

**Role of SRY-related HMG box (SOX)-7 in skeletal muscle
development**

and

**Effect of an extracellular matrix on skeletal and cardiac
muscle development**

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ABSTRACT

A complex network of transcription factors, which are regulated by signalling molecules, is responsible in coordinating the formation of differentiated skeletal and cardiac myocytes from undifferentiated stem cells. The present study aims to understand and compare the transcriptional regulation of skeletal and/or cardiac muscle development in the absence of Sox7 or in the presence of a collagen-based matrix in P19 embryonal carcinoma (EC) and mouse embryonic stem (ES) cells.

First, knock-down of Sox7, by shRNA, in muscle inducing conditions (+DMSO) and in the absence of RA (-RA), decreased muscle progenitor transcription factor and myogenic regulatory factor (MRF) levels, suggesting that Sox7 is necessary for myogenesis. However, knock-down of Sox7 in the presence of RA (+RA) and DMSO increased expression of muscle progenitor markers and MRFs, suggesting that Sox7 is inhibitory for myogenesis +RA. Furthermore, Sox7 overexpression enhanced myogenesis -RA, but inhibited myogenesis and enhanced neurogenesis +RA. These results suggest an important interplay between RA signalling and Sox7 function during P19 differentiation.

Second, Q-PCR analysis showed that compared to the mouse ES cells differentiated on the regular TC plates, differentiation on the collagen matrices had a higher expression of skeletal and cardiac precursors, MRFs and terminal differentiation markers. Collagen alone enhanced myotube formation. The enhanced collagen matrix, containing the oligosaccharide sialyl Lewis^x (sLe^x), specifically enhanced cardiomyogenesis.

These studies have added to our understanding of the transcriptional regulation of premyogenic mesoderm factors and the role of Sox7 in this process. In addition these studies provide a vision for possible use of biomaterials in directed differentiation of stem cells for the purpose of cell therapy.

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Dedicated to Mom, Dad, Soha and Abtin

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ATCC	American tissue type collection
BMP	Bone morphogenetic protein
bHLH	Basic helix-loop-helix
Ca ²⁺	Calcium ions
cDNA	Complementary deoxyribonucleic acid
Dhh	Desert Hedgehog
DM	Dermomyotome
DML	Dorso-medial lip
DMD	Duchenne muscular dystrophy
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E	Embryonic day
EB	Embryoid body
EC	Embryonal carcinoma
ECM	Extracellular matrix
EPC	Endothelial progenitor cell
ES	Embryonic stem
FGF	Fibroblast growth factor
Frz	Frizzled
GFP	Green fluorescent protein

HDAC	Histone deacetylase
Hh	Hedgehog
HMG	High mobility group
HRP	Horseradish peroxidase
Ihh	Indian Hedgehog
Lnfg	Lunatic fringe
MHC	Myosin Heavy Chain
MPC	Muscle precursor cell
MRF	Myogenic regulatory factors
mRNA	Messenger ribonucleic acid
NP-40	Nonidet-P40
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PGK	Phosphoglycerate kinase
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PSM	Pre-somitic mesoderm
Ptch1	Patched 1
Puro	Puromycin
Q-PCR	Quantitative-polymerase chain reaction
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor

RARE	Retinoic acid response element
RBP	Retinol binding protein
RDH	Retinol dehydrogenase
RNA	Ribonucleic acid
RNAi	RNA interference
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
Shh	Sonic hedgehog
sLe ^X	sialyl Lewis ^X
Smo	Smoothened
SMA _t	Spinal muscular atrophy
Sp	Splotch
sPBS	Stockholm (Na-Phosphate based) PBS
TBST	Tris-Buffered Saline with Tween
TC	Tissue culture
TCPS	Tissue culture polystyrene
VLL	Ventro-lateral lip

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CONTRIBUTION OF COLLABORATORS

Section 2.3.1

- The P19[Sox7] cell line was generated by Dr. Josée Coutu and the analysis was performed by Sara Khan.
- Figures 2.1 and 2.2 were generated by Sara Khan.

Sections 2.3.2 – 2.3.5

- All of the experiments were performed and analyzed by myself.

Section 2.3.6

- The differentiation experiments were performed by Dr. Ashraf Al-Madhoun and the analysis was performed by myself.

Section 2.3.7

- The P19[Sox7] cell line was generated by Dr. Josée Coutu and the analysis was performed by myself.

Sections 3.3.1 – 3.3.2

- All the experiments were performed and analyzed by myself.
- Drew Kuraitis, a PhD candidate in Dr. Erik Suuronen's lab, prepared the collagen- and sLe^X-coated plates and coverslips.

Appendix B

- The animals were prepared, raised and sacrificed in Dr. Erik Suuronen's lab.
- The mRNA was extracted from the hindlimb tissue by Dr. Ashraf Al-Madhoun.
- All the other experiments and analyses were performed by myself.

The thesis was written by me and edited by Dr. Ilona Skerjanc.

CHAPTER 1

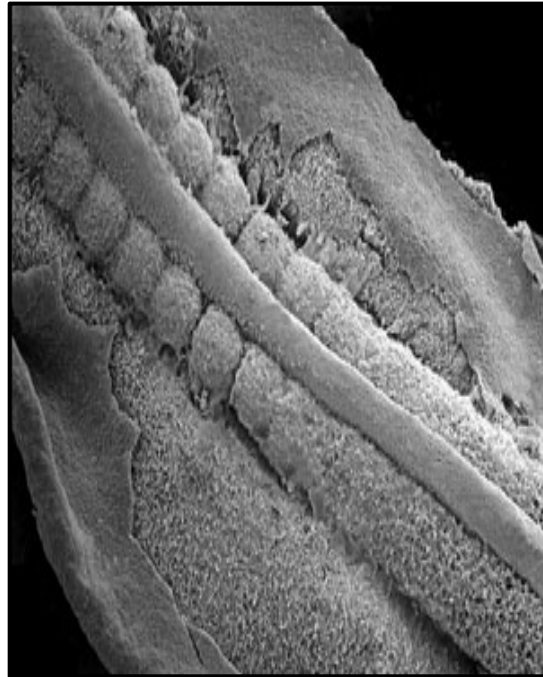
Introduction

1.1 Vertebrate Embryonic Skeletal Muscle Development

During embryonic growth the formation of skeletal muscle occurs progressively and it begins during gastrulation as shown by early transplantation studies in the chick embryo [1]. Gastrulation is defined as the transition from a simple and not very highly organized group of cells to a more complex, more organized, and multilayered embryo, which has the three germ layers, endoderm, mesoderm and ectoderm [2]. As this continues through the primitive streak, highly coordinated movements control the migration of the cells to their final destinations. Cells that migrate along the anterior-posterior axis are patterned to become the paraxial mesoderm, while the cells that travel further along the medial axis will become the lateral plate mesoderm [3].

As the primitive streak regresses, the paraxial mesoderm gets further patterned into blocks of segmented mesoderm structures, called somites, which form symmetrically on both sides of the axial structures, the neural tube and the notochord (Figure 1.1, A). The process of somite formation or somitogenesis occurs in a rostral-caudal manner and follows a “clock-wavefront” model originally proposed by Cooke, *J et al.*. This model states that a ‘clock’ that refers to an oscillator, and the ‘wavefront’ which refers to the boundary established between a newly formed somite and the pre-somitic mesoderm (PSM), set the pace for somite formation and maturation, respectively [4]. Later, it was discovered that the Notch-Delta and Wnt signalling pathways control the cyclic expression of genes during somitogenesis and allow the cells to respond to the wave of determination that establishes boundaries between somites [5]. The wave of determination is formed by a gradient of retinoic acid (RA) that opposes gradients of Wnt and fibroblast growth factor (Fgf) signalling. The Wnt and Fgf signalling decrease in the

A



B

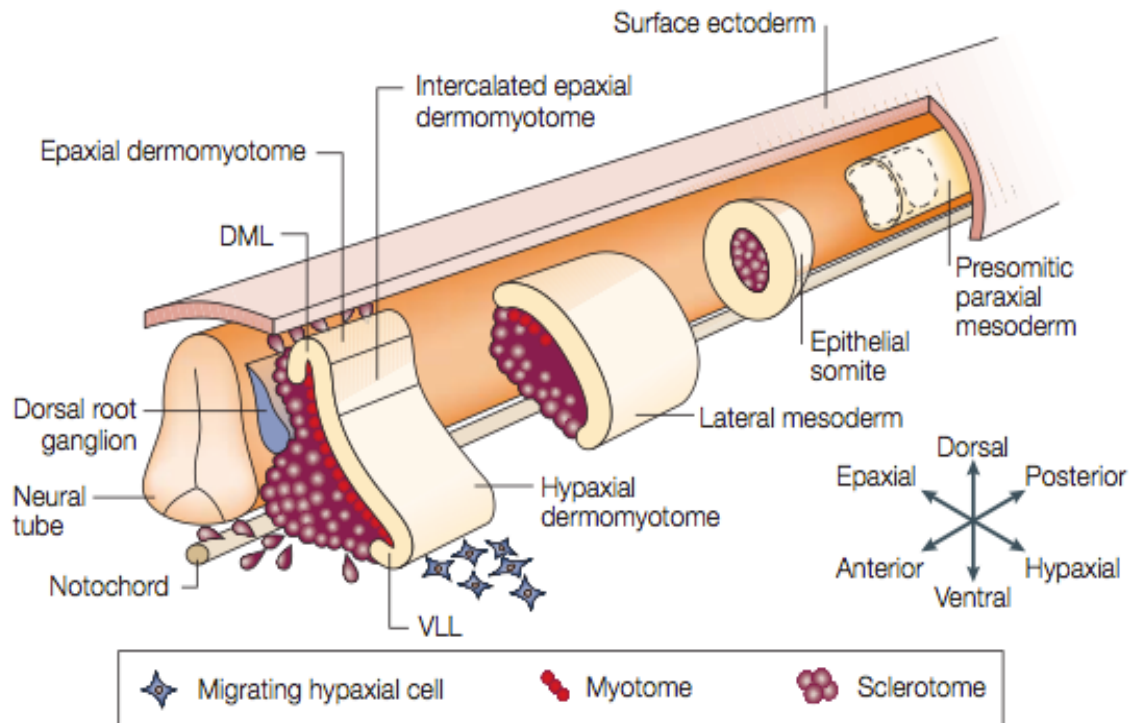


Figure 1.1- Somite formation and patterning in the vertebrate embryo. Panel A illustrates a scanning electron micrograph of the paraxial mesoderm that is undergoing segmentation in the anterior to posterior direction to form symmetrical pairs of somites flanking the axial structures, neural tube and notochord. Copyright: Gilbert S.F., *Developmental Biology 6th edition*, 2000, Figure 14.3. Panel B is a schematic depiction of vertebrate somite segmentation. The presomitic paraxial mesoderm is segmented in an anterior to posterior fashion, forming ball-like structures around the neural tube and notochord. 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 reference [17] with permission from Nature Publishing Group © 2003.

caudal-rostral direction while the expression of the RA gradient antagonizes their expression. As the level of Wnt-Fgf decrease below a threshold, being opposed by RA signalling, the front position forms and the pre-somitic mesoderm cells become competent to respond to the 'clock' and form segmental boundaries [5]. Other studies have shed light upon this model and shown Wnt signaling regulates the segmental clock by controlling the oscillating expression of Axin2 and Lunatic fringe (Lnfg) genes [6, 7]. It has also been shown that loss of RA results in loss of bilateral symmetry of the somites since the Fgf8 signalling gradient is expressed more anteriorly and genes that are required to control the oscillation of Notch expression such as Hes7 and Lnfg are expressed asymmetrically [8-10].

After segmentation as the epithelial somites mature, they differentiate into dermomyotome (DM), which is the dorsal compartment of somites and gives rise to muscles of the trunk and dermis, and into sclerotome, which is the ventral part of the somites and gives rise to bones and cartilage of the axial skeleton (Figure 1.1, B) [11]. The muscle progenitor cells that express transcription factors such as Pax3, Pax7, Meox1 and Gli2, are located in the DM and migrate ventrally to form the myotome, the site of the first fully differentiated embryonic myocytes and the source of the first skeletal muscle found in the body. It is now known that the myotome is formed in two steps as demonstrated by Gros *et al* using an electroporation of a GFP reporter construct into chick interlimb somites [12]. The first step is the formation of the primary myotome which involves migration of DM cells through the dorsomedial lip (DML). As the new myoblasts form, they displace the older myocytes and they elongate across the rostral caudal borders of the somites. This is followed by migration of the cells from all four

borders of the DM to the myotome and their elongation along the anterior-posterior axis of the embryo [13]. 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 [14, 15].

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 [16, 17]. Although morphologically continuous, the medial and lateral halves of the DM and myotome generate separate myogenic domains in the embryo [18]. It has been suggested that the epaxial-hypaxial DM and myotome are separated by a horizontal subdivision based on the expression pattern of transcription factors such as *Sim1* and *En1* [19, 20]. 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) [21-23]. 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) [17].

The differentiation of the epithelial somites into the DM and myotome is influenced by soluble signaling factors such as Wnt, Sonic hedgehog (Shh) and BMP emanating from the surrounding embryonic tissues such as the notochord, neural tube, overlying ectoderm and lateral plate mesoderm [24], (Figure 1.1, B and 1.2, B). In addition to these signaling pathways, a complex network of temporally and spatially

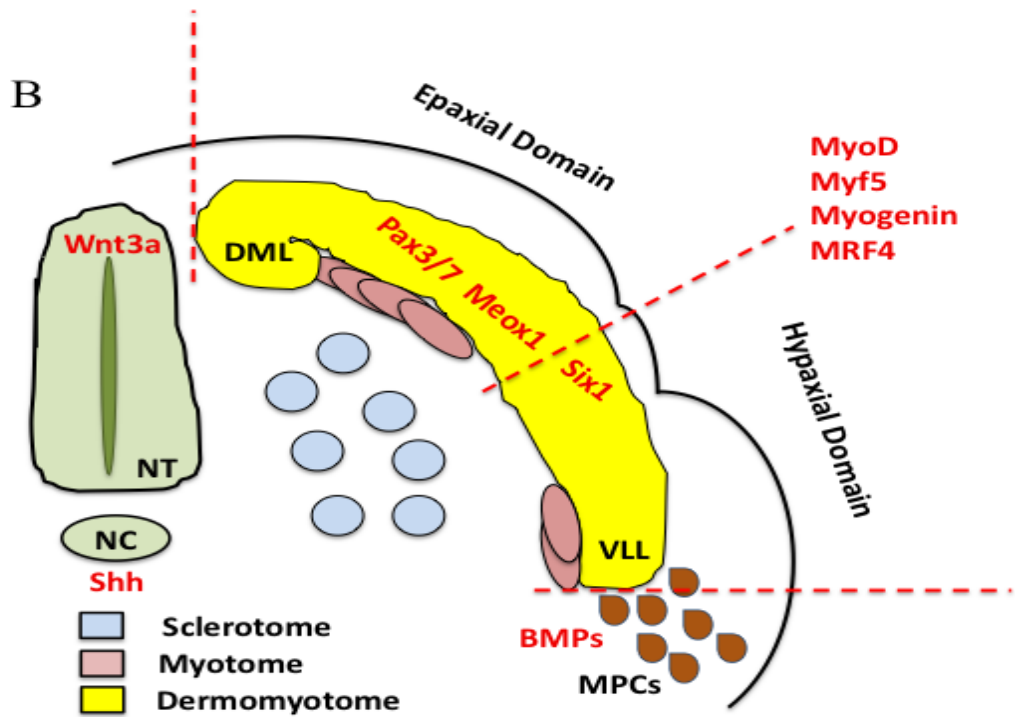
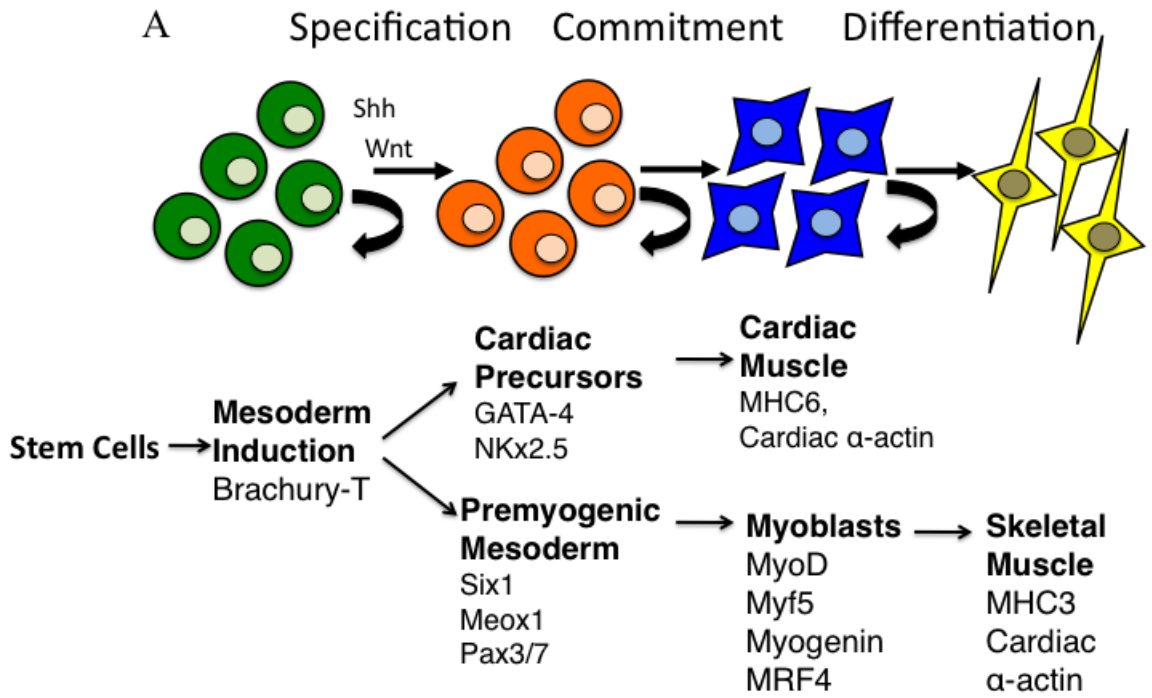


Figure 1.2- Transcriptional regulation of cardiac and skeletal muscle development. Panel A is a schematic representing the series of molecular events involved during stem cell differentiation and mesoderm patterning into cardiac and skeletal muscle. The important signalling molecules, transcription factors and structural genes involved in both cardiac and skeletal muscle formation are illustrated in this diagram. Panel B is a diagram representing the somitic division of epaxial and hypaxial domains of the dermomyotome and myotome. The expression location of important signalling molecules and key transcription factors for skeletal muscle development, are illustrated in the schematic. NT: Neural tube. NC: Notochord. MPC: Muscle precursor cells. DML: Dorso-medial lip. VLL: Vento-lateral lip.

expressed transcription factors play a role in regulating the process of muscle differentiation. Although much is known about the key molecular players in the process of skeletal muscle development, the mechanisms that regulate the expression of these genes remain unclear.

1.2 Transcriptional Regulation of Skeletal Muscle Development

Each event that occurs during the course of skeletal muscle development can be identified based on the expression of a set of molecular markers (Figure 1.2, A).

1.2.1 The Myogenic Regulatory Factors (MRFs)

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 [25-29]. 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.

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 [30, 31]. 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 [32, 33]. 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 [34]. It is thought that MyoD rescues the myogenic program in the absence of Myf5 [34].

MyoD is expressed at E9.5 mostly in the hypaxial domain of the dermomyotome [35, 36]. 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 [29, 37]. In addition, MyoD can interact with HDAC1 in order to prevent premature differentiation of myoblasts [38]. 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 [39], possibly 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 [36, 40]. These MyoD null mice display delayed hypaxial myogenesis while the epaxial myogenesis stays unaffected [40]. These observations suggest 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 [41, 42].

MRF4 is expressed in the myotome from E9.0 to E12.0, after which its expression is repressed until after birth [43]. 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 [44, 45]. 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 [34]. 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 [46]. Finally, it was shown that mice lacking Myf5, MyoD, and MRF4 are defective in myogenesis and unable to form myoblasts [34].

Myogenin mRNA is detected at E8.5 following the expression of Myf5, which is thought to regulate Myogenin's expression [47-49]. Myogenin protein however is not detected until E10.5, indicating some sort of post-transcriptional regulation or protein instability [48, 49]. 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 in which myocytes are normally formed but do not fuse efficiently into myotubes and hence these mice die perinatally [50, 51]. When Myogenin was inserted into the Myf5 locus in the Myf5^{-/-}/MyoD^{-/-} mice (which are also missing MRF4), myogenesis was only rescued to some extent, indicating that Myogenin has some ability to direct cells into the myogenic lineage while suggesting Myf5 action is required early during differentiation [52, 53].

The knowledge gained by the genetic analysis of MRFs has increased our understanding of muscle development. However we are still at the beginning of our

comprehension of the interplay of these transcription factors and their role in regulating other genes that control myogenesis.

1.2.2 The Pax Family- Pax3 and Pax7

The *Pax* family of transcription factors are involved in many developmental processes including tissue specification and organ formation [54]. These genes are characterized based on their paired box domain and paired-type homeodomain and are further classified into groups based on their expression patterns and structural characteristics [55]. 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 [56], is comprised of an octapeptide motif, a homeodomain and a paired domain key for sequence-specific DNA binding [57, 58]. In mice, Pax3 is initially expressed in the PSM prior to segmentation [57] and its expression becomes progressively limited to the lateral domain of the DM as the somites are patterned [59]. The domain of Pax3 expression extends throughout the entire epithelial somite and the extremities of DM [54] and then myotome [15] where it controls formation of hypaxial muscle as well as migration of limb muscle progenitor cells in the VLL of the limb somite [59-63]. In fact, *Spotch* (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 [61, 62, 64].

Various studies have shown that Pax3 regulates expression of MRFs during myogenesis, however, so far, only Myf5 has been identified to have an important binding site for Pax3 in its enhancer region [65, 66]. In Myf5/MRF4 double mutant mice, MyoD

expression is not impaired, however, a cross between *Splotch* and Myf5/MRF4 mutant mice, forming a Myf5/MRF4/Pax3 triple mutant mice, resulted in the loss of MyoD in the trunk, limbs and hypaxial and epaxial somites, implying that Pax3 is upstream of MyoD and is required for its activation in the absence of other MRFs [67, 68]. Furthermore, it has been shown that ectopic expression of Pax3 leads to activation of MRFs in embryonic tissues and in stem cells [69, 70].

Pax7, which is closely related to Pax3, is also expressed in the somites, however its expression is concentrated in the medial domain of the DM [71]. This expression pattern can be altered as it has been speculated that 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 [67]. Pax7 mutants appear normal at birth, but die in a few weeks for unknown reasons and they show no evident embryonic skeletal muscle defects [72] implying compensation by other *Pax* genes [67].

Several groups have identified a progenitor population of Pax3/7 positive (and MRF negative) cells, which continue to proliferate throughout embryonic development and contribute to the developing muscle. Later, these cells are found in the sublaminar layer adjacent to the muscle fibres, implying that they might in fact be a developmental source of satellite cells [54]. 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 this population of progenitor cells undergoes continued apoptosis and is inhibited from undergoing myogenesis resulting in a loss of skeletal muscle, with the exception of the early muscle of myotome [15].

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 [73]. It is important to note that although Pax7 activity is not essential for embryonic myogenesis in the presence of Pax3, its expression is extremely important for the formation of satellite cells [72, 74]. 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 [75]. Recently, however, it was shown that Pax7 labels satellite cells and ablation of Pax7^{+ve} satellite cells completely blocks regenerative myogenesis following injury, suggesting their essential role for acute injury-induced muscle regeneration [76, 77]. Another recent study suggests that a population of non-satellite cell muscle resident progenitor cells, which are PW1⁺/Pax7⁻ called interstitial cells (PICs) may play a role during adult muscle regeneration in response to injury or aging [78]. Despite their clear role during embryonic muscle development, the role of Pax3 and Pax7 in adult muscle regeneration through satellite cell activation is debatable.

1.2.3 The Meox Family- Meox1 and Meox2

Meox1 and Meox2 are members of the large family of homeodomain transcription factors and are expressed in the developing somite [79, 80]. In addition to having a highly conserved sequence homology, Meox1 and Meox2 have a highly conserved pattern of expression and function among species [81]. In mice, Meox1 expression is initiated in the PSM as early as E7.0 and as development proceeds its

transcripts are expressed throughout the entire epithelial somite and later its expression becomes restricted to the dermomyotome and the sclerotome [79, 80, 82]. Expression of Meox1 decreases during somite maturation, suggesting that downregulation of this gene must happen for the differentiation to proceed [79, 82]. The expression of Meox2 is initiated later as the somites form and its transcripts are not detected in the pre-somitic mesoderm [80]. Unlike Meox1, the expression of Meox2 remains high in the differentiating somites and interestingly it is detected at the level of limb bud [80, 83]. Consistent with this, Meox1 null mice display sclerotome-related defects such as abnormal fusions in ribs and vertebrae, while mice that lack Meox2 fail to develop limb muscle accompanied by a downregulation of Pax3 and Myf5 expression [80, 83, 84]. Meox1/2 double knockout mice show a much more severe phenotype characterized by loss of both epaxial and hypaxial muscle [84] suggesting a degree of compensation exists between these two genes and the necessity of both for normal muscle development. Further studies have revealed that Meox1 and Meox2 interact directly with Pax1 and Pax3 respectively through their homeodomains [85] suggesting that Meox factors might exert their effect on limb muscle development in cooperation with Pax factors.

1.2.4 The Sox Family of Transcription Factors

The Sox family is a subgroup of the high-mobility-group (HMG)-box superfamily of transcription regulators [86]. 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), which is the testis determining factor [86, 87]. Sry was the first Sox family member identified [88, 89]. At least 30 members of the

Sox family have been identified including the 20 Sox genes found in mice and human [90, 91]. These members are further divided into ten subgroups (A-J) based on the degree of homology in the HMG domain and the presence of conserved motifs outside the HMG box [92], even though outside of the HMG domain, the SOX sequences are actually quite variable [93]. The evolutionary conserved HMG motif exhibits sequence-specific DNA binding activity at the minor groove of the double helix with the DNA sequence 5'-(A/T)(A/T)CAA(A/T)G-3', and induces dramatic bending exposing the major groove [94-97]. Since Sox genes can induce large conformational changes in their target DNA, it has been proposed that these genes might act as architectural proteins that can organize the chromatin structure and assemble transcription factors into large, multi-protein complexes [98, 99] (Figure 1.3). Sox genes are expressed in many different cell types and tissues at multiple stages during development and they are involved in many developmental processes including hematopoiesis, chondrogenesis, neural tube development, lens development and sex determination [100]. Interaction of Sox transcription factors with tissue-specific co-factors determines the specificity of these processes [101].

Sox7 belongs to the group F of the Sox family, along with Sox17 and Sox18 [90]. Homologs of Sox7 have been characterized in *Xenopus*, zebrafish, mice and humans [102-104]. Chromosomal mapping analyses identified mouse Sox7 on band D of mouse chromosome 14, and localized human SOX7 on human chromosome 8 (8p22) [103]. In F9 EC cells, Sox7 regulates the differentiation of the parietal endoderm via induction of GATA4, GATA6 and Fgf3 activation and expression [105, 106]. In human embryonic stem cells (hESCs), stable expression of Sox7 is sufficient to induce the formation of

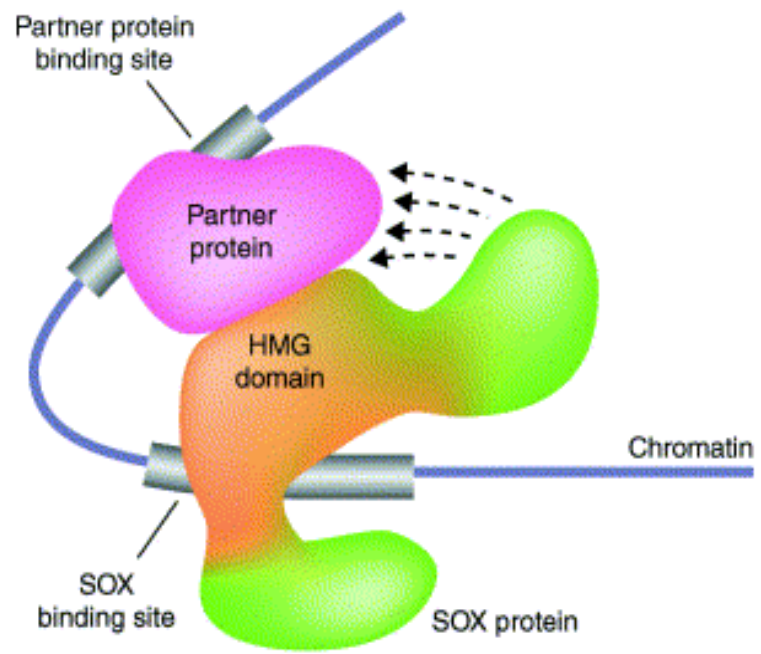


Figure 1.3-A model for the specificity of the action of SOX transcription factors. Sox transcription factors may function as activators or repressors by interacting with partner proteins that bind to adjacent binding sites in the promoter or enhancer of target genes. The Sox-partner interaction could 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 Wilson, M., and P. Koopman. 2002. Matching SOX: partner proteins and co-factors of the SOX family of transcriptional regulators. *Curr Opin Genet Dev* 12:175-177, with permission from Elsevier © 2002.

endoderm progenitor cells [107]. In *Xenopus* and zebrafish, Sox7 plays a role in regulating cardiomyogenesis and vascular development by controlling arteriovenous specification [108-110]. Sox7 transcripts are detected in the somite of mice as early as E7.5/8, suggesting a role for Sox7 in cell fate specification [103]. In *Xenopus*, xSox7 can induce the expression of mesoderm by inducing Xnr1-6 and Mixer genes [111], therefore indicating a role for Sox7 in regulating the cell fate of mesodermal cells during embryogenesis. Furthermore, recent studies in P19 EC cells have demonstrated that Sox7 induces expression of muscle precursor factors like Pax3/7, Meox1, and Foxc1, leading to skeletal muscle formation in P19 cells [112].

1.3 Signalling Pathways That Regulate Skeletal Muscle Development

The myogenic network of transcription factors described above is regulated by several signalling molecules including Retinoic acid (RA), Shh, Wnts, and Bone Morphogenic Protein (BMP). These morphogens are secreted from the tissues surrounding the developing somite such as the notochord, the neural tube and the overlying surface/dorsal ectoderm (Figure 1.2, B). The cell fate within the somite is controlled by the interplay of these pathways.

1.3.1 Retinoic acid

RA is a derivative of Vitamin A and plays a critical role in embryonic growth and patterning [113] and in regulating major developmental processes, including skeletal myogenesis [114].

1.3.1.1 RA synthesis and signalling

Inside the cell, RA is synthesized from retinol, which is supplied maternally during embryogenesis. The canonical pathway for RA synthesis is comprised of two steps. The first step is the conversion of retinol to retinaldehyde by two families of enzymes, Alcohol Dehydrogenase (ADH) and Retinol Dehydrogenase (RDH) (Figure 1.4). The second step is the oxidation of retinaldehyde to RA by the Retinaldehyde dehydrogenase (Raldh) class of enzymes [115]. Of the three Raldh family members, Raldh1,-2,-3, Raldh2 is the first to be expressed and its expression is first detected in the primitive streak during gastrulation of mesodermal cells and then becomes restricted to the posterior embryonic trunk region [116]. Consistent with this, *Raldh2* null mice die pre-natally and peri-natally and exhibit defects in several tissues including the hindbrain, forebrain, heart, limbs and somites [115]. Raldh1 and Raldh3, however, play a role in the later stages of embryogenesis, mainly during eye and nasal development, as revealed by knockout studies [115].

RA exerts its effects by binding to its nuclear receptor, retinoic acid receptor (RAR). RARs (α , β , γ) are ligand-inducible activators and heterodimerize with retinoid X receptors, RXRs (α , β , γ) on the retinoic acid response elements (RAREs) within the genome. In the absence of RA, the receptors recruit transcriptional co-repressors such as NCoRs, which in turn recruit histone deacetylases (HDACs) and methyl-transferase complexes to the DNA, making chromatin inaccessible for gene transcription. Upon binding of RA to RAR, a conformational change is induced in the RAR-ligand binding domain which results in the exchange of co-repressors for co-activators and hence activation of RA-responsive genes [115, 117].

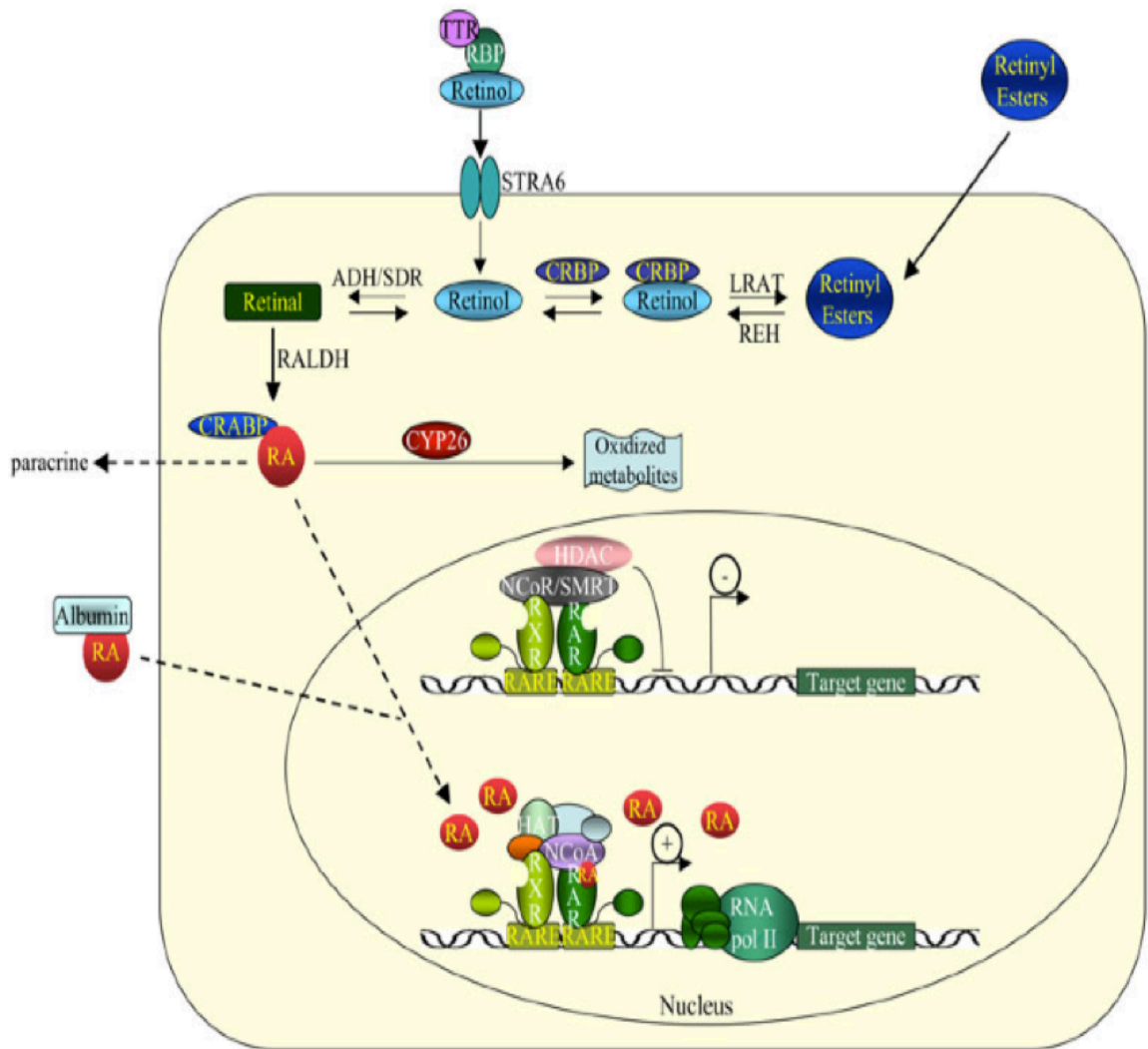


Figure 1.4- Mechanisms of RA synthesis and function- In the bloodstream retinol is bound by the retinol binding protein, RBP and the retinol:RBP complex is bound to TTR (transthyretin) to ensure delivery to target tissues. Retinol uptake occurs via the retinol receptor STRA6 (stimulated by retinoic acid gene 6). In the target cell, free retinol is oxidized to retinaldehyde by members of the ADH/SDR family of enzymes. A second oxidation catalyzed by the RALDH class of enzymes, converts retinaldehyde to RA. RA is now free to enter the nucleus where RAR/RXR heterodimers bound to RAREs are associated with a co-repressor complex. Binding of RA induces a conformational change in the RAR/RXR heterodimer that results in release of co-repressors and recruitment of co-activators and initiation of transcription allowing the expression of RA target genes. Reproduced from Maria Theodosiou • Vincent Laudet • Michael Schubert. 2010. From carrot to clinic: an overview of the retinoic acid signalling pathway. *Cell. Mol. Life Sci.* 67:1423–1445, with permission from Springer © 2010.

1.3.1.2 RA signalling and myogenesis

Several studies in different model organisms, including mouse, chick and zebrafish, have revealed that RA plays an important role in regulating segmental patterning during somitogenesis. Loss of RA signalling leads to an uncoordinated somitogenesis and loss of bilateral symmetry of somite pairs as one side of the neural tube has less number of somites compared to the other side [8, 118, 119]. This probably occurs because of anteriorization of the FGF8 caudal gradient. Normally *fgf8* is expressed in a caudal to rostral gradient, while *Raldhs* are expressed in the rostral presomitic mesoderm [10, 118]. It is believed that RA acts as a factor that prevents left-right asymmetry from occurring in the pre-somitic mesoderm. Although the role of RA is well characterized in somitogenesis, RAR/RXR knockout mice had no somite-related defects [120]. The developmental role of RARs (α , β , γ) is not clearly known since mice with single knockouts of these isoforms are viable and relatively normal [121-125]. The same observation is made when RXR β and RXR γ are knocked out in mice, however loss of RXR α is lethal because it causes cardiac dysfunction during development [126-128]. Recently, it was shown that activation of RXR by rexinoid enhances differentiation of mouse ES cells into skeletal myocytes [129].

RA is also needed for the regulation of genes involved in the differentiation of the myotome. In stem cells and myoblast cell lines, low levels of RA can enhance skeletal myogenesis [130-135]. This enhancement occurs through upregulation of Pax3/7, MRFs and through direct synergy between RARs and MRFs [131, 136, 137]. Expression of a dominant negative RXR in C2C12 myoblasts inhibits expression of MyoD while expression of a dominant negative RAR does not have this effect, implying that the

RARs and RXRs have functionally distinct roles during development [138]. Furthermore, it has been shown that RA is able to induce myogenic differentiation via enhancing MyoD expression in C2C12 myoblasts, cultured chick satellite cells and limb buds [131, 132, 137]. A more recent investigation of the role of RA in limb myogenesis revealed that the inhibition of RA synthesis which was achieved through treatment of developing chick limbs with citral, resulted in downregulation of Meox1/2, Pax3, Myf5 and MyoD and eventually an inhibition of myogenesis [139]. Similar to the effects of lack of RA, excessive amounts of RA can result in downregulation of these same genes and an inhibition of myogenesis in the developing limb. These results demonstrate the complexity of RA signalling during muscle development and also the importance of the concentration and timing of exposure of this signalling molecule during cell fate determination. Our understanding of the role of RA signalling during myogenesis is still incomplete and further detailed molecular analyses are needed.

1.3.2 Shh

Shh, a member of the hedgehog family of signalling molecules is expressed in several tissues during embryonic development, predominantly in the notochord, floor plate of the neural tube and the limb bud [140, 141]. The three members of the hedgehog family, Shh, Desert hedgehog (Dhh) and Indian hedgehog (Ihh), start their signal by binding the cell surface receptor Patched-1 (Ptch1) [142]. When hedgehogs are not present, Ptch1 inhibits the transmembrane protein Smoothed (Smo) resulting in the prevention of the cytoplasmic molecules Gli2 and Gli3 from entering the nucleus. Upon binding of the Hh ligand to Ptch1, Smo inhibition is relieved and the Gli transcription

factors, including the activators Gli1/2 and the repressor Gli3, are activated and can enter the nucleus and regulate gene expression [143]. It has been shown in the somitic tissue explants that Shh, in combination with Wnt1 or Wnt3 is sufficient to induce myogenesis [144]. Furthermore there are other studies that revealed that Shh could induce MyoD and Myf5 expression [145-147]. Activation of Gli2 or Gli3 is required for Myf5 activation in epaxial muscle progenitors while in the absence of Shh ligand, Gli3 represses Myf5 expression [148]. Double mutant mice for Gli2 and Gli3 display a mispatterning of genes in the myotome and the hypaxial dermomyotome [148]. Taken together these studies reveal that Shh is essential for gaining the myogenic cell fate.

1.3.3 Wnt

Wnt gets its name from a *Drosophila* segment polarity gene, Wingless (Wg), and its mouse homolog, Int-1 oncogene [149, 150]. Wnt ligands are secreted from the neural tube adjacent to the medial portion of the somite and play a critical role in determining the myogenic lineage [151] among their other roles during development including regulation of cell proliferation, patterning and cell fate determination [152, 153]. The Wnt family, which is composed of 19 mammalian homologs, signals through three distinct pathways, the canonical pathway which function through β -catenin, the planar cell polarity pathway (PCP), which works through the rac and rhoA pathways and the Ca^{2+} pathway through Protein Kinase C [154]. Wnt ligands are able to induce a pro-myogenic effect on the adjacent somites. It has been shown that Wnt1, Wnt3 and Wnt4, which are expressed in the dorsal part of the neural tube, can induce myogenesis in somite explants [144]. These ligands exert their effect through activation of downstream

targets. For example, Wnt1 activates Myf5 and Wnt7a induces activation of MyoD [155, 156]. When the Wnt antagonist, Frizzled (Frz)-1b was delivered transplacentally into mouse embryos, skeletal myogenesis was inhibited and Myf5 expression was downregulated while the expression of premyogenic mesoderm markers Pax3 and Meox1 was not altered, implying that these Wnts are needed for commitment and terminal differentiation, but not for myogenic specification [157]. *In vitro* studies in mesenchymal stem cells [158] and in P19 EC cells [159, 160] support the pro-myogenic effect of Wnt/ β -catenin signalling. In addition to Myf5, to date, only a few direct targets of the Wnt pathway have been identified in developing muscle, including Foxc1, which is involved in somitogenesis [161]. These findings underline the importance of Wnt ligands for muscle development, however more studies are needed to identify the downstream targets of this signalling pathway during skeletal myogenesis.

1.3.4 BMP

The name Bone Morphogenic Proteins is given to the members of the TGF- β family of peptide growth factors that can induce the formation of ectopic bone in muscle tissue [162, 163]. BMPs also play a role during chondrogenesis, adipogenesis, as well as cardiac and skeletal myogenesis [164]. These signalling proteins activate their receptors serine/threonine kinase upon binding, resulting in phosphorylation and nuclear translocation of Smad1/5/8 proteins to regulate the transcription of the downstream genes [165]. During skeletal myogenesis, BMP plays a very complex role. BMP4 that is secreted from the lateral plate mesoderm in the chick induces cells to express the hypaxial marker Sim1 while inhibiting the expression of MyoD and terminal

differentiation [166]. When derived from the dorsal neural tube, BMP signals activate Wnt1 and Wnt3a and ultimately Wnt11 in the medial lip, which plays an important role in migration of progenitor cells into the myotome [167]. Noggin, an endogenous BMP inhibitor, is known to prevent BMP signals from reaching the medial lip and it restricts the positional activity of BMP [167]. It is also believed that Noggin signalling counteracts the inhibitory effect of BMP4 on the epaxial somites [167-169]. Furthermore the interplay between levels of BMP4 and Noggin regulates Pax3 activity resulting in temporal and spatial activation of the MRFs [170]. The contribution of BMP to myogenesis is not fully understood, however it is evident that this signalling pathway works in concert with other signalling pathways such as Notch, Wnt, FGF, RA and Shh that play a role in different aspects of muscle development in the embryo [171, 172].

1.4 Vertebrate Embryonic Cardiac Muscle Development

Cardiomyogenesis is distinct from skeletal myogenesis. However, similar to skeletal muscle development, cardiac muscle also develops from mesoderm in a hierarchical fashion that is controlled by transcription factors. The progenitor cells that are not yet committed to the cardiac fate leave the primitive streak and migrate in an anterior-lateral manner to form a crescent-shaped structure right below the head folds of the embryo. The two sides of this crescent will then fuse at the embryonic midline to form the linear heart tube. As development progresses, the heart tube will undergo a series of morphogenetic changes including looping and septation which will give rise to a mature heart; which is the first organ to form during vertebrate embryonic development[173].

The cardiac crescent contains two distinct populations of progenitor cells, the primary and the secondary heart fields. At the crescent stage the secondary heart field is positioned medially to the primary heart field [173]. The secondary heart field is located anteriorly and dorsally to the heart tube as it forms later. Studies have shown that the secondary heart field lineage population of progenitor cells is highly proliferative and differentiates later than the primary heart field and it contributes to the formation of the outflow tract and the right ventricle of the heart [174, 175]. The rest of the heart including both atria, the left ventricle, and to a less extent, the right ventricle, originate from the primary heart field lineage population of progenitor cells [173].

Similar to the case of skeletal myogenesis, a network of transcription factors and signaling molecules, secreted from the surrounding tissues, regulate the proliferation and differentiation of cardiac progenitors along with the morphogenetic events that shape the heart.

1.5 Transcriptional Regulation of Cardiomyogenesis

Similar to skeletal muscle development, each event that occurs during the course of cardiac muscle development can be identified based on the expression of a set of molecular markers (Figure 1.2, A), a few of which will be discussed here.

1.5.1 The Nkx2 Family

Nkx2.5, a member of the NK family of homeodomain transcription factors, is expressed in the cardiac progenitor population of both the primary and the secondary heart fields [176]. Transcripts of Nkx2.5 are first detected in the cardiac crescent as early

as E7.5. Mouse embryos with an Nkx2.5 mutation fail to undergo heart looping which leads to their death. In these mutants, cardiomyocytes are specified normally, however genes downstream of Nkx2.5 including atrial natriuretic factor (ANF) are expressed abnormally [177, 178]. The same observation was made in mouse embryonic stem cells (mESCs) [179]. The normal specification of cardiomyocytes in Nkx2.5 mutants could be due to genetic redundancy of Nkx2.5 with other members of the NK family expressed in the developing heart, as observed in co-expression of dominant negative xNkx2.5 and xNkx2.3 in frogs which results in a complete inhibition of cardiomyogenesis [180]. In P19 embryonal carcinoma (EC) cells, Nkx2.5 activity is required for cardiomyogenesis since the expression of a dominant negative form of this factor results in inhibition of cardiac muscle formation [181]. Furthermore, Nkx2.5 overexpression is sufficient to induce cardiac differentiation in P19 cells [182]. A more in depth analysis of Nkx2.5 function in mice revealed that Nkx2.5 works in a negative feedback loop to inhibit BMP2/Smad1 signalling to ultimately regulate the balance between specification and proliferation of the progenitor population of the secondary heart field [183]. It has also been shown that Nkx2.5 regulates many of its target genes through direct interactions with other cardiac transcription factors including GATA4, Tbx5 and Mef2C [184-186] and these interactions confer target specificity and modulate Nkx2.5 activity.

1.5.2 The GATA Family

The GATA family of transcription factors belongs to the superfamily of zinc finger transcription regulators and three of the members of this family; GATA4, GATA5 and GATA6 are expressed, not exclusively, in the developing heart tissue [187-190]. The

expression of GATA factors is initiated in the mesoderm at E7 during early stages of cardiomyogenesis [190]. GATA4 is the most studied GATA factor in the heart. Similar to NKx2.5, GATA4 expression is dispensable for specification and differentiation of cardiomyocytes in vertebrates and in ES cells [191-193]. In GATA4 null mice, ventral migration of cardiac progenitors is defective leading to failure of cardiac crescent fusion in the midline and inhibition of heart tube formation [191]. Loss of function studies have shown no single GATA factor is absolutely needed for mouse cardiomyogenesis, while double knockout mutations have revealed a level of redundancy between these factors [194, 195]. When both GATA4 and GATA6 are mutated in mice, primary heart field progenitors are lost while the progenitors of the secondary heart field are normal, indicating an important role for GATA factors in progenitor specification in the primary, but not secondary heart field [196]. These double mutant mice completely lack terminally differentiated cardiomyocytes. Of the three GATA factors mentioned, only GATA4 is expressed in P19 cells and knockdown of GATA4 in these cells blocks cardiomyogenesis [197, 198]. Consistent with this, GATA4 overexpression induces and accelerates cardiomyogenesis in P19 cells [199]. Interestingly and maybe significantly for cardiac cell therapy implications, it has been found recently that GATA4, in cooperation with Mef2C and Tbx5, is able to reprogram cardiac fibroblast into cardiomyocytes [200].

1.6 Stem Cells, A Model For Myogenic Differentiation

Stem cells have the ability to proliferate, self-renew and differentiate into all three germ layers. They present a good model to study the molecular mechanisms of 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. Before efficient protocols for maintaining and differentiating mESCs and hESCs were developed, EC cell lines were used predominantly as a model to study cellular differentiation mechanisms. Particularly, P19 EC cells have served as a useful model to study cardiac and skeletal muscle development [201]. 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 they display pluripotency by their ability to contribute to tissues from all three germ layers [202, 203]. The advantage of studying the P19 cell system is based on the ability of these cells to grow quickly and maintain an undifferentiated state, their ability to be induced to differentiation depending on the culture condition, and their ability to be amenable to genetic manipulation [201]. These genetic manipulations include stable integration or knockdown of genes in the P19 cell genome.

P19 EC cells can be induced into cardiac and skeletal muscle by aggregation and exposure to low levels of dimethyl sulfoxide (DMSO) [204]. Aggregation is needed for induction of mesoderm and DMSO is needed for differentiation into muscle and hence both are necessary for muscle formation in P19 cells [205]. In addition, factors in fetal calf serum play a role in the efficiency of P19 cell differentiation [206]. 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 [204, 207]. Over the years many studies have revealed that the differentiation pathways [65, 70, 73, 208-215], the gene expression patterns [70, 160, 161, 216] and the signalling networks [70, 134, 159, 160, 217-219],

identified in P19 cells recapitulate what is found during embryonic muscle formation, supporting their validity as a model for the study of differentiation of embryonic stem cells.

mESCs and hESCs, 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 which regulate target genes for the purpose of self-renewal [220]. The cooperation of these three transcription factors for regulation of pathways involved in pluripotency is evident by the large subset of common target genes they share with each other [221]. 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 [134, 135, 222-225]. Both of these cell types readily differentiate into cardiomyocytes in a similar fashion as in the embryo, forming a progenitor population [226]. This finding may prove valuable for cell therapy purposes, however it won't be possible until the yield of "working" cardiomyocytes formed from ESCs, as opposed to "nodal" cells, is optimized. Differentiation of ESCs into skeletal muscle has been less popular, due to relative difficulty of obtaining sufficient amount of skeletal muscle *in vitro*. Most of the differentiation protocols for hESCs differentiation into muscle are not reproducible and do not induce utilization of embryonic pathways [227]. Very recently, however, a protocol was developed which allows the hESCs to employ embryonic pathways to form muscle through the formation of a Pax3/7+/MRF- progenitor population [135]. Differentiation of mESCs into skeletal muscle also follows an embryonic pathway and has been more promising for cell therapy mediated treatment of dystrophic or injured muscle tissue as they enrich mesodermal and myogenic

progenitors during differentiation [227-232]. Cell therapy will not be feasible, however, until methods for increasing the yield of skeletal myocytes from ESCs have been optimized.

1.7 Thesis Summary

In order to design future therapies for restoring damaged muscle tissues using stem cells, we need to understand the molecular networks and signalling pathways which regulate the process of muscle development and cell fate decision. This will allow us to manipulate these mechanisms to generate high yields of either skeletal myocytes or cardiomyocytes. Currently there are at least two major limitations to stem cell therapy, differentiation of these cells into the desired tissue and engraftment of these cells at the transplantation site.

In Chapter 2, the objective was to further extend our knowledge on the role of Sox7 during muscle development. My specific aim was to knockdown Sox7 using an shRNA approach to study the effect of loss of Sox7 on the expression of premyogenic markers as well as MRFs. My hypothesis was that Sox7 is essential for skeletal myogenesis in P19 cells in muscle-inducing condition (+DMSO) +/-RA. Previous data in our lab illustrated that RA and Wnt signalling pathways contribute to Sox7 expression. In addition, our lab has demonstrated that ectopic expression of Sox7 can induce the entire skeletal muscle pathway in P19 cells [112]. Here we present data showing that RA alters the function of Sox7 during skeletal muscle development and Sox7 modulates the effect of RA on cell fate decision-making.

In Chapter 3, experiments were designed to test the ability of mESCs to differentiate into cardiac and skeletal muscle on a collagen matrix with or without a ligand. My hypothesis was that these collagen-based matrices could enhance myogenesis in mESCs. The effect of these matrices on regeneration of hindlimb muscle was also tested *in vivo* in a mouse model (appendix B). These experiments were performed based on the hypothesis that these matrices can enhance the regeneration response in the injured muscle tissue. *In vitro* Collagen increases the expression of skeletal muscle precursor genes and myotube formation and collagen+ligand increases the expression of cardiac precursors and final differentiation markers. *In vivo* when either collagen alone or collagen+ligand are injected at the site of injury, the number of cells that express skeletal muscle precursor and final differentiation markers increase compared to the PBS controls.

CHAPTER 2

Role of SRY-Related HMG Box (SOX)-7 in Skeletal Myogenesis in P19 Cells

2.1 Introduction

The members of the SOX family of transcription factors are expressed in many different cell types and tissues at various stages during development [91, 100]. These transcription factors are widely recognized as key players in the determination of different cell fates during embryonic development. Sox genes are divided into smaller subgroups based on the degree of homology within their HMG domain and the presence of conserved motifs outside the HMG box [92]. Subgroup F is formed from three closely related SOX proteins, SOX7, SOX17 and SOX18. SoxF factors have a pivotal role in cardio-vascular development. They coordinate endothelial cell fate and direct cell differentiation in developing heart, blood vessels and lymphatic vessels [233]. In *Xenopus*, zebrafish and mouse a degree of redundancy is observed between the SoxF genes during embryonic events such as arteriovenous specification and cardiovascular development [108, 109, 234, 235], but not during lymphatic development [236]. To date, no redundancy between SoxF genes has been reported during skeletal myogenesis. SoxF transcription factors also play a role in germ layer specification. In *Xenopus* the F-type Sox members are involved in mesendodermal differentiation. For example, Sox7 regulates the expression of all five genes encoding mesoderm-inducing Nodals, *Xnr1-6* and its regulation of *Xnr4-6* appears to be direct [111, 235]. This observation indicates a role for Sox7 in regulating the cell fate of mesoderm cells. In mice, Sox7 is expressed throughout the somite as early as E7.5 [103]. In zebrafish, Sox7 is expressed during early somitogenesis in the posterior and anterior lateral plate mesoderm [108]. The expression of Sox7 in the lateral plate mesoderm is regulated by multiple signals such as Sonic hedgehog and VegF [110].

In vitro studies have revealed a novel role for Sox7 in regulating skeletal myogenesis under conditions that do not normally support differentiation [112]. In P19 EC cells, Sox7 can induce the expression of skeletal muscle precursor genes such as Pax3/7, Meox1, and Foxc1 as well as the MRFs leading to skeletal muscle formation [112]. In these cells, Sox7 expression precedes the expression of the skeletal muscle precursor markers, making it a potential target of RA signalling. It has also been demonstrated that Sox7 is a RA-responsive gene, whose expression accelerates and increases during RA-mediated myogenesis in P19 cells (Savage and Skerjanc, unpublished results). Many studies have shown that RA positively regulates myogenesis by increasing expression levels of the MRFs and enhancing overall muscle development in stem cells [130-132, 136]. Treatment of P19 cells with RA accelerates and enhances skeletal myogenesis while preventing differentiation into cardiac muscle cells [134]. In P19 cells, this occurs through increasing the expression of skeletal muscle precursor genes Pax3 and Meox1, and the MRFs MyoD and Myogenin. It was also shown that RARs bound directly to RAREs in the upstream and downstream regions of preskeletal mesoderm genes, Meox1 and Pax3 in a population of differentiating P19 cells thus leading to enhanced skeletal myogenesis [134]. Based on the evidence that RA and Sox7 both play an important role in skeletal myogenesis and that RA modulates Sox7 expression, the present study aims to further characterize the role of Sox7 as well as the ability of RA to modulate Sox7 function. Our findings indicate that Sox7 is necessary for skeletal myogenesis in the absence of exogenous RA, but not in its presence. Further analysis revealed that Sox7 in the presence of RA is necessary and sufficient for efficient neurogenesis in P19 EC cells.

2.2 Materials and Methods

2.2.1 Cell culture

P19 EC cells (ATCC) were cultured as previously described [206] in alpha-minimal essential media (Invitrogen, Burlington, ON, Canada) supplemented with 5% Cosmic Calf Serum and 5% Fetal Bovine Serum (PAA Laboratories, Mississauga, Ontario, Canada) or 10% Fetal bovine Serum (Wisent Inc. St-Bruno, Quebec, Canada). Differentiation was initiated by aggregating 5.0×10^5 cells for 4 days in 10 cm Petri dishes in media containing serum. The cells were aggregated in the presence of 1% DMSO (Sigma Aldrich, Oakville, ON, Canada). The aggregates were transferred into tissue culture dishes and gelatin-coated coverslips on day 4 of differentiation, and maintained until either day 8 or day 9. In the experiments that were performed in the presence of RA, the cells were treated with 3nM RA (Sigma-Aldrich, Oakville, Ontario Canada) during aggregation from day 0 until day 4. RA was dissolved in 95% ethanol and hence ethanol was added to the cells as a control in the (-RA) differentiations.

2.2.2 DNA constructs and RNA interference constructs

The P19[sox7] cell line used in some of the experiments in this study had been previously described [112]. Two shRNA vectors that were tested and used to create two stable cell lines were generated previously by Dr. Josée Coutu and Sara Khan. Briefly complementary DNA sequences targeting nucleotides 108 to 127 (shRNA1) (5'-TTTGAAGCCGAGCTGTCGGATGGTTCAAGAGACCATCCGACAGCTCGGCTTCTTTTTT-3' and 5'-CTAGAAAAAAGGAAGCCGAGCTGTCGGATGGTCTCTTGAA CCATCCGACAGCTCGGCTT-3') and nucleotides 677 to 696 (shRNA 2) (5'-TTTGAC

CTTCTTCTCGTCCTCATTTCAAGAGAATGAGGACGAGAAGAAGGTCTTTTTT
-3' and 5'CTAGAAAAAAGACCTTCTTCTCGTCCTCATTCTCTTGAAATGAGGA
CGAGAAGAAGGT-3') of *Mus musculus* Sox7 (NM_011446) (Invitrogen, Burlington,
ON, Canada) were annealed and cloned into mU6pro vector using Bbs1 and XbaI
restriction enzymes. Two control vectors that are not complementary to any sequences in
the mouse genome were used to create two control stable cell lines. One of these vectors
was described previously [112] and the other one was prepared by Dr. Josée Coutu and
Virja Mehta (5'-TTTGACAAGATGAAGAGCACCAATTCAAGAGATTGGTGCTCTT
CATCTTGTTGTTTTTT-3' and 5'-CTAGAAAAACAACAAGATGAAGAGCACCA
ATCTCTTGAATTGGTGCTCTTCATCTTGT-3'). The mU6pro vector was kindly
given by Dave Turner (University of Michigan, Ann Arbor, MI), and has been described
previously [237].

2.2.3 Stable cell line generation

P19 cells were transfected with the short hairpin constructs as described
previously [160]. Briefly 0.8µg of either shSox7 or shScrambled constructs were
transfected using FuGene 6 reagent (Roche, Mannheim, Germany) along with PGK-Puro,
a plasmid conferring puromycin resistance (Invitrogen, Burlington, Ontario, Canada).
The cells were grown under puromycin selection and colonies for each shRNA were
pooled together for further analysis.

2.2.4 Immunofluorescence

Aggregates from each differentiation were plated on day 4 on coverslips coated
with 0.1% gelatin (Fisher Scientific, Canada). On day 8 or 9 aggregates were washed
with cold PBS, fixed with -20°C methanol for 5 minutes on ice, allowed to air dry for

another 5 minutes and then rehydrated with Stockholm PBS (sPBS) for 15 minutes. Myosin Heavy Chain (MHC) expression was detected using the anti-MHC monoclonal antibody, MF20 [238] at a 1:1 dilution with sPBS. Neurons were detected with the mouse monoclonal anti-Neurofilament 68 antibody (Sigma-Aldrich, Oakville, Ontario, Canada) at 1:400 dilution with sPBS. The cells were incubated in the primary antibodies for 1 hour at room temperature or at 4°C overnight. In both cases, the cells were washed with sPBS and incubated with goat anti-mouse IgG(H+L) Cy3-linked secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:100 dilution with sPBS for 1 hour at room temperature or overnight at 4°C. The aggregates were then washed with sPBS and mounted in mounting media, 1:1 dilution of sPBS and glycerol, containing Hoechst dye at 1:100 dilution. Fluorescence was visualized using a Zeiss Axioskop2 microscope or an Olympus BX50. Images were captured on an AxioCam MRm camera and processed with Axiovision 4.42 or were captured with a Photometrics CoolSNAP camera and processed with Image-Pro Plus 5.1.

2.2.5 Reverse transcription and quantitative PCR

The cells were harvested using the RLT lysis buffer provided in the RNeasy Mini Kit and RNA was isolated according to the manufacturer's protocol (Qiagen, Mississauga, Ontario, Canada). The total RNA concentration was read by a Nanodrop ND-100 Spectrophotometer (Thermo Fisher Scientific, Canada). 1µg of the purified RNA was used during the first strand DNA synthesis reaction using the QuantiTect Reverse Transcription Kit as per manufacturer's protocol (Qiagen, Mississauga, Ontario, Canada). 1/20th of the resultant cDNA was used as a template for amplification during the Q-PCR reactions using the FastStart SYBR Green Master Mix (Roche Applied Science, Laval,

Quebec, Canada) or GoTaq qPCR Master Mix (Promega, USA). The PCR reaction was set at 2 stages. The first stage was set at 95°C for 10 min for 1 cycle. The second stage involved 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. The fluorescence was recorded at the last step of the second stage. Some of the reactions were performed using the ABI7300 system (Applied Biosystems, Streetsville, Ontario, Canada) with a total reaction volume of 25µl and the data were analyzed using the SDS analysis software of Microsoft Excel. The rest of the reactions were performed at 12.5µl total volume and were ran and analyzed on the Eppendorf Realplex2 system (Eppendorf, Canada). All reactions were performed in duplicate and the readings were normalized to β-actin levels for the corresponding sample. A list of the primers used for Q-PCR analysis can be found in Table1 in Appendix A.

2.2.6 Statistical analysis

Statistical differences between means of data from independent differentiations were calculated using the Student's t-test. P-values of at least ≤ 0.05 were considered significant.

2.3 Results

2.3.1 Sox7 enhances skeletal myogenesis in the absence of RA and neurogenesis in the presence of RA in P19 cells

Previous studies revealed that Sox7 is expressed during the precursor stage of myogenic differentiation in P19 cells and that Sox7 is sufficient to induce the entire skeletal myogenesis pathway in aggregated P19 cells in non-muscle inducing conditions [112]. Other studies in our lab have revealed that RA can enhance skeletal muscle formation in muscle-inducing conditions in P19 cells [134]. To determine if RA can effect Sox7 function, cell lines that stably expressed Sox7, termed P19[Sox7], were differentiated in the absence and presence of RA. Using immunofluorescence with an anti-MHC antibody, we were able to detect an increase in skeletal myocyte formation in P19[Control] cells in the presence of RA as well as an increase in skeletal myocyte formation in DMSO treated P19[Sox7] cell as compared to P19[Control], as expected (Figure 2.1, A-B). However RA caused a decrease in the proportion of P19[Sox7] cells that differentiated into MHC-positive skeletal myocytes (Figure 2.1, B). Given that RA can also induce formation of neurons in P19 cells [239], and that Sox7 plays a role in early germ layer specification [240], we investigated the possibility of the formation of neurons using an anti-neurofilament 68 antibody. We noticed an increase in neurogenesis in RA-treated P19[Sox7] cells as compared to P19[Control] cells (Figure 2.1, C-D). No neurons were formed when P19[Sox7] or P19[Control] cells were differentiated in the absence of RA with DMSO alone. Thus, Sox7 enhanced skeletal myogenesis in the absence of RA and neurogenesis in the presence of RA, suggesting that the interplay between RA and Sox7 changes the cell fate during differentiation in P19 cells.

To examine the molecular basis of the effect of RA, total RNA from differentiated P19[Control] and P19[Sox7] was harvested on day9. Q-PCR was used to measure the changes in gene expression that were induced by the presence of Sox7 and RA. Sox7 was overexpressed by 89-fold (± 13 , n=4) on day 0 in P19[Sox7] cell lines (Figure 2.2, A). When cells were differentiated in the presence of DMSO, a significant increase in the expression of MyoD, Myogenin, Myf5 and Cardiac α -actin, markers of myoblast and myocyte formation, was observed in aggregated P19[Sox7] cells compared to control cells (Figure 2.2, B-E). This finding is in agreement with the published results [112]. However when the cells were differentiated in the presence of both DMSO and RA, a significant decrease was observed in the mRNA expression of all the MRFs (Figure 2.2, B-D). The Q-PCR analysis revealed a significant 40-fold (± 7 , n=4) increase in the mRNA levels of NeuroD, a neuronal marker, in aggregated P19[Sox7] cells compared to a 10-fold (± 5 , n=4) upregulation in P19[Control] cell (Figure 2.2, F) by RA. Therefore Sox7 enhances the marker genes for myogenesis in the absence of RA, while it increases those for neurogenesis in the presence of RA.

2.3.2 Sox7 is necessary for efficient skeletal myogenesis in the absence of RA

In order to assess the role of endogenous Sox7 and whether it is essential to regulate the expression of premyogenic factors and myogenic regulatory factors during skeletal myogenesis, RNA interference was used to knockdown the expression of Sox7. Two P19[ShSox7] and P19[ShScrambled] cell lines were generated which stably express a unique short-hairpin construct targeting Sox7 and a scrambled sequence with no homology to any sequence in the mouse genome, respectively. All cell lines were

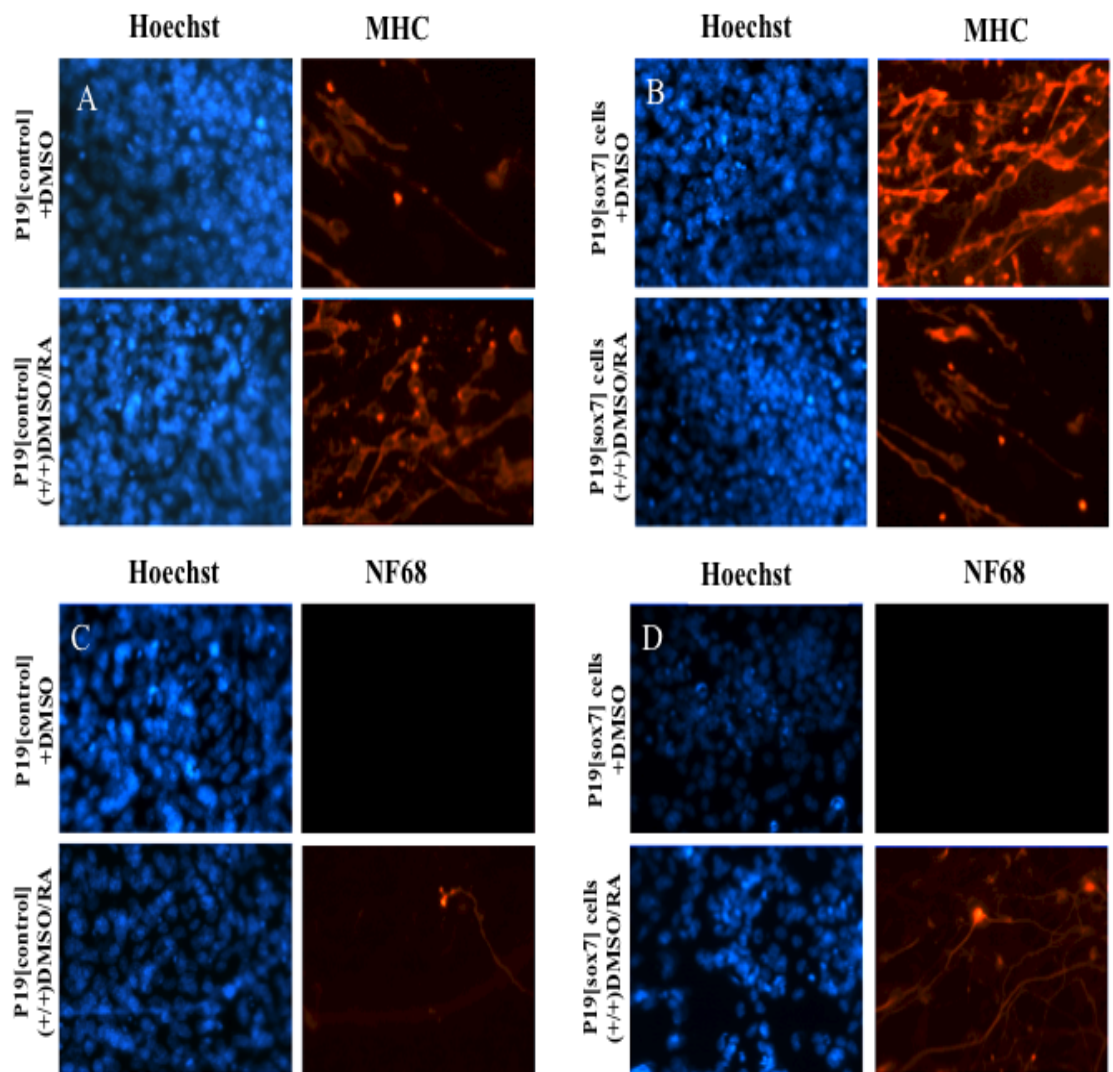


Figure 2.1- Retinoic acid inhibits myogenesis and induces neurogenesis in P19[Sox7] cells . The morphology of the cells is visualized by immunofluorescence for P19[Control] and P19 [Sox7] cell lines on day 9 of differentiation. The presence of skeletal myocyte was confirmed by using an antibody against MHC, and nuclei were visualized with Hoechst (A-B). The presence of neurons was detected by using anti-NF68 antibody (C-D). Images are shown at 400X magnification.

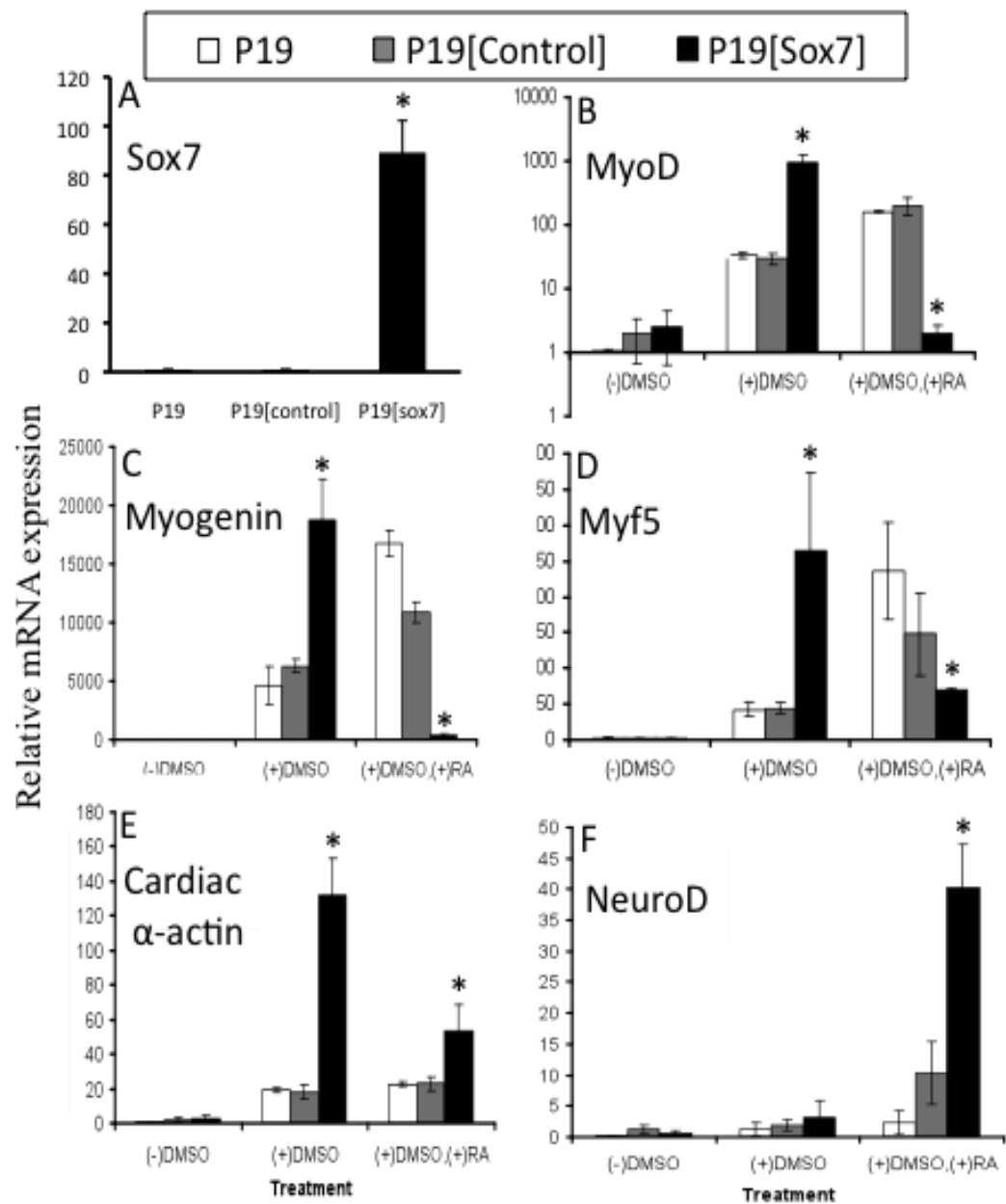


Figure 2.2- Retinoic acid downregulates myogenesis and upregulates neurogenesis in P19[Sox7] cells. P19, P19[Control] and P19[Sox7] were differentiated in the absence and presence of RA. mRNA level of Sox7 in P19[Sox7] cells was measured on day0 (A). Total RNA was harvested on day 9 of differentiation, and transcript levels of various myoblast/muscle factors (B-E) as well as NeuroD (F) were quantified using Q-PCR. Data was expressed relative to Day 0 of P19[Control] cells and was normalized to GAPDH. Error bars represent average \pm SEM of 2 P19[Control] and 2 P19[Sox7] clones from 2 independent differentiations (n=4,* p<0.05).

generated from pooled clones and due to similarity of their behaviour, both P19[ShSox7] cell lines and both P19[ShScrambled] cell lines were grouped as one for analysis. Q-PCR analysis of gene expression revealed a 60% (\pm 14%, n=4) knockdown of Sox7 on day 5 in P19[ShSox7] cells when compared to P19[ShScrambled] cells in DMSO-induced differentiation (Figure 2.3, A). Further analysis of gene expression in P19[ShSox7] cells revealed an 85% (\pm 4%, n=4) decrease in mRNA levels of the mesoderm marker Brachyury-T as compared to the control cell lines (Figure 2.3, B). This observation is in agreement with previously published results indicating Sox7 positively regulates mesoderm differentiation [240]. The expressions of muscle precursor markers Pax3, Pax7 and Meox1 were downregulated on day 5 by 76.5% (\pm 16%, n=4), 83% (\pm 9%, n=4) and 92.5% (\pm 6%, n=4), respectively (Figure 2.3, C). Given the overall downregulation of several premyogenic factors, the mRNA expression of MRFs was analyzed on day 9 and the cells were stained for MHC to determine if loss of Sox7 caused a defect in both muscle commitment and differentiation. The expression of MRFs was significantly downregulated in P19[ShSox7] cell lines by 79% (\pm 14%, n=4) for Myf5, 80% (\pm 8%, n=4) for MyoD and 85% (\pm 8%, n=4) for Myogenin, when compared to P19[ShScrambled] cells (Figure 2.3, D). Furthermore, an overall decrease in the number of skeletal myocytes was observed in P19[ShSox7] cells compared to the control cells, as observed by MHC immunostaining (Figure 2.3, E). Quantification of the number of MHC-positive cells revealed a 2.8-fold decrease in skeletal muscle formation (Figure 2.3, F). Therefore Sox7 is necessary for skeletal muscle formation in P19 cells aggregated in the absence of RA.

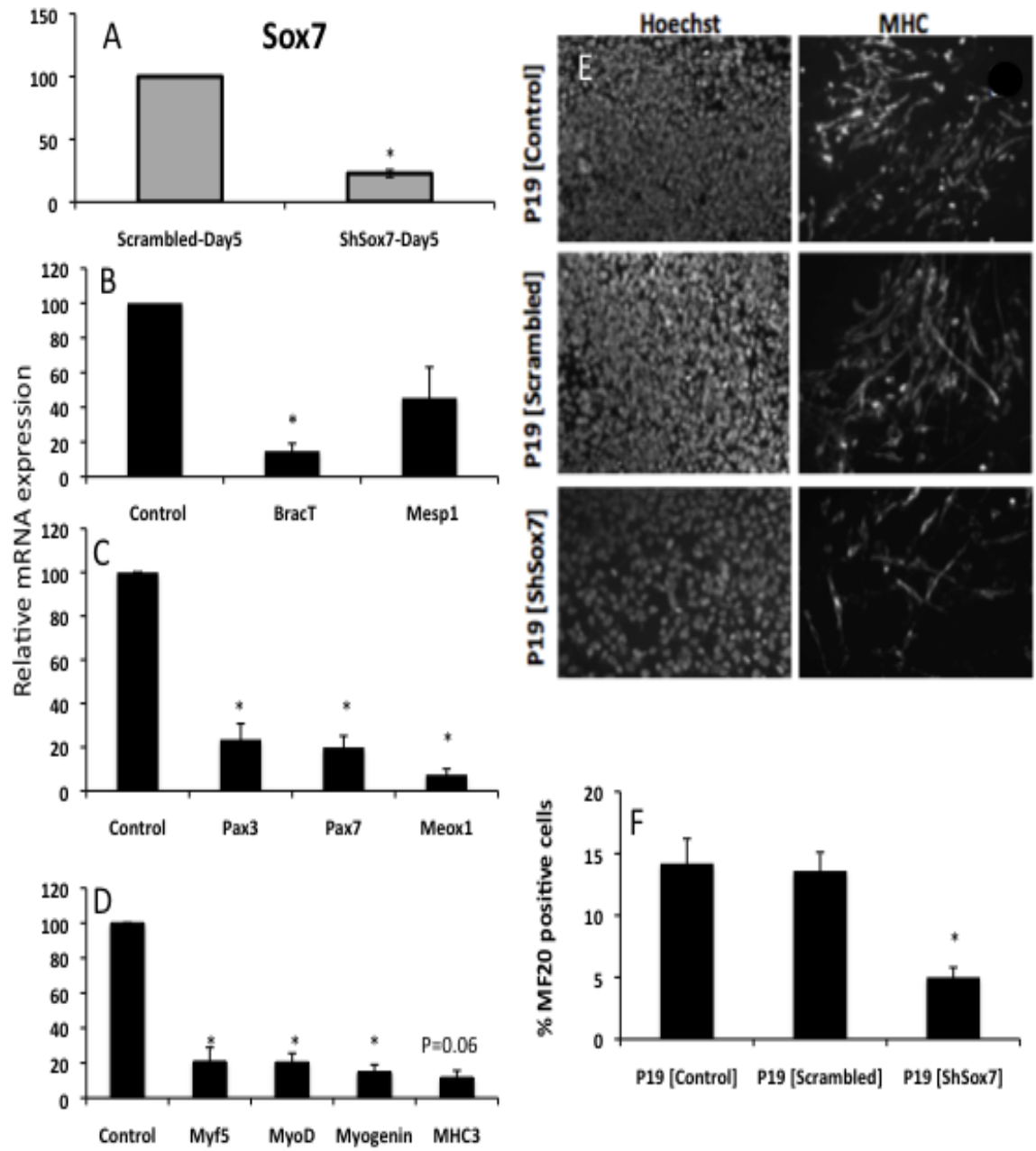


Figure 2.3- Expression of muscle precursor genes, and myogenic regulatory factors (MRFs) is downregulated in P19 [ShSox7] cell lines in the absence of RA. P19 cells were differentiated in the presence of 1% DMSO, and RNA was harvested from days 0, 5 and 9. Q-PCR analysis of gene expression was used to measure the relative mRNA levels of Sox7 (A), mesoderm markers (B) muscle precursor genes (C) on day 5 of differentiation and MRFs (D) on day 9. Immunofluorescence was performed by fixing the cells on day 9 of differentiation and staining with an anti-MHC antibody (E). Five fields of view per coverslip (in duplicates) were counted for two P19[ShSox7] cell lines, two P19[Scrambled] cell lines and P19[Control] cells (F). Changes in gene expression patterns were calculated using the comparative Ct method. Results were normalized against the internal control beta-actin, and are expressed as a percentage of control. Bars represent average \pm SEM, n=4. Statistical analysis versus control at each time point was carried out using the Student's T-test (*p<0.05)

2.3.3 Loss of endogenous Sox7 expression results in activation of skeletal myogenesis in the presence of low levels of RA through induction of mesoderm

Based on the previous observation regarding the interplay of RA and Sox7 in cell fate determination during P19 differentiation (Figure 2.1), we were interested to investigate the effects of RA on P19 myogenesis when Sox7 expression is knocked down. Two P19[ShSox7] cell lines and two P19[ShScrambled] cell lines were differentiated in the presence of DMSO and RA and each pair was grouped as one for analysis. Q-PCR analysis revealed a 62% (\pm 6%, n=10), a 62% (\pm 9%, n=8) and a 25% (\pm 6%, n=8) knockdown of Sox7 transcripts on days 5, 7 and 9, respectively, in P19[ShSox7] cells when compared to the control cells (Figure 2.4, A). Sox7 expression was not detected on day 0. Furthermore, by western blot analysis, Sox7 protein expression in P19[ShSox7] cells decreased on day 5 and day 9 when compared to the control cells (Figure 2.4, A). Expression of the muscle precursor marker, Pax3, showed a 1.9-fold (\pm 0.4, n=10) increase in the P19[ShSox7] cells treated with RA (Figure 2.4, B). Although the same trend was observed for the other premyogenic factor, Meox1, the increase in its mRNA level in the differentiated P19[ShSox7] cells was not significant when compared to the P19[Scrambled] cells (Figure 2.4, B). To investigate if increased Pax3 levels translated to change in myogenesis, P19[ShSox7] and P19[Scrambled] cells were harvested on day 9 of differentiation with RA for Q-PCR analysis of MRFs as well as the structural gene MHC3 and were stained for MHC for detecting muscle formation. Although the expression of all the MRFs showed a trend of upregulation in P19[ShSox7] cells, only Myf5 expression was significantly upregulated, 4.9-fold (\pm 2, n=8) (Figure 2.4, C).

An increase in the population of MHC-positive cells was observed in

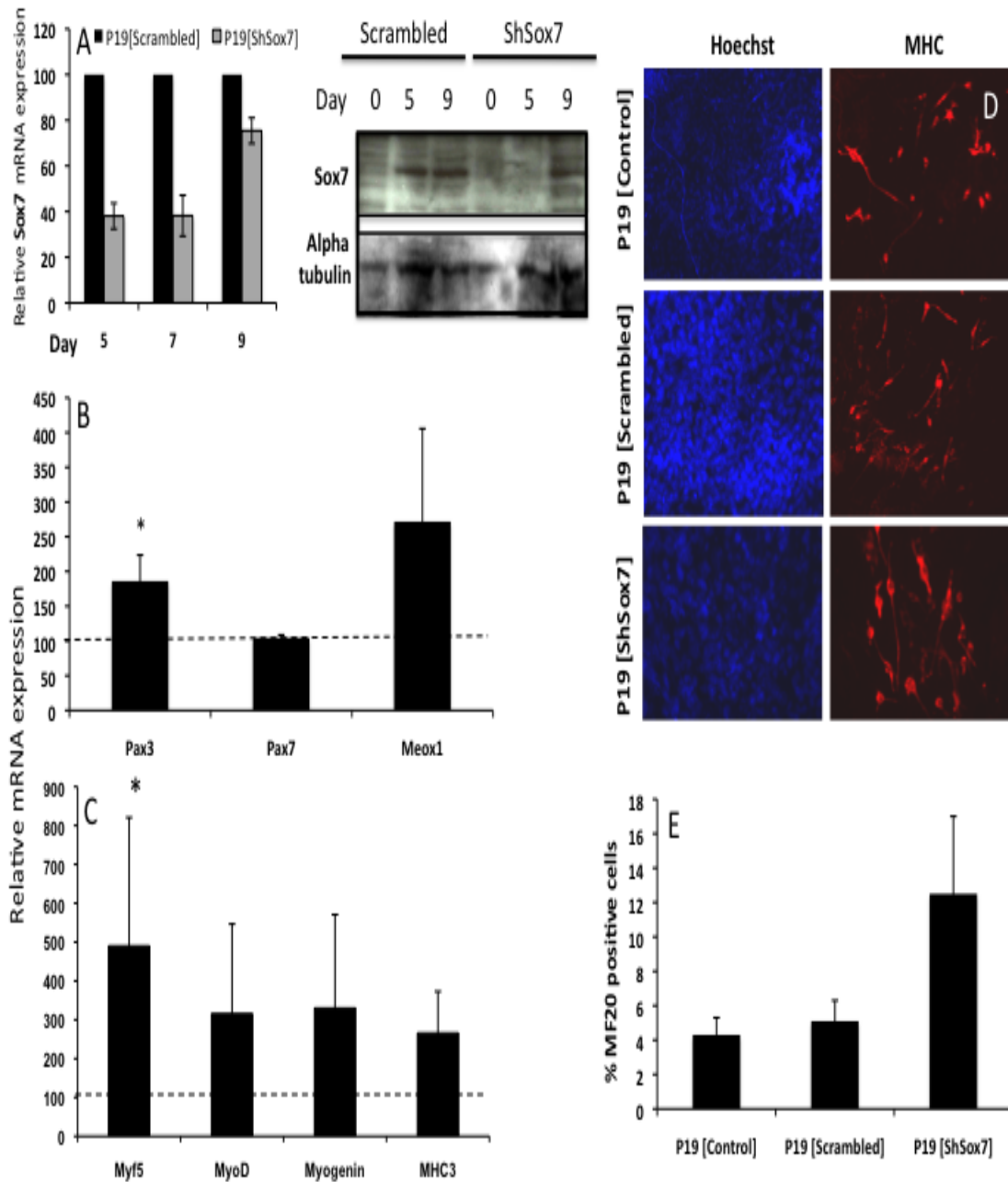


Figure 2.4- Expression of muscle precursor genes, and myogenic regulatory factors (MRFs) was upregulated in P19 [ShSox7] cell lines in the presence of RA. P19 cells were differentiated in the presence of 1% DMSO + 3nM retinoic acid, and RNA was harvested from days 0, 5 and 9. Q-PCR analysis of gene expression was used to measure the relative mRNA levels of Sox7 (A), muscle precursor genes (B) and MRFs (C). Immunofluorescence was performed by fixing the cells on day 9 of differentiation and staining with an anti-MHC antibody (D). Eight fields of view per coverslip (in duplicates) were counted for two P19[ShSox7] cell lines, two P19[Scrambled] cell lines and P19[Control] cells (E). α -tubulin was used as a loading control in panel A. Changes in gene expression patterns were calculated using the comparative Ct method. Results were normalized against the internal control beta-actin, and are expressed as a percentage of maximum expression observed for each differentiation. Bars represent average \pm SEM, n= 5 independent differentiations for two different P19[ShSox7] giving an overall n of 10. Statistical analysis versus control at each time point was carried out using the Student's T-test (*p<0.05, **p<0.005).

P19[ShSox7] cells compared to the P19[Scrambled] and P19 cells (Figure 2.4, D). However, quantification of the number of MHC-positive cells did not reveal a significant difference in the overall skeletal muscle formation (Figure 2.4, E). Variation in serum lots [206] and/or RA lots may explain the differences observed in the extent of upregulation of the precursor genes, MRFs and MHC. Thus there was a trend for enhanced skeletal myogenesis and no evidence of inhibition of myogenesis during differentiation of P19[ShSox7] cells in the presence of RA.

Given that expression of some of the muscle precursor markers and MRFs were upregulated in P19[ShSox7] cells when they were differentiated in the presence of RA, we investigated if this upregulation initiated at the level of mesoderm formation. Q-PCR analysis of mesoderm markers on day 1 revealed that expression of the mesoderm marker Brachyury-T increased by 20-fold (± 7 , $n=4$) in P19[ShSox7] cells as compared to P19[Scrambled] cells (Figure 2.5, A). Expression of the other mesoderm marker *Mesp1* also showed the same trend, however the upregulation was not significant. Previous studies have demonstrated that *Sox7* plays a role in germ layer specification during embryonic development in *Xenopus* [240]. To identify whether *Sox7* regulates the differentiation of endoderm and ectoderm, while increasing the expression of mesoderm marker Brachyury-T, RNA from differentiated P19[ShSox7] and P19[Scrambled] cells was harvested on day 5. Expression of endoderm markers GATA4 and FoxA2 decreased by 69.5% ($\pm 12\%$, $n=6$) and 75% ($\pm 13\%$, $n=6$), respectively (Figure 2.5, B). This is consistent with the published results indicating *Sox7* is required for GATA4 induction during parietal endoderm differentiation in F9 EC cells [105]. Furthermore the expression of ectoderm markers *Sox2* and *Noggin* was downregulated by 73.5% ($\pm 8\%$, $n=6$) and

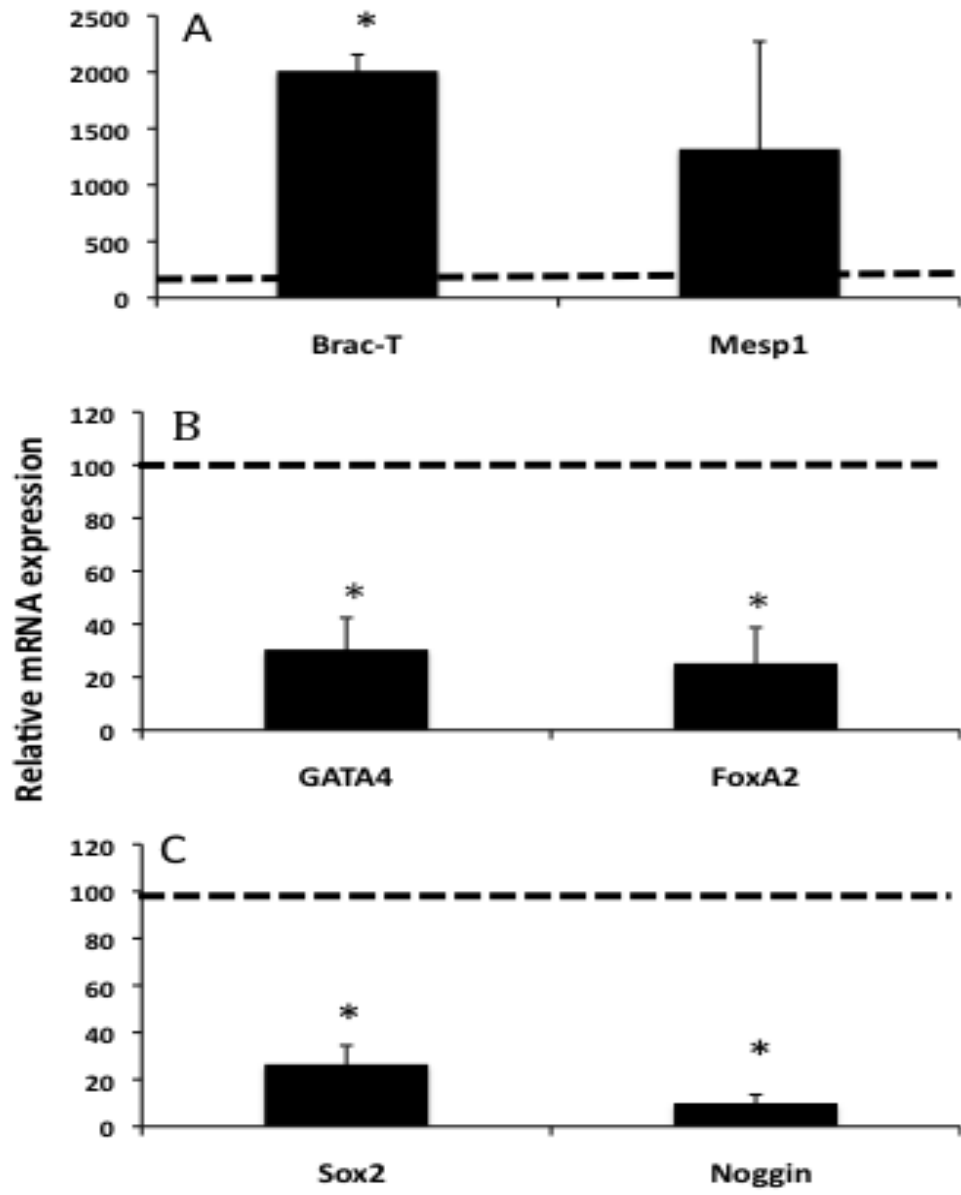


Figure 2.5- Expression of mesoderm marker BracuryT was upregulated in P19 [ShSox7] cell lines in the presence of RA while expression of endoderm and ectoderm markers was downregulated. P19 cells were differentiated in the presence of 1% DMSO + 3nM retinoic acid, and RNA was harvested from days 0 and 5. Q-PCR analysis of gene expression was used to measure the relative mRNA levels of mesoderm markers (A), endoderm markers (B) and ectoderm markers (C). Changes in gene expression patterns were calculated using the comparative Ct method. Results were normalized against the internal control beta-actin, and are expressed as a percentage of maximum expression observed for each differentiation. Bars represent average \pm SEM, n=3 independent differentiations for two different P19[ShSox7] giving an overall n of 6. The 100% line represents the P19[Scrambled] mRNA level. Statistical analysis versus control at each time point was carried out using the Student's T-test (*p<0.05, **p<0.005).

90% ($\pm 4\%$, $n=6$) in P19[ShSox7] cells when compared to P19[Scrambled] cells (Figure 2.5, C). These results indicate that loss of Sox7 in the presence of low levels of RA enhances mesoderm induction and inhibits endoderm or ectoderm formation in P19 cells.

2.3.4 Sox7 is necessary for efficient neurogenesis in the presence of RA

Given that overexpression of Sox7 enhances neurogenesis in P19 cells in the presence of RA (Figure 2.1) and loss of Sox7 results in downregulation of ectoderm differentiation (Figure 2.5 C), we further investigated the role of Sox7 in P19 neurogenesis. P19 cells, P19[ShSox7] and P19[Scrambled] cells were harvested and their total mRNA was isolated on day 9 for Q-PCR analysis of neuronal marker NeuroD and the cells were stained with an anti-neurofilament 68 antibody, NrF4. Immunostaining illustrated an overall decrease in the number of NrF-positive cells in P19[ShSox7] cells (Figure 2.6, A). Quantification of these cells revealed a 1.7-fold ± 0.3 and 1.6-fold ± 0.3 decrease in the number of NrF-positive cells in P19[ShSox7] cells when compared to P19 cells and P19[Scrambled] cells, respectively (Figure 2.6, B). Furthermore the expression of NeuroD mRNA was downregulated by 19% ($\pm 6\%$, $n=4$) in P19[ShSox7] cells as compared to the control cells (Figure 2.6, C). Therefore reduction of Sox7 results in downregulation of neurogenesis when P19 cells are differentiated in the presence of RA.

2.3.5 Sox17 and Sox18 do not compensate for loss of Sox7 during skeletal myogenesis

Previous publications have demonstrated that members of the subgroup F of Sox family of transcription factors play a redundant role during cardiovascular development

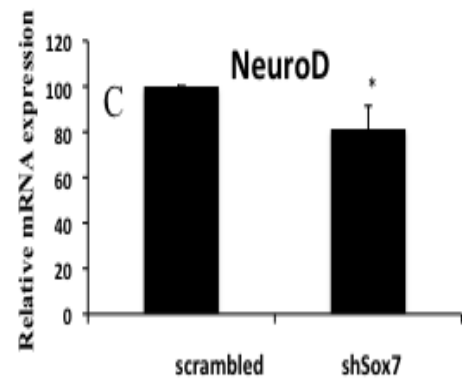
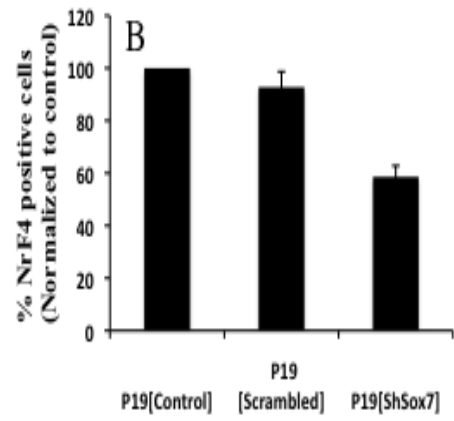
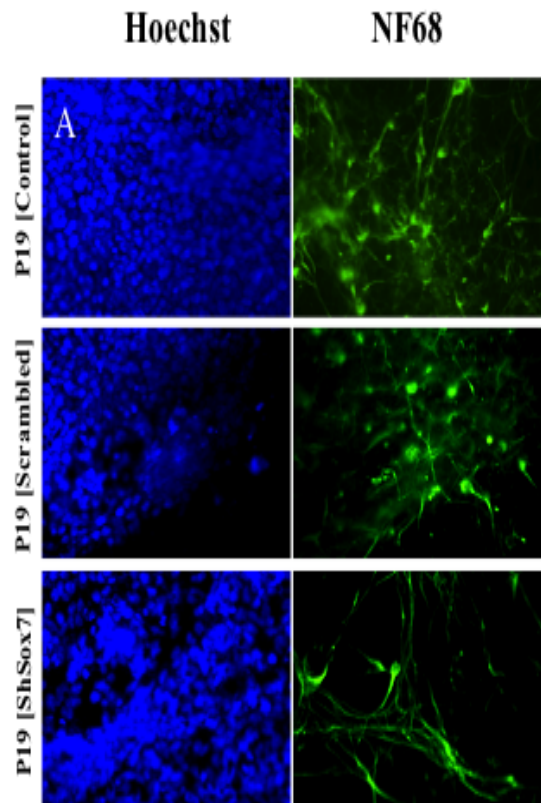


Figure 2.6- Loss of endogenous Sox7 results in downregulation of neurogenesis in the presence of RA. P19, P19[Scrambled] and P19[ShSox7] cells were differentiated in the presence of 1% DMSO + 3nM retinoic acid. Cells were lysed on day 9 for immunofluorescence analysis and total mRNA was harvested on day 9 for Q-PCR analysis. Neurons were visualized using an anti-neurofilament 68 antibody, NrF4 (A). Three fields of view per coverslip were counted for two P19[ShSox7] cell lines, two P19[Scrambled] cell lines and P19[Control] cells (B). mRNA levels of NeuroD were quantified in P19[ShSox7] cell lines using Q-PCR method (C). Changes in gene expression patterns were calculated using the comparative Ct method. Results were normalized against the internal control beta-actin, and are expressed as a percentage of P19[Scrambled] cells. Bars represent average \pm SEM, n=3 independent differentiations. Statistical analysis was carried out using the Student's T-test (*p<0.05).

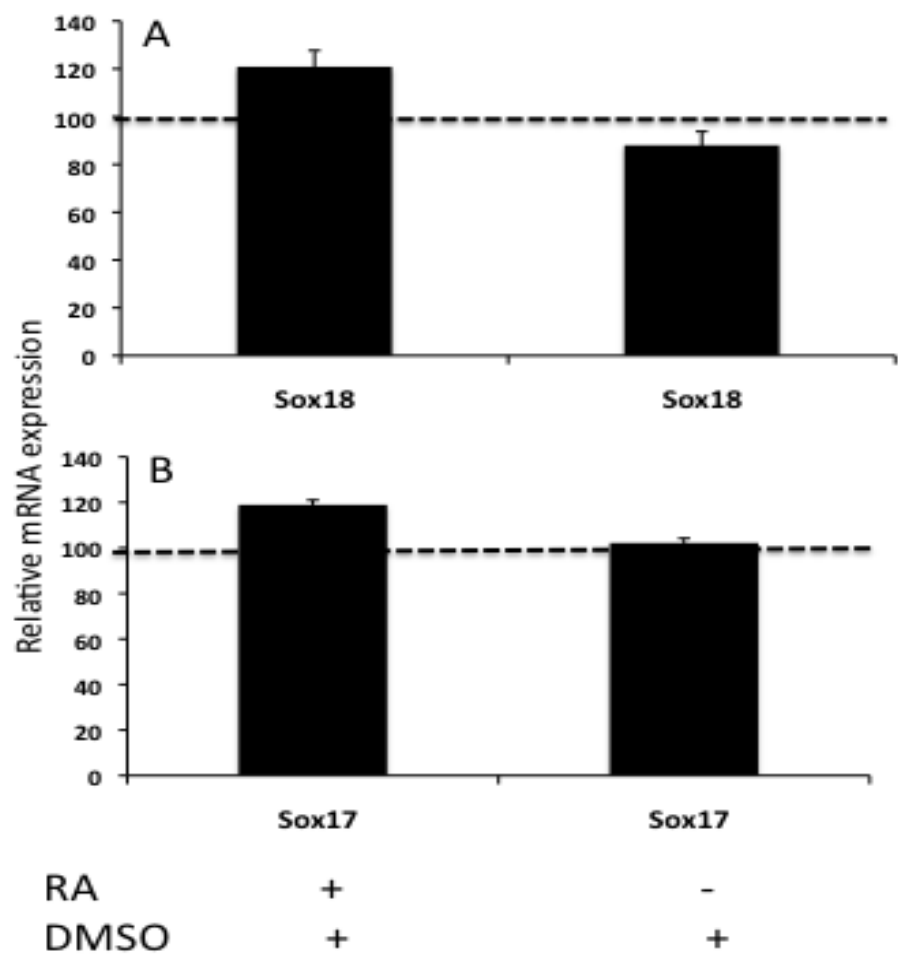


Figure 2.7- Knockdown of Sox7 does not alter expression of Sox17 and Sox18 during P19 differentiation. P19[ShSox7] and P19[Scrambled] cell lines were differentiated in the presence of 1% DMSO +/- 3nM retinoic acid, and RNA was harvested on day 0 and 5. Q-PCR analysis of gene expression was used to measure the relative mRNA levels of the genes. Changes in gene expression patterns were calculated using the comparative Ct method. Results were normalized against the internal control beta-actin, and are expressed as a percentage relative to P19[Scrambled] day 0. Bars represent average \pm SEM, of three independent differentiations for two clones of both ShSox7 and Scrambled. Statistical analysis was carried out using the Student's T-test.

in various organisms [108, 109, 234, 235]. We were interested in investigating the possibility of compensation by other SOXF factors, given the contrasting effect of loss of Sox7 on skeletal myogenesis in the presence and absence of RA. Q-PCR analysis of Sox17 and Sox18 mRNA levels on day 5, revealed no alteration in the expression of these genes in the P19[ShSox7] cells when compared to P19[Scrambled] cells (Figure 2.7 A-B). Treatment with RA did not have any effect on the expression of these genes during the differentiation of P19[ShSox7] as compared to P19[Scrambled] cells. Thus, the expression of other SOXF factors does not change when Sox7 is knocked down during +/- RA P19 differentiation, indicating the observed phenotypes were not due to compensation by the other SOXF genes.

2.3.6 Retinoic acid accelerates the expression of SOXF genes but not RA receptors

Our lab has previously demonstrated that treatment with a range of RA concentrations from 0.5-30nM is sufficient to enhance and accelerate skeletal myogenesis, while suppressing cardiomyogenesis [134]. Sox7 is found to be an RA-responsive gene whose expression is enhanced and accelerated early during differentiation in RA-treated cells (Savage, unpublished data) suggesting that Sox7 may lie downstream of retinoic acid signalling during skeletal muscle differentiation in P19 cells. In accordance with the studies of Savage et al. (unpublished observations) we showed that Sox7 expression peaked on day 3 following RA stimulation, 2 days earlier than the maximal peak observed on day 5 for cells treated with DMSO only (Figure 2.8, A). Similarly, the expressions of other members of subgroup F of Sox family were accelerated upon treatment of cells with RA. Sox17 expression peaked on day 3 and

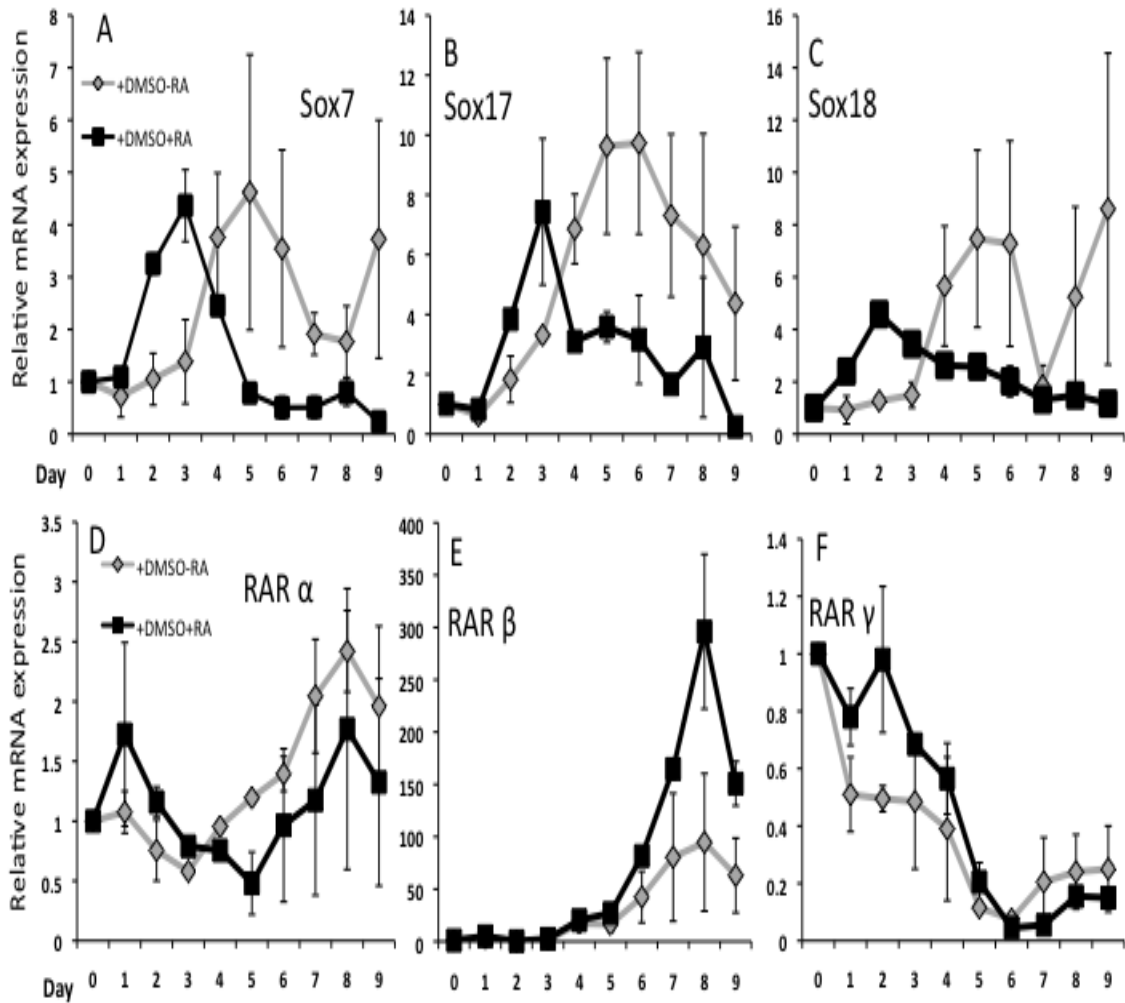


Figure 2.8- Retinoic acid precipitates expression of Sox7, Sox17 and Sox18 but not retinoic acid receptors. P19 cells were differentiated in the presence of DMSO or DMSO+/-3nM retinoic acid, and RNA was harvested on day 0-9. Q-PCR analysis of gene expression was used to measure the relative mRNA levels of Sox7 (A), Sox17 (B), Sox18 (C), RAR-alpha (D), RAR-beta (E) and RAR-gamma (F). Changes in gene expression patterns were calculated using the comparative Ct method. Results were normalized against the internal control beta-actin and are expressed relative to day 0. Bars represent average \pm SEM of two independent differentiations for each condition.

Sox18 expression peaked on day 2 in RA-treated cells, 3 days earlier than their peaks observed during DMSO alone-treated differentiation (Figure 2.8 B-C). To confirm that the changes in the expression of gene expression observed following RA treatment were correlated with the myogenic pathway, we assessed levels of MyoD and Myogenin transcripts using Q-PCR. Treatment with 3nM RA increased the expression of both MyoD and Myogenin on day 9 (data not shown), in agreement with previous findings from our lab. Furthermore the expression of all isoforms of RA-receptor during P19 differentiation was examined. RA did not change the mRNA level of RARs notably (Figure 2.8 D-F) except for RAR β . The observed expression pattern of RAR isoforms was in agreement with the previous published results indicating RAR α and RAR γ are constitutively expressed in P19 cells while RAR β expression is upregulated during DMSO-induced P19 differentiation [218]. Our studies here provide a more detailed analysis of SOXF and RAR expression in the presence and absence of RA.

2.3.7 Sox7 is sufficient but not necessary for RAR β expression

Our previous observations revealed that treatment of P19 cells with RA accelerates Sox7 expression and that either overexpression of Sox7 or knockdown of endogenous Sox7 has an effect on the cell fate determination of the RA-treated P19 cells. To further analyze the possible regulation of RA function by Sox7, we examined the expression of RA receptors during differentiation of P19[ShSox7] cells and P19[Scrambled] cells. We were interested to find out whether Sox7 modulates the upregulation of RARs in the presence or absence of RA during P19 skeletal myogenesis. The expression of RAR α , RAR β and RAR γ on day 5 did not change in the P19[ShSox7]

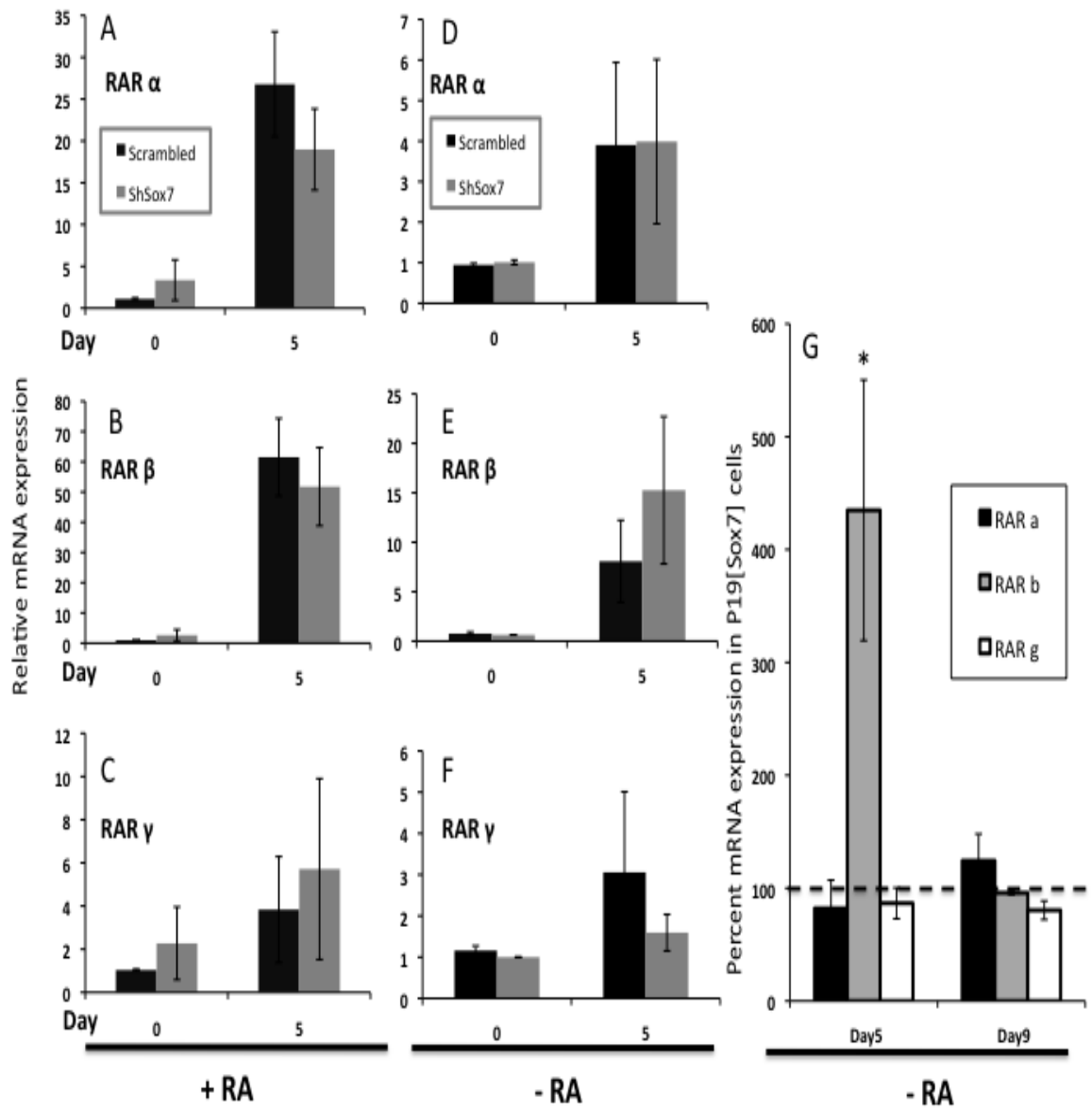


Figure 2.9- Sox7 is sufficient but not necessary for RAR β expression during P19 differentiation. P19[ShSox7], P19[Scrambled] and P19[sox7] cell lines were differentiated in the presence of 1% DMSO +/- 3nM retinoic acid, and RNA was harvested on day 0, day5 or day 9. Q-PCR analysis was used to measure the relative mRNA levels of the genes. Changes in gene expression patterns were calculated using the comparative Ct method. Results were normalized against the internal control beta-actin, and are expressed as a fold change relative to day 0 (A-F) and as a percentage of expression relative to P19 cells (G). Bars represent average \pm SEM, of three independent differentiations for two clones of both ShSox7 and Scrambled and three independent differentiations of P19[Sox7]. Statistical analysis was carried out using the Student's T-test (*p<0.05).

cells when compared to P19[Scrambled] cells as revealed by Q-PCR analysis (Figure 2.9 A-F). This observation is consistent with the previously published results showing that upregulation of RAR β was not abolished during RA-induced differentiation of Sox7-silenced F9 EC cells [105]. Further, it indicates that Sox7 is not essential for RAR β expression. Interestingly, however, overexpression of Sox7 resulted in upregulation of RAR β on day 5 of differentiation of P19 cells in the absence of RA (Figure 2.9, G). This finding indicates that Sox7 is sufficient to enhance RAR β expression in the absence of RA. Therefore, it seems that the ability of Sox7 to modulate RA function could be regulated, in part, by the expression of RAR β .

2.4 Discussion

In this study, we have demonstrated that retinoic acid can modulate the role of Sox7 during differentiation of P19 EC cells. Overexpression of Sox7 results in enhanced skeletal myogenesis and loss of Sox7 results in a defect in skeletal myogenesis in P19 cells in the absence of RA, indicating that Sox7 is both sufficient and necessary for skeletal myogenesis without RA treatment. Aggregation of Sox7 over-expressing cell lines with RA results in loss of skeletal myogenesis while enhancing neurogenesis. Furthermore, exposure to RA of cell lines in which Sox7 expression is knocked down results in activation of skeletal myogenesis through induction of mesoderm while negatively effecting neurogenesis. Expression of Sox7 and the other members of its subfamily Sox17 and Sox18 is accelerated following exposure to RA. However, Sox17 or Sox18 do not compensate for the loss of Sox7 during skeletal myogenesis in P19 cells. Finally, neither RA treatment nor Sox7 knockdown changed the RNA expression of RA-

receptors. Taken together using gain- and loss-of function approaches, RA seems to modulate Sox7 expression and function, whose mechanisms remain to be discovered.

Previously, it had been shown that RA enhanced and accelerated myogenesis in EC cells [130]. This was supported by the data from our lab demonstrating that treatment with RA resulted in upregulation of expression of Pax3, Meox1, MyoD and Myogenin [134] and also Sox7 (Savage and Skerjanc, unpublished observations). Our lab has demonstrated that Sox7 is also sufficient to enhance myogenesis in P19 cells by upregulating the expression of Pax3 and Meox1 as well as the MRFs [112]. Given the early expression of Sox7, it was speculated that Sox7 could be responsible for mediating the upregulation of muscle precursor genes, and MRFs, brought on by RA. Contrary to our initial expectation, overexpression of Sox7 downregulated MRFs when the cells were treated by RA and loss of Sox7 did not inhibit RA-directed myogenesis. To understand these results we examined the possibility of a change in cell fate specification mediated by the combination of Sox7 and RA. The diversion of cell phenotype from skeletal myocytes to neurons suggests a change in the activation of cascades of gene expression. It is well known that increasing the concentration of RA can switch cell fate from skeletal muscle to neurons during P19 cell differentiation [208], although the mechanism is not clearly understood.

RA-induced activation of RARs allows for recruitment of co-activators at the RA-response element (RARE) of target genes. Sox7 might act as a sensor that directs RA towards a different set of RAREs, modulating the genes expressed, dependent upon the relative levels of RA bound to RARs and Sox7. We cannot disregard the possibility that the RA-bound RAR/RXR heterodimer may bind to the regulatory regions of myogenic

genes, but is prevented from positively regulating transcription by some repressor factors. Further experiments are required to understand the complex mechanisms involved in the switch of cell fate by changes in the levels of Sox7 and RA.

Loss of Sox7 resulted in upregulation of Brachyury-T, Pax3 and Myf5 and MHC3 following exposure to RA but not in its absence. The upregulation of the mesodermal factor, Brachyury-T suggests that Sox7 might have a role during early mesoderm formation as well as during skeletal myogenesis. Switches in cell fate caused by Sox7/RA could be due to changing transcript levels of the RARs. However, in the absence of Sox7, no changes were identified in RAR transcript levels. In contrast, our analysis revealed that Sox7 overexpression increases the transcript levels of RAR β , suggesting a possible mechanism that Sox7 overexpression could modulate the sensitivity of cells to RA concentration. It is known that in both mouse and human there is one evolutionarily conserved RARE approximately 25kb upstream of Sox7 transcriptional start site. The possibility that the RARs bind the Sox7 protein is currently being investigated in our lab. We also demonstrated that the expression of other members of the subgroup F of the Sox family of transcription factors does not change when Sox7 is knocked down, excluding the redundancy mechanism.

In summary, the data presented here have provided insight into the role of Sox7 during RA-mediated induction of myogenesis in P19 cells. We have shown that Sox7 is necessary for skeletal myogenesis in the absence of exogenous RA, but not in its presence. Further, Sox7 in the presence of RA is necessary and sufficient for efficient neurogenesis. Understanding the complex interactions between Sox7 and RA signalling is important for enhancing our knowledge of mechanisms regulating skeletal myogenesis.

CHAPTER 3

Effect of a Collagen-Based Matrix on Skeletal and Cardiac Muscle Development in Mouse Embryonic Stem Cells

3.1 Introduction

Diseases of cardiac and skeletal muscle are the leading causes of death in developed countries. These medical conditions include ischemic heart disease, spinal muscular atrophy (SMA) and Duchenne muscular dystrophy (DMD), all characterized by irreversible muscle degeneration. Aging is another cause of muscle wasting and degeneration. Despite major advances in medicine, there are no current therapies that allow for cardiac and skeletal muscle regeneration. Recent attempts to cure muscle diseases using stem cell therapy has been somewhat promising. For example human endothelial stem cells derived from bone marrow can improve perfusion and function of myocardium when transplanted into infarcted hearts of mice [241], ES cell-derived neural stem cells can improve function and increase lifespan in a SMA mouse model [242]; and bone marrow-derived mesenchymal stem cells can improve locomotor function and prolong life when transplanted into a DMD mouse [243]. However, no optimal therapy has been found yet and the search for strategies to regenerate muscle remains significant.

There are limitations to stem cell therapy including the lack of availability of autologous cells, time needed to prepare the cells for transplantation, differentiation into desired cells, cell death and potential requirement for immunosuppressant drugs [244]. Another major problem could be attributed to the poor cell engraftment which can be improved by using implantable biomaterials [245]. To circumvent these limitations an acellular approach to tissue regeneration can be considered as an alternative. Recently, some groups have focused on using transplantable materials that can release stem cell-activating growth factors to augment tissue regeneration after ischemic damage in murine hindlimb ischemia models [246, 247]. Similarly, another group recently reported the

design of a cardiac collagen scaffold that could enrich for endogenous progenitor cell populations by presenting antibodies recognizing specific cell surface markers [248]. Others have focused on a less intervening strategy of using an implantable material that mediates and augments the body's natural responses. For example, the use of a Type 1 collagen-derived matrix that contained the oligosaccharide sialyl Lewis^X (sLe^X), the natural ligand for the receptor L-Selectin, has been reported [249]. L-selectin is expressed on the membrane surface of endothelial progenitor cells (EPCs) and has a role in regulating their homing and adhesion to the ischemic areas [250]. When used in a rat model of muscle ischemia, sLe^X augmented the generation of new vasculature and restored perfusion [249]. This is of importance since revascularization to regain sufficient blood supply represents a major limitation for a successful therapeutic approach to enhance muscle regeneration after ischemia [251].

Type 1 collagen is the major component of skeletal muscle extracellular matrix [252]. In the heart, the extracellular area around cardiomyocytes consists mainly of interstitial collagen I and III [253]. Collagen has been used for muscle cell development from myoblast cell culture [254] and embryonic stem cell culture [255]. In this study, we investigated the effect of sLe^X and collagen on differentiation of mouse ESCs into cardiac and skeletal muscle. Our findings highlight the importance of the extracellular matrix in differentiation of embryonic stem cells into both skeletal and cardiomyocytes. Additionally, we investigated the ability of the sLe^X-matrix to induce local tissue for muscle regeneration in ischemic hindlimb. The myogenic response after an ischemic-induced injury was assessed by observing the transcriptional profile up to 10 days post-injury. Our results suggest that sLe^X augments skeletal muscle regeneration *in vivo*.

3.2 Materials and methods

3.2.1 Cell culture

D3 mouse embryonic stem cells (mESCs) were grown and differentiated as previously described [134]. Briefly, the cells were aggregated at a density of 4×10^4 cells/ml for 2 days in hanging drops and 5 days in non-tissue culture plates (Fisher Scientific, Ottawa, Canada). On day 7, the embryoid bodies were transferred to standard tissue culture polystyrene (TCPS) plates or tissue culture plates coated with either collagen or sLe^X-matrix. At the same time, some aggregates were transferred to coverslips coated with 0.1% gelatin (Fisher Scientific), collagen or sLe^X-matrix for later immunofluorescence analysis. On day 10, the serum was lowered to a serum containing N2 supplement and the cells were allowed to grow for 5 more days. On day 15, the cells were harvested from the TC plates and fixed on coverslips.

3.2.2 Reverse transcription and quantitative PCR

Total RNA was extracted from cells and tissues using the RNeasy Kit (Qiagen Inc., Streetsville, Canada) following the manufacturer's protocol. The first strand of cDNA was synthesized from 1 µg RNA by reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen Inc.). qPCR reactions were performed as previously described [112]. The reactions and data analysis were performed on an Ep_gradient S system (Eppendorf, Hamburg, Germany) using RealPlex version 2.2 software (Eppendorf). Relative gene expression was calculated using the comparative Ct method as described previously [112]. Results for *in vitro* and *in vivo* tissue work were normalized to β-actin and GAPDH, respectively. *In vitro* results were standardized to results obtained on TCPS; *in vivo* results were standardized to each animal's untreated, contralateral limb.

3.2.3 Western blot

Cells were harvested for total protein extract with modified RIPA buffer (50mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA, 1mM PMSF) with a protease inhibitor cocktail (Roche, Laval, Canada). Protein was separated on a 10% SDS PAGE gel in 1× Running Buffer and transferred to an Immunoblot PVDF-membrane (BioRad, Saint-Laurent, Canada). L-selectin, Pax3 and α -tubulin proteins were detected with anti-L-selectin(1:200 dilution, Abcam, Cambridge, USA), anti-Pax3 (1:300, R&D Systems, Cat# MAB2457), anti- α -tubulin (1:10000 dilution, Sigma Cat# T6199) antibodies, and were visualized with HRP-conjugated secondary antibodies. The Pax3 blot was enhanced using Western Blot Signal Enhancer (Fisher Scientific) before blocking with 5% Milk in TBST.

3.2.4 Immunofluorescence

mESCs were fixed in cold methanol and incubated with mouse anti-myosin heavy chain (MHC) monoclonal antibody MF20 (Developmental Studies Hybridoma Bank) overnight at 4°C or for an hour at room temperature. Goat anti-mouse IgG (H+L) Cy3-linked secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, USA) was used to visualize MHC positive cells as previously described [219]. Hoechst dye was used for nuclear staining. Images were acquired with an Olympus BX50 microscope using Image Pro Plus.

3.2.5 Statistical analysis

Statistical differences between means of independent differentiations were calculated using Student's t-test. P-values of ≤ 0.05 were considered significant.

3.3 Results

3.3.1 Collagen-based matrices enhance skeletal myogenesis

Previous studies have reported on the ability of collagen to yield higher levels of skeletal myogenesis from primary myoblasts in vitro [254, 256]. Furthermore, it has been shown that collagen can facilitate the differentiation of embryonic stem cells into mature cardiomyocytes [255]. We were interested to determine if the collagen-based sLe^X matrix has the ability to enhance cardiac and skeletal myogenesis in mouse embryonic stem cell (mESC) culture in vitro. Since sLe^X mediates its effect through binding to L-selectin, the expression of this cell surface receptor by mESCs was evaluated and confirmed by western blot (Figure 3.1, A). mESCs were differentiated on the collagen matrices for 8 days, after 7 days of embryoid body (EB) formation, for a total differentiation of 15 days. Using an anti-MHC antibody, we were able to detect an increase in skeletal muscle formation in the cells differentiated on collagen matrices (Figure 3.1, B-G). Quantification of the number of MHC-positive cells revealed a 2-fold (± 0.5) and a 1.6-fold (± 0.4) increase in the number of skeletal myoblasts formed on collagen and collagen+sLe^X, respectively (Figure 3.1, H). Q-PCR analysis of gene expression revealed a 2.1 fold (± 0.4 , n=5) increase in the mRNA level of the muscle precursor marker Pax7 on collagen matrix (Figure 3.1, I). Collagen matrix and sLe^X matrix increased expression of Pax3, another premyogenic marker, by 3.2 fold (± 0.2 , n=5) and 3.5 fold (± 1.0 , n=5), respectively (Figure 3.1, J). The mRNA expression of the MRFs Myogenin and Myf5 and the terminal differentiation marker MHC3 increased in cells cultured on collagen by 1.7 fold (± 0.4 , n=5), 3.3 fold (± 0.9 , n=5) and 3.1 fold (± 0.5 , n=5), respectively (Figure 3.1, N-P). SLe^X matrix also caused an increase in

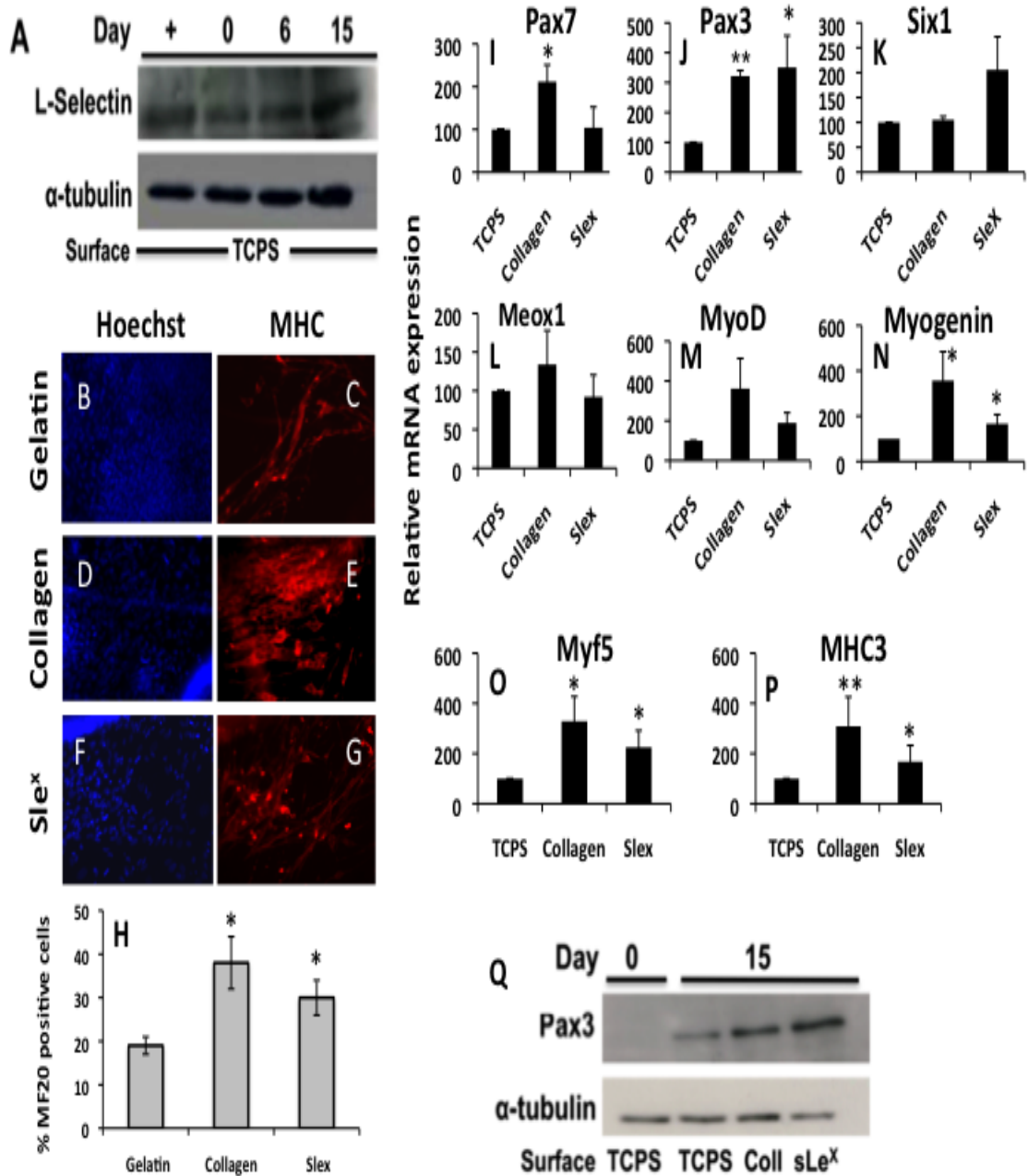


Figure 3.1- Differentiation of mouse embryonic stem cells on the collagen based matrices upregulates expression of skeletal muscle precursors and terminal differentiation markers. mES cells were aggregated in the absence of LIF for 7 days and then plated on either regular tissue culture plates, Collagen coated plates or Collagen+Sle^X coated plates. Protein was harvested on day 15 and western blot for L-Selectin was performed with peripheral blood mononuclear cells as the positive control and α -tubulin as the loading control (A). Skeletal myocytes were visualized by immunofluorescence using an anti-MHC antibody (B-G). Cell counts were performed on 3-5 fields of view (that contained cells) of three independent differentiations in duplicate (H). RNA was harvested on day 15 of differentiation and Q-PCR analysis of gene expression was used to measure the relative mRNA levels of skeletal muscle precursor genes (I-L), MRFs (M-O) and MHC3 (P). The increase in Pax3 transcripts was confirmed with Western Blot analysis (Q). Changes in gene expression patterns were calculated using the comparative Ct method. Results were normalized against the internal control beta-actin, and are expressed as a percentage of expression relative to tissue culture plates. Bars represent average \pm SEM, n= 5. Statistical analysis versus control at each time point was carried out using Student's T-test (*p<0.05, **p<0.005)

transcript levels of Myogenin (1.7 fold \pm 0.4, n=5), Myf5 (2.3 fold \pm 0.6, n=5) and MHC3 (1.7 fold \pm 0.3, n=5) (Figure 3.1, N-P). Although a trend of upregulation was observed for the mRNA level of Meox1, Six1 and MyoD on matrices, no significant differences were observed. An increase in protein level of Pax3 on the matrices was demonstrated by western blotting (Figure 3.1, Q). These results demonstrate that the matrices enhanced skeletal myogenesis in mESCs by upregulation of skeletal muscle markers.

3.3.2 Collagen enhances myotube formation

While studying the morphology of the cells by immunofluorescence, we noticed mESCs cultured on collagen formed a greater number of myofibers shaped by fusion of skeletal myocytes as compared to the ones cultured on gelatin, our standard coating for coverslips (Figure 3.2, A-D). Quantification of fused cells revealed a fusion index of 15% (\pm 2.5 %) for cells differentiated on collagen as compared to cells plated on gelatin-coated coverslips with fusion index of 1%. (Figure 3.2, E). Q-PCR analysis of fusion markers in cells cultured on collagen revealed a 1.3 fold (\pm 0.09, n=5), a 1.1 fold (\pm 0.03, n=5) and a 1.2 fold (\pm 0.06, n=5) increase in mRNA levels of CD9, CD81 and NFATc3, respectively, when compared to TCPS (Figure 3.2 F-H). Together these findings demonstrate that collagen supports the fusion of skeletal muscle myocytes into myotubes.

3.3.3. Collagen-based sLe^X matrix enhances cardiomyogenesis in mES cells

We further analyzed the effect of collagen matrices on differentiation of mESCs into cardiac myocytes using the same differentiation program as described to induce

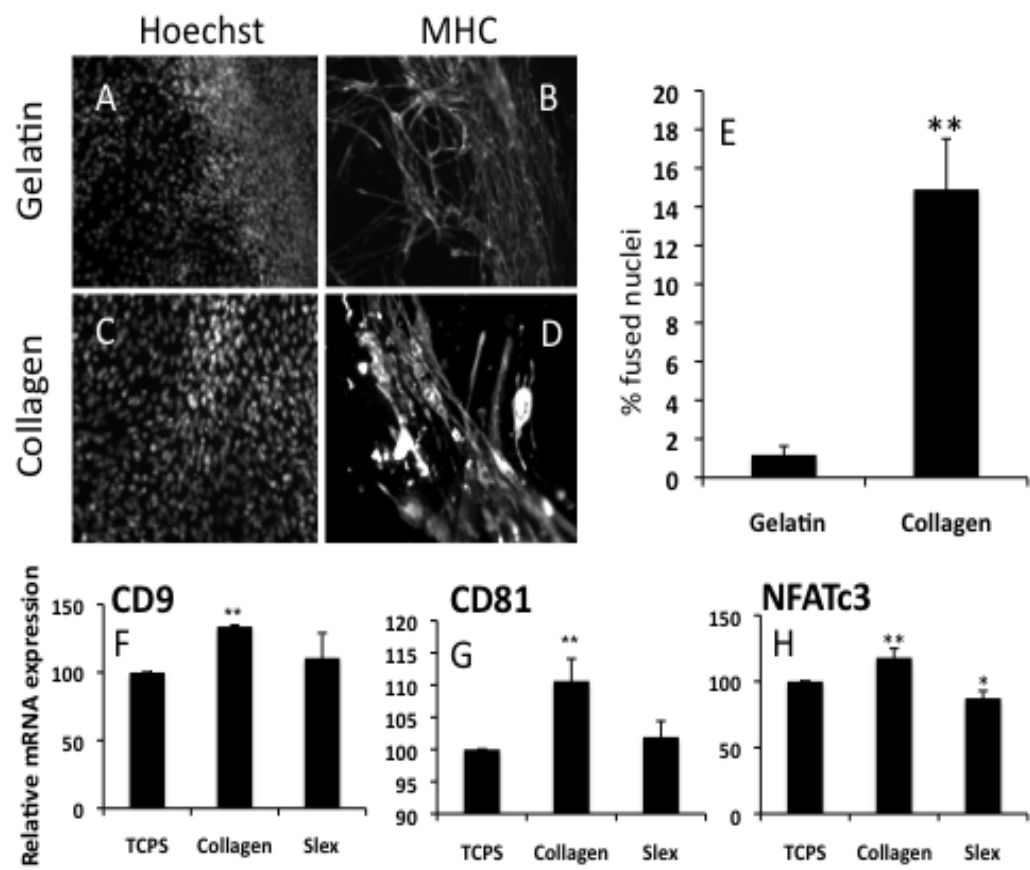


Figure 3.2- Differentiation of mouse embryonic stem cells on collagen upregulates myotube formation. mES cells were aggregated in the absence of LIF and plated on either regular tissue culture plates, Collagen coated plates or Collagen+Sle^X coated plates on day 7. Immunofluorescence confirmed the formation of myotubes on Collagen (A-D). The number of fused nuclei (where at least two cells were fused) was counted and compared to the total number of myocytes for three independent experiments performed in duplicate (E). RNA was harvested on day 15 of differentiation and Q-PCR analysis of gene expression was used to measure the relative mRNA levels of fusion markers (F-H). Changes in gene expression patterns were calculated using the comparative Ct method. Results were normalized against the internal control beta-actin, and are expressed as a percentage of expression relative to tissue culture plates. Bars represent average \pm SEM, n= 5. Statistical analysis versus control at each time point was carried out using the Student's T-test (*p<0.05, **p<0.005)

skeletal muscle. Q-PCR analysis of cardiac markers GATA4 and NKx2.5 revealed that the sLe^X-matrix increased mRNA expression of these genes by 1.9 fold (\pm 0.2, n=6) and 2.3 fold (\pm 0.4, n=5), respectively (Figure 3.3, A-B). Transcript levels of GATA4 were also increased on collagen by 1.7 fold (\pm 0.1, n=6) (Figure 3.3, A). Interestingly, the expression of the hyperpolarization-activated cyclic nucleotide-gated channel 4, HCN4, which is a maker of pace-making/nodal cells in the heart [257], was upregulated by 1.3 fold (\pm 0.1, n=5) (Figure 3.3, C). Further Q-PCR analysis of the cells differentiated on sLe^X matrix, revealed a 3.0 fold (\pm 0.9, n=5) increase in the mRNA levels of the atrial natriuretic factor (ANF), which is secreted from mature cardiomyocytes, and represents working cardiac muscle cells [258, 259] (Figure 3.3, D). No significant change in the transcript levels of MHC6, the cardiac-specific isoform of myosin heavy chain, was detected when different surfaces were compared, although an upward trend was observed (Figure 3.3, E). Both collagen and sLe^X increased the expression of cardiac α -actin by 3.2 fold (\pm 1.0, n=5) and 3.0 fold (\pm 0.8, n=5), respectively, indicating an overall increase in either skeletal or cardiac muscle formation (Figure 3.3, F).

Knowing that the sLe^X-matrix supports cardiomyogenesis by upregulating cardiac precursors and maturation markers, we were interested in analyzing the ability of the newly formed cardiomyocytes to beat. Formation of cardiomyocytes was detected on the sLe^X matrix using an anti-MHC antibody (Figure 3.3, G-H). Quantification of the number of beats per minute revealed on average 45 beats (\pm 4, n=6) on tissue culture plates, 104 beats (\pm 3, n=6) on collagen surface and 29 beats (\pm 4, n=6) on sLe^X surface (Figure 3.3, I). Further analysis of the beating area disclosed that on average 83% (\pm 10, n=3) of the field of view contained cardiomyocytes that were beating on the sLe^X

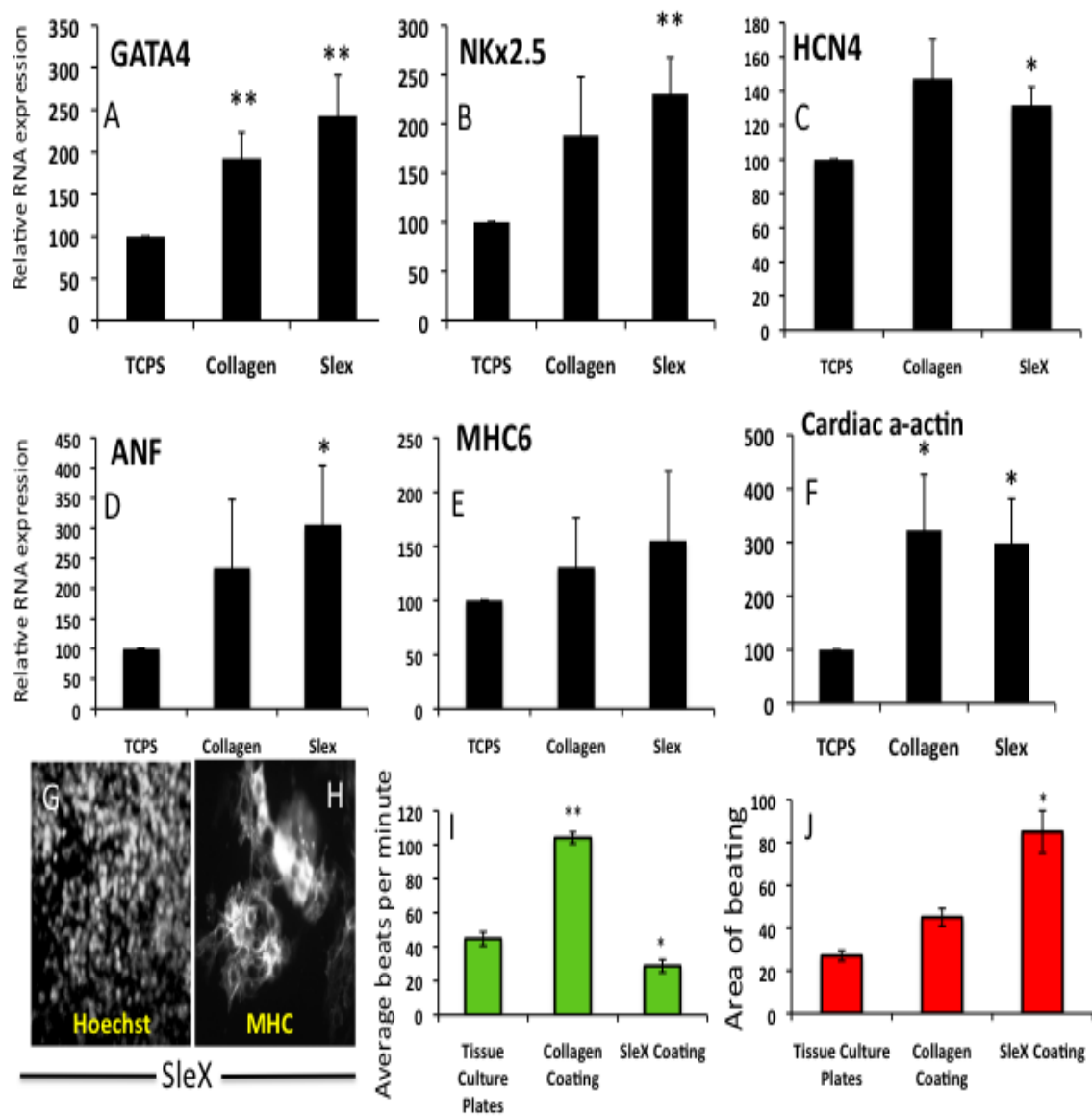


Figure 3.3- Differentiation of mouse embryonic stem cells on the collagen based sle^X matrix upregulates expression of cardiac precursors and terminal differentiation markers. mES cells were aggregated in the absence of LIF for 7 days and then plated on either regular tissue culture plates, Collagen coated plates or Collagen+Sle^X coated plates. RNA was harvested on day 15 of differentiation and Q-PCR analysis of gene expression was used to measure the relative mRNA levels of cardiac muscle genes (A-F). Cardiomyocytes formed on the sle^X surface were visualized using an anti-MHC antibody (G&H). Average beats per minute were measured for 3-5 aggregates in duplicate for 3 independent differentiations (I). The area beating was measured in the fields of view containing beating aggregates of 3 independent differentiations (J). Changes in gene expression patterns were calculated using the comparative Ct method. Results were normalized against the internal control beta-actin, and are expressed as a percentage of expression relative to tissue culture plates. Bars represent average \pm SEM, n= 5. Statistical analysis versus control at each time point was carried out using Student's T-test (*p<0.05, **p<0.005).

surface which is significantly higher than the area of beating on tissue culture plates ($25\% \pm 5$, $n=3$) (Figure 3.3, J). Note that only beating areas were examined in this calculation. Altogether these results suggest the collagen-based sLe^X-matrix can facilitate the differentiation of mESCs into cardiomyocytes.

3.4 Discussion

We were interested in examining the effect of extracellular matrix (ECM) on differentiation of mESCs into muscle cells, which is complementary to our analysis of signalling pathways and transcription factors to optimize myogenesis. We demonstrated that the culture of mESCs on the collagen-based matrix as a substrate resulted in an increased differentiation towards a myogenic lineage compared to the standard cell culture protocols on tissue culture plates. We found that the sLe^X-matrix particularly enhanced cardiomyogenesis. On day 15, mESCs cultured on collagen matrices expressed higher levels of skeletal muscle progenitor markers, Pax3 and Pax7, the myogenic regulatory factors, Myf5 and MyoD and the terminal differentiation marker, MHC3 as compared to TCPS. Therefore, similar to previous results from our lab, using retinoic acid for enhancing skeletal myogenesis [134], cultures on the matrices promoted a more efficient differentiation in mESCs that produced a premyogenic progenitor population and enhanced MRF expression and terminal differentiation into myotubes.

Given the pluripotent nature of mESCs and their capacity to differentiate into most lineages [260], it is significant that the collagen-based matrices have an ability to enhance mESC differentiation towards a Pax3/7⁺ progenitor population and skeletal muscle formation. Pax3 alone is sufficient to induce skeletal myogenesis in stem cells [70]. The

Pax3/7^{+ve} population has the ability to replenish the satellite cell niche [261, 262], and can be important for therapies targeted at treating muscle diseases. Similarly, using Q-PCR analysis we examined the expression of myogenic markers in an injured hindlimb tissue, 10 days after injury. The sLe^X-matrix induced an upregulation of transcript levels of genes involved in directing myogenesis, such as *M-cadherin* [263], *Pax7* [264], *Six1* [265], *Myf5* [266], *MyoD* [267] and *Myogenin* [51] (Appendix B). These results suggest that the collagen-based matrices enhance and enrich populations of myogenic lineage cells and promote endogenous regenerative myogenesis that may be proven useful for future therapeutic purposes.

The sLe^X-matrix supported differentiation of mESCs into cardiomyocytes. On day 15 of differentiation, mESCs cultured on the collagen-based sLe^X surface expressed higher levels of cardiac progenitor markers GATA4 and Nkx2.5 as well as cardiac maturation markers HCN4 and ANF. The physical properties of the sLe^X-matrix seem to be optimal for cardiac muscle formation and maturation as the newly formed cardiomyocytes beat slower but have a greater beating area on this surface as compared to TCPS. This finding is significant and may be useful for generating functional cardiomyocytes for transplantation without the need for genetic manipulations. Current efforts in delivering cardiomyocytes within a biomaterial to heart infarcts are still facing poor cell survival, poor functional integration of the grafts and weak mechanical coupling leading to clinically detectable improvements [268-270]. Therefore a need for an acellular injectable biomaterial that can support regeneration of infarcted heart remains significant.

In summary we have presented evidence for the ability of sialyl Lewis^X- enhanced

collagen matrix to augment differentiation of embryonic stem cells towards a myogenic lineage as well as regeneration of ischemic muscle. Considering the limited success of regenerative stem cell transplantation for both cardiac and skeletal myopathies, the need for cell-free methods of regeneration is necessary. The application of biocompatible, naturally derived materials that can support myogenesis holds great promise as a future therapy for muscle diseases.

CHAPTER 4

Discussion

The use of embryonic stem cells to repair damaged and/or dystrophic muscle has been of considerable interest. However, injection of ES cells into animals induces tumor formation and a very low percentage of the cells differentiate into skeletal muscle [271] or cardiac muscle [272]. There have been efforts to purify a population of mesodermal or muscle progenitor cells prior to injections, which have proven somewhat successful in long-term myoblast engraftment or restoring dystrophic muscle tissue in mice [227, 229-232]. Other problems such as cell delivery and engraftment as well as dealing with an induced immune response have also hindered advances in the field of stem cell therapy. All of these problems are currently considered as active areas of research. In this discussion we will focus on differentiation of stem cells as well as ways to circumvent the delivery and engraftment problems. The insight gained from the studies presented in this thesis may provide new strategies for isolating and expanding the myogenic population prior to differentiation as well as new delivery strategies using materials that augment stem cell differentiation into a myogenic fate and support natural body responses for muscle regeneration.

In Chapter 2, we report the investigation of the effect of a secreted signalling molecule, retinoic acid, in regulating the role of Sox7 during P19 differentiation. The role of Sox7 has been identified in the development of endodermal [105-107] and cardiovascular tissues [108-110]. However, to date, the only group that has reported that Sox7 is involved in the regulation of skeletal myogenesis is our lab. Previously we have demonstrated that P19[Sox7] cells are able to enhance the expression of muscle precursor genes, MRFs and MHC [112]. Here we report, through knockdown studies, that in addition to being sufficient [112], the endogenous Sox7 is also necessary for skeletal

muscle formation (Figure 4.1). Our lab has also demonstrated that RA functions by enhancing the specification of mesoderm cells to the skeletal muscle lineage [134]. In this study, we have shown that exposure to exogenous RA accelerates the expression of Sox7 and changes the cell fate during differentiation of both P19[Sox7] and P19[shsox7] cells. Specifically, Sox7 overexpression can enhance skeletal myogenesis in the absence of RA, and neurogenesis in its presence, while Sox7 knockdown inhibits skeletal myogenesis in the absence of RA but not in its presence. Therefore, it would be of interest to understand the complexity of the interplay between retinoic acid signalling and Sox7 function on a detailed molecular level.

For future directions, my first approach would be to identify more genes that are differentially expressed in each of these conditions and could account for the differences in cell fate determination. Global analysis of gene expression could be carried out in order to identify such genes. Although hundreds of genes have been identified to be RA-inducible, only a small number of them have been shown to contain functional RAREs [273]. *In silico* analysis of the Sox7 genomic DNA sequence has identified one conserved RARE approximately 25kb upstream of the transcriptional start site. However our lab has not been able to identify a direct binding of RAR to the Sox7 gene or Sox7 protein. However, we have found that overexpression of Sox7 upregulates the expression of RAR β , suggesting that the ability of RA to regulate Sox7 function could be, in part, by expression of RAR β . A global expression analysis would provide a list of all the genes that were changing under the different conditions we have used.

We could also look at the activity of Sox7 in the presence and absence of RA in the aggregated P19 cells using a luciferase assay. To further look into the ability of Sox7

to bind to RARs we will need to optimize our CoIP protocol. We can identify the list of genes that are bound by Sox7 in the presence and absence of RA by chromatin immunoprecipitation followed by high throughput sequencing (ChIP-seq). This list will help us find direct targets of Sox7 and how they change in each of the conditions we have used in our experiments in this thesis. I expect to find that the ability of Sox7 to bind to DNA will be modulated by the presence of the RARs.

It will be also interesting to determine if these observations hold true in mES and hES cells. Since there is a low conservation of RAREs between human and mouse and significant differences exist in the RA-regulation of the highly conserved RAR-target genes between species [273], I expect similar results between P19 cells and mES cells, but probably less similarity between these two cell systems and hES cells.

In summary, the data presented here have provided insight into the role of Sox7 during RA-mediated induction of myogenesis in P19 cells. We propose a model where RA signalling regulates Sox7 expression and Sox7 modulates RA binding to its receptors. The mechanisms underlying the interplay between Sox7 and RA remain to be discovered.

In chapter 3, we have demonstrated that a collagen-based biomaterial can augment myogenesis in mES cells. The need for biomaterials to act as *de novo* environments *in vivo* for stem cell and regenerative responses has been recognized [274]. As we study the signalling pathways and transcription factors regulating skeletal and cardiac myogenesis in order to discover ways to enhance myogenesis in stem cells, we need to consider possible strategies to deliver these cells to repair damaged muscle. We have found that the collagen-based matrices enhance skeletal myogenesis and specifically the sLe^x-matrix enhances cardiomyogenesis in mES cells. *In vivo*, in an ischemic hindlimb

mouse model, the sLe^X-matrix did not increase the levels of myogenic mRNA transcripts 3 days after injury, but by day 10 post-injury it had induced an upregulation of transcripts of myogenic genes as shown in appendix B. Pelosi *et al.* had demonstrated that in wild-type mice, the onset of skeletal muscle regeneration begins around 10 days after injury [275]. Interestingly as compared to animals injected with collagen-based matrices, the PBS-injected control animals demonstrated an enhanced expression of Pax7 transcripts at day 3. Pax7 is a marker for satellite cells, and after injury, the normal response for satellite cells is to proliferate after 18 hrs, peaking at 3 days and gradually reducing afterwards [264]. Our results suggest that treatment with collagen matrices may have delayed this process, however, the sLe^X-matrix was still more efficient in inducing regenerative myogenesis over the longer time as compared to the controls.

We have presented evidence for the enhanced differentiation of mESCs into skeletal and cardiac muscle on collagen matrices and enhanced regeneration of damaged ischemic muscle after the application of sLe^X injectable matrix (Figure 4.1). Based on its ability to guide pluripotent cells towards a myogenic lineage, we believe that the collagen-based matrix has an inherent ability to support muscle regeneration. This is of great interest considering the limited success of stem cell therapy in getting the cells to differentiate to the desired tissue. The non-invasive application of biocompatible naturally-derived materials that can augment myogenesis holds great promise as a future therapy for prevalent myopathies. The enhanced collagen-based biomaterial used in this *in vivo* study was injected with no cells bound to it and it supported the natural body responses for regeneration of the damaged tissue. This is important because this strategy

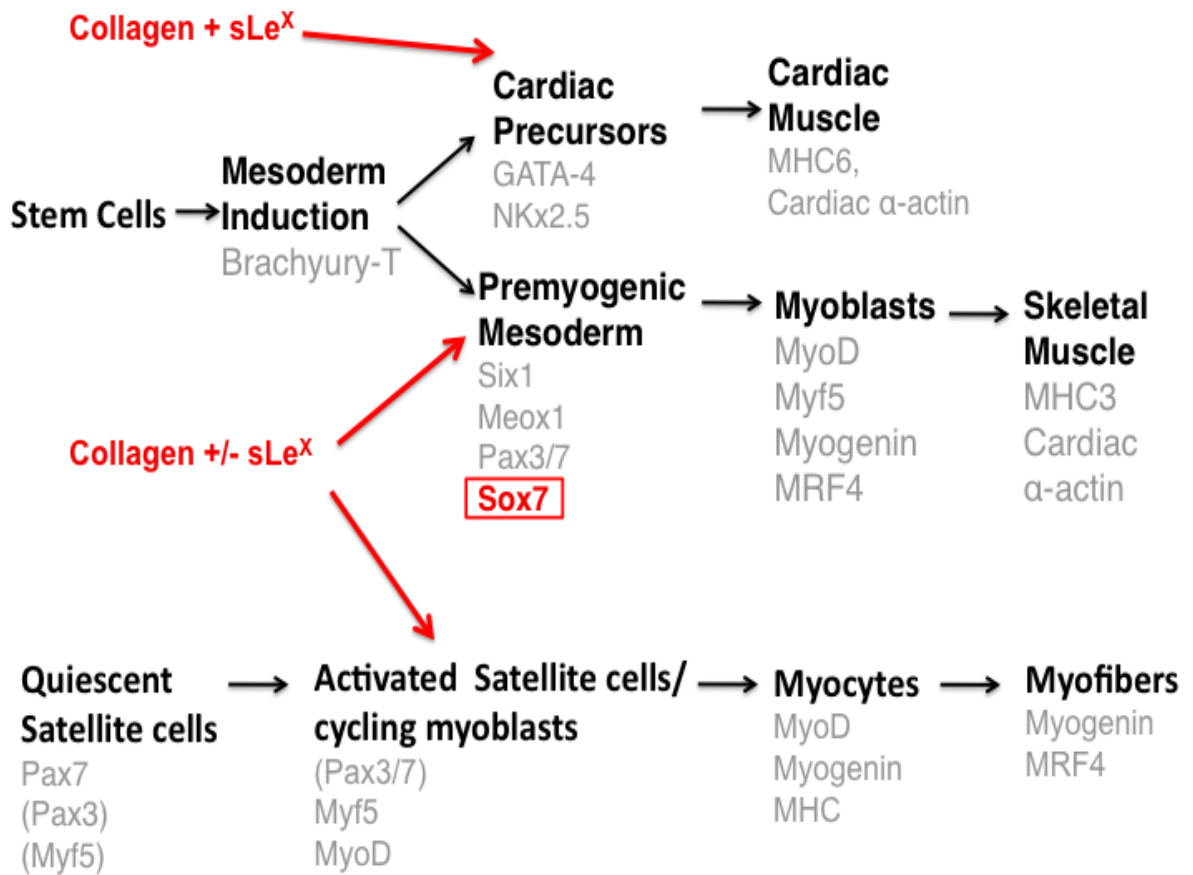


Figure 4.1- The molecular network of genes that regulate skeletal and cardiac myogenesis in stem cells and regeneration in satellite cells. The red arrows represent the positive effects we have uncovered. Sox7, shown in red, was discovered to be necessary for skeletal myogenesis in P19 cells. The black arrows and the black and grey fonts represent what was already known in the field.

addresses the other limitations of stem cell therapy including cell delivery and engraftment.

While the mechanical properties of the sLe^X-matrix have been studied in Dr. Erik Suuronen's lab where the biomaterials are prepared, it would be interesting to study the mechanism of binding of the cells to these biomaterials. It would also be interesting to study, in details, the enhanced cardiomyogenesis on the sLe^X surface. We could compare the maturity of the cardiomyocytes on each surface by performing electrophysiology.

In this thesis, we have obtained new information and insight that can potentially be used to devise new therapeutic interventions aimed at repairing muscle disease.

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APPENDICES

Appendix A

Table A1- Reverse and Forward primers used for mRNA Q-PCR analysis

GENES	FORWARD PRIMERS	REVERSE PRIMER
β -actin	AAATCGTGCGTGACATCAAA	AAGGAAGGCTGGAAAAGAGC
GAPDH	TCGGTGTGAACGGATTTG	GGTCTCGCTCCTGGAAGA
BrachyuryT	CTGGACTTCGTGACGGCTG	TGACTTTGCTGAAAGACACAGG
Mesp1	CATCGTTCCTGTACGCAGAA	TCTAGAAGAGCCAGCATGTCTG
FoxA2	CCCTACGCCAACATGAACTCG	GTTCTGCCGGTAGAAAGGGA
Sox2	GCGGAGTGGAACCTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
Noggin	GCCAGCACTATCTACACATCC	GCGTCTCGTTCAGATCCTTCTC
Pax3	TTTCACCTCAGGTAATGGGACT	GAACGTCCAAGGCTTACTTTGT
Pax7	CTCAGTGAGTTCGATTAGCCG	AGACGGTTCCTTTGTCTCGC
Meox1	TGGCCTATGCAGAATCCATTCC	TTGGATCTGAGCTGCGCATGTG
Six1	TAACTCCTCCTCCAACAAGCA	CGAGTTCTGGTCTGGACTTTG
M-Cadherin	CATCCCACCCATTAGTGTGTC	TCCAGTGAACCTTGTCGATAGA
MyoD	CCCCGGCGGCAGAATGGCTACG	GGTCTGGGTTCCCTGTTCTGTT
Myf5	CCTGTCTGGTCCCAGAAAGAAC	GACGTGATCCGATCCACAATG
Myogenin	GCAATGCACTGGAGTTCG	ACGATGGACGTAAGGGAGTG
MHC3	GCATAGCTGCACCTTTCCTC	GGCCATGTCCTCAATCTTGT
MHC6	CAACAACCCATACGACTACGC	ACATCAAAGGGCCACTATCAGTG
MHC7	GATGAGCAAGCCCTGGGCAGTC	TCAGAGCGCAGCTTCTCCACCT
Cardiac α -actin	CTGGTATTGCCGATCGTATG	CTTGCTGATCCACATTTGCT
Sox7	ACCTCAGGGGACAAGAGTTCG	GTTTTTCTCAGGCAGCGTGTTT
Sox17	GATGCGGGATACGCCAGTG	CCACCACCTCGCTTTTAC
Sox18	CCTGTCACCAACGTCTCGC	GCAACTCGTCGGCAGTTTG
RAR-alpha	ATGTACGAGAGTGTGGAAGTCG	ACAGGCCCGGTTCTGGTTA
RAR-beta	CTGCTCAATCCATCGAGACAC	CTTGTCCTGGCAAACGAAGC
RAR-gamma	ATGTACGACTGCATGGAATCG	CCAGTGGCTCTGCGTAGTAA
GATA4	AAAACGGAAGCCCAAGAACCT	TGCTAGTGGCATTGCTGGAGT
NKx2.5	AAGCAACAGCGGTACCTGTC	GCTGTCGCTTGCACTTGTAG
ANF	ACTAGGCTGAACAGCTTCC	TGACACCCACAAGGGCTTA
HCN4	GCGGACACCGCTATCAAA	GCCGAACATCCTTAGGGAGAAT
NeuroD	GCATGCACGGGCTGAACGC	GGGATGCACCGGGAAGGAAG
CD9	ATGCCGGTCAAAGGAGGTAG	GCCATAGTCCAATAGCAAGCA
CD81	GTGGAGGGCTGCACCAAAT	GACGCAACCACAGAGCTACA
NFATc3	GAGGTTGCCCTGGAGAAGA	CTGGAGTGCCGTCGTTTAC

Appendix B

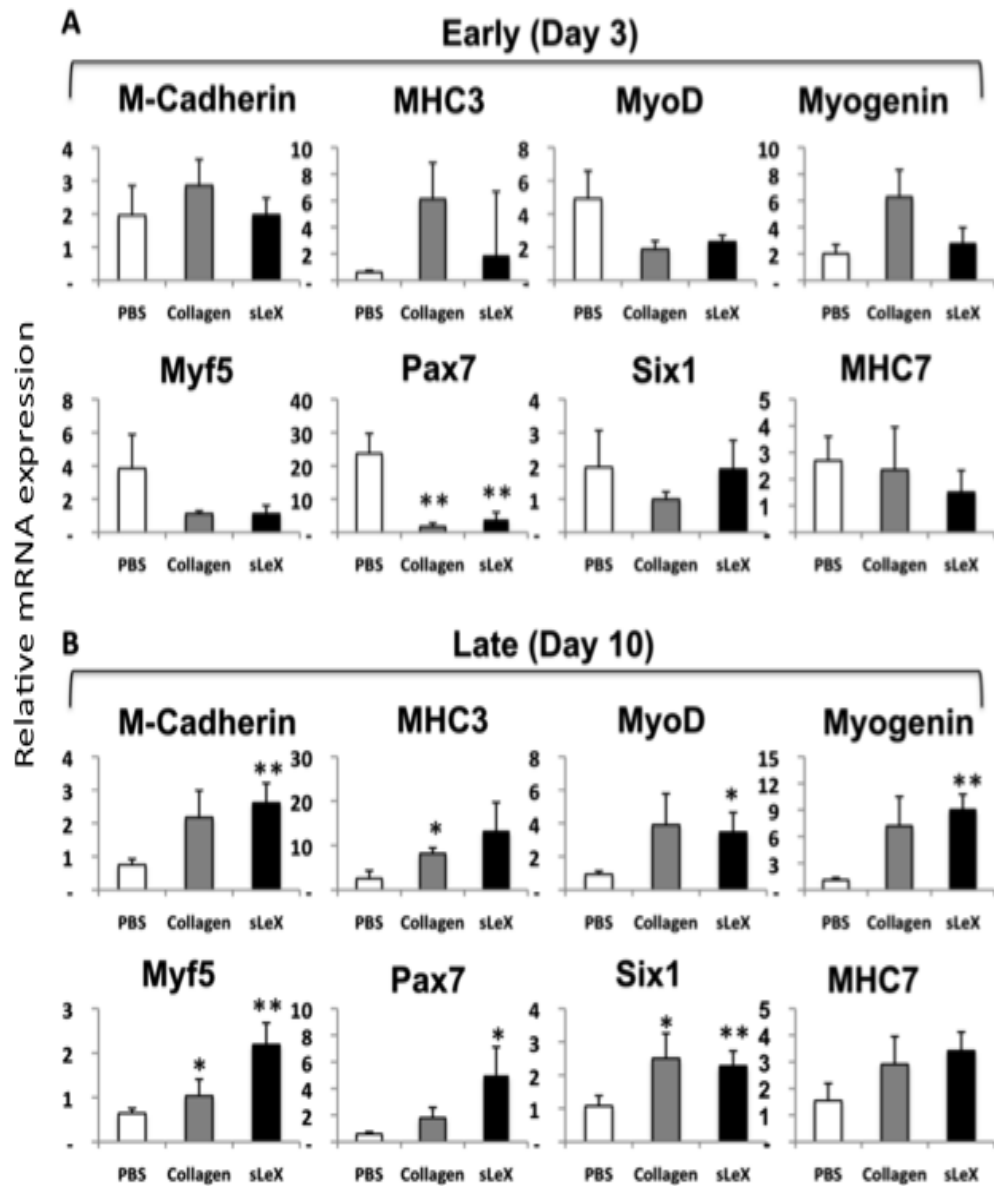


Figure B1- Collagen-based matrices helped in regeneration of mouse ischemic hind limb tissue. Animals were injured in the right hind limb with cardiotoxin. After the induction of injury, the animals were injected with PBS, Collagen or Collagen+Sle^X at the site of injury. After 3 (A) or 10 (B) days post-injury the animals were sacrificed and RNA was extracted from the hind limbs. Q-PCR analysis of gene expression was used to measure the relative mRNA levels of skeletal muscle precursor genes, MRFs and terminal differentiation markers. Changes in gene expression patterns were calculated using the comparative Ct method. Results were normalized against the internal control GAPDH, and are expressed as a percentage of expression relative to PBS. Bars represent average \pm SEM, n= 6, representing 6 different mice per group per time point. Statistical analysis versus control at each time point was carried out using the Student's T-test (* $0.05 < p < 0.1$ vs. PBS; ** $p < 0.05$ vs. PBS)

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Appendix D

CURRICULUM VITAE

Name Diba Ebadi
Citizenship Canadian
Date of Birth September 1, 1983

POST-SECONDARY EDUCATION AND DEGREES

2009 – 2011 **M.Sc. Biochemistry**
University of Ottawa
Ottawa, Ontario, Canada

2005 – 2009 **Honours B. Sc. Biochemistry
with a minor in Psychology**
University of Ottawa
Ottawa, Ontario, Canada

SCHOLARSHIPS

2011 – 2012 OGS PhD Scholarship
2010 – 2011 NSERC Master's Scholarship (CGS)
2009 – 2010 OGSST Master's Scholarship
2009 – 2011 Admission/Excellence Scholarship, University of Ottawa
2008 (May – August) NSERC/USRA summer studentship
2007 – 2009 Merit Scholarship, University of Ottawa

AWARDS

2011 – June CIHR- Musculoskeletal Health and Arthritis Travel Award
2011 – April SFB- Student Travel Achievement Recognition Honorable Mention
2010 – May BMIGSA- Poster Award
2009 – April Undergraduate Research Excellence Award, University of Ottawa
2009 – May Canadian Biomaterial Society (CBS)- Travel Award
2009 – May Summer Student Medical Research Forum Bursary
2009 – June Magna Cum Laude B.Sc. Award
2007 – 2009 Dean's Honour List, University of Ottawa

VOLUNTTEERING ACTIVITIES

2010 – Present	SUPPORT (Students undertaking a pediatric program of research training) Recruited patients for clinical trails in the ER at CHEO
2009 – present	Let’s Talk Science and Science Travels Partnership Programs Gave science workshops in classrooms, University of Ottawa
2007 – present	Canadian Cancer Society- The Ottawa Hospital General Campus Assisted patients and physicians, Ottawa, Ontario
2007 – 2009	Ottawa Youth Commission- Executive member Voiced the needs of youth to the Mayer and MPs, Ottawa, Ontario
2007 – 2009	Kiwanis/ Circle K club- member Helped in organizing fundraising events. University of Ottawa
2006 – 2009	St. John Ambulance- Medical First Responder Gave first aid as needed, University of Ottawa

TEACHING ASSISTANT EXPERIENCES

2010 (January – April)	BCH 3346 Biochemistry Laboratory II, Faculty of Science University of Ottawa
2010 (September – December)	BCH 3170 Molecular Biology, Faculty of Science, U of O
2011 (January – April)	BCH 2333 Laboratory of Introduction to Biochemistry, Faculty of Science, University of Ottawa

PUBLICATIONS

Manuscripts:

- 1- Joanne McBane, **Diba Ebadi**, Soroor Sharifpoor, J. Paul Santerre, Rosalind S. Labow. “Differentiation of monocytes to inflammatory or wound healing macrophages on a degradable polar hydrophobic ionic polyurethane: films versus scaffolds” *Acta biomaterialia* (2011) 7: 115-122
- 2- Drew Kuraitis, **Diba Ebadi**, Pingchuan Zhang, Branka Vulesevic, Donna T. Padavan, Ashraf Al Madhoun, Kimberly McEwan, Tanja Sofrenovic, Kyra Nicholson, Stewart C. Whitman, Thierry G. Mesana, Ilona S. Skerjanc, Marc Ruel and Erik J. Suuronen. “Injectable matrix stimulates marrow and local cells for neovascularization and regenerative myogenesis in mouse skeletal muscle” (Submitted to *J Biomaterials*)

Conference Abstracts:

- 1- **D Ebadi**, D Kuraitis, D Padavan, A Al-Madhoun, M Ruel, IS Skerjanc and EJ Suuronen. “An Enhanced Collagen-based Matrix Promotes Cardiomyocytic Differentiation of Embryonic Stem Cells” American Heart Association Scientific Sessions, Orlando, FL, USA. November 2011
- 2- D Kuraitis, **D Ebadi**, P Zhang, B Vulesevic, A Al-Madhoun, T Sofrenovic, TG Mesana, IS Skerjanc, M Ruel and EJ Suuronen “Injectable Matrix Activates Myogenic Differentiation in Embryonic Stem Cells and Regenerates Muscle in Ischemic Tissue” Submitted to the Canadian Cardiovascular Congress, Vancouver, BC, Canada. October 2011
- 3- B McNeill, DT Padavan, D Kuraitis, **D Ebadi**, IS Skerjanc, M Ruel and EJ Suuronen. “Physical Properties of Collagen Matrix Conducive for the Activation of Myogenic Differentiation in C2C12 Myoblasts” Submitted to the Canadian Cardiovascular Congress, Vancouver, BC, Canada. October 2011
- 4- **D Ebadi**, D Kuraitis, A Al-Madhoun, EJ Suuronen and IS Skerjanc. “A Collagen-Based Matrix that Enhances Cardiac and Skeletal Muscle Development in Mouse Embryonic Stem Cells” Society For Biomaterials Annual Meeting and Exposition, Orlando, FL, Abstract #376. April, 2011 and International Society for Stem Cell Research, Toronto, ON, Canada, Abstract # 2045. June 2011
- 5- J Savage, S Khan, **D Ebadi** and IS Skerjanc. “Canonical Wnt signaling and retinoic acid regulate Sox7 expression” Ottawa Conference on New Directions in Biology & Disease of Skeletal Muscle, Ottawa, ON, Canada, Abstract #127. May 2010
- 6- **D Ebadi**, JE McBane, S Sharifpoor, JP Santerre, RS Labow. “Monocyte differentiation on vinyl polyurethane films and scaffolds” 27th Annual Meeting of the Canadian Biomaterials Society, Quebec City, QC, Canada, Abstract #120-mVTF-71. May 2009
- 7- JE McBane, S Sharifpoor, **D Ebadi**, J. Paul Santerre, Rosalind S. Labow. “Effect of protein coating and material surface on early monocyte differentiation to inflammatory or wound healing phenotype macrophage” Annual Meeting of the Society for Biomaterials, San Antonio, TX, USA, Abstract #216. April 2009