UNIVERSITY OF CALIFORNIA, SAN DIEGO

Biomaterial-assisted myogenic differentiation of pluripotent stem cells and their *in vivo* function

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Bioengineering

by

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The dissertation of Sara Hariri is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2017

DEDICATION

I would like to dedicate my doctoral dissertation to my amazing father, Nader Hariri, for his immeasurable love, support, and encouragement. Thank you for being my lifelong role model, for teaching me the most important values in life, and for believing in me when it was hard for me to believe in myself. I owe everything I have and everything I seek to be, to you. I will forever cherish all our moments together, from the car rides to school every morning in Tehran, for making me believe that I don't suck at geometry as much I thought I did, to always keeping up with our grades in school, college, and grad school. For being the best father that you know how to be and for all your admirable dedication to Leila and I's education and future, I am forever thankful. I look up to you more than you can imagine and hope that reading this dissertation will make you proud.

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LIST OF ABBREVIATIONS

Myogenic progenitor cells	=	MPCs
Pluripotent stem cells	=	PSCs
Induced pluripotent stem cells	=	iPSCs
Gelatin methacrylate	=	GelMA
poly(sodium-4-styrenesulfonate)	=	PSS
Extracellular Matrix	=	ECM
Duchenne Muscular Dystrophy	=	DMD
Basic fibroblasts growth factor	=	bFGF
Heparin sulfate proteoglycans	=	HSPG
Phosphate buffered saline	=	PBS
Photoinitiator	=	PI
Growth medium	=	GM
Horse serum	=	HS
Dulbecco's modified Eagle's medium	=	DMEM
Insulin, transferrin, selenium	=	ITS
Sarcomeric myosin	=	MF20
Desmin	=	DES
Myogenin	=	MyoG
Bovine serum albumin	=	BSA
Real-time Polymerase Chain reaction	=	rt-PCR
Cardiotoxin	=	CTX
Tibialis anterior	=	TA

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ACKNOWLEDGEMENTS

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To Professors Prashant Mali, and Pedro Cabrales, Sylvia Evans, and Samuel Ward, thank you for taking the time to serve on my thesis committee. Without your time and expertise, the value of this work would be severely lessened.

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To Gaurav, I enjoyed collaborating with you on the skeletal-muscle-on-a-chip project and learned so much from it. I appreciate both our scientific and casual conversations and discussions and I have certainly learned a lot from it.

To Vernon, I am thankful for your mentorship and patience. I have learned so much from you. I learned that it takes more than knowing the science and the skills to be a good researcher. Your critical eye for data analysis is truly impressive. I look up to you in so many ways. Your dedication and perseverance towards research is commendable and I draw inspiration from it.

To all my other lab members Heemin, Aereas, Han, Shruti, Meng-qian, Vikram, Jasper, Nilah, and all the newbies. It was very nice to have the comradeship and collaboration of like-minded people who understand the struggles we all go through at one point or another. I wish you all the best of luck in the future.

To my amazing friend, Ana, I could have never done this without you. Thanks for being my shoulder to cry on, my coffee break buddy, my all-time supporter. You were my rock through grad school and one of the most valuable people in my life.

To my lovely sister, Leila, for her positive attitude and for making everything seem better than it is. You are the joy of our family and a true inspiration for me. I may be the older sister but I have learned so much from you.

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Chapter 1, in part, is currently being prepared for submission for publication of the material: Sara Hariri, and Shyni Varghese[*]. The dissertation author was the primary lead and author of this paper.

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Chapter 3, in part, is currently being prepared for submission for publication of the material: Sara Hariri, and Shyni Varghese[*]. The dissertation author was the primary lead and author of this paper.

VITA

CORE COMPETENCIES

- Demonstrated **Technical** skills (Tissue engineering and stem cell therapy) with **Successful leadership** of multiple parallel projects on time, budget, & resources
- Demonstrated ability in **Execution** of multiple parallel projects by **Strategic Approach** and **out of the box** thinking and focusing on excellence
- Self-motivated, independent thinker, result-oriented, and team player with **Multidisciplinary Knowledge** of **biomaterials, tissue engineering, stem cell therapy, 3D printing, micro/nano manufacturing**
- Strong problem solving capabilities with a track record of introducing innovative ideas with proactive mindset
- Excellent documentation and presentation skills

PROFESSIONAL EXPERIENCE

University of California San Diego, La Jolla, CA

Graduate research and development engineer **Sep 2014-June 2017**

Design and development of stem cell differentiation and delivery platforms to address skeletal muscle injuries

Responsibilities:

- Designed and fabricated natural and synthetic 3D scaffolds
- Developed and optimized various in vitro cell-based assays (IF, WB, ELISA)
- Designed microfluidic platforms using soft lithography approach
- Experienced in executing process validation and testing
- Highly experienced with in vivo transplantation studies
- Skilled in aseptic techniques involving various activities including cell culture and bench assays
- Experienced with imaging techniques and microscopy (ImageJ)
- Experienced in fabrication of micropatterned substrates and microbeads for stem cell differentiation
- Experienced with synthesis of biomaterials with different mechanical properties
- Skilled in cryopreservation and tissue processing
- Highly experienced in documentation practices
- Responsible for the design of system architecture, project planning, allocating resources, estimating cost, and managing budget
- Superior interpersonal and time-management skills
- Excellent technical and communication skills
- Trained undergraduate and graduate students for skills such as stem cell culture, immunofluorescence staining, gene expression analysis, and imaging

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Graduate Researcher **Sep 2013 – Sep 2014**

- Highly experienced in stem cell culture and differentiation
- Developed doxycycline-inducible cell lines expressing genes of interest
- Performed Chromatin immunoprecipitation sequencing
- Highly experienced in various cell culture techniques, gene expression analysis, and immunohistochemistry
- Experienced in lentivirus transfection, virus purification, plasmid purification, plasmid sequencing
- Developed teamwork and leadership skills by collaborating on multiple projects and mentoring undergraduate students

University of California Irvine, Irvine, CA
Undergraduate research fellow **Dec 2010 - Nov 2011**

- Investigated a novel approach to develop 3D nitinol-based tissue engineered heart valves
- Learned various design principles using SolidWorks

TEACHING EXPERIENCE

California State University, Fullerton, CA
Part-time Faculty, Bridges to Stem Cells Research (BSCR) June 2017 – Aug 2017

- Designed and taught stem cell technology techniques for various biomedical applications
- Covered topics such as CRISPR gene editing, cloning, microscopy, cell culture, immunohistochemistry, and qPCR

Teaching assistance, University of California San Diego
General Chemistry Laboratory, Chemistry Department (Chem 7L)

- Designed and taught chemistry concepts and laboratory skills
- Designed and delivered presentations, quizzes, and study guides in preparation for student's final exam
- Presented experiment demos to engage students in active learning and offered discussion time
- Received excellent feedback from my students and supervisor

Cell and Molecular biology, Bioengineering Department (BENG 230B)

- Conducted discussion sessions to review relevant scientific publications
- Assist students in data analysis and evaluation of experimental designs
- Provided study guides and conducted presentations to prepare students for various concepts such as gene expression, genetic mechanisms, cellular structure and function, cell signaling
- Designed course material and graded assignments and exams

Transport phenomena in Living systems, Bioengineering Department (BENG227)

- Designed course material, exams, and provided study guides to students on topics such as diffusion, convection, and biochemical reactions
- Provided guidance for final project design and experimental setup
- Promoted student success by making myself available to students outside of classroom and office hours through one on one meetings, additional review sessions, and email communications

EDUCATION

University of California, San Diego 2014-2017
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University of California, Irvine 2007-2012
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HONORS AND AWARDS

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- Member: Women in Bioengineering 2012-2016

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3D CAD Design: SolidWorks, AutoCAD
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Image Processing: ImageJ
Statistical analysis: Prism

PUBLICATIONS

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- Synthetic heparin matrix-assisted myogenic differentiation of pluripotent stem cells improves in vivo transplantation in cardiotoxin-injured mice (prepared for publication)
- Cell based therapies for muscular dystrophies (prepared for publication)
- iPSC-derived myogenic progenitor cells ameliorate MDX phenotype (In progress)
- A bioengineered approach to cell-based therapies for muscular injuries, poster presentation, Research Expo, UCSD 2015
- Motorneuron- and signal-mediated maturation of hiPSC-derived skeletal myospheres, poster presentation, UC System-wide Bioengineering Symposium, 2014

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ABSTRACT OF THE DISSERTATION

Biomaterial-assisted Myogenic Differentiation of Pluripotent Stem Cells and their *in vivo* function

by

Sara Hariri

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2017

Professor Shyni Varghese, Chair

Skeletal muscle function can be severely compromised by injury and disease, leading to decreased quality of life. Progressive muscle wasting due to genetic disorders results in myofiber necrosis and ultimate loss of muscle function. In case of muscular dystrophies, lack of functional regenerative machinery results in replacement of muscle fibers with connective and adipose tissue. Despite extensive efforts to develop cell-based therapies, several major limitations, such as limited expansion capacity of cells, immune rejection, and lack of cell viability hinders the therapeutic potential of transplanted cells. Previous studies and clinical trials have studied the efficacy of cell therapy for muscle regeneration and have faced disappointing results, including low levels of engraftment within host tissue and insignificant improvements in muscle function. These outcomes can be attributed to the intrinsic limitation of muscle tissue to engraftment of transplanted cells.

This doctorate dissertation worked towards developing a cell-based therapy with a focus on induced pluripotent stem cells (iPSCs) to address muscular injuries. Specifically, we developed an approach for matrix-mediated myogenic differentiation of hiPSC-derived muscle progenitor cells (MPCs) using a synthetic mimic of heparin incorporated in a gelatin-based hydrogel. We show that using this approach large quantity of progenitor cells can be derived *in vitro* and used for *in vivo* applications.

The engraftment potential of such progenitor cells is tested in immunocompromised mdx mice. Furthermore, we investigate the various stages of myogenic differentiation and identified different sub-populations present in culture. We were able to show that transplantation of a specific stage of myogenic differentiation is best suited for treatment of Duchenne Muscular Dystrophy modeled in mdx mice.

The results described in this thesis provide a proof-of-principle that derivation of myogenic progenitor cells without genetic modifications can be achieved using a simple monolayer culture system by taking advantage of biomaterials. We further demonstrate the significance of the extent of *in vitro* myogenic maturation for successful cell transplantation.

Chapter 1: Cell based therapies for treatment of muscular injuries

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Abstract

Skeletal muscle tissue has an inherent ability to regenerate itself after experiencing an injury. However, it's regenerative capacity is limited by the extent of fibrosis post injury, depletion of muscle resident stem cells, and the size of defect. A promising therapeutic strategy to address skeletal muscle defects involves transplantation of a combination of cells, biological factors, and biomaterials to facilitate functional skeletal muscle restoration. Extensive effort in identifying the right cell type for skeletal muscle regeneration has lead scientists to define some of the most important characteristics for cell-based therapies. Donor cells should be able to engraft with recipient's myofibers to enable formation of new tissue and also repopulate the satellite cells niche for future rounds of regeneration. On the other hand, Bioscaffolds can act as an artificial niche to protect donor cells against immunogenic responses, stimulate stem cell differentiation, and promote new fiber formation. Proposed strategies to date are limited by poor donor cell viability, immunogenic rejections, and inability of donor cells to migrate and engraft in host tissue. In this review, advancements in tissue engineering and regenerative medicine to address skeletal muscle defects is highlighted.

Skeletal muscle physiology and anatomy

There are approximately 640 skeletal muscles in the human body, which account for about 34% of average human body mass (Janssen et al., 2000). Skeletal muscle is a highly vascularized and innervated tissue composed of highly organized myofibers, extracellular matrix (ECM), and connective tissue. Elongated and multinucleated

myofibers are bundled together to form fascicles surrounded by a layer of connective tissue that maintains the shape of the tissue. The vasculature surrounding the fibers ensures that oxygen and nutrients are sufficiently delivered to the tissue. The striated appearance of myofibers comes from the arrangement of sarcomeres in series, which are the basic contractile element of the cells. The sarcomeres are a combination of many key proteins such as actin and myosin heavy chain, which interact to generate force during each contraction. This sophisticated architecture leads to proper voluntary and involuntary motion.

During normal embryonic development, skeletal muscle is established in a multi-step fashion by activation of key myogenic regulatory factors and development of a combination of precursor cell types (Biressi 2007, Messina 2009, and Tajbakhsh 2000). Myogenic regulatory factors (MRFs) such as Myf5, MyoD, and Mrf4 determine skeletal muscle cell identity and upon activation regulate myogenesis (Kablar 2003, Kassar-Duchossoy 2004). It has been shown that triple mutant Myf5/Mrf4/Myf5 mice fail to generate myoblasts and muscle fibers; however, a population of progenitor cells are present in the organism (Kassar-Duchossoy 2004). A population of proliferating $Pax3^{+}/Pax7^{+}$ cells mark muscle progenitor cells that reside in skeletal muscle and do not express the down-stream maturation markers such as Myogenin and Myosin heavy chain (Kassar-Duchossoy 2005) which confirms the presence of a reservoir of quiescent $Pax3^{+}/Pax7^{+}/MRF^{-}$ cells. Myogenin is another important MRF in myogenesis which plays a pivotal role in myoblast differentiation and fusion. Studies have shown that mutation in myogenin in mice results in lack of differentiated skeletal muscle and eventual death (Hasty 1993, Meadows 2008, Myer 2001).

Skeletal muscle has a robust regenerative capacity in response to injury (Carlson and Faulkner 1983). Regeneration resembles the process of embryogenesis during skeletal muscle formation and depends on the extent of injury and type of damaged muscle. During this repair process, endogenous satellite cells are activated to undergo myogenic differentiation. Activated mononucleated progenitor myoblasts migrate to the site of injury and fuse with the preexisting myofibers or to one another to form multinucleated cells called myotubes (Musaro et al 2007). These quiescent adult skeletal muscle stem cells not only participate in myogenesis to regenerate the muscle but they can also repopulate their own niche by undergoing self-renewal (Kuang 2008; Shi 2006). It is generally accepted that quiescent satellite cells in mice do not express MyoD or Myf5 while activated satellite cells express key MRFs such as MyoD (Zammit 2006). Newly formed or repaired myofibers after injury are characterized by centrally located nuclei. Upon tissue maturation and cytoskeletal remodeling the nuclei are positioned around the periphery of the myofibers.

Skeletal muscle defects

Skeletal muscle injuries can be due to sports-related trauma (strains, lesions, lacerations), age-induced muscle loss (sarcopenia), and genetic disorders (Duchenne Muscular Dystrophy). Although skeletal muscle has an intrinsic repair mechanism by which it can regenerate lost or damage tissue, the extent of injury may prevent complete regeneration and functional recovery. Muscle injury is followed by tearing of the myofibers and recruitment of mononuclear inflammatory cells to the site of injury (Tidball 1995; Toumi 2003). While inflammatory macrophages (M1) are involved in

phagocytosis of nacrotic myofibers, anti-inflammatory macrophages (M2) participate in activation and recruitment of muscle progenitor cells and are shown to facilitate their proliferation and differentiation into newly formed myotubes (Hawke 2001; Tidball 2010; Malerba 2010). Depending on the level of injury, fibrosis and scar tissue formation may happen at a faster rate than tissue regeneration and can potentially prevent complete tissue recovery. General loss of muscle mass (atrophy) and remodeling of the ECM and connective tissue can slowly affect muscle function and overall locomotion.

Duchenne muscular dystrophy (DMD) is a progressive debilitating disease that takes away physical strength, independence, and life. This disease is caused by a mutation in the dystrophin gene. Dystrophin is a key cytoskeletal protein that links the actin cytoskeleton to the extracellular matrix (ECM) proteins, such as laminin, via transmembrane dystrophin-glycoprotein complex (DGC) (Gawlic 2014; Ervasti 2008). Dystrophin is responsible for stabilizing the sarcolemma of muscle cells and its absence compromises the integrity of the sarcolemma, and results in oxidative stress, rapid cycles of degeneration/regeneration, and deposition of adipose and fibrotic tissue (Goncalves 2006).

Immune system and its role in myogenesis

Cell transplantation can either be allogeneic (donor-derived cells) or autologous (patient-specific cells). Inevitably, transplantation of cells from a donor is associated with high risk of immune rejection and significant cell death. Regardless of the nature of injury, repair and regeneration of muscle fibers follows a general trend (Jarvinen et

al., 2005). The initial phase is the destruction/inflammatory phase where injury results in myofiber rupture and fiber necrosis. Mononuclear inflammatory cells secrete chemotactic signals and recruit circulating inflammatory cells to the site of injury (Tidball et al., 1995; Toumi et al., 2003). Tumor necrosis factor- α (TNF- α), fibroblasts frowth factor (FGF), insulin like growth factor (IGF), interleukin- 1β (IL-1β), and IL-6 (Philippou et al., 2012; Chen et al., 2005) are among the cytokines and growth factors that play a critical role during the initial phase of injury.

Recruitment of marcrophages to the site of injury for phagocytosis of necrotic muscle fibers and elimination of debris occurs during the repair phase (Novak et al., 2014). Macrophages are responsible for amplification of inflammation at the time of injury and down-regulation of the immune response to protect muscle tissue from further damage (Nathan, 2005; Fujiwara and Kobayashi, 2005; Serhan and Savill; 2005). They are known for the heterogeneity and opposing roles in nature (i.e., proinflammatory vs. anti-inflammatory, immunogenic vs. tolerogenic, and tissue destructive vs. tissue-repair activities) (Stout and Suttles, 2004). As mentioned earlier, muscle injury results in massive macrophage infiltration (McLennan, 1996). It has been shown that monocyte-derived macrophages can enhance myogenic cell growth by releasing mitogenic growth factors for MPCs which prevents cell apoptosis (Chazaud et al., 2003). The role of inflammatory cells in muscle regeneration has been explored recently (Sonnet et al., 2006; Conteras-Shannon et al., 2007). Using *in vivo* tracing methods, Arnold et al., have analyzed monocyte/macrophage profiles during muscle regeneration. They have shown that pro-inflammatory macrophages (M1) are responsible for phagocytosis of ruptured fibers and recruitment of MPCs including

satellite cells, whereas anti-inflammatory macrophages (M2) stimulate proliferation and differentiation of satellite cells-derived myoblasts (Hawke et al., 2001; Tidball et al., 2010; Malreba et al., 2010; Arnold et al., 2007). Additionally, it has been shown that *in vivo* depletion of circulating monocytes and macrophages prevents muscle regeneration and decrease fiber diameter, respectively (Arnold et al., 2007).

The final stage of muscle regeneration is the remodeling phase, characterized by the reorganization of newly generated myofibers. Fibrosis and scar tissue formation is remodeled during this phase and the muscle begins to regain its contractile function. It is important to note that severe muscle damage or volumetric muscle loss is associated with higher rate of scar tissue formation and slower rate of regeneration. This contributes to the thick scar tissue formation that prevents proper healing (Turner et al., 2012).

Tissue engineering and regenerative approaches aim to address skeletal muscle defects

Many therapeutic approaches are currently being investigated to replace or repair damaged or injured skeletal muscle tissue using tissue engineering (TE) and regenerative medicine approaches. Most commonly, a combination of cells, scaffolds, and biological factors are used to form an injectable or implantable construct (Fig 1.1). Scaffolds are usually designed as structures that provide support to the cells they harness and aim to mimic the properties of the natural ECM of the tissue. The possibility of fine tuning the scaffold design with specific structural, physiochemical, biological, and mechanical properties allows scientists to use multifunctional biomaterials to regulate

stem cell fate (Discher 2005), cellular alignment (Hwang 2017; Zhao 2009), and drug delivery (Panyam 2003).

Tissue engineering of skeletal muscle defects rely on *in vitro* and *in vivo* approaches. The *in vitro* tissue engineering approaches aim to develop functional skeletal muscle tissue by seeding cells onto a scaffold to form a structure with highly aligned myofibers that can generate physiologically relevant contraction forces (Bian 2008). Some of the main challenges to this approach are (a) maintaining cellular viability in 3D constructs due to diffusion limitations (b) ability of in *vitro* construct to integrate with host tissue post transplantation (c) ensuring that the engineered tissue is functional and contraction force readout can be obtained (d) changes in gene expression profiles of cells in culture due to de-differentiation or improper differentiation. *In vivo* strategies either rely on scaffold-free cell-based transplantation or delivery of a combination of cells and biomaterial that acts as a local microvironment for the cells at the injury site. The goal of *in vivo* cell therapy is to direct donor cells to facilitate muscle regeneration by forming new muscle tissue or activating host's regenerative machinery (McCullen 2011). *In vivo* therapies require less *ex vivo* cell manipulations and reduce variation in cell phenotype and gene expression profile. However, they are susceptible to host's immune response which compromises their viability post transplantation.

Cell sources for tissue engineering and regenerative medicine applications

Extensive effort has been made in identifying the ideal cell type for skeletal muscle tissue engineering and muscle repair. Somatic cell lines and tissue specific stem cells are a popular candidate for TE and regenerative medicine applications; however,

they are limited in supply, *in vitro* expansion capacity, and ability to maintain their phenotype in a dish. Additionally, in order for donor cells to participate in new tissue formation they must be able to migrate away from their niche. The extent of donor cell migration is another key factor in determining *in vivo* therapy success. Some of the most common cell types for skeletal muscle TE and cell-based therapies is highlighted in this review.

Primary myoblasts

Primary myoblasts have the ability to give rise to dystrophin-expressing myofibers when transplanted into dystrophic muscles. As early as 1989, myoblast transplantation was tested in nude/mdx mouse and was shown to restore dystrophin expression (Patridge 1989). Following this early success, several clinical trials on myoblast transplantation in DMD patients were initiated that showed little to no positive results (Huard 1991; Tremblay 1993; Miller 1997; Mendell 1995). The success of this approach is heavily dependent on the ability of donor cells to migrate and remain viable post transplantation without eliciting an immune response. Skuk and colleagues have attempted to use donor myoblasts in a phase I clinical trial study to perform 25 injections into the tibialis anterior (TA) muscle of three immunosuppressed DMD patients (Skuk *et al*, 2004). After 1 month, between 6.8% and 11% of donor cells expressed dystrophin. Scientists have also explored myoblast transplantation in conjunction with genetic manipulation (MyoD expression) and matrix metalloproteinases to address viability and migration limitations (Smythe 2001; El Fahime 2000). Successful myoblast transplantation in mice, monkeys and humans currently require immunosuppression

therapy with FK506 which is associated with some adverse side effects including increased risk of cancer and diabetes. An alternative to this approach is using autologous genetically-corrected myoblast transplantation. In a more recent study, myoblasts corrected for the dystrophin gene using lentiviral-based ex vivo exon skipping were transplanted in mice and monkeys and showed promising results (Quenneville 2007). Despite encouraging results obtained, ability to deliver myoblasts systemically to circumvent migration limitations still remains a challenge.

Adult skeletal muscle cells for DMD treatment

Satellite cells (SCs) comprise ~2-7% muscle cell nuclei and play a pivotal role in muscle regeneration (Rudnicki 2008). This cell population is associated with self-renewal, high proliferation, and differentiation capacity. In a study done by Sacco et al. self-renewal and expansion capacity of a single luciferase-expressing muscle satellite cell was assessed in mice by bolus injection. The results show that the transplanted cell was capable of both proliferation and differentiation *in vivo* and significant donor cells engraftment with host myofibers was observed post transplantation (Sacco et al. 2008). Several protocols for satellite cell isolation has been developed either by enzymatic digestion (Conboy 2003), physical trituration (Rosenblatt 1995), or fluorescent-activated cell sorting (Bosnakosvki 2008, Day 2007). It is important to note that muscle stem cell properties of satellite cells are only maintained by direct transplantation of satellite cells attached to myofibers or injection of freshly isolated single satellite cells without *in vitro* culture (Montarras 2005; Relaix 2005; Sacco 2008). It has been showed that satellite cells residing in freshly isolated single myofibers migrate away from their

niche and start proliferating and differentiating within 48 hours of culture time (Shefer and Yablonka-Reuveni, 2005).

In summary, a lot has been learned about satellite cells in the past decade or two. The culmination of all findings confirm that satellite cells represent a potent cell source for skeletal muscle regeneration. For successful clinical applications, perhaps the greatest challenge to be circumvented is to obtain large quantities of cells for transplantation since activation and *in vitro* expansion of satellite cells reduces their engraftment *in vivo* (Montarras et al., 2005).

Mesoangioblasts as a stem cell therapy candidate for skeletal muscle regeneration

Among other mesoderm-origin stem cells for skeletal muscle regeneration, mesoangioblasts are highly proliferative progenitor cells present in the vessel wall that can differentiate into endothelial or mesodermal lineages. Researchers have shown that mesoangioblasts delivered intra-arterially from wild-type or genetically corrected dystrophic muscle of α-sarcoglycan-null mice shows functional improvement in dystrophic phenotype. In this study, donor cells were distributed throughout the capillary network and were able to migrate through the vessel wall and facilitate muscle repair (Sampaolesi 2003). More recently, myogenic differentiation of mesoangioblasts pre-conditioned with stromal cell derived factor-1 or tumor necrosis factor- α showed enhanced migration when transplanted in α-sarcoglycan-null dystrophic mice (Galvez 2006). In the same study, transient expression of α-4-integrin or L-selectin were also shown to improve migration both *in vitro* and *in vivo.* Therefore, combining stromal derived factor-1 with α-4 integrin expression resulted in more than 80% reconstituting

of α-sarcoglycan fibers (Galvez 2006). Similar cells can be derived from blood vessels that express pericyte markers such as alkaline phosphatase.

In a study published by Dellavalle et al, when pericyte-derived cells were transplanted into MDX/SCID mice via femoral artery, they colonized the host muscle and produced dystrophin positive muscle fibers. Since this distinct cell population of pericyte-derived cells express proteins such as β2 and α4 integrins then can adhere and migrate across the endothelium making intra-arterial injections possible. Finally, mesoangioblast transplantation in dystrophic dogs via arterial delivery has shown to ameliorate muscle morphology and function (Sampaolesi 2006). Taken together, mesoangioblasts are an attractive cell source for skeletal muscle differentiation and their application in clinical experimentation.

Repairing skeletal muscle with pluripotent stem cells

Due to their unlimited expansion potential, and ability to differentiate into all three germ layers, pluripotent stem cells are great candidates for cell based therapies (Zheng et al. 2006). More importantly, induced pluripotent stem cell (iPSC) technology has revolutionized personalized medicine by allowing patient's own cells to be reprogrammed for autologous transplantation. Several groups have studied myogenic differentiation of human and murine embryonic stem cells (ESCs) . Transient expression of Pax3 in hESC during embryoid body (EB) formation has been shown to induce paraxial mesoderm. Subsequent sorting for platelet-derived growth factor (PDGFRA- α) positive cells led to isolation of a homogenous population of myogenic progenitor cells. Transplantation of this subpopulation of hESC-derived myogenic

progenitor cells improved muscle function in MDX mice (Darabi et al. 2008). In a similar study, Hwang et al. have recently published a protocol for deriving progenitor cells based on isolation of cells from hESC-derived embryoid bodies that exhibit migratory behavior and express a mesoderm marker, PDGRA- α (Hwang et al. 2013). This approach does not require any genetic manipulation (i.e. overexpression of Mrfs) and is therefore favorable for clinical experimentation. In an attempt to use human induced pluripotent stem cells (hiPSCs) for skeletal muscle regeneration, large quantities of myogenic progenitor cells were derived by conditional expression of Pax7 in hiPSCs, which showed significant engraftment potential, and dystrophin production in dystrophic mdx mice (Darabi et al. 2012).

The potential application of hESCs is limited by potential immunogenic rejection and the ethical concerns associated with clinical applications. These concerns have been addressed with the discovery of iPSC technology and has opened up discussions about personalized medicine and stem-cell therapies using autologous transplantation (Takahashi et al., 2006; Yu et al., 2007; Blelloch et al., 2007).

CD133$^+$ progenitor cells can facilitate muscle repair

Freshly isolated human CD133+ cells from adult peripheral blood have shown myogenic differentiation potential when co-cultured with primary myogenic cells or by Wnt-conditioning. CD133+ cells injected intramuscularly can facilitate muscle regeneration and can contribute to the satellite cells compartment in MDX/SCID mice (Torrente et al. 2004). CD133+ cells isolated from human dystrophic muscles were genetically corrected ex vivo to restore dystrophin expression and delivered into

scid/mdx mice. Treated muscles showed improved muscle morphology, dystrophin expression, engraftment, and function (Benchaouir et al. 2007). Clinical studies using CD133+ cells without genetic engineering in human DMD patients have shown great promise with autologous transplantation (Torrente et al. cell transplant 2007). Long term viability and function of these cells in DMD patients has not been confirmed yet.

Limitations of cell-based therapies

Despite extensive efforts in developing strategies for cell-based therapies, major limitations including but not limited to cell availability, immune rejection, and donor cell survival *in vivo* seriously compromises the therapeutic potential of cell therapies. Previous studies and clinical trials have faced disappointing results such as low engraftment within the host tissue, limited contribution to the satellite cell compartment, and insignificant function muscle improvement (Hwang et al., 2013; Skuk et al., 2003). These outcomes are often associated with the intrinsic limitation of muscle tissue in engrafting donor cells. The intricate process of muscle regeneration relies on the precise coordination of various cellular events such as cell-cell and cell- matrix interactions, a condition known as anoikis. These processes are fundamental to myoblast alignment and fusion, a prerequisite to formation of multinucleated myotubes. It is a safe assumption that our limited success in cell-based therapies can be due to anoikis and lack of proper methods of in vitro differentiation as well as our partial understanding about the extent of *in vitro* differentiation and conditioning necessary for optimal *in vivo* outcome.

Myogenic differentiation of stem cells

Directed differentiation of stem cells towards myogenic lineage is very challenging and often associated with very low efficiency (Zhu et al. 2009). Many studies take advantage of a gene inducible system to over-express key MRFs to drive myogenesis in iPSCs or ESCs. Perlingero et al. have shown that mESC-derived myogenic progenitor cells induced with Pax3 can engraft in FRG1 transgenic mice, a dominant model associated with facioscapulohumeral muscular dystrophy. Their analysis of contractile properties reveals function improvement in treated muscles (Darabi et al., 2009). In another study by the same group, miPSC-derived myogenic progenitor cells induced to express Pax7 showed extensive engraftment in dystrophic mice (Darabi et al, 2011). Previous studies have also shown that MyoD overexpression in various cell types is sufficient to induce skeletal muscle differentiation (Shani et al, 1992; Goudenege et al., 2009).

Forced transgene overexpression does not mimic normal development and is not an ideal model for therapeutic purpose or disease modeling. To this end only a few groups have reported protocols to derive myogenic progenitor cells without relying on genetic overexpression. Barberi et al., takes advantage of antibodies specific to myogenic cells to generate specialized, multipotent, mesenchymal progenitors from hESCs that can be differentiated into myogenic cells (Barberi et al., 2007). Furthermore, this group has reported a simple two step differentiation protocol to differentiatie hiPSCs into skeletal muscle cells and proposed a stringent cell-sorting strategy to purify for Pax7/Pax3 precursors at high purity (Borchin et al, 2013). Varghese et al., examine the derivation and differentiation potential of PDGFRA+ progenitor cells from hESCs

without genetic manipulation and show their ability to mature *in vitro* and engraft in NOD/SCID mice (Hwang et al, 2013). Wnt signaling is shown to play a significant role in satellite cell activation and differentiation in response to skeletal muscle injury. Among 2400 chemicals screened, Xu et al has shown that GSK3B inhibitor (a Wnt agonist) in conjunction with bFGF and forskolin can induce myogenic differentiation of hiPSCs. Treatment of PDGFRA+ cells with Wnt3A protein has shown to improve myogenic differentiation and *in vivo* engraftment in cardiotoxin-injured skeletal muscles of NOD/SCID mice (Hwang et al., 2014).

Biomaterials in regenerative medicine and TE

Stem cells reside in a highly specialized microenvironment characterized by a unique combination of physiochemical and biological properties which regulate cell faith and function (Ottone 2014; Gilbert 2010; Chakkalakal 2012). While cells play an important role in skeletal muscle TE and regenerative medicine, biomaterials, although not as often mentioned, are instrumental to the development of a well-rounded TE construct or delivery vehicle for cell-based therapies. In many ways, cellular development and function is synchronized with the dynamic process of ECM production, degradation, and remodeling (Page-Mcgcaw et al. 2007 and Lu et al. 2011). Additionally, the ECM plays an instructive role by sequestering growth factors and regulating various cell signaling pathways which dictate cell growth and survival (Rozario et al. 2010).

Bioscaffolds can act as a substrate for cell adhesion (Lee 2012;) control cell proliferation and differentiation (Vorotnikova 2010; Reing 2009; Kanf 2014; Rao 2015;

Stern 2009) modulate relevant cell signaling pathways (Sangaj 2010; Kang 2015) provide spatial organization (Hwang 2017; Bian 2009; Zhao 2009), and protect cells from immunogenic responses (Murua 2008). A comprehensive summary of literature studies using biomaterials for skeletal muscle regeneration and tissue engineering approaches is highlighted in this review. These bioscaffolds can be cellular or acellular depending on the application and can range from solid, porous, to injectable networks.

Natural scaffold materials

The fundamental role of ECM and connective tissue is to support cells in a three-dimensional space to allow cells to properly function. Using biomaterials in tissue engineering aims to mimic the native microenvironment to facilitate the development of a functional tissue *in vitro* and improve viability and integration *in vivo*. Naturally derived biomaterial such as collagen, laminin, and fibronectin are biocompatible and naturally degradable which makes them an attractive candidate for TE applications. However, most natural polymers are associated with intrinsic variability and uncontrollable mechanical and structural properties as well as rapid degradation which limits the ability of tailoring the system with targeted characteristics.

Collagen hydrogels have been extensively studied for skeletal muscle tissue engineering and myogenic differentiation. 3D collagen gels packed at high density with murine myoblasts (C2C12s) exhibit myotube formation and alignment *in vitro* and demonstrate extensive capillary invasion post implantation (Okano et al. 1998). Chen et al. have reported on an approach to engineer three-dimensional (3D) micropatterened porous collagen-based scaffolds that exhibits myoblast alignment and expression of

muscle specific proteins (Chen et al. 2015). Varying the degree of crosslinking of gelatin-based hydrogels can be utilized to tailor the mechanical properties of the matrix to match native muscle to maintain stem cell faith, induce differentiation, and enhance engraftment efficiency (urciuolo 2013). In a recent study done by Quarta et al. transplantation of human muscle stem cells seeded on collagen based microscaffolds has been shown to facilitate skeletal muscle TE in case of volumetric muscle loss.

Hyaluronic acid (HA) as a major component of the ECM has been used in several skeletal muscle TE applications. A mixed population of satellite and progenitor muscle cells encapsulated in a photo-polymerizable HA hydrogel were transplanted into ablated mouse muscles. Angiogenesis and innervation were observed postoperatively with generation of new muscle fibers and a local satellite cell niche (Rossi et al. 2011). HA hydrogels have been shown to sequester and present growth factors to facilitate cell differentiation and function (Jha et al. 2015). A hybrid biomaterial made of HA grafted with 6-aminocaproic acid (6ACA) moieties have been shown to improve stem cell viability and engraftment in NOD/SCID mice by taking advantage of growth factor binding and sequestration (Kabra et al. 2014).

Scientists have been extensively studying fibrin-based Bioscaffolds for skeletal muscle differentiation and *in vivo* cell delivery. Hinds et al. have shown that the structure and function of a TE skeletal muscle construct is highly dependent on its ECM composition. The study clearly demonstrates that fibrin-based hydrogels give rise to muscle bundles with superior mechanical integrity and force generation capacity (Hinds 2011). In another effort to fabricate synthetic microfibers, Page et al. seeded microthread fibrin scaffolds with adult human muscle cells and transplanted the

construct in TA muscle of mice (Page 2011). The cell laden constructs contributed to new muscle fiber formation and showed significant reduction in fibrosis.

A naturally occurring crosslinking reagent (genipin) was used for chitosan crosslinking to produce microspheres that were subsequently injected into the skeletal muscle of a rat model. The results demonstrate that the transplanted microspheres have a slow degradation profile and superior biocompatibility when compared to glutaraldehyde-crosslinked chitosan microspheres (Mi. et al 2002).

Acellular Bioscaffolds

Although liquefying the ECM eliminates the structural components (vascular network, mechanical properties, porosity, etc), presence of protein components and cytokines will support the cellular components and future angiogenesis. ECM from decellularized tissues are also widely used as acellular scaffolds to promote skeletal muscle formation following volumetric muscle loss. Elimination of the cellular compartment while maintaining the microstructure of the ECM allows such acellular scaffolds to serve as a cell-guiding platform to recruit host cells and mediate tissue repair without eliciting an immune response (Sicari et al. 2014). A different approach in using accellular ECM components as cell delivery vehicles is conversion of the ECM network into a hydrogel. Using a simple two-step freezing and lyophilization method, decellularized skeletal muscle can be converted into an injectable hydrogel and used for tissue engineering and regenerative applications (Dequach et al 2012).

Synthetic scaffold materials

Synthetics materials can be precisely engineered with specific characteristics with greater control over physiochemical properties. Variables such as degradation rate, cell binding motifs, and structural properties can be fine-tuned to meet design constraints. Growth factors can be incorporated into synthetic scaffolds to be released at the site of injury or for the purpose of modulating endogenous regeneration (Nelson et al., 2011).

Various lithography techniques have been attempted to create nanoscale patterns on poly(lactide-co-glycolic) acid (PLGA) substrates that aim to enhance myoblast alignment and myogenic differentiation *in vitro*, while improving *in vivo* survival and engraftment (Boldrin et al 2007; Yang et al. 2014). In addition to 2-D microfabrication approaches, 3-D printing techniques extend the skeletal muscle tissue engineered design to the third dimension to improve the physiological relevance and mimic structure and function more accurately. In a recent study by Yang et al, 3D nanoscale patterns on PLGA scaffold patches aim to direct myoblast alignment and differentiation *in vitro* and improve *in vivo* engraftment of myoblasts in MDX mice (Yang et al 2014). An implantable 3D engineered muscle flap made from porous, biodegradable PLGA scaffold seeded with myoblasts, fibroblasts, and endothelial cells was developed to repair a full-thickness abdominal wall defect (Shandalov 2014).

Electrospinning is a versatile technique used to fabricate micro- and nano-fibers mimicking the elongated parallel fibers observed in native muscle tissue. Cells seeded onto electrospun nanofibers made of collagen and poly (ε-caprolactone) (PCL) oriented either randomly or aligned showed similar proliferation and fusion rates; however,

myotubes on aligned fibers were significantly longer and exhibited anisotropic mechanical properties that were more suitable for *in vivo* applications (Choi et al., 2008).

Muscle patch tissue engineering is another area of active research. Myoblasts cultured on gelatin coated PLGA nonpatterned substrates form a 2D tissue sheets that can be transplanted onto mdx mice quadriceps and contribute to new dystrophin-positive fiber formation (Yang et al. 2014). In a recent attempt to develop a biomimetic engineered muscle, Juhas et al. demonstrated that implantation of highly differentiated myofibers with quiescent satellite cells in form of a muscle patch can facilitate muscle regeneration following cardiotoxin induced injury in nude mice (Juhas et al. 2014). To address large volumetric loss muscle defects, it is often necessary to fabricate 3D scaffolds to fill structural voids and restore muscle tissue. Cells sheets grown to confluency on substrates engineered to have micropatterned grooves can be stacked on top of one another to form multilayered multicellular constructs (Neumann et al., 2003). To ensure that nutrient diffusion limitations does not compromise cell viability, sophisticated micromolding techniques can be incorporated into the tissue engineering design to allow for diffusion of oxygen and nutrients to throughout the 3D construct (Bian 2009).

Hybrid scaffolds composed of a synthetic and natural component are another attractive approach in skeletal muscle TE. Many studies take advantage of applying a natural polymer coating on synthetic materials to enhance cell attachment *in vitro* or affect host response *in vivo*. For example, polyurethane microchannel scaffolds chemically crosslinked with gelatin or silk fibroin coatings exhibited improved myotube

formation (Shen et al, 2013). In a study done by Kim et al., myoblasts seeded on nanofibers composed of gelatin and PCL showed myotube formation whereas PCL alone nanofibers did not allow myoblast differentiation (Kim et al., 2010). Scientists have shown that the extracellular matrix secreted by muscle myoblasts can be collected and used to engineer a hybrid scaffold (Hurd et al., 2015). The synthetic component of a hybrid scaffold provides tunable stiffness, flexibility, porosity, and overall mechanical support while the natural component improves attachment, biocompatibility, integration with host.

Importance of vascularization

Proper vascular supply is a crucial component of any tissue engineered skeletal muscle construct. In order to maintain the viability of donor cells *in vivo*, oxygen and nutrients must be delivered and metabolic byproducts must be removed continuously. Therefore, for any long term *in vivo* application, tissue engineered constructs must be integrated into host's vascular network. In the absence of these conditions, the constructs will likely undergo necrosis and the integration of the construct with the host will be compromised (Radisic et al., 2004). In a study by Langer's group, prevascularization of engineered skeletal muscle tissues *in vitro* improved viability, vascularization, and blood perfusion of transplanted constructs in a SCID mouse (levenberg et al. 2005). Similar work done by other groups emphasize the importance of vascularization for *in vivo* survival of skeletal muscle constructs composed of differentiated or committed cells upon transplantation (Koffler et al. 2011; Borselli et al 2011). More recently, Juhas et al. have demonstrated an approach to create 3D

biomimetic skeletal muscle tissues which can undergo robust perfusion and vascularization *in vivo*. These constructs support myogenic differentiation, functional satellite cell compartment, and improved contratile function *in vivo* (Fig 1.2) (Juhas et al., 2014).

Conclusion and future direction

As indicated by the number of publications on cell-based therapies addressing skeletal muscle regeneration it is evident that tissue engineering and cell transplantation approaches hold great promise. Despite all the great effort done by researchers, reproducible differentiation of stem cells towards myogenic lineage without genetic manipulations still remains a challenge. It is crucial to be able to derive a pure population of myogenic progenitor cells for any regenerative application. To ensure that donor cells can facilitate new muscle formation or induce endogenous regeneration, we must have a better understanding of the normal cascades of regeneration and how they are altered during muscle injuries. Additionally, there is an unmet need for improved donor cell viability, migration, and engraftment *in vivo*. To deliver cells for muscle repair, biomaterial based approaches take advantage from different delivery methods such as hydrogel encapsulation, fibrous meshes, or microporous scaffolds to mimic the stem cell niche and enhance cell survival. It is essential to work towards improving our understanding of the stem cell niche and microenvironmental factors that contribute to quiescence, stem cell activation, and modulation of endogenous regenerative cascade.

In an effort to address volumetric muscle loss defects, tissue engineering approaches have reported good advancements on functional substitutes that mimic the native target tissue. In order for such approaches to be successfully tested in a clinical setting, we must really work towards answering the key question "what does it mean for a tissue to be functional?"

In the near future, multifunctional biomaterials capable of recruiting and activating endogenous regeneration cascades and modulating host immune response will address some of these unanswered questions. Taking advantage of hiPSC technology, there is no doubt that novel tissue engineering approaches at the interface of stem cell differentiation and biomaterials can lead to the development of multicellular and multifunctional tissues. These advancements will undoubtedly improve healthcare by allowing scientists and clinicians to make breakthrough changes at the forefront of stem cell therapies, disease modeling, and drug discovery.

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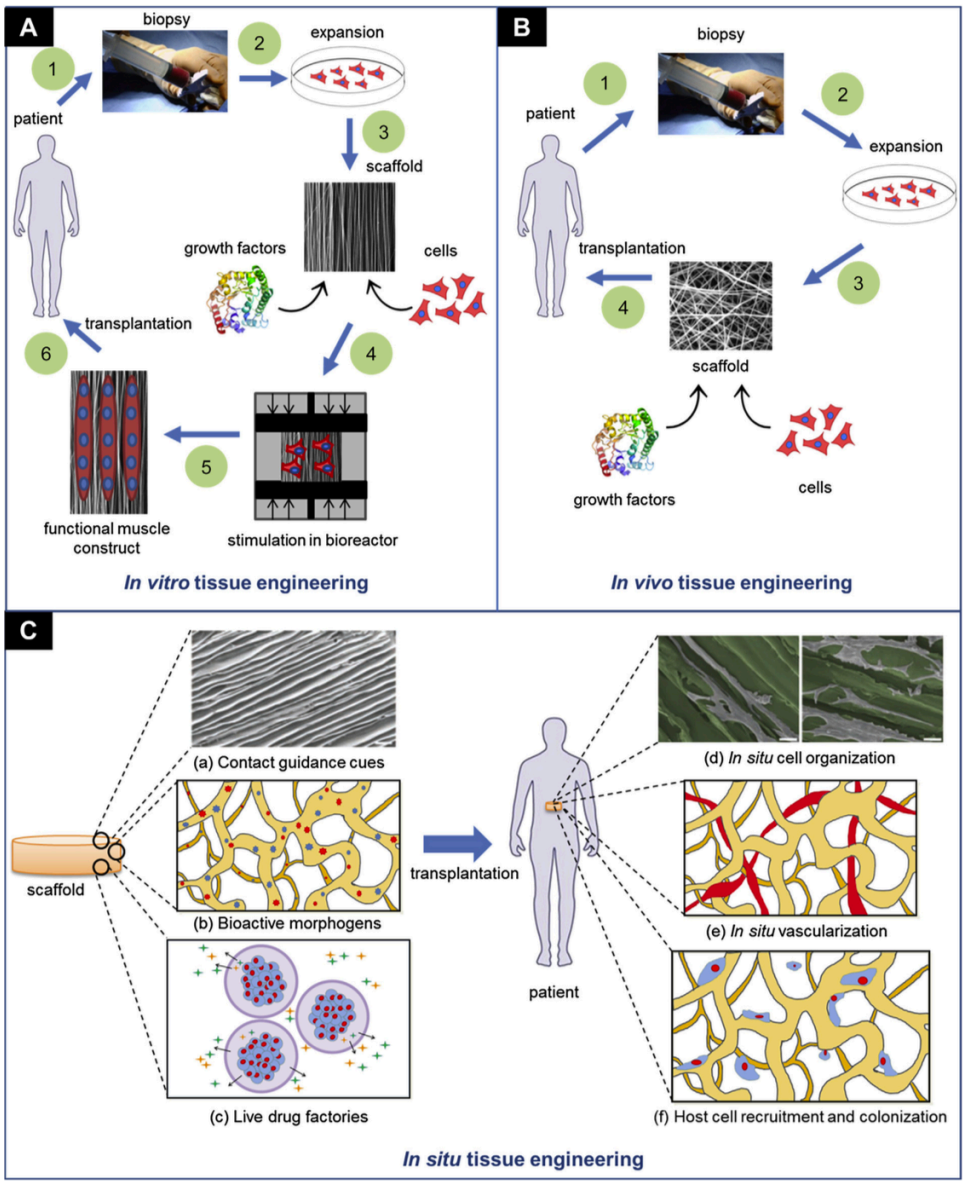

Figure 1.1 The three main approaches for skeletal muscle tissue engineering and regenerative medicine (A) In vitro tissue engineering works towards development of a functional tissue engineered muscle construct. This consists of mature muscle fibers exhibiting contractile properties, for volumetric muscle loss defects in patients (B) In vivo tissue engineering involves the transplantation of various combinations of cells, growth factors, and biomaterials into the patient. This combination provides a niche for donor cells to orchestrate the regeneration process. (C) In situ skeletal muscle regeneration typically relies on biomaterials that can guide endogenous regeneration. Picture adopted with permission from Qazi et al., 2015.

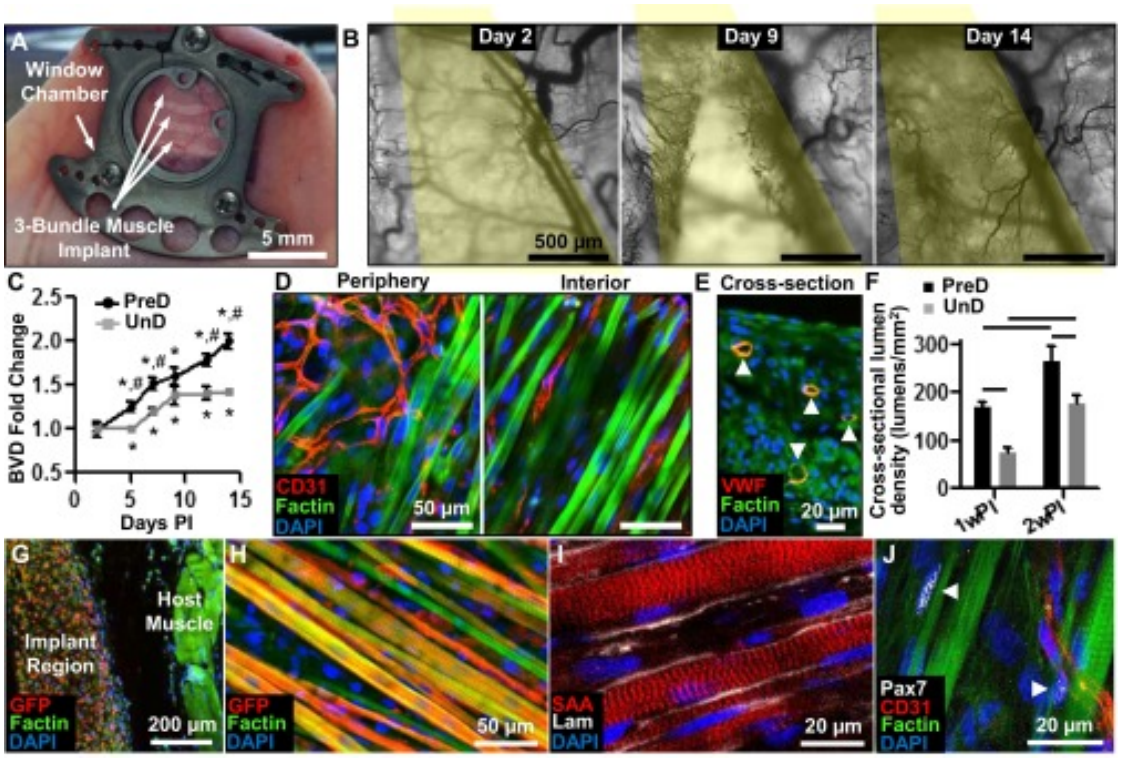

Figure 1.2 Vascular integration of implanted engineered muscle (*A*) Implanted muscle patch (*B*) Images of total hemoglobin at day 2, 9, and 14 in window chamber (yellow, implant region). (*C*) Fold change in blood-vessel density (BVD) in the implant region of predifferentiated (PreD) and undifferentiated (UnD) bundles with time PI. (*D*) Vessel organization at the periphery and interior of muscle implant. CD31 labels endothelial cells. (*E*) Cross-section of the muscle implant showing lumens of ingrown blood vessels (arrowheads). VWF, von Willebrand factor. (*F*) Increase of cross-sectional BVD from 1 wk PI to 2 wk PI. (*G*) Cross-section of implant region (GFP-positive myofibers) and underlying host muscle. (*H* and *I*) Longitudinal section of implanted bundle showing aligned and cross-striated myofibers (*H*) embedded in laminin matrix (*I*). (*J*) $Pax7^{+}$ satellite cells (arrowheads) are found at the periphery of implanted myofibers. Picture adopted with permission from Juhas et al., 2014.

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Chapter 2: Synthetic-heparin-mediated myogenic differentiation of induced pluripotent stem cells improves *in vivo* outcome

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Abstract

Designing cost effective and simple biomaterials with an inherent ability to induce lineage-specific differentiation of human induced pluripotent stem cells (hiPSCs) is a powerful tool in regenerative medicine applications. Differentiation of pluripotent stem cells into skeletal muscle cells is very challenging, often requiring genetic manipulations. In this study, we report on the development of a hydrogel-based matrix containing synthetic heparin-mimicking moieties of poly(sodium-4-styrenesulfonate) (PSS) in methacrylated-gelatin (GelMA) to drive myogenic differentiation of C2C12s and iPSC-derived myogenic progenitor cells (MPCs). Out results show that cells cultured on different concentrations of PSS undergo robust myogenic differentiation compared to those on GelMA-alone matrices. Furthermore, In vivo transplantation of these committed cells into cardiotoxin-injured tibialis anterior (TA) muscles of NOD/SCID mice reveals survival and engraftment of donor cells. The results of this study clearly demonstrate that using a unique combination of gelatin-based hydrogels with additional moieties of PSS can drive hiPSC differentiation without genetic manipulation and be used for cell transplantations in animal models.

Introduction

Human pluripotent stem cells (hPSCs) can differentiate into all three germ layers and hold great potential for cell-based therapies to address debilitating diseases and replace lost tissue (Odorico 2001). Additionally, hPSC-based tissue engineering approaches can improve our understanding of organ development and serve as an instrumental tool in drug-screening platforms. Over the past few decades, researchers

have worked towards using hPSC-derived cells for myogenic differentiation and treating muscle injuries. Differentiation of hPSCs involves a complex interplay of various factors including but not limited to medium composition, genetic factors, biological factors, and substrate physiochemical cues that work together to successfully differentiate stem cell into specific cell types. Despite efforts made in recent years, targeted differentiation of hPSCs towards myogenesis still remains a challenge.

In the past few years, many groups, including our research group, have reported protocols for derivation of myogenic progenitor cells (MPCs) from hPSCs (Barberi et al., 2007; Goudenege et al., 2012; Darabi et al., 2012; Hwang et al., 2014; Hwang et al., 2013). Substantial strides have been made in deriving MPCs using force expression of key myogenic regulatory factors (MRFs) (Darabi et al., 2008; Darabi et al., 2012; Goudenege et al., 2012). Transplantation of hPSC-derived MPCs using over-expression methods has been tested in diseased muscles of mdx mice, a model of Duchenne Muscular Dystrophy (DMD), and has been shown to contribute to muscle regeneration, despite its low engraftment efficiency (Mendell et al., 1995; Asakura et al., 2007; Montarras et al., 2005). Although genetic manipulation is an efficient method for differentiation, from a therapeutic standpoint, introducing transgenes in patients is not favorable. Borchin et al., have demonstrated that hiPSC-derived $PAX3^{+}/PAX7^{+}$ muscle precursors can be derived without transgene expression by a stringent fluorescence-activated cell sorting-based purification system (Borchin et al., 2013). The difficulty in obtaining MPCs lies in our limited knowledge about the spatio-temporal cues that regulate myogenesis. Better understanding of the expression patterns of MRFs, the

environmental inductive cues, and the appropriate markers for efficient isolation of cells of interest can be instrumental for successfully deriving MPCs.

Previous work by many groups have emphasized on the importance of the extracellular matrix (ECM) in directing myogenic differentiation and improving function, structure, and better understanding of skeletal muscle development (Huijing et al., 1999; Purslow et al., 2002; and Buck and Horwitz, 1987). Numerous studies have shown that stem cell fate and differentiation relies on the microenvironment comprised of soluble and matrix-based cues (Kang et al., 2016; Rao et al. 2015; Kang et al., 2014; Discher et al. 2009). Therefore, investigating suitable biomaterials that can drive myogenic differentiation and maturation by providing necessary biochemical cues is of great interest. Naturally occurring hydrogel-based matrices are an attractive choice due to their ability to closely mimic the native tissue. Alginate (hill et al., 2006), gelatin (Chen et al., 2012; Benton et al.,2009), collagen (Chen et al., 2015; Kroehne et al., 2008), and fibrin (Janmey et al., 2009; Grasman et al., 2017) have been heavily studied as matrices for myogenic differentiation using C2C12 cells. However, natural hydrogels are associated with batch to batch variability in properties and biofunctionality. Heparin and heparin sulfate proteoglycans (HSPG) play an important role in regulation of basic fibroblast growth factor (bFGF) signaling by interacting with FGF receptors (FGFR) and bFGF molecules (Pellergrini 2001). More importantly, bFGF signaling has been identified as a crucial component of myogenesis (Eswarakumar et al. 2005; Scata et al. 1999; Brunetti et al. 1990). Therefore, a number of studies have utilized synthetic heparin mimics for myogenic differentiation and muscle regeneration (Desgranges et al., 1999; Sangaj et al., 2010).

Synthetic matrices eliminate the batch to batch variability in properties and allow us to tailor the matrix with specific characteristics. In this study, we synthesized a methacrylated-gelatin (GelMA) based hydrogel as an adhesion substrate for C2C12s and iPSC-derived MPCs and compared the effect of conjugating styrene-sulfonate moieties within the GelMA hydrogel network at different concentrations to drive myogenic differentiation. We demonstrate that presence of poly(sodium-4-styrenesulfonate) (PSS) moieties, that act as synthetic heparin mimics, improve myogenic differentiation of murine myoblasts and hiPSC-derived MPCs as evident by expression of key myogenic proteins and fusion of myoblasts into multinucleated myotubes. When transplanted intra-muscularly, hiPSC-derived myogenic progenitor donor cells preconditioned *in vitro* with PSS-based substrates show improved *in vivo* viability and engraftment thereby contributing to muscle regeneration and formation of new myofibers.

Materials and methods

Synthesis of gelatin-based hydrogels

GelMA was prepared through methacrylation of gelatin (Sigma-Aldrich, catalog number: G1890) as described previously (Hutson et al, 2011). Briefly, 10g of gelatin was dissolved in 100mL of phosphate buffered saline (PBS) at 60°C for about 15 minutes. 8mL of methacrylic anhydride (Polysciences, catalog number: 01517) was added dropwise during ~400RPM stirring for 2 hours. The reaction mixture was then quenched using 100mL of pre-warmed PBS. The resulting GelMA was purified by dialysis (spectrum Labratories, catalog number: 132676) in miliq water at 40°C for 7

days changing the water three times a day. The mixture is then filtered through 40µm pores, lyophilized and stored at -20°C until used.

Synthesis of gelMA-PSS hydrogels

To synthesize the hydrogels, 15% (w/v) GelMA was dissolved in miliq water at 40°C and split into 4 tubes. To each tube, 0,2,5,10% (w/v) of styriene sulfonate (M_w: 80 kDa; Sigma-Aldrich, catalog number: 25704-18-1) was added. Around 0.3% (w/v) of photoinitiatior (PI), 1-[4-(2-hydroxyeth- oxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1 (Ciba Specialty Chemicals, Irgacure 2959), reconstituted in 70% ethanol was added to each tube. Each solution is then sandwiched between Bio-Rad glass plates and 1-mm spacer and allowed to polymerize under 365 nm UV light for 10 min. The polymerized hydrogels were allowed to equilibrate in PBS for 24 hours with two PBS changes. Hydrogel sheets are then punched into 2 cm^2 (area) x 1 mm (height) disks for cell seeding.

Elastic Modulus Measurements

Equilibrium swollen hydrogels in PBS were used for compression measurements. Elastic moduli were acquired using Bose electroforce 3200 Test Instrument (Bose, Minnesota, USA). Punched hydrogels were compressed between two parallel plates at a rate of 0.37mm/s. The elastic moduli were calculated using the linear region of the stress-strain curve (0-5% strain). All measurements were carried out as quadruplicates for each sample.

Swelling ratio

Swelling ratios of acellular hydrogels were calculated using a gravimetric approach. To do so, dry weight of hydrogels prior to PBS wash was measured. Hydrogels were then thoroughly washed in PBS and the weight of the hydrogels was measured in an equilibrium-swollen state. To obtain the swelling ratio, the formula $SR = W_E/W_D$ was used; where W_E and W_D are the equilibrium weight and dry weight of the hydrogels. The average and standard deviation of triplicates for each sample were calculated.

Sterilization and cell culture

Hydrogels were sterilized using 70% ethanol for 20 minutes followed by PBS washes 3 times a day for 3 days to eliminate any residual ethanol. Prior to cell seeding, hydrogels were incubated in 10% premium select fetal bovine serum (FBS) medium (Premium (FBS); Atlanta Biologicals, catalog number: S11150) for 24 hours at 37°C to enhance cell attachment. C2C12 cells were procured from ATCC (CRL-1772) and seeded at a density of 10^4 cell/cm^2 on the sterilized 2D hydrogels and cultured in growth medium (GM) containing high glucose DMEM, 10% (vol/vol) FBS (Gibco), and 1% (vol/vol) penicillin streptomycin for 3 days to reach optimum confluency for differentiation. The medium was subsequently changed to differentiation medium containing 2% (vol/vol) horse serum (HS), 1% (vol/vol) penicillin streptomycin, and 100x ITS (sigma).

hiPSC cell maintenance

L-EPCCC3 human iPSC (hiPSC) line, derived from normal human skin fibroblasts (HFBC), was kindly gifted to us from the Verma Lab at Salk Institute for Biological Sciences. The unmodified cells were cultured on matrigel (BD Bio-sciences)-coated dishes in mTeSR1 medium (Stem Cell Technologies). Cell were passaged as aggregates around 60% confluency using ReLeSR (Stem Cell Technology) for selective detachment of pluripotent cells. Each batch prepared for differentiation is passaged at least twice prior to starting day of differentiation.

Derivation and differentiation of iPSC progenitor cells

To obtain hiPSC myogenic progenitor cells, we have taken advantage of a simple three-step differentiation protocol previously established by Borchin et al. (2013). Briefly, hiPSCs colonies at low-medium (confluency ~40%) were prepared with an estimated colonies average diameter of 400-600 μm. Cells were treated with CHIR 99021, a WNT agonist and glycogen synthase kinase-3 inhibitor, at 3 μM for 4 days in serum-free Dulbecco's modified Eagle's medium F-12 (DMEM F-12) to facilitate paraxial mesoderm induction. Subsequently these mesodermal committed cells were expanded in presence of 20ng/ml of FGF2 (Sigma Aldrich catalog number: SRP4037) for an additional 14 days. Following removal of FGF2 from culture, cells are allowed to undergo myogenic differentiation in presence of insulin, transferrin, selenium (ITS) (Sigma Aldrich catalog number: I3146) medium. After 35 days of myogenic differentiation, hiPSC-derived myogenic progenitor cells acquired from the

aforementioned protocol are cultured for an additional 14 days on (i) GelMA-alone or (ii) GelMA + 2%PSS for an additional 14 days for terminal myogenic differentiation.

Immunofluorescent staining

Myogenic differentiation of C2C12s was evaluated by immunofluorescent staining for sarcomeric myosin (MF20), desmin (Des) and myogenin (MyoG). Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature followed by incubation in blocking buffer composed of 3% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Triton^{TM} X-100 in PBS for 1 hour. The cells were subsequently incubated in primary antibodies (1:200; rabbit monoclonal desmin, Abcam, catalog number ab8592), (1:100 mouse monoclocal MF20, hybdridoma bank, catalog number AB2147781), and (1:10 mouse monoclonal F5D, hybridoma bank, catalog number AB2146602) diluted in blocking buffer overnight at 4°C. The next day, primary antibodies were removed and washed using PBS and cells were incubated in blocking buffer containing secondary antibody (1:250; Alexa Fluor® 488, Life Technologies, catalog number: A12379 and Alexa Fluor® 568, Life Technologies, catalog number: A11011) for 1hr at room temperature. Nuclei were counter stained using Hoechst 33342 ((2 μg/mL; Life Technologies, catalog number: H1399) at room temperature for 10 minutes and washed with PBS. Images were acquired using an A1 Zeiss Inverted microscope and analyzed using the ImageJ software. Immunofluorescent images of all samples were acquired under the linear mode and at an exposure time of 1s. The background was uniformly subtracted from all images using a rolling ball radius method and value of 750.0 pixels.

Quantitative real-time Polymerase Chain Reaction (rt-PCR)

For RNA extraction using phenol chloroform, samples were taken from at least 4 hydrogels in each category and pooled together using TRIzol Reagent (Life Technologies, catalog number: 15596-018). For each sample, exactly 1μg of RNA was reverse transcribed to complementary DNA (cDNA) using iScript cDNA Synthesis Kit (Bio-Rad, catalog number: 17-8891) according to the manufacturer's instructions. The synthesized cDNA was analyzed via quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR Select Master Mix (Life Technologies). Expression levels of target genes were normalized to 18S expression as house-keeping gene and delta Ct values were calculated as $Ct_{target} - Ct_{18S}$. The relative gene expression profiles were presented as $2^{-\Delta\Delta Ct}$. The primers used in this study are listed in Supplementary Table S1.

Cell transplantation and histological analysis

Animal experiments were carried out according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego (UCSD), and National Institute of Health (NIH). NOD.CB17-Prkdcscid/J (hereafter, NOD/SCID) mice were anesthetized 48 hours prior to cell transplantation intraperitoneally with ketamine (100 mg/kg) and xylazine (10 mg/kg), to injure their tibialis anterior (TA) muscle with 15μl of cardiotoxin (CTX) from *Naja mossambica mossambica* (10 μM, Sigma; cat# C9759). 2 days following the injury, L-EPCCC3 hiPSC MPCs differentiated for 14 days on (i) GelMA or (ii) GelMA + 2%PSS were trypsinized for 10 mins and spun down at 1000 RPM for 5 mins to form a pellet. The pellets were re-suspended in PBS and intramuscularly injected into the CTX-injured TA

muscles at 5.0×10^5 cells/15µl/TA). Two weeks post transplantation, treated TA muscles were harvested and embedded in Optimal Cutting Temperature (OCT) for cryosectioning. Survival and engraftment of the hiPSC progenitor cells in the injured muscles were analyzed using histology and immunofluorescence imaging.

Immunofluorescence staining of cryo-sectioned tissue samples

For immunofluorescence staining of tibialis anterior (TA) muscles transplanted with donor cells, samples were first embedded in optimal temperature cutting compound (OCT) for cryosectioning. The sections (~15 µm thickness) were fixed with 4% PFA for 10 min at room temperature, permeabilized with 0.3% Triton X-100, blocked with 3% BSA for 1hr at room temperature, and stained with anti-human lamin A/C (1:100; Vector Laboratories), and rabbit anti-laminin (1:200; Abcam). The next day, primary antibodies were removed and washed using PBS and slides were incubated in blocking buffer containing secondary antibody (1:250; Alexa Fluor® 488, Life Technologies, and Alexa Fluor® 568, Life Technologies) for 1hr at room temperature. Nuclei were counter stained using Hoechst 33342 ((2 µg/mL; Life Technologies) at room temperature for 10 minutes and washed with PBS to visualize transplanted donor cell survival and their engraftment within the host tissue. To quantify the data obtained, total number of lamin A/C nuclei from five fields of view were counted. Percentage of donor cells centrally located within a myofiber or positioned under the basal lamina was calculated and reported as “percentage of engraftment”.

Statistical analysis

All values are presented as mean ± standard deviation and statistical significance was determined by two-tailed unpaired Student's t-test or single-factor analysis of variance (ANOVA) with Tukey's Multiple Comparison Test (*$p<0.05$, **$p<0.01$, and ***$p<0.001$). GraphPad Prism software was used to perform all the statistical analysis.

Results

Structural and mechanical properties of gelMA-based matrices

Mechanical property of GelMA-based hydrogels was measured in terms of elastic modulus (Young's modulus). With increasing concentration of PSS in the GelMA-based hydrogels the rigidity of the hydrogels increases as seen from the elastic modulus measurements (fig 2.1A). The structural properties of the hydrogel networks can be controlled by changing the precursor concentration and/or crosslinker. While the GelMA precursor concentration was kept constant at 15%, the PSS concentration was varied from 0%, 2%, 5%, to 10% and thereby changing the overall stiffness of the hydrogel. The equilibrium swelling ratio of the hydrogels are given (fig 2.1B). Increasing the concentration of the precursor PSS exhibited an increase in the swelling ratio of the hydrogels at 37°C, however this increase is not significant after statistical analysis.

Morphology of hiPSC-derived MPCs and C2C12s on gelMA-based matrices

The effect of PSS-based matrices on myogenic differentiation of hiPSC-derived MPCs and murine myoblast C2C12s was evaluated as a function of time. With all

culture conditions, both cell lines grew to maximum confluency in GM as a monolayer and began to elongate in shape and fuse to neighboring cells by day 3 with no obvious differences in cell shape and morphology (figs 2.2 and 2.6). Proliferation of the cells was observed to be slightly faster on GelMA alone matrices; however, all matrices supported cell attachment and proliferation. A higher percentage of C2C12 cell death (~10%) was observed on 5% and 10% PSS-based GelMA substrates.

Matrix-mediated myogenic differentiation of C2C12s

The immunofluorescence staining for sarcomeric myosin (MF20) and desmin (DES) suggest that cells cultured on substrates containing PSS moieties underwent myogenic differentiation more effectively (fig 2.3). Cells cultured on varying percentages of PSS represent an enriched population of MF20/DES double positive myotubes. Desmin expression is observed predominantly in all cells across all samples and MF20 expression is more selective. Cells grown on the control substrate (GelMA) exhibit more "choppy" myotubes with sporadic expression of MF20, 7 days post differentiation.

Myogenin staining was performed to assess myogenic maturation of C2C12s cultured on (i) GelMA (ii) GelMA + 2% PSS, (iii) GelMA + 5% PSS, and (iv) GelMA + 10% PSS (fig 2.5A). Cells grown on all PSS-based substrates underwent myogenic maturation as depicted by number of myogenin positive nuclei and formation of multinucleated myotubes 7 days post differentiation. Quantification of the staining data suggests that all PSS-based substrates support terminal differentiation and maturation

regardless of the concentration of PSS or stiffness of the substrate at a significantly higher rate as compared to the control (fig 2.4).

Extent of myogenic differentiation of C2C12s was quantified based on (i) differentiation index and (ii) fusion index which characterizes cells based on MF20/DES double positive myotubes, and MF20 positive cells containing two or more nuclei, respectively. The differentiation index showed a significantly higher number of differentiated cells on PSS-based matrices compared to the control matrix (Fig 2.4). Similarly, a significant fraction of cells was found to be multinucleated when cultured on PSS-based substrates, as highlighted in Fig 3B. Additionally, myotube width and length was quantified for C2C12 differentiation as an indication of maturation (Figs 2.4). Together, the quantification results suggest that 2% PSS is sufficient for myogenic maturation of murine myoblasts (Figs 2.4 and 2.5B).

Matrix-mediated myogenic differentiation of hiPSC-derived myogenic progenitor cells

Similar to the results obtained from C2C12s, PSS-based GelMA hydrogels mediated myogenic differentiation of hiPSC-derived MPCs more effectively than their GelMA-alone counterpart. Presence of MF20/DES double positive multinucleated myotubes and myogenin positive nuclei were only prominent on GelMAPSS substrates while the GelMA-alone cells only expressed DES after 14 days of culture (Figs 2.7 and 2.8). Since control matrices did not yield any noticeable myogenic differentiation or maturation of hiPSC-derived MPCs, we did not perform quantifications for those results.

Gene expression analysis of matrix-mediated myogenic differentiation of hiPSC-derived MPCs

Myogenic commitment of hiPSC-derived MPCs was examined by gene expression analysis of key myogenic markers such as PAX7, MyoD, MyH1, MyoG, and Des (Fig 2.9). The gene expression patterns revealed that the cells cultured on PSS-based GelMA matrices exhibit an upregulation of early MRFs, Pax7 and MyoD by Day 7 of differentiation followed by their downregulation with culture time. In contrast, late upregulation of maturity markers MyoG, MyH1, and Des compared to control confirms our previous findings using immunofluorescence analysis. The data overall demonstrates that PSS-mediated myogenic differentiation of hiPSC-derived MPCs improves the extent of myogenic commitment and differentiation as compared to the control matrix.

Cell Transplantation in NOD/SCID Mice

We next determined the effect of PSS in promoting in vivo survival and function of transplanted iPSC-derived MPCs. 14 days post transplantation, the TA muscles were harvested and characterized to assess survival and in vivo engraftment of donor cells. The muscle sections were stained for lamin A/C which marks human donor cells as well as laminin which marks host muscle fibers. Interestingly, pre-conditioning of MPCs on gelMA-PSS *in vitro* improved their in vivo survival and engraftment. Quantification of histological sections showed an approximate 40-fold increase in donor cells survival when they were differentiated on PSS-based matrices (Fig 2.10).

Discussion

Biomaterial assisted differentiation of stem cells is a unique approach with applications in stem cell therapy, wound healing, tissue regeneration, and organogenesis (Lutolf et al., 2005; Lutolf et al., 2009; Hwang et al., 2011). In recent studies, biomaterial features such as rigidity, surface topography, hydrophobicity, and presence of functional groups have been highlighted and shown to influence stem cell differentiation (Benoit et al., 2008; Ayala et al., 2011; Dalby et al., 2007; Engler et al., 2006; Huebsch et al., 2010). Gelatin-based substrates have been extensively studied as scaffolds in stem cell tissue engineering (Talwar et al., 2001; Nichol et al., 2010). Since gelatin is derived from collagen, a natural ECM component, it contains cell-binding motifs that facilitate adhesion of hiPSCs and C2C12s to the underlying matrix (Hutson et al., 2011). Additionally, Gelatin's ability to degrade in response to cell secreting factors allows cells to remodel their matrix during the course of differentiation (Hutson et al., 2011; Chen et al., 2012; Benton et al., 2009).

In this study, we have evaluated the effect of a heparin-mimicking polymer, PSS, on myogenic differentiation of C2C12s and iPSC-derived MPCs. Previous work by our group has shown that exogenous supplementation of PSS, a widely used heparin mimic, can enhance myogenic differentiation using C2C12 cells (Sangaj et al. 2010). Changes in cell morphology such as elongation and fusion of cells cultured on GelMA-based hydrogels with varying concentration of PSS was monitored during the course of differentiation. In this study, the myogenic differentiation of precursor cells was characterized using immunofluorescence staining against key myogenic markers as well as myotube formation, multinucleation, and cell morphology. In presence of low-serum

differentiation medium, myoblasts start fusing with one another to form multinucleated myotubes expressing maturity markers MF20, Des, and MyoG. The data clearly demonstrates that PSS-mediate myogenic differentiation of C2C12s and hiPSC-derived MPCs is associated with higher expression of key myogenic factors as well as increased formation of multinucleated myotubes. This could be in part explained by the presence of PSS moieties that sequester bFGF, thereby pushing cells towards terminal differentiation. Samples containing 2% PSS showed highest improvement in differentiation index; however, all samples containing PSS improved fusion and overall differentiation of C2C12s.

To examine the effect of PSS on iPSC-derived MPC, we decided to use gelMA hydrogels containing 2% PSS. Gene expression analysis and immunofluorescence characterizations shows similar trend as observed in C2C12s. Incorporation of PSS moieties improves myogenic progenitor differentiation significantly. Myogenin expression levels increase with culture time and result in formation of mature and multinucleated myotubes (Fig 2.8). Immunofluorescence staining and gene expression analysis suggests that cells cultured on gelMA are not able to undergo maturation and differentiation as effectively (Figs 2.7 and Fig 2.8). This can be partly due to lack of bFGF withdrawal in gelMA matrices.

Granted that addition of 2%PSS can improve myogenic differentiation of iPSC-derived MPCs, we aimed to investigate the *in vivo* transplantation efficacy of the cells in cardiotoxin-injured NOD/SCID mice. We have previously established that hESC-derived progenitor cells can undergo myogenic differentiation *in vitro*; however, only a small percentage survive *in vivo* and the majority of donor cells reside within the

interstitial space (Hwang et al. 2012). Upon harvesting the tissues 14 days post transplantation, we observed that muscles treated with PSS-derived MPCs show improved viability and engraftment *in vivo*. From this data, we can conclude that the extent of myogenic differentiation plays a crucial rule for *in vivo* survival and donor cell engraftment. The gene expression profiles previous obtained suggests that differentiation on 2% PSS results in a mixture of early myoblasts expressing PAX7 and MyoD as well mature myotubes expressing MyoG, Des, and MyH1. In Contrast, most cells cultures on GelMA matrices are under-differentiated and do not display significant expression of any of the myogenic markers. The information gathered from the cell transplantation study suggests that a mixed population of mature myotubes and less differentiated myoblasts is associated with enhanced in vivo outcome as compared to a population of under-differentiated progenitor cells.

In conclusion, the results summarized in this study illustrate the role of matrix-mediated myogenic differentiation of C2C12s and hiPSC-derived MPCs. It is interesting to note that addition of 2% PSS moieties in the matrix composition can have a drastic affect in expression of myogenic factors, fusion of myoblasts, and overall size of myotubes. The study further highlights the importance of the degree of *in vitro* myogenic commitment of donor cells in cell therapy applications. The findings of this study can be utilized for myogenic differentiation of hPSCs without the need for genetic modifications.

Acknowledgement

Chapter 2, in part, is currently being prepared for submission for publication of the material: Sara Hariri, Heemin Kang, Jung-mi Kang, and Shyni Varghese*. The thesis author was the primary lead and author of this paper.

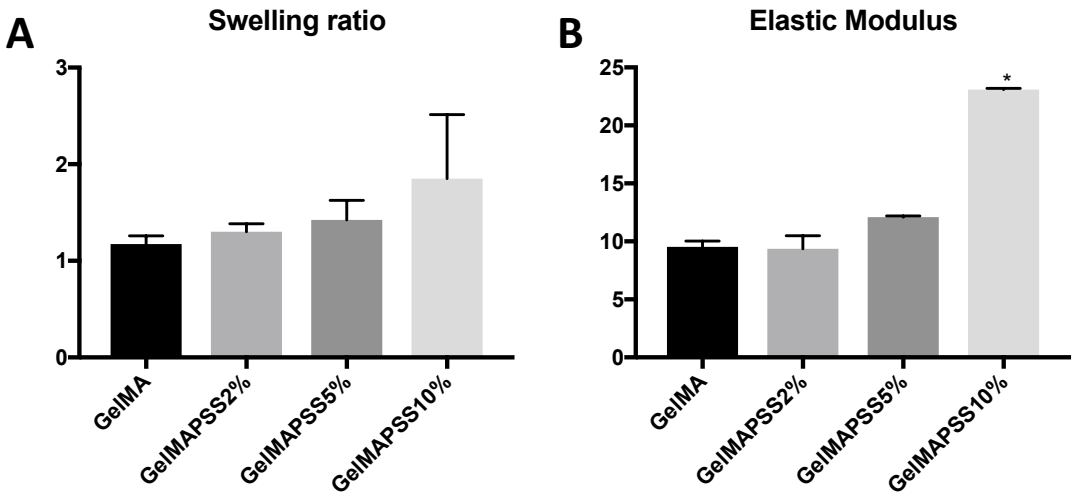

Figure 2.1 Mechanical characterization of gelati-based hydrogels. Elastic modulus of gelMA hydrogels with varying percentage of PSS (A) and equilibrium swelling ratio measured as SR = W_E/W_D (B)

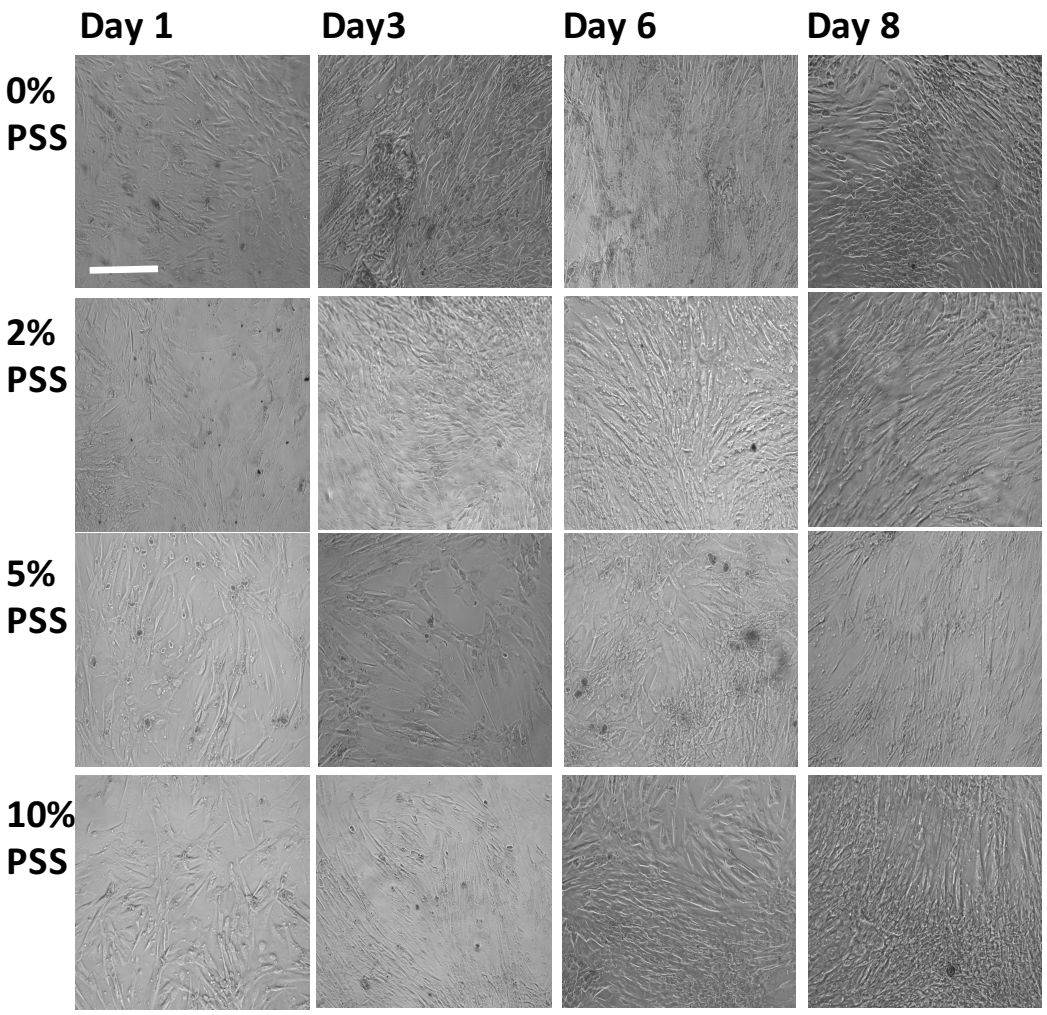

Figure 2.2 Attachment and proliferation of C2C12s on GelMA matrices. Bright-field images of C2C12s after 1, 3, 6, and 8 days of culture on gelMA hydrogels with varying concentration of PSS 0, 2, 5, and 10%. Scale bar is 50μm.

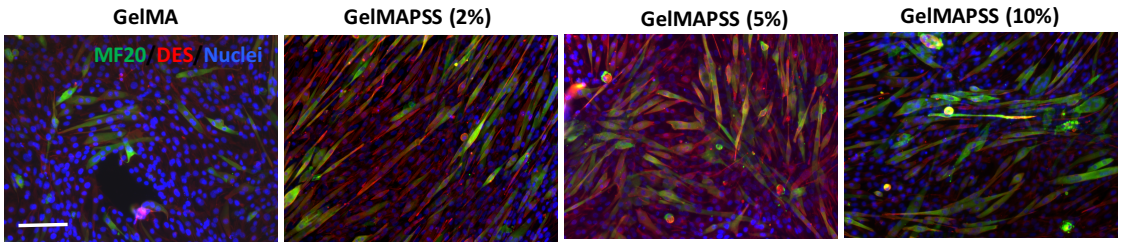

Figure 2.3 Immunofluorescence staining showing the extent of myogenic differentiation. Immunofluorescence staining of C2C12s cultured on varying concentration of PSS using MF20 (green), DES (red), and nuclei (blue). Evidence of multinucleated myotubes and MF20/DES double positive cells. Scale bar is 100um.

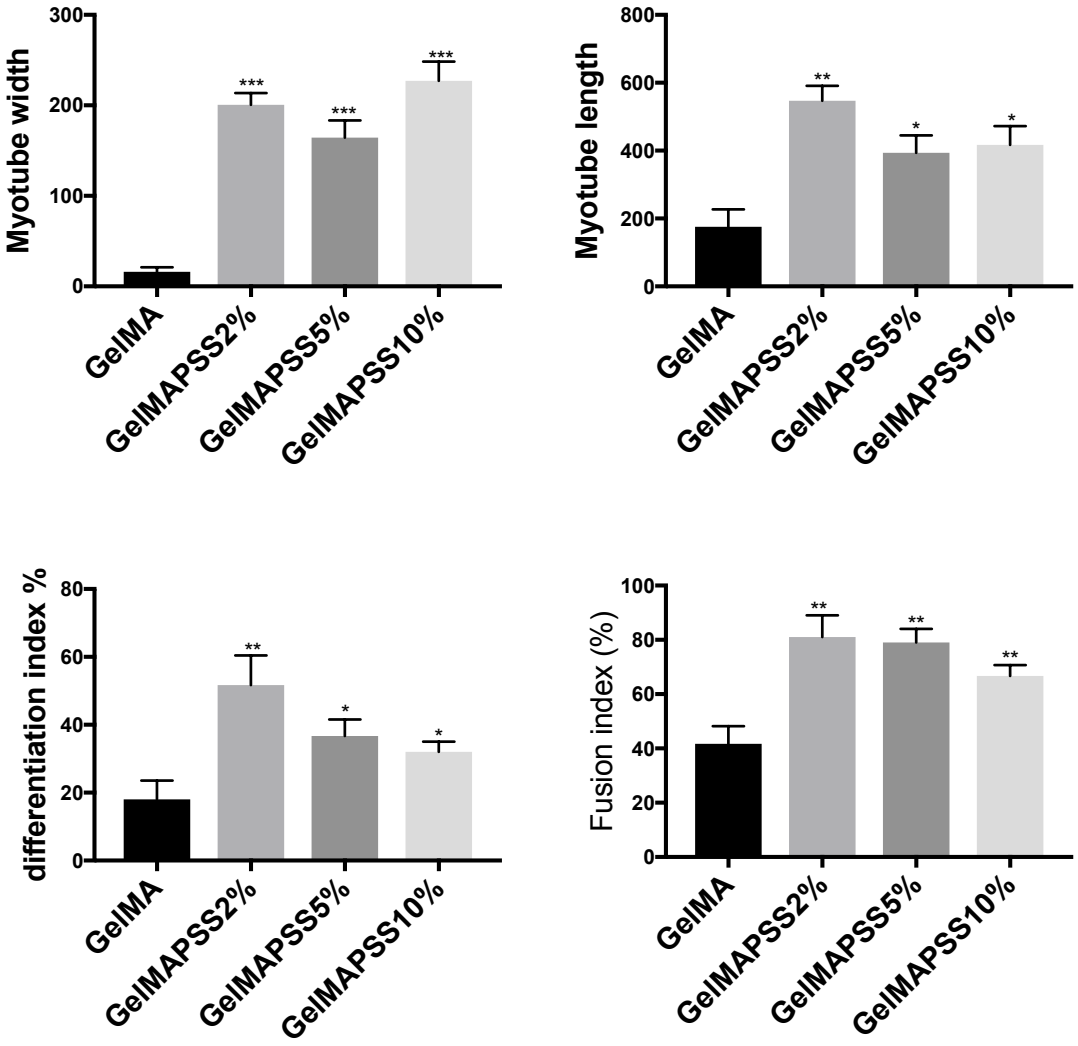

Figure 2.4 Cell shape and differentiation quantification. Quantification analysis using myotube width and length measurements (A and B). Estimated differentiation indices of PSS-derived myogenic progenitor cells (C) and estimated fusion indices of differentiated cells (MF20 positive cells) cultures on PSS-based matrices. n=341, 320, 412 and 311, respectively. *p,0.1, **p<0.01, and ***p<0.001.

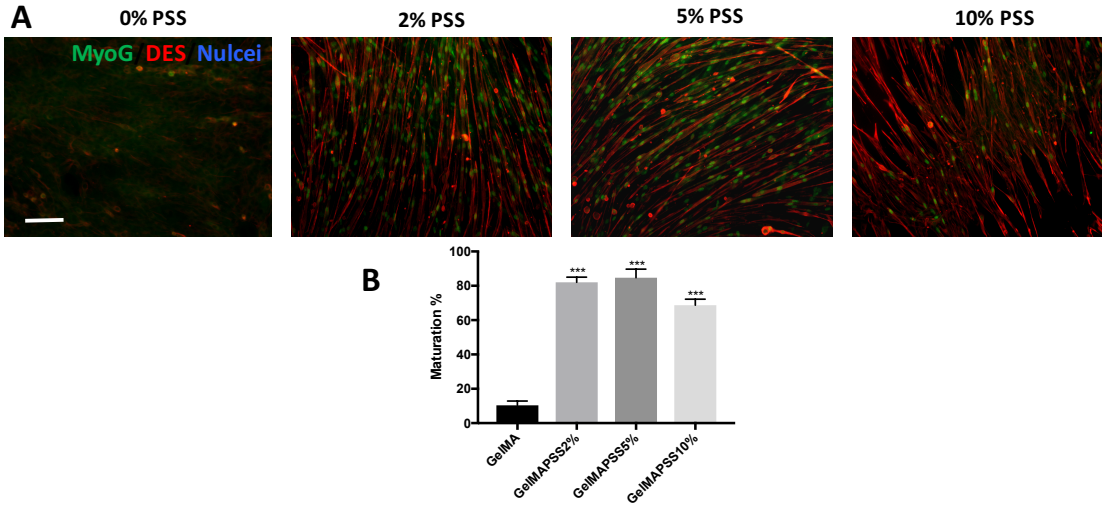

Figure 2.5 Immunofluorescence staining to show maturation of C2C12s. Staining for MyoG (green), DES (red) and nuclei (blue) of C2C12 cells cultured on 0%, 2%, 5%, 10% concentration of PSS (A). Quantification analysis of mature myotubes containing 2 or more MyoG positive nuclei. Scale bar is 50μm. n=330, 291, 280 and 319, respectively. *p,0.1, **p<0.01, and ***p<0.001.

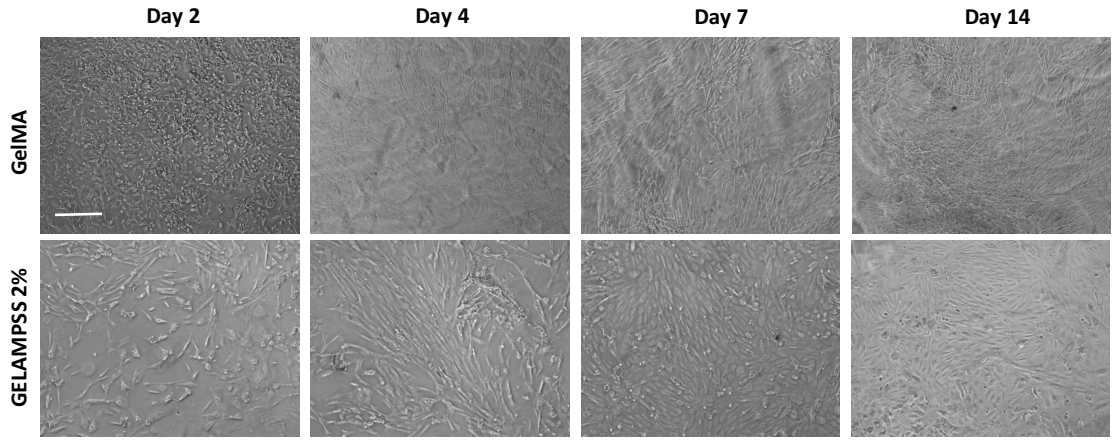

Figure 2.6 Attachment and proliferation of hiPSC-derived MPCs on GelMA-based matrices. Bright-field images of MPCs after 2, 4, 7, and 14 days culture on GelMA or GelMAPSS (2%) hydrogels. Scale bar is 50μm.

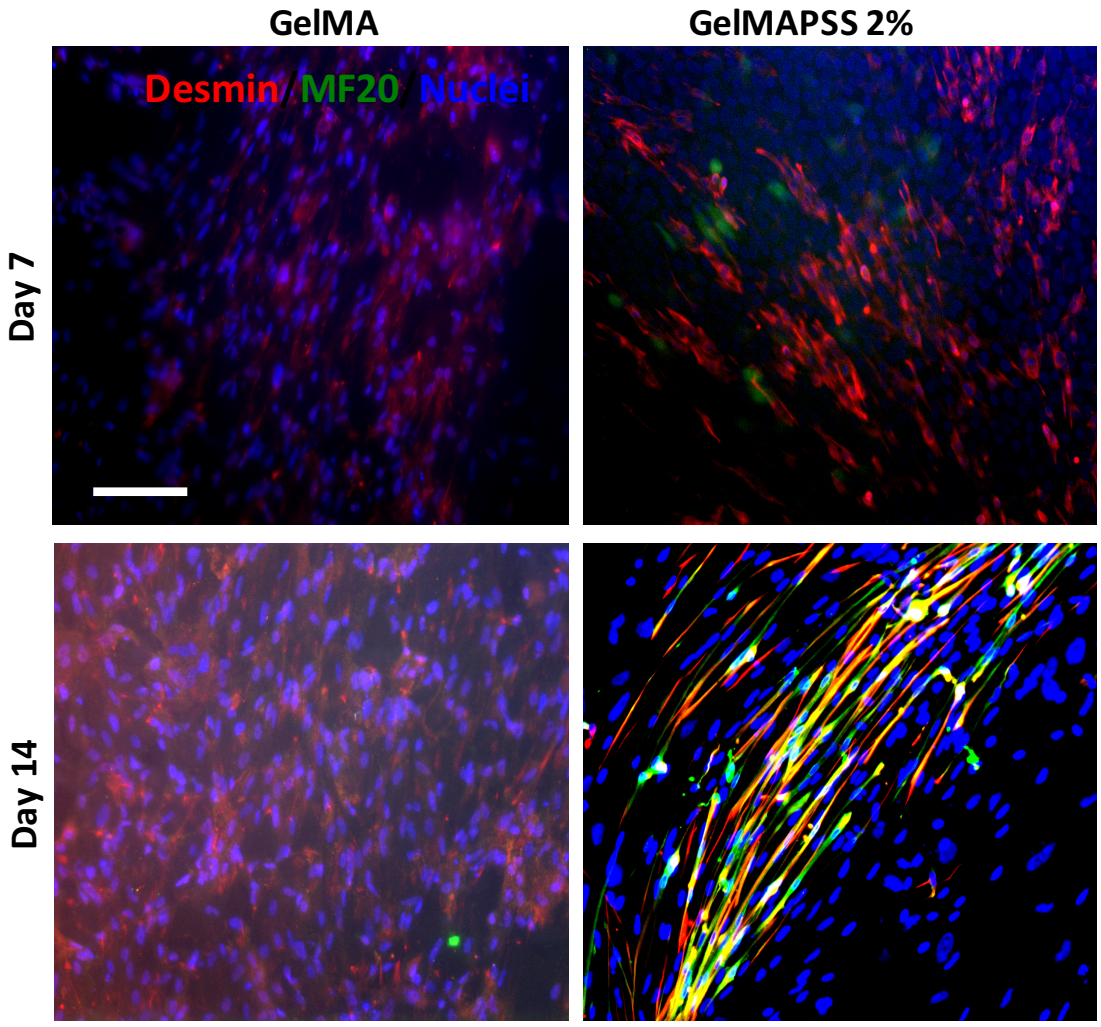

Figure 2.7 Immunofluorescence staining showing the extent of myogenic differentiation. Immunofluorescence staining of hiPSC-derived MPCs cultured on GelMA or GelMAPSS (2%) using MF20 (green), DES (red), and nuclei (blue). Evidence of multinucleated myotubes and MF20/DES double positive cells. Scale bar is 100um.

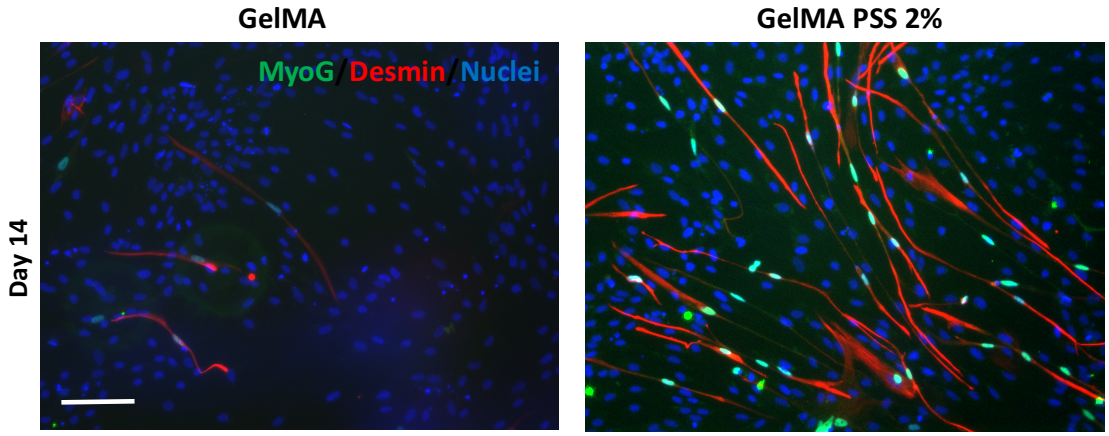

Figure 2.8 Immunofluorescence staining to show maturation of hiPSC-derived MPCs. Staining for MyoG (green), DES (red) and nuclei (blue) of MPCs cultured on GelMA or GelMAPSS (2%). Scale bar is 100 μm

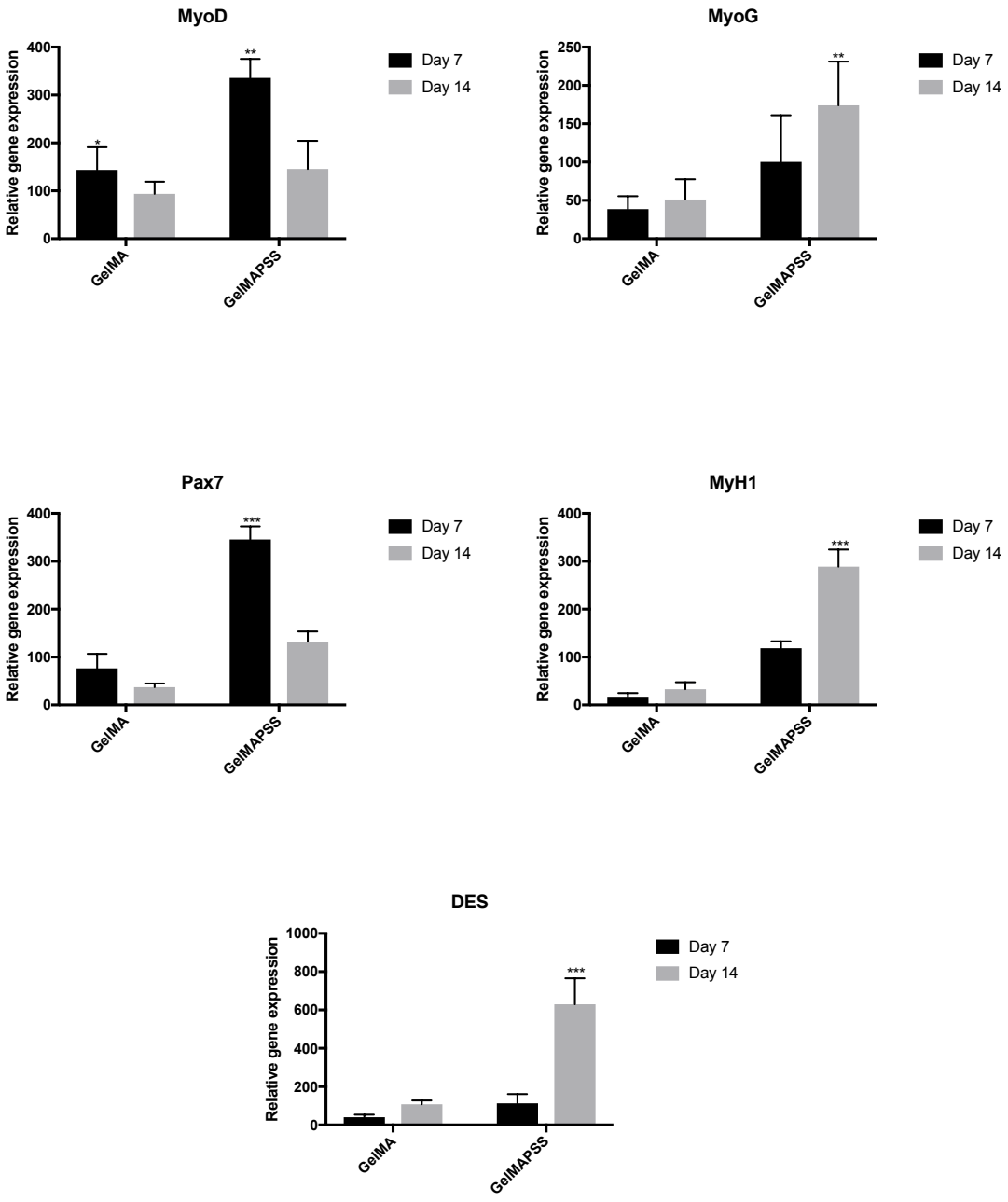

Figure 2.9 Gene expression analysis of *in vitro* differentiation. Gene expression profiles of hiPSC-derived myogenic progenitor cells differentiated on GelMA or GelMAPSS (2%) matrices. Analysis done on days 7 and 14 of differentiation. Statistical analysis was performed among cells cultured between two time points of differentiation. $p<0.05$, $**p<0.01$, and $***p<0.001$.

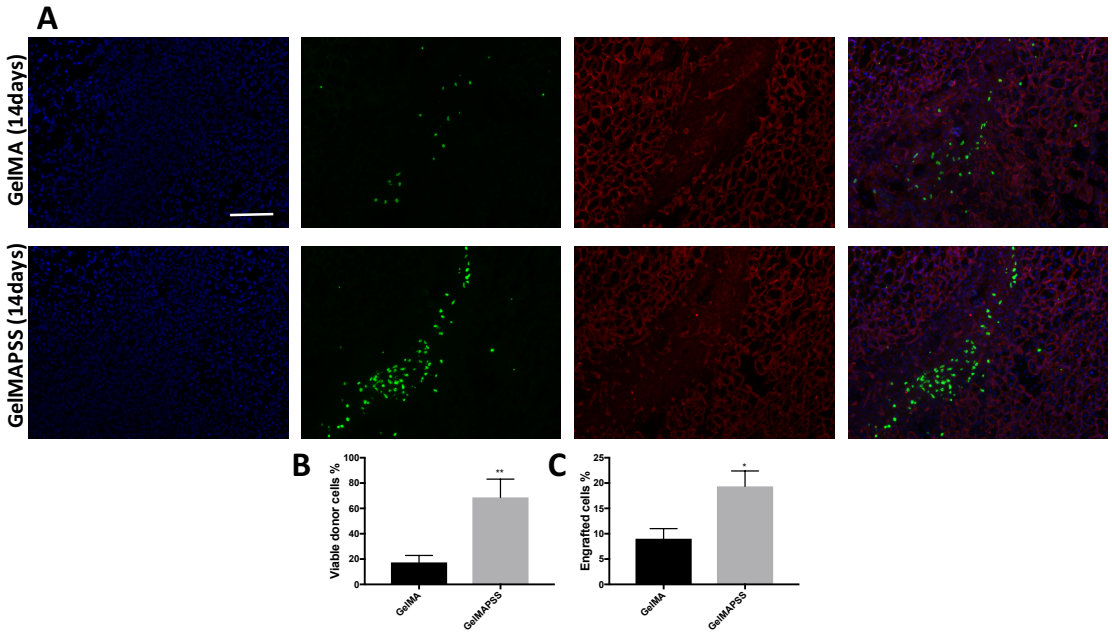

Figure 2.10 Intramuscular transplantation of hiPSC-derived myogenic progenitors in cardiotoxin injured NOD/SCID mice. Immunofluorescence staining for human-specific lamin A/C (green), laminin (red), and nuclei (blue). Scale bar is 100 μm (A) quantitative estimation of the total number of donor cells (B) quantitative estimation of the contribution of donor cells to the host myofibers 14 days post transplantation (C)

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Chapter 3: Derivation of an expandable population of hiPSC-derived myogenic progenitor cells and their *in vivo* engraftment in mdx mice

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Keywords: Human induced pluripotent stem cells, myogenic progenitor cells, Myogenic differentiation, Duchenne Muscular Dystrophy, multinucleated myotubes.

Abstract

Current approaches for generating large number of engraftable human skeletal muscle myogenic progenitor cells from pluripotent stem cells requires in vitro overexpression of key myogenic regulatory factors. To apply our understanding from animal studies to the clinic, it would be preferable to eliminate or minimize the risks associated with random genomic integration by developing differentiation protocols that do not use genetic manipulations. Here, we are reporting an approach to derive large numbers of myogenic progenitor cells that can be passaged while maintaining their phenotype and be used for transplantation in mdx/scid mice. We show that the progenitor population is a mixture of less differentiated satellite-cell-like mononucleated cells expressing Pax7 as well as more differentiated multinucleated myotubes expressing MF20, desmin, and myogenin. Transplantation of this mixed population of early and late myogenic progenitor cells results in formation of hybrid myofibers from fusion of donor cells with injured host fibers as well as contribution to the satellite cell compartment. This transgene-free derivation protocol is desirable for clinical applications to obtain large number of progenitor cells.

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked progressive skeletal muscle disease caused by mutations in the dystrophin gene. This disease presents itself in boys around the age of four years old and is associated with advanced muscle wasting and weakness. Dystrophin provides a structural link between the muscle cytoskeleton and extracellular matrix via transmemebrane dystrophin-glycoprotein complex (DGC)

and is responsible for the overall integrity of the muscle tissue (Ervasti et al., 2008). Lack of dystrophin protein results in rapid cycles of myofiber degeneration/regeneration due to instability of the overall tissue which leads to gradual replacement of muscular tissue with adipose tissue and fibrosis (Miyagoe-Suzuki et al., 2012; Goncalves et al., 2006). Patients suffering from DMD experience rapid muscle atrophy that leads to wheelchair dependency by the age of 10, assisted ventilation by 20 years of age, and premature death soon after.

To explore treatment options, animal models that can mimic the physiological and pathological disorder in humans are always needed. An appropriate animal model is one with the same genetic basis as well as comparable disease progression and pathology as in humans. To date, over 60 dystrophin-deficient animal models have been described in the literature (McGreevy et al., 2015). Innovative technologies such as gene editing, cell-based therapies, and pharmaceutical therapies can be tested using these animal models. A widely used animal model for studying DMD is the *mdx* mouse model. This model displays a mutation in exon 23 of the DMD gene that introduces a premature stop codon and leads to the absence of the full-length dystrophin protein (Bulfield et al., 1984). This model mimics many of the clinical features of the disease but it displays less serves symptoms due to upregulation of utrophin (Arechavala-Gomeza, et al., 2010). Some other animal models for studying DMD include the dystrophin/utrophin double knockout mdx/utrn$^{-/-}$) mouse, and the dystrophin-deficient golden retriever dog (GRMD) (Berry et al., 2007; Vieira et al., 2015).

Pioneering studies initiated in the late 1980s (Partidge et al., 1989) and expanded further in the 1990s (Brussee et al., 1999; Gussoni et al., 1997; Gussoni et al., 1992)

began to investigate cell based therapies to address muscular dystrophies. In the early stages of research, many groups focused on the transplantation of healthy adult myoblasts, aiming to facilitate muscle regeneration by the fusion of donor cells with recipient's cells to form new hybrid fibers. In such approach, adult myoblasts were isolated from muscle biopsies of healthy donors and expanded *ex vivo* in preparation for transplantation. Major limiting factors including limited migration capacity beyond injection site and poor viability *in vivo* as well low expansion capacity and changes in phenotype *in vitro* resulted in obtaining discouraging results both in animal models and clinical investigations (Guerette et al., 1997; Qu et al., 1998; Fan et al., 1996; Patridge et al., 1998). To date, several research groups have been working towards the goal of overcoming these barriers and finding the ideal cell source for cell based therapies to address muscular dystrophies.

Satellite stem cells are skeletal muscle specific adult stem cells residing between the basal lamina and sarcolemma of each myofibers (Montarras et al., 2005). Satellite cells are known to express a paired box homeodomain-containing transcription factor, Pax7 (Seale et al., 2000). Upon injury, quiescent satellite cells are activated to give rise to proliferative myoblasts that fuse with pre-existing myofibers to repair lost or injured fibers. Meanwhile, a small subset of satellite cells remain quiescent by asymmetric division to repopulate the stem cell niche for future rounds of regeneration (Rosenblatt et al., 1995). Many decades of rigorous work have lead scientists to identify protocols for pure isolation and preparation of mouse satellite cells (Collins et al., 2005; Montarras et al., 2005). Collins and colleagues demonstrated that transplantation of a single muscle fiber containing 7 or less satellite cells can generate over 100 new myofibers (Collins et

al., 2005). Later on, Sacco et al., showed that a single luciferase-expressing muscle stem cells can be tracked using in vivo bioluminescence imaging to observe the dynamics of muscle stem cell behavior during muscle repair. They further confirmed that the progeny of a single transplanted cell can both self-renew and generate new muscle fibers (Sacco et al., 2008). For proper isolation of satellite cells using fluorescent activated cell sorting (FACS) techniques, several surface markers such as M-Cadherin (Irintchev et al., 1994), CD34 (Beauchamp et al., 2000), syndecan-3/4 (Cornelison et al., 2004), alpha7beta1-integrin (Burkin et al., 1999), and the chemokine receptor CXCR4 (Ratajczak et al., 2003) has been identified. A highly specific population of $CD45^{-}$/Sca-1^{-}/Mac-1^{-}/$CXCR4^{+}$/β1-$integrin^{+}$/$CD34^{+}$ has been shown to have *in vitro* and *in vivo* myogenic potential (Sherwood et al., 2004; Cerletti et al., 2008).

There is great body of evidence that satellite cells are a potent cell source for skeletal muscle regeneration; However, protocols established for isolation of murine satellite cells cannot be applied to isolation of human satellite cells since surface markers are not translatable to the human model. To date, investigators have identified N-CAM (CD-56) as a surface marker of satellite cells in human tissues (Dreyer et al., 2006; Crameri et al., 2004). There is great need for a more stringent combination of positive and negative markers to obtain a pure population of muscle specific progenitor cells that can give rise to myofibers upon transplantation. Additionally, despite great effort in identifying protocols for satellite cells isolation, obtaining sufficient number of cells for transplantation applications and strategies for their *in vitro* expansion without

loss of phenotype and engraftment capacity remains a challenge (Montarras et al., 2005).

More recently, impactful work using pluripotent stem cells (PSCs) has revolutionized the idea of cell based therapies. PSCs display the ability to expand on a culture dish and differentiate into different specialized cell types. One of the most attractive advantages of using PSCs is the prospect of deriving large quantities of progenitor cells for tissue engineering and regenerative applications. The breakthrough discovery of induced pluripotent stem cell (iPSC) technology enables scientists to generate patient-specific cell populations for autologous transplantations (Park et al., 2008; Takahashi et al., 2007, Yu et al., 2007). *In vitro*, iPSC-derived progenitor cells display extensive expansion capacity and the ability to differentiate into all three germ layers. Generation of functional human skeletal myogenic progenitor cells from human embryonic stem cells (hESCs) and iPSCs has been reported by several groups (Hwang et al., 2013; Hwang et al., 2014; Darabi et al., 2008; Borching et al., 2013; Darabi et al., 2012). Transplantation of PSC-based progenitor cells in murine models has been well documented; however, most have shown minimal engraftment and migration (Barberi et al., 2007; Hwang et al., 2013; Kim et al., 2017; Zheng et al., 2006).

To date, PSC-based derivation of myogenic progenitor cells heavily depends on over-expression of myogenic regulatory factors such as Pax7 (Darabi et al., 2012; Kim et al., 2017) or MyoD (Goudenege et al., 2012; Albini et al., 2013; Tedesco et al., 2012) using lentiviral vectors. Several other groups have proposed transgene-free strategies to recapitulate myogenesis *in vitro* (Borchin et al., 2013; Shelton et al., 2014; Hwang et

al., 2013; Xi et al., 2017). Non-viral approaches are considered to be safer for clinical applications due to their integration-free nature (Hardee et al., 2017).

In this study, we take advantage of a simple three-step derivation protocol previously established by Borchin et al. (2013) to obtain pre-mature myogenic progenitor cells. The protocol is modified to increase the myogenic conversion efficiency and sub-populations of early myogenic cells expressing satellite-cell-like marker, Pax7, and late myogenic cells expressing maturity markers such as MyoG, MF20, and desmin are characterized within our culture system. The engraftment potential of these cells is tested in mdx/scid mice and shown to contribute to regeneration.

Materials and Method

Cell culture maintenance

L-EPCCC3 human iPSC (hiPSC) line, derived from normal human skin fibroblasts (HFBC), was kindly gifted to us from the Verma Lab at Salk Institute for Biological Sciences. The unmodified cells were cultured on Matrigel (BD Bio-sciences)-coated dishes in mTeSR1 medium (Stem Cell Technologies). Cell were passaged as aggregates around 60% confluency using ReLeSR (Stem Cell Technology) for selective detachment of pluripotent cells. Each batch prepared for differentiation is passaged at least twice prior to starting day of differentiation.

Generation of myogenic progenitor cells using a monolayer approach

To obtain hiPSC myogenic progenitor cells, we have taken advantage of a simple three-step differentiation protocol previously established by Borchin et al.

(2013). Briefly, hiPSCs (confluency ~40%) were treated with CHIR 99021, a WNT agonist and glycogen synthase kinase-3 inhibitor, at 3 μM for 4 days in serum-free Dulbecco's modified Eagle's medium F-12 (DMEM F-12) to facilitate paraxial mesoderm induction. Subsequently these mesodermal committed cells were expanded in presence of 20ng/ml of FGF2 (Sigma Aldrich) for an additional 14 days. Following removal of FGF2 from culture, cells are allowed to undergo myogenic differentiation in presence of insulin, transferrin, selenium (ITS) (Sigma Aldrich) medium. After 35 days of myogenic derivation, hiPSC-derived myogenic progenitor cells were trypsinized and filtered through 70μm cell strainer to get rid of debris, clumps, and excess extracellular matrix proteins and obtain a homogenous mixture of single cells. The cell pellet is then re-suspended in fresh growth medium (GM) containing high glucose DMEM, 20% (vol/vol) FBS (Gibco), 4ng/ml of FGF2 (Sigma Aldrich), and 1% (vol/vol) penicillin streptomycin and re-plated at 10k/cm^2 seeding density. 24 hours post seeding, medium is switched back to ITS-only medium to drive further myogenic maturation for additional 14-21 days. Medium is exchange every other day until cells are ready to be passaged again (confluency ~80%). The same protocol is applied for any further passages at 1:3 or 1:4 ratio. Highest passage number recommended is p3.

Immunofluorescent staining

Myogenic differentiation of hiPSC-derived myogenic progenitor cells was evaluated by immunofluorescent staining for sarcomeric myosin (MF20), desmin (DES), myogenin (MyoG), and Pax7. Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature followed by 3 times wash and incubation in blocking

buffer composed of 3% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Triton^{TM} X-100 in PBS for 1 hour. For MyoG and Pax7 staining, the blocking step was skipped. For clear visualization, an antigen retrieval step was used prior to primary antibody incubation for Pax7 staining using antigen unmasking solution (Vector Laboratories). The cells were subsequently incubated in primary antibodies (1:200; rabbit monoclonal desmin, Abcam, catalog number ab8592), (1:100 mouse monoclonal MF20, hybdridoma bank, catalog number AB2147781), (1:10 mouse monoclonal F5D, hybridoma bank, catalog number AB2146602), and (1:7 mouse monoclonal Pax7, hybridoma bank, catalog number AB528428) diluted in blocking buffer overnight at 4°C. Blocking buffer composition for MyoG and PAX7 antibodies were modified to 0.1% BSA, 2%FBS, 0.1%TX in PBS to improve staining results. The next day, primary antibodies were removed and washed using PBS and cells were incubated in blocking buffer containing secondary antibody (1:250; Alexa Fluor® 488, Life Technologies, catalog number: A12379 and Alexa Fluor® 568, Life Technologies, catalog number: A11011) for 1hr at room temperature. Nuclei were counter stained using Hoechst 33342 ((2 μg/mL; Life Technologies, catalog number: H1399) at room temperature for 10 minutes and washed with PBS. Images were acquired using an A1 Zeiss Inverted microscope and analyzed using the ImageJ software. Immunofluorescent images of all samples were acquired under the linear mode and at an exposure time of 1s. The background was uniformly subtracted from all images using a rolling ball radius method and value of 750.0 pixels. For a complete summary of reagents and steps used for immunofluorescence analysis please refer to Table S1. For quantification analysis,

percentage of positive nuclei from total nuclei in five fields of view was calculated and reported.

Quantitative real-time Polymerase Chain Reaction (rt-PCR)

For RNA extraction using phenol chloroform, samples were taken from at least 4 wells in each category and pooled together using TRIzol Reagent (Life Technologies, catalog number: 15596-018). For each sample, exactly 1μg of RNA was reverse transcribed to complementary DNA (cDNA) using iScript cDNA Synthesis Kit (Bio-Rad, catalog number: 17-8891) according to the manufacturer's instructions. The synthesized cDNA was analyzed via quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR Select Master Mix (Life Technologies). Expression levels of target genes were normalized to 18S expression as house-keeping gene and delta Ct values were calculated as $Ct_{target} - Ct_{18S}$. The relative gene expression profiles were presented as $2^{-\Delta\Delta Ct}$. The primers used in this study are listed in Table 2.

Cell transplantation

Animal experiments were carried out according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego (UCSD), and National Institute of Health (NIH). mdx mice (Jackson laboratories) and NOD.CB17-Prkdcscid/J were mated to generate an mdx/scid population. The 2.5 month old male mdx/scid mice were anesthetized 48 hours prior to cell transplantation intraperitoneally with ketamine (100 mg/kg) and xylazine (10 mg/kg), to injure their tibialis anterior (TA) muscle with 15μl of cardiotoxin (CTX)

from *Naja mossambica mossambica* (10 μM, Sigma; cat# C9759). 48 hours post injury, L-EPCCC3 hiPSC myogenic progenitor cells differentiated for 49 days were trypsinized for 10 mins and spun down at 1000 RPM for 5 mins to form a pellet. The pellets were re-suspended in PBS and intramuscularly injected into the CTX-injured TA muscles at 5.0×10^5 cells/15μl/TA. Two weeks and four weeks post transplantation, treated TA muscles were harvested and embedded in Optimal Cutting Temperature (OCT) for cryosectioning. Survival and engraftment of the hiPSC progenitor cells in the injured muscles were analyzed using histology and immunofluorescence imaging.

Immunofluorescence staining of cryo-sectioned tissue samples

For immunofluorescence staining of tibialis anterior (TA) muscles transplanted with donor cells, samples were first embedded in optimal temperature cutting compound (OCT) for cryosectioning. The sections (~15 μm thickness) were fixed with 4% PFA for 10 min at room temperature, permeabilized and blocked in 0.3% Triton X-100 with 3% BSA for 1 hr at room temperature, and incubated with primary antibodies anti-human lamin A/C (1:100; Vector Laboratories), and rabbit anti-laminin (1:200; Abcam). The next day, primary antibodies were removed and washed using PBS and slides were incubated in blocking buffer containing secondary antibody (1:250; Alexa Fluor® 488, Life Technologies, and Alexa Fluor® 568, Life Technologies) for 1hr at room temperature. Nuclei were counter stained using Hoechst 33342 ((2 μg/mL; Life Technologies) at room temperature for 10 minutes and washed with PBS to visualize transplanted donor cell survival and their engraftment within the host tissue. To quantify the data obtained, total number of lamin A/C nuclei from five fields of view were

counted. Percentage of donor cells centrally located within a myofiber or positioned under the basal lamina was calculated and reported as “percentage of engraftment”.

Hematoxylin and Eosin (H&E) staining for cryo-sectioned tissues

Tissue sections were fixed using 4% PFA for 10mins at room temperature. Hematoxylin solution is incubated for 8 mins to stain nuclei. Slides are washed in lukewarm running tap water for 2 mins and placed in distilled water. Sections are counterstained in eosin for 1min followed by dehydration using 80%, 95%, and 100% ethanol. Slides are then mounted with 200µl of 100% glycerol and eliminating bubbles by pressing down on the coverslip. Coverslips are sealed using nail polish and let dry at room temperature and stored at -20°C.

Statistical Analysis

All values are presented as mean ± standard deviation and statistical significance was determined by two-tailed unpaired Student’s t-test or single-factor analysis of variance (ANOVA) with Tukey’s Multiple Comparison Test (*$p<0.05$, **$p<0.01$, and ***$p<0.001$). GraphPad Prism software was used to perform all the statistical analysis.

Results

Derivation of skeletal muscle progenitor cells from hiPSCs

A schematic of the hiPSC differentiation and transplantation is shown (Fig 3.1). We initiated differentiation of hiPSCs at medium sized colonies (diameter 500 µm) and low colony density. During days 0-4, paraxial mesoderm specification is achieved

through GSK-3β inhibition via CHIR 99021. During phase 1 of differentiation marked as yellow in Fig 1, colonies begin to loosen up and merge with one another. The edges of each colony become less defined and single cells start to sprout out of each colony (Fig 3.2). By day 4, each well is covered with a monolayer of spindle-shaped single cells. Addition of FGF2 in phase 2 of differentiation marked as green in Fig 1 results in expansion of the mesoderm progenitor compartment within our culture system. During this phase, excessive proliferation is observed due to treatment with FGF2. The monolayer begins to reach 90% confluency with cells begin to compact, shown as dark spots in culture (Fig 3.2). Following withdrawal of FGF2 in phase 3, the myogenic progenitor cells are further differentiated in ITS medium alone. Multiple layers of cells begin to form due to continued proliferation of the progenitor cell compartment and packing of cells continues (Fig 3.2).

Terminal differentiation of iPSC-derived myogenic progenitor cells

Following the expansion of myogenic progenitors after 35 days of differentiation as passage 1 (p1) population, cells are further differentiated for additional 14 days in ITS-medium alone to reach maturation. Staining for maturation markers MF20, DES, and MyoG highlight the formation of multinucleated myotubes expressing (Fig 3.3A and 3.4). Meanwhile, a subpopulation of early progenitor cells expressing Pax7 is indicative of presence of satellite-like-cells in culture. Quantification of our findings suggest that the differentiation yields a heterogeneous population of mature myotubes and early progenitor cells ranging between 20-30% of total cells (Fig 3.3B). We did not characterize the non-myogenic cells present in culture.

It is noteworthy to mention that myogenic differentiation in a dish using our proposed protocol occurs in patches. Therefore, there are areas in the well that are saturated with myogenic cells while other areas are completely devoid of positive cells. Our quantification is an average of 3 different fields of view to account for the irregular pattern of differentiation.

Gene expression analysis of myogenic progenitors over time and passage

According to our gene expression analysis, by day 35 of differentiation there is an upregulation of Pax7, Myf5, and NCAM which are markers of early myogenesis. Additional differentiation up to 49 days results in downregulation of these early markers and simultaneously results in upregulation of maturation markers MyoD, MyoG, and DES (Fig 3.5) We also extended our analysis to look at how passaging the progenitor cells alters their gene expression profile. According to our findings, P1 progenitor population closely resembles the p0 progenitor population on days 35, 42, and 49. There seems to be an elevated relative fold induction of late markers by day 42 in P1 population compared to the p0 population. Interestingly, we find that the P2 population shows a shift in its expression profile. Early markers such as Pax7 and NCAM are significantly downregulated in the P2 population while MyoD, MyoG and DES are highly expressed. Our findings suggest that increasing the number of passages of the myogenic progenitors results in loss of early progenitor cells and selection of more differentiated cells (Fig 3.5). We were not able to observe differentiation in p3 population and therefore recommend using this protocol up to passage 2.

Marginal engraftment potential of hiPSC-derived myogenic progenitor cells

To assess the in vivo regenerative potential of hiPSC-derived myogenic progenitor cells, we transplanted cells at different time points of the differentiation protocol into cardiotoxin-injured TA muscles of mdx/scid mice. We first compared transplantation of cells differentiated for 35 days to cells differentiated for 49 days and found that cells differentiated for 49 days display improved viability and engraftment when compared to the day35 treated samples (Fig 3.6A). Quantification of this data is highlighted in Fig 3.6B. We then aimed to look at the long-term effect of cell therapy by comparing 2 weeks and 4 weeks of transplantation using Day49 cells (Fig 3.7A). According to our findings, estimated number of cells is higher after 4 weeks of transplantation. Additionally, engraftment is improved during the 4 weeks study. More cells are observed to be centrally located within myofibers or positioned under the basal lamina (3.7B).

Injury analysis with H&E

Cardiotoxin-injured TA muscles of mdx/scid mice were analyzed by histology to determine the extent of injury. Centrally located nuclei and irregular fiber diameter are prominent in DMD from rapid cycles of degeneration/regeneration to counteract the lack of dystrophin (Goncalves et al., 2006). We aimed to identify the needle track to observe the propagation of injury from the needle tip. We observed that along the needle track, fibers are irregular in shape and in some cases ruptured by day 14 post injury. We also observed that the location of donor cells is associated with higher fraction of myofibers containing centralized nuclei (Fig 3.6A). Additionally, presence of infiltrating immune cells such as macrophages is evident by excessive hematoxylin-

stained nuclei in between fibers and indication of fibrosis surrounding the injected area (Fig 3.6B).

Discussion

Transplantation of myogenic progenitor cells has been considered as an attractive option for treatment of degenerative muscle disorders such as DMD. The success in cell therapy using hiPSCs is highly dependent on a efficient differentiation protocol that can closely mimic normal development *in vivo* to generate an enriched population of progenitor cells. This will require tight control over the differentiation process. The ideal myogenic progenitor cell population should have extensive *in vitro* expansion capacity and ability to engraft with host tissue.

Although numerous protocols are available for *in vitro* differentiation of PSCs into myogenic cells, most published work relies on an artificial system of derivation using forced transgene expression (Darabi et al., 2012; Goudenege et al., 2012). At present, the controlled derivation of myogenic progenitor cells is hindered by our limited knowledge about the spatio-temporal cues, comprising both physiochemical and biological factors, required for recapitulating myogenic differentiation *in vitro*. Recently, scientists have been able to provide evidence of muscle differentiation from hPSCs without genetic modifications; however, their approaches are hindered by the use of serum in their induction medium, low myogenic conversion, and limited engraftment potential (Ryan et al., 2012; Hwang et al., 2013; Barberi et a., 2008). Therefore, it is highly desirable to deliver innovative differentiation solutions for generating clinical-grate precursor cells for cell-therapy applications.

Here, we took advantage of a very simple monolayer culture system previously established protocol by Borchin et a., (2013) and modified it to efficiently derive expandable pre-mature and mature skeletal muscle precursor cells from hiPSCs. The core idea behind this derivation approach is the activation of canonical WNT signaling by GSK3β inhibitor, CHIR. Previously, a thorough expression profiling over the course of derivation shows the progression of myogenesis from day 3 to day 30 of differentiation (Borchin et al., 2013). Somatic mesoderm induction is achieved by upregulation of PARAXIS and MSGN1, followed by FGF2 treatment resulting in expansion of the myogenic progenitor compartment. By day 21 early markers of differentiation such as PAX3, PAX7, and MYF5 should be detected (Borchin et al., 2013). However, we noticed that 35 days of differentiation is not sufficient to obtain maturation *in vitro.* Our hypothesis is that there could be a cell line differentiation variability contributing to the differences in the differentiation progression.

We decided to extend the differentiation protocol for an additional 14 days for a total of 49 days. We observed that by day 49 a significant fraction of the differentiated cells is enriched with mature myocytes and multinucleated myotubes, the function unit of skeletal muscle fibers, evident from expression of MF20, DES, and MyoG. We also noticed that this population is extremely heterogeneous since the multinucleated myotubes were co-localized with a mononucleated myoblasts and satellite cells expressing Pax7. This observation was further confirmed using gene expression analysis for key myogenic markers. Our analysis shows that peak expression of early myogenic markers is established by day 35 and it takes about 49 days to achieve myogenic maturation evident from expression of MyH1, MyoG, and DES.

Although the study reported by Borchin et al. showed differentiation into specialized myogenic precursor cells *in vitro*, the *in vivo* performance of these precursor cells remained unexplored. We have shown that transplantation of the derived progenitor cells at day 49 in mdx/scid mice results in extensive engraftment *in vivo.* As previously mentioned, satellite cells are located between the basal lamina and the sarcolemma of each myofiber. We noticed that a significant number of donor cells are positioned under the basal lamina of host myofibers (Marked with white arrow). We believe that these cells are the satellite cell population that express Pax7 *in vitro* and contribute to the stem cell niche *in vivo* (Fig 3.3A). We were also able to detect engraftment of donor cells with host tissue by locating laminA/C positive donor nuclei centrally located within host myofibers (Marked with white star). Engraftment of donor cells increased from 2 weeks to 4 weeks of transplantation. Interestingly, donor cells do proliferate in vivo since the estimated total number of donor cells is significantly higher after 4 weeks of transplantation (Fig 3.7B). It is therefore safe to assume that majority of cell death occurs within the first two weeks of transplantation from the presence of pro-inflammatory cells recruited to the site of injury and injection (Arnold et al., 2007). Anti-inflammatory macrophages activated and recruited to site of injury 48 hrs post injection are known to facilitate muscle regeneration by inducing differentiation of muscle progenitor cells (Arnold et al., 2007).

We hypothesize that injection of a mixed population of early progenitor cells and mature skeletal muscle cells can contribute to faster muscle healing. Paracrine signaling of donor cells can direct myoblasts to participate in regeneration of host myofibers and recruit Pax7 satellite-like cells to repopulate the stem cell niche for future

rounds of regeneration. Transplantation of day35 differentiated cells lead to significantly less engraftment (Fig 3.6B), which can be contributed to their less differentiation gene expression profile (Fig 3.5).

In the future, we plan on proposing a strategy to purify the skeletal muscle progenitor cells from the non-specifically differentiated cells which requires stringent FACS sorting parameters that are currently not determined. Since myotubes are more susceptible to trypsin treatment, we can also explore serial trypsinization technique to separate the mature myotubes from less-differentiated myoblasts and satellite cells. Additionally, we aim to do long-term transplantation studies up to 3 months to investigate the effect of cell therapy on dystrophin expression and pax7 expression *in vivo*. To investigate functional improvement from cell therapy, we should also develop strategies to measure force of contraction generated in response to electrical stimulations or mechanical stretching.

In summary, we presented a platform for differentiation of highly potent myogenic progenitor cells capable of engrafting in CTX injured skeletal muscle tissue of mdx mice. The proposed derivation protocol yields a heterogeneous population of cells expressing both early and late markers of differentiation. We think that the combination of satellite-like cells expressing PAX7 and mature myotubes expressing MyoG, DES, and MF20 can facilitate donor cell engraftment in the host tissue. The aforementioned cells exhibit regenerative and stem-like characteristics both *in vitro* and *in vivo* and can be used for cell-therapy applications in muscular injuries and genetic diseases such as DMD. It is conceivable that this differentiation approach can be further

adapted to three-dimensional culture conditions for organogenesis and synthetic tissue engineering applications.

Acknowledgement

Chapter 3, in part, is currently being prepared for submission for publication of the material: Sara Hariri, and Shyni Varghese*. The thesis author was the primary lead and author of this paper.

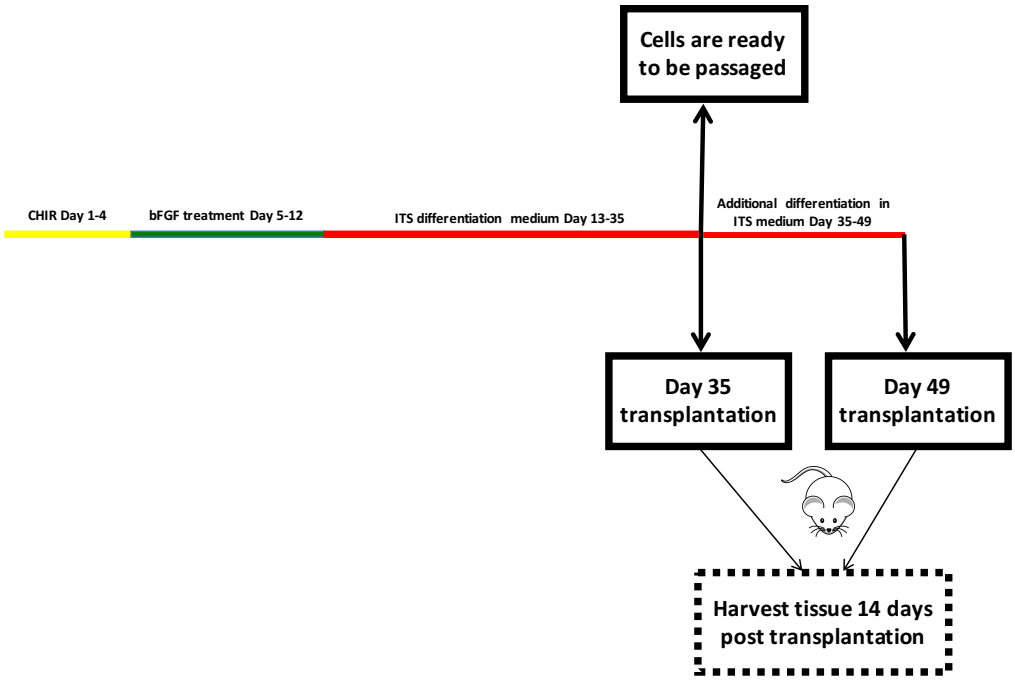

Figure 3.1 hiPSC-derived myogenic progenitor derivation and transplantation scheme. Schematic illustration of differentiation protocol used for derivation and preparation of cells for transplantation in mdx/scid mice.

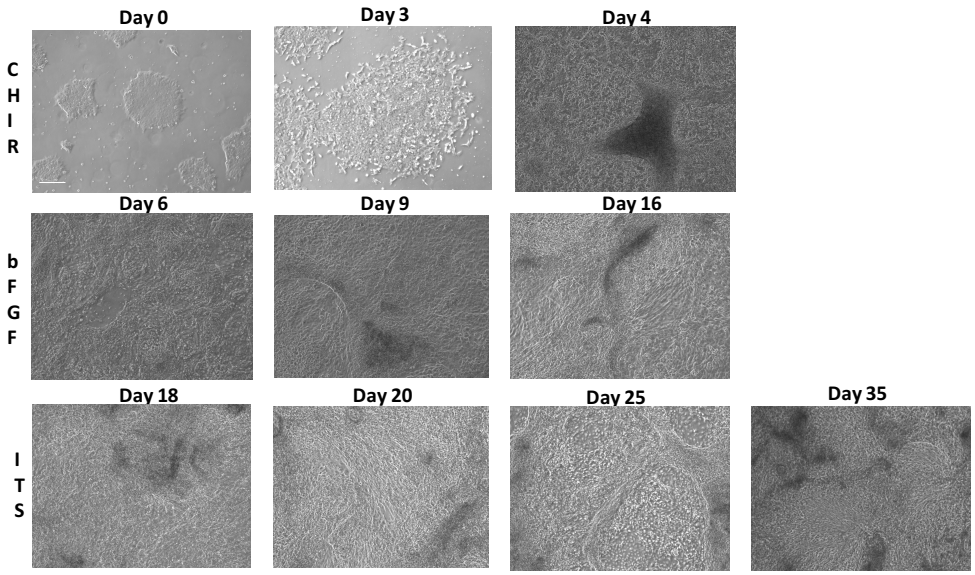

Figure 3.2 Characterization of cellular morphology. Phase contrast images of hiPSC-derived myogenic progenitor cells from various days of differentiation. Days 0-4 are GSK3β inihibition using CHIR medium, days 5-16 are progenitor expansion phase using bFGF treatment, and finally additional days up to day 35 in ITS medium alone to drive myogenic maturation. Scale bar is 50 μm.

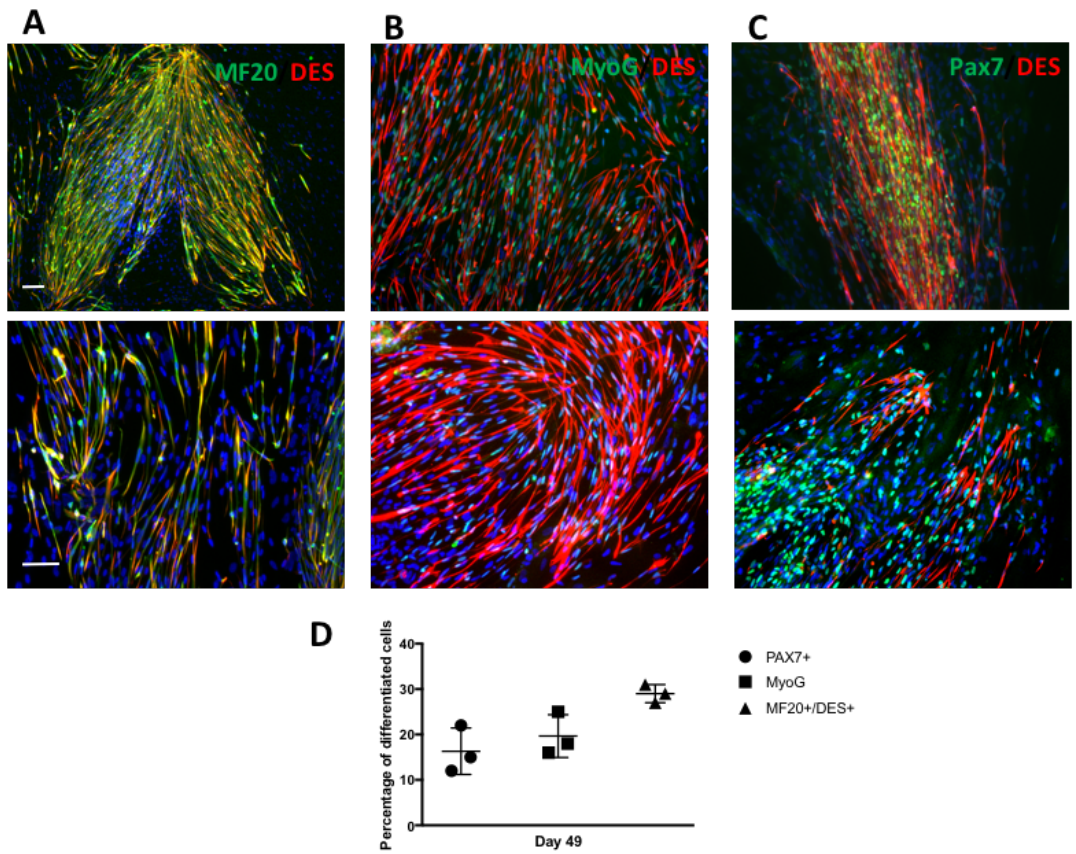

Figure 3.3 *in vitro* characterization of myogenic differentiation (A) Immunofluorescence staining for MF20 (green) and desmin (red) of myogenic progenitor cells. (B) Myogenic maturation evident from nuclear green signal from myogenin co-locolized with desmin positive myotubes (C) Presence of satellite cells in culture as evident from nuclear green signal from Pax7 positive cells (D) quantification of different cell populations 49 days post differentiation. Scale bar is 50 µm.

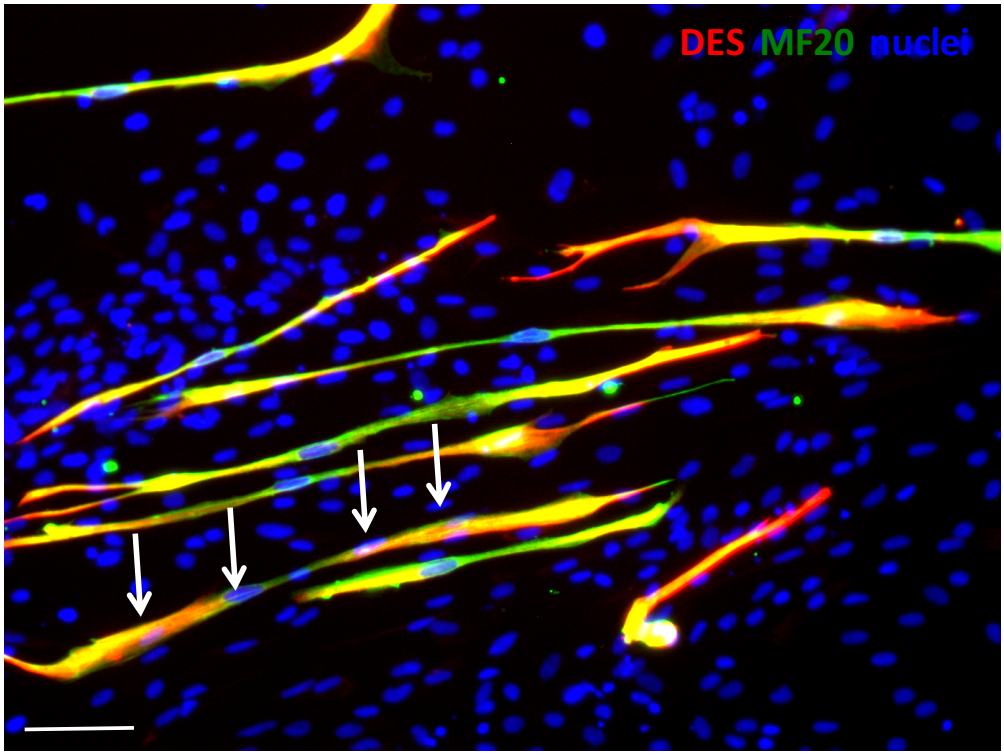

Figure 3.4 Multinucleation of hiPSC-derived myogenic progenitor cells. Immunofluorescence staining for MF20 (green), DES (red), and nuclei (blue). White arrows marking multiple nuclei contained within a single myotube. Scale bar is 50 µm.

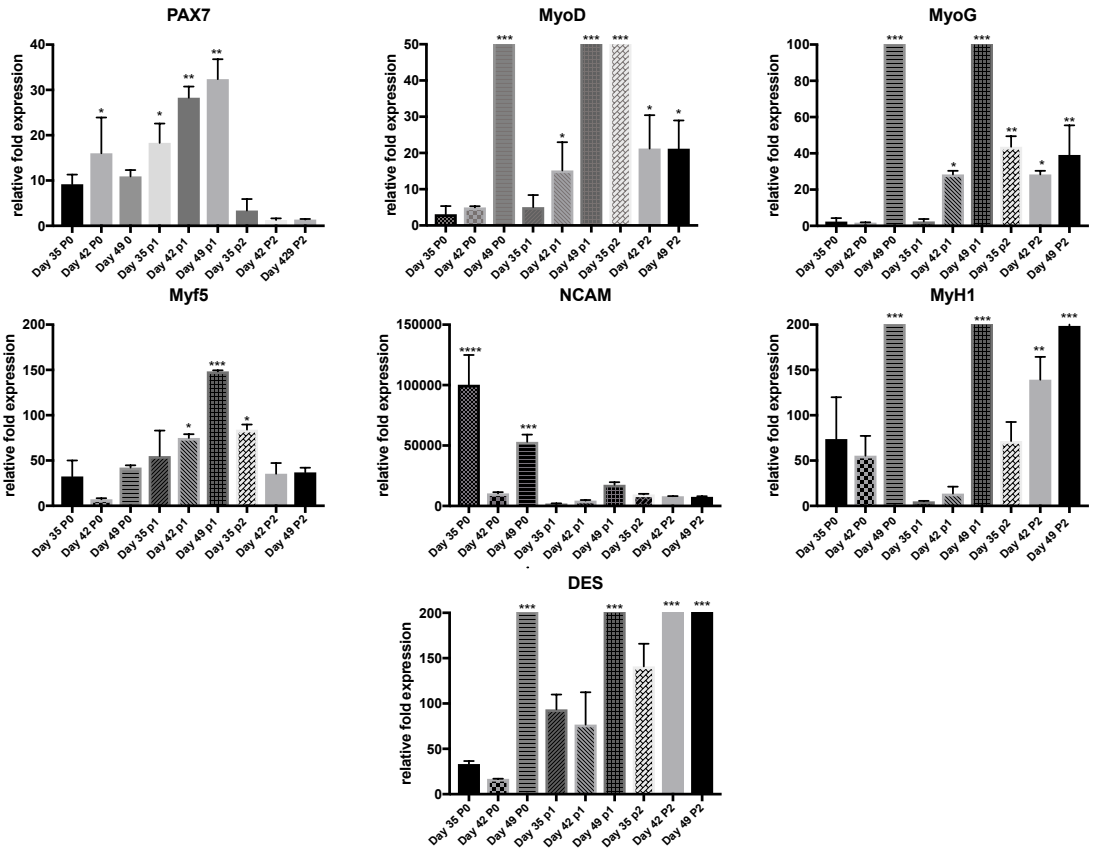

Figure 3.5 Gene expression analysis of *in vitro* differentiation. Gene expression profiles of hiPSC-derived myogenic progenitor cells freshly differentiated p0, after one round of passaging p1, and two rounds of passaging p2. Analysis done on days 35, 42, and 49 of differentiation. Statistical analysis was performed among cells cultured within the same time at various passage number and between days of differentiation. $p<0.05$, $**p<0.01$, and $***p<0.001$.

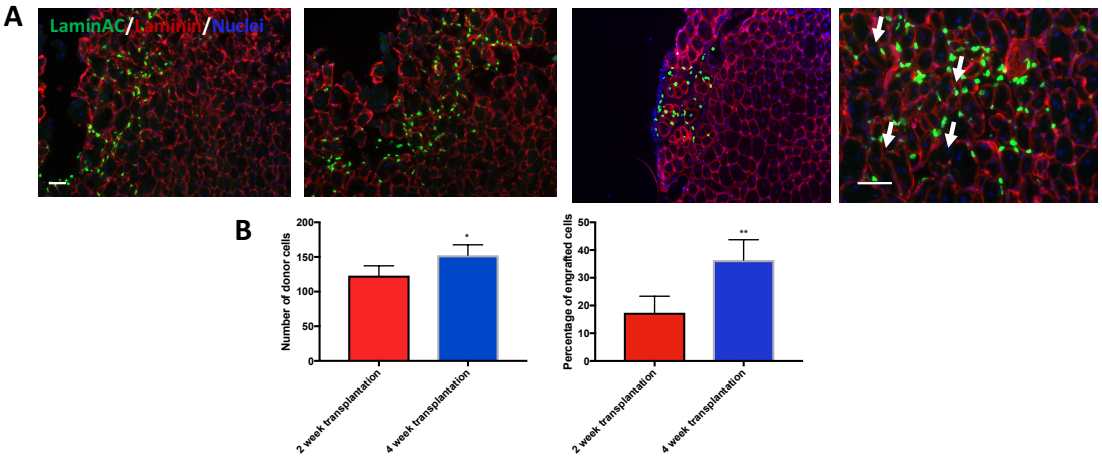

Figure 3.6 2-week *in vivo* survival and engraftment of donor cells in mdx/scid mice. (A) Immunofluorescence staining for human-specific lamin A/C (green), laminin (red), and nuclei (blue). Scale bar is 100 μm. (B) quantitative estimation of the total number of donor cells (C) quantitative estimation of the contribution of donor cells to the host myofibers 14 days post transplantation.

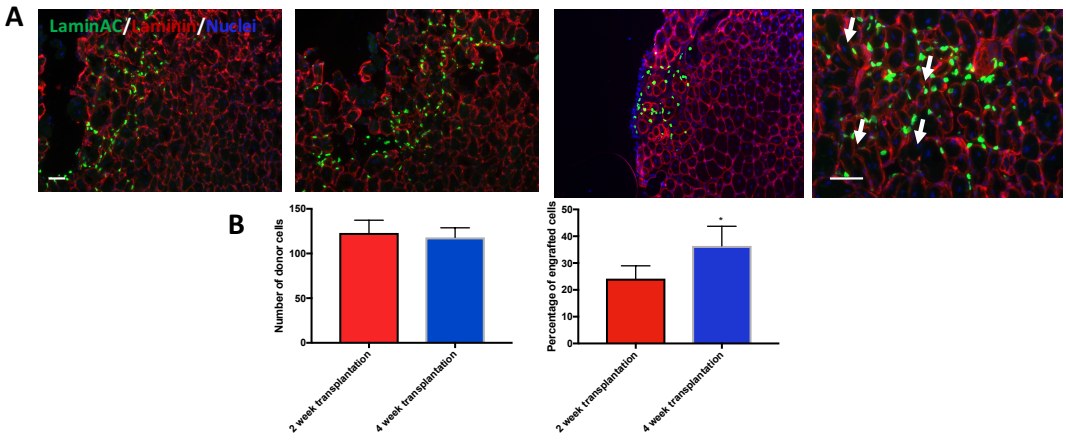

Figure 3.7 4-week *in vivo* survival and engraftment of donor cells in mdx/scid mice. (A) Immunofluorescence staining for human-specific lamin A/C (green), laminin (red) , and nuclei (blue). Scale bar is 100. (B) quantitative estimation of the total number of donor cells © quantitative estimation of the contribution of donor cells to the host myofibers.

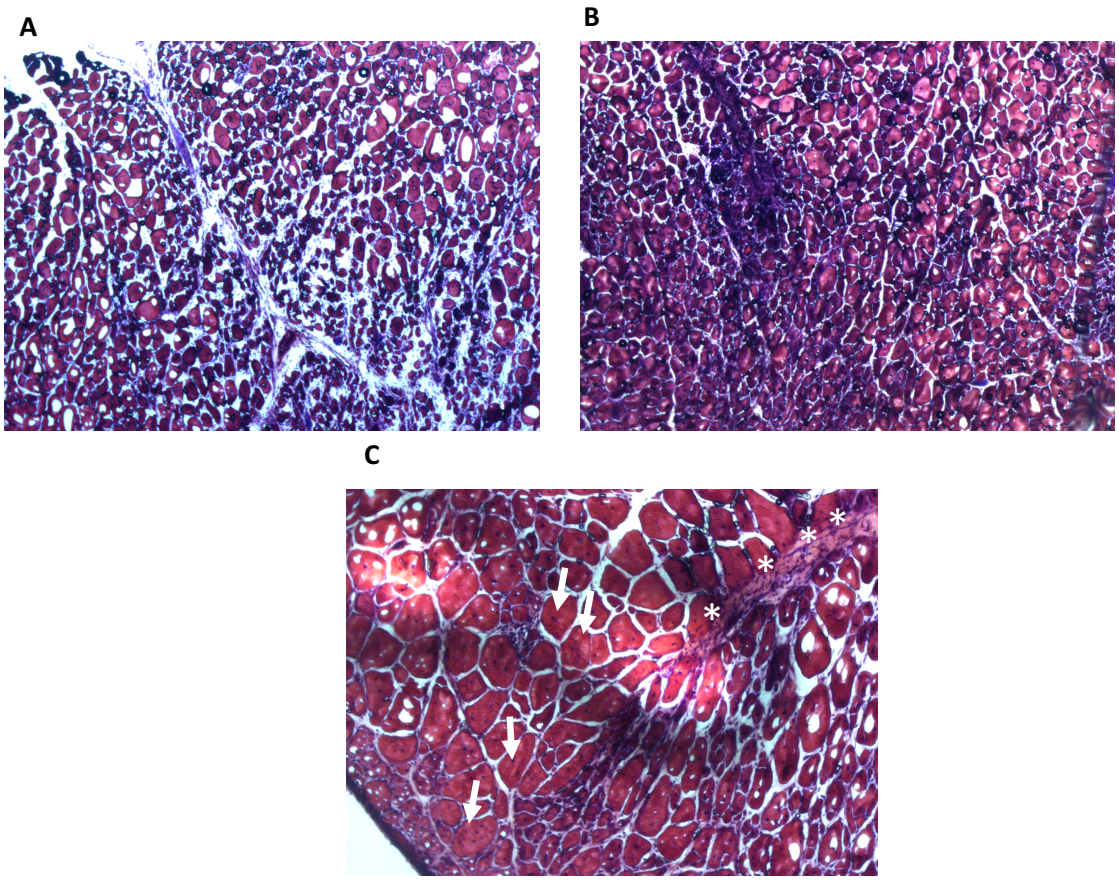

Figure 3.8 Histological analysis of mdx/scid muscle cross sections post injury. (A) Needle track 7 days post injection and presence of irregularly shaped musclr fibers and infiltrating immune cells between fibers (B) Area where donor cells are present 14days post injection has significantly more uniform fiber diameter (C) Irregular myofibers and presence of fibrosis marked with white star (*) and fibers containing centralized nuclei marked with white arrow 14 days post cell injection

Table 3.1 Complete list of primers used for gene expression analysis on hiPSC-derived myogenic progenitor cells by quantitative PCR.

Gene	Primer sequence (5’ to 3’)
Pax7	F-ACC CCT GCC TAA CCA CAT C
	R-GCG GCA AAG AAT CTT GGA GAC
MyoD	F-AGC ACT ACA GCG GCG ACT C
	R-TAG TAG GCG CCT TCG TAG CA
MyoG	F-CAG CTC CCT CAA CCA GGA G
	R-GCT GTG AGA GCT GCA TTC G
MyH1	F-TCT TGG ACA TTG CTG GCT TT
	R-TCC ACT CAA TGC CTT CCT TC
DES	F: GAAGCTGCTGGAGGGAGAG
	R: ATGGACCTCAGAACCCCTTT
Myf5	F: TTCTCCCCA TCCCTCTCGCT
	R: AGCCTGGTTGACCTTCTTCAG
18S	F: ACGCTATTGGAGCTGGAATTAC
	R: CCCTGTAATTGGAATGAGTCCAC

Table 3.2 Summary of immunofluorescence staining protocols used for characterization of hiPSC-derived myogenic progenitor populations.

Antibody	Fixation	Antigen Retrieval	Permeabilization and blocking buffer	Primary antibody dilution factor	Secondary antibody dilution factor
Pax7	10 min RT	15 min in boiling water (100°C)	Not needed	1:7, overnight, 4°C	1:250, 1hr, RT
MyoG	10 min RT	Not needed	Not Needed	1:10, overnight, 4°C	1:250, 1hr, RT
MF20	10 min RT	Not Needed	0.3% TritonX and 3% BSA, 1hr, RT	1:100, overnight, 4°C	1:250, 1hr, RT
DES	10 min RT	Not Needed	0.3% TritonX and 3% BSA, 1hr RT	1:200 overnight, 4°C	1:250, 1hr, RT
LaminA/C	10 min RT	Not Needed	0.3% TritonX and 3% BSA, 1hr, RT	1:100, overnight, 4°C	1:250, 1hr, RT
Laminin	10 min RT	Not Needed	0.3% TritonX and 3% BSA, 1hr, RT	1:200, overnight, 4°C	1:250, 1hr, RT

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