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Proteomic analysis of a decellularized human vocal fold mucosa scaffold using 2D electrophoresis and high-resolution mass spectrometry

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Abstract

Natural biologic scaffolds for tissue engineering are commonly generated by decellularization of tissues and organs. Despite some preclinical and clinical success, *in vivo* scaffold remodeling and functional outcomes remain variable, presumably due to the influence of unidentified bioactive molecules on the scaffold-host interaction. Here, we used 2D electrophoresis and high-resolution mass spectrometry-based proteomic analyses to evaluate decellularization effectiveness and identify potentially bioactive protein remnants in a human vocal fold mucosa model. We noted proteome, phosphoproteome and *O*-glycoproteome depletion post-decellularization, and identified >200 unique protein species within the decellularized scaffold. Gene ontology-based enrichment analysis revealed a dominant set of functionally-related ontology terms associated with extracellular matrix assembly, organization, morphology and patterning, consistent with preservation of a tissue-specific niche for later cell seeding and infiltration. We further identified a subset of ontology terms associated with bioactive (some of which are antigenic) cellular proteins, despite histological and immunohistochemical data indicating complete decellularization. These findings demonstrate the value of mass spectrometry-based proteomics in identifying agents potentially responsible for variation in host response to engineered tissues derived from decellularized scaffolds. This work has implications for the manufacturing of biologic scaffolds from any tissue or organ, as well as for prediction and monitoring of the scaffold-host interaction *in vivo*.

Keywords

biocompatibility; extracellular matrix; larynx; tissue engineering

1. Introduction

Decellularization of tissues and organs is widely employed for generating natural biologic scaffolds for tissue engineering. Decellularized scaffolds are appealing because they provide tissue/organ-specific extracellular cues that can help direct seeded and infiltrating cells

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toward a target phenotype [1,2], as well as geometrical and biomechanical properties that closely resemble the native construct. Progress in this area over the past 20 years has led to significant translational advances, including the routine clinical use of xenogeneic and allogeneic scaffolds as sheet and mesh materials for surgical reconstruction [3], the restoration of a human patient's airway following transplantation of a recellularized 3D tracheal segment to replace a severely impaired mainstem bronchus [4], and the regeneration of complex vascularized 3D organs including heart [5], lung [6,7] and liver [8] in preclinical models.

Despite these preclinical and clinical successes, *in vivo* scaffold remodeling and functional outcome remain difficult to predict. Evidence suggests that residual bioactive molecules within the decellularized scaffold drive the host immune response towards either favorable tissue regeneration and remodeling, or towards chronic inflammation, scaffold encapsulation and fibrosis; however, the specific molecules and mechanisms involved in this process are poorly understood [9]. Initial attention has been given to obvious targets such as DNA [10] and cell surface protein remnants [9], ligands at the scaffold surface [11,12], the α -gal epitope (for xenogeneic scaffolds intended for human transplantation) [13], and damage associated molecular pattern (DAMP) molecules [14]; as well as the relationships between these molecules, infiltrating leukocyte immunophenotype, and functional scaffold outcome [14-16]. Still, it remains unknown whether additional scaffold-specific molecules help to promote tolerance and remodeling, or contribute to undesirable host response and failed outcomes.

Scaffold decellularization is typically evaluated by preservation of extracellular matrix (ECM) protein/glycan constituents and architecture, along with removal of major nuclear and membrane proteins, particularly those with known antigenicity [5-8]. Mass spectrometry (MS)-based proteomic methodologies offer the ability to characterize low-abundance molecules that may hold an outsized influence on scaffold performance, and initial reports suggest that these approaches are feasible with decellularized materials [17,18]. The purpose of this study was to evaluate decellularization effectiveness at the proteome level, using human vocal fold (VF) mucosa as a model. VF mucosa presents a particularly worthy challenge for both tissue engineering and proteomics, as it has an exquisite, purpose-specific ECM that is critical to sustained oscillation for voice production [19,20]. We used 2D electrophoresis, high-resolution MS, and gene ontology-based enrichment analysis to probe the decellularized VF mucosa for potentially antigenic protein remnants that might account for variation in host response to this biologic scaffold.

2. Materials and methods

2.1. Tissue decellularization

VF mucosae were microdissected from fresh human cadaveric larynges (5 male, 3 female; age 51-82 years at death) obtained 3-48 h postmortem. Care was taken to remove all visible thyroarytenoid muscle fibers from each specimen. One VF mucosal sample per larynx was randomly assigned to the decellularization protocol; the contralateral sample was used as a within-subject native tissue control.

Tissue decellularization was performed using a previously validated detergent-free approach based on repeat cycles of osmotic stress, nuclease treatment and ethanol dehydration [21]. Briefly, samples were immersed in 3 M NaCl for 24 h at room temperature (RT) to induce osmotic stress, treated with 25 mg/mL DNase I and 10 mg/mL RNase A (Sigma, St Louis, MO) in PBS containing an EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) for 24 h at 37° C, and then placed in 70% ethanol for a further 24 h at RT. Next, the samples were subjected to a second round of DNase and RNase digestion for 48 h

at 37° C, followed by a final wash in PBS for 24 h at RT. All samples were subjected to continuous mechanical agitation to aid reagent infusion and incubated with 1000 U/mL penicillin and 1 mg/mL streptomycin (Sigma) to prevent microbial contamination.

2.2. Protein extraction and quantification

Samples were minced, immersed in protein solubilization buffer (4 M guanidine HCl, 65 mM DTT, 10 mM EDTA in 50 mM sodium acetate; pH 5.8) [22] with an EDTA-free protease inhibitor cocktail (Roche) and subjected to ultrasonic homogenization (300V/T; Biologics, Manassas, VA) on ice for repeated 3 min cycles at 40% power and 50% pulse rate. Following overnight incubation at 4° C, samples were then centrifuged once at 13,000 *g* for 10 min at 4° C to remove insoluble material, and again with the addition of a 100 kDa cut-off filter (Millipore, Billerica, MA), used according to the manufacturer's instructions to deplete high M_r glycans known to impair isoelectric focusing (IEF) during 2D sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [23,24]. Next, the filtrate was subjected to ethanol precipitation and reconstituted in 300 μ L SDS buffer (5% SDS, 10% glycerol, 60 mM Tris; pH 6.8). Total protein was quantified using the bicinchoninic acid (BCA) assay (Pierce Biotech, Rockford, IL).

2.3. Electrophoresis and image analysis

1D SDS-PAGE was performed using a 4-20% precast acrylamide gradient gel (BioRad, Hercules, CA) with 10 μ g protein load per well. All 1D gels were Coomassie Brilliant Blue (CBB)-stained. 2D SDS-PAGE was performed using carrier ampholine (pH 3.5–10) isoelectric focusing in a glass tube, followed by M_r separation on a 10% acrylamide slab gel, as previously described [25,26]. Protein load was 50 μ g for gels intended for silver staining and image analysis ($n = 5$ biological replicates per condition), 200 μ g for gels intended for polyvinylidene difluoride (PVDF) membrane transfer and immunoblotting ($n = 5$ biological replicates per condition), and 200 μ g for gels intended for CBB staining and spot excision for liquid chromatography (LC)-MS/MS (protein pooled across 5 biological replicates per condition).

2D gels were scanned (200 dpi, 24-bit image depth) and analyzed using Melanie 4.02 (GeneBio, Geneva, Switzerland) according to a previously reported protocol [25]. Automatic spot detection was performed using 2 smoothing passes, a 3.5 saliency value, and 18 pixels minimum spot area. Spot matching was performed both within and across native and decellularized conditions. Two expert gel analysts independently performed spot-by-spot inspection and correction of algorithmic detection/matching errors. Differences between analysts were resolved by discussion until consensus was reached. Synthetic gels were constructed using spots that were consistently detected and matched across all constituent gels. Spot intensity values were normalized to account for protein loading and staining variations across gels. Estimated M_r values for identified spots were determined by logarithmic interpolation and extrapolation of values for a series of M_r standards loaded at the basic edge of each gel. Estimated *pI* values were determined by linear interpolation and extrapolation of surface pH measurements taken from four blank IEF tube gels.

2.4. Mass spectrometry

2D gel spots of interest were excised from a CBB-stained gel containing pooled protein from all decellularized VF samples. A superimposed silver-stained replicate gel was used to guide the excision of faint spots. Surfactant-enhanced in-gel tryptic digestion was performed using ProteaseMAX™ (Promega, Madison, WI) and the manufacturer's protocol optimized for low protein amounts. Briefly, gel spots were washed, destained with 50% MeOH (if visibly blue), dehydrated with acetonitrile, treated with 50 mM dithiothreitol (DTT) for 30 min at 37° C, treated with 55 mM iodoacetamide for 20 min at RT in the dark, rewashed,

dehydrated, transferred to a LoBind tube (Eppendorf, Hauppauge, NY), and incubated with 50 ng trypsin (Promega) in 20 μ L of 25 mM ammonium bicarbonate (pH ~8.5) containing 0.01% ProteaseMAX™ at 37°C for 3 h. Next, the solutions containing released peptides were transferred to new LoBind tubes using low-retention pipet tips. The gel spots were then rinsed with 15 μ L of 2.5% trifluoroacetic acid, and the rinses added to the peptide solutions. Finally, samples were centrifuged at 14,000 g for 10 min and the supernatants transferred to autosampler vials.

Nine μ L of sample was injected into a high-performance liquid chromatography-electrospray ionization (HPLC-ESI)-MS/MS capillary system consisting of a Waters nanoAcquity HPLC (Milford, MA) connected to an ESI ion-trap/orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Scientific, San Jose, CA). Sample loading occurred for 20 min at 1 μ L/min onto a trapping column prepared by packing 5 cm of 5 μ m-diameter 300Å-pore C18 beads (Western Analytical Products, Murrieta, CA) into a 75 $\times$ 365 μ m fused silica capillary having a frit at one end. The analytical column (50 $\times$ 365 μ m) was packed with 15 cm of the same C18 beads, but instead of packing against a frit, the capillary tip was pulled to ~1 μ m with a P-2000 laser puller (Sutter Instruments, Novato, CA). Peptides were eluted at 0.2 μ L/min in an aqueous mobile phase containing 0.1 % formic acid with a gradient of increasing acetonitrile (2-15 % in the first 15 min, increasing to 45 % in the next 75 min). A full-mass scan (150-2000 m/z) was performed in the orbitrap at a resolution of 60,000. The six most intense peaks from the full scan were selected for fragmentation by both collision induced dissociation (CID) and high-energy collision dissociation (HCD) at 30% and 42% relative collision energies, respectively. The isolation width for the parent ions was 3.0 m/z , and the product ions from fragmentation were analyzed in the orbitrap detector at a resolution of 7500.

MS/MS samples were analyzed using Mascot 2.2.07 (Matrix Science, London, UK) and a database containing forward and reverse sequences for tryptic peptides derived from the UniProt homo sapiens database (<http://www.uniprot.org/taxonomy/9606>; downloaded August 2010, 70,130 entries). Searches were performed using a fragment ion mass tolerance of 0.050 Da, parent ion tolerance of 10.0 ppm, methionine oxidation as a variable modification, iodoacetamide derivative of cysteine as a fixed modification, and a maximum of one missed cleavage. Scaffold 3.00.07 (Proteome Software, Portland, OR) was used to validate MS/MS-based peptide and protein identifications: positive identifications were defined by a Peptide Prophet probability score above 95% [27], Protein Prophet probability score above 99% [28], and minimum of two peptide hits per protein. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principle of parsimony.

2.5. Immunoblotting

Gels ($n = 5$ biological replicates per condition) were placed in transfer buffer (10 mM CAPS, 10% methanol; pH 11.0) and electrotransferred to PVDF membranes overnight. Non-specific sites were blocked using 5% bovine serum albumin (BSA) in 0.05% Tween-20 Tris-buffered saline (TTBS) for 2 h, and then blots were washed in TTBS. Each blot was incubated with the primary antibody diluted in 2% BSA in TTBS overnight, followed by the secondary antibody diluted in 2% BSA (jacalin lectin) or 2% nonfat dry milk (all other blots) in TTBS for 2 h. Blots were washed three times for 10 min in TTBS following each incubation. Following the final wash, blots were treated with an enhanced chemiluminescence substrate (Pierce Biotech), and exposed to x-ray film.

Phosphotyrosine was detected using mouse anti-phosphotyrosine clone PY20 (1:1,000; $\times$ 1021, Exalpha Biologicals, Shirley, MA) followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:2,000; NA931V, GE Healthcare, Piscataway, NJ).

Phosphoserine/phosphothreonine were detected using mouse anti-phosphoserine clone Q5 (1:10,000; 37430, Qiagen, Valencia, CA) and mouse anti-phosphothreonine clone Q7 (1:10,000; 37420, Qiagen) followed by HRP-conjugated anti-mouse IgG (1:100,000; NA931V, GE Healthcare). Jacalin lectin was detected using biotin-conjugated artocarpus integrifolia lectin (0.25 µg/mL; BA-6301-5, EY Laboratories, San Mateo, CA) followed by poly-HRP-conjugated streptavidin (1:2,000; N200, Pierce Biotech).

2.6. Histology and immunohistochemistry

Tissue samples ($n = 3$ biological replicates per condition) were dehydrated using 25% sucrose in PBS overnight at 4° C, embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetech, Tokyo, Japan), and snap-frozen using acetone and dry ice. Eight µm cryosections were prepared in the coronal plane using a cryostat. Collagen content and organization were examined using Masson's trichrome (American MasterTech, Lodi, CA) according to the manufacturer's instructions. Sections intended for immunostaining were fixed with either 2% paraformaldehyde or acetone for 10 min at RT, washed with PBS, and blocked with 5% BSA for 1 h at RT. Sections were sequentially incubated with primary and then relevant secondary antibodies for 1 h each at RT, counterstained with 2 µg/mL DAPI (MP Biomedicals, Santa Ana, CA) for 15 min at RT, covered with antifade mounting medium (Vectashield, Vector Labs, Burlingame, CA), and coverslipped. Slides were washed thoroughly between each incubation step. Imaging was performed using a microscope with both bright field and fluorescent capabilities (E-600; Nikon, Melville, NY) equipped with a digital microscopy camera (DP-71; Olympus, Center Valley, PA). All images were captured with consistent exposure settings.

The primary antibodies used were mouse anti-collagen, type IV clone COL-94 (1:1,000; C1926, Sigma); rabbit anti-MHC-I clone H-300 (1:100; sc-25619, Santa Cruz Biotech, Santa Cruz, CA); mouse anti-MHC-II (1:100; ab55152, Abcam, Cambridge, MA); goat anti-dermatopontin clone S-14 (1:100; sc-160280, Santa Cruz Biotech); goat anti-biglycan clone L-15 (1:50; sc-27936, Santa Cruz Biotech); rabbit anti-lamin B2 clone H-75 (1:100; sc-134477, Santa Cruz Biotech). The secondary antibodies used were Texas Red goat anti-mouse IgG (1:500; 115075166, Jackson ImmunoResearch, West Grove, PA); Alexa 555 donkey anti-goat IgG (1:600; A21432, Invitrogen, Carlsbad, CA); and Alexa 594 goat anti-rabbit IgG (1:600; A11012, Invitrogen). Control sections stained with an isotype control, without the primary antibody, or without the secondary antibody, ensured each immunosignal was specific to the intended antigen.

2.7. Statistical analysis

2D gel spot normalized intensity data were analyzed using a paired *t*-test. Positive protein identifications from the MS database searches were based on the Peptide/Protein Prophet algorithms [27,28] and corresponded to estimated false discovery rates of 1.1% for peptides and 0.1% for proteins. Gene ontology term enrichment was calculated using BiNGO 2.44 [29] with implementation of a hypergeometric model and Benjamini-Hochberg correction to account for multiple testing. UniProt accession numbers were used as input data and evaluated against the entire ontology term set using homo sapiens annotation. Ontology term enrichment schematics were generated using Cytoscape 2.8.2 [30]. An initial (pre-correction) type I error rate of 0.01 was used for all statistical testing.

3. Results

3.1. Structural features of decellularized VF mucosa

The decellularized VF mucosa was evaluated first using standard histological and immunohistochemical approaches, as a benchmark for subsequent proteomic analyses.

Mucosae were dissected from human cadaveric larynges (Fig. 1A) and decellularized using a previously-reported detergent-free protocol based on repeat cycles of osmotic stress, nuclease treatment and ethanol dehydration [21]. We observed generally intact extracellular architecture with retention of lamina propria and epithelial collagen (Fig. 1B and C), and type IV collagen-positive subepithelial (arrows in Fig. 1D and E) and vascular (arrowheads in Fig. 1D and E) basement membrane structures. Cell nuclei were absent on both Masson's trichrome (Fig. 1C) and DAPI (Fig. 1G) stained sections. MHC class I (MHC-I) and class II (MHC-II) antigen-presenting proteins were expressed by both epithelial (arrows in Fig. 1H and J) and lamina propria (arrowheads in Fig. 1H and J) cells in the native mucosa, but were not detected in the decellularized construct (Fig. 1I and K).

3.2. Proteome simplification following tissue decellularization

To identify system-wide changes associated with the decellularization process, we performed 1D and 2D SDS-PAGE of native and decellularized VF mucosa protein extracts. As expected, our decellularization protocol resulted in simplification of the VF mucosa proteome (Fig. 2A-E), presumably due to depletion of its cellular components. Image analysis followed by synthetic composite gel construction resulted in successful matching of 66 protein spots that were consistently identified in all gels representing the decellularized condition (Fig. 2E), with significantly decreased mean protein abundance (inferred from mean spot intensity) compared to the native condition (Fig. 2F). Evaluation of posttranslational phosphorylation and glycosylation status revealed near-universal depletion of phosphotyrosine, phosphoserine, phosphothreonine and jacalin lectin (*O*-glycosylation) signals on 2D immunoblots post-decellularization (Fig. 3).

3.3. Protein species identification and gene ontology term enrichment

To fully characterize the decellularized VF mucosa proteome, we pursued high-resolution LC-MS/MS-based identification of all protein species within the 66 spots consistently resolved by 2D SDS-PAGE, as well as four blank gel regions (Fig 2E). From these 70 independent LC-MS/MS runs, we confidently assigned 73 unique proteins to 58 of the 66 gel spots of interest (Table S1). Spot assignment involved manual analysis of protein identification data from each MS run. Protein assignments to a spot were based on the number of assigned spectra and degree of correspondence of experimental M_r and pI to theoretical values (accounting for the possibility of protein fragmentation in cases where all peptide assignments were clustered at a single terminus). This strict assignment of proteins to individual 2D gel spots resulted in a list of 73 proteins; however a number of confident protein identifications were ignored during this process due to a severe mismatch between experimental and theoretical M_r and pI values. While 2D SDS-PAGE is a powerful technique for protein separation and visualization, it has inherent proteome characterization challenges due to: (i) co-migration of multiple proteins to a single spot (the majority of our gel spots contained >20 proteins each, while several high-abundance proteins were each detected in >50 gel spots), (ii) differential migration due to posttranslational processing, and (iii) fragmentation of high M_r (e.g., ECM) proteins. Therefore, we generated a comprehensive proteome library by compiling the confidently identified proteins from all 70 LC-MS/MS runs, irrespective of 2D gel assignment. These 219 protein identifications (from 38,602 spectra) are presented in Table S2 and comprise the decellularized VF mucosa proteome.

Next, we subjected both MS datasets (the subset of proteins assigned to 2D gel coordinates, and the entire decellularized VF mucosa proteome) to gene ontology-based enrichment analysis to identify overrepresented functional categories (ontology terms). Both datasets revealed greater enrichment of *cellular component* terms and lesser enrichment of *biological process* and especially *molecular function* terms, consistent with decellularization (Fig. 4,

Figs. S1-S3, Tables S3-S8). The *cellular component* terms identified in the subset of proteins assigned to 2D gel coordinates clustered into two primary functional groups associated with the ECM and muscle contractile fiber apparatus (Fig. 4, Table S3); parallel analysis using the entire proteome list yielded greater resolution of deep ontology terms/subcategories within these groups, as well as secondary clusters of enriched terms related to the cytoskeleton (e.g., intermediate filament), epithelial protein complexes (e.g., desmosome, adherens junction) and certain residual membrane-bound organelles (e.g., melanosome, mitochondrion) (Fig. S1, Table S4).

The most highly enriched *biological process* terms in the subset of proteins assigned to 2D gel coordinates related to muscle contractile function (Fig. 4, Table S5). Consideration of the entire proteome (Fig. S2, Table S6) revealed additional term clusters associated with tissue/organ patterning and structure (e.g., developmental process, tissue development, anatomical structure morphogenesis) and protein assembly/organization (e.g., macromolecular complex assembly, protein tetramerization, ECM organization). While these ontology terms are consistent with favorable decellularization, we also identified terms that are potentially unfavorable for biologic scaffold outcome, such as: oxidative stress response, biotic stimuli response, cell killing and leukocyte-mediated immunity. Closer inspection of the individual protein species driving enrichment of these terms highlighted proteins with both protective and potentially alloantigenic functions. We identified the antioxidant enzymes peroxiredoxin 1, 2 and 4 (which are also documented xenoantigens [31]), Cu/Zn superoxide dismutase and glutathione peroxidase 3; proteins with antimicrobial and immunomodulatory function, including lysozyme C, neutrophil defensin 1 (which also holds mammalian cytotoxic capacity [32]), dermcidin and lactoperoxidase; and the alpha chain of C4b-binding protein, which acts as a complement system inhibitor [33] (Table S2). We further identified the reported (auto)antigenic proteins serum albumin, haptoglobin, annexin A5, alpha-enolase, lamin A/C and lamin B2 [31,34-38].

Several enriched *molecular function* terms paralleled those identified under the *cellular component* and *biological process* domains, including structural molecule activity specific to the ECM, muscle and cytoskeleton; protein binding, including to MHC-I (although this term [GO:0042288] does not encompass binding to an antigen presented by the MHC-I protein); and oxidoreductase activity specific to the peroxiredoxin enzymes (Fig. S3, Tables S7 and S8). We further identified enriched terms related to serine-type endopeptidase inhibition and GTPase activity.

3.4. Validation of select MS-based protein identifications

Next, we performed immunohistochemical validation of two proteins and one proteoglycan identified as retained in decellularized VF mucosa by LC-MS/MS. Our candidates were selected to represent a range of biologic functions and ontology terms, localization to the cell nucleus, cytoplasm and extracellular space, and various regions within the native VF mucosa. In native mucosa, the ECM and cell adhesion protein dermatopontin was localized to cells in the lamina propria (arrowheads in Fig. 5A) and epithelium (arrows in Fig. 5C), with greater immunosignal in the superficial epithelial cell layers (Fig. 5B). Biglycan, a small leucine-rich proteoglycan generally found in the ECM but also reported to have nuclear localization/function in certain cell populations [39,40], appeared as extracellular signal in the subepithelial lamina propria (arrowheads in Fig. 5F) and within epithelial cell nuclei (arrows in Fig. 5H). Lamin B2, an intermediate filament protein of the nuclear lamina and nucleoplasm, but which disassembles and is released to the cytoplasm during mitosis [41], was detected in both lamina propria (arrowheads in Fig. 5K) and epithelial cells (arrows in Fig. 5M), with greater cytoplasmic localization in the superficial epithelial cell layers (Fig. 5L). Consistent with our MS data, dermatopontin, biglycan and lamin B2 all demonstrated positive immunosignals in decellularized VF mucosa (arrowheads in Fig. 5E,

J, O), typically localized to the subepithelial region deep to the basement membrane (Fig. 5D, I, N). We did not identify positive epithelial signals in any samples.

4. Discussion

In this study, we applied high-resolution proteomic and functional enrichment tools to the analysis of a decellularized biologic scaffold intended for therapeutic use. We observed depletion of the VF mucosa proteome, phosphoproteome and *O*-glycoproteome after decellularization, and identified >200 unique protein species within the decellularized scaffold. Enrichment analysis revealed a dominant set of ontology terms associated with ECM assembly, organization, morphology and patterning, consistent with preservation of a tissue-specific niche for later cell seeding and infiltration. Importantly however, we also identified a subset of ontology terms associated with bioactive (some of which are antigenic) cellular proteins, despite histological and immunohistochemical data indicating complete decellularization. This finding demonstrates that MS-based proteomics can provide meaningful biological insight in cases where traditional assays have limited sensitivity, such as the identification of agents and mechanisms potentially responsible for variation in outcomes after *in vivo* scaffold transplantation.

Previous work has shown that decellularized VF mucosa prepared using the protocol reported here induces a host immune response characterized by granulocyte, macrophage and lymphocyte recruitment in a xenotransplantation model [42]. The bioactive proteins identified in our dataset are potential drivers of this host immune response and therefore candidates for further investigation; however, additional work is required to identify mechanistic links between a given candidate protein, humoral or cell-mediated immune response, and functional scaffold performance *in vivo*. Our enrichment analysis suggests that certain residual proteins may confer protective (e.g., antioxidant, antimicrobial) functions, whereas others may promote an adverse immune response and scaffold rejection. Of note, a subset of the proteins found in this study have been identified as antigenic in other experimental systems using other techniques [31,34-38]. Given the large number of candidate proteins in a typical proteomic dataset, individual antigen response and functional testing may be facilitated if preceded by high-throughput screening and computational modeling of predicted responses, narrowing the need for *in vivo* experimental testing to a smaller number of high-probability candidates.

The proteome of any decellularized scaffold is unique to both the source tissue and decellularization strategy employed; as such, proteomic data offer scaffold-specific insight into the strengths and limitations of a given strategy. In this study, we utilized a relatively gentle detergent-free protocol in an attempt to remove cellular materials with minimal ECM disruption, based on prior decellularization data [21] and well-accepted evidence that the oscillatory capacity of VF mucosa is dependent upon an intact ECM [19,20]. It is clear from our proteomic data, however, that this protocol results in incomplete removal of cellular proteins. The identification of proteins and ontology terms associated with the muscle contractile apparatus, also reported previously in the native VF mucosa proteome [43], suggests the presence of invasive thyroarytenoid muscle fibers, despite no evidence of muscle disturbance during tissue microdissection. Further, the identification of mitochondrial and melanosome proteins suggests that a detergent step might be needed to more effectively solubilize these membrane-bound organelles [44,45]. The addition of a detergent step, possibly in combination with pressure cycling or perfusion delivery, might also aid the removal of typically adherent cytoskeletal proteins such as actin and myosin. Based on these observations, we are currently pursuing proteome-guided refinement of VF mucosa decellularization.

The 2D gel workflow used in this study provided the dual benefits of extensive sample fractionation prior to LC-MS/MS and parallel immunoprofiling of system-wide phosphorylation and glycosylation. Drawbacks to this 2D gel-based approach include its low throughput, the need for extensive handling that carries an associated risk of sample contamination, and the necessity of removing high M_r glycans (and consequently proteins) prior to the isoelectric focusing step. Given these constraints, we are exploring alternative sample preparation strategies that improve the solubility and detectability of all proteins, including high M_r ECM proteins and glycans. These approaches, combined with fractionation at the peptide level, promise to enable ever-wider proteome coverage of decellularized materials.

5. Conclusions

Our findings demonstrate the value of MS-based proteomic data in both fully characterizing the ECM network, and identifying agents potentially responsible for variation in host response to engineered tissues derived from decellularized scaffolds. This work has implications for the manufacturing of biologic scaffolds from any tissue or organ, as well as for the prediction and monitoring of the scaffold-host interaction *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Histological and immunohistochemical characterization of decellularized human VF mucosa. Native mucosae (A) were dissected from fresh cadaveric larynges, frozen, thawed and subjected to repeat cycles of osmotic stress (alternating hypertonic and isotonic treatments), nuclease treatment and ethanol dehydration. Frozen coronal sections were stained with Masson's trichrome to identify collagen fibers (blue in B, C), antibodies anti-type IV collagen (red in D, E), anti-MHC-I (red in H, I) and anti-MHC-II (red in J, K), and nuclear dye DAPI (blue in F, G). The type IV collagen⁺ subepithelial basement membrane is indicated by arrows, and type IV collagen⁺ vascular basement membrane structures are indicated by arrowheads, in D, E. DAPI⁺ epithelial cell nuclei are indicated by arrows, and DAPI⁺ lamina propria cell nuclei are indicated by arrowheads, in F. MHC-I⁺ epithelial cells are indicated by arrows, and MHC-I⁺ lamina propria cells are indicated by arrowheads, in H. MHC-II⁺ epithelial cells are indicated by arrows, and MHC-II⁺ lamina propria cells are indicated by arrowheads, in J. Dashed contour lines in B-K indicate the boundary of the epithelium (right) and its underlying lamina propria (left). Scale bar = 2 mm in A; 50 μ m in B-K.

Figure 2.

Decellularization simplifies the human VF mucosa proteome. Total protein extracts from native and decellularized mucosae were separated by 1D (A) and 2D SDS-PAGE (B, C). Silver-stained 2D gels were subjected to protein spot detection, spot matching, and construction of synthetic composite gels (D, E). Synthetic gels were constructed using 5 biological replicate input gels per experimental condition. Sixty-six spots, spanning $13\text{--}77 \times 10^3 M_r$ and $4.9\text{--}6.8 pI$, were consistently identified in all gels representing the decellularized condition (E). For the subset of gel spots matched to both experimental conditions, quantitative analysis revealed a significant decrease in mean spot intensity post-decellularization (F). Spots 1-66 in E indicate those selected for excision, digestion, and LC-MS/MS-based protein identification (reported in Table S1); spots B1-B4 represent blank gel regions used as control samples for LC-MS/MS.

Figure 3. Decellularization causes near-universal depletion of the human VF mucosa phosphoproteome and *O*-glycoproteome. Total protein extracts from native and decellularized mucosae were separated by 2D SDS-PAGE, electrotransferred to PVDF membranes, and immunoblotted using antibodies anti-phosphotyrosine (A, B), anti-phosphoserine/phosphothreonine (C, D), and jacalin lectin (E, F).

Figure 4.

Gene ontology terms enriched in the decellularized human VF mucosa proteome. 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 ontology term, whereas node color represents the corrected p -value (Benjamini-Hochberg false discovery rate correction) corresponding to enrichment of the term. This schematic represents the subset of proteins assigned to the synthetic composite 2D gel presented in Figure 2E, and for clarity, is limited to highly significant terms ($p < 10^{-4}$). Complete lists of enriched terms ($p < 10^{-2}$) are provided in Tables S3 (*cellular component*), S5 (*biological process*), and S7 (*molecular function*).

Figure 5.

Immunohistochemical validation of representative extracellular and cellular proteins/ proteoglycan identified as retained in the decellularized human VF mucosa, using LC-MS/ MS. Frozen coronal sections were stained with antibodies anti-dermatopontin (red in A-E), anti-biglycan (red in F-J), anti-lamin B2 (red in K-O), and nuclear dye DAPI (blue in A-O). Dermatopontin⁺ native lamina propria cells are indicated by arrowheads in A; dermatopontin⁺ native epithelial cells are indicated by arrows in C; retained dermatopontin⁺ structures are indicated by arrowheads in E. Biglycan⁺ subepithelial extracellular structures in the native lamina propria are indicated by arrowheads in F; biglycan⁺ native epithelial cell nuclei are indicated by arrows in H; retained biglycan⁺ structures are indicated by arrowheads in J. Lamin B2⁺ native lamina propria cells are indicated by arrowheads in K; lamin B2⁺ native epithelial cells are indicated by arrows in M; retained lamin B2⁺ structures are indicated by arrowheads in O. In B, D, G, I, L, N, dashed white contour lines indicate the boundary of the epithelium (right) and its underlying lamina propria (left); dashed red boxes indicate regions that have been enlarged for better illustration in A, C, E, F, H, J, K, M, O. Scale bar = 15 μm in A, C, E, F, H, J, K, M, O; 50 μm in B, D, G, I, L, N.