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Research Paper

Coronavirus Replication Does Not Require the Autophagy Gene *ATG5*

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ABSTRACT

Macroautophagy (herein autophagy) is a cellular process, requiring *ATG5*, by which cells deliver double membrane-bound packets containing cytoplasm or cytoplasmic organelles to the lysosome. This process has been reported in some cases to be antiviral, while in other cases it has been reported to be required for efficient viral replication or release. A role for autophagy in RNA virus replication has been an attractive hypothesis because of the association of RNA virus replication with complex membrane rearrangements in the cytoplasm that can generate opposed double membranes. In this study we demonstrate that *ATG5* is not required for murine hepatitis virus (MHV) replication in either bone marrow derived macrophages (BMM ϕ) lacking *ATG5* by virtue of Cre-recombinase mediated gene deletion or primary low passage murine *ATG5*^{-/-} embryonic fibroblasts (pMEFs). We conclude that neither *ATG5* nor an intact autophagic pathway are required for MHV replication or release.

INTRODUCTION

Autophagy is an evolutionarily conserved cellular process in which a series of cytoplasmic proteins generate isolation membranes that envelop cytoplasm and cytoplasmic organelles, resulting in double-membrane bound packets of cytoplasmic constituents about 0.5 to 1.5 μ m in diameter.^{12,13,20,21,32,42} This enveloped packet of cytoplasm is delivered to the lysosome for degradation via fusion of the outer membrane of the autophagosome with the lysosomal membrane. Autophagy plays an important role in multiple biological processes including development,^{12,19,20,26,31} tumor suppression,^{4,14,18,30,43} prevention of neurodegeneration,^{7,10} survival during starvation or growth factor withdrawal,^{11,17,20} and T cell homeostasis.²⁹

Autophagy requires the concerted action of a series of proteins that together generate the curved membranes responsible for envelopment of cytoplasm and delivery of captured cytoplasmic constituents to the lysosome.^{21,42} Envelopment of cytoplasm during autophagy requires two *ATG5*-dependent protein conjugation systems.^{21,22,24} The first of these generates *ATG5*-*ATG12* conjugates which become associated with the elongating isolation membrane during autophagy.²¹ This system is highly efficient; the majority of *ATG5* in cells is found conjugated to *ATG12*. A second conjugation system modifies the free C-terminal glycine of the autophagy protein *ATG8/LC3* (termed *LC3-I*) with phosphatidylethanolamine generating *LC3-II* which associates with autophagosomes. *LC3-I* and *LC3-II* can be distinguished by mobility in electrophoretic gels. The conversion of *LC3-I* to *LC3-II* depends on *ATG5*-*ATG12* conjugation.²² Homozygous deletion of *ATG5* results in neonatal lethality in mice.¹¹ Therefore mice in which the third exon of *ATG5* is flanked by *LoxP* sites have been used in combination with cell type-specific expression of the Cre recombinase to study autophagy in adult animals [*ATG5*^{fllox/fllox},^{7,11}].

Two attributes of autophagy have drawn the attention of virologists. First, the potential ability to deliver cytoplasmic virions or replication factories to the lysosome suggests that autophagy could serve as a mechanism for combating cytoplasmic viral replication. Data consistent with such an antiviral role for autophagy and autophagy genes was originally obtained for Sindbis virus¹⁵ and subsequently for herpes simplex virus^{25,36,37} and tobacco mosaic virus.¹⁶ The observation that two different herpesvirus proteins, a viral Bcl-2 family member²⁷ and the herpes simplex virulence factor ICP34.5^{25,36} inhibit autophagy indicates that viruses have evolved potent ways to antagonize the antiviral effects of

autophagy. The existence of such autophagy evasion proteins supports the concept that autophagy is an important antiviral innate immune pathway.

However, as opposed to the idea that autophagy is antiviral, the similarity in structure between the curved double membrane of autophagosomes and membrane structures observed by electron microscopy in association with RNA or poxvirus replication has suggested that the autophagic pathway, or at least specific autophagy genes, might be subverted to foster viral replication. This concept is supported by studies of coronavirus replication in embryonic stem cells²⁸ and poliovirus replication in MCF7 and HeLa cells.⁹ In each case, initial support for a role for autophagy in viral replication came from studies colocalizing viral proteins with autophagy proteins. In embryonic stem cells lacking ATG5, replication of the coronavirus murine hepatitis virus (MHV strain A59) was decreased more than 1000-fold compared to cells expressing ATG5.²⁸ In contrast to these observations, replication of vaccinia virus, a DNA poxvirus whose replication also involves a series of complex cytoplasmic membrane rearrangements, does not require ATG5.⁴⁴

We sought to further evaluate the possible role of ATG5 and an intact autophagy pathway in coronavirus replication. It is well recognized that primary cells and continuous or transformed cell lines can differ in their permissiveness for viral replication. Moreover, viruses exhibit tropism for specific cells *in vivo*,³⁸ making it important to evaluate the role of host proteins in relevant primary cell types when experimentally feasible. Since the autophagic machinery may be altered in transformed cell lines, and since embryonic stem cells are not directly involved in viral pathogenesis, we determined whether ATG5 and an intact autophagic pathway is required for coronavirus replication in both primary BMM ϕ and primary low passage MEFs. In contrast to studies in embryonic stem cells using the same strain of virus,²⁸ ATG5 was not required for MHV-A59 replication in either cell type. We conclude that, similar to studies of vaccinia virus replication,⁴⁴ neither an intact autophagy pathway nor the autophagy gene *ATG5* is required for coronavirus replication.

MATERIALS AND METHODS

Mice. *ATG5*^{-/-} and *ATG5*^{flax/flax} mice have been described.^{7,11} Mice expressing the Cre recombinase from within the lysozyme M locus (*Lyz-Cre* mice) were purchased from the Jackson Laboratory (Strain # 004781). Mice were genotyped as described,⁷ with the *ATG5* gene detected with the primers exon3-1, short2 and check2 run using PCR [94°C (4 min); 30 cycles of 94°C (30 sec), 60°C (30 sec), 72°C (1 min); 72°C (5 min)]. The *Cre* gene was detected with primers cre1 and cre2 using PCR [94°C (4 min); 25 cycles of 94°C (30 sec), 60°C (30 sec), 72°C (1 min); 72°C (5 min)].

Macrophages and viral infection of macrophages. BMM ϕ cultures were maintained in low-endotoxin DMEM with 4.5 g/ml glucose (Mediatech, Herndon VA), 100 U/ml penicillin/100 μ g/ml streptomycin (Mediatech, Herndon VA), and 10% HEPES (Mediatech, Herndon VA) at 37°C with 5% CO₂ unless otherwise noted. BMM ϕ were isolated from the femurs and tibias of *Atg5*^{flax/flax}-*Lyz-Cre* and *Atg5*^{flax/flax} mice. Bone marrow was flushed out with BM20 medium containing low-endotoxin DMEM with 4.5 g/ml glucose, 2 mM L-glutamine (Washington University School of Medicine tissue culture support center, St. Louis MO), 1 mM sodium pyruvate (Mediatech, Herndon VA), 10% defined

low endotoxin FCS (Hyclone, Logan UT), 5% defined equine serum (Hyclone, Logan UT), and 20% L929 cell conditioned medium [LCM,⁸]. Cells were plated in BM20 at 3 x 10⁵ cells/ml in 8 mls in non-tissue culture treated dishes (Fisher Scientific, Fair Lawn NJ). After four days an additional 8 mls of BM20 was added. Cells were harvested by scraping on day 7 of culture, and replated with BM10 (same as BM20 except containing 10% LCM) for experiments. For all experiments murine coronavirus strain MHV-A59 stocks were generated and titered by plaque assay using 17 Cl 1 cells.^{6,34} For detection of viral replication in BMM ϕ , 1.5 x 10⁵ cells were plated in 1.0 ml of BM10 in 12-well tissue culture treated plates (Corning, Corning NY) for two days. BM10 medium was then removed and replaced with either BM10 plus 1% MEM nonessential amino acids (Mediatech, Herndon VA) and 2% MEM amino acids (Invitrogen, Grand Island NY) ["non-starved" condition] or Earle's balanced salt solution (EBSS; Sigma, St. Louis MO) ["starved" condition] for 2 hours. Medium was then removed and replaced with 0.2 mls of starved or non-starved medium containing 7.5 x 10⁴ plaque forming units (PFU) of MHV-A59 (moi = 0.5). After incubating for 1 hr at 37°C, monolayers were washed three times with BM10 to remove unbound virus, and 1.0 ml of BM10 was added. A plate was frozen immediately for later titration as the t = 0 time point. To assess released virus, 0.5 ml of supernatant from each well was removed and stored at -80°C for viral titration. To assess total virus production, a plate was frozen for later titration after removal of 0.5 ml supernatant for "released" virus. Samples were freeze-thawed three times and titrated by plaque assay on 17 Cl 1 cells.

Fibroblasts and viral infection of fibroblasts. All fibroblast cultures were maintained in 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 mM HEPES at 37°C with 5% CO₂ unless otherwise noted. Primary *ATG5*^{+/+} and *ATG5*^{-/-} mouse embryonic fibroblasts (pMEFs) were prepared from 13.5-day embryos by carefully dissecting the embryo from associated uterine and placental tissue and mincing prior to culture. pMEFs were maintained in Dulbecco's modified Eagle's medium supplemented with 10% characterized FCS (Hyclone, Logan VA), 2 mM L-glutamine, 1% MEM non-essential amino acids, 2% MEM amino acids. pMEFs were harvested by trypsinization and replated for experiments. For detection of MHV-A59 replication in pMEFs, 5 x 10⁵ cells in 2 ml of non-starved DMEM10 medium (DMEM10 with 1% MEM nonessential amino acids, 2% MEM amino acids) were plated per well in 6 well plates (Corning, Corning NY) and cultured overnight. After washing three times with 4°C EBSS, cells were incubated in 2 ml non-starved DMEM-10 medium or "starved" medium (EBSS) for 2 hr. Medium was then removed and replaced with 0.5 ml of either starved or unstarved medium containing 2.5 x 10⁵ PFU of MHV-A59 (moi = 0.5) and incubated at 37°C for 1 hr. Infected cells were washed three times with 2 ml of DMEM10 per well to remove unbound virus and then incubated in 2 ml of non-starved DMEM10 for the indicated time prior to freezing for later titration by plaque assay as described above.

Immunoblots. Cells pellets were lysed on ice in lysis buffer [50 mM Tris-HCl (Fisher Scientific, Fair Lawn NJ), pH 7.4, 150 mM NaCl (Fisher Scientific, Fair Lawn NJ), 1% Triton X-100 (Sigma, St. Louis MO), 1mM EDTA (Fisher Scientific, Fair lawn NJ)] for 30 min in presence of 1 μ M PMSF (Sigma, St. Louis MO) and 4% protein inhibitor cocktail-III (Roche Applied Science, Indianapolis IN) and subjected to western blotting using antibodies specific

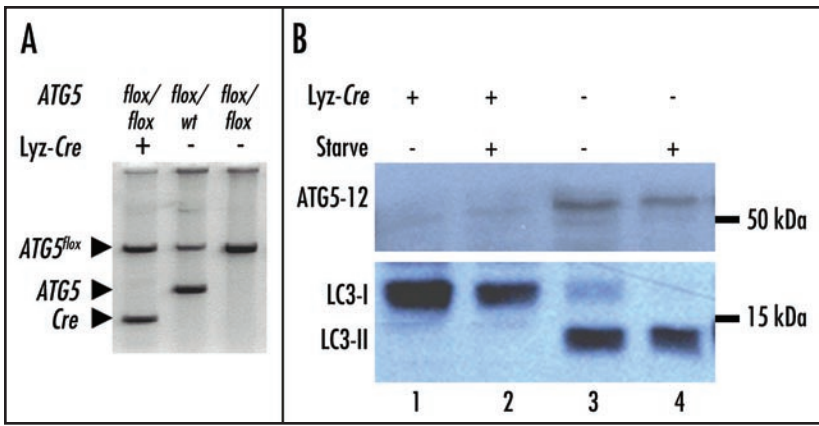


Figure 1. Bone marrow macrophages from *ATG5^{flox/flox}-Lyz-Cre* mice are *ATG5* deficient and convert LC3-I to LC3-II inefficiently. (A) PCR products from genotyping BMMφ cultured from mice of the following genotypes: *ATG5^{flox/flox}-Lyz-Cre* (left lane), *ATG5^{flox}/wild type* (middle lane) and *ATG5^{flox/flox}* (right lane). The predicted sizes of the bands generated by PCR are: *ATG5^{flox}*: 651bp; *ATG5*: 351 bp; *Cre*: 250bp. (B) Western blot with antibodies detecting *ATG5-12* conjugates (upper panel) and LC3-I/II proteins (lower panel) from either *ATG5^{flox/flox}-Lyz-Cre* (lanes 1 and 2) or *ATG5^{flox/flox}* (lanes 3 and 4) BMMφ under starved (lanes 2 and 4) or non-starved (lanes 1 and 3) conditions. These data are representative of at least two experiments.

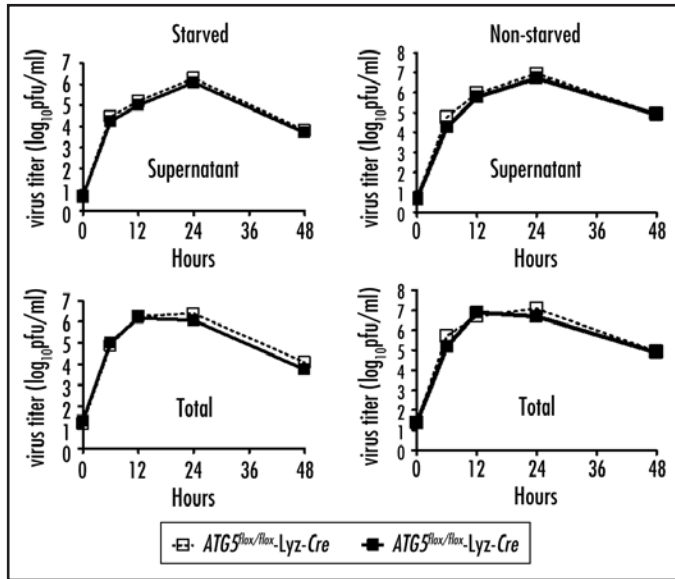


Figure 2. MHV-A59 replication in bone marrow macrophages does not require *ATG5*. MHV-A59 titers produced by BMMφ derived from either *ATG5^{flox/flox}-Lyz-Cre* (empty square, dotted line) or *ATG5^{flox/flox}* (filled square, solid line) mice. All experiments were done at *moi* = 0.5. Titers are shown separately for starved (left panels) and non-starved (right panels) conditions. Titers of released virus are shown in the upper two panels; total virus titers are shown in the lower panels. Average virus titers ± SEM are shown for at least three experiments.

for *ATG5*,²² LC3 (Novus Biologicals, Littleton CO), and β-actin (Sigma-Aldrich, St. Louis MO). Immunoblots were developed with HRP-conjugated secondary antibodies (Jackson Immunoresearch, West Grove PA) and visualized by chemiluminescence (Amersham Biosciences, Pittsburgh PA).

Statistical analysis. All data were analyzed with Prism software (GraphPad, San Diego, CA) using two-tailed unpaired t test.

RESULTS AND DISCUSSION

Mφ are involved in the pathogenesis of coronavirus infection. Mφ are infected in the lungs of patients with coronavirus-induced SARS,^{23,41} the virulence of feline coronavirus is associated with macrophage tropism,³³ macrophages are amongst the first cells infected in mice inoculated with the coronavirus MHV-A59,^{39,40} and BMMφ are permissive for MHV-A59 replication.³⁵ We therefore selected macrophages as a relevant primary cell type in which to test the hypothesis that *ATG5* and an intact autophagy pathway is required for coronavirus replication.

To generate Mφ lacking *ATG5* we bred *ATG5^{flox/flox}* mice⁷ to mice in which the *Cre* recombinase is expressed from the endogenous lysozyme M locus (*Lyz-Cre*).² Lysozyme M expression is restricted to myelomonocytic cells, specifically Mφ and granulocytes.^{1,3,5} We then cultured BMMφ from *ATG5^{flox/flox}-Lyz-Cre* and *ATG5^{flox/flox}* mice (Fig. 1A) and analyzed expression of *ATG5-ATG12* conjugates and the conversion of LC3-I to LC3-II in these cells (Fig. 1B). We observed no differences in the generation of BMMφ between *ATG5^{flox/flox}-Lyz-Cre* and *ATG5^{flox/flox}* bone marrow (data not shown). *ATG5^{flox/flox}-Lyz-Cre* BMMφ expressed significantly lower levels of *ATG5-ATG12* conjugates than *ATG5^{flox/flox}* BMMφ (Fig. 1B). In addition, we observed a significant decrease in the amount of LC3-II in *ATG5^{flox/flox}-Lyz-Cre* BMMφ compared to *ATG5^{flox/flox}* BMMφ (Fig. 1B) under both starved and unstarved conditions. These data indicated that expression of the *Cre* recombinase from the lysozyme M locus effectively deletes the *ATG5* gene in cultured BMMφ, and that, as expected, *ATG5* is required for efficient conversion of LC3-I to LC3-II in macrophages.

We next examined the replication of MHV-A59 in *ATG5^{flox/flox}-Lyz-Cre* BMMφ compared to *ATG5^{flox/flox}* BMMφ (Fig. 2). BMMφ were infected and viral titers determined at various times over a period of 48 hours. We compared the amount of virus released into the supernatant compared to the total amount of infectious virus produced because of the proposed role of autophagy in virus release.⁹ In addition, we compared BMMφ cultured under starved and unstarved conditions. MHV-A59 replicated to the same levels regardless of the presence or absence of *ATG5* or the culture conditions. There was no effect of *ATG5* on the amount of released MHV-A59. This demonstrated that neither *ATG5* nor an intact autophagic pathway is required for MHV-A59 replication in, or release from, BMMφ. Further, the induction of autophagy by starvation did not alter viral replication or release.

These results are in contrast to studies in embryonic stem cells.²⁸ It could be argued that this difference between embryonic stem cells and BMMφ was due the presence of *ATG5* or autophagic conversion of LC3-I to LC3-II at levels below those detectable by western blot. To address this possibility using cells that lack *ATG5* due to a null mutation in the *ATG5* gene rather than *Cre*-recombinase mediated deletion of the *ATG5* gene, we isolated *ATG5^{-/-}*, *ATG5^{+/-}* and *ATG5^{+/+}* murine embryonic fibroblasts. These cells were used as low passage (less than 5 passages, termed primary MEFs or pMEFs) cells in order to prevent changes in cell physiology associated with passage crisis and selection of continuous MEFs.

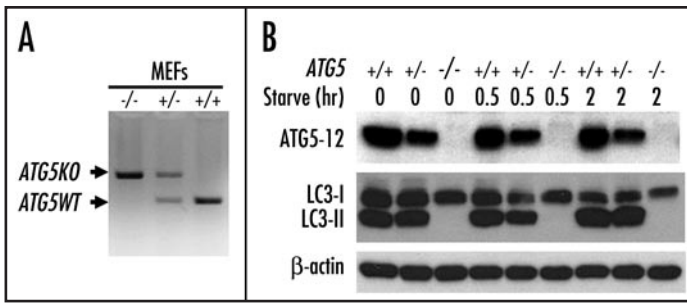


Figure 3. Low passage MEFs from *ATG5*^{-/-} mice convert LC3-I to LC3-II inefficiently. (A) Genotype of pMEFs from *ATG5*^{-/-} (left lane), *ATG5*^{+/-} (middle lane) and *ATG5*^{+/+} mice by PCR. The predicted sizes of the bands generated by PCR are: *ATG5*^{-/-}: 574bp; *ATG*^{+/-}: 351 bp. (B) Western blot with antibodies detecting ATG5-12 conjugates (upper panel), LC3-I/II proteins (middle panel) and β-actin. Cells were starved for the indicated times. These data are representative of at least two experiments.

We confirmed the genotype of these cells by PCR (Fig. 3A). Western blot revealed the expected lack of ATG5-ATG12 conjugates in *ATG5*^{-/-} MEFs (Fig. 3B). As expected, *ATG5*^{-/-} MEFs contained undetectable LC3-II even under starvation conditions (Fig. 3B). Together these data show that pMEFs had the predicted ATG5 deficiency and consequent lack of normal autophagy.

Next we performed growth experiments in starved and unstarved pMEFs. Cells were infected with MHV-A59 and virus titers determined over four days in culture (Fig. 4A and B). Consistent with results obtained in *ATG5*^{flax/flax}-*Lyz-Cre* BMMφ (Fig. 2), ATG5 was not required for replication of MHV-A59 in pMEFs. We also assessed the growth of MHV-A59 during the first 24 hours of infection (Fig. 4C). There was no role for ATG5 or an intact autophagic pathway in MHV-A59 replication at these earlier time points. Similar experiments were performed in Saint Louis, Missouri and Nashville, Tennessee, confirming that these data are representative across institutions and that they are independent of subtle differences in experimental techniques between laboratories.

These experiments indicate that, as observed for vaccinia virus,⁴⁴ ATG5 is not required for coronavirus replication. To determine if there was an alternative pattern of virus infection-induced membrane modifications in the absence of ATG5, we used electron microscopy to compare the ultrastructure of MHV infected *ATG5*^{-/-} and *ATG5*^{+/-} pMEFs 24 hours after infection at an MOI of 5 under either starved or unstarved conditions. Pleiomorphic membrane changes were noted in infected cells, including expanded ER, multilamellar membranes, and vesicles containing large numbers of virus particles (data not shown). No differences in the morphology of intracellular membranes were observed in *ATG5*^{-/-} compared to *ATG5*^{+/-} cells. Similar data were obtained at 36 hours after infection in an independent experiment. The results indicate that there are multiple ATG5-independent membrane modifications associated with MHV infection of pMEFs. Membrane rearrangements that are necessary for coronavirus replication do not require the participation of either ATG5 or the conversion of LC3-I to LC3-II in primary low passage pMEFs and BMMφ. Of note, we have confirmed the initial observation²⁸ that SARS replicase proteins and LC3 distribution overlaps in punctate cytoplasmic foci in infected Vero Cells (data not shown). The physiologic meaning of such colocalization is uncertain, but the results reported here showing that an intact autophagic pathway is

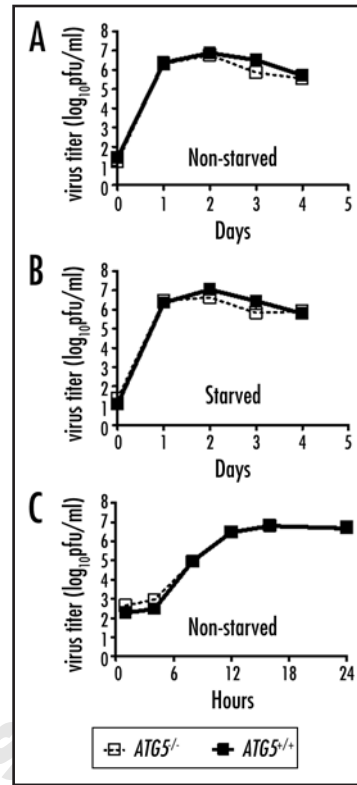


Figure 4. MHV-A59 replication in low passage MEFs does not require ATG5. MHV-A59 titers produced by pMEFs derived from either *ATG5*^{-/-} (empty square, dotted line) or *ATG5*^{+/-} (filled square, solid line) mice. (A and B) moi = 0.5. (C) moi = 5. Average virus titers ± SEM are shown for at least two independent experiments (A and B). Data is representative of at least two independent experiments (C).

not required for coronavirus replication indicates that colocalization between viral proteins and autophagy proteins in continuous cell lines should not be used as a sole indicator that autophagy is involved in viral replication. While ATG5 and an intact autophagic pathway are not required for coronavirus replication *in vitro*, it remains possible that other proteins of the autophagy pathway may play a role in coronavirus replication or pathogenesis.

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