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Embryonic Mesoderm Progenitor Cells Exhibit VEGF Induced Differentiation and In Vitro Vasculogenesis

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Abstract

Human embryonic mesoderm progenitor cells (HEMPCs) upon differentiation give rise to embryo angioblasts, which differentiate into endothelial tissue and can form a primitive vascular network; making them a prime source of cells for transplantation and tissue engineering. Here, we have analyzed the effects of vascular endothelial growth factor (VEGF-121) on previously uncharacterized lines of HEMPCs and what role it plays in their differentiation, expression of endothelial specific proteins, and formation of vascular like structures in vitro. Different lines of HEMPCs were cultured and their behavior was characterized in vitro. Cells were characterized qualitatively and quantitatively; recording morphological changes and measuring fluorescence, respectively. Upon culture the cells express an increase in expression of endothelial specific proteins such as platelet endothelial cell-adhesion molecule-1 (PECAM1) and von Willebrand Factor (vWF), upon addition of VEGF-121. Cells also exhibit elongation, migration, proliferation, and formation of vessel like structures: morphological changes characteristic of vasculogenesis. An immuno plate assay was then created to test the effect of different concentrations of VEGF-121 on endothelial specific protein expression. Protein expression was quantified using a fluorescence plate reader. Upon further experimentation, we will be able to better understand the role VEGF-121 plays in differentiation and in vitro vasculogenesis of HEMPCs, allowing us to explore their potential for use in regenerative medicine.

Keywords: Embryonic mesoderm progenitor cells, In vitro, Vasculogenesis

1. Introduction

The field of regenerative medicine is growing and advancing, with new discoveries giving promise to the field. With the developments of tissue transplantation and tissue engineering, vascularization is a key component in the success of these therapeutic applications. Because human embryonic mesoderm progenitor cells have the potential to differentiate into many different cell types they have the capability of being a good source of cells for the vascular structures needed in tissue engineering or tissue transplantation. However before these stem cells can be used they must be differentiated into endothelial cells forming vascular like structures. Endothelial cells (ECs) are thin, flattened cells that line the inside of blood vessels in a continuous monolayer in all blood vessels through the entire circulatory system. ECs are best identified by their specific location and function, but there are also various cell-surface molecules (such as vWF, CD31, CD34, CD105, vascular endothelial cadherin [VE-cadherin], vascular endothelial growth factor receptor 1 [VEGFR-1], VEGFR-2, Tie-1, Tie-2) that characterize their phenotype[2]. The formation of endothelial tissue (vasculogenesis) is a process in which the embryo angioblasts are differentiated from mesodermal cells and organized to form a primitive vascular network[8]. Although the molecular mechanisms responsible for vasculogenesis are currently not completely understood, the pivotal role of VEGF in this process is evident[8]. The interaction of VEGFs with VEGFR-2 has been widely studied and appears to play a central role in stimulating endothelial cell migration, differentiation, proliferation and survival[11]. Differentiation of embryonic stem cells into endothelial

cells and the formation of vessel structure have been studied extensively in murine embryogenesis, including maturation steps, molecular events, and the involvment of growth factors. However, lack of experimental cell systems has made it difficult to study these developmental processes in humans until now[5]. In order to better understand the role that VEGF plays on the process of vasculogenesis in vitro, it is necessary to understand how VEGF affects cell proliferation, differentiation, elongation, and migration during vasculogenesis. In this study, we analyze the effects that VEGF has on the differentiation of human embryonic mesoderm progenitor cells to endothelial cells and the role it plays in the promotion and formation of vascular like structures in vitro. The role VEGF plays in triggering portions of the vascularization process, including the role it plays in triggering the expression of various proteins for endothelial cell differentiation and vasculogenesis was explored. Our experiments seek to determine which aspects of in vitro vasculogenesis are under the control of VEGF.

2. Materials and Methods

2.1. Cell Culture

Five different lines of human embryonic mesoderm progenitor cells were received from Biotime, lines 19-22, and 24. Cells were grown in 225 cm^3 cell culture flasks (Corning) in 5% CO_2 and MV2 medium (Promocell). Cells were plated out on p-60 tissue culture dishes (Falcon) and grown in MV2 medium. Lines 21 and 24 were tested first. Each line was plated into a control plate and an experimental plate. Upon confluency of the cells in the plate, the experimental plate received 50ng/mL of VEGF-121. After three days, images of the cells were taken using a Nikon D5000 and a Leitz DMIRB. Lines 22 and 24 were then tested. Cells were plated onto p-60 plates, two for each line, one control and one experimental. Upon confluency, 25ng/mL of VEGF-121 were added to the experimental plate. After three days images were taken using a Nikon D5000 and a Leitz DMIRB and cells were placed back in the incubator at 37°C.

2.2. Immunohistochemistry

Nine days after the addition of 25ng/mL of VEGF to the experimental plates for lines 22 and 24, immunohistochemistry (IHC) was done on all four plates. Cells were fixed to the plate with an Acetone-Methanol solution for 10 minutes at room temperature. Two sections of the each plate were marked off with a pap-pen, and cells on each section of the plate were stained for 50 minutes with anti-human primary antibodies, either PECAM-1 or VWF (Santa Cruz Biotechnology). The secondary antibodies used were Alexa Fluor goat anti-mouse IgG 594 or 488 (Molecular Probes). Plates were examined with a conventional fluorescence microscope (Nikon Eclipse E800) and images were taken using a Nikon D60. Fluorescence was quantitated with ImageJ software.

2.3. Immuno Plate Assay

Two black 96-well tissue culture plates (Corning) were seeded 30 wells with line 24 cells and MV2 with 5% FBS. After four days, upon confluency, medium was changed and replaced with MV2 with 5% FBS and a specific concentration of VEGF-121. Concentrations of VEGF-121 tested were 0ng, 0.5ng, 1ng, 5ng, 10ng, 20ng, 30ng, 40ng, and 50ng. Medium with each concentration was seeded to the wells in triplicate, in which three wells received one of the 10 concentrations. For one plate, three days post addition of VEGF-121, and for the second plate, seven days post addition of VEGF-121, immunohistochemistry was done. Cells were fixed to the plate with an Acetone-Methanol solution for 10 minutes at room temperature. Cells in each well were stained for one hour at 37°C with anti-human primary antibodies for PECAM-1 (Santa Cruz Biotechnology). The secondary antibodies used were Alexa Fluor goat anti-mouse IgG 594 (Molecular Probes). Plates were analyzed using a fluorescence plate reader (FLUOstar).

3. Results

3.1. VEGF Induced Morphological Changes

Under light microscope analysis, culture of both lines 21 and 24 in the presence of 50ng/mL of VEGF-121 resulted

in a clear morphological change (Figure 1 & 2). The control for line 24 does not display any significant morphological changes, and under high magnification (200x) cells appear to be lining up in a parallel direction (Figure 1G). The control line did not show evidence of marked cell branching, migration, or formation of branched capillary like structures over the time course of incubation. Line 24 cells in the presence of VEGF-121 exhibited migration, cell elongation, and formation of branched capillary-like structures. Morphological changes were first observed at one-day post addition of 50ng/mL of VEGF-121. Migration is evident, as cells appear to be starting to create branched honeycomb structures. Elongation and branching of cells can also be seen one-day post addition of VEGF-121 (Figure 1G). At three days post addition of VEGF, cells had elongated even further, displayed additional migration, and had formed branched capillary like structures.

The control plate for line 21 began as a nearly confluent monolayer, and over the culture period demonstrated cell proliferation, but did not display elongation, migration, or formation of capillary like structures (Figure 2A-E). After the three-day time course the control plate at high magnification (200x) cells appear to be aligned in a random cobblestone arrangement (Figure 2E). Prior to addition of VEGF-121, the experimental plate for line 21 was at near confluency, and did not present any distinct morphological characteristics of differentiation or formation of capillary like structures. At one-day post addition of VEGF-121 line 21 exhibits substantial cell migration, cell branching, and cells appeared to be arranging and connecting in the beginning of tubular structures. However at day-three post addition of VEGF-121, cell proliferation was evident but the majority of the cells did not exhibit elongation, with only a few cells making connections to form early closed ring structures. Cells exhibited mostly cobblestone like organization surrounding a few ring like structures.

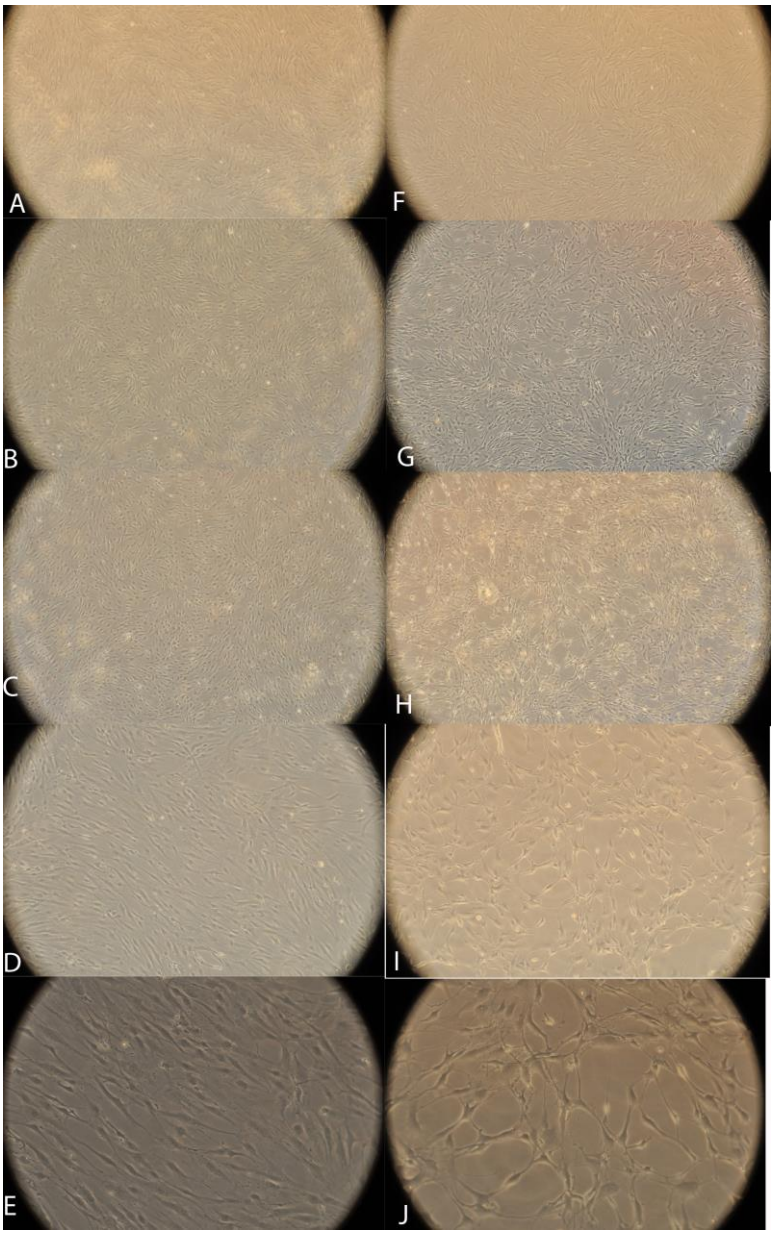

Figure 1.(A-E) Line 24 control plates and (F-J) experimental plates

Figure 1. Control plates received no VEGF-121 and experimental plates received 50 ng/mL of VEGF-121. (A) 0hrs 50x (B) 24hrs 50x (C) 72 hrs 50x (D) 72 hrs 100x (E) 72 hrs 200x (F) 0hrs 50x (G) 24 hrs 50x (H) 72 hrs 50x (I) 72 hrs 100x (J) 72 hrs 200x

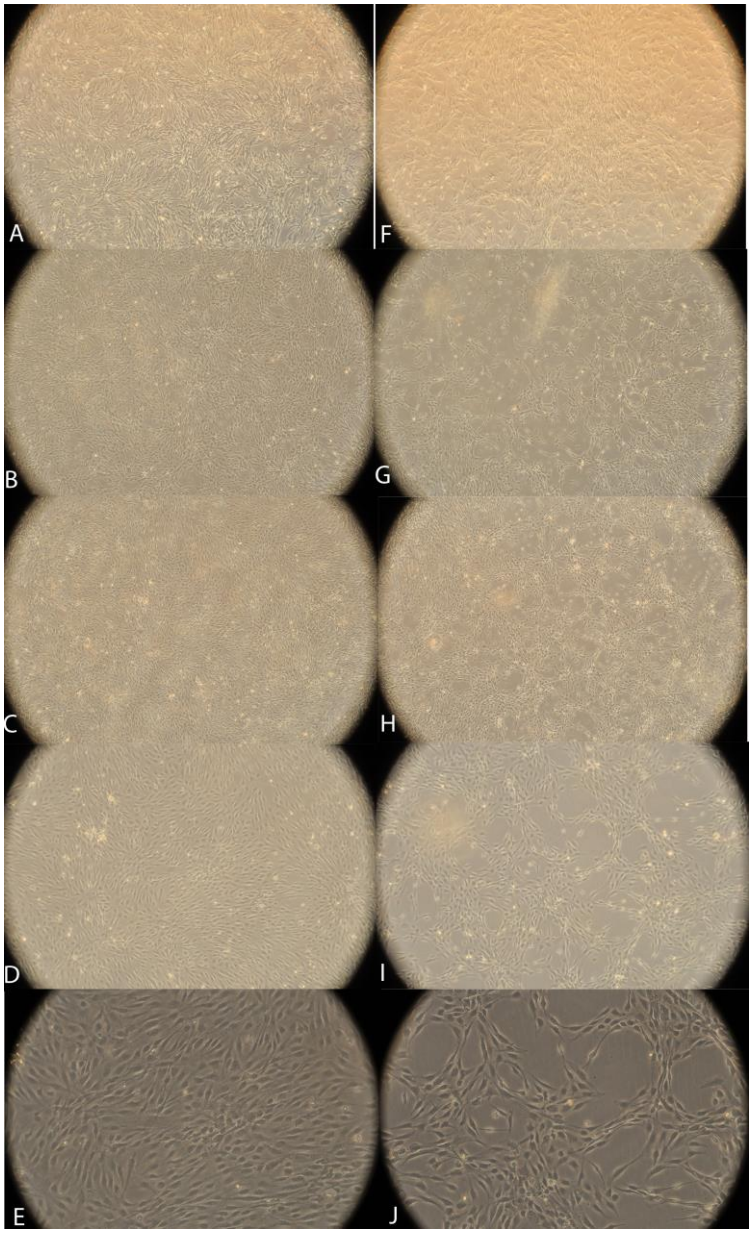

Figure 2. (A-E) Line 21 control plates and (F-J) experimental plates

Figure 2. Control plates received no VEGF-121 and experimental plates received 50 ng/mL of VEGF-121. (A) 0hrs 50x (B) 24hrs 50x (C) 72 hrs 50x (D) 72 hrs 100x (E) 72 hrs 200x (F) 0hrs 50x (G) 24 hrs 50x (H) 72 hrs 50x (I) 72 hrs 100x (J) 72 hrs 200x

3.2. Expression Of Endothelial Specific Proteins

After culture of lines 22 and 24 in 25ng/mL of VEGF-121 for nine days, immunohistochemisty was done to characterize the expression of PECAM-1 and VWF. Although neither of the lines displayed major morphological changes upon addition of 25ng/mL of VEGF-121, both lines displayed an increase in the expression of both PECAM-1 and VWF (Figures 3&4). Quantification of protein expression confirmed the small increase in expression of endothelial specific proteins (Table 1).

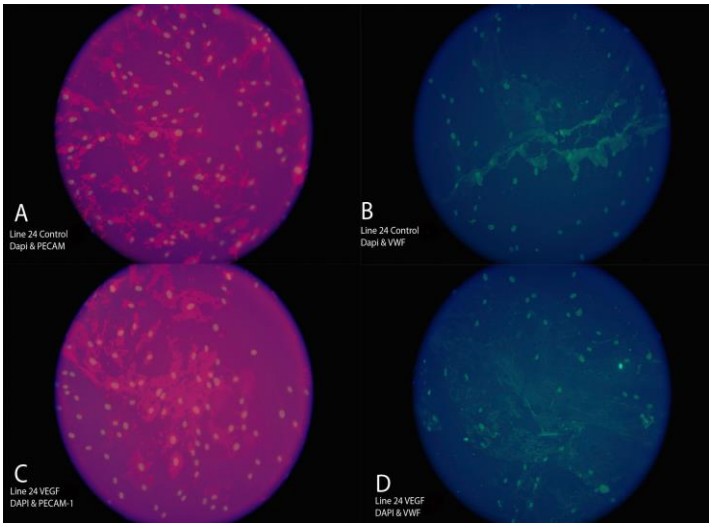

Figure 3. IHC staining of line 24 for PECAM-1 and VWF (200x)

Figure 3. (A) Line 24 control PECAM-1 & DAPI overlay (B) Line 24 control VWF & DAPI overlay (C) Line 24 VEGF PECAM-1 & DAPI overlay (D) Line 24 VEGF VWF & DAPI overlay

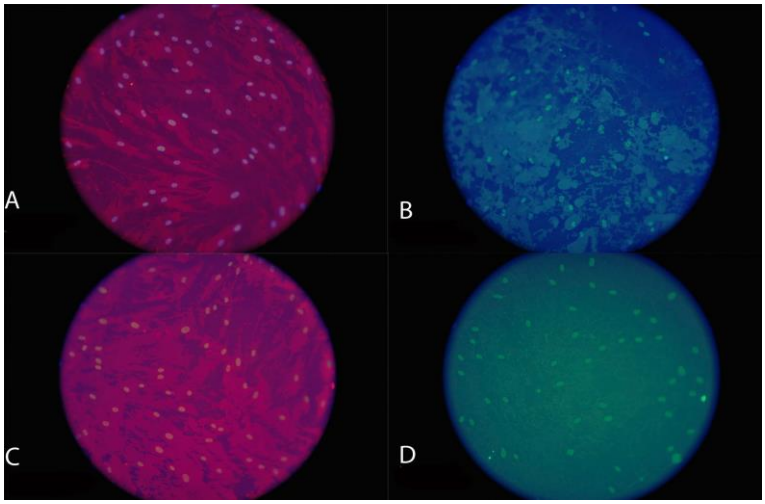

Figure 4. IHC staining of line 22 for PECAM-1 and VWF (200x)

Figure 4. (A) Line 22 control PECAM-1 & DAPI overlay (B) Line 22 control VWF & DAPI overaly (C) Line 22 VEGF PECAM-1 & DAPI overlay (D) Line 22 VEGF VWF & DAPI overlay.

Table 1. Image J quantification of PECAM-1 and VWB expression

Label	Area	StdDev	IntDen ($x10^7$)	RawIntDen	Fold Increase from VEGF/Control	Expression Increase of VEGF
Line 22 Control VWF	2509056	23.176	8.0936382	80936382		
Line 22 VEGF VWF	2509056	22.189	8.6038966	86038966	1.06304438	5.93%
Line 22 Control PECAM-1	2509056	20.491	8.260129	82601290		
Line 22 VEGF PECAM-1	2509056	21.076	8.4642935	84642935	1.02471687	2.41%
Line 24 Control PECAM-1	2509056	21.183	8.4733946	84733946		
Line 24 VEGF PECAM-1	2509056	22.293	8.8276394	88276394	1.04180672	4.01%
Line 24 Control VWF	2509056	13.375	5.7065408	57065408		
Line 24 VEGF VWF	2509056	17.09	6.4971848	64971848	1.13855049	12.17%

3.3 Immuno Plate Assay

Three days post addition of VEGF-121 immunohistochemistry was done on one plate to test the levels of PECAM-1 expression. The secondary antibody used, IgG 594, was incompatible with the filters on the plate reader and data was not able to be collected for the three day plate. For the second plate, seven days post addition of VEGF-121 immunohistochemistry was done, using IgG 488 secondary antibodies to test the levels of PECAM-1 expression. While the seven-day plate exhibited a general increase in PECAM-1 expression at increasing concentrations of VEGF-121 (Table 2), there was not a clear linear trend for the increase in expression (Figure 5).

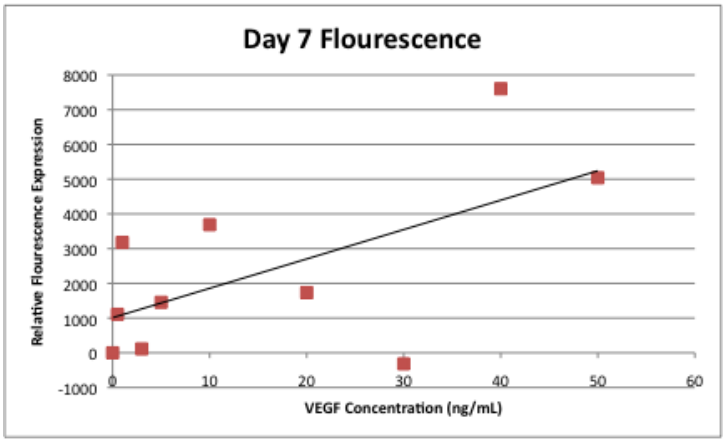

Figure 5. Day 7 Plate Flourescence Expresstion

Figure 5. Scatter plat of flourescense expression vs. concentration of VEGF-121

Table 2. Day 7 Plate Flourescence

VEGF Concentration (ng/mL)	Average Flo Read	Change in expression compared to control	Percent increase of expression from control
0	38023	0	0.00%
0.5	39131	1108	2.91%
1	41203	3180	8.36%
3	28136	113	0.30%
5	39478	1455	3.80%
10	41711	3688	9.70%
20	39756	1733	4.56%
30	37706	-317	-0.83%
40	45626	7603	20.00%
50	43063	5040	13.26%

4. Discussion

Human embryonic mesoderm progenitor cells have the potential to differentiate into many different cells types, including endothelial cells, and therefore are a prime source for use in vascular structures needed in tissue engineering. The mechanisms behind the differentiation and the vasculogenesis of theses cells are currently not fully understood, however the pivotal role of VEGF for both processes is evident. Hence, VEGF is part of all cocktails for the in vitro differentiation of endothelial progenitor cells into endothelial cells in vitro[10]. The purpose of this study was to explore the role that VEGF plays in cell proliferation, differentiation, migration and elongation during in vitro vasculogenesis. This study of the VEGF affects on previously uncharacterized HEMPCs, showed culture in VEGF-121 supplemented medium lead to HEMPCs showing an increase in expression of PECAM-1 and vWF compared to cells cultured without VEGF supplemented medium. Because the medium that the lines of HEMPCs arrived in and were cultured in, was endothelial specific MV2 medium containing 0.5ng/mL of VEGF, may have lead to subpopulations of cells becoming committed. This would explain why the cells cultured in medium without additional VEGF had some expression of endothelial specific proteins. While this may affect our analysis of the role of VEGF in HEMPC differentiation, it does not affect the clear correlation of VEGF induced increased endothelial specific protein expression.

An immuno plate assay was created to analyze the affects of various concentrations of VEGF on endothelial specific protein expression. The data from the day seven plate suggests a general correlation between the increase of VEGF concentration and PECAM-1 expression. However there was no clear linear correlation upon graphical analysis. In order to better understand the results of the assay, the procedure should be repeated, and multiple sets of data needs to be compared to see if this assay gives reliable results and if the initial data collected is typical and reproducible.

Nonetheless, there was a clear correlation between concentration of VEGF and morphological changes consistent with vasculogenesis. Even though an increase in endothelial specific protein expression was seen at VEGF concentrations of 25ng/mL, there were no morphological changes noted compared to the control even after seven days of culture. Yet when cells were cultured with 50ng/mL of VEGF, marked morphological changes such as elongation, migration and formation of capillary like structures were seen at one day post addition of VEGF, with even further morphological changes seen upon three days of culture. The difference in morphological changes was very pronounced in the lines cultured with addition VEGF compared to the control lines. The control lines exhibited some proliferation but did not show any elongation, migration or formation of capillary like structures. It becomes evident upon examination of the data that at higher concentration of VEGF, cells are signaled to begin morphological changes consistent with those of vasculogenesis. The data shows that VEGF not only plays a role in determining endothelial specific protein expression, it plays a major role, at the right concentrations, in cell elongation, migration and in the formation of capillary like structures.

While many studies of in vitro embryonic stem cell differentiation and endothelial cell vasculogenesis are done on plates coated with gelatin[6], collagen gels[10], or matrigel, we were able to successfully culture our HEMPCs on tissue culture plates without the addition of any plate coating. This makes our model for differentiation and in vitro vasculogenesis different than the model assay commonly accepted for in vitro vasculogenesis.

Upon further experimentation it will be possible to better understand the role that VEGF plays in in vitro HEMPC differentiation and vasculogenesis. Furthering our knowledge of this important relationship will allow us to be better able to

create vascular tissue for use in implantation or tissue engineering, giving much promise to the field of regenerative medicine.

5. Acknowledgment

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6. References

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