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—Mini Review—

The Role of Autophagy in Early Mammalian Embryonic Development

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Abstract: During oogenesis, the oocyte stores a large amount of maternally provided products, including mRNAs and proteins. However, after fertilization, these products are rapidly degraded and new materials are synthesized from the zygotic genome. This oocyte-to-embryo transition, also known as the maternal-to-zygotic transition, is conserved in many species and plays a pivotal role in development, because non-degraded products can hamper further embryonic development. Given that the time of early embryonic development is so rapid, ubiquitin/proteasome-mediated turnover of individual proteins is probably not sufficient to remove maternal products during the oocyte-to-embryo transition. Autophagy is an evolutionally conserved degradation system in which portions of the cytoplasm sequestered by double membrane structures called autophagosomes are delivered to lysosomes for degradation. The basic roles of autophagy are the generation of amino acids for energy and the maintenance of cellular quality. In addition to the fundamental functions of autophagy, the unique role of autophagy in removing random cytoplasmic contents including mitochondria, peroxisomes, and even lipids, may contribute to extensive cellular remodeling during the oocyte-to-embryo transition. Here we briefly review the history and molecular mechanisms of autophagy, and discuss the function of autophagy in early mammalian embryogenesis.

Key words: Autophagy, Lysosome, Fertilization, Preimplantaion embryo, Mouse

Autophagy

Since autophagy was first reported about 40 years ago, it has become a worldwide topic of research. The discovery of the genes involved in autophagy, the AuTophaGy (ATG)-related genes, and the evolutionary conservation of these genes, across species from yeast to humans, has attracted the attention of many researchers. The conservation of the genes indicates that the functions of autophagy are also preserved across species. Many of the physiological functions concerning autophagy have been elucidated in recent studies in various animal models. Autophagy is a bulk degradation process, in which cytoplasmic constituents, including proteins and organelles, are degraded by lysosomes [1]. Autophagy is categorized into three types: microautophagy, chaperone-mediated autophagy, and macroautophagy [2]. Of these, macroautophagy is by far the most commonly studied and characterized process. Here we will describe macroautophagy, hereafter referring to it as autophagy.

The process of autophagy can be grossly divided into the following steps: induction, membrane elongation, sequestration, lysosome fusion, and degradation (Fig. 1). When autophagy is induced, a double membrane structure called the isolation membrane appears, which elongates and sequesters cytoplasmic constituents to form an autophagosome. As the sequestration of cytoplasmic constituents by autophagosomes occurs more or less randomly, an autophagosome may, in addition to proteins, include organelles such as mitochondria and peroxisomes. Following this step, the outer membrane of the autophagosome fuses with the lysosomal membrane, causing the degradation of the cytoplasmic constituents within the autolysosome.

Autophagy is highly induced by starvation conditions, under which the amino acids produced by autophagic degradation are recycled as nutrients. This function is one of the most important functions of autophagy that is conserved across species. Though the induced autophagy is temporary, a low level of autophagy (hereafter referred to as basal autophagy) occurs constantly. Basal

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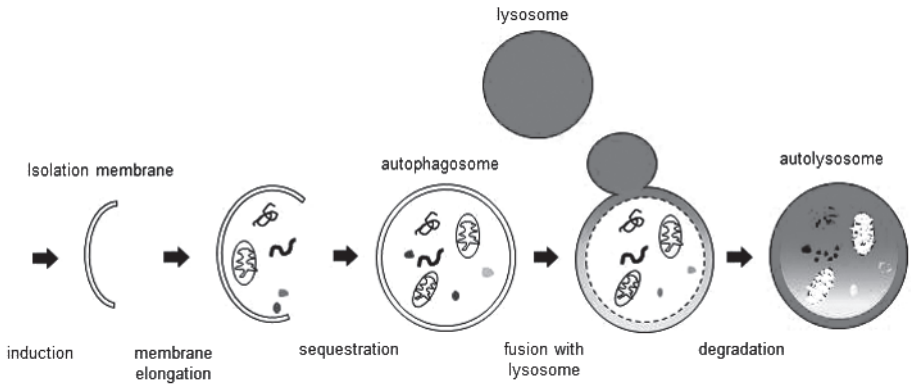

Fig. 1. The process of autophagy
When autophagy is induced, an isolation membrane appears, elongates, and encloses a portion of the cytoplasm, resulting in the formation of a double membrane structure, the autophagosome, which includes not only proteins, but also organelles such as mitochondria. After the fusion of the autophagosomal outer membrane with the lysosome, which generates the autolysosome, lysosomal enzyme enters and degrades the inner membrane of the autophagosome, resulting in the digestion of materials inside the autophagosome.

autophagy is responsible for cytoplasmic quality control. For instance, a deficiency of autophagy in neurons and hepatocytes leads to the accumulation of ubiquitinated protein aggregates and inclusion bodies [3–5]. This suggests that autophagy acts in concert with the ubiquitin-proteasome system, selectively degrading those components that are not eliminated by the ubiquitin-proteasome system, such as abnormal protein aggregates. A protein called p62 (SQSTM1), which is a target molecule of autophagy, plays an important role in this selective autophagy [6, 7].

As mentioned above, the basic functions of autophagy are the generation of amino acids during starvation and cellular quality control. Recently, however, many other roles of autophagy, such as in neurodegeneration, ageing, cancer, microbial infection, and antigen presentation, have been elucidated [8]. In addition, selective autophagy is now a hot topic in the field of autophagy research, which has led to the development of the following classifications: mitophagy, for the selective autophagy of mitochondria; pexophagy, for peroxisomes; lipophagy, for lipid droplets; reticulophagy, for endoplasmic reticula; aggrephagy, for aggregate prone or misfolded proteins; ribophagy, for ribosomes; and xenophagy, for bacteria and viruses (see the review of Johansen and Lamark [9]).

Induction Mechanisms of Autophagy

As autophagy is induced by starvation conditions, mammalian target of rapamycin (mTOR), which acts as a nutrient sensor and also controls protein synthesis and cell proliferation [10], is believed to be involved in autophagy induction. The two mTOR complexes (mTORCs) are conserved in mammals: mTORC1 and mTORC2. Although both complexes are thought to be regulated by growth factors, only mTORC1 is associated with cellular nutrient status. Thus, treatment with rapamycin, which inhibits mTORC1, induces autophagy. Although rapamycin is widely used for the investigation of mTORC1, Torin1 is highly potent and is more sensitive to both mTORC1 and mTORC2 [11].

In nutrient-rich conditions, mTORC1 suppresses autophagy by phosphorylating the ULK1 (unc-51-like-kinase 1, the mammalian homologue of yeast Atg1) protein complex, which consists of ULK1, ATG13, FIP200, and ATG101. Under starvation conditions, autophagy is thought to be triggered by the activation of ULK1, as a result of the dissociation of mTORC1 from the ULK1 complex. ULK1 is also regulated by AMP-activated protein kinase (AMPK), a serine/threonine kinase. AMPK senses the energy status within the cell by determining the AMP:ATP ratio, becoming activated when the energy status is low. Autophagy is also activated by low oxygen levels, stress, DNA damage, and depletion of hormones and growth factors. In addition, many more components are involved in the processes linking these factors and mTORC1. For current information on this topic, see also the recent review by Jewell and Guan [12].

Identification of *ATG* Genes and their Use in Autophagy Research

One of the reasons behind the recent expansion of autophagy research is the discovery of the *ATG* genes, which are a set of genes associated with autophagy. More than 30 *ATG* genes have thus far been identified, mostly through genetic studies of the yeast, *Saccharomyces cerevisiae* [13–15]. Many of these genes are conserved in eukaryotes, including those of humans. One of the *ATG* genes is microtubule-associated protein 1 light chain 3 (MAP1LC3/*LC3*), a homologue of yeast *Atg8*. This protein binds stably and specifically to the autophagosome membrane [16]. Thus, studying the localization and translocation of LC3 fused with fluorescent proteins such as green fluorescent protein (GFP) has become a standard procedure for observing autophagy induction. With the development of transgenic mice that ubiquitously express GFP-fused LC3 (GFP-LC3) [17], it has become possible to monitor autophagy in organs and tissues at the level of the organism.

Other members of the ATG family, *Atg5* and *Atg7*, are responsible for the extension of the isolation membranes and the formation of autophagosomes. An important contribution to the unknown functions of autophagy in eukaryotes has come from the mutation of these fundamental genes. For example, the genetic disruption of *Atg5* and *Atg7* completely represses autophagy. Thus, the development of *Atg5* and *Atg7* conventional knockout mice has opened up the possible study of the physiological functions of autophagy at the animal level. These conventional *Atg5* and *Atg7* knockout mice die immediately after birth due to extremely low energy production [5,18]. During pregnancy, fetuses acquire their necessary nutrients (e.g., amino acids) through the placenta. This situation ends with parturition, which suggests that autophagy is induced after birth to allow the organism to survive the short period of starvation caused by severance from the placenta. With the subsequent development of tissue-specific conditional *Atg5* and *Atg7* knockout mice, the functions of autophagy in each organ and tissue have become clear. For the physiological functions of autophagy in organs and tissues, see the current review of Mizushima and Komatsu [19].

The Role of Autophagy in Development and Differentiation

In lower organisms, the role of autophagy in the processes of development and differentiation has been studied for a long time [20]. In such organisms, the dysfunction of autophagy causes anomalies that are likely to be the result of inadequate material degradation. In *Caenorhabditis elegans*, autophagy is involved in the selective degradation of P granules in somatic cells [21]. On the other hand, the involvement of autophagy in the developmental and differentiation processes of eukaryotes remains unclear, particularly in the early stage of embryogenesis. In fact, this question was probably considered pointless, because the autophagy-deficient *Atg5* and *Atg7* knockout mice, though not viable after birth, do not show any developmental or differentiation anomalies. However, we have discovered that autophagy is highly induced during preimplantation embryo development (Fig. 2) [22], which may be explained in the following way. Conventional *Atg5* knockout mice, as discussed above, are generated by cross-breeding of heterozygotes ($Atg5^{+/-}$). Although the female mice produce haploid oocytes of either $Atg5^{+}$ or $Atg5^{-}$ genotype, the mature haploid oocytes are derived from primary oocytes of genotype $Atg5^{+/-}$. Consequently, even though the genotype of the ovulated oocyte is $Atg5^{-}$, its cytoplasm still contains Atg5 proteins derived from the $Atg5^{+}$ genome produced during oogenesis. These remaining protein products of $Atg5^{+}$ may be responsible for the autophagy that occurs after fertilization.

This effect of the maternal genotype is known as the maternal effect. It often becomes a blind spot for conventional knockout mice in which heterozygotes are crossed. To resolve this problem, we generated oocyte-specific conditional knockout mice that specifically lack autophagy in their oocytes. In these mice, it is possible to knockout the *Atg5* gene during oogenesis, which eliminates the maternal effect after fertilization. When such oocyte-specific conditional *Atg5* knockout females were crossed with *Atg5* heterozygous males, all the offspring were of the $Atg5^{+}$ genotype [22]. This indicates that all embryos fertilized by sperms with the $Atg5^{-}$ genotype (i.e., $Agt5^{-/-}$ zygotes) die sometime during embryogenesis. Further analyses revealed that $Atg5^{-/-}$ embryos are developmentally arrested at the 4-cell and 8-cell stages [22]. These results show that autophagy occurs after fertilization and is indispensable for preimplantation embryo development. As far as we have observed, autophagy does not seem to influence oogenesis or fertilization, since these processes are normal in the *Atg5* conditional knockout female.

The Reason for Embryonic Lethality in the Absence of Autophagy

Why does the autophagy-deficient embryo die before

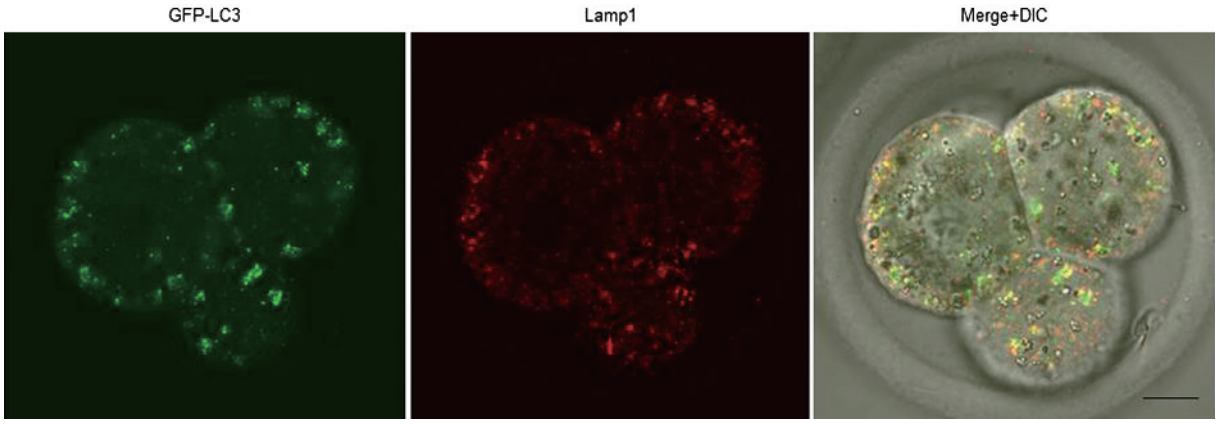

Fig. 2. Observation of autophagy in preimplantation embryos
To observe autophagy during preimplantation embryo development, mRNA encoding GFP-LC3 was microinjected into the cytoplasm of 1-cell embryos. These embryos were further cultured until the 4-cell stage, fixed, and then immunostained with anti-Lamp1 antibody. Fluorescence signals were captured under laser confocal fluorescence microscopy. The green and red dots represent autophagosomes and lysosomes, respectively. The yellow dots, which indicate the fusion of GFP-LC3 with lysosomes, represent autolysosomes. Differential interference contrast (DIC) images are also shown. The scale bar is 10 μm.

implantation? The development of preimplantation embryos immediately after fertilization is largely dependent on the maternal factors, which accumulate during oogenesis [23]. These maternal factors must be rapidly degraded to change the developmental program from that of a differentiated oocyte to a totipotent embryo, which is known as the oocyte-to-embryo transition, or the maternal-to-zygotic transition. It is reasonable to assume that autophagy is responsible for this degradation, because the amino acids generated by autophagic degradation may be used as materials for newly synthesized proteins, as well as nutrients for further embryonic development.

We confirmed that the rate of protein synthesis is low in autophagy-deficient embryos [24]. Therefore, we concluded that the low rate of amino acid synthesis is the cause of embryonic developmental arrest in the absence of autophagy. However, autophagy has a wide variety of physiological functions, which may also be important for early embryogenesis. The failure to degrade specific factors in the fertilized oocyte may cause developmental arrest. For example in mitophagy, depolarized and/or damaged mitochondria are selectively removed by autophagy. These abnormal mitochondria produce reactive oxygen species and cause cell death. Thus, mitophagy is thought to control the quality of mitochondria in the cytoplasm. In many eukaryotes, mitochondria are maternally inherited, indicating that paternal mitochondria, which are derived from spermatozoa (hereafter referred to as sperm mitochondria), are selectively eliminated. Sperm mitochondria disappear during preimplantation embryo development [25–27], and the ubiquitin-proteasome system could be involved in sperm mitochondria degradation in mammals [28, 29].

Recent elegant studies reported independently by the Sato and Galy groups, which used *C. elegans*, have revealed that sperm mitochondria are selectively degraded by autophagy induced immediately after fertilization [30, 31]. Galy's group has also shown that in mouse embryos, autophagy-related molecules are localized near sperm mitochondria [30], suggesting the preservation of this selective degradation system in the mouse embryo. In *C. elegans*, membranous organelles surrounding the paternal mitochondria are ubiquitinated after fertilization [30, 31], and this proximity is possibly related to the selectivity of autophagy. The selective autophagy of sperm mitochondria discovered by the Sato and Galy groups is named allophagy [32, 33]. Although the basic physiological functions of autophagy are evolutionarily conserved across species, from yeast to humans, the functions may be somewhat different in developmental and/or differentiation processes that involve dynamic morphological changes, particularly in early embryonic development.

Basal Autophagy for Cellular Quality Control

As mentioned above, the basal autophagy that occurs constitutively at a low level is important for cellular quality control. Interestingly, highly induced autophagy is not

observed in neurons, even in the starvation condition [17]. However, mice with a neuron-specific deficiency of autophagy accumulate abnormal protein aggregates in their neural cytoplasm, and display a neurodegeneration which is similar to that of Parkinson's disease and Alzheimer's disease [3, 4]. In the case of oocytes, though autophagy is highly induced soon after fertilization, autophagy is suppressed in ovulated oocytes; however, the underlying mechanisms and reasons for this are not yet known. Given that the cytoplasmic volume of the mature oocyte is larger than that of other mammalian cells, it is likely that basal autophagy is involved in oocyte quality control by accelerating the turnover of cytoplasmic contents.

In general, female fertility declines with advancing age [34]. Although the principal reason for this decline is the gradual depletion of the ovarian oocyte pool, the decrease in oocyte quality may also play an important role. The decline in oocyte quality is primarily determined at two levels: the genomic level and the cytoplasmic level. At the genomic level, oocyte quality decline is associated with chromosome instability, which results in the occurrence of aneuploidy. In humans, Down syndrome (trisomy 21) is a typical example of chromosome aneuploidy [35]. On the other hand, at the cytoplasmic level, abnormalities such as mitochondrial dysfunction and protein aggregate accumulation cause the decline of female fertility. Current research has revealed evidence for a decline in the level of basal autophagy with age [36], drawing attention to a possible correlation with the age-related decrease in oocyte quality.

Signaling Pathways and Factors Involved in Fertilization-triggered Autophagy

What are the regulators of the autophagy induced after fertilization? As described above, autophagy is generally regulated by an mTOR-mediated signaling pathway, and this pathway is preserved in many species, from yeast to humans. It is therefore possible that the mTOR pathway is involved in autophagy induction after fertilization. However, we cannot assume the sole involvement of mTOR, as other signaling cascades, such as the calcium-signaling pathway, are also activated immediately after fertilization. As previously mentioned, autophagy is suppressed in ovulated oocytes until fertilization occurs. Following fertilization, autophagy is highly induced within 4 h. Fertilization triggers the cyclic increases in intracellular calcium ions (Ca^{2+}), called Ca^{2+} oscillations, that continue during the interphase of the first cell cycle, ceasing at the time of pronuclei formation [37]. Thus, it is possible that the Ca^{2+} oscillation might be the trigger of autophagy after fertilization. However, autophagy induction occurs within 4 h after fertilization, whereas Ca^{2+} oscillations begin immediately. This implies that the induction of autophagy is controlled by a mechanism distinct from that of the Ca^{2+} oscillations. We found that autophagy is also induced by artificially triggered Ca^{2+} oscillations [22], which are achieved by treating an oocyte with strontium chloride. This indicates that the induction of autophagy after fertilization is controlled by factors or mechanisms that are oocyte in origin, rather than by factors of paternal (spermatozoa) origin.

The peak autophagic activity occurs at the middle of the 1-cell stage (around 12 h after fertilization). It is then completely suppressed during the first cleavage (from the late 1-cell to early 2-cell stages) and reactivated in the middle of the 2-cell stage. Cleavage is equivalent to the M phase of a cell cycle, in which the nuclear membrane is broken down and nuclear materials are dispersed within the cytoplasm. Autophagy might be strongly suppressed in this period to prevent the degradation of such nuclear materials. In relation to this, the Eggan group suggested that reprogramming factors are dispersed in the cytoplasm during the cleavage stage [38]. Taken together, the release of nuclear materials, such as reprogramming factors, during mitosis contributes to the regulation of autophagy activity through embryo development. In any case, it appears that autophagy induced after fertilization is tightly regulated, the mechanism of which will require elucidation in future studies.

The Dynamics and Functions of Lysosomes during Preimplantation Embryo Development

In the final stage of autophagy, cytoplasmic constituents isolated by autophagosomes are degraded inside autolysosomes. Staining of mouse embryos with LysoTracker Red, a specific probe for lysosomes, has revealed a number of lysosomes (Fig. 3) [39]. Interestingly, the size and number of lysosomes change before and after fertilization. These characteristics continue after fertilization, and lysosomes appear to display stage-specific morphology and localization during preimplantation embryo development.

Cathepsins are the major proteases within lysosomes. After the precursors of cathepsins are synthesized, they are transported to lysosomes while undergoing processing, before maturing in the acidic conditions of lysosomes. Though our analyses have revealed the presence of mature cathepsins in early embryos, from the unfertilized oocyte to the morula stage, the mature

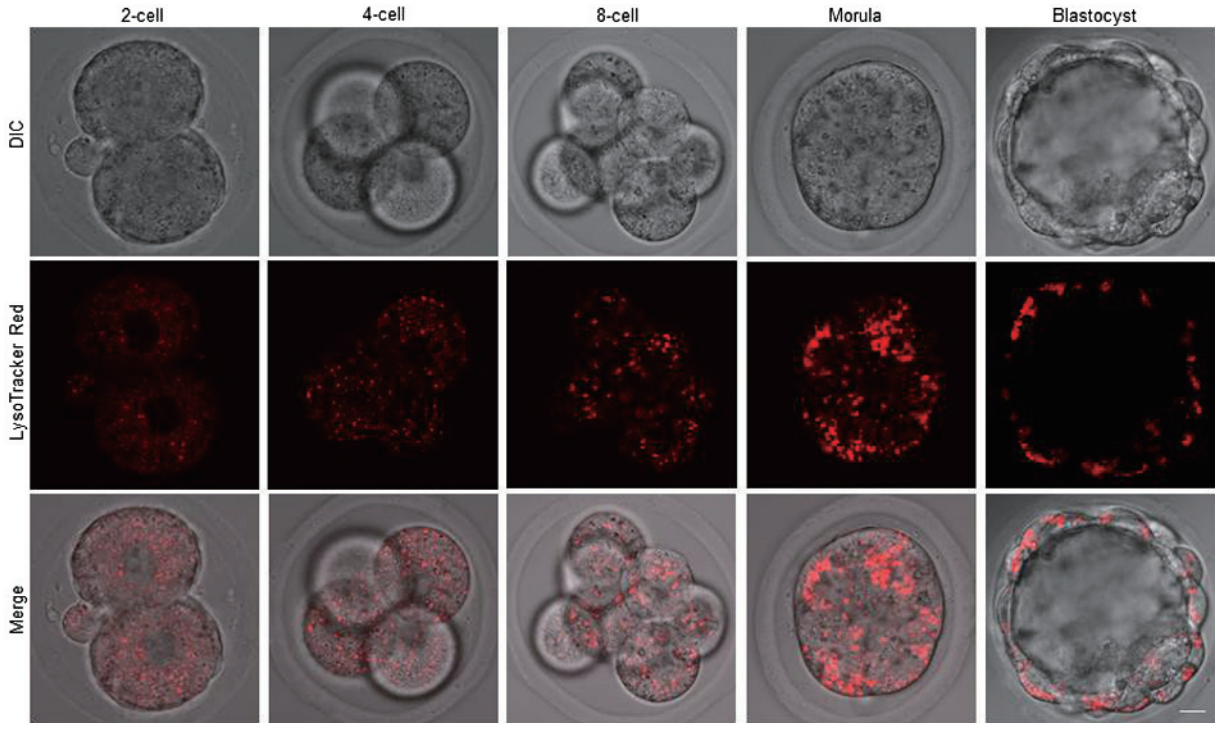

Fig. 3. Localization of lysosomes during preimplantation development
Lysosomes at the indicated developmental stages were stained with LysoTracker Red and observed under laser confocal fluorescence microscopy. The scale bar is 10 μm. Reproduced with permission of the Society for Reproduction and Development from Tsukamoto S, et al.: Functional analysis of lysosomes during mouse preimplantation embryo development. J Reprod Dev 2013; 59: 33–39 [39].

cathepsins are replaced by immature forms in blastocysts [39]. As the ratio of mature to immature cathepsins present in the blastocysts is comparable to that of other mammalian cells, e.g. mouse embryonic fibroblasts [39], it is possible that the activity of cathepsins is high from the early embryonic stage until the morula stage.

The reason for the increase in immature cathepsins between the morula and blastocyst stages is currently unknown. Changes in nutrient requirement and use are observed during the transition from the morula to the blastocyst stages [40]. Given that lysosomal activity is related to the nutrient condition, the change in the nutrient environment may influence the activity state of cathepsins. Our studies have shown that treatment of embryos with cathepsin inhibitor results in developmental arrest at the morula stage [39]. We also showed that a massive accumulation of lipofuscin-like structures occurs in the treated embryos [39], suggesting that cathepsin activity is constitutively high to promote the degradation of cytosolic constituents for the maintenance of cellular quality.

The Transcriptional Regulation of Autophagy and Lysosomal Biogenesis

Even though the components involved in the autophagy process have been rapidly identified in recent years, there is very little information on what regulates autophagy at the transcriptional level. The Ballabio laboratory recently reported that transcription factor EB (TFEB) is a master regulator of lysosomal biogenesis and autophagy [41]. TFEB regulates the functions of genes that possess coordinated lysosomal expression and regulation (CLEAR) motifs. CLEAR motifs have been found not only in the gene cluster of lysosome-associated membrane glycoproteins (such as Lamp1 and Lamp2), but also in some ATGs. In nutrient-rich conditions, TFEB is phosphorylated and mostly present in the cytoplasm, forming a complex with mTORC1 [42, 43]. In nutrient-poor conditions, TFEB becomes dephosphorylated and dissociates from mTORC1, entering the nucleus, and inducing the transcription of CLEAR motif-possessing genes. High amino acid levels also activate the small GTPase Rag, which binds and localizes TFEB to the lysosome via its

phosphorylation by mTORC1 [44]. We have observed the transient expression of TFEB in mouse early embryos (S.T., unpublished data), indicating a possible TFEB-mediated transcriptional regulation of lysosomal function and autophagy during preimplantation embryo development. However, as the regulation of autophagy and lysosome functions by TFEB requires multiple molecules functioning in concert, it is of great interest to determine whether these molecules operate in a similar manner in early embryos.

Conclusion

This review has described the physiological functions of autophagy in eukaryote development and differentiation, with a focus on early mouse embryonic development. In the processes of development and differentiation, cells alter their morphology dynamically from one state to another in short periods of time. One important requirement of these processes is to alter the cell's program in order to proceed to a new state. To do so, the cellular material and/or constituents of the original state must be broken down rapidly. Particularly in the fertilized oocyte, the majority of factors stored in the oocyte cytoplasm must be degraded because they are of maternal origin. Through the autophagy induced after fertilization, the maternal proteins are degraded in large amounts, providing amino acids that serve as essential nutrients for further embryonic development and materials for protein synthesis. This is a very effective process in which the oocyte cytoplasm is remodeled progressively in a self-sufficient manner.

The molecular mechanism of the induction of autophagy after fertilization remains unclear. There is a high possibility that this regulation is mediated by molecules and/or factors derived from the oocytes, since the other mammalian cells (somatic cells) lack the ability to stimulate fertilization-triggered signaling pathways. It has also become clear that even a widely distributed organelle, such as the lysosome, dynamically alters its morphology and function in the developmental processes of early embryos. The specific changes in the nutrient status during preimplantation embryo development are still largely unknown, especially the change in the amino acid levels. Recent studies have revealed amino acid sensors [45] and nutritional status-sensing molecules, and their dynamics in the process of embryogenesis is a point of interest. A better understanding of the function of autophagy in early embryos may allow us to provide new insights into mammalian preimplantation biology.

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