miR-142-3p balances proliferation and differentiation of mesenchymal cells during lung development

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ABSTRACT

The regulation of the balance between proliferation and differentiation in the mesenchymal compartment of the lung is largely uncharacterized, unlike its epithelial counterpart. In this study, we determined that miR-142-3p contributes to the proper proliferation of mesenchymal progenitors by controlling the level of WNT signaling. miR-142-3p can physically bind to adenomatous polyposis coli mRNA, functioning to regulate its expression level. In miR-142-3p loss-of-function experiments, proliferation of parabronchial smooth muscle cell progenitors is significantly impaired, leading to premature differentiation. Activation of WNT signaling in the mesenchyme, or Apc loss of function, can both rescue miR-142-3p knockdown. These findings show that in the embryonic lung mesenchyme, the microRNA machinery modulates the level of WNT signaling, adding an extra layer of control in the feedback loop between FGFR2C and β-catenin-mediated WNT signaling.

KEY WORDS: WNT signaling, Mesenchymal cell, miRNA

INTRODUCTION

During lung organogenesis, mesenchymal cells undergo active proliferation to support the continuous growth of the organ. Simultaneously, many cells are actively differentiating into the specific cell types that will populate the adult lung – such as parabronchial and vascular smooth muscle cells, pericytes, nerve cells, lipofibroblasts and interstitial fibroblasts. A specific balance between proliferation and differentiation must exist to guarantee the function of a functional lung. For example, parabronchial smooth muscle cell (PBSMC) progenitors are kept undifferentiated in the sub-mesothelial mesenchyme while starting to differentiate more proximally (Mailleux et al., 2005). It is crucial to understand this delicate balance in order to better characterize lung diseases involving altered mesenchymal proliferation and differentiation.

WNT signaling plays a crucial role in proper proliferation of mesenchymal cells during lung development (De Langhe et al., 2008; Yin et al., 2008). In particular, through β-catenin (CTNNB1), it promotes G1 phase progression by activation of downstream target genes such as Myc and cyclin D1 (Ccnd1) (Niehrs and Acebron, 2012). High WNT signaling and Myc activation are associated with the hyper-proliferative state of cancer of many organs, including the lung (He et al., 1998; van de Wetering et al., 2002; Van Scoyk et al., 2008).

A main regulator of WNT signaling is adenomatous polyposis coli (APC), which can directly bind to CTNNB1, antagonizing the interaction with T-cell factor (TCF). In combination with AXIN and GSK3B, APC induces ubiquitylation and degradation of CTNNB1 (Clevers and Nusse, 2012). Loss of Apc leads to accumulation of CTNNB1 in the nucleus and hyperactivation of WNT signaling. Apc was first identified as a tumor suppressor gene that, upon mutation, causes intestinal cancer (Groden et al., 1991). APC can also control cytoskeleton structure and cell migration by binding microtubules and actin filaments. For example, loss of Apc in the small intestine reduces the migration of epithelial cells and promotes the formation of polyps (Oshima et al., 1997).

Specific microRNAs (miRNAs) have been implicated in both lung development and disease (Jiang et al., 2010; Ornitz and Yin, 2012). In the epithelium, mice with loss of function of members of the miR-17 family show early lethality and hypoplastic lungs, whereas overexpression results in hyperproliferation and inhibition of differentiation of epithelial progenitors (Lu et al., 2007; Ventura et al., 2008). The miR-302 family regulates epithelial progenitor proliferation and differentiation, as well as apical-basal polarity (Tian et al., 2011). Recently, miR-375 has been shown to target WNT signaling and regulate the differentiation of alveolar epithelial cells by controlling the expression of the frizzled 8 gene (Wang et al., 2013). However, miRNAs regulating WNT signaling in the lung mesenchyme have yet to be reported. miR-142-3p is a miRNA first identified for its function in the development of the lymphoid system (Neilson et al., 2007), and was subsequently implicated in leukemia (Lv et al., 2012). In the lung, miR-142-3p is involved in malignancy and has been reported to be an early marker for aggressive and recurrent lung adenocarcinomas (Kaduthanam et al., 2013).

Herein, we detail miR-142-3p as a specific regulator of Apc expression in the mesenchyme. Using a loss-of-function approach, we analyzed the role of the miR-142-3p-Apc axis in mesenchymal cells. Using both pharmacological and genetic tools, we tested whether WNT signaling upregulation is sufficient to rescue miR-142-3p loss-of-function and whether Apc is a crucial target of this miRNA.

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RESULTS

miR-142-3p regulates mesenchymal cell proliferation and differentiation

In order to identify miRNAs with a specific function in the lung mesenchyme, we performed a microarray analysis on embryonic mouse lung tissue (Fig. 1A). We observed that 5 and 14 miRNAs were highly expressed, respectively, in the embryonic lung mesenchyme and the epithelium. Among those in the epithelium, we found several members of the miR-200 family. These miRNAs are involved in epithelial to mesenchymal transition (Brabletz and Brabletz, 2010), and their high expression in the embryonic epithelium suggests they may play a role in the plasticity of the branching tips during lung morphogenesis.

An in vitro loss of function (LOF) assay was optimized to functionally characterize the newly identified miRNAs in the mesenchyme. Embryonic day (E) 11.5 lung explants were grown in the presence of vivo-morpholino against either a scrambled sequence or a specific miRNA. We selected miR-126 as a positive control for our LOF assay because it was previously described to regulate vasculature formation. Using vivo-morpholino against miR-126 (mo126), we were able to impair vasculature formation (supplementary material Fig. S1A-L, n=3, P≤0.05), thus mimicking the phenotype observed in the miR-126 knockdown zebrafish (Fish et al., 2008). As a negative control we selected miR-144, because its inactivation does not lead to a specific lung phenotype in mice (Rasmussen et al., 2010). In our LOF assay,
**miR-142-3p regulates WNT signaling by directly controlling Apc expression**

To determine whether specific signaling pathways are involved in miR-142-3p regulation of mesenchymal cell proliferation, we utilized the prediction software miRTar and grouped the candidate genes by pathway. Interestingly, WNT signaling was the first hit (Table 1). To verify that WNT signaling was regulated by miR-142-3p in the mesenchyme, we performed a LOF assay on E11.5 lung explants obtained from a WNT reporter mouse line carrying β-galactosidase (β-Gal) expression under the endogenous control of the Axin2 promoter (Axin2\(^{−/−}\) mice). Staining of β-Gal after 24 hours showed a specific inhibition of WNT signaling in the distal mesenchyme (Fig. 2A-E; \(n=3\), \(P<0.05\)). Because miRNAs are negative regulators of gene expression, we aimed to identify WNT genes upregulated upon miR-142-3p knockdown. A qPCR screening for members of the WNT signaling pathway expressed in the lung was carried out at 12 hours and at 24 hours during our LOF assay. At 12 hours, we observed that Apc – a gene encoding a negative regulator of WNT signaling – was highly expressed. At 24 hours, Apc was still upregulated while other members were downregulated (Fig. 2F,G). Analysis of LEF1 expression by immunofluorescence confirmed a strong reduction of mesenchymal WNT signaling 24 hours after LOF assay (Fig. 2H-K). We reasoned that since the effect on proliferation, branching and WNT signaling were visible starting at 12 hours, the LOF assay confirmed an increase of mesenchymal Apc at 24 hours post-treatment showed a striking reduction of proliferation that, interestingly, was evident in the mesenchyme but not in the epithelium (Fig. 10-S; \(n=4\), \(P<0.05\)). Furthermore, inhibition of miR-142-3p led to ectopic expression of the SMC marker alpha-smooth muscle actin (\(α\)-SMA) (ACTA2) in the distal mesenchyme (Fig. 1T-X; \(n=3\), \(P<0.05\)). Together, these results suggest a function for miR-142-3p in the control of proliferation and differentiation of the lung mesenchyme.

**miR-142-3p downregulation leads to parabronchial smooth muscle cell progenitor differentiation**

Apc is a negative regulator of WNT signaling and, as a direct target of miR-142-3p, its increase can explain the drop in WNT signaling following miR-142-3p knockdown. In the embryonic lung mesenchyme, WNT, in combination with FGF signaling, creates a positive feedback loop. In fact, lack of contribution from either FGF or WNT signaling target genes such as Sox9, myostatin (Mstn) or Tbx1, 24-hour treatment showed a decrease of proliferation that, thus resulting in a decreased number of terminal buds (Fig. 1M; \(n=10\), \(P<0.05\)); supplementary material Movie 1). Reduction of lung growth, illustrated by a diminished total lung area (Fig. 1N; \(n=3\), \(P<0.05\)) was observed. Immunostaining for KI67 at 24 hours post-treatment showed a striking reduction of proliferation that, interestingly, was evident in the mesenchyme but not in the epithelium (Fig. 1O-S; \(n=3\), \(P<0.05\)). The number of branching points was decreased (Fig. 1L; \(n=3\), \(P<0.05\)) associated with impaired branching (Fig. 1C-J). The expression of Tbx1, Rock2 and Rac1 at 24 hours was among the five most highly ranked WNT signaling genes (Apc, Tbx1, Rock2, Rac1, Senp2) that were predicted as targets of miR-142-3p (Table 1). As Tbx1, Rock2 and Rac1 were not included in our first screening, we analyzed their expression 12 hours after the LOF assay. Although the expression of Apc and Senp2 was enhanced, expression of Tbx1, Rock2 and Rac1 showed no significant change (Fig. 2P; \(n=3\), \(P<0.05\)). The expression of Senp2 was reduced at 24 hours (Fig. 2G), suggesting that the modulation of its expression by miR-142-3p may not be as specific as for Apc.

We then wanted to determine whether miR-142-3p was able to physically bind the mRNA of its predicted target genes. For this purpose, we performed a pull-down experiment using a biotinylated miR-142-3p followed by qPCR to detect specific binding. The biotinylated miR-142-3p was able to cause overexpression of WNT signaling target genes such as Myc and Fgfr2 (supplementary material Fig. S2A,B), suggesting that biotinylation was not altering the property of miR-142-3p. In this assay, performed on a lung mesenchymal cell line (MLg cells), Apc was enriched more than six times compared with the control (Fig. 2M; \(n=3\), \(P<0.05\)). The same assay was reproduced in MFLM4 and HEK293 cells, suggesting that indeed miR-142-3p directly interact with Apc to regulate its expression (supplementary material Fig. S2C; \(n=3\), \(P<0.05\)). We further wanted to determine if miR-142-3p was binding Apc by specifically interacting with the predicted binding sites located at the 3′-UTR of Apc mRNA. For this purpose, we cloned Apc seed sites into two luciferase (Luc) reporter plasmids containing either a mutated binding site or a wild-type locus. We observed that the ability of miR-142-3p to target Apc was compromised in the presence of the mutated 3′UTR (Fig. 2N; \(n=3\), \(P<0.05\)).

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**Table 1. miR-142-3p targets pathway enrichment**

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Pathway</th>
<th>(P) value</th>
<th>Number of matched genes</th>
<th>Number of genes in pathway</th>
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<td>APC, TBL1X, SENP2, ROCK2, RAC1</td>
<td>Wnt signaling</td>
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<td>APC, ROCK2, RAC1</td>
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<td>73</td>
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<tr>
<td>ROCK2, RAC1</td>
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<td>9.25E-03</td>
<td>2</td>
<td>187</td>
</tr>
<tr>
<td>ROCK2, RAC1</td>
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<td>2</td>
<td>187</td>
</tr>
<tr>
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<td>191</td>
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<td>201</td>
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<tr>
<td>APC, RAC1</td>
<td>Pathways in cancer</td>
<td>5.21E-02</td>
<td>2</td>
<td>415</td>
</tr>
</tbody>
</table>
signaling observed in our miR-142-3p LOF assay was associated with perturbation of the FGF-WNT sustained regulation. Indeed, qPCR on E11.5 lung explants showed decreased Fgfr2c expression beginning 24 hours into the assay (Fig. 3D-F; n=3, \( P<0.05 \)). At 48 h, Fgfr2c reduction was associated with a specific decrease in the mesenchyme of phosphorylated ERK (pERK; Fig. 3M-Q; n=4, \( P<0.05 \)) and phosphorylated MEK (pMEK; Fig. 3R-V; n=3, \( P<0.05 \)). One of the functions of FGF9-FGFR2c signaling in the lung is to keep mesenchymal PBSMC progenitors undifferentiated and proliferating (Yi et al., 2009). Furthermore, we have previously shown that FGF10 is expressed in, and identifies PBSMC progenitors (Mailleux et al., 2005). Analysis by qPCR after the miR-142-3p LOF assay showed that Fgfr2b expression remained stable, suggesting that epithelial FGF signaling was unaffected (Fig. 3J-L; n=3, \( P<0.05 \)). However, Fgf10 started decreasing at 24 hours and was significantly reduced at 48 hours (Fig. 3G-I; n=3, \( P<0.05 \)). This result suggests that Fgf10-positive PBSMC progenitors were specifically affected by the imbalance of FGF-WNT regulation. We therefore decided to analyze the effect of our miR-142-3p LOF assay on SMC differentiation. To directly analyze PBSMC progenitors during miR-142-3p downregulation, conditional red fluorescent protein (RFP) reporter mice (\( \text{RFPf/f} \)) were crossed with mice carrying \( \text{Fgf10CreERT2} \). We will refer to these mice with genotype \( \text{Fgf10CreERT2/+; RFPf/+} \) as \( \text{RFPFgf10} \).

Live imaging of \( \text{RFPFgf10} \) E11.5 lung explants treated with miR-142-3p vivo-morpholino (mo142; supplementary material Movie 2)
showed that labeled PBSMC progenitors formed ring-like structures, reminiscent of smooth muscle cell phenotype, at the tip of the epithelial buds (Fig. 4A-D). These data are in agreement with the ectopic alpha-SMA (ACTA2) expression at the tip of the epithelial buds during mo142 treatment (Fig. 1T-W). Additionally, in mo142-treated explants, labeled cells displayed reduced motility and proliferation, but in scrambled-treated explants, they were highly motile and proliferative (supplementary material Movie 2; Fig. 4E-G; n=3, P<0.05). Our results suggest that PBSMC progenitors prematurely differentiate upon mo142 treatment. To further demonstrate that this was due to loss of FGF9-FGFR2c signaling, primary embryonic lung mesenchymal cells were cultured in vitro in different experimental conditions. Scrambled-treated explants, labeled cells displayed reduced motility and proliferation, but in scrambled-treated explants, they were highly motile and proliferative (supplementary material Movie 2; Fig. 4E-G; n=3, P<0.05). Our results suggest that PBSMC progenitors prematurely differentiate upon mo142 treatment. To further demonstrate that this was due to loss of FGF9-FGFR2c signaling, primary embryonic lung mesenchymal cells were cultured in vitro in different experimental conditions. Scrambled-treated primary mesenchymal cells grown in the presence of FGF9 were prevented from differentiating into SMCs (Fig. 4H,I). After miR-142-3p knockdown, FGF9 was no longer effective and thus differentiation into SMCs occurred (Fig. 4J-L). The role of miR-142-3p in SMC differentiation was further evaluated using a gain of function approach. We used mesenchymal cells from a mouse lung mesenchymal cell line (MLg cells). Under normal in vitro culture conditions, MLg cells differentiate into ACTA2-positive cells. Overexpression of miR-142-3p by a synthetic form of miR-142-3p that mimics its function (mi142) led to reduced ACTA2 expression and diminished elongation (Fig. 4M-O). Because Apc is a specific target for miR-142-3p (Fig. 2H-N), we wanted to determine if Apc overexpression is sufficient to mimic loss of miR-142-3p knockdown. Primary mesenchymal cells isolated from RFPFgf10 lungs were electroporated with either control plasmids (pGFP) or experimental plasmids overexpressing APC (pAPC). Apc overexpression was sufficient to mimic loss of miR-142-3p, producing reduced proliferation and motility of PBSMC progenitors (Fig. 4P-V; n=3, P<0.05; supplementary material Movie 3).

**Constitutive WNT signaling rescues miR-142-3p knockdown**

The inhibition of WNT signaling in the miR-142-3p LOF assay prompted us to attempt a rescue experiment in which WNT...
signaling was stimulated at different levels of the pathway. We began with WNT3a, a commercially available WNT ligand. Lungs treated with WNT3a display expansion of the epithelium and mesenchyme compared with BSA-treated lungs (data not shown and supplementary material Fig. S3C,D). Upon miR-142-3p attenuation, WNT3a was unable to affect mesenchymal growth (supplementary material Fig. S3A,B,D; n=3, P<0.05). Similar results were obtained by using FGF9 (Fig. 5A-D; n=3, P<0.05) and FGF9 in combination with scra (scra or mo142) administration. MLG cells were analyzed for ACTA2 expression by immunostaining after scra (M) or mi142 (N) administration. (O) Quantification of cellular morphology as a ratio of length to width. (P-S) RFP-Fgf10 primary mesenchymal cells were electroporated with a plasmid expressing GFP (pGFP) or APC (pAPC) and analyzed between 0 and 45 hours. (T-V) Quantification of the cell motility (TU) and proliferation (V) for pGFP- and pAPC-treated cells. Dashed boxes indicate the magnified areas shown in the insets. Dashed lines demarcate the epithelium-mesenchyme boundary. Scale bars: 250 μm (A,C); 50 μm (B,D); 50 μm (H-K insets); 20 μm (H-K main image); 50 μm (M,N); 75 μm (P-S). Data are means ± s.d. See also supplementary material Movies 2 and 3.
with WNT3a (supplementary material Fig. S3E-H; n=3, P<0.05).
Under these conditions FGF9 still caused expansion of the epithelium. This result further confirms that when miR-142-3p expression is inhibited the positive feedback loop between FGF and WNT signaling in the mesenchyme is impaired. To intervene downstream of WNT signaling, we used mice harboring a floxed allele in the Ctnnb1 gene (Ctnnb1(ex3)f) to generate a stable form of Ctnnb1. Crossing this line with Tbx4LMECreERT2 mice, we were able to obtain inducible constitutive activation of Ctnnb1, specifically in the mesenchyme. We will refer to the genotype Tbx4LMecreERT2/+; Ctnnb1(ex3)f/+ as Ctnnb1f/+;Tbx4, Ctnnb1f/+;Tbx4 and Ctnnb1f/+ lungs from the same mating represented WNT-activated and control lungs, respectively, and were used in the LOF assay (Fig. 5E). Lungs from Tbx4LMecreERT2/+ mice injected with tamoxifen intraperitoneally were also used as controls in the LOF assay, showing similar results to the Ctnnb1f/+ lungs (data not shown). Forty-eight hours after the miR-142-3p LOF assay, the activation of CTNNB1 signaling in Ctnnb1f/+;Tbx4 lungs, compared...
to Ctnnb1f/f lungs (supplementary material Fig. S3-M-P), led to the disappearance of mo142-induced MYH11 (supplementary material Fig. S3-L) and ACTA2 ectopic expression (Fig. 5F-I,N; n=3, P<0.05) at the tip of the epithelial buds. We also observed increased pMEK expression in mo142-treated Ctnnb1f/f Tbx4CreERT2 compared with Ctnnb1f/f lungs, suggesting recovery of FGF signaling in the mesenchyme (Fig. 5J-O; n=3, P<0.05).

**Reduction of Apc expression rescues miR-142-3p knockdown**

Having identified Apc as a direct target of miR-142-3p, we decided to test whether reduction of Apc by ablation of one of the two copies of the gene was able to rescue the effect of miR-142-3p inhibition. For this purpose we used Apc<sup>−/−</sup> mice crossed with Tbx4LME<sup>CreERT2</sup> mice. We will refer to the genotype Tbx4LME<sup>CreERT2/−</sup>; Apc<sup>−/−</sup> as Apc<sup>−/−;Tbx4</sup>. Apc<sup>−/−</sup> lungs were used as controls. Tbx4LME<sup>CreERT2/−</sup> were also used as controls with similar results to Apc<sup>−/−</sup> lungs in the LOF assay (data not shown). Forty-eight hours after the miR-142-3p LOF assay, the reduction of APC expression in Apc<sup>−/−;Tbx4</sup> compared with Apc<sup>−/−</sup> lung explants was confirmed by qPCR (data not shown) and immunohistochemistry (Fig. 5DD-GG). This reduction led to the disappearance of mo142-induced ectopic expression of ACTA2 at the tip of the epithelial buds (Fig. 5Z-C; n=3, P<0.05), as well as increased Ki67 expression in the mesenchyme (Fig. 5DD-GG; n=3, P<0.05). In addition, Apc<sup>−/−;Tbx4</sup> lungs were able to recover growth and branching (Fig. 5P-Y; n=3, P<0.05). These results suggest that the inhibition of Apc alone is sufficient to counteract miR-142-3p knockdown.

**DISCUSSION**

In this study we describe a new function of the microRNA machinery in the lung embryonic mesenchyme. We determined that miR-142-3p is a crucial regulator of WNT signaling in this compartment. By directly controlling Apc expression, miR-142-3p can fine-tune its inhibition of WNT signaling. Furthermore, miR-142-3p controls proliferation and differentiation of mesenchymal progenitor in the embryonic lung (Fig. 6A,B).

A fine balance between FGF and WNT signaling helps maintain the pool of mesenchymal progenitor cells in the lung embryonic mesenchyme. For example, evidence suggests that FGF9 from the mesothelium can signal to Fgf10-positive cells located in the submesothelial mesenchyme to prevent differentiation (Colvin et al., 2001). In this system, WNT signaling helps maintain proper levels of FGF signaling, because reduced WNT signaling creates disequilibrium in the feedback loop that maintains mesenchymal FGF-WNT signaling (De Langhe et al., 2008; Yin et al., 2011). Here, we found that miR-142-3p is implicated in the modulation of the WNT and FGF balance, adding an extra layer of control that increases the robustness of this system.

We show that miR-142-3p fulfills this function by directly controlling Apc expression, therefore acting as a negative regulator of an inhibitor of WNT signaling. Dereegulation of miR-142-3p expression uncouples WNT and FGF signaling in the mesenchyme, affecting mesenchymal progenitor cells differentiation. Specifically, the Fgf10-positive lineage which contributes to the formation of parabronchial smooth muscle cells is affected by the deregulation of miR-142-3p expression, resulting in premature differentiation and proximalization of the mesenchyme.

Recently, miR-142-3p was found to control hemangioblast specification and vasculogenesis in Xenopus (Nimmo et al., 2013). In our LOF assay we observed impaired angiogenesis of the lung explants, which was associated with enrichment of markers for undifferentiated hemangioblasts such as CD31 (data not shown). This suggests that a similar regulation could also contribute to the vasculogenesis in the lung. It will be interesting to determine if miR-142-3p controls similar pathways – for instance WNT signaling – or if the two systems are completely independent.

Although our screening identified Apc as a main target of miR-142-3p, other genes are likely to be affected. In particular, other members of WNT signaling were found to bind to biotinylated miR-142-3p to a lower degree than Apc. Nonetheless, rescue experiments with Apc<sup>−/−</sup> mice showed that the inhibition of Apc in the mesenchyme is sufficient to rescue the lung from miR-142-3p downregulation, suggesting that Apc is a crucial molecule regulated by miR-142-3p in the lung mesenchyme. We also found that in the parenchyma of the adult lungs, miR-142-3p and APC are mutually exclusive (data not shown). This suggests that the regulation here described may not be limited to the embryonic lung. In addition, our finding is consistent with the observation that inactivation of Apc in the lung mesenchyme using a Tbx4-rtTA driver mouse line leads to reduced and disorganized smooth muscle cell differentiation in the lung (personal communication from Wei Shi). This suggests that APC per se, has a critical role in the regulation of proliferation, survival, and differentiation of smooth muscle progenitor cells.

In summary, our work shows that miR-142-3p is intimately linked to mesenchymal activated WNT signaling by specific regulation of Apc. It will be of interest to determine if the miR-142-3p-Apc–β-catenin axis plays a role in lung diseases such as idiopathic pulmonary fibrosis, in which cellular proliferation and differentiation of mesenchymal cells is unbalanced.
Genes and Genomes (KEGG) analysis for pathway enrichment was performed. MFE

Laboratory as previously described (El Agha et al., 2012). Ctnnb1 flox/flox mice were generated in our laboratory as previously described (Alexer et al., 2012) and used as negative control. Tbx4LMECreERT2/ Y-box 4 large muscle enhancer cre-recombinase were used to generate Tbx4LMECreERT2/animals, which were intercrossed with Ctnnb1 flox/flox mice to obtain Tbx4LMECreERT2/;Ctnnb1 flox/flox double knockouts. Tbx4LMECreERT2/+ and Ctnnb1 flox/flox mice were used as control.

Microarray analysis and qPCR

Epithelium from E12.5 lung buds was isolated as previously described (Carraro et al., 2009). The quality of the total RNA was verified using an Agilent 2100 Bioanalyzer profile. Total RNA (1000 ng) was labeled with fluorescent Hy3 and Hy5 using the miRCURY LNA Array power labeling kit (Exiqon) following the procedure described by the manufacturer. The hybridization was performed according to the miRCURY LNA Array version 5th Generation (Exiqon). The hybridization was performed according to the miRCURY LNA array manual using an HS4800 hybridization station (Tecan). The slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies) and the image analysis was carried out using ImaGene 8.0 software (BioDiscovery). The quantified signals were background corrected (Normexp with offset value 101) (Ritchie et al., 2007), and normalized using the global Lowess (Locally Weighted Scatterplot Smoothing) regression algorithm. microRNAs that were more than fourfold differentially expressed were considered for validation by qPCR. Statistical analyses were performed using Student’s t-tests. RT-PCR for miRNA was carried out using SuperScript II reverse transcriptase (Invitrogen) with random primers, and in RT-PCR for miRNA the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) was used. In both cases, reactions were assembled following the manufacturer’s recommendations. qPCR was performed on a LightCycler 480 system (Roche). The universal probe library (Roche) was used for the analysis of mRNA expression. The Taqman microRNA assay (Applied Biosystems) was used for screening the differential expression of microRNAs. qPCR reactions and data analysis were performed as previously described (Carraro et al., 2009). Results are presented as relative expression compared with control. Results were collected from at least three lungs or pools of lungs from independent experiments.

MATERIALS AND METHODS

Mouse strains

C57BL/6 wild-type, Apc flox/+; Tmam1 cre/+ and Axin2 cre/cre mice were obtained from Jackson Laboratories. Figf cre/cre mice were generated in our laboratory as previously described (El Agha et al., 2012). Ctnnb1 flox/flox mice were generated in the Taketo laboratory (Harada et al., 1999). Tbx4LME cre/cre mice were generated in the Krasnow laboratory (M.E.K., Patrick E. Bogard, F. Hernán Espinoza, Douglas B. Menke, David M. Kingsley and Mark A. Krasnow, unpublished data). For rescue experiments, tamoxifen was administrated by intraperitoneal injection (0.1 mg/g of body weight) 24 hours before the LOF assay. Animals were housed at room temperature with a 12 hour/12 hour light/dark cycle and free access to food and water. The Federal Authorities for Animal Research of the Regierungspräsidium Giessen (Hessen, Germany) approved the study protocol (MIR-142-3p protocol 52/2012).

MicroRNA array and qPCR

Epithelium from E12.5 lung buds was isolated as previously described (Carraro et al., 2009); mesenchyme from E12.5 lung buds was physically isolated using 0.12 mm tungsten needles (Fine Science Tools). Total RNA was extracted using a miRNeasy Mini Kit (Qiagen). The quality of the total RNA was verified using an Agilent 2100 Bioanalyzer profile. Total RNA (1000 ng) was labeled with fluorescent Hy3 and Hy5 using the miRCURY LNA Array power labeling kit (Exiqon) following the procedure described by the manufacturer. The labeled RNAs were mixed pair-wise and hybridized to the miRCURY LNA Array version 5th Generation (Exiqon). The hybridization was performed according to the miRCURY LNA array manual using an HS4800 hybridization station (Tecan). The slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies) and the image analysis was carried out using ImaGene 8.0 software (BioDiscovery). The quantified signals were background corrected (Normexp with offset value 101) (Ritchie et al., 2007), and normalized using the global Lowess (Locally Weighted Scatterplot Smoothing) regression algorithm. microRNAs that were more than fourfold differentially expressed were considered for validation by qPCR. Statistical analyses were performed using Student’s t-tests. RT-PCR for miRNA was carried out using SuperScript II reverse transcriptase (Invitrogen) with random primers, and in RT-PCR for miRNA the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) was used. In both cases, reactions were assembled following the manufacturer’s recommendations. qPCR was performed on a LightCycler 480 system (Roche). The universal probe library (Roche) was used for the analysis of mRNA expression. The Taqman microRNA assay (Applied Biosystems) was used for screening the differential expression of microRNAs. qPCR reactions and data analysis were performed as previously described (Carraro et al., 2009). Results are presented as relative expression compared with control. Results were collected from at least three lungs or pools of lungs from independent experiments.

Culture of embryonic lung explants

Timed-pregnant wild-type or transgenic mice were sacrificed on post-coitum (embryonic) day 11.5 (E11.5), and the embryos were harvested. Lung primordia were placed on 8 μm Nucleopore Track-Etch membranes (Whatman) and cultured in DMEM:F12 medium (Gibco) and 0.5% fetal bovine serum (FBS). Vivo-morpholinos (Gene Tools) were added at 1-4 μM to the lung explants. Recombinant FGF9 and WNT3a (R&D) were added at 200 ng/ml and 250 ng/ml, respectively. The data are representative of at least three lungs from different timed pregnant mice.

Computational targets prediction

The mir-142-3p targets were selected on the basis of the on-line available prediction database mirTar (mirTar.mbc.nctu.edu.tw). The 5’-UTR and the 3’-UTR of target genes were included in the screening. A minimum free energy (MFE) ≤ 51 Kcal/mol and Score ≥ 140 were used. A Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis for pathway enrichment was performed.

β-galactosidase staining

Axin2 cre/cre lung explants were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C for 5 minutes with rocking, washed twice for 5 minutes in PBS at 4°C, then transferred into freshly prepared X-gal solution and stained at 37°C until a clear precipitate formed; the method was modified from that of Hogan et al. (Hogan et al., 1994). After rinsing with PBS, epithelial explants were post-fixed in 4% PFA in PBS. For vibratome sections, samples were embedded in a mixture of 300 mg/ml albumin, 5 mg/ml gelatin and 0.6% glutaraldehyde, and sectioned. The data are representative of three lungs from independent experiments.

Immunofluorescence

Tissues were fixed in 4% PFA, gradually dehydrated in ethanol, impregnated with toluene, embedded in paraffin, and sectioned into 5 μm slices on poly- L-lysine-coated slides. Antigen retrieval was performed by boiling the sample for 10 minutes in sodium citrate buffer (10 mM, pH 6.0; Vector). The following antibodies (Abs) were used: APC Ab (Abcam), K67 Ab (Novabios), phospho-ERK (pERK) Ab (Cell Signaling), phospho-MEK Ab (Cell Signaling), LEF1 Ab (Cell Signaling), phospho-Ser525-CTNNB1 Ab (Cell Signaling), ACTA2 Ab (Sigma), MYH11 Ab (Sigma). All antibodies were used at a 1:200 dilution. The data are representative of at least three lungs from independent experiments.

Image analysis

Photomicrographs of immunofluorescence staining were taken using a Leica DMRA fluorescence microscope with a Leica digital camera. Live-cell and lung explant time-lapse experiments were performed with a Leica AF6000 fluorescence imaging system. Digital analysis of staining intensity and distribution, and of live-imaging experiments was performed with MetaMorph (Molecular Devices) or ImageJ software. At least three different samples for each condition were analyzed. Phase-contrast images of the samples were recorded using a digital camera (Diagnostic Instruments) connected to a reversed phase-contrast microscope (Leica). The public domain software ImageJ was broadened by routines specifically developed by us and used to process and analyze the images (Guidolin et al., 2004). The number of branches, percentage of tissue area involved in branching, and branching complexity (i.e. the number of branching points of the binary skeleton and the distribution of branching orders) were the morphometric parameters estimated from each sample. Data were processed by using statistical analysis software (GraphPad Prism 3.03, GraphPad Software). The data are representative of at least three lungs from independent experiments.

miRNA pull-down assay

MLG, MFLM4 or HEK293 cells were cultured in six-well plates and transfected in triplicate with 3’-biotinylated miR-142-3p (Bio-miR-142) or 3’-biotinylated scrambled (Bio-scramble) (Dharmacon), at a final concentration of 30 nM using Lipofectamine RNAmax (Invitrogen) following the manufacturer’s protocol. After 48 hours, the cells were pelleted at 1000 rpm for 5 minutes. After washing twice, cell pellets were resuspended in 0.5 ml lysis buffer (50 mM Tris-HCl 2 mM EDTA, 0.1% NP40, 10% glycerol, 2 mM EGTA, diethylycarbonte (DEPC)-treated water, 50 μ RNasin (Promega) and complete mini-protease inhibitor cocktail (Roche Applied Science), and incubated at 4°C for 10 minutes. The cytoplasmic extract was isolated by centrifugation at 10,000 rpm for 10 minutes. Streptavidin-coated magnetic beads (Invitrogen) were blocked for 1 hour at 4°C in blocking buffer (10 nM Tris-HCl pH 6.5, 1 mM EDTA, 1 mg/ml yeast RNA and 1 mg/ml BSA) and washed twice with 1 ml washing buffer (10 mM Tris-HCl pH 6.5, 1 mM EDTA 0.5 M NaCl). Beads were resuspended in 0.5 ml washing buffer. Cytoplasmic extract was then added to the beads and incubated for 1 hour at 4°C with slow rotation. The beads were then washed five times with 1 ml washing buffer. RNA bound to the beads (pull-down RNA) or from 10% of the extract (input RNA), was isolated using Trizol reagent LS (Invitrogen). The level of miRNA in the Bio-mir-142-3p or Bio-scramble control pull-down was quantified by qPCR. miRNA levels were normalized to a housekeeping gene (Gapdh, H4). The enrichment ratio of the control-normalized pull-down RNA to the control-normalized input levels was then calculated. The data for each cell line are representative of three independent experiments.

Luciferase reporter assay

The predicted binding sequence on the 3’ UTR of Apc was synthetically produced by PCR and transferred into a luciferase reporter vector. To
determine the specificity of the interaction, a reporter vector containing a 'seed' mutated sequence was generated using specific primers. The vector backbone was a pmiRGLO luciferase reporter (Promega) carrying a synthetic firefly luciferase (Iuc2) and a Renilla luciferase. Our constructs were inserted downstream of the luciferase gene into a unique XbaI restriction site. The Renilla luciferase was used as a control to normalize luminescence levels. Experiments were carried out using a dual-luciferase reporter assay system (Promega) and analyzed with a Victor® Luminometer.

Cell culture
For the preparation of primary mesenchymal cells, whole lungs were dissected at E14.5 and subjected to trypsin digestion to give rise to single cells. Mesenchymal cells were separated from epithelial cells by differential adhesion as previously described (Lebeche et al., 1999). Fifty minutes after plating, 200 ng/ml FGF9 was added to the mesenchymal cells. The cells were cultured in DMEM/F12 medium containing penicillin, streptomycin and 0.5% FBS in the presence or absence of FGF9. MFLM4 (Seven Hills), MLg (ATCC) and HEK293 (ATCC) cells were grown in DMEM with 10% FBS. Plasmid expressing GFP (pGFP) and APC (pAC) were introduced into the cells by electroporation (Lonza). MLg cells were electroporated with 30 nM scramble (scra) or miR-142-3p mimic (mi142) molecules (Dharmacon).

Statistical analysis
All data are reported as means ± s.d. Statistical analyses were performed using Student’s t-tests, with P<0.05 considered significant.

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Competing interests
The authors declare no competing financial interests.

Author contributions
G.C. developed the approach, performed experiments and prepared the manuscript. A.G. developed the approach, performed experiments and prepared the manuscript. A.S., J.R., A.C., C.C., E.A. and B.M. performed experiments. S.D. and G.C. developed the approach, performed experiments and prepared the manuscript. S.B. developed the approach and prepared the manuscript prior to submission.

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Supplementary material
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