directly enclosed by direct invagination from the lysosomal membrane. B) Chaperone-mediated autophagy: the components to be degraded are selectively transported toward the lysosome after interacting with the chaperone hsc70. C) Macroautophagy (commonly called autophagy): the autophagosome containing various cytosolic proteins fuses with lysosomes; subsequently, the contents of the autophagosome is degraded by the lysosomal enzymes.

Morphologically, autophagy is evidenced by the presence of autophagic vesicles, character‐ized by a double membrane structure. In mammalian cells, autophagy is initiated by the formation or elongation of the isolation membrane, also called a phagophore. Autophagy entails a sequence of events that includes sequestering the cytoplasmic contents in the double membrane vesicle. Once formed, the autophagosomes are conducted toward lysosomes to constitute the autolysosomes in which the sequestered cellular material is degraded. To avoid degradation itself, the lysosomal membrane is enriched by specific membrane proteins called lysosomal associated membrane proteins (Lamp1 and Lamp2) (Fukuda, 1991).

Though autophagy was first identified in mammalian cells, its molecular characteristics were discovered in yeast. Identification of the participation of the autophagy *Atg* genes and the subsequent documentation of their homologues in mammals (Yang and Klionsky, 2009) made it possible to determine the molecular machinery involved in the formation and maturation of autophagosomes. TOR kinase is considered an important element in autophagy. When TOR is inhibited under stress conditions, autophagy is induced upon the activation of this kinase; then Atg13 is quickly dephosphorylated, causing a higher affinity for Atg1 and Atg17 that results in an increase in the activity of the Atg1 protein kinase (Kamada et al., 2000; Kabeya et al., 2005). Atg1 kinase plays a pivotal role in controlling autophagy, and its activity is required for the switch from cytoplasm formation to vacuole targeting vesicles (Cvt) and the emergence of autophagosomes (Scott et al., 1996; Matsuura et al., 1997). In mammals, the microtubule-associated protein 1 light chain 3 (LC3) homolog of the Atg8 yeast is an important protein involved in the autophagy process. LC3 is present in autophagosomes and is synthetized in an inactive form called LC3-I, which is later converted into an active membranous form: LC3-II, the lapidated form, which means that it bonds to phosphatidylethanolamine (Wang et al., 2009; Maiuri et al., 2007). LC3 is lapidated via an ubiquitylation-like system that is targeted to the early autophagosome membrane (Kabeya et al., 2004).

During autophagy induction, LC3 is converted from the LC3-I to the LC3-II form. It has been suggested that the amount of LC3-II correlates to the number of autophagosomes present. Autophagy is involved in stress response, developmental remodeling, organelle homeostasis, and disease pathophysiology, and this process may also be used as a host-cell response against bacteria and viruses (reviewed in Kindergaar, 2004).

Additionally, it has been suggested that the effects of autophagy can be either deleteri‐ous or protective, depending on the specific cellular context and the stage of the patholog‐ical process (reviewed in Rubinsztein et al., 2005). At present, we know that autophagy functions as a form of programmed cell death, classified as type II. One essential differ‐ence between physiological autophagy and autophagic cell death is that the levels of autophagy in dying cells are excessive. The role of autophagy as a process of cell death is interesting because it has been observed under certain experimentally manipulated systems. When the pro-apoptotic proteases are inhibited, autophagic levels increase (Yu et al, 2004). Some neurodegenerative diseases, such as Parkinson's disease, have also been associated with the autophagic cell death process (Anglade et al., 1997), and autophagic cell death has been observed as well in remodeling tissues.
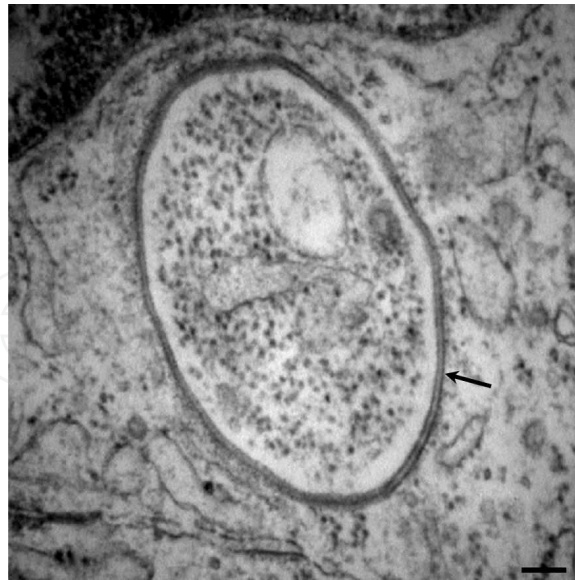
Figure 2. Electron microscopy showing an autophagosome. The arrow points to the two lipid bilayers that surround the cytoplasmatic content. Scale bar 100 nm.

Morphological evidence of autophagy has been found in electron microscopy studies that have shown vesicular structures surrounded by two lipid layers known as autophagosomes (Figure 2). Autophagosomes may contain cytoplasm and cytoplasmic organelles, such as mitochondria and peroxisomes, etc. Autophagy can be evidenced by the immune-microscopic localization of the proteins involved in this process, including LC3/Atg8, or the Lamp1 proteins (Figure 3).
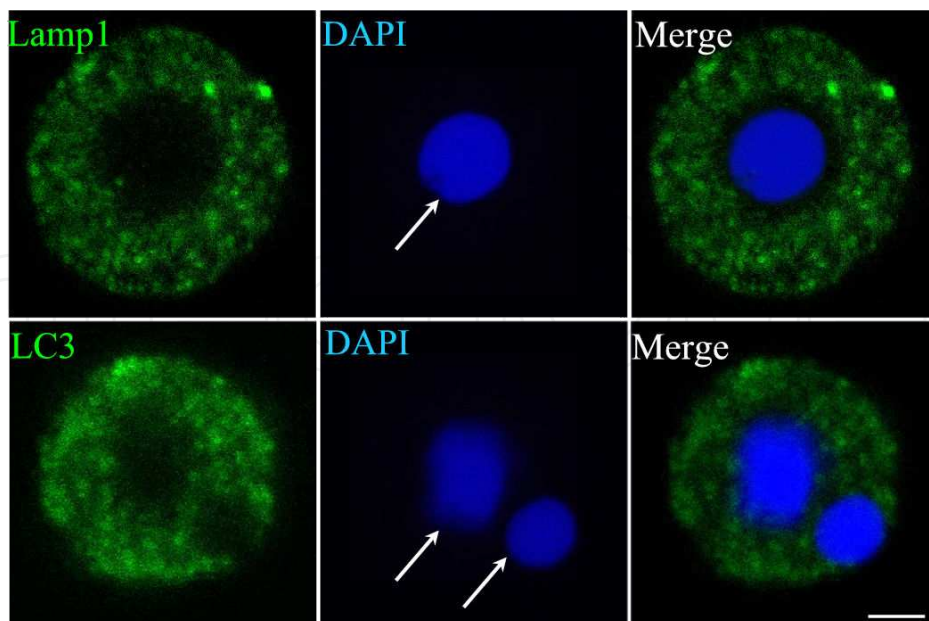
Figure 3. Immunodetection of Lamp1 and LC3. Confocal observations of cellular fractions: DAPI is evident in the nucleus (arrows). The green punctuate fluorescence distinguishes the cytoplasmatic localization of the Lamp1 and LC3 proteins. Scale bar 10 microns.

The role of autophagy as a process of cell death in diverse pathologies, including cancers, has been evaluated widely, but the results from the different studies are somewhat controversial, because at first autophagy functions as a pro-survival strategy, as in the case of tumor cells under certain stimuli; for example, low oxygen or a lack of nutrients (Lefranc et al., 2007). But cancer cells can also use autophagy as a strategy for evading cell death and a means of adapting to an adverse environment. On the other hand, under certain conditions, tumor cells use autophagy as a mechanism of cell death.

3. Follicular atresia

In mammals, the ovary is a paired organ whose principal functions are oocyte production and hormone synthesis. Structurally, the ovary is made up of a medulla and a cortical region where the follicles are generally located. The mammalian ovary is the site of oocyte maturation, which takes place inside a complex structure, the follicle, which is made up of a germinal cell –the oocyte– surrounded by somatic granulosa cells.

Follicles go through several steps before attaining maturation. During this process, various morphological and functional changes occur in the follicle that have led to its development being classified in stages: primordial, primary, secondary, early antral, and antral, according to the number of granulosa layers that surround the oocyte, the size of the follicle, and the presence of the antrum.
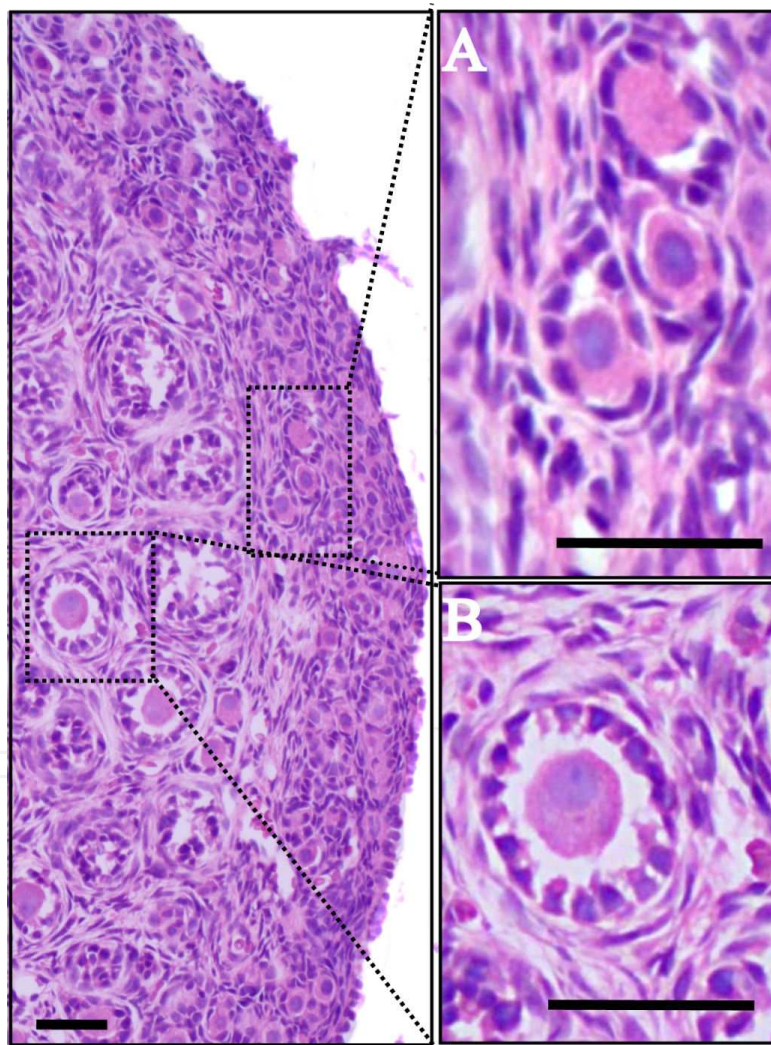
Primordial follicles consist of a single flattened cell layer surrounding the oocyte (Figure 4A). In a primary follicle, the granulosa cells around the oocyte acquire a cubical shape in a single-layer cell (Figure 4B). Secondary follicles are characterized by the presence of two or more granulosa cell layers (Figure 5). In this stage, the oocyte increases in size and the granulosa cell layers emerge through intensive proliferation that leads to the formation of the theca interna cell layer (Knight and Glister, 2006). In the secondary follicular phase, the specialized structure associated with the oocyte, called the zona pellucida, is completely discernible. Early-antral and antral follicles (Figure 6) are characterized by the development of a fluid-filled space among the granulosa cells that forms the antral cavity. Granulosa cells are in intercellular contact with neighboring cells via gap junctions (Figure 7), which allow metabolic exchange and the transport of molecules between follicular cells.

During the follicular maturation process, only a few follicles are selected for ovulation, while more than 99% are eliminated via a process denominated follicular atresia (Kaipia and Hsueh, 1977). In ovarian physiology, follicular atresia is a key mechanism for removing the follicles that are not selected for ovulation.

Numerous morphological and biochemical studies have revealed the frequent participation of apoptosis in follicular atresia; indeed, apoptosis came to be considered the cellular route that underlies this process. In caprine ovaries, ultrastructural changes in the granulosa cells show the classic morphological characteristics of apoptosis (Sharma and Bardwaj, 2009). Several pro-apoptotic factors have been identified in granulosa cells, including the FasL-Fas

system, TNF-a, and members of the Bcl-2 family of proteins (reviewed in Matsuda et al., 2006). In fact, follicular atresia has been attributed to the alteration of granulosa cells, since studies have demonstrated that these cells synthetize molecules that are essential for follicular maintenance and growth. Furthermore, the death of granulosa cells due to an apoptotic process results in follicular elimination (Matsuda et al., 2012).
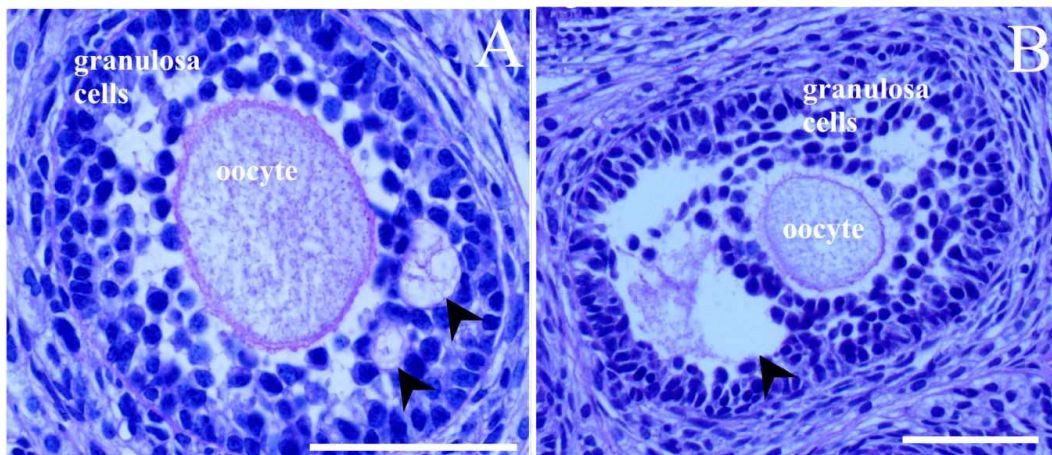

Figure 4. Histological images of a rat ovary. The dotted squares are magnified in the right panel. A) Primordial follicles with flattened pre-granulosa cells. B) Primary follicle with a single layer of cubical granulosa cells. Scale bars 20 microns.
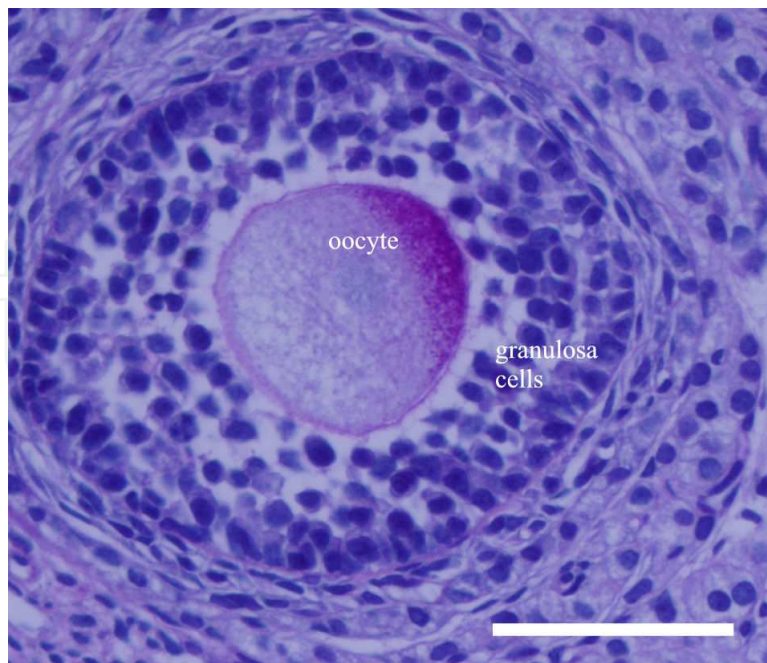
Figure 5. Secondary follicle. The oocyte is surrounded by several layers of granulosa cells. Scale bar 50 microns.


Figure 6. Histological images of a rat ovary. A) Early antral follicle: The arrows show the growing spaces that will form the antral cavity. B) Antral follicle: The arrow points to the antral cavity. Scale bars 50 microns.

4. Granulosa cell death via autophagy

While numerous studies have shown that the process of granulosa cell death is carried out mainly by apoptosis (Feranil et al. 2005; Hurst et al. 2006; Matsuda-Minehata et al. 2006, Lin and Rui, 2010), in some conditions autophagy may be induced in granulosa cells by the process of apoptosis, a process that in rat ovaries is gonadotropin-dependent. These results suggest that both apoptosis and autophagy are gonadotropin-dependent in rat ovaries, and that both

processes are involved in regulating granulosa cell death during ovarian follicular develop-
ment and atresia (Choi et al., 2010). Despite the obvious differences between apoptosis and
autophagy, they are now thought to represent points on a continuum of mechanisms of cell
death, because the induction of apoptotic cell death is regulated by the process of autophagy.
Autophagic cell death is induced by inhibiting the accumulation of autophagosomes in various
carcinoma cells, which suggests that the autophagic process may prevent apoptotic death.
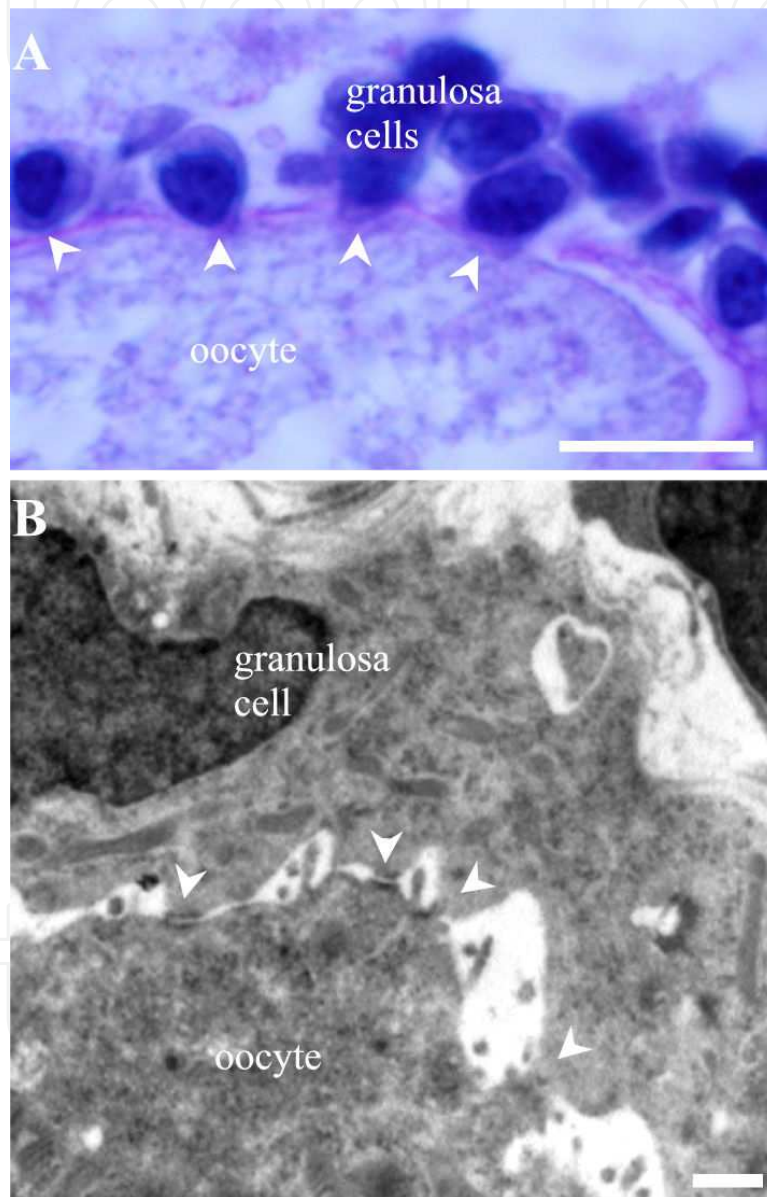
Figure 7. Gap junctions between granulosa cells and the oocyte. A) Optical micrograph showing granulosa cells in strong contact with the oocyte (arrowheads). B) Electron micrograph showing the gap junctions of granulosa cells and the oocyte (arrowheads). Scale Bars: A-50 microns; B-100 nanometers.

In order to investigate the involvement of autophagy in folliculogenesis, and its correla-
tion with apoptosis, isolated rat granulosa cells from immature animals primed with

pregnant mare serum gonadotropin were studied. LC3 and autophagic vacuoles were used as markers of autophagy, while cleaved caspase-3 served as the marker of apoptosis. In these conditions, LC3 was expressed by isolated granulosa cells in all developmental stages, and showed a similar expression pattern to the cleaved caspase-3. These results indicate that autophagy is induced in granulosa cells during folliculogenesis in correlation with apoptosis (Choi et al., 2010).

In the human ovary, lectin-like oxidized low-density lipoprotein (LOX) is localized in regressing antral follicles. Treatment with oxLDL (oxidized low-density lipoprotein) causes autophagy in granulosa cells. The process of cell death is characterized by the reorganization of the actin cytoskeleton, abundant vacuoles, autophagosome formation, the absence of apoptotic bodies, and cleaved caspase-3; thus, the reduction of granulosa cells may be mediated by autophagy (Duerrschmidt et al. 2006).

5. Oocyte cell death via autophagy

During the first two trimesters of pregnancy, the number of oocytes in human fetal ovaries increases from approximately 7,200 to 4,933,000 (Mamsen et al., 2011). However, oocyte death begins during the fetal and perinatal stages and continues in newborn, pre-pubertal (Hulas-Stassiak and Gawron, 2011) and adult mammals.

Autophagy is not only a process of cell death; it is also required for cells to survive in conditions of nutrient depletion (Han et al. 2011). Moreover, in murine ovaries it is a cell survival mechanism that maintains the endowment of female gem cells prior to establishing primordial follicle pools (Gawriluk et al. 2011). Several genes have been described as regulators of autophagy; many of them have been conserved from yeast to mammals. In vertebrates, autophagic defects may be lethal if the mutated gene is involved in the early stages of development. However, in different eukaryotes autophagy seems to be crucial during embryogenesis in a way that parallels apoptosis. The earliest autophagic event in mammalian development is observed in fertilized oocytes (Mizushima and Levine, 2010). The identification of *ATG* genes that mediate the initiation and assembly of autophagosomes and their fusion with lysosomes to form autolysosomes brought important advances in our understanding of the various functions of autophagy (Randall-Armant, 2011).

Thus, autophagy seems to be crucial during embryogenesis by acting in tissue remodeling, parallel to apoptosis (Di Bartolomeo et al., 2010). Studies in different organisms indicate that the autophagy pathway in the amoeba *Dictyostelium discoideum* is much more similar to that of mammalian cells than that of *S. cerevisiae*, despite its earlier evolutionary divergence. This indicates that in mammals the autophagic pathway is much older than was previously thought (King, 2012). MicroRNAs are involved in autophagy and are also important regulators of the crosstalk between autophagy and apoptosis (Xu et al. 2012).

ATG genes are also essential for the autophagic pathway in mammalian development (Mizushima and Levine 2010). The oocyte-specific deletion of Atg5, which removes the

maternal stores of this protein, produces oocytes that fail to develop past the eight-cell stage, thus demonstrating that autophagy is required during pre-implantation development (Randall Armant 2011). An important increase in the number of autophagosomes takes place immediately after fertilization, which shows the need for autophagosomes after fertilization, in all likelihood to destroy the existing proteins and provide amino-acids for subsequent development (Randall Armant 2011).

In 1-to-28-day-old –i.e., newborn to pre-pubertal– rats, numerous follicles undergo atresia and oocytes are eliminated by processes that include, simultaneously, features of both apoptosis and autophagy. Elements of apoptosis are present in adjacent sections of the same dying oocyte, in the form of active caspase-3 and DNA breaks, as well as large increases of the Lamp1 protein and acid phosphatase, which are present in autophagosomes (Escobar et al., 2008; Escobar et al., 2010). Studies carried out in adult rats have also demonstrated that in all phases of the estrous cycle oocytes die by processes involving features of apoptosis and autophagy simultaneously (Escobar et al., 2012). Morphological changes in atretic oocytes include vacuolization of the cytoplasm, condensation of the mitochondria and segmentation, altera-tions that are not involved in classic apoptosis (Devine et al. 2000). These analyses were carried out using classic markers of apoptosis, such as the TUNEL reaction that reveals DNA frag-mentation, immunolocalization of active caspase-3, and markers of autophagy like a large increase of acid phosphatase, lysosomal hydrolase, and immunodetection of Lamp1, a protein of the lysosomal membrane. These markers are located in the same regions of the oocyte's cytoplasm that present clear vacuoles which correspond to the autophagosomes that became visible using adjacent, semi-thin sections of the same oocyte (Escobar et al., 2008).

In newborn and pre-pubertal spiny mouse oocytes, follicular atresia was studied using markers of apoptosis like the TUNEL reaction, which demonstrate DNA fragmentation and active caspase-3, as well as with markers of autophagy, such as immunodetection of Lamp1. Numerous small clear vacuoles, autophagosomes and Lamp1 staining were found in all follicle types, especially in primordial and primary samples (Figure 8). Active caspase-3 and the TUNEL reaction were detected only in the granulosa cells, showing that both apoptosis and autophagy are involved in follicular atresia, and that these processes are both cell- and developmental-stage specific (Hulas-Stasiak and Gawron, 2011).

Follicular atresia has also been studied in fish ovaries during early and advanced stages of follicular regression. The main events assessed using light microscopy were splits in the zona radiata, yolk degradation and reabsorption, hypertrophy of follicular cells and the accumula-tion of autophagy vacuoles. Labeling for Bcl-2 and cathepsin-D was pronounced in follicular cells when they were involved in yolk phagocytosis. Immunofluorescence for Beclin-1 was significant in the follicular cells that often surround autophagic vacuoles during the advanced stages of follicular regression. TUNEL-positive reactions and immunostaining for Bax and caspase-3 showed the participation of apoptosis in advanced stages of follicular regression. These observations show that both autophagy and apoptosis are activated in some stages of follicular regression in fish ovaries (Morais et al., 2012). Inhibition of the increase of prolifer-ating cell nuclear antigen (PCNA) markedly reduces the apoptosis of oocytes and down-

regulates known pro-apoptotic genes, such as Bax, caspase-3, and TNFα, while up-regulating known anti-apoptotic genes like Bcl-2 (Xu et al. 2011).

Retraction of the prolongations of the granulosa cells that normally contacts the surface of the oocyte is one of the early signs of follicular atresia (Devine et al. 2000). Numerous unpublished observations by the authors of this chapter show that the microvilli of the oocyte are elongated after retraction of the prolongations of the granulosa cells during the process of atresia (Figure 9).
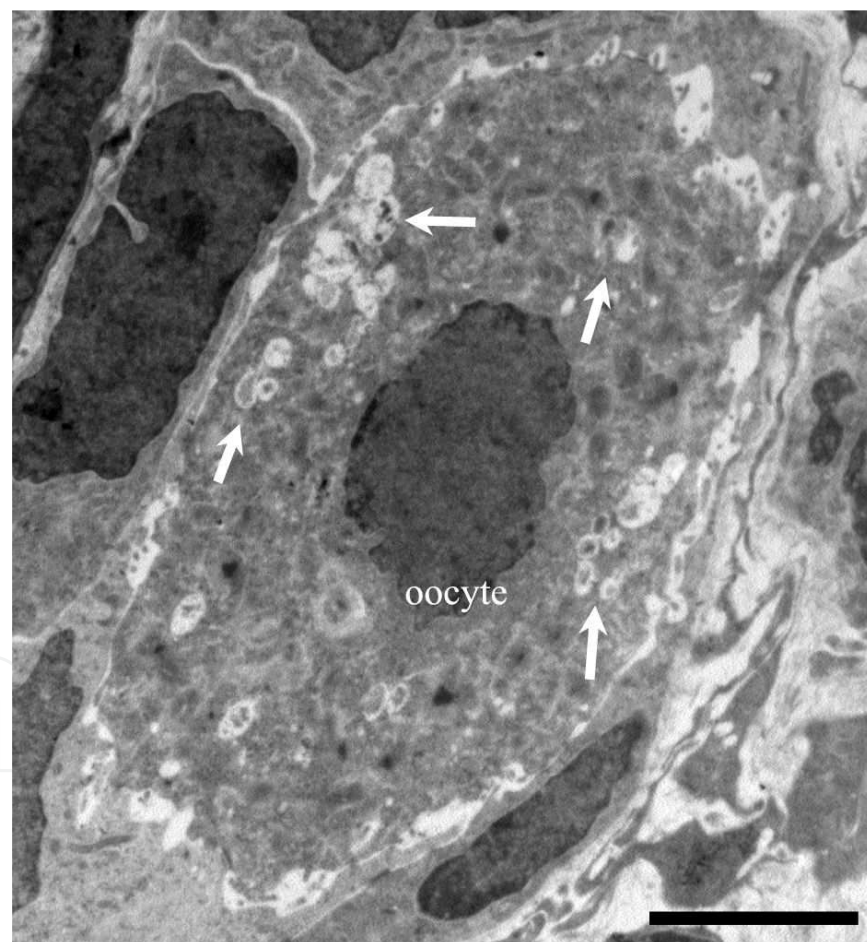
Figure 8. Primordial follicle. The cytoplasm has numerous vacuoles with cytoplasmic contents in different degrees of degradation. Scale bar 2 microns.
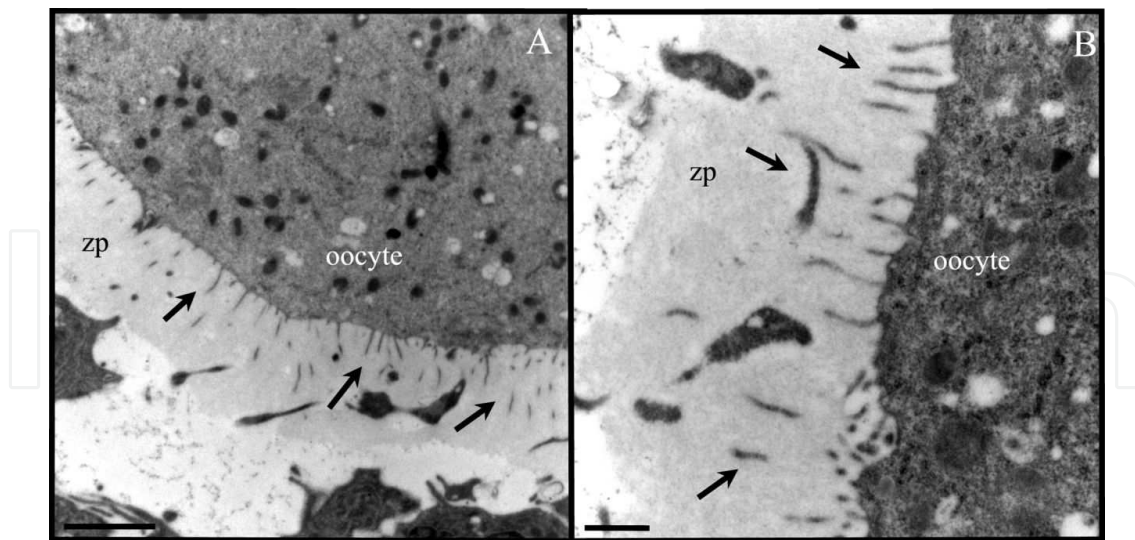
Figure 9. Retraction of the prolongation of the granulosa cells. The arrows point to several microvilli of the oocyte that are elongated after retraction of the prolongations of granulosa cells during atresia. Zp: zona pellucida. Scale bars: A-2 microns; B-500 nanometers.

6. Autophagic cell death in *corpus luteum* regression

The corpus luteum is a transitory ovarian structure formed by cells of the ovulated follicle (figure 10). After an initial proliferation of granulosa cells and closing of the antral cavity, capillaries and theca cells invade the region that was once the granulosa layer of the follicle. During its life span, the corpus luteum undergoes a period of rapid growth that involves hypertrophy, proliferation and differentiation of steroidogenic cells with extensive angiogenesis. After that, it engages in a large production of steroids. Growth factors including insulin-like factor, vascular endothelial growth factor and fibroblast growth factor are important for the development and completion of the dense network of capillaries during the formation of the corpus luteum (Berisha and Schams 2005). There is evidence to suggest that the luteinizing hormone, growth hormones and local regulators such as growth factors, peptides, steroids and prostaglandins are all important regulators of the luteal function. During early corpus luteum development, and up to the mid-luteal stage, oxytocin, prostaglandins and progesterone itself stimulate luteal cell proliferation and functioning, supported by the luteotropic action of several growth factors. High mRNA expression, protein concentration and localization of vascular endothelial growth factor, fibroblast growth factor and members of the family of insulin-like growth factors suggest that they play important roles in the maintenance of the corpus luteum. Progesterone regulates the length of survival of the corpus luteum (Berisha and Shams 2005). In addition, progesterone increases Bcl-2 expression in different stages of the estrous cycle. Treatment of luteal cells with progesterone and prostaglandin PGE2 for 24 hours decreased active caspase-3, while aminoglutethimide, spermine and staurosporine increased caspase-3 activity in luteal cells. These results suggest that progesterone concentrates

in luteal cells to protect against apoptosis, while disruption of steroidogenesis and the reduced ability of luteal cells to produce progesterone can induce cell death (Liszewska et al. 2005).
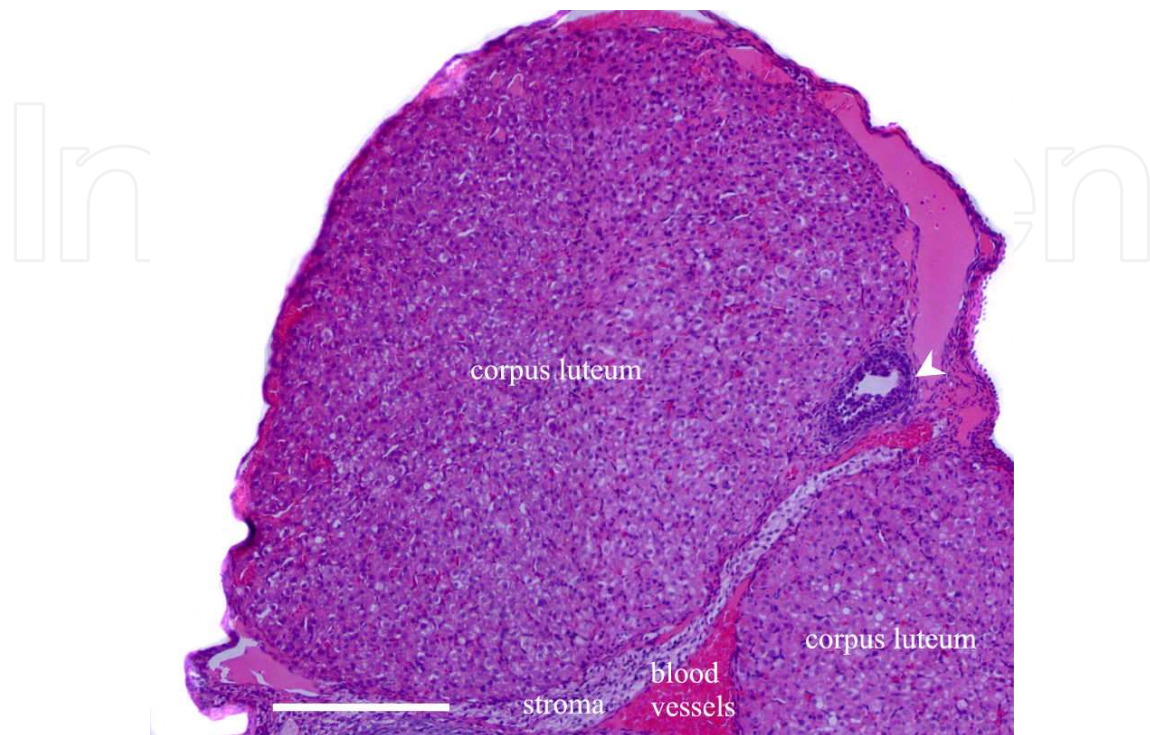
Figure 10. Corpus luteum from a rat ovary. The arrow points to a secondary follicle. Scale bar: 50 microns.

In non-fertile cycles, uterine release of prostaglandin (PG)F(2a) initiates a cascade of events that result in a rapid loss of steroidogenesis and destruction of the luteal tissue (Pate et al. 2012). Periodically, the corpus luteum regresses (luteolysis) and numerous luteal cells undergo cell death processes, mainly through apoptosis and autophagy.

Studies on the role of autophagy in corpus luteum regression have shown an increase of the protein microtubule-associated protein light chain 3 (LC3), a marker of autophagy. Apoptosis was evaluated by measuring cleaved caspase-3 expression (Choi et al. 2011). LC3 expression increases slightly from the early to the mid-luteal stage in steroidogenic cells. The expression levels of the membrane form of LC3 (LC3-II) also increase during luteal stage progression. In the same period, the expression of cleaved caspase-3 also increases. LC3-II expression rises, as do the levels of active caspase-3 in luteal cells cultured with prostaglandin F(2α), which is known to induce corpus luteum regression. These facts suggest that autophagy of luteal cells is directly involved in corpus luteum regression, and correlates with an increase of apoptosis (Choi et al. 2011). When autophagosome degradation by fusion with lysosomes was inhibited using bafilomycine A1 (Baf A1) increased apoptotic cell death. Moreover, inhibition of autophagosome formation using 3-methyladenine decreased apoptosis and cell death, suggesting that the accumulation of autophagosomes induces luteal cell apoptosis. The accumulation of autophagosomes increased apoptotic luteal cell death via an increase in the Bax/Bcl-2 ratio and subsequent caspase activation. Therefore, autophagy plays an important

role in regulating apoptotic luteal cell death by controlling the Bax-to-Bcl-2 ratio and the subsequent activation of caspases. These experimental results indicate that autophagy is involved in rat luteal cell death through apoptosis, and that it is most prominent during corpus luteum regression (Choi et al. 2011).

Luteal cell regression during the normal postpartum involution of the corpora lutea is characterized by a large increase in the number of lysosomes and the appearance of numerous double-walled autophagic vacuoles, which become evident under electron microscope cytochemistry (Paavola, 1978).

Compelling evidence indicates that both apoptotic and autophagic cell death programs are involved in corpus luteum regression in primates. Beclin-1, an autophagy-related protein, is involved in the relation between apoptosis and autophagy through interaction with the anti-apoptotic protein Bcl-2. In ovarian follicles, Beclin 1 has been found in the theca layer, but granulosa cells are negative. After ovulation, Beclin-1 is present in theca-lutein and granulosa-lutein cells. The expression of Beclin 1 is related to the functional and structural status of the corpus luteum, as it is a factor in cell survival and plays important roles in the life span of the human corpus luteum (Gaytán et al. 2008).

An endocrine type, voltage-activated sodium channel was identified in the human ovary and human luteinized cells. Whole-cell patch-clamp studies showed that the voltage-activated sodium channels in granulosa cells are functional and tetrodotoxin-sensitive. Luteotrophic hormone was found to decrease the peak amplitude of the sodium current within seconds. Treatment with hGC (human chorionic gonadotropin) for 24-48 hours suppressed not only the mRNA levels in voltage-activated sodium channels, but also the mean sodium peak currents and resting potentials. Tetrodotoxin preserves a highly differentiated cellular phenotype, whereas veratridine not only increases the number of secondary lysosomes but also leads to a reduced progesterone production. In luteinized granulosa cells in culture, abundant secondary lysosomes were evident in the regressing corpus luteum, suggesting a functional link between the voltage-activated sodium channel activity and autophagic cellular regression *in vivo* (Bulling et al., 2000).

Taken together, these data show that several factors are involved in corpus luteum regression. One type of factors includes the process of eliminating the different types of cells that form the corpus luteum, while other types of factors are those involved in destroying the structure of this transitory organ. The normal programmed cell death processes –apoptosis and autophagy– are involved in cell elimination in the corpus luteum. Most authors have found that the most frequent process of cell death is apoptosis; however, very detailed studies demonstrate that both processes are often present simultaneously, as in the case of cell elimination in other organs.

7. Conclusions

Recent years have seen interest grow in the different routes of cell death. Today, two types of programmed cell death are known: apoptosis and autophagy. Cell death in follicle structures

is a continuous event during the life of female organisms. Several studies have demonstrated the active participation of apoptosis in this process, but recent biochemical and morphological evidence has revealed the participation of autophagic cell death in oocyte elimination during this physiological process. In granulosa cell death and corpus luteum regression, experimental evidence has shown that autophagy is an active route in the process of cellular elimination. Future studies should test for different stimuli and molecular mechanisms involved in the autophagic cell death process in follicular atresia in vertebrates.

Acknowledgements

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