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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.

Keywords

acellular scaffold; reseeded; stable isotope labeling; protein turnover; tissue remodeling; vocal fold fibroblast; vocal fold mucosa

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

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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., $^{13}\text{C}_6$ -versus $^{12}\text{C}_6$ -lysine, respectively). These isotopically labeled amino acids are metabolically incorporated into each cell’s 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.

Materials and Methods

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.

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 3M 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.

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 incubating at 37 °C for 2 h to allow gel formation. All seeded scaffolds were first incubated at 37 °C in 5% CO₂ 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 (retained as negative control samples) were subject to the same culture conditions.

Hydroxyproline and sulfated glycosaminoglycan assays

Hydroxyproline content was measured using a commercial detection kit (BioVision, Mountain View, CA) following sample hydrolysis in 12 N HCl for 3 h at 120 °C. Absorbance was measured at 560 nm. sGAG content was measured using the Blyscan assay

(Biocolor, Carrickfergus, UK) following processing with a detergent removal spin column (Thermo Scientific) and papain extraction, according to the manufacturer's instructions. Absorbance was measured at 656 nm.

VFF isotopic labeling, seeding, and culture

For the isotopic labeling experiment, VFFs were cultured and expanded for 7 d in DMEM containing 100 mg/L $^{13}\text{C}_6$ -lysine and 100 mg/L $^{13}\text{C}_6$ -arginine (SILAC Protein Quantitation kit; Thermo Scientific). We also added 100 mg/L $^{12}\text{C}_6$ -proline (see Supplementary Notes). LC-MS/MS analysis confirmed nearly complete (99%) incorporation of heavy lysine and arginine into the VFF proteome by 6 d (see Supplementary Notes). Heavy-labeled cells were seeded on decellularized human VFM scaffolds using recellularization strategy 3 and then cultured. Samples were harvested for proteomic assays at 1, 2, 3, 4, 5 and 6 w post-seeding; biopsies of the 3 and 6 w samples were additionally processed for histology. The 6 w sample was examined to check for incorporation of "light" amino acids, derived from degradation of scaffold proteins, into newly synthesized proteins (see Supplementary Notes).

Histology, cell migration analysis, and cell number estimation

Samples intended for histology were rinsed in PBS and fixed in 1 mL of 4% paraformaldehyde (PFA) at 4 °C for 1 h, incubated in 1 mL of 25% sucrose at 4 °C overnight, and embedded in Tissue-Tek Optimum Cutting Temperature compound (Sakura Finetek, Tokyo, Japan). Serial frozen sections (8 μm thickness) were prepared in the coronal plane using a cryostat. For the decellularization and recellularization strategy comparison experiments, 200 serial sections (representing 1.6 mm total tissue thickness) were prepared from each sample, beginning at the midmembranous VFM transsection plane and moving towards either the anterior or posterior pole. Three sections of every 10 were stained with hematoxylin and eosin (H&E), Alcian blue (pH 2.5) and Movat's pentachrome. For the isotopic labeling experiment, 20 serial sections were prepared from each biopsy sample and stained with H&E. All sections were imaged using standard light microscopy.

For the recellularization strategy comparison experiment, cell migration was quantified using H&E-stained sections and Metamorph 7.5 (Molecular Devices, Downingtown, PA) by measuring the shortest distance between each individual cell and the epithelial seeding surface. To minimize possible artifacts due to uneven cell distribution throughout the scaffold, image analysis was performed on at least 10 sections per sample, selected from each 1.6 mm span of serial sections.

Additional cell counting was performed on 3 and 6 w sections obtained from the validation of recellularization strategy 3 using decellularized human VFM scaffolds. The mean number of cells per 8 μm section was used to estimate the total number of cells in a whole human female VFM scaffold with average length of 10 mm[27], at 3 and 6 w post-seeding. These cell numbers were 8.2×10^4 (3 w) and 2.9×10^5 (6 w). By logarithmic growth curve extrapolation, the number of cells that actually engrafted in the scaffold at the time of seeding (i.e., at 0 w) was then calculated to be 2.4×10^4 (~5% of the number of seeded cells). We used these cell count-based data to generate control samples for the isotopic labeling experiment, as follows. As the labeling experiment involved analyzing half-VF

scaffolds, we mixed a certain portion of the heavy cell peptide solution with a certain portion of the scaffold peptide solution to generate “scaffold + 1.2×10^4 cells”, “scaffold + 4.1×10^4 cells”, and “scaffold + 1.5×10^5 cells” conditions, and used them as the 0 w sample, the 3 w control, and the 6 w control, respectively.

Proteomics sample preparation

For heavy-labeled VFFs, protein was extracted with 150 μ L of SDT solution containing 4% SDS, 0.1 M Tris-HCl (pH 7.6) and 0.1 M dithiothreitol. For intact, decellularized, and reseeded human VFM, ~15 mg tissue pieces were washed with ice cold PBS, then ground with disposable pellet pestles (Kimble Chase Kontes, Vineland, NJ), before 150 μ L of SDT solution was added. Samples were then heated at 95 °C for 7 min and sonicated on ice with a probe sonicator—alternating 20 s on and 20 s off for 6 min, followed by centrifugation at 20 °C for 5 min at 16,100 g. The Filter-Aided Sample Preparation (FASP) protocol was used for SDS removal and on-filter digestion [28]. Briefly, a 30 μ L aliquot of the supernatant was added to a 30K MW Vivacon 500 filter (Sartorius, Bohemia, NY), washed, alkylated, and digested with trypsin (protein:enzyme ratio of 50:1) overnight at 37 °C. Finally, the digest was collected by centrifugation. After the digestion was quenched with 10% trifluoroacetic acid (TFA) to a final concentration of 0.5% TFA, samples were desalted using Sep-Pak C18 1 cc Vac Cartridges (Waters, Milford, MA), according to the manufacturer’s instructions. Eluate was dried down and reconstituted in 5% acetonitrile (ACN) and 2% formic acid (FA).

Mass spectrometry and data analysis

Approximately 1 μ g protein digest (estimated by BCA assay) of the heavy-labeled VFFs, or 0.05 mg tissue equivalent, 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 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 and acquired in profile mode. The ten most intense peaks were selected for fragmentation by high-energy collisional dissociation (HCD) at 42% collision energy, with a resolution of 7500, and isolation width of 2.5 m/z . Dynamic exclusion was enabled with a repeat count of 2 over 30 s and an exclusion duration of 120 s. Each experiment condition had four biological replicates; the 1 w to 6 w samples were subjected to LC-MS/MS analysis twice per biological replicate.

The acquired raw files were analyzed by MaxQuant (version 1.4.1.2)[29]. The derived peak lists were searched with Andromeda against the UniProt canonical protein database (*Homo sapiens*: 20278 sequences downloaded on December 5, 2013) supplemented with common contaminants. All biological and technical replicates for a particular sample were searched together. 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 oxidation (+15.9949 Da) were specified. A false discovery rate (FDR) 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.

The “match between runs” function was enabled. A minimum of two unique and razor peptide ratio counts was required and only unmodified peptides were used to quantify a protein. Protein groups containing matches to proteins from the reversed database or contaminants were discarded. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium[30] via the PRIDE partner repository (dataset identifier PXD002734).

Statistical analysis

Technical replicates were averaged and all statistical comparisons were performed using independent biological replicates. Gene ontology term enrichment analysis was performed using the BiNGO[31] (hypergeometric model with Benjamini-Hochberg correction) and REVIGO[32] (SimRel cutoff = 0.4) algorithms. Ontology term enrichment schematics were generated using Cytoscape 2.8.2[33]. Differences among two groups were analyzed using an independent samples *t* test; differences among multiple groups were analyzed using a one-way ANOVA. *P* values below 0.05 were considered statistically significant.

Results

Comparison of five decellularization strategies

The first step towards creating a functional tissue is to engineer a scaffold that retains much of the ECM composition and architecture of the original tissue while removing any cellular remnants that hold the potential to trigger a maladaptive immune response. Previous work has shown that optimal decellularization is tissue or organ-specific[11]. The vocal fold mucosa (VFM) is an attractive model for matrix remodeling studies because its unique ECM is biomechanically tuned for voice production; disordered VFM is recalcitrant to current clinical therapies and so represents a substantial, albeit challenging, tissue engineering need[34, 35].

We therefore decellularized porcine VFM using five different strategies, each consisting of isolated or sequential treatments with a zwitterionic (CHAPS) or anionic (SDS) detergent, osmotic stress, and nuclease digestion (Fig. 2a), in order to identify an optimal procedure. A hydroxyproline assay showed preservation of native collagen abundance with each of the five strategies ($P > 0.05$; Fig. 2b), suggesting maintenance of scaffold structural integrity and strength. Sulfated glycosaminoglycan (sGAG) abundance was significantly depleted with strategies 3 and 5 ($P < 0.05$; Fig. 2b), indicating probable impairment of tissue viscosity and biomechanical performance[36] but consistent with decellularization outcomes in other tissues[7]. The lower concentrations of both hydroxyproline and sGAG with strategy 2 compared to 1, as well as with strategy 5 compared to 4, suggest that SDS is more disruptive to the VFM ECM than CHAPS.

Hematoxylin and eosin (H&E), Alcian blue and Movat’s pentachrome histological stains were used to evaluate the effectiveness of cell removal and preservation of native ECM architecture. The consistent absence of cell nuclei in histologic sections from strategies 3-5 (Fig. 2c; Supplementary Fig. S1) support the benefit of osmotic stress and nuclease digestion in lysing cells and removing nuclear material *in situ*. Strategy 4 was selected for

decellularization of porcine and human VFM in subsequent recellularization and isotopic labeling experiments, based on its effective depletion of cells and superior quantitative preservation of collagen and sGAGs.

Comparison of five reseeding strategies

To find a seeding condition that promotes maximum cell infiltration into the scaffold, we compared five reseeding strategies in decellularized porcine VFM (Fig. 3a). VFFs were seeded on either the luminal or deep LP surface of the scaffold and treated with either gentle centrifugation, chemoattraction using platelet derived growth factor (PDGF), or encapsulation in a collagen I gel[37-39]. H&E staining and cell migration analysis were performed 3 w after reseeding (Fig. 3b and 3c). Seeding on the deep LP surface combined with either centrifugation (strategy 3) or chemoattraction (strategy 4) resulted in superior cell migration, compared to all other strategies ($P < 0.05$). With strategies 3 and 4, VFFs infiltrated the scaffold to a mean depth of $\sim 200 \mu\text{m}$ and a maximum depth of $\sim 800 \mu\text{m}$, which approximates the mean thickness of porcine VFM ($\sim 900 \mu\text{m}$)[27, 40]. Given the expense and possibility of unanticipated off-target effects from extended PDGF treatment, we selected strategy 3 for VFF reseeding in subsequent experiments. We next repeated the reseeding experiment in decellularized *human* VFM and extended the culture time to 6 w. Fig. 3d shows a substantial increase in cell density from 3 w to 6 w, suggesting ongoing VFF proliferation throughout the culture period. The final recellularized construct at 6 w contained VFFs of comparable morphology and density to those of native VFM (Supplementary Fig. S2).

Proteomic characterization of native, decellularized, and recellularized human VFM

To further characterize the selected decellularization and recellularization strategies, we performed LC-MS/MS-based proteomic analysis of native, decellularized, and recellularized (6 w post-seeding) human VFM. A total of 1028, 509 and 704 proteins, respectively, were identified using a 1% false discovery rate (FDR) (Fig 4a; Supplementary Tables S1-S3). Gene Ontology (GO) enrichment analyses were performed on the set of 429 proteins that were exclusively identified in the native condition, as well as the set of 160 proteins that were exclusively identified in the recellularized condition. Native VFM was characterized by enrichment of an array of biological process (BP) terms (Supplementary Table S4) including those associated with defense response and muscle contraction, consistent with the presence of epithelial and immune cells[41] that were not used in our recellularization experiments, as well as residual muscle cells and fibers that remain in the native VFM despite careful microdissection during sample preparation[42]. Recellularized VFM was characterized by enrichment of BP terms (Supplementary Table S5) associated with various metabolic, signaling, transport and regulatory functions, as well as a set of interconnected BP terms associated with biogenesis, morphogenesis and developmental processes (Fig. 4b). Interestingly, a number of these BP terms relate to specific tissue substructures, such as the epithelium, vascular and nervous systems, implying that VFFs in the recellularized VFM respond to regional cues and engage in remodeling these subspecialized ECMs, in addition to the primary ECM of the lamina propria.

We also specifically examined the effects of decellularization and recellularization on matrisome proteins. Note that the non-matrisome proteins (remnant cellular proteins that are incompletely removed during decellularization) still constitute a large portion of the proteome of decellularized VFM (Figs. 4a, 4c, and Supplementary Table S2); this has also been observed in other acellular scaffolds[43]. A total of 46 matrisome proteins were removed by decellularization (Fig. 4c). The majority of these proteins were ECM regulators, ECM-affiliated proteins, and secreted factors: their removal resulted in a corresponding decrease in the relative MS intensity attributed to these three matrisome categories (Fig. 4d). In contrast, core matrisome collagens, glycoproteins and proteoglycans showed generally well-preserved MS intensity following decellularization, consistent with our previous hydroxyproline and histological data (Fig. 2b and c). Sixteen of the 46 decellularization-removed proteins were replenished by recellularization (Fig. 4c; Supplementary Table S6). Most of these newly synthesized matrisome proteins were ECM regulators that contribute to matrix remodeling (e.g., cathepsins B and C, serine protease HTRA1, MMP10, serpin E2) and glycoproteins that support cell adhesion (e.g., laminins, EMILIN-2). Of the 34 matrisome proteins that were exclusively identified in recellularized VFM (Fig. 4c; Supplementary Table S7), the most abundant was thrombospondin-1, an adhesion glycoprotein that supports fibroblast migration and interaction with the ECM. The most abundant ECM regulators were the matrix metalloproteinases MMP1 and MMP2, as well as the inhibitor TIMP1, all of which are associated with matrix turnover and cell migration. Overall, the proteomics data show that decellularized VFM contains a well-preserved core matrisome, and that the recellularized VFM is characterized by tissue-appropriate biogenesis and matrix remodeling.

Matrisome and cellular protein turnover

VFFs are responsible for ECM maintenance and turnover in the functionally important LP region. To investigate these aspects of the tissue engineering process, we developed and implemented a SILAC-based strategy to differentiate between original and newly synthesized proteins. The goal of this experiment, therefore, was to quantitatively measure the rate and extent to which seeded VFFs remodel the decellularized VFM *in vitro*. As illustrated in Fig. 1, we isotopically labeled the proteome of cultured VFFs using medium containing $^{13}\text{C}_6$ -lysine (Lys) and $^{13}\text{C}_6$ -arginine (Arg) (99% incorporation of $^{13}\text{C}_6$ -Lys/Arg to the VFF proteome achieved by 6 d). We then seeded these heavy isotope-labeled cells onto decellularized VFM containing naturally occurring $^{12}\text{C}_6$ -Lys/Arg. We continued VFF culture with $^{13}\text{C}_6$ -Lys/Arg supplementation for 6 w; samples were harvested weekly for LC-MS/MS proteomic analysis.

To evaluate overall protein turnover, we first looked at the MS intensity of four broad classes of proteins: heavy (newly synthesized) and light (remnant) for both matrisome and non-matrisome (cellular) protein categories (Fig. 5a and b). The percentages of these four classes add up to 100% for each time point, but redistribution occurs over time. The percentage of newly synthesized matrisome proteins (black squares in Fig. 5a) increased steadily across the 6 w culture period ($P < 0.05$); the percentage of newly synthesized cellular proteins (black squares in Fig. 5b) increased and then plateaued ($P < 0.05$). This observation suggests that VFFs engage in more active and sustained synthesis of matrisome,

versus cellular, proteins during extended *in vitro* culture within decellularized VFM. Analysis of remnant protein forms showed no change ($P > 0.05$) in the normalized MS intensity of remnant matrisome proteins (red circles in Fig. 5a) but ~50% degradation of remnant cellular proteins (red circles in Fig. 5b) over the 6 w period ($P = 0.05$). Follow-up analysis of these remnant cellular proteins revealed the fastest degradation rates for nuclear and cytoskeletal proteins, moderate degradation of cytoplasmic proteins, and little change in already-low-abundance cell membrane proteins (Supplementary Fig. S3).

Next, we evaluated the extent to which interaction between VFFs and the decellularized VFM contributes to protein synthesis and degradation kinetics. To accomplish this, we collected tissue biopsies from 3 and 6 w samples and employed histology-based cell counts and logarithmic growth curve extrapolation to estimate the total number of cells within each scaffold throughout the experiment (including an estimate of initial VFF engraftment at 0 w). Using these cell counts, we mixed peptide digests isolated from $^{13}\text{C}_6$ -Lys/Arg VFF with peptide digests from decellularized VFM at appropriate ratios to match those of the recellularized tissue at each time point of interest. As these control samples were prepared at the peptide level, there was no opportunity for cell-scaffold interaction. Analysis at 3 and 6 w showed significantly greater MS intensity for heavy-labeled matrisome proteins in the *in vitro* culture samples compared to controls ($P < 0.05$; Fig. 5a), suggesting that exposure to the native ECM enhances matrisome synthesis by VFFs. We observed a comparable effect for heavy-labeled cellular proteins at 3 w ($P < 0.05$; Fig. 5b), as well as significantly lower MS intensity for remnant cellular proteins in the *in vitro* culture samples compared to controls at 3 w ($P < 0.05$; Fig. 5b). Overall, these data indicate that interaction between VFFs and the decellularized VFM promotes sustained matrisome synthesis, early-phase cellular activity that involves an uptick in cellular protein synthesis (consistent with VFF activation and proliferation), and early-phase enhancement of remnant cellular protein degradation.

Studies on degradation of collagens and other ECM proteins[44-46] have suggested two key pathways: a principal intracellular pathway involving phagocytosis with subsequent lysosomal cathepsin digestion, and an extracellular pathway involving secretion of MMPs by fibroblasts[47, 48]. The light-form matrisome proteins in our *in vitro* system were apparently resistant to degradation (Fig. 5a), which may be due to their extensive crosslinking, inadequate culture duration, or the absence of more effective phagocytes that would be present *in vivo*. In contrast, we observed gradual degradation of remnant cellular proteins over time. Little has been reported on the degradation mechanism of remnant cellular proteins with direct exposure to the matrix. Therefore, we fit both zero-[49, 50] and first-order[51, 52] kinetic functions to our MS intensity data (Fig. 5c), as both have been experimentally demonstrated for protein degradation. Note that the discrepancy between these functions can be explained by the assumption that the degradation rate follows a Michaelis-Menten function, and the reaction order depends upon the relative magnitude of dissociation constant and substrate concentration[53]. These functions predict complete remnant cellular protein degradation by VFFs at 16 w or beyond in our experimental system.

Matrisome protein synthesis

Based on our observation of sustained matrisome synthesis by VFFs across the 6 w culture period, we performed a follow-up analysis of each matrisome subcategory and its constituent proteins (Fig. 6; Supplementary Fig. S4). As we found no evidence of significant remnant matrisome protein degradation over time (Fig. 5a), most of the change in the percentage of MS intensity could be attributed to the net output from synthesis and degradation of heavy-labeled matrisome proteins by resident VFFs. ECM regulators and glycoproteins, important players in fibroblast migration and interaction with the matrix, exhibited the fastest increase in MS intensity during the first 4 w. Collagens and proteoglycans, extensively crosslinked core matrisome proteins that were well preserved in the original decellularized scaffold (Fig. 2b and c; Fig. 4d), increased more gradually during the same time period, then plateaued. Secreted factors, which include key growth factors and signaling molecules that bind to ECM proteins/glycans and were severely depleted during decellularization (Fig. 2b and c; Fig. 4d), exhibited an exponential increase in MS intensity from 2 to 5 w.

We examined the specific proteins driving MS intensity changes within the core matrisome (Fig. 6b-d). Most net collagen synthesis was attributable to the primary fibrillar isoform collagen I (COL1A1) as well as the collagen I-associated isoforms VI (COL6A3) and XII (COL12A1) (Fig. 6b). At the final 6 w time point, ~25% of total COL1A1 intensity was due to new protein synthesis. Several ECM glycoproteins exhibited sharp increases in net synthesis during the first week of culture (Fig. 6c). The greatest initial increase was seen for heavy-labeled fibronectin (FN1), which accounted for 75-80% of total FN1 intensity during the remainder of the experiment. Early and rapid FN1 synthesis is consistent with its critical roles facilitating the deposition of other ECM proteins and maintaining cell-ECM adhesion sites[54]. The small leucine-rich proteoglycan (SLRP) family members decorin (DCN), lumican (LUM) and biglycan (BGN), which are regulators of collagen fibril assembly and associated tissue strength[55, 56], followed a pattern of gradually increasing net synthesis from 1 to 5 w, then a decrease at 6 w (Fig. 6d).

The primary analytical advantage of our SILAC-based method in a tissue engineering context is the ability to differentiate original and newly synthesized proteins. This is particularly important when a high rate of synthesis but little overall turnover results in a dramatic difference in a given protein's heavy versus total MS intensity change. To further illustrate this, we plotted heavy, light and total MS intensity fold changes for the proteoglycan DCN at 0, 3, and 6 w (Fig. 6e), and compared these data with immunoblots of total DCN abundance at 1, 3, and 6 w (Fig. 6f). We observed a ~100-fold increase ($P < 0.05$) in heavy DCN intensity over the 6 w culture period but statistically insignificant change in light and total ($P > 0.05$) DCN intensity, or DCN abundance. This observation demonstrates the value of isotopic labeling for capturing aspects of protein synthesis kinetics that would otherwise be masked by traditional protein detection methods.

Discussion

Tissue engineering using acellular biological scaffolds is a popular and promising technique that is dependent upon a synergistic relationship between the scaffold ECM and its seeded

cells. Despite the importance of this relationship, traditional assays have been unable to capture the dynamic remodeling events that are presumably responsible for the engineered tissue function reported in prior studies[6, 7, 10]. This deficiency, in part, is due to the difficulty of interrogating the cell-matrix system comprehensively and quantitatively. In response to this challenge, we adapted a SILAC-based proteomics method to differentiate original and newly synthesized proteins in a tissue engineering context, applied the method to decellularized VFM, and herein present the first broad analysis of protein turnover after reseeding cells on an acellular scaffold.

Across the proteome, and over time, proteins in certain categories were actively degraded whereas others were actively synthesized. Our initial analysis of the decellularized VFM proteome attributed substantial MS intensity to remnant cellular proteins, despite the absence of cellular structures in histology. We observed ongoing degradation of these remnant proteins during the 6 w culture period, which theoretically corresponds to a decrease in the immunogenic potential of the final engineered tissue[57, 58]. Further, isotopic labeling enabled measurement of the degradation rates of these cellular proteins *in vitro*; we suspect the rates would be faster *in vivo*.

Biological scaffolds are not intended as permanent implants; rather, they should be biodegradable and subject to ECM turnover by resident cells[59]. In this work with VFM, we observed little degradation of the scaffold's core matrisome; it would be helpful to examine whether longer *in vitro* culture times and/or *in vivo* conditions result in the breakdown and replacement of these large structural proteins. In contrast, isotopic labeling revealed active and sustained synthesis of a myriad of matrisome proteins across the experiment. Different synthesis rates were observed among categories, with ECM glycoproteins, ECM regulators, and secreted factors exhibiting the fastest rates, confirming their importance to tissue remodeling. Notably, remnant cellular protein degradation and matrisome protein synthesis were both significantly increased compared to control samples that lacked interplay between the scaffold and its seeded cells.

In summary, the ability to differentiate residual from newly synthesized proteins at the biological system level provides a more complete understanding of ECM turnover during tissue engineering. The analytical strategy developed here is directly applicable to other tissue/organ types as well as different engineering techniques. Isotopic labeling experiments can also be conducted *in vivo*, whereby an entire organism's proteome is labeled via sustained dietary intake of heavy amino acids[60]. Such an experimental setup could be used to evaluate remodeling of a tissue engineered graft by host cells. Beyond the realm of tissue engineering, this method could also be applied to a variety of three-dimensional and organotypic culture systems, such as are used in developmental biology[61] and cancer biology[62].

Supplementary Material

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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 $^{13}\text{C}_6$ -Lys and $^{13}\text{C}_6$ -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.

Fig. 2. Comparison of five decellularization strategies. (a) Flowchart summarizing the five strategies. (b) Quantitative hydroxyproline and sulfated glycosaminoglycan (sGAG) concentrations for each decellularization strategy compared to native tissue control ($n = 3$ biological replicates, each with $n = 3$ technical replicates). *, $P < 0.05$ versus native condition; n.s., non-significant difference; error bars, s.e.m. (c) Pentachrome-stained sections of native and decellularized porcine VFM. Collagen is yellow; elastin is black; arrows indicate residual cells. H&E- and Alcian Blue-stained sections are shown in Supplementary Fig S1. Scale bar, 50 μ m.

Fig. 3.

Comparison of five reseeding strategies. (a) Schematic illustrating the five strategies plus negative control condition. (b) H&E-stained sections of recellularized porcine VFM, 3 w post-seeding. No seeding control is also shown. Tissue orientation matches that shown in panel (a); cells were seeded on the tissue surface corresponding to the top of each image. Arrows indicate cells lodged at the basement membrane in strategy 1; dashed lines indicate polymerized collagen in strategy 5. Scale bar, 100 μ m. (c) Mean and maximum cell migration observed for each seeding strategy, 3 w post-seeding ($n = 3$ biological replicates, each with $n = 3$ technical replicates). *, $P < 0.05$ compared to both strategy 3 and 4; n.s., non-significant difference; error bars, s.e.m. (d) H&E-stained sections of recellularized human VFM (strategy 3), 3 and 6 w post-seeding. No seeding control is also shown. Scale bar, 100 μ m.

Fig. 4. Proteomic analysis of native, decellularized, and recellularized (6 w post-seeding) human VFM. (a) Venn diagram summarizing number of protein identifications for each condition. (b) Functional enrichment analysis of the 160 proteins exclusively identified in the recellularized condition. Enriched gene ontology terms are depicted as nodes connected by arrows that represent hierarchies and relationships between terms. Node size is proportional to the number of proteins assigned to a given term; node color represents the Benjamini Hochberg-corrected P value corresponding to enrichment of the term. Functionally related terms are labeled and grouped using green ovals. Biogenesis terms are enlarged for better visualization. (c) Venn diagram summarizing number of matrisome protein identifications for each condition. (d) Stacked bar graph showing distribution of MS intensity among the six matrisome subcategories.

Fig. 5.

Percentage of light or heavy protein intensity, out of total protein intensity of each sample at each time point, for (a) matrisome proteins and (b, c) cellular proteins during 6 w in culture ($n = 4$ biological replicates, each with $n = 2$ technical replicates). The 0 w, and 3 w and 6 w control samples were generated by mixing cells and scaffold at the peptide level. *, $P < 0.05$ for comparisons between experimental and control samples at 3 and 6 w; error bars, s.e.m.

Fig. 6.

Synthesis of matrisome proteins. (a) Fold change in heavy (newly synthesized) protein intensity for each of the six matrisome subcategories compared to 0 w control. Turnover is shown for proteins associated with the following core matrisome subcategories: (b) collagens, (c) ECM glycoproteins, and (d) proteoglycans. Turnover is plotted as the percentage of heavy intensity out of total intensity for each individual protein. (e) Percentage of heavy, light, or total (heavy + light) intensity for the proteoglycan decorin (DCN), out of total protein intensity of each sample at each time point. (f) Immunoblots showing total DCN abundance. MS data (a-e) were collected on $n = 4$ biological replicates, each with $n = 2$ technical replicates; immunoblots (f) were performed using $n = 3$ biological replicates; error bars, s.e.m. (b-e). The discrepancy in initial time point in panels (e) and (f) is because the 0 w MS data were generated following peptide-level sample preparation, meaning that no directly comparable protein samples were available for immunoblotting.