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The Transcriptional Regulation of Premyogenic Mesoderm Factors During Skeletal Myogenesis

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The Transcriptional Regulation of Premyogenic Mesoderm Factors during Skeletal Myogenesis

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A thesis submitted to
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In Partial Fulfillment of the requirements for the degree of
Master of Science

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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 myocytes from the undifferentiated state. The present study aims to understand and compare the transcriptional regulation of skeletal muscle progenitor and MRFs through β-catenin- and MyoD-induced P19 EC cell differentiation. Furthermore, the study aims to compare the expression pattern of transcription factors that mark either the epaxial or hypaxial domain of the differentiated somite of the developing embryo in DMSO-, β-catenin- and MyoD induced P19 EC cell differentiation.

First, stable expression of an active β-catenin in P19 cells was sufficient to induce the expression of the premyogenic mesoderm factors such as Pax3/7, Meox1, Gli2, Foxc2, in addition to the somitic marker Foxc2. Knock-down of β-catenin, by shRNA, resulted in a decreased expression of these factors, and a downregulation of the MRFs, indicating that β-catenin is essential for myogenesis. DMSO-induced differentiation led to the upregulation of Sim1 and Lbx1, whereas β-catenin-induced differentiation did not show any significant changes to either.

Second, gain- and loss-of-function experiments demonstrated that MyoD directed P19 cells into the skeletal muscle lineage by inducing the expression of Meox1, Pax7, Six1, and Eya2, on day 4 and eventually Pax3 leading to the subsequent upregulation of the MRFs. Chromatin immunoprecipitation experiments
showed that MyoD bound directly to the regulatory regions of *Pax3/7, Eya2, Six1, Meox1* and *myogenin*. Furthermore, MyoD-induced differentiation upregulated the expression of Lbx1 and C-met and had relatively unchanged levels of En1 and Sim1 expression. These results demonstrate that MyoD re-directs P19 cells into the skeletal muscle lineage by directly binding to the regulatory regions of several premyogenic mesoderm genes, leading to the formation of skeletal muscle that express the transcription factors C-met and Lbx1, both of which are shown to be essential in limb, diaphragm and tongue muscle formation in the embryo.

These studies have provided an understanding of the transcriptional regulation of premyogenic mesoderm factors in both β-catenin- and Myod-induced differentiation, with further insight into the type of skeletal muscle that may be forming in P19 EC cells.
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Lastly I would like to thank everyone in the lab, past and present! I would especially like to thank Tammy, Donna, Jenny and Anastassia for their great company over the last 3 years!!! In addition, I would like to thank Carol Ann Kelly for always helping me out with my paper work! I will miss each and every one of you!
Dedicated to my Mom, Dad and Grandma
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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>ATCC</td>
<td>American tissue type collection</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>AD</td>
<td>Activation domain</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>CamKII</td>
<td>Ca²⁺-calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CKI</td>
<td>Casein kinase I</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding</td>
</tr>
<tr>
<td>Dhh</td>
<td>Desert Hedgehog</td>
</tr>
<tr>
<td>DM</td>
<td>Dermomyotome</td>
</tr>
<tr>
<td>DML</td>
<td>Dorso-medial lip</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EC</td>
<td>Embryonal carcinoma</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------</td>
</tr>
<tr>
<td>En-2</td>
<td>Engrailed 2</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Fz</td>
<td>Frizzled</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GSK</td>
<td>Glycogen synthase kinase</td>
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<tr>
<td>H/C</td>
<td>Histidine/ Cysteine</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>Ihh</td>
<td>Indian Hedgehog</td>
</tr>
<tr>
<td>JNK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>Lnfg</td>
<td>Lunatic fringe</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL receptor-related protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin Heavy Chain</td>
</tr>
<tr>
<td>MPC</td>
<td>Muscle precursor cell</td>
</tr>
<tr>
<td>MRF</td>
<td>Myogenic regulatory factors</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCAF</td>
<td>P300/CBP-associated factor</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar cell polarity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>PGK</td>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphotidylinositol</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PSM</td>
<td>Pre-somitic mesoderm</td>
</tr>
<tr>
<td>Ptch1</td>
<td>Patched 1</td>
</tr>
<tr>
<td>Puro</td>
<td>Puromycin</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RALDH</td>
<td>Retinaldehyde dehydrogenase</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RARE</td>
<td>Retinoic acid response element</td>
</tr>
<tr>
<td>RBP</td>
<td>Retinol binding protein</td>
</tr>
<tr>
<td>RDH</td>
<td>Retinol dehydrogenase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RT Q-PCR</td>
<td>Reverse transcription quantitative-polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>Smo</td>
<td>Smoothened</td>
</tr>
<tr>
<td>Sp</td>
<td>Splotch</td>
</tr>
<tr>
<td>sPBS</td>
<td>Stockholm (NaPhosphate based) PBS</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>Switch/Sucrose NonFermentable</td>
</tr>
<tr>
<td>VLL</td>
<td>Ventro-lateral lip</td>
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CONTRIBUTION OF COLLABORATORS

Section 3.1-3.3

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

Section 3.4

- One set of differentiation was performed by Anastassia Voronova, a PhD Candidate.

- The rest of the experiments were performed and analyzed by myself.

Section 3.5 – 3.7

- The P19[MyoD/EnR] cell line was generated by Dr. Peter J Gianakopoulos, Alan Ridgeway, and Michelle Waddington.

- For the P19[MyoD] ChIP experiment, the primers were designed based on ChIP sequencing information provided by Dr. Stephen Tapscott and his Laboratory.

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

The thesis was written by me and edited by Dr. Ilona Skerjanc and Anastassia Voronova, a PhD Candidate.
CHAPTER 1 - Introduction

1.1 Vertebrate Embryonic Skeletal Muscle Development – General Overview

Skeletal muscle represents a major component of muscle found in the body. Its formation occurs progressively and initial transplantation studies in the chick suggest that skeletal muscle formation is initiated during gastrulation (49, 120). The formation of the primitive streak determines the site of gastrulation, defined as the formation of a more complex, organized, and multilayered embryo, from a simple, not very highly organized group of cells, with three distinguishable germ layers, endoderm, ectoderm and mesoderm (128). As it extends through the midline, highly coordinated movements control the ingression and migration of cells to their final positions. At this stage cells patterned to become the paraxial mesoderm migrate medially along the anterior-posterior axis, while the cells that travel further medially become the lateral plate mesoderm (199).

During the regression of the primitive streak, the paraxial mesoderm is further patterned into blocks of segmented ball-like structures, referred to as somites, which flank both sides of the axial structures (neural tube and notochord) (Figure 1, 1). Somitogenesis, the process of somite formation, occurs in a rostral-caudal direction following a “clock-wavefront” model initially proposed by Cooke et al. in the 1970s (51). This model proposes that the ‘clock’, which refers to the oscillator, and the ‘wavefront’, which refers to the boundary established between a newly formed
somite and the pre-somitic mesoderm (PSM), determines the rate and maturation of a somite, respectively (51). It was later discovered that the Notch-Delta and Wnt signalling pathways control the cyclic expression of genes during somitogenesis. In addition to Wnt gradients, a gradient of retinoic acid (RA) and fibroblast growth factor (Fgf) is established to create the wave of determination front. Fgf and Wnt signalling diminishes as you approach the rostral end, which is antagonized by a gradient of RA signalling. The position of the front is established where the gradients of Fgf-Wnt and RA oppose each other, and the particular level found at the front is characterized as a threshold at which the cells become competent to respond to the segmented clock, forming segmental boundaries (reviewed in (59)). Wnt3a has been proposed to establish a gradient as knockout mice demonstrate that Wnt signalling regulates the segmental clock by controlling the oscillation of Axin2 and Lunatic fringe (Lnfg) (4, 170, 183). Loss of RA results in the loss of bilateral symmetry of the somites because the Fgf8 gradient is anteriorized and Hes7 and Lnfg, genes required to control the oscillation of Notch expression, are asymmetrically expressed (114, 219-220).

As the epithelial somites mature, they begin to differentiate into the dorsal dermomyotome (DM), giving rise to muscles of the trunk and dermis, and the ventral sclerotome, giving rise to axial cartilage and bone (Figure 1, II) (as reviewed in (117)). The muscle progenitor cells located in the DM migrate to form the myotome, the source of the first skeletal muscle found in the body. The formation of the myotome has been intensively studied and controversial at times. The most recent and accepted observations, through electroporation of a GFP reporter construct into
Figure 1 – Somitogenesis in the Vertebrate Embryo. (Panel I): A scanning electron micrograph illustrating the rostral to caudal directed segmentation of the paraxial mesoderm into bilaterally symmetric pairs of somites flanking the axial structures (neural tube and notochord). Copyright: Gilbert S.F., Developmental Biology 6th edition, 2000, Figure 14.3. (Panel II): A schematic of Vertebrate somite segmentation. The paraxial mesoderm is patterned into blocks of segmented ball-like structures, flanking the neural tube and notochord. Signals, such as Wnt and Shh, emanating from adjacent tissues, pattern the somite into the dorsal dermomyotome (DM) and ventral sclerotome. Translocation of DM cells from the dorsal medial lip, followed by the migration of cells from all four borders, form the myotome, located beneath the DM. The myotome forms a continuous sheet of epaxial and hypaxial muscle. Reproduced from reference [159] with permission from Nature Publishing Group © 2003, originally adapted from Buckingham, M., L. Bajard, T. Chang, P. Daubas, J. Hadchouel, S. Meilhac, D. Montarras, D. Rocancourt, and F. Relaix. 2003. The formation of skeletal muscle: from somite to limb. J Anat 202:59-68. with permission from John Wiley and Son’s © 2003.
chick interlimb somites, illustrate that the myotome is formed in two waves (83)).

The first wave represents the primary myotome, which forms by the translocation of DM cells from the dorso-medial lip (DML), followed by the migration of cells from all four borders of the DM. The new myoblasts from DML displace the older myocytes and elongate toward the rostral and caudal borders, while cells from all four borders elongate along the anterior posterior axis of the embryo (as reviewed in (96)). The second wave represents the secondary myotome which forms from the cells that translocate directly from the central DM to the myotome, not from those of the DML (82, 178).

The dorsomedial half of the somite gives rise to the deep back muscles, called epaxial muscles, while the cells from the ventrolateral half migrate ventrally to form the body wall muscles, called hypaxial muscles, in addition to the muscles of the limb (as reviewed in (159, 175)). Hypaxial and epaxial musculature in adult amniotes are physically separated by a distinct connective tissue sheet (49, 74-75, 79). In the early embryo, it has been demonstrated that the medial and lateral halves of the DM and myotome generate separate myogenic domains, although they are morphologically continuous (152). A horizontal subdivision has been suggested in separating the epaxial-hypaxial DM and myotome based on the expression patterns of transcription factors such as En1 and Sim1 (101, 207). Furthermore, proper migration and differentiation of the migrating hypaxial muscle precursor cells (MPCs) located in the ventro-lateral lip (VLL) of cervical, occipital and limb DM, forming the diaphragm, tongue and limb muscles, respectively (60-61, 105), require the expression of Lbx1 and C-met. The epaxial myotome of all somites are formed from
the progenitor cells that exit the cell cycle, elongate and differentiate from the DML, while the progenitor cells of the VLL similarly form the hypaxial myotome (159). The muscles of the limb, diaphragm and tongue are formed from the epithelial to mesenchymal transition that occur from the migrating cells of the VLL (159). The differentiation of the epithelial somites into the DM and myotome are influenced by factors such as Wnt, Sonic hedgehog (Shh) and Bone morphogenetic protein (BMP) secreted from adjacent embryonic tissues, including the neural tube, notochord, overlying surface ectoderm and lateral plate mesoderm (as reviewed in (34)) (Figure 1, II).

In order for a cell to acquire its fate for proper growth and development, there needs to be a coordinated balance between lineage commitment, proliferation, differentiation and cell death. For this reason, a complex network of temporally and spatially expressed transcription factors and signalling molecules, secreted from adjacent tissues is established to regulate the fate of a cell. Different transcription factors play a more predominant role during either epaxial or hypaxial muscle formation or to mark the epaxial and hypaxial domain. Signalling molecules secreted from adjacent structures influence the expression of those transcription factors. Although several studies have identified key molecular players involved in the process of skeletal muscle development, the mechanism of their regulation remains elusive. The following sections review current knowledge on the regulation of skeletal myogenesis.
1.2 Transcriptional Regulation of Skeletal Muscle Development

During the course of myogenesis, each event can be identified based on the set of molecules being expressed (Figure 2, Panel I). A cohort of transcription factors define the skeletal muscle progenitor cells located in the DM, amongst which are Pax3, Pax7, Six1, Eya2, Meox1, Gli2, and Foxc1. These factors are important in regulating the expression of the myogenic regulatory factors (MRFs), which ultimately commit and differentiate the cells of the myotome into the skeletal muscle lineage (reviewed in (34)). Furthermore, key transcription factors such as En1, Sim1, Lbx1 and C-met mark medial, lateral or migrating cells of the DM and myotome (Figure 2, Panel II).

1.2.1 The Myogenic Regulatory Factors

The myogenic regulatory factors (MRFs) are part of a superfamily of basic helix-loop-helix (bHLH) transcription factors. This highly related gene family consists of four members termed MyoD (Myf3) (57), Myf5 (28), MRF4 (Myf6/Herculin) (27, 143) and Myogenin (Myf1) (64).

1.2.1.1 Expression during embryogenesis

Myf5 is the first MRF to be expressed during embryogenesis at E8.0 and is localized in the DML of the epaxial domain of the embryo (153). Myf5 expression is followed by that of Myogenin at E8.5 (194) and MRF4 from E9.0 to E12.0 in the myotome, the latter of the two is expressed again only after birth (94). In comparison to Myf5, MyoD is expressed notably in the hypaxial domain of the myotome at E10.5 (109, 194).
Figure 2 – Transcriptional regulation of Skeletal Myogenesis. (Panel I): A schematic representing the series of molecular events involved during mesoderm patterning into skeletal muscle. The important signalling molecules, transcription factors and structural genes involved in skeletal muscle formation are illustrated on this schematic. (Panel II): A schematic representing the somitic division of epaxial and hypaxial domains and the key factors expressed in specific regions of each domain. Note the differentiated somite illustrated represents the somites found occipitally, cervically and at the level of the limb buds in the embryo, as Lbx1 is only expressed in the ventral lateral lip (VLL) and migrating hypaxial muscle precursor cells (MPC) of those somites. NT – Neural tube, NC – Notochord, MPC – muscle precursor cell.
1.2.1.2 Gene targeting studies

The MRFs, including MyoD, are sufficient to drive a wide variety of cell types into the skeletal muscle lineage (5, 28, 57, 151, 232), illustrating the importance of the MRFs as 'master regulators'. This was first evident through the forced expression of MyoD in fibroblasts (57), followed by ectopic expression in several other cell types (48). Studies indicate that the conversion occurs through chromatin remodelling by the interaction of MyoD with Brg1, the ATPase subunit of the SWI/SNF chromatin remodelling enzyme (58), and PCAF and p300 which acetylate MyoD and histones on DNA (169, 192). Furthermore, the association of MyoD with HDAC1, ensures that the cells do not differentiate prematurely (132).

The importance of each MRF through conversion studies has lead to gene targeting studies that have revealed hierarchical relationships between MyoD, Myf5, Myogenin and MRF4, and have elucidated many genetic targets of the MRFs, in particular MyoD. A MyoD\(^{-/-}\)/Myf5\(^{-/-}\)/MRF4\(^{-/-}\) triple mutant is born with no detectable skeletal muscle (111, 189, 210), while Myf5 and MRF4 double mutants (29, 111), show rib abnormalities (29) and have delayed epaxial muscle formation, with no effect on hypaxial or limb muscle development (109). However, mice, null for both MyoD and Myf5 with restored MRF4 expression, form skeletal muscle (111). In addition, a homozygous deletion of either Myf-5 or MyoD alone does not result in a loss of muscle (29, 187), whereas a homozygous deletion of Myogenin and/or MRF4 affects the formation of muscle fibres (89, 162). Interestingly, hypaxial muscle formation is delayed and Myf5 expression is found to be upregulated in MyoD null mice (109, 187). Altogether, results indicate early and late functionally redundant
roles for the MRFs in regulating skeletal myogenesis, with Myf5 and MyoD having distinct roles in epaxial and hypaxial muscle formation, respectively. Furthermore, these ablation studies have revealed that Myf5, MRF4 and MyoD are involved in the determination of the skeletal myogenic lineage, while Myogenin, in addition to MRF4, are involved in regulating differentiation.

1.2.1.3 MRF structure and function

The conserved basic domain of the MRFs, including MyoD, is important for DNA binding, while the helix-loop-helix is important for dimerization (31, 55) (Figure 3, Panel I). MyoD, along with the other MRFs, has been shown to heterodimerize with the ubiquitous E protein bHLH subfamily which on their own, cannot turn on the myogenic program (Figure 3, Panel II). Together they bind to DNA at sites known as E-boxes (CANNTG) on the promoters of many skeletal muscle-specific genes (126, 151, 231). Several studies have attempted to isolate additional genetic targets of the MRFs using C2C12 cells (adult muscle stem cells) or fibroblast myogenic conversion assays on null MRF backgrounds (16, 103, 233). Recently Cao et al. have confirmed that there is a preference for E-boxes with internal CC or GC sequences, and revealed already known and novel MyoD targets that include many of the skeletal muscle-specific genes during the course of differentiation (39). However, their studies also revealed thousands of additional sites genome-wide bound by MyoD inducing regional histone acetylation (39).

Previous studies have demonstrated that MyoD binding does not always correlate with transcriptional activation (55, 224). In fact, MyoD, with the help of other transcription factors and/or cofactors, can bind to E-box sites prior to
Figure 3. A schematic diagram of the MyoD protein. (Panel I): The key domain regions of MyoD are illustrated, including the transactivation domain (TAD), the histidine- and cysteine-rich regions, the bHLH and the helix III (amphipathic α-helix) domains. (Panel II): The schematic depicts the binding of the MyoD-E-protein heterodimer to the major groove of DNA. The basic regions bind to DNA while the helix-loop-helix domains participate in the dimerization. The histidine/cysteine rich (H/C) and the amphipathic α-helix (Helix 3) domains participate in the regulation of a subset of genes through their interaction with Pbx/Meis homeodomain proteins. Reproduced from reference [213] with permission from The Company of Biologists Ltd. © 2005.
transcriptional activation (12, 163, 174). The transcriptional activation domain of MyoD (aa 3-53) (224) is important for maximal interaction with the Histone Acetyl Transferases P300/CBP and PCAF in vitro (191), and this interaction was required for the activation of muscle-specific gene expression (173). Studies also illustrated that two amino acids in the basic domain of MyoD, an Alanine and Threonine (AT), are essential to activate the transcription of target genes (30, 56, 224-225). Furthermore, the Histidine/Cysteine rich (H/C) and the amphipathic α-helix (helix III) domains of MyoD (Figure 3) can regulate a subset of genes, distinct from the genes regulated by the classical N-terminus activation domain, through an interaction with the Pbx and Meis homeodomain proteins located adjacent to the myogenin promoter (13, 92, 214). These studies have revealed novel information on the complex hierarchy of MyoD transcriptional networks in myogenesis.

1.2.2 Pax3 and Pax7

Pax3 and Pax7 are functionally similar members of the paired box family of homeodomain transcription factors, referred collectively as Pax genes (116). Pax genes play key roles in several aspects of tissue specification and organogenesis during embryonic development (as reviewed in (35)). In particular, both Pax3 and Pax7 play an important role in skeletal muscle development.

The highly conserved Pax3 protein, which has 97% identity at the amino acid level between the chicken and mouse (78), contains an octapeptide motif, and a homeodomain and paired domain required for DNA binding in a sequence specific manner (43, 77). In mice, Pax3 is expressed in the developing nervous system, as early as E8.5, and in the PSM (77), prior to segmentation. It continues to be
transcribed throughout the epithelial somite, extending into the epaxial and hypaxial ends (as reviewed in (35)). Although the closely related Pax7 is also expressed in the somites, its expression is concentrated in the central (medial) domain of the DM (108) and is found to be essential for satellite cell specification after birth (155, 198). By E10.5, Pax3 can be detected in the myotome (178) and is required for the formation of the hypaxial body and limb musculature (76, 216, 226). In fact, embryos from homozygous Splotch (Sp) mutant mice, carrying a mutant allele for the Pax3 gene (66), fail to develop limb musculature, whereas epaxial muscles appear relatively unaffected (17, 76). Furthermore, migration of muscle progenitor cells in the VLL of the limb somites are under the control of Pax3 (17). Although only MyoD was impaired in the epaxial region (23), both MyoD and Myf5 were compromised in the hypaxial region of the somite (8). Borycki et al. speculate that expansion of Pax7 expression in epaxial regions of Splotch mice, which normally only express Pax3, may compensate for the loss of Pax3 (23). Further studies led to the identification of an important Pax3 binding site in the Myf5 enhancer. A cross between the Splotch and Myf-5/MRF4 mutant mouse revealed the important role of Pax3 in establishing both epaxial and hypaxial muscle through MyoD, as there is no detectable expression of MyoD in the trunk, limbs or hypaxial and epaxial somites (212). Compared to Pax3 mutants, Pax7 mutants appear normal at birth, but do not thrive and die in 2-3 weeks from unknown causes (135, 198). These results indicate that Pax3 is required for the activation of MyoD and essential for the survival of cells at the edges of the DM, particularly those located hypaxially (17, 76). Pax7 mutants show no evident skeletal muscle defects (135), and compensation between the Pax genes are observed (23).
A population of Pax3/7-positive cells gives rise to myogenic cells and thus muscle fibres, providing a pool of cells needed during embryonic and fetal development, in addition to satellite cells (as reviewed in (35)). For this reason, it is not surprising that a Pax3/7 double mutant embryo prevents the commitment of these progenitor cells into the myogenic program, as they undergo continued apoptosis, resulting in a major deficit of skeletal muscle formation (178). Relaix et al. further illustrated that dominant negative versions of Pax3 and Pax7, in satellite cell cultures of mice, exhibited low levels of MyoD but not Myf5, suggesting that Pax3/7 may act through a MyoD-dependent pathway in adult satellite cells (177). It is important to note that recent studies done by Lepper et al suggest that adult satellite cells do not require Pax3 or Pax7 for regeneration (127). Furthermore, later studies suggest that non-satellite cell muscle resident progenitor cells, PW1+/Pax7− interstitial cells (PICs), may play a role during adult muscle regeneration (145). Although the role of Pax3 and Pax7 during embryonic and fetal development is fairly established, its role in adult regeneration through satellite cell activation is controversial.

1.2.3 Six1 and Eya2

During myogenesis, Six1 and 4 activate Pax3 in the hypaxial cells of the DM (81), and interact with Eya2, which also acts genetically upstream of Pax3 (80). In support of this, retroviral expression of Pax3 does not activate Six1 and weakly activates Eya2, as shown using chick somite explants cultures (90). Six1 belongs to the sine oculis/Six homebox gene family of transcription factors that includes six members (Six1-6) (reviewed in (113)). Sine oculis was first identified in Drosophila
where it belongs synergistically with an eyeless (Pax6), eyes absent (Eya2) and dachshund (Dach2) network to establish proper eye development (47, 167, 200).

Although Six1, 4 and 5 are co-expressed during mouse myogenesis and mice null for either Six4 or Six5 have no developmental defects (119, 157, 190), Six1\textsuperscript{−/−} mice do not survive and have several organ defects (156, 235). In addition Six1\textsuperscript{−/−} foetuses have a selective loss of muscles, including diaphragm and limb muscles (124). Six1 and 4 have similar patterns of expression, as they are found in sensory organs of the head region and branchial arches, and extend their expression posteriorly into the dorsal root ganglia, somites and limbs (as reviewed in (113)). The initiation of Pax3 migrating hypaxial MPCs into the limb buds is not impaired in Six1\textsuperscript{−/−} embryos and subsequent Myf5 expression is initiated normally (124). Other Six factors such as Six4 and 5 may compensate for the loss of Six1\textsuperscript{−/−}, as Six4 and 5 are expressed in the migrating myoblasts of the limb (124). Mice null for Six1 and Six4 show a more severe phenotype as no muscle is detected in the limbs because of the downregulation of Pax3 and subsequent MRF expression (81).

A complete loss of eya is lethal in Drosophila (18), whereas loss of eyes absent (Eya2) show organ (7) and muscle defects (7). Eya2 is found to be expressed in the ventrolateral compartment of the DM with Pax3 and Six1 (90). Pax3 expression is lost in mice null for Eya1/2 and Six1/4, resulting in a loss of hypaxial muscle (80). Epaxial muscle is relatively unaffected in either Eya or Six double mutants (80-81). Furthermore, Grifone et al. illustrate that Six recruits Eya to drive transcription in the epaxial and hyaxial lips (80). In summary, Six1, and Eya2 are important for both epaxial and hypaxial muscle formation.
1.2.4 C-met and Lbx1

The proto-oncogene C-met, a member of the receptor tyrosine kinase family (158), encodes the receptor for its ligand hepatocyte growth factor (HGF)/scatter factor (25), and is required for limb, diaphragm and tongue development since homozygous C-met mutant mice developed all other skeletal muscles except the former (15). Particularly, expression of C-met and HGF is required for the delamination and migration of hypaxial DM cells to target sites, as C-met mutant mice lack myogenic precursors that invade the limb, diaphragm and tip of the tongue (60). Further studies demonstrate that Pax3 modulates the expression of the C-met receptor during limb muscle development, as there is a deficit of C-met expression in Sp mice and overexpression of Pax3 in cultured cells increases C-met expression (67). Interestingly, while C-met is found to be expressed in the lateral DM of all somites, including those that form the limb, diaphragm and tongue (Figure 2, Panel II) (15), expression of Lbx1, part of the ladybird family of genes, is restricted to the migrating hypaxial MPCs that delaminate from the VLL of cervical, occipital and limb DM, forming the diaphragm, tongue and limb muscles respectively (Figure 2, Panel II) (60-61, 105, 217). Lbx1 mutants lack or have reduced limbs in different limb regions (32). Particularly, Lbx1 expression is required for correct movement along migratory routes as aberrant migration patterns are observed in Lbx1 mutant mice, although delamination is observed to occur normally (32, 84, 197). Although Pax3 also lies genetically upstream of Lbx1, at the forelimb and hindlimb level (60), Lbx-1 is not directly under the control of Pax3. In comparison to Sp mutants, C-met mutant mice (receptor or ligand) still specify the migratory hypaxial MPC, as Lbx1 expression still takes place, but does not make limb muscles (60). Altogether studies
suggest that genetically C-met and Lbx1 can be placed downstream of Pax3 (17, 54, 60, 70, 76, 216).

1.2.5 Meox1

Meox1 and Meox2 are homeodomain transcription factors expressed in the paraxial mesoderm and regulate a variety of developmental processes. The Meox genes have a highly conserved sequence homology and both Meox1 and Meox2 have a conserved pattern of expression and function amongst several organisms studied so far (144). In mice, Meox1 is found as early as E7.0 in the PSM and in the differentiating somite, whereas Meox2 expression is restricted until the somites are formed (37, 176). During somitogenesis, Meox1 expression becomes restricted to the dermomytome and sclerotome, whereas Meox2 expression is upregulated later in the migrating myoblasts of the limb bud (37-38, 176). Expression of Meox1 decreases as somites mature, while Meox2 expression is maintained throughout the differentiating somite (176). While mice that lack Meox1 display no apparent defects, they do possess a few abnormalities in rib, vertebral and cranio-vertebral fusions (134, 176). Mice that lack Meox2 fail to make limb muscle, although muscles of the body wall are largely unaffected, and down-regulate the expression of Pax3 and Myf5 (133). Meox1 and Meox2 double knockout mice show severe loss of both epaxial and hypaxial muscle (134), suggesting that both family members are necessary for normal function of the DM and muscle development.
1.2.6 Gli2

Another set of transcription factors found to be important during muscle development are the zinc family of transcription factors, Gli1, Gli2 and Gli3. Its homologue Cubitis Interruptus (Ci) was first discovered as a Sonic hedgehog (Shh) effector in the fly (3). Although all three members were found in the developing somite of the mouse (100), later studies demonstrated that while Gli1 is expressed in the ventromedial somite, Gli2 and Gli3 are restricted to the dorsomedial somite, which gives rise to the epaxial myotome (21). In vitro, both Gli2 and Gli3 can activate and repress Shh target genes (53, 193). In vivo, mouse mutants illustrate that Gli2 acts primarily as a transcriptional activator (63, 139). Although Gli2 and Gli3 have redundant roles, a Gli2−/−Gli3−/− double mutant mice exhibit altered morphology of the epaxial myotome, as there is a ventromedial expansion of the hypaxial myotome (142).

1.2.7 Foxc1 and Foxc2

The Forkhead family of helix-turn-helix genes, including Foxc1 and Foxc2, have recently emerged as another important regulator of paraxial mesoderm cell fate and patterning. Foxc1/2 are expressed in the paraxial mesoderm and somites, and to some extent have redundant biological roles (93, 209, 229). Homozygous Foxc1 mutants are born with skeletal muscle defects, amongst other defects, and die pre- and perinatally, whereas Foxc2 mutants show similar prenatal abnormalities (122, 229). Mice lacking Foxc1/2 have a more severe phenotype, as the somites fail to form and the expression of MyoD is lost in the homozygous mutants, which fail to undergo myogenesis (123). Work done in P19 embryonal carcinoma cells illustrated
that Foxc1/2 expression depends on an interplay between the Shh and canonical Wnt signalling pathways (196), implicating Foxc1/2 as a transcription factor important for epaxial myogenesis. Although recent work done using genetic approaches and mouse explants cultures suggest that Foxc2 is negatively regulated by Pax3 and its removal promotes skeletal myogenesis (125).

1.2.8 En1 and Sim1

En1, a homeobox containing transcription factor, has been shown to be restricted to the medial-intercalated region of the epaxial DM, myotome and dermatome (46, 101, 207) (Figure 2, Panel II). Furthermore, Cheng et al. illustrate that En1 precursor cells originate from the dorsomedial edge of the somite and demonstrate that the bulk of En1 cells are part of the epaxial somitic programme in chick (46). Signals from the notochord/floorplate (Shh) and dorsal neural tube (Wnt1) specify En1 precursor cells (46, 101, 207), while the surface ectoderm (Wnt1-like) directly regulates and maintains the expression of En1 in the newly specified cells (46). On the other hand, the lateral plate mesoderm and possibly the roof plate, through BMP4 signals, negatively regulates En1 expression. In comparison, Sim1, a bHLH transcription factor, has been shown to be restricted in the central-ventral region of the hypaxial DM, myotome and dermatome (46, 61, 171, 207) (Figure 2, Panel II). Sim1 precursor cells originate from the lateral half of the somite and unlike En1 expression, Sim1 expression is positively regulated by the lateral plate mesoderm through BMP4 signalling (46, 61, 171, 207), and negatively regulated by the dorsal neural tube (Wnt1) (95, 136, 171). These studies place En1 and Sim1 into the epaxial and hypaxial somitic program, respectively.
1.3 Soluble Secreted Molecules that Regulate Skeletal Muscle Development

Transcriptional regulation during muscle development is governed by signalling molecules, such as Wnts, Retinoic Acid (RA), Sonic hedgehog (Shh), and BMP, which are expressed from the surrounding tissues such as the neural tube, notochord and overlying surface ectoderm (Figure 2).

1.3.1 Wnts and Signalling

The Wnt family of signalling molecules, the name originally derived from Wingless (Wg), a Drosophila segment polarity gene, and Int-1, the related mouse oncogene (9, 182), have been implicated in regulating various developmental processes, such as proliferation, asymmetric division, patterning and cell fate determination (99, 230). To date, there are 19 Wnt genes in mice and humans and are defined by a conserved primary sequence that includes 21 specifically spaced Cysteine residues. Wnts can mediate their signals via three independent mechanisms: the canonical signalling pathway, the planar-cell polarity pathway (PCP)/Wnt/JNK and the Wnt/Ca\(^{2+}\) pathway (Figure 4 (72)).

Canonical Wnt signalling is initiated in the plasma membrane when specific Wnt soluble glycoproteins bind their respective cell surface receptors of the Frizzled (Fz) family, at a Cysteine rich domain (CRD) (14), and co-receptors of the low-density lipoprotein (LDL) family, LRP5/LRP6 (129, 168, 223). A co-receptor is required in order for the Fz-mediated signal to be transduced to the β-catenin signalling cascade (129, 168, 223). In the absence of canonical Wnt signalling, β-catenin is complexed with the scaffolding proteins Adenomatosis polyposis coli
Figure 4. Mechanisms of Wnt Signaling. Wnts can mediate their signals via three independent mechanisms. **(A)** Activation of the canonical signaling pathway leads to the stabilization of β-catenin in the cytoplasm and the subsequent accumulation in the nucleus, positively regulating transcription of the Wnt target genes. **(B)** The planar-cell polarity (PCP)/ JNK pathway leads to the activation of JNK via the activation of small GTPases and Dishevelled (Dsh). **(C)** The activation of the Ca2+ pathway occurs when intracellular calcium is released, regulating the activity of enzymes such as PKC and CamKII, through the activation PI. This cascade also leads to the regulation of gene expression in the nucleus. Reproduced from reference [72] with permission from Elsevier © 2008.
(APC), AXIN, glycogen synthase kinase 3β (GSK3β) and casein kinase I (CKI). This 'destruction complex' sequesters cytoplasmic β-catenin, where GSK3β and CKI phosphorylate its N-terminal domain, and leads to its proteosomal degradation (as reviewed in (36)). The destruction complex is inactivated in the presence of Wnt through the recruitment of Dishevelled (Dsh/Dvl) to the Fz receptor, which leads to the phosphorylation of the LRP co-receptor by GSK3β and CKI and subsequent recruitment of Axin to the LRP co-receptor (as reviewed in (36)). The sequence of events results in the translocation of cytosolic β-catenin into the nucleus where it can complex with the DNA binding T-cell factor/lymphoid enhancer factor 1 (TCF/LEF) family of transcription factors, p300/CBP and Brg1 (a chromatin remodelling enzyme) turning on transcription of target genes (36, 91, 213). β-catenin is also found to recruit other cofactors such as the Legless family of docking proteins (BCL9 and BCL9L), which in turn is associated with the PYGO family of co-activators (PYGO1 and PYGO2) (as reviewed in (71)). Although TCF/LEF proteins bind to Wnt responsive genes in the absence of Wnt signalling, they are associated with a repressive complex that includes Groucho and histone deacetylases (HDACs). A combination of both this repressive complex along with chromatin remodelling machinery inhibits transcription of Wnt target genes in the absence of Wnt ligand (42, 45).

Non-canonical Wnt signalling is less understood and is β-catenin independent. The Wnt/Ca²⁺ and the Wnt/JNK (or PCP) pathways fall into the non-canonical Wnt category. In the Ca²⁺ pathway, Wnt binding to its Fz receptor leads to the release of intracellular calcium and the activation of CamKII and PKC (121, 201, 206). Studies suggest a role for the G-protein linked phosphotidylinositol (PI)
signalling in releasing the intracellular stored Ca\(^{2+}\) (121, 201, 206). The Wnt/JNK pathway involves the Rho (GTPase) mediated activation of JNK, which leads to the activation of effector proteins involved in controlling cell polarity, asymmetrical cell divisions and apoptosis (26, 130, 218).

1.3.1.1 Wnt Signalling during Development and Establishment of Myogenic Identity

As early as gastrulation and neurulation, a gradient of Wnt/\(\beta\)-catenin signalling is required for the anterior-posterior (AP) patterning of the embryo (see section 1.1) (98, 118). The expression of Wnts and their Fz receptors have been characterized during embryo development in chick and mouse. Several Fz receptors such as Fz1, -6, -7 in mouse and Fz1, -2, -7 in the chick are expressed in the newly formed somites of the embryo at all axial levels (20, 41). The expression of Fz8 in mice occurs later in epithelial somites becoming localized to the myotome in mature somites, while the expression of Fz9 occurs only in the myotome (20). Wnt1,-3a and -4 are expressed in the dorsal neural tube (41, 97, 161), while Wnt5b is expressed in the somites becoming restricted to the dorsomedial region overlapping with Myf5 and MyoD expression (41). The surface ectoderm (in addition to somites (101, 161)) expresses Wnt6 (41), and Wnt7a (211), while Wnt11 expression is found in the DML of the DM (50, 101) and in the ventral somite (41). Altogether, the expression of Wnts and Fz receptors in the axial midline structures and myotome place Wnt signalling early on during development. Further overexpression and knockout studies of Wnts and their receptors illustrate the importance of Wnt signalling in somite maturation and conferring myogenic identity (148, 208).
Ectopically expressed Wnt1, -3a, - 4 and β-catenin in chick embryos expanded the epaxial domain and the expression of Pax3 and Pax7 of the DM (40, 68, 154, 222). In addition, Wnt3a and Wnt1 null mice show a reduction of the medial compartment of the DM and of Pax3 and Myf5 expression (101, 236). In comparison, Wnt7a signalling from the dorsal ectoderm activated MyoD expression (211), whereas ectopic expression of Wnt6 was shown to regulate the expression of Pax3 and Pax7 (154). Wnt signalling, in addition to Shh signalling, was also shown to regulate the expression of Gli2 during somite patterning (21). Altogether, studies corroborate the importance of Wnt signalling in conferring myogenic identity as it effects the expression of transcription factors important for skeletal myogenesis.

The generation of separate myogenic domains from medial and lateral halves of the DM was demonstrated in the early nineties (152). In fact, later Myf5 and MyoD were shown to have distinct roles in epaxial and hypaxial muscle formation, respectively (109, 187). Therefore, it is plausible that different Wnts, expressed in different regions of the embryo, may regulate the preferential expression of Myf5 and MyoD. Mouse paraxial mesoderm explantation studies illustrate that Wnt1 signalling from the neural tube preferentially activates Myf5 expression (211). The activation is thought to be mediated preferentially via canonical Wnt signalling as studies illustrate an activated β-catenin is sufficient to upregulate Myf5 expression in the somite (19). However, later studies imply that Wnt1 can also mediate Myf5 expression non-canonically through adenylyl cyclase (AC) via PKA and CREB signalling (44). In comparison, Wnt6/7a signalling from the dorsal ectoderm activates MyoD expression (211) possibly playing a role in hypaxial myogenesis, as
Wnt7a<sup>−/−</sup> mice were demonstrated to have limb abnormalities (160). MyoD activation by Wnt is thought to occur through a non-canonical pathway, either by the activation of PKC (33) or by adenylyl cyclase (AC) signalling (44).

### 1.3.2 Retinoic Acid Synthesis and Signalling

Retinoic acid (RA), the derivative of Vitamin A, is important for the growth and patterning of the embryo. A lack of Vitamin A results in congenital malformations (reviewed in (149)). During embryogenesis, Retinol, supplied maternally, is synthesized into RA once inside the cell. There are two steps involved in canonical RA synthesis. The first, involves the conversion of retinol into retinaldehyde by two enzyme families, Retinol Dehydrogenase (RDH) and Alcohol Dehydrogenase (ADH). The second involves the oxidation of retinaldehyde to RA by the Retinaldehyde dehydrogenase (Raldh) class of enzymes. RA can now act in the nucleus as a ligand for the retinoic acid receptor (RAR). RARs (α, β, γ) heterodimerize with RXRs (α, β, γ) and bind to retinoic acid response elements (RAREs) in the absence of ligand (RA) recruiting co-repressors. These co-repressors inhibit transcription by recruiting HDACs and methyl-transferase complexes to DNA preventing its accessibility. A conformational change occurs when the ligand-inducible RAR is bound by RA resulting in the recruitment of activators and release of co-repressors (as reviewed in (149)).

Of the three Raldh family members, Raldh1,-2,-3, Raldh2 is expressed first and its expression is detected as early as the primitive streak, during gastrulation in the mesodermal cells and then finally becoming restricted to the posterior trunk region of the embryo (150). It is therefore not surprising that mice null in Raldh2
alleles die pre- and peri-natally and display several defects including those that effect the limbs and somites (reviewed in (149)). Raldh1 mutants are viable and act later during eye development, while Raldh3 are found to function in later stages of eye and nasal development (as reviewed in (149)).

1.3.2.1 RA Signalling during Development and Establishment of Myogenic Identity

RA has been shown to induce the expression of numerous genes, and several studies in different model organisms demonstrate that it plays an important role in the formation of somites. For instance, a loss of RA signalling results in the loss of the bilateral symmetry of somites as one side of the neural tube has fewer somites than the other (62, 114, 149). The antagonistic expression of Fgf8 and RA established in the PSM plays an important role for proper symmetry (Section 1.1) (62). While Fgf8 follows a caudal to rostral gradient, Raldh2 is predominately expressed in the rostral PSM (62, 219).

During the maturation of the somite, RA is needed for the differentiation of the myotome. Earlier studies demonstrated that the inhibition of RA synthesis results in the downregulation of MyoD, Myogenin and fast myosin expression in somites of zebrafish, whereas the exogenous addition of RA results in the upregulation of all three genes in addition to prematurely expressing MyoD and Myogenin in the PSM (87). In addition, myogenic differentiation is induced as MyoD expression is enhanced in C2C12 myoblasts, limb buds and cultured satellite cells of chick (2, 86, 146).
1.3.3 Shh – Another important signalling pathway

In addition to Wnt and RA, other signalling molecules, such as Sonic hedgehog (Shh) are expressed during embryonic development. Shh, part of the hedgehog family of signalling molecules, is expressed in the notochord, floor plate of the neural tube and the limb bud (107, 138). All three hedgehog family members, Shh, Desert hedgehog (Dhh) and Indian hedgehog (Ihh) signal by binding to Patched-1 (Ptc1), their cell-surface receptor (137). In the absence of hedgehog the cytoplasmic effectors Gli2 and Gli3 are kept from entering the nucleus, as Ptc1 inhibits the transmembrane protein Smoothened (Smo). In the presence of the ligand, the inhibition of Smo is relieved, and the Gli factors, which include the activators Gli1/2 and the repressor Gli3, translocate to the nucleus regulating gene expression (as reviewed in (102)). Shh in combination with Wnt1 and Wnt3 is sufficient to induce the myogenic program in somitic tissue explants (148). There is also direct evidence to illustrate that Shh can induce MyoD and Myf5 expression (24, 85, 215). Moreover, either Gli2 or Gli3 is required for Myf5 expression in muscle progenitors of the epaxial DM (see section 1.2.4) (142). It is therefore not surprising that Shh does not induce MyoD expression in the absence of Myf5 (22), in light of the fact that Myf5 expression is dependent on the presence of a Gli binding site within the epaxial enhancer of Myf5 (85).

1.4 Myogenesis in Stem Cells

The ability to proliferate, self-renew and the capability of differentiating into a wide variety of cell types are characteristic of stem cells. Although mouse and
human embryonic stem (ES) cells have not been extensively studied in terms of transcriptional regulation for differentiation in the skeletal muscle lineage, earlier studies demonstrate that mouse ES cells are capable of recapitulating the early steps of muscle development (185). Furthermore, several studies report successful mouse and human ES differentiation into muscle in culture (10, 184-185, 239).

The model stem cells used by our laboratory are the stable, euploid, pluripotent P19 embryonal carcinoma (EC) cells, derived from a teratocarcinoma formed following transplantation of a 7.5 day embryo into the testis of a male mouse (141). Teratocarcinomas can develop in some mouse strains from early embryos transplanted from the uterus into ectopic sites. The P19 cell line, containing undifferentiated stem cells, were established directly from the primary tumor which arose from the transplanted embryo (141).

The P19 cell line was found to grow readily in cell culture and appeared to differentiate using the same mechanisms as normal embryonic cells. When these cells were injected into the inner cell mass of embryos they could be detected in all tissues analyzed, illustrating that P19 cells have the potential to contribute to tissues from all three germ layers (186). Unfortunately these embryos showed atypical morphology and had tumours forming in the head. A number of characteristics make P19 cells valuable for studying early developmental events. Since these cells are immortal, they are very easy to grow and maintain in the undifferentiated state when compared to mouse or human ES cells, and they can be induced to differentiate by manipulation of culture conditions. The cells can maintain their pluripotency in culture for many passages and are amenable to genetic manipulation.
In addition, the genetic composition of these cells can be manipulated by either the selection of mutant strains or by selection of clones carrying transfected genes stably integrated into their genome.

These cells can be induced to differentiate into skeletal and cardiac muscle when aggregated and exposed to 0.5%-1% dimethyl sulfoxide (DMSO) (65). Aggregation of the cells causes induction of mesoderm and exposure to DMSO induces stem cell differentiation into muscle. Both DMSO and cell aggregation are necessary for muscle differentiation (140). Cardiac myocytes are visible after 6 days of differentiation, and bipolar skeletal myocytes after 9 days. While cardiomyocytes make up approximately 10-20% of the total cell population, skeletal myocytes only account for approximately 5-15% (65, 188). The mechanism of DMSO action on muscle differentiation is not fully understood, but studies indicate DMSO controls the release of intracellular Ca$^{2+}$ and epigenetic modification at several loci genome wide (104, 147). Factors in fetal calf serum, that have yet to be determined, have also been shown to play a role in the efficiency of P19 cell differentiation (227).

P19 cells were shown to express the muscle sarcomeric proteins MHC and cardiac-alpha actin along with the MRFs (203, 205), suggesting the possibility that P19 cells may behave similarly to embryonic cells involved in skeletal muscle differentiation. Further studies illustrate that P19 cells are able to recapitulate the expression pattern of genes found during embryonic muscle formation.

Mesoderm formation is marked by the presence of Brachyury-T (11) which is observed to be expressed as early as day 1 during DMSO-induced P19 cell differentiation (221). The Wnt signalling molecules Wnt3a and Wnt5b are also observed to be expressed as early as day 1 and 2 (179). The co-expression with
Brachyury-T reflects that the Wnt expression observed during P19 cell differentiation marks the primitive streak. As embryonic development progresses the DM (formed by the patterning of somite (see section 1.1)), the source of the muscle precursor cells, express the transcription factors Pax3, Pax7, Meox1, Gli2, Six1, Eya2, and Foxc1 (referred to as premyogenic mesoderm factors) (see section 1.1) which is also observed to follow the expression of Brachyury-T in DMSO-induced P19 differentiation (179-180, 196). A few days later the expression of Myf5, MyoD and Myogenin, which marks the myotome of the embryo (see section 1.1) are expressed in P19 cells (179-180).

Gain- and loss-of-function experiments revealed transcriptional and signalling networks involved in P19 cell differentiation. Genes and signalling pathways known to be involved in myogenesis, such as Pax3, Meox1, Gli2, Wnt3a, β-catenin and Shh were targeted. Cells stably expressing Wnt3a or an activated form of its downstream effector β-catenin are sufficient to induce the myogenic program as they express Pax3, Meox1 and Gli2 (166). The expression of Wnt3a results in the subsequent differentiation of these cells into skeletal myocytes, verified by the expression of MyoD and MHC, and activation of MRF function when co-expressed with cell lines over-expressing MyoD or myogenin in monolayer (179). Furthermore, aggregation of cells either expressing MyoD or myogenin induce the myogenic program (181, 205). A dominant-negative version of β-catenin results in a complete loss of these genes and prevents myogenesis (166). Pax3, Meox1 and Gli2 were demonstrated to act together in a regulatory loop to induce each other’s expression when P19 cells stably expressing these genes, or a dominant negative version, were examined. Ectopic Pax3 is only able to induce the expression of Meox1 and
itself (180), while Meox1 is sufficient to induce the expression of Pax3, Gli2 and itself (165). On the other hand, Gli2 is only sufficient and necessary for Pax3 and Meox1 expression (165). Of the three genes, only Gli2 and Pax3 are able to induce differentiation in the absence of DMSO (165, 180). The expression of the MRFs and MHC are lost in the dominant negative mutants of Pax3, Meox1 and Gli2 (165, 180). The observations reveal another layer of complexity in the transcriptional regulation of P19 cell differentiation.

In addition to Wnt, a role for RA signalling has also been identified in P19 cells. P19 cells were shown to constitutively express RARα and γ, while RARβ is found to be upregulated during the course of DMSO-induced differentiation (172). Furthermore, RA has been demonstrated to regulate skeletal and inhibit cardiac muscle development, by enhancing the expression of the muscle premyogenic markers and the MRFs (115). Shh signalling has also been identified in P19 cells as inducing the expression of Nkx2.5, GATA4 and Mef2c, subsequently controlling cardiac muscle development (73).

1.5 Thesis Summary

Understanding the molecular networks and signalling pathways which regulate the process of muscle development is essential in order to design future therapies based on adult or embryonic stem cells for restoring damaged muscles. The first major limitation to stem cell therapy is our inability to drive specific differentiation of these cells with any significant efficiency. Therefore, it becomes
important to study the mechanisms which control a cell's decision to proliferate or differentiate.

Previous results from our lab illustrated that MyoD is sufficient to drive aggregated P19 cells into the muscle lineage in the absence of DMSO (205). In addition, our lab has demonstrated that Wnt3a acts through the canonical pathway activating β-catenin and inducing the myogenic program. The experiments were designed based on the hypothesis that MyoD, which is thought to play a role in hypaxial muscle formation, directs P19 cells into the muscle lineage by binding the regulatory regions and activating the expression of the premyogenic mesoderm genes. While β-catenin, which is thought to play a role in epaxial muscle formation through Myf5, is essential for myogenesis by activating premyogenic mesoderm genes through direct and/or indirect mechanisms. Here we illustrate that MyoD binds to the regulatory elements of several premyogenic mesoderm genes, forming Lbx1- and C-met- positive muscle. In comparison, DMSO-induced differentiation of P19 cells significantly upregulates Sim1 and Lbx1, and slightly induces En1 expression, with no upregulation of C-met. Initiation of the canonical Wnt pathway via β-catenin activation is essential for the upregulation of premyogenic mesoderm factors, either directly or indirectly.
CHAPTER 2 - Materials and Methods

2.1 DNA Constructs

P19 EC cell lines used in these studies stably overexpress the transcription factor of interest or the dominant-negative version of the transcription factor. Dominant-negative transcription factors are chimeric proteins where the activation domain has been removed and replaced by the engrailed repressor domain, as previously described (165-166, 180). P19[Control], P19[MyoD], and P19[β-catenin*] cells lines used in the study have been previously made and described (166, 179). The P19[MyoD/EnR] cell line was generated by Dr. Peter J Gianakopoulos, Alan Ridgeway, and Michelle Waddington. Briefly, the transcriptional activation domain of MyoD (1-54 aa) (224) was removed by PCR of MyoD cDNA utilizing the oligonucleotides 5' GGATCCATGGCCCTGAAACCGGAG and 3' CTGCAGGTCGATCTCTCAAAGCACC. The 5' and 3' oligonucleotides contained BamH1 and Xhol sites (boldface) respectively, to facilitate cloning. The Δ(1-54 aa) MyoD cDNA was then cloned into the PGK-1 vector that contains the phosphoglycerate kinase-1 promoter (1). Subsequently, the 198 aa N-terminal repression domain (EnR) of the mouse En-2 protein was isolated from En-2 cDNA by PCR utilizing the oligonucleotides 5' AAGGATCCATGGGAGGAG AAGGATTCAAG and 3' AAGGATCCCGAGATGGCGTGGCTGCT and subcloned into the pGEM-T Easy™ (Promega™) vector. The 5' and 3' oligonucleotides
contained BamHI sites (boldface) to facilitate subcloning. The EnR was excised with BamHI and blunt end ligated to the BamHI site of PGK-Δ(1-54 aa)MyoD.

2.1.1 RNA interference Constructs

Complementary DNA sequences targeting nucleotides 3056 to 3076 (5’- TTTGGTTATCAAAACCCTAGCCTTCTCAAGAGAAGGCTAGGGTTTGATAACGCT TTTTT -3’ and 5’-CTAGAAAAAAAGCGTTATCAAAACCCTAGCCTTCTCCTTTGAGAAG GCTAGGGTTTGATAAC -3’ and nucleotides 312 to 334 (5’- TTTGAATCCATTCTGGTGCCACCTTCAAGAGAGGTGGCACCAGAATGGATTCTTTTTTTC -3’ and 5’- CTAGAAAAAAGGAATCCATTCTGGTGCCACCTCTCTTTGAGGTCCTTTTG TGGAGACACCAGAATG -3’) of mouse β-catenin (NM_007614) were annealed and cloned into the mU6pro vector (237) using the BbsI and Xbal restriction sites. One control vector was created using a scrambled sequence that is not complementary to any sequences in the mouse genome. (5’- TTTGACAAGATGAAGAGCACCAATTCAAGAGATTGGTGCTCTTCATCTTGTTGTTTTTT -3’ and 5’- CTAGAAAAAAACAACAGATTGAAGAGCACCAATCTCTTGAATTGGTGCTCTTCATCTTGT -3’). (Note that another scrambled control vector generated and described previously was also used (195) to create a stable cell line.) The mU6pro vector was provided by Dave Turner (University of Michigan, Ann Arbor, MI) and has been previously described (237).

2.2 Cell culture

P19 EC cells (ATCC) were cultured in alpha-essential media (Invitrogen, Burlington, Ontario, Canada) supplemented with 5% Cosmic Calf Serum (Hyclone,
Logan, UT or PAA Laboratories, Etobicoke, Canada) and 5% Fetal Bovine Serum (CanSera, Rexdale, ON, Canada or PAA Laboratories, Etobicoke, Canada) or 10% Fetal Bovine Serum (Wisent).

Differentiation was initiated by aggregating $5.0 \times 10^5$ cells for 4 days into 100mm Petri dishes/ non-tissue culture plates. Under these conditions the cells spontaneously form floating aggregates (7). The day after, day 2, the cells were transferred to a 150mm Petri dish. The aggregates were transferred into tissue culture dishes on day 4, changing media when necessary. The cells were aggregated either in the absence or presence of 1%DMSO (Sigma Aldrich, Canada). Note that the media was changed every day and fresh DMSO was only added to the aggregates from day 0 to day 3.

### 2.2.1 Transfection and Stable cell line generation

The short hairpin constructs were transfected into P19 cells as previously described (179). Briefly 0.8µg of either shβ-cat or shControl sequences were transfected utilizing the FuGene 6 reagent (Roche, Mannheim, Germany) alongside a plasmid containing puromycin (Invitrogen, Burlington, Ontario, Canada) for selection. Following puromycin selection, clones for each shRNA were pooled together and further analyzed.

### 2.3 Immunofluorescence

For detection of myosin heavy chain (MHC) expression, aggregates from each differentiation were plated on day 4 onto 35-mm tissue culture dishes
containing 0.1% gelatin-coated coverslips. On day 8 or 9, cells were washed with PBS, fixed with -20°C methanol for 5 min on ice, allowed to air dry for another 5 min, then rehydrated with Stockholm PBS (sPBS) for 15 min (or left overnight at -4°C for staining on another day). Cells were stained for myosin utilizing the anti-myosin heavy chain monoclonal antibody, MF20 (6), at a 1:1 dilution with sPBS. The cells were incubated with MF20 for 1 hour at room temperature or overnight at 4°C. Cells were then washed with sPBS and incubated with goat anti-mouse IgG(H+L) Cy3-linked antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:100 dilution with sPBS for 1 hour or overnight at 4°C.

After 2-3 washes with sPBS, the coverslips were mounted in a solution containing Hoechst dye, diluted 1:100 with sPBS and glycerol (1:1). Fluorescence was visualized utilizing a Zeiss Axioskop2 microscope. Images were captured on an AxioCam MRm camera and processed with Axiovision 4.7.2, Adobe Photoshop 7 and Canvas 11. For cell counts, MHC-positive cells were counted in 10-12 fields for each treatment per experiment over three independent experiments and data was expressed as a percentage of MHC positive cells over the total cell number.

2.4 Reverse Transcription and Quantitative PCR

The aggregates plated for differentiation were harvested on the days indicated using the RLT lysis buffer (Provided by the QIAGEN RNaseasy Mini Kit). The lysate was then homogenized with a 26G3/8 needle and RNA was isolated according to the manufacture's protocol (Qiagen, Mississauga, ON, Canada).
The total RNA was read by the Spectramax Plus 384 (or Nanodrop ND-100 Spectrophotometer (Thermo Fisher Scientific, Canada)) to determine the concentration of the RNA. The amount of RNase-free water needed to get a concentration of 0.167 µg/µl was added for each RNA sample. The RT was performed on 1 µg of RNA using the QuantiTect Reverse Transcription Kit according to the manufacture’s protocol (Qiagen, Mississauga, ON, Canada).

Q-PCR reactions were done using the FastStart SYBR Green Master Mix (Roche Applied Science) or the GoTaq qPCR Master Mix (Promega). A master mix containing 12.5 µl of the SYBR, 0.5 µl of forward and reverse primer (at a stock of 10 µM) and 6.5 µl of sterile ddH₂O was added to 5µl of cDNA, for a final volume of 25 µl. 1/20th of the resultant cDNA was used for Q-PCR. The PCR experiment was set at 2 stages. The first stage was set at 95°C for 10 min for 1 cycle. The second stage of 40 cycles was set up for 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. The fluorescence was recorded at the last step (72°C for 30 sec) of the second stage. All reactions of 25 µl were performed and analyzed on the ABI 7300 system (Applied Biosystems) using the SDS analysis software, while some experiments with a reaction volume of 12.5 µl were performed and analyzed on the Eppendorf Realplex2 system. All reactions were performed in duplicate, and Ct values for each treatment was normalized to β-actin levels for the corresponding day and treatment. Primers used for Q-PCR analysis are listed in Table 1 in Appendix A.

2.5 Northern Blots

Protocols and DNA probes utilized, with the exception of Pax3, have been
described previously (73, 165, 179-180, 204, 227). Briefly, total RNA was isolated from each differentiation utilizing the LiCl method. 12 µg of total RNA were separated on a 1% agarose and formaldehyde gel, transferred onto Hybond-N (Amersham Biosciences) by capillary action, and crosslinked by UV light irradiation. Blots were then hybridized to DNA probes labeled with (α-32P) dNTP by multi-prime labeling (Amersham Biosciences) for 16 h at 42 °C.

2.6 Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) assays were performed using P19[Control] and P19[MyoD] cell lines. The cells were aggregated for four days in the absence of DMSO, and harvested for ChIP analysis on day 0, 4 or 6. The cells were crosslinked with 1% formaldehyde for 1 hour and neutralized by adding glycine at 0.125M final concentration. Cells were washed with cold PBS and then resuspended in lysis buffer 1 (50mM HEPES-KOH pH7.4, 1mM EDTA, 140mM NaCl, 0.5% NP-40, 0.25% Triton X-100, 10% glycerol and protease inhibitor cocktail (Roche Applied Sciences, Laval, QC)) and gently rocked at 4°C for 10 min. The supernatant was removed after centrifugation and the pellet was resuspended and incubated with gentle rocking at 4°C for 10 min in lysis buffer 2 (1mM EDTA pH 8.0, 200mM NaCl, 0.5mM EGTA pH8.0, 10mM Tris pH8.0 and protease inhibitors) prior to the pellet being resuspended in 1mL of sonication buffer (1mM EDTA pH 8.0, 0.5mM EGTA pH8.0, 10mM Tris pH 8.0 and protease inhibitors). Cells were sonicated using a Sonic Dismembrator (Fisher Scientific, Canada) for a total of 15 x 20 second pulses with 1 min rest in between each pulse. Any insoluble chromatin
was removed by centrifugation at 13,000 rpm for 30 min at 4°C. The chromatin was divided into three tubes and each aliquot was brought up to 1 mL of TE buffer containing 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF and protease inhibitor cocktail. The chromatin was then pre-cleared for 2 hours by incubating with Rec-Protein-G Sepharose 4B conjugate beads (Invitrogen, Canada), which were preblocked with 1 mg/mL of BSA overnight and washed with TE three times before use. A 1% input sample of the total chromatin was set aside after pre-clearing. For immunoprecipitation, the chromatin was incubated overnight at 4°C with 5 µg of rabbit anti-mouse MyoD antibody (Santa Cruz Biotechnology Cat# sc-760x, Santa Cruz, CA), 5 µg of rabbit IgG antiserum (Zymed Laboratories, CA) or 5 µg of rabbit anti-mouse acetylated histone H3 (Millipore Cat# 06-599). The following day the chromatin was incubated with 30 µl of Protein-G Sepharose beads (1:1 bead:TE buffer) for two hours to capture the immune complexes. Consecutive 10 min washes were then performed on the beads with the following buffers: low salt (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.1), high salt (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.1), LiCl (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris pH 8.1) and TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The protein/DNA complexes were then eluted from the beads using 50 mM Tris pH 8.0, 1 mM EDTA, 1% SDS, 50 mM NaHCO₃ and reversed crosslinked overnight at 65°C with the addition of 200 mM NaCl. The following day the samples were incubated for one hour with 20 µg Rnase A at 37°C followed by a two hour incubation at 65°C with 40 µg Proteinase K. The DNA was then purified using Qiagen's PCR Purification Kit (Qiagen, Mississauga, ON). Relative enrichment of binding sites were compared to
the IgG negative control and shown as a percent input. Immunoprecipitation was analyzed from 30-60 μg of chromatin using Q-PCR, as described above. Primers used for ChIP Q-PCR analysis are listed in Table 2 in Appendix A.

2.7 Statistical Analysis

Statistical differences were calculated between means using the Student’s t-test. P-values of at least $p \leq 0.05$ were considered to be significant.
CHAPTER 3 - Results

3.1 β-catenin* was sufficient to induce the expression of premyogenic mesoderm factors but not MHC or the MRFs

Previous experiments have demonstrated, by northern blot analysis, that an activated β-catenin*, Xenopus β-catenin where the N-terminal domain is removed, was sufficient to induce the expression of the premyogenic mesoderm genes Pax3, Gli2, Meox1, and Six1 (166). To analyze the role of β-catenin in more detail, the quantitative approach of Q-PCR was used and wider selections of genes were examined.

To identify whether β-catenin* regulates the expression of premyogenic mesoderm factors, RNA from differentiated P19[Control] and P19[β-catenin*] clonal populations were harvested on day 0 and 9. Q-PCR analysis revealed that β-catenin* is sufficient to upregulate the expression of the premyogenic mesoderm genes (Figure 5, Panel I). On day 9, Meox1 and Pax3 transcripts were 714-fold (+/- 320, n=8) and 593-fold upregulated (+/-511, n=9), respectively, in P19[β-catenin*] cells, while Gli2 was upregulated 3-fold (+/- 0.4, n=9). In addition, Foxc1 and Foxc2 were upregulated 31-fold (+/- 13, n=9) and 97-fold (+/- 38, n=9), respectively, in P19[β-catenin*] cells on day 9 (Figure 5, Panel I). Interestingly, Six1 was not upregulated by β-catenin* in these experiments. These results are in agreement with published results that β-catenin* is sufficient to induce the expression of premyogenic mesoderm factors and extends them to show upregulation of Foxc1/2 (166).
Figure 5. The over-expression of β-catenin in aggregated P19 cells induced the expression of the premyogenic mesoderm genes but not the MRFs. P19[Control] and P19[β-catenin*] stable cell lines were differentiated in the absence of Me2SO. Total RNA was harvested on days 0 and 9. (Panel I, II): The genes indicated were analyzed using RT Q-PCR. For this experiment, the data has been normalized to the expression of the housekeeping gene β-actin, and is expressed relative to day 0 P19[Control] cells. Error bars represent the average +/- SEM, of three independent differentiations of three separate clones for the P19[β-catenin*] cell lines (n=9), and of one clone for the P19[Control] cell line (n=3). Statistical analysis was performed using the type three, one-tailed Student’s t test (vs. control cells), with p < 0.05 considered statistically significant. Outliers were removed for Myogenin and MEOX1 from one or more differentiations of particular P19[β-catenin*] clones, and therefore represent an n=6 and n=8, respectively. (Panel I): The premyogenic mesoderm genes. (Panel II): The Myogenic regulatory factors. (Panel III): On day 9, cells were fixed and stained with Hoechst dye to visualize the nuclei (A, C, E) and anti-MyHC antibody to visualize muscle (B, D, F). Scale bar: 10μm.
The β-catenin* expressed in these cells is an activated form, from Xenopus, as it does not contain a region of its N-terminal domain that is normally phosphorylated by GSK-3β and CKI, targeting β-catenin for proteosomal degradation (166). Q-PCR analysis demonstrated that exogenous Xenopus β-catenin* was overexpressed on day 9 of differentiation on average of $45 \times 10^4$-fold ($\pm 15 \times 10^4$, n=9) over background (Figure 5, Panel I).

Previous experiments further demonstrated that β-catenin* could convert P19 cultures into small amounts of skeletal muscle (166). To re-examine this, P19[β-catenin*] cell lines, differentiated in the absence of DMSO, were immunostained for myosin heavy chain (MHC) and total RNA was extracted and analyzed for MRF expression. As expected P19[Control] cells aggregated in the presence of DMSO formed MHC-positive skeletal myocytes, while P19[Control] cells aggregated in the absence of DMSO did not, as seen by the lack of MHC staining (Figure 5, Panel III, B compared to D). In contrast with previous results, P19[β-catenin*] did not form any amount of skeletal muscle on day 9, indicated by the lack of MHC staining (Figure 5, Panel III, F). At the level of gene transcription, the MRFs Myf5, MyoD and myogenin were not found to be upregulated (Figure 5, Panel II). Variation in serum lots may explain the differences observed in the extent of differentiation (227).

3.2 Loss of endogenous β-catenin expression results in the downregulation of premyogenic mesoderm factors

To assess the role of endogenous β-catenin and whether it is essential to regulate the expression of premyogenic and/or myogenic regulatory factors during
skeletal myogenesis, we utilized RNA interference to knockdown the expression of β-catenin. Two P19[Sh-β-cat] and P19[ShControl] cell lines were generated that stably express a unique short-hairpin construct targeting either β-catenin [Sh-β-cat] or a scrambled [ShControl] sequence with no homology to any sequences in the mouse genome. Each cell line was generated from pooled clones and both [Sh-β-cat] cell lines and [ShControl] cell lines were grouped as one for analysis. Q-PCR analysis of gene expression indicated a 55% (± 8.5%) and a 35% (± 11%) knockdown of β-catenin on day 0 and 9 in P19[Sh-β-cat] cells, when compared to P19[ShControl] cells in DMSO-induced differentiation (Figure 6, Panel I). On the other hand, a knockdown of β-catenin was not observed in P19[Sh-ft-cat] cells on day 5 when compared to P19[ShControl].

Further analysis of gene expression in P19[Sh-β-cat] cells revealed that Pax3, Six1, Eya2 and Pax7 showed a 45% (± 8.0%, n=8), 67% (± 13%, n=7), 69% (± 10%, n=8) and 83% (± 8.3%, n=8) decrease on day 9, respectively (Figure 6, Panel II). Moreover, Gli2, Foxc1, Meox1 and Foxc2 showed a 61% (± 7.7%, n=8), 64% (± 12%, n=8), 70% (±13%) and 84% (± 6.8%, n=8) decrease on day 9, respectively (Figure 6, Panel II). The results illustrate a significant downregulation of all premyogenic mesoderm factors by day 9. The downregulation of premyogenic genes is consistent with previous results performed with a dominant negative β-catenin, termed P19[β-catenin/EnR] (166).
Figure 6. Knockdown of β-catenin in P19 cells inhibited the expression of Pax3, Pax7, Eya2, Six1, Meox1, Foxc1/2 and Gli2. Pooled stable cell lines expressing shRNA targeted to β-catenin, termed P19 [Shβ-cat], or targeted scrambled sequences, termed P19[ShControl] were differentiated in the presence of 1% Me2SO, and RNA was harvested on day 0 and 9. Quantitative polymerase chain reaction was performed to determine (Panel I): The efficiency of β-catenin knockdown throughout the differentiation and (Panel II): The expression of Pax3, Pax7, Eya2, Six1, Meox1, FoxC1/2 and Gli2 on day 9 of differentiation. Error bars represent the average +/- SEM, of four independent differentiations of two clones for both β-catenin and control shRNA (n=8). The data was normalized using β-actin as an internal control and Control day 0, and each gene is represented as a percentage of the expression compared to control cell lines for the particular day. Statistical analysis was performed using the type two, one-tailed Student’s t test (compared with the control group), with p < 0.05 considered statistically significant. Outliers were removed for Six1 and Foxc2 (day9) and Foxc1(day5) from particular control or scrambled clones of one experiment and therefore represent an n=7.
3.3 Loss of endogenous β-catenin expression results in a loss of skeletal muscle and a downregulation of MRF expression

Given that there was an overall downregulation of several premyogenic factors, we performed MHC staining and analyzed mRNA expression of the MRFs to determine if there is a defect in muscle commitment and differentiation. An overall decrease in the number of skeletal myocytes was observed in P19[Sh-β-cat] cells when compared to the P19[ShControl] cells, as seen by immunostaining against MHC (Figure 7, Panel I, D compared to B). A quantification of the number of MHC-positive cells revealed a 4-fold decrease in overall myogenesis (Figure 7, Panel II). Moreover, MRF expression was found to be significantly downregulated in P19[Sh-β-cat] cells by 89-fold (± 5.0, n=8) for MyoD and Myogenin and 79-fold (± 18, n=8) for Myf5, when compared to P19[ShControl] (Figure 7, Panel III). These results illustrate that β-catenin is essential for skeletal muscle formation and MRF expression in P19 cells.

3.4 DMSO-induced P19 cell differentiation significantly upregulates the expression of Lbx1 and Sim1

We were interested in identifying the type of skeletal muscle made in DMSO-induced P19 differentiation. Total RNA, for P19 cells differentiated in the presence of 1% DMSO, was harvested on day 0, 6 and 9 and mRNA expression of Lbx1, C-met, En1, and Sim1 were analyzed using RT Q-PCR (Figure 8, Panel I). Sim1 was significantly upregulated by 15-fold (± 4.3, n=3) on day 6 relative to day 0. Whereas Lbx1 was significantly upregulated by 323-348-fold (± 83.7-185, n=3) on day 9 and 6, respectively, relative to day 0. The transcript levels of C-met were relatively
Percent fold change relative to control

- MyoD
- Myf5
- Myogenin

Shb-cat Control

Percent relative to control

Control Shb-cat

% positive MHC

Hoechst Dye
Antibody

p<0.04
p=0.05
p<0.01
p<0.001
Figure 8. DMSO-induced P19 differentiation results in the upregulation of Sim1 and Lbx1. P19 cells were differentiated in the presence of 1% Me2SO, and RNA was harvested on day 0 and 9. Quantitative polymerase chain reaction was performed to determine the mRNA expression of En1, Sim1, C-met and Lbx1. Error bars represent the average +/- SEM, of three independent differentiations (n=3). The data was normalized using β-actin as an internal control and each gene is represented as a fold change relative to day 0. Statistical analysis was performed using the type two, one-tailed Student’s t test (compared with the control group), with p < 0.05 considered statistically significant.
unchanged, while En1 transcripts were slightly upregulated on day 9, but not significantly. In comparison, the levels of Lbx1, C-met, En1, and Sim1 were not upregulated in P19[β-catenin+] cells (data not shown).

3.5 **MyoD was sufficient to induce myogenesis and the expression of premyogenic mesoderm factors**

Initial experiments have demonstrated that MyoD was sufficient to convert P19 cells into the skeletal muscle lineage (179, 205). To understand how MyoD functions as a master regulatory gene in a stem cell context, MyoD gain- and loss-of-function studies were utilized to determine direct or indirect gene targets of MyoD. Furthermore, ChIP was performed in P19[MyoD] cells in order to determine which genes are directly regulated by MyoD.

To confirm and extend previous results, P19[MyoD] cell lines were differentiated in the absence of DMSO and immunostained for myosin heavy chain (MHC). In agreement with previous results, P19[MyoD] cells had robust skeletal muscle formation, indicated by the MHC staining (Figure 9, Panel I, D), compared to P19[Control] cells which lacked MHC staining (Figure 9, Panel I, B).

Northern blot analysis illustrated that the P19[MyoD] cells over-express MyoD throughout the course of differentiation, day 0 to 6 (Figure 9, Panel II) (Work done by Dr. Peter Gianakopoulos/Alan Ridgeway). In order to assess which premyogenic mesoderm genes were upregulated during the course of differentiation, RT Q-PCR was performed on the total RNA isolated on days 0 and 4-6 from P19[MyoD] and P19[Control] cells. A few of the premyogenic mesoderm genes initially expressed
Figure 9. The over-expression of MyoD in aggregated P19 cells induced the expression of the premyogenic mesoderm genes and MRFs. P19[Control] and P19[MyoD] cells were differentiated in the absence of Me₂SO. Total RNA was harvested on days 0 and 2-6. (Panel I): On day 6, cells were fixed and stained with Hoechst dye to visualize the nuclei (A, C) and anti-MyHC antibody to visualize muscle (B, D). Scale bar: 10μm. (Panel II): Western blot with 25-50 μg of protein were blotted with the factors indicated on the right. Lanes are indicated at the bottom. (Panel III): Genes indicated above were analyzed using RT Q-PCR. The data has been normalized to the expression of β-actin, and is expressed relative to day 0 P19[Control] cells. Error bars represent the average +/- SEM of three independent differentiations of a single clone. Statistical analysis was performed using the type two, one-tailed Student’s t test (vs. control cells), with p < 0.05 considered statistically significant.
in the paraxial mesoderm and developing somite of the embryo, such as Meox1, Pax7, Six1, and Eya2, were upregulated significantly on day 4, whereas Pax3 was found to be significantly upregulated on day 6 in P19[MyoD] cells compared to P19[Control] cells (Figure 9, Panel III). Upregulation of the myoblast markers myogenin and Myf5 occurred subsequent to the early premyogenic mesoderm genes on days 5-6, in agreement with previous results. In addition, the migrating hypaxial muscle precursor markers C-met and Lbx1 were also found to be upregulated significantly by day 4 or 5 of differentiation. Altogether, these results demonstrate that over-expression of MyoD leads to the upregulation of the premyogenic genes and genes that mark the migrating hypaxial muscle prior to the upregulation of the myoblast markers in aggregated P19 cells.

3.6 The expression of premyogenic mesoderm factors are lost and myogenesis does not occur in the presence of a dominant negative MyoD

To assess the role of endogenous MyoD and whether it is essential to regulate the expression of premyogenic and/or the myogenic regulatory factors during skeletal myogenesis, we utilized a dominant negative version of MyoD, termed P19[MyoD/EnR]. This dominant negative approach should result in a loss of function of all MRFs, thus removing the complication of redundancy between the family members. Loss-of-function experiments were performed by comparing aggregated P19[MyoD/EnR] cells to P19[Control] cells (both generated previously) in the presence of 1%DMSO. P19[MyoD/EnR] cell lines overexpressed a dominant-negative MyoD fusion protein, in which the activation domain of MyoD was replaced
with the repressor domain of the mouse En-2 protein, as described previously (165-166, 180). P19[MyoD/EnR] and P19[Control] cells were differentiated in the presence of DMSO and immunofluorescence was performed on day 9 with MF20 antibodies (6). P19[MyoD/EnR] cells did not differentiate into skeletal myocytes when compared to the P19[Control] cells (Figure 10, Panel I, D compared to B). Therefore, the over-expression of MyoD/EnR in P19 cells inhibits skeletal myogenesis.

To identify genes inhibited by MyoD/EnR, cells were differentiated in the presence of DMSO and total RNA was harvested on days 0 and 9, as indicated. By Northern blot analysis there were high levels of MyoD/EnR in P19[MyoD/EnR] cell lines and undetectable levels in P19[Control] cells (Figure 10, Panel II, A and B; Panel II was performed by Dr. Peter Gianakopoulos). The P19[Control] cells showed strong mRNA expression of the premyogenic mesoderm transcription factors on day 9, while the MyoD/EnR clones showed no detectable expression of Eya2, Six1 and Meox1 (Figure 10, Panel II, C, D and E, respectively). The mRNA transcript levels of Gli2 were moderately affected by the presence of the MyoD/EnR protein. On day 9 Gli2 was down-regulated in the majority of MyoD/EnR clones compared to P19[Control] cells (Figure 10, Panel II, F). Furthermore, the utilization of Q-PCR analysis illustrated that the mRNA expression of Pax3/7, Foxc1/2, MEF2C, myogenin, and Myf-5 were all completely downregulated (Figure 10, Panel III). Altogether, the presence of the MyoD/EnR fusion protein in P19 cells ablated the expression of premyogenic mesoderm factors and myoblast markers and subsequent skeletal myogenesis during the course of differentiation.
3.7 MyoD binds directly to the regulatory regions of the premyogenic mesoderm genes

Given that the expression of the premyogenic genes are upregulated during MyoD-induced differentiation and downregulated in the presence of a dominant-negative MyoD, we sought to determine if the observed changes in gene expression were due to direct or indirect effects of MyoD. To address this, chromatin immunoprecipitation (ChIP) experiments were performed on P19[MyoD] cells. The primers for this experiment were designed based on ChIP sequencing results that identified regions bound by MyoD in C2C12 myoblasts (done by the Tapscott laboratory) (39). Results illustrated that P19[MyoD] cells bound significantly to the regulatory regions of Pax3, Eya2, Pax7, Six1, myogenin and Meox1 when compared to the IgG control, at all time points examined (Figure 11), which is in agreement with their upregulation during the course of differentiation (Figure 9, Panel III, P19[MyoD] cell line). Pax3 and Eya2 had the highest levels of MyoD binding, followed by Pax7, myogenin, Six1, and lastly Meox1. The regulatory regions of Gli2, Foxc1 and Foxc2 were not significantly bound by MyoD, which is in agreement with their lack of mRNA upregulation in P19[MyoD] cells during the six day differentiation. In conclusion, MyoD binds to the regulatory regions of the premyogenic genes, with the exception of Gli2, Foxc1 and Foxc2.
Figure 11. MyoD binds to the regulatory regions of Pax3/7 Eya2, Six1, Meox1 and myogenin. P19[Control] and P19[MyoD] stable cell lines were differentiated in the absence of Me2SO and Chromatin immunoprecipitation was performed using an anti-MyoD antibody to identify enriched MyoD targets in P19[MyoD] cells on day 0, 4, and 6 of differentiation. Q-PCR was used to analyze the chromatin isolated, and MyoD was shown to bind regulatory regions of the Pax3/7, Eya2, Six1, Meox1 and myogenin on all three days. The regions are shown to be evolutionarily conserved and were shown to be enriched by Chip sequencing of MyoD in C2C12 cells (Tapscott, unpublished). Error bars represent the average +/- SEM, of three independent differentiations of a single clone. Statistical analysis was performed using the Student’s t test (vs. IgG), with a p value of at least p < 0.03 considered statistically significant.
CHAPTER 4 - Discussion

We have shown that ectopically expressed β-catenin* or MyoD was sufficient to upregulate the expression of premyogenic mesoderm factors in P19 cells under non-muscle inducing conditions. However, while cells that ectopically expressed β-catenin* failed to progress past the myoblast stage and did not express MyoD, Myf5, myogenin or MHC, cells that ectopically expressed MyoD differentiated efficiently and expressed the MRFs and MHC. Knockdown of endogenous β-catenin or the presence of a dominant-negative MyoD resulted in a decrease in the expression of premyogenic mesoderm factors and subsequent skeletal muscle differentiation, indicated by the downregulation or absence of MyoD, Myf5, myogenin and MHC. Furthermore, ChIP experiments performed in MyoD overexpressing cells illustrated that MyoD binds to the regulatory regions of the Pax3/7, Meox1, Eya2, and Six1 genes, in addition to myogenin.

In comparison, we found that MyoD may function similarly to β-catenin, by regulating the expression of Pax3, Pax7, Meox1, Six1 and Eya2 directly, leading to the specification of these cells into the myogenic lineage. These findings support and extend a model in which β-catenin regulates the expression of Pax3, Pax7, Meox1, Gli2, and Foxc1, functioning to positively enhance the specification of these cells into the myogenic lineage. Furthermore, these findings illustrate that exogenous MyoD can bind and activate the expression of premyogenic mesoderm genes transforming multipotent stem cells into skeletal muscle (Figure 12).
Moreover, preliminary results suggest that MyoD-induced differentiation upregulates a different subset of muscle-specific genes when compared to DMSO-induced differentiation. Cmet, Sim1 and Lbx1 are differentially expressed in the hypaxial domain of the developing embryo (Figure 2, Panel II), marking different regions of the DM and myotome (Their expression provides clues towards identification of the type of muscle made in the embryo). Our results illustrate that, while MyoD-induced differentiation leads to the formation of muscle expressing the essential limb, diaphragm and tongue markers Cmet and Lbx1, DMSO-induced differentiation leads to the upregulation of Sim1 and Lbx1 transcripts.

Our current P19 skeletal muscle differentiation model (Figure 12) illustrates that Wnt signaling via β-catenin results in the activation of premyogenic mesoderm factors Gli2, Pax3, Six1, Meox1, Eya2, and Foxc1 (40, 68, 166, 196, 222). Furthermore, this model demonstrates that MyoD overexpression results in the similar activation of the premyogenic mesoderm genes.

4.1 β-catenin expression is sufficient for the expression of premyogenic mesoderm factors

Our finding that β-catenin* can regulate the expression of Pax3/7, Meox1, Gli2, and Foxc1/c2 is consistent with and extends previous results that β-catenin* and Wnt3a enhance the specification of P19 cells into the skeletal myogenic lineage (166, 196). However, unlike previous results, P19[β-catenin*] cells did not express Six1 or the MRFs and were negative for MHC staining (166) (Figure 8). First, the differences can be attributed to changes in serum conditions. The differentiation
Figure 12. A simplified model illustrating the transcriptional network of skeletal myogenesis in P19 cells. Black arrows represent direct interactions found in the MyoD study. Grey arrows represent what was already known and was further supported in the β-catenin study.
serum is not defined, and over the years different lots can be attributed to the differences observed during the course of differentiation (227). For example, Nkx2.5, important in heart development, was able to initiate cardiomyogenesis is some sera but not in others (106). In fact, variability is observed when using the same serum lot, as indicative by the large error bars (Figure 8, Panel III). Second, the differences can be attributed to the changes in expression of the stably integrated β-catenin* in the cell lines. Although Q-PCR analysis illustrates that β-catenin* expression is highly expressed on day 9 of P19[β-catenin*] cells compared to P19[Control] cells (Figure 5, Panel I), it is difficult to conclude whether the levels are comparable to previously published results (166). It is possible that the levels of β-catenin* expression may have decreased in the β-catenin* cell lines and may contribute to the lack of muscle formation observed.

Although the P19[β-catenin*] cells did differentiate into skeletal muscle previously, the extent of their differentiation was minimal in comparison to P19 DMSO-induced differentiation, or to Wnt3a-induced differentiation (166). Recent studies in satellite cells suggest that β-catenin may promote self-renewal of muscle precursors in P19 cells, with fewer cells undergoing myogenesis (164), as they demonstrate that constitutively active β-catenin expression in satellite cells results in an increase in the proportion of Pax7 positive cells, without increasing MyoD expression (164). We postulate that β-catenin may be playing parallel roles in the adult and embryo.

It is highly plausible that the premyogenic mesoderm factors are directly or indirectly regulated by β-catenin, as disruption of wild-type β-catenin function
resulted in a decrease of all premyogenic factors analyzed on day 5 and day 9, with the exception of Pax3 and Eya2 which were not found to be downregulated on day 5 (Figure 11, Panel II). Interestingly, the levels of β-catenin in P19[Sh-β-cat] cells were similar to P19[ShControl] cells on day 5, making the day 5 results difficult to interpret. In theory, β-catenin knockdown should be consistent throughout differentiation, but the inconsistency can be attributed to the site of gene integration and chromatin accessibility. Each pooled stable cell line has a mixed population of cells (from different clonal populations) that have shβ-catenin integrated in different regions of the genome. The area of integration could affect expression during the course of differentiation as chromatin accessibility may vary spatially and temporally. The unchanged Pax3 and Eya2 expression on day 5 could be a direct result of the unchanged expression of β-catenin, especially if Pax3 or Eya2 are direct targets, or can be attributed to the fact that both transcription factors are indirect targets of β-catenin. It is possible that Meox1, Gli2 and Six1 are direct downstream targets of β-catenin and their lack of expression eventually leads to the downregulation of Pax3 and Eya2 after day 5 in P19[Sh-β-cat] cells, as an autoregulatory loop is formed with these transcription factors (Figure 12).

The expression of the premyogenic mesoderm factors is important for the expression of the MRFs and subsequent muscle formation in P19 cells (Figure 12) (165, 180, 202). Therefore, it is not surprising that, P19[Sh-β-cat] cells did not differentiate into muscle to the same extent as in P19[ShControl] cells when aggregated in the presence of DMSO (Figure 7). Furthermore, these results extend previous results and demonstrate that β-catenin is essential for skeletal myogenesis.
in P19 cells. Previous results with a dominant negative β-catenin, β-catenin/EnR, illustrated the importance of the transcription factor in skeletal myogenesis, as all premyogenic muscle precursors and MRFs were inhibited (166). However, an engrailed fusion protein cannot be compensated for by other transcription factors on promoters of target genes, whereas a shRNA approach allows for compensation by other factors, as the target gene is not repressed. Thus, our results extend the dominant negative study, demonstrating that β-catenin is essential for myogenesis and cannot be compensated by other factors.

4.2 MyoD behaves similarly to β-catenin in regulating premyogenic mesoderm factors

Literature demonstrates that the MRFs, including MyoD, are sufficient to drive a wide variety of cell types into the skeletal muscle lineage, illustrating the importance of the MRFs as 'master regulators' (5, 28, 57, 151, 232). Furthermore, work done in P19 cells has demonstrated that stably expressed MyoD or Myogenin are sufficient to induce the myogenic program (181, 205). We therefore sought to determine whether the molecular mechanism behind the myogenic induction by MyoD was similar to the current P19 model (Figure 12). Our findings illustrate that MyoD can induce the expression of Pax3/7 and Meox1. Unlike β-catenin, MyoD is unable to induce the expression of Gli2 and Foxd/2 but is capable of inducing the expression of Eya2 and Six1. Furthermore, all the premyogenic mesoderm genes are found to be downregulated in the presence of a dominant-negative MyoD, with the exception of Gli2 in a few clones (Figure 10, Panel II). The question lies in understanding how MyoD initiates the same set of transcription factors as DMSO- or
β-catenin-induced myogenesis in P19 cells (Figure 12). ChIP assays demonstrate that MyoD binds directly to the regulatory regions of Pax3/7, Meox1, Eya2, Six1 and myogenin, initiating gene transcription (Figure 11).

The relevance of this novel finding becomes important when you consider the possibility of a feedback loop established between MyoD and the premyogenic mesoderm factors playing a role in the amplification and maintenance of each other's expression during normal DMSO-induced P19 differentiation. Although initially several of the premyogenic mesoderm factors found to be expressed in the somite were thought to be restricted to the DM, studies have shown that they are also found in the myotome (24, 37, 69, 81, 90, 124, 142, 178), with the exception of Foxc1/2 which are expressed only in the paraxial mesoderm and somites in mouse (93, 125, 209, 228). This idea is further supported by the activation of Six1 by forced expression of MyoD or Myf-5 into MyoD-/-;Myf-5-/- fibroblasts (103) and by the loss of MRF expression in Six1/Six4 null mice (44). Furthermore, in proliferating C212 cells Eya1, which is also expressed in somites (234), was shown to be a direct target of MyoD (16). Finally, our finding that myogenin is not upregulated by MyoD until the premyogenic mesoderm genes, including Six1, are expressed, is consistent with the recent finding that Six1 can regulate MyoD function in C2C12 cells (131).

It was originally thought that satellite cells were derived from cells that mark the fetal myoblasts in the chick and mouse (52, 88). But more recent studies have implicated a primitive satellite cell progenitor population that expresses Pax3 and Pax7 (112, 178). Furthermore, it was shown that essentially all satellite cells in an adult originate from MyoD-positive progenitors (110). This suggests a potential role
for MyoD in maintaining premyogenic mesoderm expression. Finally, Zammit et al illustrated that activated satellite cells initially co-express Pax7 and MyoD. A subset of cells lose MyoD and can return to a quiescent, Pax7-positive state (238). Thus a role for MyoD in maintaining the expression of premyogenic mesoderm genes may potentially be important at various stages of embryogenesis and/or satellite cell regeneration.

4.3 MyoD induced differentiation leads to limb muscle marker gene expression

Interestingly, P19[MyoD] cells upregulated the expression of C-met and the migrating hypaxial MPC marker Lbx1 (Figure 9, Pane III). Unlike Lbx1 which is only expressed in the cervical, occipital and limb somites (60-61, 105), C-met marks the ventro-lateral regions of all somites (15). Interestingly, both are required for limb, diaphragm and tongue muscle development (15, 32, 60-61, 105). These results demonstrate that MyoD induced the formation of skeletal muscle expressing limb markers. Furthermore, the absence of En1 and Sim1 expression (data not shown), which marks the central epaxial or hypaxial domains respectively (Figure 2, Panel II), supports the idea that MyoD-induced differentiation leads to the formation of muscle expressing the essential limb, diaphragm and tongue markers Cmet and Lbx1.

In comparison, during DMSO-induced P19 differentiation Sim1 and Lbx1 expression are both found to be significantly upregulated, whereas En1 levels are found to increase slightly (although not significantly) and C-met levels are relatively unchanged (Figure 8). C-met mutants (receptor or ligand) were shown to still
specify the migratory hypaxial MPC, as Lbx1 expression still takes place, but does not make limb, diaphragm or tongue muscles (60). Similarly, the upregulation of Lbx1 and the lack of upregulation in C-met expression in DMSO-induced P19 differentiation suggest that these cells still specify the migratory hypaxial MPC (indicative by Lbx1 expression) before they begin migrating. The presence of Sim1 suggests that the muscle formed may be that of the central-ventral (lateral) hypaxial myotome, excluding the muscle made from the migratory MPCs as C-met expression is not upregulated. Further gene analysis and staining need to be performed, in order to determine the type of muscle made during DMSO-induced and MyoD-induced P19 cell differentiation.

In summary, we have provided evidence that MyoD-induced differentiation behaves similarly to β-catenin-induced differentiation in P19 cells as both transcription factors initiate the expression of the premyogenic mesoderm factors. Furthermore, we have demonstrated that MyoD induces muscle markers that are expressed in hypaxial migratory MPCs, Lbx1 and C-met, and that MyoD directly binds and regulates the expression of several premyogenic mesoderm genes. In comparison, DMSO-induced P19 differentiation leads to the formation skeletal muscle that is Sim1 and Lbx1 positive. We thus propose differential roles for MyoD and β-catenin during myogenesis. β-catenin initiates and maintains skeletal muscle precursor cell formation either directly or indirectly, whereas MyoD appears to initiate the entire myogenic program through the direct activation of premyogenic mesoderm genes forming skeletal muscle expressing the essential cervical, occipital and limb markers C-met and Lbx1.
## APPENDICES

### Appendix A

**Table 1 - Reverse and Forward primers used for mRNA Q-PCR analysis**

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<th>FORWARD PRIMER</th>
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<td>MyoD</td>
<td>CCCCGGCCGCCAGATGGCTACG</td>
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<td>TAATCTCCTCCCAACAGCA</td>
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<td>En1</td>
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<td>Cmet</td>
<td>CGATCAGCGATCTGTCATTT</td>
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**Table 2 - Reverse and Forward primers used for ChIP Q-PCR**

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<td>Foxc1</td>
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binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell 58:823-831.


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PUBLICATIONS

Accepted, in review, submitted or in preparation Manuscripts


Conference Abstracts


