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# Targeting p16$^{INK4a}$ promotes lipofibroblasts and alveolar regeneration after early life injury

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**Author contributions**

LB, JB, and MZ conceived and designed the study.

MZ, BRB, LAE, CG, CTM, AI, MLF, RS, ST and MB performed experiments.

MZ, BRB, LAE, and AA analyzed gene expression and human expression data.

JTVN, FC, SL, MD, ML, CJ, SA, RE, and SL provided critical analysis and discussions.

MZ, JB, and LB wrote the paper with significant input and contributions.

All co-authors reviewed and approved the final manuscript.

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**Running title: p16$^{INK4a}$ deficiency drives lung regeneration**

3.24 Cell Fate, Non-Inflammatory Cell Types: Stem Cells/Tissue Regeneration

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

## ABSTRACT

**Rationale**. Promoting endogenous pulmonary regeneration is crucial after damage to restore normal lungs and prevent the onset of chronic adult lung diseases.

**Objectives**. To investigate whether the cell-cycle inhibitor $p16^{INK4a}$ limits lung regeneration after newborn bronchopulmonary dysplasia (BPD), a condition characterized by the arrest of alveolar development, leading to adult sequelae.

**Methods**. We exposed $p16^{INK4a}$ -/- and $p16^{INK4a}$ *ATTAC* transgenic mice to post-natal hyperoxia, followed by pneumonectomy of the $p16^{INK4a}$ -/- mice. We measured $p16^{INK4a}$ in blood mononuclear cells of preterm newborns and 7- to 15-year-old BPD survivors and the lungs of BPD patients.

**Measurements and main results**. $P16^{INK4a}$ levels increased in lung fibroblasts after hyperoxia-induced BPD in mice and persisted into adulthood. $P16^{INK4a}$ deficiency did not protect against hyperoxia lesions in newborn pups but promoted restoration of the lung architecture by adulthood. Curative clearance of $p16^{INK4a}$ positive cells once hyperoxia lung lesions were established restored normal lungs by adulthood. $P16^{INK4a}$ deficiency increased neutral lipid synthesis and promoted lipofibroblast and alveolar type 2 cell (AT2) development within the stem-cell niche. Besides, lipofibroblasts support self-renewal of AT2 cells into alveolospheres. Induction with a PPARγ agonist after hyperoxia also increased lipofibroblast and AT2 numbers and restored alveolar architecture in hyperoxia-exposed mice. After pneumonectomy, $p16^{INK4a}$ deficiency again led to an increase in lipofibroblast and AT2 numbers in the contralateral lung. Finally, we observed $p16^{INK4a}$ mRNA over-expression in the blood and lungs of preterm newborns, which persisted in the blood of older BPD survivors.

**Conclusions.** These data demonstrate the potential of targeting $p16^{INK4a}$ and promoting lipofibroblast development to stimulate alveolar regeneration from childhood to adulthood.



**Key words:** alveolar regeneration; $p16^{INK4a}$; lipofibroblasts

## INTRODUCTION

Lung regeneration is critical for recovering after lung damage or to protect against age-associated decline. Thus, promoting endogenous regeneration through stem cells to restore lung architecture and function may be promising for the future therapy of respiratory diseases (1-3). However, clinical trials targeting endogenous regeneration have failed to improve the onset of chronic lung diseases (4, 5). This highlights the need to better understand the molecular mechanisms that limit the regeneration of damaged lungs due to lung disease.

The induction of cell-cycle inhibitors during disease processes may limit lung regeneration. Among them, the protein $p16^{INK4a}$, encoded by the *CDKN2A* locus, is of specific interest, as it contributes to the age-associated decline of pancreatic islet, bone marrow, and neural tissues (6-12). Although $p16^{INK4a}$ accumulates in the lungs with age, whether this protein disturbs its capacity to regenerate is yet to be determined.

We hypothesized that targeting $p16^{INK4a}$ could enhance lung regeneration following injury. We addressed this hypothesis using bronchopulmonary dysplasia (BPD) as a model. BPD is a common complication of prematurity, characterized by developmental arrest of the lungs, with impaired alveolar septation. These alterations lead to lifelong sequelae in survivors, such as the alteration of lung function (13-18). No therapeutic intervention is currently available for BPD survivors to restore normal lung function in adulthood (19).

We assessed whether $p16^{INK4a}$ plays a causative role in limiting lung regeneration using an experimental mouse model of BPD induced by neonatal exposure to hyperoxia associated with morphological, physiological, and molecular analyses of the lung and pharmacological interventions in both $p16^{INK4a}$ -/- and $p16^{INK4a}$ *ATTAC* mice (11). These experiments

demonstrated a critical role for $p16^{INK4a}$ deficiency in promoting lung regeneration after hyperoxia-induced arrest of alveolarization. This process was mediated by lipofibroblast differentiation in the stem-cell niche. In addition, the induction of differentiation after hyperoxia with a PPARγ agonist restored alveolar architecture. These results were confirmed in a pneumonectomy model, leading to compensatory re-alveolarization of the remaining lung. Finally, $p16^{INK4a}$ expression was higher in the cord blood cells from preterm newborns than that from full-term controls. This was associated with the induction of $p16^{INK4a}$ in the lungs of BPD patients. Furthermore, elevated $p16^{INK4a}$ levels in blood cells persisted in 7- to 15-year-old BPD survivors. Thus, targeting $p16^{INK4a}$ could become an innovative therapeutic strategy to promote alveolar regeneration.

Some of the results of these studies have been previously reported in the form of abstracts at ERS congress in 2017, 2018 and 2019 (20-22).

## METHODS

Additional details on the methods are provided in an online data supplement.

**Animals—**Animal use was approved by the French Institutional Animal Care Committee. $P16^{INK4a}$ -/- mice were provided by A. Bern, Amsterdam (23) and *ATTAC* mice by S. Adnot (Créteil) for inducible elimination of $p16^{INK4a}$ positive ($p16^{INK4a}$+) cells (11)(Figure 3A,G).

**Hyperoxic Exposure—**Mouse pups were exposed to an 85% (hyperoxia) or 21% (room-air/normoxia) fraction of inspired oxygen (24) from D3 to D14 (Figure 1A)(25).

**Drug administration—**We injected rosiglitazone or T0901317 (Bertin©) from D14 to D60 (Figure 8E).

**Pneumonectomy procedure—**We performed left lung removal as described (26).

**Morphometry—**Standard morphometric methods were used (mean linear intercept, MLI) (27) and immunostaining was performed according to standard procedures.

**Microarray analyses—**The Mouse Gene 2.0 microarray (Affymetrix©) was used at D14 and D60.

**Real Time PCR**—Analyses were performed and gene expression presented as that relative to the expression of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase 1 (Hprt 1, Table E1).

**Lipidomics analyses—**Lipids were extracted from whole lung at D60 as previously described (28).

**Cell culture—**Primary fibroblasts were isolated from mouse lungs.

**Alveolospheres—**The total AT2 population (Sftpc+ $Tom^{high}$cells) was isolated from lungs of 6-8 weeks old *Sftpc$^{CreERT2/+}$; tdTomato$^{flox/flox}$* mice. For the isolation of mesenchymal cells of interest, we have first sorted enriched population of Cd45-Cd31-Epcam- Sca1+. LipidTOX+ and -cells were isolated from the Sca1+ population. For the isolation of epithelial cells from the *Sftpc$^{CreERT2/+}$; tdTomato$^{flox/flox}$* lungs, we first sorted a population of Cd45-Cd31-Epcam+ cells, enriched with mature AT2 cells, using Lysotracker (Invitrogen #L7526). FACS sorted cells were resuspended in media and mixed 1:1 with growth factor-reduced phenol Red-free Matrigel (Corning #356231). Spheres were counted and measured at day 14.

**Human blood samples—**We measured $p16^{INK4a}$ expression in peripheral blood mononuclear cells (PBMCs) from cord blood of very preterm newborns (<24 weeks of gestational age), matched with full-term newborns, and in PBMCs from BPD survivors from 7 to 15 years of age, matched with full-term children (NCT03540680).

**Human lung samples—**Lung sections from BPD patients were provided by Dr. R. Rottier, Rotterdam (29).

**Statistical Analysis—**GraphPad Prism6 was used for all statistical analysis. Student's t test (unpaired, two-tailed) and two-way ANOVA were used appropriately. Data are presented as the mean±SEM and differences were considered to be statistically significant if $p < 0.05$.

## RESULTS

### Exposure of immature mouse lungs to hyperoxia results in sequelae and elevated$p16^{INK4a}$ expression in adulthood.

Exposure of newborn C57BL/6J mice to 85%$O_2$ during the alveolarization period, from D3 to 14 of post-natal life (Figure 1A), radically arrested alveolar development, reducing the number of alveoli (MLI), with a concomitant disruption of secondary septation, relative to that of mice exposed to room air (Figure 1A-D, E1 A-E). This had long-term consequences, revealed by the persistence of a higher MLI and lower secondary septation at early and late adulthood (D60 and D120, respectively) in mice exposed to hyperoxia than those exposed to room air (Figure 1B,D). Lung fibroblasts, which are essential for secondary septation during alveologenesis, isolated from 14-day-old hyperoxic pups had a significantly lower wound healing velocity than those of normoxic animals (Figure 1E, E3). Lung function was also altered, with a decrease in dynamic lung elastance and exercise capacity at D60 in mice exposed to hyperoxia (Figure 2E, H).

The proportion of cells expressing $p16^{INK4a}$ in the alveolar wall increased four times immediately after hyperoxia relative to normoxia (D14, Figure 1B, F)(30). This phenomenon was amplified at early and late adulthood (Figure 1B, F). Co-staining of $p16^{INK4a}$ and PDGFRα

or pro-SpC (for fibroblasts and alveolar type 2 cells (AT2), respectively) showed that ~20% of fibroblasts and AT2 expressed $p16^{INK4a}$ in normoxic mice at D14. This percentage remained similar for AT2 in hyperoxic mice but reached more than 70% in fibroblasts (Figure 1G). Thus, $p16^{INK4a}$ was significantly induced in mesenchymal cells after hyperoxia. $P16^{INK4a}$ over-expression was associated with an induction of cell senescence in the lungs at D14 (31, 32), but this difference disappeared by adulthood (D60 and D120, Figure 1H, E1B)(33). We obtained similar results for p21 mRNA expression in whole lung homogenates (data not shown).

Overall, exposure of mouse lungs to hyperoxia results in arrested alveolarization, with sequelae at adulthood associated with$p16^{INK4a}$ over-expression in lung fibroblasts.

**$P16^{INK4a}$ deficiency does not protect against early hyperoxia-induced hypo-alveolarization but restores normal lung architecture at adulthood.**

Hyperoxia induced $p16^{INK4a}$ expression, which was amplified at adulthood. We thus assessed the structure of the lungs of wild type and $p16^{INK4a}$ -/- mice from D14 to 120. The lungs of normoxic $p16^{INK4a}$-/- and wild type lungs were similar in terms of MLI and septal crests at each time point (Figure 2B-D, E1D, E). Immediately after hyperoxia (D14), there was no difference between the lungs of $p16^{INK4a}$ -/- and wild type mice (Figure 2B-D), showing that $p16^{INK4a}$ deficiency did not protect against hyperoxia-induced hypo-alveolarization.

Two days after the end of hyperoxia (D16), $p16^{INK4a}$ deficiency was associated with a modest but significant increase in the number of septal crests relative to that of wild type mice (Figure E1C, E). This early sign of a protective effect of $p16^{INK4a}$ deletion was confirmed at early and late adulthood (D60 and D120, respectively, Figure 2 B, G, E1E). The MLI decreased

by 30% at D60 and 71% at D120 in $p16^{INK4a}$ deficient mice relative to that of wild-type mice, reaching the same level as those of both wild type and $p16^{INK4a}$ deficient room air mice (Figure 2C, E1E).

At D120, $p16^{INK4a}$ deficiency normalized elastance and improved exercise capacity (+12% running distance, Figure 2E, H). In summary, $p16^{INK4a}$ deficiency restored lung architecture and function in adult mice exposed to neonatal hyperoxia. This process was dynamic, starting early after the cessation hyperoxia but still continuing distant from the initial damage.

We evaluated collagen deposition (red Sirius, collagen 1 and 3 immunostaining) to determine whether lung restoration was associated with fibrosis (1, 34) or if it was due to true lung regeneration. Collagen deposition was not modulated in hyperoxic wild type mice at any time point. Septal thickness and collagen deposition, especially that of collagen1,was greater in the alveolar walls of hyperoxic $p16^{INK4a}$ -/- than wild type mice at D60, but this difference disappeared by D120 (Figure E5). Thus, $p16^{INK4a}$ deficiency was associated with an ongoing process of lung regeneration after early hyperoxia that led to successful *de novo* alveolarization.

**Clearance of $p16^{INK4a}$ + cells restores normal lungs at adulthood.**

We demonstrated that $p16^{INK4a}$ deficiency restores normal lungs at adulthood in mice exposed to early life hyperoxia. We thus examined whether eliminating $p16^{INK4a}$ + cells once lesions were established could also restore normal lungs. We used *INK-ATTAC* transgenic mice (12) to induce apoptosis in $p16^{INK4a}$ + cells, starting just after hyperoxia exposure (D14) to adulthood (D60, Figure 3A)(12). $P16^{INK4a}$ *ATTAC* had no specific phenotype in mice

exposed to room air. Hyperoxia dramatically altered alveolar development at D14 and this effect lasted until D60 among $p16^{INK4a}$ *ATTAC* mice treated with vehicle (Figure 3C-E). However, $p16^{INK4a}$+ cell clearance efficiently restored lung architecture, with the normalization of MLI and the number of septal crests by D60 (Figure 3B-E), whereas treating mice concurrently with hyperoxia (Figure 3G) to block the initial trigger did not modify $p16^{INK4a}$ expression at D60 and was insufficient to restore normal lung architecture (Figure 3H-J), suggesting that regular clearance of $p16^{INK4a}$ after hyperoxia is essential to lung regeneration.

**$P16^{INK4a}$ deletion confers a lipogenic switch during resolution at adulthood after murine hyperoxia injury model.**

We performed microarray analyses on whole lung homogenates after hyperoxia (D14) and at D60 to further understand the mechanisms of the regenerative process triggered by $p16^{INK4a}$ suppression. The number of genes modulated by $p16^{INK4a}$ deficiency in hyperoxic mice increased from 219 at D14 to 612 at D60 (Figure 4B). Only 14 genes modulated by $p16^{INK4a}$ deficiency were common between D14 and 60, suggesting a dynamic transcriptional process. We mainly observed increased expression of genes associated with lipogenesis pathways in $p16^{INK4a}$ -/-mice exposed to hyperoxia relative to that of wild type mice at adulthood (Figure 4A), such as sterol regulatory element-binding proteins (Srebp1 and 2), insulin-induced genes (Insig1 and 2), SREBP cleavage activating protein (Scap, Figure 4A). Pathways associated with lung development, such as FGF7 pathway, or senescence were not modulated by $p16^{INK4a}$ deficiency (Figure E6).

We confirmed that hyperoxia reduced Srebp2, Scap, and Adrp expression in both wild type and $p16^{INK4a}$ -/- mice at D14by *Q*pcr (Figure 4D-E). At D60, hyperoxia alone did not induce Srebp2 or Scap expression, but when associated with $p16^{INK4a}$ deficiency Srebp2, Scap and Adrp expression increased (Figure 4D).

We performed lipidomic analyses on whole lung at D60 to determine whether the activation of lipogenesis increased lipid content. Hyperoxia did not modulate the lipid composition in wild type mice, whereas neutral lipids were differentially expressed in $p16^{INK4a}$ -/- mice: the level of 19 triacylglicerides increased (among 24) and 20 phospholipids or sphingolipids increased and 11 decreased (among 167, Figure 4C).

Overall, these results confirm the activation of lipogenesis pathways, increasing lipid synthesis, mainly neutral lipids, in the lungs due to $p16^{INK4a}$ deficiency.

**$P16^{INK4a}$ deficiency promotes lipogenic fibroblast differentiation and the stem-cell niche**

Neutral lipids in the alveoli are primarily localized as lipid droplets in lipofibroblasts, interstitial fibroblasts involved in alveolar maturation and surfactant production during lung development (35-38). Lipid droplets consist of a neutral lipid core, mainly composed of triglycerides, essential for cellular metabolism and membrane production. We explored whether lipofibroblasts were involved in lung regeneration of$p16^{INK4a}$ -/- hyperoxic mice by analyzing isolated lung fibroblasts *in vitro* (Figure 5A). $P16^{INK4a}$ -/- fibroblasts showed augmented lipid-droplet content (Figure 5B) and up-regulation of lipogenic genes relative to those of wild-type mice (Figure 5C). $P16^{INK4a}$ deficiency provided a better migratory phenotype to fibroblasts, as wound closure was accelerated (Figure 5D, E7).

We observed more lipid droplets in p16$^{INK4a}$ -/- hyperoxic lungs than in those of wild type mice at D60 *in vitro* (Figure 5E, F). We confirmed the presence of lipofibroblasts by measuring ADRP expression surrounding the lipid droplets (39). Hyperoxia initially markedly reduced ADRP expression, both in wild type and p16$^{INK4a}$ -/- mice. On the second day after removing hyperoxia (D16), ADRP levels were similar between hyperoxic and normoxic wild type mice, but hyperoxia induced ADRP expression in p16$^{INK4a}$ -/- mice that persisted until D120 (Figure 5H, E2A). Similarly, the clearance of p16$^{INK4a}$ + cells after hyperoxia was also associated with the increase of lipofibroblasts (Figure 3F). We further confirmed that p16 $^{INK4a}$ deficiency resulted in an increase in the number of lipofibroblasts by FACS (CD45-CD31-Epcam-ADRP and/or lipidTOX+) at D60 after hyperoxia (Figure 5K). Additionally, CD45-CD31-Epcam-PDGFRα+ lipidTOX+ and/or ADRP were increased (Figure 5K, E2C).

Given the ability of lipofibroblasts to differentiate into myofibroblasts during repair and promote fibrosis (36, 40), we tested whether p16$^{INK4a}$ deficiency increased myofibroblast content. There was no difference between the groups at the various time points in terms of α-SMA expression, except transiently at D16 (Figure E5), thus excluding a fibrosis-driven process.Secondary crest myofibroblast (also known as alveolar myofibroblast) are initially PDGFRα+, and later become αSMA+ and are found localized at the tip of the alveolar septa close to elastin (41). Using co-staining of αSMA and PDGFRα we confirmed that alveolar myofibroblasts increased in septal crests from hyperoxia-exposed p16$^{INK4a}$ -/- mice compared to all other groups at D14 (Figure E2E). No difference was observed between groups at D60 (data not shown).

Altogether, p16$^{INK4a}$ deficiency was associated with lipogenic differentiation of fibroblasts in the stem cell niche after hyperoxia.

Lipofibroblasts belong to the alveolar stem-cell niche and are found next to AT2 (36, 42) to boost AT2 self-renewal and differentiation (42). We also confirmed that lipofibroblasts were adjacent to AT2 (Figure 5G). We further evaluated how lipofibroblasts formed the alveolar niche with AT2 by measuring the distance between the two types of cells using AT2 as an anchor point (43). The mean distance between lipofibroblasts and AT2 was less in the lungs of hyperoxic $p16^{INK4a}$ -/- mice than in those of all other groups and the number of lipofibroblasts close to AT2 was higher in the lungs of $p16^{INK4a}$ -/- mice exposed to hyperoxia than those of wild type mice (Figure 5J). Finally, the number of AT2 was higher in the lungs of hyperoxic $p16^{INK4a}$ -/- than hyperoxic wild type mice at D120 (Figure 5I, E2B). In addition, we observed lipid droplets in the lungs of every investigated mouse by electron microscopy (Figure E9). Lipofibroblasts in the lungs of hyperoxic wild type mice were embedded in a large quantity of elastin, whereas those in the lungs of hyperoxic $p16^{INK4a}$ -/- mice were not surrounded by elastin but rather associated with thin septa and well-preserved epithelial cells, similarly to those of the room-air wild type mice (Figure E9). Overall, $p16^{INK4a}$ deficiency was associated with the lipogenic differentiation of fibroblasts in the stem-cell niche after hyperoxia.

We next used another model of alveolarization, left pneumonectomy, which leads to compensatory neo-alveolarization of the remaining lung (Figure 6A)(44-46). Ten days after pneumonectomy, $p16^{INK4a}$ expression in the lung was higher than in that of sham wild type mice (Figure 6B). The number of lipofibroblasts but also of AT2 was higher in the alveolar wall of $p16^{INK4a}$ -/- than wild type mice (Figure 6C-F), after surgery, confirming the induction of both cell types by $p16^{INK4a}$ deficiency.

Overall, these results show that p16$^{INK4a}$ may play a major role in lung regeneration and the induction of lipofibroblasts in various models.

**P16$^{INK4a}$ deficiency induces early M2 macrophage polarization, which is however not involved in lipofibroblast differentiation**

P16$^{INK4a}$ deficiency has been reported to induce M2 macrophages (47, 48), which could regulate lung regeneration (45, 49-52). We therefore evaluated whetherp16$^{INK4a}$ deficiency induces a switch from M1 to M2 macrophages during hyperoxia to trigger lung regeneration through the induction of lipofibroblasts.

Micro-array and Luminex© analyses of whole lung homogenates showed up-regulation of inflammatory pathways at D14 in hyperoxic *versus* normoxic mice. This response was abrogated by p16$^{INK4a}$ deficiency, which was also associated with higher levels of IL-4 and arginase 1 (Figure 7A-D, E8). The results were similar concerning both M1 (CD68) and M2 (arginase 1 and CD163) markers, with higher levels of CD68 after hyperoxia in wild type than p16$^{INK4a}$ -/- mice, whereas arginase 1 and CD163 levels changed in opposite directions (Figure 7D). However, M1/M2 modulation by p16$^{INK4a}$ deficiency after hyperoxia was only transient (Figure E8B). These results show that the p16$^{INK4a}$ -/- macrophages underwent early and very short-lasting functional changes, which were temporally dissociated from lipofibroblast activation, making it unlikely that they are responsible for this process.

**The lipogenic switch of fibroblasts can induce lung regeneration after postnatal hyperoxia after lesions are established.**

We further tested the hypothesis that p16$^{INK4a}$ deficiency induces lung regeneration through lipofibroblasts. We first tested whether lipofibroblasts were able to induce a process of

alveolar regeneration by using an organoid assay. Lipofibroblasts were isolated as Sca-1+LipidTOX+ cells and compared to Sca-1+LipidTOX-resident stromal cells. They were co-cultured with Lyso+Tom$^{high}$ AT2 cells (Figure 8A). Organoids did not form with Sca-1+LipidTOX-resident stromal cells. Organoids size was range from 50 µm to 350 µm with lipofibroblasts and associated with a colony forming efficiency of 5.2. Immunofluorescence staining of organoids for DAPI, Sftpc (AT2 cell marker) and Hopx (AT1 cell marker) indicated that lipofibroblasts support AT2 stem cell differentiation into AT1 cells (Figure 8B-D).

We next treated mice with a PPARγ agonist, rosiglitazone, which induces lipofibroblasts (36, 53-55), from just after hyperoxia (D14) until adulthood (D60). In addition, we also assessed the effects of a SREBP activator, T0901317 (Figure 8E), as the SREBP pathway was highly modulated in the microarray analyses.

Rosiglitazone did not modify lung structure in normoxia-exposed mice but the MLI and number of septal crests was higher at adulthood than in non-treated mice. This was associated with higher numbers of lipofibroblasts and AT2 in the lungs of hyperoxic mice (Figure 8F-H). SREBP activation increased secondary septation but not the MLI (Figure 8F-J). Overall, inducing lipofibroblasts through a PPARγ agonist in a curative way was sufficient to induce lung regeneration after hyperoxia-induced hypo-alveolarization.

Again, these data show that p16$^{INK4a}$ deficiency promotes the alveolar regeneration process, involving lipogenesis pathways and lipofibroblasts in the stem-cell niche.

**P16$^{INK4a}$ level increases in the blood of preterm newborns, persists in BPD survivors, and are high in the lungs of BPD patients.**

We assessed $p16^{INK4a}$ levels in the blood of six preterm children, born before 24 weeks of gestational age, and six children born full term, matched for sex and date of birth (56). The subject characteristics are presented in Table 1. $P16^{INK4a}$ levels were significantly higher in preterm newborns than those born full term (Figure 9A)(57).

We next examined $p16^{INK4a}$ levels in the blood of 30 BPD survivors from 7 to 15 years old and 27 non-BPD full-term children (Table 2). The ex-BPD patients (born at a mean gestational age of 28 weeks) were mostly male, with a mean age of 8.9 years, and had small alterations of lung function (mean FEV1 post-bronchodilation1.37 ± 0.39L). Interestingly, $p16^{INK4a}$ induction persisted in the blood of BPD survivors (Figure 9B).

In addition, $p16^{INK4a}$ expression was higher in lungs damaged by BPD than those of premature infants without BPD (Figure 9C, D)(58). Most of the cells in the lung of BPD patients expressed $p16^{INK4a}$, especially within interstitial tissue but not in the alveolar epithelium. Furthermore, data from the LungMAP consortium (lungmap.net) shows nearly no lung expression of this protein before four years of age in humans.

Thus, prematurity induces $p16^{INK4a}$ in the blood and lungs and such over-expression persists in BPD survivors.

## DISCUSSION

Here, we show that: i)$p16^{INK4a}$ deficiency may play a major role in lung regeneration, independently of ageing or senescence, ii) a curative and not only preventive effect of the clearance of $p16^{INK4a}$ cells on lung structure, and iii) the ability of a PPARγ agonist to induce lung regeneration once lung lesions are established (Figure 9E). We also found that

prematurity induced p16$^{INK4a}$ expression in the blood and lungs, which persisted in BPD survivors. Overall, our results offer new therapeutic perspectives.

Previous studies have shown the accumulation of p16$^{INK4a}$+ cells during ageing, providing evidence that p16$^{INK4a}$ may be a driver of the multi-organ age-related phenotype (11, 12, 59-61). Moreover, p16$^{INK4a}$ deletion has been shown to have a regenerative potential after the damaging of slow-turn-over tissues, pancreas, and brain (7, 9). However, this effect has only been observed in aged organisms. For example, pancreatic islet proliferation and diabetes-specific survival were not affected by p16$^{INK4a}$ deficiency in young mice but increased in old p16$^{INK4a}$ -/- mice (7). Interestingly, the clearance of p16$^{INK4a}$+ cells improved pulmonary function without improving lung histology after bleomycin induced-fibrosis in mice (62). In our study, we demonstrate that p16$^{INK4a}$ deficiency drives regeneration, leading to the original organ architecture and not only partial repair with extracellular matrix accumulation (34). Alveolospheres experiment confirmed that lipofibroblasts are specifically able to support AT2 growth. The regeneration process evolves through a number of steps, starting with extracellular matrix deposition concomitant with alveolarization, which then disappears, resulting in the healthy restoration of alveolar airspaces. This suggests that an aberrant repair process, which involves fibrosis, was reversed by p16$^{INK4a}$ deficiency during late adulthood (62). Moreover, the pro-regenerative potential of p16$^{INK4a}$ deficiency was confirmed in the pneumonectomy model, which is free of any initial lung injury.

An important point highlighted by his study is the ability of the clearance of p16$^{INK4a}$+ cells to restore lung architecture in a curative manner. Clearance of p16$^{INK4a}$+ cells delays several manifestations of aging, prevents neuronal diseases and attenuates post-traumatic osteoarthritis (11, 12, 61). However, the induction of p16$^{INK4a}$+ cell clearance was not

initiated once the diseases were established. Here, we treated p16$^{INK4a}$ *ATTAC* mice after D14, once hyperoxia-induced lesions were clearly established. After 45 days of treatment, the lungs of hyperoxia-exposed mice showed the same characteristics as lungs of non-exposed mice, confirming the curative potential of p16$^{INK4a}$ + cell clearance. As p16$^{INK4a}$+ cells are mainly mesenchymal, their elimination may stop inhibitory signals and allow another population of fibroblasts to emerge and induce regeneration.

The specific regenerative role of p16$^{INK4a}$ in young lungs may be due to its involvement in pathways different than those involved during ageing. We particularly identified a role of p16$^{INK4a}$ in regulating lipogenesis in mesenchymal cells. Lipofibroblasts appeared as the main pro-regenerative cells in p16$^{INK4a}$ -/-lungs after hyperoxia. Their increased number in p16$^{INK4a}$ -/- mice following p16$^{INK4a}$+ cell clearance and their proximity to AT2 strongly suggest that they belong to the stem-cell niche and that p16$^{INK4a}$ deficiency modulates the AT2 environment in a pro-regenerative manner (42, 63, 64), confirming the essential role of mesenchymal cells (65). This phenomenon has been documented during lung development (35, 37, 38, 66). It has been recently shown that lipofibroblasts can reduce alveolar fibrosis once this disease is established (36). However, the ability of lipofibroblasts to promote lung regeneration was unknown. The effectiveness of the curative administration of a PPARγ agonist, in the same way as p16$^{INK4a}$ deficiency or p16$^{INK4a}$+ cell clearance, strongly supports a role of lipofibroblasts in lung regeneration.

A critical point that emerges from our results is the mechanism by which p16$^{INK4a}$ controls the lipofibroblast switch. Given the similarity between lipofibroblasts and fat progenitors (67,68), this lipogenic balance may occur through the control of cell senescence by p16$^{INK4a}$, as this contributes to age-dependent dysfunction of fat progenitors rescued by the clearance

of $p16^{INK4a}$+ cells (11, 69). However, at least two arguments run counter to a senescence-mediated process: i) $p16^{INK4a}$ deficiency was not associated with a decrease in hyperoxia-induced cell senescence in lungs after hyperoxia and ii) β-galactosidase activity decreased from childhood to adulthood after hyperoxia in wild type mice to reach the same level as that of normoxic mice, but was not associated with the restoration of lung architecture. Another possibility is a mechanism mediated by immune cells, such as M2 macrophages (70,71). However, first, inflammatory changes were short-lived, making them unlikely to be responsible for the late-arising fibroblast lipogenic switch. Moreover, an initial depletion of $p16^{INK4a}$+ cells did not lead to lung restoration at adulthood. Finally, $p16^{INK4a}$may modulate lipogenic differentiation through the inhibition of cyclin-dependent kinase 4 (CDK4), which can impair adipocyte differentiation through a reduction in PPARγ activity (72).

Overall, this study, including human data in preterm newborns and children with BPD to preclinical models, highlights the fact that targeting $p16^{INK4a}$ and its downstream partners may be a promising approach to promote lung regeneration following injury. More globally, our results support the concept that tissue regeneration may provide a novel strategy and therapeutic window for the correction of early-life diseases.

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## Figure Legends

**Figure 1. Exposure of immature mouse lungs to hyperoxia engenders sequelae at adulthood. P16$^{INK4a}$ levels in fibroblasts increase early and this induction is amplified in adulthood and associated with cell senescence.**

A. Timeline of exposure to hyperoxia. Mice were exposed to hyperoxia, *versus* room air, from days 3 to 14, and then to room air. Lungs were harvested at days 14, 60 and 120. B. HE staining (X20), elastin staining by the Weigert technique (X40), immunostaining of p16$^{INK4a}$ (X40), immunofluorescence of Pro-SftpC and p16$^{INK4a}$ (X40), and immunofluorescence of PDGFRα and p16$^{INK4a}$ (X40). C. Quantification of the mean linear intercept. D. Septal crest counts. E. Speed of wound healing, after scratch test. F. Quantification of the area of p16$^{INK4a}$ staining/nuclear staining. G. proportion of p16-positive cells among alveolar type 2 cells (AT2) or fibroblasts at D60. H. Quantification of the area of SA-β-galactosidase staining/nuclear staining. Black dots: room-air, white dots: hyperoxia. Scale bar =20µm. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Figure 2. P16$^{INK4a}$ deficiency does not protect against hyperoxia-induced hypo-alveolarization but restores normal lung architecture by adulthood in mice exposed to postnatal hyperoxia.**

A. Timeline of exposure to hyperoxia. Wild type and p16$^{INK4a}$ -/- mice were exposed to hyperoxia, *versus* room air, from days 3 to 14, and then to room air. Lungs were harvested at days 14, 16, 60, and120. B. HE staining (X20) and elastin staining by the Weigert technique (X40). C and D. Quantification of the mean linear intercept at days 14, and 60. E. Functional Testing; dynamic elastance. F and G. Septal crest counts at days 14, and 120. H. run distance.

Yellow dots: wild-type, blue dots: $p16^{INK4a}$-/-. Scale bar = 20µm. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Figure 3. Clearance of $p16^{INK4a}$ expressing cells restores the lung architecture by adulthood after early-life exposure to hyperoxia**

A. Timeline of exposure to hyperoxia. P16 *ATTAC* mice were exposed to hyperoxia, *versus* room air, from days 3 to 14, and then to room air. One group was treated with AP20187 another with vehicle from days 15 to 60. Lungs were harvested at day 60. B. Immunostaining of $p16^{INK4a}$ (X20) showing that clearance of $p16^{INK4a}$-expressing cells is efficient at D20, HE staining (X20) and elastin staining by the Weigert technique (X40) in early adulthood (D60). C. Quantification of $p16^{INK4a}$. D. Quantification of the mean linear intercept inearly adulthood (D60). E. Septal crest counts in early adulthood (D60). F. ADRP staining in early adulthood (D60). G. Timeline of exposure to hyperoxia. P16 *ATTAC* mice were exposed to hyperoxia, *versus* room air, from days 3 to 14, and then to room air. One group was treated with AP20187 and another to vehicle, during hyperoxia from days 3 to 15. Lungs were harvested inearly adulthood (D60). H. HE staining (X20) and elastin staining by the Weigert technique (X40) in early adulthood (D60). I. Quantification of the mean linear intercept in early adulthood (D60). J. Septal crest counts in early adulthood (D60). Black dots: vehicle, pink dots: AP20187. Scale bar = 20µm *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Figure 4. $P16^{INK4a}$ deletion confers a lipogenic switch during the resolution of BPD in adulthood.**

A. Micro-array analysis of whole lung homogenates, showing themain modulated pathway: activation of the lipogenesis pathways in $p16^{INK4a}$-/- compared to wild type hyperoxic mice. yellow: up-regulation, blue: down-regulation. B. Venn diagram of genes modulated at days

14 and 60. C. Lipidomic analysis of whole lung. red:upregulation, green: downregulation. D. Genes expression by *q*PCR: *Srebp2*, *Scap*, *Insig1* and *Adrp* relative to that of the housekeeping gene *Hprt1* at day 14. E. Gene expression by *q*PCR: *Srebp2*, *Scap*, *Insig1* and *Adrp* relative to that of the housekeeping gene *Hprt1* at day 60. Yellow dots: wild type, blue dots: $p16^{INK4a-/-}$. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Figure 5. $P16^{INK4a}$ deficiency promotes lipogenic fibroblast differentiation and development of the stem-cell niche**

A. Isolation of fibroblasts from the lung by enzymatic digestion. B. Lipid droplet staining by LipidTOX Deep-Red staining (X40), lipid droplet counts. C. Gene expression by *q*PCR of the lipid pathway genes *Adrp, Srebp2, Scap* and *Ldlr* relative to that of the housekeeping gene *Hprt1*. D. Wound healing after the scratch assay was significantly fasterforp$16^{INK4a}$-/- than wild type fibroblasts. E. LipidTOX staining in lung (X40) F. Lipid droplet counts. G. Co-staining of pro-SpC (in green) and ADRP (in red). H. Quantification of ADRP at day 14 and 60. I. Quantification of pro-SpC at day 120. J. Distribution of AT2-lipofibroblasts. Mean distance between alveolar type 2 cells and lipofibroblasts. LipidTOX. K. Flow cytometry showing greater numbers of CD45-,CD31-, EpCAM-, PDGFRα+ cells and CD45-, CD31-, EpCAM-, lipidTOX+, and ADRP+ cells at D60 in hyperoxic $p16^{INK4a}$ -/- than wild type mice. I. Scale bar = 20µm *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Figure 6.The lipogenic switch of fibroblasts can induce lung regeneration after pneumonectomy**. **$P16^{INK4a}$ deletion inducesan increase in the number of lipofibroblasts (ADRP positive cells) and AT2 cells (pro-SpC positives cells) in the lungs relative to controls.**

A. Timeline after pneumonectomy. Wild type mice underwent either a left pneumonectomy or only a left thoracotomy. Lungs were harvested 10 days later. B. Immunostaining of $p16^{INK4a}$

(X40) and quantification of $p16^{INK4a}$ staining in wild-type mice, pneumonectomy*versus* sham. C. Immunostaining of ADRP, Pro-SpC, and arginase 1 (X40). D. Quantification of the area of ADRP staining/nuclear staining. E. Quantification of the area of pro-Spc staining/nuclear staining. F. Mean linear intercept. Yellow dots: wild-type, blue dots: $p16^{INK4a}$ -/-. Scale bar = 20µm *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Figure 7. $P16^{INK4a}$ deficiency induces macrophage polarization towards the M2 phenotype and is involved in lipofibroblast differentiation.**

A. Luminex© assay for inflammatory cytokinesof whole lungfrom wildtype and $p16^{INK4a}$ just after hyperoxia. B. PCR of IL4 relative to that of the housekeeping gene HPRT1 of whole lung. C. *q*PCR of arginase 1 relative to that of the housekeeping gene HPRT1 of whole lung. D. Immunostaining and quantification of the M1 marker (CD68) and the M2 marker (arginase 1). Yellow dots: wildtype, blue dots: $p16^{INK4a}$-/-. Scale bar = 20µm. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Figure8. The lipogenic switch of fibroblastscan induce lung regeneration after postnatal hyperoxia**

A. $Sca1^{+}LT^{+}$cells support the self-renewal and differentiation of AT2 stem cells. *Sftpc*$^{CreERT2/+}$ ; *tdTomato*$^{flox/flox}$ and C57BL6 (wild-type) mice in 6-8 weeks of age were used to sort $Lyso^{+}Tom^{high}$AT2 cells and Sca-$1^{+}LT^{+}$ or Sca-$1^{+}LT^{-}$ resident stromal cells. Mixture of cells was seeded in Matrigel in 24-well Transwell. B. Fluorescence and bright field picture of representative wells at day 14. C. IF staining of organoids for DAPI, Sftpc (AT2 cell marker) and Hopx (AT1 cell marker) indicating that lipofibroblasts ($Sca1^{+}LT^{+}$) support AT2 stem cell differentiation into AT1 cells (Scale bar 100 µm). D. Quantification of organoid size (50 µm to 350 µm vs. 0, in Sca-$1^{+}LT^{+}$ vs. Sca-$1^{+}LT^{-}$, respectively) and colony forming efficiency (5.2 *vs*.

0in Sca-1$^{+}$LT$^{+}$vs. Sca-1$^{+}$LT$^{-}$, respectively). E. Timeline of exposure to hyperoxia. Wild type mice were exposed to hyperoxia,*versus* room air, from days 3 to 14, and then to room air. One group was treated with rosiglitazone (PPARγ agonist), another with T0901317 (activator of SREBP), and another to vehicle from days 15 to 60. Lungs were harvested at day 60. F. HE staining (X20) and elastin staining by the Weigert technique (X40). Immunostaining for ADRP or pro-SpC (X40). G. Quantification of the mean linear intercept. H. Septal crest counts. I. Quantification of the area of ADRP staining/nuclear staining. J. Quantification of the area of pro-SpC staining/nuclear staining. Blue dots: vehicle, red dots: T020187, green dots: rosiglitazone. Scale bar = 20µm *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Figure 9. P16$^{INK4a}$ level increasesin the blood of preterm newborns, persists in BPD survivors, and are highin the lungs of BPD patients.** A. Gene expression by *q*PCR of p16$^{INK4a}$ relativeto that ofthe housekeeping gene 36B4 at birth, using peripheral blood mononuclear cells (PBMCs) extracted from the cord blood of preterm or full-term newborns. B. Gene expression by *q*PCR of p16$^{INK4a}$ relative to that of the housekeeping gene 36B4 at 7 to 15 years old, using PBMCs extracted from the peripheral blood of former dysplasia patients matched to controlsforage and sex. C-D. Representative images of p16$^{INK4a}$ expression in the lungs of newborns who died of BPD. Scale bar = 20µm. Black dots: roomair, white dots: hyperoxia. *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001.E. Schematic diagram showing the switch of fibroblasts to lipofibroblasts, through p16$^{INK4a}$ deletion or the activation oflipogenesis, and itsrole in alveolar regeneration *via* alveolar type 2 cells.