Mig-6 is required for appropriate lung development and to ensure normal adult lung homeostasis

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Mitogen-inducible gene 6 [Mig-6; Erffi1 (ErbB receptor feedback inhibitor 1); RALT (receptor-associated late transducer); gene 33] is a ubiquitously expressed adaptor protein containing CRIB, SH3 and 14-3-3 interacting domains and has been shown to negatively regulate EGF signaling. Ablation of Mig-6 results in a partial lethal phenotype in which surviving mice acquire degenerative joint diseases and tumors in multiple organs. We have determined that the early lethality in Mig-6–/– mice occurs in the perinatal period, with mice displaying abnormal lung development. Histological examination of Mig-6–/– lungs (E15.5-P3) revealed reduced septation, airway over-branching, alveolar type II cell hyperplasia, and disturbed vascular formation. In neonatal Mig-6–/– lungs, cell proliferation increased in the airway epithelium but apoptosis increased in the blood vessels. Adult Mig-6–/– mice developed features of chronic obstructive pulmonary disease (COPD); however, when Mig-6 was inducibly ablated in adult mice (Mig-6d/d), the lungs were normal. Knockdown of Mig-6 in H441 human bronchiolar epithelial cells increased phospho-EGFR and phospho-AKT levels as well as cell proliferation, whereas knockdown of Mig-6 in human lung microvascular endothelial (HMVEC-L) cells promoted their apoptosis. These results demonstrate that Mig-6 is required for prenatal and perinatal lung development, in part through the regulation of EGF signaling, as well as for maintaining proper pulmonary vascularization.

KEY WORDS: Mitogen-inducible gene 6 (Mig-6), Gene ablation, Lung development, EGF signaling, Vascularization

INTRODUCTION

Lung development requires the appropriate integration of extracellular signaling and transcriptional regulation to ensure that appropriate morphological architecture and cellular differentiation occur to allow development of a fully functional organ (Perl and Whitsett, 1999). Although lung development is completed during the neonatal period, appropriate neonatal lung development is crucial for the formation of fully functional adult lungs (Shi et al., 2007). Dysfunctional lung development results in a higher propensity towards adult lung diseases, including chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis (Shi et al., 2007). Using mouse model systems, numerous pathways have been identified as regulating neonatal lung development.

Mouse lung development is precisely regulated by a number of spatially and temporally expressed growth factors and transcription factors (Warburton et al., 2000), which regulate the growth and differentiation of all compartments of the lung including the epithelium, stroma and vasculature. Among the essential factors, epidermal growth factor (EGF) signaling is important for both early- and late-stage lung development (Kramer et al., 2007; Le Cras et al., 2004; Miettinen et al., 1997). Ablation of epidermal growth factor receptor (EGFR) leads to reduced lung branching and altered epithelial cell differentiation (Sibilia and Wagner, 1995). Overexpression of TGFβ, an EGF ligand, in the perinatal lung disrupts alveolarization and vascularization, leading to lung fibrosis in the adult (Korthagen et al., 1994; Kramer et al., 2007; Le Cras et al., 2004; Le Cras et al., 2003). EGF can also activate the downstream signaling pathways RAS-PI3K/AKT and RAS/MAPK (Copland and Post, 2007; Kling et al., 2006; Uzumcu et al., 2002; Wang et al., 2005), and can cross-talk with other growth factors, such as TGFβ1 (Ding et al., 2007) and human growth hormone (HGF) signaling (Chess et al., 1998), all of which are important for lung development.

Vascularization, which is driven by growth factors and transcription factors, occurs at all stages of lung development (Warburton et al., 2000). Among the growth factors, VEGF plays a central role in vasculogenesis and angiogenesis (Shibuya, 2008). In addition, the angiopoietin-TIE system is crucial for the development and maturation of the vascular system (Augustin et al., 2009). Lung morphogenesis is heavily dependent on proper vascularization. Both inhibition of neovascularization (Schwarz et al., 2000) and over-vascularization (Zeng et al., 1998) disrupt branching morphogenesis of the lung. Furthermore, epithelial-endothelial interactions are required for perinatal lung development, and loss of such cross-talk results in the arrest of septation and alveolarization (DeLisser et al., 2006; Yamamoto et al., 2007). Here, we have identified the adaptor protein encoded by mitogen-inducible gene 6 [Mig-6; Erffi1 (ErbB receptor feedback inhibitor 1); RALT (receptor-associated late transducer); gene 33], a potential regulator of intracellular signaling, as a crucial regulator of vascularization and lung development.

Mig-6 is a ubiquitously expressed scaffold protein found in vertebrates but not in lower organisms (Zhang and Vande Woude, 2007). Mig-6 can be induced by a number of factors, including growth factors (Anastasi et al., 2003; Chu et al., 1988; Fiorentino et al., 2000; Hackel et al., 2001; Pante et al., 2005), hormones (Jeong et al., 2009; Kent et al., 1994; Lee et al., 1985; Xu D. et al., 2005), cytokines (Ferby et al., 2006; Zhang et al., 2005) and stress stimuli.
(Kent et al., 1994; Makkinje et al., 2000; Xu et al., 2006). Five functional regions have been identified in the MIG-6 protein and include a threonine/serine phosphorylation region, an SH3-binding domain, a CDC42/RAC interaction and binding (CRIB) domain, a 14-3-3-binding domain, and the EGFR-binding domain (Fiorentino et al., 2000; Makkinje et al., 2000). MIG-6 can negatively modulate EGF signaling (Anastasi et al., 2003; Anastasi et al., 2005; Ballaro et al., 2005; Ferby et al., 2006; Fiorentino et al., 2000; Xu D. et al., 2005), either by inhibiting the auto-phosphorylation of EGFR (Ferby et al., 2006) or by binding to GRB2 (immediately upstream of RAS) (Fiorentino et al., 2000), to dampen the downstream pathways.

Mig-6 has been shown to be a tumor suppressor gene in the lung (Ferby et al., 2006; Zhang et al., 2007). The MIG-6 locus (chromosome 1p36) is frequently associated with lung cancer in humans (Tseng et al., 2005). Downregulation of Mig-6 has been found in lung squamous cell cancer and lung adenocarcinoma (Amatschek et al., 2004). Mutations in Mig-6 have been identified in primary lung cancer and in lung tumor cell lines (Ferby et al., 2006; Korfhagen et al., 1994; Tseng et al., 2005; Zhang et al., 2007). The mechanism of Mig-6 action in lung tumorigenesis has been not fully elucidated, but may be attributed to the abnormal overactivation of EGFR or HGF signaling (Ferby et al., 2006; Pante et al., 2005). Whether Mig-6 is involved in other lung diseases remains to be determined.

We and others recently demonstrated that genomic disruption of the Mig-6 gene leads to degenerative joint diseases, skin and lung cancer, and gastrointestinal tract tumors in mice (Ferby et al., 2006; Jin et al., 2007; Zhang et al., 2007). Furthermore, 50% of the Mig-6−/− mice die before adolescence, although the cause of the lethality is not known. Here, we identify the time at which the lethality occurs as during the neonatal period of lung development. Histological analysis of Mig-6−/− mice revealed a phenotype similar to that of transgenic mice with conditional expression of TGFβ in the distal lungs. Both strains show altered alveolarization and vascularization of the lungs. Signaling pathway analysis showed that Mig-6−/− mice have altered expression of EGF signaling and vascularization genes. This demonstrates that Mig-6 is a crucial regulator of pulmonary development and vascularization.

MATERIALS AND METHODS
Animals
All animal protocols were approved by the Animal Care and Use Committee of Baylor College of Medicine. Mig-6−/− mice were generated by crossing Mig-6+/− mice as previously described (Jin et al., 2007). To induce the ablation of Mig-6 in adult mice, Rosa26-Cre-ER2/flox mice (de Luca et al., 2005) were crossed with Mig-6+/− mice. Rosa26-Cre-ER2/flox mice (Jin et al., 2007) and the resulting Rosa26-Cre-ER2/flox, Mig-6+/−flox or Mig-6+/−flox mice (2 months old) were injected peritoneally with 1 mg of tamoxifen for 5 days. These mice were subsequently maintained for 4 months before analysis. The midday of vaginal plug identification was considered E0.5 and the day when neonates were born was considered P1. Mice after weaning were considered as adult (AD).

Genotyping of pups or embryos by PCR was as previously described (Jin et al., 2007). Mice after weaning were considered as adult (AD).

Quantitative real-time PCR
Total RNA was isolated from frozen lung tissue or cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA samples were treated with DNaseI (Sigma, St Louis, MO, USA) to remove any genomic DNA contamination. One microgram of the RNA was reverse transcribed into cDNA using M-MLV (Invitrogen) in a 20-μl volume. Quantitative real-time PCR for quantification of mRNAs encoding MIG-6, EGF, HbEGF, AREG, CCSP and 18S rRNA was performed using TaqMan probes and SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Additional primer and probe sequences are listed in Table 1.

Table 1. Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegfa</td>
<td>F: CTGTACCCACCATGATGCTAGGT</td>
<td>R: CTTCGGCTGTACCATCATCA</td>
</tr>
<tr>
<td>Vegfc</td>
<td>F: GCAGAACACGACGACAGAGT</td>
<td>R: GTGATTGACCAAAAAGCTGAGAC</td>
</tr>
<tr>
<td>Vegfd (Fgfr)</td>
<td>F: TCTGAGGAGCAGACAATGAGCC</td>
<td>R: AACAATGAAACGTCTGGAGGAC</td>
</tr>
<tr>
<td>angiopoietin 1</td>
<td>F: GGGAGAAGGATTCAAGGGAGCTA</td>
<td>R: TCAGGACGTCCTCCATCAATAC</td>
</tr>
<tr>
<td>angiopoietin 2</td>
<td>F: GAGGACTACGATCTCGCTGGA</td>
<td>R: CTTTCCACGTCCTGGAAAGTC</td>
</tr>
<tr>
<td>SP-C (Sftpabc)</td>
<td>F: AGACACGACGAGAACATCCTCTTG</td>
<td>R: CTTCCGACCTCCTGGAAAGTC</td>
</tr>
<tr>
<td>T1α (Pdpn)</td>
<td>F: TCAGAAAGCTCCTGCTTTGAGGAA</td>
<td>R: ACTGCTTGGTGGTGGTGTG</td>
</tr>
<tr>
<td>Muc5ac</td>
<td>F: AGAAATCTCTTCGAGACCGCTGCT</td>
<td>R: ACACGACTGCTGAGACATCATTTT</td>
</tr>
<tr>
<td>P: CCAGCCGTTGAGAAGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

f, forward; R, reverse; P, probe.

Antibodies
Rabbit anti-MIG-6, monoclonal anti-α-tubulin, peroxidase-labeled goat anti-rabbit IgG and peroxidase-labeled rabbit anti-mouse IgG were from Sigma. Rabbit anti-EGFR and anti-α-tubulin antibodies were from Santa Cruz (Santa Cruz Biotech, Santa Cruz, CA, USA). Rabbit anti-phospho-EGFR (Tyr1173), anti-phospho-AKT (Ser473), anti-mTOR, anti-phospho-mTOR (Ser2448) and anti-cleaved caspase 3 antibodies were from Cell Signaling (Danvers, MA, USA). Rabbit anti-CCSP antibody was from this laboratory. Rabbit anti-pro-SP-C antibody was from Seven Hills (Cincinnati, OH, USA). Monoclonal anti-MAC3 antibody was from Becton Dickinson (Franklin Lakes, NJ, USA). Monoclonal anti-PECAM1 antibody was from Pharmingen (San Diego, CA, USA). Rabbit anti-α-SMA antibody was from Abcam (Cambridge, MA, USA). Rabbit anti-phospho-histone H3 antibody was from Upstate (Charlottesville, VA, USA).

Western blot
Western blot analysis was carried out using standard protocols. Lung tissue or cultured cells were homogenized in protein lysis buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 50 mM NaCl, 0.1% NP40, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Thirty micrograms of protein was electrophoresed on an SDS-PAGE gel and transferred to PVDF membrane. The membranes were blocked with 1:1000-1:2000 rabbit anti-MIG-6, anti-EGFR, anti-phospho-EGFR, anti-AKT, anti-phospho-AKT, anti-mTOR, anti-phospho-mTOR, or monoclonal anti-α-tubulin antibodies. After washing, the membranes were probed with peroxidase-labeled goat anti-rabbit IgG(1:2000) or rabbit anti-mouse IgG (1:5000). Bands were detected using ECL Reagent (Amersham, Piscataway, NJ, USA) followed by exposure to X-ray film.

Histology
Lungs were dissected from embryos, neonates or adult mice and immediately fixed in buffered formalin at 4°C overnight and processed for paraffin embedding. Histological analysis was performed on 5-μm sections. Hematoxylin and Eosin (H&E), Periodic Acid Schiff (PAS), and Masson’s trichrome staining were performed according to standard protocols.

Immunohistochemistry and immunofluorescent staining
Sections were de-paraffinized, rehydrated, and steamed in 1:10 Antigen Unmasking Solution (Dako, Carpinteria, CA, USA) or in EDTA (1 mM pH 8.0) and TE (10 mM Tris, 1 mM EDTA, pH 9.0) solutions for 20 minutes. Sections were then incubated with 3% H2O2 in methanol for 15 minutes to quench the endogenous peroxidase. After blocking the slides with Biotin-Avidin Blocking Reagent (Dako) and 10% normal goat serum, or
Mouse-on-Mouse Blocking Reagent (M.O.M.; Vector Laboratories, Burlingame, CA, USA), the slides were incubated with primary antibodies in blocking buffer at 4°C overnight. Dilutions of primary antibodies were as follows: rabbit anti-CCSP (1:3000), goat anti-pro-SP-C (1:1200), monoclonal anti-MAC3 (1:500), rabbit anti-α-SMA (1:500), rabbit anti-AKT (1:100), rabbit anti-phospho-AKT (1:100), rabbit anti-mTOR (1:500) and rabbit anti-phospho-mTOR (1:500). The slides were washed with PBS containing 0.2% Triton X-100 (PBST) and then incubated with 1:200 biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-mouse IgG (Vector Laboratories). After washing, the slides were incubated with Vectastain Elite ABC Reagent (Vector Laboratories) and developed with DAB reagent (Vector Laboratories) followed by counterstaining with Hematoxylin.

Immunofluorescent staining was performed on paraffin-embedded sections using the Tyramide Signal Amplification (TSA) system (PerkinElmer, Waltham, MA, USA). The slides were blocked with TSA Blocking Reagent and incubated with rabbit anti-Mig-6 (1:500), anti-pro-SP-C (1:1200), anti-CCSP (1:3000), anti-EGRF (1:100), anti-phospho-EGRF (1:100), monoclonal anti-PECAM1 (1:200), and rabbit anti-α-SMA (1:500) at 4°C overnight. After washing with PBST, the slides were incubated with 1:200 biotinylated goat anti-rabbit IgG or rabbit anti-mouse IgG (Vector Laboratories). Streptavidin-HRP (1:200) was then applied to the slides. Thereafter, tetramethylrhodamine or Fluorescein (1:100 in TSA amplification diluents) was used to detect the specific signals. Nuclei were stained with DAPI. For dual immunofluorescence staining, images from the red, green and blue channels were overlaid using Photoshop 8.0.

Cell proliferation and apoptosis analysis
Proliferating and apoptotic cells were detected with rabbit anti-phospho-histone H3 (1:2000) and anti-cleaved caspase 3 (1:100). For quantification of the proliferating cells on neonatal lungs, images (20× magnification) were taken of the entire left lung of four Mig-6++/+ and four Mig-6–/– mice. Cells positive for phospho-histone H3 were counted from different lung compartments, including airway epithelium, mesenchyme, blood vessels and alveoli. The ratio of proliferating to total cells in each compartment was then calculated.

Determination of alveolar airspace size, number of primitive alveoli and number of alveolar septa
The size of the alveolar airspace was determined by measuring mean chord lengths on H&E-stained lung sections (Bry et al., 2007). Images were taken at 40× from five representative non-overlapping fields of lungs from at least eight mice. A grid consisting of six lines at 35-μm intervals was overlaid on the image. Areas of bronchiolar airways and blood vessels were eliminated from the analysis. The length of lines overlapping the alveoli was measured and averaged to give the mean chord length of the alveolar space.

The number of primitive alveoli and secondary alveolar septa was determined on H&E-stained E18.5 and P3 lung sections, respectively. Three E18.5 Mig-6++/+ and Mig-6–/– mice and six P3 Mig-6++/+ and Mig-6–/– mice were used and at least six images (40×) were taken randomly from those lung sections. Primitive alveoli of E18.5 lungs were identified as the pouches or sacs with flattened epithelium that radiate from the terminal airways. Secondary septa on P3 lungs were identified as the thin membranes that folded into the alveolar sac.

RNAi
H441 human bronchiolar epithelial cells (National Cancer Institute, Frederick, MD, USA) were grown in RPMI 1640 medium until they achieved 40% confluence. Human lung microvascular endothelial cells (HMVEC-L; Lonza, Walkersville, MD, USA) were maintained in Endothelial Cell Medium containing human EGF, hydrocortisone, GA-1000, FBS, VEGF, human FGFB (FGF2), R3-IGF1 and ascorbic acid (Lonza) until they reached 60% confluency. The cells were transfected with 50 nM control siRNA or SMARTpool of human Mig-6 siRNA (Dharmacon, Chicago, IL, USA) in serum-free Opti-MEM (Invitrogen) for 6 hours. Opti-MEM was then removed and fresh growth medium added. After 24 hours, the siRNA treatment was repeated and cells were cultured for 48 hours.

MTT cell proliferation assay
MTT assays were performed on siRNA-treated H441 and HMVEC-L cells in 24-well plates following standard protocols. Briefly, upon siRNA treatment, 75 μl of Dye Solution (Promega, Madison, WI, USA) were added directly to each well. After incubation at 37°C for 4 hours, 500 μl of Solubilization Solution (Promega) were added and mixed well, and A570 measured with a spectrometer.

Statistics
Values are expressed as mean ± s.e.m. Student’s t-test was used for comparison of two group averages. When there were more than two groups, one-way ANOVA followed by least significance difference (LSD) or Tukey analysis was carried out. n≥3 and statistical significance P<0.05.

RESULTS

Mig-6–/– mice have a high neonatal mortality
Only 12% of the mice weaned from Mig-6–/– breeding cages were Mig-6–/– (see Table 2). This represents less than half of the expected Mendelian ratio (Jin et al., 2007). In order to determine the time point at which the lethality occurs in Mig-6–/– mice, we examined the genotype of mice resulting from Mig-6–/– breedings at E15.5-E18.5, P3 and P28. The results of the genotyping of these progeny, as shown in Table 2, demonstrate that prior to birth, the frequency of Mig-6–/– was 23%, which is consistent with the expected Mendelian ratio. However, the frequency of Mig-6–/– mice fell to 9.3% after birth. This indicated that the loss of Mig-6–/– mice occurred in the neonatal period.

Examination of neonatal Mig-6–/– mice showed that these animals were smaller than normal: average birth weight was only 77% of the average birth weight of their Mig-6++/+ littermates (Fig. 1A,B). Most Mig-6–/– pups were less active and less capable of feeding, as shown by the lack of milk in their stomachs (Fig. 1A, arrows). Although their lungs inflated well with air at birth (Fig. 1C), many Mig-6–/– mice became cyanotic shortly before death (Fig. 1A). These results suggest that compromised lung development might be a contributing factor to the neonatal lethality of Mig-6–/– mice.

Mig-6 is differentially expressed during lung development
Prior to investigating the pulmonary phenotype of these mice, the expression and distribution pattern of Mig-6 during development was investigated (Fig. 2A). Quantitative real-time PCR results showed that Mig-6 mRNA is expressed at relatively low levels in the lungs during the embryonic stage (E13.5-E18.5). Mig-6 mRNA levels were significantly higher in the wild-type than Mig-6–/– lungs, indicating that although low, there is still detectable expression of Mig-6 at E13.5. After birth, Mig-6 mRNA expression was dramatically, but transiently, upregulated from P1 to P3. By P4, Mig-6 mRNA expression decreased to levels equivalent to those at E18.5, which were then maintained until the adult stage (Fig. 2A). Dual immunofluorescence staining of Mig-6 with an alveolar type II cell marker, surfactant protein C (SP-C; SFTPC – Mouse Genome Informatics), and a Clara cell-specific marker, Clara cell secretory protein (CCSP; SCGB1A1), revealed Mig-6 expression to be ubiquitous in E18.5 and P3 lungs, being highly expressed in airway

<table>
<thead>
<tr>
<th>Age</th>
<th>Mig6++/+</th>
<th>Mig6–/–</th>
<th>Total n</th>
</tr>
</thead>
<tbody>
<tr>
<td>P28</td>
<td>75 (32.3)</td>
<td>129 (55.6)</td>
<td>204</td>
</tr>
<tr>
<td>P3</td>
<td>34 (39.5)</td>
<td>44 (51.2)</td>
<td>78</td>
</tr>
<tr>
<td>E15.5-18.5</td>
<td>16 (30.8)</td>
<td>24 (46.1)</td>
<td>40</td>
</tr>
</tbody>
</table>
epithelial cells (Fig. 2B-G, arrows) and alveolar type II cells (Fig. 2K-P, arrows). In P3 lungs, Mig-6 was also expressed in pericytes surrounding blood vessels and muscle cells surrounding airways, as indicated by dual immunofluorescence staining of MIG-6 with smooth muscle α-actin (α-SMA) (see Fig. S1 in the supplementary material). In adult lungs, Mig-6 was mainly expressed in the airway epithelium and in alveolar type II cells, and less so in other structures (Fig. 2H-J,Q-S).

**Perinatal Mig-6<sup>−/−</sup> mice exhibit altered lung architecture**

Histological analysis of Mig-6<sup>+/+</sup> and Mig-6<sup>−/−</sup> mouse lungs (E18.5 and P3) was performed to determine the impact of the ablation of Mig-6 on lung architecture. By E18.5, Mig-6<sup>−/−</sup> mouse lungs have formed multiple pods that end with sac-like structures on the antrum to the alveoli (Fig. 4E,F). Examination of smaller airways demonstrated normal morphology in prenatal and neonatal Mig-6<sup>−/−</sup> mice, with airways lined with CCSP-expressing cells, whereas the small airways of the Mig-6<sup>−/−</sup> mice showed an irregular morphology with hyperplastic CCSP-positive epithelium (Fig. 4C,D). Examination of the distal lungs by pro-SP-C staining showed hyperplasia of alveolar type II cells in neonatal Mig-6<sup>−/−</sup> mouse lungs, with multiple alveolar type II cells at the antrum to the alveoli (Fig. 4E,F).

**Mig-6<sup>−/−</sup> neonates exhibit over-branching and airway epithelial and alveolar type II cell hyperplasia**

In order to further investigate the impact of Mig-6 ablation on pulmonary architecture and epithelial cell differentiation, the expression of CCSP and SP-C was assayed by immunohistochemistry in the lungs of P3 Mig-6<sup>−/−</sup> and Mig-6<sup>+/−</sup> mice (Fig. 4). Staining of the large airways showed that whereas Mig-6<sup>−/−</sup> mice had normal airway architecture and expression of CCSP, Mig-6<sup>−/−</sup> mice developed over-branched large airways (Fig. 4A,B). Examination of smaller airways demonstrated normal morphology in Mig-6<sup>−/−</sup> mice, with airways lined with CCSP-expressing cells, whereas the small airways of the Mig-6<sup>−/−</sup> mice showed an irregular morphology with hyperplastic CCSP-positive epithelium (Fig. 4C,D). Examination of the distal lungs by pro-SP-C staining showed hyperplasia of alveolar type II cells in neonatal Mig-6<sup>−/−</sup> mouse lungs, with multiple alveolar type II cells at the antrum to the alveoli (Fig. 4E,F).
with the alveolar surface (Fig. 5C,D). Immunohistochemistry for α-SMA indicated that blood vessels were well developed in the Mig-6+/+ lungs, but not in the Mig-6−/− lungs (Fig. 5E,F).

Abnormal cell proliferation and apoptosis in neonatal Mig-6−/− mouse lungs

In order to investigate the reasons for the interruption of lung development in Mig-6−/− mice, we examined the cell proliferation and apoptosis status of Mig-6−/− mouse lungs at P3. In Mig-6+/+ lungs, proliferating cells were commonly found in blood vessels (Fig. 6C, arrows) but rarely in the bronchial epithelium (Fig. 6A, arrow). However, in Mig-6−/− lungs, proliferating cells were frequently found in the bronchial epithelium (Fig. 6B, arrows), but less so in the blood vessels (Fig. 6D). Quantification of the proliferating cells in four compartments of the lung, comprising airway, mesenchyme, blood vessel and alveoli, showed that the number of proliferating cells increased 3-fold in the airways, but decreased by 66% in the blood vessels of Mig-6−/− as compared with Mig-6+/+ lungs (Fig. 6E). As for cell survival, very few cells stained for cleaved caspase 3 in the Mig-6−/− lungs. By contrast, apoptotic cells were frequently found in the airway epithelium, blood vessels, mesenchyme and alveolar septa of Mig-6−/− lungs (Fig. 6F-K, arrows).

Altered pulmonary morphology in adult Mig-6−/− but not Mig-6d/d mice

In order to investigate whether altered lung development in Mig-6−/− mice impacted adult lung structure and cell differentiation, we examined the morphological changes in 3-month-old adult Mig-6−/− lungs. Histological examination of the lungs of Mig-6−/− mice (Fig. 7A) showed enlargement of the alveolar spaces as compared with the Mig-6+/+ mice, which was confirmed by quantification (Fig. 7B). Papillary hyperplastic proliferation was observed in the airway epithelium of Mig-6−/− mice (Fig. 7C,D). Masson’s trichrome staining showed a significant increase in extracellular matrix deposition around the bronchi, around the blood vessels, in the alveolar interstitia and in the plural membranes of Mig-6−/− mice (Fig. 7E-H). Mig-6−/− lungs also showed an increase in macrophage invasion (Fig. 7I,J) and mucous cell metaplasia (Fig. 7K,L). These phenotypes indicate the development of COPD in adult Mig-6−/− mice, which was not asthma as there was no airway contraction.

To determine whether this pulmonary phenotype in adult mice was a result of the developmental defect, we conditionally ablated Mig-6 in the adult mouse using a ubiquitously expressed CreErT2 mouse model (de Luca et al., 2005). Rosa26-Cre-ERT2/+; Mig-6flox/flox (Mig-6+/+) and Mig-6flox/flox (Mig-6−/−) mice were treated with tamoxifen and their lungs analyzed 4 months after the Mig-6 conditional ablation. Immunofluorescence staining and quantitative real-time PCR both showed that Mig-6 expression was successfully ablated in the adult Mig-6d/d mouse lung (Fig. 8A,B,G). In addition to the lung, other tissues, including the liver, uterus and mammary gland, also showed efficient ablation of Mig-6 (data not shown). H&E staining showed that Mig-6+/+ and Mig-6d/d mice both had normal lung structure (Fig. 8C,D) and normal alveolar spaces (Fig. 8H). PAS staining did not detect mucosal cells in Mig-6+/+ or Mig-6d/d lungs (Fig. 8E,F). Quantitative real-time PCR showed that Mig-6−/−...
lungs exhibited altered expression of lung molecular markers including SP-C, CCSP, T1α (PDPN) and MUC5AC, all of which were normal in Mig-6d/d lungs (see Fig. S2 in the supplementary material). Although there was no visible pulmonary phenotype, these mice demonstrated the dermal papilomas and uterine hyperplasia previously described (Jin et al., 2007). This confirmed the efficiency of the ablation of Mig-6 (data not shown) and the presence of a phenotype in tissues that undergo dynamic changes.

Since the lungs of the Mig-6d/d mice were normal, the COPD phenotype in the adult Mig-6–/– mice can be attributed to the developmental defects.

**Increased EGF signaling but decreased expression of angiogenetic genes in neonatal Mig-6–/– mouse lungs**

The morphological changes of the Mig-6–/– mouse lungs are highly consistent with those exhibited by transgenic mice in which TGFα is overexpressed perinatally (Korfhagen et al., 1994; Kramer et al., 2007; Le Cras et al., 2004; Le Cras et al., 2003). Therefore, we examined whether Mig-6 regulates lung development through the modulation of EGF signaling. As shown in Fig. 9, there was no major difference in the expression of total EGFR, AKT and mTOR between the Mig-6+/+ and Mig-6–/– mouse lungs. However, phospho-EGFR (Fig. 9D), phospho-AKT (Fig. 9H) and phospho-mTOR (Fig. 9L) were all increased in the airway epithelium and alveoli of Mig-6–/– as compared with Mig-6+/+ lungs. Western blot analysis confirmed the increase in phospho-AKT and phospho-mTOR in the Mig-6–/– lungs (Fig. 9M). Quantitative real-time PCR revealed that expression of the genes encoding EGF, AREG and HbEGF, which are all ligands for EGFR, was significantly increased in Mig-6–/– as compared with Mig-6+/+ lungs (Fig. 9N). These results suggest that Mig-6 might regulate lung morphogenesis through the regulation of EGF signaling, probably through an autocrine/paracrine mechanism. Because Mig-6–/– mouse lungs also exhibited reduced vascularization, we examined the expression of angiogenetic genes, including those that encode VEGF-A, VEGF-C, VEGF-D (FIGF), angiopoietin 1 and angiopoietin 2, by quantitative real-time PCR. All these angiogenic genes were downregulated in the Mig-6–/– as compared with Mig-6+/+ lungs (Fig. 9N).

**Increased epithelial cell proliferation and endothelial cell apoptosis upon Mig-6 knockdown**

To investigate the roles of Mig-6 in different lung cell types, we performed siRNA studies on human H441 bronchiolar epithelial cells and HMVEC-L lung microvascular endothelial cells. No mutation was found in the MIG-6 gene of the H441 cell line by
sequencing (data not shown). When MIG-6 was knocked down in H441 cells (1 in Fig. 10A), there was an increase in phospho-EGFR and phospho-AKT as compared with the no siRNA and control siRNA cells (2 and 3 in Fig. 10A). The MTT assay showed that the population of living cells increased significantly in siRNA-treated H441 cells (2 and 3 in Fig. 10A). The MTT assay showed that the population of living cells increased significantly in siRNA-treated H441 cells (Fig. 10C). Cleaved caspase 3 staining showed an increase in apoptosis in the MIG-6 siRNA-treated HMVEC-L cells (Fig. 10D). Cleaved caspase 3 staining showed an increase in apoptosis in the MIG-6 siRNA-treated HMVEC-L cells (Fig. 10E). No difference was found in the number of phospho-histone H3-stained HMVEC-L cells or cleaved caspase 3-stained H441 cells upon MIG-6 knockdown (data not shown). Therefore, knocking down MIG-6 expression increased EGF signaling and cell proliferation in epithelial cells, but elevated apoptosis in endothelial cells.

**DISCUSSION**

Over the last 10 years, Mig-6 has drawn increased attention for its role in stress responses and tumorigenesis (Anastasi et al., 2005; Ferby et al., 2006; Jeong et al., 2009; Makkinje et al., 2000; Xu et al., 2006; Zhang et al., 2005). As of yet, however, there is no evidence, either from tissue culture or animal models, to show a contribution of Mig-6 to organogenesis. In this report, we demonstrate that Mig-6 is constitutively expressed throughout lung development and is highly upregulated after birth. Mig-6/– mice developed abnormal branching, alveolarization and vascularization in the embryonic and neonatal lungs, as well as COPD features in the adult. Further, there was epithelial hyperplasia accompanied by elevated EGF signaling and disrupted vascularization in the neonatal Mig-6/– lungs. Our results provide the first evidence that, by controlling EGF signaling and vascularization, Mig-6 regulates lung development.

It is not surprising that disruption of Mig-6 in the mouse genome influenced all aspects of lung development and resulted in a bronchial pulmonary dysplasia (BPD) phenotype in neonates, as Mig-6 was widely expressed in lung cells throughout most, if not all, developmental stages. Furthermore, Mig-6 has been shown to actively regulate cell proliferation, transformation (Anastasi et al., 2003; Fiorentino et al., 2000), differentiation, cell migration and neurite growth (Pante et al., 2005; Wick et al., 1995), processes that are all crucial for lung development. Neonatal Mig-6/– mice also had a lower birth weight, suggesting that Mig-6 might have a broad impact on development, in addition to lung morphogenesis.

That Mig-6/– mice still formed the correct number of lung lobes, together with the fact that 50% of the Mig-6/– mice survived until adulthood, suggest that Mig-6 might not be a master gene that...
regulates the early stages of lung development (Warburton et al., 2000). Instead, Mig-6 might play a more important role during the mid-to-late stages of lung development. In support of this, the main lung phenotypes of Mig-6–/– mice, such as alveolar type II cell hyperplasia, arrested alveolarization and vascularization defects, all emerge at the terminal sacular stage (Costa et al., 2001; Inselman and Mellins, 1981). Mig-6 functions might closely associate with cellular activities. Other phenotypes of Mig-6–/– mice, including penetrant skin, skin cancer and degenerate joint diseases, all developed in highly dynamic organs (Ferby et al., 2006; Jin et al., 2007; Zhang et al., 2007).

Delaying the ablation of Mig-6 until the adult results in no overt pulmonary phenotype. One explanation for this is that the adult lungs are relatively static and the impact of Mig-6 might only be manifested during dynamic stages, such as during development or injury repair (Warburton et al., 2001). Challenging the Mig-6–/– mice with pulmonary injury would be necessary to test this hypothesis. However, the COPD phenotype of adult Mig-6–/– mice is the result of recurrent inflammation and scaring in the lung, a process similar to BPD developing into COPD when BPD is not properly controlled at the neonatal stage (Bhandari and Panitch, 2006; Coalson, 2003).

Similar to previous reports, Mig-6–/– lungs showed increased phospho-EGFR and downstream EGF signaling (Ferby et al., 2006), suggesting the direct interaction of Mig-6 with EGFR (Anastasi et al., 2003; Anastasi et al., 2005; Ferby et al., 2006; Fiorentino et al., 2000; Xu D. et al., 2005). Activation of the AKT/mTOR pathway might be a cause of the increased cell proliferation in the Mig-6–/– lungs because knockdown of Mig-6 in H441 cells led to elevated cell proliferation accompanied with increased phospho-EGFR and phospho-AKT. In addition, overactivation of the AKT/mTOR pathway has been reported in bronchial epithelium with conditional deletion of phosphatase and tensin homolog (PTEN), which brings about papillary epithelial hyperplasia analogous to that observed in Mig-6–/– lungs (Dave et al., 2007; Yanagi et al., 2007). An increase in EGFR ligands, such as EGF, HbEGF and AREG in Mig-6–/– lungs, has also been observed in human fetal and neonatal lungs with BPD and in injured lungs, suggesting that the autocrine/paracrine regulation of EGF signaling might contribute to the pathogenesis of Mig-6–/– lungs (Singh and Harris, 2005; Stahlman et al., 1989; Strandjord et al., 1995).
The mechanisms by which Mig-6 regulates lung vascularization might be complex. We detected a decrease in numerous angiogenic factor genes in the Mig-6−/− lungs, which might directly contribute to their under-vascularization. Vascular hypoplasia, together with a decrease in angiogenic factors and their receptors, have been observed in human infants with BPD (Bhatt et al., 2001; Lasus et al., 2001) and in prematurely delivered baboons (Maniscalco et al., 2002). The under-vascularization of the Mig-6−/− lungs was detected as early as E15.5, suggesting that the vascularization defect is not a secondary change that occurs with a lung defect in newborns. In addition to the effect of angiogenic factors, increased EGF signaling might partially contribute to the pulmonary vascular defects, as was concluded for transgenic mice expressing TGFRα from the SP-C promoter (Le Cras et al., 2003). EGF signaling normally promotes angiogenesis in tumors (Goldman et al., 1993; Salomon et al., 1995); however, in developing organs, EGF might act differently given that EGF/TGFRα treatment has been shown to abrogate the angiopoietic and hemangiopoietic potentials of the splanchnopleural mesoderm of avian embryos (Pardanaud and Dieterlen-Lievre, 1999). We are not sure why ablation of Mig-6 leads to the downregulation of angiogenic factors in Mig-6−/− lungs. As a scaffold protein that is ubiquitously expressed in E18.5 and P3 lungs, Mig-6 might directly influence the expression of VEGF, angiopoietin 1 and angiopoietin 2, which are mainly produced by peri-endothelial mural cells, fibroblasts and endothelial cells (Davis et al., 1996; Leung et al., 1989; Stratmann et al., 1998; Tischer et al., 1991). Furthermore, Mig-6 might be crucial for the survival of endothelial cells because knocking down Mig-6 in HMVEC-L cells promoted their apoptosis. It is not clear whether Mig-6 regulates endothelial cell apoptosis by a mechanism similar to that in breast cancer cells (Xu J., et al., 2005); however, Mig-6 possesses the functional domain to bind 14-3-3 proteins (Makkine et al., 2000), which may block apoptosis via differential regulation of MAPK pathways (Xing et al., 2000; Zhang et al., 2003). The loss of endothelial cells in the Mig-6−/− lungs might further disrupt the epithelial-endothelial interaction, which leads to septation defects in neonatal lungs (DeLisser et al., 2006; Yamamoto et al., 2007).

In summary, this study has revealed that Mig-6 is required for lung morphogenesis and homeostasis. The function of Mig-6 in the lung is programmed at the prenatal and perinatal stages, such that when Mig-6 is ablated in the adult no lung phenotype develops. Mig-6 may negatively regulate EGF receptor intracellular signaling in epithelial cells and modulate vascularization systems through different mechanisms, which might include the regulation of angiogenic factors and the control of endothelial cell apoptosis. This study may open a new field for the functional study of Mig-6 and in the development of other organs.


