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Degradation of maternal factors during preimplantation embryonic development

Satoshi TSUKAMOTO[1)] and Takayuki TATSUMI[1, 2)]

[1)]*Laboratory Animal and Genome Sciences Section, National Institute for Quantum and Radiological Science and Technology, Chiba 263-8555, Japan*

[2)]*Comprehensive Reproductive Medicine, Regulation of Internal Environment and Reproduction, Graduate School, Tokyo Medical and Dental University, Tokyo 113-8519, Japan*

Abstract. During oogenesis, oocytes accumulate a large set of proteins derived from the maternal genome. These proteins, known as maternal proteins, are not only required for oocyte maturation and fertilization, but also implicated in subsequent embryonic development. However, most maternal proteins are degraded and their amino acid components are utilized for newly synthesized proteins from the embryonic genome. This process is known as the oocyte-to-embryo transition; because it occurs over a short period, mechanisms involving massive degradation of maternal proteins have been proposed. Intracellular protein degradation mechanisms can be broadly classified into two types. The first is the ubiquitin–proteasome system, a highly selective pathway in which ubiquitylated proteins are degraded by proteasomes. The second mechanism is autophagy, which involves lysosome-mediated degradation of cytoplasmic components. In this review, we describe recent advances in the understanding of autophagy, focusing on its role in early embryonic development.

Key words: Autophagy, Embryo, Lipid droplet, Mouse, Oocyte

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Autophagy

Macroautophagy (hereafter referred to as autophagy) is a pathway that leads to massive degradation of cytoplasmic components such as proteins and organelles via the lysosome [1]. Induction of autophagy leads to the formation of a double-membrane structure, known as the isolation membrane, which develops and engulfs cytoplasmic components as it elongates, forming a structure called the autophagosome. The outer membrane of the autophagosome then fuses with a lysosome to form the autolysosome. Ultimately, lysosomal enzymes degrade the cytoplasmic components isolated by the autophagosome (Fig. 1A). The internal environment of the lysosome is acidic, with a pH of approximately 5, and contains up to 50 types of digestive enzymes [2, 3]. Proteins and lipids can be degraded into amino acids and fatty acids, respectively, and the degradation products are reutilized within the cell. More than 30 autophagy-related (ATG) genes have been identified in yeast [4, 5]. ATG genes are generally conserved in a wide range of species from yeast to humans. Autophagy functions at a low, barely detectable level when nutrition is normal, but is highly induced when a deterioration in the nutritional state occurs. This starvation response is the most physiologically conserved aspect of autophagy among species.

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Correspondence: S Tsukamoto (e-mail: tsukamoto.satoshi@qst.go.jp)

In contrast, the low-activity state of autophagy (basal autophagy) is known to play a role in intracellular clearance (intracellular protein/organelle quality control), by degrading protein aggregates or damaged organelles not fully degraded by the ubiquitin–proteasome system. Autophagy-deficient neurons show accumulation of ubiquitin-positive protein aggregates and neurodegenerative disease [6, 7]. Thus, autophagy acts in concert with the ubiquitin-proteasome system. The fundamental physiological roles played by autophagy are nutrient provision as part of the starvation response and cytoplasmic quality control as described above. In recent years, autophagy has been suggested to play other roles in vital processes such as tumor suppression, bacterial infection, cell death, and aging [8, 9]. Selective autophagic processes were also recently identified, as described in detail below.

Embryonic Development and Autophagy

Mammalian post-implantation embryonic development is supported through intrauterine placental delivery of nutrients, during which autophagic activity is low [10]. However, studies have demonstrated highly active autophagy in neonates immediately after birth [11]. This autophagic activation is attributed to transient neonatal starvation following the abrupt cessation of placental nutrition. *Atg5* and *Atg7* are related to elongation of the isolation membrane, and systemic *Atg5* and *Atg7* knockout mice die within 1 day of birth, although they are born phenotypically normal [11, 12]. The plasma and tissue amino acid concentrations in these knockout mice are normal at the time of birth but greatly decrease thereafter. Based on these findings, survival in neonates may require energy utilization of amino acids provided through autophagy directly after birth.

Atg5 and *Atg7* knockout mice do not survive for long after birth,

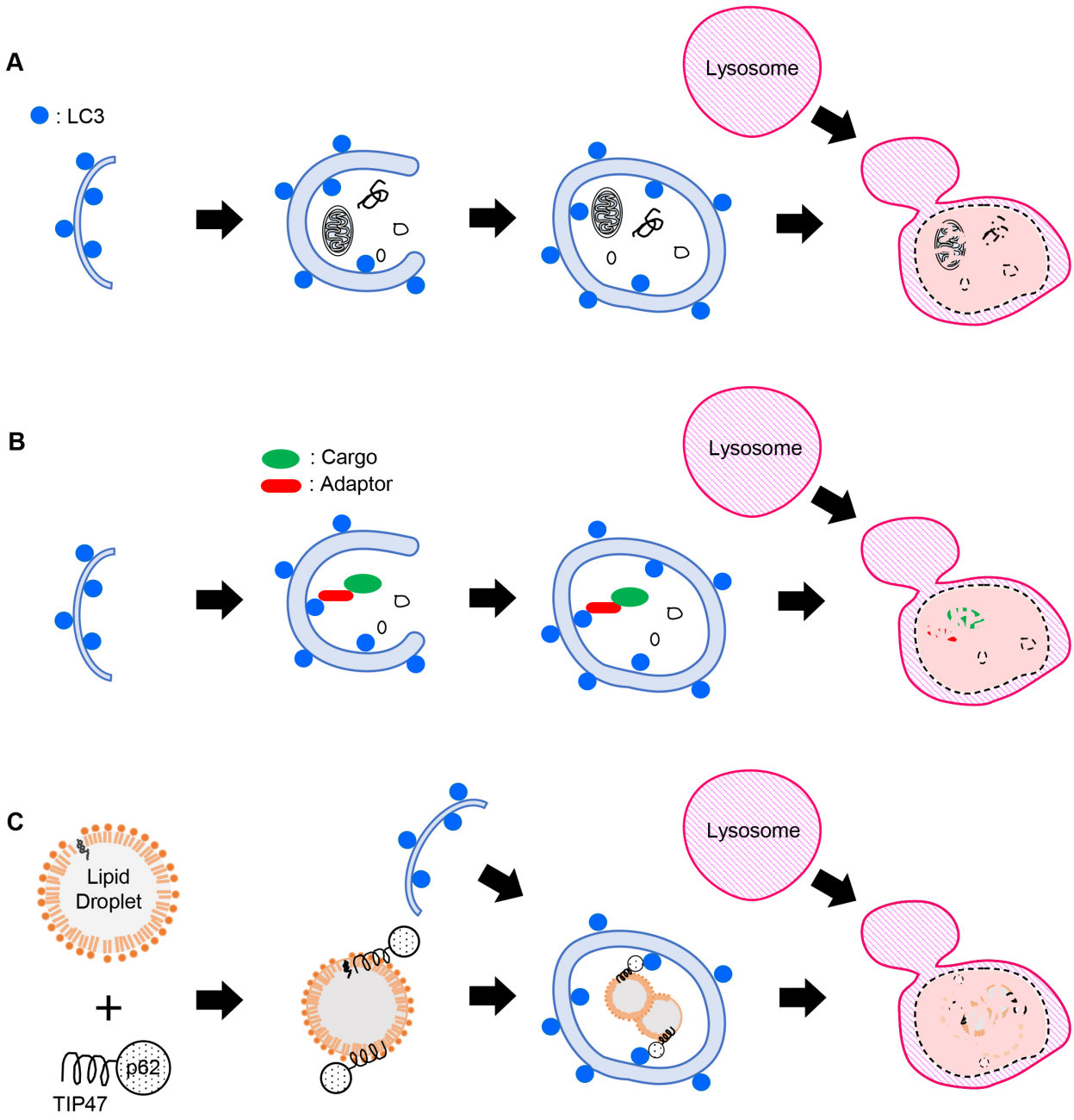

Fig. 1. (A) Overview of autophagy process. A double-membrane structure (isolation membrane) develops and encloses cytoplasmic contents, followed by formation of an autophagosome. The autophagosome then fuses with a lysosome to form an autolysosome, and lysosomal enzymes degrade the cytoplasmic contents isolated by the autophagosome. (B) Selective autophagy pathway. Autophagy adaptors recognize cargo (such as damaged organelles or misfolded proteins) and recruit the cargo into autophagosomes through a direct interaction with LC3. (C) Proposed model of forced lipophagy. To express an autophagy adaptor (p62) on the LD surface, fusion protein of p62 and TIP47 (LD coat protein) is overexpressed. p62 localized on the LD surface can be recognized as an autophagic cargo, facilitating the interaction with LC3 via LIR. LC3 associates stably with autophagosomes, forming a bridge between LDs and the membrane. The autophagosomal membrane then elongates to form the autophagosome, which encloses a portion of LDs, and fraction of LDs sequestered by the autophagosome is delivered to the lysosome for degradation.

as described above, but their phenotype is largely indistinguishable from that of wild-type mice. Accordingly, it was suggested that autophagy is not required for prepartum development. However, our subsequent research revealed that autophagy is highly induced shortly after fertilization [13]. The induction of such autophagy is fertilization-dependent, as demonstrated by the absence of autophagic activity from cultured ovulated oocytes. Autophagy has also been observed in parthenogenetic oocytes, indicating that autophagy is

induced through a fertilization-dependent, oocyte-specific mechanism. Interestingly, autophagy is rapidly activated within the first 3–4 h after fertilization and continues for a short period before it becomes largely undetectable from late in the 1-cell stage to early in the 2-cell stage [13]. A possible explanation for the reduction of autophagic activity in this phase is the need to avoid random degradation of nuclear factors crucial for embryonic development (such factors that may be exposed to degradation following nuclear membrane destruction that accompanies cell division). Autophagy is reactivated at the middle of the 2-cell stage and maintains a high activity level from the 4- to 8-cell stage before becoming largely undetectable at subsequent stages. Such post-fertilization variations in autophagic activity have also been demonstrated in *C. elegans* [14, 15]. Nevertheless, fertilization-induced autophagy, observed in *C. elegans*, has been detected only near sperm-derived mitochondria (as explained in the section "Selective autophagy during early embryonic development"). It is possible that the role of autophagy differs greatly between species.

Considering that a series of waves of embryonic genome activation begin as early as the 2-cell stage [16, 17], and major qualitative changes in the pattern of protein synthesis occur at the 4- to 8-cell stages in mice [18], maternal proteins stored in the oocyte can be rapidly degraded and replaced with embryonic-genome derived proteins during this period. Autophagy observed after fertilization is thought to be required for the conversion of proteins accompanying bulk degradation. However, the normal development in *Atg5* and *Atg7* knockout mice up to birth suggests that autophagy is not required after fertilization. The generation of *Atg5* and *Atg7* knockout mice requires mating between heterozygous (+/−) males and females. For this breeding pattern, the matured oocytes are from heterozygous (+/−) females. Although these oocytes have an Atg5-deficient genotype (Atg5−), both Atg5− and Atg5+ proteins are present during oogenesis because there is a period during which each oocyte is heterozygous (+/−). Even with a homozygous Atg5-deficient post-fertilization genotype (−/−), Atg5+-derived proteins are present in the oocytes [19]. Thus, the potential for autophagic functionality exists. We found that autophagy occurs in homozygous Atg5-deficient (−/−) embryos obtained through breeding of heterozygous mice [13]. This function of maternal proteins in the oocyte after fertilization is known as a maternal effect.

We thus generated mice in which this maternal effect was eliminated (oocyte-specific Atg5-knockout mice) to investigate of the actual role of autophagy soon after fertilization. Oocyte-specific Atg5 knockout mice showed normal oocyte growth, maturation, and fertilization, despite the complete lack of Atg5 protein. Thus, we considered that autophagy is not required for these processes. To investigate the offspring genomes, we then mated female oocyte-specific Atg5 knockout mice and male Atg5 heterozygous (+/−) knockout mice and found that all pups obtained were derived from wild-type sperm (Atg5+). These results show that complete autophagy-deficiency is lethal in embryos; however, sperm-derived Atg5 protein may affect rescue in some cases, even when autophagy is absent in early stages [19]. Detailed analysis of subsequent developmental stages showed that autophagy-deficient embryos were developmentally arrested at the 4- to 8-cell stage, before implantation [13]. Therefore, induction of autophagy after fertilization is essential for preimplantation embryonic development.

The cause of the embryonic lethality at the 4- to 8-cell stages remains unclear. One possible explanation involves the autophagic supply of amino acids required for embryonic development. Accordingly, we investigated the synthesis rates of new proteins during early embryonic development. We found that protein synthesis rates were normal up to the 2-cell stage but were significantly reduced in autophagy-deficient embryos at the 4- to 8-cell stages [13]. Thus, early embryonic development immediately after fertilization appears to be heavily dependent on protein synthesis, and the amino acids resulting from bulk autophagic degradation of maternal proteins are then utilized.

Mechanism for Induction of Autophagy Shortly After Fertilization

Starvation-induced autophagy is generally regulated by the serine-threonine protein kinase mammalian target of rapamycin (mTOR). mTOR is classified into two types of protein complexes, mTORC1 and mTORC2. Both mTORC1 and mTORC2 are large complexes (nearly 300 kDa), and each is separately controlled and has a unique function [20]. mTORC1 is involved in the induction of nutritional state-dependent autophagy [21]. mTORC1 suppresses autophagy when nutrients are available within the cell, but inactivated and autophagy is induced under nutrient-limited conditions. Our previous study revealed activation of mTORC1 prior to fertilization and a sudden subsequent shift to inactivation after fertilization. To investigate the induction or non-induction of autophagy under such conditions, we treated unfertilized oocytes (with high mTORC1 activity) with a specific mTORC1 inhibitor. We observed a decrease in mTORC1 activity, while autophagy was not observably induced [22]. The autophagosomal membrane contains the lipid phosphatidylinositol-3-phosphate (PI3P) [23]. The class III phosphoinositide-3 kinase (PI3K) produces PI3P and is known to be involved in elongation of the autophagosome membrane. In a subsequent study, we found that autophagy was completely suppressed within a short period after fertilization when embryos were treated with a PI3K inhibitor [22]. These findings demonstrated that fertilization-induced autophagy involves unique factors or a unique mechanism that does not dependent on mTORC1.

Correlation Between Autophagic Activity and Embryonic Development Viability

Is there a correlation between the level of autophagic activity in a fertilized embryo and its subsequent development? We predicted that differences exist in autophagic activity between embryos. Specifically, we hypothesized that high autophagic activity is associated with enhanced embryonic development capacity because of efficient degradation of maternal proteins. To investigate this hypothesis, we microinjected mRNA encoding GFP-LC3 (fusion protein of green fluorescent protein and microtubule-associated protein 1 light chain 3, which is associated with inner- and outer-autophagosomal membrane, thus serving as a marker for autophagosomes) and monitored the total fluorescence level of GFP-LC3 through embryo development. As GFP-LC3 stably associated with the autophagosome, GFP-LC3 itself was degraded by autophagy following subsequent fusion of

the autophagosome with the lysosome [24]. GFP fluorescence level measurements thus enable visualization of autophagic activity in viable embryos. We observed a strong fluorescent signal at the 2-cell stage on the day after microinjection, and this signal was significantly decreased at the 4-cell stage [25]. The fluorescent signal was not decreased in autophagy-deficient embryos or those with suppressed lysosome function, indicating that GFP-LC3 in embryos is abruptly degraded by autophagic activity.

Interestingly, some embryos showed the same fluorescence level at the 2-cell stage. Upon reaching the 4-cell stage, there were individual differences in fluorescence levels between these embryos. We used an imaging apparatus to determine the fluorescence level and classified embryos as having high or low autophagic activity. We then transferred these embryos into a foster mother. Those that received high-autophagic-activity embryos produced larger litters [25]. These results revealed a correlation between autophagic activity in embryos and subsequent embryonic development. Based on these results, autophagic activity is a potential indicator of embryo quality.

Selective Autophagy During Early Embryonic Development

Autophagy was originally regarded as non-selective but advances in research have revealed selective processes [26, 27]. Here, we describe the molecular mechanism of mitophagy as an example of a selective process. Mitophagy is the selective autophagic degradation of mitochondria, or more specifically a pathway for eliminating damaged mitochondria through selective autophagy. In mammalian cells, the protein PTEN-induced kinase 1 (PINK1) is stably expressed on the outer membrane of depolarized mitochondria (i.e. those with decreased membrane potential). PINK1 targets and recruits the ubiquitin E3 ligase Parkin, which translocates to the mitochondrial membrane and ubiquitylates membrane proteins [28–30]. The ubiquitylated proteins mark the mitochondria for degradation by autophagy. Mitophagy is considered a quality control mechanism for intracellular mitochondria, which proactively eliminates damaged and surplus mitochondria. Selective autophagy substrates (termed adaptors) fulfill an important role in such selectivity (Fig. 1B). Sequestosome1 (p62/Sqstm1; hereafter p62) is the most extensively researched of these adaptor proteins. p62 possesses a C-terminal ubiquitin-associated domain and LC3 interacting region (LIR) [31]. Accordingly, p62 is considered an adaptor molecule that brings some ubiquitylated proteins and organelles to autophagosomes. Because p62 binds directly to LC3 via the LIR, p62 is itself degraded by autophagy [32]. In addition to functioning as an adaptor molecule, p62 regulates multiple signals (e.g. in cell proliferation and division, and stress response) [33]. Thus, it is possible that selective autophagy is a controlled process that dependents on changes in the intracellular environment. Other than the mitochondria, peroxisomes, endoplasmic reticulum, and lipid droplets (LDs) are degraded by selective autophagy [34]. One very striking feature of nearly all currently identified adaptor proteins is that they contain an LIR, indicating that an LIR-mediated recognition mechanism is involved in selective autophagic degradation.

The role of selective autophagy in embryonic development has gradually been revealed. The research groups of Sato and Gary reported independently that sperm-derived paternal mitochondria were eliminated by fertilization-induced autophagy in *C. elegans* [14, 15]. Recent reports have also implicated adaptor molecules or kinases in this selective degradation [35]. Although the maternal inheritance of mitochondria is well-known, elimination of paternal mitochondria through post-fertilization selective autophagic degradation is a newly proposed mechanism. Subsequent research in this area has targeted embryos in mammalian species and suggested that the mechanism involving either the ubiquitin–proteasome system, selective autophagy (mitophagy), or both regulates elimination of the paternal mitochondria [36, 37]. However, the selective autophagy observed in *C. elegans* has not been clearly demonstrated in mammals. Possible mechanisms involve other degradation pathways or coordination between such pathways and autophagy, and we anticipate future progress in research in this area.

Degradation of LDs by Forced Lipophagy is Detrimental to Embryonic Development

LDs are structures with a lipid ester (mainly triacylglycerol or cholesteryl ester) core surrounded by a phospholipid monolayer. The surface layers contain LD-native proteins, of which Perilipin family proteins (Plin1, Plin2/adipophilin/ADRP, Plin3/TIP47, Plin4/S3-12, and Plin5/OXPAT) are representative examples. Recent studies demonstrated that LDs are dynamic organelles involved in proactive intracellular lipid metabolism, rather than simple inactive lipid-storing structures [38, 39]. LDs are present in tissue throughout the body, but their size and composition vary greatly. The roles of LDs include their utilization as a substrate for intracellular steroid hormone synthesis and energy production and vary according to the tissue in which they are located. LDs also accumulate in the oocyte during oocyte maturation [40]. The content of LD in oocytes vary greatly between species; the level in pigs is at least 6-fold higher that in mice [41]. Recent studies revealed the distribution of LDs in mouse oocytes/embryos and demonstrated that LDs are mostly detected as clustered structures before fertilization, but LDs tend to be dispersed throughout the cytoplasm within a short time after fertilization [42, 43]. Generally, LDs show slight dispersal and are very efficiently converted into fatty acids in the cell, and may perform some function after fertilization. We investigated the distribution of LDs using transgenic mice expressing GFP-ADRP developed in our lab. As expected, we observed a major change in LD dynamics after fertilization (ST, unpublished data). However, because numerous genes are involved in LD metabolism and there is a lack of specific inhibitors, the role of LDs in developing embryos remained unclear.

Next, we developed an approach for investigating the effect of LDs on embryonic development. The inspiration for this approach stemmed from the molecular mechanism of selective autophagy described above (Fig. 1B). The approach involves artificial degradation of LDs designed with highly active autophagy induced after fertilization. The underlying principle of this technique is as follows. The autophagy adaptor protein p62 is localized to LDs, and the marked LDs are recognized by autophagosomes (through an interaction of LC3 on the autophagosomal membrane), forming a bridge between LDs and the membrane. The autophagosomes enclose a portion of LDs and are ultimately subjected to lysosomal degradation (Fig. 1C). Our initial evaluations using cultured mammalian cells revealed an

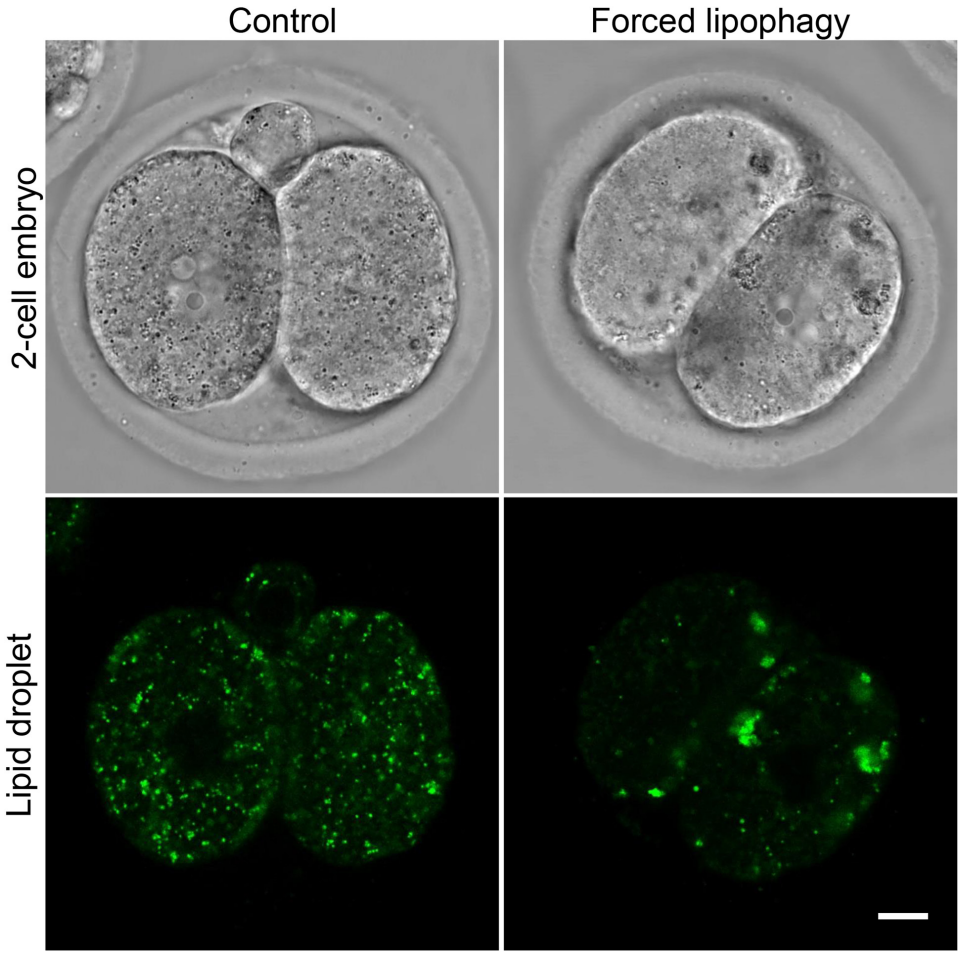

Fig. 2. Two-cell embryos microinjected at the 1-cell stage with mRNA encoding either TIP47-mCherry (control) or TIP47-mCherry-p62 (forced lipophagy-induced) were stained with LD dye: BODIPY 493/503 and observed by laser confocal fluorescence microscopy. Scale bars, 10 μm.

autophagy-dependent decrease in p62-localized LDs [44]. We named this developed method as "forced lipophagy". We then investigated the induction of forced lipophagy immediately after fertilization. LDs were expected to be dispersed and localized, but we found that they re-aggregated and migrated near the cell membrane in forced lipophagy-induced embryos (Fig. 2). The area to which they migrated contains abundant lysosomes, and thus LDs migrated for proactive degradation. Forced lipophagy-induced embryos showed a decrease in actual intracellular lipid content to approximately half that in normal embryos [44]. Furthermore, forced-lipophagy embryos showed a significantly lower blastocyst developmental rate than normal embryos. Interestingly, removal of amino acids from the culture medium caused a substantial decrease in blastocyst developmental rate for normal embryos but no decrease was observed this condition for forced lipophagy-induced embryos [44]. Based on these findings, embryonic development up to the blastocyst stage requires an existing pool of LDs in the oocyte. Additionally, a molecular mechanism may exist that enables reutilization of fatty acids produced from forced lipophagy in response to changes in the culture conditions (e.g. nutritional depletion, stress).

LD contents in mouse and human embryos are low and their functions have not been widely examined. However, further in-depth studies may reveal a new nutrient provision system which exploits LDs. The role of LDs in embryonic development may differ between species, explaining the large species differences in LD levels.

Conclusions

In preimplantation embryonic development, maternal proteins are promptly degraded, and embryonic genome-derived proteins are expressed. Considering that this expression involves the synthesis of new proteins, the balance between degradation and synthesis appears to be under sophisticated control. Particularly, focusing on degradation highlights the role of autophagy. Autophagy is highly activated to result in massive degradation of maternal proteins, and the degradation products become nutrients and raw materials for subsequent embryonic development. There are two degradation systems: the ubiquitin–proteasome pathway and autophagy. These systems are thought to maintain early embryonic development, as the presence of maternal and paternal factors to be degraded is

stage-specific after fertilization. We found that normal embryonic development requires LDs stored in oocytes. LDs may play a role as an energy source in embryonic development, although their content in human and mouse embryos are low. We anticipate that basic research in mouse preimplantation embryos such as that described here will contribute to the understanding of the mechanisms of human infertility.

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