

Sex-determining region Y-box 2-positive alveolar cells are responsive to bleomycin-induced lung injury

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As an important barrier, the pulmonary alveolar epithelium plays an important role in lung gas exchange and host defense. The alveolar epithelium is mainly comprised of alveolar type I (AT1) and type II (AT2) cells that are tightly connected to maintain the integrity of the alveolar epithelium. There are several kinds of stem/progenitor cells capable of repairing the adult alveolar epithelium in response to various injuries. Kim and colleagues identified bronchioalveolar stem cells (BASCs) that are capable of differentiating into alveolar cells and airway cells.^[1] In addition, a subset of alveolar cells expressing the laminin receptor $\alpha 6\beta 4$ but not surfactant protein C (SPC) was able to regenerate into AT2 cells upon bleomycin-induced lung injury.^[2] Rare keratin 5-positive basal cells give rise to alveolar epithelial lineages after influenza infection.^[3,4] Sex-determining region Y-box 2 (Sox2) is a transcription factor that maintains and regulates self-renewal and pluripotency in embryonic stem cells.^[5] The functional roles of Sox2 in the repair of alveolar epithelium injuries remain unknown. This study aimed to investigate whether the alveolar cells expressing Sox2 are involved in alveolar epithelial repair.

Mouse lung cells were isolated by elastase digestion and stained for fluorescence-activated cell sorting (FACS) from a transgenic surfactant protein C (*SFTPC*)-green fluorescent protein (GFP) reporter mice line to analyze SPC expression in alveolar cells. Flow cytometry was performed with primary antibodies against CD31-biotin, CD34-biotin, CD45-biotin, CD24-phycoerythrin (PE), anti-epithelial cellular adhesion molecule-PE-cyanine 7, and Sca-1-allophycocyanin. The secondary antibody was against strepta-

vidin. All the antibodies were purchased from eBioscience (San Diego, CA, USA). 7-amino-actinomycin D was added to label the dead cells. Flow cytometry was performed using an FACS Aria III sorter (BD Immunocytometry Systems, San Jose, CA, USA). LightCycler real-time polymerase chain reaction (PCR) was performed to measure gene expression (β -actin-F: 5'-GGCCAACCGTGAAAAGATGA-3'; β -actin-R: 5'-CAGCCTGGATGGCTACGTACA-3'; Sox2-F: 5'-CACATGAAGGAGCACCCGGATTAT-3'; Sox2-R: 5'-CGGGAAGCGTGTACTTATCCTTCT-3'). The relative messenger RNA (mRNA) expression level of the target genes was calculated by the comparative Ct (threshold cycle) method, normalized to β -actin mRNA in the same sample. Specific Δ Ct was calculated as follows: Δ Ct = (Ct _{β -actin}) - (Ct_{target}); relative expression was defined as $2^{-\Delta$ Ct}. The Sox2-GFP reporter transgenic mice and immunofluorescence analysis were adopted to detect Sox2-positive cells location. Primary antibodies used in the study included chicken polyclonal anti-GFP antibody (Abcam, Cambridge, MA, USA), Sox2 (Invitrogen, Carlsbad, CA, USA), and pro-SPC (Millipore Corp, Billerica, MA, USA). Secondary antibodies included Rabbit anti-Chicken immunoglobulin of yolk (Ig Y) (immunoglobulin G)-fluorescein isothiocyanate (Sigma, St. Louis, MO, USA), Donkey anti-Rat Alexa Fluor 488 (Invitrogen), and Donkey anti-Rabbit Alexa Fluor 594 (Invitrogen). Slides were mounted with Fluoromount G containing 4',6-diamidino-2-phenylindole (Southern Biotech, Birmingham, AL, USA). Stained sections were imaged using a Zeiss LSM710 confocal microscope (Carl Zeiss, Jena, Thuringen, Germany).

We next determined whether Sox2-positive alveolar cells are involved in alveolar epithelial repair using bleomycin-

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induced lung injury model. The mice were anesthetized and then intratracheally injected with 2.5 U/kg bleomycin (Nippon Kayaku Co. Ltd., Tokyo, Japan) with a 25-gauge needle inserted between the cartilaginous rings of the trachea. The control animals received phosphate-buffered saline alone. At designated time points after bleomycin injection, the mice were euthanized, and their lung tissues were harvested for immunofluorescence staining or flow cytometry analysis. All the mice were treated with strict adherence to the protocol (No. 2015HHLL06) approved by the Haihe Hospital Animal Care and Use Committee. Statistical analysis was performed using the SPSS 17.0

software (SPSS Inc., Chicago, IL, USA). The measurement data conforming to the normal distribution were expressed as mean ± standard deviation. The comparison between two groups was performed using Student's *t* test and *P* < 0.05 indicates that the difference is statistically significant.

We took advantage of a transgenic *SFTPC*-GFP reporter line mice to analyze SPC expression in alveolar cells by an FACS-based strategy. Dot plot analysis of the alveolar cell population revealed that there were SPC^{hi} (AT2 cells expressing high level of SPC) and SPC^{low} cell (alveolar cells

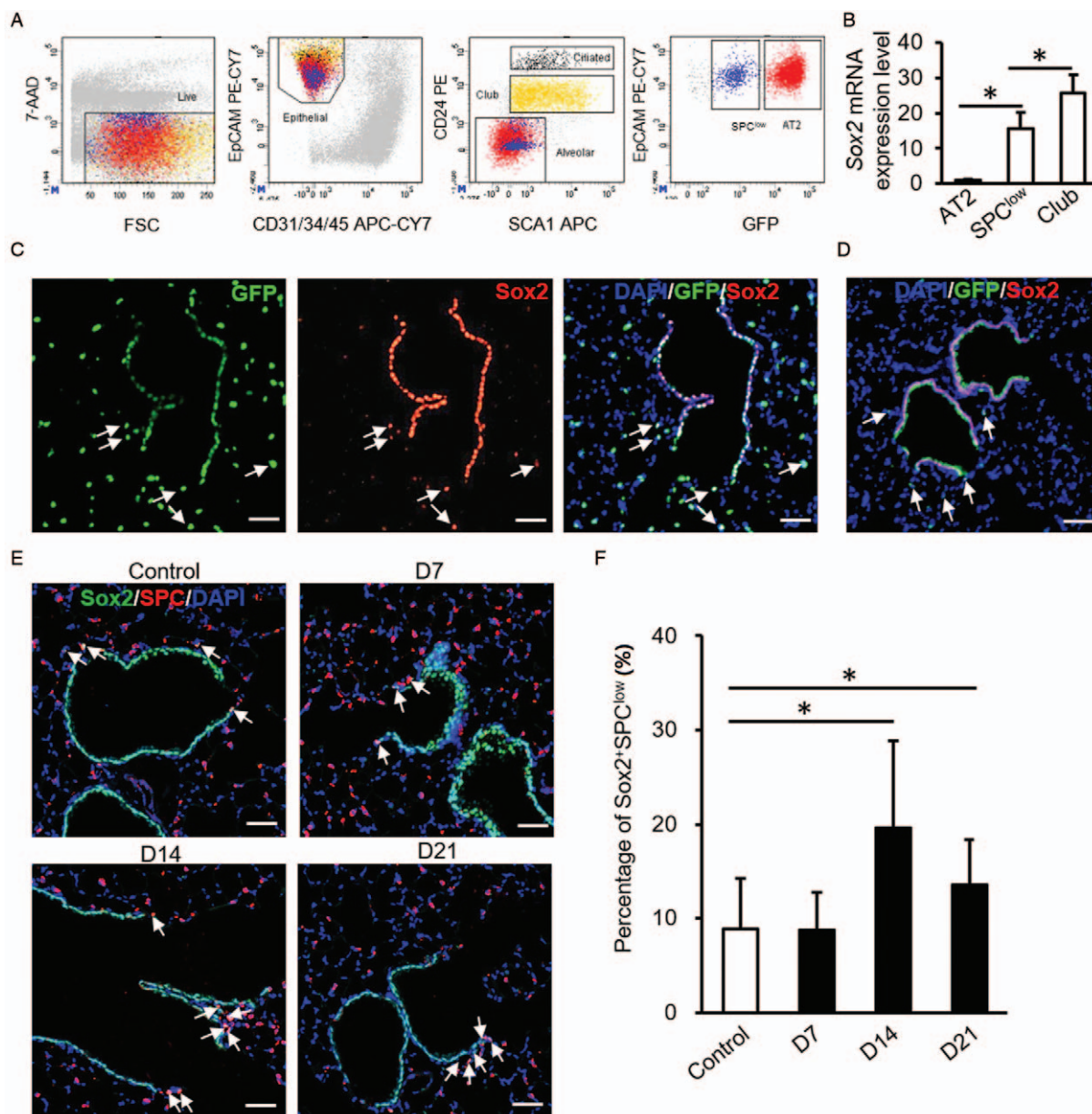


Figure 1: Expression of Sox2⁺SPC^{low} alveolar cells during bleomycin-induced lung injury. (A) Segregation of lung alveolar epithelial cells based on *SFTPC*-GFP expression. (B) *Sox2* mRNA expression in SPC^{low} alveolar, AT2, and Club cells. (C) Immunolocalization of GFP (green) and Sox2 (red) in lung tissues of *SFTPC*-GFP mice. (D) Immunostaining of GFP (green) and Sox2 (red) in lung tissues of *Sox2*-GFP mice. (E) Immunostaining of lung tissues from C57BL/6 mice for Sox2 (green) and SPC (red) with or without bleomycin treatment. (F) Quantification of Sox2⁺SPC^{low} alveolar cells. **P* < 0.05. Scale bar represents 20 μm. 7-AAD: 7-Amino-actinomycin D; APC: Allophycocyanin; AT2: Alveolar type II cell; CD31/CD34/CD45: CD31-biotin/CD34-biotin/CD45-biotin; CD24 PE: CD24-phycoerythrin; CY: Cyanine 7; DAPI: 4',6-diamidino-2-phenylindole; EpCAM PE-CY7: Epithelial cellular adhesion molecule phycoerythrin-CY7; FSC: Forward scatter; GFP: Green fluorescent protein; mRNA: Messenger RNA; SCA1 APC: Sca1 allophycocyanin; *SFTPC*: Surfactant protein C; Sox2: Sex-determining region Y-box 2; SPC: Surfactant protein C; SPC^{low}: Alveolar cells expressing low level of SPC.

expressing low level of SPC) subsets with different GFP fluorescence intensities [Figure 1A]. Quantitative real-time PCR analysis of these fractionated lung epithelial cells indicated that the level of *Sox2* mRNA was lower in SPC^{low} alveolar cells than that in Club cells (15.570 ± 3.181 vs. 25.675 ± 5.145 , $t = 7.903$, $P = 0.015$), but much higher than that in AT2 cells (15.570 ± 3.181 vs. 1.000 ± 0.274 , $t = 2.894$, $P = 0.044$) [Figure 1B]. Immunofluorescence analysis of lung tissues from *SFTPC*-GFP mice for GFP and *Sox2* expression revealed that the SPC^{low} alveolar cells were *Sox2*-positive and localized in the alveolar region [Figure 1C]. The *Sox2*-GFP reporter transgenic mice were then adopted, and it was validated that *Sox2*-positive cells exist within the alveolar space [Figure 1D].

Lung stem/progenitor cells normally survive to repair tissues after injuries. Next, we determined whether *Sox2*-positive alveolar cells are involved in alveolar epithelial injuries. Bleomycin was administered to mice by intratracheal instillation. We observed that *Sox2*⁺SPC^{low} cells resided within the alveolar space near the bronchioalveolar duct junction (BADJ) [Figure 1E]. Immunofluorescence staining of lung sections indicated that the percentage of *Sox2*⁺SPC^{low} cells in total SPC⁺ alveolar cells was increased on days 14 ($[19.58 \pm 9.23]\%$ vs. $[8.90 \pm 5.36]\%$, $t = 3.164$, $P = 0.005$) and 21 ($[13.55 \pm 4.78]\%$ vs. $[8.90 \pm 5.36]\%$, $t = 1.918$, $P = 0.036$) after bleomycin-induced injury [Figure 1F].

In our study, we identified an alveolar cell population expressing a low level of SPC in mouse lung tissue. The SPC^{low} alveolar cells express *Sox2* at a lower level than the airway progenitor Club cells. Immunofluorescent staining of lungs from *Sox2*-GFP mice confirmed that *Sox2*-expressing cells were present in the alveolar space. Importantly, we showed that *Sox2*⁺SPC^{low} alveolar cells were responsive to bleomycin-induced lung injury. It seemed that abundance of these cells was topped at day 14 after bleomycin, suggesting that alveolar repair became significant after this time. The *Sox2*⁺SPC^{low} alveolar cell population observed in this study seems to be different from epithelial stem/progenitor cells found previously to repair alveolar epithelium. The BASCs are present in BADJ and express clara cell secretory protein and SPC.^[1] *Sox2*⁺SPC^{low} alveolar cells were found to reside within the alveolar space near the BADJ. Alveolar laminin $\alpha 6\beta 4$ -positive epithelial cells with regenerative potential do not express SPC.^[2]

The molecular basis for the function of epithelial stem/progenitor cells in the lung remains largely unknown. We showed that the level of *Sox2* expression differs among AT2, *Sox2*⁺SPC^{low}, and Club cells. *Sox2* is a transcription factor expressed exclusively in tracheal epithelium and conducting airway epithelium in lung. The functional roles for *Sox2* in the regulation of alveolar *Sox2*⁺SPC^{low} cells remain unknown. *Sox2*⁺SPC^{low} alveolar cells are responsive to bleomycin-induced lung injury, but how these cells contribute to alveolar epithelial regeneration needs to be studied in the future.

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Conflicts of interest

None.

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