Fetal lung underdevelopment is rescued by administration of amniotic fluid stem cell extracellular vesicles in rodents

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One Sentence Summary: Administration of extracellular vesicles derived from amniotic fluid stem cells regenerates underdeveloped fetal lungs.

Abstract

Fetal lung underdevelopment, also known as pulmonary hypoplasia, is characterized by decreased lung growth and maturation. The most common birth defect found in babies with pulmonary hypoplasia is congenital diaphragmatic hernia (CDH). Despite research and clinical advances, babies with CDH still have high morbidity and mortality rates, which are directly related to the severity of lung underdevelopment. To date, there is no effective treatment that promotes fetal lung growth and maturation. Herein, we describe a stem cell-based approach in rodents that enhances fetal lung development via the administration of extracellular vesicles (EVs) derived from amniotic fluid stem cells (AFSCs). Using fetal rodent models of pulmonary hypoplasia (primary epithelial cells, organoids, explants, and in vivo), we demonstrated that AFSC-EV administration promoted branching morphogenesis and alveolarization, rescued tissue homeostasis, and stimulated epithelial cell and fibroblast differentiation. We confirmed this regenerative ability in in vitro models of lung injury using human material, where human AFSC-EVs obtained following good manufacturing practices restored pulmonary epithelial homeostasis. Investigating EV mechanism of action we found that AFSC-EV beneficial effects were exerted via the release of RNA cargo. miRNAs regulating the expression of genes involved in lung development, such as the miR17~92 cluster and its paralogues, were highly enriched in AFSC-EVs and were increased in AFSC-EV-treated primary lung epithelial cells compared to untreated cells. Our findings suggest that AFSC-EVs hold regenerative ability for underdeveloped fetal lungs, demonstrating potential for therapeutic application in patients with pulmonary hypoplasia.

Introduction

Fetal lung development is a crucial step during embryogenesis, which if disrupted leads to a condition called pulmonary hypoplasia. Hypoplastic lungs have fewer bronchiolar divisions, enlarged airspaces, defective alveolarization, and impaired tissue maturation (*1*). Pulmonary hypoplasia is idiopathic or secondary to associated malformations, the most common of which is congenital diaphragmatic hernia (CDH) (*1, 2*). CDH is a birth defect characterized by an incomplete closure of the diaphragm and herniation of intra-abdominal organs into the chest (*1*, *2*). Pulmonary hypoplasia secondary to CDH has a mortality rate of 40% with most babies dying within the first days of life (*3*), and with 60% of survivors suffering from long-term morbidities (*4*, *5*). There is an unmet clinical need for an effective treatment that rescues lung growth and maturation, but none of the therapies tested to date has been successful (*6*). As pulmonary hypoplasia can be diagnosed as early as at the anatomy scan (18-20 weeks of gestational age), the paradigm of treatment in the last decades has focused on promoting lung growth and maturation before birth (*6*).

Lung development is a complex process regulated by a network of signaling molecules. Particularly, some miRNAs control branching morphogenesis and epithelial and mesenchymal differentiation (*7-9*), and are missing or dysregulated in human and animal hypoplastic lungs (*10-13*). Correcting the dysregulated network of signaling molecules would be beneficial to promote lung regeneration in fetuses with pulmonary hypoplasia.

A promising strategy to deliver a heterogeneous population of small RNA species is by administering extracellular vesicles (EVs) (*14-16*). EVs are biological membrane-bound nanoparticles carrying genetic material and bioactive proteins as cargo (*17-19*). EVs are key mediators of stem cell paracrine signaling and promote tissue maturation and regeneration (*20-*

22). Amniotic fluid stem cells (AFSCs) could be the ideal source of EVs to promote lung regeneration as AFSCs integrate and differentiate into epithelial lung lineages (*23*), reduce lung fibrosis (*24*), repair damaged alveolar epithelial cells (*25*), and promote lung growth in a model of pulmonary hypoplasia secondary to CDH (*26*, *27*). AFSCs confer regenerative ability despite low engraftment rates, thus suggesting a paracrine effect (*26*-*28*), which could be partly mediated by EVs. Recently, AFSC-EVs have been reported to hold regenerative potential in several animal models, including lung, kidney, and muscle injury *(29)*.

In the present study, we investigated whether AFSC-EV administration to various models of pulmonary hypoplasia could promote growth and maturation of underdeveloped fetal lungs.

Results

Rat AFSC-EV administration promotes growth and maturation in fetal hypoplastic lungs

The most robust model of pulmonary hypoplasia relies on nitrofen administration to pregnant rats at embryonic day (E) 9.5 (*30*, *31*), which mainly targets retinoic acid synthesis (*32*). In this model, the whole litter has a degree of lung underdevelopment analogous to that of human fetuses (*2*, *30*-*33*). In utero nitrofen exposure results in fewer bronchiolar divisions and airspaces compared to lungs from uninjured fetuses (Fig. 1, A to C) (*30*). Administration of rat AFSC-EVs (rAFSC-EVs, mean size 140±5 nm) to hypoplastic lung explants harvested during the pseudoglandular stage (E14.5) resulted in improved branching morphogenesis (Fig. 1, A to C, fig. S1). Particularly, rAFSC-EV-treated lung explants had increased bud count and surface area compared to untreated hypoplastic lung explants and similar to control. This effect was specifically due to rAFSC-EVs, as administration of rAFSC conditioned medium (CM) or EV-depleted rAFSC-CM did not rescue impaired terminal branching. The beneficial effects of rAFSC-EV were dependent on the size and concentration of EVs isolated from the CM (fig. S1,

F and G) and were similar to those observed following administration of their parent cells (AFSCs; fig. S2, A and B). As larger rAFSC-EVs (mean size 363±17 nm) did not rescue branching morphogenesis (fig. S1F), in all experiments performed hereafter we used small rAFSC-EVs (mean size 140±5 nm). We also tested whether the effects obtained with rAFSC-EVs could be replicated by another EV source, namely mesenchymal stromal cells (MSCs). There is growing evidence that MSC-EVs ameliorate bronchopulmonary dysplasia (BPD) (*34*), a neonatal lung condition similar to pulmonary hypoplasia secondary to CDH. However, rMSC-EV administration to hypoplastic lung explants did not rescue branching morphogenesis (Fig. 1, A to C, fig. S1). Moreover, rAFSC-EVs did not affect branching morphogenesis in uninjured control lungs (fig. S3, A and B). Investigating pathways responsible for branching morphogenesis, we observed that compared to control, hypoplastic lungs had lower expression of *Fgf10, Vegfα* and receptors (*Flt1, Kdr*), which were increased upon rAFSC-EV administration (Fig. 1D) (*35, 36*).

Along with compromised fetal lung growth, pulmonary hypoplasia is characterized by impaired maturation, a feature replicated with in utero nitrofen exposure (*2, 30*). Hypoplastic lungs have decreased cell proliferation and delayed epithelial cell differentiation, as demonstrated by increased density of distal progenitor cells ($SOX9^+$) and reduced surfactant protein C (SPC) expression (Fig. 1, E to L, fig. S4, A and B) (*32, 37, 38*). rAFSC-EV administration rescued cell proliferation and improved epithelial cell differentiation, as evidenced by lower $SOX9^+$ cell density (Fig 1, E and F) and increased SPC expression (Fig. 1, E to L). As reported, hypoplastic lungs had similar degree of apoptosis compared to control, and rAFSC-EV administration did not alter this phenotype (Fig. 1, M and N, fig. S4C) (*33, 39*).

rAFSC-EV administration rescues homeostasis and stimulates differentiation of lung epithelial cells

A hallmark of pulmonary hypoplasia secondary to CDH is impaired respiratory epithelial homeostasis (*2*, *40*). rAFSC-EV administration to primary lung epithelial cells isolated from hypoplastic lungs of nitrofen-injured fetuses increased proliferation and reduced cell death back to control (Fig. 2, A and B). We confirmed that these effects were specific to rAFSC-EVs, as they were not reproduced by the administration of either rAFSC-CM, or EV-depleted rAFSC-CM and that they were similar to those observed with the administration of their parent cells (AFSCs; fig S2, C and D). In addition, rMSC-EVs improved proliferation of primary lung epithelial cells, but failed to rescue cell death (fig. S4, D and E). We also observed that rAFSC-EV administration to uninjured control cells did not change proliferation or cell death rates (fig. S3, C and D).

To study rAFSC-EV effects on respiratory epithelial cell differentiation, we generated fetal lung organoids from nitrofen-injured and control lungs (Fig. 2C, fig. S4F). rAFSC-EV administration to nitrofen-injured organoids resulted in proliferation rates similar to control organoids and higher than untreated nitrofen-injured organoids (Fig. 2D, fig. S4G). Moreover, rAFSC-EV administration improved respiratory epithelial differentiation, with higher SPC (marker of early distal epithelium and alveolar type II cells) and CC10 (marker of club cells) expression (Fig. 2, E and F, fig. S4, H and I).

rAFSC-EV cargo content and its effect on lung epithelium

rAFSC-EV effects on nitrofen-injured hypoplastic lungs and respiratory epithelium were associated with transfer of EV cargo. EV cargoes were detected throughout the lung parenchyma (Movie S1) and primary lung epithelial cells regardless of size and EV source (Fig. 3, A and B;

Movie S2, S3, S4, S5). To study the cargo content, we differentially analyzed rAFSC-EV and rMSC-EV protein and RNA, as we had observed different effects between the two EV sources. Proteomics analysis identified 222 differentially expressed proteins, none of which had obvious molecular functions directly related to fetal lung development (fig. S5A). rAFSC-EVs contained proteins involved in EV formation (HSPa and CD63), miRNA stabilization (Annexins and Hnrnps), and EV structure and function (table S1).

To test whether rAFSC-EV effects were due to their RNA cargo, we treated rAFSC-EVs with RNase-A. We verified that RNase-A was captured by rAFSC-EVs and degraded their RNA cargo (Fig. 3, C to I). RNase-pretreated rAFSC-EVs administered to hypoplastic lung explants or primary epithelial cells derived from hypoplastic lungs did not rescue branching morphogenesis or epithelial homeostasis (Fig. 3, J to M). We confirmed that these effects were not due to carry-over of RNase-A to the epithelial cells (fig. S5B). Taken together, these data suggested that the rAFSC-EV RNA was key for their biological effects on fetal lung development. With RNA-sequencing we found that both rAFSC-EV and rMSC-EV cargos contained messenger RNA (mRNA), transfer RNA (tRNA), microRNA (miRNA), and piwi-interacting RNA (piRNA) (Fig. 3N, fig. S5C). Among all, miRNAs were the RNA species most proportionally different between rAFSC-EV and rMSC-EV cargos. Compared to rMSC-EVs, rAFSC-EVs were enriched for miRNAs critical for lung development. These include the miR17~92 cluster and paralogues (miRs-93, -106, -250, -363; fold enrichment range: 5.1 - 9.36; table S2, Fig. 3O), that were found in higher concentrations in AFSC-EVs compared to AFSCs (fig. S2E). Moreover, rAFSC-EVs contained miRNAs that were reported to be dysregulated in hypoplastic lungs, such as miR-33 and miR-200 (table S3) (*10*, *12*).

To identify the regulatory pathways affected by rAFSC-EVs, we used mRNA-sequencing to compare gene expression between primary lung epithelial cells from nitrofen-injured lungs treated with rAFSC-EVs or rMSC-EVs. Nitrofen-injury altered the gene expression profile of primary lung epithelial cells compared to uninjured epithelial cells (fig. S5D). Gene set enrichment analysis showed that rAFSC-EV administration to nitrofen-injured primary epithelial cells altered the expression of genes related to epithelial differentiation and homeostasis maintenance, whereas rMSC-EV administration altered the expression of genes involved with cell cycle regulation and nuclear organization (Fig. 4, A and B, fig. S6, A to C). Furthermore, we manually queried these genes and identified those that are critical for lung development (table S4). We then asked if the miRNAs identified in the rAFSC-EV cargo were part of predicted regulatory networks with the mRNAs that were down-regulated in the target epithelial cells (Fig. 4C). The network that resulted from this analysis showed that there were genes important for lung development and that were regulated by miR17~92 cluster and paralogues. Small RNA-sequencing of nitrofen-injured rAFSC-EV-treated primary epithelial lung cells showed that most miRNAs in the network had higher expression compared to nitrofen-injured untreated cells (Fig. 4C). Lastly, we correlated the miRNA cargo content with the miRNA target cell content, and their validated mRNAs in the target cells (fig. S7). This triple analysis identified miRNA-mRNAs pairs that might be responsible for the phenotype observed and provided indirect evidence that the rAFSC-EV miRNA cargo was transferred to the target cells.

Towards clinical translation of AFSC-EVs as treatment for fetal lung regeneration

To test the effects of rAFSC-EVs in vivo, we used a surgical model of pulmonary hypoplasia secondary to CDH in fetal rabbits, as it allowed us to topically deliver our treatment (Fig. 5A) (*41*). To prevent rAFSC-EV egression, we performed tracheal occlusion on rabbit fetuses (Movie

S6), a procedure currently used in clinical trials for selected CDH human fetuses (*42*). rAFSC-EV administration improved lung alveolarization by increasing the number of alveoli, decreasing the alveolar wall thickness, and promoting alveolar lipofibroblast maturation (Fig. 5, B to E). Moreover, only rAFSC-EV-treated lungs had improved BMP signaling (BMP2, BMP4, Id1), important for alveolar maturation (Fig. 5F). These effects were not observed in rMSC-EV-treated lungs (fig. S8, A to C).

To investigate whether rAFSC-EV beneficial effects could be translated onto human lung tissue, we obtained human AFSCs (hAFSCs) from donated amniotic fluid following clinically compliant good manufacturing practices (GMP), as reported (*22*). hAFSC-EV administration replicated similar effects on cellular homeostasis observed in rat models on both a validated model of lung injury with nitrofen-injured human alveolar epithelial (A549) cells and on nitrofen-injured primary human pulmonary alveolar epithelial cells (HPAEpiC) isolated from a fetus at 21 weeks of gestation (Fig. 5, G to J). We verified that fluorescently labeled $PKH26^{+}$ hAFSC-EVs entered A549 cells and HPAEpiC (Fig. 5, K and L). Administration of human MSC-EVs (hMSC-EVs) to HPAEpiC did not rescue cell homeostasis, despite entering the cells (fig. S9, A to C). Lastly, when we tested hAFSC-EVs on the in vivo rabbit model, we found similar effects on alveolar wall thickness, as found with rAFSC-EV administration (fig. S9, D to F). Among the top 50 miRNAs enriched in hAFSC-EVs (*22*), 13 were evolutionarily conserved between human and rabbit species, including a member of the miR17~92 cluster (table S5).

Discussion

In this study, we have shown that AFSC-EV administration to various models of pulmonary hypoplasia promotes fetal lung regeneration. Specifically, AFSC-EVs administered to fetal hypoplastic lungs rescued branching morphogenesis and alveolarization, promoted epithelial and

mesenchymal tissue maturation, and re-established cellular homeostasis. These beneficial effects were obtained through AFSC-EV RNA cargo, including miRNAs that regulate lung development.

EVs are emerging as a successful strategy to promote tissue regeneration in various models. The regenerative potential observed in our models of fetal pulmonary hypoplasia has been reported in other models of tissue regeneration using either AFSC-EVs (*29*) or EVs from other stem cell sources (*14-16, 20, 43, 44*). In fact, there is increasing evidence that EVs secreted by stem cells carry cargo that stimulates stem cell-like paracrine functions on target cells such as renewal, differentiation, and maturation (*45-47*). In this study, we confirmed that rAFSC-EVs have similar effects on hypoplastic lung explants and lung epithelium to that exerted by their parent cells, rAFSCs. For this reason and their immunological innocuity, EVs are an advantageous and safer cell-free alternative to stem cell-based therapies (*48-50*). Although GMP-grade CM could be therapeutic in humans (*51-53*), in our study the EV fraction of the CM was more potent than the whole CM or than the EV-depleted CM fraction, as also observed by others (*54, 55*). This could be due to EVs carrying active molecules that are more concentrated than in parent cells . This is in line with our previous findings that AFSC-EV concentration is the most important parameter responsible for their regenerative potential (*29*). Likewise, in the present study, we have shown that rAFSC-EV effects are dependent on the concentration of EVs isolated from CM, as well as on the size of the vesicles that enter target cells. How size contributes to biological function of EVs remains unknown (*56*). Nonetheless, small EVs, also called exosomes (*17*), have traditionally been considered the EV subpopulation with more potent, protective, and pathological functions than larger vesicles, and therefore with more potential as diagnostic or therapeutic tools (*29*).

We used MSCs as an alternative EV source, since MSCs are being tested in several clinical trials for BPD treatment (*57*), the lung condition that is most comparable to pulmonary hypoplasia. In our study, MSC-EV administration to pulmonary hypoplasia models did not have similar beneficial effects as AFSC-EV administration, despite entering primary lung cells. The different effects obtained with the two EV populations may be due to differences in disease pathogenesis, where pulmonary hypoplasia is mainly the result of abnormal and delayed lung development, and BPD is a chronic postnatal lung disease with severe inflammatory response (*58*). Moreover, our analysis of the EV cargo identified profile differences between the two EV populations, which could explain the outcome differences observed.

In our models of pulmonary hypoplasia, we identified that rAFSC-EV RNA cargo was key to regenerate hypoplastic fetal lungs. This finding is in line with reports that EV-mediated effects occur through RNA transfer (*14-17*). When considering the rAFSC-EV specific genes in the context of miRNA cargo enriched in rAFSC-EVs, we found miRNA species important for the phenotypes observed, including branching morphogenesis, alveolarization, cell homeostasis and differentiation. Specifically, a family of miRNAs that is enriched in rAFSC-EVs compared to rMSC-EVs is the miRNA 17~92 cluster. This cluster is essential for lung branching morphogenesis (*59*), and when knocked out causes severe fetal pulmonary hypoplasia, making this a candidate mechanism that warrants further investigation (*9*). Moreover, small RNA sequencing analysis revealed that members of this cluster were up-regulated in rAFSC-EV-treated nitrofen-injured primary lung epithelial cells.

Improved fetal lung development was observed not just when the same species of AFSC-EVs was administered on the same species of target cells (that is, rAFSC-EVs on rat lung tissue or hAFSC-EVs on human lung epithelial cells), but also when we tested rAFSC-EVs or hAFSC-

EVs on the in vivo rabbit model. We speculate that the improvement in alveolarization observed in rabbit fetuses is because some miRNAs and their targets are conserved across species (*60*).

Our study provides insights into the potential use of AFSC-EVs as a therapy for fetal pulmonary hypoplasia. Using GMP-grade hAFSC-EVs, we have confirmed similar beneficial effects on damaged epithelial cells derived from a fetal lung at the gestational age when pulmonary hypoplasia and CDH are typically detected. Further steps are needed for AFSC-EVs to be used as a therapy in human fetuses, such as identifying the most effective and safest administration route. In this study, we employed topical administration via intra-tracheal injection in rabbit fetuses. This route could be further explored, also in clinical settings, as it is currently used to occlude the trachea of human fetuses with severe pulmonary hypoplasia secondary to CDH (*42*).

We acknowledge that our study has some limitations. Our findings are mainly based on the use of animal models and human lung epithelial cells to study a complex human condition with an unknown etiology. However, obtaining human lung tissues from babies with pulmonary hypoplasia is not considered ethically acceptable nor has it been reported. Moreover, in this study we mainly focused on fetal lung epithelium and mesenchyme, but we did not examine other lung compartments such as pulmonary vasculature, which is known to undergo remodeling in hypoplastic lungs. Nonetheless, we observed improvements in pathways important for lung vascular development, such as VEGF and FGF10 that suggest the opportunity for further studies. Another limitation is that little is known about factors that alter lung development and the mechanisms that are dysregulated in pulmonary hypoplasia. Similarly, it remains unknown how exactly the EV RNA cargo species function. It is hoped that our transcriptomic profiling of both hypoplastic lungs and EVs increases the understanding on the pathogenesis of pulmonary hypoplasia and on EV function.

Materials and Methods

Study Design

This study aimed to evaluate the ability of AFSC-EV administration to promote fetal lung growth and maturation in pulmonary hypoplasia. As obtaining human lung tissues from babies with CDH is not considered ethically acceptable, part of this study was conducted using animal models that closely resemble the degree of pulmonary hypoplasia observed in human fetuses. To advance towards a translational therapy, we obtained EVs from GMP-grade hAFSCs isolated from donated amniotic fluid during amniocentesis. We tested hAFSC-EVs first on a validated lung injury model using A549 alveolar epithelial cells (*29, 61*). To more closely replicate human fetal pulmonary hypoplasia, we investigated the GMP-grade hAFSC-EV effects on nitrofen-injured human pulmonary alveolar epithelial cells obtained from a healthy fetus at 21-weeks of gestation. Experimental models and sample collections were approved by appropriate regulatory committees at: The Hospital for Sick Children, Toronto (protocol #39168 and 49892); University College London Hospital, London, UK (UCL/UCLH Joint Committee for the Ethics of Human Research, REC Reference: 08/0304); Ribeirão Preto Medical School, University of São Paulo, Brazil (191/2018+40/2020). Sprague-Dawley rats and New Zealand rabbits were used for animal studies. Samples from all models were randomly assigned to treatment groups. All data including outliers are shown, and all experiments were performed in at least triplicate, with the number of replicates indicated in the figure legends. Analysis of data was conducted by at least two blinded investigators. Additional details on methods used are provided in Supplementary Materials.

Extracellular vesicles

EVs from rat and human AFSCs or bone-marrow MSCs were isolated by ultracentrifugation from CM of cells treated with exosome-depleted fetal bovine serum for 18 hours, as described

(*29*). Based on previous studies, we established that $4x10^6$ AFSCs cultured under these conditions secrete approximately $3x10^9 \pm 1x10^7$ EVs, quantified by nanoparticle tracking analysis (*29*). For all experiments, EV doses are described as v/v (EV volume in CM volume). In accordance with the International Society for Extracellular Vesicles guidelines, EVs were characterized for size (nanoparticle tracking analysis), morphology (transmission electron microscopy), and expression of canonical EV-related protein markers (Western blot analysis), as described (*29*). To track EV migration into primary lung epithelial cells and lung explants, EV cargoes were fluorescently labelled for RNA and protein using Exo-Glow and for lipid membrane using PKH26. To determine the role of RNA in rescuing pulmonary hypoplasia, RNase enzymatic digestion of rAFSC-EV cargo was conducted and confirmed by bioanalyzer and immuno-electron microscopy.

Experimental models of pulmonary hypoplasia

Ex vivo - In fetal rats, pulmonary hypoplasia was induced as described (*30, 40*) with nitrofen administration to dams (100 mg) by oral gavage on E9.5. At E14.5, the dam was euthanized, and fetal lungs were micro-dissected. Lungs were grown on nanofilter membranes, and incubated for 72 h in culture medium alone, rAFSC-CM, or medium supplemented with rAFSC-EVs or rMSC-EVs (0.5% v/v). Fetal lungs from dams that received olive oil (no nitrofen) at E9.5 served as control.

In vitro – 1) For primary epithelial cell experiments in rats, a single cell suspension was obtained at E14.5 from pooled lungs of control or nitrofen-injured rat fetuses by trypsinization. Cells were subjected to serial fibroblast depletions as described (*40, 62*). 2) For organoid studies, cells were seeded in a ratio of 60:40 semi-solid Matrigel to medium, as described (*63*). Cells from nitrofen-injured fetuses were cultured for 10 days with medium alone or medium supplemented with

rAFSC-EVs or rMSC-EVs (1.2% v/v). Lung organoids from fetuses whose mothers had not received nitrofen served as control. 3) A549 cells were treated for 24 h with nitrofen (40 μM), and treated with medium alone, or medium supplemented with hAFSC-EVs or hMSC-EVs (0.5% v/v). Uninjured and untreated A549 cells served as control. 4) HPAEpiCs were obtained from the lung of a 21-week-gestation fetus and used at first passage. Cells were injured with 400 μM nitrofen for 24 hours, and treated with medium alone, or medium supplemented with hAFSC-EVs or hMSC-EVs (0.5% v/v).

In vivo - Pulmonary hypoplasia was induced by surgical creation of CDH at E25 in New Zealand rabbits (*41*). At E27, tracheal ligation was performed alone or after intra-tracheal administration of a 50 μL bolus containing rAFSC-EVs, rMSC-EVs, or hAFSC-EVs (Movie S6). Lungs were harvested at E31 and frozen for RNA extraction or fixed in 4% paraformaldehyde and embedded in paraffin.

Outcome measures

For lung morphometry, rat fetal lung explants were imaged by differential interference contrast microscopy and independently assessed by two blinded researchers for terminal bud density and surface area using ImageJ, as described (*27*). Rabbit fetal lungs were blindly evaluated with histology (hematoxylin/eosin) to assess alveolar density and wall thickness, as described (*64, 65*). For RNA expression, factors involved in rat or rabbit lung branching morphogenesis were assessed with quantitative polymerase chain reaction (qPCR). To investigate cellular homeostasis on lung explants and organoids, we used 5-ethynyl-2'-deoxyuridine (EdU) incorporation, viability/cytotoxicity staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL assay), or $Ki67^+$ immunostaining. To assess epithelial differentiation, lung explants were immunostained and blotted for SOX9 and SPC, and organoids were

immunostained for SPC and CC10. To study rat EV protein cargo, we used nanoscale liquid chromatography coupled to tandem mass spectrometry and performed differential expression analysis (1% protein and peptide false discovery rate, FDR). For rat EV RNA cargo, we conducted small RNA-sequencing and compared RNA content using Bioconductor DESeq. To study rAFSC-EV RNA effects on target cells, total RNA was isolated from primary lung epithelial cells from each condition and used edgeR for comparative analyses. To study changes in miRNA expression patterns in the same target cells, we further analyzed the Nitrofen and Nitrofen+rAFSC-EVs treated cells using small RNA-sequencing. edgeR was used to determine miRNA expression changes (FDR < 0.1), and Spearman's correlation was used to assess potential gene targets of detected miRNAs in cells. Pathway enrichment analysis was conducted with g:profiler and miRNA-mRNA network analysis was performed with TargetScan, miRTarBase, and Cytoscape.

Statistical Analysis

Groups were compared using two-tailed Student t-test, Mann-Whitney test, one-way ANOVA (Tukey post-test), or Kruskal-Wallis (post-hoc Dunn's nonparametric comparison) test according to Gaussian distribution assessed by D'Agostino-Pearson omnibus normality test. For correlation studies, a Pearson coefficient was reported as r (confidence interval). P value was considered significant when $p<0.05$. All statistical analyses were produced using GraphPad Prism software version 6.0. mRNA and miRNA sequencing analyses in lung epithelial cells were performed in R (version 3.6.0). Package "edgeR" (version 3.26.5) was used for differential analyses between two conditions with FDR < 0.1 considered as significant for both mRNA and small RNA-sequencing.

Supplementary Materials

Fig. S1. Characterization of rAFSC-EVs and rMSC-EVs and effects on lung explants based on size and concentration.

Fig. S2. Influence of rAFSCs in co-culture with ex vivo and in vitro models of pulmonary hypoplasia and effects on rAFSC-EV miRNA cargo.

Fig. S3. Effects of rAFSC-EVs on control lung explants and primary lung epithelial cells.

Fig. S4. Effects of rMSC-EV administration on in vitro and ex vivo models of pulmonary hypoplasia.

Fig. S5. Analysis of protein and RNA cargo of rAFSC-EV and rMSC-EV.

Fig. S6. Enrichment plots for RNA-seq analysis of rMSC-EV treated primary lung epithelial cells.

Fig. S7. Correlation analysis of miRNA-mRNA sequencing in primary lung epithelial cells.

Fig. S8. Effects of rMSC-EV administration in the in vivo model of pulmonary hypoplasia.Fig. S9. Effects of hAFSC-EVs and hMSC-EVs on human fetal lung epithelial cells and in the in vivo model of pulmonary hypoplasia.

Table S1. Highlighted proteins expressed in rAFSC-EVs and rMSC-EVs.

Table S2. miRNAs related to lung development that are differentially expressed in rAFSC-EVs over rMSC-EVs.

Table S3. miRNAs known to be involved in pulmonary hypoplasia and present in rAFSC-EVs.

Table S4. Genes differentially expressed in nitrofen-injured lung epithelial cells.

Table S5. Inter-species conservation of top enriched miRNA in hAFSC-EVs

Table S6: Primer sequences used in this study.

Table S7. Details of antibodies used in this study.

Movie S1. rAFSC-EV tracking into lung explant.

Movie S2. Live tracking of rAFSC small EV RNA into lung epithelial cells.

Movie S3. Live tracking of rAFSC small EV Protein into lung epithelial cells.Movie S4. Live tracking of rAFSC medium/large EV RNA into lung epithelial cells.

Movie S5. Live tracking of rMSC small EV RNA into lung epithelial cells.

Movie S6. In vivo administration of AFSC-EVs prior to tracheal ligation in fetal rabbits.

Data file S1 (Microsoft Excel format). Differential analysis of rAFSC-EV and rMSC-EV protein cargo using proteomic analysis.

Data file S2 (Microsoft Excel format). Quality control table for all RNA-sequencing experiments used in this study.

Data file S3 (Microsoft Excel format). Full list of differentially expressed genes with pair-wise comparisons between Nitrofen vs. Nitrofen+rAFSC-EVs, and Nitrofen vs. Nitrofen+rMSC-EVs.

Data file S4 (Microsoft Excel format). Full list of pathway enrichments for mRNA-sequencing experiments.

Data file S5 (Microsoft Excel format). Data for Western blot experiments.

References and Notes

1. A. Hislop, L. Reid. Persistent hypoplasia of the lung after repair of congenital diaphragmatic hernia. *Thorax.* **31**, 450-455 (1976).

2. P. K. Donahoe, M. Longoni, F. A. High. Polygenic causes of congenital diaphragmatic hernia produce common lung pathologies. *Am. J. Pathol.* **186**, 2532-2543 (2016).

3. M. T. Harting, K. P. Lally. The Congenital Diaphragmatic Hernia Study Group registry update. *Semin. Fetal Neonatal. Med.* **19**, 370-375 (2014).

4. M. Spoel, M. H. van der Cammen-van Zijp, W. C. Hop, D. Tibboel, J. C. de Jongste, H. Ijsselstijn. Lung function in young adults with congenital diaphragmatic hernia; a longitudinal evaluation. *Pediatr. Pulmonol.* **48**, 130-137 (2013).

5. L. Montalva, G. Raffler, A. Riccio, G. Lauriti, A. Zani. Neurodevelopmental impairment in children with congenital diaphragmatic hernia: Not an uncommon complication for survivors. *J Pediatr Surg*. **55**, 625-634 (2020).

6. C. Jeanty, S. M. Kunisaki, T. C. MacKenzie. Novel non-surgical prenatal approaches to treating congenital diaphragmatic hernia. *Semin. Fetal Neonatal Med.* **19**, 349-356 (2014).

7. L. Cushing, Z. Jiang, P. Kuang, J. Lü. The roles of microRNAs and protein components of the microRNA pathway in lung development and diseases. *Am. J. Respir. Cell Mol. Biol*. **52**, 397-408 (2015).

8. M. Herriges, E. E. Morrisey. Lung development: orchestrating the generation and regeneration of a complex organ. *Development* **141**, 502-513 (2014).

9. A. Ventura, A. G. Young, M. M. Winslow, L. Lintault, A. Meissner, S. J. Erkeland, J. Newman, R. T. Bronson, D. Crowley, J. R. Stone, R. Jaenisch, P. A. Sharp, T. Jacks. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell* **132**, 875-886 (2008).

10. S. Zhu, Q. He, R. Zhang, Y. Wang, W. Zhong, H. Xia, J. Yu. Decreased expression of miR-33 in fetal lungs of nitrofen-induced congenital diaphragmatic hernia rat model. *J. Pediatr. Surg.* **51**, 1096-100 (2016).

11. T. H. Mahood, D. R. Johar, B. M. Iwasiow, W. Xu, R. Keijzer. The transcriptome of nitrofen-induced pulmonary hypoplasia in the rat model of congenital diaphragmatic hernia. *Pediatr. Res*. **79**, 766-775 (2016).

12. P. Pereira-Terra, J. A. Deprest, R. Kholdebarin, N. Khoshgoo, P. DeKoninck, A. A. Munck, J. Wang, F. Zhu, R. J. Rottier, B. M. Iwasiow, J. Correia-Pinto, D. Tibboel, M. Post, R. Keijzer. Unique tracheal fluid microRNA signature predicts response to FETO in patients with congenital diaphragmatic hernia. *Ann. Surg.* **262**, 1130-1140 (2015).

13. E. L. Sanford, K. W. Choy, P. K. Donahoe, A. A. Tracy, R. Hila, M. Loscertales, M. Longoni. MiR-449a affects epithelial proliferation during the pseudoglandular and canalicular phases of avian and mammal lung development. *PLoS One* **18**, e0149425 (2016).

14. L. Alvarez-Erviti, Y. Seow, H. Yin, C. Betts, S. Lakhal, M. J. Wood. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat. Biotechnol.* **29**, 341-345 (2011).

15. S. Rani, A. E. Ryan, M. D. Griffin, T. Ritter. Mesenchymal stem cell-derived extracellular vesicles: Toward cell-free therapeutic applications. *Mol. Ther*. **23**, 812-823 (2015).

16. B. György, M. E. Hung, X. O. Breakefield, J. N. Leonard. Therapeutic applications of extracellular vesicles: clinical promise and open questions. *Annu. Rev. Pharmacol. Toxicol*. **5**, 439-464 (2015).

17. H. Valadi, K. Ekström, A. Bossios, M. Sjöstrand, J. J. Lee, J. O. Lötvall. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol*. **9**, 654-659 (2007).

18. E. van der Pol, A. N. Böing, P. Harrison, A. Sturk, R. Nieuwland. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol. Rev.* **64**, 676-705 (2012).

19. A. F. Hill, D. M. Pegtel, U. Lambertz, T. Leonardi, L. O'Driscoll, S. Pluchino, D. Ter-Ovanesyan, E. N. Nolte-'t Hoen. ISEV position paper: Extracellular vesicle RNA analysis and bioinformatics. *J. Extracell. Vesicles* **2** (2013).

20. S. Sedrakyan, V. Villani, S. Da Sacco, N. Tripuraneni, S. Porta, A. Achena, M. Lavarreda-Pearce, A. Petrosyan, H. Soloyan, R. E. Filippo, B. Bussolati, L. Perin. Amniotic fluid stem cell-derived vesicles protect from VEGF-induced endothelial damage. *Sci. Rep.* **7**, 16875 (2017).

21. M. Riazifar, E. J. Pone, J. Lötvall, W. Zhao. Stem Cell Extracellular Vesicles: Extended Messages of Regeneration. *Annu. Rev. Pharmacol. Toxicol.* **57**, 125-154 (2017).

22. B. Mellows, R. Mitchell, M. Antonioli, O. Kretz, D. Chambers, M. T. Zeuner, B. Denecke, L. Musante, D. L. Ramachandra, F. Debacq-Chainiaux, H. Holthofer, B. Joch, S. Ray, D. Widera, A. L. David, T. B. Huber, J. Dengjel, P. De Coppi, K. Patel. Protein and molecular characterization of a clinically compliant amniotic fluid stem cell-derived extracellular vesicle fraction capable of accelerating muscle regeneration through enhancement of angiogenesis. *Stem Cells Dev.* **26**, 1316-1333 (2017).

23. G. Carraro, L. Perin, S. Sedrakyan, S. Giuliani, C. Tiozzo, J. Lee, G. Turcatel, S. P. De Langhe, B. Driscoll, S. Bellusci, P. Minoo, A. Atala, R. E. De Filippo, D. Warburton. Human amniotic fluid stem cells can integrate and differentiate into epithelial lung lineages. *Stem Cells* **26**, 2902–2911 (2008).

24. O. Garcia, G. Carraro, G. Turcatel, M. Hall, S. Sedrakyan, T. Roche, S. Buckley, B. Driscoll, L. Perin, D. Warburton. Amniotic fluid stem cells inhibit the progression of bleomycin-induced pulmonary fibrosis via CCL2 modulation in bronchoalveolar lavage. *PLoS One* **8**, e71679 (2013).

25. S. Buckley, W. Shi, G. Carraro, S. Sedrakyan, S. Da Sacco, B. A. Driscoll, L. Perin, R. E. De Filippo, D. Warburton. The milieu of damaged alveolar epithelial type 2 cells stimulates alveolar wound repair by endogenous and exogenous progenitors. *Am. J. Respir. Cell Mol. Biol.* **45**, 1212-1221 (2011).

26. F. Pederiva, M. Ghionzoli, A. Pierro, P. De Coppi, J. A. Tovar. Amniotic fluid stem cells rescue both in vitro and in vivo growth, innervation, and motility in nitrofen-exposed hypoplastic rat lungs through paracrine effects. *Cell Transplant.* **22**, 1683-1694 (2013).

27. J. Di Bernardo, M. M. Maiden, M. B. Hershenson, S. M. Kunisaki. Amniotic fluid derived mesenchymal stromal cells augment fetal lung growth in a nitrofen explant model. *J. Pediatr. Surg.* **49**, 859-864 (2014).

28. A. Zani, M. Cananzi, F. Fascetti-Leon, G. Lauriti, V. V. Smith, S. Bollini, M. Ghionzoli, A. D'Arrigo, M. Pozzobon, M. Piccoli, A. Hicks, J. Wells, B. Siow, N. J. Sebire, C. Bishop, A. Leon, A. Atala, M. F. Lythgoe, A. Pierro, S. Eaton, P. De Coppi. Amniotic fluid stem cells improve survival and enhance repair of damaged intestine in necrotising enterocolitis via a COX-2 dependent mechanism. *Gut* **63**, 300-309 (2014).

29. L. Antounians, A. Tzanetakis, O. Pellerito, V. D. Catania, A. Sulistyo, L. Montalva, M. J. McVey, A. Zani. The regenerative potential of amniotic fluid stem cell extracellular vesicles: Lessons learned by comparing different isolation techniques. *Sci. Rep*. **9**, 1837 (2019).

30. I. Iritani. Experimental study on embryogenesis of congenital diaphragmatic hernia. *Anat. Embryol. (Berl.)* **169**, 133-139 (1984).

31. L. Antounians, R.L. Figueira, L. Sbragia, A. Zani. Congenital Diaphragmatic Hernia: State of the Art in Translating Experimental Research to the Bedside. *Eur. J. Pediatr. Surg*. **29**, 317-327 (2019).

32. L. Montalva, A. Zani. Assessment of the nitrofen model of congenital diaphragmatic hernia and of the dysregulated factors involved in pulmonary hypoplasia. *Pediatr. Surg. Int.* **35**, 41-61 (2019).

33. R. Keijzer, J. Liu, J. Deimling, D. Tibboel, M. Post. Dual-hit hypothesis explains pulmonary hypoplasia in the nitrofen model of congenital diaphragmatic hernia. *Am. J. Pathol.* **156**, 1299-1306 (2000).

34. G. R. Willis, A. Fernandez-Gonzalez, J. Anastas, S. H. Vitali, X. Liu, M. Ericsson, A. Kwong, S. A. Mitsialis, S. Kourembanas. Mesenchymal stromal cell exosomes ameliorate experimental bronchopulmonary dysplasia and restore lung function through macrophage immunomodulation. *Am. J. Respir. Crit. Care Med.* **197**, 104-116 (2018).

35. H. Teramoto, A. Yoneda, P. Puri. Gene expression of fibroblast growth factors 10 and 7 is downregulated in the lung of nitrofen-induced diaphragmatic hernia in rats. *J. Pediatr. Surg.* **38**, 1021-1024 (2003).

36. A. F. Schmidt, F. L. Gonçalves, A. C. Regis, R. M. Gallindo, L. Sbragia. Prenatal retinoic acid improves lung vascularization and VEGF expression in CDH rat. *Am. J. Obstet. Gynecol.* **207**, 25-32 (2012).

37. J. Rhodes, D. Saxena, G. Zhang, G. K. Gittes, D. A. Potoka. Defective parasympathetic innervation is associated with airway branching abnormalities in experimental CDH. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **309**, L168-74 (2015).

38. P. E. Cogo, M. Simonato, O. Danhaive, G. Verlato, G. Cobellis, F. Savignoni, D. Peca, A. Baritussio, V. P. Carnielli. Impaired surfactant protein B synthesis in infants with congenital diaphragmatic hernia. *Eur. Respir. J.* **41**, 677–682 (2013).

39. E. C. Jesudason, M. G. Connell, D. G. Fernig, D. A. Lloyd, P. D. Losty. Cell proliferation and apoptosis in experimental lung hypoplasia. *J. Pediatr. Surg*. **35**, 129–133 (2000).

40. A. Tzanetakis, L. Antounians, A. Belfiore, Q. Ma, M. Stasiewicz, O. Pellerito, A. Zani. Endoplasmic reticulum stress response is activated in pulmonary hypoplasia secondary to congenital diaphragmatic hernia, but is decreased by administration of amniotic fluid stem cells. *Pediatr. Surg. Int.* **35**, 63-69 (2019).

41. R. L. Figueira, K. Costa, A. L. Marsico, T. Milani, W. A. Gonçalves, M. C. Borges, O. Silva, L. Sbragia. Vascular and ventilatory mechanical responses in three different stages of pulmonary development in the rabbit model of congenital diaphragmatic hernia. *Acta Cirurg Brasileira*. **33**, 879–888 (2018).

42. J. Deprest. P. Brady, K. Nicolaides, A. Benachi, C. Berg, J. Vermeesch, G. Gardener, E. Gratacos. Prenatal management of the fetus with isolated congenital diaphragmatic hernia in the era of the TOTAL trial. *Semin. Fetal Neonatal Med.* **19**, 338-348 (2014).

43. M. Pierro, L. Ionescu, T. Montemurro, A. Vadivel, G. Weissmann, G. Oudit, D. Emery, S. Bodiga, F. Eaton, B. Péault, F. Mosca, L. Lazzari, B. Thébaud. Short-term, long-term and paracrine effect of human umbilical cord-derived stem cells in lung injury prevention and repair in experimental bronchopulmonary dysplasia. *Thorax* **68**, 475-484 (2013).

44. L. Barile, V. Lionetti, E. Cervio, M. Matteucci, M. Gherghiceanu, L. M. Popescu, T. Torre, F. Siclari, T. Moccetti, G. Vassalli. Extracellular vesicles from human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction. *Cardiovasc Res.* **103**, 530-541 (2014).

45. M. Nawaz, F. Fatima, K. C. Vallabhaneni, P. Penfornis, H. Valadi, K. Ekström, S. Kholia, J. D. Whitt, J. D Fernandes, R. Pochampally, J. A. Squire, G. Camussi.

Extracellular vesicles: Evolving factors in stem cell biology. *Stem Cells Int.* **2016**, 1073140 (2016).

46. M. Khan, R. Kishore. Stem cell exosomes: Cell-free therapy for organ repair. *Methods Mol. Biol.* **1553**, 315-321 (2017).

47. M. Riazifar, E. J. Pone, J. Lötvall, W. Zhao. Stem cell extracellular vesicles: extended messages of regeneration. *Annu. Rev. Pharmacol. Toxicol*. **57**, 125-154 (2017).

48. S. G. Ong, J. C. Wu. Exosomes as potential alternatives to stem cell therapy in mediating cardiac regeneration. *Circ. Res*. **117**, 7-9 (2015).

49. T. R. Doeppner, M. Bähr, D. M. Hermann, B. Giebel. Concise review: Extracellular vesicles overcoming limitations of cell therapies in ischemic stroke. *Stem Cells Transl. Med*. **6**, 2044-2052 (2017).

50. M. Adamiak, G. Cheng, S. Bobis-Wozowicz, L. Zhao, S. Kedracka-Krok, A. Samanta, E. Karnas, Y. T. Xuan, B. Skupien-Rabian, X. Chen, U. Jankowska, M. Girgis, M. Sekula, A. Davani, S. Lasota, R. J. Vincent, M. Sarna, K. L. Newell, O. L. Wang, N. Dudley, Z. Madeja, B. Dawn, E. K. Zuba-Surma. Induced pluripotent stem cell (iPSCs)-derived extracellular vesicles are safer and more effective for cardiac repair than iPSCs. *Circ. Res*. **122**, 296-309 (2018).

51. S. Y. Kim, J. H. Lee, H. J. Kim, M. K. Park, J. W. Huh, J. Y. Ro, Y. M. Oh, S. D. Lee, Y. S. Lee. Mesenchymal stem cell-conditioned media recovers lung fibroblasts from cigarette smoke-induced damage. *Am. J. Physiol. Lung Cell Mol. Physiol*. **302**, L891-908 (2012).

52. B. R. Zhou, Y. Xu, S. L. Guo, Y. Xu, Y. Wang, F. Zhu, F. Permatasari, D. Wu, Z. Q. Yin, D. Luo. The effect of conditioned media of adipose-derived stem cells on wound healing after ablative fractional carbon dioxide laser resurfacing. *Biomed. Res. Int*. **2013**, 519126 (2013).

53. H. Shin, C. H. Won, W. K. Chung, B. S. Park. Up-to-date clinical trials of hair regeneration using conditioned media of adipose-derived stem cells in male and female pattern hair loss. *Curr. Stem Cell Res. Ther*. **12**, 524-530 (2017).

54. M. Monguió-Tortajada, S. Roura, C. Gálvez-Montón, J. M. Pujal, G. Aran, L. Sanjurjo, M. Franquesa, M. R. Sarrias, A. Bayes-Genis, F. E. Borràs. Nanosized UC MSC-derived extracellular vesicles but not conditioned medium exclusively inhibit the inflammatory response of stimulated T cells: implications for nanomedicine. *Theranostics* **7**, 270-284 (2017).

55. S. Sahoo, E. Klychko, T. Thorne, S. Misener, K. M. Schultz, M. Millay, A. Ito, T. Liu, C. Kamide, H. Agrawal, H. Perlman, G. Qin, R. Kishore, D. W. Losordo. Exosomes from human CD34(+) stem cells mediate their proangiogenic paracrine activity. *Circ. Res*. **109**, 724-728 (2011).

56. L. Margolis, Y. Sadovsky. The biology of extracellular vesicles: The known unknowns. *PLoS Biol*. **17**, e3000363 (2019).

57. https://clinicaltrials.gov/ct2/results?cond=Bronchopulmonary+Dysplasia&term=mesenchymal+stem+cell&cntry=&state=&city=&dist=

58. T. Shahzad, S. Radajewski, C. M. Chao, S. Bellusci, H. Ehrhardt. Pathogenesis of bronchopulmonary dysplasia: when inflammation meets organ development. *Mol. Cell Pediatr*. **3**, 23 (2016).

59. Y. Lu, J. M. Thomson, H. Y. Wong, S. M. Hammond, B. L. Hogan. Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells. *Dev. Biol*. **310**, 442-453 (2007).

60. M. Ha, M. Pang, V. Agarwal, Z. J. Chen. Interspecies regulation of microRNAs and their targets. *Biochim. Biophys. Acta.* **1779**, 735–742 (2008).

61. Q.-S. Tong, L.-D. Zheng, S.-T. Tang, G.-S. Jiang, Q.-L. Ruan, F.-Q. Zeng, J.-H. Dong. Nitrofen suppresses cell proliferation and promotes mitochondria-mediated apoptosis in type II pneumocytes. *Acta Pharmacol. Sin.* **28**, 672–684 (2007).

62. I. Caniggia, I. Tseu, R. N. Han, B. T. Smith, K. Tanswell, M. Post. Spatial and temporal differences in fibroblast behavior in fetal rat lung. *Am. J. Physiol.* **261**, L424-433 (1991).

63. M. Bilodeau, S. Shojaie, C. Ackerley, M. Post, J. Rossant. Identification of a proximal progenitor population from murine fetal lungs with clonogenic and multilineage differentiation potential. *Stem Cell Reports* **3**, 634-649 (2014).

64. J. L. Emery, A. Mithal. The number of alveoli in the terminal respiratory unit of man during late intrauterine life and childhood. *Arch. Dis. Child.* **35**, 544–547 (1960).

65. X. I. Roubliova, J. M. Biard, L. Ophalvens, D. Gallot, J. C. Jani, E. K. Verbeken, C. P. Van De Ven, D. Tibboel, J. A. Deprest. Morphology of the developing fetal lung — the rabbit experimental model. In: Modern Research and Educational Topics in Microscopy, edited by Méndez-Vilas A Díaz J. Badajoz, Spain: Formatex, 2007, p. 417–425.

66. P. De Coppi, G. Bartsch, Jr., M. M. Siddiqui, T. Xu, C. C. Santos, L. Perin, G. Mostoslavsky, A. C. Serre, E. Y. Snyder, J. J. Yoo, M. E. Furth, S. Soker, A. Atala. Isolation of amniotic stem cell lines with potential for therapy. *Nat. Biotechnol.* **25**, 100-106 (2007).

67. R. A. McCloy, S. Rogers, C. E. Caldon, T. Lorca, A. Castro, A. Burgess. Partial inhibition of Cdk1 in G 2 phase overrides the SAC and decouples mitotic events. *Cell Cycle* **13**, 1400-1412 (2014).

68. G. Y. Xiao, C. C. Cheng, Y. S. Chiang, W. T. Cheng, I. H. Liu, S. C. Wu. Exosomal miR-10a derived from amniotic fluid stem cells preserves ovarian follicles after chemotherapy. *Sci. Rep.* **6**, 23120 (2016).

69. G. Carraro, A. El-Hashash, D. Guidolin, C. Tiozzo, G. Turcatel, B. M. Young, S. P. De Langhe, S. Bellusci, W. Shi, P. P. Parnigotto, D. Warburton. miR-17 family of microRNAs controls FGF10-mediated embryonic lung epithelial branching morphogenesis through MAPK14 and STAT3 regulation of E-Cadherin distribution. *Dev. Biol*. **333**, 238-250 (2009).

70. P. Silveyra, Z. C. Chroneos, S. L. DiAngelo, N. J. Thomas, G. T. Noutsios, N. Tsotakos, J. A. Howrylak, T. M. Umstead, J. Floros. Knockdown of Drosha in human alveolar type II cells alters expression of SP-A in culture: a pilot study. *Exp. Lung Res.* **40**, 354-366 (2014).

71. P. Dravet-Gounot, P. Dravet-Gounot, C. Morin, S. Jacques, F. Dumont, F. Ely-Marius, D. Vaiman, P. H. Jarreau, C. Méhats, E. Zana-Taïeb. Lung microRNA deregulation

associated with impaired alveolarization in rats after intrauterine growth restriction. *PLoS One* **12**, e0190445 (2017).

72. S. Mujahid, T. Logvinenko, M. V. Volpe, H. C. Nielsen. miRNA regulated pathways in late stage murine lung development. *BMC Dev. Biol.* **13**, 13 (2013).

73. V. V. Sherstyuk, S. P. Medvedev, E. A. Elisaphenko, E. A. Vaskova, M. T. Ri, Y. V. Vyatkin, O. V. Saik, D. N. Shtokalo, E. A. Pokushalov, S. M. Zakian. Genome-wide profiling and differential expression of microRNA in rat pluripotent stem cells. *Sci. Rep.* **7**, 2787 (2017).

74. N. Khoshgoo, R. Visser, L. Falk, C. A. Day, D. Ameis, B. M. Iwasiow, F. Zhu, A. Öztürk, S. Basu, M. Pind, A. Fresnosa, M. Jackson, V. K. Siragam, G. Stelmack, G. G. Hicks, A. J. Halayko, R. Keijzer. MicroRNA-200b regulates distal airway development by maintaining epithelial integrity. *Sci. Rep.* **7**, 6382 (2017).

75. N. Khoshgoo, R. Kholdebarin, P. Pereira-Terra, T. H. Mahood, L. Falk, C. A. Day, B. M. Iwasiow, F. Zhu, D. Mulhall, C. Fraser, J. Correia-Pinto, R. Keijzer. Prenatal microRNA miR-200b therapy improves nitrofen-induced pulmonary hypoplasia associated with congenital diaphragmatic hernia. *Ann. Surg.* (2017).

76. M. P. Eastwood, J. Deprest, F. M. Russo, H. Wang, D. Mulhall, B. Iwasiow, T. H. Mahood, R. Keijzer. MicroRNA 200b is upregulated in the lungs of fetal rabbits with surgically induced diaphragmatic hernia. *Prenat. Diagn.* **38**, 645-653 (2018).

77. S. E. Wert, C. R. Dey, P. A. Blair, S. Kimura, J. A. Whitsett. Increased expression of thyroid transcription factor-1 (TTF-1) in respiratory epithelial cells inhibits alveolarization and causes pulmonary inflammation. *Dev. Biol*. **242**, 75–87 (2002).

78. C. Missero, M. T. Pirro, S. Simeone, M. Pischetola, R. Di Lauro. The DNA glycosylase T:G mismatch-specific thymine DNA glycosylase represses thyroid transcription factor-1-activated transcription. *J. Biol. Chem*. **276**, 33569–33575 (2001).

79. K. C. Wade, S. H. Guttentag, L. W. Gonzales, K. L. Maschhoff, J. Gonzales, V. Kolla, S. Singhal, P. L. Ballard. Gene induction during differentiation of human pulmonary type II cells in vitro. *Am. J. Respir. Cell Mol. Biol.* **34**, 727–737 (2006).

80. N. Lopez Jimenez, S. Gerber, V. Popovici, S. Mirza, K. Copren, L. Ta, G. M. Shaw, B. Trueb, A. M. Slavotinek. Examination of FGFRL1 as a candidate gene for diaphragmatic defects at chromosome 4p16.3 shows that Fgfrl1 null mice have reduced expression of Tpm3, sarcomere genes and Lrtm1 in the diaphragm. *Hum. Genet*. **127**, 325–336 (2010).

81. S. Perveen, K. Ayasolla, N. Zagloul, H. Patel, K. Ochani, D. Orner, H. Benveniste, M. Salerno, P. Vaska, Z. Zuo, Y. Alabed, M. Nasim, E. J. Miller, M. Ahmed. MIF inhibition enhances pulmonary angiogenesis and lung development in congenital diaphragmatic hernia. *Pediatr. Res.* **85**, 711–718 (2019).

82. F. Friedmacher, T. Doi, J.-H. Gosemann, N. Fujiwara, B. Kutasy, P. Puri. Upregulation of fibroblast growth factor receptor 2 and 3 in the late stages of fetal lung development in the nitrofen rat model. *Pediatr. Surg. Int.* **28**, 195–199 (2012).

83. M. A. Martorell, M. C. Calabuig, A. Llombart-Bosch, J. Esquerdo. Primary omental tumor with ultrastructural features of cellular schwannoma and absence of S-100 antigen reactivity. *Pathol. Res. Pract.* **185**, 480–487 (1989).

84. H. Glatt, W. Meinl. Use of genetically manipulated Salmonella typhimurium strains to evaluate the role of sulfotransferases and acetyltransferases in nitrofen mutagenicity. *Carcinogenesis.* **25**, 779–786 (2004).

85. J. Wang, J.-Y. Zhou, D. Kho, J. J. J. Reiners, G. S. Wu. Role for DUSP1 (dual-specificity protein phosphatase 1) in the regulation of autophagy. *Autophagy.* **12**, 1791–1803 (2016).

86. B. Yeganeh, J. Lee, L. Ermini, I. Lok, C. Ackerley, M. Post. Autophagy is required for lung development and morphogenesis. *J. Clin. Invest.* **129**, 2904–2919 (2019).

87. I. Tanida, S. Waguri. Measurement of autophagy in cells and tissues. *Methods Mol. Biol.* **648**, 193–214 (2010).

88. W. Hou, Y. Xie, X. Song, X. Sun, M. T. Lotze, H. J. 3rd Zeh, R. Kang, D. Tang. Autophagy promotes ferroptosis by degradation of ferritin. *Autophagy.* **12**, 1425–1428 (2016).

89. K. Kokame, K. L. Agarwala, H. Kato, T. Miyata. Herp, a new ubiquitin-like membrane protein induced by endoplasmic reticulum stress. *J. Biol. Chem.* **275**, 32846–32853 (2000).

90. R. B. van Loenhout, I. Tseu, E. K. Fox, Z. Huang, D. Tibboel, M. Post, R. Keijzer. The pulmonary mesenchymal tissue layer is defective in an in vitro recombinant model of nitrofen-induced lung hypoplasia. *Am. J. Pathol.* **180**, 48–60 (2012).

91. D. R. Dorscheid, K. R. Wojcik, K. Yule, S. R. White. Role of cell surface glycosylation in mediating repair of human airway epithelial cell monolayers. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **281**, L982-92 (2001).

92. Z. Qian, D. Okuhara, M. K. Abe, M. R. Rosner. Molecular cloning and characterization of a mitogen-activated protein kinase-associated intracellular chloride channel. *J. Biol. Chem*. **274**, 1621–1627 (1999).

93. Y.-T. Chang, A. Ringman Uggla, C. Osterholm, P.-K. Tran, A.-C. Eklof, M. Lengquist, U. Hedin, K. Tran-Lundmark, B. Frenckner. Antenatal imatinib treatment reduces pulmonary vascular remodeling in a rat model of congenital diaphragmatic hernia. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **302**, L1159-66 (2012).

94. L. A. Laviolette, J. Mermoud, I. A. Calvo, N. Olson, M. Boukhali, O. K. Steinlein, E. Roider, E. C. Sattler, D. Huang, B. T. Teh, M. Motamedi, W. Haas, O. Iliopoulos. Negative regulation of EGFR signalling by the human folliculin tumour suppressor protein. *Nat. Commun*. **8**, 15866 (2017).

95. M. R. Chinoy, X. Chi, R. E. Cilley. Down-regulation of regulatory proteins for differentiation and proliferation in murine fetal hypoplastic lungs: altered mesenchymal-epithelial interactions. *Pediatr. Pulmonol.* **32**, 129–141 (2001).

96. M. Adams, R. J. Simms, Z. Abdelhamed, H. R. Dawe, K. Szymanska, C. V Logan, G. Wheway, E. Pitt, K. Gull, M. A. Knowles, E. Blair, S. H. Cross, J. A. Sayer, C. A. Johnson. A meckelin-filamin A interaction mediates ciliogenesis. *Hum. Mol. Genet.* **21**, 1272–1286 (2012).

97. P. Spagnolo, A. Bush. Interstitial Lung Disease in Children Younger Than 2 Years. *Pediatrics*. **137**. (2016).

98. H. Cheng, T. Chen, M. Tor, D. Park, Q. Zhou, J. B. Huang, N. Khatib, L. Rong, G. Zhou. A High-Throughput Screening Platform Targeting PDLIM5 for Pulmonary Hypertension. *J. Biomol. Screen.* **21**, 333–341 (2016).

99. X. Huang, L. Li, R. Ammar, Y. Zhang, Y. Wang, K. Ravi, J. Thompson, G. Jarai. Molecular characterization of a precision-cut rat lung slice model for the evaluation of antifibrotic drugs. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **316**, L348–L357 (2019).

100. K. Brown, A. Filuta, M.-G. Ludwig, K. Seuwen, J. Jaros, S. Vidal, K. Arora, A. P. Naren, K. Kandasamy, K. Parthasarathi, S. Offermanns, R. J. Mason, W. E. Miller, J. A. Whitsett, J. P. Bridges. Epithelial Gpr116 regulates pulmonary alveolar homeostasis via Gq/11 signaling. *JCI Insight* **2** (2017).

101. K. C. Liu, R. E. Cheney. Myosins in cell junctions. Bioarchitecture 2, 158–170 (2012).

102. S. Averaimo, R. H. Milton, M. R. Duchen, M. Mazzanti. Chloride intracellular channel 1 (CLIC1): Sensor and effector during oxidative stress. *FEBS Lett.* **584**, 2076–2084 (2010).

103. R. Fernandez-Valdivia, Y. Zhang, S. Pai, M. L. Metzker, A. Schumacher. l7Rn6 encodes a novel protein required for clara cell function in mouse lung development. *Genetics.* **172**, 389–399 (2006).

104. K. E. Driscoll, D. G. Hassenbein, B. W. Howard, R. J. Isfort, D. Cody, M. H. Tindal, M. Suchanek, J. M. Carter. Cloning, expression, and functional characterization of rat MIP-2: a neutrophil chemoattractant and epithelial cell mitogen. *J. Leukoc. Biol.* **58**, 359–364 (1995).

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Data and materials availability: Proteomics differential analysis with rAFSC-EV and rMSC-EV cargos is in Supporting Data File S1. We submitted all EV data to EV-TRACK knowledgebase (#EV190001). RNA-sequencing data for rAFSC-EVs, rMSC-EVs, and primary lung epithelial cells are available on ArrayExpress database (#E-MTAB-8921).

Figure Legends

Fig. 1. Administration of rAFSC-EVs promotes growth, branching morphogenesis, and maturation in fetal hypoplastic lungs. (**A**) Representative light microscopy photos of lung explants harvested at E14.5 (0 h) and grown for 72 h in conditions indicated in columns. Scale bar = 750 µm. (**B** and **C**) quantification of total terminal bud count and total lung surface area measured at 72 h. ****P<0.0001, **P<0.01, ns= P>0.05. Data are quantified blindly by two investigators for the following number of biological replicates: Control (n=16), Nitrofen (n=17), Nitrofen+rAFSC-CM (n=12), Nitrofen+rAFSC-EVs (n=12), Nitrofen+EV-depleted rAFSC-CM (n=5), Nitrofen+rMSC-EVs (n=8). (**D**) Gene expression changes in lung maturation markers fibroblast growth factor 10 (*Fgf10*) and vascular endothelial growth factor (*Vegfa*), and its receptors (*Flt1* and *Kdr*) in lung explants measured at 72 h. *P<0.05, ***P<0.001. Expression values of at least n=9 technical replicates are shown (**E** to **G**) Immunofluorescence co-stain experiment of proliferating cells and distal lung epithelium progenitor cells of lung explants (SOX9, green; EdU, pink, DAPI nuclear stain, blue; scale bar = 100 µm), quantified through number of EdU^+ cell per DAPI and SOX9 fluorescence intensity (AU = arbitrary units). N=4 biological replicates were used with a total of 50x50 µm fields covering entire lung sections as indicated: Control (n=157), Nitrofen (n=222), Nitrofen+rAFSC-EVs (n=128). (**H** and **I**) Western blot analysis of SOX9 and SPC expression in lung explants grown for 72 h, quantified by signal intensity normalized to GAPDH in at least n=3 biological replicates from each condition. (**J** and **K**) Immunofluorescence experiment of surfactant protein C (SPC) expressing cells in lung explants (SPC, green; DAPI nuclear stain, blue; scale bar =100 µm), quantified by fluorescence intensity: Control (n=6), Nitrofen (n=4), Nitrofen+rAFSC-EVs (n=4). (**L**) Quantification of SPC protein expression by signal intensity normalized to GAPDH in n=3 biological replicates from

each condition. (**M** and **N**) TUNEL immunofluorescence experiments on lung explants grown for 72 h, quantified by $TUNEL^+$ cells per DAPI in n=4 biological replicates with a total of 50x50 µm fields covering entire lung sections as indicated: Control (n=311), Nitrofen (n=240), Nitrofen+rAFSC-EVs (n=107). Groups were compared using Kruskal-Wallis (post-hoc Dunn's nonparametric comparison) test for Fig. 1 B, C, F, G, I, K, L, N, and with one-way ANOVA (Tukey post-test) for Fig. 1 D, according to Gaussian distribution assessed by D'Agostino Pearson omnibus normality test.

Fig. 2. rAFSC-EVs maintain homeostasis and stimulate differentiation of the epithelium from hypoplastic fetal lungs. (**A**) Proliferation rate of primary lung epithelial cells from control and nitrofen-injured hypoplastic lungs treated with rAFSC-CM, rAFSC-EVs, EV-depleted rAFSC-CM [5'EdU labeling, Control (n=7), Nitrofen (n=5), Nitrofen+rAFSC-CM (n=3), Nitrofen+rAFSC-EVs (n=5), Nitrofen+EV-depleted rAFSC-CM (n=3)]. ****P<0.0001, ns= P>0.05. (**B**) Cell death rate of primary lung epithelial cells from control and nitrofen-injured hypoplastic lungs treated as in (**A**) [live/dead cytotoxicity assay, Control n=5, Nitrofen (n=5), Nitrofen+rAFSC-CM (n=6), Nitrofen+rAFSC-EVs (n=5), Nitrofen+EV-depleted rAFSC-CM (n=4)]. *P<0.05, ***P<0.001. (**C**) Light microscopy photos of fetal rat lung organoids derived from control lungs and nitrofen-injured hypoplastic lungs either treated with medium alone (Nitrofen) or medium supplemented with rAFSC-EVs (Nitrofen+rAFSC-EVs). Scale bar = 100 µm. Representative photo of Control (n=108), Nitrofen (n=63), Nitrofen+rAFSC-EVs (n=94). (**D**) Proliferation of cells in organoids evaluated with immunofluorescence (Ki67 staining, green; scale bar= 50 µm) and quantified as percentage of $Ki67^+$ cells per total number of DAPI (blue) stained nuclei in Control (n=8), Nitrofen (n=7), Nitrofen+rAFSC-EVs (n=9). (**E**) SPC staining in organoids (green; DAPI nuclear stain, blue; scale bar = 50 µm) quantified with fluorescence intensity calculated from total corrected cellular fluorescence from Control (n=30), Nitrofen (n=31), Nitrofen+rAFSC-EVs (n=25) (AU= arbitrary units). (**F**) $CC10^+$ cells in organoids (green; DAPI nuclear stain, blue; scale bar = 50 µm) quantified with fluorescence intensity calculated from total corrected cellular fluorescence Control (n=30), Nitrofen (n=30), Nitrofen+rAFSC-EVs (n=30). Groups were compared using Kruskal-Wallis (post-hoc Dunn's nonparametric comparison) test for Fig. 2 A, B, D, E, F according to Gaussian distribution assessed by D'Agostino Pearson omnibus normality test.

Fig. 3. The role of RNA cargo released by rAFSC-EVs. (**A** and **B**) Fluorescently labeled rAFSC-EV protein (green) and RNA (red) cargo entered primary lung epithelial cells (DAPI nuclear stain, blue; scale bar = 100 μm). Cells were outlined based on light microscopy images to highlight the border. To confirm this, cells were washed twice with PBS and fixed in 4% PFA and re-imaged after live cell imaging (shown in **Movie S2 and Movie S3**). (**C**) Bioanalyzer traces of RNase pre-treated rAFSC-EVs. (**D** to **I)** Representative photos of gold immunolabeling experiments of RNase-treated rAFSC-EVs in far field (**D**) and near field (**E** to **H**) or rAFSC-EVs in near field (**I**), using transmission electron microscopy, with TSG101 (10 nm), and RNase (25 nm), scale bar = 100 nm. Controls include single stains (**F** and **G**), secondary only antibodies (**H**), and co-stains in untreated rAFSC-EVs (**I**). Photos are representative of four biological replicates of rAFSC-EV-RNase and rAFSC-EVs (**J** to **M**) Effects of RNase pre-treated rAFSC-EVs on lung growth parameters (bud count, (**J**); and surface area, (**K**), from Control (n=16), Nitrofen (n=17), Nitrofen+rAFSC-EVs (n=12), and Nitrofen+rAFSC-EV-RNase (n=4), and on pulmonary epithelial cells (proliferation, (**L**); and cell death rate (**M**) from Control (n=7), Nitrofen (n=5), Nitrofen+rAFSC-EVs (n=5), and Nitrofen+rAFSC-EV-RNase (n=4), ****P<0.0001, ***P<0.001, **P<0.01, ns= P>0.05. (**N**) Small RNA-sequencing analysis of rAFSC-EVs and rMSC-EVs separated by type of RNA species. Solid bar represents proportion of significantly different species per type of RNA. (**O**) Heat map of miRNAs detected in rAFSC-EVs (n=3) and rMSC-EVs (n=2), ranked based on fold change and significant difference between the two populations. The two inlets report the miRNAs involved in lung development (see table S2). Right inlet: miRNAs significantly enriched in rAFSC-EVs detected within the top 50 miRNAs. Left inlet: miRNAs equally abundant in rAFSC-EVs and rMSC-EVs detected within the top 50 miRNAs. Groups were compared using Kruskal-Wallis (post-hoc Dunn's

nonparametric comparison) test for Fig. 3 J, K, L, M according to Gaussian distribution assessed by D’Agostino Pearson omnibus normality test.

Fig. 4. Effects of rAFSC-EVs exerted on primary lung epithelial cells. (**A**) Heat map of mRNA expression of genes from lung epithelial cells of nitrofen-injured lungs treated with rAFSC-EVs from n=6 biological replicates each, FDR < 0.1. Color represents row-scaled, normalized read counts (RPKM). (**B**) GSEA enrichment plot of epithelial cell differentiation generated with genes ranked by fold change between NA vs. N. "Leading edge" genes are shown on the side. (**C**) Interaction network of rAFSC-EV miRNAs and down regulated genes in nitrofen-injured lung epithelial cells treated with rAFSC-EVs. Size of node represents number of connections. Blue nodes represent genes down-regulated (FDR < 0.1) in nitrofen-injured lung epithelial cells treated with rAFSC-EVs compared to nitrofen-injured lung epithelial cells. White nodes represent miRNAs that were detected in the rAFSC-EV cargo. Green nodes represent miRNAs that were detected in rAFSC-EV cargo that had higher expression in rAFSC-EV-treated nitrofen-injured lung epithelial cells compared to untreated nitrofen-injured epithelial cells. Triangles represent miRNAs from the miR17~92 family and paralogues. Each miRNA-gene target pair is connected by a gray edge and pairs containing a miRNA from miR17~92 family or its paralogues are connected by a blue edge.

Fig. 5. Towards the clinical translation of AFSC-EVs as treatment for fetal lung regeneration. (**A**) Schematic of experimental groups from the rabbit model of CDH. (**B**) Representative histology images (hematoxylin/eosin) of fetal lungs from control rabbits and from rabbits that underwent surgical CDH creation and were either untreated (CDH), or subjected to tracheal occlusion (CDH+TO), or were administered rAFSC-EVs prior to tracheal occlusion (CDH+TO+rAFSC-EVs). Each condition included fetal lungs from n=9 experiments. Scale bar = 500 μm. (**C** and **D**) Differences in number of alveoli (radial alveolar count) were quantified in at least 12 counts per fetal lung in 2 different sections of the lung, and thickness of the alveolar wall (mean wall transection length) was measured in 10 different areas of each lung. **P<0.01, *P<0.05. (**E** and **F**) Gene expression changes in alveolar lipofibroblasts (PLIN2), BMP (bone morphogenetic protein) signaling (BMP2, BMP4, Id1) in n=9 biological replicates of each condition. ***P<0.001 (**G** and **J**) Effects of good manufacturing practice-grade hAFSC-EVs on proliferation rate and viability rate on nitrofen-injured human A549 cells (**G** and **H**), and human pulmonary alveolar epithelial cells (**I** and **J**) (n>30 technical replicates). (**K** and **L**) Uptake of hAFSC-EVs fluorescently labeled with PKH26 by A549 cells or human pulmonary alveolar epithelial cells. Scale bar = 25 μm. Groups were compared using Kruskal-Wallis (post-hoc Dunn's nonparametric comparison) test for Fig. 5 C, D, E, F, H, I, and J, and with one-way ANOVA (Tukey post-test) for Fig. 5 G according to Gaussian distribution assessed by D'Agostino Pearson omnibus normality test.