The role of SOX7 in the activation of satellite cells and regulation of skeletal myogenesis.

by

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Abstract

One of the major drawbacks of using stem cell therapy to treat muscular dystrophies is the challenge of isolating sufficient numbers of suitable precursor cells for transplantation. As such, a deeper understanding of the molecular mechanisms involved during muscle development, which would increase the proportion of embryonic stem cells that can differentiate into skeletal myocytes, is essential. In conditional SOX7-/- mice, we observed that the loss of SOX7 in satellite cells resulted in poor differentiation and fusion. *In vivo*, we observed fewer Pax7+ satellite cells in the mice lacking SOX7 as well as smaller muscle fibers. RT-qPCR data also revealed that Pax7, MRF and MHC3 transcript levels were down-regulated in SOX7 knockdown mice. Surprisingly, when SOX7 was overexpressed in embryonic stem cells, we found that there was a defect in making muscle precursor cells, specifically a failure to activate Pax7 expression. Taken together, these results suggest that SOX7 expression is required for the proper regulation of skeletal myogenesis.

Acknowledgements

I would love to take this opportunity to thank my supervisor Dr. Ilona Skerjanc for her trusting nature and always wanting to give a chance to every student, regardless. Her endless direction, support and scientific brainstorming sessions made my graduate student experience a lot easier. Her continual patience, encouragement and optimistic attitude gave me a great learning opportunity, which went beyond the scope of lab techniques and experimentation. I hope I have lived up to your potential, or at least tried.

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In addition I would like to thank the members of my Thesis Advisory Committee, Dr. Robin Parks and Dr. Kristin Baetz. Their suggestions, comments and guidance over the past two years assisted me a lot.

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Lastly, I would like to mention the source of funding for my project, which was provided by Canadian Institute Health & Research to Dr. Ilona Skerjanc.

Dedicated to Mum, Dad and Science.

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List of Abbreviations

ACTC	Actin, Alpha Cardiac Muscle
bHLH	Basic helix-loop-helix
BME	Beta- Mercaptoethanol
BMP	Bone Morphogenetic Protein
Ca2+	Calcium ions
CTX	Cardiotoxin
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
DM	Dermomyotome
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DML	Dorso-medial lip
DMEM	Dulbecco's Modified Eagle Medium
DMD	Duchenne muscular dystrophy
DGC	Dystrophin-glycoprotein complex
E	Embryonic day
EB	Embryoid body
EC	Embryonal carcinoma
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ESC	Embryonic stem cell
EDTA	Ethylenediaminetetraacetic acid

FBS	Fetal Bovine Serum
F1	First filial generation
GFP	Green Flourescent Protein
HMG	High mobility group
H&E	Hematoxylin and Eosin
hESC	human Embryonic stem cell
HRP	Horseradish peroxidase
kDa	kilo Dalton
LIF	Leukemia Inhibitory Factor
miRNA	micro Ribonucleic acid
mESC	mouse Embryonic stem cell
MHC	Myosin Heavy Chain
MPC	Muscle precursor cell
MD	Muscular Dystrophy
MRF	Myogenic regulatory factors
mRNA	Messenger ribonucleic acid
NP-40	Nonidet-P40
ORF	Open Reading Frame
PBS	Phosphate buffered saline
PGK	Phosphoglycerate kinase
PMSF	Phenyl methyl sulfonyl fluoride
PVDF	Polyvinylidene fluoride
PSM	Pre-somitic mesoderm
Puro	Puromycin

RT-qPCR	Reverse Transcriptase- quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RA	Retinoic Acid
SC	Satellite cells
SRY	Sex-determining region on the Y chromosome
SDS	Sodium dodecyl sulphate
SHH	Sonic Hedgehog
Sp	Splotch
SEM	Standard Error Mean
sPBS	Stockholm (Na-Phosphate based) PBS
TBST	Tris-Buffered Saline with Tween
VLL	Ventro-lateral lip

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Contribution of Collaborators

Project Development and Experimental Design

Dr. Ilona Skerjanc and Dr. Nadine Wiper-Bergeron developed the experimental approaches seen in this study.

Experiments

Dr. Joseé Coutu designed the plasmid used for the overexpression of SOX7 in the mouse embryonic stem cells. Dr. Coutu obtained the SOX7 open reading frame (ORF) from the pCMVScript vector, a generous gift from Y. Hayashi, Japanese Science and Technology Agency. I used this vector to generate the stable cell lines that were then used for further analysis. The $SOX7^{F/F}$ mice were provided by our collaborator Dr. Daryl Scott, Baylor College USA. We used these mice to cross with an EIIa-Pax3Cre mice, Jackson laboratory, Maine USA to get our desired mouse strain, $SOX7^{-/-}$. Jennifer MacDonald, previous technician in our lab designed the breeding scheme for the mice and carried out the initial genotyping and colony maintenance. Francois Marchildon and Neena Lala, PhD students in Dr. Wiper-Bergeron's lab provided assistance with mouse handling, TA muscle dissection and sectioning. They also helped me with the isolation of satellite cells from hind limb muscles and *in vitro* differentiation assay, H&E staining and quantification shown in this study. Émilie Lamarche, a PhD student from Dr. Wiper-Bergeron's lab provided assistance with all the western blots shown in this study. Michael Shelton, a PhD student in the Skerjanc lab assisted with tissue culture techniques and MF20 quantification.

Writing

I wrote this thesis with editing help from Dr. Nadine Wiper-Bergeron.

List of Appendices

1. INTRODUCTION

1.1. Muscular Dystrophy

Muscular dystrophies (MD) are genetic disorders which cause progressive degeneration of the skeletal muscle (1). Skeletal muscle cells, or myofibers, are multinucleated contractile, cells which constitute the minimal functional unit of all muscles in the body that function to provide mobility and stability to the body skeleton. The characteristic symptom of these muscular dystrophies is muscle weakness and muscle wasting (1). The muscle wasting arises due to defects in muscle proteins which render the muscle more susceptible to damage (1). Most common are the dystrophinopathies, which as the name suggests, are caused by mutations in the gene for dystrophin. Dystrophin is one of the largest genes in humans spanning 2.3 megabases on the X chromosome and thus comprising of 0.1% of the entire human genome (1). At 3500 amino acid residues in length, the dystrophin protein is coded for by 79 exons that make up only 0.6% of the gene sequence with the remaining 99.4% of the sequence found within intronic sequences. Dystrophin is a 427 kDa protein, with eight promoters and seven different transcripts, which can be found in skeletal, cardiac, and smooth muscle fibers along with cortical neurons, Purkinje, Schwann, glial, retinal, and kidney cells.

Dystrophin is an essential component of the dystrophin-glycoprotein complex (DGC) and in healthy muscle fibers, dystrophin acts as an actin-binding protein that links the cytoskeleton via the α/β-dystroglycan complex to the extracellular matrix protein laminin through the outer cell membrane (Fig. 1.1). (2). This transmembrane-sarcolemmal complex not only stabilizes the muscle surface thereby preventing membrane rupturing during

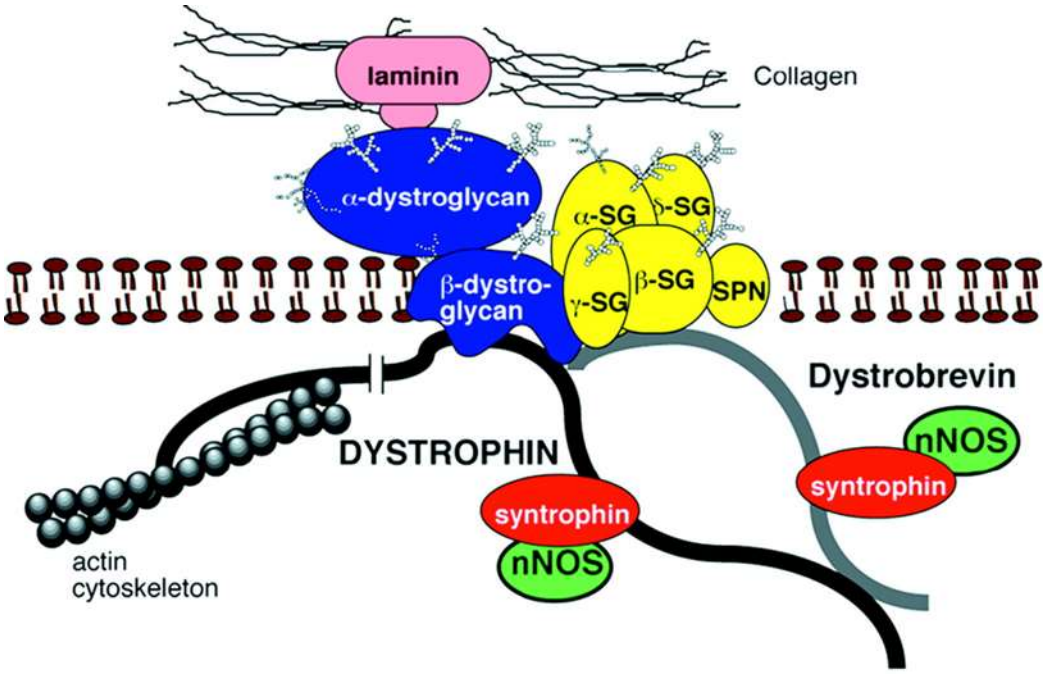

laminin
Collagen
α-dystroglycan
α-SG
δ-SG
β-SG
γ-SG
SPN
β-dystro-
glycan
Dystrobrevin
DYSTROPHIN
nNOS
syntrophin
syntrophin
nNOS
actin
cytoskeleton

Figure 1.1: Dystrophin and the dystrophin–glycoprotein complex in muscle.

Dystrophin is a large sub-sarcolemmal, actin-binding protein associated with a complex of sarcolemmal and cytoskeletal proteins in mature striated muscle cell (myofibers), both skeletal and cardiac known as the DGC (Dystrophin-Glycoprotein Complex). The DGC can be separated into three sub-complexes: dystroglycans, dystrobrevins and sarcoglycans, which have already been implicated in a number of muscle diseases, illustrating the vital role this complex plays in the maintenance of muscle integrity. Reproduced from J.Chamberlain, (2002). "Gene therapy for muscular dystrophy", Human Molecular Genetics, 11: (20) 2355–2362 with permission from Oxford University Press © 2002.

continuous excitation-contraction-relaxation cycles, but it also supports muscle fiber strength, reduces muscle stiffness, increases sarcolemmal deformability, and helps to prevent muscle fiber injury (2,3). Therefore, dystrophin plays a pivotal role in maintaining cell structure and integrity (3). In the highly progressive and debilitating muscle wasting disease Duchenne muscular dystrophy, a primary deficiency in dystrophin causes a drastic reduction in dystrophin-associated glycoproteins, which renders muscle fibers more susceptible to necrosis (2). Generally, in dystrophinopathies, where dystrophin expression is compromised, the mechanical stress of contraction can lead to muscle fiber degeneration and leakage. This leads to a calcium imbalance, with the intracellular Ca^{2+} levels increasing, leading to the symptoms typically seen in MD (4, 5).

Several forms of muscular dystrophy exist and differ based on the muscle types affected and the severity of the disease, as well as the underlying defect that causes the muscle wasting (1) (Figure 1.2). Broadly speaking there are 8 different types of muscular dystrophies (Congenital, Duchenne, Becker, Emery-Dreifuss, Limb-girdle, Facioscapulohumeral, Oculopharyngeal and distal MD) each having a mutation in a different gene and showing slight variations in symptoms and progression.

1.2 Therapeutic Options for Muscular Dystrophy

Currently, there is no known cure for muscular dystrophy or specific treatments targeting different muscle groups, although significant headway is being made with antisense oligonucleotides (6). At present physical therapy, occupational therapy, speech therapy and

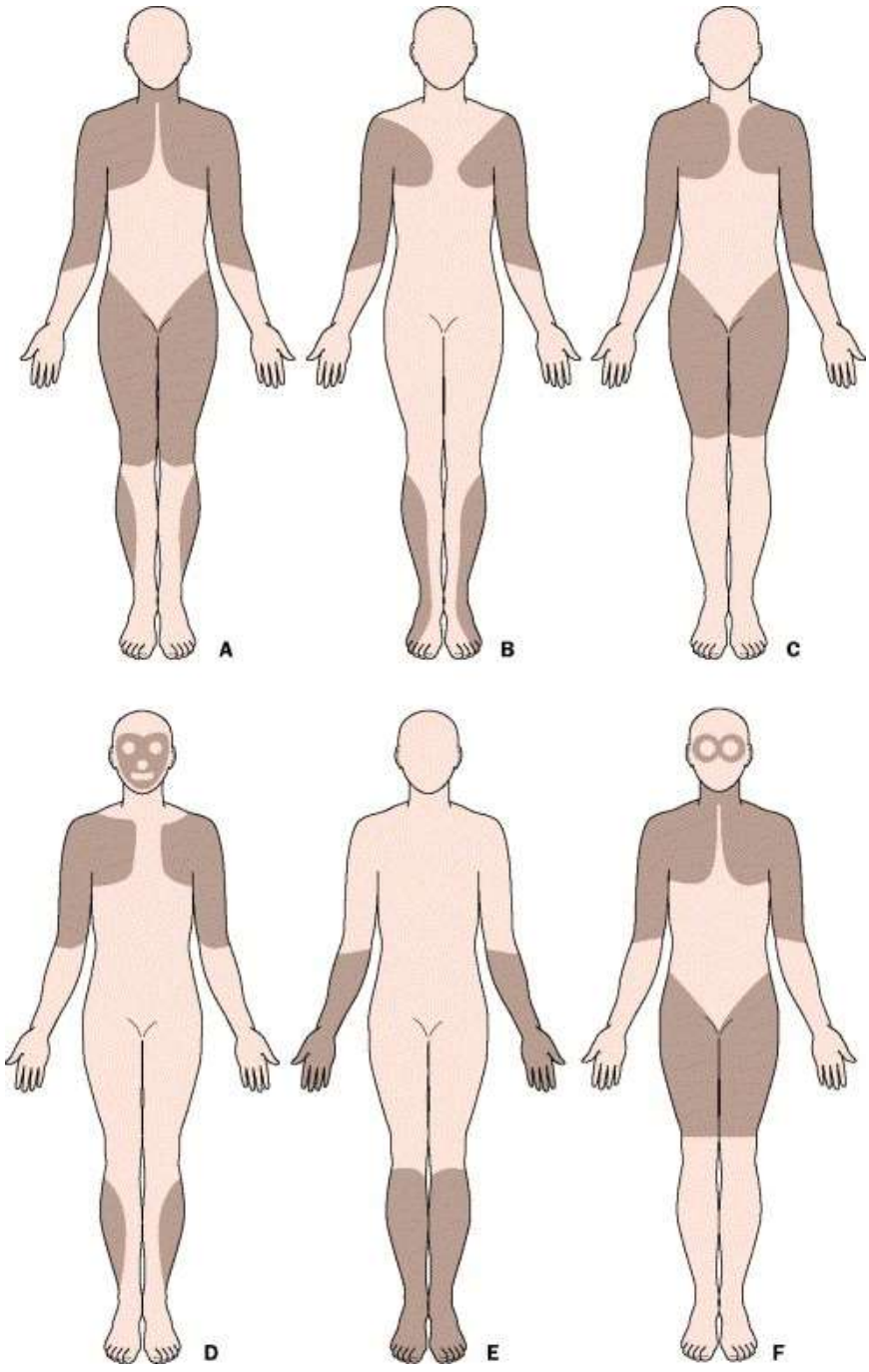

A
B
C
D
E
F

Figure 1.2: Major muscle groups affected in the different types of Muscular Dystrophy. Shading marks the affected areas in Duchenne-type and Becker-type (A), Emery-Dreifuss (B), limb-girdle (C), facioscapulohumeral (D), distal (E) and Oculopharyngeal (F) muscular dystrophies. Muscular dystrophy is an inherited (genetic) disorder causing muscle weakness. The different types of muscular dystrophy vary in severity, ranging from very mild to severe, as well as age of onset. The above mentioned schematic representation depicts all the main areas of muscle weakness in the different types of MD. Reproduced from A. Emery, (2002). "The Muscular Dystrophies". The Lancet, 359: (9307) 687 – 695 with permission from Elsevier © 2002.

orthopedic instruments such as wheelchairs, standing frames and powered mobile arm supports are proving to be the most helpful. Pluripotent embryonic stem (ES) cells derived from the inner cell mass of the blastocyst also hold tremendous potential to be used in the repair of deteriorating organs through stem cell therapy (7). Stem cells are characterized by certain qualities, mainly by their ability for long term self-renewal through mitotic cell division and their capacity to differentiate into a diverse range of cell lineages (7). They present a good model to study the molecular mechanisms driving myogenesis because the molecular pathways that regulate this process as well as the steps involved in this process are very similar to those observed in the embryo. While adult stem cells (aka satellite cells) are largely unipotent, embryonic stem cells are pluripotent, which means that embryonic stem cells have the potential to differentiate into cell types of any of the three germ layers: endoderm, mesoderm and ectoderm. Despite their differentiation potential, it is necessary to isolate sufficient numbers of myogenic precursor cells capable of reconstituting the stem cell niche within muscle to transform a stem cell therapy into a long term cure for muscular dystrophy (7).

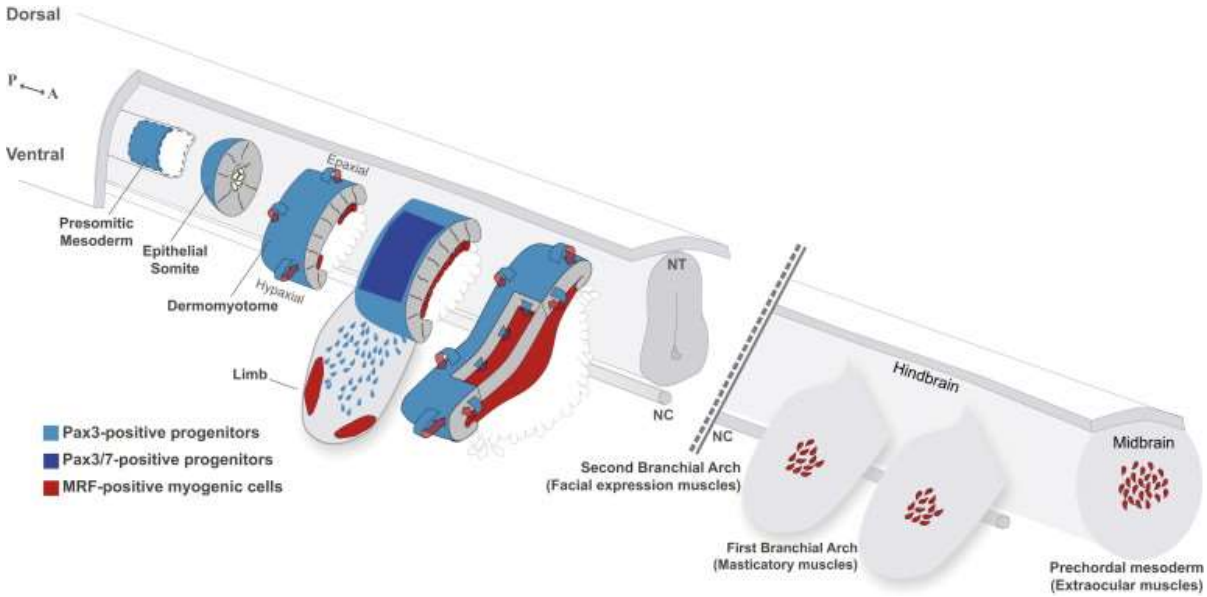
1.3 Vertebrate Skeletal Muscle Development

The union of two gametes (the ovum and the sperm cell) produces a zygote. This single diploid cell undergoes several rounds of cellular divisions to form the blastula and then the gastrula. Gastrulation is defined as the transition from a simple, unorganized group of cells to a more complexly organized, and multilayered embryo, which has the three germ layers: endoderm, mesoderm and ectoderm (8). As the formation of the primitive streak continues, highly coordinated movements control the migration of the cells to their final destinations. The mesodermal layer is important for skeletal myogenesis as it can form the paraxial

mesoderm, which is further segmented to form the somites. Cells that migrate along the anterior-posterior axis are destined to become the paraxial mesoderm, while the cells that travel further along the medial axis will become the lateral plate mesoderm (9). With the exception of a few muscles (mostly in the head), skeletal muscle is largely derived from the somites, which in vertebrates such as birds and mammals, are formed by the segmentation of the presomitic paraxial mesoderm in an anterior to posterior fashion, forming ball-like structures around the neural tube and notochord, once the primitive streak begins to regress (10) (Figure 1.3A). After segmentation as the epithelial somites mature, they differentiate into a ventral sclerotome and a dorsal dermomyotome. The sclerotome gives rise to cartilage and bone of the vertebral column and rib cage (11). The dermomyotome (DM) gives rise to muscles of the trunk and limbs and dermis of the back (11). Myogenesis in the vertebrate embryo is a multi-step process that is largely controlled by a complex network of transcription factors (12). The muscle progenitor cells expressing transcription factors Pax3, Pax7, Meox1 and Gli2, are located in the DM and migrate ventrally to form the myotome, which is the site where the first fully differentiated embryonic myocytes are found and more significantly, the source of the first skeletal muscle found in the body.

Most of our elaborate understanding and knowledge of myogenesis in the embryo is based on the manipulation of chick embryos and chick/quail chimera studies; but the findings can be extrapolated to mammals as well. By electroporating a GFP reporter construct into the inter-limb somites of a chick embryo *Gros et al* discovered that the formation of the myotome occurs via a two-step process (11). The first step is the formation of the primary myotome that involves the migration of DM cells through the dorsomedial lip (DML). As the new myoblasts are born, they displace the older myocytes and elongate across the rostral-

A


Dorsal
P
A
Ventral
Presomitic Mesoderm
Epithelial Somite
Epaxial
Hypaxial
Dermomyotome
Limb
NT
NC
NC
Hindbrain
Midbrain
Second Branchial Arch (Facial expression muscles)
First Branchial Arch (Masticatory muscles)
Prechordal mesoderm (Extraocular muscles)
Pax3-positive progenitors
Pax3/7-positive progenitors
MRF-positive myogenic cells

B

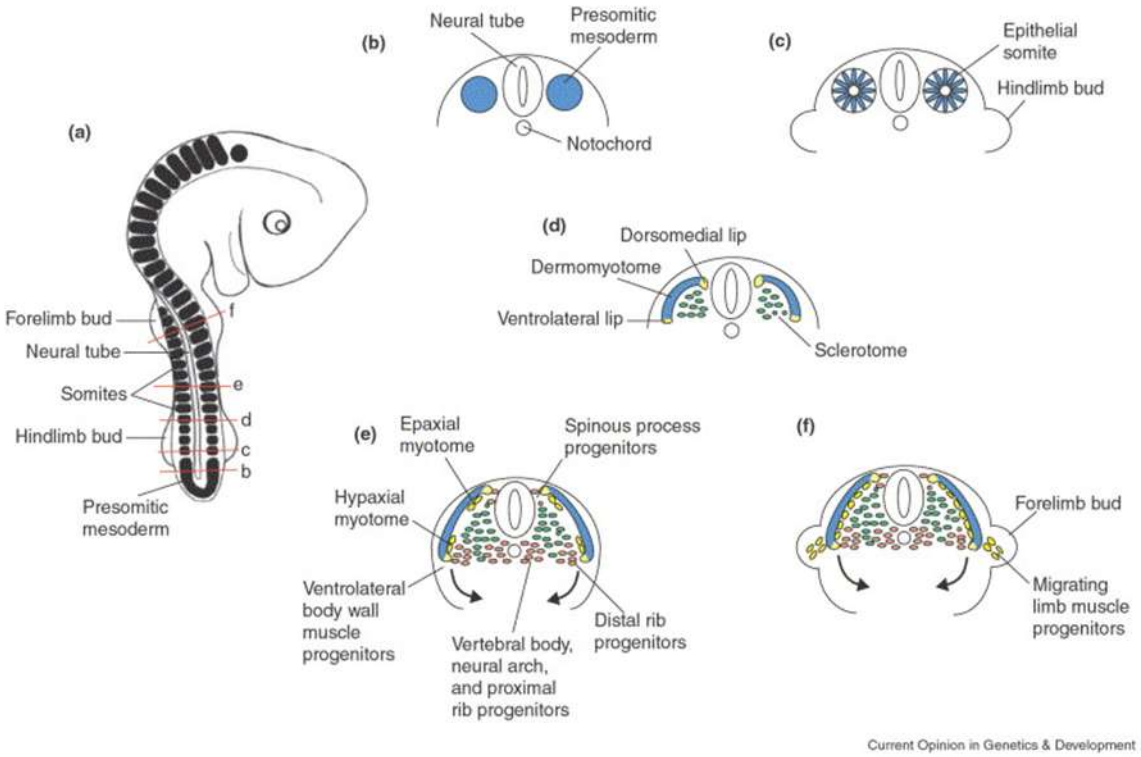

(a)
Forelimb bud
Neural tube
Somites
Hindlimb bud
Presomitic mesoderm
f
e
d
c
b
(b)
Neural tube
Presomitic mesoderm
Notochord
(c)
Epithelial somite
Hindlimb bud
(d)
Dorsomedial lip
Dermomyotome
Ventrolateral lip
Sclerotome
(e)
Epaxial myotome
Spinous process progenitors
Hypaxial myotome
Ventrolateral body wall muscle progenitors
Vertebral body, neural arch, and proximal rib progenitors
Distal rib progenitors
(f)
Forelimb bud
Migrating limb muscle progenitors
Current Opinion in Genetics & Development

Figure 1.3: Somite formation and patterning in the vertebrate embryo.

(A) Schematic representation of the presomitic paraxial mesoderm undergoing segmentation in the anterior to posterior direction to form symmetrical pairs of somites flanking the axial structures, neural tube and notochord. Each structure is represented by a specific group of progenitor cells. Reproduced from M. Parker, P. Seale, & M. Rudnicki, (2003). "Looking back to the embryo: defining adult myogenesis". National Review in Genetics, 4(7):497- 507 with permission from Nature Publishing Group © 2003.

(B) Schematic depiction of vertebrate somite segmentation. Further patterning of the somites into the dorsal dermomyotome and ventral sclerotome is induced by the signals from the surrounding tissues. Migration of cells from the dorsal medial lip (DML), followed by migration from all four borders of the dermomyotome, forms the myotome under the dermomyotome. The myotome forms a continuous sheet of epaxial and hypaxial muscle. Reproduced from A. Brent, & C. Tabin, (2002). "Developmental regulation of somite derivatives: muscle, cartilage and tendon". Current Opinion in Genetics & Development, 12(5): 548 – 557 with permission from Elsevier © 2002.

caudal borders of the somites. This is followed by migration of cells from all four borders (dorso-ventral and rostro-caudal) of the DM to the myotome and their elongation along the anterior- posterior axis of the embryo (12). The second step is the formation of the secondary myotome, which involves proliferation and differentiation of the muscle progenitor cells that migrate directly from the DM into the myotome (13, 14). Different parts of the somites give rise to different types of muscles in the body. The ventrolateral half of the somites migrate ventrally to form the limb muscles and the body-wall muscles, called hypaxial muscles, while the dorsomedial half gives rise to the deep back muscles, called epaxial muscles (15, 16). It has been suggested that the horizontal boundary marking the separation of the epaxial-hypaxial DM and myotome is based on the expression pattern of transcription factors such as Sim1 and En1 (17, 18). The expression of transcription factors Lbx1 and C-met is required for proper migration and differentiation of the migrating hypaxial muscle precursor cells (MPCs) that are located in the lateral lip of cervical, occipital and limb DM (which form the diaphragm, tongue and limb muscles, respectively) (19-21). The epaxial myotome is formed from the progenitor cells that exit the cell cycle, elongate and differentiate from the DML while the hypaxial myotome is formed similarly from the progenitor cells of the ventrolateral lip (VLL) (16). The differentiation of the epithelial somites into the DM and myotome is influenced by soluble signaling factors such as Wnt, Sonic hedgehog (SHH) and Bone Morphogenetic Proteins (BMP) emitting from the surrounding embryonic tissues such as the notochord, neural tube, overlaying ectoderm and lateral plate mesoderm (22). In addition to these signaling pathways, a complex network of temporally and spatially expressed transcription factors also play a role in regulating the process of muscle differentiation, as mentioned previously (Figure 1.3B).

1.4 Transcriptional Regulation of Skeletal Myogenesis

Embryonic myogenesis can be described as the process by which mesoderm cells undergo specification to form the premyogenic mesoderm, which contains a pool of muscle precursor cells, the commitment of these precursor molecules into myoblasts, and finally the terminal differentiation to form skeletal myocytes (14). Each stage is characterized by the expression of a group of transcription factors that regulate the transition from one state to another (Figure 1.4).

1.4.1 Mesoderm markers: Brachyury T and Mesogenin. Genetic evidence suggests that in mouse the T gene (Brachyury) has a role in the formation and organization of mesoderm (23). Mice homozygous for mutant alleles of the T gene do not generate enough mesoderm (24), and show severe disruption in morphogenesis of mesoderm-derived structures, in particular the notochord (25– 28). The cloning of the T gene has now allowed us to examine its expression pattern (28). T-gene expression occurs in both early stage mesoderm and its epithelial progenitor, and then becomes restricted to the notochord (23). The first stage of early mesoderm induction is marked by the expressed of Brachyury T (29). This factor has been shown to peak on day 3 during the normal differentiation of mouse embryonic stem cells (mESC) (30).

Another factor that is expressed in developmentally immature un-segmented paraxial mesoderm is Mesogenin (31). At the molecular level, Mesogenin null mice die during gestation due to dramatic loss of expression of the Notch/Delta pathway components and oscillating somitic clock genes within the presomitic mesoderm that are thought to control segmentation and somitogenesis (32). Subsequent patterning and specification steps for paraxial mesoderm formation also fail, leading to a complete lack of all trunk paraxial mesoderm derivatives, which include skeletal muscle, vertebrae, and ribs (32).

1.4.2 Premyogenic mesoderm markers: Pax3 and Pax7. Pax3 and Pax7 are the most important transcription factors during premyogenic mesoderm formation, marking the beginning of stage two, the specification of the mesodermal cells into the premyogenic mesoderm (33). Besides this, the Pax family of transcription factors is involved in many developmental processes including tissue specification and organ formation (34). These genes are characterized by their paired box domain and paired-type homeodomain and are further classified into groups based on similarities in their expression patterns and structural characteristics (35). Pax3 and Pax7 are expressed during skeletal muscle development and play an important role in muscle formation. The Pax3 protein, which is highly conserved among vertebrates (36), is comprised of an octapeptide motif, a homeodomain and a paired domain key for sequence-specific DNA binding (37, 38). In mice, Pax3 is initially expressed in the presomitic mesoderm prior to segmentation (37) and its expression becomes progressively limited to the lateral domain of the dermomyotome as the somites are patterned (39). The domain of Pax3 expression extends throughout the entire epithelial somite and the extremities of the dermomyotome (34) and then myotome (41) where it controls formation of hypaxial muscle as well as migration of limb muscle progenitor cells in the ventro-lateral lip of the limb somite (39-43). In fact, Splotch (Sp) mutant mice, in which a non-functional Pax3 protein is expressed, fail to develop limb and diaphragm muscle while the epaxial muscle remains unaffected (41, 42, 44).

Pax7, which is closely related to Pax3, is also expressed in the somites; however its expression is concentrated in the medial domain of the dermomyotome (45). However, Pax7 expression is expanded in the epaxial regions of the Splotch mice, where normally only Pax3 is expressed, to compensate for the loss of Pax3 (46). Pax7 mutants appear normal at birth, but die a few weeks after birth for unknown reasons, showing no evident embryonic skeletal

muscle defects (47) implying compensation by other Pax genes (46). More importantly, it is believed that the Pax3/7 population that is present in the central dermomyotome is responsible for almost all of the skeletal muscle of an organism (47).

Several groups have identified a progenitor population of Pax3/7 positive (and Myogenic Regulatory factors, "MRF" negative) cells, which continue to proliferate throughout embryonic development and contribute to the developing muscle. Myogenic regulatory factors are an important group of transcription factors that are essentially considered the master regulators of skeletal myogenesis and will be introduced in the following section. Later, these Pax3/7+ve MRF-ve cells are found in the sub-laminar layer adjacent to the muscle fibers, implying that they might in fact be a developmental source of satellite cells (adult muscle stem cells) (34), that have an important role in maintaining skeletal muscle throughout the life of an organism (33). The Pax3/Pax7 double mutants exhibit a more severe phenotype than the one observed for the single mutants implying some level of compensation between these two transcription factors. When both Pax3 and Pax7 are mutated the progenitor cell population undergoes continued apoptosis and is inhibited from undergoing myogenesis resulting in a loss of skeletal muscle, with the exception of the early muscle of the myotome (14).

1.4.3 Myoblast markers: Myogenic Regulatory Factors (MRFs): In the next stage, the premyogenic mesoderm cells must commit to form myoblasts that can terminally differentiation into mature skeletal muscle. The MRFs, including four transcription factors, Myf5, MyoD (Myf3), MRF4 (Myf6/Herculin) and Myogenin (Myf1), are members of a superfamily of basic helix-loop- helix (bHLH) transcription factors (48-52). This family of transcription factors is central to the acquisition of myogenic identity and differentiation into skeletal muscle as evidenced by their ability to drive a wide variety of cell types into the

skeletal muscle lineage. A variety of gene targeting studies have examined the function of MRF proteins revealing a complex hierarchical relationship between Myf5, MyoD, MRF4 and Myogenin (48-52).

Myf5 is the first MRF to be expressed during embryogenesis and it can be detected as early as E8.0 in the mouse somites or stage 3 in the PSM of developing chick embryo (53, 54). It is expressed in dermomyotome and in both DML and VLL where it plays a role in migration of progenitor cells into the myotome and their specification into the myogenic fate (55, 56). Myf5 is not necessary for normal myotomal muscle development although in its absence, a subset of progenitor cells migrate abnormally to sites within the sclerotome and dermomyotome and adopt the fate specific to these regions and it has been shown that Myf5 null mice exhibit defects of epaxial muscle (57). It is thought that MyoD rescues the myogenic program in the absence of Myf5 (57). MyoD is expressed at E9.5 mostly in the hypaxial domain of the dermomyotome (58, 59). Forced expression of MyoD causes the conversion of other cell types into muscle most likely by chromatin remodeling at muscle-specific loci and by transactivation of transcription of myogenic genes (52, 60). In addition, MyoD can interact with HDAC1 in order to prevent premature differentiation of myoblasts (61). It was recently shown through a high-throughput study aiming to identify MyoD binding sites that MyoD was enriched at thousands of sites within the genome and this binding was associated with increased histone acetylation at those sites (62), possibly

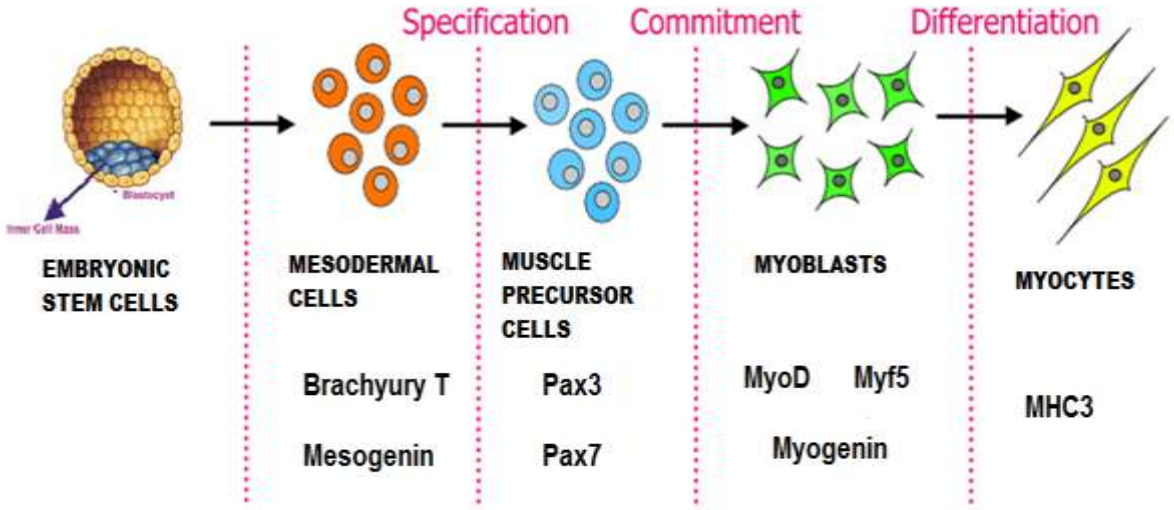

Specification
Commitment
Differentiation
Blastocyst
Inner Cell Mass
EMBRYONIC STEM CELLS
MESODERMAL CELLS
Brachyury T
Mesogenin
MUSCLE PRECURSOR CELLS
Pax3
Pax7
MYOBLASTS
MyoD
Myf5
Myogenin
MYOCYTES
MHC3

Figure 1.4: Transcriptional regulation of skeletal myogenesis in the vertebrate embryo.

A schematic representation of the series of molecular events involved during stem cell differentiation and mesoderm patterning into skeletal muscle. The important signaling molecules, pivotal transcription factors and structural genes involved in skeletal muscle formation are illustrated.

explaining why MyoD can reprogram other cell types to the myogenic lineage. As in the case of Myf5, MyoD null mice do not display any gross muscle defect (59, 63).

MyoD null mice display delayed hypaxial myogenesis while the epaxial myogenesis remains unaffected (63). These observations suggest that while MyoD and Myf5 might have redundant functions during myogenesis, they each have specific roles in regulation of hypaxial and epaxial muscle formation. An interesting observation in MyoD-/- mice is that these mice display an impaired regeneration in response to injury due to the tendency of their satellite cells towards self-renewal rather than terminal differentiation (64, 65).

MRF4 is expressed in the myotome from E9.0 to E12.0, after which its expression is repressed until after birth (66). It has been shown that MRF4-/- embryos undergo normal skeletal myogenesis and MRF4 knockout mice exhibit only minimal muscle defects likely due to compensation by high levels of Myogenin (67, 68). The presence of MRF4 in MyoD/Myf5 double mutant mice is sufficient to rescue myoblast determination and myogenesis suggesting a role for MRF4 in the specification of myogenic fate (57). Furthermore, it was shown that MRF4 (but not Myf5) can rescue skeletal muscle myogenesis in the Myogenin-/- mutants, which displayed inhibition of muscle differentiation, indicating that in addition to involvement in myoblast determination, MRF4 plays a role in regulation of differentiation (69). Finally, it was shown that mice lacking Myf5, MyoD, and MRF4 are defective in myogenesis and unable to form myoblasts (57).

Myogenin mRNA is detected at E8.5 following the expression of Myf5, which is thought to regulate Myogenins expression (70- 72). Myogenin protein however is not detected until E10.5, indicating some sort of post-transcriptional regulation or protein instability (71, 72). Although expressed early during embryonic myogenesis, Myogenin is primarily known for its role in terminal differentiation of skeletal muscle. Myogenin-/- mice display inhibition of

muscle differentiation and do not fuse efficiently into myotubes and hence these mice die prenatally (73, 74). When Myogenin was inserted into the Myf5 locus in the Myf5-/-/MyoD-/- mice (which are also missing MRF4), myogenesis was only partially rescued, indicating that Myogenin has some ability to direct cells into the myogenic lineage though Myf5 action is required early during differentiation (75, 76).

1.4.4 Mature skeletal muscle marker: Myosin Heavy Chain. The expression of the MRFs leads to the formation of terminally differentiated skeletal myocytes which express myosin heavy chain three (MHC3), an important protein in the contractile apparatus (Figure 1.4). This protein belongs to a group of proteins called myosins, which are involved in cell movement and transport of materials within and between cells. Thick myosin filaments and thin actin filaments are the primary components of muscle fibers and are important for muscle contraction.

1.5 Satellite Cell & Muscle Regeneration

The Pax3+/7+ population present in the central dermomyotome is responsible for giving rise to adult muscle stem cells, known as satellite cells, which have an important role in maintaining skeletal muscle throughout the life of an organism (34). At around E16, which marks the end of fetal myogenic development, Pax3+/7+ progenitor cells begin to accumulate under the basal lamina that forms around the muscle fibers and become quiescent satellite cells (14). These satellite cells remain dormant until after injury, where they become activated, differentiate and fuse to existing muscle fibers or together to form new fibers. Muscle satellite cells (SCs) were primarily identified by their unique anatomical position between the sarcolemma (cell membrane) and the basement membrane of myofibers and were therefore hypothesized to be the stem cell responsible for adult vertebrate muscle

regeneration (77). During development, the mesodermal progenitor cells called myoblasts fuse to form myofibers (78). In the neonatal stages, the number of myofibers remains constant, but each myofiber grows in size upon fusion of a differentiated satellite cell. Usually SCs remain quiescent, with sparse periods of activation wherein they fuse to compensate for muscle turnover caused by daily wear and tear (78). Throughout its lifespan satellite cells remain in a quiescent state, but upon muscle damage they can re-enter the cell cycle and either undergo one of two division types. Some undergo a symmetric cell division to self-renew and expand the satellite cell population; others undergo an asymmetric cell division that results in the cell on the basal lamina side maintaining its satellite cell identity, while the cell adjacent to the muscle fiber enters the myogenic differentiation program by expressing the MRFs to generate myoblasts, which terminally differentiate by fusing to each other or with damaged fibers (79, 80).

Skeletal muscle regeneration is a highly orchestrated process involving the activation of various cellular and molecular responses (78). The self-renewal of proliferating satellite cells not only maintains the stem cell population but also provides many new myogenic cells, which can proliferate, differentiate, fuse, and lead to new myofiber formation, in turn helping to reconstitute the functional contractile apparatus (78). Cell fate decisions undertaken by the satellite cells upon muscle damage are thought to be regulated through epigenetic mechanisms that modify the structure of chromatin without changing the DNA sequence (81). The antagonistic Polycomb group and Trithorax group proteins play a pivotal role in the epigenetic marking of muscle-specific genes to ensure proper temporal and spatial expression during muscle regeneration (81).

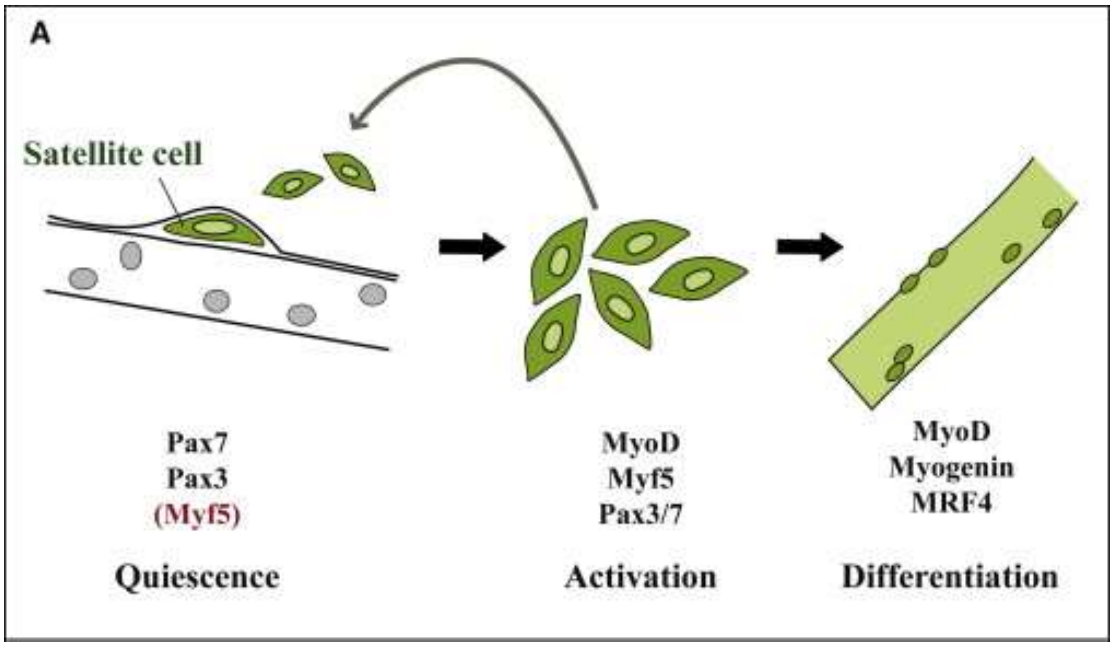

A
Satellite cell
Pax7
Pax3
(Myf5)
Quiescence
MyoD
Myf5
Pax3/7
Activation
MyoD
Myogenin
MRF4
Differentiation

Figure 1.5: Process of satellite cell activation and differentiation upon injury.

A schematic representation of adult muscle stem cells progressing through the stages of activation and differentiation, upon different stress's such as injury or acute exercise. In resting muscle, SCs are always quiescent marked with the expression of the paired box homeodomain markers. Upon injury, they get activated and turn on MRF expression and then differentiate to fuse with newly forming muscle fiber at the site of injury. Some of the SCs also undergo self-renewal to t repopulate the SC niche. Reproduced from M. Buckingham, & P. Rigby, (2014). Gene Regulatory Networks and Transcriptional Mechanisms that Control Myogenesis, Developmental Cell, 28: (3)225 – 238 with permission from Elsevier © 2014.

Relaix *et al.* have shown that in addition to their role in embryonic muscle development, Pax3 and Pax7 are involved in regulation of satellite cell function, possibly through a MyoD-dependent pathway (82). During the onset of skeletal myogenesis in the embryo, Pax3 is required for the persistence of the ventro-lateral dermomyotome, the part of the somite that gives rise to hypaxial body (82, 83) and limb (84) musculature. It is important to note that although Pax7 activity is not essential for embryonic myogenesis in the presence of Pax3, its expression is vital for the formation of satellite cells (47, 86). In fact Pax7 null mice completely lack satellite cells. Interestingly however, it has been shown that loss of Pax7 or Pax3 expression in adult mice has no impact on the ability of these mice to regenerate in response to injury, suggesting that Pax7 activity is not important for self-renewal or differentiation of satellite cells in adults (87). Recently, however, it was shown that Pax7 labels satellite cells and ablation of Pax7+ satellite cells completely blocks regenerative myogenesis following injury, suggesting their essential role for acute injury-induced muscle regeneration (88, 89). Despite a role for Pax3 and Pax7 during embryonic muscle development, their role in adult muscle regeneration through satellite cell activation remains unclear.

Studies in adult mice have shown that either Pax7+ or Pax3+ satellite cells that do not yet express MRFs can be used to repopulate the SC niche, which is a key determinant of a successful muscle engraftment (90-92). Given that the harvesting of satellite cells for transplantation can itself activate them, myoblast transplantation protocols have met with limited success (93). Hence, it is of the utmost importance to understanding the nature, origin and function of these satellite cells, so that they can be maintained *ex vivo* such that this adult stem cell population can be amplified for therapeutic use to treat muscle-wasting diseases such as MD.

1.6. SOX Superfamily of Transcription Factors

The HMG box is a novel type of DNA-binding domain found in a diverse group of proteins. The HMG box superfamily broadly comprises 5 major subfamilies: the High Mobility Group proteins HMG1 and HMG2, the nucleolar transcription factor UBF, the lymphoid transcription factors TCF-1 and LEF-1, the fungal mating-type genes mat-Mc and MATA1, and the mammalian sex-determining gene SRY. Evolutionary studies date the superfamily at least 1,000 million years ago, as its members appeared in animals, plants and yeast (94). A large portion of the eukaryotic DNA binding proteins can be identified by a set of conserved structural motifs such as: zinc finger, basic leucine zipper, homeodomains and the helix-loop-helix motif, and on the basis of this, the superfamily can be subdivided into 2 main subfamilies: TCF/SOX and UBF/HMG (94).

The SOX proteins are characterized by their 79-amino acid HMG domain that shares more than 60% sequence similarity to the HMG box of SRY (sex determining region of the Y chromosome) (95). SOX genes have shown to be involved in the regulation of embryonic development, the determination of neural progenitor fate, chondrogenesis, cardiogenesis and other cell fates. At least 30 members of the SOX family have been identified so far (96, 97). SOX genes are further divided into nine subgroups (A-I) based on their amino acid sequence (degree of homology) inside the HMG domain consisting of three α helices (98, 99) and the presence of conserved motifs outside the HMG box (100). Although the precise functions of many SOX proteins is still unknown, many have been identified as transcription factors that bend DNA upon binding to the minor groove of the double helix at the consensus sequence 5’-(A/T)(A/T)CAA(A/T)G-3’ (101, 102). The HMG box is believed to interact with DNA as a monomer and this binding widens the minor groove and causes DNA to bend towards the major groove (103). The binding of a SOX protein alone to DNA does not appear to elicit

transcriptional activation or repression, but instead requires binding of another partner transcription factor (TF) to an adjacent site on the DNA (104). This theory fits in accordance to the emerging idea that transcriptional regulation seen during developmental processes is carried out by transcriptional factor complexes, rather than by a single TF (105, 106).

There are multiple levels of regulation of SOX activity. SOX proteins function together with partner proteins to elicit their action. SOX2 can bind and activate target genes in synergy with Pax6 in the lens, or with Otx2 in the retina (107). However, the expression of SOX genes themselves is frequently subjected to auto-regulation or control by other members of the SOX superfamily. The C-terminal domains of SOX21 and SOX14 proteins function as strong and weak repression domains, respectively, when linked to the DNA binding domain. These SOX proteins strongly (SOX21) or moderately (SOX14) inhibit the activation of SOX1 or SOX2, establishing that SOX14 and SOX21 are a repressing subgroup (B2) of Group B SOX genes, whereas SOX1, 2 and 3 are part of the activating B2 subgroup (108). SOX protein expression levels are modulated post-transcriptionally by microRNAs (miRNAs, miRs), which repress the translation and/or promote the degradation of their target mRNAs. Several examples of such regulation are detailed below (109,110) miR-145 expression is highly up-regulated during the differentiation of human embryonic stem cells (HESCs). miR-145 directly targets 3′ UTRs (untranslated regions) of the mRNAs of SOX2 and other ‘core pluripotency factors’, and promotes their differentiation into mesoderm and ectoderm lineages (110). miR-200 family members are expressed at high levels in mouse embryonic stem cells (MESCs) and neural stem/progenitor cells. SOX2 is directly targeted by miR-200c, whereas SOX2 trans-activates the promoter of the miR-200c/141 gene, thereby forming a negative-feedback mechanism (109). This miR-200-mediated negative regulation might be what leads to gradual reduction in SOX2 levels during neural differentiation. SOX

protein function is known to be dose-dependent (111), so modulating SOX protein levels is an important mode of regulation. Studies revealed that there is a requirement for SOX2 in the maintenance of neural stem cells, as well as a downstream role in the differentiation of specific neuron sub-types. In both systems, SOX2 action is markedly dose-dependent (111).

In addition, SOX protein activity in a cell is modulated by covalent modification or by interaction with various proteins. Notably, SOX- dependent regulations intersect with important signaling systems such as the sonic hedgehog (SHH) and Wnt signaling pathways, in which SOX-Gli and SOX-β-catenin interactions, respectively, are implicated (112-117). Various post-translational modifications such as phosphorylation (118, 119), sumoylation (120), acetylation (121), methylation (122) and glycosylation (123) have also been reported to modulate the activity, stability and intracellular localization of many of its members.

SOX7 is a member of Subgroup F that has two other members, SOX17 and SOX18. Studies have been performed wherein the human SOX7 gene has been characterized and compared to its mouse orthologue. Chromosomal mapping analyses have localized mouse SOX7 on chromosome 14, band D, and located human SOX7 in a region of shared synteny on human chromosome 8 (8p22) (117). Quantitative PCR studies detected the presence of SOX7 mRNA during embryonic development in various tissues, but most abundantly in the brain, heart, lung, kidney, prostate, colon and spleen suggesting a role in their development and differentiation (117). Studies done in *Xenopus laevis*, a model for studying cell fate maps and lineage tracing, have assigned a role for SOX17 protein as an early endoderm inducer (125) and also proposed a role during spermatogenesis in mouse (126). Point mutations in SOX18 are implicated in cardiovascular and hair follicle defects in *ragged* mice (127). Other

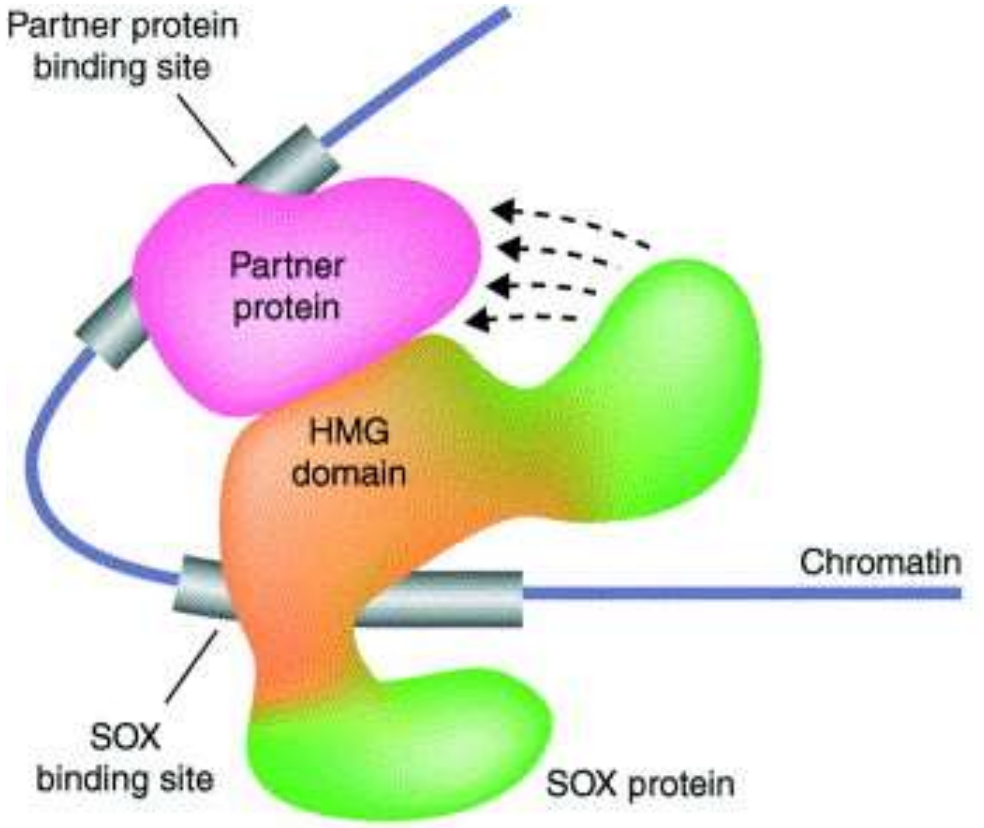


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Figure 1.6: A model for the specificity of SOX transcription factor action.

SOX transcription factors may function as activators or repressors by interacting with partner proteins that bind to adjacent binding sites in the promoter or enhancer of target genes. The SOX-partner interaction could help to stabilize binding of the transcription factor complex to DNA. The interaction of SOX factors with the partner proteins that are expressed in specific tissues may contribute to regulation of different genes by the same SOX factor expressed in different cell types. Reproduced from M. Wilson, & P. Koopman, (2002). "Matching SOX: partner proteins and co-factors of the SOX family of transcriptional regulators". Current Opinion in Genetic Development, 12:175-177, with permission from Elsevier © 2002.

characteristic features of SOX proteins include functional redundancy between members of the same subgroup and at any given time more than one SOX gene is expressed in many cell or tissue types. In addition to its role in the induction of endoderm, studies in *Xenopus laevis* demonstrated that SOX7 was able to induce the expression of mesoderm inducing genes in this model (128). This indicates that SOX7 may play a role in regulating cell fate and the formation of mesodermal cells. The total knockdown of SOX7 is embryonic lethal from E10.5–14.5 in mice further indicating the importance of this transcription factor during embryogenesis (128).

During normal development, expression of SOX7 can be detected at the 4-somite stage (8 d.p.c.) mouse embryo in the somites and head regions. At 9.5 d.p.c., SOX7 expression was clearly marked in the intersomitic vessels persisting until 11.5 d.p.c. At 17.5 d.p.c., the expression continued throughout the brain, cochlea, tongue, cartilage, lung, liver and vertebrae (117). Recently, transient SOX7 expression was detected in quiescent adult muscle satellite cells (129). Purified satellite cells from adult skeletal muscle (mainly quiescent) were compared to satellite cells from 1-week postnatal muscle and from adult dystrophic muscle of mdx mice (130) that were undergoing activation. SOX7 expression was shown to be highest in quiescent cells and down-regulated during activation.

Functionally, SOX7 has been identified as a transcriptional activator (117). Transcriptional regulation was examined with truncated mouse SOX7 constructs containing the HMG domain, but lacking different parts of the C-terminal region. Transfection experiments done in COS7 cells revealed that the full- length SOX7 (FLSOX7) (131) activated transcription by binding to a consensus DNA binding site present in the reporter plasmid SOXLUC, while the other mutants with deletions in the C-terminal domain of SOX7 failed to function as transcriptional activators (117).

1.7 Models for Myogenic Differentiation

1.7.1 P19 Embryonal Carcinoma Cells: Before efficient protocols for maintaining and differentiating mESCs and hESCs were developed, embryonal carcinoma cell lines were used predominantly as a model to study cellular differentiation mechanisms. Particularly, P19 embryonal carcinoma (EC) cells have served as a useful model to study cardiac and skeletal muscle development (132). These cells are derived from a teratocarcinoma formed after transplantation of a 7.5 day mouse embryo into the testis of a male CH3/HC mouse and display pluripotency by their ability to contribute to tissues from all three germ layers (133,134). The advantages using the P19 cell system are: they grow quickly and maintain an undifferentiated state in culture, their differentiation is controlled by the culture conditions, and they are amenable to genetic manipulation, including stable integration or knockdown of genes (132). P19 EC cells can be induced into cardiac and skeletal muscle by aggregation (needed for the induction of mesoderm) and exposure to low levels of dimethyl sulfoxide (DMSO) (135, 136). In addition, factors in fetal calf serum play a role in the efficiency of P19 cell differentiation (137). After 6 days of differentiation, cardiomyocytes will form and they make up approximately 10-20% of the total cell population, while skeletal myocytes form after 9 days of differentiation and account for 5-15% of total population (135, 137). Many studies have supported the validity of P19 EC cells as a model for the study of differentiation of embryonic stem cells. Embryonal carcinoma cells are generally considered to be the malignant counterparts of embryonic stem cells and within this resides the biggest disadvantage for using these cells as a therapeutic option. Since immortalized cell lines are often genetically abnormal and have been maintained under artificial conditions in culture for very long periods of time, they cannot be considered normal and there remains the possibility of carcinoma formation when injected into the host during transplantation.

Another issue with P19 cells is that sub-optimal culture conditions can lead to spontaneous differentiation of these cells, causing increased contaminating cell types in the differentiated culture.

1.7.2 Mouse and human embryonic stem cells: Mouse and human embryonic stem cells (ESCs), derived from the inner cell mass at the blastocyst stage of the embryo, are maintained pluripotent through the function of transcription factors SOX2, Oct4 and Nanog that regulate target genes for the purpose of self-renewal (138). mESCs and hESCs do follow embryonic stages of differentiation, albeit with slower kinetics and with a similar set of transcription factors waves at each stage as seen in the embryo. The cooperation of these three transcription factors in the regulation of pathways involved in pluripotency is evident by the large subset of common target genes they share with each other (139). Many groups have extensively studied mESCs and hESCs in the context of myogenesis and reported the successful differentiation of these cells into cardiac and skeletal muscle in culture (140, 141 , 142-145), though difficulties in obtaining sufficient amount of skeletal muscle *in vitro* have been reported. Most of the differentiation protocols for hESCs differentiation into muscle are not reproducible and do not use embryonic pathways (145). Very recently, however, a protocol was developed that allows the hESCs to form muscle through the formation of a Pax3/7+/MRF progenitor population (142). Previous studies had shown that Pax3/7-positive progenitor cells can repopulate the satellite cell niche, indicating the importance of this population niche for therapy. In this study they sought to optimize the differentiation of hESCs into skeletal muscle in order to characterize the myogenic pathway at a molecular level and shorten the time course. They treated hESCs with retinoic acid (RA) and found a specific enhancement of skeletal myogenesis, and the expression of the myogenic regulatory factors (MRFs) MyoD and Myogenin by day 25. Furthermore, they also observed that that

RA treatment expanded the distinct Pax3+ population muscle progenitor pool, which occurred prior to MRF expression. Even in P19 embryonal carcinoma cells the RA signaling pathway was shown to regulate premyogenic mesoderm formation at low dosages (3-30nM) by up-regulating Gli2, Pax3, Meox1 and Wnt3a transcript levels (141). In the differentiation of mESCs carried out during this research project, RA treatment was not applied prior to MRF formation and could potentially be used as a step towards future directions. Although promising, a major barrier facing the use of embryonic stem cell therapy is the inability of current methods to commit a sufficient proportion of these cells into a skeletal muscle lineage in order to form large homogenous pools of skeletal muscle progenitor cells required for effective therapy. Currently, only a small percentage (<10%) of skeletal myogenesis occurs when these cells differentiate and the replication of this low occurrence is very inconsistent (142). Although there are specific protocols used to induce differentiation of these cells towards a particular lineage there is always a high proportion of contaminating cells of different lineages found in these cultures. Indeed, the formation of contaminating cardiomyocytes are difficult to separate from skeletal myoblasts using a pan Myosin Heavy Chain antibody.

1.7.3 Primary Myoblasts: Myofibers are the functional contractile units of skeletal muscle. While myofibers are established during embryogenesis by fusion of myoblasts into myotubes, processes involved in their growth and repair continue throughout life (78). These processes are supported by myogenic progenitors called satellite cells that are located between the basal lamina and the sarcolemma of the myofiber (77). Skeletal muscle precursors persist in mature muscle as satellite cells, maintaining the capacity to self-renew and to differentiate (146). These adult muscle stem cells facilitate the postnatal growth, remodeling, and regeneration of skeletal muscle. Given the remarkable regenerative potential

of satellite cells, there is great promise for treatment of muscle pathologies such as the muscular dystrophies with this cell population. In response to a variety of conditions, ranging from increased muscle utilization to muscle injury, satellite cells can enter the cell cycle, producing progeny that fuse into existing myofibers, or form new myofibers (79,80). Satellite cells are considered stem cells because in addition to giving rise to progeny needed for myofiber repair; they can also self-renew (147,148). It is not known, however, if all satellite cells are identically amplified and renewed (148,149). Two main cell culture approaches have been employed in the study of satellite cells: (i) primary myogenic cultures prepared from mononucleated cells dissociated from whole muscle; and (ii) cultures of isolated myofibers where the satellite cells remain in their *in situ* position underneath the myofiber basal lamina. Protocols for obtaining primary myogenic cultures involve releasing satellite cells from their niche. Steps of mincing, enzymatic digestion and repetitive trituration of the muscle are required for breaking down both the connective tissue network and the myofibers in order to release the satellite cells from the muscle bulk. These steps are followed by procedures for removing tissue debris and reducing the contribution of non-myogenic cells typically present in primary isolates of myogenic cells, by a process called pre-plating (148,150,151,152–156). In contrast, protocols for isolating individual muscle fibers result in the release of intact myofibers that retain satellite cells in their native position underneath the basal lamina (150, 153, 157-159). These protocols allow the study of satellite cells and their progeny in their *in situ* position on the myofiber, and after they migrate from the parent myofiber. Both of these methods accurately replicate the activation and differentiation of satellite cells *in vivo*. However, while various protocols have been developed which allow for isolation, enrichment, and expansion of satellite cell derived muscle stem cells, isolated satellite cells have yet to translate into effective modalities for

therapeutic intervention. One of the major setbacks in using satellite cells for therapy is the maintenance of its "stem-ness". Typically, these cells lose their stem cell-like function as soon as they are isolated to culture. Plating down these cells turns on the MRF expression within them, causing these cells to differentiate into primary myoblast cultures. Although studies in adult mice have shown that either Pax7+ or Pax3+ satellite cells that do not yet express MRFs can be used to repopulate the satellite cell niche, which is a key determinant of a successful muscle engraftment (90-92). So given that the harvesting of satellite cells for transplantation can itself activate them, myoblast transplantation protocols have met with limited success (93). Broadening our understanding of satellite cells and their niche requirements should improve our *in vivo* and *ex vivo* manipulation of these cells to advance their use for regeneration of diseased muscle.

1.8 Rationale

In culture studies performed in our lab using P19 embryonal carcinoma (EC) cells and a 15 day differentiation assay, revealed that SOX7 expression is both essential and sufficient to trigger the formation of skeletal myocytes in conditions that do not typically support myogenesis (160). SOX7 was found to up-regulate the expression of premyogenic markers Pax3/7, Meox1, Foxc1 and induce myogenesis by enhancing the expression of the myogenic regulatory factors (Myf5, MyoD and Myogenin) and also the mature skeletal muscle marker, MHC3 (160). Furthermore, ChIP-qPCR analysis revealed enrichment of SOX7 protein to a SOX DNA binding site (HMG box) in the Pax3 promoter (Ebadi *et al.*, unpublished results). The recruitment of SOX7 to the Pax3 promoter correlated with increased Pax3 expression levels under these conditions. The Pax3 promoter region was also found to be activated in a dose-dependent manner by increasing amounts of SOX7 in a reporter assay (Ebadi, *et al.*,

unpublished results). In P19 cells overexpressing Pax3, up-regulation of SOX7 expression was observed, indicating a potential positive regulatory loop between SOX7 and Pax3/7. Studies in literature have shown that the Wnt signaling pathway plays a pivotal role in regulating skeletal myogenesis, thus using ChIP-qPCR, we found ß-catenin (a major factor of the Wnt pathway) bound to the regulatory regions of the SOX7 gene, implicating SOX7 as a ß-catenin target (161), suggesting a model of ß-catenin/SOX7/Pax3 functional interaction and in turn a role for SOX7 in the process of myogenesis. Our lab has also performed knockdown studies in P19 EC cells that show that the loss of SOX7 results in defects in skeletal myogenesis and that SOX17 and SOX18 do not compensate for this loss (Ebadi, *et al*., unpublished results).

1.9 Thesis Summary

In order to design therapies to restore damaged muscle tissues using cell transplantation approaches, we need to understand the molecular networks and signaling pathways which regulate the process of muscle development and regeneration. This will allow us to manipulate these mechanisms to generate high yields of skeletal muscle progenitors. Currently there are at least two major limitations to cell therapy, differentiation of these cells into the desired tissue and engraftment of these cells at the site of transplantation. My first objective was to further extend our knowledge on the role of SOX7 during embryonic muscle development. My specific aim was to overexpress the transcription factor SOX7 during skeletal myogenesis in mouse embryonic stem cells (mESC) using nucleofection and to study the effect of overexpressing SOX7 on the expression of mesoderm induction markers, premyogenic mesoderm markers and as well as MRFs. We hypothesized that SOX7 overexpression in the mouse embryonic stem cell model would promote skeletal myogenesis

through the up-regulation of Pax3/7, as seen previously in P19 EC cells (160). Here we present data showing that SOX7 does enhance skeletal muscle formation in mESCs but independent of Pax3/7.

My second objective was to test the effect of SOX7 knockdown *in vivo* using a mouse model. My specific aim was knockdown SOX7 in Pax3+ cells (muscle progenitors) and to measure the differentiation and regeneration capacity as compared to littermate controls. We hypothesized that the loss of SOX7 *in vivo* would cripple skeletal muscle development. Indeed, given that SOX7 transcripts are detected in quiescent muscle satellite cells (129), we predicted that SOX7 might also be playing an important role post-natally. Using a conditional $SOX7^{-/-}$ mouse model, we hypothesized that loss of SOX7 would lead to activation of fewer satellite cells which in turn would impair the differentiation and fusion of these cells. This loss would also be predicted to cause inefficient muscle regeneration after acute injury and affect the quantity and size of the muscle fibers. Here we present data showing that loss of SOX7 during post-natal stages of murine development *in vivo* resulted in defective satellite cells which were incapable of differentiating and fusing *in vitro* to the same extent as the wildtype mice. Fewer Pax7+ satellite cells were observed as compared to the wildtype. RT-qPCR data also revealed that loss of SOX7 transcript led to decreases in Pax7, MyoD, Myf5 and MHC3 transcript levels, suggesting poor induction of myogenesis.

2. MATERIALS AND METHODS

2.1 Constructs

The SOX7 expression plasmid was created by Dr. Josée Coutu. The phosphoglycerate kinase (PGK)-SOX7 was created by excision of the SOX7 open reading frame (ORF) from the pCMVScript vector (gift from Y. Hayashi, Japanese Science and Technology Agency). Ends of the excised fragment were blunted and the insert was placed in the SmaI site of the PGK vector that has been previously described (162). An empty PGK vector was used as a negative control. The PGK-SOX7 (E174) or empty PGK (V5) plasmid was co-transfected along with a PGK-Puro (E11) a plasmid conferring Puromycin resistance (Invitrogen, Carlsbad, CA, USA) (163).

The plasmids were grown in *Escherichia coli* DH5α. Transformed bacterial cells were grown on Luria Bertani agar plates and selected for by using ampicillin (Sigma-Aldrich, St. Louis, MO, USA). Pure Yield^{TM} Plasmid Maxiprep system (Promega, Madison, WI, USA) was used to purify plasmids from bacterial cells. Subcloning was confirmed by agarose gel electrophoresis. Concentration of DNA samples was measured using the Nanodrop^{TM} Spectrophotometer (ND-1000) (Thermo Fisher Scientific, Waltham, MA, USA). The PGK-SOX7 and the PGK-Puro plasmids were linearized for better nucleofection using restriction enzymes HindIII and XbaI respectively (New England Biolabs, New England, MA, USA). Digest was confirmed using agarose gel electrophoresis.

2.2 Cell Culture

D3 mouse embryonic stem cells (ATCC, #CRL-1934) were grown in 100mm culture dishes (Corning, New York, USA) at 37^{o}C and 5% CO_2 in mESC complete medium (Dulbecco's Modified Eagle Medium (DMEM) High Glucose Medium (Life Technologies, Carlsbad, CA, USA) supplemented with 13% fetal bovine serum (FBS)(Wisent Bioproducts,

St-Bruno, PQ, Canada), 1X non- essential amino acids (Invitrogen), 8mg/L β-Mercaptoethanol (J.T. Baker) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA)). To prevent cells from spontaneously differentiating, 1000U/mL of leukemia inhibitory factor (LIF) (Millipore, Billerica, MA, USA) was added to cell cultures. Cells were passed using Trypsin-EDTA at reaching roughly 70% confluency (~2 days) and nutritionally depleted media was replaced with fresh media. Cells were counted using a Coulter Counter Beckman Z Series (Beckman, Brea, CA, USA) and plated at a density of 20,000 cells/mL.

2.3 Nucleofection

For the generation of stable cell lines, 2 x 10^6 trypsinized cells were electroporated using nucleofection as per manufacturer's protocol using 2 μg of PGK-SOX7 and 0.4 μg PGK-puro and program CG-104 of the 4D-Nucleofector (Lonza, Basel, Switzerland). Similarly the mESC[Control] cells from the same culture were electroporated using the empty PGK and PGK-puro plasmids. The pmaxGFP vector supplied by Lonza was used as a positive control for nucleofection, 2 μg of this vector was added to the mESCs. Two negative controls were used for Nucleofection. This included cells that were subject to the nucleofection program without DNA and cells with 2 μg pmaxGFP vector to which no electric pulse was applied.

Following nucleofection, cells were cultured in mESC complete medium overnight, which was further supplemented with 2 mg/mL Puromycin (Invitrogen) and LIF (Millipore) on day 2 post nucleofection. Media was changed every day for the first 3 days followed by every alternate day thereafter. Colonies arising from single cells were picked using Trypsin-EDTA on Day 10 and selected. Levels of SOX7 transcript expression were measured in puromycin resistant colonies using quantitative Polymerase Chain Reaction (qPCR).

2.4 Stem Cell Differentiation

mESC[Control] and mESC[SOX7] cells were differentiated according to standard protocol. Cells were collected by trypsinization and counted using the Coulter Counter Beckman Z series (Beckman). Cells were diluted to a concentration of 4 x 10^4 cells/mL in DMEM and using a multi-channel pipette (Thermo-fisher Scientific) 20μL drops were placed on the lid of a 150mm Petri dish (Corning) containing phosphate buffered saline (PBS) to prevent droplet evaporation. Cells remained suspended for 2 days in an incubator at 37^{o}C and 5% CO_2. On day 2 the cells, now in the form of aggregates, were moved using DMEM into 100mm petri dishes (Corning) coated with 1% agarose to prevent the aggregates from adhering. Cells were allowed to grow only for 5 additional days and the media was replaced with fresh DMEM every 2 days. On day 7 aggregates resuspended in DMEM were moved into 12 well tissue culture plates. Cells were plated directly into wells for RNA or protein extraction or wells containing 0.1% gelatin coated coverslips for immunofluorescence. Cells were allowed to grow for 3 days and media was replaced with fresh DMEM every alternate day. On day 10, DMEM media was replaced with low serum N_2 media (Gibco), to promote the differentiation of cells into a skeletal muscle lineage, which was replaced every 2 days until the end of the protocol on day 15 (164).

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

Total RNA was harvested from approximately 5 x 10^5 mESC[Control] and mESC[SOX7] cells on day 0, 3, 4, 5, 6, 7, 9, 12, and 15. Extraction of mRNA from cells was completed using the E.N.Z.A. ® Micro-elute Total RNA Kit I as per manufacturer's protocol (Omega Bio-tek, USA). mRNA concentrations following extraction were calculated using a Nanodrop^{TM} Spectrophotometer (ND-1000) (Thermo Fisher Scientific). Quantitect Reverse Transcription Kit (Qiagen, Gaithersburg, MD, USA) was used to reverse transcribe 1 μg of mRNA into cDNA as per manufacturer's protocol using the T100^{TM} Thermal Cycler (Bio-

Rad, Hercules, CA, USA). For qPCR analysis, 1/40 of the cDNA generated was used as a template and amplified with the KAPA SYBR® FAST Universal qPCR Master Mix (Kapa biosystems, Woburn, MA, USA) as per manufacturer's protocol. PrimerBank (165) or Primer-BLAST (166) was used to generate primers listed in appendix B. All primers were validated before use. Reactions were performed and data collected using the Mastercycler® Realplex^2 qPCR machine (Eppendorf, Mississauga, ON). Samples were analyzed in duplicates and the average of the two values was used for further analysis. Data was analyzed using the delta-delta CT method to quantify gene expression and graphs were made using Microsoft Excel (167). For this method, values obtained were first normalized to β-actin (for mouse embryonic stem cell differentiation assay experiments) and to 18S (for satellite cell isolation and *in vitro* differentiation assay experiments) then normalized to mESC[Control] day 0 samples. These values were expressed as for each respective gene and error bars represent ± standard error of the mean (SEM) of six independent experiments.

2.6 Western Blot Analysis

Total protein was collected from approximately 1x 10^6 cells from mESC[Control] and mESC[SOX7] cultures on day 0, 3, 4, 5, 6, 7 and 15 using radioimmunoprecipitation assay (RIPA) buffer (50mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA) containing 0.5 mM PMSF (Sigma-Aldrich) and 1X complete mini EDTA-free Protease Inhibitor Cocktail (Roche). Total protein concentration was determined using the Bradford assay. Proteins were separated based on their molecular weights using a 12% sodium dodecyl sulphate (SDS) polyacrylamide gel, when a potential difference of 200V was applied for one hour. Resolved proteins were transferred to an Immunoblot polyvinylidene fluoride (PVDF) membrane (Bio-Rad) by wet-transfer and an electrical potential difference of 100V was applied for one and a half hours. Membrane was blocked using 2% non-fat dry

milk powder in TBS-T solution for 1 hour on a shaker. SOX7, Pax3, Pax7 and CyclophilinB proteins were detected with anti- SOX7 (1:1000 dilution, Abcam, Cambridge, U.K. Cat# ab49163), anti- Pax3 (1:200 dilution, Santacruz Biotechnology, Santa Cruz, CA Cat# 34916) and anti- Pax7 (1:200 dilution, Developmental Studies Hydridoma Bank, DSHB, Iowa City, IA) and anti- CyclophilinB (1:5000 dilution, Abcam, Cambridge, U.K. Cat# ab16045) antibodies. Antibodies were diluted in 1X TBS-T overnight at 4^{o}C on a shaker. Following incubation the membrane was washed 3 times for 5 minutes in 1X TBS-T buffer. SOX7, Pax3, Pax7 and CyclophilinB were visualized using horseradish peroxide (HRP)-conjugated anti- rabbit, anti- goat, anti- mouse and anti-rabbit (1:5000 dilution, GE Healthcare, Little Chalfont, U.K.) respectively. This was followed by a chemiluminescence reaction using Pierce ECL substrate and luminal solution (Fisher Scientific, Nepean, ON, Canada). Chemiluminescence images were captured using the Luminescent Image Analyzer LAS-4000 (Fujifilm Life Science) and saved using Image reader LAS-400 and Multi Gauge v3.11 Fujifilm. Western blots were edited using the software Paint.net and quantified using ImageJ. Results are shown of four independent experiments.

2.7 Immunofluorescence Analysis

Immunofluorescence analysis was performed on mESC on day 15 of differentiation. Media was aspirated from cells and cells were fixed in -20^{o}C methanol. Cells in methanol were incubated at 4^{o}C for 5 minutes. Next the methanol was aspirated and cells were allowed to air dry for 5 minutes. Cells were then rehydrated with Stockholm's phosphate buffered saline (sPBS) for 15 minutes. Myosin Heavy Chain (MHC) was detected using a monoclonal mouse anti- MHC primary antibody (MF20) (DSHB, Iowa City, IA). Antibody was diluted 1:1 in sPBS and added to the samples. Next, samples were covered with Parafilm and incubated overnight at 4^{o}C. The secondary antibody used for MF20 was a Cy3-conjugated

goat anti- mouse IgG2B antibody (Jackson ImmunoResearch, West Grove, PA, USA). This antibody was used at a 1:100 dilution in sPBS. The cells were then covered with Parafilm and incubated for 1 hour at room temperature. Coverslips were mounted onto glass slides using mounting media composed of Hoechst dye (to stain the nuclei) diluted 1:100 in a 1:1 solution of Glycerol:sPBS to visualize cell nuclei. Clear nail polish was used to fix the coverslips to the glass slides. Cells were visualized with a Leica DMI6000 B microscope (Leica Microsystems Inc., Richmond Hill, ON, Canada) and images were acquired using a Micropublisher 3.3 RTV camera (Q Imaging, Surrey, BC, Canada). Staining was quantified by performing either manual or automated cell counts using the Volocity software (PerkinElmer Inc., Waltham, MA, USA) and represented as a proportion of total nuclei. Results are shown ± S. E. of the mean of six independent experiments.

2.8 SOX7 -/- conditional knockout mice generation

A monohybrid cross was carried out between the Parental generation of 4 month old B6:129- Pax3 $^{tm1(cre)}$ Joe/J (The Jackson laboratory, Bar Harbor, Maine, USA) and SOX7$^{F/F}$ mice (kindly provided by Dr. Daryl Scott) (128) to obtain the F1 generation of offspring with the genotype SOX7$^{+/-}$Pax3$^{+/Cre}$ and SOX7$^{+/F}$Pax3$^{+/+}$. Offspring genotype was confirmed by PCR by digestion of tail samples using the KAPA Mouse Genotyping Kit (KAPA Biosystems, Wilmington, MA, USA). The SOX7$^{+/-}$Pax3$^{+/Cre}$ offspring was further self-crossed to obtain the second filial generation of offspring with the genotypes SOX$^{-/-}$Pax3$^{+/Cre}$, SOX7$^{F/F}$Pax3$^{+/+}$, SOX7$^{+/-}$Pax3$^{+/Cre}$ and SOX7$^{+/F}$Pax3$^{+/+}$, which were again confirmed by genotyping as mentioned previously. For all the animal experiments, SOX7$^{F/F}$Pax3$^{+/+}$ was used as the Wildtype group and the SOX$^{-/-}$Pax3$^{+/Cre}$ was used as the experimental knockout group. All animals were maintained in ventilated racks in a controlled step-down barrier

facility in the Roger Guindon building at the University of Ottawa, at 22°C with 30% relative humidity on a 12 hours light/dark cycle and provided food and water *ad libitum*.

2.9 Cardiotoxin Injury Experiments

For Cardiotoxin (Ctx) injury, mice were anesthetized with isofluorane before the procedure. Legs were shaved and washed with an antiseptic solution, after which 30 µl of 10µM Ctx in PBS or PBS alone was injected intramuscularly using a $26^{1/2}$ gauge insulin needle into the Tibialis anterior (TA) muscle. Mice were sacrificed 7 days post injury. For all animal work, following sacrifice, hind limb muscles from $SOX7^{F/F}Pax3^{+/+}$ and $SOX^{-/-}$ $Pax3^{+/Cre}$ mice were flash frozen in iso- butanol cooled in liquid Nitrogen and stored at -80C as mentioned in (168). Eight micron thick sections were cut and stored at -80C for histological studies.

2.10 Histological studies

For histological analysis, 8 micron thick sections were stained with hematoxylin and eosin following fixation in 10% Formalin for 15 min. Data analysis was performed manually on the total number of fibers and average fiber size (a minimum of 350 fibers from the TA muscle) using ImageJ and all graphs were made using Microsoft Excel as mentioned in (168). All animal handling procedures were in accordance to the guidelines established by the University of Ottawa Animal Care Service and the Canadian Council on Animal Care.

2.11 Isolation and Differentiation of adult muscle stem cells

Adult muscle stem cells were isolated using standard protocols (169). Lower hind limb muscles from $SOX7^{F/F}Pax3^{+/+}$ and $SOX^{-/-}Pax3^{+/Cre}$ male mice aged 6- 10 weeks were dissected and digested with collagenase and dispase (Roche Diagnostics). Digested muscle was passed through a 40 µm cell strainer to remove undigested connective tissue. After

multiple rounds of centrifugation the pellet was re-suspended in normal satellite cell growth media (DMEM with penicillin and streptomycin (Wisent) containing 20% FBS, 10% HS (Invitrogen)). These cells were pre-plated in an incubator with 37^{o}C and 5% CO_2 for 2-3 hrs. After pre-plating isolated cells from the supernatant were plated on Matrigel-coated 35mm culture dishes and allowed to attach and grow for 2 days. Media was changed every alternate day until day 4 at which point differentiation was promoted by changing the medium of 70% confluent myoblasts cultures to DMEM containing 2% FBS and 10% HS. The differentiation media was changed until day 7 every other day. mRNA was harvested on day 0, 3 and 7 for gene expression profile using qPCR analysis and immunofluorescence was performed using a monoclonal mouse anti- MHC primary antibody (MF20) (DSHB, Iowa City, IA) to stain myoblasts. Coverslips were mounted onto glass slides using mounting media composed of Hoechst dye (to stain the nuclei) diluted 1:100 in a 1:1 solution of Glycerol:sPBS to visualize cell nuclei. Clear nail polish was used to fix the coverslips to the glass slides. Cells were visualized with a Leica DMI6000 B microscope (Leica Microsystems Inc., Richmond Hill, ON, Canada) and pictures were acquired using a Micropublisher 3.3 RTV camera (Q Imaging, Surrey, BC, Canada). Differentiation and fusion indices were quantified using ImageJ software.

2.12 Fluorescent immunohistochemistry on frozen sections

Indirect immunofluorescence was performed on 8 micron thick frozen sections of the Gastrocnemius muscle, fixed in 4% paraformaldehyde. Detection was performed according to standard procedures (168) using the following antibodies: anti- mouse Pax7 (DSHB, Iowa City, IA) antibody, anti-mouse Biotin (Jackson Immunoresearch Labs, West Grove, PA,

USA Cat# 715-066-150) antibody conjugated to Alexa Fluor 594-Streptavidin (Jackson Immunoresearch Labs, West Grove, PA, USA Cat# 016-580-084).

2.13 Statistical Analysis

Statistical differences between means were calculated using the two-tailed Student's t-test. P-values of less than 0.05 were considered significant.

3. RESULTS

3.1. Validation of the animal model.

The $Pax3^{+/Cre}$ mice were crossed with the $SOX7^{F/F}$ mice in order to generate a mouse strain in which SOX7 was excised in all muscle precursor cells (Pax3+ cells). This provides a good mouse model for studying myogenesis and limb development in the absence of SOX7 by loss of function analysis. Meticulous observations made post-natally and throughout adult development revealed that the $SOX7^{-/-}$ mice were in no way impaired physically nor did they exhibit any striking abnormalities relating to limb development and/or movements. They were weighed between 4-6 months of age and no significant difference was found between them and their littermate controls ($SOX7^{F/F}$). To validate the animal model, mRNA was harvested from satellite cells isolated from $SOX7^{-/-}$ and littermate controls on Day 0, 3 (proliferating) and 7 (differentiated) post-isolation. cDNA samples made from this mRNA were analyzed using quantitative Polymerase Chain Reaction (RT-qPCR). A significant knockdown of SOX7 transcript levels (70-75%) was observed on Day 0 compared to the control mice and consistent with previously published results, the expression of SOX7 reduced dramatically with the onset of activation and differentiation (Fig. 3.1A). The expression of Pax7 and the myogenic regulatory factors (MRFs) MyoD, Myf5 were analyzed and there was significant down-regulation of MyoD and Myf5 transcript levels on Day 3 and 7 and a reduction in MHC3 of Day 7 in knockout mice. These results suggest that loss of SOX7 in satellite cells results in down-regulation of Pax7, the MRFs and significant loss of Myosin Heavy Chain 3 transcript levels, which is a marker for terminally differentiated myoblasts *in vitro.*

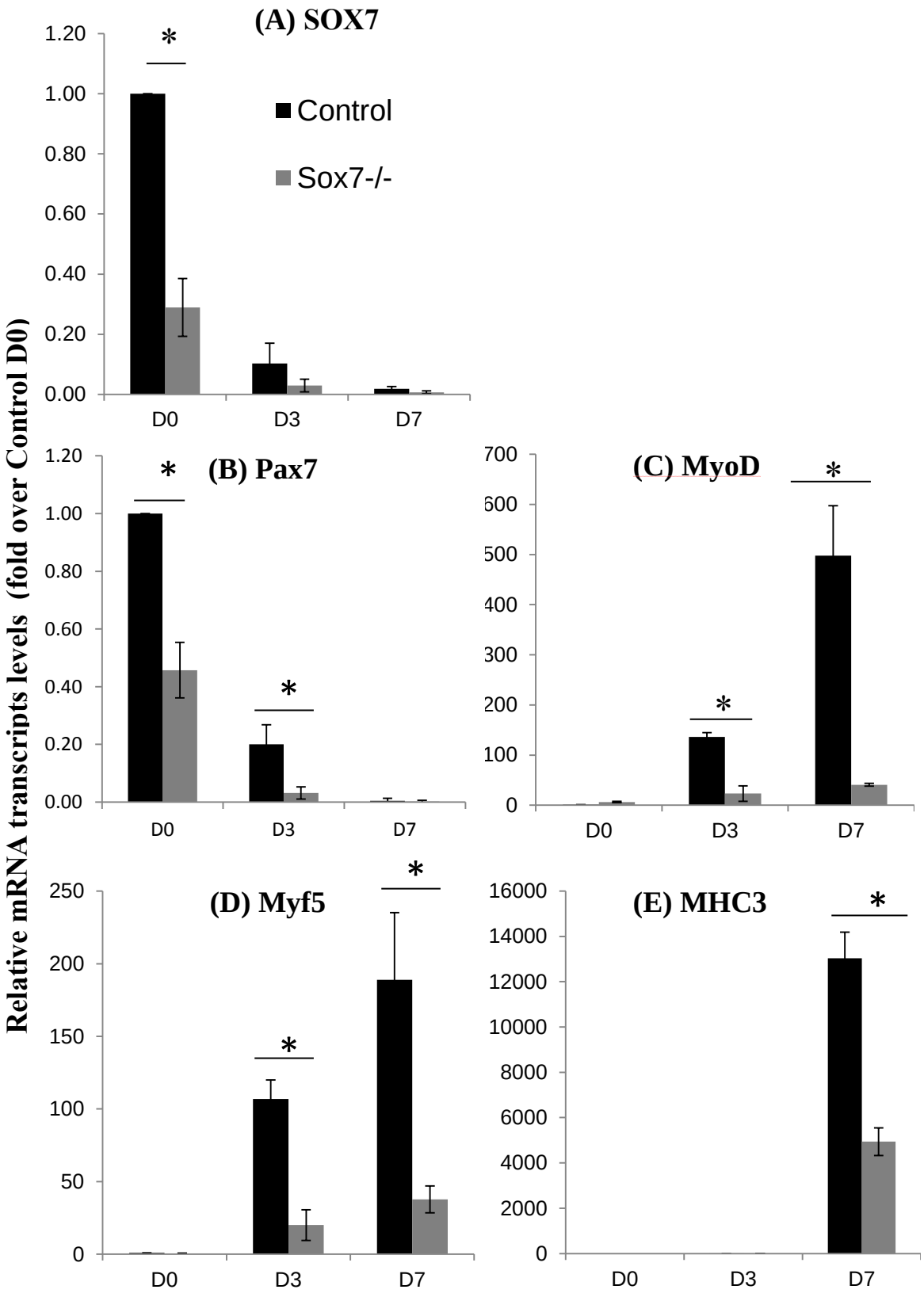
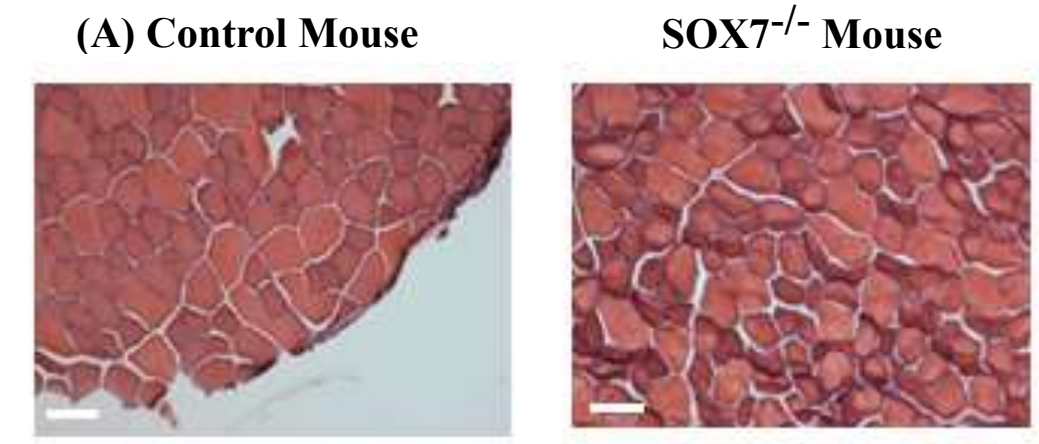
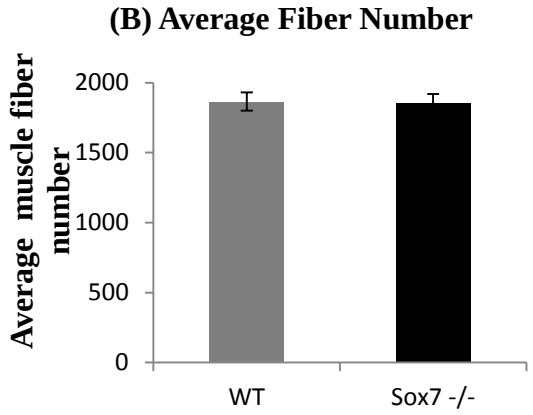

(A) SOX7
Control
Sox7-/-
Relative mRNA transcripts levels (fold over Control D0)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
D0
D3
D7
*
(B) Pax7
(C) MyoD
700
600
500
400
300
200
100
0
(D) Myf5
250
200
150
100
50
0
(E) MHC3
16000
14000
12000
10000
8000
6000
4000
2000

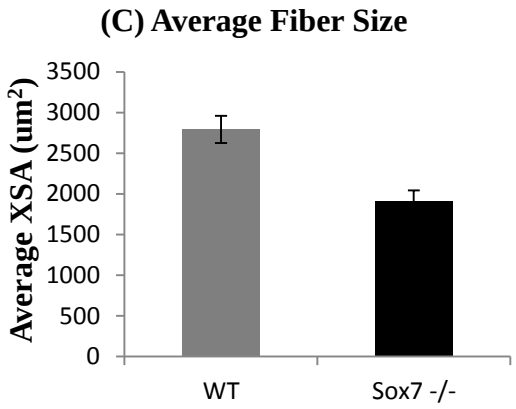
Figure 3.1: **Loss of SOX7 leads to a decrease in overall MRF transcript levels and also significantly down regulated MHC3 levels.** Transcript levels of SOX7 (A), Pax7 (B), MyoD (C), Myf5 (D) and MHC3 (E) in freshly isolated (Day 0), proliferating (Day 3) and differentiating (Day 7) satellite cells. Expression levels were first normalized to 18S rRNA then normalized to the expression on day 0 in control mice and presented as a relative mRNA levels for each gene transcript. Error bars represent ±SEM from an independent biological replicate using 3 Control and 3 $SOX7^{-/-}$ mice *$p<0.05$. n=3.

3.2 Loss of SOX7 leads to a decrease in the average muscle fiber size using the SOX7$^{-/-}$ mouse model.

At 12 weeks of age, the mice were sacrificed and the Tibialis Anterior (TA) muscle was dissected and stained using Hematoxylin and Eosin to study muscle histology (Fig. 3.2A). Staining revealed no significant difference in the total number of fibers per muscle section between the control and the SOX7$^{-/-}$ muscle (Fig. 3.2B), however the average size of the fibers was approximately 30% smaller in the SOX7$^{-/-}$ mouse than in control littermates (Fig 3.2C). These *in vivo* results lead us to investigate the potential role of SOX7 during *in vitro* primary myoblast development and fusion suggesting that this factor might be important during limb development.


(A) Control Mouse
SOX7-/- Mouse


(B) Average Fiber Number
Average muscle fiber number
2000
1500
1000
500
0
WT
Sox7 -/-


(C) Average Fiber Size
Average XSA (um2)
3500
3000
2500
2000
1500
1000
500
0
WT
Sox7 -/-

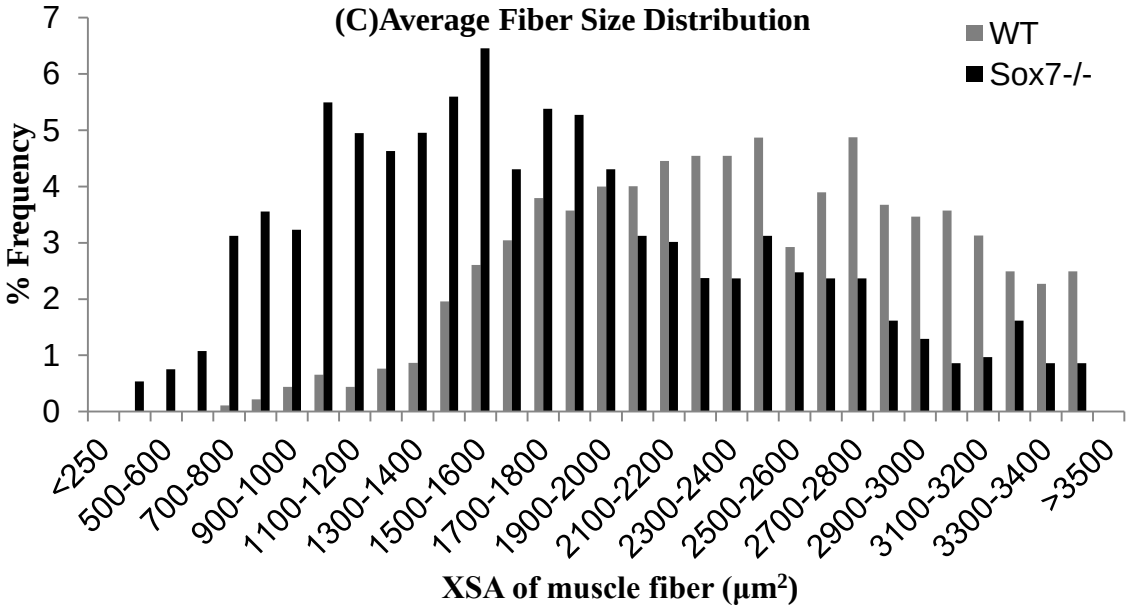
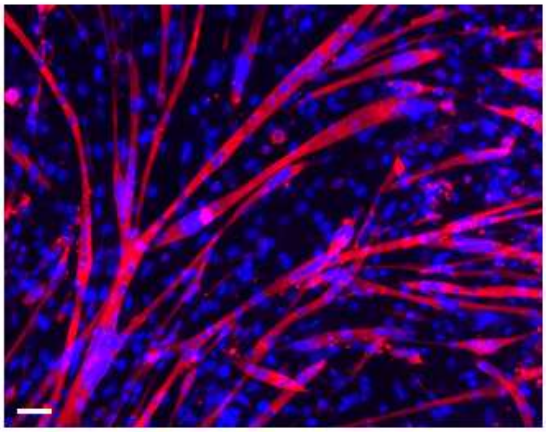

(C)Average Fiber Size Distribution
WT
Sox7-/-
% Frequency
7
6
5
4
3
2
1
0
<250
500-600
700-800
900-1000
1100-1200
1300-1400
1500-1600
1700-1800
1900-2000
2100-2200
2300-2400
2500-2600
2700-2800
2900-3000
3100-3200
3300-3400
>3500
XSA of muscle fiber (µm2)

Figure 3.2: Loss of SOX7 expression decreases muscle fiber cross-sectional area. (A) Hematoxylin and eosin staining was performed on the TA muscle of Control and $SOX7^{-/-}$ mice. (B) The average muscle fiber number in TA muscle isolated from $SOX7^{-/-}$ and control animals. (C) Average muscle cross-sectional area from $SOX7^{-/-}$ and control animals. Error bars represent ±SEM from independent biological replicates of 3 Control and 3 $SOX7^{-/-}$ mice, average muscle fiber number p= N.S. and average muscle fiber size p=N.S., n=3.

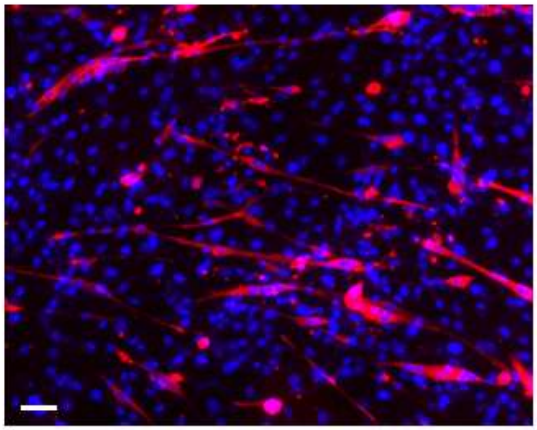
3.3 SOX7 inhibits the differentiation of satellite cells and fusion of myoblasts during an *in vitro* differentiation assay.

Previous studies showed that SOX7 transcripts were detected in quiescent adult muscle stem cells (129). We wanted to study the role of this transcription factor during satellite cell activation and during differentiation to fuse to become myofibers. For that purpose we isolated satellite cells from the hind limb muscles of the control and $SOX7^{-/-}$ mice through enzymatic digestion and selective plating. These cells were then grown on culture dishes in growth media until confluent (day 4 post-isolation) and induced to differentiate into primary myoblasts by lowering the serum for an additional 3 days (until day 7 post-isolation), a standard protocol used in the field. Following differentiation, cells were subjected to immunofluorescence analysis for myosin heavy chain expression to quantify differentiation (Fig. 3.3A). While control cells displayed a high level of differentiation, with over 50% of the nuclei within myosin heavy chain positive cells (Fig. 3.3A, B) and with on average 9-10 nuclei per myotube (Fig.3.3A, C), the satellite cells isolated from the $SOX7^{-/-}$ mouse differentiated poorly and of those that became myosin heavy chain positive, also displayed myoblast fusion defects compared to controls. The differentiation index in $SOX7^{-/-}$ cultures was lowered by approximately 50% and the fusion index was reduced by approximately 35% as compared to controls. These results indicate that loss of SOX7 causes abnormal satellite cell differentiation and affects the ability of myoblasts to fuse together resulting in the formation of fewer myofibers in the mouse.

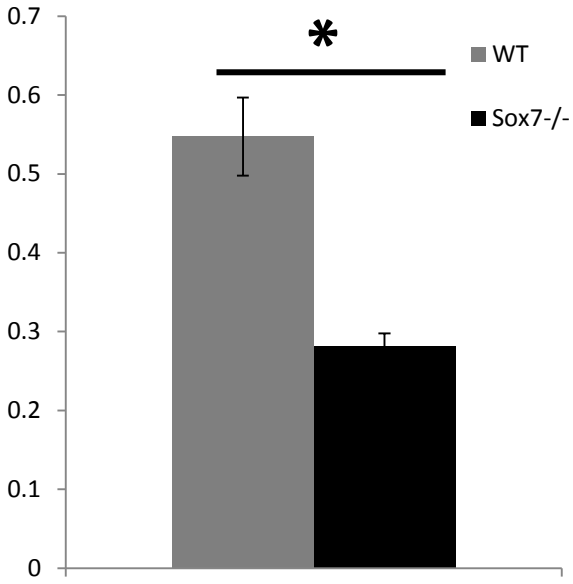
(A) Control Mouse



SOX7$^{-/-}$ Mouse



(B) Differentiation Index



(C) Fusion Index

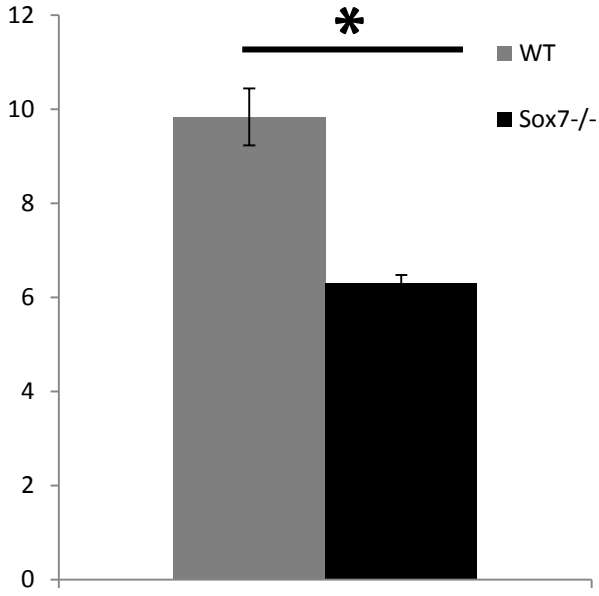
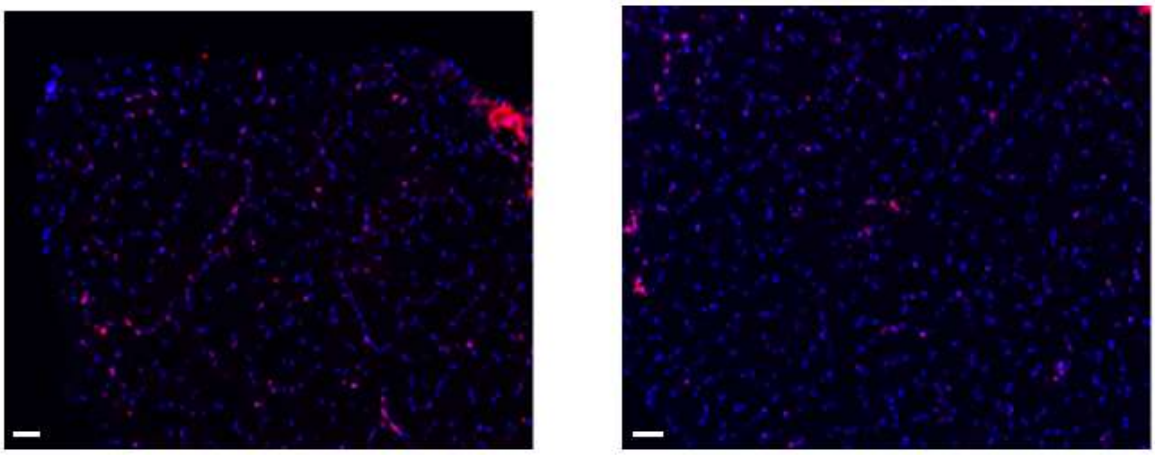


Figure 3.3: Loss of SOX7 leads to a defect in differentiation and lowered the fusion index of satellite cells. (A) Immunostaining for Myosin Heavy Chain (MHC) using a MF20 primary antibody (red) and Hoechst dye to visualize cell nuclei (blue) on day 7 of satellite cell differentiation from $SOX7^{-/-}$ and littermate controls. Images were obtained using the Leica microscope. (B) Differentiation index calculated from cultures induced to differentiate as in (A). (C) Fusion index calculated from cultures induced to differentiate as in (A). Error bars represent ±SEM from independent biological replicates of 3 Control and 3 $SOX7^{-/-}$ mice, DI and FI *$p<0.05$, $n=3$.

3.4 Loss of SOX7 leads to significantly fewer Pax7 positive satellite cells in skeletal muscle.

After confirming that loss of SOX7 leads to satellite cell (SC) differentiation and myoblast fusion defects in culture, I sought to assess the effect of loss of SOX7 expression on the Pax7+ population *in vivo*. Cryosectioned gastrocnemius muscle sections were immunostained using the anti-Pax7 antibody and Hoechst dye was used to stain the nucleus. A 77% reduction in the number of Pax7+ cells (marked by merged pink cells) were observed in the gastrocnemius muscle of the $SOX7^{-/-}$ mouse as compared to control littermates (Fig. 3.4A, B). These results suggest that loss of SOX7 leads to a decrease in the number of satellite cells marked by the transcription factor Pax7, which may, in turn, contribute to the smaller muscle fibers observed in the $SOX7^{-/-}$ muscle.

(A) Control Mouse **SOX7$^{-/-}$ Mouse**



(B) Pax7 (+)ve cells per muscle per section

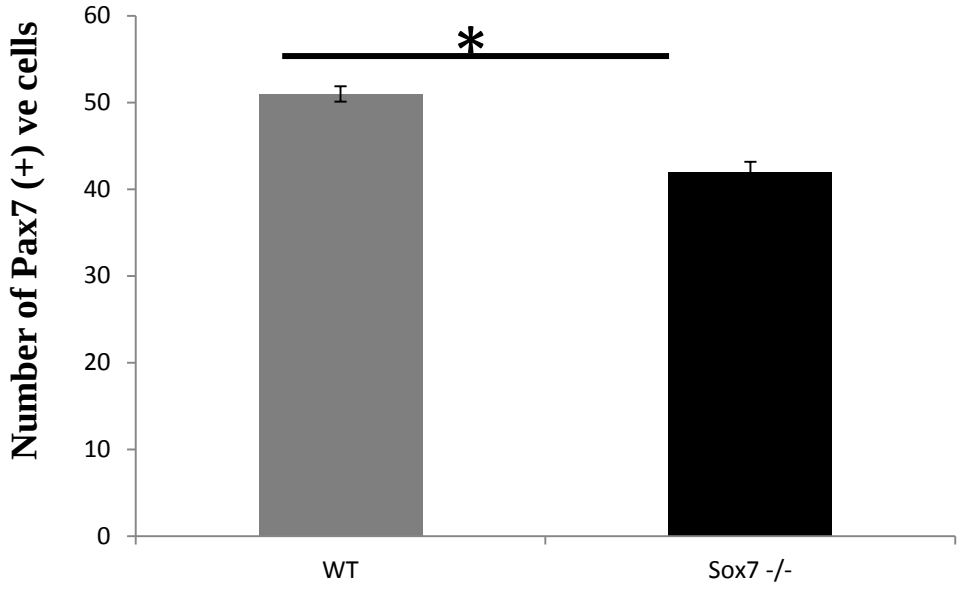
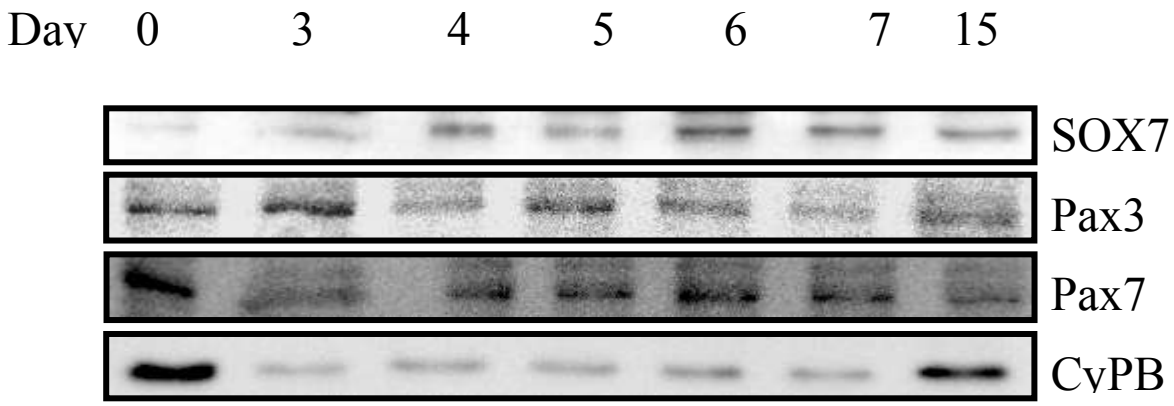


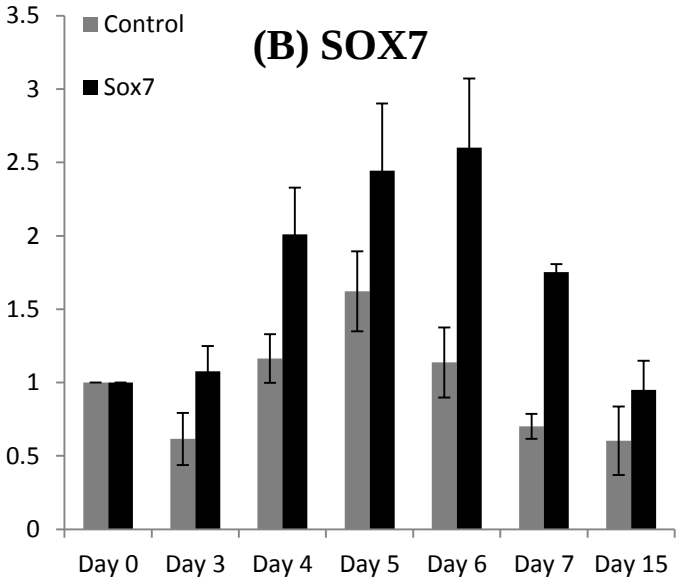
Figure 3.4: Loss of SOX7 leads to fewer numbers of Pax7+ cells in the gastrocnemius muscle. (A) Immunofluorescence for Pax7 (red) was performed on the gastrocnemius muscle of control and $SOX7^{-/-}$ mice. Hoechst dye was used to visualize cell nuclei (blue). **(B)** The average number of Pax7 positive cells was quantified using ImageJ. Error bars represent ±SEM from independent biological replicates of 3 Control and 3 $SOX7^{-/-}$ mice, *$p<0.005$, $n=3$.

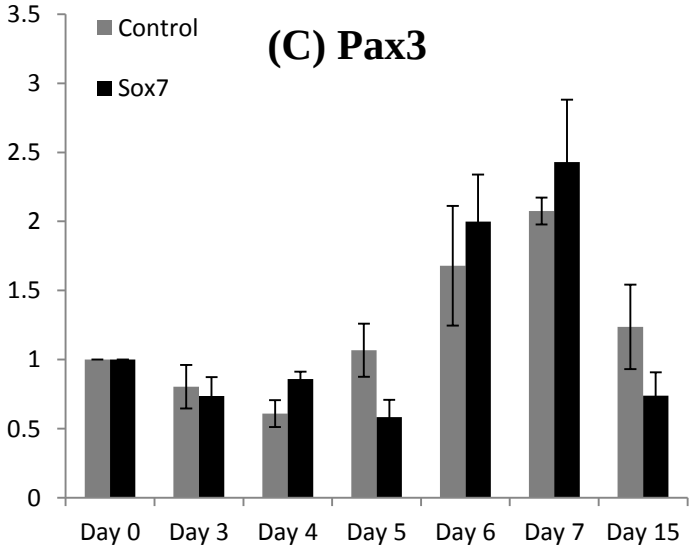
3.5 Gene expression profile during normal mouse embryonic development in D3 cells.

While loss of SOX7 in Pax3+ myogenic precursors results in smaller muscle fibers and fewer satellite cells, it is impossible to determine from these experiments whether the phenotype is due to abnormal embryonic development of skeletal muscle, for example, reduced specification of myogenic precursors, or defects in postnatal myogenesis. My experiments using isolated satellite cells suggest that there is an intrinsic defect in myogenesis in these cells, which likely contributes to the smaller muscle fibers observed in the knockout animal. However, to better understand the role of SOX7 in embryonic development, I used mouse embryonic stem cells as a model system. To better understand the regulation of SOX7 expression in this model, I first performed time course analysis of SOX7 protein (Fig. 3.5A). D3 mouse embryonic stem cells were differentiated according to the standard serum-based embryoid body *in vitro* differentiation protocol for 15 days to obtain skeletal myocytes. Protein was collected on Day 0, 3, 4, 5, 6, 7 and 15. Western blotting revealed that SOX7 protein rises early in the differentiation protocol and peaks on Day 7, similar to the Pax3 protein expression pattern (Fig. 3.5A, B). By contrast, Pax7 expression was also found to increase during early differentiation with a peak of expression noted on day 6. Since cells on day 15 were differentiated, the drop in both Pax3 and Pax7 expression at this late time point was expected. Further, down-regulation of SOX7 expression in these cells was consistent with a role for SOX7 during myogenesis. .

(A) mES D3 cells Western Blot







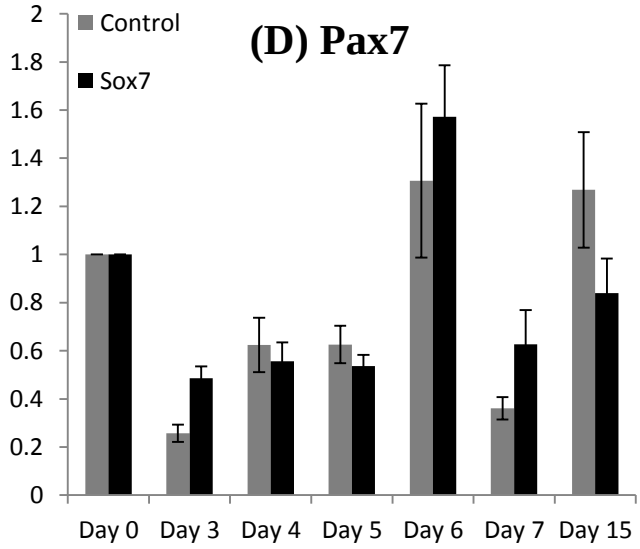


Figure 3.5: Protein expression levels of SOX7, Pax3 and Pax7 during *in vitro* mouse embryonic stem cell differentiation. (A) Western blotting was performed on the total protein extracted on Days 0, 3, 4, 5, 6, 7 and 15 from mES D3 cell lines. Proteins were detected with anti-SOX7, anti-Pax3, anti-Pax7 and anti-Cyclophilin B (loading control) antibodies, and were visualized with HRP-conjugated secondary antibodies. (B) SOX7 protein quantification was performed using ImageJ and expression levels were first normalized to the loading control Cyclophilin B and then normalized to 1 and presented as relative protein expression levels for each gene transcript. n=3. (C) Pax3 protein quantification was performed using ImageJ and expression levels were first normalized to the loading control Cyclophilin B and then normalized to 1 and presented as relative protein expression levels for each gene transcript. n=3. (D) Pax7 protein quantification was performed using ImageJ and expression levels were first normalized to the loading control Cyclophilin B and then normalized to 1 and presented as relative protein expression levels for each gene transcript. n=3.

3.6 SOX7, an early mesoderm and paraxial mesoderm induction marker, gene expression profile during mES D3 cell differentiation.

In addition to protein, mRNA was also harvested on Days 0, 3, 4, 5, 6, 7, 9, 12 and 15 of the skeletal muscle differentiation assay using mouse embryonic stem cells. The transcript levels of SOX7, BrachyuryT and Mesogenin were measured using RT-qPCR. The expression of BrachyuryT transcripts was used to observe the induction of the early mesoderm and that of Mesogenin was used to confirm the formation of the paraxial mesoderm in mES D3 cells. Both of these peak on day 5-6 of the differentiation assay (Fig. 3.6A, B). The SOX7 mRNA transcript levels rose steadily and peaked on day 9 and were down-regulated modestly by day 12 (Fig. 3.6C).

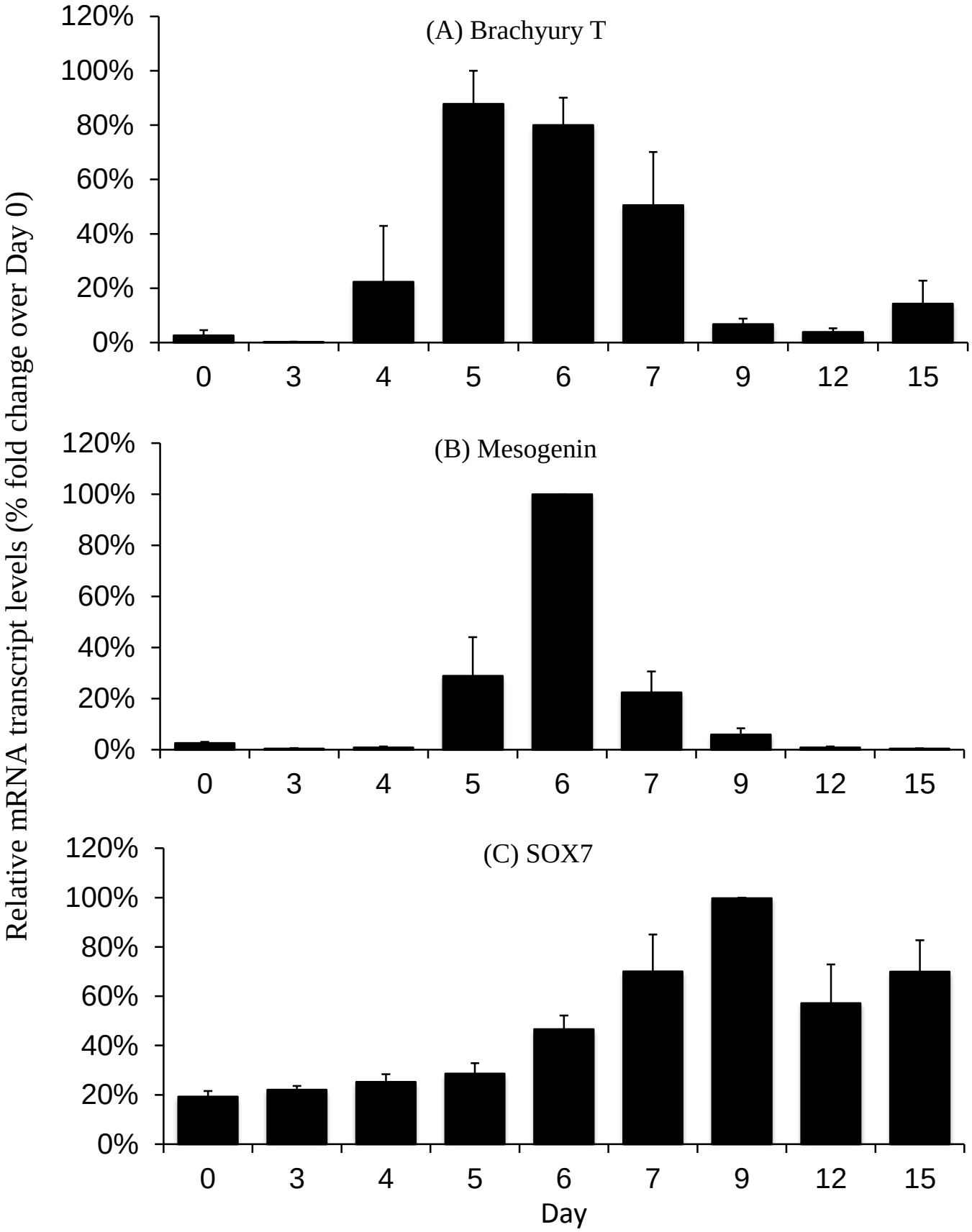

(A) Brachyury T
(B) Mesogenin
(C) SOX7
Relative mRNA transcript levels (% fold change over Day 0)
120%
100%
80%
60%
40%
20%
0%
0
3
4
5
6
7
9
12
15
Day

Figure 3.6: SOX7, early mesoderm and paraxial mesoderm induction marker gene expression profile during mES D3 cell differentiation. Transcript levels of Brachyury T (A), Mesogenin (B) (markers of early mesoderm and paraxial mesoderm induction) and SOX7 (C) in differentiating mES D3 cells were measured using RT-qPCR. Expression levels were first normalized to the housekeeping gene β-actin and then normalized to the expression on day 0 and presented as a percent of max expression for each gene transcript. Error bars represent ±SEM from an independent biological replicate using 3 mES D3 cell lines.

3.7 Premyogenic mesoderm induction marker gene expression profile during mES D3 cell differentiation.

The transcript levels of Pax3 and Pax7 were measured using RT-qPCR to verify the induction of premyogenic mesoderm in mES D3 cells. As expected, Pax3 mRNA transcript appeared after induction of mesoderm on days 5-6, and peaked on day 7 (Fig. 3.7A). At later time points, the expression of Pax3 was found to decrease with high variability (Fig. 3.7A). By contrast, the levels of Pax7 transcript expression in mES D3 cells were modestly up-regulated on day 7 of the assay, but highly up-regulated in day 15 cultures, corresponding perhaps to the creation of satellite cell-like cells in the differentiated cultures (Fig. 3.7B).

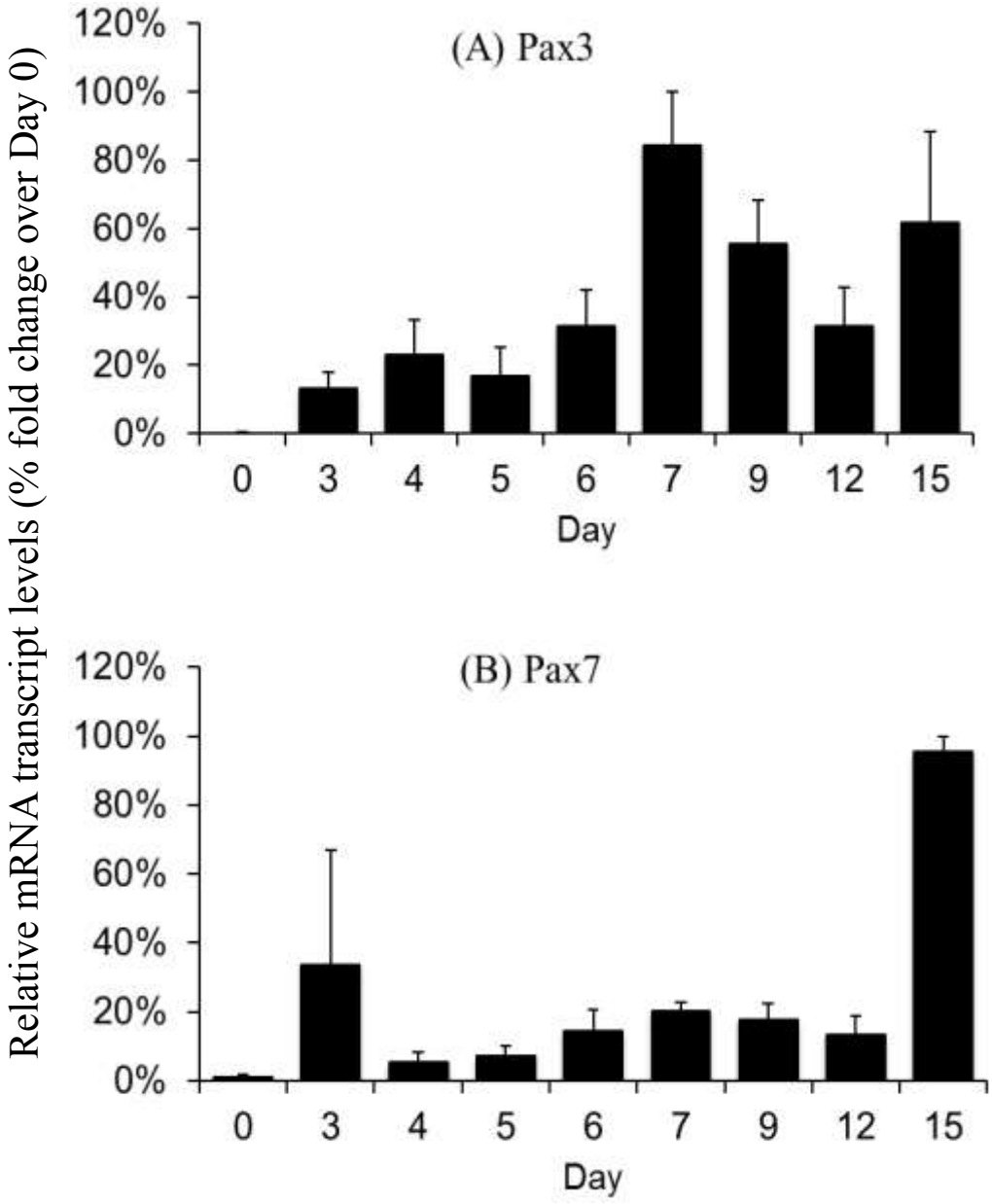
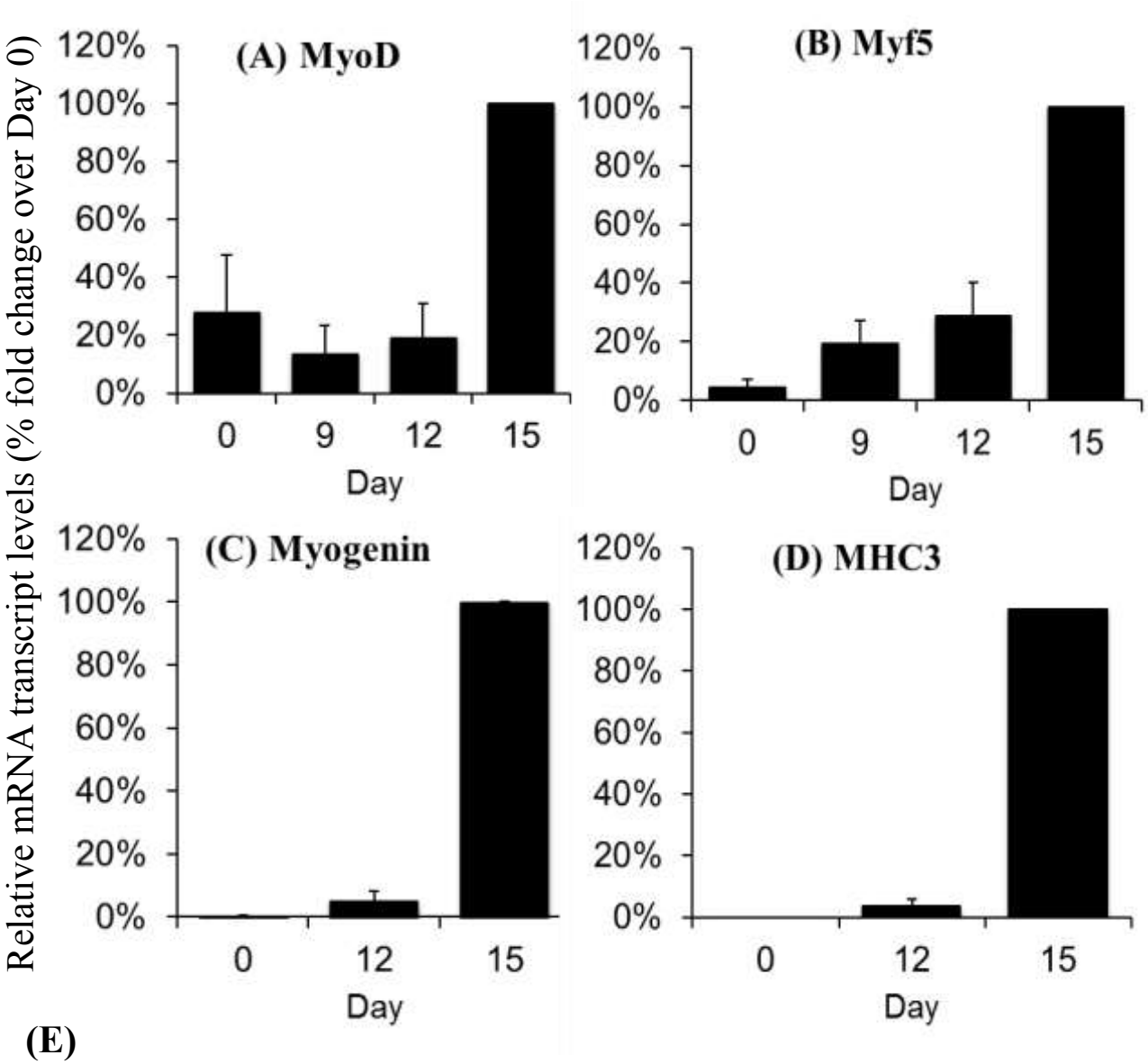

Relative mRNA transcript levels (% fold change over Day 0)
(A) Pax3
120%
100%
80%
60%
40%
20%
0%
0
3
4
5
6
7
9
12
15
Day
(B) Pax7
120%
100%
80%
60%
40%
20%
0%
0
3
4
5
6
7
9
12
15
Day

Figure 3.7: Premyogenic mesoderm induction marker gene expression profile during mES D3 cell differentiation. (A) Pax3 transcript levels in differentiating mES D3 cells quantified by RT-qPCR. Expression levels were first normalized to the housekeeping gene β-actin and then normalized to the expression on day 0 and presented as a percent of max expression for each gene transcript. (B) Pax7 transcript levels in differentiating mES D3 cells quantified by RT-qPCR. Expression levels were first normalized to the housekeeping gene β-actin and then normalized to the expression on day 0 and presented as a percent of max expression for each gene transcript. Error bars represent ±SEM from an independent biological replicate using 3 mES D3 cell lines.

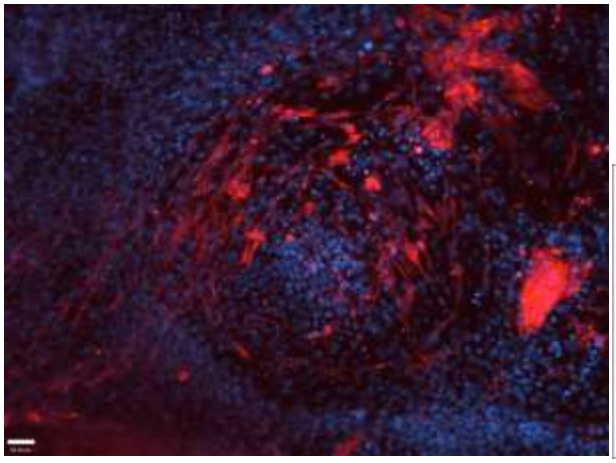
3.8 Myoblast and mature muscle marker gene expression profile during mES D3 cell differentiation.

To measure the progression of myogenesis, the transcript levels of MyoD, Myf5, Myogenin and MHC3 were measured using RT-qPCR (Fig. 3.8). Myf5, MyoD and Myogenin mark the formation of myoblast cells while MHC3 was used to examine the formation of terminally differentiated skeletal myocytes. The transcript levels of MyoD and Myf5 rose gradually from day 9 and peaked at day 15 whereas Myogenin and MHC3, more late stage markers, peaked at day 15 (Fig. 3.8A, B, C, D).

In addition to qPCR, the cells were also stained on day 15 with the anti- MHC antibody, MF20. Immunofluorescence analysis revealed that 6.5% of mES D3 cells were positive for myosin heavy chain expression, and thus differentiated towards the skeletal muscle lineage or cardiomyocyte lineage, indicating that the vast majority of cells were from alternative lineages. For this reason we decided to also look at cardiac, ectodermal and endodermal markers.


Relative mRNA transcript levels (% fold change over Day 0)
(A) MyoD
(B) Myf5
(C) Myogenin
(D) MHC3
120%
100%
80%
60%
40%
20%
0%
0
9
12
15
Day

(E)



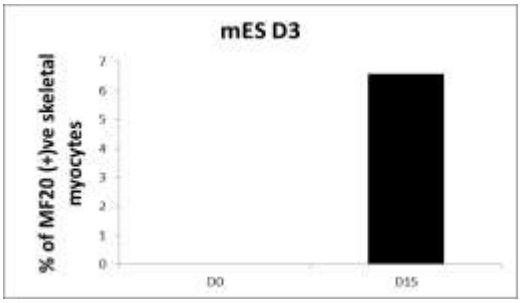

mES D3
% of MF20 (+)ve skeletal myocytes
D0
D15

Figure 3.8: Myoblast and mature muscle marker gene expression profile during mES D3 cell differentiation. Transcript levels of MyoD (A), Myf5 (B) and Myogenin (markers of myoblast formation) (C), and MHC3 (maker of mature skeletal myocyte formation) (D) in differentiating cells mES D3 cells quantified using RT-qPCR. Expression levels were first normalized to the house-keeping gene β-actin and then normalized to the expression on day 0 and presented as a percent of max expression for each gene transcript. Error bars represent ±SEM from an independent biological replicates using 3 mES D3 cell lines. (E) Immunostaining on day 15 of the mES cell differentiation for Myosin Heavy Chain (MHC) using a MF20 primary antibody (red) and Hoechst dye to visualize cell nuclei (blue). Images were obtained using the Leica microscope. Scale bar represents 60 μm. Quantification shows that 6.5% of cells differentiated into the skeletal lineage.

3.9 Early and late cardiac muscle marker gene expression profile during mES D3 cell differentiation.

To quantify the presence of cardiomyocytes in the differentiated mouse ES cell culture, the expression of Nkx2.5 (marker for early cardiac muscle), ACTC and MHC7 (late cardiac muscle markers) were determined (Fig. 3.9A, B and C). Expression of all three markers was noted in late differentiation with the Nkx2.5 transcript levels peaking on day 9, and ACTC and MHC7 rising by day 9 and peaking at day 15.

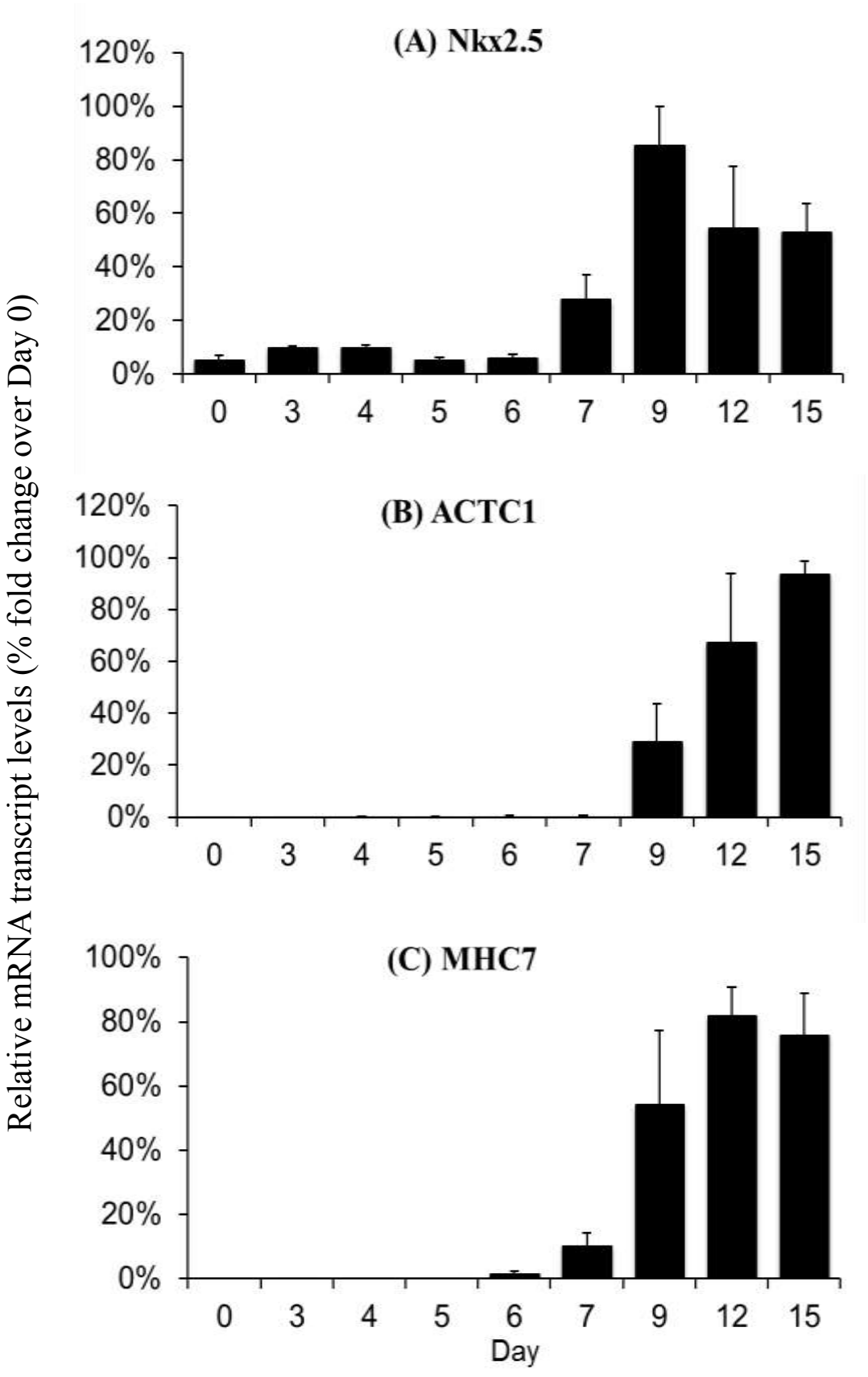

(A) Nkx2.5
120%
100%
80%
60%
40%
20%
0%
0 3 4 5 6 7 9 12 15
(B) ACTC1
120%
100%
80%
60%
40%
20%
0%
0 3 4 5 6 7 9 12 15
(C) MHC7
100%
80%
60%
40%
20%
0%
0 3 4 5 6 7 9 12 15
Day
Relative mRNA transcript levels (% fold change over Day 0)

Figure 3.9: Cardiac progenitor marker and mature cardiomyocyte marker transcript gene expression profile during mES D3 cell differentiation. Transcript levels of Nkx2.5 (early marker) (A), ACTC (B) and MHC7 (C) (late markers) of cardiac muscle genes in corresponding differentiating mES D3 cells, were quantified using RT-qPCR. Expression levels were first normalized to the housekeeping gene β-actin and then normalized to the expression on day 0 and presented as a percent of max expression for each gene transcript. Error bars represent ±SEM from an independent biological replicate using 3 mES D3 cell lines.

3.10 Gene expression profile of ectodermal and endodermal lineage markers during mES D3 cell differentiation.

To quantify the presence of ectodermal lineage in the differentiating cultures, NeuroD mRNA expression was determined. The NeuroD transcript levels peak early on day 3 and then were down-regulated gradually (Fig. 3.10A). The expression of FoxA2 was used to mark the endodermal lineage. FoxA2 was found to gradually rise until day 7 where it peaked. FoxA2 expression was then down-regulated until the end of differentiation (Fig. 3.10B).

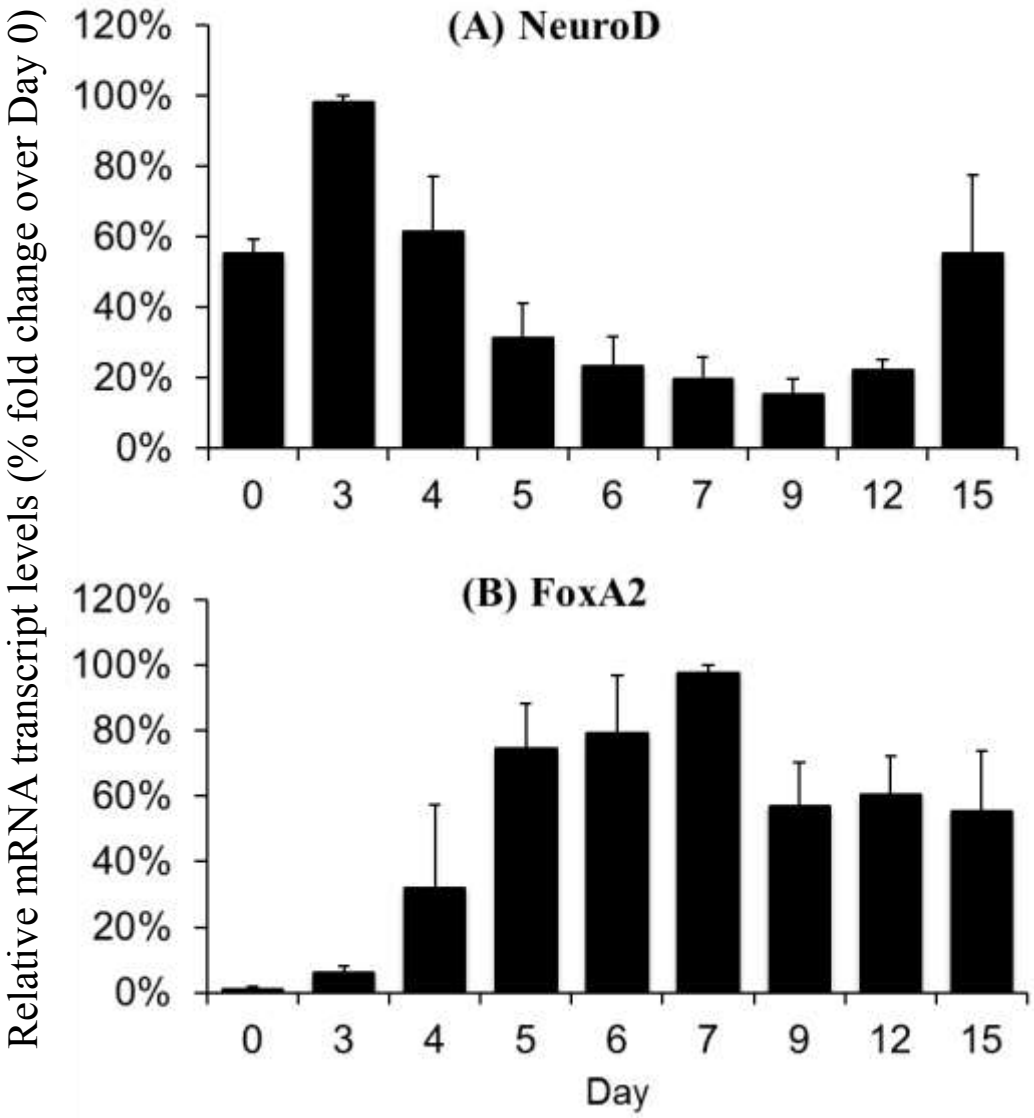

Relative mRNA transcript levels (% fold change over Day 0)
(A) NeuroD
120%
100%
80%
60%
40%
20%
0%
0
3
4
5
6
7
9
12
15
(B) FoxA2
120%
100%
80%
60%
40%
20%
0%
0
3
4
5
6
7
9
12
15
Day

Figure 3.10: Ectodermal and endodermal marker transcript gene expression profile during mES D3 cell differentiation. Transcript levels of NeuroD (marker of cells entering the ectodermal lineage) (A) and FoxA2 (marker of cells entering the endodermal lineage) (B) in differentiating mES D3 cells, were quantified using RT-qPCR. Expression levels were first normalized to the housekeeping gene β-actin and then normalized to the expression on day 0 and presented as a percent of max expression for each gene transcript. Error bars represent ±SEM from an independent biological replicate using 3 mES D3 cell lines.

3.11 Confirmation of SOX7 overexpression in puromycin resistant mES D3 cells

To determine the role of SOX7 expression in mouse ES cell differentiation, I created a mES D3 line overexpressing SOX7 and these were compared to empty plasmid controls. Following nucleofection and selection, isolated colonies arising from single cells were obtained. In total 35 distinct mES[SOX7] and 10 mES[Control] colonies were isolated. The expression of SOX7 was determined in these clones using RT-qPCR analysis. Clones with the highest level of SOX7 expression were used for further analysis (clones 9, 26, 29, and 32) (Fig. 3.11).

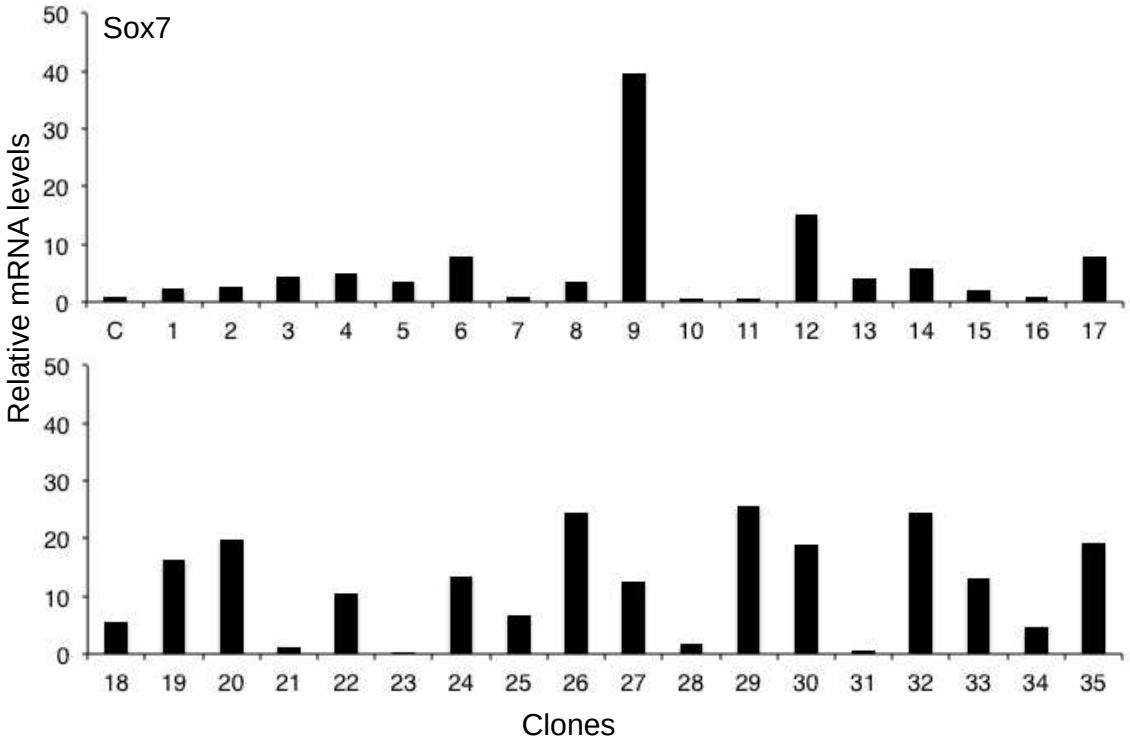

Sox7
Relative mRNA levels
50
40
30
20
10
0
C 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
Clones

Figure 3.11: Confirmation of SOX7 overexpression in Puromycin resistant mES D3 cells.

Transcript levels of SOX7 in the 35 clones that were isolated were quantified using RT-qPCR. On the x- axis "C" represents the average expression of SOX7 transcripts in 10 mES[Control] samples. The levels of SOX7 expression were first normalized to β-actin then normalized to average expression of mES[Control] samples. n=1

3.12 Overexpression of SOX7 transcripts was maintained during the differentiation of mES D3 cells.

The overexpression of SOX7 was confirmed using RT-qPCR analysis on days 0, 3, 6, 9, 12, and 15 of differentiation. Significantly higher levels of SOX7 expression were observed on all days of differentiations compared to control cells (Fig. 3.12A). Additionally, the expression of SOX7 transcripts was found to remain relatively stable in the control cell lines throughout the differentiation.

To examine SOX7, Pax3 and Pax7 protein expression, western blot analysis was performed (Fig. 3.12B). Total protein extracts were collected on day 0, 3, 4, 5, 6, 7, and 15 of the differentiation to examine the protein expression within the created cells. Samples obtained from mES[SOX7] cultures showed higher levels of SOX7 protein expression normalized to loading control. In the western blot performed only the lower band at ~43 kDa was specific to full length SOX7 protein. The higher bands are non-specific binding of the SOX7 antibody to other proteins. The lower band was confirmed to be specific to SOX7 protein through western blot analysis performed using knockdown SOX7 P19 EC cells (Ebadi et al, unpublished results). In addition the molecular mass of the lower band corresponds with the mass of SOX7 indicated in the literature (170). In addition, the expression of both Pax3 and Pax7 were increased in SOX7 overexpressing cells, as compared to controls (Fig. 3.12 B), though high variability was noted.

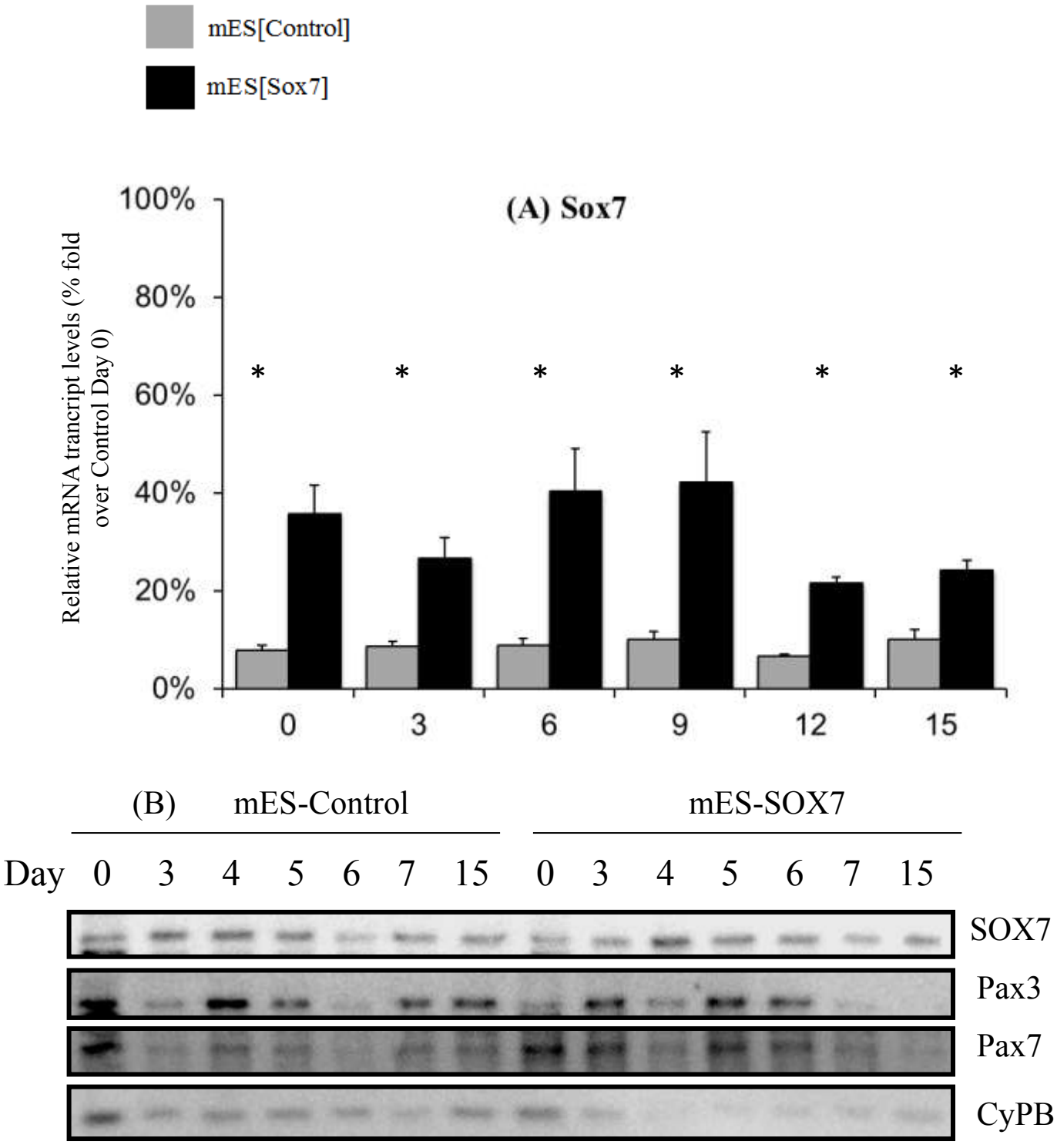

mES[Control]
mES[Sox7]
(A) Sox7
Relative mRNA trancript levels (% fold over Control Day 0)
100%
80%
60%
40%
20%
0%
*
0
3
6
9
12
15
(B)
mES-Control
mES-SOX7
Day 0 3 4 5 6 7 15 0 3 4 5 6 7 15
SOX7
Pax3
Pax7
CyPB

Figure 3.12: Created mESC[SOX7] cell lines show stable overexpression of SOX7 during differentiation. (A) Transcript levels of SOX7 in differentiating mouse ES cells were quantified using RT-qPCR. Expression levels were first normalized to β-actin then normalized to the expression on day 0 of mES[Control] and presented as a percent of max expression for each gene transcript. Error bars represent ±SEM from an independent biological replicate using 4 mES[Control] and 4 mES[SOX7] cell lines differentiated twice. *$p<0.05$; n=8. (B) Western blot analysis of the total protein extracted on Days 0, 3, 4, 5, 6, 7 and 15 from mES[Control] and mES[SOX7] cells D3 cell lines. Proteins were detected with anti SOX7, anti-Pax3, anti-Pax7 and anti- Cyclophilin B antibodies, and were visualized with HRP-conjugated secondary antibodies. Data is representative of 4 mES[Control] and 4 mES[SOX7] cell lines differentiated once. n=4.

3.13 Overexpression of SOX7 inhibits mesoderm formation in mES D3 cells.

The expression of BrachyuryT transcripts was used to examine the effect of SOX7 overexpression on the formation of the early mesoderm (Fig. 3.13A). The expression of Mesogenin transcripts was used to examine the effect of SOX7 on the formation of the paraxial mesoderm (Fig. 3.13B). mRNA was harvested on day 0, 3, 6, 9, 12 and 15 of the differentiation assay. While BrachyuryT transcript expression in mES[SOX7] were significantly increased on day 6 similar to control cultures, this expression was approximately 40% lower than that observed in control cultures (Fig. 3.13A).

The levels of Mesogenin transcript expression in mES[SOX7] samples were also found to significantly increase on day 6 relative to day 0, but were reduced by more than half as compared to control cultures (Fig. 3.13B). Taken together, these results suggest that overexpression of SOX7 in differentiating mouse ES cells inhibits the formation of mesoderm.

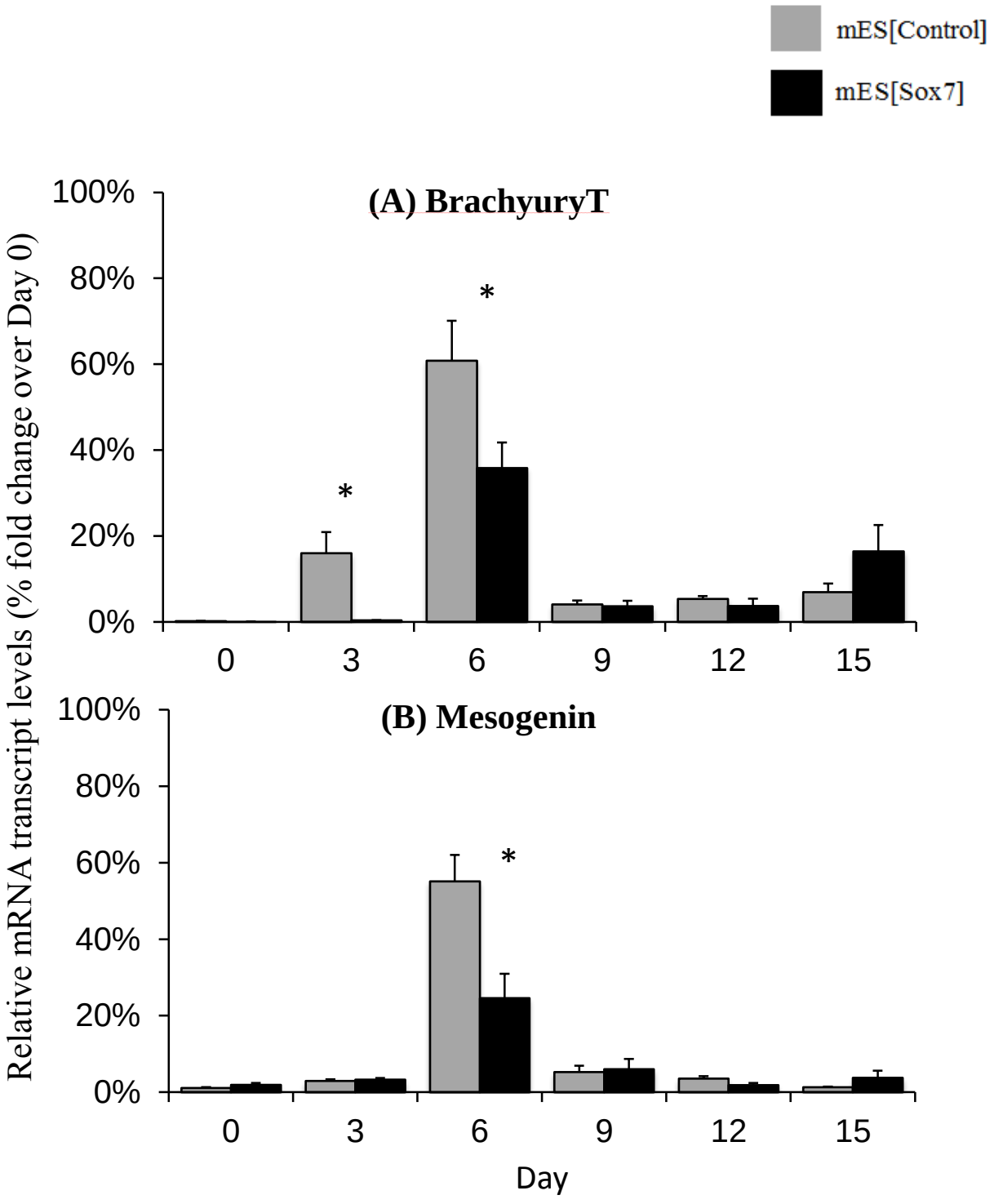

mES[Control]
mES[Sox7]
(A) BrachyuryT
(B) Mesogenin
Relative mRNA transcript levels (% fold change over Day 0)
100%
80%
60%
40%
20%
0%
0
3
6
9
12
15
Day
*

Figure 3.13: Overexpression of SOX7 inhibited the expression of mesoderm factor transcripts. Transcript levels of Brachyury T (A) and Mesogenin (B) in differentiating mES D3 cells were quantified using RT-qPCR. Expression levels were first normalized to β-actin then normalized to the expression on day 0 of mESC[Control] and presented as a percent of max expression for each gene transcript. Error bars represent ±SEM from an independent biological replicate using 4 mESC[Control] and 4 mESC[SOX7] cell lines, differentiated twice *$p<0.05$, **$p<0.01$; n=8.

3.14 Overexpression of SOX7 also inhibits premyogenic mesoderm formation in mES D3 cells.

The expression of Pax3 and Pax7 transcripts were used to determine the effect of SOX7 overexpression on the formation of the premyogenic mesoderm (Fig. 3.14). mRNA was harvested on day 0, 3, 6, 9, 12 and 15 and RT-qPCR was performed for Pax3 and Pax7 transcripts. The levels of Pax3 and Pax7 transcript expression in mES[SOX7] cells were found to significantly increase on day 6 relative to day 0, comparable to controls (Fig. 3.14 A,B). However, no significant difference was observed when the expression of Pax3 and Pax7 transcripts in the mES[Control] samples was compared to that of the mES[SOX7] samples on day 6. Further, while Pax3 and Pax7 transcripts were down-regulated in both control and SOX7 overexpressing cultures, the transcript abundance was significantly reduced further in SOX7 over-expressing cells only on day 3. These results indicate that despite a reduction in mesoderm formation and an initial lag in Pax3 and Pax7 expression, SOX7 overexpression did not significantly impair premyogenic mesoderm marker expression in mES D3 cells beyond day 6.

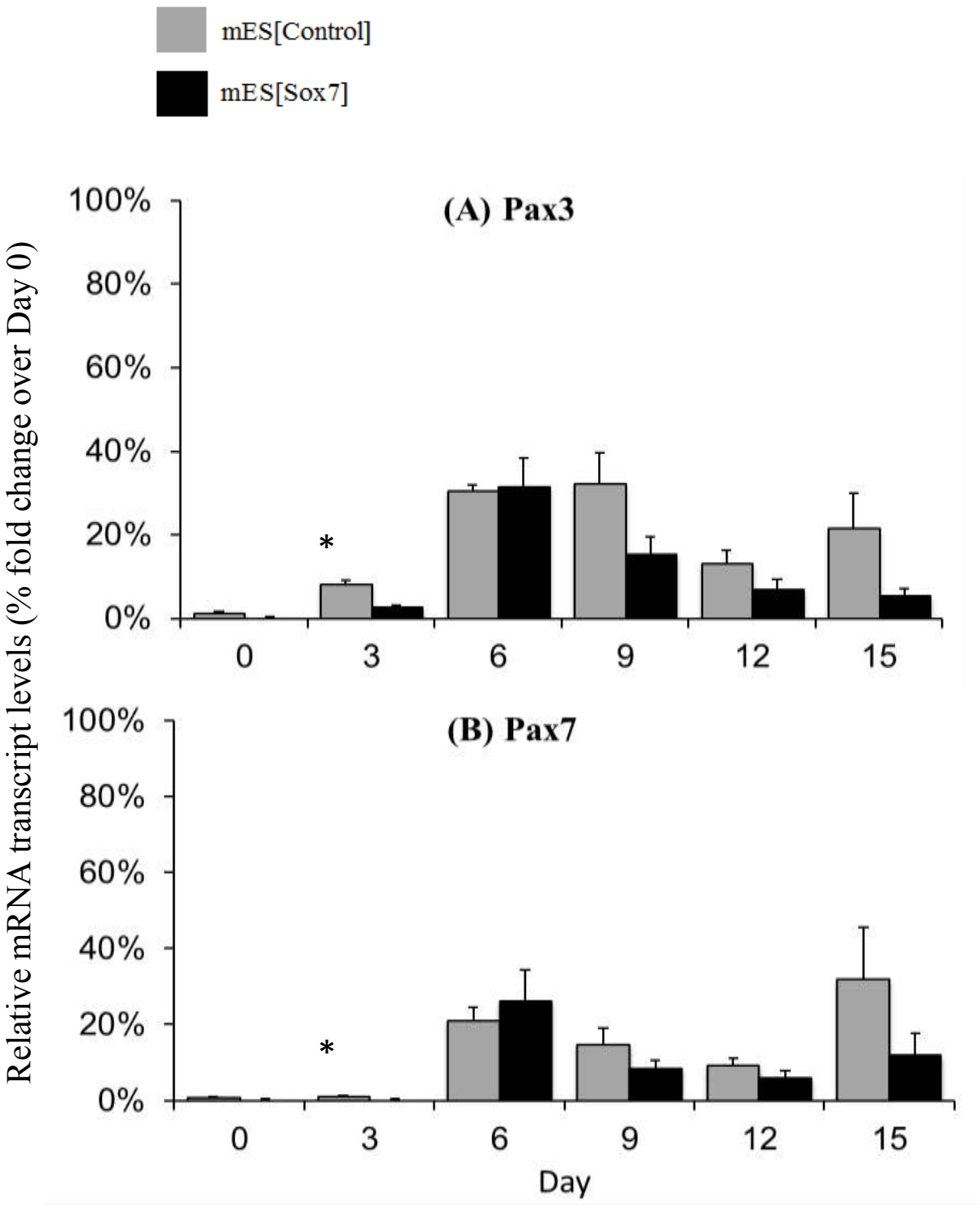
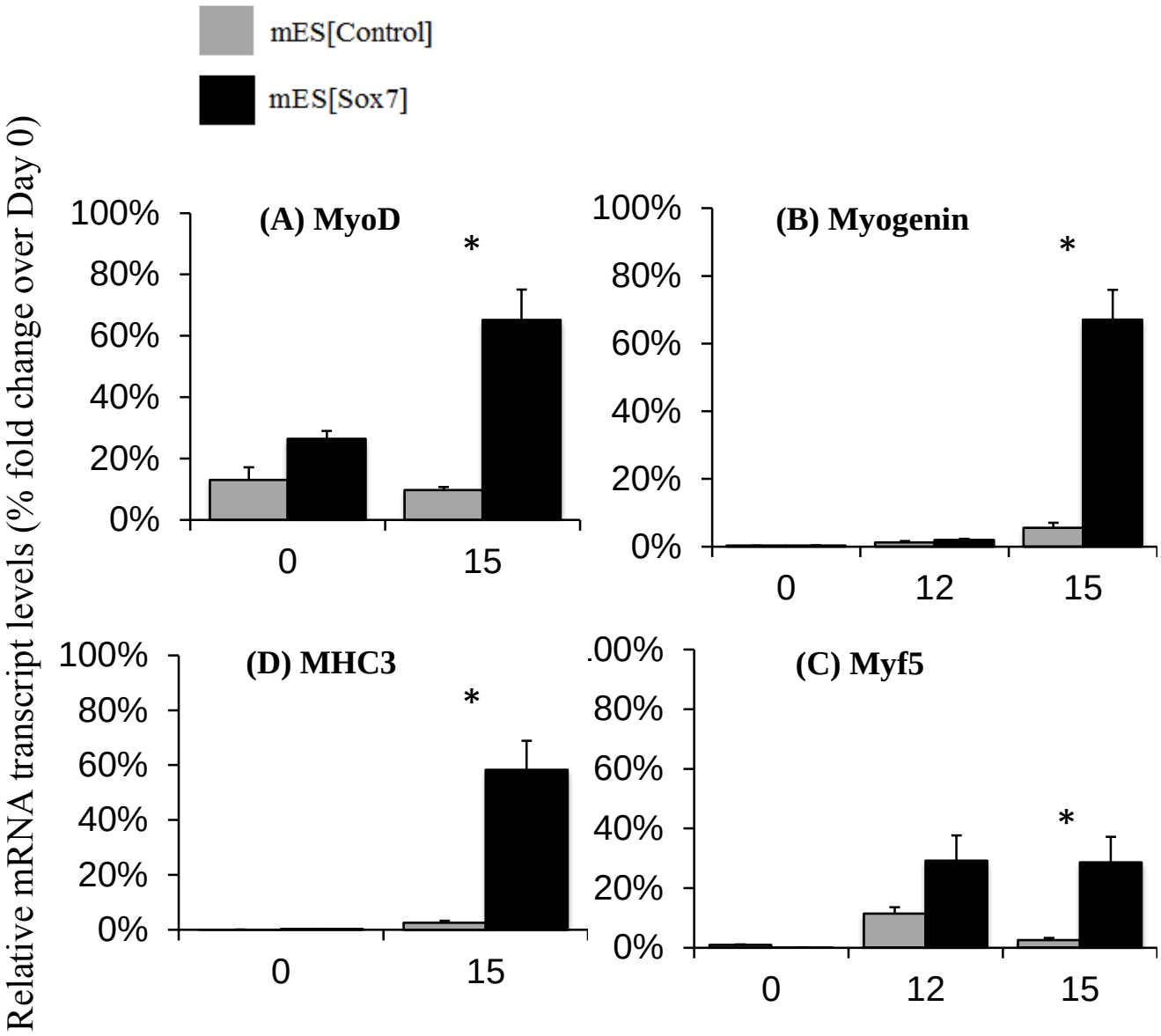

mES[Control]
mES[Sox7]
(A) Pax3
(B) Pax7
Relative mRNA transcript levels (% fold change over Day 0)
100%
80%
60%
40%
20%
0%
*
0
3
6
9
12
15
Day

Figure 3.14: Overexpression of SOX7 significantly reduced the expression of premyogenic mesoderm factor transcripts. Transcript levels of Pax3 (A) and Pax7 (B) in differentiating mES D3 cells were quantified using RT-qPCR. Expression levels were first normalized to β-actin then normalized to the expression on day 0 of mESC[Control] and presented as a percent of max expression for each gene transcript. Error bars represent ±SEM from an independent biological replicate using 4 mESC[Control] and 4 mESC[SOX7] cell lines, differentiated twice *$p<0.05$, $n=8$.

3.15 Overexpression of SOX7 significantly enhanced the expression of myoblast and mature muscle marker expression in mES D3 cells.

The expression of Myf5, MyoD and Myogenin transcripts was used to examine the effect of SOX7 overexpression on the formation of myoblasts. mRNA was harvested on day 0, 3, 6, 9, 12 and 15 of the differentiation protocol and expression was monitored by RT-PCR. The levels of MyoD, Myf5 and Myogenin transcript expression in mES[SOX7] samples were found to significantly increase on day 15 relative to day 0. Further, expression of all markers tested were consistently higher in SOX7-overexpressing cells as compared to controls at all-time points tested. On day 15 of the differentiation protocol, MyoD transcripts were increased approximately 7-fold over control expression (Fig. 3.15A). Similarly, at day 15, Myf-5 expression was increased 11-fold, and Myogenin expression increased 12-fold in mES[SOX7] cells as compared to controls (Fig. 3.15B,C). The expression of MHC3 transcripts was used to examine the effect of SOX7 on the formation of skeletal myocytes. A significant 22-fold enhancement was observed when the expression of MHC3 transcripts in the mES[SOX7] samples was compared to the mES[Control] samples on day 15 (Fig. 3.15D).

In addition to RT-qPCR analysis, cells were fixed and stained using an anti-MHC antibody, MF-20. Consistent with the RT-qPCR analysis, I observed an increase in the number of MHC+ cells following immunofluorescence analysis in mES[SOX7] cells as compared to control cultures (Fig. 3.15E).


mES[Control]
mES[Sox7]
Relative mRNA transcript levels (% fold change over Day 0)
(A) MyoD
(B) Myogenin
(D) MHC3
(C) Myf5
100%
80%
60%
40%
20%
0%
0
12
15
*

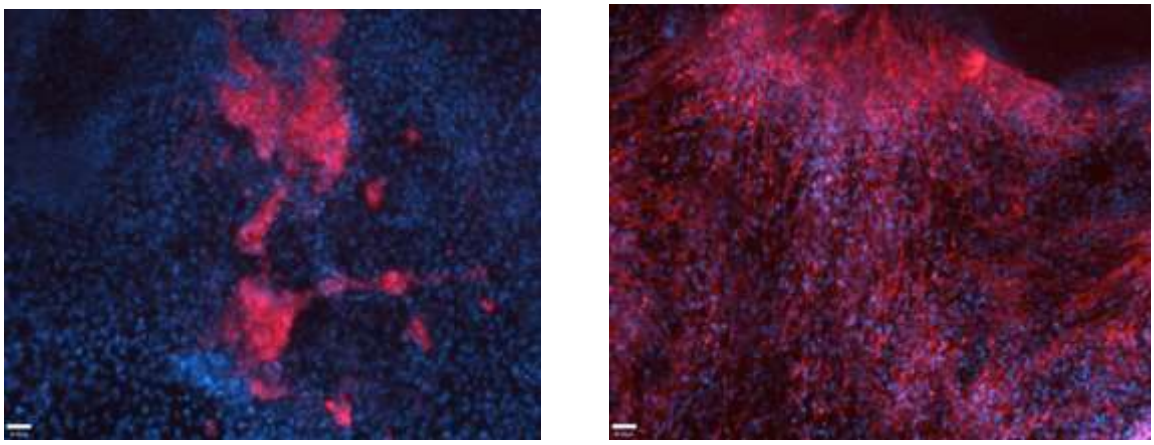

(E) mES [Control]
mES [SOX7]

Figure 3.15: Overexpression of SOX7 had a significant effect on the expression of myoblast and mature muscle marker transcripts. Transcript levels of MyoD (A), Myf5 (B), Myogenin (C) and MHC3 (D) in differentiating mES D3 cells were quantified using RT-qPCR. Expression levels were first normalized to β-actin then normalized to the expression on day 0 of mES[Control] and presented as a percent of max expression for each gene transcript. Error bars represent ±SEM from an independent biological replicate using 4 mES[Control] and 4 mES[SOX7] cell lines, differentiated twice *$p<0.05$, **$p<0.01$; n=8. (E) Immunofluorescence analysis of day 15 cultures for myosin heavy chain (MHC) using a MF20 primary antibody (red) and Hoechst dye to visualize cell nuclei (blue). Images were obtained using the Leica microscope. Scale bar represents 60 μm.

3.16 Overexpression of SOX7 inhibits cardiac muscle progenitor formation.

The expression of Nkx2.5 transcripts was used to examine the effect of SOX7 overexpression on the formation of cardiac muscle progenitors (Fig. 3.16A). mRNA collected on day 0, 3, 6, 9, 12 and 15 was analyzed RT-qPCR. Nkx2.5 expression in mES[SOX7] cells was decreased at all time-points tested except day 6, most notably reduced by approximately 50% on day 9 and by approximately 80% on day 12 as compared to mES[Control] cells (Fig. 3.16A).

Despite a reduction in the number of cardiac progenitors predicted in mES[SOX7] cultures, when the expression of the cardiomyocyte marker alpha actin found in cardiac muscle (ACTC) was unchanged in SOX7 overexpressing cells as compared to control cultures (Fig. 3.16B). Taken together, these results suggest that the formation of mature cardiac muscle is unlikely to be impaired by the overexpression of SOX7 in mouse ES cells, despite inhibition of Nkx2.5 expression.

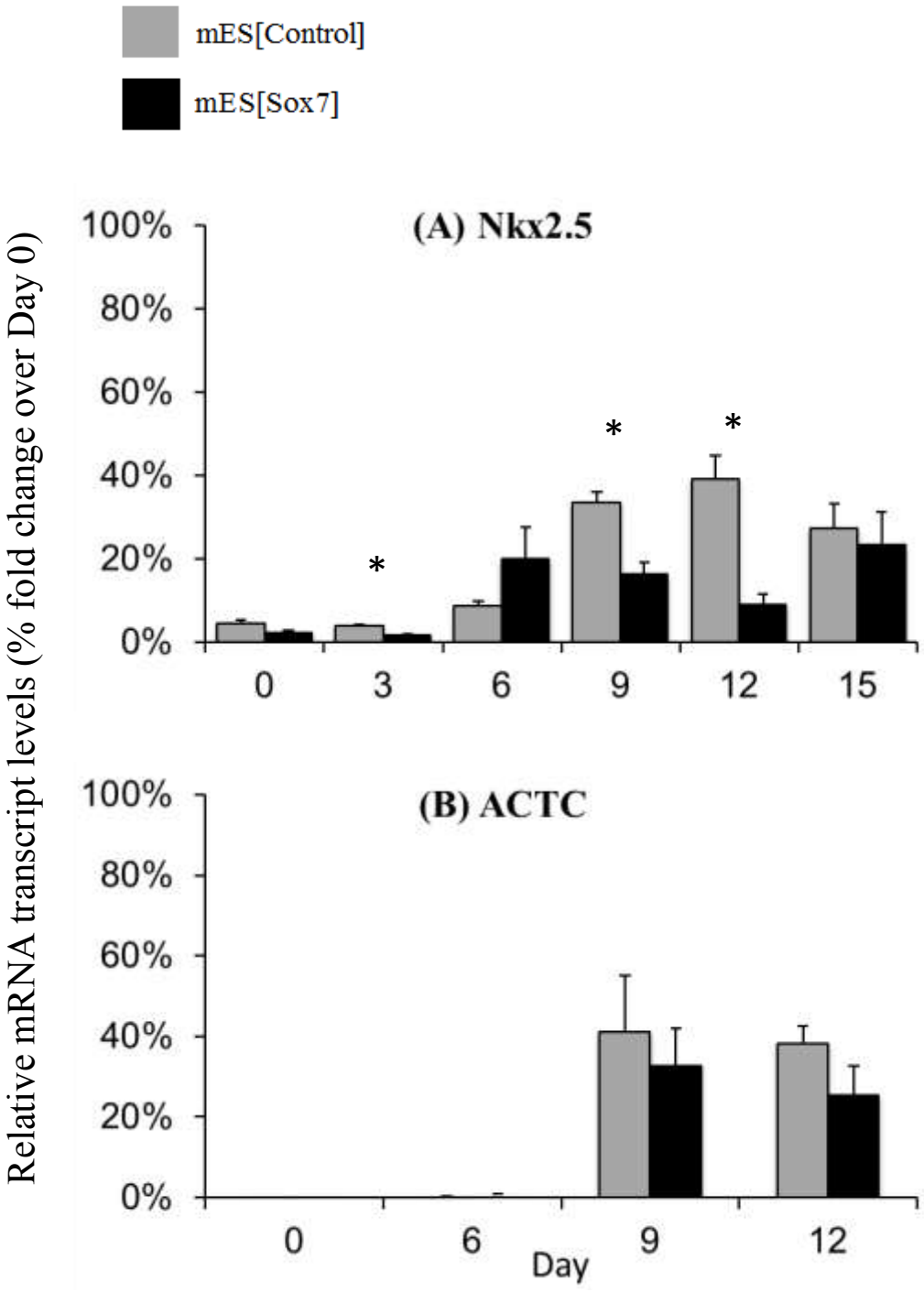

mES[Control]
mES[Sox7]
Relative mRNA transcript levels (% fold change over Day 0)
(A) Nkx2.5
100%
80%
60%
40%
20%
0%
0
3
6
9
12
15
*
*
*
(B) ACTC
100%
80%
60%
40%
20%
0%
0
6
9
12
Day

Figure 3.16: Overexpression of SOX7 inhibits the formation of cardiac muscle progenitors. Transcript levels of Nkx2.5 (A) and ACTC (B) in differentiating mES D3 cells were quantified using RT-qPCR. Expression levels were first normalized to β-actin then normalized to the expression on day 0 of mESC[Control] and presented as a percent of max expression for each gene transcript. Error bars represent ±SEM from an independent biological replicate using 4 mES[Control] and 4 mES[SOX7] cell lines, differentiated twice. *$p<0.05$, $n=8$.

3.17 Overexpression of SOX7 did not affect the expression of ectoderm and endoderm derived tissue marker transcripts.

The expression of NeuroD transcripts were used to examine the effect of SOX7 overexpression on the formation of mature neurons, an ectodermal tissue. The expression of NeuroD was unchanged in SOX7 overexpressing cultures as compared to controls (Fig. 3.17A). Similarly FoxA2 expression, which marks endoderm-derived tissues, was also unchanged by SOX7 overexpression (Fig. 3.17B). Taken together, these results suggest that the differentiation of ectodermal and endodermal lineages in mouse ES cells overexpressing SOX7 is unperturbed.

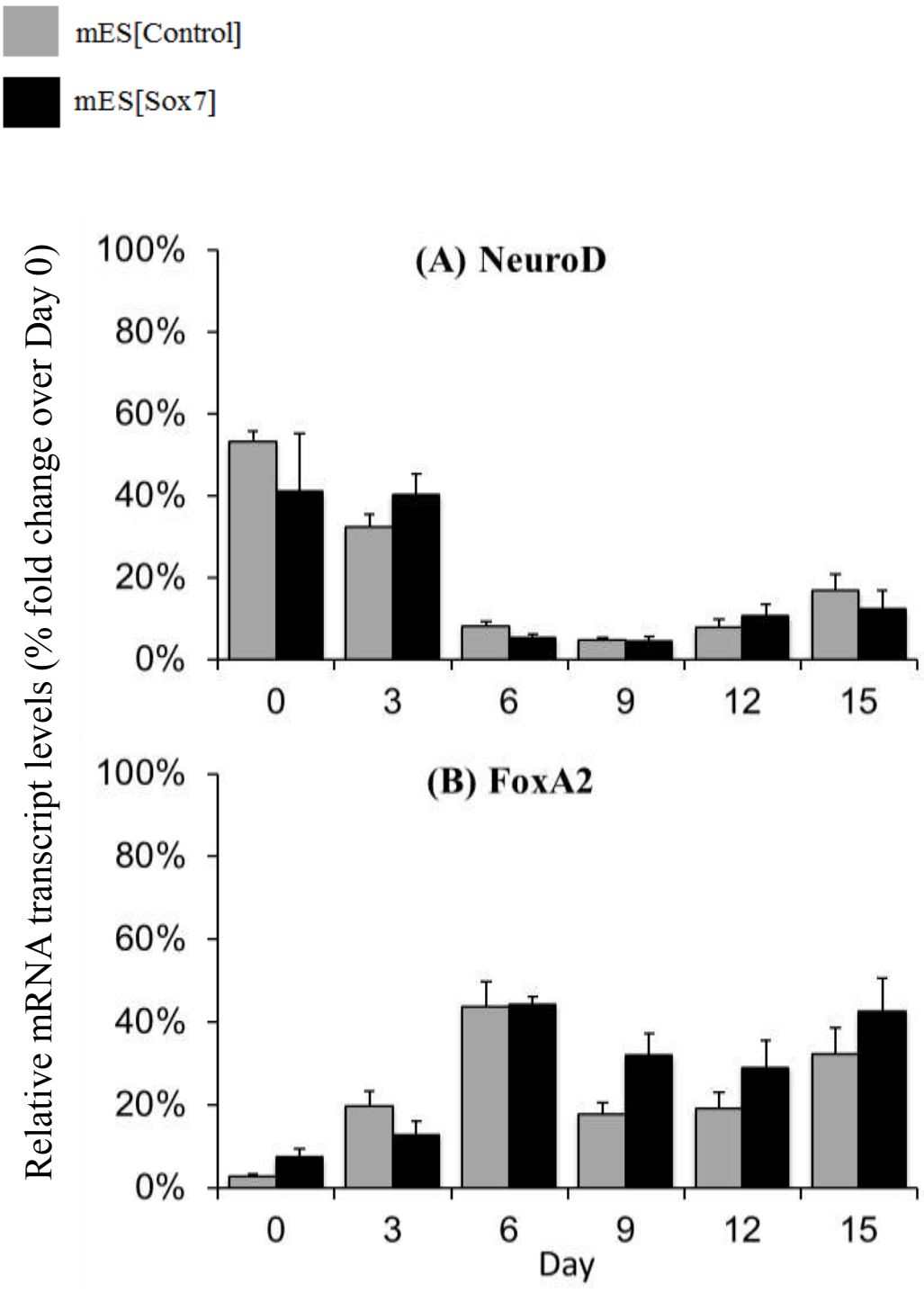

mES[Control]
mES[Sox7]
Relative mRNA transcript levels (% fold change over Day 0)
(A) NeuroD
100%
80%
60%
40%
20%
0%
0
3
6
9
12
15
(B) FoxA2
100%
80%
60%
40%
20%
0%
0
3
6
9
12
15
Day

Figure 3.17: Overexpression of SOX7 had no significant impact on the expression of ectoderm and endoderm derived tissue marker transcripts. Transcript levels of NeuroD (A) and FoxA2 (B) in differentiating mES D3 cells were quantified using RT-qPCR. Expression levels were first normalized to β-actin then normalized to the expression on day 0 of mES[Control] and presented as a percent of max expression for each gene transcript. Error bars represent ±SEM from an independent biological replicate using 4 mES[Control] and 4 mES[SOX7] cell lines, differentiated twice.

4. DISCUSSION

Muscular dystrophies are genetic, progressive, degenerative disorders with the primary symptom of muscle weakness. Duchenne, Becker, Facioscapulohumeral, and Myotonic muscular dystrophies are most prevalent and tend to have distinctive features helpful in diagnosis. The Limb-girdle, Emery-Dreifuss, and Oculopharyngeal muscular dystrophies are less common but often may also be diagnosed on the basis of phenotype. Prior to finding novel therapeutic options for patients, we need to fully understand which pathways are effective in slowing or halting disease progression, reversing or preventing underlying mechanisms, and repairing previously damaged muscle.

4.1 Overview of experimental findings

Previous studies in our lab demonstrated that overexpressing SOX7 up-regulates Pax3/7 in P19 embryonal carcinoma cells directly (160). We also know that Pax3 overexpression up-regulates SOX7 expression in P19 cells (171) creating, in this model, a positive regulatory loop between the 3 factors. As such, I predicted that loss of SOX7 expression in Pax3+ cells would perturb this, resulting in a loss of Pax3/7 maintenance and thereby defective myogenesis.

The results obtained from the animal work indicate that mice lacking SOX7 in the skeletal muscle-specific lineage have smaller muscle fiber cross-sectional areas in the Tibialis Anterior, though the number of fibers and overall muscle weights are comparable to control animals (Fig. 3.2). Further, fewer Pax7+ satellite cells were observed in SOX7 knockout mice, suggesting that this factor is important in the generation of muscle stem cells in the postnatal animal (Fig. 3.4). I observed a defect in myogenesis when satellite cells were isolated from the SOX7 knockout mice, which suggests that the smaller muscle fibers may

arise from both a lack of precursor cells for renewal and repair, as well as defective adult myogenesis (Fig. 3.1 and 3.3).

However, it remains possible that the differentiation defect observed is due to fewer precursors isolated from the SOX7 null mice, which, due to decreased cell density would impinge on normal differentiation and fusion. To explore this, it would be important to perform immunofluorescence on day 0 and 3 using anti-Pax3 and Pax7 to quantify the exact number of cells losing their paired box expression and anti-MyoD and anti-Myf5 on day 3 and 7 to quantitate the number of cells turning on MRF expression, as well as proliferation assays to detect the number of dividing cells in living tissues, such as BrdU incorporation.

The mouse model used in this study has the Pax3-cre constitutively active. Hence, in these cells, once Pax3 expression comes on in the myogenic precursors during embryonic development, the SOX7 is excised; hence the model under consideration is looking at embryonic as well as postnatal defects. All experiments performed in this study use satellite cells, which measure only the capacity for postnatal myogenesis in the mouse. Herein, lays one of the first limitations of my animal model. Since we are solely observing how the loss of SOX7 affects skeletal myogenesis in adults, and not during embryogenesis, it was impossible to determine whether the smaller population of Pax7+ cells was due to impaired specification of these precursors or a failure of self-renewal post-natally. Immunostaining for Pax7 and laminin proteins on sections of the TA muscle of newborn and 21 day old mice would clarify when the Pax7 population diverges from control animals. The development of a temporally controlled knockout model, where the activity of the Cre recombinase is controlled by tamoxifen (CreER) would also allow for the isolation of the effects of SOX7 in the postnatal animal following normal embryogenesis (172).

One of the limitations of using the primary myoblast differentiation assay is the purity of the isolated satellite cells. The crude extract of all the hind limb muscles naturally includes endothelial cells, fibroblasts and other cells along with SCs, and though the SCs are enriched through pre-plating, uneven proliferation rates between the control and knockout cultures could allow for the plating of unequal number of satellite cells, and the overtaking of the culture by contaminating fibroblasts. In both slow proliferation and culture impurity, differentiation and fusion would be expected to be negatively impacted. High purity satellite cell cultures can be obtained by using FACS sorting using cell surface markers (Integrin α7(+)/CD34(+) double positive cells and CD45, CD31, CD11b, and Sca1 negative (Lin(-)) cells (Integrin α7(+)/CD34(+)/Lin(-)) to isolate satellite cells (173).

We also have preliminary data suggesting that loss of SOX7 might be playing a role during muscle regeneration. We injected cardiotoxin into the left TA muscle of control and SOX7 knockout mice to induce injury and PBS into the right muscle of 3 wildtype and 3 $SOX7^{-/-}$ mice aged 24 weeks. The mice were sacrificed and weighed 7 days later and both their TA muscles were also weighed and dissected. Preliminary data shows that the $SOX7^{-/-}$ mice weighed significantly less than the wildtype controls after injury (See Appendix A) but there was no significance difference noted in the weights of uninjured and injured TA muscle between SOX7-/- and controls. However, these gross measurements may mask more subtle differences in regenerative capacity which would be revealed by histological analysis of the muscle and assessment of the number of regenerating myofibers and their cross-sectional areas. We predict that loss of SOX7 will affect the efficiency with which the muscle regenerates as compared to control.

The results obtained from the *in vitro* differentiation studies indicate that during normal mES D3 differentiation time course, SOX7 mRNA transcript levels peak at Day 9 gradually

(Fig. 3.6) but protein levels are at a maximum on Day 7 (Fig. 3.5) which can be quite easily explained due to the fact that since there was no protein samples collected on day 9, the highest SOX7 protein level was conferred upon day 7 and if and when day 9 protein was quantified its amount would exceed that on day 7, going in accordance to the qPCR data. The quantification of the western blot using anti-SOX7, Pax3 and Pax7 on days 0, 3, 4, 5, 6, 7 and 15 show a similar trend as that which was seen with respect to the transcript levels (Fig. 3.5). In mES D3 cell lines stably overexpressing SOX7, significant enhancement in expression of myoblast marker transcripts and mature skeletal muscle marker transcripts was observed compared to controls (Fig.3.15). However, no enhancement and on the contrary significant down-regulation on days 3 and 6 (BrachyuryT), day 6 (Mesogenin), day 3 (Pax3/7) (Fig. 3.13 and 3.14) was observed in the expression of mesoderm and premyogenic mesoderm marker transcripts, suggesting that the embryonic stem cells underwent accelerated and enhanced differentiation as compared to the control cells. This data could suggest that overexpression of SOX7 in these cultures caused precocious differentiation resulting in higher amount of skeletal muscle. Further, the SOX7 overexpressing cells do not increase their Pax7 expression on day 15 as seen in the controls (Fig. 3.14B) suggesting the poor specification of satellite cells in the culture which well explains the reason as to why there may be fewer Pax7+ cells in the $SOX7^{-/-}$ animal model.

The overexpression of SOX7 during the differentiation of mES[SOX7] clones was confirmed by RT-qPCR and western blot analysis (Fig. 3.12). Results from the qPCR analysis show that the expression of SOX7 transcripts was significantly enhanced on days 0, 3, 6, 9, 12 and 15 of the differentiation relative to control cells (Fig. 3.12). An interesting observation in the qPCR results is that the expression of SOX7 transcripts is much more variable in the mES[SOX7] samples relative to the mES[Control] samples (Fig. 3.12). This

variability is likely a consequence of using multiple mES[SOX7] lines for analysis. As these cell lines did not arise from the same single cell it is probable that a different number of copies of the SOX7 gene were integrated into the genome during the Nucleofection and/or integration occurred at different loci. Indeed, studies examining the expression of genes introduced into embryonic stem cells, known as transgenes, show heterogeneous expression of the transgene is common (174). Variation in expression may also arise from chromatin remodeling events that are known to commonly occur during embryogenesis (175). Therefore fluctuations in the expression of SOX7 may depend on the locus in which integration occurred and how chromatin-remodeling events change the expression of that locus as the cell differentiates. It is likely that the observed variations in SOX7 expression occurred due to the combined action of these mechanisms. In order to obtain more consistent levels of SOX7 expression, a single mES[SOX7] clone should be used for further differentiations and analysis.

The Western blot analysis showed a strong trend toward enhanced expression of SOX7 protein in mES[SOX7] on days 0,3,4,5,6,7,9 and 15 of the differentiation relative to control cells (Figure 3.12). Further, the expression of both Pax3 and Pax7 protein appeared up-regulated in SOX7 expressing cells, though high variability, but nonetheless supports the notion that SOX7 expression can promote Pax3 expression. These results appear to confirm those observed through RT-qPCR analysis.

The overexpression of SOX7 was not found to have a significant effect on the expression of mesoderm markers, Brachyury-T and Mesogenin, at a transcriptional level relative to control samples on day 6 of the differentiation (Figure 3.13). SOX7 has been shown to induce early mesoderm formation in Xenopus (176). However, other studies have shown that SOX7 has the ability to antagonize Wnt/β-catenin signaling (117, 177). In mouse

embryonic stem cells the expression of β-catenin and Wnt signaling is known to be important for the induction of mesoderm formation (178). Although these previous findings suggest that SOX7 may have a role in mesoderm formation this was not observed in this study (Figure 3.13). Alternatively, SOX7 may act downstream of mesoderm formation to promote the formation of myogenic precursors, such as the specification of satellite cells or myogenesis directly. Hence as mentioned earlier, a more informative alternative would be to isolate myofibers from the $SOX7^{-/-}$ and control animals to quantify the transition from quiescence to proliferation/activation, to differentiation using immunostaining for Pax7, MyoD and Myogenin. This approach would identify any inherent differences in self-renewal capacity in the $SOX7^{-/-}$ animal. Reserve cell assays could also complement this approach, allowing us to track the formation of myogenic precursors after serial rounds of differentiation. Quantification of Pax7+ cells in newborn SOX7-/- cells would also support the notion that embryonic specification of myogenic precursors is perturbed in the absence of SOX7.

The overexpression of SOX7 had no significant effect on the expression of premyogenic mesoderm factors, Pax3 and Pax7, at a transcriptional level relative to control samples on day 6 of the differentiation (Figure 3.14). As described earlier the role of SOX7 during skeletal myogenesis has previously been examined using P19 embryonal carcinoma (EC) cells, where it was found to enhance the expression of both Pax3 and Pax7 (160). Additionally our lab has shown that SOX7 can bind to the regulatory region of Pax3, by ChIP-qPCR, and a reporter assay revealed that SOX7 could functionally activate the Pax3 promoter (Ebadi, *et al* unpublished results). Based on these previous findings it was expected that the overexpression of SOX7 would result in significantly enhanced expression of Pax3 and Pax7 during the differentiation of mES D3 cells, however this was not observed. A

possible explanation for this is that the maximal levels of expression for these markers in mES[SOX7] cultures occurred on a day of the differentiation that mRNA was not analyzed. Previous studies examining the expression of the Pax3 and Pax7 genes in mESCs show that these factors tend to reach maximal expression between days 6 to 10 (142). Therefore, qPCR analysis on other days of the differentiation may show that the expression of Pax3 and Pax7 transcripts was significantly higher in mES[SOX7] cultures, if only transiently. Another potential reason for this discrepancy is the model system used for these experiments. P19 EC cells are produced by the transplantation of a 7.5 day mouse embryo into an adult rat testis (134). The P19 cell line is then derived from the pluripotent stem cells that were isolated from the teratocarcinoma that had formed (179). These cells are immortal and remain in an undifferentiated state unless induced to differentiate (180). In the study examining SOX7 the P19[Control] cells were not induced to differentiate (160). However, in the present study mES[Control] cells spontaneously differentiate when they are subjected to the 15 day *in vitro* protocol that was outlined in the methods section (162). This spontaneous differentiation may increase the expression of Pax3 and Pax7 in mES[Control] samples such that no significant enhancement of expression is observed when SOX7 is overexpressed. Although SOX7 has been shown to functionally activate the Pax3 promoter this enhancement may not significantly contribute to the total expression of Pax3 transcripts that is already present in mES cultures. In this case, the creation of a mES cell line lacking SOX7 using small hairpin RNA molecules targeting SOX7 would be informative. I predict that these cells would up-regulate mesoderm and premyogenic mesoderm markers in the absence of SOX7. Further, loss of SOX7 would be expected to perturb myogenesis. Indeed, similar studies have actually been carried out in P19[shSOX7] cells and it was found to inhibit skeletal myogenesis (Ebadi *et al*, unpublished results).

The overexpression of SOX7 significantly enhanced the expression of myogenic regulatory factors MyoD, Myf5, and Myogenin at a transcriptional level relative to control samples on day 15 of the differentiation (Figure 3.15). This result was expected as previous work with P19[SOX7] showed enhanced expression of these genes (160). Interestingly, not all the MRFs were up-regulated to the same extent, although significant enhancement of each factor was observed. One possible explanation for this finding could be that the cells differentiated into a specific muscle type. All MRFs are detectable in muscle tissue; however different types of muscle are associated with different proportions of MRF expression (181, 182). Specifically, slow twitch oxidative muscles are associated with high levels of Myogenin transcript while fast twitch glycolytic muscles are associated with high levels MyoD transcript expression (181, 182). As it appears that the expression of Myogenin is enhanced during the differentiation while MyoD levels are not to the same extent, the skeletal muscle developing in the mES[SOX7] cultures may predominantly be of a slow twitch phenotype. However, further analysis of metabolic activity and the contractile protein isoforms present in the muscle produced by the differentiation would be required to confirm this.

The overexpression of SOX7 significantly enhanced the expression of mature muscle marker MHC3 transcripts and showed a trend toward enhanced myocyte formation relative to control samples on day 15 of the differentiation (Figure 3.15). Again, these results were expected as P19[SOX7] cells showed enhanced expression of MHC3 transcripts and resulted in the formation of an increased number of myocytes (160). In the current analysis an antibody against all forms of myosin heavy chain was used, detecting the presence of both skeletal and cardiac myocytes (183). As both the mES[Control] and mES[SOX7] were found to express cardiac muscle-associated transcripts, contaminating cardiomyocytes were present

in the cultures as well (Figure 3.15). Therefore, a more accurate measure of the amount of skeletal myocytes produced would require immunofluorescence analysis to be performed with a skeletal specific anti-MHC3 antibody and further quantification.

As SOX7 plays a role in the development of many different tissues, it was necessary to assess the expression of markers from lineages other than skeletal muscle as well. In particular, the markers for the formation of cardiac muscle, neurons, and endoderm-derived tissues were analyzed using RT-qPCR. The overexpression of SOX7 significantly reduced the expression of cardiac progenitor marker, Nkx2.5, and shows a trend toward reduced expression of mature cardiomyocyte marker, ACTC, transcripts relative to control cells (Figure 3.16). Indeed the growth protocol used to generate skeletal muscle is very similar to the protocol used to generate cardiac muscle for the first 10 days of the 15 day protocol used in this study (184). Additionally cells overexpressing SOX7 did not show enhanced expression of ectodermal marker NeuroD or endodermal marker FoxA2 transcripts relative to control cells on any of the analyzed days of differentiation (Figure 3.17). These findings indicate that SOX7 can act as a positive regulator of skeletal myogenesis without also enhancing the formation of other lineages in mES D3 cell lines.

4.2 Future directions

I used mice lacking SOX7 in skeletal muscle specific progenitors to perform experiments in adult mice. As such, it is difficult to separate the effects of SOX7 on the embryonic development of skeletal muscle from the postnatal effects. We could use the same approaches used in this study at various stages throughout murine development and map out the effect SOX7 has at every stage. We could use newborn mice and embryos, at E9.5, 10.5 and 16.5 for example and examine them for SOX7, Pax3/7 and MHC mRNA and protein

expression, by *in situ* hybridization and immunofluorescence, using techniques previously employed in the lab (185, 186). We predict that SOX7 expression will be efficiently lost in the muscle lineage, resulting in reduced expression of the paired box family members and MHC. Loss of Pax7 at these different stages would suggest a reduction in satellite cells numbers and demonstrate how severely the embryonic level of murine development is affected by the loss of SOX7 and could be further supported by the creation of a temporally controlled conditional null model.

In the study regarding mouse embryonic stem cells the results were obtained using 4 mES[Control] and 4 mES[SOX7] clones, which were differentiated twice. However in the future experiments a single mES[Control] and mES[SOX7] clone should be selected and analyzed during multiple independent differentiations. This should be done to reduce the variability in the results that may arise due to the use of multiple cell lines. However, more importantly independent differentiations improve the reproducibility of the results that are obtained. This is essential to strengthen the validity of any findings that are made in a study.

Another future study may involve chromatin immunoprecipitation (ChIP)-seq experiments to determine globally which genes are direct targets of SOX7 during mES differentiation into skeletal muscle. Our lab has previously shown, using ChIP-qPCR,that SOX7 can bind to the Pax3 regulatory region (Ebadi, *et al.*, unpublished results). To date, only a few studies have examined the direct targets of SOX7 by ChIP analysis, however, these experiments were examining the formation of lineages other than skeletal muscle (187, 188). By determining which genes SOX7 directly interacts with we may gain a better understanding of the role of SOX7 during skeletal myogenesis. The results from these experiments would require further validation by ChIP-qPCR.

As SOX7 expression was stably enhanced throughout differentiation in this study it is difficult to locate the exact stage at which SOX7 is acting during skeletal muscle formation. A possible way of overcoming this boundary would be through the use of an inducible system that would enable the expression of SOX7 at specific time points during the differentiation. This system would allow us to examine the effects of SOX7 overexpression at each of the stages of skeletal muscle formation individually. Since the entire mouse embryonic work carried out in this project was through gain-of-function analysis we could look into the role of SOX7 may also be acquired through loss-of-function experiments. This would require the creation of a mES cell line expressing short hairpin that targets SOX7 (shSOX7), using lentiviral shRNAmir particles targeting SOX7, along with a non-silencing control. These cells would be differentiated and analyzed exactly as mentioned above with the SOX7 overexpression studies, using the serum based differentiation protocol. We would expect that the loss of SOX7 would inhibit skeletal myogenesis in mES cells, as seen in satellite and P19 cells. Analysis of this cell line would let us determine if SOX7 is necessary for the differentiation of mES D3 cells into skeletal muscle.

4.4 Conclusion

In summary, I have demonstrated that the loss of SOX7 during adult stages of murine development *in vivo* resulted in defective satellite cells which were incapable of differentiating and fusing *in vitro* to the same extent as the wildtype cells, which was further confirmed via immunofluorescence and quantification. These Pax7+ satellite cells were also fewer as compared to those found in the gastrocnemius muscle of the control animals. Further, SOX7 knockout mice had smaller muscle fibers. Loss of SOX7 expression led to a decrease in Pax7, MyoD, Myf5 and MHC3 transcript levels. At the embryonic stage, *in vitro*

overexpression of SOX7 in mouse embryonic stem cells resulted in enhanced skeletal myogenesis. This occurred at least in part, through the enhanced expression of myoblast marker transcripts Myf5, MyoD, and Myogenin as well as mature skeletal myocyte marker MHC3 transcripts. This enhancement was observed to occur after the premyogenic mesoderm markers Pax3 and Pax7 were expressed indicating that SOX7 may have a role downstream of this stage. SOX7 overexpression also resulted in a trend toward reduced expression of cardiac progenitor marker Nkx2.5 and mature cardiac muscle marker ACTC transcripts indicating decreased levels of cardiomyogenesis. Furthermore SOX7 did not enhance the expression of ectodermal marker, NeuroD, or endodermal marker, FoxA2, transcripts relative to control cells.

The results of this study indicate that the multi-faceted protein SOX7 is a positive regulator of skeletal myogenesis.

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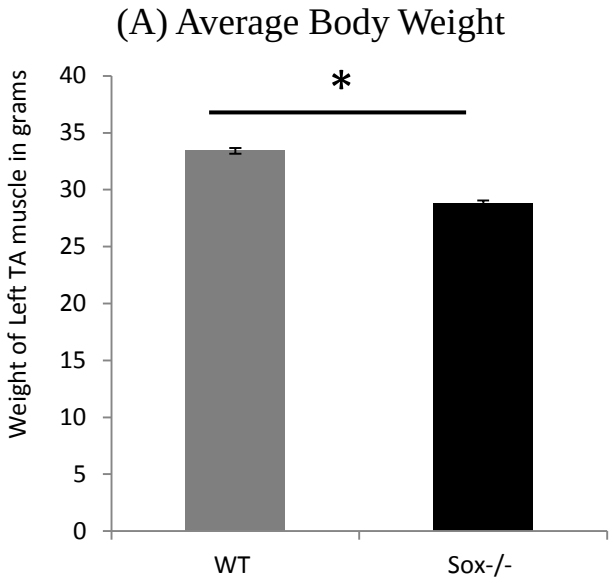
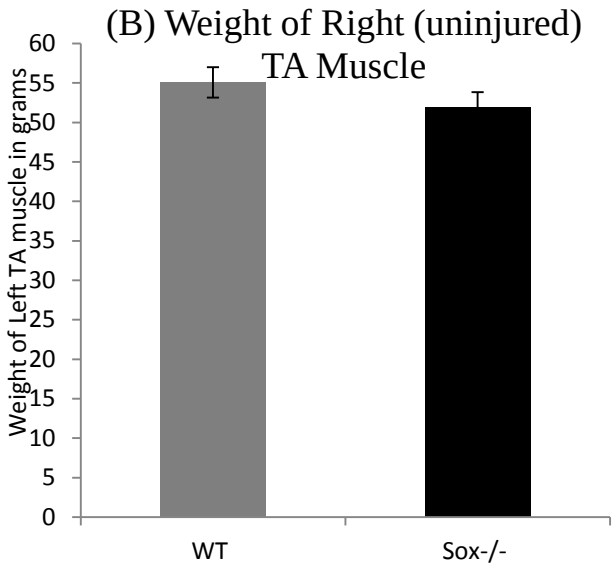
Appendices

Appendix A

Cardiotoxin Injury Experiments





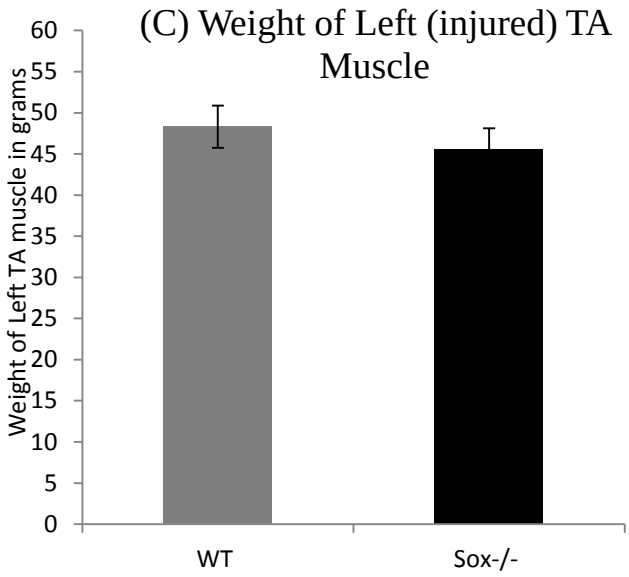


Figure A: Loss of SOX7 significantly smaller body weights in mice after Cardiotoxin injury. (A) Average body weights of Wildtype and $SOX7^{-/-}$ mice taken one week post cardiotoxin injury. (B) Average weight of right TA muscle, where PBS-control was injected in wildtype and $SOX7^{-/-}$ mice. (C) Average weight of left TA muscle, where cardiotoxin was injected in wildtype and $SOX7^{-/-}$ mice. Error bars represent ±SEM from independent biological replicates of 3 Control and 3 $SOX7^{-/-}$ mice, DI and FI *$p<0.05$, n=3.

Appendix B

Validated primers used for quantitative PCR analysis.

Target	Forward Primer (5'-3')	Reverse Primer (5'-3')
β-actin	AAATCGTGCGTGACATCAAA	AAGGAAGGCTGGAAAAGAGC
BrachyuryT	CTGGACTTCGTGACGGCTG	TGACTTTGCTGAAAGACACAGG
FoxA2	CCCTACGCCAACATGAACTCG	GTTCTGCCGGTAGAAAGGGA
Mesogenin	CTTCTGACACCGCTGGTCTG	GTGACTGCCGTAGCCATCG
Myf5	CCTGTCTGGTCCCGAAAGAAC	GACGTGATCCGATCCACAATG
MyoD	CCCCGGCGGCAGAATGGCTACG	GGTCTGGGTTCCCTGTTCTGTT
Myogenin	GCAATGCACTGGAGTTCG	ACGATGGACGTAAGGGAGTG
MHC3	GCATAGCTGCACCTTTCCTC	GGCCATGTCCTCAATCTTGT
MHC6	CAACAACCCATACGACTACGC	ACATCAAAGGGCCACTATCAGTG

NeuroD	GCA TGCACGGGCTGAACGC	GGGATGCACCGGGAAGGAAG
Nkx2.5	AAGCAACAGCGGTACCTGTC	GCTGTCGCTTGCACTTGTAG
Pax3	TTTCACCTCAGGTAATGGGACT	GAACGTCCAAGGCTTACTTTGT
Pax7	CTCAGTGAGTTCGATTAGCCG	AGACGGTTCCCTTTGTCGC
SOX7	ACCTCAGGGGACAAGAGTTCG	GTTTTTCTCAGGCAGCGTGTTC
18S	CGCCGCTAGAGGTGAAATC	CCAGTCGGCATCGTTTATGG
Cylcophilin B	GATGGCACAGGAGGAAAGAG	AACTTTGCCGAAAACCACAT

Appendix C

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Appendix D

Curriculum Vitae

RASHIDA RAJGARA

Date of Birth: 17th June 1990 Citizenship: Indian

EDUCATION

University of Ottawa, Ottawa, ON

Master's in Science **2012-Current**

Thesis: The role of SOX7 in the activation of satellite cells and regulation of skeletal myogenesis.

Department: Biochemistry, Microbiology & Immunology.

University of Carleton, Ottawa, ON

4th year Bachelor's Diploma **2011-12**

Areas of Concentration: Biology and Biochemistry

University of Mumbai, Mumbai, MH

Bachelor's in Science **2008-11**

Areas of Concentration: Life Sciences

Minor: Biochemistry

Honor's: In Chemistry

CONFERENCES

- Attended the 7th Canadian Developmental Biology Conference held at Mont Tremblant and presented a poster. **March 2014**

- Attended the "International Conference on Current Research in Biosciences" held and organized by the students of the University of Mumbai. **December 2010**

RESEARCH EXPERIENCE/EXCHANGE PROGRAMS

- Was selected to enter into a 1 year fully funded Exchange program at the University of Carleton, Canada under the Ontario-Maharashtra-Goa exchange program. **2011-12**

- "Study of chromosomal abnormalities due to heavy metal toxicity in onion root tip cells". The experiments were carried out in the Life Science Laboratory at the University of Mumbai. **2009-10**

- Carried out a summer research project "Towards understanding the rotational forces generated by the flagella of a live algal cell" under the supervision of Professor Deepak Mathur and Dr. Jacinta D' Souza. **2008-09**

LABORATORY EXPERIENCE

- Stem cell culture & differentiation assay expert.
- mRNA and Protein isolation and detection expertise.
- Worked with mice as an animal model for treating Muscular Dystrophy.
- Mouse colony breeding and genotyping. Expert in statistical tests and data analysis.
- Worked as a Laboratory Technician for 8 months, acquiring skills such as ordering lab supplies for all lab members to record keeping and periodic checks on stocks.
- Decent knowledge on mouse breeding and colony maintenance to performing genotyping and dissection experiments on test mice. Honed skills such as muscle dissections, satellite cell isolation, tissue-section staining & injury experiments.

VOLUNTEERING ACTIVITIES

- Volunteered at the "Door Step School" NGO working towards the betterment of street children in Mumbai, as part of the Social Involvement Program. **2008-11**

- Worked at "The Spastic Society" NGO in Mumbai as a volunteer for special students with disabilities in Mumbai. **2004-06**

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