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The Role of Autophagy and Apoptosis During Embryo Development

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Additional information is available at the end of the chapter

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Abstract

Programmed cell death (PCD) and cell survival are two sides of the same coin. Autophagy and apoptosis are crucial processes during embryo development of Invertebrates and Vertebrates organisms, as they are necessary for the formation of a new organism, starting from a fertilized egg. Fertilization triggers cell remodeling from each gamete to a totipotent zygote. During embryogenesis, the cells undergo various processes, thus allowing the transformation of the embryo into an adult organism. In particular, cells require the appropriate tools to suddenly modify their morphology and protein content in order to respond to intrinsic and external stimuli. Autophagy and apoptosis are involved in cell proliferation, differentiation and morphogenesis. Programmed cell death is a key physiological mechanism that ensures the correct development and the maintenance of tissues and organs homeostasis in multicellular organisms. PCD has been classified into three types, according to the morphology that the dying cells acquire and the molecular machinery involved: PCD type I or apoptosis; PCD type II or autophagy and PCD type III or necrosis (not involved in physiological development). These different types of cell death have specific features that can be used to be identified and characterized. Apoptosis is a highly conserved, genetically-controlled process through which certain cells destroy themselves. Autophagy is an evolutionarily conserved pathway used by eukaryotes for degrading and recycling various cellular constituents, such as long-lived proteins and entire organelles, that was mainly detected in those tissues where abundant cell death is required. Both autophagy and apoptosis are induced under stress conditions as an adaptive response against stress. Usually, environmental stress cause severe effects on embryonic development. Embryos of different species, exposed to different types of physical or chemical stress, temporarily suspend their development and activate several protective strategies, including PCD II and PCD III. Research has yet to elucidate the interplay between these key processes. Not all types of PCD are always detected in association with a developmental process. Unlike the degeneration of tissues of some invertebrates, the tissues of vertebrates undergo PCD preferentially through an apoptotic mechanisms. In this chapter, we will briefly describe some specific features of apoptotic and autophagic processes. We will focus our attention in some useful model systems of invertebrates and vertebrates organisms, where autophagy and apoptosis occur both in physiological and stress conditions; specifically, we will analyze embryos of: the nematode *Caenorhabditis el-*

egans, the insect *Drosophila melanogaster*, the sea urchin *Paracentrotus lividus*, the fish *Danio Rerio*, the mouse mammalian model, and finally we will consider the differentiation of the male and female embryonic germlines in humans.

Keywords: Cell death, apoptosis-autophagy crosstalk, stress, differentiation, embryo model systems

1. Introduction

Embryonic development is a dynamic and well-coordinated event that includes cell proliferation, differentiation and death influenced by internal and external signals coming from the microenvironment. Research has yet to elucidate the interplay between autophagy and apoptosis, two processes of programmed cell death, and cell proliferation and morphogenesis in embryos of invertebrates and vertebrates.

Programmed cell death is a key physiological mechanism that ensures the correct development and the maintenance of tissues and organs in multicellular organisms [1]. Similar to apoptosis, autophagy is essential for the development, growth and maintenance of homeostasis. It occurs constitutively at basal levels and appears to be increased as an adaptive response to several intracellular and extracellular stimuli. In both lower and higher eukaryotes, autophagy is a crucial event during embryogenesis. It was proposed that autophagy has a key role in insect metamorphosis, representing a dramatic developmental change associated with widespread cell death and complete disappearance of whole tissues.

In this chapter, we will discuss an emerging research field: programmed cell death and cell survival through apoptosis and/or autophagy under physiological and stressful conditions during the development of invertebrates and vertebrates.

Recently, cell death (CD) has been classified into three types according to the morphology and molecular machinery involved: PCD type I or apoptosis; PCD type II or autophagy; and PCD type III or necrosis, not involved in embryo development [2]. These different types of cell death have specific features that can be used for their identification and characterization. Not all types of PCD are always detected in association with a developmental process. Autophagy is mainly detected in those tissues where abundant cell death is required. Vertebrates' tissues undergo PCD preferentially through apoptotic mechanisms in contrast with the degeneration of tissues in some invertebrates [3].

Although many features are specific among different types of death, some overlapping exists between these different mechanisms. It is noteworthy that this crosstalk often allows the conversion of autophagy into apoptosis or vice versa. Thus, if one pathway is blocked, a cell may still die through a second biological pathway.

1.1. Apoptosis

Apoptosis is a cellular phenomenon that orchestrates cell suicide following two main pathways: cytochrome *c* liberation from the mitochondria or activation of death receptors. This

genetically controlled process is highly conserved during the evolution from nematodes to mammals, playing critical roles in both homeostasis and development during the morphogenesis and metamorphosis of invertebrates and vertebrates. Cells undergoing apoptosis show a series of physical and biochemical changes such as plasma membrane blebbing, loss of mitochondrial membrane potential, caspase-activation, DNA fragmentation in distinct ladders and, finally, cell disintegration into apoptotic bodies subsequently engulfed by specialized cells. Phosphatidylserine (PS), a phospholipid normally asymmetrically expressed in the inner leaflet of the plasma membranes in living cells during the final stages of apoptosis, is actively extruded from the internal face of the cell membrane of the dying cell; its exteriorization represents one of the markers that identify the cell as a target for phagocytosis [4] (Figure 1).

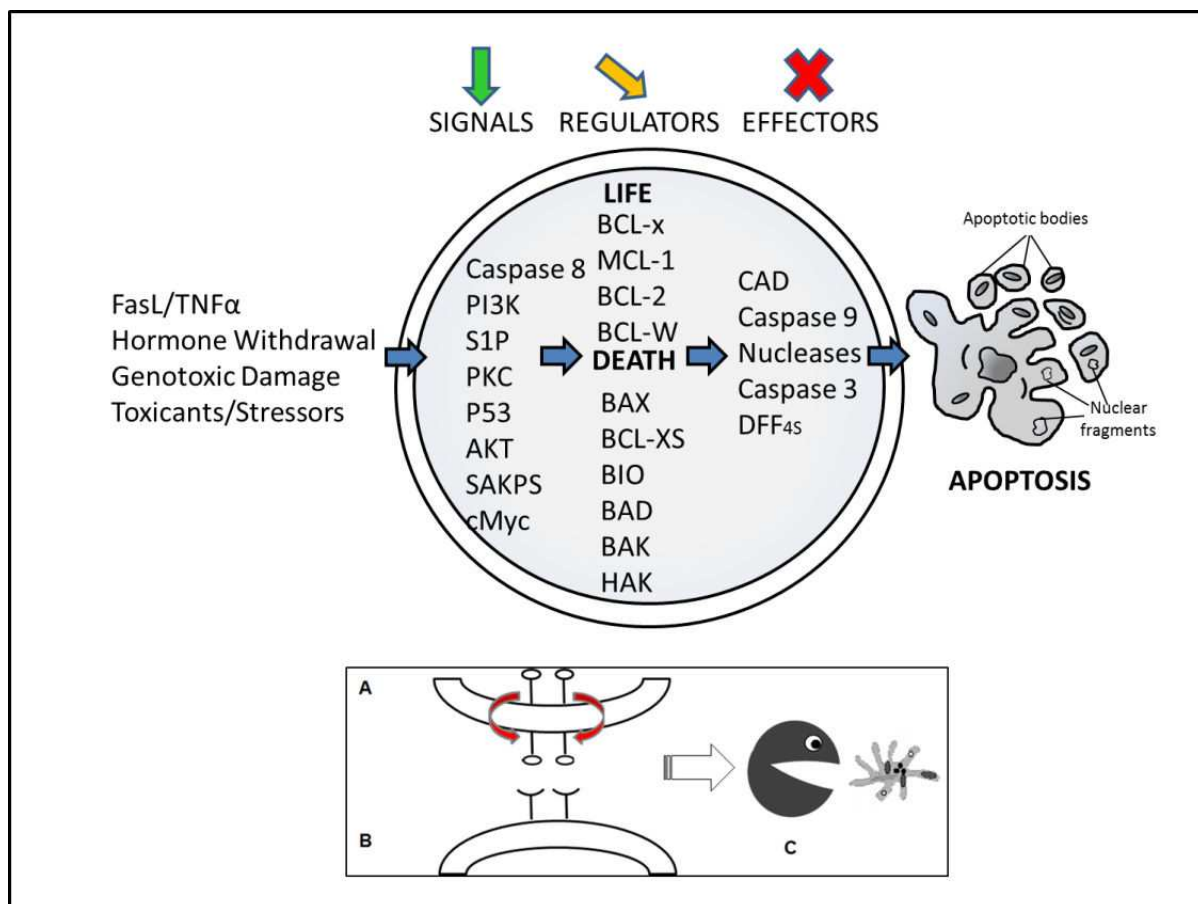


Figure 1. Top: signals, regulators and effectors involved in the balance between life and death. Left, the main inductors of apoptosis. Bottom, exemplification of the exposure mechanism of PS from the inner to the outer membrane of an apoptotic cell. (A) PS exposure in the apoptotic cell and (B) PS receptors of the phagocyte. (C) This specific recruitment consents the engulfment of the apoptotic cell or apoptotic bodies and their clearance.

This type of cell death can be greatly affected by ATP levels: if the energy level is not sufficient, cells can undergo partial apoptosis [5]. It is well known that PCD-I is required to remove transitory structures, to sculpt tissues and to eliminate damaged cells that can be harmful to the organism. On the other hand, apoptosis is also employed in response to environmental stimuli to remove cells damaged by chemical, physical and mechanical stress.

1.2. Autophagy

Autophagy is an evolutionarily conserved pathway used by eukaryotes for degrading and recycling various cellular constituents such as long-lived proteins and entire organelles [6]. Autophagy, contrary to apoptosis, can induce cell survival or cell death: it is a process of cell survival if the cellular damage is not too extensive; alternatively, it is a process of cell death if the damage/stress is irreversible. In addition, autophagy can act in association with apoptosis or as an independent pathway.

In higher eukaryotes, the lysosome compartmentalizes a range of hydrolytic enzymes and maintains a highly acidic pH in order to decompose into small molecules and then recycle the organelles and the components of cytosol targeted to them [7]. This recycling mechanism allows the cells to conserve their limited resources by minimizing the costs associated with biosynthesis or acquiring resources from the environment.

Depending on how the substrates are delivered to the lysosomal compartment, autophagy is classified into macroautophagy, microautophagy and chaperone-mediated autophagy [8,9]. Macroautophagy involves the formation of autophagosomes that will subsequently fuse with the lysosome. The molecular mechanism governing macroautophagy is highly conserved among eukaryotes. At first, a cup-shaped membranous organelle emerges and encircles a portion of the cytosol which sometimes includes various organelles. This activity results in the formation of spherical bodies, the autophagosomes, in which a double membrane sequesters the compartmentalized material. The outer membrane of autophagosomes fuses with the limiting membrane of lysosomes to release the sequestered materials into the lumen of an autophagolysosome.

In microautophagy, lysosomal compartments engulf a portion of the cytosol along with organelles, forming membrane-bound spherical bodies within lysosomes. In chaperone-mediated autophagy, the substrates, such as proteins, are translocated across the lysosome membrane and delivered directly into the lumen.

In lower eukaryotes, autophagy functions as a cell death mechanism or as a stress response during development. Autophagy's significance and the role (if any) of vertebrate-specific factors in its regulation remain unclear. In particular, in mammals, autophagy may be involved in specific cytosolic rearrangements needed for proliferation and differentiation during embryogenesis and postnatal development. Thus, autophagy is a process of cytosolic "renovation", crucial for cell fate decisions [10]. However, in both invertebrate and vertebrate organisms, it is generally thought that autophagy plays an essential dual role both in the adaptation to stress and in the starvation occurring during morphogenesis, as well as in cell elimination in concert with the apoptotic machinery.

Genetic studies have revealed the importance of autophagy during the early stages of embryogenesis; most of the genes involved, the so-called autophagy-related (ATGs) genes, have been discovered in *Saccharomyces cerevisiae* [11], and their orthologues have been isolated and functionally characterized in higher eukaryotes, indicating that autophagy is an evolutionarily conserved process [12]. Although many apoptosis and autophagy regulatory genes have been

discovered and characterized and some of them are conserved during evolution, the relationship between autophagy and apoptosis still remains rather obscure.

1.3. Cell death in stress conditions

Environmental stress can cause severe effects on embryonic development, affecting the phenotype as a result of some emergency responses and adaptive modifications. Embryos of different species, exposed to various toxicants or to physical or chemical stresses, temporarily slow down or suspend their development, eliminating the affected cells throughout apoptosis and thus altering the normal developmental program. In the long run, embryos with several accumulated damages could also die if the stressful conditions persist.

On the other hand, embryos have the ability to activate a general protective strategy against many stress-inducing agents. The accumulation of damaged proteins acts as an inductor signal that activates the stress response and the apoptotic program.

The autophagic process, similar to apoptosis, is triggered as an adaptive response to several intracellular and extracellular stimuli such as toxic stimuli, radiation, nutrient deprivation (starvation), accumulation of misfolded proteins and damaged organelles, hormonal treatments and bacterial/viral infections. Generally, autophagy seems to be crucial for cell survival in stress conditions because it promotes the recycling of damaged proteins and organelles.

A few years ago, we studied both the apoptotic and the autophagic processes in *Paracentrotus lividus* sea urchin embryos, investigating whether these events are activated as a defense strategy after cadmium exposure, a heavy metal recognized as an environmental contaminant [13,14]. Our model suggests that the temporal choice of the apoptotic or autophagic mechanism depends on the persistence of the stressful event: initially, the embryo tries to face the stress conditions using a defense strategy that is less deleterious, namely, autophagy, in an attempt to preserve the developmental program; if this strategy is not sufficient to offset the stress-induced damage, the embryo activates the mechanism of apoptosis. In addition, it can be assumed that autophagy could provide the ATP necessary for apoptosis during development, by recycling damaged cellular components. In light of these and other evidences, it can be hypothesized that there is a close interplay between autophagy and apoptosis [15].

In conclusion, apoptotic and autophagic processes may be used as alternative and/or combined defense strategies by cells exposed to many kinds of stresses. Nowadays, there is a growing interest in cell death via autophagy, which could substitute or act synergistically to the apoptotic pathway.

In this review, we will describe and compare various eukaryotic model systems that use apoptosis and autophagy during development both under physiological and stress conditions. We will also focus on the research methods employed to study the cascade of events involved in these two processes. The purpose of discussing the data in this chapter is not to review all the work in the field but rather to focus on a few arguments with the intent of re-examining some ideas and concepts.

2. Apoptosis and autophagy during embryogenesis of eukaryotes

Recent studies have shown that cell death mechanisms are used for specific purposes: morphogenesis during embryogenesis, histogenesis in the progression of metamorphosis and phylogenesis for the elimination of vestigial or larval organs. Like proliferation and differentiation, programmed cell death, PCD-I and PCD-II, play a conspicuous role during normal development as well as during disease conditions. It is essential for the removal of undesirable cells and it is critical both for restricting cell number and for tissue patterning during development.

In both lower and higher eukaryotes, autophagy seems to be crucial during embryo development by acting in tissue remodeling, in parallel with apoptosis. An increase of autophagy is observed in the embryonic stages characterized by massive cell elimination. Moreover, autophagy protects cells during metabolic stress and nutrient paucity occurring during tissue remodeling.

The study of autophagy-defective model systems has highlighted the contribution of PCD-II in the development of invertebrates, for example, during the complex events occurring in the metamorphosis of flies and worms [16]. Furthermore, it has been well documented in the early stages of the development of invertebrates that the activation of apoptotic processes contributes to the formation of different body parts and multiple organs of an organism. Using *Caenorhabditis elegans*, as well as *Drosophila* and mice, it has been demonstrated that developmental cell death is under genetic regulation as shown by mutagenesis experiments.

In vertebrates, on the other hand, there are many examples in which autophagy and apoptosis are involved in embryogenesis. For example, autophagy defects can be lethal for the animal if the mutated gene is involved in the early stages of development or it can lead to severe phenotypes if the mutation affects later stages [17].

Cell death starts at a very early stage in mammalian development. Inhibition of caspase activity leads to the arrest of embryonic development. During gastrulation, apoptosis allows the generation of a pro-amniotic cavity by the removal of the inner ectodermal cells.

Autophagy also has a crucial role during cavitation in the early stages of mammalian development [18]. Furthermore, evidence outlines the importance of autophagy during tissue differentiation in mammals [19].

Both PCD-I and PCD-II are well-controlled biological processes that play fundamental functions during development, differentiation, morphogenesis, tissue homeostasis as well as disease. The different modes of execution of cell death were investigated as separate events from each other. However, in recent times, several findings suggest that these two types of death are often regulated by similar pathways and, depending on the cellular context, can cooperate in a complementary fashion to facilitate cellular destruction. Interactions among components of the two pathways show that there is a complex crosstalk that may be induced by similar stimuli: PCD-I and PCD-II can cooperate, antagonize or assist each other affecting cell fate.

3. Apoptosis and autophagy in the development of the invertebrate model system

3.1. Nematodes: *Caenorhabditis elegans*

C. elegans has been widely recognized as a suitable model system in developmental research because of some important features: it is a simple and transparent animal with a highly reproducible development and an invariant cell lineage, it is self-fertilizing, it is easy to culture and it has a short reproductive cycle of about 3 days [20]. Recently, it was also recognized as a valuable model organism to study apoptosis and autophagy, two processes redundantly required during its embryogenesis.

Apoptosis can be observed during two stages of *C. elegans* life in two different types of tissues: the "developmental cell death" occurring during embryonic and post-embryonic development of the soma and the "germ cell death" occurring in the gonad of adult hermaphrodites. Developmental cell death can be divided into two moments: between 250 and 450 min after fertilization, the first apoptotic event removes almost a fifth (113/628) of the cells that are generated during embryonic development [21]; the second event occurs during the larval stage L2 and removes some of the newly generated neurons.

During the development of the *C. elegans* hermaphrodite soma, the embryo generates 1090 cells and exactly 131 of these undergo programmed cell death in a highly reproducible manner [22]. It was demonstrated that these dying cells are essentially invariant among individuals since they can be easily identified because of some changes in their morphology and because of their high refractivity under differential interference contrast optics [23]. To unravel the genetic pathway involved in these cell death events, a biochemical characterization of the genes was carried out. The identification of the apoptotic machinery suggested that cell suicide is performed by an evolutionarily conserved molecular program innate in all metazoan cells. Indeed, the key cell death genes of *C. elegans* have one or more mammalian homologs and all the interactions among proteins have also been described for their mammalian counterparts [24].

The first event needed to induce the apoptotic process is the transcription of the *egl-1* (egg-laying defective-1) gene coding for a BH3-only protein and directly regulated in a cell-specific manner by transcription factors of the Hox family. This protein will then bind to the Bcl-2 (B cell lymphoma-2)-like anti-apoptotic protein CED-9 (cell death defective-9), which normally protects cells from undergoing apoptosis. This will activate CED-4, the nematode ortholog of the mammalian Apaf-1 [25], which mediates the activation of the caspase CED-3 (Cell Death abnormal) from the inactive zymogen (proCED-3) into the mature protease [26,27]. The activity of CED-4 and CED-3 is essential for the execution of the apoptotic cell process. It is worthy to note that autophagy contributes to the removal of embryonic apoptotic cell corpses by promoting phagosome maturation.

Besides, almost half of the female germ cells undergo apoptosis just before exiting the pachytene stage of meiotic prophase I [28], but *egl-1* has no role in this case. During physiological germ cell apoptosis, the nuclei of the apoptotic cells are rapidly cellularized away from

the syncytium, probably to sequester apoptotic factors from the other nuclei. These results indicate that cells in different stages of development within the same organisms are able to trigger different regulatory mechanisms to control programmed cell death [29].

In 2012, *C. elegans* was introduced into the guidelines for the use and interpretation of assays for monitoring autophagy [30]. Numerous studies recognized the advantages of *C. elegans* as a useful multicellular genetic model to study the autophagic pathway. A genetic screen showed the high conservation of the autophagic machinery between yeast and *C. elegans* [31]. It has a single ortholog of most yeast Atg proteins; however, two nematode homologs exist for Atg4 and Atg8.

Autophagy has copious roles during *C. elegans* development. The sperm's entry into the ooplasm triggers the induction of basal levels of autophagy required to remove aggregate-prone proteins and paternally inherited embryonic organelles, i.e. paternal mitochondria and spermatid-specific membranous organelles, by engulfing the autophagosomes and targeting the lysosomes for degradation during early embryogenesis [32]. The localized induction of autophagy around the site of the penetrated sperm indicates that a selective degradation is occurring rather than the bulk degradation of the embryonic cytoplasm [33]. During larval development, autophagy is involved in the specification of multiple cell fates controlled by regulating miRNA-mediated gene silencing through the degradation of one of the components of the miRNA-induced silencing complex, AIN-1 [32].

Autophagy also has an important role during *C. elegans* development under stressful conditions such as starvation, high population density or hyperthermia. *C. elegans* larvae react to these stressful stimuli by arresting development at the third larval stage (L3), an event called the Dauer diapause [34]. Dauer larvae show some metabolic and morphological changes that allow long-term survival, and their cells use autophagy to generate amino acids for the neosynthesis of proteins indispensable for survival during starvation periods [16]. Autophagy was also suggested to be involved in longevity [35,36]. Finally, during the development of the hermaphrodite germline, autophagy acts coordinately with the core apoptotic machinery to execute genotoxic stress [32].

It is worthy to note that, unlike in many other model organisms, programmed cell death is not essential for *C. elegans* viability, at least under laboratory conditions [37]: mutant nematode embryos defective for the core apoptotic cell death pathway are able to develop into adulthood with superficially normal morphology and behavior. Similarly, inactivation or mutations in atg genes do not affect embryonic development and viability. An elegant work [38] showed that, strikingly, double mutant nematode embryos deficient in both autophagy and apoptosis are unable to undergo body elongation or to arrange several tissues correctly. These observations suggested that the apoptotic and autophagic processes are redundantly required and share essential developmental functions for *C. elegans* embryogenesis, revealing why single mutants embryos defective for one of the two processes are able to accomplish the embryonic development [38].

3.2. Insects: *Drosophila melanogaster*

D. melanogaster provides a powerful model system to investigate both the mechanisms and roles of PCD during animal development with a level of intermediate complexity between nematodes and mammals. The growing appreciation of the conservation of some apoptotic and autophagic responses in insects and mammals is producing an exchange of ideas that is continuing to invigorate this research field.

The development of the *D. melanogaster* embryo into an adult fly is a process that integrates cell proliferation and differentiation with programmed cell death, or apoptosis, according to circumstances. The identification of many central genes required to execute apoptosis provides the tools for the exploration of the phenotypes during development. Genetic manipulation allowed the study of apoptotic regulators and effectors in the context of the whole animal. In *D. melanogaster*, all the embryonic apoptotic processes are initiated by the activity of three genes: reaper, grim and hid. Each of these genes is independently regulated allowing developmental apoptosis to be finely controlled [39]. The role phagocytosis plays in the final stages of apoptosis and the molecular mechanisms guiding the elimination of apoptotic corpses has been outlined [40].

The initiator genes (reaper, grim and hid) in turn activate the core apoptotic machinery, including caspases, a conserved family of cysteine proteases [39]. While caspases have been characterized from many organisms, little is known about insect caspases. In *D. melanogaster*, seven caspases have been characterized, three initiators and four effectors. The availability of new insect genome sequences will provide a unique opportunity to examine the caspase family across an evolutionarily diverse phylum and will provide valuable insights into their function and regulation. This concept is supported by numerous genetic studies in *D. melanogaster*, where a single caspase, Dronc, is essential for most developmental cell death. Importantly, cell death in some cell types occurs in the absence of Dronc and the primary effector caspase, Drice, suggesting that, similar to mammals, redundancies have been built into the cell death system of insects [41].

The methods used to study apoptotic cell death in the *D. melanogaster* embryo and ovary as well as in cultured cell lines have been recently discussed. These techniques, including acridine orange staining, fragmented DNA *in situ* analysis (TUNEL), cleaved caspase staining, caspase activity assays and assays for mitochondrial fission and permeabilization, are suitable for analysis of apoptosis in normal and stress conditions [42].

In *Drosophila*, many developing tissues require programmed cell death (PCD) for proper formation. Two bcl-2 genes are encoded in the *Drosophila* genome and some studies suggested their requirement during embryonic development. However, despite the fact that many tissues in fruit flies are shaped by PCD, deletion of the bcl-2 genes does not perturb normal development. By irradiating fly mutants, it has been demonstrated that developmental PCD regulation does not rely upon the Bcl-2 proteins but that it provides an added layer of protection in the apoptotic response to stress [43].

Cells damaged by environmental insults have to be repaired or eliminated to ensure tissue homeostasis in metazoans. Recent studies on apoptosis induced by stress during embryogen-

esis of *D. melanogaster* demonstrated the implication of Jun N-terminal kinase (JNK) in the apoptotic response to stress. JNK signaling is implicated in many processes of normal development, e.g. the dorsal closure. The repression of Dpp (Decapentaplegic) signalling causes the activation of the proapoptotic role of JNK and the following activation of proapoptotic gene reaper. The protein Schnurri mediates the repression of gene reaper through Dpp. This arrangement allows JNK to control migratory behavior without triggering apoptosis. Dpp plays a dual role during dorsal closure: it cooperates with JNK in stimulating cell migration and also prevents JNK from inducing apoptosis [44].

It has been demonstrated that *Drosophila* lines overexpressing the gene *menin* or an RNA interference for this gene normally develops but are impaired in their response to several stresses including heat shock, hypoxia, hyperosmolarity and oxidative stress. Menin, the product of the multiple endocrine neoplasia type I gene, is implicated in several biological processes including gene expression control and apoptosis, modulation of mitogen-activated protein kinase pathways and DNA damage sensing or repair. In the *Drosophila* embryo subjected to heat shock, this impairment was characterized by a high degree of developmental arrest and lethality. The gene *menin* seems to be implicated in the regulation of stress response and in the preservation of protein structure and function, as suggested by the fact that a deletion of the *menin* gene causes a strong decrease of HSP70 and HSP23 synthesis and an increase of the sensibility to many kinds of stresses [45].

Concerning autophagy, the fruit fly provides an excellent model system for *in vivo* studies in the context of a developing organism. Because of its short life cycle, the well-characterized genetics of the organism and the expression of genes and their regulators, the *D. melanogaster* model system has proven to be very useful in the understanding of the physiological roles of autophagy. Atg genes and their regulators are conserved in *Drosophila* and autophagy can also be induced in response to nutrient starvation and hormones during development. Differences in the role of autophagy in specific contexts and/or cell types suggest that there may be cell context-specific regulators of autophagy and studies in *Drosophila* are well-suited to yield discoveries about this specificity. Autophagy is induced in *Drosophila* in starvation conditions or during metamorphosis in specific tissues. Consequently, the regulation of this process may change in these different contexts and circumstances; thus, this field needs further investigations [46].

During the embryogenesis of *Drosophila*, autophagy and apoptosis seem to occur contemporarily. Autophagy characterizes embryonal districts with massive cell elimination but also exerts a protective role against metabolic stress during tissue remodelling [16].

Analogous to the observations in yeast, worms and mice, Atg inactivation may result in severe phenotypes in *Drosophila*. Mutation of *atg18* and *atg6* is lethal at the larval stage. Atg1 expression is also crucial for development as *atg1* mutants show reduced larval viability and those that survive cannot develop beyond pupa [47]. In *Atg7* gene mutant flies, attenuation of autophagy occurs, but there is no impairment of vitality, fertility or morphology. Nevertheless, *atg7* mutant adults show higher sensibility to stress and decrease of life span, probably for the neuronal accumulation of ubiquitinated proteins [48].

Induction of autophagy has also been observed during two nutrient status checkpoints of oogenesis in the fruit fly: germarium and mid-oogenesis stages [49]. Mutagenesis experiments in *atg7* genes have confirmed these evidences [50]. In *D. melanogaster*, it appears clear that apoptosis and autophagy work in parallel in the disruption of certain tissues during the fly's development. The relationship between autophagic cell death and apoptosis has been examined during *Drosophila* embryogenesis by studying the elimination of an extra-embryonic tissue known as the amnioserosa (AS), demonstrating that both processes are required for its disruption. Interestingly, autophagy seems to be essential for caspase activation in the AS: a reduction of cell death and permanence of AS are related to the down-regulation of autophagy; a caspase-dependent premature AS dissociation is related to autophagic up-regulation [51].

3.3. Echinoderms: *Paracentrotus lividus*

The sea urchin embryo is one of the most important model systems for studies regarding developmental biology, cellular and/or molecular biology and toxicology. In recent years, this organism has become a model for the study of cell death both in physiological and stress conditions. Physiological apoptosis can be considered an important aspect of sea urchin development, which is regulated and controlled by specific genetic programs and is necessary to construct the animal [52].

Studies on the activation of physiological apoptosis in the sea urchin embryo were conducted for the first time in 1997 using different methodological approaches: DNA electrophoresis analysis, morphological observations and TUNEL assay. Physiological apoptosis at pluteus stage (the first larval stage) was shown by those investigations, especially in cells from specific districts: oral and aboral arms and intestine. No apoptotic signals were observed at the blastula or gastrula stage. It has been assumed that some embryonic structures known to at least partially disappear after metamorphosis can be somehow eliminated through this pathway [53].

Further studies showed the occurrence of apoptosis in *P. lividus* larvae with a specific spatial distribution in definite and discrete districts of 8 arms larvae in oral and aboral arms, intestine, ciliary bands, apical and oral ganglia. During the stage of metamorphosis (corresponding to competent and juvenile larvae), only a few cells trigger apoptosis. Therefore, it has been hypothesized that the removal of inadequate cells was the result of a programmed cell death required for the development of the adult and for the elimination of unnecessary structures [54].

Regarding early embryogenesis, the available data suggest that apoptosis is not frequent during developmental cleavage, becoming active from gastrula stages onwards. Only necrotic or pathological cell death has been observed during cleavage stages [55].

The sea urchin embryo, as well as many other embryos of marine organisms, is highly sensitive to several kinds of stressors and is able to activate different defense strategies such as apoptosis. Apoptosis was studied in sea urchin embryos and larvae during stress conditions after exposure to emetine, etoposide [56], ultraviolet radiation [57], staurosporine [58], 12-O-

tetradecanoylphorbol-12-acetate (TPA) followed by heat shock at 31°C [53,54], cadmium (Cd) [13, 15, 59, 60], 2-trans-4-trans decadiene [61,62] and chromium (Cr III) [63]. In particular, it was demonstrated that a brief treatment with TPA and heat shock or an exposure to very high Cd concentrations are able to trigger apoptosis in *P. lividus* embryos [53,59]. By TUNEL assays, immunolocalization of cleaved caspase-3, cleaved α -fodrin and cleaved lamin-A (two substrates of cleaved caspase-3), an increase of apoptotic events was observed from 15 h to 24 h of development [13]. Other studies showed that long-lasting exposure to lower Cd concentrations, similar to those found in polluted seawaters, caused severe developmental delays and abnormalities in *P. lividus* larvae. The authors reported that small Cd concentrations, if accumulated in the cells, induce several cytotoxic effects and abnormalities depending on cell loss caused by apoptosis. Numerous apoptotic cells could interfere with the developmental program causing a misregulation of cellular remodeling, which normally occurs in pre-metamorphic stages of development. Probably, the correct larval feeding behavior is perturbed by defects in arms and ciliary bands [60]. In general, apoptosis induced by stress in sea urchin embryos and/or larvae can be considered as part of a defense strategy that, by sacrificing a few cells, can save the whole organism.

Concerning autophagy, the activation of this process in sea urchin embryos was reported for the first time in 2011 [14]. In 2012, the *P. lividus* embryo was included into the “Guidelines for the use and interpretation of assays for monitoring autophagy” as a valuable model system.

Studies on whole embryos make it possible to obtain qualitative and quantitative data for autophagy and also to get information about spatial localization aspects in cells that interact among themselves in their natural environment. In such a system, it is possible to add many autophagic inducers or inhibitors to the media (seawater) that will subsequently be directly absorbed through the membrane of embryo cells [30].

Several experimental approaches have been used to detect physiological autophagy: identification of autophagolysosomes by acidotropic dyes such as neutral red (NR) and acridine orange (AO); immunodetection of LC3-II (an autophagic marker) by Western blotting and immunofluorescence. The results showed that autophagy seems to have a crucial role and it is constantly present, reaching peaks in specific points of embryonic development. This aspect was studied by analyzing the molecular autophagic flux and the dynamics of autophagic organelles (autophagosomes and autophagolysosomes). A major activation of autophagy was detected after 18 h of development, probably because there is a reorganization of the embryo at the gastrula stage as the cells begin to take strategic positions and need to recycle metabolites in order to obtain the energy necessary for the completion of development [14].

Regarding autophagy induced by stress, it has been found that sea urchin embryos are able to modulate this process as a defense strategy against Cd exposure. Analyzing the autophagosomes by LC3, an increase of the levels of this autophagic marker during development was observed in particular at late gastrula stage [14,64]. Specifically, the experiments revealed a higher level of autophagosomes for embryos treated for 18 h with high Cd concentrations, while embryos show lower levels of autophagosomes after 24 h of treatment, probably because the apoptotic process becomes significant [13,14].

Further studies on the role of autophagy during oogenesis and early stages of development and on the possible interplay between apoptosis and autophagy are in progress in our laboratory.

We proposed three different hypotheses about the homeostatic relationship between survival and death pathways in sea urchin embryos exposed to Cd stress: (a) hierarchical choice of defense mechanisms, (b) energetic hypothesis and (c) clearance of apoptotic bodies. First hypothesis: the embryo tries to face the stress conditions triggering, initially, a less deleterious defense strategy, i.e. autophagy, in order to preserve the developmental program. Second hypothesis: apoptosis is activated since autophagy is unable to offset the damage caused by stress; in this case, autophagy could provide ATP by recycling of damaged cellular components. Third hypothesis: sea urchin embryo begins the clearance of apoptotic bodies through autophagy [14]. To study the functional relationship between autophagy and apoptosis induced by Cd, we blocked autophagy by treatment of the embryos with the inhibitor 3-methyladenine and, subsequently, we analyzed the apoptotic signals. Results demonstrated that the inhibition of autophagy, inevitably, produced a concomitant reduction of apoptosis and the degeneration of the embryos, probably by necrosis.

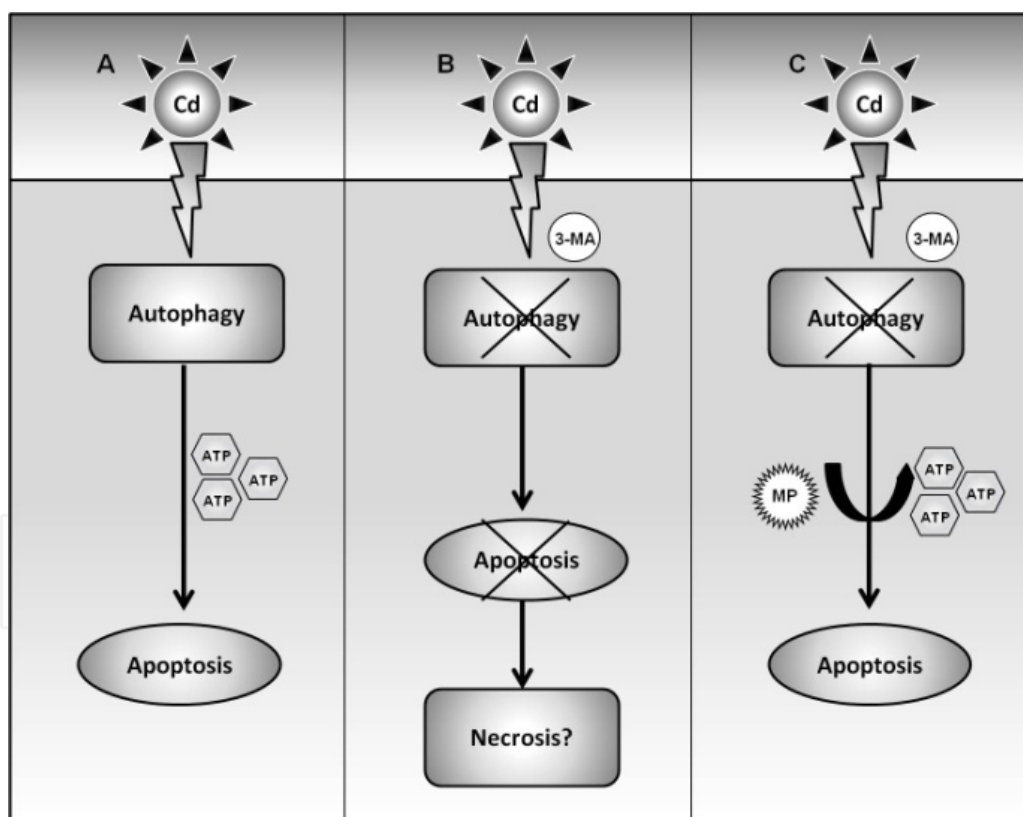


Figure 2. Summary diagram on the functional relationship between autophagy, apoptosis and probable necrosis in sea urchin embryos exposed to Cd. (A) Cd induces autophagy and then triggers apoptosis. (B) Cd-treated embryos, incubated with the autophagic inhibitor 3-MA, do not show autophagy or apoptosis activation and embryos degenerate, probably by necrosis. (C) Cd-treated embryos incubated with the autophagic inhibitor 3-MA do not show autophagy but, after incubation with the energetic substrate (MP), there is a re-activation of the apoptotic process.

We hypothesized that, in Cd-exposed embryos, autophagy could operate to supply ATP, recycling damaged cellular components necessary to sustain the apoptotic pathway, which is essential for the clearance of dying cells. This could justify the temporal relationship between autophagy and apoptosis. We showed that by administering an energetic substrate for production of ATP, methyl pyruvate (MP), the apoptotic signals were substantially restored. These data may be explained considering that autophagy could energetically contribute to the apoptotic execution program through its catabolic role [15] (Figure 2).

4. Apoptosis and autophagy in the development of the vertebrate model system

4.1. Fish: *Danio rerio*

Recently, an increasing number of studies have shown that fish express all the core components equivalent to the mammalian apoptotic machinery, suggesting that at least the central pathways of cell death are highly conserved within vertebrates [65].

Zebrafish (*Danio rerio*) is an exceptional model for developmental research: development is rapid and, thanks to the transparency of the embryo, it is now possible to visualize the apoptotic cells. Thanks to some genetic and bioinformatics screenings, it was demonstrated that zebrafish possesses most homologs of mammalian components of the extrinsic and the intrinsic pathway of apoptosis [66,67]. Since it is supposed that gene and chromosome duplication is a common event in teleosts, several duplicate Bcl-2 family genes were found in zebrafish [68].

In zebrafish embryos, the ability to activate the cell death program is obtained only at gastrulation, simultaneous with the introduction of cell cycle checkpoints, suggesting a close dialog between the cell cycle machinery and the apoptotic machinery [28]. A significant work described the temporal and spatial distribution of apoptotic cells during normal development of the zebrafish embryo from 12 to 96 h after fertilization using a TUNEL assay. The authors found transient high rates of cell death in various structures, focusing on the nervous system and associated sensory organs such as the olfactory organ, retina, lens, cornea, otic vesicle, lateral line organs and Rohon–Beard neurons but also in other non-neural structures such as the notochord, somites, muscle, the vascular and urinary systems, tailbud and fins [69].

Zebrafish also have many features that make it a suitable vertebrate model organism for the analysis of autophagy [70].

To detect autophagic induction, a widely used marker protein is the microtubule-associated protein 1-lightchain 3B protein (MAP1-LC3B) [71]. LC3 is one of the major biochemical markers of autophagy [72]; the MAP (microtubule associated protein) family comprises a group of proteins found in association with microtubules [73] with some possible key roles in interacting with other signaling proteins of the MAP kinase pathway [74]. Both the zebrafish orthologue of mammalian peptide forms, MAP1LC3A (map1lc3a) and MAP1LC3B (map1lc3b), were

identified by phylogenetic and conserved synteny analysis and their expression during zebrafish embryo development was analyzed. Both genes show maternally contributed expression during early embryogenesis; thereafter, levels of map1lc3a transcript steadily increase until at least 120 h post-fertilization. Using the autophagic inhibitor chloroquine, the authors were also able to demonstrate that the LC3II/LC3I ratio increases after the exposure of zebrafish larvae to rapamycin or sodium azide (for mimicry of hypoxia) in their aqueous medium [75].

Because of their aqueous habitat, simple drug administration can be achieved in *in vivo* zebrafish embryo cultures for screening compounds that regulate autophagy and for studying the interplay between genetic and environmental influences on autophagy [76]. The already mentioned transparency of the embryo gives the possibility to observe autophagosome formation by the use of transgenic GFP-LC3. This method was used to study the induction of autophagy in cultured zebrafish embryonic cells under starvation conditions. Analyzing the subcellular localization of endogenous MAP1-LC3B protein during amino acid starvation, the authors found that this protein translocates from the cytosol to the membranes including lysosomes and the endoplasmic reticulum indicating the induction of the autophagy–lysosome pathway [77].

Recent studies indicate that autophagy is one of the major strategies used by marine organisms to face the presence of nanoparticles in the marine environment as the concentration of these emerging contaminants is increasing year by year. Using zebrafish embryos, two papers reported the activation of the autophagy–lysosome pathway after nanoparticle exposure: one of them described the appearance of lysosome-like vesicles after multi-walled carbon nanotubes exposure at the single-cell stage of zebrafish embryos [78]; the simultaneous treatment of embryos with S-doped TiO₂ nanoparticles and simulated sunlight irradiation suggested receptor-mediated autophagy and vacuolization indicating the entrance of nanoparticles via endocytosis rather than diffusion [79].

4.2. Mammals: Mouse

In mammalian embryos, the apoptotic cell death is mainly implicated in modelling and occurs in processes such as cavitation. In all cases studied, apoptosis is under genetic control, with activation sometimes regulated by local environmental variables [80]. In mammals, a very early activation of zygotic genes and apoptosis occurs at the blastocyst stage, throughout inner cell mass differentiation, differently from most vertebrate in which cell death cannot be seen prior to gastrulation [81,82]. Phosphatidylserine (PS) has been used to identify cell death in the preimplantation of the developing embryo. Annexin V is a very specific apoptotic marker since it preferentially binds to negatively charged phospholipids like PS in the presence of Ca²⁺. Staining is visualized by fluorescence or confocal microscopy or by fluorescence-activated cell sorting. As early as the two-cell stage, cell death was found in the polar bodies. No dying cells in embryos containing 1 to 8 cells were observed, but a few cells died between the 16-cell and blastula stages. Although cell death in the developing preimplantation embryo is not caspase dependent, a generalized inhibition of the caspase activity causes the arrest of

embryonic development even at stages where there is no cell death: the inhibition of caspases causes an ulterior cell death [80,83].

One of the mechanisms responsible for the elevated levels of embryo death during the first week of *in vitro* development, developmental arrest, is often due to ROS. Approximately 10–15% of IVF embryos permanently arrest in mitosis at the 2- to 4-cell cleavage stage, showing no indication of apoptosis. A sensor protein implicated in the apoptotic response to oxidative stress is p66Shc: the deletion of the p66Shc gene in mice leads to a decrease of apoptotic cells and resistance to oxidative stress [84].

Studies on mammalian autophagy suggest the importance of this process in the regulation of cell fate decisions such as differentiation and proliferation. Many autophagy gene knockout mice have embryonic lethality at different stages of development. Furthermore, interactions of autophagy with crucial developmental pathways such as Wnt, Shh (Sonic hedgehog), TGF β (transforming growth factor β) and FGF (fibroblast growth factor) have been reported. Studying how mammalian autophagy may affect phenotypes associated with development, it was recently shown that knockout of many autophagy-related genes in mice affects early developmental stages, neonatal development and neuronal differentiation [84]. Autophagy is important during critical mammalian developmental stages in which nutrients are restricted, for example, during the preimplantation [85]. Embryogenesis is mainly governed by developmental pathways such as Shh, TGF β , Wnt and FGF, and there is an intensive crosstalk of autophagic proteins with these pathways. However, it is likely that there are further mechanisms and regulatory loops to be discovered [86].

In mammals, the autophagic process starts just after fertilization. It is known that maternal mRNAs and proteins that accumulate in oocytes during oogenesis are responsible for zygote formation [87]. Following fertilization, maternal proteins and mRNAs are largely degraded, the organelles are remodeled and the translation of zygotic mRNAs starts. Contemporarily, the autophagy seems up-regulated, as demonstrated by a significant increase of autophagosomes. Autophagic activation is probably related to the inactivation of mTOR signalling [86], which occurs after the wave of Ca²⁺ following fertilization. It has been found that the Atg5 protein is essential for the very early developmental stages of mouse. The early induction of autophagy could be necessary for the catabolism of maternal macromolecules and proteins, to obtain a pool of free amino acids to be used for zygotic protein synthesis [88,89]. During the cavitation of mouse embryo, autophagy and apoptosis occur in parallel. The removal of the inner ectodermal cells by apoptosis consents the formation of a proamniotic cavity [18]. Employing embryonic stem cells from embryoid bodies (EB), it has been found that Atg5 and Beclin1 (pro-autophagy genes) are involved in the elimination of cells died in the cavitation mechanism [90]. Recent studies showed the failure of cavitation in EB derived from Atg5- or Beclin-defective cells, probably due to the accumulation of dead cells [91].

In vertebrates, the autophagic process acts as a pro-survival or pro-death mechanism in different physiological and pathological conditions such as neurodegeneration and cancer; however, the roles of autophagy during embryonic development are still largely uncharacterized. Beclin1 is a principal regulator in autophagosome formation and its deficiency results in early embryonic lethality. A functional deficiency of Ambra1, a positive regulator of Beclin1,

is associated with autophagy reduction, increase of ubiquitinated proteins and of apoptotic death and causes serious neural tube defects [92].

A variation of cell proliferation and an impairment of autophagic process always compromises the organ size and often causes a high incidence of tumors [93]. The crosstalk between cell proliferation and differentiation needs further investigations and represents a great objective for the researchers [16].

5. Apoptosis and autophagy during differentiation in mammals

There are numerous cases in which the PCD-I and PCD-II are involved during tissue differentiation and cell homeostasis in mammals. For example, correct central nervous system development and neuroretina formation require the activation of the autophagic process [94]. On the other hand, during vertebrate brain development, 20–80% of the originally produced neurons are eliminated by apoptosis [95] and more than 80% of ganglion cells in the cat retina die shortly after birth [96]. It has also been shown that the development of the rat lens vesicle involves the apoptotic elimination of the cells between the ectoderm surface and the optic vesicle helping the invagination and facilitating the separation from the ectoderm [97].

The role of autophagy in cardiogenesis has been carefully investigated demonstrating that constitutive autophagy represents a homeostatic mechanism necessary for cardiomyocyte remodeling, maintaining cardiac size and function. In addition, up-regulation of autophagy plays a protective role for the heart in response to hemodynamic stress increasing protein turnover and preventing the accumulation of abnormal proteins and organelles [98]. Still, in cardiac morphogenesis, apoptosis is essential in generating the overall four-chambered architecture of the heart. The transformation of the endocardial cushion into valves and septa results from a region-specific balance between cellular proliferation, apoptosis and differentiation [99].

A crucial role for apoptosis is seen during morphogenesis and tissue remodeling. The areas of interdigital cell death during limb development provide a paradigmatic model of massive cell death with an evident morphogenetic role in digit morphogenesis. PCD-I sculpts the limbs of all amniotes such as humans, mouse and birds by removal of interdigital webs [100,101]. Cell death can be observed in the anterior and posterior marginal zones of the developing limb bud and in later stages in almost all of the interdigital mesenchymal tissue accompanying the formation of free and independent digits of birds and mammals [102].

5.1. From oogenesis to embryogenesis

Autophagy involvement in reproduction has still not been extensively studied, although its activity is fundamental for many processes across the reproduction spectrum from gametogenesis to embryogenesis. Malfunctions in autophagy are associated with deleterious repercussions throughout reproduction.

Death is known to strike the male and female germlines with roughly equal intensity; nevertheless, the innate male germ cells have a major self-renewing ability compared to the female ones. The reproductive life of mammals, including humans, depends on the biological activity of the female gonad that, from birth, has a defined number of oocytes. During a woman's life, most of them are eliminated following a well-defined genetic process [103].

Recent studies on the availability of mice lacking key components of this conserved cell death program were important to confirm the gene expression studies that identified certain molecules as indispensable for female germ cell survival or for death to proceed.

Inadequate nutritional and energy supplies, metabolic stress, hypoxia and growth factor insufficiency are the main inducers of autophagy. The autophagic pathway can also be either promoted or inhibited by cellular components that are involved in the induction of apoptosis. Similarly, autophagy components can also inhibit some players of the apoptotic pathway such as caspase-8 and mitochondria. At both the organism and cell levels, autophagy can (paradoxically) have pro-death or pro-survival functions depending on the context [104].

The activation of the apoptotic pathway in fertilized oocytes is an early event probably responsible for the degradation of superfluous maternal material [105]. This early activation may also be involved in the degradation of organelles derived from spermatozoa involved in fertilization, such as mitochondria, which enter the mammal oocyte after gamete fusion [106]. This autophagic turnover during the early phases of embryo development is known as the oocyte-to-embryo transition, and is a key event for preimplantation embryonic development [87].

Moreover, experimental results showed that autophagy is also activated during folliculogenesis, especially in the granulosa cells [107], as demonstrated by the presence of a strong immunoreactivity for LC3 at all stages of development [108]. By using rat ovaries as a model of follicular development, it has been demonstrated that the induction of autophagy in granulosa cells is closely related to the beginning of apoptosis [108]. In fact, in primordial, primary and pre-antral follicles, caspase 3 is not active. On the contrary, in antral follicles, cleaved caspase 3 signals were detected only in cells that showed a strong immunoreactivity for LC3II, but not in cells expressing an inactive LC3 [109]. Autophagy in granulosa cells seems to be controlled by the levels of FSH, determining the direction of biological processes toward atresia or survival, which is the prerequisite to completing the maturation of the follicle and its ovulation.

Most primordial follicles remain in the quiescent phase during the reproductive life of a woman. Autophagy seems to be regulated by the proto-oncogene KIT through activation of the PI3K-Akt-mTOR pathway. It has been shown in mice that the mutation of the gene results in a significant reduction of the primordial germ cells in the female gonad [110,111]. mTORC1 suppression seems to be the main pathway that is able to support autophagy in the maintenance of primordial follicles in a dormant state throughout a woman's reproductive life [112].

Apoptosis seems to be involved in the gonad's activity. In granulosa cells of secondary and antral follicles, the apoptotic pathway seems to operate in eliminating oocytes with chromatin

defects [113]. Apoptosis during human oogenesis acts on oogonia and oocytes in the preleptotene stage and for the oocytes only in the pachytene stage [114].

Typically, the apoptosis process in mammalian cells is characterized by morphological changes as cytoplasmic or nuclear condensation, apoptotic body formation and chromatin margination along the nuclear membrane. In granulosa cells and oocytes, apoptosis occurs with the segmentation of the oocyte and cytoplasmic vacuolization [115].

Shortly after the fourth week of gestation, primordial germ cells migrate from the yolk sac to the gonadal ridge and proliferate. In the second half of pregnancy, this number declines. Therefore, about 7×10^6 oocytes are formed in the human ovary during early fetal life. This number is sharply reduced before birth through apoptotic cell death of the oocytes. Apoptosis has its highest peak between weeks 14 and 28 and then decreases.

Mitochondria play an important role in the competence of the oocyte to support embryogenesis. It has been demonstrated that to reduce apoptosis, injection into the oocytes of mitochondria derived from other oocytes without an active apoptotic pathway in granulosa cells can be useful [116].

5.2. From spermiogenesis to embryogenesis

Autophagy also seems to be represented in the male gametogenesis, and it acts in a way that appears similar to that observed in oocytes [117,118]. An increased focus on autophagy at all stages of gametogenesis appears to be a promising area for future research which has been relatively neglected to date.

The testis produces spermatozoa from spermatogonia in a complex developmental cascade involving proliferation, meiotic maturation and subsequent differentiation of germ cells in the germinal epithelium lining the seminiferous tubules. The duration of this process varies among species.

In the testicle, apoptosis eliminates sperm cells with chromatin or genetic defects, and it regulates the optimal ratio of germ cells to Sertoli cells. It has been speculated that the impairment of apoptosis could be related to the male infertility phenotype [119].

Later in life, apoptosis is involved in the removal of germ cells that are damaged as a result of exposure to environmental toxicants, chemotherapeutic agents or heat [120].

The DNA fragmentation detected in sperm cells can be related to apoptotic events. Normally, "physiological" strand breaks are corrected by a complex process involving H2Ax phosphorylation and the subsequent activation of nuclear poly(ADP-ribose) polymerase and topoisomerase [121]. These strand breaks, achieved by endonuclease-mediated DNA cleavage, could also represent the induction of an incomplete or abortive apoptotic response during spermatogenesis, even if the cell's viability is not compromised.

Malformations and pregnancy loss seems to be influenced by DNA damage in the spermatozoa [122]. In animal models, it has been demonstrated that the genetic integrity of the spermatozoa is closely related to embryonic development and the ability to implant [123].

Apoptosis can be observed in mature spermatozoa as a result of its activation during the spermatogenesis process. In particular, the fragmented apoptotic DNA is easily detected by TUNEL assay or TdT assay (Figure 3).

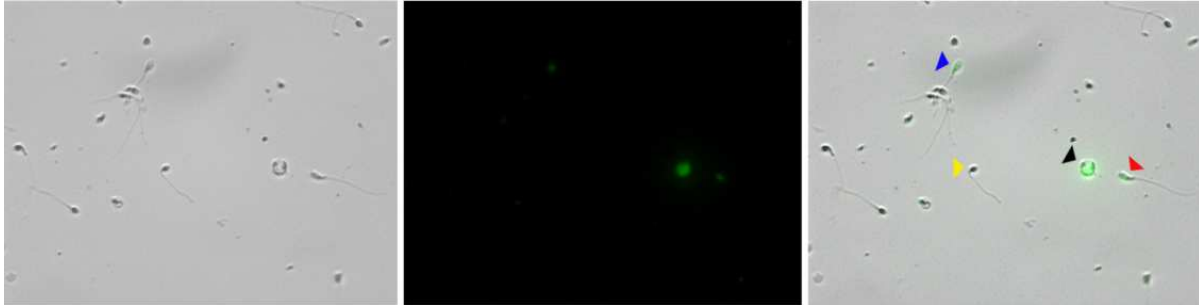


Figure 3. TdT assay: spermatozoa sample. Images captured by fluorescent microscopy in the same field. (A) Light, (B) fluorescent (green) and (C) merged images. Red arrowhead: sperm with flattened head and DNA fragmentation; yellow arrowhead: sperm with angled neck and DNA integrity; blue arrowhead: morphologically normal sperm but with DNA fragmentation; black arrowhead: round cell [124].

6. Conclusion

Recent findings have linked the major routes to programmed cell death, apoptosis and autophagy during embryo development, tissue differentiation and homeostatic balance of cells. These distinct types of PCD have been studied separately for a long time; however, recently, accumulated data have suggested that the interaction among components of the two pathways is due to a complex crosstalk. PCD-I and PCD-II are often induced by similar stimuli and even use analogous initiator and effector molecules. Depending on the cellular context, these two main modes of cell death can cooperate in a balanced interplay to facilitate cellular destruction.

In both lower and higher eukaryotes, autophagy seems to be crucial during embryogenesis by acting in tissue remodeling in parallel with apoptosis. An increase of autophagy is observed in those embryonic stages characterized by massive cell elimination.

Moreover, both apoptosis and autophagy have been described as the most important mechanisms that regulating the death of damaged cells in response to stress or in pathologic conditions. Environmental stress can alter embryo development and affect the phenotype as a result of some emergency responses and adaptive modifications which are not the same for all the species analyzed but rather depends on the organism's characteristics. Usually, damage can be restored using different defense strategies but if the cellular insult is beyond the organism's ability to repair, the only alternative can be to destroy the aberrant cell through apoptosis or autophagy.

Here, we have briefly described some specific features of the apoptotic and autophagic processes and their involvement under physiological and stress conditions; we have illustrated

the most remarkable results obtained in some model systems which use apoptosis and/or autophagy for the above-mentioned purposes.

The homeostatic relationship between apoptosis and autophagy during embryo development represents a very interesting chapter, and future studies in this direction are needed to clarify the molecular relevance of the apoptosis–autophagy crosstalk.

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