

**LEIOMYGENIC AND CARDIOMYGENIC DIFFERENTIATION POTENTIAL OF
HUMAN ADIPOSE-DERIVED STEM CELLS**

by

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Coronary artery disease is the leading cause of death in industrialized countries. Strategies to treat atherosclerotic coronary artery disease include coronary artery bypass grafting, which is often complicated by vein graft occlusion or stenosis. Severely occluded vein grafts can completely obstruct blood flow to the myocardium, resulting in a myocardial infarction, and eventually lead to heart failure. Heterotopic heart transplantation remains the treatment of choice for end-stage heart failure, however its widespread applicability is limited by the chronic shortage of donor organs.

The therapeutic potential of stem cells in cardiac repair following myocardial infarction has generated a great deal of interest. Many types of stem/progenitor cells including embryonic stem cells and bone marrow-derived mesenchymal stem cells (MSCs) have been used to regenerate the infarcted heart with promising results.

Adipose tissue is an abundant source of multipotent stem cells that can be easily obtained from liposuction waste tissue. The yield of stem cells per gram of fat is higher when compared with marrow-derived MSCs, making adipose tissue an attractive source of autologous stem cells for cardiovascular cell therapies. The goal of this research effort was to examine the differentiation potential of adipose-derived stem cells (ASCs) along the leiomyogenic and cardiomyogenic lineages.

ASCs were extracted from human subcutaneous adipose tissue from female donors during elective abdominoplasty, cultured in the presence of biomolecules responsible for vascular and cardiac development, and subjected to uniaxial cyclic strain in magnitudes comparable to the *in vivo* conditions. Protein and gene expression of smooth muscle- and cardiomyocyte-specific markers were assessed via immunocytochemistry, Western blot analysis, and RT-PCR. Our results indicated that uniaxial cyclic strain inhibited cell proliferation, resulted in alignment of ASCs perpendicular to the direction of strain, and down-regulated protein expression of early smooth muscle cell markers α -SMA and h₁-calponin. Transforming growth factor β -1 significantly up-regulated the expression of α -SMA and h₁-calponin in ASCs. Cardiac-specific proteins sarcomeric α -actinin, troponin-I, troponin-T were undetected in ASCs exposed to demethylation agent 5-azacytidine. Expression of cardiac transcription factors Nkx2.5 and GATA4 were also absent. These results suggest that human ASCs may not be capable of cardiomyogenic differentiation via exposure to 5-azacytidine.

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PREFACE

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1.0 INTRODUCTION

Stem cells have great potential for use in tissue repair and regeneration. There has been continuing and incrementing interest in both the biology and potential therapeutic applications of adult stem cells. Bone marrow-derived mesenchymal stem cells are the most extensively characterized stem cell population derived from an adult tissue, and have been shown to differentiate into a wide range of cell phenotypes, including fat, bone, cartilage, muscle, and neural progenitors. The recent discovery and characterization of multilineage cells from adipose tissue has been met with a great deal of excitement by the field of tissue engineering and regenerative medicine. Even though studies have shown that nascent stem cells exist in other adult tissues, including the brain, dermis, skeletal muscle, and vasculature, adipose tissue is the most abundant and accessible source of adult stem cells. The work of this thesis examines the potential of adipose-derived stem cells in cardiovascular repair and the differentiation capabilities of these cells along the smooth muscle and cardiac lineages.

1.1 GOALS AND CLINICAL MOTIVATION

Coronary artery disease is the leading cause of death in the United States and other industrialized countries. The high impact of its main sequelae, acute myocardial infarction and

congestive heart failure, on the quality of life of patients and the cost of health care worldwide drives the search for new therapies.

Current strategies to treat atherosclerotic coronary artery disease include coronary artery bypass grafting (CABG), in which autologous or synthetic grafts are used to bypass atherosclerotic vessels and restore blood flow to the ischemic myocardium. The grafts used include healthy arteries or veins harvested from a separate site, such as autologous saphenous vein and mammary artery grafts, or synthetic grafts made from Dacron or expanded polytetrafluoroethylene (ePTFE). Results with arterial grafts have been superior to venous grafts, however, suitable arterial grafts are scarce and harvest procedures add to morbidity and cost. Studies have shown that synthetic grafts can trigger immunologic reactions. Furthermore, autologous saphenous vein and mammary artery grafts often cannot be employed because of damage and pre-existing disease [1-2].

Complications from CABG include graft occlusion or stenosis [3], and 10-30% of patients suffer from thrombosis-related occlusions within the first month [4]. Furthermore, 95% of those patients require intervention within 10 years after the operation [4]. In fact, in patients who have had arterial or vein grafts, 40% of the grafts are severely obstructed 10 years after the initial procedure [5].

Severely occluded arteries or grafts can completely obstruct blood flow to the myocardium, leading to a myocardial infarction, accompanied by irreversible damage to the heart. Overtime, critical loss of cardiac tissue and impairment of left ventricular function can cause ineffective pumping of the heart, resulting in heart failure.

Congestive heart failure (CHF) is a major medical challenge, with 4.9 million patients in the United States currently suffering from its effects [6]. The economic cost associated with CHF

is estimated to be \$27.9 billion annually [6]. Current therapeutic strategies used to treat CHF include pharmaceutical intervention, short term mechanical cardiac support, and heart transplantation. As adult myocardium has low potential for repair and regeneration, the ability of pharmaceutical agents to improve cardiac function is limited as these reagents do not address the fundamental issue of cell loss [7].

Currently, heterotopic heart transplantation is the treatment of choice for end-stage heart failure. However, its application is severely limited by the availability of donor organs. Although the technique has been used for over 30 years, the long term outcome of patients is still complicated by problems of organ rejection, infection, and development of disorders caused by immunosuppressive regimens designed to modulate the rejection process [7]. Clearly, there is a need for improved treatment options.

The therapeutic potential of stem cells in cardiac repair following myocardial infarction has generated a great deal of interest. Many types of stem/progenitor cells including embryonic stem cells and bone marrow-derived mesenchymal stem cells (MSCs) have been used to regenerate the infarcted heart with promising results.

1.2 THESIS OVERVIEW

Adipose tissue is an abundant, accessible, and replenishable source of multipotent adult stem cells that can be easily obtained from discarded liposuction waste tissue. The yield of stem cells per gram of fat is much higher when compared with bone marrow-derived MSCs [26-27], making adipose tissue an attractive source of autologous stem cells for tissue engineering strategies and cell therapies. The goal of this research effort was to examine the leiomyogenic

and cardiomyogenic differentiation potential of human adult stem cells derived from adipose tissue. We hypothesized that human adipose-derived stem cells (ASCs) could be induced to express early smooth muscle and cardiac muscle markers via biochemical and mechanical modulation, and have potential use in cardiovascular cell therapies. The specific aims of this thesis are as follows:

1.2.1 Specific Aim 1: Examine the effects of β -mercaptoethanol, ascorbic acid, retinoid acids, dibutyl-cAMP, and transforming growth factor- β 1 on early smooth muscle protein expression in human ASCs.

Various combinations of bioactive molecules responsible for vascular development, including β -mercaptoethanol (BME), ascorbic acid (AA), retinoic acid (AA), dibutyl-cAMP (d-cAMP), and transforming growth factor- β ₁ (TGF- β ₁), were used to stimulate differentiation of ASCs into smooth muscle cells (SMCs). ASCs were extracted from superficial abdominal adipose tissue of young female donors undergoing elective abdominoplasty. Response of ASCs to various exogenous biochemical stimuli and smooth muscle cell differentiation of ASCs were characterized by changes in cell morphology and protein expression of early SMC markers α -smooth muscle actin (α -SMA) and calponin. We hypothesized that exposure to BME, AA, RA, d-cAMP, and TGF- β ₁ would result in up-regulation of α -SMA and calponin protein expression in human ASCs.

1.2.2 Specific Aim 2: Determine the effects of mechanical strain on ASC morphology, proliferation, and differentiation along the smooth muscle cell lineage.

The response of human ASCs to uniaxial cyclic strain and modulation of early smooth muscle protein expression (α -SMA and calponin) via mechanical stimulation was accessed in Aim 2. ASCs were subjected to 10% uniaxial cyclic strain for 7 days using a FlexcellTM FX-4000T tension strain unit (strain parameters were chosen to simulate *in vivo* conditions). An identical source of adipose tissue was used in this study as previously described in Aim 1. The synergistic effect of chemical and mechanical stimulation on ASCs utilizing the optimized biochemical conditions defined in Aim 1 was also examined. We hypothesized a reduction in cell proliferation, altered cell morphology/orientation, and up-regulation of α -SMA and calponin protein expression in human ASCs that were exposed to 10% uniaxial cyclic strain. We also expected amplified smooth muscle protein expression in ASCs that were stimulated with bioactive molecules in addition to strain.

1.2.3 Specific Aim 3: Define the effects of DNA-demethylator 5-azacytidine on early cardiac muscle protein and gene expression in human ASCs.

The potential of ASCs to differentiate into cardiomyocyte-like cells for myocardial repair via exposure to DNA-demethylation agent 5-azacytidine was investigated in Aim 3. ASCs were exposed to 5-azacytidine for specified time periods using repeated exposure and single transient exposure treatment methods. Expression of cardiomyogenic transcription factors Nkx2.5, GATA4, and contractile, cardiac-specific proteins such as sarcomeric α -actinin, troponin I,

troponin T, and gap junction protein connexin-43 was characterized. We hypothesized that exposure to 5-azacytidine could result in up-regulation of early cardiac-specific markers, and small populations of contractile cell clusters in human ASCs.

1.3 GUIDE TO REMAINING CHAPTERS

Chapter 2: Background information on coronary artery disease, myocardial infarction, congestive heart failure, current and alternative treatment options, adult stem cells, stem cell therapies for cardiovascular repair/regeneration, adipose-derived stem cells – characteristics and differentiation potential.

Chapter 3: Methodologies used to extract stem cell population from adipose tissue, design of chemical and mechanical stimulation experiments, molecular biology and tissue engineering techniques used to evaluate cell response to biochemical and mechanical stimuli.

Chapter 4: Results obtained from treating adipose-derived stem cells with an array of bioactive molecules reported to be significant in regulating the phenotype of cells of the vasculature, from exposing adipose-derived stem cells to uniaxial cyclic strain (in magnitudes similar to what vascular smooth muscle cells are subjected to *in vivo*), from culturing the stem cells in the presence of a DNA-demethylation reagent 5-azacytidine, are presented in this chapter.

Chapter 5: Discussion of results presented in the previous chapter; conclusion and future directions.

2.0 BACKGROUND

2.1 CORONARY ARTERY DISEASE

Despite recent progress in prevention and treatment, cardiovascular disease remains the leading cause of morbidity and mortality in industrialized countries. Coronary artery disease (CAD) is the most common form of cardiovascular disease, and accounts for over 900,000 deaths in the United States annually among both men and women in major ethnic groups [1-2]. Coronary artery disease is characterized by the presence of atherosclerosis in the epicardial coronary arteries. Atherosclerotic plaques, the hallmark of atherosclerosis, progressively narrow the coronary artery lumen and impair antegrade myocardial blood flow. The reduction in coronary artery blood flow may be symptomatic or asymptomatic, and may culminate in a myocardial infarction, depending on obstruction severity and the rapidity of development [1-2].

2.1.1 Treatment Options for CAD

The most common form of treatment for atherosclerotic CAD is coronary artery bypass graft (CABG) surgery. Such surgical procedure frequently requires multiple bypasses, and commonly employs the patient's internal mammary artery or saphenous vein to redirect blood flow around atherosclerotic vessels and restore blood flow to the ischemic myocardium.

Synthetic grafts made of Dacron or expanded polytetrafluoroethylene (ePTFE) have also been used with some success.

2.1.2 Complications of CABG

More than 500,000 CABG procedures are performed every year in the United States, making this surgery one of the most commonly performed surgical procedures in this country [1-2]. However, CABG is not without significant constraints and complications. Studies have shown that autologous saphenous vein and mammary artery grafts often cannot be used because of damage or pre-existing disease. Synthetic grafts are employed when native vessels are not ideal or available, but suffer from immunologic and thrombotic complications [3]. Graft occlusion or stenosis remains a major clinical concern: 10-30% of CABG patients suffer from thrombosis-related occlusions within the first month, and 95% of those patients require intervention within 10 years after the initial operation [4-5].

Severely occluded vein grafts can completely obstruct blood flow to the myocardium, leading to a myocardial infarction. Myocardial infarction is characterized by irreversible loss of cardiac tissue. The ischemia-induced necrosis of cardiomyocytes can result in scar formation and reduced contractility of the left ventricle, and eventually lead to total heart failure.

2.1.3 Congestive Heart Failure and Heterotopic Heart Transplantation

Despite significant progress in the prevention and treatment of cardiovascular disease in the past two decades, national statistics indicate that the incidence and prevalence of congestive heart failure (CHF) have been increasing steadily in recent years, especially in the elderly [6]. It is estimated that four to five million Americans suffer from heart failure and its related effects,

with 400,000 new cases occurring each year. CHF results in almost one million hospitalizations every year, and the economic cost associated with CHF is estimated to be \$27.9 billion annually [6]. Moreover, prognosis is poor for CHF, with a median survival after onset of only 1.7 years in men and 3.2 years in women [7].

Current therapeutic strategies used to treat CHF include pharmaceutical intervention, short term mechanical cardiac support, and heart transplantation. As adult myocardium has low potential for repair and regeneration, the ability of pharmaceutical agents to improve cardiac function is limited as these reagents do not address the fundamental issue of cell loss [7].

Currently, heterotopic heart transplantation is the treatment of choice for end-stage heart failure. Heart transplantation has a high success rate with a short-term survival of 81%, however its application is severely limited by the chronic shortage of donor organs [7]. Although the technique has been used for over 30 years, the long term outcome of patients is still complicated by problems of organ rejection, infection, and development of disorders caused by immunosuppressive regimens designed to modulate the rejection process [7]. Clearly, there is a need for improved treatment options.

2.1.4 Cell Transplantation and Alternative Therapies

A newly developed therapeutic strategy termed cell transplantation targets the issue of cell loss and consequent deterioration in heart function. Cell transplantation has been shown to be a promising alternative treatment for myocardial repair. Ideally, cell transplantation allows the replacement of diseased cardiomyocytes and scar tissue with fully functional cardiomyocytes, improving cardiac function, and thus relieving the symptoms of heart failure. To date, fetal cardiomyocytes [8-10], skeletal myoblasts [11-12], and smooth muscle cells [13] have been

successfully transplanted into normal and infarcted myocardium in various animal models. All of these transplanted cells survived initially but were slowly eliminated due to immune rejection. Other sources of transplantable cardiomyocytes that have been described in literature include stem cells derived from neonatal or embryonic tissue [14] and marrow stromal cells [15-16].

2.2 STEM CELLS

2.2.1 Embryonic Stem Cells vs. Mesenchymal Stem Cells derived from Adult Tissues

Stem cells are immature tissue precursor cells that are able to self-renew and differentiate into multiple cell lineages. In recent years, considerable activity has focused on the potential therapeutic use of stem cells for repair of damaged adult organs, such as an impaired myocardium. The candidate cell types include totipotent juvenile cells, such as embryonic stem cells (ESCs), as well as multipotent stem cells derived from adult tissues, including cells from the bone marrow compartment, liver, neural tissue, pancreas, and skeletal myoblasts. While embryonic stem cells exhibit virtually unlimited differentiation potential both *in vitro* and *in vivo*, ethical and political concerns, and potential problems of cell regulation, have limited the use of ESCs [17-18]. In contrast, there is little ethical controversy surrounding adult stem cells. Increasing evidence on the ability of mesenchymal stem cells (MSCs) derived from adult tissues to differentiate outside their predicted developmental lineage has also added to the appeal of MSCs in cell-based therapies [19-20].

2.2.2 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) have been isolated from bone marrow, skeletal muscle, skin, blood, adipose and brain tissue [21-26]. MSCs have been characterized by their plastic adherent growth and subsequent expansion under specific culture conditions, by a panel of nonspecific surface antigens and by their *in vitro* and *in vivo* differentiation potential [27]. Induction of differentiation into different cell types such as osteoblasts, adipocytes myoclasts, and chondrocytes under appropriate culture conditions has been demonstrated extensively [28-31].

2.2.3 Bone Marrow-derived Mesenchymal Stem Cells

Much of the work conducted on adult stem cells has focused exclusively on mesenchymal stem cells found within the bone marrow stroma. The existence of hematopoietic stem cells (HSCs) within the bone marrow compartment was established decades ago. The idea of a separate non-hematopoietic stem cell population was recognized with the discovery of marrow derived cells that were capable of forming new bone and cartilage [32-34]. These cells, initially referred to as marrow stromal cells, were found to have the ability to also form cells and tissue of other lineages, leading to their ultimate characterization and classification as mesenchymal stem cells.

2.2.4 Cell Yield and General Characteristics of Marrow-derived Mesenchymal Stem Cells

MSCs are rare in bone marrow, representing only ~1 in 10,000 nucleated cells [26-27]. They are isolated from the marrow by a process involving Ficoll centrifugation and adhering cell

culture in defined serum-containing medium. These cells are obtained from vigorous washing of cells from bone marrow aspirates in culture as MSCs differentially adhere to the plastic culture dish. This technique has been used to isolate MSCs from humans, rats and mice. Removing cells with HSC or endothelial progenitor cell (EPC) markers by flow cytometry analysis or FACS prior to plating initial cultures enhances the specificity of this method. The resulting cells, while not immortal, have the ability to expand many-fold in culture, and retain their growth characteristics and multilineage potential [28, 35-36]. Individual MSC clones have the ability to differentiate to cartilage, bone, muscle, adipose and stromal tissues. Phenotypically, MSCs have been defined as CD29+, CD44+, CD90+, CD105+, and negative for hematopoietic lineage markers such as CD34 and CD45 [35-36].

2.3 ADIPOSE TISSUE AS AN ALTERNATIVE SOURCE OF ADULT STEM CELLS

2.3.1 Adipose-derived Stem Cells - General Characteristics

Adipose tissue, like bone marrow, is derived from the embryonic mesenchyme and contains a fibroblast-like stem cell population known as Adipose-derived Stem Cells (ASCs). ASCs are easily isolated from discarded liposuction tissue by collagenase digestion and differential centrifugation, and can be maintained *in vitro* for extended periods with stable population doubling and low levels of senescence [21-22]. The majority of these cells are of mesodermal or mesenchymal origin with low levels of contaminating pericytes, endothelial cells, and smooth muscle cells. It has also been reported that ASCs have the ability to differentiate along mesenchymal lineages in the presence of lineage-specific induction factors. Zuk *et al* [21-

22] have demonstrated that these multipotent stromal cells could be chemically induced to differentiate into adipocytes, chondrocytes, and osteoblasts. Subsequent work by members of this group and others have confirmed and extended this work, demonstrating a capacity for myogenic [37], neuronal [38-39], and cardiomyocytic differentiation [40].

While there is some debate as to whether the cells originate in the adipose tissue or are perhaps mesenchymal or peripheral blood stem cells temporarily residing in the adipose tissue, ASCs represent a readily available source of potentially useful stem cells. The ASCs can be maintained for extended periods of time in culture, have a mesenchymal-like morphology, and demonstrate plasticity and multilineage potential *in vitro* [21-22, 37-40]. Adipose tissue is perhaps the most abundant and accessible source of adult stem cells that allows extraction of a large volume of tissue with low morbidity. The yield of stem cells per gram of fat is extraordinarily high, especially when compared to bone marrow-derived mesenchymal stem cells [26-27]. As human adipose tissue is plentiful, extraction of a large volume of tissue is possible with little patient discomfort and donor site morbidity, making adipose tissue a promising source of autologous stem cells for tissue engineering strategies and cellular therapies.

2.3.2 Adipose-derived Stem Cell Isolation Methodology

Adipose tissue is available in abundance and can be easily obtained from donors via common surgical procedures such as resection, conventional liposuction and ultrasound-assisted liposuction. All of the above surgical procedures result in minimal patient discomfort and donor site morbidity. Ultrasound-assisted liposuction was first introduced in 1987 by Scuderi *et al.* and uses ultrasonic energy to selectively destruct subcutaneous adipose tissue. Recent studies have compared the yield and growth characteristics of stem cells isolated from adipose tissue using

different types of surgical techniques and found that adipose-derived stem cells obtained by resection or traditional liposuction contain rapidly growing cell populations, whereas stem cells obtained by ultrasound-assisted liposuction exhibit low proliferative capacity (unpublished findings from our laboratory). In contrast, the surgical procedure used had no effect on the yield of viable stromal vascular fraction cells obtained from adipose tissue.

Whole adipose tissue obtained via resection requires pre-processing or mincing the tissue into small fragments. Minced or liposuctioned adipose tissue is typically digested at 37°C in a salt solution containing type II collagenase. The digested tissue is filtered to remove excessive tissue fragments and subjected to centrifugation to separate the different cellular components. The supernatant contains the mature adipocytes that are buoyant due to their lipid content, whereas the pellet contains the stromal vascular components including the adipocyte progenitor cells or multipotent stem cell population, in addition to hematopoietic lineage cells. An erythrocyte lysis buffer is used to remove the hematopoietic population. The resulting pellet is plated in regular cell culture media (1:1 DMEM/F12, supplemented with 10% FBS and antibiotics) with dexamethasone to inhibit fibroblast growth and contamination. After 4-6 hrs post cell isolation, the stem cells are washed with saline solution to remove contaminating adipocytes and fragments of adipose tissue.

2.3.3 Surface Protein Characterization and Phenotype

Adipose-derived stem cells share many properties with bone marrow-derived mesenchymal stem cells. While an extensive body of work exists pertaining to the phenotypic characterization of marrow-derived MSCs, the phenotypic characterization of ASCs is still in its infancy. ASCs express cell surface markers that are similar to those expressed by marrow-

derived MSCs, including CD105, SH3, Stro-1, CD90, and CD44, but ASCs do not express the hematopoietic marker CD45 nor the endothelial marker CD31 [41-43]. Studies in which both bone marrow and adipose tissue were obtained from the same individuals, differences in protein expression were noted: ASCs express CD49d and not CD106, whereas marrow-derived MSCs express CD106 but not CD49d [41]. There is some controversy in literature regarding the expression of CD34, a widely used marker of hematopoietic stem and progenitor cells, by ASCs. CD34 is highly expressed in vascular endothelial cells and precursors. Groups have shown that the expression of CD34 is low to absent in cultured ASCs [21,43], whereas other groups [42,44] have reported higher levels of CD34 expression.

2.4 DIFFERENTIATION CAPACITY OF ADIPOSE-DERIVED STEM CELLS

2.4.1 Adipogenesis

ASCs have the ability to revert to their original differentiation pathway, which is adipogenesis. When cultured in induction cocktails containing insulin, methylisobutylxanthine, hydrocortisone, dexamethasone, and/or indomethacin for 7-10 days, ASCs collect lipid vacuoles, as detected by Oil Red O. ASCs also express several adipocytic genes or proteins including lipoprotein lipase, aP2, PPARgamma2, leptin, and Glut4 [21-22,45-47].

2.4.2 Osteogenesis

The ability of MSCs to give rise to osteoblasts has been extensively studied [48-50]. Several groups have isolated stem cells from adipose tissue from humans and other species, and have successfully differentiated them into osteoblasts *in vitro*. ASCs have been shown to differentiate into osteoblast-like cells in the presence of ascorbate, beta-glycerophosphate, dexamethasone and vitamin D3, and bone morphogenic protein (BMP-2) [51-52]. After 2-4 weeks in culture, ASCs have been observed to deposit calcium phosphate mineral within their extracellular matrix and express osteogenic genes and proteins, including alkaline phosphatase, bone morphogenic proteins and receptors, osteocalcin, osteonectin and osteopontin [51-53].

2.4.3 Chondrogenesis

ASCs can be induced to express markers associated with mature chondrocytes. After ASCs are exposed to transforming growth factor- β (TGF- β), ascorbate, and dexamethasone for 1-2 weeks, ASCs begin to secrete the extracellular matrix proteins of cartilage, collagen type II, collagen type VI, and aggrecan (only when maintained in an appropriate 3D construct) [21,54-56]. Interestingly, when ASCs are maintained in a monolayer under the same chondrogenic culture conditions, the expression of chondrocytic markers is greatly reduced [55].

2.4.4 Neurogenesis

Early studies have suggested that ASCs can express neuronal and/or oligodendrocytic markers. When exposed to antioxidants in the absence of serum, human and murine ASCs have been shown to assume a morphology similar to that of neuronal cells. This is accompanied by the

expression of neuronal associated proteins nestin, intermediate filament M, and Neu N, as well as glial fibrillary acidic protein. Exposure to indomethacin, insulin, and isobutylmethylxanthine results in a similar phenotype [38-39,21-22]. Recently, a number of groups have challenged such findings, suggesting that the changes in cell morphology were a mere response to toxic reagents. To date, no published study has been able to demonstrate the expression of mature neural markers, nor the ability of ASCs to form synaptic junctions and the generation or transmission of an action potential.

2.4.5 Skeletal Myogenesis

Studies have shown that culturing ASCs in the presence of dexamethasone and hydrocortisone results in a time dependent increase in the expression of muscle-specific markers (MyoD1 and myosin), expression of transcription factors (myogenin) responsible for regulating skeletal muscle differentiation [21-22,37]. Under these conditions, ASCs have been shown to fuse and form multi-nucleated structures that closely resemble myotubes.

2.4.6 Epithelial Differentiation

A recent study performed by Brzoska *et al.* [57] examined the potential of ASCs to differentiate toward the epithelial lineage via the use of retinoids. In this study, the effects of all-trans retinoic acid (ATRC) on ASCs were investigated. The proliferation and viability studies with ATRA showed that at a concentration of 5 μ M an inhibitory effect on cell proliferation was evident. Further studies revealed that ASCs can be induced to express cytokeratin 18, an epithelial cytokeratin.

2.4.7 Hepatic Differentiation

Another recent study [58] examined whether human adipose tissue-derived stromal cells can differentiate into hepatocyte-like cells *in vitro* for use in hepatocyte regeneration or liver cell transplantation. Human ASCs were treated with cytokines (HGF and OSM) that have been reported to be involved in the development and differentiation of hepatocytes. The addition of DMSO to the cytokine mixture was also reported to enhance hepatic differentiation. The differentiated cells showed LDL uptake and urea production, and expressed albumin and alpha-fetoprotein.

2.5 PLASTICITY OF ADIPOSE-DERIVED STEM CELLS TOWARD CELLS OF THE CARDIOVASCULAR LINEAGE

Mature blood vessel walls have a complex three-dimensional structural organization that reflects compartments of highly differentiated cells and their associated extracellular matrix. The endothelial cells (ECs) that line the lumen are separated from the smooth muscle cells (SMCs) containing medial layer by a sub-endothelial extracellular matrix and the internal elastic lamina. Medial SMCs are arranged in concentric lamellar layers that are separated by an elastic fiber rich matrix. Fibroblasts of the outer collagen rich adventitial layer are, in turn, separated from media SMCs by the outer elastic lamina [59-60]. Recent studies have demonstrated that adult bone marrow stromal cell populations have the potential to differentiate into cells characteristic of blood vessels, in addition to bone, cartilage, muscle, fat and neural tissues. Similar studies have been applied to adipose-derived stem cells.

The adult heart is often referred to as a post-mitotic organ. Although components of the heart (endothelial cells, smooth muscles, and fibroblasts) are known to proliferate, the cells that make up the bulk of the heart, the myocardium, are thought to be terminally differentiated and have limited ability to regenerate. Bone marrow-derived mesenchymal stem cells have been shown to differentiate into cells displaying features of cardiomyocyte-like cells after exposure to a variety of chemical stimuli. However, studies conducted by other investigators have failed to reproduce these findings [115-116]. Adipose-derived stem cells from rabbit, murine and human adipose tissue have also been shown to be capable of cardiomyogenic differentiation [40, 95-96].

2.5.1 Endothelial Cells

Miranville *et al.* performed a series of studies using CD34⁺/CD31⁻ cells isolated from human adipose tissue, and demonstrated the ability of these cells to undergo *in vitro* and *in vivo* endothelial differentiation [61]. In these studies cultures of selected cells were grown in low serum medium supplemented with vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF-1), forming network-like structures that expressed endothelial markers. Cao *et al.* isolated CD31⁻, CD34⁻, CD106⁻ (VCAM-1⁻) and Flk⁺ cells from human adipose tissue and reported characteristics of endothelial progenitor cells [62]. Similarly, ASCs were shown to express endothelial markers when cultured in the presence of VEGF. Planat-Benard *et al.* [63] reported that murine ASCs can differentiate into endothelial cells, incorporate into vessels and promote post-ischemic neovascularization in nude mice and vessel-like structure formation in Matrigel. These cells (CD34⁻ and CD13⁺) were able to spontaneously express endothelial cell markers CD31 and von Willebrand factor when cultured in a semisolid medium.

2.5.2 Smooth Muscle Cells

Smooth muscle cells are an important component of the vasculature. Differentiating smooth muscle is characterized by the sequential appearance of cytoskeletal and contractile proteins to yield the variety of phenotypes that typify SMCs in the adult [76]. Two states of differentiation (“synthetic” and “contractile”) are often described in literature, representing the ends of a spectrum of phenotypes ranging from the undifferentiated state expressing few smooth muscle-specific proteins, to the fully differentiated phenotype expressing the complete repertoire of contractile proteins [76]. One of the first smooth muscle markers to be expressed is α -smooth muscle actin, though its expression is not restricted to smooth muscle cells, it is an early marker that is present in developing and mature smooth muscle. Calponin is an additional downstream marker that is more specific to SMCs. SM-22 α , caldesmon, smooth muscle tropomyosin present additional markers. The more recently discovered smoothelin and smooth muscle myosin heavy chain display a high degree of smooth muscle specificity, and are restricted to mature SMCs [76]. Expression of these late SMC markers ceases in cultured smooth muscle cells.

Smooth muscle cells are the subject of intense research in the field of cardiovascular cell therapies, however, finding a reliable source of SMCs is difficult. Biopsies of SMCs are morbid and impractical, making the search for alternative sources of healthy SMCs an urgent task. The use of stem cells has recently been suggested in smooth muscle repair. There have been numerous publications on the differentiation of marrow-derived MSCs and other progenitor cells into smooth muscle-like cells. The ability of ASCs to differentiate into SMCs has been less studied; there is only one publication to date on the capability of ASCs to differentiate into SMCs [71].

2.5.2.1 Previous Work on Smooth Muscle Cell Differentiation via Chemical Modulation

In 2000, Arakawa et al. reported that a rat bone marrow stromal cell line TBR-B differentiates into SMCs when cultured in the presence of β -mercaptoethanol (BME) and ascorbic acid (AA) [64]. A follow-up study performed by the same group further examined the dosage-dependent manner of ascorbic acid on the expression of smooth muscle-specific markers in rat VSMCs [65]. Suzuki et al., reported that pluripotent murine embryonal carcinoma P19 cells (whose developmental potential resembles that of early embryonic cells), can differentiate into SMCs when cultured in high concentrations of retinoic acid (RA) [66]. Similarly, Drab et al. demonstrated that murine totipotent embryonic progenitor cells cultured with RA and dibutyryl-cyclic adenosine monophosphate (d-cAMP) also differentiate into SMCs and express SM-specific markers [67]. In 2002, Kinner et al. reported that the α -smooth muscle actin expression in human bone marrow stroma-derived mesenchymal stem cells can be regulated by transforming growth factor- β 1 (TGF- β 1) and platelet derived growth factor-BB (PDGF-BB) [68]. Recently, Wang et al. performed an elaborate proteomic profiling on human bone marrow mesenchymal stem cells upon TGF- β 1 stimulation, and further demonstrated the role of TGF- β 1 in the differentiation of marrow-derived mesenchymal stem cells along the SMC lineage [69]. Using gene therapy, Abderrahim-Ferkoune et al. reported that the overexpression of aortic carboxypeptidase-like protein (ACLP) promotes transdifferentiation of established mouse preadipocyte clonal line 3T3-L1 cultured in BME and AA into smooth muscle-like cells, which express SM-specific markers such as SM22 α , SM α -actin, SM-MHC, and caldesmon [70].

A recent publication by Rodriguez et al. [71] examined the capacity of ASCs to differentiate into phenotypic and functional smooth muscle cells for repair of the urinary tract. The authors reported genetic expression of all smooth muscle cell markers and increased protein

expression of smooth muscle cell-specific α -actin, calponin, caldesmon, SM22, myosin heavy chain, and smoothelin, when ASCs were cultured in smooth muscle inductive medium (medium MCDB 131 from Sigma supplemented with 1% FBS and 100 units/mL heparin) for 6 weeks. In addition, ASC-derived smooth muscle-like cells were reported to exhibit the ability to contract and relax in response to pharmacologic agents [71].

2.5.3 Effects of Mechanical Forces on Vascular Cell Phenotype

It has been well documented that cells and tissues in their native environment are constantly subjected to various forms of mechanical stimulation (such as compression, tension and shear) that are necessary for their normal development and functions [72-74]. *In vivo*, the arterial vessel wall is continuously exposed to mechanical stresses. While vascular endothelial cells (ECs) are exposed to shear stress due to the nature of blood flow, vascular smooth muscle cells are subjected to significant cyclic mechanical strain in the circumferential direction throughout the cardiac cycle [75]. These mechanical stresses are likely to influence the physiology of these cells by contributing to regulation of growth and phenotypic state of these cells.

Vascular SMCs populate the media of blood vessels and play important roles in both the control of vasoactivity and the remodeling of the vessel wall. SMCs are characterized by high expression levels of an array of contractile marker proteins such as α -smooth muscle actin (α -SMA), *h1*-calponin, *h*-caldesmon, smoothelin, and smooth muscle myosin-heavy chain [76]. The phenotype and functions of vascular SMCs have been reported to be regulated by chemical factors such as TGF- β , and by mechanical factors such as cyclic strain [77-78]. Vascular SMCs

are known to change from a contractile to a synthetic phenotype in the absence of mechanical stimulation [78-79].

2.5.4 Response of Cultured Vascular Smooth Muscle Cells and Mesenchymal Stem Cells to Strain

Several experimental models have been developed to study the effects of mechanical forces on cultured vascular SMCs. Instruments such as the Flexcell Strain Unit [80] allow the examination of the cellular responses of vascular SMCs cultured on deformable substrates to mechanical strain under well-defined conditions, both in isolation and in combination with other important factors such as individual growth factors and extracellular matrices. For example, the application of cyclic strain to cultured vascular SMCs has been shown to promote the SMC contractile phenotype. Recent studies demonstrated the findings on the effects of cyclic strain alone on SMC differentiation of bone marrow-derived progenitor cells [81], and reported that mechanical forces facilitate the expression of SMC-specific cytoskeletal proteins in marrow stromal cells [82]. In addition, Park *et al.* [83] have conducted a direct comparison of the effects induced by different modes of mechanical strains on MSCs.

While the response of cultured vascular SMCs and progenitor cells such as bone marrow-derived MSCs to mechanical forces has begun to be described in literature, the effects these forces on ASCs remain unexplored. Although many groups have documented the multipotentiality or plasticity of ASCs into various cell types with chemically defined mediums, it is not well understood what role mechanical forces play in the differentiation of ASCs. Furthermore, the potential of mechanical forces in combination with bioactive molecules has also been less studied.

2.5.5 Cardiomyocytes

Cardiomyocytes are essential elements of myocardial tissue structure and function. *In vivo*, cardiomyocytes are arranged in clusters, strands, and sheets, which are surrounded by a dense network of connective tissue. Communication between cardiomyocytes is via gap junction proteins connexins. Connexin-43 is a member of the connexin family [123]. Mature cardiomyocytes express cardiac-specific troponin I and troponin T, subunits of the troponin complex that provides a calcium-sensitive molecular switch for the regulation of striated muscle contraction [122]. Tropomyosin, sarcomeric α -actinin, desmin, and α -myosin heavy chain are additional cardiac-specific markers. Cardiomyocytes also express several cardiac transcription factors, including Nkx2.5 and GATA-4. These transcription factors are expressed in pre-cardiac mesoderm and persist in the heart during development [122]. Nkx2.5 is a homeobox-containing gene that is essential for normal heart development and morphogenesis. GATA-4 is a downstream cardiac transcription factor that is hypothesized to regulate cardiomyogenic differentiation. The expression of Nkx2.5 and GATA-4 are highly restricted to the heart and heart progenitor cells even in the early developmental stage.

2.5.5.1 Cardiomyogenic Differentiation of MSCs via Exposure to 5-azacytidine

Cardiomyogenic differentiation of MSCs was first suggested by Makino et al. [84] in experiments detailing spontaneous cardiomyocyte-like contraction in isolated marrow stromal cells treated with the DNA-methylating agent, 5-azacytidine. This cardiomyogenic cell line exhibited expression of required cardiomyocyte transcription factors, GATA4, Nkx2.5, and Mef2C, organized sarcomeric structure, and ventricular cardiomyocyte-like action potentials, as well as functional adrenergic and muscarinic receptors [84]. Similar results were reported by

other investigators, including data on human MSCs based on similar treatment with 5-azacytidine [85-91, 92]. After exposure to 5-azacytidine, MSCs were reported to exhibit a myotube-like structure and a time-dependent competence to synchronously beat. In turn, electron microscopic analysis revealed a cardiomyocyte-like ultrastructure including typical sarcomeres, a centrally positioned nucleus, and atrial granules. These cells showed several functional features of developing cardiomyocytes, including the production of peptides and the expression of multiple structural and contractile proteins. They also displayed sinus node-like and ventricular cell-like action potentials [85-91, 92].

2.5.5.2 Plasticity of Adipose-derived Stem Cells Toward Cardiomyocytes

ASCs have also been shown to be capable of in vitro differentiation into cardiomyocytes [40, 95-96]. The most compelling data was obtained by Planat-Bernard et al. [95] in which fresh murine adipose-derived cells were plated into semisolid culture. After three weeks in culture, colonies of spontaneously beating cells were observed. These cells exhibited several molecular, electrophysiologic, and pharmacologic properties of cardiomyocytes. Most studies to date have focused on the use of 5-azacytidine to induce cardiomyogenic differentiation in ASCs (although these studies have reported successful transformation of rabbit or mouse, and not human ASCs into pulsating cardiomyocyte-like cells). One recent study by Gaustad et al. [96] exposed ASCs to nuclear and cytoplasmic extracts of rat cardiomyocytes transiently and reported the protein expression of cardiac specific markers such as sarcomeric α -actinin, desmin, and cardiac troponin I. The expression of the gap junction protein connexin 43 was also observed. To the best of our knowledge, there are no reports describing the effects of 5-azacytidine on human ASCs.

2.5.5.3 Regeneration of Ischemic Myocardium with Adipose-derived Stem Cells

Over the last several years, there has been a surge of data detailing the use of cell-based therapies for post-myocardial infarction treatment. While much of these initial studies focused upon the use of skeletal myoblasts or neonatal cardiomyocytes, recent evidence suggesting the cardiomyogenic potential of stem cell populations has shifted the focus of cell therapy towards myocardial regeneration with adult stem cells such as bone marrow-derived mesenchymal stem cells [124,125]. Since the first study conducted in 1999, marrow-derived mesenchymal stem cells have been shown to engraft in injured myocardium, express cardiac markers and assume cell morphology consistent with cardiac differentiation. In addition, animals that were treated with these stem cells have demonstrated improved cardiac function based on numerous clinical measures [124,125]. Several recent reports suggest that adipose-derived stem cells may be an equally promising source of cells for cardiac therapeutics. Early *in vivo* work conducted by Strem *et al.* [126] demonstrated that that intravascular injection of ASCs into mice following cryoinjury of the left ventricle resulted in positive engraftment of ASCs at the site of injury. Donor-derived cells expressed cardiac-specific transcription factor Nkx2.5, troponin I and myosin heavy chain, consistent with cardiomyocytic differentiation of ASCs *in vivo*. Cardiomyocytic differentiation is not the only mechanism by which the plasticity of ASCs could affect myocardial function after infarction. The therapeutic potential of ASC may be further enhanced through their effects on neovascularization. ASCs in culture are known to secrete a number of proangiogenic factors such as vascular endothelial growth factor (VEGF), transforming growth factor- β , and hepatocyte growth factor. Miranville *et al.* [61] showed ASCs (CD34+/CD31-) that were exposed to VEGF and insulin-like endothelial growth factor I (IGF-I) formed network-like structures expressing endothelial markers CD31 and von Willebrand's factor. These cells also increased the rate of perfusion recovery in a murine surgical model of

hind limb ischemia. Other groups have reported that growth factor supplementation is not needed with unsorted cells, suggesting that ASCs are capable of expressing the necessary pro-angiogenic factors in a paracrine fashion. Although the exact mechanisms of the pro-angiogenic response of ASCs are not well understood, their ability to secrete cytokines, as well as their direct incorporation into new vasculature, have great potential in the repair/regeneration of ischemic tissues. More research is needed to understand the mechanisms by which ASCs impart their cardio-protective or cardio-regenerative effect on injured tissue.

3.0 MATERIALS AND METHODS

3.1 ADIPOSE-DERIVED STEM CELL ISOLATION AND CULTURE

Adipose-derived stem cells (ASCs) were isolated from human subcutaneous adipose tissue harvested during elective abdominoplasty and cultured as previously described [21-22]. Briefly, the subcutaneous adipose tissue was minced and then digested in Hank's Balanced Salt Solution containing 1 mg/mL type II collagenase and 3.5% fatty acid free BSA in a 37 °C shaking water bath until the mixture was homogeneous. The digested tissue was filtered through a double-layered gauze filter (350 μ m) and centrifuged at 1000 rpm for 10 min. After centrifugation, the resulting cell pellet was treated with Erythrocyte Lysis Buffer, vortexed, and centrifuged at 1000 rpm for 10 min. The cells were resuspended in regular cell culture media (1:1 DMEM/F12, supplemented with 10% FBS and antibiotics) and plated at a density of 5×10^4 cells/cm². Cell culture media was changed every two days until confluence. Cells were either passaged and/or frozen down for future experiments. Low passage cells (<P4) from female donors (between the ages of 31 to 51 years old, mean age 42.5 years old) were utilized for the biochemical differentiation experiments in Aim 1. ASCs from six individual patients were treated with BME and AA (n=6), whereas ASCs from three patients were exposed to RA alone, RA and d-cAMP, or PDGF-BB (n=3), and ASCs from four patients were treated with two different concentrations of TGF- β_1 (n=4). We utilized P0 cells from a 20-year-old female donor and a 29-year-old female donor for all our mechanical stimulation experiments in Aim 2. Two

experiments were conducted using ASCs from the 29-year-old donor, and one additional experiment was performed using cells from the 20-year-old female (n=3, where n is the total number of experiments performed). ASCs (<P4) from the same 20-year-old female donor and an additional 35-year-old female donor were used for cardiomyogenic differentiation experiments in Aim 3. Two experiments were conducted on the 20-year-old donor, whereas one additional experiment was conducted with the 35-year-old patient (n=3, where n is the total number of experiments performed).

3.2 SMOOTH MUSCLE CELL DIFFERENTIATION EXPERIMENTS VIA CHEMICAL MODULATION

3.2.1 Effects of BME, AA, RA, d-cAMP, TGF- β_1 , and PDGF-BB on SMC Protein Expression in ASCs

Low passage ASCs (P<4) were thawed and cultured in regular plating medium for 24 hrs before the cells were lifted and seeded either on 22 x 22 mm glass coverslips in 6-well polystyrene plates (immunofluorescence staining) or in 100 mm tissue culture dishes (immunoblots). At confluence, ASCs were exposed to media with reduced serum (DMEM/F12, 5% FBS, 1% P/S, without dexamethasone) supplemented with one of the following chemicals or growth factors:

A) β -mercaptoethanol (BME), 50 μ M, and ascorbic acid (AA), 0.3mM;

B) retinoic acid (RA), 1 μ M;

C) retinoic acid (RA), 1 μ M, and dibutyl-cyclic adenosine monophosphate (d-cAMP), 0.5mM;

D) transforming growth factor- β_1 (TGF- β_1), 1ng/mL, and 10ng/mL;

E) platelet-derived growth factor-BB (PDGF-BB), 10ng/mL.

The ASCs were treated with fresh differentiation media every two days for the duration of 7 days and 14 days. Controls were ASCs cultured in plating medium with 5% serum without additional supplements. At the end of each time point, glass coverslips were fixed in 2% paraformaldehyde for immunofluorescence staining, whereas cell lysates were collected for Western blot analysis for early smooth muscle cell marker α -SMA. Plating medium with 1% serum was used for all studies involving TGF- β_1 .

3.2.2 Collagen Gel Contraction Assay

Collagen gel contraction assay was performed as an additional measure of cell contractility on treatment groups (such as exposure to TGF- β_1) with significant up-regulation in contractile smooth muscle cell protein expression. A sterile solution of acid-soluble type I collagen derived from rat tail tendon (BD Biosciences, Bedford, MA) was prepared according to the manufacturer's recommendations. ASCs that have been exposed to 1ng/mL TGF- β_1 were encapsulated in 1mg/mL type I collagen gel at 1×10^6 cells/mL. The collagen-cell mixture was dispensed into 6-well polystyrene tissue culture plates (2mL/well) and allowed to polymerize at 37 °C for 30 minutes. Immediately after polymerization, 1mL of culture medium with reduced serum (1%) was added to each well. Controls were untreated ASCs cultured in regular plating media with 1% serum. Cells embedded in collagen gels were allowed to attach for 24 hrs before the gels were released with a microspatula. Photographs of the gels were taken at different time points ($t=0$ or before release, and $t=1, 6, 24, 48$ hr). The diameter of the gel surface was measured, and the reduction in gel diameter over different time points was quantified.

3.3 MECHANICAL STIMULATION EXPERIMENTS

Following protocols described by Hamilton *et al.* [81] on the response of rat bone marrow stromal cells (BMSCs) to uniaxial cyclic strain, ASCs were subjected to 10% uniaxial cyclic strain at 1Hz (to mimic physiological conditions) for 7 days, using an FX-4000T strain unit (Flexcell Corp., McKeesport, PA, USA). Cyclic strain was supplied through membrane distension induced by air vacuum suction at the bottom of the plate, causing the flexible plate to stretch across an ArctangleTM loading post. This created a uniform uniaxial strain with negligible fluid shear stress. A computer was used to control the strain frequency, magnitude, and duration of the experiment.

ASCs were seeded on type I collagen-coated flexible bottom plates at 40,000 cells per well 3 days prior to mechanical stimulation. Controls were ASCs prepared in an identical manner and cultured on unstrained type I collagen-coated flexible 6-well plates for 7 days. ASCs (strained and controls) were cultured in plating medium with 1% serum with medium changes every 2 days. The effects of strain on cell morphology, cell proliferation, cytoskeletal organization, and early smooth muscle cell protein expression (α -SMA and calponin) were examined.

In order to study the combined effect of chemical and biomechanical stimulation on cell differentiation, unstrained and strained ASCs were also treated with media containing 1 ng/mL TGF- β_1 (R & D Systems, Minneapolis, MN, USA). Experiments conducted in Aim 1 demonstrated that TGF- β_1 at 1ng/mL and 10ng/mL induced similar levels of α -SMA expression in ASCs at 24, 48 h, and 7 days.

3.3.1 Cell Morphology

After 7 days of uniaxial cyclic strain, strained and unstrained ASCs were fixed in 2% paraformaldehyde for 15 min, washed with PBS, then stained with Coomassie blue. Images were captured at a magnification of 20x. Cell morphology was qualitatively observed and quantified using Scion Image image analysis software (version 4.02 beta; Scion Image, Frederick, MD). Ten images from each culture condition were used. Standard gray-level thresholding techniques were applied to each image to identify the cell boundaries, with manual editing required. The cell long axis of each cell was identified and measured against a fixed axis at the base of each image to determine an angle of orientation, ranging from 0° (parallel to strain) to 90° (perpendicular to strain). Each membrane was held in the same global orientation.

3.3.2 Cell Proliferation

Unstrained and strained ASCs were fixed in 2% paraformaldehyde for 15 min, washed with PBS, permeabilized with 0.1% Triton X-100, and stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) at 0.6 µg/mL to visualize the cell nuclei. Ten random fields of view of cells from each culture condition were captured at a magnification of 20x. The number of cells was counted and averaged to provide a single cell count.

3.3.3 Visualization of Cytoskeleton

Strained and unstrained ASCs were fixed in 2% paraformaldehyde for 15 min, rinsed with PBS, and permeabilized with 0.1% Triton X-100 for 15 min. The cells were then be incubated with FITC-phalloidin (Sigma Aldrich, St. Louis, MO) at a dilution of 1:500 in PBS

for 1 hour. The cells were counter-stained with DAPI for visualization of the cell nuclei. Ten images were captured for each culture condition using an Olympus Provis fluorescence microscope (AX70). F-actin labeling resulted in visualization of the alignment of the F-actin stress fibers.

3.4 CARDIOMYOGENIC DIFFERENTIATION EXPERIMENTS VIA EXPOSURE TO 5-AZACYTIDINE

ASCs used in this study were extracted from two donors: 20-year-old and 35-year-old female patients. ASCs were cultured as previously described in Section 3.1. Low passage ASCs (P<4) were seeded in 6-well polystyrene plates coated with type I collagen at 50,000 cells/well, 100,000 cells/well, and 150,000 cells/well. ASCs were exposed to regular plating media (with 10% serum, and without dexamethasone) supplemented with 5 μ M or 10 μ M of 5-azacytidine (Sigma Aldrich, St. Louis, MO). 5-azacytidine was dissolved in acetic acid:water (1:1 v/v) at 5mg/mL. Once dissolved, this stock solution was further diluted in aqueous plating media, pH'ed, and filtered. ASCs were exposed to 5-azacytidine transiently for 24 hrs or repeatedly (one or three treatments per week) for up to 8 weeks in culture. Controls consisted of ASCs cultured under the same conditions without exposure to 5-azacytidine. Parallel experiments were conducted on non-collagen coated polystyrene well plates. Untreated and 5-azacytidine treated ASCs were examined daily using light microscopy to monitor changes in cell morphology. Protein expression of sarcomeric α -actinin, cardiac troponin I, troponin T, gap junction protein connexin 43 and cytoskeletal protein α -SMA at different time points was assessed via

immunocytochemistry and Western blot analysis. Expression of cardiac transcription factors Nkx2.5 and GATA4 was also examined.

3.5 CHARACTERIZATION OF SMC AND CM PROTEIN EXPRESSION

3.5.1 Immunofluorescence Staining

At the termination of each experiment, cells were fixed in 2% paraformaldehyde for 15 min at room temperature and immunostained according to standard immunocytochemistry protocols. Briefly, after washing twice with PBS, 0.1% Triton-X made in PBS was applied to the cells for 15 min, and then non-specific binding was blocked using Normal Donkey Serum (NDS) or Normal Goat Serum (NGS). Monoclonal antibody specific for α -SMA, h₁-calponin, sarcomeric α -actinin (Sigma Aldrich, St. Louis, MO), connexin 43 (BD Biosciences, Bedford, MA), or troponin I/troponin T (abcam, Cambridge, MA) was applied for 60 min at room temperature, followed by incubation with fluorescent-tagged secondary antibody (Alexa 488) for 60 min. After subsequent washes with PBS, stained cells were viewed by fluorescent microscopy (Olympus Provis). To provide a quick assessment of cell distribution, 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (0.6 μ g/mL) was used to stain the nuclei of cells. Protein expression was qualitatively observed through the inspection of immunofluorescent images.

3.5.2 Western Blot Analysis

Cell lysates were collected at the termination of each experiment from each culture condition. Briefly, cells were washed with PBS, then 300 μ L detergent-based lysis buffer (M-

PER Mammalian Protein Extraction Reagent, Pierce, Rockford, IL) and protease inhibitor cocktail (1:100 dilution, Sigma Aldrich, St. Louis, MO) were added to each 100 mm dish for collection of total cellular protein (or 100uL of lysis buffer + protease inhibitor cocktail/well of a 6-well plate). Equal amounts of protein (5-20 μ g, depending on the protein being probed) from each sample were loaded into a 10% SDS-PAGE gel for gel electrophoresis. The separated proteins were transferred to a nitrocellulose membrane. The membrane was blocked in 5% non-fat milk/PBS-Tween 20 solution at 4°C overnight followed by the application of monoclonal antibody specific for α -SMA, h₁-calponin, sarcomeric α -actinin (Sigma Aldrich, St. Louis, MO), connexin 43 (BD Biosciences, Bedford, MA), or troponin I/troponin T (abcam, Cambridge, MA) in 5% non-fat milk/PBS-Tween 20. After incubation at room temperature for 1-1.5 hours, the secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in 5% non-fat milk/PBS-Tween 20 was applied for 1 hour. The membrane was washed three times with 0.01% PBS/Tween 20 for 10 min after each antibody application. The proteins on the nitrocellulose membrane were detected with the ECL Plus detection system (Amersham Pharmacia Biotech, Piscataway, NJ), according to the manufacturer's protocol. The resulting protein bands were quantified by volume summation of image pixels with NIH ImageJ 1.30v.

3.6 RNA EXTRACTION AND RT-PCR ANALYSIS

Total RNA was extracted from control ASCs and 5-azacytidine treated ASCs using RNeasy Mini RNA extraction kit (Qiagen Inc., Alameda, CA) according to the manufacturer's protocol.

cDNA was synthesized from 120ng of total RNA using 200U of reverse transcriptase (SuperScript II), 50ng of random hexamers, 10mM dNTP, and 40U of RNase inhibitor in buffers containing 25mM of MgCl₂.

cDNA samples were subjected to PCR amplification with human-specific primers for cardiac transcription factors Nkx2.5 and GATA4, and housekeeping gene GAPDH. 2μL of DNA template was added to a PCR buffer mix containing 25mM MgCl₂, 10mM dNTP, and 5U of Taq polymerase (Promega). cDNA of human fetal cardiomyocytes was used as a positive control.

PCR was performed over 42 cycles for Nkx2.5 and GATA4 (36 cycles for GAPDH) consisting of an initial denaturation at 95 °C for 5 minutes, then 95°C for 1 minute, 60 °C (Nkx2.5) or 55 °C (GATA4) for 30 seconds, 72 °C for 30 seconds, and was terminated by a final extension at 72 °C for 7 minutes. Housekeeping gene GAPDH was used as the control. The PCR products were size-fractionated by 2% agarose gel electrophoresis.

3.7 STATISTICAL ANALYSIS

Statistical analysis was performed by use of unpaired, two-tailed *t* tests on different combinations of variables. Data were represented as mean ± SD. The threshold for statistical significance was set at $p \leq 0.05$. In Aim 1 and Aim 2, optical density (OD) values of Western blot bands of α-SMA and calponin were normalized to the OD values of corresponding bands for GAPDH (which was used as an internal loading control). The resultant OD values of different treatment groups were then normalized to the control, where the percentage of increase/decrease in α-SMA or calponin protein expression relative to the control was computed. Statistical analysis was conducted on the normalized OD values, hence resulting in groups with zero

standard deviation. For Aim 3, statistical analysis was performed on OD values of the protein of interest divided by OD values of GAPDH (ratios of α -SMA/GAPDH or Cx-43/GAPDH). The resultant OD values were not normalized to the control.

4.0 RESULTS

4.1 ISOLATION OF ADIPOSE-DERIVED STEM CELLS FROM HUMAN ADIPOSE TISSUE

Adipose-derived stem cells were extracted from human subcutaneous adipose tissue harvested during elective abdominoplasty from healthy female subjects. Briefly, whole adipose tissue was minced, and subjected to enzymatic digestion and differential centrifugation. The resultant cell pellet contains a fibroblast-like stem cell population as depicted in **Figure 1**. The stem cells were cryopreserved until they were ready for use.

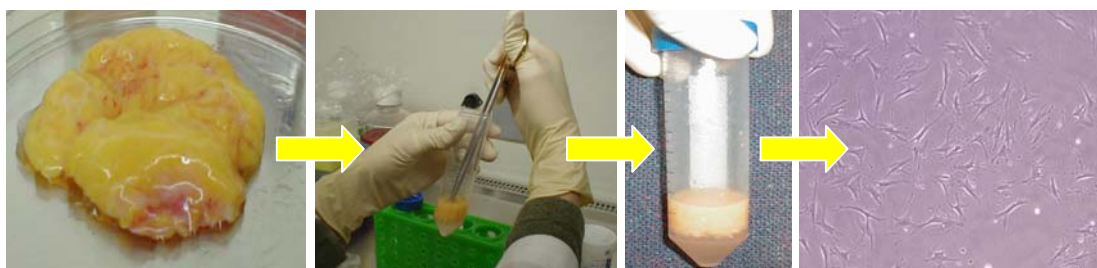


Figure 1 Culture and isolation of adipose-derived stem cells from human adipose tissue.

4.2 EFFECTS OF BME, AA, RA, D-CAMP, TGF- β_1 , and PDGF-BB ON ASCS

4.2.1 Characterization of SMC Protein Expression via Immunocytochemistry and Western Blot Analysis

Isolated ASCs (P<4) were seeded into the wells of a 6-well tissue culture plate at confluence from female donors between the ages of 20-51. Following a protocol previously described by Arakawa *et al.* [64-65] for rat bone marrow stromal cells, we have exposed ASCs to culture medium supplemented with β -mercaptoethanol (BME) (50 μ M) and ascorbic acid (AA) (0.3 mM) for 14 days with medium changes every two days. After 14 days, the medium was removed and the cells were fixed for immunofluorescence staining. Our immunofluorescence staining results indicated that ASCs treated with BME and AA were positive for early SMC markers such as α -SMA.

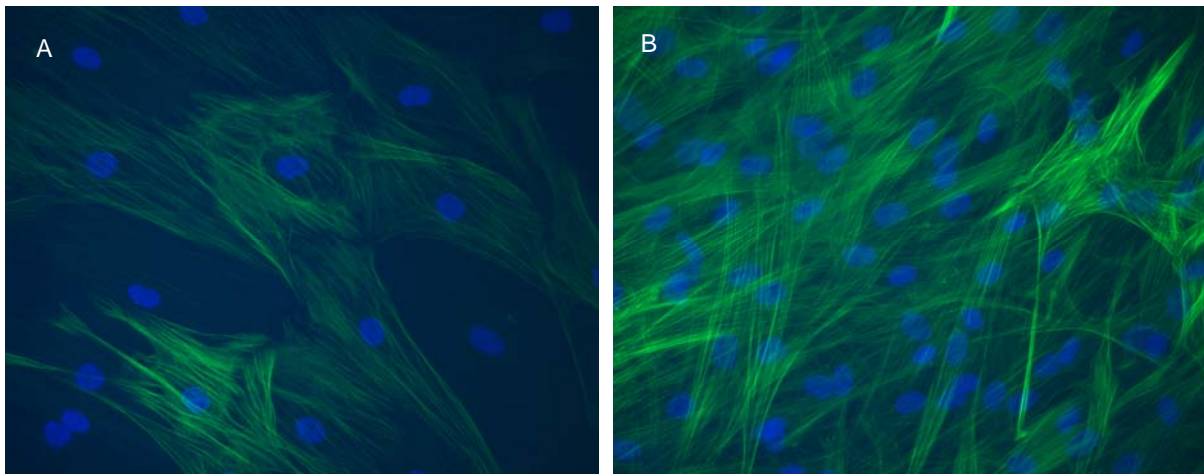


Figure 2 α -SMA immunofluorescence staining (green) counterstained with DAPI (blue) after 14 days. (a) Control, (b) BME and AA-treated ASCs.

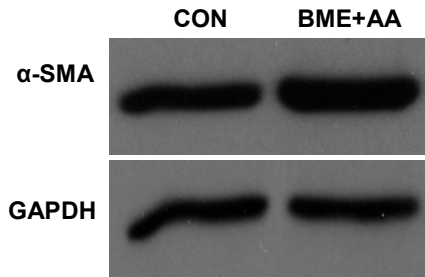


Figure 3a Representative Western blot for α -SMA and corresponding GAPDH from ASCs treated with BME and AA for 14 days.

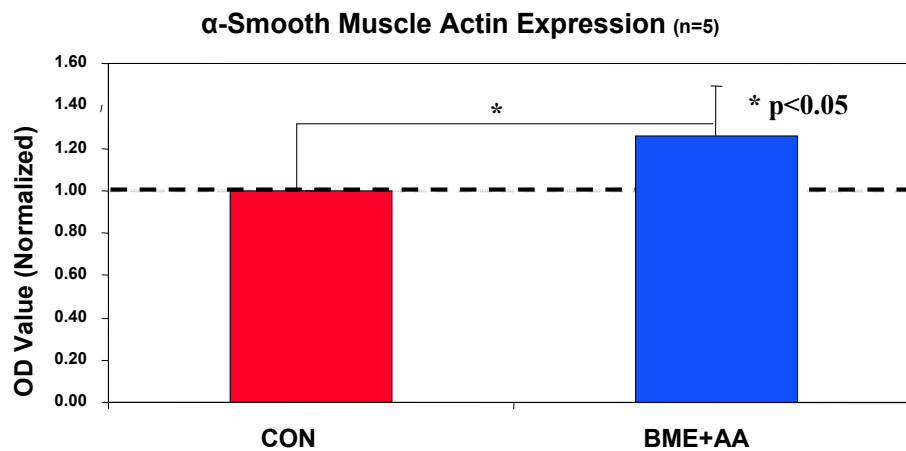


Figure 3b The optical densities (OD) of the Western blot bands were measured using Image J analysis software. BME and AA increased the expression of α -SMA by 26% over the controls. ASCs from six individual patients were treated with BME and AA (n=6). Overly saturated bands may have resulted in an underestimated difference.

From **Figure 2**, it is clear that both the controls and the experimental group were positive for α -SMA, although the cells treated with both BME and AA were marked by the appearance of pronounced stress fibers, and appeared to be more confluent. Western blot analysis demonstrated that the addition of BME and AA to ASCs induced an average increase of 26% in α -SMA expression (**Figure 3a** and **Figure 3b**).

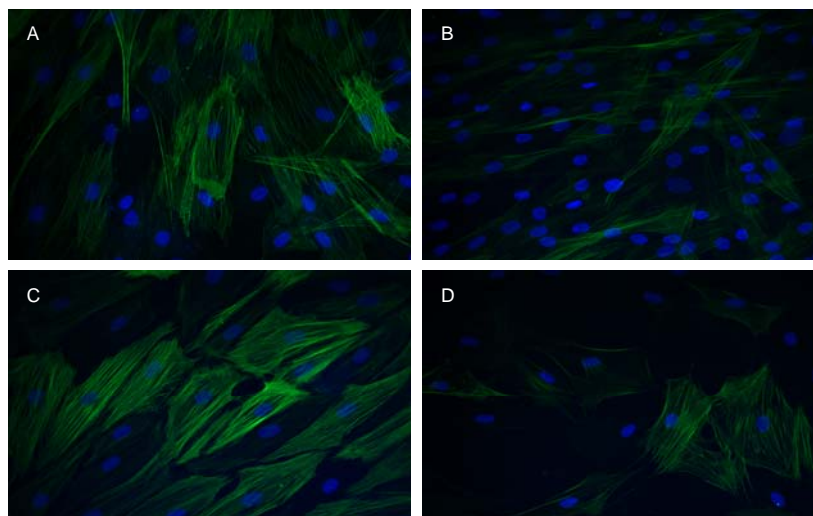


Figure 4 Immunofluorescence of ASCs for α -SMA (green) counterstained with DAPI (blue). (A) Control ASCs after 14 days. (B) ASCs exposed to PDGF-BB for 14 days. (C) ASCs treated with RA. (D) ASCs treated with RA and d-cAMP.

Our immunofluorescence staining (**Figure 4**) and Western blot results also suggested that PDGF-BB and d-cAMP may play a role in down-regulating the expression of α -SMA (**Figure 5a** and **Figure 5b**). Treatment of high concentration of RA without d-cAMP resulted in a slight increase (9%) in the expression of α -SMA. Overly saturated bands may have resulted in an underestimated difference.

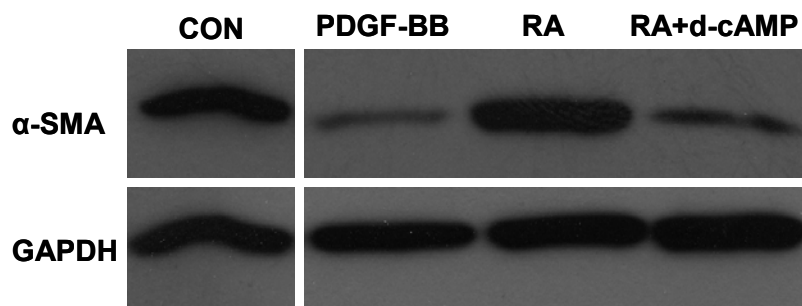


Figure 5a Representative Western blot for α -SMA and GAPDH from ASCs treated with PDGF-BB, RA, RA and d-cAMP.

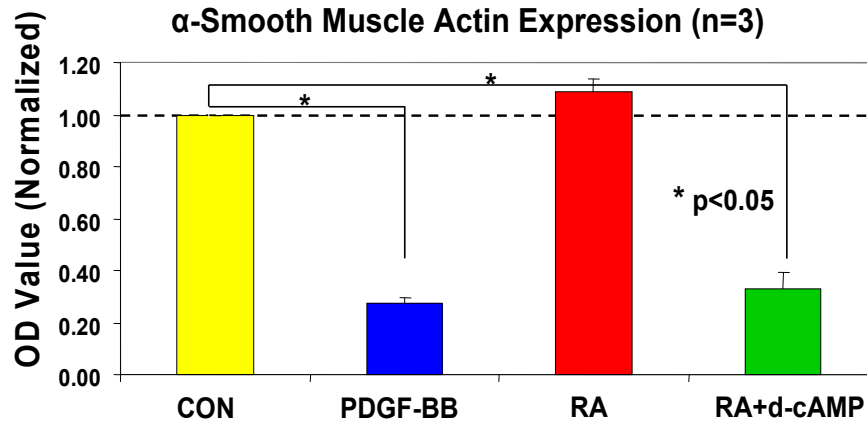


Figure 5b Both PDGF-BB and RA and d-cAMP decreased the expression of α -SMA significantly (n=3, p<0.05). Treating ASCs with high concentration of RA alone slightly increased the expression of α -SMA by 9% over the controls (n=3, p<0.05). Overly saturated bands may have resulted in an underestimated difference.

Furthermore, TGF- β_1 alone had significant effects on α -SMA expression. Two different dosages of TGF- β_1 (1 ng/mL and 10 ng/mL) induced similar levels of α -SMA expression in ASCs after only 24 hrs. Cells treated with 1 ng/mL or 10 ng/mL of TGF- β_1 demonstrated a 100% increase in the expression of α -SMA (n=4, p<0.05) (**Figure 6a and Figure 6b**).

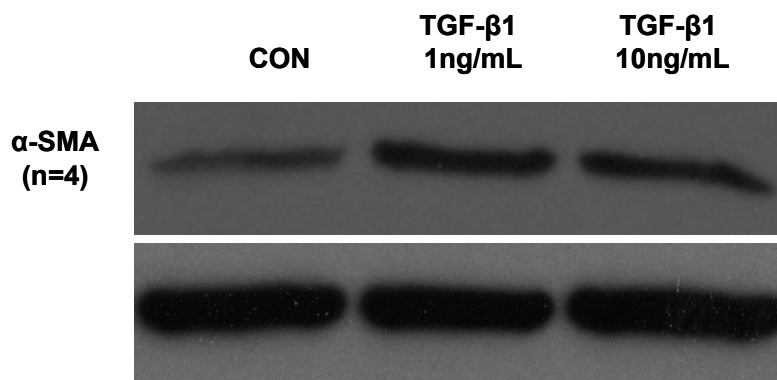


Figure 6a Representative Western blot for α -SMA and GAPDH from ASCs treated with different dosages of TGF- β_1 (1ng/mL, 10ng/mL) for the duration of 24 hours.

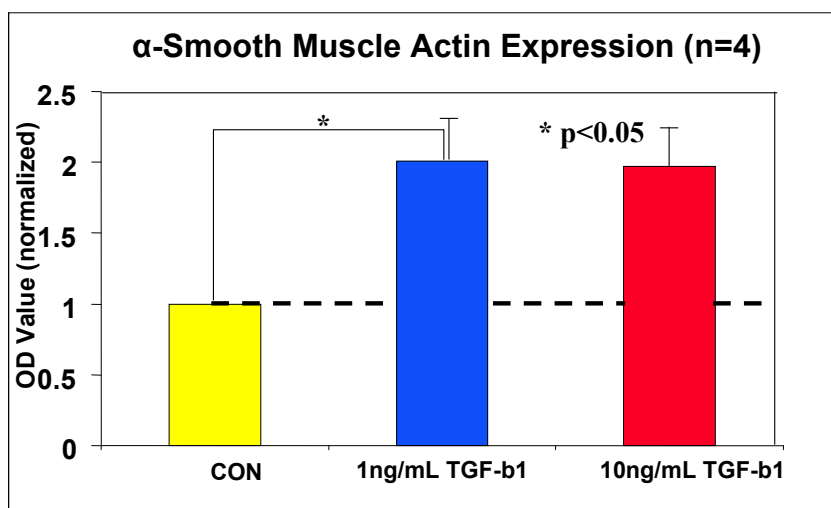


Figure 6b TGF- β_1 at 1ng/mL and 10ng/mL induced similar levels of α -SMA expression in ASCs after 24hrs. Cells treated with 1ng/mL or 10ng/mL of TGF- β_1 showed a 100% increase in the expression of α -SMA (n=4, p<0.05).

4.2.2 Characterization of Cell Contractility via Collagen Gel Contraction Assay

Collagen gel contraction assay was performed as an additional measure of cell contractility on treatment groups with significant up-regulation in contractile smooth muscle cell protein expression. Since TGF- β_1 (at either 1ng/mL or 10ng/mL) induced a 100% increase in the expression of α -SMA over the control or untreated ASCs after 24hrs, ASCs that have been exposed to 1ng/mL TGF- β_1 were encapsulated in 1mg/mL type I collagen gel, and subjected to the collagen gel contraction assay. The cells embedded in gels were allowed to attach for 24 hrs before the gels were released with a microspatula. Photographs of the gels were taken at different time points, before release (t=0), at 1hr, 6hr, 24hr, and 24hr after the gel was released as shown in **Figure 7**. The diameter of the gel surface was measured from the digital images, and the reduction in gel diameter over different time points was quantified and presented as a graph in **Figure 8**. TGF- β_1 treated ASCs showed greater cell contraction over time as compared with control or untreated ASCs.

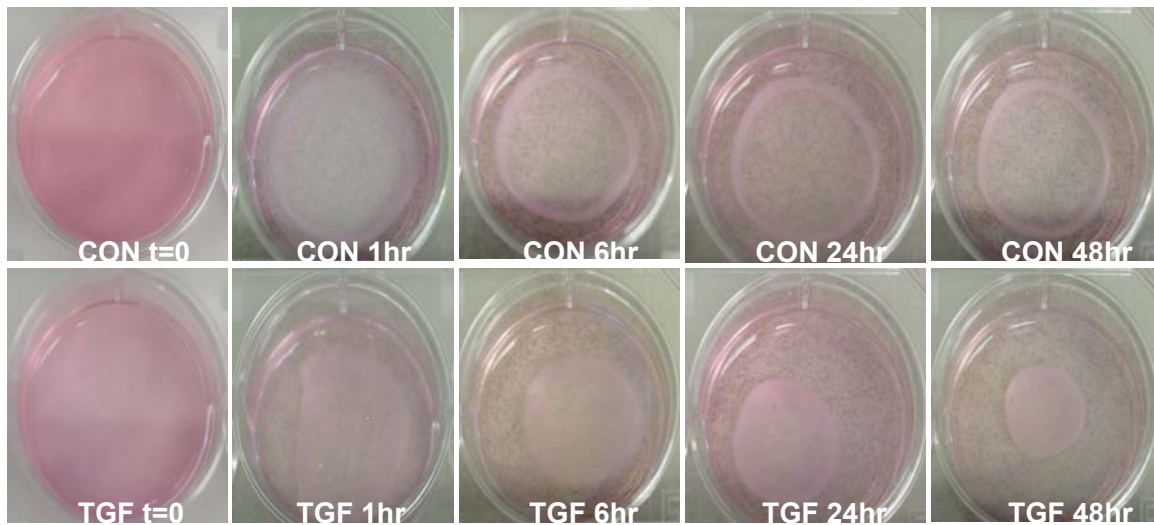


Figure 7 Representative digital photographs demonstrating the effects of TGF- β_1 on collagen gel contractility of ASCs over time (at t=0, prior to gel release, and at t=1, 6, 24, 48hr). ASCs were treated with 1ng/mL TGF- β_1 for 24hr. Controls were untreated ASCs cultured under the same conditions.

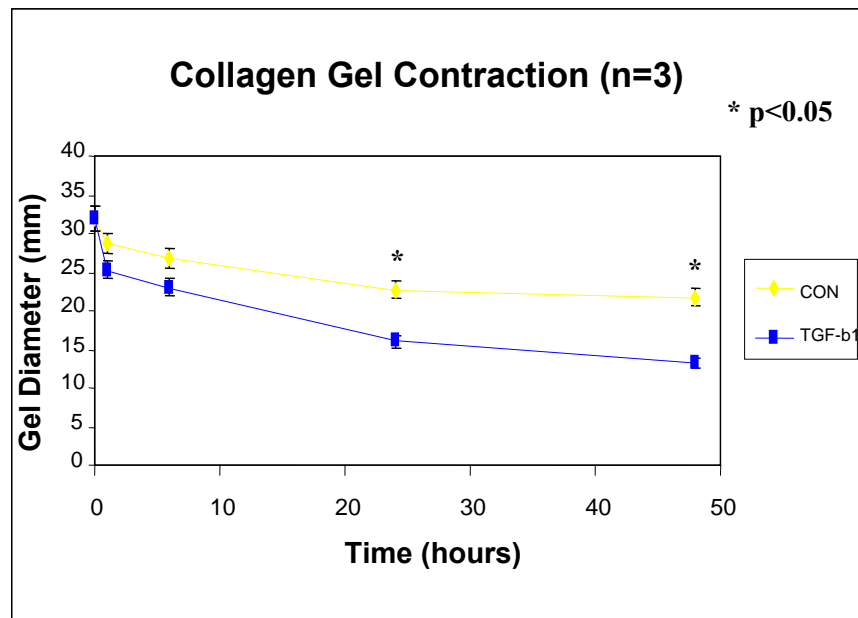


Figure 8 Time course of the stimulatory effect of TGF- β_1 on cell-mediated collagen gel contraction. Control ASCs or TGF- β_1 treated ASCs were encapsulated in collagen gels and released, after which the change in gel diameter was measured. Data are mean gel diameter \pm SD from three independent experiments.

4.2.3 Summary of Results

The objective of this work was to study the response of ASCs to exogenous biochemical stimulation, and the potential of ASCs to differentiate toward the SMC lineage. Immunofluorescence staining and Western blot analysis detected protein expression of early SMC marker α -SMA in both control and experimental groups. Expression of α -SMA in ASCs significantly increased when treated with TGF- β_1 , while α -SMA expression only slightly increased in the presence of RA, BME and AA. Treatment with PDGF-BB, RA and d-cAMP decreased the expression of α -SMA significantly. Cell contractility of TGF- β_1 treated cells was further characterized with collagen gel contraction assay. TGF- β_1 treated ASCs showed greater cell contraction over time as compared with control or untreated ASCs.

4.3 RESPONSE OF ASCS TO UNIAXIAL CYCLIC STRAIN

4.3.1 Cell Morphology and Proliferation

Adipose-derived stem cells demonstrated responsiveness to mechanical loading by altering their morphology and proliferation and regulating their protein expression of early SMC markers. To determine the effect of uniaxial cyclic strain on cell morphology and organization, ASCs exposed to uniaxial cyclic strain for 7 days were analyzed with Scion image analysis software. Cyclic strain of 10% markedly altered the morphology and orientation of cells. After exposure to strain for a day, ASCs reoriented themselves perpendicular to the direction of strain, and the stress fibers also aligned in the same direction (**Figure 9 and Figure 10**).

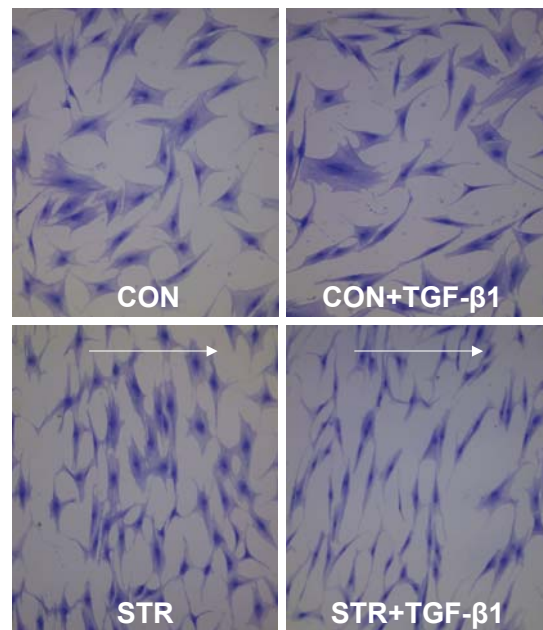


Figure 9 Phase-contrast images of ASCs cultured under static conditions (with or without TGF- β_1) and ASCs that were subjected to 10% uniaxial strain for 7 days. The arrows point in the direction of strain.

In contrast, control ASCs were randomly oriented. The orientation angle frequency distribution shown in **Figure 11** further confirmed these observations. In addition, strain caused alignment of the f-actin cytoskeleton, perpendicular to the direction of applied strain. This may be due to an adaptation process of the cells to minimize the amount of forces applied on cell bodies.

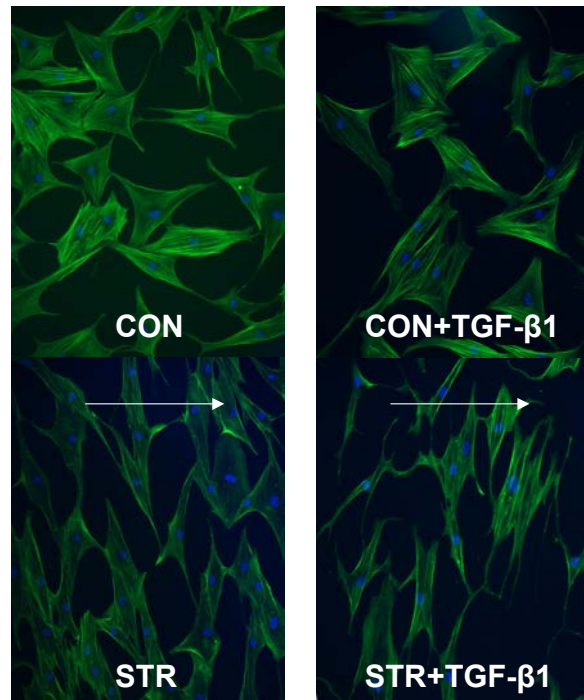


Figure 10 F-actin arrangement of ASCs in static culture or 10% uniaxial strain for 7 days, cultured with or without TGF- β_1 . F-actin staining results of control ASCs or strained ASCs treated with TGF- β_1 were counter-intuitive. Increased F-actin stress fibers were not observed in TGF- β_1 treated cells.

Moreover, unstrained and strained ASCs continued to proliferate throughout the duration of the experiment, although the strained ASCs seemed to proliferate at a lower rate compared with the controls. A trend towards a decrease in cell proliferation was observed and is shown in **Figure 12**.

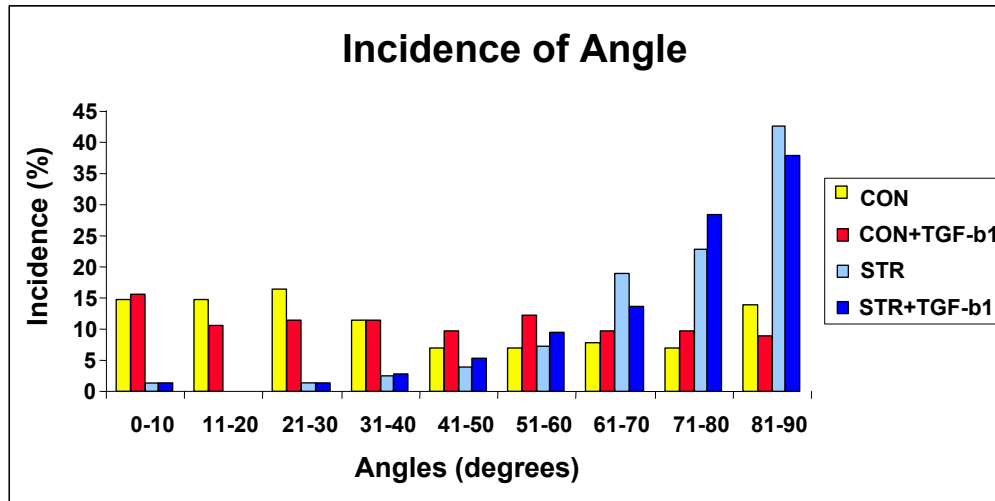


Figure 11 Orientation angle frequency distribution of each culture condition.

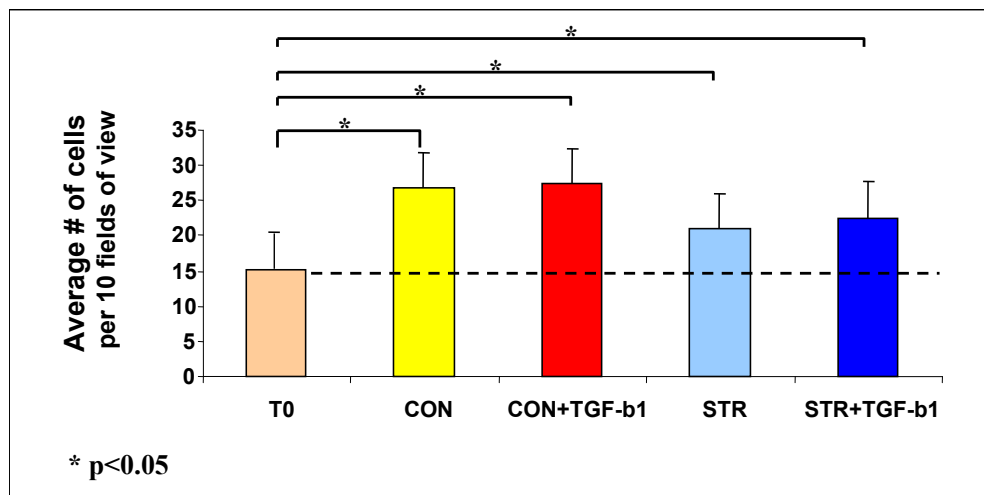


Figure 12 Average number of cells from 10 fields of view for each culture condition. TGF-β₁ had no effect on cell proliferation (n=3).

4.3.2 Characterization of SMC Protein Expression via Immunocytochemistry and Western Blot Analysis

Immunofluorescence staining and Western blot analysis detected protein expression of early smooth muscle cell markers such as α-SMA and h1-calponin in both strained and

unstrained ASCs. Immunostaining and Western blot results showed that 10% uniaxial strain alone downregulated α -SMA and h-1calponin protein expression.

Results from Western blot analysis indicated that uniaxial cyclic strain significantly decreased α -SMA expression by 40-50% after 7 days when compared with unstrained static controls (**Figure 14**).

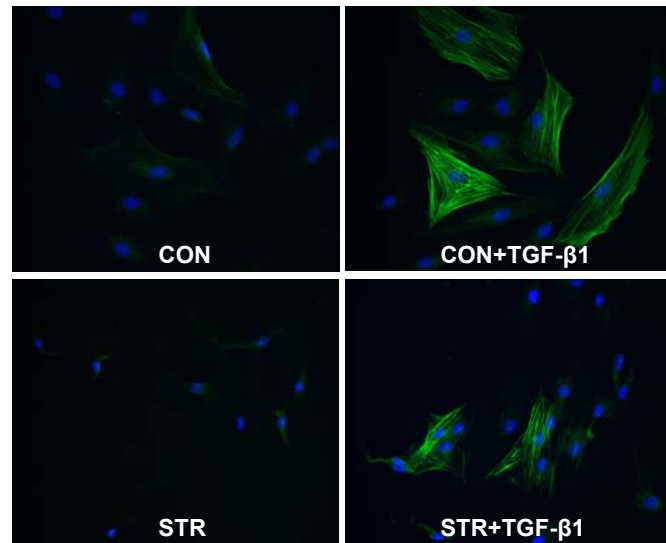


Figure 13 Immunofluorescence staining for α -SMA. After 7 days of 10% uniaxial cyclic strain, the controls & strained ASCs were stained for α -SMA.

Western blot analysis for α -SMA further confirmed the immunofluorescence staining results. GAPDH was used as an internal loading control. The optical densities (OD's) of the Western blot bands were measured using Image J & normalized to the control. ASCs that were treated with TGF- β ₁ had about a 1.5 fold increase in the expression of α -SMA over the control ASCs. Strain alone reduced the expression by about 50%. Strain & TGF- β ₁ combined increased the expression to about 50% above that of the control ASCs (~1 fold over that of the strain only group).

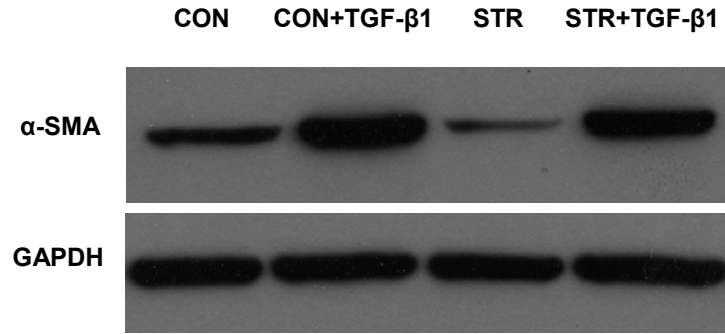


Figure 14a Western blot analysis for α -SMA further confirmed the immunofluorescence staining results. GAPDH was used as an internal loading control.

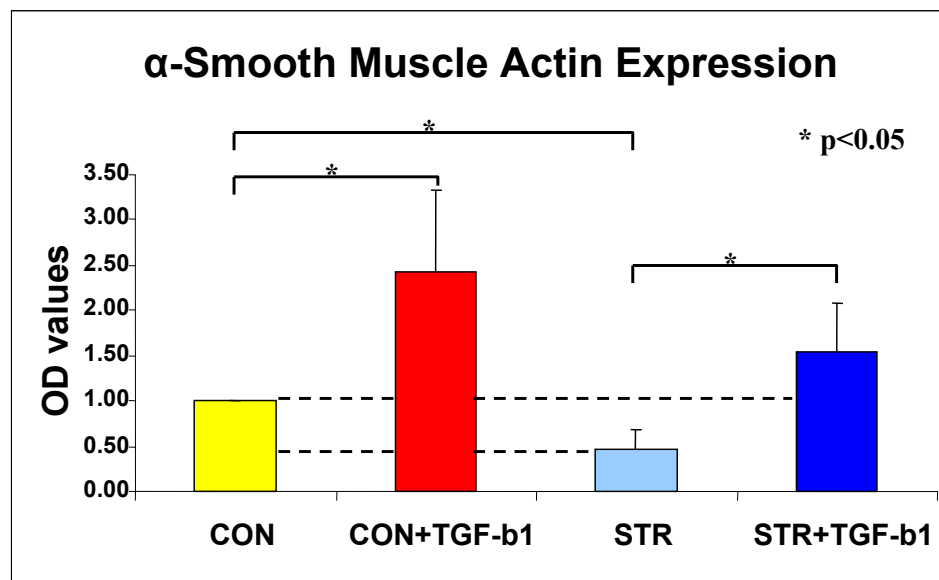


Figure 14b Western blot analysis for α -SMA further confirmed the immunofluorescence staining results. GAPDH was used as an internal loading control. The data shown here were based on three experiments (n=3) with cells from two donors. Overly saturated bands may have resulted in an underestimated difference.

In addition, h_1 -calponin was downregulated by ~50% as evidenced by immunofluorescence staining (**Figure 15**) and Western blot analysis (**Figure 16**). Cells cultured under static conditions in media supplemented with TGF- β_1 demonstrated a significant increase in the expression of α -SMA and h_1 -calponin, marked by the presence of pronounced actin stress

fibers. ASCs cultured under strained conditions in media supplemented with TGF- β_1 expressed α -SMA and h-1calponin at levels nearly comparable to that of the static controls treated with TGF- β_1 .

Figure 15 depicts immunofluorescence staining results for h₁-calponin. Control ASCs & strained ASCs (with or without TGF- β_1) were stained positive for calponin. Similar to the expression of α -SMA, strain alone reduced the expression of calponin significantly, whereas the addition of TGF- β_1 increased the expression drastically.

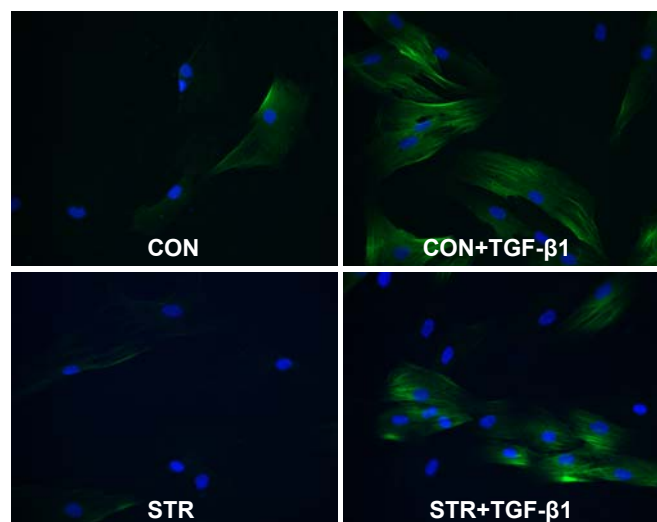


Figure 15 Immunofluorescence staining for h₁-calponin.

Again, the immunofluorescence staining results for calponin were confirmed with Western blot analysis. The bands were quantified using the same methods. Similarly, the addition of TGF- β_1 induced a 2 fold increase over the control. Strain alone reduced the expression of calponin by 40%. Strain & TGF- β_1 combined brought the expression of calponin back to about 3.5 fold over the control.

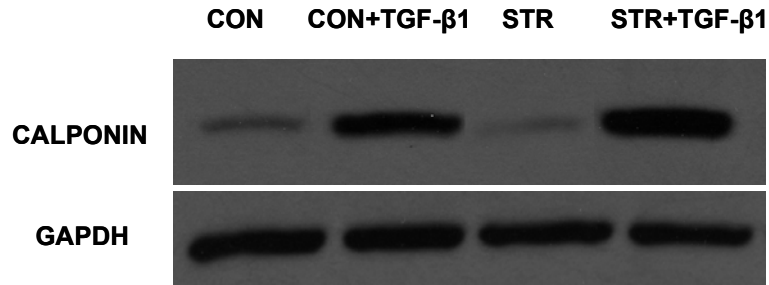


Figure 16a Western blot analysis for calponin further confirmed the immunofluorescence staining results.

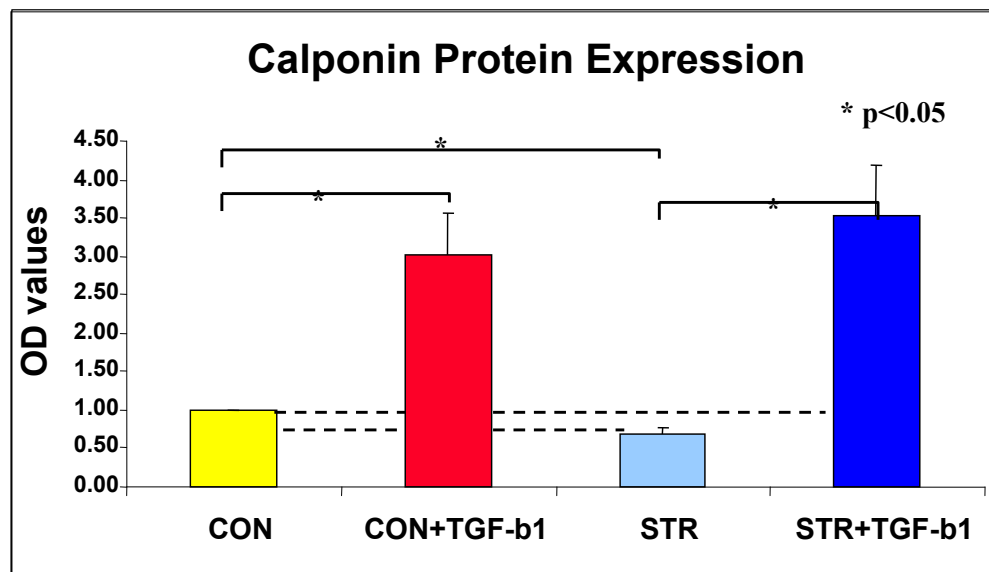


Figure 16b Western blot analysis for calponin further confirmed the immunofluorescence staining results. The data shown here were based on three experiments (n=3) with cells from two donors. Overly saturated bands may have resulted in an underestimated difference.

4.3.3 Summary of Results

In this study, we demonstrated that cyclic strain inhibited proliferation, and caused alignment of the cells and of the F-actin cytoskeleton perpendicular to the direction of strain. Cyclic strain also regulated α -SMA and h₁-calponin protein expression in ASCs. 10% uniaxial

cyclic strain alone down-regulated α -SMA and h₁-calponin protein expression in ASCs, whereas TGF- β ₁ up-regulated the expression of α -SMA and h₁-calponin. In addition, F-actin staining results of control ASCs or strained ASCs treated with TGF- β ₁ were counter-intuitive. Not only were increased F-actin stress fibers not observed in TGF- β ₁ treated cells, F-actin staining results also did not correspond with α -SMA or calponin immunostaining results.

4.4 CARDIOMYOGENIC DIFFERENTIATION POTENTIAL OF ASCS

4.4.1 Effects of 5-azacytidine on ASCs

We have conducted studies on the potential of ASCs to differentiate into cardiomyocyte-like cells via exposure to 5-azacytidine. ASCs were seeded at different cell densities, and were treated with 5-azacytidine for 24 hr, 1 week, 4 weeks, and up to 8 weeks using repeated exposure and single exposure treatment methods as described in literature. Spontaneously and synchronously pulsating cardiomyocyte-like cells were not observed. **Figure 17** depicts phase-contrast images of control ASCs and ASCs treated with 10 μ M of 5-aza after 4 weeks in culture (via repeated 5-azacytidine treatment). As shown in this figure, ASCs treated with 5-aza seemed less confluent and demonstrated a different morphology. ASCs that were repeated exposed to 5-azacytidine for 4 weeks were indistinguishable from control ASCs.

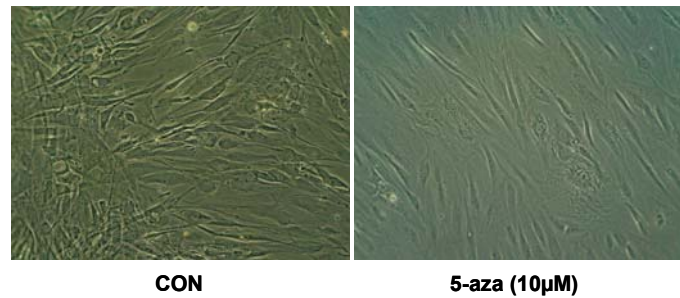


Figure 17 Phase-contrast images of control ASCs and ASCs treated with 10 μ M of 5-aza for 3 weeks in culture (from repeated treatment group). ASCs treated with 5-aza are less confluent and have a different morphology.

Untreated ASCs and 5-azacytidine treated ASCs were stained for cytoskeletal protein and early smooth muscle cell marker α -SMA. Control ASCs and ASCs that were transiently exposed to 5-azacytidine expressed high levels of this protein, whereas the expression of α -SMA was significantly reduced in ASCs that were repeated treated with 5-azacytidine (as shown in **Figure 18**). Western blot analysis further confirmed the reduction in α -SMA expression (**Figure 19**).

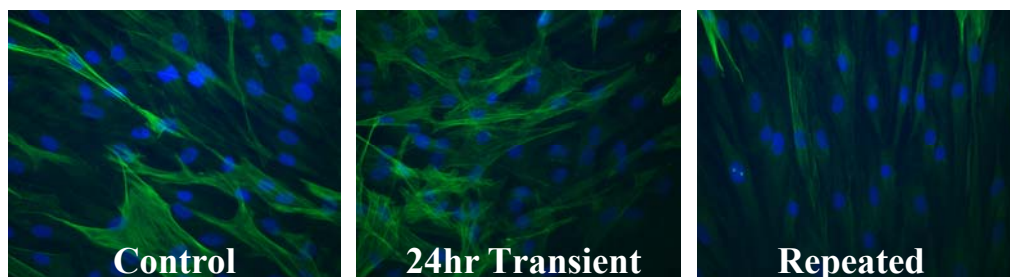


Figure 18 Immunofluorescence staining results for α -SMA (green), counter-stained with DAPI (blue). (A) Control ASCs that were cultured in the absence of 5-azacytidine for 4 weeks. (B) ASCs that were transiently exposed to 5-azacytidine for 24 hrs, and cultured in regular plating media with 10% serum for 4 weeks. (C) ASCs that were repeatedly treated with 5-azacytidine for 4 weeks in culture.

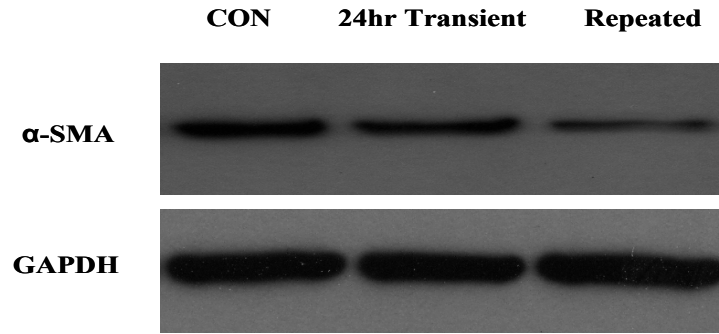


Figure 19 Western blot results for α -SMA. There was a significant reduction in the expression of α -SMA in ASCs that were repeatedly exposed to 5-azacytidine.

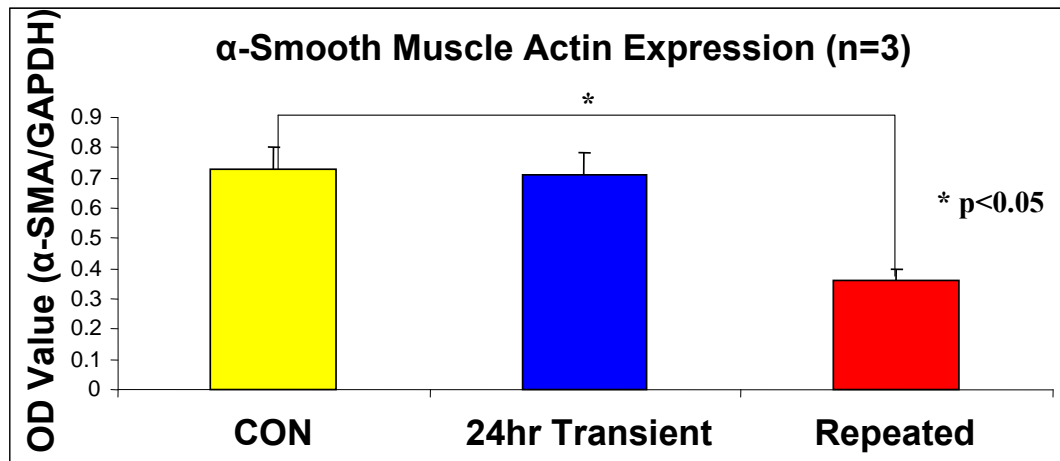


Figure 20 Optical density (OD) values of Western blot bands for α -SMA were measured and quantified. The OD value of α -SMA was divided by the OD value of GAPDH for bands representing each treatment group. There was no significant difference in the expression of α -SMA between CON and ASCs that were transiently exposed to 5-azacytidine. ASCs that were repeatedly treated with 5-azacytidine showed a significant decrease (-48%) in α -SMA expression as compared with control ASCs.

Control ASCs and 5-azacytidine treated ASCs were also stained for gap junction protein connexin-43. After 1 week in culture, control ASCs and ASCs that were transiently exposed to 5-azacytidine expressed low levels of connexin-43. The expression of Cx-43 was merely present

in ASCs that were repeatedly exposed to 5-azacytidine after 1 week (3 treatments of 5-azacytidine). After 4 weeks in culture, there was a significant increase in the expression of Cx-43 in all treatment groups, as shown in **Figure 21**. The expression of Cx-43 seemed independent of exposure to 5-azacytidine, but dependent on cell density or confluency.

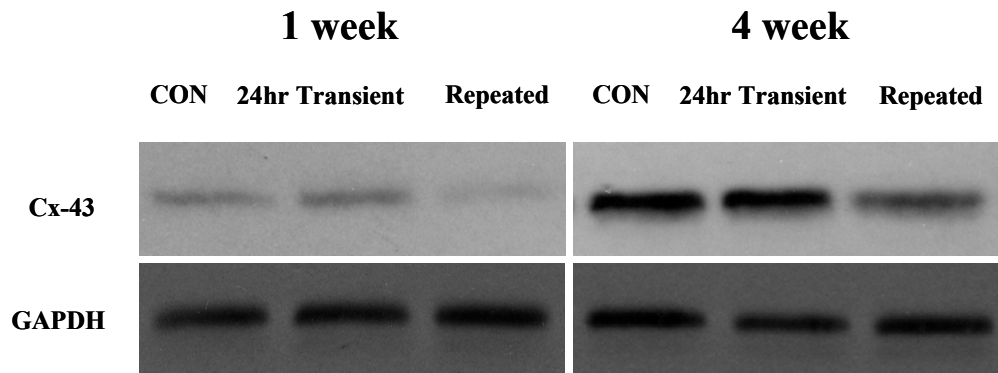


Figure 21 Western blot results for gap junction protein connexin 43. GAPDH was used as an internal loading control. There was a significant increase in the expression of Cx-43 after 4 weeks in culture. The increase in Cx-43 protein expression was dependent on cell density.

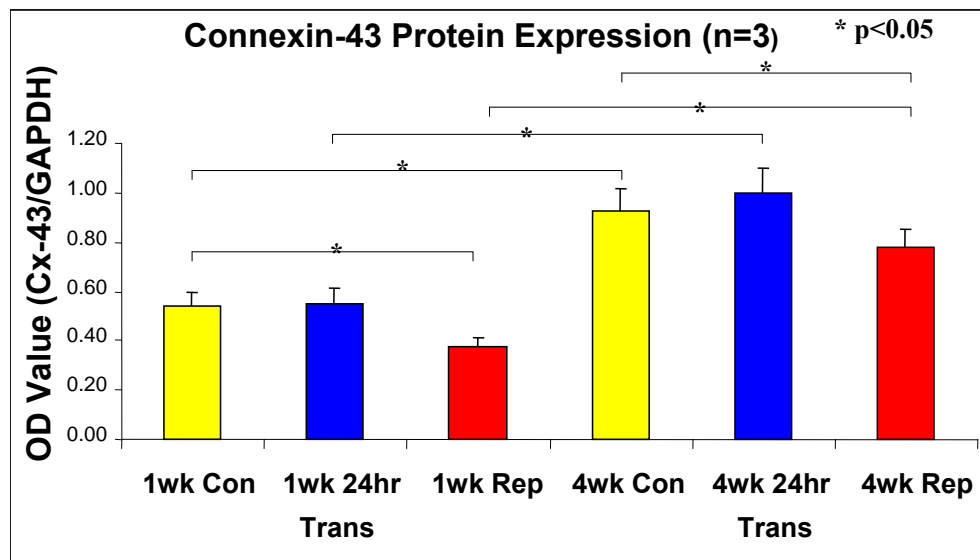


Figure 22 Ratios of OD values (Cx-43/GAPDH). Data were collected from three separate experiments. There was a 30% reduction in the expression of Cx-43 in ASCs that were repeatedly exposed to 5-azacytidine after 1 week or 4 weeks in culture. A 70% increase in Cx-43 expression was noted between corresponding 1 week and 4 week samples from all treatments.

Control ASCs and 5-azacytidine treated ASCs were also stained for cardiac specific markers sarcomeric α -actinin (**Figure 23**). Control ASCs, ASCs that were transiently or repeated exposed to 5-azacytidine for 4 weeks were negative for α -actinin. Western blot data confirmed the negative findings (**Figure 24**). The 5-azacytidine treated ASCs were also negative for cardiac troponin I and troponin T. Sections of the human heart were used as positive control. Protein collected from cultured fetal human cardiomyocytes was used as positive control for Western blot analysis.

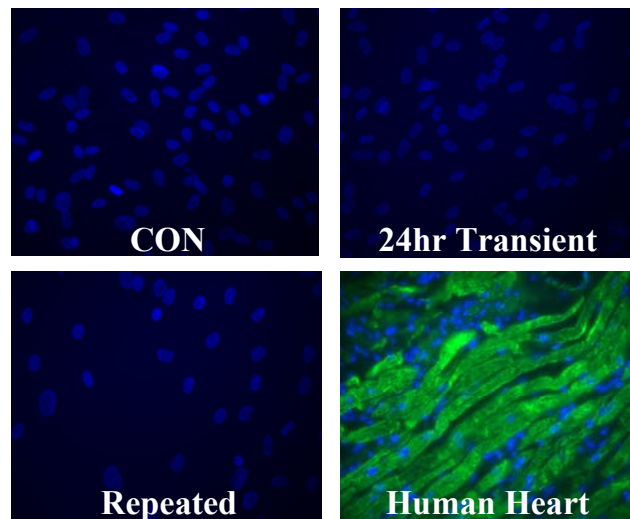


Figure 23 Immunofluorescence staining results for α -actinin (green), counter-stained with DAPI (blue). (A) Control ASCs that were cultured in the absence of 5-azacytidine for 4 weeks. (B) ASCs that were transiently exposed to 5-azacytidine for 24 hrs, and cultured in regular plating media with 10% serum for 4 weeks. (C) ASCs that were repeatedly treated with 5-azacytidine for 4 weeks in culture. (D) Human heart (positive control).

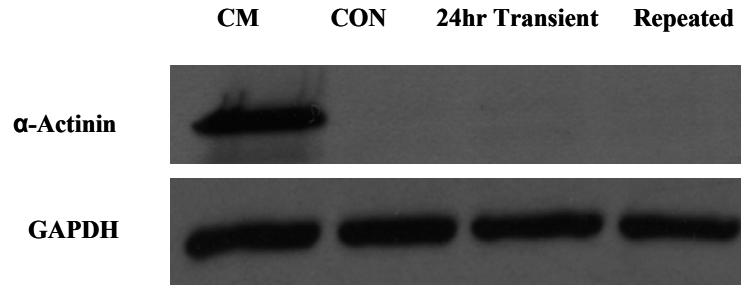


Figure 24 Western blot analysis results for α -actinin. α -actinin protein expression was undetected in control ASCs and 5-azacytidine treated ASCs.

The expression of cardiac specific transcription factors Nkx2.5 and GATA4 in control ASCs and 5-azacytidine treated ASCs was also examined. ASCs (regardless of exposure to 5-azacytidine) were negative for both Nkx2.5 and GATA4, as shown in **Figure 25** and **Figure 26**.

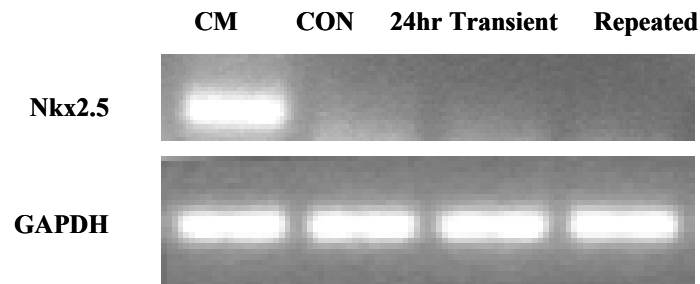


Figure 25 Gene expression of cardiac transcription factor Nkx2.5. Control ASCs and 5-azacytidine treated ASCs were negative for Nkx2.5. cDNA from fetal human heart was used as the positive control.

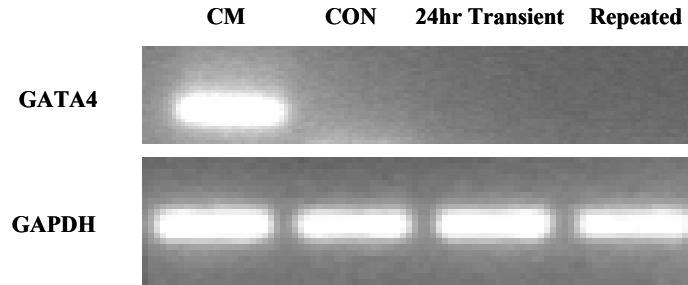


Figure 26 Gene expression of cardiac transcription factor GATA4. Control ASCs and 5-azacytidine treated ASCs were negative for GATA4.

4.4.2 Summary of Results

In summary, repeated exposure of ASCs to 5-azacytidine inhibited cell growth and significantly reduced α -SMA protein expression. The expression of gap junction protein connexin-43 was up-regulated in ASCs in long term cultures, independent of 5-azacytidine exposure, but dependent on cell density. Protein expression of sarcomeric α -actinin, cardiac troponin I and troponin T was undetected in 5-azacytidine treated ASCs. Expression of cardiac transcription factors Nkx2.5 and GATA4 was also undetected.

5.0 DISCUSSION

5.1 RESPONSE OF ASCS TO TGF- β_1 , PDGF-BB, BME, AA, RA, AND D-CAMP

There is an extensive body of literature describing the role of specific growth factors or cytokines in the regulation of smooth muscle cell differentiation. Some of these factors include transforming growth factor- β_1 (TGF- β_1), platelet-derived growth factor BB (PDGF-BB), β -mercaptoethanol (BME), ascorbic acid (AA), retinoic acid (RA), and dibutyl-cyclic adenosine monophosphate (d-cAMP). Differentiating smooth muscle is characterized by the sequential appearance of cytoskeletal and contractile proteins to yield the variety of phenotypes that typify adult smooth muscle cells [78]. One of the first smooth muscle cell markers to be expressed during smooth muscle cell differentiation is α -smooth muscle actin (α -SMA) [78].

The specific objectives of the first aim of this thesis work were to investigate the effects of TGF- β_1 , PDGF-BB, BME, AA, RA, and d-cAMP on the expression of α -SMA during ASC differentiation. These growth factors have been demonstrated to either up or down-regulate α -SMA expression in marrow-derived MSCs and embryonic progenitor cells in several previous studies [64-70].

Protein characterization conducted in this study demonstrated that the expression of α -SMA in ASCs significantly increased in the presence of TGF- β_1 , while α -SMA expression only slightly increased with RA, BME and AA. Exposure to PDGF-BB, RA and d-cAMP resulted in a dramatic decrease in the expression of α -SMA.

5.1.1 Transforming Growth Factor- β_1

Transforming growth factor- β_1 is secreted by many cell types, including vascular endothelial and smooth muscle cells, and essentially all cells have receptors for TGF- β_1 [97]. TGF- β_1 can have an inhibitory effect on smooth muscle cell proliferation, but can also have a mitogenic effect on the cells under certain conditions [98]. There are reports that TGF- β_1 can increase extracellular matrix production by smooth muscle cells [99-100]. Contractile protein expression can be increased by TGF- β_1 , not only in SMCs, but also in neural crest cells, embryonic stem cells, and mesenchymal stem cells [101, 68-69], suggesting that TGF- β_1 may play a role in vascular tissue development. The effect of TGF- β_1 in up-regulating the expression of α -SMA in ASCs as observed in this study is consistent with prior findings of similar effects of TGF- β_1 on other cell types, including progenitor cells.

5.1.2 Platelet-derived Growth Factor BB

Platelet-derived growth factor is secreted by a number of cell types found in the vasculature, including endothelial cells, smooth muscle cells, and activated macrophages [97], and PDGF-BB can produce a mitogenic response in cultured VSMCs [98]. PDGF-BB has also been shown to have potent effects on cells of mesenchymal origin [102]. There is growing evidence that PDGF-BB is involved in the pathogenesis of arteriosclerosis, in which PDGF is hypothesized to promote smooth muscle cell migration, proliferation and matrix production typically associated with intimal thickening [98]. Li *et al.* reported that PDGF-BB caused decreased α -SMA expression in vascular smooth muscle cells via a pathway separate from its effect on proliferation (which is hypothesized to be responsible for the shift toward the synthetic

phenotype) [100]. Consistent with literature findings, we have observed that ASCs respond to PDGF-BB in similar fashions as VSMCs and MSCs. After exposure to PDGF-BB for two weeks, ASCs were highly proliferative, and demonstrated a marked decrease in the expression of α -SMA.

5.1.3 Retinoic Acid

Retinoic acid, an active form of retinoids, is involved in signal transduction pathways regulating embryonic development [103]. Retinoids influence differentially the features of distinct SMC phenotypes. Additionally, all-trans retinoic acid can modulate proliferation, migration, plasminogen activator activity, and α -smooth muscle actin expression in SMCs [104]. d-cAMP can act synergistically with RA in several molecular and developmental processes; however, molecular mechanisms of cooperation between RA and d-cAMP is not known. Suzuki *et al.* [66] have shown that the treatment of a P19 cell line resulted in significant smooth muscle cell differentiation enhancement, whereas Drab *et al.* [67] reported the formation of spontaneously contracting smooth muscle cell-like cell clusters in the presence of high concentration retinoic acid and d-cAMP. These results were not observed in this study, indicating that ASCs and totipotent embryonic stem cells are fundamentally different, and respond differently to biochemical stimuli such as RA and d-cAMP. A slight increase in α -smooth muscle actin expression was observed in ASCs cultured with RA alone, whereas a significant decrease in α -smooth muscle actin expression was noted with cells treated with RA and d-cAMP.

5.1.4 Ascorbic Acid

L-Ascorbic acid (L-Asc) and its derivatives, L-Ascorbic acid 2-phosphate, have been shown to have some effects on the cardiovascular system, including growth inhibition of VSMCs [105]. Moreover, Arakawa *et al.* [64-65] have found that L-Asc stimulates the differentiation of pluripotent stem cells into smooth muscle-like cells, which is similar to what we have observed (a significant increase in the expression of α -SMA) when culturing ASCs in the presence of L-Asc and BME (a reducing agent that breaks down toxic cell metabolites).

5.1.5 Conclusion and Future Studies

It is poorly understood what factors are involved in mesenchymal cell differentiation toward the SMC lineage. The complete set of biochemical and mechanical signals that regulate SMC function *in vivo* is not clear, though it has been demonstrated that changes in environmental conditions can lead to phenotype shifts. Removal of VSMCs from their native environment alters the biochemical and mechanical signals responsible for maintaining normal cell function, causing a shift from a quiescent, contractile phenotype to a more proliferative, synthetic state.

The function of SMCs in their native environment is influenced by a wide variety of biochemical factors, many of which interact to create complex signaling mechanisms. Recreating this level of control *in vivo* is a challenging task. It is possible to use selected biochemicals to modulate cell function and phenotype for vascular tissue engineering applications; however it is difficult to select the appropriate bioactive molecules for the homing of these cells into cells of the vasculature. Factors that need to be taken into account include optimal concentration of each molecule, duration, succession of incidence. Furthermore, it may not be possible for such simple

growth factor-driven differentiation events to occur *in vitro* in the absence of other important physiological signals experienced by cells in their native environment. Future studies include examining the role of hemodynamic forces in cell differentiation and phenotype modulation.

5.2 EFFECTS OF MECHANICAL STRAIN ON ASCS

It has been shown that ASCs can differentiate into a variety of cells via an array of bioactive molecules that have been well defined, and have immense potential as a cell source for regenerative medicine. However, it is not well known how these cells respond to the forces that they would be subjected to *in vivo*. A recent study by Knippenberg *et al.* [106] examined whether ASCs are responsive to mechanical loading via pulsating fluidflow (PFF) and reported osteogenic differentiation of ASCs. This study was one of the first to address the effects of mechanical forces on ASCs; however, the effects of other types of forces such as cyclic strain on ASCs remain unexplored.

Prior studies have demonstrated that MSCs in long-term culture can differentiate into SMCs and that TGF- β_1 , enhances the expression of SMC-specific markers [68, 107]. The effects of the vascular mechanical environment on MSCs have been recently examined [81-83]. Hamilton *et al.* [81] demonstrated that cyclic strain resulted in the expression of vascular smooth muscle α -actin and *h1*-calponin in rat bone marrow-derived progenitor cells. Kobayashi *et al.* [82] reported that long term incubation and subsequent compressive strain, or cyclic uniaxial strain alone (not equiaxial strain) promotes the expression of smooth muscle-like properties in MSCs [83].

In aim 2 of this thesis work, we demonstrated that mechanical strain regulated α -SMA and calponin protein expression in ASCs and that uniaxial strain alone induced a decrease in the α -SMA and calponin protein expression. Our results suggest that uniaxial strain downregulates the expression of SMC contractile marker in ASCs. Cyclic strain of 10% at 1Hz for 7 days downregulated α -SMA and α 1-calponin protein expression in ASCs, whereas TGF- β_1 upregulated α -SMA expression and resulted in prominent actin stress fibers.

5.2.1 Importance of Cell Orientation on Cellular Response to Strain

Mechanical forces have been shown to induce various biological responses, including cell reorientation, actin cytoskeletal remodeling, altered cell proliferation, gene expression, and protein synthesis [108]. It is also well recognized that mechanical forces can alter cellular phenotype. Various *in vitro* model systems, such as the Flexcell Strain Unit used in this study, have been developed to examine the effects of mechanical forces on cells. Typically, these systems utilize deformable materials with smooth surfaces to culture cells. There are limitations to these systems. Cells randomly orient themselves prior to mechanical stimulation, and once exposed to mechanical strain, cells reorient in a direction with minimal deformations to minimize strain experienced by the cell bodies. Smooth culture surfaces do not allow control of cell orientation [108].

We postulate that the dramatic decrease of protein expression under uniaxial cyclic strain alone may be partly due to the reorientation of cells cultured on smooth surfaces, as reported by other investigators [83]. It may be possible that sustained uniaxial strain was not sensed by the cells. Cells *in vivo* are well organized, have a defined shape or morphology, and remain in a certain orientation. For example, vascular SMCs in their native environment assume an

elongated morphology, align in the circumferential direction, and are parallel to the direction of strain exposed on them due to the pulsatile nature of blood flow. Additionally, vascular SMCs upregulate their expression of contractile SMC markers when exposed to strain. Therefore, the responses of cells to uniaxial strain may not closely mimic those observed *in vivo*.

As cell organization, morphology, and orientation appear to influence cell function and phenotype, it is necessary to utilize an *in vitro* system that can readily mimic *in vivo* mechanical loading conditions. Using microfabrication technology, investigators have developed microgrooved silicone surfaces and/or extracellular matrix micropatterning to keep cells ranging from human patellar tendon fibroblasts [108] to vascular SMCs and cardiomyocytes [109-110] aligned in the direction of strain *in vitro* while exposed to repetitive mechanical stretching.

Our results also suggest that although uniaxial cyclic strain closely mimics the type of mechanical strain experienced by native vascular SMCs, and upregulates SMC specific protein markers when applied to bone marrow-derived MSCs [81-83], it is possible that the parameters (i.e., 10% strain at 1Hz, 7 days) of mechanical stimulation used in this experiment may not be optimal for ASCs.

5.2.2 Role of TGF- β_1 and Mechanical Strain

The last variable in our study, growth factor addition, is as important as both the mechanical stimulation and surface matrix. We incorporated TGF- β_1 into our studies, both with and without strain. Transforming growth factor- β is secreted by many cell types, and essentially all cells have receptors for TGF- β . TGF- β is secreted by cells in an inactive form and must be cleaved by the action of proteolytic enzymes or other methods to become active. Complex positive and negative control mechanisms exist to regulate the activation and function of TGF- β

in vivo. TGF- β plays an important role in cell growth, differentiation, migration, and extracellular matrix production. It is unclear from our study whether mechanical loading is an independent factor in the regulation of smooth muscle specific protein expression or its effect is mediated by factors such as TGF- β_1 . Grainger *et al.* [111] and Hautmann *et al.* [112] have reported that the SMC contractile phenotype is regulated by TGF- β , and that TGF- β can increase α -SMA expression in MSCs [68-69]. The addition of TGF- β_1 to each of the different culture conditions in our experiments caused a significant increase in the protein expression of α -SMA and h_1 -calponin.

5.2.3 Conclusion and Futures Studies

In summary, the results of this study indicate that cyclic mechanical strain influences ASC orientation, growth and phenotype. The effect of mechanical forces on the growth and differentiated properties of ASCs is not well understood. Little is known about how individual cells respond to mechanical strain or the underlying molecular mechanisms by which mechanical events are transduced into physiological responses. Recent studies, together with our current results, suggest that the type of mechanical strain, matrix environment, and/or additional chemical factors may result in different cellular responses. Future studies include controlling cell orientation *in vitro* to ensure sustained uniaxial cyclic strain is sensed by the cells, optimizing the magnitude and frequency of strain, and duration of experiment.

5.3 CARDIOMYOGENIC DIFFERENTIATION POTENTIAL OF ASCS

5.3.1 Cardiomyogenic Differentiation in Marrow-derived MSCs via 5-Azacytidine Stimulation

The ability of 5-azacytidine to induce cardiomyogenic gene and protein expression in stem cells or progenitor cells has been well-documented by several different groups over the last few decades. Although Constantinides *et al.* [113] were the first to describe the appearance of multinucleated striated muscle cells in non-myoblast precursors following 5-azacytidine treatment, it was Makino *et al.* [84] who initially described the appearance of cardiomyocytes from immortalized murine bone marrow-derived MSCs. Following this report, Tomita *et al.* [114] reported that rat MSCs in a primary culture were also capable of achieving this phenotype. However, recent studies by other investigators have failed to reproduce these findings. Zhang *et al.* reported passage-restricted differentiation potential of rat MSCs into cardiomyocyte-like cells [115]. Only P4 but not P1 and P8 bone marrow-derived rat MSCs showed formation of myotubes and expressed cardiomyocyte-specific markers after exposure to 5-azacytidine. In addition, Liu *et al.* were unable to obtain spontaneously beating cells after exposing primary and passaged Wistar rat MSCs to various concentrations of 5-azacytidine using single or repeated treatment methods [116].

5.3.2 Effects of 5-azacytidine on ASCs

Bone marrow is not the only source of cells that are capable of cardiomyogenic differentiation. Recently, three groups have reported *in vitro* differentiation of stem cells derived

from the adipose tissue into cardiomyocyte-like cells [40, 95-96]. Although distinct induction methods were used, each group reported spontaneously beating cells and evidence of expression of cardiomyocytic cell surface markers. Planat-Bernard *et al.* [95] cultured fresh murine ASCs in semi-solid methylcellulose medium supplemented with cytokines, and observed colonies of beating cells after three weeks in culture. However, passaged cells could not be induced into beating cell colonies exhibiting molecular and electrophysiologic properties of cardiomyocytes using identical semi-solid medium. Gaustad *et al.* [96] reported protein expression of cardiac specific markers in human ASCs after transient exposure to nuclear and cytoplasmic extracts of rat cardiomyocytes.

Rangappa *et al.* [40] reported that mesenchymal stem cells derived from rabbit fatty tissue could transform into contractile cardiomyocyte-like cells via exposure to 5-azacytidine. A similar approach was applied in the present study as an attempt to further assess the effects of 5-azacytidine on the fate of stem cells derived from human adipose tissue. The novel conversion of rabbit ASCs into beating cardiomyocyte-like cells as reported by Rangappa *et al.* was not observed in our study with human ASCs. Three different concentrations of 5-azacytidine (5, 9, 10 μ M), different cell densities, and treatments methods were examined, but neither the formation of myotubes nor presence of spontaneously beating cells were noted after 8 weeks in culture. Protein expression of cardiac-specific markers such as α -actinin, cardiac troponin I, troponin T was undetected. Expression of cardiac transcription factors Nkx2.5 and GATA4 were also absent. The specificity and reliability of the detection techniques were confirmed using human fetal cardiomyocytes. Gap junction protein connexin-43 was present, although the level of expression was independent of exposure to 5-azacytidine, but dependent on cell density/confluency. Cytoskeletal protein α -SMA was present in control ASCs and 5-azacytidine

treated ASCs, although the expression of α -SMA was significantly reduced in ASCs that were repeated exposed to 5-azacytidine.

5.3.3 Non-specific Activity of 5-azacytidine

5-azacytidine is a chemical analogue of the cytosine nucleoside used in DNA and RNA. Cells in the presence of 5-azacytidine incorporate it into DNA during replication and RNA during transcription. The incorporation of 5-azacytidine into DNA or RNA inhibits methyltransferase thereby causing demethylation in that sequence, affecting the method that cell regulation proteins are able to bind to the DNA/RNA substrate [117].

Genetic reprogramming by 5-azacytidine may not be specific. As a cytosine analogue, 5-azacytidine has been reported to cause phenotypical changes in a number of cell types by activating novel gene expressions both in vitro and in vivo [118]. Treatment with 5-azacytidine causes phenotypic conversions of fibroblasts to adipocytes and myocytes [119] and induces erythroid differentiation in erythroleukemia cells [120]. Although the precise mechanisms and effects of 5-azacytidine are not well-understood, it has been suggested that the cytosine analogue may activate a number of silent genes by inhibiting DNA methylation or induce changes in other specific genes to trigger their response to exogenous stimulation [121]. As Liu et al. [116] have speculated, it is possible that 5-azacytidine-induced cardiomyogenic differentiation can only occur in certain immortalized cell lines of specific animal species due to random reprogramming of gene expression in response to the effects of 5-azacytidine.

5.3.4 Conclusion and Future Studies

DNA demethylation agent 5-azacytidine has been widely described in literature as an effective chemical stimulus used to promote cardiomyogenic differentiation in various cell types, ranging from embryonic stem cells, P19 cells, bone marrow-derived mesenchymal stem cells, and recently to adipose-derived stem cells. Of interest and importance is the observation that human ASCs may not be capable of differentiating into cardiomyocyte-like cells via exposure to 5-azacytidine. Our results indicate that exposure to 5-azacytidine is not likely to be clinically applicable due to its low efficacy and potentially harmful effects of its non-specific demethylating activity.

Future studies include examining the effects of other chemical stimuli and direct cell-cell contact on ASC differentiation along the cardiomyogenic lineage. A recent study conducted by Behfar *et al.* [127] identified an array of recombinant cardiogenic agents that were successful in collectively inducing a cardiogenic phenotype in human bone marrow-derived mesenchymal stem cells after 21 days in culture. The cardiogenic agents employed include transforming growth factor- β_1 , bone morphogenetic protein 2, insulin-like growth factor 1, fibroblast growth factor 4, interleukin-6, leukemia inhibitory factor, α -thrombin, vascular endothelial growth factor A, tumor necrosis factor- α , and retinoic acid [127]. It is possible that ASCs may respond similarly to the cardiogenic agents as the marrow-derived MSCs.

In addition to chemical stimuli, it has been hypothesized that intercellular crosstalk may play a critical role in directing the progression of stem cells into differentiated cells [93-94]. Recent studies have shown that intercellular interaction between adult human mesenchymal stem cells and adult human cardiomyocytes could induce stem cells to acquire the phenotypical characteristics of functional cardiomyocytes. By co-culturing human MSCs with human

cardiomyocytes, it was demonstrated that MSCs acquired a cardiomyocyte-like phenotype characterized by the expression of myosin heavy chain and troponin T [93-93]. However, when MSCs were incubated with a cardiomyocyte-conditioned medium, cardiac specific protein expression was not observed. Thus, direct cell-to-cell contact may be obligatory in relaying cardiac environmental or microenvironmental signals for successful MSC differentiation along the cardiomyogenic lineage [93-94]. These data suggest that human ASCs may also benefit from environmental cues resulting from direct cell-cell contact with cultured cardiomyocytes.

5.4 SUMMARY

In summary, adipose tissue, like bone marrow, contains a population of cells that has extensive self-renewal capacity and the ability to differentiate along multiple lineages. These cells can be obtained in large numbers at high frequency from a tissue source that can be extracted in large quantities with minimal morbidity. Like marrow-derived mesenchymal stem cells, adipose-derived stem cells have been shown to differentiate into cells that express markers of endothelial, smooth muscle, and cardiomyocytic differentiation.

The differential response of ASCs to biomolecules such as BME, AA, and RA suggests that although adipose tissue and bone marrow are both derived from embryonic mesoderm and contain a heterogeneous stromal cell population and share many similarities, stem cells isolated from adipose tissue and bone marrow are fundamentally different and respond to exogenous biochemical stimulation differently. In addition, our data on the effects of cyclic strain on ASCs led us to conclude that ASCs also respond to mechanical stimulation differently than marrow-derived MSCs. Furthermore, species differences and non-specific activity of DNA demethylation

agent 5-azacytidine could have led to the unexpected results with cardiomyogenic differentiation of human ASCs.

This study has advanced our understanding of the biochemical and mechanical regulation of adipose-derived stem cells. These findings, combined with the high yield of cells from adipose, suggest that adipose tissue represents a potential clinically useful source of cells for cellular therapy and tissue engineering applications targeting at cardiovascular disorders. Although the effects of mechanical loading, exogenous biochemical stimulation, and the crosstalk between mechanical and chemical factors still remain to be elucidated, further investigation is underway.

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