Endothelial Nitric-oxide Synthase Antisense (NOS3AS) Gene Encodes an Autophagy-related Protein (APG9-like2) Highly Expressed in Trophoblast*

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Takahiro Yamada‡, Andrew R. Carson§§, Isabella Caniggia†, Kyoei Umebayashi, Tamotsu Yoshimori, Kazuhiko Nakabayashi, and Stephen W. Scherer§§**

From the ‡Program in Genetics and Genomic Biology, Research Institute, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada, §Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario M5G 2M9, Canada, ¶Program in Development and Fetal Health, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada, and **Department of Cell Genetics, National Institute of Genetics, Mishima 411-0801, Japan

Macroautophagy is an intracellular degradation system for the majority of proteins and some organelles that is conserved in all eukaryotic species. The precise role of autophagy in mammalian development and potential involvement in disease remain to be discerned. Yeast Atg9p is the first integral membrane protein shown to be essential for the cytoplasm to vacuole targeting (Cvt) pathway and autophagy, whereas its mammalian functional orthologue has yet to be identified. We have identified two human genes homologous to yeast Atg9p and designated these as APG9L1 and APG9L2. We have previously identified APG9L2 as NOS3AS, which participates in the post-transcriptional regulation of the endothelial nitric-oxide synthase (NOS3) gene on chromosome 7 through its antisense overlap. In human adult tissues, APG9L1 was ubiquitously expressed, whereas APG9L2 was highly expressed in placenta (trophoblast cells) and pituitary gland. In transient transfection assays we found that both proteins were primarily localized to the perinuclear region and also scattered throughout the cytosol as dots, a subset of which colocalized with an autophagosome-specific marker LC3 under starvation conditions. Finally, by the small interfering RNA-mediated knockdown of APG9L1 in HeLa cells, we demonstrated that APG9L1 is essential for starvation-induced autophagosome formation. In addition, APG9L2 can functionally complement APG9L1 in this process. These results, taken together with those of phylogenetic and sequence analyses, suggest that both APG9L1 and APG9L2 are functionally orthologous to the yAtg9 in autophagosome formation. Moreover, APG9L2 is a vertebrate-specific gene that may have gained critical roles in mammalian-specific developmental events, such as placentation, through rapid evolution.

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** An Investigator of the Canadian Institutes of Health Research and International Scholar of the Howard Hughes Medical Institute, with support from Genome Canada. To whom correspondence should be addressed: Rm. 9107, Program in Genetics and Genomic Biology, 555 University Ave., Toronto, Ontario, M5G 1X8, Canada. Tel.: 416-813-7613; Fax: 416-813-8319; E-mail: steve@genet.sickkids.on.ca.

Macroautophagy (or simply autophagy) is an intracellular degradation system used to recycle the majority of proteins and some organelles (1–3). In yeast, the autophagic pathway begins with the nonselective sequestering of cytoplasm into a double-membrane vesicle called the autophagosome. The outer membrane of the autophagosome is then targeted to the vacuole, where it fuses with the vacular membrane and releases the inner membrane with its contents (the autophagic body) into the vacuole lumen. Inside the vacuole, a variety of hydrolases breakdown the vesicle and degrade the cytoplasmic material (1). The molecular mechanism of autophagy has been extensively studied through genetic screening and characterization of yeast genes required for autophagosome formation. Many of the 27 yeast autophagy-related genes identified so far are found to have homologues in higher eukaryotes, including mammalian species (4). Although studies of such homologues at the cellular level have revealed the conservation of autophagosome formation mechanisms from yeast to mammalian species, the roles of autophagy at the organismal level have just begun to be understood.

Autophagy is suggested to be involved in development, differentiation, growth regulation, and tissue remodeling in multicellular organisms (5). Studies using mutants disrupted in autophagy genes have demonstrated a variety of phenotypes such as defective fruiting body formation in Dictyostelium discoideum (6), premature death from the third larval to pupal stages in Drosophila melanogaster (7), abnormal dauer formation and short life span in Caenorhabditis elegans (8), and accelerated senescence in Arabidopsis thaliana (9, 10). Recent generation of transgenic and knock-out mouse models for autophagy genes has provided new knowledge about the roles of autophagy in mammalian development and its possible links with other physiological events such as apoptosis. Analysis of transgenic mice expressing a fluorescent autophagosome marker (GFP1-LC3) revealed that autophagy is induced in almost all tissues by nutrient starvation but with different patterns of response and that autophagy occurs actively without starvation treatments in some tissues (11). Homozygous knock-out mice for Atpg6β (Becn1) are lethal at embryonic day 7.5, and heterozygous mice exhibit increased tumorigenesis (12). Conversely, mice deficient for Atpg5, which is shown to be es-
sential and solely responsible for autophagosome formation (13), appear almost normal at birth but die within 1 day of delivery due to energy depletion (14). This suggests autophagy is seemingly dispensable for mouse early developmental events.

In humans, mostly from morphological studies, possible roles of autophagy have been suggested in several disease conditions such as cancer, liver disease, muscular disorders, neurodegeneration, and pathogen infection (15, 16). However, whether autophagy might protect from or cause disease is unclear for the majority of cases. In plant and mammalian species, several autophagy genes such as APG1, APG4, and APG8 are shown to have paralogous gene copies (10, 17, 18). Although it remains to be determined whether all or a part of these genes function in autophagosome formation, some of those have been demonstrated to show tissue-specific differential expression patterns (10, 17), which may contribute to the developmental regulation of autophagic activity. Further characterization of the paralogous members would provide clues about how autophagy is involved in mammalian development, homeostasis, and possibly disease.

We have previously reported that NOS3AS overlaps with NOS3 at chromosome 7q36 (19) and that it is involved in its post-transcriptional regulation (20). The NOS3 locus has been suggested to be associated with a number of diseases including pre-eclampsia (OMIM 189800), which is characterized by pregnancy-induced hypertension, proteinuria, and edema (21, 22). However, the association was not consistently observed in disease-induced hypertension, proteinuria, and edema (21, 22). We have previously reported that NOS3AS encodes a putative 924-amino acid protein having significant sequence similarity with the yeast Atg1p/Agg9p protein (yAtg9p hereafter) and that the human genome contains two homologues of the yeast ATG9 gene: APG9L1 (Autophagy 9-like 1) on 2q35 and APG9L2 (NOS3AS) on 7q36 (20). yAtg9p is the first integral membrane protein shown to be essential for the cytoplasm to vacuole targeting (Cvt) pathway and autophagy (26), whereas its mammalian functional orthologue has yet to be identified. In yeast, yAtg9p localizes to pre-autophagosomal structure (PAS) but is not found on completed autophagosomes (26), indicating that it is retrieved during the autophagosome maturation process. yAtg9p has also been shown to localize to other cytosolic punctate compartments (26, 27). It has been postulated that yAtg9p recycles between PAS and these other distinct structures (28).

In this study, as a first step in understanding the potential roles of APG9L2 and autophagy in placental development and disease, we determined if APG9L2 functions in mammalian autophagy. Our results suggest that both APG9L1 and APG9L2 are functionally orthogonal to the yeast ATG9 gene in the cellular autophagosome formation. APG9L2, however, is a vertebrate specific gene that may have gained critical roles in mammalian-specific development.

**EXPERIMENTAL PROCEDURES**

*mRNA Sequences for the Human and Mouse APG9L1 and APG9L2 Genes*—The full-length mRNA sequence (GenBank™ accession number BK004019) for the human APG9L2 gene was assembled from two mRNA sequences (AK027791 and AK096734), three ESTs (BM829733, BI055880, and AI952090), and putative exons identified by human-mouse homology (>75%) using mVISTA (www.gsl.lbl.gov/vista) and verified by RT-PCR. The mRNA sequences for the mouse Apg9l1 and Apg9l2 genes (BK004020 and BK004021) were predicted by the comparison of the mouse genomic DNA sequence with the human mRNA sequences and verified by RT-PCR.

**Collection of APG9 Homologues**—The yeast Atg9p sequence (NP_010132.1) was queried against the nr protein database (NCBI) using blastp and tblastn programs to retrieve the following APG9 homologues: *Anopheles gambiae* (XP_393558.1), *Caenorhabditis briggsae* (CAE58273.1), *A. thaliana* (NP_850164.1), *Ciona intestinalis* (AK114827), *C. elegans* APG9L1 (NP_501781.1), *D. melanogaster* APG9L1 (NP_611114.1). The other APG9 homologues from chimpanzee, dog, rat, chicken, Fugu, and zebras were deduced from hypothetical mRNA sequences assembled from our own sequence annotation (the deduced protein sequences are available upon request). Partial EST sequences, predicted gene models by Ensembl (www.ensembl.org), and exon-intron structures supported by the sequence conservation between the human APG9L1 or APG9L2 mRNA sequence and the genomic DNA sequence of each species (available from the UCSC genome browser at genome.ucsc.edu) were used for the sequence assembly.

**Multiple Alignment of APG9 Homologues**—The assembled nucleotide sequences of yAtg9 homologues were initially translated into proteins using Open Reading Frame Finder (ORF Finder; www.ncbi.nlm.nih.gov/orffinder.html). ClustalW (29) was then used to align all of the identified protein homologues. This protein alignment, along with the nucleotides sequences, was used to create a multiple, nucleotide sequence alignment that maintained the correct reading frame for all the sequences. This nucleotide alignment was then used to create the phylogenetic tree in Fig. 1B.

**RT-PCR**—Two micrograms of total RNA were used for reverse transcription with random hexamers using SuperScript II (Invitrogen). The following conditions were used for PCR: initial denaturation at 94 °C for 4 min, followed by 30–35 cycles of denaturation at 94 °C for 40 s, annealing at 56 °C for 40 s, and extension at 72 °C for 1–2 min. The primers (product size) used for human genes are 5′-GATGAACC-TACCCCTCAAGGG-3′ and 5′-ACAGCCGACAGCTGTCATC-3′ for APG9L1 (484 bp), 5′-CAGTGATCAAGACCAGCACT-3′ and 5′-CTTCGCTGCGCTGCTACTAC-3′ for APG9L2 (1014 bp), and 5′-CTGTG-GCACACAGCTA-3′ and 5′-AAAGCATTGCCAATCTACTAC-3′ for β-actin (399 bp).

**Quantitative Real-time RT-PCR**—ABI PRISM 7900 HT sequence detection system (PE Applied Biosystems) was used with SYBR Green detection method. The primers used and amplon size (in parentheses) are 5′-GCAAGATCTCCTGACCAATATAAGAA-3′ and 5′-CAGTGG-AAGCCATTTCTCTTTGGTAGCAG-3′ (85 bp) for APG9L1, 5′-CACATCCAA-GTCCTAGACAGTTTCTC-3′ and 5′-CCGGCTGTTGTGCTTAGTCTT-GGG-3′ (54 bp) for APG9L2, 5′-GAGGCTGAAGGCTGAGATC-3′ and 5′-GAAAGCTGTGATGAGGTTTCTC-3′ (265 bp) for glyceraldehyde-3-phosphate dehydrogenase, 5′-CATTGGCCGCGCCGACGCCTTGG-3′ and 5′-CTGTGGCCTGTGTTGGACGACTTGTG-3′ (75 bp) for the human cytochrome A. PCR conditions were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The PCR reactions were performed in separate tubes for each gene. The results from six independent reactions in two experiments were used to determine the absolute and relative expression levels of APG9L1 and APG9L2 transcripts. The absolute quantification was performed using a standard curve based on the amplification levels of a dilution series of transcript-specific purified PCR products. The relative quantification was performed according to the manufacturer’s instructions (PE Applied Biosystems) using glyceraldehyde-3-phosphate dehydrogenase and cyclophilin A as normalization controls.

**Isolation of Human Trophoblast Cells**—Human trophoblast cells were prepared as described previously (30). Briefly, normal term human placenta was obtained after spontaneous vaginal delivery. It was washed in phosphate-buffered saline and digested with trypsin (Invitrogen) and DNase I (Sigma) to release free cells. Unwanted cells were removed from the resulting cell suspension by centrifugation through a 5–75% Percoll gradient. The purity of resulting cytotrophoblast cells (single cell cytotrophoblast) was assessed immunocytochemically using anti-cytokeratin 7 antibody (DakoCytomation, Glostrup, Denmark), and >95% cells were found to be cytokeratin 7-positive. A subset of cytotrophoblast cells was plated in Dulbecco’s modified Eagle’s medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (HyClone) and cultured for 3 days at 37 °C. Under this condition, cytotrophoblast cells aggregate and form a spheroid-like structure that was assessed by phase contrast microscopy.

cDNA Constructs for Fluorescent Protein-tagged Mouse APG9L1 and APG9L2 Proteins—The Apg9l1 ORF was generated by PCR using primers mcApg9-F-NheI (5′-GCTAGGCTGCACCATGTTAGAAGA-3′) and mcApg9-R-HindIII (5′-AAGCTTCTCTGTTGACTGAGG-3′). The Apg9l2 ORF was amplified as three overlapping cDNA fragments by PCR. The primer sets used are 4upF-NheI (5′-GCTAGGCTGCACCATGTTAGAAGA-3′) and 4upR-HindIII (5′-AAGCTTCTCTGTTGACTGAGG-3′) and 7upF-NheI (5′-GCTAGGCTGCACCATGTTAGAAGA-3′) and 7upR-HindIII (5′-AAGCTTCTCTGTTGACTGAGG-3′).
Characterization of Mammalian APG9 Proteins

TABLE I
Pairwise p-distance analysis between hAPG9L1, hAPG9L2, and yeast Atg9p

| Region of comparison        | hAPG9L1 | hAPG9L2 |
|-----------------------------|---------|---------|
| hAPG9L2                     |         |         |
| APG9 domain                 | 0.436   |         |
| Outside domain              | 0.604   |         |
| Whole protein               | 0.517   |         |
| yAtg9p                      |         |         |
| APG9 domain                 | 0.719   |         |
| Outside domain              | 0.832   |         |
| Whole protein               | 0.774   | 0.789   |
| hAPG9L2                     | 0.436   |         |
| APG9 domain                 | 0.436   |         |
| Outside domain              | 0.604   |         |
| Whole protein               | 0.517   |         |

*The positions of the APG9 domains are amino acids 173–550 of hAPG9L1 (DAA02272), amino acids 320–701 of hAPG9L2 (DAA02273), and amino acids 405–798 of yAtg9p (NP_010132).

GGGAGACTG-3', mousegsp-F (5'-TCTGGGACATCAGGTGTGGT-3') and 2R (5'-CAGCCCATCAGCTCAG TGGA-3'), and 9upF-BamHI (5'-GATCCTCTGGTCTTCTCTGGGAG-3') and R2-BamHI (5'-GGATCCCTGGTCTTCTCTGGGAG-3'). Restriction enzyme sites in the primer sequences used for subcloning are underlined. PCR was performed with PfuTurbo Hotstart DNA polymerase (Stratagene) using mouse placenta cDNA as template. The Apg9l1 ORF fragment was excised with NheI and HindIII and cloned in pEGFP-N1 and pDest2-N1 vectors (Clontech) to generate cDNA constructs that express the mouse APG9L1 protein tagged with a fluorescent protein (EGFP or DsRed2) at the C terminus of APG9L1 (mAPG9L1-EGFP and mAPG9L1-DsRed2). The three Apg9l2 ORF fragments were successively cloned in the same vectors to generate cDNA constructs for mAPG9L2-EGFP and mAPG9L2-DsRed2.

Tissue Culture and DNA Transfection—Cells were grown in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% bovine growth serum (Hyclone) at 37 °C in 5% CO2. HeLa, COS7, and NIH3T3 cells stably expressing EGFP, EYFP or ECFP fused with LC3 (Si) were established and maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine growth serum and 300–400 μg/ml G418. Cells were transfected with expression vectors using Lipofectamine reagent (Invitrogen). Forty-eight hours after transfection, to induce autophagy by nutrient starvation conditions, cells were washed three times with Hank's balanced salt solution (HBSS, catalog number 14025-092, Invitrogen) and incubated in HBSS for 2 h (NIH3T3) or 4–24 h (for COS7 and HeLa) at 37 °C.

Small Interfering RNA (siRNA)-mediated Gene Silencing of APG9L2—siRNA duplex (5'-TCTGGGACATCAGGTGTGGT-3') targeted to the human APG9L1 mRNA sequence was designed by Qiagen (HiPerformance 2-For-Silencing siRNA Duplexes). The duplex or scrambled control siRNA (Qiagen) was transfected in the HeLa cells stably expressing LC3-EGFP using RNAiFect Transfection Reagent (Qiagen) following the manufacturer's instructions. The non-silencing control siRNA labeled with Alexa Fluor 488 from the RNA interference reagent (Invitrogen). Forty-eight hours after transfection, to observe the evolution of APG9 genes through multiple lineages, we collected APG9-like nucleotide and protein sequences from a wide range of organisms, from mammalian species to many lower eukaryotes, using the BLAST programs (NCBI). We then used MEGA5 (32) to construct a neighboring phylogenetic tree from the nucleotide sequence alignment using a Kimura 2-parameter model (Fig. 1B). This analysis indicated that a vertebrate-specific duplication event occurred before the divergence of fish. This has led to the evolution of two APG9 paralogues in all vertebrates examined, including fish, amphibian, bird, and mammalian lineages. Lower eukaryotes have only a single APG9 gene. As was reported previously, the human and mouse APG9L2 homologues overlap with NOS3 in a 3'-to-3' orientation (20). This orientation appears to be conserved in all mammals investigated and in Xenopus tropicalis. Although we were unable to confirm this orientation in chicken and fish due to the incomplete nature of their genome sequence builds, our data indicate that APG9L2 homologues are consistently adjacent to NOS3 homologues in vertebrates.
APG9L2 proteins are much more similar to the mammalian mouse than the identity between the non-mammalian homologues of the APG9L1 and APG9L2 proteins, and found that performed Northern and RT-PCR analyses on human adult in critical developmental events such as placentation. This rapid divergence of the mammalian lineage may have led to the evolution of novel mammalian-specific functions that could be important in critical developmental events such as placenta.

Expression Patterns of APG9L1 and APG9L2 Genes—We performed Northern and RT-PCR analyses on human adult tissues to compare the expression patterns of APG9L1 and APG9L2, and found that APG9L1 is more widely expressed than APG9L2, whose expression appears to be limited to placenta and pituitary gland with minor levels in testis and uterus (Fig. 2, A and B). This tendency was also observed for the mouse Apg9l1 and Apg9l2 genes in the RT-PCR analysis on the adult tissues. Mouse Apg9l2 was found to be more widely expressed at embryonic stages than the adult stage (data not shown). One goal underlining this study was to examine if APG9L2 has any role in placental development and disease. Therefore, we performed quantitative RT-PCR to measure the expression levels of APG9L1 and APG9L2 in human trophoblast cells isolated from term placenta, a choriocarcinoma cell line JEG3, and three additional human cell lines of non-placental origin: HeLa (cervical carcinoma), VA13 (SV40-transformed fibroblast), and A549 (lung carcinoma). Consistent with its ubiquitous expression in human and mouse tissues, APG9L1 was found to be expressed in all cell lines and trophoblast cells with various levels. However, APG9L2 was found to be only in placenta-derived cells, highly expressed in cytotrophoblast and syncytiotrophoblast cells, expressed in JEG3 at a very low level, but not expressed in the other three cell lines (Fig. 2C). Through the differentiation from cytotrophoblast to syncytiotrophoblast cells, APG9L2 showed a 4.3-fold decrease in its expression level, whereas APG9L1 showed a 1.5-fold increase, suggesting that APG9L2 expression is not only tissue-specific but also possibly developmentally regulated.

Subcellular Localization of APG9 Proteins—To elucidate the cellular functions of the two mammalian APG9 proteins and their possible functional differences, we examined their subcellular localizations in cultured cells by transient transfection of cDNA constructs carrying a fluorescent protein-tagged mouse Apg9l1 or Apg9l2 protein (mAPG9L1-EGFP or mAPG9L2-EGFP, see “Experimental Procedures”). It should be noted that yAtg9p tagged with EGFP on its C terminus was previously shown to be functional (26). mAPG9L1-EGFP or mAPG9L2-EGFP was co-transfected with a mitochondrial marker (DsRed2-Mito, Clontech) into HeLa, COS7, and NIH3T3 cells. pDsRed2-Mito encodes the mitochondrial targeting sequence of the human cytochrome c oxidase subunit VIII fused with DsRed2. Subcellular localization of expressed fluorescent proteins was observed under normal and starvation (Hanks’ balanced salt solution) conditions. We noticed that although 2 h of starvation culture induced autophagosome formation efficiently in NIH3T3 cells, a longer starvation culture was required for HeLa and COS7 cells. Because we did not observe apparent cell death of HeLa cells upon 36 h and COS7 cells upon 24 h of starvation culture, we used 4–24 h of Hanks’ balanced salt solution culture to induce autophagy in these cells. mAPG9L1 and mAPG9L2 showed indistinguishable subcellular localization patterns, being primarily localized at a certain perinuclear region and also localized throughout the cytoplasm under both normal and starvation conditions (Fig. 3 and data not shown). The ratio of intensity levels between the perinuclear and cytoplasmic signals varied within the cell population. However, neither of them colocalized with DsRed2-Mito. We also transiently expressed the predicted mitochondria signal sequence of mAPG9L2 tagged with EGFP and found the fusion protein did not colocalize with DsRed2-Mito (data not shown). Therefore, although three independent programs detected a candidate mitochondrial signal sequence at the N terminus of APG9L2 but not in APG9L1, we failed to detect any clear difference between the two proteins in their subcellular localization patterns with respect to mitochondria in our experimental conditions. However, this is unremarkable because the reliabilities of prediction programs of mitochondrial signal sequence are still controversial (33). Future studies should examine the subcellular localization of endogenous APG9 proteins to directly determine the functional property, if any, of the predicted mitochondrial signal sequence.

A Subset of APG9 Proteins Colocalizes with an Autophagosome-specific Marker LC3—To assess if APG9 protein is involved in autophagosome formation in mammalian cells, we compared the subcellular localization of these proteins with that of LC3, which targets to the isolation membrane throughout the course of membrane elongation and remains on the autophagosomal membrane after the completion of autophagosome formation (3). mAPG9L1-DsRed2 or mAPG9L2-DsRed2 was transiently expressed in the following cells stably expressing some-specific Marker LC3: HeLa with EGFP-LC3, COS7 with EYFP-LC3, and NIH3T3 with EGFP-LC3. Subcellular localization patterns were analyzed under starvation conditions. Both mAPG9L1 and mAPG9L2 were found to be partially co-localized with LC3 to various extents among a cell population in all three cell lines (Fig. 4 and data not shown). This localization pattern is similar to that of yAtg9p, which is shown to localize both to pre-autophagosomal structure and to other cytosolic punctate compartments (26, 27). To further assess if APG9L1 or APG9L2 are in the same protein complex with LC3 when they are colocalized, we performed time-lapse imaging of these fluorescent protein-labeled structures. We observed that a portion of colocalized signals moved in concert with LC3 when they are colocalized, we performed time-lapse imaging of these fluorescent protein-labeled structures. We observed that a portion of colocalized signals moved in concert with LC3 when they are colocalized. Taken together, these results suggest the possibility that both APG9L1 and APG9L2 proteins are involved in autophagosome formation.

Both APG9 Proteins Are Functional in Mammalian Autophagosome Formation—To determine whether APG9 proteins are required for mammalian autophagosome formation, we initially performed functional complementation assays using the yeast Atg9 mutant strain in which autophagic activity can be measured by alkaline phosphatase assays (34). Neither introduction of mouse Apg9l1 nor Apg9l2 complemented the function of yATG9 (data not shown), consistent with the previous report on Arabidopsis APG9 (10). Mammalian and plant APG9 proteins are likely to be too diverged.
from the yAtg9p to complement the yeast mutant even though they are orthologous.

To directly assess the function of APG9L1, we knocked down APG9L1 expression in HeLa cells using siRNA. We chose HeLa cells because our quantitative RT-PCR analysis (Fig. 2C) showed that only APG9L1 but not APG9L2 is expressed in this cell line. HeLa cells stably expressing EGFP-LC3 were treated with targeted or scrambled (control) siRNA for 48 h, further cultured either in the normal or the starvation condition for 24 h, and used to analyze the effects of siRNAs on the APG9L1 expression level and autophagosome formation. The transfection efficiency of siRNA in our optimized experimental conditions was typically near 100% (Fig. 5A). siRNA targeted to APG9L1 was found to cause an 80% decrease in its expression level compared with the control (Fig. 5B). Starvation-induced autophagosome formation (EGFP-LC3-positive dots) was found to be drastically reduced by APG9L1-targeted siRNA compared with the control (87.5% decrease) (Fig. 5, C and D).

Subsequently, we examined if mAPG9L2 expression recovers the suppression of autophagosome formation by siRNA-mediated knockdown of APG9L1. APG9L1-targeted siRNA and the cDNA construct for mAPG9L2-DsRed2 were co-transfected in the HeLa cells stably expressing EGFP-LC3. In the starvation condition, apparently higher numbers of EGFP-LC3 dots were observed in mAPG9L2-DsRed2-positive cells than negative cells (Fig. 6), indicating that mAPG9L2 is functional in autophagosome formation. The mean ± S.E. values of the numbers of EGFP-LC3 dots in mAPG9L2-DsRed2-positive and negative cells were 37.1 ± 1.7 and 5.1 ± 0.4, respectively (n = 10, p < 0.001) (Fig. 6B). It should be noted that transient expression of fluorescent protein-tagged mAPG9L2 (as well as mAPG9L1) was detected in a small proportion of the transfected cells consistently through the experiments in this study as shown in Fig. 6 (the middle panel), whereas the transfection efficiency of siRNA typically approached 100% (Fig. 5A). Our results indicate that APG9L1 is essential, and APG9L2 is complementary to APG9L1 in mammalian autophagy.

DISCUSSION

Both APG9L1 and APG9L2 Are Functionally Orthologous to the Yeast ATG9—We have identified two mammalian APG9-like genes and have shown that both encoded proteins are involved in autophagosome formation. This is the first demonstration of the essential role of APG9-like genes in mammalian autophagy. APG9L1 is ubiquitously expressed and evolutionarily conserved from yeast to mammalian species, whereas APG9L2 is tissue-specifically expressed in adult tissues and exists only in vertebrate species (from fish to mammals) but not

FIG. 2. Expression patterns of human APG9L1 and APG9L2 genes. A, Northern blot analysis. A human MTN blot containing poly(A)+ RNA of human adult tissues (Clontech, number 7760-1) was hybridized with [α-32P]dCTP-labeled probes. cDNA fragments used as probes correspond to nucleotides 2664–3147 of BK004018 for APG9L1 and nucleotides 309–509 of AK027791 for APG9L2. B, RT-PCR analysis. C, quantitative RT-PCR on human trophoblast cells and cell lines. C.T and S.T denote cytotrophoblast and syncytiotrophoblast cells, respectively. The upper panels show relative expression levels of APG9L1 and APG9L2. The lower panels show absolute expression levels of APG9L1 and APG9L2 in each cell type or line. See “Experimental Procedures” for the normalization methods used.
We postulated that the assays, it is possible that between cancer (36, 37). Although we did not find significant differences is shown to be required for both autophagy and protection from is shown to be well conserved from yeast to mammalian species, autophagy mechanisms at the organismal level are not well understood. Higher multicellular organisms seem to have gained paralogues for autophagy genes through evolution, and such paralogues have diverged to encode different tissue-specific expression patterns and possibly differentiated functions in autophagy. Among the autophagy genes found to have paralogues in mammals, although autophagy can be induced during stress conditions such as nutrient starvation, it also occurs at basal levels in most tissues and contributes to the turnover of cytoplasmic components (15). In GFP-LC3 transgenic mice autophagic degradation of mitochondria because a mitochondrial target sequence was predicted at the N terminus of mammalian APG9L2 proteins but not in the APG9L1 proteins. However, under the experimental conditions in our study, we observed neither colocalization of mAPG9L2 with a mitochondrial marker nor an obvious difference in the subcellular localization patterns between mAPG9L1 and mAPG9L2.

Evolution of Autophagy-related Genes—Although fundamental mechanisms of cellular autophagosome formation are shown to be well conserved from yeast to mammalian species, the regulatory role of the organelle appears to be quite different between these two species. We found that the mammalian APG9L2 gene has a WD-repeat domain that is not present in its yeast orthologue ATG16 (35). Another instance is Atg6/Becn1, which is shown to be required for both autophagy and protection from cancer (36, 37). Although we did not find significant differences between APG9L1 and APG9L2 in the subcellular localization assays, it is possible that APG9L2 has additional vertebrate-specific functions.

Subcellular Localization of Mammalian APG9-like Proteins—We postulated that the APG9L2 protein might be involved in the autophagic degradation of mitochondria because a mitochondrial target sequence was predicted at the N terminus of mammalian APG9L2 proteins but not in the APG9L1 proteins. However, under the experimental conditions in our study, we observed neither colocalization of mAPG9L2 with a mitochondrial marker nor an obvious difference in the subcellular localization patterns between mAPG9L1 and mAPG9L2.

Roles of APG9L2 in Placental Development and Disease—Although autophagy seems to be dispensable for mammalian embryonic development, it remains to be elucidated if it has a causative role in the pathogenesis of late onset diseases in which autophagic activity is observed to be elevated. In mammals, although autophagy can be induced during stress conditions such as nutrient starvation, it also occurs at basal levels in most tissues and contributes to the turnover of cytoplasmic components (15). In GFP-LC3 transgenic mice autophagy is shown to be constitutively active in several tissues regardless of nutrient conditions (11), suggesting that...
section, autophagosome formation was not remarkable. This observation indicates the possibility that the high levels of APG9L2 expression in placenta and pituitary gland are not necessarily involved in autophagic activity but some other membrane trafficking events. Yeast Atg9p is shown to be essential for both Cvt pathway and autophagy (26), although there is no clear evidence for the Cvt pathway in any eukaryotic species other than Saccharomyces cerevisiae (5). APG9L2 may be involved in an unknown mammalian membrane-trafficking pathway(s) in the tissues such as placenta and pituitary gland. Analyses of autophagy and APG9L2 in the placenta of normal and disease conditions would yield insight into their roles at the organ level.

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