Fabrication and Characterization of an Acellular Human Kidney ECM-derived Hydrogel

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Abstract

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Today, more than 900,000 patients in the United States live with end-stage renal disease, with many more suffer from poor renal regeneration[1]. Current methods commonly use synthetic tissue-engineered constructs to deliver cells, drugs, or as scaffolds for reconstruction of injured tissues[2, 3, 4, 5, 6]. However, these constructs usually lack vascularization *in vivo,* and have poor nutrient diffusion abilities that limit their effectiveness[7]. On the other hand, we have found that extracellular matrix (ECM) hydrogels can be used as scaffolds to facilitate the repair and reconstruction of various tissues. The objective of this study is to fabricate and characterize the mechanics and cell response *in vitro* of ECM hydrogels prepared from decellularized human kidney tissues. Our preliminary data indicate that Sodium dodecyl sulfate (SDS) decellularization of human kidney tissue sections gives the most optimal results in terms of cellular material removal and ECM structure preservation; decellularized human kidney ECM, when mixed with collagen gel on a 1:1 ratio, shows similar mechanical properties as commonly used type I collagen gel. These data suggest that

decellularized human kidney ECM hydrogels are able to provide the structural support necessary for cellular activities. Given these findings and the fact that ECM hydrogels are derived from naturally occurring kidney materials, I hypothesize that decellularized human kidney ECM hydrogel is able to not only provide structural support for cell growth and proliferation, but also to enhance bioactivity and vascularization. To test this hypothesis, I performed angiogenesis and tubulogenesis studies by seeding human umbilical vein endothelial cells and human kidney microvascular endothelial cells onto or in the decellularized human kidney ECM hydrogel as well as type I collagen gel.

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ACKNOWLEDGEMENTS

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INTRODUCTION

The Kidneys and End-Stage Renal Disease (ESRD)

Kidneys, the bean-shaped organs located at the rear of the abdominal cavity in the retroperitoneal space, play key roles in a wide variety of functions that are essential to the normal functioning of the human body—from the regulations of extracellular fluid volume, blood pressure, and osmolarity, the homeostatic regulation of pH, and the maintenance of ion balance, to the excretion of wastes and the production of hormones.

The malfunctioning of the kidneys has huge impacts on the global population. All over the world, chronic kidney disease is a leading cause of mortality and morbidity, affecting an estimate of 13% of the world's population[8]. End-stage renal disease (ESRD) is the last stage of chronic kidney disease. During this stage, the kidneys can no longer perform their functions at a level that supports the normal functioning of daily activities. At the end of 2009, more than 871,000 patients in the United States were being treated for end-stage renal disease, with over 100,000 new diagnoses every year[8]. Hemodialysis and kidney transplantation are the last resorts for ESRD patients. Hemodialysis, not being a curative treatment, is extremely time- and energy-consuming and oftentimes requires trained personnel to initiate and monitor the process in an outpatient facility. As a consequence of the limited treatment options that are currently available, an estimate of 100,000 people in the United States are awaiting a donor kidney—a significant number when compared to the 18,000 kidney transplantations that are performed each year. Even after kidney transplantation, despite advances in renal transplant immunology, 20% of recipients experience an episode of acute rejection

within 5 years of the transplantation, and approximately 40% of the recipients will die or lose graft function within 10 years[8].

One reason why there is a lack of treatment options for ESRD patients is that kidneys, like hearts, have very limited self-regenerating capacities. Consequently, a curative treatment which could result in kidney tissue regeneration, ultimately achieving the regeneration of kidney functions, is in urgent need.

Vascularization and Tissue Formation: Important Bioactive Proteins

Vascularization of the injured kidney site is the first step towards kidney tissue regeneration. Original vasculature formations during the development of mammalian tissues are achieved with various types of bioactive components within the extracellular matrix that guide cellular behavior, activities, and ultimately, vasculature formation. Certain bioactive components are shown to be essential to the vascularization of the human kidney tissue.

Laminin, a protein of the ECM, is a major component of a protein network foundation called the basal lamina. Fibronectin is a glycoprotein of the ECM that binds to integrins, membrane-spanning receptor proteins. Both laminin and fibronectin are shown to impose great influence on cell differentiation, migration, and adhesion. They also play important roles in determining cell phenotype and cell survival rates. Collagen IV, a type of collagen primarily found in the basal lamina, is abundant in the human kidney ECM and is believed to be the major structural protein and a main component of connective tissue. Another important ECM component, heparan sulfate regulates a wide variety of biological activities, including various developmental processes and

angiogenesis. Versican is yet another ECM protein that plays a crucial role in cell adhesion, migration, and proliferation.

Decellularization

Decellularization is a process that separates the extracellular matrix of a tissue from its inhabiting nucleic contents. The ideal result is a naturally-occurring ECM scaffold with its intact tissue architecture and bioactive components. There are different approaches to decellularizing tissues, including physical methods, chemical methods that use various chemical detergents, enzymatic methods, methods using protease inhibitors, and methods using antibiotics. Among these various approaches for tissue decellularization, the physical, chemical, and enzymatic approaches were the most robust and effective[9]. SDS is one of the most commonly used ionic detergents for decellularization and is very effective in terms of removing cellular components from their inhabiting tissues. There is a possibility that the use of SDS could disrupt the native tissue architecture and collagen integrity; however, previous studies done by different groups have shown ideal results of decellularization using SDS[9, 10, 11].

In a study performed by Song et al., histology of decellularized cadaveric rat kidneys using renal artery perfusion with 1% SDS at a constant pressure of 40 mm Hg has shown both preservation of the rat kidney tissue architecture and the complete removal of nuclei and cellular components[9]. Furthermore, a study on the more human kidney size-relevant porcine kidneys performed by Sullivan et al. shows that porcine kidneys perfused with SDS also showed both the preservation of native ECM architecture and the removal of nucleic components[11].

Our Approach

Our approach utilizes the fact that natural human kidney ECM, after decellularization, is able to retain most, if not all, of its important bioactive components. Therefore, we propose that a hydrogel derived from the acellular human kidney ECM, when later on re-popularized with cells, will have the abilities to enhance cellular activities and vascularization, ultimately achieving tissue regeneration and kidney function restoration.

MATERIALS AND METHODS

Decellularization of Human Kidney

Human kidneys that were rejected from or not used in transplantation were obtained from Pacific Northwest Life Science Center. Human kidneys were cut into smaller sections with a thickness of approximately 1 cm using sterile scalpel blades in bio-hood. 1% SDS decellularizing solution was made by dissolving SDS powder (SIGMA-ALDRICH) in deionized water with the appropriate ratio. A magnet is then placed into the solution and the solution was then placed on top of a magnetic stir plate for thorough mixing. 1N Sodium hydroxide (NaOH) was added to achieve a pH of 7.4 for the SDS solution. Human kidney sections were then placed in the 1% SDS solution for 5-7 days until complete decellularization was achieved.

Characterization of the Decellularized Human Kidney Scaffold

Immunofluorescent staining was performed to characterize the decellularized human kidney scaffold. The decellularized human kidney tissue was further processed into smaller pieces with dimensions of approximately 1 cm x 1 cm x 1 cm. The tissue section was then embedded in Optimal Cutting Temperature Compound (Fisher HealthCare) embedding medium and was cryosectioned into thin tissue sections with a thickness of 8 μm. 3.7% paraformaldehyde (SIGMA-ALDRICH) was applied for 15 minutes to fix the tissue section, 0.1% Triton X-100 (SIGMA-ALDRICH) was applied for 2 minutes to permeate the tissue section, and 2% bovine serum albumin (BSA; SIGMA-ALDRICH) was applied for 1 hour to block the tissue section. All solutions were applied at room temperature. Primary antibodies were diluted with the 2% BSA blocking buffer. The primary antibodies rabbit anti-laminin (Abcam), rabbit anti-fibronectin (Abcam),

rabbit anti-collagen IV (Abcam), mouse anti-heparan sulfate (Abcam), and rabbit anti-versican (Abcam) were applied to the tissue sections overnight at 4°C. After primary antibody incubation, slides with tissue sections were washed in 1XPBS for 15 minutes, and corresponding secondary antibodies were subsequently applied at dilutions of 1:200 for 1 hour (Alexa Fluor). Hoechst stain for deoxyribonucleic acid (DNA) detection was also performed. Cover slips were mounted to the stained slides using permount (Fisher). Wide-field microscopy was performed to image the untreated human adult kidney sections (control) and the stained decellularized human kidney tissue sections.

Gel Fabrication

The resulting acellular scaffolds of human kidney sections from SDS decellularization were lyophilized and homogenized, resulting in a decellularized ECM (d-ECM) solution. M199, a reddish pH indicator that turns yellow under acidic conditions, and Endothelial Growth Media (EGM) were added into the d-ECM solution on ice. 1N NaOH was added in a dropwise fashion and mixed with the solution for neutralization purposes. The entire neutralized solution was incubated under 37°C for approximate 30 minutes, until complete decellularized human kidney ECM (d-hk ECM) gel formation.

Characterization of Acellular ECM Gel Content

Immunofluoresent staining was performed to characterize the ECM components of the decellularized hman kidney ECM hydrogel. Refer to “Characterization of the Decellularized Human Kidney Scaffold” for detailed a procedure.

Characterization of the Mechanical Properties of the d-hk ECM Hydrogel

Mechanics studies was performed to test the mechanical properties of the d-hk ECM gel. The results were compared to the commonly used type I collagen gels with different collagen concentrations. Rheology studies were performed on the d-hk ECM hydrogel, a 1:1 mixture gel, and type I collagen gels of concentrations of 0.2%, 0.4%, and 0.75%. The viscoelastic measurements were obtained using parallel plate geometry (Physica MCR 301). The volume of each sample for all measurements was 1000 μl. Oil was applied around the samples as a solvent trap to avoid evaporation. All samples were measured at 37°C in oscillatory mode at a fixed frequency of 1 Hz with varying strain from 0.01% to 1,000%. Storage moduli (G') and loss moduli (G'') were measured, and complex moduli were calculated from the two using the equation

$$\text{Complex Modulus} = \text{sqrt}(G'^2 + G''^2).$$

Cellular Activity Assessment of the d-hk ECM Hydrogel

Angiogenesis studies and tubulogenesis studies were performed to study the d-hk ECM hydrogel's abilities to promote vascularization. Human umbilical vein endothelial cells (HUVECs) and human kidney microvascular endothelial cells (hkMECs) were seeded onto the d-hk ECM hydrogel as wells as onto the type I collagen gel that had similar mechanical properties shown from the previous viscoelastic measurements. Each piece of polydimethylsiloxane (PDMS) mold with an approximate 2 mm thickness were punched with three 5 mm-diameter holes. Another piece of PDMS

of an approximate 1 mm in thickness was adhered to one side of the previous PMDS mold, forming 3 wells in each PDMS mold.

Angiogenesis assays were performed to assess the number of angiogenic sprouting. D-hk ECM hydrogel, 1:1 mixture gel, and type I collagen gel were loaded into the wells of the PDMS mold. HUVECs and hkMECs were trypsinized, centrifuged, re-suspended, and diluted to a final concentration of 10^6 cells/mL in growth medium and were seeded onto each gel type. The PDMS molds were then placed into a 24-well plate, submerged in EGM containing vascular endothelial growth factor (VEGF) at 37°C for 7 days. Medium was changed every other day.

Tubulogenesis assays were performed to assess the number of tube formations within the d-hk ECM hydrogel, 1:1 mixture gel, and type I collagen gel. HUVECs and hkMECs were trypsinized, centrifuged, re-suspended, diluted to a final concentration of 10^6 cells/mL in growth medium, and thoroughly mixed with each gel solution. The cell-gel solutions were then loaded into the wells of the PDMS molds. The molds were the placed into a 24-well plate, submerged in EBM-2 with 40ng/mL VEGF at 37°C for 7 days. Medium was changed every other day.

RESULTS

Characterization of Decellularized Human Kidney

Human kidney were decellularized with 1% SDS at room temperature. Upon visual inspection, all kidney sections appeared translucent and devoid of nucleic material. Biochemical quantification of residual nucleic material showed DNA content to be 210 +/- 10 ng/μl/10 mg dry tissue in decellularized tissue compared to 900 +/- 50 ng/μl/10 mg dry tissue in untreated tissue samples (Figure 1).

High percentage liquid chromatography mass spectrometry showed the presence of the various important ECM components following SDS decellularization (Figure 2). Furthermore, immunofluorescent staining against laminin, fibronectin, collagen IV, heparan sulfate, and versican, together with hoechst stain for deoxyribonucleic acid detection, showed complete removal of nucleic material and good preservation of the natural human kidney architecture (Figure 3). After washing with deionized water, the decellularizing detergent was not detected in the decellularized human kidney ECM scaffolds.

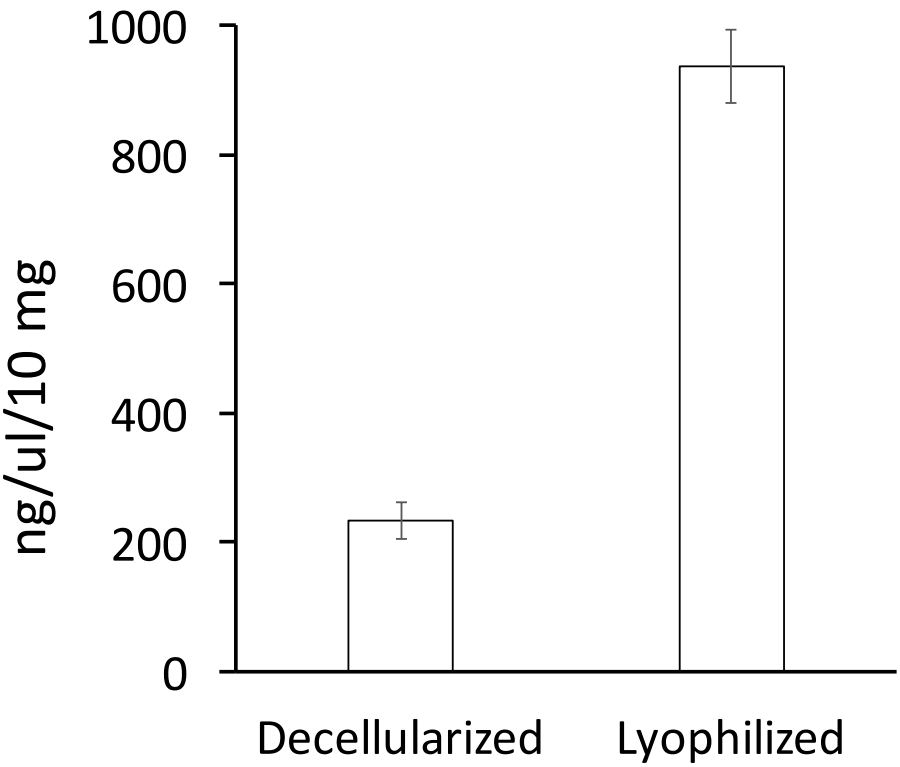


Figure 1. Biochemical quantification of nucleic material in decellularized and lyophilized (control) human kidney tissue showing a reduction of nucleic material after SDS-decellularization.

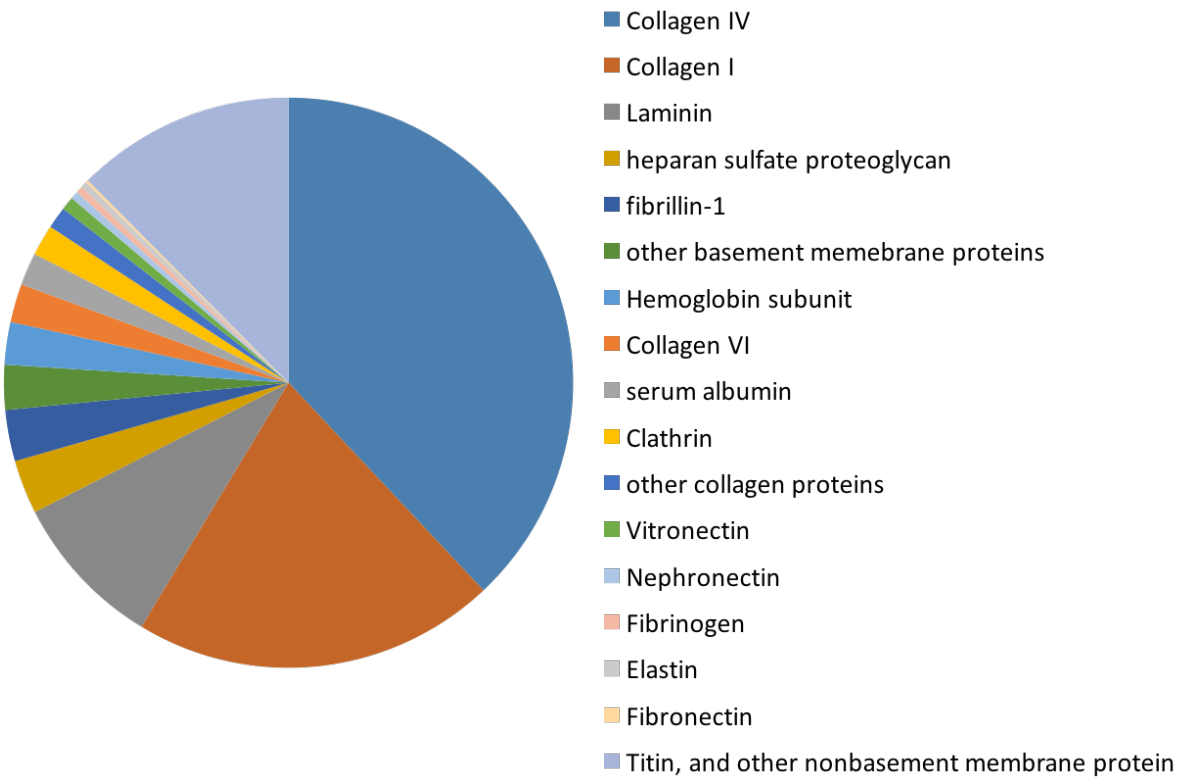
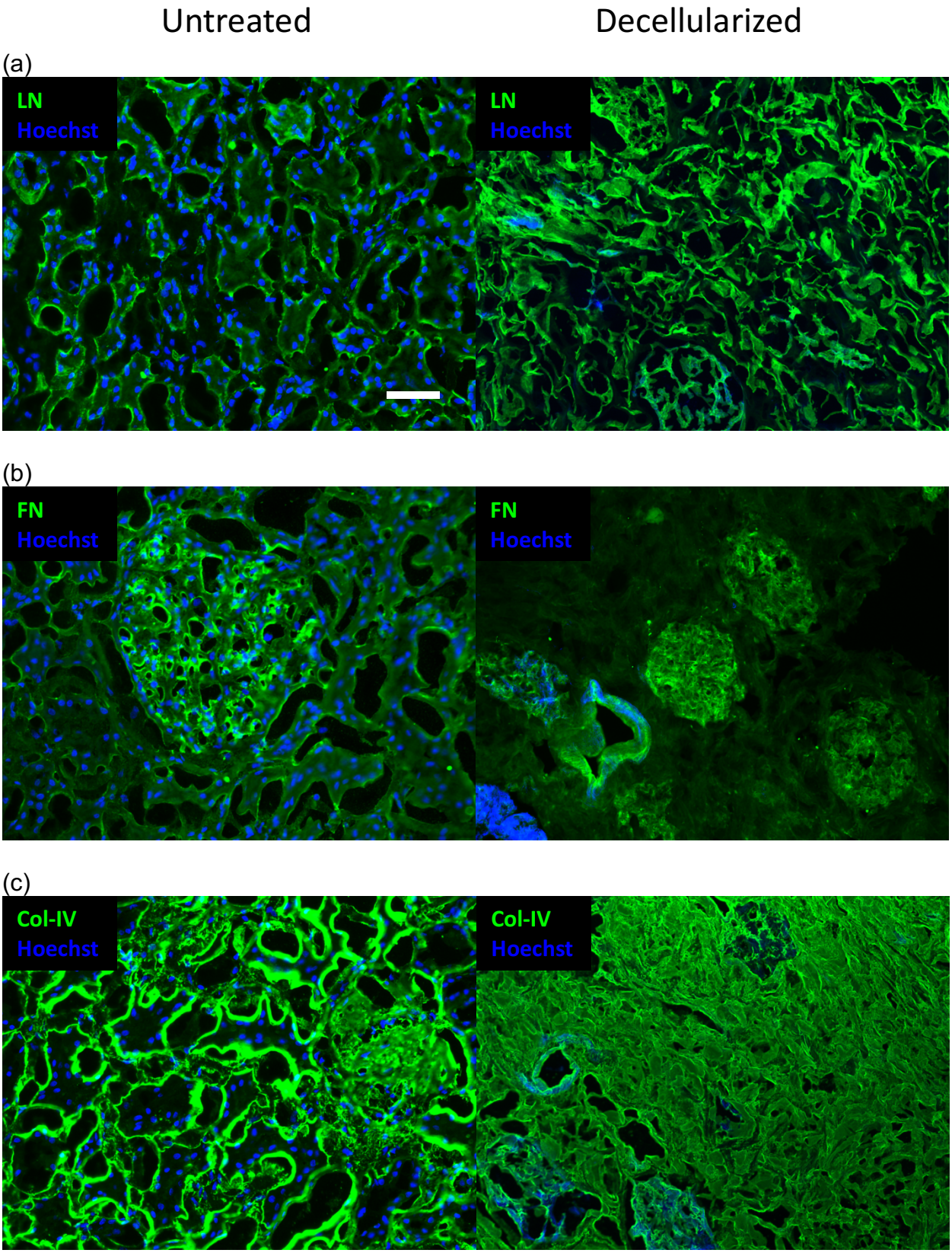


Figure 2. HPLC-MS quantified key ECM components after decellularization.


Untreated
Decellularized
(a)
LN
Hoechst
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(b)
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(c)
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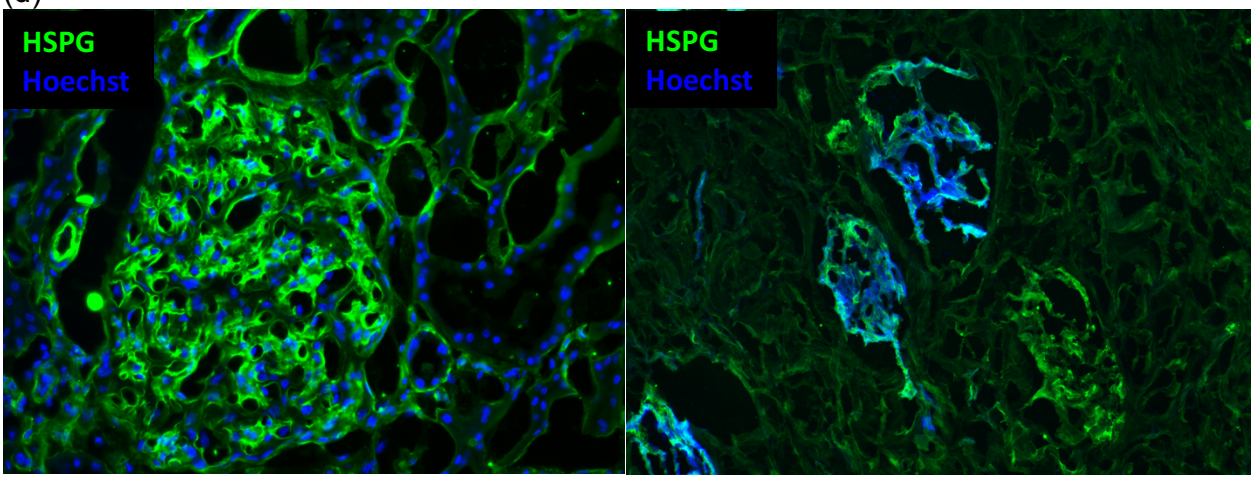
Col-IV
Hoechst

(d)



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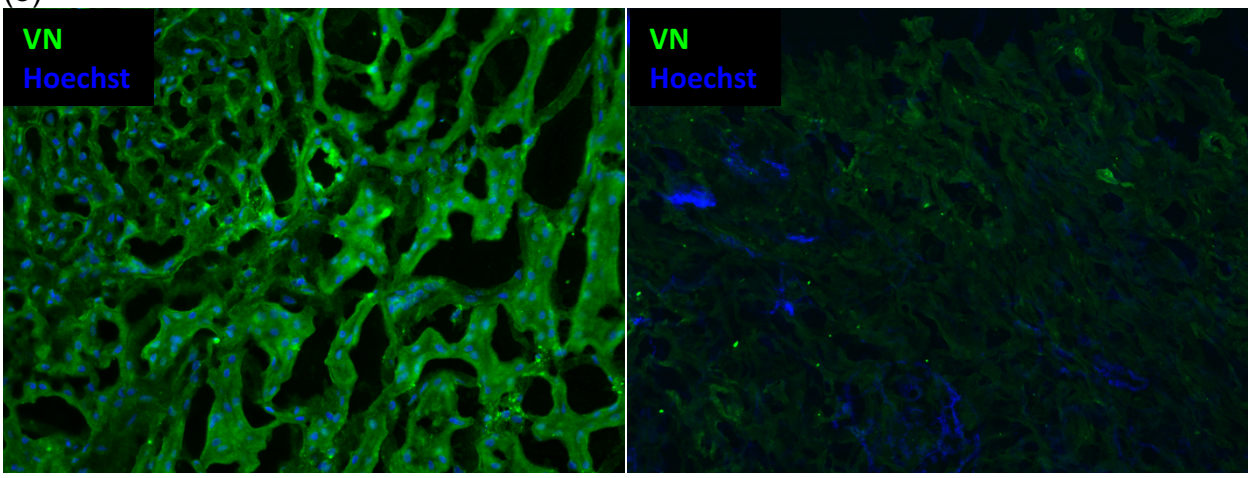


Figure 3. Fluorescence micrographs showing the distributions of (a) laminin, (b) fibronectin, (c) collagen IV, (d) heparan sulfate, and (e) versican in decellularized and untreated (control) human kidney ECM, confirming the preservation of important bioactive ECM components in the absence of nucleic materials. Scale bars 100 μm.

Fabrication of d-hk ECM Hydrogel and 1:1 Mixture Gel

Decellularized human kidney ECM hydrogels and 1:1 mixture gels were successfully fabricated from decellularized human kidney ECM scaffolds at a concentration of 7.5 mg/ml. Macroscopically, 1:1 mixture gel had a more rigid structure with better defined edges. The 1:1 mixture gel was also able to withstand greater forces when compared to the d-hk ECM hydrogel of the same final concentration of 7.5 mg/ml.

The d-hk ECM hydrogels could not be handled with force using forceps, and could completely dissolve in 1x PBS over time, or if not handled with caution (Figure 4).

Scanning electron micrographs of the surfaces of the type I collagen gel, 1:1 mixture gel, and d-hk ECM hydrogel showed qualitatively and microscopically that all three gel types tested possess randomly oriented fibrillar structures, indicating similarities in their microscopic structures (Figure 5).

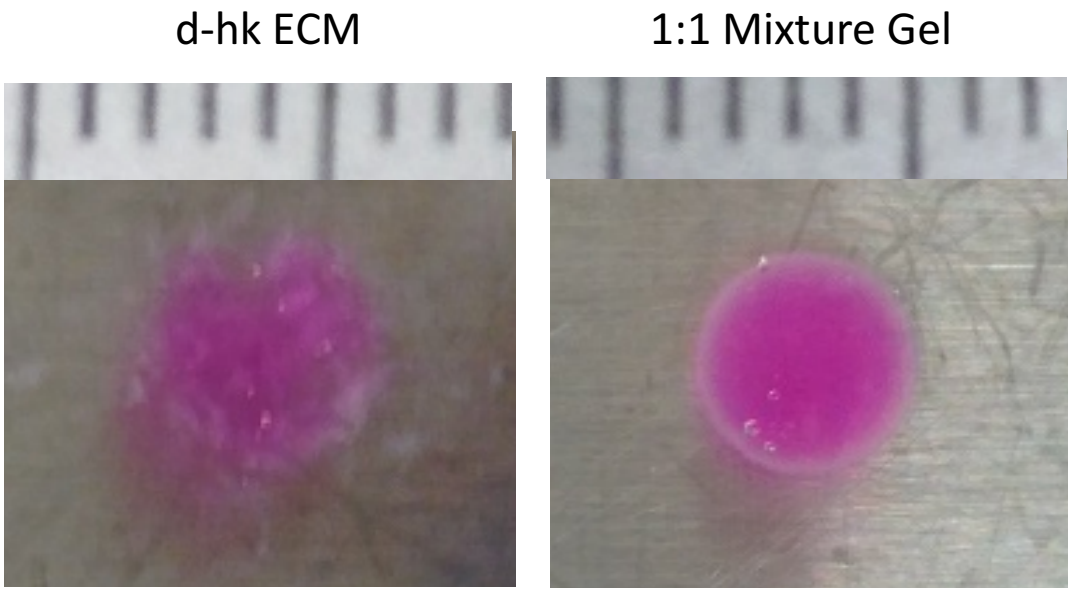


Figure 4. Macroscopic appearance of d-hk ECM hydrogel and 1:1 mixture gel.

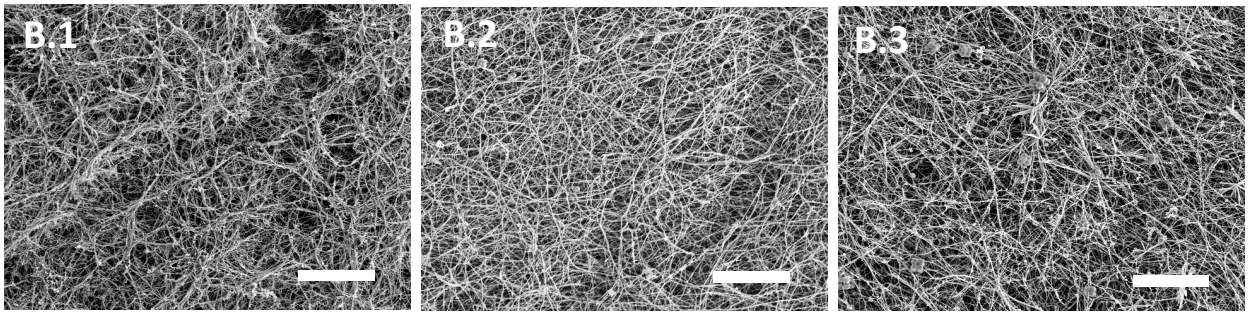
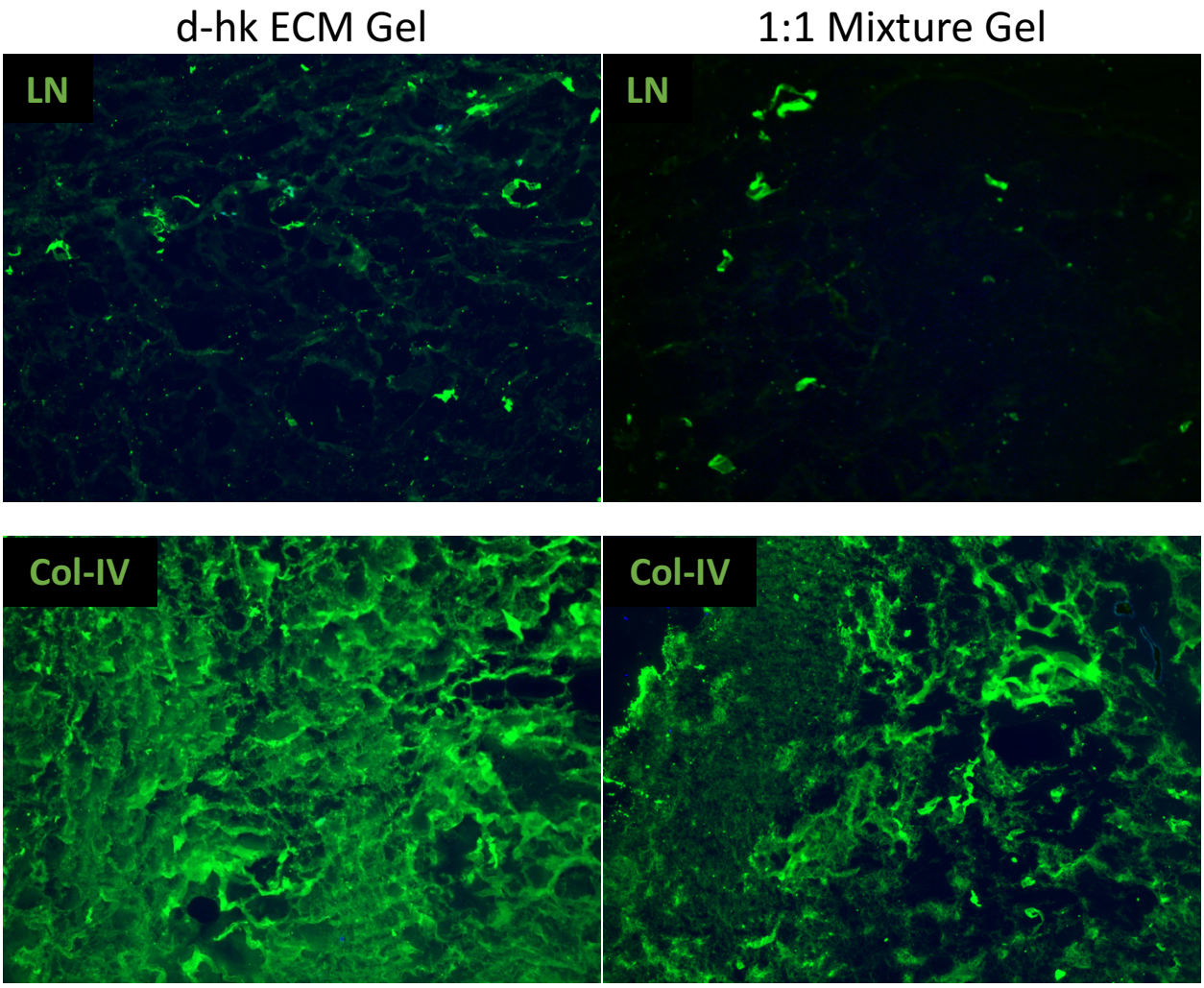


Figure 5. Scanning electron micrographs of left: type I collagen gel, middle: 1:1 mixture gel, and right: d-hk ECM hydrogel. Scale bar 10 μm.

Characterization of ECM Components of d-hk ECM Hydrogel and 1:1 Mixture Gel

Immunofluorescent staining against laminin, collagen IV, and heparan sulfate showed preservation of key ECM components within the decellularized human kidney ECM hydrogel and the 1:1 mixture gel (Figure 6).



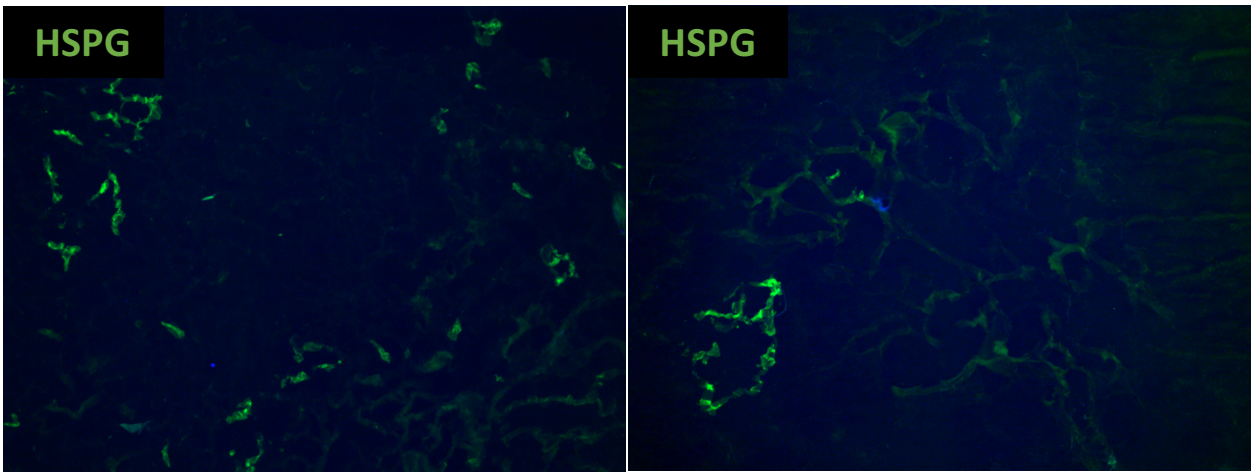


Figure 6. Fluorescence micrographs showing the distributions of laminin, collagen IV, and heparan sulfate in decellularized human kidney ECM hydrogel and in 1:1 mixture gel, confirming the preservation of important bioactive ECM components within each gel type.

Complex Moduli of Tested Gels

The mechanical properties of the type I collagen gel (concentrations: 2 mg/ml, 4 mg/ml, and 7.5 mg/ml), the 1:1 mixture gel (final concentration = 7.5mg/ml), and d-hk ECM hydrogel (concentration = 7.5 mg/ml) were determined using a parallel plate rheometer. Type I collagen gels showed an increase in complex modulus with increasing collagen concentration. 1:1 mixture gel and 4 mg/ml type I collagen gel had comparable complex moduli. 7.5 mg/ml d-hk ECM hydrogel had the lowest complex modulus of 15 +/- 5 Pa, suggesting the possibility that 7.5 mg/ml d-hk ECM hydrogel not possessing the desirable mechanical properties and physical environment for cellular activities and cell proliferation (Figure 7).

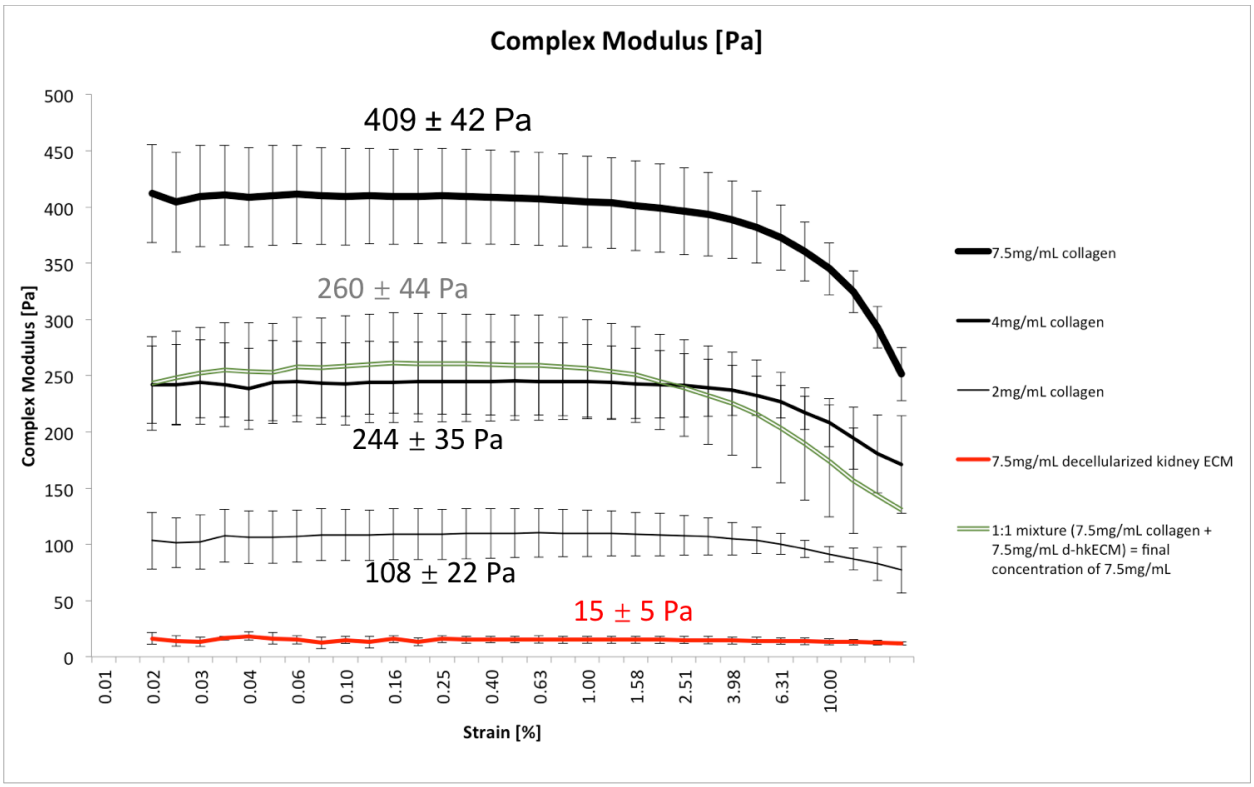


Figure 7. Parallel-plate rheological characterization of d-hk ECM hydrogel, 1:1 mixture gel, and type I collagen gels at concentrations of 2 mg/ml, 4 mg/ml, and 7.5 mg/ml. Storage (G') and loss moduli (G'') were determined by monitoring the responses of the gels to applied strain (varying from 0.1% to 1,000%). Complex moduli were calculated from the storage and loss moduli.

Angiogenesis Studies

HUVECs and hkMECs were cultured on the surface of 4 mg/ml type I collagen gel and on the surface of 1:1 mixture gel with a final concentration of 7.5 mg/ml. Proliferation of the two cell types, as well as the number of angiogenic sproutings were assessed after 7 days of *in vitro* cell culture at 37°C. Confocal microscopy were performed subsequently. HUVECs were confluent on the surface of both the 4mg/ml type I collagen gel and the 1:1 mixture gel.

Immunofluorescent staining against CD 31, an indicator for the prescence of endothelial cells in histological tissue sections, and phalloidin, which functions by binding and stabilizing filamentous actin, was performed (Figure 8).

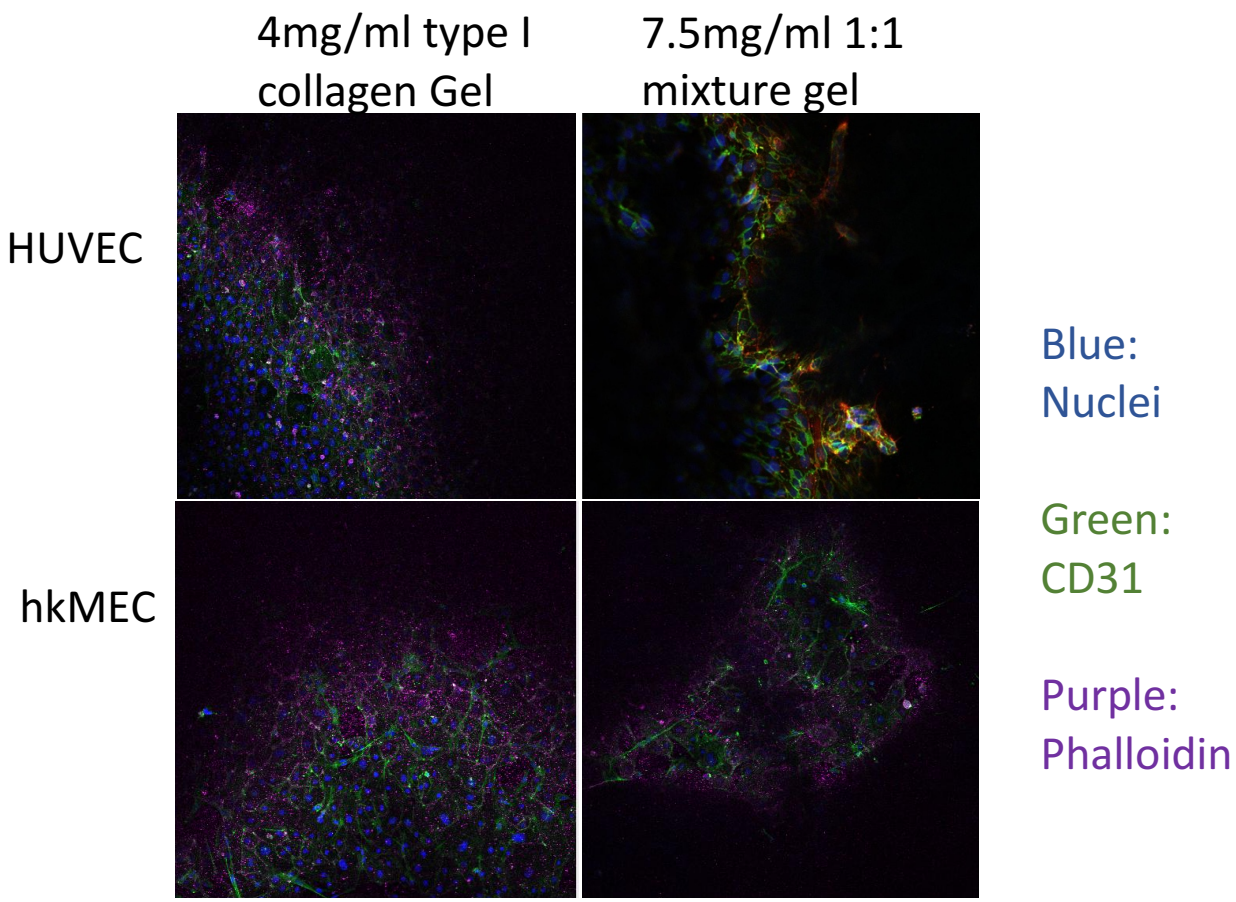


Figure 8. Confocal micrographs for HUVECs and hkMECs seeded on 4 mg/ml type I collagen gel and 7.5 mg/ml 1:1 mixture gel after 7 days of cell culture.

Tubulogenesis Studies

HUVECs and hkMECs were mixed thoroughly with the gel solution before complete gel formation. The mixtures were then incubated under 37°C for complete gel formation. Proliferation of the two cell types, as well as the number of tube formations were assessed after 7 days of *in vitro* cell culture at 37°C. Confocal microscopy were performed subsequently.

More tube formation was observed for HUVECs within both 4 mg/ml type I collagen gel and 7.5 mg/ml 1:1 mixture gel then for hkMECs (Figure 9).

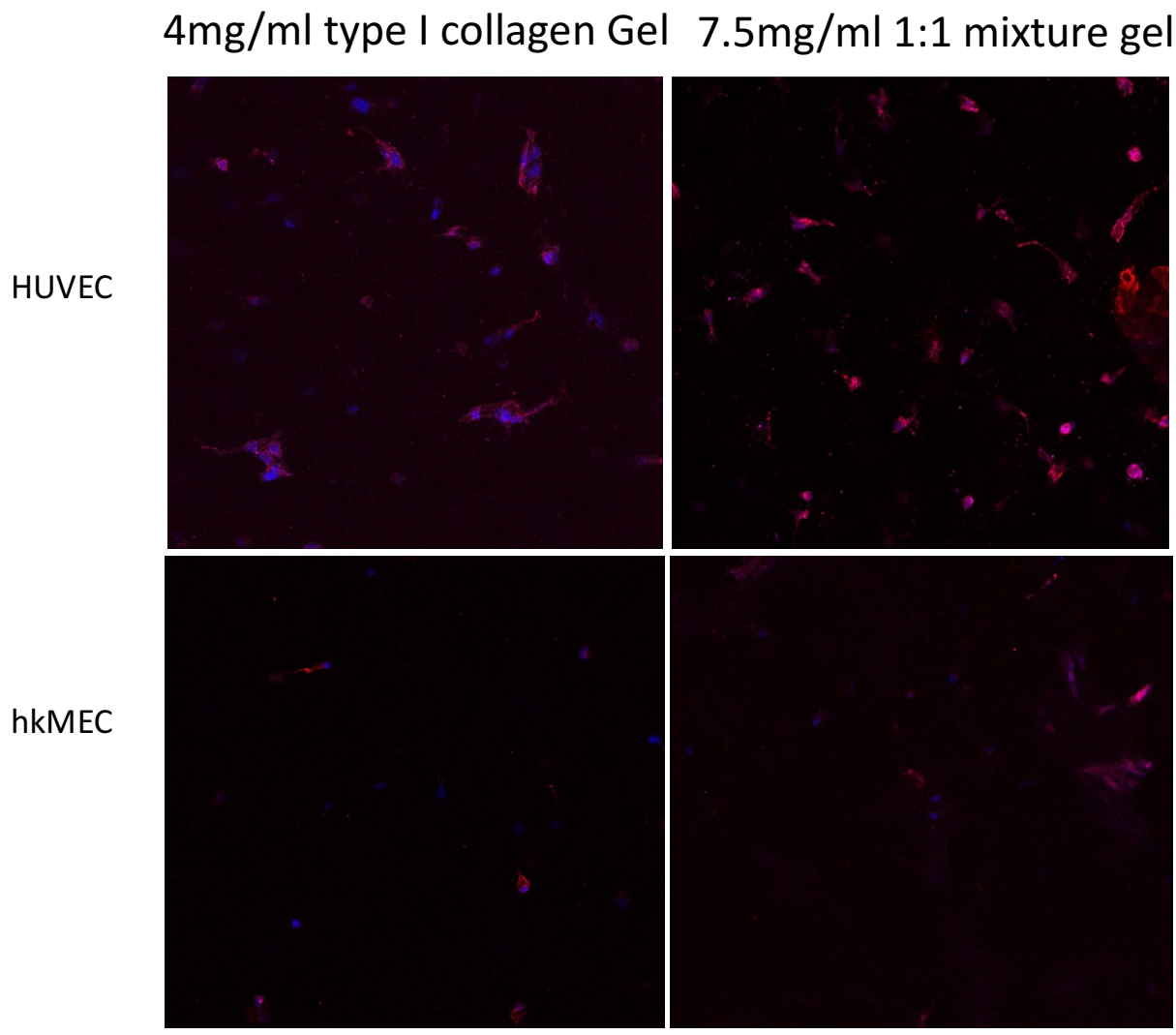


Figure 9. Confocal micrographs for HUVECs and hkMECs seeded within 4 mg/ml type I collagen gel and 7.5 mg/ml 1:1 mixture gel after 7 days of cell culture.

Characterization of Endothelial Cells Within ECM Hydrogel

Figure 10 compares the various biomarkers expressed by hkMECs within type I collagen gel and d-hk ECM hydrogel. The expression levels of the following biomarkers were measured using polymerase chain reaction (PCR): von willebrand factor (VWF), a product and indicator of endothelial cells; eNOS, nitric oxide synthase, an important cellular signaling molecule that is involved in angiogenesis and neural development; MMP, matrix metalloproteinases, which responds to VEGF in endothelial cells; VCAM 1, vascular cell adhesion molecule 1, which functions as a cell adhesion molecule; and JAM 2, junctional adhesion molecule, which represents one mode of cell-to-cell adhesion in endothelial sheets.

The expression levels of the various biomarkers within the type I collagen gel were set to 1. Comparing to the basal levels of expression within type I collagen gel, the increases in VWF, eNOS expression levels, and the decreases in MMP10, VCAM1, JAM2, and PCNA (indicating proliferation) expression levels demonstrated more mature and quiescent kidney endothelial cells within the d-hk ECM hydrogel.

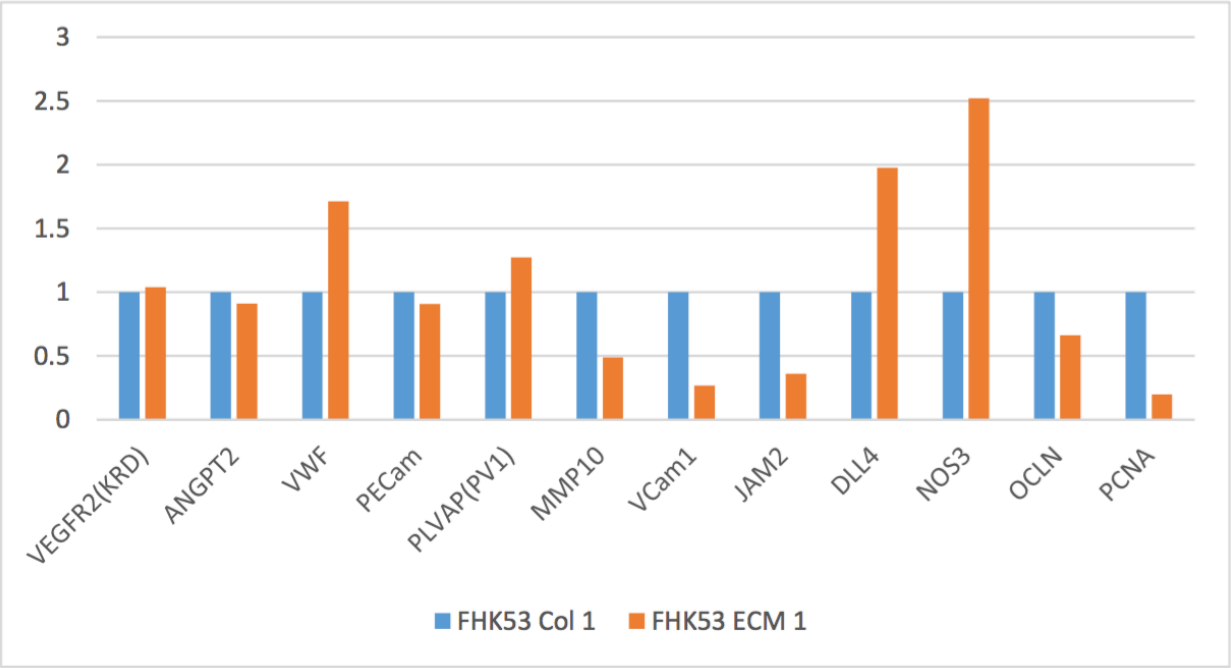


Figure 10. Expression levels of various biomarkers of hkMECs within type I collagen gel and d-hk ECM hydrogel, by PCR.

DISCUSSION

The overall goal of this research study was to develop a naturally-occurring, acellular human kidney ECM scaffold with the abilities to enhance cellular activities and to promote vascularization in vitro, with the potential to promote vascularization and tissue regeneration *in vivo*. The various key bioactive ECM components reside in native kidney tissues play crucial roles in guiding cellular activities, including developmental processes and angiogenesis. Additionally, our data on decellularized human kidney tissue demonstrated complete removal of nucleic material and overall good preservation of key ECM components. Decellularized human kidney ECM fabricated into injectable hydrogel under physiologically relevant pH and temperature is able to support cell growth and activities. The d-hk ECM hydrogels, together with 1:1 mixture gels, were evaluated for structural and mechanical characteristics, as well as for *in vitro* cellular activity enhancements.

In line with the results of kidney decellularization from previous studies performed by other research groups, this study confirmed that SDS detergent decellularization of adult human kidney sections successfully removes cells and cellular debris from their inhabiting tissue environment, and preserves of the native kidney tissue architecture and ECM components important in guiding cellular activities. High percentage liquid chromatography mass spectrometry and immunofluorescent staining of the decellularized human kidney tissue sections further confirmed the preservation of ECM components after SDS decellularization. This study also showed that hydrogels can be derived from the naturally-ocurring, decellularized human kidney ECM scaffolds. Furthermore, immunofluorescent staining of the decellularized human kidney ECM

hydrogel and the 1:1 mixture gel confirmed the preservation of ECM components after lyophilization, homogenization, and gel formation.

Further examination of the d-hk ECM hydrogel and 1:1 mixture gel showed that, microscopically, the decellularized human kidney ECM hydrogel, 1:1 mixture gel, and the commonly used type I collagen gel possess similar fibrillar structures. However, compared to hydrogels composed of solely decellularized human kidney tissue, 1:1 mixture gels possess different and distinct mechanical stability that is more desirable in supporting cellular activities. Furthermore, despite the fact that the 7.5 mg/ml 1:1 mixture gel and the 4 mg/ml type I collagen gel possess similar mechanical properties shown from rheological characterizations, the two gel types gave rise to different results in cellular studies. These results indicate that the mechanical properties and the biologic components of a hydrogel are responsible for different cellular activities. Consequently, altering the mechanical properties and the biologic components of a hydrogel can be used to achieve desirable cellular response. Specifically, a hydrogel with the desirable mechanical properties and the appropriate biologic component, as is the case of our 1:1 mixture gel, should be able to support cell growth, to enhance cellular activities, as well as to promote vascularization within the hydrogel.

The greatest advantage of the d-hk ECM hydrogel and 1:1 mixture gel, namely their similarities to native kidney tissues in terms of ECM composition, has great therapeutic implications. Due to enhanced cellular activies, increased numbers of angiogenic sprouting and tube formations, d-hk ECM hydrogel and 1:1 mixture gel are able to promote robust biologic activity, ultimately achieving vascularization and tissue

regeneration *in vivo*. The fact that both the d-hk ECM hydrogel and the 1:1 mixture gel are syringe injectable indicate the ease of delivery in real-life therapeutic applications.

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