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Fibrillin-2 and Tenascin-C bridge the age gap in lung epithelial regeneration

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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.

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1. 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

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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 α3 and α4 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.

2. Methods

2.1. Study Approval

Human donor lungs otherwise unsuitable for transplantation were obtained from the New England Organ Bank under sterile conditions within 60 min 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.

2.2. 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].

2.3. 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 (250–300 g, >8 weeks of age, Charles River Laboratories) and decellularized by perfusion of 0.1% SDS solution through the pulmonary artery at 40 mmHg, 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.

2.4. 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 (1 mg of pepsin per mL of 0.1 M sterile HCl) at 10 mg/mL for 20 h at room temperature, using a sterile M tube (MACS Miltenyl Biotech). Subsequently, the pepsin digested tissue was diluted in 0.1 M acetic acid to a final concentration of 0.1 mg/mL, and used to coat cell culture plates for 1 h at 37 °C. The coating was added to tissue culture plates and centrifuged at 300 × g for 5 min. A total of 1×10^6 BESCs (identified by p63 and Krt5 expression) were added to each well of a 24-well plate, and cultured for 7 d in SAGM. BESCs 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 BESCs added to each

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

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