Disruption of Sorting Nexin 5 Causes Respiratory Failure Associated with Undifferentiated Alveolar Epithelial Type I Cells in Mice

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Abstract

Sorting nexin 5 (Snx5) has been posited to regulate the degradation of epidermal growth factor receptor and the retrograde trafficking of cation-independent mannose 6-phosphate receptor/insulin-like growth factor II receptor. Snx5 has also been suggested to interact with Mind bomb-1, an E3 ubiquitin ligase that regulates the activation of Notch signaling. However, the in vivo functions of Snx5 are largely unknown. Here, we report that disruption of the Snx5 gene in mice (Snx5-/- mice) resulted in partial perinatal lethality; 40% of Snx5-/- mice died shortly after birth due to cyanosis, reduced air space in the lungs, and respiratory failure. Histological analysis revealed that Snx5-/- mice exhibited thickened alveolar walls associated with undifferentiated alveolar epithelial type I cells. In contrast, alveolar epithelial type II cells were intact, exhibiting normal surfactant synthesis and secretion. Although the expression levels of surfactant proteins and saturated phosphatidylcholine in the lungs of Snx5-/- mice were comparable to those of Snx5+/+ mice, the expression levels of T1α, Aqp5, and Rag, markers for distal alveolar epithelial type I cells, were significantly decreased in Snx5-/- mice. These results demonstrate that Snx5 is necessary for the differentiation of alveolar epithelial type I cells, which may underlie the adaptation to air breathing at birth.

Introduction

During lung development, the proximal-distal axis is formed by the elongation, expansion, and bifurcation of lung buds during branching morphogenesis, and the functional units for gas exchange are generated during alveologenesis. During late gestation, alveolar development occurs and type I and type II alveolar epithelial cells undergo differentiation and maturation to form functional alveoli [1,2,3,4,5,6]. Alveolar epithelial type I cells are in close contact with endothelial cells in alveolar capillaries and form an efficient gas exchange area [7]. Alveolar epithelial type II cells undergo marked biochemical and ultrastructural changes, including depletion of glycogen content, enhanced synthesis of surfactant proteins and lipids, increased numbers of lamellar bodies, and surfactant secretion. In neonates, deficiency of surfactant protein production or secretion due to pulmonary immaturity can cause clinical disorders, such as respiratory distress syndrome or bronchopulmonary dysplasia. Respiratory distress syndrome is a major cause of high morbidity and mortality in premature infants [8,9,10].

Deletion or mutation of genes encoding surfactant proteins (SP-B, SP-C, and ATP-binding cassette (ABC) A3 (ABCA3) in mice causes respiratory failure or severe lung disease soon after birth [11,12,13,14]. In addition, recently, mutant mice with knock-in of the thyroid hormone receptor repressor silencing mediator of retinoid and thyroid hormone receptors (SMRT) have provided a novel model for alveolar epithelial type I cell-associated respiratory distress syndrome [15]. Although functional maturation of alveolar epithelial type I and II cells is critical for the adaptation of air breathing at birth, the molecular mechanisms that control the functional maturation of alveolar epithelial cells, particularly type I cells, are poorly understood [16,17].

Sorting nexins (SNXs) are a large family of proteins that contain the phosphoinositide-binding Phox homology domain. Previous studies have reported that SNX5 plays a role in endosomal trafficking of epidermal growth factor receptor (EGFR) and cation-independent mannose 6-phosphate receptor (CL-MPR)/insulin-like growth factor II receptor (IGF2R) [18,19,20,21]. Overexpression of SNX5 inhibits the degradation of EGFR, whereas the overexpression of SNX1 or SNX6 enhances EGFR degradation, suggesting that EGFR degradation may be finely
regulated by SNX1, SNX5, and SNX6 [18,22,23]. Knockdown experiments of SNX5 and SNX6 using RNA interference (RNAi) show dispersed CI-MPR trafficking in HeLa cells into early endosomes [20], suggesting that CI-MPR is also regulated by SNX5 in endosome-to-trans-Golgi network (TGN) retrieval [24]. In addition, SNX5 interacts with Mind bomb-1 (Mib1), a key regulator of Notch ligands in mammalian development [25,26,27,29,30,31]. In zebrafish, depletion of snx5 by morpholino leads to severe defects in vascular formation and hematopoietic cell generation [21], suggesting that Snx5 may be involved in Notch signaling through regulation of Mib1. Despite a decade of studies examining the role of SNX5, the precise physiological function of SNX5 in mammals is still not known.

To study the in vivo functions of SNX5, we generated mice lacking the Snx5 gene. These Snx5−/− mice showed partial perinatal mortality, with approximately 40% of the animals dying within a day of birth. Thirty percent of Snx5−/− mice survived until adulthood, but displayed severe growth inhibition. Downstream signaling of EGF, EGFR degradation and endosome-to-TGN retrieval of CI-MPR were not affected in Snx5−/− murine embryonic fibroblasts (MEFs). In addition, EGF-R signaling was not altered in isolated primary lung epithelial cells (PLEs). Neonatal Snx5−/− mice showed significant breathing defects associated with cyanosis and reduced pulmonary air space in the lungs at birth. Histological analysis revealed that Snx5−/− mice had reduced alveolar epithelial type I cells, while alveolar epithelial type II cells were intact. Consistently, Snx5−/− mice had decreased levels of the alveolar epithelial type I cell markers T1 and Aqp5, and RAGE, whereas the expression levels of surfactant proteins and saturated phosphatidylcholine were comparable to wild-type mice. The results of our study indicate that Snx5 is necessary for the differentiation of alveolar epithelial type I cells during perinatal murine lung development.

Materials and Methods

Ethics statement

Mice used in this study were housed under specific pathogen-free conditions. All animal protocols were approved by the Seoul National University Institutional Animal Care and Use committee (Approval number: SNU080623-4).

Mouse experiments

The XA153 gene-trapped ES cell line, which contained an insertion of β-geo in intron 7 of Snx5, was used to generate chimeric mice (BayGenomics, San Francisco, CA). Following a standard protocol (http://www.genetrap.org/info/protocols/baygenomics/blastocyst.html), XA153 ES cells were microinjected into C57BL/6 mouse blastocysts. The resultant chimeric mice were mated with C57BL/6 mice to obtain heterozygous Snx5+/− mice. The heterozygous mutant mice were backcrossed to C57BL/6 mice 5 times. Heterozygous mice were mated with C57BL/6 mice to obtain heterozygous Snx5+/− embryos. MEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone), 2-mercaptoethanol (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS; Hyclone). Mouse experiments of SNX5 and SNX6 using RNA interference (RNAi) and Western blot analysis and measurement of saturated phosphatidylcholine (Sat PC) were performed as described previously [25]. The relative concentration of each mRNA was normalized to the concentration of 18S rRNA in each sample. PCR primer sequences are shown in Table S2.

Isolation and culture of primary mouse lung epithelial cells

Lung tissues from Snx5−/− intercrosses were collected by caesarean section at E18.5. Primary lung epithelial cells were cultured with the methods described by Warshamana and Matsui [32,33] with slight modifications, and were used for EGF treatment experiments. Briefly, isolated lungs were removed and incubated with dispase (5 mg/mL, STEMCELL Technologies, Inc.) for 45 min at room temperature. The lungs were minced and transferred to Dulbecco’s modified Eagle’s medium (DMEM) with 0.01% DNase I (1 mg/mL, STEMCELL Technologies, Inc.) for 10 min at 37°C. The dissociated cells were filtered through a 40-μm nylon cell strainer and centrifuged at 1000 rpm for 10 min. The resuspended cells were plated into cell culture dishes at 37°C for 1 h in order to remove the macrophages and fibroblasts. After a 1-h incubation, nonattached cells were transferred into new cell culture dishes. This step was repeated 4 times. Final nonattached cells were filtered through a 40-μm strainer and centrifuged. The resuspended cells were plated on type-I collagen-coated 35 mm culture dishes (BD BioCoat) in DMEM/Ham’s F-12 50/50 mixed medium (Cellgro) supplemented with 15 mM HEPES, 0.8 mM CaCl2, 0.25% BSA, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite, and 2% mouse serum. After 24 h, the culture medium was changed to serum-free DMEM/Ham’s F-12 mixed medium supplemented as described above and maintained in the same medium. The isolated cells were almost all type II cells, as determined by qRT-PCR and immunocytochemistry analysis of T1α and pro-SP-C.

Cell culture, EGF stimulation, and CHX treatment

Primary MEFs were isolated from embryonic day 13.5 (E13.5) Snx5−/− and Snx5+/− embryos. MEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone) containing 10% fetal bovine serum (FBS; Hyclone), antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, USA), and 2-mercaptoethanol (Invitrogen) in a humid atmosphere of 5% CO2 at 37°C. For EGF treatments, MEFs were serum-starved for 12 h and then treated with 100 ng/mL EGF for 0, 5, 10, 20, 60, 120 and 120 min and 0, 5, 15, 30, 60 and 120 min. For CI-MPR protein detection, cells were treated with 40 μg/mL cycloheximide (CHX; Sigma, St. Louis, MO, USA) for 17 h to inhibit biosynthesis of CI-MPR protein.

RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated from lung tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA) and complementary DNA synthesis was performed according to the manufacturer’s instructions (iScript cDNA Synthesis Kit, Bio-Rad) using SYBR Green PCR mixture (Takara Bio Inc.) as described previously [25]. The relative concentration of each mRNA was normalized to the concentration of 18S rRNA in each sample. PCR primer sequences are shown in Table S2.

Western blot analysis and measurement of saturated phosphatidylcholine (Sat PC)

Protein detection in murine embryonic fibroblasts (MEFs), primary lung epithelial cells (PLEs) and total lung lysates of Snx5+/− and Snx5−/− mice by western blot has been described previously [27]. Proteins were detected using specific primary antibodies at 1:1000 dilution, including rabbit anti-epidermal growth factor receptor (EGFR), anti-cation-independent mannose 6-phosphate receptor (CI-MPR), anti-phospho-Akt, anti-Akt, anti-phospho-Erk1/2,
anti-Erk1/2 (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-tubulin (Abcam), rabbit anti-β-actin (Sigma), rabbit anti-Snx5, goat anti-CC-10, mouse anti-PCNA, goat anti-Pecam-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-proSP-C (Chemicon), Syrian hamster anti-T1α (1:100, Developmental Studies Hybridoma Bank), rabbit anti-pro-SP-C (1:2000, Chemicon), goat anti-CC-10 (1:2000, Santa Cruz Biotechnology), rabbit anti-Ki67 (1:500, Novo Castra), and goat anti-Pecam-1 (1:100, Santa Cruz Biotechnology). The slides were washed with PBS and then incubated with Alexa 594 goat anti-rabbit IgG at a 1:200 dilution, Alexa 488 goat anti-rabbit IgG at a 1:200 dilution, Alexa 498 goat anti-mouse IgG at a 1:200 dilution, Alexa 594 goat anti-mouse IgG at a 1:200 dilution, Alexa 594 donkey anti-goat IgG at a 1:200 dilution, Alexa 488 goat anti-hamster IgG at a 1:200 dilution (Invitrogen), or reagents from a Vectastain Elite ABC kit (Vector Laboratories) for secondary detection. Immunocytochemistry was performed in Snx5 MEF cell lines and PLEs. MEFs on gelatin-coated cover glass and PLEs on type I-coated cover glass were fixed in 4% PFA for 15 min on ice and then washed with 0.1% Triton X-100 in PBS. The samples were incubated with primary rabbit anti CI-MPR (1:100, Abcam), hamster anti-T1α (1:100, Developmental Studies Hybridoma Bank), or rabbit anti-pro-SP-C (1:2000, Chemicon) antibodies for 2 h at room temperature followed by incubation with secondary antibodies, i.e., AlexaFluor 594 goat anti-rabbit IgG (1:200, Invitrogen) and Alexa 488 goat anti-hamster IgG, for 1 h at room temperature. The cover glasses were mounted with Vectashield Mounting Medium with DAPI (VECTOR, H-1200). Images were obtained using an Axio Imager A2 microscope (Carl Zeiss, Germany) and Olympus software (Olympus micro DP71/DP70/DP30 BW Ver.03.03).

Transmission electron microscopy (TEM)

To examine the ultrastructure of E18.5 lung morphology, E18.5 lungs were collected and fixed in modified Karnovsky’s fixative overnight at 4°C. Fixed tissues were washed 3 times with 0.05 M sodium cacodylate buffer (pH 7.2) and postfixed with 1% osmium tetroxide in 0.05 M sodium cacodylate buffer for 2 h. Tissues were dehydrated in graded ethanol solutions and propylene oxide and then embedded in Spurr’s resin. Ultrathin sections were stained with uranyl acetate and Reynolds’ lead citrate. The sections were imaged with a LIBRA 120 TEM (Carl Zeiss, Germany).

Microarray analysis

Expression profiles of Snx5+/+ and Snx5−/− in E18.5 mouse lungs were generated using an Illumina microarray system (Illumine Beadchip Array MouseRef-8 v2), which includes 25,697 probes corresponding to 17,214 annotated genes. Total RNA was isolated from Snx5+/+ and Snx5−/− lungs using the RNeasy MiniKit (Qiagen). RNA integrity numbers (RIN) ranged from 9.3 to 9.7 when analyzed using an Agilent 2100 Bioanalyzer. RNA was reverse transcribed and amplified using Illumina Total Prep RNA amplification kit (Ambion). In vitro transcription was then carried out to prepare cRNA. The cRNAs were hybridized to the array and then labeled with Cy3-streptavidin (Amersham Bioscience). The fluorescent signal on the array was measured using a BeadStation 500 System (Illumina). In this study, two biological replicates of Snx5+/+ and Snx5−/− lungs were analyzed. The microarray data used in this study were deposited in the GEO database (GSE57080).

Statistical analysis of gene expression data

The probe intensities from the arrays were converted to log2 intensities. The log2 intensities were then normalized using a quantile normalization method [41]. The probes were annotated using (GPL6883.annot). A gene was considered “expressed” if the probe intensity of the gene was larger than a cutoff intensity in at least one condition. The cutoff intensity (in this study, intensity = 8) was determined by mixture modeling using 2 Gaussian
distributions, one for the “absent” probes and one for the “present” (expressed) probes [42]. To identify differentially-expressed genes (DEGs) by comparing \( \text{Snx}5^{+/+} \) vs. \( \text{Snx}5^{-/-} \) in E18.5 lungs, the following integrative statistical hypothesis-testing was performed: (1) Student’s t-test and log2 median ratio test were performed to compute \( T \)-values and median ratios for all the genes [42,43]; (2) \( P \)-values from each test were computed using an empirical distribution of the null hypothesis (that the means of the genes were not different), which was obtained from random permutations of the samples; (3) the individual \( P \)-values were combined to compute the false discovery rate (FDR) using Stouffer’s method [43]; and (4) the DEGs were selected as the genes with an FDR of less than 0.05 and log2-fold change larger than the cutoff of 0.3531 (1.27-fold change at the original scale). This fold-change cutoff value corresponded to the 95th percentile of the fold changes obtained from random permutation experiments using 2-tailed settings. Finally, functional enrichment analysis of DEGs was performed using DAVID software to identify cellular processes overrepresented by the DEGs governed by \( \text{Snx}5 \).

Statistics

In this study, all quantitative graphs are expressed as means ± standard deviations (SDs). \( P \)-values were calculated by Student’s \( t \)-tests. A \( P \)-value ≤ 0.05 was considered statistically significant.

Results

Generation of \( \text{Snx}5^{-/-} \) mice and expression of \( \text{Snx}5 \)

To determine the physiological role of \( \text{Snx}5 \), we generated \( \text{Snx}5 \) heterozygous mice (\( \text{Snx}5^{+/+} \)) using the gene-trapped ES cell line XA155, containing a \( \beta \)-galactosidase-neomycin cassette (\( \beta \)-geo) between exons 7 and 8 (Figure 1A). Germline transmission of the trapped allele was confirmed by Southern blot analysis with an \( \text{Snx}5 \)-specific probe (Figure S1A). Inactivation of the \( \text{Snx}5 \) gene was confirmed by genomic DNA PCR (Figure S1B), RT-PCR (Figure S1C), and western blotting (Figure S1D). Through \( \beta \)-galactosidase staining of \( \text{Snx}5^{+/+} \) mice expressing the \( \beta \)-galactosidase gene, we examined the expression patterns of \( \text{Snx}5 \). \( \beta \)-galactosidase staining of E11.5 \( \text{Snx}5^{+/+} \) tissues (Figure 1B) and adult tissues (data not shown) revealed ubiquitous expression of \( \text{Snx}5 \). Western blot analysis of adult tissues from \( \text{Snx}5^{+/+} \) mice also showed that \( \text{Snx}5 \) was expressed in various adult tissues (Figure 1C), suggesting that \( \text{Snx}5 \) may play a role in embryonic and adult developmental stages in various tissues.

Perinatal lethality and respiratory failure in \( \text{Snx}5^{-/-} \) mice

Examination of the survivorship of \( \text{Snx}5^{-/-} \) mice showed that 40% of mutant mice died at birth. Until E17.5, embryos from \( \text{Snx}5^{-/-} \) intercrosses showed the expected Mendelian ratio. However, at E18.5, \( \text{Snx}5^{-/-} \) embryos were born at a frequency of 19.4%, lower than the expected Mendelian ratio of 25% (Table 1).

Interestingly, 40% of \( \text{Snx}5^{-/-} \) mice died within 24 h of birth (Figures 1D and 2D) and an additional 20% of \( \text{Snx}5^{-/-} \) mice died within a month of birth (Figure 1D). The surviving \( \text{Snx}5^{-/-} \) mice had a smaller body size compared to that of \( \text{Snx}5^{+/+} \) and \( \text{Snx}5^{-/-} \) mice after birth (Figure S1E and F). Newborn \( \text{Snx}5^{+/+} \) and \( \text{Snx}5^{-/-} \) mice were pink and exhibited a normal sucking reflex and well-inflated lungs (Figure 2A–C). However, most \( \text{Snx}5^{-/-} \) mice were cyanotic (Figure 2D and G) and had condensed lungs (Figure 2E and F), although some \( \text{Snx}5^{-/-} \) lungs were partially inflated with air at birth (Figure 2H and I). These results suggested that \( \text{Snx}5^{-/-} \) mice may experience respiratory failure at birth.

### Table 1. Embryonic survival frequency of \( \text{Snx}5^{+/+} \), \( \text{Snx}5^{-/-} \) and \( \text{Snx}5^{-/-} \) littermates.

| Genotype n (%) | Age of embryos (day) |
|----------------|----------------------|
|                | \( \text{Snx}5^{+/+} \) | \( \text{Snx}5^{-/-} \) | \( \text{Snx}5^{+/+} \) | Total n |
| E14.5 ~ E16.5  | 33 (20.3)            | 86 (54.3)              | 41 (25.3)              | 162     |
| E17.5          | 18 (23.3)            | 38 (49.3)              | 21 (27.2)              | 77      |
| E18.5          | 124 (30.2)           | 207 (50.3)             | 80 (19.4)*             | 411     |

Embryos from timed matings were genotyped on embryonic day 14.5 ~ 16.5, 17.5 and 18.5 day. Fourth column shows the observed percentage of \( \text{Snx}5^{-/-} \) mice (the Mendelian percentage is 25%). *Calculated using \( \chi^2 \) test comparing the expected and observed frequency of viable \( \text{Snx}5^{-/-} \) mice. (\( p = 0.0089 \))

In order to investigate the causes of respiratory failure in \( \text{Snx}5^{-/-} \) mice, we examined the palate, trachea, esophagus, diaphragm, intercostal muscles, and heart. In \( \text{Snx}5^{-/-} \) mice, the palate was closed well (Figure S2A and B), and the structures of the trachea and esophagus were formed and separated properly (Figure 2C–F and 2K and L). The structure of the tracheal cartilage ring was also properly formed in mutant mice (Figure S2M and N). Histological analysis of other respiratory organs, such as the diaphragm (Figure S2G and H) and intercostal muscles (Figure S2I and J) of \( \text{Snx}5^{-/-} \) mice showed well-formed morphologies. In addition, the heart showed the normal morphological structure in \( \text{Snx}5^{-/-} \) mice (Figure S2O and P). Collectively, these results suggested that the respiratory failure of \( \text{Snx}5^{-/-} \) mice was not due to malformations of the palate, trachea, esophagus, diaphragm, intercostal muscles, or heart.

Impaired alveolar formation in \( \text{Snx}5^{-/-} \) mice

Considering that the structures of palate, trachea, esophagus, diaphragm, intercostal muscles, and heart were intact, we hypothesized that the respiratory failure observed in these mice could be due to defects in lung development. To test this possibility, we performed an air-breathing test after caesarean section at E18.5. After removing the pups from their mother, we measured the time until death from the point the pup began normal breathing in a 37°C chamber, intended to help the mouse maintain body temperature. While \( \text{Snx}5^{+/+} \) and \( \text{Snx}5^{-/-} \) mice survived after beginning to breathe, approximately 60% of mutant mice died within 1 h (Figure 3A and B). Moreover, while the body weights of \( \text{Snx}5^{-/-} \) mice was similar to those of control mice at E18.5, lung weights and lung-to-body weight ratios of \( \text{Snx}5^{-/-} \) mice were slightly lower than those of \( \text{Snx}5^{+/+} \) and \( \text{Snx}5^{-/-} \) mice (Figure 3C). Together with the results of the air-breathing test, these data suggested that lung defects in \( \text{Snx}5^{-/-} \) mice could lead to the observed high mortality rate at birth.

Next, in order to investigate whether \( \text{Snx}5 \) was expressed in the lung, we performed \( \beta \)-galactosidase staining on E18.5 \( \text{Snx}5^{-/-} \) lungs. As expected, \( \text{Snx}5 \) was expressed throughout the lung (Figure 4A), suggesting that \( \text{Snx}5 \) may play a role in lung development. In addition, hematoxylin and eosin staining revealed that \( \text{Snx}5^{-/-} \) lungs had thickened alveolar walls and reduced air space in the alveolar sacs at E18.5. Alveolar walls and air space were normal in E14.5 and E16.5 lungs (Figure 4B), suggesting that \( \text{Snx}5 \) was required for alveolar formation in the saccular phase of lung development.

To examine whether the observed impaired alveolar formation was due to defective differentiation of pulmonary epithelial cells or...
defective vascular formation, we performed immunohistochemistry with specific antibodies against alveolar epithelial type II cells (proSP-C), Clara cells (CC-10), smooth muscle cells (α-SMA), and blood vessels (Pecam-1). In E18.5 Snx5+/− mice, proSP-C-expressing cells were similar to those of controls (Figure 5A and B). Western blot analysis also showed similar levels of proSP-C in Snx5+/− lungs (Figure 5I). The expression of CC-10 in mutant bronchioles was comparable to that of Snx5+/+ mice (Figure 5C and D), suggesting that proximal lung development was intact in mutant mice. In addition, immunohistochemical staining of α-SMA and Pecam-1 did not show significant differences between Snx5+/+ and Snx5+/− lungs (Figure 5E–H and J), indicating that the terminal vascular architecture was intact in the mutant lungs. Furthermore, we also investigated whether thickened alveolar septa were correlated with increased proliferative cells in Snx5+/− lungs. At E18.5, Ki67 staining of the Snx5+/− lungs showed similar amounts of proliferating cells relative to controls (Figure S3A and B), indicating that the thickened alveolar septa of Snx5+/− lungs were not caused by increased proliferation of epithelial cells.

Most lung atelectasis is caused by defective maturation of alveolar epithelial type II cells [44]. Mature alveolar epithelial type II cells secrete surfactants that reduce pulmonary surface tension and prevent atelectasis at the end of expiration [45]. These surfactants are composed of a complex mixture of proteins and lipids. To investigate whether the impaired alveolar formation was due to a maturation defect of alveolar epithelial type II cells, we compared the expression levels of surfactant proteins and lipids in Snx5+/+ and Snx5+/− lungs. Alveolar epithelial type II cells expressed the surfactant proteins SP-A, -B, -C, and -D. qRT-PCR analysis showed that the mRNA expression levels of these surfactant proteins were not significantly different between Snx5+/+ and Snx5+/− lungs (Figure 5K). The amount of SP-B in 15 μL of supernatant from lung homogenates (total volume 2 mL) was analyzed by western blotting (Figure S4A and B). SP-B protein/lung weight ratios were not altered by deletion of Snx5 (Figure 5L). Levels of Sat PC, the most abundant component of surfactant lipids, were slightly lower in Snx5+/− lungs than in Snx5+/+ lungs (Figure S4C). However, Sat PC content relative to lung weight was similar between groups (Figure 5M). Likewise, ultrastructure analysis by TEM revealed that Snx5+/− lungs had normal alveolar epithelial type II cells with compacted, well-organized lamellar bodies and had secreted surfactants in the alveolar air-space (Figure 5N). Taken together, it can be concluded that deletion of Snx5 does not appear to affect the maturation of alveolar epithelial type II cells.

Impaired differentiation of alveolar epithelial type I cells in Snx5+/− lungs

In order to further investigate the causes of respiratory failure in the Snx5+/− mice, we examined whether the differentiation of alveolar epithelial type I cells was intact. Immunohistochemical analysis of T1α, a marker of typical alveolar epithelial type I cells, revealed that the expression of T1α was markedly decreased in Snx5+/− lungs, whereas T1α expression extended along the alveolar wall in control lungs (Figure 6A). Consistently, the expression levels of T1α in lung lysates from E18.5 Snx5+/− mice were dramatically decreased compared to those of controls (Figure 6B).
Additionally, the typical markers for distal alveolar epithelial type I cells, such as T1α, AQP5, and RAGE were examined [46]. Indeed, the mRNA expression levels of T1α, Aqp5, and Rage were significantly decreased in E18.5 Snx5-/- lungs compared to those of controls (Figure 6C). Thus, we hypothesize that loss of Snx5 leads to impaired differentiation of alveolar epithelial type I cells.

Reduced mRNA expression of T1α in Snx5-/- lungs was evident from E14.5 (Figure 6D). Next, we examined the expression levels

Figure 3. Respiratory failure in Snx5-/- mice. (A) Survival after air-breathing tests in Snx5+/-, Snx5-/-, and Snx5-/- mice. Within 30 min, nearly 60% of Snx5-/- mice had died (asterisk). (B) Appearance after air-breathing tests in E18.5 mice. Cyanotic appearance and condensed lungs in Snx5-/- mice. (C) Body and lung weights of Snx5+/-, Snx5-/-, and Snx5-/- mice. Lung weight and lung to body weight ratios of Snx5-/- mice were decreased by 22.9% and 14.9%, respectively, relative to Snx5+/- mice. *P < 0.0001; data represent Snx5+/-, n = 24; Snx5-/-, n = 27; and Snx5-/- mice, n = 17.

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Figure 4. Expression of Snx5 and morphological malformations in Snx5-deficient mice at E18.5. (A) Whole and sectional X-gal staining of E18.5 lungs. The Snx5-/-geo reporter gene was expressed ubiquitously in Snx5+/- lungs. Scale bars: 1 mm, 100 μm. (B) H&E staining of E14.5, E16.5, and E18.5 Snx5+/- and Snx5-/- lungs. Snx5-/- lungs exhibited thickening of the alveolar walls and reduced alveolar space at E18.5. Scale bar: 50 μm.

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of lectin, galactoside-binding, soluble, 3 binding protein (Lgals3bp) and indolethylamine N-methyltransferase (Inmt), which have been shown to be reduced in a defective type I epithelial cell-associated mouse model, SMRTmRID mice [15]. Pei and colleagues reported that differentiation-defect of type I pneumocytes promoted respiratory distress syndrome at perinatal stages. In this paper, genome-wide analysis of mutant mice showed that several genes involved in Lgals3bp and Inmt signaling were reduced in mutant mice. To determine whether decreased T1α affected the expression of these genes, we performed qRT-PCR. We observed that Lgals3bp and Inmt transcripts were significantly reduced in Snx5−/− lungs (Figure 6E). Next, to further investigate the morphological differentiation of alveolar epithelial type I cells, we performed ultrastructure analysis by using TEM. Although Snx5−/− lungs showed a less alveolar epithelial type I cells compared to Snx5+/+ lungs (data not shown), the alveolar epithelial type I cells of Snx5−/− lungs represented slightly thicker nucleus and cytoplasm (Figure S5B and D) compared to Snx5+/+ lungs (Figure S5A and C). However organelles enriched in alveolar epithelial type I cells, such as mitochondria (Figure S5A and B; white arrowhead) and vesicles (Figure S5A and B; black arrow), existed well in Snx5+/+ lungs (Figure S5B). Also, extended cytoplasmic

Figure 5. Snx5 was not required for maturation of alveolar epithelial type II cells. (A–H) Immunohistochemical analysis of proSP-C, CC-10, α-SMA, and Pecam-1 in sections from Snx5+/+ and Snx5−/− lungs (n = 5). Scale bars: 50 μm. (A and B) proSP-C, a maker of surfactant protein C precursor in alveolar epithelial type II cells; (C and D) CC-10, a marker of Clara cells; (E and F) α-SMA, a marker of smooth muscle cells; and (G and H) Pecam-1, a maker of blood vessels, showed similar expression levels. The nuclei were stained with Hoechst. (I and J) Western blot analysis of proSP-C (I, n = 5) and Pecam-1 (J, n = 2) proteins in E18.5 Snx5+/+ and Snx5−/− lung whole lysates. (K) qRT-PCR analysis of surfactant proteins (SP-A, -B, -C, and -D) performed on RNA samples collected from total lungs (n = 3). Similar expression levels of surfactants were seen. (L) Mature SP-B did not differ in Snx5−/− lungs (n = 5). (M) Sat PC were similar between Snx5+/+, Snx5+/−, and Snx5−/− mice (n = 6). (N) Transmission electron microscopy (TEM) of distal alveolar epithelial type II cells at E18.5. Apical microvilli (Mv; arrowhead), condensed lamellar bodies (LB), and secreted surfactants (Sf; arrow) were observed in Snx5−/− lungs. Scale bars: 2 μm.

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Snx5 has been implicated in endosomal trafficking of EGFR and CI-MPR [18,19,20,47]. In order to investigate whether impaired differentiation of alveolar epithelial type I cells in Snx5−/− lungs was due to defective endosomal trafficking of EGFR, we examined EGFR signaling after EGF stimulation in Snx5−/− MEFs and isolated PLEs (Figure S6A-C). Expression levels of EGFR were comparable between Snx5+/+ and Snx5−/− MEFs (data not shown). To further investigate the EGFR signaling, the downstream signaling molecules were measured by using immunoblot in EGF stimulated Snx5−/− and Snx5+/+ MEFs. Phosphorylation of Akt and Erk1/2, the key effectors of EGFR signaling, was comparable between Snx5−/− and Snx5+/+ MEFs in response to EGF treatment (Figure 7A and C). In addition, after long-term exposure to EGF, there was no difference in the downregulation of EGFR (Figure 7B and D). The Snx5 deletion did not affect the endosomal signaling and degradation of EGFR in MEFs and PLEs and the expression levels of EGFR in the E18.5 Snx5−/− lungs were also similar between two groups (Figure S7A and B).

Next, we examined whether endosome-to-TGN retrieval of CI-MPR was affected by disruption of Snx5. CI-MPR has been reported to be a major retromer component in retrograde endosome-to-TGN transport [24]. Knockdown of Snx5 using small interfering RNA inhibits trafficking of CI-MPR from endosomes to the TGN, resulting in increased degradation of CI-MPR [20,48]. To establish whether the local alteration of CI-MPR resulted from Snx5 depletion, we performed immunocytochemistry analysis of CI-MPR (Figure 7E). Additionally, to determine CI-MPR stability in Snx5−/− MEFs, we examined the degradation rate of CI-MPR (Figure 7F). Because the biosynthetic rate of CI-MPR protein varies in efficiency, we treated cells with 40 μg/mL CHX for 17 h to compare the degradation ratio of CI-MPR in Snx5+/+ and Snx5−/− MEFs. Unexpectedly, however, when we examined the localization and stability of CI-MPR in Snx5−/− MEFs, there was no significant alteration in Snx5−/− MEFs compared to Snx5+/+ MEFs (Figure 7E and F). This finding suggests that the loss of Snx5 alone in a retromer complex does not affect the localization and lysosomal degradation of CI-MPR in MEFs.

No studies have reported the correlation between Snx5 and the differentiation of alveolar epithelial type I cells. Since there was no clear molecular explanation for the defective differentiation of alveolar epithelial type I cells in Snx5−/− mice, a genome-wide cRNA microarray analysis using the RNA isolated from E18.5 Snx5−/− and Snx5+/+ lungs was performed. Microarray analysis data using an Agilent 2100 Bioanalyzer [49] revealed the decreased expression of more than 60 genes in Snx5−/− lungs compared to Snx5+/+ lungs (data not shown). The altered genes were members of several functional categories, including genes for pulmonary epithelial cell markers, development, maturation, regulation of endocytosis and transport, serine/threonine kinases, enzyme-linked receptor signaling pathways, cell adhesion, and cell migration. Among these downregulated genes, we found that expression of the alveolar epithelial type I cell markers T1α, Aqp5, and Rage was significantly decreased (data not shown). In addition, several genes involved in ribosome biogenesis, RNA processing, translation and oxidative phosphorylation exhibited upregulated observed in both groups (Figure S5C and D). It seems that there were no obvious differences in ultrastructural morphologies of alveolar epithelial type I cells. From these data, we concluded that Snx5 was required for proper differentiation of alveolar epithelial type I cells.

Snx5 as a novel mediator required for differentiation of alveolar epithelial type I cells

The Functions of Snx5 in Lung Development

Figure 6. Impaired differentiation of alveolar epithelial type I cells in E18.5 Snx5−/− lungs. (A) Immunohistochemical analysis of T1α at E18.5. T1α, a marker of alveolar epithelial type I cells, was significantly decreased in Snx5−/− compared to Snx5+/+ lungs (n = 5). The nuclei were stained with Hoechst. Scale bars: 50 μm. (B) Decreased protein expression of T1α in E18.5 (n = 4). Densitometric analysis showed that average expression levels of T1α in Snx5−/− lungs were reduced compared to those in Snx5+/+ lungs. (C–E) qRT-PCR analysis of type I cell-related genes from lung tissue. (C) Markers of alveolar epithelial type I cells, i.e., T1α, Aqp5, and Rage, were decreased in Snx5−/− lungs (n ≥ 3). (D) T1α mRNA during the embryonic stage. From E14.5, mRNA expression of T1α was decreased. (E) Decreased expression levels of Lgals3bp and Inmt (n ≥ 3). **, P < 0.01, ***, P < 0.001 using the Student’s t-test.

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expression (Table S1). However, from microarray analysis data and qRT-PCR, we concluded that there was no significant difference in Notch signaling-related genes, such as *Hes1*, *Hes5*, *Hey1*, *Hey2*, and *HeyL*, between groups (data not shown). Thus, these data raise the possibility that Snx5 may be involved in other signaling pathways in addition to the Mib1-Notch signaling pathway. To address this question, we performed a literature search for previous studies on Snx5. Recently, Pei et al. reported that defective differentiation of alveolar epithelial type I cells led to a respiratory distress syndrome-like phenotype in SMRT mutant mice and that 21 genes, including *Klf2*, *Lgal3bp*, *Klf4*, *Inmt*, *Zfp36*, and *Dusp1*, were significantly decreased [15]. Interestingly, when we examined the expression levels of these genes in *Snx5*−/− lungs, we also found that these genes were significantly downregulated (data not shown). Taken together, although more molecular studies on Snx5 are required to determine the precise role of this protein in lung development, these findings suggest that Snx5 could be a novel mediator for proper differentiation of alveolar epithelial type I cells.

**Discussion**

Here, we report that *Snx5* is necessary for the differentiation of alveolar epithelial type I cells. Targeted inactivation of the *Snx5* gene led to neonatal mortality caused by respiratory failure, in which differentiation of alveolar epithelial type I cells was impaired, while maturation of alveolar epithelial type II cells was intact. Although SNX5 has been posited to interact with Mib1, an E3 ubiquitin ligase that regulates the activation of Notch signaling, it is unlikely that these defects are caused by Mib1-Notch signaling. In addition, other related molecules, EGFR and CI-MPR, were not affected by Snx5 depletion. Collectively, our data reveal an essential role of Snx5 in the differentiation of alveolar epithelial type I cells.

Maturation defects of alveolar epithelial cells can cause neonatal respiratory failure. Thus far, various factors, such as C/EBPα, co-activator-associated arginine methyltransferase 1 (CARM1), Erk3, Foxa2, Foxm1, Fstl1, Mig6, and Pdpn (T1α), have been implicated in alveolar formation at the saccular stage of lung development. Disruption of these factors can lead to perinatal lethality caused by a respiratory distress syndrome (RDS)-like phenotype, which includes pulmonary atelectasis. This phenotype is largely caused by the excessive proliferation of immature alveolar epithelial type II cells [17,35,39,50,51,52,53]. In contrast, *Snx5*−/− lungs did not show a significant difference in cellular proliferation (Figure S3A and B), although cellularity was slightly increased. In addition, there were no significant differences between *Snx5*+/+ and *Snx5*−/− lungs in the production of surfactant proteins and Sat PC. Moreover, *Snx5*−/− lungs had normal alveolar epithelial type II cells with compacted, well-organized lamellar bodies and secreted surfactants in the alveolar air-space. This suggests that deletion of *Snx5* did not affect the maturation of alveolar epithelial type II cells and that the respiratory failure in the *Snx5*−/− mice was not due to functional defects of alveolar epithelial type II cells. However, *Snx5*−/− lung showed a significantly reduced expression of alveolar epithelial type I cell markers. Through these phenotypes, we could suggest that intact alveolar epithelial type II cells may reduce the severity of lung defects in *Snx5*−/− lung.

In present study, *Snx5*−/− mice showed the variability in survival rates. To determine whether leaky expression of *Snx5* could affect a different survival rates, we investigated the expression level of *Snx5* gene. In addition, we observed surviving and non-surviving *Snx5*−/− mice at birth and checked the expression levels of alveolar epithelial cell markers. Although we could not observed the differences between survivors and non-survivors, all mutant mice showed reduced expression of type I epithelial cell markers ; T1α, AQ5 and Rage (data not shown). So far, we did not define other causes of perinatal mortality in this study, we could suggest that the survivor mice have other complex compensatory mechanisms. Further studies will be required to address this possibility. In addition, the embryonic studies of *Snx5*−/− mice showed the significantly reduced numbers of mutant embryos at E18.5. Although the causes of embryonic death are ambiguous and need further studies, ubiquitous expressed of Snx3 suggests that Snx5 may play a role in various developmental contexts in embryonic stage. Previous studies have demonstrated that EGFR-knockout mice exhibit growth retardation and epithelial immaturity, which shows partial similarity to the phenotype of *Snx5*−/− mice. *Egfr*−/− mice can be classified into 3 groups: runted, small-sized, and normal-
ized pups [54]. The second group, small-sized pups, were shown to have breathing problems (gasping) and lived for up to 2 days. Dead EGFR−/− mice exhibited condensed and immature lung morphologies resembling neonatal respiratory distress syndrome. Although significant differences in EGFR signaling and degradation in MEFs after EGF stimulation were not observed in the current study, Snx5+/− and EGFR−/− mice display partially similar phenotypes such as lung immaturity, growth retardation and strain-dependent survivability. Although we did not go into details for postnatal phenotypes, we could observe that Snx5−/− mice had a longer lifespan and growth inhibition on other genetic background mice (data not shown). The inability to detect prominent differences in EGFR signaling and degradation in Snx5−/− MEFs may be due to the presence of a redundant signaling mechanism in MEFs and PLEs or the limited sensitivity of this experiment to detect subtle changes. Nevertheless, phenotypic similarities between Snx5−/− and EGFR−/− mice suggest that Snx5 may be involved in EGFR signaling in the differentiation of alveolar epithelial type cells.

Knockdown of Snx1 using RNA interference (RNAi) has been shown to cause dispersed cytosolic localization and degradation of CI-MPR in HeLa cells [22]. However, Snx1+/−/− deficient MEFs exhibit similar CI-MPR sublocalization and stability compared to wild-type MEFs [55,56]. Similarly, although depletion of Snx5 and Snx6 in HeLa cells leads to dispersed CDS-CI-MPR trafficking into early endosomes [20], no significant differences in the localization and degradation of CI-MPR were observed in Snx5−/− MEFs in the current study. The lack of consensus in these findings suggests that the retrograde activity of CI-MPR may be regulated in a context-dependent manner in different cells and tissues.

We previously reported that Snx5 interacts with Mib1, which plays a role in Notch signaling in various contexts of mammalian development [25,26,27,28,29,30]. Snx5 morpholino injection into zebrafish embryos resulted in hematopoietic and blood vessel defects, which could be due to defective Notch signaling [21]. We hypothesized that Snx5 could play an important role in Notch signaling and that Snx5+/− mice would exhibit Notch phenotypes; however, we did not observe Notch phenotypes, such as morphological defects of the somite, brain, vessels, intestines, kidneys, or hematopoietic cells in which Mib1-Notch signaling is required [25,26,27,28,29,30]. When we transplanted fetal liver cells from Snx5+/− and Snx5−/− mice into lethally irradiated recipient mice, we did not observe any developmental alteration of immune cells, including T and marginal zone B lymphocytes (data not shown) [26,29]. Moreover, in this experiment, E18.5 Snx5+/− lungs did not show a significant alteration in Notch signaling-related genes, such as Hes1, Hes5, Hey1, Hey2, and Hey4, as assessed by qRT-PCR (data not shown). Thus, we conclude that disruption of the Snx5 gene in lungs does not alter Notch signaling.

In summary, this study demonstrated that deletion of the Snx5 gene in mice can cause neonatal death, most likely due to sacculum immaturity. Snx5 is essential for the differentiation and maturation of distal alveolar epithelial type I cells during lung development. Although the underlying mechanisms of Snx5 in lung development are unknown, it is plausible that Snx5 mediates lung maturity. Future studies of Snx5 with new binding molecules will deepen our understanding of the critical mechanisms involved in mouse lung development and provide new insight into the functions of SNXs in the cellular regulation of endocytic trafficking. Moreover, elucidation of the function of Snx5 in lung development may provide targets for novel therapeutic strategies for the prevention and treatment of respiratory distress syndrome.

### Supporting Information

**Figure S1** Characterization of Snx5-trapped mice. (A) Southern blot analysis of gene-trapped mouse tails. The wild-type (WT) allele (4.2 kb) and the mutant (MT) allele (5.8 kb) were generated by EcoR1 restriction enzyme digestion. (B) PCR-based genotyping analysis of the progenies of Snx5 heterozygous crosses. The WT band was detected using W-L (exon7) and W-R (exon7) primers (Figure 1A). The MT band was detected using W-L (exon7) and M-R (β-geo cassette) primers. (C) RT-PCR and (D) Western blot of mouse lung tissue. Mouse Snx5 mRNA and protein were absent in tissue derived from Snx5−/− mice. (E) A representative growth retardation phenotype in Snx5−/− mice at P30. (F) Graph shows a reduced body weight of Snx5−/− mice at P21.

**Figure S2** Normal structure of the respiratory organs and heart. (A–J) Hematoxylin and Eosin (H&E) staining of respiratory organs at E18.5. (A and B) Palates, (C–F) morphology of sagittal and transverse sections of the trachea and esophagus, (G and H) diaphragm and (I and J) intercostals muscles showing similar structures in Snx5−/− mice and controls. (A and B) Scale bars: 1 mm, (C and D) scale bars: 400 μm, (E and F) scale bars: 200 μm, (G–J) scale bars: 100 μm. (K and L) Light micrograph of the separated trachea and esophagus in E18.5 Snx5−/− mice. Scale bars: 1 mm. (M and N) Alcian blue staining of tracheal cartilage at E18.5. Scale bars: 0.5 mm. (O and P) Normal morphological structure of Snx5−/− heart compared to controls. (O and P) Scale bars: 1 mm.

**Figure S3** Proliferation was not altered in Snx5−/− mice. (A) Ki67 and Hoechst double staining of Snx5+/− and Snx5−/− lungs at E18.5. Scale bars: 50 μm. (B) Graph of Ki67-positive cell counts showed similar proliferation rates in Snx5+/− and Snx5−/− lungs. Ki67-positive cells were counted in 3 random 400× microscope fields. Means ± SDs were determined using 4 Snx5+/− embryos and 7 Snx5−/− embryos in each group.

**Figure S4** Mature SP-B and lung Sat PC content were not decreased in Snx5−/− mice. (A) Relative density of SP-B expression in isolated control and Snx5−/− lungs. (B) Graph shows similar SP-B expression levels in Snx5+/− and Snx5−/− lungs. Western blotting analysis of mature SP-B in 15 lung homogenate supernatant (n = 5). Supernatant was recovered and processed using RIPA buffer. (C) Western blot analysis of Sat PC content and Sat PC to body weight levels in Snx5+/−, Sat PC−/−, and Sat PC−/− mice. Scale bars: 0.5 mm, (G–J) normal morphological structure of Snx5+/−, (TIF) Scale bars: 1 mm.

**Figure S5** Ultrastructure of alveolar epithelial type I cells in Snx5−/− lungs. (A–D) Transmission electron microscopy (TEM) was used to determine ultrastructural morphology of the alveolar epithelial type I cells in Snx5−/− and Snx5+/− lungs at E18.5. (A and B) Vesicle (Vv; black arrow) and mitochondria (Mt; white arrowhead) were observed in Snx5+/− (A) and Snx5−/− (B) lungs. Scale bars: 2 μm. (C and D) Extended cytoplasm was also observed in Snx5+/− (C) and Snx5−/− (D) mice. Scale bars: 400 nm.

**Figure S6** Identification of primary lung epithelial cell (PLE) characteristics. (A) Western blot analysis of lysates (10 μg) from MEFs and isolated PLEs. Isolated PLEs exhibited E-cadherin but not vimentin expression. (B) Mean relative log_{10} mRNA expression for vimentin, E-cadherin, SP-C, and T1α from MEFs and PLEs using qRT-PCR. Graphs revealed dramatic increases in E-cadherin and SP-C expression in isolated PLEs. Also, T1α was expressed at low levels in PLEs compared to the
negative control, MEFs. (C) Bright-field and fluorescence images of PLEs. Bright-field images showed a type II epithelial cell-like morphology. Fluorescence images revealed partial TlZ and strong proSP-C expression. Scale bars: 100 μm. (TIF)

Figure S7 EGFR expression in E18.5 Snx5+/– lungs was not altered. (A) Western blotting of EGFR protein in whole-lung lysates from E18.5 Snx5+/– and Snx5–/– mice (n = 10 each). (B) Densitometric analysis showed that the ratio of EGFR to β-tubulin was similar between E18.5 Snx5+/– and Snx5–/– lungs. (TIF)

Table S1 Biological processes enriched with up or down-regulated DEGs of E18.5 Snx5+/– lungs. Genome-wide analysis of E18.5 Snx5+/– lungs showed the biological processes enriched with up or down-regulated DEGs. (TIF)

Table S2 Used primers for qRT-PCR in lungs. The qRT-PCR primers for analysis of changed mRNA expression levels in Snx5+/– compared to Snx5–/– lung. (TIF)

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Author Contributions
Conceived and designed the experiments: SKI YYK. Performed the experiments: SKI HBJ HWJ MI. Analyzed the data: SKI HBJ HWJ DH MI YYK. Contributed reagents/materials/analysis tools: SKI HBJ DH MI YYK. Wrote the paper: SKI KTK YYK.

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