TEA-2013-0196-ver9-Bara_1P
Type: research-article

TISSUE ENGINEERING: Part A
Volume 00, Number 00, 2013

DOI: 10.1089/ten.tea.2013.0196

Original Article

Bone Marrow-Derived Mesenchymal Stem Cells Become Antiangiogenic When Chondrogenically or Osteogenically Differentiated: Implications for Bone and Cartilage Tissue Engineering

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AU2▶ Jennifer J. Bara, PhD,[1] Helen E. McCarthy, PhD,[2] Emma Humphrey, PhD,[3] William E.B. Johnson, PhD,[4] and Sally Roberts, PhD[5]

Osteochondral tissue repair requires formation of vascularised bone and avascular cartilage. Mesenchymal stem cells stimulate angiogenesis both *in vitro* and *in vivo* but it is not known if these proangiogenic properties change as a result of chondrogenic or osteogenic differentiation. We investigated the angiogenic/antiangiogenic properties of equine bone marrow-derived mesenchymal stem cells (eBMSCs) before and after differentiation *in vitro*. Conditioned media from chondrogenic and osteogenic cell pellets and undifferentiated cells was applied to endothelial tube formation assays using Matrigel™. Additionally, the cell secretome was analysed using LC-MS/MS mass spectrometry and screened for angiogenesis and neurogenesis-related factors using protein arrays. Endothelial tube-like formation was supported by conditioned media from undifferentiated eBMSCs. Conversely, chondrogenic and osteogenic conditioned media was antiangiogenic as shown by significantly decreased length of endothelial tube-like structures and degree of branching compared to controls. Undifferentiated cells produced higher levels of angiogenesis-related proteins compared to chondrogenic and osteogenic pellets. In summary, eBMSCs produce an array of angiogenesis-related proteins and support angiogenesis *in vitro* via a paracrine mechanism. However, when these cells are differentiated chondrogenically or osteogenically, they produce a soluble factor(s) that inhibits angiogenesis. With respect to osteochondral tissue engineering, this may be beneficial for avascular articular cartilage formation but unfavourable for bone formation where a vascularised tissue is desired.

Introduction

The clinical success of osteochondral tissue engineering relies upon the generation of a functional tissue of physiologically relevant size that is capable of integration at the repair site. Bone has an extensive blood supply that is critical to the tissue's viability and function. In the clinic, disruption or lack of blood supply to bone causes osteonecrosis and is implicated in the pathogenesis of nonunion fractures.[1] Conversely, the presence of blood vessels in articular cartilage is associated with various pathologies, including osteoarthritis[2,3] rheumatoid arthritis,[4] and inferior repair tissue in autologous chondrocyte implantation.[5] One approach to osteochondral tissue engineering is to create a biphasic construct *in vitro* whereby constituent cells have reached a suitable level of chondrogenic and osteogenic differentiation before implantation.[6] However, nutritional limitations posed by diffusion gradients currently limit the size of viable tissues that can be engineered.[7–9] Moreover, the viability and successful integration of such constructs into osteochondral defects relies upon vascular ingrowth after *in vivo* implantation. Therefore, much attention is now focused on tissue engineering vascularised constructs. Current strategies include incorporating vascular cell types,[10,11] or the addition of slow release angiogenic growth factors bound to scaffolds.[12,13] Vascularised bone constructs have also been engineered *in vivo* by endocultivation, whereby a construct is implanted intramuscularly to allow vascular ingrowth before reimplantation into the defect site.[14]

Bone marrow-derived mesenchymal stem cells (BMSCs) represent a potential cell source for osteochondral tissue engineering. In the clinic, implantation of *in vitro* expanded

[1]Musculoskeletal Regeneration Group, AO Research Institute, Platz, Switzerland.
[2]Connective Tissue Biology Research Group, Cardiff School of Biosciences, Cardiff, United Kingdom.
[3]RJAH Orthopaedic Hospital, Keele University Mass Spectrometry Facility, Oswestry, United Kingdom.
[4]Life and Health Sciences, Aston University, Birmingham, United Kingdom.
[5]Spinal Studies & Institute for Science and Technology in Medicine, RJAH Orthopaedic Hospital, Keele University, Oswestry, United Kingdom.

BMSCs has been used to repair long bone[15,16] and cartilage defects.[17–19] However, it is not clear if, or how, *in vitro* differentiated BMSCs may affect vascular ingrowth once reimplanted into osteochondral defects. Undifferentiated BMSCs elicit potent angiogenic effects which appear to be due to a combination of direct cellular interactions and by the release of soluble factors. *In vitro*, BMSCs secrete VEGF, FGF-2, interleukin-6, PIGF, angiopoeitin-1, PDGF, plasminogen activator, MMP-9 and monocyte chemotactic protein-1 (MCP-1).[20,21] BMSC conditioned media stimulates endothelial cell proliferation and migration[20] and tube-like formation.[21] Neutralising antibody experiments have demonstrated that the mitogenic effects of BMSC secretome were not solely due to the presence of one particular growth factor, but a combination of factors.[20] *In vivo*, improved vascular ingrowth and osteogenesis at the fracture site was observed after implantation of gelatin sponges containing MSC conditioned media in rats.[22] Similarly, injection of BMSC conditioned media improved blood flow in a mouse model of hindlimb ischaemia.[20] BMSCs have also been reported to stimulate vascularisation in animal models of myocardial infarction.[23,24] Conversely, there is also evidence to suggest that BMSCs may inhibit angiogenesis. Otsu *et al.* reported that direct cell–cell contact between rat BMSCs and endothelial cells caused apoptosis and destruction of endothelial tube-like structures on Matrigel™. Furthermore, in their mouse melanoma model, BMSCs appeared to abrogate tumour growth by inhibiting tumour angiogenesis.[25]

It is currently unknown whether these predominantly proangiogenic properties of undifferentiated BMSCs are conserved after *in vitro* chondrogenic or osteogenic differentiation and the accompanying change from a 2D to 3D environment. In the present study, equine BMSCs (eBMSCs) were differentiated along chondrogenic and osteogenic lineages in 3D pellet culture as previously described.[26] Angiogenic properties of undifferentiated and differentiated cells were analyzed by collecting serum-free conditioned media and (1) screening it for angiogenesis-related proteins and (2) applying it to an *in vitro* angiogenesis assay, which measures the degree of endothelial tube-like formation on Matrigel. Our results show that conditioned media from undifferentiated eBMSCs supports endothelial tube-like formation *in vitro* and contains an array of angiogenic, antiangiogenic and neurotrophic factors. We present novel data showing that when differentiated along chondrogenic and osteogenic lineages, eBMSCs reduce production of angiogenesis and neurogenesis-related proteins and produce a soluble factor(s) that inhibits endothelial tube-like formation *in vitro.*

Materials and Methods

Cell culture

eBMSCs were isolated from the bone marrow of the third metacarpal of distal forelimbs from four horses (mean age 7 years) as previously described.[26] Four days after isolation, flasks were washed to ensure removal of haematopoietic cells. eBMSCs were incubated at 37°C at 5% CO_2 and received media changes with eBMSC medium (DMEM + GlutaMAX^{TM-1}, 100 μg mL^{-1} gentamicin, 10% foetal calf serum [FCS] [PAA]) three times a week. At each passage, population doublings were calculated using the following formula: LOG10 (final cell number/initial cell number))*3.33. Subsequent differentiation assays and generation of conditioned media were performed using eBMSCs that had undergone approximately 25 population doublings (p2–3).

Monolayer adipogenic and osteogenic differentiation

eBMSCs were seeded into six-well plates at 3×10^3 cells/cm^2 and cultured in eBMSC medium until 80% confluent. For adipogenesis, cells were incubated with adipogenic media (DMEM + GlutaMAX^{TM-1}, 100 μg mL^{-1} gentamicin, 10 μg mL^{-1} insulin [Sigma], 1 μM dexamethasone [Sigma], 100 μM indomethacin [Sigma], 500 μM 3-isobutyl-1-methyl xanthine [Sigma] and 15% normal rabbit serum [Sigma]) for 6 days. Cells were fixed in 10% formalin and treated with 0.5% Oil red O (Sigma) in 60% IPA for 1 h.

For osteogenesis, eBMSCs were incubated for 21 days with DMEM + GlutaMAX^{TM-1}, 0.1 mM L-ascorbic-acid-2-phosphate (Sigma), 100 μg mL^{-1} gentamicin, 10 nM dexamethasone, 10 mM β-glycerophosphate (Sigma), and 10% FCS. Cells were fixed with 10% formalin and treated with the following solution for 1 h in to detect alkaline phosphatase activity: 10% naphthol solution (50 mg mL^{-1} napthol AS-BI phosphate [Sigma] in dimethyl formamide [BDH] pH 8) in 0.2 M Tris–HCl (Sigma) buffer pH 9 + 1 mg mL^{-1} Fast Red TR (Gurr). eBMSCs treated with standard eBMSC media served as controls.

Chondrogenic and osteogenic differentiation in 3D pellet culture

eBMSCs were chondrogenically and osteogenically differentiated in defined media using a previously described pellet culture system.[26] To make cell pellets, eBMSCs, suspended in either chondrogenic or osteogenic differentiation media, were transferred into 1.5 mL Eppendorf tubes (0.5×10^6 cells per Eppendorf) and centrifuged at 500 *g* for 5 min. Chondrogenic differentiation medium consisted of: DMEM + GlutaMAX^{TM-1}, 2% FCS, 100 μg mL^{-1} gentamicin, 10 μg mL^{-1} Insulin-Transferrin-Selenium-X (Gibco), 0.1 mM L-ascorbic-acid-2-phosphate, 10 nM dexamethasone and 10 ng mL^{-1} TGF-β1 (Peprotech). Osteogenic differentiation medium was as described for monolayer differentiation. Pellets were incubated at 37°C and 5% CO_2 for 21 days with media changes three times a week.

Generation of conditioned media

Serum-free conditioned media was generated from undifferentiated cells, chondrogenic and osteogenic pellet cultures of eBMSCs.[27] Undifferentiated eBMSCs were cultured until 70% confluent, washed twice with phosphate-buffered saline (PBS) and incubated with 20 mL of conditioning media (serum free DMEM + GlutaMAX^{TM-1}, 100 μg mL^{-1} gentamicin supplemented with 10 μg mL^{-1} Insulin-Transferrin-Selenium-X and 1% MEM nonessential amino acids [Gibco]) per T75 flask. Conditioning media was incubated with cells for 4 days, filtered through a 0.45 μm filter (Sarstedt) and stored at −20°C. Upon collection of conditioned media, cells were passaged and a cell count performed. For chondrogenic/osteogenic pellets, at day 21, pellets were washed twice in PBS and incubated with 1 mL of conditioning media per pellet. Conditioned media was collected after 4 days and processed as described above. Pellets were snap frozen in liquid nitrogen cooled hexane and stored at −80°C.

Histological and immunohistochemical analysis of pellet cultures

Histology and immunohistochemistry was performed on 7 μm cryosections from a minimum of four pellets per horse (16 chondrogenic & 16 osteogenic). Toluidine blue was applied to cryosections for 1 min, washed and air dried overnight before being mounted in Pertex (Histolab®). For Von Kossa staining, 5% silver nitrate (VWR) in distilled water was applied to cryosections and placed under a UV light until brown colour development (~2 min). Sections were washed with 2% sodium thiosulphate (BDH) in distilled water, dehydrated then mounted with Pertex.

Immunohistochemistry was carried out at room temperature unless otherwise stated. For type II collagen, cryosections were pretreated with 4800 U/mL ovine testicular hyaluronidase (Sigma) and 0.25 U/mL chondroitinase ABC (MP Biomedicals) in Tris acetate buffer pH 8 for 2 h. Sections were fixed in 10% formalin, blocked with 5% goat serum (Vector), then incubated with mouse anticollagen type II (CIIC1, DSHB) (1:10 in PBS) overnight at 4°C. Negative controls were incubated with mouse IgG1a (Dako). The secondary antibody goat anti-mouse Alex Fluor 488 (Invitrogen) (1:200 in PBS) was applied to sections for 1 h. After washing in PBS, sections were mounted with Vectashield mounting medium for fluorescence containing DAPI (Vector) and observed using a Leitz Diaplan light microscope. For type X collagen, cryosections were fixed in a solution of methanol and acetone for 10 min, washed in PBS, then treated with 1 mg/mL protease in PBS (Sigma; P-6911) for 30 min. Sections were blocked with 5% bovine serum albumin (BSA) in PBS for 30 min, then incubated with mouse anticollagen type X antibody (a kind gift from Gary J. Gibson, Henry Ford Hospital, Detroit, MI) (1:300 in PBS containing 1% BSA) overnight at 4°C. An isotype matched mouse immunoglobulin was used as a negative control. Sections were incubated with goat anti-mouse Alexa Fluor 594 (Invitrogen) (1:200 in PBS containing 1% BSA) for 45 min. Slides were washed in PBS then mounted with Vectashield mounting medium for fluorescence containing DAPI (Vector) and observed using Leica TCS SP2 AOBS confocal microscope.

DNA quantification assay

DNA content of undifferentiated, chondrogenically and osteogenically differentiated eBMSCs that generated a standard volume of conditioned media was calculated using the Quanti-iT™ PicoGreen® assay (Invitrogen). Pellets/undifferentiated eBMSCs were digested in 1.25 mg/mL Proteinase K (Sigma) in 100 mM ammonium acetate (Sigma) containing 7% EDTA (BDH) in distilled water, at pH 7 at 60°C overnight. Briefly, 200 μL of pellet/cell digest were diluted in Tris–EDTA buffer and pipetted in triplicate into a 96-well plate. PicoGreen reagent was incubated with samples for 5 min at room temperature. Fluorescence was measured using a fluorescent microplate reader (FLx800 microplate fluorescence reader; Bio-Tek Instruments) at (excitation wavelength 480 nm, emission wavelength 520 nm).

Angiogenesis array

A sample of conditioned media from undifferentiated, chondrogenically, and osteogenically differentiated eBMSCs from each horse was screened for angiogenesis-related proteins using a Human Angiogenesis Proteome Profiler™ antibody array (R&D Systems) according to the manufacturer's instructions. Positive signals were detected by chemiluminescence (UptiLight US WBlot HRP chemiluminescent detection reagent; Interchim) and visualised with ChemiDOc™ EQ (Bio-Rad). Array data was quantified by measuring the sum of the intensities of the pixels within the spot boundary × pixel area with image analysis software (Quantity One® version 4.6.3; Bio-Rad). Array data was normalized to background then DNA content as calculated for each culture from each respective horse.

Endothelial tube-like formation assay

Endothelial tube-like formation assays were performed using Matrigel (growth factor reduced, phenol red-free [BD Biosciences]). Twenty-four-well tissue culture plates were coated with 230 μL Matrigel per well and allowed to solidify at 37°C for 30 min. The human dermal microvascular cell line, HMEC-1, was seeded onto Matrigel (1×10^5 per well) in MCDB 131 medium, 0.05% penicillin and streptomycin (both Gibco) and 10% FCS. After 4 h plates were washed gently thrice before application of either undifferentiated, chondrogenically or osteogenically differentiated eBMSC conditioned media. Conditioning media that had not been in contact with cells was used for controls. Time lapse images were captured over a 24 h period using a video camera set up to an Olympus CK2 microscope. After 24 h, wells were washed and viewed in phase using a Nikon TS100 fluorescent microscope.

Total tube length in five fields of view per well was quantified by drawing over and measuring tube-like structures using image analysis software (IPLab version 3.6; Becton Dickinson) (Fig. 1A). Complexity of tube-like networks was scored using an adapted scoring system by Mcilroy *et al.* (Fig. 1B).[28] Two cells sharing a common branching point were scored as 1 ("a" in Fig. 1B); three cells sharing a common branching point were scored as 2 ("b" in Fig. 1B) and so on. Angiogenic sprouts, classified as an unconnected tube-like structure 25–100 μm in length were scored as 1. Data for total tube length and branching score were normalized to the DNA content calculated for each culture from each respective horse.

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Mass spectrometry

Undifferentiated, chondrogenically, and osteogenically differentiated eBMSC conditioned media from three horses was analysed by LC-MS/MS mass spectrometry. Conditioned media was concentrated 10× using Amicon Ultra-2 centrifugal filter devices (10 kDa molecular weight cut off) according to the manufacturer's instructions (Millipore), digested with trypsin at 37°C then separated by liquid chromatography. MS/MS analysis was performed on fractionated peptides using a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems). The search engine "MASCOT" was used to identify peptides from the NCBI database. Peptides with a total ion score >95% confidence interval or with a peptide count of >2 were excluded. Unamed or hypothetical proteins were identified using an online bioinformatics database (DAVID).

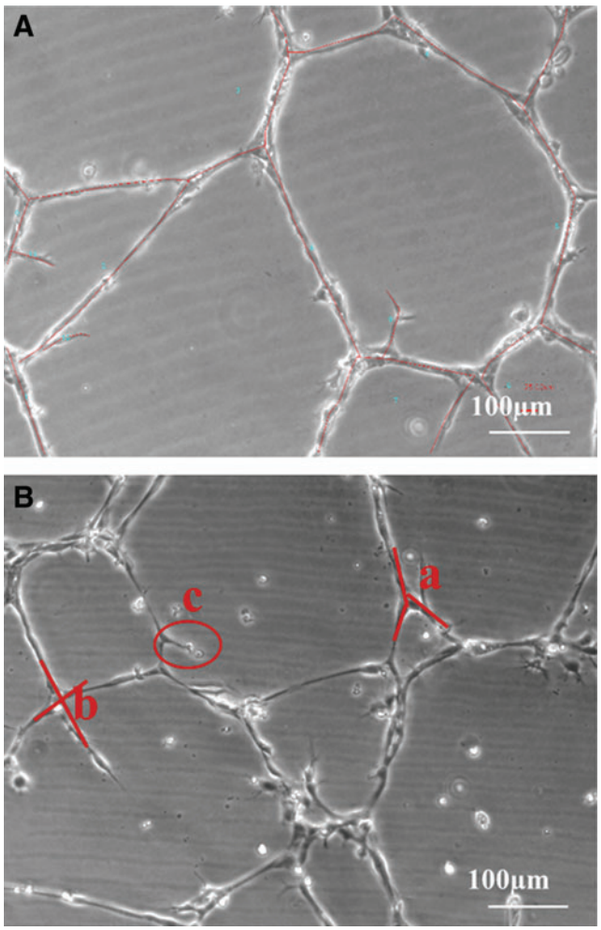


FIG. 1. Analysis of endothelial tube-like formation. **(A)** Lengths of tube-like structures were drawn manually over the image (shown in red). Total tube-like length per field of view was automatically measured using image analysis software (IPLab version 3.6, Becton Dickinson). **(B)** Complexity of tube-like networks scored using an adapted scoring system by Mcilroy *et al.*, which measures cell branch points. (a) Two (b) Three cells sharing a common branching point were scored as 1 and 2, respectively. (c) Angiogenic sprouts, classified as unconnected tube-like structure 25–100 µm in length were scored as 1. Color images available online at www.liebertpub.com/tea

Statistical analysis

With the exception of growth kinetics, data from all horses were pooled before statistical analysis. Differences between treatment groups and controls were tested for statistical significance using Analyse-it®. All data presented a non-normal distribution as determined using the Anderson Darling test for normality and Levene's test for equality of variances. Data from the three treatment groups were tested for significant differences using a Kruskal–Wallis one-way analysis of variance test.

Results

Growth kinetics and mutipotency of eBMSCs

eBMSCs demonstrated a characteristic "MSC" morphology and grew rapidly, undergoing approximately 20 population doublings in the first 12 days of culture (Fig. 2A). ◀F2 eBMSCs from all 4 horses demonstrated adipogenic and osteogenic differentiation in 2D differentiation assays. Adpiogenic differentation of eBMSCs was confirmed by the presence of intracellular lipid droplets as shown by oil red O staining (Fig. 2B, C). Differentiated cells were larger and less bipolar in morphology compared to controls. Osteogenic differentiation of eBMSCs was indicated by the presence of alkaline phosphatase (Fig. 2D, E). Notably, differentiated cells lost their bipolar morphology, became larger and demonstrated a jagged-like border.

eBMSCs chondrogenically and osteogenicially differentiated in pellet culture

Osteogenic pellets stained intensely pink with eosin (Fig. ◀F3 3A), suggesting mineralisation, compared to chondrogenic pellets where staining was paler (Fig. 3B). Osteogenic pellets contained little sulphated glycosaminoglycan (sGAG) (Fig. 3C), whereas chondrogenic pellets demonstrated strong metachromatic staining with toluidine blue indicating a sGAG-rich matrix (Fig. 3D). All osteogenic pellets displayed a mineralised matrix as shown by Von Kossa staining (Fig. 3E). In contrast, none of the chondrogenic pellets stained positively with Von Kossa (Fig. 3F). Immunohistochemistry demonstrated positive labelling of type II collagen in chondrogenic pellet cultures (Fig. 3G–H). Type X collagen was detected in discrete regions of chondrogenic pellets (Fig. 3J), as confirmed by an equine growth plate positive control (Fig. 3K). Neither collagen type II or type X were found immunohistochemically in osteogenic pellets (not shown). Appropriately matched isotype controls for both type II and type X collagen antibodies were negative (Fig. 3I, L, respectively).

Conditioned media from chondrogenically and osteogenically differentiated eBMSCs inhibited endothelial tube-like formation

HMEC-1 formed complex networks of tube-like structures when seeded onto Matrigel (Fig. 4). A time course of images ◀F4 showing tube-like formation may be viewed in Supplementary Figure S1 (Supplementary Data are available online at ◀SF1 www.liebertpub.com/tea). Fewer tube-like networks formed ◀AU5 when HMEC-1 were cultured for 24 h with either chondrogenic (Fig. 4A) or osteogenic (Fig. 4B) conditioned media compared to both controls (conditioned media from undifferentiated cells and control media that had not been in contact with cells). Total tube length (Fig. 4E) and branching score (Fig. 4F) were significantly less when endothelial cells were cultured with chondrogenic or osteogenic conditioned media compared to controls (Fig. 4E, all comparisons: $p < 0.0001$, Kruskal–Wallis).

Chondrogenically and osteogenically differentiated eBMSCs reduced production of both angiogenic and antiangiogenic proteins

Conditioned media from undifferentiated, chondrogenically and osteogenically differentiated eBMSCs was screened for the presence of angiogenic/antiangiogenic factors using an antibody array, which detects the relative levels

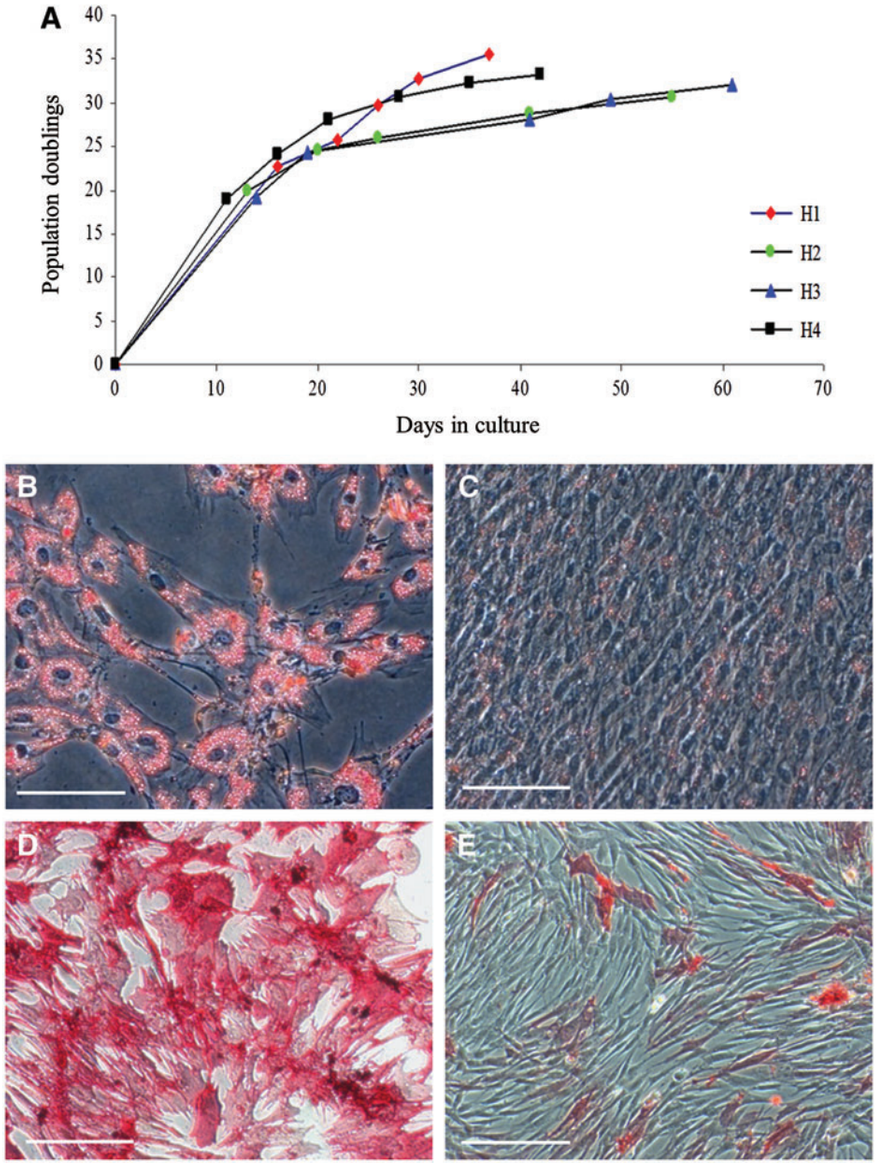
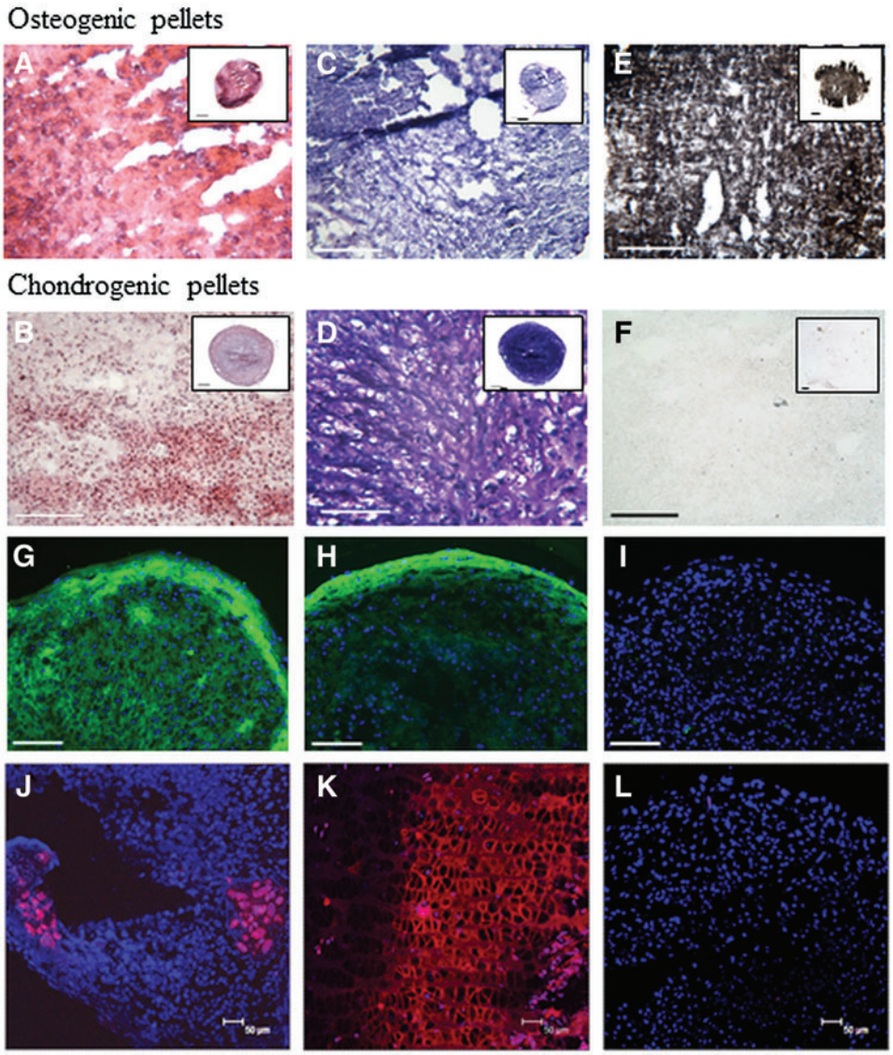


FIG. 2. Multipotent phenotype of equine bone marrow-derived mesenchymal stem cells (eBMSCs). **(A)** Population doublings against time in culture. Each data series represents a cell population isolated from a different horse. Oil red O staining of lipid droplets in **(B)** adipogenically differentiated and **(C)** undifferentiated control eBMSCs. Alkaline phosphatase staining of **(D)** osteogenically differentiated and **(E)** undifferentiated control eBMSCs. Scale bars 100 μm. Color images available online at www.liebertpub.com/tea

of 55 different angiogenesis related proteins. Six angiogenic AU4 ▶ (DPPIV, MMP-9, endothelin-1, PDGF-AA, UPA, and VEGF) and four antiangiogenic proteins (TIMP-1, IGFBP-2, PF4 and angiopoietin-2) were detected at higher levels compared to F5 ▶ all other proteins (Fig. 5A, E, respectively). Undifferentiated eBMSC conditioned media contained significantly higher levels of angiogenesis-related proteins compared to chondrogenic and osteogenic conditioned media, with the exception of DPPIV, VEGF, and PDGF-AA, which were not significantly different between undifferentiated and chondrogenic conditioned media (Fig. 5A–G). Interestingly, chondrogenic conditioned media contained higher levels of angiogenic factors, PDGF-AA ($p=0.0079$), VEGF ($p=0.0497$), FGF-2 ($p=0.0363$), FGF-4 ($p=0.0126$), FGF-7 ($p=0.0225$), MCP-1 ($p=0.0363$) and activin A ($p=0.0230$), and the antiangiogenic factor, IGFBP-3 ($p=0.0188$), compared to osteogenic conditioned media.

Chondrogenically and osteogenically differentiated eBMSCs reduced production of neurotrophic proteins

Undifferentiated eBMSC conditioned media also contained higher levels of neurogenic factors compared to chondrogenic and osteogenic secretome (Fig. 5H). Levels of artemin were significantly higher in undifferentiated compared to both chondrogenic ($p=0.0209$) and osteogenic ($p=0.0079$) conditioned media. Undifferentiated eBMSC secretome contained significantly more persephin than osteogenic conditoned media ($p=0.0044$). Both chondrogenic ($p=0.0126$) and osteogenic ($p=0.0002$) conditoned media



AU7 ▶ **FIG. 3.** Haematoxylin and eosin staining of **(A)** osteogenic and **(B)** chondrogenic pellets. Metachromasia of **(C)** osteogenic and **(D)** chondrogenic pellets using toluidine blue indicates a sulphated glycosaminoglycan rich matrix in chondrogenic pellets. **(E)** Osteogenic pellets stained strongly with Von Kossa indicating a calcium rich mineralised matrix, whereas chondrogenic pellets appeared unmineralised **(F)**. **(G–L)** Immunohistochemical analysis of type II and X collagen in chondrogenic pellet cultures. Chondrogenic pellets labelled positively but variably for type II **(G–H)** and type X **(J)** collagen. Equine growth plate type X collagen positive control **(K)**. IgG and IgM controls were negative for type II **(I)** and X **(L)** collagen, respectively. Cell nuclei counterstained blue with DAPI, scale bars are 100 μm unless stated otherwise. Color images available online at www.liebertpub.com/tea

contained less glial cell-derived neurotrophic factor (GDNF) compared to undifferentiated conditioned media, with chondrogenic conditioned media containing higher levels of the neurotrophin compared to osteogenic secretome ($p = 0.0363$).

Proteomic analysis of eBMSC conditioned media

After applying a stringent set of exclusion criteria, almost all peptides detected in eBMSC conditioned media by mass spectrometry were identified as ECM proteins. Type I collagen peptides were identified in all samples (Table 1). Chondrogenic conditioned media contained multiple fibronectin peptides. Fibronectin was also detected in the secretome of undifferentiated and osteogenically differentiated eBMSCs (two of the three BMSC populations tested). Peptides of cartilage-associated matrix proteins biglycan and decorin were identified in chondrogenic conditioned media. Similary, type II collagen and lumican were identified in two out of the three samples of chondrogenic secretome. The presence of these peptides are indicative of a cartilaginous ECM; thus, further confirming the chondrogenic phenotype of differentiated eBMSCs. Peptides of the antiangiogenic molecule thrombospondin were detected in two out of three samples of chondrogenic and in one sample of both undifferentiated and osteogenic conditioned media. Bone sialoprotein-2 was also present in the chondrogenic conditioned media from two out of the three BMSC populations tested and at 94.6% confidence interval in the third sample.

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Discussion

In the present study, we show for the first time, that when either chondrogenically or osteogenically differentiated in a 3D culture system, eBMSCs reduce production of angiogenesis and neurogenesis-related proteins and inhibit angiogenesis *in vitro*. With respect to osteochondral tissue engineering, this supports the use of BMSCs for avascular cartilage formation; however, maybe of concern for the generation of vascularised bone.

Initially, eBMSCs proliferated rapidly in culture, which is concominant with other studies examining the growth kinetics of eBMSCs *in vitro*.[26,29] We have previously shown that these cells express the putative stem cell markers CD90, CD166 and STRO-1.[26] eBMSCs demonstrated multipotency

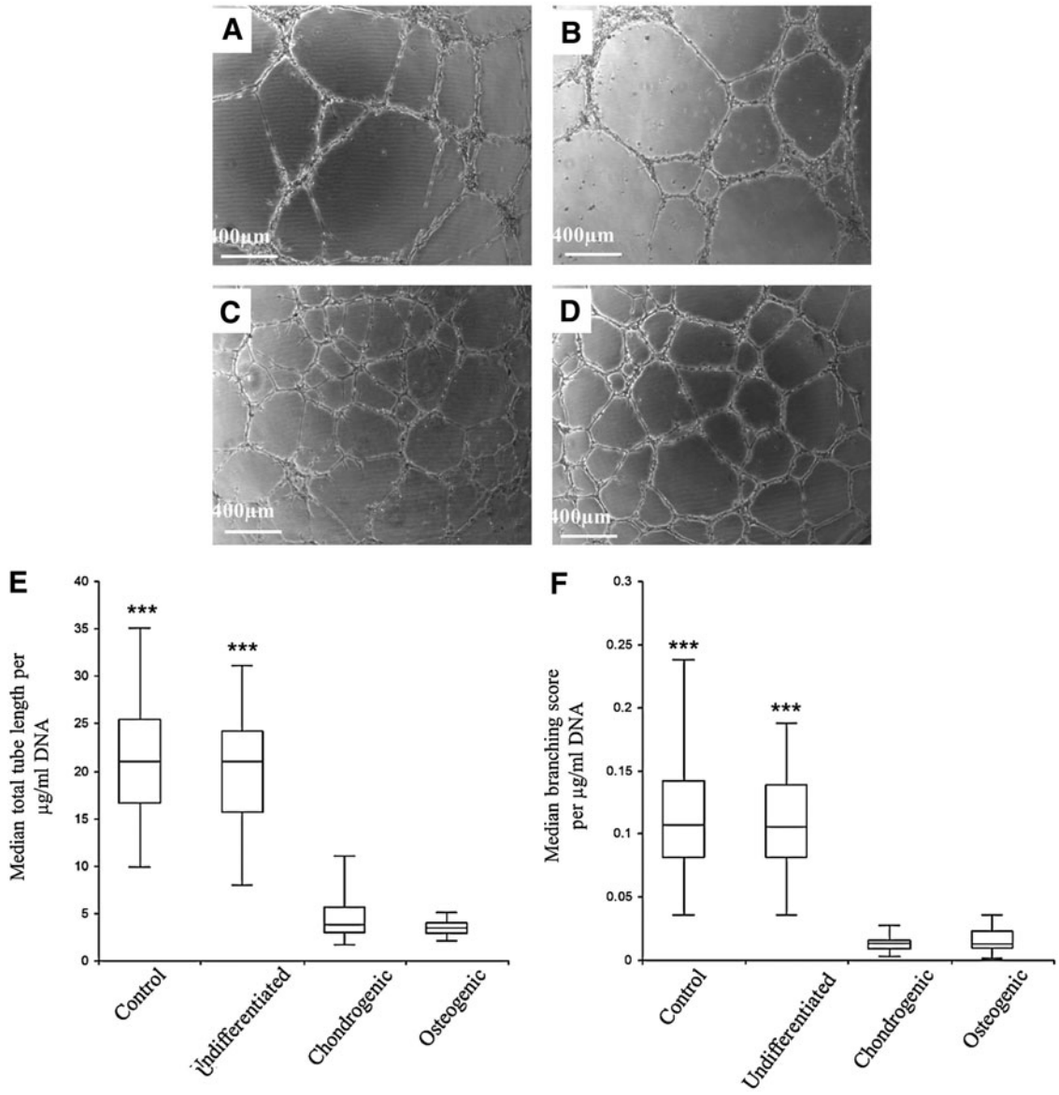

FIG. 4. Matrigel assays 24 h after treatment with eBMSC conditioned media. Representative images showing endothelial tube-like formation after treatment with **(A)** chondrogenic, **(B)** osteogenic, **(C)** undifferentiated, and **(D)** control conditioned media. **(E)** Tube-like length and **(F)** branching score were significantly greater when endothelial cells were cultured with control media or undifferentiated eBMSC conditioned media compared to both chondrogenic and osteogenic conditioned media. Data shown are medians, error bars represent minimal and maximal data points (***$p < 0.0001$, Kruskal–Wallis, $n = 12$).

as shown by their ability to differentiate along adipogenic, osteogenic and chondrogenic lineages. In addition to standard differentiation in monolayer, osteogenic differentiation was performed in a 3D pellet culture system as previously described.[30] Mineralization of osteogenic pellets shown by Von Kossa and haematoxylin and eosin suggest that eBMSCs differentiated osteogenically. Previous 3D osteogenic differentiation of these cells has also identified the presence of osteocalcin immunohistochemically.[26]

Chondrogenic differentiation was demonstrated by the presence of sGAG and type II collagen; however, discrete regions of type X collagen immunostaining indicate areas of hypertrophy. This, together with the identification of bone sialoprotein in chondrogenic conditioned media, suggest differentiation of eBMSCs toward a hypertrophic phenotype. Hypertrophy of chondrogenically differentiated BMSCs in pellet culture is widely reported in the literature.[26,31–34] In endochondral ossification, hypertrophic chondrocytes are proangiogenic, producing VEGF, which leads to vascular invasion and ossification of the cartilage anlagen.[35] Although we observed a degree of hypertrophy, chondrogenic eBMSC secretome was antiangiogenic. Our findings highlight important differences between the phenotypes of nascent hypertrophic chondrocytes compared to MSC that have been manipulated to differentiate chondrogenically *in vitro*.

Conditioned media from undifferentiated eBMSCs supported the formation of endothelial tube-like structures, although this was comparable to controls. This suggests angiogenic factors detected in undifferentiated eBMSC conditioned media did not further enhance tube-like formation.

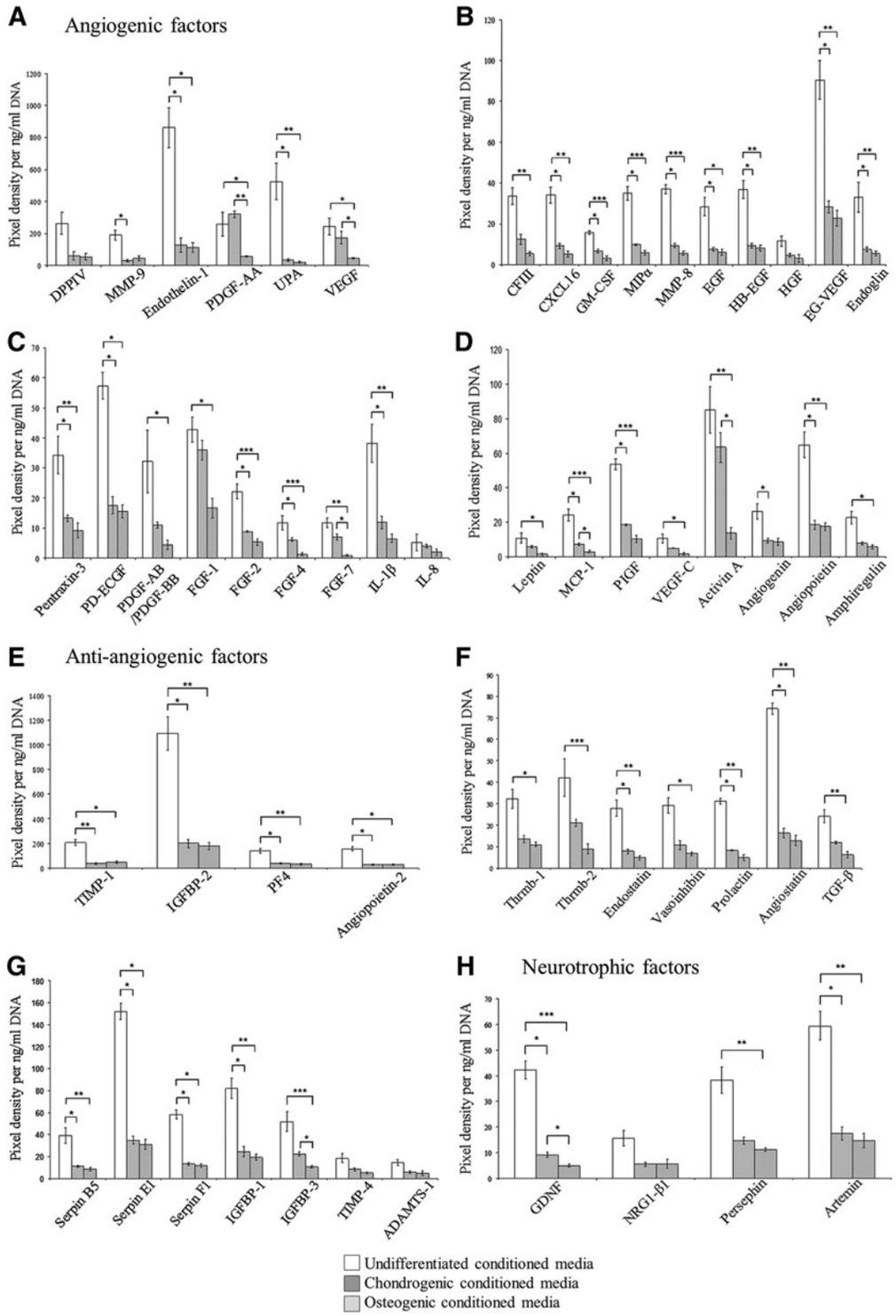

FIG. 5. Angiogenic, antiangiogenic, and neurotrophic factors identified in eBMSC conditioned media, normalised to DNA content. Undifferentiated eBMSC secretome generally contained higher levels of angiogenic, antiangiogenic, and neurotrophic factors compared to both chondrogenic and osteogenic conditioned media. **(A)** Six angiogenic (Dipeptidyl Peptidase IV [DPPIV], MMP-9, endothelin-1, PDGF-AA, UPA, and VEGF) and **(E)** four antiangiogenic (TIMP-1, insulin-like growth factor binding protein-2 [IGFBP-2], platelet factor-4 [PF4], angiopoietin-2) were detected at high levels compared to all other proteins. Conditioned media from chondrogenically differentiated eBMSCs contained higher levels of angiogenic factors PDGF-AA ($p=0.0079$), VEGF ($p=0.0497$), FGF-2 ($p=0.0363$), FGF-4 ($p=0.0126$), FGF-7 ($p=0.0225$), monocyte chemotactic protein-1 (MCP-1) ($p=0.0363$), activin A ($p=0.0230$), the antiangiogenic factor IGFBP-3 ($p=0.0188$) and glial derived neurotrophic factor (GDNF) ($p=0.0363$) compared to osteogenic conditioned media. Data shown are means ± SEM (*$p<0.05$, **$p<0.01$, Kruskal–Wallis, conditioned media from four horses [$n=4$]). AU3 AU8 AU3

Table 1. Summary of Proteins Identified from Peptides Detected in Equine Bone Marrow-Derived Stem Cell Conditioned Media by LC-MS/MS Mass Spectrometry

Peptides detected in eBMSC conditioned media by LC-MS/MS mass spectrometry				
		Peptide count		
Peptide	*Accession number*	*Horse 1*	*Horse 2*	*Horse 3*
Undifferentiated				
	gi|50978774	28	22	17
	gi|22328092	27	21	17
Collagen type I (multiple)	gi|27806257	14	10	20
	gi|50978940	17	10	17
	gi|32451581	10	8	15
	gi|111120329	8	7	10
	gi|2894106	14	11	—
	gi|16758080	7	—	13
	gi|470674	19	—	10
Collagen type V	gi|32822777	4	—	17
Procollagen alpha 2 (V)	gi|2370202	5	—	15
Type V preprocollagen alpha 2 chain	gi|16197600	5	—	17
Fibronectin	gi|31874109	—	7	5
Thrombospondin-1	gi|37138	—	2	—
Chondrogenically differentiated				
	gi|50978774	20	11	14
	gi|22328092	19	11	15
	gi|27806257	7	2	7
Collagen type I (multiple)	gi|50978940	9	—	6
	gi|16758080	4	—	6
	gi|32451581	5	—	9
	gi|2894106	—	5	9
	gi|179631	3	—	3
	gi|2497972	5	4	6
Fibronectin (multiple)	gi|1675365	2	3	3
	gi|31874109	8	7	—
Bone sialoprotein 2	gi|160358833	2	2	1 *(94.%CI)*
Biglycan	gi|20137008	5	—	7
Collagen type II	gi|30410850	5	—	7
Clusterin	gi|126352584	—	3	3
Decorin (multiple)	gi|126352546	—	4	6
	gi|160333372	—	2	2
Lumican (multiple)	gi|57097203	—	4	5
	gi|21542114	—	3	3
Thrombospondin-1 (multiple)	gi|37138	—	2	—
	gi|899229	—	—	2
Osteogenically differentiated				
	gi|50978774	17	7	18
	gi|27806257	6	7	11
	gi|50978940	8	5	12
	gi|111120329	3	—	5
Collagen type I (multiple)	gi|2894106	—	7	11
	gi|22328092	—	7	18
	gi|470674	12	7	13
Clusterin	gi|126352584	4	—	4
Collagen type III	gi|56711254	2	—	4
Fibronectin (multiple)	gi|31874109	3	—	3
	gi|1675365	1	—	0
	gi|2497972	3	—	0
Thrombospondin-1	gi|37465	1	—	—

eBMSCs, equine bone marrow-derived mesenchymal stem cell; —, peptide not detected.

This is contrary to a study by Hung *et al.*, who reported stimulation of endothelial tube formation by human BMSC secretome.[21] Hung *et al.* used a different cell line and method for the generation of conditioned media and performed the Matrigel assay under hypoxic conditions, which could account for such discrepancies. Our data show significant inhibition of endothelial tube-like formation when HMEC-1 were cultured with conditioned media from chondrogenically or osteogenically differentiated eBMSCs. eBMSCs produced lower levels of antiangiogenic factors following

differentiation; therefore, this antiangiogenic activity cannot be accounted for by the presence of these inhibitory molecules. Therefore, we propose, that differentiated cells produced one or more soluble factor(s) not present on our protein-antibody arrays that inhibited endothelial cells by either an angiogenic specific or nonspecific mechanism. It is possible that antiangiogenic proteins previously reported in cartilaginous tissues, such as chondromodulin[36,37] may have been responsible. Endothelial tube formation assays using Matrigel are highly sensitive, reproducible assays, used to model angiogenesis *in vitro* (reviewed by Staton *et al.*).[38] We used growth factor-reduced Matrigel, to avoid any interference from endogenous factors. However, being composed of murine tumor eCM proteins, Matrigel does not precisely model the vascular microenvironment associated with osteochondral tissues. Species difference is also a noteworthy consideration of our study (equine-human). Whether the observed antiangiogenic effects of eBMSCs would be recapitulated by human BMSCs *in vivo* is unclear and requires further investigation.

With the aim of identifying this soluble antiangiogenic factor(s), conditioned media was analysed by mass spectrometry. The only known antiangiogenic peptide identified was thrombospondin in two samples of chondrogenic and in one sample of osteogenic and undifferentiated conditioned media, respectively. However, thrombospondin was detected by protein-antibody array at higher levels in undifferentiated compared to differentiated eBMSC secretome, making it unlikely to be responsible for the inhibition of endothelial tube-like formation. Mass spectrometry did not identify any other angiogenesis-related proteins, which is likely due to masking by more abundant ECM proteins, such as the collagens.

Angiogenic factors have been previously identified in undifferentiated BMSC conditioned media.[20,21] The results presented here demonstrate, for the first time, reduction of angiogenesis-related proteins when BMSCs are differentiated in a 3D culture system toward chondrogenic and osteogenic lineages. This finding does not appear to have been reported previously in the literature with any species of BMSC. It could be argued that the change in culture environment rather than the differentiation status of the cells reduced secreted levels of angiogenesis-related proteins. However, this is not supported by a previous study, which reported an increase in production of angiogenic factors by BMSCs upon transition from a 2D to 3D culture system.[39] Moreover, our data reveal significant differences in levels of angiogenesis-related proteins between chondrogenic and osteogenic cultures. Thus, it is clear that the state of cellular differentiation affects the angiogenesis-related protein secretome profile of eBMSCs. The effect of 2D versus 3D culture could be further investigated by culturing eBMSC pellets in standard media as a control. However, this is complicated by the fact that BMSCs may spontaneously differentiate chondrogenically in pellet culture in the absence of chondrogenic growth factors, such as TGF-β.[40,41]

Angiogenesis and osteogenesis are coupled *in vivo,* which is reflected by cross-talk between vascular and osteogenic cell types *in vitro* (reviewed by Grellier *et al.*).[42] Osteoblasts produce VEGF,[43] an important angiogenic growth factor, which may also influence osteogenesis as it can function to recruit osteoprogenitor cells and stimulate their differentiation into osteoblasts.[44,45] Recently, endothelial cells have been shown to promote osteogenic differentiation of BMSCs in a 3D spheroid coculture model.[46] Thus, cellular communication between bone/cartilage forming and vascular cells may not only influence the vascularisation of osteochondral tissues but may also affect repair tissue composition. Considering the above, we were surprised to observe antiangiogenic properties of osteogenically differentiated eBMSCs. However, our findings are supported by a recent study in which BMSC secretome reduced endothelial tube-like formation and angiogeneic factor production after osteogenic differentiation in monolayer.[47] Importantly, we and the aforementioned author examined the angiogenic properties of *in vitro* differentiated BMSCs at one stage of cellular differentiation. It is possible that the angiogenic properties of differentiating BMSCs change in a temporal manner as the cells become more committed.

Our findings have important implications for the development of vascularised osteochondral constructs, utilizing the regenerative capacity of BMSCs. Antiangiogenic paracrine activity of chondrogenically and osteogenically differentiated BMSCs may prevent subchondral vascular ingrowth upon *in vivo* transplantation into the defect site. This would reduce viability, function, and integration of the osseous portion of the construct and potentially compromise the nutritional supply to the overlying articular cartilage. There are several strategies that may negate this problem by promoting vascularisation: (1) refine/manipulate osteogenic differentiation of BMSCs to promote their angiogenic capacity (2) prevascularise the osseous portion of the construct by either incorporating vascular cells[10,11] or by intramuscular endocultivation techniques[14,48] or (3) angiogenic priming of the osseous portion of the construct by slow release angiogenic factor delivery systems.[12,13,49]

Undifferentiated human BMSCs elicit neurogenic effects, which are attributed, in part, to production of neurotrophins.[27,50–52] Here eBMSCs produced neurogenic proteins, GDNF, NRG1-β1, persephin and artemin, when chondrogenically/osteogenically differentiated. The fact that chondrogenic and osteogenic differentiation resulted in reduced angiogenic and neurogenic properties of eBMSCs is noteworthy, since during development angiogenesis and neurogenesis often occur simultaneously and are regulated by similar molecular mechanisms.[53,54] For future work it would be interesting to investigate the effect of conditioned media from chondrogenically and osteogenically differentiated BMSCs on *in vitro* models of nerve growth and to examine potential interplay between soluble angiogenesis and neurogenesis-related factors.

In conclusion, when either chondrogenically or osteogenically differentiated, eBMSCs exert antiangiogenic effects by paracrine activity. With respect to *in vitro* osteochondral tissue engineering this would be beneficial for maintaining an avascular articular cartilage but undesirable for bone formation. Future work in this field is required to differentially regulate vascularisation in tissue engineered bone and cartilage to improve osteochondral tissue engineering therapies for clinical use.

Acknowledgments

The monoclonal antibody, CIIC1, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The

University of Iowa, Department of Biology, Iowa City, IA 52242. The authors would like to thank the European Union (MyJoint Project FP-6 NEST 028861), Keele University and the Institute of Orthopaedics, RJAH Orthopaedic Hospital for funding.

Disclosure Statement

No competing financial interests exist.

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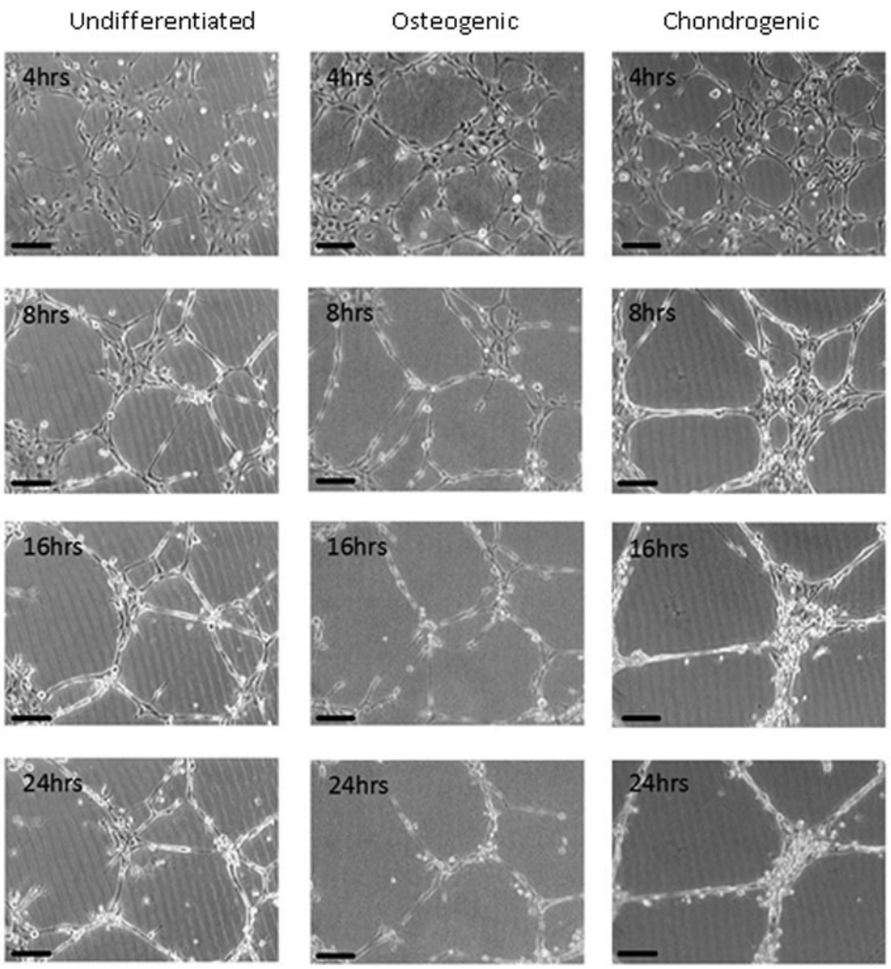
Address correspondence to:
Jennifer J. Bara, PhD
Musculoskeletal Regeneration Group
AO Research Institute
Clavadelerstrasse 8
Davos 7270
Switzerland

E-mail: jennifer.bara@aofoundation.org

Received: March 27, 2013
Accepted: July 17, 2013
Online Publication Date:

AU6 ►

Supplementary Data



SUPPLEMENTARY FIG. S1. Time lapse images of Matrigel assays taken over a 24 h period during treatment with equine bone marrow-derived mesenchymal stem cells conditioned media. Representative images showing endothelial tube-like formation after application of undifferentiated, osteogenic, and chondrogenic conditioned media. Scale bars 100 μm.

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