Developmental Biology 382 (2013) 110–123

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Abrogation of Eya1/Six1 disrupts the saccular phase of lung morphogenesis and causes remodeling

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ARTICLE INFO

Article history:
Received 12 March 2013
Received in revised form
27 June 2013
Accepted 22 July 2013
Available online 26 July 2013

Keywords:
Eya1
Six1
SHH
Lung development
Saccular phase

ABSTRACT

The Eya1 gene encodes a transcriptional co-activator that acts with Six1 to control the development of different organs. However, Six1-Eya1 interactions and functional roles in mesenchymal cell proliferation and differentiation as well as alveolarization during the saccular stage of lung development are still unknown. Herein, we provide the first evidence that *Six1* and *Eya1* act together to regulate mesenchymal development as well as alveolarization during the saccular phase of lung morphogenesis. Deletion of either or both *Six1* and *Eya1* genes results in a severe saccular phenotype, including defects of mesenchymal cell development and remodeling of the distal lung septae and arteries. Mutant lung histology at the saccular phase shows mesenchymal and saccular wall thickening, and abnormal proliferation of α-smooth muscle actin-positive cells, as well as increased mesenchymal/fibroblast cell differentiation, which become more sever when deleting both genes. Our study indicates that SHH but not TGF-β signaling pathway is a central mediator for the histologic alterations described in the saccular phenotype of $Eya1^{-/-}$ or $Six1^{-/-}$ lungs. Indeed, genetic reduction of SHH activity in vivo or inhibition of its activity in vitro substantially rescues lung mesenchymal and alveolar phenotype of mutant mice at the saccular phase. These findings uncover novel functions for Six1–Eya1–SHH pathway during the saccular phase of lung morphogenesis, providing a conceptual framework for future mechanistic and translational studies in this area.

Introduction

Lung development is a conserved process and well studied in mice, which represent an ideal model for investigating mechanisms controlling lung morphogenesis and congenital respiratory diseases in humans. The development of murine lung starts at embryonic day (E) 9.5, and is divided histologically into pseudoglandular stage (E9.5–E16.5), canalicular stage (E16.6–E17.4), saccular stage (E17.5—post-natal day (P5) and alveolar stage (P5–P30) (Warburton et al., 2010).

Mammalian lung development begins when two primary buds, each consisting of three cell layers: the inner epithelium, the surrounding mesenchymal stroma, and a thin outer mesothelium, arise from the laryngotracheal groove in the ventral foregut. The cellular composition of the pulmonary mesenchyme is complex and involves the distinct patterning of different cell types. Early distal lung mesenchyme differentiates to smooth muscle, cartilages, endothelial cells of blood vessels and pericytes (deMello et al., 1997; Partanen et al., 1996). Postnatally, the distal mesoderm gives rise to myofibroblasts in the tips of the alveolar septae (Bostrom et al., 1996; Kapanchi and Gabbiani, 1997). Other cell types, including stromal fibroblasts, interstitial lipofibroblasts, and lymphatic vessels are also found in the lung (McGowan and Torday, 1997). Yet little is known about the functional roles of transcription factors in mesodermal lung development.

In mammals, Eya1-4 and homeobox sine oculis (Six) transcription factor gene families are co-expressed and regulate the development of several organs (Xu et al., 1997a, 1997b; Ford et al., 1998; Coletta et al., 2004). $Eya1^{-/-}$ or $Six1^{-/-}$ mouse embryos have defects in the proliferation and survival of the precursor cells of multiple organs, and die at birth (Xu et al., 1999, 2002; Li et al., 2003; Zou et al., 2004). Haploinsufficiency for human EYA1 or SIX1 leads to branchio-oto-renal syndrome (Abdelhak et al., 1997; Ruf et al., 2004). *Eya1* and *Six1* gene products participate in protein-protein interactions (Buller et al.,

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http://dx.doi.org/10.1016/j.ydbio.2013.07.019

2001). We have recently shown that genetic deletion of either *Eya1* or *Six1* gene in mice results in severe lung hypoplasia, which is characterized by a severe reduction of epithelial branching, and a marked increase of both mesenchymal cellularity and SHH signaling activity (El-Hashash et al., 2011a, b). Yet the synergistic regulation of mesenchymal and epithelial cell proliferation/differentiation, as well as SHH signaling activity by *Six1* and *Eya1* genes during lung morphogenesis is unknown.

Herein, we tested the involvement of Six1 and Eya1 transcription factors, together with SHH signaling pathway in the control of mesenchymal cell proliferation/differentiation as well as alveolarization during saccular lung development. Our results from loss- and gain-of-function analyses indicate that *Eya1* and *Six1* genes act together to regulate mesenchymal and epithelial cell proliferation/differentiation in the developing lung. Eya1 and Six1 probably function together to regulate saccular lung development by controlling SHH signaling activity levels.

Materials and methods

Animals

Eya1$^{-/-}$, Six1$^{-/-}$ and *Shh*$^{f/f}$, as well as *Spc-rtTA*$^{+/-}$-transgenic and tet(O)CMV-Cre-transgenic mice, and their genotyping have been published (Xu et al., 1999, 2002; Perl et al., 2002; Laclef et al., 2003a, b; Bassères et al., 2006). *Eya1;Six1* compound homozygous or heterozygous mice were generated by crossing mice carrying mutant alleles of *Eya1* and *Six1*. Conditional *Eya1*$^{-/-}$; *Spc-rtTA*$^{+/-}$*-tet(o) Cre*$^{+/-}$*-Shh*$^{\Delta/WT}$ (hereafter known as *Eya1*$^{-/-}$*-Shh*$^{\Delta/WT}$) mutant mice were generated as described (El-Hashash et al., 2011a).

Six1$^{+/-}$*;Shh*$^{f/WT}$ female mice were generated by intercrossing *Six1*$^{+/-}$ mice with the *Shh*$^{f/f}$ mouse line. *Six1*$^{+/-}$*-Spc-rtTA*$^{+/-}$*-tet*(*o*) *Cre*$^{+/-}$ mice were generated by intercrossing *Six1*$^{+/-}$ mice with the *Spc-rtta*$^{+/-}$*-tet(o)Cre*$^{+}$/$^{+}$ mouse line previously generated in our laboratory. The resulting *Six1*$^{+/-}$*-Spc-rtTA*$^{+/-}$*-tet(o)Cre*$^{+/-}$ mouse males were intercrossed with *Six1*$^{+/-}$*;Shh*$^{f/WT}$ females to decrease 50% of SHH activity in the distal epithelium by generating *Six1*$^{-/-}$; *Spc-rtTA*$^{+/-}$*-tet(o) Cre*$^{+/-}$*-Shh*$^{\Delta/WT}$ (hereafter known as *Six1*$^{-/-}$*-Shh*$^{\Delta/WT}$) mutant mice for analysis. Pregnant *Six1*$^{+/-}$*; Shh*$^{f/WT}$ females were maintained on doxycycline containing food from E6.5 until sacrificed. Reduction of SHH expression in compound mutant lungs was confirmed by real-time PCR/Western blot as compared to lungs of *Six1*$^{-/-}$ or *Eya1*$^{-/-}$ littermates. For all experiments, wildtype littermates were used as controls. In addition, no changes in lung phenotype or protein expression were evident in controls: DOX-fed *Spc-rtTa* and *Spc-rtTa-tet*(*O*) *Cre* mice (data not shown).

Phenotype analyses, antibody staining, Western blot and co-immunoprecipitation

Fluorescent staining on paraffin sections or fixed MLE-15 cells, immunoperoxidase staining, Western blot and co-immunoprecipitation were performed in triplicates using commercially available antibodies following the manufacturer's instructions and standard protocols as we described (El-Hashash et al., 2011a, b, c).

Embryonic lung culture and treatment with cyclopamine

Murine lungs were dissected from E14.5 embryos and cultured in 12-well cluster dishes on a rocker platform for 4 to 5 days as described (Rubin et al., 2004). They were exposed to 5 μmol/l cyclopamine, which proved to be not toxic by us (El-Hashash et al., 2011b), for 4 days in culture.

Cell culture and transfection

MLE15 cells were grown in culture as described by Tefft et al. (2002). Transfection of cells with siRNAs was performed as we described (El-Hashash et al., 2011c). There is no change in cells of blank controls or Lipofectamine controls, and their data are not presented. The knockdown/overexpression efficiency was analyzed by Western blotting/immunostaining of targeted protein.

Lung morphometry and proliferation index

The mean linear intercept (MLI) on E18.5 lungs was calculated following previously published protocols (Thurlbeck, 1967; Neptune et al., 2003; Shu et al., 2007). Briefly, we captured digital images at both 200 × and 400 × magnification. Then, horizontal, vertical and diagonal grid lines were overlaid and used to count the number of saccular septa intersections. We calculated MLI as follows: length of grid lines divided by the number of intersections with saccular septa. Data are from three samples of each indicated genotype.

Using the same images, we drew approximately 30–50 lines per field perpendicular to the narrowest segment of saccular septa in order to measure saccular wall thickness. The mean length of lines crossing the septa was determined using Scion Image (Scion Corp.)

We generated proliferation index for mesenchymal cells by counting the percentage of PCNA-positive mesenchymal cells in 4–5 fields of view from 3 different lungs.

Video image, statistical and densitometry analyses

Video image analysis of vacular wall remodeling was performed for PECAM stained sections as previously described (Mushaben et al., 2012). Briefly, we used Metamorph imaging software (v6.2; Universal Imaging/Molecular Devices) to measure the medial wall thickness and external diameter of each vessel. For each vessel, wall thickness and the external diameter were measured twice, along two different (crossed) axes. Percent wall thickness was calculated as [(wall 1a thickness+wall 1b thickness)/external diameter 1] × 100 (Mushaben et al., 2012). We perform this calculation for each axis. Each axis measurement was averaged together to determine the final wall thickness. Between 4 and 6 vessels were measured for each mouse. Statistical analysis was performed as described previously (El-Hashash et al., 2005, 2011a,c). Protein quantification was produced by densitometry analysis with the Image J software as described (El-Hashash et al., 2011c). Data were analyzed using ANOVA-Dunnett's test and values considered significant if $P < 0.05$.

Results

Six1 and Eya1 act together to regulate lung development

We recently reported that the expression of *Eya1* and *Six1* overlaps at the distal mesenchyme and epithelium of the developing lung and that *Eya1*$^{-/-}$ or *Six1*$^{-/-}$ mouse embryos exhibit severe lung hypoplasia, which is characterized by a reduction of epithelial branching, but increased mesenchymal cellularity (El-Hashash et al., 2011a, b). Therefore, to further test whether these genes function in a molecular pathway during murine lung development, we generated and examined the lungs of compound homozygotes of *Eya1*$^{-/-}$;*Six1*$^{-/-}$ (Fig. 1). *Eya1*$^{-/-}$;*Six1*$^{-/-}$ homozygous lungs exhibited hypoplasia that was more severe than the degree of *Eya1*$^{-/-}$ or *Six1*$^{-/-}$ lungs (Fig. 1A and D–G). The newborn pups of these mice gasped for breath, appeared cyanotic and died at birth similar to *Eya1*$^{-/-}$ or *Six1*$^{-/-}$ mice. Histological sections of E14.5 and E18.5 *Eya1*$^{-/-}$;*Six1*$^{-/-}$ hypoplastic lungs