

# **ENGINEERED HUMAN CARDIAC TISSUE FROM MUSCLE DERIVED STEM CELLS**

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Submitted to the Graduate Faculty of  
Swanson School of Engineering in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy in Bioengineering

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH  
SWANSON SCHOOL OF ENGINEERING

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University of Pittsburgh, 2014

Heart failure results in significant cardiomyocyte (CM) loss, and post-natal mammalian heart has limited regenerative capacity. Cellular cardiomyoplasty has emerged as a novel therapy to restore contractile function. A number of cell types illicit functional benefits through paracrine mechanisms, but cardiac stem cells are unique in their ability to preferentially differentiate down a cardiac lineage to replace lost CMs. However, cardiac stem cell isolation is highly invasive. Alternatively, skeletal myoblasts can be safely isolated and showed some benefits in clinical trials as donor muscle cells, but arrhythmias occurred due to lack of electric coupling with host cells. This limitation could be overcome by differentiating cells toward a cardiomyogenic lineage. Multipotent muscle derived stem cells (MDSC) are different from skeletal myoblasts and possess greater phenotypic plasticity. Our studies showed that cardiac and skeletal muscle share major genes/proteins during development in rodents, so it may be possible for human MDSCs to differentiate into CM-like cells under the appropriate conditions. My dissertation aims to develop approaches to differentiate human MDSCs into CM-like cells. Specifically, my work focuses on three aims: (I) to characterize the biochemical and functional properties of human MDSCs cultured in a 3-dimensional engineered muscle tissue (EMT) and examine whether it recapitulates properties of developing striated muscle; (II) to determine the potential for further CM differentiation under defined biophysical and chemical conditions; (III) to evaluate the potential of human MDSC derived cardiac progenitors to improve cardiac function in a human-rat xenograft model.

The results of my studies showed that human MDSCs in EMT beat spontaneously, displayed calcium transients, expressed cardiac-specific genes/proteins, and exhibited pharmacological responses similar to iPS cell-derived CMs. They also possessed characteristics of skeletal muscle including expression of MyoD, myogenin, and sk-fMHC. Their electrical

coupling also remained immature. By temporally treating EMT with 4 chemical factors (4CF: miR-206 inhibitor, IWR-1, BMP4, and LiCl) and improving aggregation conditions, 4F-AEMT showed better muscle tissue formation and cardiac-like morphology with improved contractility, pharmacological responses, and electrical coupling. Although 4F-AEMT expressed MyoD and myogenin, it exhibited more cardiac-like function. Finally, human MDSC-aggregates showed evidence of survival and improved cardiac function in vivo.

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## ACKNOWLEDGEMENTS

The work presented here in this dissertation would not have been possible without the contributions of the people I would like to acknowledge here. First, I would like to thank my advisor, Dr. Kimimasa Tobita, for his continuous guidance and support. I would also like to thank the other members of my committee, Dr. Sanjeev Shroff, Dr. Lei Yang, and Dr. Johnny Huard for their constructive feedback and expertise of their respective labs.

I would also like to thank the other members of the lab, past and present, Dr. Lijun Liu, Shivam Verma, Jillian Chen, and Narmeen Hassan. I would like to specifically thank Dr. Bo Lin and Dr. Lu Han from Dr. Lei Yang's lab for providing human iPS cell derived cardiomyocytes and several reagents used in these studies. I would also like to acknowledge Dr. Jong Jin Kim and Dr. Richard Francis for their help with the calcium transient imaging experiments and Dr. Cecilia Lo for use of her lab's microscope for live cell calcium imaging.

I would like to acknowledge the sources of funding that allowed me to pursue and complete this work, The National Institutes of Health Cardiovascular Bioengineering Training Grant (CBTP, T32 HL076124), The American Heart Association, and the Commonwealth of Pennsylvania.

Lastly I would like to thank my parents and family for their unwavering support through this long and challenging process.

## 1.0 INTRODUCTION

In the United States, an estimated 15.4 million people have coronary heart disease, of which 7.6 million are affected by myocardial infarction and 2.1 million by congestive heart failure [1]. In the last two decades, the classic concept of the heart as an organ with extremely limited regenerative capacity has been analyzed. The cell therapy approaches were limited at the turn of the new millennia to the transfer of non-cardiac cell types like smooth muscle cells, embryonic stem cells, mesenchymal bone marrow stromal cells, and hematopoietic stem cells [2], and skeletal myoblasts [3] into an injured heart. The idea of these engraftments was to stimulate repair and regeneration of the injured myocardium [4-6]. The field of cardiac regenerative medicine was brought into greater focus with the recognition of cardiac stem cells in 2001 [7] and the ability to antigenically select cardiac progenitor cells experimentally [8]. The capacity to generate cardiomyocytes in the adult human heart is 1% at the age of 25, which decreases to 0.45% at the age of 75 [9]. This rationale, together with published research reports and experimental studies [10], indicated that the heart is in fact capable of regeneration, but therapeutic strategies are needed to stimulate this process in cardiac pathologies. The use of fetal cardiac cells poses ethical, logistical, and technical issues, and there still is no means for effectively mobilizing a hypothetical pool of resident stem cells. Long term functional improvement requires stem cells with true cardiomyogenic properties and angiogenic potential. Presently, it is not clear whether such a ‘perfect’ stem cell exists. However, a number of stem

cell types show promise for autologous cardiomyoplasty, including multipotent muscle derived stem cells.

## **1.1 STEM CELLS ARE A PROMISING SOURCE FOR CARDIOMYOPLASTY**

Unspecialized cells that are capable of continuous self-renewal, while maintaining the ability to differentiate into multiple different cell types, are defined as stem cells. There are broadly two major categories of stem cells: adult stem cells and pluripotent stem cells, which include embryonic (ES) and induced pluripotent (iPS) stem cells. Embryonic stem cells are undifferentiated cells, which are present during embryonic development and possess pluripotent differentiation capacity. Adult stem cells can be isolated from postnatal tissues and are multipotent. Reinecke et al in 2002 [3] and Murry et al in 2004 [11] concluded that none of the adult stem cell sources convincingly demonstrated a potential for significant long-term engraftment and differentiation into functional cardiomyocytes. The technique developed by Yamanaka in 2006 demonstrated that the introduction of quartet of transcription factors, Oct4, Sox2, Klf4 and c-Myc, into terminally differentiated cells (e.g. skin fibroblast) changed these cells into an embryonic stem cell-like state known as iPS cells. Human iPS cells are similar in morphology, proliferation, surface antigens, gene expression, telomerase activity, epigenetic status of pluripotent cell specific genes [12], and cardiac potential to human embryonic cells [13]. However, maturation of iPS cell derived cardiomyocytes remains non-uniform [14, 15]. Human implantation of embryonic and iPS cell derived cardiomyocytes is limited by significant safety and ethical issues. Studies have revealed significant genetic and epigenetic abnormalities in iPS cells, higher than embryonic stem cells or fibroblasts. The mutation rates are estimated to

be 10 times higher compared to fibroblasts [16]. Chromosome p12 has been found to be overrepresented in iPS cell cultures, a characteristic associated with testicular germ cell tumors [16]. Other mutations are also associated with cell cycle regulation and oncogenesis, which raises the risk of teratogenesis in vivo. High-throughput functional genomics approaches are needed to better understand the nature and consequences of these mutations. iPS technology avoids the ethical dilemma of embryo destruction related to ES cells, but other ethical issues including informed consent and genetic anonymity of donors must still be carefully considered. While iPS technology holds great promise for the regeneration of the heart and other organs, clinical use of iPS technology in ischemic heart disease remains untested. From this point of view, adult stem cells remain a more practical option at the present time.

Significant efforts in the field of cardiac cell therapy have focused on bone-marrow derived cells, especially bone marrow mononuclear cells such as hematopoietic and mesenchymal stem cells. Mesenchymal stem cells have numerous desirable characteristics, including positive paracrine effects, angiogenic potential, and an immunoprivileged profile. Studies have shown that mesenchymal stem cells but not hematopoietic stem cells can differentiate into cardiomyocytes in-vivo [17, 18] with slightly to significantly positive results in clinical trials [19, 20]. However, bone marrow mesenchymal stem cells do not differentiate into cardiomyocytes in significant numbers in vitro, suggesting that their therapeutic benefit is predominantly paracrine rather than through transdifferentiation [21]. Challenges include expanding these stem cells in clinically relevant numbers and developing safe methods for cardiomyocyte differentiation. (5-azacytidine used for in vitro differentiation of mesenchymal stem cells into cardiomyocytes is toxic [17].) More recent work has focused on cardiac stem cells, which are found in niches within the heart and can be isolated by myocardial biopsies.



Studies using immunosuppressed rats have shown that human cardiac stem cells can give rise to myocardium with improved function [22]. In light of these results, clinical trials have been initiated [23, 24]. While initial results of the SCIPIO trial are promising, the CADUCEUS trial showed no difference in ventricular function compared to control despite a reduction of infarct size. While cardiac stem cells are unique and advantageous in their ability to preferentially differentiate into cardiac lineages, including cardiomyocytes, isolation of cardiac stem cells is also invasive and carries higher risk compared to isolation from other tissues. Further investigation is needed to safely and effectively isolate, expand, differentiate, and deliver these stem cells.

Cellular cardiomyoplasty of autologous skeletal muscle cells into the myocardium to reinforce its structure and function can be used as an alternative to heart transplantation [25]. Transdifferentiation directly converts a specialized cell type to another specialized cell type, bypassing a pluripotent state. In the 1980s, it was shown that fibroblasts can be converted into skeletal muscle cells with the use of the MyoD transcription factor [26]. In recent years, direct reprogramming of cardiac fibroblasts into functional cardiomyocytes by transcription factors GATA4, Tbx5, and Mef2c both in vitro and in vivo has been reported [15, 27]. However, the low reprogramming efficiency of this approach and the use of viral vectors are undesirable characteristics. It is interesting to speculate that direct reprogramming to cardiomyocytes might be more effective in skeletal muscle stem cells, which are pre-endowed with myogenic potential. But once they are in one committed lineage, it is a challenge to make them convert to another unrelated cell type, and transdifferentiation efficiency is often low. Szabo et al. also proved that blood cells can be generated from fibroblasts, making it likely that transcription factors can induce 'large jumps' between distantly related cell types [28]. This opened up the prospect that

any desired specialized cell could be generated from essentially any other cell type. Since skeletal and cardiac muscle both arise from myogenic, mesodermal lineages and share some characteristics, transdifferentiation from skeletal muscle progenitors to cardiac muscle would require less comprehensive alteration compared to distantly related cell types, at least in theory. Cells are considered ideal if they are derived from relatively easy to obtain tissue, readily grown in large numbers, available “off the shelf” for acute indications, nonimmunogenic, nonarrhythmogenic, and able to regenerate healthy myocardium [29]. Although the cell isolation procedure is relatively invasive, skeletal muscle comprises the largest percentage of total body mass, thus providing a major pool of stem cells [30]. These desirable characteristics and developmental similarity to cardiac muscle, described below, make skeletal muscle derived cells a strong candidate to repopulate damaged myocardium.

## **1.2 SKELETAL MUSCLE AND CARDIAC MUSCLE: DIFFERENCE AND SIMILARITY DURING DEVELOPMENT**

It is a widely accepted notion that terminally differentiated mature cardiac muscle does not express proteins that are specific to skeletal muscle. However, developing skeletal muscle has been proven to be similar to cardiac muscle by various studies, suggesting that they have a similar cell lineage. Different isoforms of myosin heavy chain (MHC) are present in different muscle fiber types to meet their functional needs. There are four major MHC isoforms in rat skeletal muscle. I/ $\beta$  is a slow type and IIa, IIx/IIc, and IIb are fast types. These are equivalent to skeletal muscle specific fast MHC (sk-fMHC) [31-33]. In the adult myocardium,  $\alpha$ -MHC and  $\beta$ -MHC are present, with the latter being identical to I/ $\beta$ -MHC found in skeletal muscle. Non-cardiac MHCs play a major role during the early myofibrillogenesis of nascent cardiomyocytes

in assembling sarcomere structure [34]. Recently, it was found sk-fMHC was expressed in developing rat myocardium, and its expression was attenuated in post-natal life [35].

The thin filament system in skeletal and cardiac muscle is regulated by a family of proteins known as troponins, which form part of the troponin complex. Troponin I is encoded by three different genes. Cardiac troponin I was previously shown to be exclusively expressed in the myocardium in adults [36], but recently, it was revealed that it is also expressed in developing skeletal muscle [35]. It is distinct from the fast and slow forms in skeletal muscle [36]. Cardiac troponin T is a component of the troponin complex. It is encoded by the gene *TNNT2* and allows actomyosin interaction and contraction to occur in response to  $Ca^{2+}$ . *TNNT2* is a common mutation in familial hypertrophic cardiomyopathy, but surprisingly, it has been found that distinct *TNNT2* mutations also lead to dilated cardiomyopathy [37]. It is also expressed in skeletal muscle during injury. Apart from this, skeletal muscle specific troponins are transiently present in the immature heart [38]. In the early phases of myogenesis in skeletal muscle, cardiac-like excitation-contraction coupling mechanisms dominate, while skeletal muscle-like excitation-contraction coupling dominates in more mature muscle [39, 40]. Thus, between cardiac and skeletal muscle, there is a strong overlap in the genes encoding key proteins responsible for contractility, which is a hallmark of striated muscle.

Cardiac and skeletal muscles also share common metabolic regulatory proteins. Fatty acid binding protein 3 (FABP3) is a member of a family of binding proteins and is mainly expressed in cardiac and skeletal muscle cells, and it has been linked to fatty acid metabolism, trafficking, and signaling [41]. UDP-N-acetylglucosamine 2 epimerase/N-acetylmannosamine kinase (GNE) is involved in the early development of both cardiac and smooth muscle. GNE deficient cardiac cells degraded very soon, and their beating capacity decayed rapidly. Skeletal

muscle committed cells were identified in the GNE deficient embryonic stem cell cultures by the expression of Pax7, MyoD, and MHC markers. GNE is strongly involved in cardiac tissue and skeletal muscle early survival and organization as shown by these results [42].

A common finding in certain types of muscular dystrophy, including Emery-Dreifuss, Duchenne, and Becker type dystrophies, is chronic cardiac diseases [43]. Some types are caused by mutations in the  $\beta$ -sarcoglycan associated proteins [44], which are expressed both in cardiac and skeletal muscle. Limb-girdle muscular dystrophy type 2E is caused by mutations in *Sgcb* gene, and *Sgcb* deficient mice develop severe cardiomyopathy and severe muscular dystrophy, suggesting that cardiac and skeletal muscle pathologies arise from a common origin [45]. All these studies indicate how closely cardiac and skeletal muscle are interlinked, especially in their immature phase. Recently, our group found that skeletal and cardiac muscle share similar transcription factors and structural proteins during development [35]. However, the molecular mechanisms that regulate the fate determination between these two lineages of striated muscle remain poorly understood.

### **1.3 SKELETAL MYOBLASTS: AN EARLY CANDIDATE FOR CELLULAR CARDIOMYOPLASTY**

Given their phenotypic similarity to cardiac muscle, ease of isolation/expansion, and relative resilience to hypoxia, skeletal myoblasts were considered an appealing cell source for cellular cardiomyoplasty [46-53]. Skeletal myoblasts have shown promise for ischemic heart regeneration, making them potential candidates for early cardiomyoplasty studies [54]. Skeletal myoblasts were the first cell type to enter the clinical arena of heart regeneration. Lavine et al. in 1937 performed the first of its kind surgery in which his team treated myocardial ischemia by

using a skeletal muscle graft to the heart. The patient was typically acromegalic. Before the procedure, he could not walk 5 yards without any rest, but after the surgery, he was able to walk 140 yards without any shortness of breath. [55]. Early animal studies conducted in porcine animal models showed that myoblast-derived muscle grafts remained functionally isolated from the surrounding myocardium. The outcome did not reveal an increased frequency in ventricular tachycardia or fibrillation [56]. Menasche et al in 2001 transplanted autologous skeletal myoblasts into the post infarction scar during coronary artery bypass grafting of remote myocardial areas. However, in the distinct case of skeletal myoblasts, the results of several preclinical and clinical studies have shown improved functional outcomes following transplantation of these myogenic progenitors into post infarction scar.

Arrhythmogenic complications have raised concern over the safety of adoptive transfer of skeletal muscle cell therapy [3, 11, 45-47, 57]. There is a risk of ventricular arrhythmias [48, 58-61]. The fact remains that myoblast transplantation has its limitations, as implanted cells have limited survival and do not spread uniformly at the site of injury [47]. Skeletal myoblasts do not differentiate into cardiomyocytes but rather into multinucleated myotubes after injection into the heart. These myotubes form islands of conduction block in the heart and lack gap junctions. This results in electrical inhomogeneities that slow conduction velocity and predispose patients to reentrant ventricular arrhythmias [62]. While skeletal myoblasts (and other skeletal muscle derived cells) show promise of improved myocardial contractile function, risk of arrhythmia is an issue that needs to be addressed to advance this therapy.

#### **1.4 CARDIOMYOCYTE DIFFERENTIATION POTENTIAL FROM SKELETAL MUSCLE DERIVED CELLS**

Since skeletal myoblasts do not differentiate into functioning cardiomyocytes *in vivo*, efforts have been made to isolate cells from skeletal muscle which can differentiate into cardiomyocytes *in vitro* or *in vivo*. Skeletal muscle derived stem cell (MDSC) is a somatic stem cell that is different from skeletal myoblasts (satellite cell) in its multipotent properties [63-65]. MDSCs are obtained from skeletal muscle via a pre-plate method or fluorescent activated cell sorting of surface markers and are easily expanded under *in vitro* culture environment as an autologous cell source [53, 64, 65]. MDSCs differentiate into skeletal muscle, bone, tendon, nerve, endothelial, hematopoietic, and smooth muscle cells [53, 63-66]. They have long-term proliferation capacity and enhanced resistance to hypoxia. Several studies have shown that MDSCs can differentiate into functioning cardiomyocytes using various strategies. Clause et al. demonstrated that rat MDSCs could differentiate into cardiomyocyte-like cells expressing cardiac specific genes and proteins by combining cell aggregation with 3D culture in a collagen bioreactor [67]. Winitzky et al. isolated non-adherent cells; SPOC cells (skeletal-based precursors of cardiomyocytes), from adult murine skeletal muscle that become beating floating cells. They are CD34<sup>-</sup>/CD45<sup>-</sup>/c-kit<sup>-</sup>. [68]. The muscle-derived Side Population (SP) cells express the surface marker Sca-1 (65%) and the beating cells develop out of the Sca-1<sup>-</sup> pool, which comprises 20%–40% of the total isolated cells. Cells expressing the cardiac-specific marker Nkx2.5 and spontaneously contracting in culture were detected in RT-PCR analysis. The presence of specific mRNA coding markers for skeletal (myogenin, Myf5) and cardiac muscle (GATA4) and smooth muscle actin was seen in differentiated cells. Interestingly, all the above-mentioned markers were also detected in floating proliferating progenitor cells. This supports the idea that stem cells can express markers of multiple lineages in their differentiating state [69].

Skeletal Muscle Interstitium-Derived Multipotent Stem Cells (Sk-34 and Sk-DN cells) differentiated into cardiomyocytes in a cardiac environment [49, 70]. After 4 weeks, the transplanted cells became cardiomyocytes with desmosomes and intercalated discs associated with gap-junctions [49]. Myotube formation was not observed. Sk-34 and Sk-DN cell differentiation is based on a cardiac environment. Taken together, these studies emphasize the importance of isolation techniques and culture conditions in obtaining the desired target cell. For example, Parker et al. showed that activation of Notch signaling during *ex vivo* expansion of muscle derived cells can significantly improve their engraftment [71].

There are a number of problems faced during isolation of these cells. Cell surface markers define Muscle-derived stem and progenitor cell populations. Some of these markers are restricted to these populations, whereas other markers are shared with other cell populations. The marker profile-based isolation methods exclusively depend on the expression of cell surface markers that are variable and may change under *in vitro* cell culture conditions. Thus, these methods have their limitations. Presently, the most prevalent method for isolating MDSCs is the modified pre-plate technique. It is a marker profile-independent technique which is based on variable adherence of freshly dissociated muscle cells to collagen-coated flasks. However, the isolated populations are heterogeneous [72]. Multiple non-satellite stem cell fractions reside in skeletal muscle, though it remains unclear how these different populations are related. Okada et al. showed that slowly adhering cells showed greater regenerative potential and oxidative/inflammatory stress tolerance compared to rapidly adhering cells [73]. Myogenic-endothelial cells, cells which express both myogenic and endothelial markers (CD56, CD34, and CD144), can be isolated from skeletal muscle and have been shown to have superior regenerative properties and stress tolerance compared to myoblasts [74, 75]. The blood vessels of skeletal

muscle also contain stem cells, perivascular stem cells, which demonstrate myogenic and multipotent potential [76]. They are isolated on the basis of CD146 expression and lack of CD34, CD45, CD144, and CD56 expression.

*In vitro* generated MDSC derived cardiomyocytes are more similar functionally and biochemically to fetal cardiomyocytes [77]. However, the rate of cardiomyocyte differentiation alone is likely not sufficient to explain the functional improvements gained from MDSC transplantation. Another possible mechanism by which MDSCs exert therapeutic benefit is by acting as a reservoir to secrete paracrine factors which promote angiogenesis and cell survival within the injured microenvironment [53]. Transplanted undifferentiated human skeletal myoblasts (CD56+) in an immunodeficient nude rat model demonstrated expression of human-specific MMP-2, TNNI3, CNN3, PGF, TNNT2, PAX7, TGF- $\beta$ , and IGF-1 1 month after transplant. Gene expression showed upregulation of pro-angiogenic factors (PGF), antiapoptotics (BAG-1, BCL-2), heart development (TNNT2, TNNC1) and extracellular matrix remodeling (MMP-2, MMP-7) in skeletal myoblasts. The data suggested that myoblast-secreted factors may contribute to the beneficial effects of myogenic cell transplantation in infarcted myocardium [78].

The “paracrine effect” exerted by MDSCs and other stem cells has a positive effect within the injured myocardium. Bioactive molecules such as bFGF, VEGF, PDGF, SDF-1, HGF, and IGF-1 confer cardioprotective effects. In addition, secretion of molecules such as VEGF, Angiopoietin, and bFGF increase blood vessel formation in the ischemic region, improving perfusion and function. HGF and IGF-1 also activate resident stem cells and promote endogenous repair mechanisms. Additional factors such as TGF- $\beta$ , MMPs, and TIMPs reduce fibroblast proliferation, fibrosis, and left ventricular dilation [79, 80]. Okada et al. showed that a



subpopulation of slowly adhering MDSCs showed enhanced paracrine factor secretion, increased stress tolerance, engraftment, and angiogenesis compared to rapidly adhering MDSCs. They also found increased cardiomyocyte proliferation within the infarct region, suggesting the enhancement of endogenous repair mechanisms [81]. The selective enrichment of certain types of MDSCs may improve their regenerative effects through enhancement of paracrine effects. Studies of MDSC transplantation have shown that only a small fraction of donor cells demonstrate long-term engraftment, and the majority of cells within the regenerating tissue come from the host via the chemo-attraction of post-natal stem cells from the vasculature [82]. Thus, the paracrine or “by-stander” effect of MDSCs may be equally, if not more important than myogenic differentiation in contributing to cardiac repair.

## **1.5 TISSUE ENGINEERING APPROACHES TO CARDIAC REPAIR**

Transvascular methods of cell delivery are commonly used to treat recently infarcted myocardium. Intracoronary artery infusion can deliver a high concentration of cells homogeneously to the site of injury. Direct needle injection into the ventricular wall is preferred for patients late in the disease process when occlusion of the coronary artery prevents transvascular delivery [83]. However, it often results in poor engraftment, survival, and further damage by needle injury. Tissue engineering approaches may provide a more effective means of cell transplantation. Two main engineered tissue transplantation approaches for cardiac tissue are three dimensional scaffolds and cell sheets. One of the most challenging issues in tissue engineering is the reconstruction of myocardium in three-dimensions (3-D). 3-D scaffolds are now available [84, 85]. Cell sheets are used for treating advanced heart failure. Myocardial cell

sheets decrease fibrosis and increase vascularization in the injured heart [81, 86-88]. In 2006, Zimmerman et al. produced convincing evidence that a heart tissue can be produced with enough contractile force to support a failing heart. Neonatal rat heart cells were seeded with liquid collagen type I and Matrigel in molds. The rings were grafted onto the infarcted rat heart and after a month showed positive results. Further dilation of the heart was prevented, and systolic wall thickness increased [89]. In 2010, Fujimoto et al. also proved that engineered cardiac tissue from immature cardiomyocytes can functionally integrate into damaged myocardium. In this study, engineered fetal cardiac tissue proved to be more successful than neonatal tissue. Engineered fetal cardiac tissue implantation increased left ventricle contraction and showed higher cardiomyocyte proliferation than engineered neonatal cardiac tissue [90].

The major issues concerning the therapeutic application of stem cells remain unresolved due to 3 primary factors: poor survival, marginal proliferation, and limited functional engraftment/commitment within the host tissue. Furthermore, the cells should be prepared to combat against apoptotic, necrotic, and hypoxic conditions prevalent within the damaged tissue. Finally, if cells manage to proliferate, they can be functionally incapable of appropriate lineage commitment and can end in an oncogenic transformation. [91].

The *in vivo* studies with MDSCs show promising results for meeting some of these challenges. Repair of the infarcted heart with MDSCs is more effective than conventional myoblasts [53]. MDSCs injected into the myocardium of infarcted heart showed outstanding survival and engraftment, promoted angiogenesis, and increased left ventricular function in a more effective manner in comparison to the transplantation of skeletal myoblasts [92]. In a post-infarcted myocardium, transplanted MDSCs sustained its contractile function, preventing left ventricular chamber remodeling. Some transplanted donor MDSCs differentiated into both sk-

fMHC and/or cTn-I positive muscle cells [53, 74]. Clause et al. demonstrated that MDSCs cultured within a three-dimensional engineered tissue construct differentiated into an immature functioning cardiomyocyte phenotype [67]. Transplantation of tissue-engineered cell sheets has negated some of the limitations by eliminating use of trypsin, direct needle injury to the heart, and by covering larger infarcted area [93, 94]. Reduction in left ventricle (LV) dilation and sustained LV contraction were displayed with the implanted MDSC sheet. It yielded better functional recovery of chronic infarcted myocardium compared with direct MDSC injection without any significant arrhythmic events, indicating that this cell sheet delivery system could markedly improve the myocardial regenerative potential of the MDSCs [81]. These studies suggest that tissue engineering approaches may provide a means to further improve upon the functional benefits derived from MDSC transplantation.

Tissue engineering approaches help to address one of the major limitations of current delivery modalities – the immediate and massive attrition of “naked” cells that occurs after cell delivery, which leads to low retention of implanted cells even 24 hours post-procedure. However, current tissue engineering approaches focus on building complex 3D structures, which precludes catheter-based delivery and necessitates surgery. Using the cell patch approach, transplanted cells are initially localized to the epicardial region. A recent development, which improves cell retention, is the delivery of hydrogel-cell liquid suspensions via catheter [95]. The hydrogel polymerizes en route and traps cells in the target tissue. If successful, this method can combine the demonstrated safety of catheter-based delivery with the ability of a tissue-engineered scaffold to improve engraftment.

## 1.6 FUTURE CHALLENGES AND OPPORTUNITIES

Although skeletal muscle stem/progenitor cells have benefits, a large number of injected cells undergo early death. The first prospective randomized placebo-controlled phase II skeletal myoblast trial (MAGIC trial) exhibited lack of efficacy and was discontinued prematurely [47]. The trial's disappointing results were due to several reasons: a low rate of initial cell retention, a high rate of subsequent cell death, and the inability of engrafted myoblasts to establish functional electromechanical connections with the host cardiomyocytes [3]. In addition, a trend towards excess arrhythmias was observed in myoblast-treated patients despite the use of the prophylactic antiarrhythmic drug, Amiodarone. This raised safety concerns that had already been raised by earlier phase 1 trials. In contrast, SEISMIC trial [62] argued that injection of autologous skeletal myoblasts in heart failure patients is safe and may provide symptomatic relief, as a trend towards increased exercise tolerance was observed in the cell-treated group. Nevertheless, no significant effect on global left ventricular ejection fraction (LVEF) was detected. All this taken together indicates that more work needs to be done to bridge the gap between preclinical animal models and clinical trials.

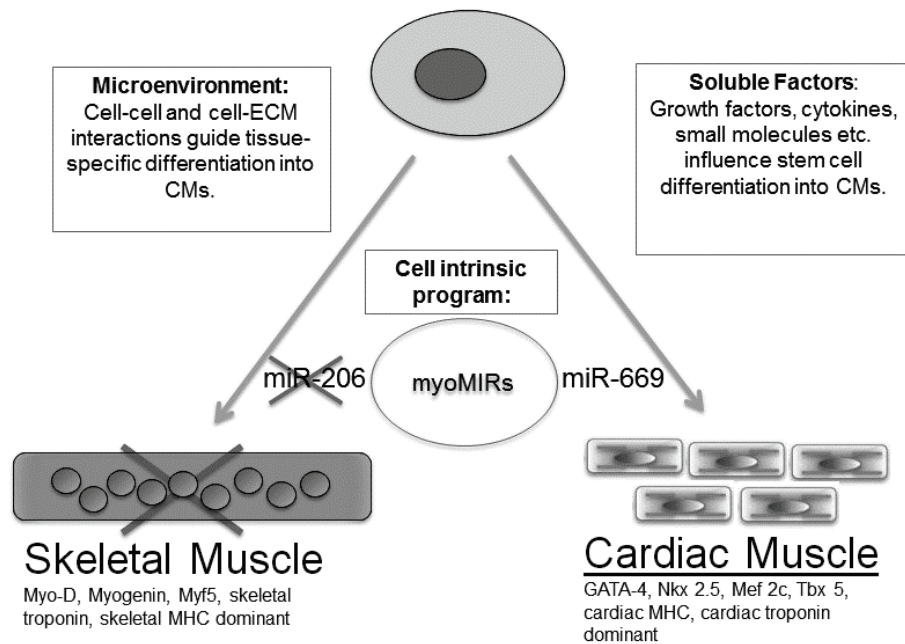
In light of the mixed results from clinical trials, further work has gone into eliminating the heterogeneity of transplanted cells and improving electromechanical coupling between transplanted and host myocytes. Transplantation of Myogenic-endothelial cells which express CD56, CD146, and Ulex, purified from human skeletal muscle into the ischemic heart drastically improved left ventricular function, reduced scar tissue and promoted angiogenesis [53]. Connexin-43 is the predominant gap junction of the ventricular myocardium. Skeletal myoblasts lack connexin-43 after fusion into elongated contractile myotubes. In cellular monolayers, conduction velocity was slowed and reentry-induced arrhythmias were promoted when skeletal

myoblasts were co-cultured with neonatal cardiomyocytes *in vitro* and studied with high-resolution optical mapping. The proarrhythmic effect was reduced when engineered cells over-expressed connexin-43 [96]. The findings were later tested in an animal model [11]. Methods to improve electromechanical compatibility between engrafted muscle and host myocardium are currently under investigation.

Issues related to electromechanical compatibility between cardiac and skeletal muscle tissue could be ameliorated by generating cells from MDSCs which have a cardiomyocyte-like phenotype. The heart also contains resident stem cells. Oh et al. identified in 2003 an independent population of Sca-1<sup>+</sup> cardiac stem cells as a subgroup of cells (constituting ≈14%) isolated in the non-cardiomyocyte cell fraction of the adult mouse heart in a whole heart digestion. Sca-1<sup>+</sup> cells co-expressed CD31 and CD38, and lacked c-Kit, CD34, and CD45 when freshly isolated. 93% of the side population were Sca-1<sup>+</sup>. Freshly isolated Sca-1<sup>+</sup> cells did express the early cardiac-specific transcription factors GATA4, Mef2C, and Tef-1 but not Nkx2.5 or genes encoding cardiac sarcomeric proteins. Sca-1<sup>+</sup> cells engrafted at a much higher rate than Sca-1<sup>-</sup> cells in a mouse model of ischemia-reperfusion after 2 weeks and could be found forming new cardiomyocytes [97]. Cardiac stem cells in bulk culture up-regulated GATA-4 expression resulting in enhanced cardiomyocyte differentiation, suggesting that the GATA-4 high c-kit<sup>+</sup> cardiac stem cells have potent cardiac regenerative potential. The study also demonstrated spontaneous differentiation into skeletal myocytes [98]. Hasan et al. established cardiac pluripotent stem cell-like cells from the left atrium of adult rat hearts that could differentiate into beating cardiomyocytes in the methylcellulose-based medium containing interleukin-3 and stem cell factor, which contributed to the differentiation into cardiac troponin I-positive cells. Distinctly small populations of pluripotent stem cell-like cells from the left atrium

co-expressed GATA4 and myogenin, which are markers specific to cardiomyocytes and skeletal myocytes, respectively. These could differentiate into both cardiac and skeletal myocytes [99]. These studies suggest the possibility that cardiac and skeletal muscle can arise from a common myogenic progenitor, and stem cells purified from skeletal muscle may have similar differentiation potential, as demonstrated by studies of cardiomyocyte differentiation from MDSCs. However, the pathways which determine if a cell differentiates into a cardiomyocyte or skeletal muscle cell are only beginning to be unraveled.

A hypothesis is presented in **Figure 1-1** showing how skeletal muscle stem/progenitor cells can be induced to become cardiac muscle with post-transcriptional modification. Microribonucleic acids (micro-RNAs, miRs) are post-transcriptional regulators of cardiac and skeletal myogenesis, including miR206, which specifically promotes skeletal myogenesis [100-102] as part of an intrinsic cell-regulatory program. Crippa et al. isolated cardiac progenitors from neonatal sarcoglycan-null mouse hearts affected by dilated cardiomyopathy, and they spontaneously differentiated into skeletal muscle fibers both in vitro and when transplanted into regenerating muscles or infarcted hearts. The absence of expression of miR669q and down-



**Figure 1-1.** MicroRNAs post-transcriptionally regulate myogenesis. miR-206 specifically promotes skeletal muscle differentiation, while miR-669 promotes cardiac differentiation by inhibiting MyoD, a skeletal muscle transcription factor. By modulating expression of these and other muscle-specific microRNAs, stem cells can be induced to differentiate into cardiac or skeletal muscle. The local microenvironment and soluble factors also play important roles in stem cell differentiation.

regulation of miR669a were associated with differentiation potential. Skeletal myogenesis was prevented by miR669a and miR669q acting upstream of myogenic regulatory factors by directly targeting the MyoD untranslated region. [103]. Successful conversion of cardiac cells into skeletal muscle fibers opened a huge area of discussion if the reverse is possible. MDSCs express similar genes to cardiac and skeletal muscle, and cardiac and skeletal muscles are interchangeable because of miR669. The physical environment may also present important environmental cues [104]. 3-dimensional cultures and cardiac-specific extracellular matrix (ECM) have been shown to promote cardiomyocyte induction [35, 67, 105].



## **1.7 SUMMARY**

In any endeavor as complex and perplexing as stem cell research, controversies and disagreements are to be expected. While early clinical trials of skeletal myoblast transplantation yielded mixed results and did not meet initial expectations, more recent works have shown that improved isolation methods, novel tissue engineering approaches, and differentiation strategies may improve the efficacy of myogenic stem cell transplantation. Skeletal muscle derived stem cells remain the only class of stem cell which can preferentially differentiate into a muscle phenotype and can be isolated easily in abundant numbers. Cardiovascular regenerative medicine is still in its early stages but recent studies show renewed promise. The field of regenerative medicine has been quite progressive, as a search on PubMed for the key words “cardiac, stem cell, heart,” yields over 9000 references as of today with almost 2000 review articles to summarize all current understanding. This field was nonexistent a little over a decade ago.

## **1.8 AIMS OF THE STUDY**

Given the multi-lineage potential of MDSCs, the overall goal of this thesis is to develop a functioning cardiac graft using human MDSCs with the long-term goal of treating congenital and acquired heart diseases. Specifically, the objectives of the study are to 1) characterize the CM differentiation potential of human MDSCs in a 3-dimensional engineered muscle tissue compared to pluripotent stem cell derived CMs, 2) modify the biophysical and soluble environment of 3-dimensional engineered muscle tissue to improve CM differentiation, and 3) determine the effect of MDSC graft implantation on recipient cardiac function.

## **2.0 DEVELOPING CARDIAC AND SKELETAL MUSCLE SHARE FAST-SKELETAL MYOSIN HEAVY CHAIN AND CARDIAC TROPONIN-I EXPRESSION**

### **2.1 INTRODUCTION**

Muscles are composed of different fiber types to fulfill various functional needs. Fiber types are categorized generally according to their specific myosin heavy chain (MHC) isoforms. In rats, there are four major isoforms of MHC, one slow type (type I/ $\beta$ ) and three fast types: IIa, IIx/IIc, and IIb, which is equivalent to skeletal muscle specific fast myosin heavy chain (sk-fMHC). An individual muscle fiber can contain just one myosin isoform or mixtures of two or more different isoforms [31-33]. An additional MHC isoform,  $\alpha$ , is present in the myocardium. Different MHC isoforms are expressed in both tissue and stage-specific manners, and much work has been done to show the relative change in the expression ratio of  $\alpha$ -MHC:  $\beta$ -MHC in the myocardium during development and with intervention. During the early myofibrillogenesis of nascent cardiomyocytes, non-cardiac MHC plays an important role in assembling sarcomere structure [34]. However, little investigation into the expression of skeletal muscle specific MHCs in the developing myocardium have been done.

Troponins are proteins that regulate the thin filament system in skeletal and cardiac muscle and form part of the contractile complex. Troponin-I is encoded by 3 different genes and is expressed differentially in various types of tissue. However, cardiac troponin I (cTn-I) is uniquely expressed in the heart and is distinct from the fast and slow forms in skeletal muscle [36].

It has been widely accepted that terminally differentiated mature cardiac muscle does not express proteins that are specific to skeletal muscle. However, studies have shown that several skeletal muscle specific proteins, such as skeletal muscle specific troponins, are transiently present in the developing heart [38]. Similarly, “cardiac” and “skeletal” excitation-contraction coupling mechanisms co-exist in developing skeletal muscle with the “cardiac” type dominant in the early phases of myogenesis and the “skeletal” dominating in more mature muscle [39, 40]. These studies suggest the coexistence of many cardiac and skeletal muscle specific proteins and excitation-contraction coupling mechanisms within both developing tissue and cultured cells, particularly those that are considered to be immature. While the idea that skeletal and cardiac muscle share partially overlapping developmental profiles is not new, reports of expression of specific structural protein isoforms have varied across studies. For example, Fougousse et al. concluded that the cardiac isoform of myosin binding protein C, cardiac MyBP-C, is strictly specific to the heart during murine and human development [106]. However, it is reported to be expressed in developing chick skeletal muscle [107-109]. Factors such as experimental conditions, differences across species, and developmental time points examined can lead to these differences in results. Moreover, while these phenotypic changes have been studied during early cardiac morphogenesis period, few have focused on the transition that occurs between late fetal and early postnatal life.

In our previous studies of murine or human skeletal muscle derived stem cell (MDSC) transplantation into infarcted mouse myocardium, we found that MDSC transplanted post-infarcted myocardium sustains its contractile function, preventing left ventricular chamber remodeling, and some transplanted donor MDSCs differentiate into both sk-fMHC and/or cTn-I positive muscle cells [53, 74]. We have recently shown MDSCs can differentiate into cells with

an immature functioning cardiomyocyte phenotype within a three-dimensional engineered tissue construct [67]. However, MDSC derived cardiomyocytes generated *in vitro* are biochemically and functionally more similar to neonatal rather than mature cardiomyocytes. While stem cell derived cardiomyocytes show promise, the sequence of events that lead to terminal cardiomyocyte differentiation and functional maturation is as of yet poorly understood [90]. The use of stem-cell derived cardiomyocytes for tissue engineering and regenerative medicine applications would clearly benefit from a better understanding of the biochemical and structural changes that lead to cardiac and skeletal muscle specification during natural development. However, it remains to be seen whether MDSC derived sk-fMHC positive cells are terminally differentiating skeletal muscle cells or potentially differentiating cardiomyocytes. In order to help address the question, we investigated the presence of sk-fMHC and cTn-I within the native developing myocardium and skeletal muscle in a rat animal model.

## **2.2 MATERIALS AND METHODS**

The presence of sk-fMHC and cTn-I from embryonic day (ED) 13 and 20, neonatal day (ND) 0 and 4, postnatal day (PND) 10, and 8 week-old adult female Lewis rat ventricular myocardium and skeletal muscle (hind limb in ED13, ED20, ND0 and ND4, and gastrocnemius and soleus muscles in PND10 and adult) was assessed using immunohistochemistry, western blot, and RT-PCR. Our research protocol followed the National Institutes of Health (NIH) guidelines for animal care and was approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

### **2.2.1 Construction of 3-dimensional Collagen Gel Bioreactor from Rat Skeletal Muscle Derived Stem Cells (MDSC)**

We isolated skeletal muscle derived stem cells (MDSCs) from 3 post-natal day 10 Lewis rat gastrocnemius muscle using a preplate technique. Isolated MDSCs were expanded on a rat-tail collagen type-1 (Invitrogen, Carlsbad, CA, USA) coated flask (T-75, Fisher Scientific, Pittsburgh, PA) and a 3-dimensional collagen gel bioreactor was constructed using a mixture of MDSCs and rat tail collagen type-I (Invitrogen) with Matrigel (BD bioscience, San Jose, CA, USA), and a Flexcell 4000TT system (Flexcell International, Inc. Hillsborough, NC, USA). The constructed collagen gel tissue was cultured for 9 days and the tissue was harvested for histological assessment and the gene expression.

### **2.2.2 Immunohistochemical staining**

Heart and skeletal muscle samples were fixed with 4% paraformaldehyde/PBS for 15 minutes (EDs 13 and 20) to 12 hours (ND0 to adult) and embedded in 13% polyacrylamide gel. The 100 (ND4 and adult) to 150 $\mu$ m (ED13, ED20, ND0) thick sections were made using a vibratory microtome (Vibratome-1000, Vibrotome.com) [110] and stained for mouse monoclonal sk-fMHC (Sigma MY32, St. Louis, MO, USA, 1:200 dilution) and mouse monoclonal cardiac specific Troponin-I [cTn-I, Abcam (19C7), Cambridge, MA, USA, 1:100 dilution] primary antibodies and Alexa Fluor 488 IgG<sub>2b</sub> or Alexa Fluor 594 IgG<sub>1</sub> goat anti-mouse secondary antibodies (Invitrogen, Carlsbad, CA, USA, 1:200 final concentration). All samples were scanned and visualized by Z-stack imaging protocol using a standard laser scanning confocal microscope (FV1000, Olympus, Tokyo, Japan) [110].

### 2.2.3 SDS-PAGE and Immunoblotting

Freshly frozen heart and skeletal muscle samples were used. Protein was extracted from pooled samples, and immunoblotting was carried out using routine protocols. Each lane contained 20 $\mu$ g of total protein. Mouse monoclonal  $\alpha$ -sarcomeric actinin (Sigma, EA53, 1:500 dilution), mouse monoclonal cardiac troponin-I (Abcam, 1:100 dilution), and mouse monoclonal sk-fMHC (Sigma MY32, 1:500 dilution) were visualized with IR-Dye 800 donkey anti-mouse secondary antibody (IgG<sub>1</sub> and IgG<sub>2b</sub>, Rockland Immunochemicals, Gilbertsville, PA, USA, 1:10,000 dilution). All proteins were visualized using an infrared western blot imaging system (Odyssey, LI-COR Biosciences Lincoln, NE, USA). Immunoblots were performed in either quintuplicate or triplicate (sample n=5 for ED 13, ED 20, and ND 4; n=3 for ND0, PND 10, and adult rats) [110].

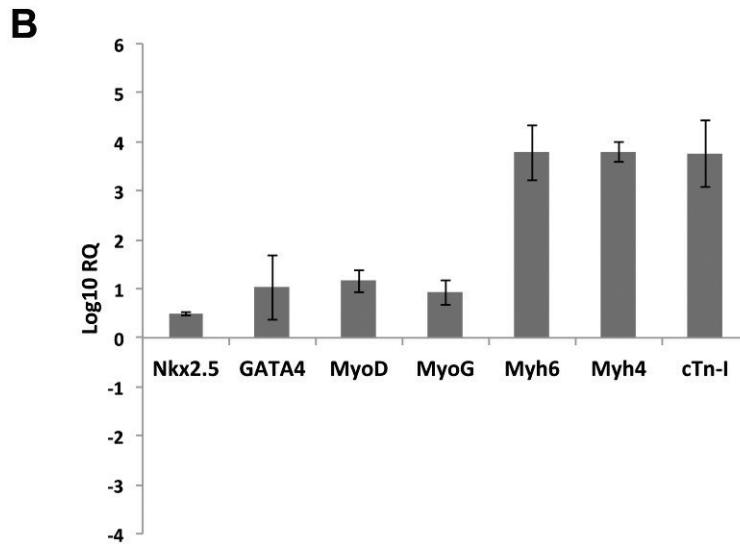
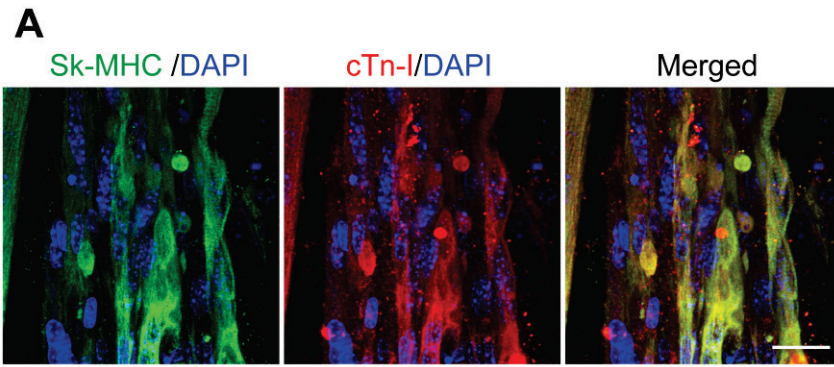
### 2.2.4 RT-PCR and real-time RT-PCR

Freshly frozen pooled ventricular myocardium and skeletal muscle samples were used. Total RNA was prepared using Trizol solution (Invitrogen) and treated with TURBO DNA-free kit (Ambion, Austin, TX, USA). Primers, whose target genes were, Nkx2.5, GATA4, MyoD, Myogenin, sk-fMHC (MYH4),  $\alpha$ -MHC (MYH7), cardiac troponin-I (Tnn3), were obtained from Qiagen Quanti-Tect Primer Assay (Qiagen, Valencia, CA). One-step reverse transcription (RT) was performed with a total amount of 1 $\mu$ g RNA in a total volume of 25 $\mu$ L that used MuLy (Roches, Pleasanton, CA, USA). The following program was used for RT: 42 $^{\circ}$ C 15 minutes, 99 $^{\circ}$ C 5minutes, 5 $^{\circ}$ C 5minutes, for 1 cycle. The produced cDNA (1 $\mu$ L) was used for RT-PCR (94 $^{\circ}$ C 2minutes, 95 $^{\circ}$ C 50seconds, 58 $^{\circ}$ C 30 seconds, 72 $^{\circ}$ C 1 minute, for 30 cycles followed by 72 $^{\circ}$ C 7 minutes extension) or real-time RT-PCR (50 $^{\circ}$ C for 2 minutes, 95 $^{\circ}$ C for 10 minutes, 95 $^{\circ}$ C for 15 seconds, 60 $^{\circ}$ C for 1 minute, 95 $^{\circ}$ C for 15 minutes, 60 $^{\circ}$ C for 15 minutes for 40 cycles). For

normalization of real-time PCR results,  $\beta$ -actin was used as an internal control. All PCR products were confirmed by University of Pittsburgh DNA Sequence Core Facilities, performed by Eppendorf Mastercycles. All real-time PCR assays were completed in triplicate (n=3 in each developmental stage). Real-time PCR samples were processed using Applied Biosystems HT7900 system.

### 2.3 RESULTS

Differentiating MDSCs in a 3-dimensional collagen/extracellular matrix gel construct displayed spontaneous beating activity from culture day 5 and formed a muscle tissue by culture day 7 similarly to our previous study. Confocal microscopy showed that the differentiating cardiomyocyte-like cells co-expressed skeletal muscle specific fast myosin heavy chain (sk-fMHC) and cardiac specific troponin-I (cTn-I) as a typical striated muscle pattern (**Figure 2-1A**). Real time RT-PCR showed that both cardiac and skeletal muscle transcription factors, cardiac sk-fMHC, cardiac  $\alpha$ -myosin heavy chain, and cTn-I genes are up-regulated compared to undifferentiated MDSC (**Figure 2-1B**).

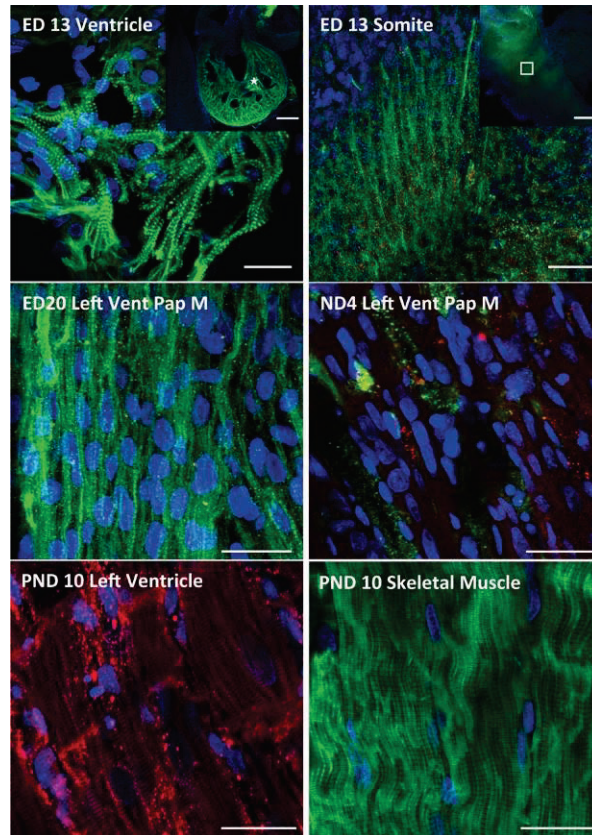


**Figure 2-1.** sk-fMHC (green color) and cardiac troponin-I (red color) expression (Panel A) and gene expression (Panel B) of skeletal muscle derived stem cell 3D collagen gel bioreactor (MDSC-3DGB).

MDSC-3DGB showed co-localized expression of sk-fMHC and cardiac troponin I. Transcription factor and structural gene expression was increased compared to 2D undifferentiated MDSCs. Scale in panel A indicates 20  $\mu$ m.

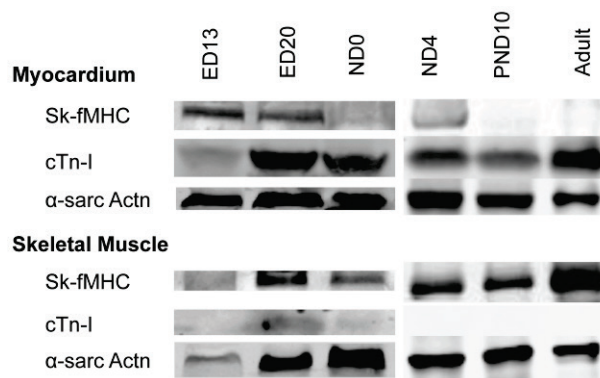


In the native rat heart, sk-fMHC was expressed as a typical striated muscle pattern throughout the entire ventricular myocardium at ED13, and cTn-I was very weakly expressed (close to background) (**Figure 2-2**). Within ED13 somite, sk-fMHC was expressed as a striated thin fiber-like pattern, while cTn-I was diffusely expressed. At ED20 and ND0, striated sk-fMHC expression was restricted to the ventricular papillary muscle and losing striated muscle pattern in the myocardial wall (**Figure 2-2**). cTn-I was very weakly expressed without striation in the majority of the myocardial wall and some striated cTn-I expression pattern was identified in the myocardium close to LV apex (Data not shown). At ND4, sk-fMHC was still expressed in the LV myocardium (Figure 2-2). However, striated pattern was completely lost. cTn-I expression increased. The PND10 LV myocardium did not express sk-fMHC, whereas cTn-I was expressed with a typical striated pattern. Conversely, PND10 skeletal muscle expressed sk-fMHC as a typical striated muscle pattern and did not express cTn-I (**Figure 2-2**).



**Figure 2-2.** sk-fMHC (green color) and cardiac troponin-I (red color) expression within developing ventricular myocardium and skeletal muscle. The skeletal muscle fast myosin heavy chain (sk-fMHC) and cardiac troponin-I (cTn-I) expression within the embryonic day (ED13) heart (ED13 Ventricle insert, scale indicates 500  $\mu\text{m}$ ). The sk-fMHC was expressed as a typical striated muscle pattern in the developing ventricle and cTn-I expression was near background level at ED13. Somites also express sk-fMHC as a fiber structure and cTn-I was also expressed very weakly similar to the heart (ED13 somite panel). Scales in ED13 ventricle and somite panels indicate 20  $\mu\text{m}$ . At ED20, both heart muscle (ED20 Left Vent Pap M) and skeletal muscle (not shown) express sk-fMHC and cTn-I as a striated muscle pattern. Scales in ED20 and ND4 Vent Pap M panels indicate 20  $\mu\text{m}$ . In the heart, sk-fMHC expression is significantly decreased, and cTn-I expression was increased with striation pattern after neonate day 4, and skeletal muscle significantly decreases its cTn-I expression after neonate day 4 (data not shown). At postnatal day 10 (PND10), left ventricular myocardium does not express sk-fMHC and cTn-I displayed a typical striation pattern (PND10 Left Ventricle panel). Conversely, gastrocnemius muscle expressed sk-fMHC as a typical striation pattern and cTn-I was negative (PND10 skeletal muscle panel). Scales in PND10 panels indicate 20  $\mu\text{m}$ .

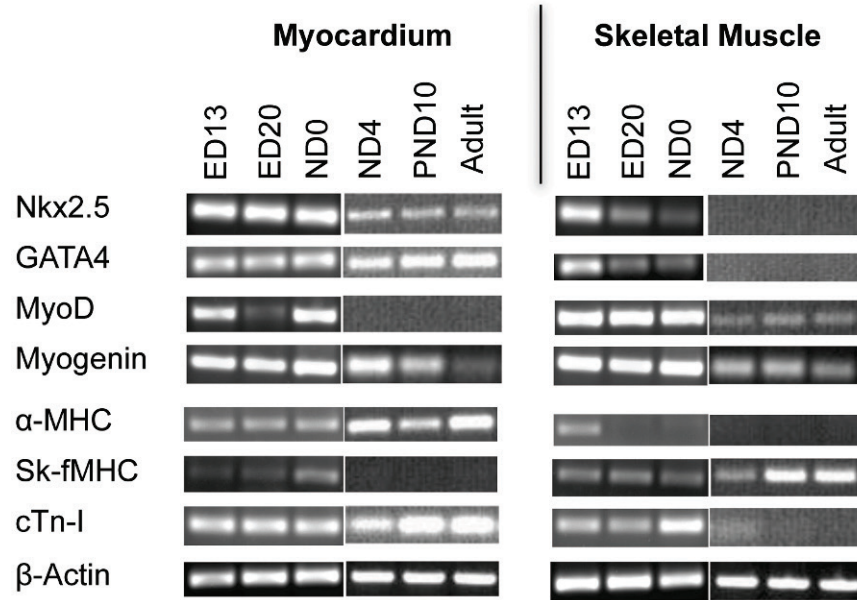
Western blot analysis indicated that in the myocardium, sk-fMHC was expressed in decreasing amounts until PND10 (juvenile) and was not expressed in the adult myocardium (8 week-old), whereas cTn-I was expressed in increasing amounts at all developmental stages. In skeletal muscle, sk-fMHC was expressed at all developmental stages, while cTn-I was expressed very weakly at ED13 and the expression increased at ED20, decreased at ND0, and became negative after ND4 (**Figure 2-3**).



**Figure 2-3.** Western blot analysis of developing myocardium and skeletal muscle. Lane 1: ED13; Lane 2: ED20; Lane 3: ND0; Lane 4: ND4; Lane 5: PND10; Lane 6: Adult (8 week-old). Top panel: Left ventricular myocardium, Bottom panel: hind limbs at ED13, ED20, ND0, and ND4, and gastrocnemius muscle in PND10 and adult. At ED13 and ED20, left ventricular myocardium expressed sk-fMHC and cTn-I expression was very weak while skeletal muscle expressed sk-fMHC. During development, sk-fMHC expression in the left ventricular myocardium decreased and increased in skeletal muscle, whereas cTn-I is expressed in the left ventricular myocardium and was negative in skeletal muscle.  $\alpha$ -sarcomeric actinin ( $\alpha$ -sarcActn) was used to adjust the total protein loading for the electrophoresis.

RT-PCR analysis (30 PCR cycles, **Figure 2-4**) showed that in ventricular myocardium, cardiac transcription factors, Nkx2.5 and GATA4, were expressed through all developmental stages and were also expressed in gradually decreasing levels in skeletal muscle. Skeletal muscle

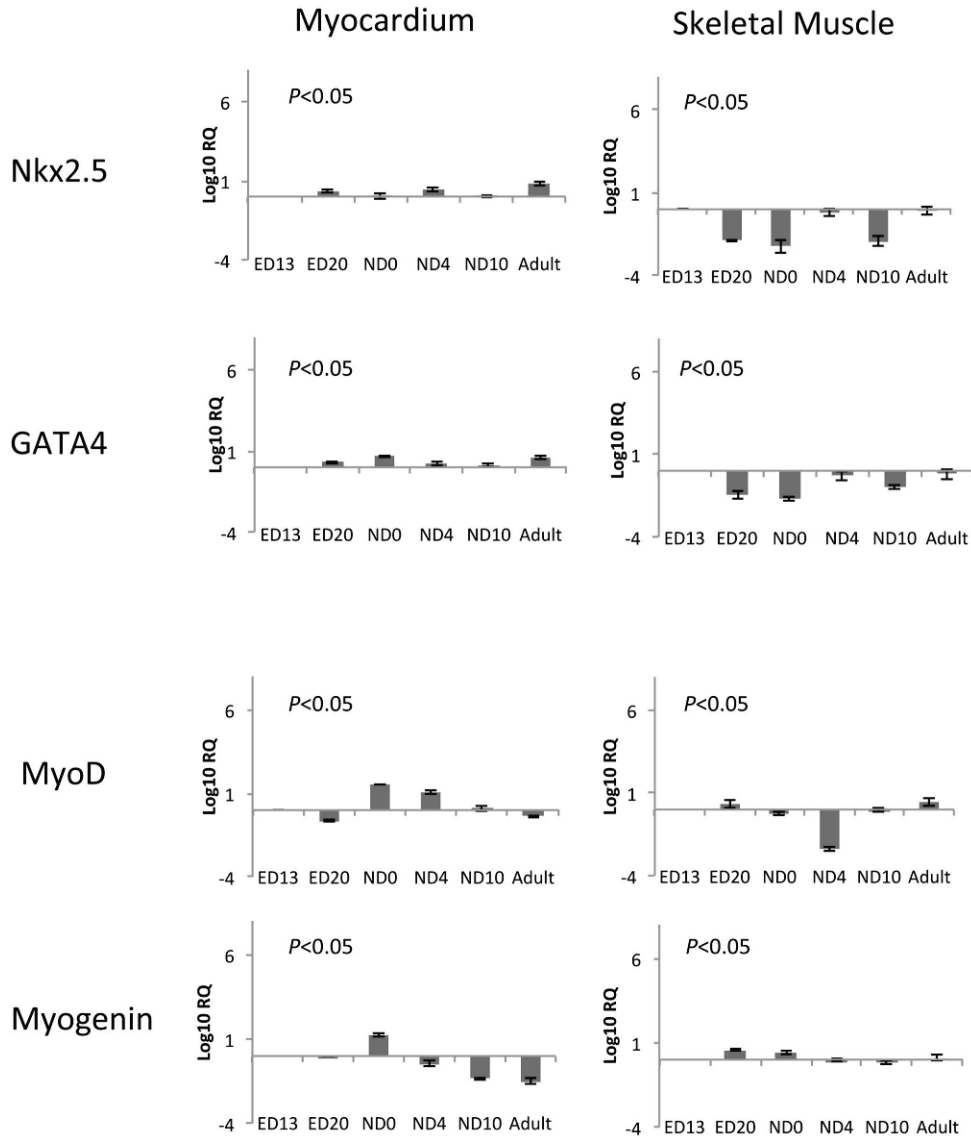
transcription factor MyoD was detected at 30 PCR cycles in skeletal muscle at all developmental stages and in ventricular myocardium until ND0. Another major skeletal muscle transcription factor, myogenin, was expressed in both ventricular myocardium and skeletal muscle in all developmental stages. The sk-fMHC mRNA was expressed in ND0 myocardium, and the expression was significantly decreased at ND4. It was then negative in PND10 and adult ventricular myocardium. The sk-fMHC mRNA was expressed at all developmental stages in the skeletal muscle. The cardiac specific  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) was present in the ventricular myocardium at all developmental stages and ED13 skeletal muscle. Conversely, postnatal and adult skeletal muscle did not express  $\alpha$ -MHC. cTn-I mRNA was expressed in ventricular myocardium in all developmental stages and ED13, ED20 and ND0 skeletal muscle, and became negative in PND10 and adult skeletal muscle.



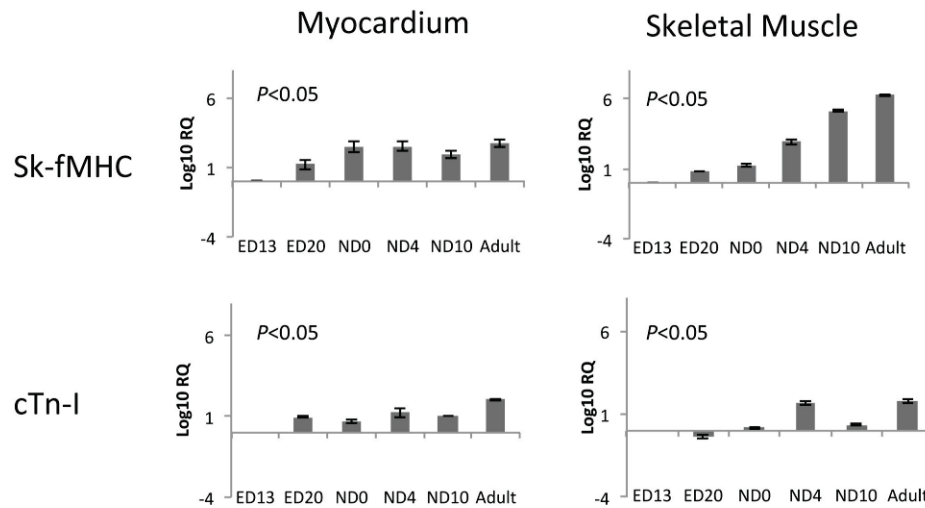
**Figure 2-4.** RT-PCR analysis of cardiac and skeletal muscle transcription factors, sk-fMHC, cTn-I mRNA expression. Lane 1: ED13 ventricle, Lane 2: ED20 ventricle; Lane 3: ND0 ventricle; Lane 4: ND4 ventricle; Lane 5: PND10 ventricle; Lane 6: Adult ventricle; Lane 7: ED13 hind limbs; Lane 8: ED20 hind limbs, Lane 9: ND0 hind limbs; Lane 10: ND4 hind-limbs; Lane 11: PND10 gastrocnemius muscle; Lane 12: Adult gastrocnemius muscle. We note that ED20 skeletal muscle (hind limbs) mRNA expression patterns are similar to ED20 ventricular myocardium.

We semi-quantified the changes in mRNA levels of the genes described above by real-time RT-PCR. Although the Ct value of each transcription factor and structural protein mRNA varied, these gene expression patterns overlapped between both types of muscle tissues. During development, cardiac transcription factor, Nkx2.5 and GATA4, expression levels in postnatal ventricular myocardium remained at the same levels as ED13. The mRNA level of the skeletal muscle transcription factor, MyoD in the ventricular myocardium was elevated at ND0 and ND4 but remained the same as ED13 in postnatal stages, whereas the mRNA level of another skeletal muscle transcription factor, myogenin, was significantly decreased postnatally. In developing skeletal muscle, Nkx2.5 and GATA4 expression levels were significantly decreased, while

MyoD and myogenin expression levels were transiently decreased at ND4 and were maintained at the same levels during the development (**Figure 2-5**). The sk-fMHC level in the myocardium increased from ED13 to ED20 and maintained a steady level thereafter, while it continuously increased in skeletal muscle. Conversely, cTn-I and  $\alpha$ -MHC levels were significantly increased in postnatal ventricular myocardium. In skeletal muscle, cTn-I was increased at ND4 and adult, while  $\alpha$ -MHC was decreased at ED20 and thereafter it was unchanged in postnatal development (**Figure 2-6**).



**Figure 2-5.** Changes in mRNA levels of cardiac and skeletal muscle specific transcription factors within ventricular myocardium and skeletal muscle. Data are expressed as average  $\pm$  SD. Cardiac transcription factors, Nkx2.5 and GATA4, and skeletal muscle transcription factor MyoD expression did not change in ventricular myocardium in adulthood, whereas myogenin expression was significantly decreased in ventricular myocardium ( $P<0.05$ , ANOVA). Nkx2.5 and GATA4 were significantly decreased during development, and MyoD and myogenin were significantly increased in skeletal muscle during the development ( $P<0.05$ ). Log10RQ: Relative Quantification of mRNA level compared to the mRNA level in ED13. The ratio is expressed as logarithm with base value of 10 (Log10).



**Figure 2-6.** Changes in mRNA levels of sk-fMHC, cTn-I, and cardiac  $\alpha$ -MHC within ventricular myocardium and skeletal muscle. Data are expressed as average  $\pm$  SD. The sk-fMHC mRNA level was significantly decreased and cTn-I and  $\alpha$ -MHC levels were significantly increased in ventricular myocardium ( $P<0.05$ , ANOVA). Conversely, sk-fMHC mRNA level was significantly increased and cTn-I and  $\alpha$ -MHC were significantly decreased in skeletal muscle. Log10RQ: Relative Quantification of mRNA level compared to the value in ED13. The ratio is expressed as logarithm with base value of 10 (Log10).

## 2.4 DISCUSSION

In the current study, we found that skeletal muscle specific fast myosin heavy chain (sk-fMHC) is transiently expressed in the developing immature myocardium, and cardiac troponin I (cTn-I) is transiently expressed in developing skeletal muscle. A number of previous studies have looked at an assortment of myosin and troponin expression patterns in both the cardiac and skeletal muscle.  $\alpha$ -MHC was found in both normal adult diaphragm and stimulated fast twitch muscles in the rabbit, human masseter and extraocular muscles, as well as the bag fibers of human, rat, and cat muscle spindles [111, 112]. From the opposite perspective, anti-anterior latissimus dorsi myosin antibodies showed specific reactivity with the cells of the conduction system in the rabbit



heart [113]. Similarly, there is abundant evidence that myosin expression undergoes transitions during the development of both skeletal and cardiac muscle in a number of species. Sweeney et al. found that the earliest MHC detectable in both forms of striated muscle in chick embryos (cardiac and skeletal) was indistinguishable from cardiac ventricular myosin expressed in the adult heart but immunologically distinct from myosins expressed in later embryonic and adult skeletal muscle. This suggests that the initial developmental program for myosin expression in differentiating skeletal muscle may be related to that for cardiac ventricular myosin [114]. In a similar study, cardiac ventricular MHC was found in developing as well as regenerating anterior latissimus dorsi avian skeletal muscle [115]. In the case of cardiac muscle though, it was unclear whether the developmental program involved the co-expression of skeletal as well as cardiac isomyosins. In the current study, we found that skeletal muscle specific fast MHC was in fact transiently expressed during the initial developmental program of the developing fetal myocardium.

Cardiac troponin-I and -T are normally specific to the adult cardiac muscle. However, cTn-T isoforms have been shown to be expressed in human skeletal muscle in individuals suffering from Duchenne muscular dystrophy, polymyositis, and end stage renal disease [36] and in developing and regenerating rat skeletal muscle [116]. Fredericks et al. showed that cTn-I and cTn-T are expressed in adult rat skeletal muscle in response to denervation, but the protein expression levels were several thousand times lower than in the heart [117]. Messner et al. showed that mRNA from cTn-T and cTn-I are expressed in skeletal muscle in patients with Duchenne muscular dystrophy and several other myopathies [118]. In the present study, we found that cTn-I was transiently expressed during the early development of skeletal muscle. To our knowledge, this is the first comprehensive study of the time course expression of cTn-I

during the normal development of skeletal muscle. Sutherland et al. examined cTn-I mRNA expression in the developing rat hind limb by Northern blot but did not detect any cTn-I in skeletal muscle [119]. This may be due to the low sensitivity of Northern blot relative to RT-PCR. We have shown that cTn-I is expressed in early developing skeletal muscle at both the gene and protein level (by immunohistochemical staining but not western blot) and visually shown changes in its structural organization. As for why cTn-I was detected in skeletal muscle by immunohistochemical staining but not western blot, it is possible that there are regional differences in cTn-I expression in skeletal muscle, and the total amount of cTn-I relative to total protein is below the detectable threshold of western blot. However, regional expression can still be visualized by immunohistochemical staining.

As development of the myocardium progressed, sk-fMHC became negative, and the myocardium then expressed cTn-I. Conversely, as development of skeletal muscle progressed, cTn-I became negative, and the skeletal muscle then expressed sk-fMHC. Fast skeletal muscle is more prone to fatigue, utilizes ATP quickly, and uses less oxygen, whereas cardiac muscle is made to resist fatigue and generally needs more oxygen. Thus, from a physiological standpoint, the benefit of sk-fMHC in the immature developing fetal myocardium may be shorter and quicker contraction for quicker blood ejection throughout the system, which is necessary for supporting rapid fetal growth prior to the establishment of coronary artery circulation, cardiac autonomic, and conduction systems. As the myocardium matures, the contraction pattern shifts towards less rapid but continuous contraction under aerobic conditions, at which point the coronary circulation and conduction as well as cardiac autonomic system are established, and sk-fMHC expression decreases. Continued expression of sk-fMHC would be detrimental because if fast muscle tissue were to persist in the postnatal and adult myocardium, it would fatigue much

faster, potentially increasing the risk of cellular acidosis. A recent study by Rutland and colleagues reported the expression of embryonic myosin heavy chain (eMYH), a fast skeletal specific isoform, in the developing chick heart. Its functional homologue in humans is believed to be Myh3 [120]. While they did not detect the presence of other skeletal myosin heavy chain genes in human fetal and adult hearts, we speculate that sk-fMHC may serve a similar role in rats as Myh3 in humans during heart development. Moreover, while their study focused on changes that occur during early development, we place a specific emphasis on the events that occur during the transition from late fetal to early postnatal life.

One potential limitation of the current study is the use of the Sigma MY32 antibody and the lack of a more specific commercially available antibody. Sigma reports that it stains the fast (type II) and neonatal myosin molecules found in skeletal muscle. It is possible that MY32 may cross react with the embryonic and neonatal myosin heavy chain isoforms. The reactivity of this antibody with the embryonic and neonatal forms has not been fully characterized. However, Lyons et al. have reported that the embryonic and neonatal forms are not expressed in the developing mouse embryo heart [121], which would indicate that sk-fMHC is the only fast skeletal muscle MHC expressed in the developing heart in rats.

The N-terminal extension of cTn-I contains two phosphorylatable serines (Ser-23 and Ser-24), which are targets of protein kinase A and protein kinase C and are not present in the fast and slow skeletal isoforms of troponin I [122]. These additional sites confer a greater degree of regulation of Calcium sensitivity and contractility [122, 123]. Thus, the presence of the cardiac isoform in developing skeletal muscle might provide an additional level of control of muscle activity prior to the full development of the nervous system, which would increase energy efficiency in the metabolically active developing fetus.

Real-time RT-PCR analysis of gene expression in the developing heart and skeletal muscle supports the idea of developmental commonality between both types of tissue. Cardiac specific genes such as Nkx2.5, GATA4,  $\alpha$ -MHC, and cTn-I are expressed in developing skeletal muscle. Expression levels of certain genes within the heart and skeletal muscle change over time, while others, notably Nkx2.5 and GATA4 within the myocardium, remain relatively constant over time. Interestingly, sk-fMHC mRNA continues to be expressed at low levels in adult myocardium, and cTn-I mRNA continues to be expressed at low levels in adult skeletal muscle, despite the absence of protein expression. It is possible that these genes are post-transcriptionally suppressed in adulthood, but the continued expression at the transcript level provides a faster means of response in response to external events such as injury such as myocardial infarction, which leads to recapitulation of the fetal gene program. Sassoon et al. reported that MyoD and myogenin were detected by in-situ hybridization in embryonic skeletal muscle but not heart [124]. However, they only specify that they examined expression in the heart at embryonic day 7.5 during heart tube formation. They do not specify the range of time points that they examined. MyoD and myogenin may only be expressed in the heart during later development, as our results indicate. Di Lisi et al. reported that GATA4 is expressed in C2C12 muscle cells at the transcript level but not at the protein level [125]. However, they used C2C12 cells terminally differentiated into myotubes in vitro under conventional 2D culture for their analysis, which does not accurately represent in vivo development. Overall, our results suggest a shared developmental program between skeletal and cardiac muscle, which is lost as they mature into distinct tissue types. We speculate that skeletal muscle development (maturation) occurs at later development compared to the cardiac muscle, and the skeletal muscle differentiation temporarily requires the cardiac muscle phenotype (cyclic twitch) to acquire the function towards highly controlled

contraction and relaxation under the regulation of the nervous system, which may be supported by the previous report from Fredericks et al. [117].

In our previous work, we showed that stem cells isolated from juvenile rat skeletal muscle could differentiate into cardiomyocyte like cells in a 3D collagen gel bioreactor (MDSC-3DGB) [67]. With respect to the current work, we showed that MDSC-3DGB expresses cTn-I at higher levels than ED20 skeletal muscle but lower than ED20 myocardium. Skeletal muscle derived stem cells (MDSCs) isolated from skeletal muscle express biochemical markers present in both developing skeletal and cardiac muscle. The findings presented here support the idea that MDSCs have the capacity to differentiate into a more cardiac phenotype in-vitro. Fate decisions are ultimately influenced by biochemical and biomechanical signals from the environment. Providing the appropriate environment for stem cell differentiation remains a topic of ongoing research.

## **2.5 CONCLUSION**

In conclusion, the current study provides novel evidence that skeletal muscle specific fast myosin heavy chain is transiently expressed in the developing immature myocardium, and cardiac troponin I is transiently expressed in developing skeletal muscle. The similarities between cardiac and skeletal muscle may be linked to their common mesodermal origin. These findings shed light on a previously unknown aspect of muscle development. Further studies are necessary to examine the role of these contractile proteins during development and how they are regulated.

## 2.6 ACKNOWLEDGEMENTS

This research was supported by NIH RO1HL085777, NIH R21 HL094402, and NIH T32-HL76124.

**Table 2-1. List of Abbreviations Used in Chapter 2**

### **Abbreviations**

MHC:	Myosin Heavy Chain
Sk-fMHC:	Fast skeletal myosin heavy chain
cTn-I:	Cardiac troponin I
MDSC:	Muscle derived stem cell
ED:	Embryonic day
ND:	Neonatal day
PND:	Postnatal day
EA53:	Alpha sarcomeric actinin
MDSC-3DGB:	3D collagen gel bioreactor

### **3.0 ENGINEERED HUMAN MUSCLE TISSUE FROM SKELETAL MUSCLE DERIVED STEM CELLS AND INDUCED PLURIPOTENT STEM CELL DERIVED CARDIAC CELLS**

#### **3.1 INTRODUCTION**

The adult heart is largely a non-regenerative organ. Although cardiomyocytes (CMs), the contractile cells of the heart, have a modest rate of turnover, ranging from 1% in youth to less than 0.5% in old age [9], this level is not enough to compensate for the large number of cardiomyocytes which are lost as a result of heart injury. Combined with the fact that heart disease is the leading cause of death in the United States [126], this has prompted the search for novel therapies to replace damaged myocardium. Muscle derived stem cells (MDSCs) and induced pluripotent (iPS) stem cells are among the types of stem cells under investigation for cardiac repair. MDSCs are a multipotent, somatic stem cell which can be obtained from skeletal muscle via a modified pre-plate method [127]. MDSCs can be rapidly expanded *in vitro* to obtain clinically relevant numbers of cells, which can be transplanted as an autologous graft. They are also advantageous because they are resistant to hypoxia, attenuate fibrosis, and readily differentiate into contractile cells [30]. We previously showed that rodent MDSCs differentiate into CM-like cells with cardiac-like electrophysiological, biochemical, and contractile properties using cell aggregate formation and 3-dimensional (3D) culture in a collagen-based scaffold [67], but engineered tissue models of human MDSCs in the context of their relationship to cardiac development and disease has not been investigated before. Studies have shown that cell

aggregate culture can enhance cell-cell interactions and modulate gene expression, facilitating differentiation. Use of 3D engineered tissues as a vehicle for cell transplantation has been shown to provide a microenvironment which is optimal for cell survival and integration [90]. The iPS cells can be obtained from theoretically any somatic cell type by virus-mediated transfection of a quartet of reprogramming factors [128, 129]. These cells can then be differentiated into CMs or other cell types using established protocols [130, 131], which combine 3-dimensional culture with sequential growth factor and cytokine treatments. This approach ideally provides an unlimited source of CMs, but the modification of the genome of the host cell poses a challenge to clinical translation [132]. While cell therapy for heart disease remains a long-term goal in the field, our current aim is to provide a versatile and robust test bed to study striated muscle differentiation from stem cells towards this long-term goal.

Fetal gene expression is reactivated in the heart in response to various myocardial insults and disease states [133, 134], which includes expression of skeletal muscle specific proteins. However, this process remains poorly understood. The process may vary in different species, limiting the translatability of animal models, and conventional 2D *in vitro* models do not faithfully represent complex tissue architecture or allow for assessment of function at the tissue-level. Direct biochemical and functional analyses on human myocardial tissue cannot take place due to limited tissue access *in vivo*. Therefore, it is necessary to develop better *in vitro* models of human cardiac muscle in order to better understand the relationship between striated muscle development (of both cardiac and skeletal muscle) and the pathogenesis of heart failure, which may lead to the development of better cell-based therapies. Creating better *in vitro* models to study human cardiac muscle development will not only broaden our understanding of



developmental biology, it may enable us to develop better cell-based therapies for heart disease in the future.

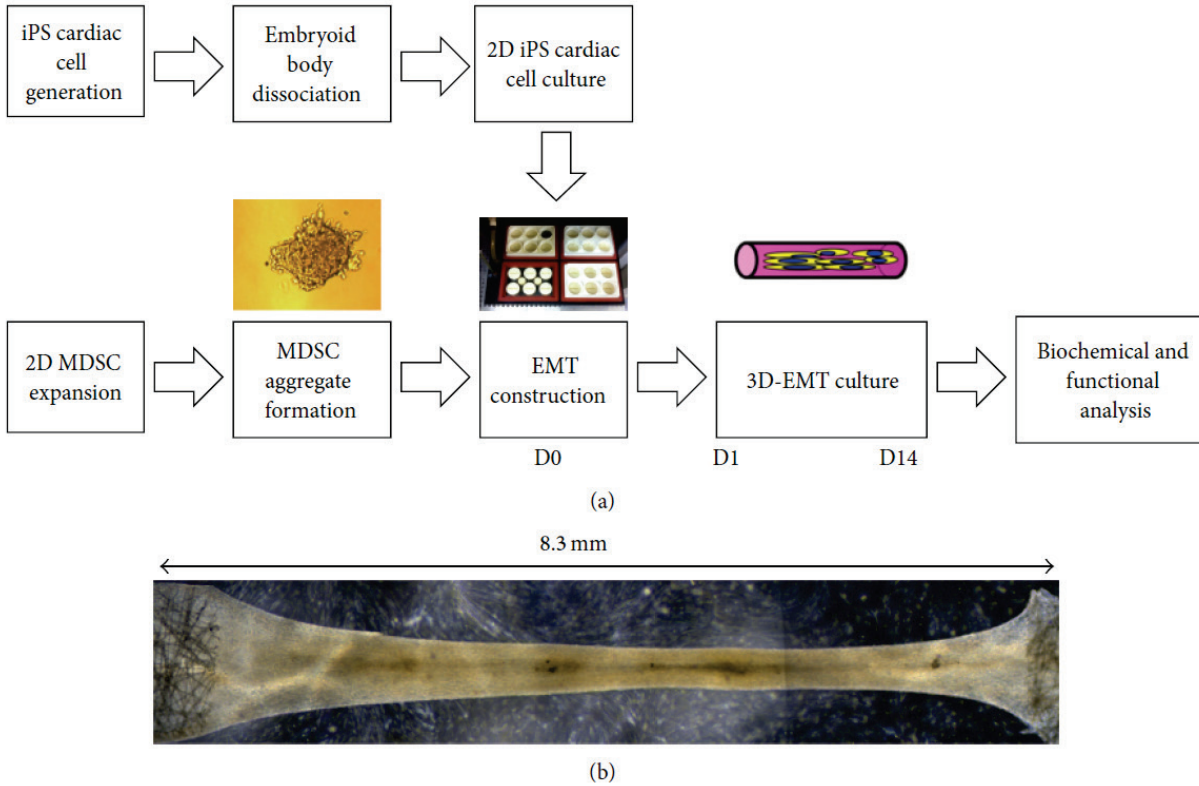
Several studies have shown that developing cardiac and skeletal muscle share expression of major cardiac or skeletal muscle specific contractile proteins and transcription factors [35, 36, 38, 135]. However, this phenomenon has not been investigated in *ex vivo* engineered muscle tissues (EMT). Our current understanding of the nature of engineered muscle tissues is based on established models of mature cardiac and skeletal muscle. Thus, the objective of the current study was to investigate the occurrence of this “hybrid” phenotype in EMTs using two stem-cell based models, human MDSCs and iPS-cell derived cardiac cells. Our hypothesis was that both MDSC-EMT and iPS-EMT possess properties of cardiac and skeletal muscle. Our results indicate that MDSC-EMT and iPS-EMT share a number of biochemical similarities, but iPS-EMT has a better degree of electrical coupling and adrenergic responsiveness.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Cell Culture**

MDSCs of 3 different human subjects (from 10 to 30 years old) were purchased from Cook Myosite, Inc. Cells were cultured in Cook Basal Media (Cook Myosite, Inc., Pittsburgh, PA, USA) supplemented with 10% Growth Supplement (Cook Myosite, Inc., Pittsburgh, PA, USA) and 1% Antibiotic-Antimycotic solution (AAS, Invitrogen) on collagen-I coated flasks. Cells were used for no more than 4 passages and maintained below 50% confluence prior to initiation of differentiation. Human Y1 iPS cells were previously established in L.Y’s lab from healthy fibroblasts [131] and used as the control. iPS cells were maintained on mouse embryonic

fibroblast (MEF) feeder layers with regular human embryonic stem cell medium containing 10 ng/mL FGF-2 [130]. iPS cells were differentiated into cardiomyocytes using our previously established protocol [130, 131]. All growth factors were from R&D systems. Following differentiation, embryoid bodies were dissociated using 0.05% trypsin (Invitrogen) and plated on Matrigel (BD Bioscience) coated 6 well plates in High-Glucose Dulbecco's Modified Eagle Medium (DMEM, Cellgro) supplemented with 10% Fetal Bovine Serum (FBS), L-Glutamine (1:100, Invitrogen), and Penicillin Streptomycin (1:100, Invitrogen) (Figure 3-1A).



**Figure 3-1. EMT Culture Platform.** (a) Model system of 3D engineered muscle tissue culture. MDSCs undergo cellular aggregation prior to EMT construction. EMT is formed by mixing MDSC aggregates with collagen/ECM in a Flexcell Tissue Train Culture System. MDSC aggregates are cultured as EMT under low-serum conditions to induce differentiation. In an analogous manner, iPS-EMT is constructed by mixing dissociated iPS cardiac cells with collagen/ECM. (b) Composite phase contrast image of EMT. Cells in EMT aligned in the longitudinal direction and formed a muscle tissue during the 14-day culture period. Tissue morphology is maintained by anchors at each end of the tissue train plate.

### 3.2.2 Fluorescence activated cell sorting

Cryopreserved MDSCs from Cook Myosite were thawed and expanded to ~60% confluency. Cells were trypsinized and stained with CD45-APC (BD), CD56-PE-Cy7 (BD), CD146-PE (BD), and UEA-1-FITC (Ulex, BD) antibodies. Samples were analyzed on a Becton Dickinson

(San Jose, CA) FACS Aria flow cytometer. The sorter is equipped with a standard configuration of three excitation sources. The FACS Aria contains blue, red and violet lasers which excite at 488, 633 and 407 nm respectively. Dead cells were initially eliminated based on 7-AAD, a dead cell exclusion dye. Cells were then analyzed on the basis of forward scatter (FSC-A) vs side scatter (SSC-A) for the selection of single cells and for the elimination of debris and aggregates of cells. This population was also analyzed on the basis of height vs width for both forward scatter and side scatter. Doublets of cells were eliminated on the basis of a high width measurement. Subsequent analysis of fluorescent populations was limited to this group. Unstained cells and isotype controls were used to set the background fluorescence. Single fluorochrome stained cells were used as compensation controls to eliminate spectral bleed over between the dyes. Cells were separated into CD56(+) and CD56(-) subpopulations. These were also determined to be CD45 (-), eliminating hematopoietic subpopulations. The CD56(+)/CD45(-) and CD56(-)/CD45(-) were then analyzed for CD146 and Ulex expression. The FACS Aria is equipped with an 85 micron nozzle and the cells were sorted at a pressure of 35 psi. The larger nozzle size and lower pressure were used to increase the viability of the cells. Sterile PBS is run through the instrument as sheath fluid. Cells are collected into Cook Basal Medium with the populations as previously described [75, 76, 136, 137].

### **3.2.3 Engineered Muscle Tissue (EMT) Construction**

MDSCs were trypsinized, and MDSC-cell aggregates were obtained by rotation culture (Labnet Orbit 1000) on a suspension culture plate at 50 rpm for 24 hours prior to tissue construction [67]. iPS cardiac cells were trypsinized and counted on the day of EMT construction. Liquid rat tail collagen type I (3 mg/mL, Invitrogen) was neutralized with 0.1N NaOH and mixed with Matrigel

(BD Bioscience) with a collagen: Matrigel ratio of 0.8 and a final collagen concentration of 0.67 mg/mL. Cells were seeded in a collagen/Matrigel mixture at a density of 0.5 million cells per construct using a Flexcell Tissue Train Culture system (FX-4000, Flexcell International) with a total volume of approximately 200  $\mu$ L per construct to form a linear shaped construct (Figure 3-1B). To induce differentiation, MSDC-EMTs were cultured in Cook Basal Media supplemented with 5% growth supplement, 1% AAS, and human FGF-2 (5ng/mL, Sigma). iPS-EMTs were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS), L-Glutamine (1:100, Invitrogen), and Penicillin Streptomycin (1:100, Invitrogen).

#### 3.2.4 Real-time Polymerase Chain Reaction

Total RNA was prepared using Trizol solution (Invitrogen) and treated with TURBO DNA-free kit (Ambion, Austin, TX, USA). Primers, whose target genes are Nkx2.5, GATA4, Mef2A, Tbx5, GJA1, Myh6, Myh7, MyoD, myogenin, and Beta-Actin were obtained from Qiagen Quanti-Tect Primer Assay with the target fragment sizes approximately 100 base pairs. One step RT was performed using Applied Biosystems High Capacity RNA-to-cDNA Kit (Applied Biosystems) with a total of 2  $\mu$ g RNA in a total volume of 20  $\mu$ L with the following program: 37°C for 1 hour, 95°C for 5 minutes, 4°C hold. cDNA (1  $\mu$ L) was used for RT-PCR using the following program: 50°C for 2 minutes, 95°C for 10 minutes. This was followed by 95°C for 15 seconds and 60°C for 1 minute, repeated for 40 cycles. The final stage was 95°C for 15 seconds, 60°C for 15 seconds. SYBR Green was used as a detector. 3 samples were run for each target gene per group from pooled samples using Applied Biosystems 7900HT system. Relative expression (RQ) was calculated using the *ddCt* method with  $\beta$ -actin as an internal control. A Ct threshold of 38 was set based on the vendor's instructions.

### 3.2.5 Immunohistochemical Staining

EMTs were fixed using 4% paraformaldehyde/PBS for 30 minutes and embedded in 13% polyacrylamide gel. 150  $\mu\text{m}$  thick sections were made using a vibratory microtome (Vibratome-1000, Vibratome.com) and stained for cardiac troponin-I (cTn-I, Abcam), cardiac troponin-T (cTn-T, Abcam), fast-skeletal muscle heavy chain (sk-fMHC, Sigma), connexin-43 (Cx-43, Abcam), CD31 (R&D Systems), Nkx2.5 (Santa Cruz), MyoD (Santa Cruz), and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA, Abcam) primary antibodies and Alexa Fluor 488 or Alexa Fluor 594 secondary antibodies. Stained samples were scanned using a confocal microscope (Olympus Fluoview FV1000) and used to generate 3D projection images of the tissue sections in ImageJ.

### 3.2.6 Mechanical Testing

The active force of EMTs was measured using a customized setup. Constructs were transferred to a muscle testing station perfused with warmed Ringer solution containing 2mM  $\text{CaCl}_2$ , 135mM NaCl, 4mM KCl, 10mM Trizma-HCl, 8.3 mM Trizma-base, and 11mM glucose. The constructs were attached to a force transducer (403A, Auroura Scientific, Auroura, Canada) using 10-0 monofilament nylon sutures. The other end of each construct was attached to a micromanipulator. Field stimulation was applied using a stimulator (Grass S48 Stimulator, Grass Medical Instruments) at 5ms duration, 100V. The parameters were set at 10% above the threshold required to induce visible contraction of all constructs. The construct length was adjusted from 0% to 15% elongation. Force was measured at stimulation rates of 1-5 Hz and in response to isoproterenol (ISP, 1 $\mu\text{M}$ ) and extracellular calcium (Calcium Chloride, 5 mM).

Skeletal muscle stimulation was applied as follows to test if tetanus could be induced: 500ms train rate, 1000ms duration, 20Hz stimulation rate.

### **3.2.7 Intracellular Calcium Transient Measurement**

Samples were loaded for 10 to 15 minutes at 37°C with Rhod 2-AM (Biotium) at a concentration of 5 µg/mL. After the dye was loaded, the samples were placed on the heated stage of a Leica (DM LFSA) microscope. Optical mapping was performed with high spatiotemporal resolution (64x64 pixels, 176 fps) at 37°C using a Hamamatsu EM-CCD camera (Model C9100-12). Bipolar stimulation (20V, 5ms, 1-3Hz, Grass S48 Stimulator, Grass Medical Instruments) was applied to electrically pace EMTs when necessary. The parameters were set at 10% above the threshold required to induce visible transients of all constructs. Calcium transients were recorded during spontaneous beating and during bipolar stimulation. Changes in signal were measured in response to isoproterenol (ISP, 1 µM). Videos were processed in ImageJ (NIH) and analyzed using densitometry techniques. A 3x3 pixel area was selected within each cell. The average pixel intensity within the region was calculated and normalized to the baseline resting value.

### **3.2.8 Statistical Procedures**

One-way ANOVA was used to compare gene expression among experimental groups. A paired t-test was used to compare changes in force in response to isoproterenol and changes in force in response to extracellular calcium. An independent t-test was used to compare changes in calcium transient frequency in response to isoproterenol. A two-way repeated measure ANOVA was performed to compare active stress-strain relations. Data were expressed as mean ± standard

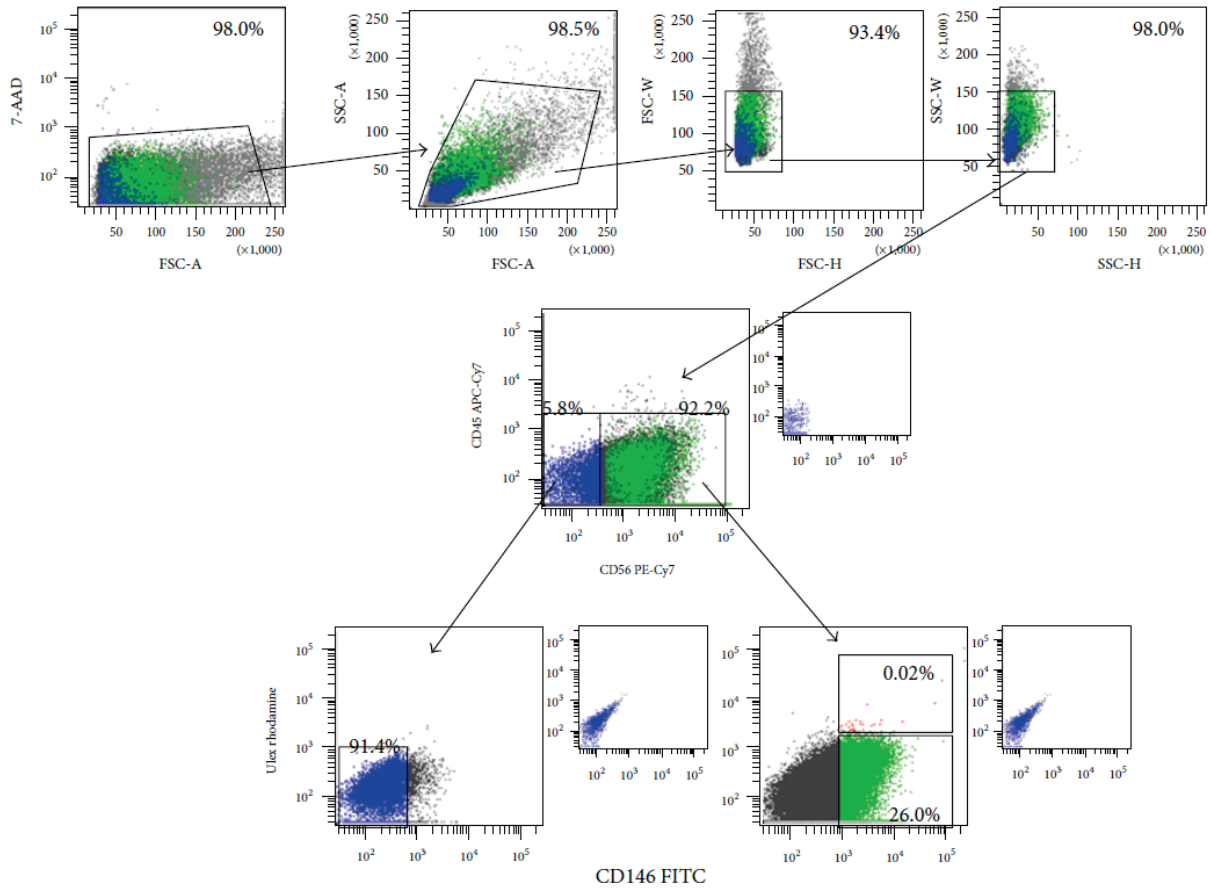
error. Statistical significance was defined by a value of  $P < 0.05$ . All calculations were performed using Sigma Stat 3.0 software (Systat Software Inc.).

### **3.3 RESULTS**

#### **3.3.1 Surface Marker Expression of MDSC**

We examined the surface marker expression profiles of undifferentiated MDSCs. MDSCs predominantly express CD56, a marker of myogenic lineage. A large number of cells also expressed CD146, a marker of mesenchymal stem cells. A smaller fraction of cells expressed endothelial cell marker Ulex (Figure 3-2, Table 3-1). The majority of cells were myogenic-mesenchymal cells, co-expressing CD56 and CD146 but not Ulex. Smaller fractions of cells were myoblasts (CD45-/CD56+/CD146-/Ulex-) and myogenic-endothelial cells (CD45-/CD56+/CD146+/Ulex+). Small populations of pericytes (CD45-/CD56-/CD146+/Ulex-) and endothelial cells (CD45-/CD56-/CD146-/Ulex+) were also present in sorted populations (Table 3-1). These data indicate that MDSCs are a heterogeneous population, which contains myogenic and supporting non-myogenic cells.





**Figure 3-2. Fluorescence Activated Cell Sorting Analysis of undifferentiated human MDSCs.** The majority of cells expressed myogenic (CD56) and mesenchymal (CD146) markers. A small number of cells expressed hematopoietic (CD45) and endothelial (Ulex) markers. Human MDSC population is composed of myoblasts (CD45-/CD56+/CD146-/Ulex-), myogenic-endothelial cells (CD45-/CD56+/CD146+/Ulex+), pericytes (CD45-/CD56-/CD146+/Ulex-), endothelial cells (CD45-/CD56-/CD146+/Ulex-), and myogenic-mesenchymal cells (CD45-/CD56+/CD146+/Ulex-).

**Table 3-1. FACS Analysis of MDSC**

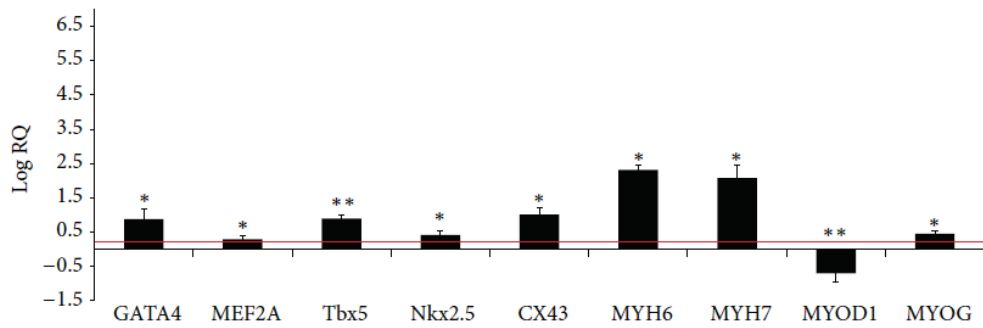
	<b>Positive %</b>
CD45	1.1 ± 1.0 ( <i>n</i> = 3)
CD56	93.1 ± 6.2 ( <i>n</i> = 5)
CD146	84.3 ± 5.9 ( <i>n</i> = 5)
Ulex	1.9 ± 1.8 ( <i>n</i> = 5)
Cell type	
CD45-/CD56+/CD146-/Ulex-	2.87 ± 0.68
CD45-/CD56+/CD146+/Ulex+	1.79 ± 0.78
CD45-/CD56-/CD146+/Ulex-	1.55 ± 0.98
CD45-/CD56-/CD146-/Ulex+	0.02 ± 0.01
CD45-/CD56+/CD146+/Ulex-	81.26 ± 3.12

### 3.3.2 Cardiac and skeletal muscle-specific gene and protein expression of EMT

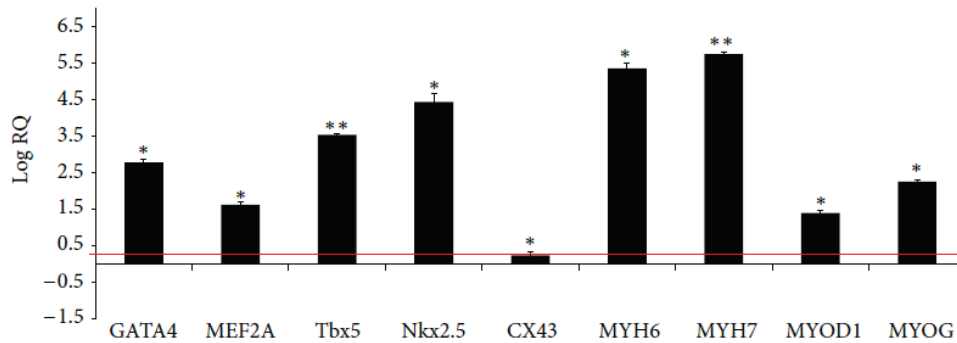
In this study, a healthy Y1 human iPS cell line was differentiated into cardiac cells using our established method [131], which was modified from our previous cardiac differentiation protocol with human embryonic stem cells [130]. iPS cell derived cardiac cells and MDSCs were cultured in EMTs for 14 days.

We examined cardiac and skeletal muscle specific gene expression in MDSC-EMT and iPS-EMT at culture day 14 by quantitative RT-PCR. MDSC-EMT (Figure 3-3A) significantly upregulated expression of both cardiac (Nkx2.5, GATA4, MEF2A, Tbx5, Cx-43, Myh6, Myh7) and skeletal muscle (myogenin) genes compared to undifferentiated MDSCs. Note that relative quantification values are expressed as log<sub>10</sub> scale. Interestingly, a major skeletal muscle transcription factor, MyoD, was downregulated (*n*=9, *p*<0.05). iPS-EMT (Figure 3-3B) significantly upregulated expression of both cardiac and skeletal muscle related genes compared

to undifferentiated iPS cells (n=6). While expression levels of certain genes vary between the two tissues, the key point is that they share a common gene expression profile at the transcriptional level, which opens up the possibility that MDSCs can transdifferentiate into cardiomyocyte-like cells under the appropriate conditions. On a similar note, the expression of skeletal muscle specific genes in cardiomyocytes derived from iPS cells using a widely used, established protocol suggests that the cardiac and skeletal muscle lineages are not mutually exclusive. These data show that MDSC-EMT and iPS-EMT share a common gene expression profile of cardiac and skeletal muscle transcription factor and structural protein genes similar to developing cardiac and skeletal muscle [35] and differentiating mesenchymal stem cells [138].



(a)



(b)

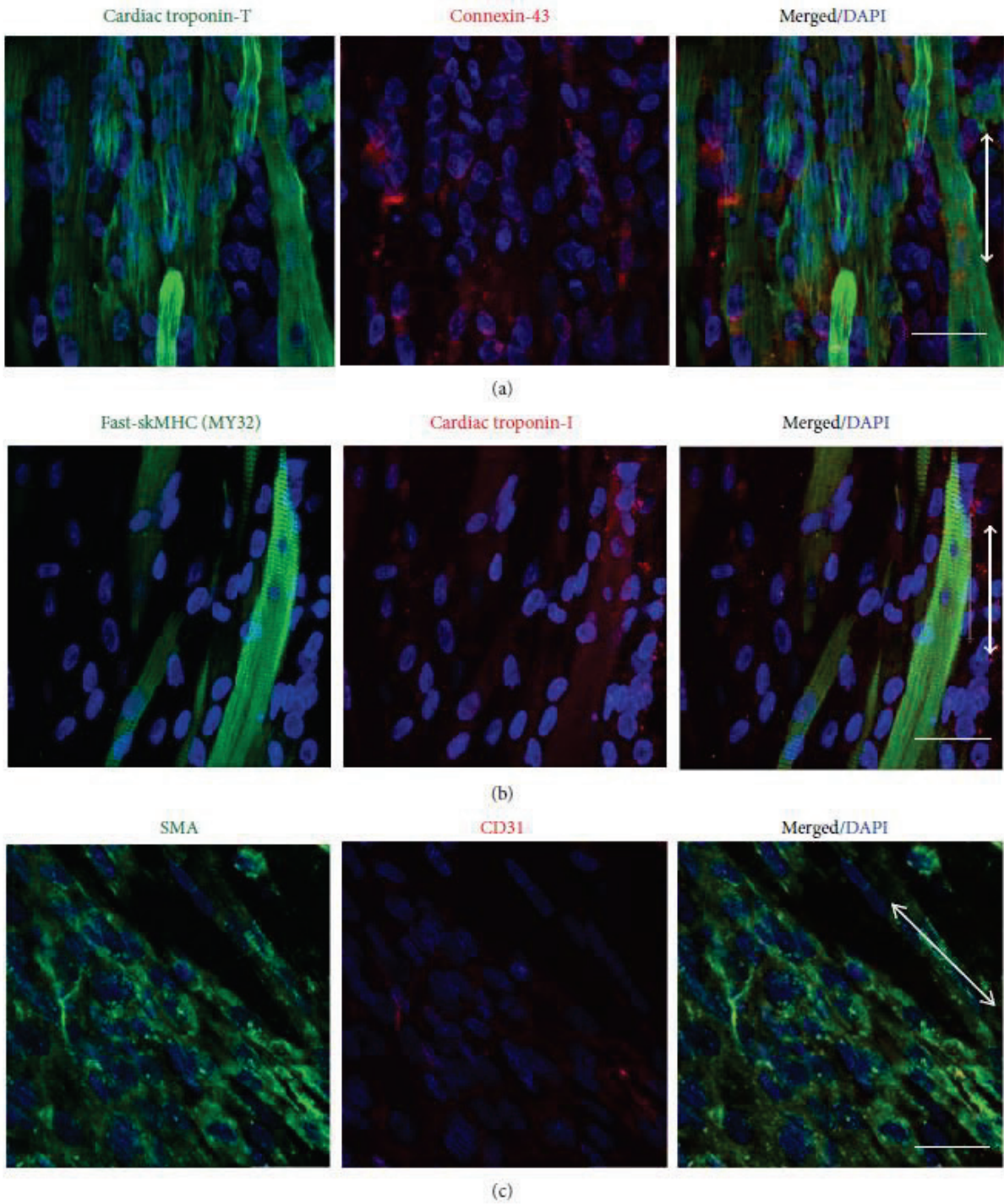
	$\beta$ -Actin	GATA4	MEF2A	TBX5	Nkx2.5	CX43	MYH6	MYH7	MYOD1	MYOG
MDSC-EMT	16.1	35.6	21.9	35.7	35.4	23.0	17.4	17.6	21.7	18.0
iPS-EMT	15.6	19.9	19.9	20.8	20.9	22.2	14.8	16.9	30.8	27.9

(c)

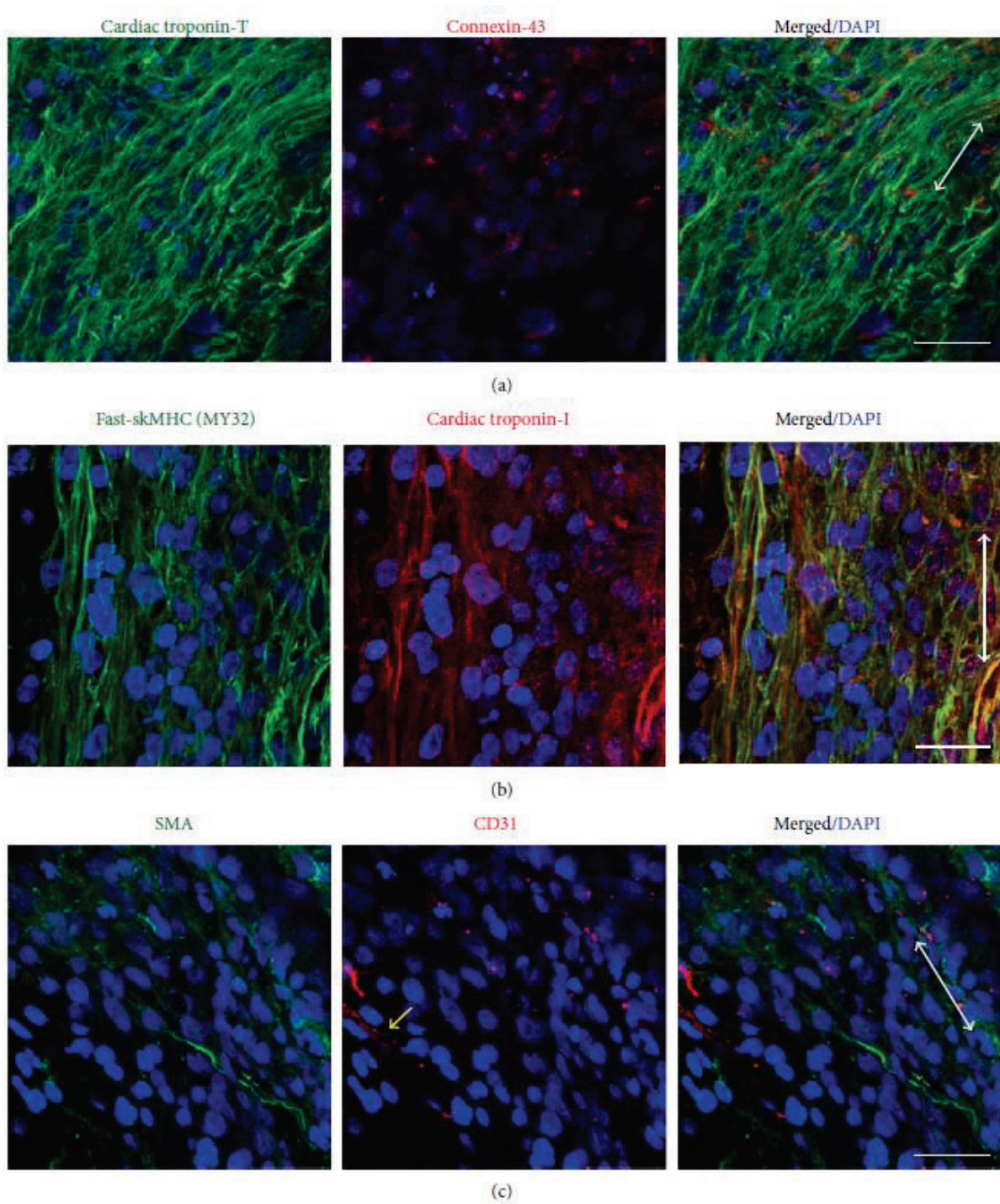
**Figure 3-3. RT-PCR analysis of EMT Gene Expression.** (a) Cardiac and skeletal muscle specific mRNA expression of MDSC-EMT at culture day 14 after tissue construction. Values are normalized to undifferentiated MDSCs. (b) Cardiac and skeletal muscle specific mRNA expression of culture day 14 iPS-EMT. Values are normalized to undifferentiated iPS cells. \* $P < 0.05$  . \*\* $P < 0.001$  . Red line indicates twofold increase. (c) Representative Ct values for MDSC-EMT and iPS-EMT. Although expression levels vary between the tissues, their transcriptional and structural gene profiles overlap.

Histologically, it is evident that MDSC-EMT and iPS-EMT co-express cardiac and skeletal muscle structural proteins. Both EMTs express cTn-T in a striated pattern with gap junction protein, Cx-43 (Figure 3-4A, Figure 3-5A). Notably, the expression pattern of Cx-43 in

iPS-EMT resembles neonate myocardium. On the other hand, Cx-43 in MDSC-EMT appears to be more diffusely expressed and with less discrete localization at cellular junctions mimicking immature fetal myocardium [67]. In line with our previous findings, cTn-I and skeletal muscle specific sk-fMHC were found to be co-expressed (Figure 3-4B, Figure 3-5B) similar to developing myocardium or skeletal muscle. Finally, non-muscle proteins are also expressed within the tissue. We detected endothelial (CD31) and smooth muscle/mesenchymal cell ( $\alpha$ -SMA) marker expression in iPS-EMT and MDSC-EMT (Figure 3-4C, Figure 3-5C). The presence of CD31 and  $\alpha$ -SMA together with the detection of a mixture of endothelial, mesenchymal, and myogenic surface markers by FACS, support the notion that EMT is complex, containing multiple cell types, which support cellular processes at the tissue level.



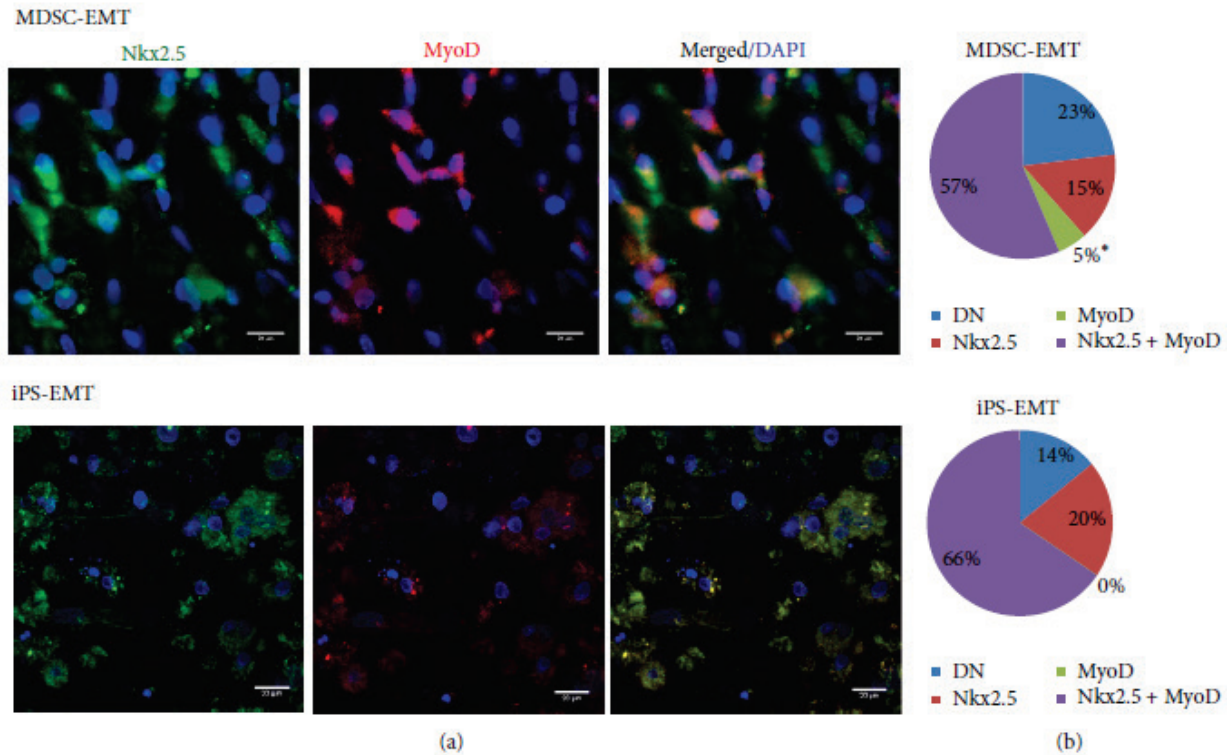
**Figure 3-4. Immunohistochemical Analysis of culture day 14 MDSC-EMT.** (a) Cardiac troponin-T (green) and gap junction protein Cx-43 (red). (b) sk-fMHC (green) and cardiac troponin-I (red). (c)  $\alpha$ -Smooth muscle actin (green) and CD31 (red). Arrow indicates tissue orientation. Blue staining (DAPI) indicates nuclei. Scale bar indicates 30 $\mu$ m.



**Figure 3-5. Immunohistochemical Analysis of culture day 14 iPS-EMT.** (a) Cardiac troponin-T (green) and gap junction protein Cx-43 (red). (b) sk-fMHC (green) and cardiac troponin-I (red). (c)  $\alpha$ -Smooth muscle actin (green) and CD31 (red). Yellow arrow shows representative CD31 staining. Arrow indicates tissue orientation. Blue staining (DAPI) indicates nuclei. Scale bar indicates 30 $\mu$ m.

To investigate whether myocytes share cardiac and skeletal muscle-specific transcription factors at the protein level or exist as distinct populations of cells, we stained EMTs with Nkx2.5, a cardiac transcription factor, and MyoD, a skeletal muscle transcription factor (Figure 3-6A, n=3 fields each). In both MDSC-EMT and iPS-EMT, many cells expressed both Nkx2.5 and MyoD, suggesting that these cells have a true “hybrid” phenotype that does not simply arise from skeletal muscle contamination of cardiac cell cultures or vice versa. We did not observe any MyoD positive cells that did not express Nkx2.5 in iPS-EMT, demonstrating that MyoD expression is not the result of skeletal muscle contamination of iPS cultures. 15% of Nkx2.5 expressing cells in MDSC-EMT did not express MyoD, which may be related to the downregulation of MyoD at the transcriptional level (Figure 3-6B). While it is tempting to speculate that these cells may be differentiating towards a more cardiac-like lineage, further studies are required to determine the final fate of these cells. A fraction of cells in MDSC-EMT expressed only MyoD, which was not observed in iPS-EMT. This fraction may contribute to some of the functional differences that were observed between MDSC and iPS-EMT.





**Figure 3-6. Muscle Transcription Factor Expression in EMT.** (a) Expression of Nkx2.5 and MyoD in MDSC-EMT and iPS-EMT. Both MDSC-EMT and iPS-EMT expressed Nkx2.5 (green) and MyoD (red) transcription factors at the protein level. Scale bar indicates 20 $\mu$ m. (b) Distribution of Nkx2.5 and MyoD positive cells in EMT. The majority of cells expressed both Nkx2.5 and MyoD. DN denotes double negative cells. \*P<0.05 by Chi-square test.

### 3.3.3 Contractile properties of MDSC-EMT and iPS-EMT

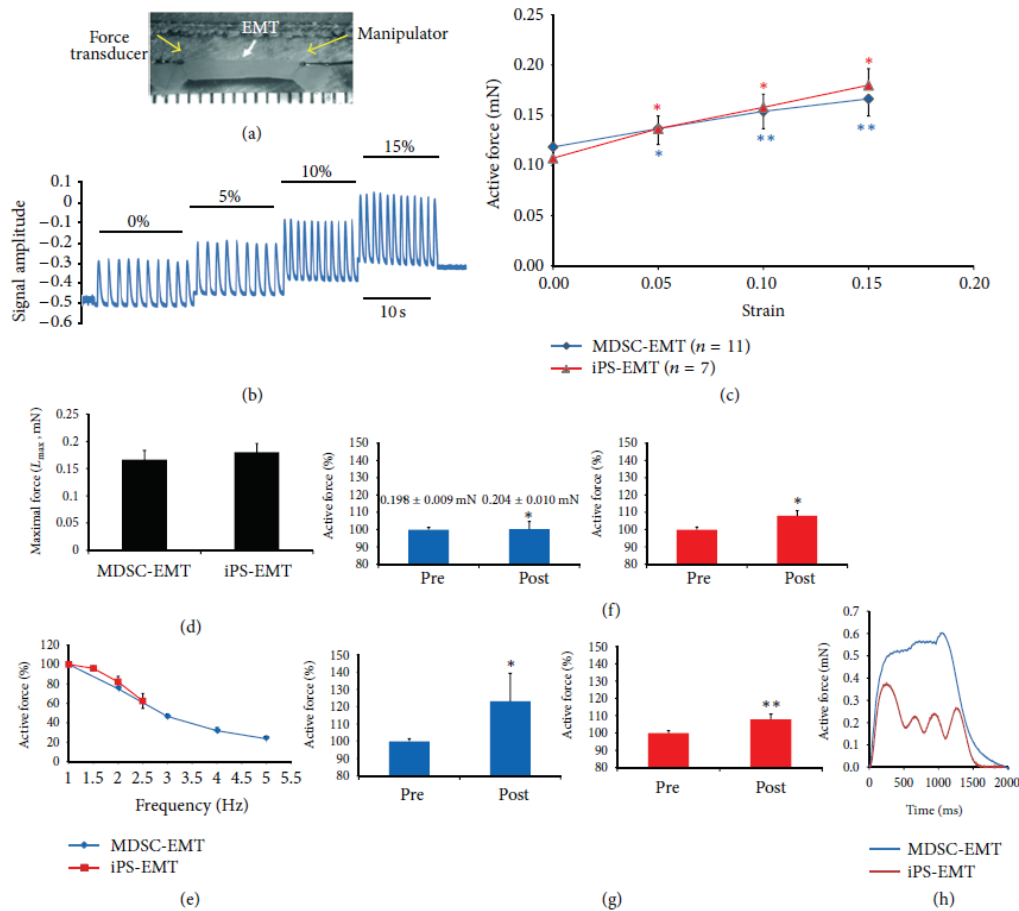
Both MDSC-EMT and iPS-EMT exhibited spontaneous beating activity. Single spontaneously beating cells were observed in MDSC-EMT as early as culture day 4, and spontaneous beating at the tissue level was observed by day 7 and sustained throughout the 14 day culture period. iPS-EMT exhibited spontaneous beating at day 3 which was also maintained for the duration of culture. EMT was attached to a force transducer in order to measure contractile force (Figure 3-

7A). In response to field stimulation, both MDSC-EMT and iPS-EMT demonstrated the ability to generate contractile force. Both MDSC-EMT (n=11) and iPS-EMT (n=7) exhibited a Frank-Starling relationship, displaying a proportional increase in force with increasing construct length (Figure 3-7B, Figure 3-7C). Some reports have stated that the force-strain relation is steeper in cardiac vs. skeletal muscle [139], while others have found them to be similar [140]. Nonetheless, we have found that both share the same fundamental relationship with a similar slope. A greater steepness in cardiac muscle may manifest itself in mature tissues. iPS-EMT ( $0.179 \pm 0.016$  mN, n=7) and MDSC-EMT ( $0.166 \pm 0.017$  mN, n=11) exhibited similar contractile force at maximal length,  $L_{\max}$  (Figure 3-7D). Both MDSC-EMT (n=11) and iPS-EMT (n=7) displayed negative force-frequency relationships in the range of 1 to 5 Hz stimulation rates (Figure 3-7E). iPS-EMT failed to respond above stimulation rates of 2.5Hz, instead maintaining its own spontaneous cycling frequency. In response to non-selective  $\beta$ -adrenergic receptor agonist isoproterenol, iPS-EMT ( $107.91 \pm 3.07\%$ , n=7,  $P < 0.05$ ) and MDSC-EMT ( $101.44 \pm 3.19\%$ , n=8,  $P < 0.05$ ) showed a modest but significant increase in contractile force (Figure 3-7F). MDSC-EMT ( $123.05 \pm 16.18\%$ , n=11,  $P < 0.05$ ) and iPS-EMT ( $110.99 \pm 5.76\%$ , n=7,  $P < 0.001$ ) showed a significant increase in force in response to increased extracellular calcium ion (Figure 3-7G), suggesting that force generation is sensitive to extracellular calcium influx.

The negative force-frequency relationship is indicative of immature excitation-contraction coupling. Skeletal muscle can respond to adrenergic stimulation with increased inotropy [141], particularly under fatigued conditions [142]. However, under normal conditions, the response of skeletal muscle is small [143] but similar to early embryonic tissue [144]. On the other hand, the cardiac contractile response to ISP is much greater in later stages in cardiac muscle [144]. From this, we can infer that immature cardiac and skeletal muscle both respond to

ISP with modest positive inotropy. The extracellular calcium challenge is designed to test the acute response of the EMT to calcium influx. Addition of calcium leads to a transient increase in force [145] as calcium ions enter through cell membrane-bound calcium channels. Cardiac and skeletal muscle share isoforms of these calcium handling proteins during early myogenesis [146-148], and expression of certain proteins, particularly SERCA, is weak in early embryogenesis and increases during development. As a result, contraction is more dependent on extracellular calcium entry during the early phases of both cardiac and skeletal myogenesis [149-151]. Although we did not examine the expression of calcium handling proteins in this study, the acute extracellular calcium response suggests that the ability of myocytes within EMT to regulate cytosolic calcium is still immature and similar to embryonic or fetal myocytes [144]. While mature cardiac or skeletal muscle can also respond to extracellular calcium, the small inotropic response to ISP relative to the greater response to calcium demonstrate that beta adrenergic receptor signaling and calcium cycling are not yet fully developed. Taken together with the negative force frequency relationship, these contractile responses to pharmacological and electrical stimulation indicate that EMT has contractile properties of immature striated muscle.

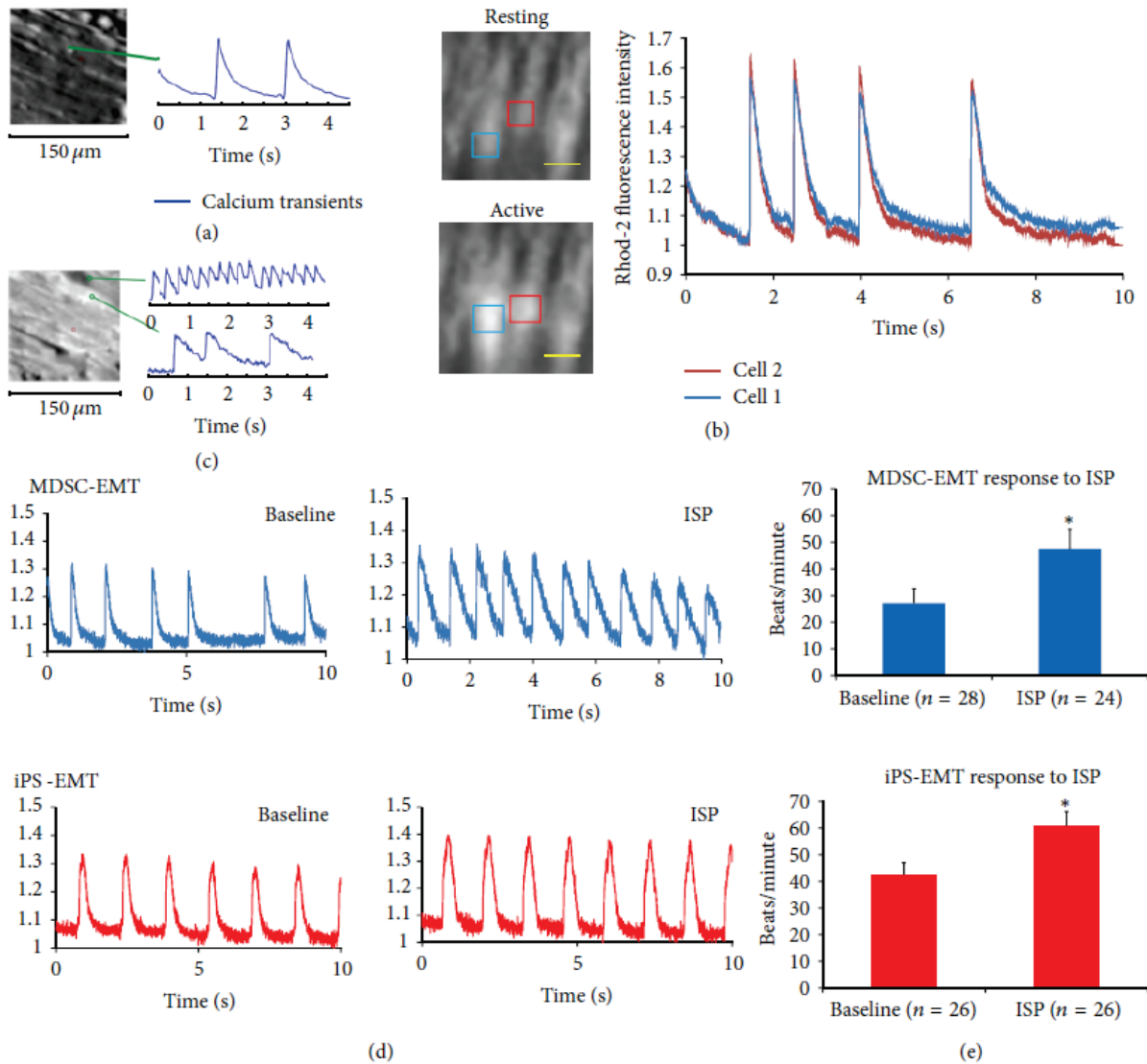
To further investigate the dependence of contraction on extracellular calcium, we treated EMTs with Cadmium Chloride (1 mM), a calcium channel blocker. After 5 minutes, contraction was significantly suppressed. The majority of MDSC-EMT and iPS-EMT constructs stopped contracting by visual inspection under a light microscope. (Data not shown.) This behavior does not occur in mature skeletal muscle [68]. Train stimulation at 20Hz elicited a tetanic response from MDSC-EMT, while it did not in iPS-EMT (Figure 3-7H). In general, tetanus cannot be triggered in cardiomyocytes due to the unique electrical properties of myocardium, although it is possible under certain conditions [152, 153].



**Figure 3-7. Biomechanical testing of EMT.** (a) EMTs were mounted on a customized mechanical testing station using monofilament nylon sutures at the points indicated by the yellow arrows. (b) Representative contractile force traces of EMT at 0, 5, 10, and 15% strain. (c) Active force-strain relations of culture day 14 MDSC-EMT and iPS-EMT at 0 to 0.15 strain. (d) Active force increased in response to increased strain (\* $P < 0.05$ , \*\* $P < 0.001$  versus baseline, ANOVA). (d) Active force at maximal strain, (15%). (e) Force frequency relationship of EMT. Culture day 14 MDSC-EMT and iPS-EMT electrically field stimulated at rates of 1 to 5 Hz at 100V and 5ms duration. EMTs showed decreased active force at increasing pacing rates. (f) Contractile response of EMT to ISP. Culture day 14 MDSC-EMT (blue) and iPS-EMT (red) were treated with  $1\mu\text{M}$  ISP for 3 minutes and showed increased active force \* $P < 0.05$  versus pretreatment. (g) Contractile response of EMT to extracellular calcium. Culture day 14 MDSC-EMT (blue) and iPS-EMT (red) were treated with  $2\text{mM}$   $\text{CaCl}_2$  for 3 minutes and showed increased active force. \* $P < 0.05$  versus pretreatment. \*\* $P < 0.001$  versus pretreatment. (h) Representative train stimulation of MDSC-EMT and iPS-EMT.

### 3.3.4 Intracellular Calcium Ion Transients

To examine the electrophysiological properties of MDSC-EMT and iPS-EMT, we recorded intracellular calcium ion transients from culture day 14 EMTs. Transients were recorded using calcium indicator dye Rhod2-AM during spontaneous beating or under electrical pacing (1Hz, 20V, 5ms). EMTs exhibited spontaneous calcium transients and responded to electrical pacing (Figure 3-8A). We noted that while iPS-EMT maintained steadier, rhythmic contractions (Figure 3-8E) compared to MDSC-EMT, MDSC-EMT contained a mixture of cells whose transients were synchronized (Figure 3-8B) and not fully synchronized (Figure 3-8C) with surrounding cells within the same engineered muscle tissue. These responses indicate immature calcium handling. Positive chronotropic effects of ISP on calcium transients of randomly selected cells within each EMT were observed in both EMTs (Figure 3-8D, Figure 3-8E). We observed an increase in the frequency of calcium transients in both MDSC-EMT ( $P<0.05$ ) and iPS-EMT ( $P<0.05$ ).



**Figure 3-8. Intracellular Calcium Transients of EMT.** (a) MDSC-EMT calcium transient under electric pacing at 1Hz, 20V, 5ms. (b) Synchronized calcium transients at rest (top) and during spontaneous beating (bottom) of cells within MDSC-EMT. (c) Unsynchronized spontaneous calcium transients in MDSC-EMT. Scale bar indicates 50µm. (d) Representative calcium transients of MDSC-EMT (blue) and iPS-EMT (red) at baseline and after addition of 1µM ISP. (e) Chronotropic effect of ISP on calcium transient activity of EMT. Treatment of culture day 14 MDSC-EMT and iPS-EMT with 1µM ISP increased spontaneous calcium transient activity. \*P<0.05 versus baseline.

### 3.4 DISCUSSION

The widespread burden of cardiovascular disease has prompted the investigation of cell sources for cellular cardiomyoplasty. Among these, cells derived from skeletal muscle are an attractive source for cells. Unlike myocardium, which has limited regenerative ability, MDSCs have a robust potential for self-renewal as well as tolerance for hypoxic conditions and ability to reduce fibrosis and stimulate angiogenesis. In addition, unlike most other cell sources, they are naturally disposed to contractility. Although there are fundamental differences between mature cardiac and skeletal muscle (gap junction coupling, degree of multinucleation, morphology), there are multiple lines of evidence that support strong similarities between cardiac and skeletal muscle during development, which makes skeletal muscle a compelling source of stem cells. The Mef2 family of genes regulates many genes related to cardiac and skeletal muscle. Mef2c is first expressed in cardiac mesoderm and is used as an early marker of the cardiac lineage [154], but it is also expressed in MDSCs. Cardiac isoforms of Tn-T (cTn-T) are present in fetal skeletal muscle but are not expressed in healthy adult skeletal muscle [155]. Cardiac and skeletal actin are co-expressed in developing cardiac and skeletal muscle [156]. Skeletal muscle specific troponins are expressed in the developing heart [38, 157]. Cardiac-like excitation-coupling mechanisms are present in early skeletal myogenesis, while the “skeletal” excitation-coupling mechanisms dominate in more mature skeletal muscle [39]. Among the various cell types, which have been investigated for cardiac cell therapy, several studies have shown that cells isolated from skeletal muscle can transdifferentiate into a CM-like phenotype [49, 68] or through fusion events. There have been several previous reports of CM differentiation from rodent MDSCs, including those from our lab [49, 67, 68, 158]. To our knowledge, cardiomyocyte differentiation from human skeletal muscle derived stem cells has only been reported once [159]. Invernici and

colleagues reported that retinoic acid induces cardiomyocyte differentiation in MDSCs. However, retinoic acid also enhances skeletal muscle differentiation in skeletal myoblasts [160], embryonic stem cells [161], and rhabdomyosarcoma cells [162]. While retinoic acid has been shown to accelerate CM differentiation [163], other studies have shown that it may even play an inhibitory role in cardiac specification [164]. Invernici et al. did not examine the potential for concurrent skeletal muscle differentiation. It is possible that skeletal muscle differentiation overlaps with cardiomyogenic differentiation, as we have reported here. An interesting study by Crippa et al. showed that MyoD expression in the heart is regulated by miR-669 [103], and ablation of this microRNA causes cardiac progenitor cells to differentiate into skeletal muscle. Taken together, these studies show that (1) developing cardiac and skeletal muscle share many similarities and (2) stem cells from skeletal muscle can differentiate into a cardiac-like phenotype and vice versa. However, the underlying mechanisms remain poorly understood. The current study was motivated by the need to develop a better model to study this phenomenon.

Recently, iPS cell derived cardiac cells have garnered interest as a source of cells to repopulate damaged myocardium. However, issues such as teratogenicity and viral-based genome modification limit clinical translation of this technology. We recently showed that developing cardiac and skeletal muscle share expression of cTn-I and sk-fMHC and major transcription factors [35]. To date, the question of whether engineered tissues created *in vitro* also exhibit this hybrid cardiac-skeletal phenotype has not been explored in detail. Gaining a better understanding of the nature of human EMT in the broader context of skeletal and cardiac muscle development, rather than exploring each one exclusively, may provide key insights towards advancing current cell therapies. In order to further probe this question, we studied EMT constructed using human iPS cell derived cardiac cells and human MDSCs.



Our strategy for differentiation of human MDSCs builds on our previous work with differentiation of rodent MDSCs [67]. The combination of low-serum conditions, cell aggregate formation, and 3-D culture enhanced cardiac or skeletal muscle-specific gene expression and contractile properties in a way that better represents native cardiac or skeletal muscle tissue. This strategy, namely using 3D culture, is analogous, though not identical, to use of embryoid bodies (EB) to facilitate cardiac or skeletal muscle differentiation from pluripotent stem cells such as embryonic or iPS cells. It is worth noting that this strategy (EB formation) can be used to obtain both cardiac and skeletal muscle cells from pluripotent stem cells [165]. We found that both cardiac and skeletal muscle specific genes were upregulated during MDSC-EMT differentiation and iPS-EMT differentiation. This mirrors the co-expression of cardiac and skeletal muscle transcription factors that occurs during development of both muscle types, which become divergent in mature cardiac and skeletal muscle. The expression of skeletal muscle transcription factors in iPS cell derived CMs is interesting, since these transcription factors are generally considered to be skeletal muscle specific and not present in cardiac muscle [166]. The role that these transcription factors play in cardiac cells and their potential relation to the fetal gene program in heart failure remain to be investigated. We also found that most iPS cell-derived cardiomyocytes and MDSCs (>50%) co-expressed Nkx2.5 and MyoD protein, suggesting that these cells appear to be a hybrid phenotype of skeletal and cardiac muscle. The presence of MyoD in iPS-cell derived cardiomyocytes is interesting. There have been a few reports of MyoD expression in the hearts of adult, developing, or diseased organisms [103, 167, 168], but the prevailing consensus is that MyoD is specific to skeletal muscle [15, 169]. However, these reports are based on animal studies, and we are not aware of any reports of MyoD expression in the human fetal heart, either positive or negative. Thus, while there are numerous biochemical

and functional similarities between engineered and native tissue, there may be more subtle differences between in vitro stem cell differentiation and developmental courses seen in native embryonic tissue. It is also possible that human heart development differs from other species in some respects. While fibroblasts can be programmed to cardiomyocyte-like cells by 3 factors (GMT) in mice, generation of cardiomyocyte-like cells from human fibroblasts required a total of 7 factors to achieve a similar level of reprogramming [167]. This study highlights the as of yet poorly understood differences between cardiovascular development in humans and animals, which are important for translating basic research towards clinical therapies. Therefore, studies using human cells are important to complement the results of animal models.

While there were differences in expression of cardiac and skeletal muscle genes between iPS-EMT and MDSC-EMT, gene expression tells only one part of a much bigger story. Fibroblasts can be directly reprogrammed into cardiomyocytes by constitutive overexpression of 3 cardiac transcription factors (GATA4, Tbx5, and Mef2c) [15]. Yet, only a small subset of these transduced cells develops into functioning cardiomyocytes despite high levels of expression of transduced genes. In this case, it is possible that more is not necessarily better. Generation of functional cells requires an overall balance of gene expression in the context of the source cell, desired target cell, and posttranscriptional factors. It has been suggested that ratios of selected genes rather than their absolute levels allows them to contribute to the development of functional cellular phenotypes [169]. In accordance with this study, the cardiac transcription factors Nkx2.5, GATA4, and Tbx5 are expressed in a ratio of approximately 1:1:1 in both iPS-EMT and MDSC-EMT. This balance may contribute to the cardiac-like phenotypes that were observed in both EMTs.

These trends also manifest themselves histologically. We found that cTn-I and sk-fMHC are co-expressed by both MDSC-EMT and iPS-EMT, similar to developing cardiac and skeletal muscle [35]. Both EMT types also express cTn-T and Cx-43. A noteworthy difference is that the expression of Cx-43 in MDSC-EMTs at the cell-cell interface as discrete gap junctions is less pronounced compared to iPS-EMT. However, we did observe synchronous calcium transients between neighboring myocytes in MDSC-EMT, suggesting the potential for electrical coupling. Gap junction coupling is an important requirement for synchronized contraction between myocytes, and lack of electromechanical integration was identified as a major hurdle for the use of skeletal myoblasts for myocardial repair [170]. While cells in MDSC-EMT did not form gap junctions to the same extent as iPS-EMT during the culture duration in the current study, connexin-43 gap junction formation is upregulated prior to myoblast alignment, and overexpression of connexin-43 in myoblasts [171-173] and mechanical preconditioning [174, 175] have been shown to enhance electrical coupling with cardiomyocytes. Thus, strategies to improve cell-cell coupling in skeletal muscle cells are ongoing.

Both MDSC-EMT and iPS-EMT demonstrated a chronotropic response to ISP similar to cardiac tissue and distinct from mature skeletal muscle [68]. Both MDSC-EMT and iPS-EMT exhibit positive force-strain (Frank-Starling) and negative force-frequency relationships, which are indicative of immature excitation-contraction coupling. Taken together, our functional assessments show that MDSC-EMT and iPS-EMT mimic immature or developing striated muscle.

A number of studies have shown that a mixture of cell types is optimal for efficient CM differentiation and tissue formation [176]. Our flow cytometry data indicates that MDSC is a predominantly myogenic-mesenchymal-like population with smaller numbers of skeletal

myoblasts, myogenic-endothelial cells, pericytes, and endothelial cells [136]. We also show expression of smooth muscle ( $\alpha$ -SMA) and CD31 in the construct. iPS-EMT also contains a mixture of cardiomyocytes, smooth muscle, and endothelial cells [131]. Thus, both tissue types contain a naturally heterogeneous mixture of cells which supports efficient muscle differentiation and tissue formation. The role that these heterotypic cell interactions play in striated muscle development and growth requires further investigation.

There are a number of limitations to the current study. Our study compared the properties of two types of artificial human striated muscle and showed that both share cardiac and skeletal muscle proteins/genes and functional properties. We did not have access to native human tissues for this study, so it remains to be seen if these properties are also present in either developing or mature human tissues. However, our findings are consistent with previous *in vitro* and animal studies, and we believe these findings provide new insight into previously unknown properties of engineered tissues. Given our previous findings, further studies are needed to better understand the role of the “hybrid” phenotype in cell fate decisions. Our study also calls into question the specificity of commonly used cardiac and skeletal muscle markers. When evaluating cell differentiation, the possibility of differentiation into other, closely related cell types should also be considered. This also adds complexity to evaluating cardiac and skeletal muscle differentiation. Differentiated myocytes likely express a combination of cardiac and skeletal muscle specific proteins. Post-transcriptional and epigenetic factors may be an important key in generating more functional CM-like cells. Finally, we identified both muscle and non-muscle cell types within each EMT as part of a heterogeneous population. Whether differentiated muscle cells arise from a single subpopulation or multiple subpopulations is unclear, and the role of these cell interactions in cell differentiation and tissue formation requires further investigation.

### 3.5 CONCLUSION

In summary, we have shown that MDSC-EMT and iPS-EMT share major cardiac and skeletal muscle specific gene and structural protein expression patterns and highlighted their functional similarities/differences. These 3D muscle tissue models recapitulate elements of developing cardiac and skeletal muscle. While the optimization of cell sources for cardiomyoplasty remains an area of ongoing work in the field, our results show that EMT mimics developing cardiac and skeletal muscle and can serve as a versatile *in vitro* model to study and better understand cell differentiation and tissue development as a functional human engineered muscle towards the long-term goal of cellular cardiomyoplasty.

### 3.6 ACKNOWLEDGEMENTS

This study was supported by NIH R21 HL094402 (K.T.), Commonwealth of Pennsylvania Grant (K.T.), NIH Cardiovascular Bioengineering Training Program T32 HL076124 (J.T.), AHA Pre-doctoral Fellowship 13PRE15030000 (J.T.), University of Pittsburgh start-up (L.Y.), and AHA SDG Grant 11SDG5580002 (L.Y.). We would like to thank Alison Logar for assistance with the Flow Cytometry experiments.

**Table 3-2. List of Abbreviations Used in Chapter 3**

**Abbreviations**

CM:	Cardiomyocyte
MDSC:	Muscle derived stem cell
EMT:	Engineered muscle tissue
iPS cell:	Induced pluripotent stem cell
ISP:	Isoproterenol
Cx-43:	Connexin-43
cTn-I:	Cardiac specific troponin I
cTn-T:	Cardiac specific troponin T
Sk-fMHC:	Fast skeletal myosin heavy chain
$\alpha$ -SMA:	Alpha smooth muscle actin.

## **4.0 CARDIOMYOCYTE DIFFERENTIATION FROM HUMAN MUSCLE DERIVED STEM CELLS BY TEMPORAL MODULATION OF SOLUBLE AND BIOPHYSICAL FACTORS**

### **4.1 INTRODUCTION**

In chapter 2, we saw that developing cardiac and skeletal muscle share major transcription factors and structural proteins during development [35]. In chapter 3, we extended these findings and found that in vitro-based 3D engineered muscle tissues made using cardiac (iPS cardiac progenitors) and skeletal muscle derived stem cells (MDSC) also share these biochemical properties and are also functionally similar [177]. Keeping in mind the risks associated with native cardiac stem cell isolation and the untested nature of iPS cells for human cell therapy, MDSCs remain an important candidate cell source for replacing damaged myocardial tissue. However, in order to achieve more positive therapeutic outcomes, the transplanted cells/tissues should resemble those which they are intended to replace. Following this biomimetic approach, MDSCs should be induced to differentiate into cells which are similar to cardiomyocytes (CM). Unfortunately, significant knowledge gaps still exist regarding how stem cells are regulated at the genetic and epigenetic levels and particularly how the cardiac and skeletal myogenic programs are tied to one another. The goal of the current study is to integrate soluble and biophysical factors which are known to promote cardiomyogenesis in order to improve the existing EMT protocol. To that end, we have introduced a combination of biophysical and soluble factor-based stimuli in a controlled manner in order to provide an integrated approach for

induction of MDSCs into more functionally competent CM-like cells, which are capable of contraction and intercellular integration. We have identified a sequential treatment of four soluble factors, which, combined with optimized biophysical conditions, produced cardiac-like tissue capable of coordinated contractions, improved force generation properties, and better response to isoproterenol (ISP). While iPS cells are triggered to differentiate by similar signals, the use of MDSCs bypasses a pluripotent state and eliminates the need for viral reprogramming.

#### 4.1.1 **miR-206 Inhibitor**

MicroRNAs (miRNAs) are small, non-coding RNAs averaging 22 nucleotides in length. They regulate gene expression at the transcriptional and post-transcriptional levels. While many miRNAs are ubiquitously expressed, others are tissue specific. Several miRNAs, including miR-1, -133, -and -206, have been shown to be highly enriched in striated muscle and are termed myoMIRs [178]. Subsequent studies confirmed that miR-206 is primarily expressed in skeletal muscle. It plays a role in somite development, myogenesis, and fiber type specification [100]. One of the experimentally verified downstream targets of miR-206 is connexin-43 (cx-43) [179]. In the adult heart, cx-43 is the predominant gap junction protein in ventricular CMs [180] and is responsible for proper electrical propagation. In skeletal muscle, cx-43 is required for proper myoblast alignment and fusion, after which cx-43 is rapidly downregulated [179]. It is believed that abnormal, prolonged expression of cx-43 in skeletal muscle can interfere with neuromuscular junction function [100]. However, expression of cx-43 is desirable for stem cells to be used in the heart. The miR-206 inhibitor is a chemically modified, single stranded RNA that specifically binds to endogenous miR-206 and inhibits its activity. By inhibiting its activity,



cx-43 gap junctions can be preserved and skeletal muscle differentiation and fusion could be abrogated in favor of a more cardiac-like phenotype.

#### 4.1.2 **Lithium Chloride**

Lithium Chloride (LiCl) is an ionic compound with high water solubility. Lithium compounds are commonly used as psychiatric medication [181], suggesting that they can be safely used to treat cells in vitro. LiCl can activate Wnt signaling by inhibiting GSK-3 $\beta$  [182]. This allows for the accumulation of  $\beta$ -catenin and the activation of  $\beta$ -catenin dependent genes, including cx-43. LiCl treatment has been shown to enhance cx-43 gap junction expression in skeletal myoblasts [183] and differentiating ES-cell derived CMs [184]. GSK-3 $\beta$  is also involved in other signaling pathways, so it may also have effects in addition to Wnt activation.

#### 4.1.3 **Inhibitor of Wnt Response-1 (IWR-1)**

IWR-1 is a small molecule that acts as a Wnt pathway inhibitor via interaction with Axin [185]. The Wnt pathway plays a critical role during cardiac differentiation. Temporal control of Wnt signaling by small molecule agonists and antagonists produces highly pure cultures of CMs from human pluripotent stem cells [186]. Wnt signaling is active during early cardiac differentiation and later suppressed during commitment of early mesoderm to cardiac mesoderm in directed pluripotent stem cell differentiation protocols [187] and in the embryo [188]. There is also evidence that Wnt inhibition can prevent hypertrophic myotube formation in skeletal muscle cells [189]. We reasoned that since MDSCs are already derived from adult mesoderm tissue, inhibition of Wnt at the onset of differentiation rather than later stages would aid in cardiac specification.

#### 4.1.4 **Bone Morphogenic Protein-4 (BMP-4)**

Bone morphogenic protein-4, BMP-4, is a cytokine of the TGF- $\beta$  family that is involved in cardiac differentiation. BMP-4 can activate cardiac genes in combination with Wnt signals at the expense of other lineages such as skeletal muscle in mesenchymal stem cells [138]. The combination of small molecule Wnt inhibitors with BMP-4 can synergistically induce cardiac differentiation in iPS cells [190]. It has also been shown that Wnt and BMP signals activate distinct sets of cardiac transcription factors [191], suggesting that both are required for efficient cardiac differentiation. BMP signals also have a role in regulating cx-43 expression in osteoblasts [192], chondrocytes [193], and neurons [194]. Although it has not been experimentally verified, this suggests that BMP may also have a role in gap junction regulation in muscle. Appropriate timing and dosage are also important, since these signals can have stage-dependent effects [195]. For example, higher dosages [196] or sustained treatment [197] can push cells toward the osteogenic lineage.

#### 4.1.5 **Biophysical Aggregation**

Cell aggregate formation is important for CM differentiation from stem cells. ES cells are routinely aggregated into embryoid bodies and cultured in suspension using cardiac differentiation protocols for pluripotent stem cells [198]. It is believed that aggregate culture of stem cells recapitulates important physical aspects of embryonic development such as cell-cell interactions and can mediate processes including differentiation, proliferation, and apoptosis. For example, aggregate formation kinetics can affect  $\beta$ -catenin signaling and subsequent differentiation through cadherin junctions [199]. Rotary orbital suspension culture is a common method to generate stem cell aggregates. In addition to cell-cell contacts, this method also

introduces hydrodynamic shear, which can also alter gene expression [200]. Muscle derived stem cells cultured as 3D aggregates, termed myospheres, also display electrophysiological properties similar to CMs, which are modulated by culture conditions [201]. Aggregate culture can also significantly reduce proliferation [67], and affect apoptosis [202]. In light of these multivariate effects, aggregate culture conditions should be carefully selected to achieve the desired outcome. In the current study, we would that short-term aggregate culture for several hours proved superior to longer (24 hour) culture periods for generating functional tissue.

Thus, the objective of the present study is to develop an advanced culture method that induces CM differentiation from human MDSCs at the tissue level in vitro. We tested the hypothesis that timed soluble factor and cell aggregate formation in conjunction with 3D culture could induce MDSCs to differentiate into CM-like cells as a functioning cardiac-like 3D tissue. While the full reconstitution of lost myocardial tissue by MDSCs, or any stem cell, remains more of an ideal than an attainable goal, our findings represent a stepwise advancement over existing cell therapy modalities.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 Cell Culture**

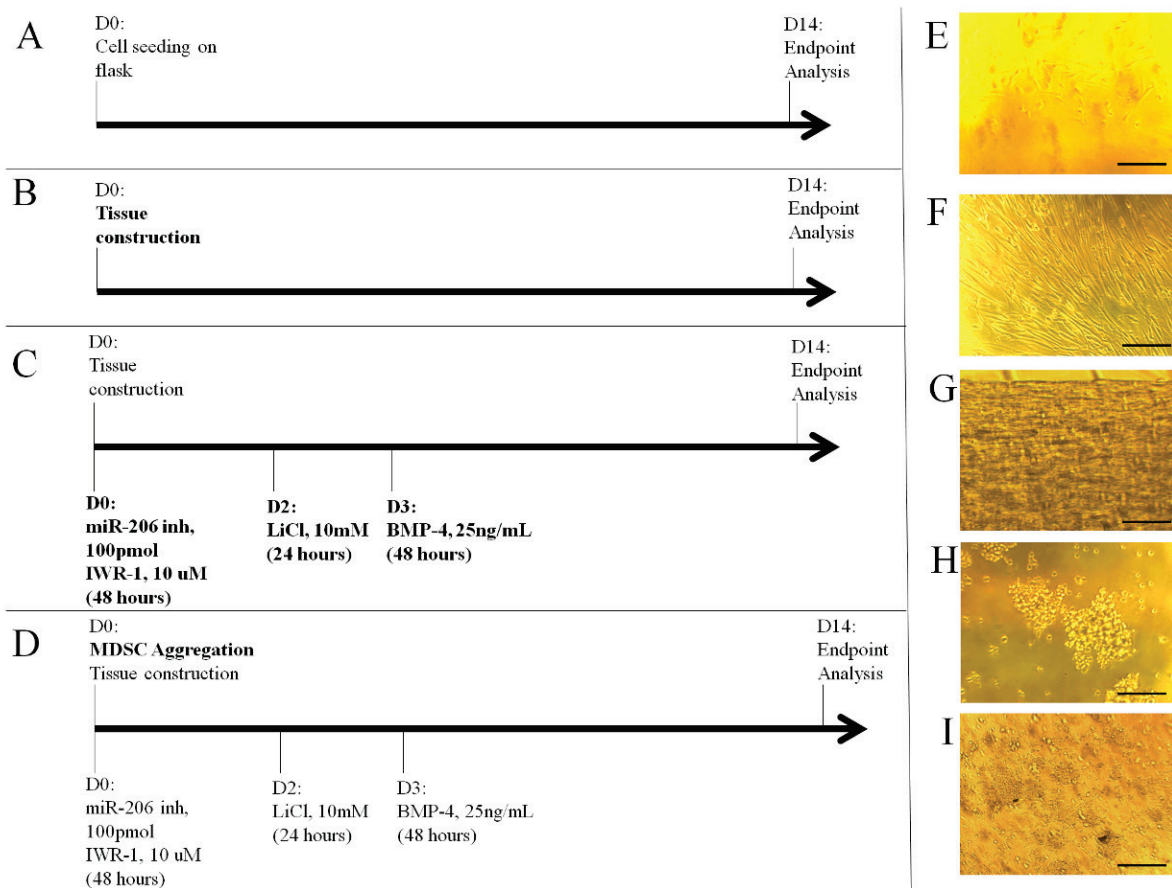
MDSCs of 3 different human subjects (from 10 to 30 years old) were purchased from Cook Myosite, Inc. Cells were cultured in Cook Basal Media (Cook Myosite, Inc., Pittsburgh, PA, USA) supplemented with 10% Growth Supplement (Cook Myosite, Inc., Pittsburgh, PA, USA) and 1% Antibiotic-Antimycotic solution (AAS, Invitrogen) on collagen-I coated flasks. Cells

were used for no more than 4 passages and maintained below 50% confluence prior to initiation of differentiation.

#### 4.2.2 Engineered Muscle Tissue (EMT) Construction and MDSC Differentiation

Undifferentiated MDSCs underwent 4 different differentiation protocols. In the 2D differentiation protocol (2D, Figure 4-1A), undifferentiated MDSCs (Figure 4-1E) were plated at high density (40000 cells/cm<sup>2</sup>) and exposed to differentiation media (DM) consisting of Cook Basal Media supplemented with 5% growth supplement, 1% AAS, and human FGF-2 (5ng/mL, Sigma) for 14 days (Figure 4-1F). In the 3-dimensional engineered muscle tissue protocol (EMT, Figure 4-2B), MDSCs were cultured in DM within the 3D engineered muscle tissue (Figure 4-2G). To construct EMT, Liquid rat tail collagen type I (3 mg/mL, Invitrogen) was neutralized with 0.1N NaOH and mixed with Matrigel (BD Bioscience) with a collagen: Matrigel ratio of 0.8 and a final collagen concentration of 0.67 mg/mL. Trypsinized MDSCs were seeded in a collagen/Matrigel mixture at a density of 0.5 million cells per construct using a Flexcell Tissue Train Culture system (FX-4000, Flexcell International) with a total volume of approximately 200  $\mu$ L per construct to form a linear shaped construct. In the 4 factor engineered muscle tissue protocol (4F-EMT, Figure 4-1C), EMT was constructed as described, cultured in DM, and treated with 4 chemical factors: miR-206 inhibitor (100pmol, Life Technologies), IWR-1 (10uM, Sigma), LiCl (10mM, Sigma), and BMP-4 (25ng/ml, Prospec-Bio) at different time points as illustrated in Figure 4-1C. For transfection of miR-206 inhibitor, X-treme Gene siRNA transfection reagent (Roche) was used according to the manufacturer's instructions. Using 100pmol/construct, the majority of cells were transfected (~80% by Cy3 labeled small RNA, Supplementary Figure 4-8B). In the MDSC aggregate 4 factor engineered muscle tissue protocol

(4F-AEMT, Figure 4-1D), MDSCs were trypsinized, and MDSC-cell aggregates were obtained by rotation culture (Labnet Orbit 1000) on a suspension culture plate at 50 rpm for 4 hours prior to tissue construction (Figure 4-1H) [67]. Aggregates were mixed with collagen and matrix factors (Figure 4-1I) to form a construct as previously described and treated with 4 chemical factors as illustrated in Figure 4-1D. 14 days was used as the end point for all protocols, and the media was changed every other day.



**Figure 4-1. Differentiation of human MDSC.** (A) **2D**: MDSCs are seeded on a tissue culture flask at high density and allowed to differentiate. (B) **EMT**: MDSCs are cultured within 3D engineered muscle tissue. (C) **4F-EMT**: MDSCs are cultured in 3D engineered muscle tissue and treated with 4 chemical factors. (D) **4F-AEMT**: MDSC

aggregates are cultured in 3D engineered muscle tissue and treated with 4 chemical factors. (E) Undifferentiated MDSCs on tissue culture flask. (F) 2D Differentiated MDSCs at day 14. (G) MDSCs in EMT. (H) MDSC aggregates after 4 hours of rotary suspension culture. (I) MDSC aggregates in EMT immediately after tissue construction. Scale bar indicates 250  $\mu\text{m}$ .

#### 4.2.3 Real-time Polymerase Chain Reaction

Total RNA was prepared using Trizol solution (Invitrogen) and treated with TURBO DNA-free kit (Ambion, Austin, TX, USA). Primers, whose target genes are Nkx2.5, GATA4, Mef2c, Tbx5, GJA1, Myh6, Myh7, MyoD, myogenin, and  $\beta$ -Actin were obtained from Qiagen Quanti-Tect Primer Assay with the target fragment sizes approximately 100 base pairs. One step RT was performed using Applied Biosystems High Capacity RNA-to-cDNA Kit (Applied Biosystems) with a total of 2  $\mu\text{g}$  RNA in a total volume of 20  $\mu\text{L}$  with the following program: 37°C for 1 hour, 95°C for 5 minutes, 4°C hold. cDNA (1  $\mu\text{L}$ ) was used for RT-PCR using the following program : 50°C for 2 minutes, 95°C for 10 minutes. This was followed by 95°C for 15 seconds and 60°C for 1 minute, repeated for 40 cycles. The final stage was 95°C for 15 seconds, 60°C for 15 seconds. SYBR Green was used as a detector. 3 samples were run for each target gene per group from pooled samples using Applied Biosystems 7900HT system. Relative expression (RQ) was calculated using the *ddCt* method with  $\beta$ -actin as an internal control.

#### 4.2.4 Immunohistochemical Staining

EMTs were fixed using 4% paraformaldehyde/PBS for 30 minutes and embedded in 13% polyacrylamide gel. 150  $\mu\text{m}$  thick sections were made using a vibratory microtome (Vibratome- 1000, Vibratome.com) and stained for cardiac troponin-T (cTn-T, Abcam), alpha

sarcomeric actinin ( $\alpha$ -Actinin, Sigma), connexin-43 (Cx-43, Abcam), active caspase-3 (Abcam), and phospho histone H3 (P-histone3, Abcam) primary antibodies and Alexa Fluor 488 or Alexa Fluor 594 secondary antibodies. Stained samples were scanned using a confocal microscope (Olympus Fluoview FV1000) and used to generate 3D projection images of the tissue sections in ImageJ. Proliferation and apoptosis were assessed by calculating the percentage of total nuclei positive for phospho histone H3 and active caspase-3, respectively. Myotubes were identified as cells containing 5 or more nuclei, since human CMs can contain up to 4 nuclei [203].

#### 4.2.5 Mechanical Testing

The active force of EMTs was measured using a customized setup. Constructs were transferred to a muscle testing station perfused with warmed Ringer solution containing 2mM  $\text{CaCl}_2$ , 135mM NaCl, 4mM KCl, 10mM Trizma-HCl, 8.3 mM Trizma-base, and 11mM glucose. The constructs were attached to a force transducer (403A, Auroura Scientific, Auroura, Canada) using nylon meshes. The other end of each construct was attached to a micromanipulator. Field stimulation was applied using a stimulator (Grass S48 Stimulator, Grass Medical Instruments) at 5ms duration, 100V. The parameters were set at 10% above the threshold required to induce visible contraction of all constructs. The construct length was adjusted from 0% to 15% elongation. Force was measured at stimulation rates of 1-5 Hz and in response to isoproterenol (ISP, 1 $\mu$ M) and extracellular calcium (Calcium Chloride, 5 mM).

#### 4.2.6 Intracellular Calcium Transient Measurement

Samples were loaded for 10 to 15 minutes at 37°C with Rhod 2-AM (Biotium) at a concentration of 5 µg/mL. After the dye was loaded, the samples were placed on the heated stage of a Leica (DM LFSA) microscope. Optical mapping was performed with high spatiotemporal resolution (64x64 pixels, 176 fps) at 37°C using a Hamamatsu EM-CCD camera (Model C9100-12). Calcium transients were recorded during spontaneous beating. Videos were processed in ImageJ (NIH) and analyzed using densitometry techniques. A 3x3 pixel area was selected within each cell. The average pixel intensity within the region was calculated and normalized to the baseline resting value.

#### 4.2.7 Statistical Procedures

One-way ANOVA was used to compare gene expression among experimental groups, proliferation rates, active caspase-3 expression, multinucleation percentages, changes in force in response to isoproterenol, and changes in force in response to extracellular calcium. A two-way repeated measures ANOVA was performed to compare active stress-strain relations. A post-hoc Tukey test was used to determine individual differences between experimental groups. Data were expressed as mean ± standard error. Statistical significance was defined by a value of  $P < 0.05$ . All calculations were performed using Sigma Stat 3.0 software (Systat Software Inc.).

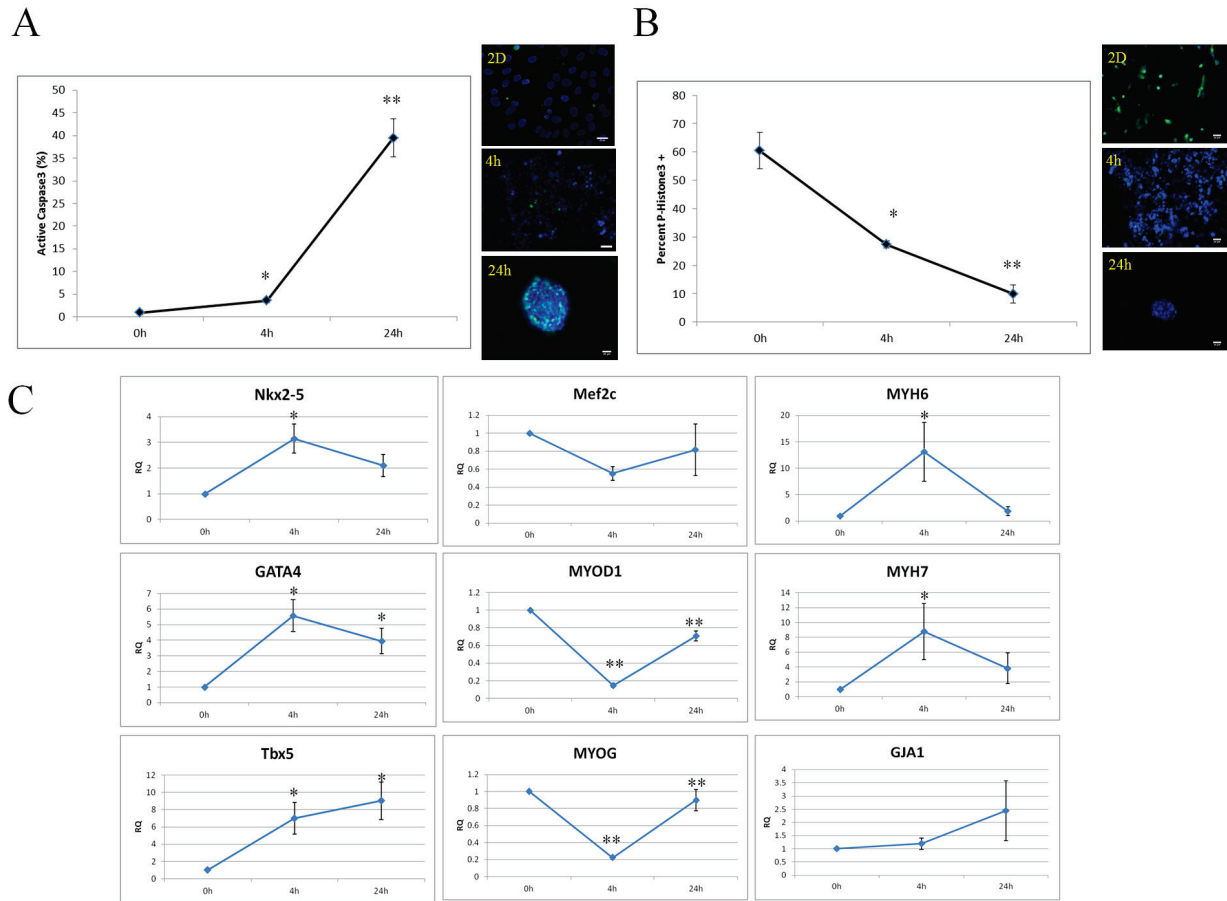


## 4.3 RESULTS

### 4.3.1 Temporal Effects of MDSC Aggregate Formation

First, we examined the effects of MDSC aggregate formation on cell death, proliferation, and differentiation. We noted that active caspase-3 expression increased significantly at 4 hours ( $3.6 \pm 0.4\%$ ,  $n=4$  fields,  $p < 0.05$ ) and 24 hours ( $39.5 \pm 4.2\%$ ,  $n=4$ ,  $p < 0.001$ ) of aggregate formation compared to 0 hours ( $0.9 \pm 0.5\%$ ,  $n=4$ ) indicating that cells were entering an apoptotic fate at longer culture periods. We also examined proliferation by phospho histone H3 expression and noted that proliferation decreased in a time dependent manner at 4 hours ( $27.4 \pm 1.2\%$ ,  $n=3$ ,  $p < 0.05$ ) and 24 hours ( $9.9 \pm 3.3\%$ ,  $n=6$ ,  $p < 0.001$ ) relative to actively proliferating cells on a standard tissue culture surface ( $60.5 \pm 6.5\%$ ,  $n=8$ ). To examine differentiation in aggregates, we looked at muscle specific gene expression at different stages of aggregate formation. Aggregate formation upregulated cardiac transcription factor (Nkx2.5, GATA4, and Tbx5) gene expression ( $n=6$ ). It also upregulated later cardiac differentiation markers Myh6 and Myh7 ( $n=6$ ). Interestingly, skeletal muscle transcription factors MyoD and myogenin were maximally suppressed at 4 hours but began to return to basal level by 24 hours ( $n=6$ ). To determine if MDSCs can differentiate into a muscle phenotype within aggregates, we cultured aggregates for 2 weeks in suspension culture in DM. We found that MDSCs within 2 week old aggregates stained positive for troponin-T (Supplemental Figure 4-8C). However, a striated muscle pattern was not present, and the cells did not beat spontaneously. These results suggest that aggregate formation is important for triggering the switch from proliferation to differentiation and early activation of muscle gene expression. Short term aggregate formation appears to maintain cell viability and favors CM differentiation at the gene level. While MDSC aggregate formation is

important for functional CM differentiation, long-term aggregate culture may not be the optimal environment for MDSCs.



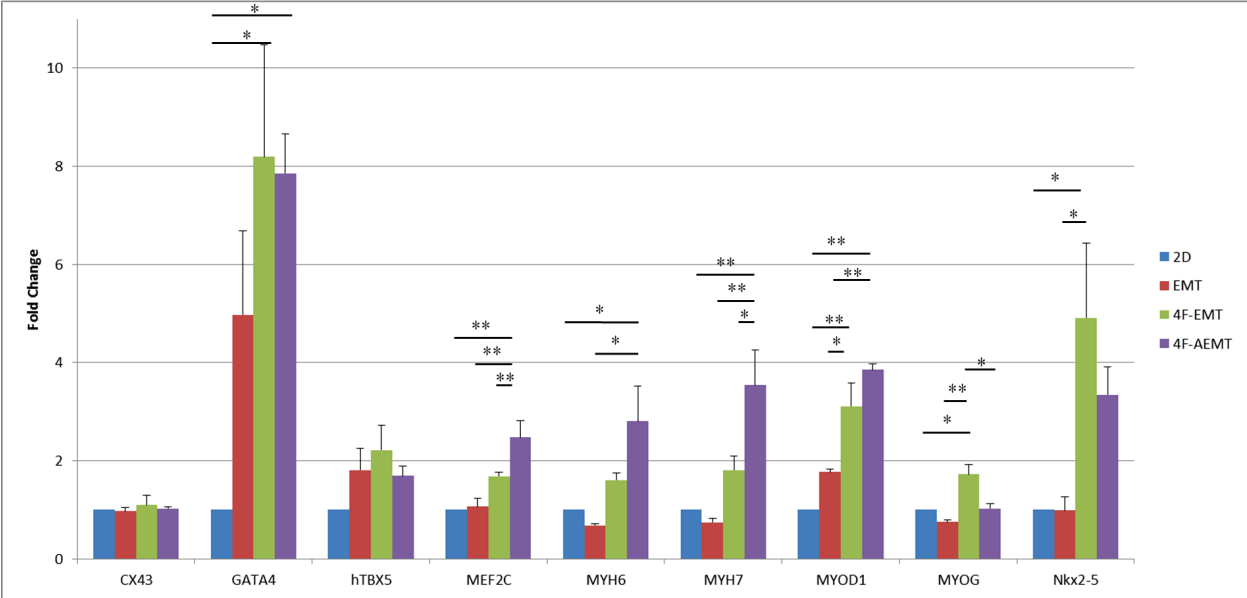
**Figure 4-2. Temporal Effects of Aggregate Formation on MDSCs.** (A) Aggregate formation markedly increased active caspase-3 expression (green) of MDSCs. (B) Aggregate formation significantly decreased MDSC phospho histone H3 (green) expression and proliferation. (C) Aggregate formation altered myocyte-specific gene expression patterns. \*  $P < 0.05$ . \*\*  $P < 0.001$  vs. baseline. Blue indicates nuclear DAPI staining. Scale bar indicates 20  $\mu\text{m}$ .

### 4.3.2 Gene and Protein Expression of EMT

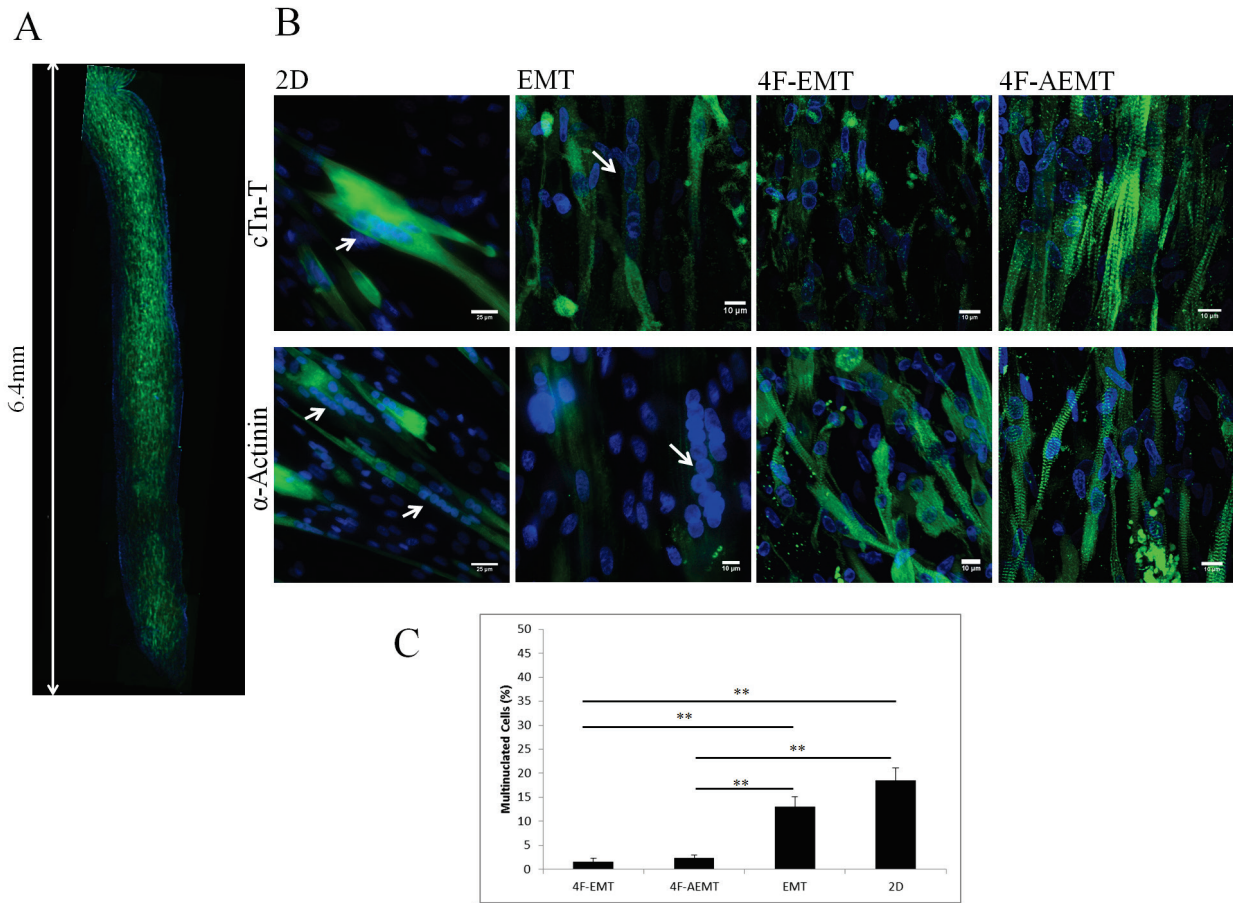
We examined gene expression of MDSCs differentiated under the 4 different protocols. All genes were expressed regardless of the protocol used. 4F-EMT increased expression of cardiac transcription factors Nkx2.5, GATA4, and to a lesser extent, Tbx5 (n=6). Aggregate formation

combined with 4 factor treatment (4F-AEMT) increased Mef2c, Myh6, and Myh7 expression in addition to the aforementioned genes (n=6). Interestingly, MyoD also increased under the 4F-EMT and 4F-AEMT protocols (n=6). Myogenin was also elevated in the 4F-EMT protocol (n=6). Connexin-43 expression remained unchanged across the differentiation protocols (n=6). These data indicate that human MDSCs express both cardiac and skeletal muscle genes, a characteristic shared with iPS cell derived cardiomyocytes and immature developing cardiac and skeletal muscle [35, 177].

Histological assessments confirmed that MDSCs differentiate into a muscle phenotype in EMT (Figure 4-4A). MDSCs expressed  $\alpha$ -actinin and cTn-T, both sarcomeric proteins, in all culture conditions (Figure 4-4B). However, a striated pattern was not visible under 2D differentiation by either  $\alpha$ -actinin or cTn-T staining. MDSCs in 2D also formed elongated fibers containing many (more than 5) nuclei, which resemble myotubes ( $18.5\pm 2.5\%$ , n=18). In EMT, some cells retained the appearance of myotubes ( $13.1\pm 2.1\%$ , n=33). In EMT treated with 4 factors (4F-EMT [ $1.6\pm 0.6\%$ , n=38] and 4F-AEMT [ $2.4\pm 0.7\%$ , n=38]), myotubes were not readily apparent. 4F-AEMT formed a muscle tissue with closely apposed muscle fibers that had a clear striated pattern by cTn-T staining, which was less apparent in the 4F-EMT (no aggregate) group. These findings suggest that a combination of MDSC aggregate formation and 4 chemical factors was necessary for optimal differentiation towards a CM-like phenotype.



**Figure 4-3. Gene Expression of Day 14 Differentiated MDSCs.** Muscle-specific gene expression of MDSCs is dependent on culture conditions. Values are expressed as fold change in gene expression and normalized to 2D differentiation protocol (2D). \*P < 0.05. \*\*P < 0.001.

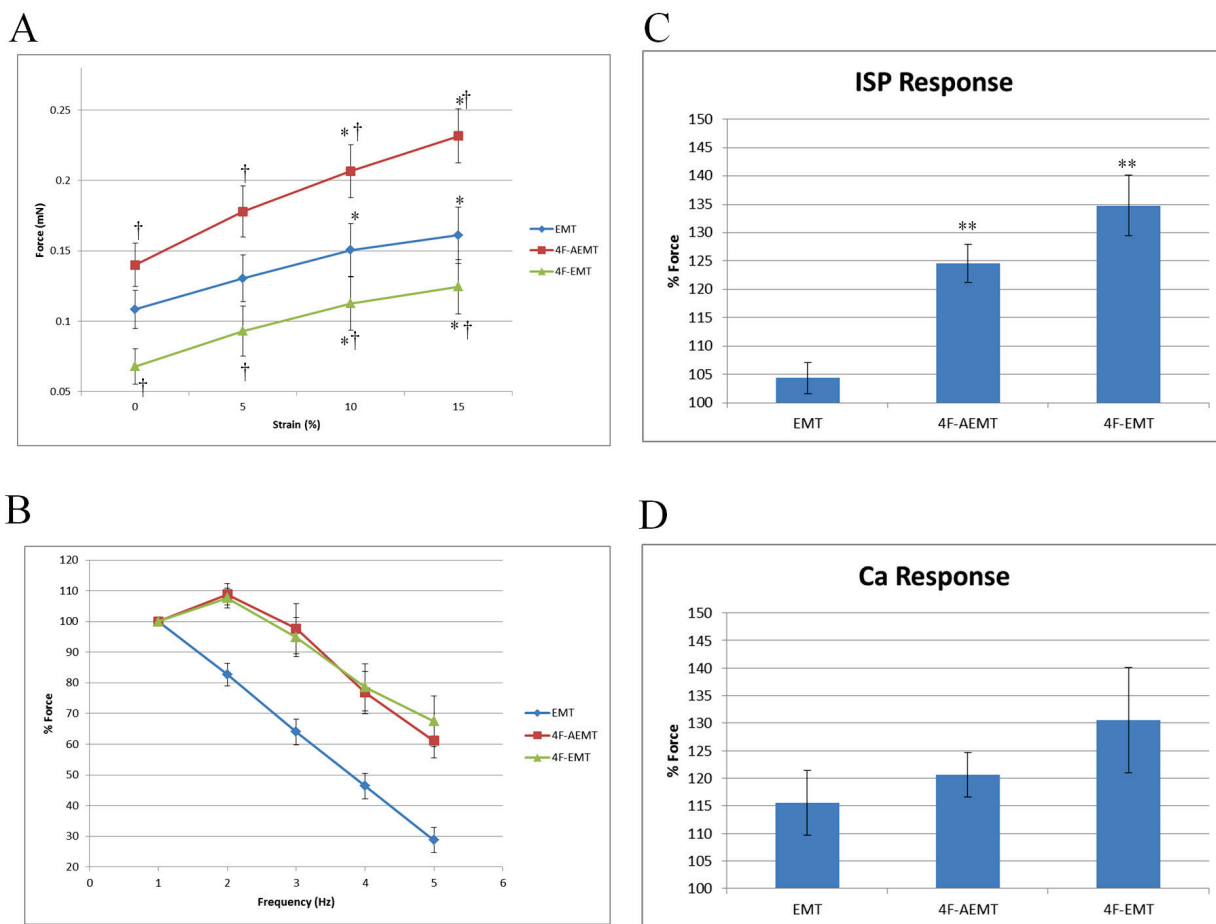


**Figure 4-4. Histological Analysis of Day 14 Differentiated MDSCs.** (A) Low magnification image of EMT showing  $\alpha$ -Actinin expression throughout the construct, indicating MDSCs acquire a muscle phenotype. (B) Expression of  $\alpha$ -Actinin and cTn-T in MDSCs differentiated under various protocols. Scale bars indicate 10  $\mu$ m or 20  $\mu$ m as stated in each image. Arrows point to multinucleated cells. Blue indicates nuclear DAPI staining. (C) Percentage of multinucleated cells ( $n > 4$  nuclei). 4-factor treatment significantly reduced the percentage of multinucleated cells. \*\*  $P < 0.001$ .

### 4.3.3 Contractile Properties of EMT

All EMTs showed spontaneous beating activity from day 4, which gradually increased over the culture period and was sustained until day 14. We observed that the spontaneous beating activity of 4F-AEMT was visibly more vigorous than other groups. The presence of spontaneous beating

activity confirms the differentiation of MDSCs into a functioning muscle phenotype. EMTs in all groups generated contractile force in response to electrical stimulation. The isometric force increased with strain (Frank-Starling mechanism) in all groups. 4F-AEMT ( $0.23 \pm 0.02$  mN at  $L_{\max}$ , n=20) generated significantly higher force than EMT ( $0.16 \pm 0.02$  mN at  $L_{\max}$ , n=16), and 4F-EMT generated less force than EMT ( $0.12 \pm 0.02$  mN at  $L_{\max}$ , n=15) (Figure 4-5A). EMT showed a negative force-frequency relationship. In contrast, 4F-EMT and 4F-AEMT displayed a modestly positive force-frequency relationship at low frequencies (1-2Hz) before becoming negative at higher stimulation frequencies (Figure 4-5B). 4F-EMT ( $134.7 \pm 5.4\%$ , n=14,  $P < 0.001$ ) and 4F-AEMT ( $124.6 \pm 3.4\%$ , n=24,  $P < 0.001$ ) showed an increased positive inotropic response to isoproterenol compared to control untreated EMT ( $104.4 \pm 2.8\%$ , n=23,  $P < 0.001$ ) (Figure 4-5C). All EMTs had a positive inotropic response to increased extracellular calcium, but there was no significant difference among groups (Figure 4-5D).

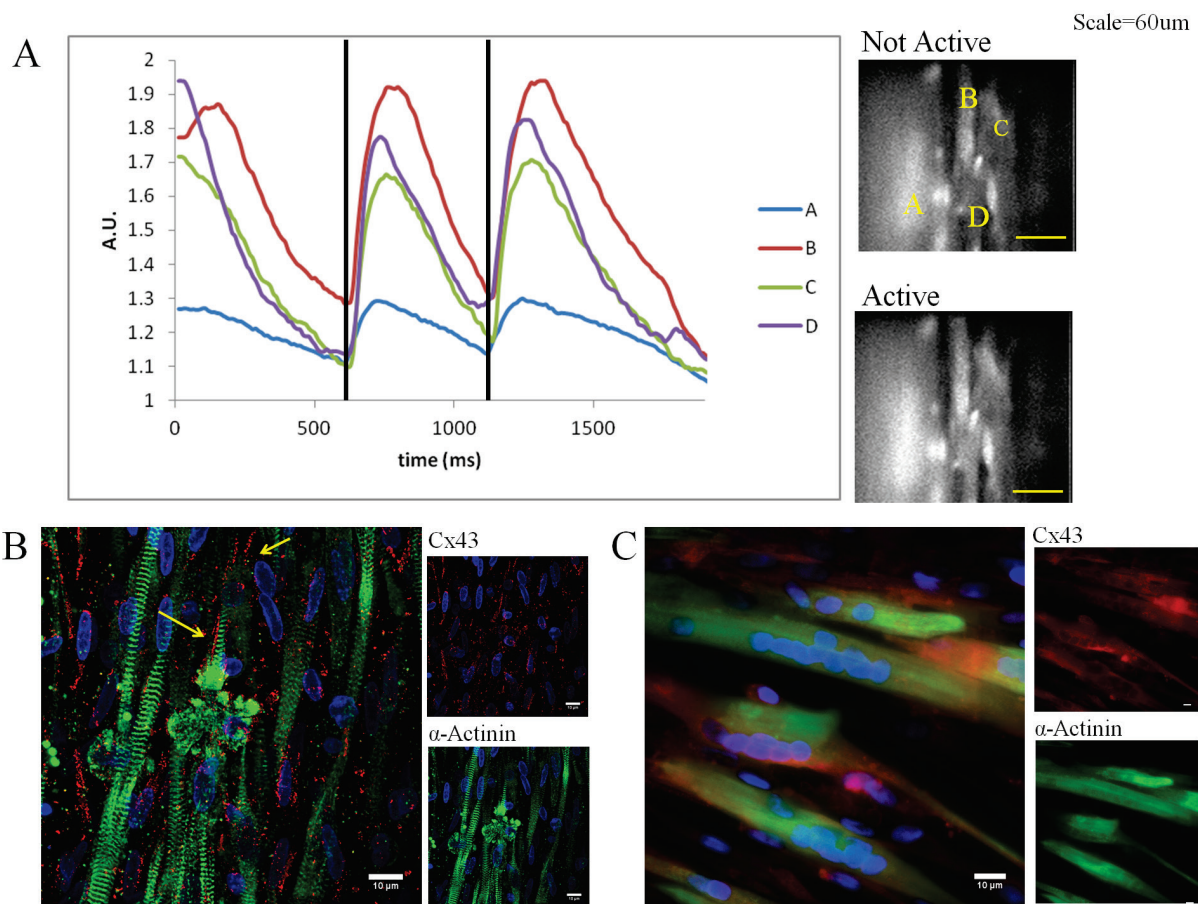


**Figure 4-5. Contractile Properties of Engineered Muscle Tissue.** (A) Force-strain relation of EMT. EMT increased force in response to increasing strain (Frank-Starling). \*  $P < 0.05$  vs. baseline. †  $P < 0.05$  vs EMT. (B) Force-frequency relation of EMT at stimulation rates of 1-5 Hz. (C) Contractile response of EMT to isoproterenol. Values are expressed relative to pre-treatment values. \*\*  $P < 0.001$  vs. EMT. (D) Contractile response of EMT to extracellular calcium. Calcium concentration of the bathing solution was increased from 2mM to 5mM.

#### 4.3.4 Intercellular Coupling

We assessed functional intercellular coupling by looking at intracellular calcium transients in EMT during spontaneous beating. EMT treated with 4 factors (4F-EMT and 4F-AEMT) showed that calcium transients in each cell were synchronous over the entire field of view (Figure 4-6A). We co-stained cells for connexin-43 and  $\alpha$ -Actinin to identify gap junction intercellular coupling

between differentiated myocytes. We observed a distinct dot-like pattern of connexin-43 staining in cells treated with 4 factors, which included concentrated staining at the border between adjacent cells (Figure 4-6B, yellow arrows). In contrast, cells that were not treated stained positive for connexin-43 and  $\alpha$ -Actinin, but a distinct staining pattern was not observed (Figure 4-6C). Taken together, these data suggest that treatment of EMT with chemical factors can improve intercellular coupling.



**Figure 4-6. Intercellular Coupling of MDSCs.** (A) Intracellular calcium transients of spontaneously beating MDSC-CMs recorded using Rhod-2 AM dye from 4 different locations. Trace shows that the transients begin and end in a synchronous manner (vertical lines). (B) Co-staining of 4-factor treated day 14 EMT with cx-43 (red),  $\alpha$ -Actinin (green), and DAPI (blue). Arrows show gap junction formation between cells. Scale indicates 10  $\mu$ m. (C) Co-staining of day 14 2D Differentiated MSDCs with cx-43 (red),  $\alpha$ -Actinin (green), and DAPI (blue). Scale bar indicates 10  $\mu$ m.



## 4.4 DISCUSSION

The repair of predominantly non-regenerative organs such as the heart is the holy grail of regenerative medicine. Direct injection of stem cells and other cell types has yielded modest functional benefits through what are believed to be paracrine effects, which stimulate revascularization of the infarcted area, promote cell survival, and reduce scarring [204]. However, true regeneration requires remuscularization of the infarcted region. Despite recent advances, this goal remains elusive for the heart. Protocols exist to generate CMs from embryonic stem cells, induced pluripotent stem cells, and fibroblasts (direct reprogramming), but the use of these cells clinically is limited by safety and ethical issues. In the arena of adult stem cells, hematopoietic stem cells and bone marrow mesenchymal stem cells do not differentiate into muscle efficiently or require direct contact with CMs [205], which are already depleted in the injured area [206]. True regeneration requires cells which can readily differentiate into a muscle phenotype. Stem or progenitor cells from cardiac and skeletal muscle tissue meet this criterion. However, cardiac stem cells require invasive isolation and are difficult to expand in vitro, and skeletal myoblasts have limited functional integration with host tissue due to differences in terminally differentiated cardiac and skeletal muscle [207]. MDSCs, given their plasticity and myogenic nature, are a promising cell source and may have the potential to differentiate into CM-like cells.

Realizing this goal requires recognition of the complexities of the stem cell niche and the factors that drive stem cell differentiation. Stem cell biology and tissue engineering have gradually moved away from traditional cell culture on a plastic dish and implemented novel approaches such as bioreactors and cell aggregate culture in order to better recapitulate physiological conditions. In this study, we attempted to create a biomimetic environment for

MDSC differentiation by providing an external environment containing collagen and other extracellular matrix factors (EMT) and dynamically regulating cell-cell interactions and soluble factor signaling by temporally controlling cell aggregate formation and treatment of signaling molecules. By using this integrative approach, we have successfully generated functional CM-like cells in vitro from human-origin MDSCs.

Multiple pathways regulate heart organogenesis including BMP, Wnt, and Notch signaling [208]. These signals are delivered in a temporally controlled manner, and the same signal can have opposing effects at different stages of development [188, 209, 210]. Reflecting this idea, we found in our initial studies that addition of LiCl or BMP-4 at day 0 inhibited muscle differentiation if they were added too early (Supplemental Figure 4-8A). This is reasonable given that these pathways are also involved with maintenance or expansion of stem cell pools in certain contexts [211, 212]. However, they did not interfere with muscle differentiation if they were added sequentially after initial IWR-1 and miR-206 inhibitor treatment. We attempted to mimic this temporal aspect in vitro. While these signals may not be fully representative of in vivo conditions, we were able to generate cells which are able to generate force as a functional muscle tissue while preserving their intercellular connectivity. miR-206 inhibition should theoretically inhibit skeletal muscle differentiation and prevent subsequent fusion. This combination of cytokines and small molecules with miR-206 inhibition blocked terminal skeletal muscle differentiation while simultaneously promoting CM differentiation. 4-factor treatment increased expression of cardiac transcription factors Nkx2.5 and GATA4. Visually, we observed a reduced incidence of fusion into myotubes in 4 factor treated EMTs. We also saw a connexin-43 distribution pattern similar to fetal CMs. With respect to function, we observed a cardiac-like force-frequency relationship with a small positive slope in the range of physiological human

heartbeat similar to neonate CMs [213]. The EMTs also showed an increased contractile response to isoproterenol, a cardiotropic compound. We also observed synchronous spontaneous calcium transient activity, which reflects improved spatial cx-43 expression at gap junctions. Without treatment, cells lose this distinct staining pattern and show a diffuse cytoplasmic distribution. Since we did not observe a difference in cx-43 expression at the mRNA level, the stability and localization of cx-43 and subsequent gap junction formation may be post-transcriptionally regulated as it is after birth [214]. Taken together, these data suggest that timed cytokine/small molecule treatment and transient miR-206 inhibition were sufficient to produce the desired phenotype.

Surprisingly, 4-factor treatment increased the expression of MyoD, a skeletal muscle specific transcription factor. Although MyoD is generally considered a skeletal muscle specific factor, we have previously reported that MyoD is expressed at the gene level in the heart during murine development and is upregulated during differentiation of human iPS cells and rat MDSCs into CMs [35, 177]. These findings have also been supported by studies in other species [215]. The role that MyoD may play during cardiac differentiation is unknown. Further studies are needed to elucidate its role. 4F-EMT also generated less force overall compared to EMT. This could be a result of differences in sarcomere length, which is proportional to force generation capacity. As cells differentiate into skeletal muscle, they elongate and some fuse, forming even larger fibers. However, the improvements in the force-frequency relationship and response to isoproterenol may reflect changes in excitation-contraction coupling and calcium handling at the cellular level.

MDSC aggregate formation had dynamic effects on cell proliferation, apoptosis, and differentiation. We previously used 24 hour aggregate formation time in our studies with rat

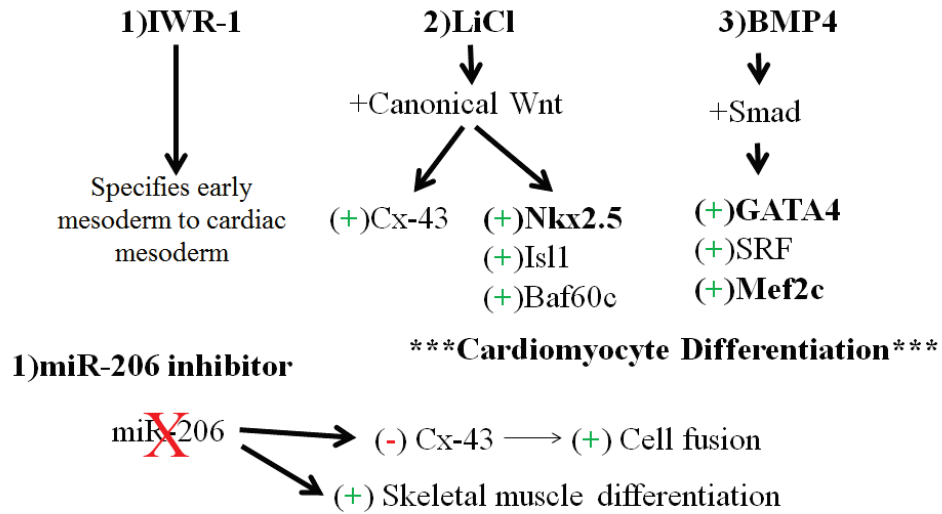
MDSCs [67]. However, in our current study with human MDSCs, we found that a short period of aggregate formation was sufficient to trigger early cardiac differentiation markers and suppress skeletal muscle differentiation markers. Although the mechanisms by which cell-cell contact within aggregates influence cell differentiation are not well understood, it is evident that it plays an important role. One study found that aggregate formation can directly impact  $\beta$ -catenin signaling within embryoid bodies during cardiac differentiation [199]. Direct contact of mesenchymal stem cells with CMs is also a prerequisite for their differentiation into CMs [216]. Cell aggregation occurs in two stages: an initial stage of aggregate formation driven by integrin-ECM binding followed by a period of spheroid compaction driven by cadherin-cadherin interactions [217]. It is possible that downstream effectors of integrin-ECM binding can affect genes involved in CM differentiation. Cell death increased significantly in aggregates after 24 hours. Cell death may occur as a result of diffusion limitations of nutrients and oxygen or by hydrodynamic shear during rotary suspension culture. Proliferation also decreased in a time dependent manner. While differentiation is the end goal of this study, maintenance of proliferative activity is desirable for tissue formation, and fetal-like proliferative myocytes have better therapeutic benefit in vivo [90]. We also tested long-term culture of MDSCs in aggregates to see if they could differentiate into CMs. After 2 weeks of culture as aggregates in suspension, MDSCs expressed cTn-T protein. However, they did not beat spontaneously, and a striated pattern was not visible. These findings suggest that aggregate formation is important, but additional factors are required to trigger full differentiation of MDSCs into functional CMs.

Combined with EMT culture and 4-factor treatment, short term aggregate formation resulted in robust differentiation of MDSCs into functional myocytes. In addition to Nkx2.5 and GATA4, 4F-AEMT upregulated cardiac transcription factor Mef2c and sarcomere related genes

Myh6 and Myh7 compared to other groups. 4F-AEMT also showed good tissue formation and prominent striations by both cTn-T and  $\alpha$ -Actinin staining. In agreement with these results, we saw an overall increase in force generation in the 4F-AEMT group compared to other groups. These findings suggest that the 4F-AEMT protocol provided the best conditions for functional muscle differentiation, and the differentiated muscle cells were morphologically distinct from typical skeletal myotubes. In fact, human skeletal myotubes do not normally spontaneously contract in vitro [218]. Persistent, rhythmic spontaneous beating is further evidence of both functional differentiation and more cardiac-like behavior. Embryoid bodies and other stem cell aggregate systems recapitulate elements of early embryonic development. However, as tissues develop into more complex structures, additional factors may be required to drive their development into functional tissues. EMT contains collagen and other extracellular matrix factors, which may be critical for functional differentiation of MDSCs.

Recent efforts in cardiac tissue engineering have focused on embryonic stem cells and induced pluripotent stem cells. While these cells may serve as useful models for developmental studies or drug screening, questions concerning their safety and genomic stability limit their clinical use [219, 220]. Adult stem cells such as bone marrow and adipose derived mesenchymal stem cells can be differentiated into CMs by co-culture with CMs or treatment with 5-azacytidine [205]. However, the rate of differentiation is low, and functional characterization has been limited. These cells may also co-express cardiac and skeletal muscle genes [138]. To our knowledge, we are the first group to report the creation of engineered cardiac tissue from human origin adult stem cells in vitro with the ability to generate contractile force and coordinated intracellular calcium transients at the tissue level.

There are a number of limitations to the current study. We were unable to perform detailed electrophysiological measurements of EMTs beyond measurement of calcium transients. However, others have reported that MDSCs cultured as aggregates display electrophysiological properties similar to bona-fide CMs [201]. We also did not assess the effects of each of the 4 factors on an individual basis. We chose the timing and dosage of the 4 factors based on the combination that permitted functional muscle differentiation while preserving intercellular connectivity. Figure 4-7 illustrates the hypothetical effects of 4 factors on MDSC differentiation. They may also have overlapping effects. The idea of preconditioning adult stem cells with a cocktail of cardiogenic compounds to enhance their therapeutic performance has been tested in the C-CURE clinical trial [221]. It was found to be feasible and safe. Such lineage guided cell therapy could be further enhanced by introducing signals in a timed manner as we have done. Finally, MDSCs differentiated under our protocol continue to express a combination of cardiac and skeletal muscle markers. Additional factors such as physical (electrical stimulation, cyclic stretch) or chemical (miRNAs, cytokines) stimuli may be necessary to further increase differentiation and maturation of MDSCs towards the cardiac lineage.



**Figure 4-7. Theoretical Framework for 4-factor Differentiation of MDSCs into Cardiomyocytes.** Inhibition of miR-206 preserves gap junctions and prevents fusion. Modulation of Wnt and BMP signaling promotes CM differentiation.

## 4.5 CONCLUSION

In summary, we have built upon the established importance of EMT by temporally controlling direct cell contact and soluble signals, two of the mechanisms by which cells communicate. As a result, the engineered tissue showed better contractile properties and preserved intercellular connectivity. These findings highlight the importance of the temporal, physical, and soluble aspects of the cellular environment for functional cardiac tissue engineering.

## 4.6 ACKNOWLEDGEMENTS

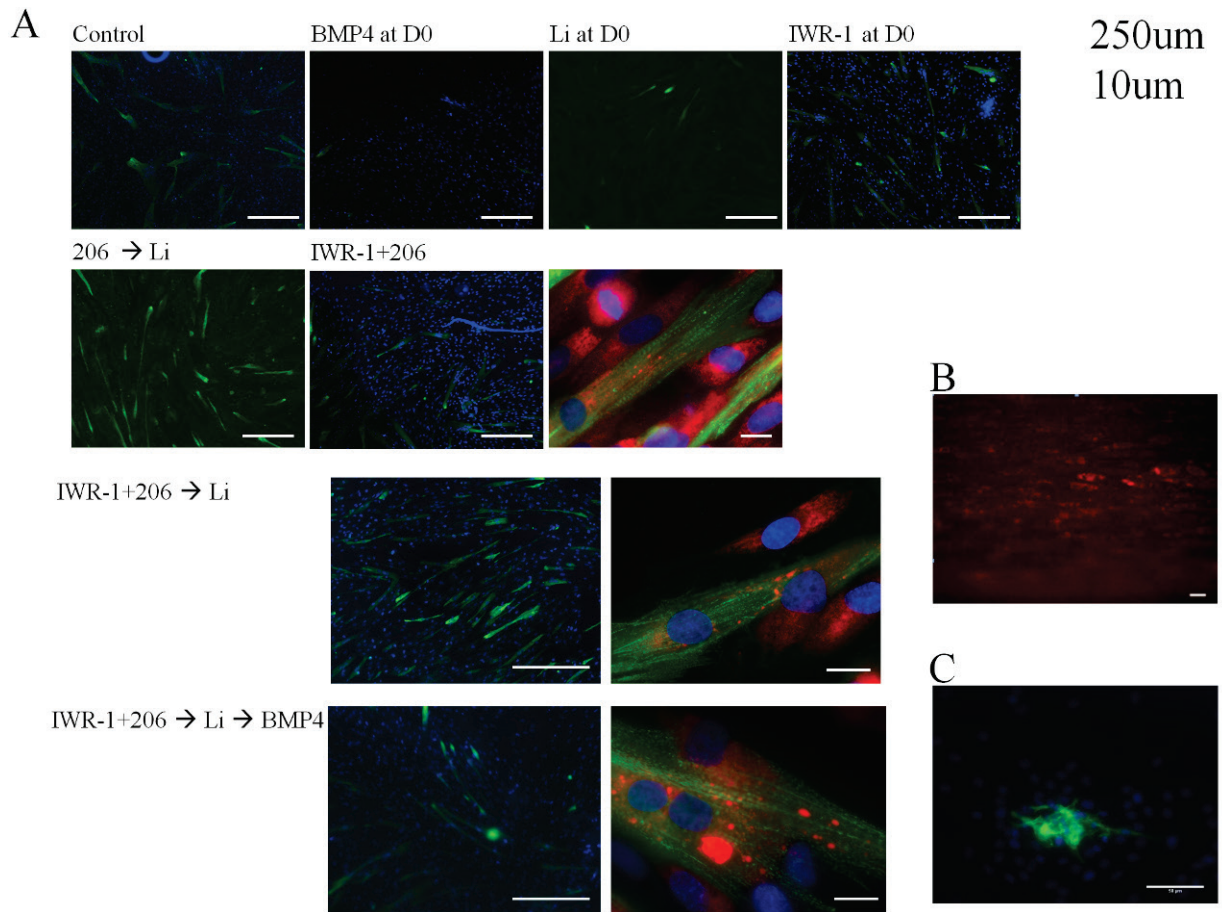
This study was supported by NIH R21 HL094402 (K.T.), Commonwealth of Pennsylvania Grant (K.T.), NIH Cardiovascular Bioengineering Training Program T32 HL076124 (J.T.), and AHA Pre-doctoral Fellowship 13PRE15030000 (J.T.), We would like to thank Lei Yang's lab for use of cTn-T antibody.

**Table 4-1. List of Abbreviations Used in Chapter 4**

### **Abbreviations**

iPS cell:	Induced pluripotent stem cell
MDSC:	Muscle derived stem cell
CM:	Cardiomyocyte
EMT:	Engineered muscle tissue
ISP:	Isoproterenol
miRNA:	MicroRNA
Cx-43:	Connexin-43
LiCl:	Lithium Chloride
GSK-3 $\beta$ :	Glycogen synthase kinase 3 beta
IWR-1:	Inhibitor of Wnt Response 1
BMP-4:	Bone morphogenic protein 4
ES cell:	Embryonic stem cell
DM:	Differentiation media
4F-EMT:	EMT treated with 4 factors
4F-AEMT:	MDSC Aggregate EMT treated with 4 factors
cTn-T:	Cardiac Troponin T
$\alpha$ -Actinin:	Alpha sarcomeric actinin
P-histone3:	Phospho Histone H3





**Figure 4-8. Supplementary Information.** (A) 2D Differentiated MDSCs at day 6 stained for  $\alpha$ -Actinin (green) and cx-43 (red). Cells were treated for the following durations beginning at day 0: BMP4 (48 hrs), LiCl (24 hrs), miR-206 inhibitor (48 hrs), IWR-1 (48 hrs). Forward arrow indicates sequential treatment. + sign indicates simultaneous treatment. Scale bar of low magnification images indicates 250  $\mu$ m. Scale bar of high magnification indicates 10  $\mu$ m. (B) EMT transfected with Cy3 labeled 100pmol small RNA. Approximately 80% of cells were transfected. Scale bar indicates 20  $\mu$ m. (C) MDSC aggregates cultured for 14 days and stained for cTn-T (green). Scale bar indicates 50  $\mu$ m.

## **5.0 IN-VIVO POTENTIAL OF PRECONDITIONED HUMAN MDSC 3D AGGREGATE CELL SHEET PATCHES FOR CARDIAC REPAIR**

### **5.1 INTRODUCTION**

In previous chapters, we saw that human MDSCs have potential for CM differentiation in vitro. Others studies have shown that undifferentiated MDSCs have potential to improve cardiac function when introduced via direct injection or as engineered cell sheets in vivo [73, 81]. However, the delivery of preconditioned MDSC aggregates has never been tested in vivo. In this chapter, we seek to investigate if preconditioned MDSC aggregates can survive, differentiate, and improve cardiac function in vivo. Our preliminary results show potential for MDSC cell therapy. However, additional work is necessary to verify the results and better optimize cell preconditioning and implantation techniques. Human MDSCs have already entered clinical trials for cardiac repair and urinary incontinence [222]. At the present time, it is unclear whether transplantation of undifferentiated MDSCs, which secrete paracrine factors, or differentiated MDSCs, which form new muscle tissue but may secrete fewer paracrine factors, is the better approach for cardiac repair.

## 5.2 METHODS

### 5.2.1 Experimental animals

8 week old female athymic nude rats weighing 140 to 170g were used as recipient animals for MDSC cardiac patches. The athymic nude rats were maintained within the animal facility of the Rangos Research Center Facility, Children's Hospital of Pittsburgh of UPMC. All experimental protocols followed the National Institutes of Health guidelines for animal care and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

### 5.2.2 MDSC Patch Construction

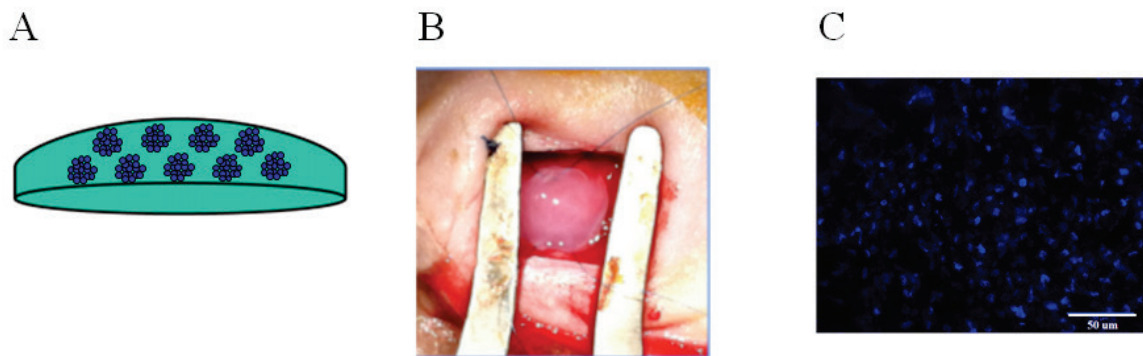
MDSCs were purchased from Cook Myosite, Inc. Cells were cultured in Cook Basal Media (Cook Myosite, Inc., Pittsburgh, PA, USA) supplemented with 10% Growth Supplement (Cook Myosite, Inc., Pittsburgh, PA, USA) and 1% Antibiotic-Antimycotic solution (AAS, Invitrogen) on collagen-I coated flasks. Cells were used for no more than 4 passages. Once cells reached ~80% confluence, cells were treated with Inhibitor of Wnt Response-1 (IWR-1, Sigma, 10  $\mu$ M) and miR-206 inhibitor (206inh, Life Technologies, 200pmol). MDSCs were then trypsinized and underwent rotary orbital suspension culture (Labnet Orbit 1000) at 50 rpm for 24 hours. During this time, MDSCs were treated with Lithium Chloride (LiCl, Sigma, 10 mM). To construct tissue patches, liquid rat tail collagen type I (3 mg/mL, Invitrogen) was neutralized with 0.1N NaOH to reach a final collagen concentration of 1.5 mg/mL. MDSC aggregates were seeded in collagen at a density of 2 million cells per construct using a Flexcell Tissue Train Culture system with the tissue mold removed (FX-4000, Flexcell International) with a total volume of approximately 600  $\mu$ L per construct to form a lens shaped construct (Figure 5-1A).

### **5.2.3 Chronic left ventricular infarction model**

The chronic left ventricular infarction was made as previously described [90]. Recipient adult athymic nude rats were anesthetized using 3.0% isoflurane inhalation with 100% oxygen gas. Next, rats underwent endotracheal intubation and connection to a rodent volume controlled mechanical ventilator (Model 683; Harvard Apparatus). The heart was exposed through a left thoracotomy. The proximal left anterior descending coronary artery was ligated using 7-0 polypropylene sutures. Regional cyanosis and changes in electrocardiogram (ST-segment elevation) were used to confirm myocardial ischemia. The incision was then closed with 4-0 silk continuous sutures. A total of 10 rats underwent left coronary artery ligation, and 2 animals died within 24 h of permanent coronary artery ligation, and 8 animals survived. These surviving rats were recipient candidates for MDSC patches.

### **5.2.4 MDSC Patch Implantation**

Animals were anesthetized with isoflurane, and the infarction size and cardiac function were assessed by 2 weeks after permanent coronary artery ligation. Left ventricle (LV) infarction size was estimated in a standard LV short-axis view by the percentage of scar area (akinetic or dyskinetic regions) to LV free wall area. Animals were studied at 6 weeks after graft implantation. The LV anterior wall was exposed through left thoracotomy, and the anterior infarcted myocardium was covered with the MDSC patch and sutured using 7-0 polypropylene (Figure 5-1B). For the infarction control group, a thoracotomy was performed 2 weeks after coronary ligation, but no implantation was performed.



**Figure 5-1. Construction and Implantation of MDSC Patch.** (A) Schematic representation of MDSC aggregates (blue) embedded in lens-shaped collagen construct (green). (B) MDSC patch implanted on top of infarcted rat myocardium. (C) MDSC patch at day 0 stained negative for cTn-T (green), confirming the undifferentiated state of MDSCs prior to implantation. Nuclei are stained with DAPI (blue). Scale bar indicates 50  $\mu\text{m}$ .

### 5.2.5 Histological Assessment

At 6 weeks after the MDSC patch implantation, the rats were exposed under 3.0% isoflurane inhalation with 100% oxygen gas, and the heart was arrested by apical injection of 2mL of a hypothermic arresting solution (28mM KCl). The embedded frozen LV tissues were sectioned in the LV transverse direction using a standard cryo-microtome (Leica). Sections were permeabilized with 0.1% Triton X-100 and stained with Alexa Fluor 488 conjugated human anti-nuclei antibody (235-1, EMD Millipore), alpha sarcomeric actinin ( $\alpha$ -Actinin, Sigma) with Alexa Fluor secondary antibodies, and DAPI (Vecta-Shield; Vector Labs).

### 5.2.6 Assessment of in vivo recipient cardiac function

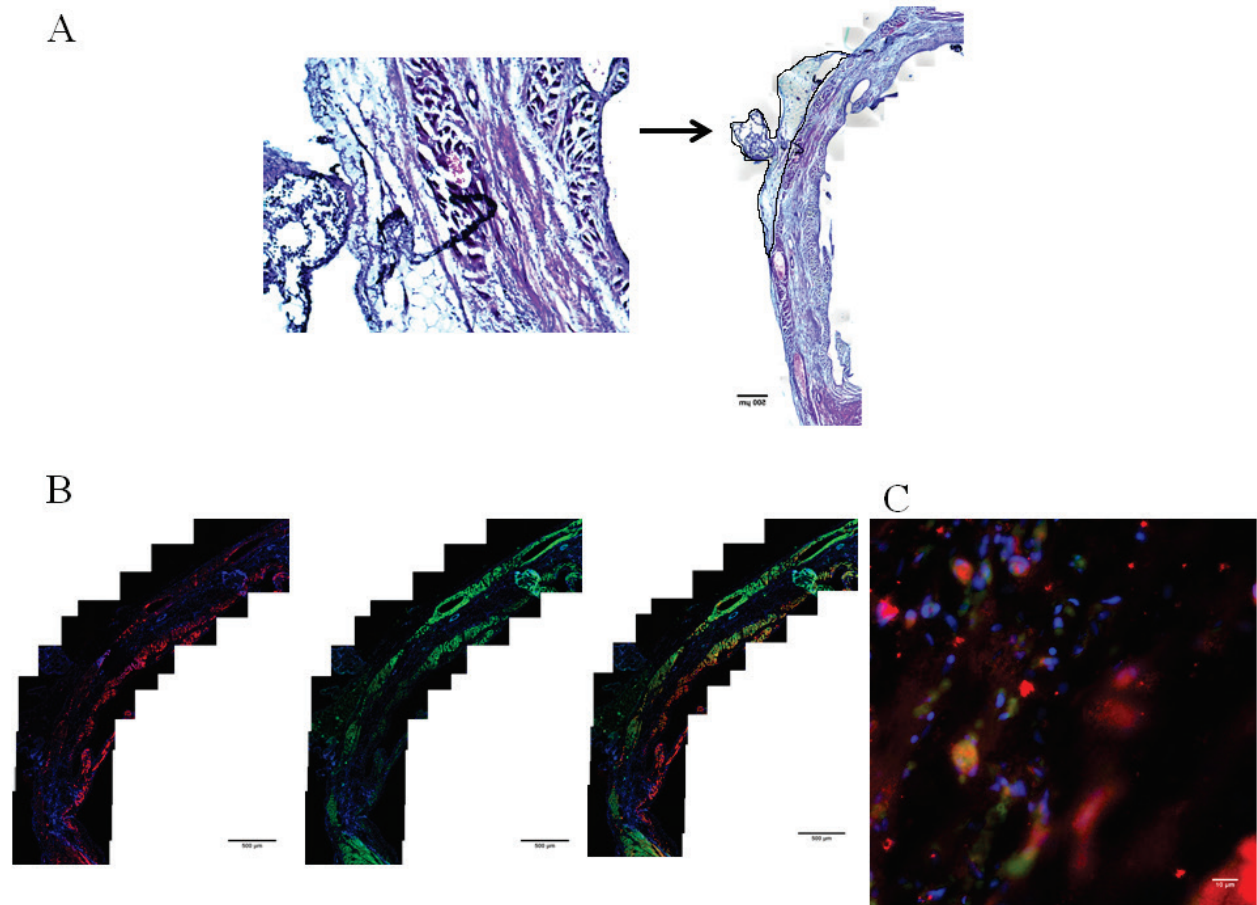
LV function was measured by tagging cardiac MRI at preimplantation and 6 weeks after graft implantation. End-diastolic area (EDA) and end systolic area (ESA) of the LV cavity were

measured, and % LV fractional area change (FAC) was measured as  $[(LVEDA - LVESA)/LVEDA]*100\%$ .

## 5.3 RESULTS

### 5.3.1 Histological Assessment

Six weeks after implantation, the MDSC patch was still visible on the surface of the myocardium (Figure 5-2A). We performed double-labeling for human nuclei (green) and  $\alpha$ -Actinin (red) (Figure 5-2B). Due to high green auto fluorescence (possibly caused by lipofuscin accumulation [223]), detection of human nuclei was difficult. However, at higher magnification, we were able to detect cells which were double positive for human nuclear antigen and DAPI within  $\alpha$ -Actinin positive regions, confirming their differentiation into a muscle phenotype. The  $\alpha$ -Actinin staining lacked a clear striated pattern, indicating a nascent muscle phenotype. These results show that MDSCs can survive and differentiate into a muscle phenotype by tissue engineered transplantation of MDSC aggregates.



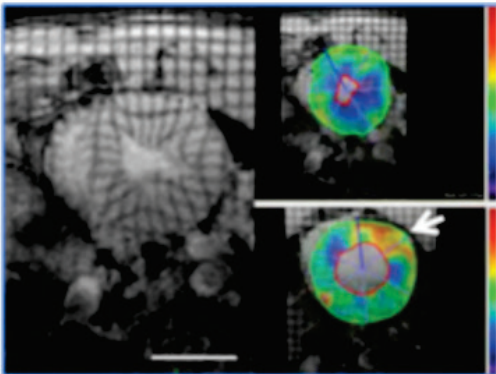
**Figure 5-2. Histological Assessment of MDSC Patch Implantation.** (A) Hematoxylin and Eosin staining of MDSC patch implanted rat myocardium. MDSC patch is outlined in black. Scale bar indicates 200 μm (B) Immunohistochemical staining of MDSC patch implanted rat myocardium for  $\alpha$ -actinin (red), human nuclear antigen (green), and DAPI (blue). Scale bar indicates 500 μm. (C) High magnification image showing staining of MDSC patch implanted rat myocardium for  $\alpha$ -actinin (red), human nuclear antigen (green), and DAPI (blue). Scale bar indicates 10 μm.

### 5.3.2 Cardiac Function

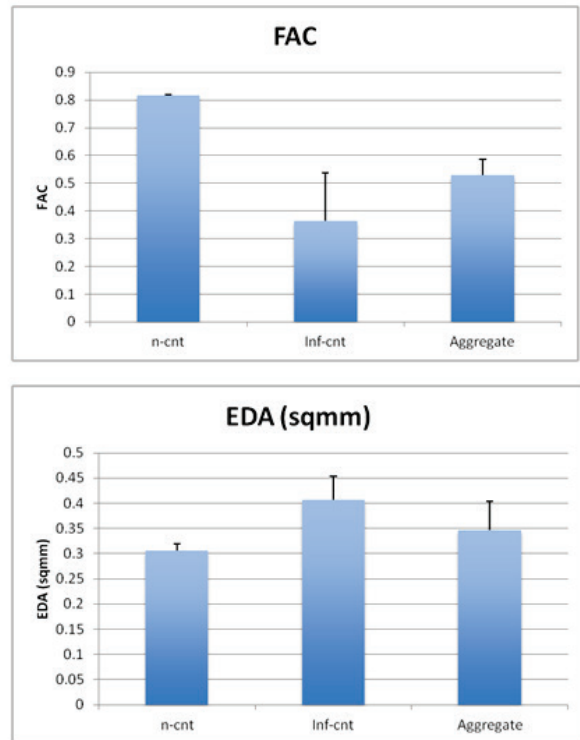
Cardiac function was assessed by MRI (Figure 5-3A). The fractional area change (FAC) of the MDSC aggregate implanted group ( $0.529 \pm 0.056$ ,  $n=3$ ) was higher than the infarcted control ( $0.365 \pm 0.171$ ,  $n=5$ ) but lower than the non-infarcted control group ( $0.817 \pm 0.003$ ,  $n=2$ ). The end

diastolic area (EDA) of the MDSC aggregate implanted group ( $0.346 \pm 0.057$ ,  $n=3$ ) was lower than the infarcted control group ( $0.407 \pm 0.046$ ,  $n=5$ ) but higher than the non-infarcted control group ( $0.817 \pm 0.003$ ,  $n=2$ ) (Figure 5-3B). These results suggest that MDSC patch implantation can potentially improve cardiac function, although function is not fully recovered to normal levels.

A



B



**Figure 5-3. In-vivo Evaluation of Cardiac Function.** (A) Representative MRI recording of LV. Scale indicates 5mm. (B) Cardiac performance parameters fractional area change (FAC) and end diastolic area (EDA). Abbreviations: n-cnt, non-infarcted control group; inf-cnt, infarcted control group; aggregate, infarcted group receiving MDSC patch.



## 5.4 DISCUSSION

Previous small animal studies have demonstrated the feasibility and efficacy of MDSC transplantation for cardiac repair [73, 81]. However, current therapy modalities have their limitations. The benefits of MDSC transplantation are believed to be derived from secretion of paracrine factors that stimulate angiogenesis and promote survival of existing CMs [204]. Few MDSCs differentiate into CMs. Furthermore, cellular retention remains low due to a hostile environment and lack of an anchorage matrix. Tissue engineering offers an alternative to overcome some of these challenges. We have developed a novel tissue engineered construct for the delivery of MDSCs *in vivo*. Collagen provides a natural anchorage matrix and shields MDSCs from the surrounding hypoxic and pro-inflammatory environment. The circular lens shape allows for easier implantation and coverage of the infarcted area compared to our previously used linear shaped construct. In the last chapter, we showed that aggregate formation and soluble factor pre-conditioning improved the cardiomyogenic potential of MDSCs. In this study, we tested whether preconditioned MDSC aggregates could survive, differentiate into a muscle phenotype, and improve cardiac function in a human-athymic nude rat xenograft model. Due to limitations on available resources, we were only able to perform a limited number of animal experiments. However, these preliminary data suggest that this method can potentially aid in MDSC survival and differentiation as well as restoration of cardiac function.

A number of improvements can be implemented in future studies. First, the *in vivo* animal study was performed before the 4 factor treatment protocol described in chapter 4 was fully developed. We did not include BMP-4 pretreatment in the animal study. Adding BMP-4 may further improve the effectiveness of the patch implantation. Second, we chose a 24 hour aggregation time based on our previous studies with rat MDSCs. However, we later found that a

shorter aggregation time (4 hours) was sufficient to trigger muscle differentiation while minimizing apoptosis and preserving some proliferative activity. Future studies should be performed with the 4 hour aggregate formation protocol. Third, intense endogenous green auto fluorescence made identification of human cells difficult. The human nuclei should be stained with a different color to facilitate their identification. Other important cellular processes such as angiogenesis, proliferation, and apoptosis should also be investigated. Finally, studies have shown that implantation of cell-free biomaterials can improve cardiac function without the addition of exogenous cells [224]. Although the mechanisms are not well understood, it is thought that functional improvement is at least partially attributable to the mechanical wall support afforded by the implanted biomaterial. In the case of this study, it is not clear if the functional benefits are due to muscle differentiation, paracrine secretion, the scaffold itself, or a combination of these factors. Future studies should include experimental groups for scaffold-only and MDSC-only injection to better understand the contribution of each in addition to including more animals in all experimental groups. Treatment of MDSC patches with BMP4, IWR-1, LiCl, and miR-206 inhibitor may also improve its effect.

## **5.5 CONCLUSION**

In summary, we have provided preliminary evidence that preconditioned MDSC aggregates can be safely delivered via collagen patch while retaining the ability to survive and differentiate. This study highlights the potential for cardiac tissue engineering using MDSCs.

## 5.6 ACKNOWLEDGEMENTS

This study was supported by Commonwealth of Pennsylvania Grant (K.T.) and NIH Cardiovascular Bioengineering Training Program T32 HL076124 (J.T.),

**Table 5-1. List of Abbreviations Used in Chapter 5**

### **Abbreviations**

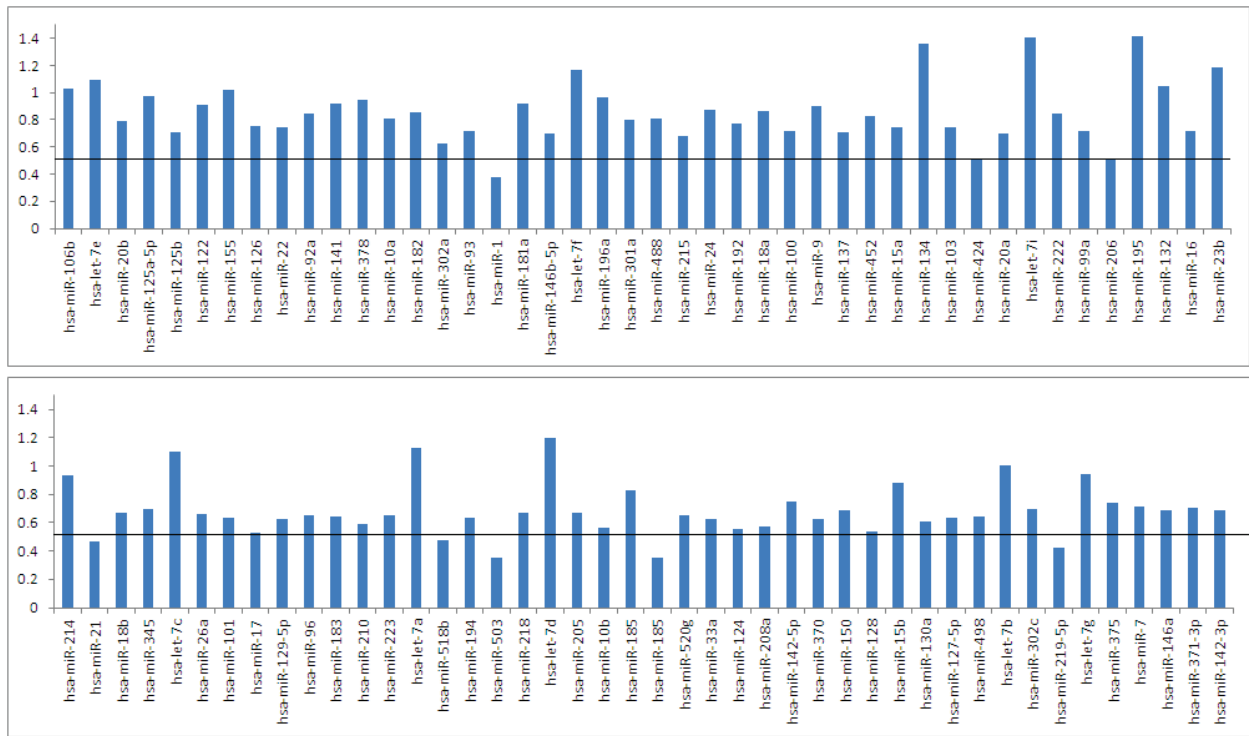
MDSC:	Muscle derived stem cell
CM:	Cardiomyocyte
IWR-1:	Inhibitor of Wnt Response 1
206inh:	miR-206 inhibitor
LiCl:	Lithium Chloride
LV:	Left ventricle
EA53:	Alpha sarcomeric actinin
cTn-T:	Cardiac troponin-T
EDA:	End diastolic area
ESA:	End systolic area
FAC:	Fractional area change
n-cnt:	Non-infarcted control group
Inf-cnt:	Infarcted control group
Aggregate:	Infarcted group receiving MDSC patch

## 6.0 CLOSING REMARKS AND FUTURE DIRECTIONS

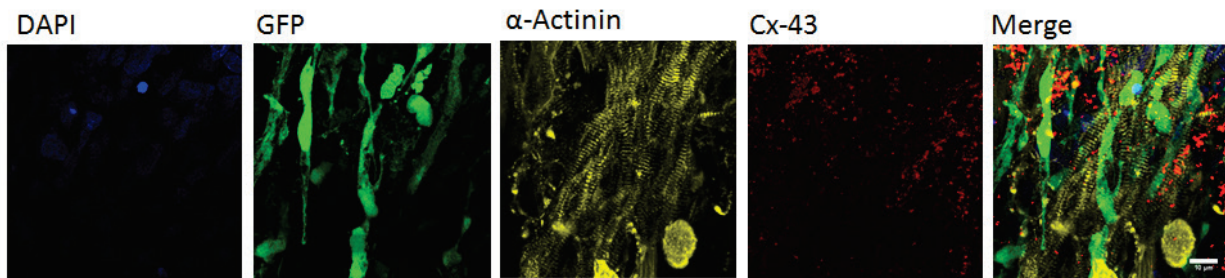
In this dissertation, we investigated the properties of cardiac and skeletal muscle as a means to galvanize the use of skeletal muscle stem cells (MDSCs), which are relatively more abundant and easier to isolate than their cardiac counterparts, for cardiac repair. (The rationale for using MDSCs has been discussed previously.) First, we studied developing rat cardiac and skeletal muscle and found that they share a similar, albeit not identical, set of transcription factors and contractile proteins, particularly cardiac troponin-I and fast skeletal myosin heavy chain, before birth. This was also true of MDSCs that were isolated and differentiated in vitro as an engineered tissue. Next, we extended these findings to humans, comparing human MDSC-derived engineered muscle tissue (MDSC-EMT) to cardiac tissue from human iPSc CMs. They were remarkably similar in terms of biochemical and contractile properties. However, MDSC-EMT suffered some deficits such as incomplete intercellular coupling. Then, we employed two strategies to improve CM differentiation of MDSCs. First, we improved the conditions for forming MDSC aggregates. Second, we introduced a sequence of factors which promote CM differentiation, stimulate gap junction formation, or prevent skeletal muscle differentiation. These approaches recapitulate important physical and temporal aspects of development and improve intercellular coupling and contractile function. Finally, we put human MDSCs to the test in vivo using a xenograft animal model. Although the data are preliminary, it suggests that cell therapy based on MDSCs holds promise.

Thus, our contributions to the field are twofold. First, we have extended our previous results in rats to humans, which is a major step towards clinical translation. Second, we have created a growth factor and small molecule based protocol for functional differentiation of MDSCs into beating CM-like cells with intercellular coupling. (Lack of coupling was previously cited as a concern for use of myoblasts.) This has been achieved without the use of any genetic modification currently used in iPS cells and direct reprogramming. MDSC derived CMs in this protocol also demonstrate more advanced functional properties compared to other common adult stem cell (bone marrow, adipose stem cells) derived CMs, since they are able to contract as an integrated tissue and generate force.

However, there are still numerous improvements to be made in the future. The contractile force of MDSC engineered tissue is still much lower than that of native CMs [67], though it is within the range reported for pluripotent stem cell derived engineered cardiac tissues [225]. Future advancements may come from manipulation of epigenetic factors and the environment. 4-factor treatment induces changes in expression of a large number of miRNAs (Figure 6-1), which are post-transcriptional regulators. The environment is also a potent effector of cellular changes. In addition to well-documented roles in differentiation, a recent study showed that changes in the environment are sufficient to induce pluripotency in somatic cells [226]. Along those lines, factors such as electrical stimulation, stretch, and flow may positively influence CM differentiation. Finally the in vivo potential of pre-conditioned human MDSCs should be investigated more rigorously. BMP-4 expressing MDSCs may be more cardiogenic. In vitro co-culture of MDSCs with CMs can be used to study interactions between stem cells and CMs in a controlled environment (Figure 6-2). For example, stress conditions could be simulated by culture in a hypoxic environment or addition of inflammatory cytokines.



**Figure 6-1. microRNA analysis of 4F-EMT at culture day 5.** Values are normalized to EMT (untreated) and expressed as fold change. 4-factor treatment influences the expression of a large number of miRNAs.



**Figure 6-2. Co-culture of MDSCs with iPS CMs in EMT at culture day 21.** 400,000 MDSCs were mixed with 100,000 iPS-CMs per EMT. Co-cultures were stained for DAPI (blue), α-Actinin (yellow), iPS-CMs (green), and cx-43 (red). Scale bar indicates 10μm.

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