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Deriving Functional Endothelial Cells from Embryonic Stem Cells in Chemically Defined Conditions

By

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B.S. (California State University, Fresno) 2004

Dissertation submitted in partial satisfaction of the requirements for the degree of

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In the

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At

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Curriculum Vitae

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2008 — **Reviewer for *Current Molecular Medicine***

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In Review:

Blancas AA, McCloskey KE. Specialized Tip- and Phalanx-like Endothelial Cells Derived from Embryonic Stem Cells. Angiogenesis.

Relevant Presentations

Role of the Extracellular Matrix in Vascular Differentiation
Dec. 2008: Poster, Tissue Engineering & Regenerative Medicine International Society

Serum-Free Derivation of Endothelial Cells from Embryonic Stem Cells
June 2008: Oral, University of California Bioengineering Symposium

Serum-Free Endothelial Derivation and Expansion of Murine Embryonic Stem Cells
Jan. 2008: Poster, Society for Biological Engineering's first International Conference on Stem Cell Engineering

Role of Extracellular Matrix Proteins in Vascular Differentiation in Chemically Defined Conditions
March 2009: Poster, Gordon Research Conference (Vascular Biology)

Modeling Adhesive Forces in Embryonic Stem Cell Morphology: A Testimony of Integrins and Cadherins

June 2009: Oral, University of California Bioengineering Symposium

Adhesive Forces Dominating Morphology of Embryonic Stem Cell Cultures

July 2009: Poster, International Society for Stem Cell Research, Barcelona, Spain

Vascular Differentiation: The Role of Matrix in Controlling Stem Cell Fate

October 2009: Poster, Emergent Behavior of Integrated Cellular Sytems (EBICS), MIT

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2009	**International Society for Stem Cell Research (ISSCR)**
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2003-2004	**Golden Key International Honours Society** (Lifetime Membership) California State University, Fresno Chapter *Public Relations Director: Spring 2003, Co-President: Fall 2003-Spring 2004*
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Chapter 1

1 Introduction

Stem cell therapy has been heralded as a possible treatment for a myriad of ailments. Their capacity to differentiate into various cell types allows them to, not only, repair or replace damaged tissue, but also to be used to model the developmental processes of different tissue systems. Our laboratory is interested in the process of vascular differentiation from undifferentiated stem cells into mature endothelial cells. Potential applications for these cells include seeding tissue engineered vascular grafts [1]. Also, damaged and ischemic tissue from myocardial infarcts could be "patched" with scaffolds containing EC, aiding in the restoration of cardiac function [2-4]. Moreover, the development of engineered tissues in vitro has been limited in respect to size due to their dependence on diffusion for nutrients. The prevascularization of these tissues would enable the development of larger grafts, but will still require connection to the host vasculature once implanted [5, 6]. Since murine embryonic stem cells (ESC) are relatively easy to maintain in culture compared with human ESC, we chose to use this culture system for our studies. However, we expect that information from this study may be applicable to mammalian systems in general.

*The overall **goal** of this project was to generate a chemically defined culture and differentiation scheme for the generation of functional endothelial cells. The stem cell-derived cells were then thoroughly characterized and subphenotyped correlating with specific differentiation/isolation methodologies and functional assays.*

1.1 Background

1.1.1 Vascular Development

During murine embryonic development, cells of mesodermal origin first begin to express fetal liver kinase-1 (Flk-1) at day 7 of embryonic development, considered to be the bipotential precursors of vascular and hematopoietic cells [7-14]. As these cells begin to differentiate, they self-assemble into structures called blood islands. These blood islands consist of endothelial cells surrounding hematopoietic progenitors. As blood islands interconnect, they form the primary capillary plexus [15, 16]. This formation of blood vessels, where there previously were none, is called vasculogenesis. In a murine system, this occurs at day 8 of development and is the first organ to develop in the embryo. As development continues, the vascular system expands by a process called “angiogenesis”, new vessels sprouting from preexisting vessels [7, 17-20]. Angiogenesis plays a major role in many physiological processes such as wound healing, menstruation, and tumor growth.

1.1.2 Endothelial Cells from Embryonic Stem Cells

Embryonic stem cells are isolated from the inner cell mass (ICM) of an embryonic blastocyst [21]. They are pluripotent cells, are able to self-renew, and retain the potential to differentiate into cells from all three germ layers. Although it is possible to obtain stem cells from a variety of adult sources such as bone marrow and adipose tissue, these adult stem cells are not pluripotent and generally can only be differentiated into a limited range of cell types [22-24]. Moreover, adult stem cells

can be difficult to isolate and expand in culture. Alternatively, ESC can be more easily maintained and expanded in culture.

Methods for the successful differentiation of endothelial cells (EC) from ESC in vitro have been previously described [12, 25-31]. One common method of vascular differentiation involves the formation of a 3D cell aggregate called an embryoid body (EB). This structure often contains differentiating cell types from all germ layers, but cell fate and visualization of individual cells with the EB remains challenging. Efficiencies of EC numbers from EB derivation methods are also very low [32]. Alternatively, a 2D monolayer differentiation system allows for better microenvironmental control and easy cell visualization facilitating morphological analysis of the differentiating cells [30, 33, 34]. Endothelial cell promoting growth factors, such as vascular endothelial growth factor (VEGF), may also be added to the differentiation medium. These growth factors may also be present in the serum component of the medium at unknown levels and combined with non-EC generating growth factors, making serum-containing mediums more challenging for use in stem cell derivation methods.

Moreover, comparisons between primary endothelial cells and ESC-derived EC have shown that ESC-derived EC may lack some functions observed in naturally occurring EC such as decreased low density lipoprotein (LDL) uptake and improper localization of vascular endothelial (VE) cadherin [29]. However, they also produce nitric oxide and display permeability comparable levels to primary EC [35]. We

hypothesized that the use of chemically defined conditions, that can provide better control over microenvironmental signals for EC fate, will generate more pure and EC with appropriate EC functions.

1.1.3 Endothelial Progenitor Cells

In our attempts to characterize an endothelial progenitor cell compared with mature endothelial cells from embryonic stem cells, we became aware that the term "endothelial progenitor cell" is only loosely defined by its potential to generate some EC and form tubes in vivo, and a specific identifying marker profile has not yet been identified. Currently, the definition of an EPC relies on the expression levels of endothelial markers relative to a known terminally differentiated EC control such as human umbilical vein EC (HUVECS) or murine aortic endothelial cells (MAEC) [29, 36, 37], or by assessing endothelial colony formation [38]. Published reviews have also compiled characteristics associated with adult-derived endothelial progenitor cells and circulating endothelial cells, but a unifying group of identifying variables remains elusive [39, 40]. Possible origins of EPC include both the lumen of vessels and bone marrow [41, 42], however, the CD34+/Flk-1+ cells that were once thought to contain EPC have been shown to include only non-endothelial hematopoietic cell populations [43]. Characterizing EC and EPC derived in vitro is additionally challenging due to the many different derivation protocols for generating EPC and CEC, leading to differences in EC phenotype and functionality. This further complicates the identification of progenitor and mature EC in derived populations since no definitive characterization is available for EPC [44].

1.1.4 Chemically-Defined EC Induction

When inducing ESC to differentiate, the most common approaches incorporate serum as well as growth factors specific for the desired cell lineage. However, serum is not a chemically-defined ingredient, varies from batch-to-batch, and contains factors promoting non-desired cell types as well as our desired cell lineages. This lends an element of unpredictability in culture conditions and introduces a factor that cannot be controlled experimentally. It is possible to secure an entire batch of serum from a manufacturer, ensuring the research is at least reproducible in one's own laboratory. When the supply is exhausted, it becomes necessary to perform labor intensive batch testing by running replicates of experiments in order to determine which batch yields the most favorable results.

Serum replacements have been used successfully in the maintenance and differentiation of ESC [12, 45-49]. Although most formulations are proprietary, they are generally free from animal components or are available in xeno-free forms and facilitate experimentation in a chemically defined condition. Although our lab chose to use Knockout Serum Replacement (Invitrogen) and Nutridoma-CS (Roche), in other serum free culture systems, bovine serum albumin (BSA) is used alone instead of KSR [50]. By using chemically defined medium, it is possible to more accurately control the cell's microenvironment and to evaluate the response to added signals.

The initial stage of induction requires the addition of factors that promote the differentiation of cells into the desired population. Flk-1, also called VEGF receptor 2, is considered to be the first lineage commitment marker for cells that will become vascular progenitors or hematopoietic progenitors [7, 8, 10, 11, 14, 15, 18, 20, 25, 51-55]. Induction of Flk-1 expression in ESC, for the purpose of studying the process of vascular development, in serum free medium has allowed the investigations of additional growth factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and bone morphogenic proteins (BMP), that promote early mesoderm formation [56].

VEGF mediates vasculogenesis and angiogenesis in various ways. It binds to Flk-1, which promotes EC survival by activating the PI3 kinase/Akt pathway ultimately leading to the inhibition of caspase activity. Also by binding to Flk-1 and causing the phosphorylation of Tyr-1173, VEGF activates the PLCγ-PKC-MEK-MAPK pathway which enhances proliferation and a PLCγ-PKC-eNOS pathway[57-59]. Endothelial nitric oxide synthase (eNOS) produces nitric oxide (NO), which affects vascular permeability and vasodilation, both key functions in the vascular system (Figure 1.1). Activation of the PI3 kinase/Akt pathway may also affect vascular permeability [59]. VEGF is also secreted by endothelial and smooth muscle cells (SMC) suggesting a positive feedback mechanism [57, 60]. Although Flk-1 is not expressed by SMC, these cells also respond to VEGF stimulation. Specifically, the binding of VEGF-to the VEGF-receptor 1 (fms-like tyrosine kinase 1, or Flt-1), causes the upregulation of

matrix metalloproteinase-9 [61] in SMC. This facilitates the degradation of the extracellular matrix during angiogenesis [62].

Basic fibroblast growth factor (bFGF) is also considered to be an angiogenic factor. However, it did not increase mesoderm formation under serum free conditions [63]. Alternatively, bone morphogenetic protein-4 (BMP-4) and activin A succeed in promoting mesodermal specific gene expression in serum free differentiation conditions [64]. BMP-4, in concert with VEGF, is used in our current serum free differentiation medium (Figure 1.2) since it promotes lateral mesoderm and hematopoietic development while simultaneously inhibiting neuronal development [63, 65, 66].

1.1.5 Angiogenic Signaling

VEGF is the most well studied angiogenic factor. There are several isoforms that bind to different VEGF receptors (Figure 1.3). VEGFA is the isoform that is commonly referred to as VEGF. Its expression is largely driven by hypoxic conditions that allow hypoxia-inducible factors to bind to the promoter of VEGFA [57]. Hypoxic conditions also trigger the upregulation of Flk-1 and Flt-1 [57, 61]. Knockouts of Flk-1 result in embryonic lethality at day 8-9 with a lack of normal blood island formation [16, 57]. Tumor cells also secrete VEGF to promote angiogenesis to meet the high nutrient demand of the proliferating tissue [19].

VEGF receptor 1 (Flt-1) has a higher affinity for VEGF than Flk-1 [67, 68]. In Flt-1 knockout studies, embryonic lethality occurs at day 9 with excessive EC proliferation and disorganization [57]. Flt-1 expression during development suggests that it negatively regulates the pro-angiogenic response triggered by VEGF-Flk-1 binding by acting as a "trough" for excess VEGF [68].

Tie-2 is a receptor tyrosine kinase that is expressed later in development than Flk-1 and Flt-1 [52]. Angiopoietin-1 has been identified as its ligand [69, 70]. The binding of Ang-1 to Tie-2 is essential for vessel stability possibly due to the recruitment of SMC [71]. Tie-1 is an orphan receptor, having no specific ligand, and its function is not completely understood. However, it is thought to affect venous system symmetry and play a part in fluid exchange in capillaries [69, 70]. Angiopoietin-2 is the antagonist of Ang-1 [72]. When binding to Tie-2, Ang-1 promotes vessel destabilization and inflammatory responses [73]. It has been proposed that Ang-2 is expressed earlier in tumor cells than VEGF to render the vessels more "plastic" to allow for angiogenic activity [19], playing an important role in the progression and metastasis of various cancers [74-77]. Moreover, when combined with VEGF, Ang-2 promotes tumor vascularization [78, 79] via a positive feedback mechanism.

Peripheral cell populations can provide additional sources of pro- and anti-angiogenic factors. Spherical SMC/EC coculture assays indicate that the presence of SMC may inhibit vessel sprouting by signaling EC to remain in a quiescent state

[73], but SMC may be necessary for vascular stability [80] and for the production of angiogenic factors [81].

1.1.6 Mimicking the Extracellular Matrix In Vitro

In addition to soluble signals, the cell-matrix signaling may also affect the differentiation fate and kinetics of ESC. Endothelial cells are known to express various integrins that allow adhesion between various ECM components, leading to events that effect adhesion, proliferation, and angiogenesis [82].

Collagen type-IV (CIV) is a network forming collagen that is the most abundant factor in the basement membrane of blood vessels providing structural stability [82]. Integrins α2β1 and α1β1 facilitate cell binding to CIV, stimulated by VEGF [83-85]. During angiogenesis, CIV is proteolytically cleaved by matrix metalloproteases, which reveals cryptic sites that promote binding to αvβ3, necessary for angiogenesis, while the α1β1 binding is lost [84].

Collagen type-I, a fiber forming collagen, is the most common ECM component in the body outside the basement membrane of vessels. Degradation of the basement membrane around vessels exposes EC to collagen I. Binding of collagen I to certain integrins has been proposed as a mechanism that enhances the migration of EC and therefore facilitating angiogenesis [86, 87].

Laminin, specifically isoforms -8 and -10, are a major components of the basement membrane lining blood vessels [82]. Studies have shown that laminin is also required for SMC maturation [64], the cells responsible for the contractility necessary for the vasodilation and vasoconstriction actions of large vessels.

Fibronectin is an extracellular matrix protein that has been used to culture isolated EC and EPC [88] and promotes the continued differentiation of EPC into EC [89]. It has also been demonstrated that primary EC generate networks of fibronectin [35, 90]. Since this is also a key component of the endothelial basement membrane, fibronectin may play a critical role in cell fate signaling, and is an excellent candidate in studying differentiation of EC from ESC. It has been shown to enhance VEGF induced differentiation via the α5β1 integrin [89].

In addition to cell-substrate, cell-cell interactions can affect cellular responses. A mathematical model can be used to approximate the strength of cell-substrate interactions and cell-cell interactions. This can facilitate further optimization of microenvironmental mimicry, using the “natural” adhesive forces on the cells as a guide [91]. Thus nanotopographical cues, which are already in use for tissue engineering [92-94], can be customized based on cell type.

1.1.7 Scaffolding for Cell Incorporation

In order to investigate the therapeutic applications of endothelial cells and/or progenitors, it is often necessary to incorporate the EC into scaffolding material.

Scaffolding materials are also valuable in facilitating 3D angiogenesis in vitro. For cell incorporation, there are three main types of scaffolds: 1) natural scaffolds consisting of components derived from the native ECM, 2) permanent synthetic scaffolds, and 3) biodegradable polymers.

Although natural scaffolding materials can have the benefit of low thrombogenicity (clot formation), they are derived from animal products and must therefore go through an extensive purification process to avoid immune reaction from the recipient [95]. Permanent synthetic scaffolds can be mechanically sturdy, but since they become a permanent fixture in the recipient, there have been reports of calcification around the scaffolds [96].

We chose to use Gelfoam due to its popular use in angiogenic studies. Gelfoam's honeycomb-like structure provides a suitable scaffold for EC [97]. Cells can be seeded into the Gelfoam, and embedded in a gelled substance for an in vitro angiogenesis assay or implanted into an animal model for in vivo studies. Gelfoam can also be used to deliver angiogenic factors. Previous studies have involved the implantation of Gelfoam structures loaded with VEGF and bFGF to observe if the host EC migrate towards the source of the angiogenic signals [98]. EC then invade the Gelfoam, which is later removed, sectioned, and imaged to measure the amount of EC in the scaffold. However, factors loaded in Gelfoam are not secreted in a controlled manner, making it better suited as a cell-only scaffold.

Additionally, a substance called Matrigel is also commonly used as a cell delivery mechanism in various animal models. Matrigel also serves as a well-established in vitro model of tubulogenesis and sprouting activity of EC. Cells can be embedded in the Matrigel or on top and the subsequent angiogenic activity can be measured. Matrigel is derived from Engelbreth-Holm-Swarm (EHS) mouse sarcomas and contains a rich mixture of ECM proteins. However; because Matrigel is derived from tumorogenic mouse tissue, it cannot be used in human therapeutics.

1.2 Endothelial Cell Characterization

1.2.1 Characterization Using Flow Cytometry

Commonly used EC and SMC markers are listed below.

Marker (Source)	**Rationale**
Sca-1 (BD Pharmingen)	Sca-1+ cells shown to give rise to EC and SMC[99, 100].
Oct 4 (Santa Cruz)	Transcription factor found in ESC[101].
Flk-1 (RDI/Fitzgerald)	First indicator of hematopoietic and vascular differentiation[16, 102]. Key to triggering several biochemical pathways involved in cell migration, survival, and proliferation[33, 53, 68, 103].
PECAM 1 (CD 31) (Santa Cruz)	Platelet endothelial cell adhesion molecule. Once thought to be localized at cell-cell junctions[104, 105] it may actually be more diffuse[106].
VE-cadherin (Santa Cruz)	Localized in cell-cell junctions. Prevents disassembly of blood vessels[107]. Associates with Flk-1 to induce cell survial via PI3kinase/AKT signaling[108].
Flt-1 (Santa Cruz)	Flt-1 knock-outs show increased amounts of endothelial progenitors and vascular disorganization. Die at day 9[68].
Tie-1 (Santa Cruz)	Mature EC marker[69, 70]. No specific ligand. Double knock-outs of Tie-1 and Ang-1 lead to disruption in right hand side venous system formation, but not on the left hand side[109, 110].
Tie-2 (Chemicon)	Mature EC marker[69, 70]. Receptor for angiopoietin-1. Maintains interaction between EC and SMC[111].
vonWillebrand factor	Clotting at vascular injury sites[112]. Also located in the

(AbCam)	basement membrane of vessels[113].
α-smooth muscle actin (Sigma)	Show how much of the population is comprised of smooth muscle cells[27-29].
Calponin (Santa Cruz)	Early marker for SMC development[114-116].
SM22α (AbCam)	Late marker for SMC development[114-116].
EphB4 (Santa Cruz)	Marker for venous EC[13].
ephrinB2 (Santa Cruz)	Marker for arterial EC[13].
CD45 (BD Pharmingen)	Lymphocyte marker. Negative control[117].

Several known endothelial and smooth muscle markers are used to characterize each stage of stem cell commitment in order to develop a scheme to make the isolation of the population reproducible for possible clinical studies. Since the expression timeline of several of these markers are known, the approximate maturity of derived EC can be estimated. For example, a progenitor stage may express VE-cadherin and Tie-2, but not Tie-1 [52]. On the same note, the smooth muscle progenitors in culture may express calponin, an early SMC marker, but not SM22α [114-116, 118-120]. Thorough characterization would allow for detection of sub-populations in the intermediate development stages.

1.2.2 Animal Models of Angiogenesis/Vasculogenesis

Several types of in vivo studies have been used to observe angiogenesis. While in vitro assays provide an estimation of the angiogenic and vasculogenic potential of a cell population, the in vivo assays additionally determine whether the transplanted cells are able to successfully integrate with the host vasculature.

One type of angiogenesis assay that allows for relatively simple visualization is the dorsal skinfold chamber. This chronic transparent chamber model involves the

usage of a steel frame attached to the back of a mouse or hamster to hold the dorsal skin in place [121, 122]. Angiogenic activity is then monitored in the translucent skin through a window in the frame using epi- or trans-illlumination [121]. This study must be conducted using immuno-deficient rodents in order to reduce immune reactions to the implanted tissue or cells.

The cranial window assay is another form of chronic transparent chamber model that has the benefit of being performed in an immuno-priviledged site [121, 123]. It lasts longer than other chamber assays, but the surgery necessary for implantation requires a high level of technical skill. In addition, monitoring of angiogenic activity may require the use of fluorescent markers or cells that ubiquitously express fluorescent protein.

Due to the complete lack of background vasculature, the corneal pocket angiogenesis assay has become a popular model [121, 124]. A pocket is made in the cornea and tissue or scaffolding loaded with cells or angiogenic factors is introduced [121, 124, 125]. Vessel growth is three dimensional, which makes visualization and quantification challenging. This assay was originally designed to be performed on rabbits, but has been adapted for use with mice and rats [121, 124]. Like the cranial window assay, this procedure also requires significant training.

A Matrigel plug assay is a relatively simple in vivo assay. Cells or angiogenic factors are suspended in a solution of Matrigel and injected subcutaneously. The solution

forms a plug within seconds at body temperature [121, 124, 126]. The plug is recovered one to three weeks after injection. It can be analyzed histologically both for vessel growth and hemoglobin levels. The latter method is less reliable since it can vary with vessel size or the presence of blood pools in the plug[127]. Vascularization can be visualized by injecting fluorochrome labeled dextran into the tail vein and using UV-illumination.

The chick chorioallantoic membrane (CAM) assay has become popular due to its ease and cost effectiveness. It requires little technical skill and the embryo can be fixed and sectioned for staining [124]. Fertilized eggs are cracked and placed into petri dishes or a small window is made in the shell to allow for manipulation [121, 128, 129]. Cells can be directly implanted on the CAM or seeded onto a scaffold pre-implantation. Although a dissecting scope can be used to image vessel growth, fluorescently labeled cells facilitate the distinction between implanted cells and host cells. AngioQuant, a software specifically developed for vessel/tubule quantification, was used to analyze images of the formed tubules. The software automatically converts the image into binary form, measures tubule length, and branch points [130].

1.2.3 EC Mobility and Sprouting

EC migration is a key component in angiogenic activity. This process involves focal adhesion turnover and cytoskeletal organization, which is activated by the VEGF pathway [131] discussed previously Another candidate in the angiogenic activity of

cells is their production of matrix metalloproteinases (MMPs). Matrix metalloproteinases (MMPs) are a group of enzymes that degrade various extracellular matrix proteins, required for angiogenic sprouting. A few MMPs have been shown to be involved in angiogenesis, specifically MMP2 and MMP9, also known as gelatinase A and B, respectively. The structures of MMP2 and MMP9 are quite similar. Both contain repeating fibronectin II-like structures, which allows them to interact with and degrade collagen types I, IV, and V [132]. MMP2 has been shown to activate MMP9 [133] and both can induce the secretion of VEGF [134]. Additionally, MMP2 and MMP9 play a role in the metastasis and malignancy of several types of cancers [135-137].

A more recent addition to the MMP family is MMP19. Like MMP2 and MMP9, it is capable of digesting collagen IV. However it can also degrade nidogen, laminin, and aggrecan [138, 139]. Although the exact functions of MMP19 is still being examined, it has been shown to affect the invasiveness of breast cancer [140]. MMP19 also has the potential to inhibit angiogenesis while breaking down plasminogen [141].

Another candidate, phosphorylation of heat shock protein-27, HSP27, has been recently shown to be heavily involved in actin organization and migration of EC. HSPs traditionally behave as chaperone proteins, where they assist in protein folding and localization [142] and protect against apoptosis resulting from stress [143]. Like many proteins, HSP27 becomes active via phosphorylation. Although there are many potential locations for phosphorylation, serine 82 on HSP27 has

been identified as the crucial component in EC migration and angiogenic activity [144-146] (Figure 1.1). Without this activation, EC migration can be severely restricted. HSP27 activity is induced by the activation of protein kinase D as a response to VEGF signaling [147-149]. It can also be activated through p38 signaling activated by TNF alpha [145, 149, 150]. HSP27 has also been shown to play a role in certain cancers [151], which has made it a potential target for anti-angiogensis therapies [152].

The most angiogenic types of endothelial cells include “tip” and “stalk” cells (Figure 1.4). Tip cells are located at the leading end of angiogenic sprouts and respond to various proangiogenic signals, including VEGF. They have been shown to express Neuropilin-1, which assists in vessel guidance [153], delta-like ligand 4, necessary for notch signaling which also guides tip cells [154], in addition to CXCR4 [155] and UNC5B [156]. In addition, they respond to angiogenic stimulus by releasing F-actin caps and facilitating polymerization and extension of fillipodia [157]. These cellular projections help the cell “crawl” along ECM proteins, facilitating sprouting migration [158]. Stalk cells share many characteristics of tip cells, including the expression of certain markers. However, the Dll4/Notch signaling in stalk cells has been shown to decrease expression levels of neuropilin 1, Flk-1, and CXCR4, as opposed to increasing expression as in tip cells [159]. The exact mechanism behind this difference is still being investigated.

Although many EC will exhibit some migration, there is a population of cells known as phalanx endothelial cells in the lumen of the vessels that remain largely non-migratory (Figure 1.3). A distinguishing characteristic of phalanx EC is the high level of soluble Flt-1 production and high level of VE-cadherin expression [160]. As stated before, Flt-1 has a higher affinity for VEGF than Flk-1 and is hypothesized to serve as a "trough" for excess VEGF [161, 162]. Since VEGF signaling triggers migration of EC, the secretion of additional sFlt-1 dampens the signal, helping to maintain the phalanx EC within the vessel lumen. Often angiogenic potential is used as a gauge of the functionality of derived EC. However, if generating EC that demonstrate a phalanx phenotype, as described here, then the traditional views of EC functionality may need to be expanded to include specifics regarding EC subpopulations.

1.3 Conclusion and Significance

Stem cell therapy has the potential to be used in a wide range of clinical applications. The goal of this project was to 1) develop optimized methods for generating EC from ESC, and 2) determine which stage of differentiation, from ESC to EC, displays the most angiogenic potential. These cells can be used for a variety of applications including vascular grafts, pre-vascularizing tissue transplants, and repairing ischemic tissue after myocardial infarction. Since cardiovascular disease is so prevalent in the United States and around the world, ongoing research in this area has the potential to have a tremendous impact not only in the medical field as a whole, and specifically in the lives of those who benefit from the latest advances.

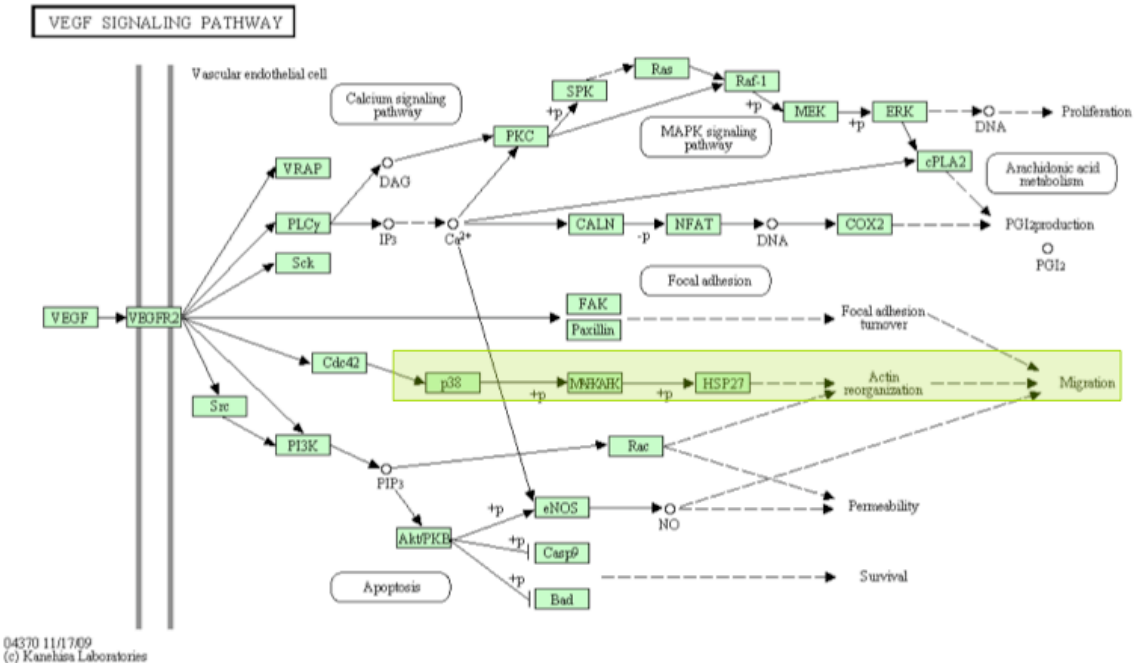


Figure 1.1: VEGF signaling modulates endothelial behavior and function. Proliferation, migration, and survival of endothelial cells are all in response to VEGF. In order to determine the mechanism behind in vitro angiogenic behavior, we examined HSP27 phosphorylation, an important factor for EC migration (pathway highlighted in green). Modified from Kanehisa Laboratories (http://www.genome.jp/kegg/pathway/hsa/hsa04370.html)

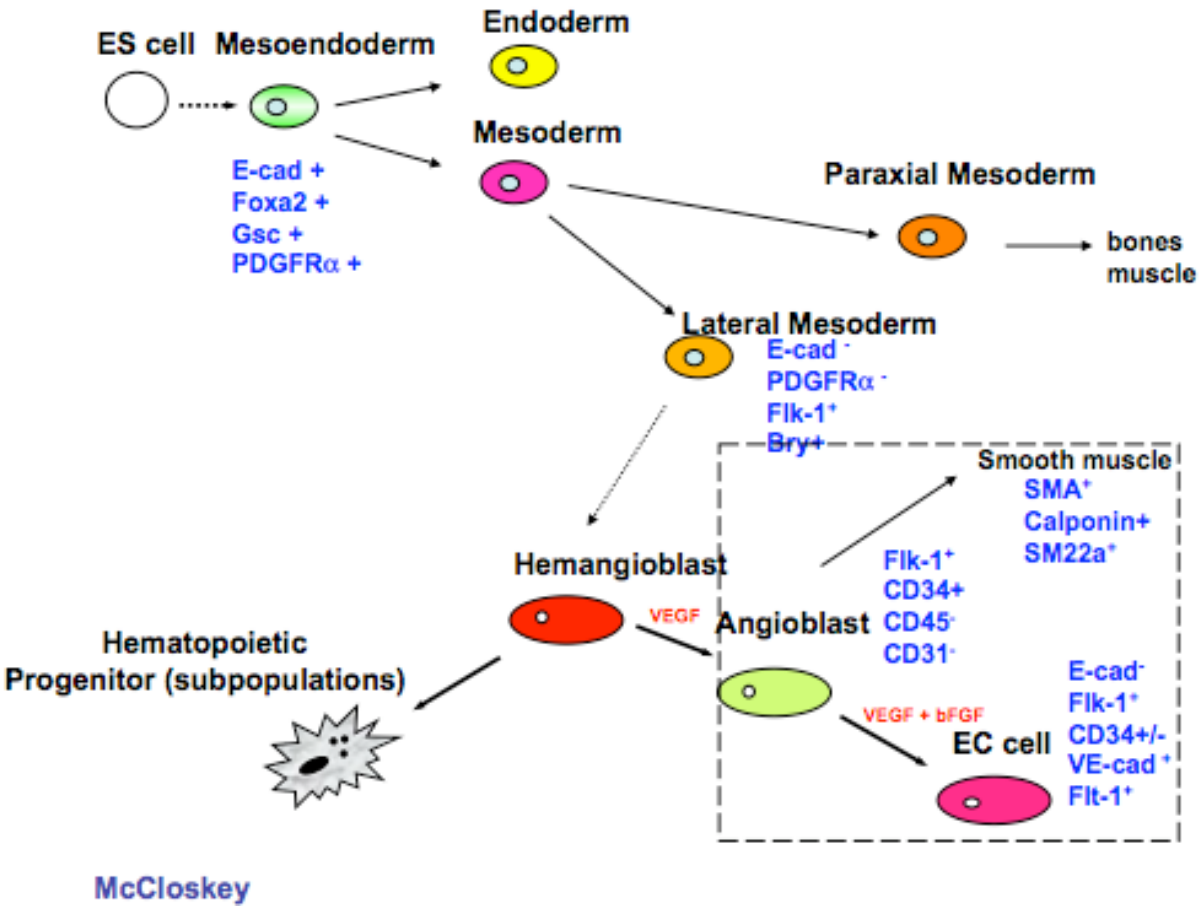


Figure 1.2: The differentiation of Vascular cells. Vascular cells (smooth muscle and endothelial) are of the mesodermal lineage and share a common Flk-1+ progenitor. Several markers can be used to differentiate between smooth muscle cells (SMC) and endothelial cells (EC), facilitating the generation of purified cell populations. Although the co-culture of SMC and EC can be used to stabilize vessel formation, SMC can also inhibit the proliferation of EC. Original image courtesy of Dr. Kara E. McCloskey. Modified by A.A. Blancas.

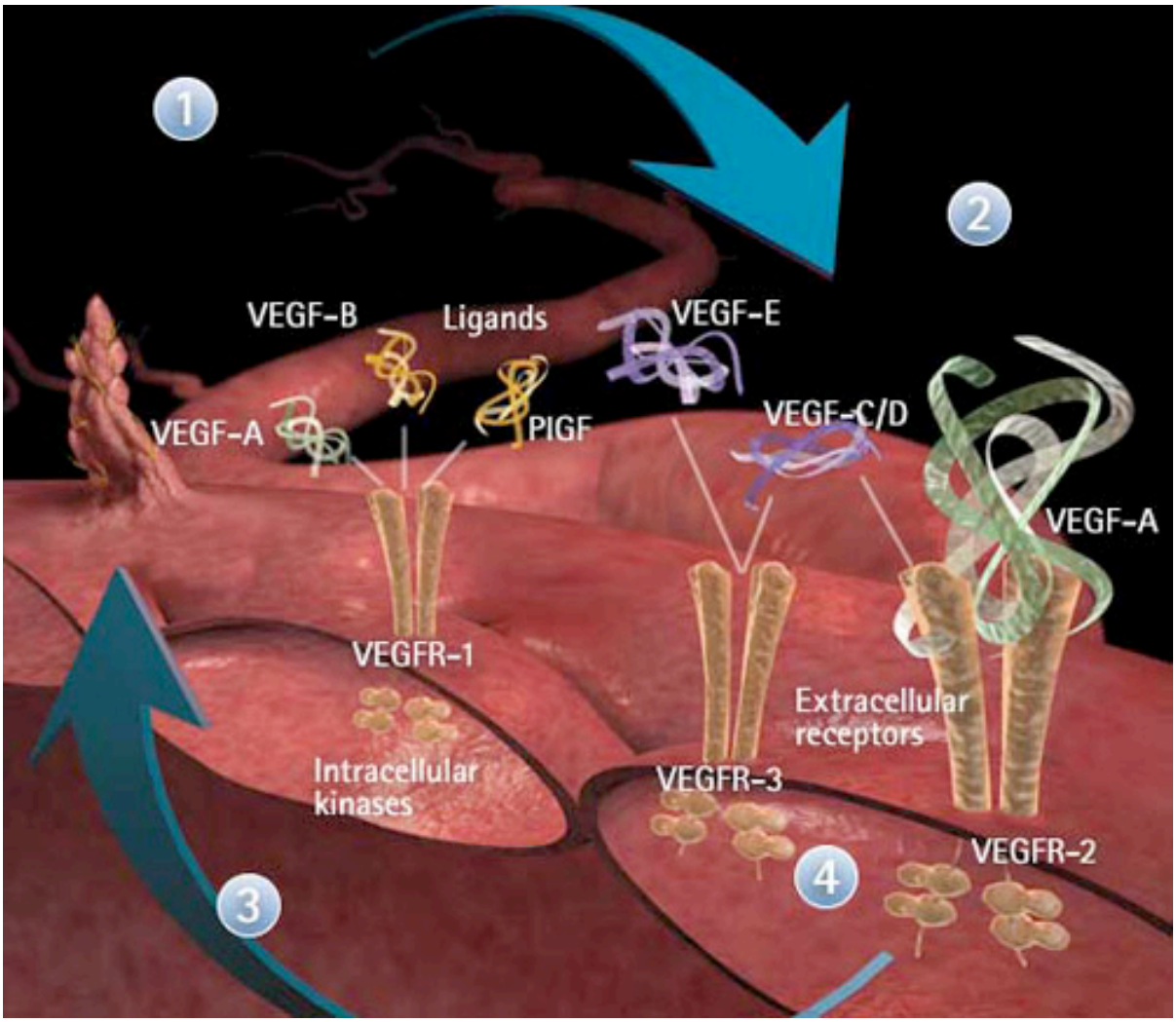


Figure 1.3: Angiogenic signaling. **1)** Upstream processes stimulate the production of VEGF, **2)** VEGF binds to receptors on endothelial cells, **3)** angiogenesis is mediated primarily through the interaction of VEGF-A with VEGFR2 (Flk-1), **4)** other variants of the VEGF ligand and receptor play a secondary role in this process. Image and description © Genetech USA.

http://www.biooncology.com/research-education/vegf/vegf/vegf-pathway/index.html

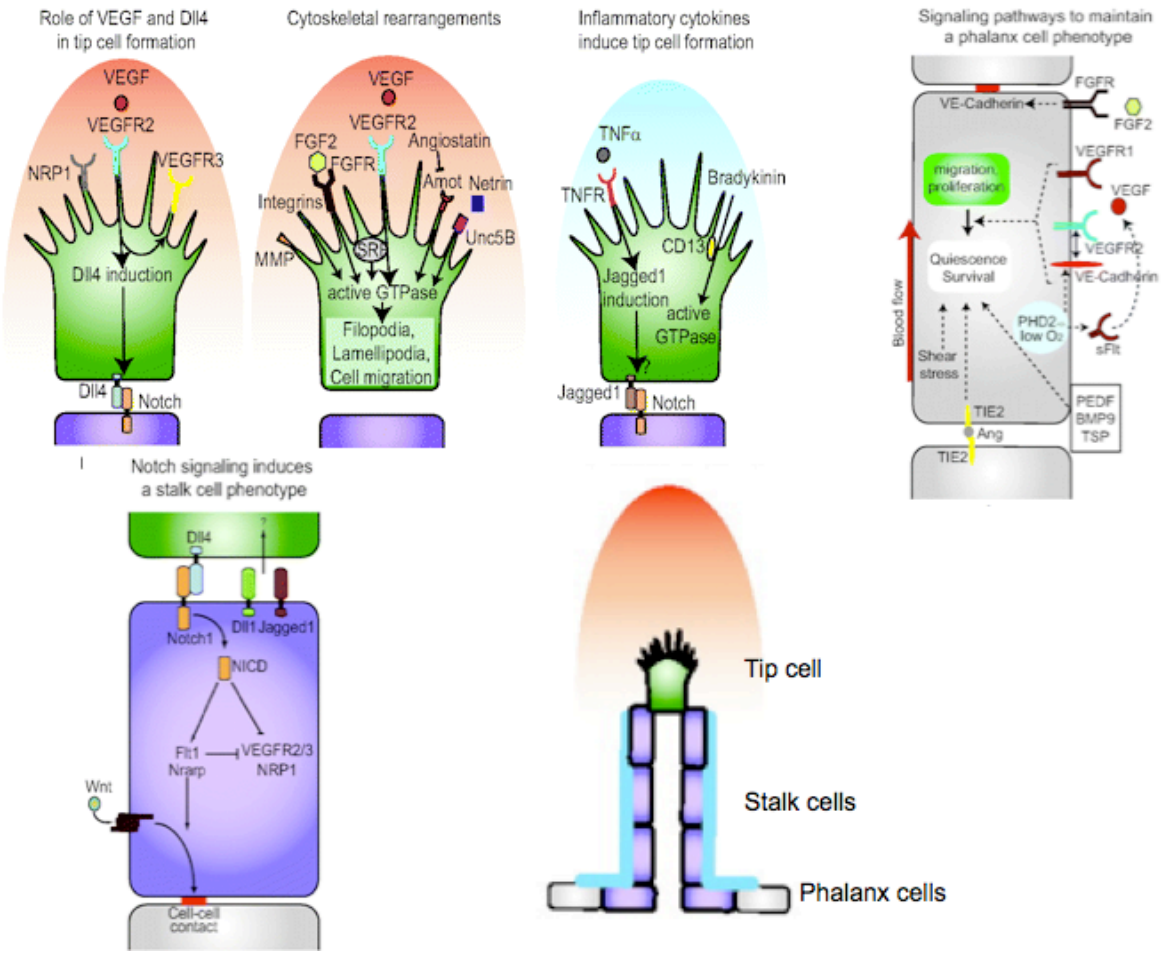


Figure 1.4: Identifying tip, stalk, and phalanx EC. Tip cells phenotype can be triggered by Notch/Dll4 signalling. Migration of the tip cells requires cytoskeleton reorganization, generally in response to VEGF. Inflammatory cytokines, such as TNFα can also promote tip cell migration. Stalk cell migration is hampered by Notch/Dll4 signaling, which ensures that the tip cell retains the lead position in a new vascular sprout. Stalk cells are responsible for forming the lumen of new sprouts and depositing ECM. Phalanx cells remain quiescent in the lumen of vessels. High levels of soluble Flt-1 are produced, which helps dampen proangiogenic signals from VEGF. Image from De Smet et al.

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Chapter 2

Endothelial Cells from Embryonic Stem Cells in Chemically Defined Medium

Abstract

Endothelial cells (ECs) are desired for their therapeutic potential in variety of areas including: gene therapy, cardiac regeneration, development of tissue engineered vascular grafts, and pre-vascularized tissue transplants. Pluripotent embryonic stem cells (ESCs) can be induced to differentiate into ECs in vitro using embryoid bodies, monolayer cultures, or by genetic manipulation and immortalization. However, obtaining homogeneous cultures of proliferating ESC–derived ECs without genetic manipulation is a challenging undertaking and often requires optimization of protocols and rigorous purification techniques. Moreover, current differentiation methods that use mediums containing fetal calf or bovine serum components introduce additional challenges due our limited ability to control the differentiation signals and batch-to-batch variations of serum. We have explored the development of new medium formulations for deriving ECs from murine embryonic stem cells (mESCs) using only chemically defined reagents. We present two different medium formulations along with the detailed methodologies, including the optimization of extracellular matrix-derived substrates known to play a role in cell attachment and proliferation, as well as cell differentiation. Characterization of the ESC-derived ECs indicate that 1) chemically defined medium formulations reproducibly generate superior ECs compared with previous serum-containing formulations, 2) fibronectin, and not collagen-type IV, is the optimal substrate for EC induction in our chemically defined medium formulations, 3) without additional activation of Notch-signaling,

ESC-ECs develop predominantly into venous ECs, and 4) using these medium formulations, a second rigorous selection step is not required to generate proliferating ECs from ESCs, but does enhance the final purity of the ECs.

2.1 Introduction

Endothelial cells (ECs) are highly dynamic cells that participate in the regulation a variety of tissue system functions including: vascular, cardiovascular, as well as the immune system. ECs regulate blood pressure through controlling vasodilation and vasoconstriction via synthesis of nitric oxide. ECs also regulate the permeability of the endothelium for recruiting and permitting transmigration of leukocytes in response to inflammation. It is well known that ECs also help inhibit platelet adhesion and clotting, and are key players in initiating new blood vessel growth and assembly.

Vascular ECs or endothelial progenitor cells derived from stem cells could potentially lead to a variety of clinically relevant therapeutic applications [1]. Endothelial progenitor cell transplantation has been shown to induce new vessel formation in ischemic myocardium and hind limb [2-4], supporting enthusiasm that these cells could be used in strategies for the repair and revascularization of ischemic tissue in patients exhibiting vascular defects [4, 5]. Additionally, because ECs inhibit platelet adhesion and clotting, lining the lumen of a synthetic or tissue-engineered vascular graft may aid in patency of vascular grafts [6, 7], or in the development of pre-vascularized tissue-engineered materials. Moreover, because ECs line the lumen of blood vessels and can release proteins directly into the blood stream, they are ideal candidates to be used as vehicles of gene therapy.

2.1.1 EC Differentiation from ESC

Human and murine embryonic stem cells (ESCs), isolated from the inner cell mass (ICM) of a developing blastocyst, are pluripotent cells that are also capable of self-renewal, as well as, able to differentiate into cells from all three germ layers [8]. ESCs are an especially attractive cell culture system because they can be easily maintained and expanded in culture. Although it is possible to obtain stem cells from adult sources, such as bone marrow and adipose tissue, adult cells exhibit limited pluripotency compared with ESCs or induced-pluripotent stem cells (iPSC). Additionally, adult stem cells can be difficult to identify, isolate and expand in culture. For these reasons, ESCs are an ideal cell culture system for studying stem cell fate and vascular development.

Successful methods for the *in vitro* differentiation of ECs from ESCs [9-16] and adult stem cells [17-19] have been previously described. One common method used in the derivation of several cell types from ESCs, including ECs, involves the formation of a three-dimensional (3-D) aggregate called an embryoid body (EB) [9, 14]. This structure allows the differentiation of ESCs towards various cell types from all three germ layers. Unfortunately, it is difficult to control the cells' microenvironment within the EB. Conversely, a two-dimensional (2-D) monolayer induction system allows for easier cell visualization real time and better control over the cells' microenvironment [12, 13, 15]. Endothelial promoting growth factors, such as vascular endothelial growth factor (VEGF), can be also be added to the differentiation medium to increase cell differentiation and proliferation of a specific cell phenotype.

Our laboratory and others have published methods for the differentiation of ECs from ESCs [9-13, 15]. These methods incorporate treatment with vascular endothelial growth factor (VEGF) to promote EC specification, however; they also rely on fetal bovine serum to further promote differentiation and proliferation of the EC populations. Unfortunately, the presence of serum in the induction medium formulations often leads to problems with reproducibility due to uncontrolled variations from batch-to-batch of serum, and also limits one's ability to directly control stem cell fate.

For these reasons, serum-free replacements have been explored and successfully used in the maintenance of ESCs and stem cell differentiation [20-24], but this has not yet been accomplished for EC induction from mouse ESCs. Although most formulations of serum replacements are proprietary, they are generally free from animal components and do not demonstrate the batch-to-batch variation seen in serum. By using a chemically defined medium, one can more accurately control the cell's microenvironment and therefore, better evaluate the response of a particular biochemical or physical signal. In addition, the final yield and quality of functionally mature tissue-specific cells derived *in vitro* from stem cells may be improved by using chemically defined medium formulations that allow directed differentiation, rather than serum-containing formulations.

We set out to explore methodologies for directed differentiation of ESCs towards ECs using only chemically-defined medium formulations. Here, we present our novel medium formulations for the induction and culture of these cells at well-defined stages of maturation. Moreover, we explored optimal time points and matrix substrates required for the initial generation of high numbers of Flk-1+ vascular progenitor cells and Flk-1+ outgrowths. The methods presented in this paper allow a more consistent and robust generation of ECs with appropriate expression of endothelial markers, as well as, improved low density lipoprotein (LDL)-uptake compared with previous derivation methods that included the use of serum [25].

2.2 Materials and Methods

2.2.1 ESC Culture

R1 and D3 mESCs (ATTC) and E14 mESCs (gift from Bruce Conklin) were cultured on 0.5% gelatin coated-cell culture dishes in serum-free medium [26]. This medium contains Knockout Dulbecco's Modified Eagle Medium (KO-DMEM; Invitrogen), 15% Knockout Serum Replacer (KSR; Invitrogen), 1x Penicillin-Streptomycin (Invitrogen), 1x Non-essential Amino Acids (Invitrogen), 2mM L-glutamine (Invitrogen), 0.1mM 2-mercaptoethanol (Calbiochem), 2000 Units/ml of leukemia inhibitory factor (LIF-ESGRO; Chemicon), and 10 ng/ml of bone morphogenetic protein-4 (BMP-4; R&D Systems).

2.2.2 Induction of Flk-1+ Cells in Chemically Defined Medium

Undifferentiated R1 and E14 mESCs were harvested from gelatin-coated dishes using 0.25% Trypsin/2.21mM EDTA (Mediatech) and plated on cell culture plates coated with various commercially available substrates including: 0.5% gelatin, 50ug/ml fibronectin, 50ug/ml collagen type-I, 50ug/ml collagen type-IV, and 50ug/ml laminin as per manufacturer instructions (BD Biosciences). The initial induction medium optimized by our laboratory was named "NS1D2b". This consists of alpha-MEM (Cellgro), 20% KSR (Invitrogen), 1x Penicillin-Streptomycin (Invitrogen), 1x Non-essential amino acids (NEAA; Invitrogen), 2mM L-glutamine (Invitrogen), 0.05 mM 2-mercaptoethanol (Calbiochem), 30ng/ml of vascular endothelial growth factor (VEGF; R&D Systems) and 5ng/ml BMP-4 (R&D Systems). Because the optimal induction lengths for generating Flk-1+ vascular progenitor cells does vary between cell lines [26], undifferentiated ESC were also cultured on plates in NS1D2b medium for 2, 3, 4 and 5 days in order to determine the optimal number of days for initial induction of Flk-1+ vascular progenitor cells.

In addition to verifying induction time, we also optimized the substrate for induction of Flk-1+ cells by using culture plates coated in either gelatin, fibronectin, collagen-type I, collagen-type IV, and laminin. The adherent cells were dissociated, counted, stained with AlexaFluor 647-conjugated anti-Flk-1 antibodies (Biolegend) and analyzed for Flk-1 expression using a BD LSRII flow cytometer and FlowJo software (TreeStar).

2.2.3 Purification and expansion of ESC-derived ECs in Chemically Defined Conditions

After the initial induction period, the cell population was enriched for Flk-1+ vascular progenitor cells using either fluorescent activated cell sorting (FACS) or a MiniMACS (Miltenyi Biotec). AlexaFluor 647 conjugated anti-mouse Flk-1 antibodies (Biolegend) and anti-AlexaFluor 647 magnetic beads (Miltenyi Biotec) were used to label the Flk-1+ expressing cells. Post enrichment, the Flk-1+ cells were re-plated on dishes coated with either fibronectin, laminin, collagen-type I, collage-type IV, or gelatin. These cells were expanded in another chemically defined medium developed in our laboratory that we have named "LDSk". The medium consists of 70% alpha-MEM (Mediatech) and 30% DMEM (Invitrogen) plus 100ng/ml VEGF (R&D Systems), 2x Nutridoma CS (Roche), 50ng/ml bFGF (Sigma), 2mM L-glutamine (Invitrogen), 1x penicillin-streptomycin (Invitrogen), 1x nonessential amino acids (Invitrogen), and 0.1mM 2-mercaptoethanol (Calbiochem). The cells were expanded for 2 weeks until clear cobblestone morphology became visible.

2.2.4 Second Purification of ESC-derived ECs

We have previously published that a second purification is necessary for proliferation of the D3 ESC-derived ECs produced using medium formulations containing serum [10, 25, 26]. Although we have rigorously examined a variety of purification techniques including magnetic and fluorescent activated cell sorting (MACS and FACS), the manual picking of cobblestone colonies consistently results in the most pure EC cultures (>95% purity [10]). Moreover, we found that obtaining relatively

homogeneous EC cultures is a critical factor allowing the expansion of the maturing EC [10]. Here, we also compare ECs generated with and without this second purification step under serum-free conditions.

ECs with cobblestone morphology were manually picked with flame-pulled micro-tip Pasteur pipettes in a sterile laminar flow hood outfitted with a stereoscope (Zeiss). The 9" Pasteur pipettes (VWR) were flame-pulled to a thin point and attached to a mouth aspirator line (Sigma-Aldrich) with a 0.22um filter (Whatman) for performing the sterile manual selection. The culture plates were washed with phosphate buffered saline (PBS) followed by 10 minutes in Cell Dissociation Buffer (Invitrogen) in order to allow gentle cell scraping and aspiration of the picked cells with the micro-tip pipette. The manually picked cells were then plated onto fibronectin-coated dishes in LDSk medium.

2.2.5 Flow Cytometry Analysis

Vascular cells, both with and without the second manual selection, were stained for the following endothelial markers: Flk-1 (Biolegend), vascular endothelial-cadherin (VE-cad; eBioscience), Flt-1 (Santa Cruz), EphB4 (Santa Cruz), ephrin-B2 (Santa Cruz), and Tie-1 (Santa Cruz). Some cells were also stained for calponin (Santa Cruz), a marker indicating smooth muscle cells. The secondary antibodies include anti-rat PE (Abcam), donkey anti-rabbit PE (Fitzgerald), and donkey anti-goat FITC (Abcam). All samples were analyzed using a BD LSRII flow cytometer and FlowJo software (TreeStar).

2.2.6 Low Density Lipoprotein Uptake

ESC-derived ECs derived in serum containing medium and those derived under chemically defined conditions were plated on Permanox microscope slides (NUNC). Commercially available Alexa Fluor 488 Acetylated-LDL (Invitrogen) was diluted to 1:100 in DMEM (Invitrogen) and incubated with the cells for 4 hours at 37°C. The slides were then stained with DAPI and fixed with 4% formaldehyde. The slides were imaged with a Leica fluorecent scope.

2.3 Results

R1-ESCs were induced towards vascular progenitor cells in chemically defined NS1D2b medium on various substrates over 5 days, and then analyzed for the expression of the vascular progenitor cell marker, Flk-1, using flow cytometry analysis (Fig. 2.1). Unfortunately, the ESCs at day 1 exhibited very low levels of cell adhesion/proliferation and we were not able to consistently obtain enough cells for flow cytometry analysis. The percent of Flk-1+ cells from R1-ESCs cultured for 0, 2, 3, 4, and 5 days on fibronectin under the chemically defined conditions peaked on day 2 (over 70%), continually decreasing over the next 5 days. We also examined the effect of cell-matrix signaling during the 2 day induction of Flk-1+ progenitors. We plated these cells on collagen-type I, -type IV, laminin, fibronectin, and gelatin. Figure 2.2A shows that, contrary to currently accepted reports that collagen-type IV is the optimal substrate for the induction of Flk-1+ vascular cells [12, 13, 15], the actual percentages of Flk-1+ cells induced from R1-ESCs under chemically defined conditions did not vary significantly between substrates. We subsequently counted

the adherent cells on the various substrates at the end of the 2 day induction period to quantify their ability to promote adhesion and proliferation of the differentiating ESCs (Fig. 2.2B). Combining Flk-1+ cell percentages with total cell numbers, the optimal substrates yielding the greatest total number of Flk-1 cells are gelatin and fibronectin (Fig. 2.2C). We suspect that these results might not necessarily be due to directed differentiation, but enhanced proliferation of the adherent cells on gelatin and fibronectin. The results of this study shown in Figure 2.2 is particularly important to note, as many previous studies, including ours [10, 25], use collagen-type IV for differentiation of vascular cells in serum-containing mediums. The culture of the ESC on collagen type-IV in chemically defined medium without serum actually yields the lowest number of Flk-1+ cells of all the substrates tested. We hypothesize that the superior results on fibronectin, and not collagen IV, is due to the fact that important adhesion-related proteins, like fibronectin, found in serum would not be available in our serum-free cultures.

The Flk-1+ cells were then enriched and plated onto either collagen-type I, collagen-type IV, laminin, gelatin, and fibronectin coated-dishes in LDSk medium. After two weeks, the outgrowths from the Flk-1 expressing cells were analyzed for markers of venous endothelium (EphB4), arterial endothelium (ephrin-B2), endothelial VE-cadherin, and vascular smooth muscle (calponin). Here we do see marked differences in the induction of vascular cells on different substrates (Fig. 2.3). Laminin generates the largest number of venous endothelial cells, while both collagen IV and fibronectin generate the most number of arterial endothelial cells

(Fig. 2.3D). However, all of these percentages are still quite low at this early stage of endothelial maturation. Looking at an earlier endothelial marker, VE-cadherin, we see that collagen I, IV, and fibronectin yield the largest percentages of VE-cadherin + cells (Fig. 2.3A and D). We then looked closely at the smooth muscle cell (SMC) outgrowths and observed that the cells cultured on fibronectin also contained the lowest percentage of contaminating SMCs (Fig. 2.3B) and that the greatest cell yield was again observed in cells cultured on fibronectin (Fig. 2.3C). Based on this data, we continued to expand ESC-derived ECs on fibronectin in order to reduce the percentage of SMCs in the cultures while promoting optimal adhesion and proliferation of our endothelial cells.

The expanded Flk-1+ cells sub-cultured in LDSk medium on fibronectin robustly supported the differentiation and proliferation of ECs, and some of the Flk-1+ exhibited distinct cobblestone-like morphologies (Fig. 2.4) allowing manual selection to be used for further enrichment of the ECs. The expanded ESC-derived ECs were then analyzed for EC markers Flk-1, VE-cad, Flt-1, Tie-1, venous-specific marker, EphB4, and the arterial-specific marker, ephrin-B2 using FACS analysis (Fig. 2.5). Here, the differentiation and expansion procedures of the ESC-R1 cells derived in our chemically-defined mediums formulations, as described here, expressed very high levels of the most of the EC markers that were examined, but did not exhibit a high level of ephrin-B2 arterial surface marker, indicating a largely venous phenotype (EphB4) for these cells (Fig. 2.5; green). We also included characterization of our ESC-EC derived in serum-containing medium for two

different ESC lines. Comparing columns II (aqua) and III (red), the serum-containing induction protocol worked well for the ESC-D3 cell line (Fig. 2.5; red) as previously described [10, 25, 27], but the same serum-containing medium was not able to generate the same level of quality ECs from the ESC-R1 cell line (Fig. 2.5; aqua). We expect that this difference is due to the inherent heterogeneity between ESC lines.

All three of the ESC-ECs presented in the first 3 columns (green, aqua, and red) followed a rigorous differentiation and expansion methodology including two distinct isolations. These include an early isolation of vascular progenitor cells plus a late isolation that purifies the progenitor cells further into relatively pure (> 95%) ESC-ECs [10]. In addition, the second manual selection (or another method of post Flk-1+ sorting enrichment) of late developing ECs was required for EC proliferation of the ESCs derived using serum-containing medium [10]; we expect, due to the high levels of contaminating smooth muscle cells in serum-containing cultures. However, the EC induction methodology presented in this manuscript uses chemically-defined mediums that allow the generation of proliferative ECs with minimal contaminating SMCs. Therefore, we sought to characterize the outgrowths of the non-enriched Flk-1+ in order to explore whether the second late stage selection is a necessary manipulation under these chemically defined conditions (Fig 2.5; gold). The data indicate that although these cells do exhibit equivalent expression of EC molecules Flk-1 and VE-cad, the expression levels of Flt-1, Tie-1, and EphB4 were lower compared with the ESC-ECs that did undergo the second isolation, indicating that

this second selection process does generate better quality, and potentially more mature, ECs. Figure 6 examined the co-expression of Flk-1 with endothelial markers, EphB4 and Ephrin-B2, as well as the smooth muscle marker, calponin. Interesting, 45% of the Flk-1+ cells co-express the marker for venous endothelium, EphB4, but only 8% of these cells express Ephrin-B2. This indicated that the remaining 37-45% of the Flk-1+ cells have not yet specified an endothelial subphenotype (previous studies indicate that none of the cells are lymphatic, not shown) and may retain some level of plasticity.

Lastly, the entire derivation procedure was repeated in a third ESC line (E14) in order to verify that the chemically-defined medium formulations presented in this manuscript can be applied towards other mouse ESCs. These ESC were also able to generate ECs, but require a second manual selection for purification of the EC (Fig. 2.5; purple).

Functional assays are also an important indicator of EC quality. The uptake of low-density lipoproteins (LDL) is considered an important EC function that was not seen in our ESC-ECs derived from D3-ESCs in serum-containing medium [10, 25], however, as seen in Fig. 2.7 A and B, our ESC-ECs derived in chemically defined conditions were able to take up LDL. We also observed that some of the ESC-ECs that did not undergo the second isolation (Fig 2.5; gold) could still take up LDL (Fig. 2.7C). However, not all of the cells in the culture could take up LDL (Fig. 2.7D),

indicating that these unpurified cells were also remaining somewhat heterogeneous, even in the chemically defined medium.

2.4 Discussion

Our initial stage of induction focused on promoting the differentiation of ESCs into Flk-1+ cells similar to studies using serum-containing medium formulations [10-13, 15, 16]. Our rationale is supported by the fact that the Flk-1 surface molecule is currently considered to be the first lineage commitment marker expressed on vascular and hematopoietic progenitors [28-41]. BMP-4 and VEGF are also known to promote ventral mesoderm and hematopoietic development while inhibiting neuronal development [42-44], therefore; these were also incorporated into to our serum-free differentiation medium. Conversely, basic fibroblast growth factor (bFGF), considered to be a pro-angiogenic factor, was not required in our early stage of induction (i.e. generation of Flk-1+ cells) [42], but was incorporated with VEGF at the later, more mature, stages of development for enhancing EC proliferation.

We also examined the effect of ECM substrate on the EC inductions. Our results using chemically-defined medium, indicate that early stage EC inductions focused on generating Flk-1+ mesodermal cells can take place on any of the substrates examined, including gelatin. However, the substrates' role in directing the specification of the Flk-1+ cells towards various cell phenotypes was much more enlightening. It seems that fibronectin directs the most EC differentiation leading to

the greatest number of VE-cadherin+ endothelial cells, whereas culture on gelatin, laminin, and collagen I result in more SMCs. If one wanted to study the co-development of EC and SMC, collagen-type IV, would remain the optimal substrate that allows the simultaneous proliferation and differentiation of both cell types. This data from ESC-EC derivations in chemically-defined medium also challenges the currently accepted belief that collagen IV substrate generates largest number of vascular progenitors and ECs [13, 15].

Lastly, it has been proposed that the venous lineage is the default pathway during EC development [16], presumably due to insufficient Notch activation [45]. Based on the high EphB4 and limited ephrin-B2 expression level of our cells, we found that our ESC-derived ECs also resembled venous endothelium compared with ephrin-B2 expressing arterial endothelium. One study, using cells cultured in serum and VEGF found that >90% of the cells became venous EC, but when Notch signaling was activated by stimulating the cyclic adenosine monophosphate (cAMP) pathway (by adding either 8-bromo-cAMP or adrenomedullin - a cAMP-elevating factor - to the serum and VEGF containing medium), up to 70% of the cells expressed ephrin-B2 arterial marker [46]. Based on this study, one would expect that specifically activating Notch signaling in our chemically defined cultures may also lead to the generation arterial ECs.

The generation of ECs from ESCs in chemically defined conditions is valuable for potential use of these cells in a variety of therapeutic applications. By eliminating

various unknown animal contaminants and removing unspecified and uncontrollable elements found in serum, chemically defined culture conditions allow better controlled studies of the effects of various biological and mechanical signaling variables. Although we still see some developmental variations between different ESC lines, the reproducibility and quality of ESC-derived ECs were increased significantly using the chemically defined conditions described.

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Base Medium	NS1D2b	LDSk
	Alpha MEM 1x Penicillin-Streptomycin 1x Non-essential Amino Acids 2mM L-glutamine 0.05mM 2-mercaptoethanol	70% Alpha MEM, 30% DMEM 1x Penicillin-Streptomycin 1x Non-essential Amino Acids 2mM L-glutamine 0.1mM 2-mercaptoethanol
Serum Replacement	20% KnockOut Serum Replacement (KSR)	2x Nutridoma-CS
Additional Factors	30ng/ml VEGF 5ng/ml BMP-4	100ng/ml VEGF 50ng/ml bFGF

Table 2.1: ***Chemically-defined medium formulations for the derivation of EC from mESC.*** The first induction medium optimized for the generation of Flk-1+ vascular progenitor cells is called “NS1D2b”. The second medium formulation, called “LDSk” is optimized for EC specification and expansion.

	R1 (no FBS)	R1 (w/FBS)	D3 (w/FBS)	R1 (not purified)	E14 (not purified)
Flk-1	83%	23%	99%	94%	72%
VE-cad	91%	22%	99%	98%	66%
Flt-1	89%	23%	33%	14%	48%
Tie-1	90%	25%	22%	25%	35%
EphB4	91%	25%	63%	25%	44%
ephrinB2	11%	0%	1%	4%	3%

Table 2.2: ***Quantitative expression of various EC markers.*** The percent positive cells expressing the listed EC markers for the ECs derived using our chemically defined medium compared with old serum formulations (quantitative data from Fig. 2.5). Also included are data comparing the percent positive cells with and without a second rigorous manual selection for optimal purification of the ECs.

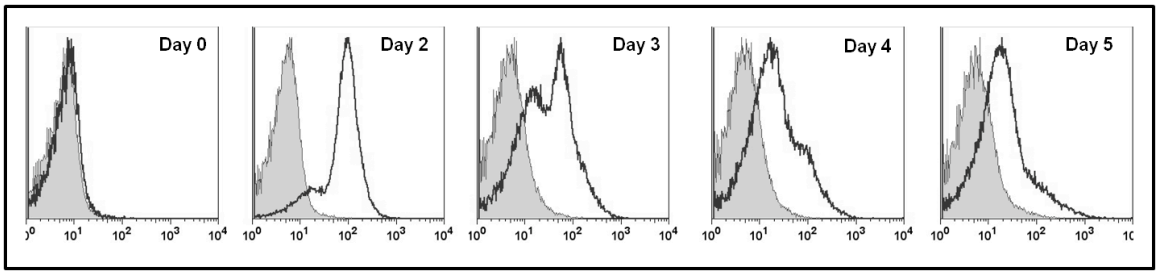


Figure 2.1: ***The largest numbers of Flk-1+ vascular progenitor cells is seen at day 2 of induction from ESC.*** Histograms of the Flk-1+ expression of R1 mouse ESC on day 0 (prior to induction) and those induced in chemically-defined medium on days 2, 3, 4, and 5. The optimal expression of Flk-1+ cells occurs at day 2 for this cell line and subsequently decreases.

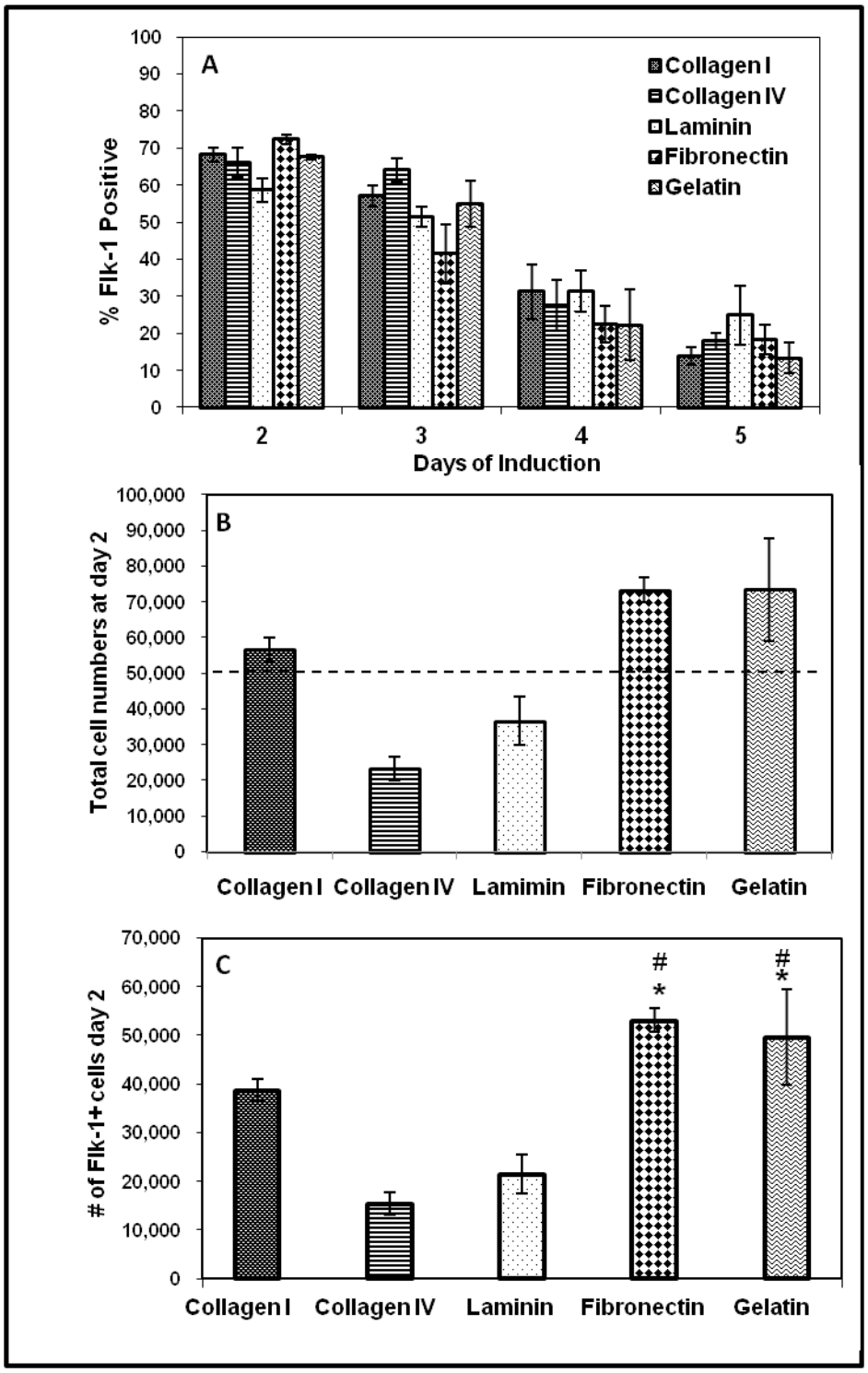
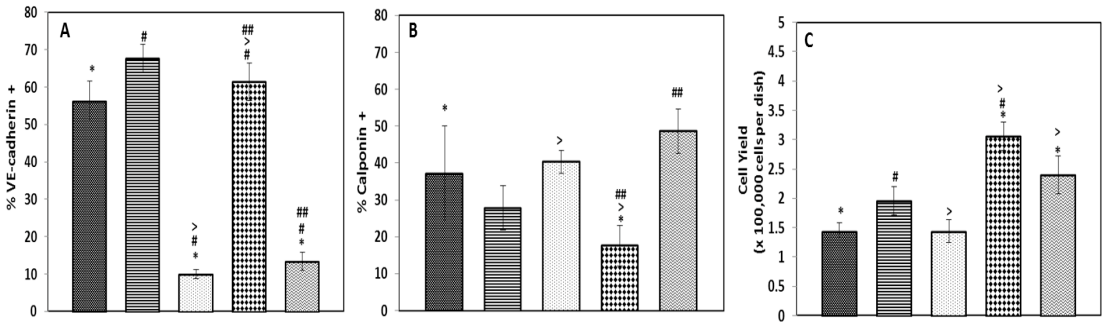

A
% Flk-1 Positive
Days of Induction
Collagen I
Collagen IV
Laminin
Fibronectin
Gelatin
B
Total cell numbers at day 2
C
# of Flk-1+ cells day 2
#
*

Figure 2.2: ***Culture on fibronectin and gelatin yield the largest numbers of Flk-1+ cells at day 2.*** **(A)** The levels of Flk-1 expression at various initial induction periods. Lack of adequate cell adhesion forbids the testing of Day 1. Flk-1+ expression decreases as the induction period is lengthened. Shown here is the expression profile for induction on fibronectin coated dishes at Days 2-5. Error bars represent SEM. **(B)** Total number of adherent cells at Day 2 of induction for the various substrates. The dashed line represents the initial seeding number of 50,000 cells. Note that the fibronectin and gelatin substrates encourage the largest proliferation of adherent cells. Error bars represent SEM. **(C)** Graph depicts the number of Flk-1+ adherent cells at Day 2 of induction. Note that fibronectin results in the best combination of cell yield (B) and Flk-1 expression (C). One-way ANOVA and TUKEY tests were used to analyze statistically significant differences between substrates. The number of Flk-1+ cells generated on gelatin and fibronectin were both statistically greater than on collagen IV (*) or laminin (#).



D

	EphB4 (venous)	Ephrin-B2 (arterial)	VE-cadherin (endothelial)	Calponin (smooth muscle)
Collagen I	7%	4%	56%	37%
Collagen IV	8%	6%	68%	32%
Laminin	11%	9%	10%	40%
Fibronectin	9%	4%	61%	18%
Gelatin	2%	5%	13%	49%

Figure 2.3: ***Flk-1+ outgrowths cultured on fibronectin generate the largest number of EC with minimal smooth muscle cell contamination.*** **(A)** Expression of endothelial VE-cadherin in outgrowths of Flk-1+ vascular progenitors cultured on collagen-type I, collagen-type IV, laminin, fibronectin, and gelatin. **(B)** Expression of calponin, a marker expressed early on smooth muscle cells, culture on the same 5 material substrates. **C)** The cell yield (total cell numbers per dish) on the various substrates.

D) Table summarizing the quantitative differences in vascular cell specification between groups. Note that although collagen-type IV and fibronectin allow induction of equivalent percentages of EC, collagen-

type IV also allows the differentiation/proliferation of more calponin-expressing smooth muscle cells (SMCs) while fibronectin does not lead to cultures with as many contaminating SMCs. Student T-tests were used to analyze statistically significant differences between substrates. Statistical significance ($P<0.05$) between cells cultured on the various substrate are indicated by various symbols: collagen I (*), collagen IV (#), laminin (>), and, fibronectin (##).

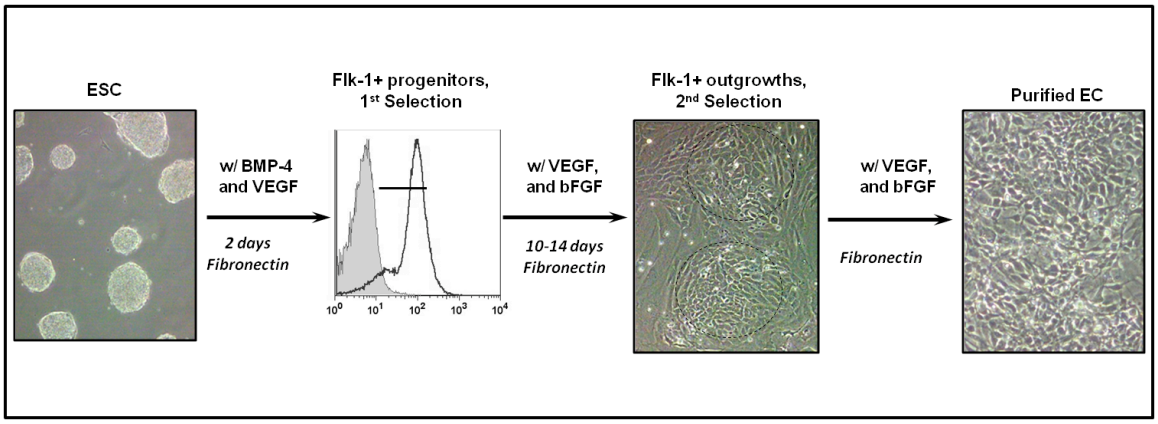


Figure 2.4: ***Flowchart of EC differentiation procedure.*** Undifferentiated ESC are expanded on gelatin, and then transferred to fibronectin for 2 days of induction with BMP-4 and VEGF treatment. The Flk-1+ cells are then isolated and re-plated on fibronectin in medium containing VEGF and bFGF. The Flk-1+ outgrowths generate cobblestone-like cell sheets. These sheets are manually selected for optimal EC purification.

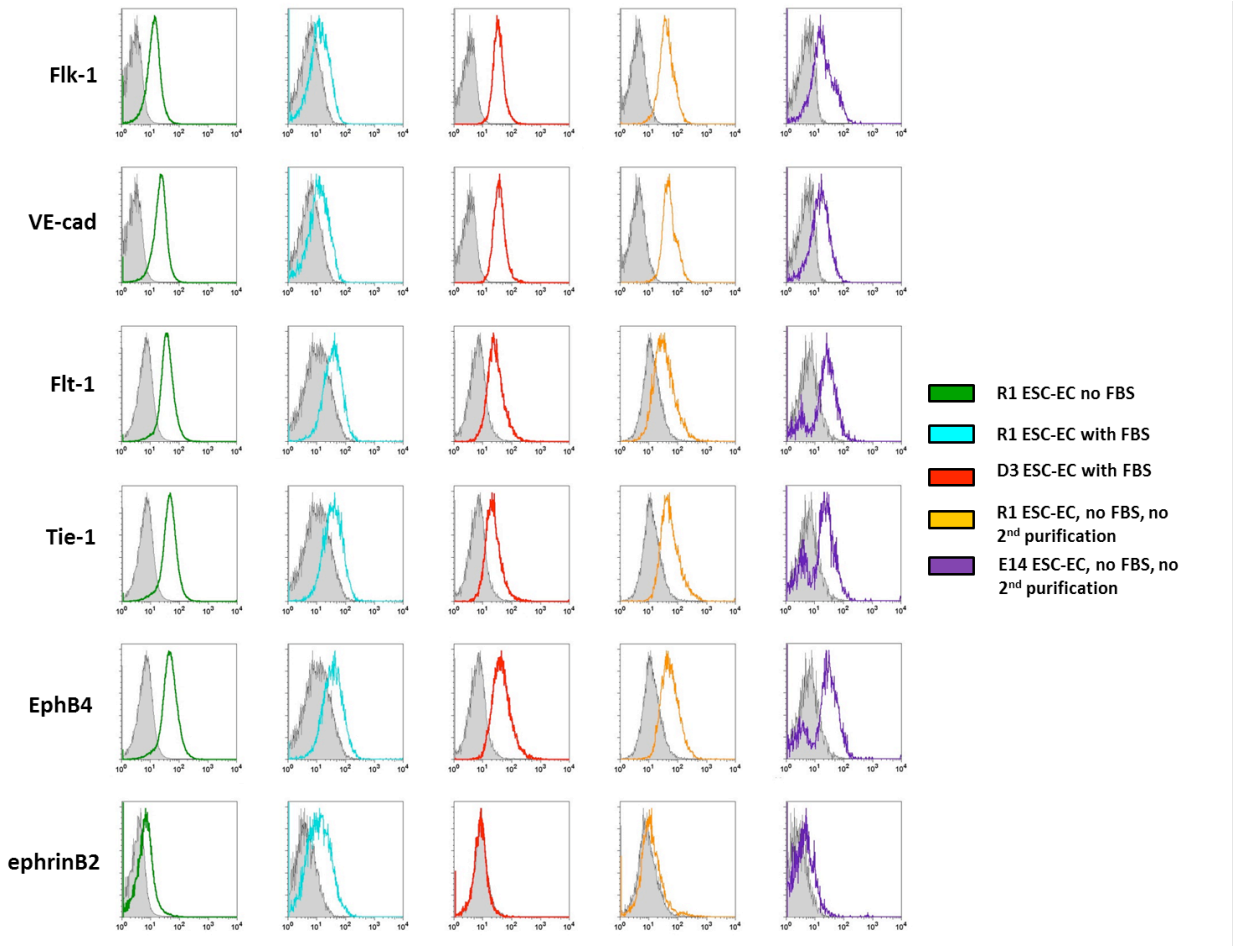


Figure 2.5: ***Endothelial cells derived in chemically defined conditions express appropriate endothelial markers.*** We examined the expression of endothelial markers Flk-1, VE-cadherin, Flt-1, Tie-1, EphB4 (venous), and ephrin-B2 (arterial). The histograms include the ECs derived using R1 ESC under chemically defined conditions (green). Also included are ECs derived from R1-ESCs (aqua) and D3-

ESCs (red) using our previous medium formulation that contained FBS. The last two columns include comparisons of the EC marker expression for the ECs from R1-ESCs (gold) and E14-ESCs (purple) derived under the new chemically defined conditions, but these did not include the second EC purification.

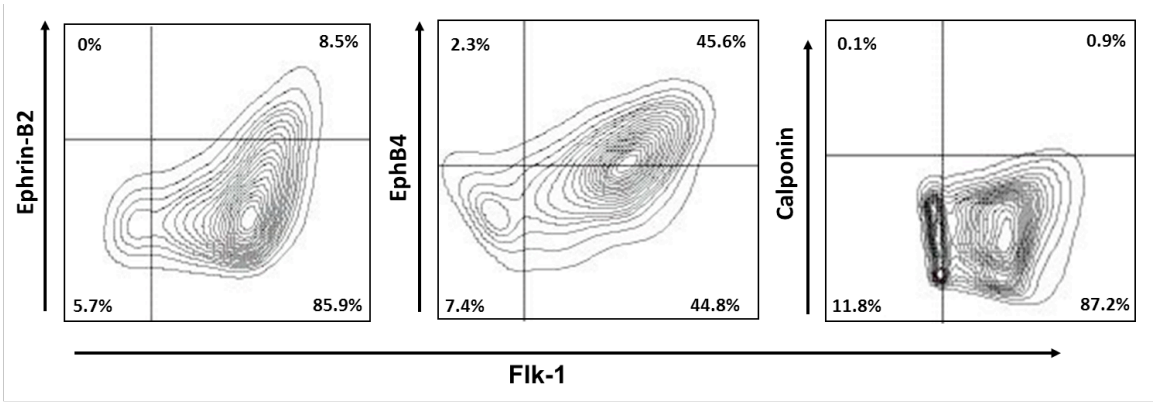


Figure 2.6: ***Endothelial cells derived in chemically defined conditions co-express Flk-1 and EphB4 (venous).*** These cultures contain very few ephrin-B2+ arterial cells and do not contain calponin+ smooth muscle cells.

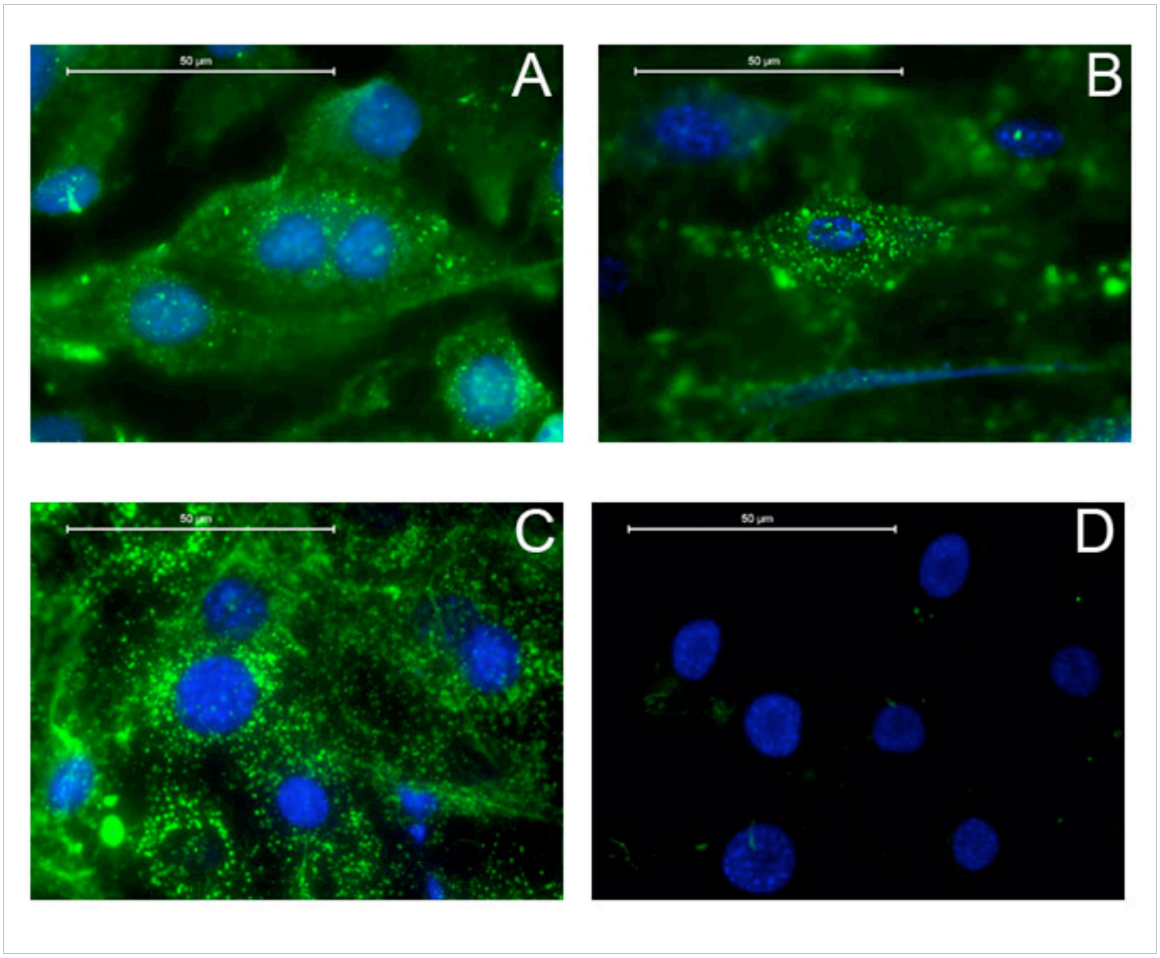


Figure 2.7: ***Endothelial cells derived in chemically defined conditions take up low density lipoprotein (LDL).*** The two **A and B)** distinct isolations of ECs derived from R1-ESCs both take up LDL. We also examined the LDL uptake of the R1s derived in the same chemically defined conditions, but not non-manually selected for additional purification. **C)** Some of the cells did take up LDL, but **D)** some of the cells in the same culture do not take up LDL. These results indicate that the non-purified cultures still contained some heterogeneity. (LDL = green and DAPI = blue).

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Chapter 3

Specialized Tip- and Phalanx-like Endothelial Cells Derived from Embryonic Stem Cells

Abstract

Endothelial cells (EC) generated in vitro from stem cells are desirable for their potential in a variety of cell-based therapeutic approaches. In this study, distinct EC subpopulations are generated from embryonic stem cells (ESC): the first resemble proangiogenic migrating “tip” EC, while the second appears to be composed of purified “phalanx” EC typically found lining vessel lumen. Both ESC-derived EC subpopulations arose from outgrowths of Flk-1+ vascular progenitor cells in our chemically defined medium formulations. However, the phalanx-like ESC-EC are selected early in differentiation for “cobblestone” shape, while the others do not undergo any further selection processing. Here, we show that the unselected “angiogenic” endothelial cells (ESC-AEC) exhibit increased levels of in vitro angiogenic sprouting activity compared with ESC-EC. Moreover, the ESC-AEC demonstrate increased migration in response to angiopoietin-2, increased F-actin stress fiber numbers and organization, and increased HSP27 phosphorylation. The ESC-AEC also exhibit lower numbers of Flt-1 and Tie-1 positive cells compared with the phalanx-type ESC-EC, while expressing the same high levels of Flk-1 and VE-cadherin. We deduce that the “angiogenic” ESC-AEC are consistent with EC populations that would include the specialized “tip” EC found at the leading edge of sprouting blood vessels, while the ESC-EC represent the Flt-1 expressing, but less migratory, “phalanx” EC found lining the lumen of blood vessels. Perhaps most intriguing, these specialized and functionally distinct EC (tip, stalk, and

phalanx) subtypes typically found within the developing blood vessel can also be found within the EC populations derived in vitro from embryonic stem cells.

3.1 Introduction

The generation of functional endothelial cells (EC) in vitro continues to be a major topic of interest due to the myriad of potential therapeutic applications. Successful methods for the *in vitro* differentiation of EC from both adult stem cells [1-6] and embryonic stem cells (ESC) [7-15] and have been previously described. More recently, serum-free [16, 17] culture conditions have also been developed for the derivation of EC from ESC. These serum-free methods allow improved control over stem cell microenvironments and fate, leading to more pure EC populations. The cleaner EC populations have also uncovered some more subtle phenotypical and functional differences within specific stem cell-derived EC populations [16]. Although, the ability to generate specialized EC would impact the specific applications for which these EC may be best suited, little work has been conducted examining and controlling the phenotypical specifications of stem cell-derived EC. To date, only two studies have emphasized the specificity of venous, arterial, and lymphatic EC lineages from ESC [15, 18], while a neglected aspect of endothelial functionality in these stem cell derived EC populations is the angiogenic proclivity of these EC populations, including the specialized EC found within a branching blood vessel.

Although a fairly recent advance to the field of vessel branching, the various specialized EC required to build a functional vessel sprout have been identified [19]. The first specialized EC type within a sprouting vessel is called the “tip” EC. Tip EC are found at the leading edge of a sprouting vessel, and are distinct from other EC in their DLL4/Notch 1 signaling [20], CXCR4 [21], and neuropilin-1 [22] expression. Tip cells

also exhibit more organized stress fibers with numerous probing filopodia, and readily migrate towards an angiogenic stimulus [23], but do not form lumens and proliferate minimally [19, 24, 25]. The “stalk” cells then trail behind the tip cells during angiogenic sprouting forming the stalk of the sprout. Moreover, the Notch signaling from the tip cells dampens the VEGF-induced expression of Dll4 on stalk cells [20], thereby allowing the tip cells to maintain their position at the leading edge of the sprouting vessel. Unlike tip cells, stalk cells can also proliferate, form lumens, and lay down extracellular matrix, but do not extend filopodia [25].

There is also a third population of less migratory EC, called a “phalanx” EC [26], that may be best suited for seeding the lumens of small-diameter vascular grafts [27, 28]. These cells have been identified in previous studies by their high levels of soluble and membrane bound Flt-1, known to mitigate the otherwise proangiogenic signals of VEGF [26]. Although, phalanx-type EC are capable of responding to VEGF signaling, VEGF signaling in phalanx EC acts as an apoptosis rescue from serum-deprived conditions, rather than as the proliferative and mitogenic response seen in tip cells [26].

Here we show that we are able to generate both tip- and phalanx-like EC from ESC in vitro using our previously developed chemically-defined culture methods for generating highly purified EC from murine embryonic stem cells [16, 29]. Both EC populations include outgrowths of Flk-1+ vascular progenitor cells, but only some ESC-derived EC also undergo an additional manually selection based on cobblestone shape. While the unselected ESC-derived EC cell populations are composed of pure EC populations based on Flk-1 (94%) and VE-cadherin (98%) expression levels [16], the Flt-1 and Tie-1

expression numbers are lower, 14% and 25% respectively, in the unselected ESC-derived EC compared with the selected ESC-derived EC exhibiting 90% expression of these molecules. Here, we show that the unselected ESC-derived EC, named ESC-derived "angiogenic" endothelial cells (ESC-AEC; unsorted) exhibit significantly increased angiogenic activity compared with our manually selected cobblestone-shaped ESC-derived EC. Moreover, the angiogenic activity in ESC-AEC also correlates with the increased migration, proliferation, actin organization, and HSP27 phosphorylation that would be expected in tip-like EC while the high Flt-1 expression in the sorted ESC-EC is seen in phalanx-like EC.

3.2 Materials and Methods

3.2.1 Cell Culture

R1 murine embryonic stem cells were maintained on 0.5% gelatin coated plates in serum-free medium containing Knockout Dulbecco's Modified Eagle Medium (KO-DMEM; Invitrogen), 15% Knockout Serum Replacer (KSR; Invitrogen), 1x Penicillin-Streptomycin (Invitrogen), 1x Non-essential Amino Acids (Invitrogen), 2mM L-glutamine (Invitrogen), 0.1mM 2-mercaptoethanol (Calbiochem), 2000 Units/ml of leukemia inhibitory factor (LIF-ESGRO; Chemicon), and 10 ng/ml of bone morphogenetic protein-4 (BMP-4; R&D Systems). ESC-derived EC were generated as described previously [16]. Briefly, ESC-EC underwent two different derivation and purification steps using 3 different medium formulations. The initial induction step was induced using a medium

optimized by our laboratory was named “NS1D2b”. This consists of alpha-MEM (Cellgro), 20% KSR (Invitrogen), 1x Penicillin-Streptomycin (Invitrogen), 1x Non-essential amino acids (NEAA; Invitrogen), 2mM L-glutamine (Invitrogen), 0.05 mM 2-mercaptoethanol (Calbiochem), 30ng/ml of vascular endothelial growth factor (VEGF; R&D Systems) and 5ng/ml BMP-4 (R&D Systems). After 2 days, we enriched for Flk-1+ cells using a MiniMACS (Miltenyi Biotec) or Fluorescence Activated Cell Sorting (FACS). The Flk-1+ cells were then seeded onto fibronectin-coated dishes in “LDSk” medium containing 70% alpha-MEM (Mediatech) and 30% DMEM (Invitrogen) plus 100ng/ml VEGF (R&D Systems), 2x Nutridoma CS (Roche), 50ng/ml bFGF (Sigma), 2mM L-glutamine (Invitrogen), 1x penicillin-streptomycin (Invitrogen), 1x nonessential amino acids (Invitrogen), and 0.1mM 2-mercaptoethanol (Calbiochem) [16]. A second optional purification was sometimes also incorporated after expansion of the Flk-1+ cells. This second selection included only EC exhibiting distinct cobblestone cell morphologies. Alternatively, the ESC-AEC were purified based on the Flk-1+ expression only without the second selection for the cobblestone only shape. Both cell lines were subsequently maintained in a medium comprised of 50% LDSk and 50% serum-free EGM-2 (Lonza), containing a propriety mix of hydrocortisone, bFGF, VEGF, IGF, ascorbic acid, hEGF, heparin, and GA-1000. This mixture will be termed “LDSF” for the duration of this manuscript. An immortalized murine cardiac endothelial cells (MCEC; CELLutions Biosystems) control cell line was also used, expanded in EGM-2 medium (Lonza) on 0.5% gelatin coated dishes.

3.2.2 In Vivo Angiogenesis

Fertilized eggs were generously donated from a local hatchery (Waterford, CA) at day 2 post-fertilization. On day 3 post-fertilization, the eggs were windowed to facilitate visualization. First, a small hole was made at the large blunt end of the egg at the air sac. A 16-gauge needle with fitted syringe was inserted into the hole to remove 4-5 ml of albumenal fluid, thus creating a false air sack on the long side of the egg for cutting a small window. Clear tape was then placed over both the hole and window and the eggs were placed in a 37°C incubator with 1% CO_2.

On day 6 post-fertilization, a Matrigel scaffold was generated as the vehicle for cell transplantation. Briefly, 40ml of Matrigel was mixed with 5,000 EC and allowed to gel for 30 minutes at 37°C on top of coverslips. The coverslips were then placed gel-side down onto the CAM. Controls also included a blank coverslip and cell-free Matrigel.

On day 10 post-fertilization, the vascular pattern of the CAM was imaged using a Leica stereoscope using Axiovision software. These images were manually skeletonized using Adobe Photoshop and subsequently analyzed with Angioquant software. Tissue was also harvested from the CAM assays on day 10 post-fertilization and fixed in 4% formaldehyde. The samples were then incubated in 0.3% H_2O_2 in methanol, rinsed for 3 minutes in PBS, and incubated in buffer (0.1% BSA in PBS) for 20 minutes. The samples were then rinsed in PBS and incubated in the buffer with an anti-mouse horseradish peroxidase (Abcam) conjugated antibody at room temperature for 30 minutes. The tissue was rinsed again with PBS and subsequently stained with 3,3-diaminobenzidine (DAB) substrate kit (Abcam).

3.2.3 In Vitro Angiogenesis

In a 24 well plate, 30ml of growth factor-reduced Matrigel was added to each well and allowed to gel at 37°C. After 30 minutes, 50,000 cells were added to each well. The cells were observed at intervals for 12, 24, and 48 hours and imaged using a phase contrast microscope with a 25x objective (Fisher) using Micron software.

3.2.4 Cell Migration

The EC were harvested from fibronectin coated dishes, pelleted, and re-suspended in EGM-2 medium (Lonza) without serum. Approximately 200,000 cells were added to a fluoroblock transwell insert (Millipore) and placed in a well of a 24-well plate. In order to facilitate migration of the EC, 500 ml of EGM-2 with 10% FBS with and without 100ng/ml of angiopoietin-2 was added to the wells below each transwell. After 24 hours, the inserts were fixed, permeablized, and fluorescently stained with Phalloidin 488 (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI). The transwells were then imaged using a Leica DMI6000 scope. Migration was measured by counting the number of intact nuclei in the field of view. A student's t-test was used to compare the migration of cells in both conditions.

3.2.5 Cytoskeleton Analysis

Coverslips were coated with fibronectin and seeded with either ESC-AEC or ESC-EC. The coverslips were then fixed, permeablized, and fluorescently stained with Phalloidin 488 (Invitrogen) and DAPI. The coverslips were then mounted on glass slides using mounting medium (Dako) and imaged with a Leica DMI6000 scope.

3.2.6 Flow Cytometry

The ESC-AEC and ESC-EC were harvested and stained for HSP27 phosphorylation at serine 82 following an established protocol [30] Briefly, the cells were pelleted, fixed and permeablized using ice cold methanol. The cells were then incubated with pHSP27 primary antibody (Santa Cruz) and then an anti-goat FITC secondary antibody (AbCam) or anti-calponin primary antibody (Santa Cruz) and FITC-conjugated secondary antibody (AbCam). The cells were then analyzed using a BD LSRII. The results were analyzed using FlowJo software (TreeStar).

3.2.7 Cell Proliferation

EC were harvested and resuspended in LDSF medium and plated onto fibronectin coated wells in a 6-well plate. Approximately 200,000 cells were seeded into each well. This process was repeated with MCEC onto gelatin coated wells. Cells were allowed to expand for 72 hours. They were subsequently harvested and counted using a hemacytometer.

3.2.8 MMP Analysis using RT-PCR

ESC-AEC and ESC-EC were cultured on collagen-type IV-coated dishes. After reaching confluence, total RNA was isolated using an RNAeasy Kit (Quiagen). RT-PCR was performed using a GeneAmp Thermostable RNA PCR kit using the primers provided in the supplemental data (Table 3.2) [31]. The PCR cycle utilized an annealing period of 30 seconds at 60°C and an elongation period of 30 seconds at 72°C.

3.3 Results

Our lab previously reported, also highlighted in Table 3.1, that the unsorted population of ESC-derived EC (renamed “angiogenic” ESC-AEC in this manuscript) and the manually selected (ESC-EC) express many of the same endothelial markers, but also some at varied levels of expression [16]. The distinction in expression level of ESC-AEC is most apparent their reduced expression of Flt-1and Tie-1.

We then examined the ability for these cells to promote angiogenesis and vasculogenesis in vivo. The results of the CAM assay indicate that all EC populations and Matrigel were able to promote vascular development in vivo (Figure 3.1B-E). Additionally, the vascular activity, measured in average length of vessels, of all of the EC treatments (MCEC, ESC-AEC, and ESC-EC) increased significantly compared with controls (Figure 3.1K).

Another important indication of the functional potential of the ESC-derived EC is whether or not they can be successfully integrated into the host vasculature. Using the metallic DAB substrate to identify the murine graft cells, we note that all the cell populations can integrate into the chick vasculature (Figure 3.1G-J). However, this trait was not exclusive to the EC populations (MCEC, ESC-AEC, and EAC-EC). The undifferentiated R1 ESC also clearly exhibited integration into the chick vasculature (Figure 3.1G).

Although the ESC-AEC and ESC-EC behave similarly in vivo, their sprouting behavior in vitro differs significantly. Once seeded onto Matrigel, both EC populations migrate into

cord-like structures with some additional sprouting within 12 hours (Figure 3.2A and B). However, after 48 hours (Figure 3.2C and D), the ESC-EC are regressing into cell clumps, while the ESC-AEC continue to proliferate and sprout robustly for over 48 hours and do not exhibit signs of regression for up to 5 days (not shown).

Based on the facts that 1) endothelial and smooth muscle cells are known to be derived from a common progenitor [15] and 2) studies indicate increased angiogenesis with smooth muscle cell or fibroblast co-culture [32, 33], we proceeded to examine the unsorted ESC-AEC for the presence of vascular smooth muscle cells. However, Figure 3A clearly indicates a lack calponin-positive smooth muscle cells in both ESC-EC and ESC-AEC populations. Because matrix metalloprotease (MMP)-2, -9, and -19 are known to play a role in the degradation of matrix at the leading edge of new blood vessel growth [34-36], we then examined the enzymatic activity of these MMP in our EC monolayer cultures. However, the RT-PCR analysis of the MMP activity indicates that both cell populations generate similar amounts, expressing low levels of MMP-9, but not any MMP-2 or MMP-19 (Figure 3.3B) in monolayer cultures.

Probing further to identify the reason for the functional differences in our EC populations, we stained the EC for F-actin in order to examine the stress fibers in the two EC populations more closely. Here, we were able to detect differences in the number of F-actin stress fibers, as well as, the organization of these fibers (Figure 3.4A-B). The ES-AEC exhibited a more organized phenotype (Figure 3.4A) with extensive and complex actin networks compared with the ESC-EC (Figure 3.4B).

It has been previously shown that the phosphorylation of HSP27 is required to release the cap ends from actin filaments, thus allowing the generation of new polymerization required for cell migration [37, 38], therefore, we also stained our cells for HSP27 phosphorylation at serine 82. A large percentage of ESC-AEC express HSP27phosphorylation (Figure 3.4C) while the ESC-EC express virtually no HSP27 phosphorylation (Figure 3.4D). This data suggests that the increased in vitro sprouting behavior of the ESC-AEC compared with the sorted ESC-EC is due to HSP27 phosphorylation in the ESC-AEC, leading to increased F-actin polymerization and cell migration.

Because angiogenesis also requires some EC proliferation, the ESC-AEC and ESC-EC were analyzed for their potential differences in the proliferation rates of these two cell populations. Interestingly, the average proliferation rate of ESC-AEC was greater than the ESC-EC and MCEC, though the differences were not statistically significant (Figure 3.4E).

We then examined the migration of EC in response to a 1) serum nutrient or 2) specific chemotactic chemical signal. For these studies, fetal bovine serum (FBS) was used as a general nutrient attractant, and angiopoeitin-2 was used as the specific chemotactic migration signal. As expected, all cells migrated through a Boyden chamber transwell (Figure 3.5A) towards both the FBS nutrient and angiopoeitin-2, exhibiting a stronger response to angiopoeitin-2 compared to the FBS (Figure 3.5B). Although the addition of angiopoeitin-2 increased the migration activity by approximately 1.7 fold in the ESC-EC, the ESC-AEC migrated only sparingly towards the FBS attractant while migrating more

strongly towards the specific angiopoeitin-2 chemokine, resulting in a much larger, 5-fold, increase in migration compared with FBS alone. Interestingly, the MCEC control exhibited migration, even in the absence of a specific migration signal.

3.4 Discussion

The generation of functional vascular cells continues to be of interest for various potential therapeutic applications. Here, we sought to identify and characterize the functionality of EC subpopulations generated from mESC in our previously established chemically defined differentiation medium formulations [16]. Using these medium formulations combined with our differentiation/purification methods, we were able to generate two EC populations with some notable functional differences. Both the ESC-AEC and ESC-EC underwent an initial induction and purification based on the Flk-1 markers for early in vascular progenitor cells, while only the ESC-EC required a second selection based on EC exhibiting a distinct cobblestone morphology. Although both EC populations expressed high levels of Flk-1 and VE-cadherin, only the ESC-EC contained a high number of EC cells expressing Flt-1 and Tie-1.

In the in vivo CAM assays, the EC populations performed similarly in their contributions to the native vasculature. Since this was an in vivo assay, we suppose that the sophisticated microenvironment in the CAM was able to compensate for the subtle differences between the graft cell populations. However, during the in vitro Matrigel angiogenic assays, our ESC-AEC consistently exhibited increased sprouting activity compared with ESC-EC, and these differences were not due to smooth muscle cell contamination in the ES-AEC or differences in MMP activity [34, 36, 39-41] in the cells.

However, a limitation of analyzing MMP levels in monolayer cultures is that active sprouting does not occur. Using a 3-dimensional ECM hydrogel and an MMP inhibitor, such as GM6001, would allow for the observation of angiogenic activity with and without MMP inhibition [42]. Results of such studies would facilitate the comparison of the in vitro sprouting behavior of derived cells to known physiological conditions. For example, increases of MMP expression have been observed to increase tumor angiogenesis [43], but also to inhibit wound healing angiogenesis [44].

Investigating further, we also noted that cytoskeletal differences between the two cell populations were apparent in the organization of the actin cytoskeleton. ESC-AEC demonstrated more F-actin stress fibers with increased cytoskeletal organization compared with the ESC-EC. ESC-AEC also exhibited increased phosphorylation of HSP27, correlating with the increased actin organization of EC [45-47], and increased motility towards angiopoetin-2.

Previously, we suspected that the cobblestone selected ESC-EC with increased EC marker expression of Flt-1 and Tie-1 indicated that the ESC-EC represented a more mature EC population. However, the newer studies presented in this manuscript suggest that the “angiogenic” ESC-AEC may actually contain the three distinct endothelial subphenotypes (including “tip”, “stalk”, and “phalanx” type EC) typically represented in the developing vessel (Figure 6), whereas, the ESC-EC that were purified based on cobblestone morphology would contain only the “phalanx” EC.

Tip EC are found at the leading edge of a sprouting vessel, exhibiting increased expression of actin stress fibers, numerous probing filopodia, and readily migrate

towards an angiogenic stimulus [23]. The tip cells also express lower levels of Flt-1 compared with phalanx EC [26], consistent with the ESC-AEC population. Conversely, our cobblestone-shaped ESC-EC expressed higher levels of Flt-1, consistent with a "phalanx-like" phenotype [26], but did not exhibit greater VE-cadherin expression compared with ESC-AEC. However, the high levels of Flt-1 expression on our ESC-EC phalanx-type EC is congruous with the proposed function for Flt-1, serving as a VEGF repository (with greater binding affinity to VEGF) and mitigating EC migration in response to VEGF signaling [48].

The role of Tie-1 expression in our ESC-EC, and EC in general, remains poorly understood. In fact, a high affinity binding ligand has not yet been conclusively identified for this molecule. However, it has been shown that overexpression of Tie-1 in EC leads to the upregulation of the proinflammatory cell adhesion molecules (CAMs) VCAM-1, E-selectin, and ICAM-1 through a p38-dependent mechanism, as well as increased monocyte attachment in high expressing Tie-1 cells [49]. This data indicates a proinflammatory role for Tie-1 in endothelial cells and may, conversely, suggest a role for low Tie-1 expressing cells, like our ESC-AEC, in specific therapeutic strategies.

3.5 Conclusions

Here, we describe the generation of functionally distinct EC subpopulations derived from ESC that correlate with tip/stalk, and phalanx EC subtypes typically found within the developing blood vessel. Our two EC populations display noticeable differences in angiogenic behavior and quantifiable expression of some endothelial molecules [16]. The data indicate that the manually selected "cobblestone" ESC-EC more closely

resemble purified phalanx cells, whereas, the unselected, but proangiogenic ESC-AEC would also include tip and stalk cells within the EC populations. Furthermore, we present HSP27 phosphorylation and organized actin fibers as additional parameters for distinguishing angiogenic “tip” containing EC populations (ESC-AEC). Perhaps most importantly, our studies support the growing body of literature identifying the specialized EC subpopulations within the sprouting blood vessel, and show that these specialized EC also may be derived in vitro from ESC.

Acknowledgments

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	ESC-EC	ESC-AEC
Flk-1	+++	+++
VE-cadherin	+++	+++
Flt-1	+++	+
Tie-1	+++	++
EphB4	+++	++
ephrin-B2	+	-

+++ represents high expression, ++ represents moderate expression, + represents low expression

Table 3.1: ***Expression of EC markers varies between ESC-EC and ESC-AEC.*** As shown previously (Blancas et al., Stem Cells and Development, 2011), the purified ESC-derived EC express high levels of multiple endothelial markers (left). However, the unsorted ESC-derived EC, named ESC-derived "angiogenic" endothelial cells (ESC-AEC), display the same high levels of VE-cadherin and Flk-1 and lower levels of Flt-1 and Tie-2 molecules.

	Sense Primer	Antisense Primer
MMP2	AGA TCT TCT TCT TCA AGG ACC GGT T	GGC TGG TCA GTG GCT TGG GGT A
MMP9	CCC ACA TTT GAC GTC CAG AGA AGA A	GTT TTT GAT GCT ATT GCT GAG ATC CA
MMP19	TGG GCC ACT GGA GAA AGA AG	TCA GCC CAA CCA GCT TTC AC
GAPDH	GGT GGC AGA GGC CTT TG	TGC CCA TTT AGC ATC TCC TT

Table 3.2: ***List of primers used for RT PCR analysis.*** List was modified from Maquoi et al., Diabetes, 2002.

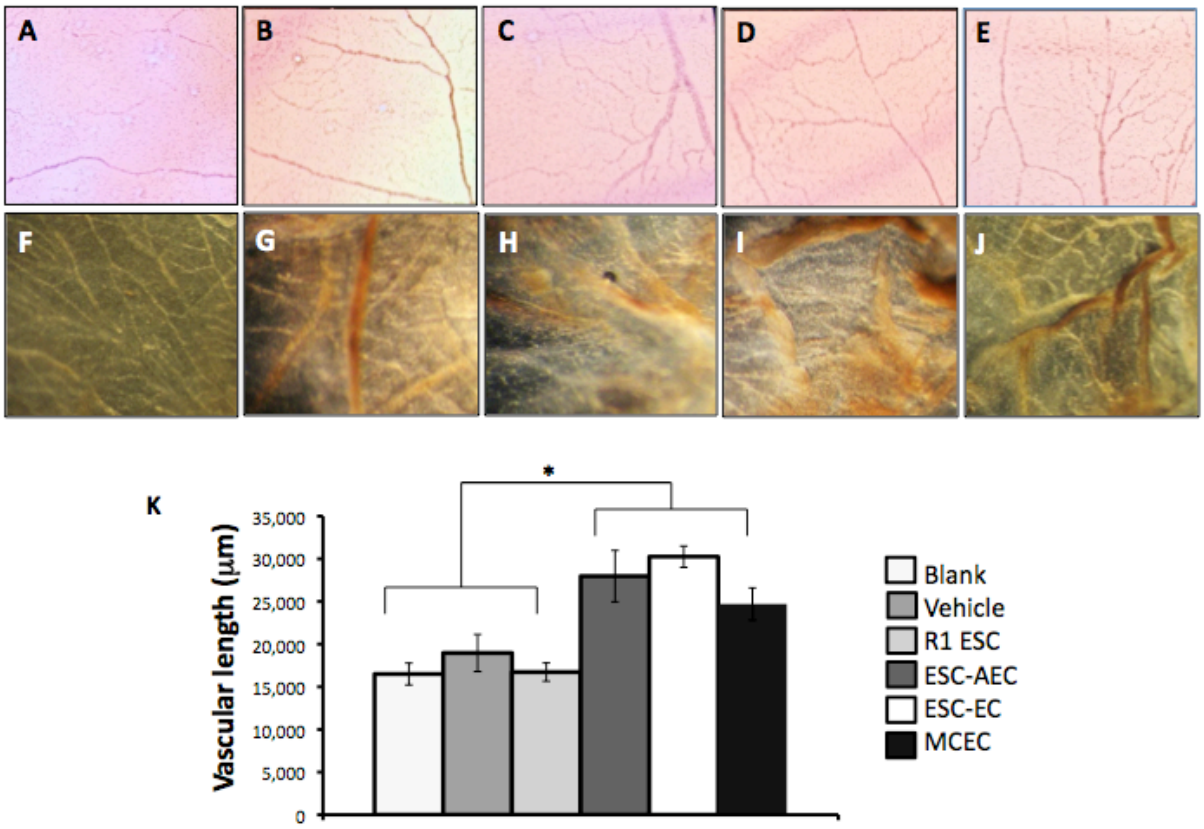


Figure 3.1: ***ESC-AEC and ESC-EC behave similarly in chick chorioallantoic membrane (CAM) assays.*** Images of CAM vasculature resulting from the **(A)** Matrigel vehicle control, **(B)** Undifferentiated R1-ESC, **(C)** ESC-AEC, **(D)** ESC-EC, and **(E)** MCEC. The CAMs were then stained for murine cell incorporation (brown), and as expected, the **(F)** vehicle control stained negative for incorporation of murine cells into the CAM tissue, while the incorporation of murine cells into the CAM tissue vasculature is

observed for the **(G)** R1 mESC, **(H)** ESC-EPC, **(I)** ESC-EC, and **(J)** MCEC. **(K)** The quantification of vessel lengths for blank, vehicle control, and undifferentiated R1-ESC populations yield comparable amounts of vascular activity while the ESC-AEC, ESC-EC, and MCEC yield significantly increased vascular activity. Statistical significance differences in average vessel lengths is not seen between the three EC populations, but all three EC grafts do significantly increase vasculature in vivo compared with the three control groups.

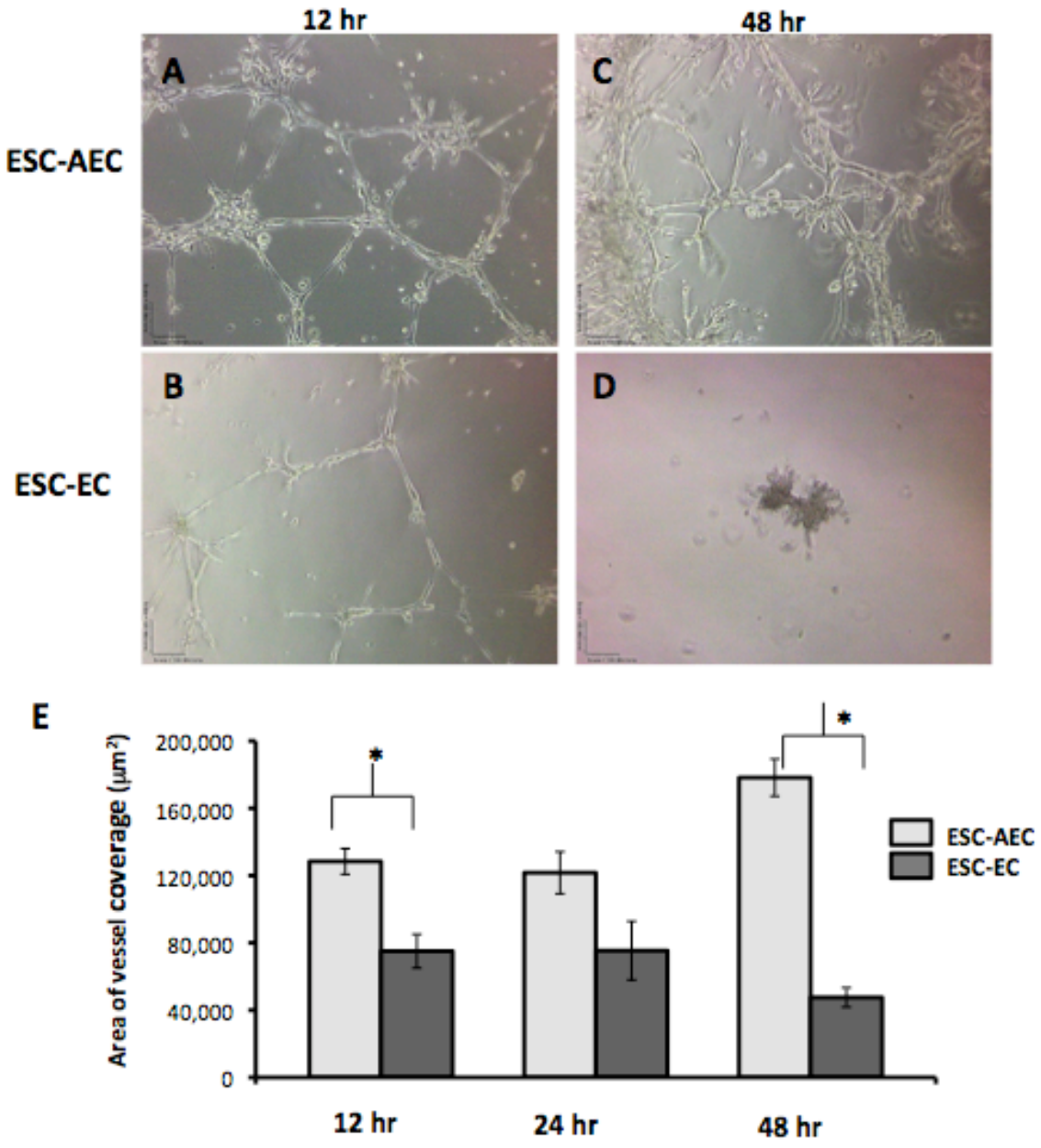


Figure 3.2: ***Sprouting activity of ESC-AEC and ESC-EC on Matrigel.*** **(A-B)** After 12 hours on Matrigel, both cell populations exhibit angiogenic sprouting activity. However, after 48 hours, the **(C)** ES-AEC sprouts are increasing is number and size while the **(D)** ESC-EC sprouts shown signs of regression. **(E)** The quantification of vessel coverage area indicates that a

significance increase in “angiogenic” sprouting of the ESC-AEC is evident after only 12 hours, and become even more pronounced after 48 hours when the sprouts from AEC continue to increase compared with the regressing ESC-EC. Statistically significant differences (*, $p > 0.05$) between the 2 cell populations were observed at 12 and 48 hr.

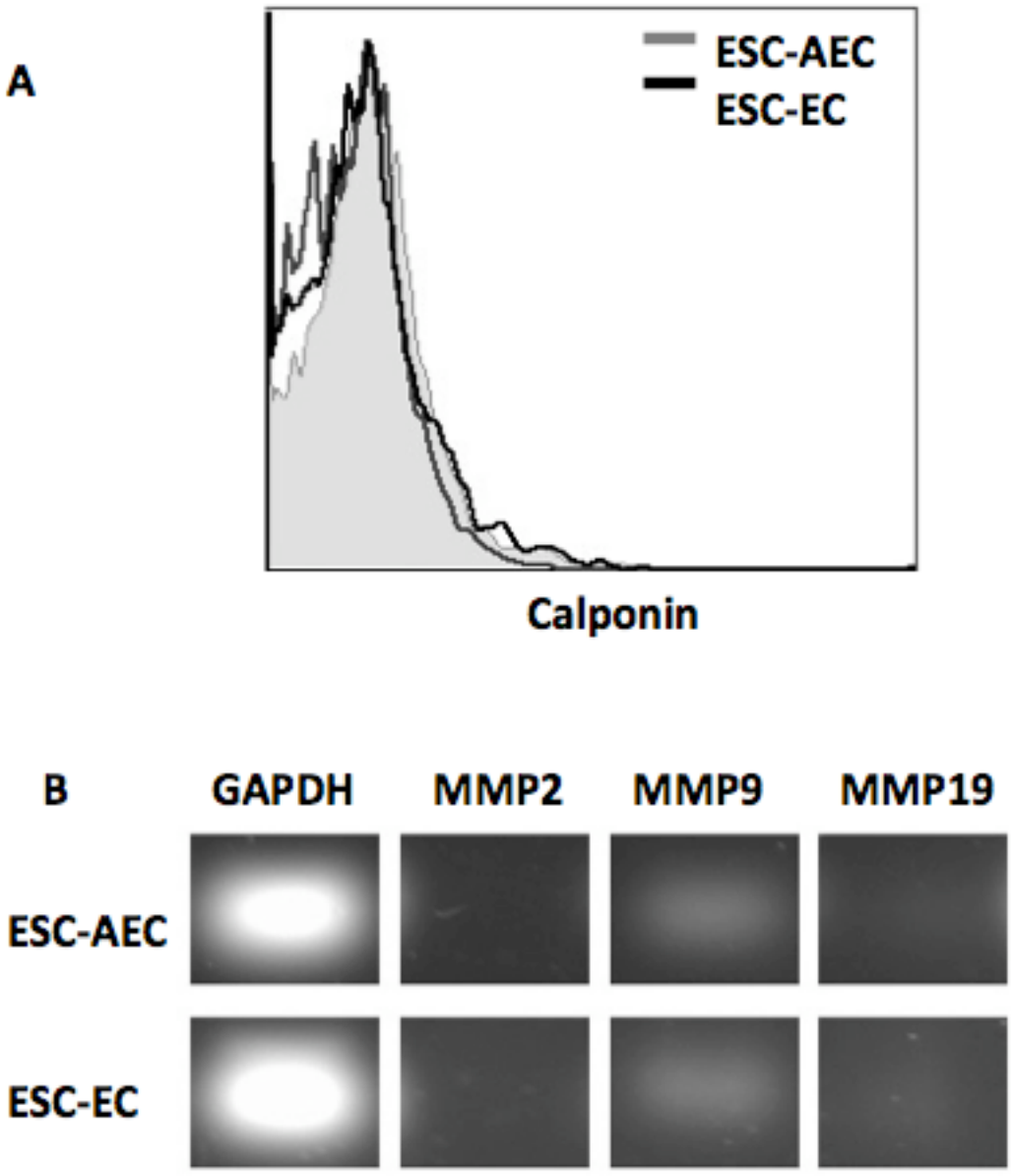


Figure 3.3: ***Calponin expression and MMP production were not significant.*** The ESC-AEC and ESC-EC were examined for the presence of calponin positive smooth muscle cells. **(A)** Based on the calponin staining, neither EC populations contained smooth muscle cells. **(B)** RT PCR of MMP-2, -9,

and -19 activity was also examined. MMP-9 production is observed in both cell populations, but did not differ between the EC populations. Note: MMP activity is generally low for monolayer cultures.

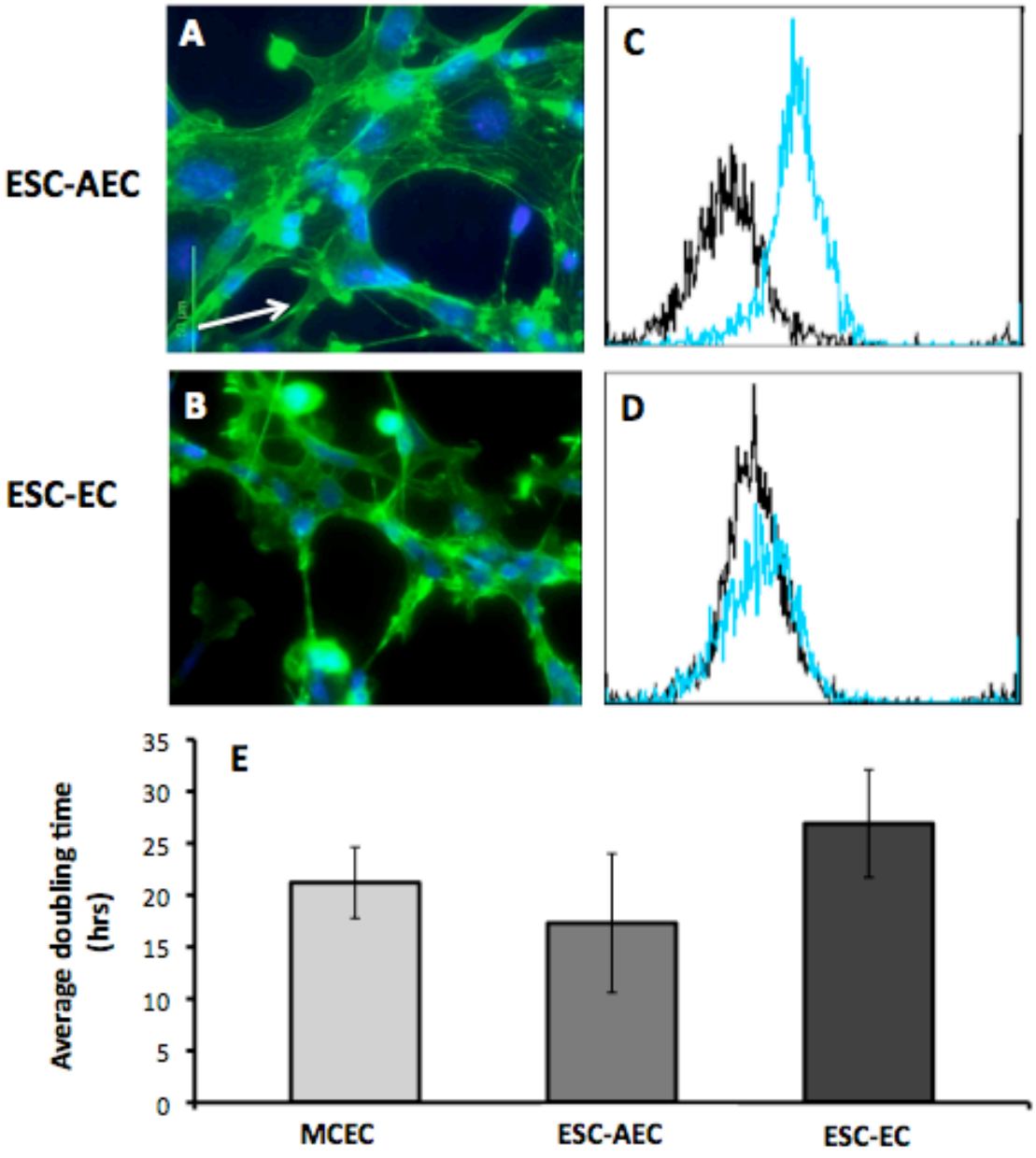


Figure 3.4: ***ESC-AEC express increased levels of actin stress fibers and HSP27 phosphorylation.*** The cytoskeleton organization and presence of stress fibers of the **(A)** ESC-AEC is noticeably increased compared with the **(B)** ESC-EC. Blue = DAPI, Green = phalloidin 488. Scale bar = 50um.

HSP27 phosphorylation, a factor in endothelial actin organization and migration, expression is **(C)** high in ESC-AEC compared with **(D)** almost no expression in the ESC-EC. **(E)** Graph shows average population proliferation rates of the MCEC controls, ESC-AEC, and ESC-EC. The ESC-AEC proliferate at a faster rate compared with the other EC, but the differences between EC populations were not statistically significant.

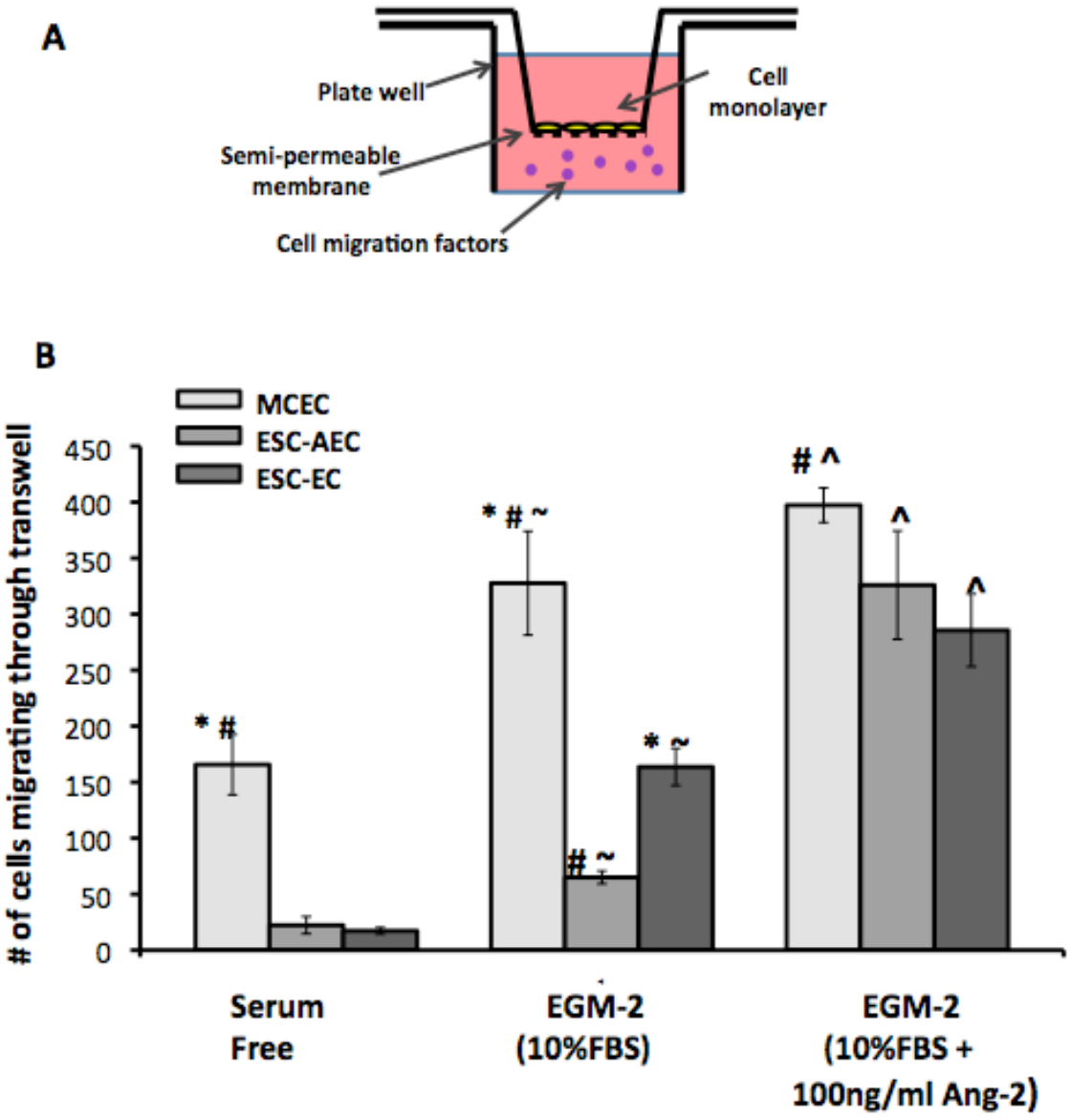


Figure 3.5: ***ESC-AEC and ESC-EC both migrate towards chemoattractant.*** **(A)** Diagram of the transwell assay shows the chemoattractants are added to the bottom well. The cells migrate from the top transwell though the pores in the transwell insert onto the bottom surface of the insert well. **(B)** The

number of migrating cells was then quantified. Note that ESC-AEC were much more responsive to the proangiogenic chemoattractant compared to serum only. All cell populations were compared for statistical significance. * = statistically significant from ESC-AEC, # = statistically significant from ESC-EC, ~ = statistically significant from serum-free, ^ = statistically significant from 10% FBS

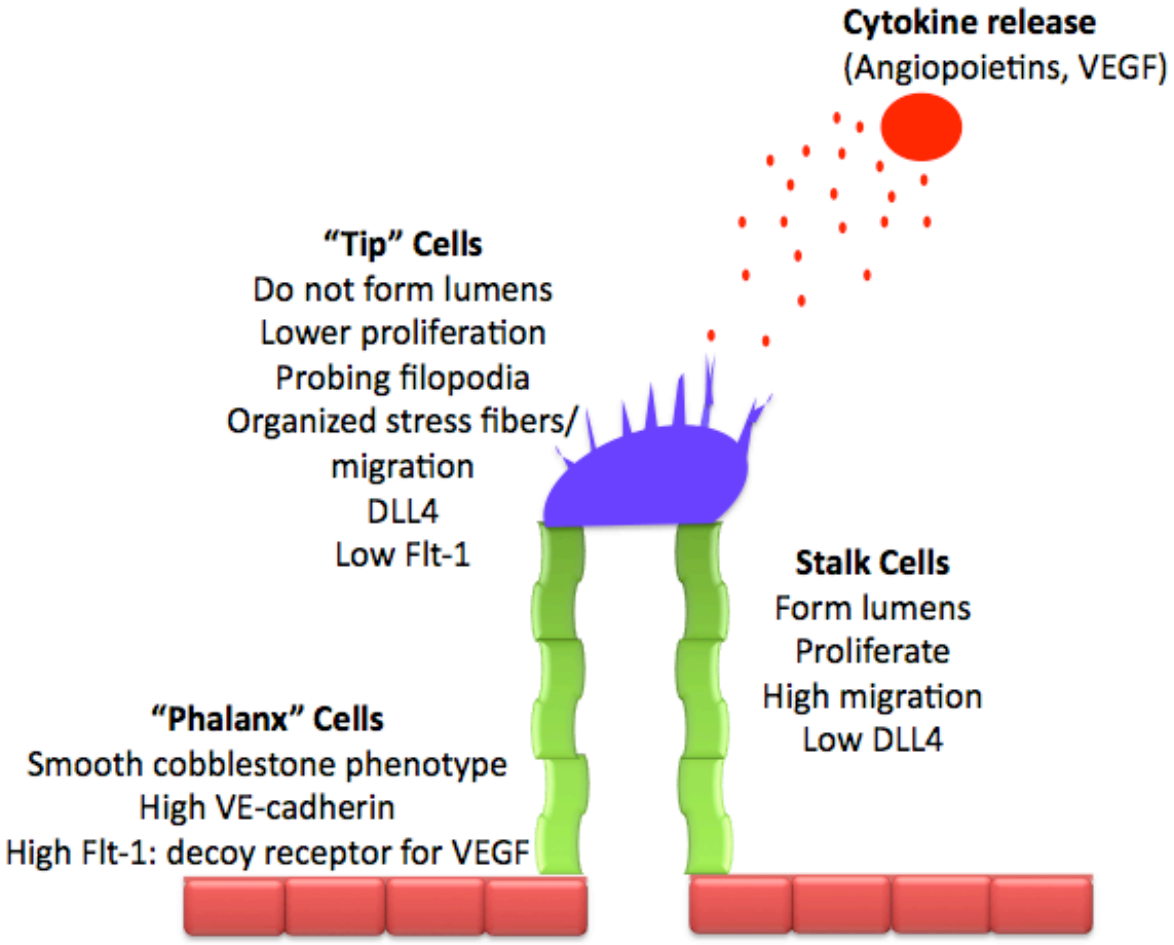


Figure 3.6: ***Specialized tube-forming EC subtypes.*** The diagram depicts the tip, stalk, and phalanx EC within a sprouting blood vessel. Included is also a summary of the phenotypic and functional differences in the three specialized EC.

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Chapter 4

Adhesive Forces in Embryonic Stem Cell Cultures

Abstract

Most cell culture systems grow and spread as contact-inhibited monolayers on flat culture dishes, but the embryonic stem cell (ESC) is one of the cell phenotypes that prefer to self-organize as tightly packed three-dimensional (3D) colonies. ESC also readily form 3D cell aggregates, called embryoid bodies (EB) that partially mimic the spatial and temporal processes of the developing embryo. Here, the rationale for ESC aggregation, rather than "spreading" on gelatin-coated or mouse embryonic fibroblast (MEF)-coated dishes, is examined through the quantification of the expression levels of adhesion molecules on ESC and the calculation of the adhesive forces on ESC. Modeling each ESC as a dodecahedron, the adhesive force for each ESC-ESC binding was found to be 9.1×10^5 pN, whereas, the adhesive force for ESC-MEF binding was found to be an order of magnitude smaller at 7.9×10^4 pN. We also show that E-cadherin is the dominating molecule in the ESC-ESC adhesion and blocking E-cadherin leads to a significant reduction in colony formation. Here, we mathematically describe the preference for ESC to self-assemble into ESC-ESC aggregates and 3D colonies, rather than to bind and spread on gelatin or MEF-coated dishes, and have shown that these interactions are predominantly due to E-cadherin expression on ESC.

4.1 Introduction

Many in vitro tissue cell culture systems classically grow as contact-inhibited monolayers on 2D tissue culture dishes. Conversely, the ESC cultures are morphologically distinct when cultured on these same surfaces. ESC form tightly packed 3D colonies in standard tissue culture dishes (Figure 4.1A). These ESC colonies also readily and spontaneously assemble into unattached floating cell aggregates, called embryoid bodies (EB; Figure 4.1B), which partially mimic the spatial and temporal processes of the developing embryo [1, 2]. We were interested in quantitatively exploring why the ESC prefer to adhere to one another and grow as 3D colonies compared to attaching and spreading on a flat surface like many other cell types.

4.1.1 Cadherins

Calcium-dependent cell-cell adhesion junctions, called cadherins, are known to play a crucial role in a multitude of cellular processes including cell-cell adhesion, motility, and cell sorting in maturing organs and tissues [3]. It has been thought that much of self-cell sorting is mediated through the expression of specific cadherin cell surface molecules, largely because the varying expression of cadherin proteins during ESC differentiation and between tissue-specific cell phenotypes. For example, vascular endothelial (VE) cadherin denotes the endothelial lineage, while neural (N) cadherin is present on neural cells. Alternatively, the primary cadherin expressed on murine ESC (mESC) is the epithelial (E) cadherin [4, 5]. Interestingly, E-cadherin is also the expressed on MEF [6] and may pay a key role in the adhesion of ESC-to-MEF feeder cells in the ESC co-

cultures. When cells expressing different cadherin molecules are co-cultured, the cells expressing E-cadherin will self-separate into distinct aggregates from the cells expressing N-cadherin [7]. However, it has been more recently shown that both the cadherin quantity and affinity control tissue segregation [8].

4.1.2 Integrins

Integrins are another family of adhesion proteins that exist as combinations of ab subunits, largely distinguished by their β subunits: β1 (CD28), β3 (CD18), β5 (CD16), etc. [9]. Integrins are foremost molecules responsible for cell binding to extracellular matrix, as well as some cell-cell interactions crucial for many physiological processes signaling cell growth and differentiation. Their ability to bind ligand is regulated by cellular signaling mechanisms and involves conformational changes of the extracellular domains and/or changes in their cell surface distribution [10, 11]. It has been recently established that embryonic stem cells express a specific subset of integrins, all in the β1 family [12], which are also present on the MEF feeders cells [9, 13].

4.1.3 Tissue Morphogenesis

The spontaneous self-organization of cells and tissues is an intriguing phenomenon. The morphogenic process of “shifting of cell associations and subsequent segregation” into tissue arrangements was first described by Towens and Holtfreter [14] and then later developed by Steinberg in 1994 [15]. The mechanisms that underlie these goal-directed rearrangements were suggested to be a combination of directed movements and selective adhesion, called the differential adhesion hypothesis (DAH) [16]. The

DAH suggests that “mobile, cohesive subunits will spontaneously tend to rearrange so as to maximize their mutual adhesive bonding and the relative bonding energies.” According to DAH, the self-sorting process of individual entities do so with certain affinity for each other as a consequence of maximizing the ‘strength’ of mutual binding, thus minimizing the adhesive free energy of a system. Although DAH was formulated to explain the sorting and spreading behaviors of cells and tissues, it has been subsequently demonstrated to play a role in embryogenesis [17-20], wound healing [21], vasculogenesis [22, 23], and malignancy [24].

Here, we quantitatively explore the relative roles of cadherins and integrins and their corresponding binding forces in order to mathematically describe the resulting 3D cell morphology of ESC colonies. Our method examines the expression levels of E-cadherin and Integrin-β1 on mouse ESC cultured on a) the MEF feeder cells, and b) a gelatin substrate without feeders, as well as, the expression levels of these molecules on the MEF cells. The surface area for ESC-ESC binding is calculated by modeling the cell as a dodecahedron bordered by 12 other cells. The results mathematically describe the preference for ESC to self-assemble into ESC-ESC aggregates and 3D colonies, rather than to bind and spread on gelatin or MEF-coated dishes.

4.2 Results

4.2.1 ESC adhesion

By modeling the ESC as a dodecahedron, it is possible to map out the adhesive forces acting on the “sides” of a cell. The dominant adhesion molecule expressed on an undifferentiated ESC is the E-cadherin [25]. In addition, MEF also produce extracellular

matrix molecules including laminin and fibronectin for integrin binding [26, 27]; therefore the integrin expression on ESC is also considered in the ESC binding to the MEF feeder layer. Other adhesion molecules are also present on ESC, but these will be shown to play a negligible role in the cell-cell adhesion of ESC compared with the cadherins and integrins.

4.2.2 Cell Surface Area

The spherical ESC will be modeled as dodecahedrons with the following assumptions: the ESC are spherical in shape, tightly packed, and exhibit close spherical regular packing such that each sphere is bordered by 12 other spheres [28, 29]. This dodecahedral model was chosen because it has the same packing as the regular spherical packing model, but also gives us a simple and nicely quantifiable cell-cell contact area. Although models like Kelvin's Conjecture [30] and a slightly revised version provided by Weaire and Phelan [31] suggest using a tetrakaidecahedron, these models focus on finding a solution to the tightly packed, space-filling, equivolumetric polyhedral of minimal surface area. However, the close packing of our cell aggregates require diffusion of nutrients, etc., and are therefore, not as tightly packed as spheres made of inorganic materials.

The 12-sided 3-D shapes divide equilaterally and equiangularly in space. Also, the surface that adheres to neighboring cells, called a "face", can be quantified based on cell size. When in a fluid suspension, the diameters of murine ESC range from 6 to 10μm. Using the formula for the volume, **V**, of a sphere: $\mathbf{V} = (4/3)\pi r^3$, the following

values are obtained (Table 4.1): for a 6μm diameter cell: **V** = 113 μm³, for 8μm: **V** = 268 μm³, and for 10μm: **V** = 523 μm³. Next, we calculate the edge length of the dodecahedron model. This can be done by solving for '***a***' in the following equation:

$$V = \frac{1}{4}\left(15 + 7\sqrt{5}\right)a^3 \qquad (2)$$

Using the edge length, the surface area, **SA**, of our cells can be calculated using the equation:

$$SA = 3\sqrt{\left(25 + 10\sqrt{5}\right)}a^2 \qquad (3)$$

Using edge length for the dodecahedron, the estimations for the surface area of an ESC were calculated for 3 different sizes of stem cells using 1) the **SA** of a perfect sphere and 2) the **SA** for the dodecahedron. The data indicates that the **SA** measurements are 10% larger in the dodecahedron model compared to the **SA** of a perfect sphere (Table 4.1). Given the range of cell sizes present in mESC cultures, the differences in the calculated surface area between these 2 models are not statistically significant.

4.2.3 MEF synthesis of ECM

We first examined the ECM proteins produced by our MEF in order to verify that the appropriate ECM proteins were available for ESC integrin-β1 binding. The immunofluorescent analysis indicates that the MEF layer actively produces the ECM proteins fibronectin and, to a lesser extent, laminin, collagen type I and type IV (Figure 4.2), verifying that these cells are generating specific sites for ESC integrin binding.

4.2.4 ABC of E-cadherin and Integrin-β1 expression

The ABC values for QSC microbeads stained with E-cadherin and integrin-β1 (Figure 4.3A and B) were obtained by labeling the QSC microbeads with the corresponding antibodies that were also used to label the cells. From this data, we established correlations between ABC and fluorescence intensity (FI) values for each of the antibody labels (Figure 4.3C and D). MEF and mESC (cultured on both MEF and gelatin) were then stained with identical E-cadherin and integrin-β1 antibodies. The mean fluorescence intensity (FI) values corresponding to the E-cadherin expression on MEF and ESC was then calculated. On MEF, the E-cadherin FI = 5, while FI = 140 and 160 for the ESC cultured on gelatin and on MEF-coated dishes respectively MEF-coated dishes respectively (from Figure 4.4A). The procedure is repeated for the integrin β1 antibody. The mean FI value correlating to integrin-β1 expression on MEF was found to be FI = 4.7, while FI = 9.6 and 4.7 for ESC cultured on gelatin and MEF-coated dishes respectively (from Figure 4.4B). The values were then inserted into the calibration equations (Figure 4.3C and D) to obtain the ABC values for each molecule.

4.2.5 Modeling Cell Adhesion

In order for the ESC-ESC to adhere one another more strongly than their MEF or ECM-coated surfaces, and grow vertically into a 3-D colony, we expect that the adhesive forces between the ESC-ESC are greater than the adhesive forces between an ESC-MEF and its ECM.

$$F_1 > F_2 \qquad (4)$$

where F_2 represents the total adhesive force of ESC binding to the MEF feeder layer and ECM, and F_1 represents the total adhesive force for ESC-ESC binding. For simplification, the model assumes all adhesive forces are binding at their maximum strengths and that 100% of the available adhesive bonds are in their "bound" state. The maximum binding force of an E-cadherin/E-cadherin homophillic bond on mouse L-M(TK-) cells was measured to be 73 pN [7]. The bond strength of an individual a5bı/fibronectin interaction on K562 cell lines was previously calculated to be 100 pN [32]. We are assuming that these bonds strength values are relatively consistent across cell lines, and therefore, applicable to our ESC and MEF as well.

4.2.6 ESC-to-MEF adhesion

The key components of the binding of ESC to MEF include E-cadherin/E-cadherin cell-cell adhesions, $F_{E\text{-}cad}$, and ESC integrin-binding to ECM components, F_{int}.

$$F_2 = F_{E\text{-}cad} + F_{int} \qquad (5)$$

Interestingly, MEF express very low levels of E-cadherin, under 2,000 ABC (calculated from Figure 5A). Returning to the dodecahedron model where we consider twelve sides per ESC: 2,000ABC /12 = 166 E-cadherin per "face" of the ESC.

$$F_{E\text{-}cad} = (166)*(73\ pN) \quad = 1.2 \times 10^4\ pN \qquad (6)$$

The ESC analyzed express β1-integrin molecules at a fairly low levels as well, under 500 ABC for ESC cultured on MEF and approximately 4,000 ABC for ESC cultured on gelatin. Because MEF are often plated on gelatin, the 4,000 ABC value is used for

calculations. Returning to the dodecahedron model where we consider the 12 sides of an ESC binding to a MEF (and assuming that the ESC does not "spread" when binding to the MEF surface): 4,000/12 = 333 integrins on each "face" of the ESC. However, we also know that the integrins tend get recruited towards anchoring sites (i.e. the ECM binding surface), as well as contribute to the generation of focal adhesion complexes that can enhance adhesion strength by up to 30% [33]. Due to integrin clustering, we estimate that we will have double the number of integrins at the binding surface: 2 x 333 = 666 integrins per binding "face" [34]. If one "face" is in contact with the MEF layer and the maximum strength of an integrin bond is 100pN [32, 35-37]:

$$F_{int} = (666)*(100\ pN) \quad = 6.7 \times 10^4\ pN \qquad (7)$$

Thus,

$$F_2 = F_{E\text{-}cad} + F_{int} = 7.9 \times 10^4\ pN \qquad (8)$$

4.2.7 ESC-to-ESC adhesion

F_1 represents the total adhesive force for ESC-ESC binding, due to cadherin-cadherin binding only. The quantitative flow cytometry data for E-cadherin expression on our mouse ESC indicates approximately 150,000 ABC of E-cadherin molecules per ESC regardless of whether they were cultured on MEF or on gelatin. The surface area of one pentagonal side of an ESC of approximately 8μm in diameter is 221 μm²/12 = 18 μm² and the maximum binding force of an E-cadherin/E-cadherin bond is 73pN [7]. Once again assuming that one side of an ESC binds to only 1 side of another ESC:

$$F_1 = F_{E\text{-}cad(ESC)} = [(150{,}000)/12]*(73pN) = 9.1 \times 10^5 pN \qquad (9)$$

Note, that we do not estimate for migration of E-cadherins toward the "binding face" because, unlike monolayer cultures, the E-cadherin/E-cadherin binding is expected to exist equally on all sides of the ESC, due to its 3-D colony morphology.

4.2.8 E-cadherin and Integrin Blocking

The E-cadherin receptors on the ESC were blocked with an E-cadherin blocking antibody. After 48 hours, the ESC with the E-cadherin blocking antibody exhibited significantly reduced colony sizes with many more ESC growing as single cells spreading on the culture dish (Figure 4.5). Likewise, we expect that the blocking of β1-integrin on the ESC would interfere with the ESC-to-MEF binding. Although we did not run this experiment ourselves, a previous study has shown that β1-integrin null ESC did not adhere well on fibroblasts [38].

4.3 Discussion

It is already well-known that the relative binding energies per unit area determine the most stable cell culture configurations [39]. The presented calculations make use of this concept, estimating the adhesion forces between embryonic stem cells with each other, and between embryonic stem cells and the fibroblasts feeders. Combining the known adhesive forces for each molecule with the measured number of molecules per cell allows new calculations of the relative adhesive forces between the ESC-ESC and ESC-MEF. The results indicate that the strength of adhesion of ESC-ESC, F_1 = 9.1 x

10^5pN, is an order of magnitude larger than the strength of adhesion of ESC-MEF, F_2 = 7.9 x 10^4pN, thus; mathematically describing the preference for ESC to adhere to each other in 3D colonies, rather than growing and spreading as monolayer cultures on MEF or gelatin-coated plates.

The examined cadherin and integrin adhesion molecules were carefully chosen due to their known expression levels on ESC. Although additional adhesion molecules such as: connexin-43 [40], zona occluden-1 (ZO-1) [41], platelet endothelial cell adhesion molecule 1 (PECAM-1) [42], and EpCAM [43, 44] are often expressed on ESC, the integrins and cadherins are the dominating adhesion molecules on these cells. The cell separation force for E-cadherin, 30nN, is an order of magnitude larger than the cell separation forces for occludins, 1 nN [45]. Moreover, whether a specific receptor is considered and adhesion molecule at all depends on the magnitude of adhesive force and the time scale of the cell contact [46]. Although PECAM-1, due to its tendency to localize at cell-cell borders, was originally described as an adhesion molecule, the primary role of PECAM-1 is in transmitting signals leading to an upregulation of β1 integrin function. It is now accepted that PECAM-1 itself cannot support strong adhesion [47]. Connexin-43 is also expressed on the developing embryo for facilitation of intercellular coupling, cell morphology, and very recently, exhibited a role in the modulation of cell-cell assembly as well [48]. EpCAM is the final known adhesion molecule that can be expressed on ESC, but the whole cell separation forces for this molecule could not be found in the literature. This body of literature indicates other adhesion molecules exist at lower levels or exhibit lower binding energies compared

with E-cadherin and integrin-β1. In addition, our E-cadherin blocking study shows that the E-cadherin molecule is the most influential molecule determining the ESC colony-like morphology.

Integrin clustering and the presence of focal adhesion complexes on the ESC binding surface could also increase the strength of adhesion to the substratum by up to 30% [33]. Although the presented calculations did account for integrin clustering (by doubling the number of integrins per binding surface), potential increases in binding strengths and affinities due to assembly of focal adhesions was not assessed in detail. If we consider that focal adhesions could increase the integrin binding forces by an additional 30%, our calculated value for ESC-MEF binding, F_2, might be as large as 1 x 10^5pN, but is still not a large enough force to overcome the F_1 = 9.1 x 10^5pN for ESC-ESC binding.

It has also been shown that integrins possess the ability to mediate strong intercellular cohesion when cells are grown as 3D aggregates [49]. In this mechanism, the cell-cell cohesion enhances the adhesion and compaction of the cells in the 3D aggregate via a5β1-integrins, but fibronectin synthesis by the cells in the 3D aggregate is required for the integrin-ECM cohesion [49]. Since the integrin-β1 expression on our ESC was measured to be very low, cohesion would not amount to a very large adhesive force in our ESC cultures.

This manuscript is the first of its kind to quantitatively explore the role of cadherins and integrins the cell morphology of ESC cultures. Here, the exploration of the relative binding forces of the cell-cell and cell-substratum (with and without MEF co-cultures), the 3-D cell morphology of ESC colonies is mathematically described. We have shown that the cell-cell adhesive forces of the ESC aggregates are much greater than the cell-substratum adhesive forces of the ESC and the E-cadherin molecule is responsible for the colony-like morphology of ESC, revealing mathematically, why these cells do not tend to spread significantly on the tissue culture plates.

Although the model presented in the manuscript was developed for homogeneous populations of ESC, we expect that the model would also remain valid for other types of homogeneous cell aggregates, including neurospheres - assuming that the neurospheres also contain homogeneous populations of neural progenitor cells. The model would only need to be altered for the different cell size, adhesion molecules, and binding strengths. However, the model would not be applicable to cells in a developing embryo, embryoid body, or differentiating neurosphere, since these cell aggregates contain inhomogeneous cell populations of varying sizes, morphologies, and adhesion molecules.

4.4 Materials and Methods

4.4.1 Stem Cell Culture

E14 murine ESC (generously donated from Dr. Bruce Conklin, UCSF) were cultured on either 0.5% gelatin coated-tissue culture plates or on mitotically inactivated MEF plated

at $4x10^5$ cells per 35-mm dish in chemically defined medium containing: 15% KnockOut Serum Replacement (KSR; Gibco), 1x Penicillin-Streptomycin (Invitrogen), 2mM L-glutamine (Invitrogen), 1x Non-essential Amino Acids (Invitrogen), 0.1mM b-mercaptoethanol (Calbiochem), 2000 units/ml Leukemia Inhibitory Factor (LIF;Chemicon), 10ng/ml bone morphogenic protein-4 (BMP-4; R&D Systems), and Knock-Out Dulbecco's Modified Essential Medium (KO-DMEM; Gibco). ESC were harvested using 1ml of cell dissociation buffer (Invitrogen) per 35mm-dish for 20 minutes and separated from the MEF feeder layer by differential sedimentation of the MEF. The smaller and less sticky mESC are then removed with the supernatant. This separation method for removing MEF from ESC cultures consistently yields high purity mESC populations.

4.4.2 Immunofluorescent Analysis of Extracellular Matrix (ECM) Proteins

The mitotically inactivated MEF were plated into 8-well chamber slides (Nunc) at 20,000 cells per well. Confluent MEF were subsequently labeled with unconjugated primary antibodies against rabbit polyclonal fibronectin, laminin, collagen I and collagen IV antibodies (Abcam) followed by an anti-rabbit PE or anti-rabbit FITC conjugated secondary antibody (Research Diagnostics). Images were recorded with a Leica DFC 350FX fluorescent microscope.

4.4.3 Quantification of Adhesion Molecules

Antibody binding capacity (ABC) is a measure of the number of primary antibodies binding to a cell or microbead, and is linearly proportional to the number of specific

surface molecules on a cell including the variables: valence of antibody binding, steric hindrance, binding affinity, and non-specific antibody binding. Thus we have:

$$ABC = n_1\theta_1\lambda_1 \quad (1)$$

where n_1 is the number of binding sites per cell, including specific and non-specific binding sites (n_s + n_{sp}), θ_1 is the fraction of binding sites on the surface bound by the primary antibody, and the parameter λ_1 represents the valence of the primary antibody binding. The combined term is equivalent to the antibody binding capacity (ABC) of a cell population [50-52].

4.4.4 Quantitative Flow Cytometry

The Quantum Simply Cellular® microbeads (QSC, Bangs laboratories) used in this study were uniform, 8.0 μm-diameter, polystyrene mcirobeads with calibrated numbers of goat anti-mouse (GAM) antibodies bound to their surfaces. Individual sets of these microbeads are coated with four distinct populations of GAM antibodies by the manufacturer. These GAM antibodies bind the Fc region of IgG1, IgG2a, and IgG2b isotypes of mouse monoclonal antibodies. The QSC microbeads used in this study expressed antibody binding populations of median ABC values of: 0, 5,400, 33,000, 200,000, and 440,000.

4.4.5 Cell staining

Cells were then fluorescently labeled with either a rat monoclonal anti-mouse E-cadherin-PE conjugated antibody (R&D Systems) or a mouse monoclonal anti-integrin

β1-FITC conjugated antibody (Abcam) using a buffer containing 0.5 % bovine serum albumin (BSA), 2mM EDTA, and phosphate buffered saline without calcium and magnesium (PBS). After staining, the cells were strained using a 70mm cell strainer (BD Falcon) to ensure single cell suspensions. Flow cytometry analysis (FACS) was performed using a BD LSRII (Becton Dickinson) and data were analyzed using FlowJo software (TreeStar). For quantitative flow cytometric analysis, antibody binding capacity (ABC) was determined using Quantum Simply Cellular (QSC) anti-mouse IgG microbeads (Bangs Laboratories) [53].

4.4.6 E-cadherin Blocking

The E14 mESC were grown in serum-free maintenance medium on 0.5% gelatin coated plates. The cells were then harvested using Cell Dissociation Buffer (Invitrogen), pelleted, and resuspended in either serum-free maintenance medium or maintenance medium with E-cadherin blocking antibody (Invitrogen) at a 1:500 dilution. Individual wells in a 24-well plated are coated with. Cells were then seeded into 24-well plates coated with 0.5% gelatin at a 10,000 cells/cm^2 and imaged using a phase contrast microscope after 48 hours.

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Diameter (μm)	Volume (μm³)	a (μm)	SAsphere (μm²)	SAdodecahedron (μm²)
6	113	2.45	113	124
8	268	3.27	201	221
10	523	4.09	314	347

Table 4.1: The surface area (SA) of a sphere and a dodecahedron calculated for a range of cell sizes. The differences between calculation methods vary by less than 10% for our range of cell sizes.

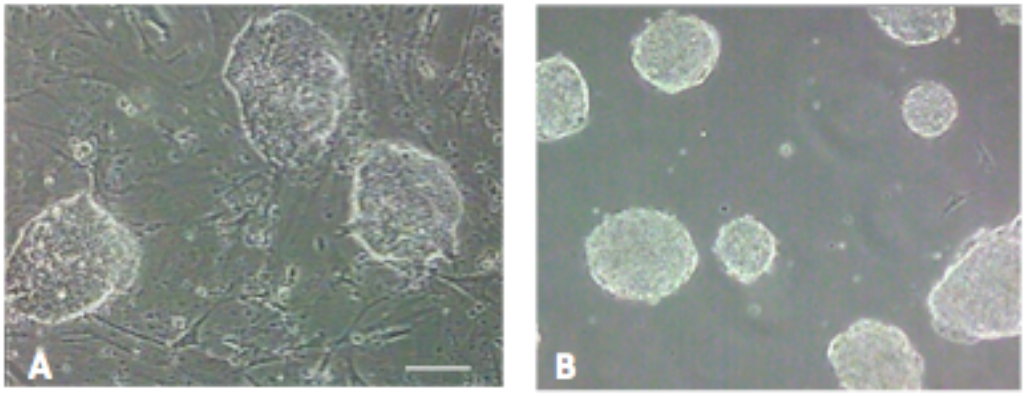


Figure 4.1: Embryonic stem cell colonies grow as 3D structures. (A) Micrographs of dome-shaped murine embryonic stem cell (mESC) colonies growing on top of murine embryonic fibroblasts (MEF) and **(B)** embryonic stem cell (ESC) aggregates in forming embryoid bodies (EB) in suspension culture. Scale bar = 50mm

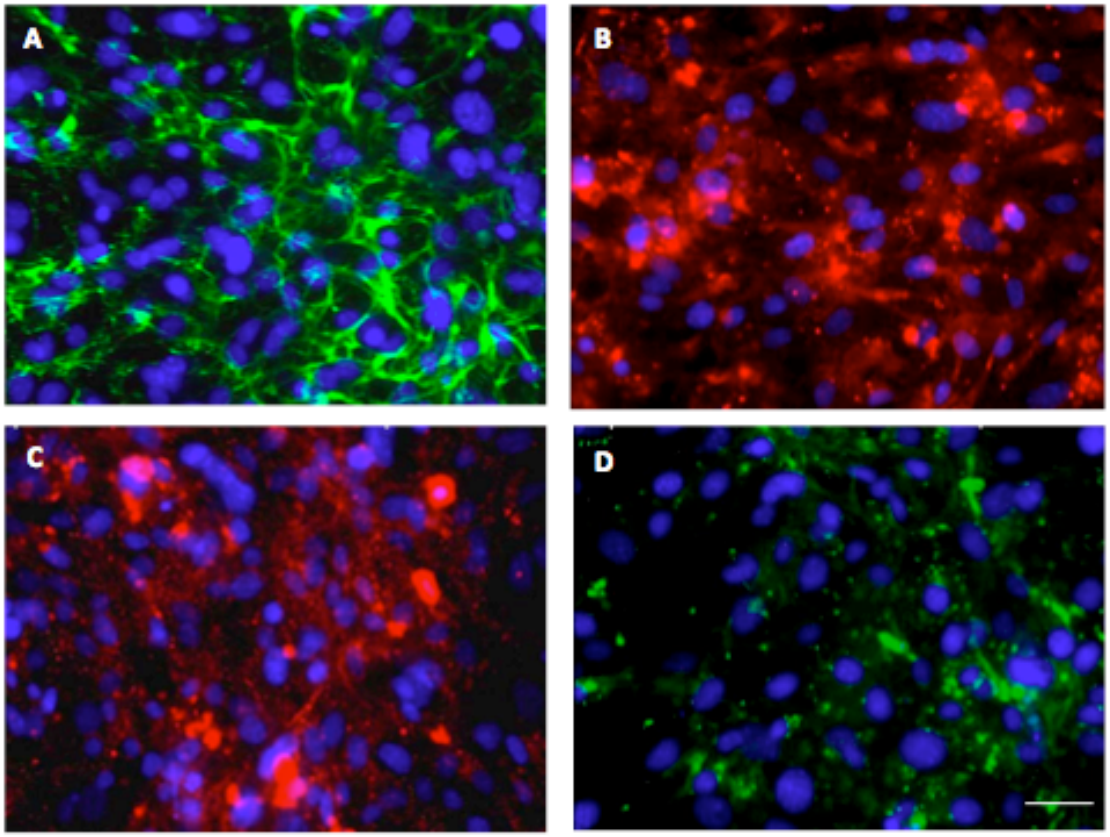


Figure 4.2: Mouse embryonic fibroblasts synthesize various extracellular matrix proteins. Extracellular matrix (ECM) proteins produced by MEF cells include: **A)** fibronectin, **B)** laminin, **C)** collagen-type I, and **D)** collagen-type IV. Scale bar = 50 mm.

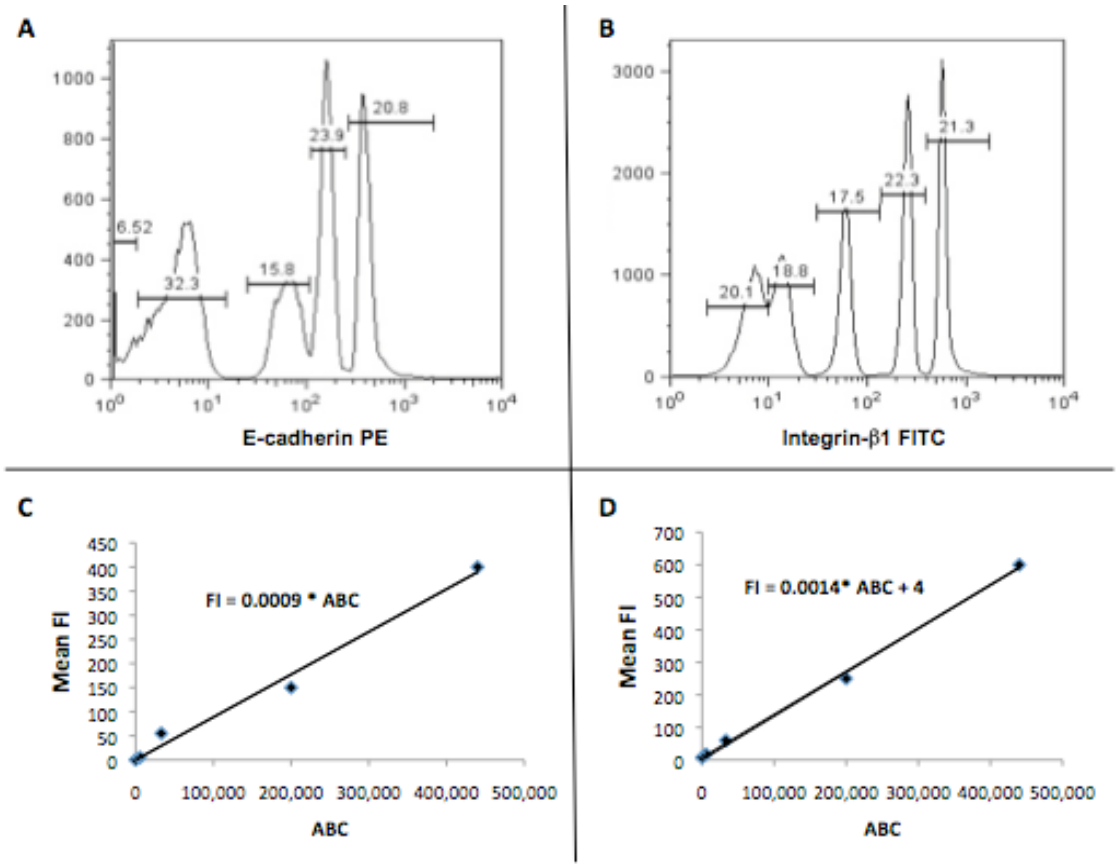


Figure 4.3: The antibody binding capacity values correlate with fluorescence intensity values for specific monoclonal antibodies. A&B) Histograms of the Quantum Simply Cellular (QSC) flow cytometry calibration microbeads stained with **A)** PE-conjugated E-cadherin antibodies and **B)** FITC-conjugated integrin-β1 antibodies. **C&D)** The linear relationship of the measured mean fluorescence intensity (FI) values for a range of antibody binding capacities (ABC) on QSC flow cytometry calibration microbeads were obtained for QSC microbeads stained with **C)** PE-conjugated E-cadherin antibodies and **D)** FITC-conjugated integrin-β1 antibodies.

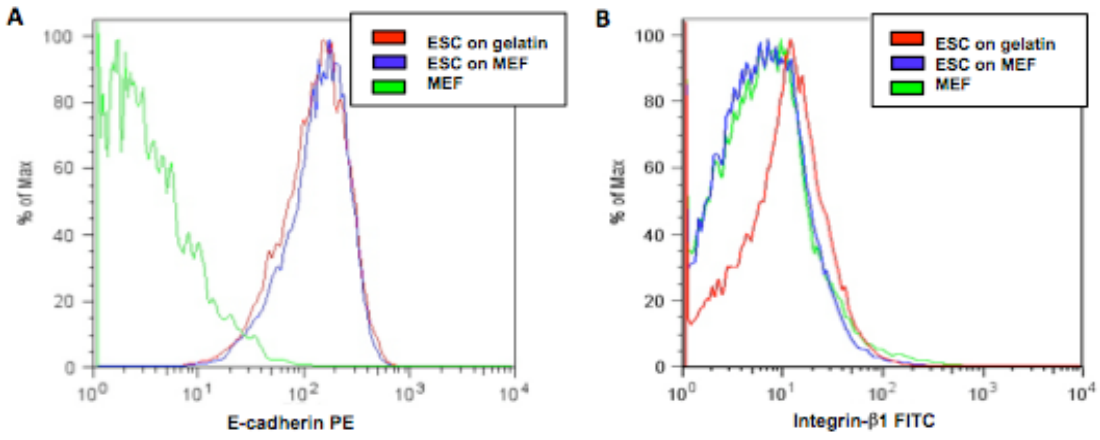


Figure 4.4: ESC express larger numbers of E-cadherin molecules. Histograms of the mESC and MEF stained with **A)** PE-conjugated E-cadherin antibodies and **B)** FITC-conjugated integrin-β1 antibodies. The MEF do not express either molecule. The mESC express more integrin-β1 when cultured on gelatin (FI = 10) compare with when cultured on MEF (FI =5, approximately equivalent to autofluorescence for the cells and calibration beads). The ESC express high levels of E-cadherin while cultured on MEF (mean FI = 139) or gelatin (mean FI = 148).

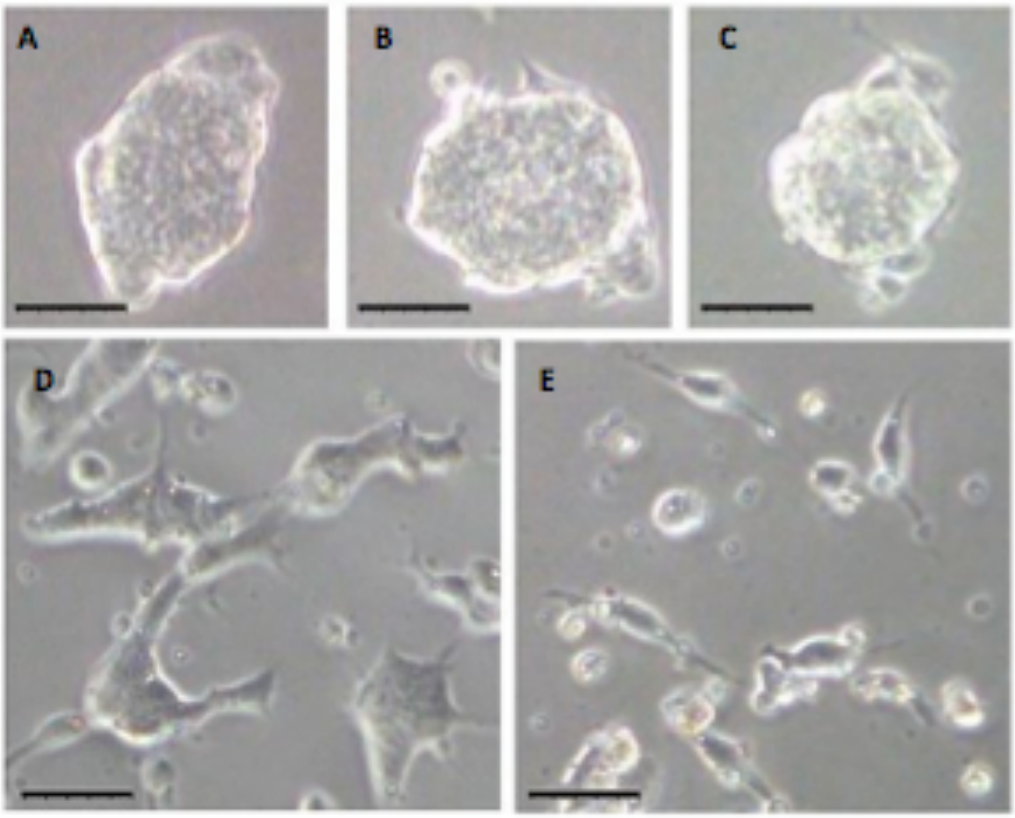


Figure 4.5: E-cadherin blocking reduces ESC-ESC contacts. Images of ESC colonies cultured **(A-C)** without E-cadherin blocking antibody and **(D-E)** with the blocking antibody. Note that the ESC colonies cultured with the blocking antibody are significantly smaller with more cells growing as single cells. Scale bar = 50mm

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Chapter 5

Conclusions

5.1 Serum free stem cell maintenance

Murine and human embryonic stem cells are often co-cultured on murine embryonic fibroblast (MEF) in order to maintain pluripotency. However, serum free maintenance cultures have been developed [1-4], which lead to the decision to try serum free cultures for our cells. The R1 mESC line was chosen as the experimental mESC line. R1 mESC grew robustly in the serum free stem cell maintenance medium. Moreover, the cells did not require the use of fibroblasts to maintain pluripotency, and were maintained on 0.5% gelatin coated dishes. The maintenance medium was developed with the influence of other previous formulations with minor adjustments for our particular cell line [1-4], specifically the addition of BMP-4 to inhibit neural differentiation, which can be triggered by LIF added to maintain pluripotency in ESC [5].

Moreover, we have demonstrated that E-cadherin is the key cell-to-cell adhesion molecule in undifferentiated murine ESC using a mathematical model based on a dodecahedron, as opposed to a sphere, and quantification beads to determine cell-cell adhesion strength. In addition, the calculations confirmed that ESC-ESC bonds are an order of magnitude stronger than both ESC-MEF and ESC-gelatin bonds [6].

5.2 Serum free endothelial cell differentiation

Our previously published derivation scheme for generating endothelial cells from ESC was influenced by the monolayer differentiation protocol developed by the Nishikawa laboratory [7]. Alternative methods of ESC differentiation utilized the formation of cell aggregates, celled embryoid bodies (EB), which mimic the formation of the three germ layers in vitro. One of the major advantages of monolayer technique is that a higher percentage of the derived cells of the lineage(s) of interest can be obtained due to increased microenvironmental control in the monolayer compared with the generation of EBs, with less contamination from cells from other germ layers. Moreover, due to the inconsistent results from serum containing mediums, we chose to develop serum-free differentiation formulations to better control the biochemical signaling components in the medium during differentiation.

5.3 Developing a differentiation scheme

The initial induction stage involved the generation of Flk-1+ vascular progenitor cells. Although Flk-1 is the first lineage marker for the vascular lineages, it is also present on hematopoietic stem cells and other mesodermal cell types. The serum free formulation for this initial induction used, NS1D2b medium, was a modification of a differentiation medium used previously [8, 9]. In lieu of fetal bovine serum, KnockOut serum replacement (KSR) (Invitrogen) was used, as well as, adding VEGF and BMP-4 components.

In order to purify the Flk-1+ cells, magnetic activated cell sorting (MACS) was often used instead of Fluorescence Activated Cell Sorting (FACS) for enhanced cell survival. Initially fluorescence activated cell sorting was considered an ideal sorting method since it would theoretically yield the highest purity of Flk-1+ cells. Unfortunately during the early stages of serum free differentiation, the cells were too fragile to survive the stress from a high speed fluidics system.

The Flk-1+ outgrowth medium required many steps of optimization. Multiple formulations of basal medium were examined, and the final formulation resulting in the greatest cell proliferation at this stage consisted of 70% Alpha MEM and 30% DMEM. We also found that the KSR used in the initial induction medium was not rich enough in nutrients to support the continued viability of the serum-free Flk-1+ cells. After attempting to boost the efficacy of the KSR by adding albumen and glucose, we decided to search for other commercially available substitutes. Nutridoma-CS (Roche), a supplement originally meant for serum-free culture of cancer and immortalized lines, was a more effective replacement. The resulting formulation, LDSk, can be used as a medium alone or can be mixed in a 1:1 ratio with serum-free EGM-2 (Lonza) medium for long term maintenance.

In order to generate EC, Flk-1+ cells were expanded until cobblestone colonies became visible, usually 10-14 days. These colonies were then manually selected and replated. A separate population of EC were not manually selected for cobblestone morphology and merely expanded in our EC-promoting medium formulation after Flk-1+ enrichment.

5.4 Cell-Matrix Signaling in EC fate

Published studies using serum claimed that collagen type IV was the optimal substrate for EC differentiation [7, 10, 11]. Since we were working with novel serum-free differentiation media, the question as to whether or not collagen type IV was indeed the best substrate was again examined. Five different commercially available substrates: collagen type I, collagen type IV, laminin, fibronectin, and gelatin, were tested for differentiation efficacy.

In the first stage of differentiation, Flk-1 expression peaks for all substrates. At two days of induction, fibronectin and gelatin both yielded the highest number of Flk-1+ cells, however fibronectin yields slightly more. Therefore it was selected as the substrate of choice for the initial stage of differentiation.

After Flk-1+ enrichment, all of the aforementioned five substrates were tested again for maximum yield of VE-cadherin+ (EC marker) cells and minimal calponin+ smooth muscle cell (SMC) contamination. Previous studies have shown that under monolayer EC differentiation conditions, the resulting population contains a mix of smooth muscle cell and EC. Collagen type IV and fibronectin both yielded high percentages of VE-cadherin+ cells. However, fibronectin also yielded the smallest percentage of calponin+ cells. Thus, fibronectin was determined to be the optimal substrate for our EC serum-free differentiation scheme. This was not surprising due to the fact that primary EC generate a vast fibronectin network in culture [12] and therefore it is a major component of the EC niche.

5.5 Morphology and marker expression

The generated EC cells that underwent two purification steps (named ESC-EC) and the EC that only underwent the initial Flk-1+ enrichment (named ESC-AEC) both express high levels of Flk-1 and VE-cadherin. ESC-EC also express high levels of Flt-1, Tie-1, and Eph B4 (venous lineage), while ESC-AEC express these markers in lower numbers. Both are negative for calponin smooth muscle cells, and both populations can uptake low density lipoprotein, another essential function of EC [13].

5.6 Angiogenic behavior

In a whole animal assay, the chick chorioallantoic membrane assay, both populations of EC perform similarly to each other and to an immortalized EC positive control (MCEC). All three populations yield a significantly higher amount of vascular activity than both the vehicle control and R1 mESC control, demonstrating that EC have a larger effect on in vivo vascular activity than undifferentiated ESC.

The ESC-EC and ESC-AEC were also compared in an angiogenesis Matrigel assay. Clear differences in angiogenic behavior between the two populations were observed. Specifically, the ESC-AEC demonstrated robust sprouting activity over several days while the ESC-EC only sprouted for 12-24 hours and then regressed by 48 hours. EC tube regression is a common occurrence in many in vivo and vitro assays [8], but further investigation was performed to elucidate the reason for the pronounced difference in sprouting behavior between two ESC-EC types.

As mentioned, flow cytometry analysis of ESC-EC and ESC-AEC shows that both populations contain a minimal amount of SMC contamination. Since the expression levels were virtually identical, this clearly was not the reason for the angiogenic differences. RT-PCR analysis was also performed to determine if ESC-AEC produce a high level of matrix metalloprotienases (MMPs), which would facilitate migration and sprouting [14]. In this assay as well, both ESC-AEC and ESC-EC produce similar levels of MMPs.

Fluorescent analysis of the cytoskeleton structure was performed to determine if actin organization was responsible for the different behavior between the two cell populations. It was apparent that the actin cytoskeletons of the ESC-AEC were more organized, forming extensive networks with neighboring cells, which the ESC-EC lacked. A key factor in actin organization related to EC migration and angiogenesis is the phosphorylation of HSP27, specifically at serine 82 [15, 16]. Flow cytometry analysis revealed that ESC-AEC exhibit robust phophorylation of HSP27 while ESC-EC expressed virtually no HSP phosphorylation.

5.7 Human stem cell derivation

Although generating a chemically defined differentiation scheme for murine cells can shed light on EC differentiation in general, the real potential lies in the implementation of a similar scheme for human ESC. There has been a recent study using a serum-free differentiation system for EC [17]. We have also conducted our own testing of our

serum free differentiation scheme on H9 human embryonic stem cells (hESC). Thus far, we have shown that hESC can be differentiated into EC using our medium formulations and differentiation system. The resulting EC population expressed moderate to high levels of pan-endothelial markers and can form sprouts on Matrigel (Figure 5.1).

In contrast to their murine counterparts, the EC generated from hESC expressed high levels of ephrin-B2, an arterial lineage marker and low levels of EphB4, the venous lineage marker. This was highly unusual since the venous lineage is considered to be the default differentiation pathway for EC generated in static conditions [18]. An external stimulus like shear stress or a chemical stimulus, like cAMP, is generally needed for arterial lineage selection [18, 19].

5.8 Future direction

It has been well established that the differentiation kinetics of various cell lines differ from each other [13, 20]. Identifying the optimal conditions for EC derivations across ESC lines is a tremendous undertaking. Our laboratory is continuing the optimization process in several mESC and hESC lines in order to generate high quality EC and to determine similarities and critical differences amongst the cell lines.

EC derived in chemically-defined conditions have many potential therapeutic and basic science applications. One application that is currently being developed in our laboratory is the production of a pre-vascularized cardiac patch. Prevascularizing a tissue

transplant would potentially eliminate the size restriction since the graft can inosculate with the host vasculature and thereby reduce healing time dramatically [21].

An additional application for derived EC could be in seeding tissue engineered vascular grafts. Currently, research into the optimal biomaterial or natural materials attempt to develop grafts that combine structural stability with biocompatibility. Regardless of the graft type used, generating functional aortic EC to seed the grafts is of critical importance in reducing thrombogenicity often associated with small diameter vascular grafts.

Although not directly related to EC differentiation, the model of ESC adhesion we generated can be applied to EC and SMC in co-culture conditions to determine adhesion properties. It can also be used to investigate various cell-substrate pairings to generate conductive and inductive scaffolding materials [22, 23].

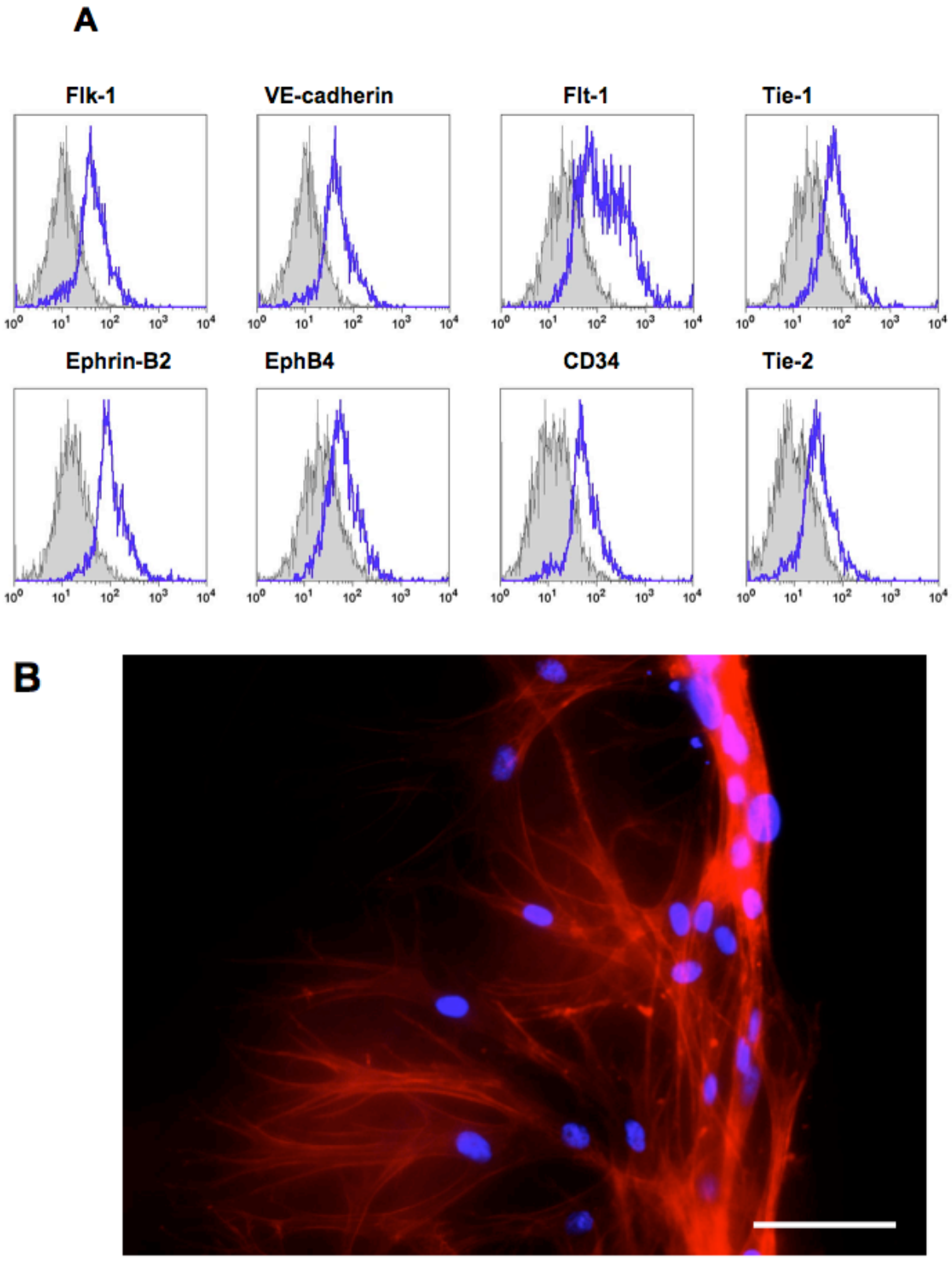

A
Flk-1
VE-cadherin
Flt-1
Tie-1
Ephrin-B2
EphB4
CD34
Tie-2
B

Figure 5.1: Endothelial cells derived from human H9 ESC in chemically defined conditions. (A) Expression of various EC markers. Only an initial Flk-1+ enrichment was performed, followed by expansion. Although derived in static conditions, the cells express high levels of ephrin-B2, an arterial marker. (B) EC derived from human EC are capable of forming sprouts in Matrigel. Phalloidin actin stain = red. DAPI nuclear stain = blue. Scale bar = 50 microns.

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