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Protein turnover during *in vitro* tissue engineering

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ABSTRACT

Repopulating acellular biological scaffolds with phenotypically appropriate cells is a promising approach for regenerating functional tissues and organs. Under this tissue engineering paradigm, reseeded cells are expected to remodel the scaffold by active protein synthesis and degradation; however, the rate and extent of this remodeling remain largely unknown. Here, we present a technique to measure dynamic proteome changes during *in vitro* remodeling of decellularized tissue by reseeded cells, using vocal fold mucosa as the model system. Decellularization and recellularization were optimized, and a stable isotope labeling strategy was developed to differentiate remnant proteins constituting the original scaffold from proteins newly synthesized by reseeded cells. Turnover of matrix and cellular proteins and the effects of cell–scaffold interaction were elucidated. This technique sheds new light on *in vitro* tissue remodeling and the process of tissue regeneration, and is readily applicable to other tissue and organ systems.

1. Introduction

Acellular biological scaffolds obtained from tissue/organ decellularization are appealing platforms for tissue/organ regeneration. They promote immunologic tolerance and retain three-dimensional architectures and biochemical cues that can facilitate the adhesion, migration, proliferation, and differentiation of seeded cells that, in a clinical situation, may be autologous to the tissue recipient. The acellular scaffold contains tissue-specific extracellular matrix (ECM) that has been shown to direct stem and progenitor cells towards a target fate [1–3], as well as maintain the functional phenotype of somatic cells in extended culture [4,5]. Recellularization of decellularized whole organs such as heart [6], liver [7], lung [8,9], and kidney [10] have partially restored the contractile, metabolic, gas exchange, and urine production function of these respective organs *in vitro*. These studies demonstrate the strong clinical potential of acellular biological scaffolds.

Beyond regulating cell behavior, the scaffold itself is also continuously remodeled by its resident cells. This dynamic reciprocity constitutes an advantage of acellular biological scaffolds over synthetic materials for tissue reconstruction [11]. Prior work on matrix remodeling has focused primarily on accumulation of individual structural matrix proteins (e.g., collagens) [12,13] and/or cellular secretion of known matrix-remodeling enzymes (e.g., matrix metalloproteinases [MMPs]) [14–16]. However, the current human matrisome (i.e., all ECM and ECM-associated proteins) consists of >1000 proteins [17]. This large number of proteins, especially when combined with the various complex interactions and signaling networks formed between the ECM and its resident cells, creates a significant analytical challenge. A proteomic analysis can address this challenge by characterizing the complex and synergistic biological events that comprise the remodeling process. Moreover, since tissue remodeling is a dynamic process, it is desirable to reveal protein turnover by differentiating between original and newly-synthesized proteins.

Mass spectrometry (MS) offers the opportunity to characterize protein identity and abundance at the whole-proteome level. Stable isotope labeling with amino acids in cell culture (SILAC) is a quantitative proteomics method [18,19], wherein two cell populations are cultured in media that are identical except that one contains a “heavy” and the other a “light” form of a particular amino acid (e.g., $^{13}C_6$- versus $^{12}C_6$-lysine, respectively). These isotopically labeled amino acids are metabolically incorporated into each cell's

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proteome, and the two populations are mixed prior to MS sample preparation and analysis. The resulting MS peak ratios between "heavy" and "light" forms indicate relative protein abundances. SILAC has been used to study protein turnover in cells [20,21], animals [22] and plants [23], and protein half-lives can be calculated [24].

In the present work, using vocal fold mucosa (VFM) as the model system, we compared different decellularization and recellularization approaches. We then developed a novel strategy using SILAC to differentiate between proteins originally present in the acellular scaffolds and newly synthesized ones, thereby assessing active protein synthesis and *in vitro* remodeling of the ECM. The entire workflow is summarized in Fig. 1. This study is the first to analyze the dynamic relationship between the matrix and its resident cells, providing biological system-wide insight into the protein turnover that is central to tissue remodeling.

2. Materials and methods

2.1. Porcine and human VFM preparation

Porcine larynges were harvested from female market pigs (age 6–8 mo) and snap frozen within 2 h of death. Human larynges were harvested from female cadavers (age 27–73 y) under IRB exemption and snap frozen within 3–48 h of death. Prior to experimentation, larynges were thawed overnight at 4 °C and each VFM specimen (epithelium and lamina propria [LP]) was microdissected from its underlying thyroarytenoid muscle.

2.2. VFM decellularization

Porcine VFM were assigned to five decellularization protocols, as detailed in Fig. 2a. Strategies 1 and 2 consisted of immersion in 1% CHAPS or 1% SDS, respectively, for 24 h at room temperature (RT, 22 °C), followed by PBS wash for 24 h at RT. Strategy 3 was previously reported by Xu et al. [25]. Briefly, osmotic stress was first applied by immersing the samples in a highly hypertonic 3 M NaCl solution for 24 h at RT. Samples were then treated with 25 μg/mL DNase I and 10 μg/mL RNase A in an isotonic PBS-containing EDTA-free mini-protease inhibitor cocktail for 24 h at 37 °C, followed by 70% ethanol for 24 h at RT. Another round of DNase and RNase digestion (at the concentrations noted above) was performed for 48 h at 37 °C, followed by PBS wash for 24 h at RT. Strategies 4 and 5 involved the addition of either 1% CHAPS or 1% SDS treatment to strategy 3 following the second round of enzyme treatment. For all conditions, a shaker applied continuous mechanical agitation, and 1000 U/mL penicillin and 1 mg/mL streptomycin in PBS were added at each step to mitigate potential bacterial contamination of the decellularized tissue.

Following the initial experiment, strategy 4 was selected for decellularization of porcine and human VFM specimens in subsequent recellularization experiments. In these later experiments, DNase I and RNase A concentrations were adjusted to 500 U/mL and 20 μg/mL respectively.

2.3. Recellularization of decellularized VFM with immortalized VFFs

A previously characterized vocal fold fibroblast (VFF) cell line [26] was used for all recellularization experiments. Five cell seeding protocols were evaluated, as illustrated in Fig. 3a. Each decellularized scaffold was placed in the apical chamber of a culture insert with either its luminal (strategy 1) or deep LP (strategies 2–5) surface facing upwards. DMEM (1.7 mL, containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B) was added to the basolateral chamber and 5×10^5 VFFs in 0.5 mL DMEM was pipetted onto the seeding surface of each scaffold. Strategies 1 and 3 included a post-seeding centrifugation step, performed at 170 *g* for 8 min. Strategy 4 involved placement of a platelet derived growth factor (PDGF)-infused gel into the basolateral chamber at the time of cell seeding, followed by replacement of the PDGF gel at 24 h and 1 w post-seeding. The PDGF-infused gel was prepared by adding 50 ng/mL PDGF (R&D systems, Minneapolis, MN) to a type I collagen solution (pH 7.2) and incubating at 37 °C for 2 h to allow gel formation. Strategy 5 involved soaking the scaffold in 1 mL type I collagen solution (pH 7.5) with agitation at 4 °C overnight, and then incubating at 37 °C for 2 h to allow gel formation. All seeded scaffolds were first incubated at 37 °C in 5% CO_2 overnight, transferred to a new well after 24 h, and cultured for 6 w. Half of each sample, corresponding to the anterior VFM, was harvested under sterile conditions at 3 w. Unseeded scaffolds

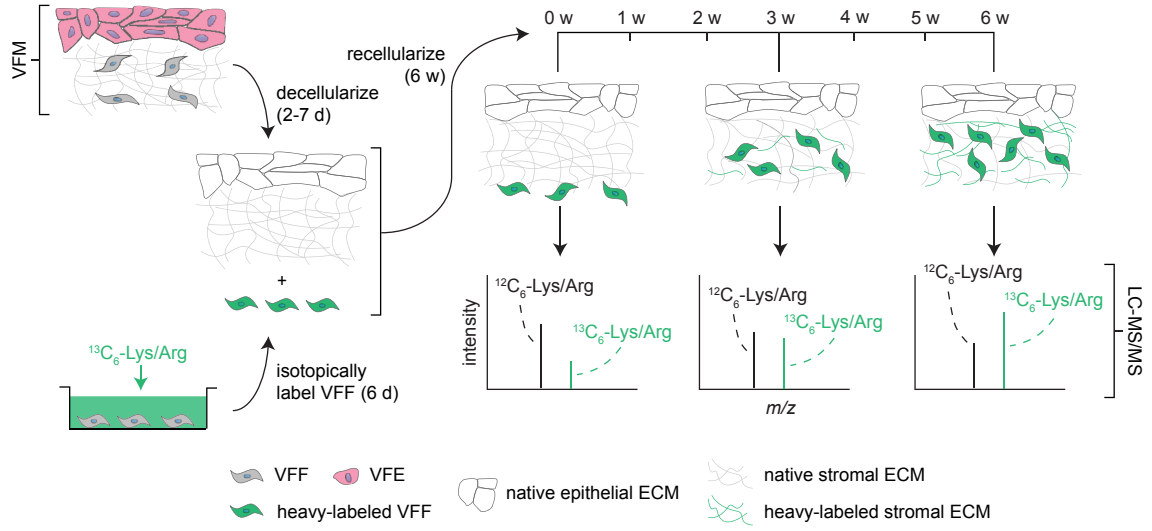

Fig. 1. Summary of the entire experimental workflow. Vocal fold mucosae (VFM) are decellularized using one of five strategies for 2–7 d. Vocal fold fibroblasts (VFFs) are isotopically labeled for sufficient time to ensure full-proteome incorporation of $^{13}C_6$-Lys and $^{13}C_6$-Arg. Next, the labeled VFFs are seeded and cultured for up to 6 w in decellularized VFM, with liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based analysis at each of 6 w. Representative cell proliferation and new ECM synthesis are shown for 0, 3, and 6 w timepoints only. VFE, vocal fold epithelial cell; ECM, extracellular matrix.