**High content and high throughput screening to assess the angiogenic and neurogenic actions of mesenchymal stem cells *in vitro***

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**Abstract**

Mesenchymal stem cells are universally regarded across many fields of medicine, as one of the most promising cell types for use in cell-based therapies. Although not yet fully understood, the therapeutic effects of these cells are largely attributed to the trophic actions of growth factors and cytokines present in the cell secretome. Specifically, the angiogenic and neurogenic properties of these cells make them attractive for the repair of vascularised and innervated tissues. In this study, we investigate the effect of mesenchymal stem cell conditioned media on *in vitro* assays of angiogenesis and nerve growth. We describe the use of two state of the art high content and high throughput

 cell analysis systems and compare them against manual analysis techniques. Mesenchymal stem cell secretomes stimulated angiogenesis and nerve growth *in vitro* in a donor dependant manner. Levels of neuroregulin, platelet-derived growth factor-AA and glial-derived neurotrophic factor, positively correlated with the observed angiogenic effects of these cells. High content and high throughput cell analysis systems such as the ones used in this study, may provide rapid screening tools to assist not only with patient selection but the identification of predictive therapeutic markers to support clinical outcome monitoring for patients treated with stem cell therapies.

**Keywords:** Mesenchymal stem cell, high content, high throughput, secretome, conditioned media, angiogenesis, neurogenesis.

**Introduction**

Bone marrow-derived mesenchymal stem cells (BMSCs) are fast becoming the most widely used cell type for tissue repair and regeneration. In the clinic, BMSCs have been used for the treatment of bone defects and non-union fractures [1-3], spinal cord injury (SCI) [4-6], stroke [7,8] acute myocardial infarction [9-11] and chronic wounds [12,13]. In such pathologies, co-ordinated angiogenesis and neurogenesis are vital for the repair and correct functioning of the damaged tissue and ultimately, recovery of the patient.

Both the angiogenic and neurogenic properties of BMSCs are well reported in the literature and are attributed to direct cell-cell interactions and the trophic actions of cell-secreted factors. *In vitro*, BMSCs stimulate endothelial tube formation [14] and when intravenously [15] or intramuscularly [16] injected, were reported to increase capillary density in rats post-myocardium infarction. Furthermore, application of BMSC conditioned media was reported to enhance vascular perfusion in a mouse model of lower limb ischaemia [17]. BMSC secretomes stimulate the proliferation, migration and tube-like formation of endothelial cells in a paracrine manner [17,18]. BMSCs produce a plethora of angiogenic factors *in vitro* including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), interleukin-6 (IL-6), placental growth factor (PIGF), angiopoeitin-1, platelet-derived growth factor (PDGF), matrix metalloproteinase-9 (MMP-9) and monocyte chemoattractant protein-1 (MCP-1) [17,18]. Neutralising antibody experiments have demonstrated that the mitogenic effects of BMSC secretomes are not solely due to the presence of one particular growth factor, but as a result of a combination of factors [17]. More recently, comprehensive protein analysis of equine BMSC secretomes has identified the presence of additional secreted angiogenic factors not previously reported including dipeptidyl peptidase-4, endothelin-1, urokinase-type plasminogen activator, activin A, platelet-derived endothelial cell growth factor, pentraxin-3 and IL-1β [19].

BMSCs demonstrate neurogenic properties as shown by their ability to stimulate nerve growth *in vitro* [20-23] and by promoting functional recovery in animal models of SCI [24]. Monolayer cultured BMSCs produce neurotrophic factors including brain-derived neurotrophic factor (BDNF) [21,25], nerve growth factor [21], neuroregulin-1 (NRG1-β1) [26], glial-derived neurotrophic factor (GDNF) [27], artemin and persephin [19]. So far, it is unclear from the literature whether individual or the combined activities of trophic factors present in BMSC secretomes are important for the observed angiogenic and neurogenic activity of these cells.

Variation in the *in vitro* behaviour of BMSCs from different donors is widely reported in the literature and the uncertainty of how such variation may affect therapeutic potential, represents a significant problem with regards to the development of reliable, predictable BMSC cell-based therapies [28]. Donor variation may contribute to minimal rates of improvement in clinical outcome and discrepancies seen between similarly designed clinical trials. Currently, there are no standardised tools available to screen stem cells prior to transplantation. High content and high throughput automated cell analysis techniques may offer fast, reproducible, and economically viable methods for screening the suitability of stem cells, including BMSCs for clinical use. Moreover, such systems could be used to identify predictive markers to determine the mechanisms responsible for the therapeutic efficacy of these cells. Previously, we have reported the development and validation of neuronal algorithms allowing high content screening of neural cell lines and primary cultures, exposed to human BMSC secretomes [29]. Here, we have investigated both the neurogenic and angiogenic properties of BMSC secretomes from a cohort of patients undergoing spinal fusion. BMSC conditioned media was applied to neurite outgrowth and endothelial tube formation assays. Data was acquired and assessed by established manual analysis techniques and then compared to data acquired using automated high content and high throughput image analysis systems. Finally, we screened BMSC secretomes for a wide panel of neurogenesis and angiogenesis-related proteins and correlated levels of detected factors to the neurogenic and angiogenic effects observed in our *in vitro* assays.

**Materials and Methods**

**Isolation of BMSCs and generation of conditioned media**

Bone marrow aspirates were obtained from 7 patients (4 female & 3 male, age range 33-55) undergoing spinal fusion with written informed consent and Local Research Ethics Committee approval (Shropshire and Staffordshire Strategic Health Authority, Reference number:04/02/RJH). BMSCs were isolated by Lymphoprep™ (Fresenius Kabi AG, Germany) density centrifugation. The interphase containing the mononuclear cell fraction was collected and plated at a cell seeding density of 20x106 per T175 flask in DMEM F12, 10% foetal calf serum (FCS) and 0.05% penicillin and streptomycin (all GIBCO, UK). After 3 days, non-adherent haematopoietic cells were removed. BMSCs were expanded in monolayer (5x103cells/cm2) at 37°C and 5% CO2 and received media changes three times a week. Conditioned media was generated from cells at passage 2. When 70% confluent, BMSCs were washed with phosphate buffered saline (PBS) and incubated with conditioning media (serum free DMEM F12, 0.05% penicillin and streptomycin, 10µgml-1 Insulin-Transferrin-Selenium-X and 1% MEM non-essential amino acids (all GIBCO, UK) for 48hours. Conditioned media was filtered through a 0.45µm sterile filter and stored at -20°C. Following collection of conditioned media, BMSCs were passaged and a cell count performed.

**DRG culture and stimulation with BMSC conditioned media**

Dorsal root ganglia (DRG) explants were obtained from chick embryos (embryonic day 10) and cultured on a type I collagen substrate as previously described [30]. Briefly, 24 well plates were treated with nitrocellulose (Schleicher & Schuell, Dassel, Germany), air dried and then coated with 100µg/ml type 1 collagen (Cat: C8919, Sigma, UK). Excess collagen was removed by washing three times with PBS. DRG explants were cut into three equally sized segments and cultured in either standard DRG culture media (DMEM F12 containing 10% FCS, 10µgml-1 Insulin-Transferrin-Selenium-X and 1% MEM non-essential amino acids and 50ng/ml nerve growth factor (Cat: 13290-010, Invitrogen, UK) BMSC conditioned media (n=5 donors) or BMSC control media (BMSC culture media which had not been in contact with cells) for 48hours at 37°C and 5% CO2.

**Immunocytochemical characterisation of DRG explants**

Fixation and immunohistochemistry were performed at room temperature. DRG explants were fixed by gently adding an equal volume of neutral buffered 20% paraformaldehyde to the culture media for 30 minutes. Wells were washed with immunobuffer (0.05% bovine serum albumin + 0.1% Triton X-100 in PBS) twice for 10 minutes. A blocking buffer (immunobuffer containing 10% goat serum (Dako, UK) was applied for 1 hour. DRG explants were then incubated with either monoclonal anti-neurofilament 200 (Clone NE14, Sigma, UK) at 1:200 in PBS and 2% goat serum or monoclonal mouse anti-chick 1E8 antibody (DSHB, USA) at 1:20 in PBS and 2% goat serum for 1 hour. After washing, DRG explants were incubated with goat anti-mouse Alexa Fluor 488 secondary antibody at 1:200 in PBS containing Hoechst 33342 (both Invitrogen, UK) for 40 minutes. Wells were washed with PBS and visualised by fluorescence microscopy using a Nikon TS100 microscope.

**Manual analysis of DRG neurite outgrowth**

Neurite outgrowth was quantified from DRG cultures immunostained for neurofilament. Multiple images were taken at x10 magnification and then aligned to create a montage of the entire explant. A grid was positioned over the montage and defined regions of the explant were analysed (Supplemental Figure S1). Neurite length was measured from the tip of the neurite along the axon until the path was obscured by neighbouring neurites, at which point, a straight line was traced back to the DRG explant perimeter (Supplemental Figure S1). Analysis was performed using four DRG explants per donor with conditioned media from a total of 5 donors (n=5). Neurite length from each patient secretome is presented as the mean ± standard deviations.

**High content analysis of DRG neurite outgrowth**

Neurite outgrowth from DRG explants was additionally quantified by high content analysis using a Thermo Scientific Cellomics® ArrayScan® VTI HCS Reader, a modular high content screening instrument designed for high capacity automated fluorescence imaging and quantitative analysis of both fixed and live cells. The instrument features optics by Carl Zeiss®, broad white-light source, scientific grade digital camera and integrated acquisition and analysis software. Entire DRG explants were analysed by automated image capture in multiple fields of view in two fluorescent channels. Complementary Cellomics Neuronal Profiling BioApplication software was then used to perform cell-based measurements by analysing images taken in these two fluorescent channels. All cells (neuronal and non-neuronal) were identified on the basis of nuclear staining in Channel 1 by the nuclear stain, Hoechst 33342. Neurites were identified in Channel 2 by immunofluorescence using a primary antibody raised against neurofilament. Following optimisation, the BioApplication creates a neurite mask using Channel 2, allowing quantitation of parameters such as total length, area and number of branch points. Total neurite length and branching were quantified from multiple images taken of the entire DRG explant. Analysis was performed using four DRG explants per donor with conditioned media from a total of 5 donors (n=5).

**Endothelial tube-like formation assay**

The human umbilical cord endothelial cell line (HUVEC) was cultured in monolayer at 5x103 cells/cm2 with endothelial cell growth medium (Promocell, Heidelberg, Germany). HUVEC were maintained in 5% CO2 at 37°C with media changes every 2-3 days. Endothelial tube-like formation assays were performed using Matrigel™ (growth factor reduced, phenol red-free (BD Biosciences, France). Briefly, 24-well tissue culture plates were coated with 230μl Matrigel™ per well and allowed to solidify at 37°C for 30 minutes. HUVEC were seeded onto Matrigel™ (5x104 per well) in endothelial cell growth media. After 4 hours, plates were gently washed in PBS before application of either BMSC conditioned media (n=5 donors), HUVEC control media or BMSC control media that had not been in contact with cells. After 24 hours, wells were washed and viewed in phase using a Nikon TS100 fluorescent microscope.

The total tube length in 5 fields of view from duplicate wells per donor, using 5 donors (n=5) was quantified by tracing and measuring tube-like structures using image analysis software (IPLab version 3.6, Becton Dickinson). Complexity of tube-like networks was scored using a scoring system as previously described [19]. In brief, two cells sharing a common branching point were scored as 1 three cells sharing a common branching point were scored as 2 and so on. Angiogenic sprouts, classified as unconnected tube-like structures 25-100µm in length were scored as 1.

**Cell-IQ™ analysis of tube-like formation**

Formation of endothelial tube-like structures was monitored for 24 hours in the Cell-IQ™ live cell imaging system (CM Technologies Ltd, Tampere, Finland). The Cell-IQ™ comprises a computer controlled microscope and camera set-up in a temperature controlled environment. Regions of interest are identified and the software (Cell IQ Imagen™) records the x, y and z co-ordinates of the in-focus image. Cells were maintained at 37°C and 5% CO2, 21% O2 using pre-mixed gas (BOC Ltd, Guildford, UK). Nine regions of interest (in a 3x3 grid) per well were continuously imaged for a 24hour period with a x10 objective and 60µm z-stack. Following image capture, z-stacks of the 9 regions of interest per well were stitched together using the Cell-IQ Imagen™ software to generate a larger region for analysis. Total tubule length per region and branching complexity were quantified from these 9 fields of view, from wells in duplicate using conditioned media from a total of 5 donors (n=5), using the accompanying Cell IQ Analyser™ software. Using image thresholding tools, which may be finely tuned by the user at the set-up phase of the analysis, this software is able to create a mask over the endothelial tube-like structures which are then automatically quantified.

**Angiogenic and neurogenic protein arrays**

Conditioned media generated from BMSCs (n=7) was screened for a panel of 30 neurogenic and angiogenic factors using custom designed antibody arrays (RayBiotech Inc, USA). All steps were carried out according to the manufacturer's instructions. Briefly, array membranes with protein antibodies spotted in duplicate were incubated with blocking buffer for 30 minutes at room temperature. Samples of conditioned media were thawed and incubated with the membranes overnight at 4°C. Membranes were washed with wash buffer provided in the kit then incubated with a Biotin-conjugated antibody for 1 hour at room temperature. Wash steps were repeated as before and membranes incubated with HRP-conjugated streptavidin for 2 hours at room temperature. Following another series of wash steps, membranes were incubated with a chemiluminescent detection reagent provided in the kit for 2 minutes. Positive signals were visualised with a chemiluminescence imaging system (ChemiDOc™ EQ, Bio-Rad Laboratories Srl, Italy). Array data was semi-quantified by measuring the sum of the intensities of the pixels within each spot boundary x pixel area, with image analysis software (Quantity One® version 4.6.3, Bio-Rad, Italy). A signal from a clear part of the array was subtracted from all data to account for background signal. A mean was taken from the two duplicate spots for each factor. Levels of neurogenic and angiogenic factors were normalised to positive controls (provided in the kit) and to the cell number of BMSCs that had generated a standard volume of conditioned media.

**Statistical Analysis**

Statistical analysis was performed using SPSS Version 21. Data acquired from individual patient secretomes are presented separately. Protein array data is pooled and therefore appropriately normalised to cell number. All data presented non-normal distribution as determined by Kolmogorov-Smirnov normality tests. Non-parametric data from the three treatment groups were tested for significant differences using Kruskall-Wallis one-way analysis of variance tests. All correlation analysis, including the relationship between the levels of angiogenesis and neurogenesis related proteins detected in BMSC conditioned media to the data acquired by Matrigel™ and neurite outgrowth assays, were performed on un-normalised data and tested for significance using Spearman rank correlation tests (n=5).

**Results**

**Mesenchymal stem cell secretome stimulated neurite outgrowth from chick dorsal root ganglia**

Extensive neurite outgrowth, as visualised by neurofilament staining, was observed from DRG explants that had been cultured in standard DRG growth media (Figure 1 A). BMSC conditioned media stimulated DRG neurite outgrowth from the DRG body (Figure 1 B) compared to control media where neurite outgrowth was comparably poor (Figure 1 C). Importantly, the degree of neurite and cellular outgrowth varied considerably between BMSC secretome derived from different patients (Figure 1 H). Cells located within the DRG body stained positively for the monoclonal antibody 1E8 which recognises the antigen ‘Po’, an early marker of the Schwann cell lineage (Figure 1 D-E). Upon application of BMSC conditioned media, these neural support cells appeared to migrate out of the DRG body, together with associated neurites (Figure 1 F-G). Neurite outgrowth was significantly greater when DRGs were cultured with BMSC conditioned media compared to BMSC control media for donor 2 (p<0.05) donors 4 and 5 (p<0.0001) (Figure 1 H, Kruskall-Wallis). In the case of donors 1-3, neurite outgrowth was greater following application of DRG growth media compared to BMSC conditioned media (Figure 1 H, Kruskall-Wallis, donor 1 and 3: p<0.0001, donor 2: p<0.001). However, with regards patients 4 and 5, BMSC conditioned media stimulated significantly greater neurite outgrowth compared to both BMSC control media and DRG growth media (Figure 1 H, Kruskall-Wallis, p<0.0001).

**High content analysis permitted rapid assessment of neurite outgrowth**

High content analysis of DRG neurite outgrowth was performed on identical explant cultures that had previously been assessed manually. Following optimisation of the algorithm, the system was capable of rapid, image capture and analysis. A single field of view could be imaged in just 0.1seconds, which allowed imaging and analysis of an entire DGR explant in under 10 seconds. The Bioapplication analysis tool was successfully applied to fluorescent images of the DRG explant cultures, as shown by analysis overlays, which created a trace of neurite outgrowth and branch points (Figure 2 A-D). Whereas manual analysis considered only partial analysis of the DRG explant, giving mean neurite length, high content analysis allowed quantitation of the entire explant generating data for total neurite length (Figure 2 E). High content analysis also gave the opportunity to quantify additional parameters including neurite branching (Figure 2 F) which was not feasible using manual analysis techniques. Neurite length and branching, as determined by high content analysis, correlated significantly (Spearman rank correlation rs = 0.900, p=0.037). In terms of donor trends, neurite length and branching data acquired by high content analysis was very similar, although not statistically significant to that obtained manually (Spearman rank correlation rs= 0.800, p=0.104).

**Mesenchymal stem cell secretome stimulated endothelial tube-like formation *in vitro***

HUVEC formed a stable endothelial tube-like network on Matrigel™ when cultured in standard culture media (Figure 3 A), whereas tube-like formation was limited following incubation with control conditioned media, that had not been in contact with BMSCs (Figure 3 B). BMSC conditioned media stimulated endothelial tube-like formation (Figure 3 C-D). Tube-like formation was greater following application of BMSC conditioned media compared to control conditioned media in all donors and was statistically significant in donors 1 (p<0.05) and 3 (p<0.0001) (Figure 3 E, Kruskall Wallis). A similar, corresponding donor trend for tubule branching was also observed in Matrigel™ assays (Figure 3 F).

**Cell-IQ™ analysis of endothelial tube-like formation**

The Cell-IQ™ analysis tool was successfully applied to HUVEC cultures to provide measurements of tube-like length and branching score (Figure 3 G-H). Quantitation of tube-like length by Cell-IQ™ generated similar data to that acquired using manual techniques (Figure 3 I-J). Correlations between tube-like length and branching score were significant, as measured manually and by Cell-IQ™ (Figure 3 K, Spearman correlation coefficient rs: 0.900 p-value=0.037 and rs: 1.00 p-value= 0.001 respectively). Both endothelial tube-like length and branching score quantified by Cell-IQ™, positively correlated, with data obtained by manual analysis however this was not statistically significant (Figure 3 K, Spearman correlation coefficient rs: 0.800). A clear trend identifying concomitant angiogenic and neurogenic effects of BMSC conditioned media was evident in this study. Specifically, the effects of patient BMSC secretomes on endothelial tube-like length and branching positively correlated, although not significantly, with neurite length, as acquired by both manual (Spearman rs: 0.800, p=0.164) and high content analysis (Spearman rs: 1.00, p=0.08).

**Levels of NRG1, GDNF and PDGF-AA in BMSC secretome positively correlated with endothelial tube-like formation**

BMSCs secreted a multitude of angiogenic and neurogenic factors as determined by protein array (Figure 4). The highest levels of factors detected were that of BDNF, PDGF-AA, VEGF, epidermal growth factor, FGF4 and PIGF. Notably, levels of NRG1, GDNF and PDGF-AA correlated significantly with endothelial tube-like length and branching score acquired by manual analysis (Figure 4, Spearman correlation coefficient, NRG1: p=0.037, GDNF: p=0.037, PDGF-AA: p=0.001). Positive correlations between levels of Activin-A, SCF and NT4 and Matrigel™ assay parameters were also evident but not statistically significant (Figure 4, Spearman correlation coefficient, rs:0.700, p-value=0.188 for all three factors). Positive relationships between these factors mentioned above and endothelial tube-like formation and branching score, as acquired by Cell-IQ™ were also observed (data not shown). No statistically significant correlations were identified between angiogenesis/neurogenesis related proteins and our neurite outgrowth assay.

**Discussion**

The trophic actions of BMSCs are thought to constitute a significant part of their therapeutic potency with regards to cell therapy. Here, we demonstrate coupled angiogenic and neurogenic effects of human BMSC conditioned media *in vitro*. In this study, we have utilised and compared state of the art high throughput and high content cell analysis technologies against manual analysis techniques. By performing wide screen protein analysis, we were able to identify several growth factors as candidate regulatory molecules responsible for, in part, the angiogenic properties of BMSC secretome.

The stimulatory effect of BMSC conditioned media on neurite outgrowth seen here, is in accordance with previously published works [20-22]. In the case of BMSC secretomes from two patients, DRG neurite outgrowth was stimulated above that achieved with standard DRG culture media supplemented with NGF. By performing immunohistochemical studies, we show that BMSC conditioned media not only stimulated the growth of neurites, but also stimulated the proliferation and/migration of neural support cells expressing a lineage specific Schwann cell marker. This suggests that BMSC may support neurogenesis by multiple modes of action i) by directly stimulating neurite growth and ii) by enhancing the proliferation/migration of neuronal support cells which in turn guide neurite outgrowth. The neuroprotective effects of MSC secretome is reported in primary neuronal support cell cultures *in vitro* [31-33]. Similarly, we found that BMSC secretomes were angiogenic, as demonstrated by the stimulation of endothelial tube-like formation on Matrigel™. As with our *in vitro* assays of nerve growth, this was very much patient dependent and in certain cases, tube-like formation was greater with BMSC secretomes compared to HUVEC culture medium.

We observed coupled angiogenic and neurogenic effects of BMSC conditioned media, in that patient secretomes that were highly neurogenic was also highly angiogenic. In developmental and physiological processes, nerves and blood vessels often grow together and appear to be regulated by similar environmental cues [34,35]. With respect to BMSC therapy, it is imperative that the intricate relationship between angiogenesis and neurogenesis is maintained in order to promote effective and functional tissue repair. For example, it has been shown that regenerating axons grow alongside blood vessels [36] and that increased blood vessel density is associated with greater functional recovery in rat models of SCI [37,38]. Thus, the coupled angiogenic and neurogenic effects of BMSC conditioned media make the secretome and the cells themselves attractive candidates for use in cell-based therapies where vascularisation and innervation are desired. The beneficial effects of hypoxia preconditioned BMSC secretome on blood vessel and nerve growth has been demonstrated in an *in vivo* model of stroke [39].It is important to consider, that the angiogenic and neurogenic actions of BMSCs are likely to be affected by different *in vitro* culture conditions which are known to affect many aspects of MSC phenotype [19]. For example, the angiogenic properties of these cells have been shown to be affected by 3D culture conditions [40] and *in vitro* differentiation status [19,41].

In the present study, BMSCs secreted a multitude of growth factors, cytokines and chemokines, as previously reported [17,42,43]. We present novel data showing significant correlations between the levels of neurogenic and angiogenic factors: NRG1, GDNF, PDGF-AA, and endothelial tube-like formation. Of course, in order to further investigate the individual roles of these growth factors, either receptor knock-out or neutralization antibody experiments could be performed. Our findings are supported by previous work suggesting that the stimulation of angiogenesis *in vitro* by BMSCs is not dependent upon the presence of one secreted protein, but many [17]. In previous work, we correlated the stimulation of neurite outgrowth from chick motor neurons with levels of GCSF, FGF-4 and MMP-8 in BMSC secretome [23]. Others have associated levels of cell-secreted BDNF [21] and recombinant GDNF with *in vitro* assays of nerve growth [44]. In this study, we did not find any positive correlations between the levels of secreted proteins detected in our array and neurite outgrowth. This discrepancy between other published works using chick DRG and ours is likely due to differences such as how BMSC conditioned media was generated, the tissue source of MSC and possibly due to competitive binding between growth factors/cytokines.

We identified NRG1 as an important factor contributing to the angiogenic effects of BMSC conditioned media. NRG1 plays an important role in vascular biology, acting as a mitogen of cardiomyocytes, endothelial progenitor cells and mature endothelial cells [45]. Up-regulation of NRG1 by vascular cell types following vascular insult and improved cardiac performance following its endogenous application in animal models of myocardial infarction, suggests it's potential for therapeutic intervention [46-48]. *In vivo*, NRG1 is important for the early stages of nerve re-myelination following injury [49] and as such, has shown neuro-regenerative effects when delivered in its recombinant form or by viral gene transfer [50-52]. Taken together, NRG1 represents a potential candidate for the promotion of both vascular and neural repair in the clinic.

Levels of PDGF in BMSC conditioned media also strongly correlated with the stimulation of endothelial tube-like formation. PDGF is a ubiquitously expressed growth factor involved in developmental and physiological processes of many tissues including the cardiovascular, peripheral and central nervous systems. The neuroprotective effects of BMSCs on retinal ganglion cells have previously been attributed to PDGF-AA [53]. Very recent work, indicates a role for PDGF-AA in MSC secretome induced migration of resident cardiac atrial appendage stem cells in an *in vitro* model of injured myocardium [54]. PDGF has a long history of safety and therapeutic efficacy in wound healing and peridontal regeneration [55]. Furthermore, the combined osteogenic and angiogenic effects of BMSCs and PDGF make this cell and growth factor combination attractive for bone healing strategies [56]. Lastly, we found a strong correlation between levels of GDNF and endothelial tube-like formation, not previously reported in the literature. The mechanism responsible for this effect is not yet clear, however, previous work describing the angiogenic effects of GDNF upon HUVEC in a tissue engineered angiogenesis model suggests a role of the tropomyosin-receptor kinase A, TrkB GFRalpha-1 and c-ret receptors signaling pathways [57]. Little is known regarding the role of GDNF in physiological and pathological angiogenesis, therefore determining whether its effect *in vitro* translates *in vivo* is certainly worthy of investigation. From a neurogenesis perspective, GDNF is a key regulatory growth factor functioning to promote the survival and differentiation of cortical neurons. Direct infusion/virally delivered GDNF exerts neuroprotective and neuroregenerative effects of animal models of Parkinson's disease [58,59]. BMSC secreted GDNF has previously been correlated with the survival of cortical rat neurons [60]. Interestingly, in the aforementioned study, the authors report beneficial effects of GDNF in BMSC secretome at low levels (200pg/ml). Moreover, the ED50 of GDNF for dopamine uptake by rat cortical neurons was found to be just 20pg/ml. In these *in vivo* studies, levels of the growth factor above 10ng/ml did not lead to any improvement in therapeutic efficacy. This suggests that only small quantities of GDNF may be required in order to achieve a therapeutic response. With regards growth factor therapy, we do not know whether or not excessive amounts of trophic factors may have negative or even harmful effects in a clinical setting. Considering the apparent therapeutic effects of MSC secretome *in vivo*, we postulate that it is the combination of many angiogenesis and neurogenesis related proteins in small, physiologically relevant concentrations that are important with respect to the trophic actions of these cells upon tissue regeneration. Moreover, the innate homing capacity of BMSCs may facilitate targeted delivery and growth factor release at the injury site.

The effects of BMSC secretomes upon *in vitro* assays of nerve and blood vessel growth in this study was very much patient dependent and, in accordance with other published works, could not be explained by characteristics such as age or sex [61]. We did not find any correlation between the number of BMSCs associated with each conditioned media and the observed effects of the secretome on our *in vitro* assays. This suggests that donor variation in cell secreted proteome between different patients exerted a greater influence overall than that attributed to cell number. As such, here we presented un-normalised data and directly compared each patient secretome to internal controls. (For additional information, data normalized to the cell number of BMSCs that were responsible for producing each respective conditioned media is shown in Supplemental Figure S2.) Whether growth rate or the total number of population doublings that an MSC population undergoes, would correlate with the angiogenic or neurogenic potential of a cell population and/or it's secretome *in vivo* is unclear. Work by Deskins et al., suggests that *in vitro* characteristics such as growth kinetics and cell survival may serve as predictive markers for *in vivo* potency [61]. However, we are a long way away from defining a set of criteria for predicting the therapeutic efficacy of MSCs and/or their secretome *in vivo*. Such criteria will be dependent upon which *in vivo* effects are desired, which will vary according to the tissue aiming to be repaired as well as other aspects of the animal study/ trial.

In this study, we compared cell secretome from different donors on the protein level only. However, it would certainly be of interest to investigate donor difference at the genomic level. It is widely known that the phenotype of primary human MSC cultures varies considerably between donors and is attributed in part to artificial culture conditions that the cells experience *in vitro* [28]. Investigation of the intracellular signalling pathways that contribute to these differences and the identification of gene expression signatures may not only help us to better understand why such donor differences exist, but also help us to develop predictive screening tools that could be used for cell transplantation in a clinic setting.

 Altogether, our data suggests, that autologously transplanted BMSCs may exert a range in therapeutic efficacy between patients. Phenotypic differences between primary BMSC cultures are widely reported in the literature and are thought to be most likely attributed to sampling variation at the time of aspirate harvest. Specifically, bone marrow contains a heterogeneous population of MSCs leading to discrepancies between monolayer cultured cells, even from serial aspirates from the same patient [62]. The wide variation in neurogenic and angiogenic response seen here and in reported clinical trials of BMSC transplantation still suggests that patient responsiveness to treatment with BMSCs constitutes a notable problem. Thus, not all patients may be suitable candidates for BMSC therapy, or may require additional treatment in order to achieve the same clinical outcome as seen by 'responders'.

High throughput and high content analysis technologies have recently emerged as promising tools for both basic and clinical research. The Cell-IQ™ system allowed for accurate analysis of endothelial tube-like formation *in vitro*. Previous work by our group and others has also demonstrated the real time image capture and analysis capabilities of Cell-IQ™ [23,29,63,64].

Manual quantitation of neurite outgrowth was time consuming, taking on average, 3 hours per explant which included image capture, creation of an image montage, and analysis. The time demanding nature of this manual analysis method meant that only a portion of the explant could be feasibly measured, which limited the reliability of the data compared to high content analysis where total neurite outgrowth could be quantified. Use of the Thermo Scientific Cellomics® ArrayScan® VTI HCS Reader allowed fast, accurate and comprehensive quantitation of neurite outgrowth. Moreover, this system is capable of generating data on multiple analysis parameters that may not be possible to acquire manually, in this case, neurite branching. Importantly, when comparing manual vs. automated techniques the same donor trends were evident. The speed and ability to measure multiple parameters not achievable by manual methods of analysis suggest that automated cell analysis systems may be particularly advantageous for use in both fundamental and clinical research settings.

We have shown that automated, high throughput and high content systems offer fast, economical and reliable methods of screening BMSCs and their trophic effects on other cell types. In a clinical context, such tools have great potential for patient selection, identifying prognostic markers and in the evaluation of clinical outcomes achieved by cell-based therapies. Analysis of cell secretome offers the potential for a non-destructive method of phenotypic characterisation and patient screening that could be readily applied to cell-based therapies. Although a great proportion of BMSC therapeutic efficacy is attributed to the cell secretome it would certainly be of interest to further investigate the importance of direct cell-cell interactions. Effective analysis of fibroblast and keratinocyte co-cultures has been achieved previously using Cell-IQ™ [63]. The effect of BMSC secretome on primary human nerves and vascular cell types would also be interesting to investigate, however, would only be truly relevant in the case of donor matched cells, which are not readily available.

**Conclusions**

In this study we have investigated the angiogenic and neurogenic properties of human BMSC secretomes *in vitro*. The trophic stimulatory effects that BMSCs have on blood vessel and nerve growth, supports their use in the cell-based repair of vascularized and innervated tissues. Our work shows that such trophic actions of BMSCs on vascular, neural and indeed other cell types following transplantation is likely to be patient dependent. The use of high content and high throughput screening platforms may help us to identify predictive markers of therapeutic efficacy. Such tools will become increasingly important to the stem cell engineering and biotechnology industries as we strive to develop robust and reproducible stem cell therapies.

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**Figure legends**

Figure 1. DRG neurite outgrowth visualised immunocytochemically using an antibody for neurofilament following application of (A) DRG media, (B) BMSC conditioned media and (C) control media. Fluorescent and phase contrast images of cellular and neurite outgrowth from DRG bodies with immunolocalisation of the Schwann cell marker Po on neural support cells growing out from the DRG body following application of (D,E) control media and (F,G) BMSC conditioned media. (H) Manual quantitation of neurite length, data presented are means ± standard deviations, \*p<0.05, \*\*p<0.01, \*\*\*p<0.0001.

Figure 2. High content analysis of DRG neurite outgrowth. (A,B) DRG immunostained with neurofilament and Hoescht for neurites and cell nuclei respectively, (C,D) corresponding high content analysis tool overlays of neurite length and branch points. Quantitation of (E) total neurite length and (F) neurite branching. Data presented are means ±standard deviations.

Figure 3. Endothelial tube-like formation on Matrigel™ 24hrs after stimulation with (A) HUVEC control media (B) conditioned media control media, (C) BMSC conditioned media. (D) Corresponding manually traced overlay following application of BMSC conditioned media. Manual quantitation of (E) endothelial tube-like length and (F) branching score. (G,H) Representative images of endothelial tube-like formation captured by Cell-IQ™ with corresponding analysis overlay. Quantitation of (I) tube-like length and (J) branching score as assessed by Cell-IQ™. (K) Spearman rank correlations between manual and Cell-IQ™ analysis of endothelial tube-like length and branching score. All scale bars 200µm. Data presented are means ±standard deviations, \*p<0.05 \*\*p<0.01 \*\*\*p<0.001.

Figure 4. (A) Angiogenic and neurogenic factors identified in BMSC secretome by protein array. Data are means ±standard deviations, n=7 BMSC donors. (B) Spearman rank correlations between angiogenesis and neurogenesis-related proteins in BMSC secretome and parameters of endothelial tube-like formation.