Reserve Autophagic Capacity in Alveolar Epithelia Provides a Replicative Niche for Influenza A Virus

David R. Hahn, Cheng-Lun Na, and Timothy E. Weaver

Perinatal Institute, Section of Neonatology, Perinatal and Pulmonary Biology, Cincinnati Children's Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, Ohio

Abstract

Autophagy contributes to cellular homeostasis through metabolite recycling and degradation of cytotoxic protein aggregates and damaged organelles. Although recent studies have established that the requirement for basal autophagy is largely tissue specific, the importance of autophagy for alveolar epithelial cell homeostasis remains an important knowledge gap. In the present study we generated two mouse models, with > 90% or > 50% recombination at the Atg5 locus in the distal respiratory epithelium, to assess the effect of dose-dependent decreases in autophagy on alveolar homeostasis. A 90% decrease in autophagy was well tolerated in young adult mice but resulted in alveolar septal thickening and altered lung mechanics in aged animals, consistent with accumulation of damage over time. By comparison, a 50% decrease in autophagy had no effect on alveolar structure or function throughout the murine life span, indicating that basal autophagy in this compartment exceeds that required for homeostasis. A 50% decrease in autophagy in the bronchoalveolar epithelium

significantly attenuated influenza A/H3N2 viral replication, leading to improved lung structure and function and reduced morbidity and mortality after infection. The reserve of autophagic capacity in the alveolar epithelium may provide a niche for replication of influenza A virus.

Keywords: Atg5; influenza A; viral burden; lung injury; cilia

Clinical Relevance

This work identifies a reserve (excess) autophagic capacity in the alveolar epithelium that is not required for alveolar homeostasis. This reserve, amounting to as much as 50% of alveolar autophagic activity, provides a replicative niche for Influenza A virus. Pharmacologic inhibition of the autophagic reserve could provide an antigen-independent adjunct to current antiviral therapies.

Macroautophagy (hereafter referred to as autophagy) is a catabolic process by which eukaryotic cells engulf cytoplasmic contents within double-membraned vesicles (autophagosomes) that deliver sequestered cargo to lysosomes for degradation. Autophagosome formation requires the activity of more than 18 autophagy-related (Atg) proteins and is a critical step in a pathway that functions broadly to maintain cellular homeostasis. By channeling substrates to the lysosome through autophagy, cells are able to maintain metabolic stability during periods

of starvation, cytoprotection against accumulation of toxic protein aggregates and/or damaged organelles, and host defense against invading intracellular microbes (1–3). The autophagic pathway is highly conserved in eukaryotic organisms, and there is abundant evidence linking autophagic dysfunction to pathogenesis in mammalian organ systems, including the brain (4, 5), kidney (6), skeletal and cardiac muscle (7, 8), liver (9), and lung (10–13).

Autophagy degrades organelles (mitochondria, peroxisomes, endoplasmic reticulum [ER], and ribosomes) as well as

cytosolic pathogens and protein aggregates via nonspecific (bulk) or selective incorporation of substrates into autophagosomes. Selective recruitment of substrates is accomplished by adaptor proteins (p62/SQSTM1 and NBR1) that bind ubiquitinated protein aggregates and dock them to LC3 (microtubule-associated protein 1 light chain 3A, the mammalian homolog of yeast Atg8) on the autophagosome membrane (14), ultimately leading to lysosomal degradation of the adapter protein and the client substrate. Anchoring of the LC3 docking protein in

(Received in original form October 15, 2013; accepted in final form March 18, 2014)

This study was supported by the National Institutes of Health/National Heart, Lung and Blood Institute grant R01HL103923 (T.E.W.).

Correspondence and requests for reprints should be addressed to Timothy E. Weaver, Ph.D., 3333 Burnet Ave., Cincinnati, OH 45229. E mail: tim.weaver@cchmc.org

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 51, Iss 3, pp 400–412, Sep 2014

Copyright © 2014 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2013-0437OC on March 24, 2014

Internet address: www.atsjournals.org

the autophagosome membrane requires conversion of cytosolic LC3-I to the lipidated LC3-II form, a process that involves seven distinct Atg proteins, including Atg5 and Atg7. Inhibition of autophagy, by genetic deletion of Atg5 or Atg7, blocks the generation of LC3-II and leads to accumulation of p62, both of which are widely used to assess autophagic activity.

Disruption of basal autophagy in the airway epithelium by targeted deletion of Atg7 in bronchiolar epithelial cells resulted in altered cell morphology and increased airway reactivity to methacholine (15). This finding supports a link between autophagic function and the development of asthma, a hypothesis that is supported by correlation of *Atg5* SNPs with the incidence of childhood asthma (16, 17). The results of these studies suggest that basal autophagy may be required for maintenance of airway epithelial homeostasis; however, the role of basal autophagy in the alveolar epithelium remains an important knowledge gap. Autophagy was previously implicated in the biogenesis and/or maintenance of lamellar bodies in alveolar type II epithelial cells (18, 19), but this hypothesis has not been directly tested. It is also not known if loss of autophagic capacity during aging contributes to age-related changes in alveolar structure and/or function.

The alveolar epithelium is an important target of influenza A virus strains, which are responsible for the majority of seasonal epidemic infections and all known pandemic outbreaks of influenza. Results of *in vitro* studies established that influenza A virus strains subvert the autophagic pathway of host cells during infection and hint at a complex relationship between viral replication, autophagy, and apoptosis (20). To date, a single *in vivo* study has demonstrated that impairment of autophagy in the lung, by chemical inhibition or small interfering RNA-mediated gene silencing, improved morbidity and mortality in mice infected with influenza A/H5N1 (21). However, it is not clear if selective loss of autophagy in the respiratory epithelium is protective and, if it is, to what extent autophagy must be inhibited to provide protection or if protection extends to other viral subtypes *in vivo*.

To assess the importance of autophagy for alveolar homeostasis, *Atg5* was selectively deleted in the respiratory

epithelium of mice. The use of different Cre drivers allowed us to manipulate *Atg5* expression in alveolar epithelial cells, resulting in a dose-dependent decrease in alveolar autophagic capacity. Reduced expression of *Atg5* did not alter alveolar structure and function during normal growth and aging; however, decreased *Atg5* expression did confer protection against influenza A virus, leading to a significant reduction in morbidity and mortality.

Materials and Methods

Animals

All mouse studies were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee at the Cincinnati Children's Research Foundation. Lung epithelial cell-specific deletion of *Atg5* was achieved by crossing mice carrying a floxed *Atg5* locus (*Atg5^{flx/flx}*) to mice carrying human *SFTPC* promoter-driven *rtTA* (*SPC-rtTA*) and (*tetO*)₇*CMV-Cre* transgenes or *sonic hedgehog* promoter-driven *Cre* (*Shh-Cre*). Detailed methodology is available in the online supplement.

PCR Analyses

Allele-specific DNA primers were used for PCR-based genotyping of transgenic animals using genomic DNA isolated from tail clips. *XBPI* splicing was assayed in cDNA prepared from type II epithelial cells isolated from *Atg5^{flx/flx}* and *Atg5^{epiΔ/Δ}* mice (as described below). Quantitative PCR (qPCR) analyses were performed on cDNA prepared from isolated type II epithelial cells and snap-frozen lung tissue using TaqMan Universal PCR Master Mix (Life Technologies, Grand Island, NY) and TaqMan or PrimeTime (IDT, Skokie, IL) qPCR primer/probe assays. A complete list of primers and probes is presented in Table E1 in the online supplement.

Preparation of Lung Tissue for Histological Analysis and Electron Microscopy

Procedures for processing lung tissue for histological analysis and electron microscopy are provided in the online supplement.

Immunofluorescence, Confocal Microscopy, and Airway Cilia Morphometry

Procedures for immunofluorescent staining of lung tissue sections and morphometric analysis of motile cilia length are provided in the online supplement.

Type II Epithelial Cell Isolation

Alveolar type II epithelial cells were isolated from lungs as previously described (22). Purity of cell preparations was assessed by modified Papanicolaou stain of isolated cells and ranged from 86 to 93%.

Western Blot and ELISA Analyses

Preparation of tissue lysates and Western blot analyses were performed as previously described (23) using antibodies specific for *Atg5* (Novus, Littleton, CO), p62/SQSTM1 (Abcam, Cambridge, MA), LC3B, poly (ADP-ribose) polymerase (PARP) (Cell Signaling, Danvers, MA), β -actin, mature SP-B, or mature SP-C (all from Seven Hills Bioreagents, Cincinnati, OH). Tissue lysates were analyzed for TNF- α content by ELISA per the manufacturer's instructions (BioLegend, San Diego, CA).

Adult Lung Morphometrics

Histological sections of lungs were analyzed by hematoxylin and eosin staining followed by point counting as previously described (24). Details are provided in the online supplement.

Mouse Viral Infection and Bronchoalveolar Lavage Cytometry

Procedures for mouse viral infection and bronchoalveolar lavage (BAL) cytometry are provided in the online supplement.

Analysis of Lung Function

Mice were anesthetized and cannulated intratracheally with 18-gauge luer stub adaptors. Baseline lung function and airway reactivity to methacholine challenge were assessed using a FlexiVent system (SCIREQ, Montreal, PQ, Canada). Methacholine was prepared in ice-cold PBS in concentrations of 0, 25, and 50 mg/ml and administered to mice via an in-line nebulizer.

Measurement of Viral Burden

The methodology for determining viral RNA, viral protein, and live virus titers in mouse lung tissues is described in the online supplement.

Statistical Analysis

Data are reported as mean values $\pm$ SEM, with significance reported as $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$. Multiple-group comparisons, unless noted otherwise, were made by one-way ANOVA with *post hoc* analysis performed by Tukey's honest significance test.

Results

Analyses of Mice in which Atg5 Expression Is Decreased by 50% (Atg5^{epiΔ/Δ} Mice)

Generation and characterization of Atg5^{epiΔ/Δ} mice. To determine the role of autophagy in the alveolar epithelium, triple-transgenic mice were generated bearing the SPC-rtTA (line 2), TetO-Cre, and floxed Atg5 (Atg5^{flx/flx}) alleles. Matings were arranged to produce progeny heterozygous for the rtTA and Cre alleles and homozygous for the floxed Atg5 allele. Recombination at the Atg5 locus in lung epithelium (Atg5^{epiΔ/Δ}) was achieved by *in utero* exposure to doxycycline between embryonic Days (E)6.5 and E14.5. Atg5^{epiΔ/Δ} mice were viable, born at the expected Mendelian ratio, and did not exhibit overt signs of respiratory dysfunction. qPCR analyses performed on cDNA prepared from isolated alveolar type II epithelial cells demonstrated that Atg5 mRNA levels were reduced by 58% in young adult (6–8 wk of age) Atg5^{epiΔ/Δ} mice compared with wild-type (WT) Atg5^{flx/flx} littermates ($P < 0.0005$ [$n = 11$]) (Figure 1A). Decreased type II cell expression of Atg5 mRNA was stable over the normal murine lifespan, with a 56% reduction at 9 months ($P = 0.002$ [$n = 4$]) and a 50% reduction at 22 months ($P = 0.01$ [$n = 5$]) (Figure 1A). Similarly, Atg5 protein in type II epithelial cells was decreased by 62% in 6- to 8-week-old Atg5^{epiΔ/Δ} mice ($P < 0.0005$ [$n = 12$]) and by 51% at 9 months of age ($P = 0.002$ [$n = 4$]) as demonstrated by Western blot of the Atg5/Atg12 complex (Figures 1B and 1C). Loss of Atg5 was also associated with impaired generation of LC3-II (Figure 1B) and with an 11- to 12-fold increase in p62 in type II epithelial cells isolated from Atg5^{epiΔ/Δ} mice at 6 to 8 weeks ($P < 0.0005$ [$n = 8$]) and 9 months of age ($P = 0.0007$ [$n = 4$]) compared with Atg5^{flx/flx} littermates (Figures 1B and 1D). Accumulation of p62 in

lung epithelial cells of Atg5^{epiΔ/Δ} mice was confirmed by immunohistochemistry at 6 to 8 weeks, 9 months, and 22 months of age (Figure 1E). p62 localized to type II epithelial cells and subsets of bronchiolar epithelial cells in Atg5^{epiΔ/Δ} mice but was undetectable or detected at very low levels in Atg5^{flx/flx} mice. Collectively these results indicate that autophagy is significantly, selectively, and permanently decreased in epithelial cells of alveoli and peripheral airways of Atg5^{epiΔ/Δ} mice, consistent with the previously reported expression pattern of embryonically activated SPC-rtTA/CMV-Cre (25).

Lung structure and function is not altered in Atg5^{epiΔ/Δ} mice. Histological analyses of lung tissues from Atg5^{epiΔ/Δ} mice showed no evidence of alveolar remodeling or interstitial thickening compared with Atg5^{flx/flx} littermates at 6 to 8 weeks, 9 months, or 22 months of age (Figures 2A and 2B). Baseline lung mechanics were not different between Atg5^{epiΔ/Δ} and Atg5^{flx/flx} mice at 6 to 8 weeks, 9 months, and 22 months of age (Figures 2C–2E); however, lung compliance (Figure 2C) in Atg5^{epiΔ/Δ} and Atg5^{flx/flx} animals increased 2.3-fold at 22 months of age when compared with 6- to 8-week-old animals ($P = 0.0002$ for Atg5^{flx/flx} mice; $P = 0.007$ for Atg5^{epiΔ/Δ} [$n = 4$ –6]). Total lung resistance (Figure 2D) and lung elastance (Figure 2E) was not significantly different between Atg5^{flx/flx} and Atg5^{epiΔ/Δ} mice at any age; however, lung elastance declined with age in Atg5^{flx/flx} and mutant mice ($P = 0.0002$ for Atg5^{flx/flx} mice; $P = 0.02$ for Atg5^{epiΔ/Δ} [$n = 4$ –6]) (Figure 2E). Thus, age-related changes in lung function occur independently of Atg5 expression.

To further assess the potential effects of a 50% decrease of autophagy in the alveolar epithelium, ER stress, surfactant protein expression, and inflammation were analyzed in Atg5^{epiΔ/Δ} mice. ER stress, as assessed by Western blot analysis of BiP concentration (not shown) or RT-PCR analysis of XBP1 splicing was not altered in type II epithelial cells isolated from 6- to 8-week-old, 9-month-old, or 22-month-old animals (Figure E1A). Western blot analyses of mature SP-B (Mr $\sim$ 18k) and SP-C (Mr $\sim$ 4k) protein levels in BAL fluid and isolated type II epithelial cells detected no difference at 6 to 8 weeks or 9 months of age in Atg5^{epiΔ/Δ} mice (Figure E1B), consistent with normal lung function in these animals (Figures 2C–2E). The total number of

airway cells recovered from BAL fluid was not significantly different between Atg5^{epiΔ/Δ} mice and Atg5^{flx/flx} littermates at 6 to 8 weeks and 9 months of age (Figure E1C), and only macrophages were recovered (not shown).

Inflammatory cell infiltration was not detected in histological sections at any age (Figure 2A), indicating that bronchoalveolar deficiency of Atg5 does not induce an inflammatory response in Atg5^{epiΔ/Δ} mice.

Atg5^{epiΔ/Δ} mice are protected against Influenza A virus infection.

Pharmacological inhibition of autophagy or gene silencing of core autophagy proteins has previously been shown to reduce viral replication *in vitro* (26, 27). Treatment of mice with 3-methyladenine before infection with Influenza A/H5N1 virus was reported to result in increased lung epithelial cell viability (21). To determine if decreased autophagic function in bronchioles and alveoli would improve lung structure and function in infected animals, 8-week-old Atg5^{epiΔ/Δ} mice and Atg5^{flx/flx} littermates were inoculated with the HKx31 strain of H3N2 Influenza A virus. When inoculated with a high dose of virus (7.2×10^3 TCID₅₀), only 1 of 16 Atg5^{flx/flx} mice survived (animals were removed from the study when they became moribund or if weight loss exceeded 25% of original body mass) compared with 6 of 14 Atg5^{epiΔ/Δ} mice ($P = 0.025$) (Figure 3A). At a lower, sublethal dose (5.7×10^3 TCID₅₀), Atg5^{epiΔ/Δ} mice presented with reduced morbidity compared with Atg5^{flx/flx} littermates, as indicated by attenuated weight loss (Figure 3B), which was most apparent in the first 5 days after inoculation (Day 2: $P = 0.017$; Day 3: $P = 0.012$; Day 4: $P = 0.012$ [$n = 5$]). After sublethal inoculation, TNF- α protein levels were significantly reduced in lungs of Atg5^{epiΔ/Δ} mice compared with Atg5^{flx/flx} littermates at 3 and 5 days after infection but were indistinguishable at 7 days after infection (Day 3: $P = 0.037$; Day 5: $P = 0.041$ [$n = 5$]) (Figure 3C). Cellular apoptosis, as indicated by cleavage of PARP in lungs of infected Atg5^{epiΔ/Δ} and Atg5^{flx/flx} mice, was minimally detected at 3 and 5 days after infection but was strongly up-regulated at 7 days after infection, with significantly enhanced levels detected in Atg5^{flx/flx} animals compared with Atg5^{epiΔ/Δ} littermates ($P = 0.009$ [$n = 5$]) (Figure 3D). Thus, reduced Atg5 expression was

Figure 1. Generation and characterization of Atg5^{epiΔ/Δ} mice. (A) Quantitative RT-PCR analyses of *Atg5* mRNA expression in alveolar type II epithelial cells isolated from Atg5^{flx/flx} and Atg5^{epiΔ/Δ} mice at 6 to 8 weeks (***) $P < 0.0005$ [$n = 11$], 9 months (** $P = 0.002$ [$n = 4$]), and 22 months of age (* $P = 0.01$ [$n = 5$]). (B) Western blot of type II cell lysates at 6 to 8 weeks and 9 months of age. Cell lysates (50 μ g) were analyzed by SDS-PAGE followed by Western blotting for Atg5 (resolved as Atg5/12 complex), p62/SQSTSM, LC3B, and actin. Mass markers (in kD) appear to the left of each gel. Densitometric analyses of Atg5/12 complex (C) and p62 protein (D) in Atg5^{flx/flx} and Atg5^{epiΔ/Δ} type II epithelial cells at 6 to 8 weeks and 9 months of age, normalized to actin. ** $P < 0.005$; *** $P < 0.0005$ ($n = 4$ –12 per group). (E) Accumulation of p62 in alveolar type II epithelial cells and bronchiolar epithelial cells of Atg5^{flx/flx} (top panel) Atg5^{epiΔ/Δ} mice (lower panel) at 6 to 8 weeks, 9 months, and 22 months of age. Scale bar, 100 μ m.

associated with decreased cell death and reduced morbidity and mortality after infection.

At 14 days after infection, histological analyses of tissue sections from left and right-inferior lung lobes of Atg5^{epiΔ/Δ} and

Atg5^{flx/flx} mice revealed varying degrees of tissue injury and remodeling. Both groups presented with moderate to severe

Figure 2. Lung structure and function is not altered in $Atg5^{epi\Delta/\Delta}$ mice. (A) Representative images of hematoxylin and eosin–stained lung sections from $Atg5^{flx/flx}$ and $Atg5^{epi\Delta/\Delta}$ mice at 6 to 8 weeks (top), 9 months (center), and 22 months (bottom) of age. Scale bar, 50 μ m. (B) Percentage of fractional (%Fx) area of lung parenchyma in $Atg5^{flx/flx}$ and $Atg5^{epi\Delta/\Delta}$ mice at 6 to 8 weeks, 9 months, and 22 months of age ($n = 4$ –6 per group at each age group). (C) Lung compliance in $Atg5^{flx/flx}$ and $Atg5^{epi\Delta/\Delta}$ mice at 6 to 8 weeks, 9 months, and 22 months of age. *** $P = 0.0002$ for $Atg5^{flx/flx}$ comparison; * $P = 0.007$ for $Atg5^{epi\Delta/\Delta}$ comparison ($n = 4$ –6). (D) Lung resistance in young and aged $Atg5^{epi\Delta/\Delta}$ and $Atg5^{flx/flx}$ littermates ($n = 4$ –6). (E) Lung elastance in young and aged $Atg5^{epi\Delta/\Delta}$ and $Atg5^{flx/flx}$ littermates. *** $P = 0.0002$ for $Atg5^{flx/flx}$ comparison; * $P = 0.02$ for $Atg5^{epi\Delta/\Delta}$ comparison ($n = 4$ –6).

bronchiolitis with leukocytic infiltration and deposition of extracellular matrix around the airways (Figure 4A); however, inflammation tended to extend further into the lungs of $Atg5^{flx/flx}$ animals, resulting in increased prevalence of alveolitis and subsequent peripheral and pleural extracellular matrix deposition in these animals. Consistent with these findings, total lung hydroxyproline content in infected $Atg5^{flx/flx}$ lungs was 2.45 μ mol ($n = 5$) compared with 1.57 μ mol in PBS-treated $Atg5^{flx/flx}$ lungs ($P = 0.001$ [$n = 4$]); in contrast, hydroxyproline content in infected $Atg5^{epi\Delta/\Delta}$ lungs (1.95 μ mol

[$n = 7$]) was significantly decreased relative to infected $Atg5^{flx/flx}$ mice ($P = 0.015$) and was not significantly different from noninfected mice ($P = 0.07$) (Figure 4B). Lung compliance was decreased in virus-infected $Atg5^{flx/flx}$ mice ($n = 6$) by 47% compared with PBS-treated control littermates ($P = 0.0003$ [$n = 4$]); however, compliance was preserved in virus-infected $Atg5^{epi\Delta/\Delta}$ animals ($n = 7$) and was not significantly different from PBS-treated control mice (Figure 4C). Lung resistance and elastance were not significantly altered in virus-infected $Atg5^{epi\Delta/\Delta}$ or $Atg5^{flx/flx}$ mice compared

with PBS-treated control animals (Figures 4D and 4E). Taken together, these results indicate that decreased autophagy in alveolar and bronchiolar epithelial cells significantly protects lung structure and function in mice infected with H3N2 virus.

Viral burden is decreased in infected $Atg5^{epi\Delta/\Delta}$ mice. Three days after infection with a sublethal titer of Influenza A virus, levels of the viral matrix protein M2 were 4-fold higher in lung tissues of $Atg5^{flx/flx}$ mice compared with $Atg5^{epi\Delta/\Delta}$ littermates ($P = 0.01$ [$n = 5$]) (Figures 5A and 5B). At 7 days after infection, M2 protein levels

Figure 3. Morbidity and mortality is decreased in Atg5^{epiΔ/Δ} mice infected with Influenza A virus. (A) Survival of Atg5^{flx/flx} and Atg5^{epiΔ/Δ} mice after infection with 7.2×10^3 TCID₅₀ of H3N2 virus. Six of 14 Atg5^{epiΔ/Δ} mice survived compared with 1 of 16 Atg5^{flx/flx} littermates. $*P = 0.025$, representative of two independent experiments. (B) Atg5^{flx/flx} and Atg5^{epiΔ/Δ} littermates were infected with 5.7×10^3 TCID₅₀ of Influenza A/H3N2 virus, and weight loss was tracked as a measure of morbidity. Day 2: $*P = 0.017$; Day 3: $*P = 0.012$; Day 4: $*P = 0.012$ ($n = 5$), representative of three independent experiments. (C) TNF- α protein concentration was determined by ELISA in lysates of whole lungs of Atg5^{flx/flx} and Atg5^{epiΔ/Δ} mice infected with Influenza A. Values are normalized to total protein content. Day 3: $*P = 0.037$; Day 5: $*P = 0.041$ ($n = 5$). (D) Representative Western blots for poly(ADP-ribose) polymerase (PARP) in whole lung lysates (75 μ g) of Atg5^{flx/flx} and Atg5^{epiΔ/Δ} mice at 3, 5, and 7 days after infection (upper panel) and Western blot densitometry for cleaved PARP (lower panel, $*P = 0.009$) ($n = 5$ each time point). Cleaved PARP signal was normalized to actin and expressed relative to 3 days after infection Atg5^{flx/flx} levels.

Figure 4. Improved lung structure and function in *Atg5^{epiΔ/Δ}* mice infected with Influenza A virus. (A) Trichrome staining of lung sections from *Atg5^{flx/flx}* and *Atg5^{epiΔ/Δ}* mice 14 days after infection. Images are representative of analyses from three mice (scale bar, 1 mm; inset scale bar, 50 μm). (B) Total lung hydroxyproline content of lungs from H3N2-infected *Atg5^{flx/flx}* ($n = 5$) animals compared with uninfected (PBS-treated) *Atg5^{flx/flx}* animals ($n = 4$) and infected *Atg5^{epiΔ/Δ}* ($n = 7$) mice. * $P = 0.015$; ** $P = 0.001$. (C–E) Lung mechanics were assessed 14 days after infection of 8-week-old mice ($n = 4$ –7 per group). (C) Lung compliance in infected *Atg5^{flx/flx}* and *Atg5^{epiΔ/Δ}* littermates compared with PBS-treated control animals. ** $P = 0.007$; *** $P = 0.0003$. (D) Lung resistance in infected *Atg5^{flx/flx}* and *Atg5^{epiΔ/Δ}* littermates compared with PBS-treated control animals. (E) Lung elastance in infected *Atg5^{flx/flx}* and *Atg5^{epiΔ/Δ}* littermates compared with PBS-treated control animals.

Figure 5. Viral burden is decreased in infected *Atg5^{epiΔ/Δ}* mice. (A) Western blot of Influenza A matrix protein M2 in lung lysates of infected *Atg5^{flx/flx}* and *Atg5^{epiΔ/Δ}* mice at 3 days (upper panel) and 7 days (lower panel) after infection. (B) Densitometry of M2 protein levels at 3 and 7 days after infection. Western blot data were normalized to actin, and protein levels are recorded as relative concentration compared with *Atg5^{flx/flx}* mice at 3 days after infection. * $P = 0.01$ ($n = 5$). (C) Influenza M2 RNA levels in lungs of *Atg5^{flx/flx}* and *Atg5^{epiΔ/Δ}* mice at 3, 7, and 14 days after infection were assessed by quantitative RT-PCR ($n = 5$ for each time point). Viral RNA levels were normalized to β -actin mRNA and recorded as relative expression compared with *Atg5^{flx/flx}* mice at 3 days after infection. * $P = 0.017$. (D) Viral M2 and nucleoprotein RNA levels in isolated alveolar type II epithelial cells of *Atg5^{flx/flx}* and *Atg5^{epiΔ/Δ}* mice at 8 and 18 hours after infection ($n = 4-6$). Viral RNA levels were normalized to *Atg5^{flx/flx}* animals at each time point. (E) Type I interferon *INFα1* and *INFβ1* mRNA fold induction in isolated alveolar type II epithelial cells of *Atg5^{flx/flx}* and *Atg5^{epiΔ/Δ}* mice at 8 and 18 hours after infection ($n = 4-6$). Fold induction calculated relative to uninfected *Atg5^{flx/flx}* and *Atg5^{epiΔ/Δ}* mouse levels (baseline). (F) Lung viral titers in H3N2-infected *Atg5^{flx/flx}* and *Atg5^{epiΔ/Δ}* mice at 3, 5, and 7 days after infection ($n = 5$ for each time point). Whole lung lysate TCID₅₀ (dilution factor required to infect 50% of cell culture samples) was calculated and recorded as TCID₅₀/mL. Day 3: ** $P = 0.001$; Day 5: ** $P = 0.0006$.

were greatly diminished in all animals, with no significant difference between genotypes. M2 RNA was increased 2.3-fold in infected *Atg5^{flx/flx}* mice compared with

Atg5^{epiΔ/Δ} littermates at 3 days after infection ($P = 0.017$ [$n = 5$]) and was similar in both groups at 7 days after infection (Figure 5C). Viral RNA was still detectable at 14 days

after infection in lungs from both genotypes but was six orders of magnitude lower than at Day 3 after infection. RNA levels of M2 and viral nucleoprotein were similar in type II

epithelial cells isolated from $Atg5^{epi\Delta/\Delta}$ and $Atg5^{flx/flx}$ mice at 8 and 18 hours after infection ($n = 4-6$) (Figure 5D), indicating that decreased autophagic function did not alter cell infectivity. Expression of type I interferon $INF-\alpha 1$ and $INF-\beta 1$ mRNA was also assessed in isolated type II epithelial cells after infection. Compared with uninfected $Atg5^{epi\Delta/\Delta}$ and $Atg5^{flx/flx}$ littermates (baseline), interferon expression levels were largely up-regulated (> 2 orders of magnitude) at 8 hours after infection and to a lesser degree (> 1 order of magnitude) at 18 hours after infection ($n = 4-6$) (Figure 5E). Expression was not different between groups at baseline or after infection, indicating that loss of $Atg5$ did not contribute to hyperproduction of type I interferon (28). Lung viral titers were determined to be 24-fold higher in $Atg5^{flx/flx}$ mice compared with $Atg5^{epi\Delta/\Delta}$ littermates at 3 days after infection ($P = 0.001$ [$n = 5$]), 11-fold higher at 5 days ($P = 0.0006$ [$n = 5$]), and similar between groups at 7 days (Figure 5F). Collectively, these results suggest that increased viral replication contributes to morbidity and mortality in WT mice (Figure 3).

Total BAL cell counts were significantly increased 3- to 4-fold in $Atg5^{flx/flx}$ and $Atg5^{epi\Delta/\Delta}$ mice at 3 days after infection. BAL cell numbers were not significantly different between groups at 3, 7, or 14 days after infection ($n = 5-8$) (Figure E2A). At 3 and 14 days after infection, there was no significant difference in the proportion of neutrophils recovered from the airway of $Atg5^{flx/flx}$ and $Atg5^{epi\Delta/\Delta}$ mice; however, there was a small but significant increase in neutrophil concentration in $Atg5^{epi\Delta/\Delta}$ lungs at 7 days after infection ($P = 0.002$ [$n = 5$]) (Figure E2B). Collectively, these data indicate that decreased autophagy in alveolar type II epithelial cells and bronchiolar epithelial cells does not appreciably alter recruitment of inflammatory cells to the site of infection. However, total cell and neutrophil counts at 14 days after infection suggest a trend toward earlier resolution in $Atg5^{epi\Delta/\Delta}$ animals, which is in concordance with morbidity data (Figure 3B) and decreased inflammatory cell infiltrates in tissue sections (Figure 4A).

Analyses of Mice in which $Atg5$ Expression Is Decreased by 90% ($Atg5^{ShhCre}$ Mice)

Generation and characterization of $Atg5^{ShhCre}$ mice. The unexpected finding

that a 50% decrease in autophagy did not alter lung structure/function in unchallenged mice prompted us assess the effect of a larger deficit in autophagic capacity on alveolar homeostasis. To achieve more efficient deletion of $Atg5$, $Atg5^{flx/flx}$ mice were crossed with Shh -Cre driver mice to generate $Atg5^{ShhCre}$ progeny. Expression of $Atg5$ mRNA ($P = 0.01$ [$n = 4$]) (Figure E3A) and protein ($P = 0.001$ [$n = 4$]) (Figures E3B and E3C) was reduced by more than 90% in type II epithelial cells isolated from $Atg5^{ShhCre}$ mice at 6 to 8 weeks of age. Conversion of LC3-I to LC3-II was decreased, and p62 accumulated in type II epithelial cells isolated from $Atg5^{ShhCre}$ mice ($P = 0.002$ [$n = 4$]) (Figures E3B and E3D), confirming inhibition of autophagy. p62 accumulated in epithelial cells throughout the respiratory epithelium (Figure E3E), consistent with the reported expression pattern of Shh in the lung (29). Aged $Atg5^{ShhCre}$ animals developed significant morbidity by 16 months, presenting with tachypnea and hind limb ataxia, which increased in severity over the span of several weeks. Body mass was reduced 19% in female mice and 23% in male $Atg5^{ShhCre}$ mice compared with WT littermates at this age (female mice: $P = 0.0013$ [$n = 6-8$]; male mice: $P = 0.0005$ [$n = 5-10$]) (Figure E3F). These phenotypes, which are consistent with the reported expression pattern of Shh in lung, neural, and intestinal epithelial cells (29-31), precluded analyses of animals older than 16 months in this study.

Lung structure and function is altered in aging $Atg5^{ShhCre}$ mice. Autophagy was previously suggested to play a role in the biogenesis of lamellar bodies (LB) in developing type II epithelial cells (18, 19). Accumulation of p62 was detected in fetal type II epithelial cells of $Atg5^{ShhCre}$ mice, but not in control littermates, at E18.5, indicating that autophagy is indeed active in type II epithelial cells at this developmental stage (Figure E4). Ultrastructural analyses identified numerous lamellar bodies with normal patterns of lamellation and limiting membranes in type II epithelial cells of $Atg5^{ShhCre}$ mice at Day E18.5 and at 6 to 8 weeks of age (Figure E5). No obvious changes in LB structure were detected in these animals (not shown), suggesting that autophagy is not required for LB biogenesis.

Histological analyses of E18.5 lungs detected bulging of ciliated bronchiolar epithelial cells (Figures 6A and 6B), similar to the phenotype previously reported for adult mice with conditional deletion of $Atg7$ in the bronchiolar epithelium (15). The "bulging" phenotype was transient and was not detected in $Atg5^{ShhCre}$ mice that were 6 to 8 weeks old and older. Cilia of bronchiolar epithelial cells were shortened, and this phenotype persisted postnatally in $Atg5^{ShhCre}$ mice ($P < 0.005$ [$n = 4-6$]) (Figures 6C and 6D) and $Atg5^{epi\Delta/\Delta}$ mice (not shown). p62 was most highly accumulated in ciliated cells in airways of $Atg5^{ShhCre}$ (Figure 6C) and $Atg5^{epi\Delta/\Delta}$ mice (Figure E6). In addition, mitochondria encircled by concentric membrane structures were evident in club cells (Clara cells) (Figure E7A), as previously reported for $Atg7$ -deficient lung (15) and liver (9). Mitochondrial defects, including loss of cristae and limiting membrane, were observed in ciliated bronchiolar epithelial cells (not shown). Similar mitochondrial defects and concentric membrane structures were detected in ciliated bronchiolar epithelial cells of 21-month-old $Atg5^{epi\Delta/\Delta}$ mice (Figure E7B). These findings are consistent with region-specific requirements for autophagy in the respiratory epithelium.

Histological analyses of alveolar regions showed no evidence of remodeling or interstitial thickening in $Atg5^{ShhCre}$ mice at 6 to 8 weeks of age (Figure 7A). The overall proportion of respiratory parenchyma and airspace was not different between $Atg5^{flx/flx}$ and $Atg5^{ShhCre}$ mice at 6 to 8 weeks of age (Figure 7B). In contrast, $Atg5^{ShhCre}$ mice aged to 16 months developed focal regions of alveolar septal thickening (Figure 7A) associated with a small but significant increase in lung parenchyma fractional area ($P = 0.0035$ [$n = 3$]) (Figure 7B). In addition, the number of cells recovered by lavage was increased 6-fold in $Atg5^{ShhCre}$ mice ($P = 0.02$ [$n = 4$]) (Figure E8A), and neutrophils were detected in $Atg5^{flx/flx}$ and $Atg5^{ShhCre}$ mice, with a trend toward increased presence of neutrophils and activated macrophages in the airspaces of $Atg5^{ShhCre}$ mice (Figure E8B). Thus, lung structure is altered in an age-dependent manner in $Atg5^{ShhCre}$ mice.

No significant changes were detected in lung compliance, resistance, or elastance in 6- to 8-week-old $Atg5^{ShhCre}$ mice compared with littermate control mice (Figures 7C-7E); however, 16-month-old $Atg5^{ShhCre}$ mice had significantly reduced

Figure 6. Altered bronchiolar epithelial cell morphology in *Atg5^{ShhCre}* mice. (A) Immunohistochemistry for p62 in E18.5 lung sections of *Atg5^{flx/flx}* and *Atg5^{ShhCre}* mice. Arrows indicate swollen bronchiolar cells. Scale bar, 100 μ m, inset: original magnification, $\times 3$. (B) Electron micrograph of bronchiolar epithelial cells showing bulging ciliated epithelial cell (asterisk). Scale bar, 5 μ m. (C) Immunofluorescent staining of 6- to 8-week-old *Atg5^{flx/flx}* and *Atg5^{ShhCre}* bronchiolar epithelium showing cilia (green), p62 (blue), and Clara cell secretory protein (CCSP; red). Scale bar, 20 μ m. (D) Population distribution of cilia length in bronchioles of 6- to 8-week-old *Atg5^{flx/flx}* and *Atg5^{ShhCre}* mice for distal airways < 100 μ m diameter. ** $P < 0.005$ ($n = 4-6$).

lung compliance ($P = 0.009$) (Figure 7C) and increased lung resistance ($P = 0.037$) (Figure 7D) and elastance ($P = 0.0037$) (Figure 7E) compared with *Atg5^{flx/flx}* littermates ($n = 6-9$). In concordance with observations for *Atg5^{epi Δ / Δ}* mice, baseline lung function was similarly altered with age in *Atg5^{flx/flx}* and *Atg5^{ShhCre}* littermates. Lung compliance was increased 2.2-fold ($P < 0.0005$) and lung elastance was decreased 54% ($P < 0.0005$) in 16-month-old *Atg5^{flx/flx}* mice compared with 6- to 8-week-old animals ($n = 6-9$) (Figures 7C and 7E). No significant difference was observed in lung central airway response at baseline or in response to methacholine challenge

in *Atg5^{epi Δ / Δ}* mice at any age (Figure E9A); however, there was a trend toward increased airway resistance in aged *Atg5^{ShhCre}* mice (Figure E9B). Taken together, these data indicate that the loss of autophagic function in the lung epithelium is well tolerated, with changes to lung structure and function appearing only after a prolonged period in animals with > 90% loss of *Atg5* expression.

Discussion

The current study was designed to assess the role of autophagy in alveolar homeostasis.

Two loss-of-function models were generated using SPC-rtTA (line2)/tetO-Cre (*Atg5^{epi Δ / Δ}*) or Shh-Cre (*Atg5^{ShhCre}*) drivers to delete *Atg5* in the lung epithelium. Embryonic deletion of *Atg5* in *Atg5^{epi Δ / Δ}* mice resulted in > 50% decrease in *Atg5* mRNA and protein in alveolar type II epithelial cells that was maintained throughout the lifespan of the mouse, indicating that there was no selective advantage to maintaining targeted versus nontargeted progenitor cells. Furthermore, because type II epithelial cells serve as progenitors for type I epithelial cells (32), it is likely that *Atg5* expression is similarly decreased throughout the alveolar

Figure 7. Age-dependent changes in lung structure and function in Atg5^{ShhCre} mice. (A) Representative images of hematoxylin and eosin-stained lung sections from Atg5^{flx/flx} and Atg5^{ShhCre} mice at 6 to 8 weeks and 16 months of age. Arrowheads indicate regions of septal thickening. Scale bar, 50 μ m. (B) Percent fractional area (%Fx) of lung parenchyma in Atg5^{flx/flx} and Atg5^{ShhCre} mice at 6 to 8 weeks ($n = 4$) and 16 months of age (** $P = 0.0035$ [$n = 3$]). (C) Lung compliance in 6- to 8-week-old and 16-month-old Atg5^{ShhCre} and Atg5^{flx/flx} littermates. *** $P < 0.0005$ for Atg5^{flx/flx} comparison; * $P = 0.009$ for 16-month comparison ($n = 6$ – 9 each group/age). (D) Lung resistance in 6- to 8-week-old and 16-month-old Atg5^{ShhCre} and Atg5^{flx/flx} littermates. * $P = 0.037$. (E) Lung elastance in 6- to 8-week-old and 16-month-old Atg5^{ShhCre} and Atg5^{flx/flx} littermates. *** $P < 0.0005$ for Atg5^{flx/flx} comparison. ** $P = 0.0037$ for 16-month comparison.

epithelium in these animals. As expected, decreased Atg5 expression was associated with reduced autophagic activity, as indicated by accumulation of p62 and inhibition of LC3 lipidation in type II epithelial cells. A 50% deficit in autophagic function did not affect alveolar structure or lung function in fetal, adult, or aged Atg5^{epiΔ/Δ} mice. Age-related changes in

lung function were evident in WT and Atg5^{epiΔ/Δ} mice; however, compliance and elastance were similarly altered in WT and mutant mice, suggesting that decreased autophagy did not significantly contribute to the age-related decline in lung function. Moreover, decreased alveolar autophagy was not associated with ER stress in type II epithelial cells, inflammatory cell

infiltration, or changes in surfactant protein expression. Collectively, these results indicate that 50% loss of autophagic function in the alveolar epithelium is well tolerated in young and aged mice.

Embryonic deletion of Atg5 in Atg5^{ShhCre} mice resulted in > 90% decrease in Atg5 mRNA and protein in type II epithelial cells at 8 weeks of age. Although

we did not assess Atg5 levels in older Atg5^{ShhCre} mice, it is likely that this deficiency persisted, given the elevated levels of p62 and significant lung pathology in older animals. Atg5^{ShhCre} mice at 16 months of age presented with alveolar septal thickening and altered lung mechanics compared with age-matched control mice; in contrast, alveolar structure and lung function were not altered in 6- to 8-week-old Atg5^{ShhCre} mice compared with WT littermates. Consistent with the latter results, LB structure was intact in fetal and postnatal type II epithelial cells. These findings cannot exclude a potential role for Atg5-independent, noncanonical autophagic pathways (e.g., Rab9-dependent autophagy) (33, 34), which may contribute to preservation of lung structure and function in younger mice. However, the observation that Atg5^{epiΔ/Δ} mice are significantly more resistant to viral infection (discussed below) taken together with age-dependent lung pathogenesis in Atg5^{ShhCre} mice suggest that compensatory, noncanonical autophagy is unlikely. Collectively, these results indicate that basal, canonical (Atg5-dependant) autophagy is sufficient to maintain alveolar homeostasis (including lamellar body structure) in young mice even in the face of a 90% decrease in Atg5 expression, consistent with the concept of reserve autophagic capacity in this compartment. However, it is equally clear from these studies that some autophagic activity (> 10% of basal activity) is required to maintain alveolar homeostasis in aging mice.

In contrast to the alveolar epithelium, decreased autophagy in bronchiolar epithelial cells resulted in altered cell morphology. Inoue and colleagues (15) reported that postnatal deletion of Atg7 in bronchiolar epithelial cells, using the *CCSP-rtTA* (line1)/*tetO-Cre* driver, resulted in 50% reduction of Atg7 expression and swollen bronchiolar epithelial cells. In response to methacholine challenge, baseline lung function was normal, but airway resistance increased. In the current study, embryonic activation of *SPC-rtTA* (line2)/*tetO-Cre* or *Shh-Cre* resulted in recombination in alveolar and bronchiolar epithelial cells, as expected (25, 29). Although we did not directly

assess the efficiency of Atg5 recombination in the bronchiolar epithelium of Atg5-deficient mice, accumulation of p62 was readily detected in bronchiolar epithelial cells. Decreased autophagy was associated with deformed mitochondria, shorter cilia, and concentric membrane structures in bronchiolar epithelial cells of Atg5^{epiΔ/Δ} and Atg5^{ShhCre} mice, similar to the phenotype described by Inoue and colleagues (15). However, swollen bronchiolar cells were detected only in fetal lung, and a significant increase in airway resistance after methacholine challenge was not detected in Atg5^{epiΔ/Δ} or Atg5^{ShhCre} mice. The latter discrepancies may be related to toxicity associated with expression of the *CCSP-rtTA* (line1) allele (35) and/or strain differences. Taken together, the results of these two independent studies suggest that there are likely region-specific requirements for basal autophagy in the alveolar and bronchiolar compartments. p62 accumulation was markedly increased in ciliated epithelial cells and was correlated with significant reduction in motile cilia length. These observations suggest an elevated requirement for autophagy in these bronchiolar cells and are consistent with recent reports implicating the p62/SQSTM1 protein in HDAC6-dependent autophagic turnover of ciliary components (36, 37).

It is well established that Influenza A virus can subvert autophagy to facilitate infection of cultured cells (20). Translation of these findings to the role of autophagy in influenza pathogenesis *in vivo* has been hampered by a paucity of animal studies and by recognition of cell line-dependent responses and virus subtype-specific effects on autophagic function (38). A key *in vivo* study by Sun and colleagues (21) demonstrated that pharmacologic or small interfering RNA-mediated inhibition of autophagy ameliorated lung injury in mice inoculated with avian Influenza A/H5N1 virus. Inhibition of autophagy did not alter viral replication but rather blocked autophagic cell death mediated by the hemagglutinin protein of H5N1. In the current study, decreased autophagy significantly inhibited replication of H3N2 virus. In contrast to H5N1-infected mice (21), improved lung structure and

function in Atg5^{epiΔ/Δ} mice was strongly associated with reduced H3N2 viral burden in addition to reduced inflammatory signaling and apoptosis. These results do not exclude a role for autophagic cell death in H3N2 pathogenesis; however, they do indicate that autophagy promotes H3N2 replication within alveolar epithelial cells, a major cell target of Influenza A virus (39–42), and that this event is linked to morbidity and mortality. A permanent (genetic) 50% reduction in alveolar autophagy was sufficient to reduce viral replication without affecting alveolar homeostasis. We did not assess the susceptibility of Atg5^{ShhCre} mice to viral infection because of significant comorbidities in these mice and the potential for reactivation of *ShhCre* in response to infection-induced lung injury (43).

In summary, 90% loss of autophagic function in alveolar epithelial cells resulted in septal thickening and altered lung mechanics in aged mice. This degree of ablation did not alter alveolar structure and function in young adult mice, underscoring the cumulative effect of sustained inhibition of autophagy during aging. In contrast, 50% loss of autophagic function had no effect on lung structure and function in young or aged mice, consistent with reserve or excess autophagic capacity in the alveolar epithelium. This reserve autophagic capacity provided a niche for viral replication, resulting in increased susceptibility to infection by Influenza A virus. These results support the concept that partial inhibition of autophagy in the lung epithelium may be a useful adjunct to current antiviral therapies that operates independently of virus subtype or antigen. Finally, these studies support the concept of region-specific requirements for autophagy in the respiratory epithelium with a higher requirement in bronchiolar cells linked to the turnover or remodeling of cilia. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. Kevin S. Harrod of the Infectious Diseases Program of Lovelace Respiratory Research Institute for providing the HKx31 Influenza A/H3N2 virus.

References

- Devenish RJ, Klionsky DJ. Autophagy: mechanism and physiological relevance 'brewed' from yeast studies. *Front Biosci* 2012;4:1354–1363.
- Shanware NP, Bray K, Abraham RT. The PI3K, metabolic, and autophagy networks: interactive partners in cellular health and disease. *Annu Rev Pharmacol Toxicol* 2013;53:89–106.
- Yuk JM, Yoshimori T, Jo EK. Autophagy and bacterial infectious diseases. *Exp Mol Med* 2012;44:99–108.
- Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H, *et al.* Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 2006;441:885–889.
- Nishiyama J, Miura E, Mizushima N, Watanabe M, Yuzaki M. Aberrant membranes and double-membrane structures accumulate in the axons of Atg5-null Purkinje cells before neuronal death. *Autophagy* 2007;3:591–596.
- Hartleben B, Gödel M, Meyer-Schwesinger C, Liu S, Ulrich T, Köbler S, Wiech T, Grahmmer F, Arnold SJ, Lindenmeyer MT, *et al.* Autophagy influences glomerular disease susceptibility and maintains podocyte homeostasis in aging mice. *J Clin Invest* 2010;120:1084–1096.
- Masiero E, Agatea L, Mammucari C, Blaauw B, Loro E, Komatsu M, Metzger D, Reggiani C, Schiaffino S, Sandri M. Autophagy is required to maintain muscle mass. *Cell Metab* 2009;10:507–515.
- Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, Omiya S, Mizote I, Matsumura Y, Asahi M, *et al.* The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. *Nat Med* 2007;13:619–624.
- Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, *et al.* Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol* 2005;169:425–434.
- Araya J, Kojima J, Takasaka N, Ito S, Fujii S, Hara H, Yanagisawa H, Kobayashi K, Tsurushige C, Kawaishi M, *et al.* Insufficient autophagy in idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2013;304:L56–L69.
- Mizumura K, Cloonan SM, Haspel JA, Choi AM. The emerging importance of autophagy in pulmonary diseases. *Chest* 2012;142:1289–1299.
- Patel AS, Lin L, Geyer A, Haspel JA, An CH, Cao J, Rosas IO, Morse D. Autophagy in idiopathic pulmonary fibrosis. *PLoS ONE* 2012;7:e41394.
- Ryder SW, Choi AM. Autophagy in the lung. *Proc Am Thorac Soc* 2010;7:13–21.
- Kirkin V, McEwan DG, Novak I, Dikic I. A role for ubiquitin in selective autophagy. *Mol Cell* 2009;34:259–269.
- Inoue D, Kubo H, Taguchi K, Suzuki T, Komatsu M, Motohashi H, Yamamoto M. Inducible disruption of autophagy in the lung causes airway hyper-responsiveness. *Biochem Biophys Res Commun* 2011;405:13–18.
- Martin LJ, Gupta J, Jyothula SS, Butsch Kovacic M, Biagini Myers JM, Patterson TL, Ericksen MB, He H, Gibson AM, Baye TM, *et al.* Functional variant in the autophagy-related 5 gene promoter is associated with childhood asthma. *PLoS ONE* 2012;7:e33454.
- Poon A, Eidelman D, Laprise C, Hamid Q. ATG5, autophagy and lung function in asthma. *Autophagy* 2012;8:694–695.
- Hariri M, Millane G, Guimond MP, Guay G, Dennis JW, Nabi IR. Biogenesis of multilamellar bodies via autophagy. *Mol Biol Cell* 2000;11:255–268.
- Weaver TE, Na CL, Stahlman M. Biogenesis of lamellar bodies, lysosome-related organelles involved in storage and secretion of pulmonary surfactant. *Semin Cell Dev Biol* 2002;13:263–270.
- Dumit VI, Dengjel J. Autophagosomal protein dynamics and influenza virus infection. *Front Immunol* 2012;3:43.
- Sun Y, Li C, Shu Y, Ju X, Zou Z, Wang H, Rao S, Guo F, Liu H, Nan W, *et al.* Inhibition of autophagy ameliorates acute lung injury caused by avian influenza A H5N1 infection. *Sci Signal* 2012;5:ra16.
- Rice WR, Conkright JJ, Na CL, Ikegami M, Shannon JM, Weaver TE. Maintenance of the mouse type II cell phenotype in vitro. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L256–L264.
- Bridges JP, Wert SE, Noguee LM, Weaver TE. Expression of a human surfactant protein C mutation associated with interstitial lung disease disrupts lung development in transgenic mice. *J Biol Chem* 2003;278:52739–52746.
- Wert SE, Yoshida M, LeVine AM, Ikegami M, Jones T, Ross GF, Fisher JH, Korfhagen TR, Whitsett JA. Increased metalloproteinase activity, oxidant production, and emphysema in surfactant protein D gene-inactivated mice. *Proc Natl Acad Sci USA* 2000;97:5972–5977.
- Perl AK, Zhang L, Whitsett JA. Conditional expression of genes in the respiratory epithelium in transgenic mice: cautionary notes and toward building a better mouse trap. *Am J Respir Cell Mol Biol* 2009;40:1–3.
- Shin YK, Liu Q, Tikoo SK, Babiuk LA, Zhou Y. Effect of the phosphatidylinositol 3-kinase/Akt pathway on influenza A virus propagation. *J Gen Virol* 2007;88:942–950.
- Zhou Z, Jiang X, Liu D, Fan Z, Hu X, Yan J, Wang M, Gao GF. Autophagy is involved in influenza A virus replication. *Autophagy* 2009;5:321–328.
- Jounai N, Takeshita F, Kobiyama K, Sawano A, Miyawaki A, Xin KQ, Ishii KJ, Kawai T, Akira S, Suzuki K, *et al.* The Atg5 Atg12 conjugate associates with innate antiviral immune responses. *Proc Natl Acad Sci USA* 2007;104:14050–14055.
- Harris KS, Zhang Z, McManus MT, Harfe BD, Sun X. Dicer function is essential for lung epithelium morphogenesis. *Proc Natl Acad Sci USA* 2006;103:2208–2213.
- Haldipur P, Bharti U, Govindan S, Sarkar C, Iyengar S, Gressens P, Mani S. Expression of Sonic hedgehog during cell proliferation in the human cerebellum. *Stem Cells Dev* 2012;21:1059–1068.
- Madison BB, Braunstein K, Kuizon E, Portman K, Qiao XT, Gumucio DL. Epithelial hedgehog signals pattern the intestinal crypt-villus axis. *Development* 2005;132:279–289.
- Barkauskas CE, Cronce MJ, Rackley CR, Bowie EJ, Keene DR, Stripp BR, Randell SH, Noble PW, Hogan BL. Type 2 alveolar cells are stem cells in adult lung. *J Clin Invest* 2013;123:3025–3036.
- Nishida Y, Arakawa S, Fujitani K, Yamaguchi H, Mizuta T, Kanaseki T, Komatsu M, Otsu K, Tsujimoto Y, Shimizu S. Discovery of Atg5/Atg7-independent alternative macroautophagy. *Nature* 2009;461:654–658.
- Shimizu S, Arakawa S, Nishida Y. Autophagy takes an alternative pathway. *Autophagy* 2010;6:290–291.
- Sisson TH, Hansen JM, Shah M, Hanson KE, Du M, Ling T, Simon RH, Christensen PJ. Expression of the reverse tetracycline-transactivator gene causes emphysema-like changes in mice. *Am J Respir Cell Mol Biol* 2006;34:552–560.
- Yan J, Seibenhener ML, Calderilla-Barbosa L, Diaz-Meco MT, Moscat J, Jiang J, Wooten MW, Wooten MC. SQSTM1/p62 interacts with HDAC6 and regulates deacetylase activity. *PLoS ONE* 2013;8:e76016.
- Lam HC, Cloonan SM, Bhashyam AR, Haspel JA, Singh A, Sathirapongsasuti JF, Cervo M, Yao H, Chung AL, Mizumura K, *et al.* Histone deacetylase 6-mediated selective autophagy regulates COPD-associated cilia dysfunction. *J Clin Invest* 2013;123:5212–5230.
- Comber JD, Robinson TM, Siciliano NA, Snook AE, Eisenlohr LC. Functional macroautophagy induction by influenza A virus without a contribution to major histocompatibility complex class II-restricted presentation. *J Virol* 2011;85:6453–6463.
- Korteweg C, Gu J. Pathology, molecular biology, and pathogenesis of avian influenza A (H5N1) infection in humans. *Am J Pathol* 2008;172:1155–1170.
- Kumar PA, Hu Y, Yamamoto Y, Hoe NB, Wei TS, Mu D, Sun Y, Joo LS, Dagher R, Zielonka EM, *et al.* Distal airway stem cells yield alveoli in vitro and during lung regeneration following H1N1 influenza infection. *Cell* 2011;147:525–538.
- Wang J, Nikrad MP, Phang T, Gao B, Alford T, Ito Y, Edeen K, Travanty EA, Kosmider B, Hartshorn K, *et al.* Innate immune response to influenza A virus in differentiated human alveolar type II cells. *Am J Respir Cell Mol Biol* 2011;45:582–591.
- Yin L, Xu S, Cheng J, Zheng D, Limmon GV, Leung NH, Rajapakse JC, Chow VT, Chen J, Yu H. Spatiotemporal quantification of cell dynamics in the lung following influenza virus infection. *J Biomed Opt* 2013;18:046001.
- Crosby LM, Waters CM. Epithelial repair mechanisms in the lung. *Am J Physiol Lung Cell Mol Physiol* 2010;298:L715–L731.