

HUMAN MESENCHYMAL STEM CELLS
EXPRESS A MYOFIBROBLASTIC PHENOTYPE *IN VITRO*

BY

MELANIE ALLISON NGO

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FOR THE THREE ANGELS IN MY LIFE

MOM, AUNTIE DARLENE & CHAU

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ABSTRACT

There is emerging evidence to suggest that cardiac myofibroblasts (CMyfbs) participating in cardiac fibrosis represent a heterogeneous population in origin. We hypothesized that bone marrow derived mesenchymal stem cells (MSCs) readily adopt a myofibroblastic phenotype in culture.

We assessed and compared human primary MSCs and human CMyfbs with respect to their phenotypic and functional characteristics by examining their gene expression profile, ability to contract collagen gels, and ability to synthesize collagen. We also examined the role of non-muscle myosin II (NMMII) in modulating the myofibroblast function using siRNA and blebbistatin to inhibit NMMII activity.

The data revealed that MSCs adopt a myofibroblastic phenotype in culture and demonstrate the capability to contract collagen gels and synthesize collagen similar to human CMyfbs. Inhibition of NMMII activity with blebbistatin completely inhibits gel contractility without affecting cell viability. Thus, MSCs exhibit similar physiological and functional characteristics as CMyfbs, and may contribute to cardiac fibrosis.

TABLE OF CONTENTS

Dedication	ii
Acknowledgements	iii
Abstract	iv
Table of Contents	v
List of Tables	viii
List of Figures	ix
List of Abbreviations	xi
I. REVIEW OF THE LITERATURE	1
1. Cardiac fibrosis	1
2. Myofibroblasts	3
2.1. Origin	3
2.2. Function	5
3. Mesenchymal stem cells	6
3.1. Origin	6
3.2. Therapeutic potential	7
3.3. Plasticity and differentiation	8
4. Actin-myosin cytoskeleton	10
5. Function of non-muscle myosin II	11
6. Rationale for study	14

II. MATERIALS AND METHODS	17
1. Isolation and culture of human MSCs and human CMyfbs.....	17
2. Differentiation of MSCs	18
2.1 Adipogenesis	18
2.2 Osteogenesis	19
2.3 Chondrogenesis	19
3. Immunofluorescence	19
4. Fluorescence-activated cell sorting analysis.....	20
5. Immunoblot analysis	20
6. Real-time PCR Analysis	21
6.1 RNA extraction	21
6.2 Real-time PCR	22
7. Inhibition of NMMII	22
7.1 Blebbistatin administration	22
7.2 Knockdown of NMMII siRNA	22
8. Collagen gel contraction assay	23
9. Measurement of type I collagen synthesis	24
10. Live dead assay	25
11. Reagents	25
12. Antibodies	27
13. Statistical analysis	27

III. RESULTS	29
1. Human MSCs functionally express a myofibroblastic phenotype <i>in vitro</i>	29
1.1. Phenotypic characterization of human MSCs	29
1.2. Functional characterization of human MSCS	37
2. Myosin expression in hMSCs	42
3. Effect of NMMII inhibition on hMSC phenotype	43
3.1. Blebbistatin	43
3.2. siRNA knockdown	45
4. Effect of NMMII inhibition on gel contractility	50
5. Effect of NMMII inhibition on cell viability	53
IV. DISCUSSION	54
1. Significance of standard cell culture conditions in stimulating acquisition of a myofibroblast phenotype by human MSCs <i>in vitro</i>	54
2. Significance of NMMII inhibition on adoptive myofibroblast phenotype and function	59
V. CONCLUSION AND FUTURE DIRECTIONS	63
VI. REFERENCES	66

List of Tables

Table 1: Primer sequences	28
Table 2: Gene and protein names	28
Table 3: On-target SMART pool siRNA target sequences	28

List of Figures

Figure 1:	<i>In vitro</i> differentiation of hMSCs	30
Figure 2:	hMSCs display similar morphology to their cardiac derived myofibroblasts	31
Figure 3:	Bone marrow derived hMSCs display myofibroblast markers	33
Figure 4:	Early passage hMSCs express myofibroblast proteins	35
Figure 5:	Early passage hMSCs express myofibroblast mRNA	36
Figure 6:	hMSCs contract collagen gels to a similar degree as CMyfbs and display TGF- β 1 responsiveness	38
Figure 7:	hMSCs display increased contractility under serum free conditions	39
Figure 8:	hMSCs synthesize mature collagen type I independently of TGF- β 1 stimulation	41
Figure 9:	Myosin expression in hMSCs and CMyfbs	42
Figure 10:	Chronic NMMII inhibition with blebbistatin alters expression of myofibroblast markers over serial passage	44
Figure 11:	NMMIIA and IIB siRNA knockdown is efficient and specific	46
Figure 12:	NMMIIA and IIB protein knockdown expression is stable over 96 hours	48

Figure 13:	Inhibition of NMMII alters α -SMA and F-actin organization	49
Figure 14:	NMMII inhibition with blebbistatin reduces gel contractility in hMSCs	51
Figure 15:	Inhibition of NMMII with blebbistatin reduces collagen gel contractility greater than NMMII specific isoform knockdown	52
Figure 16:	Inhibition of NMMII does not affect cell viability	53

List of Abbreviations

α -SMA	alpha-smooth muscle actin
ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BB	blebbistatin
BCA	bicinchoninic acid
BM-MSC	bone marrow-derived mesenchymal stem cells
BSA	bovine serum albumin
CICP	carboxyterminal propeptide of type I collage
CMyfb	cardiac myofibroblast
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ECM	extracellular matrix
ED-A	extra domain A
EDTA	ethylene diamine tetraacetic acid

EGTA	ethylene glycol tetraacetic acid
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
hCMyfb	human cardiac myofibroblast
hMSC	human mesenchymal stem cell
MI	myocardial infarction
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
NMMII	non-muscle myosin II
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFA	paraformaldehyde
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
rpm	rotations per minute
RT-PCR	real-time polymerase chain reaction
SEM	standard error mean

SMemb	embryonic smooth muscle myosin heavy chain
SMM	smooth muscle myosin
TGF- β 1	transforming growth factor beta ₁

I. REVIEW OF THE LITERATURE

1. Cardiac fibrosis

Heart failure is a frequent complication of myocardial infarction (MI) that is associated with adverse ventricular remodelling (Katz, 1995). The progression of heart failure occurs as a result of loss of ventricular muscle and the subsequent architectural alterations in both infarcted and noninfarcted areas, leading to reduced ventricular function (systolic and diastolic) and increased ventricular wall stress (Tomasek et al., 2002). In order to compensate, the injured heart adapts with increased ventricular mass (myocyte hypertrophy) and ventricular dilatation (Edgley et al., 2010). The ability of the adult heart to regenerate new, fully functional muscle is limited, and thus wound healing consists primarily of a fibrotic response. Myocardial healing and the development of fibrosis is a complex process that is multifactorial and involves an intricate interplay between several cell types, including inflammatory cells, endothelial cells, fibroblasts and myofibroblasts (van den Borne et al., 2010). Wound healing occurs in three overlapping phases: inflammation, proliferation and remodeling. In the inflammatory phase, local release of pro-inflammatory cytokines and recruitment of neutrophils and macrophages to the injured area, remove debris, pathogens and necrotic myocytes from the host tissue (Midwood et al., 2004). During the proliferative phase, fibroblasts deposit collagen in the injured area, granulation tissue is formed, endothelial cells promote angiogenesis and myofibroblasts contract the wound (Midwood et al., 2004). Tissue remodeling of the extracellular matrix (ECM) occurs and cells that are no longer needed are removed by apoptosis.

In the normal heart, there is a dynamic balance that exists between collagen deposition by fibroblasts and collagen degradation by matrix metalloproteinases (Edgley et al., 2010). In the injured heart, however, cardiac fibrosis occurs when the regulation of both ECM synthesis and degradation is disrupted, leading to an excess deposit of collagen fibers (type I and type III) with an increased degree of cross-linking of collagen fibers (van den Borne et al., 2010).

Maintaining the ECM in the scar is essential and can prevent dilation of the infarct area leading to heart failure. However, maladaptive ECM deposition at sites remote from the infarct area can lead to cardiac stiffness and provide the basis for the development of heart failure (van den Borne et al., 2010). Excessive accumulation of collagen within the ECM in the heart leads to increased stiffness of the myocardium that can impair ventricular diastolic relaxation (Swynghedauw, 1999). Moreover, by altering the cellular architecture of the ECM and cell-cell connections between myocytes, electrical conductance is interrupted, resulting in arrhythmias (Weber et al., 1994). Heterogeneity of the myocardium due to cardiac fibrosis may also affect systolic function (Wolk et al., 1999).

There are two types of pathological fibrosis: reactive fibrosis and reparative fibrosis. Reactive fibrosis occurs in response to loss of myocardial tissue due to myocyte death, whereas reparative fibrosis occurs in response to the inflammatory processes that accompany tissue injury (Swynghedauw, 1999; Beltrami et al., 1994; Anderson et al., 1979). Following acute MI, reactive fibrosis in the infarcted region is crucial to maintain structural integrity of the myocardium, but is also accompanied by reparative fibrosis both in the infarcted region and the remaining viable myocardium (Edgley et al., 2010).

Thus, pathological cardiac hypertrophy is associated with interstitial and perivascular fibrosis in the heart.

2. Myofibroblasts

2.1 Origin

The key cell mediators in myocardial fibrosis are cardiac myofibroblasts that are contractile and hypersecretory cells. There are no reports of myofibroblast activity in the healthy, uninjured heart (Porter and Turner, 2009). Cardiac interstitial fibroblasts differentiate into myofibroblasts upon activation following injury and subsequently migrate to the infarct area where they participate in scar formation and ECM remodeling, and are capable of persisting for many years after infarction (van den Borne et al., 2010). In addition to this pool of cells, there is evidence to suggest that myofibroblasts may also be derived from blood-borne mesenchymal stem cell progenitors (fibrocytes), epithelial cells, endothelial cells and smooth muscle cells (van den Borne et al., 2010; van Amerongen et al., 2008; Mollmann et al., 2006; Haudek et al., 2006).

Bucala et al. characterized a population of circulating cells with fibroblast properties (termed “fibrocyte”) that mobilize to sites of tissue injury and contribute to scar formation. Using sex-mismatched bone marrow chimeric mice, they showed that fibrocytes (collagen+/vimentin+/CD34+) were present in connective tissue scar, and were readily identified in subcutaneously implanted wound chambers (Bucala et al., 1994). Correspondingly, Mollmann et al. transplanted bone marrow from enhanced green fluorescent protein (eGFP)-transgenic mice into sublethally irradiated mice and showed

that 57.4% of myofibroblasts (detected by costaining with α -smooth muscle actin and vimentin) were eGFP-positive in the infarct zone. Meanwhile, eGFP positive myofibroblasts were absent in the remote areas (Mollmann et al., 2006).

Using transgenic mice, Duan and colleagues showed that following acute cardiac injury, the epicardium becomes Wnt responsive and undergoes epithelial-mesenchymal transition to generate fibroblasts that localize in the subepicardial space and promote a pro-fibrotic response (Duan et al., 2011). Zeisberg et al. used *Tie1Cre:R26RstoplacZ* mice which display lacZ expression in cells of endothelial origin, and FSP1-GFP transgenic mice which expressed GFP under the control of the fibroblast-specific protein 1 (FSP1) promoter. Using these two independent mouse models of heart disease, this group was able to show that TGF- β 1 induced endothelial-mesenchymal transition in adult human coronary endothelial cells, thus contributing to the total pool of cardiac fibroblasts and could be inhibited by rhBMP-7 (Zeisberg et al., 2007).

Myofibroblasts of different origins along with resident cardiac myofibroblasts are thought to participate together in repairing the injured heart, however, the relative contribution of each of these populations remains largely unknown (van den Borne et al., 2010). Myofibroblasts may be isolated from a number of different organs and although they all participate in tissue fibrosis, it is unlikely that myofibroblasts from different origins will behave in precisely the same way (Chang et al., 2002).

2.2 Function

Mainly differentiated from fibroblasts, myofibroblasts express contractile proteins and exhibit migratory, proliferative and secretory properties (van den Borne et al., 2010). In contrast, cardiac fibroblasts do not possess contractile microfilaments or stress fibers, display few or no actin-associated cell-cell and cell-matrix contacts and produce little ECM (Tomasek et al., 2002). Myofibroblast differentiation is a two-step process and requires the actions of transforming growth factor beta-1 (TGF- β 1), specialized ECM molecules such as the ED-A splice variant of fibronectin, and a mechanically stressed environment (Tomasek et al., 2002; Hinz and Gabbiani, 2003). In the initial step, under mechanical stress, fibroblasts adopt a proto-myofibroblast phenotype and form actin-containing stress fibers that terminate in fibronexus, a specialized adhesion complex that links intracellular actin with the ECM, and express cellular fibronectin at the cell surface (Tomasek et al., 2002). Functionally, proto-myofibroblasts can generate contractile force, which is further amplified in the mature myofibroblast. In the second step, proto-myofibroblasts differentiate into myofibroblasts, a process that is characterized by the increased expression of ED-A fibronectin (via TGF- β 1 stimulation), expression of α -smooth muscle actin (α -SMA), and increased assembly and complexity of stress fibers and focal adhesions (Tomasek et al., 2002).

The most studied marker of the differentiated myofibroblast is the *de novo* expression of α -SMA, the actin isoform that is also found in smooth muscle cells (Hinz and Gabbiani, 2003). Expression of α -SMA confers at least a twofold stronger contractile activity compared to undifferentiated fibroblasts in culture (Hinz et al., 2001). Unlike

smooth cells, myofibroblasts are usually negative for desmin, smooth muscle myosin heavy chain, h-caldesmon, and smoothelin (Schurch et al., 2007).

The nature of the contraction generated by the myofibroblasts is fundamentally distinct from that generated by cardiomyocytes. Myofibroblasts typically generate a sustained tonic contraction resembling that of smooth muscle cells that is regulated by circulating factors and neurohormones (Tomasek et al., 2002). By contrast, cardiomyocytes contract and relax cyclically upon electrical activation (Tomasek et al., 2002).

3. Mesenchymal stem cells

3.1 Origin

Mesenchymal stem cells (MSCs), otherwise known as ‘mesenchymal stromal cells’, are a rare, non-hematopoietic cell population that can be isolated from a number of different sources. Although the primary source of MSCs is the bone marrow, there has been numerous reports showing that these cells can also be isolated from several cell sources such as adipose tissue, umbilical cord blood, peripheral blood, connective tissues of the dermis and skeletal muscle, as well as from the heart, liver and other organs and tissues (Pittenger et al., 1999; Zuk et al., 2001; Bieback et al., 2004, Kuznetsov et al., 2001; Jiang et al., 2002; Beltrami et al., 2007). The International Society for Cellular Therapy defines human MSCs based on the following three criteria: (1) adherence to plastic in standard culture conditions, (2) specific surface antigen expression and (3) *in*

vitro differentiation to the osteoblast, adipocyte and chondroblast lineages (Dominici et al., 2006).

3.2 Therapeutic potential

In an effort to promote myocardial regeneration and improve ventricular function after ischemic injury, stem cell based therapy has been proposed as an alternative treatment strategy for end-stage heart failure (Boyle et al., 2006). Specifically, human mesenchymal stem cells (hMSCs) are attractive candidates for cellular cardiomyoplasty and other tissue regeneration, because they offer several practical advantages. Human MSCs are easily isolated and expanded in culture, are less immunogenic than other cell lines, and retain growth and multilineage potential over several passages (Muraglia et al., 2000; Pittenger et al., 1999). In addition, MSCs have been shown to possess anti-apoptotic, anti-fibrotic and proangiogenic features (Li et al., 2007; Mias et al., 2009; Kasper et al., 2007). Early clinical and basic study evidence for the therapeutic use of hMSCs in cardiac regeneration has suggested that these cells are safe and feasible to use in treating heart failure (Van Linthout et al., 2011; Chen et al., 2004; Hare et al., 2009; Katritsis et al., 2005; Schuleri et al., 2007; Hou et al., 2007; Nesselmann et al., 2008). However, results from these studies indicate that the benefits of stem cell therapy are modest, the generation of new cardiac tissue is limited, and the predominant mechanisms of action of these transplanted cells appear to involve favorable paracrine effects on the injured myocardium rather than functional trans-differentiation into cardiomyocytes (van den Borne et al., 2010).

Toma and colleagues reported that only 0.44% of human bone marrow derived mesenchymal stem cells (BM-MSCs) transfected with β -galactosidase reporter gene and injected into immunodeficient adult mouse hearts were identified in the myocardium, and that these cells had adopted a morphology indistinguishable from host cardiomyocytes (Toma et al., 2002). In a study using the porcine experimental model, Quevedo *et al.* injected male BM-MSCs into female swine and reported differentiation of these cells into cardiomyocytes, smooth muscle cells, and endothelium cells. However, cardiomyocyte differentiation was present in only 14% of engrafted BM-MSCs (Quevedo et al., 2009). In another study, Silva and colleagues, using a canine model, reported that canine MSCs engraft and differentiate into cells with a vascular phenotype but did not differentiate into functional cardiomyocytes (Silva et al., 2005). One plausible explanation for the observed limited trans-differentiation of hMSCs into functional cardiomyocytes could be attributed to the acquisition of a phenotype in culture that does not support further differentiation into cardiomyocytes.

3.3 Plasticity and differentiation

Human MSCs represent a heterogeneous population and its differentiation can be influenced by its origin, culture conditions and its microenvironment. MSCs residing in different tissues may already be primed to differentiate towards a specific lineage to form tissue specific cell types (Nesselmann et al., 2008). In fact, it has been reported that the differentiation potential and functions vary among MSCs isolated from different sources (Kern et al., 2006; Wagner and Ho, 2007; Wagner et al., 2006). Kern and colleagues characterized and compared MSCs isolated from bone marrow, umbilical cord blood and

adipose tissue. While MSCs from all three sources shared typical MSC characteristics, they differed significantly in their expression of CD90, CD105, and CD106, cell surface markers associated with hematopoiesis and cell migration (Kern et al., 2006). Moreover, MSCs derived from umbilical cord blood did not differentiate to the adipogenic lineage. In another similar study, Sakaguchi and colleagues compared the properties of patient-matched human MSCs isolated from bone marrow, synovium, periosteum, skeletal muscle and adipose tissue and found differences in colony number, self-renewal capacity, expansion ability, and ability to differentiate to the adipogenic, osteogenic and chondrogenic lineages (Sakaguchi et al., 2005).

The plasticity of MSCs to differentiate to specific lineages by soluble stimuli has been well described (Gang et al., 2004; McBeath et al., 2004; Pittenger et al., 1999). In addition to differentiating into osteoblast, adipocytes and chondrocytes, MSCs can also be induced to differentiate *in vitro* into cardiomyocytes (when treated with 5-azacytidine and amphotericin B) as well as neuron-like cells (Wakitani et al., 1995; Woodbury et al., 2000). Wagner et al. studied the effects of two different expansion media, BM-MS-C-M1 and BM-MS-C-M2 on human mesenchymal stem cells isolated from bone marrow (Wagner et al., 2006). They reported significant differences in terms of predisposition to specific differentiation pathways, genomic and proteomic make-up, and cell morphology (Wagner et al., 2006). Genes regulating metabolism and mitochondria were more highly expressed in BM-MS-C-M1 whereas genes involved in muscle development, actin binding, neurogenesis, cell differentiation, morphogenesis, skeletal development, and

development were more highly expressed in BM-MS-C-M2 and these cells were more committed towards myogenic differentiation (Wagner et al., 2006).

It has also been shown that MSCs display great sensitivity to tissue-level elasticity when committing to specific phenotypes (Engler et al., 2006). Forces generated and imposed on the cell's cytoskeleton from their microenvironment are central to predicting differentiation (Engler et al., 2004; Hinz et al., 2001; McBeath et al., 2004). In particular, matrix elasticity is now known to strongly influence the lineage specification of human MSCs (Engler et al., 2006). Human MSCs that were plated on soft matrices adopted a neurogenic phenotype, while cells grown on stiffer matrices were myogenic, and increasing elasticity resulted in osteogenic cells (Engler et al., 2006). This group also showed that matrix elasticity was more selective in driving differentiation than soluble induction factors, thus suggesting that optimization of the matrix elasticity is important in fostering tissue regeneration (Engler et al., 2006).

4. Actin-myosin cytoskeleton

Fibroblasts and myofibroblasts are able to sense their surrounding microenvironment through both indirect stresses from nearby fibroblasts pulling on the collagen matrix, and from direct cell-cell interactions (Bond et al., 2010). Non-muscle myosins are involved in tensioning cortical actin structures, which are in turn linked to focal adhesions that act as mechano-transducers by transmitting the force from inside the cell to the surrounding ECM (Kim et al., 2005; McBeath et al., 2004; Wang et al., 2002; Beningo et al., 2001; Tamada et al., 2004). Cellular mechano-transduction process generates biochemical

signals, which then signal to the cell to adjust the cytoskeletal structure, and activate actin-myosin contractility needed to deform the matrix (Engler et al., 2006; Bond et al., 2010).

The actin-myosin cytoskeleton is a dynamic system that is needed for various cellular functions, including contraction, motility, and tissue organization (Evan-Ram et al., 2007; Ivanov, 2008; Ivanov et al., 2007; Montell, 2008). The contractile apparatus consists of actin microfilaments, non-muscle myosin heavy chains and bundles of specialized adhesion complexes (Tomasek et al., 2002). Cell contraction requires the hydrolysis of ATP to generate energy needed to slide the non-muscle myosin along the actin filaments. Phosphorylation of the non-muscle myosin light chains through kinases such as Rho-associated kinase or myosin light chain kinase, convert the myosin into an active form, allowing it to bind to actin and facilitate contractility (Quintin et al., 2008; Tyska and Warshaw, 2002; Sellers and Knight, 2007). Regulation of actin-myosin contraction forces and opposing anchoring forces are crucial to maintaining normal cell morphology and function (Lecuit and Lenne, 2007; Montell, 2008; Okeyo et al., 2008). Disruption of this balance can lead to cell death, as seen in human pluripotent stem cells and embryonic stem cells (Ohgushi et al., 2010; Chen et al., 2010).

5. Function of non-muscle myosin II

Non-muscle myosin II (NMMII) is a protein found in both fibroblasts and myofibroblasts. This protein is composed of two heavy chains, two essential light chains, and two regulatory light chains (Vicente-Manzanares et al., 2009). Its activation via

phosphorylation is the end target of many contractile signaling pathways, and thus plays a pivotal role in many cellular processes that require force and translocation, including cell adhesion, cell migration and cell contraction (Vicente-Manzanares et al., 2009; Even-Ram et al., 2007; Meshel et al., 2005; Abe et al., 2003).

Three isoforms of non-muscle myosin II exist in human, NMMIIA, IIB and IIC. The human genes MYH9, MYH10 and MYH14 encode for NMMIIA, IIB, and IIC respectively. All three isoforms are well conserved and display great homology (Golomb et al., 2004; Simons et al., 1991). However, differences in their enzyme kinetics, subcellular localization and tissue expression, allow them to have distinct functions within the same cell (Flynn and Helfman, 2010; Kovacs et al., 2003; Tullio et al., 1997; Conti et al., 2004).

In human scar tissue, NMMIIA and IIB were highly expressed throughout the remodeling phase of wound repair and contraction of collagen lattices was greater in scar fibroblasts compared to normal fibroblasts (Bond et al., 2010). *In vitro* kinematic studies have shown that NMMIIA probably has a role in rapid contractility, whereas NMMIIB is more likely to maintain isometric tension since it spends a longer time being strongly attached to actin (Wylie and Chantler, 2001; Wylie et al., 1998, Saitoh et al., 2001; Lo et al., 2004; Rosenfeld et al., 2003). In one study, NMMIIB expression in MSCs was shown to be more sensitive to matrix elasticity than NMMIIA expression (Engler et al., 2006). In contrast, Bond et al. observed greater NMMIIA expression in fibroblasts with increasing matrix stiffness (Bond et al., 2010). Both studies suggest that as matrix stiffness increases, NMMII expression is altered in order to generate greater forces on the ECM, and as a result, greater contractility occurs. In addition, NMMIIA is functionally

different than NMM IIB as it is involved in Rho-dependent function, actin stress fiber formation and focal adhesion formation, but IIB is not (Conti et al., 2004; Lo et al., 2004; Sandquist et al., 2006).

There have been several reports that show NMMII regulates cell survival, and that actin-myosin contractility is associated with reduced viability (Walker et al., 2010; Chen et al., 2010; Ohgushi et al., 2010; Flynn and Helfman, 2010). One group reported that knockdown of NMMIIB but not IIA in HeLa cells, impaired caspase 3 cleavage and nuclear condensation in response to TNF-alpha (Flynn and Helfman, 2010). In another study, inhibition of myosin heavy chain ATPase, and downregulation of both myosin heavy and light chains all increased survival and cloning efficiency in human embryonic stem cells, thus showing that actin-myosin contraction is critical in elucidating the cell death response (Chen et al., 2010). Moreover, mutations and deletions in the various NMMII genes have been linked to human diseases, such as autosomal dominant hearing loss, and defects in embryonic patterning, and heart and brain development in mice (Donaudy et al., 2004; Marini et al., 2006; Conti et al., 2004; Tullio et al., 1997).

Blebbistatin (BB), a small molecule drug, inhibits all NMMII isoforms, but does not inhibit other myosins found in MSCs, other than myosin VI (Limouze et al., 2004). Blebbistatin works by inhibiting the actin-activated ATPase activity by preventing the release of inorganic phosphate from ADP and requires a specific alanine or serine residue only expressed in class II and VI myosins (Kovacs et al., 2004; Limouze et al., 2004; Straight et al., 2003). This inhibition disrupts NMMII's association with actin, leading to destabilization of the actin cytoskeleton (Flynn and Helfman, 2010). Blebbistatin is cell permeable and its effects are reversible upon removal of the drug from cell culture media.

6. Rationale for study

Mesenchymal stem cells were first identified as colony-forming unit fibroblast-like cells in the 1970s and have previously been compared to fibroblasts with respect to gross cell morphology and growth patterns in culture (Friedenstein et al., 1976). Gene expression profiles used to define various types of cell cultures as MSCs are also expressed by cultures of fibroblastic cells from any tissue (Covas et al., 2008; Bianco et al., 2008). Covas et al. compared morphologically and functionally human MSCs isolated from several different fetal and adult tissues to that of fibroblasts isolated from 4 different sources. Human MSCs and fibroblasts were found to be similar in regards to morphology, immunophenotype, and differentiation capacity (Covas et al., 2008). Both MSCs and fibroblasts displayed large nuclei with prominent nucleoli, abundant rough endoplasmic reticulum, and numerous mitochondria. Moreover, only three of the four fibroblasts cultures could be induced to differentiate (Covas et al., 2008). Wagner and Ho showed that human fibroblast cell lines (HS68 and NHDF) displayed an identical phenotype panel of 22 CD surface markers used to identify MSCs (Wagner and Ho, 2007). In another study, Ball and colleagues directly co-cultured human MSCs with human dermal fibroblasts and demonstrated that MSCs induced fibroblast to myofibroblast cells with well-organized α -SMA filaments, thus suggesting that resident tissue cells are key players in determining the fate of recruited MSCs (Ball et al., 2004).

However, direct comparison of primary human adult BM-MSCs and human cardiac myofibroblasts has not previously been reported. Specifically, a functional comparison between the two cell types in regards to contractility and collagen synthesis

does not appear in the literature and is therefore a novel issue to be addressed. In support of the notion that bone-marrow derived cells contribute to fibrosis, several groups have reported the presence of these cells in cardiac fibrosis secondary to myocardial infarction (Mollmann et al., 2006), myocarditis (Kania et al., 2009) and angiotensin-II induced fibrosis (Sopel et al., 2011). Furthermore, cardiac resident MSCs may also act as a source of fibroblasts that mediate scar formation after MI (Carlson et al., 2011). Thus it has been suggested that fibroblasts are the end-stage lineage of self-renewing multipotential MSCs (Sarugaser et al., 2009).

We hypothesized that human BM-MSCs acquire a myofibroblastic phenotype in culture that is similar to cardiac myofibroblasts and that this myofibroblastic function can be attenuated with the inhibition of NMMII. We suggest that because of their propensity to adopt a myofibroblastic phenotype, hMSCs may contribute to cardiac fibrosis following myocardial infarction, and may therefore represent a novel therapeutic target in controlling fibrosis. Furthermore, this issue should be taken into consideration when expanding these cells *in vitro* for therapeutic use in myocardial regeneration.

In this study, our objectives were as follows: (1) to isolate and characterize human BM-MSCs from patients with ischemic heart disease, (2) to compare their phenotype and function to human cardiac myofibroblasts with respect to gene expression profile, collagen gel contractility and collagen synthesis, and (3) to determine whether inhibition of NMMII using blebbistatin, (small molecule that specifically inhibits all NMMII activity), and specific knockdown of MYH9 and MYH10 (NMMIIA and IIB respectively) can attenuate the myofibroblast phenotype conversion. These comparisons

were observed under standard two dimensional cell culture conditions without the addition of specific induction factors.

II. MATERIALS AND METHODS

1. Isolation and culture of human MSCs and human cardiac myofibroblasts

The Bannatyne Campus Research Ethics Board of the University of Manitoba approval was obtained for the collection of bone marrow, left ventricular and atrial tissue from patients undergoing a cardiac procedure. Written informed consent was obtained from each patient prior to tissue collection.

Human MSC cultures were prepared based on plastic adherence according to the methods developed by Caplan and Friedenstein (Friedenstein et al., 1976; Caplan, 1991) with some modifications. Bone marrow aspirates of 0.5 to 3 ml were taken from the sternum of patients undergoing a cardiac surgery requiring a full median sternotomy. The collected fresh bone marrow specimens were immediately placed in a tube containing 10 ml of phosphate-buffered saline (PBS) and subjected to mechanical disaggregation. The cells were then diluted with Dulbecco's modified Eagle's medium (DMEM) low glucose media, supplemented with 20% fetal bovine serum (FBS), 100U/ml penicillin, 100 µg/ml streptomycin, and 100 mM ascorbic acid. Cells were plated onto 10 cm plastic culture dishes and maintained at 37°C in a humidified atmosphere containing 5% CO₂. After 24 hours, non-adherent cells (hematopoietic cells) were discarded, and the adherent cells were thoroughly washed twice with PBS. Fresh complete medium was added and replaced every 3 or 4 days. After 10 to 14 days of cultivation, primary cultures were 60-80% confluent. Cells were then dissociated with TrypLE Express and seeded at low densities for expansion through successive passages. Samples were collected at early passages (P0-P2) for analysis.

Atrial appendage and left ventricular tissue from the apex of the heart was taken from patients undergoing mechanical ventricular assist device surgery and subjected to collagenase digestion to isolate cardiac myofibroblasts. Minced ventricular and atrial tissue was treated with 2 mg/ml collagenase II in SMEM media and incubated for 3 hours at 37°C. Collagenase was neutralized by the addition of an equal volume of medium containing 20% FBS and liberated cells were collected by centrifugation at 2000 rpm for 7 minutes. Cells were resuspended in fresh complete medium containing 20% FBS, seeded onto plastic culture dishes and incubated at 37°C in 5% CO₂ and 95% humidity. The digestion was repeated with the remaining tissue pieces. Non-adherent cells (myocytes) were removed the next day and fresh medium was added to the adherent cells and replaced every 3 or 4 days. Samples were harvested at early passages (P0-P2) for analysis.

2. Differentiation of MSCs

The stem cell characteristics of human MSCs were assessed through targeted differentiation to fat, bone and cartilage using the appropriate induction medium. Induced differentiation was performed at passage 0, 1, and 2.

2.1 Adipogenesis

For adipogenic differentiation, hMSCs were cultured in basal medium supplemented with 0.5 µM dexamethasone, 0.5 µM isobutyl methylxanthine, and 50 µM

indomethacin for up to 2 weeks. The presence of intracellular lipid accumulation was evaluated with Oil Red O stain to assess adipogenesis.

2.2 Osteogenesis

For osteogenic differentiation, hMSCs were maintained in basal medium supplemented with 0.05 mM ascorbic acid-2-phosphate, 0.1 μ M dexamethasone, and 100 mM β -glycerophosphate and cells were cultured for 3 weeks. Evidence of calcium deposits was detected by Alizarin red staining.

2.3 Chondrogenesis

The STEM PRO chondrogenesis differentiation kit was used to induce chondrogenesis. Cells were cultured for 3 weeks and Alcian blue staining was used to verify synthesis of proteoglycans, an indication of chondrogenic differentiation.

3. Immunofluorescence

Cells cultured in 24-well plates overlaid with glass coverslip were fixed in 4% paraformaldehyde for 15 minutes and permeabilized in 0.2% Triton-X-100 in PBS for 5 minutes. Cells were subsequently washed with PBS and blocked with 5% bovine serum albumin (BSA) for 30 min at room temperature. Fixed cells were incubated with 50 μ L of the appropriate primary antibody overnight at 4°C and detected with a biotinylated secondary antibody for 1 hour at room temperature. Cells requiring dual staining for filamentous actin were washed with PBS after incubation with the secondary antibody,

and incubated with 50 μ L of phalloidin for 30 min at room temperature. Vectashield with DAPI was used to mount the coverslips and cells were visualized with an epifluorescent microscope with appropriate filters.

4. Fluorescence-activated cell sorting analysis

Cells were grown to 80-90% confluency, trypsinized with TrypLE, centrifuged at 3000 rpm for 5 min, washed with PBS and counted. 1×10^6 cells were used for each marker stained. Cells were permeabilized with 0.1% Triton-X-100 for 15 min and blocked with 2% BSA in PBS for 30 min at room temperature. Primary antibodies were added to the cells and incubated for 45 min on ice. Cells were then washed three times, centrifuged, and incubated with the appropriate fluorescent secondary antibody for 30 min. Equal volumes of 0.1% BSA and 4% paraformaldehyde (PFA) were added to each tube and the cells were kept at 4°C overnight. Cells were centrifuged and resuspended in 0.1% BSA before running analysis on the Becton Dickinson LSR2 cytometer.

5. Immunoblot analysis

Cells were washed twice in cold PBS and lysed in RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1mM EDTA, 1 mM EGTA, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Triton X-100) and protease inhibitor cocktail (0.1 M phenylmethylsulfonyl fluoride, 5 μ g/ml leupepetin, 2 μ g/ml aprotinin, and 1 μ g/ml pepstatin). Protein concentrations of whole cell lysates were determined using the BCA method (Smith et

al., 1985) and equal amounts of each protein sample (15 μ g) were separated on an 8% sodium dodecyl sulphate-polyacrylamide gel at 130V. Separated proteins were then transferred to a polyvinylidene difluoride membrane for 1 hour at 150V. After blocking the membrane with 5% nonfat dried skim milk powder for 2 hours at room temperature, the membrane was incubated with primary antibody for 1 hour at room temperature or overnight at 4°C. The membrane was washed three times with 0.05% PBS-Tween for 15 minutes each and then incubated for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody. After extensive washing with PBS-T, protein bands were visualized by ECL Plus according to the manufacturer's instructions and developed on film. Membranes were subsequently stripped and reprobed for β -tubulin as a loading control. Blot densities were measured using Quantity One software and normalized to β -tubulin blot densities.

6. Real-time PCR Analysis

6.1 RNA extraction

Cells were washed once with sterile PBS and lysed with TRIzol reagent for 5 min at room temperature and subsequently stored at -80°C until ready to process. Isolated RNA was then subjected to a phase separation with chloroform and the aqueous upper phase was transferred into a new tube. Total RNA was then precipitated with isopropanol and washed twice with 75% ethanol. Total RNA isolation was further purified with Ambion's DNA-free kit according to the manufacturer's instructions. RNA concentration and quality was analyzed using the Agilent 2100 Bioanalyzer.

6.2 Real-time PCR

We assessed the expression of NMMIIA, NMMIIB, α -SMA, ED-A Fibronectin and vimentin by quantitative real-time PCR in both hMSCs and cardiac myofibroblasts. Real-time PCR was performed on the Bio-Rad Mini Opticon detection system using the iscript one-step RT-PCR kit. Gene expression was normalized relative to the endogenous gene ACTB (β -actin). All primers were designed using the Primer3 Plus program (Table 1 and Table 2).

7. Inhibition of NMMII

7.1 Blebbistatin administration

Blebbistatin, a specific small molecule inhibitor of NMMII was used to treat cells for 24-48 hours at a concentration of 5, 10 and 50 μ M. Culture media was changed every 2 days and fresh blebbistatin was added to cells chronically treated with blebbistatin from isolation. Cells treated with blebbistatin were protected from light at all times as blebbistatin is a light sensitive compound.

7.2 Knockdown of NMMII siRNA

Optimal cell density and siRNA transfection reagent 1 dose was determined using Dharmacon siGLO Green Transfection Indicator. Successful transfection was assessed by

visual fluorescent RNA duplex signal uptake and localization to the nucleus. MSCs were passaged into 20% FBS media containing no antibiotics one day before the transfection at 5×10^4 cells/well in 6-well plates. Specific siRNA (25 nM, 50 nM or 100 nM) was mixed with 4.8 μ l Dharmacon siRNA Transfection Reagent 1 and the cells were incubated with the transfection medium for 24 hours as suggested in the standard protocol. Cells were then washed once with PBS and fresh media was added. Knockdown efficiency was analyzed with RT-PCR at 24 and 48 hours after transfection and compared to a non-targeting negative control siRNA. For protein, contractility and viability experiments, cells were harvested at 48, 72 and 96 hours post-transfection.

8. Collagen gel contraction assay

Collagen gels were prepared from purified bovine type I collagen (3 mg/ml) in 24-well culture dishes and incubated overnight at 37°C to induce gelation. Cells were plated at a density of 5×10^4 cells (2.5×10^4 cells for siRNA transfection) per well and allowed to attach overnight. For phenotype studies, the cells were serum starved for 24 hours before treatment with or without 10 ng/ml of recombinant human TGF- β 1 for an additional 24 hours. For transfection studies, cells were transfected with negative control, NMMIIA and NMMIIB siRNA the following day for an additional 24 hours in 20% FBS media with no antibiotics. The cells were then washed once with PBS and fresh media was added. Treatment with 10 ng/ml of TGF- β 1 and 50 μ M of blebbistatin were added at 24, 48 and 72 hours post transfection for 24 hours and gel contractility was assessed.

Collagen gels were physically detached from the side of the wells immediately after treatment and the diameters of collagen lattices were measured using digital photographs taken at time 0 and 24 hours after stimulation. Three replicates were used for each group. IDL based MeasureGel software was used to analyze the reduction in collagen gel surface area.

9. Measurement of type I collagen synthesis

Synthesis of mature type I collagen was determined by measuring the concentration of the carboxyterminal propeptide of type I collagen (CICP) in conditioned media according to the manufacturer's specification. Briefly, cells were plated at a density of 5×10^4 cells per well in a 6 well culture dish, allowed to attach overnight and subsequently serum starved for 24 hours. Cells were then stimulated with or without TGF- β 1 (1 ng/ml and 10 ng/ml) for an additional 48 hours at which point the medium was collected and cells were trypsinized and counted. Each group was performed in triplicates. Analysis was carried out in a microtitre plate format utilizing a monoclonal anti-CICP antibody coated on the plate, a rabbit anti-CICP antiserum, a goat anti-rabbit alkaline phosphatase conjugate, and a pNNP substrate to quantify CICP in the conditioned media. CICP concentrations were normalized to total cell count and compared to controls as a fold change.

10. Live dead assay

Cells plated onto collagen gel substrates were assessed for viability 24 hours after cells were allowed to contract. The LIVE/DEAD Viability/Cytotoxicity kit was used to detect both live and dead cells using a two-colour fluorescence cell viability assay according to the manufacturer's protocol. Viable cells are detected by the presence of ubiquitous intracellular esterase activity determined by the enzymatic conversion of non-fluorescent to intensely fluorescent cell-permeant calcein AM. The calcein dye is well retained within live cells and produces an intense uniform green fluorescence. Non-viable cells are detected by the uptake and binding of ethidium homodimer-1 to exposed nucleic acids by cells with damaged membranes. Ethidium homodimer produces a bright red fluorescence in dead cells. Briefly, cells were washed once with PBS and 300 μ L of the live/dead solution was added to each well and incubated for 30 min at 37°C in the dark. Gels were then manually detached from the wells, transferred to a glass slide, cover slipped and visualized with an epifluorescent microscope with appropriate filters. Each treatment group was run in triplicate, and 3 representative images were taken for each triplicate. Live and dead cells were counted and quantified using ImageJ software.

11. Reagents

Cell culture media and reagents used for hMSCs and cardiac myofibroblasts growth and differentiation were purchased from GIBCO (Grand Island, NY) unless otherwise specified. FBS was purchased from Hyclone Laboratories Inc. (Logan, UT) and antibiotics (penicillin and streptomycin) were purchased from LONZA (Walkersville,

MD). Cell culture plates and coverslips were purchased from BD Falcon VWR (Franklin Lakes, NJ). BSA was purchased from Fisher Scientific (Fair Lawn, NJ) and Vectashield mounting medium with DAPI was purchased from Cedarlane (Burlingame, CA). A BCA kit used for protein assay and pre-stained protein ladder for Western blot analysis were purchased from BIO-RAD (Hercules, CA), PVDF membrane was obtained from Millipore (Etobicoke, ON). The enhanced chemiluminescence (ECL Plus) detection system was purchased from Amersham Biosciences (Buckinghamshire, UK) and collagenase type 2 used to digest atrial and ventricular tissue was purchased from Worthington Biochemical Corp. (Jackwood, NJ). Purified bovine collagen type I used for collagen gel contraction assay was obtained from Advanced BioMatrix (San Diego, CA) and the MicroVue CICP EIA kit used to measure collagen synthesis was purchased from QUIDEL (San Diego, CA). TGF- β 1 and Blebbistatin used to treat cells were purchased from R&D Systems (Minneapolis, MN) and EMD (Gibbstown, NJ) respectively. All ON-TARGET smart pool and ON-TARGET plus non-targeting pool siRNAs and DharmaFECT 1 transfection reagents were purchased from Dharmacon (Lafayette, CO). All primers used for RT-PCR were purchased from Sigma-Genosys (Oakville, ON). DNA-free kit and TRIzol reagent were both purchased from Ambion (Carlsbad, CA), iscript one-step RT-PCR kit was purchased from BIO-RAD (Hercules, CA) and RNA Nano chips and reagents were purchased from Agilent (Mississauga, ON). The LIVE/DEAD Viability/Cytotoxicity kit used to assess cell viability was purchased from Molecular Probes (Eugene,OR).

12. Antibodies

Mouse monoclonal non-muscle myosin heavy chain myosin, non-muscle myosin IIA and rabbit polyclonal beta-tubulin used as our loading control for Western blots were purchased from Abcam (Cambridge, MA). Polyclonal antibodies to non-muscle myosin IIC, vimentin and CD90 were purchased from Santa Cruz (Santa Cruz, CA). Mouse monoclonal ED-A Fibronectin was purchased from Millipore (Etobicoke, ON), mouse monoclonal α -SMA was purchased from Sigma (Saint Louis, MO) and mouse monoclonal CD45 was purchased from BD Bioscience (Franklin Lakes, NJ). Mouse monoclonal antibody against procollagen (SP1.D8) was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA). Secondary antibodies used for Western blotting (goat anti-rabbit IgG, rabbit anti-mouse IgG and rabbit anti-goat IgG, conjugated to horseradish peroxidase) were purchased from Jackson ImmunoResearch Laboratories (Eugene, OR). Phalloidin used to stain filamentous actin and all Alexa fluor 488 secondary antibodies for immunofluorescence staining were purchased from Invitrogen (Eugene, OR).

13. Statistical analysis

All data are expressed as the means \pm SEM. Means between two groups were compared using the two-tailed Student's *t*-test. Differences between multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Neumann-Keuls test using SigmaStat 3.5 software program. A *p* value less than 0.05 was considered statistically significant.

Table 1: Primer Sequences

Gene Name	Primers 5'-3' Forward	Primers 5'-3' Reverse
MYH9	ACACCGCCTACAGGAGTATGA	ACACCGCCTACAGGAGTATGA
MYH10	AGGTGGACTATAAGGCAGATGAG	CTGTCTGATGACTGGTGCAAAA
ED-A FN	CCAGTCCACAGCTATTCCTG	ACAACCACGGATGAGCTG
VIM	GAGAACTTTGCCGTTGAAGC	TGGTATTCACGAAGGTGACG
ACTA2	TGTAAGGCCGGCTTTGCT	CGTAGCTGTCTTTTTGTCCCATT
ACTB	AGGCCAACCGCGAGAAGATG	CAGAGGCGTACAGGGATAGCAC

Table 2: Gene and protein names

Gene name	Protein name	Alternative name
MYH9	NMMIIA	
MYH10	NMMIIB	SMemb
ED-A FN	ED-A fibronectin	
VIM	Vimentin	
ACTA2	α -SMA	
ACTB	β -actin	

Table 3: On-target SMART pool siRNA target sequences

Gene	Duplex name	Target sequence
MYH9	MYH9 on-target SMART pool	MYH9-5, 9-6, 9-7, 9-8 pool
MYH9	MYH9-5	GUAUCA AUGUGACCGAUUU
MYH9	MYH9-6	CAAAGGAGCCCUGGCGUUA
MYH9	MYH9-7	GGAGGAACGCCGAGCAGUA
MYH9	MYH9-8	CGAAGCGGGUGAAAGCAA
MYH10	MYH10 on-target SMART pool	MYH10-5, 10-6, 10-7, 10-8 pool
MYH10	MYH10-5	CCAUUUACUCUGAGAAUA
MYH10	MYH10-6	GGCCACUCUACAAAGAAU
MYH10	MYH10-7	GAGCAGCCGCCAACAAUU
MYH10	MYH10-8	GGAAGAAGCUCGACGCGCA

III. RESULTS

1. Human MSCs functionally express a myofibroblastic phenotype *in vitro*

1.1 Phenotypic characterization of human MSCs

To confirm the multipotency starting population of MSCs, we induced our bone marrow derived cells to undergo differentiation into the adipogenic, osteogenic, and chondrogenic lineages, an established critical requirement of MSC (Pittenger et al., 2000; Dominici et al., 2006). Human MSCs at each passage were cultured in the appropriate induction medium for up to 3 weeks. Evidence of adipogenic differentiation was noted by the presence of intracellular lipid vacuoles when stained with Oil Red O (Figure 1B). Positive alizarin red staining for alkaline phosphatase and minerals was indicative of osteogenic cells (Figure 1C). Glycosaminoglycans and proteoglycans indicating chondrogenic differentiation was confirmed with Alcian blue stain (Figure 1D). Induced differentiated hMSCs were morphologically distinct from undifferentiated cells (Figure 1A). The successful differentiation of hMSCs at each passage provides evidence that the starting cell population were indeed multipotent and can maintain their capacity for differentiation over serial passages. To further confirm that the collected bone marrow derived cells were mesenchymal stem cells and not hematopoietic stem cells, cells were stained for the hematopoietic markers CD45 and CD34. At each passage, FACS analysis showed that less than 17% of the entire cell population stained positive for both CD 45 and CD 34, meanwhile immunofluorescent data revealed negative staining for each marker.

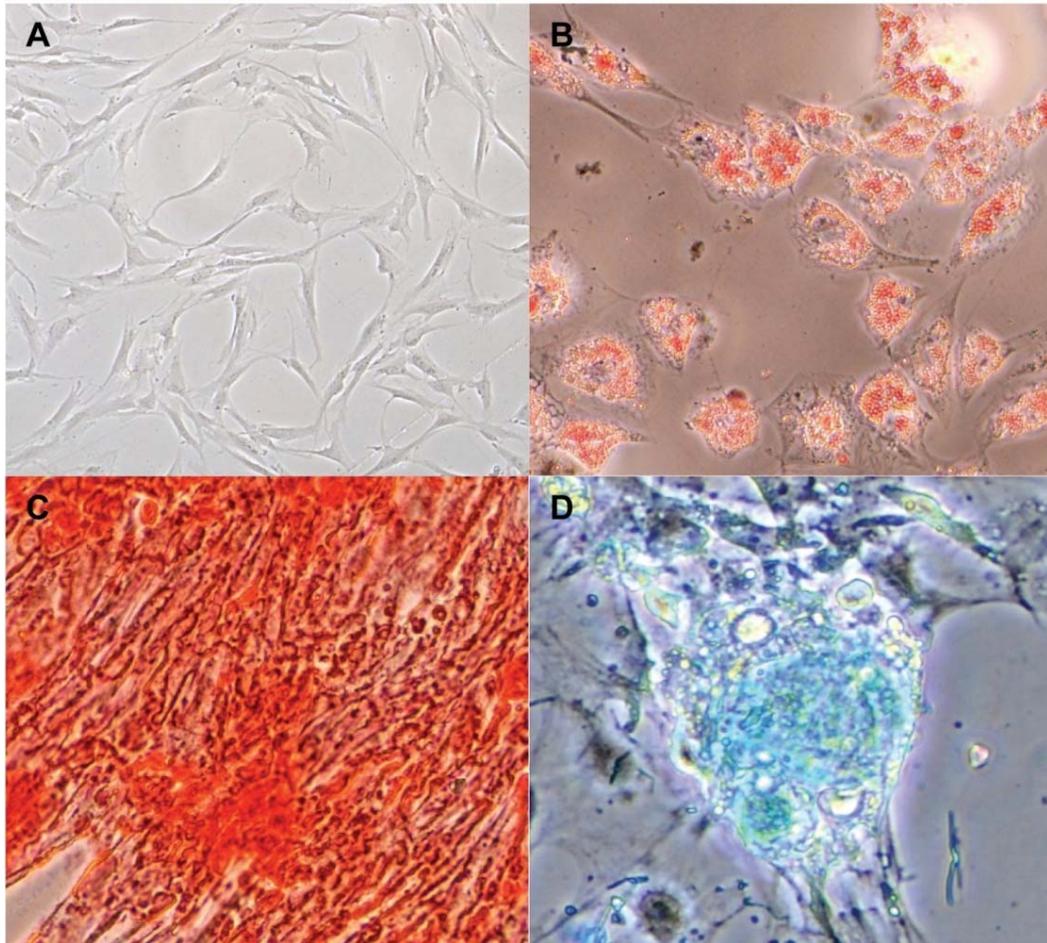


Figure 1: *In vitro* differentiation of hMSCs.

Cultured hMSCs displayed trilineage differentiation capacity when cultured in the appropriate induction medium: adipogenic (B), osteogenic (C) and chondrogenic (D) lineages. Undifferentiated control hMSCs (A). Abbreviations: human mesenchymal stem cells (hMSCs).

We examined the effects of standard culture conditions on bone marrow derived hMSC differentiation and found that hMSCs have phenotypic characteristics that are very similar to human CMyfbs. Morphologically, hMSCs display an adherent spindle shape that became increasingly flattened throughout successive passages that was similar to that observed in human CMyfbs (Figure 2).

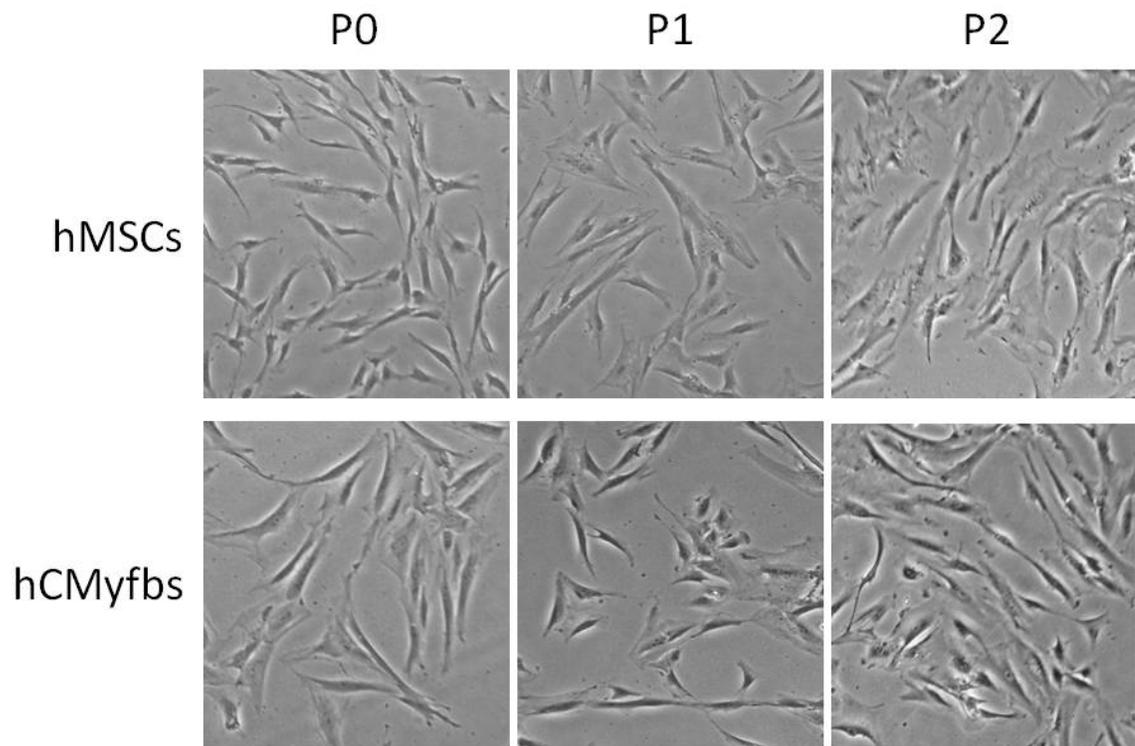


Figure 2: hMSCs display similar morphology to their cardiac derived myofibroblasts.

Bone marrow derived hMSCs consistently displayed spindle shaped morphology over serial passages (A-C). This morphology was comparable to that observed in ventricular cardiac myofibroblasts isolated from the same patient (D-F). Abbreviations: human mesenchymal stem cells (hMSCs) and human cardiac myofibroblasts (hCMyfbs).

To confirm whether these hMSCs expressed a myofibroblastic phenotype in culture, we performed immunofluorescent staining and Western blot analysis on cultured cells for a panel of known myofibroblast markers. Currently, the expression of α -SMA is considered to be the most reliable and pivotal hallmark of the differentiated myofibroblast (Pittenger et al., 2000). However, a number of other cells express α -SMA and thus, the upregulated expression of vimentin, of embryonic smooth muscle myosin heavy chain (SMemb), and of focal adhesion proteins like paxillin, vinculin, tensin, and the extra domain A (ED-A) splice variant of fibronectin are further distinguishing markers of myofibroblasts (Rohr, 2011; Eyden, 2008). Furthermore, the role of myofibroblasts in ECM remodelling and consequently fibrosis, is marked by their ability to synthesize collagen. Similar to CMyfbs, primary human MSCs isolated from patients undergoing heart surgery were stained positive for α -SMA, vimentin, SMemb, ED-A fibronectin, and sp1D8 (collagen type I) (Figure 3).

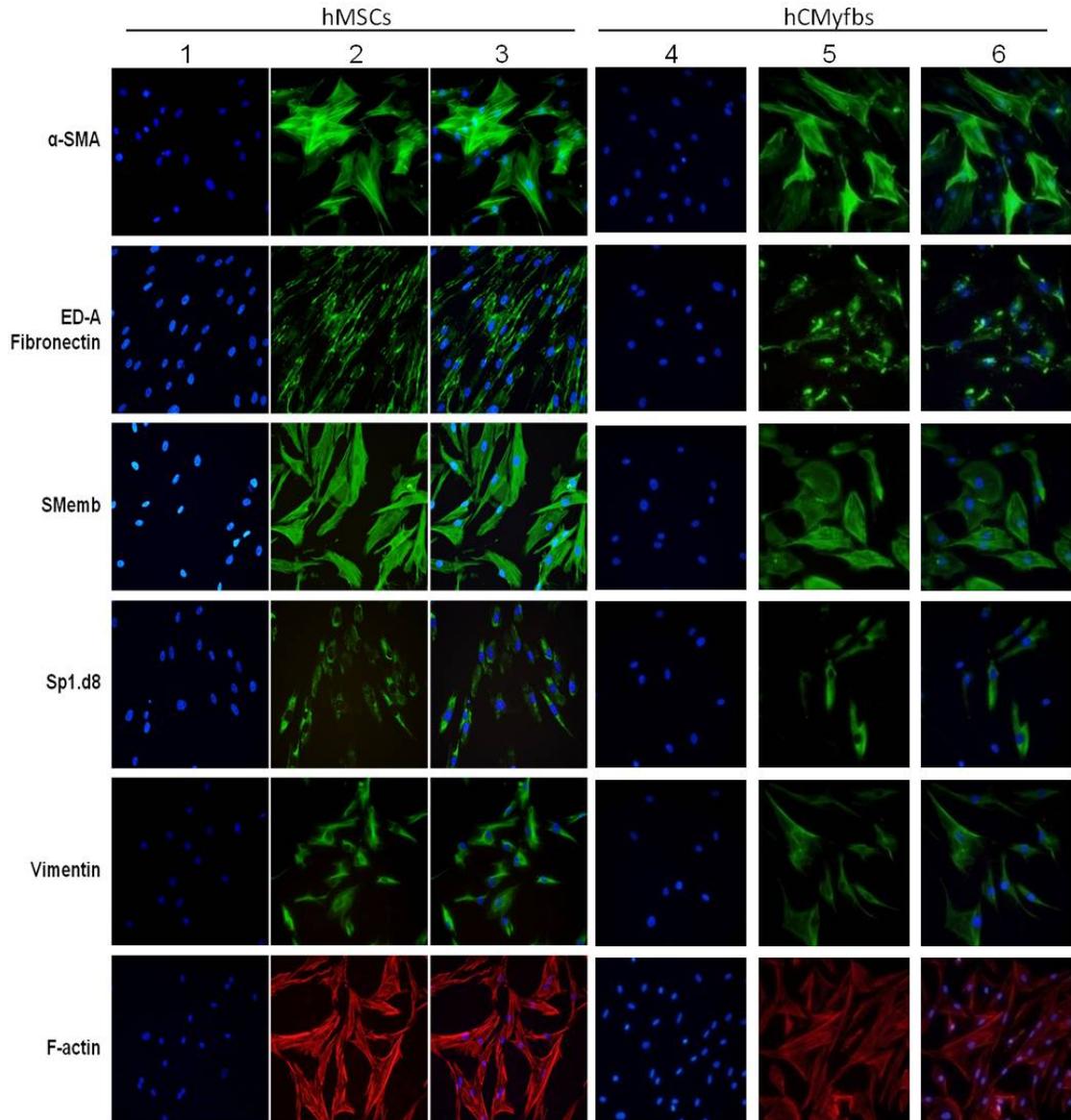


Figure 3: Bone marrow derived hMSCs display myofibroblast markers. Similar to ventricular cardiac myofibroblasts, hMSCs stained positive for various myofibroblast markers (green) and filamentous-actin (red). Lanes 1 and 4: nuclei stained with DAPI. Lanes 2 and 5: myofibroblast markers. Lanes 3 and 6: merged image. Abbreviations: human mesenchymal stem cells (hMSCs), human cardiac myofibroblasts (hCMyfbs), alpha smooth muscle actin (α -SMA), collagen I (Sp1.D8), and non-muscle myosin heavy chain IIA (SMemb).

Western blot and PCR analysis confirmed that expression of these myofibroblast markers was consistently expressed soon after isolation and throughout serial passage in both hMSCs and CMyfbs (Figure 4 and 5). These results were obtained in multiple experimental repetitions with both hMSCs and hCMyfbs (n=3-10). However, it is important to note that variable expression levels of myofibroblast markers occurred not only between patients, but also between cell passage and cell type.

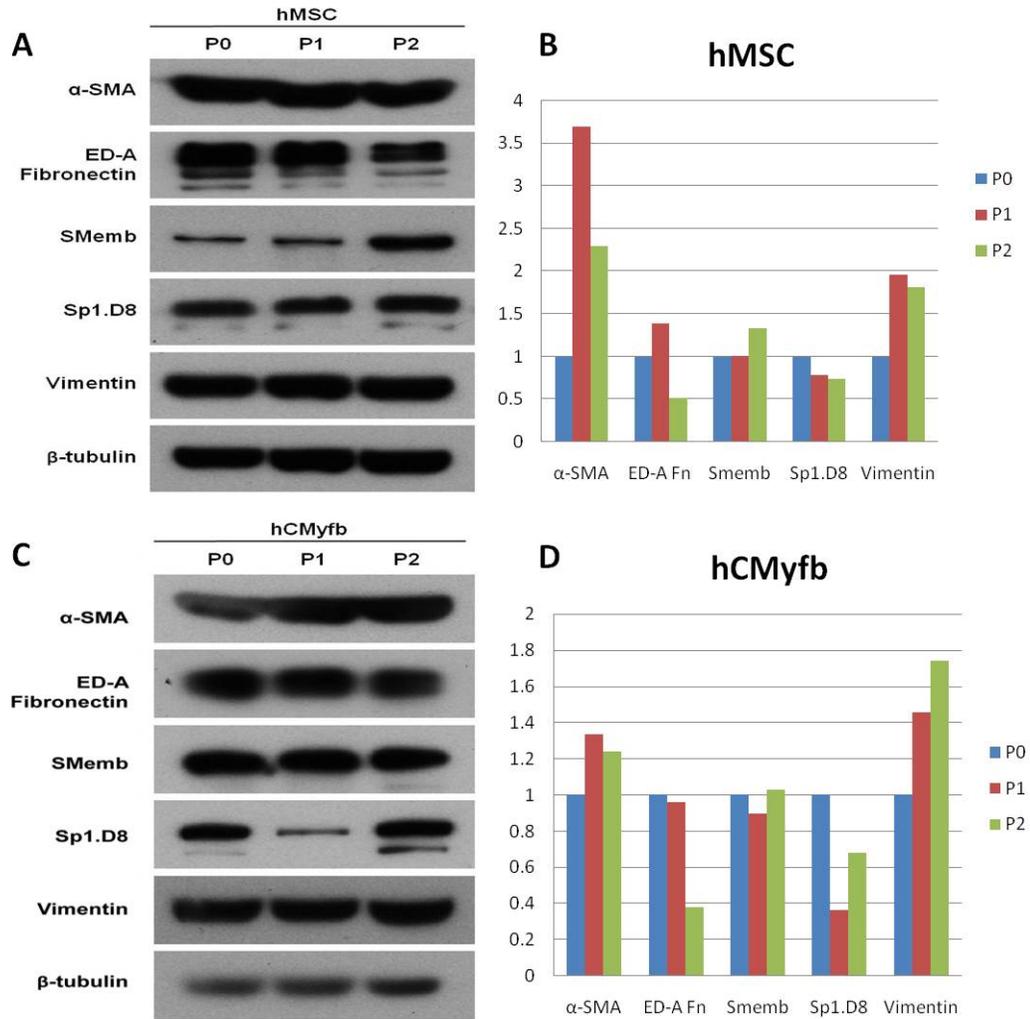


Figure 4: Early passage hMSCs express myofibroblast proteins.

Representative Western blots from the same patient were probed for various myofibroblasts markers over serial passage (A and C). Primary human MSCs (B) consistently express myofibroblast proteins throughout early passages (P0-P2), similar to ventricular cardiac myofibroblasts (D). β -tubulin was used as a loading control. Fold change expression was compared to P0 cells. Comparable results were obtained in multiple experimental repetitions with both hMSCs (n=10) and hCMyfbs (n=3). Results are displayed as mean \pm SEM. Abbreviations: human mesenchymal stem cells (hMSCs), human cardiac myofibroblasts (hCMyfbs), alpha smooth muscle actin (α -SMA), procollagen I (Sp1.D8), and non-muscle myosin heavy chain IIA (SMemb).

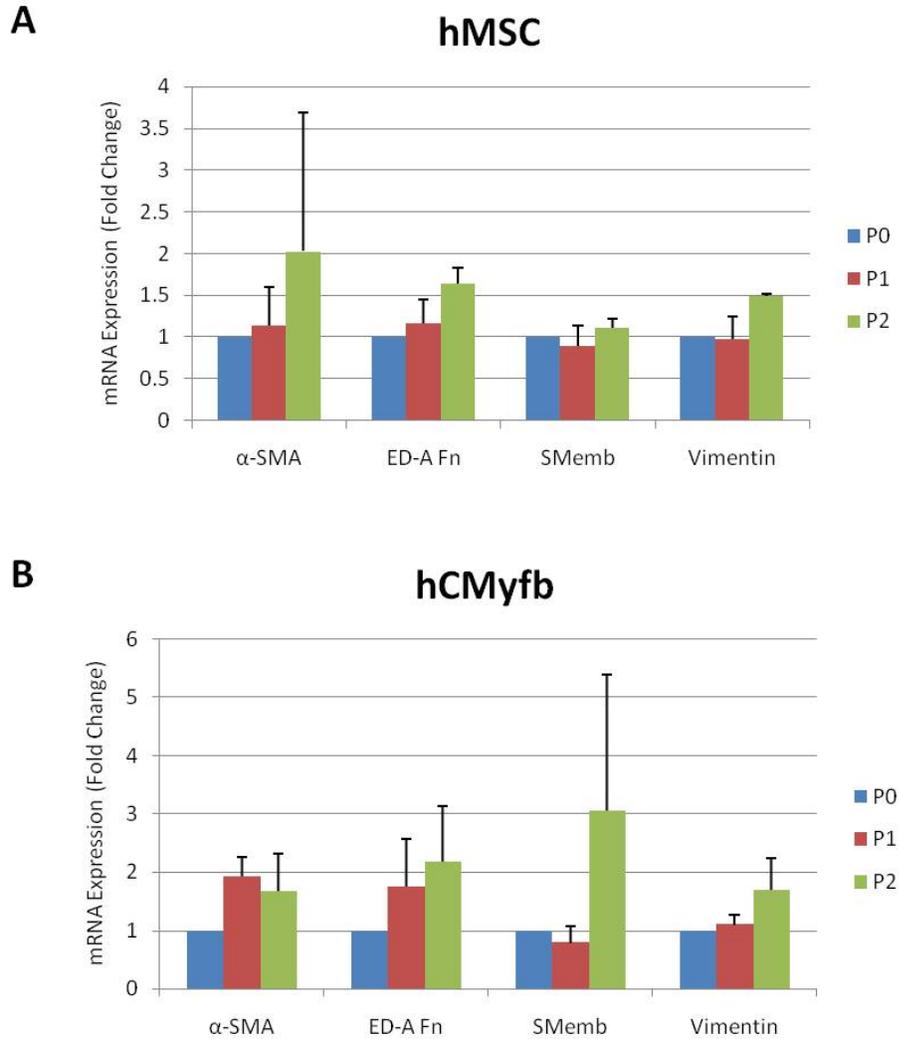


Figure 5: Early passage hMSCs express myofibroblast mRNA.

Human MSCs express an mRNA profile consistent with a myofibroblastic phenotype throughout serial passages (A) similar to atrial cardiac myofibroblasts (B). Fold change expression was compared to P0 cells. Comparable results were obtained in multiple experimental repetitions with both hMSCs (n=3-5) and hCMyfb (n=4). Results are displayed as mean \pm SEM. Abbreviations: alpha smooth muscle actin (α -SMA), ED-A fibronectin (ED-A Fn), human cardiac myofibroblasts (hCMyfb), human mesenchymal stem cells (hMSCs), and non-muscle myosin heavy chain IIB (SMemb).

1.2 Functional characterization of human MSCs

Collagen gel contraction assays, which mimic the infarct scar that is rich in collagen and TGF- β 1, was used to assess the physiological function of BM-MSCs. We compared the ability of hMSCs to exert sustained tonic contractions on collagen gels, to that of ventricular CMyfbs, and observed the effect of TGF- β 1 (10 ng/ml) on gel contractility (Figure 6). Samples were taken from 4-8 patients and run in triplicates. Non-treated hMSCs displayed a basal level of contraction that was similar to that observed in cardiac myofibroblasts (34.9% \pm 1.4%, 32.8% \pm 1.4%, 31.8% \pm 1.8%, 48.5% \pm 2.5% reduction in surface area in P1 hMSCS, P1 hCMyfbs, P2 hMSCs and P2 hCMyfbs respectively). Moreover, TGF- β 1 treated cells displayed significant increased contractile function as compared to non-treated cells (41.1% \pm 1.3%, 44.7% \pm 2.5%, 38.2% \pm 1.7%, 48.5% \pm 2.5% reduction in surface area in P1 hMSCS, P1 hCMyfbs, P2 hMSCs and P2 hCMyfbs TGF- β 1 treated cells respectively).

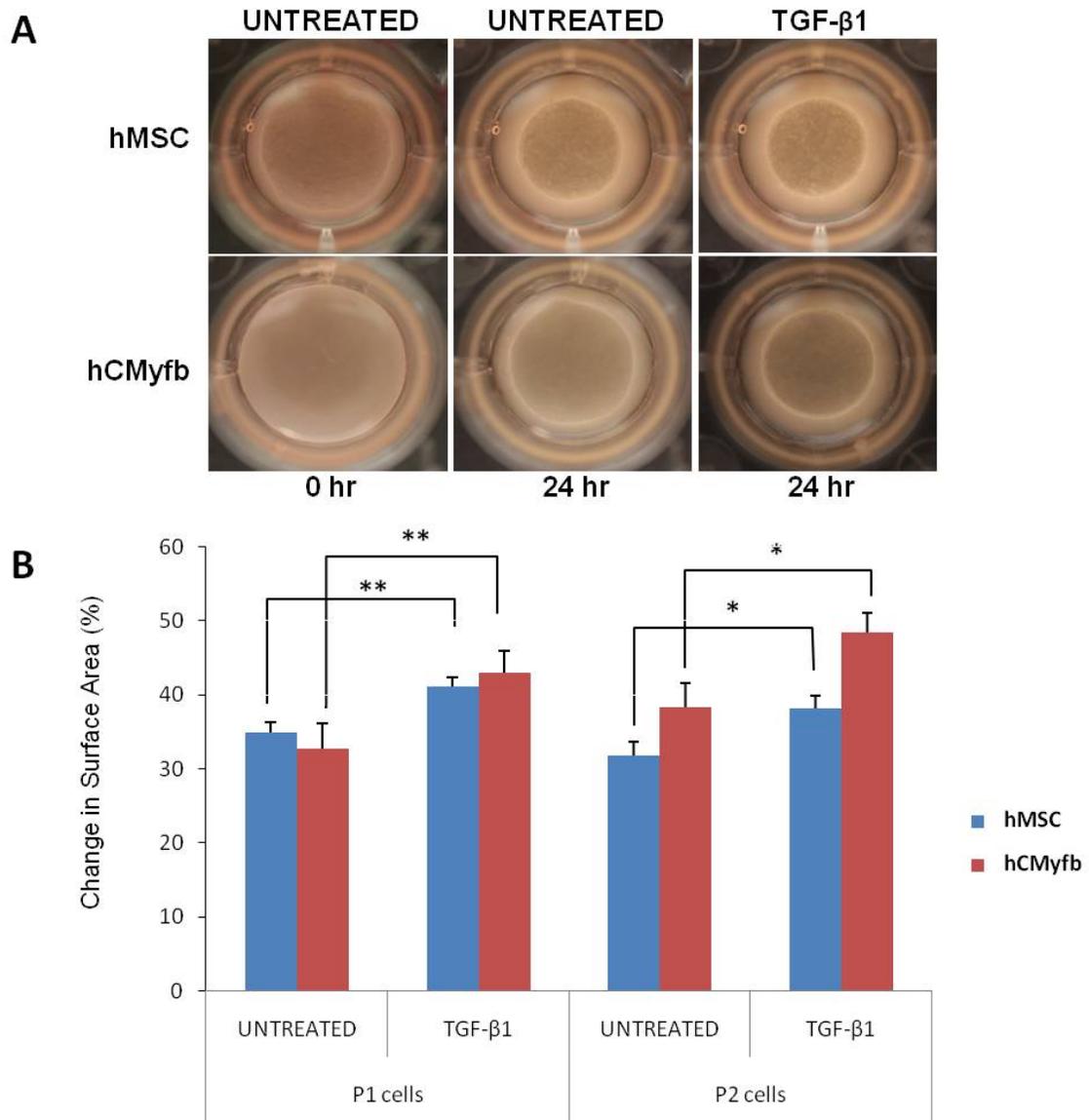


Figure 6: hMSCs contract collagen gels to a similar degree as hCMyfbs and display TGF-β1 responsiveness.

Representative images of hMSCs and hCMyfbs from a single patient showing reduced collagen gel surface area after 24 hours with or without TGF-β1 (10 ng/ml) treatment (A). hMSCs seeded onto collagen gel matrixes functionally contract to the same extent as ventricular cardiac myofibroblasts and displayed a significant increase in contractility with TGF-β1 treatment (B). Samples were run in triplicate and comparable results were obtained in multiple experimental repetitions with both hMSCs (n=8) and hCMyfbs (n=4-6). Results are displayed as mean \pm SEM. * $p < 0.05$ vs. non-stimulated control. ** $p < 0.01$ vs. non-stimulated control. Abbreviations: human mesenchymal stem cells (hMSCs), human cardiac myofibroblasts (hCMyfbs) and transforming growth factor beta 1 (TGF-β1).

Furthermore, the effects of different serum conditions on gel contractility were compared. P1 hMSCs were either serum starved or cultured in low serum (1%FBS) media one day prior to treatment with TGF- β 1 (10 ng/ml). Cells cultured in serum free conditions significantly increased contractility by 26.9% as compared to cells cultured in low serum conditions (Figure 7). Thus, cells cultured in serum free media exhibit a more differentiated myofibroblast phenotype associated with increased contractility. These findings suggest that hMSCs functionally behave like cardiac myofibroblasts *in vitro* and display TGF- β 1 responsiveness when cultured on collagen matrices.

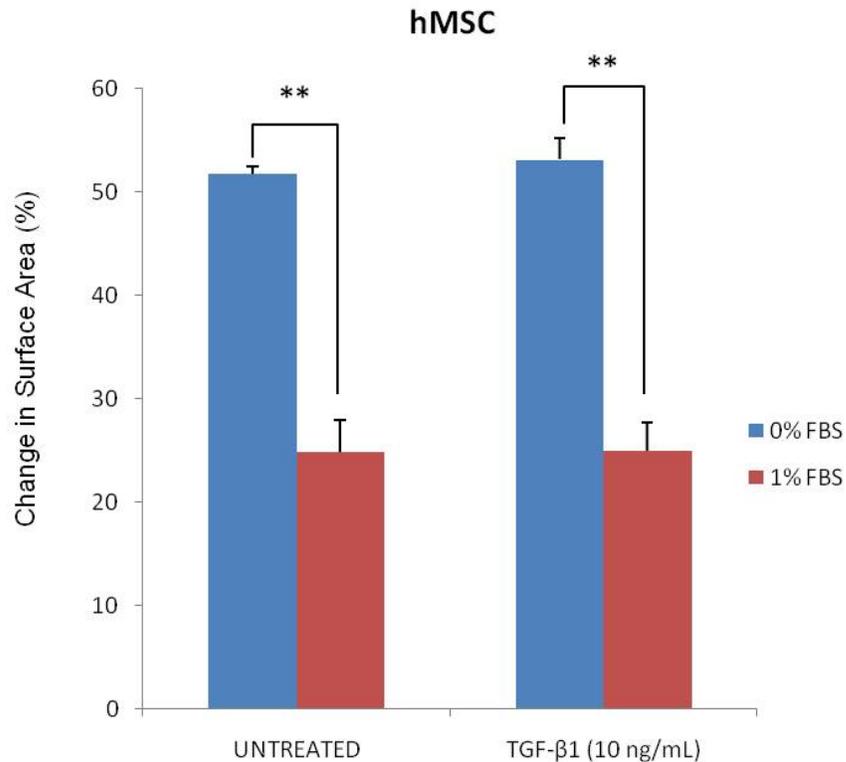


Figure 7: hMSCs display increased contractility under serum free conditions. Passage 1 hMSCs cultured on collagen gel substrates under serum free conditions are significantly more contractile in comparison to cells cultured under 1% serum conditions for 48 hours. Samples were run in triplicate and comparable experiments were repeated (n=3). Results are displayed as mean \pm SEM. ** $p < 0.01$ vs. non stimulated control. Abbreviations: human mesenchymal stem cells (hMSCs), transforming growth factor beta 1 (TGF- β 1) and fetal bovine serum (FBS).

The physiological function of hMSCs was further assessed by measuring the production of mature type I collagen *in vitro*. Collagen is synthesized and secreted by myofibroblasts as procollagen precursors, and is converted to mature collagen by proteolytic reactions catalyzed by specific procollagen proteinases (Prockop and Kivirikko, 1995). During collagen production, the carboxyterminal propeptide of type I collagen (CICP) is cleaved and released into the cell culture media. Thus, measurement of CICP is a reliable indicator of mature type I collagen production. All patient samples (n=4-7) were run in triplicates. The level of CICP in cells treated with or without TGF- β 1 (1 ng/ml and 10 ng/ml) for 48 hours was measured at each passage. Similar levels of collagen production between hMSCs and hCMYfbs at different passages was observed (Figure 8). However, in contrast to the observed TGF- β 1 responsiveness of hMSCs on collagen gel substrates, hMSCs did not show a significant increase in collagen production with either dose of TGF- β 1 treatment. Thus, hMSCs synthesize mature type I collagen independent of TGF- β 1 stimulation.

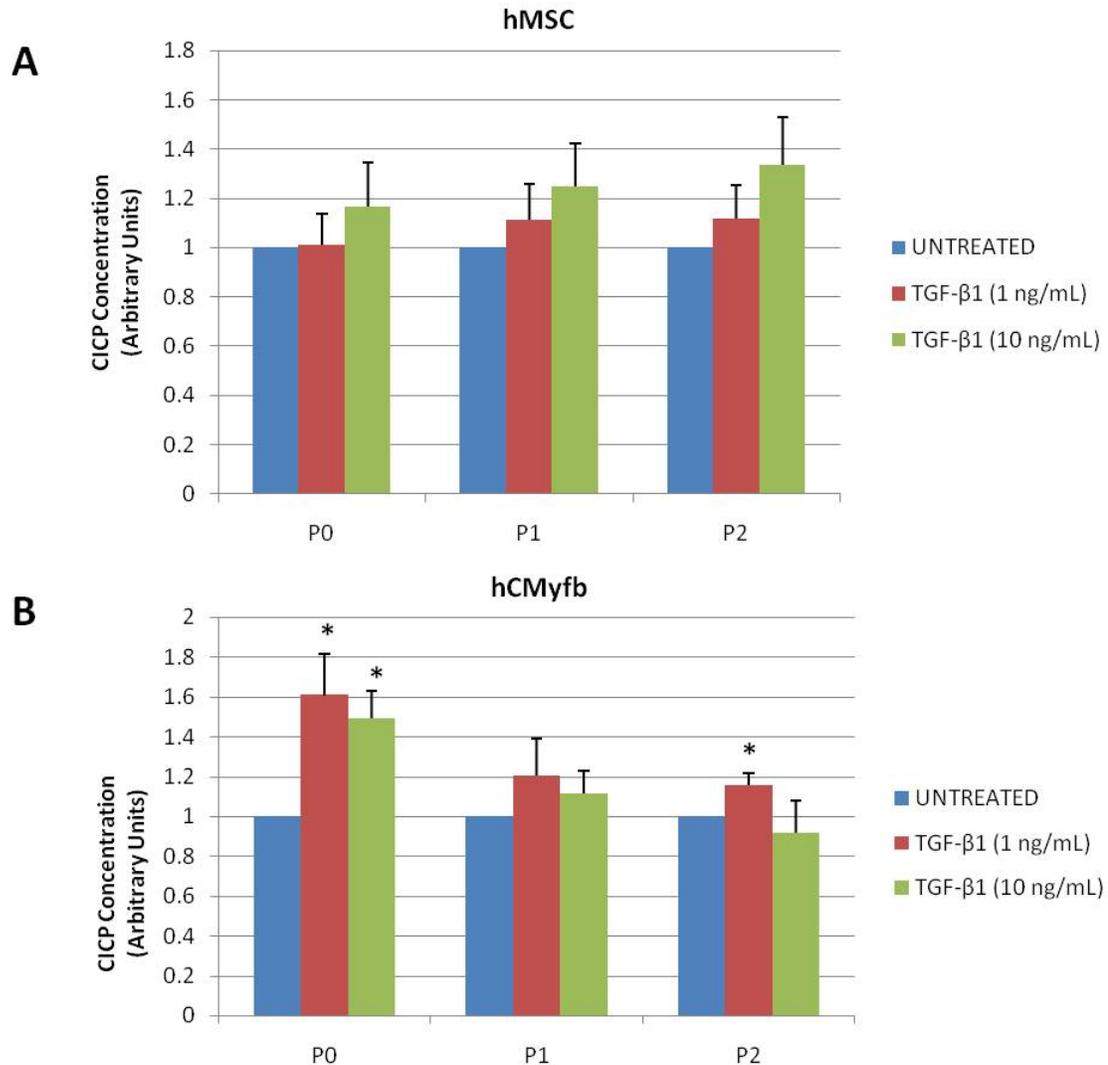


Figure 8: hMSCs synthesize mature collagen type I independently of TGF-β1 stimulation.

Levels of C1CP measured in the cell culture media showed comparable levels of mature collagen production between hMSCs (A) and their respective ventricular cardiac myofibroblast counterparts (B). hMSCs treated with TGF-β1 (1 ng/ml and 10 ng/ml) for 48 hours did not result in significant increased collagen synthesis. Samples were analyzed in triplicate and comparable results were obtained in multiple experimental repetitions with both hMSCs (n=7) and hCMyfb (n=4). Results are displayed as mean ± SEM. * $p < 0.05$ vs. non-stimulated control. Abbreviations: Collagen I carboxyterminal propeptide (C1CP), human cardiac myofibroblast (hCMyfb), human mesenchymal stem cells (hMSCs), and transforming growth factor beta 1 (TGF-β1).

2. Myosin expression in hMSCs

To determine which myosins are expressed in hMSCs, we probed for the presence of all three isoforms of non-muscle myosin II (IIA, IIB, and IIC) and smooth muscle myosin (SMM). Figure 9 demonstrates that both hMSCs and CMyfbs expressed the non-muscle myosin isoforms IIA and IIB, but did not express the IIC isoform or SMM. To ensure that our antibodies were specific, we used skeletal muscle and smooth muscle tissue as positive controls for NMMIIC and SMM respectively.

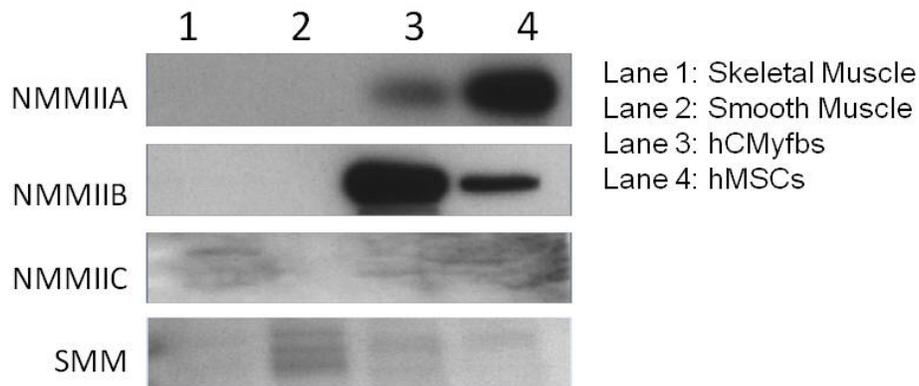


Figure 9: Myosin expression in hMSCs and cardiac myofibroblasts

Western blot analysis showing specificity of the different myosin antibodies. Both hMSCs and ventricular cardiac myofibroblasts showed expression of NMMIIA and NMMIIB isoforms, but lack of NMMIIC and SMM. Skeletal muscle and smooth muscle tissue were used as a positive control for NMMIIC and SMM respectively. Abbreviations: human cardiac myofibroblasts (hCMyfbs), human mesenchymal stem cells (MSCs), non-muscle myosin II (NMMII) and smooth muscle myosin (SMM).

3. Effect of NMMII inhibition on hMSC phenotype

3.1 Blebbistatin

Blebbistatin, a small molecular inhibitor specific for muscle and NMMII, binds to the myosin-ADP-Pi complex with high affinity and interferes with the phosphate release process required for rigid actomyosin cross-linking (Limouze et al., 2004). Thus, treatment with blebbistatin was used to assess the change in both function and phenotype associated with the inhibition of NMMII's activity. We chronically treated our cells with blebbistatin (5 μ M and 10 μ M) immediately after isolation. Chronic treatment with blebbistatin resulted in an increased expression of NMMIIA, NMMIIB, α -SMA, vimentin and decreased expression of ED-A fibronectin at all passages as compared to untreated cells in a dose dependent manner (Figure 10). These findings were confirmed by repeat experiments (n=3). Thus, inhibition of NMMII alters the expression of myofibroblast markers by hMSCs over serial passage.

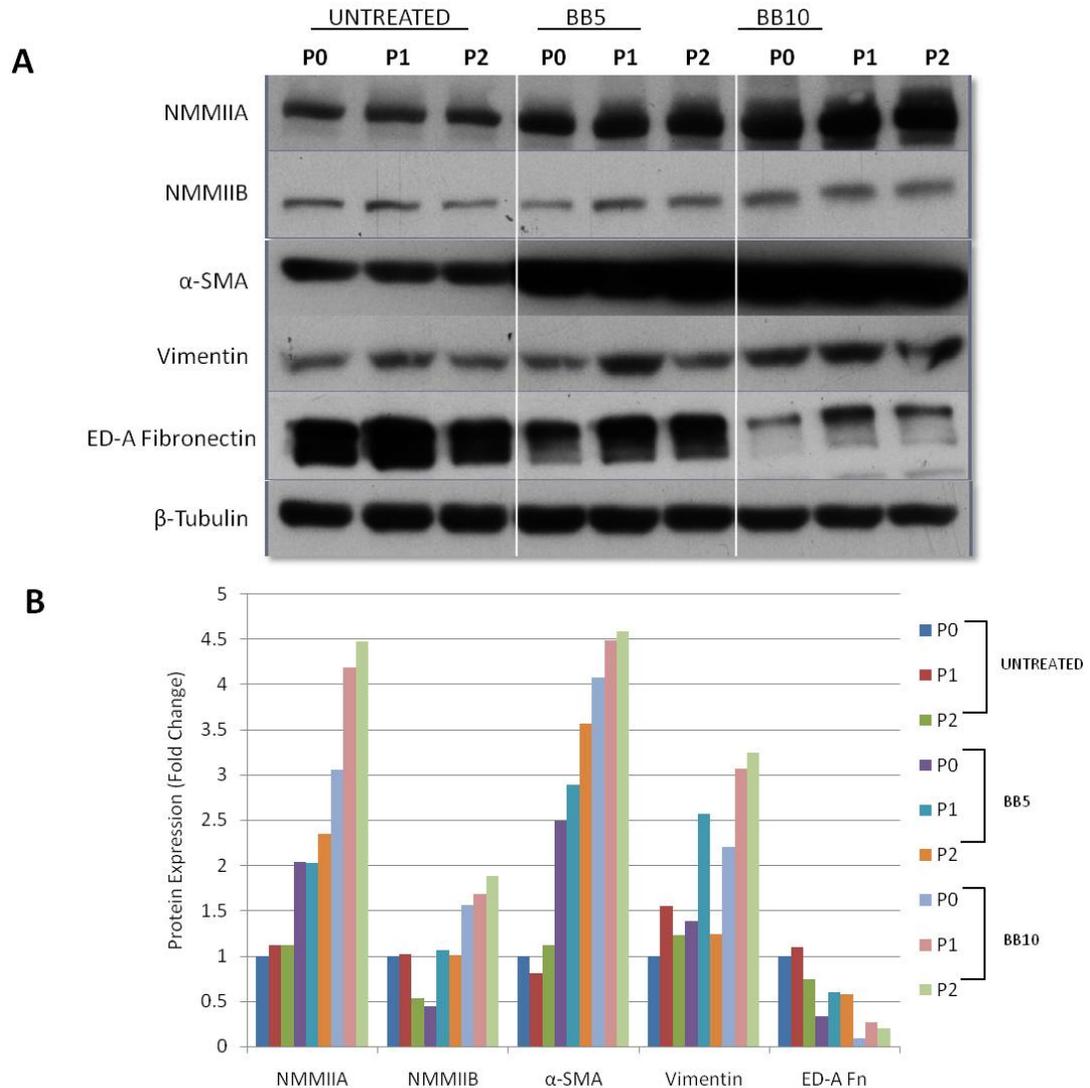


Figure 10: Chronic NMMII inhibition with blebbistatin alters expression of myofibroblast markers over serial passage.

hMSCs chronically treated with the NMMII inhibition drug blebbistatin (5 μ M and 10 μ M) from isolation showed an increased expression of NMMIIA, NMMIIB, α -SMA, vimentin and decreased expression of ED-A fibronectin compared to untreated P0 cells. Representative Western blots from a single patient (A) and corresponding histogram (B). The result was confirmed by repeat experiment (n=3). Abbreviations: human mesenchymal stem cells (hMSCs), non-muscle myosin II (NMMII), alpha smooth muscle actin (α -SMA), ED-A fibronectin (ED-A Fn) and blebbistatin (BB).

3.2 siRNA knockdown

MYH9 and MYH10 siRNA were used to knockdown NMMIIA and NMMIIB (NMMIIC was not tested as its expression was not detected in our cells) to examine the effects of each specific NMMII isoform on hMSC phenotype and function. We employed SMART Pool siRNAs that contained four different target sequences for greater target specificity knockdown. All patient samples (n=3-4) were run in triplicates. Quantitative real-time PCR analysis displayed that a knockdown efficiency greater than 80% in both MYH9 (100 nM) and MYH10 (50 nM) was achieved at 24 and 48 hours post-transfection (Figure 11). Gene expression of MYH9 and MYH10 was compared to non-targeting negative control siRNA (50 and 100 nM) treated cells as a change in fold. Knockdown of each MYH gene was target specific and simultaneous knockdown of MYH9/10 (50 + 50 nM) was greater than 65% at both time points.

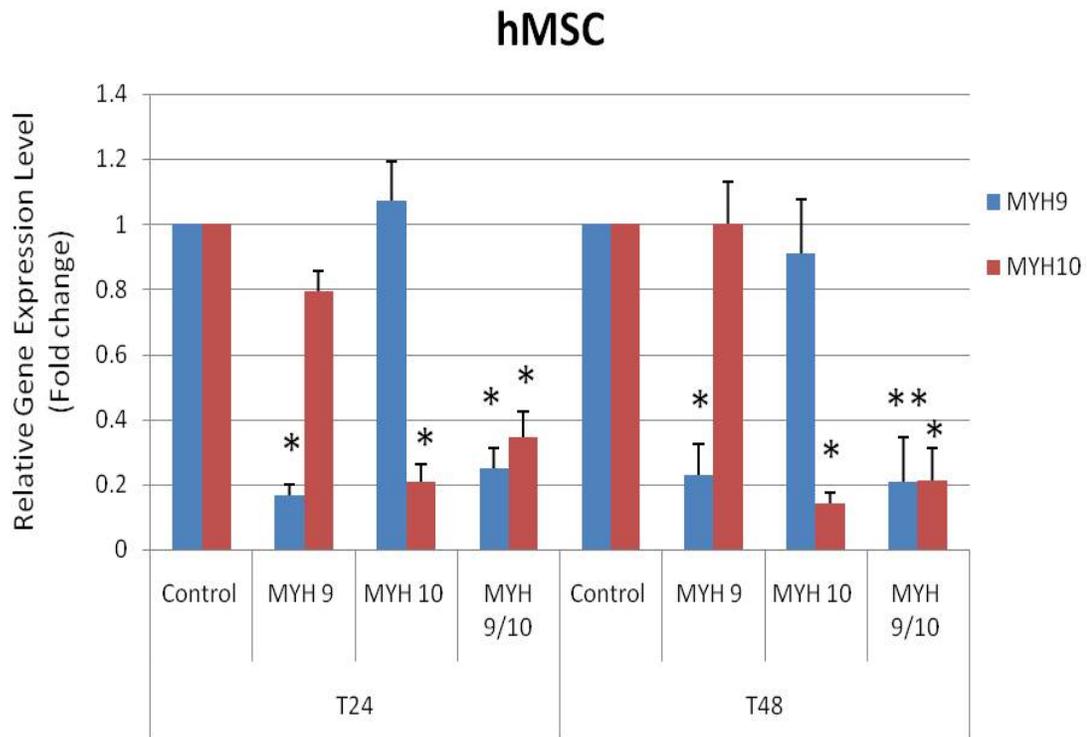


Figure 11: NMMIIA and IIB siRNA knockdown is efficient and specific.

NMMIIA and IIB expression was knocked down with the Dharmacon on-target SMART pool MYH9 and MYH10 siRNA respectively in P1 hMSCs. Both MYH9 (100 nM) and MYH10 (50 nM) siRNA pools were efficient and specific at both 24 and 48 hours post-transfection when compared to control siRNA (50 and 100 nM) treated cells (blue columns indicate MYH9 expression, and red columns indicate MYH10 gene expression). MYH9/10 (50 + 50 nM) siRNA knockdown was also efficient at both time points. The result was confirmed by repeat experiment (n=3-4). Results are displayed as mean \pm SEM. * $p < 0.01$ vs non stimulated control and ** $p < 0.05$ vs non stimulated control. Abbreviations: human mesenchymal stem cell (hMSC) and non-muscle myosin II (NMMII).

Knockdown efficiency and specificity was further confirmed using Western blot analysis. P1 hMSCs display decreased NMMIIA expression with MYH9 (100 nM) and MYH9/10 siRNA knockdown over a span of 96 hours (Figure 12). MYH9 knockdown was specific and MYH10 (50 nM) knockdown showed increased NMMIIA expression, consistent with results observed in RT-PCR. MYH10 (50 nM) and MYH9/10 siRNA knockdown displayed decreased expression of NMMIIB over a span of 96 hours. Moreover, MYH9 (100 nM) knockdown showed increased expression of NMMIIB at both 24 and 48 hours, an effect that was not observed with RT-PCR. High MYH9/10 (50 nM + 50 nM) siRNA concentrations resulted in further decreased expression of NMMIIB compared to low MYH9/10 (25 nM + 25 nM) siRNA knockdown.

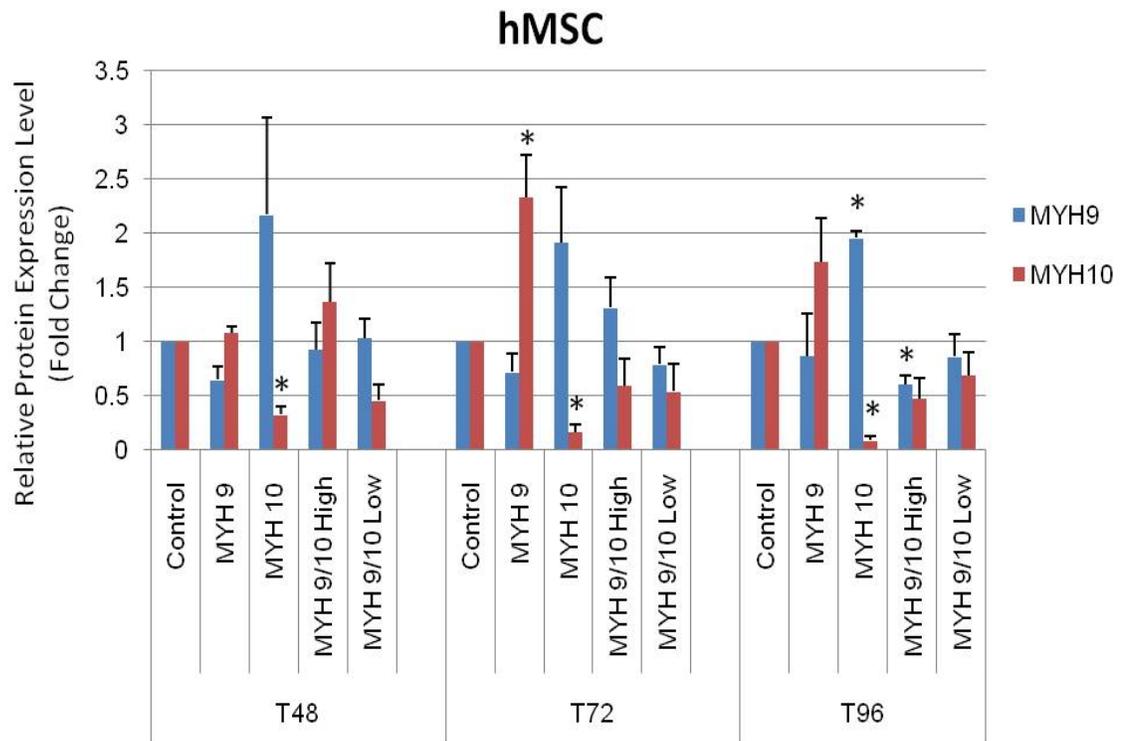


Figure 12: NMMIIA & IIB protein knockdown expression is stable over 96 hours. P1 hMSCs display decreased NMMIIA and IIB expression with MYH9 and MYH10 siRNA knockdown respectively over a span of 96 hours when compared to control siRNA treated cells. Both MYH9 (100 nM) and MYH10 (50 nM) knockdown was specific. MYH9 knockdown showed increased NMMIIB expression and vice versa. Decreased NMMIIA and IIB expression was seen with both high (100 nM) and low (50 nM) dose MYH9/10 siRNA at 96 hours. The result was confirmed by repeat experiment (n=3-6). Results are displayed as mean \pm SEM. * $p < 0.05$ vs. *non stimulated control*. Abbreviations: human mesenchymal stem cell (hMSC) and non-muscle myosin II (NMMII).

Human MSCs transfected with both MYH9 (50 nM) and MYH10 (50 nM) siRNA showed reduced and less prominent α -SMA and F-actin fibers 96 hours post-transfection compared to control siRNA treated cells (Figure 13). A complete change in morphology was observed with blebbistatin (50nM) treatment, an effect that may be associated with the disassociation of the actin-myosin complex.

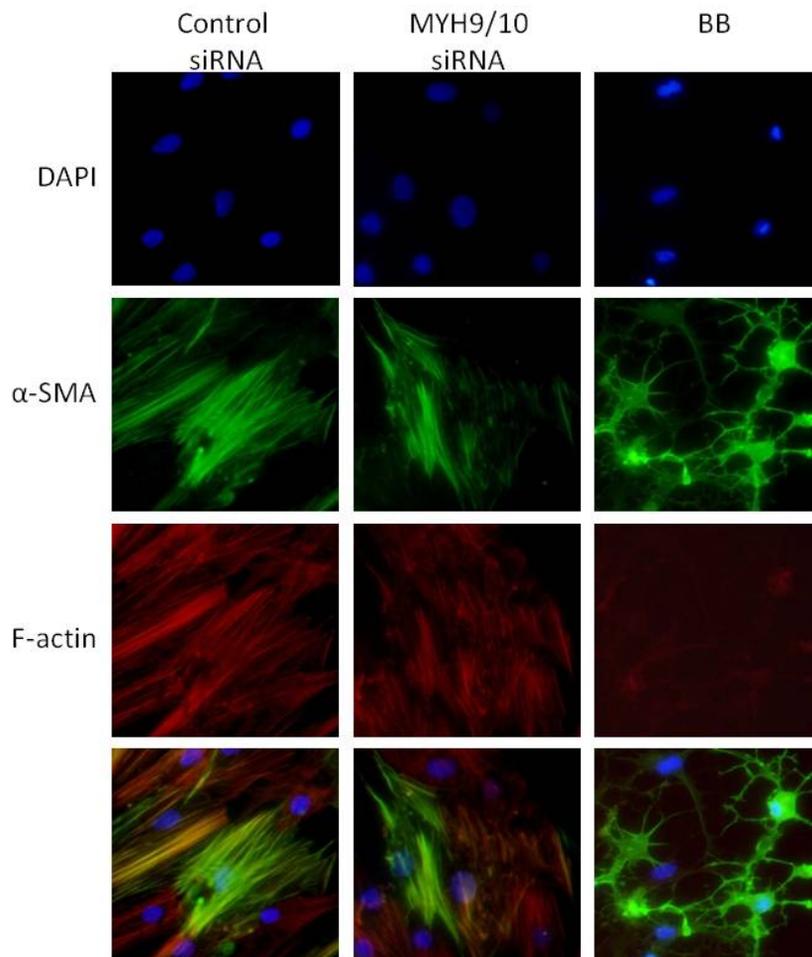
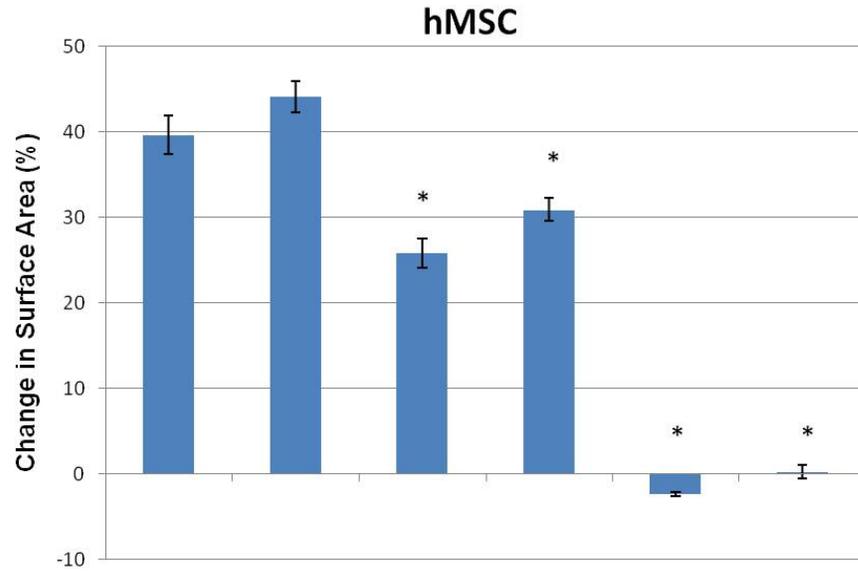


Figure 13: Inhibition of NMMII alters α -SMA and F-actin organization

Inhibition of NMMII with MYH9/10 siRNA (100 nM) displayed reduced and less prominent α -SMA (green) and F-actin (red) fibers in hMSCs 96 hours post-transfection compared to non-targeting negative control siRNA (100 nM) treated cells. Complete change in morphology was observed with blebbistatin (50 μ M) treatment. Bottom panel displays merged image. Abbreviations: non-muscle myosin II (NMMII), alpha smooth muscle actin (α -SMA), filamentous actin (F-actin) and blebbistatin (BB).

4. Effect of NMMII inhibition on gel contractility

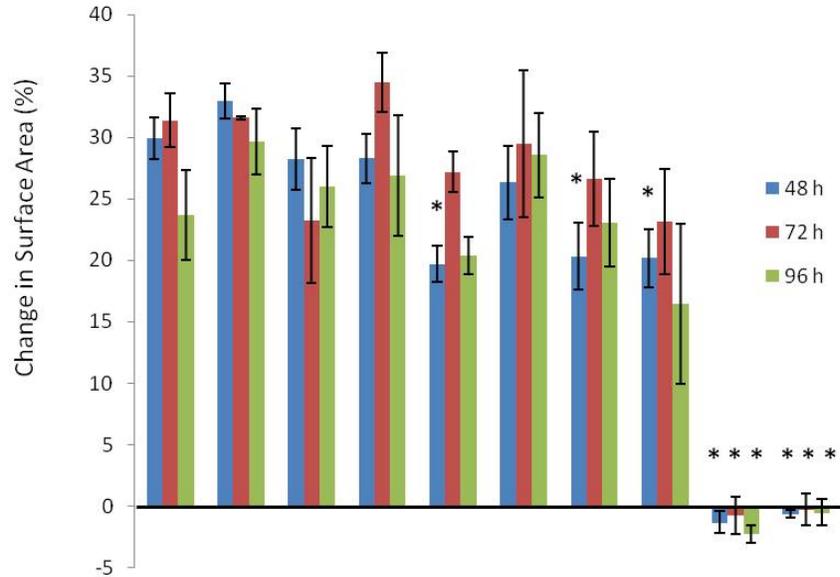
To determine if NMMII inhibition could functionally affect contractility in hMSCs, we plated P1 hMSCs onto collagen gel substrates and treated the cells with blebbistatin (10 μ M and 50 μ M) for 24 hours. Treatment with low dose blebbistatin reduced cell contractility by 17.8% compared to untreated cells and did not affect the cell's TGF- β 1 responsiveness (Figure 14). Complete inhibition of gel contractility was observed with high dose blebbistatin treatment. Knockdown of MYH9, MYH10 and MYH9/10 at 48, 72, and 96 hours post transfection did not significantly reduce gel contractility when compared to cells transfected with a non-targeting control siRNA (Figure 15). The discrepancy observed between blebbistatin and siRNA treated cells may be a reflection of the differences in NMMII inhibition – blebbistatin inhibits NMMII's ATPase activity meanwhile siRNA reduces the gene expression.



TGF-β1 (10 ng/mL)		+		+		+
Blebbistatin (10 μM)			+	+		
Blebbistatin (50 μM)					+	+

Figure 14: NMMII inhibition with blebbistatin reduces gel contractility in hMSCs.

Cell contractility was enhanced with either TGF-β1 (10 ng/mL) treatment alone or attenuated by NMMII inhibition with blebbistatin treatment alone in P1 hMSCs. Treatment with blebbistatin (50 μM) for 48 hours completely inhibited basal and TGF-β1 induced contractility. Samples were run in triplicate and comparable experiments were repeated (n=6-16). Results are displayed as mean ± SEM. * $p < 0.01$ vs. non stimulated control. Abbreviations: human mesenchymal stem cell (hMSC), non-muscle myosin II (NMMII), and transforming growth factor beta 1 (TGF-β1).



TGF-β1 (10 ng/mL)		+		+		+		+		+
Non-targeting Negative Control siRNA (100 nM)	+	+								
MYH9 siRNA (100 nM)			+	+						
MYH10 siRNA (100 nM)					+	+				
MYH9 siRNA (50 nM)							+	+		
MYH10 siRNA (50 nM)							+	+		
Blebbistatin (50 μM)									+	+

Figure 15: Inhibition of NMMII with blebbistatin reduces collagen gel contractility greater than NMMII inhibition using specific isoform knockdown.

Significant reduction in P1 hMSC contractility was seen with blebbistatin (50 μM) treatment but not MYH9 (100 nM), MYH10 (100 nM) or MYH9/10 (100nM) siRNA knockdown when compared to non-targeting control siRNA (100 nM) treated cells at 48, 72, and 96 hours post-transfection. Samples were run in triplicate and comparable experiments were repeated (n=3). Results are displayed as mean ± SEM. * $p < 0.01$ vs. non stimulated control for corresponding time point. Abbreviations: Non-muscle myosin II (NMMII), human mesenchymal stem cells (hMSCs), and transforming growth factor beta 1 (TGF-β1).

5. Effect of NMMII inhibitor on cell viability

To assess whether the observed reduction in gel contractility was a result of NMMII inhibition and not reduced cell viability, cells plated on the collagen gel substrates were analyzed using a LIVE/DEAD assay kit 24 hours after the cells were allowed to contract. As shown in Figure 16, cell viability was preserved (> 99%) in both transfected cells (MYH9, MYH10 and MYH9/10) and blebbistatin (50 μ M) treated cells at 48, 72 and 96 hours post-transfection. Thus, inhibition of NMMII does not affect cell viability.

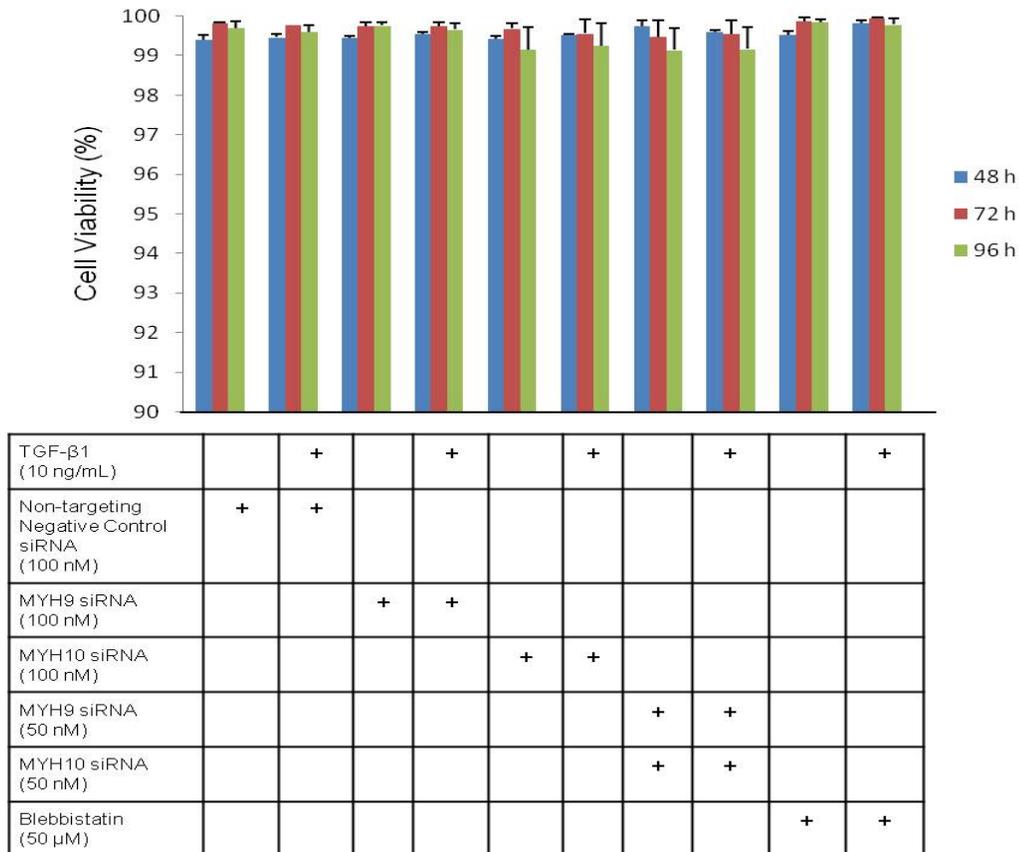


Figure 16: Inhibition of NMMII does not affect cell viability.

No change in cell viability was observed with NMMII inhibition with either siRNA or blebbistatin compared to non-targeting negative control siRNA cells at 48, 72 and 96 hours. P1 hMSC samples were run in triplicate and comparable experiments were repeated (n=3). Results are displayed as mean \pm SEM. Abbreviations: Non-muscle myosin II (NMMII), human mesenchymal stem cells (hMSCs), and transforming growth factor beta 1 (TGF- β 1).

IV. DISCUSSION

1. Significance of standard cell culture conditions stimulating acquisition of a myofibroblast phenotype by human MSCs *in vitro*

Bone marrow extracts contain heterogeneous cell populations. MSCs represent only 0.001-0.01% of the total population of nucleated cells in the bone marrow (Pittenger et al., 1999). Expansion of plastic adherence MSCs is the most widely used method of obtaining MSCs for cardiac repair (Williams and Hare, 2011). Cultures of hMSCs, unlike murine cells (Phinney et al., 1999), become devoid of hematopoietic precursors after a couple of passages and can be extensively expanded before they senesce (Sekiya et al., 2002). Furthermore, the choice of culture medium employed is an important factor for obtaining MSC of good quality. The most commonly employed reagents are α -MEM or DMEM with low glucose content, containing fetal bovine serum at concentrations of 10% or 20% (Chen et al., 2009). In this study, we employed DMEM F12 media supplemented with 20% FBS as our standard culture condition and characterized hMSCs and their functional properties within these conditions.

Previous investigators have demonstrated that hMSCs can differentiate into fibroblasts by cyclic mechanical stimulation and treatment with several growth factors, including connective tissue growth factor and fibroblast growth factor 2 (Altman et al., 2002; Noth et al., 2005; Moreau et al., 2005; Hankemeier et al., 2005; Lee et al., 2010). Our data indicate that hMSCs readily adopt a functional myofibroblastic phenotype in culture without the use of any additional stimulation or inducing growth factors. It is known that MSCs produce an array of endogenous growth factors and cytokines, including the basic fibroblast growth factor (Schuleri et al., 2007). It is possible that basic

fibroblast growth factor secreted into the conditioned medium from cultured MSCs may promote this MSC-myofibroblast differentiation. In addition, the effects of culture expansion on hard plastic surfaces as well as the stress from mechanical isolation may also be key players in modulating this MSC-myofibroblast phenomenon (Tse and Engler, 2011). In one study, hMSCs assumed morphological patterns and gene expression patterns consistent with differentiation into distinct tissue-specific cell types when exposed to polyacrylamide gels with different ranges of stiffness (Engler et al., 2006). MSCs cultured extensively *in vitro* displayed up regulation of genes involved in cell differentiation, apoptosis and cell death, whereas expression of genes associated with mitosis and proliferation were down regulated (Schallmoser et al., 2010). It has been suggested that in an attempt to adapt to their new environment, MSCs detached from their *in vivo* niche undergo changes in gene expression and partial differentiation (Pevsner-Fischer et al., 2011). Thus, one can postulate that the disruption of the microenvironment niche when isolating MSCs from bone marrow, combined with the drastic increase in stiffness matrix elasticity owing to the rigidity of plastic culture dishes used, may initiate a cascade of signalling events that induces these primary cells to undergo differentiation to myofibroblasts *in vitro*.

Moreover, numerous parameters of culture isolation and expansion conditions on the differentiation capacity of hMSCs were considered when comparing results between different studies. Factors such as the source of bone marrow, isolation technique, the initial plating density, incubation times between passages, culture media employed, type of culture flask and scaffolds used, and exogenous growth factors added, among many other factors, have an enormous impact on the yield, quality and differentiation fate of

hMSCs. The lack of a standardized method to culture hMSCs is especially problematic as slight modifications might lead to completely different cell populations, and thereby hinder the reproducibility of one study to the next (Wagner and Ho, 2007).

The disparity between the results reported in this study compared with other studies could potentially be attributed to the source of bone marrow used, individual patient characteristics and cell passage used for analysis. While the majority of studies obtained their bone marrow aspirates from the iliac crests of normal healthy adult donors, we isolated bone marrow from the sternum of adult donors with various confounding heart diseases. Although MSC are immunologically tolerated, making them a very attractive candidate of allogeneic use, autologous stem cells are still the safest to use in clinical trials. Healthy donors typically are young in age and their bone marrow composition can significantly differ from that of our aging patient population. It is not yet well understood how and whether patient attributes such as age, gender, race, cardiovascular disease risk factors, medication, and co morbidities influence MSCs growth and differentiation. Further, most MSC studies use later cell passages (P4 and higher) for analysis where as in this study, we used early passages (P0-P2). Despite these differences, the strength of this study is that a direct comparison of bone marrow derived hMSCs and ventricular myofibroblasts from patients with heart disease was used, the very same patients that cellular cardiomyoplasty aims to help.

Our study showed that hMSCs isolated from patients with heart disease maintained their *in vitro* differentiation capacity into adipogenic, osteogenic, and chondrogenic lineages throughout serial passages despite their myofibroblast phenotype, a phenomenon that is not observed with human fibroblasts (Pittenger et al., 1999; Wagner

et al., 2005). This suggests that although these cells phenotypically and physiologically behave like myofibroblasts, they are unique in the sense that they still retain proliferative and differentiation properties characteristic of MSCs.

Observed differences in the relative expression of key myofibroblast markers in MSCs between patient samples may potentially be attributed to the heterogeneity of the starting population as colony forming units in the bone marrow represent a mixed population of progenitors at different stages of commitment. Differences in the composition of undifferentiated MSCs, and mature and immature differentiated myofibroblasts during culture are most likely due to variability among the bone marrow composition of each patient. Mature myofibroblasts are characterized by the expression of α -SMA in more extensively developed and organized stress fibres and by supermature focal adhesions *in vitro* (Tomasek et al., 2002). Notably, although the level of myofibroblast expression varied between patients and between passages, these markers were consistently expressed in each patient at all passages studied. We suspect that the change in phenotype occurs within days and possibly hours after isolation and culturing, although due to the lengthy time required to culture MSCs to obtain adequate numbers for characterization, we were unable to detect a precise time frame in which this phenotype switch occurs.

Myofibroblasts can sustain a tonic contractile force over prolonged periods of time. This force is generated by contractile stress fibers composed of bundles of actin microfilaments with associated non-muscle myosin and other actin-binding proteins (Tomasek et al., 2002). Treatment of TGF- β 1 is known to induce differentiation of protomyofibroblasts to differentiated myofibroblasts in collagen lattices and to induce

collagen synthesis (Roberts et al., 1986). Our data indicate that hMSCs treated with TGF- β 1 displayed increased contractility on collagen gels and that this effect may be attributed to an increased expression of α -SMA (Tomasek et al., 2002). In 2002, Kinner et al. demonstrated for the first time that expression of α -SMA enabled contraction of human MSCs. They reported a high correlation between α -SMA content and contractility, and that TGF- β 1 treatment up regulated α -SMA expression in these cells (Kinner et al., 2002).

Using pro-COL1A2 chimeric mice, van Amerongen and colleagues were able to show via β -galactosidase staining that bone marrow derived myofibroblasts expressed collagen I in the heart, and that this expression was confined to the infarct area, thus showing that bone marrow derived myofibroblasts actively participate in scar formation after MI, although their presence was transient (van Amerongen et al., 2008). Similar to their results, data presented herein demonstrates that hMSCs were able to synthesize and secrete collagen I in culture and that these levels were comparable to that of cardiac myofibroblasts. However, significant increase in collagen production when hMSCs were treated with TGF- β 1 was not observed. This lack of TGF- β 1 responsiveness in collagen I secretion may be attributed to age. In a recent study comparing fibroblasts from young and aged mice (4 and 30-month-old mice respectively), it was reported that fibroblasts derived from older mice under the same culture conditions as young fibroblasts, demonstrated no substantial increase in connective tissue growth factor and collagen type I mRNA expression in response to TGF- β 1 (Cieslik et al., 2011). In contrast to the TGF- β 1 responsiveness observed with hMSCs plated on collagen gels, this lack of increased collagen secretion with TGF- β 1 treatment may reflect differences in downstream TGF- β 1

signaling pathway targets between cell contraction and collagen synthesis. In addition, this discrepancy in TGF- β 1 responsiveness might also be accredited to differences in matrix substrate (plastic vs. gel) as studies have shown that matrix elasticity plays a greater role than addition of soluble induction factors in stem cell differentiation (Engler et al., 2006). Cellular tension is modulated by matrix stiffness, with force transmission occurring via focal adhesions (Engler et al., 2006). Associated with the focal adhesion complexes are a number of well-known signaling molecules that act as the mechano-transducers, relaying information about the cell's microenvironment to the cell and ultimately influencing their differentiation fate (Engler et al., 2006). We speculate that the stiffness of culture dishes used to determine collagen synthesis induced a phenotype that is less sensitive to TGF- β 1 compared to that of the much softer elasticity of collagen gels used to assess contractility. Moreover, recent evidence suggests that aging and diabetes significantly alter the cellular responses to TGF- β 1 (Wu et al., 1999; Broadley et al, 1989).

2. Significance of NMMII inhibition on adoptive myofibroblast phenotype and function

Our results show that hMSCS derived from bone marrow, express NMMIIA and IIB, but not IIC. Engler and colleagues reported reduced transcript levels of NMMIIA (to 50%) and IIB (to 8%) in MSCs when chronically treated with 50 μ M of blebbistatin (Engler et al., 2006). In contrast to their findings, our results demonstrate that chronic treatment of hMSCs with 5 and 10 μ M of blebbistatin revealed a remarkable dose-dependent increase in NMMIIA, NMMIIB, α -SMA and vimentin expression, consistent

with an up regulated myofibroblast phenotype. The observed differences may be related to the different chronic blebbistatin doses (5 and 10 μM vs. 50 μM) employed. Since blebbistatin has a half maximal inhibition (IC_{50}) value of 5.1 and 1.8 μM for NMMIIA and IIB respectively (Limouze et al., 2004), it may be that blebbistatin used at 5 and 10 μM were not sufficiently high enough doses to induce a significant reduction in NMMII expression. In addition, since low dose chronic blebbistatin treatment does not attain maximal NMMII inhibition values, it is possible that the cells may secrete factors or signals that induced an increase in myofibroblast marker expression as a compensatory mechanism. Moreover, since NMMII is a key cytoskeletal motor involved in exerting force through focal adhesions in mechanisms of the matrix-elasticity sensing that drives lineage specification (Engler et al., 2006), it is possible that disruption of this sensing at isolation, relays signals to the cell causing it to adopt this altered phenotype.

Interestingly, expression of ED-A fibronectin was decreased with chronic blebbistatin treatment. Fibronectin is an adhesive ECM protein that is involved in cell adhesion, migration, proliferation and differentiation (van der Straaten et al., 2004). It is produced by fibroblasts, endothelial cells, adipocytes and to a lesser extent, by hepatocytes (van der Straaten et al., 2004). Under mechanical stress, ED-A fibronectin and TGF- β 1, are known to induce the myofibroblast phenotype through increased expression of α -SMA and collagen type I expression in fibroblasts (Desmouliere et al., 1993; Rønnov-Jessen and Petersen, 1993; Vyalov et al., 1993; Jarnagin et al., 1994; Zhang et al., 1994; Serini et al., 1998). It has therefore been suggested that ED-A fibronectin is a marker of fibrosis (van der Straaten et al., 2004). Thus, decreased expression of ED-A fibronectin with chronic

blebbistatin treatment may indicate that inhibition of NMMII may play a role in attenuating fibrosis.

Human MSCs plated on collagen gel substrates demonstrated reduced gel contractility when treated with low dose blebbistatin (10 μ M), but complete contractile inhibition was only obtained with high dose blebbistatin (50 μ M). Low dose blebbistatin acts by inhibiting the ATPase activity of myosin, but does not inhibit the movement of all actin filaments as less than 100% of the NMMIIs are inhibited at 10 μ M. Blebbistatin at 50 μ M, a concentration much higher than the IC_{50} is thus needed to inhibit most or all of the myosin interactions with actin filament.

Despite the fact that smooth muscle myosin is more closely related to NMMIIA and IIB, than to any striated muscle myosin heavy chain gene, blebbistatin does not inhibit SMM (Berg et al., 2001). Complete inhibition of gel contractility at 50 μ M of blebbistatin suggests that the involvement of other myosins, such as smooth muscle myosin, is not involved in the actin-myosin contractility of myofibroblasts, further supporting our results that smooth muscle myosin is not expressed in our cells.

As blebbistatin inhibits all of the NMMII isoforms, the specific contribution of NMMIIA and IIB in reducing gel contractility was tested. Knockdown of NMMIIB with MYH10 siRNA resulted in increased NMMIIA expression, and vice versa. However, NMMIIA expression was much greater in MYH10 knockdown cells than NMMIIB expression in MYH9 knockdown cells. We suggest that this disparity in compensatory NMMII expression may be related to differences in enzyme kinetics as studies have reported that NMMIIB has a higher duty ration than IIA, therefore making it more suitable for structural roles since it can exert tension on actin filaments for a longer length

of time without utilizing as much ATP as IIA does (Flynn and Helfman, 2010). Thus it is possible that NMMIIB expression adapts less quickly than NMMIIA expression and hence, would provide insight into why NMMIIA expression was more highly expressed with MYH10 knockdown than NMMIIB expression with MYH9 knockdown.

In contrast to the observed effects of blebbistatin treatment on collagen gel contractility, knockdown of specific NMMII isoforms did not show the same results. It is possible that although we saw greater than 80% knockdown expression in both NMMIIA and IIB, the low level expression of NMMII that remains is sufficient enough to compensate. Moreover, in comparing reduced gel contractility in cells treated with low dose blebbistatin with NMMII expression knockdown, it may be possible that inhibition of NMMII activity is more potent than decreased expression of NMMII in regulating contractility. Another possible confounding effect may be the possible off target actions of blebbistatin that result in complete contractile inhibition.

Contrary to what other studies have reported, we showed that cell viability was preserved with both inhibition and knockdown of NMMII expression, and that cell viability was maintained over a span of 96 hours after post-transfection. This is therefore congruent with the notion that reduction in gel contractility with NMMII inhibition is not due to reduced cell viability.

Taken together, the observations made in this study suggest that NMMII, specifically the NMMIIA and IIB isoforms, may play a role in attenuating the acquired MSC-myofibroblastic contractile function without affecting cell viability and thus, may provide a potential therapeutic target in regulating MSCs differentiation and fibrosis associated with myofibroblasts.

V. CONCLUSION AND FUTURE DIRECTIONS

In conclusion, we have shown that bone marrow derived hMSCs (under standard culture conditions) demonstrate gene expression profiles that are similar to human CMyfbs and exhibit similar functional characteristics, with respect to collagen synthesis and TGF- β 1 induced collagen gel contraction. Moreover, it was also shown that inhibition of NMMII can attenuate the contractile function of these MSC-myofibroblasts. The implications of these findings in regards to therapeutic strategies for cardiac repair are twofold: contribution to cardiac fibrosis and implications for cellular cardiomyoplasty. However, the results reported here must be interpreted with caution. Although hMSCs adopt a myofibroblastic phenotype in culture, we have not shown that (1) these cells can contribute to fibrosis and (2) these cells are unable to further differentiate into functional myocytes both *in vitro* and *in vivo*. Thus, we can only continue to speculate that hMSCs are unlikely to further differentiate as others have suggested that the fibroblast is the end-stage lineage of multipotential MSCs (Sarugaser et al., 2009). Additional studies investigating the fibrotic contribution of cultured MSCs and their potential for further differentiation *in vivo* are warranted.

Intense efforts during the past decade have shown that cellular cardiomyoplasty using stem cell therapy holds great promise as an adjunctive treatment for systolic heart failure. The ultimate goal of stem cell transplantation is formation of new and functional myocardial tissue (Segers and Lee, 2011). The ideal stem cell should be easy to isolate, should have a high survival rate after delivery, and should form contractile and non-arrhythmogenic cardiac tissue (Segers and Lee, 2011). Among the different types of stem

cells, MSCs are recognized as the best potential candidates for treatment of cardiac diseases due to their beneficial properties, such as easy isolation, rapid expansion *in vitro*, unique immune-privileged status and rare formation of teratomas (Kumar et al., 2008). Various clinical trials have reported moderate improvement in ventricular function after transplantation of bone marrow derived stem cells. Most studies typically report an increase in left ventricular ejection fraction of only a few percent, usually ranging from 0-15% (Wei et al., 2009). Although these results yield a statistically significant increase in left ventricular ejection fraction, clinically, these numbers reflect a very marginal benefit and do not obtain clinical significance (ejection fraction greater than 50%) (Bhatia et al., 2006). Furthermore, the lack of differentiation of MSCs into functionally integrated cardiomyocytes remains an obstacle to this effective implantation (van den Borne et al., 2010; Orlic et al., 2001).

The infarcted myocardium is an ischemic and hostile environment to most cells. Regardless of the delivery route, MSC engraftment and retention in the myocardium remains low (Williams and Hare, 2011). Biomaterials can mimic or include naturally occurring ECM and also instruct stem cell function in different ways (Segers and Lee, 2011). For the purpose of cardiac regeneration after MI, biomaterials could be designed with the optimal stiffness for a particular stem cell to engraft, and may contain signals for attachment, proliferation and differentiation (Segers and Lee, 2011). MSC expression of α -SMA and their ability to contract is important with respect to the adverse effects that α -SMA-enabled contraction can have if the cells distort scaffolds into which they are seeded or distort newly synthesized matrix *in vivo* in which they are employed for cell therapy (Kinner et al., 2002).

Whether the use of MSCs will be beneficial or detrimental to cardiac repair remains controversial. On one hand, it has been shown that bone marrow derived stem cells can contribute to cardiac fibrosis and understanding the mechanisms behind this recruitment of MSCs to the injured heart will prove beneficial to providing novel therapeutic targets. On the other hand, the use of MSC in cellular cardiomyoplasty has shown to be promising, although there are many obstacles left to overcome, such as long term engraftment and differentiation into functional myocytes. The observations reported here underscore the importance of proper selection and characterization of BM-MSCs that are ultimately selected for use in cell therapy. Moreover, whether bone marrow derived MSCs must be kept in an undifferentiated state if they are to adopt a functional cardiac myocyte phenotype upon transplantation warrants further investigation.

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