Gene Level Analysis of Endothelial Progenitor Cells in Co-Culture

by

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Thesis submitted in partial fulfillment of
the requirements for the degree of Master of Science in the Department of
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ABSTRACT

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Abstract

hCB-ECs show varying morphology under co-culture conditions. They are known to form networks when co-cultured on matrices like Matrigel, collagen gels and SMCs. Optimizing the co-culture model for the formation of networks can enable better understanding of angiogenesis and can aid in the area of tissue engineered organs while creating a model with no networks can help in producing a smooth layer of cells for tissue engineered blood vessels. Additionally, the study of networks on smooth muscle cell surfaces gives a better approach to understanding the in vivo phenomena.

The main goal of this study was to identify conditions that would support the formation of networks and to study the gene level alterations in the hCB-EC cells between co-culture and monoculture during the formation and absence of networks. To study the morphological changes co-cultures were setup by varying the hCB-EC densities at 26,316 cells/cm^2, 52,632 cells/cm^2, 80,000 cells/cm^2 and 105,263 cells/cm^2 on SMCs. Lower seeding densities of hCB-ECs led to network formations while a confluent layer was observed at the highest density. Medium components were altered to identify factors which contributed to network formation and it was found that absence of VEGF led to delayed cell migration and network formation while absence of heparin produced sparser networks in co-culture. Microarray analysis using four different hCB-EC sources plated at the highest and lowest densities resulted in higher expression of ECM

remodeling and endothelial cell migration genes consistent to the low density conditions were networks were abundant while high density conditions expressed downregulation of cell cycle associated genes.

Dedication

I would like to dedicate this thesis to my parents and my grandmother. My parents have always encouraged me and supported every decision I have made. They have worked very hard to ensure that I have a better future and all the achievements I have made or will make will always be related to them. My grandmother has been a second mother to me and has had a huge role in molding me to be the person I am today.

I would also like to dedicate this thesis to my thesis advisor Dr. Truskey for his constant support and guidance throughout my graduate school years. His words of encouragement have been a great motivation throughout this work.

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I would like to thank Dr. Satterwhite for all her help in the microarray analysis without which this work would not have been complete. Her interest and enthusiasm has always been a great motivation. I would also like to thank Dr. Cao without whose training this work would not have been possible. I would like to thank all my friends Pavithra, Gouthami, Gayathri, Vineela and Vidhya for always having been there when I needed them and for giving me all the moral support I needed. I am also thankful to all the lab members for their help and for making my graduate school life memorable.

1. Introduction

Endothelial cells which line the tunica intima of blood vessels are known to play a significant role in blood vessel formation through processes such as angiogenesis and vasculogenesis. Lining the inner surface of the blood vessels, these cells also provide a smooth surface for blood flow preventing platelet adhesion and clotting. Circulating endothelial cells aid the process of neovascularisation.[1] Endothelial dysfunction is linked to several diseases such as arteriosclerosis, coronary heart disease, hypertension and diabetes.

Capillaries are the smallest of blood vessels with a highly permeable surface that enables transport of oxygen and nutrients to tissues. Capillary formation occurs through angiogenesis, the development of capillaries from existing ones, or through vasculogenesis, the formation of blood vessels in the embryo by incorporating endothelial cells. [6] Muscle capillaries can adjust the amount of blood flowing through them depending upon the amount required by the muscles. [2] Capillaries contain an inner endothelial layer. They also change in appearance with age. In infants the capillaries appear as a network of tubes without many loops while loops are more profound in adults tending to a more tortuous appearance towards senescence. An increase in tortuosity is also related to high blood pressure conditions while low blood pressure conditions are associated with long, straight, dilated capillary loops. [3]

Capillaries play a significant role in wound healing by providing amino acids and oxygen to the cells involved in the wound healing process. Soluble signals like cytokines and metabolites as well as insoluble signals like the ECM substrate play a significant role in this process. The soluble signals, such as growth factors like VEGF, PDGF, proteases like plasminogen activator, collagenase, and urokinase or metabolic gradients like oxygen and lactate initiates endothelial cell migration proliferation and ECM synthesis to initiate blood flow to the direction of the wound. [4]. Capillaries also play a role in tumor angiogenesis and supply nutrients to the tumor cells.

In the field of tissue engineering where biologically compatible substitutes to tissues are made, vascularization by the means of capillaries can help in the survival of the implants. The study of angiogenesis and capillary formation can thus enable significant insights to the field of tissue engineering. Also prevention of capillary formation can significantly reduce the propagation of tumors.

It has also been observed that endothelial cells when cultured in matrices of mural cells like vascular smooth muscle cells are capable of forming networks.[23] A proper understanding of network formation adds to the understanding of angiogenesis and would thus provide valuable insights to the area of tissue engineered blood vessels.

The hypothesis of this work is to create a suitable co culture model involving smooth muscle cells and late growth endothelial progenitor cells to study capillary

formation and to identify the key genes which play a role. A potential advantage is producing controlled network formations and a better understanding of endothelial cell behavior in co culture.

1.1 Angiogenesis and Vasculogenesis.

The two means by which blood vessels are formed are angiogenesis and vasculogenesis. Angiogenesis, or the process of development of new capillary vessels from pre-existing ones, occurs by a series of steps with endothelial cells as the key players. In case of sprouting angiogenesis, initial activation of the endothelial cells occurs by attachment of growth factors to their cell surface receptors. This is then followed by degradation of the extracellular matrix and basement membrane by proteases. The endothelial cells then migrate and proliferate within the matrix, get polarized and result in the formation of a lumen. The blood vessels thus formed are then stabilized by recruiting mural cells and by producing an extracellular matrix. The extra cellular matrix plays a key role in maintaining contact and in allowing permeability between the endothelial and mural cells. Angiogenesis mostly occurs during wound healing, during the menstrual cycle [49] and is also known to play a role in cancer, cardiovascular ischemic complications and rheumatoid arthritis. Growth factors known to play a major role in activating endothelial cells are VEGF, FGF and angiopoietin amongst others. [5]

Vasculogenesis is the process of formation of blood vessels in the embryo by incorporating endothelial cells.[6] In vasculogenesis, the early mesodermal cells differentiate to form the endothelial progenitor cells.[6] These cells aggregate to form blood islands which in turn fuse leading to the formation of a honeycomb shaped primary capillary plexi in the yolk sac and the embryo. These then form the arteries, capillaries and the veins. Mural cells like smooth muscle cells, fibroblasts are associated with these blood vessels as well for their stabilization. Circulating bone marrow derived endothelial progenitor cells are also thought to play a role in regeneration of blood vessels. [7]

Chemotaxis plays a major role in the formation of networks by the endothelial cells. In chemotaxis cells tend to migrate to regions of higher concentration of cells. [5] A significant change in the cells during capillary formation is the change in their cell shape. The cells become more elongated in the case of capillary formation than in the case of a confluent layer covering the basement membrane.[6] Gene expression of endothelial cells is thought to play a role in this change in cell shape leading to network formation. Some of these genes include the Dll4-notch signaling pathway activated by VEGF-A. Class 3 semaphorins and plexin D1 expressed in ECs guide the migratory pattern of ECs. [7]

1.2 Role of Growth Factors in Angiogenesis

VEGF plays a significant role in mediating angiogenesis. VEGF are classified as VEGF -A, VEGF-B, VEGF-C, VEGF-D and VEGF-E based on their functions and specificity towards the tyrosine kinase receptors VEGFR1, VEGFR2 and VEGFR3.

VEGF-B, expressed mostly in the heart and skeletal muscles of adult tissues, is located on the human chromosome 11q13. [8, 9] VEGF-B binds to receptor VEGFR1 with a high affinity and provides a poor mitogenic signal for endothelial cells. [9] VEGF-C expressed in the heart, ovary, placenta, thyroid and small intestine of humans is present on the human chromosome 4q34. VEGF-C binds specifically to receptors VEGFR2 and VEGFR3. It activates mitosis, migration of endothelial cells and increases their permeability. VEGF-D located on the human chromosome Xp22.31 is expressed in the lung heart, skeletal muscle, colon and small intestine. VEGF-D binds specifically to the receptor VEGFR2 and VEGFR3. [10]. VEGF-D acts as a mitogen for endothelial cells. [10] VEGF-C and VEGF-D on binding to VEGFR-3 play a significant role in lymphatic angiogenesis. VEGF-E is expressed by parapoxvirus Orf virus and binds specifically to VEGFR2 but not VEGFR1. It is a potent angiogenic factor. [17]

VEGFA is a mitogen for angiogenesis that plays a significant role in chemotaxis, EPC differentiation and EC proliferation. [7] VEGF A is also a chemoattractant to monocytes and thus produces chemotaxis between endothelial cell layers. [7] VEGFA

influences cell shape or cell elongation during the process of network formation. [18] Amongst the five different types of VEGFs, VEGF-A is known to be conserved amongst several species like teleost fish, mammals, birds and fish. [11] Disruption of the VEGF-A gene in the embryonic stem cells of mice was found to be lethal and also resulted in impaired angiogenesis and blood island formation. [12]

The human VEGFA gene is present in the chromosome 6p21.3. Its coding sequence is present as eight exons separated by seven introns. Alternate splicing of these exon regions in the mRNA of the VEGF gene result in the formation of six isoforms consisting of different number of amino acids. [13] The different isoforms are 125, 145, 165, 183, 189, 206. Amongst these isoforms, VEGF 165, 189, 121 are known to be present predominantly. [6] VEGF 165 is found to be expressed by most of the normal and transformed human cells while a majority of the tissues and cells express genes coding for VEGF 121 and 189. VEGF 206 has however only been found to be expressed in the human fetal liver cDNA library. [13] Vascular smooth muscle cells have been found to express all three isoforms VEGF 121, 165 and 189. [14]

The predominant VEGFA isoforms vary structurally and thus have different affinities towards binding to heparin. While VEGF 121 which lacks the sequence coded by exon 6 and 7 does not bind to heparin, VEGF 189 which has all the sequences coded from exon 2-8 intact, binds with a strong affinity and VEGF 165 which only lacks the

sequence coded by exon 6, binds with a moderate affinity to heparin. [19] VEGF 165 has a 44 basic amino acid sequence when compared to VEGF 121, which helps it bind to heparin or heparin like molecules in the matrix and cell surfaces. This sequence also helps VEGF-165 to bind with the cell surface of neuropilin-1 which in turn increases its affinity 10 fold to bind with the VEGF receptor KDR /VEGFR2 thus enabling it to be the strongest signal transducer amongst the VEGF isoforms. [14, 15] VEGF 165 is found mostly in the extracellular matrix bound to the heparin sulphate chains of the proteoglycans and also binds to the VEGFR2 receptor of endothelial cells which then activates these cells to undergo proliferation, differentiation and even sprouting required for angiogenesis. While VEGF 165 and 121 are secreted proteins, VEGF189 remains sequestered to the extra cellular matrix and requires plasmin-mediated proteolysis to convert it into a more diffusible form. [11]

VEGFA receptors are VEGFR1 and VEGFR2 predominantly present on the endothelial cell surfaces. [9] VEGFR3 receptor is present in endothelial precursor cells and in lymphatic endothelial cells. [17] Neuropilin 1 and 2 co receptors are present on endothelial cells which bind specifically to VEGF 165. [16] VEGFR1 and VEGFR2 receptor binding depends on VEGF binding to heparin. Heparin and heparin sulphate unmask receptor binding sites present in VEGFR2 promoting stronger VEGF 165 binding to the receptor. [20] VEGFA binding to VEGFR2 phosphorylates the Y1175

binding site on VEGFR2 and leads to activation of the MAP kinase pathway. Y1175 has a binding site for PLC γ. PLC γ or phospholipase C gamma is a protein kinase C- Raf-mitogen activated protein kinase (MAPK) pathway component. Thus binding of VEGF to VEGFR2 leads to phosphorylation of PLC- γ and MAP kinase which play a significant role in endothelial cell proliferation through the MAP kinase pathway. [21] VEGF 121 can aid in endothelial cell propagation but not tip cell guidance or vascular branching unlike VEGF 165. [7]

Ashikari-Hada [22] has reported that VEGF induced phosphorylation of VEGFR2 increased 1.7 fold when activated by heparin binding compared to VEGF-A without activation. Binding to VEGFR2 on the endothelial cell surface was completely inhibited when treated with heparinase. Also, when endothelial cells were cultured on collagen gel increased tube formation was observed when higher levels of heparin were added externally keeping the amount of VEGF 165 added constant.

Co-cultures can also be induced by plating ECs on SMCs or MSCs. Evensen *et al* created a co-culture by trypsinizing HUVECs and SMCs and mixing them and studied the effect on VEGF on network formation. VEGF 165 isoform was found to be predominantly secreted by smooth muscle cells. [23] Sh RNA mediated silencing of this isoform led to no network formation in a model where the human aortic smooth muscle cells and the human umbilical vein endothelial cells were mixed and plated down on a

well containing fibronectin. [23] VEGF supplemented by the external media did not produce any significant changes in this model either. Similarly when endothelial cells were plated on hyaluronic acid based hydrogels, higher levels of VEGF added to the medium increased endothelial cell tubulogenesis whereas low levels of VEGF promoted proliferation of these cells. [19]

VEGF and its receptors also play a key role in mobilizing the endothelial cells in the bone marrow and in their recruitment during angiogenesis. The role of EPCs in angiogenesis is, however, small. [7] VEGF-A is also reported to support tumor angiogenesis by binding to the its receptors present in the tumor sites thereby providing neovascularisation and thus transport of nutrients to support the growth of tumors.[24]

1.3 In Vitro Angiogenesis

In vitro angiogenesis has been studied for several applications such as creation of tissue-engineered blood vessels, creation of synthetic vascular grafts, vascularization of skin grafts and other tissue engineered grafts and tissues.

The formation of networks by the endothelial cells when grown on a matrix in vitro enabled better understanding of the process of angiogenesis in vivo. Much of the studies of tubulogenesis have been done on Matrigel. Matrigel matrix consists of a solid gel of basement membrane proteins from murine tumors containing most of the growth factors that the endothelial cells would come into contact. Matrigel matrices have been

used to conduct studies on EC migration and recruitment into network formation. [25] This was done by coating endothelial cells on a Matrigel and studying the cells every few hours. This also has helped prove chemotaxis to play a significant role in the cell migration.

Formation of networks by endothelial cells on Matrigel, consisting of basement membrane proteins from EHS mouse tumors, has also been proven to occur in vivo where mice when fixed subcutaneously with the Matrigel implants of the endothelial cells resulted in the formation of networks as they had in vitro. This has opened up the possibilities on enabling neovascularisation for tissue engineered human tissues and organs. [26] The effect of VEGF on the formation of networks has been tested by plating the endothelial cells on a Matrigel surface and adding different amounts of VEGF and studying the network formation. [19]

The use of Matrigel for studying network formation in vitro has the disadvantage of being produced from mouse tumor cells which make their incorporation into humans ethically unsafe. Matrigel also provide all the growth factors necessary for ECs but they do not provide the environment produced by cell interactions. Matrigel does not allow penetration of the networks formed within the matrix, thus forming a two dimensional network formation platform. [18]

Matrigel derived from mouse tumors result in rapid attachment and formation of networks within 4-12 hours of endothelial cell seeding. This has been increased by using Matrigel with reduced amounts of cytokines and growth factors present. Whereas in case of co culture, where the endothelial cells are mixed with fibroblasts or smooth muscle cells, network formation may take up to 6-8 days as it involves migration, proliferation and differentiation of the endothelial cells to form networks. [29] In co-culture the basement membrane is formed between the smooth muscle cells and the endothelial cells unlike in Matrigel where the basement membrane proteins are already present in the matrix but not associated with cells. Comparing the tubules formed on Matrigel and on co culture with the tubules formed in vivo. Networks formed by co culture appear similar to the ones formed in vivo [25]. The tubules formed on Matrigel are shorter and have more branching points and a 'honey comb'- like structure while those formed by co culture are well extended. The shorter tubule formation on Matrigel (>20μm) also shows the participation of only 2-3 endothelial cells in the network formation. Endothelial cells have tubules four times the size in length of those formed in Matrigel. The tubules formed in co culture are heterogeneous in length with some tubules formed from the long existing ones consistent with the conditions in vivo. The tubules formed on Matrigel are however homogenous in length and are short indicating only the tubules formed immediately upon seeding. More branching observed in

Matrigel indicates joining of endothelial cell aggregates where plated than significant migration as observed under co culture. Matrigel is also found to promote formation of tubules of fibroblasts, breast cancer cell line while this does not occur on co culture systems indicating that Matrigel is not specific for endothelial cell differentiation.

A different approach to this is the direct injection of smooth muscle cells and endothelial cells to the ischemic site resulting in better neovascularisation [27] and an increased incorporation of ECs than when ECs were injected alone. Several studies have also been done to study the role of different components on the formation of these networks when cultured on different matrixes. Effect of Tie-2 on these networks was studied by growing the SMC and the ECs on Matrigel. [27]

Another method of co culture used has been reported by Evensen *et al.* [23] where the human aortic smooth muscle cells and the human umbilical vein endothelial cells have been trypsinized and mixed before plating the cells onto a layer of fibronectin. Well-formed networks have been reported in this case as well. The EC cells have been reported to stop dividing and become more elongated and migratory after 48 hours and images of network formations on day 5 have been imaged.

In order to design a co culture system to study capillary formation of endothelial progenitor cells, we selected a lamellar direct co culture setup. Our lab has successfully produced co cultures of endothelial cells on a quiescent smooth muscle cell surface

plated on a fibronectin surface. [30] The smooth muscle cells are initially plated down and placed in a quiescent medium before the endothelial cells are plated on to the SMCs. These provide the additional advantage of being capable of implanting within the human system without much ethical problems and also the smooth muscle cells allow penetration of the networks within their layer and thus produce a more similar environment to that present in vivo. The smooth muscle cells and the endothelial cells being in contact with each other also produce a suitable extra cellular matrix which enables cell-cell interactions.

The production of networks when endothelial progenitor cells were plated on smooth muscle cells after 3-4 days was reported by Brown *et al.* [29] while working on producing a smooth endothelial cell layer in co culture. The networks formed were also found to be consistent for several different culture conditions under static co- culture and varied in the case of experiments conducted under flow.

In this work we have studied the contribution of density of cord blood endothelial progenitor cells, VEGF and heparin to network formation when in co-culture with smooth muscle cells. Gene expression studies of endothelial cells in monoculture or co-cultured with smooth muscle cells provides insights to genes which contribute to network formation and this can help optimize network formation and also help regulate the genes to aid in the fields of tissue engineering and tumor angiogenesis.

2. Methods

2.1 Cell Culture

Human Umbilical Cord Blood was obtained from the Carolina Blood Bank, Duke University. Early outgrowth cells were isolated as per protocols approved by the Duke University Institutional Review Board. hCB-ECs were isolated from the late outgrowth EPCs in umbilical cord blood samples as described by Brown. [29] The hCB-ECs were maintained in the endothelial basal media-2 (Lonza/Clonetics) supplemented with the Endothelial Growth Media-2 SingleQuots and 1 X Antibiotics/Antimycotics Solution. The hCB-ECs were used between passages 4-9 for all experiments.

Human Aortic SMCs (Lonza) are cultured with SMC Growth Media (Lonza/Clonetics) containing Smooth Muscle Growth Media-2 SingleQuots (Lonza/Clonetics) and 1X Antibiotics/Antimycotics Solution. The SMCs were used at passages 6-10 for all experiments.

All cells were cultured in a tissue culture incubator with 95% air, 5%CO_2 at 37^0C.The cells are detached from surfaces using 0.025% Trypsin/EDTA (Clonetics) and incubated at 37^0 C for 5 minutes and neutralized with equal amount of the respective culture medium.

2.2 Co-Culture Setup

All co-cultures were setup on 12 well plates (BD Falcon). The SMCs were plated at 80,000 cells/cm^2, with SMC growth media on wells that were incubated with 3.3μg/ml of fibronectin in 1ml of Dulbecco's Phosphate Buffered Saline with Ca, Mg (DPBS +/+) for one hour. After 24 hours the growth medium was switched to the serum free quiescent medium which is composed of DMEM/F-12 (Lonza/Clonetics) supplemented with 1X Insulin- Transferrin- Selenium (Invitrogen) and 1X Antibiotics/Antimycotics Solution. The stained hCB-ECs were plated at varying densities on the SMCs a day after the quiescent medium was added. The co-cultures were maintained in the EBM-2 medium for 4 days with media change on alternate days.

Table 1: Experimental setup to study the effect of seeding density of hCB-ECs on their morphology. The different densities used to study the morphological changes in hCB-ECs when in co-culture with SMCs. Numbers in parentheses represent the total number of cells added to the 3.9 cm^2 12 well plate.

Sample	SMC density (cells/cm^2)	hCB-EC density (cells/cm^2)
1	80,000 (300,000 cells)	26,316 (100,000 cells)
2	80,000	52,632 (200,000 cells)
3	80,000	80,000 (300,000 cells)
4	80,000	105,263 (400,000 cells)
Monoculture	0	80,000 (300,000 cells)

.

Table 2: Densities used for microarray analysis.

Sample	SMC density (cells/cm^2)	hCB-EC density (cells/cm^2)
Low density co-culture	80,000 (300,000 cells)	26,316 (100,000 cells)
High density co-culture	80,000	105,263 (400,000 cells)
Monoculture	0	80,000 (300,000 cells)

2.3 Cell Staining

The hCB-ECs were stained with Cell Tracker Orange (Invitrogen, 2μM). The stock solution is diluted to 8μM with DPBS+/+ and incubated with the hCB-ECs for 10 minutes. The T-75s are washed thrice with DPBS+/+ thrice before re-suspending the cells in basal media for 30 minutes. The cells are then washed with DPBS without Ca and Mg (DPBS-/-) and plated at different densities onto the SMCs in EBM-2 media. The images are viewed using a Nikon Eclipse TE 2000DU microscope and the quantification was done using the Angiogenesis Tube Formation Application of the Metamorph Software (LSRC, Duke University).

2.4 Quantification of Networks

The networks obtained under the lower density conditions were quantified using the Metamorph Premier software (LSRC 32 bit workstation, Duke University). The quantification was done using the Angiogenesis Tube Formation Application of the

Metamorph software. Various parameters such as mean tube length, number of nodes, segments and branch points were measured after normalizing the minimum and maximum width of the networks. The Angiogenesis Tube Application requires a minimum and a maximum tube diameter to be entered. The regions which exceed the maximum diameter are denoted in green as 'nodes'. The branch points represent regions where the networks branch and are denoted as orange dots. A segment is the region between two branch points. Quantification was done for two sets of experiments with three replicates each. Graphs were plotted for the average of values obtained in each experiment.

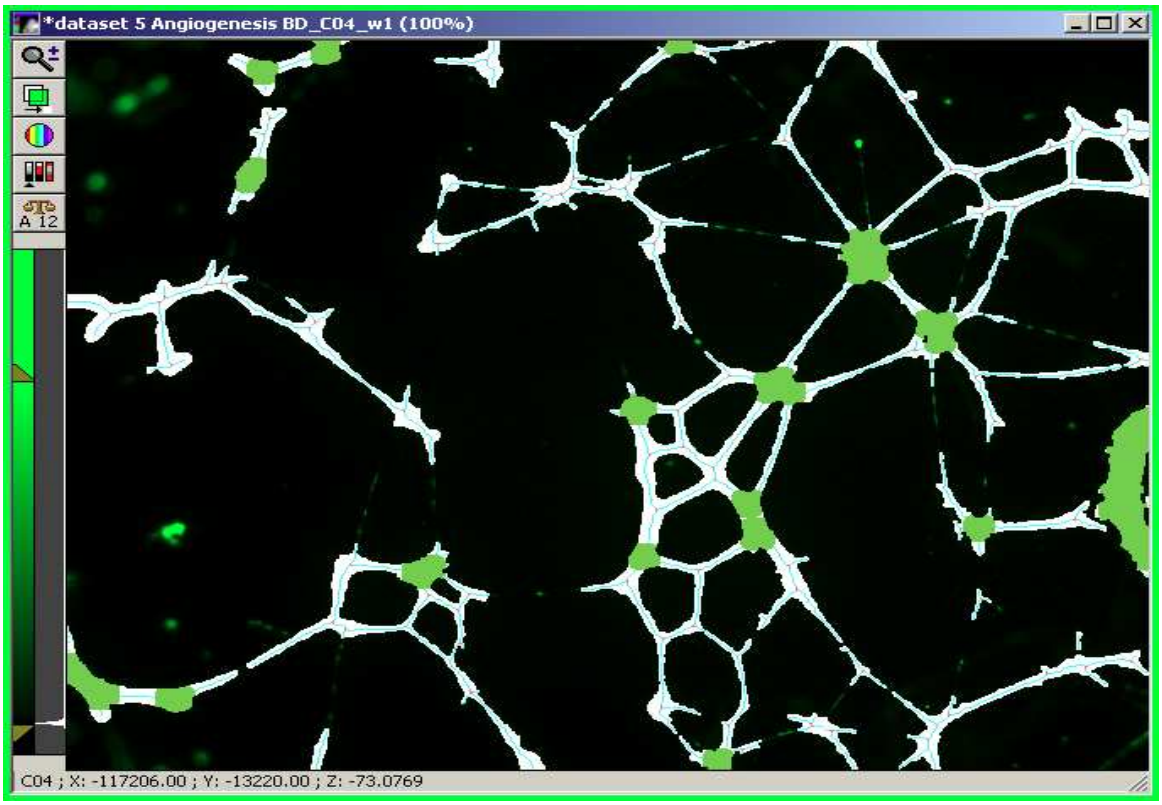

Figure 2.1: Quantification using Angiogenesis Tube Formation Application on Metamorph Premier Software. The green represent the nodes and the orange points denote the branch points.

2.5 Detection of lumen formation with dextran.

The cells were placed in co-culture using the EBM-2 medium changed on alternate days for five days. Texas Red conjugated Dextran (MW 10,000, D1828, Invitogen) was made up to 0.5 mg/ml with culture medium and incubated overnight. The cell densities used for this experiment were 80,000 cells/cm^2 of SMCs and 26,316 cells/cm^2 of hCB-ECs

2.6 Cell Separation.

The separation of hCB-ECs from SMCs in co-culture were done as described by Wallace [46] using CD 31 Endothelial Dynabeads (Invitrogen) and the Dynal MPC magnet. Following cell separation using the Dynal MPC magnet, the supernatant was discarded and the beads were washed four times in 1 ml of 0.1% bovine serum albumin (BSA, Sigma-Aldrich) in DPBS+/+.

2.7 RNA Isolation

Total RNA was isolated from the hCB-ECs in lamellar co-cultures and from the monocultures using a Qiagen RNeasy Kit (#74104) and RNase-free DNase treatment (#79254). Isolated cells were disrupted with Lysis buffer (200 ml) and the slurry was transferred to a Qiashredder column (#79654, Qiagen, Valencia, CA) and processed according to manufacturer s instructions. Mircroarray analysis was performed by the microarray core in the Institute for Genome Sciences and Policy at Duke University. The RNA samples were checked for quality control using the Agilent Lab-on-a-Chip assay and Agilent 2100 Bioanalyzer G2939A (Agilent Technologies, Santa Clara, CA) and the Nanodrop 8000 spectrophotometer (Thermo Scientific/Nanodrop, Wilmington, DE). The RNA isolated was run on electrophoresis apparatus to check the presence of bands using a marker. Figure 2.2 shows the distinct bands obtained

indicating no impurities. The RNA isolated was also measured using the nanodrop and were found to contain significant amounts of RNA (Table 3). A minimum of 50ng/μl of RNA is required for the microarray analysis. mRNA was amplified using the Ambion MessageAmp Premier and MessageAmp III RNA Amplification Kit (Applied Biosciences, Austin) and hybridized to Affymetrix Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA) according to the Affymetrix Technical Manual. Data were collected using an Affymetrix GC3000 scanner 7G. Image processing was done using the AGCC version 3.1.1.

2.8 Statistical Analysis

In preparation for statistical analysis, data was normalized onto a single array using the Robust Multichip Average (RMA) algorithm in Partek Genomics Suite (Partek Inc., St. Louis). ANOVA was used to test for differentially expressed genes between endothelial cells in monocultures and the lamellar co-cultures. A false discovery rate (FDR) of 0.01 was used to correct for the multiple hypothesis testing. Gene lists were created for the most significantly expressed genes on comparison of the low density co-culture to the monoculture and the high density co-culture condition to the monoculture. Heat maps were generated for the changes in gene expression between the three conditions. Pathway analysis was done in GeneGo (MetaCore, Thomas

Reuters, St. Joseph, MI) and a list of statistically significant pathways at an FDR of 0.01 was generated.

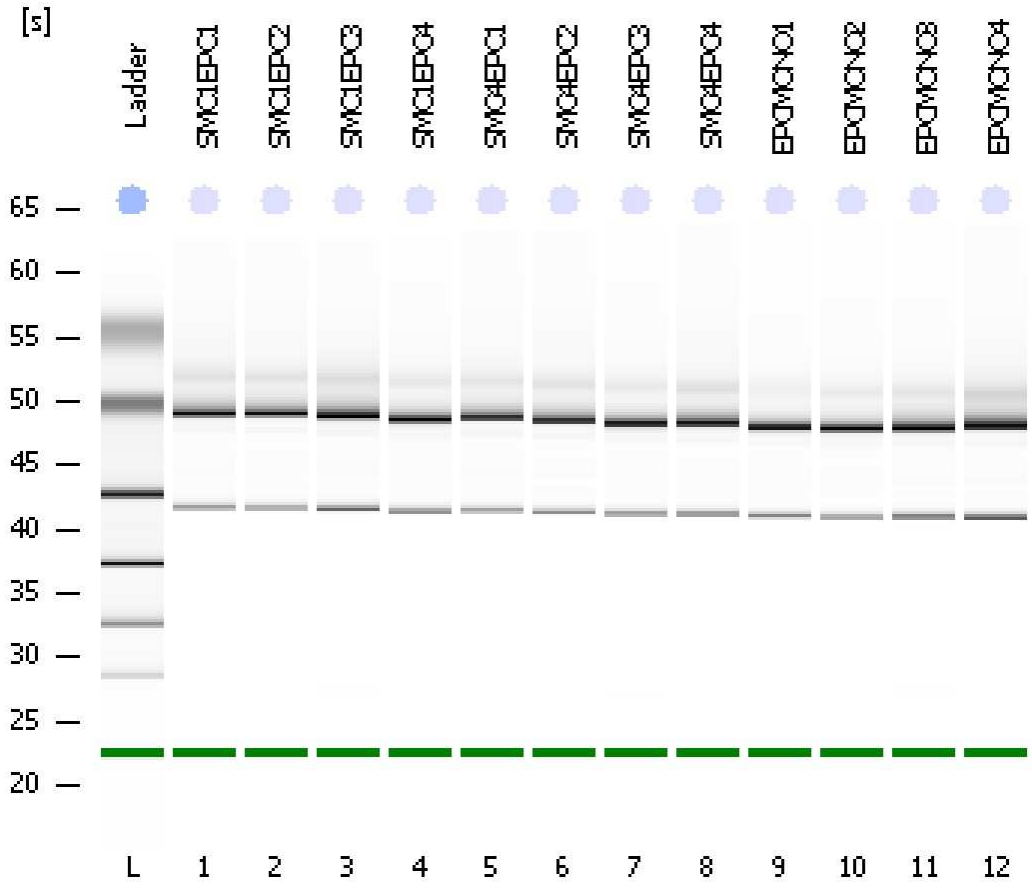

Figure2.2: Electrophoresis results for the RNA samples. The samples SMC1EPC1, SMC1EPC2, SMC1EPC3, SMC1EPC4 denote the low density co-culture samples. The SMC4EPC1, SMC4EPC2, SMC4EPC3, SMC4EPC4 represent the high density co-culture samples and the EPCMONO1, EPCMONO2, EPCMONO3, EPCMONO4 represent the monoculture samples.

Table 3: Results of QC analysis. The SMC1EPC 1-4 samples represent the samples in low density monoculture, SMC4EPC1-4 represent high density co-cultures and the EPCMONO1-4 represent the monoculture conditions.

Sample	Concentration of RNA (ng/μl)	A260	A280	260/280	260/230	RIN number
SMC1EPC1	121.61	2.432	1.194	2.04	1.20	10
SMC1EPC2	213.97	4.279	2.130	2.01	1.82	10
SMC1EPC3	390.5	9.763	4.654	2.1	1.87	10
SMC1EPC4	562.2	14.055	6.813	2.06	1.83	10
SMC4EPC1	215.2	5.379	2.566	2.1	1.1	10
SMC4EPC2	314.3	7.857	3.741	2.1	1.07	10
SMC4EPC3	568.5	14.213	6.847	2.08	1.55	10
SMC4EPC4	425.4	10.634	5.057	2.1	1.39	10
EPCMONO1	487.7	12.193	5.902	2.07	2	10
EPCMONO2	552.1	13.803	6.744	2.05	1.05	10
EPCMONO3	454.7	11.367	5.419	2.1	1.41	10
EPCMONO4	422.7	10.568	5.052	2.09	1.57	10

3. hCB-EC morphology in co-culture

3.1 Introduction

hCB-EC have been found to produce networks when plated on surfaces like Matrigel and collagen. Recent findings by Brown [29] suggested that these cells are also capable of network formation under co-culture conditions on SMCs. Capillary formations formed by hCB-ECs in co-culture with SMCs can help understand the process of angiogenesis much more significantly than studies done with the same in Matrigel as it is closer match to the in vivo process. Studying network formation could provide new insights to endothelial cell migrations and ECM remodeling. Formation of capillary networks can also help in providing vascularization for tissue engineered organs and grafts. Eliminating network formations in co-culture can also help in creating a confluent layer of hCB-ECs required for tissue engineered blood vessels. Thus, studying the changes in morphology of hCB-ECs and optimizing them can aid in identifying conditions under which networks form.

3.2 Results

The effect of seeding density of hCB-ECs in co-culture was studied by seeding four different densities of hCB-ECs in co-culture with SMCs. The co-culture was setup with the different densities as mentioned in 2.1. The co-culture was maintained in EBM-2 growth media and imaged after 4 days of seeding the hCB-ECs. (figure 3.1)

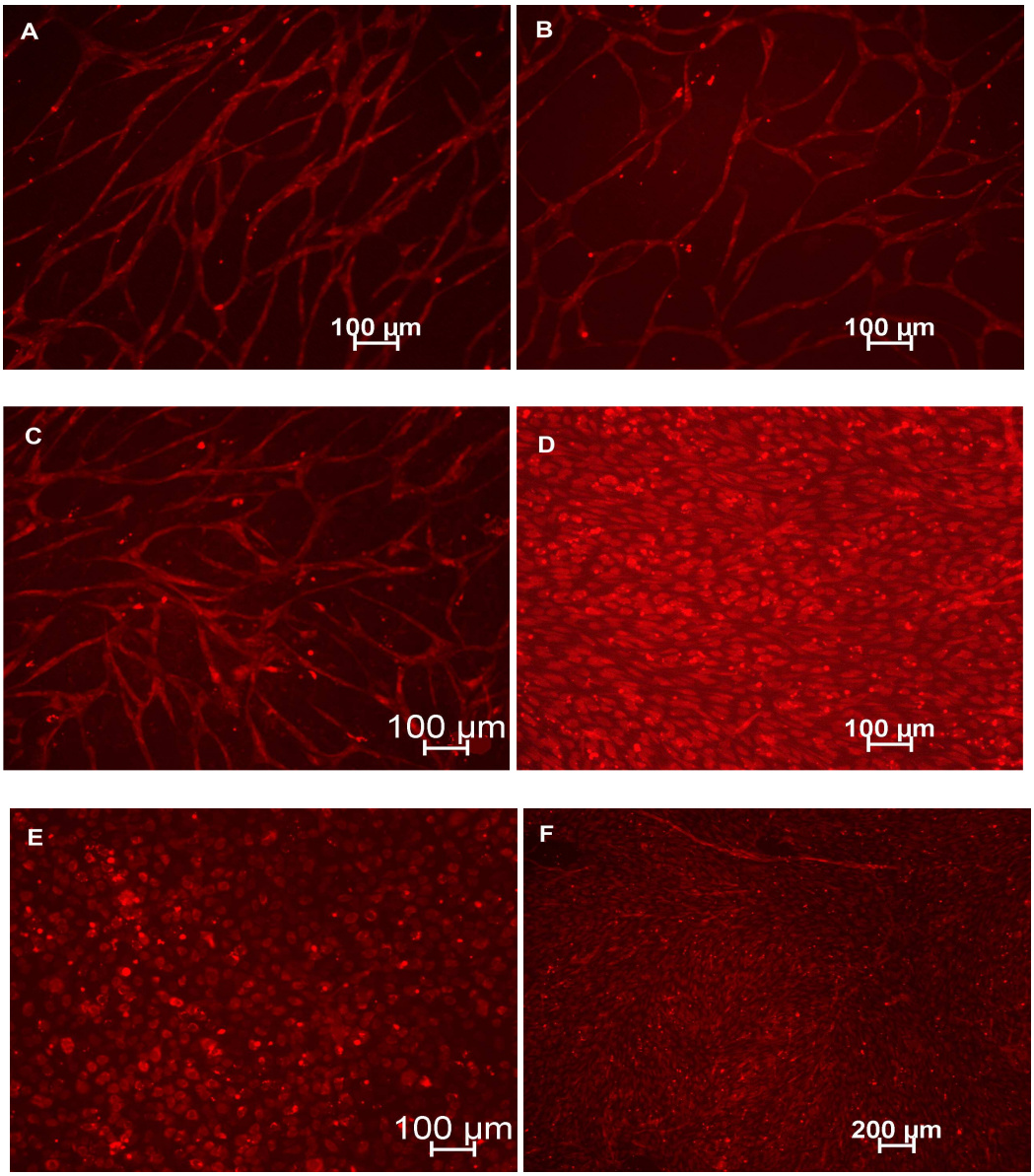

Figure 3.1: Varying morphology of hCB-ECs under different densities. The effect of seeding density on morphology viewed by using 4 different densities of hCB-ECs in co-culture with SMCs after 4 days in co-culture. (A) hCB-ECs at 26, 316 cells/cm^2 at 10X, (B) hCB-ECs at 52, 632 cells/cm^2 at 10X, (C) hCB-ECs at 80,000 cells/cm^2 at 10X, (D) hCB-ECs at 105263 cells /cm^2 at 10X, (E) hCB-EC monoculture at 80,000 cells/cm^2 10X, (F) hCB-ECs at 105, 263 cells/cm^2 in co-culture at 4X magnification.

Network formation was observed under the hCB-ECs seeding densities of 26,316 cells/cm^2, 52,632 cells/cm^2 and 80,000 cells/cm^2 (Figure 3.1 A, B and C) while there was no network formation associated at a density of 105,263 cells/cm^2 (Figure 3.1 D, F). At conditions of 80,000 cells/cm^2 partial confluent regions of hCB-ECs were also observed. The morphology of hCB-ECs in the high-density condition also varied with the morphology obtained by hCB-EC monoculture conditions (Figure 3.1E). The cells in high-density co-culture were aligned based on the SMC matrix and appeared elongated compared to the hCB-ECs in monoculture.

Quantification was done using the Angiogenesis Tube Formation application of the Metamorph Premier Software (LSRC 32- bit workstation, Duke University) for the networks observed under the first three density conditions. The images obtained after 4 days of seeding hCB-ECs on SMCs were used for quantification (Figure 3.1). The minimum and maximum width of the networks was measured and the software was used to identify the number of nodes, branch points and segments and to measure other parameters. The experiment model was repeated twice using three replicates each and the measurements obtained were represented graphically. The segments represent the total number of vessel segments connecting the branch points. Branch points are total number of junctions connecting segments. Nodes represent the number of connecting blobs whose thickness exceed the maximum width of the vessel. Mean tube length is the

total number of tubes divided by the number of segments. Total tube area is the total square microns of vessel area excluding nodes and the percentage tube area is the total vessel area divided by the total area of the image.

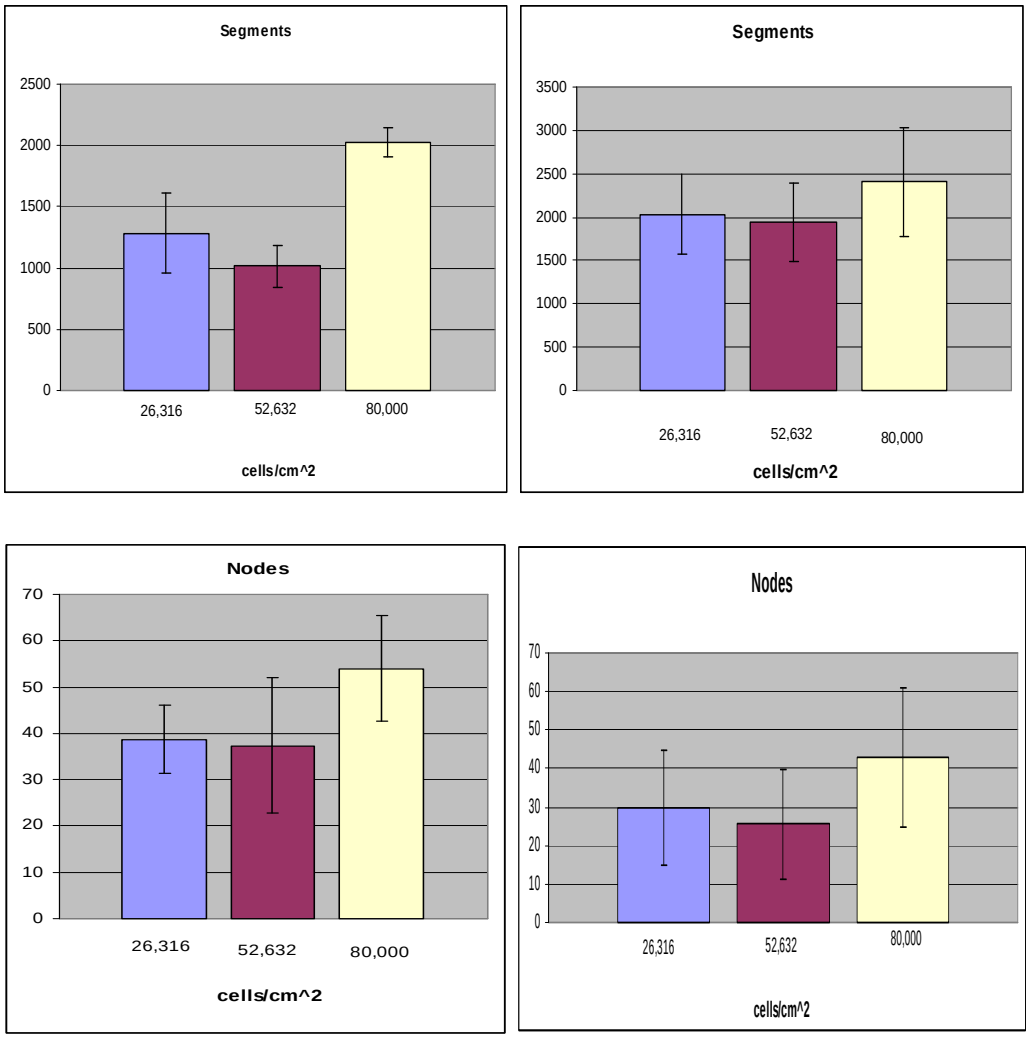

Figure 3.2: Quantification results of networks obtained under differential seeding densities of hCB-ECs

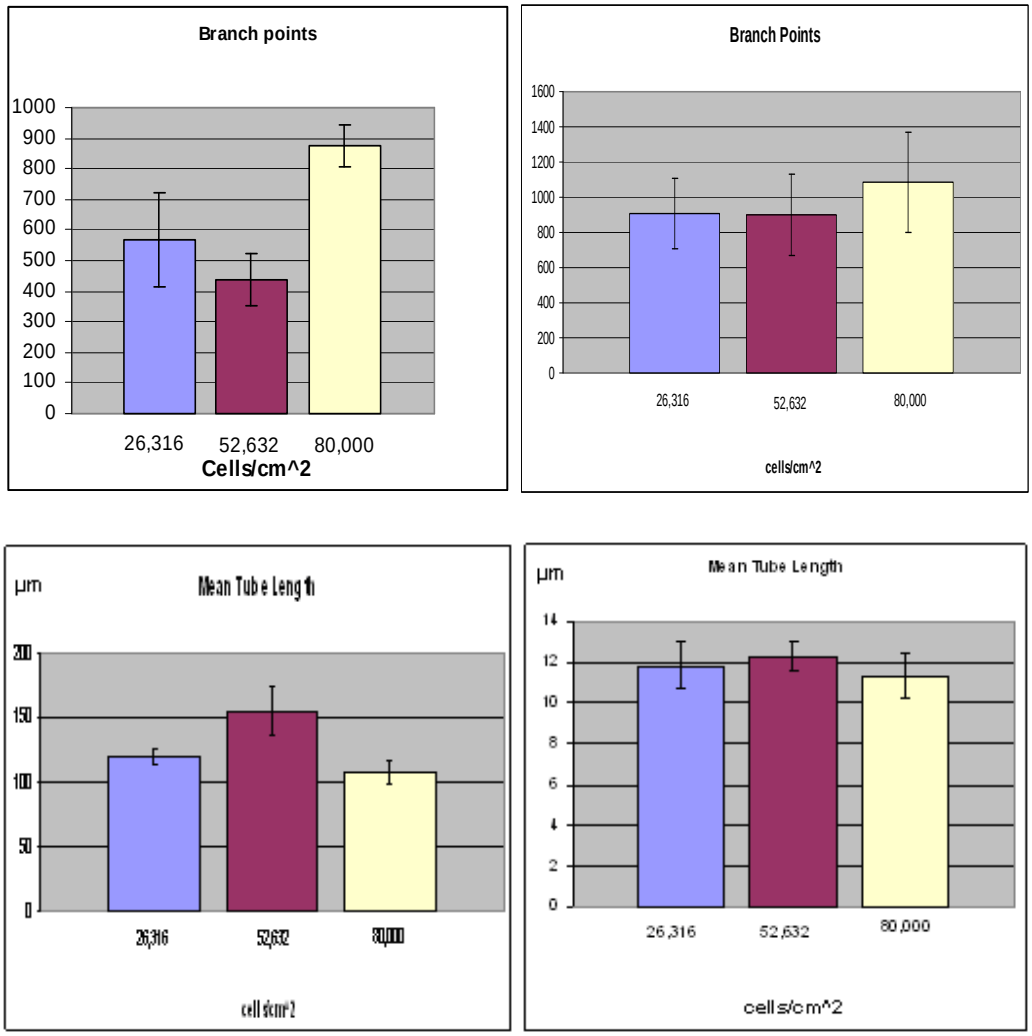

Figure 3.2 (Continued)

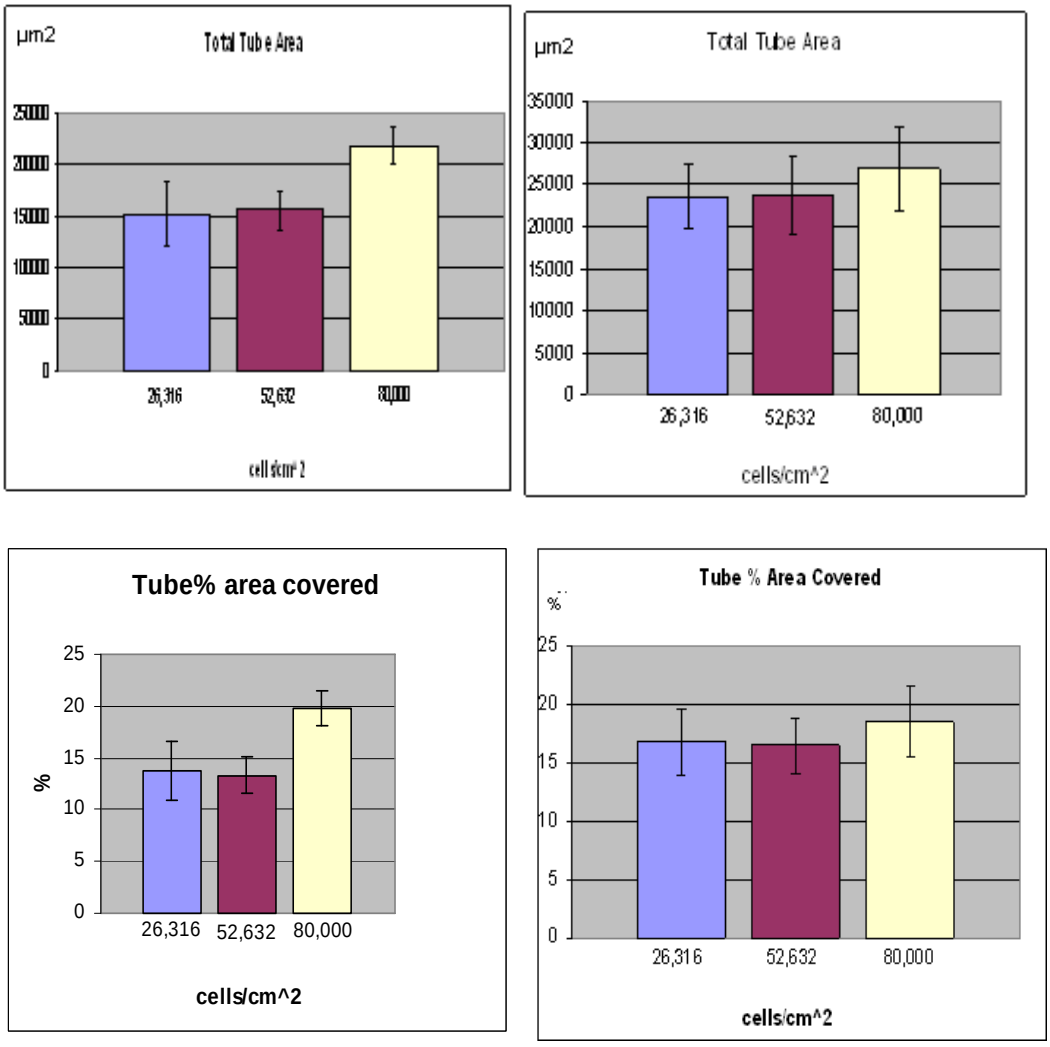

Figure 3.2: Quantification results of networks obtained under differential seeding densities of hCB-ECs. The quantification was done on images obtained after four days of seeding hCB-ECs on SMCs. Two experiments with different SMC and hCB-EC sources are compared in the above figure. The averages of values obtained from each experiment are plotted in the graph. The arrow bars represent standard deviation. The graphs on left from values of the same experiment while those on the right are those of the second experiment done with the same sample conditions

The quantification results suggested that under the density condition of 52,632 cells/cm^2, the networks obtained were elongated with fewer nodes and branch points. Under this density condition there was lesser coverage of tube area with respect to the image area but the tubes formed were continuous with lesser branching. Under the lowest density condition of 26,316 cells/cm^2, there were a greater number of networks present with greater branching relative to the density condition of 52,632 cells/cm^2 condition. The condition at 80,000 cells/cm^2, had denser and shorter network formation. There were more nodes and branches under this density condition than the other two conditions. At some places, "honeycomb"-like structures of networks were also obtained. The area covered by the tubes is the greatest in the 80,000 cells/cm^2 condition. It has the lowest tube lengths compared to the other two density conditions. The results obtained from both sets of experiments had consistent patterns of results while comparing the three density conditions.

Studies done by Brown [29] used different medium conditions to study network formations under static and flow conditions and reported networks formed under static conditions when hCB-ECs were plated at a density less than that needed to achieve confluence. Several previous studies reported the role of VEGF in aiding network formation in co-cultures done in Matrigel and collagen gels. [19,25] Evensen *et al.* [23] mentioned the role of VEGF 165 produced by SMCs aiding in network formation in co-

culture conditions. Studies by Evensen also reports externally added VEGF to have no role in the formation of networks in mixed co-cultures. The role of heparin in formation of networks was brought out by Ashikari-Hada *et al.* [22] by studying network formation of HUVECs in type 1 collagen gels. The study also showed that inhibition of heparin by heparinase led to 75% decreased binding of VEGF 165 to VEGFR2.

In order to study the effect of VEGF and heparin on network formation, co-culture wells were setup under different medium conditions. The Singlequots added to the EBM-2 basal medium include aliquots of VEGF and heparin. EBM-2 medium was aliquoted under different conditions with the addition of VEGF and no heparin, with the addition of heparin and no VEGF and in the absence of both VEGF and heparin. Co-cultures were also setup with the co-culture medium containing the EBM-2 medium with no SingleQuots and increased serum content (10%). The co-cultures were setup with a density of 80,000 cell/cm^2 of SMC and 26,316 cells/cm^2 of hCB-ECs. The cells were maintained in co-culture for 4 days and the images were acquired on the fourth day. (Figure 3.3) No network formation was observed under the co-culture medium. The networks obtained under the other conditions were not well extended relative to the networks obtained in the EBM-2 medium with SingleQuots. The absence of VEGF in the medium had a greater effect on network formation when compared to the absence of heparin or heparin and VEGF in the medium.

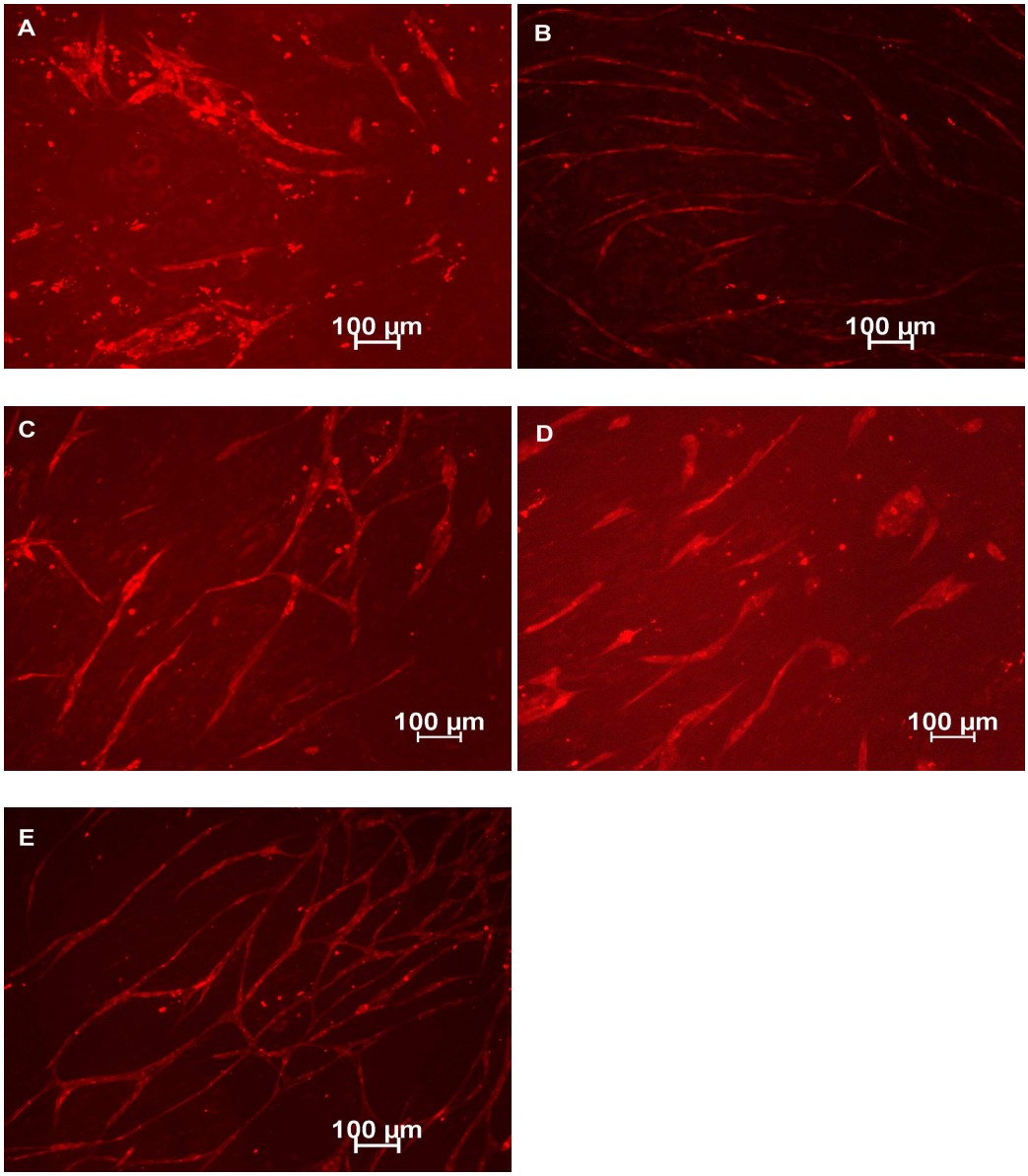

Figure 3.3: Network formation under different culture conditions. The network formations observed under different co-culture medium conditions. (A) the cells in co-culture medium, (B) cells in EBM-2 medium without heparin, (C) cells in EBM-2 medium without VEGF and heparin, (D) EBM-2 medium without VEGF, (E) EBM-2 medium. The cells were placed in the different medium conditions for four days before imaging. Images were taken at 10X magnification.

The network formations were affected by the absence of medium components like VEGF and heparin and other growth factors present in the SingleQuots. VEGF is a mitogen of angiogenesis and its absence in the medium created more reduction in network formation. The images obtained under no VEGF conditions indicate a cell migration morphology and might suggest delayed network formations as the cells depends on endogenous sources of VEGF. Heparin aids in binding of VEGF to the VEGFR2 receptor present in endothelial cell. Absence of heparin in the media produced networks but they were much sparser compared to the formation of networks under EBM-2. Absence of VEGF and heparin in the medium also lead to reduced network formation and lesser coverage of networks in the wells. Absence of VEGF in the medium produced a more profound effect in the formation of networks than in the absence of heparin. In the absence of VEGF and heparin networks formed were sparser than under no VEGF or no heparin conditions. Thus, endogenous VEGF and heparin provided exogenously through the medium contributed to network formations.

Evensen *et al.* [23] had used Texas Red conjugated 10,000MW dextran to detect the presence of a lumen as the lumen concentrates exogenously available dextran. The cells in co-culture were provided with dextran in the medium on day 5 and were observed after an overnight incubation. The co-cultures did not show an accumulation

of dextran in the lumen. Evensen *et al.* used co-cultures setup by trypsinizing and mixing SMCs and hCB-ECs while the co-culture technique used in our experiments were different and the images were taken by Evensen on networks at day 9 which may have enhanced a better lumen formation. It appears that the dextran accumulated in smooth muscle cells.

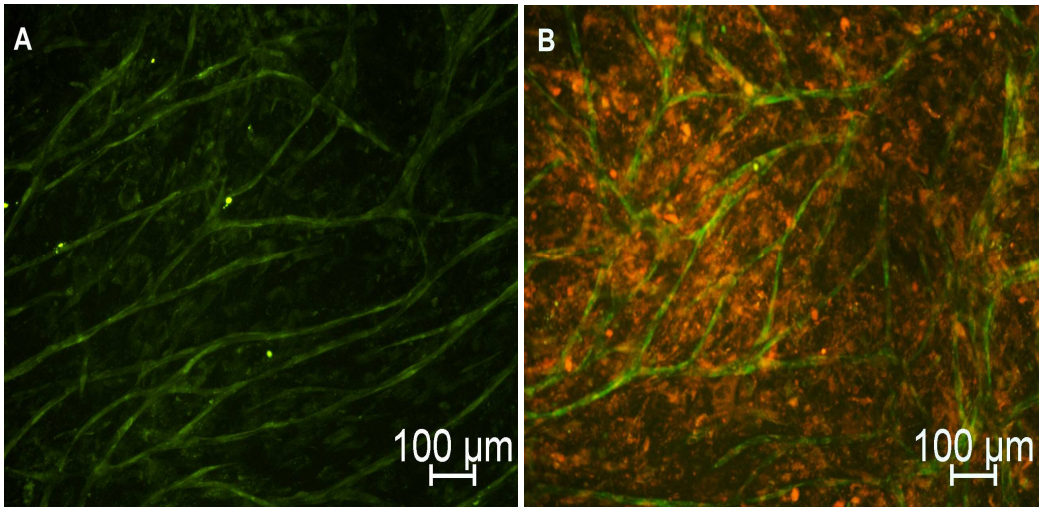

Figure 3.4: Dextran Experiment: Image got after 6 days of co-culture. (A) The hCB-ECs stained with cell tracker green, (B)Image after dextran incorporation. The red represents the Texas Red conjugated Dextran. The hCB-ECs were plated at a density of 26,316 cells/cm^2 on SMCs at 80,000 cells/cm^2.

3.3 Discussion

hCB-EC morphology depends greatly on the seeding densities of hCB-ECs on SMCs in co-culture. At lower densities of seeding increased network formations are observed. The cells also have a more elongated morphology with increased cell migratory patterns. Under high density conditions of hCB-EC plating (105, 263 cells/cm^2)

the cells do not form networks but appear elongated and aligned relative to the cells in monoculture. With gradual increase in density variation from network formation to confluent layer was observed. In case of hCB-ECs plated at 80,000 cells/cm^2, areas of confluent regions were observed along with regions of network formations.

The networks formed by the lowest density condition were elongated with fewer nodes compared to the seeding density of 80,000 cells/cm^2 co-culture condition. Networks formed under 56,316 cells/cm^2 have the least number of nodes and branch points and has lesser percentage of image area covered with networks. Thus the condition at 26,316 cells/cm^2 seeding density was taken as a model to represent network formation in co-culture for further studies done on understanding network formations of hCB-ECs.

Different conditions of culture medium were also used to study the effect of culture media components on hCB-EC morphology. Components such as VEGF and heparin were found to affect network formation when removed from the culture media. The presence of singleQuots in the medium, containing growth factors like VEGF, FGF and components like heparin enhanced the formation of networks. The co-culture condition without VEGF in the culture medium showed cell migration but no network formation. Growth factors and heparin provided exogenously affect cell growth and morphology at lower densities of co-culture. In case of co-culture in the high serum

medium with no SingleQuots there is very less growth and cell migration of hCB-ECs observed when compared to the other co-culture conditions.

4. Microarray Analysis

4.1 Introduction

The identification of gene alterations in co-culture can help in better understanding the processes that lead to hCB-EC network formations. Studies done so far in understanding the genes involved in angiogenesis have mostly been done on Matrigel or collagenase gels with HUVEC cells. The use of smooth muscle cells as a matrix for studying angiogenesis related genes is an area less explored. Many studies also indicate growth factors like VEGF to play a key role in network formations in vitro. We were interested in identifying the pathways that may regulate these growth factors in enabling ECM remodeling and endothelial cell migration to form capillary networks. Microarray analysis was performed in order to identify the genes alterations between monoculture and co-culture conditions as well as between the high and low density conditions of the co-culture. This unbiased approach allowed us to examine whether genes involved in angiogenesis and other cellular processes were altered by co-culture.

4.2 Results

The lamellar co-cultures were setup with the four different sources of hCB-ECs in which either 26,315 cells/cm^2 (low density co-culture) and 105,000 cells /cm^2 (high-

density co-culture) were seeded onto quiescent SMCs. The SMC sources were not varied. Monocultures of the four sources of hCB-ECs were also setup as a control at a density 80,000 cells/cm^2. The hC-ECs were stained with cell tracker orange. The wells were observed and imaged after 4 days (Figure 4.1) and the cell separation RNA extraction using dynabeads and MACS was carried out on the fourth day. RNA extraction was done using the Qiagen RNEasy kit (#74104) and RNase free DNase treatment (#79254). The samples were submitted to the Institute for Genome Sciences and Policy at Duke University for microarray analysis. RNA samples were assessed for quality using the Agilent Lab-on-a-Chip assay and using the Agilent 2100 Bioanalyzer and Expert Software. The samples which passed the quality control check were amplified using the Ambion MessageAmp Premier and MessageAmp III RNA Amplification Kit (Applied Biosciences, Austin) and hybridized to Affymetrix Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA) according to the Affymetrix Technical Manual. Data were collected using an Affymetrix GC3000 scanner with 7G upgrade with Factory PMT settings. The AGCC version 3.1.1 was used for imaging processing.

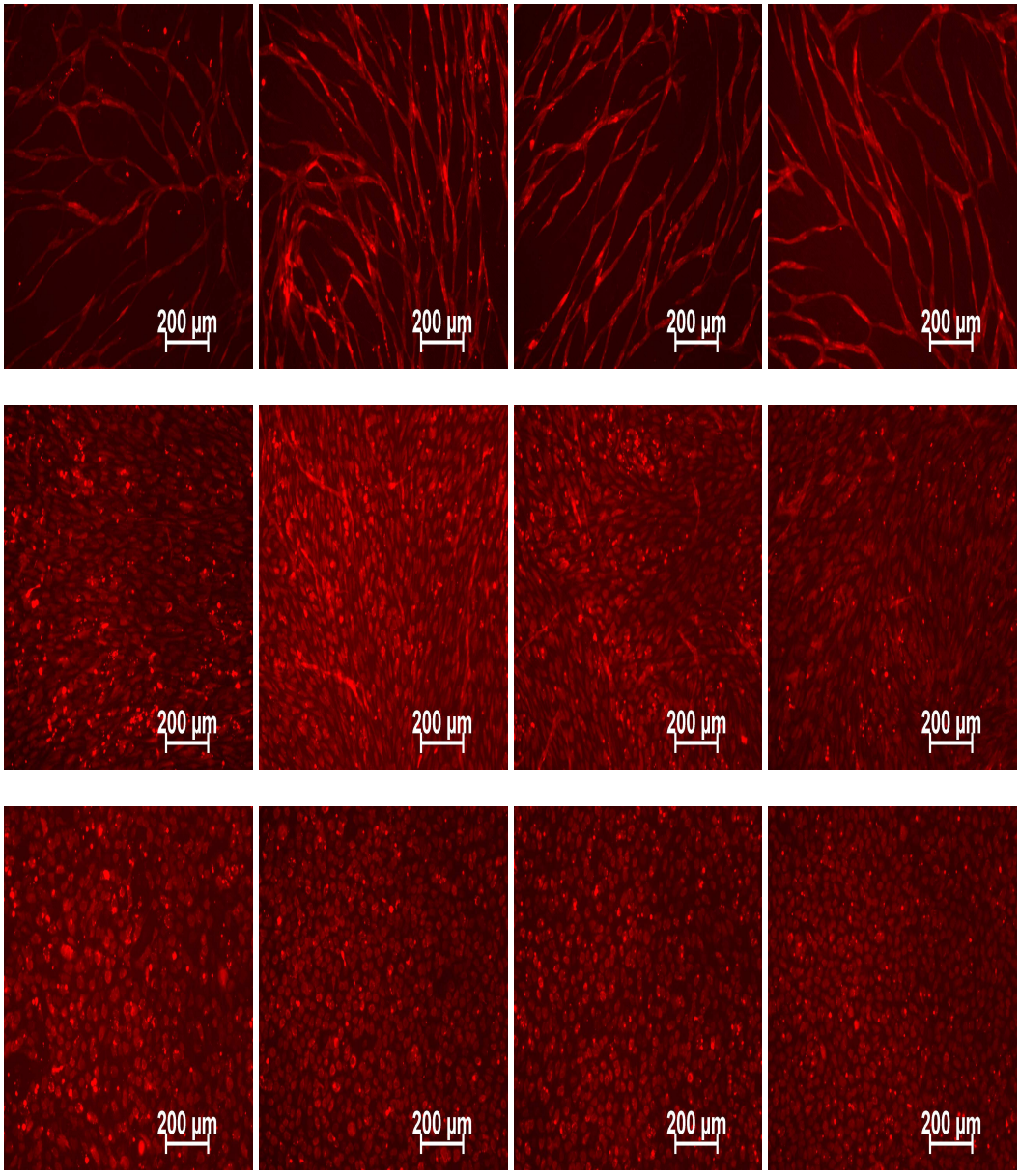

Figure 4.1: Images of the four sources of hCB-ECs in co culture and monoculture prior to RNA isolation. The top panel represents the hCB-ECs in low density co-culture condition (26,316 cells/cm^2), the middle panel represents the hCB-ECs in co-culture at the higher density (105, 263 cells/ cm^2) and the lower panel represents the control hCB-ECs in monoculture (80,000 cells/ cm^2). The images were taken after four days of seeding the hCB-ECs on SMCs.

The microarray data were analyzed using the Partek Genomic Suite (Partek Inc., St. Louis) and GeneGo (MetaCore, Thomas Reuters, St. Joseph, MI) software. The data were initially normalized onto a single array using the Robust Multichip Average (RMA) algorithm in Partek Genomics Suite. ANOVA was carried out to compare the samples in high-density co-culture vs. samples in monoculture, the samples at a low density in co-culture vs. monoculture and the samples in high-density co-cultures vs. low density of co-culture. Gene lists were created and 1021 statistically significant genes were obtained for the comparison of low density co-culture vs. monoculture while 381 genes were obtained for the comparison of high density co-culture vs. monoculture at a false discovery rate (FDR) of 0.01. There were no statistically significant genes obtained between the comparison of low and high density co-cultures at an FDR of 0.01 and a single gene, plakophilin4, appeared significant at an FDR of 0.05.

4.2.1 Comparison of gene expression and generation of heat maps.

The heat maps were generated for the gene lists that were obtained. (Figure 4.2) The rows in the heat map represent the genes and the columns represent the samples. The samples denoted in green are the co-cultures of four different donor hCB-ECs under high density conditions and the samples denoted in blue represent the co-cultures in low density conditions. The samples denoted in red are the monoculture controls from the four different donors of hCB-ECs. The genes denoted by red in the heat map are up

regulated and the genes in blue are down regulated. The grey area denotes genes with no change in expression. The comparisons are based on the t-test results done on all three samples.

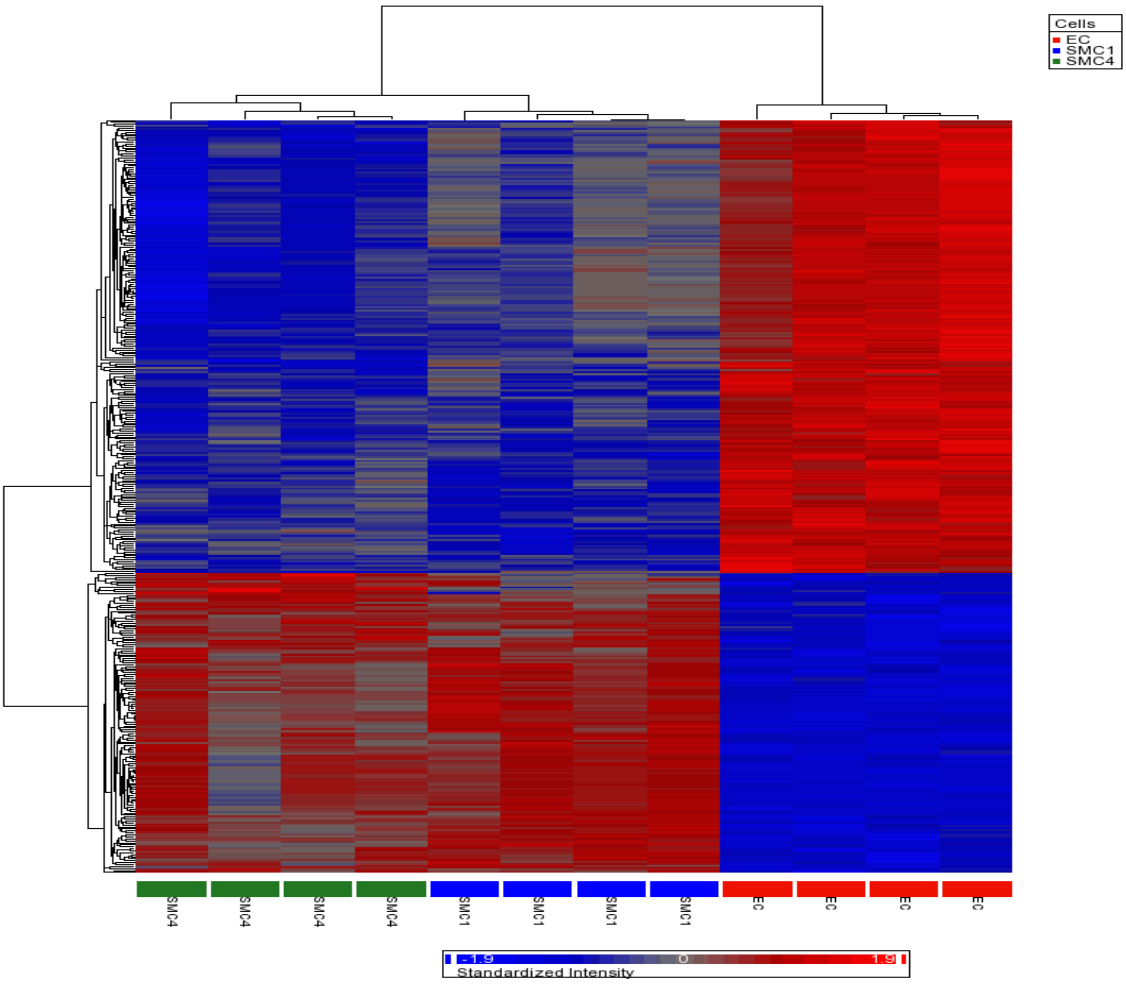

Figure 4.2: The heat map of differentially expressed genes in the monoculture and co-culture conditions. EC denotes the monoculture samples, SMC1 the low density co-culture and SMC 4 the high density co-culture samples. The rows represent genes. The blue represents a down regulation of the gene and red represents an up regulation of the gene.

The heat map generates a clear distinction amongst the genes expressed in co-culture and monoculture. The genes expression patterns of all four sources of hCB-EC in the monoculture appear uniform with less variation than in co-culture. The gene expression patterns of the different density conditions of co-culture appear similar with very few differences. The top rows of genes in the low and high density conditions of co-culture shows no change in expression in most sources of the low density co-culture condition denoted by SMC1 but the same genes appear to be down regulated under high density condition of co-culture denoted as SMC4. Most of the genes down regulated in the monoculture condition are up regulated in the co-culture conditions.

On comparing the list of genes obtained on comparison of the co-cultures of both densities to the monoculture at an FDR of 0.01, 224 genes were found to be in common to both lists and 784 unique genes were found in the low-density co-culture gene list. Many ECM related genes like collagen type I alpha I, collagen type III alpha I, collagen type VI alpha I, versican (VCAN), decorin (DCN), desmoplakin (DSP) were found to be increased by several folds in co-culture than in monoculture. Genes like Pregnancy associated plasma protein A, pappalysin A (PAPPA) which is associated in increased levels in patients with acute coronary syndrome, is also found in increased folds in lamellar co-culture conditions than in monoculture. [30] Other noteworthy genes which have an increased fold change include cytochrome P450, family 1, subfamily B,

polypeptide1 (CYP1B1), IL6, Insulin Growth Factor Binding Protein (IGFBP)-3, IGFBP- 5, SNAI2, periostin (PSN) and α-macroglobulin (A2M). Most of these genes are increased at a higher fold under the lower density co-culture conditions than in the high density co-culture conditions indicating these genes may have a role in endothelial cell migration and network formation. The expression level of decorin, which is an extracellular dematan sulphate proteoglycan, is three fold greater in the lower density co-culture conditions than in the higher density co-culture conditions.

Previous studies done by Nellimarrka *et al.,* have shown that ECs produce decorin during inflammation associated angiogenesis. [31] Other studies by Jarvelainen [32] and Schönherr [33] have shown that bovine aortic ECs accumulate decorin when changing morphology during sprouting and also that the presence of decorin when ECs are grown in collagenous matrices increased tubule formation and their absence reduced corneal neoangiogenesis in mice. Decorin is also majorly involved in the cell matrix interactions leading to endothelial cell sprouting and alignment through IGF-IR binding and in lumen formation and maturation through activation of Rac and $\alpha_2\beta_1$ integrin. [34] Decorin is also however known to inhibit angiogenesis on binding to thrombospondin-1 [35] and is thus considered a regulator of angiogenesis. [34]

Amongst genes which were uniquely expressed in the lower density co-culture conditions include the notch homolog 2, Wnt-5A, Wnt-5B, MMP14, PAK1,

Thrombospondin-2, polo like kinase , TIMP metallopeptidase inhibitor 1, EGFR and the chemokine (C-X-C) motif ligand. A list of the most significant commonly expressed genes in the two co-culture conditions is presented in the Appendix A.

4.2.2 Pathway Analysis

Pathway analysis of the statistically significant genes in the comparison of low density co-culture vs. monoculture and high density co-culture condition vs. monoculture were obtained using the GeneGo software. At an FDR of 0.01 the low density co-culture produced a list of seven pathways while ten significant pathways were obtained for the genes under the high density co-culture condition vs. monoculture. The lists are presented in Table 4 for low-density co-culture condition vs. monoculture and Table 5 in high density co-culture condition vs. monoculture. The genes involved in these pathways that were present in the gene list obtained are listed in the Appendix A.

Table 4: List of significant pathways generated for the low density co- culture condition vs. monoculture. The list of pathways was created using the GeneGo software.

#	Maps	P Value	Number of genes in the dataset involved in the pathway	Number of genes in the pathway
1	Development: Regulation of epithelial-to-mesenchymal transition (EMT)	2.646E-06	11	64
2	Membrane-bound ESR1: interaction with G-proteins signaling	1.754E-05	9	51
3	Muscle contraction: Regulation of eNOS activity in endothelial cells	1.830E-05	10	64
4	Cell adhesion: ECM remodeling	2.066E-05	9	52
5	Transport: Macropinocytosis regulation by growth factors	9.963E-05	9	63
6	Cytoskeleton remodeling: TGF, WNT and cytoskeletal remodeling	1.179E-04	2	111
7	Immune response: Signaling pathway mediated by IL-6 and IL-1	1.211E-04	6	27

Table 5: List of significant pathways generated for the high density co-culture condition vs. monoculture. The pathways were generated using the GeneGo software at an FDR of 0.01.

#	Maps	P value	Number of genes in the dataset in the pathway	Number of genes in the pathway
1	Cell cycle: Role of APC in cell cycle regulation.	4.320E-19	14	32
2	Cell cycle: The metaphase checkpoint	8.638E-15	12	36
3	Cell cycle: Spindle assembly and chromosome separation	1.202E-13	11	33
4	Cell cycle: Chromosome condensation in prometaphase	1.659E-07	6	21
5	Cell cycle: Role of Nek in cell cycle regulation	2.474E-06	6	32
6	Reproduction: Progesterone-mediated oocyte maturation	9.655E-06	6	40
7	Cell cycle: Initiation of mitosis	1.284E-05	5	25
8	Cell cycle: Nucleocytoplasmic transport of CDK/Cyclins	2.203E-05	4	14
9	Immune response: IL-6 signaling pathway	2.757E-05	5	29
10	Cell cycle: Sister chromatid cohesion	1.489E-04	4	22

Most of the pathways generated with the gene list of the high-density condition co- culture were related to cell cycle regulation. The genes involved in these pathways were down regulated when compared to their expression in monoculture indicating that co-cultures of high density inhibited the cell cycle and proliferation of cells. These results were consistent results done by previous studies done in our lab in which hCB-EC growth was inhibited on SMCs. [39] The predominant genes involved in the cell cycle pathways involved the down regulation of cyclins B1, B2 and A2.

Most of the pathways elevated in the low density co-culture are signaling pathways involved with ECM remodeling and cell migration. One interesting pathway is the epithelial to mesenchymal transition pathway which generally occurs during the formation of mesenchymal cells and mesoderm during embryo development. They are also known to play a significant role in tumor progression and providing stem cell like characteristics to cancer cells. Several signaling pathways lead to triggering EMT, some of which are the Notch signaling pathway and the Wnt signaling pathway, genes of which have been found in the low density co-culture gene list. EMT is also triggered by the increased levels of transforming growth factor β, receptor tyrosine kinases and matrix metalloproteinases (MMPs). [36] EMT can affect in remodeling of the extracellular matrix as EMT inducing factors upregulate the expression of ECM proteins like fibronectin, collagens and MMPs. [36] SNAI2 gene which is a major transcription

factor in the EMT pathway is found to be increased several folds in co-culture when compared to monoculture.

The genes obtained in the gene lists were compared to angiogenesis related genes reported in previous studies. A comparison was done with the study done by Abe *et al.,* [37] where HUVECs were plated on collagen coated plates and incubated in endothelial basal medium containing SingleQuots and 10% calf serum. The confluent cells were then incubated with M-199 medium containing 1%FCS and1nM VEGF. The genes that were induced by VEGF were then studied at different time points using c-DNA microarray analysis. The following genes (Table 6) were found to be common to the list simulated in the studies.

Table 6: The list of genes induced by VEGF. The list of genes induced by VEGF obtained on comparison with the studies done by Abe *et al.*[37] The genes from the studies were compared with the gene lists obtained on comparison with the high density and the low density co-cultures with the monoculture.

Gene name	P value in the low density co-culture vs. monoculture	Fold change in the low density co-culture vs. monoculture.	Presence in the gene list of high density co-culture vs. monoculture at FDR0.01
N- met hylpurine DNA glycosylase	8.13E-05	1.438	Absent
Myosin VII A	0.1521	-6.572	Absent
Cytochrome P450 family 1, subfamily B, polypeptide 1	1.35E-07, 2.09E-07, 2.96E-07	63 37.51 36.855	Present with p = 1.45E-06, 3.75E-06, 5.28E-06 and fold changes of 23, 13.20, 13.
Cerebellar degeneration-related protein 2 like	1.72E-05	1.499	Absent
Alpha 2-macroglobulin	1.05E-05	11.459	Present with p = 4.93E-06 and fold change of 14.484.

The results obtained were also compared to the previous studies done by Chiu *et al.* [38] where ECs were grown in co-culture with SMCs separated by a 10μm thick porous membrane. The co-culture was plated on a parallel plate flow chamber and was subjected to stress following which microarray analysis was done in an inflammation

relevant gene array. The gene expression was also compared to the expression under EC monoculture conditions and co-culture conditions under static using western blots. On comparing these genes with the ones obtained in our study similar expression patterns for genes like IL6, caveolin, SCARA3, TNFRSF11b were found. A majority of the inflammatory genes that were found to be upregulated due to the role of NF-κB in the co-culture used by Chiu *et al.* were not present in the gene lists obtained. In addition it was also noticed that there was a downregulation of the gene TNFRSF 11a, an NF-κB activator, in both co-culture conditions.

Comparisons were also done to studies reported by Tressel [39], where shear stress sensitive and insensitive angiogenesis genes were identified by using an angiogenesis array. The co-culture was setup by using HUVEC, HMEC-1 or BAEC on Matrigel. No comparisons to the gene expression in monoculture were done in this study. Angiogenesis genes found in common include IL-6, thrombospondin-2, ephrin A5, sphingosine kinase 1 and chemokine (C-X-C) motif ligand. It is also noted that with the exception of IL-6 all other genes were expressed only in the low density co-culture conditions where the formation of networks are observed

4.3 Discussion

We showed in this study that the lamellar co-culture model alters the gene expression considerably relative to the gene expression of the confluent hCB-ECs in

monoculture. In the comparison of low density co-culture vs. monoculture a total of 1021 genes were statistically significant at an FDR of 0.01 amongst which 541 genes were upregulated while the expression of 480 genes were downregulated. Comparing the high-density co-culture condition to the monoculture, it was found that amongst a total of 381 genes, the expression 152 genes were upregulated and 229 genes were downregulated. Twenty one genes are upregulated several folds commonly amongst the low and high density co-cultures in comparison to the monoculture. It is also noted that the gene expression changes in the co-culture model compared to the monoculture are significant even at a sample size of four. Though there are changes in gene expression between the low- and high-density co-culture conditions the differences are not statistically significant and can be seen only when comparing the gene lists obtained by comparing each of the co-culture conditions to the monoculture.

Another key observation is the variation in significant pathways created with the gene lists obtained for the low and high-density co-culture conditions. The high-density co-culture conditions had most of its significant pathways involved in down regulation of cell cycle while the lower density co-culture condition has most of its genes involved in the EMT pathway and cell signaling pathway involving ECM remodeling and cell migration. The difference in morphology of the hCB-ECs amongst the low and high density co-cultures (Figure 4.1) mediates for this change in gene expression. The low

density co-culture had significant formation of networks consistent with increased EC migration. In contrast the high-density co-culture resembles a confluent monolayer with no network formation. Correspondingly, cell proliferation and migration are expected to be low.

Amongst the genes that were common between the high- and low-density co-culture which were expressed at significantly higher fold than at monoculture condition is versican, a proteoglycan present in the extracellular matrix. Versican is up regulated in cases of PDGFR and TGFβ-1 activation and has roles in cell adhesion, proliferation and in expansion of pericellular ECM. Higher amounts of versican are involved during ECM remodeling during developmental events. [40] Particular isoforms of versican are expressed during endothelial cell migration. Insulin dependent growth factor binding protein (IGFBP)-5 is also increased several folds in co-culture than in monoculture. IGFBP-5 is expressed by endothelial cells and is known to be upregulated by VEGF which is in turn a mitogen of angiogenesis. [41] SNAI-2 or the SLUG gene is also increased under co-culture conditions. This gene is involved in cell adhesion and migration and is activated by the NOTCH ligand during angiogenesis. [42]

Amongst the genes that were different in the two density conditions of co-culture, the Wnt-5A gene though expressed 38X times higher in the low-density co-culture condition than in monoculture and was absent in the list of statistically

significant genes in the high density co-culture condition. Wnt- 5A expression is known to help endothelial cell proliferation through the non-canonical pathway and reduction of its expression was related to reduce capillary formations by the endothelial cells. [43] Amongst the metallopeptidases that are expressed, MMP 14 is expressed in the low-density co-cultures while MMP 11 is expressed under high-density conditions. MMP 14, also known as MT1-MMP, is associated with tumor angiogenesis and up regulates the production of VEGF. [44] Thus, most of the genes expressed in the low-density co cultures relate to endothelial cell migration and angiogenesis.

The SMCs used for this work were from a single donor while the EPC sources were varied over four donors to test variability. The different density conditions for hCB-ECs were tested to study the changes in morphology and an optimal density of 27,000 cells/cm^2 and 105,000 cells/cm^2 were chosen based on the drastic change in morphology observed between the two conditions. The hCB-ECs in monoculture control are plated at a concentration of 80,000cells/cm^2 similar to the density at which the SMCs are plated. The media used for co-culture and the monoculture were consistent. The experiment was done after four days of plating the cells as the networks were found to be well established in four days when viewed at a 24 hr time gap. The hCB-ECs were used for the microarray analysis and not the SMCs as the hCB-ECs underwent a morphological change on changing the density conditions.

The significant pathways for the high-density co-culture indicate a down regulation of the cell cycle. These results are consistent with the results reported from previous studies done in our lab with the BrdU assay to measure cell proliferation of the hCB-ECs in co-culture. [29] The up regulation of genes like tensin in co-culture and the down regulation of caveolin [45] in co-culture condition are also consistent to previous results obtained.

Appendix A

Table 7: List of the most significant commonly expressed genes amongst the low and high density co-culture conditions on comparison with the monoculture. The list of genes expressed commonly by both co-culture conditions. The p value and fold changes are obtained on comparison to the monoculture condition. These genes are significant at an FDR of 0.01.

Gene Symbol	Gene Title	p-value(co-culture high density vs. mono)	Fold-Change(co-culture high density vs. mono)	p-value(co-culture low density vs. mono)	Fold-Change(co-culture low density vs. mono)
PAPPA	pregnancy-associated plasma protein A, pappalysin 1	3.84E-07	20.4947	6.42E-08	40.9627
PAPPA	pregnancy-associated plasma protein A, pappalysin 1	6.14E-07	44.7122	1.81E-07	79.8064
MAP2K3	mitogen-activated protein kinase kinase 3	7.73E-06	1.33466	9.32E-08	1.62563
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	1.45E-06	23.1189	1.35E-07	63.0106
DSP	desmoplakin	4.08E-07	34.148	2.94E-07	39.2012
IGFBP5	insulin-like growth factor binding protein 5	1.13E-06	24.3436	1.81E-07	52.1765
PAPPA	pregnancy-associated plasma protein A, pappalysin 1	7.71E-07	42.3532	2.20E-07	76.3846
PAPPA	pregnancy-associated plasma protein A, pappalysin 1	9.05E-07	37.1123	2.30E-07	69.4973

IGFBP3	insulin-like growth factor binding protein 3	1.05E-06	17.4912	2.37E-07	30.1217
IGFBP3	insulin-like growth factor binding protein 3	1.18E-06	17.3072	2.44E-07	30.8832
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	3.75E-06	13.2035	2.09E-07	37.5124
VCAN	versican	1.02E-06	156.099	4.12E-07	274.469
PAPPA	pregnancy-associated plasma protein A, pappalysin 1	2.25E-06	15.1894	2.95E-07	316274
DCN	decorin	1.42E-06	80.2666	3.66E-07	171.254
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	5.28E-06	13.0027	2.96E-07	36.855
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	9.93E-07	6.73354	1.02E-06	6.69661
COL3A1	collagen, type III, alpha 1	1.38E-06	203.636	8.15E-07	285.619
VCAN	versican	1.77E-06	102.768	7.37E-07	170.199
COL1A1	collagen, type I, alpha 1	1.49E-06	272.523	1.19E-06	317.566
ADAMTS5	ADAM metallopeptidase withthrombospondin type1 motif, 5	1.78E-05	5.80474	5.14E-07	14.7477
SERPINE2	serpin peptidaseinhibitor, clade E (exin, plasmiogen activator inhibitor type	4.35E-06	7.87348	9.21E-07	11.9708
DCN	Decorin	5.68E-06	60.5918	9.68E-07	159.057
GNG2	guanine nucleotide binding protein (G protein), gamma 2	2.87E-06	39.013	1.42E-06	53.6339

IL6	interleukin 6 (interferon, beta 2)	6.66E-06	7.89773	1.02E-06	13.264
VCAN	versican	3.80E-06	93.8768	1.40E-06	166.846
COL1A1	collagen, type I, alpha 1	4.40E-06	267.927	2.83E-06	362.968
STC1	stanniocalcin 1	1.37E-05	9.29148	2.34E-06	15.8075
COL3A1	collagen, type III, alpha 1	7.09E-06	135.658	3.51E-06	209.746
STC1	stanniocalcin 1	1.12E-05	13.1191	3.00E-06	20.4881
MFAP5	microfibrillar associated protein 5	1.03E-05	54.2473	3.70E-06	91.9605
COL1A2	collagen, type I, alpha 2	8.55E-06	15.6267	4.45E-06	19.6022
A2M	alpha-2-macroglobulin	4.93E-06	14.4846	1.05E-05	11.4569
VCAN	versican	1.53E-05	50.1114	4.22E-06	97.5346
COL1A1	collagen, type I, alpha 1	1.06E-05	168.862	5.36E-06	22.886
DCN	decorin	2.14E-05	42.9155	3.92E-06	102.212
DCN	decorin	3.02E-05	25.0945	4.44E-06	59.2318
GPNMB	glycoprotein (transmembrane) nmb	4.33E-05	12.1708	4.30E-06	27.821
SNAI2	snail homolog 2 (Drosophila)	1.98E-05	19.4665	8.14E-06	27.4524
MFAP5	microfibrillar associated protein 5	2.11E-05	49.2972	7.84E-06	81.8938

Table 8: List of genes involved in the pathways generated for the high density co-culture genelist.

Pathway 1: Cell cycle_Role of APC in cell cycle regulation

#	Gene name	P value
1	aurora kinase B	1.13693E-5
2	budding uninhibited by benzimidazoles 1 homolog (yeast)	2.48908E-5
3	budding uninhibited by benzimidazoles 1 homolog beta (yeast)	2.33211E-6
4	cyclin A2	4.2174E-6
5	cyclin B1	3.02699E-5
6	cyclin B2	5.08655E-5
7	cell division cycle 20 homolog (S. cerevisiae)	6.03158E-5
8	cell division cycle associated 3	9.12695E-6
9	cyclin-dependent kinase 1	5.27439E-5
10	CDC28 protein kinase regulatory subunit 1B	2.43496E-5
11	F-box protein 5	5.89419E-6
12	geminin, DNA replication inhibitor	1.55709E-5
13	MAD2 mitotic arrest deficient-like 1 (yeast)	0.0000623262
14	NIMA (never in mitosis gene a)-related kinase 2	6.42837E-5
15	pituitary tumor-transforming 1	2.74285E-5

Pathway 2: Cell cycle_The metaphase checkpoint

#	Gene name	P value
1	aurora kinase B	1.13693E-5
2	budding uninhibited by benzimidazoles 1 homolog (yeast)	2.48908E-5
3	budding uninhibited by benzimidazoles 1 homolog beta (yeast)	2.33211E-6
4	cancer susceptibility candidate 5	1.97298E-5
5	cell division cycle 20 homolog (S. cerevisiae)	6.03158E-5
6	centromere protein A	2.47863E-5
7	centromere protein E, 312kDa	5.50908E-5
8	MAD2 mitotic arrest deficient-like 1 (yeast)	8.96987E-6
9	NIMA (never in mitosis gene a)-related kinase 2	6.23262E-5
10	NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)	6.42837E-5
11	NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)	3.89014E-5

12	SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae)	5.24907E-5

Pathway 3: Cell cycle: Spindle assembly and chromosome separation

#	Gene name	P value
1	aurora kinase B	1.13693E-5
2	cyclin B1	3.02699E-5
3	cyclin B2	5.08655E-5
4	cell division cycle 20 homolog (S. cerevisiae)	6.03158E-5
5	cyclin-dependent kinase 1	5.27439E-5
6	extra spindle pole bodies homolog 1 (S. cerevisiae)	1.11078E-5
7	kinesin family member 11	6.5736E-5
8	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	2.15515E-5
9	MAD2 mitotic arrest deficient-like 1 (yeast)	6.23262E-5
10	NIMA (never in mitosis gene a)-related kinase 2	6.42837E-5
11	pituitary tumor-transforming 1	2.74285E-5
12	TPX2, microtubule-associated, homolog (Xenopus laevis)	1.52444E-5

Pathway 4: Cell cycle: Chromosome condensation in prometaphase

#	Gene name	P value
1	aurora kinase B	1.13693E-5
2	Cyclin A2	4.2174E-6
3	Cyclin B1	3.02699E-5
4	Cyclin B2	5.08655E-5
5	cyclin-dependent kinase 1	5.27439E-5
6	Non-SMC condensin I complex, subunit G	1.94062E-5
7	non-SMC condensin II complex, subunit G2	2.83433E-5

Pathway 5: Cell cycle: Role of Nek in cell cycle regulation

#	Gene name	P value
1	cyclin B1	3.02699E-5
2	cyclin-dependent kinase 1	5.27439E-5
3	MAD2 mitotic arrest deficient-like 1 (yeast)	6.23262E-5
4	NIMA (never in mitosis gene a)-related kinase 2	6.42837E-5
5	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)	8.39217E-6
6	TPX2, microtubule-associated, homolog (Xenopus laevis)	1.52444E-5

Pathway 6: Reproduction: Progesterone-mediated oocyte maturation

#	Gene name	P value
1	adenylate cyclase 7	5.70676E-6
2	budding uninhibited by benzimidazoles 1 homolog (yeast)	2.48908E-5
3	cyclin B1	3.02699E-5
4	cell division cycle 20 homolog (S. cerevisiae)	6.03158E-5
5	cyclin-dependent kinase 1	5.27439E-5
6	SHC (Src homology 2 domain containing) transforming protein 1	3.42417E-5

Pathway 7: Cell cycle: Initiation of mitosis

#	Gene name	P value
1	Cyclin B1	3.02699E-5
2	Cyclin B2	5.08655E-5
3	Cyclin dependent kinase 1	5.27439E-5
4	Forkhead box M1	4.24242E-5
5	Kinesin family member 11	6.5736E-5

Pathway 8: Cell cycle: Nucleocytoplasmic transport of CDK/Cyclins

#	Gene name	P value
1	Cyclin A2	4.2174E-6
2	Cyclin B1	3.02699E-5
3	cyclin-dependent kinase 1	5.27439E-5
4	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	2.15515E-5

Pathway 9: Immune response: IL-6 signaling pathway

#	Gene name	P value
1	CCAAT/enhancer binding protein (C/EBP), beta	3.81864E-5
2	interleukin 6 (interferon, beta 2)	6.65542E-6
3	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	8.62528E-6
4	SHC (Src homology 2 domain containing) transforming protein 1	3.42417E-5
5	suppressor of cytokine signaling 3	2.40557E-7

Pathway 10: Cell cycle: Sister chromatid cohesion

#	Gene name	P value
1	cyclin B1	3.02699E-5
2	cyclin B2	5.08655E-5
3	cyclin-dependent kinase 1	5.27439E-5
4	extra spindle pole bodies homolog 1 (S. cerevisiae)	1.11078E-5
5	pituitary tumor-transforming 1	2.74285E-5

Table 9: List of genes involved in the pathways generated for the gene list of the low density co-culture condition

Pathway 1: Development: Regulation of epithelial-to-mesenchymal transition (EMT)

#	Gene name	P value
1	Cyclic AMP-responsive element-binding protein 1	5.23817E-5
2	Endothelin-1	9.75532E-6
3	Endothelin-1 receptor	4.01231E-5
4	Epidermal growth factor receptor	8.03403E-6
5	Occludin	1.02734E-4
6	Alpha- type platelet -derived growth factor	1.27674E-4
7	28-gene expression cancer panel	1.27674E-4
8	Ski-like protein	2.36931E-5
9	Zinc finger protein SNAI2	8.13585E-6
10	Protein Wnt 5A	3.33772E-5
11	Protein Wnt 5B	2.65266E-5
12	Zinc finger E- box- binding homeobox 1	5.84862E-5

Pathway 2: Membrane- bound ESR1: interaction with G-proteins signaling

#	Gene name	P value
1	ADAM metallopeptidase domain 12	5.7858E-8
2	adenylate cyclase 7	1.5018E-6
3	caveolin 1, caveolae protein, 22kDa	3.33971E-5
4	cyclin D2	5.84981E-5
5	cAMP responsive element binding protein 1	5.23817E-5
6	Epidermal growth factor receptor	8.03403 E-6
7	guanine nucleotide binding protein (G protein), gamma 10	2.55097E-5
8	guanine nucleotide binding protein (G protein), gamma 2	1.42298E-6
9	inositol 1,4,5-triphosphate receptor, type 2	1.51396E-5
10	Matrix metallopeptidase 14 (memberane inserted)	9.021E-6
11	SHC (Src homology 2 domain containing) transforming protein 1	6.35845E-6

Pathway 3: Muscle contraction: Regulation of eNOS activity in endothelial cells

#	Gene name	P value
1	Bradykinnin receptor B2	8.17472E-5
2	caveolin 1, caveolae protein, 22kDa	3.33971E-5
3	cAMP responsive element binding protein 1	5.23817E-5
4	endothelin 1	9.75532E-6
5	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	7.37108E-5
6	FBJ murine osteosarcoma viral oncogene homolog B	1.07437E-4
7	guanine nucleotide binding protein (G protein), gamma 10	2.55097E-5
8	guanine nucleotide binding protein (G protein), gamma 2	1.42298E-6
9	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	1.35074E-5
10	inositol 1,4,5-triphosphate receptor, type 2	1.51396E-5
11	Phosphoinositoid -3- kinase, regulatory subunit 3 (gamma)	6.76253E-6
12	Sp3 transcription factor	7.39137E-5

Pathway 4: Cell adhesion: ECM remodeling

#	Gene name	P value
1	collagen, type I, alpha 1	2.83154E-6
2	collagen, type I, alpha 2	1.89384E-5
3	collagen, type III, alpha 1	8.15476E-7
4	epidermal growth factor receptor	8.03403E-6
5	ezrin	6.05609E-5
6	matrix metallopeptidase 14 (membrane-inserted)	9.0241E-6
7	plasminogen activator, urokinase receptor	1.73473E-4
8	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	9.2113E-7
9	TIMP metallopeptidase inhibitor 1	8.78576E-5
10	versican	4.12238E-7

Pathway 5: Transport: Macropinocytosis regulation by growth factors

#	Gene name	P value
1	BAI1-associated protein 2	3.39154E-5
2	epidermal growth factor receptor	8.03403E-6
3	insulin receptor substrate 1	1.3792E-4
4	p21 protein (Cdc42/Rac)-activated kinase 1	3.50557E-5
5	platelet-derived growth factor receptor, alpha polypeptide	1.27674E-4
6	protein kinase, AMP-activated, beta 2 non-catalytic subunit	1.05019E-4
7	SHC (Src homology 2 domain containing) transforming protein 1	6.35845E-6
8	WAS/WASL interacting protein family, member 1	1.67962E-4

Pathway 6: Cytoskeleton remodeling: TGF, WNT and cytoskeletal remodeling

#	Gene name	P value
1	Caveolin 1, caveolae protein, 22kDa	3.33971E-5
2	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	1.01739E-6
3	mitogen-activated protein kinase kinase 3	1.01467E-6
4	myosin, light chain 6B, alkali, smooth muscle and non-muscle	1.67768E-4
5	myosin light chain kinase	5.92326E-6
6	p21 protein (Cdc42/Rac)-activated kinase 1	3.50557E-5
7	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)	6.76253E-6
8	plasminogen activator, urokinase receptor	1.73473E-4
9	ribosomal protein S6 kinase, 90kDa, polypeptide 5	9.90624E-5
10	SHC (Src homology 2 domain containing) transforming protein 1	6.35845E-6
11	wingless-type MMTV integration site family, member 5A	3.33772E-5
12	wingless-type MMTV integration site family, member 5B	2.65266E-5

Pathway 7: Immune response: Signaling pathway mediated by IL-6 and IL-1

#	Gene name	P value
1	CCAAT/enhancer binding protein (C/EBP), beta	3.53122E-6
2	interleukin 1 receptor, type I	1.67338E-4
3	interleukin 1 receptor accessory protein	9.09919E-5
4	interleukin 6 (interferon, beta 2)	1.0234E-6
5	SHC (Src homology 2 domain containing) transforming protein 1	6.35845E-6
6	suppressor of cytokine signaling 3	1.16077E-6

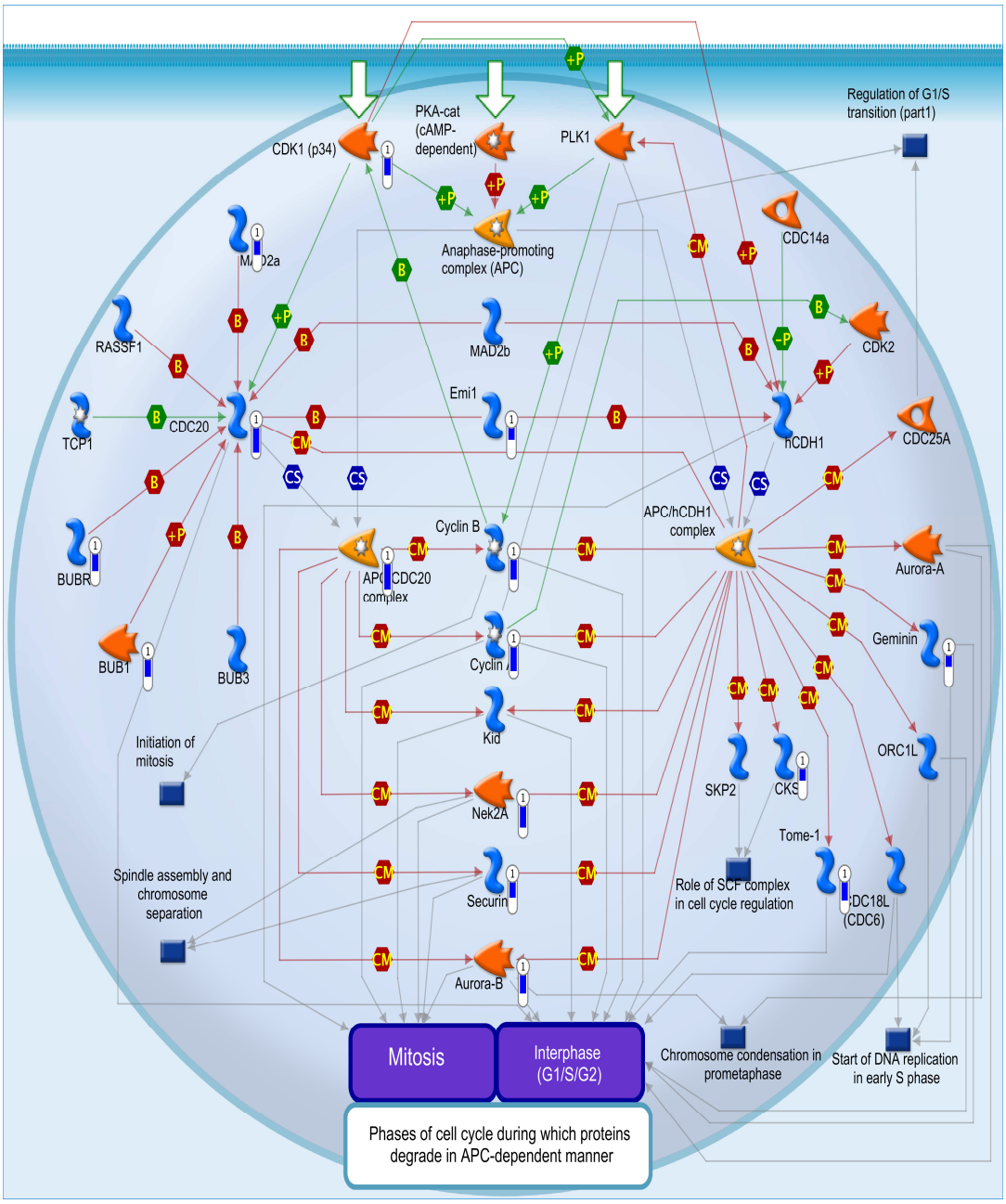

Figure A.1: The Role of APC in Cell Cycle Regulation. The most significant pathway obtained on comparison of the high density co-culture to the monoculture. The genes present in the gene list have a blue bar next to them indicating their down regulation.

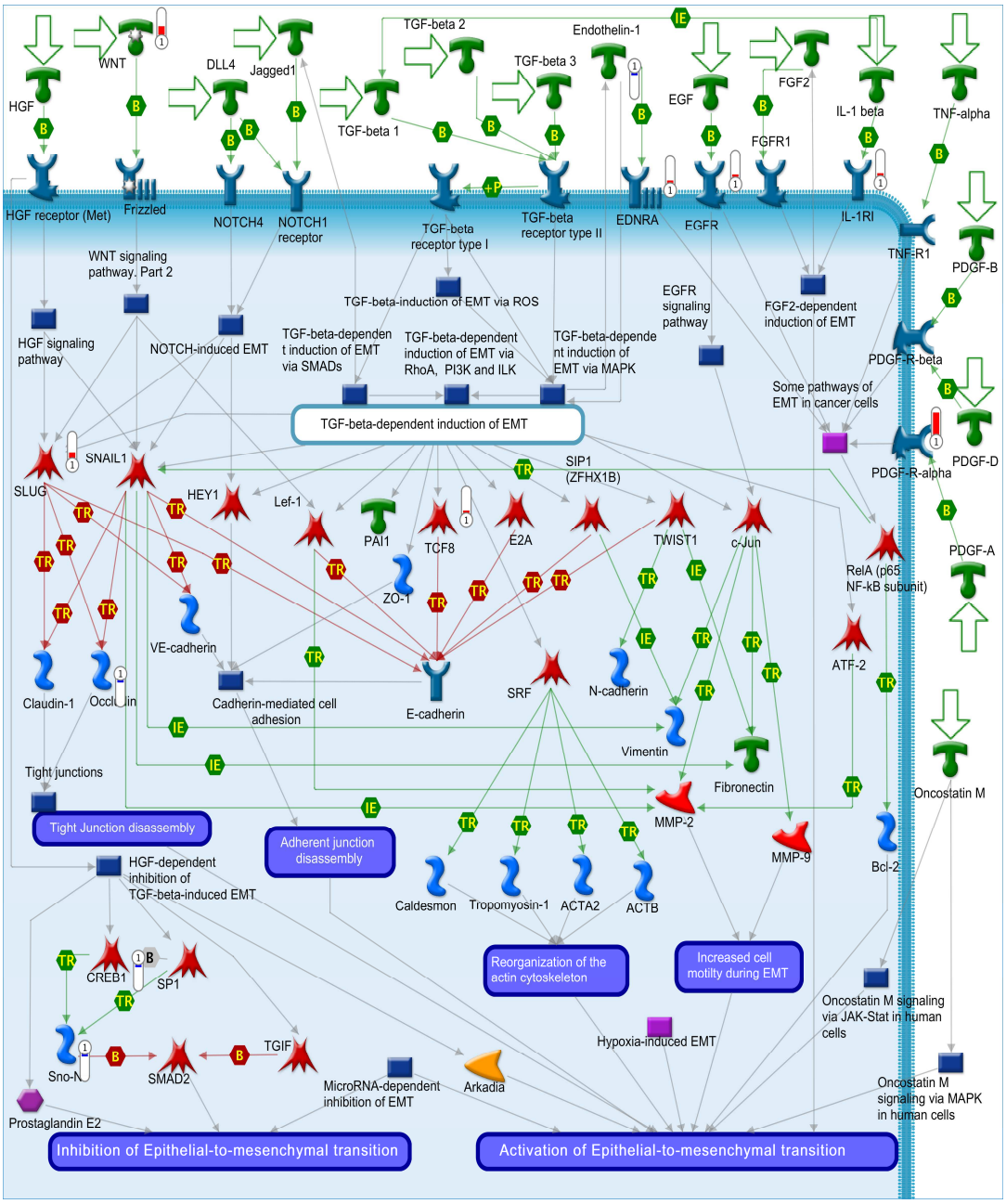

Figure A.2: The Epithelial to Mesenchymal Transition pathway. The most significant pathway generated on the comparison of low density co-culture gene list to the monoculture. The genes present in the gene list have a bar next to them. Red indicates their up regulation while blue indicates a down regulation of the gene.

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