

However, one of the areas that have lagged is a mechanistic understanding of autophagy and the function of the autophagy-related (Atg) genes. An additional issue is that there is essentially no quantitative information on the relative expression of the Atg genes in early embryo. There are two types of autophagy, macroautophagy or microautophagy, but the best characterized type is macroautophagy. During autophagy process, a portion of the cytoplasm is sequestered by an expanding membrane sac, phagophore. Upon completion, the phagophore generates a double-membrane vesicle termed an autophagosome. The autophagosome then fuses with lysosome in eukaryotes, releasing the inner vesicle, the autophagic body. The autophagic body, along with its cargo, is then degraded by vacuolar hydrolases, and the resulting macromolecules are released back into the cytosol for recycle.

Although autophagy in yeast originally identified as a nonselective degradative pathway, it can also mediate a selective biosynthetic pathway called the cytoplasm-to-vacuole targeting (Cvt) pathway, which uses much of the same protein machinery to transport the resident vacuolar hydrolase aminopeptidase 1[2]. The Cvt pathway has only been described in fungi; however, there are many examples of selective types of autophagy in higher eukaryotes[3]. For both autophagy and the Cvt pathway, the phagophore assembly site (PAS) is thought to be the organization center for the formation of the Cvt vesicle and autophagosome during growth and starvation, respectively[4]. As detected by fluorescence microscopy, most Atg proteins show a specific localization at this punctate perivacuolar structure in cytosol. Although the function of the PAS is still not fully understood, it is known that the sequestering of Atg proteins to this site is essential for their normal function. In the Cvt pathway, Atg11 is reported to play a predominant role in PAS formation because the knock-out of this gene results in no detectable PAS under vegetative conditions[5]. According to our current model, Atg11 acts as a scaffold protein and participates in several steps of the Cvt pathway. For example, Atg11 plays a role in cargo recognition by binding Atg19, the receptor of precursor Ape1[6]. Atg19 also interacts with Atg8, which is normally localized at the PAS[7]. In an atg11 knock-out mutant, however, the Atg19 and prApe1 proteins interact and form a complex,

but this complex localizes away from the PAS. Similarly, Atg8 primarily shows a diffuse cytosolic localization in this mutant strain. Another interaction partner of Atg11 is Atg9, and the interaction between them mediates the movement of Atg9 from peripheral pools (potential membrane sources) to the PAS[8].

It is not known to what autophagy plays a role in the preimplantation embryo. The overall objective of the present study was to evaluate association of autophagy in preimplantation mouse embryo.

2. Materials and Methods

2.1 Collection and Culture of Mouse One-Cell Embryos

To obtain fertilized embryos, 6-week-old ICR female mice were superovulated by intraperitoneal injections of 5 IU of pregnant mare serum equine chorionic gonadotropin (eCG, Sigma, St. Louis, MO), followed by 5 IU human chorionic gonadotropin (hCG, Sigma) 48 hr later. They were then caged individually with ICR male mouse. The presence of a vaginal plug the following morning confirmed successful mating (Day 1 of pregnancy). Experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. One-cell (1C) zygotes were collected from the ampullae of superovulated females mated at 20 hr after hCG injection. Cumulus cells were removed by pipetting with 0.1 mg/ml hyaluronidase (Sigma) in M2 (Sigma) medium. The embryos were cultured in mouse culture medium (M16) at 37°C under an atmosphere containing 5% CO₂.

2.2 Real-Time RT-PCR

mRNAs from groups of 20 embryos derived by in vitro culture were isolated with Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway). The first-strand cDNA synthesis was achieved by reverse transcription of mRNA using Oligo(dT)₁₂₋₁₈ primer and SuperScript™ III Reverse Transcriptase (Invitrogen). Real-time PCR using a DNAEngine OPTICON2 (MJResearch, Waltham, MA) instrument was performed in a final reaction volume of 20ul with SYBR Green, a fluorophore which binds to all

double-strand DNA (qPCR kit from FINNZYMES, Finland). The primers used for PCR are shown in Table 1. PCR conditions were as follows: 10min at 94°C followed by 39 cycles of 30sec at 94°C, 30sec at 55 or 60°C, 55sec at 72°C, and a final extension of 5min at 72°C. Finally, the relative quantification of gene expression was analyzed using the 2-ddCt method by normalization to internal mouse histone H₂AmRNA levels

2.3 Statistical analysis

The general linear models (GLM) procedure in the Statistical Analysis System (SAS User's guide, 1985, Statistical Analysis System, Inc., Cary, NC) was used to analyze the data from all experiments. Significant differences were determined using Tukey's multiple range test and $p < 0.05$ was considered significant.

3. Results

3.1 Expression of autophagy-related genes (ATGs) in mouse embryo

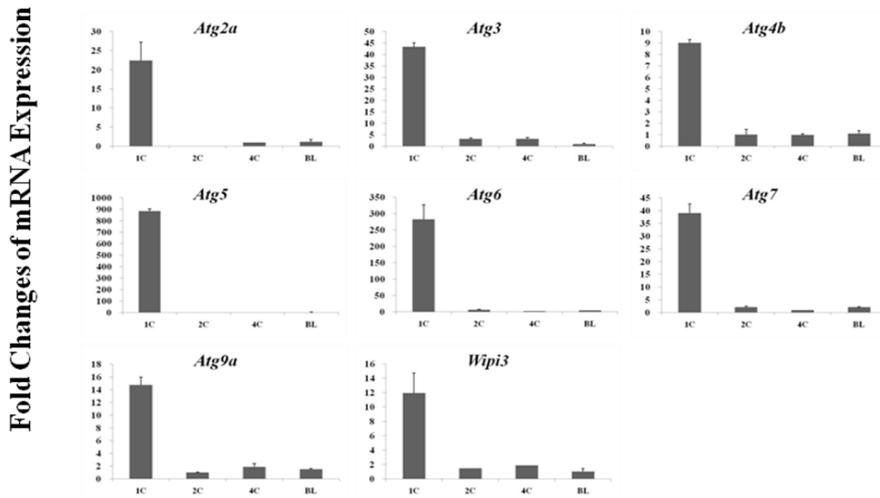
Expression of autophagy-related genes (ATGs) were analyzed from 1-cell to blastocyst stage embryos to understand relationship of autophagy in early embryo development. Using real-time RT-PCR technique with

primers listed in Table 1, ATGs including *Atg2a*, *Atg3*, *Atg4b*, *Atg5*, *Atg6*, *Atg7*, *Atg9a*, and *Wipi3* were generally up-regulated in 1-cell embryo stage before zygotic genomic activation (ZGA), and then those expressions were decreased during 2-cell, 4-cell and blastocyst embryo stage, despite *Atg4b*, *Atg7*, *Atg9a*, and *Wipi3* are a little expressed in 2-cell, 4-cell and blastocyst embryo stage Fig. 1. Relative mean expression of *Atg2a* is 22.3, 0, 1 or 1.19, *Atg3* is 43.4, 3.39, 3.2 or 1, *Atg4b* is 9.03, 1.01, 1 or 1.09, *Atg5* is 885.2, 1.87, 1 or 2.64, *Atg6* is 282.08, 6.89, 2.06 or 4.54, *Atg7* is 38.98, 2.18, 1 or 2.12, *Atg9a* is 14.72, 1, 1.89 or 1.54, and *Wipi3* is 11.95, 1.44, 1.86 or 1 in 1-cell, 2-cell, 4-cell, or blastocyst stage embryo.

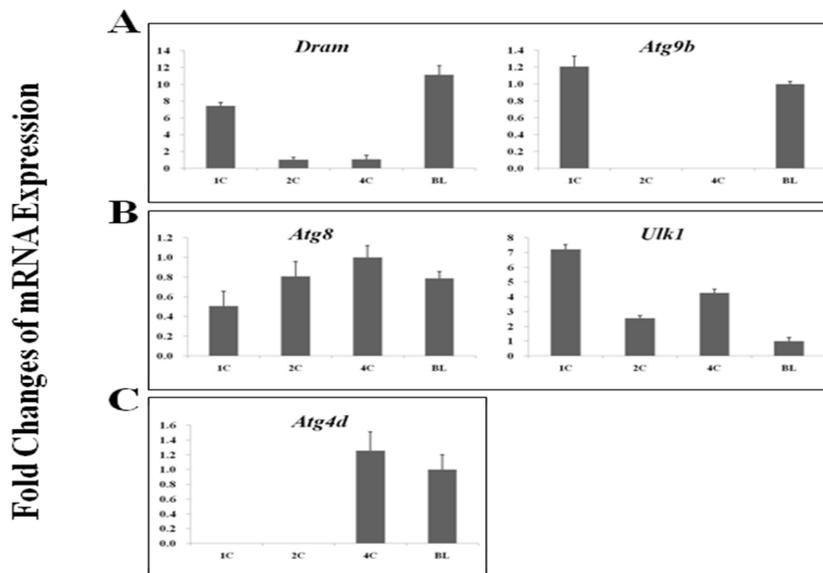
ATGs including *Dram* and *Atg9b* were only expressed in 1-cell embryo state and in blastocyst embryo stage (Fig. 2A). Relative mean expression of *Dram* is 7.43, 1, 1.06 or 11.11, and *Atg9b* is 1.2, 0, 0 or 1 in 1-cell, 2-cell, 4-cell, or blastocyst stage embryo. And, *Atg8/lc3* and *Ulk1* were constantly expressed during preimplantation stage (Fig. 2B). Relative mean expression of *Atg8/lc3* is 0.5, 0.8, 1 or 0.78 and *Ulk1* is 7.21, 2.54, 4.27 or 1 in 1-cell, 2-cell, 4-cell, or blastocyst stage embryo. However, *Atg4d* was only up-expressed from 4-cell to blastocyst stage (Fig. 2C). Relative mean expression of *Atg4d* is 0, 0, 1.25 or 1 in 1-cell, 2-cell, 4-cell, or blastocyst stage embryo.

[Table 1] List of primers used for real-time PCR

Gene name	Sense primer sequence (5'-3')	Antisense primer sequence (5'-3')	PCR product (bp)	GenBank ac. number
Ulk1	CCCAGAGTACCCGTACCAGA	GTGTAGGGTTTCCGTGTGCT	245	NM_009469
Atg2a	CCAGCCTAGCAGCCAGTATC	CAGCAGAGCCTCTTGAGCTT	331	NM_194348
Atg3	TGATGGGGGATGGGTAGATA	TAGCTTTGCAGGCTTCCACT	245	NM_026402
Atg4b	TGCTTTGAGAACCCAGACCT	CTCCTGACCCACTGCTCTTC	250	NM_174874
Atg5	GGAGAGAAGAGGAGCCAGGT	TGTTGCCCTCCACTGAACTTG	227	NM_053069
Atg6	GGCCAATAAGATGGGTCTGA	GCTTTTGTCCACTGCTCCTC	333	NM_019584
Atg7	CCAGGACACCCTGTGAACTT	CAGGTCAGCAGGTGCTACAA	285	NM_028835
Atg8	TTCTTCTCTCTGGTGAATGG	GTGGGTGCCTACGTTCTCAT	251	NM_026160
Atg9a	GGTCACTCTATGGCCGTTGT	GAAGGACCTGCACACAGTCA	278	NM_001003917
Atg9b	ACTAGGGGTGGGGAATATGG	TGAACAGGTGCAGTCCTCAG	217	NM_001002897
Wipi3	CACAGAGGTCAAGGCTGTCA	TGTACGTGGCCAGTGTGAGT	204	NM_025793
Dram	CCTCCTTTGGCTGCTAAGTG	CCAGCTTCCACAGGAAATGT	224	NM_027878
H2a	GATCCTGGAATTGGCTGGTA	TCGGTCTTCTTGGGCAGTAG	180	NM_178218



[Fig. 1] Real-time RT-PCR analysis of autophagy-related genes. *Atg2a*, *Atg3*, *Atg4b*, *Atg5*, *Atg6*, *Atg7*, *Atg9a*, and *Wipi3* that are abundantly upregulated in 1C compared with 2C, 4C, and BL stage embryos in mouse. Mouse *H2a* mRNA expression was used as an internal standard. Fold differences of mRNA from the same numbers of 1C, 2C, 4C, and BL stages after normalization to the internal standard (mouse *H2a*). The lowest messenger RNA expression of stage embryos was arbitrarily set to onefold. 1C, 1-cell stage embryos; 2C, 2-cell stage embryos; 4C, 4-cell stage embryos; BL, blastocyst stage embryos.



[Fig. 2] Real-time RT-PCR analysis of Autophagy-related genes A, *Dram* and *Atg9b* gene that are upregulated in 1C and BL compared with 2C and 4C stage embryos in mouse. B, *Atg8* and *Ulk1* that are constantly upregulated in 1C, 2C, 4C, and BL stage embryos in mouse. C, *Atg4d* that are constantly upregulated from 4C to BL stage embryos in mouse. Mouse *H2a* mRNA expression was used as an internal standard. The lowest messenger RNA expression of stage embryos was arbitrarily set to onefold. 1C, 1-cell stage embryos; 2C, 2-cell stage embryos; 4C, 4-cell stage embryos; BL, blastocyst stage embryos.

4. Discussion

Autophagy are highly conserved in many eukaryotes. In the first step of autophagosome formation, cytoplasmic constituents are sequestered by a unique membrane called the phagophore or isolation membrane. Complete sequestration by the elongating phagophore results in formation of the autophagosome, which is typically a double-membraned organelle. In yeast, 31 Atg (autophagy-related) proteins have been identified, and many of them gather at a site that can be identified by fluorescence microscopy as a punctate spot very close to the vacuolar membrane. Among the 31 Atg proteins, 18 Atg proteins (*Atg1 - 10*, *Atg12 - 14*, *Atg16 - 18*, *Atg29*, and *Atg31*) are involved in autophagosome formation and are called AP-Atg proteins[9].

Atg17 was found to be a scaffold for PAS organization. The recently identified *Atg29* and *Atg31*[10] appear to function together with *Atg17*. *Atg11* is also important for PAS organization but is essential only for the Cvt pathway. Although the precise function of each protein remains to be understood, an unexpected function was shown for yeast *Atg8*. *Atg8*, a ubiquitin-like protein, is present on autophagic membranes as a phosphatidyl ethanolamine (PE)-conjugated form (*Atg8-PE*). *Atg8* mediates tethering and hemifusion of liposomes containing *Atg8-PE* in an in vitro system[11]. This unique membrane fusion process was proposed to account for membrane elongation of phagophore/isolation membranes in vivo. Although many Atg proteins are conserved between yeast and mammals, several mammalian-specific factors that modulate the functions of Atg proteins have been identified. The most well studied is Beclin 1, which is a mammalian *Atg6/Vps30* (vacuolar protein sorting 30) ortholog and a subunit of the class III PI3-kinase complex. Beclin 1 was originally identified as an interaction partner of *Bcl-2*, an anti-apoptotic protein[12]. This *Bcl-2 - Beclin 1* interaction is mediated through a BH3 domain in *Beclin 1* and is reduced upon starvation, freeing *Beclin 1* to activate autophagy.

Humans have approximately seven *Atg8* homologs, including microtubule-associated protein 1 light chain 3 (*LC3*) isoforms A, B and C, gamma-aminobutyric acid A receptor-associated protein (*GABARAP*) and its L3 isoform, Golgi-associated ATPase enhancer of 16 kDa

(*GATE-16*) and *Atg8*-like protein (*ATG8L*),¹⁵ and the best studied of these is *LC3*. Yeast *Atg8* and its mammalian homolog *LC3* specifically label the growing phagophores and completed autophagosomes[13]. *GABARAP*, a second mammalian homolog of *Atg8*, is another modifier in lipidation reactions mediated by *Atg7*, *Atg3* and *Atg4* in the same manner as *Atg8* and *LC3*, and localizes to autophagosome membranes when overexpressed.

In the present study, we explored the expression patterns of autophagy-related genes (Atgs) and their possible role in mouse embryo preimplantation development using quantitative real time RT-PCR. After normalization of real time RT-PCR results against *H2a* expression[14], we found that ATGs including *Atg2a*, *Atg3*, *Atg4b*, *Atg5*, *Atg6*, *Atg7*, *Atg9a*, and *Wipi3* as maternal transcripts were only up-regulated in 1-cell embryo stage before zygotic genomic activation (ZGA). ATGs including *Dram* and *Atg9b* were expressed in 1-cell embryo state and in blastocyst embryo stage, indicating regulation as maternal transcripts in addition of expression by zygotic genomic activation (ZGA). And, *Atg8/lc3* and *Ulk1* were constantly expressed during preimplantation stage. However, *Atg4d* was only up-expressed from 4-cell to blastocyst stage, indicating expression by ZGA. Taken together, these results suggest that autophagy is related in mouse embryo, which possibly give a role for early development.

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<Research Interests>

Animal Reproductive Physiology