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Novel human-derived extracellular matrix induces *in vitro* and *in vivo* vascularization and inhibits fibrosis

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ABSTRACT

The inability to vascularize engineered organs and revascularize areas of infarction has been a major roadblock to delivering successful regenerative medicine therapies to the clinic. These investigations detail an isolated human extracellular matrix derived from the placenta (*h*PM) that induces vasculogenesis *in vitro* and angiogenesis *in vivo* within bioengineered tissues, with significant immune reductive properties. Compositional analysis showed ECM components (fibrinogen, laminin), angiogenic cytokines (angiogenin, FGF), and immune-related cytokines (annexins, DEFA1) in near physiological ratios. Gene expression profiles of endothelial cells seeded onto the matrix displayed upregulation of angiogenic genes (TGFB1, VEGFA), remodeling genes (MMP9, LAMA5) and vascular development genes (HAND2, LECT1). Angiogenic networks displayed a time dependent stability in comparison to current *in vitro* approaches that degrade rapidly. *In vivo*, matrix-dosed bioscaffolds showed enhanced angiogenesis and significantly reduced fibrosis in comparison to current angiogenic biomaterials. Implementation of this human placenta derived extracellular matrix provides an alternative to Matrigel and, due to its human derivation, its development may have significant clinical applications leading to advances in therapeutic angiogenesis techniques and tissue engineering.

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1. Introduction

The ability to initiate and control angiogenesis in a determinant fashion would have a significant impact in a wide range of clinical applications including tissue engineering, regenerative medicine, *in situ* regeneration after myocardial infarction, and the inhibition of cancer. Most approaches to initiate angiogenesis innately and within biomaterials have either had limited success or are simply not transferable to the clinic due to their non-human or tumor-derivation, limited biomolecular composition in comparison to the native *in vivo* environment, or lack of genetic regulation as in the case of methods to control gene expression [1,2]. Further, once implanted these approaches often cause immunological reactions and fibrotic capsule formation that prevents coupling with the native vasculature. As such, a clinically applicable approach to induce and control angiogenesis remains an extremely high priority [3].

Angiogenesis is a complex process that is both location and stimuli dependent, and in each instance the capacity to modulate these processes may involve a unique combination of regulatory molecules [4,5]. Control of vessel formation is further complicated by different mechanisms of formation, with the two most understood being intussusception and sprouting [6,7]. Intussusception is characterized by the insertion of interstitial cellular columns into the lumen of preexisting vessels [8], and sprouting is characterized by endothelial cells sprouting toward an angiogenic stimulus in tissue previously devoid of microvessels [9]. The innate control of angiogenesis is complex with a significant number of regulatory molecules identified [10], with more likely to be discovered. This diversity has driven researchers to identify modulators that control vascular development for drug screening, regenerative medicine and direct clinical therapies [1].

Common models to study angiogenesis use non-human or human recombinant proteins, animal-derived stimulators, or are entirely dependent on the use of live animals for evaluation [11]. *In vivo* animal models are commonly used to study angiogenesis because they provide an accurate model that compares to the complexity of multiple biomolecular pathways that occur during vessel formation. Current *in vivo* angiogenesis models include the rabbit corneal neovascularization assay, the *in vivo/in vitro* chick chorioallantoic membrane assay, the zebrafish assay, and the rat

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mesentery window assay. However, these models are primarily intended for molecular pathway studies and are not ideal for clinical translation due to interspecies differences [12,13].

When possible, *in vitro* angiogenesis models are chosen as they control complex biological phenomena by limiting the number of molecular species that are needed to represent the complexity that exists *in vivo*. For example, human-derived recombinant growth factors have been used *in vitro* to model angiogenesis, and are typically based on single modulators such as FGF, TGF-β, VEGF [14]. Further approaches have used drug delivery techniques to control the delivery of combinations of modulators [15,16]. These methods, while showing positive outcomes, lack the variety of cytokines and chemical gradients associated with the native *in vivo* environment [17]. As such, in order to replicate the complex molecular cascades that occur *in vivo*, it is hypothesized that a multifactorial approach is necessary in order to promote competent vascularization.

The murine derived basement membrane matrix (BMM) or 'Matrigel' assay has been the preferred *in vitro* angiogenesis model (although *in vitro* may more closely represent vasculogenesis mechanisms) as it brings a degree of *in vivo* complexity to an *in vitro* model and results appear to be more comparable to *in vivo* data. However, due to the derivation of Matrigel from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells the product is not clinically relevant and as such is limited to *in vitro* testing. Further limiting desirability is that production of Matrigel requires the sacrifice of large numbers of animals [18].

Given interspecies differences associated with animal-derived angiogenesis models [12,13], and the complexity of creating multi-protein formulations from human recombinant proteins, a robust human derived approach (consisting of multiple proteins at near physiological ratios) would be desirable. Such an approach would have a significant impact in mechanistic studies, drug screening, and most importantly numerous clinical applications. For example, organ and tissue regeneration is largely restricted due to the inability to vascularize developing constructs. The capacity to modulate angiogenic processes to represent the different mechanisms and stages of formation would provide an improved platform to characterize key molecules and molecular pathways during vascularization.

To date, the majority of successful attempts to induce angiogenesis innately and within bioengineered tissues have relied upon non-human animal compounds, such as Matrigel and other animal derived angiogenesis modulators [19]. Due to their human derivation and capacity to remodel and adapt to changing fetal demands, we hypothesized that full-term placentas would yield a material with a complex extracellular matrix component in addition to pro-angiogenic compounds at near physiological ratios [20]. Here, directed fractionation and separations techniques were used to isolate a matrix containing a complex of active angiogenic biomolecules. Then, using cell-seeded human placenta matrix (*h*PM) and *h*PM-dosed vascular graft bioscaffolds, the matrix was shown to induce *in vitro* vasculogenesis and *in vivo* angiogenesis, to significantly enhance capillary formation, and to inhibit implant fibrosis. In addition, by modulating cell densities within this matrix, specific control of the rate of microvessel maturation as well as selective modeling of microvessel network morphology was achieved.

2. Methods

2.1. Human placental matrix derivation

Full-term placental tissues were collected at the delivery suite at Shands Hospital at the University of Florida (Gainesville, FL) in accordance with UF IRB #642010 within 12 h of birth. The umbilical cords and fetal membranes were removed and the placenta was dissected into 2 cm cubes and frozen. 12 h after progressive freezing to −86 °C at a rate of −1 °C/min, the placental cubes were transported to a cold room maintained at 4 °C where the rest of the procedures were completed. In preparation of each *h*PM batch, placental tissue from 3 separate donors was pooled (100 g total) prior to sample homogenization. Once at 4 °C, 100 g of the tissue was mixed with 150 mL cold 3.4 M NaCl buffer (198.5 g NaCl, 12.5 ml 2 M tris, 1.5 g EDTA, and 0.25 g NEM in 1 L distilled water). The NaCl buffer/tissue mix was homogenized into a paste then centrifuged at 7000 RPM for 15 min and separated from the supernatant. This NaCl washing process was repeated two additional times, discarding the supernatant each time to remove blood.

Next, the pellet was homogenized in 100 mL of 4 M urea buffer (240 g urea, 6 g tris base, and 9 g NaCl in 1 L distilled water), stirred on a magnetic stirplate for 24 h, and then centrifuged at 14000 RPM for 20 min (Sorvall RC6+ Centrifuge, Thermo Scientific, NC, USA). The supernatant was removed and dialyzed using 8000 MW dialysis tubing (Spectrum Laboratories, Inc., CA, USA) placed in 1L of TBS (6 g tris base and 9 g NaCl in 1 L distilled water) and 2.5 ml of chloroform for sterilization. The buffer was replaced with fresh TBS 4 more times, each at 2 h intervals. Finally, contents of the dialysis tubes were centrifuged at 3000 RPM for 15 min (Allegra X-12R Centrifuge, Beckman Coulter, Inc., CA, USA) to remove polymerized proteins, and the supernatant (pink viscous matrix) was collected and stored at −86 °C until use.

2.2. Biomolecular composition analysis

Relative cytokine levels were determined using a sandwich immunoassay array from RayBiotech, Inc. (Human Cytokine Antibody Array C Series 1000, Inc, GA, USA). Chemiluminescence was detected using a Foto/Analyst Luminaryfx Workstation (Fotodyne Incorporated, WI, USA) and the signal intensities were measured using TotalLab 100 software (Nonlinear Dynamics, Ltd, UK). The relative abundance of basement membrane and immune related biomolecules was performed (with 4 pooled samples) by MSBioworks (Ann Arbor, MI) using nano LC/MS/MS with a Waters NanoAcquity HPLC (Waters, Milford, MA) system interfaced to a Orbitrap Velos Pro (ThermoFisher, Waltham, MA). Proteins were identified from primary sequence databases using Mascot database search engine (Boston, MA). Total protein content was determined using a micro BCA protein assay (Pierce, Rockford, IL), and measured at 562 nm using a Synergy II microplate reader (BioTek, Winooski, VT).

2.3. RT-PCR analysis of cells from hPM-induced vasculogenic networks

Relative angiogenic gene expression was determined using 384-well RT^2 Human Angiogenesis RT^2 Profiler PCR Arrays (PAHS-024A, Quiagen, CA, USA). ECs were detached from culture plates using Accutase (Innovative Cell Technologies, San Diego, CA) and immediately stored in 100 μl of RNA*later*. RNA was extracted using the RNeasy Mini Kit (Quiagen, CA, USA), and genomic DNA was digested using an RNase-Free DNase kit (Quiagen, CA, USA). Purified RNA was reverse transcribed to cDNA using the RT^{2}First Strand Kit (SA Biosciences, TX, USA) with incubation at 42 °C for 15 min followed by incubation at 95 °C for 5 min to stop the reaction. Next, cDNA was mixed with RT^2 SYBR Green Mastermix (SA Biosciences, TX, USA) and loaded into 384-well Human Angiogenesis PCR Arrays. Using the Bio Rad CFX384 Real-Time System (Bio-Rad, CA, USA) the loaded array plates went through a denaturation cycle for 10 min at 95 °C, 40 cycles of 30 s annealing/extension cycles at 60 °C, and finally melting curves were obtained by ramping from 60 °C to 95 °C at a rate of °C per second. Data was analyzed the -using $\Delta\Delta C_t$ method and the RT^2 Profiler PCR Array Data Analysis Template v4.0 software package (Quiagen, CA, USA).

2.4. Human umbilical vein endothelial cell isolation and smooth muscle cell culture

Endothelial cells were derived from human umbilical veins (collected from UF Health Shands Hospital, Gainesville, FL) by detachment from the vessels walls as previously described using a 1 mg/ml solution of bovine Type-I Collagenase (Gibco, Invitrogen, NY, USA) in phosphate buffered saline [21]. The primary derived human umbilical vein endothelial cells (HUVEC) were used between passages 1–3 for all experiments. For comparison between the control group and the hPM group, endothelial cells in both sample groups were cultured using complete EC media which was prepared by adding 25 ml of glutamine, 0.5 ml of hydrocortisone, 0.5 ml of ascorbic acid, 10 ml of FBS, and 1.25 μl of bFGF to 500 mL VascuLife Basal media (Lifeline, MD, USA). Human smooth muscle cells (ATCC PCS-100-012) were obtained from the ATCC and used between passages 5 and 10 (Manassas, VA), and cultured under standard conditions at 37 °C and 5% CO_2. SMC were cultured in standard culture media consisting of high-glucose Dulbecco's Modified Eagle Medium (DMEM) (HyClone, Rockford, IL) supplemented with 10% FBS (Gibco, Carlsbad, CA), and cells were detached from culture plates using Accutase (Innovative Cell Technologies, San Diego, CA). To compare the morphologies of SMC culture on Matrigel and hPM, cells were seeded at 40,000 cells/cm^2 and cultured with unsupplemented high-glucose Dulbecco's Modified Eagle Medium (DMEM) (HyClone, Rockford, IL).

2.5. Preparation of placenta matrix-derived angiogenesis assays

Unless otherwise stated, 32 μl of placental matrix was thawed and pipetted into each well of a 96 well plate. The matrix was evenly coated onto the bottom of each well using an orbital shaker at 30 RPM for 1 min. The coated plate was then incubated at 37 °C for 30 min. HUVEC were then plating by direct pipetting at 20000 cells/cm^2, 40000 cells/cm^2, or 80000 cells/cm^2. Multiple time points were investigated at each concentration including at days 1, 3, and 5. Thrombospondin-1 was tested as an angiogenesis inhibiting drug using final concentrations 0, 5, 10, 20, and 35 μg/μL diluted in endothelial cell media.