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# Preserved extracellular matrix components and retained biological activity in decellularized porcine mesothelium

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ABSTRACT

Mesothelium tissues such as peritoneum and pleura have a thin and strong layer of extracellular matrix that supports mesothelial cells capable of rapid healing. Decellularized porcine mesothelium was characterized for strength, composition of the matrix and biological activity. The tensile strength of the material was 40.65 ± 21.65 N/cm. Extracellular matrix proteins collagen IV, fibronectin, and laminin as well as glycosaminoglycans were present in the material. Cytokines inherent in the extracellular matrix were preserved. Vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and transforming growth factor β (TGF-β) were retained and the levels of VEGF and TGF-β in the decellularized mesothelium were higher than those found in decellularized small intestinal submucosa (SIS). The decellularized mesothelium also stimulated human fibroblasts to produce more VEGF than fibroblasts grown on tissue culture plastic. Decellularized mesothelium is a sheet material with a combination of strength and biological activity that may have many potential applications in surgical repair and regenerative medicine.



## 1. Introduction

Extracellular matrix-based biomaterials have many inherent advantages over synthetic polymer materials for surgical and regenerative medicine applications. These protein-based materials have significant mechanical strength, retained biological activity and can promote regeneration of tissue. Decellularized tissues for these applications began with the development of decellularized porcine small intestine submucosa (SIS) as a novel material for surgical repair [1–3]. Recently porcine and human decellularized dermis products have been developed for hernia repair applications [4–6] and breast reconstruction [7,8]. Likewise, a porcine urinary bladder matrix (UBM) material [9,10], and bovine pericardium have also been developed [11]. As the options of these ECM materials grow, their applications continue to expand into new tissue engineering and reconstruction applications including heart valve tissue engineering [12] and urologic repair [13] and pelvic floor reconstruction [14]. Decellularization of natural tissues has progressed beyond simple sheets for surgical applications. Whole organs are now being decellularized and subsequently recellularized with hope of discovering an architecturally appropriate scaffold for organ replacement. The heart [15,16], the liver [17,18] and even the trachea [19,20] have been decellularized and reseeded. Although clinical application for solid organs is still a distant hope, these sheet-based and whole organ decellularized tissues have demonstrated the complex biological stimulus that remains after the cells have been removed.

After removal of cellular components, an array of extracellular matrix proteins present in the native tissue remains in the acellular sheets of SIS, dermal, pericardial, and UBM materials. Collagen I, II, III, IV and VII can be preserved in these materials [21–24]. Likewise, glycosaminoglycans [25,26] and other ECM proteins like fibronectin [22,27] and laminin [22,23] also remain. Cytokines stored in tissue or in cells at the time of decellularization have also been shown to be preserved [23,28,29]. As the materials are remodeled, these cytokines may be released into the regenerating tissue at the surgical site, further promoting tissue growth.

Mesothelial tissues are composed of a mesothelial layer of cells over a basement membrane. This tissue layer is found surrounding the abdominal cavity, chest cavity and heart. The mesothelium over this basement membrane can heal very rapidly following surgery.

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This thin, strong membrane may have surgical and regenerative medicine applications if its inherent strength and presumed significant biological activity is preserved as an acellular matrix material. Decellularized porcine mesothelium was developed and reported here is the initial evaluation of the material including the preserved extracellular matrix components and retained biological activity of this natural structure.

## 2. Materials and methods

All materials were purchased from Sigma (St. Louis, MO) unless otherwise noted.

### 2.1. Decellularization of porcine mesothelium

The mesothelial layer was harvested from market weight pigs. Decellularization of the material was performed by Kensey Nash Corporation using proprietary methods. The mesothelial layer was dissected from the surrounding connective tissue and decellularized through a series of agitations involving organic solvents, detergents, salt solutions, and enzymes with multiple rinsings between steps. Agitation times and speeds were optimized for the thickness of the tissue. Following the final rinse, samples are lyophilized, packaged in gas permeable packaging, and sterilized by ethylene oxide sterilization. When required, the material was hydrated in saline or tissue culture medium for at least 5 min prior to testing.

### 2.2. PicoGreen analysis of residual DNA content and H&E staining

Lysates of the ECM material were prepared by digestion in papain solution (20 μg/ml papain, Worthington Biochemical Corp, Lakewood, NJ), 1 mM EDTA, 7.25 mM cystein, 1 M NaCl in PBS at 100 μl solution/mg dry weight at 60 °C for 48 h on shaker. Residual DNA in the lysates were measured using the Quant-iT PicoGreen kit (Invitrogen, Carlsbad, CA) according to manufacture's instructions. The samples were read on F-7000 Fluorescence Spectrophotometer (Hitachi, Schaumburg, IL). Analysis was done on native tissue and decellularized ECM, controlled for tissue weight. Hematoxylin and eosin staining was also performed on the material to demonstrate removal of nuclear components.

### 2.3. Analysis of residual glycosaminoglycans

Alcian blue staining was performed on rehydrated paraffin sections of the decellularized ECM material and the native tissue from which it was derived in order to compare distribution of retained glycosaminoglycans (GAGs).

### 2.4. Immunofluorescent staining for retained ECM proteins

Immunofluorescent staining was performed on frozen sections of the extracellular matrix after decellularization. Frozen sections were cut 3 to 5 μm thick and mounted. Primary antibodies for collagen IV (T59106R, Biodesign International Saco, ME), fibronectin (MAB 1926, Millipore, Billerica, MA), and laminin (L9393, Sigma) were used to stain for ECM proteins. Alexa Fluor 488- and Alexa Fluor 594-labeled secondary antibodies (Invitrogen) were used. In the preparation of samples for immunofluorescent staining, some of the samples were sectioned from their state immediately prior to freeze drying to eliminate the antigen changes that can occur with drying. The materials were not fixed prior to any of the analysis. A novel antigen retrieval step utilizing sodium dodecyl sulfate [30] was also important in achieving epitope exposure. Negative controls utilizing secondary antibodies only were performed for each antibody.

### 2.5. Scanning electron microscopy

As decellularized ECM samples were lyophilized as part of standard processing, fixation and dehydration were not performed prior to scanning electron microscopy (SEM). Cross-section samples were sharply cut as freeze fracture resulted in significant structural damage due to fibers being pulled out of the soft, fibrous materials. Samples were mounted on aluminum stubs, sputter coated with gold and imaged ISI DS130 SEM (International Scientific Instruments, Pleaston, CA) at 14 kV. SEM was performed at Nanographics (Andover, MA).

### 2.6. Tensile strength analysis

Tensile testing on dog-bone samples was conducted in accordance with ASTM D1708 to determine mechanical integrity. The samples were fully hydrated in 0.9% saline prior to testing. Samples were pulled to failure at 50 mm/min using a mechanical test stand with a 100 N load cell. Tensile strength is reported in N/cm of sample width.

### 2.7. Suture retention testing

Suture retention testing was conducted to determine the strength required to pull out suture through the material as previously described [31]. The samples were fully hydrated in 0.9% saline prior to testing. A 2 cm × 1 cm sample was cut and 2-0 Silk suture (Ethicon, Sommerville, NJ) was inserted at a 2 mm bite distance centered along the short edge. Samples were loaded onto the top grip of a mechanical test stand with a 100 N load cell. The sutures through the sample were attached to the bottom grip, with a grip separation of 10 cm. Samples were stretched at 125 mm/min and the peak force was recorded as the suture retention strength.

### 2.8. Analysis of retained cytokines

Cytokines retained in the decellularized ECM were quantified by ELISA analysis. Soluble molecules were extracted from the matrix using Tissue Extraction Reagent I (FNN0071, Invitrogen) with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and homogenized. The extracted lysates were centrifuged at 10,000 RPM for 5 min and the supernatant collected. Lysates of two available versions of the SIS material were also made for comparison; an 8-layer air dried SIS material (C-SAH-8P-7 × 10, Cook Biotech, West Lafayette, IN) and a 4-layer lyophilized SIS material (SLH-4S-7 × 10, Cook Biotech, West Lafayette, IN). All materials were normalized by weight prior to extraction.

ELISA analysis of the extracted lysates was performed ($n = 2$–4 per material) for vascular endothelial growth factor (VEGF), specifically for VEGF-165, basic fibroblast growth factor (FGF) and transforming growth factor beta (TGF-β) according to the manufacturer's instructions (all from R&D Systems, Minneapolis, MN).

### 2.9. Conditioned medium testing of fibroblast stimulation

The decellularized matrix was evaluated for release of soluble signals that stimulate enhanced biological cellular activity. Seven mm diameter disks were punched from the decellularized ECM and the two SIS materials outlined in Section 2.8. The disks ($n = 6$ for each material) were incubated in 0.5 ml of Eagle minimal essential medium (EMEM) (Invitrogen) for 24 h at 37 °C to create conditioned medium volumes for each material. Additional EMEM without scaffold disks was similarly processed to provide non-conditioned controls. The disks were removed providing a medium that was fortified with the soluble components that had eluted from the ECM materials. Human foreskin fibroblasts [32] were expanded in culture then trypsinized, pelleted and resuspended in fresh EMEM or the conditioned medium. The cells were then plated in wells of a 24 well plate (6 per material and 6 EMEM only controls) at 75,000 cells per well. After 24 h in culture, the culture medium from each well was sampled and measured in triplicate for VEGF by ELISA, specifically VEGF-165 (R&D systems, Minneapolis, MN). The conditioned medium directly from the ECM samples was also measured for VEGF by the same ELISA, shown to react with porcine VEGF [33].

### 2.10. Cell culture

Human umbilical vein endothelial cells (HUVEC) (Lonza, Allendale, NJ) and human foreskin fibroblasts [32] were separately cultured directly on the decellularized ECM material. The cells were seeded at $0.2 \times 10^6$ cells/cm$^2$ on disks of ECM material adhering to 6.5 mm transwell inserts (Corning Inc., Corning, NY) and cultured in Endothelial Cell Growth Medium (EGM-2) (HUVEC) (Lonza) or DMEM with 10% FBS and 1% Pen-Strep (fibroblasts). After 3 and 7 days of culture, the cells on the ECM were fixed with 4% paraformaldehyde. The HUVECs were stained with CD31 (MAB1393MI, Fisher Scientific, Pittsburgh, PA) primary and goat anti-mouse AlexaFluor 488 (Invitrogen) and counterstained with the nuclear dye DAPI. Fibroblasts were stained with Alexa Fluor 568 Phalloidin (Invitrogen) for f-actin in order to visualize cells and counterstained with the nuclear dye DAPI. Cells were imaged on Nikon 80i microscope (Nikon, Melville, NY).

### 2.11. Statistical analysis

Results are expressed as mean ± standard deviation. The ELISA results were compared between groups using Student's $t$ test.

## 3. Results

### 3.1. H&E staining and PicoGreen analysis of residual DNA content

The protocol for decellularization of the mesothelial tissue was effective in removal of the cellular components. H&E staining of the tissue before and after the decellularization process demonstrates preservation of the native tissue architecture while removing the cellular components (Fig. 1). Analysis with the quantitative Pico-Green assay for residual DNA content demonstrated efficient

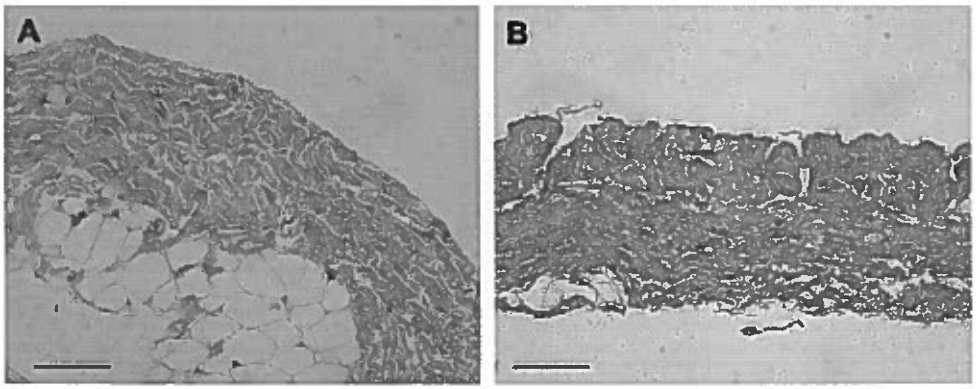


Fig. 1. Hematoxylin and eosin staining of native and decellularized epithelial tissue. The mesothelial side is at the top of the images. Comparatively, A) native mesothelial tissue and B) decellularized mesothelium ECM demonstrate elimination of the nuclear staining material after decellularization but preservation of the architecture of the extracellular matrix. Scale bars = 100 μm.

removal of DNA from the decellularized tissue. The native mesothelium tissue had 1360.1 ± 80.4 ng/mg ECM of DNA whereas the decellularized mesothelium ECM was reduced to 88.7 ± 8.5 ng/mg ECM.

### 3.2. Analysis of residual glycosaminoglycans and immunofluorescent staining for retained ECM proteins

Preservation of the extracellular matrix proteins and glycosaminoglycans of the native tissue was a paramount goal in the development of this material. GAGs are present in mesothelial tissue and were preserved following decellularization as demonstrated by Alcian blue staining (Fig. 2). Collagen IV, fibronectin and laminin are present in the decellularized ECM material (Fig. 3). As evidenced in Fig. 3, the pattern of staining for each of these proteins is on the surface of the material as well as interspersed throughout the thickness of the material.

### 3.3. Scanning electron microscopy and material tensile and suture retention strength

Scanning electron micrographs of the surface and cross-section of the material demonstrates the fibrous architecture of the material with variable porosity (Fig. 4). In the cross-sectional images of Fig. 4C and D, the different regions of the single layered structure of the material are evident. This fibrous architecture is consistent with the significant strength of the material demonstrated by the dog-bone specimens used in tensile test. The tensile strength was 40.65 ± 21.65 N/cm. This result is for a single layer of the material. For applications requiring higher strength than this, multiple layers may be used. The suture pull through strength was 9.12 ± 3.62 N. This is higher than the suture pull through strength of ePTFE at 5.36 ± 0.25 N [31].

### 3.4. Analysis of retained cytokines

The retention of cytokines and growth factors naturally occurring in the mesothelium tissue was demonstrated by ELISA analysis of protein extractions from the decellularized ECM. Shown in Fig. 5, VEGF, FGF and TGF-β are present in the decellularized ECM. The levels of VEGF and TGF-β were significantly higher than those in the SIS materials. The SIS air dried sample had higher levels of FGF than the mesothelium ECM.

### 3.5. Conditioned medium testing of fibroblast stimulation

The VEGF conditioned medium study demonstrated there are soluble factors released by the decellularized ECM that can stimulate dermal fibroblasts to have increased secretion of VEGF. The production of VEGF by fibroblasts in the decellularized

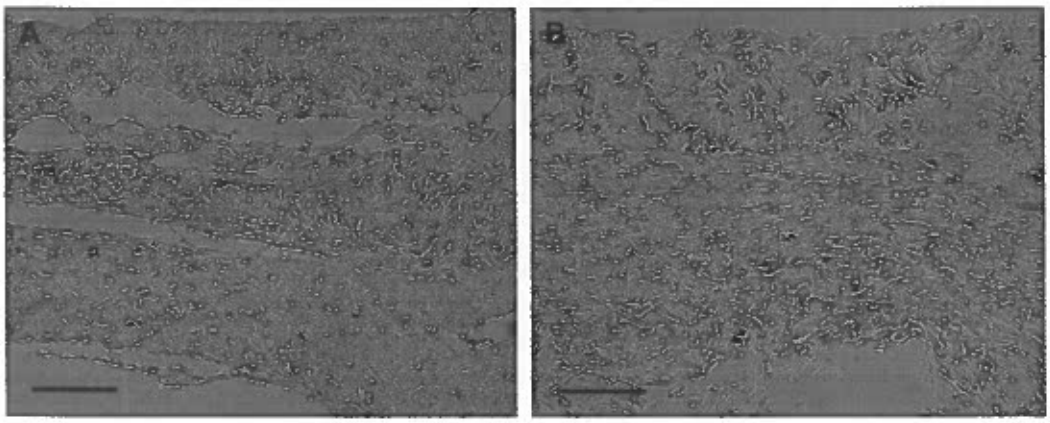


Fig. 2. Alcian blue staining of native and decellularized mesothelial tissue. The epithelial side is at the top of the image for A) native mesothelial tissue and B) decellularized mesothelial ECM. There is preservation of GAG staining in the decellularized ECM with reduced intensity but similar pattern of staining throughout the material. Scale bars = 100 μm.


Fig. 3. Immunofluorescent staining of the decellularized mesothelium ECM for retained ECM proteins. Shown are residual A) collagen IV, B) laminin and C) fibronectin within the mesothelium ECM material. The top of the image represents the epithelial side of the mesothelium. There is deposition of all three proteins at the mesothelial interface with other distributions throughout the cross-section of the material. Scale bars = 100 μm.

mesothelium conditioned medium (Fig. 6) was significantly higher than the control medium (EMEM on Fig. 6) or the conditioned medium from two different SIS material formulations. Importantly, there was minimal VEGF detected (only 4.0 ± 0.001 pg/ml) in the decellularized mesothelium ECM condition medium samples, measured after soaking but prior to adding them to the fibroblasts. This indicates that the majority of the VEGF measured was fibroblast derived and not simply eluted from the ECM.

### 3.6. Cell culture

To investigate the cellular response to this material *in vitro*, human foreskin fibroblasts and HUVECs were cultured directly on the materials *in vitro* (Fig. 7). When seeded at a moderate cell density, both cell types became confluent within three days. There was a degree of natural alignment of the fibroblasts on the side of the material opposite the mesothelial side (Fig. 7A). Endothelial cells grown on the mesothelial side of the material were phenotypically normal with expression of CD31 at the cell junctions (Fig. 7B).

## 4. Discussion

Decellularized ECM-based materials have shown significant promise as surgical implants and scaffolds for regenerative medicine. Understanding the composition of these complex natural

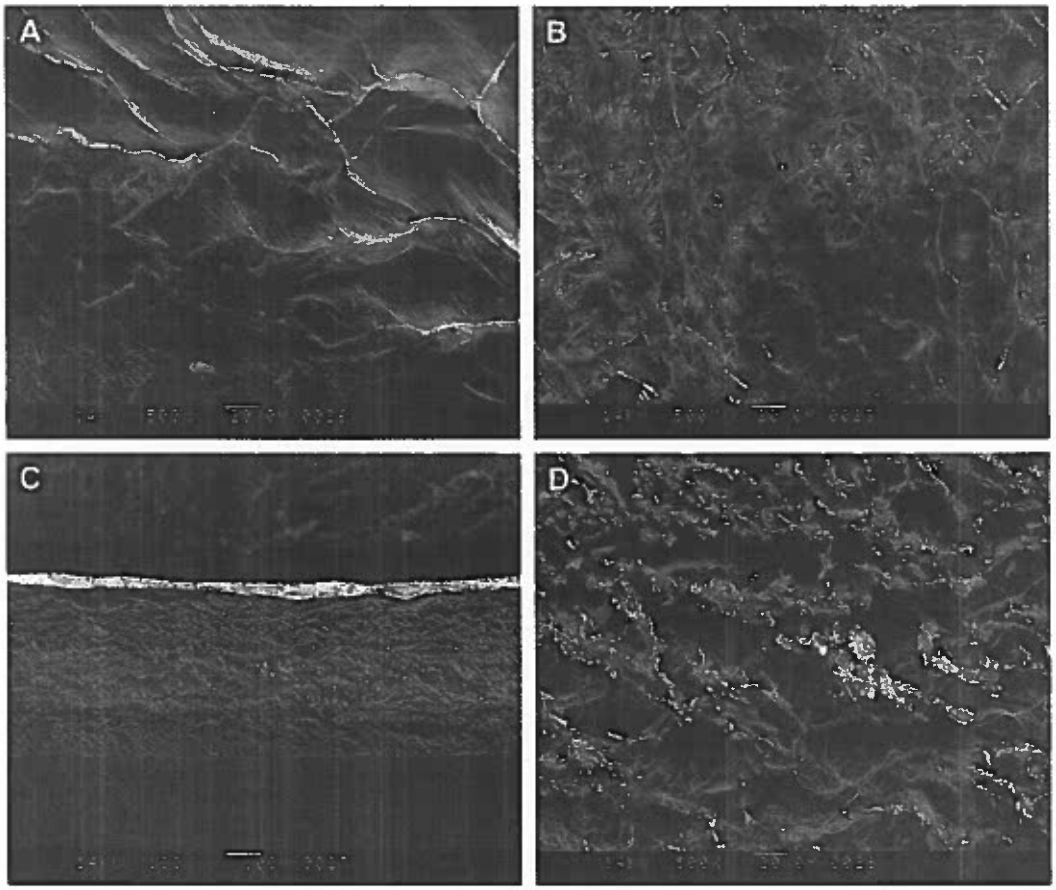


Fig. 4. Scanning electron micrograph images of decellularized mesothelium ECM. Shown is A) the mesothelial side, B) the side opposite the mesothelium and C) and D) cross-sectional images.

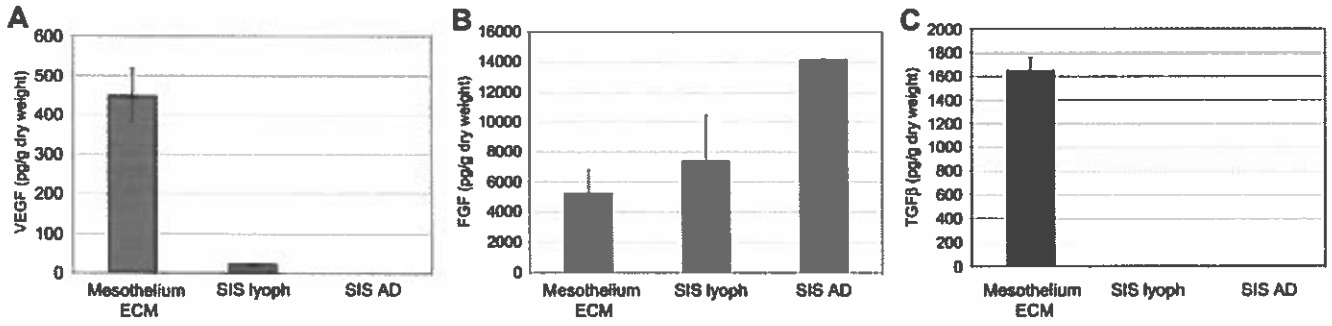


Fig. 5. Extraction of retained cytokines from the decellularized materials. A) VEGF, B) FGF and C) TGF-β were extracted from the mesothelium ECM or two commercially available formulations of SIS; lyophilized 4-layer SIS (SIS lyoph) or air dried 8-layer SIS (SIS AD). VEGF and TGF-β levels were significantly higher in the decellularized mesothelium than either SIS formulation. The air dried SIS material had significantly higher FGF than the mesothelium ECM, $p = 0.0037$.

materials and the biological stimuli they can produce will be a key in maximizing their potential for therapies. In this study, an ECM-based biomaterial was characterized. Mesothelium is the basement membrane of the quickly healing lining of the abdominal and chest cavities. This basement membrane, when decellularized, retains significant biological components that may be useful for regenerative medicine applications.

The efficiency of decellularization of the material was quantified by the PicoGreen assay, in addition to H&E staining. The PicoGreen assay quantifies the amount of residual double stranded DNA in the material. Although the preparation methods were slightly different, the residual amount of DNA in this material was significantly lower than the native material, as was described for other decellularized ECM materials [34]. The PicoGreen assay was run without a control accounting for the residual protein in the material possibly elevating the results. When the controls were made with ECM material treated with DNase to create a DNA free protein control, there was no detectable DNA in the decellularized mesothelium. Thus, this reported amount of residual DNA run without a protein control may represent an overestimation of the actual DNA remaining. The effect of residual DNA in these materials is not known but residual DNA has been used as a marker of decellularization.

As retention of ECM proteins was a central goal, it is significant that the collagen IV, fibronectin and laminin are present in the material and their distribution in the decellularized material is consistent with reported natural mesothelium [35,36]. SIS has been demonstrated to have fibronectin throughout the thickness of the material [37,38] but staining was negative for collagen IV and laminin [37]. Similar to the retained ECM proteins, retention of VEGF, FGF and TGF-β in the decellularized ECM is an important aspect of its biological activity as an implant or scaffold material. It is important to note the presence of GAGs in the material along with the cytokines. Glycosaminoglycans including heparin both protect cytokines such as basic FGF from denaturation and modulate their activity [39]. Glycosaminoglycans also sequester growth factors [40,41] so their presence in decellularized ECM materials may be important in the retention of naturally present cytokines. For example, the demonstrated presence of heparin and heparan sulfate in SIS [26] may be centrally related to the high amount of basic FGF retained in SIS as shown in this study and previously[29]. As the material is remodeled *in vivo*, it is expected that the VEGF

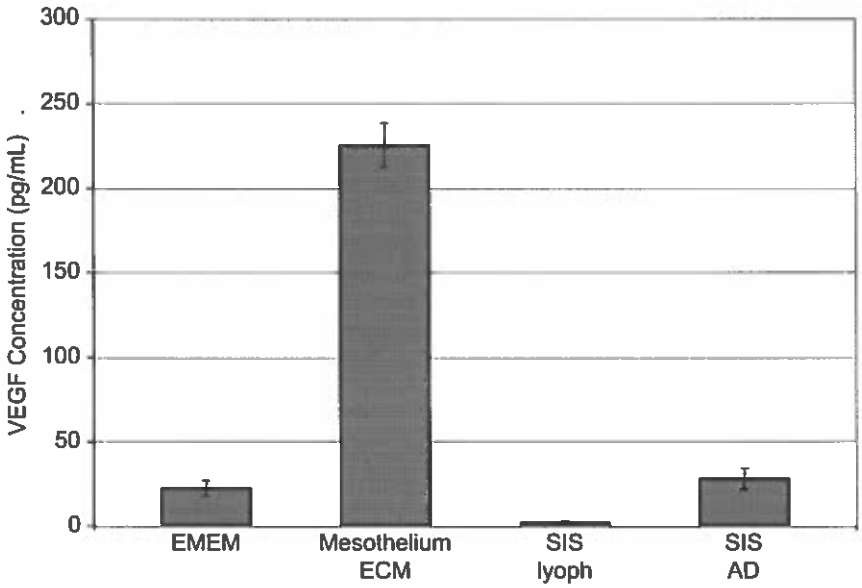


Fig. 6. VEGF production by fibroblasts grown in conditioned medium. VEGF production compared between control medium (EMEM) or medium conditioned in mesothelium ECM or two commercially available formulations of SIS; lyophilized 4-layer SIS (SIS lyoph) or air dried 8-layer SIS (SIS AD). The fibroblasts in the mesothelium ECM conditioned medium had significantly more VEGF production than the SIS conditioned medium or control EMEM medium. For mesothelium ECM compared to each of the other samples, $p < 0.05$.

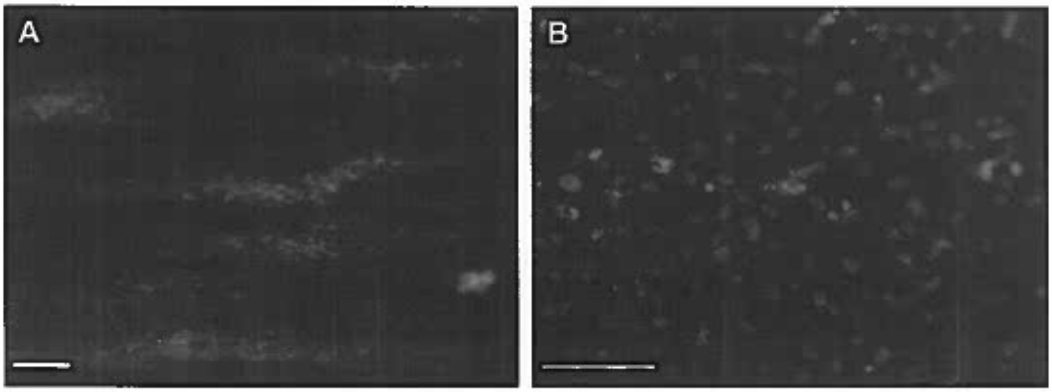


Fig. 7. Cell culture directly on the decellularized mesothelium ECM material. A) Human fibroblasts were grown on the ECM for 3 days and stained for f-actin and counter stained with the nuclear stain DAPI. B) Human umbilical vein endothelial cells were grown on the ECM for 3 days and stained for CD31 and counter stained with DAPI. Scale bars = 100 μm.

and FGF will be released and promote angiogenic ingrowth of vessels into the material. TGF-β is a known mitogen for fibroblasts and induces collagen production [42]. Depending on the application, the release of TGF-β may be useful for creating a mechanically strong replacement tissue. The inherent TGF-β in the material may also make the material a potential scaffold or scaffold component for *in vitro* or *in vivo* differentiation of mesenchymal stem cells into chondrocytes for cartilage regenerative medicine applications. It should be noted that although the ELISA measures retained cytokines, only the TGF-β assay is specific for those molecules that have the potential to be biologically active.

In addition to the retained cytokines, a very intriguing aspect of this material is the soluble factor(s) that are released into the conditioned medium that stimulates fibroblasts to increase their secretion of VEGF. The VEGF retained in the matrix was not released with an aqueous-based extraction thus the VEGF in the conditioned medium study was produced by the fibroblasts. The factors that caused the increase in secretion of VEGF were not identified in this study but will be an important aspect in the next level of evaluation of this material. The stimulated production of other factors by the fibroblasts and the stimulated production of cytokines by other cells will also be investigated. In particular, basic FGF and hepatic growth factor/scatter factor are of principle interest. The ability of the material to stimulate cytokines from other cell types, including mesenchymal stem cells and endothelial cells will be studied. Any difference in biological stimulus by the two different sides of the material will also be a central component of future investigation. Qualitatively more robust cell growth was observed on the side of the ECM that hosted the native mesothelium compared to the side adjacent to adipose tissue. There may be a direct stimulus for cell growth based on the residual ECM proteins and cytokines on the mesothelial side. Certainly this is consistent with surgical observations that this is a quickly healing natural tissue. The nature of this biological stimulus will continue to be elucidated as it may hold keys to combinations of ECM proteins and other factors that naturally signal rapid cell growth and repair. These physiologic keys may be applied to other scaffold materials and regenerative medicine applications.

This sheet-based material may have initial clinical applications as a soft tissue repair surgical material for hernias and other soft tissue reconstruction. It has excellent mechanical strength as a single layer and that could be increased by layering multiple sheets for particular clinical applications. As the biological responsiveness of this material becomes better understood, it may have multiple regenerative medicine applications. As demonstrated by the phenotypically normal endothelial cell growth on the once mesothelial side of the material in Fig. 7, the ECM could be used to create vascular grafts or conduits. The end mechanical responsiveness of such grafts after remodeling *in vivo*, including compliance, would be paramount in understanding the utility of the material. Given the high degree of flexibility and ease of handling, the ECM material may work well as a biological patch material for vessels or intracardiac applications (e.g. ventricular septal defect patches). If the combination of the retained angiogenic and TGF-β cytokines has a significant *in vivo* effect, the material may be an excellent reinforcement material to seal surgical staple lines and provide a biological stimulus for accelerated wound healing.

## 5. Conclusions

Decellularized porcine mesothelium is a thin flexible sheet material with significant mechanical strength that retains several types of collagen and other ECM proteins and glycosaminoglycans. Multiple cytokines are present in the material including VEGF, FGF and TGF-β which may promote remodeling of the material when clinically applied. The stimulation of increased VEGF secretion by fibroblasts exposed to the material demonstrates retained biologically active factors within the material. Further work will identify the mechanism of the robust cellular growth on this material and define therapeutic clinical applications.

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