Published in final edited form as:

Proteomics Clin Appl. 2016 January ; 10(1): 75–83. doi:10.1002/prca.201500048.

Quantification of Decellularized Human Myocardial Matrix: A Comparison of Six Patients

Todd D. Johnson^{#1}, Ryan C. Hill^{#2}, Monika Dzieciatkowska², Vishal Nigam³, Atta Behfar⁴, Karen L. Christman^{1,*}, and Kirk C. Hansen^{2,*}

¹Department of Bioengineering, Sanford Consortium for Regenerative Medicine, University of California at San Diego, La Jolla, CA, USA

²Department of Biochemistry and Molecular Genetics, School of Medicine, University of Colorado, Aurora, CO, USA

³Department of Pediatrics (Cardiology), University of California San Diego and Rady Children's Hospital, San Diego, La Jolla, CA, USA

⁴Division of Cardiovascular Diseases, Mayo Clinic, Rochester, MN, USA

These authors contributed equally to this work.

Abstract

Purpose—The purpose of this study was to characterize and quantitatively analyze human cardiac extracellular matrix (ECM) isolated from six different cadaveric donor hearts.

Experimental Design—ECM was isolated by decellularization of six human cadaveric donor hearts and characterized by quantifying sulfated glycosaminoglycan content (sGAG) and via polyacrylamide gel electrophoresis (PAGE). The protein content was then quantified using ECM-targeted Quantitative conCATamers (QconCAT) by Liquid Chromatography - Selected Reaction Monitoring (LC-SRM) analysis using 83 stable isotope labeled (SIL) peptides representing 48 different proteins. Non-targeted global analysis was also implemented using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Results—The sGAG content, PAGE, and QconCAT proteomics analysis showed significant variation between each of the six patient samples. The quantitative proteomics indicated that the majority of the protein content was composed of various fibrillar collagen components. Also, quantification of difficult to remove cellular proteins represented less than 1% of total protein content, which is very low for a decellularized biomaterial. Global proteomics identified over 200 distinct proteins present in the human cardiac ECM.

Conclusions and Clinical Relevance—In conclusion, quantification and characterization of human myocardial ECM showed significant patient-to-patient variability between the six investigated patients. This is an important outcome for the development of allogeneic derived biomaterials and for increasing our understanding of human myocardial ECM composition.

*Corresponding Authors: Karen L. Christman, PhD, Sanford Consortium for Regenerative Medicine, Room 2006, 2880 Torrey Pines Scenic Drive, La Jolla, CA 92037, Telephone: (858) 822-7863, Fax: (858) 534-5722, Christman@eng.ucsd.edu; Kirk C. Hansen, PhD, University of Colorado, RC-1S, L18-9403J, 12801 E 17th Ave., Aurora, CO 80045, Telephone: (303) 724-5544, Fax: (303) 724-1580, Kirk.Hansen@UCDenver.edu.

Keywords

Decellularization; Extracellular Matrix; Mass Spectrometry; Proteomics; QconCAT

1. Introduction

Decellularized biomaterials have been designed to treat numerous diseases and applied towards regenerative medicine therapies either as a patch, an implantable scaffold, or an injectable hydrogel [1, 2]. Some of these materials have been derived from human tissues as either autologous or allogeneic biomaterials containing a complex composition of human proteins, proteoglycans, and glycosaminoglycans. Due to the complexity and physical properties, the composition of these materials is challenging to identify and quantify. Here we were interested in quantifying the composition of decellularized myocardial ECM derived from six different human patients to characterize the biomaterials and ascertain potential patient-to-patient differences as well as provide a greater understanding of human myocardial ECM protein composition.

Significant patient-to-patient variability in proteins from human derived biomaterials has previously been reported, and this can potentially be correlated with differences in genetics, diet, environment, and shifts due to aging [3-5]. This variability was recently observed in a study comparing an injectable hydrogel derived from human myocardial ECM to a previously investigated hydrogel derived from porcine myocardial ECM [4], both of which were developed as potential minimally invasive therapies for myocardial infarction (MI) [6]. The porcine myocardial matrix hydrogel was shown to increase cardiac muscle, reduce fibrosis, and improve cardiac function in animal models [7, 8]. In comparing the porcine derived hydrogel to the human material, variability between patient samples was observed in the gross anatomy with significant adipose tissue deposition, in processing during decellularization, and finally in the ability to form a hydrogel via self-assembly [4]. Thus, variations in the biomolecular composition between the different decellularized matrices dramatically impacted their material properties.

Previously, proteomics analysis of complex decellularized materials, such as the myocardial matrix, has only produced a list of identified ECM proteins present in the material [4, 5, 9-12]. Unfortunately these methods often under-represent structurally and functionally relevant ECM proteins [13] and do not allow for quantitative comparisons between samples. Here we were interested in analyzing six different patient samples of decellularized myocardium to investigate their similarities and differences in biomolecular composition. We characterized the pepsin digested material (injectable) and also implemented proteomic analysis of the pre-digested decellularized matrix. More specifically we employed stable isotope labeled (SIL) internal standards to obtain accurate peptide levels as surrogate measures of individual protein abundance. The SIL peptides uniquely represent ECM and ECM-associated proteins, in addition to abundant cellular proteins that are often difficult to remove during decellularization. These peptide standards were generated as Quantitative conCATamers (QconCATs) [14], which are added during sample processing prior to LC-MS/MS analysis for more accurate quantification of the endogenous peptides. In addition to

quantifying proteins within the matrix, we fractionated these samples prior to global LC-MS/MS analysis to gain a more comprehensive qualitative profile of these matrices.

The aim of this study was to more fully characterize the injectable form of the human myocardial matrix and to quantify the absolute protein content of the decellularized human cardiac tissue. To the best of our knowledge, this is the first study to perform absolute proteomics quantification of a human derived decellularized biomaterial using QconCAT approach with mass spectrometry. This targeted approach will potentially provide standardization for characterizing decellularized biomaterials not only for internal quality control, but will allow for cross-comparison between different methods of processing and between different tissue sources. Here we quantitatively investigated the protein content between decellularized myocardial matrixes derived from six different human patients along with providing a compositional profile of human ECM proteins by global proteomics analysis.

2. Materials and Methods

2.1. Decellularization of Human Myocardium

Human cardiac tissue was collected under an institutionally approved protocol from donors who were not eligible for heart transplantation and whose deaths were not cardiovascular related. Only patient age was provided for the six patients (p1-6) and is as follows: p1-43, p2-52, p3-63, p4-69, p5-63, and p6-34. The methods for decellularizing human myocardial tissue have been previously reported [4], and were based on methods developed for decellularizing porcine myocardium [6]. In brief, human cardiac tissue was collected from 6 different patients. The left ventricular myocardial tissue was isolated and chopped into small pieces. The tissue was rinsed in water and then spun on a stir plate at 85 rpm in 800 mL of phosphate buffered saline (PBS) with 1% sodium dodecyl sulfate (SDS) (Fisher Scientific, Fair Law, NJ) and 0.5% penicillin streptomycin (PS) of 10,000 U/mL (Gibco Life Technologies, Grand Island, NY) with daily solution changes for 6-8 days until the tissue was fully white as an indicator of decellularization. The tissue was rinsed in water and then treated with isopropyl alcohol (IPA) (Fisher Scientific, Fair Law, NJ) for 12-24 hours to decrease the lipid content. Next, the tissue was rinsed again in water then treated with a DNase/RNase (Sigma-Aldrich, St. Louis, MO)/(Qiagen, Hilden, Germany) in a buffered solution and incubated at 37°C with agitation for 24 hours. Finally, the tissue was spun in the SDS solution to remove DNA/RNA fragments after digestion and shaken with water. After decellularization the material was rinsed again in water, frozen at -80°C, lyophilized, and then milled into a fine powder using a Wiley® Mini-Mill.

2.2. DMMB and PAGE Characterization of the Injectable Matrix

Milled ECM at 10 mg/ml was digested with pepsin at 1 mg/ml in 0.1M HCl for 48 hours at room temperature. Once the material was digested into a liquid form, the matrices from six human patients were analyzed with a dimethyl-methylene blue (DMMB) assay and with polyacrylamide gel electrophoresis (PAGE). The DMMB assay was used to quantify the sulfated glycosaminoglycan (sGAG) content of each human matrix and the assay was implemented in technical triplicate (n=3). The protocol was implemented as previously

described with chondroitin sulfate (Sigma-Aldrich, St. Louis, MO) used for the standard [15]. Also, the molecular weight of the proteins in the injectable liquid form were visualized via a PAGE run with a NuPAGE 12% Bis-Tris Gel (Novex, Life Technologies, Carlsbad, CA) by loading 2.5 µg of ECM per well. Rat tail collagen type-1 (BD Biosciences, San Jose, CA) was used as a control.

2.3. Sample preparation for LC-MS/MS

Milled myocardial samples were dissolved in 400 µl of 100 mM CNBr in 86% TFA. Chemical digestion was carried out in the dark for 24 hours at room temperature and was then stopped by adding 500 µL of water. The sample volume was reduced under vacuum, and formic acid was removed by solvent exchange with water. This procedure was repeated three times. The samples were neutralized with 1 M Tris-HCl pH 8.0. Protein concentration was measured by Bradford protein assay, and 50 µg of samples were digested according to the FASP protocol using a 10 kDa molecular weight cutoff filter. Samples were supplemented with 1 pmol of ¹³C₆ labeled QconCAT peptides representing ECM and ECM-associated proteins as described here [13]. In brief, samples were mixed in the filter unit with 8 M urea, 0.1 M Tris-HCl, pH 8.5 and centrifuged at 14 000g for 15 min. The proteins were reduced with 10 mM DTT for 30 min at RT, centrifuged, and alkylated with 55 mM iodoacetamide for 30 min at RT in the dark. Following centrifugation, samples were washed 3× with Urea solution, and 3× with 50 mM Ammonium Bicarbonate, pH 8.0. Protein digestion was carried out with sequencing grade modified Trypsin (Promega) at 1/50 protease:protein (wt:wt) in 0.02 % of ProteaseMax (Promega, Madison, WI) surfactant at 37°C overnight. Peptides were recovered from the filter using 30% Acetonitrile. Samples were dried in Speed-Vac and stored at –80°C until LC- SRM analysis or high pH reversed phase fractionation.

High pH reversed phase chromatography was performed on a Gemini-NH C18, 50 × 2 mm analytical column containing 3 µm particles. The solvent consisted of 20 mM ammonium bicarbonate (pH 10) as mobile phase (A) and 20 mM ammonium bicarbonate and 75% ACN (pH 10) as mobile-phase B. Sample separation was accomplished using the following linear gradient: from 0 to 5% B in 10 min, from 5 to 50% B in 30 min, from 50 to 100% B in 5 min, and held at 100% B for an additional 10 min at a flow rate of 0.2 mL/min. Thirty two fractions were collected and pooled into 6 fractions by combining fractions 1, 7, 13, 19, 25 for fraction 1 of 6, and serially repeated for the remaining fractions. Samples were dried in Speed-Vac and stored at –80°C until global LC-MS/MS analysis.

2.4. LC-MS/MS Analysis

Global LC-MS/MS was performed on the LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) and Selected Reaction Monitoring (SRM) was performed on the QTRAP®5500 triple quadrupole mass spectrometer (ABSciex) coupled with an Eksigent nanoLC-2D & Agilent 1200 LC system, respectively. Instrument parameters and run conditions were described previously [13], with the following modifications. For global runs, 8 µl of each of the six pooled fractions from high pH RP chromatography was injected. For targeted SRM analysis, these 6 fractions were pooled and 10 µl of each sample (5 µg) was injected per duplicate run.

2.5. Data analysis

For global proteomics, peak lists were generated from the RAW files using PAVA (UCSF) and searched using an in house Mascot server (Version 2.3, Matrix Science). Peptide tolerance was set at ± 10 ppm with MS/MS tolerance set at ± 0.6 Da from spectra acquired on the LTQ Orbitrap Velos. Full trypsin specificity was required and one missed cleavage was allowed; carbamidomethylation on cysteine was defined as a fixed modification; $^{13}\text{C}_6$ Arg and Lys were defined as Heavy Labels; methionine oxidation and proline hydroxylation were defined as variable modifications for the database searches. Files were searched against *Homo Sapiens* filtered Swissprot database containing 20,268 proteins (updated October 23rd, 2014). Result files from MASCOT were consolidated using Scaffold (Version 4.0, Proteome Software), where Peptide Spectral Match (PSM), and Total Ion Current (TIC) result files were exported for further analysis. This export included 134418 spectra mapping to peptides at a 99% confidence interval and 260 proteins at a 95% confidence interval with at least 2 peptides per protein resulting in a FDR of 0.1%.

All data files generated on the triple quadrupole mass spectrometer during LC-SRM analyses were imported to Skyline v2.2 software [16] for data processing. Transition quality, peak shape, and peak area boundaries were manually validated. Integrated peak areas were calculated by the software after Savitsky-Golay smoothing, and quantification was based on the ratio of the ^{12}C peptide representing the endogenous sample to the corresponding ^{13}C peptide from QconCATs. Linear range, limits of detection, and limits of quantification were defined as described previously [13], and peptides outside of these ranges were not included in the analysis.

2.6. Statistical Analysis

All data, unless otherwise stated, is presented as mean $\pm$ standard deviation (SD). The sGAG quantification was analyzed with a one-way ANOVA and a Tukey post-hoc test with $p < 0.05$. Global proteomics data was directly exported and normalized to sample maximum. Peak area ratios for the SRM data was normalized to an internal standard spike, and then coefficients of variance were determined between the biological replicates.

3. Results and Discussion

Decellularized biomaterials can be derived from numerous tissue sources [1, 17]. These materials are composed of the native ECM, which is inherently complex in nature. Even though collagen is the most prevalent protein in human ECM, a tissue can be composed of numerous different types of collagen and potentially hundreds of other proteins as well as polysaccharides. This complexity makes characterization of each material challenging, especially since each tissue of the body has a unique composition. Here we aimed to more fully characterize a previously developed human myocardial matrix biomaterial derived from six different human donor hearts [4].

3.1. Sulfated GAG Content and PAGE

First, we wanted to characterize two important parameters for the injectable form of the human myocardial matrices, the sulfated glycosaminoglycan (sGAG) content and the protein

molecular weights. A DMMB assay was used to quantify sGAG content in each material (Fig. 1). The six different patient samples (p1-6) had a range of 0.79-9.59 μg of sGAG per mg of dry ECM. Each material had a significantly different sGAG content ($p < 0.05$), but the range of values overall was relatively small. Next, we wanted to visualize the protein molecular weights of the different matrices in their digested form by 1D PAGE visualized via coomassie staining (Fig. 1). Material from all six patients (lanes C-H) showed numerous molecular weight bands over the full range of the gel, which showed characteristic collagen banding as seen in the rat tail type 1 collagen control (lane B). Overall, there were minimal differences in observed bands between the six patients. There were some differences in band intensities; however, this could be a result of slight variations in digestion or sample loading. Overall, sGAG quantification showed distinct differences between the six different human myocardial ECMs; however, PAGE analysis highlights the need for higher resolution determination of protein characterization.

3.2. Protein Quantification

The aforementioned complexity of the ECM is what makes naturally derived bio-scaffolds such an attractive alternative to synthetic scaffolds. However, until now, our understanding of this complexity has been limited to gross quantitative assays and qualitative readouts. Mass spectrometry approaches have been previously utilized to characterize decellularized cardiac ECM; however, these were not quantitative [4, 10, 18]. Here, we utilized an ECM targeted proteomics method to define the molecular components of human derived myocardial matrices at a quantitative level. This analysis resulted in the quantification of forty-eight proteins from eighty-three SIL peptides. To gain an understanding of the compositional profile of these myocardial matrices we calculated the total molar quantity of each protein, and graphed the representative proteins as a percentage of the total by gene ontology classification (Fig. 2). Not surprisingly, these decellularized matrices are predominantly composed of fibrillar collagens, which represent over 70% of the quantified protein. It should be noted that this value is likely under-represented because our SIL peptide probe for COL3A1, which was previously identified in the material and is known to be a significant component of cardiac ECM [4, 19], was not used for quantification due to irreproducibility of the COL3A1 QconCAT peptide across samples. Encouragingly, cytoskeletal components represented less than 1% of the quantified protein, which confirms the efficiency of the decellularization procedure previously outlined [4]. Biological variability between the patient samples indicated that fibrillar collagen, and basement membrane proteins vary by as much as 10-20%. The ratio of fibrillar collagen to basement membrane proteins ranges from 2:1, all the way up to 6:1. ECM-derived matrices are subject to both compositional and mechanical variability depending on age, tissue-type, and species of origin. Whether the variation between ECM profiles in these myocardial matrices is due to natural variance of ECM composition between different donors, or an artifact introduced during the decellularization process is not known. Despite the origin of variance, altering local concentrations of basement membrane collagens and laminins can modulate cardiomyocyte behavior [20]. Additionally, constructive ECM remodeling at the site of therapeutic intervention has been shown to facilitate ultimate successful implantation. This response is dependent on the host immune response, which can be modulated by unknown compositional differences within the ECM [21]. For the first time, we utilized this method to

begin to quantitatively define these molecular variations within a myocardial matrix (as shown in Table 1). Absolute quantitative characterization of the ECM has the potential to correlate alterations in protein abundance with functional outcomes to drive further development.

Matricellular proteins responsible for many of the cell-ECM interactions that drive cell differentiation and adhesion have an average coefficient of variance (CV) of nearly 60%. For example, collagen VI has been shown to play a pivotal role in cardiac myofibroblast differentiation post-infarction [22], and is nearly two orders of magnitude lower in concentration for all three isoforms (COL6A1, COL6A2, COL6A3) in p6 than any of the other matrices, despite identical sample handling and anatomical tissue origins. Cellular proteins have the largest CV's of any group assessed. Consistent with previous reports, the majority of cellular proteins we examined were removed with decellularization. However, the amount of specific cellular proteins required to elicit an immune response in the host is currently poorly defined. Data from this method could be used as a readout to help establish standardized maximum thresholds for individual cellular protein concentrations to minimize the potential for negative immune responses. In addition, the method offers an improvement over the current standards for estimating the extent of decellularization, which involve measuring total dsDNA.

Successful fabrication for myocardial matrix hydrogels has been defined by the matrices ability to self-assemble into a hydrogel at physiological conditions [4]. Johnson TD et al. noted that p4 & p6 were the only samples out of the six patients investigated here to self-assemble into hydrogels under physiological conditions [4]. To determine whether our quantitative ECM profile could differentiate these two groups, we ran an unsupervised Principal Component Analysis (PCA) comparing all six samples against the concentration of ECM proteins within the sample (Fig. 3). While the two samples that gelled did not cluster, based on their proximity in the sample plot (right), the samples that did not gel were readily distinguished from those that formed a hydrogel. For ECM hydrogels, it was previously hypothesized that the driving force for self-assembly was due to collagen components [23]. Based on the loading plot, COL1A1, COL1A2 and several matricellular proteins contribute to a distinct profile that may play a role in the ability of these matrices to self-assemble into a hydrogel. It is unlikely that a single component or even a small subset of components in the ECM are wholly responsible for functional outcome. However, this method provides the opportunity to begin to identify the components that correlate to eventual therapeutic outcome.

3.3. Global Proteomics Analysis

The targeted LC-SRM approach used to generate this data is robust in its potential to accurately quantify hundreds of peptides, and as a result, proteins, in a single analytical run. However, a caveat of this method is that we can only quantify the proteins included in our original QconCAT design. To expand upon our LC-SRM data we also performed a global LC-MS/MS analysis to gain a more comprehensive profile of sample protein. Proteins were organized by spectral counting, a common relative quantitative proteomic comparison (Table 2). Although global LC-MS/MS can result in a more comprehensive profile of

protein identifications within a sample, differences in ionization efficiency and stochastic sampling of peptides in the mass spectrometer can confound semi-quantitative results. Thus, spectral counting should not be used to make definitive comparisons between protein levels within a sample, and instead, quantitative interpretation should be limited to relative comparisons between samples. The top 20 proteins identified by global LC-MS/MS, based on spectral counts, indicates that our QconCAT method is covering a majority of the most abundant proteins in the myocardial matrix (italicized proteins in Table 2 are covered in QconCATs). Additionally, biological variability of individual proteins is similar; basement membrane and fibrillar proteins collectively have the lowest CV's between biological replicates while matricellular and cellular proteins are largely variable (>40% CVs). Titin, a major protein component in heart tissue responsible, in part, for the architecture of striated muscle cells, varies by nearly 2 orders of magnitude between samples. Thus, absolute quantification of titin should be investigated in future experiments to help correlate the impact of this potential cellular protein contaminate on functional outcomes.

Global proteomics identified more than 200 proteins in the decellularized myocardial matrix (Supplementary Table 1). While many of these proteins are identified by only a few peptides, there are several notable ECM proteins that were not included in our targeted assay that are likely to be of importance in characterizing and understanding the mechanism of action for myocardial hydrogels. For instance, COL3A1, which was not included in the quantitative analysis, is a major fibrillar collagen known to play a role in myocardial remodeling [24], and was previously identified in a porcine model of ischemia as a potential TGF β -1 antagonist [25]. As seen here, global proteomics offers us the advantage to see a more comprehensive compositional profile within the matrix, but with the caveat that inter-protein quantitative comparisons are imprecise using this method. By looking at Peptide Spectral Matches (PSMs), it would at first appear that these samples are poorly decellularized, and that Fibrillin, Perlecan, and Myosin-7 are as abundant as Collagen alpha-1(1). However, from our quantitative proteomics, we can conclude that these proteins are over-represented and that the samples are in fact highly decellularized. For example, p1 contained 634.07 nmol/g of COL1A1, but only contained 0.69 nmol/g of Myosin – 3, 4, 6, 7 combined. Thus global proteomics, although limited in its quantitative application, improves protein coverage and gives some insight to protein variability, thus providing additional targets for future QconCAT design.

4. Concluding Remarks

Ultimately, biological scaffold based therapies depend upon a complex ECM framework that dictates the host immune response and eventual therapeutic success. Here, we have utilized robust quantitative proteomic methods to quantify and characterize six cadaveric human heart derived matrices. The methods presented here provide a means to potentially determine whether the protein variability observed between matrices contributes to physiological outcome. Future work will involve using these methods to correlate protein composition with functional testing and outcomes of biologically derived therapeutic matrices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Lifesharing for their assistance in obtaining the human cardiac tissue. Funding for this study was provided by the NIH Heart, Lung, Blood Institute RO1HL113468 (KLC) and by the NIH/NCI IMAT program grant R21CA132741 and NCRR grant S10RR024599 (KCH). TDJ received funding during this project from the NSF as a graduate student fellow, the NHLBI as a training grant recipient, and as a Powell Fellow from the Powell Foundation.

KLC is co-founder, board member, and holds equity interest in Ventrix, Inc.

Abbreviations

DMMB	Dimethyl-methylene Blue
ECM	Extracellular Matrix
IPA	Isopropyl Alcohol
MI	Myocardial Infarction
PS	Penicillin Streptomycin
PSM	Peptide Spectral Match
QconCAT	Quantitative conCATamers
sGAG	Sulfated Glycosaminoglycans
SIL	Stable Isotope Labeled

References

- [1]. Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. *Biomaterials*. 2011; 32:3233–3243. [PubMed: 21296410]
- [2]. Johnson TD, Christman KL. Injectable hydrogel therapies and their delivery strategies for treating myocardial infarction. *Expert Opin. Drug Deliv.* 2012; 10:59–72. [PubMed: 23140533]
- [3]. Seif-Naraghi SB, Horn D, Schup-Magoffin PA, Madani MM, Christman KL. Patient-to-Patient Variability in Autologous Pericardial Matrix Scaffolds for Cardiac Repair. *J. of Cardiovasc. Trans. Res.* 2011; 4:545–556.
- [4]. Johnson TD, DeQuach JA, Gaetani R, Ungerleider J, et al. Human versus porcine tissue sourcing for an injectable myocardial matrix hydrogel. *Biomater. Sci.* 2014; 2:735–744.
- [5]. Gilpin SE, Guyette JP, Gonzalez G, Ren X, et al. Perfusion decellularization of human and porcine lungs: bringing the matrix to clinical scale. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation*. 2014; 33:298–308.
- [6]. Singelyn JM, DeQuach JA, Seif-Naraghi SB, Littlefield RB, et al. Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering. *Biomaterials*. 2009; 30:5409–5416. [PubMed: 19608268]
- [7]. Singelyn JM, Sundaramurthy P, Johnson TD, Schup-Magoffin PJ, et al. Catheter-deliverable hydrogel derived from decellularized ventricular extracellular matrix increases endogenous cardiomyocytes and preserves cardiac function post-myocardial infarction. *Journal of the American College of Cardiology*. 2012; 59:751–763. [PubMed: 22340268]

- [8]. Seif-Naraghi SB, Singelyn JM, Salvatore MA, Osborn KG, et al. Safety and efficacy of an injectable extracellular matrix hydrogel for treating myocardial infarction. *Science translational medicine*. 2013; 5:173ra125.
- [9]. Wallis JM, Borg ZD, Daly AB, Deng B, et al. Comparative assessment of detergent-based protocols for mouse lung de-cellularization and re-cellularization. *Tissue engineering. Part C, Methods*. 2012; 18:420–432. [PubMed: 22165818]
- [10]. DeQuach JA, Mezzano V, Miglani A, Lange S, et al. Simple and high yielding method for preparing tissue specific extracellular matrix coatings for cell culture. *PloS one*. 2010; 5:e13039. [PubMed: 20885963]
- [11]. Welham NV, Chang Z, Smith LM, Frey BL. Proteomic analysis of a decellularized human vocal fold mucosa scaffold using 2D electrophoresis and high-resolution mass spectrometry. *Biomaterials*. 2013; 34:669–676. [PubMed: 23102991]
- [12]. Didangelos A, Yin X, Mandal K, Baumert M, et al. Proteomics characterization of extracellular space components in the human aorta. *Molecular & cellular proteomics : MCP*. 2010; 9:2048–2062. [PubMed: 20551380]
- [13]. Hill RC, Calle EA, Dzieciatkowska M, Niklason LE, Hansen KC. Quantification of extracellular matrix proteins from a rat lung scaffold to provide a molecular readout for tissue engineering. *Molecular & cellular proteomics : MCP*. 2015; 14:961–973. [PubMed: 25660013]
- [14]. Beynon RJ, Doherty MK, Pratt JM, Gaskell SJ. Multiplexed absolute quantification in proteomics using artificial QCAT proteins of concatenated signature peptides. *Nature methods*. 2005; 2:587–589. [PubMed: 16094383]
- [15]. Barbosa I, Garcia S, Barbier-Chassefière V, Caruelle J-P, et al. Improved and simple micro assay for sulfated glycosaminoglycans quantification in biological extracts and its use in skin and muscle tissue studies. *Glycobiology*. 2003; 13:647–653. [PubMed: 12773478]
- [16]. MacLean B, Tomazela DM, Shulman N, Chambers M, et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*. 2010; 26:966–968. [PubMed: 20147306]
- [17]. Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: Structure and function. *Acta biomaterialia*. 2009; 5:1–13. [PubMed: 18938117]
- [18]. de Castro Bras LE, Ramirez TA, DeLeon-Pennell KY, Chiao YA, et al. Texas 3-step decellularization protocol: looking at the cardiac extracellular matrix. *Journal of proteomics*. 2013; 86:43–52. [PubMed: 23681174]
- [19]. McClain PE. Characterization of cardiac muscle collagen. Molecular heterogeneity. *The Journal of biological chemistry*. 1974; 249:2303–2311. [PubMed: 4362072]
- [20]. Higuchi S, Lin Q, Wang J, Lim TK, et al. Heart extracellular matrix supports cardiomyocyte differentiation of mouse embryonic stem cells. *Journal of bioscience and bioengineering*. 2013; 115:320–325. [PubMed: 23168383]
- [21]. Londono R, Badylak SF. Biologic Scaffolds for Regenerative Medicine: Mechanisms of In vivo Remodeling. *Ann Biomed Eng*. 2015; 43:577–592. [PubMed: 25213186]
- [22]. Naugle JE, Olson ER, Zhang X, Mase SE, et al. Type VI collagen induces cardiac myofibroblast differentiation: implications for postinfarction remodeling. *American journal of physiology. Heart and circulatory physiology*. 2006; 290:H323–330. [PubMed: 16143656]
- [23]. Johnson TD, Lin SY, Christman KL. Tailoring material properties of a nanofibrous extracellular matrix derived hydrogel. *Nanotechnology*. 2011; 22:494015. [PubMed: 22101810]
- [24]. Fan D, Takawale A, Lee J, Kassiri Z. Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. *Fibrogenesis & tissue repair*. 2012; 5:15. [PubMed: 22943504]
- [25]. Barallobre-Barreiro J, Didangelos A, Schoendube FA, Drozdov I, et al. Proteomics analysis of cardiac extracellular matrix remodeling in a porcine model of ischemia/reperfusion injury. *Circulation*. 2012; 125:789–802. [PubMed: 22261194]

Statement of Clinical Relevance

Here we investigated the composition of human tissue sourcing for a decellularized biomaterial derived from myocardium. For the first time, a decellularized biomaterial from human tissue has been characterized using targeted proteomics for absolute quantification. This is an important pre-clinical characterization step for the design of new decellularized biomaterial therapies and for studying both isolated healthy and diseased human cardiac extracellular matrix (ECM) proteins. Analysis of six different patient preparations showed significant patient-to patient variability, which is a concern when developing clinical therapies from aged human cadaveric donor tissue. Thus, this study has pre-clinical implications not just for the myocardial matrix presented here, but also for the development of decellularized biomaterials in general. This quantitative proteomics approach advances the field and can be utilized as a means for an internal control, as standard for comparing between materials of different labs and tissue sources, as a tool for assisting the investigation of the mechanism of action for complex naturally-derived biomaterials, and as an instrument to provide insight into protein variations in diseased ECM.

Figure 1. sGAG Quantification and PAGE on the Injectable Human Myocardial Matrices
The sGAG content of each ECM was quantified with a DMMB assay. The matrix samples were significantly different from one another ($p < 0.05$). PAGE was used to visualize the molecular weight bands of the standard ladder in kDa (A), the rat tail type 1 collagen control (B) and for p1 (C), p2 (D), p3 (E), p4 (F), p5 (G), and p6 (H).

QconCAT Quantification of Human Cardiac ECM

Figure 2. QconCAT Quantification of Injectable Human Myocardial Matrices

Pie chart represents the average percentage of total protein from six injectable human myocardial matrices as assayed by an ECM targeted QconCAT LC-SRM assay. Individual percentages of total protein from the matrices are shown by column. Proteins were consolidated into their respective functional gene ontology class for each graph. Values representing less than 2% are not labeled in the column chart.

Figure 3. PCA Analysis of Myocardial Matrices Grouped by Gelation
Loading plot (left) highlights the top 10 proteins that discriminate the two groups of samples based on principle component analysis (PCA) of absolute protein quantification between samples that gelled (p4 & p6) and samples that did not gel (p1-3, p5). Sample plot (right) portrays the ability to discriminate sample groupings based on PCA analysis.

Table 1

ECM Protein Concentration in Human Myocardial Matrices

Absolute quantification of 48 ECM, ECM associated, and common cellular contaminant proteins. Embedded blue bar in the average (Avg) column represents relative protein abundance between quantified proteins in the table. The coefficients of variances (CVs) are color coded on a gradient scale relative to all proteins in the table from green-white-red, representing low-medium-high respectively.

Protein Abundance in Myocardial Matrix (nmol/g)										
Protein	GENE	Annotation	p1	p2	p3	p4	p5	p6	Avg	CV (%)
Agrin	AGRN	Basement Membrane	0.02	0.02	0.03	0.03	0.02	0.01	0.02	24.3%
Agrin (iso 2,3,4,5,&6)	AGRN*	Basement Membrane	0.03	0.03	0.04	0.04	0.03	0.02	0.03	22.0%
Collagen alpha-1(IV) chain (Arresten/Core Protein)	COL4A1*	Basement Membrane	87.78	110.56	136.51	68.59	107.20	80.39	98.51	24.9%
Collagen alpha-1/5(IV) chain (Arresten/Core Protein)	COL4A1/5*	Basement Membrane	82.26	87.97	122.28	68.46	97.30	102.37	93.44	19.8%
Collagen alpha-2(IV) chain (Canstiatin/Core Protein)	COL4A2*	Basement Membrane	46.34	84.83	88.96	33.72	64.70	22.51	56.84	47.9%
Collagen alpha-5(IV) chain	COL4A5	Basement Membrane	0.95	3.29	0.75	0.59	0.87	0.47	1.15	91.9%
Perlecan	HSPG2	Basement Membrane	4.71	4.86	7.11	4.07	3.78	2.11	4.44	36.9%
Laminin alpha-2	LAMA2	Basement Membrane	0.54	0.50	0.43	0.29	0.28	0.01	0.34	56.1%
Laminin alpha-5	LAMA5	Basement Membrane	0.66	0.48	0.78	0.42	0.42	0.12	0.48	47.3%
Laminin beta-1	LAMB1	Basement Membrane	0.65	0.78	0.76	0.54	0.79	0.24	0.63	33.7%
Laminin beta-2	LAMB2	Basement Membrane	1.42	0.94	1.48	0.68	0.96	0.15	0.94	52.5%
Laminin gamma-1	LAMC1	Basement Membrane	3.17	2.85	3.22	1.97	2.62	1.79	2.60	23.2%
Nidogen-1	NID1	Basement Membrane	0.56	0.58	0.58	0.51	0.37	0.17	0.46	35.8%
Nidogen-2	NID2	Basement Membrane	0.51	0.51	0.76	0.42	0.39	0.22	0.47	38.4%
Actin (All Isoforms)	ACT	Cytoskeletal	2.01	2.26	3.37	5.78	0.46	0.28	2.36	86.3%
Actin, cytoplasmic 1/2	ACTB	Cytoskeletal	0.63	0.49	0.55	0.66	0.30	0.19	0.47	39.9%
Desmin	DES	Cytoskeletal	0.25	0.37	0.41	1.76	0.04	0.06	0.48	133.5%
Myosin (Myosin-3,4,6,7)	MYH*	Cytoskeletal	0.69	1.45	6.20	8.26	0.36	0.27	2.87	120.6%
Tubulin beta-4B chain (4b & 5 chain)	TUBB*	Cytoskeletal	0.16	0.21	0.21	0.66	0.05	0.03	0.22	102.9%
Vimentin	VIM	Cytoskeletal	0.26	0.23	0.21	0.13	0.05	0.12	0.17	47.4%
Transglutaminase 2	TGM2	ECM regulator	1.71	1.09	0.79	1.81	0.27	0.08	0.96	75.1%
Collagen alpha-1(XI) chain	COL12A1	FACIT Collagen	0.19	0.13	0.13	0.12	0.05	0.02	0.11	57.9%

<i>Protein Abundance in Myocardial Matrix (nmol/g)</i>										
<i>Protein</i>	<i>GENE</i>	<i>Annotation</i>	<i>p1</i>	<i>p2</i>	<i>p3</i>	<i>p4</i>	<i>p5</i>	<i>p6</i>	<i>Avg</i>	<i>CV (%)</i>
Collagen alpha-1(I) chain	COL1A1	Fibrillar Collagen	634.07	465.73	711.42	671.24	332.43	569.40	564.05	25.3%
Collagen alpha-2(I) chain	COL1A2	Fibrillar Collagen	376.00	279.69	387.12	342.31	202.23	349.06	322.74	21.7%
Collagen alpha-1(V) chain	COL5A1	Fibrillar Collagen	27.06	24.27	24.20	19.74	14.75	15.33	20.89	24.4%
Collagen alpha-2(V) chain	COL5A2	Fibrillar Collagen	13.35	11.48	12.61	15.42	8.83	10.97	12.11	18.5%
Collagen alpha-1(XVIII) chain	COL18A1	Matricellular	0.14	0.14	0.12	0.14	0.09	0.03	0.11	39.1%
Collagen alpha-1(VI) chain	COL6A1	Matricellular	15.61	17.89	11.65	18.26	6.67	0.21	11.71	60.9%
Collagen alpha-2(VI) chain	COL6A2	Matricellular	18.51	22.12	14.47	19.08	6.38	0.27	13.47	62.7%
Collagen alpha-3(VI) chain	COL6A3	Matricellular	6.77	6.47	3.65	5.89	1.91	0.13	4.14	65.5%
Dermatopontin	DPT	Matricellular	4.91	4.69	4.37	2.90	3.47	4.29	4.11	18.7%
Emilin 1	EMILIN1	Matricellular	0.30	0.30	0.52	0.47	0.15	0.18	0.32	46.3%
Fibulin 5	FBLN5	Matricellular	0.82	0.55	1.02	1.37	0.42	0.72	0.82	42.0%
Fibronectin 1 (type-III 4 domain)	FN1 *	Matricellular	3.02	6.04	7.05	4.09	1.26	1.23	3.78	64.1%
Lumican	LUM	Matricellular	1.30	0.93	1.11	1.84	0.53	0.20	0.98	58.9%
Periostin	POSTN	Matricellular	2.24	0.80	5.51	7.25	0.59	0.16	2.76	106.8%
Prolargin	PRELP	Matricellular	0.58	0.56	0.59	0.57	0.24	0.19	0.45	41.5%
Thrombospondin 2	THBS2	Matricellular	0.21	0.13	0.79	0.13	0.16	0.14	0.26	100.1%
TnxB Protein	TNXB	Matricellular	0.15	0.24	0.16	0.19	0.09	0.07	0.15	42.3%
Annexin A2	ANXA2	Other ECM	0.03	0.04	0.03	0.04	0.03	0.03	0.03	17.0%
Galectin-1	LGALS1	Other ECM	0.29	0.20	0.26	0.55	0.12	0.05	0.25	71.3%
Mimcan/Osteoglycin	OGN	Other ECM	0.47	0.34	0.37	0.24	0.14	0.11	0.28	50.6%
Transforming growth factor-beta-induced protein ig-h3	TGFBI	Secreted	1.75	1.82	2.26	2.52	1.15	1.09	1.77	32.5%
Biglycan	BGN	Structural ECM	0.36	0.32	0.30	0.25	0.18	0.08	0.25	41.5%
Decorin	DCN	Structural ECM	2.65	1.82	2.37	1.61	1.43	1.59	1.91	25.5%
Fibrillin 1	FBN1	Structural ECM	58.88	43.06	61.50	63.54	42.95	9.54	46.58	43.6%
Latent transforming growth factor beta binding protein 1	LTBP1	Structural ECM	0.24	0.25	0.43	0.61	0.13	0.14	0.30	62.6%
Microfibrillar-associated protein 2	MFAP2	Structural ECM	3.62	2.75	5.50	4.13	2.38	2.30	3.45	35.9%

* Indicates that peptide probe used for quantification is homologous to multiple isoforms of a single protein; isoforms are noted in parenthesis

Table 2
Top 20 Proteins of Human Myocardial Matrices by Global Proteomics

Table represents top 20 proteins identified based on the number of unique peptide spectral matches from global LC-MS/MS for a given protein. CV's are color coded as previously described in Table 1. Italicized proteins are represented in absolute quantitative proteomics data.

Top 20 Proteins of Human Myocardial Matrix by Global Proteomics											
Proteins	Gene	Functional Classification	Accession	Unique PSM's							CV (%)
				p1	p2	p3	p4	p5	p6		
Collagen alpha-1(IV) chain	COL4A1	Basement Membrane	P02462	30	34	34	25	36	25	16%	
Collagen alpha-2(IV) chain	COL4A2	Basement Membrane	P08572	47	53	49	51	62	34	19%	
Perlecan	HSPG2	Basement Membrane	P98160	71	86	78	80	116	76	19%	
Laminin subunit alpha-2	LAMA2	Basement Membrane	P24043	27	30	24	29	38	14	29%	
Laminin subunit alpha-5	LAMA5	Basement Membrane	O15230	30	29	36	27	39	18	25%	
Laminin subunit beta-2	LAMB2	Basement Membrane	P55268	44	40	48	34	50	35	16%	
Laminin subunit gamma-1	LAMC1	Basement Membrane	P11047	41	37	35	38	38	21	20%	
Desmoplakin	DSP	Cytoskeletal	P15924	46	17	23	25	31	58	47%	
Filamin-C	FLNC	Cytoskeletal	Q14315	7	24	39	0	4	62	107%	
Myosin-7	MYH7	Cytoskeletal	P12883	36	54	101	21	31	96	61%	
Collagen alpha-1(I) chain	COL1A1	Fibrillar Collagen	P02452	102	107	100	84	94	96	8%	
Collagen alpha-2(I) chain	COL1A2	Fibrillar Collagen	P08123	96	96	92	91	96	78	8%	
Collagen alpha-1(III) chain	COL3A1	Fibrillar Collagen	P02461	90	95	78	75	85	72	11%	
Collagen alpha-2(V) chain	COL5A2	Fibrillar Collagen	P05997	25	24	15	20	19	19	18%	
Collagen alpha-3(VI) chain	COL6A3	Matricellular	P12111	57	57	45	25	50	58	26%	
Perlestin	POSTN	Matricellular	Q15063	29	16	35	17	25	45	40%	
Titin	TTN	Other Cellular	Q8WZ42	20	79	214	2	11	289	118%	
Serum albumin	ALB	Secreted	P02768	26	35	26	26	47	28	27%	
Elastin	ELN	Structural ECM	P15502	17	14	16	22	26	28	28%	
Fibrillin-1	FBN1	Structural ECM	P35555	117	114	119	106	131	133	9%	