Regulation of alveolar septation by microRNA-489

Nelida Olave,¹ Charitharth V. Lal,¹ Brian Halloran,¹ Kusum Pandit,² Alain C. Cuna,³ Ona M. Faye-Petersen,⁴ David R. Kelly,⁴ Teodora Nicola,¹ Panayiotis V. Benos,⁵ Naftali Kaminski,⁶ and Namasiyavam Ambalavanan¹,⁴
¹Department of Pediatrics, University of Alabama at Birmingham, Birmingham, Alabama; ²Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; ³Department of Pediatrics, University of Missouri-Kansas City School of Medicine, Kansas City, Missouri; ⁴Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama; ⁵Department of Computational & Systems Biology, University of Pittsburgh, Pittsburgh, Pennsylvania; and ⁶Division of Pulmonary, Critical Care and Sleep Medicine, Yale School of Medicine, New Haven, Connecticut

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BRONCHOPULMONARY DYSPLASIA (BPD) is a major cause of morbidity and mortality in premature infants. BPD is characterized by impaired alveolar septation, in combination with varying degrees of lung fibrosis and abnormal vascular remodeling, which is mimicked by neonatal animal models of hyperoxia exposure (1, 2, 5, 14, 46). The critical gene regulatory pathways involved in alveolar septation are not well determined, but likely depend on integration of numerous signals from multiple cross-talking pathways (e.g., Wnt, transforming growth factor-β, PTEN, hedgehog, retinoid pathways) affecting gene expression in a coordinated manner, leading to phenotypic reprogramming of lung parenchymal cells (7, 8, 21, 22, 27, 32, 36). There is a need to understand regulation of normal alveolar development and how these pathways are dysregulated in BPD. MicroRNA (miRNA) are small noncoding RNA that regulate gene expression of the majority of protein-coding genes, mainly by inhibition of translation and mRNA stability of target genes. Differential expression of miRNA regulating target miRNA have been noted in lung development, with dysregulated expression observed in multiple disorders (23, 33). We, therefore, hypothesized that specific miRNA are critical in regulating normal alveolar development, and hyperoxia may dysregulate the expression of these miRNA. As miRNA can be specifically targeted and either inhibited or overexpressed, this may yield insights into potential therapeutic strategies for disorders, such as BPD.

Our laboratory recently performed bioinformatic analyses of miRNA and mRNA expression data in lungs of C57BL/6 wild-type mice at different stages of alveolar septation and developed dynamic miRNA-regulated interaction networks that indicated that multiple miRNA are potentially involved in regulation of normal alveolar septation (35). Evaluation of the miRNA and the target genes that were differentially expressed over the course of alveolar septation suggested a potential role for miR-489 as it is predicted to regulate target genes insulin-like growth factor-1 (Igf1) and tenascin C (Tnc), which are known to be involved in lung development (40, 47). To gain a better understanding of the role of miR-489 in normal and abnormal alveolar septation, we evaluated the effects of inhibition and overexpression of miR-489 on normal alveolar septation and hyperoxia-induced inhibition of alveolar septation in the mouse and determined if miR-489 was associated with human BPD.

METHODS

All animal procedures were approved by the Institutional Animal Care and Use Committee and were consistent with the Public Health Service policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare, 2002). All experiments were done with a minimum of six mice from at least two litters for each condition. The use of human samples was approved by the Institutional Review Board.
Table 1. Primers used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Stem Loop RT Primer (5'-3')</th>
<th>Forward 5' to 3'</th>
<th>Reverse 5' to 3'</th>
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</thead>
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<tr>
<td>mmu-miR-489</td>
<td>CACCTTTCCCCCAGTGGTGCTGCTGCC</td>
<td>CCCCAGCAATGACACCCATA</td>
<td>CCAAGTGGTGCTGGCATAT</td>
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<tr>
<td>Mouse Igf1</td>
<td>ATGACTTTTGGTCTTGGGAGGCT</td>
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<tr>
<td>Mouse Tnc</td>
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<td>hsa-miR-489</td>
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<td>CCCCAGGAAGACAGCTGTACAT</td>
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<tr>
<td>Human Igf1</td>
<td>CTTTACAGCTTGCTTGGAGCACAG</td>
<td>TGGAGGCCCGCTGGACGTCTG</td>
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</tbody>
</table>

See text for definition of acronyms.

Mouse Studies

miRNA profiling. As described earlier (35), we quantified miRNA extracted from whole lung homogenates of newborn C57BL/6 mice at five developmental time points (postnatal day 0 (P0), P4, P7, P14, and P42). Four to five biological replicates were collected for each time point. Analyses were done using the Agilent Mouse miRNA microarray, which contains 627 mouse miRNAs and 39 mouse viral miRNAs (Sanger miRBase release 12.0), following the manufacturer’s protocol (Agilent). Data analysis was performed using GeneSpring GX 11.0 (Agilent). The raw signal intensities were thresholded to 1, transformed to log base 2, followed by quantile normalization and baseline transformation to mean of P1 samples.

Neonatal hyperoxia exposure model. Newborn C57BL/6 mice, along with their dams, were exposed to normoxia (21%: control group) or hyperoxia (85% O2) from 4 to 14 days of age in a sealed Plexiglas chamber with continuous oxygen monitoring (18). Dams were alternated every 24 h from hyperoxia to air to reduce oxygen toxicity. Daily animal maintenance was carried out, with exposure of the animals to room air for <10 min/day. A standard mouse pellet diet and water were provided ad libitum.

In vivo LNA-miR-489. For in vivo miR-489 knockdown we used locked nucleic acid (LNA) miRNA technology (16, 20). LNA oligonucleotides have higher affinities for their targets than regular DNA- or RNA-based oligonucleotides (16, 20). Mice were treated daily from 4 to 14 days of age by intranasal administration of LNA Mus musculus (mmu)-miR-489 corresponding to 5 μg·g⁻¹·day⁻¹ dissolved in 5 μl water (16).

The LNA for mmu-miR-489 (accession no. MIMAT0003112) (Exiqon, Woburn, MA) is 15-nucleotide long with the following sequence, TATATGTGGTGTCAT, and contains phosphorothioate backbone modifications.

In vivo overexpression of miR-489. For in vivo miR-489 overexpression, we used pCMV-miR-489 (OriGene, Rockville, MD). The pCMV-miRNA system has been used successfully in vitro to over-express miRNAs (11, 38), and we adapted this system for in vivo use. The miR-489 clone was sequenced to ensure that the pre-miR sequence matched the reference sequence in miRBase (http://www.mirbase.org/). pCMV-miR-489 was mixed with TransIT-TKO Transfection Reagent (Mirus, Madison, WI) and PBS and then incubated at room temperature for 15–30 min. Mice were treated intranasally every other day from P4 to P14 with 0.7 mg/kg pCMV-miR-489. Control mice were treated with empty vector.

Lung function. After completion of hyperoxia or air exposure, mouse pulmonary function was evaluated on a flexVent, as described previously (18).

Quantitative RT-PCR. Total RNA from homogenized lung was extracted using TRIzol (Invitrogen, Carlsbad, CA), and reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative RT-PCR (qPCR) for Igf1, Tnc, and miR-489 on homogenized lung were performed as described previously (18) using specific primers for miR-489 (Table 1). Real-time PCR was performed on the MyiQ Single-Color Real-Time PCR detection System (Bio-Rad) using SYBR Green PCR Master Mix (Applied Biosystems). Two microlecters of each cDNA sample were subjected to real-time PCR in a total volume of 20 μl using 100 nM of each primer. Thermocycling of miRNA cDNA was performing after 10 min of initial denaturation at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min annealing/extension at 60°C. miR-489 expression levels were normalized to U6 small nuclear RNA (snRNA). Expression levels of genes were normalized to 18S RNA.

We performed qPCR analysis using an alternative method for samples from cell cultures, flow-sorted cells, and human samples isolated from lung sections. Total RNA, including small nucleic acids, was extracted using Qiagen’s miRNeasy kit. Reverse transcription was performed using TaqMan miRNA Reverse Transcription Kit (Life Technologies). qPCR analysis used Universal Master Mix (Life Technologies), no uracil N-glycosylase (UNG), along with TaqMan miRNA assays for mmu-miR-489 (001302) and control assay U6 snRNA. Messenger RNA analysis was also performed on this total RNA via Takara/Clontech Primerscript RT Master Mix for cDNA synthesis and Probe qPCR (Probe qPCR), along with Taqman Gene Expression Assays [human IGF-1 (Hs01547656_m1), human TNC (Hs01115665_m1), mouse IGF-1 (Mm00439560_m1), and mouse TNC (Mm00495662_m1) (Life Technologies/ThermoFisher)].

Western blot. Newborn mouse lungs were homogenized in a tissue protein extraction reagent (Pierce Biotechnology, Rockford, IL) plus protease inhibitors (Roche Diagnostics, Basel, Switzerland), centrifuged at 7,000 g × 5 min, and supernatant frozen at −80°C. Protein concentrations were measured by Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA). Western blots were done as described previously (18). The primary antibody was rabbit anti-IGF-1 (1:100) or anti-TNC-1 (1:100; Abcam) in 1% BSA/1× Tris-buffered saline/0.1% Tween 20 overnight at 4°C. The secondary antibody was a goat anti-rabbit horseradish peroxidase (HRP) secondary antibody (Abcam) used at 1:10,000 dilution for 2 h at room temperature.

Lung morphometry. Lung alveolar morphometry was performed as described previously, with measurements of inflation-fixed lung sections for mean linear intercepts (MLI) and radial alveolar counts (RAC) being performed by an observer masked to sample identity (18).

Immunohistochemistry. Five-micrometer paraffin sections were immunostained for IGF-1 and TNC as described previously (3, 28). Antibodies used were rabbit anti-IGF-1 or anti-TNC (Abbiotec, San Diego, CA) used at 1:400 dilution, followed by HRP-labeled polymer conjugated with secondary antibody and diamobenzidine staining, as described in the product manual [DakoCytomation EnVision+ System-HRP (diamobenzidine), Dako North America, Carpinteria, CA]. For quantitation, six ×400 fields from three mice per group were evaluated. Thresholds for positive antibody staining compared with nonimmune serum controls were defined using image analysis software (MetaMorph version 7.8, Universal Imaging, Downingtown, PA). Positive pixels were expressed as percentages of total tissue area, excluding air spaces, as previously described by Brey et al. (9) and modified by our laboratory for use in the lung (4).
Localization of miR-489. In situ hybridization for miR-489. In situ hybridization for miR-489 was done on 8-μm frozen sections. Slides were warmed to room temperature and then dried at 50°C for 15 min. The tissue sections were fixed in 4% paraformaldehyde-Dubecque’s PBS (DPBS) at room temperature for 20 min and washed with DPBS at room temperature for 5 min. The sections were treated with proteinase K (10 μg/ml) for 10 min at room temperature. The sections were washed with DPBS and acetylated with triethanolamine-acetic acid for 10 min at room temperature. After the acetylation step, the sections were washed with DPBS and prehybridized for 30 min at 55°C. The hybridization was carried out at 55°C for 2 h with LNA U6 short hairpin RNA (3 nM) or LNA-miR-489 antisense oligonucleotides labeled with digoxigenin (DIG; 50 nM) (39246-15 Exiqon) or no probe. After the hybridization, the slides were washed with 5× SSC, 1× SSC, and 0.2× SSC at 55°C. Tissue sections were then washed in DPBS, blocked (2% sheep serum in DPBS-Tween 20) for 1 h at room temperature, and then incubated with sheep anti-DIG-alkaline phosphatase (Roche, Indianapolis, IN) at 1:800 overnight at 4°C. The slides were washed with DPBS-Tween 20 and incubated with alkaline phosphatase substrate (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate; Roche) overnight at room temperature. The slides were incubated with KTBT buffer (Tris·HCl 50 mM, NaCl 150 mM, KCl 10 mM), and then washed with water and air-dried. Finally, the slides were mounted with Permount and examined under oil immersion.

Mouse lung cell lines. To determine cell origin of miR-489, IGF-1, and TNC, we used mouse lung microvascular endothelial cells (mPMVEC) (17, 30), murine lung epithelial (MLE-12, ATCC CRL-2110) (43), and newborn mouse lung fibroblasts (NMLFs) isolated by explant culture, as described previously (30). qPCR for miR-489, Igf1, and Tnc was then done on RNA isolated from the cells. In addition, exosomes were isolated from serum-free conditioned media of MLE-12 using the miR-curry exosome isolation kit (Exiqon), 24 h after fresh media were added. qPCR was done for miR-489 on RNA isolated from the exosomes using the miRcurry RNA isolation kit (Exiqon).

Flow cytometry. To confirm results from flow cytometry, cell suspensions were made from mouse lungs minced and digested in collagenase [0.1% collagenase (Worthington), 0.05% trypsin/EDTA (Corning), 50 mM HEPES, pH = 7.4 (Coming)], followed by dissociation. Flow cytometry was performed for epithelial and endothelial cells, as described earlier (28), using specific [rat-anti-mouse CD31/platelet endothelial cell adhesion molecule 1 (PECAM-1)-Alexa-488 and rat-anti-E-cadherin-phycocyanin] and isotype control antibodies. Cell lines of endothelial cells (mPMVEC) and epithelial cells (MLE-12) were also labeled for use as positive controls. Gates for flow sorting were set using both isotype controls and positive controls. qPCR for miR-489 was then done on RNA from the isolated epithelial (E-cadherin+/PECAM-1−) and endothelial (E-cadherin−/PECAM-1+) cells.

Elastic fiber staining. Deparaffinized minced lung sections from room air, hyperoxia, LNA-miR-489, hyperoxia/LNA-miR-489, and pCMV-miR-489 were stained for elastin using Hart’s elastic stain.

In vitro studies to confirm miR-489 targeting of IGF-1 and TNC. IGF-1 and TNC are conserved targets for miR-489 in silico, as IGF-1 is an 8mer match and TNC is a 7mer-m8 match in TargetScan Mouse (http://www.targetscan.org/mmu_61/). To confirm miR-489 targeting of IGF-1 and TNC, mouse lung epithelial cells (MLE-12, ATCC CRL-2110) were seeded overnight and subsequently cotransfected with 1 μg pCMV-miR-489 and 2 μg pEZX-Gaussia Luciferase (GLuc)/secreted alkaline phosphatase (SEAP) reporter plasmid containing the mouse TNC or IGF-1 3′ untranslated region (UTR) using TransIT-TKO transfection reagents (Mirus, Madison, WI). As controls, MLE-12 were transfected with empty pEZX-GLuc/SEAP or cotransfected with pEZX-GLuc/SEAP plus pCMV-miR-489 or TNC/IGF-1 3′ UTR plus LNA-miR-489 or scramble. After 48 h, cell culture media was collected for GLuc and SEAP luminescence assay. The Secretre-Pair Dual Luminescence Assay Kit (GeneCopoeia, Rockville, MD) was used to read luminescence. Three independent experiments were performed.

Human Studies

RNA was isolated using Qiagen RNeasy FFPE kit (Qiagen, Valencia, CA) from paraffin-embedded formalin-fixed samples of lungs collected at autopsy from extremely preterm infants (24–28 wk gestation) who died soon after birth, term stillborn infants, and preterm infants who died due to BPD at term-corrected age (36–44 wk postmenstrual age) (n = 4/group). RNA quality was evaluated on the Agilent 2100 bioanalyzer instrument. miR-489 and Igf1 were evaluated in relation to 18S rRNA using specific primers (Table 1) and Tnc (Hs01115665_m1) was evaluated in relation to 18S rRNA (4310893E) by TaqMan Gene Expression assays (Life Technologies, Grand Island, NY).

RESULTS

Evaluation of miRNA Involved in Lung Development Identifies miR-489 and Its Targets IGF-1 and TNC as Key Molecules

The miRNA microarray analysis on mouse lungs obtained from different stages of alveolar septation identified 291 miRNAs demonstrating altered expression by one-way ANOVA at P < 0.05 (asymptotic P value computation and no correction for multiple testing). One hundred eighty-one of these miRNAs had a fold change of ≥2 (heat maps in Fig. 1A). We used 2K-means clustering on these two lists to form clusters with miRNA expressions either increasing or decreasing during the 6 wk, with a major deflection between the 1st and 2nd wk (alveolar septation) (Fig. 1B). Time series expression data have been deposited at the Gene Expression Omnibus database with accession GSE41412, and critical miRNA alterations at different time points and probable regulating transcription factors have been recently described by our laboratory (35). Conserved miRNA targets were determined by feeding miRNAs with a fold change of ≥2 into TargetScan (www.targetscan.org), with a context percentile of 99 and matching with the conserved targets database. These proteins were analyzed for transcriptional regulation (Fig. 1C) and biological processes (Fig. 1D). Different biological processes known to be important in alveolar septation, such as epithelial cell differentiation, mesenchymal cell proliferation, myoblast differentiation, and stem cell differentiation, were identified by this process. Igf1, a highly conserved target of miR-489 (now miR-425/425-5p/489 in TargetScan 6.2) was noted to be highly connected to other processes (organ growth along with Vegfa, and mesenchymal cell proliferation along with Ptc1), indicating a possible key role in alveolar septation.

We monitored the steady-state accumulation of the mature and functional miR-489 through qPCR with RNA from mouse lungs at different stages of development (P1, P14, and P42). There was an upregulation of miR-489 between P14 and P42, when alveolar septation normally ends (Fig. 2A). qPCR for Igf1 and Tnc mRNA showed that these two genes were maximally expressed on P1, with reduction in expression by P14, and almost absent expression by P42 (Fig. 2). Western blotting showed maximal IGF-1 and TNC at P14, and marked reduction of IGF-1 at P42, and no detectable TNC at P42 (Fig. 2, D and E).

Neonatal Hyperoxia Reduces miR-489 and Increases Igf1 and Tnc Expression

Exposure to hyperoxia decreased miR-489 by ~80% at P4, 25% at P7, and 60% at P14 compared with air-vehicle mouse
MicroRNA (miRNA) alterations during normal murine lung development. 

**A**: miRNA in mouse lung development [postnatal day 1 (P1), P4, P7, P14, P42]. The left heat map shows statistically significant ($P < 0.05$) differentially expressed miRNAs with $>2$-fold change clustered by hierarchical clustering, whereas the right heat map represents that organized by self-organizing maps (SOM). Upregulated miRNAs are in progressively brighter shades of yellow, depending on fold difference, and downregulated miRNAs are shown in progressively brighter shades of purple. Gray: no difference in miRNA expression between the groups. 

**B**: the differentially expressed miRNAs are clustered into two clusters by K-means. Clusters 1 and 2 represent miRNAs gradually decreasing and increasing over time, respectively. The black line represents the mean expression of the miRNAs at each time point. 

**C**: a graphical representation of the targets of differentially expressed miRNAs ($>2$-fold change) involved in transcriptional regulation. 

**D**: a graphical representation of the targets of differentially expressed miRNAs ($>2$-fold change) involved in different biological processes, such as epithelial cell differentiation, mesenchymal cell proliferation, myoblast differentiation, and stem cell differentiation. The arrow indicates insulin-like growth factor (IGF)-1, regulated by miR-489, which was chosen for further analysis.
pups (Fig. 3, A and B). At P14, *Igf1* and *Tnc* mRNA were significantly higher in hyperoxia-vehicle compared with air-vehicle mice (Fig. 3, C and D). Immunohistochemical staining for IGF-1 and TNC at P14 determined that IGF-1 was primarily localized to bronchial epithelium and pulmonary vessels (arteries and veins), with lesser amounts in the interstitium, while TNC was primarily in the alveoli, but not in the bronchi or pulmonary arteries (Fig. 3). Exposure to hyperoxia did not appreciably alter IGF-1 staining in bronchi and pulmonary arteries, but increased IGF-1 staining in alveolar macrophages (increased in numbers with hyperoxia) (Fig. 3). TNC staining in alveoli did not change with hyperoxia exposure, but interpretation was complicated by the reduction in alveolar density and lung parenchymal volume.

Fig. 2. miR-489, IGF-1, and tenascin C (TNC) during lung development. A: expression of miR-489 on P1, P4, P7, P14, and P42. B: expression of *Igf1* by quantitative RT-PCR (qPCR) at P1, P14, and P42, showing reduced *Igf1* at P42, when miR-489 is increased. C: expression of *Tnc* by qPCR at P1, P14, and P42, showing reduced *Tnc* at P42, when miR-489 is increased. D: Western blot demonstrating reduced IGF-1 protein at P42, when miR-489 is increased. E: Western blot demonstrating markedly reduced TNC protein at P42, when miR-489 is increased. Values are means ± SE; *n* = 6 mice/group. *P* < 0.05 vs. other time points.
Inhibition of miR-489 Using LNA to miR-489 Increases Igf1 and Tnc, While Attenuating Hyperoxia-induced Inhibition of Lung Development and Alterations in Lung Function

No change in mortality or growth was noted with exposure to LNA-miR-489. qPCR for miR-489 in mouse lungs treated with LNA-miR-489 demonstrated a decrease of ∼85% in air-LNA-miR-489 mice and ∼95% in hyperoxia-LNA-miR-489 mice, compared with air-vehicle mice (Fig. 3B).

Igf1 mRNA was increased by hyperoxia. LNA-miR-489 decreased Igf1 mRNA in both air- and hyperoxia-exposed mice compared with corresponding vehicle-exposed mice (Fig. 3C, D and F). IGF-1 staining was increased by hyperoxia and LNA-miR-489, while TNC staining was decreased by hyperoxia and LNA-miR-489.

However, LNA-miR-489 during hyperoxia attenuated hyperoxia-induced decreases in MLI (Fig. 3C, D) and RACs (Fig. 3F, H). Exposure to LNA-miR-489 during hyperoxia also reduced RACs (Fig. 3H). Exposure to LNA-miR-489 during air exposure did not affect lung histology (Fig. 3G, H).

LNA-miR-489 during hyperoxia also reduced lung compliance and increased total lung resistance in hyperoxia-vehicle mice compared with air-vehicle mice (Fig. 3I, J and K). Exposure to LNA-miR-489 during air exposure did not affect lung function compared with air-vehicle mice (Fig. 3I, J and L). Exposure to LNA-miR-489 during hyperoxia attenuated hyperoxia-induced decrease in lung compliance, although not to the level seen in air-vehicle mice (Fig. 3J, L).

IGF-1 staining was increased by hyperoxia and LNA-miR-489, while TNC staining was decreased by hyperoxia and LNA-miR-489. Figure 4 shows the effects of hyperoxia, LNA-miR-489, and pCMV-miR-489 on Igf1 and Tnc. A: lung homogenates from mice at 4, 7, and 14 days of age exposed to air or hyperoxia (85% O2) were analyzed by qPCR for miR-489. Values are means ± SE; n = 6 mice/group. Immunohistochemistry for IGF-1 (E, F, I, J, M, N, Q, and R) and TNC (G, H, K, L, O, P, S, and T) in mouse pups exposed to air or hyperoxia in combination with either vehicle (F–L), LNA-anti-miR-489 (M–P), or pCMV-miR-489 (Q–T). IGF-1 staining is noted in alveolar epithelium, blood vessels, lung parenchyma, and macrophages, while TNC staining is mainly in lung parenchyma interstitium and not in airways or blood vessels. Magnification ×400. Calibration bar = 50 μm. Ab, antibody; Br, bronchiole; PA, pulmonary artery. Quantitation of immunohistochemical staining for IGF-1 (U) and TNC (V) as percentage of stained area is shown. Values are means ± SE; n = 3 mice/group. *P < 0.05 vs. corresponding air. †P < 0.05 vs. corresponding vehicle.
miR-489 and Lung Development

Overexpression of miR-489 decreases Igf1 and Tnc, induces inhibition of lung development, and reduces lung compliance

No change in mortality or growth was noted with exposure to pCMV-miR-489. qPCR for miR-489 in mouse lungs treated with pCMV-miR-489 demonstrated an increase of miR-489 expression at 14 days, compared with air-vehicle mice (Fig. 3A). This was accompanied by reductions in Igf1 mRNA compared with corresponding vehicle-exposed mice. (Fig. 3, B and C). Tnc mRNA was also reduced in air-pCMV-miR-489 compared with air-vehicle mice, but Tnc mRNA in hyperoxia-vehicle and hyperoxia-pCMV-miR-489 mice was not different. IGF-1 and TNC staining was reduced by pCMV-miR-489 both in air and hyperoxia, compared with vehicle-exposed mice. Overexpression of miR-489 inhibited alveolar development, as indicated by larger alveolar spaces with reduced seption on histological examination, increased MLi, and reduced RACs compared with air-vehicle mice (Fig. 4). Lung compliance was also reduced in air-pCMV-miR-489 compared with air-vehicle mice (Fig. 4I). However, no changes in total lung resistance were noted between air-pCMV-miR-489 and hyperoxia-pCMV-miR-489 and air-vehicle mice, as the expected increase in lung resistance with hyperoxia or with pCMV-miR-489 was not seen (Fig. 4I).

Localization of miR-489

In situ hybridization for miR-489 demonstrated punctate staining in alveolar septae, as well as in base of alveoli and interstitium (Fig. 5). Staining was also noted in bronchiolar epithelium (Fig. 5).

In cell lines, expression of miR-489 was primarily in epithelial cells (miR-489/U6 snRNA: MLE-12: 2.3 ± 0.3 vs. NMLF: 0.008 ± 0.001 and mPMVEC: 0.004 ± 0.002; P < 0.05), while IGF-1 (Igf1/Gapdh mRNA: NMLF: 0.53 ± 0.03 vs. MLE-12: 0.0003 ± 0.00001 and mPMVEC: 0.001 ± 0.0001, P < 0.05) and TNC (Tnc/Gapdh mRNA: NMLF: 5.3 ± 0.2 vs. MLE-12: 0.04 ± 0.01 and mPMVEC: 0.001 ± 0.0004, P < 0.05) were mainly expressed in fibroblasts. miR-489 was also found in exosomes isolated from conditioned media of MLE-12 cells. In flow-sorted cells, miR-489 was

Air

85% O2

Vehicle

LNA to miR-489

pCMV-miR-489

Fig. 4. LNA-miR-489 attenuates hyperoxia-induced inhibition of neonatal mouse lung development and increased lung resistance, while miR-489 overexpression inhibits normal lung development and reduces lung compliance. A, B, E, F, G, and H: representative photomicrographs of H&E-stained sections of lungs from mice at 14 days of age administered either vehicle (A and B), LNA-miR-489 (E and F), or pCMV-miR-489 (G and H) during 14 days of air (A, E, and G) or hyperoxia (B, F, and H) exposure (magnification ×100, calibration bars = 250 μm). Alveolar size was larger and secondary septae were fewer in hyperoxia-vehicle (B), air-pCMV-miR-489 (G), and hyperoxia-pCMV-miR-489 (H) mice compared with air-vehicle (A), air-LNA-miR-489 (E), and hyperoxia-LNA-miR-489 (F) mice. Hyperoxia-exposed mice administered LNA-miR-489 (F) had significantly smaller alveoli, as indicated by a smaller mean linear intercept (C), and more alveoli, as indicated by a greater radial alveolar count (D), than hyperoxia-vehicle mice (B), although the alveolar development was still diminished compared with air-vehicle mice (A). I: lung compliance was reduced by hyperoxia, and LNA-miR-489 partly attenuated the hyperoxia-induced reduction in compliance. J: total lung resistance was increased in hyperoxia-vehicle mice, and LNA-miR-489 reduced resistance to the level seen in air-vehicle mice. Air-exposed pCMV-miR-489 (G) had significantly larger alveoli, as indicated by a larger mean linear intercept (C), and fewer alveoli, as indicated by a greater radial alveolar count (D), than air-vehicle mice (A), although the alveolar development was still less compared with hyperoxia-vehicle mice (B). J: lung compliance was reduced in air-pCMV-miR-489 mice compared with air-vehicle mice, although hyperoxia-vehicle and hyperoxia-pCMV-miR-489 mice had similar reduction in compliance. Values are means ± SE; n = 6 mice/group. *P < 0.05 vs. corresponding air. †P < 0.05 vs. corresponding vehicle.
primarily expressed in epithelial cells (miR-489 expression 72 ± 23-fold higher in E-cadherin+/PECAM-1− cells vs. E-cadherin−/PECAM-1+ cells or NMLF).

**Hyperoxia and Overexpression of miR-489 Reduces Tropoelastin mRNA Expression and Results in Abnormal Elastin Deposition, While miR-489 Inhibition Increases Tropoelastin mRNA and Improves Elastin Deposition During Hyperoxia**

Compared with air-vehicle or air-LNA-miR-489 mice, hyperoxia-vehicle mice and pCMV-miR-489 mice had reduced tropoelastin mRNA. Distribution of elastin by Hart’s staining was also abnormal with hyperoxia and pCMV-miR-489, as there was reduced elastin in alveolar septae and increased amounts in alveolar walls. In contrast, hyperoxia-LNA-miR-489 mice had increased tropoelastin mRNA, as well as a more normal pattern of elastin staining (Fig. 6).

**In Vitro Studies to Confirm miR-489 Targeting of IGF-1 and TNC**

Transfection with pCMV-miR-489 decreased reporter expression of IGF-1 3’ UTR by 60% (3’ UTR-Igf1: 1.0 ± 0.2 vs. 3’ UTR-Igf1+pCMV-miR-489: 0.4 ± 0.1 vs. 3’ UTR-Igf1+scramble: 1.0 ± 0.05) and TNC 3’ UTR by 50% (3’ UTR-TNC: 1.0 ± 0.1 vs. 3’ UTR-TNC+pCMV-miR-489: 0.5 ± 0.1 vs. 3’ UTR-TNC+scramble: 1.1 ± 0.05).

**Human Studies**

Expression of miR-489 was decreased in BPD lung compared with term lung. Expression of Igf1 and Tnc were increased in human BPD lung compared with preterm lung or term lung (Fig. 7).

**DISCUSSION**

In this study, we first determined overall patterns of change in miRNA during murine postnatal lung development. Our unbiased miRNA transcriptomic analysis indicated specific transcriptional regulation and biological processes and pointed to target genes potentially contributing to alveolar septation, the major postnatal change that occurs in the murine lung. As it is not possible to investigate a large number of potential miRNA and target genes simultaneously in a mechanistic manner, we decided to focus on molecules that appeared to be involved in multiple biological processes. Hence, we focused on IGF-1 regulated by miR-489, which is involved in the biological processes of mesenchymal cell proliferation and organ growth. We first identified that miR-489 was minimally expressed until after completion of most of alveolar septation (P42). The peak expression of IGF-1 and TNC (conserved targets of miR-489 known to be involved in lung development) was during alveolar septation, following which there was a marked reduction in their concentration. Taken together, these results suggest that the increase in miR-489 after P14 may serve to inhibit IGF-1 and TNC and is associated with completion of alveolar septation (earlier changes in IGF-1 and TNC may be mediated by other regulatory mechanisms). We noted that hyperoxia reduced miR-489, accompanied by increased IGF-1 and TNC. We initially suspected that this hyperoxia-induced reduction in miR-489 and increases in IGF-1 and TNC contributed to inhibition of alveolar septation. However, contrary to our expectations, further inhibition of miR-489 using LNA-miR-489 improved alveolar septation, while miR-489 overexpression in newborn mice inhibited alveolar septation. Our results suggest that miR-489 is an inhibitor of alveolar septation, and reduced miR-489 and increased IGF-1 and TNC
may be compensatory during hyperoxia or BPD. The hyperoxia-induced inhibition of miR-489 appears to be an inadequate attempt at compensation to maintain alveolar septation during hyperoxic exposure. Further inhibition of miR-489 using LNA-miR-489 may permit alveolar septation to proceed. Our results suggest that increased miR-489 is a marker of completion of lung development. The finding that LNA-miR-489 attenuated hyperoxia-induced inhibition of alveolar septation and abnormalities of lung function is of potential therapeutic relevance for disorders characterized by impaired alveolar development and lung inflammation, such as BPD in preterm neonates. Our finding that miR-489, IGF-1, and TNC are increased in human BPD further supports the role of these molecules in normal and abnormal lung development.

Strengths of our study include the unbiased profiling of miRNA during murine lung development and the validation of the importance of miR-489 using in vivo inhibition and overexpression. In addition, we evaluated both lung structure and function, in addition to estimating expression of miR-489, as well as its main targets. However, there are also limitations of our study. A single miRNA may target many genes, and each gene may in turn be regulated by multiple miRNAs. Mouse models may not closely simulate human disorders due to interspecies differences. However, substantial similarity has been noted in lung miRNA expression profiles between mouse and humans, with similar relative expression levels (44). In addition, our results showing a reduction in miR-489 in human BPD compared with term lung, and the increase in IGF-1 and TNC in BPD compared with preterm and term lung, support the relevance of our animal model. IGF-1 and TNC are abundant in the lung, involving many cell types and with multiple possible interactions, and it is difficult to determine which cell types are primarily responsible for pathophysiology. Our results suggest that miR-489 is epithelial derived (as miR-489 expression is primarily in epithelial cells and not in fibroblasts or endothelial cells) and affects its targets IGF-1 and TNC in fibroblasts (as epithelial cells and endothelial cells did not express much IGF-1 or TNC). We found that exosomes derived from epithelial cells contain miR-489, suggesting that epithelial cells may possibly interact with subjacent fibroblasts.

Fig. 6. Elastin staining. A–J: representative photomicrographs of elastin staining in sections of lungs from mice at 14 days of age administered either vehicle (A, D, F, and I), LNA-miR-489 (B, E, G, and J), or pCMV-miR-489 (C and H) during 14 days of air (A–C, F–H) or hyperoxia exposure (D, E, I, and J) (top panels at ×100 magnification, calibration bars = 250 μm; bottom panels at ×400 magnification, calibration bars = 50 μm). K: tropoelastin mRNA by qRT-PCR of RNA from lung homogenates of mice at 14 days of age administered either vehicle, LNA-miR-489, or pCMV-miR-489 during 14 days of air or hyperoxia exposure. L: tissue area (T), elastin stained area (E), and tissue/elastin stained area (E/T) measured by image analysis in sections of lungs from mice at 14 days of age administered either vehicle, LNA-miR-489, or pCMV-miR-489 during 14 days of air or hyperoxia exposure. Values are means ± SE; n = 6 mice/group. *P < 0.05 vs. corresponding air. †P < 0.05 vs. corresponding vehicle. Data from pCMV-miR-489 mice are similar to that from 85% O2-vehicle mice (not shown).
and other cell types in a paracrine manner via secreted exosomes. Future studies evaluating such epithelial-mesenchymal interactions mediated via exosomes and miRNA are indicated. Additional studies targeting specific cell types are also required, as morphogenesis is determined by cell-specific expression of regulatory genes.

miRNAs are known to be important mediators of lung development (6, 15). Dong et al. (15) comprehensively profiled miRNA and mRNA from embryonic day 12 to adulthood in mice and observed that 117 miRNAs and 11,220 mRNA probes were dynamically regulated and clustered into six distinct temporal expression patterns. In this study, 55% of proteins were regulated with a direct correlation between miRNA and protein concentration, but without detectable change in mRNA levels, indicating that miRNA may directly downregulate target proteins without any changes in corresponding mRNA (15).

Cox et al. (12) did a global proteomic survey during mouse lung development from embryonic day 13.5 to adult life. Large groups of gene products were identified with significant correlation as well as divergence of protein and transcript levels during lung development (12), again indicating regulation of protein levels independent of transcription. Bhaskaran et al. (6) and Williams et al. (44) also profiled miRNA expression during rodent lung development, but with fewer miRNA being evaluated, primarily because more miRNA have been identified in the past decade and techniques have advanced. Recently, we used mirDREM, a probabilistic modeling method that reconstructed dynamic regulatory networks that explained how temporal gene expression is jointly regulated by miRNAs and transcription factors (35). We now extend this understanding of gene regulation with a focus on one specific miRNA (miR-489) and its targets that are known to be involved in lung development.

Relatively little has been described about miR-489 in literature to date. It is known that miR-489 is highly conserved among species and is important in the regulation of differentiation of mesenchymal stem cells, maintaining muscle stem cell quiescence (11), negatively regulating osteogenesis (34), and inhibiting a cytoplasmic protein tyrosine phosphatase (PTPN11) that is a tumor suppressor (24). Genomewide gene expression analysis in a tumor cell line identified 53 putative gene targets significantly downregulated by miR-489 transfection, of which 32 genes had miR-489 target sites (24). In TargetScan (www.targetscan.org; release 6.2: June 2012), mmu-miR-425/425-5p/489 has 130 conserved targets, with a total of 133 conserved sites and 44 poorly conserved sites. Obviously, it is not feasible to evaluate all possible targets in vivo, and we chose to evaluate the two most biologically plausible targets with relevance to alveolar septation (IGF-1 and TNC). It is quite possible that other highly conserved targets, such as ephrin-B2 are also important (37). Our results indicate miR-489 is of epithelial origin, while IGF-1 and TNC are derived from fibroblasts. We speculate that miR-489 from epithelial cells has paracrine effects (perhaps via exosomes) on subjacent fibroblasts that mediate downstream effects.

We observed that miR-489 overexpression inhibited lung alveolar septation and reduced compliance, but did not increase lung resistance. Further reduction of miR-489 during hyperoxia using LNA-miR-489 attenuated hyperoxia-induced inhibition of septation and improved lung compliance and resistance. It is possible that hyperoxia induces alterations in signaling pathways that are not operative during normoxia, which can be suppressed by reducing miR-489. IGF-1 and TNC, the targets of miR-489 that we investigated, are well known as regulators of lung development. Lung concentrations of IGF-1 (40) and TNC (47) peak during the height of alveolar formation. Inhibition of IGF-1 signaling (using a truncated soluble IGF-1 receptor to decoy ligand away from IGF-1 receptor, or using a neutralizing antibody to IGF-1) inhibits alveolar formation in neonatal rats (25). Increased IGF-1 has been observed in models of accelerated lung growth (29), and a reemergence of a fetal pattern of greater IGF-1 expression has been observed during hyperoxic lung injury (39). TNC contributes both to branching morphogenesis (31, 47) and alveolar septation (32), although the TNC knockout mouse is viable without a major pulmonary phenotype (26). During alveolar septation, TNC is mainly produced by lung fibroblasts and is localized to alveolar septal walls and concentrated at secondary septal tips, suggesting a role in formation of secondary septa (48). In preterm infants with RDS and BPD, there is increased IGF-1 in airway and alveolar epithelia and in mesenchymal cells (10), and increased TNC in alveolar walls (19), indicating relevance to our model of hyperoxia-exposed newborn mice. While we identified IGF-1 and TNC as being regulated by miR-489, IGF-1 and TNC are also regulated by other miRNAs. They are also affected by multiple transcription factors that are known to be involved in lung development.
factors and DNA methylation (13) during lung development. It is also important to note the lack of effect of LNA-miR-489 during normoxia and overexpression (pCMV-miR-489) during hyperoxia. LNA-miR-489 during normoxia does not accelerate alveolar septation beyond normal, because, while normal amounts of IGF-1 and TNC permit normal alveolar septation to proceed and their deficiency inhibits alveolar septation, excessive amounts of IGF-1 and TNC cannot improve alveolarization beyond normal, as other components required for septation do not increase concordantly. Similarly, overexpression of miR-489 during hyperoxia cannot make alveolar septation any worse, as it is already maximally inhibited by 85% O2 exposure.

In conclusion, multiple miRNA and their targets are involved in normal lung alveolar septation, and their dysregulation may contribute to hyperoxia-induced inhibition of lung development and human disorders, such as BPD. We have identified miR-489 as one of the negative regulators of alveolar septation, which, in adult life, may act as a "brake" to inhibit continued development of new alveoli. We have also identified a potential novel mechanism of epithelial-mesenchymal interaction in which epithelial-derived exosomes containing miRNA may affect signaling in fibroblasts. Our work suggests that modulation of miR-489 and similar miRNAs may be potential therapeutic strategies for neonatal lung diseases, and perhaps other disorders characterized by impaired alveolar development, or conditions when it may be advantageous to regenerate alveoli in adults (e.g., in chronic obstructive pulmonary disease). The ability to administer miRNA selectively to the lung by instillation (e.g., similar to surfactant therapy) or inhalation and affect multiple target genes is an advantage, and miRNA-based therapeutics for disorders such as lung cancer are under development (42, 45). Additional research is needed in larger mammalian models with evaluation of possible toxicity and longer term effects.

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