



# miR-200 family members reduce senescence and restore idiopathic pulmonary fibrosis type II alveolar epithelial cell transdifferentiation

Silvia Moimas<sup>1,9</sup>, Francesco Salton<sup>2,9</sup>, Beata Kosmider<sup>3,4,5,9</sup>, Nadja Ring<sup>6</sup>, Maria C. Volpe<sup>2,7</sup>, Karim Bahmed<sup>3,4</sup>, Luca Braga<sup>1</sup>, Michael Rehman<sup>6</sup>, Simone Vodret<sup>6</sup>, Maria Laura Graziani<sup>2</sup>, Marla R. Wolfson<sup>3,4,5,8</sup>, Nathaniel Marchetti<sup>4</sup>, Thomas J. Rogers<sup>3,4</sup>, Mauro Giacca<sup>1,7</sup>, Gerard J. Criner<sup>4,10</sup>, Serena Zacchigna<sup>6,7,10</sup> and Marco Confalonieri<sup>2,7,10</sup>

## ABSTRACT

**Rationale:** Alveolar type II (ATII) cells act as adult stem cells contributing to alveolar type I (ATI) cell renewal and play a major role in idiopathic pulmonary fibrosis (IPF), as supported by familial cases harbouring mutations in genes specifically expressed by these cells. During IPF, ATII cells lose their regenerative potential and aberrantly express pathways contributing to epithelial–mesenchymal transition (EMT). The microRNA miR-200 family is downregulated in IPF, but its effect on human IPF ATII cells remains unproven. We wanted to 1) evaluate the characteristics and transdifferentiating ability of IPF ATII cells, and 2) test whether miR-200 family members can rescue the regenerative potential of fibrotic ATII cells.

**Methods:** ATII cells were isolated from control or IPF lungs and cultured in conditions promoting their transdifferentiation into ATI cells. Cells were either phenotypically monitored over time or transfected with miR-200 family members to evaluate the microRNA effect on the expression of transdifferentiation, senescence and EMT markers.

**Results:** IPF ATII cells show a senescent phenotype (p16 and p21), overexpression of EMT (ZEB1/2) and impaired expression of ATI cell markers (AQP5 and HOPX) after 6 days of culture in differentiating medium. Transfection with certain miR-200 family members (particularly miR-200b-3p and miR-200c-3p) reduced senescence marker expression and restored the ability to transdifferentiate into ATI cells.

**Conclusions:** We demonstrated that ATII cells from IPF patients express senescence and EMT markers, and display a reduced ability to transdifferentiate into ATI cells. Transfection with certain miR-200 family members rescues this phenotype, reducing senescence and restoring transdifferentiation marker expression.



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**Idiopathic pulmonary fibrosis alveolar epithelial type II cells show senescence and EMT features, but miR-200b and miR-200c can restore the ability of type II cells to transdifferentiate *in vitro* into type I alveolar epithelial cells** <http://bit.ly/359lit>

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The datasets analysed during the current study are available from the corresponding author on reasonable request.

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## Introduction

Idiopathic pulmonary fibrosis (IPF) is a devastating progressive fibrotic disease of the lungs, leading to chronic respiratory failure and death within 2–5 years from diagnosis in most patients [1]. Gradual loss of lung function and increased exercise limitation correspond to progressive spreading of the typical histopathological findings that show the usual interstitial pneumonia pattern, which is characterised by patchy involvement of distal airways and lung parenchyma with areas of alveolar damage and fibrotic remodelling [2].

Despite the recent introduction of two antifibrotic drugs for the treatment of IPF, lung transplantation remains the only intervention able to improve survival [3]. The incidence of IPF increases with age and ageing-related mechanisms such as cellular senescence may be pathogenic drivers [2]. Prior studies focused on activated fibroblasts to induce excessive deposition of extracellular matrix that causes fibrosis and scarring for targeting therapy [4]; nevertheless, recent evidence suggests that alveolar type II (ATII) cells may have a central role in the pathogenesis of IPF due to a loss of regenerative potential [5, 6]. A pathogenetic relationship between ATII cell dysfunction and the development of scarring is indicated by the discovery that patients with familial pulmonary fibrosis harbour mutations in genes that are specifically expressed in ATII cells [7]. These data suggest that alveolar epithelial dysfunction may be a key driver to induce the fibrotic response [8, 9].

In normal lung which has been injured, ATII cells act as stem cells that enhance alveolar type I (ATI) cell renewal through transdifferentiation [10]. Conversely, ATII cells isolated from IPF patient lung explants showed impaired *in vitro* colony-forming capacity that suggests ATII stem cell failure [11]. Immunohistochemistry staining of IPF lung specimens shows aberrant activation of major developmental pathways (e.g. canonical Wnt/ $\beta$ -catenin signalling, zinc finger E-box binding homeobox 1 (ZEB1), transforming growth factor (TGF)- $\beta$  and  $\beta$ -tubulin III) [12, 13].

All these pathways contribute to dysfunction of epithelial–mesenchymal transition (EMT) in the alveolar epithelium, which is a possible pathogenic mechanism that leads to pneumocyte loss, myofibroblast accumulation and lung fibrosis [14, 15], although the role of EMT in murine models is less established [16]. Aberrant EMT can also be triggered by ageing-related mechanisms, including alveolar epithelial cell injury alone [17], endoplasmic reticulum stress and unfolded protein response [18], overexpression of TGF- $\beta$  [19], and premature apoptosis of ATII cells [14], as well as through the differential expression of microRNAs (miRNAs) [20].

Interestingly, these pathways have been shown to be controlled by miR-200 family members [21]. YANG *et al.* [22] demonstrated that miR-200 family members are significantly downregulated in the lungs of both mice with bleomycin-induced pulmonary fibrosis and patients with IPF. Furthermore, they reported that introduction of miR-200c into the lungs diminishes experimental pulmonary fibrosis in mice, suggesting that restoring miR-200c levels may be a novel approach for treating lung fibrosis [22]. Nevertheless, the similarity of the bleomycin-induced lung fibrosis model with IPF is frequently questioned [23] and the applicability of miRNA mimics in humans has yet to be proven [24].

The availability of human ATII cells isolated from explanted IPF lungs provides a unique opportunity to study the effect of different miRNAs on alveolar epithelial cells [25, 26]. Herein, we provide evidence of the failure of human ATII cells harvested from patients with IPF to transdifferentiate into ATI cells when compared with ATII cells obtained from healthy normal human subjects. Secondly, we evaluate whether the miR-200 family can restore normal regenerative function in exhausted senescent IPF pneumocytes.

## Methods

Human lung tissue from unidentified patients with IPF or recently deceased donors was obtained from the Lung Tissue Bank at Temple Lung Center (Dept of Thoracic Medicine and Surgery, Temple University,

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**Affiliations:** <sup>1</sup>Molecular Medicine Laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy. <sup>2</sup>Pulmonology Dept, University Hospital of Cattinara, Trieste, Italy. <sup>3</sup>Center for Inflammation, Translational and Clinical Lung Research, Lewis Katz School of Medicine, Temple University, Philadelphia, PA, USA. <sup>4</sup>Dept of Thoracic Medicine and Surgery, Lewis Katz School of Medicine, Temple University, Philadelphia, PA, USA. <sup>5</sup>Dept of Physiology, Temple University, Philadelphia, PA, USA. <sup>6</sup>Cardiovascular Biology Laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy. <sup>7</sup>Dept of Medical, Surgical and Health Sciences, University of Trieste, Trieste, Italy. <sup>8</sup>CENTRE: Collaborative for Environmental and Neonatal Therapeutics, Katz School of Medicine at Temple University, Philadelphia, PA, USA. <sup>9</sup>These authors contributed equally to this work (co-first authors). <sup>10</sup>These authors contributed equally to this work (co-last authors).

**Correspondence:** Marco Confalonieri, Pulmonology Dept, University of Trieste, Ospedale di Cattinara, Strada di Fiume 447, Trieste 34149, Italy. E-mail: mconfalonieri@units.it

Philadelphia, PA, USA). ATII cells were isolated as we previously described [26], obtaining a yield of  $2\text{--}3 \times 10^6$  and  $2\text{--}6 \times 10^4$  cells·g<sup>-1</sup> (wet weight) for controls and IPF lungs, respectively. Purity of the cells, as assessed by flow cytometry and immunostaining, is shown in supplementary figures S1 and S2. The selected donors had a reasonable lung function: an arterial oxygen tension/inspiratory oxygen fraction ratio of >250, limited time on a ventilator, a clinical history and radiography that did not reveal infection.

Institutional written informed consent was obtained for each lung specimen studied which was donated for lung research from patients undergoing lung transplantation for IPF or from family members for brain-dead normal subjects whose family members donated lung tissue for human research from the Gift of Life Donor Program (control). The study was approved by the Institutional Review Board (approval 4407) at Partners Healthcare and Temple University, and conformed to the Declaration of Helsinki protocols. ATII cells were harvested and grown in culture from lungs of either patients affected by end-stage IPF or recently deceased human donors whose lungs were not suitable for transplantation. Patients' data are reported in supplementary table S1.

#### **Alveolar epithelial cell culture**

The isolated ATII cells were resuspended in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine,  $2.5 \mu\text{g}\cdot\text{mL}^{-1}$  amphotericin B,  $100 \text{ U}\cdot\text{mL}^{-1}$  penicillin,  $100 \mu\text{g}\cdot\text{mL}^{-1}$  streptomycin and  $10 \mu\text{g}\cdot\text{mL}^{-1}$  gentamicin (all reagents from Thermo Fisher Scientific, Waltham, MA, USA), and plated on plates previously coated with rat tail collagen (200 000 cells per well in 96-well plates). To transdifferentiate the ATII cells to ATI cells, the cells were kept in DMEM and 10% FBS for 2 days, and cultured in DMEM supplemented with 5% FBS in addition to glutamine, amphotericin B, streptomycin, penicillin and gentamicin. The culture medium was replaced every other day. These culture conditions promote the transdifferentiation of ATII cells into ATI cells, as we previously described [25, 26].

#### **Cell transfection with small interfering RNA and miRNA**

All small interfering RNAs (siRNAs) (siGENOME siRNAs) and all miRNAs (miRIDIAN miRNA mimics) were purchased from Dharmacon (Lafayette, CO, USA). In order to identify the best transfection conditions, ATII cells were plated in 96-well plates and cultured as previously described. ATII cell transfection was performed using DMEM and 5% FBS without antibiotics and according to a standard forward transfection protocol with either a scramble siRNA (siScr; Dharmacon D-001210-0X), designed not to target any known human genes, or an siRNA targeting the polyubiquitin C gene (siUBC; Dharmacon M-019408-01) at a final concentration of 50 nM. Two different amounts of transfection reagent (Lipofectamine RNAiMAX; Thermo Fisher Scientific) were tested, *i.e.* 0.4 or 0.6  $\mu\text{L}$ , in a final volume of 150  $\mu\text{L}$ . Briefly, the reagent was diluted in Opti-MEM (Thermo Fisher Scientific), added to the siRNA and, after 30 min of incubation at room temperature, the transfection mix was added to the cells. At 12 h after transfection the medium was replaced by fresh medium. ATII cells were fixed after an additional 3 days for the assessment of transfection efficiency. The siUBC targets the polyubiquitin C gene that is essential for cell survival; thus the efficiency of transfection was assessed as we previously described [27]. The setup experiment led to the identification of the condition which utilises 0.6  $\mu\text{L}$  of the transfection reagent as the most efficient (transfection efficiency >80%). The miRNA transfection was performed as described earlier, using the miRNA mimics corresponding to the 11 members of the miR-200 family and the miRNA mimic negative controls, which have minimal sequence identity in the human genome. Cells were either fixed for immunofluorescence analysis or lysed in TRIzol reagent (Thermo Fisher Scientific) for subsequent gene expression analysis, 6 days after transfection.

#### **RNA isolation and quantitative real-time PCR**

Total RNA from either freshly isolated or cultured ATII cells was extracted with TRIzol reagent according to the manufacturer's instructions. RNA concentration was assessed by means of a NanoDrop fluorospectrometer (Thermo Fisher Scientific), 1  $\mu\text{g}$  was treated with DNase I (Roche Diagnostic, Indianapolis, IN, USA) and reverse transcription was performed with Moloney murine leukaemia virus reverse transcriptase (Thermo Fisher Scientific) in the presence of hexameric random primers (Thermo Fisher Scientific). Gene expression analysis was performed by SYBR Green technology using the GoTaq qPCR master mix (Promega, Madison, WI, USA) on a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The relative expression levels were calculated according to the  $2^{-\Delta C_t}$  method, by using the equation  $\Delta C_t = C_{t(\text{target})} - C_{t(\text{housekeeping})}$ . The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used for normalisation.

#### **Immunofluorescence**

Cells were fixed with 4% paraformaldehyde for 10 min, permeabilised with 0.1% Triton X-100 (Sigma, St Louis, MO, USA) diluted in PBS solution for 10 min, followed by blocking in 2% bovine serum albumin

(Roche Diagnostic) for 2 h at room temperature. Cells were then stained overnight at 4°C with the following primary antibodies diluted in blocking solution: anti-AQP5 1:100 (clone EPR3747; Abcam, Cambridge, UK), anti-pro-SPC 1:1000 (Millipore, Burlington, MA, USA), anti-p21 1:100 (clone C-19; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-Ki-67 (clone D3B5; Cell Signaling Technology, Danvers, MA, USA). Cells were washed with PBS and incubated for 2 h with the respective secondary antibodies conjugated to Alexa Fluor 594 (Thermo Fisher Scientific). Cell nuclei were counterstained with Hoechst 33342 (Thermo Fisher Scientific).

#### Image acquisition and analysis

Image acquisition was performed using an ImageXpress Micro (Molecular Devices, San Jose, CA, USA) automated high-content screening fluorescence microscope at  $\times 10$  magnification; a total of 16 images were acquired per wavelength. Image analysis was performed using MetaXpress (Molecular Devices).

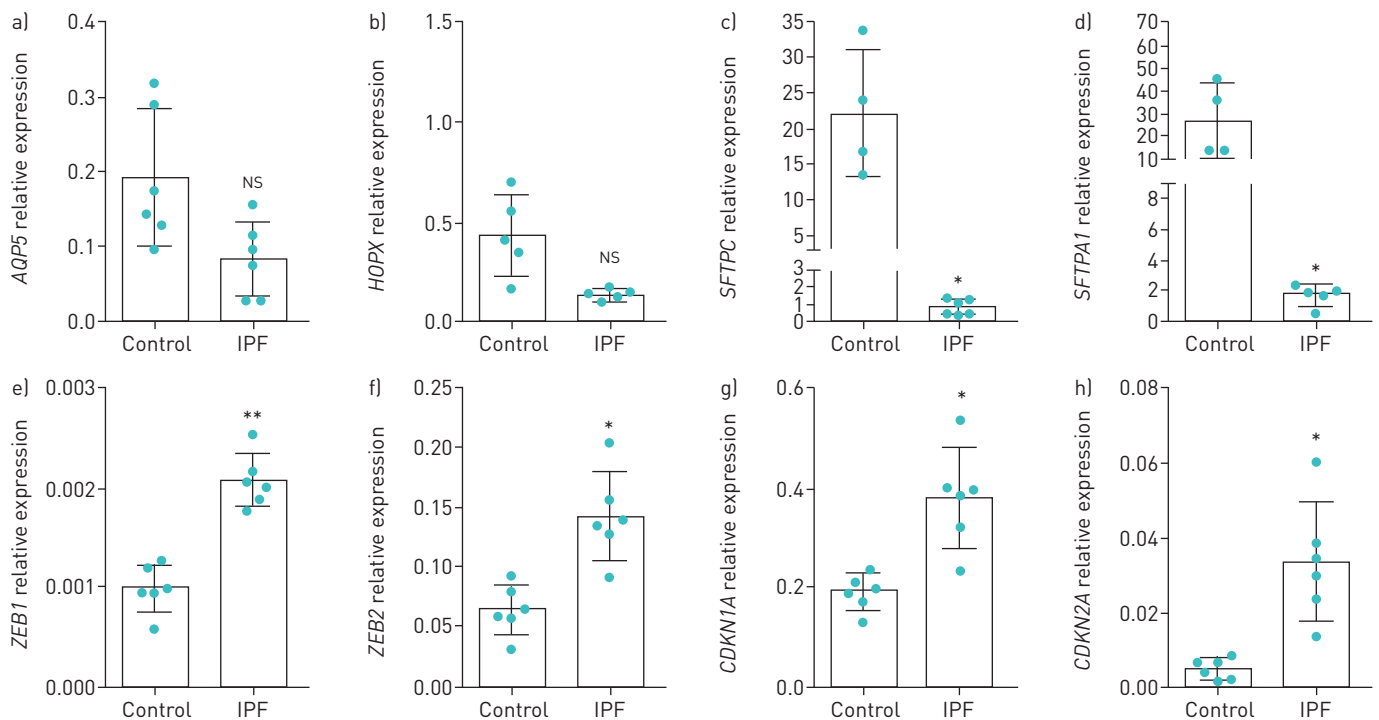
#### Statistical analysis

All data are presented as mean with standard deviation. Statistical analysis was performed using Prism version 6 (GraphPad, San Diego, CA, USA) considering a p-value  $< 0.05$  as statistically significant. For gene expression analysis at a single time-point, statistical significance of the differences between groups was determined using the unpaired t-test. For multiple group comparison (more than two), results were analysed using ANOVA followed by Fisher's least significant difference test. For gene expression and immunofluorescence analysis among the time-points within each group and among the groups within each time-point, we used two-way ANOVA followed by Šidák's multiple comparison *post hoc* test.

## Results

### ATII cells harvested from IPF patients show a senescent phenotype and impaired expression of epithelial markers

ATII cells expressed negligible levels of ATI markers, including aquaporin 5 (AQP5) and HOP homeobox (*HOPX*) [10]; no significant differences were observed between control and IPF cells (figure 1a and b). In contrast, we detected significantly reduced levels of ATII cell markers, namely pro-surfactant protein C



**FIGURE 1** Quantitative analysis of differentiation and senescence markers in freshly isolated alveolar type II (ATII) cells from control and idiopathic pulmonary fibrosis (IPF) lungs. Real-time quantification of the expression levels of the following genes in ATII cells harvested from control (n=6) and IPF (n=6) patients: a) aquaporin 5 (*AQP5*), b) HOP homeobox (*HOPX*), c) surfactant protein C (*SFTPC*), d) surfactant protein A1 (*SFTPA1*), e) zinc finger E-box binding homeobox 1 (*ZEB1*), f) zinc finger E-box binding homeobox 2 (*ZEB2*), g) cyclin dependent kinase inhibitor 1A (*CDKN1A*) and h) cyclin dependent kinase inhibitor 2A (*CDKN2A*). Data are presented as mean  $\pm$  SD relative expression normalised to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). NS: nonsignificant; \*, p < 0.05; \*\*, p < 0.01.

(pro-SPC; *SFTPC*) and surfactant protein A1 (*SFTPA1*), in IPF when compared with control cells (figure 1c and d). In addition, IPF cells expressed abundant levels of zinc finger E-box binding homeobox 1 (*ZEB1*) and 2 (*ZEB2*), both markers of EMT (figure 1e and f). Finally, these cells also exhibited significantly increased levels of the cell cycle inhibitors cyclin dependent kinase inhibitor 1A (*CDKN1A*) and 2A (*CDKN2A*), coding for the proteins p21 and p16, respectively (figure 1g and h).

#### ***ATII cells harvested from IPF patients fail to efficiently transdifferentiate into ATI cells in culture***

IPF and control ATII cells were isolated and subsequently cultured on rat tail collagen-coated dishes to promote their transdifferentiation into ATI cells [25] with subsequent characterisation of their markers over the following 6 days. While *AQP5* and *HOPX* were significantly upregulated over time in control cells, IPF cells had negligible expression of either marker nor were they increased over time (figure 2a and b). Inversely, the ATII markers *SFTPC* and *SFTPA1* were progressively downregulated over time, with very low expression at day 6, while IPF cells consistently showed low expression (figure 2c and d). The EMT markers *ZEB1* and *ZEB2* were expressed in cultured control cells and remained high in IPF cells, with a peak of *ZEB1* expression at day 6 (figure 2e and f). As expected, the senescence markers *CDKN1A* and *CDKN2A* progressively increased in expression over time in both control and IPF cells, although the IPF cells expressed significantly higher levels of both proteins, with the highest increase for p16 (figure 2g and h).

We validated the expression of several proteins by immunofluorescence in cells from at least three patients per condition, by quantifying the percentage of positive cells. Consistent with the mRNA data, at day 2 control cells expressed higher pro-SPC (coded by the *SFTPC* gene) levels compared with IPF cells and both progressively downregulated expression of these markers (figure 3a and b). In contrast, *AQP5* was nearly undetectable in both control and IPF cell lines at day 2, and was progressively upregulated, reaching a significantly higher level in control compared with IPF cells at day 6 (figure 3c and d). The senescence marker p21 was also progressively upregulated in both control and IPF cells over the 6 days, with a significantly higher expression in IPF cells compared with control (figure 3e and f). The coexpression of p21 and SPC in ATII cells from IPF lungs at day 2 is shown in supplementary figure S3. Consistently, the expression of marker of proliferation Ki-67, which labels proliferating cells, was reduced in IPF cells compared with controls at all time-points, with marked differences at days 4 and 6 (figure 3g and h).

#### ***miRNA-200 family members enhance IPF ATII cell transdifferentiation into ATI cells and reverse senescence without rescuing proliferation***

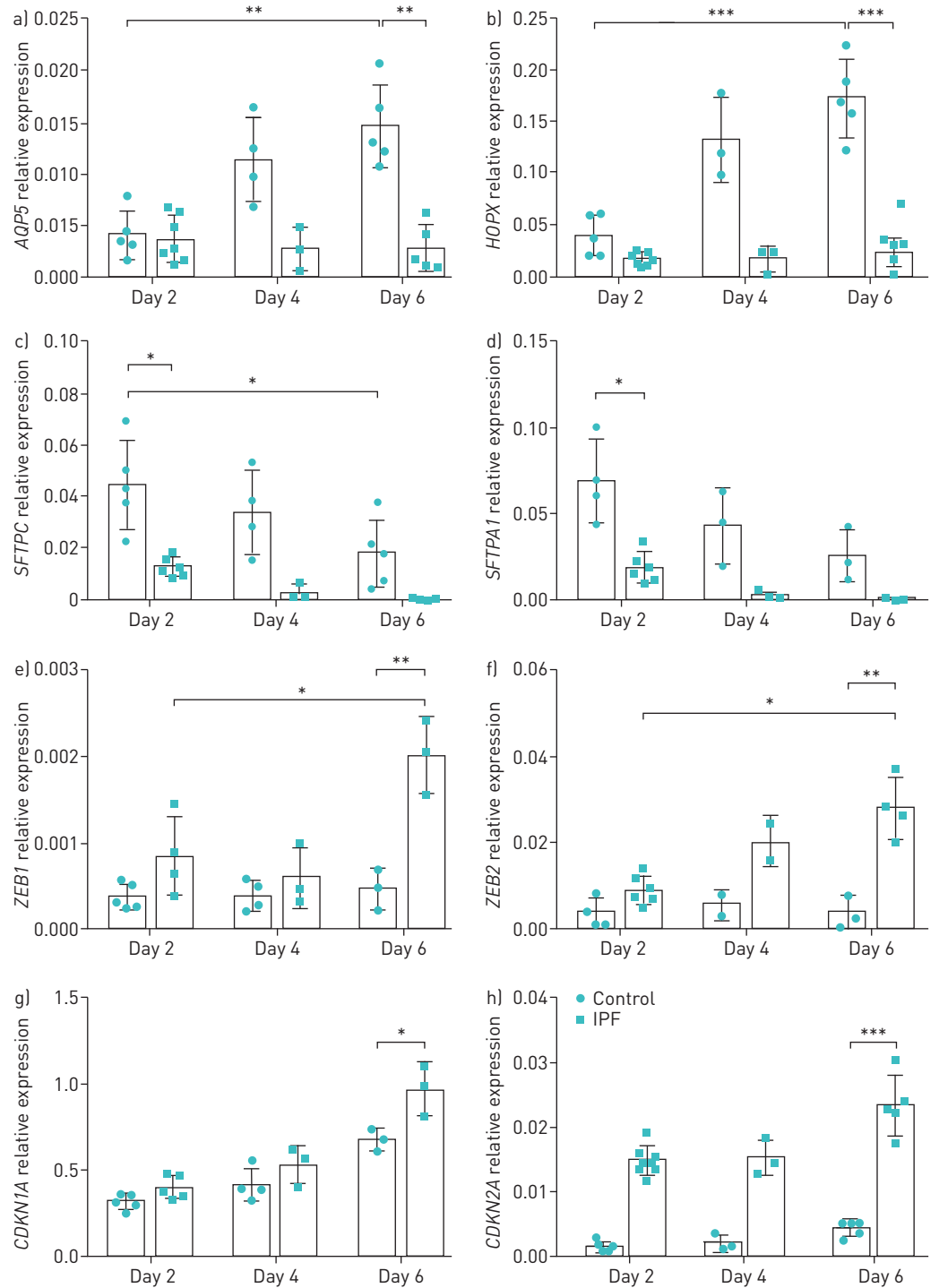
We tested the effect of various members of the miRNA-200 family on ATII cells isolated from four IPF patients, divided into two pools, by transfecting miRNA on day 2 and assessing the expression of differentiation and senescence-associated genes on day 8. We identified four interesting miRNAs that regulate the expression of markers of ATII cell transdifferentiation and senescence, and validated them in an additional four IPF patients. These selected miRNAs contained very similar seed sequences, with the seed sequence of miR-200b/c-3p, AAUACU, differing from the seed sequence of miR-200a/141-3p, AACACU, by only one nucleotide (figure 4a). All four miRNAs significantly downregulated their direct and validated targets *ZEB1* and *ZEB2* [12, 28], which are also implicated in EMT (figure 4b and c).

Interestingly, miR-200b-3p and miR-200c-3p, which share the same seed sequence, were most effective in promoting the expression of *AQP5* (figure 4d and e), consistent with their capacity to foster transdifferentiation of ATII into ATI cells. A similar trend, although less pronounced, was observed for the additional ATI cell marker *HOPX* (figure 4f). No significant difference in the expression of ATII markers was induced by any of the tested miRNAs.

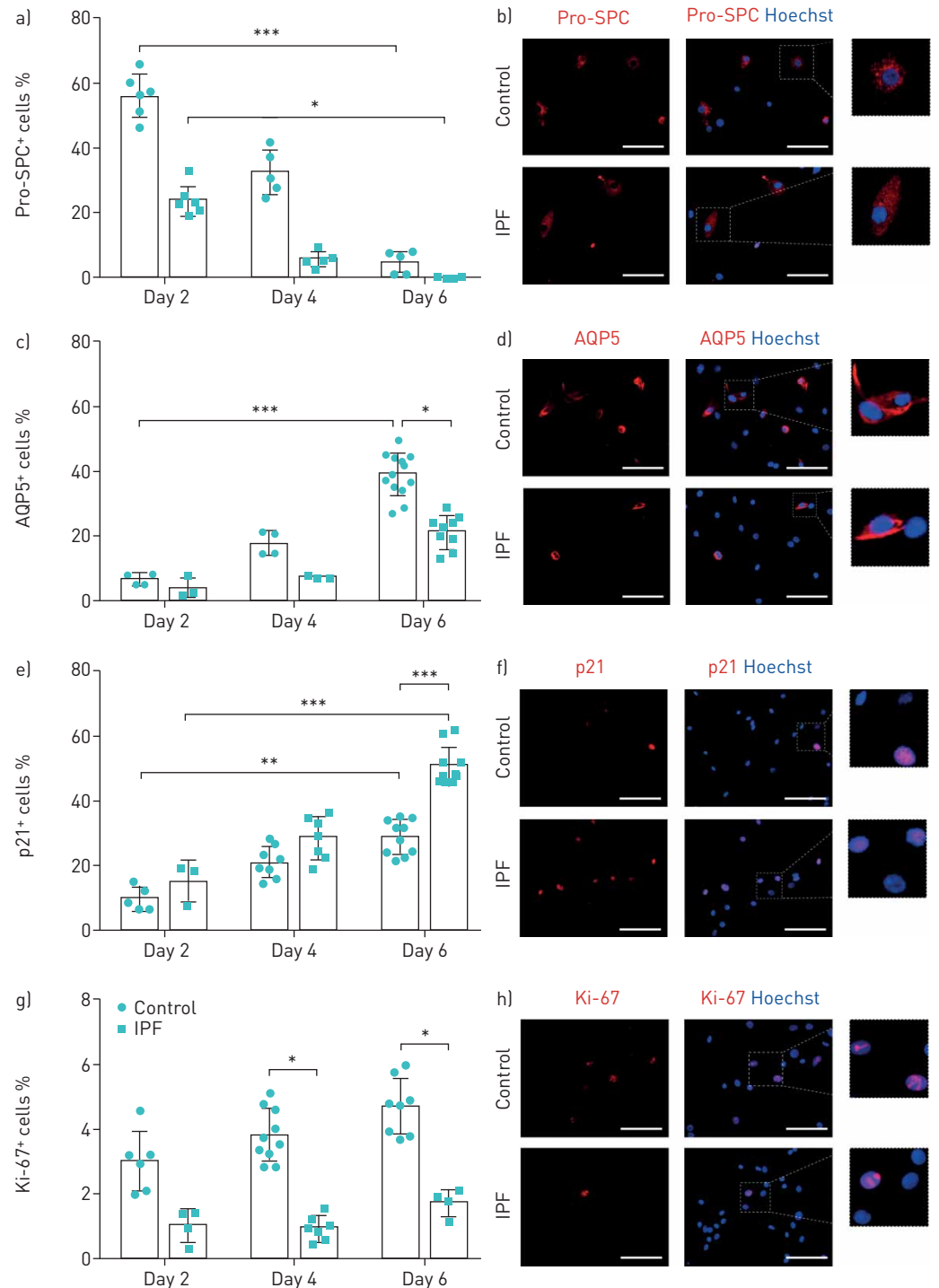
In contrast, miR-200a-3p and miR-141-3p, which also share the same seed sequence, markedly reduced the expression of the senescence-associated genes *CDKN1A* and *CDKN2A*, coding for p21 and p16, respectively (figure 4g–i). However, no significant difference in the proliferation rate of ATII cells, as assessed by immunofluorescence staining for Ki-67, was observed upon treatment with any of the miRNAs (figure 4j).

## **Discussion**

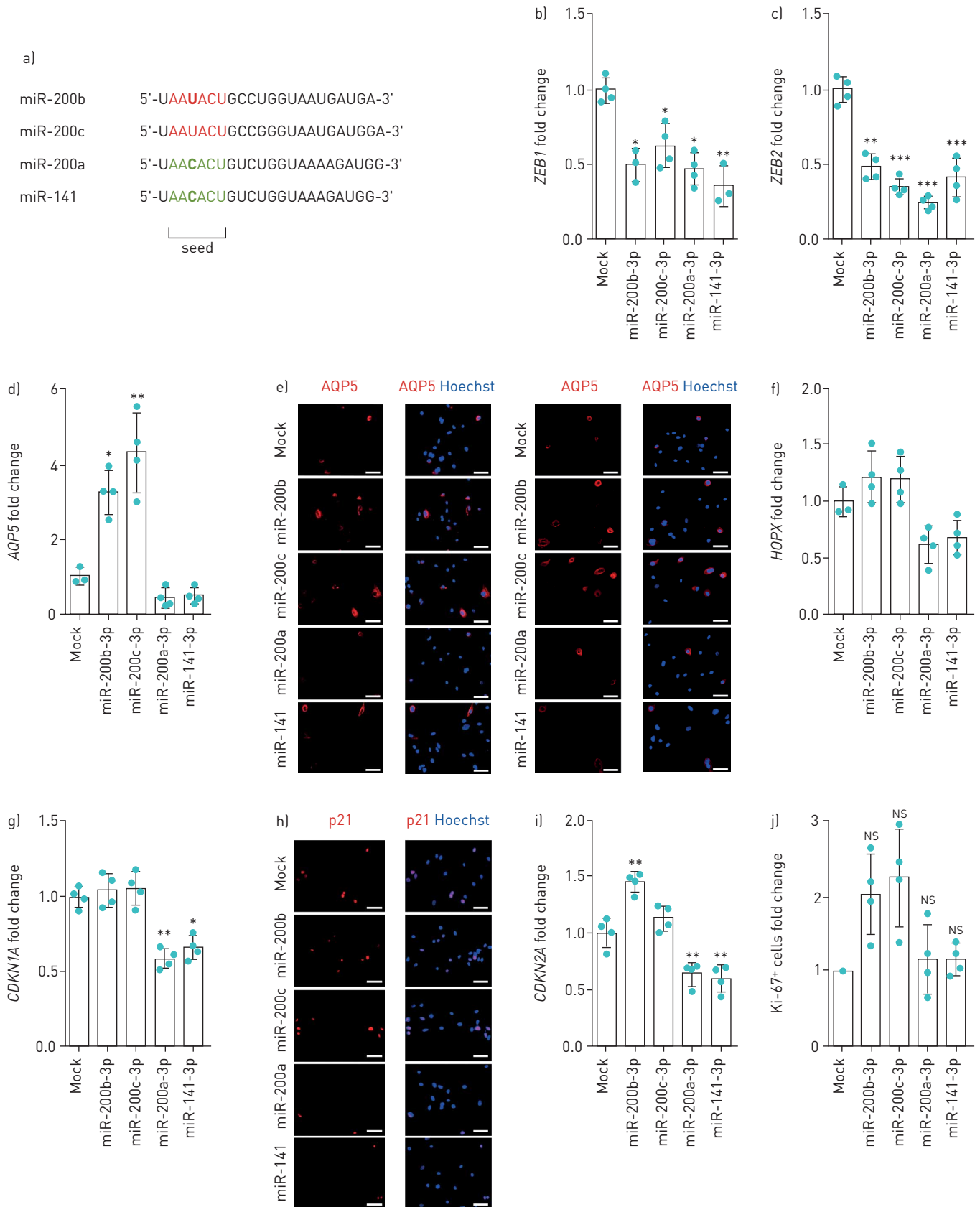
Our study provides the first characterisation of human ATII cells harvested from explanted lungs of patients with IPF who underwent lung transplantation in comparison with control ATII cells isolated from organ donors. We used an established protocol to obtain a pure population of ATII cells from human lungs [26] and harvested these cells from patients with end-stage IPF. This gave us the unique opportunity to culture cells that until now were considered too fragile to be isolated from the extremely fibrotic end-stage IPF lungs and establish a viable culture. Culturing these so-called “fibrotic” human ATII cells [29] in conditions favouring their transdifferentiation to ATI allowed us to study both their gene and protein expression, as well as their ability to transdifferentiate to ATI cells. Furthermore, we transfected ATII cells



**FIGURE 2** Time-course analysis of differentiation and senescence markers in alveolar type II (ATII) cells upon *ex vivo* transdifferentiation into alveolar type I (ATI) cells. IPF: idiopathic pulmonary fibrosis. Real-time quantification of the expression levels of the following genes in ATII cells harvested from control (n=6) and IPF (n=6) patients and kept in culture for 2, 4 and 6 days in conditions promoting their transdifferentiation into ATI cells: a) aquaporin 5 (*AQP5*), b) HOP homeobox (*HOPX*), c) surfactant protein C (*SFTPC*), d) surfactant protein A1 (*SFTPA1*), e) zinc finger E-box binding homeobox 1 (*ZEB1*), f) zinc finger E-box binding homeobox 2 (*ZEB2*), g) cyclin dependent kinase inhibitor 1A (*CDKN1A*) and h) cyclin dependent kinase inhibitor 2A (*CDKN2A*). Data are presented as mean±SD relative expression normalised to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001.



**FIGURE 3** Validation by immunofluorescence of the expression of differentiation and senescence markers in alveolar type II cells upon *ex vivo* transdifferentiation into alveolar type I cells. SPC: surfactant protein C; IPF: idiopathic pulmonary fibrosis; AQP5: aquaporin 5. a) Quantification of cells expressing pro-SPC at day 2, 4 and 6 of culture in differentiating conditions obtained from control (n=6) and IPF (n=6) patients. b) Representative images of cells expressing pro-SPC (red) after 2 days of culture in differentiating conditions. c) Quantification of cells expressing AQP5 as in (a). d) Representative images of cells expressing AQP5 (red) after 6 days of culture in differentiating conditions. e) Quantification of cells expressing p21 as in (a). f) Representative images of cells expressing p21 as in (d). g) Quantification of cells expressing Ki-67 as in (a). h) Representative images of cells expressing Ki-67 as in (d). Data are presented as mean $\pm$ SD percentage of positive cells. \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001. Nuclei are stained in blue with Hoechst 33342. Scale bar: 100  $\mu$ m.



**FIGURE 4** Effect of miR-200 family members on the differentiation, senescence and proliferation of alveolar type II (ATII) cells from idiopathic pulmonary fibrosis patients. miRNA: microRNA. a) Sequences of the selected miR-200 family members, with indication of the seed sequence and the unique single nucleotide difference between miR-200b/c-3p and miR-200a/141-3p, highlighted in green and red, respectively. b, c) Real-time



quantification of the expression of the described targets zinc finger E-box binding homeobox 1 (*ZEB1*) and 2 (*ZEB2*) at day 8 after the delivery of the indicated miRNAs. d) Real-time quantification and e) representative images of the expression of aquaporin 5 (*AQP5*) at day 8 after the delivery of the indicated miRNAs. f) Real-time quantification of the expression of HOP homeobox (*HOPX*) at day 8 after the delivery of the indicated miRNAs. g) Real-time quantification and h) representative images of the expression of cyclin dependent kinase inhibitor 1A (*CDKN1A*, p21) at day 8 after the delivery of the indicated miRNAs. i) Real-time quantification of the expression of cyclin dependent kinase inhibitor 2A (*CDKN2A*) at day 8 after the delivery of the indicated miRNAs. j) Quantification of marker of proliferation Ki-67<sup>+</sup> cells at day 8 after the delivery of the indicated miRNAs. Data are presented as mean $\pm$ sd expressed as fold change over mock (cells transfected with lipid only). ns: nonsignificant; \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001. Nuclei are stained blue with Hoechst 33342. Scale bar: 50  $\mu$ m.

with an miRNA family found to be downregulated in IPF. Consistent with our hypothesis, our results showed that ATII cells derived from IPF patients display higher levels of senescence and EMT markers and inability to transdifferentiate into ATI cells [30]. Our observation further confirms the hypothesis that ATII cells in IPF are exhausted and unable to transdifferentiate into ATI cells, although the role of senescence in modulating ATII into ATI differentiation is still unclear [31].

Our results are supported by the findings of Xu *et al.* [32] who used single-cell RNA sequencing to demonstrate that human epithelial cells from IPF lungs follow an abnormal differentiation programme that leads to a disrupted alveolar architecture. Available data indicates that cells derived from IPF lung tissue suppress the miRNA processing machinery, which leads to a global miRNA dysregulation that favours progressive lung scarring [33].

A number of miRNA networks have been implicated in the complex pathogenesis of IPF [34], among them the miR-200 family members which are dysregulated in fibrotic alveolar epithelial cells [22]. Importantly, our study supports previous publications showing a critical role of the miR-200 family members in regulating multiple aspects of ATII cell physiology in IPF. It is known that levels of miR-200 family members are reduced in the lungs of IPF patients and are downregulated in the lungs of mice with experimental lung fibrosis [22]. PECOT *et al.* [35] showed that miR-200 family members can either limit or reverse the progression of experimental lung fibrosis.

Our predominant finding is that specific members of the miR-200 family induce transdifferentiation of primary human IPF ATII cells into ATI cells. To demonstrate this, we transfected IPF ATII cells with synthetic mimics of the entire miR-200 family and were able to demonstrate that two miRNAs, *i.e.* miR-200b-3p and miR-200c-3p, are able to restore the capability of exhausted senescent IPF ATII cells to transdifferentiate into ATI cells. Probably, we are the first to demonstrate that diseased “fibrotic” and exhausted human ATII cells obtained from end-stage IPF may improve their ability to transdifferentiate into ATI cells when transfected with miR-200 family members.

A further finding of our study was that two additional miR-200 family members, *i.e.* miR-200a-3p and miR-141-3p, significantly reduce the expression of senescence markers in IPF ATII cells, without reverting their inability to proliferate. To what extent this is due to either intrinsic properties of primary human ATII cells, which spontaneously tend to become ATI cells when put in culture, or to the culture conditions promoting transdifferentiation rather than proliferation is still an open question [36, 37].

miRNAs are key players in the transdifferentiation of somatic cells by regulating gene expression in development, tissue regeneration and disease [38]. As regulatory molecules, miRNAs may be involved not only in the processes of cell proliferation and apoptosis, but also in EMT. Recently it was demonstrated that the miR-200 family, upregulated in human fetal lung, promotes differentiation of epithelial alveolar cells by targeting *ZEB1* and *ZEB2* [39]. Transcription factors of the *ZEB* protein family form a double-negative feedback loop with several miRNAs, predominantly miR-200 family members, which control EMT and mesenchymal-to-epithelial programmes during repair that normally generate fibroblasts and other related cells in order to reconstruct tissue following injury [40]. Previous evidence indicates that miR-200a–c inhibit EMT [22], which is in line with our experiments showing that the same miRNAs downregulate *ZEB1/2*. We have observed that miR-200a blocks EMT without increasing transdifferentiation, while miR-200b and miR-200c block EMT and increase transdifferentiation. This suggests that the inhibition of EMT is not sufficient for induction of transdifferentiation.

The observation that the miR-200 family is downregulated in human IPF lungs [12], together with our results, supports the perspective for an expanding view of the role of miRNA cellular reprogramming in the pathogenesis of IPF. The failure of ATII cells to complete the normal re-epithelisation process after injury may be a key mechanism leading to pulmonary fibrosis. While it is emerging that the dysregulation of miRNAs plays an important role in the development of IPF, they may also serve as potent tools to rescue the pathological process of injury and the response of aberrant repair that is observed in patients with IPF. The fact that some members of the miR-200 family are able to promote ATII cell transdifferentiation to ATI cells without stimulating cell proliferation suggests a possible therapeutic strategy to rescue the regeneration process.

### Conclusions

Our study pioneers the isolation and culture of ATII cells from human fibrotic lungs that are a valuable resource for comparative biology experiments and effective translational treatments of IPF. Moreover, we describe the phenotype of ATII cells from patients with IPF and how this may be rescued with miR-200 family members. We demonstrated that ATII cells from IPF patients express senescence and EMT markers, and display a reduced ability to transdifferentiate into ATI cells. Transfection with certain miR-200 family members rescues this phenotype, reducing senescence and restoring transdifferentiation marker expression.

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**Author contributions:** S. Moimas and F. Salton designed, discussed, conceived and performed the study experiments, participated in cell transfection and microscopy, collected data, produced figures, and helped to draft the manuscript. B. Kosmider and K. Bahmed participated in the study design, performed cell isolation and purification, shared expertise in assays, and produced figures. N. Ring and M.C. Volpe performed study experiments, microscopy, data collection and statistical analysis, produced figures, and reviewed the final manuscript. L. Braga, M. Rehman, S. Vodret and M.L. Graziani assisted in the design of the study, participated in the experiments and reviewed the final manuscript. N. Marchetti, T.J. Rogers and M.R. Wolfson participated in the study design, coordinated study procedures, and selected and collected lung samples. M. Giacca assisted in the design of the study, provided reagents and other resources, and reviewed the drafted manuscript. M. Confaloni, S. Zacchigna and G.J. Criner conceived the study and participated in its design, drafted the manuscript, and were responsible for the supervision of the project. All authors read and approved the final manuscript.

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