



HAL
open science

Targeting p16 INK4a Promotes Lipofibroblasts and Alveolar Regeneration after Early-Life Injury

Maéva Zysman, Bruno Ribeiro Baptista, Laure-Aléa Essari, Sara Taghizadeh, Charlotte Thibault de Ménonville, Clément Giffard, Amelle Issa, Marie-Laure Franco-Montoya, Marielle Breau, Rachid Souktani, et al.

► **To cite this version:**

Maéva Zysman, Bruno Ribeiro Baptista, Laure-Aléa Essari, Sara Taghizadeh, Charlotte Thibault de Ménonville, et al.. Targeting p16 INK4a Promotes Lipofibroblasts and Alveolar Regeneration after Early-Life Injury. American Journal of Respiratory and Critical Care Medicine, 2020, 202 (8), pp.1088-1104. 10.1164/rccm.201908-1573OC . inserm-03029237

HAL Id: inserm-03029237

<https://inserm.hal.science/inserm-03029237>

Submitted on 2 Dec 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Targeting p16^{INK4a} promotes lipofibroblasts and alveolar regeneration

after early life injury

Maéva Zysman^{1,2}, Bruno Ribeiro Baptista^{1,2,3}, Laure-Aléa Essari³, Sara Taghizadeh⁴, Charlotte Thibault de Ménonville^{1,2}, Clément Giffard^{1,2}, Amelle Issa⁵, Marie-Laure Franco-Montoya^{1,2}, Marielle Breau^{1,12}, Rachid Souktani^{1,2}, Abdel Aissat^{1,2}, Laurence Caeymaex⁶, Muriel Lizé⁷, Jeanne Tran Van Nhieu^{1,2,8}, Camille Jung^{5,9}, Robert Rottier¹⁰, Marcio Do Cruzeiro¹¹, Serge Adnot^{1,12,13}, Ralph Epaud^{1,2,9}, François Chabot³, Sophie Lanone^{1,2}, Jorge Boczkowski^{1,2,#} and Laurent Boyer^{1,2,13,#}

¹Université Paris-Est, UMR S955, UPEC, F-94000, Créteil, France

²Inserm, U955, Team4, F-94000, Créteil, France

³Département de Pneumologie-CHRU Nancy-Université de Lorraine, Vandœuvre-lès-Nancy, Inserm, U1116; Université de Lorraine, Nancy, France

⁴Key laboratory of interventional Pulmonology of Zhejiang Province, Department of Pulmonary and Critical Care Medicine, First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China.

⁵Clinical Research Center, Biological Center Ressources Centre Hospitalier Intercommunal de Créteil, France

⁶Neonatal Intensive Care Unit, Centre Hospitalier Intercommunal de Créteil, France

⁷Molecular & Experimental Pneumology, Clinic for Cardiology and Pneumology, University Medical Center Goettingen, Germany

⁸AP-HP, Hôpital Henri Mondor, Département de Pathologie, Créteil, France

⁹Centre des Maladies Respiratoire Rares, Centre Hospitalier Intercommunal de Créteil, Service de Pédiatrie, Créteil, France

¹⁰ Department of Pediatric Surgery, Erasmus Medical Center-Sophia Children's Hospital, Rotterdam, Netherlands. Department of Cell Biology, Erasmus Medical Center, Rotterdam, Netherlands.

¹¹ Inserm, U1016, Institut Cochin, Paris, 75014, France, CNRS UMR 8104, Paris, 75014, France, Université Paris Descartes, Université Sorbonne Paris Cité, Paris, 75006, France

¹²INSERM U955 IMRB Team 08, and Université Paris Est (UPEC), UMR U955, Faculté de Médecine, Créteil, France

¹³AP-HP, Hôpital Henri Mondor, Département de physiologie, Créteil, France

#Equal contribution

*Address correspondance to: Institut Mondor de Recherche Biomédicale, Université Paris-Est, 94000 Créteil, France, laurent.boyer@aphp.fr

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.

MZ was supported by a fellowship from la Fondation pour la Recherche Médicale (bourse Marianne Josso, Paris, France) and by a grant from Novartis.

LAE and BRB were supported by Lorraine University, Nancy, France.

CG was supported by Novartis and Oxyvie grants.

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 PPAR γ 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 PPAR γ 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%O₂ 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 alveologensis, 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 PDGFR α

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 D14 by Qpcr (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 triacylglycerides 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-PDGFR α + 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 PDGFR α +, and later become α SMA+ and are found localized at the tip of the alveolar septa close to elastin (41). Using co-staining of α SMA and PDGFR α 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 $p16^{\text{INK4a}}^{-/-}$ mice than in those of all other groups and the number of lipofibroblasts close to AT2 was higher in the lungs of $p16^{\text{INK4a}}^{-/-}$ mice exposed to hyperoxia than those of wild type mice (Figure 5J). Finally, the number of AT2 was higher in the lungs of hyperoxic $p16^{\text{INK4a}}^{-/-}$ 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 $p16^{\text{INK4a}}^{-/-}$ 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, $p16^{\text{INK4a}}$ 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, $p16^{\text{INK4a}}$ 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 $p16^{\text{INK4a}}^{-/-}$ than wild type mice (Figure 6C-F), after surgery, confirming the induction of both cell types by $p16^{\text{INK4a}}$ 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 whether p16^{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 PPAR γ 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 PPAR γ 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-bronchodilation 1.37 ± 0.39 L). 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 PPAR γ 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 PPAR γ 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 PPAR γ 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.

Acknowledgments

The authors thank R. Rottier (Department of Pediatric Surgery, Erasmus Medical Center–Sophia Children’s Hospital, 3015 Rotterdam, The Netherlands; Department of Cell Biology, Erasmus Medical Center, 3015 Rotterdam, The Netherlands) for his gift of lung samples from BPD patients. The authors thank also G Pryhuber from lungmap consortium (Division of Neonatology, Department of Pediatrics, University of Rochester Medical Center, Rochester, NY 14642, USA).

The authors also thank Xavier Decrouy, Christelle Micheli (Plateforme Imagerie, IMRB - Inserm U955, Créteil, France), Sebastien Jacques (Plateforme Séquençage et Génomique, Cochin Institut, Paris, France), Marie Lhomme (ICAN, Institute of Cardiometabolism and Nutrition, Pitié-Salpêtrière Hospital, Paris, France), Claire Szczepaniak (Microscopie Électronique Centre Imagerie Cellulaire Santé, Clermont-Ferrand, France), and Philippe Caramelle for all animal procedures. The expert technical assistance of M. Surenaud (Luminex® platform, IMRB - Inserm U955, Créteil, France) is also gratefully acknowledged. The assistance of Patrick Ledudal, Amelle Issa, Diane Redel, Mélanie Vassal, Camille Jung (Clinical Research Center, Biological Center Resources Centre Hospitalier Intercommunal de Créteil, France) and Adeline Henry and Aurelie Guguin (Flow Cytometry facility, IMRB - Inserm U955, Créteil, France) is also sincerely acknowledged.

Finally, the authors thank Christophe Delacourt and Arnaud Mailleux for their advice.

References

1. Hogan BL, Barkauskas CE, Chapman HA, Epstein JA, Jain R, Hsia CC, Niklason L, Calle E, Le A, Randell SH, Rock J, Snitow M, Krummel M, Stripp BR, Vu T, White ES, Whitsett JA, Morrissey EE. Repair and regeneration of the respiratory system: complexity, plasticity, and mechanisms of lung stem cell function. *Cell stem cell* 2014; 15: 123-138.
2. Kotton DN, Morrissey EE. Lung regeneration: mechanisms, applications and emerging stem cell populations. *Nature medicine* 2014; 20: 822-832.
3. Rock JR, Barkauskas CE, Counce MJ, Xue Y, Harris JR, Liang J, Noble PW, Hogan BL. Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition. *Proceedings of the National Academy of Sciences of the United States of America* 2011; 108: E1475-1483.
4. Stolk J, Stockley RA, Stoel BC, Cooper BG, Piitulainen E, Seersholm N, Chapman KR, Burdon JG, Decramer M, Abboud RT, Mannes GP, Wouters EF, Garrett JE, Barros-Tizon JC, Russi EW, Lomas DA, MacNee WA, Rames A. Randomised controlled trial for emphysema with a selective agonist of the gamma-type retinoic acid receptor. *The European respiratory journal* 2012; 40: 306-312.
5. Roth MD, Connett JE, D'Armiento JM, Foronjy RF, Friedman PJ, Goldin JG, Louis TA, Mao JT, Muindi JR, O'Connor GT, Ramsdell JW, Ries AL, Scharf SM, Schluger NW, Sciruba FC, Skeans MA, Walter RE, Wendt CH, Wise RA, Investigators FS. Feasibility of retinoids for the treatment of emphysema study. *Chest* 2006; 130: 1334-1345.
6. Krishnamurthy J, Torrice C, Ramsey MR, Kovalev GI, Al-Regaiey K, Su L, Sharpless NE. Ink4a/Arf expression is a biomarker of aging. *The Journal of clinical investigation* 2004; 114: 1299-1307.
7. Krishnamurthy J, Ramsey MR, Ligon KL, Torrice C, Koh A, Bonner-Weir S, Sharpless NE. p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature* 2006; 443: 453-457.
8. Janzen V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, Cheng T, DePinho RA, Sharpless NE, Scadden DT. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* 2006; 443: 421-426.
9. Molofsky AV, Slutsky SG, Joseph NM, He S, Pardal R, Krishnamurthy J, Sharpless NE, Morrison SJ. Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. *Nature* 2006; 443: 448-452.
10. Baker DJ, Dawlaty MM, Wijshake T, Jeganathan KB, Malureanu L, van Ree JH, Crespo-Diaz R, Reyes S, Seaburg L, Shapiro V, Behfar A, Terzic A, van de Sluis B, van Deursen JM. Increased expression of BubR1 protects against aneuploidy and cancer and extends healthy lifespan. *Nature cell biology* 2013; 15: 96-102.
11. Baker DJ, Childs BG, Durik M, Wijers ME, Sieben CJ, Zhong J, Saltness RA, Jeganathan KB, Verzosa GC, Pezeshki A, Khazaie K, Miller JD, van Deursen JM. Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. *Nature* 2016; 530: 184-189.
12. Baker DJ, Wijshake T, Tchkonja T, LeBrasseur NK, Childs BG, van de Sluis B, Kirkland JL, van Deursen JM. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* 2011; 479: 232-236.
13. Simpson SJ, Turkovic L, Wilson AC, Verheggen M, Logie KM, Pillow JJ, Hall GL. Lung function trajectories throughout childhood in survivors of very preterm birth: a longitudinal cohort study. *The Lancet Child & adolescent health* 2018; 2: 350-359.
14. Wong PM, Lees AN, Louw J, Lee FY, French N, Gain K, Murray CP, Wilson A, Chambers DC. Emphysema in young adult survivors of moderate-to-severe bronchopulmonary dysplasia. *The European respiratory journal* 2008; 32: 321-328.
15. Gough A, Linden M, Spence D, Patterson CC, Halliday HL, McGarvey LP. Impaired lung function and health status in adult survivors of bronchopulmonary dysplasia. *The European respiratory journal* 2014; 43: 808-816.

16. Islam JY, Keller RL, Aschner JL, Hartert TV, Moore PE. Understanding the Short- and Long-Term Respiratory Outcomes of Prematurity and Bronchopulmonary Dysplasia. *American journal of respiratory and critical care medicine* 2015; 192: 134-156.
17. Jobe AH, Abman SH. Bronchopulmonary Dysplasia: A Continuum of Lung Disease from the Fetus to the Adult. *American journal of respiratory and critical care medicine* 2019; 200: 659-660.
18. Moschino L, Stocchero M, Filippone M, Carraro S, Baraldi E. Longitudinal Assessment of Lung Function in Survivors of Bronchopulmonary Dysplasia from Birth to Adulthood. The Padova BPD Study. *American journal of respiratory and critical care medicine* 2018; 198: 134-137.
19. Narayanan M, Beardsmore CS, Owers-Bradley J, Dogaru CM, Mada M, Ball I, Garipov RR, Kuehni CE, Spycher BD, Silverman M. Catch-up alveolarization in ex-preterm children: evidence from (3)He magnetic resonance. *American journal of respiratory and critical care medicine* 2013; 187: 1104-1109.
20. Ribeiro Baptista B, Zysman M, Essari LA, Giffard C, Franco-Montoya ML, Epaud R, Chabot F, Lanone S, Boczkowski J, Boyer L. Lipogenic switch of fibroblast to lipofibroblast induce lung regeneration in a model of bronchopulmonary dysplasia. *European Respiratory Journal* 2019 54: PA4119; DOI: 10.1183/13993003congress-2019PA4119; 2019.
21. Ribeiro Baptista B, Zysman M, Souktani R, Giffard C, Lize M, S. P, Lanone S, Boczkowski J, Boyer L. Increased proliferation of type 2 alveolar epithelial cells in a pneumonectomy model mice with a deletion of p16ink4a gene. *European Respiratory Journal* 2018 52: PA590; DOI: 10.1183/13993003congress-2018PA590; 2018.
22. Zysman M, Essari LA, Franco-Montoya ML, Vibhushan S, Caramelle P, Souktani R, Delacourt C, Chabot F, Epaud R, Lanone S, Boczkowski J, Boyer L. P16 in bronchopulmonary dysplasia: Early determinant of respiratory disease? *European Respiratory Journal* 2017 50: PA4186;; 2017.
23. Krimpenfort P, Quon KC, Mooi WJ, Loonstra A, Berns A. Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice. *Nature* 2001; 413: 83-86.
24. Lopez E, Boucherat O, Franco-Montoya ML, Bourbon JR, Delacourt C, Jarreau PH. Nitric oxide donor restores lung growth factor and receptor expression in hyperoxia-exposed rat pups. *American journal of respiratory cell and molecular biology* 2006; 34: 738-745.
25. Lee PJ, Alam J, Sylvester SL, Inamdar N, Otterbein L, Choi AM. Regulation of heme oxygenase-1 expression in vivo and in vitro in hyperoxic lung injury. *American journal of respiratory cell and molecular biology* 1996; 14: 556-568.
26. Takahashi Y, Izumi Y, Kohno M, Kimura T, Kawamura M, Okada Y, Nomori H, Ikeda E. Thyroid transcription factor-1 influences the early phase of compensatory lung growth in adult mice. *American journal of respiratory and critical care medicine* 2010; 181: 1397-1406.
27. Burri PH, Dbaly J, Weibel ER. The postnatal growth of the rat lung. I. Morphometry. *The Anatomical record* 1974; 178: 711-730.
28. Kayser BD, Lhomme M, Prifti E, Da Cunha C, Marquet F, Chain F, Naas I, Pelloux V, Dao MC, Kontush A, Rizkalla SW, Aron-Wisnewsky J, Bermudez-Humaran LG, Oakley F, Langella P, Clement K, Dugail I. Phosphatidylglycerols are induced by gut dysbiosis and inflammation, and favorably modulate adipose tissue remodeling in obesity. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2019; 33: 4741-4754.
29. Kool HM, Burgisser PE, Edel GG, De Kleer I, Boerema-de Munck A, de Laat I, Chrifi I, Cheng C, van Cappellen WA, Kremers GJ, Tibboel D, Rottier RJ. Inhibition of retinoic acid signaling induces aberrant pericyte coverage and differentiation resulting in vascular defects in congenital diaphragmatic hernia. *American journal of physiology Lung cellular and molecular physiology* 2019.
30. Tsuji T, Aoshiba K, Nagai A. Alveolar cell senescence in patients with pulmonary emphysema. *American journal of respiratory and critical care medicine* 2006; 174: 886-893.
31. Dagouassat M, Gagliolo JM, Chrusciel S, Bourin MC, Duprez C, Caramelle P, Boyer L, Hue S, Stern JB, Validire P, Longrois D, Norel X, Dubois-Rande JL, Le Gouvello S, Adnot S, Boczkowski J. The

- cyclooxygenase-2-prostaglandin E2 pathway maintains senescence of chronic obstructive pulmonary disease fibroblasts. *American journal of respiratory and critical care medicine* 2013; 187: 703-714.
32. Amsellem V, Gary-Bobo G, Marcos E, Maitre B, Char V, Validire P, Stern JB, Noureddine H, Sapin E, Rideau D, Hue S, Le Corvoisier P, Le Gouvello S, Dubois-Rande JL, Boczkowski J, Adnot S. Telomere dysfunction causes sustained inflammation in chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine* 2011; 184: 1358-1366.
 33. Barnes PJ, Baker J, Donnelly LE. Cellular Senescence as a Mechanism and Target in Chronic Lung Diseases. *American journal of respiratory and critical care medicine* 2019; 200: 556-564.
 34. Beers MF, Morrissey EE. The three R's of lung health and disease: repair, remodeling, and regeneration. *The Journal of clinical investigation* 2011; 121: 2065-2073.
 35. Al Alam D, El Agha E, Sakurai R, Kheirollahi V, Moiseenko A, Danopoulos S, Shrestha A, Schmoltdt C, Quantius J, Herold S, Chao CM, Tiozzo C, De Langhe S, Plikus MV, Thornton M, Grubbs B, Minoo P, Rehan VK, Bellusci S. Evidence for the involvement of fibroblast growth factor 10 in lipofibroblast formation during embryonic lung development. *Development* 2015; 142: 4139-4150.
 36. El Agha E, Moiseenko A, Kheirollahi V, De Langhe S, Crnkovic S, Kwapiszewska G, Szibor M, Kosanovic D, Schwind F, Schermuly RT, Henneke I, MacKenzie B, Quantius J, Herold S, Ntokou A, Ahlbrecht K, Braun T, Morty RE, Gunther A, Seeger W, Bellusci S. Two-Way Conversion between Lipogenic and Myogenic Fibroblastic Phenotypes Marks the Progression and Resolution of Lung Fibrosis. *Cell stem cell* 2017; 20: 571.
 37. Walther TC, Farese RV, Jr. Lipid droplets and cellular lipid metabolism. *Annual review of biochemistry* 2012; 81: 687-714.
 38. Tatsumi T, Takayama K, Ishii S, Yamamoto A, Hara T, Minami N, Miyasaka N, Kubota T, Matsuura A, Itakura E, Tsukamoto S. Forced lipophagy reveals that lipid droplets are required for early embryonic development in mouse. *Development* 2018; 145.
 39. McGowan SE, McCoy DM. Regulation of fibroblast lipid storage and myofibroblast phenotypes during alveolar septation in mice. *American journal of physiology Lung cellular and molecular physiology* 2014; 307: L618-631.
 40. Fu X, Khalil H, Kanisicak O, Boyer JG, Vagnozzi RJ, Maliken BD, Sargent MA, Prasad V, Valiente-Alandi I, Blaxall BC, Molkenstin JD. Specialized fibroblast differentiated states underlie scar formation in the infarcted mouse heart. *The Journal of clinical investigation* 2018; 128: 2127-2143.
 41. Li C, Lee MK, Gao F, Webster S, Di H, Duan J, Yang CY, Bhopal N, Peinado N, Pryhuber G, Smith SM, Borok Z, Bellusci S, Minoo P. Secondary crest myofibroblast PDGFRalpha controls the elastogenesis pathway via a secondary tier of signaling networks during alveologensis. *Development* 2019; 146.
 42. Barkauskas CE, Crouce MJ, Rackley CR, Bowie EJ, Keene DR, Stripp BR, Randell SH, Noble PW, Hogan BL. Type 2 alveolar cells are stem cells in adult lung. *The Journal of clinical investigation* 2013; 123: 3025-3036.
 43. Zepp JA, Zacharias WJ, Frank DB, Cavanaugh CA, Zhou S, Morley MP, Morrissey EE. Distinct Mesenchymal Lineages and Niches Promote Epithelial Self-Renewal and Myofibrogenesis in the Lung. *Cell* 2017; 170: 1134-1148 e1110.
 44. Green J, Endale M, Auer H, Perl AK. Diversity of Interstitial Lung Fibroblasts Is Regulated by Platelet-Derived Growth Factor Receptor alpha Kinase Activity. *American journal of respiratory cell and molecular biology* 2016; 54: 532-545.
 45. Lechner AJ, Driver IH, Lee J, Conroy CM, Nagle A, Locksley RM, Rock JR. Recruited Monocytes and Type 2 Immunity Promote Lung Regeneration following Pneumonectomy. *Cell stem cell* 2017; 21: 120-134 e127.
 46. Chung MI, Bujnis M, Barkauskas CE, Kobayashi Y, Hogan BLM. Niche-mediated BMP/SMAD signaling regulates lung alveolar stem cell proliferation and differentiation. *Development* 2018; 145.

47. Fuentes L, Wouters K, Hannou SA, Cudejko C, Rigamonti E, Mayi TH, Derudas B, Pattou F, Chinetti-Gbaguidi G, Staels B, Paumelle R. Downregulation of the tumour suppressor p16INK4A contributes to the polarisation of human macrophages toward an adipose tissue macrophage (ATM)-like phenotype. *Diabetologia* 2011; 54: 3150-3156.
48. Cudejko C, Wouters K, Fuentes L, Hannou SA, Paquet C, Bantubungi K, Bouchaert E, Vanhoutte J, Fleury S, Remy P, Tailleux A, Chinetti-Gbaguidi G, Dombrowicz D, Staels B, Paumelle R. p16INK4a deficiency promotes IL-4-induced polarization and inhibits proinflammatory signaling in macrophages. *Blood* 2011; 118: 2556-2566.
49. Kalymbetova TV, Selvakumar B, Rodriguez-Castillo JA, Gunjak M, Malainou C, Heindl MR, Moiseenko A, Chao CM, Vadasz I, Mayer K, Lohmeyer J, Bellusci S, Bottcher-Friebertshauser E, Seeger W, Herold S, Morty RE. Resident alveolar macrophages are master regulators of arrested alveolarization in experimental bronchopulmonary dysplasia. *The Journal of pathology* 2018; 245: 153-159.
50. Chamoto K, Gibney BC, Ackermann M, Lee GS, Lin M, Konerding MA, Tsuda A, Mentzer SJ. Alveolar macrophage dynamics in murine lung regeneration. *Journal of cellular physiology* 2012; 227: 3208-3215.
51. Chamoto K, Gibney BC, Lee GS, Ackermann M, Konerding MA, Tsuda A, Mentzer SJ. Migration of CD11b+ accessory cells during murine lung regeneration. *Stem cell research* 2013; 10: 267-277.
52. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature* 2013; 496: 445-455.
53. Milam JE, Keshamouni VG, Phan SH, Hu B, Gangireddy SR, Hogaboam CM, Standiford TJ, Thannickal VJ, Reddy RC. PPAR-gamma agonists inhibit profibrotic phenotypes in human lung fibroblasts and bleomycin-induced pulmonary fibrosis. *American journal of physiology Lung cellular and molecular physiology* 2008; 294: L891-901.
54. Rehan VK, Sugano S, Wang Y, Santos J, Romero S, Dasgupta C, Keane MP, Stahlman MT, Torday JS. Evidence for the presence of lipofibroblasts in human lung. *Experimental lung research* 2006; 32: 379-393.
55. Dasgupta C, Sakurai R, Wang Y, Guo P, Ambalavanan N, Torday JS, Rehan VK. Hyperoxia-induced neonatal rat lung injury involves activation of TGF- β and Wnt signaling and is protected by rosiglitazone. *American journal of physiology Lung cellular and molecular physiology* 2009; 296: L1031-1041.
56. Escribano A, Pastor S, Reula A, Castillo S, Vicente S, Sanz F, Casas F, Torres M, Fernandez-Fabrellas E, Codoner-Franch P, Dasi F. Accelerated telomere attrition in children and teenagers with alpha1-antitrypsin deficiency. *The European respiratory journal* 2016; 48: 350-358.
57. Vassallo PF, Simoncini S, Ligi I, Chateau AL, Bachelier R, Robert S, Morere J, Fernandez S, Guillet B, Marcelli M, Tellier E, Pascal A, Simeoni U, Anfosso F, Magdinier F, Dignat-George F, Sabatier F. Accelerated senescence of cord blood endothelial progenitor cells in premature neonates is driven by SIRT1 decreased expression. *Blood* 2014; 123: 2116-2126.
58. Chao CM, Moiseenko A, Kosanovic D, Rivetti S, El Agha E, Wilhelm J, Kampschulte M, Yahya F, Ehrhardt H, Zimmer KP, Barreto G, Rizvanov AA, Schermuly RT, Reiss I, Morty RE, Rottier RJ, Bellusci S, Zhang JS. Impact of Fgf10 deficiency on pulmonary vasculature formation in a mouse model of bronchopulmonary dysplasia. *Human molecular genetics* 2019; 28: 1429-1444.
59. Bussian TJ, Aziz A, Meyer CF, Swenson BL, van Deursen JM, Baker DJ. Clearance of senescent glial cells prevents tau-dependent pathology and cognitive decline. *Nature* 2018; 562: 578-582.
60. Farr JN, Xu M, Weivoda MM, Monroe DG, Fraser DG, Onken JL, Negley BA, Sfeir JG, Ogrodnik MB, Hachfeld CM, LeBrasseur NK, Drake MT, Pignolo RJ, Pirtskhalava T, Tchkonja T, Oursler MJ, Kirkland JL, Khosla S. Targeting cellular senescence prevents age-related bone loss in mice. *Nature medicine* 2017; 23: 1072-1079.
61. Jeon OH, Kim C, Laberge RM, Demaria M, Rathod S, Vasserot AP, Chung JW, Kim DH, Poon Y, David N, Baker DJ, van Deursen JM, Campisi J, Eliseieff JH. Local clearance of senescent cells

- attenuates the development of post-traumatic osteoarthritis and creates a pro-regenerative environment. *Nature medicine* 2017; 23: 775-781.
62. Schafer MJ, White TA, Iijima K, Haak AJ, Ligresti G, Atkinson EJ, Oberg AL, Birch J, Salmonowicz H, Zhu Y, Mazula DL, Brooks RW, Fuhrmann-Stroissnigg H, Pirtskhalava T, Prakash YS, Tchkonja T, Robbins PD, Aubry MC, Passos JF, Kirkland JL, Tschumperlin DJ, Kita H, LeBrasseur NK. Cellular senescence mediates fibrotic pulmonary disease. *Nature communications* 2017; 8: 14532.
 63. Torday J, Hua J, Slavin R. Metabolism and fate of neutral lipids of fetal lung fibroblast origin. *Biochimica et biophysica acta* 1995; 1254: 198-206.
 64. Schultz CJ, Torres E, Londos C, Torday JS. Role of adipocyte differentiation-related protein in surfactant phospholipid synthesis by type II cells. *American journal of physiology Lung cellular and molecular physiology* 2002; 283: L288-296.
 65. Sucre JMS, Vickers KC, Benjamin JT, Plosa EJ, Jetter CS, Cutrone A, Ransom M, Anderson Z, Sheng Q, Fensterheim BA, Ambalavanan N, Millis B, Lee E, Zijlstra A, Königshoff M, Blackwell TS, Guttentag SH. Hyperoxia Injury in the Developing Lung is Mediated by Mesenchymal Expression of Wnt5A. *Am J Respir Crit Care Med*. 2020
 66. El Agha E, Herold S, Al Alam D, Quantius J, MacKenzie B, Carraro G, Moiseenko A, Chao CM, Minoo P, Seeger W, Bellusci S. Fgf10-positive cells represent a progenitor cell population during lung development and postnatally. *Development* 2014; 141: 296-306.
 67. Green H, Kehinde O. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell* 1975; 5: 19-27.
 68. Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell* 1995; 83: 813-819.
 69. Xu M, Palmer AK, Ding H, Weivoda MM, Pirtskhalava T, White TA, Sepe A, Johnson KO, Stout MB, Giorgadze N, Jensen MD, LeBrasseur NK, Tchkonja T, Kirkland JL. Targeting senescent cells enhances adipogenesis and metabolic function in old age. *eLife* 2015; 4: e12997.
 70. Qiu Y, Nguyen KD, Odegaard JI, Cui X, Tian X, Locksley RM, Palmiter RD, Chawla A. Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell* 2014; 157: 1292-1308.
 71. Goh YP, Henderson NC, Heredia JE, Red Eagle A, Odegaard JI, Lehwald N, Nguyen KD, Sheppard D, Mukundan L, Locksley RM, Chawla A. Eosinophils secrete IL-4 to facilitate liver regeneration. *Proceedings of the National Academy of Sciences of the United States of America* 2013; 110: 9914-9919.
 72. Abella A, Dubus P, Malumbres M, Rane SG, Kiyokawa H, Sicard A, Vignon F, Langin D, Barbacid M, Fajas L. Cdk4 promotes adipogenesis through PPARgamma activation. *Cell metabolism* 2005; 2: 239-249.

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 PDGFR α 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 =20 μ m. *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, and 120. 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 in early 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 in early 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 the main 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 qPCR: *Srebp2*, *Scap*, *Insig1* and *Adrp* relative to that of the housekeeping gene *Hprt1* at day 14. E. Gene expression by qPCR: *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 qPCR 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 faster for $p16^{INK4a-/-}$ 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⁻, PDGFR α ⁺ cells and CD45⁻, CD31⁻, EpCAM⁻, lipidTOX⁺, and ADRP⁺ cells at D60 in hyperoxic $p16^{INK4a-/-}$ than wild type mice. I. Scale bar = 20 μ m * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 6. The lipogenic switch of fibroblasts can induce lung regeneration after pneumonectomy. $P16^{INK4a}$ deletion induces an increase in the number of lipofibroblasts (ADRP positive cells) and AT2 cells (pro-SpC positive 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 cytokines of whole lung from wildtype and p16^{INK4a} just after hyperoxia. B. PCR of IL4 relative to that of the housekeeping gene HPRT1 of whole lung. C. qPCR 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.

Figure 8. The lipogenic switch of fibroblasts can 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.

0 in 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 (PPAR γ 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 = 20 μ m *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 9. P16^{INK4a} level increases in the blood of preterm newborns, persists in BPD survivors, and are high in the lungs of BPD patients. A. Gene expression by qPCR of p16^{INK4a} relative to that of the 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 qPCR 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 controls for age and sex. C-D. Representative images of p16^{INK4a} expression in the lungs of newborns who died of BPD. Scale bar = 20 μ m. Black dots: room air, 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 of lipogenesis, and its role in alveolar regeneration *via* alveolar type 2 cells.