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SHORT COMMUNICATION

# Lung tissue regeneration after induced injury in Runx3 KO mice

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**Abstract** Runx3 is essential for normal murine lung development, and Runx3 knockout (KO) mice, which die soon after birth, exhibit alveolar hyperplasia. Wound healing, tissue repair, and regeneration mechanisms are necessary in humans for proper early lung development. Previous studies have reported that various signaling molecules, such as pErk, Tgf-ß1, CCSP, pJnk, Smad3, and HSP70 are closely related to wound healing. In order to confirm the relationship between lung defects caused by the loss of function of Runx3 and wound healing, we have localized various wound-healing markers after laser irradiation in wild-type and in Runx3 KO mouse lungs at post-natal day 1. Our results indicate that pERK, Tgf-β1, CCSP, pJnk, and HSP70 are dramatically down-regulated by loss of Runx3 during lung wound healing. However, Smad3 is up-regulated in the Runx3 KO laser-irradiated lung region. Therefore, the lung wound-healing mechanism is inhibited in the Runx3 KO mouse, which shows abnormal lung architecture, by reduced pErk, Tgf-β1, CCSP, pJnk, and HSP70 and by induced Smad3.

**Keywords** Runx3 KO · Wound healing · PErk · Tgf-β1 · CCSP · PJnk · Smad3 · HSP70 · Mouse (Runx3 knockout)

## Introduction

Mammalian lungs are branched networks containing thousands to millions of airways arrayed in intricate patterns that are crucial for respiration (Metzger et al. 2008). Airway branching, airway size control, airway cell differentiation, alveolus formation, and pulmonary blood vessel patterning are required to build a lung (Hislop 2002). For proper lung maturation, remodeling is needed during lung development (Ravikumar et al. 2009). Moreover, lung disease such as idiopathic pulmonary fibrosis is caused by a disorder of abnormal alveolar wound repair and remodeling (Selman et al. 2001).

Previous studies have reported that various signaling molecules such as pErk, transforming growth factor-beta 1 (Tgf-β1), Clara cell secretory protein (CCSP), pJnk, Smad3, and heat shock protein 70 (HSP70) are closely related to wound healing (Ashcroft et al. 1999; Rämet et al. 2002; Reynolds et al. 2005; Mushtaq et al. 2007, 2009; Teranishi et al. 2009; Xing et al. 2010; Zhou et al. 1996). Phosphorylated ERK1/2 was detected in human corneal epithelial cells at the wound margin during wound healing (Teranishi et al. 2009). Tgf-β1 is a major regulator of re-epithelialization and wound closure during wound healing (Reynolds et al. 2005). Clara cells are

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known as progenitor or stem cells during regeneration after injury, and lung epithelial cell differentiation is closely related to CCSP (Xing et al. 2010; Zhou et al. 1996). Jun kinase is phosphorylated in wounded epidermal tissues, suggesting that a signal mediated by epidermal wounding activates the JUN N-terminal kinase signaling pathway (Rämet et al. 2002). Mice lacking Smad3 show accelerated wound healing (Ashcroft et al. 1999). In addition, HSP70 plays a possible role during corneal epithelial wound repair (Mushtaq et al. 2007, 2009).

Mammalian RUNX3, a member of the runt-domain family of transcription factors, regulates gene expression in several important developmental pathways (Levanon et al. 2003). In mammals, the RUNX family of genes includes *RUNX1*, *RUNX2*, and *RUNX3* (Eggers et al. 2002; Kalev-Zylinska et al. 2002; Levanon et al. 2003). RUNX1 and RUNX2 play pivotal roles in hematopoiesis, osteoblast differentiation, and bone ossification (Okuda et al. 1996; Otto et al. 1997), whereas RUNX3 is closely involved in neurogenesis (Inoue et al. 2002; Levanon et al. 2002) and thymopoiesis (Taniuchi et al. 2002; Woolf et al. 2003) and functions as a tumor suppressor (Bae and Lee 2006; Chi et al. 2009; Guo et al. 2002; Ito et al. 2008; Kim et al. 2005; Li et al. 2002). Runx3 is essential for normal murine lung development and Runx3 knockout (KO) mice, which die soon after birth, exhibit alveolar hyperplasia (Lee et al. 2010). Moreover, the intra-alveolar septum structure is not observed in the Runx3 KO mouse (Lee et al. 2010). In order to understand the relationship between wound healing and the lung defect in Runx3 KO mice, we have examined the localization of various wound-healing markers after physical injury by laser. In the Runx3 KO laser-irradiated lung, the localization of pERK, Tgf-β1, CCSP, pJnk, Smad3, and HSP70 dramatically changes compared with that of wild-type (WT). pERK, Tgf-β1, CCSP, pJnk, and HSP70 are down-regulated, whereas Smad3 is up-regulated in the Runx3 KO lung. Thus, Runx3 might play a crucial role during lung wound healing. Our results provide comprehensive evidence that signaling via Runx3 is of importance during proper lung development.

## Materials and methods

All experiments were performed according to the guidelines of the Yonsei University College of Dentistry, Intramural Animal Use and Care Committee.

### Runx3 KO mice

The generation of Runx3 heterozygous C57BL/6 mice was as described earlier (Li et al. 2002). Runx3 heterozygous FVB mice were generated by interbreeding with Runx3 heterozygous C57BL/6 mice, followed by backcrossing with FVB mice for >10 generations. The animals were maintained under pathogen-free conditions and monitored daily (Lee et al. 2010). In this experiment, lungs were extracted from WT and Runx3 KO mice at post-natal day 1 (PN1).

### In vitro organ culture after physical injury by laser

A $CO_2$ laser system (Panalas CO5; Panasonic Dental, Japan) was used at a continuous wavelength of 1,064 nm. After dissection of the lungs, only the left lobes were irradiated with a single beam of laser light for 2 s at an average fluence rate of 208 W/cm$^2$. They were then cultured in DMEM/F12 containing 10% fetal bovine serum for 48 h according to the method described previously (Parrish et al. 1995). Laser-irradiated left lobes were cultivated in a 50-ml penicillin bottle containing 10 ml filter-sterilized culture medium. One laser-irradiated left lobe was placed into one bottle, and the bottle was sealed airtight with a rubber stopper and a metal clamp. The bottles were flushed for approximately 2 min with a gas mixture of 50% $O_2$, 45% $N_2$, and 5% $CO_2$. The bottles were incubated at 37°C on a roller device (20 rpm) for 48 h. The culture bottles were flushed every 24 h with the same gas mixture. Each experiment was carried out at least 10 times.

### Immunohistochemistry

Samples were fixed with 4% (w/v) paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline (PBS, pH 7.4), sectioned at 7 μm, and stained with hematoxylin-eosin. For immunohistochemistry, sections were blocked in 0.3% hydrogen peroxide for 15 min. The tissue sections were boiled in 10 mM citrate buffer (pH 6.0) for 20 min and cooled at room temperature for 20 min. The slides were incubated with primary antibody, viz., with antibodies against Phospho-p44/42 Map Kinase (dilution, 1:50; cat. no. 9101S; Cell Signaling, USA), Tgf-β1 (dilution, 1:50; cat. no. sc-146; Santa Cruz, USA), Clara cell secretory protein (dilution, 1:2000; cat. no. 07-623; Millipore, USA), Phospho-SAPK/JNK (dilution, 1:100; cat. no. 9251; Cell Signaling), Smad3 (dilution, 1:50; cat. no. ab51177; Abcam, USA), or HSP70 (dilution, 1:50; cat. no. 4876; Cell Signaling), at 4°C overnight. After being washed with PBS, the specimens were incubated with secondary antibody and streptavidin peroxidase at room temperature for 10 min each. Finally, the specimens were visualized by using a diaminobenzidine reagent kit (Invitrogen, USA). The immunostained sections were counterstained with hematoxylin.

## Results and discussion

Runx3 KO mice show alveolar hyperplasia at PN1 (Lee et al. 2010). The intra-alveolar septum structure and well-expanded alveoli have been observed in WT mice (Fig. 1a). However, alveoli are not expanded, and the intra-alveolar septum structure is not apparent in Runx3 KO mice (54/54; Fig. 1b). We hypothesize that an aberrant wound-healing mechanism induces abnormal lung structure in the Runx3 KO mouse.

Previous studies have reported that pErk and Tgf-β1 play a crucial role during normal development including the

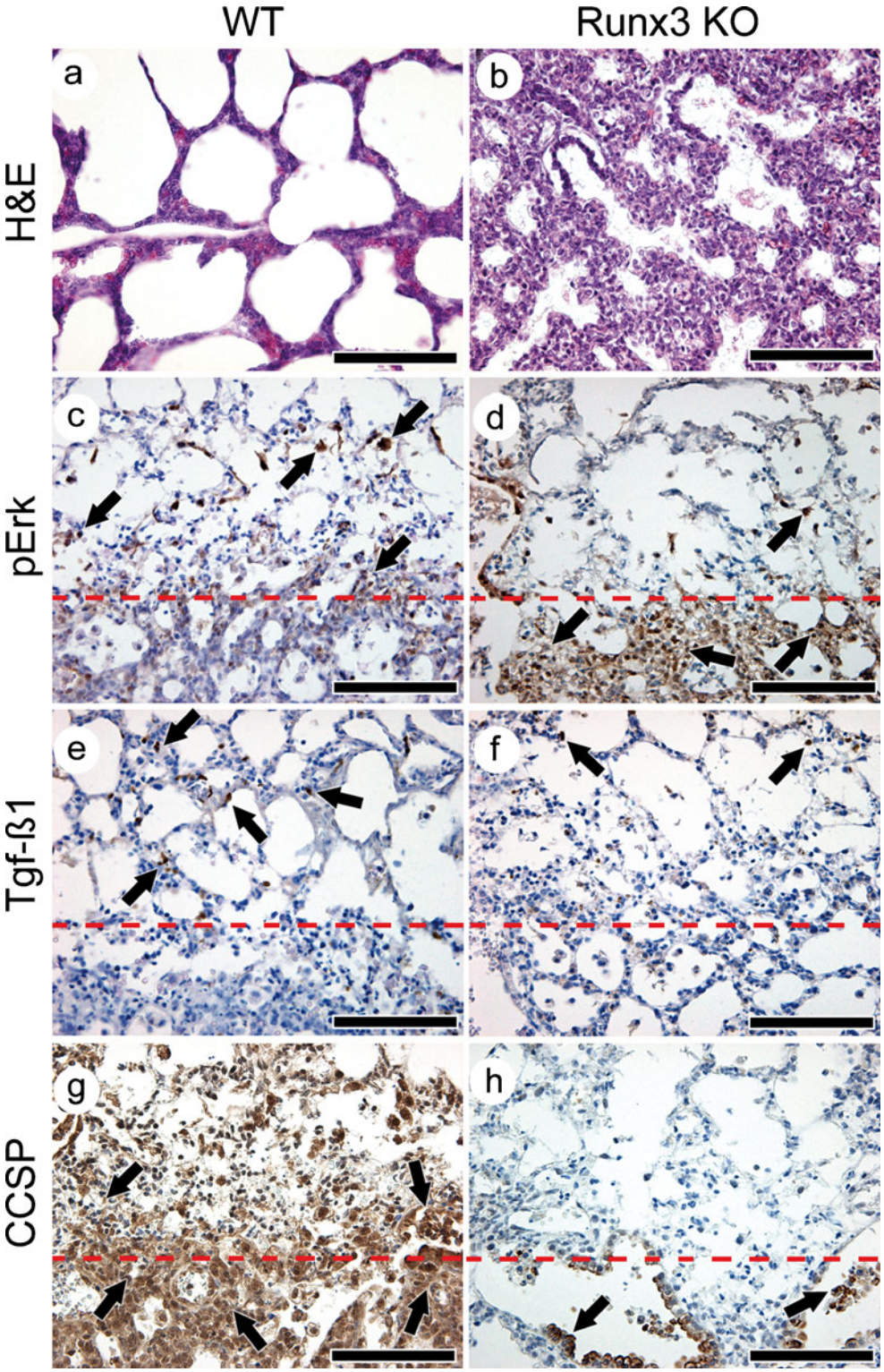


**Fig. 1** General morphology and localization of pErk and Tgf-β1 in wild-type (*WT*) and Runx3 knockout (*Runx3 KO*) lung after physical injury by laser irradiation (*red dotted line* wound margin). **a** Well-expanded alveoli and the intra-alveolar septum structure can be seen in a WT mouse (*H&E* hematoxylin and eosin staining). **b** Non-expanded alveoli are present in a Runx3 KO mouse. **c** In WT, pErk-positive cells are localized in the wounded lung region (*arrows*). **d** A small number of pErk-positive cells are observed in the laser-irradiated lung area, whereas pErk-positive cells are strongly detected in the non-wounded lung region in Runx3 KO (*arrows*). **e** Tgf-β1-stained cells are localized in the wounded region in WT (*arrows*). **f** Few Tgf-β1-positive cells (*arrows*) are observed in the Runx3 KO wounded lung compared with those in WT. **g** Cells that are strongly CCSP-positive (*arrows*) are observed in WT. **h** In the Runx3 KO lung, CCSP is detected in the bronchiole region but not around the laser-irradiated area. *Bars* 100 μm

wound-healing mechanism (Reynolds et al. 2005; Teranishi et al. 2009). Moreover, CCSP is also closely related to lung regeneration (Xing et al. 2010; Zhou et al. 1996). In order to clarify the relationship between pErk and the lung defect in the Runx3 KO mouse, pErk expression was examined after physical injury by laser. pErk-positive cells were observed in the laser-wounded region in WT mice (Fig. 1c). However, in the Runx3 KO lung tissue, pErk was strongly detected in non-injured lung regions beneath the wound margin and was only weakly detected in the wounded area (Fig. 1d). A previous study has reported that the Runx3 KO mouse lung shows excessive cell proliferation (Lee et al.

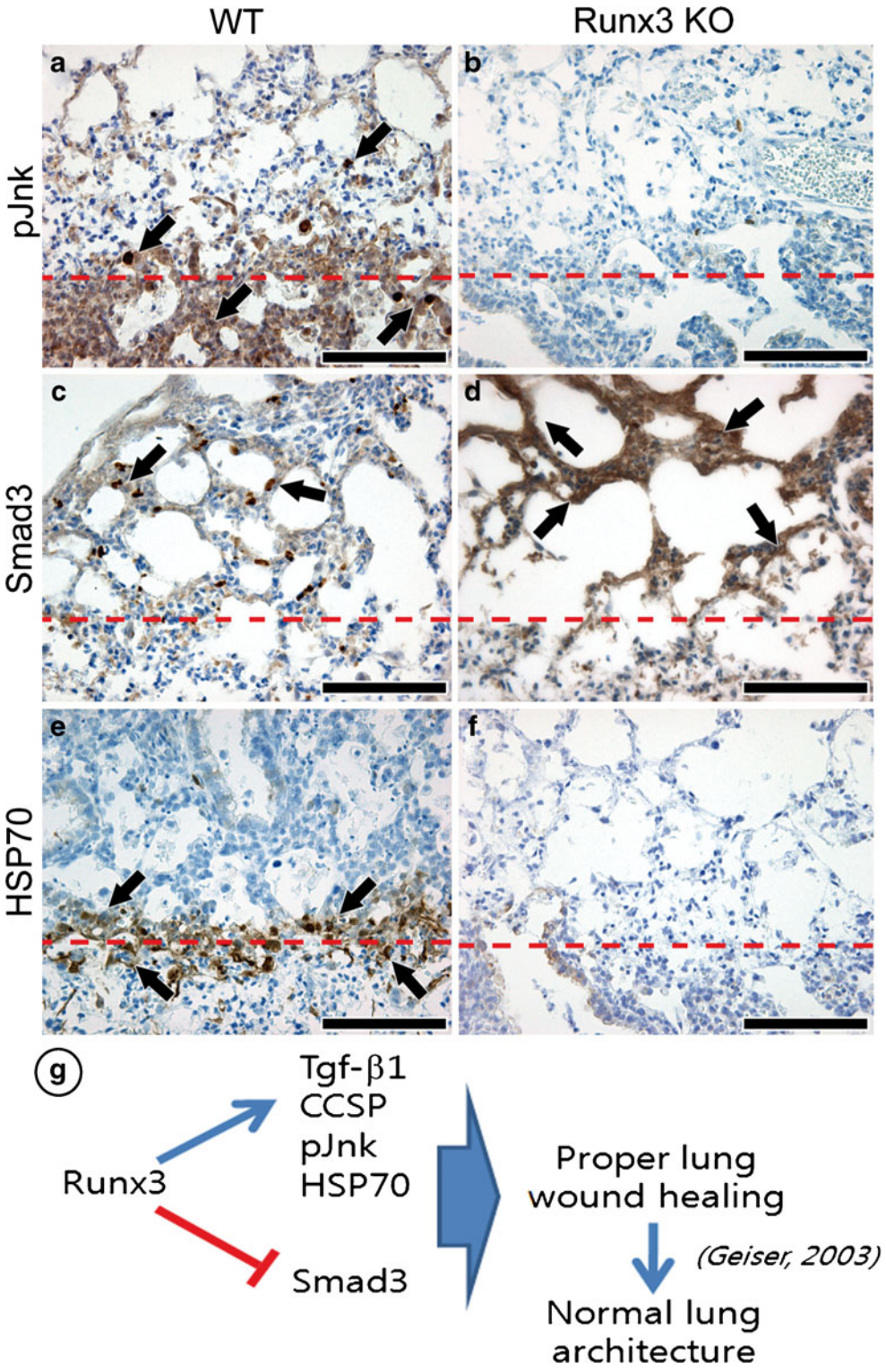


**Fig. 2** Localization of pJnk, Smad, and HSP70 in the WT and Runx KO lungs after laser irradiation (*red dotted line* wound margin). **a** In WT, pJnk-positive cells are detected around the wounded region (*arrows*). **b** pJnk-stained cells are not observed in the Runx3 KO laser-wounded lung. **c** A small number of Smad3-positive cells are localized in the laser-wounded region in WT (*arrows*). **d** Strong Smad3 is detected in the laser-irradiated Runx3 KO lung (*arrows*). **e** In WT, HSP70-positive cells are observed along the wound margin (*arrows*). **f** HSP70-positive cells are not detected in the Runx3 KO wounded lung. **g** Representation of Runx3-mediated lung wound healing, according to Geiser (2003). *Bars* 100 μm

2010). The ERK pathway is one of the most important regulators of cell proliferation (Chang and Karin 2001; Sandra et al. 2004). On the basis of this result, excessive cell proliferation in the Runx3 KO mouse lung might therefore be closely related to the high pErk level.

Tgf-β1-positive cells were localized in the WT laser-irradiated lung (Fig. 1e), whereas they were faintly observed in the Runx3 KO laser-wounded lung (Fig. 1f). CCSP-positive cells were strongly detected both in the laser-irradiated region and beneath the wound margin in WT mice (Fig. 1g). However, in the Runx3 KO lung tissue, CCSP was observed only in the bronchiole (Fig. 1h). In WT, pErk and Tgf-β1 were detectable around the laser-irradiated region but not beneath the wound margin, whereas they were weakly or similarly observed both in the laser-wounded area and in the non-wounded lung region in Runx3 KO mice (Fig. 1c–f). CCSP was strongly up-regulated in WT after laser irradiation but not in Runx3 KO mice (Fig. 1g, h). These results indicate that abnormal wound healing induced by the deletion of Runx3 might be one of the key factors that leads to lung anomaly in Runx3 KO.

The JNK signaling pathway is required for efficient wound healing (Rämet et al. 2002). In order to confirm the efficiency of wound healing in Runx3 KO, the localization of pJnk was examined in both WT and Runx3 KO mice (Fig. 2a, b). In WT, pJnk was strongly detected around the laser-irradiated region and wound margin (Fig. 2a). However, pJnk-positive cells were not observed around the wounded region in the Runx3 KO laser-irradiated region (Fig. 2b). A previous study had revealed that TGF-β negatively regulated Smad3 expression and contributed to the resolution of the inflammatory process during wound healing. Moreover, Smad3 null mice were found to show accelerated cutaneous wound healing characterized by an increased rate of re-epithelialization (Ashcroft and Roberts 2000). To confirm the relationship between Runx3 and Smad3, the localization of Smad3 was examined in WT and Runx3 KO lungs after laser irradiation. In WT, a small number of Smad3-positive cells were observed in the laser-wounded region (Fig. 2c). However, a strong level of Smad3 was detected in the Runx3 KO laser-irradiated lung area (Fig. 2d). These data indicate that Runx3 negatively regulates Smad3 directly or indirectly during lung wound healing. The increased protein expression of HSP70 detected during the active phase of cell migration in the corneal epithelium suggests its possible role in wound healing (Mushtaq et al. 2007, 2009). Moreover, HSP70 plays an important role in gastric wound healing (Odashima et al. 2007). To examine the relationship between HSP70 and Runx3, we performed immunohistochemistry in WT and Runx3 KO lungs after physical injury by laser (Fig. 2e, f). In WT, HSP70 was strongly observed along the wound margin (Fig. 2e). However, in the Runx3 KO lung, HSP70 was not detected around the laser-wounded region (Fig. 2f). These results suggest that Runx3 might be a crucial regulator of wound healing in the lung.

Our results suggest that pERK, Tgf-β1, CCSP, pJnk, and HSP70 are positively regulated by Runx3 during normal lung wound healing. pERK, Tgf-β1, CCSP, pJnk, and HSP70 are down-regulated in the Runx3 KO lung. Smad3, however, is induced in the Runx3 KO laser-irradiated lung region. These data indicate that pErk, Tgf-β1, CCSP, pJnk, Smad3, and HSP70 have lost their wound-healing functions during lung development in Runx3 KO.

In conclusion, lung wound healing is inhibited in the Runx3 KO mouse, which shows abnormal lung architecture, by reduced pErk, Tgf-β1, CCSP, pJnk, and HSP70 and induced Smad3 (Fig. 2g). These fundamental data suggest that Runx3 is necessary for lung wound healing to ensure proper early lung development. Further studies with human lung cancer cells will be needed to understand the relationship between the human lung wound-healing mechanism and Runx3.

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