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Identification and Characterization of Multiple EGF-Repeat Containing Protein 10 and its Role in Regulating Satellite Cell Function during Muscle Regeneration

by

Chet Elliot Holterman

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements

for a PhD in

Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

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Abstract

The regeneration of adult skeletal muscle is mediated by a small population of cells known as satellite cells. While a great deal is known about the events that occur following satellite cell activation, relatively little is known about the mechanisms of activation or maintenance of this population of cells. Previous work from our lab has demonstrated that primary myoblasts lacking MyoD, represent a primitive form of activated myogenic precursor, closely related to quiescent satellite cells. In an attempt to gain insight into the activation and maintenance of quiescent satellite cells we performed representational difference analysis to identify genes that function in these processes.

This screen led to the isolation a large number of candidates for examination. One of the clones, identified as mouse Megf10, is a multiple EGF-repeat transmembrane protein that is expressed in adult skeletal muscle, specifically in quiescent satellite cells. Retroviral expression of Megf10 in C_2C_{12} myoblasts resulted in enhanced proliferation and inhibition of terminal differentiation. Furthermore, following serum withdrawal, Megf10 overexpressing cells entered a state of quiescence rather than undergoing terminal differentiation and remained capable of re-entering the cell cycle following serum restimulation. While no distinct effects on cell cycle regulation were observed, ectopic expression of Megf10 resulted in transcriptional upregulation of *Myf5* and downregulation of *Pax7* as well as post-transcriptional downregulation of MyoD. Importantly, ectopic expression of a truncated version of Megf10, lacking the cytoplasmic domain, had no effect on proliferation, differentiation or expression of transcriptional regulatory factors.

Investigation of several key signaling pathways that directly affect myogenic progression revealed that ectopic expression of Megf10 resulted in increased Rac1 activity and MEK1 phosphorylation providing a possible explanation for the inhibition of differentiation observed in Megf10 overexpressing cells. These results indicate that Megf10 impinges upon these signaling pathways supporting a role for Megf10 in regulating myogenic progression.

Our results clearly demonstrate that Megf10 is expressed in quiescent satellite cells in adult mouse skeletal muscle and indicate that Megf10 is capable of regulating the proliferation and differentiation of myogenic cells. Furthermore, our preliminary results implicate Megf10 as being a modulator of Rac1 and MEK1 signaling in myoblasts.

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List of Abbreviations and Chemical Formulae

-	negative
+	positive
2N	diploid
3H	tritium
4N	tetraploid
5' RACE	five prime random amplification of cDNA ends
a	alpha or anti
a.a.	amino acid
Amp	ampicillin
APC	allophycocyanin
ATP	adenosine triphosphate
BCIP/NBT	5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium
bHLH	basic helix-loop-helix
BMP	bone morphogenic protein
BrdU	5-bromo-2-deoxyuridine
CCR2	chemokine receptor 2
CD31/PECAM	Platelet Endothelial Cell Adhesion Molecule-1
CD34	hematopoietic progenitor cell antigen CD34
CD3e	CD3E antigen, epsilon polypeptide (TiT3 complex)
Cdc42	cell division cycle 42 (GTP binding protein, 25kDa)
cdk	cyclin dependent kinase
cDNA	coding deoxyribonucleic acid
CED-1	cell death abnormality protein 1
CED-6	cell death abnormality protein 6
cm	centimeter
cre	cre-recombinase
ctx	cardiotoxin
CXCR4	chemokine (C-X-C motif) receptor 4
dCTP	deoxycytosine triphosphate
DIG	digoxigenin
DMEM	Dulbecco's Modified Eagle Medium
DML	dorsal medial lip
DNA	deoxyribonucleic acid
DTT	dithiothreitol
e	embryonic
EDL	extensor digitorum longus
EDTA	ethylenediaminetetraacetic acid

EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FGF	fibroblasts growth factor
fig	figure
FITC	fluorescein isothiocyanate
G1	growth phase 1
G2	growth phase 2
GAPDH	glyceraldehyde phosphate dehydrogenase
GFP	green fluorescent protein
Grb10	growth factor receptor-bound protein 10
Grb2	growth factor receptor-bound protein 2
GST	glutathione S transferase
GST-PBD	glutathione S transferase fused with PAK binding protein
GST-RB	
HA	hemaglutinin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HGF/SF	hepatocyte growth factor/scatter factor
HOAc	acetic acid
hrs	hours
HS	horse serum
HUGE	human unidentified gene expression
IGF	insulin growth factor
IPTG	isopropyl-beta-D-thiogalactopyranoside
ISH	in situ hybridization
JNK	c-jun N-terminal activated kinase
KCl	potassium chloride
LB	Luria-Bertani
LDL	low density lipoprotein
Lgr6	leucine-rich repeat-containing G protein-coupled receptor 6
Lin	lineage
M	mitosis
MAPK	Mitogen-activated protein kinase
mdx	dystrophic
Megf	multiple egf-repeat
MeOH	methanol
mg	milligram

MgCl2	magnesium chloride
min	minute
ml	milliliter
mM	millimolar
MMP	matrix metalloproteinase
MNF	myocyte nuclear factor
MRFs	myogenic regulatory factor
MyHC	myosin heavy chain
ng	nanogram
NO	nitric oxide
NOS-I	nitric oxide synthetase-1
NP-40	nonidet P-40
o/n	overnight
OCT	optimum cutting temperature
PBS	phosphate-buffered saline
PBS-FV	PBS with sodium fluoride and sodium vanadate
pc	post coitum
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PE	Phycoerythrin
penn	penicillin
pfu	plaque forming units
PI	propidium Iodide
PI3'K	phosphatidylinositol 3' kinase
PIN	protease inhibitors
PKB/Akt	protein kinase B
PMSF	phenylmethyl sulfonyl fluoride
pRb	retinoblastoma protein
PVDF	Polyvinylidene fluoride
Rac1	ras-related C3 botulinum toxin substrate 1
RacGAP1	Rac GTPase activating protein 1
RDA	representational difference analysis
RICS	Rho GTPase-activating protein
RLT-PCR	real-time polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
S	synthesis
Sca1	stem cell antigen-1
SCID	severe combined immuno-deficient

sec	second
Shh	sonic hedgehog
SMART	simple modular architectural research tool
SP	side population
strep	streptomycin
syn-3	syndecan-3
syn-4	syndecan-4
TA	tibialis anterior
TAOK2	TAO kinase 2
TBP	TATA Binding protein
TIMAP	TGF-beta-inhibited membrane-associated protein
TPA	phorbol 12-myristate 13-acetate
TrisHCL	pH-adjusted tris(hydroxymethyl)aminomethane
U	units
UTR	untranslated region
VCAM1	vascular cell adhesion molecule
VE-cadherin	vascular endothelial cadherin
VEGF-R2	vascular endothelial growth factor receptor 2
VLL	ventro-lateral lip
wt	wildtype
γATP	gamma adenosine triphosphate
μCi	microcurie
μg	microgram
μl	microlitre
μM	micromolar
°C	degrees Celsius

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Chapter 1 – Literature Review and Project Rationale

1.1 Skeletal Muscle: Overview

Skeletal muscle provides the framework that allows for the movement of joints and locomotion of the human body. As a whole, skeletal muscle represents the largest tissue mass in the body constituting almost 50% of total body weight. Thus the study of skeletal muscle development, as well as the repair of skeletal muscle in response to trauma, injury, and specific disease states is of utmost importance to human biology.

Skeletal muscle provides an excellent model in which to study a variety of developmental, cellular, and molecular events including proliferation, differentiation, and asymmetric cell division. Distinct molecular markers such as MyoD, Myf5, myogenin, MRF4, Pax3, Pax7, c-met, and myosin heavy chain (MyHC) have allowed for detailed analysis of the development and determination of the myogenic lineage, myoblast proliferation, terminal differentiation and skeletal muscle regeneration. The discovery of skeletal muscle stem cells has led to experiments examining the pluripotency of stem cells, the maintenance of stem cell populations, and cell based therapies for the treatment of muscle diseases. Not only do these studies advance our understanding of muscle biology, but extend our understanding of numerous biological systems.

1.2 Embryonic Origin of Skeletal Muscle

The majority of vertebrate skeletal muscle is derived from progenitor cells that originate in epithelial spheres of mesodermal cells known as somites (Christ and Ordahl, 1995). The skeletal muscles of the head and neck are an exception, and originate from

the cephalic paraxial mesoderm (somitomeres) and prechordal mesoderm (Noden, 1991; Trainor et al., 1994). Somite formation occurs at approximately day 7.5 postcoitum in the mouse when the paraxial mesoderm begins to undergo segmentation forming pairs of epithelial spheres on either side of the neural tube. These epithelial spheres, or somites are progressively generated along the anterior-posterior axis, giving rise to a series of somites along each side of the neural tube (reviewed in Asakura and Rudnicki, 2002). As somite development continues, the cells in the ventral portion of the somite lose their epithelial morphology and give rise to loosely connected mesenchymal cells, referred to as the sclerotome. The cells of the sclerotome will eventually migrate around the neural tube and give rise to the ribs and vertebral structures.

The cells in the dorsal portion of the somite maintain their epithelial morphology and form a columnar sheet of cells referred to as the dermomyotome. Between 8.0 and 8.5 days postcoitum the cells in the most dorsomedial portion of the dermomyotome, referred to as the dorsomedial lip, begin to extend laterally beneath the existing dermomyotome (Kaufman, 1992). By day 8.5 the dorsomedial lip (DML) derived cells in the rostral most somite exit the cell cycle and undergo terminal differentiation giving rise to the first terminally differentiated myocytes of the dorsal myotome (Christ and Ordahl, 1995).

In the interlimb regions of the embryo, the cells in the ventrolateral portion of the somite undergo a similar process with cells from the ventrolateral lip (VLL) extending laterally beneath the dermomyotome. By day 9.75 pc in the mouse the cells derived from the ventrolateral lip exit the cell cycle and undergo terminal differentiation forming the ventral myotome (Christ and Ordahl, 1995). The cells of the dorsal or epaxial myotome

give rise to the epaxial musculature or muscles of the deep back while the ventral or non-migrating hypaxial myotome gives rise to the hypaxial musculature consisting of intercostal muscles and body wall muscles. As development proceeds, the epaxial and hypaxial myotome extend beneath the dermomyotome to form a planar myotomal structure (Kahane et al., 1998a). The cells of the ventral myotome also form migratory muscle progenitor cells that undergo epithelial to mesenchymal transition, delaminate from the dermomyotome and migrate into the ventral region of the trunk in the interlimb region of the embryo giving rise to the pectoralis, abdominal and diaphragm muscles. These migrating precursors also populate the developing limb buds where they give rise to the musculature of the limbs.

1.3 Developmental Myogenesis Occurs in Waves

Cells from DML and VLL extend laterally beneath the dermomyotome where they withdraw from the cell cycle and undergo terminal differentiation. As these cells undergo differentiation, they elongate along the rostro-caudal axis, giving rise to the first terminally differentiated myocytes in the myotome termed muscle pioneers (Denetclaw et al., 1997; Kahane et al., 1998b). These muscle pioneers form the primary myotome. Once the primary myotome has been established, cells from the edges of the dermomyotome begin to withdraw from the cell cycle and migrate beneath the dermomyotome where they assume positions along the periphery of the primary myotome. These cells then elongate along the rostro-caudal axis between the primary myofibers giving rise to the secondary myotome (reviewed in Asakura and Rudnicki 2002). This is the second wave of myogenesis.

In the limb bud, myogenesis also occurs in distinct waves. Cells from the VLL of the dermamyotome undergo an epithelial to mesenchymal transition and delaminate allowing for their migration into the developing limb bud. Studies indicate that the limb bud is populated by two distinct lineages of migrating precursors. The first wave of limb bud myogenesis establishes the primary myofibers that give rise mainly to slow twitch muscle fibers (Kahane et al., 1998b). The second wave of myogenesis arises from a second, distinct, set of migrating precursors that gives rise to mainly fast twitch fibers (Kahane et al., 1998a).

The third wave of myogenesis occurs in the late stages of fetal life. A distinct population of proliferative myogenic precursors migrates from the somite into the developing myotome (Armand et al., 1983; Schultz, 1996). These progenitors appear to be responsible for the late stages of fetal muscle growth as well as the majority of post-natal skeletal muscle growth. These progenitors are distinct from the primary and secondary precursors and are believed to be the cells that give rise to adult satellite cells, the resident myogenic stem cells of adult muscle.

1.4 Molecular Basis of Muscle Development

Perhaps one of the most important advances in the molecular biology of skeletal muscle was the discovery of MyoD and the myogenic regulatory factors (MRF). Using a subtractive cloning method, Weintraub and colleagues identified the basic helix-loop-helix transcription factor and demonstrated that expression of MyoD in a variety of non-muscle lineages, *in vivo* and *in vitro*, was capable of converting these cells to skeletal muscle (Davis et al., 1987; Weintraub et al., 1991). These results implicated *MyoD* as a

master regulator of skeletal muscle determination. The cloning of three highly related genes *Myf5*, *MRF4*, and *myogenin*, all of which displayed similar ability to convert cells to the myogenic lineage lead to the establishment of the myogenic regulatory factors (Braun et al., 1990; Braun et al., 1989; Edmondson and Olson, 1989; Rhodes and Konieczny, 1989; Wright et al., 1989). Subsequent studies demonstrated that the MRFs form heterodimers with other bHLH proteins and that these heterodimers bound to the consensus sequence CAnnTG, referred to as the E-box. It was further demonstrated that binding of these factors to E-boxes in the promoter regions of muscle specific genes leads to their activation.

The expression patterns of the individual MRFs were examined using *in situ* hybridization. The first MRF to be detected during development is *Myf5*. Its expression can be detected in the dorsomedial lip of the rostral most somites at embryonic day 8.0 (Ott et al., 1991). This expression is maintained and expanded into the developing epaxial myotome and expands along the myotome in a rostro-caudal progression as the embryo develops. Expression is maintained in the myotome throughout development but is down-regulated in the mature myotome. It is important to note that early *Myf5* expression corresponds to the initial expansion of the DML underneath the myotome and it is expressed in the epaxial myotome that is derived from progenitors of the DML (Asakura and Rudnicki 2002). At later stages, e9.5, Myf5 expression can be detected in the lateral domain of the somite.

MyoD is first detected at day 9.75 in the hypaxial myotome at approximately the same time as *Myf5* expression (Faerman et al., 1995; Sassoon et al., 1989). *MyoD* expression spreads throughout the myotome and is maintained throughout development.

Myogenin expression is first detected in the most rostral somites at day 8.5, shortly after *Myf5* expression, and is maintained throughout fetal development and in adult muscle. *MRF4* expression in the myotome is transiently detected between embryonic days 9.0-11.5 and is re-expressed at e16 to eventually become the most highly expressed MRF in adult muscle (Bober et al., 1991). Interestingly, MRF expression is not detectable in the VLL migrating precursors that populate the limb buds until after they have arrived at the site of presumptive muscle. At approximately e10.5 the migrating precursors begin to express *MyoD* and *Myf5* followed shortly there after by *myogenin* and *MRF4* (Buckingham et al., 1992). This occurs slightly later in the hindlimb.

To gain more insight into the function of the MRFs in the developing myotome, individual knockouts of the MRFs were generated. Mice lacking a functional *MyoD* are viable with no obvious skeletal muscle phenotype although they do express *Myf5* at about a four-fold higher level (Rudnicki et al., 1992). Originally, mice lacking *Myf5* were found to display normal skeletal muscle despite a minor delay in the development of trunk musculature, however these mice died perinatally due to severe rib defects leading to suffocation (Braun et al., 1992). More recently, viable Myf5 null mice have been generated that do not display rib abnormalities but due display a delay in the development of trunk musculature (Kaul et al., 2000). Importantly when these two knockouts are combined giving rise to mice lacking both functional *Myf5* and *MyoD* a complete absence of myoblasts and myofibers is observed (Rudnicki et al., 1993). Interestingly, the putative muscle progenitor cells are present but are unable to give rise to myogenic cells. These results indicate that *MyoD* and *Myf5* are required for skeletal muscle determination of the progenitor cells within the developing myotome.

Myogenin null mice die at birth and display an almost complete absence of differentiated myotubes (Hasty et al., 1993; Nabeshima et al., 1993). Importantly, normal numbers of myogenic precursor cells expressing MyoD or Myf5 are detectable in these animals. Interestingly, primary fiber formation occurs normally but secondary fiber formation is disrupted (Venuti et al., 1995).

Targeted disruption of the *MRF4* locus results in a variety of phenotypes depending on the specific targeting event. The resulting phenotypes range from no overt muscle phenotype with dramatic upregulation of myogenin, to perinatal lethality most likely arising from disruption of the adjacent *Myf5* locus (Braun and Arnold, 1995; Patapoutian et al., 1995; Rawls et al., 1995; Zhang et al., 1995). These results suggest that myogenin may be able to compensate for MRF. In support of this theory, mice that lack both *MRF4* and *MyoD* display a phenotype similar to mice lacking *myogenin* suggesting that myogenin is only capable of compensating for *MRF4* loss in the presence of MyoD (Rawls et al., 1998). Taken together, these results are the basis for a model in which MyoD and Myf5 are primary MRFs required for the establishment and determination of the myogenic lineage while MRF4 and myogenin are secondary MRFs required for the terminal differentiation of myogenic precursors (Fig. 1 adapted from (Megeney and Rudnicki, 1995). Interestingly, recent evidence also indicates that MRF4 may play a role in myogenic determination in the developing embryo (Kassar-Duchossoy et al., 2004).

Introduction of the bacterial beta-galactosidase gene under the control of *MyoD* promoter elements has provided further insight into the roles of the MRF in the establishment of epaxial versus hypaxial muscle lineages. Using transgenic mice, Kablar

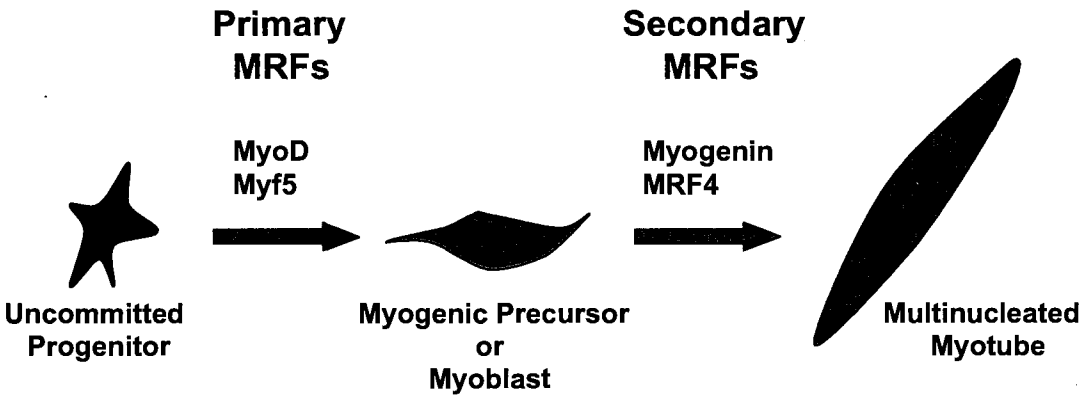

Figure 1. Myogenic Regulatory Factor Hierarchy. MyoD and Myf5 are the primary MRFs and are required for myogenic commitment of progenitor cells. Myogenin and MRF4 are secondary MRFs and are required for myogenic differentiation.

and colleagues demonstrated that in *Myf5*-null mice the formation of the epaxial myotome is delayed by approximately 2.5 days, corresponding to the time at which *MyoD* expression is first observed in this domain (Kablar et al., 1997). Hypaxial musculature develops normally in these mice. In contrast, *MyoD*-null mice display normal development of the epaxial myotome but hypaxial development is delayed by approximately 2 days (Kablar et al., 1997). Interestingly, despite the fact that development of the limb musculature is delayed, the migration of myogenic precursors into the developing limb bud occurs in a normal manner. These results demonstrate unique requirements for MyoD and Myf5 in the establishment of epaxial and hypaxial musculature during development. Thus, while the two primary MRFs are capable of compensating for one another, there are specific functions that are unique to each of these MRFs during development.

1.5 Inductive Mechanisms Regulating Developmental Myogenesis

Distinct myogenic populations arise during development and give rise to distinct muscle lineages. Quail/chick transplantation experiments clearly demonstrated that early somites respond to specific environmental cues to establish polarity. By transplanting individual somites from quails into chicks and rotating them in a manner so as to place the dorsal portion in a ventral position, it was shown that the myogenic fate of donor cells within the transplanted somite was dictated by the surrounding chick structures (Ordahl and Le Douarin, 1992). Attempts to identify specific factors regulating myogenic fate have lead to the identification of a variety of signals from surrounding structures that lead to activation or repression of *Myf5* and *MyoD*. Specialized structures such as the neural

tube, notochord, lateral plate mesoderm and dorsal ectoderm all play a role in regulating MRF expression in the developing myotome (reviewed in Asakura and Rudnicki, 2002). These structures are the source of signals including Sonic Hedgehog (Shh), Wnts, bone morphogenic proteins (BMPs) as well as specific growth factors, all of which have specific effects on MRF expression.

The neural tube/notochord have been shown to regulate the formation and survival of the dorsal myotome during early chick embryogenesis through the secretion of sonic hedgehog [Shh] (Teillet et al., 1998). Interestingly, mice that lack *Shh* undergo normal development of the hypaxial myotome but display a reduced formation of the epaxial myotome due to decreased levels of *Myf5* expression (Borycki et al., 1999). Furthermore, several Wnt family members appear to function synergistically with Shh in controlling myotome formation (Marcelle et al., 1997). These results suggest that the neural tube and notochord provide inductive cues required for appropriate formation of the myotome.

1.6 Fiber Structure

As previously discussed, skeletal muscle development occurs in a series of waves during which myocytes elongate along a particular plane and fuse with other myocytes to form multinucleated myotubes or myofibers. Multinucleated myofibers are mainly composed of two large filamentous proteins, myosin and actin, which are arranged in a highly organized manner. The overlapping myosin/actin structure provides the mechanical force for muscle contraction. Surrounding each myofiber is a framework of connective tissue known as the basal lamina or endomysium (reviewed in Gillespie et al.,

2006). The basal lamina provides a supportive framework that maintains the fiber structure during contraction. Located between the basal lamina and the sarcolemma (plasma membrane) of individual fibers are quiescent, mononuclear cells known as satellite cells (Fig 2). These quiescent cells are activated in response to a variety of events and are responsible for the post-natal growth and repair of skeletal muscle. Individual fibers and associated satellite cells are clustered into groups of tens to several hundred fibers, referred to as fasicles, and each fasicle is surrounded by a second layer of connective tissue referred to as the perimysium (Fig 2). Individual muscle groups are composed of multiple fasicles surrounded by a third layer of connective tissue referred to as the epimysium.

1.7 Satellite Cell Morphology and Distribution

Satellite cells are classically defined by their position within myofibers. Located within depressions in the fiber, satellite cells occupy the space between the basal lamina and the sarcolemma of the fiber. Satellite cells are further characterized by a sparse amount of cytoplasmic material, few organelles, and a high ratio of heterochromatin to euchromatin, indicative of their quiescent state (Schultz, 1976). Once activated, the morphology of these cells changes dramatically. The cytoplasmic volume of the cells increases substantially and cytoplasmic extensions become apparent (Schultz and McCormick, 1994). The number of organelles begins to increase and the amount of euchromatin increases indicating an increase in transcriptional activity (Schultz and McCormick, 1994).

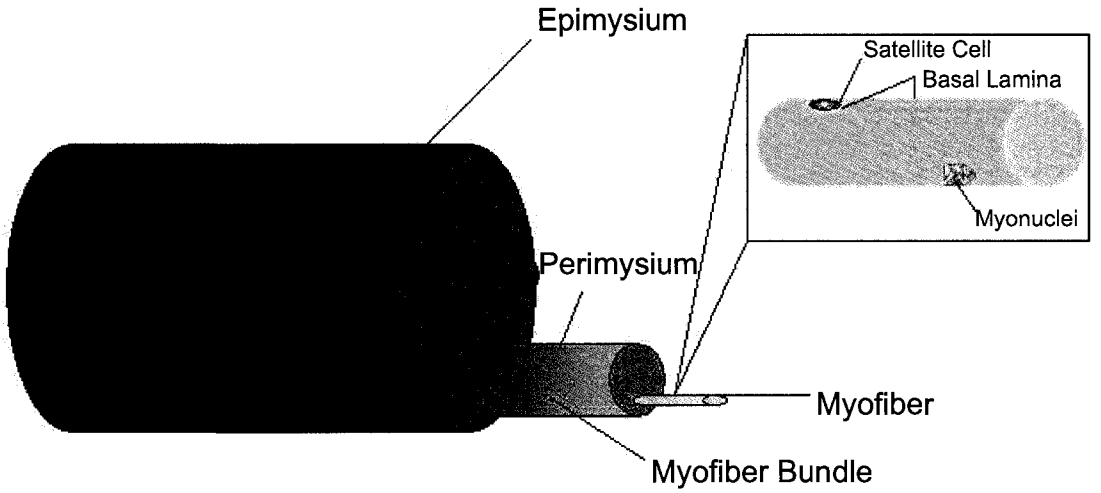

Figure 2. Skeletal Muscle Ultrastructure and satellite cell location. Individual skeletal muscles are composed of thousands of individual myofibres that are grouped into bundles surrounded by the perimysium. Each muscle is composed of multiple myofiber bundles surrounded by the epimysium. Individual fibres contain myonuclei that lie beneath the basal lamina and satellite cells that are located above the basal lamina but beneath the sarcolemma.

Satellite cells are present throughout adult skeletal muscle although their distribution varies between different muscle groups. Oxidative slow twitch fibers generally have higher numbers of associated satellite cells than glycolytic fast twitch fibers (Charge and Rudnicki, 2004). As well, satellite cells are more prevalent around neuromuscular junctions and adjacent capillaries resulting in unequal distribution within individual myofibers (Kelly, 1978). Despite these differences, satellite cells account for approximately 5% of myofiber associated nuclei within skeletal muscle. This number remains constant throughout the life span of adult mice. Furthermore, this number is maintained following multiple rounds of degeneration/regeneration suggesting that a mechanism exists for the maintenance or self-renewal of the quiescent satellite cell compartment.

1.8 Molecular Markers of Quiescent Satellite Cells

In recent years, the identification of quiescent satellite cells has been made much easier by the discovery of molecular markers that label quiescent and activated satellite cells. These markers include cell surface proteins such as c-met, VCAM1, Syndecan3, Syndecan4, and M-cadherin, as well as transcription factors such as Pax7 and MNF (Cornelison et al., 2001; Cornelison and Wold, 1997; Garry et al., 2000; Garry et al., 1997; Irintchev et al., 1994; Jesse et al., 1998; Seale et al., 2000; Tatsumi et al., 1998).

One of the first molecular markers of quiescent satellite cells to be discovered was the c-met receptor tyrosine kinase. Detectable in both resting and regenerating muscle *in vivo*, *c-met* expression in quiescent satellite cells was first demonstrated by multiplex PCR (Cornelison and Wold, 1997). Importantly, c-met is the receptor for hepatocyte

growth factor/scatter factor that has previously been shown to be capable of activating quiescent satellite cells (Allen et al., 1995; Bischoff, 1986b; Tatsumi et al., 1998).

During development, VCAM1 is widely expressed throughout the mouse embryo, but its expression is much more restricted in the adult organism where it appears to be limited to endothelial cells stimulated by inflammation, as well as quiescent satellite cells and activated myogenic precursors in skeletal muscle (Jesse et al., 1998). A possible function for VCAM1 in satellite cell biology would be to initiate interactions between VCAM1 expressing satellite cells and infiltrating lymphocytes resulting in localized accumulation of cytokines produced by the infiltrating lymphocytes.

Syndecan 3 and Syndecan 4 are transmembrane heparin sulfate proteoglycans that function in FGF signaling (Rapraeger, 2000). Syndecan 3 and 4 expression within skeletal muscle completely overlaps with that of c-met and is found strictly in cells located between the basal lamina and sarcolemma verifying syndecan expression in quiescent satellite cells (Cornelison et al., 2001). Deletion of either syndecan 3 or syndecan 4 results in severe, but distinct deficits in satellite cell activation and muscle regeneration (Cornelison et al., 2004).

M-cadherin is a calcium dependent cell adhesion molecule that marks quiescent satellite cells and proliferating myogenic precursors (Irintchev et al., 1994; Moore and Walsh, 1993). Importantly, while M-cadherin is expressed in the majority of quiescent satellite cells and activated mpcs, it is not required for satellite cell function as demonstrated by the presence of normal skeletal muscle development and regeneration in M-cadherin null mice (Hollnagel et al., 2002). Furthermore, it has been demonstrated that a small subset of quiescent satellite cells do not express detectable levels of M-

cadherin indicating that heterogeneity exists within the quiescent satellite cell compartment (Beauchamp et al., 2000). Interestingly, CD34 expression is detected in quiescent satellite cells expressing M-cadherin, but is not detectable in those cells that do not express M-cadherin (Beauchamp et al., 2000). The M-cadherin expressing population of cells was also found to express *Myf5* based on expression of β-galactosidase under control of the *Myf5* promoter region further demonstrating the heterogeneity within the satellite cell compartment (Beauchamp et al., 2000). One possibility is that this heterogeneity is a reflection of different origins of the quiescent satellite cells.

By *in situ* hybridization, the paired box transcription factor Pax7 localizes to nuclei in positions consistent with that of quiescent satellite cells (Seale et al., 2000). Furthermore, the number of *Pax7* expressing cells corresponds well with the expected number of satellite cells (Seale et al., 2000). By Northern blotting, immunohistochemistry, and immunocytochemistry, Pax7 is expressed in proliferating mpcs *in vivo* and myoblasts *in vitro* and is down-regulated upon serum withdrawal (Seale et al., 2000). Thorough electron microscopic examination of skeletal muscle isolated from adult *Pax7-/-* mutant mice revealed a striking lack of quiescent satellite cells. As well, attempts to culture primary myoblasts from adult *Pax7* mutant animals were unsuccessful further supporting the notion that *Pax7* plays a critical role in satellite cell biology (Seale et al., 2000).

The winged helix transcription factor, myocyte nuclear factor (MNF), is expressed in quiescent satellite cells and is also detected in central nuclei of newly regenerated myotubes where its expression is down-regulated during the late stages of

regeneration (Garry et al., 1997). Two isoforms of MNF, termed *MNF-α* and *MNF-β,* are detected in skeletal muscle where they display distinct expression patterns. *MNF-α* is expressed in proliferating mpcs while *MNF-β* expression appears to be limited to quiescent satellite cells (Garry et al., 2000; Hawke and Garry, 2001). Targeted disruption of the *MNF* locus results in significant impairment of skeletal muscle regeneration, most likely due to a dramatic decrease in the number of quiescent satellite cells (Garry et al., 2000; Hawke and Garry, 2001). MNF-β has been demonstrated to form a transcriptionally repressive complex with mSin3 family members and may be responsible for the repression of specific target genes in quiescent satellite cells (Yang et al., 2000). Upon activation isoform switching may disrupt this repression allowing for transcriptional activation of the previously repressed genes.

1.9 Satellite Cell Origins

Satellite cells are first detectable in the limb muscles of the mouse at approximately embryonic day 17 (Bischoff, 1994; Charge and Rudnicki, 2004). During the late stages of development, activated satellite cells can be distinguished from migrating embryonic and fetal myoblasts based on sensitivity to TPA, as well as their expression of acetylcholine receptors and expression of distinct MyHC isoforms upon differentiation in culture (Feldman and Stockdale, 1992). While the temporal appearance of satellite cells during development in most vertebrates has been well established, the origin of these cells remains controversial.

1.9.1 Somitic Origins

Early fate mapping experiments using quail/chick chimeras suggest that satellite cells are somitic in origin (Armand et al., 1983). In these experiments, somites derived from quail embryos were transplanted into chick recipients and contribution of quail cells to the developing structures was examined. Based on distinct ultrastructural characteristics, it was observed that quail cells were capable of migrating from the somite into the developing chick limb bud where they contributed to terminally differentiated myofibers (Armand et al., 1983). Furthermore, donor cells were observed within the quiescent satellite cell population indicating a possible somitic origin for satellite cells (Armand et al., 1983). However, the specific area of the somite from which these cells were derived was never characterized.

Interestingly, it has been demonstrated that the migrating myoblasts that populate the limb during development do not express MyoD or Myf5 but do express c-met, an established marker of quiescent satellite cells as well as Pax3 (Amthor et al., 1998; Pourquie et al., 1996). In the absence of c-met, myogenic precursors fail to migrate into the limb buds resulting in an absence of limb musculature (Bladt et al., 1995). Importantly, the muscles of the back and body wall develop normally suggesting that c-met expression is required for the appropriate migration of progenitors into the regions of presumptive musculature in the developing organism (Bladt et al., 1995). Importantly, the migrating myoblasts that give rise to the limb musculature are in a non-proliferative state during their migration. This raises the possibility that satellite cells are derived from

migrating myogenic precursors which remain quiescent rather than proliferating and differentiating to contribute to muscle development.

More recent evidence demonstrates that a novel population of Pax7 and Pax3 expressing skeletal-muscle progenitors arises from the central domain of the dermomyotome (Gros et al., 2005; Relaix et al., 2005). These cells migrate into the developing myotome where they are capable of proliferating without expressing the MRFs (Gros et al., 2005; Relaix et al., 2005). Importantly, these cells move into satellite cell positions accounting for more than 90% of cells in this position (Gros et al., 2005; Relaix et al., 2005). These results support the hypothesis that satellite cells arise from migrating precursors of somitic origins, however they do not exclude the possibility that satellite cells also arise from other origins.

1.9.2 Endothelial Origin

A second possible origin for satellite cells comes from data showing that myogenic cells resembling satellite cell derived mpcs can be isolated from the embryonic dorsal aorta of mice (De Angelis et al., 1999). Although these cells are of endothelial origin as indicated by their expression of vascular-endothelial markers, they also express myogenic markers including MyoD, Myf5, c-Met, desmin and M-cadherin (De Angelis et al., 1999). Interestingly, activated mpcs isolated from adult muscle express many of the markers observed in myogenic cells isolated from the dorsal aorta, including VE-cadherin, VEGF-R2, and PECAM (De Angelis et al., 1999). These results support a possible endothelial origin for adult satellite cells. This hypothesis is further supported by the fact that cells with myogenic potential can be isolated from the developing limbs

of *Pax3* null and *c-Met* null mouse embryos that display an absence of somitically derived migrating myogenic progenitors in the limb buds (De Angelis et al., 1999). Importantly, transplantation experiments reveal that myogenic cells derived from the dorsal aorta are capable of contributing to muscle growth and regeneration *in vivo;* however the ability of these cells to contribute to the quiescent satellite cell compartment remains to be clearly demonstrated (De Angelis et al., 1999). While the ability of these cells to contribute to developing and regenerating skeletal muscle is clear, the ability of these cells to contribute to the quiescent satellite cell compartment or give rise to satellite cells during development have not been established.

Importantly, the possibility that an endothelial origin for satellite cells exists does not exclude the traditional belief that satellite cells are somitic in origin. Several lines of evidence point to a heterogeneity within the quiescent satellite cell population. Satellite cells display diversity in their expression of specific molecular markers, including CD34 and M-cadherin (Beauchamp et al., 2000; Cornelison and Wold, 1997). Furthermore differences in the kinetics of proliferation have been observed within the satellite cell compartment (Schultz, 1996). This heterogeneity within the satellite cell population may, in part, be explained by the possibility of distinct developmental origins.

1.10 Activation of Quiescent Satellite Cells

While it is clear that satellite cells are the major effectors of skeletal muscle regeneration in adult organisms, very little is known about the events that lead to the activation of these cells. Early attempts to identify the factor or factors responsible for activating satellite cells demonstrated that while several well characterized mitogens such

as EGF, FGF, IGF, and PDGF were able to enhance the proliferation of mpcs, they were unable to activate quiescent satellite cells (Bischoff, 1986a, b; Johnson and Allen, 1995; Sheehan and Allen, 1999). The first insight into the factor(s) that activate satellite cells came from experiments demonstrating that extract from gently crushed muscle was capable of activating quiescent satellite cells *in vitro* (Bischoff, 1986b). Unfortunately, these experiments did not identify the specific factor in the extract that possessed activating activity. Further investigation of specific fractions of muscle extract revealed that HGF was the critical activating factor in crushed muscle extract (Bischoff, 1986a). Importantly, it was demonstrated that HGF/SF was present in the basal lamina of skeletal muscle fibers (Jennische et al., 1993; Tatsumi et al., 1998) and that injection of HGF directly into the tibialis anterior of adult mice resulted in activation of quiescent satellite cells in the absence of trauma (Tatsumi et al., 1998). These results verified the ability of HGF/SF to activate quiescent satellite cells *in vivo*. As well, incubation of crushed muscle extract with anti-HGF abolished the ability of crushed muscle extract to activate quiescent satellite cells further implicating HGF as the critical factor for activation (Tatsumi et al., 1998; Tatsumi et al., 2001).

Recent studies suggest that nitrous oxide may play a role in releasing HGF/SF from the extracellular matrix following trauma (Anderson, 2000). It has been demonstrated that *in vivo* inhibition of nitric-oxide synthase I (NOS-I) results in a reduction of satellite cell activation in response to muscle injury (Anderson, 2000). Furthermore, it has been demonstrated that NO is produced in response to fiber stretching *in vitro* and that inhibition of nitric-oxide synthase, in these experiments, reduces the amount of HGF released following fiber stretching (Tatsumi et al., 2002). While these

results implicate NO in satellite cell activation, it has not been determined if this is a direct or indirect effect of NO on HGF release.

While much remains to be learned about the events surrounding the initial activation of quiescent satellite cells, our understanding of the events that occur immediately following their activation is much greater. As in development, the MRFs play a critical role in the proliferation and differentiation of activated satellite cells. Immediately following activation, quiescent satellite cells begin to upregulate the primary MRFs (Fig 3). Within twenty-four hours of activation the majority of satellite cells have up-regulated either *MyoD* or *Myf5* (Cornelison and Wold, 1997). This is followed by co-expression of these two factors by forty-eight hours (Cooper et al., 1999; Cornelison and Wold, 1997; Smith et al., 1994). The ability to upregulate either *MyoD* or *Myf5* may in part be due to the heterogeneous nature of the quiescent satellite cell compartment, or alternatively, it may be due to unique responses to distinct cues following trauma. As in development, where MyoD and Myf5 appear to play distinct roles in the development of hypaxial and epaxial musculature, distinct roles for these factors in muscle regeneration are also apparent. MyoD-/- mice display a severe deficit in their ability to repair muscle following injury (Megeney et al., 1996). These mice display an increase in the number of mononuclear cells in the damaged area and the cells fail to fuse and differentiate (Megeney et al., 1996). *In vitro* experiments demonstrate that following serum withdrawal, MyoD-/- myogenic precursor cells (mpcs) continue to proliferate rather than undergoing terminal differentiation, suggesting that in the absence of MyoD, activated mpcs have a propensity for proliferation and self-renewal (Sabourin et al., 1999). A critical requirement for MyoD in myoblast differentiation is further supported by the

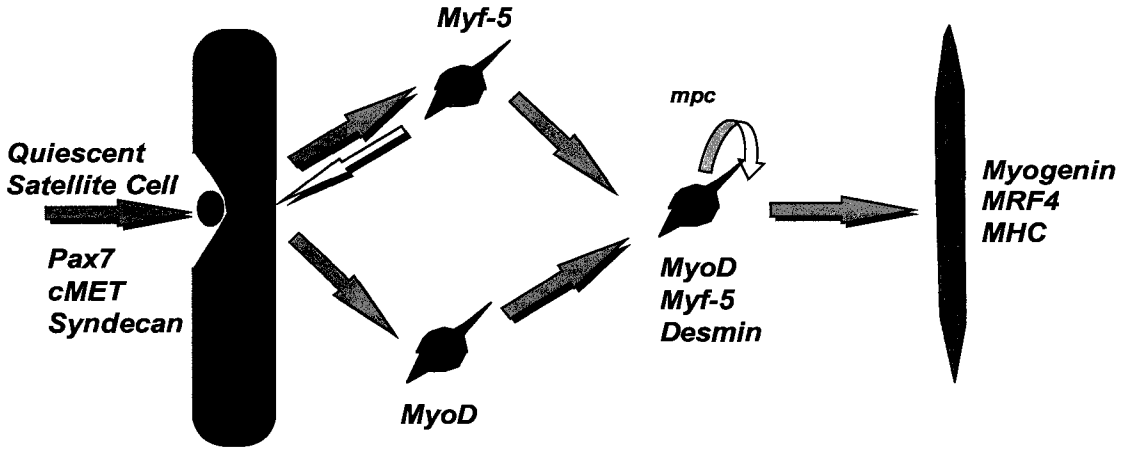

Figure 3. MRF expression following satellite cell activation. Following activation, satellite cells upregulate either MyoD or Myf5. Within 48 hours of activation, satellite cells enter a highly proliferative phase during which they express both Myf5 and MyoD. Upon appropriate cues the cells withdraw from the cell cycle, upregulate myogenin and MRF4 and undergo terminal differentiation.

observation that differentiating mpcs express either MyoD alone or Myf5 and MyoD, but are never found to express Myf5 alone (Cooper et al., 1999). These results have led to a model in which MyoD is required for differentiation of satellite cells following activation while Myf5 is most likely required for the proliferation and potentially, the self-renewal of satellite cells. Indeed it has been demonstrated that MyoD is able to preferentially activate genes required for progression towards terminal differentiation while Myf5 appears to regulate genes that function mainly in proliferation (Ishibashi et al., 2005).

1.11 Satellite Cell Maintenance and Self-Renewal

It has been clearly demonstrated that skeletal muscle has a remarkable capacity to regenerate following repeat rounds of degeneration/regeneration suggesting that the satellite cell compartment is capable of self-renewal or maintenance. Several different hypotheses exist as to how the quiescent satellite cell compartment is maintained in adult skeletal muscle. Asymmetric cell division, the return of proliferating mpcs to the quiescent state, and recruitment or contribution of other resident stem cells to the satellite cell compartment, have all been proposed. Individual lines of evidence support each of these models and importantly, none of the models are mutually exclusive.

1.11.1 Asymmetric Cell Division

Recent results suggest that following activation, satellite cells are capable of undergoing asymmetric cell division. It has been proposed that this asymmetric division results in the production of two daughter cells, with one being a committed myogenic precursor and the second returning to become a quiescent progenitor. This model

provides an attractive mechanism for the maintenance of the quiescent satellite cell compartment.

Interestingly, it has been observed that the plasma membrane associated protein Numb is segregated in an asymmetric manner in activated mpcs providing a potential mechanism for asymmetric cell division of activated satellite cells (Conboy and Rando, 2002). Increases in the level of the Notch ligand, Delta, as well as levels of activated Notch occur following satellite cell activation in response to trauma. At the same time, expression of the Notch inhibitor, *Numb*, is initially decreased in response to trauma but gradually increases as regeneration occurs (Conboy and Rando, 2002). It was found that as regeneration progressed, a proportion of mononuclear mpcs asymmetrically localized Numb giving rise to daughter cells that expressed either high levels of Numb or little to no Numb (Conboy and Rando, 2002). Furthermore, it was found that cells expressing high levels of Numb underwent differentiation while cells that expressed little to no Numb continued to proliferate (Conboy and Rando, 2002). Furthermore, expression of constitutively active Notch results in continued proliferation and inhibition of differentiation. These results suggest that specific cellular localization of Numb may provide a mechanism for the asymmetric cell division of activated mpcs and subsequently, Numb localization may play a pivotal role in the maintenance of the quiescent satellite cell compartment.

To investigate the *in vivo* role of Notch signaling in muscle regeneration, a comparison of the regenerative potential of young versus aged muscle was performed. The results demonstrated that while there was no difference in satellite cell numbers between young and old muscle, there was a dramatic decrease in satellite cell activation

and regeneration in aged muscle (Conboy et al., 2003). The decrease in regenerative potential was attributed to a failure to upregulate Delta-1 and to activate Notch. Furthermore, inhibition of Notch activation in young muscle resulted in decreased regenerative capacity, while forced activation of Notch signaling, in aged muscle, resulted in increased regenerative capacity. These results provide strong support for the role of Notch signaling in skeletal muscle regeneration (Conboy et al., 2003). While these results demonstrate the importance of Notch activation in skeletal muscle regeneration through the activation of satellite cells, it does not provide direct evidence for a role in satellite cell self-renewal. Notch signaling may be required to maintain activated satellite cells in a proliferative state in order to give rise to daughter cells required for the repopulation of the satellite cell compartment. Alternatively, the Notch signaling pathway may be required for asymmetric cell division following activation, resulting in a proliferative daughter cell expressing Notch, as well as a quiescent progenitor that repopulates the satellite cell compartment independent of Notch signaling.

1.11.2 Control of Satellite Cell Quiescence

Radiolabeling experiments have demonstrated that activated satellite cells are capable of contributing to fiber regeneration as well as to the quiescent satellite cell compartment (Charge and Rudnicki, 2004). Furthermore, it has been demonstrated that there are two distinct satellite cell populations based on their proliferative rate following activation. The majority of activated satellite cells undergo rapid division and contribute to fiber regeneration, however a small percentage divide at a much slower rate and it is hypothesized that these cells are responsible for replenishing the satellite cell pool

(Schultz, 1996). Whether the difference in proliferative capacity is due to distinct satellite cell populations, differential activation, or alternative expression of the MRFs has yet to be demonstrated.

Differential expression of the myogenic regulatory factors following activation supports a model whereby activated satellite cells can return to a quiescent state and repopulate the satellite cell compartment. Previous work has demonstrated that upon activation, satellite cells up-regulated either *Myf5* or *MyoD* followed by co-expression of these factors in mpcs (Cornelison and Wold, 1997). Strikingly, myoblasts derived from *MyoD-/-* mutant mice, display an increased propensity for self-renewal, accompanied by a failure to undergo terminal differentiation (Megeney et al., 1996; Sabourin et al., 1999). These results suggest that in the absence of MyoD, activated satellite cells are trapped in an intermediate stage between quiescent satellite cells and committed myoblasts. A similar phenomenon has been observed *in vitro* using C_2C_{12} myoblasts. Following serum withdrawal, a small proportion of cells express Myf5 but not MyoD and fail to undergo terminal differentiation (Baroffio et al., 1996; Yoshida et al., 1998). Furthermore, these cells retain the ability to proliferate and give rise to differentiation-competent progeny following serum restimulation (Yoshida et al., 1998).

Recent experiments performed by Olguin *et al.* demonstrate that forced expression of Pax7, in established myoblasts, results in the downregulation of MyoD, inhibition of differentiation, and cell cycle withdrawal (Olguin and Olwin, 2004). It has also been demonstrated that *in vitro*, Pax7+/MyoD+ mpcs are capable of downregulating MyoD and exiting the cell cycle and similar to C_2C_{12} reserve cells, these cells can re-enter a proliferative state upon restimulation (Zammit et al., 2004). These results support

a model in which activated satellite cells enter a proliferative stage during which they undergo symmetric division giving rise to a population of daughter cells, some of which down regulate MyoD and re-populate the quiescent satellite cell compartment. Those cells that maintain MyoD expression terminally differentiate and contribute to muscle repair.

1.11.3 Recruitment of multipotent stem cells

While satellite cells are thought to be the major effectors of skeletal muscle regeneration, the ability of stem cells from sources other than skeletal muscle to contribute to muscle regeneration has been demonstrated in recent years (Ferrari et al., 1998; Gussoni et al., 2002; Gussoni et al., 1999). Furthermore it has now been demonstrated that bone marrow-derived stem cells are capable of contributing to muscle regeneration as well as to the quiescent satellite cell pool, firmly establishing that non-muscle stem cells are capable of contributing to the satellite cell compartment (LaBarge and Blau, 2002). These results provide a third possible mechanism for the maintenance of the quiescent satellite cell compartment. However it should be noted that the extent to which this occurs under physiologically relevant conditions remains to be clearly established.

A muscle-derived source of stem cells known as SP cells has been shown to possess hematopoietic potential as well as the ability to contribute to muscle regeneration (Asakura et al., 2002; Gussoni et al., 1999). These cells are readily isolated from adult skeletal muscle using fluorescence activated cell sorting based on the exclusion of Hoechst 33342 dye. Interestingly, muscle SP cells do not express the established satellite

cell marker Pax7, nor do they express Myf5-nLacZ suggesting that these cells are not committed muscle progenitors (Asakura et al., 2002). Furthermore, muscle SP cells can be isolated from *Pax7-/-* muscles that lack quiescent satellite cells (Asakura et al., 2002). Therefore, muscle SP and quiescent satellite cells are believed to represent distinct stem cell populations. The ability of muscle SP cells to give rise to myogenic colonies when co-cultured with primary myoblasts, *in vitro*, has been clearly demonstrated (Asakura et al., 2002). Furthermore, muscle SP cells are capable of contributing to the quiescent satellite cell compartment following intramuscular transplantation (Asakura et al., 2002). Taken together these results suggest that muscle SP cells may represent one source of satellite cell progenitors. However, it has recently been demonstrated byt transplantation of single intact myofibers into radiation-ablated skeletal muscles that satellite cells are self-sufficient as a source of regeneration (Collins and Partridge, 2005). This study demonstrates that a small number of satellite cells associated with one transplanted myofiber can generate over 100 new myofibers as well as undergo vigorously self-renew, repopulating the host muscle with new satellite cells of donor origin(Collins and Partridge, 2005). Thus, while SP cells may be capable of contributing to the quiescent satellite cell compartment, it would appear that satellite cells themselves are sufficiently capable of maintaining the quiescent compartment.

1.12 Other Stem Cells in Muscle Regeneration

While skeletal muscle satellite cells appear to be the major contributors to skeletal muscle regeneration under physiological conditions, it has been clearly demonstrated that under certain circumstances other cells are also capable of contributing to skeletal muscle

regeneration. Stem cells from a variety of sources including bone marrow, the neuronal compartment, various mesenchymal tissues and even skeletal muscle (non-satellite cell) have been shown to be capable of contributing to muscle regeneration (Charge and Rudnicki, 2004).

1.12.1 Non-muscle Resident Stem Cells

The first indication that stem cells from sources other than skeletal muscle were capable of contributing to muscle regeneration was the demonstration that following intramuscular or intravenous injection, bone marrow from donor transgenic mice was able to contribute to the myogenic compartment of recipients (Ferrari et al., 1998). While the number of donor derived myogenic cells was extremely low, there was a definite contribution to the skeletal muscle compartment. Gussoni and colleagues were able to demonstrate that a highly purified population of hematopoietic stem cells isolated from bone marrow, and referred to as the side population, was able to contribute to muscle regeneration and restore low levels of dystrophin expression following tail vein injection into mdx mice (Gussoni et al., 1999). Interestingly, SP populations have since been shown to exist in a wide variety of tissues but the ability of this general population to contribute to muscle regeneration depends on the tissue from which it is isolated (Asakura and Rudnicki, 2002). More recent experiments have demonstrated that bone marrow derived cells are capable of contributing to the quiescent satellite cell compartment. Tail vein injection of bone marrow derived cells expressing the green fluorescent protein (GFP) marker have been shown to occupy satellite cell positions following injection and furthermore, clonal expansion of GFP positive cells isolated from

skeletal muscle expressed satellite cell markers and were capable of undergoing myogenic differentiation (LaBarge and Blau, 2002; Shi et al., 2004). These results clearly demonstrate that non-muscle resident stem cells can contribute to skeletal muscle regeneration as well as to the quiescent satellite cell compartment, however the biological relevance of these findings remains unclear.

1.12.2 Muscle Resident Stem Cells

Similar to other tissues, skeletal muscle also has a population of stem cells referred to as the muscle SP. This population of cells is capable of reconstituting the hematopoietic compartment of mice following lethal irradiation. Furthermore, muscle SP can contribute to the myogenic lineage *in vivo* (Asakura et al., 2002). Importantly, these cells do not express satellite cell markers such as Pax7, desmin, or Myf5-nLacZ suggesting that satellite cells are not present in the SP population (Asakura et al., 2002). Further support for this comes from the fact that muscle SP can be isolated from *Pax7-/-* skeletal muscles that lack quiescent satellite cells and are unable to undergo regeneration (Asakura et al., 2002). Interestingly, muscle SP from *Pax7-/-* mice are unable to undergo myogenic conversion and display an increased ability to undergo hematopoietic conversion indicating a requirement for Pax7 in muscle determination of SP cells (Asakura et al., 2002). One possibility is that muscle SP cells represent an upstream progenitor of quiescent satellite cells and that Pax7 expression is required for these cells to commit to the myogenic lineage.

A second population of stem cells with hematopoietic and myogenic capabilities has been isolated from adult skeletal muscles. These cells are isolated based on their

expression of Sca1 and CD34 (Montarras et al., 2005; Torrente et al., 2001). Interestingly, it was demonstrated that following tail vein injection these cells associated closely with the endothelium and that following subsequent muscle trauma, involving damage to the vasculature, the transplanted cell migrated from the vasculature into the skeletal muscle and contributed to regenerating myofibers (Torrente et al., 2001). Thus it is apparent that under extreme circumstances, cells other than quiescent satellite cells are able to respond to and repair muscle damage.

1.13 Project Rationale

Clearly skeletal muscle satellite cells are critical for the regeneration of skeletal muscle in response to injury and specific disease states. While our ability to identify these cells through the use of molecular markers has been greatly enhanced in the past several years, we are only just beginning to understand much of the basic biology of these cells. Until recently, the true origins of these cells remained elusive. However, through the use of the recently identified satellite cell marker Pax7, it has become apparent that the majority of these cells are of somatic origin. While it has long been known that HGF is capable of activating satellite cells, relatively little is known regarding the mechanism by which this occurs. Furthermore, it is unlikely that HGF secretion is the only mechanism by which satellite cells are activated, yet this remains the only pathway that has been clearly identified. It would appear that Syndecans may play a critical role in the activation of satellite cells but this remains to be clearly demonstrated. Indeed, the mechanism(s) by which the quiescent satellite cell population is maintained following repeat injury has not been clearly established. Therefore, we attempted to identify genes that function in the activation, proliferation and maintenance of the quiescent satellite cell

population. We hypothesized that ***MyoD-/-* myoblasts represent a primitive stage of myogenic cell immediately downstream of quiescent satellite cells but upstream of myogenic progenitors** (Fig 4) and that **using *MyoD-/-* primary myoblasts subtracted against 10T1/2 fibroblasts to perform representational difference analysis would result in the identification of genes that function in the activation, proliferation, and maintenance of the satellite cell compartment**. In support of the theory that *MyoD-/-* myoblasts represent primitive myogenic cells, we identified numerous hematopoietic markers in these cells indicating a stem-like state of these cells (Seale et al., 2004a). Furthermore, the screen identified Pax7 as being expressed in quiescent satellite cells and further investigation revealed a critical requirement for Pax7 expression in satellite cells, verifying the validity of this approach (Seale et al., 2000).

From the list of candidate genes identified in the RDA screen a small number of clones were chosen for closer examination based on their expression profiles. Genes that showed higher expression in MyoD-/- myoblasts than wildtype myoblasts or that were downregulated during differentiation in wildtype myoblasts but not MyoD-/- myoblasts were chosen for further examination. These studies led to a focus on the clone identified as Megf10, a putative signaling molecule localized to the plasma membrane of myogenic cells. Our research demonstrates that Megf10 is expressed in quiescent satellite cells and that it is capable of regulating the proliferative potnetial of myogenic cells.

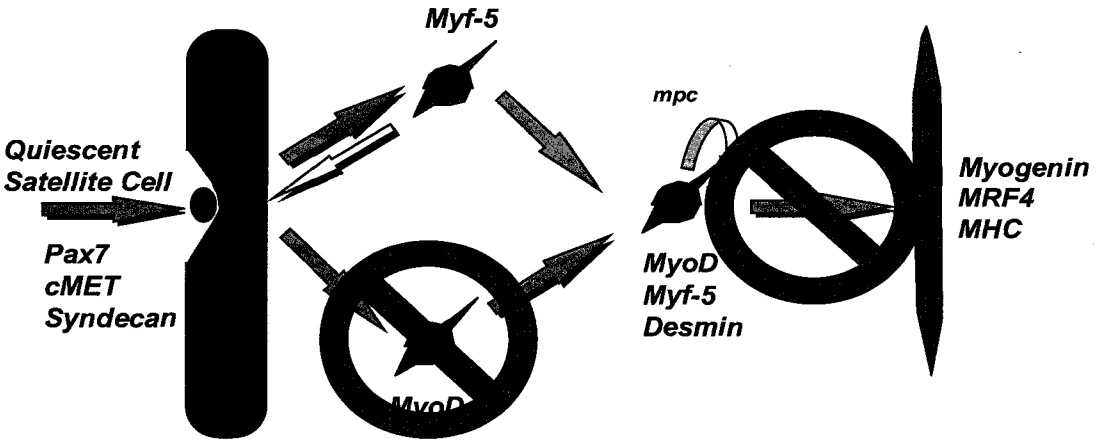

Figure 4. Satellite cell activation in the abscence of MyoD. Following activation, satellite cells that lack MyoD upregulate Myf5 and enter a highly proliferative phase. Unlike wildtype cells, MyoD-/- mpcs do not express desmin, and fail to undergo terminal differentiation which suggests these cells are a primitive form of mpc, closely related to quiescent satellite cells.

Chapter 2 –Expression Analysis and Cloning of RDA Clones of Interest

2.1 Introduction

Analysis of individual MRF knock-outs as well as different combinations of MRF knock-outs demonstrated that MyoD and Myf5 function as primary MRFs required for the initial establishment of myogenic precursors and appropriate muscle development (Braun et al., 1992; Rudnicki et al., 1992; Rudnicki et al., 1993). While removal of MyoD results in delayed development of the hypaxial muscle lineage, Myf5 is able to compensate for the loss of MyoD and normal hypaxial muscles do develop in *MyoD-/-* mice (Rudnicki et al., 1992).

The absence of MyoD has a more dramatic effect on skeletal muscle regeneration. Histological examination of skeletal muscle isolated from *MyoD-/-* mice revealed an increased number of quiescent satellite cells in two month old mice (Megeney et al., 1996). However, when challenged by freeze crush injury or cardiotoxin injection, *MyoD-/-* animals displayed a dramatic decrease in their ability to repair and regenerate skeletal muscle (Megeney et al., 1996). Large numbers of mononuclear cells persisted at the site of injury and these cells failed to undergo terminal differentiation to repair the damage (Megeney et al., 1996). Furthermore, when *MyoD-/-* mice were crossed into the mdx background in which constant rounds of degeneration and regeneration occur, a more dramatic mdx phenotype was observed. Mutant *MyoD-/- mdx* mice displayed a profound dorsal-ventral curvature of the spine, an abnormal waddling gait and premature death

indicating increased penetrance of the mdx phenotype in the absence of MyoD (Megeney et al., 1996). Furthermore, muscle mass in the *mdx/MyoD-/-* mice was dramatically decreased compared to mdx controls. These results reveal a crucial requirement for MyoD in postnatal satellite cell function.

In vitro analysis of primary myoblasts isolated from *MyoD-/-* animals revealed that following serum withdrawal, the majority of *MyoD-/-* primary myoblasts failed to undergo terminal differentiation as indicated by a lack of myogenin and myosin heavy chain expression and failure to form multinucleated myotubes (Sabourin et al., 1999). Furthermore, it was found that these cells continued to proliferate under low serum conditions as determined by continued 3H-thymidine incorporation following serum withdrawal, indicating continued DNA replication, an event that is mutually exclusive of terminal differentiation (Sabourin et al., 1999). Interestingly, in the absence of Myf5, myoblasts were found to have a very low proliferative capacity, and underwent precocious differentiation under growth conditions (Montarras et al., 2000). It has also been demonstrated that in proliferating cells, MyoD and Myf5 function to activate the expression of target genes such as L-myc, m-cadherin, Mcpt8, Runx1, Spp1, Six1, IGFBP5, and Chrnbeta1 (Ishibashi et al., 2005). However, MyoD appears to be more effecient than Myf5 at inducing differentiation-phase target genes(Ishibashi et al., 2005). These results support the notion that Myf5 functions in proliferation, whereas MyoD plays a more critical role in differentiation. These results demonstrate that MyoD and Myf5 perform unique functions in satellite cell biology.

Based on the *in vivo* and *in vitro* data from *MyoD-/-* muscle and data demonstrating the temporal expression of MyoD and Myf5 following satellite cell

activation, we proposed that mpcs or myoblasts that lacked MyoD represented a more primitive form of myogenic cell, downstream of a quiescent satellite cell but upstream of a typical mpc (Fig 4). We hypothesized that if *MyoD-/-* myoblasts represented a primitive form of mpc they would prove a useful tool for identifying genes that function in the early activation, proliferation, and potentially the maintenance of the quiescent satellite cell compartment.

To test this, we utilized representational difference analysis, a subtractive cloning technique, to identify genes that were uniquely expressed in *MyoD-/-* myoblasts (Seale et al., 2004a). As detailed in Appendix B, a large number of difference products were cloned into pCDNA3 vectors, sequenced, and subjected to northern blotting to determine their expression pattern during skeletal muscle regeneration. A list of over 50 individual products was generated including previously identified myogenic factors such as Myf5, as well as large number of genes previously shown to be expressed in hematopoietic stem cells (Seale et al., 2004a). The enrichment of genes expressed in other stem cell lineages provides support for the hypothesis that *MyoD-/-* myoblasts represent a primitive form of myogenic precursor, closely related to muscle stem cells.

Detailed analysis of one of the difference products, clone dp3-7, lead to the identification of Pax7 as a crucial factor in satellite cell maintenance (Seale et al., 2000). Adult mice lacking Pax7 display a dramatic absence of quiescent satellite cells and a subsequent inability to repair damage induced by cardiotoxin (ctx) injection (Seale et al., 2000). Furthermore, retroviral infection of non-committed stem cells isolated from skeletal muscle, with Pax7, leads to myogenic conversion of these cells further demonstrating a crucial role for Pax7 in satellite cell determination and maintenance

(Seale et al., 2004b). These results verify that our RDA experiments provide useful information into the activation, proliferation and maintenance of the satellite cell compartment.

To determine if individual RDA products were of potential interest, we examined their expression profile under growth and differentiation conditions in wildtype and *MyoD-/-* primary myoblasts. Difference products that displayed high expression under growth conditions and were downregulated during differentiation were chosen for further examination. As well, difference products that displayed high expression in *MyoD-/-* myoblasts and little or no expression in wildtype myoblasts were also further examined.

2.2 Materials and Methods

2.2.1 Myoblast Isolation and Cell Culture

Primary myoblasts were isolated from hindlimb muscles of 4-6 week old mice as previously described (Megeney et al., 1996). Briefly, hindlimb muscles were extracted and subjected to collagenase/dispase disruption for 12 minutes. The resulting suspension was triturated using a 5ml plastic pipette. This was repeated twice. Following the second trituration, the suspension was passed through a 45μm filter to remove debris. The collected suspension was centrifuged at 1000 rpm for 5 min. The supernatant was removed and the pellet was resuspended in myoblast media. The resulting suspension was plated on plastic tissue culture dishes for 20 minutes prior to switching to collagen-coated dishes. Primary myoblasts were maintained in Ham's F10 media supplemented with 20% fetal bovine serum, 100U/ml penicillin and streptomycin and 2.5ng/mL bFGF.

Cells were routinely grown to 60% confluency and passaged 1:3 on collagen coated 10cm tissue culture dishes.

Established C_2C_{12} myoblasts were maintained in Dulbeco's MEM (DMEM) media supplemented with 10% fetal bovine serum and 100U/ml penicillin and streptomycin. Cells were routinely passaged 1:5 on 10cm tissue culture plates. Under differentiation conditions, cells were maintained in DMEM supplemented with 2% horse serum and 100U/mL penicillin and streptomycin.

2.2.2 RNA Extraction and Northern Blotting

RNA was isolated using the RNEasy Miniprep kit (Qiagen). Samples were run on 0.8% agarose gels prepared with 4% formaldehyde. Northern blots were carried out using standard protocols as previously described (Seale et al., 2004a). MessageMap tissue polyA+ RNA northern blots (Stratagene) were hybridized in Miracle*Hyb* (Stratagene) as per manufacturers instructions. RDA products were utilized to generate random-primed 32P dCTP labeled probes.

2.2.3 Cardiotoxin injection

For CTX-induced muscle regeneration, mice were anesthetized with Halothane and injected with 25 µl CTX (10 µM; Latoxan) into the tibialis anterior muscles. Mice were sacrificed 4 days following injection and the TA muscle was surgically removed.

2.2.4 Isolation of myoblasts by flow cytometry

Resting and 4 days post-cardiotoxin injected TA muscles was isolated from 6-10 week old wild-type Balb/C mice. Myoblasts were recovered as described in section 2.2.1 (Megeney et al., 1996). Cells were resuspended in DMEM with 10% fetal bovine serum and incubated on ice for 20 min with with either CD45-PE (BD Pharmingen) or Sca1-APC (Caltag), or both at 1 μg per $1x10^6$ cells. Immuno-labeled cells were four-way sorted based on the presence or absence of Sca-1 and CD45 on a MoFlo cytometer (DakoCytomation). Gates were set by analyzing cells stained for Sca1 alone, CD45 alone, or unstained cells. Cell fractions obtained were $Sca1^+/CD45^+$, $Sca1^+/CD45^-$, $Sca1^-/CD45^+$, $Sca1^-/CD45^-$. RNA from the sorted fractions was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Data points represent means +/- Standard deviation (n=2).

2.2.5 RT-PCR

Total RNA was extracted using RNEasy kits (Qiagen), according to manufacturer's instructions. RT-PCR analysis was performed using the GeneAmp PCR Core kit (Perkin-Elmer) as per manufacturer's instructions. The following primers were used: Hox B4; For TCAAGACAAGATGCCCTGAAAACATAAGGT; Rev GCA TCGGCTGTGTCCGTCAGTCTGTCCTCC; HoxC4; For CTCCCTCCTGTTCCT CAAAGCTGAGG; Rev CGCTCTGGGAGTGGTCTTCAGAAGTG; HoxD4; For GCC AAGGACCACCATACG; Rev AGGAAGGTAACCTAGTCCGAGG; HoxC6 For CGCCTATGATCCAGTGAGGC Rev CTTCATGCGGCGGTTCTGGG; HoxC10 For CCAGCTGCGAGAGGTAGGA Rev CTCGCAATGTAACTCCGAAC; Megf10; For

ACCAACTGTAGCAGTGCTTGT; Rev CAGTGGCAAGCACCGGTGAT; β-Actin For TCTTTGCAGCTCCTTCGTT Rev GACCAGCGCAGCGATA.

2.2.6 *In Situ* hybridization

In situ hybridizations were performed on 8-μm cryosections of mouse TA muscles isolated from 8-week-old wildtype as per previously described procedures (Braissant and Wahli, 1998). Sense and antisense *in situ* probes were synthesized from RDA products using DIG-UTP labeling mix (Roche) with SP6 or T7 RNA polymerase (Roche). DIG labeled probes were detected with alkaline phosphatase-conjugated anti-DIG antibody (Roche) followed by reaction with BCIP/NBT (Roche).

2.2.7 Phage Library Screen and 5'RACE

A commercially prepared mouse e10.5 Lambda cDNA phage library (Stratagene) was plated at $5x10^4$ pfu/plate. A total of $5x10^5$ plaques were plated and lifted as per manufacturer's instructions. Plaques were screened using radiolabeled probe generated using the original MD p67 RDA clone. Positive plaques were cored and eluted as per manufacturer's instructions, and secondary and tertiary screens were performed at $1x10^3$ and $1x10^2$ pfu/plate respectively. The two positive clones were sequenced and compared/aligned using DNAStar software. We utilized the 5'RACE Kit (Clontech) to amplify the 5'UTR and remaining coding sequence from RNA isolated from primary MyoD-/- myoblasts as per manufacturer's instructions.

2.2.8 Retroviral Production and Infection

Retrovirus was generated using a modified version of the three-plasmid HIT system (provided by V. Sartorelli, NIH) as previously described (Ishibashi et al., 2005; Soneoka et al., 1995). For retroviral infection, C_2C_{12} cells were seeded at $1x10^5$ cells per 10cm plate. Eight hours following plating, cells were incubated with retrovirus for 12 hours with 8 μg/ml polybrene. Cells were then washed twice with PBS and maintained in growth media for 24 hours prior to the addition of antibiotics for selection. Cells were continuously maintained under antibiotic selection.

2.2.9 Protein Isolation and Cellular Fractionation

Protein was isolated as previously described (Perry et al., 2001). For cellular fractions cells were washed twice in ice-cold PBS-FV and scrapped off plates in 1ml PBS-FV. Cells were pelleted, resuspended in 5ml ice-cold hypotonic buffer (10mM HEPES [pH8.0], 15mM KCl, 2mM MgCl2, 0.1mM EDTA) and incubated for 5 minutes. Following incubation, cells were lysed using a 7ml Dounce homogenizer (B pestel) on ice. Supernatant was then centrifuged at 65000rpm (4°C) for 1 hour using NVT100 rotor in an ultracentrifuge (Beckman). Supernatant was collected and the membrane pellet was resuspended in NP-40 lysis buffer (0.5% NP-40 [v/v], 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10 mM sodium pyrophosphate, 1 mM EDTA)with protease inhibitors (1mM DTT, 1mM PMSF, 10mg/ml pepstatin A, aprotinin, leupeptin, and 1mM sodium vanadate). The nuclear pellet was washed once in Buffer A (10 mM HEPES [pH 7.8], 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.1% NP-40) and then incubated on ice for 20 min in buffer C (20 mM HEPES [7.8], 25% glycerol, 420 mM

NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT,0.5 mM PMSF, 0.1% NP-40). Nuclear lysates were centrifuged and supernatants were diluted with buffer D (20 mM HEPES [pH 7.8], 20% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5mM DTT, 0.5mM PMSF), with protease inhibitors.

2.3 RESULTS

A cDNA sample generated from *MyoD-/-* primary myoblasts was subtracted against cDNA from 10T1/2 fibroblasts in an attempt to identify genes that function in the activation, proliferation, and maintenance of the satellite cell compartment. Candidate genes identified in the RDA screen were screened for specific expression patterns in MyoD-/- and wildtype myoblasts. Specifically, genes that showed higher expression in MyoD-/- myoblasts than wildtype myoblasts or that were downregulated during differentiation in wildtype myoblasts but not MyoD-/- myoblasts were considered to be of interest. Genes that were putatively expressed on the cell surface such as MDp67 and MDp40, or genes that had previously been implicated in regeneration such as HoxC10 were chosen for closer examination (Carlson et al., 2001; Christen et al., 2003).

2.3.1 Examination of Clone MD p286

Clone MD p286, identified through BLAST analysis as HoxC10, was found to be highly expressed in *MyoD-/-* myoblasts under growth and differentiation conditions (Fig 5). HoxC10 was also expressed to a lesser extent in wildtype myoblasts under growth conditions but was not detected following differentiation (Fig 5). To further examine *HoxC10* expression, we performed Northern blots on polyA+ RNA isolated from a series

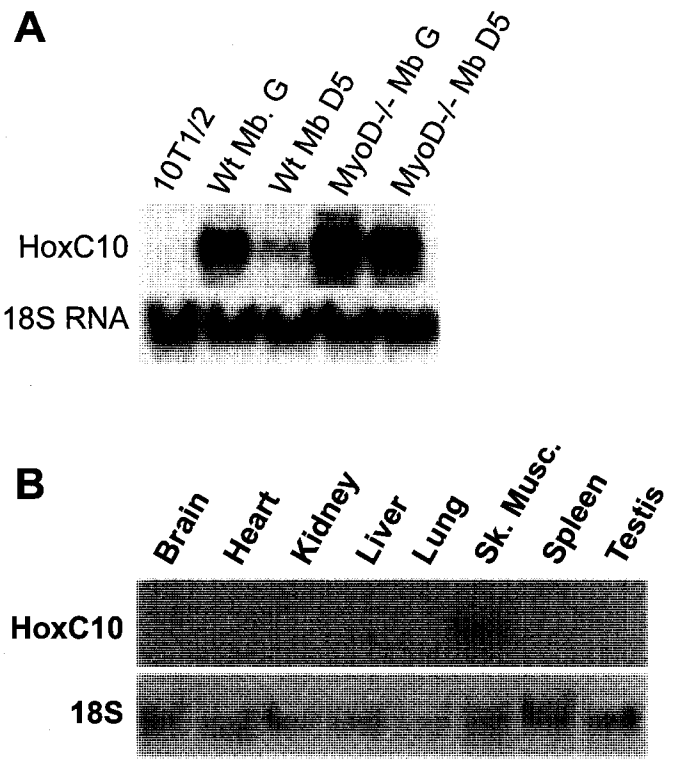

Figure 5. Northern Blot analysis of HoxC10 expression in Myoblasts and Mouse Tissue. (A) Total RNA was isolated from wildtype (wt) and MyoD-/- myoblasts from growth (G) conditions and day 5 of differentiation (D5). HoxC10 expression was detected in MyoD-/- myoblasts under both growth and differentiation conditions, but was down-regulated in wt myoblasts following differentiation. (B) MessageMap PolyA+ RNA Northern blots reveal HoxC10 expression in skeletal muscle. 18S was used as a loading control.

of adult mouse tissues. Interestingly, high levels of *HoxC10* mRNA were detected in skeletal muscle but expression was not detected in the other tissues examined (Fig 5). Previous reports examining the expression of a similar Hox family member, HoxA10, in skeletal muscle indicate that HoxA10 is specifically expressed in proliferating myoblasts and overexpression of HoxA10 as well as maintained HoxA10 expression inhibit differentiation (Houghton and Rosenthal, 1999). We next examined if *HoxC10* was up-regulated during skeletal muscle regeneration using semi-quantitative RT-PCR. Hindlimb muscles were isolated from resting and 4 days post-ctx injection adult (2 month old) mice and the RNA isolated. RT-PCR using *HoxC10* specific primers revealed that as previously demonstrated *HoxC10* was expressed in resting adult hindlimb muscle. Interestingly our results indicated that *HoxC10* was down-regulated during regeneration in hindlimb musculature (Fig 6). During development Hox paralogs display strict temporal and spatial expression patterns (Mark et al., 1997). Hox paralogs of the 10 cluster are expressed at later stages of development in a more caudal position. To examine the possibility that *HoxC10* was identified in the RDA screen due to the posterior origin of the original myoblasts used for RNA isolation we examined forelimb musculature for *HoxC10* expression. We were unable to detect *HoxC10* expression in the forelimb musculature of adult mice (Fig 6). Furthermore, *HoxC10* upregulation was not detected in regenerating forelimb musculature by RT-PCR (Fig 6). These results suggest that the identification of *HoxC10* in our RDA screen may be due to the posterior nature of the myoblasts used to generate the initial RNA population.

To further address the question as to which population of cells in resting and regenerating skeletal muscle expressed *HoxC10*, we sorted cells using FACS and

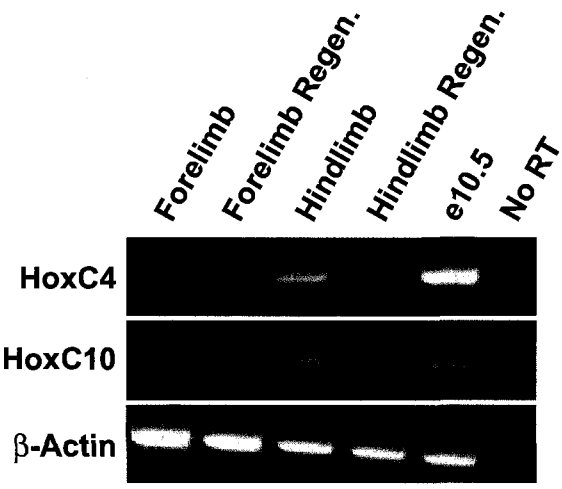

Figure 6. RT-PCR analysis of HoxC10 expression in mouse forelimb and hindlimb. Total RNA was extracted from resting and regenerating mouse limb musculature and subjected to RT-PCR for HoxC10 expression. HoxC10 is detectable in resting hindlimb and to a lesser extent in regenerating hindlimb. HoxC10 is not detected in the forelimb during rest or regeneration. HoxC4 is detectable in both forelimb and hindlimb. β-Actin was used as a loading control.

performed quantitative RT-PCR on RNA isolated from these cells. Resting TA muscle was digested and subjected to FACS sorting based on the presence or absence of Sca1 and CD45 staining. Cells were sorted into four separate fractions based on the presence or absence of Sca1 and CD45 staining (Sca1+/CD45+, Sca1+/CD45-, Sca1-/CD45+, Sca1-/CD45-). Previous work in our laboratory has demonstrated that quiescent satellite cells are limited to the Sca1-/CD45- fraction. Our RT-PCR results demonstrated *HoxC10* expression in each of the single positive populations but *HoxC10* was not detectable in the Sca1-/CD45- population in which quiescent satellite cells reside, or the Sca1+/CD45+ populations which contains multipotent stem cells, indicating that *HoxC10* was most likely not expressed in stem cells or quiescent satellite cells in skeletal muscle (Fig 7). We also examined several other members of the Hox family implicated in stem cell function and did not detect significant levels of expression in the Sca-/CD45- population (Fig 7).

2.3.2 Isolation of Full length cDNAs

The initial RDA clones, MD p40 and MD p67, were used as probes to screen a λ–phage mouse skeletal muscle cDNA library in order to obtain full-length cDNAs. In order to ensure complete coverage of the library the initial screen was performed at $5x10^4$ plaques per 150 mm plate resulting in a total of $5x10^5$ plaques being screened with each probe. Our primary screen resulted in the recovery of two positive plaques for MD p67 and three plaques for MD p40. Positive clones identified in the primary screen were cored, eluted and re-plated at $1x10^3$ for secondary screening. Again, positive plaques for each of the initial clones were cored and eluted. To ensure isolation of single cDNAs, the

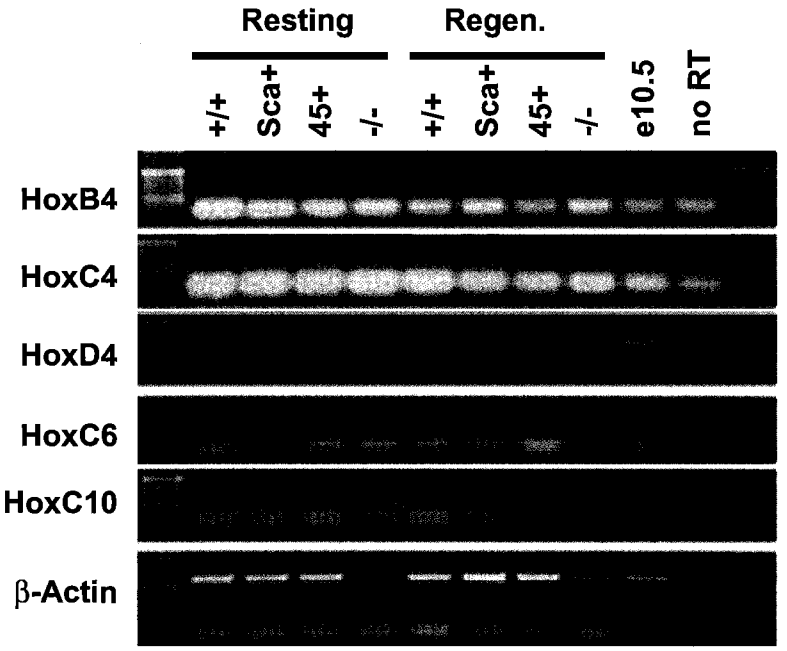

Figure 7. HoxC10 is not expressed in the cell population that contains quiescent satellite cells. Resting and regenerating hindlimb musculature was disrupted and subjected to FACS based on the presence of Sca1 and CD45. HoxC10, along with a panel of other Hox family members, were not detected in the Sca1-/CD45- population that contains quiescent satellite cells.

plaques cored in the secondary screen were plated at $1x10^2$ and re-probed. Individual plaques were again cored and eluted. The resulting phage were recovered and their DNA sequenced.

Two of the three plaques obtained using the MD p40 probe showed a large portion of overlapping sequence. However, the third sequence showed no overlap with the other two. To determine if the third sequence was a false positive, we attempted to align the recovered sequence with that of the MD p40 probe. Our results revealed no homology between the probe used and the sequence obtained indicating that the third sequence was indeed a false positive. BLAST analysis of the contig generated by aligning the remaining two sequences revealed a low degree of homology to a G-protein coupled receptor. Alignment with the MD p40 probe verified that these two sequences were true positives.

Sequencing of the two clones that hybridized to the MD p67 probe resulted in the recovery of two sequences with a large portion of identical overlapping sequence. To verify that these sequences were true positives, the individual sequences were aligned with the MD p67 probe. Alignment and subsequent BLAST analysis of the obtained sequence verified our previous results that identified MDp67 as the mouse homolog of human MEGF10 (KIAA1780 HUGE Database). Our analysis showed >94% identity at the amino acid level between Megf10 and MEGF10, revealing a high level of conservation between the mouse and human genes (Fig 8). These two sequences are also similar to probable orthologs CED-1 (*C. elegans*) and Draper (*D. melanogaster*).

Comparison of the sequence recovered for *Megf10* to that for *MEGF10* suggested that we were still lacking the 5' most portion of *Megf10*. Therefore, we employed

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                MAISLSSCLGLICLLLCHWVGTASSLNLEDPNVCSHWESYSVTVQESYPHPFDQIYYTSC
                        10        20        30        40        50        60
dp67fullpro2.P  MAISSSSCLGLICSLLCHWVGTASSLNLEDPNVCSHWESYSVTVQESYPHPFDQIYYTSC  60
megf10pr.PRO    MVISLNSCLSFICLLLCHWIGTASPLNLEDPNVCSHWESYSVTVQESYPHPFDQIYYTSC  60

                TDILNWFKCTRHRVSYRTAYRHGEKTMYRRKSQCCPGFYESGDMCVPHCADKCVHGRCIA
                        70        80        90       100       110       120
dp67fullpro2.P  TDILNWFKCTRHRISYRTAYRHGEKTMYRRKSQCCPGFYESRDMCVPHCADKCVHGRCIA 120
megf10pr.PRO    TDILNWFKCTRHRVSYRTAYRHGEKTMYRRKSQCCPGFYESGEMCVPHCADKCVHGRCIA 120

                PNTCQCEPGWGGTNCSSACDGDHWGPHCSSRCQCKNGALCNPITGACHCAAGFRGWRCED
                       130       140       150       160       170       180
dp67fullpro2.P  PNTCQCEPGWGGTNCSSACDGDHWGPHCSSRCQCKNRALCNPITGACHCAAGYRGWRCED 180
megf10pr.PRO    PNTCQCEPGWGGTNCSSACDGDHWGPHCTSRCQCKNGALCNPITGACHCAAGFRGWRCED 180

                RCEQGTYGNDCHQRCQCQNGATCDHVTGECRCSPGYTGAFCEDLCPPGKHGPQCEQRCPC
                       190       200       210       220       230       240
dp67fullpro2.P  RCEQGTYGNDCHQRCQCQNGATCDHITGECRCSPGYTGAFCEDLCPPGKHGPHCEQRCPC 240
megf10pr.PRO    RCEQGTYGNDCHQRCQCQNGATCDHVTGECRCPPGYTGAFCEDLCPPGKHGPQCEQRCPC 240

                QNGGVCHHVTGECSCPSGWMGTVCGQPCPEGRFGKNCSQECQCHNGGTCDAATGQCHCSP
                       250       260       270       280       290       300
dp67fullpro2.P  QNGGVCHHVTGECSCPSGWMGTVCGQPCPEGRFGKNCSQECQCHNGGTCDAATGQCHCSP 300
megf10pr.PRO    QNGGVCHHVTGECSCPSGWMGTVCGQPCPEGRFGKNCSQECQCHNGGTCDAATGQCHCSP 300

                GYTGERCQDECPVGSYGVLCAEACQCVNGGKCYHVSGACLCEAGFAGELCEARLCPEGLY
                       310       320       330       340       350       360
dp67fullpro2.P  GYTGERCQDECPVGSYGVRCAEACRCVNGGKCYHVSGTCLCEAGFSGELCEARLCPEGLY 360
megf10pr.PRO    GYTGERCQDECPVGTYGVLCAETCQCVNGGKCYHVSGACLCEAGFAGERCEARLCPEGLY 360

                GIKCDKRCPCHLDNTHSCHPMSGECGCKPGWSGLYCNETCSPGFYGEACQQICSCQNGAD
                       370       380       390       400       410       420
dp67fullpro2.P  GIKCDKRCPCHLDNTHSCHPMSGECGCKPGWSGLYCNETCSPGFYGEACQQICSCQNGAD 420
megf10pr.PRO    GIKCDKRCPCHLENTHSCHPMSGECACKPGWSGLYCNETCSPGFYGEACQQICSCQNGAD 420

                CDSVTGKCACAPGFKGIDCSTPCPLGTYGINCSSRCGCKNDAVCSPVDGSCICKAGWHGV
                       430       440       450       460       470       480
dp67fullpro2.P  CDSVTGRCACAPGFKGTDCSTPCPLGRYGINCSSRCGCKNDAVCSPVDGSCICKAGWHGV 480
megf10pr.PRO    CDSVTGKCTCAPGFKGIDCSTPCPLGTYGINCSSRCGCKNDAVCSPVDGSCTCKAGWHGV 480

                DCSIRCPSGTWGFGCNLTCQCLNGGACNTLDGTCTCAPGWRGAKCELPCQDGTYGLNCAE
                       490       500       510       520       530       540
dp67fullpro2.P  DCSIRCPSGTWGFGCNLTCQCLNGGACNTLDGTCTCAPGWRGAKCEFPCQDGTYGLNCAE 540
megf10pr.PRO    DCSIRCPSGTWGFGCNLTCQCLNGGACNTLDGTCTCAPGWRGEKCELPCQDGTYGLNCAE 540

                RCDCSHADGCHPTTGHCRCLPGWSGVHCDSVCAEGRWGPNCSLPCYCKNGASCSPDDGIC
                       550       560       570       580       590       600
dp67fullpro2.P  RCDCSHADGCHPTTGHCRCLPGWSGVHCDSVCAEGRWGPNCSLPCYCKNGASCSPDDGIC 600
megf10pr.PRO    RCDCSHADGCHPTTGHCRCLPGWSGVHCDSVCAEGRWGPNCSLPCYCKNGASCSPDDGIC 600
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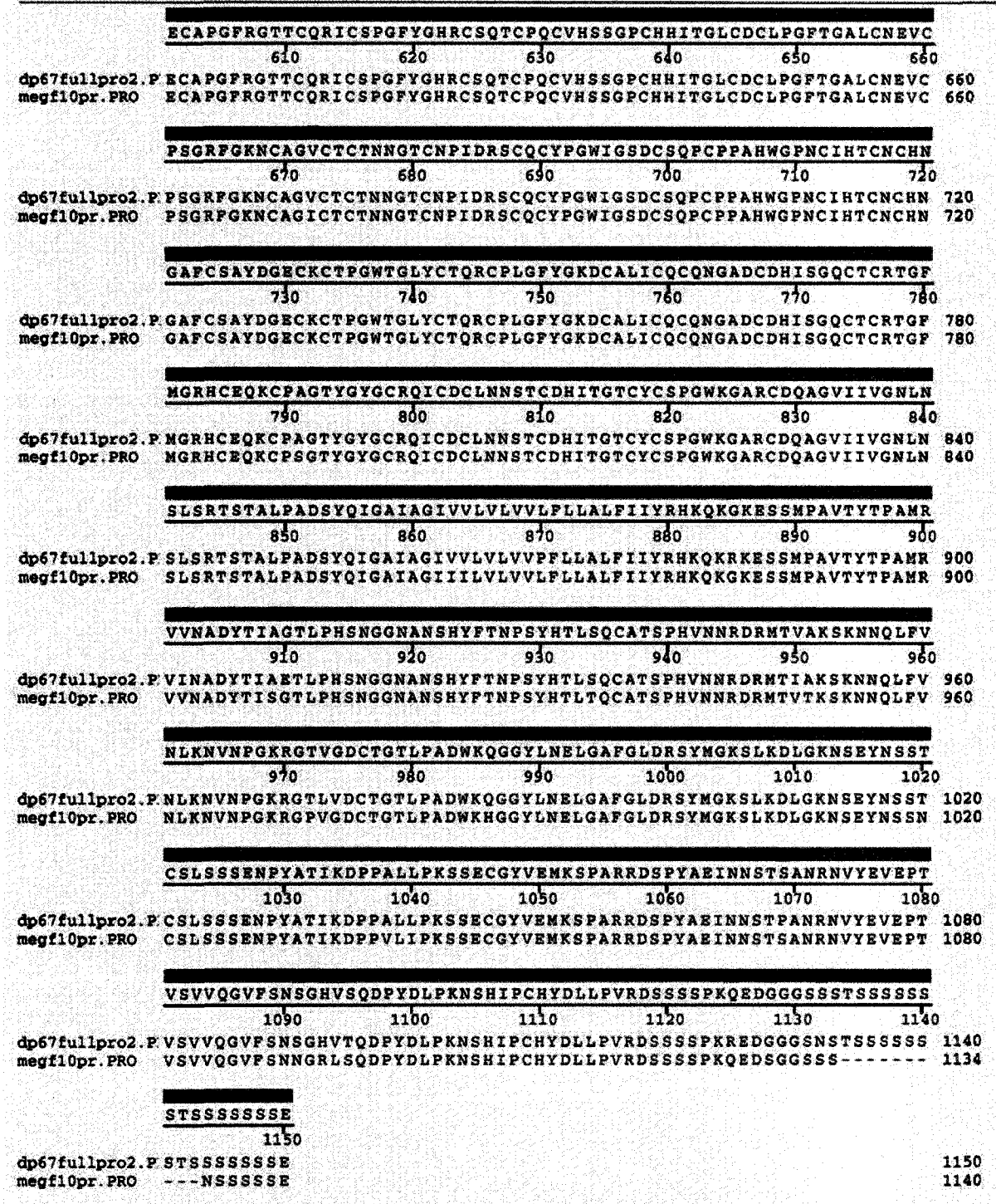

Figure 8. Amino Acid Homology of MEGF10 and MD p67. Human MEGF10 and mouse Megf10 amino acid sequences were compared for homology using DNAStar software. Over 94% homology is observed. Areas overscored by red represent complete homology. Regions overscored in blue are not conserved.

5'RACE to isolate the entire cDNA sequence. Once the full length cDNA was obtained it was cloned into the pCDNA3 backbone for subsequent use. Sequence analysis using the Simple Modular Architectural Research Tool (SMART) to identify conserved domains within Megf10 revealed the presence of a signal sequence at the amino-terminal end, multiple EGF-repeats, and a transmembrane domain preceding the carboxy-terminus (Fig 9). Furthermore, the results indicated that Megf10 most likely localized to the plasma membrane given the transmembrane domain and multiple EGF repeats.

2.3.3 Examination of Clone MD p40

BLAST analysis of the initial RDA MD p40 clone revealed no homology to known genes. Initial northern blotting of RDA clone MD p40 revealed expression in *MyoD-/-* myoblasts under growth conditions but not in differentiation conditions (Fig 10). MD p40 was not detected in wildtype myoblasts under either growth or differentiation conditions (Fig 10). Furthermore, MD p40 expression was not detected in non-myogenic cells such as HELA, mouse embryonic fibroblasts, and 10T1/2 fibroblasts (Fig 10). Northern blotting of polyA+ RNA isolated from a variety of tissues demonstrated MD p40 expression in the heart but expression was not detectable in skeletal muscle or other tissues examined (Fig 10).

Given that satellite cell specific RNAs represent a relatively low percentage of mRNAs isolated from total skeletal muscle, we performed *in situ* hybridization on tibialis anterior muscle isolated from 2 month old mice to determine whether MD p40 was truly expressed in resting skeletal muscle. MD p40 was detected in approximately 5% of total nuclei in cross section, a number consistent with restricted expression in quiescent

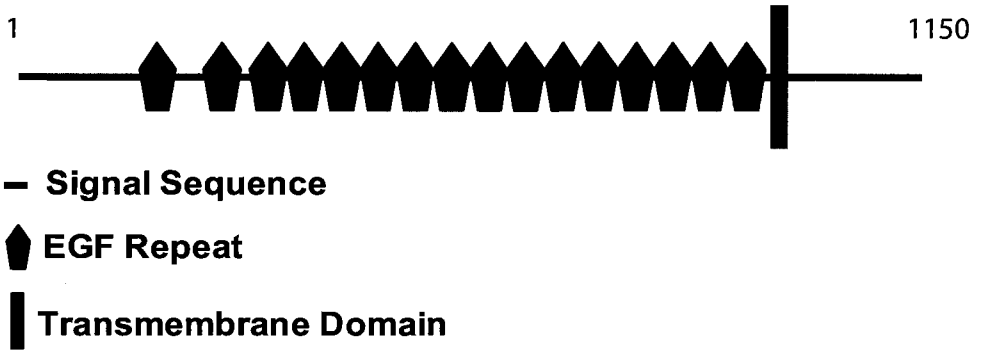

Figure 9. SMART protein modeling of Megf10 predicts multiple EGF-repeat domains. Megf10 amino acid sequence was subjected to the Simple Modular Architectural Research Tool to identify highly conserved domains. The predicted structure of Megf10 contains an amino-terminal transport signal sequence, multiple EGF-repeat domains, a single-pass transmembrane domain, and a short carboxy-terminal cytoplasmic domain.

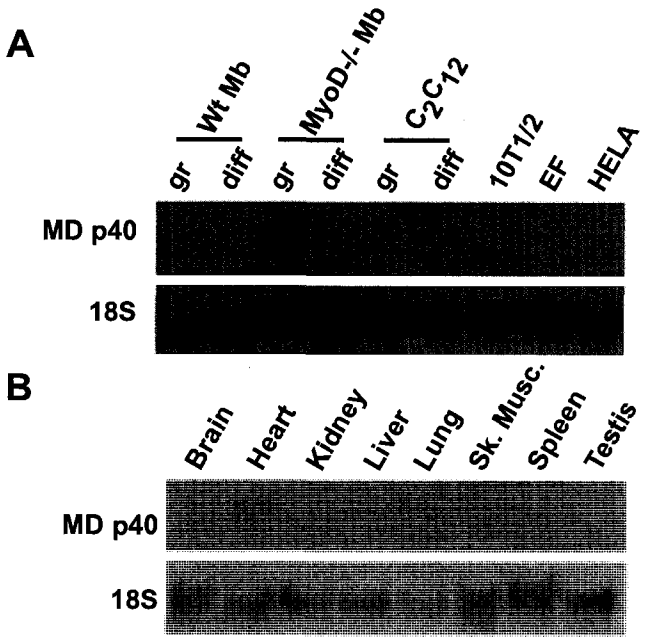

Figure 10. Northern blot analysis of MD p40 expression in cell lines and tissues. (A) Northern blotting of total RNA isolated from primary myoblasts and non-myogenic cells reveals MD p40 expression in MyoD-/- myoblasts under growth conditions. MD p40 is not detected in wildtype myoblasts or non-myogenic cell lines. (B) MessageMap PolyA+ RNA Northern blots reveal MD p40 expression in the heart and possibly the lung. MD p40 is not detected in skeletal muscle. 18S was used as a loading control.

satellite cells (Fig 11). Furthermore, positive cells were only found at the periphery of fibers in a position reminiscent of satellite cells. Given the numbers and location of positive cells, our initial results suggest that MD p40 expression may be restricted to quiescent satellite cells.

2.3.4 Examination of Clone MD p67

BLAST analysis of the initial MD p67 clone revealed high homology to KIAA1780, also referred to as *MEGF10*, previously identified by Nagase et al., in a large scale screen of unidentified genes expressed in the brain (Nagase et al., 1998). Given the high degree of similarity between mouse MD p67 and human *MEGF10*, we will refer to MD p67 as mouse *Megf10* from here forward. Results obtained by Nagase et al., demonstrated that *MEGF10* is expressed in the brain and to a lesser extent in skeletal muscle but is not expressed in the other tissues examined.

To define the expression pattern of *Megf10* we again performed Northern blots of total RNA isolated from wildtype and *MyoD-/-* primary myoblasts and C_2C_{12} myoblasts under growth and differentiation condition. We also examined a variety of non-myogenic cell lines including 10T1/2 and embryonic fibroblasts, HELA, MCF7 and P19 cells. The results revealed high expression of *Megf10* in proliferating myoblasts with highest expression detected in *MyoD-/-* myoblasts under growth conditions (Fig 12). *Megf10* expression was downregulated following serum withdrawal (Fig 12). Interestingly *Megf10* expression was not detectable in the non-myogenic cell lines examined suggesting that *Megf10* is expressed in a lineage specific manner (Fig 12). To determine if *Megf10* was expressed ubiquitously or in a tissue-specific manner we

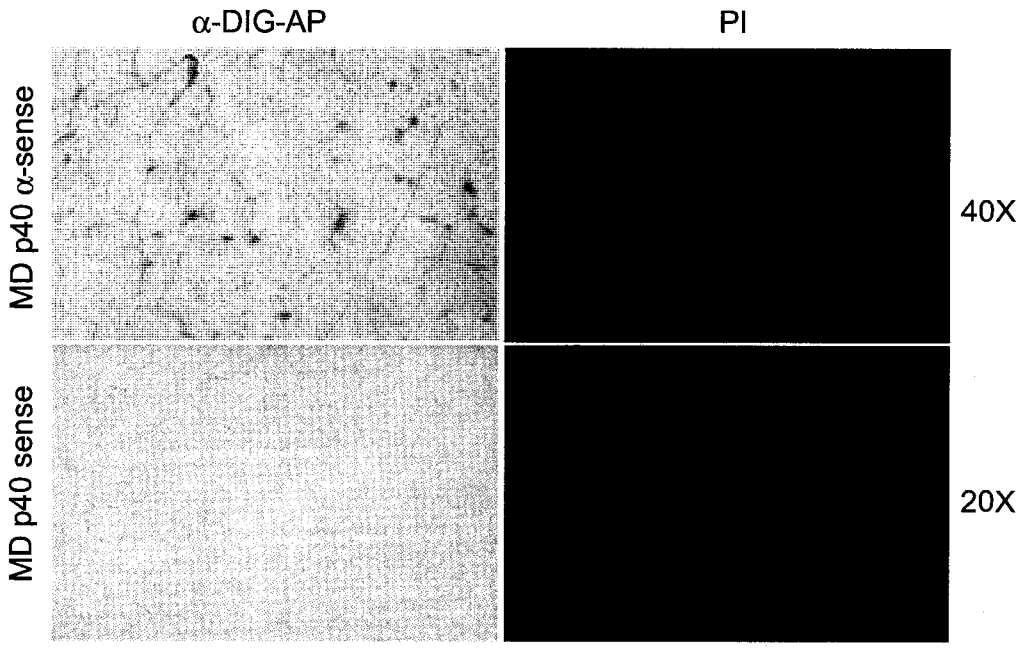

Figure 11. MD p40 is detectable in tibialis anterior muscle by in situ hybridization. Cross sections of tibialis anterior muscle subjected to in situ hybridization for MD p40 reveal multiple cells expressing MD p40 located along the periphery of fibers. Probes were labelled with DIG-UTP and visualized using alkaline phosphatase conjugated anti-DIG antibody. Total nuclei were visualized by propidium iodide (PI) staining. Sense control probes were negative.

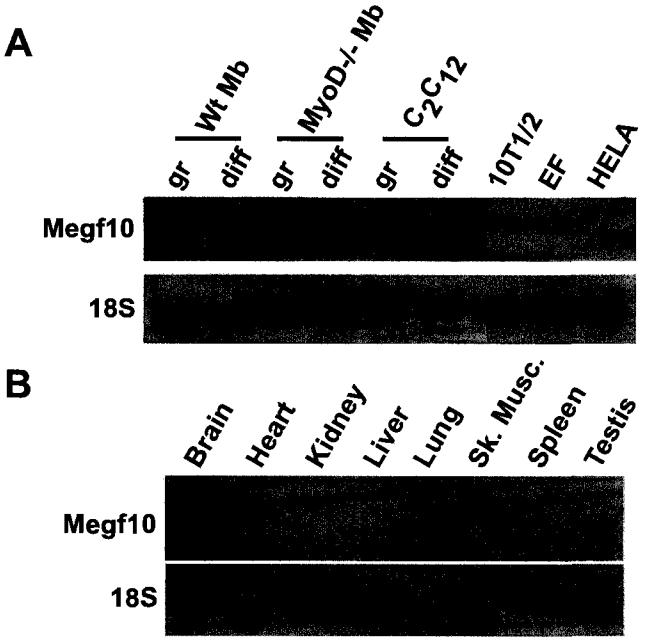

Figure 12. Northern blot analysis of Megf10 expression in cell lines and tissues. (A) Northern blotting of total RNA isolated from primary myoblasts and non-myogenic cells shows high levels of Megf10 expression in MyoD-/- myoblasts under growth conditions. Low levels of Megf10 are detected in wildtype (wt) myoblasts. Megf10 is down-regulated during differentation (diff). Megf10 is not detected in non-myogenic cell lines. (B) MessageMap PolyA+ RNA Northern blots reveal high levels of Megf10 expression in the brain. Low levels of Megf10 are detectable in skeletal muscle. 18S was used as a loading control.

performed Northern analysis of mouse polyA+ tissue RNA. *Megf10* expression was detected in the brain and at lower levels in skeletal muscle as well as testis (Fig 12). *Megf10* was not expressed at detectable levels in the other tissues examined. These results are in agreement with the previously observed expression pattern of MEGF10.

We next examined the expression of *Megf10* during skeletal muscle regeneration. Hindlimb muscles were excised from resting or 4 days post-cardiotoxin injected adult mice. RNA was extracted and subjected to semi-quantitative RT-PCR. *Megf10* was detectable in resting skeletal muscle and was found to be upregulated in regenerating muscle (Fig 13).

To determine the exact location of *Megf10* expression in adult skeletal muscle we performed *in situ* hybridization on tibialis anterior muscle isolated from 2-month-old adult mice. In cross section, approximately 5-7% of nuclei were found to express detectable levels of *Megf10* (Fig 14). Sense control probes showed no staining verifying that the hybridization of the anti-sense probe was specific. The majority of cells that were found to express *Megf10* were located adjacent to myofibers in locations characteristic of quiescent satellite cells or myonuclei. Occasionally cells expressing *Megf10* were detectable in interstitial spaces or near non-myogenic structures such as capillaries although such events were rare. These results verify that *Megf10* is expressed in resting adult skeletal muscle potentially in quiescent satellite cells but also in a small number of cells that are not in typical satellite cell position. Furthermore, *Megf10* is up-regulated in response to muscle injury indicating a potential role for *Megf10* in skeletal muscle regeneration.

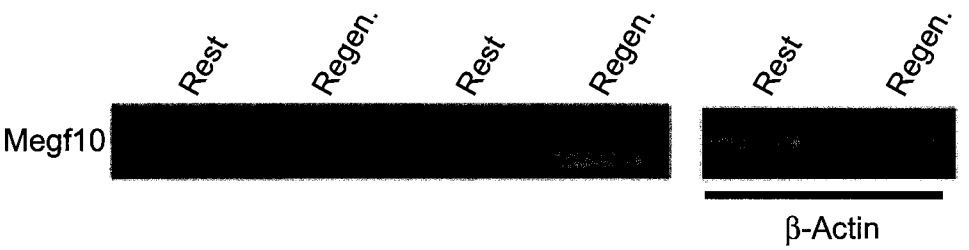

Figure 13. Megf10 is upregulated during regeneration. Semi-quantitative RT-PCR was performed on total RNA extracted from resting and 4 days post cardiotoxin injected tibialis anterior muscles. Low levels of Megf10 were detected in resting muscle. The level of Megf10 expression in regenerating muscle is dramatically increased.

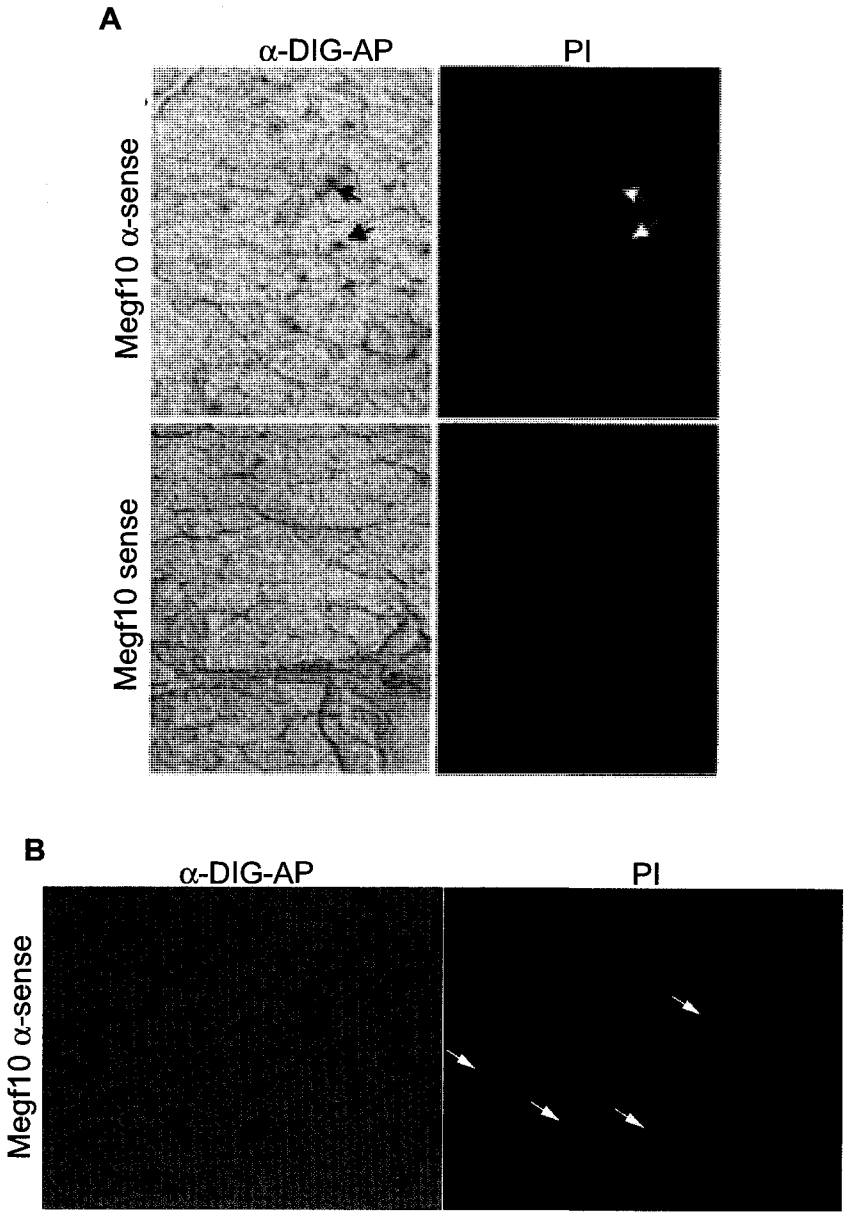

Figure 14. Megf10 is detectable in tibialis anterior muscle by in situ hybridization. (A) Cross sections of tibialis anterior muscle subjected to in situ hybridization for Megf10 reveal multiple cells expressing Megf10 located along the periphery of fibres (10X magnification). Probes were labelled with DIG-UTP and visualized using alkaline phosphatase conjugated anti-DIG antibody. Total nuclei were visualized by propidium iodide (PI) staining. Sense control probes were negative. (B) 40X mag.

2.3.5 Megf10 Localizes to the Plasma Membrane

The full length *Megf10* cDNA was cloned into the pHIT and pHAN retroviral plasmids and used for the subsequent generation of retrovirus. We utilized antibiotic selection to generate stable pools of cells expressing an HA-tagged version of Megf10. Immunocytochemistry indicated that infected cells did indeed express Megf10 although there was a significant difference in the expression levels between 10T1/2 fibroblasts that appeared to express high levels of Megf10 and C_2C_{12} myoblasts that expressed much lower levels of Megf10.

To determine if Megf10 localized to the plasma membrane as predicted, we performed cellular fractionation and subjected protein from each of the fractions to Western blotting. Our results clearly demonstrate expression of Megf10 in the plasma membrane fraction as well as the cytoplasmic fraction (Fig 15). Interestingly, two distinct bands were detectable in the plasma membrane fraction both of which appeared slightly larger that the band detected in the cytoplasmic portion. As well, there was a low level of Megf10 detected in the nuclear membrane fraction. Fraction purity was verified by blotting for Grb-2, Integrin α4, Lamin-A, and TBP that are uniquely expressed in the cytoplasm, plasma membrane, nuclear membrane and nuclear fractions respectively (Fig 15). Megf10 does localize to the plasma membrane as predicted. Thus, our results indicate that Megf10 is a multiple EGF-repeat containing transmembrane protein that shows restricted expression within adult skeletal muscle, most likely being expressed in quiescent satellite cells.

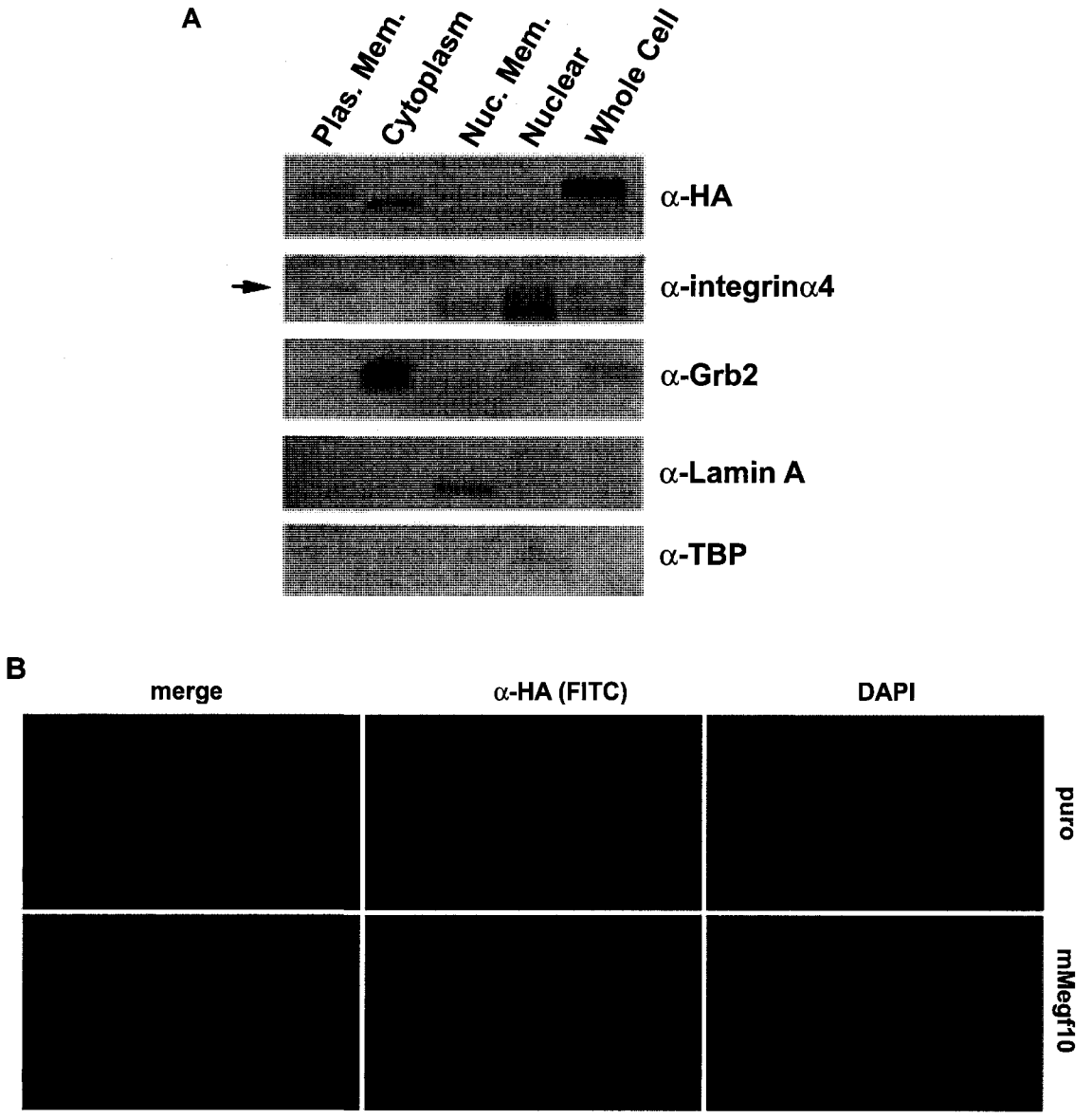

Figure 15. Megf10 localizes to the plasma membrane. C2C12 myoblasts and 10T1/2 fibroblasts were infected with retrovirus designed to allow for the expresion of an HA-tagged version of Megf10. Infected cells were selected based on antibiotic resistance. (A) C2C12 cells were fractionated and protein extracts from each fraction were subjected to Western blotting. Monoclonal HA-7 antibody detected the tagged Megf10 in the plasma membrane and cytoplasmic fractions. Fraction purity was determined by blotting for Lamin A (nuclear membrane), TBP (nuclear), Grb2 (cytoplasmic) and Integrin α4 (plasma membrane). (B) Immunocytochemistry on infected 10T1/2 cells detects the HA-tagged Megf10 while empty vector controls are negative. DAPI stains total nuclei.

2.4 Discussion

The goal of the RDA screen was to identify genes expressed in quiescent satellite cells that may function in the activation or maintenance of the satellite cell compartment. Given the primitive nature of *MyoD-/-* myoblasts, genes that showed high expression in *MyoD-/-* myoblasts and little or no expression in primary myoblasts were considered to be prime candidates for closer examination. As well, genes that were expressed under growth conditions but down-regulated during differentiation were also of potential interest.

The fact that clone MD p286, identified as *HoxC10*, was highly expressed in *MyoD-/-* myoblasts under both growth and differentiation conditions and was expressed at lower levels in proliferating primary myoblasts and not in differentiated primary myoblasts made this an intriguing candidate for further examination. While our initial analysis of tissue expression showed muscle specific expression of *HoxC10,* our RT-PCR results did not demonstrate upregulation of *HoxC10* during regeneration of adult hindlimb musculature and our FACS analysis demonstrated that *HoxC10* was not detectable in the cell population known to contain satellite cells or mpcs. This finding suggests that *HoxC10* may be expressed in myonuclei of differentiated muscle fibers in resting muscle and potentially in mpcs undergoing differentiation during regeneration. Alternatively, *HoxC10* may be expressed in a stem cell population other than satellite cells that is capable of contributing to muscle regeneration. Given that satellite cells are the main effectors of skeletal muscle regeneration and that non-satellite cell stem cells are relatively rare in skeletal muscle it is unlikely that *HoxC10* RNA would be readily detectable in skeletal muscle if it was limited to a small population of stem cells.

Therefore it is more likely that *HoxC10* is expressed by myonuclei of differentiated fibers.

While previous results have demonstrated that forced expression of HoxA10 in myoblasts *in vitro* inhibits differentiation, no role for Hox family members has been established in skeletal muscle regeneration (Houghton and Rosenthal, 1999). It is well established that Hox genes are expressed in a temporal and spatially restricted manner during development and this pattern appears to be maintained throughout the organism's lifespan (Prince, 2002). It has been clearly demonstrated that by determining the expression pattern of Hox genes within a cell, one can accurately predict where along the rostro-caudal access a cell was isolated from (Nowicki and Burke, 2000). Hox10 paralogs are expressed mainly in the posterior structures of developing organisms and are not expressed in the anterior most structures such as the head and forelimbs. Interestingly, our RT-PCR results clearly demonstrated that *HoxC10* was expressed in hind limb musculature, but not in resting or regenerating forelimb musculature. We must therefore consider the source of cells utilized in the initial RNA isolation for the RDA protocol. The *MyoD-/-* myoblasts used were isolated from the hind limb musculature while the mouse embryonic fibroblast line utilized was isolated from whole embryos. Given the ability of RDA to amplify genes that are disproportionately represented between two populations, one must give serious consideration to the possibility that the cloning of *HoxC10* was due to unequal representation of *HoxC10* between the two populations due to the posterior nature of the muscles used to isolate the primary myoblasts. For this reason, further examination of *HoxC10* was not undertaken.

RDA clone MD p67 (Megf10) is highly expressed in *MyoD-/-* myoblasts under growth conditions and is downregulated during differentiation. *Megf10* is also expressed in wildtype myoblasts in a similar manner, but expression levels are much lower than in *MyoD-/-* myoblasts. Furthermore, *Megf10* is not detectable in non-myogenic cell lines. Analysis of tissue polyA+ RNA demonstrates that *Megf10* is expressed in the brain at high levels as well as in skeletal muscle to a lesser extent. These results are similar to those observed by Nagase et al. for the probable human ortholog *MEGF10* or KIAA1780. By *in situ* hybridization, *Megf10* appears to be expressed in cells that occupy positions characteristic of satellite cells but it is also detectable in cells that do not occupy satellite cell positions. Furthermore, the number of cells that express *Megf10* in resting muscle (5-7%) is slightly higher than would be expected if expression was limited to quiescent satellite cells, suggesting that expression of *Megf10* mRNA is not limited to quiescent satellite cells *in vivo*. One possibility is that *Megf10* is expressed in quiescent satellite cells and other resident stem cells or satellite cell progenitors within the skeletal muscle. Given the demonstrated ability of hematopoietic stem cells to contribute to muscle regeneration and the identification of *Megf10* expressing cells in non-satellite cell locations by *in situ* hybridization, it is possible that *Megf10* is expressed in hematopoietic stem cells that are capable of contributing to muscle regeneration.

It should be pointed out that *in situ* hybridization identifies cells that express *Megf10* RNA and does not necessarily label cells that express the protein. Thus, while *Megf10* RNA is detectable in 5-7% of cells, the protein may not be expressed in all of these cells. One possibility is that satellite cells express both *Megf10* RNA and protein, while satellite cell progenitors or other stem cells with myogenic potential, may express

the RNA but not the protein. The generation of antibodies to Megf10 should help to determine whether or not protein expression is limited to quiescent satellite cells.

Our cellular fractionation experiments verify the prediction that Megf10 localizes to the plasma membrane. Given that we are overexpressing Megf10 it is not surprising that it was also detected in the cytoplasmic fraction. An interesting observation is the difference in size of the protein between the cytoplasmic and plasma membrane fractions and the fact that there are two distinct bands in the plasma membrane fraction. Computer analysis of the Megf10 sequence reveals the presence of several potential glycosylation sites within the protein. Given that the protein does localize to the plasma membrane, glycosylation of the protein is quite probable and this may explain the observed difference in size between the cytoplasmic and plasma membrane proteins. As well as the potential for glycosylation, there are several potential phosphorylation sites within the cytoplasmic portion of Megf10. We may be recovering phosphorylated and unphosphorylated Megf10 within the plasma membrane, explaining the observed doublet.

Megf10 orthologs appear to be expressed in a wide variety of organisms including *R. norvegicus*, *D. melanogaster*, *C. elegans*, and *H. sapiens* with the degree of conservation between human and mouse being over 94% at the amino acid level. Studies examining the function of Megf10 orthologs, CED-1 in *C. elegans* and Draper in *D. melanogaster*, have revealed a critical function in neuronal apoptosis (Kinchen et al., 2005; Manaka et al., 2004; Zhou et al., 2001). CED-1 is expressed in cells engulfing apoptotic cells of the developing nervous system and mutants that lack CED-1 display overt neurological defects that lead to death (Kinchen et al., 2005; Zhou et al., 2001). Our expression analysis, and that of others, clearly shows Megf10 expression in the brain

as well as skeletal muscle. While CED-1 and Draper clearly play a role in neural development, their potential role in myogenesis has yet to be examined. Given the limited tissue expression pattern of *Megf10*, the fact that it is not detectable in the non-myogenic cell lines examined, and the fact that it appears to be expressed in quiescent satellite cells makes Megf10 a primary candidate for further study.

Low levels of MD p40 expression were detected in proliferating wildtype myoblasts, and high levels were detected in primary *MyoD-/-* myoblasts under growth and differentiation conditions. While Northern blots of polyA+ tissue RNA did not detect MD p40 expression in adult skeletal muscle, *in situ* hybridization verified that a small percentage of cells in resting skeletal muscle do express MD p40. Given the low number of total cells expressing MD p40 within adult skeletal muscle, it is not surprising that we were unable to detect MD p40 by Northern analysis of total muscle poly A+ RNA. Given the primitive state of the *MyoD-/-* myoblasts and the limited expression of MD p40 in wildtype myoblasts, one could hypothesize that MD p40 is expressed in quiescent satellite cells and may play a role in the activation of satellite cells or the maintenance of quiescence.

Blast analysis of the partial cDNA obtained for MD p40 reveals homology to leucine-rich repeat-containing G protein-coupled receptor 6 (Lgr6). Unfortunately, relatively little is known regarding the expression or function of Lgr6. The potential for MD p40 to function as a G-protein coupled receptor is intriguing given that MD p40 is highly expressed in the primitive *MyoD-/-* lineage but is only expressed at very low levels in wildtype myoblasts. Other G-coupled receptors have been shown to be expressed in skeletal muscle during development as well as in adult muscle. CXCR4 has been shown

to be expressed in satellite cells, and its ligand, stromal-derived factor 1, induces chemotaxis and activation of mitogen-activated protein kinase p42/p44 in myoblasts (Ratajczak et al., 2003). Mice that lack the CCR2 receptor display impaired regeneration, increased fat infiltration and calcification following injury indicating a role for this G-coupled receptor in skeletal muscle regeneration (Warren et al., 2005). Expression of the P2X and P2Y nucleotide receptor families in skeletal muscle have also been demonstrated (Ryten et al., 2004). These results suggest that G-coupled protein receptors may play an integral role in satellite cell biology.

Our initial investigation into the expression patterns of the three RDA clones, MD p286, MD p40, and MD p67 revealed that indeed all three of these clones are truly expressed in skeletal muscle. Given the high expression of *Megf10* in MyoD-/- myoblasts, its down-regulation during differentiation, its limited tissue expression pattern and the fact that it localizes to the plasma membrane we chose to focus our attention on Megf10.

Chapter 3 – Megf10 is Expressed in Quiescent Satellite Cells

3.1 Introduction

The goal of the initial RDA experiments was to identify factors that functioned in the specification, activation, and maintenance of the quiescent satellite cell compartment. Indeed, the screen identified the paired-box transcription factor Pax7 as being expressed in quiescent satellite cells (Seale et al., 2000). Subsequent experiments revealed a unique requirement for Pax7 in satellite cell specification and maintenance and Pax7 expression is now considered the gold standard by which quiescent satellite cells are identified (Seale et al., 2004b; Seale et al., 2000). Other markers such as Syndecan 3 and Syndecan 4, c-met, and M-cadherin are also used as standard markers of satellite cells (Cornelison et al., 2001; Cornelison and Wold, 1997; Irintchev et al., 1994); however, Pax7 remains the marker of choice.

While Pax7 is a specific immunological marker for identifying satellite cells, it is an intracellular protein, which limits its use in the purification of myoblasts by standard cell sorting techniques. Thus, the identification of cell surface markers expressed by quiescent satellite cells should provide a useful tool for the purification of quiescent satellite cells by standard cell sorting methods. Indeed, it has been clearly demonstrated that the expression profiles of markers such as CD34, Sca1, CD45, and CD31 can be used to isolate purified preparations of satellite cells by FACS (Montarras et al., 2005).

Unpublished results from our lab have demonstrated the existence of two distinct satellite cell populations within resting skeletal muscle. By crossing R26R-YFP mice, that do not express YFP unless exposed to cre-recombinase, with Myf5-cre mice we have

generated mice in which myogenic cells that have previously expressed Myf5 also express YFP while cells that have not expressed Myf5 remain YFP negative. Careful examination of skeletal muscle from these mice revealed the existence of two distinct satellite cell populations, one that has previously been exposed to Myf5 and one that has not (Kuangand Rudnicki, unpublished observation). The first population represents satellite cells that contribute mainly to regeneration following activation (Kuang and Rudnicki, unpublished observation). We refer to these cells as satellite myogenic cells. These cells express Pax7 and are positive for YFP indicating that they have also expressed Myf5 at some point during their existence (Kuang and Rudnicki, unpublished observation). The second population referred to as satellite stem cells also express Pax7, however they do not express YFP indicating that these cells have not previously expressed Myf5 (Kuang and Rudnicki, unpublished observation). This suggests that satellite stem cells are more primitive than satellite myogenic cells and may represent an upstream progenitor of satellite myogenic cells (Kuang and Rudnicki, unpublished observation). Indeed, examination of satellite cell proliferation on isolated individual muscle fibers in culture revealed that while Pax7+/YFP+ cells give rise to only Pax7+/YFP+ cells, the satellite stem cell population gives rise to Pax7+/YFP+ cells while maintaining a Pax7+/YFP- profile indicating that these cells undergo asymmetric division and may be responsible for maintaining the quiescent satellite cell compartment (Kuang and Rudnicki, unpublished observation).

Megf10 is a cell surface marker that would function as a valuable tool for the isolation and purification of satellite cells by FACS if present on quiescent satellite cells. The goal of this chapter was to determine if Megf10 expression was limited to quiescent

satellite cells. To verify that Megf10 was expressed in quiescent satellite cells we attempted to isolate a population of cells that contained the majority of quiescent satellite cells and activated myogenic precursors in order to demonstrate that Megf10 was expressed in this population. We also attempted to generate antibodies to Megf10 to confirm that Megf10 is expressed in quiescent satellite cells by co-staining with known satellite cell markers such as syndecan-4, M-cadherin, and Pax7. Our results indicate that Megf10 is expressed in approximately 80% of quiescent satellite cells. Having demonstrated that Megf10 marks the majority of satellite cells, we attempted to ascertain whether Megf10 expression was limited to satellite myogenic cells (Pax7+/YFP+) or if it was expressed in both satellite myogenic cells (Pax7+/YFP+) and satellite stem cells (Pax7+/YFP-). Our results demonstrate that Megf10 expression is limited to the satellite myogenic population.

3.2 Materials and Methods

3.2.1 FACS Analysis

Resting and 4 days post-cardiotoxin injected TA muscle was isolated from 6-10 week old wild-type Balb/C mice. Muscle cells were recovered as previously described (Megeney et al., 1996). Cells were resuspended and incubated on ice for 20 min with conjugated antibodies at 1 μg per $1x10^6$ cells. Cells were either stained with CD31-PE (BD Pharmingen), Sca1-APC (Caltag), and lineage-FITC antibodies (BD Pharmingen). Lineage marker antibodies included the blood cell surface markers Mac-1, Gr-1, Ter119, CD45R/B220, and CD3e. Immuno-labeled cells were four-way sorted for Sca-1 and

CD31 with Lin exclusion on a MoFlo cytometer (DakoCytomation). Cell fractions obtained were $Sca1^+/CD31^+/Lin^-$, $Sca1^+/CD31^-/Lin^-$, $Sca1^-/CD31^+/Lin^-$, and $Sca1^-/CD31^-/Lin^-$. RNA from the sorted fractions was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Values expressd as means +/- standard deviation (n=2).

3.2.2 Real-Time PCR

Real-Time PCR was carried out as previously described (Ishibashi et al., 2005). Transcript levels were normalized to GAPDH transcript levels. Primer sequences for Megf10 were as follows; Forward; 5' ACCAACTGTAGCAGTGCTTGT 3' Reverse; 5' CAGTGGCAAGCACCGGTGAT 3'

3.2.3 Isolation of GST-fusion protein

The 290 carboxy-terminal amino acids of Megf10 were cloned into the pGEX 4T-1 vector. Cultures were inoculated from frozen stocks and grown overnight at 37°C. The next day, bacterial cultures were diluted 1:10 in LB/Amp and grown for 1 hour at 37°C. IPTG was added to a final concentration of 0.3mM and cultures were grown overnight at room temperature with agitation. Cultures were spun down at 4000rpm 4°C for 10min and supernatant discarded. Pellets were resuspended in 1% Triton-X in PBS with Protease inhibitors (1mM PMSF, 10mg/ml pepstatin A, aprotinin, leupeptin, and 1mM sodium vanadate). Suspensions were sonicated 3 times for 10sec each time at 30% amplitude. Samples were then centrifuged for 10 minutes at 4^0C 4000rpm. Supernatant was incubated at 4°C overnight with 50µl glutathione sepharose beads. Beads were pelleted and washed three times with PBS + PIN. Fusion proteins were eluted in 10mM

glutathione in 50mM Tris-HCl (pH 8.0) 30 minutes at room temperature. The sample was then dialyzed overnight in 50mM Tris-HCl (pH8.0) to remove the glutathione.

3.2.4 Fiber Isolation and Immunohistochemistry

Tibialis anterior muscles were isolated from 2-month-old mice. Muscles were incubated overnight through a series of sucrose solutions (4%, 15%, 30%). Following incubation in 30% sucrose/PBS the muscles were embedded in OCT and flash frozen in liquid nitrogen. Sections were cut at 8 μm using a cryostat (Leica), blocked in 5% donkey serum 1hr at romm temperature, and subsequently incubated with primary antibody overnight (α-Megf10 Washington Biotech 1:200; α-syndecan-4 1:200). FITC-conjugated α-chicken and TRITC-conjugated α-rabbit secondaries were used for visualization. For staining of individual fibers, isolation was performed as previously described (Kuang et al., 2006). Individual fibers were incubated with primary antibodies at the following dilutions; α-Megf10 (1:200), α-Pax7 (1:10), α-m-cadherin (1:200), α-syndecan-4 (1:200).

3.3 Results

3.3.1 Megf10 is expressed in the same population of cells as Pax7

We employed FACS analysis to help determine if *Megf10* was expressed in satellite cells. Cell suspensions derived from resting or regenerating hindlimb musculature were stained for expression of Sca1, CD31, and a panel of lineage (Lin) markers [Mac-1 (macrophage), Gr-1 (granulocyte), Ter119 (erythroid), CD45R/B220 (B-

cell), and CD3e (T-cell)]. Lin staining was utilized to identify cells of hematopoietic origin. Cells were sorted into four fractions based on the presence or absence of Sca1 and CD31 staining and cells of hematopoietic origin were excluded from the fractions ($Sca\text{-}1^{+}/CD31^{+}Lin^{-}$, $Sca\text{-}1^{+}/CD31^{-}Lin^{-}$, $Sca\text{-}1^{-}/CD31^{+}Lin^{-}$, $Sca\text{-}1^{-}/CD31^{-}Lin^{-}$). RNA was isolated from each of the fractions and subjected to real-time PCR to detect *Megf10* expression. As well, each fraction was examined for expression of *Pax7* to identify the fractions that contained satellite cells and activated mpcs. *Megf10* was found to be expressed in the Sca1-/CD31-/Lin- fraction and was upregulated during regeneration (Fig 16). *Megf10* expression was not detected in the other three fractions. Importantly, *Pax7* expression was also limited to the Sca1-/CD31-/Lin- population and was up-regulated during regeneration verifying that *Megf10* expression was limited to the fraction of cells containing satellite cells and activated myogenic precursors.

3.3.2 Megf10 co-localizes with Syndecan-4 in tibialis anterior muscle

Based on our FACS results, we hypothesized that Megf10 would be expressed in quiescent satellite cells in adult muscle. To test this hypothesis we generated antibodies against specific domains within Megf10. In our first attempt, we identified potential sequences for generating antibody to peptides based on the hydrophobicity, accessibility, antigenicity, and homology to other proteins of the potential peptides. Two candidate sequences were identified, one in the extracellular domain comprised of amino acids 75-96. The second sequence was located within the cytoplasmic domain of Megf10 comprised of amino acids 932-957. The extracellular location of the first peptide made it an excellent choice as the subsequent antibody could be used for FACS sorting of live

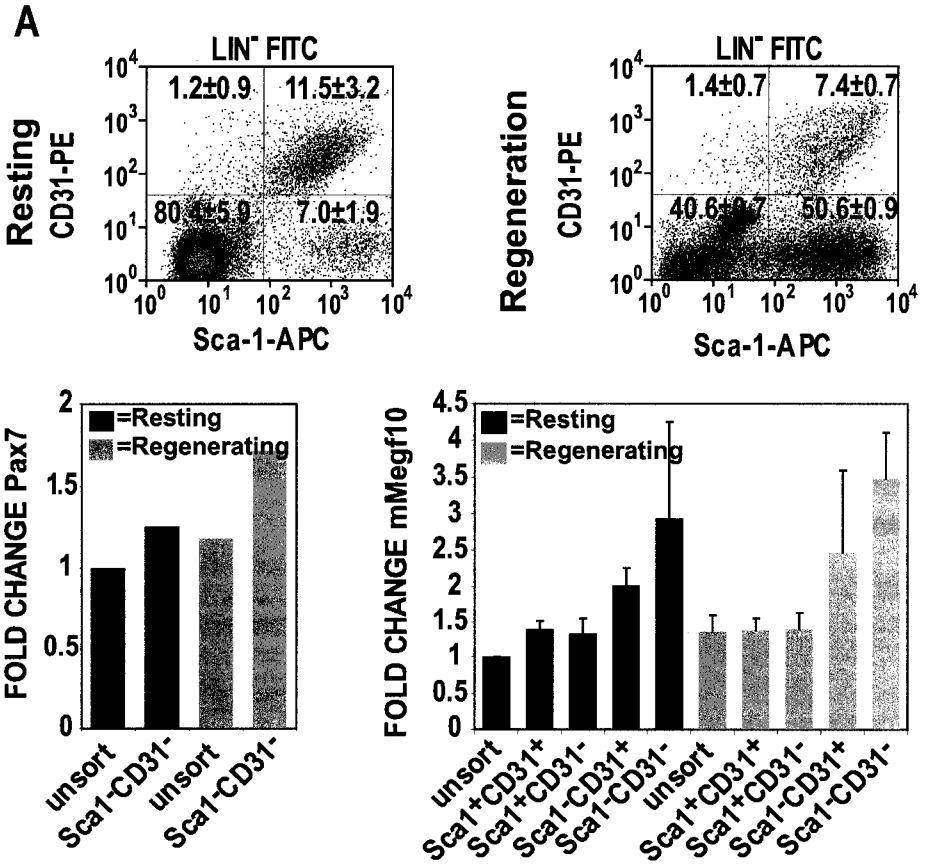

Figure 16. Megf10 is expressed in the same population of cells that contains quiescent satellite cells. FACS analysis was utilized to sort cells isolated from resting and regenerating mouse hindlimbs. Hematopoetic cells were excluded based on their expression of lineage markers. Remaining cells were divided into 4 fractions based on their expression of Sca1 and CD31. RLT-PCR for Megf10 and Pax7 expression was performed on each fraction. Pax7 was detected in the Sca1-/CD31- population and was upregulated during regeneration. Megf10 was also detected primarily in the Sca1-/CD31- fraction and also appeared to be upregulated during regeneration (n=2, error bars are Std. Dev.).

cells as well as other standard immunological processes. Thus we attempted to generate antibody to this region. Peptide was generated by Alpha Diagnostic and subsequently injected into rabbits to generate antibody following standard protocol. Initial attempts to perform western blots with the rabbit α-Megf10 antibody were unsuccessful. Given the inability of the rabbit α-Megf10 antibody to recognize Megf10 from whole cell protein extract we *in vitro* translated an HA-tagged version of Megf10 and performed western blots using the rabbit α-Megf10 antibody and mouse monoclonal HA-7 antibody. While the HA-7 antibody was able to recognize the *in vitro* translated product, the rabbit α-Megf10 antibody did not. To determine if the rabbit α -Megf10 recognized the original peptide to which is was synthesized, the peptide was subjected to serial dilutions and dotted onto PDFV membrane and subjected to western analysis. The rabbit α-Megf10 antibody readily detected the peptide to which it was raised against suggesting that the antibody could not recognize Megf10 in its native conformation.

A second antibody was generated to the c-terminal cytoplasmic portion of Megf10. Amino acids 876-1140 were cloned into a GST-fusion vector (pGEX4T1) and GST-fusion protein was generated. The GST-fusion protein was purified from bacterial lysates and shipped to Washington Biotech Ind. for the generation of rabbit antisera to Megf10. Prior to use, the antibody was pre-adsorbed against purified GST and pre-incubated with glutathione beads in order to remove any antibody that recognized GST. Specificity of the antibody was examined by Western blot (Fig 17a). We performed immunohistochemistry using the pre-cleared rabbit polyclonal antibody that recognized the carboxy-terminal 290 amino acids of Megf10. Frozen sections of tibialis anterior muscle from two-month old mice were co-stained for Syndecan-4 (syn-4), a known

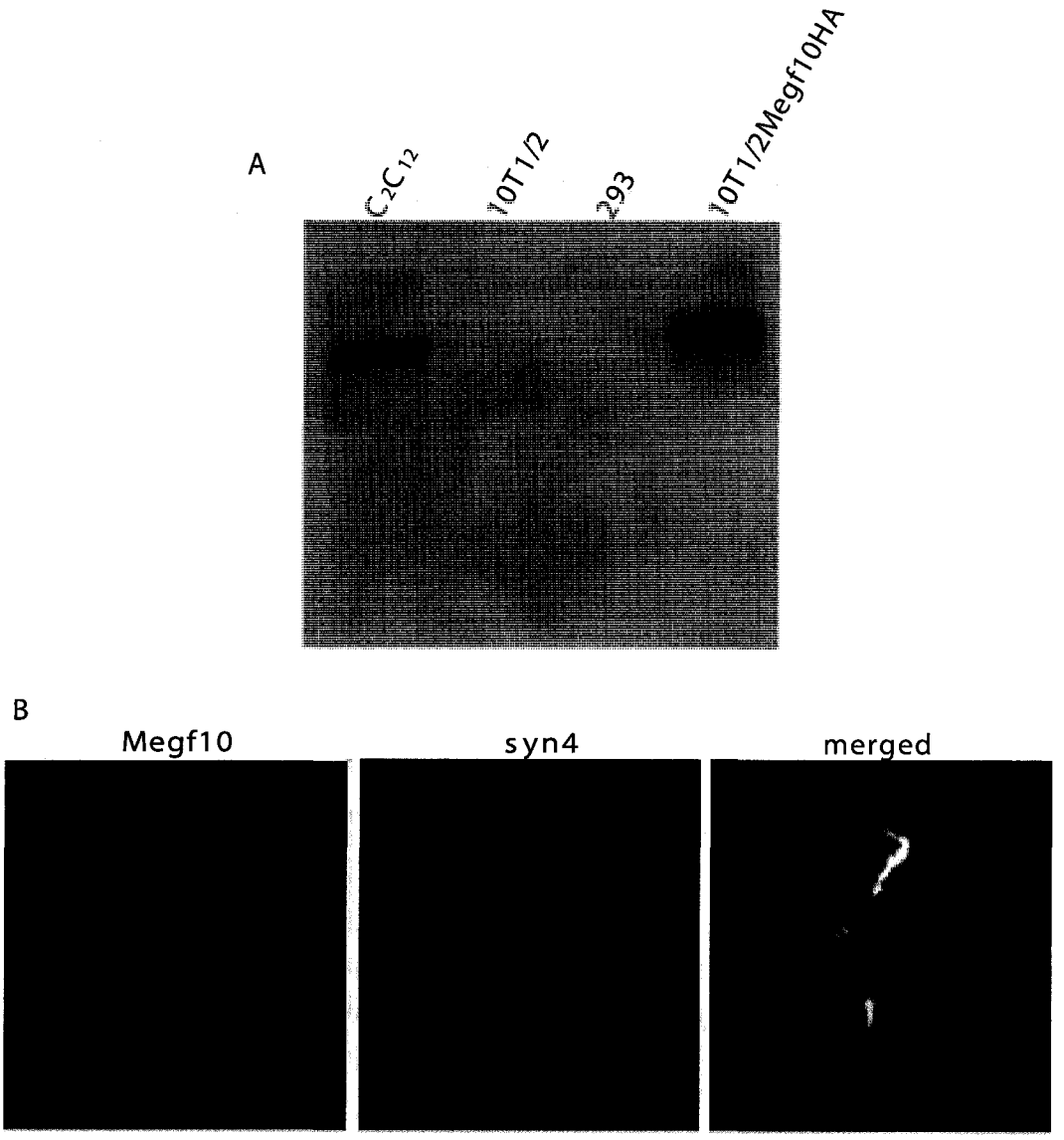

Figure 17. Megf10 is expressed in quiescent satellite cells in adult mouse skeletal muscle. (A) Western blot on cell extracts using the newly generated rabbit α-Megf10 antibody (1:50) demonstrating specificity for Megf10. (B) Cross sections of frozen tibialis anterior muscle isolated from 2 month old mice were co-stained with rabbit anti-Megf10 and chicken anti-syndecan 4. Rhodamine-conjugate α-rabbit and FITC-conjugated α-chicken secondary antibodies were used for visualization. Megf10 staining was found to overlap with syn4 staining along the periphery of fibers indicating that Megf10 is expressed in quiescent satellite cells.

marker of quiescent satellite cells, and Megf10 expression (Cornelison et al., 2001). Our results show that Megf10 expression is detectable in TA muscle using the antibody generated to the GST fusion protein and that the Megf10 staining overlapped with syn-4 staining along the periphery of muscle fibers (Fig 17b).

3.3.3 Megf10 is expressed in satellite cells in Single Fiber Cultures

To further demonstrate that Megf10 is expressed in quiescent satellite cells we isolated individual myofibers with their associated satellite cells and co-stained for Megf10, Pax7, and M-Cadherin or syn-4. As previously observed, Megf10 expression was limited to quiescent satellite cells (Fig 18). The majority (80%) of Pax7 and M-cadherin-expressing cells expressed Megf10 and Megf10+/Pax7- cells were never observed, verifying that Megf10 expression is restricted to quiescent satellite cells in resting muscle.

Examination of fibers isolated from the progeny of R26R-YFP crossed with Myf5-Cre, in which satellite cells that have previously expressed Myf5 also express the YFP protein demonstrated that Megf10 localized to Pax7+/YFP+ cells but not Pax7+/YFP- cells, suggesting that Megf10 is expressed in satellite myogenic cells rather than satellite stem cells (Fig 19). To further verify this, FACS analysis was performed to separate Pax7+/YFP- and Pax7+/YFP+ satellite cells. RT-PCR on the isolated populations demonstrated that Megf10 expression was limited to the Pax7+/YFP+ population.

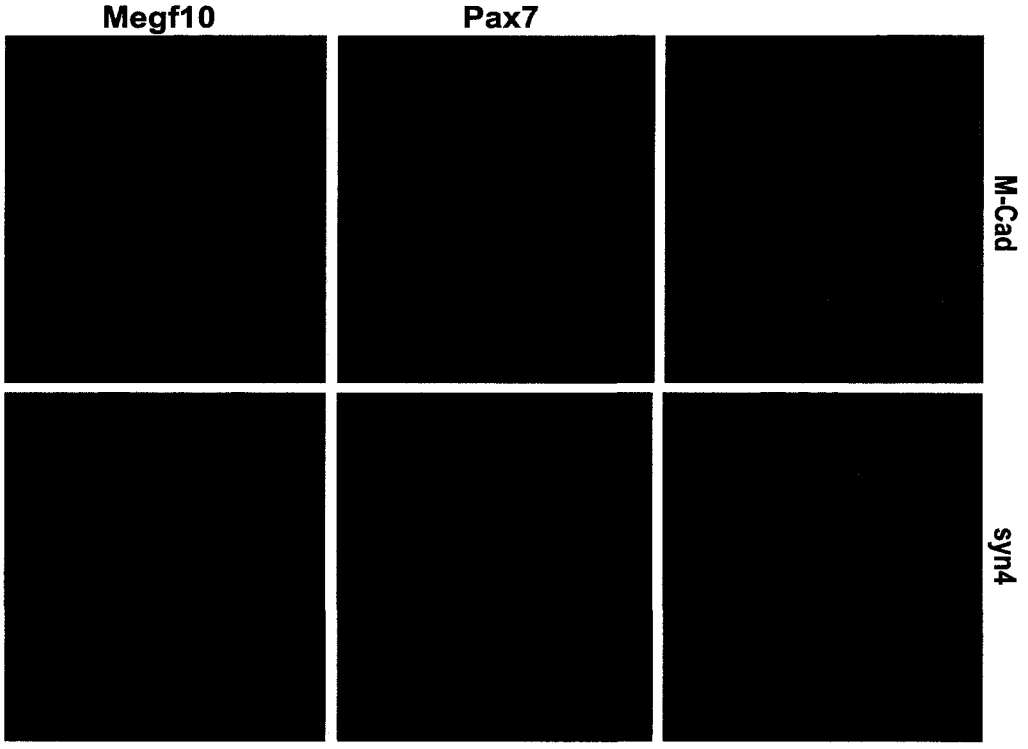

Figure 18. Single fiber staining verifies that Megf10 is expressed in quiescent satellite cells. Individual fibers were isolated from the EDL muscle of 6 week old mice and stained for expression of Megf10, Pax7, and M-Cadherin or Syndecan 4. In all instances, Megf10 positive cells were Pax7 positive and M-cadherin or syn4 positive. However, not all Pax7 positive cells expressed Megf10 indicating that a subset of satellite cells express Megf10.

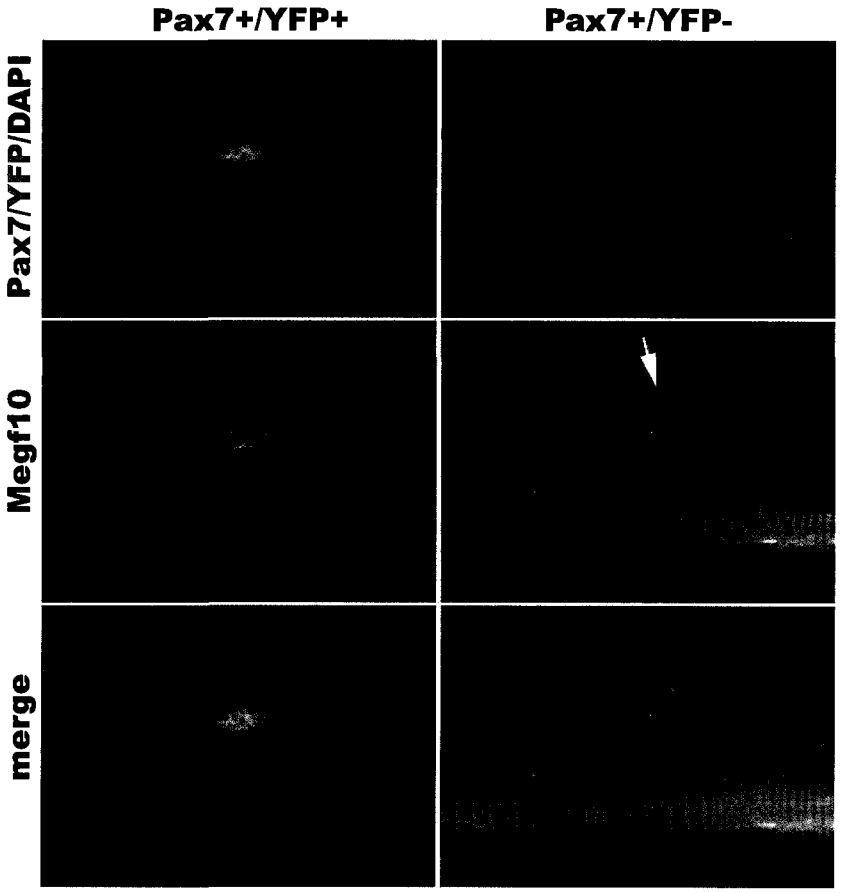

Figure 19. Immunostaining of Megf10 in Pax7+/YFP+ and Pax7+/YFP- satellite cells. Individual fibers isolated from Myf5 cre/R26R YFP mice reveal that Megf10 is expressed in satellite cells that are Pax7+/YFP+ but not in satellite stem cells that are Pax7+/YFP-.

3.4 Discussion

It has previously been demonstrated that FACS analysis can be used to isolate satellite cells based on the lack of expression of specific cell surface markers such as Sca1, CD31, and CD45 (Montarras et al., 2005). Based on these findings we attempted to isolate the Sca1-/CD31-/Lin- fraction of mononuclear cells from adult muscle, that contained satellite cells and their activated descendants. Our Real-time PCR results demonstrate that of the four fractions obtained, *Pax7* was only expressed in the Sca1-/CD31-/Lin- fraction indicating that this was the only fraction containing satellite cells. As would be expected, the level of *Pax7* expression was increased in this fraction in regenerating muscle further verifying that this fraction contains satellite cells and their activated descendants. Amplification of *Megf10* was detected in the Sca1-/CD31/Lin- fraction suggesting that *Megf10* is expressed in satellite cells. Furthermore, *Megf10* expression was elevated in this fraction when cells were isolated from regenerating muscle indicating that *Megf10* is expressed in satellite cells and their activated progeny. When combined with our previous *in situ* results this data suggests that *Megf10* is expressed in quiescent satellite cells and their activated descendants.

Isolation of the full-length cDNA for *Megf10* allowed us to generate antibodies to a GST-Megf10 fusion protein. Immunohistochemistry using this antibody indicated that Megf10 was indeed expressed in quiescent satellite cells as determined by co-staining with other known satellite cell markers such as syn-4, m-cadherin and Pax7. Interestingly, in cross sections of tibialis anterior, Megf10 was only detected in cells that expressed syn-4. Our *in situ* hybridizations suggested that *Megf10* was expressed in cells that occupied a non-satellite cell location. It is possible that *Megf10* is expressed in cells

other than quiescent satellite cells but that the protein is not expressed in these cells. This would explain the discrepancy between our *in situ* and immunohistochemical results. Other examples of this phenomenon include high levels of *Myf5* in the brain with no detectable levels of the protein. Regardless of this discrepancy, our results clearly demonstrate that Megf10 marks a large proportion of quiescent satellite cells in resting adult muscle.

The fact that Megf10 does not mark all quiescent satellite cells correlates with previously published data indicating that certain markers such as CD34 and Myf5-LacZ mark a subset of quiescent satellite cells (Beauchamp et al., 2000). This heterogeneity amongst satellite cells, with respect to the expression of specific markers may reflect the origins of these cells. Alternatively, the heterogeneity may reflect distinct regenerative functions. Indeed, recent work suggests that following activation satellite cells are capable of repopulating the quiescent satellite cell compartment as well as repairing damage (Collins and Partridge, 2005; Zammit et al., 2004). This finding is supported by recent work in our lab that demonstrates the existence of a satellite stem cell population that gives rise to Pax7+/GFP+ (Myf5 expressing) cells that contribute to regeneration. Interestingly, the satellite stem cells, which are Pax7+/YFP- (having not expressed Myf5) appear to undergo asymmetric division, remaining YFP negative following activation, and give rise to GFP+ myogenic progeny (Kuang and Rudnicki, unpublished result). Our results clearly demonstrate that Megf10 expression is limited to the myogenic Pax7+/YFP+ population. Thus, Megf10 expression appears to correlate with whether a satellite cell is a stem cell responsible for maintaining the quiescent satellite cell compartment, or a myogenic cell responsible for damage repair. Indeed RT-PCR

analysis of RNA isolated from Pax7+/YFP+ satellite myogenic cells and Pax7+/YFP- satellite stem cells indicate that *Megf10* expression is limited to the myogenic population. These results supported by staining on individual fibers that demonstrates Megf10 expression in Pax7+/YFP+ cells but not Pax7+/YFP- cells. One possible explanation is that Megf10 expression is required to maintain satellite cells in a proliferative state and inhibit progression towards differentiation. Once a satellite cell has been activated in response to trauma, it may upregulate Megf10 expression and enter a proliferative phase. Down regulation of Megf10, upon appropriate environmental cues would allow for the subsequent differentiation of these cells. If however, Megf10 expression is maintained, the cell may adopt an alternative fate and return to the quiescent compartment in order to maintain the satellite cell number following regeneration. If this is indeed the case, the absence of Megf10 expression in the Pax7+/YFP- population may reflect the fact that these cells are the initial migrating progenitors.

Chapter 4 – Megf10 Regulates Myoblasts Proliferation and Differentiation

4.1 Introduction

The classical example of an epidermal growth factor repeat-containing protein is Epidermal Growth Factor (EGF). It contains a sequence of approximately 30-40 amino acids that is conserved in a wide variety of proteins including urokinase, laminin B1, and low density lipoprotein (LDL) receptor (Appella et al., 1988). Within this conserved sequence are six cysteine residues that have been shown to be involved in disulphide bond formation (Hommel et al., 1992). Interestingly, proteins that share this conserved sequence display diverse biological functions. For example, urokinase catalyzes the proteolytic cleavage and activation of plasminogen, Laminin functions as a basement membrane protein that promotes cell adhesion and proliferation, LDL receptor binds to and internalizes low density lipoprotein, and EGF functions as a diffusible mitogen (Baur et al., 1995; Sodetz and Castellino, 1975; Stolt and Bock, 2006). The common link between the functions of these proteins is that they are all involved in protein-protein interactions indicating that EGF-repeats are most likely involved in receptor/ligand interactions (Appella et al., 1988).

Myoblasts respond to decreases in growth factor concentration by withdrawing from the cell cycle and undergoing terminal differentiation, thus precise control of the cell cycle machinery is crucial for appropriate myoblast differentiation. During early G1 myoblasts express cyclin D1 and cdk4 which form an active complex allowing for G1 progression (Bates et al., 1994). As the cell cycle progresses, cyclin E and cdk2 complexes become predominant. These complexes are crucial for S phase entry and the

initiation of DNA replication (Koff et al., 1992). During G1, the cyclinD/cdk4(6) and cyclinE/cdk2 complexes are crucial for maintaining pRb in a hyperphosphorylated, inactive state (Kato et al., 1993; Zarkowska and Mittnacht, 1997). Previous work in our lab has demonstrated a critical requirement for pRb in the switch from proliferation to differentiation in myoblasts *in vitro* and *in vivo* (Huh et al., 2004). Thus, regulation of pRb phosphorylation and activity by the cyclin/cdk complexes is critical during myogenic progression.

As cells progress through the G1/S boundary they begin to express cyclin A which complexes with cdk2 as well as cdc2 (Elledge et al., 1992; Pines and Hunter, 1990). The cyclinA/cdk2 and cyclinA/cdc2 complexes are required for appropriate S phase transition and control of DNA replication (Cardoso et al., 1993). In the final stages of cell cycle progression, cyclin B1/cdc2 complexes become predominant in late S phase but remain inactive until late G2 when their activity is required for entry into mitosis (Nurse, 1990). Since appropriate withdrawal from the cell cycle is required for terminal differentiation to occur, stringent control of these processes is crucial in the myogenic lineage.

The focus of this chapter was to determine the biological function of Megf10 in myoblasts. We utilized retrovirus to overexpress an HA-epitope-tagged (hemagglutinin-epitope-tagged) version of Megf10 in proliferating myoblasts and examined the effects of overexpression on the growth and differentiation of infected cells. Our results clearly demonstrate that overexpression of Megf10 enhances the proliferation and inhibits differentiation of myoblasts. Given the enhanced proliferation of cells we examined the cell cycle kinetics by FACS sorting PI/BrdU stained cells in order to obtain a cell cycle

profile. We also examined the expression of key cell cycle regulating proteins such as the cyclins D, E, A, and B, as well as cyclin dependent kinases (CDKs), p21, p27, and Rb. The expression profiles of key myogenic proteins were analyzed in an attempt to understand the increased proliferation and inability to undergo terminal differentiation.

4.2 Materials and Methods

4.2.1 Cell Culture and Growth Curves

Cells were plated at an initial density of $1x10^5$ cells per 10cm tissue culture dish. Cells were maintained in DMEM supplemented with 10% FBS with 10U/ml Penn/strep. At set time points (24, 48, 72, 96 hours) following plating, cells were trypsinized and counted using a hemocytometer. Counts were done in triplicate at each time point. Values expressed as the mean +/- standard deviation of three independent experiments on three independent infections for n=9. For extended growth curves, cells were plated at $1x10^5$ cells per 10cm tissue culture dish. Cells were trypsinized 48 hours following initial plating and counted as above. $1x10^5$ cells were re-plated and grown for 48 hours before subsequent counts. Values are expressed as mean +/- standard deviation (n=3). For conditioned media, Megf10 expressing cells were palted at a density of $1X10^5$ cells per 10cm plate. Media was harvested 24 hours following plating and filtered through 0.45um filter. Media was then used to feed previously plated C_2C_{12} myoblasts. Cellular proliferation was assessed as above. Values are expressed as mean +/- standard deviation (n=3).

4.2.2 Protein Isolation and Western Blotting

Protein was isolated as previously described (Perry et al., 2001). Western analysis was performed as previously described (Sabourin et al., 1999). Antibodies used for these studies were as follows: α-MyoD (C-20; Santa Cruz), α-Myf5 (C-20; Santa Cruz), α-Pax7 (hybridoma supernatant; Developmental Studies Hybridoma Bank), α-myogenin (F5D; Developmental Studies Hybridoma Bank), α-myosin heavy chain (MF20; Developmental Studies Hybridoma Bank) α-HEB (A-20; Santa Cruz), α-desmin (D33; DAKO), α-cyclin D1 (C-20; Santa Cruz), α-cyclin D2 (M-20; Santa Cruz), α-cyclin D3 (H-292; Santa Cruz), α-cdk2 (M2; Santa Cruz), α-cdk4 (C-22; Santa Cruz), α-Rb (G3-245; BD Pharmingen). α-Megf10 antibody was generated in rabbits against a GST-fusion protein containing the carboxy-terminal 290 amino acids of Megf10 (Washington Biotech).

4.2.3 BrdU Incorporation and Immuncytochemistry

C_2C_{12} myoblasts were grown in DMEM supplemented with 10% FBS with 10U/ml penn/strep. For differentiation, cells were cultured in DMEM supplemented with 2% horse serum and 10U/ml penn/strep. For BrdU incorporation, cells were incubated for 30 minutes with 30μg/ml BrdU. Cells were then fixed and stained using the BD Bioscience BrdU *In situ* kit as per manufacturer's instructions.

4.2.4 BrdU/PI FACS Analysis

Cells were maintained in DMEM supplemented with 10% FBS and 10U/ml penn/strep for a minimum of two weeks prior to experiments. Cells were incubated with

25μM BrdU for 25 minutes, trypsinized and spun down. Cells were then stained using the BD Bioscience BrdU Flow Kit as per manufacturer's instructions. Cells were sorted on a Beckman Coulter FACS STAR machine.

4.2.5 RNase Protection Assay

RNase protection assay was performed using the RiboQuant kit along with the multi-probe template sets m-CYC-1 and m-CC-1 (BD Biosciences). 2 μg of total RNA isolated from C_2C_{12} cells expressing Megf10HA or empty vector controls from each time point in a differentiation time course was used for the ribo-probe hybridization. The RNase protection assay was completed according to the manufacturer's instructions.

4.2.6 Real-Time PCR

Real-Time PCR was carried out as previously described (Ishibashi et al., 2005). Transcript levels were normalized to GAPDH transcript levels. Primer sequences for Megf10 were as follows;

Forward; 5' ACCAACTGTAGCAGTGCTTGT 3'

Reverse; 5' CAGTGGCAAGCACCGGTGAT 3'

Pax7, MyoD, and Myf5 primers as previously described (Ishibashi et al., 2005).

4.2.7 Cell Transplantation and Immunohistochemistry

Two days prior to cell transplantation, SCID beige mice were anesthetized and the tibialis anterior muscle of the right hind leg was subjected to cardiotoxin insult (30μl/TA). Mice were allowed to recover and maintained on acidified water. Two days

following injury, cell transplantations were performed. C_2C_{12} cells expressing Megf10HA (3 animals) or GFP (3 animals) were maintained under normal growth conditions. Prior to injection, cells were washed in PBS, trypsinized, spun down and resuspended at $1x10^6$ cells in 50μl PBS. Mice were anesthetized and cells were injected into the previously injured TA muscle. Mice were allowed to recover and TA muscles were harvested 21 days following cell transplantation. Muscles were incubated through a series of sucrose dilutions (4%, 15%, 30%). Following incubation in 30% sucrose/PBS the muscles were embedded in OCT and flash frozen in liquid nitrogen. Sections were cut at 8 μm using a cryostat (Leica) and subsequently incubated with primary antibody overnight [α-HA7 (Sigma) 1:200; a-laminin 1:200). Visualization was performed using FITC-conjugated and Rhodamine-conjugated secondary antibodies 1:2000). GFP was visualized directly (n=2).

4.3 Results

4.3.1 Forced Expression of Megf10 Enhances Myoblast Proliferation

To examine the effects of Megf10 expression on myoblast proliferation we infected C_2C_{12} myoblasts with retrovirus designed to express an HA-tagged version of Megf10. Stable pools were generated by antibiotic selection and their proliferative capacity examined. Strikingly, cells overexpressing Megf10 were found to proliferate at a 16% faster rate than cells infected with empty vector control (Fig 20). Using extended growth curves we were able to calculate the doubling time of control and Megf10 overexpressing cells. Control cells doubled once every 13.7 hours while Megf10

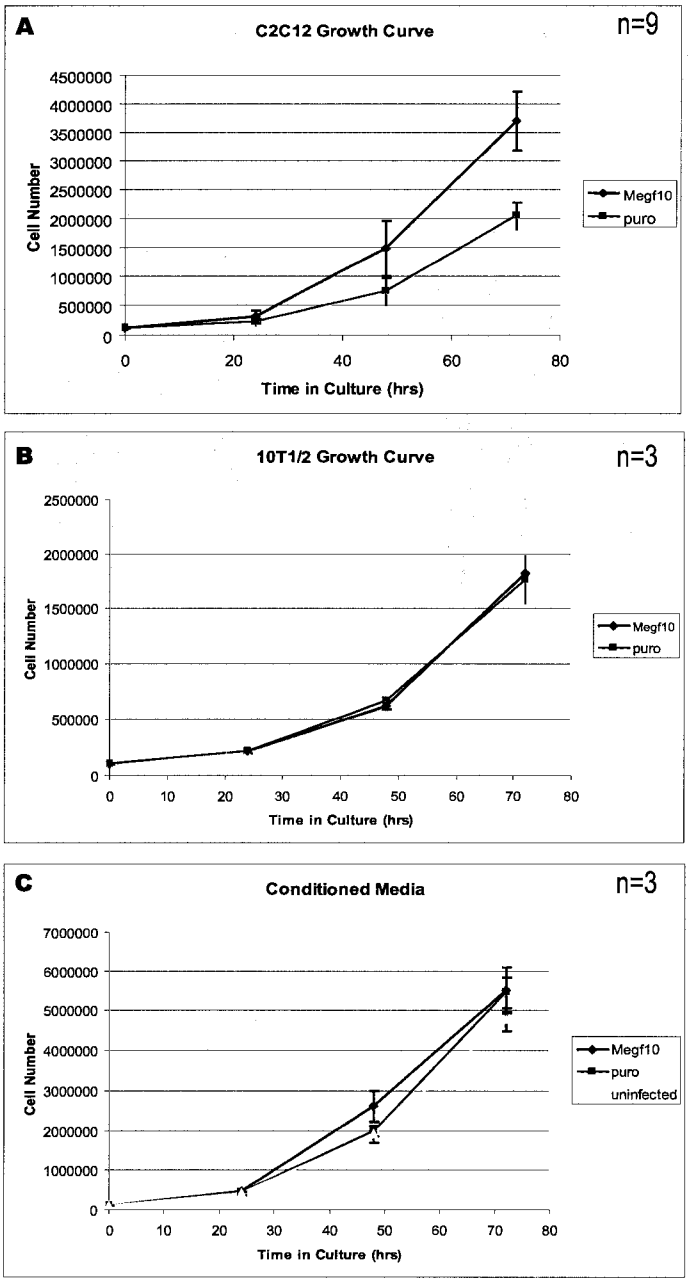

Figure 20. Megf10 over-expression specifically enhances myogenic cell proliferation in a cell autonomous manner. (A) C2C12 myoblasts were infected with HA-tagged Megf10 or empty vector controls. Cells expressing HA-tagged Megf10 proliferated at a higher rate than empty vector controls. ($p<0.02$) (B) 10T1/2 cells infected with HA-tagged Megf10 did not proliferate at a faster rate than cells containing the empty vector control. (C) Conditioned media from HA-tagged Megf10 expressing cells did not alter the growth rate of C2C12 cells when compared to conditioned media from empty vector controls or unconditioned media. Error bars represent Std. Dev.

overexpressing cells doubled every 11.6 hours, a difference of 2.1 hours. A double-tailed t-test gave a p value of 0.017 demonstrating the statistical significance (n=9).

Given that Megf10 expression was limited to myogenic cells in our previous studies we next asked whether Megf10 expression in non-myogenic cells would have a similar affect. We repeated the infections in 10T1/2 fibroblasts and analyzed the growth rates of stable pools. Unlike myoblasts, forced expression of Megf10 in fibroblasts did not alter the proliferative capacity of the cells suggesting that Megf10 requires specific factors expressed in myoblasts to exert its effect on proliferation (Fig 20).

To determine if Megf10 was functioning as a diffusible ligand, we tested if conditioned media could enhance the proliferation of uninfected myoblasts. C_2C_{12} cells overexpressing Megf10HA were plated at a density of $3x10^5$ and the media was harvested from the plates 24 hours following initial plating. This media was then used to feed uninfected myoblasts. The rate of proliferation of uninfected cells grown in conditioned media was examined over a period of 4 days and no significant changes were observed suggesting that Megf10 does not function as a diffusible ligand (Fig 20).

4.3.2 Forced Expression of Megf10 Inhibits Differentiation

Our preliminary results demonstrated that Megf10 expression was downregulated during differentiation of myoblasts. Since overexpression of Megf10 enhanced the proliferation of myoblasts we asked if it would also alter the differentiation of cells. Stable pools of Megf10HA expressing myoblasts and empty vector-infected controls were subjected to serum deprivation in order to induce differentiation. As expected, control cells underwent terminal differentiation and fusion to form multinucleated

myotubes as determined by staining for MyHC (Fig 21). In contrast, the majority of Megf10HA expressing cells remained mononuclear and failed to upregulate MyHC indicating that these cells were unable to undergo terminal differentiation (Fig 21). Five days following serum withdrawal, over 75% of empty-vector control cells had fused to form multinucleated MyHC-positive cells while only 15% of cells overexpressingmMegf10 had undergone terminal differentiation (as assessed by MyHC staining) at the same time point (Fig 22). Western blots confirmed that Megf10 overexpressing cells expressed dramatically lower levels of MyHC than controls. Interestingly, approximately 50% of the Megf10HA expressing cells that were able to undergo terminal differentiation remained mononuclear, suggesting that Megf10 overexpression may also affect myoblast fusion.

To further examine the deficit in terminal differentiation of Megf10 overexpressing cells, we performed BrdU incorporation experiments. While similar numbers of control and experimental cells incorporated BrdU under growth conditions, a significant difference was observed twenty-four hours following serum withdrawal at which time 25% of control cells incorporated BrdU while only 5% of Megf10 overexpressing cells incorporated BrdU (n=3). By forty-eight hours following serum withdrawal, less than 5% of cells in both control and experimental populations incorporated BrdU indicating that the cells had withdrawn from the cell cycle (Fig 23). This number did not change at later time points.

Given that the majority of Megf10HA overexpressing cells failed to undergo terminal differentiation, but had withdrawn from the cell cycle we asked if these cells could be restimulated to enter the cell cycle. When cells were exposed to low-serum

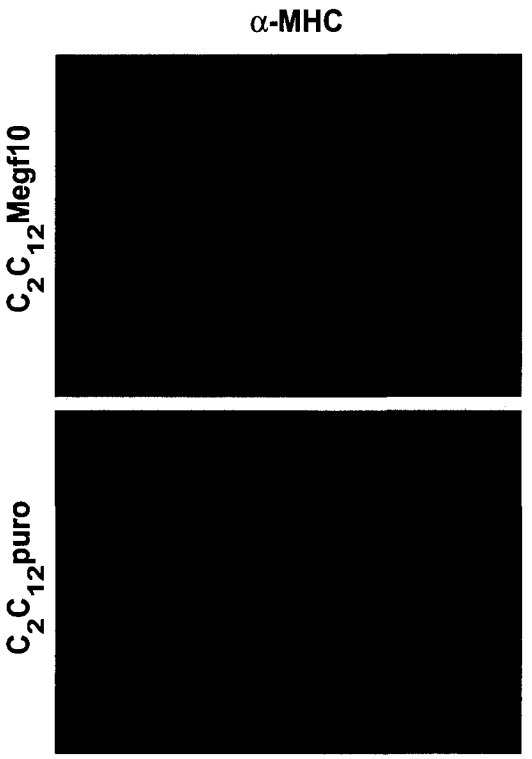

Figure 21. Megf10 over-expression inhibits terminal differentiation of C2C12 myoblasts. Staining for myosin heavy chain expression using the MF20 antibody reveals that following serum withdrawal, myoblasts overexpressing Megf10 fail to upregulate myosin heavy chain and do not form multinucleated myotubes. Cells containing empty vector control differentiate appropriately.

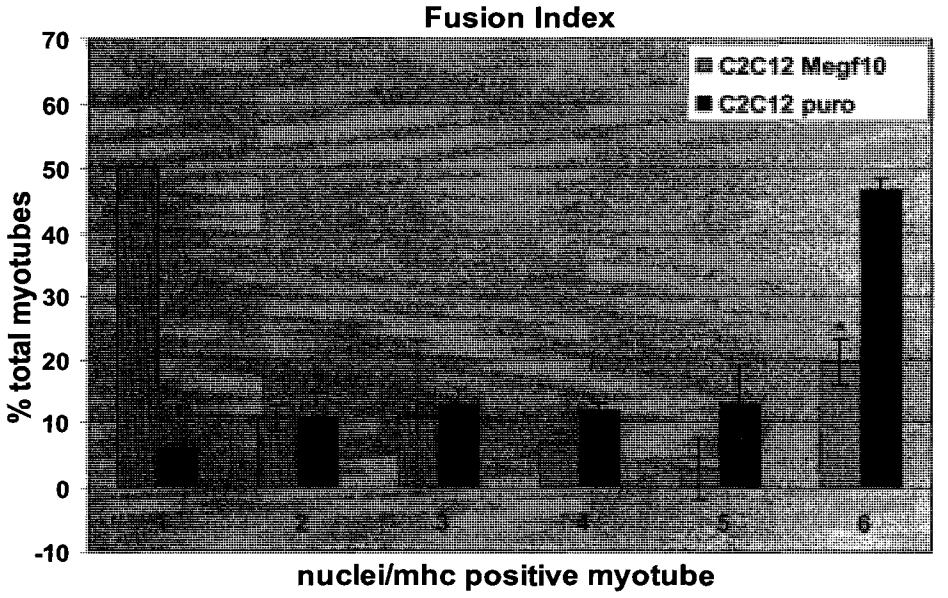

Figure 22. Megf10 over-expressing myoblasts that are capable of upregulating myosin heavy chain following serum withdrawal fail to fuse to form multinucleated myotubes. While the majority of Megf10 over-expressing cells failed to upregulate myosin heavy chain expression following serum withdrawal, the Megf10 over-expressing cells that did upregulate myosin heavy chain failed to form appropriate multinucleated myotubes. While the majority (>80%) of myosin heavy chain positive bodies in control cultures contained 3 or more nuclei, 50% of myosin heavy chain positive bodies were mononuclear in Megf10 overexpressing cultures. Error bars represent Std. dev. (n=3, * = p,0.05).

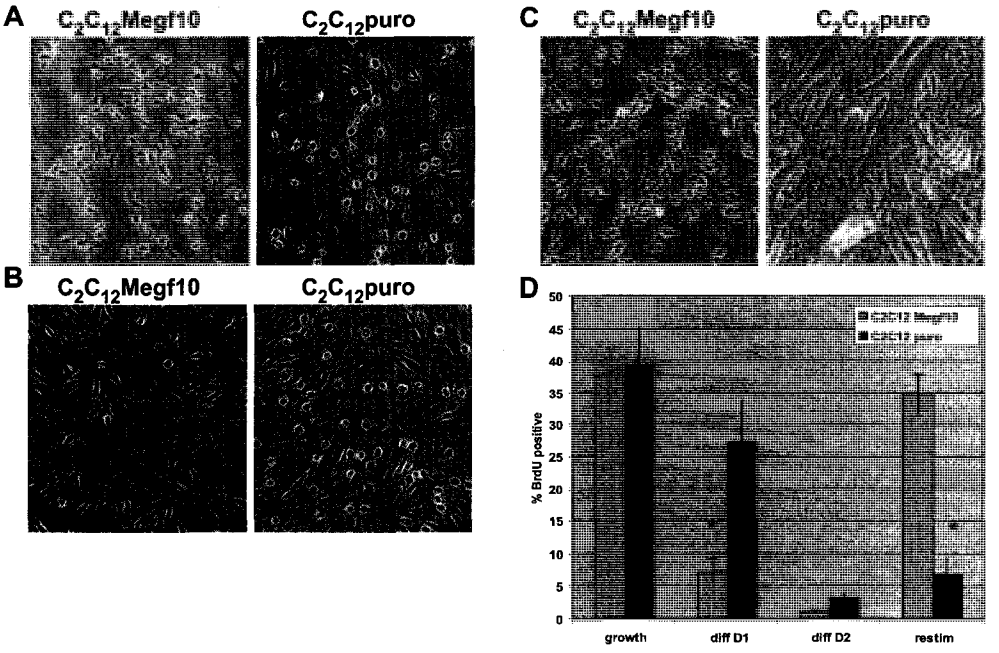

Figure 23. Megf10 over-expressing cells enter a state of quiescence following serum withdrawal. C2C12 myoblasts infected with Megf10HA or empty vector control were maintained in growth media or differentiated for 1-5 days. Prior to fixation for staining, cells were incubated for 30 min. with 30ug BrdU/ml media. Cells were stained for BrdU incorporation. Panel (A) reveals 30% of cells incorporate BrdU under growth conditions. (B) 24 hours following serum withdrawal only 8% of Megf10HA expressing cells incorporate BrdU indicating withdrawal from the cell cycle while 25% of control cells continue to incorporate BrdU. (C) A large percentage of cells expressing Megf10 that were subjected to low serum for 5 days and restimulated with growth media for 24 hours prior to BrdU incubation were able to re-enter the cell cycle, while the majority of control cells were terminally differentiated and were unable to incorporate BrdU. (D) Bar graph demonstrating the number of cells that incorporated BrdU during a 30-minute pulse Error bars represent Std. dev. (n=3, * =$p<0.01$).

conditions for 5 days and then re-stimulated with growth serum for 24 hours, 35% of Megf10 overexpressing cells were able to re-enter the cell cycle as demonstrated by their ability to incorporate BrdU (Fig 23). As expected, less than 10% of control cells were able to re-enter the cell cycle (Fig 23). The ability of Megf10 overexpressing cells to re-enter the cell cycle following extended serum withdrawal suggests that maintained expression of Megf10 under low serum conditions allows cells to enter a state of quiescence following withdrawal from the cell cycle rather than undergoing terminal differentiation.

4.3.3 Cell Cycle Kinetics are not Affected by Megf10 Overexpression

Given that Megf10 overexpression enhanced the proliferation of myoblasts we wanted to determine which, if any portion of the cell cycle was altered by Megf10 overexpression. In order to analyze the cell cycle kinetics of myoblasts overexpressing Megf10 we performed BrdU and PI staining on control and Megf10HA overexpressing myoblasts under growth conditions and then subjected the cells to sorting on a Beckman Coulter FACS STAR. BrdU labeling was used to determine the proportion of cells that were in S phase while PI staining distinguished between cells that were 2N or 4N. When combined, the results provide an overview of the percentage of cells in each stage of the cell cycle. Changes in cell cycle kinetics are represented by a shift in the staining pattern and subsequent change in the number of cells in a particular stage of the cell cycle. Our results did not reveal any significant changes in the number of cells in any particular stage of the cell cycle suggesting that the overall rate of cycling is enhanced rather than a change in one particular portion of the cell cycle.

4.3.4 Megf10 Overexpression Does not Alter Cell Cycle Protein Expression

Since we were unable to detect any shifts in the cell cycle kinetics as a result of Megf10 overexpression, we examined the expression profile of key regulators of cell cycle progression. Western blots revealed only minor changes in the expression levels of cyclin D1 under growth conditions (Fig 24). The level of cdk2 expression appeared to be slightly decreased under growth and differentiation conditions in Megf10 overexpressing cells (Fig 24). Interestingly, under growth conditions, the level of cyclin D2 expression was comparable between Megf10 overexpressing cells and puro controls, but following serum withdrawal, unphosphorylated cyclin D2 was observed in puro controls and Megf10 overexpressing cells however the level of cyclin D2 expression was much lower in Megf10 expressing cells. These results were supported by RNase protection assays. The assays demonstrated similar changes in RNA levels for *cdk2* and *cyclin D2*. There was no observable difference in the level of *cyclin D1* transcripts (Fig 25). These results indicate that there may be minor changes in the levels of specific cyclins and cdks as a result of Megf10 overexpression, however our previous FACS analysis suggests that these changes in protein levels have minor, if any effect on overall cell cycle kinetics.

Our lab has previously demonstrated that the presence of Rb and its state of phosphorylation dramatically affect the ability of myoblasts to undergo terminal differentiation (Huh et al., 2004). Therefore, we examined the expression of Rb and the ratio of hypo to hyper phosphorylated Rb in cells overexpressing Megf10HA. At one and two days following serum withdrawal, the majority of pRb was found to be in the hypophosphorylated state in cells over-expressing Megf10 while controls had equal

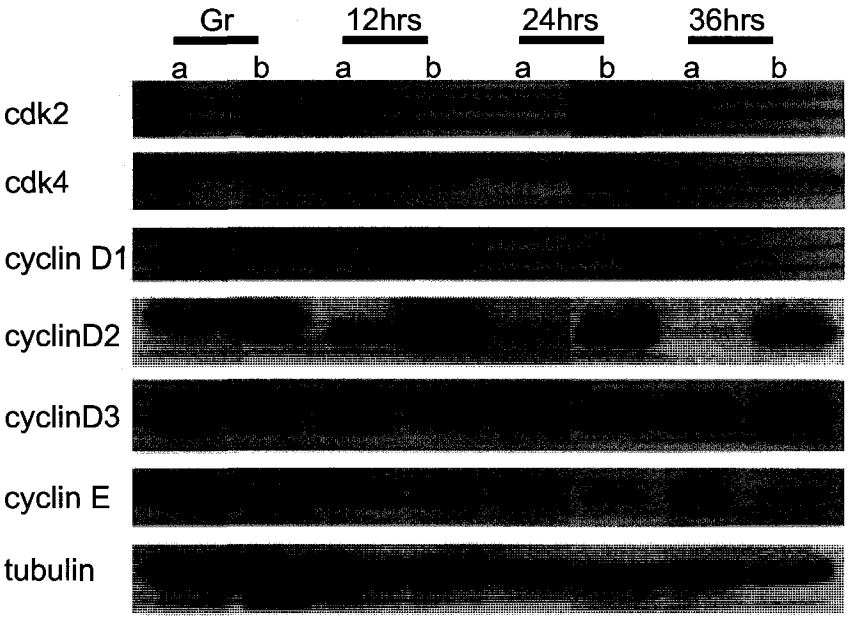

Figure 24. Over-expression of Megf10 decreases cdk2 and cyclin D1 expression. Western blot analysis of cyclin and cdk levels in C2C12 cells over-expressing Megf10 (lanes marked a) reveals that cdk2 and cyclin D1 expression levels are lower under growth conditions than in control cells (lanes marked b). Following serum withdrawal, cdk2 and cyclin D1 levels are comparable between the two populations. Following serum withdrawal cyclin D2 appears to shift to an unphosphorylated form in control cells. A similar shift is observed in Megf10 overexpressing cells however the overall expression level dramatically decreases.

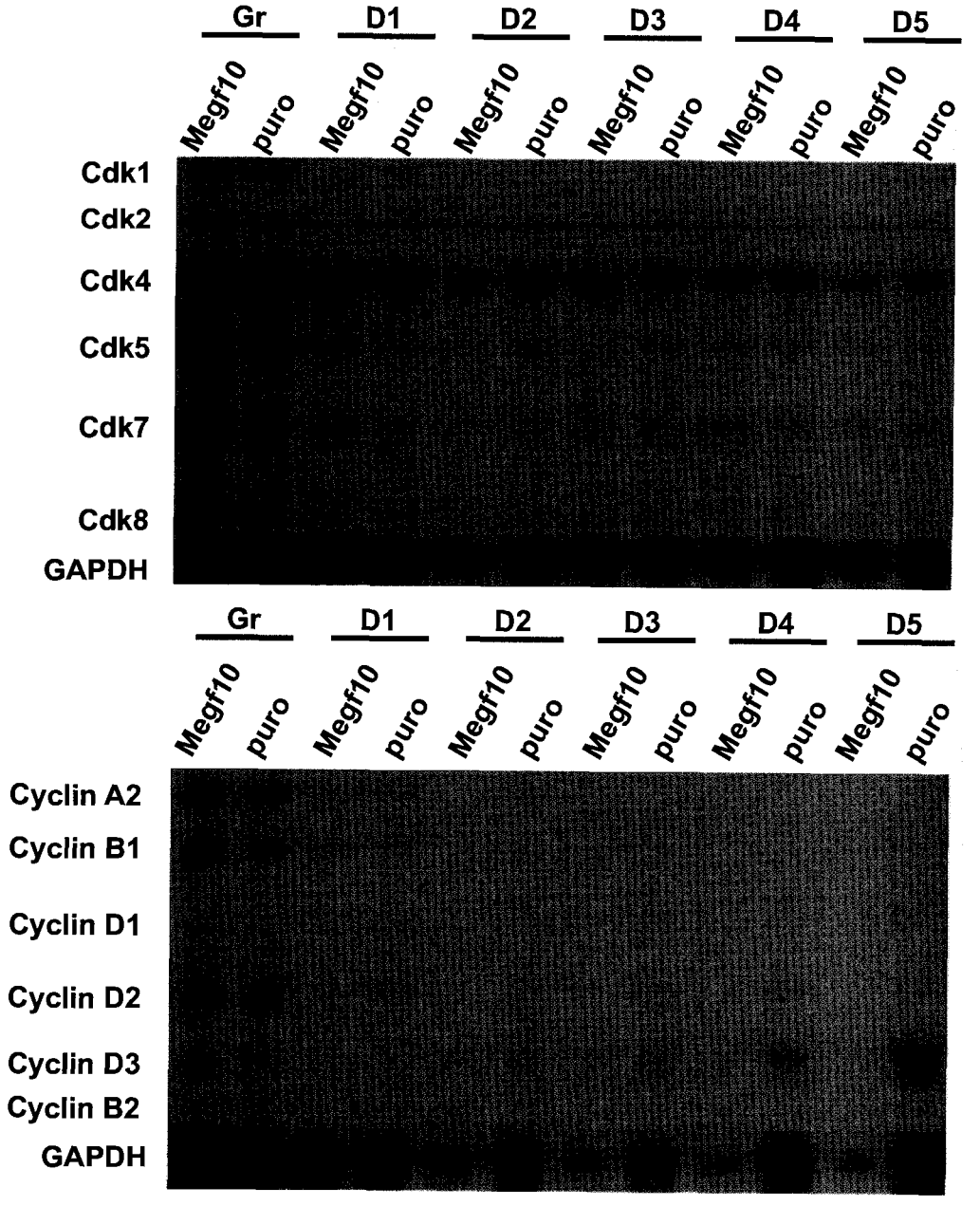

Figure 25. RNase protection assays verify decreases in cyclin D2 and cdk2. RNase protection assays performed on extracts from C2C12 cells overexpressing Megf10 or empty vector controls verifies a decrease in the level of cyclin D2 expression and cdk2 expression. No significant differences are observed in the other family members examined.

amounts of hypo- and hyperphosphorylated pRb. By three days follwing serum withdrawal, pRb was hypophosphorylated in all cells (Fig 26). Thus, while the overall rate of proliferation of cells overexpressing Megf10 appears to be enhanced and terminal differentiation is inhibited, there appears to be only minor changes in the expression of cell cycle regulatory proteins and the overall kinetics of cell cycle progression.

4.3.5 Overexpression of Megf10 alters the levels of Key Myogenic Proteins

While overexpression of Megf10 dramatically altered the differentiation and proliferation of myoblasts, no major changes were observed in the expression of cell cycle regulators. Therefore, we examined the expression of key myogenic proteins. Western blotting of protein extracts from control and Megf10HA overexpressing cells revealed dramatic changes in the level of several myogenic factors. Most strikingly, Myf5 protein levels were dramatically elevated in Megf10 overexpressing cells, while MyoD and Pax7 protein levels were decreased (Fig 27). Following serum withdrawal, Myf5 protein levels decreased in both Megf10HA overexpressing cells and controls (Fig 27). MyoD expression was maintained in control cells for the first 48 hours following serum withdrawal and was downregulated at subsequent time points. MyoD protein levels remained undetectable in Megf10HA overexpressing cells (Fig 27). Pax7 underwent similar regulation. Myogenin expression was readily detectable in control cells within 72 hours of serum withdrawal (Fig 27). Megf10HA overexpressing cells failed to upregulate myogenin to the extent observed in control cells.

To determine the level at which Megf10 regulation of MyoD, Myf5 and Pax7 was occurring, we performed Real-Time PCR to examine the level of transcripts. Our results

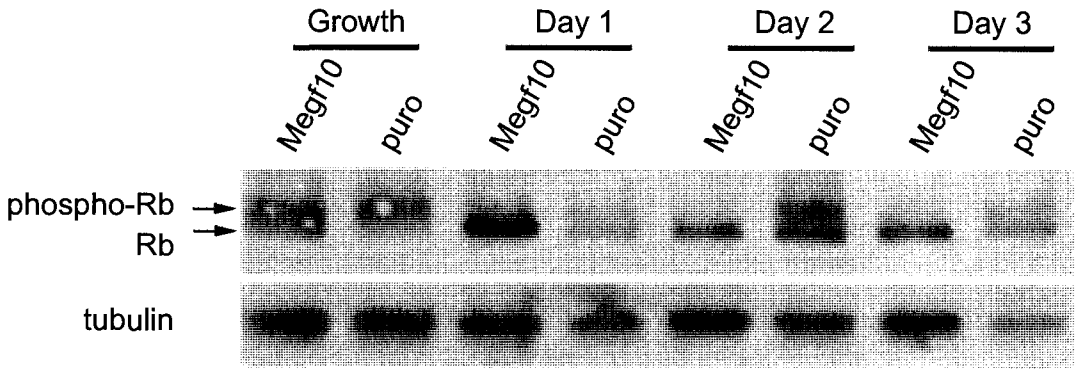

Figure 26. The phosphorylation status of pRb is unaltered in C2C12 cells over-expressing Megf10 under growth conditions. Protein was isolated from cells maintained under growth or differentiation conditions. Over-expression of Megf10 under growth conditions did not alter the phosphorylation status of pRb. At 1 and 2 days following serum withdrawal, the majority of pRb is in the hypo-phosphorylated state in cells over-expressing Megf10 while emtpy vector controls appear to have equal amounts of hypo- and hyperphosphorylated pRb. By day 3 following serum withdrawal, the majority of pRb is hypophosphorylated in both Megf10 over-expressing cells and controls.

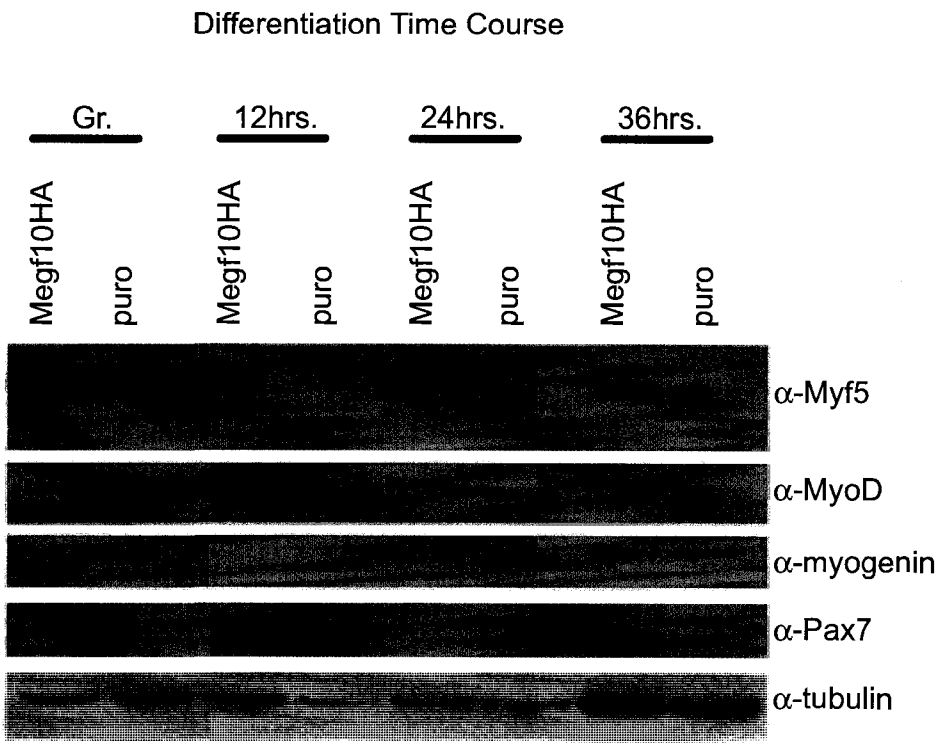

Figure 27. Over-expression of Megf10 alters the expression of key myogenic proteins. Western blot analysis of protein extracts from C2C12 cells over-expressing Megf10 or empty vector controls reveals a significant increase in the level of Myf5 protein in Megf10 cells. Myf5 protein levels decrease in Megf10 over-expressing cells following serum withdrawal. MyoD and Pax7 protein levels are significantly lower in Megf10 over-expressing cells. Following serum withdrawal, Megf10 cells fail to upregulate myogenin expression.

demonstrated a two-fold increase in the level of *Myf5* transcripts in Megf10 overexpressing cells (Fig 28). As well, a significant decrease in the level of *Pax7* transcripts was also observed suggesting that these two factors are affected at a transcriptional level. Interestingly, the level of *MyoD* transcripts appeared unaltered between control and Megf10HA overexpressing cells suggesting that MyoD expression is modified post-transcriptionally.

4.3.6 Transplantation of Megf10 Overexpressing Myoblasts

Megf10 overexpressing cells mimic *MyoD-/-* myoblasts in several ways including their enhanced proliferation and reduced ability to differentiate, their elevated levels of Myf5 and the absence of desmin expression. Since *MyoD-/-* mice have increased number of satellite cells, we examined whether or not cells overexpressing Megf10 would contribute to the quiescent satellite cell compartment upon transplantation into regenerating muscle *in vivo*.

SCID beige mice were subjected to cardiotoxin injection and two days following injury $1x10^6$ C_2C_{12} cells were injected into the regenerating muscle as well as uninjured controls. C_2C_{12} cells expressing GFP were used as controls. The mice were allowed to regenerate for a full 21 days prior to sacrificing the animals. The regenerated skeletal muscle was recovered and frozen sections were stained for laminin and Megf10HA, or laminin alone in the case of GFP expressing controls.

In mice that received injections of Megf10HA overexpressing cells, regeneration appeared to be inhibited. Large areas of unrepaired muscle remained, however very few Megf10 expressing cells were detectable at the site of injury (Fig 29). Similarly,

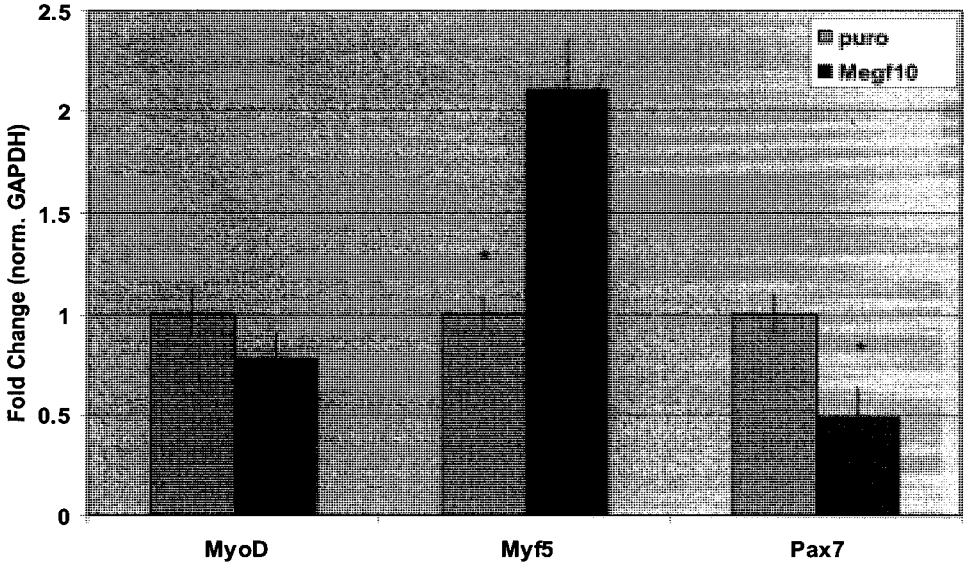

Figure 28. Real-Time PCR analysis reveals that Megf10 over-expression alters Myf5 and Pax7 expression on a transcriptional level. RLT-PCR was utilized to analyze the level of transcriptional activity of MyoD, Myf5, and Pax7 in C2C12 cells over-expressing Megf10. Expression levels were normalized to GAPDH and the expression level of each gene in control cells was set to 1. The fold change for each gene is relative to its own expression in control cells. Myf5 transcripts were elevated 2 fold while Pax7 levels were decreased 0.5 fold indicating that Megf10 expression affects the transcription of Pax7 and Myf5. MyoD transcript levels were similar between control and Megf10 over-expressing cells indicating that Megf10 alters MyoD in a post-transcriptional manner. Error bars represent Std. dev. (n=3, * = $p<0.02$).

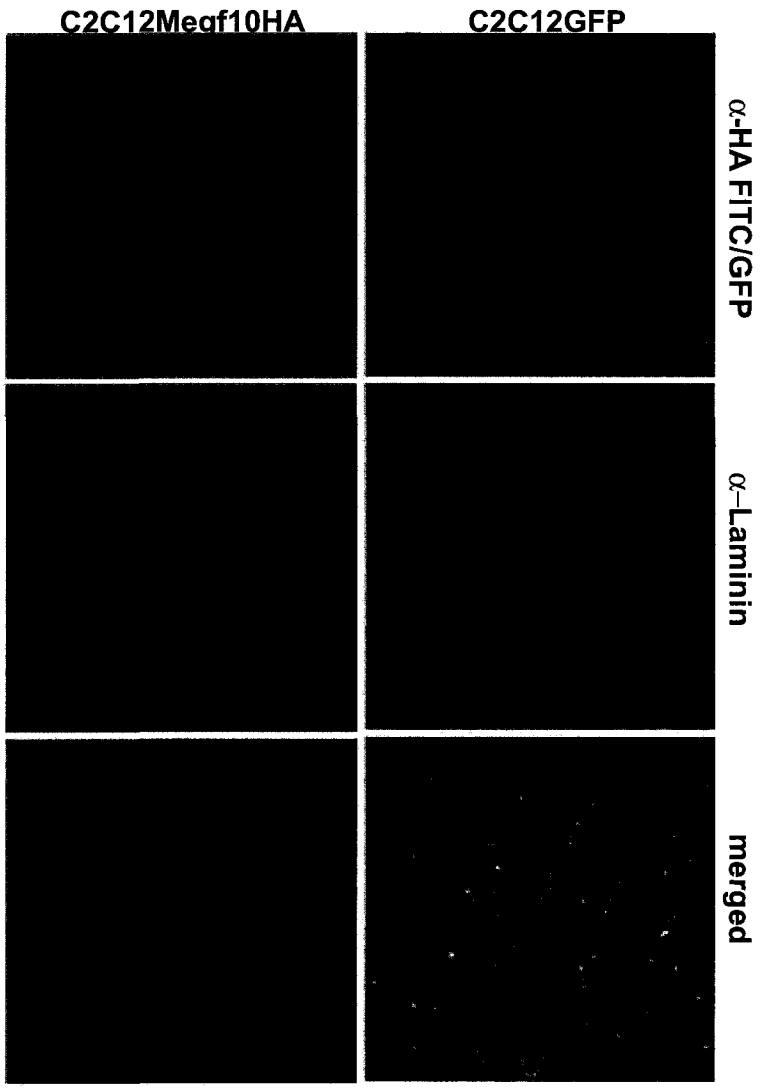

Figure 29. C_2C_{12} myoblasts over-expressing Megf10 do not contribute to muscle regeneration and do not occupy satellite cell positions upon transplantation into regenerating hindlimb musculature. $1x10^6$ C_2C_{12} cells over-expressing Megf10, or expressing GFP were transplanted into ctx injured hindlimb muscles of SCID beige mice. Muscles were harvested 21 days post injection. 8μm frozen sections were stained for Laminin and HA or examined for GFP expression. Muscle injected with myoblasts expressing Megf10HA failed to undergo appropriate regeneration. Experiments were repeated twice using three control and three experimental animals each time.

regeneration in mice that received control GFP expressing cells also appeared to have extremely low levels of regeneration. However, there were small numbers of regenerated fibers within the damaged area. Again, very few GFP positive cells were visible in the damaged area.

Examination of the areas surrounding the site of injury revealed somewhat higher numbers of injected cells in both instances, however, laminin staining indicated that these cells were most likely myonuclei rather than satellite cells. Thorough examination failed to reveal any injected cells that appeared to be located in satellite cell like positions.

4.4 Discussion

Our sequence analysis of Megf10 determined that it contained multiple EGF-repeat motifs and most likely localized to the plasma membrane. Through immunocytochemistry and cellular fractionation experiments we were able to verify that Megf10 does localize to the plasma membrane as expected. Previous work examining the function of the C. elegans and D. melanogaster orthologs of Megf10 indicated that this molecule functions in the recognition of apoptotic cells in the nervous system however its role in skeletal muscle has not been examined (Manaka et al., 2004; Zhou et al., 2001). To determine the function of Megf10 in myogenic cells, we retrovirally infected C_2C_{12} cells with an HA-tagged form of Megf10 and examined the effect. Under growth conditions, overexpression of Megf10 resulted in enhanced cellular proliferation. Our growth curves indicated that cells overexpressing Megf10 underwent division on average once every 11.6 hours while empty vector control cells divided once every 13.7 hours, a difference of 2.5 hours. Double tailed t-test revealed this to be a significant

difference with a p-value of 0.017. Analysis of the rate of apoptosis in experimental and control cells under growth conditions using TUNEL assays revealed that the basal rate of apoptosis in cells overexpressing Megf10 (0.64%) compared to empty vector controls (0.60%) was not statistically significant ($p=0.45$, $n=3$). Therefore, an increased resistance to apoptosis is likely not the cause of the increased number of cells, indicating that this is indeed an effect on proliferation rather than survival. Importantly, similar infection of fibroblasts did not result in enhanced proliferation indicating that increased proliferation due to Megf10 overexpression is a function specific to myogenic cells. This suggests that a factor or factors present in myoblasts, but not non-myogenic cells, is required for Megf10 to exert its effect on proliferation.

Several growth factors are expressed as precursor proteins that span the plasma membrane and are then processed by extracellular factors to produce active diffusible mitogens. Our initial sequence analysis of Megf10 did not reveal the presence of potential cleavage sites in the extracellular domain suggesting that Megf10 is not cleaved to form an active peptide. Our conditioned media experiments did not reveal enhanced proliferation further verifying that Megf10 does not function as a diffusible mitogen.

Cellular proliferation is a tightly controlled process with several checkpoints through which a cell must pass in order to proceed with division, enter quiescence, or undergo terminal differentiation. To determine if cells overexpressing Megf10 were proceeding through a particular stage of the cell cycle at a faster rate than controls, we performed FACS analysis on cells that were stained with BrdU to identify cells undergoing DNA replication, and PI to determine the total DNA content of cells. Based on this staining we were able to determine the percentage of cells in each portion of the

cell cycle. If a particular checkpoint was being affected by Megf10 overexpression we should have noticed a change in the percentage of cells in a particular stage. While our growth curves clearly demonstrated an increased proliferative rate, our FACS analysis failed to show any differences in the percentage of cell in a particular stage of the cell cycle. It is possible that the increased rate of proliferation is not significant enough to be able to identify a particular stage that is altered upon Megf10 overexpression. Alternatively, the enhanced proliferation may be a result of a general increase in cell cycle progression rather than a change in one particular stage.

Given that our FACS analysis did not reveal any significant changes in cell cycle progression, we examined the expression of critical cell cycle proteins to ensure that the cell cycle was not being significantly altered. Examination of the early G1stage Cyclin D1/cdk4 complex proteins revealed no significant changes in cdk protein levels and cyclin D1 levels were only slightly decreased. As well, the late G1/S stage cyclin E was also unaffected while expression of its binding partner, Cdk2, appeared to be slightly decreased. Interestingly, there appeared to be a significant difference in the level of cyclin D2 expression following serum withdrawal. While puro control cells expressed significant levels of dephosphorylated cyclin D2, Megf10 expressing cells did not express detectable levels of cyclin D2 following serum withdrawal. RNase protection assays revealed no changes in the expression of *cyclin A* or *cyclin B1* and are in agreement with the changes observed in protein levels of *cyclins D1* and *D2* as well as *cdk2*.

Previous work from our lab has demonstrated a critical requirement for pRb in myogenic proliferation and differentiation (Huh et al., 2004). During proliferation pRb is hyperphosphorylated and does not bind to E2F, thereby allowing transcription of growth

promoting genes. Upon serum withdrawal, pRb becomes hypophosphorylated and remains bound to E2F resulting in cell-cycle arrest. Given the enhanced proliferation and decreased differentiation of Megf10 overexpressing cells we examined the expression and phosphorylation pattern of pRb. Overall expression levels of pRb were similar between Megf10 overexpressing cells and empty vector controls. Furthermore, upon serum withdrawal, the shift from hyper to hypophosphorylated Rb was observed in both Megf10 overexpressing cells and controls indicating that the observed changes in proliferation and differentiation were not due to alterations in the expression or phosphorylation of pRb. Thus, the increased rate of proliferation and inhibition of differentiation by overexpression of Megf10 does not appear to be a consequence of altered cell cycle kinetics or pRb expression or phosphorylation status.

In vitro analysis of *MyoD-/-* myoblasts revealed that these cells are highly proliferative and fail to undergo terminal differentiation following serum withdrawal (Sabourin et al., 1999). Interestingly, these cells were shown to express high levels of *Myf5*, previously shown to be highly expressed during myoblast proliferation and down-regulated following serum withdrawal (Sabourin et al., 1999). As well, it has been demonstrated that MyoD is capable of binding to and activating the myogenin promoter while Myf5 is much less effective (Edmondson and Olson, 1989; Ishibashi et al., 2005). The binding of MyoD to the *myogenin* promoter is a crucial step in initiating terminal differentiation of myoblasts. Interestingly, Western blotting revealed that Megf10 overexpressing cells expressed elevated levels of Myf5 and little to no detectable MyoD, mimicking *MyoD-/-* myoblasts. Following serum withdrawal, Megf10 overexpressing cells did not express MyoD and failed to upregulate myogenin. Thus it appears that

overexpression of Megf10 results in downregulation of MyoD and subsequently, cells are unable to activate myogenin following serum withdrawal resulting in inhibition of terminal differentiation. Immunocytochemistry verified that following serum withdrawal, the majority of Megf10 overexpressing myoblasts failed to undergo terminal differentiation, as determined by MyHC expression, and did not fuse to form multinucleated myotubes.

RLT-PCR analysis revealed that the transcription of *MyoD* was not affected by Megf10 overexpression, however western blotting revealed decreased levels of MyoD protein indicating that MyoD was affected post-transcriptionally. Interestingly, Myf5 expression was increased at both the RNA and protein level. It has previously been suggested that MyoD may repress *Myf5* transcription and that the absence of MyoD results in increased *Myf5* transcription (Megeney et al., 1996). Indeed, *MyoD-/-* myoblasts express elevated levels of *Myf5* and Megf10 overexpressing myoblasts, which have dramatically reduced levels of MyoD also display increased *Myf5* expression.

Interestingly, RLT-PCR analysis also revealed that the level of *Pax7* transcripts were lower in cells overexpressing Megf10 than empty vector controls. This is in agreement with our observation that Pax7 protein levels are decreased in Megf10 overexpressing cells by western blotting. There is no previous evidence to suggest that MyoD or Myf5 control *Pax7* expression. In fact, most studies suggest that *Pax3* and *Pax7* act genetically upstream of *MyoD* and *Myf5* (Maroto et al., 1997; Olguin and Olwin, 2004; Tajbakhsh et al., 1997; Zammit et al., 2006). Interestingly our observations in single fiber culture and by immunohistochemistry on tibialis anterior muscle sections revealed that Megf10 expression was only observed in cells that expressed Pax7. Thus it

would appear that while Megf10 overexpression may result in downregulation of *Pax7 in vitro*, the expression of Pax7 and Megf10 are not mutually exclusive *in vivo*. Furthermore, Megf10 expression was only detected in cells that had previously expressed Myf5. Cells that had not expressed Myf5 (YFP-) but were Pax7 positive were not found to express Megf10. One possibility is that Pax7+/YFP- satellite cells represent a primitive pool of progenitor satellite cells that do not express a subset of the markers expressed by committed satellite myogenic cells. Alternatively, Pax7+/YFP- satellite cells may have expressed Megf10 at some point, and down-regulated its expression upon return to quiescence. However, this seems unlikely as we do not observe Pax7+/YFP-/Megf10+ cells in activated single fiber cultures suggesting that satellite cells that remain Pax7+/YFP- do not upregulate Megf10 at any point. One could hypothesize that following activation, satellite myogenic cells respond to specific cues via signaling through Megf10 that in turn regulates MyoD expression and myogenic progression.

The inability of myoblasts overexpressing Megf10 to differentiate is clearly demonstrated by their inability to upregulate myogenin and MyHC as well as their inability to fuse to form multinucleated myotubes. In order for myoblasts to undergo terminal differentiation, they must withdraw from the cell cycle. It has clearly been demonstrated that following serum withdrawal myoblasts undergo a final round of cellular division, withdraw from the cell cycle, and undergo terminal differentiation (Clegg et al., 1987). We clearly demonstrate 24 hours following serum withdrawal, C_2C_{12} cells begin to withdraw from the cell cycle as indicated by a decreased percentage of cells incorporating BrdU. By 48 hours following serum withdrawal, the majority of cells have withdrawn from the cell cycle. The cells undergo terminal differentiation and

are unable to re-enter the cell cycle when serum stimulated. Unlike control myoblasts, C_2C_{12} cells overexpressing Megf10 withdraw from the cell cycle within 24 hours of serum withdrawal. However, after 5 days in low serum culture, conditions that normally lead to terminal differentiation, cells over expressing Megf10 are able to re-enter the cell cycle as indicated by there ability to incorporate BrdU. Our preliminary examination of Megf10 expression indicates that during differentiation Megf10 expression is normally down-regulated. In cells retrovirally induced to express Megf10 throughout differentiation, cells appear to enter a quiescent state following serum withdrawal and are able to re-enter the cell cycle upon re-stimulation. This raises the possibility that Megf10 regulates the myogenic progression of activated satellite cells, potentially controlling the repopulation of the satellite cell compartment by either allowing cells to undergo terminal differentiation following downregulation of Megf10, or forcing cells to enter a quiescent state and return to the satellite cell compartment via maintenance of Megf10 expression.

The ability of transplanted myoblasts to contribute to muscle regeneration and fiber repair has been well established (Partridge et al., 1989; Rando et al., 1995). Despite the ability of these transplanted cells to repair skeletal muscle, only in rare instances do these cells take-up satellite cell like positions (Yao and Kurachi, 1993). The observation that maintained Megf10 expression during low serum conditions allowed myoblasts to enter a state of quiescence rather than proceeding with differentiation raised the possibility that these cells may be capable of repopulating the quiescent satellite cell compartment during muscle regeneration. To test this possibility we performed cardiotoxin insult on the tibialis anterior muscle of SCID/beige mice and subsequently transplanted C_2C_{12} myoblasts expressing either Megf10 or GFP and attempted to follow

their fate. Our results indicate a very low survival rate for injected cells, and those that do survive do not appear to contribute to regeneration or the satellite cell compartment. Satellite cells can be identified by their position beneath the basal lamina (marked by laminin A expression) and above the sarcolemma (Marked by dystrophin expression). Immunohistochemistry failed to identify Megf10 or GFP expressing cells beneath the basal lamina indicating a failure of cells to occupy satellite cell positions. Furthermore, we were unable to identify regenerated fibers expressing either Megf10 or GFP. Small numbers of GFP or Megf10 expressing mononuclear cells were detectable in interstitial spaces adjacent to the site of injection indicating that a small proportion of cells survived the transplantation but did not contribute to muscle repair or regeneration. While these experiments did not demonstrate the ability of Megf10 expressing myoblasts to contribute to the satellite cell compartment, the fact that very few cells were found to survive the transplantation in both experimental and control trials make these experiments inconclusive.

Our results clearly demonstrate that overexpression of Megf10 in myoblasts results in enhanced proliferation and inhibition of differentiation. While the overall cell cycle kinetics appear to be unaltered, and the expression of key cell cycle regulatory proteins appear to be unchanged there is a dramatic effect on the expression of key myogenic regulatory proteins, particularly Myf5 and MyoD. Strikingly, Megf10 overexpression results in a dramatic decrease in MyoD protein levels, quite possibly explaining the inability of these cells to undergo terminal differentiation. Strikingly, Megf10 overexpressing cells appear to be able to enter a quiescent state following serum withdrawal rather than undergoing terminal differentiation. Given the ability of Megf10

expression to downregulate MyoD, and to force cells into quiescence following serum withdrawal, it may be that Megf10 regulates the myogenic potential of satellite cells. Following activation, satellite myogenic cells may down regulate Megf10 expression and proceed with terminal differentiation. Alternatively, Megf10 expression may be maintained allowing cells to either proliferate, or to return to a quiescent state and repopulate the satellite cell compartment.

Chapter 5 – Megf10 Functions as a Transmembrane signaling Molecule

5.1 Introduction

Yeast two-hybrid experiments performed using the cytoplasmic portion of KIAA1780 have revealed that this portion of MEGF10 interacts with a variety of intracellular signaling intermediates such as TAOK2, TIMAP, and Grb10 (Nagase et al., 2001). Furthermore, it has been demonstrated that CED-1 signals through CED-6/GULP in *c. elegans* to activate Rac1 resulting in actin remodeling during engulfment of apoptotic cells (Kinchen et al., 2005). These results clearly demonstrate a role for Megf10 in intracellular signaling.

The Rac-1/Cdc42 signaling pathway has been demonstrated to play a role in regulating the proliferation and differentiation of myogenic cells, although there are conflicting reports as to the exact effects on myogenesis (Bryan et al., 2005). Previous studies suggest that constitutively active Rac1 results in the accumulation of Myf5 and the inhibition of myogenin, troponin T, and MyHC expression following serum withdrawal (Meriane et al., 2000). Inhibition of differentiation via Rac1 occurs through the activation of c-jun N-terminal kinase (JNK). Importantly, the temporal activity of Rac1 appears to be tightly regulated during myogenesis, with active Rac1 being found during proliferation and a subsequent decrease in Rac1 activity as differentiation occurs (Heller et al., 2001).

The activation of the MAPK signaling pathway has been shown to be a critical regulator of cellular proliferation and differentiation (Bennett and Tonks, 1997; Jones et al., 2001). During the early stages of differentiation, MAPK activity decreases, in part

due to upregulation of the Map Kinase Phosphatase, MPK1 (Bennett and Tonks, 1997). As well, overexpression of c-Raf, the upstream effector of MAPK activity, results in inhibition of differentiation further underscoring the importance of MAPK activity in regulating myogenic progression (Dorman and Johnson, 1999). Interestingly, work from our lab has demonstrated that activated MEK1 is capable of decreasing MyoD's ability to activate target genes and subsequently inhibits the initiation of differentiation (Perry et al., 2001). Thus MAPK signaling not only regulates the proliferation and differentiation of myogenic cells, but it directly affects the ability of specific MRF's to regulate muscle specific gene expression.

The phosphatidylinositol 3-kinase (PI3'K) pathway has been implicated in myogenic progression. Insulin-like growth factor stimulation leads to an increase in PI3'K activity that in turn can induce myogenic differentiation (Conejo et al., 2001). Increased PI3'K activity is accompanied by an increase in PKB/Akt activity (Conejo et al., 2001). Activated PKB can phosphorylate c-Raf effectively inactivating the MEK/MAPK pathway, which when active can inhibit differentiation (Suzuki and Takahashi, 2000; Wandzioch et al., 2004). Thus, signaling through the PI3'K pathway and its downstream effectors is also important in myogenic progression. Indeed, several papers have demonstrated that cross talk between the PI3'K pathway and MAPK pathways occurs during myogenic proliferation and differentiation (Glass, 2005; May et al., 2006).

Given our previous results demonstrating that Megf10 overexpression enhances proliferation and inhibits differentiation and results from other groups indicating that Megf10 orthologs function as signaling molecules, we asked which signaling pathways

Megf10 impinged upon in myoblasts. The MAPK and PI3'K pathways were examined due to their critical roles in regulating myogenic progression. As well, the Rac1/Cdc42 pathway was examined given previous demonstrations of CED-1's interaction with Rac1. Our results demonstrate that, like CED-1, Megf10 is able to activate Rac-1. As well, Megf10 overexpression results in increased levels of phosphorylated MEK1 indicating that Megf10 may activate the MAPK signaling pathway.

5.2 Material and Methods

5.2.1 Truncated Megf10

A truncated version of Megf10 lacking the c-terminal 290 amino acids was PCR amplified and cloned into a pHIT backbone modified to express a c-terminal FLAG tag. Primers used for PCR amplification were as follows;

Megf10ΔCT Forward CGGGATCCATGGCGATTTCTTCAAGTTCGTGC;

Reverse CGCTCGAGCATGGCGGGGGTGTAGGTCAC.

5.2.2 Rac1/Cdc42 Activity Assays

Initial Cdc42 activity assays were performed using the Cell Signaling Cdc42/Rac1 Activity Assay as per manufacturers instruction. Cells were lysed using the Cell Signaling Cdc42/Rac1 Activity Assay Kit as per the manufacturer's instructions. 500ml aliquots of cell lysate were incubated with Pak-PBD for 30 minutes at 4°C. Samples were briefly spun at 4°C and supernatant removed. The pellet was resuspended in Laemlli buffer with 10μM DTT and boiled for 5 minutes. Samples were then loaded and

run on polyacrylamide gels. Western blots were performed as previously described using α-Rac1 (Cell Signaling) antibody.

5.2.3 Western Blotting

Western blots were performed as previously described using the following antibodies; α-MyoD (C-20; Santa Cruz), α-Myf5 (C-20; Santa Cruz), α-Pax7 (hybridoma supernatant; Developmental Studies Hybridoma Bank), α-Akt (06-558; Chemicon), α-phospho-Akt (06-801; Chemicon), α-MEK1 (C-18; Santa Cruz), α-phospho-MEK1/2 (#9121l; Cell Signaling Technologies), α-ERK1/2 (#9102; Cell Signaling Technologies), α-phospho-ERK1/2 (#9101S; Cell Signaling Technologies).

5.2.4 *In vitro* Kinase Assays

Cells were lysed in NP40 lysis buffer containing protease inhibitors. α-JNK (C-17; Santa Cruz) and α-p38 (N-20; Santa Cruz) were immunoprecipitated from 500μg of protein extract overnight at 4°C. Antibody-protein complexes were pulled down by the addition of 25μl of G-sepharose beads for 2 hours at 4°C followed by centrifugation. The pellet was washed two times in 500μl NP40 lysis buffer. Pellets were then washed two times in freshly prepared Kinase buffer (25mM HEPES pH 7.6, 20mM $MgCl_2$, 10mM β-glycerophosphate, 2mM DTT, 0.1mM Sodium Vanadate, 1mM Sodium Fluoride). Pellets were resuspended in 20μl Kinase buffer with 25μM cold ATP. 2.5μg Histone H1 was used as a substrate and incubated with 5 μCi 32P-γATP at 30°C for 30 minutes with occasional gentle mixing. Reactions were stopped by adding 25μl Laemmli loading buffer with 10μM DTT to the samples and incubating at 100°C for 4 minutes. Beads

were pelleted and supernatant was loaded and run on polyacrylamide gels. Once complete, gels were incubated in Destain solution (30% MeOH, 10% HOAc) two times for 30 minutes. Gels were dried at 80°C for 2 hours, wrapped in saran wrap in placed in autoradiography cassettes with BioMax MS film and placed at –80°C until exposed. Densitometry was performed using ImageJ software.

5.3 Results

5.3.1 Amino Acids 851-1141 are required for Megf10 Function

Previous work done by Nagase et al., using a yeast two-hybrid system indicated that Megf10 interacted with several known intracellular signaling molecules including Cdc42, TAO-1, TIMAP, and Grb10 (Nagase et al., 2001). Given the plasma membrane localization of Megf10 we predicted that removal of the cytoplasmic portion of Megf10 would disrupt Megf10 signaling and restore the differentiation capacity of myoblasts infected with a truncated version of Megf10. Therefore, we generated a truncated form of Megf10, Megf10ΔCT, lacking the 290 carboxy-terminal amino acids predicted to represent the cytoplasmic domain. C_2C_{12} myoblasts were infected with retrovirus expressing FLAG-tagged version of Megf10ΔCT and stable pools were obtained by antibiotic selection. The growth kinetics were examined as in previous experiments. As expected, expression of Megf10ΔCT did not alter the growth kinetics of infected cells (Fig 30C). As well, cells infected with Megf10ΔCT were capable of undergoing differentiation at a rate similar to controls, indicating that Megf10 requires its cytoplasmic tail to alter the proliferation and differentiation of myoblasts (Fig 30B).

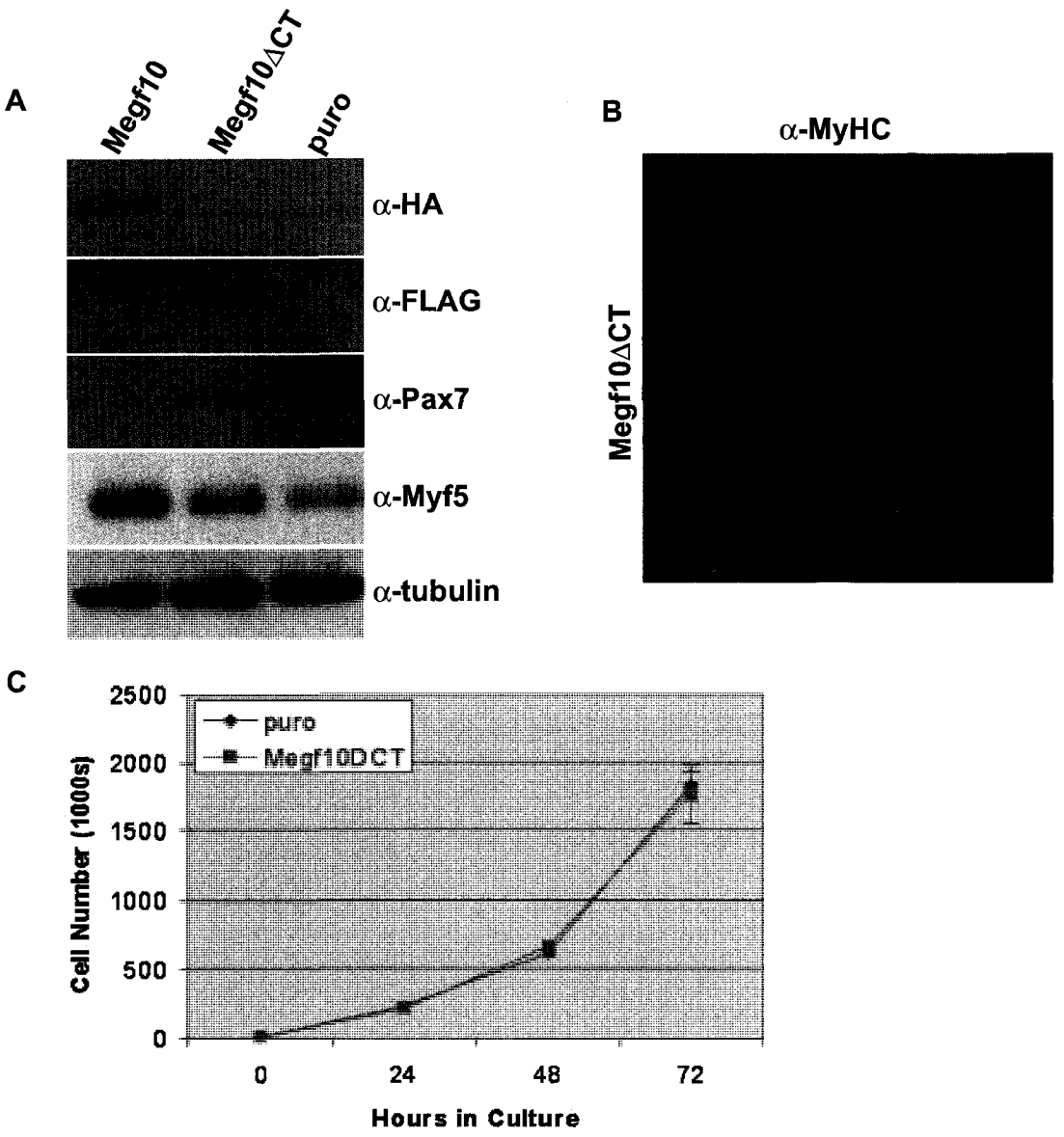

Figure 30. Removal of the cytoplasmic domain of Megf10 restores Myf5 and Pax7 expression and allows for terminal differentiation. (A) Western Blot of protein extracts from C2C12 cells over-expressing Megf10HA, a truncated FLAG-tagged Megf10 lacking the 290 carboxy-terminal amino acids (Megf10ΔCT), and empty vector controls. While over expression of Megf10 alters levels of Myf5 and Pax7, over-expression of Megf10ΔCT does not have a similar effect. (B) Immunocytochemistry for MHC demonstrates that C2C12 cells over-expressing Megf10ΔCT are capable of undergoing terminal differentiation (7 days low serum). (C) Growth curves do not indicate a difference in proliferative rates between control and Megf10ΔCT expressing cells (n=3)

Examination of the myogenic proteins MyoD, Myf5 and Pax7 revealed that the levels of these proteins in Megf10ΔCT expressing cells were similar to controls (Fig 30A). These results verify the requirement of the cytoplasmic domain for Megf10 function in myoblasts.

5.3.2 Megf10 Affects Rac1 Activity

Examination of the function of CED-1, the *c. elegans* ortholog of Megf10 revealed that CED-1 was capable of signaling through the G-protein Rac1. Since Rac1 has been shown to play a critical role in myoblast proliferation and differentiation we chose to examine whether or not overexpression of Megf10 resulted in increased Rac1 activity during proliferation. C_2C_{12} cells overexpressing Megf10 were compared to empty vector controls for the level of Rac1 activity using the Upstate G-protein Activity Assay. Cells were maintained under growth conditions, lysed and protein extract was incubated with Pak-PBD, which binds to active Rac1 and Cdc42. Western blots for Rac1 revealed equal levels of total Rac1 epxression between control and over-expressing cells and higher levels of Rac1 being pulled down by Pak-PBD in cells overexpressing Megf10 than empty vectors controls (Fig 31). These results indicate that over-expression of Megf10 in myoblasts results in increased Rac1 activity.

5.3.3 Megf10 Increases MEK1/2 Phosphorylation

A wide variety of signaling pathways are known to function in myoblasts proliferation and differentiation. Since we were able to demonstrate that overexpression of Megf10 results in increased Rac1 activity, we decided to examine the two key

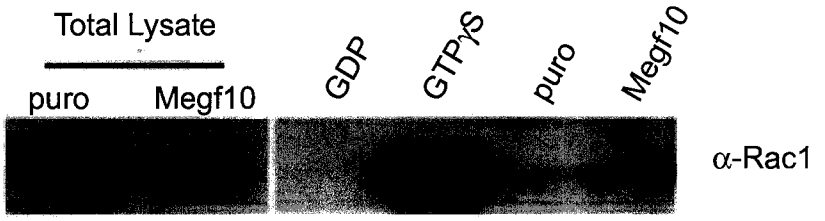

Figure 31. Overexpression of Megf10 in C2C12 myoblasts results in increased Rac1 activity. Pak-PBD pull down assays reveal elevated levels of activated Rac1 in Megf10 overexpressing cells. GDP and GTPγS loaded lysates were used as negative and positive controls respectively. IP lysate was used as a loading control for total Rac1 levels.

pathways affected by G-protein activity, the PI3' kinase pathway and the mitogen associated protein kinase (MAPK) pathway. To determine if overexpression of Megf10 resulted in increased activation of the PI3Kinase pathway, we performed Western blots to determine the level of phosphorylated Akt. Our results did not demonstrate a significant difference in the levels of phosphorylated Akt between cells overexpressing Megf10 and empty vector controls suggesting that there was no difference in the level of PI3Kinase activity.

We next examined the MAPK pathway by establishing the level of total and phosphorylated MEK1/2 in control cells and cells over-expressing Megf10 maintained under growth conditions. While the total levels of MEK1/2 appeared to be slightly lower in cells overexpressing Megf10, the levels of phosphorylated MEK1/2 were dramatically increased in these cells as compared to controls (Fig 32). We next examined the downstream target of MEK1/2, ERK1/2. Our results demonstrated that the levels of phosphorylated ERK1/2 were lower in Megf10 over-expressing cells than in control cells (Fig 32), suggesting that while MEK1/2 phosphorylation was enhanced, the major downstream target of MEK1/2 was not targeted.

5.3.4 Megf10 Increases JNK and p38 Kinase Activity

Since phosphorylated MEK1/2 did not appear to be targeting ERK1/2 we examined activity of the c-jun N-terminal kinase (JNK) and p38 kinase pathways. Cell lysates were incubated with α-p38 or α-JNK antibodies overnight and immunoprecipitated proteins were utilized to perform *in vitro* kinase assays. Our results demonstrated that the level of JNK and p38 activity were enhanced in cells

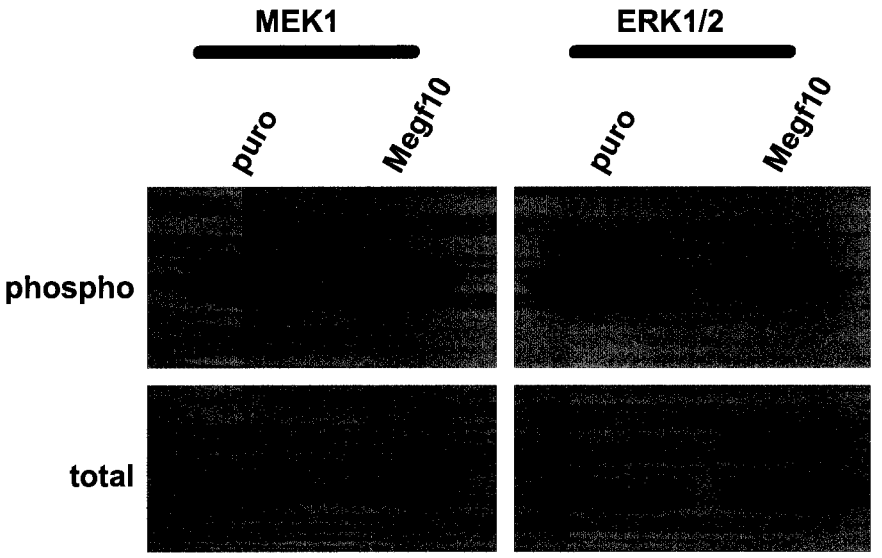

Figure 32. Over-expression of Megf10 in C2C12 myoblasts results in MEK1 phosphorylation but not ERK1/2 phosphorylation. Western Blot analysis using phospho-specific antibodies indicates elevated levels of phosphorylated MEK1 in cells over-expressing Megf10. Total levels of MEK1 appear lower in Megf10 over-expressing cells. Similar Westerns for phosphorylated ERK1/2 reveal that while total ERK1/2 levels are similar between controls and Megf10 over-expressing cells, the level of phosphorylated ERK1/2 is much higher in controls than in cells over-expressing Megf10.

overexpressing Megf10 as judged by phosphorylation of histone H1 (Fig 33). Densitometric analysis revealed a 21% increase in JNK kinase activity and a 97% increase in p38 kinase activity.

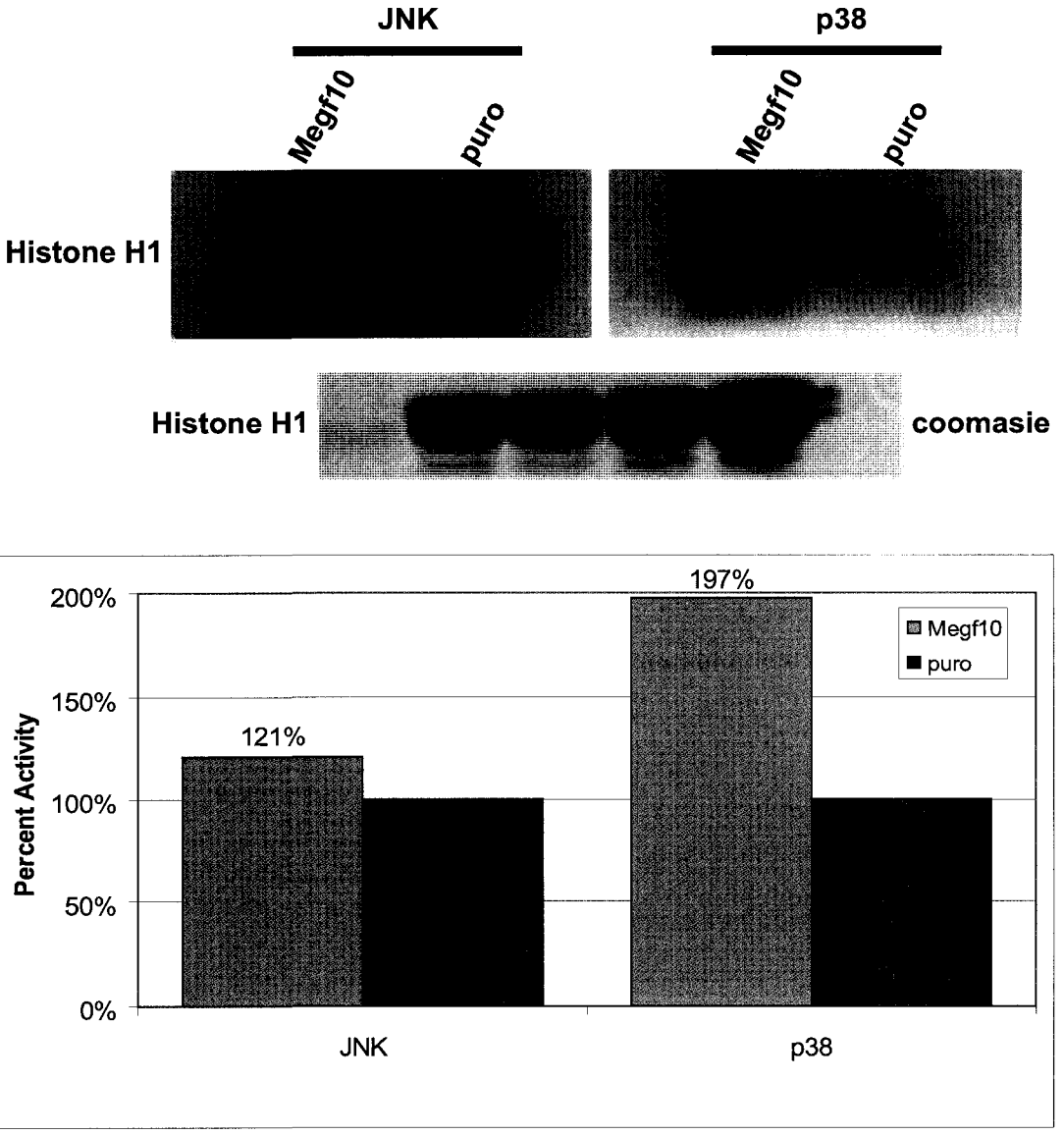

Figure 33. In Vitro Kinase Assays reveal increase JNK and p38 kinase activity in C2C12 cells overexpressing Megf10. Protein extracts from C2C12 cells expressing Megf10HA or empty vector controls were subjected to immunoprecipitation for JNK and p38. Immunoprecipitated proteins were subjected to in vitro kinase assays using histone H1 as a substrate. A 21% increase in JNK activity and a 97% increase in p38 activity were detected in extracts from Megf10 overexpressing cells via densitometric analysis. Coomasie staining of histone H1 substrate prior to in vitro kinase assays was used for normalization.

5.4 Discussion

Examination of the function of CED-1 in *C. elegans* and Draper in *d. melanogaster* have revealed that these molecules signal through their cytoplasmic tails to the small G-coupled proteins Rac1 and Cdc 42 (Kinchen et al., 2005). Furthermore, yeast two-hybrid analysis of the corresponding region of KIAA1780 (human MEGF10) demonstrated an interaction with Rho GTPase-activating protein (RICS) and Rac GTPase Activating protein 1 (RacGAP1), as well as several other signaling intermediates such as TIMAP, TAOK2 and Grb10 (HUGEppi; www.kazusa.or.jp/tech-cgi/dodirect.ppi.cgi). We have established that overexpression of Megf10 results in increased MEK1/2 phosphorylation as well as enhanced Rac-1 activity. Activation of the MAPK signaling pathway has long been known to be a crucial regulator of cellular proliferation (Bennett and Tonks, 1997; Jones et al., 2001). As well, the Rac1/Cdc42 pathway has also been demonstrated to alter the proliferative capabilities of myogenic cells although there are conflicting reports as to their exact effect on myogenesis (Bryan et al., 2005). Previous studies suggest that constitutively active Rac1 results in accumulation of Myf5 and inhibits the expression of myogenin, troponin T, and MyHC following serum withdrawal (Meriane et al., 2000). Furthermore, it blocks myogenesis via activation of c-jun N-terminal kinase (JNK). The temporal activity of Rac1 appears to be tightly regulated during myogenesis, with active Rac1 being found during proliferation and a dramatic decrease in Rac1 activity as cells progress through terminal differentiation (Heller et al., 2001). The authors suggested that active Rac1 inhibits differentiation by preventing complete withdrawal from the cell cycle. These results correspond well with our

observations. Forced expression of Megf10 results in increased Rac1 activity that, in turn, explains the decreased differentiation capacity of these cells and may also explain their inability to completely withdraw from the cell cycle as demonstrated by their ability to incorporate BrdU following serum withdrawal and restimulation. Furthermore, overexpression of Megf10 results in enhanced JNK and p38 activity, downstream effectors of Rac1 activity. Thus, it appears that Megf10 plays a critical role in modulating Rac1 signaling during satellite cell activation potentially providing a mechanism for the self-renewal and expansion of satellite cells following injury.

Typically, increases in p38 kinase activity are associated with progression towards terminal differentiation (reviewed in (Lluis et al., 2006). However, terminal differentiation requires MyoD to occur. While Megf10 overexpressing cells have elevated p38 kinase activity, there is no MyoD present for the progression of differentiation.

Interestingly, we do see a low percentage of Megf10 expressing cells undergo terminal differentiation; however the majority of these cells fail to fuse to form multinucleated myotubes. This may be explained in part by the elevated levels of activated Rac1. While controversy remains regarding the exact effect of active Rac-1 on terminal differentiation, it has been well demonstrated that activated Rac1 alters actin cytoskeleton remodeling (Bryan et al., 2005). The inability of Megf10 cells to fuse may be in part due to activated Rac1 inhibiting actin cytoskeleton re-arrangement required for appropriate fusion of differentiating myoblasts.

In addition to Rac1, MEK1/2 activity is also increased by Megf10 overexpression. We hypothesize that it may be responsible, at least in part, for the decreased

differentiation capacity observed. Previous work from our lab has demonstrated that constitutively active MEK1 is capable of inhibiting terminal differentiation, specifically by inhibiting MyoD activity (Perry et al., 2001). Furthermore, constitutively activated MEK1 can translocate to the nucleus, rather than targeting its downstream effector ERK1/2. This parallels the activation of MEK1/2 without ERK1/2 activation observed in cells overexpressing Megf10. Given the requirement of MyoD for terminal differentiation of myogenic cells through activation of myogenin and downstream targets, this is an attractive explanation for the reduced differentiation observed in cells overexpressing Megf10. One could hypothesize that forced expression of Megf10 results in maintained activation of MEK1/2 under differentiation conditions resulting in downregulation of MyoD and inhibition of terminal differentiation.

As previously stated, CED-1/Megf10 has previously been shown to interact with Rac1/Cdc42, Rho GTPase-activating protein (RICS) and Rac GTPase Activating protein 1 (RacGAP1), as well as other signaling intermediates through its c-terminal portion. We have demonstrated that Megf10 is a transmembrane protein expressed in quiescent satellite cells. Furthermore, we have demonstrated that Megf10 expression results in modulation of MEK1 and Rac1 signaling. Based on these findings we asked if removal of the cytoplasmic tail of Megf10 would alleviate any effects on growth and differentiation. Strikingly, overexpression of Megf10ΔCT had no effect on differentiation or proliferation compared with wildtype cells. Furthermore, Myf5 and Pax7 levels were identical to those of empty vector controls. These results clearly demonstrate the requirement of the carboxy-terminal 290 amino acids of Megf10. Of particular interest is the NPXY motif previously identified in CED-1 and Draper and also

present in Megf10 (Callebaut et al., 2003). Previous work suggests that this domain is crucial for interactions with signaling intermediates (Su et al., 2002). Removal of this domain in Megf10 would be a logical step to take to try and further identify the specific domains within the carboxy-terminal portion that are required for its myogenic function.

These results clearly demonstrate that the transmembrane protein, Megf10, modulates two key myogenic signaling pathways. The ability of Megf10 overexpression to activate Rac1 provides a plausible explanation for the ability of these cells to enter a quiescent state, as well as their inability to undergo terminal differentiation by inhibiting the complete withdraw of these cells from the cell cycle. Furthermore, it may also provide an explanation as to why the few Megf10 overexpressing cells that are able to undergo terminal differentiation fail to fuse to form multinucleated myotubes. Thus Megf10 signaling through Rac1 may be a crucial step in satellite cell fate. Secondly, the ability of Megf10 overexpression to enhance MEK1/2 phosphorylation may provide insight into the altered MyoD protein levels and subsequent increase in Myf5 transcription. In combination, the increased MEK1/2 phosphorylation and Rac1 activation provide a strong inhibitory mechanism for terminal differentiation and may be responsible for forcing satellite cells to return to a quiescent state rather than contributing to fiber regeneration.

Chapter 6 – Concluding Remarks

The goal of our initial RDA experiment was to identify genes that functioned in the specification, activation, and maintenance of the quiescent satellite cell compartment. The screen identified a large number of genes that were specifically expressed in myoblasts, many of which have been implicated in a variety of myogenic processes (Seale et al., 2004a). Perhaps the best example of the success of this screen is the identification of Pax7 as being critical to myoblast specification and maintenance (Seale et al., 2000). An extensive list of potential candidates was generated in the RDA screen and these candidates were examined for expression within the myogenic lineage under growth and differentiation conditions, as well as non-myogenic lineages, to identify genes that were expressed primarily in the primitive MyoD-/- myoblasts under growth conditions and down-regulated during differentiation (Seale et al., 2004a). Following the preliminary screens three clones, MD p286, MD p40, and MD p67 were chosen for further examination. It was the goal of this PhD thesis to identify these clones, determine if they were expressed in a satellite cell specific manner, and to investigate their potential role in satellite cell activation and maintenance.

The first clone to be identified (MD p286) represented HoxC10. While this clone was clearly expressed within adult skeletal muscle our results indicated that it was not expressed in quiescent satellite cells. Furthermore, careful examination revealed that HoxC10, while present in muscles from the posterior portion of mice, was not detectable in muscles isolated from the more anterior forelimbs. Based on the spatial expression patterns of Hox genes it is most likely that HoxC10 was identified due to the posterior nature of the muscle cells used in the initial RDA screen. While HoxC10 is expressed in

skeletal muscle and its expression appears to be regulated with respect to proliferation and differentiation, it is only expressed in a specific muscular domain, and does not appear to be expressed in quiescent satellite cells. Therefore, further examination of HoxC10 was not undertaken.

Clone MD p40 expression was limited to MyoD-/- myoblasts under growth conditions and was not detected in other myogenic or non-myogenic cells. Interestingly, Northern blots revealed MD p40 expression in the heart, but no expression was detected in skeletal muscle. However, *in situ* hybridization indicated that MD p40 was expressed in cells in resting tibialis anterior muscles. Approximately 5% of nuclei associated with fibers were found to express MD p40, a number consistent with expression in quiescent satellite cells. Given the low number of cells expressing MD p40 within total skeletal muscle and the differences in sensitivity between the two techniques it is not surprising that MD p40 expression was detected in skeletal muscle by *in situ* hybridization but not by Northern blotting. BLAST analysis of the initial MD p40 clone revealed homology to Lgr6, a G-coupled protein about which relatively little is known. The expression and specific functions of Lgr6 in satellite cells will need to be explored in future research.

The major research focus of this thesis was the RDA clone MDp67 identified as Megf10. Megf10 is a transmembrane protein that is expressed in the majority of quiescent satellite cells as determined by immunohistochemistry. A small population of satellite stem cells that have never expressed Myf5 and display a striking capacity to repopulate the satellite cell compartment do not express Megf10. These satellite stem cells give rise to satellite myogenic cells, a population of quiescent myogenic satellite

cells that contribute mainly to muscle regeneration. Importantly, the majority satellite myogenic cells that have expressed Myf5, also express Megf10.

Based on the restricted expression pattern of Megf10 in satellite myogenic cells and proliferating myoblasts, but not satellite stem cells or differentiated myocytes, we have proposed a model whereby Megf10 regulates the myogenic progression of satellite cells following activation. Upon activation, myogenic stem cells give rise to two distinct progeny, satellite stem cells and satellite myogenic cells. The daughter cells that become satellite myogenic cells upregulate Megf10 expression and respond to environmental cues that determine whether the cells are to proliferate or differentiate. Under appropriate conditions, Megf10 expression is maintained in the satellite myogenic cells despite surrounding signals to differentiate, the result being a return to quiescence and the subsequent maintenance of the satellite myogenic cell compartment. One possible mechanism by which to test this hypothesis would be to isolate the individual satellite cell populations, infect them with retrovirus designed to stably express Megf10 and inject these cells into regenerating muscle. Previous results from our lab have indicated that while satellite stem cells contribute mainly to both the satellite stem and satellite myogenic compartment, satellite myogenic cells contribute mainly to the regeneration of damaged fibers. Presumably the injected satellite myogenic cells downregulate Megf10 expression, as do primary myoblasts following serum withdrawal. Maintained expression of Megf10 in the injected satellite myogenic cells should result in a return to quiescence rather than terminal differentiation and a subsequent increase in their contribution to the satellite cell compartment. Alternatively, disruption of the Megf10 locus through gene targeting could be employed. In this instance the absence of Megf10 should result in a

substantial decrease in the number of satellite myogenic cells within null individuals. While satellite stem cells should be present, their ability to give rise to quiescent satellite myogenic cells in the absence of Megf10 would be disrupted resulting in depletion of the satellite cell compartment.

Our results clearly demonstrate that overexpression of Megf10 in myogenic cells enhances their proliferation while inhibiting their differentiation. Furthermore, Megf10 is capable of altering the expression of MyoD and Myf5, critical factors in myogenic progression and differentiation. The manner in which it does this has yet to be elucidated. Our results indicate Megf10 impinges on two critical myogenic signaling pathways. Based on previous studies examining the role of Megf10 orthologs we hypothesized that Megf10 would interact with the Rac1/Cdc42 signaling pathway. Through the use of G-protein activity assays we were able to demonstrate increased Rac1 activity in Megf10 overexpressing cells. Interestingly, Rac1 activity has been associated with inhibition of terminal differentiation by activating JNK and inhibiting expression of myogenin, troponin T and MyHC (Meriane et al., 2002; Meriane et al., 2000; Strle et al., 2006). Thus increased Rac1 activity is a possible cause for the inability of Megf10 overexpressing cells to differentiate.

We have also been able to demonstrate an increase in the phosphorylation of MEK1 in Megf10 overexpressing cells. The ability of MEK1 to alter MyoD activity has been previously demonstrated (Perry et al., 2001). It may be that the increased levels of MEK1 phosphorylation lead to alterations in MyoD protein levels by altering MyoD stability. Interestingly Rac1 is able to activate MEKK1 which in turn can lead to increased MEK1 phosphorylation as well as JNK and p38 activation (Minden et al.,

1995; Xu and Cobb, 1997; Xu et al., 1995). Interestingly, phosphorylation of MEK1 by MEKK1 does not lead to ERK activation, a phenomenon similar to that observed in Megf10 overexpressing cells (Xu et al., 1995). Therefore, MEK1 phosphorylation that may lead to altered MyoD, p38 and JNK activation, may be linked through Rac1 activity. This would provide a dual mechanism through which Megf10 signaling can modulate myoblast proliferation and differentiation. Megf10 activation of Rac1 activates MEKK1 that in turn has a dual function in activating JNK1, leading to inhibition of myogenin and MyHC, and phosphorylating MEK1, which in turn alters MyoD activity (Fig 34). Our results clearly demonstrate the activation of Rac1, p38, and JNK as well as increased MEK1 phosphorylation following Megf10 overexpression. The subsequent inhibition of differentiation observed in these cells corresponds to previously obtained results in which activated Rac1, JNK, and MEK are able to inhibit terminal differentiation.

If indeed Megf10 exerts its effects on myogenesis through Rac1 and MEKK1, specific inhibition of Rac1 using NSC23766 (Calbiochem) or siRNAs, or introduction of dominant negative versions of MEKK1 should rescue the differentiation deficit. As well, inhibition of MEK1 using UO126 may help to discern whether or not the increased phosphorylation of MEK1 in Megf10 overexpressing cells is responsible for the decrease in MyoD protein levels. Furthermore, if MEK inhibition rescues MyoD protein levels, it would be possible to determine whether the increased levels of Myf5 transcription are a result of relieving repression by MyoD or if Megf10 expression enhances Myf5 transcription via an alternative mechanism.

Our results clearly demonstrate the ability of Megf10 to activate Rac1 and increase MEK1 phosphorylation, indicating that Megf10 functions as a transmembrane

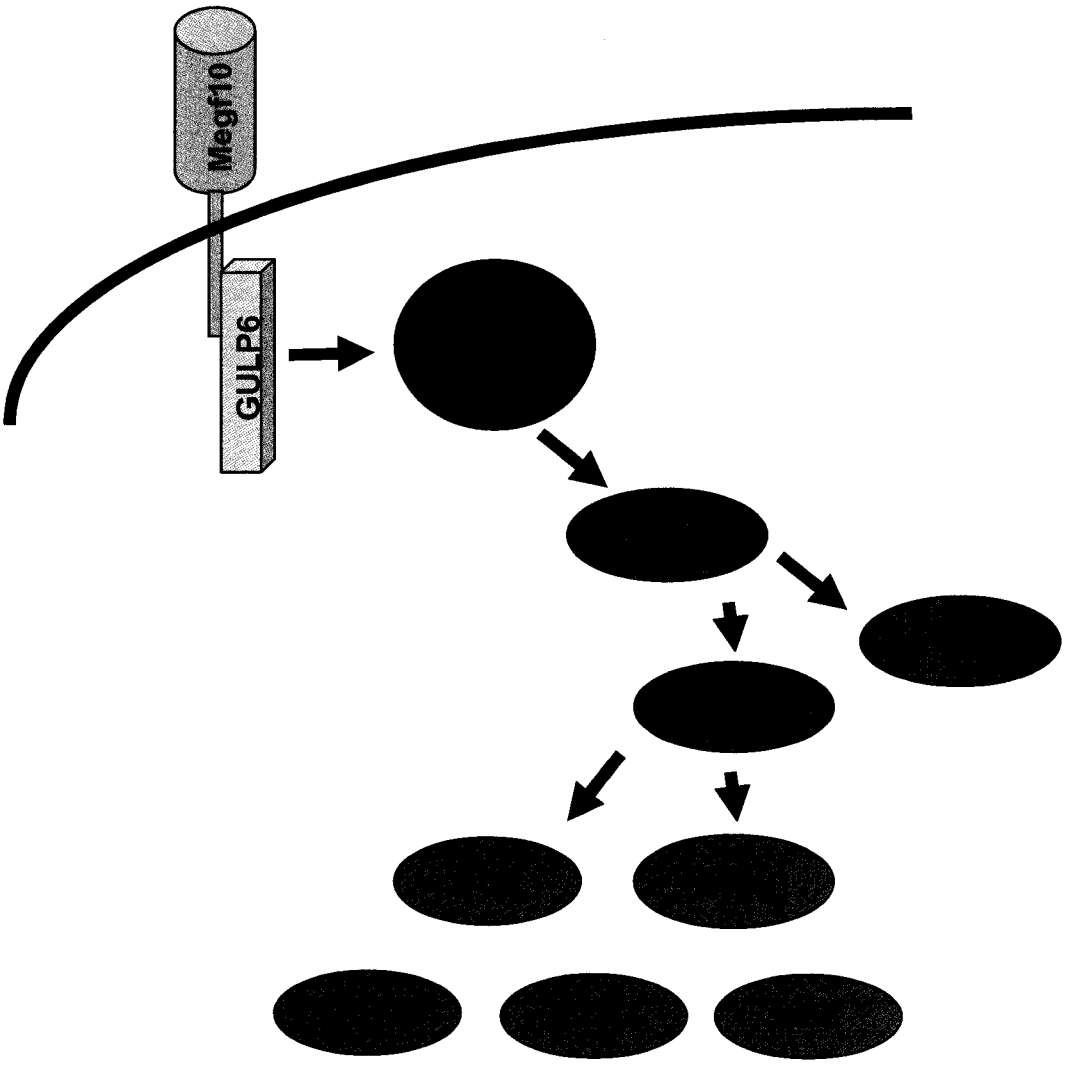

Figure 34. Proposed model of Megf10 Signaling in myogenic cells. Our results clearly indicate that Megf10 impinges on MEK and JNK. It is likely that this is mediate by Rac1 via MEKK signaling. Published results link Megf10 orthologs to Rac1 through the adaptor protein GULP. Megf10 signaling through MEK1 may be responsible for altering MyoD expression which may in turn inhibit terminal differentiation. Megf10 may also inhibit differentiation by maintaining JNK activity following serum withdrawal.

signaling molecule. Experiments in which the cytoplasmic domain of Megf10 were removed abolished the differentiation deficit observed using full length Megf10 and Myf5 and MyoD levels were similar to those observed in wildtype cells indicating a crucial requirement for the cytoplasmic domain of Megf10. Examination of Megf10 and its orthologs reveals the presence of a conserved NPXY motif within the cytoplasmic tail (Callebaut et al., 2003). Previous studies have demonstrated that this domain is crucial for CED-1 function, most likely due to its interaction with CED-6/GULP, an intermediate between CED-1 and Rac1 (Su et al., 2002; Zhou et al., 2001). Disruption of the NPXY domain in Megf10 should result in a disruption of Rac1 activation. If this is the primary mechanism through which Megf10 exerts its effect in myogenic cells, Megf10 with a mutated NPXY motif should function in a manner similar to the Megf10ΔCT used in our experiments. These experiments would help to solidify the mechanism through which Megf10 regulates the myogenic potential of satellite cells.

Recent work has established the ability of satellite cells to return to a quiescent state following activation in order to maintain the quiescent satellite cell pool. Here we describe Megf10 as being expressed in a specific satellite cell population and demonstrate its ability to regulate myogenic progression. This work provides the first insight into a potential mechanism through which committed satellite myogenic cells are able to return to a quiescent state following activation. Identification of Megf10 ligands as well as specific genes targeted through the Megf10 signaling pathway will provide critical insight into the maintenance and commitment of skeletal muscle satellite cells. Furthermore, generation of antibodies to the extracellular domain of Megf10 will provide

a useful tool for the analysis of the distinct satellite stem and satellite myogenic populations.

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APPENDIX A

Appendix A – Megf10 Yeast Two Hybrid Experiments

In order to identify proteins that interact with Megf10 and gain insight into its function, a yeast two-hybrid experiment was initiated. A Gal4DBD/Megf10 extracellular domain fusion was created that does not transactivate the Gal4 reporter or affect the viability of the yeast or their mating efficiency (Fig. 35). Furthermore, the Gal4 fusion protein is detectable in yeast protein extracts by Western blot (Fig. 35). The AH109 strain containing the Megf10 fusion protein was mated with a mouse e17.5 embryo library in the Y187 yeast strain. The mating efficiency of this cross was approximately 90% and the total number of clones screened was 2.1×10^7. Positive clones from the initial screen were picked and re-plated individually to verify transactivation of the Gal4 reporter. DNA was isolated from clones that were verified to be positive and transformed into bacteria. Subsequently, the bacteria were grown under nutritionally selective conditions to isolate the Gal4AD fusion plasmids. Once selected, clones were sequenced and the sequence data was subjected to BLAST analysis as well as verification of open reading frames. Of the 76 clones originally isolated, 50 were eliminated based on the absence of an open reading frame. Of the remaining 26, only 12 showed open reading frames that corresponded to the matching BLAST hits (Table 1).

Of the 12 potential interacting proteins identified, the most interesting is basigin, also referred to as CD147 or EMMPRIN. Basigin is a transmembrane protein implicated in the upregulation of matrix metalloproteinases (MMPs) in a wide variety of cells.

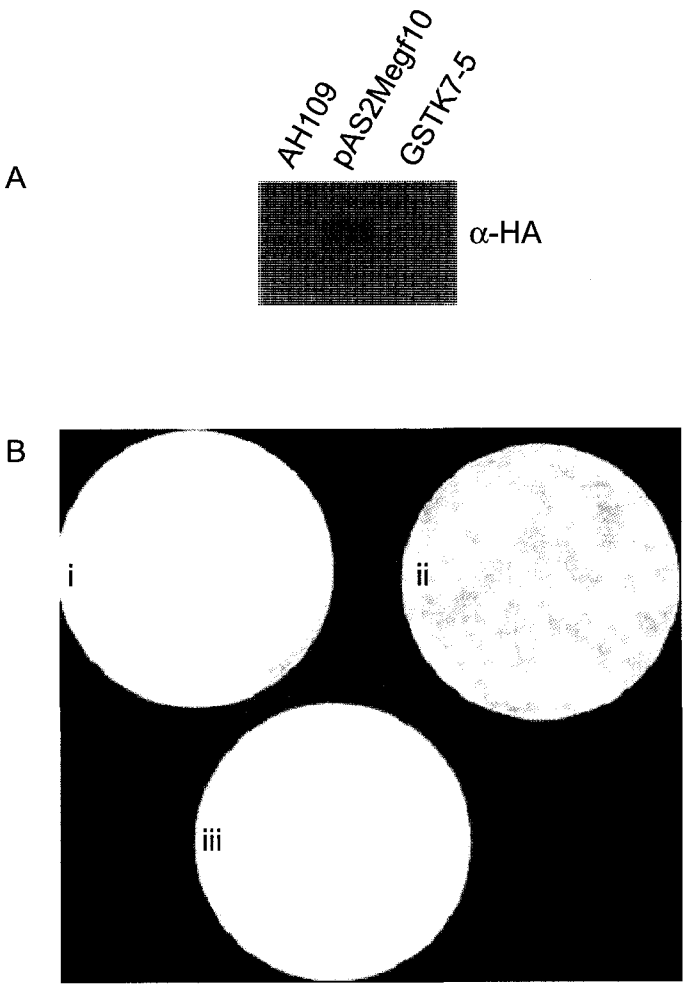

Figure 35. Yeast two-hybrid viablility and transactivation controls. (A) Western blot of protein extracted from untransformed AH109, AH109 transformed with pAS2Megf10 (HA-tagged), and mating strain GSTK7-5 yeast. The HA-tagged Megf10 product is clearly detected in transformed cells. (B) Yeast lifts were performed and subjected to B-galactosidase assays to examine the transactivation potential of Megf10Gal4DBD. The TD1-1 negative control (i) and AH109 transformed with Megf10Gal4DBD (iii) do not transactivate the Gal-4 reporter. TD1-1 crossed with GSTK7-5 was used as a positive control (ii) for B-gal reporter activity.

Basigin is expressed both in muscle tissue *in vivo*, and in C_2C_{12} myoblasts *in vitro*. MMPs play an important role in the break down of the extracellular matrix during wound repair and have also been implicated in increased metastatic ability of tumor cells. One possibility is that Megf10, expressed on the surface of quiescent satellite cells, interacts with basigin expressing macrophages and stimulates the satellite cells to produce MMPs. The upregulation of MMPs may in turn result in the release of satellite cells from the basal lamina and subsequent muscle regeneration.

While I have performed a yeast two hybrid cross using the extracellular domain of Megf10, the group that identified the human homolog, mEGF10 (KIAA1780), has performed a yeast two hybrid using the cytoplasmic domain of Megf10. The library they screened consisted only of clones that had been previously identified in a screen for unidentified human genes. Several of the proteins found to interact with the cytoplasmic domain of Megf10 are known to function in signaling pathways. These include Grb10, translokin, TIMAP, and TAOK2. Grb10 is a member of the Grb7 family of adapter proteins and encodes several functional domains including a pleckstrin homology domain and an SH2 domain (Jahn et al., 2002). Grb10 splice variants have been implicated in signal transduction of growth factors, in particular, the epidermal growth factor receptor, insulin and insulin-like growth factors (O'Neill et al., 1996; Ooi et al., 1995). TAOK2 is a serine/threonine protein kinase of the Ste-20 family. TIMAP is a TGF-β inhibited membrane associated protein that is highly expressed in hematopoietic cells (Cao et al., 2002). Translokin is an intracellular mediator of FGF-2 trafficking and may be required for FGF-2 stimulated proliferation. All of these proteins function in cell signaling in some manner, further supporting a role for Megf10 as a signaling molecule.

Protein ID	Function	Accession	Size (aa)
2610028L19Rik	DNA-directed RNA polymerase	NM_027002	124
actin, alpha 1, skeletal muscle	major component of microfilaments	BC014877	80
albumin 1		BC024643	237
ATP synthase mitochondrial F1 complex gamma 1	hydrogen ion transporter activity	NM_020615	>277
ATPase subunit 6 (Atpase6)	mitochondrial protein	AF093677	89
basigin	proposed receptor of cyclophilin A	XM_125721	252
glycyl-tRNA synthetase (Gars),	charge tRNAs with their cognate amino acids	NM_180678	148
Haymaker (Tomm40)	mitochondrial import receptor	AF316404	145
lamin A	major component of the nuclear lamina	AK004619	164
menage a trois 1 (Mnat1)	assembly factor for CDK7-cyclin H complex	NM_008612	265
methyl-CpG binding domain protein 2 (Mbd2)	bind methylated DNA and repress transcription	NM_010773	261
neuronatin-2	putative transmembrane protein	X83569	124
1110013H04Rik	unknown	BC019521	111
ATP synthase mitochondrial complex isoform 3	hydrogen ion transporter activity	NM_175015	88
calreticulin (Calr)	Ca(2+)-binding chaperone	BC003453	79
HLA-B-associated transcript 1A (Bat1)	nuclear RNA helicase	BC011067	160
nucleophosmin 1 (Npm1)	ribosome biogenesis factor	NM_008722	33
retinoid-inducible serine caroboxypetidase	secreted protein	BC021399	40
ribosomal protein S11		XM_133477	45
serine proteinase inhibitor, clade C member 1	serine proteinase inhibitor	NM_080844	23
succinate-CoA ligase	functions in energy production => glycolysis	AK005080	96
type XVIII collagen (COL18A1)/endostatin	extracellular matrix component	BC043697	36
ubiquitin-conjugating enzyme E2 variant 1	protein modification/degradation	BC003449	30
Pumilio 2 (Pumm2)	sustain mitotic proliferation of stem cells	BC041773	70
zinc finger homeodomain 4 (Zfh4-pending)	zfh family of putative transcription factors	XM_192930	48

Table 1. List of Megf10 interacting proteins identified by yeast two-hybrid. A yeast two-hybrid mouse cDNA library was screened for proteins that interacted with the extracellular domain of Megf10. The identified proteins along with their potential functions and accession numbers are listed. The size of the amino acid open reading frame of the interacting protein is also given.

APPENDIX B

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Muscle satellite cell-specific genes identified by genetic profiling of *MyoD*-deficient myogenic cell

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Abstract

Satellite cells are committed myogenic progenitors that give rise to proliferating myoblasts during postnatal growth and repair of skeletal muscle. To identify genes expressed at different developmental stages in the satellite cell myogenic program, representational difference analysis of cDNAs was employed to identify more than 50 unique mRNAs expressed in wild-type myoblasts and $MyoD^{-/-}$ myogenic cells. Novel expression patterns for several genes, such as *Pax7*, *Asb5*, *IgSF4*, and *Hoxc10*, were identified that were expressed in both quiescent and activated satellite cells. Several previously uncharacterized genes that represent putative MyoD target genes were also identified, including *Pwl*, *Dapk2*, *Sytl2*, and *NLRR1*. Importantly, many genes such as IgSF4, *Neuritin*, and *Klra18* that were expressed exclusively in $MyoD^{-/-}$ myoblasts were also expressed by satellite cells in undamaged muscle in vivo but were not expressed by primary myoblasts. These data are consistent with a biological role for activated satellite cells that induce Myf5 but not MyoD. Lastly, additional endothelial and hematopoietic markers were identified supporting a nonsomitic developmental origin of the satellite cell myogenic lineage.

Keywords: Representational difference analysis; Skeletal muscle; Satellite cell; Regeneration; Pax7; MyoD; Myf5

Introduction

Muscle satellite cells are specialized myogenic progenitors that are activated during postnatal growth and regeneration of skeletal muscle. Consequently, the majority of adult myonuclei are derived from satellite cells following the growth that occurs postnatally (Schultz, 1996). In undamaged adult muscle, most satellite cells are quiescent, contain highly condensed nuclei, and are located beneath the basal lamina of mature muscle fibers (Armand et al., 1983; Mauro, 1961; reviewed by Bischoff, 1994; Hawke and Garry, 2001; Seale and Rudnicki, 2000). In response to a variety of stimuli including exercise, stretching, and injury, satellite cells are activated and give rise to committed myogenic precursor cells (MPCs) that proliferate and differentiate to form new myofibers (Appell et al., 1988; Darr and Schultz, 1987; Grounds and Yablonka-Reuveni, 1993; Rosenblatt et al., 1994; Schultz, 1989; Schultz et al., 1985). Activated satellite cells are also thought to generate progeny that remains undifferentiated, hence restoring the pool of quiescent satellite cells (Bischoff, 1994; Seale and Rudnicki, 2000).

Due to their low abundance in mature muscle (2–5% of sublaminar nuclei), it has been difficult to molecularly investigate early events associated with satellite cell activation. Additionally, there remains a paucity of genetic markers unique to the satellite cell lineage. Many satellite cell markers such as Neural Cell Adhesion Molecule-1 (Ncam1) (Bischoff, 1994), Foxk1 (Garry et al., 1997), c-met (Cornelison and Wold, 1997), and Syndecans 3–4 (Cornelison et al., 2001) are also expressed in other lineages. The characterization of novel genes expressed in satellite cells is essential for elucidating the molecular pathways implicated in their development and function during tissue growth and regeneration.

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Quiescent satellite cells do not express detectable mRNA or protein for any of the myogenic regulatory factors (MRFs) (Cooper et al., 1999; Cornelison and Wold, 1997; Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994). Following activation, satellite cells upregulate either *MyoD* or *Myf5* mRNA before the initiation of DNA synthesis (Smith et al., 1994). RT-PCR experiments of single cells on isolated muscle fibers showed that activated satellite cells first express either *MyoD* or *Myf5* before coexpressing both factors (Cornelison and Wold, 1997). Analysis of regenerating muscle confirmed that myoblasts express either MyoD or Myf5 alone or coexpress both factors (Cooper et al., 1999). These observations suggest that activated satellite cells possess differential biological properties depending upon whether they initially activate *MyoD* or *Myf5*.

Previous work identified a unique requirement for MyoD in the satellite cell lineage (Megeney et al., 1996). Specifically, $MyoD^{-/-}$ muscles display a severe regeneration deficit following crush-induced damage or on a dystrophic (*mdx*) background. Importantly, $MyoD^{-/-}$ muscle contains an increased number of satellite cells suggesting an increased propensity for $MyoD^{-/-}$ satellite cells to self-renew rather than terminally differentiate. Consistent with these findings, satellite cell-derived myoblasts from *MyoD*-deficient muscle display a profound differentiation deficit and an increased growth rate in vitro (Cornelison et al., 2000; Sabourin et al., 1999; Yablonka-Reuveni et al., 1999). Significantly, $MyoD^{-/-}$ myogenic cells express increased levels of Myf5 mRNA and protein (Sabourin et al., 1999), demonstrating the inability of Myf5 to compensate for the absence of MyoD during differentiation.

The phenotype of *MyoD*-deficient adult myoblasts suggested the hypothesis that $MyoD^{-/-}$ myogenic progenitors are similar to activated satellite cells that express Myf5 alone (i.e., Myf5+:MyoD−) and are developmentally upstream of cells that express MyoD (Sabourin et al., 1999; Seale and Rudnicki, 2000). Therefore, cultured $MyoD^{-/-}$ myogenic cells are a unique resource to identify satellite cell-specific mRNAs. In this study, we employed representational difference analysis of cDNAs (Hubank and Schatz, 1994) to identify markers expressed specifically in wild-type primary myoblasts and *MyoD*-deficient myogenic cells. These experiments provide a collection of novel cDNAs whose expression defines different developmental stages in the satellite cell lineage.

Materials and methods

Cell cultures

Primary myoblast cultures were prepared from adult (6–8 weeks old) $MyoD^{-/-}$ (Rudnicki et al., 1992) and Balb/c (Jackson Laboratories) control animals as described previously (Sabourin et al., 1999). Primary low passage (<p6) myoblast cultures derived from multiple (>3) animals were pooled for use in gene expression studies to control for biological variability and to maintain their primary characteristics. Desmin and Myf5 immunoreactivity confirmed that cell cultures were >98% pure myoblasts. Myoblasts were propagated on collagen-coated dishes in Ham's F-10 medium (Invitrogen) supplemented with 20% FBS and 2.5 ng/ml bFGF (Invitrogen). Myoblast cultures were induced to differentiate in DMEM supplemented with 5% horse serum. Mouse embryonic fibroblasts (MEFs) were obtained from E14.5 Balb/c mouse embryos using standard procedures (Robertson, 1987) and maintained as primary cultures in 10% FBS/DMEM. C3H10T1/2 fibroblasts and C2C12 myoblasts were obtained from ATCC and maintained in 10% FBS/DMEM.

Representational difference analysis

Representational difference analysis of cDNAs was performed as described previously (Hubank and Schatz, 1994). Briefly, double-stranded cDNA was digested with the four-cutter *Dpn*II (New England Biolabs) and ligated with R-Bgl-24 adaptors. PCR was used to amplify the cDNA pools before subtractive hybridizations. R-Bgl-24 adaptors were subsequently removed from the cDNA pools, and J-Bgl-24 adaptors were then ligated only to "tester" cDNA pools. For subtractive hybridizations, wild-type myoblast cDNA "tester" was subtracted against MEF cDNA at 1:100 and 1:400 to yield wtDP1 and wtDP2, respectively. DP3 was generated by subtracting DP2 against cDNA prepared from uninjured whole skeletal muscle at a ratio of 1:400. $MyoD^{-/-}$ cDNA tester was subtracted against C3H10T1/2 fibroblast cDNAs at 1:100 and 1:400 to generate mdDP1 and mdDP2, respectively. Final difference products were cloned directly into pCR2.1 (Invitrogen) for sequence analysis.

Affymetrix array analysis

Total RNA was harvested from two independent isolations of low-passage cultures of wild-type myoblasts and $MyoD^{-/-}$ myogenic cells (as described above for RDA analysis). Hindlimb muscles from three 6- to 8-week-old mice were used for each isolate to control for biological variability between animals. Standard Affymetrix protocols were used to yield fluorescently labeled cRNA fragments, which were hybridized to the Mu11k-SubA and SubB GeneChips (Affymetrix). The hybridized GeneChips were scanned, and the raw image files analyzed using Affymetrix Microarray Suite 4.0's empirical algorithm to generate numeric average difference values and qualitative absent/marginal/present call values. The resulting processed data were further examined using Microsoft Excel and Access. Negative and low average difference values produced by the empirical algorithm were arbitrarily reassigned threshold values of 50 for purposes of calculating fold changes. The average fold change value used to compare the $MyoD^{-/-}$ data against

the wild-type data was calculated as ratios of the means of the replicates.

RNA isolation and synthesis of double-stranded cDNA

Total RNA was prepared from cell cultures and tissues using GIT method as previously described (Birnboim, 1988) or by using Qiagen RNeasy kits for Affymetrix profiling experiments. PolyA+ mRNAs were prepared by two rounds of selection with oligo d(T) cellulose (Amersham Bioscience). Double-stranded cDNAs were generated using the Universal RiboClone cDNA synthesis kit (Promega). The yield of double-stranded cDNA was determined by radioactive monitoring of first and second strand synthesis reactions.

Expression analysis of RDA clones

Northern blot studies were performed according to standard techniques using random-primed ^{32}P-dCTP radiolabeled RDA products as probes (Maniatis et al., 1982). Total RNA (15 μg) from various tissues and cell lines was electrophoresed in denaturing Formaldehyde gels and transferred to Hybond-N filters (Amersham Bioscience). In situ hybridizations were performed on 10-μm cryosections of mouse TA muscles from 8-week-old wild-type or 3-week-old *mdx* mice (Jackson Laboratories) according to previously described procedures (Braissant and Wahli, 1998). Sense and antisense in situ probes were synthesized from RDA products using the DIG labeling mix (Roche) with SP6 or T7 RNA polymerase (Roche). Alkaline phosphatase-conjugated anti-DIG antibody (Roche) followed by reaction with BCIP/NBT (Roche) was used to detect hybridized cRNA probes.

Immunohistochemistry was performed on paraformaldehyde (PFA)-fixed, 10-μm cryosections using goat anti-Vcam1 antibody (Santa Cruz), followed by staining with a biotin-conjugated secondary antibody (Zymed) and streptavidin-HRP (Zymed). Immunoreactive cells were visualized using aminoethyl carbazole (AEC) substrate (Sigma). Immunohistochemistry on cultured cells was performed by fixation with 4% PFA for 5 min, followed by permeabilization with 0.5% Triton X-100 for 5 min. Cells were incubated with primary antibodies against Desmin (DAKO), Pax7 (Developmental Studies Hybridoma Bank), Myf5 (C20, Santa Cruz), β-Gal (Molecular Probes), and Vcam1 (Santa Cruz). Secondary detection was performed with FITC- or HRP-conjugated antibodies (Sigma).

Results

Expression profiling of satellite cell-derived myoblasts

Representational difference analysis of cDNAs (cDNA RDA) was employed to generate libraries of expressed cDNAs in primary myoblasts from wild-type muscle. cDNAs from wild-type primary myoblasts were subtracted sequentially against mouse embryonic fibroblast (MEF) cDNAs at ratios of 1:100 and 1:400 to obtain the first and second difference products (wtDP1 and wtDP2), respectively (Fig. 1A). To refine the products further and eliminate markers of terminal myocyte differentiation, including structural genes (e.g., *myosins*, *dystrophin*, etc.), an additional subtractive step against cDNAs from whole adult skeletal muscle (1:400) was performed to generate wtDP3. As expected, the complexity of the wild-type myoblast cDNA mixture was progressively reduced in wtDP1 and wtDP2, resulting in the appearance of several distinct cDNA products in wtDP3 (Fig. 1B).

A similar strategy was used to identify genes responsible for the behavior of *MyoD*$^{-/-}$ satellite cells including their increased capacity for self-renewal (Megeney et al., 1996; Sabourin et al., 1999). *MyoD*$^{-/-}$ myoblast cDNAs were subtracted twice against cDNAs from C3H10T1/2 fibroblasts (at 1:100 and 1:400) to generate mdDP1 and mdDP2, respectively (Fig. 1C). C3H10T1/2 cDNA was used in this screen to avoid losing genes coexpressed by activated *MyoD*$^{-/-}$ myogenic cells and primary multipotent MEFs. After two rounds of subtraction, mdDP2 contained several distinct cDNA species (Fig. 1D).

Pools of cDNA from wtDP3 and mdDP2 were cloned into pCR2.1 (Invitrogen). To recover lower abundance difference products, 400 individual clones from both subtracted wild-type and *MyoD*$^{-/-}$ libraries were screened. Dot-blots consisting of PCR-amplified RDA products were hybridized with mixtures of labeled clones to identify redundant sequences. After dot-blot and sequence analysis, 18 difference products were identified from wild-type myoblasts and 34 difference products from *MyoD*$^{-/-}$ myogenic cells (Table 1). Comparison of cDNA sequences to database entries in GenBank established the identity of cloned products (accession numbers provided in Table 1).

Expression analyses of RDA products from wild-type primary myoblasts

From reverse Northern blot experiments, 51 of the 52 genes identified were differentially expressed in the starting pools of amplified cDNAs (data not shown), thus validating the efficacy of RDA subtractions. The expression profile of 40 genes during primary adult myoblast differentiation was assessed by Northern blot analysis as outlined in Table 1. The expression patterns for the remaining 11 clones were not determined because of technical difficulties in obtaining nonrepetitive cDNA probes or due to the identity of the cDNA as a gene involved in protein translation, mitochondria, or metabolism that was not the focus of this study (see supplementary material for information).

Northern analysis revealed that *Pax7*, *L-myc*, *Pb99*, and *Asb5* were expressed in proliferating wild-type and *MyoD*$^{-/-}$ myoblasts with no upregulation observed during myotube differentiation. As previously demonstrated, *Pax7* was

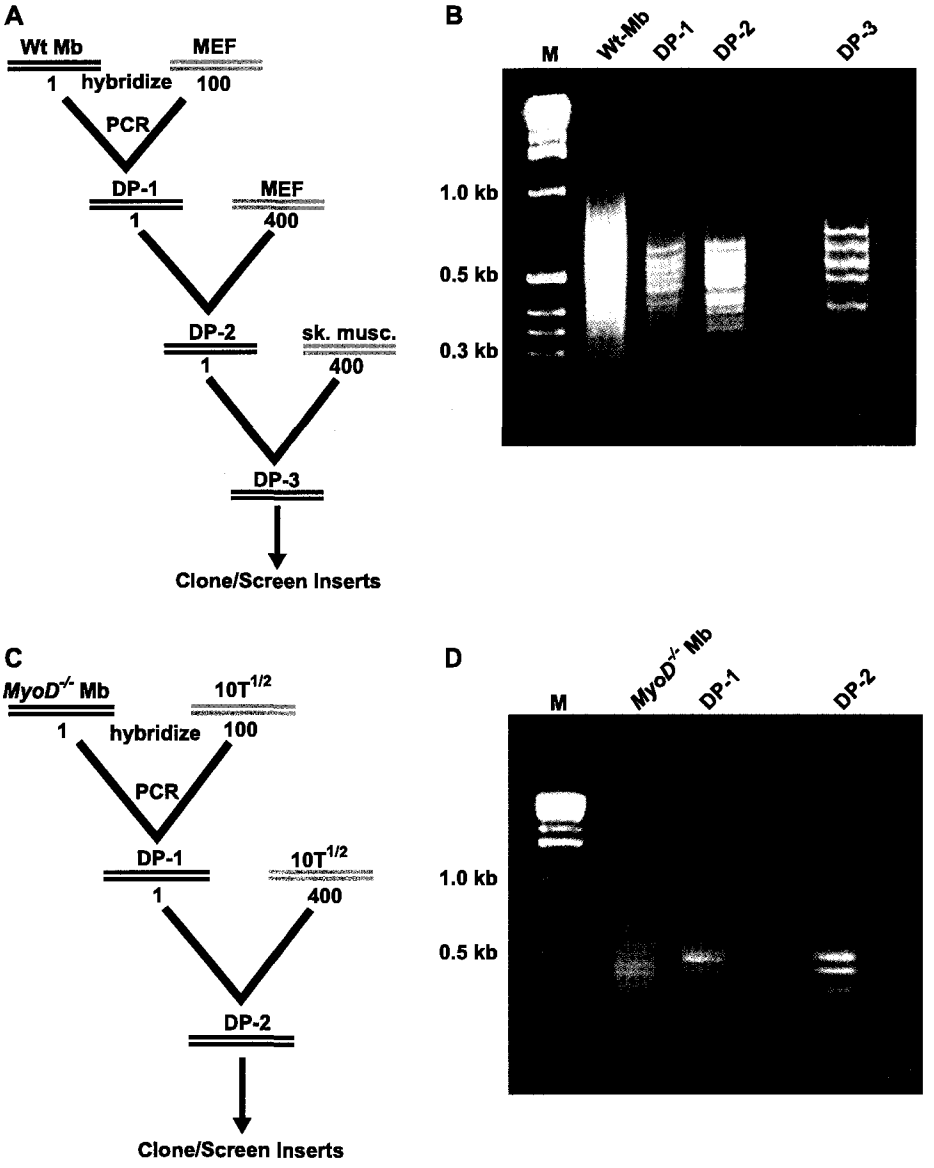

Fig. 1. Experimental strategy for identifying myoblast-specific mRNAs. (A) Wild-type myoblast cDNAs were subtracted against cDNAs prepared from mouse embryonic fibroblasts (MEF) and whole adult skeletal muscle (sk. musc) to generate the third difference product (DP-3). Ratios used for hybridizations are indicated. (B) Agarose gel electrophoresis of the starting pool of wild-type myoblast (Wt-Mb) cDNAs and subtracted DP-1, DP-2, and DP-3 showed a progressive enrichment of specific cDNA molecules. (C) *MyoD*$^{-/-}$ myogenic cell-specific cDNAs were cloned after subtraction against C3H10T1/2 cDNAs. (D) Agarose gel analysis of the starting *MyoD*$^{-/-}$ myogenic cell cDNA pool (*MyoD*$^{-/-}$Mb), DP-1, and DP-2 revealed the appearance of distinct cDNAs in DP-2.

expressed in proliferating wild-type and *MyoD*$^{-/-}$ myoblasts but rapidly downregulated upon differentiation (Seale et al., 2000). *Pb99*, a gene that encodes a seven-pass transmembrane protein was expressed exclusively in undifferentiated myoblast cultures (Fig. 2A). Analogous to *Pax7*, *Pb99* was not detected in total RNA from a panel of cell lines or mouse tissues (Fig. 2A). Furthermore, in C2C12 myoblasts, *Pb99* mRNA was downregulated within the first day of differentiation (data not shown).

Asb5, encoding an Ankyrin-repeat SOCS box containing protein, was expressed at similar levels in proliferating and differentiating myogenic cells including wild-type, *MyoD*$^{-/-}$, and C2C12 myoblasts (Fig. 2A). *Asb5* mRNA was detected in total RNA from adult skeletal muscle but was not expressed in other adult mouse tissues (Fig. 2A). These data therefore suggest previously unrecognized roles for Pb99 and Asb5 in the myogenic satellite cell lineage.

About 10% of cells in cultures of primary growing myoblasts express myosin heavy chain and are presumed to represent a basal level of differentiation (Sabourin et al., 1999). Accordingly, several RDA clones identified from cultures of wild-type myoblasts were defined as differ-

Table 1
Summary and expression of RDA clones

Clone	Title	GenBank	Expression by Northern Blot				Comments/Reference
			Wt-Mb	Wt-D	MD-/- M	MD-/- D	
Transcription Factors							
* MD p286	Hoxc10	NM_017409	++++	++	++++	++++	Expressed in regenerating limbs of Axolotls (Carlson et al., 2001).
dp3-7	Pax7	AF254422	++++	-	++++	-	Paired-box transcription factor required in the development of muscle satellite cells (Seale et al., 2000).
wt-73	Lmyc1	X13945	+	+	+	+	bHLH-Leucine Zipper protein
dp3-9	Myogenin	D90156	++	++++	-	++	Muscle-specific transcription factor that regulates differentiation (Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994).
Receptors and Integral Membrane Proteins							
MD 1	Tcrb-V13	BC034887	+/-	-	+++	-	T cell receptor β, variable 13.
* MD p12	Neuritin-1	BC035531	-	-	++++	-	Expressed exclusively in neural tissue and *MyoD*$^{-/-}$ skeletal myoblasts. GPI- membrane anchored protein that promotes neurite outgrowth (Naeve et al., 1997).
* MD 41	Itm2a	L38971	+	++	++	++++	Integral membrane protein also expressed in chondrogenic progenitors (Deleersnijder et al., 1996).
* MD p259	Klra18	NM_053153	-	-	+++	+++	Killer cell lectin like receptor-18. Cell surface molecule expressed in pre-B cells and T cells (Chan and Takei, 1989).
* wt-30	Pb99	AF249738	++++	-	++++	-	Seven-pass transmembrane protein expressed in pre-B cells (Sleckman et al., 2000).
MD p67	similar to MEGF10	XM_140362	++	-	+++	-	Multiple EGF repeat containing protein.
MD p31	Ptk7	NM_175168	+++	++	+++	++	Protein tyrosine kinase-7 belongs to a novel subclass of receptor tyrosine kinases (Park et al., 1996).
* dp3-1	Lrrn1	D45913	++	+++	+/-	++	Neuronal Leucine Rich Region-1. Expressed in developing nervous system (Taguchi et al., 1996).
dp3-8	Chrnd	BC052153	+	++++	-	++	Cholinergic receptor, nicotinic, delta polypeptide.
* wt-23	Chrna1	NM_007389	+	++++	-	+	Cholinergic receptor, nicotinic, alpha polypeptide-1.
* wt-141	Sytl2	NM_031394	+	+++	-	+	Synaptotagmin-like-2. Contains Slp-homology domain (SHD).
Adhesion/Extracellular Matrix Proteins							
MD 42	Cdh6	NM_007666	+	-	+++	-	Cadherin-6. Cell adhesion molecule expressed in hematopoietic cells and motorneurons (Marthiens et al., 2002; Mbalaviele et al., 1998).
* MD 44	Lama5	XM_203796	+	+	++++	++	Laminin-α5. Adhesive protein for hematopoietic cells (Gu et al., 1999). Expressed in developing and dystrophic muscle (Ringelmann et al., 1999; Sorokin et al., 1997)
* MD p3	Igsf4a	NM_018770	-	-	+++	-	Immunoglobulin superfamily-4. Similar to Synaptic cell adhesion molecule-1 (Biederer et al., 2002)
* MD p168	Vcam1	BC029823	+	-	++++	-	Vascular Cell Adhesion Molecule-1. Muscle satellite cell marker (Rosen et al., 1992).
MD p18	Itgβ4	L04678	++++	++	++	-	Integrin-α6β4 binds components of the extracellular matrix; and is implicated in adhesion and proliferation (Murgia et al., 1998).
MD p16	Itgα7	NM_008398	+	++++	++	++++	Integrin-α7, marks satellite cells (Blanco-Bose et al., 2001; LaBarge and Blau, 2002).

(continued on next page)

Table 1 (*continued*)

Clone	Title	GenBank	Expression by Northern Blot				Comments/Reference
			Wt-Mb	Wt-D	MD-/- M	MD-/- D	
Structural/Cytoplasmic Proteins							
MD p39	Nestin	NM_016701	++	+++	++	++	Intermediate filament protein expressed in myoblasts and regenerating muscle (Carlsson et al., 1999; Vaittinen et al., 2001).
dp3-3	Scga	NM_009161	++	++++	+/-	+/-	α-sarcoglycan, Dystrophin associated glycoprotein. Mutations cause limb girdle muscular dystrophy (Duclos et al., 1998; Liu et al., 1997).
MD p26	Caldesmon	BC019435	+	++++	++	++++	Functions in stabilization of microfilament networks (Matsumara and Yamashiro, 1993).
dp3-13	Tnnt1	NM_011618	+	+++	-	+	Troponin T1 slow. Expressed in differentiated muscle.
Cell Cycle/Apoptosis							
* MD p42	Mcm6	D86726	+	-	+++	+	Cdc21 binding protein expressed in proliferating intestinal crypt cells (Kimura et al.,1996; Sykes and Weiser, 1995).
* MD p123	GoS2	NM_008059	+	+	+++	+	G0/G1 switch gene-2. Target for BMP signals in mesenchymal cells (Bachner et al., 1998).
MD p249	Peg3 (Pw1)	AF038939	+++	n/d	+/-	n/d	Expressed in mesoderm and differentiated muscle (Relaix et al., 1996). Implicated in p53-dependent apoptosis (Coletti et al., 2002).
wt-18	Dapk2	XM_134847	+	++++	-	+	Death-associated protein kinase 2. Calcium/calmodulin regulated kinase implicated in apoptosis (Kawai et al., 1998).
Signaling/Secreted Proteins							
* MD 52	Plgf	BC016567	++	-	++++	+++	Placenta derived growth factor. Closely related to VEGF. Promotes growth of early hematopoietic cells (Luttun et al., 2002).
* wt-17	Asb5	AF398966	++	++	++	++	Ankyrin repeat SOCS-box protein-5. Expressed in satellite cells (Boengler et al., 2003).
MD p158	PAI-2	AJ000386	++	-	++	-	Plasminogen activator inhibitor-2. Plasmin activity is required for muscle regeneration (Lluis et al., 2001; Suelves et al., 2002).
Miscellaneous and Unknown mRNAs							
MD p40	Unknown	XM_129466	-	-	++	-	Putative G-protein coupled receptor.
MD p87	Unknown	BC020031	+/-	+	++	+	Contains CAG repeat region.
9 clones	Neomycin		-	-	+++	+++	Expressed highly from PGK1 promoter in *MyoD*-deficient cells.
MD 62	Unknown		+	++	+	++	Contains S100 calcium binding domain.
MD p35	Unknown	AK008210	+	++	+	++	Putative Androgen-induced protein
MD p11	H19 mRNA	BC025150	++	++++	+	++	Untranslated and imprinted mRNA. Expressed during myoblast and ES cell differentiation (Bartolomei et al., 1993; Leibovitch et al., 1995).
* dp3-2	Unknown	BC024685	+/-	+++	-	+	Hypothetical protein
* dp3-5	Unknown	XM_132832	+	+++	-	-	Hypothetical protein similar to Arachidonate 5-Lipoxygenase. Not expressed in *MyoD*-deficient cells.

*Northern blots are shown in Figures 2 and 3.

mRNAs expressed at elevated levels in $MyoD^{-/-}$ cells relative to wildtype cultures

mRNAs upregulated during myoblast differentiation and myotube formation

References for the RDA clones: Bachner et al., 1998; Bartolomei et al., 1993; Carlsson et al., 1999; Chan and Takei, 1989; Coletti et al., 2002; Deleersnijder et al., 1996; Duclos et al., 1998; Gu et al., 1999; Kawai et al., 1998; Kimura et al., 1996; Leibovitch et al., 1995; Liu et al., 1997; Lluis et al., 2001; Luttun et al., 2002; Marthiens et al., 2002; Matsumura and Yamashiro, 1993; Mbalaviele et al., 1998; Murgia et al., 1998; Park et al., 1996; Relaix et al., 1996; Ringelmann et al., 1999; Sorokin et al., 1997; Suelves et al., 2002; Sykes and Weiser, 1995; Taguchi et al., 1996; Vaittinen et al., 2001.

entiation specific (Fig. 2B and hatched rows in Table 1), including *Dapk2*, *Lrrn1*, *Sytl2*, *α-sarcoglycan*, *myogenin*, *troponin T1 slow*, and three unknown genes. Interestingly, these genes were all expressed at lower levels in $MyoD^{-/-}$ cells, consistent with a requirement for MyoD during adult myoblast differentiation (Cornelison et al., 2000; Sabourin et al., 1999; Yablonka-Reuveni et al., 1999). *Dapk2*, *Lrrn1*, *Sytl2*, and the unknown genes remain to be studied

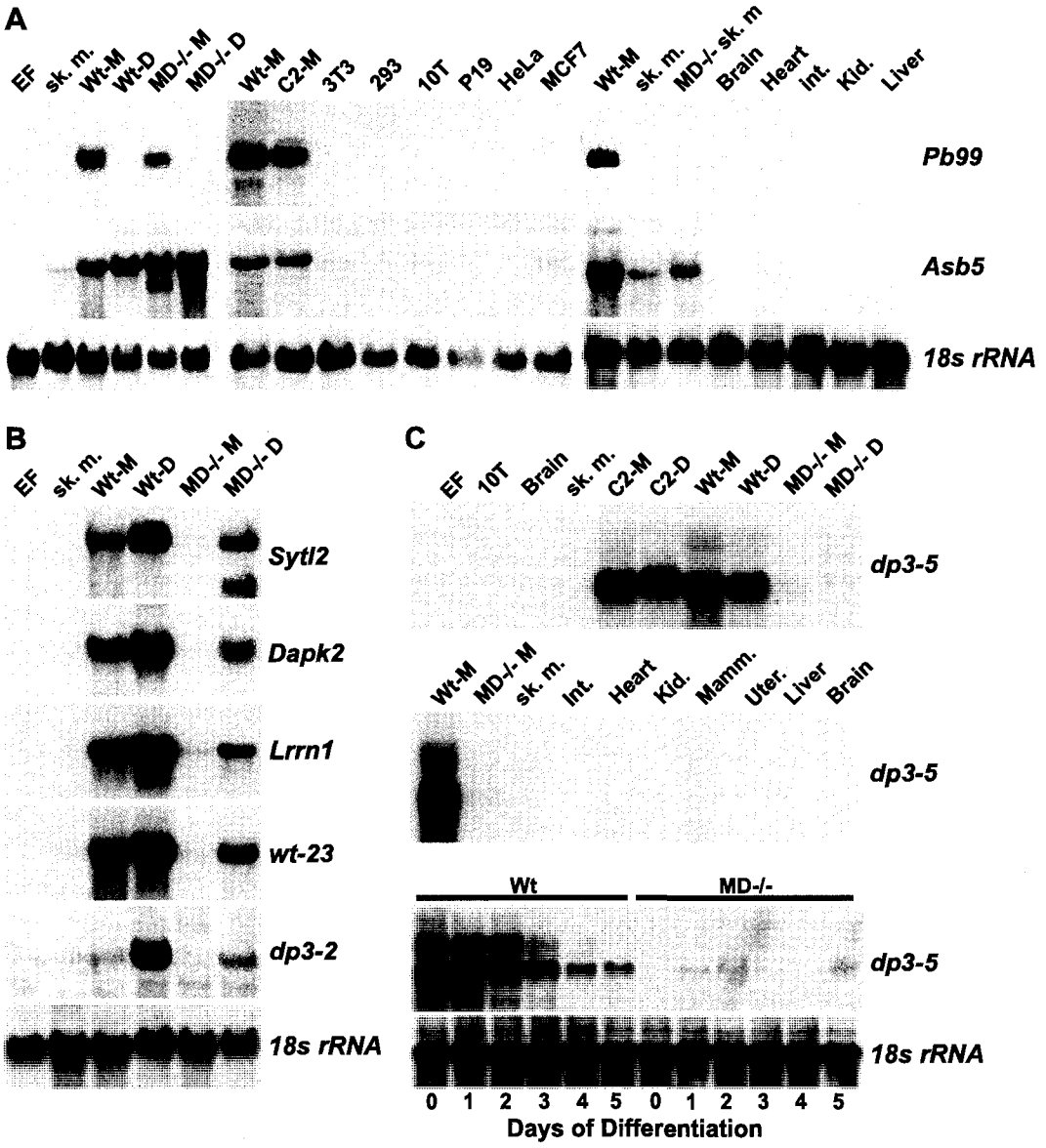

Fig. 2. Expression analysis of wild-type myoblast specific-genes. (A) *Pb99* and *Asb5* were specifically expressed in proliferating wild-type (Wt-M), *MyoD*$^{-/-}$ (MD$^{-/-}$M), and C2C12 (C2-M) myoblasts. *Asb5* was also expressed after 3 days of differentiation (Wt-D and MD$^{-/-}$D). *Asb5* mRNA was also detected in skeletal muscle tissue (sk. m). *Pb99* and *Asb5* mRNAs were not detected in a panel of cell lines. (B) *Sytl2*, *Dapk2*, *Lrrn1*, and two unknown genes (wt-23 and dp3-2) were upregulated during myogenic differentiation in wild-type (Wt) myoblasts. Reduced expression levels were observed in differentiating *MyoD*$^{-/-}$ (MD−/−) cultures. (C) Unknown gene, dp3-5 was specifically expressed in proliferating and differentiating wild-type and C2C12 (C2) myoblasts. However, dp3-5 was not expressed in *MyoD*$^{-/-}$ cultures either during growth conditions (day 0) or throughout differentiation (days 1–5). *18s rRNA* was used to control for loading.

in the context of myogenesis and may play important roles in the differentiation process downstream of MyoD.

Clone *dp3-5*, an unknown gene related to *Arachidonate 5'-lipoxygenase*, was highly expressed in wild-type and C2C12 myoblasts during proliferation and differentiation (Fig. 2C). Furthermore, expression of *dp3-5* was not detected in total RNA isolated from a panel of adult mouse tissues (Fig. 2C). Strikingly, *dp3-5* mRNA was completely absent in proliferating as well as differentiating *MyoD*$^{-/-}$ myoblasts (Fig. 2C). These data therefore suggest that this novel gene is induced downstream of MyoD in skeletal myoblasts.

Expression analyses of MyoD$^{-/-}$ *RDA clones*

Transcriptional profiling of *MyoD*-deficient myogenic cells was employed to identify markers expressed by a more primitive myogenic progenitor. Northern analyses revealed several genes expressed at higher levels in *MyoD*$^{-/-}$ cultures relative to their wild-type counterparts (see gray-shaded rows in Table 1). Importantly, the established satellite cell markers *Vcam1* (Fig. 3A) and *Integrin-α7* (not shown) were expressed at higher levels in *MyoD*$^{-/-}$ cells compared to wild-type primary myoblasts and C2C12

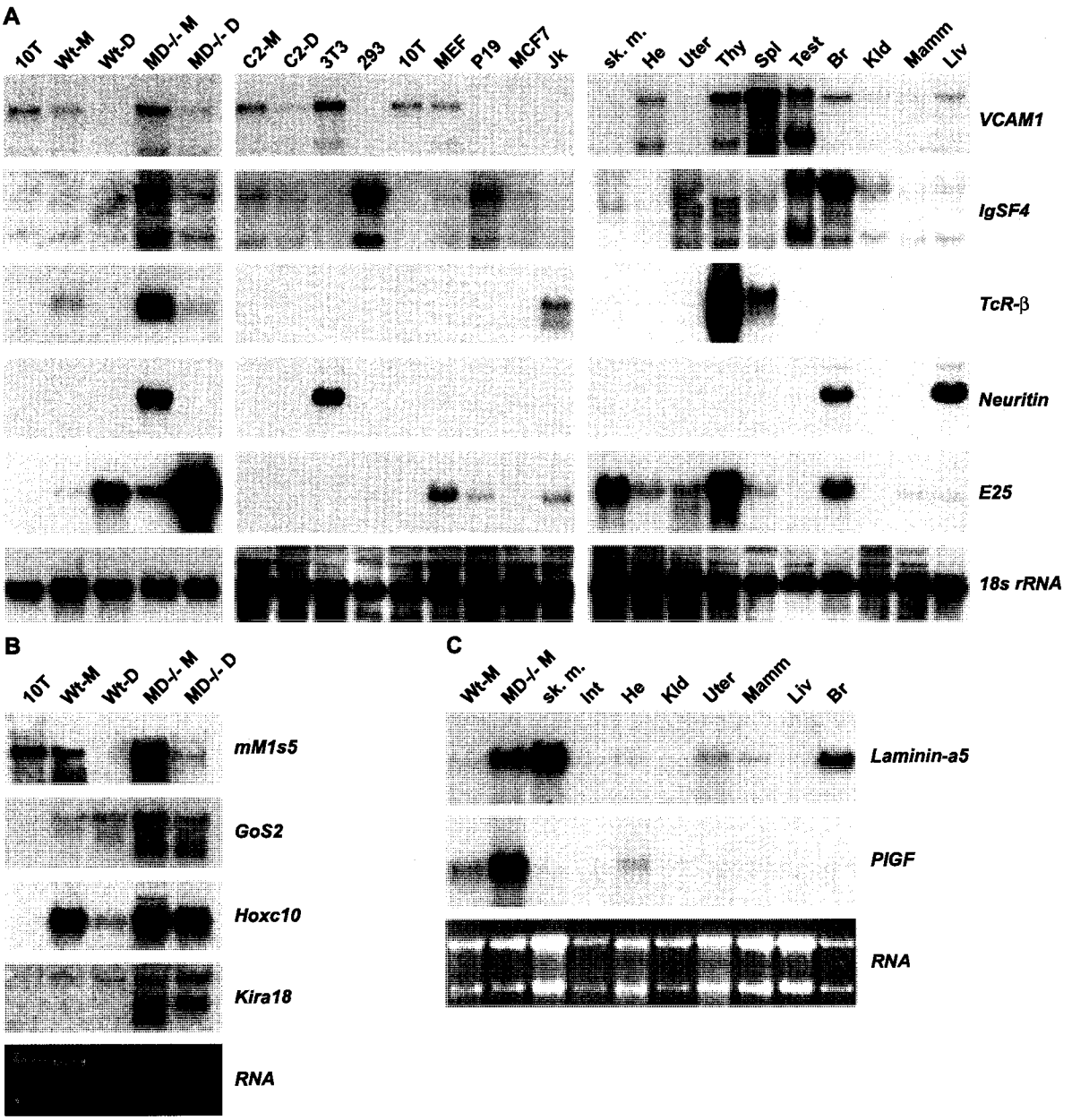

Fig. 3. Expression analysis of $MyoD^{-/-}$-specific genes. (A) *Vcam1* was highly expressed in $MyoD^{-/-}$ (MD−/−M) myogenic cells with low levels detected in wild-type myoblasts. *Vcam1* transcripts were also detected in various cell cultures including MEFs and C2C12 myoblasts (C2-M). *IgSF4* was expressed in $MyoD^{-/-}$ myoblasts, 293 cells, P19 cells, as well as brain (Br) and testis (Test), but not in wild-type myoblasts. *TcR-β* was expressed at surprisingly high levels in $MyoD^{-/-}$ cells as well as the Jurkat T cell line (Jk), Thymus (Thy), and Spleen (Spl). *Neuritin* mRNA was only detected in $MyoD^{-/-}$ myoblasts, NIH 3T3 fibroblasts, brain, and liver. *Itm2a* was expressed at dramatically higher levels in $MyoD^{-/-}$ compared to wild-type myoblasts, with a marked upregulation in differentiating cells. The *Itm2a* transcript was not detected in C2C12 myoblasts. *18s rRNA* was used as loading control. (B) *Mcm6*, *G_0S2*, and *Hoxc10* were all expressed at higher levels in $MyoD^{-/-}$ (MD−/−M) relative to wild-type myoblasts (Wt-M). *Klra18* mRNA was only detected in $MyoD^{-/-}$ myoblasts. Ethidium-stained RNA samples show loading. (C) *Laminin-α5* and *PlGF* were highly expressed in $MyoD^{-/-}$ myoblasts with low levels detected in wild-type cells. *Laminin-α5* mRNA was readily detected in skeletal muscle and brain tissue, while PlGF was expressed at low levels in heart. Ethidium-stained RNA shows loading.

myoblasts (Blanco-Bose et al., 2001; LaBarge and Blau, 2002; Rosen et al., 1992).

RDA facilitated the identification of additional genes expressed in satellite cells. *Immunoglobulin Superfamily-4* (*IgSF4*) was expressed at high levels in $MyoD^{-/-}$ cells, with a complete absence of expression in wild-type primary myoblasts and C2C12 myoblasts (Fig. 3A). *IgSF4* transcripts were also detected in 293 and P19 cells as well as several adult tissues. Similarly, *Neuritin* was expressed in $MyoD^{-/-}$ myogenic cells and not primary or C2C12 myoblasts. *Neuritin* transcripts were also observed in NIH 3T3 fibroblasts, brain, and liver (Fig. 3A).

Surprisingly, *T-cell-Receptor-β* (*TcR-β*) was highly expressed by *MyoD*$^{-/-}$ myogenic cells, with very low levels detected in wild-type myoblasts (Fig. 3A). Similarly, *Placenta Growth Factor* (*PlGF*) was expressed at high levels by *MyoD*$^{-/-}$ myogenic cells relative to wild-type myoblasts (Fig. 3C). Northern analysis of *Laminin-α5* demonstrated expression of the transcript in *MyoD*$^{-/-}$ myogenic cells, adult skeletal muscle, and brain (Fig. 3C). *Hoxc10*, *Killer-cell Lectin Receptor* (*Klra18*), *Mcm6*, *G$_0$/G$_1$ switch gene-2* (G$_0$S2), and *Caldesmon* also displayed elevated expression levels in *MyoD*$^{-/-}$ myogenic cells relative to wild-type cultures (Fig. 3B and gray shaded rows in Table 1).

Several genes cloned by RDA from activated *MyoD*$^{-/-}$ satellite cells were also expressed at similar or higher levels in wild-type myoblasts and throughout differentiation. *Protein tyrosine kinase-7* (*PTK7*), *Plasminogen activator inhibitor-2* (*PAI2*), and a novel EGF repeat containing gene similar to *MEGF10* (MD p67) were expressed at high levels in proliferating wild-type and *MyoD*$^{-/-}$ myogenic cells and downregulated during differentiation (see Table 1). *Integrin β4* exhibited elevated expression in wild-type compared to *MyoD*-deficient cells and was downregulated upon differentiation (see Table 1). *H19* mRNA, *Nestin*, and two unknown genes (MD p35 and MD 62) are examples of genes cloned from *MyoD*$^{-/-}$ cells that were induced upon myogenic differentiation (see Table 1). These results have thus identified a significant number of novel genes that are implicated in various stages of satellite cell myogenesis.

Transcriptional profiling by Affymetrix arrays

GeneChip microarray analysis was used to examine the expression patterns of genes identified by the RDA protocol. Seven of fourteen genes (7/14; 50%) identified in the wild-type myoblast RDA and 20 of 26 genes (14/26; 54%) identified in the *MyoD*$^{-/-}$ RDA experiments were represented in Mu11K probesets. The primitive, undifferentiated state of the *MyoD*$^{-/-}$ myoblasts is well illustrated by the expression patterns of muscle-specific genes identified in the wild-type myoblast RDA screen including *Troponin T1 slow*, *Chrna1*, *Lrrn1*, and *Dapk2* (Table 2). These findings are consistent with the Northern blot data summarized in Table 1.

Similarly, expression of *MyoD*$^{-/-}$ RDA-identified genes corroborates the qualitative Northern blot assessments showing that certain genes such as *Vcam1*, *Laminin-α5*, and *Plgf* are expressed at markedly higher average levels in *MyoD*$^{-/-}$ vs. wild-type myoblasts (see Table 2). A subclass of cDNAs identified in the *MyoD*$^{-/-}$ RDA screen including *H19*, *Integrin-β4* (Itgb4), *Nestin*, and *Peg3* was expressed at elevated levels in wild-type myoblasts relative to *MyoD*-deficient cells (Table 2). These genes are thought to be muscle-specific genes that were not represented in the wild-type myoblast RDA screen because of their expression in skeletal muscle tissue that was used in the subtraction procedure (see Fig. 1).

Table 2
Expression of RDA candidates by Mu11K GeneChip

Gene	GenBank	Avg. Diff./Call[a]		wt/MD
		wt	MD	Fold
Wild-type myoblast RDA candidates				
Chrna1	X03986	19325P	191A	101.4
Tnnt1	W08218	3608P	50A	72.2
Lrrn1	D45913	2919P	50A	58.4
Lmyc1	X13945	257P/A	50A	5.1
Chrnd	L10076	199P/A	50A	4.0
Dapk2	W82116	149P/A	50A	3.0
Myog	D90156	1837P	1319M/A	1.4
MyoD$^{-/-}$ *myoblast RDA candidates*				
Informative				
Vcam1	X67783	1037P	4612P	−4.4
Lama5[b]	U37501	182P/A	731P	−4.0
		596P	1653P	−2.8
Pgf	X80171	799P	1704P	−2.1
Itm2a	L38971	556P	981P	−1.8
Tcrb-V13[b]	X00619	8541P	14345P	−1.7
	AA608090	50A	50A	1.0
Muscle specific				
Mcm6	D86726	171P	230P	−1.3
G0s2[b]	AA036037	450A	593P/M	−1.3
	X95280	762P	610P	1.3
Nes[b]	AA057994	9100P	7427P	1.2
		7453P	4225P	1.8
H19[b]	X58196	12594P	3884P	3.2
		10124P	3062P	3.3
Itgb4	L04678	1244P	212P/A	5.9
Peg3[b]	U48804	2386P	175P/A	13.7
	AA172673	10315P	599P/A	17.2
Uninformative				
Ptk7	W46016	178P	218A	−1.2
Cdh6	D82029	69A	85A	−1.2
Itga7	L23423	50A	50A	1.0

[a] Call = (P)resent, (M)arginal, (A)bsent.
[b] Two distinct probesets were associated with these genes.

Several genes identified by RDA had low average difference values or "Absent" calls on the GeneChips (e.g., *Cadherin6*, *Integrin-α7*), highlighting the utility of the RDA procedure for sensitive detection of differential but low-level expression that may not be effectively detected by the probesets on the microarray. In summary, the Affymetrix array data are consistent with most of the expression data derived from Northern blot studies of clones identified in our RDA screens. We have thus provided a more complete listing of all the genes that were identified in the Affymetrix Mu11K hybridizations as having a consistent >2-fold change after pair-wise comparisons (Supplemental Table S1).

MyoD$^{-/-}$*–specific transcripts are expressed by satellite cells in vivo*

To determine whether the genes expressed predominantly by *MyoD*$^{-/-}$ myogenic cells were also expressed in

uninjured wild-type muscle and regenerating *mdx* muscle in vivo, immunohistochemistry and in situ hybridization studies were employed. Immunohistochemistry revealed expression of Vcam1 in *MyoD*$^{-/-}$ cells where it was concentrated at specific regions in the plasma membrane and cytoplasm (Fig. 4A). Moreover, Vcam1 was expressed in satellite cells as well as monocuclear interstitial cells in uninjured muscle (Figs. 4B and C).

IgSF4 mRNA was expressed in 2–3% of nuclei associated with uninjured wild-type muscle fibers (Figs. 5A and B), with increased expression in regenerating areas of *mdx* muscle (Figs. 5C and D). This result suggests that *IgSF4* is specifically expressed in quiescent satellite cells and their activated descendents. Transcripts for *Neuritin* were also detected in association with approximately 10% of nuclei in uninjured wild-type muscle inside as well as outside the basal lamina of muscle fibers (Fig. 5E). As with *IgSF4*, expression of *Neuritin* was markedly increased in regenerating *mdx* muscle (Fig. 5F). The satellite cell marker *Integrin-α7* was also detected in uninjured muscle, with upregulated expression during regeneration (Fig. 5H). In addition, *Hoxc10* (Fig. 5I), *TcR-β* (Fig. 5J), *Klra18*, *Itm2a*, and G_0S2 (data not shown) were all expressed in a similar pattern by cells in regenerating muscle. In situ hybridization of muscle sections with nonspecific sense riboprobes for

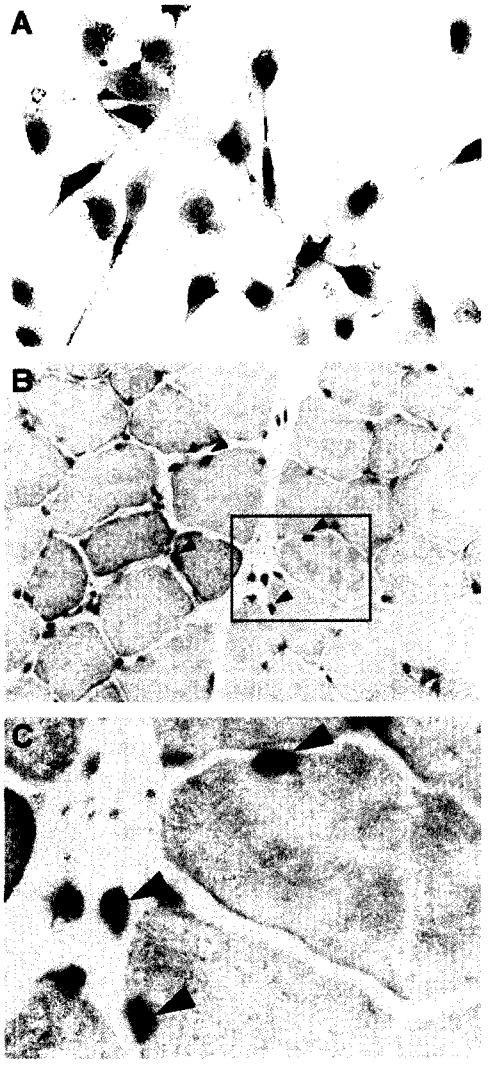

Fig. 4. Vcam1 protein is expressed in *MyoD*$^{-/-}$ myogenic cells and satellite cells. (A) Immunohistochemistry demonstrates localization of Vcam1 in distinct areas of the plasma membrane in *MyoD*$^{-/-}$ myoblasts. Hematoxylin (blue) was used to counterstain nuclei. (B) Vcam1 protein was detected in satellite cells in cross-sections of uninjured skeletal muscle (arrowheads). (C) Magnified view of boxed area in B.

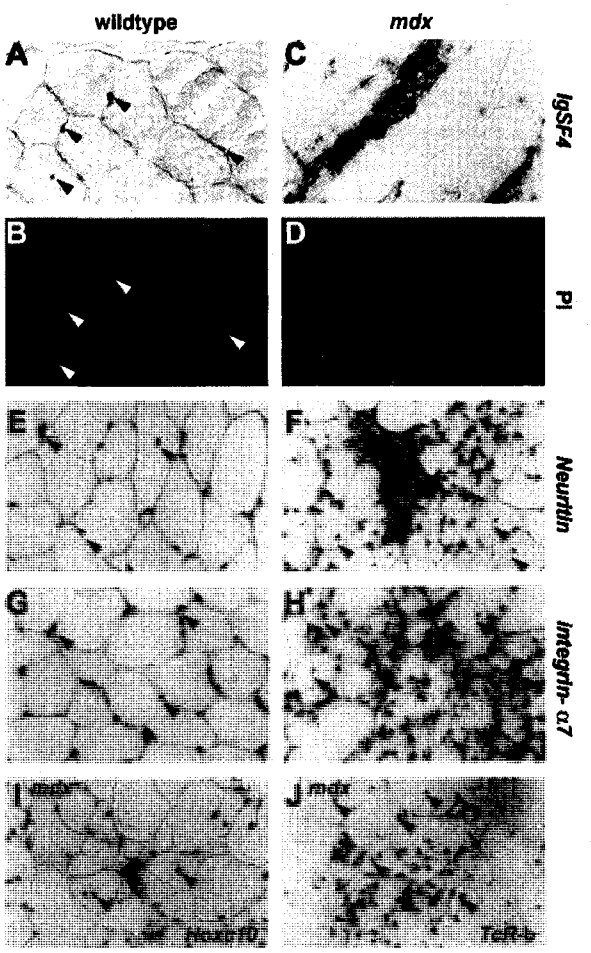

Fig. 5. *MyoD*$^{-/-}$-specific mRNAs are expressed by satellite cells in vivo. (A and B) *IgSF4* transcripts were detected in uninjured skeletal muscle by in situ hybridization. The expression of *IgSF4* was associated with 2–3% of the sublaminar nuclei visualized by PI staining (B). Arrowheads show *IgSF4* expressing cells and corresponding nuclei. (C and D) *IgSF4* was upregulated in regenerating *mdx* muscles demonstrating its expression in activated satellite cells and myoblasts. (E and F) *Neuritin* mRNA was expressed in uninjured (E) and regenerating skeletal muscle (F). Many *Neuritin*-expressing cells were located beneath the basal lamina of muscle fibers in uninjured muscle (E) (arrowheads) and associated with central nuclei in regenerated fibers (F) (arrowheads). (G and H) *Integrin-α7* was expressed in satellite cells in uninjured muscle (G) and upregulated during regeneration (H). (I) *Hoxc10* was highly expressed in regenerating *mdx* muscle. (J) *TcR-β* transcripts were also readily detected in regenerating muscle.

these genes did not show any nonspecific staining (not shown). Taken together, these studies suggest that many genes expressed specifically in *MyoD*$^{-/-}$ myogenic cells were also expressed by wild-type satellite cells in vivo.

Discussion

Satellite cells play a central role in postnatal muscle growth and regeneration of skeletal muscle. However, the molecular mechanisms responsible for their activation, expansion, and self-renewal remain to be elucidated. A major impediment in the study of satellite cells has been the difficulty in identifying genes expressed specifically in this compartment. In this report we have employed a straightforward experimental strategy to identify novel markers for the satellite cell lineage (Fig. 1).

Transcriptional mRNA profiling of *MyoD*$^{-/-}$ myogenic cells by representational difference analysis revealed that markers expressed by quiescent satellite cells were also expressed in *MyoD*-deficient cells (Fig. 5). This indicates that *MyoD*$^{-/-}$ myogenic cells represent a unique and tractable in vitro system to identify genetic pathways important for the function and maintenance of the satellite stem cell compartment in adult skeletal muscle.

Interestingly, several cDNAs expressed specifically in *MyoD*$^{-/-}$ myogenic cells were also expressed in hematopoietic or endothelial cells including *Vascular cell adhesion molecule-1* (*Vcam1*), *Asb5*, *Immunoglobulin superfamily-4* (*IgSF4*), *T-cell-receptor β-chain* (*TcR-β*), *Killer Lectin Receptor-18* (*Klra18*), *Laminin-α5* (*Lama5*), *Placenta-derived growth factor* (*PlGF*), and *Cadherin-6* (see references given in Table 1). This result supports the suggested developmental relationship between satellite cells and hematopoietic–endothelial lineages that has been proposed previously (De Angelis et al., 1999; Ordahl, 1999).

The developmental origin of satellite cells remains unclear. Quail-chick grafting experiments originally suggested that satellite cells originated from the somitic mesoderm along with embryonic and fetal myogenic cells (Armand et al., 1983). However, De Angelis et al. (1999) described the isolation of satellite cell myogenic clones from explanted dorsal aorta but not from the somites of mouse embryos. Notably, the aorta-derived myogenic cells express markers characteristic of the hematopoietic and endothelial cell lineages. The stem cells associated with blood vessels termed mesangioblasts are multipotential and readily participate in the repair of dystrophic muscle (Minasi et al., 2002). In the present study, we demonstrate that several genes expressed in hematopoietic lineages are also expressed by adult muscle satellite cells in vivo. These findings are consistent with the hypothesis that progenitors associated with blood vessels can give rise to adult muscle satellite cells.

The use of both wild-type myoblasts and *MyoD*-deficient myogenic progenitors in our study identified distinct subsets of genes based on their expression during in vitro satellite cell myogenesis (see Table 1). For example, the paired-box transcription factor *Pax7* was identified as a gene expressed specifically in proliferating myogenic cells. Based on its expression profile defined by this study, we then investigated the role of Pax7 in satellite cells (Seale et al., 2000). Importantly, analyses of *Pax7*-deficient mice revealed a requirement for Pax7 in satellite cell ontogeny. The requirement for Pax7 in the development of satellite cells is the basis of many ongoing studies aimed at identifying the origin of satellite cells and molecular pathways involved in their specification and function. The cloning and functional analysis of *Pax7* in adult progenitors highlights the importance of identifying differentially expressed genes in refined progenitor cell populations.

The Ankyrin repeat SOCS box containing gene *Asb5* was expressed at high levels in wild-type myoblasts and *MyoD*$^{-/-}$ myogenic cells as well as skeletal muscle tissue (Fig. 2A). *Asb5* was independently identified as a gene expressed in smooth muscle and endothelial cells during arteriogenesis (Boengler et al., 2003). Interestingly, immunolocalization of Asb5 protein in skeletal muscle revealed its expression in muscle satellite cells (Boengler et al., 2003). *Pb99* was expressed specifically in proliferating myoblasts and was completely downregulated after 24 h in differentiation conditions (Fig. 2A). Pb99, a putative G-protein-coupled receptor, is also expressed in pre-B cells and thymocytes but not in mature lymphocytes (Sleckman et al., 2000).

Several other genes identified in wild-type satellite cell-derived myoblasts were expressed in a differentiation-dependent manner, including *Synaptotagmin-like 2 (Sytl2)*, *Death-associated kinase 2 (Dapk2)*, and *Neuronal Leucine Rich Region-1* (Lrrn1) (Fig. 2B and Table 1). This subset of genes was expressed at lower levels in *MyoD*-deficient myogenic cells, suggesting specific roles in the differentiation process downstream of MyoD. In particular, an unknown gene related to *Arachidonate 5′-Lipoxygenase* (5-Lox) (dp3-5) was expressed in proliferating and differentiating wild-type and C2C12 myoblasts, but notably absent from *MyoD*$^{-/-}$ cultures (Fig. 2C). The expression pattern of dp3-5 is suggestive of a role for this gene in myogenic differentiation that will require further investigation. Taken together, several genes with previously undescribed roles in myogenesis were identified in wild-type myoblasts.

The increased self-renewal capacity and enhanced proliferation of *MyoD*-deficient myoblasts suggested that their gene expression profile would closely resemble that of satellite cells in vivo. The identification of known satellite cell markers including *Vcam1* (Figs. 3 and 5) and *Integrin-α7* as genes expressed highly in *MyoD*-deficient myoblasts illustrates that *MyoD*$^{-/-}$ cells express genes that are also expressed by satellite cells but that are low or absent in wild-type myoblasts. Moreover, transcripts for *IgSF4* and *Neuritin*, genes not expressed in wild-type myoblasts, were detected in cells within uninjured skeletal muscle (Fig. 5). The frequency of cells expressing *IgSF4* and *Neuritin* and

their overall expression levels were markedly increased in regenerating muscle consistent with their expression in muscle satellite cells. Several other genes expressed at high levels in *MyoD*$^{-/-}$ myogenic cells including *Hoxc10*, *TcRβ*, *Klra18*, and *G_0S2* were similarly activated during muscle regeneration (Figs. 5I and J). These results indicate a specific induction of these markers during muscle regeneration and support the hypothesis that MyoD-:Myf5+ myogenic progenitors represent a distinct developmental stage in the satellite cell program during regenerative myogenesis.

IgSF4 is a recently identified member of the immunoglobulin protein superfamily (IgSF) of proteins related to *Synaptic Cell Adhesion Molecule* (SCAM) that functions at neuronal synapses (Biederer et al., 2002). *IgSF* genes encode a diverse group of proteins characterized by the Ig homology domain that regulate several processes including cell adhesion and signal transduction cascades (reviewed by Rougon and Hobert, 2003). Neuritin is a GPI-anchored protein that is highly expressed in the brain and induced in response to neural activity (Naeve et al., 1997). Nestin is an intermediate filament protein that serves as a marker for neural stem cells and adult pancreatic stem cells (Lendahl et al., 1990; Sawamoto et al., 2001; Zimmerman et al., 1994; Zulewski et al., 2001). The function of these proteins in the myogenic lineage remains to be defined.

Hox genes have been implicated in embryonic growth and pattern formation. Notably, *Hoxc10* is specifically expressed in the developing hindlimbs but not forelimbs of the Axolotl (salamander), mouse, and chick (Carlson et al., 2001; Peterson et al., 1992, 1994). *Hoxc10* is also induced early during the regeneration of Axolotl forelimbs coincident with the appearance of the undifferentiated blastema cells, suggesting a role for Hoxc10 in dedifferentiation (Carlson et al., 2001). The expression of *Hoxc10* in undifferentiated amphibian limb progenitors is consistent with its expression in "more primitive" MyoD-negative myogenic precursors during muscle regeneration. Further studies are required to assess the functional role of *Hoxc10* in skeletal muscle development and regeneration.

Analysis of gene expression in satellite cells by RT-PCR demonstrates that either *Myf5* or *MyoD* is induced upon activation before their coexpression in committed myogenic precursors (Cornelison and Wold, 1997). Analysis of regenerating muscle confirmed these findings, indicating that 50% of activated satellite cells coexpress MyoD and Myf5, 30% express MyoD alone, and 20% express Myf5 alone 3 h postinjury (Cooper et al., 1999). Our study supports these findings and suggests a specific role for myogenic cells expressing Myf5 but not MyoD in satellite cell self-renewal. Taken together, our data indicate that cultured *MyoD*$^{-/-}$ myogenic cells provide a unique opportunity to elucidate genetic networks activated in this adult progenitor compartment.

Methodology to identify differentially expressed genes between RNA preparations has been revolutionized with the advent of spotted cDNA and oligonucleotide array technologies. These procedures are becoming increasingly robust for assessing global changes in gene expression. However, the use of independent methods like RDA remains powerful approaches to identify differentially expressed genes that are novel or are present at low levels. The ability to clone novel genes and tailor cDNA subtractions by altering input cDNA ratios is not possible with microarray methodology. Furthermore, the sensitivity of RDA for identifying genes expressed at low levels (e.g., tissue-specific transcription factors) offers a critical advantage. Finally, inherent to RDA is the "physical" retrieval of differentially expressed cDNA fragments that can then be used in expression studies and screening of cDNA or genomic libraries. In this study, we have successfully identified several specific genes using RDA that will now form the basis of further research into the biology of satellite cells. Future functional and genetic analyses of the genes identified in this survey will help elucidate the mechanisms acting during regenerative myogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.07.034.

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