Regulation of lung endoderm progenitor cell behavior by miR302/367

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SUMMARY
The temporal and spatial control of organ-specific endoderm progenitor development is poorly understood. miRNAs affect cell function by regulating programmatic changes in protein expression levels. We show that the miR302/367 cluster is a target of the transcription factor Gata6 in mouse lung endoderm and regulates multiple aspects of early lung endoderm progenitor development. miR302/367 is expressed at early stages of lung development, but its levels decline rapidly as development proceeds. Gain- and loss-of-function studies show that altering miR302/367 expression disrupts the balance of lung endoderm progenitor proliferation and differentiation, as well as apical-basal polarity. Increased miR302/367 expression results in the formation of an undifferentiated multi-layered lung endoderm, whereas loss of miR302/367 activity results in decreased proliferation and enhanced lung endoderm differentiation. miR302/367 coordinates the balance between proliferation and differentiation, in part, through direct regulation of Rbl2 and Cdkn1a, whereas apical-basal polarity is controlled by regulation of Tiam1 and Lis1. Thus, miR302/367 directs lung endoderm development by coordinating multiple aspects of progenitor cell behavior, including proliferation, differentiation and apical-basal polarity.

KEY WORDS: Lung, MicroRNA, Progenitor, Mouse

INTRODUCTION
The proper balance between proliferation and differentiation of endodermal progenitors is crucial for development and function of endodermally derived tissues such as the lung. Characterization of the gene regulatory networks that regulates the expansion and differentiation of these progenitors has a profound impact on our understanding and treatment of lung disease. The entire epithelium of the lung is initially generated from a small pool of undifferentiated progenitor cells that is present in the anterior ventral foregut endoderm at E9.5 of mouse development (Morrisey and Hogan, 2010). By E11.5 in the mouse, distinct progenitors are found in the proximal versus distal regions of the branching airways (Morrisey and Hogan, 2010). These progenitors will differentiate into distinct epithelial lineages during lung development that are required for proper gas exchange in the adult lung. Disruption in these developmental processes can lead to severe neonatal lung disease.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate mRNA translation and stability at a post-transcriptional level. Many miRNAs are expressed in groups or clusters from a single primary transcript which is further processed by the Dicer-Drosha complex into mature 22 nucleotide fragments which bind to complementary regions within the 3’ untranslated (UTR) regions of target genes. An important role for the miRNA pathway in lung development has been demonstrated using a conditional deletion of Dicer in embryonic lung epithelium (Harris et al., 2006). The resulting mutant lungs exhibited severebranching defects and increased epithelial apoptosis. Despite this dramatic effect, only a few studies have shown an important role for specific miRNAs in endoderm development. In the lung, transgenic overexpression of miR17-92 promotes proliferation and inhibits differentiation of lung endoderm progenitor cells, whereas deletion of miRNA17-92 leads to a hypoplastic lung phenotype (Lu et al., 2007; Ventura et al., 2008).

We have previously shown that the transcription factor Gata6 plays an important role in regulating lung endoderm progenitor differentiation and proliferation (Zhang et al., 2008). In these studies, we have identified the miR302/367 cluster as a target of Gata6 function in early lung endoderm. We have found that the miR302/367 cluster is a direct target of Gata6 and is expressed at high levels up to E12.5 lung development, but declines rapidly as development proceeds. Increased or decreased miR302/367 expression leads to an altered balance of lung endoderm progenitor proliferation and differentiation, as well as to disruption in apical-basal polarity. This balance between proliferation, differentiation and the disruption in progenitor cell polarity is controlled, in part, by expression of several key targets of miR302/367, including the tumor suppressor Rbl2, the cell cycle regulator Cdkn1a and the cell polarity factors Tiam1 and Lis1 (Paflahb1 – Mouse Genome Informatics). Together, these studies demonstrate that miR302/367 controls multiple aspects of lung endoderm progenitor cell behavior that regulate their ability to form a single-layered epithelium required for functional gas exchange and postnatal respiration.

MATERIALS AND METHODS
RNA purification, RT-QPCR and miRNA microarray analysis
Total RNA was isolated from embryonic lungs at the indicated ages using Trizol reagent, reverse transcribed using SuperScript First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA), and used in quantitative real time
Fig. 1. See next page for legend.
microarray analysis revealed decreased expression of several members of the miR302/367 cluster, including miR302a, miR302b and miR302d in Gata6flox/flox:Sftpc-rtTA:tetO-cre mutant mouse lungs at E14.5. Expression of miR302/367 was assessed in Gata6 mutant and control littermate lungs at E12.5 by Q-PCR. Data are mean±s.e.m. (E-G) Whole-mount in situ hybridization of miR302b, miR302a and miR367 LNA probes showing the expression of miR302/367 in epithelial cells at E14.5. (F-H) Control in situ hybridization in the absence of a primary probe. (I) Relative expression of miR302/367 cluster members during lung development by RT-qPCR. (J) Cross-species conservation of miR302/367 5’ proximal genomic region showing the putative Gata6 DNA-binding sites (red arrows). (K) ChIP assays showing binding of Gata6 to sites 1, 2 and 3 in the miR302/367 promoter region. Data are mean±s.e.m. (L) Gata6 transactivation of the miR302/367 promoter in a dose-dependent manner. Data are mean±s.e.m.

**Results**

Gata6 regulates transcription of the miR302/367 cluster during lung development.

Western blot analysis

Total protein extracts (20 μg) from transgenic lungs at E18.5 were resolved on SDS-PAGE gels and transferred to PVDF membranes for western blotting. Antibodies against Rbl2 (Abcam), Cdkn1a (Santa Cruz), Tiam1 (Abcam), Lis1 (Abcam), β-actin (Chemicon) and GAPDH (Chemicon) were used.

Cell transfection and chromatin immunoprecipitation (ChIP) assays

The upstream proximal promoter region of mouse miR302/367 has been described previously. The region between –710 to –130 bp was subcloned into the pGL3 promoter luciferase reporter vector to generate pGL3-miR302/367/luc vector. This plasmid was transfected with a Gata6 expression plasmid into HEK293 cells using Fugene 6 and luciferase activities were determined using a commercially available kit (Promega). For ChIP assays, chromatin was extracted from 15 wild-type embryonic lungs at E13.5 using a ChIP assay kit (Upstate). Chromatin was immunoprecipitated with Gata6 antibody and purified chromatin was subjected to PCR using the oligonucleotides listed in Table S1 in the supplementary material.

**Results**

Gata6 regulates transcription of the miR302/367 cluster during lung development.

To address the mechanism of Gata6 regulation of early lung endoderm progenitor development, we performed a miRNA expression microarray screen analysis at E14.5 comparing RNA from Gata6flox/flox:Sftpc-rtTA:tetO-cre mutant lungs with control lungs (Sftpc-rtTA:tetO-cre). This experiment identified decreased expression of three members of the miR302/367 cluster, miR302a/b/d, suggesting that this cluster of miRNAs is a direct target of Gata6 regulation in the lung (Fig. 1A). Quantitative real-time PCR (Q-PCR) showed decreased expression of all five members of the miR302/367 cluster in Gata6flox/flox:Sftpc-rtTA:tetO-cre mutant lungs (Fig. 1A,B).

To determine the spatial and temporal pattern of expression of the miR302/367 cluster, whole-mount in situ hybridization and Q-PCR was performed. As all five members are expressed from a single primary transcript, we determined expression of three members, miR302a, miR302b and miR367, to provide a representative expression pattern for the miR302/367 cluster. In situ hybridization with locked nucleic acid (LNA) probes showed that miR302a, miR302b and miR367 were highly expressed in the endoderm of the E12.5 lung (Fig. 1C-F). Expression of miR302a and miR367 was also detected at lower levels in the mesenchyme of the E12.5 lung (Fig. 1E). Q-PCR analysis was performed on RNA isolated from mouse embryonic lungs at developmental
stages between E11.5 and postnatal day 5 for all five members of the miR302/367 cluster. The expression of all five members of the cluster was greatest at E11.5 and decreased significantly after E14.5 (Fig. 1I). At E18.5 and postnatal stages, the expression of these miRNAs was very low to undetectable in the lung (Fig. 1I).

To determine whether the miR302/367 cluster is a direct transcriptional target of Gata6, we analyzed the putative promoter region of miR302/367 for Gata6-binding sites. The miR302/367 cluster is found in intron eight of the Larp7 gene and a previous study identified a ~500 bp promoter region upstream of miR302/367 that contains binding sites for Oct4 and Sox2, which regulates miR302/367 expression in embryonic stem cells (Card et al., 2008) (Fig. 1J). Analysis of this promoter region and flanking sequences identified three potential Gata6 DNA-binding sites that are conserved in mammals (Fig. 1J). Chromatin immunoprecipitation assays were performed to determine whether Gata6 bound these three sites in the embryonic lung. Both gel electrophoresis and Q-PCR studies showed robust association of Gata6 with all three DNA-binding sites (Fig. 1K). Moreover, Gata6 can efficiently transactivate this promoter in a dose-dependent manner when linked to a luciferase reporter (Fig. 1L). Together, these data indicate that the miR302/367 cluster is expressed in the early lung endoderm and is a direct target of Gata6.

Abnormal lung development in miR302/367 transgenic embryos
To assess the role miR302/367 plays in lung development, gain-of-function experiments were performed using the human SFTPC promoter to overexpress miR302/367 in early lung endoderm (Fig. 2A). Sftpc:miR302/367 transgenics had larger lungs when examined at E18.5 (Fig. 2B). Histological analysis on E18.5 control lungs showed that the normal embryonic lung contains many small distal saccules or primitive alveoli, which are ready to open for respiration (Fig. 2C-E). By contrast, Sftpc:miR302/367 transgenic lungs contain a thickened epithelial lining and decreased sacculation (Fig. 2F-H). Overexpression of all members of the miR302/367 cluster in the transgenic lungs versus control littermates was confirmed by quantitative RT-PCR (Q-PCR) at E18.5 (Fig. 2I). Expression of Nkx2.1 highlights the disrupted nature of the distal airways and reveals a thickened epithelium in Sftpc:miR302/367 transgenics (Fig. 2J,K). This change in airway morphology decreased the mean linear intercept between alveolar septae at E18.5 (Fig. 2L), and correlated with an increase in the average thickness of alveolar septa (Fig. 2M). These changes also result in decreased airway lumen size (see Fig. 4H). No Sftpc:miR302/367 transgenics were ever found alive after the first day of life, indicating that they died in the perinatal period (data not shown).
The miR302/367 cluster regulates lung endoderm progenitor proliferation and differentiation

Given the severe disruption in endoderm development in Sftpc:miR302/367 transgenics, we performed immunohistochemical staining for Sox2 and Sox9 to determine the effect of increased expression of miR302/367 on lung endoderm progenitors. In the developing lung, Sox2 is normally expressed in endoderm progenitors of the proximal airways and is absent in the distal endoderm (Fig. 3A) (Que et al., 2007). Conversely, Sox9 is expressed at the highest levels in the distal lung endoderm progenitors and expression in distal progenitors decreases significantly so that by E16.5, Sox9 is detected in only a small subset of cells in the terminal distal endoderm (Fig. 3B) (Okubo et al., 2005; Lu et al., 2007). In Sftpc:miR302/367 transgenics, Sox2-expressing progenitors are expanded into the distal airways (Fig. 3C). Moreover, there is an increase in the number of Sox9+ endoderm progenitors in the distal airways of Sftpc:miR302/367 transgenics (Fig. 3D). Q-PCR confirms the increased expression of Sox2 and Sox9 in transgenic lungs, as well as an additional marker of distal endoderm progenitors, Id2 (Fig. 3E). These data show that increased expression of miR302/367 leads to expansion of both proximal and distal endoderm progenitors within the distal airway endoderm.

To assess whether the miR302/367 cluster influences endoderm differentiation in the lung, immunostaining for markers of proximal airway Clara epithelial cells (CC10) and distal epithelial cells (SP-C) was performed at E18.5. In Sftpc:miR302/367 transgenic lungs, both CC10 and SP-C expression is significantly downregulated (Fig. 3F-I). Q-PCR confirms these results and also shows reduction of additional markers of lung epithelial differentiation and maturation, including SP-A, SP-B and Aqp5 (Fig. 3J). Together, these data indicated that increased expression of miR302/367 promotes expansion of both proximal and distal lung progenitors but inhibits their subsequent differentiation.

Abnormal endoderm-mesoderm signaling in Sftpc:miR302/367 transgenic lungs

Paracrine signaling between the lung endoderm and mesoderm is crucial for development of pulmonary smooth muscle (Shu et al., 2002; Weaver et al., 2003; Miller et al., 2004). To assess lung
mesodermal differentiation in *Sftpc:miR302/367* transgenic lungs, we used immunohistochemistry to analyze smooth muscle 22α (SM22α) expression. In the normal lungs, SM22α-positive smooth muscle cells encircle the endoderm of the proximal airways and the blood vessels, but are absent from the advancing tips of distal airways (Fig. 3K,L) (Tollet et al., 2001). However, *Sftpc:miR302/367* transgenic lungs displayed a dramatic increase in SM22α-positive staining around the primitive alveolar structures (Fig. 3M,N). Q-PCR also confirmed the upregulation of smooth muscle α-actin (α-SMA) expression in transgenic lungs (Fig. 3O). Expression of several other key signaling molecules and transcription factors known to be important for pulmonary smooth muscle development, including Wnt7b, Shh, Fgf10 and Fgf9, was significantly increased in transgenic lungs (Fig. 3O). These data show that increased *miR302/367* expression and the resulting expansion of lung endoderm progenitors leads to increased endoderm-mesoderm signaling that promote smooth muscle development in the lung.

**miR302/367 regulates lung progenitor proliferation through repression of Cdkn1a and Rbl2**

The increased numbers of lung endoderm progenitors in *Sftpc:miR302/367* transgenics suggested that these miRNAs promote proliferation of these progenitors. Phospho-histone H3 (PO4-H3) immunostaining showed a significant increase in proliferation in the developing endoderm of *Sftpc:miR302/367* transgenics (Fig. 4A-C). Careful analysis of the Hematoxylin and Eosin-stained slides showed an increase in mitotic figures in the epithelium of *Sftpc:miR302/367* transgenics at E16.5 (Fig. 4D,E). This increase in proliferation correlates with the formation of a multi-layered epithelium that lines the airways of *Sftpc:miR302/367* transgenics, as shown by Nkx2.1 expression (Fig. 4F,G). This multi-layered epithelial lining also decreases the average airway lumen size (Fig. 4H).

Using multiple databases, including TargetScan, MiRanda and PicTar (Krek et al., 2005; Grimson et al., 2007; Betel et al., 2008), a large subset of *miR302/367* target genes associated with cell proliferation according to their gene ontology annotation was identified for further characterization. Potential *miR302/367* targets included Cdkn1a, Rbl2, Kat2b, Mnt, Mecp2 and Pten (see Table S2 in the supplementary material). As miRNAs are known to decrease the stability and steady-state levels of mRNAs (Guo et al., 2010), Q-PCR was used to assess the expression of these proliferation associated factors. These analyses found that expression of all but Mnt were downregulated in *Sftpc:miR302/367* transgenic lungs (Fig. 4I). Rbl2 and Cdkn1a were further selected for analysis because of their importance in the regulation of the cell cycle, as well as in malignant tumor progression (Tanaka et al., 2001; Russo et al., 2003; Wikenheiser-Brodkamp, 2004; Simpson et al., 2009). Western blots and immunohistochemistry demonstrated significant reduction in protein expression of Rbl2 and Cdkn1a in *Sftpc:miR302/367* transgenic lungs (Fig. 4J-N). Luciferase reporter constructs were generated using the 3′ UTR regions of Rbl2 and Cdkn1a cloned downstream of the luciferase cDNA and were used to determine whether expression of *miR302/367* could repress Rbl2 and Cdkn1a expression via conserved *miR302/367*-binding sites (Fig. 4O). Expression of *miR302/367* inhibited expression of these luciferase reporters in these assays and mutation of the *miR302/367*-binding site resulted in alleviation of this inhibition (Fig. 4P,Q). These data indicate that *miR302/367* promotes progression through the cell cycle and increased proliferation in early lung progenitors by repressing the cell cycle inhibitors Cdkn1a and Rbl2.

**Regulation of endoderm progenitor apical-basal polarity by miR302/367**

Gross examination of E16.5–E18.5 transgenic lung sections revealed numerous layers of irregularly packed endoderm cells lining the developing airways of *Sftpc:miR302/367* transgenic lungs (Fig. 4; Fig. 5A,C). This contrasted with the control lung endoderm cells in which the nuclei of the single-layer endoderm are orderly aligned perpendicularly to the basement membrane (Fig. 4; Fig. 5B,D). To determine whether the altered epithelial morphology influenced the epithelial apical-basal polarity in transgenic lungs, we examined the localization of Par3, an integral member of the Par-aPKC complex (Lin et al., 2000; Hirose et al., 2002; Patalano et al., 2006). As expected, Par3 expression was mostly restricted to the apical and tight junctions in control lung endoderm (Fig. 5A,C). Strikingly, transgenic lung endoderm displayed a disorganized apical layer, suggesting that the apical surface of cells is highly variable, with many of the cells found in the airway lumens exhibiting increased Par3 expression throughout their plasma membranes (Fig. 5B,D). Moreover, increased expression of Par3 was observed in the cells directly adjacent to the basement membrane of the airways (Fig. 5D). Examination of the potential target list for *miR302/367* revealed Tiam1 and Lis1 as predicted targets of *miR302/367* (see Table S2 in the supplementary material). The Rac activator Tiam1, a T-lymphoma invasion and metastasis-inducing protein, acts as a crucial component of the Par complex in regulating neuronal and epithelial apical-basal polarity (Chen and Macara, 2005; Mertens et al., 2005; Nishimura et al., 2005). Lis1 (β-subunit of platelet-activating factor acetylhydrolase) is essential for proliferation and the precise control of mitotic spindle orientation in both neuroepithelial stem cells and radial glial progenitor cells (Yingling et al., 2008; Yamada et al., 2010). Expression of both Tiam1 and Lis1 mRNA is decreased in *Sftpc:miR302/367* transgenic lungs (Fig. 5E). Western blot analysis shows decreased expression of Tiam1 and Lis1 proteins (Fig. 5F). To further assess whether Tiam1 and Lis1 are direct targets of *miR302/367*, we cloned the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for *miR302a* and *miR367* binding was found in the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for *miR302a* and *miR367* binding was found in the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for *miR302a* and *miR367* binding was found in the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for *miR302a* and *miR367* binding was found in the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for *miR302a* and *miR367* binding was found in the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for *miR302a* and *miR367* binding was found in the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for *miR302a* and *miR367* binding was found in the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for *miR302a* and *miR367* binding was found in the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for *miR302a* and *miR367* binding was found in the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for *miR302a* and *miR367* binding was found in the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for *miR302a* and *miR367* binding was found in the 3′ UTR region of each mRNA downstream of a luciferase reporter. A single site for *miR302a* and *miR367* binding was found in the 3′ UTR region of each mRNA downstream of a luciferase reporter.

**Loss of miR302/367 function results in decreased lung endoderm proliferation with enhanced differentiation**

To determine the necessity of *miR302/367* in lung endoderm development, we generated a transgenic loss-of-function model using a ‘sponge’ or decoy construct to inhibit *miR302/367* function. Previous studies both in vivo and in vitro have shown the usefulness of miRNA decoys in competitively inhibiting miRNA function (Care et al., 2007; Ebert et al., 2007; Cohen, 2009; Ma et al., 2010). We generated a transgenic construct using the human *SFTPC* promoter driving a EGFP cDNA with two tandemly arrayed miRNA-binding sites in the 3′ UTR.
region, each of them containing seven repetitive sequences complementary to miR302a-d and miR367 seed sequences with mismatches at positions 9-12 for enhanced stability (Fig. 6A) (Ebert et al., 2007).

Sftp::miR302/367 decoy transgenic lungs appeared slightly smaller at E18.5 (Fig. 6B,C). Hematoxylin and Eosin-stained sections showed that, as expected, the distal airways of wild-type embryos had begun the process of alveolarization with a thinning

Fig. 4. Lung progenitor proliferation is controlled by miR302/367 through regulation of Cdkn1a and Rbl2. (A,B) PO4-H3 immunostaining showing increased proliferation in the epithelium of Sftp::miR302/367 transgenic mouse lungs at E18.5. (C) Quantitation of PO4-H3-positive cells. Data are mean±s.e.m. (D,E) Increased mitotic figures in the epithelium of Sftp::miR302/367 transgenic lungs at E16.5 (arrowheads and dotted outline). (F,G) Presence of a multi-layered epithelium lining the airways of Sftp::miR302/367 transgenic lungs at E16.5 as denoted by Nkx2.1 immunostaining (arrowheads indicate Nkx2.1-positive cells within the airways). (H) Average airway lumen size was decreased in Sftp::miR302/367 transgenic lungs at E16.5. Error bars indicate s.e.m. (I) Decreased expression of putative miR302/367 target genes related to cell proliferation measured by Q-PCR. All quantitative changes are significant (P<0.01) in I, with the exception of Mnt expression. Data are mean±s.e.m. (J) Decreased expression of Rbl2 and Cdkn1a in Sftp::miR302/367 transgenic lungs by western blot in Sftp::miR302/367 transgenic lungs. (K-N) Decreased expression of Rbl2 and Cdkn1a in Sftp::miR302/367 transgenic lungs by immunostaining (arrowheads indicate positively stained cells). (O) Schematic of 3’ UTR luciferase reporters with alignment of miR302/367 target sequences in Rbl2 and Cdkn1a mRNAs. (P) miR302/367 expression can repress luciferase expression through the Rbl2 3’ UTR and mutation of this site abolishes this repression. (Q) miR302/367 expression can repress luciferase expression through the Cdkn1a 3’ UTR and mutation of this site abolishes this repression. Statistical significance of miR302/367 repression on 3’ UTR regions in O and P are shown. Data are mean±s.e.m. Scale bars: 100 μm in A,B; 50 μm in D-G,K-N.
maturation were increased in expression of markers of lung endoderm differentiation and Aqp5 was assessed by Q-PCR. These data show that during lung development, expression of CC10, SP-C, SP-B, SP-A Rbl2 and Cdkn1a was also increased in at E18.5 (Fig. 6L). Moreover, expression of the miR302/367 targets narrowed of the airway lumen in transgenics. Of note, the lung endoderm in transgenics (Fig. 6F,G; see Fig. S1 in the supplementary material). Q-PCR confirms the downregulated expression of all members of the miR302/367 cluster, whereas the expression of another miRNA expressed in the lung, miR200b (Gregory et al., 2008; Dong et al., 2010), is not significantly affected (Fig. 6H). These data suggest that expression of the miR302/367 decoy results in a specific inhibition of miR302/367 expression and activity in vivo.

In contrast to the enhanced lung endoderm progenitor proliferation observed in Sftpc:miR302/367 decoy transgenics (Fig. 6G; see Fig. S1 in the supplementary material). Of note, the lung endoderm in Sftpc:miR302/367 decoy transgenics remained as a single layer, unlike the gain-of-function Sftpc:miR302/367 transgenics (Fig. 6D,E; see Fig. S1 in the supplementary material). Q-PCR confirms the downregulated expression of all members of the miR302/367 cluster, whereas the expression of another miRNA expressed in the lung, miR200b (Gregory et al., 2008; Dong et al., 2010), is not significantly affected (Fig. 6H). These data suggest that expression of the miR302/367 decoy results in a specific inhibition of miR302/367 expression and activity in vivo.

of the distal epithelium, whereas the airways of Sftpc:miR302/367 decoy were more narrow and condensed, and the epithelium had not begun alveolarization-mediated thickening (Fig. 6D,E). Immunostaining for Nkx2.1 expression reveals this narrowing of the airway lumen in Sftpc:miR302/367 decoy transgenics (Fig. 6F,G; see Fig. S1 in the supplementary material). How miRNAs affect tissue specific progenitor cell behavior is poorly understood. In this report, we show that a single miRNA cluster, miR302/367, can coordinate multiple aspects of lung endoderm progenitor behavior by regulating cell proliferation and polarity, which in turn affects overall endoderm differentiation in the developing lung. miR302/367 regulates a core set of cell cycle and cell polarity genes, and in doing so affects the ability of lung endoderm progenitors to exit their highly proliferative and undifferentiated state and differentiate into the single-layered mature lung epithelium, which is required for proper postnatal respiration. These studies highlight the potent ability of miRNAs to control cellular processes by coordinately regulating multiple cellular pathways that affect progenitor cell behavior in endoderm-derived tissues.

The mechanisms promoting differentiation of multipotential anterior foregut endoderm down the organ-specific pathways is only partially understood. Key signaling pathways, such as Wnt and Fgf signaling, are important during early stages of lung specification and endoderm differentiation. Previous studies have shown that Gata6 helps to regulate early lung endoderm differentiation through regulation of Wnt signaling (Zhang et al., 2008). Gata6 expression levels decrease during lung development and its regulation of miR302/367 is likely to influence the overall proliferation of early lung progenitors through suppression of the cell cycle inhibitors Cdkn1a and
Rbl2, Gata6, along with Gata4, are important regulators of early foregut endoderm development. Both Gata6/4 have been shown to regulate cell proliferation in the lung, heart, liver and intestine (Ketola et al., 2004; Zhao et al., 2005; Haveri et al., 2008; Zhang et al., 2008; Agnihotri et al., 2009; Singh et al., 2010). Previous studies have also shown a correlation between Gata factor and Wnt signaling activity in both the developing lung and heart (Zhang et al., 2008; Tian et al., 2010). Given the combined pro-proliferative abilities of miR302/367 and the Wnt pathway, one of the major roles of Gata factor function in development is likely to promote proliferation of tissue-specific progenitors through regulation of these pathways.

Fig. 6. Loss of miR302/367 function in lung endoderm progenitors leads to decreased proliferation and enhanced differentiation. (A) Schematic diagram of decoy or ‘sponge’ Sftpc:miR302/367<sup>decoy</sup> construct. (B-E) Hematoxylin and Eosin staining of miR302/367 decoy transgenic lungs at E16.5. (F-G) Altered expression pattern of Nkx2.1, showing disruption in airway branching of Sftpc:miR302/367<sup>decoy</sup> transgenics. (H) All members of the miR302/367 cluster were significantly downregulated in Sftpc:miR302/367<sup>decoy</sup> transgenic lungs at E14.5, whereas expression of miR200b was not significantly affected. All changes in H are significant (P<0.01) except for miR200b expression, which is not significant (n.s.). Data are mean±s.e.m. (I,J) Decreased cell proliferation in Sftpc:miR302/367<sup>decoy</sup> transgenic lungs as shown by PO4-H3 immunostaining. (K) Quantitation of cell proliferation shown in I and J, which is statistically significant (P<0.01). Data are mean±s.e.m. (L) Increased expression of CC10, SP-A, SP-B, SP-C and Aqp5 in Sftpc:miR302/367<sup>decoy</sup> transgenic lungs at E18.5 by Q-PCR. Data are mean±s.e.m. (M) Increased expression of Cdkn1a, Rbl2, Tiam1 and Lis1 in Sftpc:miR302/367<sup>decoy</sup> transgenic lungs at E18.5. Data are mean±s.e.m. All expression changes in L and M are statistically significant (P<0.05). Scale bars: 100 μm.
Little is known about the ability of miRNAs to regulate tissue-specific progenitor populations. In the skin, miR203 promotes differentiation of skin basal progenitor cells by repressing the important skin progenitor maintenance factor p63 (Lena et al., 2008; Yi et al., 2008; Wei et al., 2010). However, less is known in the lung. Loss of Dicer in early lung endoderm results in defective differentiation of early lung endoderm and increased apoptosis (Harris et al., 2006). However, which miRNAs play an important role in regulating early lung development is less clear. One of the few miRNAs that have been studied in lung development is miR17-92. Increased miR17-92 expression leads to increased lung progenitor proliferation, similar to miR302/367 expression (Lu et al., 2007). Conversely, loss of miR17-92 activity leads to lung hypoplasia (Ventura et al., 2008). Like miR302/367, miR17-92 also targets Rbl2 (Lu et al., 2007). Thus, miRNAs such as miR302/367 and miR17-92 may play a crucial role in the regulation of early lung progenitors by targeting a core set of factors that negatively regulate the cell cycle. This could directly or indirectly antagonize progenitor differentiation during early development and allow for the generation of the proper number of progenitors. As expression of miR302/367 decreases, the progenitors exit this highly proliferative state to differentiate into mature lung epithelium.

The ability of miR302/367 to regulate lung endoderm progenitor apical-basal polarity suggests that this attribute of mature polarized epithelium is required to be repressed in an early progenitor state to allow for rapid cell proliferation. Previous studies have shown that the combined activity of miR338-3p and miR451 is required for proper apical-basal polarity in colon cancer cell lines (Tsuchiya et al., 2009). miR29a has also been shown to play an important role in maintaining apical-basal polarity of epithelial cells in culture (Gebeshuber et al., 2009). However, little was known about whether or how miRNAs affected epithelial polarity in vivo. The loss of apical-basal polarity upon overexpression of miR302/367 in the lung points to an important correlation between high proliferation rates and the ability of epithelial cells to maintain a motile and less polarized phenotype.

miR302/367 is highly expressed in embryonic stem cells and is a target of the pluripotency factors Oct4 and Sox2 (Card et al., 2008). Interestingly, Sox2 and other Sox factors play key roles in early lung development by regulating lung progenitor expansion and differentiation in a spatially specific manner (Que et al., 2009). Loss of Sox2 in the lung leads to decreased proximal airway epithelial development characterized by loss of the secretory cell lineages (Tompkins et al., 2009). Increased Sox2 leads to the converse phenotype with increased secretory lineage expansion at the expense of the more distal progenitor compartment and upon extended overexpression can lead to lung cancer (Lu et al., 2010). Our data suggest that increased levels of miR302/367 lead to an increase in both Sox2- and Sox9-positive progenitors. Future studies into whether miR302/367 is associated with lung cancer might shed more light onto the multiple roles for this miRNA cluster.

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miR302/367 and lung endoderm development


