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RESEARCH ARTICLE

Dehydrated human amniotic membrane regulates tenocyte expression and angiogenesis *in vitro*: Implications for a therapeutic treatment of tendinopathy

Sarah E. Moreno | Michelle Massee | Thomas J. Koob

MiMedx Group, Inc., Marietta, Georgia, USA

Correspondence
Michelle Massee, MiMedx Group, Inc., 1775 West Oak Commons Court NE, Marietta, GA 30062, USA.
Email: mmassee@mimedx.com

Funding information
MiMedx Group, Inc.

Abstract

Tendon injuries are among the most common ailments of the musculoskeletal system. Prolonged inflammation and persistent vasculature are common complications associated with poor healing. Damaged tendon, replaced with scar tissue, never completely regains the native structural or biomechanical properties. This study evaluated the effects of micronized dehydrated human amnion/chorion membrane (μdHACM) on the inflammatory environment and hypervascularity associated with tendinopathy. Stimulation of human tenocytes with interleukin-1 beta (IL1β) induced the expression of inflammatory and catabolic markers, resulting in secretion of active MMPs and type 3 collagen that is associated with a degenerative phenotype. Treatment with μdHACM diminished the effects of IL1β, reducing the expression of inflammatory genes, proteases, and extracellular matrix components, and decreasing the presence of active MMP and type 3 collagen. Additionally, a co-culture model was developed to evaluate the effects of μdHACM on angiogenesis associated with tendinopathy. Micronized dHACM differentially regulated angiogenesis depending upon the cellular environment in which it was placed. This phenomenon can be explained in part through the detection of both angiogenic protagonists and antagonists in μdHACM. Observations from this study identify a mechanism by which μdHACM regulates inflammatory processes and angiogenesis *in vitro*, two key pathways implicated in tendinopathic injuries.

KEYWORDS
amniotic membrane, angiogenesis, inflammation, tendinopathy, tenocytes

1 | INTRODUCTION

Tendon is dense fibrous connective tissue, made up of a collagenous matrix that contains few cells and is noticeably devoid of vasculature. The structure and composition of the tendon is designed to have high tensile strength, however, the constant strain on this tissue also makes it vulnerable to injury, with limited intrinsic capacity for regeneration.[1,2] In general, tendon repair follows the typical wound healing course orchestrated by signaling cues secreted from surrounding cells. The healing process is relatively slow and is often interrupted by repetitive injuries, trauma, aging, and/or degenerative pathology causing prolonged inflammation and persistent vascularization.[1]

While inflammation is a necessary step during reparative processes, excessive inflammation is thought to significantly impair healing.[3]

Modulators of inflammation, including interleukin-β (IL1β), are found to be elevated in injured tendon tissue.[4-6] Increased production of these inflammatory cytokines can alter the phenotype of resident cells resulting in persistent changes to their secretome.[7] Additionally, IL1β can trigger the catabolic degradation of the extra cellular matrix (ECM) through the activation of matrix degrading enzymes such as matrix metalloproteinase (MMP).[8-10]

In line with upregulated inflammatory processes, temporary vessels emerge in the typically avascular tendon to facilitate delivery of nutrients and cell signaling molecules to the injury site.[11] However, prolonged inflammation stimulates the release of inflammatory factors that act directly or indirectly on the tendon vasculature, as confirmed by the presence of vascularization and elevated levels of angiogenic stimuli in injured tendon tissue.[12-14] Although several molecules have been shown to be important in angiogenesis, vascular endothelial growth factor (VEGF) functions as a key regulator of physiologic as well as pathologic angiogenesis and is detectable at higher concentration in diseased tendon tissue.[14-17] A balanced management of the vascular response may be required to overcome the limited regenerative capacity in tendon pathologies.

Resolution of abnormal inflammatory and angiogenic pathways are promising targets for new drug development for the treatment of tendon injuries and augmenting their repair. Micronized dehydrated human amnion/chorion membrane (μdHACM; MiMedx Group, Inc., Marietta, GA) is PURION® processed amniotic membrane allograft available in an injectable format. Previous studies evaluating the membrane configuration have demonstrated this proprietary process retains well-known regulatory proteins and preserves the bioactivity to stimulate cellular activities.[18-23] Clinical studies demonstrate efficacy in diseases with varying etiologies suggesting the complex nature of μdHACM may prove useful in a multitude of applications.[24-29]

In this study, specific pathological processes from a chronic tendon injury were modeled *in vitro*. The inflammatory environment of a tendon injury was mimicked through IL1β stimulation of tenocytes followed by evaluation of the effect of μdHACM on the ensuing expression of inflammatory signals, proteases, collagen, as well the MMP activity. Additionally, a co-culture system was developed to mimic hypervascularity and evaluate the effect of μdHACM on vascular network formation and disruption. It is hypothesized that the regulatory proteins, contained within μdHACM, facilitate regulation of the inflammatory and angiogenic processes.

2 | MATERIALS AND METHODS

2.1 | Micronized dehydrated human amnion/chorion membrane (μdHACM)

μdHACM (MiMedx, Marietta, GA) is a dehydrated human allograft comprised of laminated amnion and chorion membranes, derived from the amniotic sac. Birth tissue was donated under informed consent, following cesarean sections, in compliance with the Food and Drug Administration's (FDA) Good Tissue Practice and American Association of Tissue Banks (AATB) standards. All donors were tested and confirmed to be free of infectious diseases, including human immunodeficiency virus (HIV), human T-lymphotropic virus (HTLV), hepatitis B and C, and syphilis. Amnion and chorion were separated from the placenta and processed in accordance with the proprietary PURION® process, in which the amnion and chorion layers are gently cleansed, laminated, and dehydrated under controlled conditions. The bi-layer tissue is cryomilled and the resulting particles are collected. The final product is subjected to terminal sterilization to ensure a sterility assurance level of less than 10^{-6}.

To prepare extracts of soluble molecules from μdHACM for cell culture experiments, individual donors of μdHACM were extracted overnight at 4°C with gentle agitation at 10 mg of tissue per milliliter of medium, as specified for each individual cell type/assay below. The tissue residue was removed by centrifugation at 3,000 rpm for 15 min at room temperature and the resultant fluid was passed through 0.22 μm filter (Millipore Sigma, St. Louis, MO). The filtrate was collected in a sterile container to serve as the extract treatment. Extracts were then diluted in the appropriate medium to the desired testing concentrations.

2.2 | *In vitro* tenocyte inflammatory model

Cryopreserved human primary tendon cells (tenocytes) were purchased from ZenBio (Research Triangle Park, NC). Tenocytes from three individual donors were used in the experiments. Donor information is provided in Table 1. Tenocytes, at passage 3, were cultured in tenocyte growth medium (ZenBio, Research Triangle Park, NC) on collagen coated flasks (Corning, Corning, NY) at 37°C, 5% CO_2 until 80% confluent. Cells were detached using TrypLE cell dissociation solution (Gibco, Thermo Fisher Scientific, Waltham, MA). Tenocytes were seeded at a density of 2,500 cells per well in a 96-well plate and 75,000 cell per well in a six-well plate and cultured in tenocyte growth media (ZenBio, Research Triangle Park, NC) containing 10% FBS for 72 hr. IL1β, a potent inflammatory cytokine, was used to induce cellular inflammation according to previous studies.[30,31] The cells were then stimulated with either basal medium or basal medium, containing 1 ng/ml of IL1β (Sigma, St. Louis, MO) for 48 hr. Basal medium is defined as Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA) containing 1% penicillin streptomycin (Fisher Scientific, Waltham, MA) and 1% sodium pyruvate (Thermo Fisher Scientific, Waltham, MA). Following stimulation, the medium was removed and fresh culture medium, supplemented with one of the following was added: Group #1 basal medium; Group #2 IL1β; Group #3 IL1β + 5 mg/ml μdHACM; Group #4 IL1β + 2.5 mg/ml μdHACM; Group #5 IL1β + 0.2 mg/ml μdHACM. μdHACM treatments were prepared as previously described in basal media ($n = 3$ μdHACM donors). Each treatment group was tested in triplicate in each of the three tenocyte donors. Treatment groups containing μdHACM tested individual μdHACM donors at the indicated concentrations. All treatment groups were tested with three technical replicates for gene expression. For MMP activity and western blot, each treatment group was tested individually.

TABLE 1 Tenocyte donor demographic data

Donor number	Donor ID	Age (years)	Gender	Ethnicity	Origin
1	TENM021617C	72	Male	Caucasian	Achilles tendon
2	TENM030817E	96	Male	Caucasian	Achilles tendon
3	TENM051619A	63	Female	Caucasian	Achilles tendon

2.3 | Quantitative polymerase chain reaction

RNA and complimentary DNA (cDNA) was prepared utilizing the Cells-2-Ct Kit (Thermo Fisher Scientific, Waltham, MA), per the manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) amplification for each gene target was performed on a QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) using predesigned TaqMan Gene Expression Assays for *IL6* (Hs00174131_m1), *MCP1* (Hs00234140_m1), *MMP1* (Hs00899658_m1), *MMP3* (Hs00968305_m1), *COL1A1* (Hs00164004_m1), *COL3A1* (Hs00943809_m1), and eukaryotic 18 s (4319413E) purchased from Thermo Fisher Scientific (Waltham, MA). The $2^{-\Delta\Delta Ct}$ method was used to determine relative expression of μdHACM-treated tenocytes compared to IL1β-treated tenocytes with eukaryotic 18s as an endogenous control.

2.4 | Western blotting

Proteins were isolated in radio immunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA) complemented with a cocktail of protease inhibitors (Millipore Sigma, Burlington, MA). Cell debris was pelleted by centrifugation at 14,000 rpm at 4°C for 10 min; supernatants were harvested and protein concentrations were determined with Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, MA). Equal protein amounts were resolved by 4–12% gradient sodium dodecylsulfate poly-acrylamide gel electrophoresis and transferred onto nitrocellulose membranes using iBlot2 device (Thermo Fisher Scientific, Waltham, MA). Membranes were blocked for 1 hr in 5% non-fat dry milk 1× Tris buffered saline 0.05% Tween 20 and probed with antibodies against type 1 collagen (Abcam, Cambridge, MA) and type 3 collagen (Novus Biologicals, Centennial, CO) or β-Actin (R&D Systems, Minneapolis, MN) overnight at 4°C. Membranes were washed in 1× Tris buffered saline 0.05% Tween 20 and incubated with anti-mouse or anti-rabbit IgG HRP-conjugated secondary antibodies (Abcam, Cambridge, MA). Immunoreactive proteins were detected using chemi-luminescence (Thermo Fisher Scientific, Waltham, MA) and imaged on the GE Healthcare Imager.

2.5 | MMP activity assay

The SensoLyte 520 Generic MMP Assay (AnaSpec, Fremont, CA) was used to measure MMP activity in conditioned media (CM) according to manufacturer's instructions. In this assay, a quenched fluorescence resonance energy transfer (FRET) peptide is used as an MMP substrate. Upon cleavage of the intact peptide by an MMP, fluorescence is generated and can be measured by a plate reader. Relative fluorescent units (RFUs) are directly correlated to MMP activity. The peptide used in this assay is a generic substrate and can be cleaved by MMPs −1, −2, −3, −7, −8, −9, −10, −12, −13, and −14. CM was centrifuged at 500*g* for 5 min. Positive controls of Human MMP1 and Human MMP3 (AnaSpec, Fremont, CA) were activated with 1 mM 4-aminophenylmercuric acetate (APMA) at 37°C for 3 hr. APMA was omitted from CM test samples to allow for measurement of endogenous MMP activity. Following addition of CM test samples, standards, and positive control, the quenched peptide substrate was incubated with the samples in a 96-well format. RFUs were quantitated at excitation/emission wavelengths (490/520 nm) with the Synergy™ Mx Microplate Reader (BioTek). The fluorescence reference standard was used to convert the RFU at each time point to the concentration of the enzymatic reaction product. Enzymatic rate of activity per minute was calculated using the following formula: (concentration at 60 min—concentration at 0 min)/60 min, where activity is defined as the amount of product resulting from proteolytic cleavage of the substrate.

2.6 | Network formation *in vitro*

Preliminary evaluation of network formation was performed using a co-culture system of normal human dermal fibroblasts (NHDF) and human umbilical vein endothelial cells (HUVEC) provided in the Angiogenesis PrimeKit (Essen Biosciences, Ann Arbor, MI), according to the manufacturer's instructions. The effect of network formation specific to tendinopathy was further assessed by establishing a novel co-culture model using normal human tenocytes (ZenBio Research Triangle Park, NC) and HUVEC provided in the Angiogenesis PrimeKit (Essen Biosciences, Ann Arbor, MI). Briefly, the HUVECs, which are tagged with green fluorescent protein to allow fluorescent visualization of the tubule formation, were plated on top of a tenocyte monolayer and allowed to form networks under different experimental models. Media and supplements were used from the Angiogenesis PrimeKit. In both culture systems, three models for assessing the effect of μdHACM of angiogenesis were evaluated (Figure 1): I. Angiogenesis or the ability to stimulate vascular networks, II. Anti-angiogenesis or the ability to prevent the formation of vascular networks, III. Vascular disruption or the ability to interrupt established vascular networks. μdHACM extract was prepared, as previously described, in assay medium provided in the Angiogenesis PrimeKit. Three different tenocytes donors were tested is each model.

Model I: Treatments were added on Day 2 until Day 8 in culture. Treatment groups included: assay medium + 4 ng/ml VEGF (positive

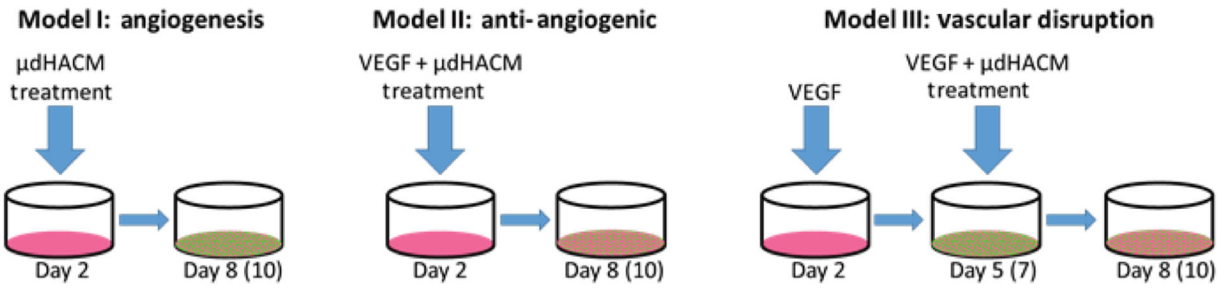

FIGURE 1 Angiogenesis models. A co-culture, of NHDF or tenocytes and HUVEC, was used to test the effect of µdHACM on network formation. Three models were used for assessing the effect of µdHACM on angiogenesis; (Model I) to determine the angiogenic response; (Model II) the anti-angiogenic potential of µdHACM; (Model III) to model vascular disruption. HUVEC, human umbilical vein endothelial cells; NHDF, normal human dermal fibroblasts; µdHACM, micronized dehydrated human amnion/chorion membrane

control), assay medium + 4 ng/ml VEGF +100 µM suramin (negative control), assay medium alone (vehicle control), or µdHACM extracts at 5, 2.5, and 0.2 mg/ml concentrations in assay medium ($n = 3$ µdHACM donors). Each treatment was performed in triplicate. *Model II*: The cells were cultured with treatment in the presence of 4 ng/ml of VEGF, including: assay medium + 4 ng/ml VEGF (negative control), assay medium + 4 ng/ml VEGF + 100 µM suramin (positive control), assay medium alone (vehicle control), or µdHACM extracts at 5, 2.5, and 0.2 mg/ml concentrations in assay medium containing 4 ng/ml VEGF ($n = 3$ µdHACM donors) from Day 2 until Day 8. Each treatment was performed in triplicate. *Model III*: Tubular networks in the co-culture system were established by addition of 4 ng/ml VEGF from Day 2 until Day 5 of culture. On Day 5, the cells were treated with one of the following: assay medium + 4 ng/ml VEGF (negative control), assay medium + 4 ng/ml VEGF + 100 µM suramin (positive control), assay medium alone (vehicle control), or µdHACM extracts at 5, 2.5, and 0.2 mg/ml concentrations in assay medium ($n = 3$ µdHACM donors). Each treatment was performed in triplicate.

The culture plates were placed into the IncuCyte system (S3; Essen Biosciences, Ann Arbor, MI) and imaged every 12 hr for 8 days. Angiogenesis was assessed by quantifying network branch points, network length, and average network length. These measurements were calculated using the Angiogenesis module provided in the InCucyte system (Essen, version 2019B REV2), which performs quantitative scoring of network formation.

2.7 | Evaluation of angiogenic antagonists in µdHACM extract

The presence of angiogenic antagonists was evaluated in µdHACM extract ($n = 6$ µdHACM donors). Extracts were prepared, at 5, 2.5, and 0.2 mg/ml, as previously described in assay medium provided in the Angiogenesis PrimeKit. High Performance Luminex Assays (R&D Systems, Minneapolis, MN) were used for quantification of VEGFR1/sFlt-1, endostatin, VEGFR2/s-Flk-1, and thrombospondin-2 in µdHACM extract. Assay was performed according to the manufacturer's instructions and each sample was tested in duplicate.

The ability of antagonists to bind or antagonize soluble VEGF was evaluated by incubating recombinant VEGF (4 ng/ml) with the treatment groups for 5 min at 37°C, followed by immediate quantification of the unbound VEGF by ELISA (DVE00; R&D Systems, Minneapolis, MN). The treatment groups were prepared as follows: assay medium only (negative control), assay medium + 4 ng/ml VEGF (positive control), µdHACM extracts at 5, 2.5, 0.2 mg/ml, or µdHACM extracts at 5, 2.5, and 0.2 mg/ml + 4 ng/ml VEGF ($n = 6$ µdHACM donors). ELISA assay was performed according to the manufacturer's instructions and each sample was tested in duplicate.

2.8 | Statistical analysis

All values are reported as mean +/− *SD* and statistical analyses were performed using the GraphPad Prism software. For gene expression and MMP activity, values were compared within each tenocyte donor using a one-way ANOVA. For angiogenesis models, Day 8 (tenocyte) or Day 10 (NHDF) values were compared using a one-way ANOVA. For each ANOVA, pairwise comparisons were made using a Tukey test. Significant difference was assigned when $p < .05$.

3 | RESULTS

3.1 | Treatment with µdHACM reduces IL1β induced changes *in vitro*

Tenocytes from three different donors were individually stimulated with 1 ng/ml of IL1β to induce a state of cellular inflammation. The extent of the response to IL1β stimuli differed between donors, demonstrating expected variability; nevertheless, the general trends were identical across all donors. Comparison of control groups, from individual tenocyte donors, confirmed the intended inflammatory response to IL1β treatment as follows: in the positive control, IL1β treatment of tenocytes resulted in elevated gene expression of inflammatory mediators *IL6*, *MCP1*, *MMP1*, and *MMP3* and the ECM component type 3 collagen compared to unstimulated control (basal) (Figure S1). No change was observed in type 1 collagen upon IL1β

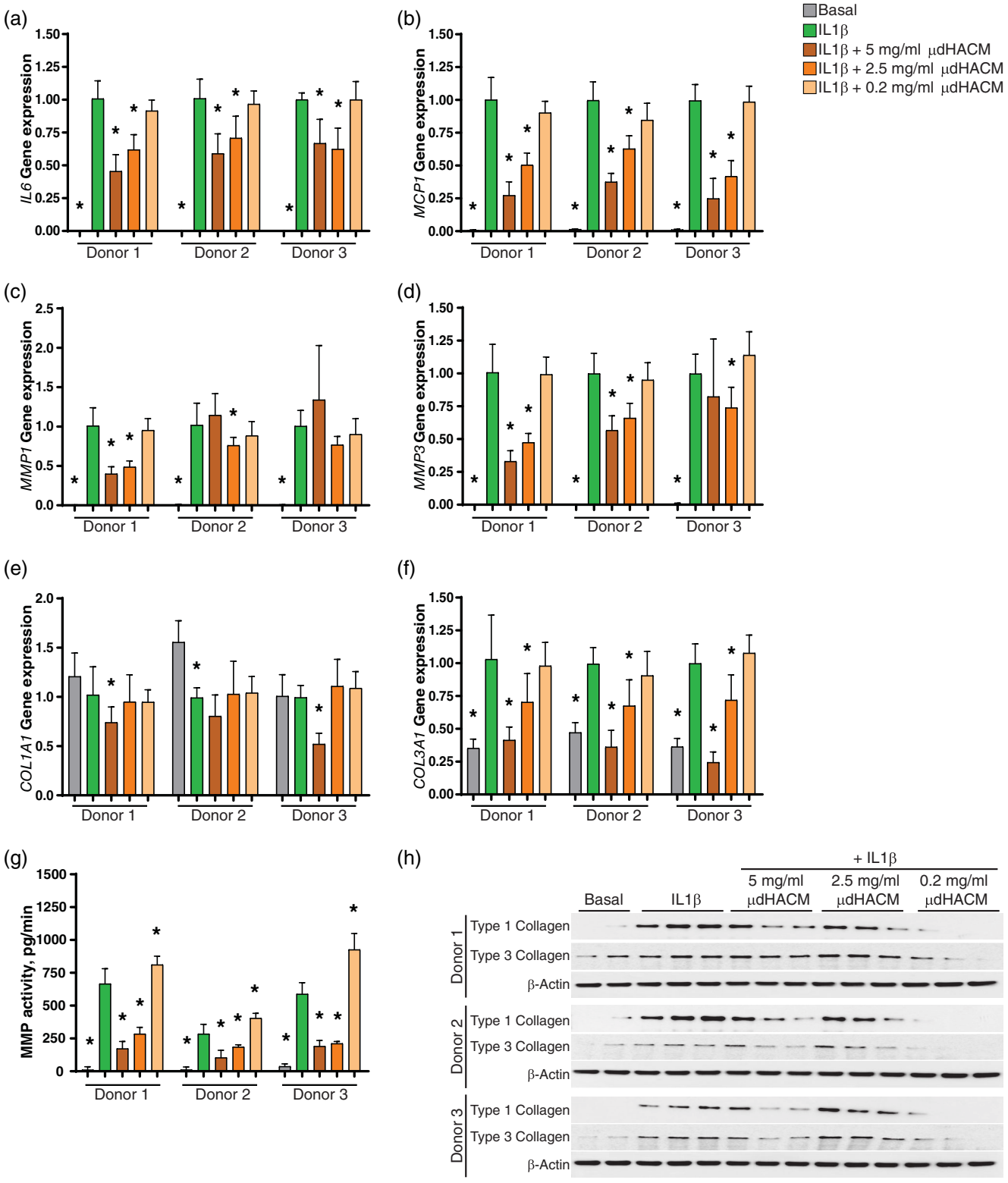

FIGURE 2 Effects of micronized dehydrated human amnion/chorion membrane (μdHACM) treatment on modulators of inflammation and ECM proteins. Fold change in gene expression in tenocytes following 48 hr stimulation with IL1β and 48 hr treatment with IL1β + μdHACM. (a) *IL16*; (b) *MCP1*; (c) *MMP1*; (d) *MMP3*; (e) *COL1A1*; (f) *COL3A1*. (g) MMP activity in the conditioned media from tenocytes stimulated with IL1β followed by treatment with IL1β + μdHACM. (h) Expression of type 1 collagen and type 3 collagen in tenocytes assessed by western blot analysis. Errors bars represent the *SD*. * $p < .05$ versus IL1β using one-way ANOVA, $n = 3$ μdHACM donors

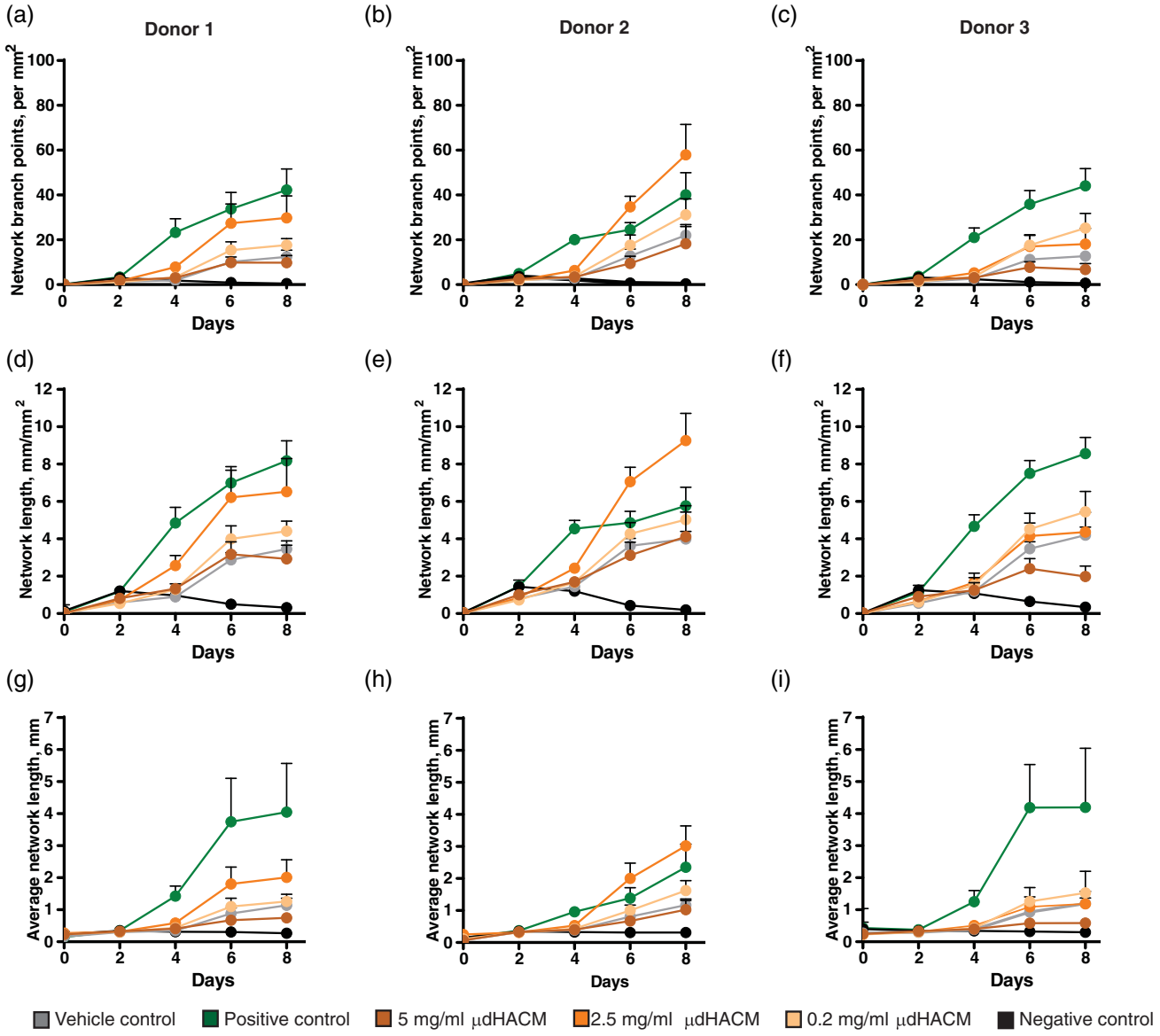

FIGURE 3 Pro-angiogenic effects of μdHACM. On Day 2, treatments, including assay medium + 4 ng/ml VEGF (positive control), assay medium + 4 ng/ml VEGF + 100 μM suramin (negative control), assay medium alone (vehicle control), or μdHACM extracts at 5, 2.5, and 0.2 mg/ml concentrations in assay medium were added to the co-cultures using each tenocyte donor. Time course image analysis was performed measuring (a–c) network branch points (per mm^2), (d–f) network length (mm/mm^2), and (g–i) average network length (mm) in response to μdHACM treatment. Error bars represent the *SD* from the mean values. $n = 3$ μdHACM donors. VEGF, vascular endothelial growth factor; μdHACM, micronized dehydrated human amnion/chorion membrane

stimulation in Donor 1 and Donor 3. IL1β stimulation of cell Donor 2 decreased the expression of type 1 collagen (Figure S1).

The μdHACM treatment at 5 mg/ml and 2.5 mg/ml significantly down regulated the expression of inflammatory markers *IL6* and *MCP1* and ECM component *COL3A1* (Figure 2a, b, and f). The effects of μdHACM treatment on the expression of *IL6*, *MCP1*, and *COL3A1* were consistent between tenocyte donors. Changes in gene expression of *MMP1*, *MMP3*, and *COL1A1* were dependent on μdHACM dose and tenocyte donor (Figure 2c–e). Reduced MMP3 expression was observed at both 5 mg/ml and 2.5 mg/ml in tenocyte Donors 1 and Donor 2. However, only 2.5 mg/ml μdHACM decreased the expression of MMP3 in Donor 3 (Figure 2d). Tenocyte Donors 1 and 3 demonstrated reduced expression of type 1 collagen at 5 mg/ml, while *COL1A1* expression in tenocyte Donor 2 was not impacted by μdHACM treatment (Figure 2e). The effects on *MMP1* were limited to treatment with 2.5 mg/ml of μdHACM, while 5 mg/ml only showed changes in gene expression in Donor 1 (Figure 2c). IL1β induced gene expression was not impacted by treatment with 0.2 mg/ml of μdHACM.

Additionally, the presence of active MMPs in the CM was analyzed using a quenched FRET peptide that can be cleaved by multiple MMPs to produce a fluorescence signal. IL1β increases

TABLE 2 Effect of μdHACM on vascular network formation

Cell donor	Model I: Angiogenesis			Model II: Anti-angiogenic			Model III: Vascular disruption		
	Network branch points[a]	Network length[b]	Average network length[c]	Network branch points[a]	Network length[b]	Average network length[c]	Network branch points[a]	Network length[b]	Average network length[c]
1 (Vehicle control)	12.39 ± 2.97	3.45 ± 0.44	1.14 ± 0.21	12.39 ± 2.97#	3.45 ± 0.44#	1.14 ± 0.22#	16.35 ± 2.84#	3.86 ± 0.51#	1.62 ± 0.20#
1 (Positive control)	42.22 ± 9.35*	8.18 ± 1.07*	4.05 ± 1.52*	0.50 ± 0.32#	0.32 ± 0.11#	0.27 ± 0.03#	3.19 ± 0.88#	1.10 ± 0.23#	0.49 ± 0.04#
1 (Negative control)	0.50 ± 0.32*	0.32 ± 0.11*	0.27 ± 0.03*	42.22 ± 9.35	8.18 ± 1.07	4.05 ± 1.52	39.20 ± 7.05	6.86 ± 0.81	2.57 ± 1.12
1 (μdHACM, 5 mg/ml)	9.76 ± 3.20	2.92 ± 0.73	0.75 ± 0.15	16.77 ± 4.11#	4.13 ± 0.92#	1.03 ± 0.23#	24.47 ± 3.71#	4.46 ± 0.55#	1.52 ± 0.39
1 (μdHACM, 2.5 mg/ml)	29.76 ± 9.79*	6.52 ± 1.78*	2.01 ± 0.55*	40.13 ± 5.98	7.95 ± 0.73	3.06 ± 1.15	32.60 ± 6.33	5.93 ± 0.84	1.95 ± 0.66
1 (μdHACM, 0.2 mg/ml)	17.64 ± 2.86	4.41 ± 0.54	1.26 ± 0.22	42.46 ± 6.94	8.08 ± 0.77	2.84 ± 0.81#	38.71 ± 7.21	7.09 ± 0.86	2.75 ± 0.86
2 (Vehicle control)	22.03 ± 4.73	3.99 ± 0.41	1.17 ± 0.19	22.03 ± 4.73#	3.99 ± 0.41#	1.17 ± 0.19#	19.04 ± 2.54#	3.65 ± 0.31#	1.04 ± 0.11#
2 (Positive control)	40.05 ± 9.87*	5.76 ± 1.00*	2.35 ± 0.72*	0.38 ± 0.32#	0.20 ± 0.10#	0.31 ± 0.06#	3.05 ± 1.15#	1.00 ± 0.15#	0.43 ± 0.10#
2 (Negative control)	0.38 ± 0.32*	0.20 ± 0.10*	0.31 ± 0.06*	40.05 ± 9.87	5.76 ± 1.00	2.35 ± 0.72	47.74 ± 8.45	6.93 ± 0.99	2.70 ± 0.58
2 (μdHACM, 5 mg/ml)	18.22 ± 7.38	4.12 ± 1.32	1.02 ± 0.29	31.21 ± 6.42	5.60 ± 0.82	1.57 ± 0.41	30.19 ± 5.40#	4.87 ± 0.68#	1.58 ± 0.29#
2 (μdHACM, 2.5 mg/ml)	57.87 ± 13.58*	9.26 ± 1.45*	3.02 ± 0.63*	66.82 ± 9.41#	9.88 ± 0.84#	4.04 ± 1.15#	40.59 ± 7.84	6.05 ± 0.72	2.30 ± 0.55
2 (μdHACM, 0.2 mg/ml)	31.13 ± 7.11	5.45 ± 1.09	1.62 ± 0.31	44.94 ± 7.42	6.72 ± 0.83	2.34 ± 0.61	38.29 ± 8.03	5.92 ± 0.72	1.95 ± 0.44#
3 (Vehicle control)	12.73 ± 2.15	4.20 ± 0.43	1.19 ± 0.17	12.73 ± 1.60#	4.20 ± 0.43#	1.19 ± 0.17#	16.99 ± 2.57#	4.59 ± 0.40#	1.46 ± 0.24#
3 (Positive control)	44.05 ± 7.69*	8.56 ± 0.86*	4.20 ± 1.85*	0.63 ± 0.37#	0.34 ± 0.17#	0.30 ± 0.04#	2.69 ± 0.53#	1.03 ± 0.08#	0.43 ± 0.04#
3 (Negative control)	0.63 ± 0.37*	0.34 ± 0.17*	0.30 ± 0.04	44.05 ± 7.36	8.56 ± 0.86	4.20 ± 1.85	49.44 ± 2.62	8.47 ± 0.53	3.30 ± 0.71
3 (μdHACM, 5 mg/ml)	6.74 ± 2.65	1.98 ± 0.56*	0.59 ± 0.10	12.78 ± 3.48#	3.35 ± 0.68#	0.81 ± 0.15#	16.97 ± 4.13#	3.30 ± 0.60#	1.05 ± 0.14#
3 (μdHACM, 2.5 mg/ml)	18.06 ± 6.99	4.37 ± 1.05	1.18 ± 0.39	36.62 ± 9.01#	7.19 ± 1.29#	2.40 ± 0.68#	31.68 ± 4.43#	5.56 ± 0.59#	1.87 ± 0.39#
3 (μdHACM, 0.2 mg/ml)	25.22 ± 6.53*	5.45 ± 1.09*	1.53 ± 0.68	49.05 ± 6.15	9.48 ± 0.84	3.46 ± 0.94	43.79 ± 5.18	7.93 ± 0.51	2.25 ± 0.54#

Note: * $p < .05$ versus the vehicle control; # $p < .05$ versus the negative control; all data represent the average ± the *SD*.

[a]Indicates per mm^2.

[b]Indicates mm/mm^2.

[c]Indicates mm.

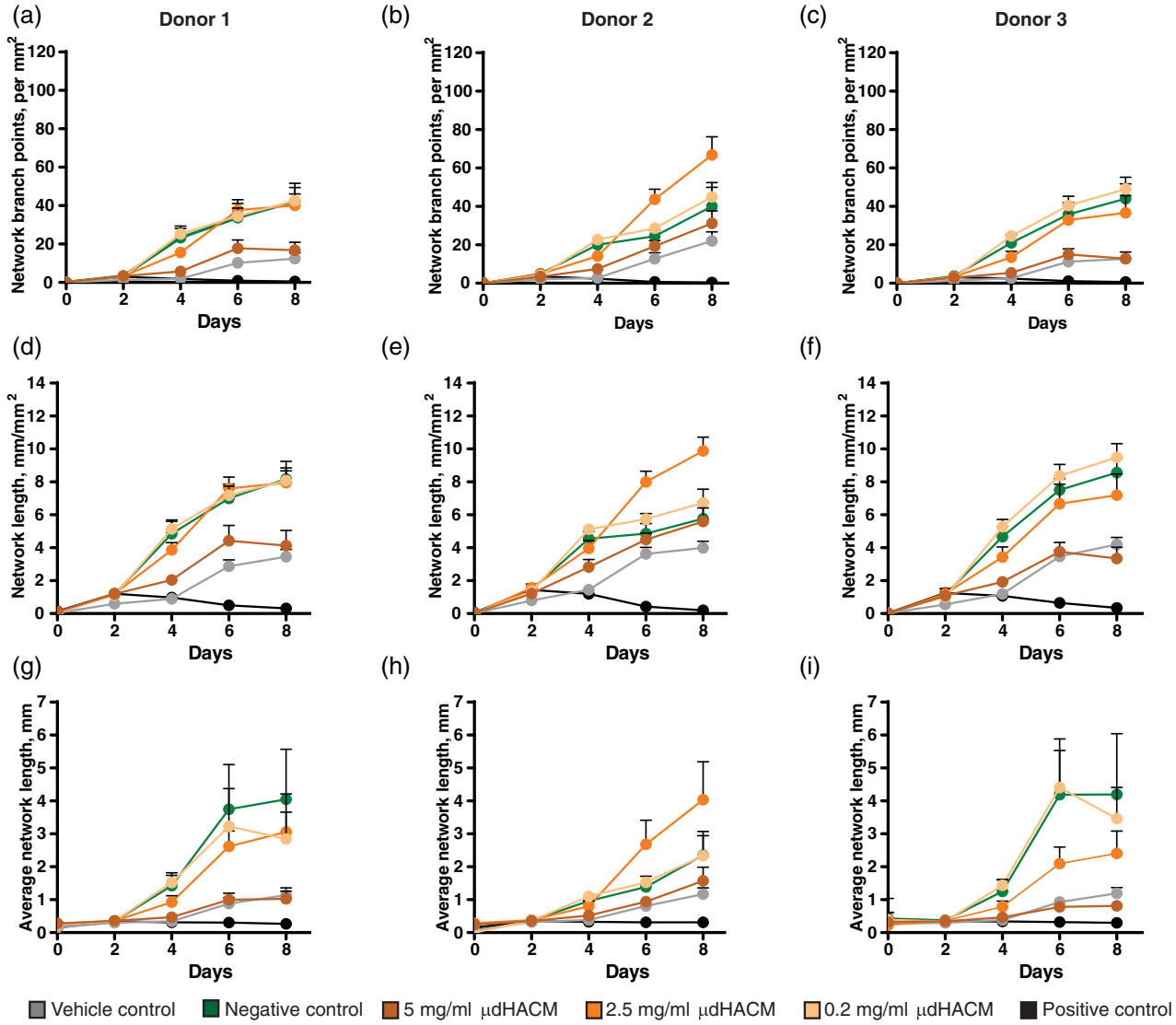

FIGURE 4 Anti-angiogenic response of μdHACM in the presence of VEGF. On Day 2 of co-culture, μdHACM treatments were added in the presence of 4 ng/ml VEGF. Time course image analysis was performed in co-culture with the three different tenocyte donors measuring (a–c) network branch points (per mm^2), (d–f) network length (mm/mm^2), and (g–i) average network length (mm) in response to μdHACM treatment. Error bars represent the *SD* from the mean values. $n = 3$ μdHACM donors. VEGF, vascular endothelial growth factor; μdHACM, micronized dehydrated human amnion/chorion membrane

the production the active MMPs in the CM in the three tenocyte donors. μdHACM treatment at 5 mg/ml and 2.5 mg/ml reduced the level of active MMPs secreted by tenocytes. Treatment with 0.2 mg/ml increased the amount of active MMPs in the CM (Figure 2g). Although the amount of active MMPs induced by IL1β varies between tenocyte donors, the reduction in active MMPs elicited by μdHACM treatment was consistent.

Consistent with gene expression, type 3 collagen protein level was increased by IL1β. Treatment with μdHACM decreases type 3 collagen in tenocytes stimulated with IL1β (Figure 2h). However, type 1 collagen was elevated by IL1β stimulation and μdHACM reduced the protein secretion of type 1 collagen induced by IL1β (Figure 2h).

3.2 | Angiogenic effects of μdHACM

The effects of μdHACM on network formation were established using a co-culture model of HUVECS and NHDF. Model I demonstrated that μdHACM enhances network formation at 2.5 mg/ml; whereas in Model II, network formation was inhibited with μdHACM treatment at 5 mg/ml (Figures S2 and S3). Additionally, disruption of an established vascular network was demonstrated at all concentrations of μdHACM tested (Figure S4). The established angiogenic models, with NHDF, demonstrated the applicability of these test systems to monitor the effect of μdHACM. Next, the use of tenocytes in the co-culture system with HUVECS was examined to elucidate the impact tenocytes

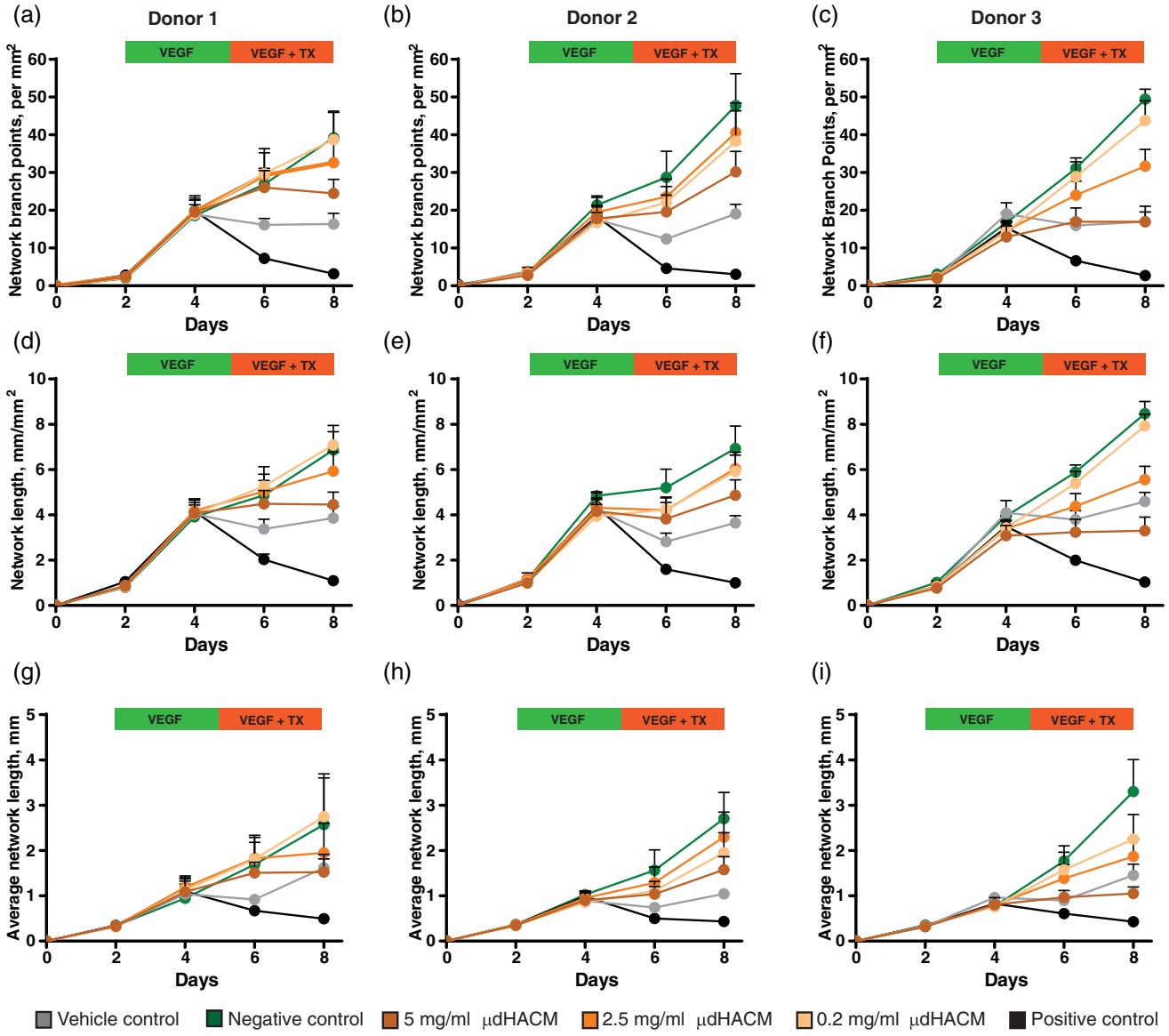

FIGURE 5 Vascular disruption potential of µdHACM. HUVECs formed networks in the presence of VEGF over 3 days followed by treatment with µdHACM + VEGF for 3 days. Time course image analysis was performed measuring (a–c) network branch points (per mm^2), (d–f) network length (mm/mm^2), and (g–i) average network length (mm) in response to µdHACM treatment. Error bars represent the *SD* from the mean values. $n = 3$ µdHACM donors. HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; µdHACM, micronized dehydrated human amnion/chorion membrane

TABLE 3 Level of angiogenesis antagonists in µdHACM extract

µdHACM concentration	Endostatin, pg/ml	VEGFR1/Flt-1, pg/ml	VEGFR2/Flk-1, pg/ml	Thrombospondin-2, pg/ml
5 mg/ml	6,225 ± 2,227	1,760 ± 341	770 ± 692	690 ± 201
2.5 mg/ml	3,002 ± 1,420	969 ± 188	699 ± 421	496 ± 48
0.2 mg/ml	387 ± 18	LLOQ	LLOQ	LLOQ

Note: LLOQ, lower limit of quantitation; VEGF, vascular endothelial growth factor; µdHACM, micronized dehydrated human amnion/chorion membrane.

have in facilitating network formation and determine any alternations in treatment effects.

Network formation was enhanced by the addition of µdHACM to a co-culture model of HUVECS and tenocytes in the absence of VEGF. The positive control of VEGF resulted in an increase in all measured parameters in the three tenocyte donors (Figure 3a–i; Table 2). µdHACM was tested at 5, 2.5, and 0.2 mg/ml, with the 2.5 mg/ml concentration showing statistically significant increases in network

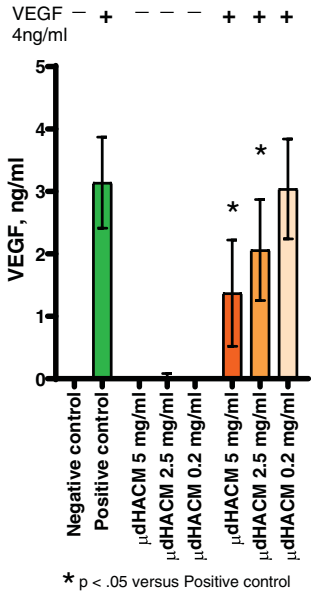

FIGURE 6 VEGF bioavailability. Soluble factors in μdHACM extract bind VEGF. Error bars represent the *SD* from the mean values. * $p < .05$ versus positive control, $n = 6$ μdHACM donors. VEGF, vascular endothelial growth factor; μdHACM, micronized dehydrated human amnion/chorion membrane

branch points, network length, and average network length in Donor 1 (Figure 3a, d, g; Table 2) and Donor 2 (Figure 3b, e, h; Table 2). μdHACM treatment at 0.2 mg/ml significantly increased the network branch points and network length in tenocyte Donor 3 (Figure 3c, f, i; Table 2).

When μdHACM and VEGF were added simultaneously to the co-culture model, the effect of treatment differed from that observed when μdHACM was added alone (Figure 4). Addition of 5 mg/ml μdHACM plus 4 ng/ml of VEGF inhibited network formation, in tenocyte Donors 1 and 3, resulting in a decrease in angiogenesis parameters, similar to the vehicle control supplemented with VEGF (Figure 4; Table 2). No observable inhibition was seen with tenocyte Donor 2; however, 2.5 mg/ml treatment increased the angiogenic parameters.

The effect of μdHACM on the disruption of an established vascular network was examined in the 8 day tenocyte and HUVEC co-culture model (Figure 5). Over the first 5 days with VEGF treatment, all groups increased the angiogenic parameters. Variable results were observed with the addition of treatments on Day 5 through Day 8. The vehicle control group, assay media with no VEGF, resulted in a decrease in vascular network parameters. This result necessitated culturing treatment groups in the presence of VEGF to measure vascular disruption, as the network will gradually degrade without sustained VEGF administration. The negative control group, VEGF, maintained the established vascular network, while the positive control group, suramin + VEGF, resulted in a significant decrease in the number of angiogenic parameters within the first day (Figure 5; Table 2). These results were observed with the three tenocyte donors. The addition of μdHACM treatment in the presence of VEGF similarly decreased the angiogenic parameters. At Day 8, a significant reduction in the number of network branch points, network length and the average network length was observed in cells from all tenocyte donors treated with 5 mg/ml μdHACM compared to the negative control, while 2.5 mg/ml decreased all network parameters in tenocyte Donor 3 (Figure 5; Table 2).

3.3 | Anti-angiogenic effects of μdHACM

The presence of endogenous angiogenesis inhibitors in μdHACM extract was evaluated. Results show the presence of the angiogenic inhibitors endostatin, VEGFR1/Flt-1, VEGFR2/KDR/Flk-1, and thrombospondin-2, are present in μdHACM extract (Table 3). Endostatin is the most abundant angiogenesis antagonist with an average of 6,225 ± 2,227 pg/ml, followed by VEGFR1/s-Flt-1 (1,760 ± 341 pg/ml), VEGFR2/KDR/Flk-1 (770 ± 692 pg/ml), and thrombospondin-2 (690 ± 201 pg/ml; Table 3).

To investigate the ability of an antagonist to inhibit VEGF activity, μdHACM extract was incubated with and without VEGF for 5 min and the unbound VEGF was quantified. The level of available or unbound VEGF (4 ng/ml) was reduced by more than 50% when incubated with 5 mg/ml μdHACM extract and μdHACM extract at 2.5 mg/ml decreased the amount of VEGF by 30% (Figure 6), confirming a direct inhibition of VEGF as a contributing mechanism for vascular disruption.

4 | DISCUSSION

While persistent tendon injury may arise from various etiologies (i.e., overuse, diabetes, etc.), there are commonalities on a cellular level that can be extrapolated for the purposes of studying potential therapeutic pathways. The primary goal of this work was to better understand the potential role of μdHACM in regulating the various pathways known to contribute to tendon pathology. In this study, two *in vitro* models were developed with the intent of exploring tenocyte inflammation and hypervascularization, hallmarks of essentially all tendonopathies.

Aberrant inflammation in a tendon is attributed to an influx of invading inflammatory cells, secreting factors such as IL1β. Downstream of these inflammatory cues, tenocytes begin to degrade surrounding ECM and replace type I collagen for type III, weakening the overall structure of the tendon repair.[1] When μdHACM was added in an inflammatory tenocyte model, a significant decrease in inflammatory factors and proteases was observed. Gene expression of *IL6*, *MCP1*, *MMP1*, and MMP3 were effectively diminished. These inflammatory mediators, IL6 and MCP1, are implicated directly in tendinopathy-related inflammation and angiogenesis; therefore, downregulation of these factors is desirable for offsetting the effects of injury.[4-6] MMP regulation is essential for establishing a balance between ECM synthesis and degradation and controlling degenerative changes associated with injury.[32] *In vitro*, IL1β-treated tenocytes induced expression of *MMP1* and *MMP3*, as well as increased the amount of active MMPs secreted into the CM; however, μdHACM treatment counteracted this effect. Additionally, μdHACM treatment reversed the increased proinflammatory-induced expression of type 3 collagen, but did not

impact type 1 collagen expression. At the protein level, μdHACM decreased both type 1 and type 3 collagen levels induced by IL1β, suggesting a potential regulation of type I collagen despite no observed impact on gene expression. The elevated expression of type 3 collagen is indicative of immature matrix formation and associated with weakened mechanical properties; therefore, μdHACM may facilitate a shift to a lower collagen type III composition in relation to collagen type I in the ECM.[1] μdHACM may not only influence the catabolic processes through MMP regulation, but also directly affect the extracellular matrix composition of a damaged tendon.

Normal tendon tissue is sparsely vascularized; whereas in tendon injury, a temporary increase in vascularization is essential for the healing process.[11] The maintenance of tendon avascularity involves a balance in the production of anti-angiogenic factors and/or inhibitors of angiogenesis. Persistent vasculature is a hallmark of the chronic disease state and associated with disease progression.[14] The angiogenesis model used in this study allowed for testing the effects of μdHACM on a vascular network representative of both a normal and a diseased tendon. μdHACM not only promotes vessel formation, but can also effectively disrupt an established vascular network depending upon the environment in which it is placed. This novel and dynamic property was discovered through the addition of μdHACM treatment in the absence and presence of exogenous VEGF. Micronized dHACM treatment without additional VEGF, stimulated a low-level of angiogenesis. Whereas, when VEGF is abundant, as in an injured tendon, μdHACM prevented or disrupted an established network. This in vitro mechanism suggests that μdHACM may function to maintain appropriate levels of angiogenesis when in an avascular tendon environment; but, decrease angiogenesis when in a hypervascular environment; ultimately, driving homeostasis.

This concept has been explored previously in studies identifying both pro and anti-angiogenic factors in amniotic membranes; however, the mechanism by which μdHACM achieves this dichotomy has yet to be explored.[22,33-36] μdHACM contains quantifiable levels of several key angiogenic cytokines and Koob et al. further demonstrated that μdHACM supports the formation of blood vessels in an *in vivo* injury model.[22] However, in this study, a panel of factors associated with inhibition of angiogenesis/lymphangiogenesis were measured in μdHACM extract and confirmed the presence of endostatin, VEGFR1, VEGFR2, and thrombospondin.[37-40] To verify the proposed function of these factors in contributing to the observed in vitro vascular disruption, exogenous VEGF was incubated with μdHACM extract and the resultant bioavailability of VEGF was measured by ELISA. VEGF levels were significantly decreased with increasing concentrations of μdHACM. This result provides one possible mechanism by which μdHACM disrupts an existing vascular network.

These data demonstrate the role of μdHACM in regulating the inflammatory and angiogenic responses in models relevant to tendinopathy. *In vitro* neutralization of proinflammatory cytokines and proteases may facilitate the restoration of ECM components, giving rise to tissue with improved structural integrity. Additionally, reduction in vascularity may reduce the influx of inflammatory cells, further mitigating inflammation. The results of these studies are promising for the use of μdHACM in the treatment of tendinopathy; however, the limitations of *in vitro* studies necessitate further investigation. Preclinical and clinical *in vivo* studies will be necessary to better understand these pathways and validate these effects in a clinical setting. This marks the first study highlighting the dynamic nature of μdHACM and its ability to elicit multiple biological changes required to effectively achieve tissue homeostasis.

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CONFLICT OF INTEREST

All authors are employees of MiMedx Inc. All authors hold equity in MiMedx Inc.

AUTHOR CONTRIBUTIONS

Sarah E. Moreno, Michelle Massee: Drafting of manuscript. **Sarah E. Moreno**: Data collection. **Sarah E. Moreno, Michelle Massee, Thomas J. Koob**: Revision and approval of manuscript.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

ORCID

Sarah E. Moreno https://orcid.org/0000-0002-6726-7223
Michelle Massee https://orcid.org/0000-0001-9025-0879
Thomas J. Koob https://orcid.org/0000-0001-6162-4202

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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