Journal of the Korea Academia-Industrial cooperation Society
Vol. 12, No. 8 pp. 3541-3546, 2011

http://dx.doi.org/10.5762/KAIS.2011.12.8.3541

Characterization of Embryo-specific Autophagy during Preimplantation

Jae-Dal Lee[1*]

[1]**Department of Pet Care, Hyejeon College**

착상전 난자 자식작용의 특성규명

이재달[1*]

[1]혜전대학교 애완동물관리과

Abstract Autophagy is an evolutionarily conserved lysosomal pathway for degrading cytoplasmic proteins, macromolecules, and organelles in addition to recycling protein and ATP synthesis. Although autophagy is very important during embryogenesis, the mechanism underlying the dynamic development during this process remains largely unknown. In order to obtain insights into autophagy in early embryo development, we analyzed gene expression levels of autophagy-related genes (ATGs) in mouse embryos developing in vitro. Using real time RT-PCR technique, 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), and then expression decreased from 2-cell to blastocyst embryo stage. ATGs including *Dram* and *Atg9b* were expressed abundantly in 1-cell embryo state and in blastocyst embryo stage, athough *Atg8* and *Ulk1* were constantly expressed during preimplantation stage. However, *Atg4d* were only up-expressed from 4-cell to blastocyst stage. These results suggest that autophagy is related in mouse embryo, which possibly gives an important role for early development.

요 약 자식작용은 난자 세포질의 단백질 고분자 물질과 세포 소기관 분해를 위해서 세포질 리소좀 통로에 유전적으로 작용하고 있으며 ATP합성과 단백질 재활용에 관여하고 있다. 이러한 자식작용은 난자 발달 과정에서 매우 중요하지만 세포질 내 자식작용의 동적 발달 과정의 근원적인 기전은 잘 알려지지 않고 있다. 따라서 본 연구에서는 초기 난자 발달 과정의 자식작용을 이해하기 위해서 쥐 난자 체외 성숙 과정에서 자식작용과 관련된 유전자들의 유전적 발현 수준을 분석하였다. Real Time RT-PCR 기법을 이용하여 유전자 *Atg2a, Atg3, Atg4b, Atg5, Atg6, Atg7, Atg9a,* 그리고 W*ipi3* 같은 모계에서 유전된 ATGs 군들의 유전자들은 수정난 유전체 활성화(ZGA) 이전 단계인 1세포기 에서 높게 발현되었고, 그 후 이들 유전자들의 발현은 배반포 단계와 2세포기 4세포기 단계에서는 감소함을 알 수 있었다. *Dram*과 *Atg9b* 유전자들은 배반포와 1세포기 단계에서 발현됨으로서 모계 유전자이면서 ZGA에 의해서 발현되는 유전자임을 알 수 있었다. 한편 *UIK1*의 유전자 발현은 착상 전 단계에서 일정하게 나타남을 알 수 있었다. 하지만 *Atg4d* 유전자의 경우 4세포기에서부터 배 반포 단계까지 높게 나타남을 알 수 있었다. 이러한 결과로부터 생쥐 난자 발달 과정에서 자식작용과 관련된 유전자들은 초기 난자 발달과정에서 중요한 역할 과정임을 알 수 있었다.

Key Word : Preimplantation, Embryo, Autophagy, Real time RT-PCR, ZGA

1. Introduction

Autophagy is an intralysosomal degradation pathway conserved from yeast to plants and animals. Increasing evidence has informed the connection between autophagy and various physiological processes in higher eukaryotes[1].

This work was supported by research grant of the Hyejeon college in 2010.
*Corresponding Author : Jae-Dal Lee(jdlee@hj.ac.kr)
Received June 14, 2011 Revised June 28, 2011 Accepted August 11, 2011

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 hyalulonidase (Sigma) in M2 (Sigma) medium. The embryos were cultured in mouse culture medium (M16) at 37°C under an atmosphere containing 5% CO_2.

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^{TM} 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℃ followed by 39 cycles of 30sec at 94℃, 30sec at 55 or 60℃, 55sec at 72℃, and a final extension of 5min at 7 2℃. Finally, the relative quantification of gene expression was analyzed using the 2-ddCt method by normalization to internal mouse histone H_2AmRNAlevels

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

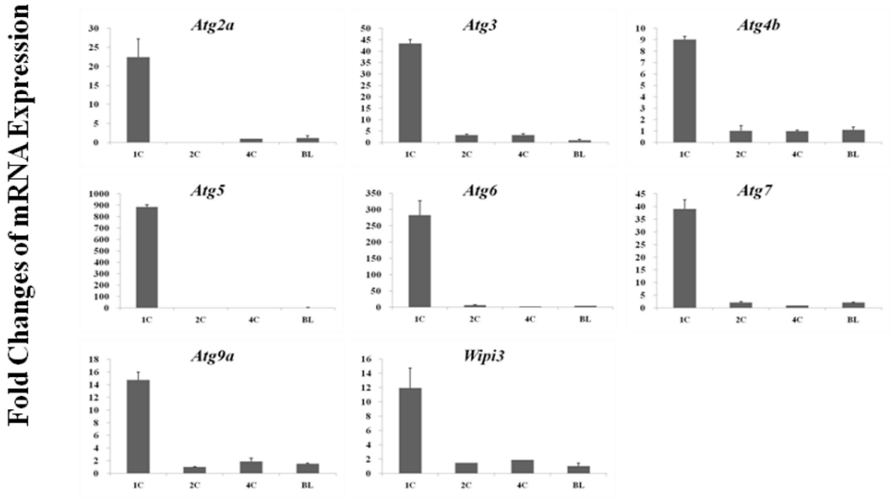
3.1 Expression of autophagy-related genes (ATGs) in mouse embryo

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.

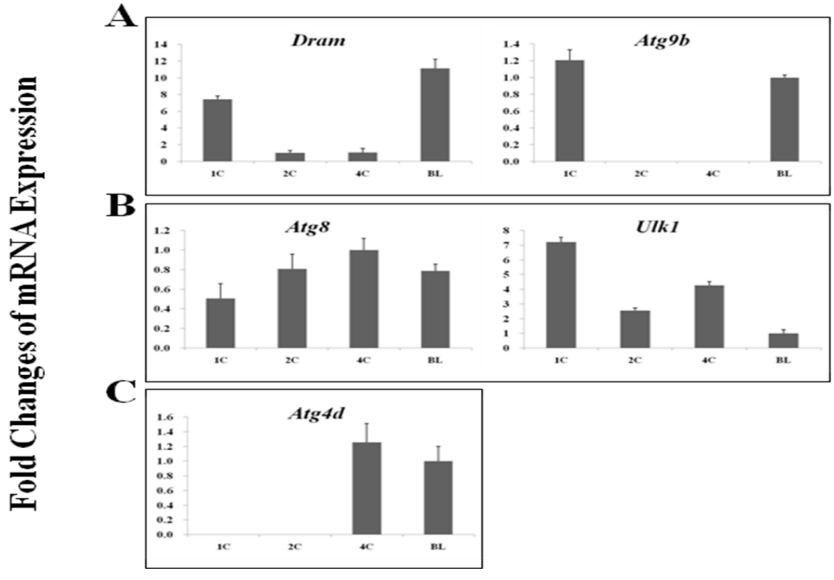
ATGs including Dram and *Atg9b* were only expressed in 1-cell embryo state and in blastocyst embryo stage (Fig. 2A). Relative mean expression of *Dram* is 7.43, 1, 1.06 or 11.11, and *Atg9b* is 1.2, 0, 0 or 1 in 1-cell, 2-cell, 4-cell, or blastocyst stage embryo. And, *Atg8/lc3* and Ulk1 were constantly 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	TGTTGCCTCCACTGAACTTG	227	NM_053069
Atg6	GGCCAATAAGATGGGTCTGA	GCTTTTGTCCACTGCTCCTC	333	NM_019584
Atg7	CCAGGACACCCTGTGAACTT	CAGGTCAGCAGGTGCTACAA	285	NM_028835
Atg8	TTCTTCCTCCTGGTGAATGG	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 exression 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.

References

[1] Shintani, T. and D, J. Klionsky. utophagy in health and disease: a double-edged sword, Science, 306:990–995, 2004.

[2] Klionsky, D.J, R. Cueva, and D.S. Yaver. Aminopeptidase I of Saccharomyces cerevisiae is localized to the vacuole independent of the secretory pathway. J. Cell Biol,119:287–299, 1992.

[3] Dunn, W.A. Jr, J.M. Cregg, J.A.K.W. Kiel, I.J. van der Klei, M. Oku, Y. Sakai, A.A. Sibirny, O.V. Stasyk, and M. Veenhuis. Pexophagy. the selective autophagy of peroxisomes. Autophagy. 1:75–83. 2005.

[4] Suzuki, K., T. Kirisako, Y. Kamada, N. Mizushima, T. Noda, and Y. Ohsumi.. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. EMBO

J, 20:5971 – 5981, 2001.

[5] Shintani, T. W-P Huang. P.E. Stromhaug. and D.J. Klionsky. Mechanism of cargo selection in the cytoplasm to vacuole targeting pathway. Dev. Cell, 3:825 – 837, 2002.

[6] Scott, S.V. J. Guan. M.U. Hutchins, J. Kim, and D.J. Klionsky. Cvt19 is a receptor for the cytoplasm-to-vacuole targeting pathway, Mol. Cell, 7:1131 – 1141, 2001.

[7] Kim J. Y. Kamada. P.E. Stromhaug. J. Guan. A. Hefner-Gravink, M. Baba. S.V. Scott. Y. Ohsumi, W.A. Dunn. and D J. Klionsky. Cvt9/Gsa9 functions in sequestering selective cytosolic cargo destined for the vacuole. J. Cell Biol, 153:381 – 396. 2001.

[8] He, C. H. Song, T. Yorimitsu I. Monastyrska. W - L. Yen E. Legakis . and D J Klionsky . 6. Recruitment of Atg9 to the preautophagosomal structure by Atg11 is essential for selective autophagy in budding yeast . J. Cell Biol, 175:925 – 935, 2006.

[9] Suzuki, K, Ohsumi, Y. Molecular machinery of autophagosome formation in yeast, Saccharomyces cerevisiae. FEBS Lett, 581:2156 – 2161, 2007.

[10] Kabeya, Y, Kawamata, T, Suzuki, K, and Ohsumi, Y. Cis1/Atg31 is required for autophagosome formation in *Saccharomyces cerevisiae*. Biochem, Biophys, Res. Commun 356, 405 – 410, 2007.

[11] Nakatogawa, H. Ichimura, Y. Ohsumi, Y. *Atg8*, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion . Cell 130:165 – 178 , 2007.

[12] Liang, X.H. Kleeman, L K. Jiang, H.H. Gordon, G. Goldman, J. E. Berry, G. Herman, B. Levine, B. Protection against fatal Sindbis virus encephalitis by Becli a novel Bcl-2-interacting protein. J. Virol. 72:8586 – 8596, 1998.

[13] Kirisako T, Baba M, Ishihara N, Miyazawa K, Ohsumi M, Yoshimori T, et al. Formation process of autophagosome is traced with Apg8/Aut7p in yeast. J Cell Bio. 147:435 – 46, 1999.

[14] Cui X-S. Shin M R . Lee KA. Kim NH. Identification of differentially expressed genes in murine embryos at the blastocyst stage using annealing control primer system. Mol Reprod Dev, 70:278 – 287, 2005.

Jae-Dal Lee [Regular member]

- Feb. 1977 : ChungNam National Univ., Dept. of Textile Engineering
- Feb. 1990 : ChungNam National Univ., Dept. of Textile Engineering, MS
- Feb. 1999 : ChungNam National Univ., Dept. of Textile Engineering, PhD
- Aug. 2007 : ChungBuk National Univ., Dept. of Animal Science Graduate, MS
- Mar. 1995 ~ current : Hyejeon College, Dept. of Pet Care, Professor

<Research Interests>
Animal Reproductive Physiology