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—Original Article—

Functional Analysis of Lysosomes During Mouse Preimplantation Embryo Development

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Abstract. Lysosomes are acidic and highly dynamic organelles that are essential for macromolecule degradation and many other cellular functions. However, little is known about lysosomal function during early embryogenesis. Here, we found that the number of lysosomes increased after fertilization. Lysosomes were abundant during mouse preimplantation development until the morula stage, but their numbers decreased slightly in blastocysts. Consistently, the protein expression level of mature cathepsins B and D was high from the one-cell to morula stages but low in the blastocyst stage. One-cell embryos injected with siRNAs targeted to both lysosome-associated membrane protein 1 and 2 (LAMP1 and LAMP2) were developmentally arrested at the two-cell stage. Pharmacological inhibition of lysosomes also caused developmental retardation, resulting in accumulation of lipofuscin. Our findings highlight the functional changes in lysosomes in mouse preimplantation embryos.
Key words: Cathepsin, Lipofuscin, Lysosome, Lysosome-associated membrane protein (LAMP), Preimplantaion embryo
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Lysosomes are specialized intracellular organelles. Because lysosomes function at pH 4.5 to 5, they use vacuolar proton ATPase pumps to maintain acidic conditions [1]. Lysosomes typically constitute 0.5% to 5% of the cell volume [2] and contain many enzymes, which are all classified as acid hydrolases. Among them, lysosomal proteases, which belong to the aspartic, cysteine or serine proteinase families, catalyze the hydrolysis of proteins. Many lysosomal proteases are ubiquitously expressed, like the aspartic proteinase cathepsin D and the cysteine proteinases B, H and L; others exhibit more tissue-specific expression patterns. Lysosomes receive degraded substrates through several pathways, including endocytosis, phagocytosis and autophagy (review by Saftig and Klumperman [3].) Accumulating evidence has revealed that lysosomal function is not restricted to degradation; lysosomes participate in many other physiologic processes. Therefore, mutation of the genes involved in lysosomal function can lead to lysosomal dysfunction and disease; examples are Danon disease and lysosomal storage disorders [3]. Despite the progress in our understanding of lysosomal function, there have been very few studies of the role of lysosomes in mammalian early embryo development.

In most species, fertilization triggers a series of cytoplasmic events, likely though Ca^{2+} oscillation. Considering that these events occur rapidly, bulk degradation systems could be an ideal way of eliminating the cytoplasmic contents, including mRNAs, proteins and organelles, over a short period of time. This could advance the transformation of the highly differentiated oocytes to totipotent zygotes and may be important for further embryo development. We recently showed that autophagy, in which the cytoplasmic contents are engulfed by autophagosomes and delivered to the lysosomes [4], is highly induced after fertilization and is essential for preimplantation development [5]. This highlights the great importance of lysosome-mediated degradation in early embryo development. However, the distribution and function of lysosomes during preimplantation embryo development remain unclear.

The focus of this study was lysosomal function during preimplantation embryo development in the mouse. We analyzed the distribution of lysosomes and the expression pattern of cathepsins B and D during preimplantation embryo development. We further examined the effect of lysosomal dysfunction on embryo development by using genetic knockdown and pharmacological inhibition. We demonstrated that the character of lysosomes varies during preimplantation development and that lysosomal dysfunction has adverse effects on preimplantation embryos.

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Materials and Methods

Embryo collection and culture

For *in vitro* fertilization, 8- to 12-week-old female C57BL/6J

mice (SLC, Shizuoka, Japan) were superovulated by intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG, ASKA Pharmaceutical, Tokyo, Japan) followed 46 to 48 h later by intraperitoneal injection of 5 IU human chorionic gonadotropin (hCG, Asuka Pharmaceutical). Metaphase II (MII) oocytes were collected 14 to 16 h after the hCG injection, cultured in mHTF medium [6] and inseminated with spermatozoa collected from the cauda epididymis of >12-week-old C57BL/6J males. One-cell embryos were collected 3 h after insemination and used for microinjection. To inhibit lysosome function, one-cell embryos were cultured with either 10 μM E64d [7] and 10 μg/ml pepstatin A [8] (Peptide Institute, Osaka, Japan) or with 0.2 μM bafilomycin A_1 [9] (Wako Pure Chemical Industries, Osaka, Japan), each of which was diluted in KSOM-AA medium. Embryo culture was performed under paraffin oil (Sigma-Aldrich, St. Louis, MO, USA) in an atmosphere of 5% CO_2 in air at 37 C. All animal handling was approved by the Animal Care and Use Committee of the National Institute of Radiological Sciences (Chiba, Japan).

Fluorescence microscopy

To label lysosomes, oocytes or embryos were cultured with 100 nM LysoTracker Red (Molecular Probes, Eugene, OR, USA) for 30 min at 37 C. The oocytes or embryos were then washed twice in PB1 [6], transferred to glass-bottomed dishes (Matsunami Glass, Osaka, Japan) and immediately viewed under a laser confocal fluorescence microscope (FV1000, Olympus, Tokyo, Japan).

RNA preparation and microinjection

For microinjection, we used 10 μM lysosome-associated membrane protein 1 (LAMP1) small interfering RNA (siRNA) (sc-35790, Santa Cruz Biotechnology, Santa Cruz, CA, USA), lysosome-associated membrane protein 2 (LAMP2) siRNA (sc-35791, Santa Cruz Biotechnology) or negative Control siRNA-A (sc-37007, Santa Cruz Biotechnology). Before microinjection, the siRNAs were filtered using an Ultrafree-MC centrifugal filter (Merck Millipore, Billerica, MA, USA) to remove insoluble materials. Microinjection was performed under an inverted microscope (DMI3000B, Leica Microsystems, Wetzlar, Germany) equipped with a Leica micromanipulation system. Typically, 10 to 12 pl of RNA was injected into each 1-cell embryo. Injection needles were made from borosilicate glass capillaries using a P-97 micropipette puller (Sutter Instrument, Novato, CA, USA). Injection was completed within 20 min in each experiment.

Quantitative real-time PCR

For the siRNA experiment, total RNA from 20 pools of embryos injected with the siRNAs described above was extracted with a CellAmp Direct RNA Prep Kit (TaKaRa, Otsu, Japan) and directly subjected to real-time PCR using One-Step SYBR Premix Ex Taq II and a Thermal Cycler Dice Real Time System (TaKaRa). Primers for LAMP1 (MA072898, TaKaRa), LAMP2 (MA127844, TaKaRa) and β-actin (MA050368, TakaRa) were used. Primer sequences were obtained from the Perfect Real Time support system (http://www.takara-bio.co.jp/prt/intro.htm). Relative expression of LAMP1 and LAMP2 was calculated by using the comparative CT method and normalized against that of an internal control (β-actin).

Western blotting

For cathepsin B and D blotting, 400 embryos were collected, lysed in 10 μl cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) and boiled in 4× Laemmli sample buffer (Sigma-Aldrich). Half the volume of each sample was subjected to SDS-PAGE and blotted onto a PVDF membrane using a Trans-Blot Turbo system (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked in 5% skim milk in Tris-buffered saline (Bio-Rad Laboratories). Polyclonal anti-cathepsin B and monoclonal anti-cathepsin D antibodies (Santa Cruz Biotechnology) were used at 1:500 dilution. Actin was detected by using anti-actin antibody conjugated with horseradish peroxidase (GenScript, Piscataway, NJ, USA). Signals were obtained using a ChemiDoc-It imaging system with a BioChemi camera (UVP, Upland, CA, USA); signal intensity was analyzed with VisionWorks software (UVP).

Electron microscopic analysis

For electron microscopic analysis, we used embryos treated with E64d and pepstatin A or injected with a mixture of LAMP1 and LAMP2 siRNAs. Embryos were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer (PB) (pH 7.4) for 30 min at room temperature. After being washed three times (for 5 min each time) with PB containing 3 mg/ml bovine serum albumin, the fixed embryos were placed on a cover glass and embedded in agar. The specimens were washed with PB, postfixed in 2% OsO_4 in PB for 60 min at 4 C, and washed again with PB. The specimens were dehydrated in a series of graded ethanol solutions and embedded in epoxy resin. After being stained with uranyl acetate and lead staining solution (Sigma-Aldrich), ultrathin sections were viewed under a JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

Results

Lysosome distribution in ovulated oocytes and preimplantation embryos

To investigate whether lysosome morphology changes after fertilization, we collected MII oocytes, one-cell embryos and two-cell embryos and stained them with LysoTracker Red, which labels lysosomes with high specificity. Lysosomes in the MII oocytes were small, and those in one-cell embryos were slightly larger. Notably, lysosome size was considerably increased in the two-cell embryos (Fig. 1A). Lysosome abundance gradually increased from the two-cell stage onward and peaked at the morula stage. At the blastocyst stage, the amount of lysosomes was slightly lower than at the morula stage (Fig. 1B). These results indicate that lysosome size and number change dynamically during early embryo development.

Maturation of lysosomal enzymes during preimplantation embryo development

Cathepsins are proteases that are the main class of lysosomal hydrolases. Most, if not all, cathepsins are transported from the Golgi via late endosomes to the lysosomes. During this transportation, inactive (immature) proenzymes are converted to active (mature) enzymes through a series of proteolytic processes (reviewed by Reiser *et al.* [10]). Therefore, we considered that cathepsin maturation could be a good marker of the status of lysosomal function during preimplantation

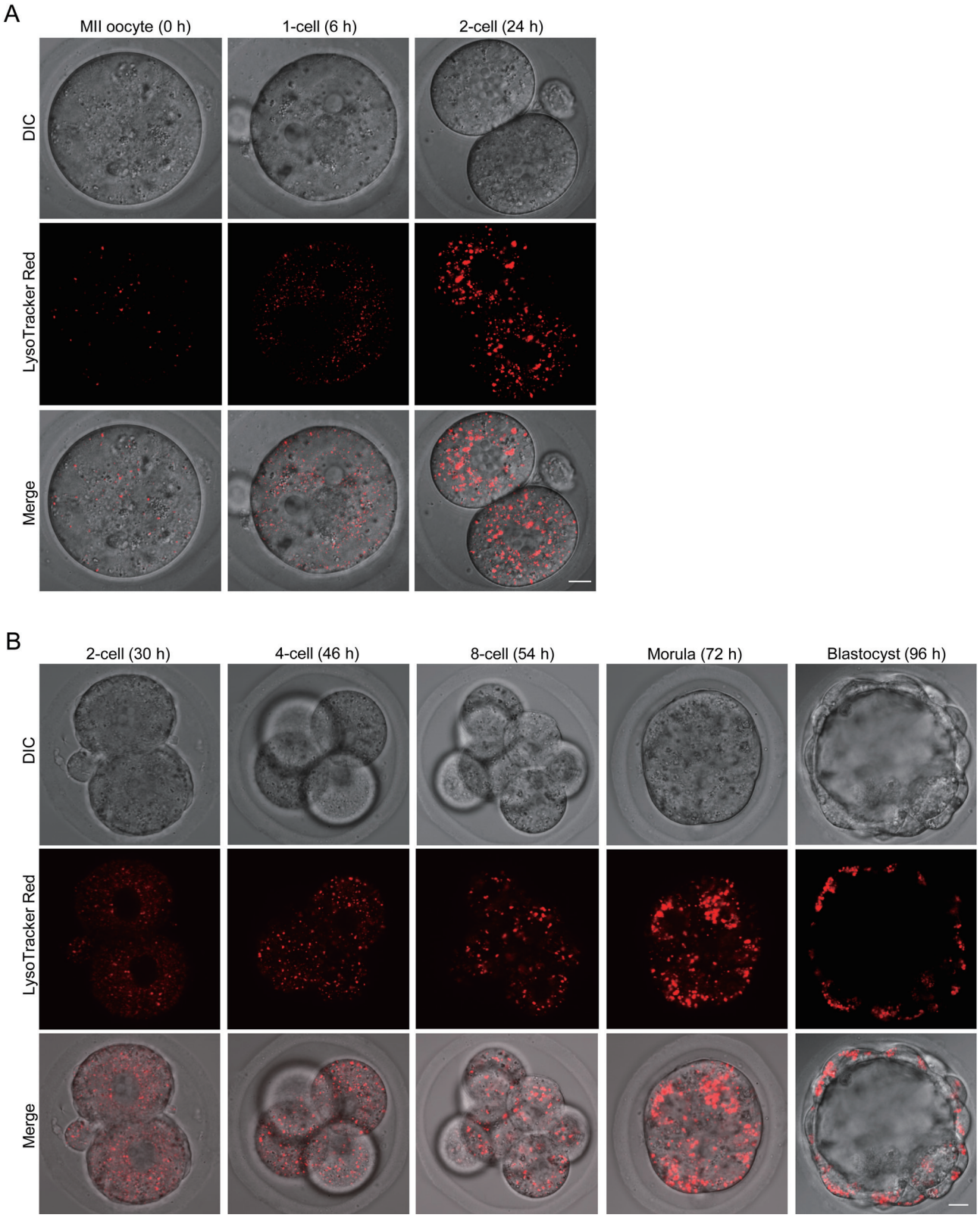

Fig. 1. Distribution of lysosomes during preimplantation embryo development. A: Lysosome distribution before and after fertilization. B: Lysosome distribution during preimplantation embryo development. Differential interference contrast (DIC) images of LysoTracker Red-stained oocytes and embryos at different developmental stages. The scale bar is 10 μm.

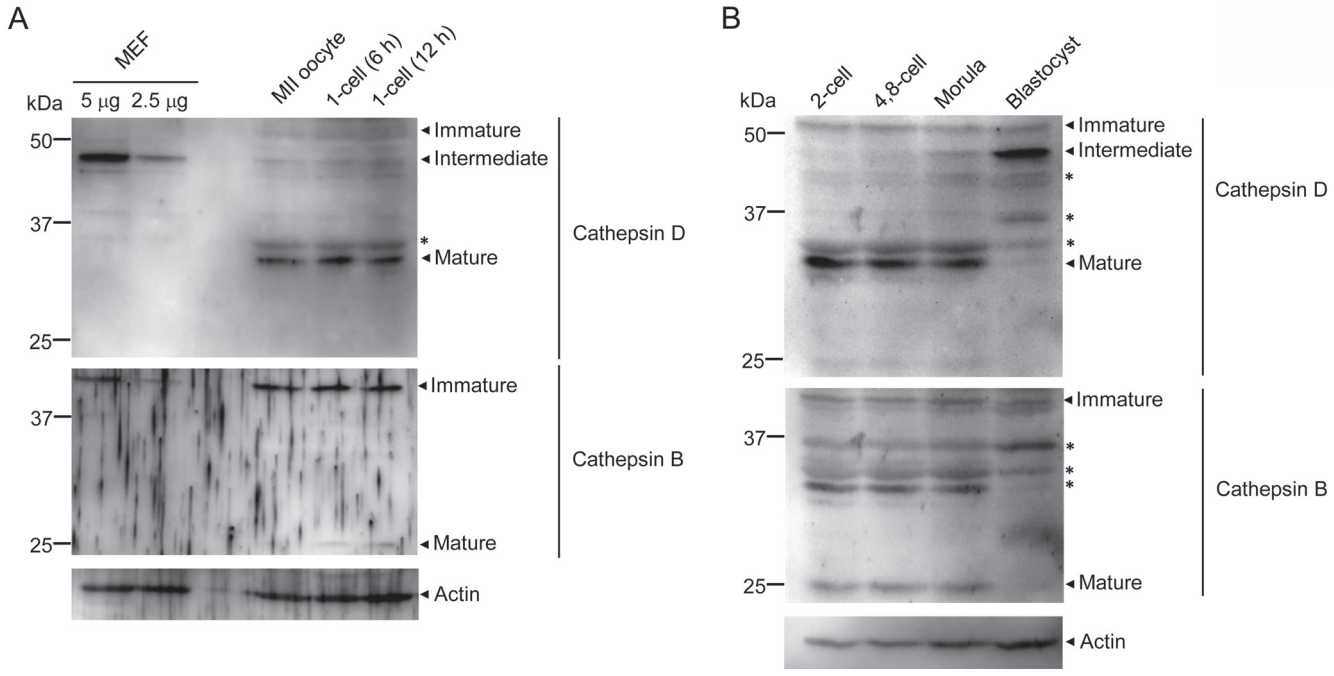

Fig. 2. Changes in cathepsin maturation during preimplantation embryo development. A: Cathepsin maturation before and after fertilization. Whole-cell lysates from MII oocytes or *in vitro* fertilized oocytes were loaded and blotted with anti-cathepsin B or D antibodies. MEF represents the lysates from mouse embryonic fibroblasts. B: Cathepsin maturation during preimplantation development. Whole-cell lysates were loaded and blotted with anti-cathepsin B or D antibodies. Asterisks show nonspecific bands. Anti-actin antibody was used as a control.

development. In addition, using the UniGene database (http://www.ncbi.nlm.nih.gov/unigene/), we found that cathepsins B (the major cysteine protease) and D (the major aspartyl protease), which are the most abundant lysosomal proteases, are highly expressed in early embryos. We therefore analyzed the maturation and expression of cathepsins B and D during preimplantation development. We first collected MII oocytes and one-cell embryos 6 or 12 h after fertilization and performed Western blotting using anti-cathepsin antibodies. Expression of the mature form of cathepsin D (33 kDa) was high in MII oocytes and one-cell embryos compared with that of the immature form of cathepsin D (52 kDa; Fig. 2A). The mature form of cathepsin B (25 kDa) was present in MII oocytes and one-cell embryos. In contrast, mature forms of both cathepsin B and cathepsin D were barely detectable in cell lysates from mouse embryonic fibroblasts, although the immature forms of these enzymes were clearly detected (Fig. 2A).

As observed in MII oocytes and one-cell embryos, expression of the mature form of cathepsin D was high from the two-cell to morula stage, unlike that of the immature form, but it was greatly reduced in the blastocyst stage. In blastocysts, the intermediate form of cathepsin D (46 kDa) appeared instead of the mature form (Fig. 2B). Mature cathepsin B was detected from the two-cell to morula stage, whereas the amount was dramatically decreased in the blastocyst stage (Fig. 2B). These data suggest the functional conversion of cathepsins B and D during preimplantation development. These results also suggest that the disposition of lysosomes is functionally regulated during preimplantation embryo development.

Effects of inhibition of lysosomal function on embryo development

To assess the importance of lysosomal function during preimplantation embryo development, we first performed a knockdown experiment using siRNAs targeted to LAMP1 and LAMP2, which are the major components of the lysosomal membrane and are essential for lysosomal biogenesis [11]. About 30% to 40% of the embryos injected with either LAMP1 or LAMP2 siRNA developed normally to the blastocyst stage. Small populations of the embryos were developmentally arrested at the two-cell stage or died and fragmented (Fig. 3A and B). In contrast, about half of the embryos injected with a mixture of LAMP1 and LAMP2 siRNAs were developmentally arrested at the two-cell stage (Fig. 3A and B). About 75% of the embryos injected with control siRNA developed normally into blastocysts. Quantitative real-time PCR analysis confirmed that LAMP1 and LAMP2 mRNA expression was effectively suppressed by siRNA injection, and this effect was cumulative when both siRNAs were injected together (Fig. 3C). Electron microscopy showed that degenerated membrane structures were present in the embryos injected with both LAMP1 and LAMP2 siRNAs (Fig. 3D). These structures resembled autophagic vacuoles, which have been observed in LAMP1 and LAMP2 double-deficient tissues [12], indicating that siRNA injection effectively inhibited the function of LAMP1 and LAMP2 in early embryos.

To confirm that the developmental retardation observed in siRNA-injected embryos was due to lysosomal dysfunction, we treated embryos with the lysosome inhibitors E64d and pepstatin A, which block the major lysosomal proteases cathepsins B, H and L (E64d)

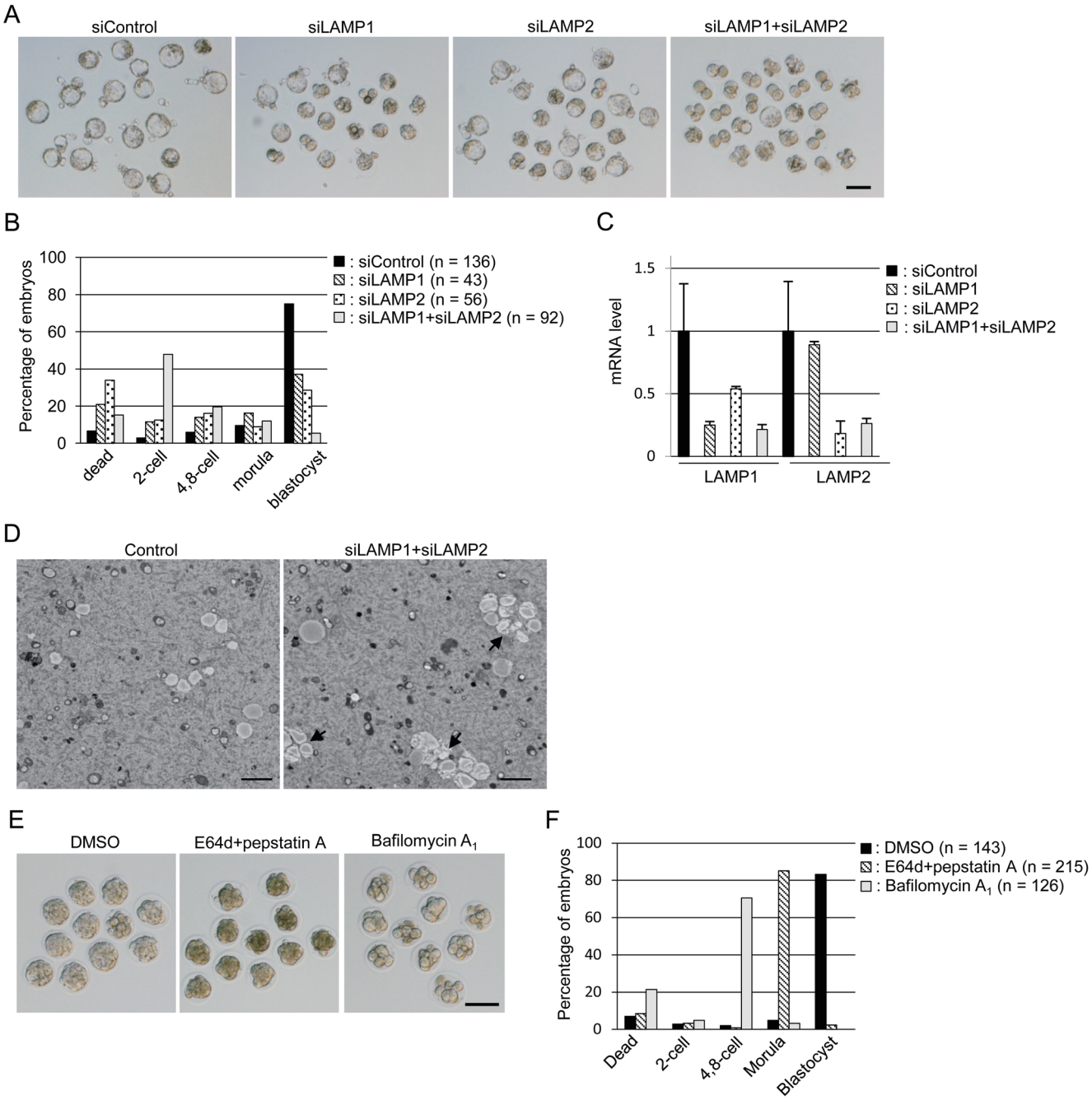

Fig. 3. Lysosomal dysfunction results in retardation of embryonic development. A: Blastocyst-stage embryos developed after injection of one-cell embryos with either LAMP1 or LAMP2 siRNA or a mixture of the two. Control siRNA-injected embryos (siControl) were used as injection controls. The scale bar is 100 µm. B: Percentages of embryos that developed successfully to the blastocyst stage. C: Levels of expression of LAMP1 and LAMP2 mRNAs in two-cell embryos developed from one-cell embryos injected with either LAMP1 or LAMP2 siRNA or a mixture of the two. siControl-injected embryos were used as controls. mRNA expression was normalized relative to actin mRNA levels. Data are means from two independent experiments. D: Electron microscopic analysis of two-cell embryos injected with both LAMP1 and LAMP2 siRNAs at the one-cell stage. Abnormal membrane structures (arrows), resembling autophagic vacuoles, were observed in the siRNA-injected embryos. The scale bar is 2 µm. E: Morula-stage embryos cultured with the indicated chemicals from the one-cell stage onward. DMSO was used as a control. The scale bar is 100 µm. F: Percentages of embryos that successfully developed to the blastocyst stage. N represents the total number of embryos examined (B and F).

and D and E (pepstatin A) [7, 8]. One-cell embryos treated with E64d and pepstatin A developed normally to the morula stage but showed no further development (Fig. 3E and F). We next treated embryos with bafilomycin A_1, which selectively inhibits vacuolar proton ATPase [9], thus potentially disturbing intralysosomal acidification. We confirmed that the LysoTracker signals rapidly disappeared when

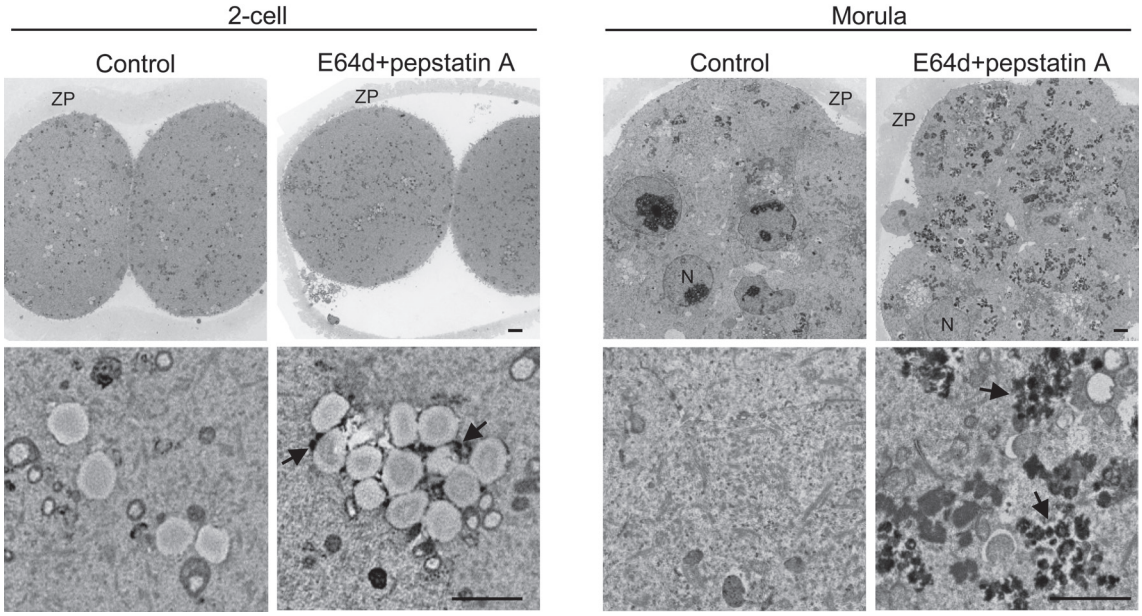

Fig. 4. Lysosome inhibition causes accumulation of lipofuscin during embryo development. Electron micrographs of two-cell- and morula-stage embryos after treatment with E64d and pepstatin A from the one-cell stage or after no treatment. Arrows represent lipofuscin-like granules. ZP, zona pellucida; N, nucleus. The scale bar is 2 μm.

the embryos were treated with bafilomycin A_1 (data not shown), indicating that bafilomycin A_1 effectively raised the lysosome pH. One-cell embryos treated with bafilomycin A_1 developed normally to the 4,8-cell stage, but their development thereafter was arrested and they fragmented (Fig. 3E and F). Preimplantation development was not affected when the one-cell embryos were treated with dimethyl sulfoxide (DMSO) as a control. Taking these findings together, we conclude that lysosomal dysfunction is involved in early embryonic death.

Lysosomal dysfunction causes massive accumulation of lipofuscin

To better understand the effect of lysosomal dysfunction at the ultrastructural level, we treated embryos with E64d and pepstatin A and analyzed them by electron microscopy. We found electron-dense materials close to vacuoles in the embryos at the two-cell stage (Fig. 4). Excessive amounts of these materials accumulated at the morula stage, whereas no visible accumulation was observed in untreated control embryos. Because lipofuscin accumulation may be involved in the decreased lysosomal activity that has been described in nondividing cells [13, 14], we speculated that these electron-dense materials were lipofuscin. We showed that the color of the embryos treated with E64d and pepstatin A changed to yellow-brown (Fig. 3E) during development. This color is an important property of lipofuscin [13], thus supporting the hypothesis that the electron-dense material was lipofuscin. These results indicated that lysosomal degradation was highly activated as early as the two-cell stage.

Discussion

Lysosomes are specialized organelles involved mainly in macromolecular degradation processes. There is accumulating evidence that lysosomes have many physiological functions. Mutations of many of the genes that are involved in lysosomal biogenesis have been shown to be associated with lysosomal dysfunction [3, 10]. In addition, nonmammalian and mouse models of these genes have been used to give an in-depth understanding of lysosomal biogenesis. However, little is known about lysosomal function in early mammalian embryos. We examined the role of lysosomes during preimplantation development in the mouse embryo. We found that the number of lysosomes increased after fertilization. Lysosomes were abundant in preimplantation embryos until the morula stage, but their numbers were slightly decreased in blastocysts. Consistent with these observations, the expression levels of mature cathepsins B and D were high from the one-cell to morula stage but low in the blastocyst stage. We also demonstrated that perturbation of lysosomal function was detrimental to preimplantation embryo development. Our findings suggest that lysosome biogenesis is essential for normal mammalian embryo development. Considering that lysosomes typically constitute 0.5% to 5% of the cell volume [2], early embryos contain a surprisingly large amount of lysosomes. This indicates that massive degradation via lysosomes may be highly activated in early embryos.

The amount of mature cathepsin was consistently high until the morula stage but decreased at the blastocyst stage (Fig. 2B), indicating that lysosomal function changed during this period. Consistently, the localization of lysosome at the blastocyst stage changes dynamically: lysosomes are scattered throughout the morula but then move to the periphery of the blastocyst (Fig. 1B). A recent study demonstrated that lysosomal positioning is dependent on cellular nutrient status [15]. Additionally, aspects of energy metabolism, such as glucose requirement and oxygen consumption, may change dynamically at the blastocyst stage [16], suggesting that lysosomal activity and

localization change with energy status during preimplantation development.

LAMP1 and LAMP2 are major protein components of the lysosome membrane [17]. LAMP1 knockout mice have a nearly normal phenotype [18], whereas LAMP2 knockout mice have elevated postnatal mortality and accumulation of autophagic vacuoles in several tissues [19]. In addition, LAMP1 and LAMP2 double-knockout mice die between embryonic days 14.5 and 16.5 [12]. However, we speculate that LAMP1 and LAMP2 are functional during preimplantation development, because transcription factor EB, a master protein that regulates lysosomal biogenesis [20], is highly expressed after fertilization (S.T., unpublished data). In the siRNA experiment, more than 40% of the embryos injected with a mixture of LAMP1 and LAMP2 siRNAs were developmentally arrested at the two-cell stage. Moreover, injection of a mixture of LAMP1 and LAMP2 siRNAs resulted in the observation of several degenerated membrane structures resembling autophagic vacuoles, which have been observed in several tissues in LAMP1 and LAMP2 double-deficient mice [12]. Our results therefore suggest that proper expression of both LAMP1 and LAMP2 is important for preimplantation embryo development.

Embryos injected with a mixture of siRNAs targeted to LAMP1 and LAMP2 were mainly arrested at the two-cell stage, whereas embryos treated with lysosomal inhibitors died at the morula stage (with treatment of E64d and pepstatin A) or the 4, 8-cell stage (with bafilomycin A_1 treatment). As described above, these inhibitors block lysosomal function in different ways; thereby, their inhibitory effects on embryo development might appear at different stages. These results indicate the pivotal role of lysosomes during preimplantation embryo development. However, the exact mechanisms underlying the phenotypes that we observed in the embryos injected with siRNAs or treated with lysosomal inhibitors remain unknown.

It is generally believed that a decrease in oocyte or embryo quality during maternal aging is a major cause of female infertility [21, 22]. One possible mechanism underlying this decline in quality may be a decrease in the ability to maintain efficient protein and organelle turnover, resulting in the accumulation of undigested materials such as lipofuscin. Lipofuscin accumulates in aged human oocytes [23]. Considering that these physiological changes are a hallmark of aging, we hypothesize that monitoring lysosomal activity might be a way of determining whether oocytes or embryos have relatively high or low developmental ability. Alternatively, strategies to regulate intracellular lysosomal activity might have therapeutic potential in improving embryonic development. Further studies are needed to determine whether lysosomal activity is related to developmental competence in mouse embryos.

Acknowledgments

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