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Fibrillin-2 and Tenascin-C Bridge the Age Gap in Lung Epithelial Regeneration

Sarah E. Gilpin¹, Qiyao Li², Daniele Evangelista-Leite¹, Xi Ren¹, Dieter P. Reinhardt³, Brian L. Frey², and Harald C. Ott¹

¹Center for Regenerative Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA

²Department of Chemistry, University of Wisconsin-Madison, Madison, WI

³Department of Anatomy and Cell Biology, and Faculty of Dentistry, McGill University, Montreal, QC

Abstract

Organ engineering based on native matrix scaffolds involves combining regenerative cell populations with corresponding biological matrices to form functional grafts on-demand. The extracellular matrix (ECM) that is retained following lung decellularization provides essential structure and biophysical cues for whole organ regeneration after recellularization. The unique ECM composition in the early post-natal lung, during active alveologenesis, may possess distinct signals that aid in driving cell adhesion, survival, and proliferation.

We evaluated the behavior of basal epithelial stem cells (BESCs) isolated from adult human lung tissue, when cultured on acellular ECM derived from neonatal (aged < 1 week) or adult lung donors (n=3 donors per group). A significant difference in cell proliferation and survival was found. We next performed in-depth proteomic analysis of the lung scaffolds to quantify proteins significantly enriched in the neonatal ECM, and identified the glycoproteins Fibrillin-2 (FBN-2) and Tenascin-C (TN-C) as potential mediators of the observed effect. BESCs cultured on Collagen Type IV coated plates, supplemented with FBN-2 and TN-C demonstrated significantly increased proliferation and decreased cellular senescence. No significant increase in epithelial-to-mesenchymal transition was observed. *In vitro* migration was also increased by FBN-2 and TN-C treatment. Decellularized lung scaffolds treated with FBN-2 and TN-C prior to re-epithelialization supported greater epithelial proliferation and tissue remodeling. BESC distribution, matrix alignment, and overall tissue morphology was improved on treated lung scaffolds, after 3 and 7 days of *ex vivo* lung culture. These results demonstrate that scaffold re-epithelialization is enhanced on neonatal lung ECM, and that supplementation of FBN-2 and TN-C to the native scaffold may be a valuable tool in lung tissue regeneration.

Introduction

The goal of regenerating whole organs for transplantation, as an alternative to cadaveric organ donation, presents a promising therapeutic option for many end-stage diseases. One exciting approach to this aim involves combining biologically suitable scaffolds with new, multipotent cell populations that can repopulate the native organ matrix. To this end, several methods have been developed to decellularize organs and tissues, leaving the extracellular matrix (ECM) intact for subsequent regeneration. We have previously described and validated the methods for perfusion decellularization of whole lungs from rodent, porcine, and human sources (1, 2). The decellularization process aims to retain the essential ECM components to support recellularization, while maximizing the removal of immunogenic cellular material (3). We have previously reported that the acellular scaffold retains many collagens, laminin, fibronectin, and other matrix proteins after decellularization, while some soluble collagens and glycosaminoglycans are lost during the procedure (1).

The optimal scaffold for lung organ engineering would not only provide the necessary structure, but would additionally guide the organization and function of new lung tissue. The ECM is a complex entity that participates in many biological processes, including tissue development and repair (4). When considering the ECM in whole organ regeneration, the source of native lung tissue used to prepare the scaffold can have a direct impact on subsequent regeneration. Several studies have shown that underlying lung pathologies can cause changes in the ECM that are retained following decellularization, and can perpetuate during tissue repair (5). This has been demonstrated for both pulmonary fibrosis and emphysema (6, 7). Age of the lung can also contribute important differences to the decellularized scaffold. It has been shown that growth on aged ECM leads to significantly lower cellular expression of laminin $\alpha 3$ and $\alpha 4$ chains, which recapitulates the laminin deficiency that is observed in aged lung ECM. These data further highlight the deep biological information that is contained in the lung scaffold, and the feedback loops that exists between reparative cell populations and the underlying protein matrix (8).

Lung development actively continues following birth, and ECM remodeling is an essential aspect of the post-natal process of alveolarization. This mechanism functions to dramatically increase the gas exchange surface area, as the lung further refines the immature alveolar structure and undertakes secondary septation to generate a greater number of smaller sized alveoli (9). The consequences of this process and the specific differences in ECM composition have not been well studied in the context of *ex vivo* tissue regeneration. Fetal wounds repair at a faster rate than adults, with little or no scarring (10). Regrowth of lung is possible after lobectomy in infancy, with restoration of airway function and total recovery of lung volume (11). Conversely, dysregulation of the ECM is an important driving factor for ageing, and age-related alterations in the ECM can be directly communicated to the surrounding cells, contributing to the development of chronic lung diseases such as emphysema and pulmonary fibrosis (12). Another consequence of aging is the phenomenon of stem cell dysfunction and exhaustion, where the multipotent pool of progenitors progressively declines and becomes increasing senescent (13). These interactions between the stem cell and the niche, including ECM, can contribute to this decrease in regenerative capacity.

We have previously reported the isolation and expansion of human basal epithelial stem cells (BESCs) from lung tissue, and investigated their capacity in whole lung epithelial regeneration (14). In normal tissue, the basal epithelial cell lies proximal to the basal lamina, and functions to aid attachment of the epithelium to the basement membrane. Basal cells can be identified by expression of the transcription factor p63, cytokeratins 5 and 14, and the epidermal growth factor receptor (EGFR), in both mice and humans (15). Basal cells additionally possess the ability to act as an endogenous adult stem cell population, capable of self-renewal and multi-lineage differentiation (16). Recent evidence also suggests that during tissue repair, the Krt5⁺ BESC population can also contribute to distal alveolar regeneration (17, 18). This capacity makes BESCs an exciting and promising cell source for whole lung epithelial tissue engineering and regeneration.

In this study, we aimed to investigate the differences in ECM from neonatal lungs actively undergoing alveolarization, compared to adult lung donors, and evaluate the consequences of these differences on *ex vivo* lung epithelial repair. We found an increase in developmentally associated proteins Fibrillin-2 (FBN-2) and Tenascin-C (TN-C) in the neonatal human lung ECM, and report that supplementation of these two proteins both *in vitro* and in *ex vivo* lung regeneration on acellular lung scaffolds enhances epithelial proliferation, decreases senescence, aids cell attachment and migration, and ultimately improves regenerated tissue morphology and structure. We believe that these results are the first report of reconditioning the acellular lung scaffold in order to recapitulate the neonatal ECM, to better support *ex vivo* lung tissue repair and regeneration.

Methods

Study Approval

Human donor lungs otherwise unsuitable for transplantation were obtained from the New England Organ Bank under sterile conditions within 60 minutes of cessation of cardiovascular circulation (see Table 1), following informed consent. Donor criteria included age < 75 years, negative serologies, non-smoker, and no known lung disease, pneumonia, aspiration, or trauma. All neonatal donors were supported by mechanical ventilation after birth.

Experiments were approved by the Massachusetts General Hospital Internal Review Board (#2011P002433) and Animal Utilization Protocol (#2014N000261). Donor demographics are listed in Table 1.

Cell Isolation and Expansion

Epithelial cells were isolated from adult donor lung peripheral tissue as previously described (14), and maintained *in vitro* on human Collagen IV (Sigma-Aldrich, C7521)-coated flasks in Small Airway Growth Media (SAGM, Lonza, CC-3118) until used for experiments at passage 3. These cells maintain their basal phenotype, as well as proliferation and differentiation capacity at air-liquid-interface (14).

Lung Decellularization

Rat and human donor lungs were decellularized as previously described (1, 2). Briefly, cadaveric rat lungs were explanted from male Sprague-Dawley rats (250–300g, >8 weeks of age, Charles River Laboratories) and decellularized by perfusion of 0.1% SDS solution through the pulmonary artery at 40mmHg, followed by washing. Human lung decellularization was performed by perfusion of 0.5% SDS solution through the pulmonary artery at a constant pressure between 30 mmHg and 60 mmHg.

Lung ECM Digestion for *In Vitro* Coating and Culture

Tissue samples from the periphery of the decellularized lungs (approximately 0.5 inch squared, neonatal, n=3 and adult, n=3), excluding the main airways and vessels, were lyophilized and mechanically homogenized in pepsin buffer (1mg of pepsin per mL of 0.1 M sterile HCl) at 10mg/mL for 20h at room temperature, using a sterile M tube (MACS Miltenyl Biotech). Subsequently, the pepsin digested tissue was diluted in 0.1M acetic acid to a final concentration of 0.1mg/mL, and used to coat cell culture plates for 1h at 37°C. The coating was added to tissue culture plates and centrifuged at 300xg for 5 min. A total of 1×10^6 BECs (identified by p63 and Krt5 expression) were added to each well of a 24-well plate, and cultured for 7 d in SAGM. BECs isolated from the same, adult donor were used.

Cytotoxicity assay was performed in a 96-well plate, coated with ECM as described above, with a total of 1×10^5 BECs added to each well. After 5 days of culture, MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega) was performed per manufacturer's instructions, and live-cell fluorescence read at 400Ex/505Em; dead-cell fluorescence measured at 485Ex/520Em.

Proteomic sample preparation

Decellularized neonatal and adult lung tissues were prepared for proteomic analyses as previously described (19, 20). Approximately 90 mg of each tissue was minced on ice, and ground with disposable pellet pestles for 1 min in 1.5-mL tubes, followed by addition of 300 μ L SDT solution—4% SDS, 0.1 M Tris-HCl (pH 7.6) and 0.1 M dithiothreitol (DTT) (all reagents from Sigma-Aldrich, St. Louis, MO). Samples were then heated at 95 °C for 7 min and sonicated on ice with a probe sonicator (Misonix XL2015, Misonix microtip PN/418, Farmingdale, NY)—alternating 20 seconds on and 20 seconds off for 6 min, followed by centrifugation at 22 °C for 5 min at 16,100xg. Aliquots (2x30 μ L) of the sample supernatant were mixed with 2x200 μ L of 8M urea/0.1 M Tris buffer (pH 8.0) in a 30K MW Vivacon 500 filter (Sartorius, Bohemia, NY). The sample was washed, alkylated with iodoacetamide, washed further, then digested with trypsin (Promega, Madison, WI; protein:enzyme ratio of 50:1 (w/w)) overnight at 37 °C, and the digested peptides were collected by centrifugation. Digestion was then quenched with 10% trifluoroacetic acid (TFA) to a final concentration of 0.5% TFA.

The quenched digests were subjected to high pH fractionation on an HPLC system (Shimadzu, Columbia, MD) using a Kinetex® C18 column (5 μ m, 100 Å, 250x4.6 mm, Phenomenex, Torrance, CA). Mobile phase A was aqueous 20 mM ammonium formate and mobile phase B was 20 mM ammonium formate in 70% acetonitrile (ACN); the gradient of

0–100% mobile phase B occurred over 20 min. The HPLC flow rate was 1 mL/min and the eluent was collected and combined into 6 fractions, each of which was evaporated to dryness in a SpeedVac and reconstituted in 5% ACN, 2% formic acid (FA).

Proteomic Analysis with Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Reconstituted peptide solution was injected into a Waters nanoAcquity HPLC coupled to an ESI ion-trap/Orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Scientific, Waltham, MA). Peptides were separated on a 100 μ m inner diameter column packed with 20 cm of 1.7 μ m BEH C18 particles (Waters, Milford, MA), and eluted at 0.3 μ L/min in 0.1% FA with a gradient of increasing ACN over 2.5 h. A heater cartridge was used to keep the capillary column at 60 °C. A full-mass scan (300–1500 m/z) was performed in the Orbitrap at a resolution of 60,000. The ten most intense peaks were selected for fragmentation by higher-energy collisional dissociation (HCD) at 42% collision energy, then analyzed with a resolution of 7,500 and an isolation width of 2.5 m/z . Dynamic exclusion was enabled with a repeat count of 1 over 30 s and an exclusion duration of 120 s.

Proteomic Data Analysis

The acquired raw files were analyzed by MaxQuant version 1.5.2.8 (21). The UniProt database used contained 20,278 reviewed sequences from Homo sapiens downloaded on December 5, 2013, supplemented with 262 common contaminants. Precursor and fragment ion mass tolerances were set to 4.5 ppm and 20 ppm, respectively. Static cysteine carbamidomethylation (+57.0215 Da) and up to 7 variable methionine and proline oxidations (+15.9949 Da) were specified. A false discovery rate of 1% at both the peptide and the protein level was allowed. Up to two missed cleavages were allowed and a minimum of two unique peptides per protein was required. Protein groups containing matches to proteins from the reversed database or contaminants were discarded. Only unique and razor peptides were used for quantification and a minimum count of two was required. Relative abundances of proteins within each sample were measured by intensity-based absolute quantification (iBAQ), and the label-free quantification (LFQ) algorithm embedded in the MaxQuant software package was employed for comparing the abundances of proteins between different samples. Perseus software (version 1.5.0.15) was used for downstream data processing. Proteins were filtered by requiring at least two valid values in at least one sample group (neonatal or adult). The corrected intensities were log₂ transformed and missing values were replaced using data imputation by employing a width of 0.3 and a downshift of 0.9. Two-sample t-tests with Benjamini-Hochberg correction were performed to statistically compare the LFQ values of individual proteins in the neonatal and adult tissues.

In Vitro Culture and Migration Assay

24-well plates were pre-coated with human Collagen IV (10 μ g/ml) Sigma-Aldrich C7521) for 2 h at 37°C. After removal of the collagen solution, TN-C (10 μ g/ml, R&D 3358-TC-050) or the recombinant N-terminal half (FBN-2-N) or C-terminal half (FBN-2-C) of human FBN-2 (10 μ g/ml) (22) were then added to select wells, and incubated for 2 h at 37°C. A total of 1 $\times$ 10⁵ BSCs from a single adult donor were subsequently plated to each well and cultured for 7 d in SAGM.

For migration assay, after coating as above, a small inset (IBIDI) was added to the wells prior to cell seeding. For migration assay the N-terminal and C-terminal halves of FBN-2 were combined for coating. A total of 1×10^4 cells were seeded within the insert and incubated for 12 h, before the insert was removed. Bright-field images were taken every 30 min for 180 min to track cell migration. Images were analyzed with ImageJ software (23) to quantify the change in cell-free area.

Ex Vivo Rat Lung Recellularization and Culture

Decellularized lung scaffolds were pre-coated with (A) PBS control, (B) TN-C, 10 μ g/ml, (C) FBN-2, (10 μ g/ml each of N- and C-terminal fragment of FBN-2), or (D) TN-C+FBN-2 (10 μ g/ml each of TN-C and of N- and C-terminal fragment of FBN-2), by delivery through the trachea (10ml volume). Solution was recycled to the trachea for 90 min at 37°C. A total of 20×10^6 BECs from the same adult donor (passage 3) were then delivered to the scaffold airways in 20ml of SAGM by gravity. Constant media perfusion of SAGM through the pulmonary artery was maintained at 4ml/min (pressure 15–20mmHg) and changed daily. Recellularized lungs were maintained in culture for 7 days, with the right lung removed on Day 3 for time point analysis.

Quantitative PCR

mRNA was isolated (Qiagen RNeasy Plus Kit) and transcribed to cDNA (Invitrogen SuperScript III). Gene expression was analyzed using Taqman probes and the OneStep Plus system (Applied Biosystems). Each biological sample was analyzed in experimental replicate (n=2 repeated wells of the qPCR reaction) and the Ct value of each replicate was averaged and handed as n=1 unique biologic sample. Expression for each sample was normalized to β -Actin (ACTA1) gene expression (Ct) and relative to peripheral lung tissue control samples (Ct), and fold change calculated by 2^{-Ct} (24). A total of n=3 unique biological samples were analyzed for each experiment.

Immunostaining

After de-paraffinization and rehydration, 5 μ m tissue sections were permeabilized with 0.1% Triton X-100 for intracellular antigens, when appropriate. Cells in culture were fixed with ice-cold methanol prior to staining. All samples were blocked with 1% donkey serum for 1h. Primary antibodies all 1:100 diluted: p63 (Biocare Medica, CM163A), Krt5 (Abcam, ab24647), E-cadherin (BD Biosciences, 610181), Ki67 (Abcam, ab16667), Fibrillin 2 (Abcam, ab128026), Tenascin C (Abcam, ab108930). Secondary antibodies all 1:400 diluted: Donkey anti-Mouse, Rabbit, or Goat, conjugated to Alexa Fluor 488 or 594 (Life Technologies). Samples were stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nucleus and imaged using a Nikon Ti-Eclipse microscope. Image analysis was performed using ImageJ software (NIH). Septal thickness and linear intercepts were measured on n=3 unique sections, with n=5 areas measured per section (see Supplemental Figure 3).

Statistical Analysis

For all experiments, the n value stated represent an independent biological sample. Data were analyzed by 1-way or 2-way ANOVA, as appropriate, using GraphPad Software. All statistical significance is reported accordingly. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Results

Extracellular matrix protein scaffolds were generated from donated human lungs deemed otherwise unsuitable for clinical transplantation (previously described, (1, 2)). The lungs were first decellularized by constant-pressure vascular perfusion of 0.5% sodium dodecyl sulfate (SDS) solution (see Supplemental Figure 1), followed by extensive washing to remove residual detergent and cellular components. A total of n=3 neonatal (less than 1 week of life) lung scaffolds and n=3 adult lung scaffolds were prepared in this manner for subsequent analyses (See Table 1).

We first aimed to evaluate the response of primary donor tissue-derived basal epithelial stem cells (BESCs) when cultured on ECM derived from neonatal versus adult lungs. To this end, acellular lung ECM from each neonatal and adult donor was prepared as a coating for *in vitro* epithelial cell culture (Figure 1A). After culture of BESCs on each substrate for 7 d, it was found that cells on neonatal ECM were significantly more proliferative (Ki67 and PCNA expression), and less senescent (CDKN2A expression) compared to cells grown on adult lung ECM (Figure 1B). No significant differences in epithelial phenotype were found (E-Cadherin, p63 expression), and no increase in expression of the mesenchymal marker smooth muscle actin (SMA) was observed. By total cell assessment, significantly more live cells engrafted on neonatal ECM coating than on adult lung ECM, by 7 d of culture, while no difference in the number of dead cells was found (Figure 1C).

To then investigate the difference in protein composition that may be mediating this effect, we evaluated acellular lung scaffolds from neonatal versus adult donor lungs by proteomic analysis with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The heat map in Figure 2A shows the change in abundance of each protein, from each biological sample. In both groups, many low-abundance proteins were measured (green), in addition to a smaller number of high-abundance proteins (red). Further analysis of the subcategories of the matrisome (Fig. 2B) showed that neonatal lung scaffolds contained a larger number of collagens, while the other subcategories (glycoproteins, proteoglycans, ECM regulators, etc.) are more abundant in the adult scaffolds.

The measured abundance of each individual matrix protein was then compared between neonatal and adult scaffolds. A volcano plot was generated, showing fold change in protein abundance (neonatal versus adult) plotted against statistical p-values. Selected proteins that are enriched in neonatal or adult scaffolds are highlighted in Figure 3A and listed with details in Figure 3B. A full list of all matrisome proteins is presented in Supplemental Table 1.

Fibrillin-2 and Fibrillin-3 were found to be enriched in the neonatal scaffold, relative to the adult samples (200-fold and 60-fold change, $p=2.8 \times 10^{-2}$ and $p=3.5 \times 10^{-2}$, respectively).

Fibrillins are glycoproteins that are essential for the deposition of elastin and the formation of elastic fibers, which supports alveolar development and structure (25). Specifically, the expression pattern of FBN-2 is largely restricted to developing fetal tissues (26). In addition, FBN-2 has been shown to interact with TN-C, both in development and in tissue repair (27). TN-C is also found in the developing and post-natal lung ECM and has been shown to aid the process of branching morphogenesis (28). A 2.64-fold increase in TN-C protein was measured in the neonatal lung ECM. Enrichment of both FBN-2 and TN-C in the neonatal lung scaffolds prompted us to further analyze their role as potential mediators of the enhanced epithelial repair response found for the neonatal lung ECM coating. The difference in abundance of both TN-C and FBN-2 was confirmed by staining of both adult and neonatal decellularized lung tissue, in comparisons to Elastin, which was not significantly different (Figure 3C).

We tested whether these individual proteins could recapitulate the beneficial effects of neonatal ECM on BESC *in vitro*. B ESCs were cultured on plates first coated with Collagen IV, and then supplemented with TN-C and/or FBN-2, and compared this to culture on uncoated wells (Figure 4A). The recombinant FBN-2 was produced as individual N- and C-terminal peptides (22), which were first evaluated separately and in combination with TN-C. As observed when B ESCs were cultured on isolated neonatal ECM coating, we found significantly greater proliferation (Ki67 and PCNA gene expression) and less senescence (CDKN2A gene expression) by B ESCs grown on FBN-2 and TN-C coated plates, with the most significant response measured on TN-C+FBN-2-C-terminal half coating. No differences in epithelial phenotype was found, when compared to uncoated or Collagen IV coated wells. No significant change in gene expression was detected for TN-C, FBN-2, or Vimentin expression in response to the different protein coatings (Figure 4A). Also, no evidence of epithelial-to-mesenchymal transition (EMT) was identified on the different coatings, as assessed by smooth muscle actin (SMA) expression and the transcription factors SNAIL and ZEB (Figure 4A). Immunofluorescent staining of B ESCs grown on the different coatings confirmed the findings of the gene expression analysis, and when Ki67 expression was quantified, a significant difference was found on FBN-2 and TN-C coated plates (Figure 4B–C). B ESC migration was also investigated on the various protein coatings, by quantifying the cell migration assay. As no significant difference was found between the N- and C- terminal halves on FBN-2, a mixture of both halves was used for the migration assay. Significantly higher rates of B ESC migration were measured on FBN-2 and TN-C coated plates over 3 h, when compared to Collagen IV coating alone ($p < 0.001$, Figure 4D–E). In addition, gene expression of Focal Adhesion Kinase (FAK), an additional indicator of cell migration (29), was measured on the various coatings, with a significantly higher expression level in B ESC grown on TN-C+FBN-2 C-terminal fragment coated plates (Figure 4F). This was further confirmed by immunofluorescent staining for phosphorylated FAK, at Y397, which also demonstrated an increase in phospho-FAK in B ESCs grown on TN-C and FBN-2 coated lung scaffolds (Figure 4G).

To ultimately assess these findings in the context of whole lung epithelial tissue regeneration, we evaluated the effect of FBN-2 (mixed N and C-terminal fragment) and TN-C pre-treatment of the acellular lung scaffold prior to epithelial recellularization (See supplemental Figure 2). After pre-coating and B ESC re-epithelialization, the lungs were

maintained in *ex vivo* biomimetic culture for 7 d, with the right lung removed on Day 3 for time-point analysis.

Tissue analysis again identified significantly more epithelial proliferation, on both Day 3 and 7 of regeneration, with scaffold pre-treatment (Figure 5A). The increase in cellular senescence on Day 7 of culture was significantly reduced by FBN-2+TN-C scaffold coating. An increase in E-Cadherin expression was measured on Day 3 following FBN-2+TN-C treatment, but otherwise epithelial fate was unchanged by scaffold coating. Expression of neither FBN-2 or TN-C were upregulated by the treatment. No increase in mesenchymal phenotype or EMT-associated transcription factor expression was noted. Gross morphologic analysis of the re-epithelialized lung tissue by hematoxylin and eosin staining revealed improved tissue structure, cell alignment to the matrix, and less cell hypertrophy in the coated lungs, when compared to untreated (Figure 5B). These observations were most apparent at Day 7 of culture.

Immunofluorescent staining was performed to assess epithelial fate and proliferation. Quantification of Ki67 expression of Krt5+ BESCs confirmed a significant increase in proliferation when lung scaffolds were pre-coated with FBN-2 and TN-C, with the greatest cellular response found when both proteins were combined for scaffold coating (Figure 5C–D).

Quantification of tissue morphology by measurement of septal thickness, also confirmed the observation that pre-coating of the scaffolds resulted in more cell alignment and less septal thickening in the regenerated lung tissue (Figure 5E, and Supplemental Figure 3). This resulted in an alveolar structure with an appearance more similar to native lung tissue, in both size and structure. Linear intercept was also manually calculated as a measurement of airspace characteristic and alveolar structural units, which was also greater in treated lung tissue (Figure 5F). Distribution of elastin in the lung scaffold, both after treatment and following re-epithelialization demonstrated no significant differences (Supplemental Figure 4).

Together, these results demonstrate that the treatment of acellular lung matrices with FBN-2 and TN-C proteins enhances basal epithelial stem cell migration and proliferation, and also aids *ex vivo* lung tissue repair.

Discussion

The ECM is an essential determinant of cell survival, differentiation, and repair. In this study, we aimed to identify compositional differences between adult and neonatal lung ECM, and its significance, during epithelialization. We identified two key proteins FBN-2 and TN-C, that actively participate in lung development and repair, which are expressed at higher levels in human neonatal lung scaffolds. We further investigated whether these ECM proteins have the potential to actively influence basal epithelial stem cell behavior and lung tissue repair.

Elastogenesis plays a significant role in the development and maintenance of normal lung structure and function. Fibroblasts secrete fibrillins into the ECM, where it becomes

incorporated into insoluble microfibrils, providing the scaffold for elastin deposition. Interfering with normal elastin fiber formation has been shown to result in abnormal alveolar architecture (30). Treatment of developing lung explants with antisense FBN-2 oligodeoxynucleotide can interfere with branching morphogenesis, resulting in smaller lung bud branches, collapsed conducting airways, and loose expanded mesenchyme (31). Pathologically, the destruction of functional elastic fibers in the lung is responsible for development of emphysema (32). The replacement of FBN-2 in acellular adult lung scaffolds may aid reparative elastin deposition and facilitate epithelial remodeling during *ex vivo* lung regeneration. The addition of mesenchymal support cells would further aid the regeneration process. Fibrillins are also involved in the storage and regulation of growth factors such as TGF- β and BMPs (33), which may support cellular survival and proliferation during epithelial repair. Fibrillins further function in cell attachment to the ECM, which is an essential component of scaffold recellularization (34). This is achieved primarily through interactions with integrins $\alpha 5 \beta 1$ and $\alpha v \beta 3$ and heparin sulfate (35, 36). In our studies, we first investigated the effect of the individual C-terminal and N-terminal portions of the FBN-2 protein. While the C-terminal portion resulted in slightly higher proliferation and less senescence than the N-terminal portion, when combined with TN-C, these differences were not statistically significant (Figure 4). For this reason, we chose to combine the two protein fragments for subsequent migration and whole lung regeneration experiments. The FBN-2 C-terminal fragment contains one RGD integrin site in TB4, and the N-terminal fragment contains two RGD integrin sites in TB3 and TB4 (22). By combining these two fragments, all sites are therefore available for epithelial binding.

Tenascin-C is a large ECM glycoprotein that is expressed during development and also upon tissue damage, where it is up-regulated within 24 h of injury (37). It has been reported that TN-C is enriched in the rat lung at post-natal week 1, during active alveolarization, and then declines to barely detectable levels by the third week of life (28). The current study utilized adult (>8 weeks old) rats, which should be depleted in TN-C.

It has been also shown that TN-C is specifically localized to the wound edge under migrating keratinocytes and fibroblasts after tissue injury, and that FBN-2 co-localizes with TN-C during wound healing (27). These results support our observation that pre-treatment with FBN-2 and TN-C increases BESC migration and tissue injury repair. An increase in focal adhesion kinase (FAK) expression also supported the observed increase in epithelial migration. Previous studies have reported that Tenascin-C can promote fibroblast migration (38) and regulates proliferation and migration of cultured astrocytes in a scratch wound assay (39). Greater proliferation and migration is of benefit in whole lung scaffold regeneration, which requires prolonged remodeling and repair of the lung tissue.

Persistent expression of tenascin-C has been associated with fibrotic diseases such as pulmonary fibrosis, scleroderma, and liver cirrhosis. This observed fibrogenesis may be facilitated by the interaction of TN-C with FBN-2 during tissue repair (27), and the capacity of FBN-2 to bind latent TGF- β binding proteins and thus store TGF- β , a known driver of fibrosis (40). In our hands, pre-treatment of the scaffold with TN-C or FBN-2 did not result in increased mesenchymal transition of the BESC population or up-regulation of Tenascin-C expression. We have previously described the utility and potential of tissue-derived BSCs

in lung epithelial tissue engineering (14). The ability to reduce cellular senescence and increase proliferation during *ex vivo* epithelial tissue regeneration presents a direct advantage to this process. During the early stages of epithelial repair, progenitor cells migrate along the underlying matrix that is then remodeled during the regeneration process (41). Enhancement of the acellular lung scaffold by treatment with FBN-2 and TN-C was shown to convey a significant benefit to epithelial migration, alignment, and tissue remodeling during lung tissue regeneration.

The aim of creating on-demand organs for transplantation requires the optimization of many essential factors, including the generation of an ideal lung scaffold to support the formation of new, functional lung tissue (42). The unique cross-talk between the ECM and cells is an essential component of tissue development, homeostasis, and repair, and the ability to harness these key factors that support these processes can have direct benefit to whole tissue engineering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Gilpin SE, Guyette JP, Gonzalez G, Ren X, Asara JM, Mathisen DJ, Vacanti JP, Ott HC. Perfusion decellularization of human and porcine lungs: bringing the matrix to clinical scale. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation*. 2014; 33(3):298–308.
2. Guyette JP, Gilpin SE, Charest JM, Tapias LF, Ren X, Ott HC. Perfusion decellularization of whole organs. *Nature protocols*. 2014; 9(6):1451–68. [PubMed: 24874812]
3. Tapias LF, Ott HC. Decellularized scaffolds as a platform for bioengineered organs. *Current opinion in organ transplantation*. 2014; 19(2):145–52. [PubMed: 24480969]
4. Balestrini JL, Niklason LE. Extracellular matrix as a driver for lung regeneration. *Annals of biomedical engineering*. 2015; 43(3):568–76. [PubMed: 25344351]
5. Burgess JK, Mauad T, Tjin G, Karlsson JC, Westergren-Thorsson G. The extracellular matrix - the under-recognized element in lung disease? *The Journal of pathology*. 2016; 240(4):397–409. [PubMed: 27623753]
6. Booth AJ, Hadley R, Cornett AM, Dreffe AA, Matthes SA, Tsui JL, Weiss K, Horowitz JC, Fiore VF, Barker TH, et al. Acellular normal and fibrotic human lung matrices as a culture system for in vitro investigation. *American journal of respiratory and critical care medicine*. 2012; 186(9):866–76. [PubMed: 22936357]
7. Sokocevic D, Bonenfant NR, Wagner DE, Borg ZD, Lathrop MJ, Lam YW, Deng B, Desarno MJ, Ashikaga T, Loi R, et al. The effect of age and emphysematous and fibrotic injury on the re-cellularization of de-cellularized lungs. *Biomaterials*. 2013; 34(13):3256–69. [PubMed: 23384794]
8. Godin LM, Sandri BJ, Wagner DE, Meyer CM, Price AP, Akinnola I, Weiss DJ, Panoskaltis-Mortari A. Decreased Laminin Expression by Human Lung Epithelial Cells and Fibroblasts

- Cultured in Acellular Lung Scaffolds from Aged Mice. *PloS one*. 2016; 11(3):e0150966. [PubMed: 26954258]
9. Whitsett JA, Nogue LM, Weaver TE, Horowitz AD. Human surfactant protein B: structure, function, regulation, and genetic disease. *Physiological reviews*. 1995; 75(4):749–57. [PubMed: 7480161]
 10. Yates CC, Hebda P, Wells A. Skin wound healing and scarring: fetal wounds and regenerative restitution. *Birth defects research Part C, Embryo today : reviews*. 2012; 96(4):325–33.
 11. McBride JT, Wohl ME, Strieder DJ, Jackson AC, Morton JR, Zwerdling RG, Griscom NT, Treves S, Williams AJ, Schuster S. Lung growth and airway function after lobectomy in infancy for congenital lobar emphysema. *The Journal of clinical investigation*. 1980; 66(5):962–70. [PubMed: 7430353]
 12. Meiners S, Eickelberg O, Konigshoff M. Hallmarks of the ageing lung. *The European respiratory journal*. 2015; 45(3):807–27. [PubMed: 25657021]
 13. Thannickal VJ, Murthy M, Balch WE, Chandel NS, Meiners S, Eickelberg O, Selman M, Pardo A, White ES, Levy BD, et al. Blue journal conference. Aging and susceptibility to lung disease. *American journal of respiratory and critical care medicine*. 2015; 191(3):261–9. [PubMed: 25590812]
 14. Gilpin SE, Charest JM, Ren X, Tapias LF, Wu T, Evangelista-Leite D, Mathisen DJ, Ott HC. Regenerative potential of human airway stem cells in lung epithelial engineering. *Biomaterials*. 2016; 108:111–9. [PubMed: 27622532]
 15. Rock JR, Randell SH, Hogan BL. Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. *Disease models & mechanisms*. 2010; 3(9–10):545–56. [PubMed: 20699479]
 16. Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, Randell SH, Hogan BL. Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106(31):12771–5. [PubMed: 19625615]
 17. Zuo W, Zhang T, Wu DZ, Guan SP, Liew AA, Yamamoto Y, Wang X, Lim SJ, Vincent M, Lessard M, et al. p63(+)-Krt5(+) distal airway stem cells are essential for lung regeneration. *Nature*. 2015; 517(7536):616–20. [PubMed: 25383540]
 18. Vaughan AE, Brumwell AN, Xi Y, Gotts JE, Brownfield DG, Treutlein B, Tan K, Tan V, Liu FC, Looney MR, et al. Lineage-negative progenitors mobilize to regenerate lung epithelium after major injury. *Nature*. 2015; 517(7536):621–5. [PubMed: 25533958]
 19. Li Q, Uygun B, Geerts S, Ozer S, Scalf M, Gilpin S, Ott H, Yarmush M, Smith L, Welham N, et al. Proteomic analysis of naturally-sourced biological scaffolds. *Biomaterials*. 2016; 75:37–46. [PubMed: 26476196]
 20. Li Q, Chang Z, Oliveira G, Xiong M, Smith L, Frey B, Welham N. Protein turnover during in vitro tissue engineering. *Biomaterials*. 2016; 81:104–13. [PubMed: 26724458]
 21. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotech*. 2008; 26(12):1367–72.
 22. Lin G, Tiedemann K, Vollbrandt T, Peters H, Batge B, Brinckmann J, Reinhardt DP. Homo- and heterotypic fibrillin-1 and -2 interactions constitute the basis for the assembly of microfibrils. *The Journal of biological chemistry*. 2002; 277(52):50795–804. [PubMed: 12399449]
 23. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature methods*. 2012; 9(7):671–5. [PubMed: 22930834]
 24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25(4):402–8. [PubMed: 11846609]
 25. Pierce RA, Mariani TJ, Senior RM. Elastin in lung development and disease. *Ciba Foundation symposium*. 1995; 192:199–212. discussion-4. [PubMed: 8575258]
 26. Zhang H, Apfelroth SD, Hu W, Davis EC, Sanguineti C, Bonadio J, Mecham RP, Ramirez F. Structure and expression of fibrillin-2, a novel microfibrillar component preferentially located in elastic matrices. *The Journal of cell biology*. 1994; 124(5):855–63. [PubMed: 8120105]
 27. Brinckmann J, Hunzelmann N, Kahle B, Rohwedel J, Kramer J, Gibson MA, Hubmacher D, Reinhardt DP. Enhanced fibrillin-2 expression is a general feature of wound healing and sclerosis:

- potential alteration of cell attachment and storage of TGF-beta. Laboratory investigation; a journal of technical methods and pathology. 2010; 90(5):739–52. [PubMed: 20195245]
28. Young SL, Chang LY, Erickson HP. Tenascin-C in rat lung: distribution, ontogeny and role in branching morphogenesis. *Developmental biology*. 1994; 161(2):615–25. [PubMed: 7508872]
 29. Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. *Nature reviews Molecular cell biology*. 2005; 6(1):56–68. [PubMed: 15688067]
 30. Kida K, Thurlbeck WM. Lack of recovery of lung structure and function after the administration of beta-amino-propionitrile in the postnatal period. *The American review of respiratory disease*. 1980; 122(3):467–75. [PubMed: 7416621]
 31. Yang Q, Ota K, Tian Y, Kumar A, Wada J, Kashiwara N, Wallner E, Kanwar YS. Cloning of rat fibrillin-2 cDNA and its role in branching morphogenesis of embryonic lung. *Developmental biology*. 1999; 212(1):229–42. [PubMed: 10419698]
 32. Shifren A, Mecham RP. The stumbling block in lung repair of emphysema: elastic fiber assembly. *Proceedings of the American Thoracic Society*. 2006; 3(5):428–33. [PubMed: 16799087]
 33. Zeyer KA, Reinhardt DP. Fibrillin-containing microfibrils are key signal relay stations for cell function. *Journal of cell communication and signaling*. 2015; 9(4):309–25. [PubMed: 26449569]
 34. Ritty TM, Broekelmann TJ, Werneck CC, Mecham RP. Fibrillin-1 and -2 contain heparin-binding sites important for matrix deposition and that support cell attachment. *The Biochemical journal*. 2003; 375(Pt 2):425–32. [PubMed: 12837131]
 35. Bax DV, Bernard SE, Lomas A, Morgan A, Humphries J, Shuttleworth CA, Humphries MJ, Kielty CM. Cell adhesion to fibrillin-1 molecules and microfibrils is mediated by alpha 5 beta 1 and alpha v beta 3 integrins. *The Journal of biological chemistry*. 2003; 278(36):34605–16. [PubMed: 12807887]
 36. Jovanovic J, Takagi J, Choulier L, Abrescia NG, Stuart DI, van der Merwe PA, Mardon HJ, Handford PA. alphaVbeta6 is a novel receptor for human fibrillin-1. Comparative studies of molecular determinants underlying integrin-rgd affinity and specificity. *The Journal of biological chemistry*. 2007; 282(9):6743–51. [PubMed: 17158881]
 37. Midwood KS, Orend G. The role of tenascin-C in tissue injury and tumorigenesis. *Journal of cell communication and signaling*. 2009; 3(3–4):287–310. [PubMed: 19838819]
 38. Trebault A, Chan EK, Midwood KS. Regulation of fibroblast migration by tenascin-C. *Biochemical Society transactions*. 2007; 35(Pt 4):695–7. [PubMed: 17635125]
 39. Nishio T, Kawaguchi S, Yamamoto M, Iseda T, Kawasaki T, Hase T. Tenascin-C regulates proliferation and migration of cultured astrocytes in a scratch wound assay. *Neuroscience*. 2005; 132(1):87–102. [PubMed: 15780469]
 40. Tatler AL, Jenkins G. TGF-beta activation and lung fibrosis. *Proceedings of the American Thoracic Society*. 2012; 9(3):130–6. [PubMed: 22802287]
 41. Im D, Shi W, Driscoll B. Pediatric Acute Respiratory Distress Syndrome: Fibrosis versus Repair. *Frontiers in pediatrics*. 2016; 4:28. [PubMed: 27066462]
 42. Gilpin SE, Charest JM, Ren X, Ott HC. Bioengineering Lungs for Transplantation. *Thoracic surgery clinics*. 2016; 26(2):163–71. [PubMed: 27112255]

Figure 1. Epithelial Culture on Isolated Human ECM

(A) Method for preparation of matrix coating for *in vitro* culture. (B) Quantitative gene expression analysis of BECs grown on neonatal (N1-N3) and adult (A1-A3) matrix coating. Expression normalized to B-Actin, and expressed relative to normal adult lung tissue. (C) Cytotoxicity assay measuring total live (400Ex/505Em) and dead (485Ex/520Em) cell fluorescence on Day 7.

Figure 2. Neonatal and Adult Lung Composition by Proteomic Analysis

(A) Heat map of detected proteins in each sample. (B) Summary matrix composition in neonatal vs adult matrix (n=3/group)

Figure 3. Quantitative comparison of the matrix proteins in adult and neonatal lung scaffolds
(A) Volcano plot of detected matrix proteins. (B) Details of proteins highlighted in (A). (C) Immunofluorescent staining for Tenascin-C (TN-C), Fibrillin-2 (FBN-2), and Elastin. Scale bar = 100µm

Figure 5. *Ex vivo* lung epithelial regeneration on pre-treated matrices

(A) Quantitative gene expression of re-epithelialized lung scaffolds (B) Hematoxylin and eosin assessment of lung tissue. Native rat lung is shown for reference. Scale bar = 50µm. (C) Immunofluorescent staining of lung tissue on Day 3 and 7 of regeneration. Scale bar = 50µm. (D) Quantification of Ki67 positive cells on Day 3 and 7 of lung epithelial regeneration (E) Quantification of tissue morphology by septal thickness. (F) Quantification of airspaces characteristics by Linear Intercept.

Table 1

Donor demographics

Age in Day (D, neonatal) and Years (adult). Gender listed as Male (M) or Female (F). Body Mass Index (BMI) listed for both group. Gestational time at birth (weeks) listed for neonatal donors.

	Neonatal (n=3)			Adult (n=3)		
Age	7D	2D	6D	48	64	47
Gender	M	F	M	M	F	M
BMI	11	9	14	24	48	21
Gestation (weeks)	36.2	40	38.4	N/A	N/A	N/A