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A Novel Adult Marrow Stromal Stem Cell Based 3-D Postnatal De Novo Vasculogenesis for Vascular Tissue Engineering

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1. Introduction

Vascular diseases are one of the leading causes of significant morbidity and mortality worldwide. Vascular diseases not only occur at all levels of vascular tree but also affect multiple organs and organ systems. Organ tissue engineering, including vascular tissue engineering, has been an area of intense investigation. The current major challenge to these approaches has been the inability to vascularize and perfuse in vitro engineered tissue constructs. Attempts to provide oxygen and nutrients to cells contained in biomaterial constructs have met with varying degrees of success. Engineering a tissue of clinically relevant magnitude requires the formation of extensive and stable microvascular networks within the tissue (Brey et al., 2005). Since most in vitro engineered tissue constructs do not contain the intricate microvascular structures of native tissue, the cells contained in scaffolds heavily rely on simple diffusion for oxygenation and nutritional delivery. The majority of cells need to be within 100-200 μm of a blood supply to receive adequate oxygen and nutrients for survival (Carmeliet & Jain, 2000). Otherwise, due to diffusion gradients, the cells in the interior regions of the artificial scaffold can experience hypoxia or anoxia and undergo cellular degeneration and necrosis. Hence, this necessitates the formation of appropriate in vitro three-dimensional (3-D) plexuses of new blood vessels within the pre-implanted biomaterial constructs through the process of in situ de novo vasculogenesis/angiogenesis for organ tissue engineering.

Development of postnatal new blood vessels occurs essentially by two temporally distinct but interrelated processes, vasculogenesis and angiogenesis. Vasculogenesis is the process of blood vessel formation occurring by a de novo production of endothelial cells in an embryo (primitive vascular network) or a formerly avascular area when endothelial precursor cells (angioblasts, hemangioblasts or stem cells) migrate and differentiate in response to local cues (such as growth factors and extracellular matrix) to form new intact blood vessels (Risau & Flamme, 1995). Angiogenesis refers principally to the sprouting of new blood vessels from the differentiated endothelium of pre-existing vessels. These vascular trees or plexuses are then pruned, remodeled and extended through angiogenesis to become larger caliber vessels (Carmeliet, 2000). In addition, there exists yet another unique mechanism of neovascularization, the postnatal vasculogenesis, where

new blood vessels are formed by the process of fusion and differentiation of endothelial progenitors of bone marrow origin (Valarmathi et al., 2009). This indicates a potential role for bone marrow-derived progenitor cells in postnatal neovasculogenesis and/or neoangiogenesis. This implies that additional mechanisms besides angiogenesis can occur in the adult, and has opened up the possibility to investigate the embryonic origin and development of these putative progenitor cells.

The adult bone marrow contains two subsets of multipotential stem cells, hematopoietic stem cells (HSCs) and bone marrow stromal cells or mesenchymal stem cells (BMSCs/MSCs). BMSCs are a readily available heterogeneous population of cells that can be directed to differentiate into multiple mesenchymal and non-mesenchymal cells either in vitro or in vivo (Wakitani et al., 1995; Pittenger et al., 1999; Makino et al., 1999; Fukuda et al., 2001; Bianco et al., 2001; Valarmathi et al., 2009, 2010). Most noticeably BMSCs have been induced to undergo maturation and differentiation towards vascular endothelial and smooth muscle cell lineages. Previous reports indicate that BMSCs and bone marrow-derived multipotent adult progenitor cells (MAPCs) can be differentiated into endothelial-like cells in vitro and contribute to neoangiogenesis in vivo (Oswald et al., 2004; Reyes et al., 2002; Al-Khalidi et al., 2003). Additionally, it has been shown that BMSCs can augment collateral remodeling and perfusion in ischemic models through paracrine mechanisms rather than by cellular incorporation upon local delivery (Kinnaird et al., 2004). Therefore, the identification of bone-marrow-derived (hematopoietic and non-hematopoietic stem cells) and non-bone-marrow-derived (tissue-resident stem/progenitor cells – adipose, neural, heart, skeletal muscle; peripheral and cord blood-derived stem cells) endothelial progenitors cells (EPCs) has led to the realization of potential postnatal vasculogenesis (Urbich & Dimmeler, 2004).

For the above mentioned reasons, embryonic, fetal and postnatal stem cells as well as various types of progenitor cells, can be a potential cellular source for vascular tissue engineering (Levenberg, 2005). However, the source for the early-stage developmental cells is restricted. The utility of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) in facilitating vascularized tissue/organ regeneration is still in its incipient stages. A number of issues, including a propensity for some implanted ESCs/iPSCs to form benign teratomas and/or malignant teratocarcinomas in the regenerating tissue/organ, remain to be addressed. In contrast to both ESCs/iPSCs, it has been well established that the adult stem cell, BMSCs exhibit multilineage differentiation potential in a well-controlled, predictable fashion. Moreover, unlike ESCs derivation, obtaining autologous or allogeneic BMSCs is feasible and can potentially be exploited to develop tissue-engineered blood vessel constructs for therapeutic purposes. Similarly, when compared to bone marrow-derived BMSCs, repeated isolation and rapid expansion of sufficient yield of autologous and/or allogeneic non-bone-marrow-derived resident stem cells/progenitors, especially from vital organs for routine therapeutic purposes are highly constrained. On the contrary, to certain extent autologous and/or allogeneic bone marrow-derived BMSCs are amenable for repeated isolation and reasonable in vitro expansion from the patients. In addition, the significant advantage of using these BMSCs is their low immunogenicity. And these autologous or allogeneic BMSCs have been reported to be immunomodulatory and immunotolerogenic both in vitro as well as in vivo. (Aggarwal & Pittenger, 2005). Taken together, these data strongly indicate that BMSCs can represent the potential cell of choice for adult autologous and/or allogeneic stem cell based vascularized tissue regeneration.

Extracellular molecules initiate biological signals and play a critical role in the control of cellular proliferation, differentiation, and morphogenesis. Many parameters, such as the presence and amount of soluble factors such as hormones, growth factors, and cytokines or the insoluble factors such as the physical configuration of the matrix which mediate the cell-cell interactions and cell-matrix interactions, exerts strong influence on the success of angiogenic processes *in vitro* and presumably *in vivo* (Even-Ram & Yamada, 2005; Carlson, 2007). The likelihood and ultimate success of *in vitro* cellular differentiation depends on how closer the cell-matrix interactions and relationships' mimic to those found during normal development or regeneration. In vascular tissue engineering, the application of these principles *in vivo* will be important to ensure that the matrix/scaffold to be implanted can support endothelial cell proliferation and migration resulting in endothelial tube formation (Ingber & Folkman, 1989). The vital issue for realistic clinical application is whether these scaffolds with preformed network of endothelial capillaries/microvessels can survive implantation into tissue defects and subsequently be able to anastomose to the host vasculature.

We therefore hypothesized that under appropriate *in vitro* physicochemical microenvironmental cues (combination of growth factors and extra cellular matrix, ECM) multipotent adult BMSCs could be differentiated into vascular endothelial and smooth muscle cell lineages. To test this hypothesis, we characterized the intrinsic vasculogenic differentiation potential of adult BMSCs when seeded onto a three-dimensional (3-D) tubular scaffold engineered from aligned type I collagen strands and cultured either in vasculogenic or non-vasculogenic growth medium. In these culture conditions, BMSCs differentiated and matured into both endothelial and smooth muscle/pericyte cell lineages and showed microvascular morphogenesis. We also explored the potential of the 3-D model system to undergo postnatal de novo vasculogenesis.

2. Experimental approach

The differentiation of rat BMSCs was carried out on a 3-D tubular scaffold made up of aligned type I collagen-gel fibers. Rat BMSCs were isolated from the tibial and femoral bone marrow of adult rats. The BMSCs isolated from the bone marrow were expanded, maintained and passaged to make sure that the attached marrow stromal cells were devoid of any non-adhering populations of cells. Phenotypic characterization of the BMSCs for cell surface markers was performed by confocal microscopy (qualitative evaluation) and single-color flow cytometry (quantitative analysis). This adherent population of cells were further purified and enriched by indirect magnetic cell sorting. The cells were subjected to CD90 positive selection. The resultant enriched CD90⁺/CD34⁻/CD45⁻ fractions were expanded by subculturing and subjected to flow cytometric analysis to validate the proper phenotype. This population of purified BMSCs was used in all experiments. For vasculogenic differentiation of BMSCs, the expanded and purified population of CD90⁺ BMSCs was seeded into the collagen-gel tubular scaffold and cultured either in vasculogenic or non-vasculogenic culture medium for 28 days. At regular intervals of 7, 14, 21 and 28 days the tube cultures were assayed by RT-qPCR, immunofluorescence, ultrastructural and biochemical analyses for various endothelial and smooth muscle cell differentiation markers as shown in table 1 and 2. These times were chosen for the following reasons: these time points cover the range of both vasculogenic and angiogenic processes seen *in vivo* and/or *in vitro* and mimic the progression of microvascular development.

3. Research methods

3.1 Fabrication of tubular scaffold

Briefly, a 25 mg/ml solution of bovine collagen type I was extruded with a device that contained two counter-rotating cones. The liquid collagen was fed between the two cones and forced through a circular annulus in the presence of an NH_3 -air (50-50 vol/vol) chamber. This process results in a hollow cylindrical tube of aligned collagen fibrils with an inner central lumen. The dimensions of tubes produced for this set of experiments had a length of 30 mm with a luminal diameter of 4 mm and an external diameter of 5 mm, leaving a wall thickness of 1 mm. The collagen tubes had a defined fiber angle of 18° relative to the central axis of the tube and had pores ranging from 1 to 10 μm . The tubes were sterilized using gamma radiation 1200 Gy followed by Stratalinker UV crosslinker 1800 (Stratagene) and then placed in Mosconas's solution (in mM: 136.8 NaCl, 28.6 KCl, 11.9 NaHCO_3 , 9.4 glucose, 0.08 NaH_2PO_4 , pH 7.4) (Sigma-Aldrich) containing 1 $\mu\text{l/ml}$ gentamicin (Sigma-Aldrich) and incubated in 5% CO_2 at 37°C until cellular seeding (Valarmathi et al., 2010). The rationale for the particular orientation of collagen fiber was based on our previous work on cardiovascular tissue engineering (Yost et al., 2004). When proepicardial organ cells (PECs) were seeded onto this scaffold, they underwent maturation and differentiation and produced elongated vessel-like structures reminiscent of in vivo-like phenotype (Valarmathi et al., 2008).

3.2 BMSCs isolation, expansion and maintenance

The initial step is to isolate the mononuclear cells from the bone marrow by aspiration and centrifugation followed by plating and isolation of the cells based on differential adherence capacity to tissue culture dishes (passage 0 cells). Rat BMSCs were isolated from the bone marrow of adult 300g Sprague Dawley[®] SD[®] rats (Harlan Sprague Dawley, Inc.). Briefly, after deep anesthesia, the femoral and tibial bones were removed aseptically and cleaned extensively to remove associated soft connective tissues. The marrow cavities of these bones were flushed with Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) and combined. The isolated marrow plugs were triturated, and passed through needles of decreasing gauge (from 18 gauge to 22 gauge) to break up clumps and cellular aggregates. The resulting single-cell suspensions were centrifuged at 200g for 5 minutes. Nucleated cells were counted using a Neubauer chamber. Cells were plated at a density of 5×10^6 – 2×10^7 cells per T75 cm^2 flasks in basal medium composed of DMEM supplemented with 10% fetal bovine serum (FBS, lot-selected; Atlanta Biologicals, Inc.), gentamicin (50 $\mu\text{g/ml}$) and amphotericin B (250 ng/ml) and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 7 days. The medium was replaced, and changed three times per week until the cultures become ~70% confluent (between 12 and 14 days). Cells were trypsinized using 0.05% trypsin-0.1% EDTA and replated at a density of 1×10^6 cells per T75 cm^2 flasks. After three passages, attached marrow stromal cells were devoid of any non-adhering population of cells. These passaged BMSCs were cryopreserved and stored in liquid nitrogen until further use (Valarmathi et al., 2011).

3.3 Immunophenotyping of BMSCs by flow cytometry and confocal microscopy

BMSCs are a heterogeneous population of cells with varying degrees of cell shapes and sizes. Stringent characterization of BMSCs used in experimental procedures is required for various cell surface markers; this is to ensure that the employed population of cells contains solely stem/progenitor cells. This will obviate the possible contaminating marrow-derived

endothelial cells and macrophages that are part of the adherent population of cultured cells. Therefore, characterization of BMSCs included qualitative evaluation for various cell surface markers and was performed on cells grown in the Lab-tek™ chamber slide system™ (Nunc) using a Zeiss LSM 510 Meta confocal scanning laser microscope (Carl Zeiss, Inc.), and quantitative analysis of the same set of markers was performed by single-color flow cytometry using a Coulter® EPICS® XL™ Flow Cytometer (Beckman Coulter, Inc.) as previously described (Valarmathi et al., 2009).

Immunophenotyping of undifferentiated BMSCs for various cell surface markers by flow cytometry revealed that the fluorescent intensity and distribution of the cells stained for CD11b, CD31 and CD45 were not significantly different from the intensity and distribution of cells stained with isotype controls (Figure 1E-G), indicating that these cultures were devoid of any possible hematopoietic stem and/or progenitor cells as well as differentiated bone-marrow-derived endothelial cells. In contrast, BMSCs exhibited high expression of CD90 surface antigens (Figure 1H), which is a consistent characteristic of undifferentiated BMSCs. Phenotypic characterization using the same set of markers on BMSCs by confocal microscopy also revealed that these cells were negative for CD11b, CD31 and CD45 (Figure 1A-C) and, strongly positive for CD90 (Figure 1D). The expression profiles of these surface molecules were consistent with previous reports and the minimal criteria for defining multipotent mesenchymal stromal cells, enunciated by the international society for cellular therapy (ISCT) position statement (Dominici et al., 2006; Valarmathi et al., 2009; Reyes et al., 2002).

3.4 Purification and enrichment of CD90⁺ BMSCs by magnetic-activated cell sorting (MACS)

Purification and enrichment of input BMSCs (such as CD45⁻, CD34⁻, CD90⁺/CD105⁺) are mandatory either using MACS (magnetic activated cell sorter) or FACS (fluorescent activated cell sorter). Since the unpurified fraction may contain sizable number of contaminating adherent macrophages and bone marrow-derived endothelial progenitors and differentiated endothelial cells. The adherent populations of BMSCs were further purified by indirect magnetic cell labeling method using an autoMACS™ Pro Separator (Miltenyi Biotech). Thus, these cells were subjected to CD90 positive selection by incubating the cells with FITC- labeled anti-CD90 antibodies (BD Pharmingen), followed by incubation with anti-FITC magnetic microbeads (Miltenyi Biotech), and passed through the magnetic columns as per the manufacturer's instructions. The resultant enriched CD90⁺/CD34⁻/CD45⁻ fractions were expanded by subcultivation and subjected to flow cytometric analysis as described previously (Valarmathi et al., 2010).

3.5 BMSCs vasculogenic differentiation

For vasculogenic differentiation of BMSCs, the purified population of CD90⁺ BMSCs were seeded into the collagen-gel tubes at a density of 0.5×10^6 cells/30 mm tube length and cultured either in mesenchymal stem cell growth medium supplemented with 10% FBS, penicillin and streptomycin (Poietics® MSCGM™ BulletKit®; Lonza Ltd.) or microvascular endothelial cell growth medium (Clonetics® EGM®-MV Bullet Kit®; Lonza Ltd.) supplemented with 5% FBS, bovine brain extract, human epidermal growth factor (hEGF), hydrocortisone, amphotericin B and gentamicin for 28 days. These BMSCs seeded tubes were cultured either in vasculogenic or non-vasculogenic medium for the defined time periods of 7, 14, 21 or 28 days. In addition, BMSCs were seeded in 65-mm Petri dishes at a density of 3×10^3 cells/cm² and cultured either in non-vasculogenic (MSCGM) or vasculogenic (EGMMV) medium for 7, 14, 21 or 28 days.

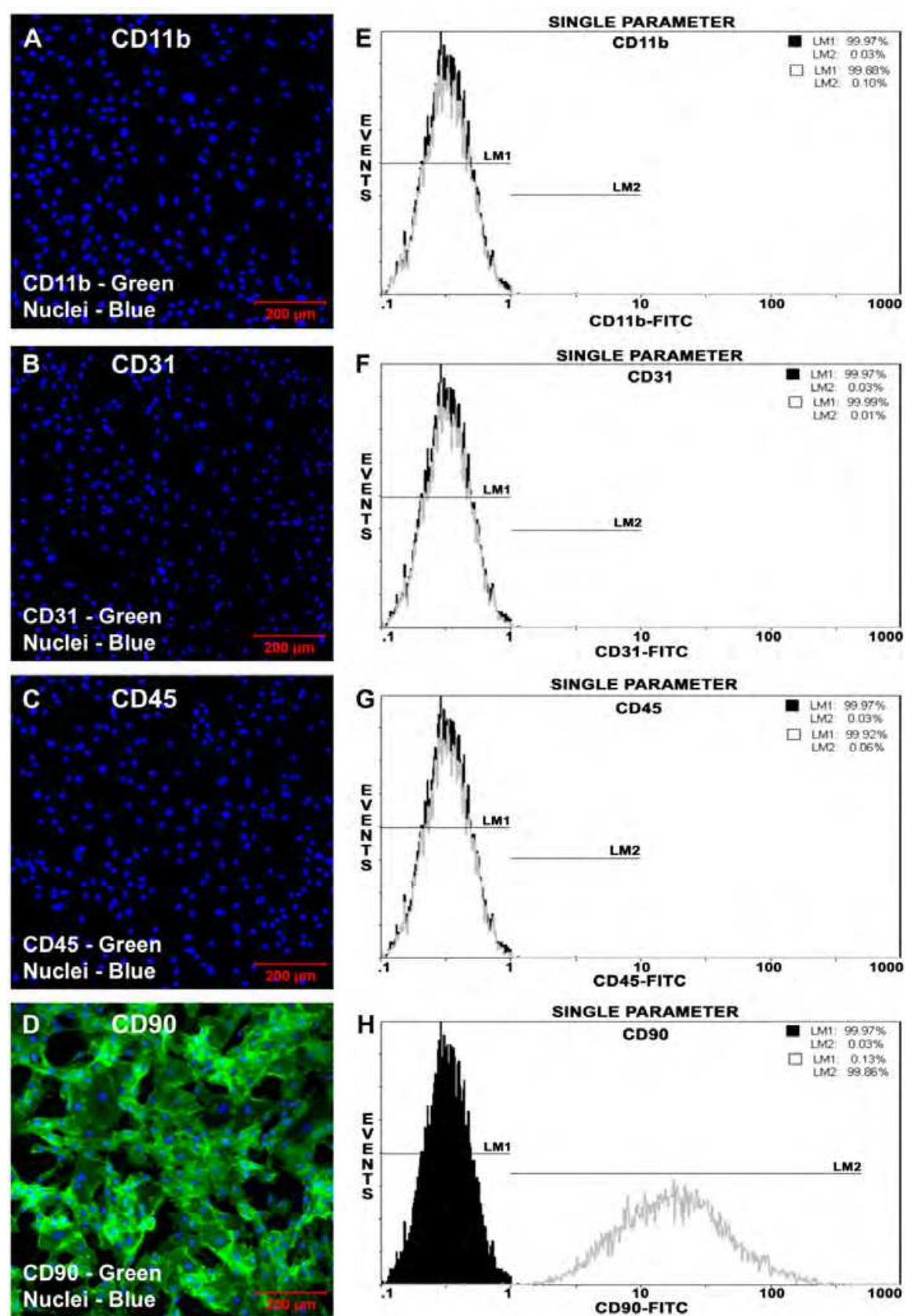


Fig. 1. Immunophenotyping of undifferentiated rat BMSCs by confocal microscopy and flow cytometry. Immunostaining and confocal microscopy revealed that BMSCs were

negative for CD11b (A), CD 31 (B) and CD45 (C) and; were uniformly positive for CD90 surface antigen (D), consistent with their undifferentiated state. Isotype and/or negative controls were included in each experiment to identify the level of background staining. Cells were also stained for nuclei (blue – DAPI). Additionally, the same population of BMSCs was subjected to flow cytometric analysis. Single parameter histograms showing the relative fluorescence intensity of staining (abscissa) and the number of cells analyzed, events (ordinate). Isotype controls were included in each experiment to identify the level of background fluorescence (black, shaded peaks). The intensity and distribution of cells stained for hematopoietic and endothelial markers; CD11b, CD31 and CD45 (grey, open peaks) were not significantly different from those of isotype control (black, shaded peaks) (E-G), indicating that these cultures were devoid of any potential contaminating hematopoietic and/or endothelial cells of bone marrow origin. The fluorescent intensity was greater (shifted to right) when BMSCs were stained with CD90 (grey) compared to isotype control (black) (H). The predominant population of BMSCs consistently expressed CD90 surface molecule, a property of rat bone marrow-derived mesenchymal/stromal stem cells. (DAPI - 4',6-diamidino-2-phenylindole). Merged images A-D (A-D, scale bar 200 μ m).

4. BMSCs based postnatal de novo vasculogenesis and in situ vascular regeneration

The 3-D collagen-gel tubular scaffold has previously been used to create vascularized bone elements (Valarmathi et al., 2008 a, b). Here we report the utility of a 3-D tubular construct for its ability to support the vasculogenic differentiation of BMSCs culminating into microvascular structures, which are similar to those structures resulting from postnatal de novo vasculogenesis and angiogenesis (Valarmathi et al., 2008 a, b).

In the developing vertebrate embryo, the initial event of blood vessel formation is the differentiation of vascular endothelial cells, which subsequently cover the entire interior surface of all blood vessels. Angioblasts are a subpopulation of primitive mesodermal cells that are committed to differentiate into endothelial cells and later on form the primitive vascular labyrinth (Risau & Flamme, 2000). In addition, endothelial cells can also arise from hemangioblasts, a common precursor for both hematopoietic and endothelial cells (His et al., 1900).

In adults, endothelial precursor cells have been identified in bone marrow, peripheral blood and blood vessels (Prater et al., 2007). Two subsets of multipotential stem cells, HSCs and BMSCs/MSCs are resident in the postnatal bone marrow. Of these cells, BMSCs can be differentiated into osteoblasts, chondrocytes, adipocytes, smooth muscle cells and hematopoietic supportive stroma either in vitro or in vivo (Bianco et al., 2001). Previous studies have provided substantial evidence that bone-marrow-derived stem and/or progenitor cells can be differentiated into either endothelial or smooth muscle cells in vitro and in pathological situations are capable of contributing to neoangiogenesis in vivo by cellular integration (Carmeliet & Luttun, 2001).

Although there are a plethora of studies focused on developing viable scaffolds for osteogenic, chondrogenic, adipogenic and musculogenic differentiation of BMSCs (Lanza et al., 2000), the optimal scaffolds that are capable of inducing and supporting the growth and differentiation of BMSCs into vascular cell lineages are yet to be identified and characterized. Despite the much known vasculogenic potential and

transgermal plasticity of BMSCs; none of these studies explicitly demonstrated the postnatal de novo vasculogenic potential of BMSCs in vitro (Reyes et al., 2002; Oswald et al., 2004).

When compared to 2-D planar cultures, the potential 3-D models of vasculogenesis allow us to understand the role of specific factors under more physiological and spatial conditions with respect to dimensionality, architecture and cell polarity. Nevertheless, the molecular composition and the natural complexity and diversity of in vivo extra cellular matrix (ECM) organization cannot be easily mimicked or reproduced in vitro (Vailhe et al., 2001). In addition, even though quite a few in vitro 3-D models of vasculogenesis based on fibrin and collagen gels are in vogue (Folkman & Haudenschild, 1980); none have explored the behavior of BMSCs and their intrinsic vasculogenic differentiation potential on a topographically structured 3-D tubular scaffold made of uniformly aligned type I collagen fibers.

Previous studies demonstrated that the formation of endothelial tubes in vitro was largely influenced by the nature of the substrate (Kleinman et al., 1982). The formation of endothelium lined tubular structures was enhanced when the substrate was rich in laminin (Madri et al., 1988), whereas a matrix rich in type I collagen would not promote rapid tubulogenesis (Montesano et al., 1983; Ingber & Folkman, 1989). Similarly, Ingber & Folkman (1989) documented that under a given cocktail of growth factors, the local physical nature of the interaction between endothelial cells and the underlying matrix/substrate ultimately determined the tubular morphogenesis. Substrates containing abundant fibronectin promoted adhesion, spreading and growth of endothelial cells. In contrast, less adhesive substrate or matrix materials that were arranged three-dimensionally permitted the endothelial cells to retract and form tubes (Ingber & Folkman, 1989).

In general, successful in vitro differentiation of cells depends on cell-cell as well as cell-matrix interactions. Therefore, we hypothesized that under appropriate in vitro local environmental cues (combination of growth factors and ECM) multipotent postnatal BMSCs could be induced to undergo microvascular development. Hence, we developed a 3-D culture system in which a pure population of CD90⁺ rat BMSCs was seeded and cultured on a highly aligned, porous, biocompatible collagen-fiber tubular scaffold for differentiation purposes. Here, we utilized two types of growth media for vasculogenic differentiation purpose, MSCGM (non-vasculogenic) as control and EGMMV (vasculogenic) preferentially for microvascular differentiation. Both of these culture media consistently promoted the vasculogenic differentiation of BMSCs and also supported the formation of endothelium lined vessel-like structures within the constructs.

A number of early and late stage markers associated with rodent vascular development in vivo were used in this study to characterize the rat BMSCs derived microvascular structures at mRNA and protein levels, which included: CD31/Pecam1, Flt1 (Vegfr1), Flk1 (Vegfr2/Kdr), VE-cadherin (CD144), CD34, Tie1, Tek (Tie2), and Von Willibrand factor (Vwf). Platelet/endothelial cell adhesion molecule, also known as CD31, is a transmembrane protein expressed abundantly early in vascular development that may mediate leukocyte adhesion and migration, angiogenesis, and thrombosis (Albelda et al., 1991). The other early stage differentiation markers Flk1 and Flt1, which are receptors for the vascular endothelial cell growth factor-A (Vegf) essentially, play a vital role in embryonic vascular and hematopoietic development (Shalaby et al., 1997). Similarly, VE-

cadherin, a member of the cadherin family of adhesion receptors, is a specific and constitutive marker of endothelial cell plays an important role in early vascular assembly. Vascular markers that are expressed at a later stage include CD34 and Tie-2 (Bautch et al., 2000). CD34 is a transmembrane surface glycoprotein that is expressed in endothelial cells and hematopoietic stem cells. Tie1 and Tek are receptor kinases on endothelial cells that are essential for vascular development and remodeling in the embryo and may also mediate maintenance and repair of the adult vascular system. In late phases of vasculogenesis, the mature endothelial cells will synthesize and secrete Vwf homolog, a plasma protein that mediates platelet adhesion to damaged blood vessels and stabilizes blood coagulation factor VIII.

In any type of in vitro cellular differentiation, the cytodifferentiated cells need to be critically evaluated for their maturation and differentiation at transcriptional, translational and functional levels. Therefore, to study the expression pattern of key vasculogenic gene transcripts in the 3-D tube constructs; we examined the time-dependent expression pattern of Pecam1, Kdr, Tie1, Tek and Vwf at mRNA level in the tube constructs by real-time PCR (Table 1, Figure 2A-D).

Genes	Forward primer	Reverse primer	Product length (bp)	Annealing temperature (°C)	GenBank accession No
Pecam1	5'-CGAAATCTAGGCCTCAGCAC-3'	5'-CTTTTGTCCACGGTCACCT-3'	227	56	NM_03159.1
Kdr	5'-TAGCGGGATGAAATCTTTGG-3'	5'-TTGGTGAGGATGACCGTGTA-3'	207	56	NM_013062.1
Tie1	5'-AAGGTCACACACACGGTGAA-3'	5'-TGGTGGCTGTACATTTTGGGA-3'	174	56	XM_233462.4
Tek	5'-CCGTGCTGCTGAACAACCTTA-3'	5'-AATAGCCGTCCACGATTGTC-3'	201	56	NM_001105737.1
Vwf	5'-GCTCCAGCAAGTTGAAGACC-3'	5'-GCAAGTCACTGTGTGGCACT-3'	163	56	XM_342759.3
Gapdh	5'-TTCAATGGCACAGTCAAGGC-3'	5'-TCACCCCATTTGATGTTAGCG-3'	101	56	XR_007416.1

Table 1. RT-qPCR primer sequences used in this study (Valarmathi et al., 2009; Rozen and Skaletsky, 2000).

Constitutive expressions of these markers were detected at low to very low levels in undifferentiated BMSCs. RT-qPCR results showed that differentiation of BMSCs under vasculogenic tube culture conditions for 28 days resulted in increased expression of transcripts coding for various endothelial cell associated proteins such as Pecam1, Kdr, Tek and Vwf. The peak expression of Vwf, the endothelial specific protein occurred around day 21 (over 400 fold) indicating that the differentiating cells acquired a distinctive phenotype and biosynthetic activity of differentiated and matured endothelial cells (Figure 2D). The upregulation of Tek during this period may represent the continual development and remodeling of the developing microvessels within the tubular constructs. Whereas differentiation of BMSCs under non-vasculogenic tube culture conditions for 14 days showed signs of early and rapid induction of transcripts coding for both early and late stage endothelial cell markers such as Kdr, Tie1, Tek and Vwf. The peak expression of Vwf occurred during day 14 (over 20 fold) (Figure 2B) (Valarmathi et al., 2009).

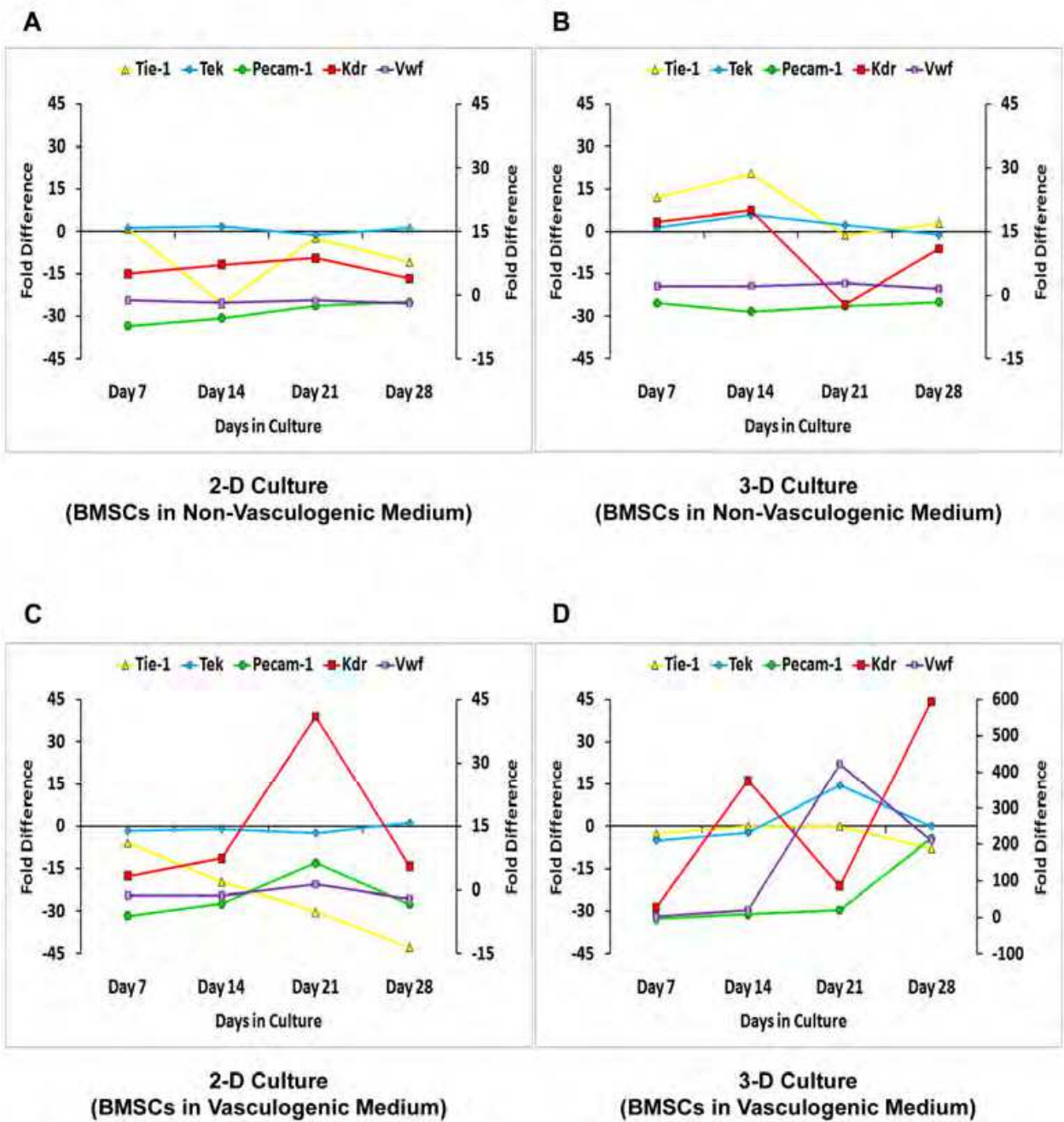


Fig. 2. Real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) analysis of various key vasculogenic markers. RT-qPCR analysis of various key vasculogenic markers such as tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie1), endothelial-specific receptor tyrosine kinase (Tek/Tie2), platelet/endothelial cell adhesion molecule 1 (Pecam1), kinase insert domain protein receptor (Kdr/Flk1/Vegfr-2), and Von Willebrand factor homology (Vwf) as a function of time (abscissa). BMSCs cultured in Petri dishes (2-D culture) in mesenchymal stem cell growth medium (A) and, in microvascular growth medium (C). BMSCs cultured in

collagen-gel tubular scaffolds (3-D culture) in mesenchymal stem cell growth medium (B) and, in microvascular growth medium (D). The calibrator control included BMSCs day 0 sample and; the target gene expression was normalized by a non-regulated reference gene expression, Gapdh. The expression ratio (ordinate) was calculated using the REST-XL version 2 software. The values are means \pm standard errors for three independent cultures ($n=3$). (Tie-1 and Tek – plotted with respect to 1° Y-axis; Pecam-1, Kdr and Vwf – plotted with respect to 2° Y-axis) (Pfaffl, 2001, 2002; Valarmathi et al., 2008 a).

As revealed by immunostaining for various vasculogenic markers, day 21 vasculogenic and non-vasculogenic tube cultures showed that BMSCs were able to adhere, proliferate, migrate and, undergo complete maturation and differentiation into microvascular structures (Figure 3A-C). BMSCs derived microvessel formation is a combination of de novo vasculogenesis i.e., in situ endothelial cell differentiation and endothelium-lined tube formation, and angiogenesis, endothelial sprouting from existing endothelial tubes. In addition, these microvessels are stabilized by association with BMSCs derived smooth muscle cells and/or pericytes.

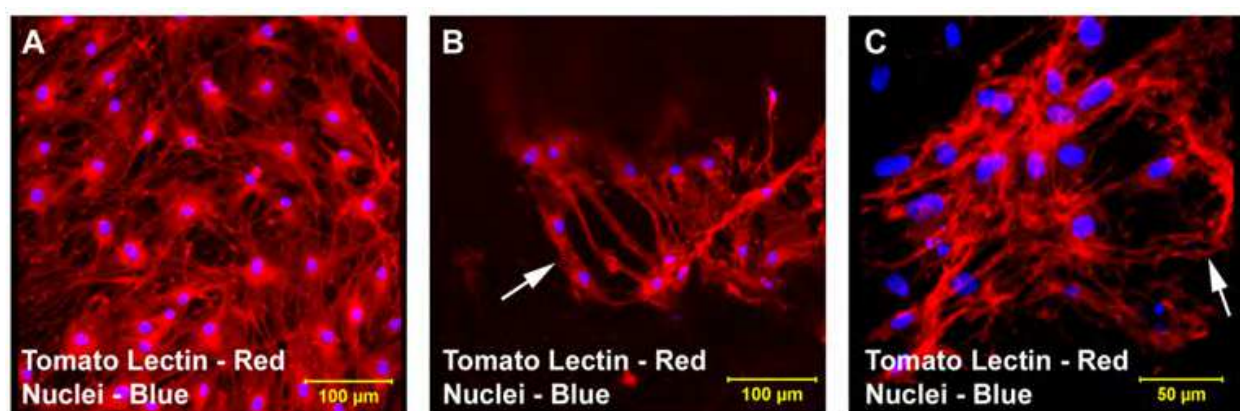


Fig. 3. Localization of BMSC-derived endothelial cells by Texas Red labeled Lycopersicon Esculentum lectin/Tomato Lectin (LEL/TL) staining. BMSCs cultured in collagen-gel tubular scaffolds under vasculogenic or non-vasculogenic culture conditions were incubated with tomato lectin (1:50 in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5; 0.15 M NaCl) to identify endothelial cells. Confocal laser scanning microscopic analysis of day 14 tubular scaffolds in these media conditions demonstrated the typical cobblestone appearance of differentiating endothelial cells (A), fusion and self-assembly (B), and evolving primitive capillary plexus with attempted lumen formation (B-C, white arrows). Cells were also stained for nuclei (blue, DAPI). Image (A) shows a projection representing 19 sections collected at 5.05 μ m intervals (90.90 μ m). Image (B) shows a projection representing 13 sections collected at 4.05 μ m intervals (48.60 μ m). Image (C) shows a projection representing 15 sections collected at 6 μ m intervals (84.00 μ m). Merged images (A-C). (A-B, scale bar 100 μ m; C, scale bar 50 μ m).

To validate the findings of mRNA expression pattern of important vasculogenic markers in these tube cultures and to determine whether these messages were in fact translated into proteins, immunostaining of the BMSC tube culture was carried out (Table 2; Figure 4A-L; Figure 5A-L).

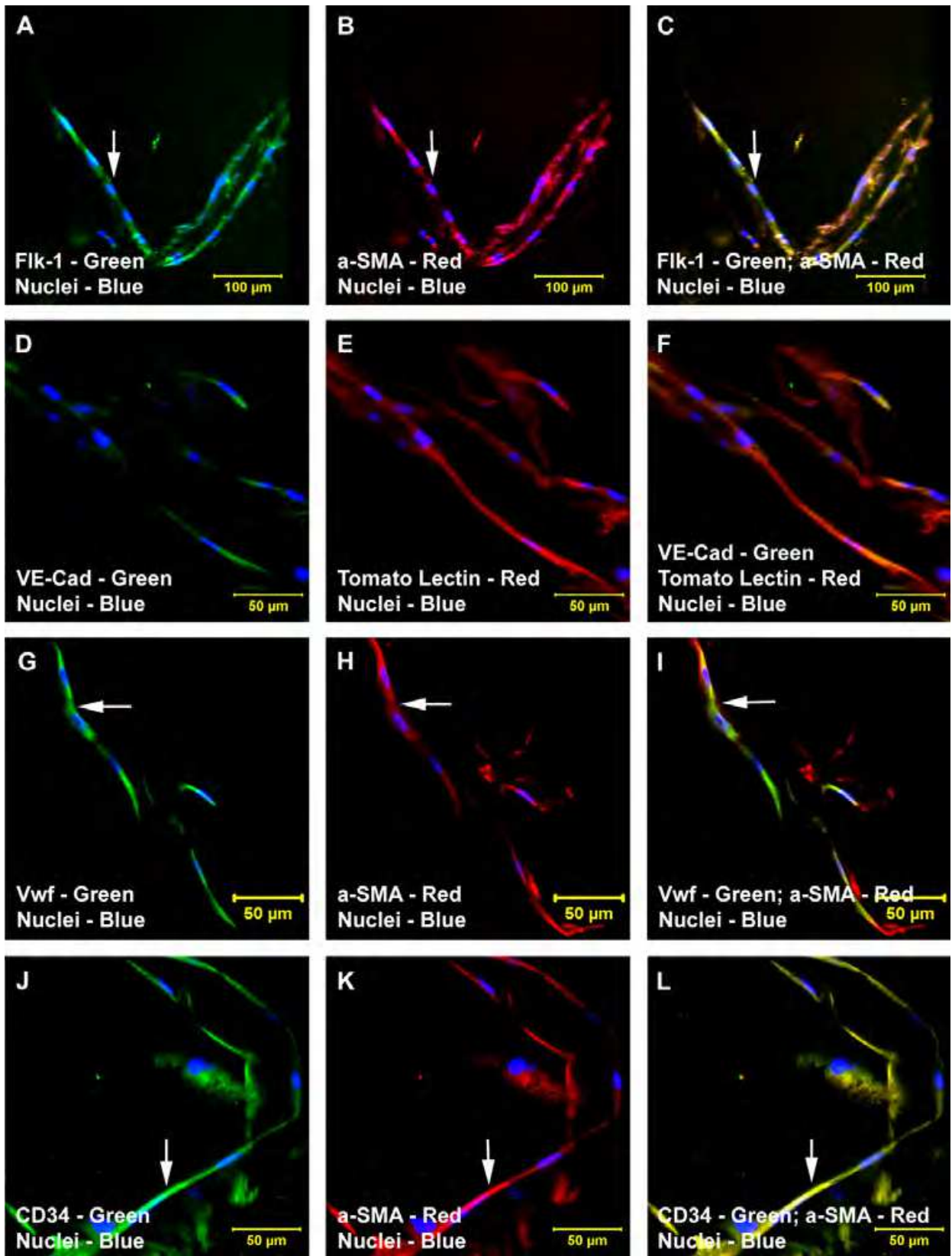


Fig. 4. Expression pattern of various vasculogenic markers in tubular scaffold by confocal microscopy. Localization of key endothelial and smooth muscle cell phenotypic markers of day 21 vasculogenic and non-vasculogenic tube cultures demonstrated the expression of

Flk1 (A, C), VE-cadherin (D, F), Vwf (G, I), CD34 (J, L), tomato lectin (E, F) and α -SMA (B-C, H-I, K-L). Dual immunostainings of these tube cultures (mesenchymal stem cell growth media, MSCGM or microvascular endothelial growth medium, EGMMV) revealed areas of elongated and flattened cells composed of varying degrees of mature endothelial and smooth muscle cells (A-L). These cells were organized into a loose delicate monomer network of nascent capillary-like structures composed of mature endothelial and smooth muscle cells. In addition, tube-like structures were emanating from the mixed population of differentiating vasculogenic cells represented by their distinct morphology and phenotypic expression (white arrows, A-C; white arrows, G-L). Cells were also stained for nuclei (blue, DAPI). Images (A-C) show a projection representing 15 sections collected at 3.05 μ m intervals (42.70 μ m). Images (J-L) show a projection representing 15 sections collected at 5.05 μ m intervals (70.70 μ m). Merged images (A-L). (A-C, scale bar 100 μ m; D-L, scale bar 50 μ m). Adapted from Valarmathi et al., 2009.

Primary antibodies	Dilutions	Source	Cell target
BMSCs characterization markers			
CD11b	1:50	BD Pharmingen	Leukocytes
CD31	1:10	Abcam	Endothelial
CD45	1:50	BD Pharmingen	Hematopoietic
CD90	1:50	BD Pharmingen	BMSCs
Endothelial cell differentiation markers			
CD34	1:100	Santa Cruz Biotechnology	Endothelial
Flk-1	1:100	Santa Cruz Biotechnology	Endothelial
VE- cadherin	1:100	Santa Cruz Biotechnology	Endothelial
Pecam1	1:100	Santa Cruz Biotechnology	Endothelial
Vwf	1:100	Santa Cruz Biotechnology	Endothelial
Tomato lectin	1:50	Vector Laboratories	Endothelial
Smooth muscle cell differentiation markers			
α -SMA	1:100	Sigma-Aldrich	Smooth muscle

Table 2. Primary antibodies used in this study (Valarmathi et al., 2009).

It is well known that endothelial cells share a large majority of their characteristic antigenic markers with other types of hematopoietic and mesenchymal cells (Bertolini et al., 2006). Therefore, antigens such as CD31, CD34, CD144 (VE-cadherin), CD146, Vwf or CD105 are not only expressed by endothelial cells but also expressed by hematopoietic cells (specifically HSCs), platelets and certain subpopulations of fibroblasts. Hence to identify the differentiated and matured endothelial cells in the tubular scaffold a battery of various early and late stage vasculogenic markers such as Pecam1, CD34, Flt1, Flk1, VE-cadherin and Vwf were employed. In addition, tomato lectin, another marker specific for rat vascular endothelial cells, was found closely associated with Flk1 and Vwf staining. These endothelial associated markers localized to endothelial cell clusters and capillary-like structures that were present throughout the tubular construct. This suggests that BMSC-derived endothelial cells assembled into endothelium-lined tube-like structures and initiated the process of vasculogenesis, consistent with our previous report (Valarmathi et al., 2008). In addition, the BMSC-derived cells and the microvessel-like structures expressed the smooth muscle antigens, α -SMA. These α -SMA positive cells were recruited in juxtaposition to the tandemly arranged endothelial cells and, were attached and wrapped around in such a way that is reminiscent of in vivo microvessel morphogenesis.

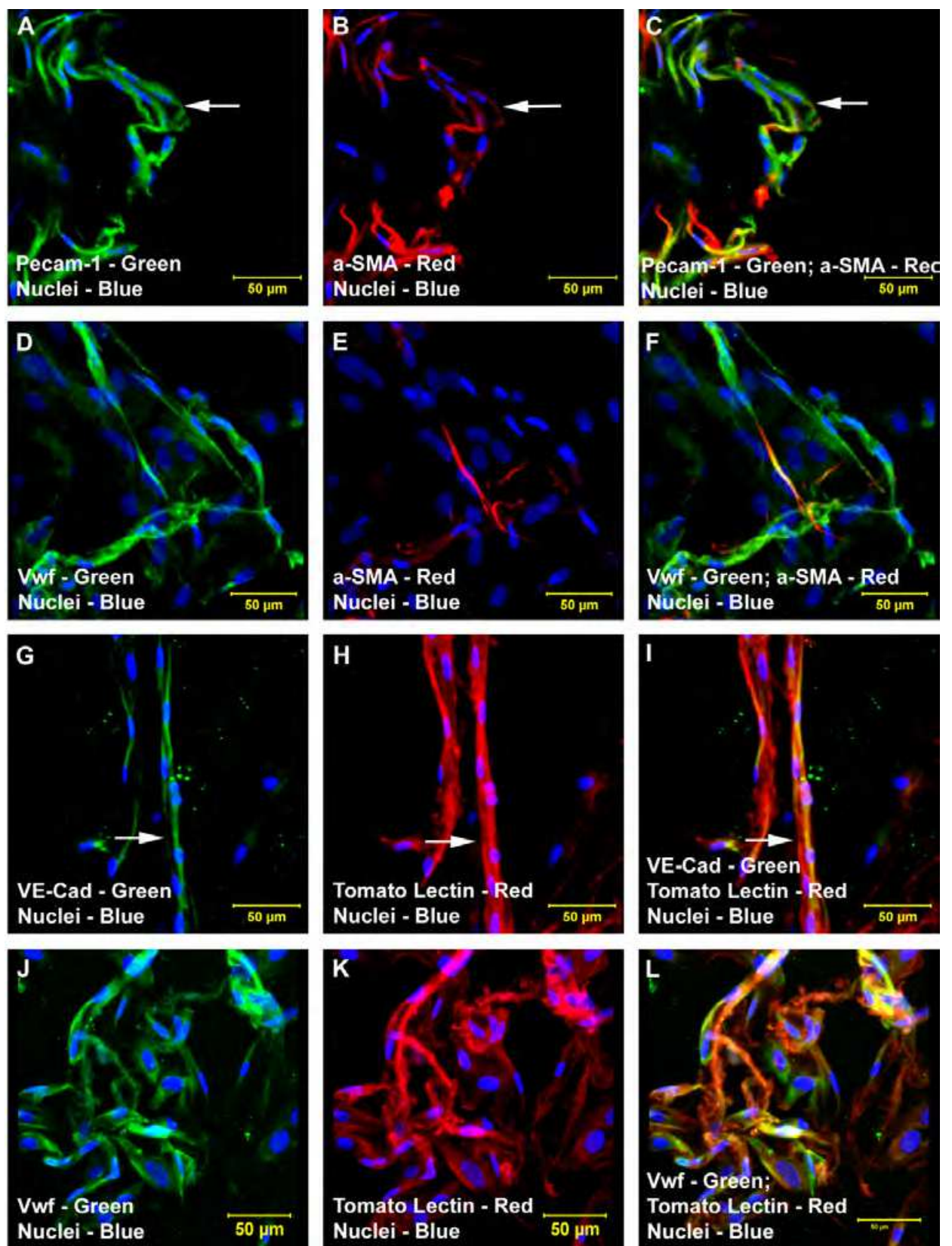


Fig. 5. Expression pattern of various vasculogenic markers in tubular scaffold by confocal microscopy. Localization of key endothelial and smooth muscle cell phenotypic markers of day 21 vasculogenic and non-vasculogenic tube cultures demonstrated the expression

of Pecam1 (A, C), Vwf (D, F; J, L), VE-cadherin (G, I), tomato lectin (H-I; K-L) and α -SMA (B-C, E-F). Dual immunostainings of these tube cultures (mesenchymal stem cell growth media, MSCGM or microvascular endothelial growth medium, EGMMV) revealed areas of elongated cells composed of both mature endothelial and smooth muscle cells (A-L). These cells were organized into a loose delicate network of nascent capillary-like structures composed of mature endothelial and smooth muscle cells and showed evidence of central lumen formation (white arrows, A-C, G-I). These cells formed developing microvessel-like structures (D-L). The linear nascent capillary-like structures showed translucent central lumen (white arrows, G-I). In addition, the cells were organized into a loose network of vascular cells and were in a ribbon-like configuration (D-F). These aligned vascular cells transformed into thin tube-like structures reminiscent of *in vivo* microvessel morphogenesis (D-L). Cells were also stained for nuclei (blue, DAPI). Images (A-C) show a projection representing 19 sections collected at 5.05 μm intervals (90.90 μm). Images (D-F) show a projection representing 16 sections collected at 4.05 μm intervals (60.75 μm). Images (G-I) show a projection representing 13 sections collected at 3.05 μm intervals (36.60 μm). Images (J-L) show a projection representing 23 sections collected at 4.05 μm intervals (89.10 μm). Merged images (A-L). (A-L scale bar 50 μm). Adapted from Valarmathi et al., 2009.

Similarly, it is critically important to characterize the ultrastructural morphology of any stem cells that are directed to differentiate into vascular lineage cells. Scanning electron microscopic (SEM) analysis of the tubular constructs depicted the pattern of microvessel morphogenesis and maturity. These formed nascent capillary-like structures and elongated tube-like structures revealed patent lumen-like structures, elucidating the vessel-maturation (Figure 6A-H). Besides, transmission electron microscopic (TEM) analysis revealed elongated capillary-like structures lined by differentiating endothelial cells (Figure 7A-F). These cells showed electron dense bodies as well as numerous small pinocytotic vesicles adjacent to the endothelial cell membranes as well as in their cytoplasm (Figure 7B, black arrows). In addition these cells exhibited variously sized cell-cell junctions, which have the appearance of typical *in vivo* endothelial tight junctions (Figure 7C-F).

Furthermore, the ability to identify endothelial cells based on their increased metabolism of Ac-LDL was examined using Ac-LDL tagged with the fluorescent probe, Dil-Ac-LDL. BMSC-derived endothelial cells and the nascent capillary-like structures were brilliantly fluorescent whereas the fluorescent intensity of smooth muscle cells/pericytes was barely detectable as reported previously (Valarmathi et al., 2009). This suggests that the formed endothelial cells were not only fully differentiated but also functionally competent and matured (Figure 8A-C).

This behavior of BMSCs and their exhibition of vasculogenic differentiation potential can be attributed to the nature of microenvironmental factors in this culture conditions. The preconditioned factors in the growth microenvironment rendered by the aligned type I collagen fibers of the tubular scaffold and the soluble differentiating factors provided by the vasculogenic or non-vasculogenic medium may be behind the BMSC fate determination. Further work is ongoing to determine whether our prevascularised tubular scaffolds can survive implantation into a tissue defect and is able to anastomose promptly with vascular sprouts emanating from the host. Finally, our morphological, molecular, immunological and biochemical data reveal the intrinsic vasculogenic differentiation potential of BMSCs under appropriate 3-D environmental conditions.

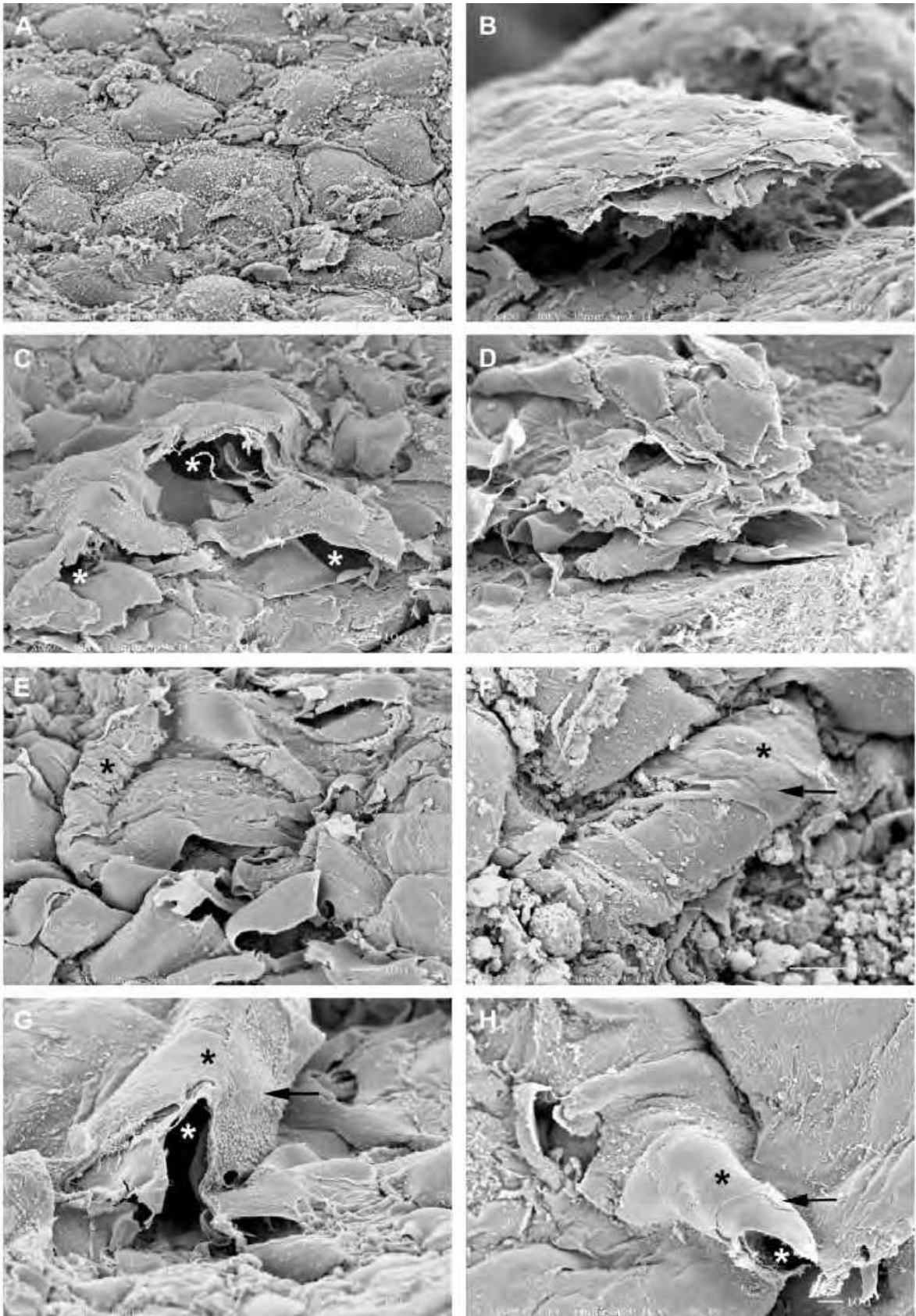


Fig. 6. Scanning electron microscopic (SEM) analysis of tubular constructs. SEM analysis of day 28 tubular constructs under vasculogenic or non-vasculogenic culture conditions

showed the typical cobblestone appearance of differentiating endothelial cells (A), stratification and networking (B-D), and the presence of smooth-walled tube-like structures with its attached smooth muscle cells and/or pericytes (black arrows, F-H). Multiple smooth muscle-like cells were wrapping around these tube-like structures (black asterisks, Figure E-H). These cylindrical structures revealed the presence of evolving patent lumens (white asterisks, C, G, H). (A-H, scale bar 10 μm). Adapted from Valarmathi et al., 2009.

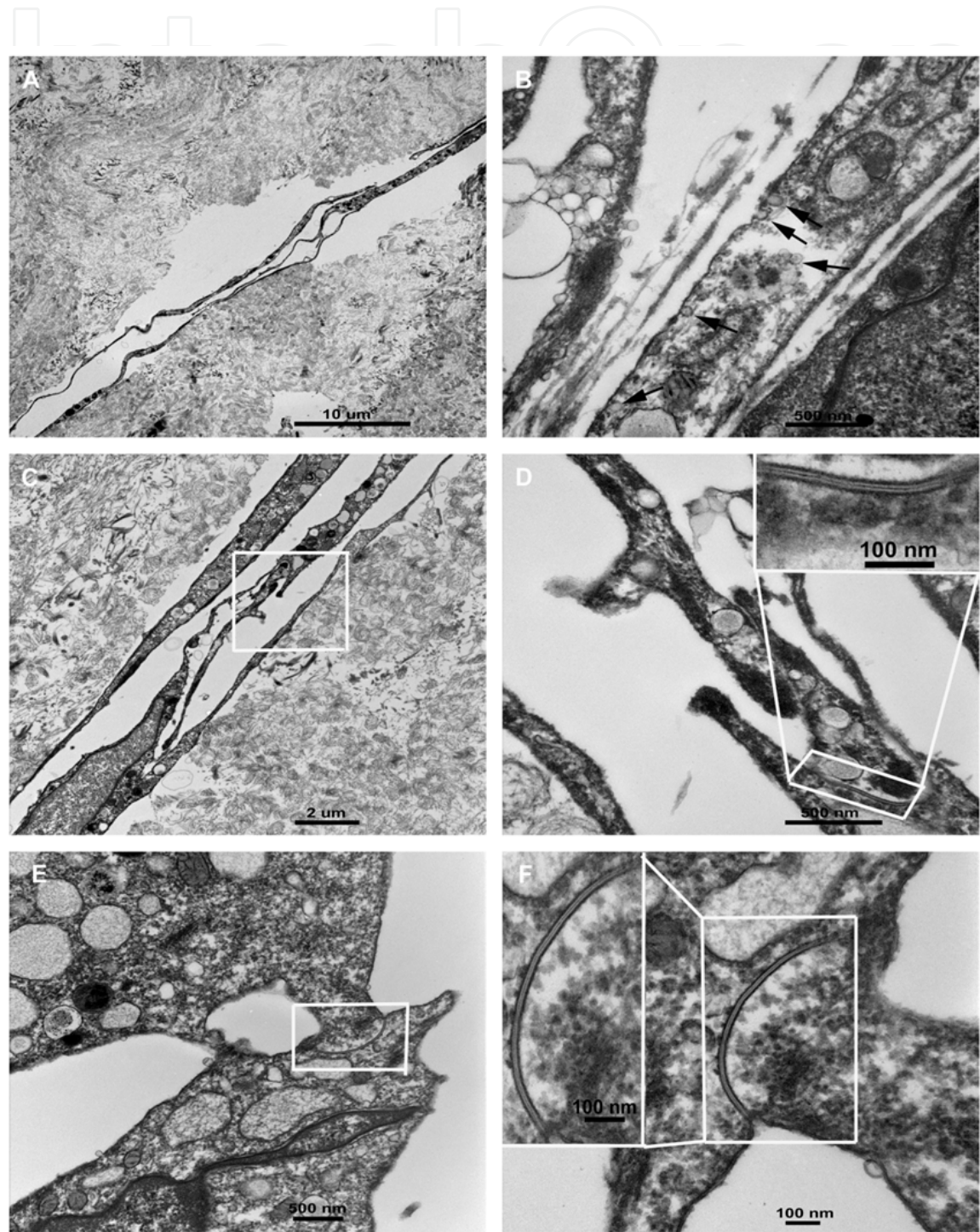


Fig. 7. Transmission electron microscopic (TEM) analysis of tubular constructs. TEM analysis of day 28 tubular constructs under vasculogenic or non-vasculogenic culture conditions showed a

vessel-like structure containing many small dense bodies within endothelial cells on either side of the lumen (A). Note the most obvious feature of endothelial cells, the concentration of small vesicles (pinocytotic vesicles) adjacent to the endothelial cell membranes and cytoplasm (B, black arrows). The interdigitating endothelial cells showing junctional regions (C, E, inserts, lower magnification). The typical adherent junction could be visualized between two overlapping endothelial cell processes (D, F, inserts, higher magnification). (Hanaichi et al., 1986).

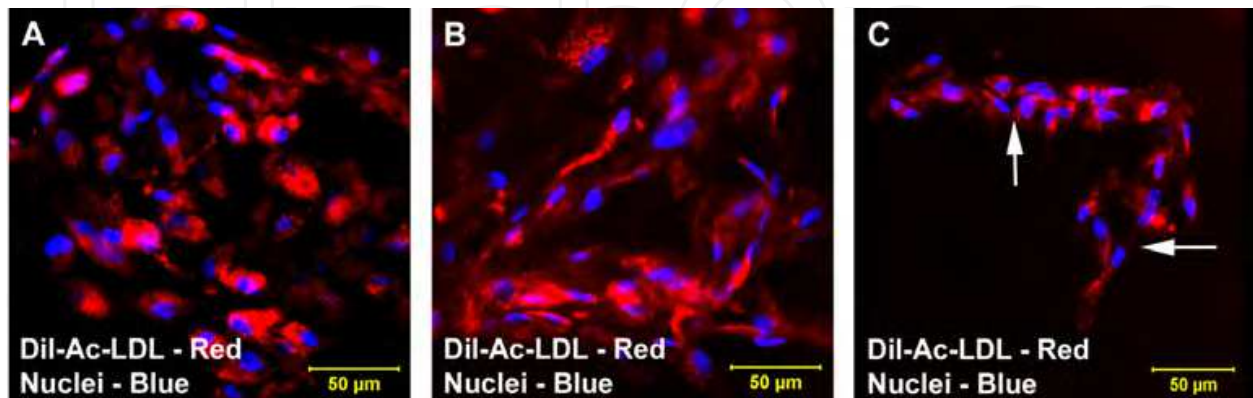


Fig. 8. Characterization of BMSC-derived endothelial cells by Dil-Ac-LDL uptake. BMSCs cultured in collagen-gel tubular scaffolds in vasculogenic or non-vasculogenic culture conditions were incubated with 10 µg/ml of Dil-Ac-LDL for 4 to 6 hours. Confocal laser scanning microscopic analysis of day 21 tubular scaffolds in microvascular endothelial cell growth medium (EGMMV) revealed typical abundant punctate perinuclear bright red fluorescence of the differentiated and matured endothelial cells (A). These labeled vascular cells were self-organized into tangled nascent linear capillary-like structures (B), assembled into solid cord of cells and, transformed into tube-like structure with attempted lumen formation (C, white arrows). Cells were also stained for nuclei (blue, DAPI). Image (C) shows a projection representing 22 sections collected at 5 µm intervals (105.00 µm). Merged images (A-C). (A-C, scale bar 50 µm). (Voyta et al., 1984) (Adapted from Valarmathi et al., 2009)

Previously, it has been shown that mature vascular endothelium can give rise to smooth muscle cell (SMC) via endothelial-mesenchymal transdifferentiation, coexpressing both endothelial and SMC-specific phenotypic markers (Frid et al., 2002). Recently, it has been shown that Flk1-expressing blast cells derived from embryonic stem cells can act as precursors that can differentiate into both endothelial and mural cell populations of the vasculature (Yamashita et al., 2000). In this study, clonal analyses revealed the bi-lineage potential of BMSCs, suggesting that both endothelial and smooth muscle/pericytes could be derived from single colonies. However, in general, BMSCs-derived colonies are clonal or nearly clonal. The colonies of BMSCs resultant from a number of cells may represent co-existence of several subclones, each capable of differentiating into specific lineages. Hence, single cell-derived colonies that are stably transfected with lineage specific markers are needed to gain more meaningful insights and address the origin of both lineages.

Our results indicate that the 3-D tubular scaffold with its unique characteristics provides a favorable microenvironment that permits the development of in situ microvascular structures. Moreover, this is the first ever documentation that explicitly demonstrates that adult BMSCs under appropriate in vitro environmental cues can be induced to undergo vasculogenic differentiation culminating in microvessel morphogenesis (Valarmathi et al.,

2009). Our model recapitulates many aspects of in vivo de novo vasculogenesis. Thus, this unique culture system provides an in vitro model to investigate the maturation and differentiation of BMSC-derived vascular endothelial and smooth muscle cells in the context of postnatal vasculogenesis. In addition, it allows us to elucidate various molecular mechanisms underlying the origin of both endothelial and smooth muscle cells and especially to gain a deeper insight and validate the emerging concept of 'one cell and two fates' hypothesis of vascular development (Yamashita et al., 2000).

5. Conclusions

Here we report a unique 3-D culture system that recapitulates many aspects of postnatal de novo vasculogenesis. This is the first comprehensive report that evidently demonstrates that BMSCs under appropriate in vitro environmental conditions can be induced to undergo vasculogenic differentiation culminating in microvessels. Since BMSCs differentiated into both endothelial and smooth muscle cell lineages, this in vitro model system provides a tool for investigating the cellular and molecular origin of both vascular endothelial cells and smooth muscle cells. In addition, this system can potentially be harnessed to develop in vitro engineering of microvascular trees, especially using autologous bone-marrow-derived BMSCs for therapeutic purposes in regenerative medicine.

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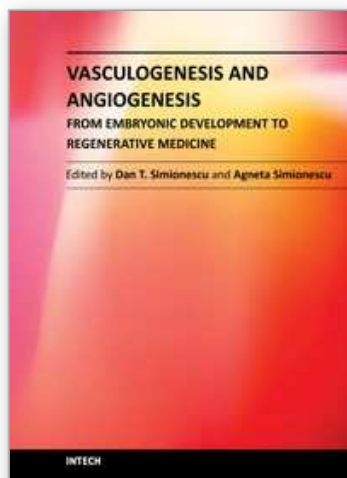
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Vasculogenesis and Angiogenesis - from Embryonic Development to Regenerative Medicine

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Vasculogenesis is the process of new blood vessel formation during embryonic development of the cardiovascular system. This is followed by formation of a vascular tree and finally the cardiovascular system with the myriad of blood vessels that nourish all tissues and organs. Angiogenesis, on the other hand is the process by which new blood vessels take shape from existing blood vessels by "sprouting" of endothelial cells thus expanding the vascular tree. Both scenarios are based on activation, migration, proliferation and maturation of unique precursor cells. The study of blood vessel formation is an essential component of embryonic development, congenital malformations, degenerative diseases, inflammation and cancer and thus has widespread appeal to the biomedical field. Moreover, scientists are now harnessing this information for the purpose of building living blood vessel substitutes for replacement of diseased arteries and veins. This book highlights novel advances in the field of vasculogenesis and angiogenesis, including embryogenesis and development, regulation of progenitor cells, cancer and blood vessel regeneration. We consider this book a good initial source of information for graduate students, medical students and scientists interested in the intricacies of blood vessel formation, maturation, disease and replacement.

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