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Inducible disruption of autophagy in the lung causes airway hyper-responsiveness

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ABSTRACT

Autophagy is a highly conserved process primarily known for its role in cellular adaptation to nutritional stress. This bulk protein degradation pathway relocates nutrients during starvation. Recent studies, however, have revealed essential roles of autophagy in various organs under normal conditions. Especially, autophagy is now recognized as the pathway responsible for the elimination of damaged proteins resulting from environmental stress. Lungs are constantly exposed to high oxygen tension and environmental chemicals. To investigate the importance of autophagy in lung physiology, we used an inducible system to ablate Atg7 expression, which is a protein essential for autophagy, in the respiratory epithelial cells of adult mice. We found that Atg7 deficiency caused swelling of bronchiolar epithelial cells and accumulation of p62, which links substrate proteins to the autophagy machinery. Bronchiolar epithelial cells, isolated by micro-dissection of lung tissues, had elevated expression of cytoprotective genes that are typically activated by Nrf2. Interestingly, Atg7-deficient lungs displayed hyper-responsiveness to cholinergic stimuli without apparent inflammatory signs. Swollen bronchiolar epithelial cells may have lead to mechanical airway constriction and lowered the threshold for the increase of airway resistance. This study demonstrates the critical role of autophagy in the lungs for the maintenance of pulmonary homeostasis.

1. Introduction

Macroautophagy (autophagy) is a cellular homeostatic process involving lysosome-dependent turnover of organelles or proteins and was originally known as the main pathway for nutrient redistribution during the survival response to starvation [1]. Upon nutrient deprivation, autophagy is rapidly induced in yeast and mammalian cells. In addition to this classical "adaptive autophagy", recent studies have uncovered a significant role of basal levels of autophagy in the maintenance of cellular homeostasis under normal physiological condition [1]. "Constitutive autophagy" is considered as a cellular strategy for the quality control of proteins and organelles. Long-lived proteins and organelles are constantly degraded by autophagy to prevent accumulation of oxidative damages and other adverse changes within cellular components, which is particularly important for non-dividing cells such as neurons and hepatocytes [2,3].

The respiratory tract is constantly exposed to relatively higher levels of oxygen and various chemicals present in external environments. Thus, quality control mechanisms for proteins and organelles to protect against oxidative and xenobiotic stresses are important for lung homeostasis. However, the contribution of autophagy to the maintenance of lung homeostasis has not previously been studied.

p62/A170/SQSTM1 is a key molecule in the selection of substrates for autophagy [4]. p62 binds to polyubiquitinated proteins and LC3 on the autophagosome membrane, linking substrate proteins to the autophagy machinery. A deficiency in autophagy deficiency results in the formation of intracellular inclusion bodies containing polyubiquitinated proteins and p62. In various cell lineages, including hepatocytes, neurons, cardiac muscles, and pancreatic β cells, p62 accumulation is observed when autophagy is inhibited [4–7]. These results indicate that p62 mediates selective autophagy and that p62 and substrate proteins are constantly

Abbreviations: rtTA, the reverse tetracycline transactivator; Nqo1, NAD(P)H:quinoneoxidoreductase 1; Txnrd1, thioredoxin reductase 1; DAB, diaminobenzidine; FOT, forced oscillation technique; PV, pressure/volume; TLC, total lung capacity; BAL, bronchoalveolar lavage; DOX, doxycycline.

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being degraded by autophagy under normal physiological conditions.

We recently found that p62 activates the Keap1-Nrf2 stress response pathway by disrupting the association between Keap1 and Nrf2 [8]. Nrf2 is a master regulator of cytoprotective genes involved in detoxification and elimination of reactive oxygen species [9]. During quiescence, Nrf2 is ubiquitinated by Keap1, which is complexed with Cullin3, and degraded in proteasomes. p62 interacts with Keap1 and inhibits the ubiquitination of Nrf2 by Keap1, resulting in Nrf2 stabilization and the activation of Nrf2 target genes. Thus, inhibition of autophagy activates Nrf2 as a result of p62 accumulation [8].

To determine the role of autophagy in the adult lung, we inducibly disrupted *Atg7* gene, whose product is essential for autophagy, utilizing adoxycycline-inducible double transgenic system; the reverse tetracycline transactivator (rtTA) driven by rat CCSP promoter (CCSP-rtTA) and Cre recombinase driven by the recognition sequence of doxycycline-bound rtTA (tetO-Cre) [10]. After administration of doxycycline for 3 months, Atg7 expression in bronchiolar epithelial cells was decreased, and the bronchiolar epithelial cells were swollen and had p62 accumulation. The expression of cytoprotective genes was elevated, suggesting increased activity of Nrf2, which is consistent with our previous study [8]. Intriguingly, the mice with autophagy-deficient lungs displayed marked hyper-responsiveness to a cholinergic stimulus. These results demonstrate the important role of basal autophagy in the lungs for the maintenance of pulmonary homeostasis.

2. Materials and methods

2.1. Mice

CCSP-rtTA/(otet)7 CMV-Cre transgenic mice [10] were kindly provided by Dr. Jeffrey Whitsett. Generation of $Atg7^{Flox/Flox}$ mice, $Keap1^{Flox/Flox}$ mice, and Nrf2-null mice was previously described [2,11,12]. Six weeks after birth, mice were given water containing 1 mg/ml doxycycline (Dox). These mice were sacrificed for analysis after 3 months of continuous administration of doxycycline. The weight of the male mice was measured every 4 weeks for 16 weeks, after starting doxycycline treatment. Oxygen saturation (SpO_2) was measured by pulse oxymetry, the Mouse Oxsystem (Starr Life Science Corp). Mice were kept in the specific-pathogen-free conditions and were treated according to the regulations of *The Standards for Human Care and Use of Laboratory Animals of Tohoku University* and *Guidelines for Proper Conduct of Animal Experiments* by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

2.2. Laser micro-dissection (LMD) microscopy

LMD microscopy was conducted as previously described [13]. Briefly, lung tissue was removed and embedded in Tissue Mount (OCT Compound, Chiba Medical Co., Ltd.) and frozen in liquid nitrogen. 20-μm frozen sections were mounted on membrane slides (Leica). The frozen sections were fixed in ethanol containing 5% acetic acid for 3 min, and stained with 0.05% Toluidine Blue. Air-dried sections were used for micro-dissection of the bronchiolar epithelia with the LMD 7000 system (Leica).

2.3. RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from the bronchiolar epithelial cells, dissected by laser dissection microscopy, and purified using an Arcturus PicoPure RNA isolation Kit (Applied Biosystems). cDNA was synthesized using random primers. Real time PCR was performed with qPCR Mastermix (Eurogentec) using an ABI7300 Sequence Detection System. Primers and probes used for the amplification of cDNAs of *NAD(P)H:quinone oxidoreductase 1* (*Nqo1*) and *Thioredoxin reductase 1* (*Txnrd1*) were previously described [14]. Ribosomal RNA control reagents (Applied Biosystems) were used for amplifying rRNA as an internal control.

2.4. Histological examination

For light microscopic analysis, lungs were fixed by an intratracheal injection of Mildform® 10 N (WAKO) and embedded in paraffin. 4-μm sections were prepared and stained with hematoxylin and eosin (HE) or with anti-p62 antibody (Progen, GP62). For immunostaining with anti-Nqo1 antibody, lung samples were fixed by an intra-tracheal injection of a mixture of an isovolume of OCT compound and 4% paraformaldehyde in PBS and frozen in liquid nitrogen as previously described [15]. About 8-μm frozen sections were stained with anti-Nqo1 antibody (Abcam, ab2346). Positive reactivity was visualized by sequential incubation with HRP-conjugated anti-rabbit IgG and then by diaminobenzidine (DAB) staining. Hematoxylin was used for nuclear counterstaining.

2.5. Electron microscopy

After cardiac perfusion with saline, lungs were fixed by an intratracheal injection of 0.1 M phosphate buffer containing 4% paraformaldehyde and 4% glutaraldehyde, followed by cardiac perfusion with the same solution. Lungs were further fixed with 0.1 M cacodylic acid buffer (pH 7.2–7.4) with 2.5% glutaraldehyde for 2 h at 4 °C. After being washed in 0.2 M phosphate buffer, lung tissues were post-fixed with 1% osmium tetroxide for 2 h, embedded in Epon812 (Shell Chemical, USA) and sectioned as previously described [16] with a few modifications.

2.6. Pulmonary function tests

Pulmonary function of the mice was assessed by forced oscillation technique (FOT) using the FlexiVent system (Scireq). FlexiVent software version 5 was used as previously described [17]. FlexiVent was calibrated for open- and closed-tube systems for every pulmonary test. Each mouse was anesthetized and briefly allowed to acclimate to the ventilator. The maximum pressure was set at 20 cmH_2O for pressure/volume (PV) analysis and determination of total lung capacity (TLC). The positive end-expiratory pressure remained constant at approximately 2.5 cmH_2O. Airway resistance, airway compliance and airway hyper-responsiveness were sequentially measured in each mouse. The values obtained were normalized against body weight. Female mice were used for the pulmonary function test.

2.7. Evaluation of bronchoalveolar lavage (BAL) fluid

BAL was performed by injecting 0.5 ml of ice cold PBS into the left lung and then gently aspirating the fluid. BAL was repeated twice with fresh PBS. These three fluid samples were pooled, centrifuged and separated into the supernatant and pellet. The supernatant was used to determine protein concentration and quantify MIP-2 and IL-17 levels. The protein concentration was measured with the BCA protein assay kit (23225, Pierce). The concentrations of MIP-2 and IL-17 were measured by enzyme-linked immunostaining assay (ELISA) according to the manufacturer's protocol (MM200 and MN55413, R&D systems, MN, respectively). The pellet was used to determine the total cell number using a hemocytometer to count the cells. Differential cell counts were performed after staining the cells with Diff-Quik stain kit (16920, Sysmex, Kobe, Japan).

3. Results

3.1. Generation of an inducible respiratory epithelial cell-specific disruption of Atg7

Atg7 is an indispensable component of the autophagy machinery [2,18]. To determine the importance of autophagy in the maintenance of lung homeostasis, we used the doxycycline-inducible system to disrupt Atg7 expression in adult lungs. CCSP-rtTA::tetO-Cre double transgenic mice are known to express Cre after doxycycline exposure in the epithelial cells of the conducting airway including Clara cells [10]. We generated triple compound mutant mice by crossing CCSP-rtTA::tetO-Cre double transgenic mice with $Atg7^{Flox/Flox}$ mice. $Atg7^{Flox/Flox}$::CCSP-rtTA::tetO-Cre mice and $Atg7^{Flox/+}$::CCSP-rtTA::tetO-Cre mice (herein designated as $Atg7^{F/F}$::CCSP and $Atg7^{F/+}$::CCSP, respectively) were obtained and treated with drinking water containing doxycycline starting at the sixth week after birth. After 3 months of doxycycline administration, the mice were sacrificed for analysis.

Atg7 expression was reduced by approximately half in the bronchiolar epithelia of $Atg7^{F/F}$::CCSP mice compared with $Atg7^{F/+}$::CCSP mice, which were collected using LMD microscopy (Fig. 1A). The observed reduction may have been lower than expected because the Cre-expressing cells were not the only cell types isolated from the whole bronchiolar epithelia micro dissected for RNA purification. This interpretation was supported by histological analysis, which demonstrated conspicuous bulging of

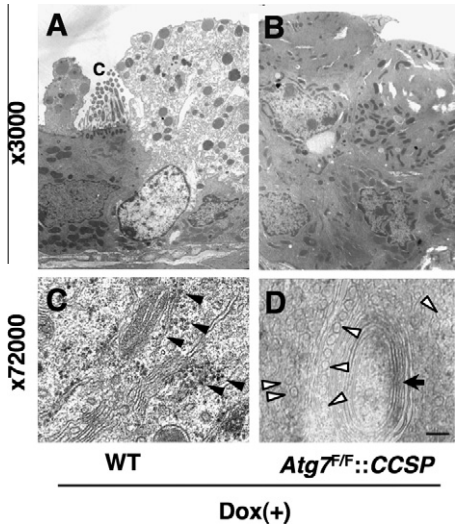

Fig. 2. Ultrastructures of bronchiolar epithelia studied by transmission electron microscopy. Wild-type (A and C) and $Atg7^{F/F}$::CCSP (B and D) mice treated with doxycycline (Dox (+)) were examined. Black arrowheads indicate ribosomes aligned on the endoplasmic reticulum, which are mostly absent in Atg7-deficient cells. An arrow and white arrowheads indicate a concentric membranous structure and spherical sac-like structures, respectively, which are observed characteristically in Atg7-deficient cells. (C) Cilia. Original magnification is ×3000 (A and B) and ×72,000 (C and D). A scale bar corresponds to 2.4 μm (A and B) and 100 nm (C and D).

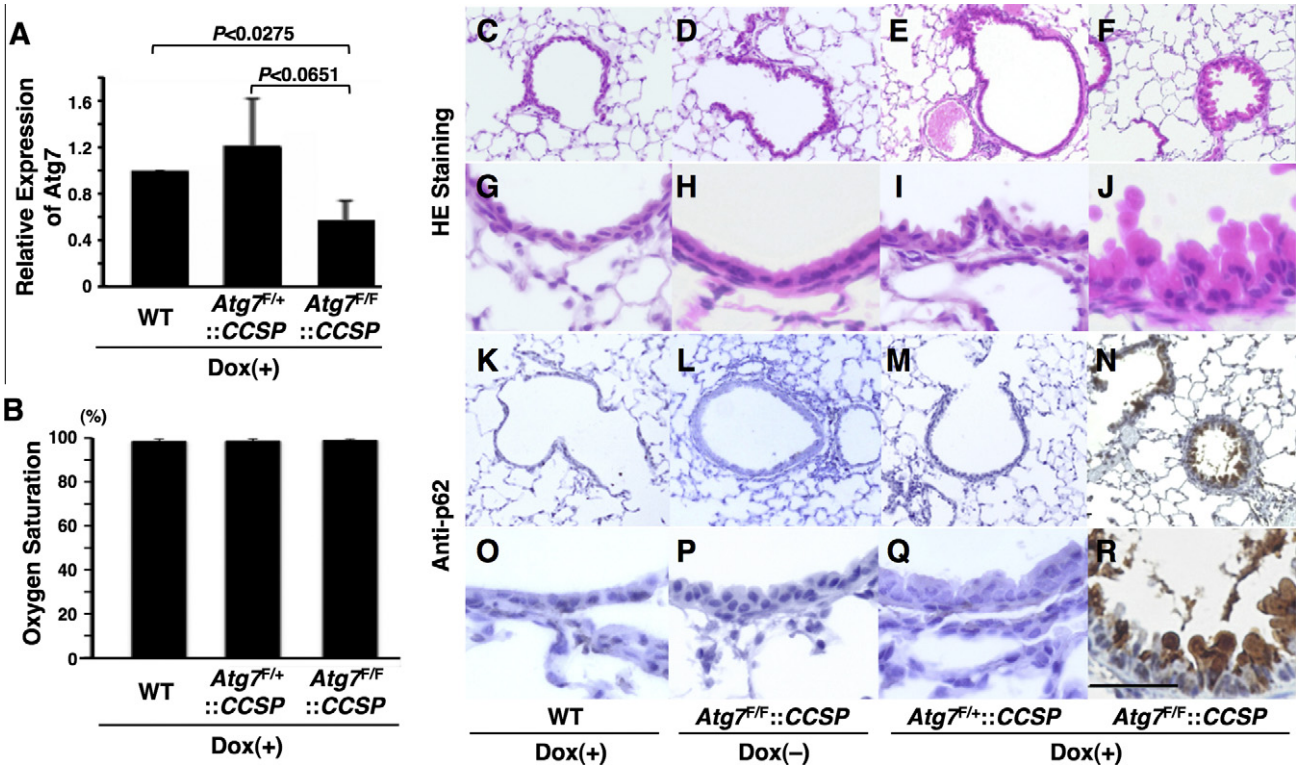

Fig. 1. Inducible deletion of Atg7 in pulmonary Clara cells in adult mice. (A) *Atg7* expression in bronchiolar epithelial cells of mice treated with doxycycline for 3 months (Dox (+)). Atg7 mRNA was detected in microdissected bronchiolar epithelial cells using quantitative RT-PCR. The average expression level of wild-type mice is set to one. Error bars indicate standard deviation of three independent mice. The student's *t*-test was used to calculate statistical significance (*P*). (B) Oxygen saturation measured by pulse oxymetry in the mice treated with doxycycline for three months (Dox (+)). Average values and standard deviations of three independent mice are shown. (C–R) Histological analysis. Staining with HE (C–J) and anti-p62 antibody (K–R) of pulmonary tissues and bronchiolar epithelial cells. Wild type (C, G, K, O), $Atg7^{F/+}$::CCSP (E, I, M, Q) and $Atg7^{F/F}$::CCSP (F, J, N, R)mice treated with doxycycline (Dox (+)) and $Atg7^{F/F}$::CCSP mice without doxycycline (Dox (–)) (D, H, L, P) were examined. A scale bar corresponds to 300 μm (C–F, K–N) and 50 μm (G–J, O–R).

cells in the bronchiolar epithelia of *Atg7*$^{F/F}$::CCSP mice compared with control mice of other genotypes or *Atg7*$^{F/F}$::CCSP mice without doxycycline (Fig. 1C–J). The similar swelling was also observed in the bronchial epithelial cells (data not shown). Doxycycline treatment appears to disrupt the *Atg7* gene effectively in single cells, inducing their respective morphological changes.

To examine the effect of Atg7 deletion on autophagy, accumulation of p62 was examined by immunohistochemistry using anti-p62 antibody. As p62 is degraded selectively by autophagy, any inhibition of autophagy results in intracellular accumulation of p62 [19]. As expected, we observed high levels of p62 in swollen bronchiolar epithelial cells of *Atg7*$^{F/F}$::CCSP mice (Fig. 1N and R). p62 reactivity was completely absent in the control mice with other genotypes and in*Atg7*$^{F/F}$::CCSP mice not exposed to doxycycline (Fig. 1K–M and O–Q). These results indicate that autophagy is suppressed by *Atg7* disruption in a subset of bronchiolar epithelial cells of doxycycline-treated *Atg7*$^{F/F}$::CCSP mice. Oxygen saturation levels in these mice were comparable to control mice (Fig. 1B).

3.2. Changes in the ultrastructure of bronchiolar epithelial cells in Atg7-deficient mice

Electron microscopy of *Atg7*$^{F/F}$::CCSP bronchioles revealed characteristic ultrastructures (Fig. 2). Few ciliated cells were found in the bronchioles of*Atg7*$^{F/F}$::CCSP mice that had been given doxycycline (Fig. 2A and B). Since CCSP-rtTA-mediated recombination is active in ciliated cells in bronchiolar epithelia [10], lack of autophagy may have disturbed the development of ciliated cells. The cytoplasm of epithelial cells in *Atg7*$^{F/F}$::CCSP bronchioles mostly lacked rough endoplastic reticulum (Fig. 2C and D) and Golgi apparatus (data not shown). Instead, the cytoplasm of *Atg7*$^{F/F}$::CCSP cells was filled with numerous spherical sac-like structures and aberrant concentric membranous structures (Fig. 2D). Similar abnormal structures were observed in previous studies of Atg7 deficiency in liver [2].

3.3. Atg7 deficiency increases the expression of cytoprotective genes

Based on our previous report that p62 disrupts the interaction between Keap1 and Nrf2 leading to constitutive activation of Nrf2, we next examined whether the Keap1-Nrf2 pathway is activated in *Atg7*$^{F/F}$::CCSP mice. Considering that p62 accumulation was only observed in bronchiolar epithelial cells, we used LMD to isolate cells from the bronchiolar epithelia and purified the RNA. The expression level of *Nqo1*, which is a typical target gene of Nrf2, was elevated in *Atg7*$^{F/F}$::CCSP mice compared to *Atg7*$^{F/+}$::CCSP and *Atg7*$^{F/F}$::CCSP mice without doxycycline (Fig. 3A). The difference in expression levels of *Txnrd1*, another Nrf2 target, between *Atg7*$^{F/F}$::CCSP and *Atg7*$^{F/+}$::CCSP mice was not statistically significant, but we found that the former tended to be higher than the latter.

The increased expression of *Nqo1* was further confirmed by immunohistochemistry using an anti-Nqo1 antibody. *Atg7*$^{F/F}$::CCSP bronchioles had more intense signals from the Nqo1 antibody than *Atg7*$^{F/+}$::CCSP bronchioles (Fig. 3D and E). The reactivity of the antibody was considered to be specific because the signal was almost

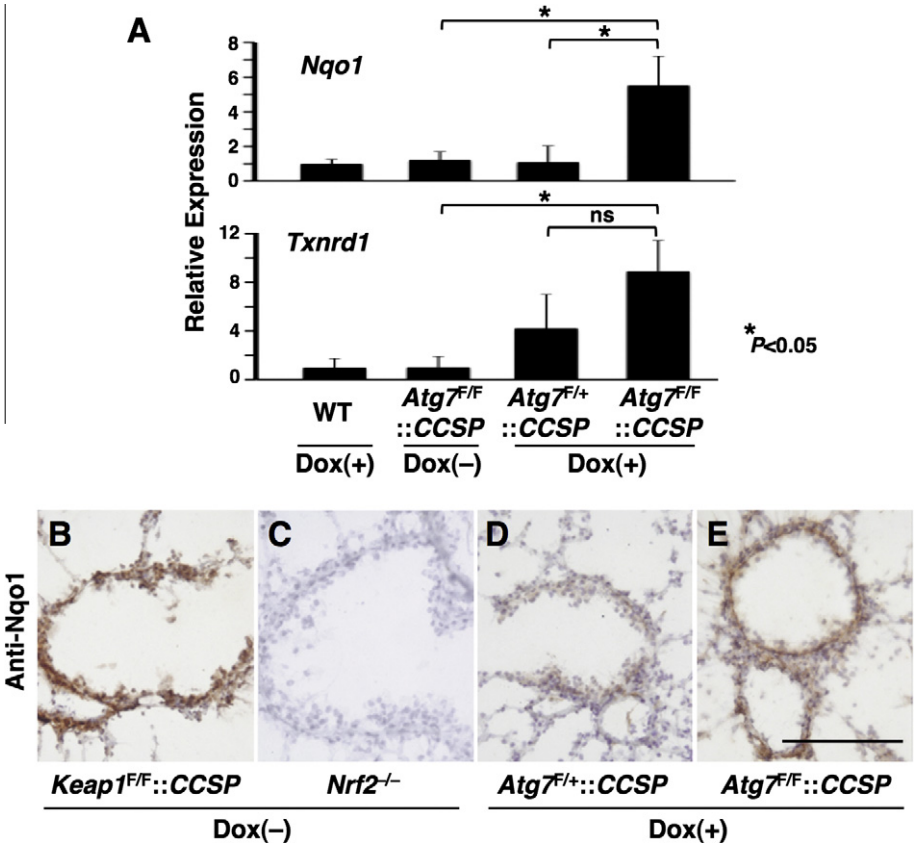

Fig. 3. Expression of cytoprotective genes in the bronchiolar epithelia of Atg7-deficient mice. (A) *Nqo1* and *Txnrd1* expression in bronchiolar epithelial cells. *Nqo1* and *Txnrd1* mRNAs were detected in quantitative RT-PCR using microdissected bronchiolar epithelial cells. Wild type ($n = 4$), *Atg7*$^{F/+}$::CCSP ($n = 5$) and *Atg7*$^{F/F}$::CCSP ($n = 4$) mice treated with doxycycline (Dox (+)) and *Atg7*$^{F/F}$::CCSP mice ($n = 5$ for *Nqo1* and $n = 4$ for *Txnrd1*) without doxycycline (Dox (−)) were examined. The average expression level of the wild-type mice is set to one. Error bars indicate the standard deviation. The student's *t*-test was used to calculate statistical significance (*P*). ns; not significant. (B–E) Immunostaining with anti-Nqo1 antibody of pulmonary tissues and bronchiolar epithelial cells. *Atg7*$^{F/+}$::CCSP (D) and *Atg7*$^{F/F}$::CCSP (E) mice treated with doxycycline (Dox (+)) were examined. *Keap1*$^{F/F}$::CCSP (B) mice and *Nrf2*$^{-/-}$(C) mice were examined as a positive and negative controls, respectively. The scale bar corresponds to 250 μm (B–E).

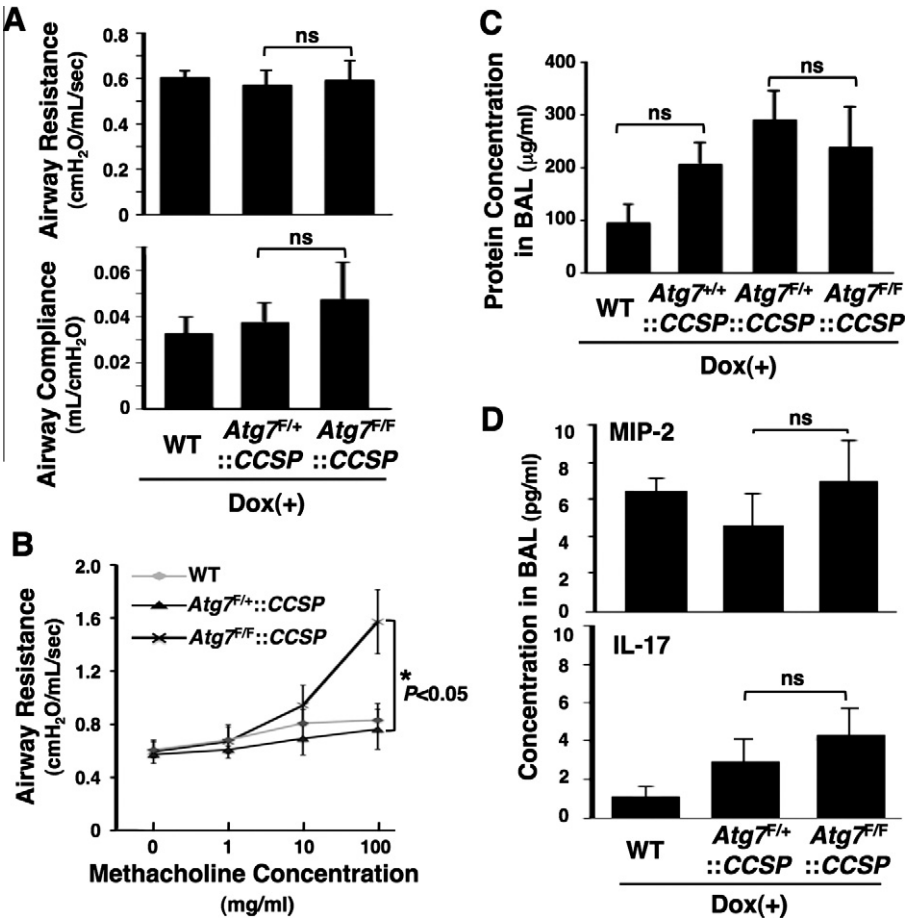

Fig. 4. Effects of Atg7 deficiency on pulmonary function in wild type, *Atg7*$^{F/+}$::CCSP and *Atg7*$^{F/F}$::CCSP mice treated with doxycycline (Dox (+)). (A) Airway resistance and compliance were examined in wild type ($n = 3$), *Atg7*$^{F/+}$::CCSP ($n = 6$ for resistance and $n = 4$ for compliance) and *Atg7*$^{F/F}$::CCSP ($n = 6$ for resistance and $n = 4$ for compliance) mice treated with doxycycline (Dox (+)). Average values and standard deviations of three independent mice are shown. (B) Airway resistance after methacholine administration was examined in wild type ($n = 3$), *Atg7*$^{F/+}$::CCSP ($n = 6$) and *Atg7*$^{F/F}$::CCSP ($n = 6$) mice treated with doxycycline (Dox (+)). Average values and standard deviations are shown. (C) Protein concentration of the BAL was examined. (D) ELISA of MIP-2 and IL-17was performed in the BAL. Average values and the standard deviations of three independent mice are shown in panels C and D. The student's *t*-test was used to calculate statistical significance (*P*). ns; not significant.

absent in *Nrf2*-null mice (Fig. 3C) and was augmented in *Keap1* knockdown (*Keap1*$^{F/F}$::CCSP without Dox) mice (Fig. 3B). These results suggest that autophagy deficiency in the respiratory epithelia activates Keap1-Nrf2 pathway through accumulation of p62.

3.4. Atg7 deficiency causes airway hyper-responsiveness to methacholine challenge

We then examined the effect of the thickened bronchiolar epithelia on pulmonary function. Forced oscillation techniques were used to measure the pulmonary function. No apparent changes in airway resistance or compliance in the presence or absence of Atg7 were observed (Fig. 4A). A substantial increase in airway resistance was observed in *Atg7*$^{F/F}$::CCSP mice after challenge with a high concentration of methacholine, whereas no change was observed in wild type and *Atg7*$^{F/+}$::CCSPmice (Fig. 4B).

We suspected that the hyper-responsiveness to methacholine observed in the *Atg7*$^{F/F}$::CCSP mice could be a general result of inflammation. Therefore, we examined the BAL collected from mice of each genotype. Alveolar macrophages constituted more than 90% of cells in the BAL fluid, and there was no significant difference among the groups (data not shown). Eosinophil was not detected in the BAL fluid (data not shown).No difference was observed in protein concentrations or MIP-2 and IL-17 levels measured by ELISA (Fig. 4C and D). Thus, *Atg7*$^{F/F}$::CCSP mice displayed a unique airway hyper-responsiveness that is not accompanied by apparent inflammation of the respiratory tract.

4. Discussion

In this study, we showed that loss of autophagy in conducting airway epithelial cells results in cell swelling, p62 accumulation and airway hyper-responsiveness to a cholinergic stimulus. Consistent with our previous report that p62 activates Nrf2 pathway by disrupting Keap1–Nrf2 interaction, expression levels of Nrf2 target genes were elevated in bronchioles of *Atg7*::CCSP mice. This is the first report demonstrating the importance of autophagy in the adult lung.

Conditional disruption of a gene using the Cre-loxP system is a powerful tool to study loss-of-function. CCSP-rtTA::tetO-Cre double transgenic system provides a well-controlled doxycycline-inducible Cre expression in airway epithelial cells [10]. CCSP-rtTA allele, however, has been shown to cause emphysema-like changes after 30 weeks of age, irrespective of doxycycline administration, suggesting an emphysematous predisposition in mice carrying the CCSP-rtTA transgene [20]. Protein concentrations in the BAL of mice carrying CCSP-rtTA allele tended to be higher than that of wild-type mice (see Fig. 4C), although the difference was not statistically significant. This might be a sign of adverse effect of CCSP-rtTA allele, which eventually leads to emphysema-like changes [20].

Airway hyper-responsiveness is a common signs of asthma and chronic inflammatory diseases of lower airways, but its precise pathophysiological mechanisms still remain to be elucidated. Airway thickening observed in *Atg7*$^{F/F}$::CCSP mice may be one of the reasons for the hyper-responsiveness from the aeromechanic point of view [21,22]. The emphysematous predisposition linked to the CCSP-rtTA allele may also exaggerate the effect of autophagy deficiency.

Target genes of Nrf2 were up-regulated in bronchiolar epithelial cells of *Atg7*$^{F/F}$::CCSP mice, suggesting that Nrf2 is stabilized by accumulated p62. In *Keap1*$^{F/F}$::CCSP mice treated with doxycycline, where Nrf2 is stabilized, the bronchiolar epithelia become thickened and multi-layered, but the swelling of each epithelial cell was not obvious (JM, DI, HM and MY, unpublished observation). Thus, *Keap1*-deficient phenotype is not completely the same with *Atg7*-deficient phenotype probably due to intact autophagy in *Keap1*$^{F/F}$::CCSP mice. Considering that hepatocytic damage caused by liver-specific disruption of *Atg7* depends on Nrf2 [8], we could expect that the bronchiolar cell swelling and subsequent airway hyper-responsiveness caused by lung-specific disruption of *Atg7* might be restored by simultaneous deletion of Nrf2.

Recent studies have revealed a remarkable degree of evidence for a role of tumor suppression by autophagy [23]. Reduced autophagic activity was found reduced in primary rat hepatocellular carcinoma [24]. Beclin 1, another essential component of the autophagy machinery, plays an important role in tumor suppression [25]. We surmise that autophagy deficiency may lead to carcinogenesis in the lung, which will be determined by long-term observation of *Atg7*$^{F/F}$::CCSP mice.

In contrast to the previous reports in other organs [2–4,6,7], the effect of lung-specific disruption of autophagy was modest but evident, causing swelling of affected cells and hyper-responsiveness to methacholine challenge. This work revealed the substantial contribution of the constitutive autophagy to the maintenance of lung homeostasis. Insufficiency of autophagy might be one of the underlying pathogeneses of airway hyper-responsiveness.

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