

Human mesenchymal stem cells stimulate EaHy926 endothelial cell migration: combined proteomic and *in vitro* analysis of the influence of donor-donor variability

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Mesenchymal stem cells (MSCs) stimulate angiogenesis within a wound environment and this effect is mediated through paracrine interactions with the endothelial cells present. Here we report that human MSC-conditioned medium (n=3 donors) significantly increased EaHy-926 endothelial cell adhesion and cell migration, but that this stimulatory effect was markedly donor-dependent. MALDI-TOF/TOF mass spectrometry demonstrated that whilst collagen type I and fibronectin were secreted by all of the MSC cultures, the small leucine rich proteoglycan, decorin was secreted only by the MSC culture that was least effective upon EaHy-926 cells. These individual extracellular matrix components were then tested as culture substrata. EaHy-926 cell adherence was greatest on fibronectin-coated surfaces with least adherence on decorin-coated surfaces. Scratch wound assays were used to examine cell migration. EaHy-926 cell scratch wound closure was quickest on substrates of fibronectin and slowest on decorin. However, EaHy-926 cell migration was stimulated by the addition of MSC-conditioned medium irrespective of the types of culture substrates. These data suggest that whilst the MSC secretome may generally be considered angiogenic, the composition of the secretome is variable and this variation probably contributes to donor-donor differences in activity. Hence, screening and optimizing MSC secretomes will improve the clinical effectiveness of pro-angiogenic MSC-based therapies.

Key Words: Mesenchymal stem cell, EaHy-926 Endothelial cell, Angiogenesis, Secretome, Donor variability

Introduction

Many potential therapies for severe and/or chronic wounds fail as a result of poor vasculature^[1]. Hence, strategies to improve blood vessel supply into a wound bed are thought to promote wound healing. Transplantations of mesenchymal stem cells (MSCs) have shown great potential as a therapeutic agent for the treatment of a range of disorders, including wound healing, and have become the subject of numerous clinical trials^[2]. However, whilst the safety of MSC transplantation does not seem to be an issue^[3, 4], the effectiveness of such treatment has exhibited considerable variability. This variation in effect is problematic when translating preclinical research into MSC-based clinical therapy.

We recently demonstrated that human MSC-conditioned medium (MSC-CM) was stimulatory to epidermal and fibroblast cell adherence and migration^[5]. Other reports suggest that MSC are pro-angiogenic also through their paracrine activity on endothelial cells^[6, 7]. Whilst there are reports that MSC are capable of endothelial differentiation^[8,9], engraftment into new vasculature is low *in vivo*^[10,11]. Hence, these and other studies have contributed to recent thought that the predominant regenerative activity of MSCs is due to their secretion of factors that stimulate endogenous cells at wound sites.

In this investigation, we have examined the effects of MSC-CM on endothelial cells, using the cell line EaHy-926 as a

model system^[12]. We report that MSC-CM promotes endothelial cell adhesion and migration, but that these effects show considerable donor-donor variability. Further, we have identified extracellular matrix (ECM) proteins that are secreted by MSCs using MALDI/TOF-TOF mass spectrometry. We provide data to suggest that ECM composition plays a major role in the donor-donor variation we have seen. These findings demonstrate proof of principle of the need to screen the MSC secretome in order to optimise the application of MSCs in the clinic.

Methods

Cell culture and mass spectrometry of conditioned medium

EaHy-926 endothelial cells were maintained in DMEM/F12 culture medium (Invitrogen, Paisley, UK) supplemented with 10% (v/v) fetal calf serum (FCS) (Invitrogen) and 1% (v/v) penicillin and streptomycin (Invitrogen), incubated at 37°C and in a humidified atmosphere containing 5% (v/v) CO₂. Passaging was performed at ~90% confluence and cells were re-seeded at 1 x 10⁴ cells/cm². MSCs were isolated from the iliac crest biopsies of bone marrow donors following ethical approval and with informed consent, as previously described^[13]. The adherent cell population obtained was consistent with the characteristics of MSCs laid out by the International Society for Cellular Therapy (ISCT)^[14]. Conditioned medium was generated from MSC cultures of equal cell number in serum free

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conditions, using DMEM/F12 supplemented with insulin, transferrin and selenium (Invitrogen). Protein content of MSC CM was determined by MALDI-TOF/TOF mass spectrometry as previously described^[5].

Characterization of MSC

After three passages in culture, bone marrow derived cells were assessed by immunoprofiling for CD markers and by examining their differentiation potential to form osteoblasts, adipocytes and chondrocytes, by staining with alkaline phosphatase, oil red O, and toluidine blue respectively as per the criteria established for a MSC phenotype by the ISCT^[14].

Coating of culture plates

Culture plates were coated with MSC-CM or type-I collagen, decorin, or fibronectin (all Sigma-Aldrich). Protein solutions were diluted in PBS to 0.2mg/ml and added to each well (50µl for 96-well plates, 500µl for 24-well plates). These were refrigerated for 24 hours before being rinsed with PBS immediately prior to use.

Cell adherence/spreading

Coated 24-well tissue culture plates were seeded with 2×10^5 EaHy-926 endothelial cells in DMEM/F12 supplemented with 1% penicillin/streptomycin (Sigma-Aldrich) and 1% ITS-X (Sigma-Aldrich) and incubated for 2 hours at 37°C and 5% CO₂ before digital images were captured (ProgRes CF, Jenoptik) and analysed using Image-J software.

Scratch-wound assays

Scratch assays were established using previously published methods^[5], in protein-coated tissue culture plates (as described above). Cell migration was automatically captured

and analysed at hourly intervals using an Incucyte™ Live-Cell Imaging System (Essen Bioscience).

Statistical analysis

Data is presented as means (\pm SEM). Data were tested for significance using the Mann-Whitney U test. Those differences that fell within a 95%, 99% or 99.9% confidence interval were considered to be significant, indicated by asterisks within Figures (* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$).

Results

Bone marrow cells obtained from iliac crest biopsies showed characteristics consistent with those expected of MSC (Figure 1). MSC-CM coating of culture plates resulted in the significant enhancement of the spreading of EaHy-926 endothelial cells upon the culture surface. This result was subject to a marked inter-donor variability, with conditioned media generated by both MSC-1 and MSC-3 resulting in a significantly greater degree of EaHy-926 endothelial cell spreading than that generated by MSC-2 (Figure 2a, 2b). EaHy-926 endothelial cell adherence on fibronectin coated plates was most advanced after two hours, compared to plates coated in either type I collagen or with decorin (Figure 3). On type I collagen-coated plates, the presence of MSC-CM appeared to enhance the rate of EaHy-926 endothelial cell migration into scratch-wounds compared to unconditioned media (Figure 4a, left). EaHy-926 endothelial cells in MSC-CM closed scratch-wounds to a significantly greater degree than those in unconditioned media over a 12-hour time course (Figure 4b, left). Once again, the degree of this MSC-CM mediated enhancement of EaHy-926 endothelial cell migration was subject to inter-donor variability. Conditioned medium generated by MSC-1 (and MSC-3) elicited a greater degree of scratch-wound closure than MSC-2 over 12 hours (Figure 4b, left).

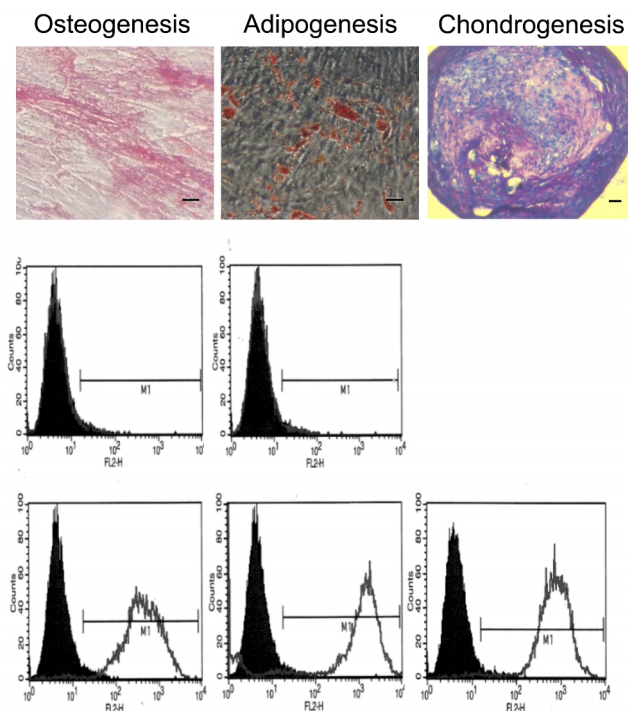


Figure 1 Cells isolated from bone marrow meet the minimum criteria for identification as MSC. Cells are immunoreactive for CD73, CD90 and CD105, whilst lacking immunoreactivity for CD14, CD34 and CD45, as shown by histograms obtained by flow cytometry, and readily differentiate to form osteoblasts, adipocytes and chondrocytes in vitro. Representative phase contrast images are shown of cells following tri-lineage differentiation and staining with alkaline phosphatase, oil red O, and toluidine blue (top, left to right). Scale bars = 50µm.

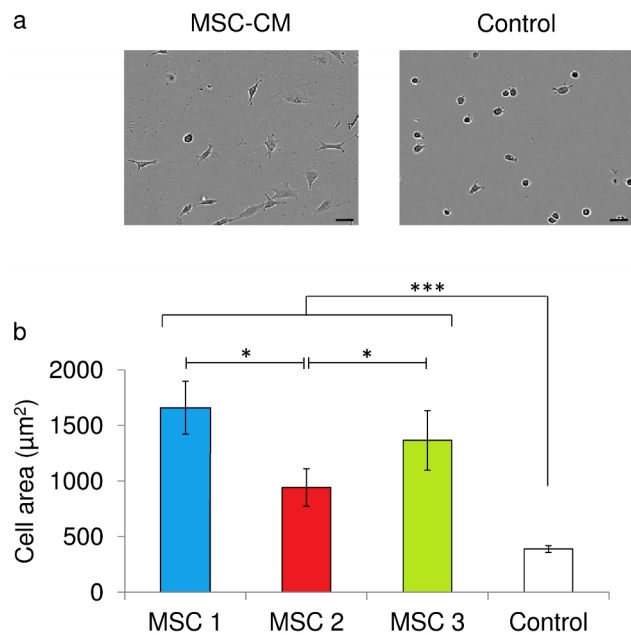


Figure 2 MSC-CM coating of culture plates influences EaHy-926 endothelial cell adherence and spreading. A: MSC-CM coating of culture plates influences EaHy-926 endothelial cell adherence and spreading. After 4 hours in culture EaHy-926 endothelial cells were observably more spread on culture plates coated with MSC-CM than on plates coated with unconditioned (control) medium. Representative phase contrast images are shown of EaHy-926 endothelial cells 4 hours post seeding. B: After 4 hours in culture the average cell area was significantly greater for EaHy-926 endothelial cells on MSC-CM coated culture plates and $389\mu\text{m}^2$ than those cells on unconditioned (control) medium coated culture plates. Data shown are means \pm SEM in relative units (**= $p<0.001$ Mann Whitney U test).

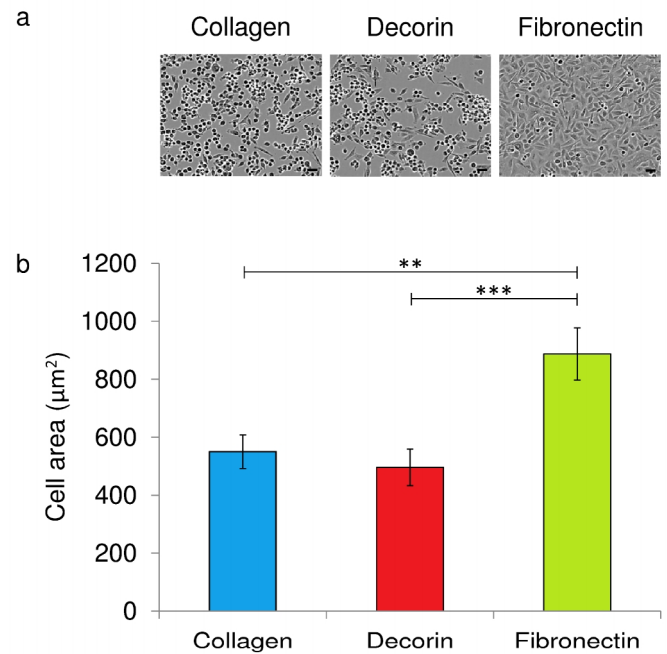


Figure 3 ECM protein coating of culture plates influences EaHy-926 endothelial cells adherence and spreading. After 4 hours in culture EaHy-926 endothelial cells were observably more spread on culture plates coated with fibronectin than on plates coated with type I collagen or decorin. Representative phase contrast images are shown of EaHy-926 endothelial cells 4 hours post seeding. B: After 4 hours in culture the average cell area was significantly greater for EaHy-926 endothelial cells on fibronectin compared to those cells on either type I collagen or decorin. Data shown are means \pm SEM in relative units (**= $p<0.01$, ***= $p<0.001$, Mann Whitney U test).

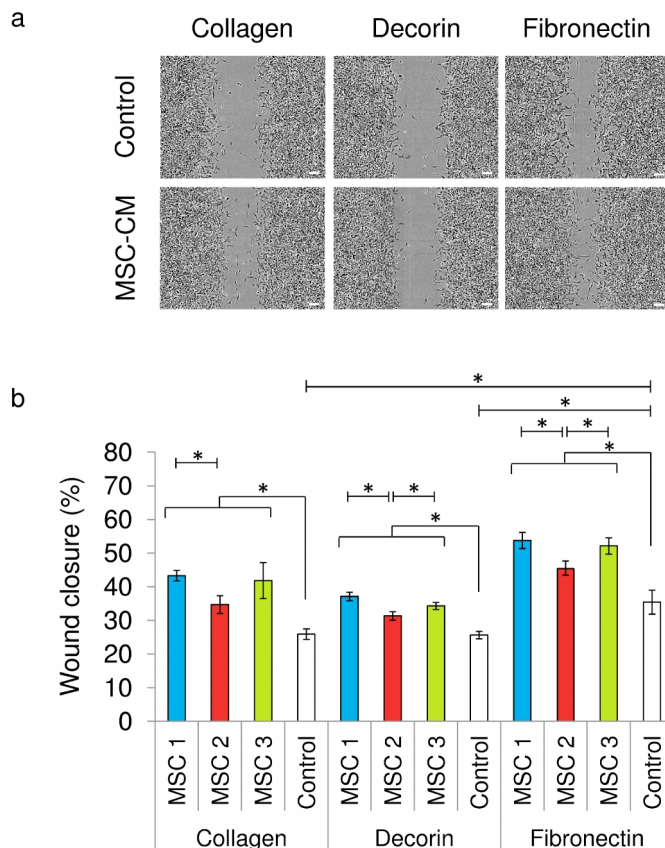


Figure 4 EaHy-926 endothelial cells closed scratch wounds faster in MSC-CM than in unconditioned medium on type I collagen, fibronectin, and decorin coated culture plates. A: EaHy-926 cells on fibronectin coated culture plates closed scratch wounds significantly faster than those on either type I collagen or decorin. Representative phase contrast images are shown of EaHy-926 scratch assays 12 hours post scratching. B: After 12 hours EaHy-926 endothelial cells in MSC-CM had closed scratch wounds by a significantly greater degree than those in unconditioned control medium. 12 hours post-scratching, EaHy-926 endothelial cells on plates coated fibronectin had closed scratch wounds by a significantly greater degree than those on either type I collagen or decorin. On fibronectin and decorin, medium conditioned by MSC-2 was associated with significantly reduced scratch closure compared to either MSC-1 or MSC-3. On type one collagen, medium conditioned by MSC-2 was associated with significantly reduced scratch closure compared to MSC-1. Data shown are means \pm SEM. (* = $p<0.05$ by Mann Whitney U test).

These MSC-CM were examined by MALDI/TOF/TOF mass spectrometry. All three were found to contain fibronectin, collagen type I, collagen type VI, and lumican, whilst cartilage oligomeric matrix protein (COMP) and SPARC were present in two out of three MSC-CM and laminin, decorin, heparan sulphate proteoglycan (HSPG) and IGFBP-1 were each only observed in one MSC-CM (Table 1). Of these, decorin, along with HSPG were only present in medium conditioned using MSC-2. Hence, not only was there clear inter-donor variability in the ECM components of the MSC secretome, but the presence of these proteoglycans seemed to be associated with a reduction in the efficacy of MSC-CM.

When type I collagen, decorin and fibronectin were used as culture substrata, the degree of EaHy-926 endothelial cell spreading appeared to be similar upon both type I collagen

and decorin and greatly enhanced upon fibronectin (Figure 2a, 2b).

Similarly, scratch-wound closure by EaHy-926 endothelial cells in unconditioned media appeared to be similar upon type I collagen and decorin, but markedly greater upon fibronectin (Figure 4a, 4b). In the presence of each ECM substrate the presence of MSC-CM resulted in significantly enhanced scratch wound closure compared to unconditioned media (Figure 4b). As seen previously upon type I collagen coated culture plates, the degree of closure upon both decorin and fibronectin coated plates was either significantly or near significantly ($p=0.057$ by Mann Whitney U test) less in the presence of medium conditioned by MSC-2, compared to medium conditioned by either MSC-1 or MSC-3 (Figure 4b).

**Table 1: EXTRACELLULAR MATRIX PROTEINS DETECTED IN MSC
CONDITIONED MEDIUM USING (MALDI-TOF/TOF) MASS SPECTROMETRY**

MSC-CM 1	MSC-CM 2	MSC-CM 3
Fibronectin	Fibronectin	Fibronectin
Type I Collagen	Type I Collagen	Type I Collagen
Type VI Collagen	Type VI Collagen	Type VI Collagen
Laminin	-	-
COMP		COMP
Lumican	Lumican	Lumican
-	Decorin	-
-	HSPG	-
SPARC	SPARC	-
IGFBP-1	-	-

Mass spectrometry of MSC-CM from 3 separate donors. MALDI-TOF/TOF mass spectrometry of MSC-CM detected variable protein content between media conditioned by MSC from three different patient donors.

Discussion

We have previously shown that MSC-CM promotes the migration of skin cells in a wound healing model, and identified numerous potentially beneficial factors that may contribute to this effect. *In vivo* wound healing is, however, a complex process influenced by a host of cellular events, including angiogenesis. If MSC can stimulate endothelial cells as suggested here and elsewhere^[7] this supports their potential use in the treatment of cutaneous wounds.

In these experiments, MSC-CM was found to be stimulatory to endothelial cell migration *in vitro*. This is similar to previous

studies in which MSC-CM has been shown to stimulate angiogenesis^[7], supporting the investigation of MSC-CM as a pro-angiogenic agent. Although it is possible for proliferation of EaHy-926 endothelial cells to have contributed somewhat to the closure of the scratch wounds, these scratch assay experiments were performed over the course of 12 hours. The reported doubling time for these cells is over 25 hours^[15]. To significantly affect the rate of scratch wound closure, those cells at the leading edge of the scratch margins would be required to undergo repeated doublings, and this is unlikely to have had a major influence on scratch wound closure over the time course of these studies.

Mass spectrometry of MSC-CM revealed numerous factors (including fibronectin and collagen) in medium conditioned by each of the three MSC examined and some, including laminin and decorin, in one or two but not all three of the MSC-CM samples. Of these ECM components, fibronectin, collagen and laminin are known to promote or support angiogenesis^[16]. Unusually, in these experiments collagen did not seem to induce any observable cell response when compared to decorin. This result may be elucidated through further study. Fibronectin and collagen contain protein motifs are known to mediate angiogenesis by integrin receptor signaling *in vivo* and *in vitro*^[17-19]. Laminin peptides have also been shown to be pro-angiogenic^[20]. Conversely decorin and lumican have been shown to inhibit angiogenesis. Decorin inhibits endothelial cell migration and tubule formation *in vitro*^[21] and inhibits the pro-angiogenic effects of VEGF^[22]. Lumican interferes with $\alpha 2\beta 1$ receptor activity and inhibits angiogenesis both *in vitro* and *in vivo*^[23, 24]. Whilst lumican was present in each of the MSC-CM used in these investigations, decorin was only found within the MSC-CM that consistently showed significantly less enhancement of EaHy-926 endothelial cell migration. As the method (MALDI-TOF/TOF mass spectrometry) used to detect these protein components within MSC-CM was not quantitative, it was not possible to determine whether differences in concentrations of each ECM component were related to efficacy of the conditioned media.

MSC-CM coating of culture plates enhanced EaHy-926 endothelial cell adherence, as did coating with fibronectin. Cell adherence to these substrata was examined in an indirect fashion, by assessing the average area of cells in the immediate-early period after seeding. As cells settle upon a permissive substrate they spread out from the rounded morphology observed in suspension to adopt a flattened morphology that is usually seen *in vitro* in adherent cell populations. After prolonged periods of time, cells in culture, including endothelial cells, synthesize matrix molecules that may also promote cell adhesion, potentially masking the initial effects of the original substrate being investigated. Of the matrix proteins detected in MSC-CM by MALDI-TOF/TOF mass spectrometry, fibronectin was observed to have a profound effect upon cell adherence, similar to those findings concerning cell migration.

Individually, fibronectin appears contribute to the MSC-CM effects observed. However, the addition of MSC-CM to endothelial cell scratch assays performed upon fibronectin coated culture plates resulted in a further enhancement of endothelial cell migration, suggesting that fibronectin may not be solely responsible for the entire effect. Further experiments investigating the effects of MSC secreted growth factors and cytokines individually, and in combination, may reveal their relative contribution to the enhancement of endothelial cell migration. The relatively low number of individual donors examined during this study is a clear limitation, and although the results presented support the conclusions that (i) MSC donor variation and secretome composition may account for differential effects of MSCs on endothelial cells, and (ii) that this variation should be taken into consideration in MSC-based regenerative medicine, subsequent investigation of greater numbers of samples is required to further authenticate these findings.

Conclusion

Angiogenesis depends on endothelial cell migration and the actions of endothelial cell chemotactic factors, e.g. VEGF and IL-8^[25-26], and ECM proteins such as collagens, fibronectin and laminin^[15]. Many of these pro-angiogenic factors have been found to be present within the MSC secretome both within this investigation and in previous studies^[5]. These experiments have showed that MSC-CM enhances both the rate of endothelial cell migration and the adherence of these cells to their culture surface, and that this effect was, in part, mediated by the presence of fibronectin. Other studies have also highlighted a role for fibronectin in stimulating angiogenesis^[16, 17]. The effects of MSC-CM upon endothelial cell migration were not entirely induced by fibronectin, as suggested by the further enhancement of EaHy-926 endothelial cell migration by MSC-CM in the presence of exogenous fibronectin. Factors such as Interleukin (IL)-8, VEGF, and laminin have all been shown to be stimulatory to endothelial cells^[19, 24] and these factors have been shown to be present within MSC-CM^[7]. It seems likely that these known mediators of angiogenesis might contribute to the effects of MSC-CM upon EaHy926 endothelial cells observed in these investigations.

Overall, the data presented here support the hypothesis that MSC may stimulate the formation of new vasculature, and that this may be an important aspect of MSC-mediated enhancement of wound healing.

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Abbreviations

CD:	Cluster of Differentiation
COMP:	Cartilage Oligomeric Matrix Protein
DMEM:	Dulbecco's modified Eagle's Medium
ECM:	Extracellular Matrix
FCS:	Fetal Calf Serum
HSPG:	Heparan Sulphate Proteoglycan
IGFBP:	Insulin-like Growth Factor Binding Protein
IL:	Interleukin
ISCT:	International Society for Cellular Therapy
MALDI-TOF/TOF:	Matrix-Assisted Laser Desorption/Ionization-Time Of Flight/Time Of Flight
MSC:	Mesenchymal Stem Cell
MSC-CM:	Mesenchymal Stem Cell-Conditioned Medium
PBS:	Phosphate Buffered Saline
SEM:	Standard Error of the Mean
SPARC:	Secreted Protein, Acidic and Rich in Cysteine
VEGF:	Vascular Endothelial Growth Factor

Potential Conflicts of Interests

None

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