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miR-17 family of microRNAs controls FGF10-mediated embryonic lung epithelial branching morphogenesis through MAPK14 and STAT3 regulation of E-Cadherin distribution

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ABSTRACT

The miR-17 family of microRNAs has recently been recognized for its importance during lung development. The transgenic overexpression of the entire miR-17–92 cluster in the lung epithelium led to elevated cellular proliferation and inhibition of differentiation, while targeted deletion of miR-17–92 and miR-106b–25 clusters showed embryonic or early post-natal lethality. Herein we demonstrate that miR-17 and its paralogs, miR-20a, and miR-106b, are highly expressed during the pseudoglandular stage and identify their critical functional role during embryonic lung development. Simultaneous downregulation of these three miRNAs in explants of isolated lung epithelium altered FGF10 induced budding morphogenesis, an effect that was rescued by synthetic miR-17. E-Cadherin levels were reduced, and its distribution was altered by miR-17, miR-20a and miR-106b downregulation, while conversely, beta-catenin activity was augmented, and expression of its downstream targets, including *Bmp4* as well as *Fgfr2b*, increased. Finally, we identified *Stat3* and *Mapk14* as key direct targets of miR-17, miR-20a, and miR-106b and showed that simultaneous overexpression of *Stat3* and *Mapk14* mimics the alteration of E-Cadherin distribution observed after miR-17, miR-20a, and miR-106b downregulation. We conclude that the mir-17 family of miRNA modulates FGF10–FGFR2b downstream signaling by specifically targeting *Stat3* and *Mapk14*, hence regulating E-Cadherin expression, which in turn modulates epithelial bud morphogenesis in response to FGF10 signaling.

Introduction

Lung development is under the control of a complex network of regulatory proteins including those that participate in the FGF, TGFbeta, SHH, and WNT pathways (Cardoso and Lu, 2006; Warburton et al., 2005). Branching and growth of the epithelium are principally controlled by the FGF10–FGFR2b signaling pathway, wherein a mesenchymal FGF10 gradient acts as a chemoattractant for the endoderm and promotes its proliferation (Bellusci et al., 1997; Park et al., 1998) *Fgf10* expression is strictly controlled by endodermal SHH pathways that function to finely tune *Fgf10* expression, assuring the correct size and shape of the growing buds (Pepicelli et al., 1998; Warburton, 2008). The growth of the developing lung must be continuously calibrated. In the mesenchyme, for example, fibronectin deposition is strictly controlled by WNT signaling during the early stages of branching morphogenesis, and alterations of such regulation lead to impaired branching with enlarged terminal buds (De Langhe et al., 2005).

Cadherins are a large family of glycoproteins that mediate specific cell–cell adhesion in a Ca^{2+}-dependent manner (Van Roy F. and Berx G., 2008). E-Cadherin (CDH1) is an important cadherin that is highly expressed in the epithelium throughout lung development. Structurally, CDH1 is comprised of a single transmembrane domain, an ectodomain for binding to other adjacent cells (Leckband and Prakasam, 2006), and a cytoplasmic domain that interacts with a variety of molecules that link CDH1 to the actin cytoskeleton, and hence to cell signaling and trafficking mechanisms (Bryant and Stow, 2004; Mege et al., 2006; Yap and Kovacs, 2003). *Cdh1* expression is promoted by transcription factors that bind cis-regulatory elements present in introns 1 and 2 (Goomer et al., 1994; Hennig et al., 1996; Liu et al., 2005), and can also be downregulated by specific transcription factors and corepressors (Peinado et al., 2007; Shi et al., 2003). CDH1 can undergo endocytosis and recycling through an endosomal

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pathway that modulates its expression at the cell surface during dynamic morphogenetic processes (Bryant and Stow, 2004). CDH1 endocytosis, ubiquitination, and transport to the cell surface are finely regulated, adding another level of control over its activity (Davis et al., 2003; Palacios et al., 2002; Palacios et al., 2005). The regulation of *Cdh1* and its network of regulatory molecules has been widely investigated at the transcriptional and post-translational levels, but the effect of translational regulation is poorly understood.

An emerging understanding of the functional roles of microRNAs (miRNAs) in the post-transcriptional regulation of gene expression has shed light on the growing importance of these sub-cellular interactions. MiRNAs are small non-coding RNAs that can bind the 3′-UTR of target mRNAs and thus specifically inhibit their translation. The function of miRNAs can vary from acting as a molecular switch for specific events, to finely tuning or maintaining the stability and robustness of specific states. MiRNAs can also function as a control to eliminate specific gene expression on a translational level in specific locations (Brennecke et al., 2003; Cohen et al., 2006; Hornstein et al., 2005; Johnston and Hobert, 2003; Kwon et al., 2005; Sokol and Ambros, 2005).

In the lung, tissue-specific deletion of Dicer, the enzyme responsible for producing active miRNAs, results in branching arrest, revealing the importance of miRNAs for epithelial morphogenesis (Harris et al., 2006). The miR-17 family of miRNA, is comprised of three paralog clusters (miR-17–92, miR-106a–363, and miR-106b–25). The miR17–92 cluster has recently been described for its importance during lung development. In mice the miR-17–92 cluster is located on chromosome 14 and contains six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92–1). The miR-106a–363 cluster is located on mouse chromosome X and contains six miRNAs (miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92–2, and miR-363), while miR-106b–25 is located on mouse chromosome 5 and contains three miRNAs (miR-106b, miR-93, and miR-25). Transgenic over-expression of the miR-17–92 cluster has been produced, showing its importance in controlling differentiation and proliferation of lung progenitor cells (Lu et al., 2007). Targeted deletion of the miR-17–92 cluster has been shown to produce smaller embryos with post-natal lethality, characterized by severely hypoplastic lungs. Furthermore during combined deletion of miR-17–92 cluster and its paralog miR-106b–25, mice showed earlier lethality before embryonic day 15 (Ventura et al., 2008).

Herein we report a specific functional role for miR-17, miR-20a, and miR-106b, in maintaining the structural homeostasis of developing lung epithelium. We show that miR-17, miR-20a, and miR-106b, target *Mapk14* and *Stat3* and thereby contribute to correctly modulating CDH1 expression in the epithelium in response to FGF10–FGFR2b signaling.

Materials and methods

RT-PCR and Real-time PCR

Total RNA was extracted using a miRNesasy Mini Kit (Qiagen) following the manufacturer's recommendations. For RNA extraction from lung tissue a pool of three samples was used. RT-PCR for mRNA was carried out using SuperScript II reverse transcriptase (Invitrogen) with random primers, while 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.

Real-time PCR was performed on a LightCycler 480 system (Roche). The universal probe library (Roche) was used for the analysis of mRNA expression. The miRNA Early Access Kit or Taqman microRNA assays (Applied Biosystems) were used for the screening of miRNAs differentially expressed during different stages of lung development. Primers and probes were used at a final concentration of 250 nM and 125 nM respectively. Following denaturation for 10 min at 95 °C, 45 cycles were performed with 10 s at 95 °C and 30 s at 60 °C. A relative quantification was performed using LightCycler 480 Software, and data were normalized with Actb for mRNA and U6 RNA for miRNA.

Immunoblotting

Samples were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing 1:100 dilution of protease inhibitor cocktail set III (Calbiochem). Immunoblotting was performed as previously described (Ding et al., 2007). The following primary antibodies were used: CDH1 (BD Biosciences) was used at 1:1000, STAT3 (Cell Signaling) was used at 1:2000, and MAPK14 (Cell Signaling) was used at 1:2000. ACTB (Chemicon) was used as a loading control and was used at 1:2000. Protein quantification was produced by densitometry analysis with the software Image J (Rasband W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij).

In situ hybridization

Embryonic lung and epithelial lung explants were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) with rocking at 4 °C for 2 h and 5 min respectively, washed twice for 5 min in PBS at 4 °C, dehydrated in several washes with 70% ethanol (EtOH), and stored in absolute EtOH at −20 °C. For the in situ hybridization of miR-17 we used a modified protocol (Winnier et al., 1995) after taking the short size of mature miRNA into consideration. Samples were washed in *Tris*-Buffered Saline (TBS)/Tween-20 0.1% (TBST), digested with Proteinase K, fixed again in 4% PFA and washed in TBST. The samples were then transferred to hybridization buffer (Formamide 50%, SSC 5×, tRNA 0.05 mg/ml, sodium dodecyl sulfate 1%, Heparin 0.05 mg/ml) for 1 h at 50 °C and then hybridized with the probe at 55 °C over night. Locked Nucleic Acid (LNA) probes (Exiqon) were used to increase the stability of the hybridization. The next day the lung was washed with standard washing solutions. All washes were performed at 50 °C. After the washes the lungs were added to blocking solution made with sheep serum for 1 h and then incubated over night a 4 °C with anti-DIG antibody. The color reaction was performed with BM Purple solution after washes with TBST and NTMT (NaCl 100 mM, Tris pH9.5 100 mM, Tween-20 0.1%).

Isolated epithelial explant culture

Lungs from E11.5 embryos were treated with dispase (BD Biosciences) for 5 min at 4 °C and then transferred to Dulbecco's Modified Eagle's Medium:Nutrient Mixture F-12 (DMEM:F12) with 10% fetal bovine serum (FBS) (Invitrogen) to block the enzymatic reaction. The epithelial buds were separated from mesenchyme using tungsten needles (Fine Science Tools), embedded in 200 µl of Matrigel (BD Biosciences) diluted 1:1 with DMEM:F12 serum-free media containing 250 ng/ml FGF10 (R and D Systems), and left to polymerize by incubation at 37 °C for 20 min . The same media used for the matrigel mix was then added to the surface of the polymerized gel, and the samples were incubated at 37 °C (modified from Bellusci et al. (1997)). Locked nucleic acid against miR-17 (LNA17) or scrambled (SCRA) (Exiqon), or miR-17 mimic molecules (MIM17) (Thermo Scientific), were added at 1.5 µM to the isolated epithelium in presence of 0.3% lipofectamine RNAiMAX (Invitrogen).

Image analysis

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