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Copolymer-mediated Cell Aggregation Promotes a Pro-angiogenic Stem Cell Phenotype *In Vitro* and *In Vivo*

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Abstract

Material-induced cell aggregation drives a pro-angiogenic expression profile Copolymer substrates containing cell-repellent and cell-adhesive domains force the aggregation of human mesenchymal stem cells, which results in enhanced tubulogenesis *in vitro* and stabilization of vasculature *in vivo*. These findings can be used to design instructive biomaterial scaffolds for clinical use.

Keywords

mesenchymal stem cells; synthetic copolymers; cell-material interface; angiogenesis; inflammation; outside-in signaling

Human bone marrow-derived mesenchymal stem cells (hMSCs) have become immensely popular for tissue engineering and regenerative therapies over the past two decades. hMSCs have been used not only to generate new bone^[1] or cartilage^[2] to replace degenerate tissues, they have also been injected into damaged heart^[3] or peripheral arteries^[4], which are highly vascularized. Choosing hMSCs to regenerate tissues that require significant vasculature is based on the fact that hMSCs are pro-angiogenic, likely due to their perivascular localization within the physiological niche^[5], and can both stimulate and stabilize the formation of vasculature over long periods of time^[6]. Furthermore, hMSCs have moved rapidly into clinical trials for modulation of the immune system in graft-versus-host disease^[7] due to their immunomodulatory capabilities. To reach relevant numbers for clinical therapy, hMSCs must undergo serial *in vitro* expansion on tissue culture polystyrene (TCPS) plates, which alters their transcriptional profile similar to natural aging^[8]. Application of biomaterial systems that impart therapeutically-beneficial properties to hMSCs during this expansion phase would prime cells prior to *in vivo* implantation. Therefore, identifying a material type

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that regulates the pro-angiogenic and anti-inflammatory capabilities of hMSCs would enhance successful outcomes of hMSC-derived therapies.

Previous work has demonstrated that forced aggregation of hMSCs, also known as “hanging drops” (HDs), stimulates a drastic increase in both their anti-inflammatory^[9] and pro-angiogenic^[10] expression profiles. A range of techniques have been leveraged to enhance and exploit these characteristics for both *in vitro* and *in vivo* applications^[11], and encapsulation of hMSC HDs for therapeutic delivery has become an area of recent interest^[12]. Similarly, a series of papers has investigated chitosan-based substrates that promote hMSC aggregation, demonstrating that gene regulation and differentiation capacity are affected by the semi-repellent surfaces^[13]. To date these studies have not addressed the therapeutic capacity of hMSC HDs although a pro-angiogenic, anti-inflammatory cellular system would have excellent clinical benefit. Since the anti-inflammatory profile of HDs is clearly established, and inflammation and angiogenesis are intrinsically linked processes^[14], we hypothesized that synthetic copolymers of moderate surface repellency could promote the HD-like aggregation of hMSCs, and that this would correlate with a pro-angiogenic, anti-inflammatory phenotype. Here, we demonstrate that formation of hMSC clusters on copolymers that contain competing cell-repellant and cell-adhesive units results in a functional pro-angiogenic phenotype both *in vitro* and *in vivo*. These findings provide new insight into material-mediated control of the therapeutic capacity of adult-derived stem cells and can be used for improving the design of cell-instructive scaffolds for tissue engineering and regenerative medicine.

We first synthesized block copolymers comprised of a cell-repellant unit, poly(ethylene glycol) (PEG), and a cell-adhesive unit, poly(ϵ -caprolactone) (PCL) at varying compositions (Figure 1A). Specifically, the length and molar percentage (mol%) of the repellent PEG unit was varied to modulate the cellular response. Copolymer names are abbreviated by the mol % and chain length of the PEG unit, and the remaining content is PCL (i.e. 5%PEG750Da and 5%PEG2kDa are both 95mol% PCL). Based upon our previous findings that PEG chain length and mol% impacts the expression of stemness genes and redox capacity (manuscript under review), we sought to investigate how these factors influence the expression of tissue-reparative, pro-angiogenic factors. Copolymers containing the shorter 750Da PEG chain allowed for moderate cell attachment and spreading (Figure 1B–C), but cells began aggregating at higher PEG mol%. In contrast the longer 2kDa PEG chains forced cell aggregation or extreme elongation at both PEG mol% tested (Figure 1D–E). Of note, PEG2kDa copolymers greater than 10 mol% abrogated all cell attachment and were not used for these studies. Representative confocal (Figure 1F–G) and scanning electron (Figure 1H–I) microscopy images verify the pseudo-three dimensionality of these aggregates; a cell-matrix interactive layer attaches to the material surface, upon which hMSCs aggregate into three-dimensional clusters with limited cytoskeletal staining.

We next investigated how culture on copolymer substrates modulated the expression profile of beneficial factors. Since HD cultures have been shown to generate a strong anti-inflammatory/pro-angiogenic profile, we first tested how expression of these genes was regulated in our system. TCPS was used as a control substrate since this is the most common culture material used in both laboratory and clinical settings. As seen in Figure 2, expression

of the anti-inflammatory cytokines, tumor necrosis factor-inducible gene 6 protein (*TSG-6*), stanniocalcin 1 (*STC1*), and interleukin-6 (*IL-6*) was drastically enhanced for hMSCs cultured on all copolymer types (Figure 2A–C). Similarly, expression of the pro-angiogenic factors interleukin-8 (*IL-8*), angiopoietin-1 (*ANGPT1*), epidermal growth factor (*EGF*), vascular endothelial growth factor A (*VEGF-A*), and angiogenin (*ANG*) was all enhanced to varying levels on all substrates tested (Figure 2D–H). Akin to blood vessel formation in the body, hMSCs can organize endothelial cells into tube-like formations and maintain abluminal localization^[15]. Therefore, a functional test for proangiogenic potential was performed to verify the regenerative phenotype. hMSCs were cultured on copolymer substrates and TCPS for 3 days and subsequently removed to be co-cultured with endothelial cells on growth factor-reduced Matrigel. Red fluorescent protein (RFP)-expressing human umbilical vein endothelial cells (HUVECs) were used to track endothelial tubes and hMSCs were given a green counterstain (PKH-67) (Figure 2I). After 8 hours in culture, tube formation was observed for all groups tested. HUVECs alone (EC only) formed an elaborate tube network, and the presence of hMSCs from any material type did not interfere with this behavior. However, at 24 hours, HUVECs alone did not maintain tube formation, and hMSCs from TCPS or 5%PEG750Da substrates did not aid in tube stabilization, which led to the formation of large heterogeneous cell clumping. However, hMSCs that had been cultured on 20%PEG750Da, 5%PEG2kDa, or 10%PEG2kDa strongly supported tube stabilization and assumed an abluminal localization, similar to what is observed for hMSCs within their *in vivo* niche^[5]. In Figure 2J, the cumulative tube length per image confirms the functional pro-angiogenic capability of hMSCs cultured upon 20%PEG750Da, 5%PEG2kDa, or 10%PEG2kDa copolymer substrates.

Finally, we tested if this effect was functionally conserved hMSCs when cultured within three-dimensional (3D) scaffolds and implanted *in vivo*. For these experiments, 10%PEG2k was fabricated into 3D scaffolds (PEG-PCL) and compared against 100%PCL scaffolds as a “PEG-free” control. As seen in Figure 3, the formation and stabilization of vasculature over a three-week period was the strongest for hMSC-loaded PEG-PCL scaffolds, as evidenced by optical coherence tomography (OCT) imaging (Figure 3A); of note, these images were collected from the same animal over time. When quantified (N = 7 animals, two-way ANOVA), hMSC-loaded PEG-PCL scaffolds demonstrated a higher density of vessels in each OCT image, as compared to hMSC-loaded PCL scaffolds (Figure 3B, $p = 0.05$). The increased presence of vasculature was verified *post mortem* by two techniques: fluorescence microangiography (Figure 3C) and positive staining for the endothelial-specific marker CD31 (Figure 3D). For both imaging analyses, staining was weakest for PCL scaffolds without hMSCs and strongest for hMSC-loaded PEG-PCL scaffolds. For hMSC-loaded PCL scaffolds, vasculature appeared immature (Figure 3C) and did not penetrate into the interior of the scaffold (INT, Figure 3D). In contrast, both the blank and hMSC-loaded PEG-PCL scaffolds promoted strong, stable vascular networks that traversed throughout the material interior. It can be hypothesized that the blank PEG-PCL scaffolds allowed for potent vascularization due to the enhanced degradability derived from PEG-mediated wettability; however, material may also promote a pro-angiogenic response by the host cells with which it interacts.

In summary, the data presented here verify the functionality of PEG-PCL in enhancing a pro-angiogenic, anti-inflammatory phenotype of hMSCs, in both two- and three-dimensional formats, *in vitro* and *in vivo*. Future work involving these scaffolds will center on co-culture of hMSC-loaded PEG-PCL scaffolds with other cell types, as well as implantation of hMSC-loaded PEG-PCL scaffolds at highly vascularized injury sites such as skin ulcers. In a separate manuscript, we have demonstrated the nanoscale surface characteristics that regulate how cells respond to the PEG-PCL copolymers used in this study. In particular, longer PEG chains (i.e. 2kDa) cannot intercalate with PCL lamellae and therefore mushroom out at the material surface, which creates a large, hydrated area to which the cells cannot bind; in contrast, shorter PEG chains (i.e. 750Da) are able to remain within the PCL domain and their effect is subsequently less pronounced. Ongoing work aims to investigate how this behavior occurs in other polymer systems, and additional processing parameters such as annealing and addition of additives will be used to further alter the cell-material interface. Furthermore, since the pro-angiogenic effect on 2D films in Figures 1 and 2 was derived from cell aggregation at the material surface, cells cultured within 3D scaffolds likely benefited from an inherent bias toward three-dimensionality that encouraged a pro-angiogenic phenotype. The chemical composition of the materials remained the same despite fabrication method, but the cell-cell interactions present in the 2D aggregates resulted in a pseudo-3D environment that encouraged the beneficial phenotype observed, and these effects were likely enhanced in the salt-leached scaffolds since the probability of cell-cell interactions in the added dimension was increased. Therefore, investigation of how cell-adhesive/-repellant polymers can be used to regulate three-dimensional cell behaviors is an important area of research that warrants immediate attention. The findings presented here provide a new paradigm for regulating the therapeutic capacity of hMSCs and are currently being used for ongoing optimization of cell-instructive scaffolds in the context of tissue engineering and regenerative medicine.

Experimental Section

Substrate Preparation

Copolymers were synthesized by methods previously described, and compositions were verified by ^1H NMR. The specific compositions of PEG in each copolymer was 5.6% (5%PEG750), 20.6% (20%PEG750), 5.5% (5%PEG2k), and 9.3% (10%PEG2k); percentages were rounded to 5/10/20% for simplicity of presentation and discussion. For two-dimensional cell culture experiments, glass cover slips (15 mm diameter, Fisher Scientific, Waltham, MA, USA) were cleaned with 100% ethanol (Sigma Aldrich, St. Louis, MO, USA), rinsed with dH_2O , and heated to 80°C for 20 min to dry. A 1% weight/volume copolymer solution in tetrahydrofuran (THF, Sigma Aldrich) was spun for 30 seconds at 3,000 RPM. Excess solvent was then removed from samples by exposure to a constant cold-trap vacuum for 30 min. Samples were UV sterilized for 30–60 min on each side before use and secured with an autoclaved silicon O-ring (McMaster Carr, Atlanta, GA, USA) during cell culture experiments.

Cell Culture

hMSCs were purchased from Lonza (Walkersville, MD, USA), maintained in complete media (CM) composed of alpha-minimum essential media with nucleosides (α MEM, Life Technologies, Carlsbad, CA, USA) with 16.7% fetal bovine serum (Life Technologies), 1% penicillin/streptomycin (Life Technologies), and 4 μ g/ml plasmocin (InvivoGen, San Diego, CA, USA). Cells were kept in a humidified incubator at 37°C and 5% CO₂, and media was replaced twice each week. When ~80% confluent, hMSCs were detached with 0.05% trypsin-EDTA, re-seeded at a density of 100 – 500 cell/cm², and cultured for 7–14 days before reaching confluence. For all experiments, hMSCs (passage < 6) were seeded at a density of 10,000 viable cells/cm², as determined by exclusion of Trypan blue, and cultured for three to four days.

Immunocytochemistry and Scanning Electron Microscopy

hMSCs were fixed with 4% paraformaldehyde (PFA, Sigma Aldrich) for 15 min, permeabilized with 0.3% Triton-X (Sigma Aldrich) for 15 min, and blocked with 10% goat serum (Sigma Aldrich) for >2 hours, all at room temperature. hMSCs were then incubated with Alexa488-phalloidin (1:40 v/v in PBS, Life Technologies) for 10 minutes followed by counterstaining with Hoechst (Sigma Aldrich, 2 μ g/ml) for 20 minutes at room temperature. Imaging was performed with either a Nikon Ti inverted microscope (Nikon Instruments Inc., Melville, NY, USA) or a Zeiss LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany), and images were processed with ImageJ (National Institutes of Health, Bethesda, MD, USA).

For SEM, hMSCs were fixed and then dehydrated with a series of graded ethanol washes (25%, 50%, 60%, 70%, 80%, 90%, 95%, 100% $\times$ 3; 10 minutes each) before being stored in a desiccator overnight. Samples were then sputter coated with gold (Cressington Scientific, Watford, United Kingdom) and imaged under SEM (Hitachi S-4200, Tokyo, Japan) at an accelerating voltage of 1–10 kV.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

hMSCs on spin-coated substrates were homogenized with the Trizol reagent (Life Technologies), mixed with chloroform (1:5 Trizol:chloroform), and separated by centrifugation (12,000x g, 15 min, 4°C). The RNA contained within the aqueous phase was then isolated with RNeasy columns (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. cDNA was synthesized using a kit (Applied Biosystems, Life Technologies), and qRT-PCR was performed with a SYBR Green master mix (Bio-Rad) with 15 – 20ng cDNA and 500nM each of forward and reverse primers, using a CFX Real-Time PCR System (Bio-Rad). The qRT-PCR protocol included: 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 58°C 30 sec, and extension at 72°C for 30 sec. For cPCR, products were separated on a 2% agarose gel containing ethidium bromide for 15 minutes at 100V, and bands were visualized with a gel imaging station. For qRT-PCR, the expression of each gene of interest was normalized to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, generating the $C(t)$ value, and expression of $2^{-C(t)}$ relative to the TCPS control with n 3 biological replicates for each experiment is reported. Primer sequences are listed in Table

1 and only those that showed single, specific amplicons were used for qRT-PCR experiments.

In Vivo Experiments

Three-dimensional scaffolds of either 100%PCL or 10%PEG-90%PCL were cut into 6 mm discs, sterilized by UV exposure for 1 hour on each side, placed into 96-well plates, and held in place with silicon O-rings. The bottom of the 96-well plates was first coated with 1% agarose to prevent cell attachment, following previously published protocols [16]. hMSCs were seeded at a density of 10^5 cells/scaffold (or left in culture media for “blank” scaffolds) and cultured for one week in complete media before subcutaneous implantation into the ventral region of athymic, NU/J Foxn1^{nu/nu} male nude mice (6–8 weeks of age, $n = 7$ animals, The Jackson Laboratory, Bar Harbor, ME, USA). Four types of scaffolds were used for this experiment: 100%PCL without cells (“blank”), 100%PCL seeded with hMSCs, 10%PEG-90%PCL without cells (“blank”), and 10%PEG-90%PCL seeded with hMSCs. One of each scaffold type was implanted into the same animal, totaling four scaffolds per animal into eight animals. Four of the eight animals were used for fluorescence microangiography to visualize functional vasculature, as previously described [14]. Scaffolds were then excised and fixed in 4% PFA overnight at 4 °C before being subjected to graded soaks in optimal cutting temperature compound (OCT, Tissue-Tek) with sucrose and snap-frozen in OCT. Samples were cryosectioned to 5 μ m-sections, and stained with goat anti-mouse CD31 antibody (sc-1505, Santa Cruz Biotechnology) and IRdye 680 tagged anti-goat secondary antibody (926-68024, Li-Cor Biosciences) before confocal imaging on Zeiss LSM 710 microscope.

Speckle variance optical coherence tomography (OCT) was used for *in vivo* imaging^[17]. To visualize projection images of the vasculature within the implanted scaffolds, volume data sets were processed with a speckle variance technique that computes the variance in each pixel over seven repeated A-lines at each lateral position. The resulting speckle variance images represent OCT flow signal for perfused vessels within the imaged volume. Noise due to signal fall-off was excluded by thresholding the corresponding structural images to create a mask, and an average signal projection over the depth (~1 mm) of the speckle variance OCT data was used to visualize all vessels present in the volume. Images were enhanced with an optimized, modified Frangi filter^[18] to enhance vessel-like features, and the enhanced image was multiplied by the initial image to accentuate the vessels. Quantification of vessel density was performed using MATLAB (MathWorks, Natick, MA, USA) built-in Canny edge detection^[19] to find edge features, and active contours to mask in edges, and the vessel density was calculated as the mask pixels over total pixels in the image.

Statistical Analysis

Comparisons between two groups were performed with a Student's unpaired t test. Comparisons between multiple groups were performed with a one- or two-way analysis of variance (ANOVA) with a Tukey *post hoc* test to adjust p-values for multiple comparisons. In all cases, $p < 0.05$ is considered statistically significant. Mean $\pm$ standard deviation is reported, unless otherwise noted.

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Figure 1. Copolymers composed of cell-repellent and cell-adhesive domains differentially regulate cell attachment and aggregation

(A) Copolymer structure: a repellent poly(ethylene glycol) (PEG) unit was copolymerized with adhesive poly(ϵ -caprolactone) (PCL). The length and molar percentage (mol%) of the PEG unit was altered to modulate the degree of repellency. (B–F) After four days in culture, hMSCs on PEG750Da substrates were able to attach and spread, but moderate aggregation was observed as the PEG mol% was increased; in contrast, cells on PEG2kDa were highly elongated at the material surface and mostly formed aggregates (green – phalloidin; blue – DAPI). Scale bars = 100 μ m. (F–G) Representative confocal z-stack projection of hMSC aggregate on 10%PEG2kDa copolymer demonstrating that a matrix-interactive layer of cells forms at the material surface with strong actin staining, and hMSC aggregates with reduced actin signal form on top. Scale bars = (F) 100 μ m and (G) 50 μ m. Scanning electron micrographs of (H) a cell aggregate, demonstrating that multiple cells cluster atop a thin cell layer, and (I) a cellular protrusion that interacts directly with the material surface. Scale bars = (H) 20 μ m and (I) 5 μ m.

Figure 2. Copolymers promote a pro-angiogenic expression profile that is functional *in vitro* qRT-PCR analysis of (A–C) anti-inflammatory factors, *TSG-6*, *STC1*, and *IL-6* and (D–H) pro-angiogenic factors, *IL-8*, *ANGPT1*, *EGF*, *VEGF-A*, and *ANG* demonstrates that culture on all copolymer substrates enhances the expression of the tested genes. (I–J) For tube formation and stabilization assay with RFP-expressing HUVECs, hMSCs were cultured on the different substrates for three days and then moved to co-culture with RFP HUVECs for the indicated time period. After 8 hours, all conditions exhibited tube network formation; however, after 24 hours, only hMSCs that had been cultured on 20% PEG750Da, 5% PEG2kDa, or 10% PEG2kDa were able to stabilize endothelial tube formation. For each time point, the yellow box indicates the area magnified for the image immediately below. Scale bars = 200 μ m. The cumulative tube length per image is quantified, verifying these data. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ versus TCPS control or between groups as indicated by the lines.

Figure 3. Pro-angiogenic capacity of hMSCs is retained during culture and implantation within three-dimensional (3D) PEG-PCL scaffolds *in vivo*

Porous, 3D scaffolds were fabricated from 10% PEG2kDa (PEG-PCL) or 100% PCL (PCL) by salt-leaching, and were either left without cells (Blank) or loaded with hMSCs prior to subcutaneous implantation (+ hMSCs). **(A)** Optical coherence tomography (OCT) was used to non-invasively monitor the formation and retention of vasculature over a three-week period. Of note, all OCT images were collected from the same animal over time. **(B)** When quantified, vessel density per image was shown to be highest for the hMSC-loaded PEG-PCL scaffolds, although hMSC-loaded PCL scaffolds demonstrated a similar response. **(C)** Fluorescent microangiographs reveal limited vasculature (red) in blank PCL scaffolds, which was enhanced by the presence of hMSCs; in contrast, both blank and hMSC-loaded PEG-PCL scaffolds exhibited strong signal from the vasculature. All microangiographs were taken from within the scaffold area. **(D)** Immunohistochemistry for CD31 (red) reveals that the interior (INT) of PCL scaffolds for both blank and hMSC-loaded conditions contained relatively poor vascular networks, whereas the PEG-PCL material demonstrated pronounced vasculature throughout the scaffold. Scale bars = 100 μ m. * $p < 0.05$, ** $p < 0.01$ between groups indicated by the lines.

Table 1

Primer sequences used for qRT-PCR

Gene	Accession Number	Forward Primer (5' – 3')	Reverse Primer (3' – 5')	Amplicon (bp)
<i>TSG-6</i>	NM_007115.3	CTCAGCCCTCTTCAAAAATTCT	TTCACACACCGCCTTAGCTT	95
<i>STC1</i>	NM_003155.2	CACTCAGGGAAAAGCATTCGT	GAAAGTGGAGCACCTCCGAA	97
<i>IL-6</i>	NM_000600.4	CAATGAGGAGACTTGCCTGGTG	GGTTGGGTCAGGGGTGGTTA	193
<i>IL-8</i>	NM_00584.3	ACTCCAAACCTTTCCACCCC	CTCAGCCCTCTTCAAAAATTCT	173
<i>ANGPT1</i>	NM_001146.3	GCTGACAGATGTTGAGACCCA	TTCTCCCTTTAGTAAAACACCTTC	395
<i>EGF</i>	NM_001963.4	TGTCCCTTTTGGTGACCGT	TGTCCCTTTTGGTGACCGT	197
<i>VEGF-A</i>	NM_001025366.2	CGAGAAGTGCTAGCTCGGG	CTGGGACCACTTGGCATGG	377
<i>ANG</i>	NM_001145.4	GGATCCCAGGCTCGTTCTTT	CTTCCAACACAGGCTCCTCG	378
<i>GAPDH</i>	NM_002046.4	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA	138