Developmental Biology 427 (2017) 93–105

Contents lists available at ScienceDirect

Developmental Biology

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

Original research article

Endothelial cells are not required for specification of respiratory progenitors

Jamie A. Havrilak[a,b,1], Kristin R. Melton[b], John M. Shannon[a,b,*]

[a] Molecular and Developmental Biology Graduate Program, University of Cincinnati College of Medicine, 3333 Burnet Ave, Cincinnati, OH 45229, United States

[b] Section of Neonatology, Perinatal and Pulmonary Biology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Ave, Cincinnati, OH 45229, United States

ARTICLE INFO

Keywords:
Lung development
Lung specification
Endothelial cells

ABSTRACT

Crosstalk between mesenchymal and epithelial cells influences organogenesis in multiple tissues, such as lung, pancreas, liver, and the nervous system. Lung mesenchyme comprises multiple cell types, however, and precise identification of the mesenchymal cell type(s) that drives early events in lung development remains unknown. Endothelial cells have been shown to be required for some aspects of lung epithelial patterning, lung stem cell differentiation, and regeneration after injury. Furthermore, endothelial cells are involved in early liver and pancreas development. From these observations we hypothesized that endothelial cells might also be required for early specification of the respiratory field and subsequent lung bud initiation. We first blocked VEGF signaling in E8.5 cultured foreguts with small molecule VEGFR inhibitors and found that lung specification and bud formation were unaltered. However, when we examined E9.5 mouse embryos carrying a mutation in the VEGFR *Flk-1*, which do not develop endothelial cells, we found that respiratory progenitor specification was impeded. Because the E9.5 embryos were substantially smaller than control littermates, suggesting the possibility of developmental delay, we isolated and cultured foreguts from mutant and control embryos on E8.5, when no size differences were apparent. We found that both specification of the respiratory field and lung bud formation occurred in mutant and control explants. These observations were unaffected by the presence or absence of serum. We also observed that hepatic specification and initiation occurred in the absence of endothelial cells, and that expansion of the liver epithelium in culture did not differ between mutant and control explants. Consistent with previously published results, we also found that pancreatic buds were not maintained in cultured foreguts when endothelial cells were absent. Our observations support the conclusion that endothelial cells are not required for early specification of lung progenitors and bud initiation, and that the diminished lung specification seen in E9.5 *Flk*$^{-/-}$ embryos is likely due to developmental delay resulting from the insufficient delivery of oxygen, nutrients, and other factors in the absence of a vasculature.

1. Introduction

Early lung organogenesis comprises the processes of progenitor specification and bud initiation. Specification of respiratory progenitors, which is the commitment and demarcation of endodermal cells fated to become lung or trachea, is evident on embryonic day (E) 8.5–9.0 in the mouse. The respiratory progenitors are identified by expression of the transcription factor *Nkx2-1*, which is the earliest known marker of lung specification, along the ventral foregut endoderm. Following specification, lung morphogenesis ensues with the formation of two primary buds that evaginate into the surrounding splanchnic mesoderm at E9.5, followed by elaboration of the conducting airways through branching morphogenesis (Havrilak and Shannon, 2015a; Herriges and Morrisey, 2014; Metzger et al., 2008).

Events in early lung development are driven by reciprocal tissue interactions between the mesenchyme and the epithelium (Havrilak and Shannon, 2015a; Hines and Sun, 2014; McCulley et al., 2015; Shannon et al., 1998). These interactions are mediated by diffusible paracrine factors, and intensive research over the past two decades has identified many of the conserved signaling pathways governing early lung specification and morphogenesis. The retinoic acid (RA), WNT/β-catenin, fibroblast growth factor (FGF), hedgehog (HH), and bone morphogenetic protein (BMP) signaling pathways form gene regulatory networks that interact to orchestrate lung development (Herriges and

* Corresponding author at: Section of Neonatology, Perinatal and Pulmonary Biology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Ave, Cincinnati, OH 45229, United States.
E-mail address: jmshannon8@gmail.com (J.M. Shannon).
[1] Present address: Department of Biological Sciences, Lehigh University, 111 Research Drive, Bethlehem, PA 18015, United States.

http://dx.doi.org/10.1016/j.ydbio.2017.05.003
Received 15 November 2016; Received in revised form 21 April 2017; Accepted 8 May 2017
Available online 10 May 2017

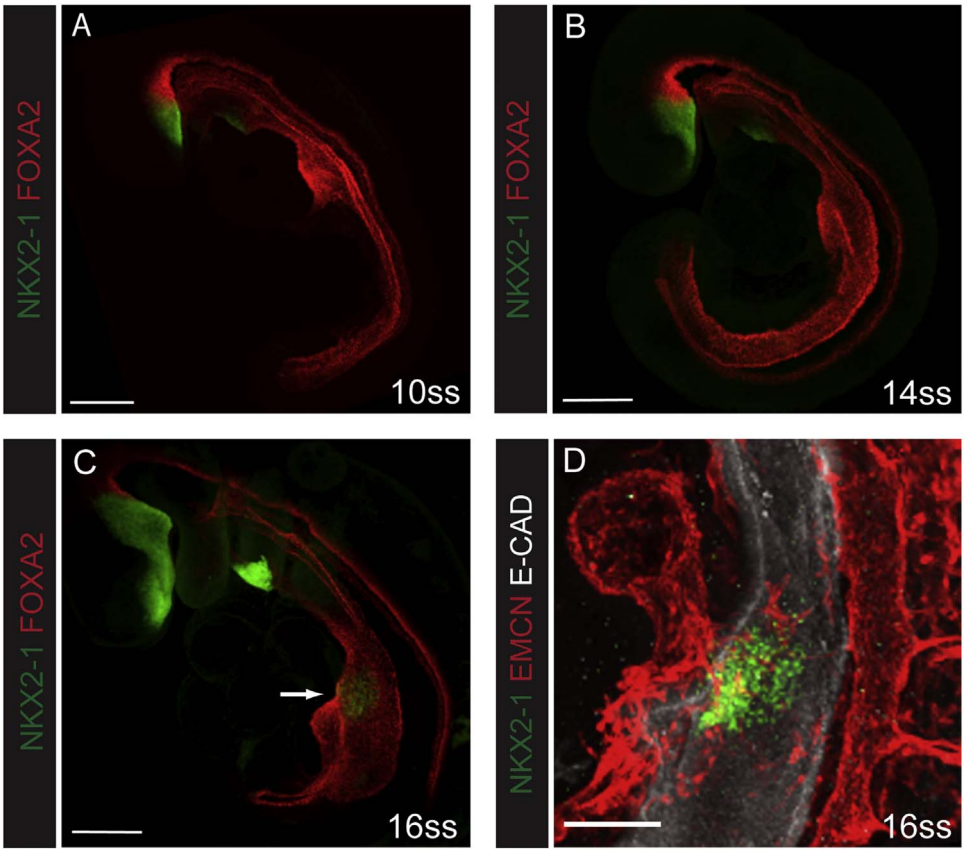

Fig. 1. Endothelial cells are associated with respiratory progenitors. Early (E8-8.5) embryos were stained by whole mount immunofluorescence for NKX2-1 (green) to visualize the emergence of the respiratory progenitors. At 10ss (A) or 14ss (B), NKX2-1 is only detected in the forebrain and thyroid region. At 16ss NKX2-1 positive cells are evident in the presumptive lung field (C, arrow). FOXA2 (red) stains the gut tube endoderm on the ventral side of the embryo, and the notochord and floor plate dorsally (A-C). Higher magnification of a 16ss embryo (D) stained for EMCN (red) demonstrates that endothelial cells are in proximity to respiratory progenitors emerging from the ventral side of the foregut; E-CAD staining (white) marks the foregut endoderm in this panel. Scale bars: A-C =200 μm, D =100 μm.

Morrisey, 2014; Hines and Sun, 2014; Rankin and Zorn, 2014). For example, RA produced by splanchnic mesenchyme cells stimulates SHH expression, which in turn supports both mesenchymal survival (Weaver et al., 2003) and its expression of *Wnt2/2b* (Rankin et al., 2016).

Although much has been learned about the gene regulatory networks controlling lung specification and development, precise identification of the cell types within the surrounding mesenchyme that produce critical signaling factors for the epithelium in early lung development remains unknown. Of the myriad of different cell types found in the developing lung mesenchyme (Kumar et al., 2014), some available evidence suggests a potential role for endothelial cells. Several studies have shown that endothelial cells contribute to some processes in the developing lung, such as patterning and alveoligenesis (Lazarus et al., 2011; van Tuyl et al., 2005; Zhao et al., 2005). A requirement for endothelial cells for early lung branching is not absolute, however, since we have recently demonstrated that embryonic (E12.5) lung endoderm is fully capable of branching *in vitro* in the absence of endothelial cells (Havrilak and Shannon, 2015b). In the adult lung, endothelial cells act to influence adult lung stem cell differentiation (Lee et al., 2014), as well as in promoting alveolar regeneration following unilateral pneumonectomy (Ding et al., 2011).

Observations from other endoderm-derived tissues, notably the pancreas and liver, have suggested that endothelial cells are required for organ initiation, specifically during bud formation. Utilizing recombination techniques, Lammert et al. showed that endoderm recombined with dorsal aorta initiated expression of the pancreas markers *Pdx1* and insulin (Lammert et al., 2001). They also found that *Xenopus* embryos lacking a dorsal aorta showed a significant decrease in pancreatic gene expression. Gain of function experiments using the *Pdx1* promoter to drive VEGF expression demonstrated that increased vascularization led to hypertrophy of pancreatic islets, ectopic expression of insulin expressing cells in the stomach near the areas of increased vascularization, and ectopic pancreatic buds in the anterior duodenum (Lammert et al., 2001). Studies examining liver initiation in mouse embryos null for the VEGF receptor *Flk-1*, which lack mature endothelial cells, showed that although early liver genes such as *Alb*, *Ttr* and *Hex* were expressed in these embryos, liver epithelial cells did not migrate into the adjacent septum transversum, either *in vivo* or *in vitro* (Matsumoto et al., 2001).

The known role of endothelial cells in some aspects of lung development, combined with observations in the pancreas and liver, raised the possibility that endothelial cells might also play a critical role in respiratory field specification and lung bud initiation. In the present study we have examined the role of endothelial cells during lung specification and bud initiation. Using pharmacological inhibitors of VEGF signaling, as well as *Flk-1* mutant mouse embryos lacking endothelial cells, we show here that a lack of endothelial cells does not interfere with specification of respiratory progenitors or subsequent lung bud initiation.

2. Results

2.1. Endothelial cells are associated with early respiratory progenitors

Previous studies from our lab have shown that pulmonary vascular development begins in the lateral plate mesoderm as soon as the lung