Stem Cell Reports
Article

ISSCR

OPEN ACCESS

Identification of a Proximal Progenitor Population from Murine Fetal Lungs with Clonogenic and Multilineage Differentiation Potential

Mélanie Bilodeau,[1] Sharareh Shojaie,[2,3] Cameron Ackerley,[2,4] Martin Post,[2,3] and Janet Rossant[1,5,*]
[1]Program in Developmental and Stem Cell Biology, Peter Gilgan Centre for Research and Learning, The Hospital for Sick Children, Toronto ON M5G 0A4, Canada
[2]Program in Physiology and Experimental Medicine, Peter Gilgan Centre for Research and Learning, The Hospital for Sick Children, Toronto ON M5G 0A4, Canada
[3]Department of Physiology, University of Toronto, Toronto ON M5S 1A8, Canada
[4]Department of Paediatric Laboratory Medicine, The Hospital for Sick Children, Toronto ON M5G 1X8, Canada
[5]Department of Molecular Genetics, University of Toronto, Toronto ON M5S 1A8, Canada
*Correspondence: janet.rossant@sickkids.ca
http://dx.doi.org/10.1016/j.stemcr.2014.07.010

SUMMARY

Lung development-associated diseases are major causes of morbidity and lethality in preterm infants and children. Access to the lung progenitor/stem cell populations controlling pulmonary development during embryogenesis and early postnatal years is essential to understand the molecular basis of such diseases. Using a *Nkx2-1*^{mCherry} reporter mouse, we have identified and captured *Nkx2-1*-expressing lung progenitor cells from the proximal lung epithelium during fetal development. These cells formed clonal spheres in semisolid culture that could be maintained in vitro and demonstrated self-renewal and expansion capabilities over multiple passages. In-vitro-derived *Nkx2-1*-expressing clonal spheres differentiated into a polarized epithelium comprised of multiple cell lineages, including basal and secretory cells, that could repopulate decellularized lung scaffolds. *Nkx2-1* expression thus defines a fetal lung epithelial progenitor cell population that can be used as a model system to study pulmonary development and associated pediatric diseases.

INTRODUCTION

The primitive trachea and two distal lung buds emerge from the anterior foregut endoderm around embryonic day 9.5 (E9.5) (Kimura and Deutsch, 2007). Already at stage E10.5, the trachea comprises epithelial cells expressing the basal cell marker P63, and they increase in number until stage E15.5 (Que et al., 2007, 2009). Branching morphogenesis, characterized by SOX9 expression in the distal lung epithelium, gives rise to the conducting airway and the gas exchange regions throughout the prenatal period (Alanis et al., 2014). Before E15.0, the proximal branches downregulate SOX9, activate SOX2, and undergo conducting airway differentiation (ending at E17.0) (Alanis et al., 2014). ASCL1-expressing neuroendocrine cells become detectable at E12.5 (Li and Linnoila, 2012). The ciliated (*Foxj1*$^+$, β-tubulin$^+$) and club cell (SCGB1A1$^+$) markers are expressed around E14.5–E16.5 (Rawlins et al., 2007, 2009b). In addition, the heterogeneous club cell population expresses early markers (*Scgb3a2*, *Cyp2f2*, and others) and region-specific transcripts (*Reg3g*, *Gabrp*, *Hp*, *Upk3a*, and others) (Guha et al., 2014). After the specification of the bronchioalveolar duct junctions at stage E17.0, alveolar type 1 (AT1) and 2 (AT2) cells differentiate during the sacculation process and mature into functional alveoli in the distal lung (Alanis et al., 2014; Desai et al., 2014; Treutlein et al., 2014). Branching morphogenesis and alveolar differentiation are oppositely regulated by KRAS, SOX9, and others (Chang et al., 2013). Mature basal cells are found postnatally (P63$^+$, KRT14$^+$, KRT5$^+$, BS-I-B4$^+$) (Daniely et al., 2004). Shortly after birth, submucosal glands emerge underneath the proximal airway epithelium, with acini comprised of secretory cells (e.g., mucous and serous cells) and myoepithelial basal cells connected to the surface by ducts made up of basal and ciliated cells (Wansleeben et al., 2013, 2014). The submucosal glands share expression of several markers with surface epithelium (e.g., P63, KRT5, MUC5AC, LTF, and others) but distinctively comprise P63$^+$ KRT5$^+$ SMA$^+$ myoepithelial basal cells (Wansleeben et al., 2014).

In the adult mouse and human lungs, distinct region-specific epithelial progenitor cells have been described (Wansleeben et al., 2013), but their fetal counterparts remain undercharacterized. At E10.5–E12.5, *Id2*-expressing distal tip cells of the fetal lung buds are multipotent and contribute to the conducting airways (e.g., club, ciliated, neuroendocrine) and alveolar (AT1 and AT2) lineages (Rawlins et al., 2009a). Later at stage E15.0, AT1 and AT2 cells derive from a bipotent progenitor (Desai et al., 2014). Inducible lineage tracing regulated by the *Cgrp* promoter (neuroendocrine cell marker) at E12.5–E14.5 labels neuroendocrine and alveolar (AT1 and AT2 cells) descendants (Song et al., 2012). However, *Ascl1*-expressing cells are reported to give rise to airway, AT2, and nonepithelial cells, a finding to be clarified by clonal analysis (Li and Linnoila, 2012). Also, secretory cells contribute to club and ciliated lineages postnatally (Guha et al., 2012). Although these studies reveal the origin of intrapulmonary airways and

alveoli lineages (i.e., distal lung), the progenitor relationships in the proximal trachea and extrapulmonary bronchi (i.e., proximal lung) remain mostly unresolved. Inducible lineage tracing driven by the human *SPC* promoter suggests a distinct origin for proximal and distal lungs (Perl et al., 2002). Moreover, fetal human tracheal tissue can mature into basal, mucociliary, and submucosal gland cells after serial xenotransplantation, suggesting progenitor/stem cell activity (Delplanque et al., 2000).

To better understand lineage relationships in fetal lung development, we knocked an mCherry reporter gene into the *Nkx2-1* locus to isolate purified primary lung epithelial cells that we submitted to in vitro clonogenic progenitor assays. NKX2-1 is the earliest marker of pulmonary fate and is broadly expressed in the proximal and distal fetal lung epithelium (Kimura and Deutsch, 2007). *Nkx2-1*-deficient mice are stillborn and show severe distal lung epithelium branching and cytodifferentiation defects (Kimura et al., 1996; Minoo et al., 1999). Also, their trachea epithelium fails to separate from the esophagus and adopts an esophagus-like phenotype, with high expression of SOX2 and P63 (Minoo et al., 1999; Que et al., 2007). Our molecular characterization of *Nkx2-1*-expressing cells reveals an underappreciated broad cellular diversity in the airways, including progenitor cells with long-term clonogenic and differentiation potential in vitro. These cells self-renew and engraft when seeded onto decellularized lung scaffolds. Overall, these results suggest that the *Nkx2-1*-expressing population in the fetal proximal airways contains cells that can act as self-renewing multilineage progenitors in vitro.

RESULTS

In Vitro Colony-Forming Potential of *Nkx2-1*-Expressing Cells

To capture mouse pulmonary cells expressing *Nkx2-1*, a nondisruptive fluorescent mCherry knockin allele was generated by gene targeting in embryonic stem cells (ESCs) (Figure 1A; Figure S1A available online). Both heterozygous and homozygous animals generated were normal and fertile and could be used for experiments. mCherry was detected by fluorescent microscopy in *Nkx2-1*-expressing tissues: the lung, brain, and thyroid (Figures 1B and S1B) (Lazzaro et al., 1991). At the cellular level, confocal imaging revealed coexpression of cytoplasmic mCherry and immunostained nuclear NKX2-1 in fetal and adult lungs (Figures 1C and S1C). The percentage of *Nkx2-1*-mCherry-positive (mC$^+$) cells varied from 9% to 20% in total lung tissue isolated from E10.5 to E15.5 (Figures S1D and S1E). To better understand the cell diversity expressing *Nkx2-1* in the developing lung (E11.5–E15.5), pan-epithelial and lineage-specific markers were monitored by quantitative real-time PCR in *Nkx2-1*-mC$^+$ and *Nkx2-1*-mCherry-negative (mC$^-$) sorted cells and by coimmunostaining at fetal and adult stages (Figures S1F and S2A–S2J; data not shown). NKX2-1 was expressed in most of the specialized airway cells (basal, club, and ciliated), in the tracheal submucosal glands, and in distal AT2 cells (Figures S2A–S2J). Therefore, NKX2-1 expression is not restricted to club or AT2 cells, as generally assumed (Kimura and Deutsch, 2007).

To assess whether *Nkx2-1*-expressing cells have progenitor activity, an in vitro colony assay was optimized. The stage E14.5 was initially selected for two reasons: (1) the amount of material available and (2) the expression profile of lineage-specific markers suggested that cytodifferentiation started around this time (data not shown). Proximal and distal lung epithelial cells were separately sorted based on *Nkx2-1*-mCherry expression and embedded in a Matrigel-based semisolid medium supplemented with growth factors, without supporting cells (Figures 1D and 1E). Morphologically distinct colonies were derived at a higher frequency in the mC$^+$ fraction in contrast to the mC$^-$ fraction (52-fold or 99-fold enrichment of mC$^+$ over mC$^-$ colonies, from proximal or distal lung regions, respectively; Figures 1E and 1F). Colonies derived from the proximal epithelium had compact or spheroid shapes of various sizes with cells tightly connected together (Figure 1E). Colonies derived from the distal lung epithelium were small with irregular morphologies (Figure 1E). Within the context of the provided culture conditions, only the colonies derived from the proximal lung could be propagated in long-term culture (e.g., >150 days, 15 passages [p.15]), as illustrated by cell numbers at first passage postsorting (Figure 1G). Quantitative real-time PCR was used to assess gene expression in pooled populations of 12- to 13-day-old colonies from the second passage (Figure 1H). Colonies derived from both the proximal and distal lung expressed *Nkx2-1*, *Scgb3a2*, *Scgb1a1*, *Muc5ac*, *Id2*, *Pdpn*, *Aqp5*, and *Ltf* (Figure 1H). However, expression of basal and ciliated cell markers (e.g., *Trp63*, *Krt5*, and *Foxj1*) and the distal lung/AT2 cell marker *Sftpc* was restricted to colonies derived from the proximal or the distal lung, respectively (Figure 1H). Expression of several cell markers was higher in cultured cells than in freshly sorted E14.5 mC$^+$ parental cells, a feature more reminiscent of later developmental stages (Figure 1H). The neuroendocrine (*Ascl1*) cell marker was expressed at low level in both colony types (Figure 1H). Overall, at E14.5, the *Nkx2-1*-expressing cells gave rise to colonies with distinct characteristics depending on their region of origin. We focused on the characterization of the putative progenitor cells derived from the fetal upper airways because of their relevance for several pediatric diseases, including cystic fibrosis, asthma, and others.