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Characterization and multilineage potential of cells derived from isolated microvascular fragments

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ABSTRACT

Background: A number of therapies are being developed that use microvessels isolated from adipose tissue (microvascular fragments [MVFs]) to improve tissue perfusion and implant survival. Because it has been demonstrated that stem cells are associated with micro vessels, the purpose of these studies was to gain further insight into the stem cells asso ciated with MVFs to better understand their therapeutic potential.

Materials and methods: Cells derived from MVF explants were compared with adipose derived stem cells (ASCs) based on the expression of cell surface proteins for mesenchymal stem cells and their capacity for angiogenic, neurogenic, adipogenic, and osteogenic differentiation.

Results: The expression of cell surface proteins for mesenchymal stem cell markers was similar between MVF derived cells and ASCs; however, the increase in markers consistent with endothelial cells and pericytes was accompanied by an improved ability to form capillary like networks when cultured on matrigel. MVF derived cells had increased neu regulin, leptin, and osteopontin expression compared with ASCs when exposed to neurogenic, adipogenic, and osteogenic induction media, respectively.

Conclusions: The stem cell functionality of cells derived from MVFs is retained after their isolation. This helps to explain the ability of MVFs to improve tissue perfusion and has implications for the use of MVFs as a means to deliver stem cells within their niche.

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1. Introduction

Tissue engineering strategies for tissue and organ replace ment will lead to significant improvements in surgical out comes for a large number of diseases. A limitation to the success of tissue engineered biomaterials is the presence of a vascular supply capable of sustaining perfusion and main taining implant viability [1,2]. To address this void, a number of prevascularization strategies have been developed that use a combination of cells derived from various tissues (e.g., fi broblasts, mesenchymal stem cells [MSCs], human umbilical vein endothelial cells) for vascular development *in vitro* before their implantation *in vivo* [1,3]. An alternative approach has been the delivery of intact microvessels (arterioles, venules, and capillaries) isolated from adipose tissue, hereafter referred to as microvascular fragments (MVFs), which

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effectively circumvents the need for *in vitro* vessel development [4–7]. MVFs improve perfusion for cardiac and skin tissues and have also been demonstrated to be an effective prevascularization strategy to improve viability of orthopedic and islet implants [4–8]. The observation that freshly isolated MVFs can be used lends to the clinical relevance of their application [6]. Despite the relationship between the therapeutic usefulness of MVFs and the wealth of data ascribing a vascular location to stem cells [9,10], a thorough characterization of the stem cells associated with MVFs is lacking.

The robust angiogenic potential of MVFs even in the absence of supplemental proangiogenic factors (e.g., vascular endothelial growth factor) supports the idea that potent stem cells reside within the vessels that contribute to their vigorous network formation *in vitro* and dynamic remodeling *in vivo* [4–6,11]. Nunes *et al.* [11] suggested that the inherent angiogenic capacity of MVFs may be due, at least in part, to regenerative cells residing within them, a concept supported by a number of studies where MSCs have been shown to reside within the vascular wall [12] or in a perivascular location [9]. The abundant microvasculature within adipose tissue, and accordingly, abundant supply of stem cells that can be derived from adipose tissue are in direct agreement with these concepts. Although adipose derived stem cells (ASCs) and the freshly isolated stromal vascular fraction have been thoroughly studied, given the differences in the methodologies to procure them, it is not prudent to rely solely on their characterizations to make conclusions regarding the stem cell identity of MVFs.

Given the growing body of literature supporting the idea that the microvasculature is a source of stem cells and the interest in improving vascularization with MVFs, in the present study cells derived from MVFs were characterized using an explant culture method. Comparison with ASCs revealed that the MVF derived cells (MVF DC) are heterogeneous and in some regards exhibit evidence of a greater regenerative potential. More importantly, the findings herein support the idea that the isolation of MVFs does not negatively impact resident vascular cells and stem cells. This lends to the possibility that the application of MVFs not only has the advantage of supplying intact microvessels to support tissue perfusion but also supply cellular factors critical for tissue regeneration.

2. Materials and methods

This study has been conducted in compliance with the Animal Welfare Act and the Implementing Animal Welfare Regulations and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals and was conducted in the animal facility at the US Army Institute of Surgical Research. Rats were housed individually in a temperature controlled environment with a 12 h light–dark cycle.

2.1. MVF-DC and ASC isolation

MVFs were isolated from the epididymal fat pads of wild type male Lewis rats (350–400 g) as previously described [4]. Briefly, adipose tissue from the epididymal fat pads of rats were subjected to a limited collagenase (Worthington Biochemical Corporation, Lakewood, NJ) digestion (~8 min) at 37° with agitation, washed, and filtered through 500 and 30 μm filters to remove large debris and minimize cell contamination, respectively (Fig. 1). MVFs were plated on tissue culture–treated plastic dishes similar to that described for the isolation of pericytes from human placental tissue [13] in growth media consisting of Dulbecco Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS) and 100 U/mL each of penicillin and streptomycin (Life Technologies, Grand Island, NY). The cells that emanated from the MVFs, referred to as MVF derived cells (MVF DCs), were subjected to characterization as described in the following. For ASC isolation, adipose tissue was digested with collagenase for 45 min at 37° with agitation followed by filtration through 100, 70, and 40 μm mesh filters to remove debris. Remaining filtrate was washed and centrifuged and pelleted cells seeded on tissue culture–treated plastic dishes in growth media [14].

2.2. Cell growth curve

Cell growth curves of MVF DC and ASC cells were determined by seeding 2.5×10^4 cells per well (n 4 wells per group per time point) in 12 well tissue culture plates. Cells were allowed to attach overnight, and then cells were trypsinized to remove all adherent cells and counted using a hemocytometer for 8 d.

2.3. Multilineage differentiation potential

ASCs (passage 1) and cells derived from MVFs (passage 1) were grown to subconfluency and their ability to differentiate toward various lineages analyzed as described in the following.

2.3.1. Adipogenesis

Adipogenic differentiation of cells was induced by replacing growth media with preinduction adipogenic media consisting of DMEM, 10% FBS, 1% antibiotics, 0.5 mM isobutylmethylxanthine, 200 μM indomethacin, 0.1 μM dexamethasone, and 1 μM insulin (Sigma–Aldrich, St. Louis, MO) for 24 h followed by 2 wk of culture in adipogenic media (same as preinduction media minus the isobutylmethylxanthine). Cells were either harvested for RNA as described in the following or fixed with 4% paraformaldehyde for 20 min followed by staining with Oil Red O for 1 h at room temperature (RT). Excess stain was removed by extensive washes with phosphate buffered saline (PBS) and cells imaged with an Olympus (Center Valley, PA) IX 71 inverted microscope.

2.3.2. Osteogenesis

Osteogenic differentiation of cells was achieved by replacing growth media with osteogenic media composed of DMEM, 10% FBS, 1% antibiotics, 10 mM β glycerophosphate, 10 nM dexamethasone, and 150 μM ascorbic acid 2 phosphate (Sigma–Aldrich) for 3 wk. Cells were either harvested for RNA as described in the following or fixed with 4% paraformaldehyde for 20 min followed by staining with Alizarin Red S (40 mM, pH 4.1, 20 min followed by extensive washes with dH_2O) to examine mineralization activity. Images were collected as described previously.

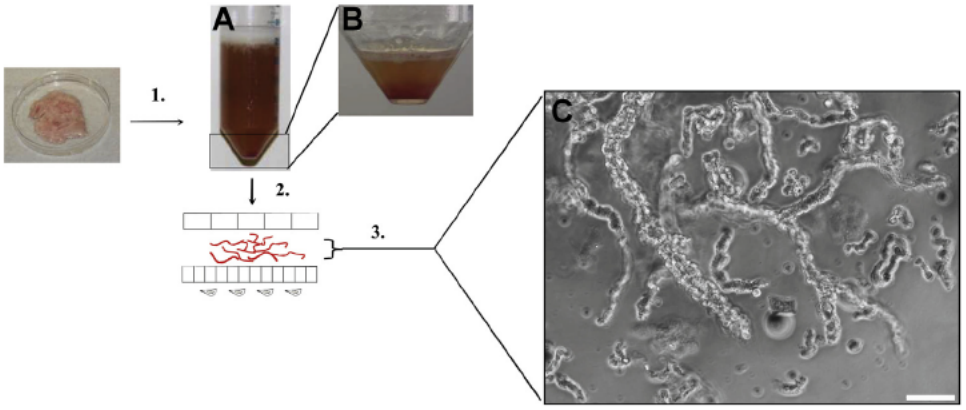

Fig. 1 – Schematic depicting the MVF isolation procedure used in the current and previous studies [4,5]. (1) Epididymal fat is minced, digested in collagenase for ~8 min, and centrifuged (400*g* × 4 min), which results in a floating layer of adipocytes and a pellet containing a heterogenous mixture of cells and MVFs (A). (B) Magnified view of pellet after aspiration of supernatant. (2) The pellet is suspended in PBS containing 0.1% bovine serum albumin and filtered through 500- and 30-μm filters to remove large debris and minimize cell contamination, respectively. (3) MVFs are washed off the 30-μm filter and suspended in growth medium for culture. (C) An aliquot of MVFs after washing was pipetted on tissue culture plastic. Scale bar = 100 μM. (Color version of figure is available online.)

2.3.3. *Neurogenesis*

Neurogenic differentiation of cells was induced by replacing growth media with preinduction neurogenic media composed of DMEM, 20% FBS, 1% antibiotics, and 1 mM β mercaptoe thanol (Sigma–Aldrich) for 24 h followed by neurogenic media (DMEM, 1% antiobiotics, and 5 mM β mercaptoethanol) for an additional 24 and 48 h. Cells were either harvested for RNA as described in the following or fixed with 4% paraformaldehyde for 20 min followed by Nissl staining (0.5% Cresyl Violet for 30 min followed by PBS wash three times) and imaged as described previously.

2.4. *Immunocytochemistry*

Cells grown in monolayers were fixed with 4% para formaldehyde for 20 min at RT. After washing in PBS, cells were permeabilized in 0.2% Triton X 100 for 20 min at RT, blocked with 10% normal serum (from animal in which sec ondary antibody was generated) for 30 min at RT and either primary antibody or nonspecific IgG applied overnight at 4°C. The antibodies used were anti CD31 (1:100; Millipore, Temecula, CA), anti CD44 (1:200; Cell Signaling, Danvers, MA), anti CD45 (1:200; Millipore), anti CD73 (1:100), anti CD90 (1:100; BD Biosciences, San Jose, CA), anti CD144 (1:200; Abbiotec, San Diego, CA), and anti NG2 (1:200; Millipore). The following day, cells were washed with PBS and incubated with the appropriate AF488 conjugated secondary antibodies (1:500; Life Technologies) for 2 h at RT. After washing cells with PBS, the nuclei were stained with 4′,6 diamidino 2 phenylindole (Life Technologies) and visualized by fluores cent microscopy (Olympus IX 71). Additionally, cell mono layers were incubated with fluorescein labeled (495 nm/ 515 nm) GS Lectin I (1:20; Vector Laboratories, Burlingame, CA) for 30 min, washed with PBS, 4′,6 diamidino 2 phenylindole stained and visualized as described previously.

2.5. *RNA isolation, complementary DNA synthesis, and quantitative real-time polymerase chain reaction*

Total RNA was isolated from ASC and MVF DC cells (Qiagen, Valencia, CA), quantified using spectrophotometry and 1 mg of total RNA used for reverse transcription reaction (Taqman;

Table 1 – Stem cell marker primer sequences used for qPCR.

Gene	Sense primer (5′→3′)	Antisense primer (5′→3′)
18S	AGACCTGGAGCGACTGAAGA	AGAAGTGACGCAGCCCTCTA
CD29	GAAGGTGGCTTTGATGCAAT	AGCAAAGTGAAACCCAGCAT
CD44	AGCACAACAGAAGAAGCAGCTA	ACATCCTCTTGACTCTGTGTTGTC
CD45	TATGTTATTGGGAGGGTGCAA	CAGGGCCATTAATTTCATAAGG
CD73	TTGCAGCCTGAAGTGGATAA	GTACTTCCCAGCAGGCACTT
CD90	CGGAGCTATTGGCACCATGA	AACTCATGCTGGATGGGCAA
CD105	TCCCTCTGACCAGTGATGTCT	TGACGTCATTGCCACACTTT
CD117	TAACGATTCCGGAGTGTTCAT	CCTCGAACTCAACAACCAAGT
Sca 1	ACTGTGGAGAGGATCGAGGA	GAGTTTGGAACACGGCAGAT

Table 2 – Differentiation marker primer sequences used for qPCR.

Gene	Lineage	Sense primer (5′→3′)	Antisense primer (5′→3′)
Leptin	Adipogenic	CAAAGTCCAGGATGACACCA	ATGAAGTCCAAACCGGTGAC
Glut4	Adipogenic	GCTTCTGTTGCCCTTCTGTC	GCCAGTGCATCAGACACATC
SPARC	Osteogenic	TGTGCAGCAATGACAACAAG	AATCGGTCAGCTCAGAATCC
OPN	Osteogenic	TCCTTCACTGCCAGCACA	AGGTCCTCATCATCTGTGGCATC
ALPL	Osteogenic	CGCCTATCAGCTAATGCACA	AGCTCTTCCAAATGCTGATGA
Runx2	Osteogenic	CTGAACTCAGCACCAAGTCCT	GTGGTGGAATGGATGGATG
NRCAM	Neurogenic	TCCTGTCAACCGGACGTT	GAGGTTGTGAGGTGCAACAA
Neuregulin	Neurogenic	TCCACCAGTCATTACACTTCCA	ACTGCTGTGCCTGCTGTTC
CD31	Angiogenic	ACCTCCAAGCAAAGCAAAGA	GACGGCTGGAGGAGAGTTC
CD144	Angiogenic	TCAGAACCGGATGACCAAAT	CGATGTGGAACGTGTACTGC
CD146	Angiogenic	AAGATCAGGTGTCTGACTGACG	CAAGTCTAGGCTCTGACATTGGT
NG2	Angiogenic	CTGATCCGATACGTGCATGA	GGAGGGATGGGCACAATAG
eNOS	Angiogenic	AGAGCATACCCGCACTTCTG	AGCAGCCTTGGCATCTTCT

Life Technologies) following manufacturer's recommendations. To determine relative expression of stem cell markers, primers were designed to span introns ensuring genomic DNA was not amplified (Table 1). Quantitative polymerase chain reaction (qPCR) of transcripts and the endogenous control, 18S ribosomal RNA, was performed for each template using IQ SYBR Green Supermix and the CFX96 Real Time PCR Detection System (Bio Rad, Hercules, CA) as follows: an initial 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Analysis of multilineage potential was conducted as described previously using primers specific for the various lineages (Table 2). All experiments were conducted independently at least three times with triplicate reactions for each complementary DNA tested and results were normalized to 18S ribosomal RNA using the $\Delta\Delta C_t$ method [15].

2.6. *Angiogenesis assay*

Triplicate wells were loaded with 150 μL Matrigel (BD Biosciences) and set to gel at 37°C for 1 h. Then MVF DCs or ASCs (both passage 1) were seeded on top of the gels at 20,000 cells per well. Cells were cultured in either growth media (DMEM, 10% FBS, and 1% antibiotics) or endothelial induction media (VascuLife VEGF Cell Culture Medium; Lifeline Cell Culture Technology, Frederick, MD). At 2, 6, and 16 h, images were taken at ×40 and ×100 magnification with an Olympus IX 71 inverted microscope to analysis the development of capillary like networks using phase contrast microscopy. Image analysis was performed using Image J V1.44p software (US National Institutes of Health, Bethesda, MD) with images converted to binary format and then the binary threshold

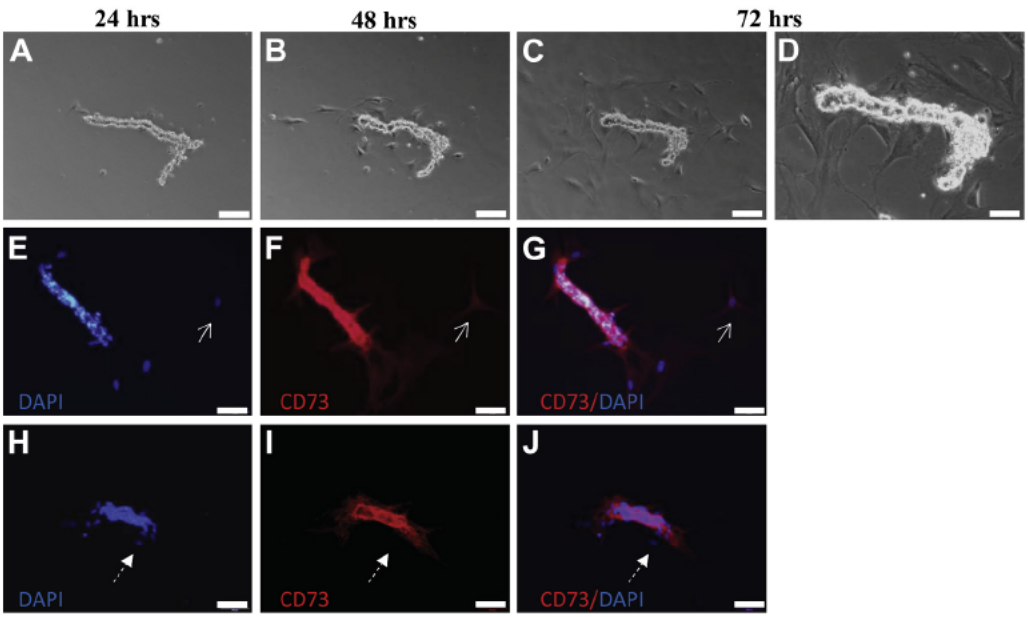

Fig. 2 – Representative images of MVF explant cultures to isolate MVF-DCs. Cells start to emanate from the MVFs within a few days of plating (A) and continue to detach and proliferate over time (B–D) until they reach subconfluency at, which time they were harvested for analyses. (E–J) MVFs and cells that emanate from them (solid arrow) are positive for the MSC marker CD73. Dashed arrow indicates CD73 negative cells. A–C, E–J scale bar = 100 μm, D scale bar = 50 μm. (Color version of figure is available online.)

function was adjusted to obtain the best contrast of capillary like tubules. The degree of tube formation was assessed at each time point by counting the number of capillary like tubules that resulted from cell to cell tube connections at branching points from the total number of cells in the threshold images as previously described [16,17].

2.7. Statistical analysis

Two way analysis of variance procedures or a Student *t* test were used to analyze experimental results using GraphPad Prism 5.01 for Windows (GraphPad Software, La Jolla, CA) followed by Tukey *post hoc* analyses where appropriate. Differences were considered significant when $P < 0.05$. All values are presented as mean ± standard error of mean.

3. Results

3.1. Isolation and culture of MVFs and ASCs

An important difference between the procurement of ASCs and MVFs is that during the isolation of ASCs, there is a longer digestion time (45 *versus* ~8 min). The shorter digestion time for the extraction of MVFs allows for vessel structure to be maintained (Fig. 1). MVFs seeded on tissue culture plates had several cells emanating from them within 48 h (Fig. 2). Consistent with previous reports identifying the association of stem cells with microvessels [9], and the expression of CD73 in digested MVFs [7], cells emanating from MVFs (MVF DCs) were positive for the MSC marker CD73 (Fig. 2). By day 7 MVF DCs were approximately 80%–90% confluent. Intense staining was present in the areas surrounding MVF remnants. ASCs were treated and displayed behavior well characterized by others, that is, attachment to tissue culture–treated plastic within 24 h after which time nonadherent cells were removed [18].

3.2. MSC characterization of ASCs and MVF-DCs

MVF DCs demonstrated an increased growth rate with the number of MVF DCs being significantly higher than the ASCs by day 3 ($P < 0.05$), and both reached plateau phase by day 8 (Fig. 3). Expression of traditional stem cell markers was observed via qPCR analysis in both cell types with no significant differences (Fig. 3). MVF DCs and ASCs were compared for their expression of MSC markers (CD29, CD44, CD73, CD90, and CD105) and hematopoietic stem cells HSCs (CD117 and SCA 1) and lack of CD45 expression. MVF DCs and ASCs demonstrated immunolocalization of the common MSC markers CD44, CD73, and CD90. Both cell types were negative for the hematopoietic marker CD45 (Fig. 3).

3.3. Expression of angiogenic markers

To assess the expression of angiogenic markers by MVF DCs and ASCs, qPCR was used using primers designed to amplify sequences specific to the genes CD31, CD144, CD146, NG2, and endothelial nitric oxide synthase (eNOS). The level of expression was 53 , 39 , 1.8 , and 26 fold higher ($P < 0.05$) for

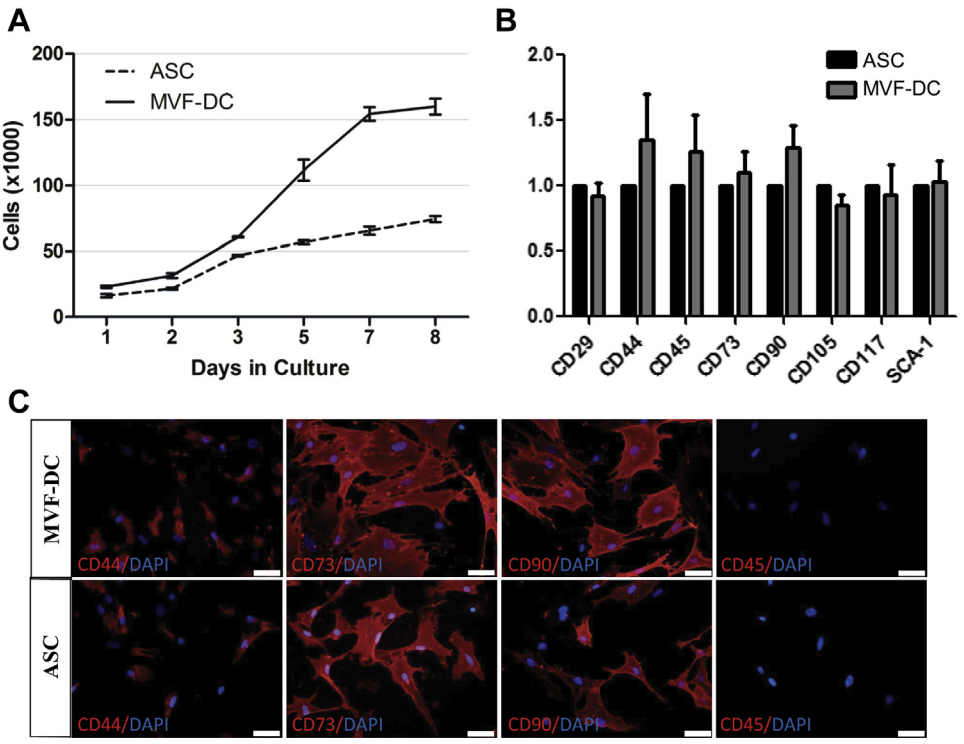

Fig. 3 – (A) Growth curve comparison between passage 1 MVF-DCs and ASCs, $n = 4$ wells per group per time point. (B) qPCR analysis of markers for MSC markers and hematopoietic stem cell markers. (C) MVF-DCs and ASCs demonstrated immunolocalization of the common MSC markers CD44, CD73, and CD90. Both cell types were negative for the hematopoietic marker CD45. Scale bar = 50 μm. *P < 0.05, **P < 0.01. (Color version of figure is available online.)

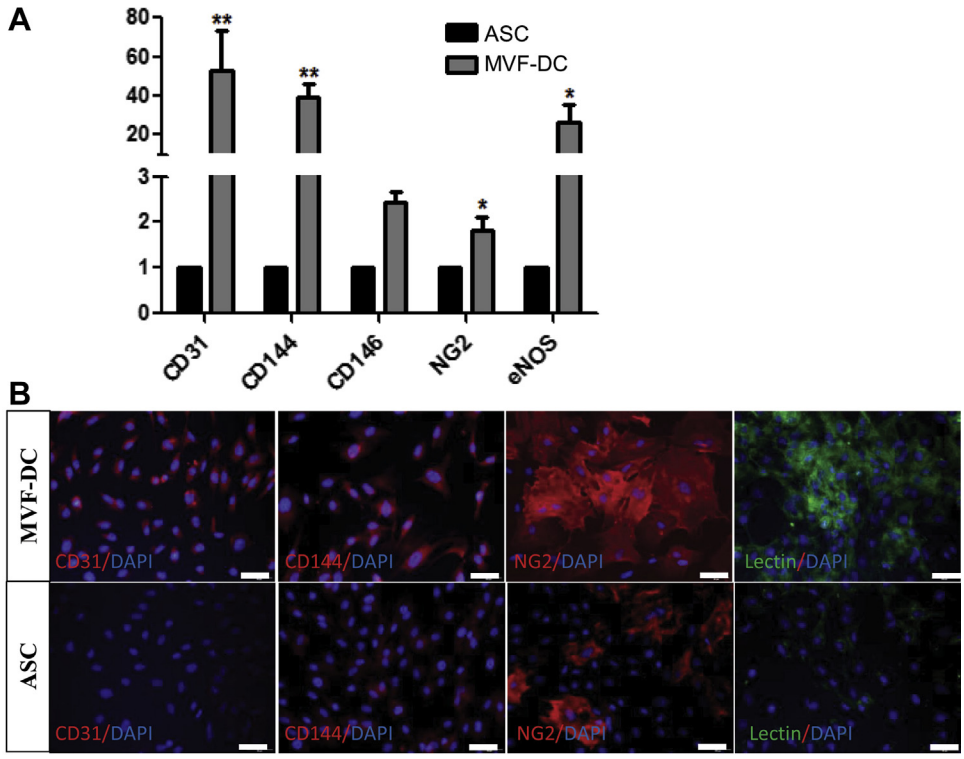

Fig. 4 – (A) MVF-DCs and adipose-derived cells (ASCs) were subjected to quantitative polymerase chain analysis for CD31, CD144, CD146, NG2, and eNOS. (B) A greater number of MVF-DCs stained positive for CD31, CD144, NG2, and Lectin compared with ASCs. $^{**}P < 0.01$, $^{*}P < 0.05$. Scale bar = 50 μm. (Color version of figure is available online.)

CD31, CD144, NG2, and eNOS, respectively, in MVF DCs than ASCs (Fig. 4). The level of CD146 was 2.4 fold higher in MVF DCs than ASCs but was not significant (P 0.14). Qualitatively, the protein expression corresponded well with the qPCR with an increased number of cells expressing CD31, CD144, and NG2 in MVF DCs than ASCs (Fig. 4). Consistent with an endothelial phenotype, qualitatively there appeared to be a higher number of cells that stained positive for GS 1 lectin in MVF DCs than ASCs (Fig. 4).

3.4. *Angiogenesis assay*

The angiogenic capacity of MVF DCs in growth media alone was higher in MVF DCs compared with ASCs as demonstrated by the tube formation by 6 h after seeding (Fig. 5; $P < 0.01$). Similar to that observed with growth media, MVF DCs cultured in endothelial induction media had a higher percentage of tube formation compared with ASCs 16 h after seeding (Fig. 5; $P < 0.01$). Both ASCs and MVF DCs displayed an increase in their angiogenic activity when treated with induction medium at 6 and 16 h compared with their counterparts grown in growth media alone (Fig. 5; $P < 0.001$).

3.5. *Multilineage differentiation potential*

To determine the differentiation potential of MVF DCs compared with ASCs, the two cell types were induced through adipogenic, osteogenic, and neurogenic lineages. Both MVF DCs and ASCs displayed a neuronal phenotype after only 2 h of induction. After 24 h of induction, qPCR revealed that MVF DCs expressed fivefold ($P < 0.05$) more transcripts for neuregulin, a marker for neuronal differentiation, than ASCs, whereas expression for neuronal cell adhesion molecule was not significantly different between the two cell types (Fig. 6). Adipogenic differentiation of MVF DCs and ASCs was demonstrated by Oil Red O staining after 14 d of culture in induction media. Gene expression analysis revealed leptin was upregulated by MVF DCs compared with ASCs after adipogenic induction; however, there was no significant difference in GLUT4 expression between the cell types (Fig. 6). After 14 d in osteogenic induction media, both MVF DCs and ASCs showed nodule formation that stained positive for mineralization with alizarin red. Transcript expression for markers of osteogenic differentiation were essentially equal between MVF DCs and ASCs with the exception of osteopontin, which demonstrated a more than threefold ($P < 0.05$) increase in MVF DCs compared with ASCs after induction (Fig. 6).

4. Discussion

Prevascularizing scaffolds, that is, creating mature microvessels and/or microvascular networks before implantation, is an encouraging means to improve vascularization to improve implant viability [19]. Future developments in this line of research will have a profound impact on surgical outcomes across a wide variety of tissues. The use of MVFs is a logical means to support prevascularization given that the

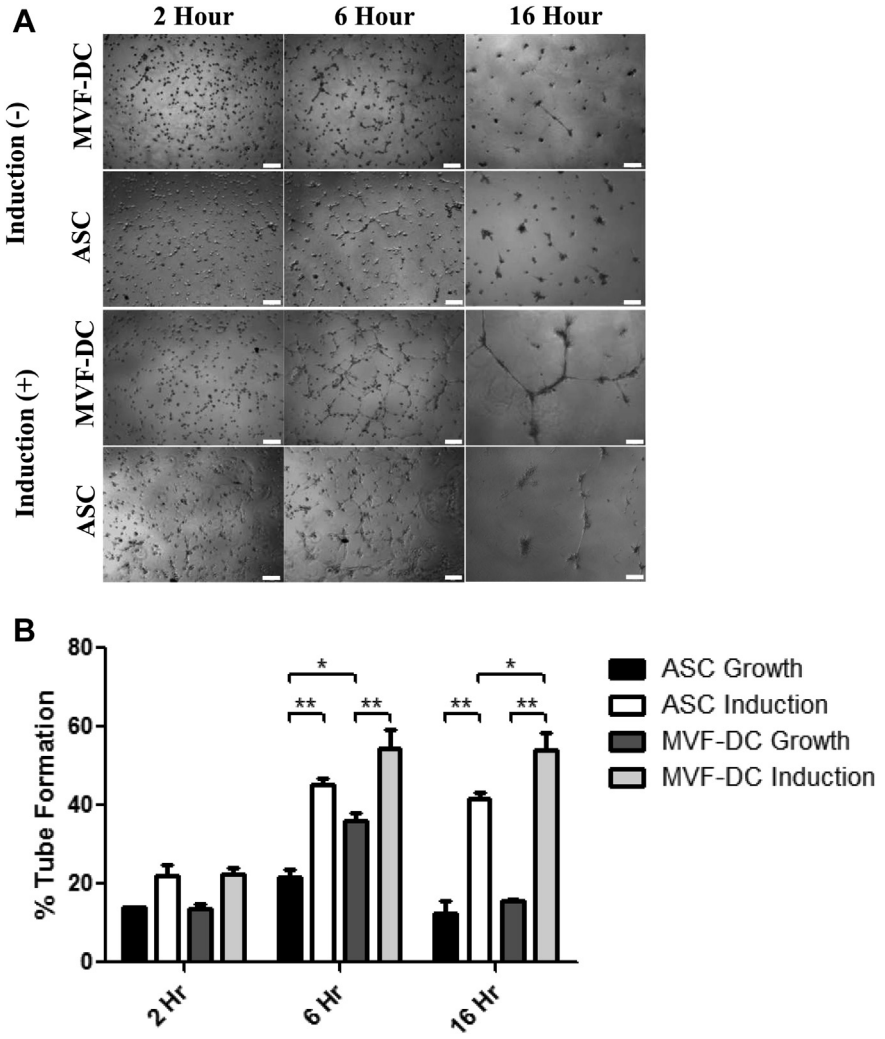

Fig. 5 – MVF-DCs and ASCs were seeded on matrigel-coated wells and analyzed during culture without (induction−) and with (induction+) endothelial induction media. (A) Representative light microscopic images of MVF-DCs and ASCs 2, 6, and 16 h of culture. (B) Quantitative analysis of tube formation of MVF-DCs and ASCs during exposure of either growth media only or growth media containing inductive factors. Scale bar = 200 μm. *$P < 0.01$, **$P < 0.001$.

need for *in vitro* manipulation to create microvessels is circumvented because intact microvessels are isolated. Because vessels are a source of resident MSCs [9,20,21], it is possible that the MVFs may produce beneficial effects that extend beyond their ability to support blood flow. In this regard, it has been suggested by others that multipotent cells associated with MVFs may augment the regenerative potential of implants [7]. The presence of stem cells within MVFs (Fig. 2 of the present study and [7]), and the improved expression of angiogenic, neurogenic, adipogenic, and osteogenic genes (Figs. 4–6) after induction supports this concept.

The isolation of stem cells from adipose tissue using a variety of methods has led to a better understanding of stem cell biology. Herein we describe results based on another methodology for the procurement of stem cells from adipose tissue, that is, the explant culture of MVFs. Early studies using adipose microvessel explant cultures derived from were directed toward isolating endothelial cells, documented the difficulty of culturing endothelial cells on tissue culture plastic (which led to substrate coating to enrich for endothelial cells), and generally considered connective tissue cells a contaminant [22,23]. The existence of plastic adherent "fibroblast like" cells observed in these early microvessel explant studies and the well described fibroblast like appearance of MSCs provides a historical connection to the current findings, that is, in the present study, plastic adherent cells from MVF explants had a fibroblast morphology, were positive for MSC cell surface markers, and exhibited multidifferentiation potential.

Because ASCs are well defined, they were used as a control to facilitate the characterization of MVF derived cells, specifically with regards to their MSC phenotype. Several observations related to ASCs are confirmatory of previous findings including the gene and/or protein expression for cell surface markers, ability to form capillary like networks on matrigel, neural, adipogenic, and osteogenic differentiation [24,25]. Despite the strong similarities between ASCs and MVF DCs with regards to their MSC attributes, MVF DCs exhibited a higher proliferative rate, increased expression for genes

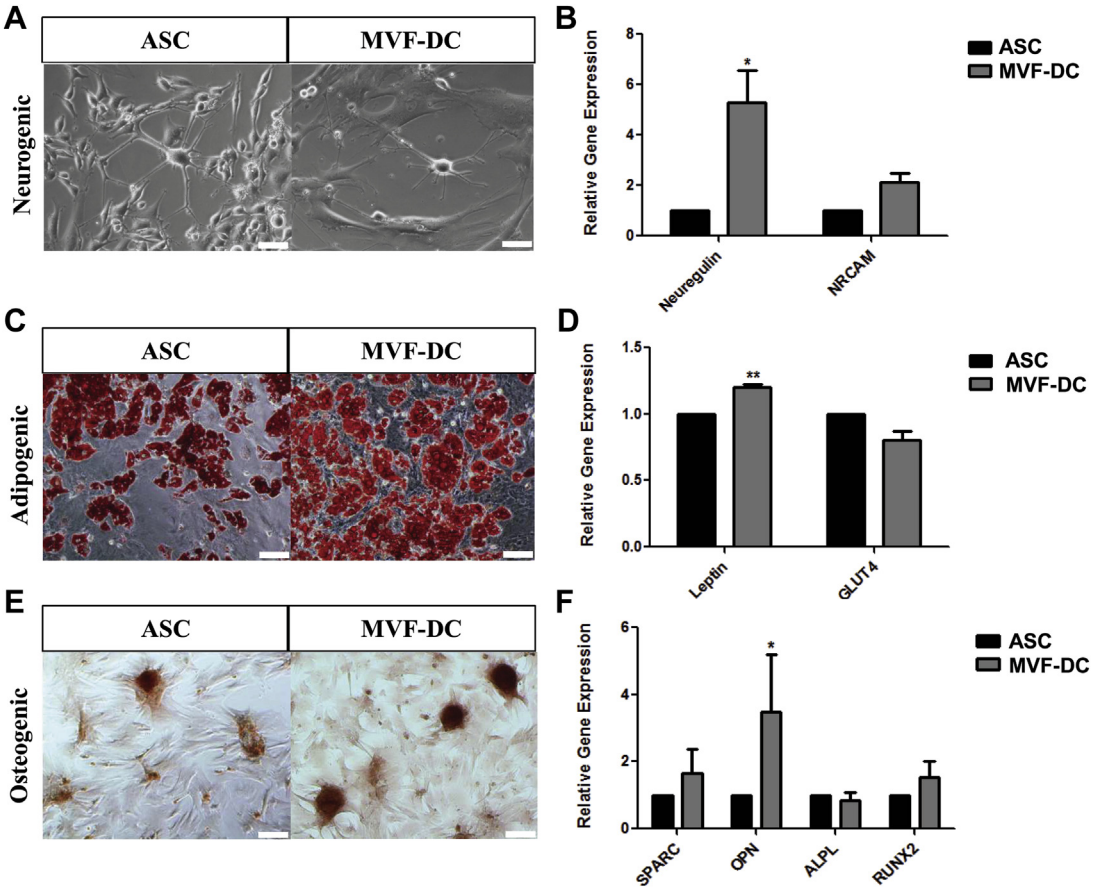

Fig. 6 – Analyses of multidifferentiation potential of MVF-DCs and ASCs using histologic (A, C, and D) and gene expression analyses (B, D, and F) after neurogenic (A and B), adipogenic (C and D) and osteogenic (E and F) induction. Scale bar = 50 μm. *P < 0.05. (Color version of figure is available online.)

involved with neurogenic, osteogenic, adipogenic lineages, and their ability to form capillary like networks (Figs. 4–6). Of these differences, the increased expression of genes and expression of markers indicative of vascular cell content (i.e., endothelial cells (CD31, CD144, and eNOS), and to a lesser extent, pericytes (NG2 and CD146) are particularly relevant to the usefulness as a therapeutic for improving vascularization *in vivo*. Accordingly, this was accompanied by an improved ability to form capillary like networks, even in the absence of endothelial growth me dium (Fig. 5). Collectively, these findings support the idea that MVF delivery supplies a number of regenerative cells that may augment tissue vascularization above that achieved with MVF delivery alone. In other words, MVF delivery not only supplies intact vessels but also supplies endothelial cells, pericytes, and MSCs that have been shown by others to effectively vascularize tissue when supplied in tandem [26,27].

The current findings raise an intriguing possibility for the advancement of cell based therapies. An exciting area of research is directed toward emulating an *in vivo* niche to improve cell survival and maximize regenerative potential [28]. Based on the data herein, it then follows that the delivery of MSCs via MVFs could be a means to deliver MSCs within their niche, a scenario that parallels the delivery of single muscle fibers for the transplantation of skeletal muscle satellite cells [29]. In fact, there are a number of striking sim ilarities between skeletal muscle fibers, that contain satellite cells, and MVFs, that contain MSCs. With regards to the former, this concept has been exploited to improve cell de livery in that the conveyance of cells within their niche is more effective than implantation after culture expansion [30]. Another similarity is apparent when one considers the isola tion strategy and subsequent *in vitro* analyses. Single muscle fibers can be isolated with satellite cells found to be emanating within days after plating allowing for a means to study satellite cells as they are directly derived from their niche [31]. In the present study, MVFs were plated and MVF derived stem cells studied in a manner that may more closely resemble their native environment.

A limitation to this speculation is that in the absence of *in vivo* experimentation, the current findings to not fully support the concept of MSC delivery via MVFs *in vivo*. Future experiments directed toward identifying the quali ties of the stem cells that are either resident on or emanating from transplanted MVFs *in vivo* are required to effectively make this conclusion. Nonetheless, the current findings support the exploration of this concept and pro vide valuable information to help explain the therapeutic potential of MVFs.

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Authors' contributions: J.S.M, C.L.W, B.P, and C.R.R conceived and designed the experiments. J.S.M, M.P, C.L.W, and B.P performed the experiments. J.S.M, B.P., and C.R.R analyzed and interpreted data. J.S.M and C.R.R wrote and critically revised the manuscript.

Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in the article.

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