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THE EFFECTS OF ANGIOGENIC GROWTH FACTORS ON MESENCHYMAL STEM CELL PROLIFERATION AND DIFFERENTIATION TO VASCULAR CELL FATES

by

William Anthony D’Angelo

A Thesis Submitted to the Graduate School of The University of Southern Mississippi in Partial Fulfillment of the Requirements for the Degree of Master of Science

Approved:

Dean of the Graduate School

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ABSTRACT

THE EFFECTS OF ANGIOGENIC GROWTH FACTORS ON MESENCHYMAL STEM CELL PROLIFERATION AND DIFFERENTIATION TO VASCULAR CELL FATES

by William D’Angelo

May 2013

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into several cell lineages, including mural cells, which surround and support blood vessels, and possibly endothelial cells, which form the blood vessel walls. In this study, we investigated the effects of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), two of the best-characterized angiogenic factors, on MSC proliferation and differentiation. We hypothesized that treatment with these two factors could inhibit mural cell character and promote differentiation toward an endothelial cell fate. C3H/10T1/2 cells (a line of MSCs derived from mouse embryonic tissues) were treated with bFGF and VEGF, either alone or in combination, over a 9-day course. The effects on cell proliferation and cell type-specific marker expression were determined by cell cycle analysis, quantitative real-time PCR (RT-qPCR) analysis, and flow cytometry. bFGF significantly stimulated MSC proliferation and inhibited expression of mural cell markers, with no apparent effect on endothelial marker expression. VEGF alone or in combination with bFGF had no significant effects on expression of mural cell or endothelial cell differentiation markers. We conclude that these angiogenic factors, although critical in maintaining the properties of endothelial cells, are not sufficient to promote C3H/10T1/2 cell differentiation to endothelial cells.
TABLE OF CONTENTS

ABSTRACT..............................................................................................................................................ii
LIST OF TABLES......................................................................................................................................iv
LIST OF ILLUSTRATIONS......................................................................................................................v

CHAPTER

I. INTRODUCTION..................................................................................................................................1

Vascular Development
Angiogenic Factors
Mesenchymal Stem Cells (MSC)

II. OBJECTIVE AND SIGNIFICANCE.......................................................................................................18

III. METHODS.......................................................................................................................................20

Mesenchymal Stem Cell Culture
HoxB5 Stable Transfection
Spectrophotometric Analysis of Cell Number
RNA Extraction
Reverse Transcription
Real-time Semi-quantitative Polymerase Chain Reaction (RT-qPCR)
Flow Cytometry Analysis of Cell Cycle and Protein Expression

IV. RESULTS.........................................................................................................................................24

bFGF Stimulates Proliferation
bFGF Inhibits Mural Differentiation
VEGF Treatment has no Significant Effects on Proliferation or Differentiation
Treatment with bFGF+VEGF did not Stimulate Endothelial Differentiation
HoxB5 transfection did not Induce Flk1 Expression or Endothelial Differentiation

V. DISCUSSION.....................................................................................................................................34

REFERENCES.........................................................................................................................................38
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cycling Protocol for RT-qPCR</td>
<td>22</td>
</tr>
<tr>
<td>2.</td>
<td>Sequences of Gene-specific Primers</td>
<td>23</td>
</tr>
</tbody>
</table>
LIST OF ILLUSTRATIONS

Figure

1. Angiogenic Process and Proposed Role of Mesenchymal Stem Cells..............3
2. Signaling Pathways Activated by bFGF......................................................8
3. Signaling Pathways Activated by VEGF.....................................................10
4. Typical Differentiation Potential of MSCs...............................................13
5. Spectrophotometric Analysis of Cell Proliferation....................................24
6. Cell Cycle Analysis by Flow Cytometry....................................................25
7. Analysis of Cyclin D1 mRNA Levels........................................................26
8. Analysis of Mural Cell Markers at mRNA Level.........................................26
9. Analysis of SMA Protein Levels...............................................................27
10. Effects of bFGF on Endothelial Marker Expression..................................28
11. Effects of VEGF on Proliferation............................................................28
12. Effects of VEGF on Endothelial Marker Expression..................................29
13. Effects of Combined Treatment with bFGF and VEGF on Differentiation Marker Expression.................................................................29
14. C3H/10T1/2 Cell Morphology..................................................................30
15. Effects of Growth Factor Treatment on Flk1 Expression..........................30
16. Analysis of Flk1 Protein Levels................................................................31
17. Hoxb5 and Flk1 Expression in Naïve and HoxB5-Transfected Cells...........32
18. Effects of Growth Factor Treatment on HoxB5-Transfected Cells.............32
19. Hoxb5 and Flk1 Expression in Naïve and Hoxb5-Transfected D3 Embryonic Stem Cells.................................................................33
CHAPTER I
INTRODUCTION
Vascular Development

Vessel Structure

The mammalian vasculature is an intricate organ that supplies all cells of the body with necessary oxygen and nutrients and is able to sense and respond to dynamic tissue needs. Blood vessels are composed of three main parts: a layer of endothelial cells that makes up the luminal surface of the vessel; a layer of mural cells, which surround the vessel and function in regulating vessel diameter to control blood flow, stabilizing contacts between endothelial cells, and secretion of extracellular matrix components; and a basement membrane, a layer of collagen and other connective tissue proteins that provides structural support and participates in signaling with endothelial and mural cells.

In large vessels, vascular smooth muscle cells (vSMCs) make up the mural cell component and are found in a continuous abluminal sheath separated from the vessel endothelium by the basement membrane. In capillaries, the microvessels where gas and nutrient exchange occurs, the mural cells are pericytes, which are embedded within the basement membrane and make focal contacts with multiple endothelial cells. Pericyte coverage of capillaries is discontinuous and variable, reportedly ranging between 10% and 50% depending on the tissue (Shepro & Morel, 1993). Pericytes are positioned on the vessel to minimally inhibit gas and nutrient exchange with the tissue, and their variable abundance probably reflects different functional requirements of capillary beds in different tissues (Gerhardt & Betsholtz, 2003).
Vasculogenesis and Angiogenesis

Vascular development proceeds through two related processes: vasculogenesis, the initial, de novo formation of vessels; and angiogenesis, the sprouting of new vessels outward from the walls of already-formed vessels. Vasculogenesis occurs mainly during embryonic development, when multipotent vascular progenitor cells (hemangioblasts) in the yolk-sac differentiate to so-called blood islands containing endothelial and hematopoietic precursors. As these precursors differentiate, blood islands coalesce and organize into the vessels of the primary vascular plexus.

After the primary vascular plexus is formed by vasculogenesis, further vascular development proceeds via the process of angiogenesis, as new vessels sprout and grow to fill out the primitive vasculature, which is then subjected to pruning and remodeling. Unlike vasculogenesis, angiogenesis continues to some extent into adulthood, for instance during wound healing. Sprouting angiogenesis occurs when a growth factor signal (for example, vascular endothelial growth factor, VEGF) activates endothelial cells of an existing vessel wall, as illustrated in Figure 1. Before the new sprout can form, the basement membrane must be degraded to allow an opening for new cells to grow into. Activated endothelial cells begin to produce proteases that break down basement membrane proteins. If many or all of the endothelial cells in a given area of a vessel were to become activated and migratory, the extant vessel would be destroyed, interrupting local circulation. So to prevent vessel destruction and disorganized growth, a mechanism of lateral inhibition by Notch signaling selects a single endothelial cell to adopt the invasive tip cell phenotype and migrate toward the VEGF gradient (Gridley, 2007). As tip endothelial cells continue to migrate along the growth factor gradient, the
trailing stalk cells proliferate and form into a tube structure to make the walls of the new vessel. The final stage of vessel growth involves the recruitment of mural cells which surround the vessel, stabilize contacts between endothelial cells, and secrete a new basement membrane to result in a mature vessel.

Figure 1. Angiogenic process and proposed role of mesenchymal stem cells. (a) Endothelial cells are activated and begin migrating toward a growth factor gradient. (b) A specialized tip cell leads the vessel sprout while trailing stalk cells proliferate to form new vessel walls. A primitive vascular network is formed (c), followed by recruitment of mural cells to stabilize the vessel (d), possibly differentiated from mesenchymal stem cells (MSCs). Alternatively, MSCs may differentiate to endothelial cells and incorporate directly into the vessel walls (figure adapted from Wood, Kamm, & Asada, 2011).

Roles of Mural Cells in the Vasculature

While endothelial cell behavior has been extensively studied for many years, mural cells have received comparatively little attention. However, these cells have several important roles in angiogenesis and vessel maintenance and function. Several studies have shown that pericyte investment of new vessels is concurrent with vessel maturity (von Tell, Armulik, & Betsholtz, 2006) and is apparently necessary for vessel stability, as animal models with faulty pericyte recruitment show vessel regression and defects in circulation (Hall, 2006). The chronically inflamed, malformed, and leaky
vasculature characteristic of tumors is often marked by a decrease in pericyte coverage (Hall, 2006), and pericyte loss is one of the early events in diabetic retinopathy (Hammes, 2005).

The presence of pericytes is thought to stabilize vessels by several different mechanisms. One method of pericyte/endothelium interaction is through paracrine signaling. For example, knockout studies have shown that PDGF-B production by endothelial cells and PDGFR-β expression by pericytes are required for pericyte recruitment to new vessels (Enge et al., 2002), and TGF-β production by vessel-associated pericytes is thought to induce differentiation of endothelial cells and maintain their quiescence (Armulik, Abramsson, & Betsholtz, 2005). The angiopoietin/Tie-2 signaling loop between pericytes and endothelial cells is also involved in vessel maturation and stability, as evidenced by embryonic death due to cardiovascular failure in animals where this pathway is inactivated (Suri et al., 1996).

Direct cell-cell contacts are another important mechanism for proper vessel maintenance and function. Peg-and-socket junctions between endothelial cells and pericytes are characterized by the presence of the cell-cell adhesion protein N-cadherin. Administration of N-cadherin-blocking antibodies results in severe vascular defects characterized by inefficient pericyte-endothelial contact, and interestingly, disrupted endothelial-endothelial contacts (Gerhardt, Wolburg, & Redies, 2000). It seems that N-cadherin signaling upregulates endothelial expression VE-cadherin (the major cell adhesion molecules between endothelial cells)—thus pericyte-endothelial contacts directly promote stable connections between endothelial cells to maintain vessel integrity (von Tell et al., 2006).
Pericytes also have indirect effects on vascular stability through their secretion of basement membrane components. Interactions between endothelial cells and extracellular matrix proteins are largely mediated through integrin signaling. Integrins are cell-surface receptors that can bind matrix components such as collagens, laminins, and fibronectin. Integrins have an intracellular domain that can interact with several signaling proteins in the cytoplasm, thus signaling to the cell information about the extracellular environment. Because different combinations of integrin α and β subunits can bind different matrix substrates, the sum of the integrin signals that a cell receives allows it to sense its surroundings in detail. The importance of these signals in vessel stability and endothelial quiescence has been shown in many studies (Stratman & Davis, 2012).

Angiogenic Factors

Although many growth factors, cytokines, and other regulatory molecules are involved in the regulation of blood vessel formation and maintenance, bFGF and VEGF are by far the two best studied growth factors that are pivotal for vasculogenesis and angiogenesis. The following sections briefly review their model of action and signaling pathways that they activate.

**bFGF Signaling**

Basic fibroblast growth factor (bFGF) or FGF2 is a pleiotropic growth factor belonging to the fibroblast growth factor superfamily, which contains 22 members (in humans) organized into 7 subfamilies. bFGF was originally isolated from the bovine pituitary gland in 1974 and characterized as a potent mitogen for fibroblasts and an inducer of angiogenesis in vivo (Ornitz, 2000). The bFGF protein ranges from 18-34 kDa depending on the translation start site. High and low molecular weight (HMW and LMW)
isoforms are reported to localize to different intracellular compartments and may lead to different physiological effects (Liao et al., 2009; Reiland & Rapraeger, 1993; Dow & White, 2000). While HMW bFGF is thought to localize mainly to the nucleus, LMW bFGF is found in both the cytosol and the nucleus, as well as in the extracellular matrix (Liao et al., 2009).

There are four high-affinity cell surface FGF receptors (FGFR1-4), which belong to the receptor tyrosine kinase (RTK) family. FGF receptors are subject to alternative splicing to generate different isoforms, each with different ligand binding specificities, expression patterns, and physiological effects. These receptors share the same general structure, including two or three extracellular immunoglobulin-like (Ig-like) repeat domains which determine ligand-binding specificity, an acid box region, a heparin binding motif, a single transmembrane domain, and an intracellular split tyrosine kinase domain near the C-terminal end (Eswarakumar, Lax, & Schlessinger, 2005).

Ligand binding induces receptor dimerization and autophosphorylation of seven tyrosine residues in the intracellular tyrosine kinase domain, resulting in its activation (Vecchione et al., 2007). Signaling pathways activated downstream of the receptor are shown in Figure 2. Phosphorylated tyrosine residues of the receptor serve as binding sites for downstream signal proteins, including the adapter protein FRS2α, which recruits Grb2 and Shp2 via their SH2 domains. The FRS2α/Grb2/Shp2 complex recruits SOS, which then activates Ras, leading to downstream activation of the MAPK pathways ERK1/2, JNK, and p38. Grb2 can also complex with Gab1, which then recruits PI3K to activate the Akt pathway. Phospholipase C-gamma (PLC-γ) can also bind to the activated FGFR, leading to DAG and IP₃ production, intracellular Ca²⁺ release, and PKC activation
(Eswarakumar et al., 2005). Activation of these pathways can alter gene expression to regulate different cellular events, such as cell proliferation and differentiation.

In addition to FGFRs, bFGF is also known to bind heparin and heparan sulfate proteoglycans (HSPGs) such as perlecan and syndecan. HSPGs are found on the surface of most cells and are major components of the extracellular matrix. Evidence suggests that these ECM proteins are essential for efficient receptor activation, as cells that cannot produce HSPGs require exogenously added heparin for FGF signaling to occur (Omitz et al., 1992). It is thought that HSPGs facilitate signaling by stabilizing transient binding between a single FGF molecule and receptor long enough for receptor dimerization to form an active signaling complex (Omitz, 2000).

Besides their direct role in receptor activation, HSPGs also sequester secreted bFGF in the basement membrane by preventing its diffusion and protecting from thermal degradation and proteolysis (Omitz, 2000). The presence of bFGF in the basement membrane has implications for angiogenesis: as activated endothelial cells secrete proteases to degrade the basement membrane, bFGF (as well as other matrix-bound growth factors) is released to regulate endothelial cell and mural cell behaviors.
Figure 2. Signaling pathways activated by bFGF. Ligand binding is facilitated by extracellular heparan sulfate proteoglycans (HSPGs) and induces receptor dimerization, autophosphorylation, and the assembly of a functional intracellular signaling complex that can activate several downstream signaling pathways, including PLC-γ/PKC, PI3K/Akt, and the MAPK pathways (figure from Lanner & Rossant, 2010), which coordinately regulate different cellular events.

**VEGF Signaling**

Vascular endothelial growth factor (VEGF) is a family of growth factors with major roles in angiogenesis. VEGF-A is the best characterized, but five related proteins have been identified (VEGF-B, C, D, and E, as well as placenta growth factor, PlGF). VEGF-A is a well-characterized inducer of angiogenic sprouting and endothelial cell proliferation, migration, and survival (Greenberg, 2008). VEGF-A is crucial for proper vasculogenesis; the loss of even a single VEGF-A allele results vascular malformation and embryonic death (Carmeliet et al., 1996). Additionally, three transmembrane receptor tyrosine kinases (VEGFR1-3) have been described. While VEGF-A binds to VEGFR-1 and -2, VEGFR-2 is responsible for most of the angiogenic effects of VEGF signaling (Ball, Shuttleworth, & Kielty, 2007). VEGFR-1 may serve as a decoy or sink for VEGF-A (Fong, Rossant, Gertsenstein, & Breitman, 2002).
VEGF receptors function similarly to other RTKs: ligand binding induces receptor dimerization and autophosphorylation of tyrosine residues in the cytoplasmic domain, followed by endocytosis of the receptor/ligand complex into a signaling endosome (Matsumoto & Mugishima, 2006). The downstream signaling pathways in endothelial cells have been well-characterized (Fig. 3). Phosphorylated tyrosines serve as binding sites for PLC-γ, Grb2, and Shb. PLC-γ activation leads to production of DAG and IP3, which leads to intracellular Ca2+ release and PKC activation. PKC activation leads to activation of the MEK/ERK pathway via Raf and stimulates cell proliferation. Binding of Grb2 and Shb adapter proteins to activated VEGFRs leads to downstream p38 and PI3K activation, cytoskeletal reorganization, and migration (Matsumoto & Mugishima, 2006). Additionally a complex composed of VEGF-A/VEGFR2 with VE-cadherin, β-catenin, and PI3K is essential for endothelial cell survival (Carmeliet et al., 1999).

VEGF is produced by cells in response to hypoxia, or low levels of oxygen, via a mechanism involving the transcription factor hypoxia-inducible factor-1 (HIF-1). At normal oxygen levels, the α subunit of HIF-1 is quickly hydroxylated, which flags it for ubiquitination and degradation by the proteasome. But the enzyme that hydroxylates HIF-1α cannot function under hypoxic conditions, resulting in increased stability of HIF-1α, which forms a dimer with the constitutively produced β subunit and binds to promoters of responsive genes, including the VEGF gene, stimulating its transcription and leading to angiogenesis (Neufeld, Cohen, Gengrinovitch, & Poltorak, 1999).

Mesenchymal Stem Cells (MSCs)

Stem Cells

The term, stem cell, is applied to cells with two main characteristics: self-renewal and multipotency. Self-renewal refers to a cell’s ability to reproduce itself without differentiation, while multipotency is the ability to differentiate into multiple cell types when induced. There are several different types of cells that fall under the category of stem cells, and each type has its own unique properties, such as characteristic differentiation capacity, tissue of origin, marker expression profiles, morphology, and cell
cycle control mechanisms. Stem cells can be classified into two broad types, embryonic stem cells and adult stem cells. These categories are briefly discussed below.

Embryonic stem cells (ESCs) are cells derived from the inner cell mass (ICM) of a blastocyst, an early stage of embryonic development. ESCs are pluripotent, or able to differentiate into all cell types from all three germ layers (ectoderm, mesoderm, endoderm). The discovery and isolation of ESCs has generated tremendous excitement over their potential use in regenerative medicine. However, several barriers to clinical implementation remain, including ethical concerns about the destruction of embryos during harvest and questions about the purity, tumorigenic potential, and immunogenicity of ESC-derived cells. For these reasons, the greatest contributions of ESCs to date have been in the field of basic research, such as cell and developmental biology (Wobus & Boheler, 2005).

Self-renewing and multipotent cells that are derived from post-blastocyst tissues are collectively called adult stem cells. Hematopoietic stem cells, which can differentiate into all blood cell types, were the first to be discovered when they were isolated from bone marrow aspirates (Till & McCulloch, 1961). Since that time, stem cells have been isolated from a wide variety of postnatal tissues, such as the brain, skeletal muscle, cardiac muscle, intestine, skin, dental pulp, and fat. It seems that most if not all tissues and organs contain rare, relatively undifferentiated progenitor cells that are normally quiescent but can differentiate to mature cells of that tissue when induced. For instance, neural stem cells give rise to neurons and glial cells in the central nervous system, and stem cells in the skin and intestines differentiate to replace epithelial cells that are continuously ageing. Additionally, recent findings suggest that some adult stem cells
have a higher plasticity of differentiation potential than was originally appreciated. For example, mesenchymal stem cells have been reported to differentiate into non-mesodermal lineages such as neurons (mesoderm to ectoderm) (Jiang et al., 2002), and neural stem cells have been reported to differentiate to hematopoietic progenitors (ectoderm to mesoderm) (Bjornson, Rietze, Reynolds, Magli, & Vescovi, 1999).

**Mesenchymal Stem Cells**

Mesenchymal stem cells (MSCs) are multipotent cells that are characterized by their ability to differentiate into cells of various mesodermal tissues, such as bone, cartilage, and fat. Physiologically, MSCs are thought to serve as a reservoir for the replenishment of injured or diseased mesodermal cells (Minguell, Erices, & Conget, 2010). Other possible roles include maintenance of microenvironment via production of growth factors, cytokines, and extracellular maintenance components, and regulation of immune cell function (Uccelli, Moretta, & Pistoia, 2008). In vitro, they are easily cultured, maintained, and induced to differentiate to several functional phenotypes. For these reasons, MSCs have attracted attention for their potential roles in medicine and for their utility as tools for development and differentiation studies.
Figure 4. Typical differentiation potential of MSCs. The classic differentiation potential of MSCs includes chondrocytes, osteocytes, and adipocytes. In addition, MSCs from various sources have also been induced to differentiate to myocytes, astrocytes, mural cells, fibroblasts, and endothelial cells (figure from Grassel 2007).

MSCs may not comprise a single homogeneous population, and cells with the hallmarks of MSCs have been isolated from different tissues and developmental stages. Similar populations have been variously described as fibroblast colony forming units (Castro-Malaspina et al., 1980), mesenchymal stem cells, bone marrow stem cells, marrow stromal cells, mesenchymal adult progenitor cells, and marrow-isolated adult multilineage inducible cells (Minguell et al., 2010).

In vitro, MSCs are plastic-adherent and form proliferative colonies. Cells have a fibroblast-like morphology with long thin cytoplasmic processes (Pinney & Emerson, 1989), and are sensitive to post-confluence inhibition of growth (Reznikoff, Brankow, & Heidelberger, 1973). Many profiles of molecular marker expression have been described, and it seems not all MSCs display all the markers all the time. However, as part of the minimum criteria for MSCs proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, MSCs should express
CD105, CD73, and CD90, and should not express CD45, CD34, CD14, CD11b, CD79α, CD19, or HLA-DR (Dominici et al., 2006). MSCs have been classically described as able to differentiate to osteoblasts, chondrocytes, and adipocytes (Pinney & Emerson, 1989). In addition, some papers report differentiation to smooth muscle/mural cells, skeletal muscle, cardiac muscle, endothelial cells, and neural cells (Minguell et al., 2010; Wang, et al., 2010; Woodbury, Schwarz, Prockop, & Black, 2000). MSCs are distinct from hematopoietic stem cells (HSCs) and thus do not differentiate into blood cells.

MSC isolation is usually achieved using either the colony-forming unit-fibroblast (CFU-f) assay or by fluorescence-activated cell sorting (FACS). First, tissue samples (often bone marrow aspirates) are separated using density gradient centrifugation. Cells at the plasma-solution interface are collected and then subjected to one of the above assays. The CFU-f assay involves plating of the primary cells in a tissue culture dish and washing away non-adherent cells to obtain adherent, colony-forming cells with a fibroblast-like morphology. These cells typically display accepted MSC markers and trilineage potential (Oswald, 2004). For isolation by FACS, primary cells are incubated with fluorophore-tagged antibodies that bind to consensus MSC markers, and then a flow cytometer is used to separate cells with the desired marker expression profile. For instance, Crisan et al. used this technique to sort CD146+/CD34- CD45- CD56- perivascular cells from several tissues. The sorted cells showed characteristic MSC morphology and differentiation potential (Crisan et al., 2008).

Differences in tissue source, developmental stage, and reported molecular marker expression profile, as well as variation in isolation, culture, and differentiation protocols, have led to ambiguity about the in vivo origin and identity of MSCs. That putative MSCs
have been isolated from many different vascularized tissues may be evidence of a perivascular origin (Caplan, 2008). A study by Crisan et al. characterized a population of perivascular cells in the microvasculatures of various tissues that displayed canonical MSC markers and tri-lineage differentiation capability, which implies that pericytes and MSCs are closely related, if not equivalent (Crisan, et al., 2008). It is possible that MSCs/pericytes play different physiological roles under different conditions. In stable adult tissues, these cells may function as pericytes, maintaining integrity and homeostasis in blood vessel walls. During early development or after tissue damage, the same cells might be induced to activate more MSC-associated functions, such as migration to an area and subsequent differentiation to a required cell type, deposition of extracellular matrix (ECM) components, or regulation/modulation of the behavior of other cells through the release of paracrine factors (Caplan, 2008).

**C3H/10T1/2 Cells**

C3H/10T1/2 cells are a clonal cell line derived from 14-17 day C3H mouse embryos by Reznikoff et al. (1973). Although the tissue of origin is unknown, 10T1/2 cells display the characteristic marker expression and differentiation potential of typical mesenchymal stem cells. In vitro, they are plastic-adherent, sensitive to contact inhibition, and display a fibroblast-like morphology when subconfluent. In confluent cultures they take on a more rounded appearance, and cultures have a characteristic cobblestone appearance. Their genome is hypertetraploid for mice, with a chromosome number of 81 (normal diploid number is 40). They are non-tumorigenic when injected into mice, and do not express C-type retroviruses (Reznikoff et al., 1973; Pinney & Emerson, 1989).
C3H/10T1/2 cells have been differentiated to several cell types: most commonly the three MSC hallmark lineages of adipocytes, chondrocytes, and osteocytes (Pinney & Emerson, 1989), but also to skeletal muscle (Kubo, 1991) and pericytes/smooth muscle cells (Proweller, Pear, & Parmacek, 2005). C3H/10T1/2 cells express smooth muscle/pericyte markers, and have been used in coculture studies of angiogenesis to model mural cells (Ding, Darland, Parmacek, & D'Amore, 2004). Additionally, these cells have been reported to differentiate to endothelial cells induced by shear stress (Wang et al., 2005) or angiogenic factors (Wang et al., 2010) as induction stimuli.

**Effects of Angiogenic Factors on MSCs**

**Basic fibroblast growth factor.** bFGF has been shown to stimulate proliferation in several cell types, including endothelial cells and MSCs (Martin, 1997; Tsutsumi, 2001; Ramasamy, et al., 2012). In oligodendrocyte precursor cells, bFGF was found to stimulate proliferation by promoting cyclin D1 transcription by MAPK pathway activation, and by suppressing levels of p27, a CDK inhibitor (Frederick, Min, Altieri, Mitchell, & Wood, 2007; Frederick & Wood, 2004; Li & DiCicco-Bloom, 2004). c-Jun pathway activation by activated FGFR is important for cyclin D1 induction (Reilly & Maher, 2001). Several studies have found that MSCs do not lose their differentiation potential after bFGF-induced proliferation (Lee, 2012; Tsutsumi, 2001) and one study found that osteogenic differentiation was actually increased after bFGF-induced expansion compared with untreated controls (Martin, 1997), suggesting that bFGF may prime cells for subsequent differentiation.

bFGF has also been shown to inhibit mural differentiation of MSCs. Papetti, Shujath, Riley, & Herman (2003) showed that TGF-β-mediated expression of SMC genes
in retinal pericytes is interrupted by treatment with bFGF. Another group found similar results in C3H/10T1/2 cells, and reported that bFGF-mediated suppression of SMC genes was dependent on MEK/ERK pathway activation (Kawai-Kowase, 2004).

Aside from its effects on MSC proliferation and differentiation, bFGF can also control migration of MSCs. Schmidt et al. (2006) found that bFGF at low doses was attractant for bone marrow-derived MSCs, while at high doses MSCs were repelled. They also found that these behaviors were mediated through Akt/PKB pathway activation (Schmidt et al., 2006).

**Vascular endothelial growth factor.** VEGF has been reported to induce endothelial differentiation of bone marrow MSCs (Oswald, 2004) and osteogenic differentiation of dental pulp MSCs (D'Alimonte et al., 2011). It has also been reported that VEGF in combination with bFGF can stimulate C3H/10T1/2 cell endothelial differentiation (Wang et al., 2010). VEGF also stimulates proliferation in dental pulp MSCs (D'Alimonte et al., 2011) and bone marrow MSCs in a MEK/ERK dependent manner (Kong et al., 2010). Although MSCs do not express VEGF receptors (Wang et al., 2010; Ball et al., 2007), it has been shown that VEGF can stimulate PDGF receptors, which are expressed in MSCs (Ball et al., 2007). The contribution of the PDGF receptors to VEGF-mediated differentiation of MSCs is unknown.
CHAPTER II

OBJECTIVE AND SIGNIFICANCE

Mesenchymal stem cells have generated much excitement in the field of regenerative medicine. MSCs can differentiate to several lineages, and their tissue regenerative properties have been well-documented in animal models (Wakitani et al., 1994; Wakitani et al., 2007). They can home to sites of injury, and not only differentiate into required cell types but can also promote restoration of injured tissues by production of microenvironmental components (Haynesworth, Baber, & Caplan, 1996) and the suppression of inflammation (Uccell et al., 2008). MSCs can be isolated from a wide variety of sources, and they are easily cultured and expanded in vitro, raising the possibility of autologous transplantation (the use of a patient’s own stem cells to treat disease, reducing the risk of immune rejection). Although embryonic stem cells have a greater potential for differentiation than MSCs, ESCs are also harder to isolate and maintain in culture. Additionally, it has proven difficult to direct ESC differentiation to specific cell types and to obtain large quantities of pure ESC-derived cells, which makes MSCs an attractive alternative. Clarifying the potential for differentiation to various cell lineages and the mechanisms involved is necessary for establishing the generation of clinically useful MSC-derived cells.

Because pathological angiogenesis is a contributing factor to a wide range of diseases, a great amount of effort has been devoted to understanding the mechanisms of this process. However, the contribution of MSCs to angiogenesis is still incompletely characterized. The similarities between putative MSCs and mural cells have been shown in detail, and it seems likely that MSCs can take part in angiogenesis by differentiating to
mural cell phenotypes. But a few studies indicate that MSCs also have the potential to become endothelial cells and incorporate into new vessel walls. Growth factor signaling is a major regulator of cell behavior during angiogenesis. The objective of this study is to clarify the effects of angiogenic growth factors on C3H/10T1/2 cell differentiation to vascular cell types. Based on our preliminary observations of the individual effects of bFGF and VEGF, the current research proposes a more efficient method for the differentiation of C3H/10T1/2 cells to an endothelial lineage than that used by Wang et al. (2010). Optimizing this method would potentially provide a new source for efficient generation of endothelial cells and a model for the study of endothelial induction, and could shed light on the participation of MSCs in the angiogenic process.
CHAPTER III

METHODS

Mesenchymal Stem Cell Culture

The cells used in this study were C3H/10T1/2 cells, described by Reznikoff et al. (1973). Cells were cultured in 10% FCS MEMα in a 37°C humidified incubator with 5% CO₂ atmosphere. For differentiation experiments, cells were seeded to 6-well culture dishes at low density (~2000 cells/cm²) and growth factors (VEGF and bFGF, alone or in combination) were added to the medium at concentrations ranging from 5-10 ng/mL. Medium was refreshed every 3 days.

HoxB5 Stable Transfection

Cells were transfected with pcDNA3-HoxB5 plasmid using Lipofectamine (Invitrogen) transfection reagent. In a 1.5 mL microcentrifuge tube, 0.5 mL of Opti-MEM (Invitrogen) and 5 µg of pcDNA3 plasmid DNA were incubated for 5 minutes at room temperature. In another tube, 0.5 mL Opti-MEM and 20 µL Lipofectamine were also incubated for 5 minutes at room temperature. These two mixtures were combined and further incubated at room temperature for 25 minutes before being added to the cells. After 48 hours, G418 was added to the culture dish at 375 µg/mL to eliminate growth of untransfected cells. After two weeks, cells that survived were stably transfected, which was confirmed by RT-qPCR analysis for HoxB5.

Spectrophotometric Analysis of Cell Number

Treated C3H/10T1/2 cells were fixed with methanol for 15 minutes, then washed three times with PBS, followed by staining with toluidine blue (TB) for 30 minutes. Cells were rinsed with tap water to remove excess TB and allowed to air dry. The TB was
extracted from the cells by a 2% SDS wash. Optical density was measured with a Bio-Tek Instruments ELX 800 microplate reader and KCjunior software.

RNA Extraction

Total RNA was extracted using Tri-reagent (Sigma). Medium was aspirated from the culture dish and Tri-reagent was added, followed by 3 minutes of shaking. The sample was transferred to a 1.5 mL microcentrifuge tube and stored at -70°C. Chloroform (0.2 mL per mL of Tri-reagent) was added, then samples were vortexed for 15 seconds and incubated at room temperature for 10 minutes. Next, samples were centrifuged at 10000 rpm for 10 minutes at 4°C. The aqueous supernatant containing RNA was transferred to a new 1.5 mL microcentrifuge tube and an equal volume of isopropyl alcohol was added. After a 10 minute incubation at room temperature, samples were centrifuged at 12000 rpm for 15 minutes at 4°C to precipitate a pellet of RNA. The supernatant was aspirated and samples were washed with 0.7 mL cold 75% ethanol, vortexed, and placed in -20°C for at least one hour. Samples were then centrifuged at 10000 rpm for 10 minutes at 4°C, ethanol was removed, and RNA pellets were allowed to dry for 5 minutes on ice. Finally, RNA was dissolved in 20 μL DEPC-water, RNA concentration was measured by a Spectronic Genesys 10 Bio spectrophotometer, and RNA integrity was checked by agarose gel electrophoresis.

Reverse Transcription

For each sample, 1 μg of RNA was mixed with dNTPs and a random primer, mixed, and held at 70°C in a thermal cycler for 5 minutes to melt secondary structure within the RNA template. Then 5x buffer (Fisher), M-MLV reverse transcriptase (Fisher), RNase inhibitor (Fisher) were added for a total volume of 20 μL per reaction
and samples were held at 42°C for a 1 hour extension step, followed by 10 minutes at 95°C to inactivate the reverse transcriptase enzyme. Resulting cDNA was diluted in 200 μL DEPC-water.

Real-Time Semi-Quantitative Polymerase Chain Reaction (RT-qPCR)

For each reaction, 10 μL of SYBR Green (Bio-Rad), 3 μL of nuclease-free water, and 5 μL of cDNA were mixed with 2 μL of gene-specific primers. Samples were run in a Stratagene MS3000P real-time PCR thermal cycler according to the protocol below (Table 1). Relative mRNA levels were calculated using the comparative Ct method \(2^{\Delta\Delta Ct}\). For all samples, β-actin was used as an internal reference control gene. Sequences of gene-specific primers are listed in Table 2.

Table 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature and Duration</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95°C for 3 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td></td>
<td>95°C for 15 seconds</td>
<td></td>
</tr>
<tr>
<td>Amplification</td>
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<td>35 cycles</td>
</tr>
<tr>
<td></td>
<td>72°C for 30 seconds</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>95°C for 45 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70°C for 30 seconds</td>
<td>1 cycle</td>
</tr>
<tr>
<td></td>
<td>95°C for 30 seconds</td>
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### Table 2

**Sequences of Gene-specific Primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>NM_008268</td>
<td>CCTGCACCTAAGCGC</td>
<td>TGGCCTCGTCTATTTTCG</td>
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<td></td>
<td>GACAG</td>
<td>GTGA</td>
</tr>
<tr>
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<td>NM_007392</td>
<td>GGACGTACAATCTGG</td>
<td>CGGCGAGTAGACGAGAG</td>
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<tr>
<td></td>
<td></td>
<td>TATTGTGC</td>
<td>GAAT</td>
</tr>
<tr>
<td>SM22α</td>
<td>NM_011526</td>
<td>AGGGATCGAAGGCA</td>
<td>ACTGCTGCGCATATCTCTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTGAAG</td>
<td>ACCT</td>
</tr>
<tr>
<td>PECAM1</td>
<td>NM_008816</td>
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<td>CTTCATCCACCGGGGCT</td>
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<tr>
<td></td>
<td></td>
<td>ACCACC</td>
<td>ATC</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>NM_009868</td>
<td>ATGAATCGCTGCC</td>
<td>CATTCCCTGTGTAGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CACTATG</td>
<td>TCGAC</td>
</tr>
<tr>
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<td>GCAAAACACTCACCC</td>
<td>GAGGTTTGAAATCGACC</td>
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<td></td>
<td></td>
<td>ATCCCACC</td>
<td>CTCG</td>
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<tr>
<td>mouse HoxB5</td>
<td>NM_008268</td>
<td>CCTGCACCTAAGCGC</td>
<td>TGGGCTCGTCTATATTTCG</td>
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<td></td>
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<td>GGAAGCGGGAAG</td>
<td>TATCATG</td>
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</table>

**Flow Cytometry Analysis of Cell Cycle and Protein Expression**

Treated C3H/10T1/2 cells were detached with trypsin, fixed with ethanol, blocked with 2% BSA, and incubated with primary antibody at 1:100 dilution for either 2 h at room temperature or overnight at 4°C, followed by incubation with the secondary antibody at 1:200 dilution for 1 h in the dark. Propidium iodide (PI, a DNA binding dye) was added during the second antibody incubation for analysis of cell cycle profiles. Samples were then analyzed using an Accuri C6 flow cytometer and Cflow software.
CHAPTER IV
RESULTS

bFGF Stimulates Cell Proliferation

As reported in other studies (Ramasamy et al., 2012; Tsutsumi, 2001), we found that treatment with bFGF stimulated proliferation of C3H/10T1/2 cells, as measured by three different methods. First, we used a spectrophotometer to measure the optical density of bFGF-treated and untreated cultures after staining with toluidine blue (Fig. 5). Increased optical density of the treated culture indicates a higher number of cells relative to untreated cells (Con).

![Figure 5](image_url)

*Figure 5.* Spectrophotometric analysis of cell proliferation. Cells were grown in 1% FBS MEM and treated with 10 ng/mL bFGF for 48h, then optical density was measured with a spectrophotometer after the cells were stained with TB. The values are means ± SD (n = 3, P < 0.05).

Next, we used flow cytometry to analyze the cell cycle profiles of treated and untreated cells (Fig. 6). By treating the cells with the DNA-binding fluorescent dye propidium iodide (PI), we measured the relative amounts of DNA in cells from treatment and control groups. Cells in S and G2/M phases of the cell cycle have a higher DNA content than cells in G1 as they replicate their genomes in preparation for mitosis, and
thus will have higher fluorescence (farther to the right on the x-axis) as measured by flow cytometry. Figure 6 shows a higher proportion of cells in S and G2/M phases in the bFGF-treated group than in the control group, indicating increased proliferation in response to bFGF.

![Cell cycle analysis by flow cytometry](image)

Figure 6. Cell cycle analysis by flow cytometry. Cells were treated with 10 ng/mL bFGF for 48h, then stained with propidium iodide, and flow cytometry was used to measure fluorescence as an indicator of DNA content. The cell populations at different phases of the cell cycle are indicated by bars.

Finally, we measured the expression of cyclin D1 as an indicator of proliferation. Cyclin-dependent kinases (CDKs) and the cyclin proteins are major regulators that control cell cycle progression. CDKs and cyclins form complexes that phosphorylate numerous targets to allow progression through cell cycle checkpoints. While CDKs are relatively stably expressed, cyclins are only expressed at specific times in the cell cycle, which provides a mechanism to prevent uncontrolled cell growth. Thus, cyclin expression can serve as an indicator of active proliferation. As seen in figure 7, bFGF-
treated cells show increased cyclin D1 mRNA levels relative to controls, which is responsible for the increased cell proliferation.

![Graph showing cyclin D1 mRNA levels](image)

**Figure 7.** Analysis of cyclin D1 mRNA levels. Cells were treated with 10 ng/mL bFGF for 48h and collected for RT-qPCR. The values are means ± SD (n = 3, P < 0.05).

bFGF Inhibits Mural Differentiation

Consistent with other reports in the literature (Papetti et al., 2003; Kawai-Kowase, 2004), we found that bFGF strongly inhibits transcription of the mural cell marker genes smooth muscle α-actin (SMA) and SM22α (Fig. 8).

![Graph showing SMA and SM22α mRNA levels](image)

**Figure 8.** Analysis of mural cell markers at mRNA level. Cells were treated with 10 ng/mL bFGF for 72h and collected for RT-qPCR. The values are means ± SD (n = 3, P < 0.01).

We then used flow cytometry to verify the downregulation of SMA at the protein level. By incubating the cells with a primary antibody that binds to the SMA protein, and then incubating with a secondary antibody that binds the primary antibody and is
conjugated with a fluorophore (FITC), we fluorescently labeled the SMA protein in treated and untreated cells, then analyzed the intensity of fluorescence (which correlates with the expression level of SMA) in each group with a flow cytometer. As seen in figure 9, the bFGF-treated group had lower fluorescence than the control group (left-shift of bFGF-treated curve), confirming reduced expression of SMA at the protein level.

![Figure 9. Analysis of SMA protein levels. Cells were treated with 10 ng/mL bFGF for 48h, then fixed and incubated with SMA-specific antibodies and analyzed by flow cytometry.](image)

Next, we examined the expression of endothelial markers in response to bFGF treatment. Untreated C3H/10T1/2 cells have extremely low baseline expression levels of the endothelial markers PECAM-1 and VE-cadherin, and these levels were not significantly increased after 9 days of treatment with bFGF (Fig. 10).
Figure 10. Effects of bFGF on endothelial marker expression. Cells were treated with 5 ng/mL bFGF for 9 days and collected for RT-qPCR. The values are means ± SD (n = 3, P < 0.01).

VEGF Treatment has no Significant Effects on Proliferation or Differentiation

VEGF is a major growth factor regulator of angiogenesis, and has been reported to contribute to endothelial differentiation of MSCs in several studies (Oswald et al., 2004; Wang et al., 2010). According to our results, treatment with VEGF alone did not cause any significant effects on proliferation (Fig. 11) or expression of mural or endothelial differentiation markers (Fig. 12).

Figure 11. Effects of VEGF on proliferation. Cells were grown in 1% FBS MEM and treated with 10 ng/mL VEGF for 48h, then optical density was measured with a spectrophotometer after the cells were stained with TB. The values are means ± SD (n = 3, P < 0.05).
Figure 12. Effects of VEGF on endothelial marker expression. Cells were treated with 10 ng/mL VEGF for 9 days and collected for RT-qPCR. The values are means ± SD (n = 3, P < 0.01).

Treatment with bFGF+VEGF did not Stimulate Endothelial Differentiation

Following the protocol of Wang et al. (2010), we treated C3H/10T1/2 cells with a combination of bFGF and VEGF (5 ng/mL and 10 ng/mL, respectively) for 9 days. This treatment was reported to induce endothelial differentiation in this cell line, but our results show that the endothelial markers PECAM-1 and VE-cadherin are not significantly upregulated at the transcriptional (Fig. 13) or protein levels (not shown), and cells are morphologically similar to cells treated with bFGF alone (Fig. 14).

Figure 13. Effects of combined treatment with bFGF and VEGF on differentiation marker expression. Cells were treated with 5 ng/mL bFGF and 10 ng/mL VEGF for 9 days and collected for RT-qPCR. The values are means ± SD (n = 3, P < 0.01).
Figure 14. C3H/10T1/2 cell morphology. Left panel, untreated cells. Middle panel, cells treated with 10 ng/mL bFGF for 3 days. Right panel, cells treated with 5 ng/mL bFGF and 10 ng/mL VEGF for 3 days.

Because of the apparent low responsiveness to VEGF, we decided to examine the expression of Flk1, the primary receptor for VEGF. In untreated cells, Flk1 mRNA levels were negligible, and treatment with bFGF, VEGF, or a combination of both over a 9 day course did not significantly increase Flk1 transcription (Fig. 15) or protein levels (Fig. 16).

Figure 15. Effects of growth factor treatment on Flk1 expression. Cells were treated with 5 ng/mL bFGF, 10 ng/mL VEGF, or a combination of both for 9 days and collected for RT-qPCR. The values are means ± SD (n = 3, P < 0.01).
HoxB5 Transfection did not Induce Flk1 Expression or Endothelial Differentiation

In order to promote endothelial differentiation, we attempted to induce expression of Flk1 so that cells would be more responsive to VEGF. Toward this end, we transfected C3H cells with HoxB5, a transcription factor that has been shown to bind to an intronic enhancer region of the Flk1 gene and increase endothelial differentiation in HoxB5-transfected embryonic stem cells (Wu, Moser, Bautch, & Patterson, 2003). Although transfection was successful and HoxB5 was expressed at high levels, we did not observe a significant increase in Flk1 transcription (Fig. 17). HoxB5 transfected cells did show some upregulation of endothelial markers after growth factor treatment, but the increases were not statistically significant and absolute expression levels remained extremely low (Fig. 18).
Figure 17. HoxB5 and Flk1 expression in naïve and HoxB5-transfected cells. Inset: RT-PCR result after gel electrophoresis. (-) and (+) represent untransfected and transfected cells, respectively.

Figure 18. Effects of growth factor treatment on HoxB5-transfected cells. Cells were treated with 5 ng/mL bFGF, 10 ng/mL VEGF, or a combination of both for 9 days, then collected for RT-qPCR. The values are means ± SD (n = 3, P < 0.01).

Finally, to verify that the HoxB5 gene we transfected was functional, we transfected D3 embryonic stem cells and analyzed expression of HoxB5 and endothelial markers. As seen in Figure 19, HoxB5 transfection resulted in increased transcription of Flk1, which is consistent other reports (Wu et al., 2003).
Figure 19. HoxB5 and Flk1 expression in naïve and HoxB5-transfected D3 embryonic stem cells. Inset: RT-PCR result after gel electrophoresis. (-) and (+) represent untransfected and transfected cells, respectively.
The differentiation capacity of mesenchymal stem cells has not been fully elucidated. MSCs classically give rise to cartilage, fat, and bone-producing cells, but have also reportedly been differentiated to smooth and cardiac muscle cells, neurons, and endothelial cells (Pinney & Emerson, 1989; Minguell et al. Conget, 2010; Wang et al., 2010; Woodbury et al., 2000; Oswald et al. 2004). Endothelial cells typically develop from an early precursor cell type called a hemangioblast, which gives rise to blood cells as well as endothelial progenitor cells (Ferguson, Kelley, & Patterson, 2005). Although the ontogeny of MSCs remains unclear, they are distinct from both hematopoietic stem cells and endothelial progenitor cells, so reports of MSC differentiation to ECs deserve further attention and clarification.

Several papers report in vivo differentiation of MSCs to ECs (Davani, et al., 2003; Tang, et al., 2006), usually involving injection of DAPI-labeled MSCs into infarcted cardiac tissue, then performing immunohistochemistry to characterize the phenotype of labeled cells after several days or weeks. However, this experimental design does not allow analysis of the molecular mechanisms that lead to endothelial differentiation. There are a few reports of endothelial induction in vitro, including two that used the C3H/10T1/2 cell line (Wang et al., 2005; Wang et al., 2010). One study used shear stress as an induction stimulus (Wang et al., 2005). This result is not surprising, as shear forces have been shown to have strong effects on cell morphology, gene expression, and differentiation of endothelial progenitor cells (Yamamoto et al., 2003). Two studies reported endothelial induction after treatment with angiogenic growth factors. Oswald et
al. (2004) reported that human bone marrow-derived MSCs could be differentiated to endothelial cells after treatment with high doses of VEGF, and Wang et al. (2010) reported endothelial induction of C3H/10T1/2 cells using a combination of bFGF and VEGF. In the present study, we attempted to validate and expand on these reports by analyzing the effects of individual angiogenic factors on differentiation to vascular cell types.

We found that the two major effects of bFGF were to stimulate proliferation and to inhibit mural differentiation markers such as α-SMA and SM22α. These effects have been previously reported in C3H cells and other MSCs (Papetti et al. 2003; Kawai-Kowase, 2004), and are consistent with a role for bFGF as an inhibitor of mural differentiation. The endothelial markers PECAM-1 and VE-cadherin are not expressed in untreated C3H cells, and bFGF did not induce significant transcription of these genes.

Although VEGF is a major regulator of vascular development, and is used as a stimulus in almost all reports of endothelial differentiation of MSCs, we did not observe any significant effects on proliferation or transcription of mural or endothelial differentiation markers after 9 days of stimulation with VEGF. Because naïve C3H/10T1/2 cells express mural cell markers at relatively high levels—indeed, these cells are often used as de facto mural cells in coculture with endothelial cells for modeling angiogenesis—it is possible that VEGF is not able to easily overcome this inherent mural character. It was thought that the addition of bFGF to the protocol to inhibit mural cell markers and de-differentiate the cells might enhance VEGF-induced endothelial induction. Following the protocol of Wang et al. (2010), we treated cells with a combination of 5 ng/mL bFGF and 10 ng/mL VEGF for 9 days. The effects were
similar to treatment with bFGF alone: increased proliferation and decreased transcription of mural cell makers, with no induction of endothelial markers. Because the effects of bFGF seemed to predominate when both factors were used simultaneously, we also tried a step-wise treatment method, in which cells were pre-treated with bFGF for 48h before treatment with VEGF or a VEGF+bFGF combination, but we obtained results similar to those of simultaneous treatment (data not shown).

The apparent inability of these cells to respond to VEGF could be attributable to the lack of expression of Flk1 (a.k.a. VEGFR2), the major cell surface receptor for VEGF. Despite the use of VEGF in many studies on MSCs, most published expression profiles show that MSCs, including C3H/10T1/2 cells, do not express VEGF receptors. One paper reports that VEGF can bind to and activate the surface receptor for the closely related platelet-derived growth factor (PDGF), which is expressed in MSCs (Ball et al., 2007); however, the intracellular effects of this activation would presumably be identical to those of PDGF, which is not involved in endothelial differentiation.

To determine whether Flk1 is in fact the missing link in the capacity for endothelial differentiation of C3H cells, we attempted to induce Flk1 by expressing the transcription factor HoxB5. This protein has been reported to bind to an intronic enhancer region of the Flk1 gene and increase Flk1 expression in embryonic stem cells (Wu et al. 2003). Although we successfully transfected the cells with HoxB5 (Fig. 17), we did not observe any increase in Flk1 transcription after real-time PCR analysis. However, when PCR products were subjected to gel electrophoresis, a very faint band of the expected size for Flk1 amplicons was visible in transfected cells (Fig. 17, inset), indicating that some low-level induction did occur. It may be that the Flk1 gene in
C3H/10T1/2 cells is mostly unavailable for HoxB5 binding (or binding of other necessary transcription factors) due to epigenetic modifications such as DNA methylation or histone modifications. Consistent with this theory, we found that HoxB5 did increase Flk1 transcription in D3 ESCs, which are undifferentiated and thus should not be epigenetically restricted in terms of differentiation capacity. Cell reprogramming methods, such as those used to generate iPSCs (Takahashi & Yamanaka, 2006), generally involve the use of master regulatory transcription factors that reset genome-wide DNA methylation patterns, causing a reversion to an undifferentiated state. In this case, it seems that the change from MSC to endothelial lineage may be too drastic to be induced by our methods.

In conclusion, our results indicate that C3H/10T1/2 cells do not differentiate to endothelial cells in vitro in response to two prototypical angiogenic factors. It seems that this cell line is intrinsically closer to a mural cell phenotype. Simple stimulation with bFGF and VEGF is unable to cause transdifferentiation to the endothelial lineage, even in the cells expressing HoxB5, which upregulates Flk1 in embryonic stem cells. While C3H/10T1/2 cells theoretically have the potential to differentiate into endothelial cells, that will need a more rigorous reprogramming strategy that involves manipulating multiple transcription factors driving endothelial cell differentiation.
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