Targeting miR-34a/Pdgfра interactions partially corrects alveologenesis in experimental bronchopulmonary dysplasia

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Abstract

Bronchopulmonary dysplasia (BPD) is a common complication of preterm birth characterized by arrested lung alveolarization, which generates lungs that are incompetent for effective gas exchange. We report here deregulated expression of miR-34a in a hyperoxia-based mouse model of BPD, where miR-34a expression was markedly increased in platelet-derived growth factor receptor (PDGFR)α-expressing myofibroblasts, a cell type critical for proper lung alveolarization. Global deletion of miR-34a; and inducible, conditional deletion of miR-34a in PDGFRα+ cells afforded partial protection to the developing lung against hyperoxia-induced perturbations to lung architecture. Pdgfra mRNA was identified as the relevant miR-34a target, and using a target site blocker in vivo, the miR-34a/Pdgfра interaction was validated as a causal actor in arrested lung development. An anti-miR directed against miR-34a partially restored PDGFRα+ myofibroblast abundance and improved lung alveolarization in newborn mice in an experimental BPD model. We present here the first identification of a pathology-relevant microRNA/mRNA target interaction in aberrant lung alveologenesis and highlight the translational potential of targeting the miR-34a/Pdgfра interaction to manage arrested lung development associated with preterm birth.

Keywords bronchopulmonary dysplasia; hyperoxia; lung development; miR-34a; platelet-derived growth factor

Subject Category Respiratory System

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Introduction

Bronchopulmonary dysplasia (BPD), a serious complication of preterm birth (Jobe, 2016), is characterized by arrested alveologenesis of lungs of infants, arising from oxygen toxicity and mechanical injury during oxygen supplementation to manage respiratory failure. How these insults impair lung alveolarization is unclear (Surate Solaligue et al., 2017; Morty, 2018).

Lung development includes progressive subdivision of airspaces to expand alveoli number, thereby increasing gas-exchange surface area; and progressive thinning of septa to minimize gas diffusion distance (Pozarska et al., 2017). Alveolar myofibroblasts, which express SMa, facilitate alveologenesis (Vaccaro & Brody, 1978; Morrisey & Hogan, 2010; Hogan et al., 2014) by generating elastin cables that drive formation of secondary septa, which divide existing airspaces by squeezing the pre-existing alveoli with an elastin net, or pulling septal invaginations into airspaces (Branchfield et al., 2016). Myofibroblasts localize to alveolar entry rings during alveologenesis (McGowan et al., 2008; Ntokou et al., 2015), exhibit phenotypic plasticity (Endale et al., 2017; McGowan & McCoy, 2017) and are marked by platelet-derived growth factor (PDGF) receptor (PDGFR)α, a mediator of normal (Boström et al., 1996, 2002; Gouveia et al., 2018) and aberrant (Oak et al., 2017) alveologenesis. Reduced levels of PDGFRα have also been noted in mesenchymal cells from human neonates that develop BPD (Popova et al., 2014).

How myofibroblast function is disturbed during aberrant alveologenesis is not known, but a role for microRNA has been proposed, since deregulation of microRNA has been noted in clinical and experimental BPD (Nardiello & Morty, 2016), although no study has validated a causal role for any microRNA/mRNA interaction in alveologenesis or BPD. We report here that the miR-34a/Pdgfра interaction...
interaction is disease relevant, and can be therapeutically targeted to partially restore lung alveolarization under pathological conditions. These data highlight a new mediator, and druggable target, in arrested alveolarization associated with preterm birth.

Results and Discussion

miR-34a is the most deregulated lung microRNA species in experimental BPD

BPD is modeled by exposure of newborn mice to hyperoxia (Nardiello et al., 2017a,b). Changes in microRNA expression during hyperoxia (85% O₂) exposure were detected by microarray (GEO accession number GSE89666). The steady-state levels of 10 and four microRNA species, respectively, were deregulated at post-natal day (P)5 and P14 (Fig 1A). These time-points represent the peak and near-completion phases, respectively, of bulk secondary seption in normally developing lungs (Morrissey & Hogan, 2010; Warburton et al., 2010). Levels of miR-34a-5p were the most consistently and appreciably increased of all microRNA species, implicating miR-34a-5p as a candidate mediator of arrested alveolarization. Independent validation by real-time RT–PCR revealed that miR-34a-5p levels were increased at P3, P5, and P14 in hyperoxia-exposed lungs (Fig 1B), with little or no impact on miR-34b-5p or miR-34c-5p (Fig 1B), or miR-34a-3p, miR-34b-3p, or miR-34c-3p (Fig 1C) levels noted. Levels of miR-34a-5p were consistently elevated over the P3-P14 hyperoxia-exposure time-course, in comparison with normoxia (21% O₂)-exposed lungs that exhibited normal alveolarization (Fig 1B). Together, these data highlight miR-34a-5p as a candidate mediator of arrested alveolarization.

Global loss of miR-34a partially restores lung alveolarization in experimental BPD

Consistent with the arrested alveolarization that forms the hallmark of the BPD animal model, a 71% decrease in total alveoli number (Fig 2A and B; Appendix Table S1) and 10% increase in mean septal thickness (Fig 2A and C; Appendix Table S1) were noted in hyperoxia-exposed wild-type mouse lungs at P14, mimicking perturbations to lung structure noted in clinical BPD cases (Jobe, 2016; Nardiello et al., 2017b). Ablation of miR-34a (miR-34a−/− mice) partially protected against the impact of hyperoxia on alveolarization (Fig 2A; Appendix Table S1), with alveoli numbers increased by 47% (Fig 2B); and septal thickness decreased to even thinner than that noted in healthy mice (Fig 2C), compared to wild-type hyperoxia-exposed controls. No compensatory increase in miR-34b or miR-34c levels was noted in miR-34a−/− mice (Appendix Fig S1A). In contrast, dual ablation of miR-34b/miR-34c (miR-34bc−/− mice), without a compensatory increase in miR-34a levels (Appendix Fig S1B), did not impact alveoli number during hyperoxia-driven arrest of alveolarization (Fig 2D and E; Appendix Table S2). However, protection against hyperoxia-driven septal thickening in miR-34bc−/− mice was noted (Fig 2F), perhaps related to the increased levels of the 3p strands of miR-34b and miR-34c in the lungs of hyperoxia-exposed mice (Appendix Fig S1B). These data implicate miR-34a as mediator of arrested alveolarization associated with hyperoxia, an idea reinforced by detection of miR-34a expression with a lacZ-tagged miR-34a gene-trap in the septa of developing lungs, with increased β-galactosidase staining evident after hyperoxia exposure (Fig 2G; Appendix Fig S2).

miR-34a in PDGFRα+ cells contributes to aberrant lung alveolarization

An in silico analysis identified two miR-34a-binding sites in the Pdgfra 3′-UTR (Fig 3A) (Silber et al., 2012; Garofalo et al., 2013). The PDGF-AA ligand and PDGFRα are key mediators of alveolarization (Boström et al., 1996, 2002), and reduced PDGFRα levels in mesenchymal cells are reported in human neonates that develop BPD (Popova et al., 2014). A synthetic miR-34a mimic reduced PDGFRα protein levels in vitro in MLg cells, a mouse lung fibroblast cell line, suggesting that a miR-34a/Pdgfra interaction occurs in mouse lung fibroblasts (Fig 3B), where increased miR-34 family microRNA transcripts (Fig 3C) and reduced Pdgfra mRNA transcripts (Appendix Fig S3) were noted in hyperoxia-exposed MLg cells. To explore this idea in vivo, exposure of newborn mice to hyperoxia (85% O₂) reduced lung PDGFRα protein levels at P5 (Fig 3D), which is the peak phase of bulk alveolarization (Morrissey & Hogan, 2010; Warburton et al., 2010). Treatment of MLg cells in vitro with antimiR-34a, which neutralizes miR-34a, partially protected steady-state PDGFRα protein levels against the impact of hyperoxia exposure, while an inert (“scrambled”) antimiR did not (Fig 3E). These data support the contention that hyperoxia-driven elevations in miR-34a levels negatively regulated PDGFRα abundance. PDGFRα+ cells were isolated from P5 mouse lungs by FACS (Appendix Fig S4A), where in vivo hyperoxia exposure had driven a dramatic increase in miR-34a levels in PDGFRα+ cells (Fig 3F, Appendix Fig S5), accompanied by reduced Pdgfra (Appendix Fig S4B) and Acta2 (Appendix Fig S4C) mRNA levels. The magnitude of the impact of hyperoxia on miR-34a levels in PDGFRα+ cells was considerably larger than that observed in lung homogenates, highlighting the PDGFRα+ cell as being particularly susceptible to hyperoxia-driven effects on miR-34a during alveologenesis.

To address miR-34a function in PDGFRα+ cells, a mouse strain carrying a conditional, tamoxifen-inducible deletion of miR-34a in Pdgfra-expressing cells was generated (denoted miR-34aAPC/−/APC−, Fig 3G) and was validated by demonstrating reduced miR-34a expression in PDGFRα+ cells (Fig 3H). Ablation of miR-34a in PDGFRα+ cells protected against hyperoxia-driven arrest of alveolarization (Fig 3I; Appendix Table S3), where approximately double the number of alveoli was noted in hyperoxia-exposed mice in which miR-34a expression was blocked in PDGFRα+ cells (Fig 3J). Ablation of miR-34a expression in PDGFRα+ cells did not impact hyperoxia-provoked perturbations to septal thickness (Fig 3K), which we attribute to the tamoxifen solvent, Miglyol, a complex fatty acid-derivative mixture, which we propose limited the impact of hyperoxia on septal thickening analogous to that reported for chemically related cottonseed oil (Nardiello et al., 2017b), since Miglyol alone is known to attenuate normal lung development (Fehl et al., 2019). Alternatively, it may be epithelial miR-34a that regulates septal thickening, since miR-34a regulates lung epithelial cell (notably, type II pneumocyte) apoptosis (Syed et al., 2017) in experimental BPD. These data validate a role for miR-34a in PDGFRα+ cells in mediating the inhibitory effects of hyperoxia on alveolarization.
Figure 1. miR-34a is the most impacted microRNA species in developing mouse lungs after hyperoxia exposure.

A Microarray analysis of microRNA expression changes in newborn mouse lungs exposed to 21% O₂ versus 85% O₂, at post-natal day (P) 5 and P14. Microarray data are available at the GEO database under accession number GSE89666.

B Quantitative RT-PCR detection of microRNA-34a/b/c-5p family members in the lung over the course of normal (21% O₂) and aberrant (85% O₂) alveolarization.

C Quantitative RT-PCR detection of microRNA-34a/b/c-3p family members in the lung over the course of normal (21% O₂) and aberrant (85% O₂) alveolarization.

Data information: For (A), a Welch’s approximate t-test was employed to determine P values (n = 4 animals for each experimental group), which were corrected using the algorithm of Benjamini and Hochberg, as described in the Materials and Methods under the heading “Power and statistical analyses”. For (B) and (C), data represent mean ± SD (n = 6 animals for each experimental group). P values were determined by one-way ANOVA with Tukey’s post hoc modification, and all P values < 0.05 for 21% O₂ versus 85% O₂ comparisons at each developmental stage (P3, P15, and P14) are indicated.
The miR-34a/Pdgfra interaction plays a causal role in aberrant lung alveolarization

MicroRNA/mRNA interactions can be interrupted using target site blocker (TSB) technology. We employed two synthetic TSBs (TSB1 and TSB2) to protect both of the miR-34a-binding sites in the Pdgfra 3’-UTR (Fig 4A). Both TSBs protected PDGFRα expression from miR-34a regulation in MLg cells in vitro (upper panels, Fig 4B and C). Both TSBs exhibited specificity for the miR-34a/Pdgfra interaction, since neither TSB interfered with the impact of a synthetic miR-34a mimic on levels of c-Kit (middle panel, Fig 4B), a validated miR-34a target (Siemens et al, 2013), or of SIRT1 (middle panel, Fig 4C), another validated miR-34a target (Yamakuchi et al, 2008). A TSB cocktail of an equimolar TSB1:TSB2 mixture effectively protected PDGFRα expression from miR-34a regulation in MLg cells (Appendix Fig S6). In vivo, TSBs afforded some protection against...
**Figure 3.**

**A** Interaction type: 7mer-A1

| Interaction | 2602 | 2608 |
|-------------|------|------|
| 7mer-m8     | 2631 | 2637 |

**B**

| PDGFRα | βactin |
|--------|--------|
| Mock   |        |
| SCR    |        |
| MIM34a 60 nM |        |
| MIM34a 80 nM |        |

**C**

| ΔCt | 34a-5p | 34b-5p | 34c-5p |
|-----|--------|--------|--------|
| 21% O₂ |        |        |        |
| 85% O₂ |        |        |        |

**D**

| PDGFRα | βactin |
|--------|--------|
| 21% O₂ |        |
| 85% O₂ |        |

**E**

| PDGFRα | βactin |
|--------|--------|
| 21% O₂ |        |
| 85% O₂ |        |

**G**

**Pdgfra-CreER¹²**

**Pdgfra-specific miR-34a deletion**

| CreERT²miR-34a⁵⁺⁺ | +Tmxfn |
|--------------------|--------|
| miR-34a.UsePC/IPC |        |

**I**

21% O₂

85% O₂

**J**

**Number of alveoli (x10⁶)**

| 34a⁵⁺⁺        | 34a⁵⁺⁺ | 34a.UsePC/IPC |
|---------------|--------|---------------|
| 21% O₂        | 85% O₂ |               |

**K**

**Septal thickness (μm)**

| 34a⁵⁺⁺        | 34a⁵⁺⁺ | 34a.UsePC/IPC |
|---------------|--------|---------------|
| 21% O₂        | 85% O₂ |               |
Through partial restoration of PDGFRα data, validate a causal role for the miR-34a/PDGFRα interaction in BPD. Application of the TSB1,2 cocktail increased the abundance of both BPD (Fig 4D; Appendix Table S4), where increased alveoli number dampened functional miR-34a levels in experimental BPD; therefore, an antimiR directed against miR-34a was applied therapeutically (concomitantly with hyperoxia exposure; Fig 5A), which decreased functional miR-34a levels in mouse lungs by P5 (Appendix Fig S8A, with no impact on miR-34b, and a moderate impact on miR-34c (Appendix Fig S8B and C). The effect of antimiR-34a on miR-34a was maintained up to P14 (Fig 5B). AntimiR-34a protected alveolarization from hyperoxia (Fig 5C; Richardson-stained plastic-embedded lung sections from wild-type mouse pups at post-natal day (P)14, treated with either scrambled target site blocker (SCR) or the TSB1,2 cocktail during aberrant alveolarization (n = 5 animals for each group). The effect of antimiR-34a on miR-34a was maintained up to P14 (Fig 5B). AntimiR-34a protected alveolarization from hyperoxia (Fig 5C; Richardson-stained plastic-embedded lung sections from wild-type mouse pups at post-natal day (P)14, treated with either scrambled target site blocker (SCR) or the TSB1,2 cocktail during aberrant alveolarization (n = 5 animals for each group). The effect of antimiR-34a on miR-34a was maintained up to P14 (Fig 5B).

Figure 3. miR-34a-5p acts in PDGFRα⁺ cells to block lung alveolarization.

- In silico identification of miR-34a binding sites in the PDGfra 3′-UTR.
- Immunoblot detection of PDGFRα levels in MLg cells after treatment with scrambled microRNA (SCR) or a miR-34a (MIM34a) mimic (n = 3 separate cell cultures for each group).
- Quantitative RT-PCR detection of miR-34a/bc-5p levels in MLg cells in vitro, maintained under 21% O₂ or 85% O₂ (n = 3 separate cell cultures for each group).
- Immunoblot detection of PDGFRα levels in the lungs of mouse pups (n = 6 animals for each group) at post-natal day (P)5, during normal (21% O₂) and aberrant (85% O₂) alveolarization.
- Immunoblot detection of PDGFRα levels in MLg cells in vitro, maintained under 21% O₂ or 85% O₂, where cells had been transfected with a scrambled (SCR) antimiR, or an antimiR directed against miR-34a (A34a) (n = 3 separate cell cultures for each group).
- Quantitative RT-PCR detection of miR-34a-5p levels in PDGFRα⁺ cells, sorted by FACS from the lungs of mouse pups (n = 4 animals for each group; data from an independent repetition are presented in Appendix Fig S5) at P5, maintained under 21% O₂ or 85% O₂, from birth.
- Quantitative analysis of lung structure in Richardson-stained plastic-embedded lung sections from 34a+/+/ or 34a−/− mice at post-natal day (P)14 during aberrant (85% O₂) alveolarization, compared with 34a+/+ mice during normal (21% O₂) alveolarization (scale bar, 50 μm). Data are representative of observations made in four other experiments.
- Quantification of total number of alveoli by design-based stereology in 34a+/+ or 34a−/− mouse pups at P14, during normal and aberrant alveolarization (n = 5 animals for each group).
- Quantification of mean septal thickness by design-based stereology in wild-type mouse pups at P5, treated with either scrambled target site blocker (SCR) or the TSB1,2 cocktail during aberrant alveolarization (n = 5 animals for each group).

Data information: For immunoblots (B, D, E), protein loading equivalence was controlled by β-actin levels. (C, F, H, J, K) Data represent mean ± SD. In (C, F, and H), P values for pair-wise comparisons were calculated by unpaired Student’s t-test. In (J and K), P values for selected comparisons were calculated by one-way ANOVA with Tukey’s post hoc modification. Source data are available online for this figure.

Figure 4. Disrupting the miR-34a/Pdgfra interaction restores myofibroblast abundance and limits hyperoxic damage to the developing alveolar architecture in mouse lungs.

A. Generation of two target site blocker (TSB) locked nucleic acid (LNA) antimiRs (Patrick et al., 2010). We theorized that

the impact of hyperoxia on lung alveolarization in experimental BPD (Fig 4D; Appendix Table S4), where increased alveoli number (Fig 4E) and decreased mean septal thickness (Fig 4F) were noted. Application of the TSB1,2 cocktail increased the abundance of both PDGFRα⁺ cells (Fig 4G; Appendix Fig S7) and PDGFRα⁺/αSMA⁺ myofibroblasts (Fig 4H) in hyperoxia-exposed mouse lungs. These data validate a causal role for the miR-34a/Pdgfra interaction in arrested lung development provoked by hyperoxia, most likely through partial restoration of PDGFRα⁺/αSMA⁺ myofibroblasts.

MicroRNA function may be modulated using locked nucleic acid (LNA) antimiRs (Patrick et al., 2010). We theorized that
described in Appendix Fig S9A,B), revealed that the abundance of both PDGFRα+ cells (Fig 5F; Appendix Fig S10A) and PDGFRα+/αSMA+ myofibroblasts (Fig 5G; Appendix Fig S10B), both of which were depleted by hyperoxia, was partially restored by antimiR-34a. However, the abundance of αSMA+ cells per se was not changed (Appendix Fig S9C and D). These data imply that antimiR-34a partially restored myofibroblast numbers in injured, developing lungs (schematically presented in Fig 5H). Consistent with this idea, increased elastin foci and improved elastin fiber organization were noted in antimiR-34a-treated mice (Appendix Fig S11).

To further explore the role of hyperoxia and miR-34a on PDGFRα+ cell abundance, apoptosis was assessed in PDGFRα+ cells from hyperoxia-treated mouse pups by flow cytometry (Appendix Fig S12A), where increased apoptosis of PDGFRα+ cells was noted at P5.
Figure 5.

**A**

A34a 10 mg/kg

21% O₂ or 85% O₂

**B**

ΔCt miR-34a-5p

S A34a A34a S A34a

21% O₂ 85% O₂

**C**

S

A34a

**D**

Number of alveoli (x10⁶)

S A34a A34a S A34a

21% O₂ 85% O₂

**E**

Septal thickness (μm)

S A34a A34a S A34a

21% O₂ 85% O₂

**F**

PDGFRα+ cells (%)

S S A34a A34a

21% O₂ 85% O₂

**G**

PDGFRα+; αSMA+ cells (%)

S S A34a A34a

21% O₂ 85% O₂

**H**

O₂

A34a/TSB

miR-34a

PDGFRα

BPD-like phenotype:

Myofibroblasts

Elastin foci

Secondary septation

Improved alveolarization:

Myofibroblasts

Elastin foci

Saccules
In turn decreases the abundance of PDGFRα cells that are resident in the developing mouse lung (Fig 3F), which in turn decreases the density of PDGFRα (Appendix Fig S12B), but not at P14 (Appendix Fig S12C). Further- more, the density of PDGFRα (Appendix Fig S12D; ostensibly, PDGFRα/a) during normal and aberrant alveolarization (n = 5 animals for each group).

H Quantification of total number of alveoli by design-based stereology in wild-type mouse pups at post-natal day (P)14, treated with either scrambled antimiR (S), or anti-miR-34a (A34a), during normal and aberrant alveolarization (n = 5 animals for each group).

A role for miR-34a in septal thickening as well?

BPD is also characterized by septal thickening (Jobe, 2016). As a secondary observation in this study, we demonstrate here that miR-34a/b/c impacted septal thinning during alveolarization. Thickened septa arose in this model from multilayered stacking of cells, which revert to the normally observed single cell layer after anti-miR-34a treatment (Fig 7). Almost all septal cells stained for aquaporin 5 (Aqp5), a type I pneumocyte marker—a cell type that exhibits tremendous plasticity during alveogenesis (Yang et al., 2016)—in both thickened and restored (thinner) septa (Fig 7). In the background of hyperoxia, anti-miR-34a treatment did not impact the number or apoptosis (Appendix Fig S15A–G) of type I pneumocytes; or whole-lung gene expression assessed by mRNA microarray at P5 and P14 (Appendix Table S6; GEO accession number GSE89730; validated in Appendix Fig S16). Thus, anti-miR-34a most likely affected gene expression in a rare cell population, such as PDGFRα+ myofibroblasts, and not broadly throughout the alveolar epithelium, composed largely of type I pneumocytes. We suggest that changes in septal complexity arose not from loss or gain of epithelial cells, but rather from the spatial organization of the type I pneumocytes, that is directed by PDGFRα+ myofibroblasts. This may be related to the production of extracellular matrix (ECM) by PDGFRα+ myofibroblasts, where perhaps ECM laid down and remodeled during alveogenesis provides migration cues to epithelial cells organize themselves within the newly-generated septa. Such cues might possibly include receptor-mediated interactions between the epithelial cells and the ECM, or matrixine gradients, the latter having been recently implicated in epithelial remodeling in asthma (Patel et al., 2018). This general idea is consistent with the observation that PDGFRα+ lung fibroblasts decline in number during septal thinning (McGowan & McCoy, 2011) and is in-line with current thinking that epithelial–mesenchymal interactions drive lung development (Hogan et al., 2014).

To date, pivotal roles for microRNA processing by Dicer (Harris et al., 2006) and Argonaute (Lü et al., 2005) in lung branching suggested microRNA control of early (embryonic) lung development (Metzger et al., 2008), where functional roles for the miR-17 family have been demonstrated (Carrao et al., 2014). In contrast, in late
Figure 6. Assessment of proliferation status of PDGFRα+ cells in developing mouse lungs.

A Mice expressing nuclear-localized GFP under the control of the Pdgfra promoter were maintained under normoxic (21% O2) or hyperoxic (85% O2) conditions, and lungs were harvested, processed, and immunostained for Ki67 to determine proliferation status. DAPI staining revealed nuclei of all cells present in the section. Low-magnification images from individual channels are presented to the right of the merged (large) image first row of images. The area demarcated by the white box in the merge image of the first row is magnified in the second and third rows to allow for visualization of greater magnification of the demarcated region of the merged image, as well as visualization of a single Ki67+, GFP+ cell (white arrowhead) in all three channels separately. Scale bar: 100 μm.

B The number of PDGFRα+ cells in four microscopic fields was assessed for co-staining with an anti-Ki67 antibody to reveal proliferating cells. P values were calculated by unpaired Student’s t-test (n = 4 fields for each group, trends are representative of those observed in two other experiments). Data represent mean ± SD.

C The Ki67 staining and GFP fluorescence was controlled for by examining lungs from wild-type mice that were treated with an isotype-matched control IgG used for the Ki67 staining experiments. Sections were examined for GFP fluorescence as well as in the red channel used to detect the Ki67 staining. Scale bar: 100 μm. DAPI, 4’,6-diamidino-2-phenylindole; GFP, green fluorescent protein.
lung development, which is relevant to BPD, several microRNA candidates have been proposed as pathogenic players, including miR-150 (Narasaraju et al., 2015), miR-489 (Olave et al., 2016), miR-29b (Durrani-Kolarik et al., 2017), the miR-19/72 cluster (Rogers et al., 2015; Robbins et al., 2016), and epithelial miR-34a (Syed et al., 2017), but transgenic mouse studies have only validated a causal role for epithelial miR-34a (most likely by targeting angiopoietin) in arrested alveolarization, where miR-34a levels were also documented to be elevated in the lungs of BPD patients (Syed et al., 2017). Ours is the first report of a causal role being validated for any microRNA/mRNA target interaction in aberrant lung alveolarization, as well as the first-in-mouse use of a TSB in vivo in an animal

Figure 7. The primary cell type in the normally and aberrantly developing septa are type I alveolar epithelial cells.

The impact of administration of scrambled antimiR (S) or an antimiR directed against miR-34a (A34a) on the abundance of type I alveolar epithelial cells (marked by aquaporin 5, Aqp5) and type II alveolar epithelial cells (marked by pro-surfactant protein C, Sftpc) was assessed in 3-µm sections of paraffin-embedded lung tissue from P5 mice undergoing normal (21% O2) or aberrant (85% O2) lung alveolarization. DAPI, 4',6-diamidino-2-phenylindole. In the DAPI images, white lines delineate tissue from airspaces, and in the 85% O2 groups demarcate septa. Antibody specificity was validated by rabbit IgG isotype control primary antibodies. The control experiments for the Aqp5 and Sftpc staining runs are illustrated here. Scale bars, 50 µm.
model of human disease. Notably, translation of these findings into the use of anti-miR treatment of mice in the BPD model documented marked benefit in this preclinical model. We, therefore, highlight a potentially druggable pathway to manage arrested alveolarization following preterm birth.

Materials and Methods

Regulatory authority compliance and legal approvals

Animal experiments reported in this study were approved by the Regierungspräsidium Darmstadt, under approval numbers B2/277, B2/1002, and B2/1060.

Mice

Wild-type *Mus musculus* C57BL/6J mice were obtained from The Jackson Laboratory. The generation and characterization of a tamoxifen-inducible *Pdgfra*-Cre driver mouse strain [Tg(Pdgfra-cre/ERT2)1Wdr; MGI:3832569] referred to herein as *Pdgfra*-CreERT², on a C57BL/6J background has been described previously (Rivers et al, 2008; Ntokou et al, 2015). A *lacZ*-tagged miR-34a gene-trap strain (Mir34a^tm1.1Lhe; MGI:5308792) (Choi et al, 2011), herein referred to as miR-34a::*lacZ* or miR-34a^−/− (on a C57BL/6J background) was always employed in the homozygous state and was used interchangeably as a miR-34a global knockout and a miR-34 *lacZ* reporter, was obtained from the Jackson Laboratory. A miR-34bc global knockout strain (Mir-34bc^tm1.1Aven; MGI:3766768) (Hamilton et al, 2003) was obtained from the Jackson Laboratory, and was always employed in the heterozygous state, and allowed the detection of *Pdgfra*-expressing cells through nuclear-localized GFP fluorescence. A strain carrying a floxed miR-34a allele (Mir34a^flox/flox; MGI:5320795) (Concepcion et al, 2012) on a C57BL/6J background was always employed in the homozygous state, is referred to herein as miR-34a^−/−, and was obtained from the Jackson Laboratory. A mouse strain expressing a human histone 2B-enhanced green fluorescent protein fusion protein under the control of the *Pdgfra* promoter (B6.129S4-Pdgfra^tm1(B2F5 صغ)/J; MGI:3766768) (Hamilton et al, 2003) was obtained from the Jackson Laboratory, and was always employed in the heterozygous state, and allowed the detection of *Pdgfra*-expressing cells through nuclear-localized GFP fluorescence.

Gene and protein expression analysis

Changes in gene expression were assessed by SYBR green-based real-time RT–PCR (using *Ratu6* and *Polr2a* as a reference for microRNA and mRNA, respectively) as described previously, after miRNA isolation with a miRNeasy Mini kit (Qiagen), and microRNA expression was assessed using an Agilent-035430 mouse miRNA array platform (miRBase release 17 miRNA ID version; Mouse_8x60K-v17). For an unbiased analysis of mRNA expression over the course of normal and aberrant lung development, mRNA was isolated with a peqGOLD total RNA kit (Peqlab), and mRNA expression was assessed using an Agilent-028005 SurePrint G3 Mouse GE 8 × 60K Microarray platform. Microarray analyses were undertaken by IMGM Laboratories (Munich).

The hyperoxia-based mouse model of bronchopulmonary dysplasia

Bronchopulmonary dysplasia was modeled in mice in a protocol well established in our laboratory (Nardiello et al, 2017b) where newborn mouse pups, randomized to litters of equal numbers of pups per nursing dam, are exposed to 85% O₂ from P1 to P14, while control mouse pups with normal lung development are exposed in parallel to 21% O₂. In the case of tamoxifen-induced gene expression where mice received tamoxifen on P1, hyperoxia exposure was initiated on P2. Both male and female animals were used, since no sex bias has been noted in studies on perturbations to lung development of C57BL/6J mice in response to hyperoxia (Nardiello et al, 2017b). Nursing dams were rotated between normoxia and hyperoxia at 24-h intervals, to limit oxygen toxicity. At either P3 (prior to bulk lung alveolarization), at P5 (the peak period of bulk lung alveolarization), or at P14 (after completing of the bulk alveolarization phase), mice were killed by pentobarbital overdose (500 mg/kg, intraperitoneal) and lungs were removed en bloc for further analysis. The investigators were not blinded to group allocation, but were blinded to outcome assessment.

Microarray analyses

For an unbiased analysis of microRNA expression over the course of normal and aberrant lung development, microRNA was isolated with a miRNeasy Mini kit (Qiagen), and microRNA expression was assessed using an Agilent-035430 mouse miRNA array platform (miRBase release 17 miRNA ID version; Mouse_8x60K-v17). For an unbiased analysis of mRNA expression over the course of normal and aberrant lung development after antimiR administration, mRNA was isolated with a peqGOLD total RNA kit (Peqlab), and mRNA expression was assessed using an Agilent-028005 SurePrint G3 Mouse GE 8 × 60K Microarray platform. Microarray analyses were undertaken by IMGM Laboratories (Munich).
were developed either with a donkey anti-goat HRP-conjugated (Santa Cruz, sc-2020; 1:2,500) or goat anti-rabbit (ThermoFisher, 31460; 1:3,000) secondary antibody.

**Stereological analysis of lung structure**

Lung structure was assessed by design-based stereology with systematic uniform random sampling, on mouse lungs that were pressure fixed at 20 cm H$_2$O, and treated with arsenic, osmium and uranium, and embedded in plastic (Technovit 7100) resin, sectioned at 2 µm, stained with Richardson’s stain, and image captured in a Nanozoomer-XR C12000 (Hamamatsu), exactly as described previously (Madurga et al., 2014; Mižiková et al., 2015; Nardiello et al., 2017b). Lung volume was determined by the Cavalieri principle (Madurga et al., 2014). Stereological analyses were undertaken using the NewCast PLUS version VIS4.5.3. computer-assisted stereology system (Visiopharm) and facilitated the determination of $\text{inter alia}$ total number of alveoli in the lung, the mean septal thickness, and total gas-exchange surface area.

**In situ $\beta$-galactosidase activity detection**

Cryosections (10 µm) from developing mouse lungs attached to glass microscope slides were fixed in 0.5% (m/v) glutaraldehyde in PBS (10 min, 4°C), washed (by immersion in 1 mM MgCl$_2$ in PBS, 2 × 15 min, RT), and pre-incubated in 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside (X-Gal) buffer [5 mM potassium ferricyanide (II), 5 mM potassium ferricyanide (III), 1 mM MgCl$_2$ in PBS, pH 7.0; 10 s, RT] followed by incubation overnight with 1 mg/ml X-Gal in X-Gal buffer at 37°C in the dark. Slides were then washed with 1 mM MgCl$_2$ in PBS (15 min, RT), followed by fixation in 4% (m/v) paraformaldehyde in PBS (4 min); and dehydrated in a graduated ethanol series [100% (v/v), 96% (v/v), 70% (v/v); 5 min each, RT], followed by immersion in PBS (5 min, RT), followed by eosin counterstaining 1% (m/v) in dH$_2$O-Ethanol 20:80 (30 s, RT). Slides were washed by immersion in dH$_2$O (2 s, RT) and mounted with PERTEX (Histolab). Sections were examined using a Leica DM6000B light microscope (Leica).

**Cell isolation and cell culture**

Primary mouse lung fibroblasts were isolated from C57Bl/6J mice. Briefly, lungs were minced with sterile scissors, and the tissue suspension was dispersed by repeated gentle passage through a 40-gauge needle. The cell suspension was then passed through a 40-µm filter into a new 50-ml Falcon tube. The cell suspension was centrifuged at 120 × g for 8 min at 4°C. The supernatant was discarded, and the cell pellet was resuspended in 5 ml of pre-warmed (37°C) high-glucose DMEM containing 10% (v/v) FCS, 100 U/ml penicillin (ThermoFisher), 100 µg/ml streptomycin (ThermoFisher), and seeded into a T-75 cell culture flask (1 flask per lung) and passaged in low-glucose DMEM containing 10% (v/v) FCS, 100 U/ml penicillin (ThermoFisher), 10 µg/ml streptomycin (ThermoFisher). Primary mouse lung fibroblasts were employed throughout this study, with the exception of in vitro hyperoxia exposure and when Lipofectamine® 2000 was used as transfection reagent. In the latter two cases, the MLg mouse lung fibroblast cell line (ATCC® CCL-206™) was employed, and was obtained from the American Type Culture Collection, and maintained in EMEM supplemented with 10% (v/v) FBS. Cultures of primary cells and cell lines were routinely (monthly) screened for mycoplasma contamination.

**MicroRNA mimic, antimiR, and target site blocker interventions in vitro and in vivo**

A synthetic scrambled miR mimic and a miR-34a mimic (catalog numbers S103650318 and MSY0000542, respectively; Qiagen) were transfected into primary mouse lung fibroblasts with HiPerFect (Qiagen) or MLg cells with Lipofectamine® 2000 or 3000, as per manufacturer’s instructions. Locked nucleic acid (LNA) oligonucleotides (purchased from Exiqon) included a scrambled (inert) sequence (5’-ACGTCTATACGCCCA-3’); an antimiR directed against miR-34a (5’-AGCTAAGACACTGCC-3’) and miRCURY LNA™ microRNA Target Site Blockers (herein referred to as target site blockers) directed to target the interaction between the two miR-34a binding sites in the mouse Pdgfra 3’-UTR and miR-34a: 5’-TTGGCAGATTTCTC-3’ (TSB1) and 5’-AGGCCACGTACGCT-3’ (TSB2) (see Fig 3A). In vitro, synthetic oligonucleotides were transfected into MLg cells with Lipofectamine® 2000. When combined, synthetic microRNA mimics and LNA target site blockers were applied together as a cocktail, at a final cumulative concentration of 160 nM (Fig 4B). In vivo, both target site blockers (applied as a cocktail of a 1:1 mixture of TSB1 and TSB2) and a scrambled or miR-34a-specific antimiR were all applied by intraperitoneal injection at a dose of 10 mg/kg at P1 and P3, in ddH$_2$O.

**Flow cytometry and FACS**

All flow cytometry protocols and gating strategies are indicated in the relevant supplementary figures in the Appendix. Antibody conjugates, dilutions, and commercial sources are detailed in Appendix Table S9. Flow cytometry was performed to estimate apoptosis (by annexin V staining) and to quantify cell populations in developing mouse lungs, using the antibodies listed in Appendix Table S9. Flow cytometry and FACS were performed with an LSR Fortessa or an FACSArria III cell sorter, respectively, operated with DIVA software (BD Bioscience). Single-cell suspensions were prepared from mouse pup lungs by instilling approximately 300 µl of Dispase (50 U/ml; BD Bioscience) followed by incubation for 30 min at 37°C. Lungs were dissociated in a gentleMACSTM Dissociator (Millenyi) in 5 ml (per lung) DMEM supplemented with 10% (v/v) FCS, 100 U/ml penicillin (ThermoFisher), 100 µg/ml streptomycin (ThermoFisher), and 320 U/ml bovine pancreatic DNAse 1 (Serva). To remove cell debris and blood clots, whole-lung cell suspensions were filtered through 100 µm and 40-µm filters. After centrifugation at 266 × g for 10 min at 4°C, cell pellets were resuspended in Flow Cytometry Staining Buffer (eBioscience; 00-4222-26), blocked with 1:100 Mouse BD Fc Block™ (BD Biosciences), and incubated with the appropriate primary antibodies or
isotype controls diluted in Flow Cytometry Staining Buffer for 20 min at 4°C in the dark. After washing with Flow Cytometry Staining Buffer, whole-lung cell suspensions were incubated with secondary antibodies diluted in Flow Cytometry Staining Buffer for 20 min at 4°C in the dark. In the case of intracellular staining for myofibroblasts, cells were fixed in 0.15% (m/v) paraformaldehyde in PBS for 10 min at 4°C and permeabilized with 0.2% (m/v) saponin (Calbiochem) diluted in PBS for 15 min at 4°C prior to addition of antibodies. For assessment of epithelial cell apoptosis, stainings were carried out on fresh non-permeabilized cells, where cells were washed and resuspended in annexin V buffer (BD Biosciences) prior to annexin V-Alexa Fluor 647 conjugate (ThermoFisher, A23204; 1:100) incubation in annexin V buffer for 20 min at 4°C in the dark. Where a fluorophore-conjugated secondary antibody was not employed, cells that had been labeled with an unconjugated primary antibody were treated with a biotin-conjugated secondary antibody, followed by a Streptavidin-phycoerythrin conjugate (Biolegend, 405204; 1:300). For S-phase analysis, live-cell determinations were made by incubation of cell suspensions Hoechst 33342 (Sigma B2261, 5 µg/ml) in PBS for 45 min at 37°C in the dark. For assessment of PDGFRα+ cell apoptosis, live cell single-cell suspensions were prepared as described above, up to and including the step employing Mouse BD Fc Block™, after apoptosis was detected in cell suspensions with an Annexin V kit (Biolegend, 640906).

For isolation of PDGFRα+ cells by cell sorting, the anti-PDGFRα-APC conjugate was coupled to microbeads using 30 µl of anti-APC Microbeads (Miltenyi 130-097-143) for 20 min at 4°C in the dark, and separated in a AutoMacs separator (Miltenyi) prior to cell sorting. The RNAqueous-Micro kit (Thermo Fisher) was employed to isolate mRNA from PDGFRα+ cells, which are present in low numbers. To exclude dead cells and debris, 1 µl of 5 mg/ml DAPI or 5 µl of 50 µg/ml 7-ADD (Biolegend) was pipetted into the whole-lung cell suspensions just before the cell analysis.

Histochemistry and immunofluorescence

Histochemical staining for elastin was undertaken on 3-µm sections from P14 mice, exactly as described previously (Mižíková et al., 2015). Immunofluorescence analysis of aquaporin 5 (for type I alveolar epithelial cells), pro-SP-C (for type II alveolar epithelial cells), and 4',6-diamidino-2-phenylindole (DAPI; to detect cell nuclei) staining was undertaken as described previously (Ntokou et al., 2015), using the primary antibodies listed in Appendix Table S9, and Alexa Fluor 647-conjugated goat anti-rabbit IgG (Thermofisher, A23204; 1:300). For S-phase analysis, live-cell determinations were made by incubation of cell suspensions Hoechst 33342 (Sigma B2261, 5 µg/ml) in PBS for 45 min at 37°C in the dark. For assessment of PDGFRα+ cell apoptosis, live cell single-cell suspensions were prepared as described above, up to and including the step employing Mouse BD Fc Block™, after apoptosis was detected in cell suspensions with an Annexin V kit (Biolegend, 640906).

Assessment of apoptosis and cell proliferation in vitro

Primary mouse lung fibroblasts were seeded at 4,000 cells (in 100 µl) per well of a 96-well tissue culture plate (Greiner, 655180, for proliferation; Greiner, 655098, for apoptosis), incubated overnight, and starved in serum-free OptiMEM (Gibco, 31985-062) for 1 h.

For assessment of proliferation, cells were transfected either with a scrambled microRNA mimic or a miR-34a-5p mimic (80 nM final concentration, as described above), and proliferation was monitored by BrdU incorporation using a colorimetric Cell Proliferation ELISA kit (Roche, 11647229001) after a 1-h serum starvation period in DMEM GlutaMAX (Gibco, 21885-025), followed by 24 h in DMEM GlutaMAX supplemented with 10% (v/v) FCS and 1% Penicillin-Streptomycin solution. Signal was allowed to develop over 5–30 min, as was read in an Infinite M200 Pro spectrophotometer (Tecan).

For assessment of apoptosis, cells were transfected with a scrambled microRNA mimic or a miR-34a-5p mimic as described above, and caspase 3 and caspase 7 activity was detected as a surrogate for apoptosis, using a Caspase-Glo® 3/7 Assay System (Promega, G8091) after 24 h. For a positive control, medium was supplemented with staurosporine (Cayman Chemical, 62996-74-1; 0.5 µM) for the last 6 h of the 24-h period. Luminescence was determined for 60 min, in an Infinite M200 Pro luminometer (Tecan).

Power and statistical analyses

A prospective power analysis was undertaken for all animal studies, to assess the sample size required. Samples sizes were calculated...
using G*Power 3.1.9.2 (Faul et al, 2007). For changes in microRNA and mRNA expression assessed by real-time RT–PCR in mouse lung homogenates, a ΔCt of (0.5) was considered relevant, resulting in an effect size of $d = 2.70$ (using miR-34a-5p expression as reference values), where $d$ is Cohen’s effect size, and using $\alpha = 0.5$ (where $\alpha$ is the Type I error), and a power (1- $\beta$) of 0.8, where $\beta$ is the Type II error; required a sample size of $n = 4$ animals per group. For cells sorted by FACS from mouse lungs and processed for microRNA or mRNA analyses, where a pronounced change in gene expression was anticipated, a ΔCt of (1.0) was considered relevant, resulting in an effect size of $d = 2.79$ (using miR-34a-5p levels in FACS-sorted PDGFR$\alpha^+$ cells as reference values), and using $\alpha = 0.5$ and a power (1- $\beta$) of 0.8, required a sample size of $n = 4$ animals per group. For changes in rare cell populations in single-cell suspensions from whole lungs of mice, assessed by flow cytometry, a doubling of the cell population (100% increase) was considered relevant, resulting in changes in rare cell populations in single-cell suspensions from (1– $D_{d}$ values), where $D$ and mRNA expression assessed by real-time RT–PCR were considered relevant, resulting in an effect size of $d = 2.79$ (using miR-34a-5p levels in FACS-sorted PDGFR$\alpha^+$ cells as reference values), and using $\alpha = 0.5$ and a power (1- $\beta$) of 0.8, required a sample size of $n = 4$ animals per group. Assessment of lung structure included two parameters (total number of alveoli in the lung and the mean septal thickness), both assessed by design-based stereology in the same lungs from the same animals. For assessment of total number of alveoli, a 50% increase in the total number of alveoli was considered relevant, resulting in an effect size of $d = 4.13$ (using the hyperoxia-treated wild-type mouse lungs as reference values), and using $\alpha = 0.5$ and a power (1- $\beta$) of 0.8, required a sample size of $n = 3$ animals per group. For assessment of mean septal thickness, an increase of 2 µm in mean septal thickness was considered relevant, resulting in an effect size of $d = 11.11$ (using the hyperoxia-treated wild-type mouse lungs as reference values), and using $\alpha = 0.5$ and a power (1- $\beta$) of 0.8, required a sample size of $n = 2$ animals per group. Since both the total number of alveoli in the lung and the mean septal thickness are measured in the same animals, a sample size of $n = 3$ animals per group was required, which was extended to four animals per group, in the event of an outlier arising from technical issues related to tissue processing during embedding for stereological analysis.

Data are presented as mean ± SD. Differences between groups were evaluated by one-way ANOVA with Tukey’s post hoc test for multiple (more than two) comparisons, while two-group comparisons were performed with an unpaired Student’s $t$-test. All statistical analyses were performed with GraphPad Prism 6.0. For microarray studies, a Welch’s approximate $t$-test was used to determine $P$ values which were corrected using the algorithm of Benjamin and Hochberg, to generate the corrected $P$-value, $P\text{(corr)}$ (Benjamini & Hochberg, 1995). The presence of statistical outliers was tested by Grubbs’ test, and no outliers were found. In general, data sets were too small to test normal distribution, and normality was assumed.

**Data Availability**

Microarray data comparing microRNA steady-state levels in lungs of mouse pups exposed hyperoxia are available at the GEO database under accession number GSE89730 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89730).

**Expanded View** for this article is available online.

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**Author contributions**

JR-C performed transgenic animal, and in vivo target site blocker, mimic, and antimiR studies. JR-C, EL, and CN performed in vitro hyperoxia, target site blocker, mimic, and antimiR studies. JR-C, EL, and CN performed the stereology analyses. JR-C, JQ, FP, and SH performed flow cytometry studies. EL and ES performed and analyzed the microarray studies and performed bioinformatics analyses. DESS performed cryosection immunofluorescence studies. PFA performed selected cell-culture experiments. IM, IV, JAR-C, and KA assisted with transgenic animal, target site blocker, and antimiR animal experiments. JR-C, JQ, ES, CN, IV, JAR-C, WDR, KA, SH, WS, and REM conceived experiments; analyzed data, supervised experiments, and...
provided essential reagents, equipment, and infrastructure. JR-C, WS, and REM conceived the study, directed the study, and wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

For more information
(i) The American Lung Association (English Language) information page on bronchopulmonary dysplasia: https://www.lung.org/lung-health-and-diseases/lung-disease-lookout/bronchopulmonary-dysplasia/
(ii) The British Lung Foundation (English Language) information page on bronchopulmonary dysplasia: https://www.blf.org.uk/support-for-you/bronchopulmonary-dysplasia-bpd/what-is-it
(iii) The KidsHealth patient (English Language) information page for parents of infants with bronchopulmonary dysplasia: https://kidshealth.org/en/parents/bpd.html
(iv) The (German Language) information page of the Federal Association “The Preterm Infant”: https://www.fruegeborene.de/
(v) The microRNA database: http://www.mirbase.org/
(vi) The Mouse Genome Informatics international database resource for the laboratory mouse: http://www.informatics.jax.org/
(vii) GenBank human miR-34a entry: https://www.ncbi.nlm.nih.gov/gene/407040
(viii) Genbank mouse miR-34a entry: https://www.ncbi.nlm.nih.gov/gene/723848
(ix) The microRNA database: http://www.mirbase.org/

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