Studies on autophagy during the embryonic and early neonatal periods

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Summary

Autophagy is a non-selective degradation process in which long-lived proteins and organelles are sequestered within double-membrane vesicles, termed autophagosomes that deliver the contents to the lysosome/vacuole for degradation. The lysosome/vacuole is an acidic compartment that contains various hydrolytic enzymes. Autophagy is fundamental and evolutionarily conserved function in eukaryotes. More than 20 genes, which are essential for autophagy (ATG genes; AuTophaGy), have been identified by genetic screens in yeast, and most of the ATG genes are conserved in higher eukaryotes.

The major function of autophagy is response to starvation. For example, autophagy is suppressed to undetectable levels under nutrient rich conditions in yeast, but it is rapidly induced under nutrient starvation conditions. In autophagy deficient mutants of yeast, the survival rate decreases under starvation conditions. Similarly, autophagy also plays a response to starvation in mammals. Autophagy is induced in many tissues in response to food withdrawal in adult mice. Autophagy-defective mutant mice, Atg5−/− mice, appear almost normal at birth, but most of the Atg5−/− neonates died within 1 day of delivery. I thought that phenotype of Atg5−/− mice is related to nutrient starvation. However, the kinetics of autophagy is unclear in wild-type embryos and neonates. In this study, to study the significance of mammalian autophagy in vivo, I observed occurrence of autophagy in embryos and neonates using transgenic mouse model in which autophagosomes are labelled with GFP-LC3.

I observed that autophagy remained at low levels throughout the embryonic period. Formation of autophagosomes was extensively induced in various tissues after a natural birth and is maintained at high levels for 3-12 h after birth. The number of autophagosomes gradually decreased to basal levels by day one or two. Soon after birth, mammals face with the first, and probably the most severe, starvation during their lifespan, because trans-placental nutrients supply is suddenly terminated. These results suggest that induction of autophagy is important for survival during neonatal starvation.

In mammal, embryos are under the nutrient rich conditions. However, I observed that low levels of autophagy occur constantly at embryonic period. It is
unclear that the basal autophagy plays an important role during mouse embryogenesis. In this study, I examined the role of basal autophagy using the autophagy indicator mice and autophagy deficient mice. In histological examination of $\text{Atg}5^{-/-}$ neonates, I detected ubiquitin-positive aggregates (inclusion bodies; IBs) only in a few tissues, including neural cells, hepatocytes, and anterior pituitary gland cells. These results suggest that function of autophagy is not only maintenance of viability during starvation, but also intracellular quality control. Recently, a similar observation was reported in the hepatocytes of liver-specific conditional $\text{Atg}7^{-/-}$ mice. I demonstrated that time from the liver genesis to the birth is sufficient to generate large IBs in hepatocytes. In addition, I revealed that protein quality control is highly dependent on basal levels of autophagy in hepatocytes, neural cells and anterior pituitary cells.

Autophagy can degrade not only proteins but also intracellular organelles such as mitochondria, peroxisomes and endoplasmic reticulum. Dramatic degradation of organelles is observed in the processes of lens organelle free zone (OFZ) formation. The mechanism by which these organelles are destroyed during this process is not fully understood. It is suggested that autophagy is involved in lens organelle degradation. However, organelle degradation during the differentiation of lens occurred normally in $\text{Atg}5^{-/-}$ mice. Therefore, autophagy is dispensable for OFZ formation in lens epithelial cells.

In this study, I showed significance of basal- and induced-autophagy during embryogenesis and neonatal starvation. These findings of in vivo analyses will help to understand physiological significance of autophagy in mammals.
**Introduction**

*Protein degradation in eukaryote*

Cell homeostasis is maintained by precisely regulated balance between synthesis and degradation of cellular components. In eukaryotic cells, intracellular proteins are degraded by two major processes; the ubiquitin-proteasome and the lysosome/vacuole pathway. The ubiquitin-proteasome pathway selectively degrades most intracellular short-lived, damaged or misfolded proteins (Hochstrasser, 1996; Hershko and Ciechanover, 1998). In this pathway, proteins are targeted for degradation by covalent ligation to ubiquitin, a highly conserved small protein. On the other hand, long-lived proteins and some cytoplasmic organelles are degraded within a specific compartment, the vacuole in yeast and the lysosome in mammalian cells (Klionsky and Ohsumi, 1999).

*Lysosome/vacuole protein transport*

The lysosome/vacuole is the major catabolic compartment in eukaryotic cells and contains a range of hydrolases capable of degrading most cellular constituents. This organelle is the terminal destination for endocytic, autophagic and secretory materials targeted for destruction. There are at least three different pathways for vacuole transport of cytosolic contents in yeast: Cvt (cytosol to vacuole targeting pathway) (Scott et al., 1996), Vid (vacuolar import and degradation pathway) (Shieh et al., 1998) and autophagy pathway (Takeshige et al., 1992). The Cvt and Vid pathway are found only in yeast. In the Cvt pathway, two vacuolar proteins, aminopeptidase I and α-mannosidase, are selectively transported to vacuoles. Under nutrient-rich conditions, this process is mediated by an autophagosome-like structure called a Cvt vesicle. In the Vid pathway, the gluconeogenic enzyme fructose-1, 6-bisphosphatase (FBPase) is rapidly degraded following a shift from low glucose condition to high glucose conditions. FBPase trafficks from the cytosol to the vacuole via Vid vesicles. Only autophagy pathway is an evolutionarily conserved process that occurs in virtually all eukaryotic cells, ranging from yeast to mammals.
**Autophagy**

Autophagy (from Greek auto, self, and phagos, to eat) was first described ultrastructurally as the sequestration of cytoplasm into closed, membrane-delimited vacuoles called autophagosome (de Duve and Wattiaux, 1966). Autophagy has three major pathways, chaperon-mediated autophagy (Schworer et al., 1981; Mortimore et al., 1989; Dunn, 1994), microautophagy (Ahlberg and Glaumann, 1985; Mortimore et al., 1988) and macroautophagy (Dice, 1990; Seglen and Bohley, 1992). Chaperon-mediated autophagy is the process in which substrates to be degraded are translocated across the lysosome/vacuole. Microautophagy, degradation substrates are engulfed by lysosome/vacuole directly. By contrast, macroautophagy (hereafter simply referred to as autophagy) is the main route to lysosomes/vacuole. Substrates are nonselectively encompassed by autophagosomes and delivered to lysosomes/vacuole.

**Autophagy pathway**

Autophagy is a membrane trafficking process that transports bulk cytoplasm and entire organelles to the lysosome (Figure 1). Autophagy is found ubiquitously in eukaryotes and involved in amino acids production, prevention of disease and aging (including removal of damaged organelles), regulation of metabolism and developmental pathway. Although autophagy occurs constitutively, it is induced under both extracellular stress conditions (nutrient deprivation, hypoxia) (Mortimore and Poso, 1986) and intracellular stress conditions (accumulation of damaged organelles, protein aggregated) (Ravikumar and Rubinsztein, 2004). The initial event in autophagy, triggered, for example, by nutrient starvation and hormones (such as glucagons) (Mortimore and Schworer. et al., 1975) is the sequestration and enclosure of a piece of cytoplasmic constituents, including organelles such as mitochondria, by one or more specialized membrane cisternae of uncertain origin, called isolation membrane. Closure of the isolation membrane results in formation of double membrane structures, called autophagosomes or initial autophagic vacuoles (AVi). Autophagosomes then fuse with endosomes, and eventually fuse with lysosomes to become autolysosomes or degradative autophagic vacuoles (AVd). Lysosomal hydrolases degrade the
Figure 1. A schematic overview of autophagy steps

Autophagy is a membrane trafficking process. Cytosol and organelles are enwrapped by membrane, termed isolation membrane. Afterwards, a double-membrane vesicle, the autophagosome, is formed. The autophagosome acquires hydrolytic enzymes by fusing with the lysosome to generate an autolysosomes, and the inner vesicle of autophagosome is released into the lumen. The resulting autophagic body is broken down.
cytoplasm-derived components of the autophagosome, together with its inner membrane.

**Molecular mechanism of autophagy**

Yeast genetic studies have identified more than 20 genes (AutophGy: ATG genes) required for autophagy, most of which function in autophagosome formation (Klionsky et al., 2003). Included in this group are two sets of components involving ubiquitin-like (Ubl) protein conjugation systems that are essential for autophagosome formation (Ohsumi, 2001). The first Ubl is Atg12, which is covalently attached to Atg5 (Mizushima et al., 1998; Mizushima et al., 2001; Mizushima et al., 2003). The C-terminal glycine residue of Atg12 is activated by Atg7 in ATP-dependent manner. Atg12 is then transferred to Atg10 to form a thioester again. The function of Atg10 likely is equivalent to that of E2 ubiquitin-conjugation enzymes. The C-terminal glycine of Atg12 is covalently attached to lysine 149 of Atg5 via an isopeptide bond. The second Ubl is Atg8, called LC3 in mammals, that undergoes proteolytic processing prior to modifying the lipid phosphatidylethanolamin (PE) (Ichimura et al., 2000; Kirisako et al., 2000). Following synthesis, the C-terminus of Atg8 is cleaved by a cysteine protease, Atg4, leaving a glycine residue at C terminus. Cleaved Atg8 is activated by an E1 protein, Atg7, which is shared with the Atg12 system. Atg8 is transferred subsequently to E2 enzyme Atg3. Atg8 is modified by PE on the C-terminus (LC3-II form) and binds tightly to autophagosomal membrane.

Formation of the Atg12-Atg5 conjugate is essential for proceeding of autophagy. The covalent modification of Atg5 with Atg12 is essential for elongation of the isolation membranes (Mizushima et al., 2001; see Figure 2). It was observed that Atg12-Atg5 conjugate initially associates with a small crescent-shaped vesicle evenly in mammalian cells. As the membrane elongates, Atg12-Atg5 associates with the outer side of the membrane asymmetrically. Finally, Atg12-Atg5 dissociates from the membrane upon completion of autophagosome formation. Furthermore, this conjugate is required for targeting of Atg8 to the isolation membranes. LC3, a mammalian homologue of yeast Atg8, was shown to localize on autophagosome membrane (Kabeya
Figure 2. Diagram of the autophagosome formation

Atg12-Atg5 conjugates initially associates with a small crescent-shaped vesicle evenly. When the membrane elongates, this conjugates associates with the outer side of the membrane asymmetrically. Finally, this conjugates dissociates with the membrane upon completion of autophagosome formation. LC3 was shown to localize on autophagosome membrane. LC3 remains on autophagosome even after Atg12-Atg5 conjugate dissociates. Dissociated Atg12-Atg5 and LC3 are reused, its associates with other isolation membrane again.
et al., 2000). LC3 remains on autophagosome even after Atg12-Atg5 conjugate dissociates, so that it is used as an autophagosome marker.

**Response to starvation**

In yeast, autophagy is suppressed to undetectable levels under growing conditions, but it is rapidly (within 30 min) induced during nitrogen starvation (Takeshige et al., 1992). Autophagy was first discovered under nitrogen starvation condition in the yeast. Then carbon, sulfate, phosphate, and even single auxotrophic amino acid depletion were also shown to induce the same membrane dynamics, although the extent of the response varied. Autophagy mutants of yeast have defect in bulk protein degradation in vacuoles during starvation. Furthermore, mutants lost its viability faster than wild-type cells in starvation conditions. The critical role played by autophagy in maintaining viability during starvation has been shown in several species, such as *S. cerevisiae* (Tsukada and Ohsumi, 1993), *D. discoideum* (Otto et al., 2003; Otto et al., 2004), *D. melanogaster* (Scott et al., 2004) and *C. elegans* (Melendez et al., 2003).

A number of developmental defects have also been found in autophagy mutants in several species. In *S. cerevisiae*, autophagy mutants are defective in spore formation (Tsukada and Ohsumi, 1993). Atg1, Atg5 and Atg7 mutants of *D. discoideum* are not grossly affected in growth, but survival during nitrogen starvation is severely reduced (Otto et al., 2003; Otto et al., 2004). Bulk protein degradation during starvation-induced development is reduced in the autophagy mutants. Moreover, development is aberrant; the autophagy mutants do not aggregate in plaques on bacterial lawns, but they do proceed further in development on nitrocellulose filters, forming defective fruiting bodies. Premature death from the third larval to pupal stages was reported in *D. melanogaster* mutants (Scott et al., 2004; Juhasz et al., 2003; Baehrecke 2003). Draut1, the *Drosophila* homolog of yeast Atg3, loss of function larvae is unable to induce autophagy in fat body cells before pupariation and die during metamorphosis (Juhasz et al., 2003). In wild-type, massive autophagy is observed in dying larval tissues such as the salivary glands (Baehrecke, 2003). In *Drosophila*, the mutant third larvae
leave food sources and starve as a result. During the pupal stage, larval tissues are degraded and used as nutrients to generate adult tissues. Dauer formation is abnormal in *C. elegans* autophagy mutants (Melendez et al., 2003). Using nematodes with a loss-of-function mutation of *bec-1*, the *C. elegans* ortholog of the yeast and mammalian autophagy gene *ATG6/VPS30/beclin1*, is essential for normal daure morphogenesis and life span extension. Dauer formation is associated with increased autophagy. These reports raise the possibility that these developmental defects of autophagy mutants reported in various species are related to nutrient starvation (Tsukada and Ohsumi, 1993; Otto et al., 2003; Juhasz et al., 2003; Scott et al., 2004; Melendez et al., 2003).

In mammalian cells, autophagy is also induced by nutrient starvation (Meijer and Codogno, 2004). Recently, Mizushima et al. have developed an autophagy indicator mouse model, in which autophagosomes are labeled with LC3 fused to green fluorescent protein (GFP) (Mizushima et al., 2004; Mizushima, 2004). Through a systematic analysis of this mouse model, autophagy was found to be activated in almost all organs following initiation of starvation *in vivo* (Figure 3). These findings suggest that autophagy in mammals also plays an important role in maintaining viability during starvation conditions in adult mice (Mizushima et al., 2004). Kuma et al. determined the significance of autophagy by analyzing mice deficient in *Atg5*, a gene essential for elongation of the isolation membrane (Mizushima et al., 2001). *Atg5*<sup>−/−</sup> mice appear almost normal at birth, but most of the *Atg5*<sup>−/−</sup> neonates died within 1 day of delivery (Kuma et al., 2004). It is known that neonates face with nutrient limitation, because neonates are suddenly terminated trans-placental nutrient supply (Medina et al., 1992). However, the kinetics of autophagy is unclear in the mammalian embryos and neonates. The aim of this study is to determine whether autophagy occurs in embryos and neonates. I will reveal that how much autophagy occurs in the mammalian embryonic and neonatal period.

**Role of basal autophagy**

It is known that autophagy is a non-selective protein degradation process, and it degrades long-lived proteins and organelles (including abnormal proteins and
**Figure 3. Autophagy in muscle tissues in adult**

GFP images of extensor digitorm longus (EDL) muscle of GFP-LC3 mice following (A) 0-, (B) 24-h starvation. Bar, 10 μm.
damaged organelles). Autophagy-defective yeast cells (Tsukada and Ohsumi, 1993), embryonic stem (ES) cells (Mizushima et al., 2001) and embryonic fibroblasts (Kuma et al., 2004) are quite healthy and show no apparent abnormalities under growing conditions. Recently, the hepatocytes of liver-specific conditional Atg7−/− mice develop ubiquitin-positive aggregates (inclusion bodies; IBs) (Komatsu et al., 2005). These results suggest that function of autophagy is not only as maintenance of viability during starvation, but also as an intracellular quality control system.

It is thought that the embryos are not starved, because nutrients are provided through the placenta from mothers. Accordingly, autophagy would play a role independent from its function in starvation adaptation. In this study, I analyzed autophagy deficient mice embryos and neonates to discover that the basal autophagy is an important mechanism for intracellular clearance during embryogenesis.

Cell death and autophagy

It has been reported that autophagy may be also involved in cell death. It is now apparent that there are several types of programmed cell death other than typical apoptosis. One type, the so called ‘autophagic cell death’ or ‘type 2 cell death’, is characterized by the appearance of cytoplasmic vacuoles, which are thought to be related to autophagy (Baehrecke, 2003; Gozuacik and Kimchi, 2004). In classical apoptotic or Type 1 programmed cell death, there is early collapse of cytoskeletal elements, but preservation of cytoplasmic organelles until late in the process. In contrast, in autophagic or Type 2 cell death, there is early degradation of cytoplasmic organelles by autophagy, but preservation of cytoskeletal elements until late stages, presumably because of their role in autophagy (Bursch et al., 2004; Clarke, 1990). However, it is unclear that whether autophagy acts fundamentally as a cell survival or cell death pathway or both. It is known that the cell death has occurred actively during embryogenesis. In this study, I determined whether cell death is up regulated in the absence of autophagy.

Organelle degradation and autophagy
In contrast to the ubiquitin-proteasome system, autophagy can degrade not only proteins but also intracellular organelles such as mitochondria (Takeshige et al., 1992; Rodriguez-Enriquez et al., 2004), peroxisomes (Tuttle et al., 1995; Sakai et al., 1998) and endoplasmic reticulum (Masaki et al., 1987; Hamasaki et al., 2005). Some of these organelles are recognized selectively (Tuttle et al., 1995; Sakai et al., 1998). Moreover, abnormal organelles were observed in autophagy-deficient hepatocytes, suggesting that autophagy might be involved in quality control of intracellular organelles (Komatsu et al., 2005).

In addition to the normal turnover of intracellular organelles, dramatic degradation of organelles is observed in the processes of lens and erythroid development. The lens contains two types of cells, the epithelial cells covering the anterior surface of the lens and the fiber cells that differentiate from the epithelial cells. During late embryogenesis, organelles within the epithelial cells are rapidly lost, which allows fiber cells to be transparent (David and Shearer, 1989; McAvoy et al., 1999; Bassbett, 2002). Similarly, intracellular organelles are eliminated during erythroid cell maturation. The mechanism by which these organelles are destroyed during this process is not fully understood. The presence of autophagic vacuoles in these cells during differentiation suggested the involvement of autophagy in the organelle loss (Tooze and Davies, 1965; Kent et al., 1966; Walton and MacAvoy, 1984; Heynen et al., 1985; Takano-Ohmuro et al., 2000). Therefore I addressed this issue using autophagy-indicator mice (Mizushima et al., 2004) and autophagy-deficient mice (Kuma et al., 2004).

**Aim of this issue**

As described above, autophagy plays important roles as starvation response. Autophagy-defective mutant mice, Atg5−/− mice, appear almost normal at birth, but most of the Atg5−/− neonates die within 1 day of delivery. It is unclear how much autophagy occurs in mammalian embryos and neonates. I have two questions. One; how much does autophagy occur in the embryonic and the neonatal period?

Another important question; what is the role of basal autophagy? Autophagy is
a non-selective protein degradation process, and it degrades long-lived proteins and organelles (including abnormal proteins and damaged organelles). If abnormal proteins accumulate in the cell, the cells will be damaged. Consequently, I thought that intracellular clearance by autophagy is very important. In the embryonic period, it is not necessary to consider about induced autophagy, because the nutrient condition is not limited in embryos.

In this study, I analyzed significance of autophagy in embryos and neonates by using autophagy indicator mice and autophagy deficient mice. I observed that autophagy was transiently induced after birth. I also found an important role for basal autophagy in constitutive turnover of intracellular components, and loss of autophagy resulted in accumulation of ubiquitinated proteins. This study will help to understand the physiologically significance of autophagy during embryogenesis and neonatal survival.
Materials and methods

Antibodies

An antibodies against recombinant rat LC3b (anti-LC3#1) was generated as described (Kabeya et al., 2000). A polyclonal anti-GFP antibody and AlexaFluor 488- and 660-conjugated goat anti-rabbit IgG (H+L) antibodies were purchased from Molecular Probes. A mouse monoclonal anti-ubiquitin antibody (1B3), purchased from Medical & Biological Laboratories (MBL), was used histochemistry. A rabbit polyclonal antibody against ubiquitin, used for immunoelectron microscopy, was purchased from DakoCytomation. A mouse anti-KDEL monoclonal antibody was purchased from Stressgen.

Mouse

All mice were C57BL/6 background. Atg5+/– mice were interbred to obtain Atg5+/− mice. Atg5+/− mice were crossed with GFP-LC3 transgenic mice to produce Atg5−/− mice expressing GFP-LC3 (Atg5−/− GFP-LC3/+). For caesarian delivery, pregnant mother were injected on 17.5 day post conception (dpc) and 18.5 dpc with 2 mg progesterone (luteum injection, Teikoku Hormone Mfg. Co.) obtained at 19.5 dpc. All animal experiments were approved by institutional committees of the Tokyo Metropolitan Institute of Medical Science.

Artificial feeding

Newborn pups were obtained by caesarean delivery at 19.5 dpc and placed in a humidified, thermostat-controlled chamber (30°C). For artificial milk feeding, a fine tube was inserted into the stomach, and 30 μl of 0.13 mg/ml infant formula for human neonates (Haihai, Wakodo Co.) was fed through the tobe every 3-6 h.

Observation of GFP-LC3 transgenic mice tissues

Tissue sample for GFP-LC3 observation were prepared from E13.3 to neonate, and fixed with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PB; pH
7.4) for at least 12 h, followed by treatment with 15% sucrose in phosphate-buffered saline (PBS; pH 7.4) and then with 30% sucrose solution overnight. Tissue samples were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and stored at -80 °C. The samples were sectioned at 5 μm thickness with cryostat (CM3050 S, Leica, Deepfield, IL), air-dried for 30 min, and stored at -80 °C until use. Cryosections were washed with PBS for 5 min and air-dried for 10 min. Samples were mounted using SlowFade Light Antifade Kit. (Molecular Probes Inc.). The mounted sections were directly observed by a fluorescence microscope (Olympus IX81, Tokyo, Japan) equipped with a CCD camera (ORCAER, Hamamatsu Photonics, Hamamatsu, Japan). U-MGFPHQ mirror unit (Olympus IX81, Tokyo, Japan), which is dichromatic mirror, was used for GFP observation and U-MWIG2 mirror unit (Olympus IX81, Tokyo, Japan) was used to check autofluorescence.

**Quantification of GFP-LC3 dots**

Cryosections were prepared from tissues isolated at the E18.5, postnatal day (P) 0, 0.5 h, 3 h, 6 h, 12 h, 24 h and P 2.5. The ratio of the total area of GFP-LC3 dots to the total cellular area is shown as percentage. These analyses were analyzed with software named MetaMolph (Universal Imaging Co.).

**Immunofluorescence and immunohistochemical analysis**

For immunofluorescent microscopy, mice were fixed with 4% PFA for overnight, and tissues were removed and immersed in sucrose solution or in 70% Ethanol for paraffin embed. Cryosections were prepared as described above. Paraffin samples were embedded in paraffin with universal manner. Paraffin sections were deparaffinized with xylene and dehydrate in a graded series of ethanol (absolute, 95%, 90%, 80%, 70%, diluted in distilled water) and were rinsed well with PBS for 5min, 3 times. After rehydrate, cryo- and paraffin-sections were treated with microwave in 0.01 M citrate buffer/0.01 M Na₂HPO₄ buffer for 10 min and cool down to room temperature. After blocking with 5% bovine serum albumin (BSA) in PBS, sections were incubated with primary antibody for 1h, followed by 30 min incubation of 2nd
antibody. Samples were mounted using SlowFade Light Antifade Kit. (Molecular Probes Inc.).

For immunohistochemical analysis, antigen activated samples were treated with 0.3% H$_2$O$_2$/methanol for intrinsic peroxidase inactivation. After blocking with BEAT Blocking Solution (Histomouse-Plus Kit; Zymed Laboratories Inc.), sections were incubated with primary antibody for 1 h. Sections were followed by addition of biotinylated secondary antibody, which serves as a linker between the primary antibody and the streptavidin-peroxidase conjugate. The presence of peroxidase can be revealed by addition of DAB substrate-chromogen solution.

Detection of cell death by TUNEL (TdT-mediated dUTP-biotin Nick End Labeling) method

Paraffin sections were prepared as described above. After rehydrate, paraffin sections were treated with 20 mg/ml proteinase K, nuclease free, in 10mM Tris-HCl, pH 7.6, for 15 min at 37°C. Cell death was detected by In Situ Cell Death Detention Kit, Fluorescein (Roche Diagnostics) according to the appended protocol. Samples were mounted using SlowFade Light Antifade Kit. (Molecular Probes Inc.). The mounted sections were directly observed by a fluorescence microscope (Olympus IX81, Tokyo, Japan) equipped with a CCD camera (ORCAER, Hamamatsu Photonics, Hamamatsu, Japan). U-MGFPHQ mirror unit (Olympus IX81, Tokyo, Japan).
**Results**

*Autophagy was extensively induced in various tissues after natural birth*

Mizushima *et al.* generated a transgenic mouse model in which autophagosomes were labelled with GFP–LC3 in almost all tissues. LC3 is one of the mammalian proteins homologous to yeast Atg8. In this mouse model, it was observed that autophagy was induced in many tissues in response to food withdrawal in young to adult mice. To study the kinetics of mammalian autophagy during development, I observed GFP-LC3-labelled structures (GFP-LC3 ‘dots’) in the embryonic and perinatal stages (Fig. 4, Fig. 5 and Fig. 6). First, I observed heart, because autophagy is massively increased following food withdrawal in heart of adult mice (Mizushima *et al.*, 2004). In day 13.5 embryos, I detected a small, but significant, number of GFP-LC3 dots (Fig. 4A). I obtained similar findings from E15.5 and E18.5 (Fig. 4B, Fig. 4C and Fig. 5A). However, the formation of the GFP–LC3 dots was extensively induced after a natural birth. The formation of GFP-LC3 dots was upregulated within 30 min after birth (Fig. 4D). The number of GFP-LC3 dots reached its maximal level 3-6 h after birth (Fig. 4E and F and Fig. 5C), although the neonatal mice began suckling before that time. The number of dots gradually decreased to basal levels by day one or two (Fig. 4G and H).

In embryonic period, a few autophagosomes were present in other tissues, such as the diaphragm (Fig. 6A), skeletal muscle (Fig. 6C), liver (Fig. 5M and Fig. 6E), lung (Fig. 6G), pancreas (Fig. 5E and Fig. 6I) and skin (Fig. 6K). However, the autophagosomes of these tissues were also increased after birth. Particularly, the diaphragm (Fig. 7B), skeletal muscle (Fig. 6D), liver (Fig. 5O and Fig. 6F), lung (Fig. 6H), pancreas (Fig. 5G and Fig. 6J) and skin (Fig. 6L) displayed massive dots. Autophagosomes were induced beginning 30 min after birth, and the number of dots reached its maximal level, though the degree of various level, at 3-6 h after birth (Fig. 7). The remarkable exception was thymic epithelial cells, which possessed large numbers of GFP-LC3-positive dots, even in embryos (Fig. 8A and B). The opposite phenomenon was observed in neural cells within the brain (Fig. 9A and B). These cells exhibited few or no GFP-LC3 dots in embryos.
To rule out the possibility that the LC3-positive dots represented a structure unrelated to autophagosomes, I examined GFP-LC3 localization in animals on the *Atg5*<sup>−/−</sup> background (Fig. 5). As expected, the small green dots disappeared in the embryonic tissues of *Atg5*<sup>−/−</sup> GFP-LC3/+ mice. These data confirmed that GFP-LC3 dots represent autophagosomes.

The appearance of autophagic vacuoles was confirmed by electron microscopy (Fig. 10A; produced by Dr. Yamamoto, Department of Bio-Science, Nagahama Institute of Bio-Science and Technology). Morphometric analysis of electron micrograph images revealed that autophagic vacuoles occupied 0.12% and 1.00% of the total cytoplasmic area in hearts isolated from neonates 0 h and 6 h after birth, respectively (Fig. 10B).

**Autophagy occurs constitutively active in the thymus**

In the thymic epithelial cells, autophagy was observed high basal levels from embryo to neonate (Fig. 8A and B), suggesting that autophagy was occurred independently of nutrient conditions. To study this possibility, I observed thymic epithelial cells of adult mice in nutrient rich conditions (Fig. 8C-E). GFP-LC3 dots were observed high level in stromal cells with stellate shapes in both the cortex and medulla. Because they stained with anti-cytokeratin antibody, these cells were identified as epithelial reticular cells. Small GFP-LC3 dots were present in both the cell bodies and processes of the reticular cells. It is noteworthy that autophagy in the thymic epithelial cells occurs even without starvation treatment. The number of GFP-LC3 dots was not induced in food withdrawal in these cells. Therefore, autophagy in the thymic epithelial cells is constitutively active, irrespective of nutrient conditions.

**GFP-LC3 is aggregated in neonatal hepatocytes in Atg5<sup>−/−</sup> mice**

I next examined neonatal tissues of *Atg5*<sup>−/−</sup> GFP-LC3/+ mice. As in the embryonic tissues, the small GFP-LC3 dots representing autophagosomes could not be detected in *Atg5*<sup>−/−</sup> GFP-LC3/+ newborns. Larger, more intense fluorescent dots, however, appeared frequently in hepatocytes (1-3 dots per cell; Fig. 5P) and DRG neurons (4-8 dots per cell; Fig. 5T). While these large dots appeared occasionally in
cells of the anterior lobe of the pituitary gland (Fig. 5X), they were rarely seen in cardiac muscle or thymic epithelial cells. In the pituitary, these structures were seen as early as E15.5 (Fig. 5V). All of these structures were homogeneously fluorescent, not ring-shaped, even when quite large, suggesting that these structures are not true autophagosomes. This hypothesis is supported by the observation that LC3-II, the phosphatidylethanolamin (PE)-conjugated form of LC3, was not generated in Atg5\(^{-/-}\) liver or other tissues (Kuma et al., 2004). Such large GFP-LC3-positive structures were not observed in pancreatic acinar cells (Fig. 5H) and skeletal muscle (data not shown) of Atg5\(^{-/-}\) neonates.

I hypothesized that these large fluorescent structures likely represented inclusion bodies (IBs) containing the unconjugated GFP-LC3 protein, which was not membranous structures. As intracellular IBs usually contain ubiquitin (Orth et al., 2003; Watanabe et al., 2001), I determined whether these structures were also positive for ubiquitin antibody. Indirect fluorescence microscopy using an anti-ubiquitin antibody showed the colocalization of GFP-LC3 and ubiquitin in Atg5\(^{-/-}\) hepatocytes (Fig. 11A).

The overexpression of GFP-LC3 may have caused the artificial aggregation of GFP-LC3. I thus examined the presence of IBs in the hepatocytes of wild-type and Atg5\(^{-/-}\) mice who did not express GFP-LC3 (Fig. 11B). While ubiquitin-positive IBs were observed in Atg5\(^{-/-}\) hepatocytes, these structures were absent from wild-type hepatocytes and dorsal root ganglion (DRG) neurons. Endogenous LC3 was detected in the IBs of Atg5\(^{-/-}\) neonate DRG neurons (Fig. 11C), suggesting that LC3 may be incorporated into protein aggregates regardless of its PE-conjugation status. The aggregation of endogenous LC3 was unclear in hepatocytes, potentially due to the lower expression of LC3 in hepatocytes. These results suggest that the absence of autophagy leads to the formation of IBs, which contain ubiquitin, in some tissues.

**Accumulation of IBs in Atg5\(^{-/-}\) mice**

Next, I determined the tissues of Atg5\(^{-/-}\) neonates that exhibited IB formation by immunohistochemistry using an anti-ubiquitin antibody. As observed in Atg5\(^{-/-}\) GFP-LC3/+ neonates, ubiquitin-positive IBs accumulated extensively in the liver (Fig.
12B), some regions of the brain (Fig. 12F), and the anterior pituitary gland (Fig. 12J) and less frequently in the heart and thymic epithelium (data not shown). In the nervous system, large aggregates were primarily observed in large neurons of the spinal cord (ventral horn cells, Fig. 12D), the pons (Fig. 12F), DRG neurons (Fig. 12H), the hypothalamus, the mid brain, and the trigeminal ganglia (data not shown). The neurons within DRG exhibited the most extensive accumulation of IBs; more than 10 aggregates were observed in a single cell slice. A subset of the IBs was as large as 2 μm in diameter. These IBs were evenly distributed throughout the cytoplasm; they were not clustered in the perinuclear regions or around the nuclei. In contrast, neural cells in the cortex (Fig. 12L) and cerebellum (data not shown) did not contain ubiquitin-positive IBs. Other tissues, such as lung, spleen, pancreas, intestine, and skeletal muscle, also did not contain intracellular aggregates that labeled with ubiquitin (data not shown).

Immunoelectron microscopy using a gold-conjugated anti-ubiquitin antibody of DRG neurons isolated from Atg5−/− neonates demonstrated the specific association of gold particles with amorphous intracellular structures and compact structures surrounding filamentous materials (Fig. 13A and B; produced by Dr. Yamamoto, Department of Bio-Science, Nagahama Institute of Bio-Science and Technology). As these structures were not observed in wild-type littermates, these structures may correspond to the large inclusions seen by light microscopy. These data suggest that the suppression of constitutive autophagy for only a short embryonic period is sufficient to impair protein quality control in embryonic cells, particular hepatocytes and neural cells.

**Normal cell death in autophagy-deficient mice**

The presence of cytoplasmic IBs prompted us to question if cell death occurred more frequently in Atg5−/− neonates. Conventional hematoxylin and eosin staining did not reveal any significant changes in the brain or other tissues in Atg5−/− neonates (data not shown). I determined if there was excess apoptotic cell death in Atg5−/− newborns by TUNEL labeling. TUNEL-positive cells did not increase in Atg5−/− tissues, even within DRG, the pons, or the hypothalamus, all sites in which ubiquitin-positive aggregates
accumulated (Fig. 14 and data not shown). These data suggest that, while the defect in autophagy during embryogenesis could accumulate IBs, it does not lead to increased cell death.

**Organelle degradation in lens fiber cells occurs normally**

In addition to the clearance of abnormal intracellular proteins, autophagy is required for the degradation of normal proteins and organelles in a subset of specialized cells, such as lens and erythroid cells. Next, I examined a possible role of autophagy in organelle degradation during lens differentiation. The lens contains only two types of cells, the epithelial cells covering the anterior surface of the lens and the fiber cells that differentiate from the epithelial cells. During differentiation, entire organelles within the epithelial cells are lost, which allows fiber cells to be transparent. Similarly, intracellular organelles are eliminated during erythroid cell maturation. Several studies have suggested the involvement of autophagy in these processes. Since the organelle free zone (OFZ) in the lens is created between 17.5 days post conception (dpc) and birth, I first determined the occurrence of autophagy at this stage by using of GFP-LC3 transgenic mice (Fig. 15B and C). In lens of 17.5 dpc embryos, a number of GFP-LC3 dots were observed at the center region. Fewer GFP-LC3 dots were observed at the border of developing OFZ. To rule out the possibility that these LC3-positive dots represented structures unrelated to autophagosomes, I examined GFP-LC3 localization in lens at the same stage on the \textit{Atg5} \textsuperscript{-/-} background (Fig. 15E and F). As expected, these small green dots were not detected in the lens epithelial cells of GFP-LC3 \textit{Atg5} \textsuperscript{-/-} mice. These data confirmed that autophagy occurred continuously in embryonic lens, particularly at the center region.

To determine whether autophagy is involved in organelle degradation, I examined the existence of organelle in lens by immnohistochemical analysis during embryogenesis. At lens of 17.5 dpc wild-type (\textit{Atg5} \textsuperscript{+/+}) embryos, nuclei and endoplasmic reticulum (ER), stained with Hoechst 33258 and anti-KDEL antibody, respectively, were present abundantly (Fig. 16A). Both the ER and the nuclei at the center region of the lens disappeared after birth, and OFZ was created. At day 0.5
neonates, no ER and only a small amount of nuclear debris could be observed in the OFZ (Fig. 16C). I next analyzed this rapid process under autophagy-deficient conditions using Atg5−/− mice. At 17.5 dpc, lens of normal size and morphology were generated in the mutant mice (Fig. 16B), suggesting that autophagy is not required for lens morphogenesis from the optic primordial. In addition, OFZ was created at day 0.5 neonates in Atg5−/− mice in a manner similar to that in wild-type mice (Fig. 16D). I prolonged the survival of neonates by artificial feeding and analyzed the OFZ formation up to 2 days after birth, but there was no significant difference between Atg5+/+ and Atg5−/− mice (Fig. 16E and F).

The generation of normal lens fibers was confirmed by electron microscopy. The cytoplasm of lens fibers in OFZ of both Atg5+/+ and Atg5−/− consisted of fine granular crystalline substance, where organelles were not observed. These data suggest that autophagy is dispensable for OFZ formation in the lens.
Discussion

Induction of autophagy in neonatal period

In this study I examined autophagy kinetics in embryonic and prenatal stage, and observed that autophagy remained at a low level throughout the embryonic period. However, the formation of the autophagosomes was extensively induced in various tissues after a natural birth (Fig. 4, Fig. 6 and Fig. 7). Such an induction pattern is different from that of starved adult mice. This might be because the energy requirements of the heart and diaphragm suddenly increase at birth, and the external environments of lung and skin are drastically changed; that is, from the amniotic fluid to the air. The plasma amino acid concentration was measured under fasting condition, because the major role of autophagy is the degradation of proteins into amino acids (Kuma et al., 2004). Soon after caesarean section, the concentration of amino acid in the plasma of $Atg5^{-/-}$ neonates was not different from that of wild-type littermates. However, at 10 h after the caesarean delivery, the total amino acid concentration of $Atg5^{-/-}$ mice was significantly lower than that of wild-type mice. In the organs, such as heart, liver and brain, the total amino acid concentration did not differ significantly among littermates at birth. However, after 10 h, significant differences were observed in those organs. Mammalians encounter the first, and probably the most severe, period of starvation during their lifespan in soon after birth by the sudden termination of the trans-placental nutrient supply. To overcome this life-threatening problem unique to mammals, it has been known that carbohydrate and lipid reserves are used during period. In addition, autophagy must be activated to maintain an adequate amino acid pool until the nutrient supply from milk reaches a steady state. Amino acids produced by autophagy can be directly used as an energy source or converted to glucose in the liver. Alternatively, amino acids can also be used for synthesis of proteins required for the proper starvation response. Considering that the developmental defects of autophagy mutants reported in other species are related to nutrient starvation, it is thought that mammalian autophagy play very important role also for neonatal survival (Fig. 17).
Constitutively active autophagy in the thymic epithelial cells

Several tissues, including thymic epithelial cells (Fig. 8) and some exocrine gland cells such as pituitary gland cells (Fig. 5) showed high basal levels of autophagy from embryo to neonates. Although the volume of thymic epithelial cells is difficult to estimate by fluorescence microscopy, the number of autophagosomes in these cells might be the highest of any nonstarved tissue. One report has also demonstrated the extensive acid phosphatase activity in some vacuoles in the thymic epithelial cells (Bowen and Lewis, 1980). Younger mice (8 weeks old) and late-stage embryos (15.5-19.5 dpc embryo) show more active autophagy in the thymus. In general, cytoplasmic proteins are processed by the proteasome, delivered into the lumen of the ER and presented by the MHC class I pathway. However, there is a growing amount of data demonstrating that endogenous proteins are also presented on MHC class II (Lechler et al., 1996). How cytoplasmic proteins are loaded on MHC class II has not been well studied, but autophagy is likely responsible for this pathway (Paludan et al., 2005; Dengjel et al., 2005; Nimmerjahn et al., 2003). During positive and negative selections, thymic epithelial cells present self-antigens to lymphocytes. Because thymic epithelial cells are not thought to have phagocytic activity, it is reasonable to hypothesize that they provide self-antigens from their own cytoplasm. In this scenario, autophagy might be involved in T-cell development and central tolerance in thymic epithelial cells.

Intracellular clearance by constitutive autophagy

In this study, I have described the role of autophagy in mammalian embryo and neonates. As yeast cells defective in autophagy do not exhibit obvious abnormalities until cultured under nutrient medium condition (Takeshige et al., 1992), the role of basal level autophagy has been ignored. In a similar manner, autophagy-deficient mammalian cells, such as Atg5−/embryonic stem cells (Mizushima et al., 2001) and embryonic fibroblasts (Kuma et al., 2004), also do not exhibit significant abnormalities, despite low level of constitutive autophagy in the wild-type parental cells. As these cells can divide rapidly, abnormal proteins would be quickly diluted and would not
accumulate, even if not degraded. Thus, the importance of basal autophagy likely depends on cellular context. In this study, I demonstrated that requirement for autophagy differs between embryonic tissues. Extensive IB (inclusion body) formation was detected only in a few tissues, including neural cells, hepatocytes, and anterior pituitary gland cells (Fig. 12). Recently, basal autophagy was demonstrated to be important for liver homeostasis using mice bearing a tissue-specific deficiency of Atg7 (Komatsu et al., 2005). I demonstrated that a deficiency of autophagy over a much shorter time, from liver genesis to birth, sufficient to generate large inclusions in hepatocytes; these ubiquitin-positive aggregates were already present in a subset of hepatocytes at E15.5 in Atg5−/− embryos. In addition, I revealed that neural cells and anterior pituitary cells are additional cell types in which protein quality control is highly dependent on basal levels of autophagy (Fig. 17).

The reason that autophagy is particularly important in these cells remains unclear. The basal levels of autophagy may be particularly high in these tissues. DRG neurons and anterior pituitary gland cells do exhibit higher levels of autophagy than other tissues (Fig. 5). The basal level of autophagy, however, does not always correlate with the degree of IB formation in Atg5−/− tissues. Thymic epithelial cells exhibit the most extensive autophagy seen throughout embryos, but only a few IBs are generated in this tissue in autophagy-deficient animals (Fig. 5). In contrast, while autophagosomes are not observed in regions of the central nervous system, such as the hypothalamus and pons, in wild-type mice, IBs accumulate in these tissues in Atg5−/− mice. The time needed to reach the postmitotic state may also influence the dependence of individual tissues on autophagy. The longer the time period that passes after cells stop dividing, the more abnormal proteins could accumulate within a cell. The lack of significant numbers of IBs in cortical neurons may be due to the young developmental age of these cells in comparison to neurons within sensory roots. In addition, cell types may differ in their expression of proteins that easily aggregate or whose quality is tightly regulated. Further detailed analysis of the protein composition of these aggregates may be needed to address this possibility.
Mechanism of Ub-positive aggregate formation

The mechanism governing the accumulation of ubiquitin-positive aggregates in Atg5\(^{-/-}\) tissues is not clear. Autophagy is thought to be a non-selective process. The turnover of long-lived proteins is impaired under autophagy-defective conditions (Komatsu et al., 2005), which would make these proteins more prone to damaged or misfolding. Under these conditions, these proteins would have an increased chance of being both ubiquitinated and aggregated. This scenario does not imply any selectivity of autophagy. In autophagy-competent cells, microaggregates could also be sequestered into autophagosomes by chance.

Recently, selective or preferential sequestration/degradation of cytosolic contents by autophagy was reported. In yeast, the Ald6 protein was degraded in an autophagy-dependent manner more rapidly than other cytosolic proteins (Onodera et al., 2004). Although it remains unknown if a similar preferential degradation of cytosolic contents occurs by autophagy in mammals, invading bacteria that enter into the cytosol seem to be selectively enclosed (Nakagawa et al., 2004; Ogawa et al., 2005). Proteins on the inner face of autophagosomal membranes may participate in the specific recognition of a subset of molecules. During the autophagic degradation of intracellular Shigella species, the bacterial VirG protein may be recognized by autophagic membranes (Ogawa et al., 2005). This kind of substrate recognition is observed in an autophagy-related system in yeast. Two vacuolar proteins, aminopeptidase I and \(\alpha\)-mannosidase, are selectively transported to vacuoles by the CVT pathway. Under nutrient-rich conditions, this process is mediated by an autophagosome-like structure called a Cvt vesicle (Klionsky, 2005). During starvation, these two enzymes are delivered to the vacuole by autophagosomes. A common receptor (Atg19) for these proteins is present on both types of sequestration membranes. Although no equivalent pathway has been discovered in other species, this process may be the prototype of selective sequestration. As a number of studies have suggested the selective delivery of ubiquitinated proteins to lysosomes (Schwartz et al., 1988; Doherty et al., 1989; Laszlo et al., 1990; Ueno and Kominami, 1991; Lenk et al., 1999), ubiquitinated or misfolded...
proteins may also be recognized by as-yet unknown proteins on autophagic membranes.

Another, but not mutually exclusive, possibility is that autophagy selectively, via p62, eliminates IBs that have already formed (Bjorkoy et al., 2005). I have not, however, observed such a selective enclosure of large IBs in wild-type cells. On the other hand, autophagosome is recruited to IBs via microtubules (Iwata et al., 2005). Although several studies demonstrated the co-localization of LC3 and IBs, this phenomenon may reflect the incorporation of LC3 into IBs, rather than autophagosome sequestration (Fig. 11C). In addition, because IBs are usually larger than typical autophagosomes (about 1 μm), it is unlikely that autophagosomes enclose inclusions of this size efficiently. I cannot, however, rule out the possibility that small aggregates are preferentially sequestered into autophagosomes.

Relevance to pathophysiology of neurodegeneration

I demonstrated that the neural cells of Atg5−/− mice exhibit morphological alterations (accumulation of IBs). The presence of multiple inclusions in certain types of neurons in Atg5−/− mice suggests that soluble abnormal proteins accumulate due to a general impairment in protein turnover. A recent study indicated that the soluble forms of mutant proteins, rather than the aggregated forms, are the major cause of toxicity for neurons (Saudou et al., 1998; Taylor et al., 2003; Arrasate et al., 2004). Therefore, the contribution of autophagy to neuronal homeostasis could be much greater than that perceived from the presence of intracellular inclusions.

The ubiquitin-proteasome system is considered to be the primary means for degradation of misfolded proteins. This study, however, indicates that autophagy collaborates with this process to prevent the accumulation of abnormal proteins. This finding has implications in the pathophysiology of neurodegenerative disease. Multiple studies have suggested that the inhibition of autophagy induces the aggregation of mutant proteins that are detected in human diseases; rapamycin-induced upregulation of autophagy reduces this aggregate formation (Teckman et al., 2000; Ravikumar et al., 2002; Fortun, 2003; Ravikumar and Rubinsztein, 2004). This study demonstrates that inhibition of autophagy causes aggregation, even in the absence of mutant protein
expression.

**Organelle degradation in lens fiber cells**

Intracellular organelles are lost during the differentiation of lens fiber cells and erythrocytes. The mechanism by which these organelles are destroyed during this process is not fully understood. The presence of autophagic vacuoles in these cells during differentiation suggested the involvement of autophagy in organelle degradation (Tooze and Davies, 1965; Kent et al., 1966; Walton and McAvoy, 1984; Heynen et al., 1985; Takano-Ohmuro et al., 2000). Recently, the degradation of lens nuclei was reported to depend on DNase II-like acid DNase I/DNase IIβ (Nishimoto et al., 2003), suggesting that chromatin degradation occurs in an acidic organelle. I demonstrated that autophagy is not essential for either lens OZF formation or organelle elimination, at least before birth (Fig. 16). I do not know if further lens maturation occurs normally, because of the early lethality of Atg5−/− mice. This data suggest, however, that an autophagy-independent mechanism of OFZ formation must exist. 15-lipoxygenese was suggested to participate in organelle degradation in lens cells by permeabilization of the organelle membranes (van Leyen et al., 1998). This enzyme is highly expressed in sites at which lens organelles are degraded. The ubiquitin-proteasome system may also be involved in this form of organelle degradation (David and Shearer, 1989). Thus, I demonstrated that the degradation of nuclei in lens fiber cells is independent of autophagy. This is reasonable, because nuclei are too large to be enclosed by typical autophagosomes.
Figure 4. Localization of GFP-LC3 in the heart of embryos and neonates mice

Hearts were isolated from GFP-LC3 transgenic mice at multiple stages, including at embryonic day (A) 13.5, (B) 15.5, (C) 18.5 and (D) neonatal day 3-h, (E) 24 h and (F) 2.5 day, immediately fixed, cryosectioned, and analyzed by fluorescence microscopy. Bar, 10 μm.
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Figure 5. Localization of GFP-LC3 in various tissues of embryos and neonates

Various organs were isolated from embryonic day 15.5 embryo and 6-h neonates of heterozygous GFP-LC3 transgenic mice on the Atg5+/+ or Atg5-/- backgrounds. Tissues were immediately fixed, sectioned, and analyzed by fluorescence microscopy. (A-D) Heart, (E-H) exocrine pancreas, (I-L) thymus, (M-P) liver, (Q-T) dorsal root ganglia, (U-X) anterior lobe of pituitary gland. (A, E, I, M, Q and U) embryonic day 15.5 embryos of Atg5+/+ GFP-LC3/+ mice, (B, F, J, N, R and V) embryonic day 15.5 embryos of Atg5-/- GFP-LC3/+ mice, (C, G, K, O, S and W) 6-h neonates of Atg5+/+ GFP-LC3/+ mice, and (D, H, L, P, T and X) 6-h neonates of Atg5-/- GFP-LC3/+ mice. Bar, 10 μm.
Figure 6. Autophagy is induced in various neonatal tissues

Detection of autophagy in (A and B) diaphragm, (C and D) skeletal muscle, (E and F) liver, (G and H) lung, (I and J) pancreas and (K and L) skin. These tissues were isolated from GFP-LC3 transgenic mice at embryonic day 18.5 (A, C and E) and 3 h after birth (D, D and F), immediately fixed, cryosectioned, and analysed by fluorescent microscopy. Bar, 10 μm.
Figure 7. Quantification of GFP-LC3 dots in neonatal tissues

Cryosections were prepared from tissues isolated at the indicated times. The ratio of the total area of GFP-LC3 dots to the total cellular area is shown percentage. Values represent mean ± s.d. of three mice. Diaph., diaphragm; Panc., pancreas; Muscle; gastronemius muscle.
**Figure 8. Localization of GFP-LC3 in the thymus of embryos and adults**

Autophagosome formation in the thymus by GFP-LC3. Thymus were isolated from GFP-LC3 transgenic mice at multiple stages, including at (A) embryonic day 15.5 embryos, (B) 3-h after birth neonates and (C-E) feeding adult mice. (C) GFP-LC3 fluorescence, (D) anti-cytokeratin antibody staining and (E) merged image are shown. Bar, 10 μm.
Figure 9. Localization of GFP-LC3 in the brain of embryos and neonates

Brain were isolated from GFP-LC3 transgenic mice at (A) embryonic day 15.5 embryos and (B) 3-h after birth neonates, and immediately fixed, cryosectioned, and analyzed by fluorescence microscopy. Bar, 10 μm.
Figure 10. Electron microscopic analysis of the hearts from wild-type neonates

(A) Typical autophagosomes (arrows) were observed in the heart at 10 h after birth. Bar, 1 μm.

(Produced by Dr. Yamamoto, Department of Bio-Science, Nagahama Institute of Bio-Science and Technology) (B) Autophagosomes were quantitated by electron microscopy and morphometric analysis. The ratio of the total area of autophagic vacuoles to the total cytoplasmic area is shown.
Figure 11. LC3 dot structures in Atg5-/- represent IBs

Neonatal liver from Atg5-/- GFP-LC3/+ (A) and Atg5-/- (without GFP-LC3 expression) mice (B) and dorsal root ganglion from Atg5-/- (without GFP-LC3 expression) mice (C) were examined by immunohistchemistry. OCT-embedded (A and B) and paraffin-embedded (C) sections were antigen-retrieved and stained with anti-GFP antibody (A, left), anti-ubiquitin antibody (A, middle) (B) (C, left). Merged images are shown (A, right) (C, right). Bar, 10 μm.
Figure 12. Ubiquitin-positive IBs accumulate in Atg5-/- tissues

0-h neonatal tissues were fixed and decalcified. Whole mount paraffin sections of Atg5+/+(A, C, E, G, I and K) and Atg5-/- (B, D, F, H, J and L) neonates were prepared and stained with an anti-ubiquitin antibody. (A and B) Liver, (C and D) anterior horn of the spinal cord, (E and F) pons, (G and H) dorsal root ganglia, (I and J) anterior lobe of pituitary gland and (K and L) cerebral cortex. Bar, 10 μm.
Figure 13. Immunoelectron micrograph of ubiquitin-positive IBs

DRG neurons isolated from Atg5-/- neonates were analyzed by immunoelectron microscopy using a gold-conjugated anti-ubiquitin antibody. (Produced by Dr. Yamamoto, Department of Bio-Science, Nagahama Institute of Bio-Science and Technology) Gold particles were associated with (A) amorphous intracellular structures and (B) compact structures surrounding filamentous materials. Bar, 500 nm.
Figure 14. Normal cell death in autophagy-deficient mice

Brain was isolated from Atg5-/− mice at postnatal day 0, and samples were fixed with 4% PFA. Cell death was detected by TUNEL method. (A-F) hypothalamus. (A-C) Atg5+/+, (D-F) Atg5-/− mice. Nuclei were stained with Hoechst 33258 (shown in red). Bar, 50 μm.
Figure 15. Localization of GFP-LC3 in embryonic lens

Lens were isolated from embryonic day 17.5 embryo of heterozygous GFP-LC3 transgenic mice on the Atg5+/+ (A-C) or Atg5-/- (D-F) backgrounds. Lens were immediately fixed and sectioned. Nuclei were stained with Hoechst 33258 (shown in red in A and D) and analyzed by fluorescence microscopy. (B, C, E and F) Higher magnification images of GFP signals resions in (A and D). Bar, 100 µm (A and D) and 10 µm(B, C, E and F).
Figure 16. Normal OFZ formation in lens of Atg5−/− mice

Mid sagittal lens slices were prepared from embryonic day 17.5 embryo (A and B) and day 0.5 neonates (C and D) and day 2.5 neonates (E and F) of Atg5+/+ (A, C and E) and Atg5−/− (B, D and F) mice. The endoplasmic reticulum was visualized by immnostaining using an anti-KDEL antibody combined with an AlexaFluor 488-conjugated goat anti-rabbit IgG antibody (shown in green). Nuclei were stained with Hoechst 33258 (shown in red). Superimposed images are shown. Ep, epithelium; OFZ, organelle-free zone. Bar, 100 μm.
Figure 17. Roles of induced and baseline autophagy

The level of autophagy is usually low but can be upregulated by starvation such as birth and fasting. The induced autophagy is important for intracellular generation of amino acids. On the other hand, the baseline autophagy is crucial for intracellular quality control.
References


Toxic Res. 6(4): 725-747.


