# PAX7 IN REGENERATIVE MYOGENESIS

# PAX7 IS REQUIRED FOR MUSCLE SATELITE CELL SPECIFICATION AND REGENERATIVE MYOGENESIS

By

PATRICK SEALE, HONS. B.SC.

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AUTHOR: Patrick Seale, Hons. B.Sc. (McMaster University)

SUPERVISOR: Dr. Michael A. Rudnicki

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**Abstract**

Muscle satellite cells are a distinct population of myogenic progenitors that mediate the postnatal growth and regeneration of skeletal muscle. To gain insight into the genetic regulation of satellite cell function during muscle regeneration, genes expressed specifically in these cells were identified by representational difference analysis of cDNAs. Notably, the paired-box transcription factor *Pax7* was isolated as a gene specifically expressed in quiescent and activated satellite cells. Cell culture and histological analysis of *Pax7*-deficient muscle revealed a complete absence of satellite cells. This result demonstrates a requirement for Pax7 upstream of MyoD and Myf5 in the specification of muscle satellite cells. Consistent with their lack of satellite cells, adult *Pax7*$^{-/-}$ mice displayed an aggravated muscle wasting phenotype characterized by spinal kyphosis and reduced muscle mass. Acute muscle damage led to extensive calcification and deposition of adipose and fibrotic tissues with the appearance of rare regenerated myofibers. Importantly, analysis of *Pax7*$^{-/-}$ muscle suspensions indicated that myogenic cells expressing Pax3 and MyoD were responsible for this low level of regeneration.

To characterize the role of adult stem cells in skeletal muscle, we investigated the myogenic potential of muscle-derived CD45$^{+}$:Sca1$^{+}$ cells *in vivo* during regeneration and *in vitro* using coculture assays. CD45$^{+}$ and Sca1$^{+}$ cells isolated from uninjured muscle were uniformly non-myogenic. Strikingly, 7-10% of CD45$^{+}$:Sca1$^{+}$ cells purified from

regenerating muscle activated the myogenic program by a Pax7-dependent mechanism in response to activation of the Wnt signaling pathway. Furthermore, expression of Pax7 was sufficient to induce myogenic commitment in $CD45^{+}$:$Sca1^{+}$ cells from uninjured muscle. This result demonstrates that non-satellite cell derived myogenic progenitors possess a physiological role in muscle regeneration and tissue homeostasis.

Taken together, this work establishes a requirement for Pax7 in the specification of muscle satellite cells and for the myogenic recruitment of adult stem cells populations during tissue repair. Importantly, these studies also suggest that targeted therapies to activate Wnt signaling and Pax7 expression in adult stem cells will be effective for promoting muscle regeneration in patients with degenerative neuromuscular diseases or muscular dystrophies.

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First, I want to thank Michael for being a wonderful mentor for me during my time in his lab. He has been constant in his encouragement and support. I am very appreciative of the confidence that he showed in my abilities right from the beginning. I hope that we will continue to remain friends and colleagues. I am extremely thankful to Luc, who taught me so much in the lab and shared many of his technical skills and "secrets" with me. I am fortunate that his passion and enjoyment of science rubbed off on me. I would also like to thank all past and present members of the Rudnicki lab for making it such a fun place to work. I enjoyed our scientific discussions as well as social outings at the Phoenix or at an Ottawa pub. In particular, I would like to thank Claire for her help, support and many constructive criticisms. I would also like to thank Jeff for being so giving of his time in helping with experiments as well as for his constructive, albeit sometimes sarcastic criticisms. In the past couple of years, I have really enjoyed collaborating on projects with Sophie. I have learnt a great many techniques and organizational skills from her. By far the most important contribution is from Neera, my loving wife, who has been there for me through thick and thin. I have enjoyed sharing all my successes with her and she has helped me deal with all the hardships. I can honestly say that I would not have made it through these last few years in one piece were it not for her constant encouragement and support.

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## List of Abbreviations

| | |
|---|---|
| BM | bone marrow |
| cDNA | complementary DNA |
| CDSC | $CD45^+$:$Sca1^+$ cells |
| CDSC-Pax7 | $CD45^+$:$Sca1^+$ cells expressing Pax7 |
| ctx | cardiotoxin |
| DMD | Duchenne Muscular Dystrophy |
| DMEM | Dulbecco's modified Eagle Medium |
| DNA | deoxyribonucleic acid |
| EDL | Extensor Digitorum Longus |
| FACS | Fluorescence Activated Cell Sorting |
| H&E | hematoxylin and eosin |
| HSC | hematopoietic stem cell |
| MD | Muscular Dystrophy |
| MyHC | Myosin Heavy Chain |
| mpc | muscle precursor cell |
| MRF | Myogenic Regulatory Factor |
| mRNA | messenger RNA |
| MT | myoblast transplantation |
| muSP | muscle SP cell |
| NSC | neural stem cell |
| PCNA | Proliferating Cell Nuclear Antigen |
| PI | propidium iodide |
| PMNL | Polymorphonuclear lymphocyte |
| RNA | ribonucleic acid |
| Sca1 | Stem Cell Antigen-1 |
| SP | side population |
| TA | Tibialis Anterior |

# Chapter 1

## Introduction

### 1.1 Overview

Skeletal muscle is an established developmental system for studying the molecular mechanisms that regulate stem cell commitment and differentiation. Embryonic skeletal myogenic cells arise from somitic progenitors in response to signaling molecules from adjacent structures. Adult myoblasts, active during the postnatal growth and regeneration of muscle, are derived from a specialized myogenic lineage known as satellite cells and possibly from other adult stem cell populations. Studies in embryonic muscle indicate that the paired-domain transcription factor, Pax3 acts upstream of the primary myogenic basic helix-loop-helix (bHLH) transcription factors, Myf5 and MyoD in myogenic specification.

### 1.2 The MyoD-family of bHLH transcription factors

The development of the skeletal muscle lineage is regulated by a family of myogenic basic helix-loop-helix (bHLH) transcription factors consisting of MyoD, Myf5, MRF4 and Myogenin. (Megeney and Rudnicki, 1995; Olson, 1993; Pownall et al., 2002; Rudnicki and Jaenisch, 1995; Weintraub et al., 1991a). The MRFs are defined by their ability to convert a wide range of cell types into the myogenic lineage (Braun et al., 1990; Braun et al., 1989; Davis et al., 1987; Miner and Wold, 1990). The MRFs share a homologous basic DNA-binding domain that binds the E-box, a DNA motif characterized

by the core CANNTG sequence. The HLH domain mediates dimerization with ubiquitously expressed members of the E-protein family of HLH containing transcription factors (Hu et al., 1992; Lassar et al., 1991; Weintraub et al., 1991b). MRF-E protein heterodimers bind E-box elements in the promoters of many genes to regulate muscle-specific expression. The DNA binding and transactivation activity of the MRFs is tightly controlled by microenvironmental stimuli (reviewed by Puri and Sartorelli, 2000).

## 1.3 Embryonic origin of vertebrate skeletal muscle

Multipotent stem cells within the prechordal and somitic mesoderm give rise to committed myogenic cells that subsequently differentiate to form the skeletal muscle of the body wall, limbs and head (Hauschka, 1994). Somites arise as transient condensations of paraxial mesoderm that develop into spherical structures on either side of the neural tube (Figure 1.1A). The somites differentiate into the epithelial dermomyotome dorsally and the mesenchymal sclerotome ventrally (reviewed in Asakura and Rudnicki, 2002; Pownall et al., 2002). The sclerotome develops into the cartilage and bone of the vertebrae and the ribs. The dermomyotome is further compartmentalized into dermatome and myotome. The dermatome gives rise to the dermis and other mesenchymal cell types while the myotome is the source of all trunk and limb muscles. Fate mapping experiments demonstrate that deep back muscles (epaxial muscles) are derived from the dorsal myotome whereas abdominal and limb muscles (hypaxial muscles) arise from small numbers of migratory cells originating in the ventral myotome (Christ and Ordahl, 1995; Ordahl and Le Douarin, 1992; Williams and Ordahl, 1997). The different somitic

origins for epaxial and hypaxial muscles suggest the existence of two distinct myogenic lineages during embryonic myogenesis.

### 1.4 Embryonic function of the MRFs

The myogenic regulatory factors (MRFs) regulate the commitment and differentiation of embryonic myoblasts during development. Myf5 and MyoD are required for myogenic commitment of somitic progenitors in response to signaling molecules produced by the neural tube and paraxial mesoderm (Bober et al., 1994a; Cossu et al., 1996a; Cossu et al., 1996b; Munsterberg and Lassar, 1995; Pownall et al., 1996; Tajbakhsh et al., 1998; Williams and Ordahl, 1997). Sonic hedgehog (Shh), secreted by the notochord, and several proteins of the Wnt family (wingless), secreted by the dorsal neural tube (Wnt1, Wnt3) and surface ectoderm (Wnt4, Wnt6, Wnt7a), are required for induction of embryonic myogenesis (Munsterberg and Lassar, 1995; Tajbakhsh et al., 1998). These signals culminate in the expression of Pax3 and the myogenic determination factors Myf5 and MyoD. Myogenin and MRF4 function downstream in terminal myocyte differentiation and fusion (reviewed by Buckingham, 1994; and Megeney and Rudnicki, 1995).

Gene targeting in mice has revealed the hierarchical role of the MRFs during embryonic and postnatal myogenesis. Importantly, mice lacking both *MyoD* and *Myf5* display a complete absence of skeletal muscle, illustrating the essential role for MyoD or Myf5 in myogenic specification (Rudnicki et al., 1993). *MyoD*$^{-/-}$:*Myf5*$^{-/-}$ knockout mice have been made that carry a *LacZ* transgene expressed under the control of regulatory

elements from the *MyoD* gene (Kablar et al., 1999). In these embryos, presumptive LacZ-expressing muscle precursors acquire non-muscle cell fates, indicating that MyoD or Myf5 protein is necessary for the specification of myoblasts (Figure 1.1B). The temporal-spatial patterning of myogenesis in *MyoD*$^{-/-}$ and *Myf5*$^{-/-}$ embryos also demonstrates the predominance of MyoD and Myf5 activity in the formation of hypaxial and epaxial muscle, respectively (Kablar et al., 1997; reviewed by Ordahl and Williams, 1998).

Following their commitment to the myogenic lineage, myoblasts differentiate and fuse with one another to form multinucleated myofibers. Mice lacking myogenin die perinatally due to a severe loss of differentiated muscle fibers, yet possess a normal number of myoblasts. This phenotype illustrates an essential role for myogenin in regulating differentiation downstream of Myf5 and MyoD (Hasty et al., 1993; Nabeshima et al., 1993). *Mrf4/Myf6* deficient mice display a range of phenotypes consistent with a role in terminal differentiation, fusion and myofiber maintenance (Braun and Arnold, 1995; reviewed by Olson et al., 1996; Patapoutian et al., 1995; Zhang et al., 1995).

## 1.5 Myocyte enhancer family-2 (Mef2) transcription factors

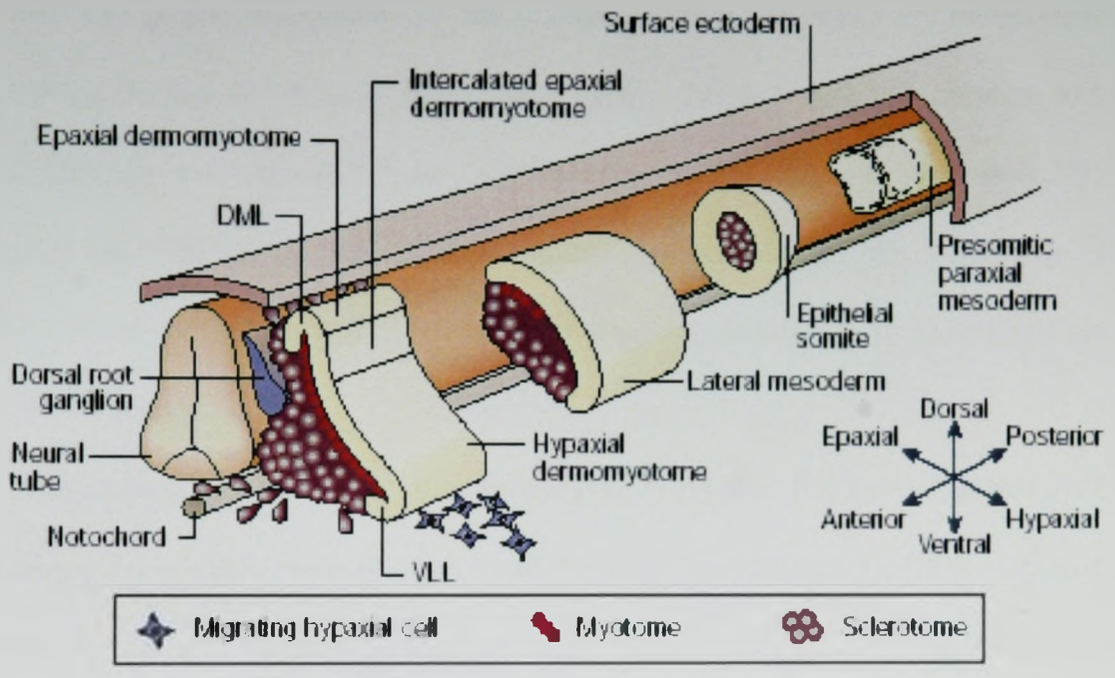
In addition to the MRFs, the Myocyte enhancer-factor 2 (Mef2) class of transcription factors direct muscle specific transcription (Kaushal et al., 1994; Molkentin et al., 1995; Molkentin and Olson, 1996; Naya et al., 1999; reviewed by Naya and Olson, 1999). Importantly, *in vitro* studies have demonstrated that Mef2 and MRFs synergistically activate muscle promoters containing both E-boxes and Mef2 binding

**Figure 1.1 Embryonic myogenesis and the myogenic regulatory factors**

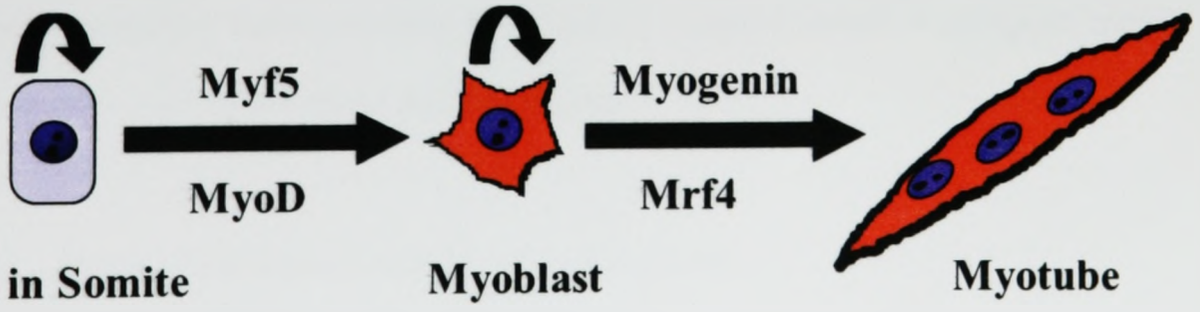
(**A**) Condensation of the paraxial mesoderm occurs in a rostral-caudal direction to give rise to the somites on either side of the neural tube in vertebrate embryos. Specialization of the somites in response to signaling molecules results in the formation of the dermomyotome and sclerotome. The dermomyotome gives rise to the dermis and the muscles of the trunk and limbs while the sclerotome develops into bone and cartilage of the vertebrae and ribs. Progenitors in the dorsal margin of the dermomyotome give rise to epaxial-body wall and deep back muscles. Migratory cells originating from the ventral dermomyotome produce the hypaxial- limb and diaphragm muscles. (Reprinted from Nat. Rev. Genet, Maura H. Parker, Patrick Seale and Michael A. Rudnicki, Looking Back to the Embryo: Defining Transcriptional Networks in Adult Myogenesis, p 495-505, Copyright 2003, with permission from Elsevier).

(**B**) MyoD or Myf5 is required for the specification of myogenic precursors from stem cells in the somitic mesoderm. Myogenin and MRF4 regulate the differentiation and fusion of myoblasts into multinucleated myofibers.

**A**


Surface ectoderm
Intercalated epaxial dermomyotome
Epaxial dermomyotome
DML
Presomitic paraxial mesoderm
Epithelial somite
Dorsal root ganglion
Lateral mesoderm
Neural tube
Hypaxial dermomyotome
Notochord
VLL
Dorsal
Epaxial
Posterior
Anterior
Hypaxial
Ventral
Migrating hypaxial cell
Myotome
Sclerotome

**B**


Myf5
MyoD
Myogenin
Mrf4
Cell in Somite
Myoblast
Myotube

elements (Kaushal et al., 1994; Molkentin et al., 1995; Naidu et al., 1995). The coregulation and potentiation of MRF activity by Mef2 proteins was suggested to be regulated by direct interaction in the DNA binding domains of these different transcription factors (Molkentin and Olson, 1996). Moreover, Mef2 proteins and the MRFs form an autoregulatory loop, such that expression of myogenin and Mrf4 is dependent on both sets of transcription factors (Naidu et al., 1995; Ridgeway et al., 2000b). After birth, expression of Mef2 is downregulated, but is re-induced during skeletal muscle regeneration.

Interestingly, a recent report indicates that Mef2 also functions together with the Peroxisome Proliferator-Activated Receptor Gamma Co-Activator-1α (PGC-1α) to direct slow-muscle specific gene expression in adult muscle (Lin et al., 2002). PGC-1α is upregulated during exercise, and is targeted by calcineurin signaling to promote the specialization of adult muscle fibers. The role of PGC-1α as a regulator of the slow muscle developmental program presents a novel strategy for transcriptional regulation whereby cofactors lacking intrinsic DNA binding activity themselves use tissue specific transcription factors to activate their target genes.

## 1.6 Role of Pax3 upstream of MyoD in myogenesis

Although MyoD or Myf5 define the identity of the skeletal myoblast, somitic precursors may be "*pre-committed*" to the myogenic lineage prior to MRF expression. In the embryo, Pax3, a paired-box transcription factor, is expressed in presomitic mesoderm and early epithelial somites (Goulding et al., 1994; Williams and Ordahl, 1994).

Moreover, myogenic precursors in the dermomyotome express *Pax3*, *Pax7* and low levels of *Myf5* (Goulding et al., 1994; Jostes et al., 1990; Kiefer and Hauschka, 2001). *Pax3*-expressing cells in the dermomyotome are maintained as a proliferative, undifferentiated population by signals secreted from the lateral plate mesoderm and surface ectoderm (Amthor et al., 1999). In *Pax3*-deficient *splotch* mice, the limb and diaphragm muscles do not form due to a defect in lateral migration and reduced proliferation in the dermomyotome (Bober et al., 1994b; reviewed by Borycki and Emerson, 1997; Daston et al., 1996; Franz et al., 1993; Tremblay et al., 1998). *Pax7*, the paralogue of *Pax3*, is not expressed in presomitic mesoderm but is induced during somite maturation (Jostes et al., 1990; Mansouri et al., 1996).

In presomitic explants from *splotch* embryos, neural tube and surface ectoderm signals fail to activate expression of *MyoD* (Borycki et al., 1999). Furthermore, Pax3 is required for expression of *MyoD* in the absence of Myf5 during hypaxial muscle development, indicating that both *Pax3* and *Myf5* function upstream of *MyoD* (Tajbakhsh et al., 1997). Importantly, ectopic expression of Pax3 in embryonic tissues activates *MyoD*, *Myf5* and *myogenin* expression (Bendall et al., 1999; Maroto et al., 1997). Pax3 is also necessary and sufficient for myogenesis in pluripotent embryonal P19 cell cultures (Ridgeway and Skerjanc, 2001). Interestingly, *MyoD* is not activated by Pax3 in the dermomyotome, suggesting either specific coactivators are required for myogenic induction, or that competing inhibitory signals, such as the BMPs, Delta/Notch and Msx1 repress *MyoD* activation. Together, these experiments indicate a critical role for Pax3 in myogenic specification upstream of the MRFs.

Interestingly, Borycki and colleagues noted increased apoptosis in somites from *splotch* embryos, in the areas that do not express *Pax7*, suggesting that Pax activity is crucial for the survival of progenitor populations (Borycki et al., 1999). The selective survival of Pax-expressing precursor populations may be a conserved developmental mechanism for the specification of myogenic cells. Alternatively, failure to activate the myogenic program may be responsible for the observed apoptotic response of undetermined cells. To distinguish between these possibilities, it will be necessary to identify the DNA binding targets of Pax3 in mesenchymal stem/progenitor cells.

The molecular activity of Pax3 in regulating myogenesis remains poorly understood. By themselves, Pax3 and Pax7 are remarkably weak transcriptional activators and have been observed to function as transrepressors under certain conditions (Magnaghi et al., 1998). Mice expressing an activated form of Pax3, in which the Forkhead (FKHR) transactivation domain is fused to the Pax3 DNA binding domain, display malformations of some but not all hypaxial muscles (Lagutina et al., 2002). In cell culture systems, Pax3-FKHR induces expression of *MyoD, myogenin* and *Six1*, a homeobox-containing transcription factor (Khan et al., 1999).

Studies in P19 embryonal carcinoma cells have uncovered some of the molecules that appear to function upstream and downstream of Pax3 in myogenic specification. In particular, Pax3 regulates expression of *Six1* and *Eya2* prior to activation of *MyoD* and myogenesis (Ridgeway and Skerjanc, 2001). In turn, activation of *Pax3*, as well as *Six1* and *Gli2*, is regulated through Wnt-3a or β-catenin (Petropoulos and Skerjanc, 2002; Ridgeway et al., 2000a). Six1 synergizes with Eya2, and Eya2 interacts with Dach2, to

activate expression of *MyoD* and *myogenin in vivo* and *in vitro* (Heanue et al., 1999; Kardon et al., 2002; Spitz et al., 1998). A recent report describes severe muscle hypoplasia and deficient hypaxial muscle development in *Six1* deficient mice, indicating a clear role for Six1 in regulating embryonic myogenesis (Laclef et al., 2003). The expression dynamics of Pax3, Six1, Eya2 and Dach2 and their interactions with one another for activating muscle specific genes remains to be carefully analyzed.

In summary, these observations suggest that Wnt-mediated activation of β-catenin activates *Pax3* expression, which in turn regulates expression of *MyoD, Six1* and *Eya2*, culminating in the expression of *myogenin* and myogenic differentiation. It also suggests that *Pax3*-mediated myogenesis requires an environment in which *Dach2, Eya2,* and *Six1* can be activated. Differential expression or activity of these factors may account for the ability of Pax3 to induce myogenesis in paraxial mesoderm explants and P19 cells but not in C3H10T1/2 fibroblasts (Maroto et al., 1997; Ridgeway and Skerjanc, 2001).

### 1.6.1 Pax3, c-met and Lbx1 in migration

The expression of *Pax3* in migrating myoblasts and the migratory deficit observed in *splotch* embryos indicates a requirement for Pax3 in this process. Delamination and migration also depend on the activity of the c-met receptor and its ligand, hepatocyte growth factor (HGF). Importantly, mice that lack either *c-met* or *HGF* have no limb muscle (Dietrich et al., 1999; reviewed by Birchmeier and Gherardi, 1998). Furthermore, the expression of *c-met* is regulated by Pax3 (Epstein et al., 1996; Yang et al., 1996b). A recent study also reveals that Pax3 controls the expression of *Lbx1* (ladybird), a

homeobox-containing transcription factor expressed in migrating limb muscle precursor cells (Mennerich et al., 1998). *Pax3*-deficient mice display a complete lack of *Lbx1* expression in the limb bud. Interestingly, Pax3 and Lbx1 appear to function together to specify the number of limb muscle precursors prior to the induction of myogenesis (Mennerich and Braun, 2001).

## 1.7 Muscle satellite cells

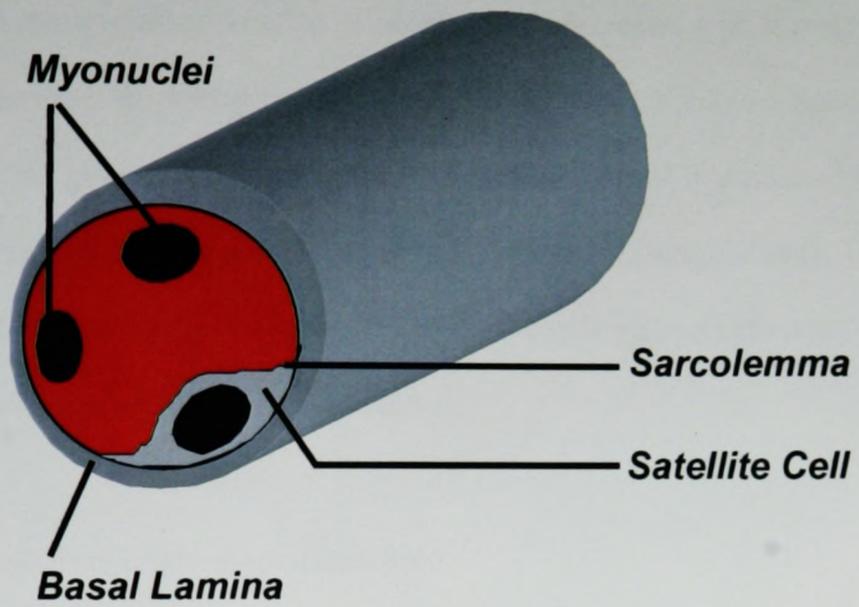
Skeletal muscle satellite cells are defined by their morphology and anatomical location relative to mature myofibers. Satellite cells adhere to the surface of myotubes prior to the formation of the basal lamina (Figure 1.2) (Armand et al., 1983; Bischoff, 1990; Bischoff, 1994; Mauro, 1961; Schultz, 1976). They reside in grooves or depressions between the basal lamina and sarcolemma of mature fibers. The association of satellite cells with adjacent myofibers is mediated by cell-cell interactions involving the activity of M-Cadherin (muscle Cadherin), N-CAM (Neural Cell Adhesion Molecule) and other cell-adhesion molecules (Cifuentes-Diaz et al., 1993; Irintchev et al., 1994). Adult satellite cells are also characterized by high nuclear to cytoplasmic ratios, heterochromatic nuclei and a paucity of cytoplasmic organelles (see Figure 1.2) (Armand et al., 1983; Schultz, 1976).

In newborn mice, satellite cells constitute the only myogenic cells in skeletal muscle with proliferative capacity (Moss and Leblond, 1971). Thus, the satellite cell compartment mediates postnatal muscle development and is the primary means by which the adult muscle mass is formed (Moss and Leblond, 1971; Schultz, 1989; Schultz,

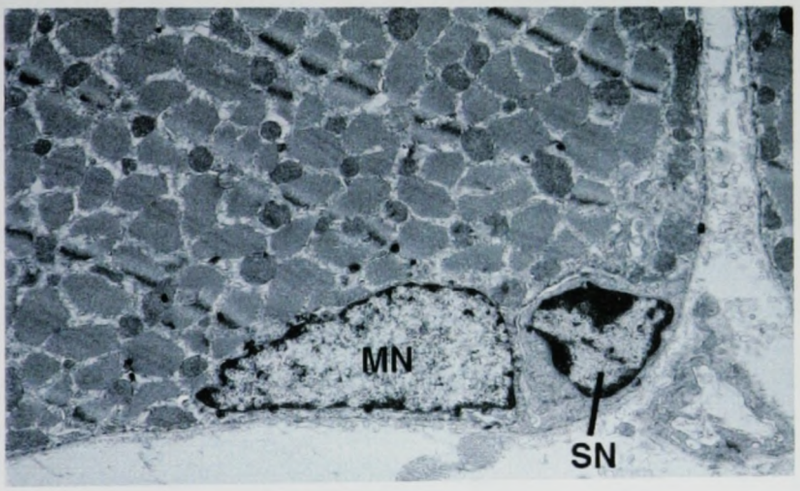
**Figure 1.2 Satellite cells in adult muscle**

(**A**) Mononuclear satellite cells are located between the basal lamina and sarcolemma of mature fibers. (**B**) Transmission electron micrograph of skeletal muscle depicts a satellite cell (SN) and fiber myonucleus (MN). Satellite cells are characterized by densely stained heterochromatic nuclei and organelle-poor cytoplasms. (**C**) Cultured single muscle fiber with activated satellite cells that have migrated from the fiber to form myogenic precursor cells. (Reprinted from Stem Cells: A Cellular Fountain of Youth, Patrick Seale and Michael A. Rudnicki, Adult Skeletal Muscle Growth and Regeneration, p 117-200, Copyright 2003, with permission from Elsevier).

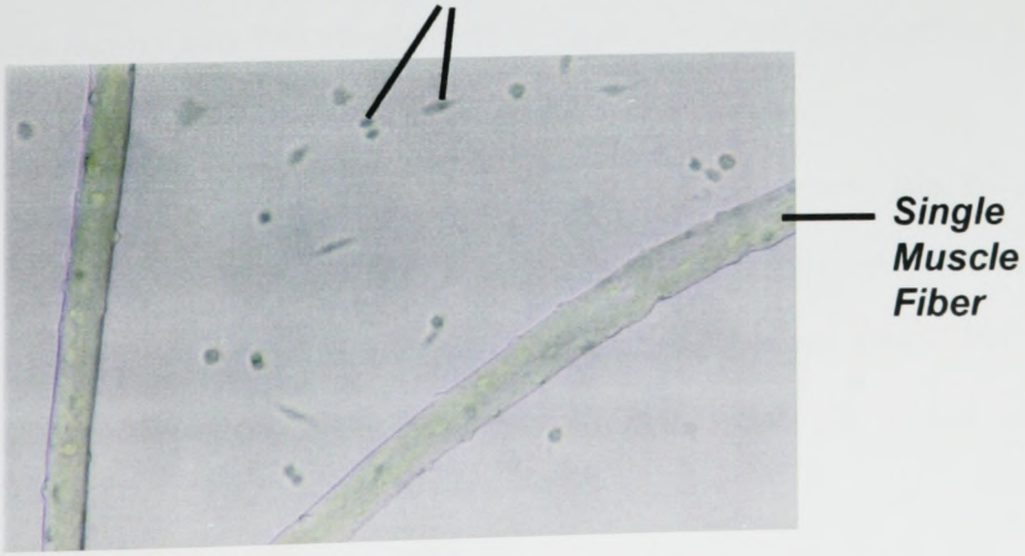
A


Myonuclei
Sarcolemma
Satellite Cell
Basal Lamina

B


MN
SN

C


Muscle Precursor Cells
Single Muscle Fiber

1996). As such, the overall population of satellite cells decreases with increasing age in developing muscles (Gibson and Schultz, 1983; Grounds and Yablonka-Reuveni, 1993; Schultz, 1996). At birth, satellite cells account for about 32% of muscle nuclei followed by a drop to less than 5% in the adult (2 months for mice) (Bischoff, 1994). The decline in satellite cell number as the postnatal muscle develops is a direct reflection of satellite cell fusion into new or pre-existing myofibers.

### 1.7.1 Developmental origin of satellite cells

Satellite cells are distinct from the embryonic myogenic lineages and first appear in the limbs of mouse embryos at about 17.5 days post coitum (dpc) (Bischoff, 1994; Cossu et al., 1985; De Angelis et al., 1999; Feldman and Stockdale, 1992; Hartley et al., 1992). The birth of satellite cells during vertebrate development constitutes the third wave of myogenesis following the formation of both embryonic and fetal myogenic cells (Feldman and Stockdale, 1992; Hartley et al., 1992). Satellite cells can be distinguished from these earlier myogenic lineages on the basis of: myosin heavy chain isoform expression; distinct morphological appearance; and their capacity to differentiate in the presence of the phorbol ester TPA (Bischoff, 1994; Cossu et al., 1985; De Angelis et al., 1999; Feldman and Stockdale, 1992; Hartley et al., 1992).

Embryonic and fetal myoblasts are committed to the myogenic fate during somitogenesis in response to signals from adjacent tissues including the mesoderm and notochord (Cossu et al., 1996a; Cossu et al., 1996b; Tajbakhsh and Cossu, 1997), however the origin of satellite cells, which arise later in development, is less well

characterized. Classical quail-chick chimerae experiments in which quail somitic mesoderm was grafted into 2-day chick embryos revealed the presence of quail satellite cells associated with host chick myofibers (Armand et al., 1983). Based largely on this study, it has been assumed that satellite cells or their precursors originate from the myotome, as do earlier myogenic progenitors, however follow-up studies specifically addressing this question have not been reported.

A recent study by DeAngelis et al. challenged previous notions concerning the origin of satellite cells (De Angelis et al., 1999). Their report convincingly demonstrates that clonal satellite cell derived myogenic precursors are readily isolated from the embryonic dorsal aorta but not from the somite of mouse embryos at different developmental stages. Moreover, analogous myogenic precursors are found in the limbs of later-stage *c-met*$^{-/-}$ and *Pax3*$^{-/-}$ mutant embryos that do not possess somite derived myoblasts (Dietrich et al., 1999; Tremblay et al., 1998). Myoblasts isolated from these mutant mice may thus arise from blood vessels or associated cells within developing limb-buds. Based on this work, it is speculated that the differentiation of multipotential precursors associated with the embryonic vasculature occurs as a function of tissue perfusion. For example, vessels which colonize skeletal muscle, contain progenitors that give rise to satellite cells. Consistent with this hypothesis, aorta-derived myogenic cells express myogenic and endothelial markers that are also expressed in adult satellite cells (reviewed by Bianco and Cossu, 1999; De Angelis et al., 1999; and Ordahl, 1999).

Developing embryonic vessels are colonized by migratory populations of angioblasts which arise in the paraxial mesoderm (in somite) and differentiate from the

mesoderm as solitary cells before fusing to form primitive blood vessels (Dzierzak, 1999; Pardanaud and Dieterlen-Lievre, 1999; Pardanaud et al., 1996). The embryonic origin of blood vessels thus raises the possibility that a proportion of satellite cells are derived from progenitors in the paraxial mesoderm, which also give rise to endothelial cells. Furthermore, aortic precursor cells, which form the primitive dorsal aortae, are closely associated with the ventral surface of the somite (Pardanaud et al., 1996). The anatomical proximity of vasculogenic progenitors to the somite suggests that the explants used in Armand's experiments (Armand et al., 1983), may have contained aortic precursors with the capacity to differentiate into satellite cells. The aorta-derived myoblasts may be derived from from pericytes (specialized vessel supporting cells), blood vessel endothelial cells or circulating endothelial cells (Cossu and Mavilio, 2000). Collectively these results support a model for satellite cell development, which occurs independently of myogenic specification within the somite.

### 1.7.2 Satellite cells in adult muscle regeneration

Satellite cells in adult skeletal muscle are normally mitotically quiescent but are activated (i.e. initiate multiple rounds of proliferation) in response to a variety of stimuli including weight bearing exercise, trauma, stretching and denervation (Appell et al., 1988; Darr and Schultz, 1987; Grounds and Yablonka-Reuveni, 1993; Rosenblatt et al., 1994; Schultz, 1989; Schultz et al., 1985). In response to signals in regenerating muscle, satellite cells can cross the myofiber basal lamina and migrate to distal sites of injury (Bischoff, 1997; Hughes and Blau, 1990; Watt et al., 1987). The descendants of activated

satellite cells, called myogenic precursor cells (mpcs), undergo multiple rounds of division prior to fusing with existing or new myofibers (Figure 1.3 and 1.4) (Bischoff, 1994; Grounds and Yablonka-Reuveni, 1993; McGeachie, 1985; McGeachie, 1989; Schultz and Lipton, 1982). It is important to stress the non-equivalence of satellite cells and their daughter mpcs as determined by various biological and biochemical criteria. By definition a satellite cell is quiescent, does not express muscle determination genes and resides beneath the basal lamina of intact fibers. By contrast, mpcs are determined myogenic cells, which express a wide array of muscle specific transcription factors and structural proteins.

The essential role of satellite cells in muscle regeneration, muscle hypertrophy and post-natal muscle growth is well documented. However, an understanding of the molecular mechanisms that regulate the activation and function of myogenic stem cells remains poorly understood. Nevertheless, studies characterizing the expression and function of the myogenic regulatory factors (MRFs) in satellite cells have provided insights into the regulation of their activity during regeneration.

### 1.7.3 The myogenic regulatory factors in satellite cells

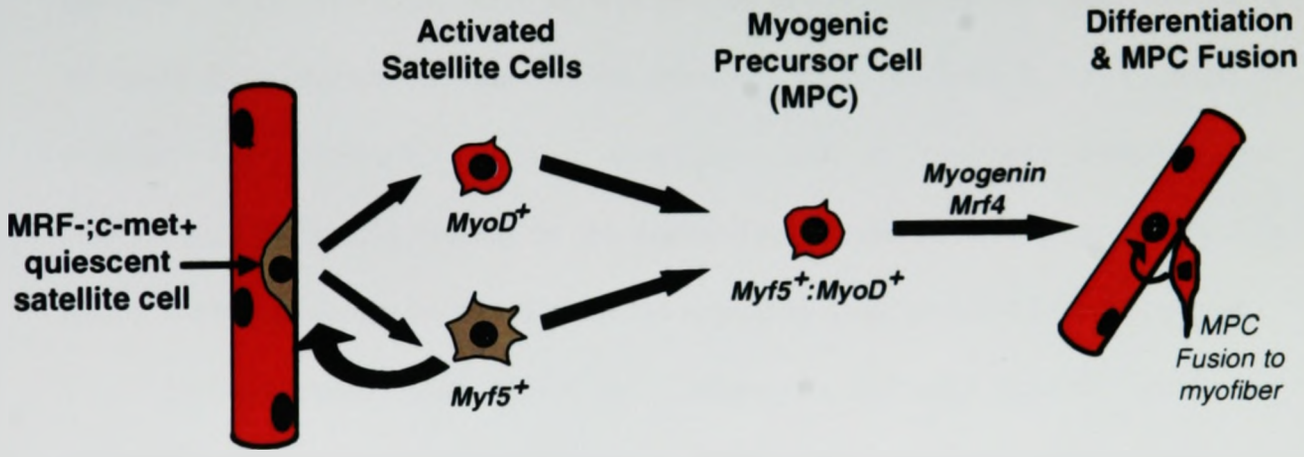
The MRF expression program during satellite cell activation, proliferation, and differentiation appears analogous to the program manifested during the embryonic development of skeletal muscle. Quiescent satellite cells express no detectable levels of MRF mRNA or protein (Cooper et al., 1999; Cornelison and Wold, 1997; Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994). MyoD or Myf5 is rapidly upregulated within

3 hours of experimentally induced muscle injury prior to expression of Proliferating Cell Nuclear Antigen (PCNA), a marker for cell proliferation (Figure 1.3) (Cooper et al., 1999). However, the presence of LacZ positive satellite cells in freshly isolated muscle fibers from *Myf5nLacZ* mice (LacZ expressed from *Myf5* gene locus) has been suggested to indicate expression of *Myf5* in quiescent cells (Beauchamp et al., 2000; Tajbakhsh et al., 1996). It remains to be determined whether satellite cell expression of *Myf5nLacZ* is allele-specific or whether single muscle fiber preparation results in low level expression detectable due to the long half-life of LacZ protein. *Myogenin* is expressed later during the time associated with myoblast fusion and differentiation (Figure 1.3) (Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994).

Analysis of gene expression by RT-PCR within single satellite cells on intact myofibers showed expression of *c-met* (receptor for Hepatocyte Growth Factor) but no detectable levels of *MRF* mRNA in quiescent cells (Cornelison and Wold, 1997). Interestingly, activated satellite cells (satellite cells entering the cell cycle) first express either *Myf5* or *MyoD* followed by a developmental stage in which *Myf5* and *MyoD* are coexpressed (Figure 1.3). *In vivo* analysis of MRF expression by immunohistochemistry in regenerating muscle shows that 50% of activated satellite cells coexpress MyoD and Myf5, 30% express MyoD alone and 20% express Myf5 alone 3 hours postinjury (Cooper et al., 1999). Collectively these results suggest independent entries into the satellite cell developmental program, through activation of either *MyoD* or *Myf5*, analogous to the pattern of MRF expression in embryonic progenitors that give rise to the MyoD-dependent hypaxial and Myf5-dependent epaxial muscles (Kablar et al., 1997).

**Figure 1.3 Myogenic regulatory factors in satellite cells**

Quiescent satellite cells expressing c-met but no detectable levels of MRFs give rise to activated satellite cells that express either Myf5 or MyoD. Proliferative myogenic precursor cells coexpress MyoD and Myf5. Terminal differentiation and fusion of myogenic precursor cells into developing myofibers involves the activity of Myogenin and Mrf4.


Activated
Satellite Cells
Myogenic
Precursor Cell
(MPC)
Differentiation
& MPC Fusion
MRF-;c-met+
quiescent
satellite cell
MyoD+
Myf5+
Myf5+:MyoD+
Myogenin
Mrf4
MPC
Fusion to
myofiber

### 1.7.4 MyoD is required for regenerative myogenesis

To investigate the role of MyoD in satellite cell function, *MyoD*$^{-/-}$ mice (Rudnicki et al., 1992), were interbred with *mdx* mice. The *mdx* mice carry a loss-of-function point mutation in the X-linked *dystrophin* gene, and thus represent an animal model for human Duchenne and Becker muscular dystrophy (Bulfield et al., 1984; Sicinski et al., 1989). The compound mutant mice exhibit markedly increased penetrance of the *mdx* phenotype characterized by muscle atrophy and increased myopathy leading to premature death (Megeney et al., 1996). By three to five months of age, *mdx:MyoD*$^{-/-}$ mice develop a profound dorsal-ventral curvature of the spine similar to the lordosis and kyphosis of patients with Duchenne muscular dystrophy, and an abnormal waddling gait characterized by weight bearing on the hocks. The animals become progressively less active, with concomitant weight loss prior to premature death around 12-months of age.

Skeletal muscle from *MyoD*$^{-/-}$ mice displays a strikingly reduced capacity for regeneration following injury (Megeney et al., 1996). Electron microscopic examination of *MyoD*-deficient muscle reveals increased numbers of morphologically normal satellite cells whose numbers are increased 1.8-fold in *MyoD*$^{-/-}$ muscle and 13-fold in *mdx:MyoD*$^{-/-}$ muscle. However, cellular proliferation during regeneration of *mdx:MyoD*$^{-/-}$ muscle is not detected by 3H-thymidine incorporation or immunohistochemistry with antibody reactive to PCNA. These data suggest a model in which upregulation of MyoD is required for satellite cells to enter the mpc proliferative phase that precedes terminal differentiation. In the absence of MyoD, myogenic progenitors undergo an apparent

increase in numbers as a consequence of an increased propensity for self-renewal rather than progression through their normal developmental program.

Satellite cell-derived primary cultures were generated from adult *MyoD*$^{-/-}$ hindlimb muscle for analysis of their proliferative and differentiation potential. *MyoD*$^{-/-}$ satellite cell derived myogenic cells exhibit a stellate flattened morphology distinct from the compact rounded morphology of wildtype myoblasts (Sabourin et al., 1999). Myogenic cells lacking MyoD express c-met, but do not express Desmin, an intermediate filament protein typically expressed in cultured myoblasts *in vitro* and mpcs *in vivo* (Sabourin et al., 1999). Under conditions that normally induce differentiation of wildtype myoblasts, *MyoD*$^{-/-}$ cells continue to proliferate and only after several days yield reduced numbers of predominantly mononuclear myocytes (Cornelison et al., 2000; Sabourin et al., 1999; Yablonka-Reuveni et al., 1999a). Interestingly, expression of *IGF1* (Insulin-like Growth Factor-1) is markedly increased in *MyoD*$^{-/-}$ myogenic cells cultured in low-mitogen conditions, suggesting that MyoD normally negatively regulates *IGF1* expression in primary myoblasts (Sabourin et al., 1999). Therefore, IGF1 may promote proliferation and inhibit differentiation in *MyoD*$^{-/-}$ myoblasts via an autocrine loop. In addition, expression of *M-cadherin* is notably decreased in *MyoD*$^{-/-}$ myogenic cells and a requirement for M-cadherin has been reported for cell-cycle withdrawal and myoblast fusion (Irintchev et al., 1994; Zeschnigk et al., 1995). Taken together, these data suggest that *MyoD*$^{-/-}$ myogenic cells represent an intermediate stage between satellite cells and mpcs.

1.7.5 Satellite cells as stem cells

Satellite cells are often described as stem cells due to their apparent capacity for self-renewal, their enormous potential for expansion and their ability to generate genetically distinct daughter cells that differentiate as skeletal myocytes. The absence of *MRF* mRNA in satellite cells prior to activation is consistent with the hypothesis that satellite cells represent a stem cell with an identity distinct from myoblasts. It is interesting therefore to speculate that the *de novo* activation of *Myf5* and *MyoD* transcription occurs in response to inductive signals analogous to those that occur during the specification of the myogenic lineage during embryonic development (Cossu et al., 1996b; Reshef et al., 1998).

Although activated satellite cells are restricted to the development of determined muscle cells *in vivo*, satellite cells within intact muscle fibers have the ability to activate alternate developmental programs in cell culture (Asakura et al., 2001; Wada et al., 2002). Furthermore, satellite cell derived myoblasts transdifferentiate to adipocytes or osteocytes in response to various culture conditions (Hu et al., 1995; Lee et al., 2000; Teboul et al., 1995). These results highlight a degree of plasticity for the satellite cell myogenic lineage, and demonstrate the importance of *in vivo* microenvironment cues in promoting efficient myogenic differentiation of satellite cells during skeletal muscle growth and regeneration.

### 1.7.6 Satellite cell self-renewal

The number of quiescent satellite cells in adult muscle remains relatively constant over multiple cycles of degeneration and regeneration, suggesting an inherent capacity for self-renewal (Grounds and Yablonka-Reuveni, 1993; Schultz and Jaryszak, 1985). In addition, the relative number of satellite cells remains constant between adult (2 months) and old mice (> 2 years) (McGeachie and Grounds, 1995). By investigating the replication of satellite cells in immature postnatal muscles, Schultz identified a population of "reserve" satellite cells that divide more slowly and remain largely in the $G_0$ phase of the cell cycle (Schultz, 1996). It was hypothesized that reserve satellite cells function primarily to maintain a pool of quiescent stem cells, which are maintained into adulthood and old age, whereas the more rapidly dividing, metabolically active cells fuse to growing myofibers.

The concept of reserve muscle cells has also been described in cell culture models. Non-fusing mononuclear myogenic cells in differentiating muscle cultures can be cloned and then propagated as fusion competent myoblasts in response to serum stimulation. Subsequent differentiation of these cultures again give rise to a discrete population of non-fusing reserve cells (Baroffio et al., 1996; Carnac et al., 2000; Yoshida et al., 1998). Interestingly, MyoD and Myf5 are downregulated following mitogen withdrawal in non-fusing reserve cells suggesting that they have acquired a stem cell phenotype more closely resembling *in vivo* quiescent satellite cells (Yoshida et al., 1998).

The mechanisms responsible for satellite cell self-renewal in adult muscle are unknown however they may involve asymmetric cell division of a multipotent satellite

cell, which divides and gives rise to a committed mpc and a repopulating "self". A recent study suggests that asymmetric distribution of Numb and differential Notch activity in daughter cells are implicated in asymmetric cell divisions from activated satellite cells (Conboy and Rando, 2002). Numb already has a well-established role in regulating asymmetric cells divisions in the *Drosophila* and mammalian CNS (Dooley et al., 2003; Rath et al., 2002; Shen et al., 2002). Specifically, in satellite cells, activated *Notch-1* may promote the proliferation of reserve, self-renewing cell types (expressing *Pax3* but not *Numb*, *Desmin*, *Myf5* and *MyoD*) whereas its inhibition results in myogenic commitment as indicated by the expression of *Numb*, *Desmin*, *Myf5*, but not *Pax3*. In an analogous manner, activation of the Notch pathway in embryonic development specifically inhibits MyoD expression, yet has no effect on Myf5 or Pax3 expression, preventing differentiation and maintaining proliferation. Further experiments are necessary to determine whether Numb+ and Numb- satellite cell progeny exhibit functional differences *in vivo* with regards to proliferation and differentiation.

Alternatively, activated satellite cells that express Myf5 but not MyoD (i.e. Myf5+MyoD- cell) may exist transiently in regenerating muscle and function primarily for self-renewal. This mechanism is postulated based on the phenotype of *MyoD*$^{-/-}$ satellite cells, which express high levels of Myf5 but have reduced differentiation capacity and an increased propensity for self-renewal.

A third possibility would involve the de-differentiation of committed mpc to satellite cells. Interestingly, overexpression of the homeobox containing msx1 transcription factor in terminally differentiated C2C12 myotubes can cause a small

number of nuclei within myotubes to de-differentiate, and re-enter the cell cycle to generate viable mononuclear myoblasts (Odelberg et al., 2000). Lastly, the possibility that adult stem cells replenish the satellite cell compartment is discussed later. A more complete understanding of the mechanisms responsible for the self-renewal of satellite cells *in vivo* may lead to the development of novel therapeutic approaches to promote muscle cell replacement.

### 1.7.7 Regulation of satellite cell activation and expansion

Satellite cells are activated in response to both modest and severe stressors suggesting that several mechanisms can activate satellite cells. The physiological triggers that lead to satellite cell activation have yet to be defined. However a number of downstream molecules and mechanisms have been studied in regard to both activation of quiescent satellite cells and mpc expansion (Figure 1.4).

#### Hepatocyte Growth Factor (HGF)

Although, the physiological stimuli that trigger satellite cell activation are largely unknown, experimental evidence suggests the involvement of HGF in this process (Figure 1.4). HGF is a potent mitogen and chemotactic agent for satellite cells both *in vivo* and *in vitro* (Allen et al., 1995; Gal-Levi et al., 1998; Miller et al., 2000; Sheehan and Allen, 1999; Sheehan et al., 2000; Tatsumi et al., 1998). Experimentally induced muscle damage results in the rapid colocalization of HGF and c-met *in vivo* (Tatsumi et al., 1998). Intramuscular infusion of HGF followed by BrdU injection, results in

increased recovery of BrdU positive mpcs after 30 hours in culture. Taken together these results suggest that HGF directly induces the activation of satellite cells. The chemotactic activity of HGF suggests that it not only activates satellite cells but also induces their migration to sites of muscle damage (Bischoff, 1997).

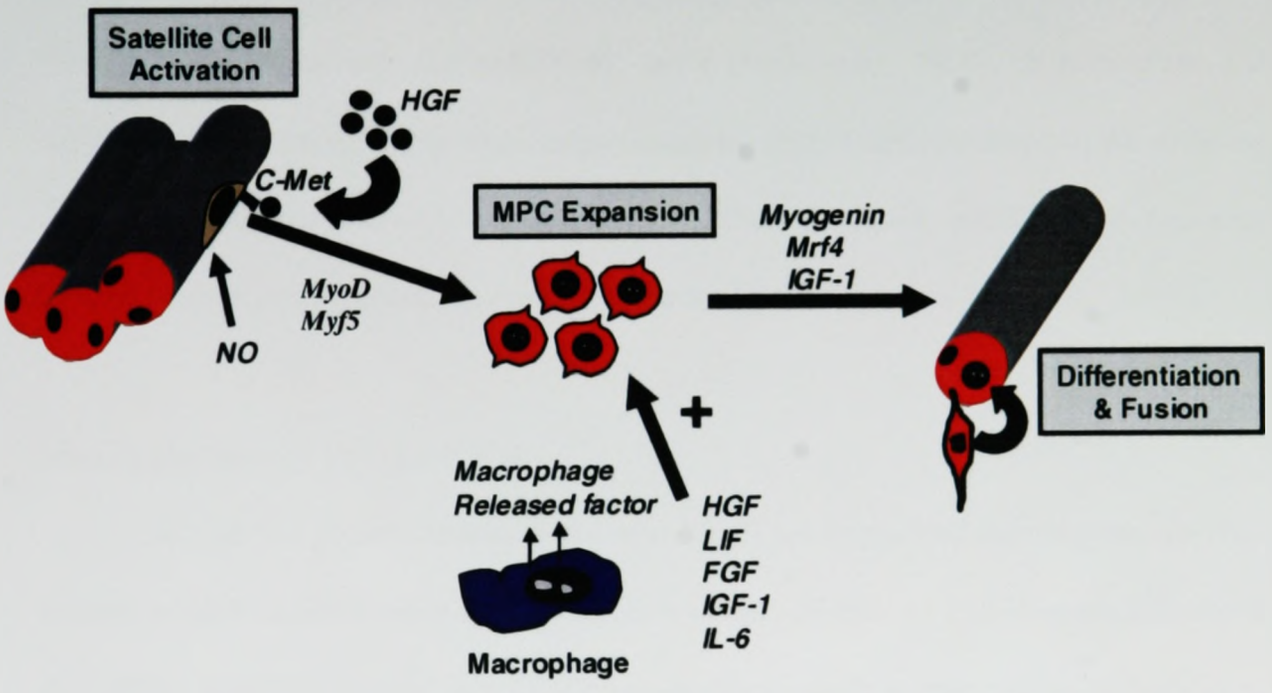
Interestingly, administration of exogenous HGF into regenerating muscle is only able to increase the size of the mpc pool for up to one day following injury (Miller et al., 2000). The exogenous HGF actually reduces the overall efficiency of muscle regeneration suggesting that HGF also inhibits myoblast differentiation (Gal-Levi et al., 1998; Miller et al., 2000).

Fibroblast Growth Factors (FGFs)

Several members of the FGF family are expressed in developing skeletal muscle including FGF-1, 2, 4 5, 6, 7, 8 (Mason, 1994). In addition, FGFR-1 and -4 (FGF-Receptor-1,-4) are expressed in quiescent muscle satellite cells suggesting a direct role for FGF signaling in satellite cell activation. FGF2 (bFGF) positively regulates myoblast proliferation and also inhibits terminal differentiation and myotube formation in culture (Doumit et al., 1993; Kuschel et al., 1999; Sheehan and Allen, 1999; Yablonka-Reuveni et al., 1999b). Furthermore, expression levels of FGF2 in damaged mouse muscle correlates with the regenerative efficiency observed in different genetic backgrounds (Anderson et al., 1995). Interestingly, infusion of FGF2 into regenerating muscle stimulates a significant increase in the concentration muscle IGF1 and total DNA but does not lead to increased protein concentration (Mitchell et al., 1996). These results

**Figure 1.4 Regenerative myogenesis**

The mechanism for satellite cell activation likely requires the activity of HGF, which binds its cognate receptor, c-met expressed on quiescent satellite cells. Release of Nitric Oxide (NO) from damaged fibers has also been suggested to activate satellite cells. Following activation, satellite cells give rise to daughter myogenic precursor cells (MPCs) which undergo multiple rounds of division in response to a variety of growth factors. Following the expansion of MPCs, they differentiate and fuse into regenerating myofibers. (Reprinted from Stem Cells: A Cellular Fountain of Youth, Patrick Seale and Michael A. Rudnicki, Adult Skeletal Muscle Growth and Regeneration, p 117-200, Copyright 2003, with permission from Elsevier)


Satellite Cell Activation
HGF
C-Met
NO
MyoD
Myf5
MPC Expansion
Myogenin
Mrf4
IGF-1
Differentiation & Fusion
+
Macrophage Released factor
HGF
LIF
FGF
IGF-1
IL-6
Macrophage

suggest that excess FGF2 antagonizes the differentiation of myogenic cells thereby preventing their fusion into myofibers.

A role for Fibroblast Growth Factor-6 (FGF6) in regulating either the proliferation or differentiation of mpcs is proposed based on the muscle regeneration deficit in mice carrying a targeted null mutation in FGF6 (Floss et al., 1997). This study suggests that FGF6 positively regulates mpc expansion during muscle regeneration. A recent report by Fiore et al., however, demonstrates no impairment in satellite cell function in independently derived *FGF6*$^{-/-}$ mice (Fiore et al., 2000). It is possible that different genetic backgrounds and compensation by other FGFs may explain the differing results. The extent to which other members of the FGF family participate in postnatal muscle growth and regeneration remains to be analyzed.

Insulin-like Growth Factors (IGFs)

Insulin-like growth factors (IGF1 and IGF2) are implicated as mitogens, survival factors as well as differentiation factors for a variety of diverse cell lineages (Benito et al., 1996). Evidence for the role of IGFs in skeletal muscle is exemplified in transgenic mice lacking the IGF1-Receptor (IGF1R) or IGF1 and IGF2, which display severe muscle hypotrophy resulting in premature death (Liu et al., 1993; Powell-Braxton et al., 1993a; Powell-Braxton et al., 1993b). Furthermore, IGF1 concentrations increase in muscle undergoing a hypertrophic response in response to functional overload (Adams and Haddad, 1996; Adams and McCue, 1998; Edwall et al., 1989; Rosenblatt et al., 1994). Consistent with a specific requirement for IGF1 in myofiber growth, direct

infusion of IGF1 into the tibialis anterior muscles of adult rats is sufficient to induce a hypertrophic response (increase in protein content and size of myofibers) (Adams and McCue, 1998). The IGF1 induced hypertrophy leads to increased total muscle protein as well as increased DNA content indicating that the response is at least partially mediated by recruitment of satellite cell nuclei to the muscle (Adams and Haddad, 1996; Rosenblatt et al., 1994). Conversely, intravenous administration of IGF1 had no effect on skeletal muscle demonstrating the requirement for local IGF1 production during regenerative and hypertrophic responses.

Recently, IGF1 has been demonstrated to induce the Calcineurin-NFAT (Nuclear Factor of Activated T cells) signaling pathway leading to activation of GATA2, a transcription factor whose up regulation is associated with myofiber hypertrophy (Musaro et al., 1999; Semsarian et al., 1999; Shibasaki et al., 1996). Additionally, IGF1 has been shown to promote myoblast survival by two independent PI3' Kinase (Phosphatidyl-Inositol-3' Kinase) pathways that both result in increased expression of the p21 cdk inhibitor (Lawlor et al., 2000; Lawlor and Rotwein, 2000a; Lawlor and Rotwein, 2000b). Therefore, IGF1 likely stimulates both mpc proliferation, and increased myofiber protein synthesis during muscle hypertrophy. Whether IGF1 directly activates satellite cells or alternatively whether IGF1 induced anabolic effects stimulate satellite cells via HGF or FGFs remains to be determined.

Nitric Oxide

A role for Nitric Oxide (NO) mediated satellite cell activation has recently been described (Anderson, 2000). By this model, bioactive NO, released locally from the membrane bound Dystrophin complex within damaged myofibers, induces the rapid activation of nearby satellite cells (Figure 1.4). Consistent with this model is the observation that inhibition of NOS (Nitric Oxide Synthase) activity was detrimental to experimentally induced regeneration (Anderson, 2000). However the normal histological appearance of NOS deficient muscle suggests that this mechanism is not absolutely required. Further examination of the role of NO signaling in satellite cell function is thus warranted.

The Inflammatory Response

Polymorphonuclear lymphocytes (PMNL) and macrophages (activated monocytes) migrate to sites of tissue damage within a few hours after trauma to muscle. Macrophages however, are the dominant immune cells present within regenerating muscle at 48 hours postinjury (Orimo et al., 1991; Tidball, 1995). The role of macrophages in muscle regeneration is two fold in that macrophages phagocytose necrotic cell debris as well as secrete a soluble growth factor, which exerts a specific mitogenic effect on myoblasts (Figure 1.4) (Cantini and Carraro, 1995; Cantini et al., 1994; Merly et al., 1999). A vital role for macrophages in muscle regeneration is supported by the observation that myogenesis is markedly impaired in the absence of monocyte/macrophage infiltration (Lescaudron et al., 1999).

The cytokines IL6 (Interleukin-6) and LIF (Leukemia Inhibitory Factor) stimulate the proliferation of mpcs in culture (Figure 1.4) (Austin et al., 1992; Kurek et al., 1997; Kurek et al., 1996). In contrast to the late induction of *IL6*, LIF expression is markedly increased 3 hours after muscle injury (Kurek et al., 1996). Moreover *LIF* mRNA is expressed in cultured myoblasts (Kurek et al., 1996), suggesting that damaged muscle secretes LIF prior to infiltration of immune cells. Importantly, *LIF*-deficient mice display a markedly reduced capacity for muscle regeneration demonstrating that secretion of LIF in damaged muscles improves myofiber repair (Kurek et al., 1997). Continuous infusion of LIF to the dystrophic diaphragm of *mdx* mice results in reduced degeneration and increased myofiber diameter demonstrating a potential clinical application for LIF in promoting muscle growth and repair (Austin et al., 2000).

A role for leukocytes in satellite cell activation has also been proposed based on the observation that quiescent satellite cells express Vascular Cell Adhesion Molecule-1 (VCAM1) (a cell surface integrin molecule), whereas infiltrating leukocytes express the specific co-receptor VLA-4 (Integrin $\alpha 4\beta 1$) (Jesse et al., 1998; Rosen et al., 1992; Yang et al., 1996a). These observations suggest a model by which cell-cell interactions mediated by VCAM1/VLA-4 initiates responses in satellite cells and immune cells to promote regeneration.

## 1.8 Adult stem cells in muscle repair

Although satellite cells have a clear role in the postnatal growth and regeneration of skeletal muscles, independent stem cell populations with myogenic potential have

recently been purified from skeletal muscle and other tissues. The identification and characterization of adult stem cells with myogenic potential has promising therapeutic applications for the treatment of degenerative muscle disease. The rapid death and insufficient dissemination of satellite cell myogenic progenitors following their transplantation into muscle indicates that a viable cellular therapy for muscle requires the ability to deliver progenitors to skeletal muscles via the circulation (reviewed by Partridge, 2000). A study by Ferrari et al., first demonstrated the ability of bone marrow cells to give rise to myogenic cells that participate in muscle regeneration. In this study, bone marrow cells were isolated from mice carrying a muscle specific *LacZ* transgene and subsequently transplanted into immunodeficient recipients (Ferrari et al., 1998). Small numbers of X-Gal expressing myofibers were formed after experimentally induced muscle injury. A similar study demonstrated the recruitment of donor derived bone marrow cells to skeletal and cardiac muscles of *mdx* mice (Bittner et al., 1999).

More recently, La Barge et al. demonstrate that bone marrow derived cells can form satellite cells in skeletal muscle following several months of exercise induced regeneration (LaBarge and Blau, 2002). The capacity for bone marrow stem cells to first differentiate as satellite cells, thereby providing a renewable source of myogenic progenitors, has profound implications for cell-based therapies.

In addition to myogenic progenitors derived from marrow, stem cell populations resident to skeletal muscle have also been defined. The inability for circulating cells to restore stem cell pools and promote muscle regeneration following local limb irradiation

argues that stem cells responsible for muscle regeneration are resident to the tissue (Heslop et al., 2000; Pagel and Partridge, 1999; Wakeford et al., 1991).

The side-population (SP) phenotype, based on the differential exclusion of Hoechst-dye, enriches for long-term repopulating stem cells from bone marrow (Goodell et al., 1996; Goodell et al., 1997). Gussoni et al. demonstrated that this procedure also enriched for progenitors in skeletal muscle, which efficiently reconstitute the blood system of irradiated mice and also contribute to regenerative myogenesis (Gussoni et al., 1999). Muscle SP cells also have the capacity to form satellite cells following intramuscular or intravenous transplantation (Asakura et al., 2002; Gussoni et al., 1999). Other muscle cell populations isolated on the basis of cell-surface marker expression, including CD34 and Sca1, have been described as possessing stem cell characteristics (Lee et al., 2000; Torrente et al., 2001). All of these populations however have yet to be rigorously studied. For example, it is unclear whether these stem cell populations possess a physiological role in normal skeletal muscle development and regeneration. Another outstanding question is whether a common muscle-derived progenitor gives rise to both blood and muscle lineages. Clonal analysis of these various skeletal muscle cell compartments will be required to address this issue.

The inability for adult stem cells to undergo myogeneseis in culture indicates that regenerating muscle provides the requisite microenvironmental cues to permit myogenic differentiation. Recently, small numbers of cultured neural stem cells were shown to differentiate as skeletal myogenic cells following exposure to myoblasts in coculture experiments (Galli et al., 2000). This study suggests that cell-cell interactions and growth

factors secreted by myoblasts are sufficient to allow neural stem cells to adopt a myogenic fate. Identification of the cell-surface molecules and soluble protein factors that drive stem cells along different developmental pathways may provide new approaches to stimulate regeneration and treat degenerative diseases.

### 1.9 Muscle atrophy and aging

Aging results in a progressive loss of muscle mass and strength due to the degeneration of myofibers possibly in response to neurodegeneration (Carlson, 1995; Lamberts et al., 1997). Between the ages of 30 and 80 in humans it is estimated that there is a reduction of ~30% in muscle mass with a selective loss of fast fiber types (Tzankoff and Norris, 1977). It is also clear that old muscle is more susceptible to injury and incomplete regeneration following exercise (Brooks and Faulkner, 1994; Faulkner et al., 1995). Related to loss of muscle mass and function is the reduced activity of the growth hormone (GH)/IGF1 axis and an increase in muscle catabolism (Lamberts et al., 1997).

The replicative capacity of mpcs from old muscle is seemingly intact in spite of an increased lag phase prior to the onset of DNA synthesis relative to cells from younger hosts (Dodson and Allen, 1987; Johnson and Allen, 1993; McGeachie et al., 1993; Schultz and Lipton, 1982). Furthermore, the stability of telomeres in satellite cells from young and old human muscle alike indicates that satellite cells undergo minimal turnover throughout adulthood and further suggests that a competent satellite cell population is present in aging muscles (Decary et al., 1997). Moreover, the relative proportion of satellite cells present in old muscle however remains stable (McGeachie et al., 1993).

The inefficient regenerative processes in old muscle may thus result from alterations to *in vivo* microenvironmental components required for efficient activation and subsequent differentiation of satellite cells (Grounds, 1998; Nnodim, 2000). Thus the degeneration of aging muscle seemingly reflects an inability to activate resident satellite cells rather than a defective stem cell compartment.

The development of strategies to prevent the aging associated loss in muscle mass is an active area of investigation. Several groups report protection against muscle loss following intramuscular delivery of IGF1. For example, viral mediated expression of IGF1 blocks the loss of skeletal muscle function in old mice (Barton-Davis et al., 1998). Moreover, treatment of atrophied old muscle with IGF1 leads to recovery of satellite cell activity and a substantial increase in muscle mass (Chakravarthy et al., 2000). Preventing the loss of muscle mass is also achieved by Growth Hormone (GH) administration which is associated with increased levels of IGF1 (Papadakis et al., 1996).

## 1.10 Muscular dystrophies

Muscular Dystrophy refers to a collection of more than 20 inherited disorders affecting approximately 1/3500 live births worldwide (Ozawa et al., 1998; van Essen et al., 1992). About 2/3 of muscular dystrophies are classified as Duchenne Muscular Dystrophy (DMD) or its milder allelic form, Becker's Muscular Dystrophy caused by loss of function mutations in the X-linked Dystrophin gene. Dystrophin is anchored to the cytoplasmic surface of the myofiber sarcolemma where it forms subsarcolemmal cytoskeletal networks (Bonilla et al., 1988). As such, Dystrophin and its associated

proteins (including the dystroglycan complex, and the α-sarcoglycan complex) links the actin-based cytoskeleton of muscle to the extracellular matrix (ECM), thus protecting muscle fibers from contraction induced damage (Ervasti et al., 1990; Ozawa et al., 1998). Dystrophin also interacts with cytoplasmic proteins including members of the Syntrophin family (Bredt; 1999; Brenman et al., 1995). Syntrophins in turn, are associated with NOS, which mediates intramuscular signaling by releasing NO (Bredt, 1999). Therefore, the Dystrophin complex not only protects myofibers from sarcolemmal damage, but also regulates aspects of muscle function through intracellular signaling.

The contraction induced myofiber damage caused by loss of function of the Dystrophin complex continuously activates resident satellite cells. Repeated cycles of degeneration and regeneration in the skeletal muscle of DMD patients ensues, eventually exhausting the replicative capacity of satellite cells (Blau et al., 1983; Ontell, 1981; Ontell, 1986; Ontell et al., 1984; Reimann et al., 2000; Wright, 1985). Thus, in contrast to muscle pathology in old muscle which reflects inefficient satellite cell activation, the final stages of DMD results from a reduction in satellite cell replication. These observations suggest that engraftment of wildtype (dystrophin+) satellite cells in dystrophic muscles is a viable therapy for DMD.

## 1.11 Cell-based therapies for muscle disease

Cell mediated therapies in which determined myoblasts are introduced into regenerating dystrophic muscle have had limited success (Blau and Springer, 1995; Gussoni et al., 1997; Gussoni et al., 1992; Partridge et al., 1998; Tremblay et al., 1993;

Vilquin et al., 1996). The inefficacy of this approach is due to the rapid loss of transplanted mpcs from the host muscle immediately after injection as well as the confinement of injected myoblasts to the area of injection. It is estimated that <1% of transplanted cells survive 4 days following transplantation (Beauchamp et al., 1999). Therefore, to achieve therapeutic benefit, MT would require numerous myoblast injections. These results suggest that efficient MT therapies require the introduction of myogenic cells with "stem cell" properties for repopulation of the satellite cell compartment. In this regard, recent reports describe the isolation of myogenic cells with "stem cell" characteristics (Lee et al., 2000; Qu et al., 1998; Smith and Schofield, 1997; Torrente et al., 2001). An elegant study by Beauchamp et al., describes the characteristics of slowly dividing mpcs with increased capacity for survival following MT (Beauchamp et al., 1999). Their results suggest that the ~1% of cells that survive MT may represent the *in vivo* counterparts of reserve cells previously described in myogenic cell cultures.

The inability of wildtype myogenic cells to persist in dystrophic muscle may also reflect defects in the ability of *dystrophin*$^{-/-}$ fibers to recruit satellite cells. For example, the absence of Dystrophin may modulate the structure of the sarcolemma and interfere with the homing and integration of satellite cells. Although MT still holds promise for clinical application, the biological properties of adult stem cells may hold the key for efficient cell mediated therapy in MD and other degenerative conditions.

The documented plasticity of adult stem cells isolated from a number of tissues raises the potential for these cells to be isolated and used for the treatment of a variety of

degenerative conditions including MD. Given the capacity for adult stem cells to circulate in the bloodstream and home to areas of regeneration, it may be feasible to deliver stem cells intravenously and allow them to infiltrate degenerating target tissue. Stem cells may also have the distinct advantage of providing a long-term source of myogenic progenitors in patients with MD. Stable engraftment of stem cells that give rise to satellite cells would provide a source of myogenic progenitors to repair damaged fibers throughout a patient's lifespan.

Several issues will need to be resolved before stem cell transplantation can become a clinical reality. It remains to be determined if stem cell transplantations can achieve high degrees of stable engraftment into host tissues. Furthermore, isolating large numbers of stem cells, which maintain their capacity to differentiate, will be essential for therapeutic application. Stem cell therapies may also be improved by identifying growth factors or molecules, which facilitate subsequent differentiation. For example, the introduction of developmental control genes into stem cells may direct their differentiation along a particular pathway.

## 1.12 Summary of intent

Satellite cells represent a tissue specific somatic stem cell population activated during muscle regeneration and hypertrophic responses. As outlined in the introduction, the biological role of satellite cells has been documented; however the genetic and molecular mechanisms regulating their function remain to be studied.

To gain insight into the developmental program initiated by quiescent satellite cells in response to injury, we aim to identify genes expressed specifically by primary wildtype and *MyoD*$^{-/-}$ satellite cell derived myogenic cells. Expression and functional analyses of satellite cell specific genes will be used to elucidate their role in myogenic specification, differentiation and muscle repair processes. Genes identified may represent satellite cell specific markers, genes involved in satellite cell activation and putative MyoD target genes. The identification of satellite cell specific markers will provide an invaluable resource for studies into satellite cell function. In chapter 2, a description of the methodology employed to identify and characterize the expression of satellite cell specific genes is presented.

Chapter 3 describes the function of the paired-box transcription factor, Pax7, (identified in Chapter 1) in postnatal muscle. Specifically, examination of *Pax7*-deficient muscle revealed a requirement for this factor in the development of muscle satellite cells. In Chapter 4, *Pax7*$^{-/-}$ animals were bred into a different genetic background to prolong survival and study acute muscle regeneration in the absence of Pax7. This work confirmed the absence of satellite cells and revealed a Pax3-expressing, Pax7-independent progenitor population with a reduced ability to participate in regeneration.

To determine a role for endogenous adult stem cells during skeletal muscle repair, the myogenic capacity of cells expressing the hematopoietic antigens CD45 and Sca1 was assessed in uninjured and regenerating muscles. Results from this work are presented in Chapter 5 and revealed a specific induction of myogenesis in $CD45^{+}$:$Sca1^{+}$ cells during regeneration in response to Wnt signals. The myogenic activation of these CD45+ progenitors was dependent of the activity of Pax7 as shown in chapter 6. The capacity for Pax7 to induce the myogenic activation of resident adult stem cells is also described in chapter 6.

In summary, this body of work demonstrates the key role for Pax7 in the specification of adult myogenic progenitors required for skeletal muscle regeneration and tissue maintenance. Future experiments ongoing in the lab will elucidate the biochemical pathways that regulate the induction of Pax7 and its downstream target genes.

# Chapter 2

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

**Preface:**

The results presented in Chapter 2 were prepared as a manuscript to be submitted for publication. I performed the majority of the experiments described in this chapter, assembled the data and figures, and wrote the manuscript. Chet Holterman provided expression data for some of the genes described in the article including Myod p67, MyoD p40, and *Hoxc10* that appear in Table II. Considerable advice and direction for the project and contribution to the final version of the manuscript were given by my supervisor, Dr. M.A. Rudnicki.

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

Patrick Seale[1,2], Chet Holterman[2] and Michael A. Rudnicki[1,2,3]

[1]Department of Biology
McMaster University
Hamilton, Ontario
Canada, L8S 4K1

[2]Ottawa Health Research Institute
Molecular Medicine Program
501 Smyth Road,
Ottawa, Ontario
Canada, K1H 8L6
Tel: 613-739-6740
Fax: 613-737-8803

[3]Correspondence should be addressed to M.A.R.

E-mail: mrudnicki@ohri.ca

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## 2.1 Abstract

Muscle satellite cells are a distinct adult progenitor population in postnatal muscle that mediate growth and repair processes. In this study, representational difference analysis of cDNAs was employed to identify genes expressed in satellite cell-derived myoblasts from wildtype and *MyoD*$^{-/-}$ muscle. The capacity for *MyoD*$^{-/-}$ cells to engraft skeletal muscle and be maintained as myogenic progenitors after transplantation was exploited to define satellite cell-specific markers. Our analysis revealed a number of genes expressed in quiescent and activated satellite cells including: *Pax7*, *Asb5*, *IgSF4* and *Hoxc10*. Expression analysis also suggested roles for several previously uncharacterized genes downstream of MyoD during differentiation including: *Dapk2*, *Sytl2* and *NLRR1*. Moreover, genes expressed at high levels in *MyoD*$^{-/-}$ myoblasts relative to wildtype myoblasts were also expressed by satellite cells *in vivo*. These results imply that myogenic cells expressing Myf5 but not MyoD (Myf5+:MyoD-) may play a specific role during muscle growth and regeneration.

## 2.2 Introduction

Muscle satellite cells are specialized myogenic progenitors that are activated during the postnatal growth and regeneration of skeletal muscle. In particular, satellite cells are highly proliferative in growing postnatal muscles and contribute the majority of adult myonuclei (Schultz, 1996). In 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; reviewed in Bischoff, 1994; Hawke and Garry, 2001; Mauro, 1961; Seale and Rudnicki, 2000). In response to a variety of stimuli including exercise, stretching and injury, activated satellite cells 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).

Due to their low abundance in adult muscle (2-5% of sublaminar nuclei) it has been difficult to investigate early events associated with satellite cells activation. Additionally, there remains a paucity of genetic markers unique to the satellite cell lineage as 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 their myogenic derivatives and other lineages. The identification and characterization of novel genes expressed in satellite cells is essential for elucidating the molecular pathways associated with satellite cell function during tissue growth and regeneration.

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; Sabourin et al., 1999; Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994). However, the expression of LacZ in satellite cells on freshly isolated fibers from *Myf5nLacZ* transgenic mice suggests that *Myf5* transcripts are induced at low levels in quiescent satellite cells (Beauchamp et al., 2000). Following activation, satellite cells upregulate either *MyoD* or *Myf5* mRNA prior to 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* prior to coexpressing both factors (Cornelison and Wold, 1997). These observations suggest the hypothesis that activated satellite cells possess differential biological properties depending upon whether they initially activate *MyoD* or *Myf5*.

Studies performed in our laboratory 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 satellite cells to self-renew rather than differentiate. Consistent with these findings, *MyoD*-deficient satellite cell-derived myoblasts display a profound differentiation deficit and an increased growth rate *in vitro* (Cornelison et al., 2000; Sabourin et al., 1999; Yablonka-Reuveni et al., 1999a). Significantly, $MyoD^{-/-}$ cells express increased levels of Myf5 mRNA and protein

(Sabourin et al., 1999), demonstrating the inability of Myf5 to compensate for the loss of MyoD during differentiation.

The phenotype of *MyoD*-deficient adult muscle suggested the hypothesis that activated satellite cells expressing Myf5 alone (i.e. Myf5+:MyoD-) are arrested at a primitive developmental stage (Sabourin et al., 1999; reviewed by Seale and Rudnicki, 2000). Therefore, cultured *MyoD*$^{-/-}$ myoblasts may serve as a unique genetic model 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 satellite cell-derived myoblasts from wildtype and *MyoD*-deficient muscle.

Our goal was to identify genes that define the identity of proliferating myogenic precursors. In our first experiment, myoblast specific mRNAs were identified by subtracting wildtype myoblast cDNAs against fibroblast and whole muscle cDNA. In this screen, the paired-box domain transcription factor Pax7 was identified as a satellite cell specific gene.

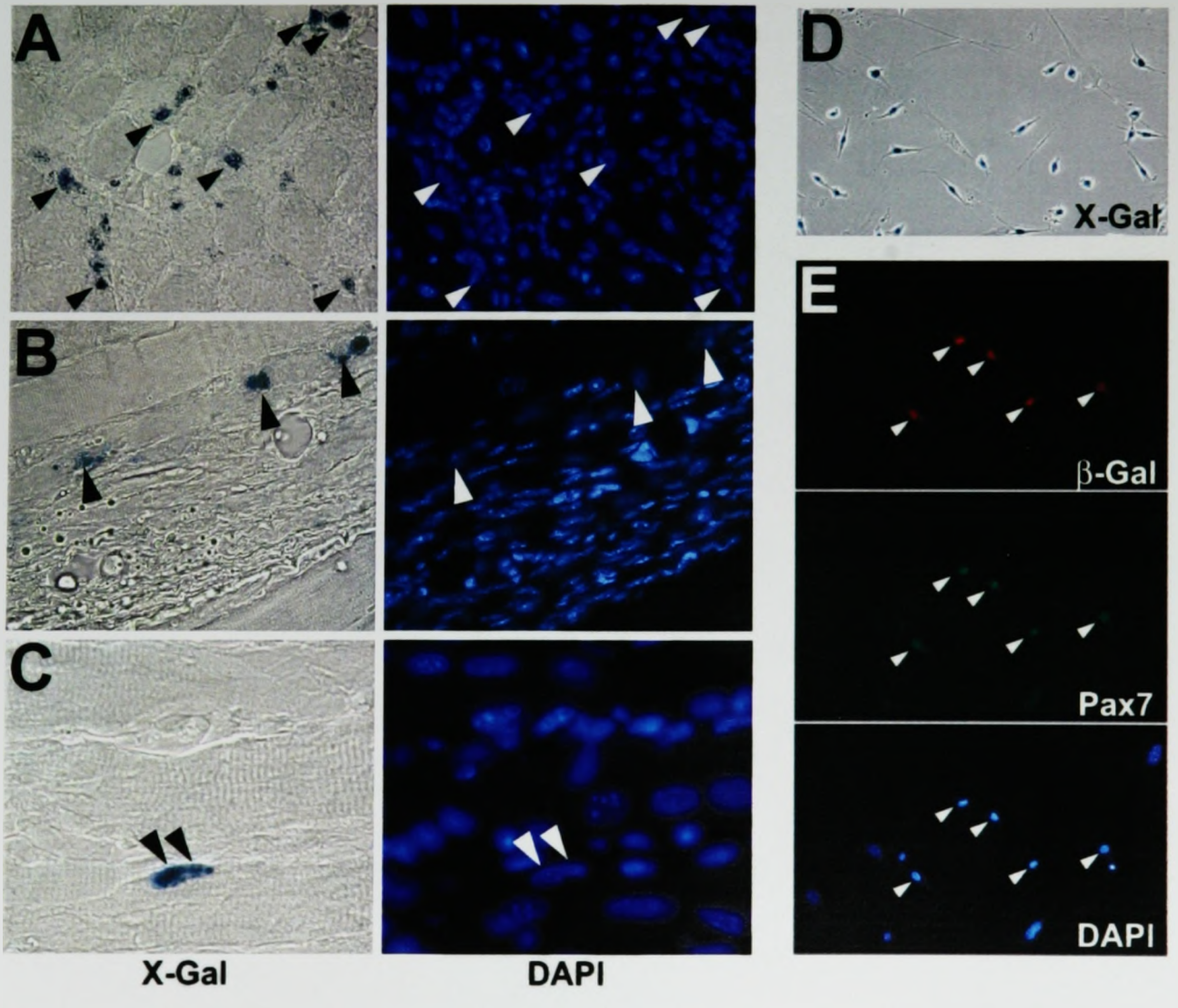
An independent set of genes was isolated by a similar genetic screen performed with *MyoD*$^{-/-}$ myoblasts. Interestingly, several genes isolated from *MyoD*$^{-/-}$ myoblasts were not expressed or expressed at reduced levels in wildtype cells. Importantly, these genes were expressed *in vivo* by satellite cells and myoblasts in uninjured and regenerating *mdx* muscle respectively. Our experiments thus present a collection of cDNAs expressed in the satellite cell lineage, which will form the basis of further studies investigating satellite cell biology and muscle regeneration.

## 2.3 Results

### 2.3.1 *MyoD*$^{-/-}$ cells occupy a stem cell niche in skeletal muscle

Previous studies suggested that *MyoD*$^{-/-}$ myoblasts model an intermediate cell type in regenerating muscle downstream of quiescent satellite cells but not yet committed to undergo terminal differentiation (Megeney et al., 1996; Sabourin et al., 1999). Based on this hypothesis, we investigated whether LacZ marked *MyoD*$^{-/-}$ myoblasts were capable of stable engraftment into muscle following regeneration. For this experiment, $5x10^5$ *MyoD*$^{-/-}$ myoblasts stably transfected with *PGK-nLacZ* were transplanted into regenerating tibialis anterior (TA) muscles of immuno-compromised *Scid/bg* mice (n=4). Two months after transplantation, muscles were processed for immunohistochemical detection of β-Gal activity to identify donor cells *in situ*. *MyoD*$^{-/-}$ cells were readily detected in host TA muscles two months after transplantation within muscle fibers and also in the interstitial areas (Figure 1A-C). The percentage of donor *MyoD*-deficient nuclei detected in transplanted muscles was approximately 5-fold increased over the level of engraftment observed with wildtype cells. These data suggest a survival and/or proliferative advantage of transplanted *MyoD*$^{-/-}$ as compared to wildtype myoblasts.

To determine whether *MyoD*$^{-/-}$ cells were capable of contributing to the satellite cell compartment, myoblasts were derived from muscles two months after cell transplants (n=2). Strikingly, a large proportion of LacZ-expressing *MyoD*-deficient myogenic cells (Figure 1D) were readily cultured from single transplanted TA muscles. By 10 days in myoblast growth conditions, 65% of isolated myogenic Pax7-expressing cells (5.4% of


A
B
C
D
E
X-Gal
β-Gal
Pax7
DAPI
X-Gal
DAPI

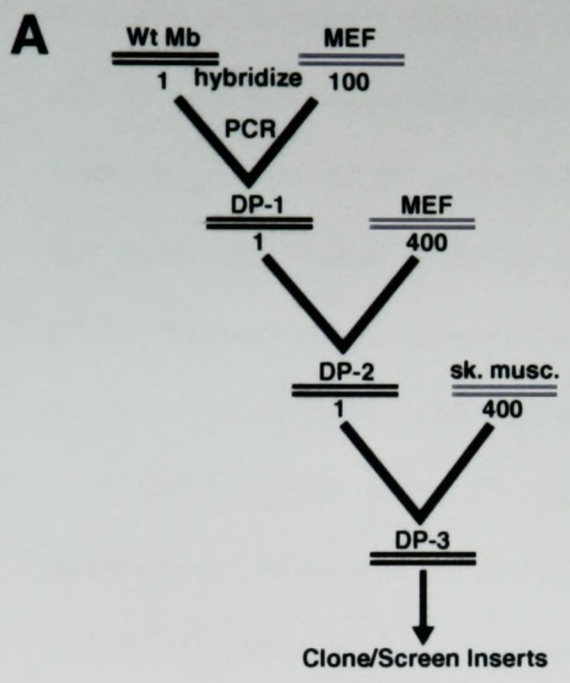
total cells) were donor-derived *MyoD*$^{-/-}$ myoblasts. By contrast, LacZ-expressing myoblasts were not detected in cell cultures prepared from TA muscles transplanted with LacZ-marked wildtype cells. Importantly, β-Gal expressing *MyoD*$^{-/-}$ cells had maintained their myogenic identity as revealed by expression of the satellite cell markers Pax7 (Figure 1E) and c-Met (not shown). Our results thus indicate a capacity for *MyoD*$^{-/-}$ cells to occupy a niche in skeletal muscle associated with maintenance of myogenic and proliferative potential.

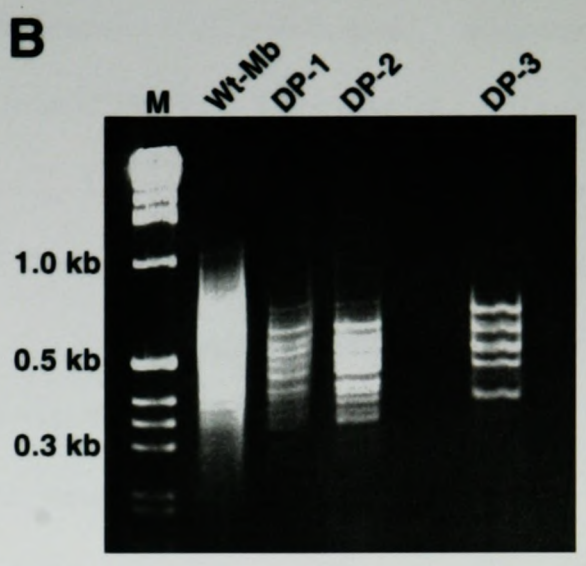
### 2.3.2 Expression profiling of wildtype and *MyoD*$^{-/-}$ myoblasts

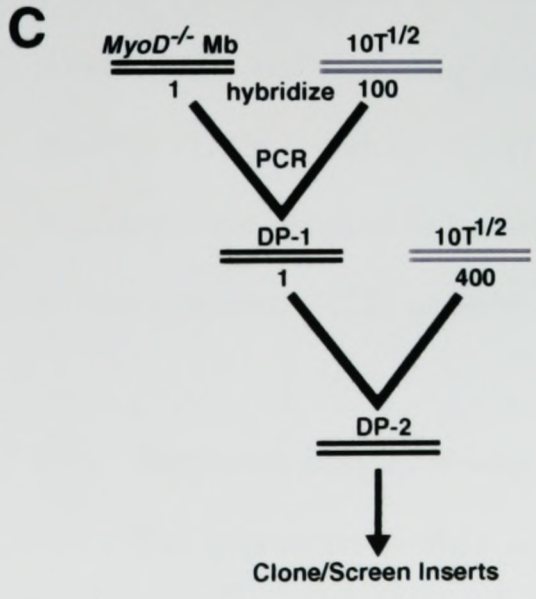
Representational difference analysis of cDNAs (cDNA RDA) was employed to generate libraries of genes expressed in cultured satellite cell derived myoblasts from wildtype and *MyoD*$^{-/-}$ muscle in separate experiments. For wildtype myoblasts, double stranded cDNAs were subtracted against cDNAs obtained from mouse embryonic fibroblasts (MEFs) at ratios of 1:100 and 1:400 to obtain the first and second difference products (wtDP1 and wtDP2) respectively (Figure 2A). 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 wildtype myoblast cDNA mixture was reduced progressively in wtDP1 and wtDP2, resulting in the appearance of several distinct cDNA products in wtDP3 (Figure 2B).

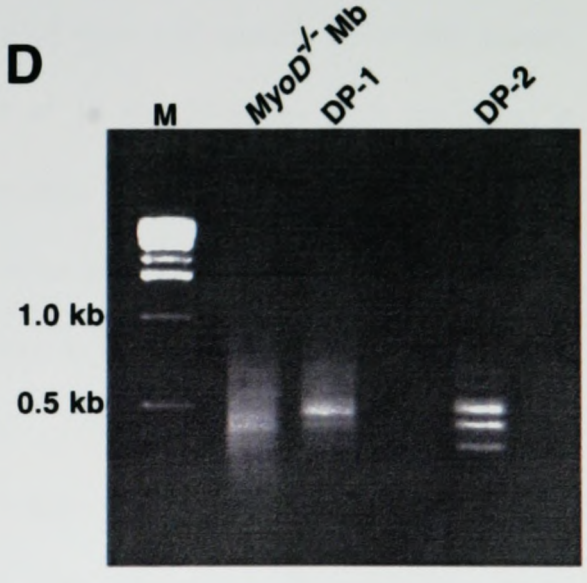
**Figure 2 Experimental strategy for identifying myoblast specific mRNAs**

(**A**) Wildtype 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 in hybridizations are indicated. (**B**) Agarose gel electrophoresis of the starting pool of wildtype myoblast (Wt-Mb) cDNAs and subtracted DP-1, DP-2 and DP-3 showed a progressive enrichment of specific cDNA molecules. (**C**) *MyoD*$^{-/-}$ myoblast specific cDNAs were cloned after subtraction against C3H10T1/2 cDNAs. (**D**) Agarose gel analysis of the starting *MyoD*$^{-/-}$ myoblast cDNA pool (*MyoD*$^{-/-}$ Mb), DP-1 and DP-2 revealed the appearance of distinct cDNAs in DP-2.


A
Wt Mb
MEF
1
hybridize
100
PCR
DP-1
MEF
1
400
DP-2
sk. musc.
1
400
DP-3
Clone/Screen Inserts


B
M
Wt-Mb
DP-1
DP-2
DP-3
1.0 kb
0.5 kb
0.3 kb


C
MyoD-/- Mb
10T1/2
1
hybridize
100
PCR
DP-1
10T1/2
1
400
DP-2
Clone/Screen Inserts


D
M
MyoD-/- Mb
DP-1
DP-2
1.0 kb
0.5 kb

A similar strategy was used to identify genes characteristic of proliferating *MyoD*$^{-/-}$ myoblasts which may contribute to their "stem cell" properties demonstrated in this study and earlier reports (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 (Figure 2C). C3H10T1/2 fibroblasts were used in this experiment instead of MEFs to prevent the exclusion of genes coexpressed in primary multipotent mesenchymal "stem cells" and activated *MyoD*$^{-/-}$ satellite cells. After two rounds of subtraction, mdDP2 contained several distinct cDNA species (Figure 2D).

cDNA pools in wtDP3 and mdDP2 were randomly cloned into pCR2.1 (Invitrogen). To saturate the analysis, 400 individual clones from subtracted wildtype and *MyoD*$^{-/-}$ libraries were screened. Dot-blots consisting of PCR amplified RDA products were hybridized with mixtures of labeled clones to identify redundant inserts. After dot-blot and sequencing analysis, a total of 18 and 34 genes were identified in wildtype (Table I) and *MyoD*$^{-/-}$ (Table II) myoblast screens respectively. All cDNA sequences were compared to known database entries in GenBank to establish the identity of cloned products (NCBI accession numbers given in Tables I and II).

### 2.3.3 Expression analyses of wildtype RDA products

The expression profiles of the genes with no previously ascribed role in myogenesis were assessed by Northern blot analysis (see Table I and II for summary of expression data). Importantly, 52 of 53 genes were confirmed to be differentially

**Table I: Summary of wildtype myoblast RDA clones**

| Clone | Identity | Wt-Mb | Wt-D | *MD*$^{-/-}$ M | *MD*$^{-/-}$ D | Comments/Reference |
|---|---|---|---|---|---|---|
| dp3-7 | *Pax7*<br>AF254422 | ++++ | - | ++++ | - | -Paired-box transcription factor.<br>-Required for development of muscle satellite cells (Seale et al., 2000) |
| wt-30* | *Pb99*<br>AF249738 | ++++ | - | ++++ | - | -Seven pass transmembrane protein<br>-Expressed in pre-B cells and thymocytes (Sleckman et al., 2000). |
| wt-17* | *Asb5*<br>AF398966 | ++ | ++ | ++ | ++ | -*Ankyrin repeat-containing SOCS box protein 5.*<br>-Expressed in satellite cells (Boengler et al., 2003). |
| wt-73 | *L-myc*<br>X13945 | + | + | + | + | -bHLH-leucine zipper protein |
| dp3-1* | *NLRR1*<br>D45913 | ++ | +++ | +/- | ++ | -*Neuronal Leucine Rich Region-1*<br>- Membrane protein, expressed in developing nervous system (Taguchi et al., 1996). |
| dp3-2* | Unknown<br>BC024685 | +/- | +++ | - | + | -Hypothetical membrane protein |
| dp3-3 | *α-sarcoglycan*<br>NM_009161 | ++ | ++++ | +/- | +/+ | -Dystrophin associated glycoprotein<br>-Mutations cause limb-girdle muscular dystrophy (Duclos et al., 1998; Liu et al., 1997). |
| dp3-5 * | Unknown<br>XM_132832 | + | ++ | - | - | -Hypothetical protein similar to *Arachidonate 5-Lipoxygenase*<br>-Complete Absence in *MyoD*$^{-/-}$ cultures |
| dp3-8 | *Cholinergic Receptor-nicotinic*<br>BC052153 | + | ++++ | - | ++ | -Absent in *MyoD*$^{-/-}$ myoblasts (Affymetrix) |
| dp3-9 | *Myogenin*<br>D90156 | ++ | ++++ | - | ++ | -Up-regulated during differentiation (Sabourin et al., 1999; Smith et al., 1994). |
| dp3-13 | *Troponin T1 slow*<br>NM_011618 | + | +++ | - | + | -Muscle differentiation marker<br>-Absent in *MyoD*$^{-/-}$ myoblasts (Affymetrix) |

**Table I continued:**

| Clone | Identity | Wt-Mb | Wt-D | *MD*$^{-/-}$ M | *MD*$^{-/-}$ D | Comments/Reference |
|---|---|---|---|---|---|---|
| wt-18* | *Dapk2*<br>XM_134847 | + | ++++ | - | + | -*Death-associated kinase 2*<br>-Calcium/calmodulin dependent kinase (Kawai et al., 1998). |
| wt-23* | Unknown<br>AK029177 | + | ++++ | - | + | -Reduced expression in *MyoD*$^{-/-}$ myoblasts |
| wt-141* | *Sytl2*<br>NM_031394 | + | +++ | - | + | -*Synaptotagmin-like 2*<br>-Slp-homology domain (SHD) binds Rab27a- GTP binding protein (Kuroda et al., 2002). |
| wt-128 | *MCA-32*<br>NM_021585 | n/d | n/d | n/d | n/d | -*Mast cell antigen-32*, surface protein |
| wt-52 | *Dbh*<br>NM_138942 | n/d | n/d | n/d | n/d | -*Dopamine β-Hydroxylase* |
| wt-261 | Unknown | n/d | n/d | n/d | n/d | |
| dp3-15 | *Mgl2-pending*<br>NM_145137 | n/d | n/d | n/d | n/d | -*Macrophage galactose N-acetyl-galactosamine specific lectin 2* |

RDA clones were sequenced and compared to known entries in GenBank (NCBI) to determine their identity. Accession numbers for known genes are given. Expression levels are shown qualitatively based on Northern analysis in wildtype myoblasts (Wt-Mb), differentiating wildtype myoblast cultures 3 days after stimulation (Wt-D), proliferating *MyoD*$^{-/-}$ myoblasts (*MD*$^{-/-}$ M) and differentiating cells (*MD*$^{-/-}$ D).

* Northern analysis illustrated in figures.

**Table II: Summary of *MyoD*$^{-/-}$ myoblast RDA clones**

| Clone | Identity | Wt-M | Wt-D | *MD*$^{-/-}$ M | *MD*$^{-/-}$ D | Comments/ Reference |
|---|---|---|---|---|---|---|
| MD p168* | *VCAM1* BC029823 | + | - | ++++ | - | -*Vascular Cell Adhesion Molecule-1*<br>-Satellite cell specific marker (Rosen et al., 1992).<br>-Increased in *MyoD*$^{-/-}$ myoblasts by average of 4.9 fold (Affymetrix). |
| MD p3* | *IgSF4* NM_018770 | - | - | +++ | - | -*Immunoglobulin superfamily-4*<br>-Only expressed in proliferating *MyoD*$^{-/-}$ cultures.<br>-Similar to *Synaptic cell adhesion molecule-1* (Biederer et al., 2002). |
| MD 44* | *Laminin α-5* XM_203796 | + | + | ++++ | ++ | -Adhesive proteins for multipotent hematopoietic cells (Gu et al., 1999). Expressed in developing and dystrophic muscles (Ringelmann et al., 1999; Sorokin et al., 1997).<br>- Increased in *MyoD*$^{-/-}$ myoblasts by average of 5.6 fold (Affymetrix). |
| MD 1* | *T cell receptor-β* BC034887 | + | - | +++ | - | -Highly expressed in proliferating *MyoD*$^{-/-}$ myoblasts. |
| MD p12* | *Neuritin-1* BC035531 | - | - | ++++ | - | -Expressed exclusively in *MyoD*$^{-/-}$ Mb and neural tissue<br>-GPI membrane anchored protein, promotes neurite outgrowth (Naeve et al., 1997). |
| MD p123* | $G_0S2$ NM_008059 | + | + | +++ | + | -*G0/G1 switch gene-2*<br>-Highly expressed in *MyoD*$^{-/-}$ Mb.<br>-BMP target in mesenchymal cells (Bachner et al., 1998). |
| MD 41* | *E25* L38971 | + | +++ | ++ | +++++ | -Elevated expression in differentiating *MyoD*$^{-/-}$ myoblasts.<br>-Integral membrane protein expressed in chondrogenic progenitors (Deleersnijder et al., 1996). |
| MD p42* | *mM1S5* D86726 | + | - | +++ | + | -Cdc21 binding protein; MCM protein<br>-Expressed in proliferating intestinal crypt cells (Kimura et al., 1996; Sykes and Weiser, 1995). |
| MD 52* | *PlGF* BC016567 | ++ | - | ++++ | +++ | -*Placenta Derived Growth Factor*<br>-Promotes growth of early hematopoietic cells; closely related to -VEGF; active on endothelial cells (Luttun et al., 2002).<br>-Elevated average of 2.14 fold in *MyoD*$^{-/-}$ myoblasts (Affymetrix). |

**Table II continued:**

| Clone | Identity | Wt-M | Wt-D | *MD*$^{-/-}$ M | *MD*$^{-/-}$ D | Comments/ Reference |
|---|---|---|---|---|---|---|
| MD p259* | *Klra18* NM_053153 | - | - | +++ | +++ | -*Killer Cell Lectin-Like Receptor-18* -Murine T-cell surface antigen, also expressed in pre-B cells (Chan and Takei, 1989). |
| MD p286* | *Hoxc10* NM_017409 | ++++ | ++ | ++++ | ++++ | -Expressed in regenerating limbs of Axolotls (Carlson et al., 2001). |
| MD p26 | *Caldesmon* BC019435 | + | + | +++ | +++ | -Functions in stabilization of microfilament network (Matsumura and Yamashiro, 1993). |
| MD 42 | *Cadherin-6* NM_007666 | + | - | +++ | - | -Cell adhesion molecule; expressed in hematopoietic cells (Mbalaviele et al., 1998) and motorneurons (Marthiens et al., 2002). |
| MD p16 | *Integrin α7* NM_008398 | + | ++++ | ++ | ++++ | -Satellite cell marker (Blanco-Bose et al., 2001; LaBarge and Blau, 2002). |
| MD p87 | Unknown | +/- | + | ++ | + | -Contains CAG repeat region (EST- mouse 15 day embryo). |
| MD p40 | Unknown XM_129466 | - | - | ++ | - | -Putative G-protein coupled receptor |
| MD p31 | *PTK-7* NM_175168 | +++ | ++ | +++ | ++ | -*Protein tyrosine kinase-7* -Novel subclass of receptor tyrosine kinases (Park et al., 1996). |
| MD p158 | *P.412 AJ000386* | ++ | - | ++ | - | -*Plasminogen Activator Inhibitor-2* -Plasmin activity is required for muscle regeneration (Lluis et al., 2001; Suelves et al., 2002). |
| MD p67 | Unknown | ++ | - | +++ | - | -Novel EGF repeat containing protein. |
| MD p18 | *Integrin β-4* L04678 | ++++ | ++ | ++ | - | -α6β4 binds components of extracellular matrix; implicated in adhesion, cell proliferation (Murgia et al., 1998). |
| MD p249 | *Peg3(Pw1)* AF038939 | +++ | n/d | +/- | n/d | -Zinc Finger protein; implicated in myogenesis (Coletti et al., 2002; Relaix et al., 1996). Involved in p53 dependent apoptosis (Deng and Wu, 2000; Johnson et al., 2002; Relaix et al., 2000; Relaix et al., 1998; Yamaguchi et al., 2002). -Expression 18 fold reduced in *MyoD*$^{-/-}$ myoblasts (Affymetrix). |

**Table II continued:**

| Clone | Identity | Wt-M | Wt-D | $MD^{-/-}$ M | $MD^{-/-}$ D | Comments/ Reference |
|---|---|---|---|---|---|---|
| MD p11 | *H19 mRNA* BC025150 | ++ | ++++ | + | ++ | -Expressed during myoblast and ES cell differentiation (Bartolomei et al., 1993; Leibovitch et al., 1995). |
| MD p39 | *Nestin* NM_016701 | ++ | +++ | ++ | +++ | -Intermediate filament expressed in myoblasts and regenerating muscle (Bischoff, 1994; Carlsson et al., 1999; Vaittinen et al., 2001). |
| MD p35 | Unknown AK008210 | + | ++ | + | ++ | -Putative androgen induced protein homolog. |
| MD 62 | Unknown | + | ++ | + | ++ | -Contains S100 Calcium binding domain. |
| MD p77 | *IGF2bp3* BC049082 | n/d | n/d | n/d | n/d | *-Insulin-like growth factor 2, binding protein 3* |
| MD p10 | *UCP-2* BC012967 | n/d | n/d | n/d | n/d | -Uncoupling protein, roles in energy metabolism (Wolf, 2001). |
| MD p23 | *Ribosomal S3* BC010721 | n/d | n/d | n/d | n/d | |
| MD 5 | *Mgl2-pending* NM_145137 | n/d | n/d | n/d | n/d | -Macrophage galactose N-acetyl-galactosamine specific lectin 2 |
| MD p122 | *Cytochrome-β 558* BC026791 | n/d | n/d | n/d | n/d | -core microbicidal component of phagocytic cells |
| MD p6 | *Lysyl tRNA synth.* BC036289 | n/d | n/d | n/d | n/d | |
| MD p131 | Unknown | n/d | n/d | n/d | n/d | |
| MD p217 | Unknown | n/d | n/d | n/d | n/d | -Putative GTP-binding protein. |
| 9 clones | *Neomycin* | - | - | +++ | +++ | -Highly expressed from targeting vector in *MyoD*$^{-/-}$ cells |

-See legend for Table I. *Northern analysis illustrated in figures.

-Shaded rows are genes expressed at elevated levels in *MyoD*$^{-/-}$ cells relative to wildtype myoblasts

expressed in the starting pools of amplified cDNAs by reverse Northern blot experiments (not shown) thereby validating the efficacy of subtractions.

Northern analysis revealed that *Pax7*, *L-myc*, *Pb99* and *Asb5* were expressed in proliferating wildtype and *MyoD*$^{-/-}$ myoblasts with no dramatic upregulation observed during their differentiation into myotubes. *Pax7* is expressed in proliferating wildtype and *MyoD*$^{-/-}$ myoblasts but is rapidly downregulated upon differentiation (see Chapter 3). *Pb99*, a seven-pass transmembrane protein, is expressed exclusively in undifferentiated myoblast cultures (Figure 3A). Similar to the expression profile of *Pax7*, *Pb99* was not detected in total RNA from a panel of cell lines or mouse tissues (Figure 3A). Furthermore, in C2C12 myoblasts, expression of *Pb99* was downregulated within the first day of differentiation (not shown). These analyses therefore suggest a previously unrecognized role for *Pb99* in proliferating myoblasts.

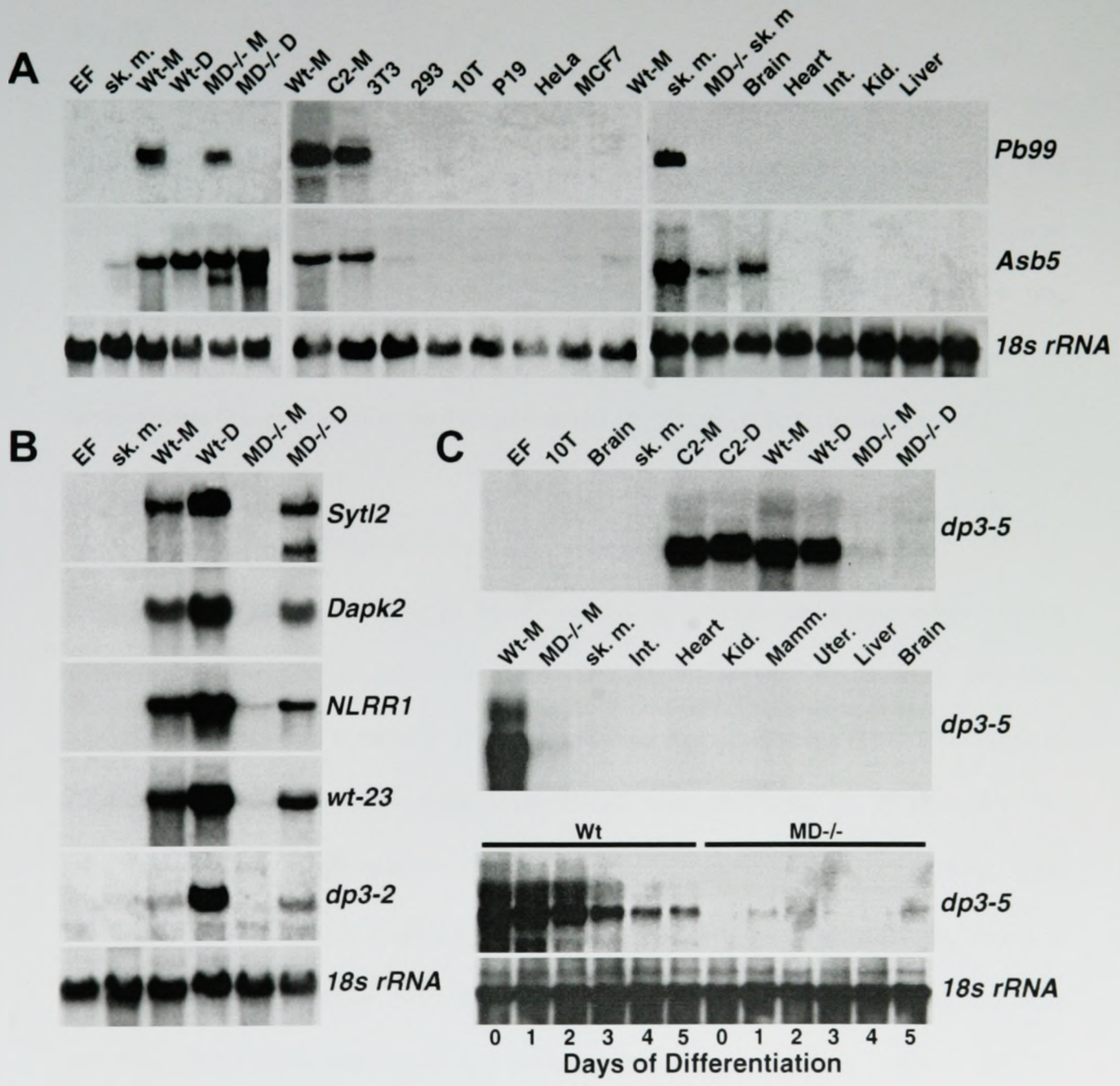
*Asb5*, encoding an Ankyrin-repeat SOCS box containing protein, was expressed at equal levels in proliferating and differentiating myogenic cells including wildtype, *MyoD*$^{-/-}$ and C2C12 myoblasts (Figure 3A). *Asb5* mRNA was detected in total RNA from adult skeletal muscle but was not expressed in other adult mouse tissues (Figure 3A). These data therefore indicate a specific role for Asb5 in muscle cells.

Several RDA clones from wildtype myoblasts were defined as differentiation specific as demonstrated by Northern blot analysis (Figure 3B) including *Dapk2*, *NLRR1*, *Sytl2*, *α-sarcoglycan*, *myogenin*, *troponin T1 slow* and three unknown genes (*dp3-2*, *dp3-5* and *wt-23*) (Table 1). These genes were all expressed at lower levels in *MyoD*$^{-/-}$ cells, consistent with the requirement for MyoD in the myogenic differentiation of adult

**Figure 3 Expression analysis of wildtype myoblast specific cDNAs**

(**A**) *Pb99* and *Asb5* were specifically expressed in proliferating wildtype (Wt-M), *MyoD*$^{-/-}$ (MD$^{-/-}$ M) and C2C12 (C2-M) myoblasts. *Asb5* was also expressed after 3 days of differentiation (Wt-D and MD$^{-/-}$ D) and in adult skeletal muscle tissue (sk. m). *Pb99* and *Asb5* mRNAs were not detected in a panel of cell lines.

(**B**) *Sytl2*, *Dapk2*, *NLRR1* and two unknown genes (wt-23 and dp3-2) were upregulated during myogenic differentiation in wildtype (Wt) myoblasts. Reduced expression levels were observed in differentiating *MyoD*$^{-/-}$ (MD-/-) cultures. (**C**) Unknown gene, dp3-5 was specifically expressed in proliferating and differentiating wildtype and C2C12 (C2) myoblasts. However, dp3-5 was not expressed in *MyoD*$^{-/-}$ cultures in growth conditions (day 0) or throughout differentiation (days 1-5). *18s rRNA* was used to control for loading.


A
EF
sk. m.
Wt-M
Wt-D
MD-/- M
MD-/- D
Wt-M
C2-M
3T3
293
10T
P19
HeLa
MCF7
Wt-M
sk. m.
MD-/- sk. m
Brain
Heart
Int.
Kid.
Liver
Pb99
Asb5
18s rRNA
B
EF
sk. m.
Wt-M
Wt-D
MD-/- M
MD-/- D
Sytl2
Dapk2
NLRR1
wt-23
dp3-2
18s rRNA
C
EF
10T
Brain
sk. m.
C2-M
C2-D
Wt-M
Wt-D
MD-/- M
MD-/- D
dp3-5
Wt-M
MD-/- M
sk. m.
Int.
Heart
Kid.
Mamm.
Uter.
Liver
Brain
dp3-5
Wt
MD-/-
dp3-5
18s rRNA
0 1 2 3 4 5 0 1 2 3 4 5
Days of Differentiation

myoblasts (Sabourin et al., 1999; Cornelison et al., 2000; Yablonka-Reuveni et al., 1999a). *Dapk2*, *NLRR1*, *Sytl2* and the unknown genes remain to be studied 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 wildtype and C2C12 myoblasts during proliferation and differentiation (Figure 3C). Expression of *dp3-5* was not detected in total RNA isolated from a panel of adult mouse tissues (Figure 3C). Significantly, *dp3-5* mRNA was completely absent in proliferating and differentiating *MyoD*$^{-/-}$ myoblasts. These data indicate that this gene is induced downstream of MyoD specifically in muscle cells.

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

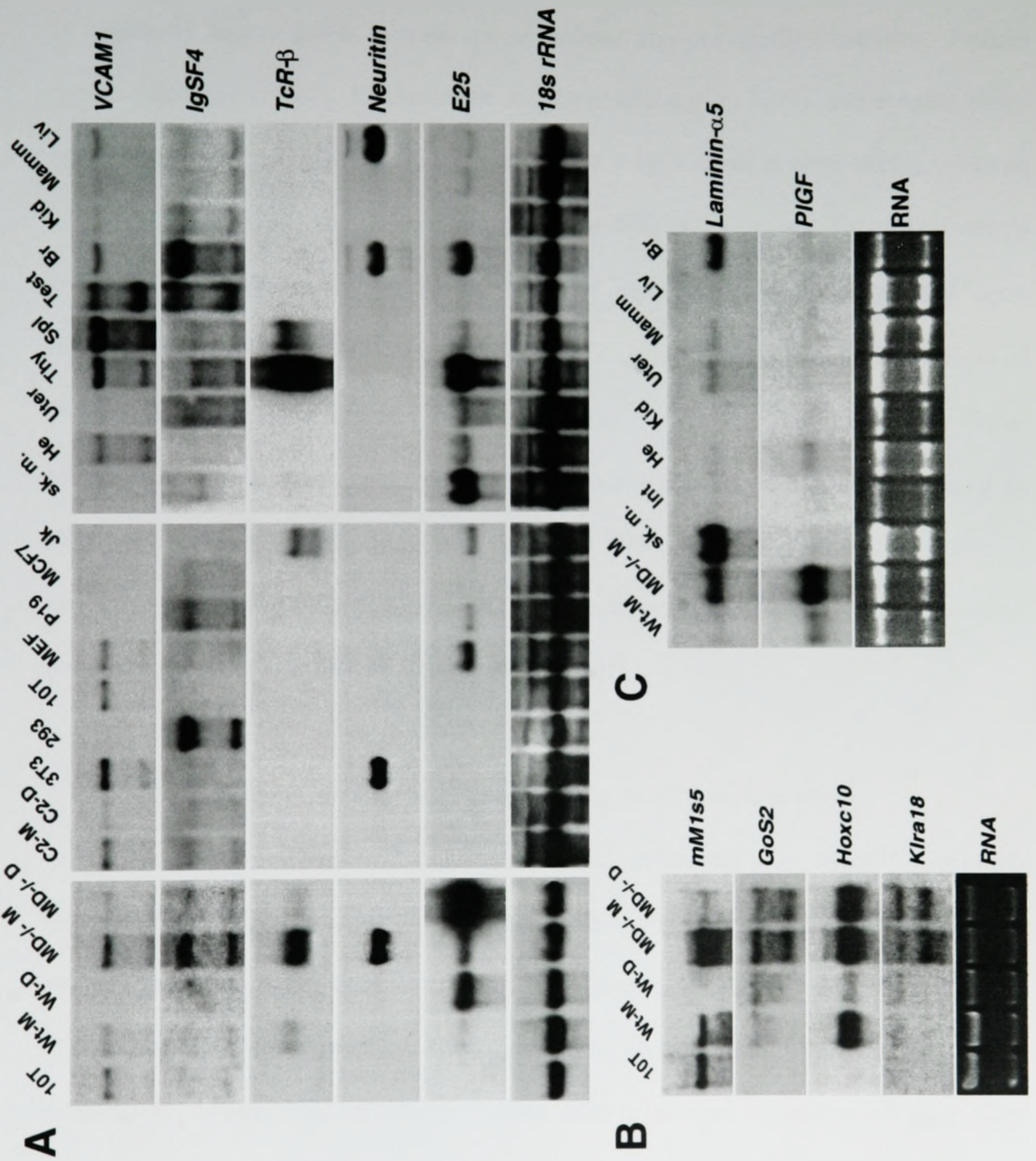
Transcriptional profiling of *MyoD*-deficient myoblasts was employed to identify markers that may be expressed by less committed myogenic progenitors. Northern analysis revealed that a number of genes were expressed at higher levels in *MyoD*$^{-/-}$ cultures relative to wildtype cells (see shaded rows in Table II). Importantly, the established satellite cell specific markers *VCAM1* (Figure 4A) and *Integrin-α7* (not shown) were expressed at higher levels in *MyoD*$^{-/-}$ cells by Northern analysis, with low levels detected in wildtype and C2C12 myoblasts (Blanco-Bose et al., 2001; LaBarge and Blau, 2002). Affymetrix profiling revealed an average 4.9 fold increase of *VCAM1* transcripts in *MyoD*$^{-/-}$ compared to wildtype myoblasts. *VCAM1* was downregulated in

differentiating myogenic cell cultures (Figure 4A). These data demonstrate that *MyoD*$^{-/-}$ myoblasts express genes that are also expressed in satellite cells.

A number of additional genes displayed elevated expression in *MyoD*$^{-/-}$ myoblasts relative to wildtype cells by Northern analysis (see shaded rows in Table II). *Immunoglobulin Superfamily-4 (IgSF4)* was expressed at high levels in *MyoD*$^{-/-}$ cells, with a complete absence of expression in wildtype myoblasts and C2C12 cells (Figure 4A). *IgSF4* transcripts were also detected in 293 and P19 cells as well as several adult tissues. Similarly, *Neuritin* was only expressed in *MyoD*$^{-/-}$ myogenic cells with high expression also observed in NIH 3T3 fibroblasts, brain and liver (Figure 4A). Surprisingly, *T-cell-Receptor-β (TcR-β)* was highly expressed in *MyoD*$^{-/-}$ myoblasts, with low levels also found in wildtype cells (Figure 4A). As expected, *TcR-β* was expressed in the Jurkat T cell line as well as in spleen and thymus. *mM1s5*, *$G_0/G_1$ switch gene-2* ($G_0S2$), *Hoxc10* and *Killer-cell Lectin Receptor (Klra18)* also displayed elevated expression levels in *MyoD*$^{-/-}$ relative to wildtype cultures (Figure 4B). Expression of *Caldesmon* was elevated in *MyoD*$^{-/-}$ cells relative to wildtype cultures, including expression of an alternate transcript, that was specific to *MyoD*$^{-/-}$ myoblasts (not shown). Northern analysis of *Laminin-α5* demonstrated elevated expression of the transcript in *MyoD*$^{-/-}$ myoblasts, adult skeletal muscle, and brain (Figure 4C). Affymetrix array analysis confirmed an average 5.6 fold increased expression of *Laminin α5* in *MyoD*$^{-/-}$ versus wildtype myoblasts. *Placenta Growth Factor (PlGF)* was highly expressed in *MyoD*$^{-/-}$ myoblasts with low levels observed in wildtype myoblasts (Figure 4C). By Affymetrix profiling, *PlGF* was expressed 2.1 fold higher in *MyoD*$^{-/-}$ cultures. In

**Figure 4 Expression analysis of *MyoD*$^{-/-}$ specific mRNAs**

(**A**) *VCAM1* was highly expressed in *MyoD*$^{-/-}$ (MD-/- M) myoblasts with low levels detected in wildtype 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, brain (Br) and testis (Test) but not in wildtype myoblasts. *TcR-β* was expressed at surprisingly high levels in *MyoD*$^{-/-}$ cells, Jurkat T cells (Jk), Thymus (Thy) and Spleen (Spl). *Neuritin* mRNA was only detected in *MyoD*$^{-/-}$ myoblasts, NIH 3T3 fibroblasts, brain and liver. *E25* was expressed at dramatically higher levels in *MyoD*$^{-/-}$ compared to wildtype myoblasts, with a marked upregulation in differentiating cells. The *E25* transcript was not detected in C2C12 myoblasts. *18s rRNA* was used as a loading control. (**B**) *mM1s5*, *$G_0$S2*, *Hoxc10* and were all expressed at higher levels in *MyoD*$^{-/-}$ (MD-/- M) relative to wildtype myoblasts (Wt-M). *Klra18* mRNA was only detected in *MyoD*$^{-/-}$ myoblasts. Ethidium stained RNA samples shows loading. (**C**) *Laminin-α5* and *PlGF* were highly expressed in *MyoD*$^{-/-}$ myoblasts with low levels detected in wildtype 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.


A
VCAM1
IgSF4
TcR-β
Neuritin
E25
18s rRNA
10T
Wt-M
Wt-D
MD-/- M
MD-/- D
C2-M
C2-D
3T3
293
10T
MEF
P19
MCF7
Jk
sk. m.
He
Uter
Thy
Spl
Test
Br
Kid
Mamm
Liv
B
mM1s5
GoS2
Hoxc10
Klra18
RNA
C
Laminin-α5
PlGF
RNA

summary, our expression analyses demonstrates that *MyoD*$^{-/-}$ cells express satellite cell markers and a number of novel genes not previously implicated in myogenesis.

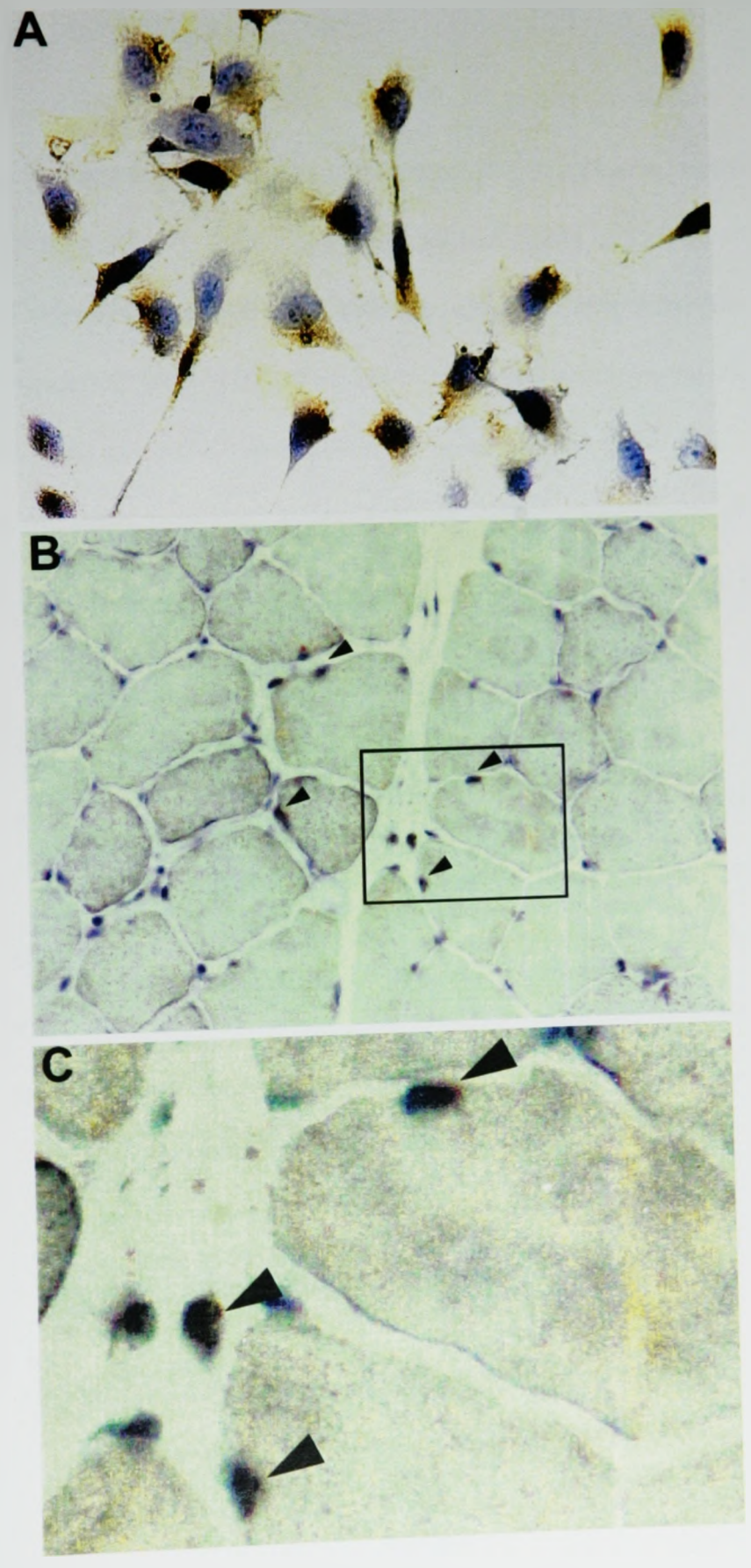
A number of genes isolated in the *MyoD*$^{-/-}$ RDA experiment were also expressed at similar or higher levels in wildtype myoblasts and during differentiation. *Protein tyrosine kinase-7* (*PTK7*), *Plasminogen activator inhibitor-2* (*PAI2*), and a novel EGF-repeat containing gene) (MD p67) were expressed at high levels in proliferating wildtype and *MyoD*$^{-/-}$ myoblasts and downregulated during differentiation (see Table II). *Integrin β-4* was expressed at high levels in wildtype myoblasts and downregulated upon differentiation (see Table II). Interestingly, *MyoD*$^{-/-}$ myoblasts expressed lower levels of *Integrin β-4* than did wildtype cells (not shown). Similarly, *Peg3/Pw1*, a zinc finger transcription factor, was expressed at 18-fold reduced levels in *MyoD*$^{-/-}$ compared to wildtype myoblasts by Affymetrix array analysis. *H19* mRNA, *Nestin* and two unknown genes (MD p35 and MD 62) represent genes cloned from *MyoD-/-* cells that were induced upon myogenic differentiation (see Table II).

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

To determine whether the genes expressed preferentially by *MyoD*$^{-/-}$ myoblasts were also expressed in uninjured wildtype muscle and regenerating *mdx* muscle *in vivo*, immunohistochemistry and *in situ* hybridization studies were employed. Immunochemical detection of VCAM1 revealed its expression in *MyoD*$^{-/-}$ cells where it localized to distinct regions of the plasma membrane and cytoplasm (Figure 5A).

**Figure 5 VCAM1 protein is expressed in satellite cells**

(**A**) Immunohistochemistry demonstrates accumulation of VCAM1 in specific domains of the plasma membrane in *MyoD*$^{-/-}$ myoblasts. Hematoxylin (blue) was used to counterstain nuclei. (**B**) VCAM1 protein was detected in satellite cells and interstitial cells in cross-sections of uninjured skeletal muscle (arrowheads). (**C**) Magnified view of boxed area in (**B**).

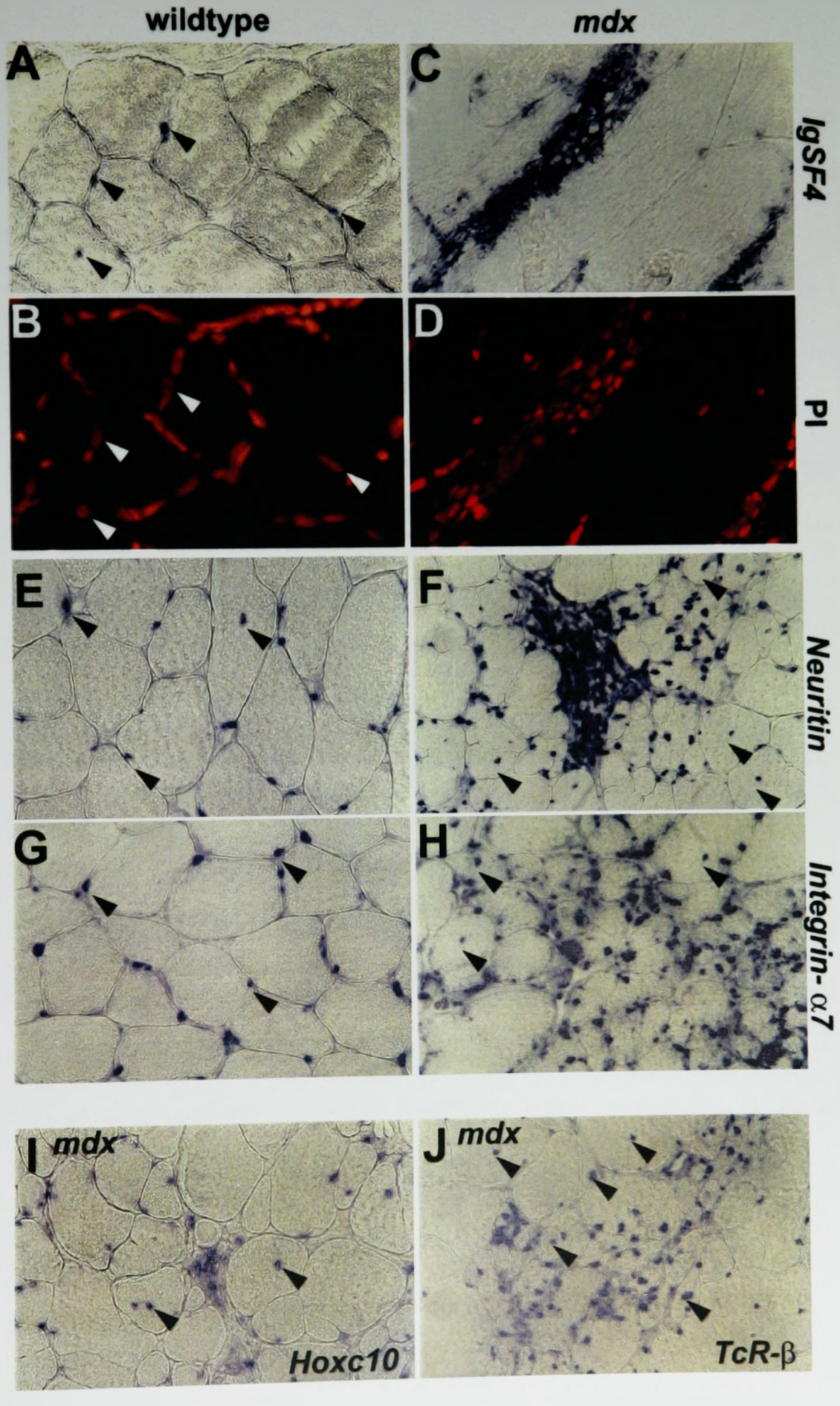

A
B
C

Moreover, VCAM1 was expressed in satellite cells in uninjured muscle as previously reported (Figure 5 B,C) (Rosen et al., 1992).

*IgSF4* mRNA was expressed in 2-3% of nuclei associated with uninjured wildtype muscle fibers (Figure 6A, B), with increased expression in regenerating areas of *mdx* muscle (Figure 6C, D). This result suggests that *IgSF4* is specifically expressed in quiescent and activated descendents of satellite cells. Transcripts for *Neuritin* were also detected in association with ~10% of nuclei in uninjured wildtype muscle within as well as outside the basal lamina of muscle fibers (Figure 6E). As with *IgSF4*, expression of *Neuritin* was markedly increased in regenerating *mdx* muscle (Figure 6F). The satellite cell marker, *Integrin* $\alpha 7$ was also detected in uninjured muscle, with increased levels observed during regeneration (Figure 6H). *Hoxc10* (Figure 6I), *TcR-*$\beta$ (Figure 6J), *Klra18*, *E25*, and $G_0S2$ were all expressed by cells in regenerating *mdx* muscle. *in situ* hybridization of muscle sections with non-specific sense riboprobes for these genes did not show any staining (not shown). Taken together, these expression studies suggest that many genes expressed specifically in *MyoD*$^{-/-}$ myoblasts are also expressed by wildtype satellite cells *in vivo*. These data are thus consistent with the existence of a Myf5-expressing, MyoD negative myogenic cell compartment in regenerating muscle.

**Figure 6** ***MyoD*$^{-/-}$ specific mRNAs are expressed by satellite cells *in vivo***

(**A**, **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**, **D**) *IgSF4* was upregulated in regenerating *mdx* muscles demonstrating its expression in activated satellite cells and myoblasts. (**E**, **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**, **H**) *Integrin-*$\alpha 7$ 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-*$\beta$ transcripts were also readily detected in regenerating muscle.


wildtype
mdx
A
C
IgSF4
B
D
PI
E
F
Neuritin
G
H
Integrin-α7
I mdx
Hoxc10
J mdx
TcR-β

## 2.4 Discussion

The hypothesis that *MyoD*$^{-/-}$ myoblasts represent an intermediate developmental stage between quiescent satellite cells and activated myogenic precursor cells was further investigated by intramuscular transplantation assays. Specifically, these experiments revealed an increased survival and integration of transplanted *MyoD*$^{-/-}$ cells in host muscle (Figure 1 A-C). Interestingly, transplanted *MyoD*$^{-/-}$ cells were localized within muscle fibers as well as in the interstitial spaces. The recovery of Pax7 expressing *MyoD*-deficient myoblasts from muscle after regeneration is complete (two months after injury), suggests a capacity for *MyoD*$^{-/-}$ cells to be maintained as myogenic progenitors (Figure 1 D, E). Transcriptional profiling of *MyoD*$^{-/-}$ cells revealed that many markers expressed by quiescent satellite cells were also expressed in *MyoD*-deficient myoblasts (Figure 6). *MyoD*$^{-/-}$ myoblast cultures thus represent a unique and tractable *in vitro* system to identify genetic pathways important for the function and maintenance of the satellite cell compartment in adult skeletal muscle.

Since the identification of satellite cells in 1961 (Mauro, 1961), their role in postnatal muscle growth and regeneration has been well established. 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 that has identified numerous markers for the satellite cell lineage (Figure 1).

The use of both wildtype and *MyoD*-deficient myoblasts for transcriptional profiling identified distinct subsets of genes based on their expression during differentiation (see Tables I and II). For example, the paired-box transcription factor, *Pax7* was identified as a gene expressed specifically in proliferating myogenic cells. On the basis of this work, we pursued the role of Pax7 in satellite cells, demonstrating its expression in quiescent and activated satellite cells (Seale et al., 2000). Analyses of *Pax7*-deficient mice revealed a requirement for Pax7 in the development of satellite cells. The cloning and functional analysis of *Pax7* in adult progenitors highlights the importance of identifying differentially expressed genes in refined progenitor cell populations. The requirement for Pax7 in satellite cell ontogeny has formed the basis of many ongoing studies aimed at identifying the origin of satellite cells and molecular pathways involved in their specification and function. The identification and characterization of Pax7 as a satellite specific marker demonstrates the strength and validity of this study and suggests that other genes presented here possess similarly important roles in adult muscle.

The Ankyrin repeat SOCS box containing gene, *Asb5*, was expressed at high levels in wildtype and *MyoD*$^{-/-}$ myoblasts as well as skeletal muscle tissue (Figure 3A). *Asb5* was independently identified as a gene expressed in smooth muscle and endothelial cells during arteriogenesis (Boengler et al., 2003). Interestingly, Asb5 protein appears to be expressed in skeletal muscle satellite cells by immunohistochemistry (Boengler et al., 2003).

*Pb99* was expressed specifically in proliferating myoblasts and was completely downregulated after 24 hours in differentiation conditions (Figure 3A). This putative G-

protein coupled receptor, is also expressed in pre-B cells and thymocytes but not mature lymphocytes (Sleckman et al., 2000).

Several other genes identified in wildtype 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* (NLRR1) (Figure 3B and Table I). This subset of genes was expressed at lower levels in *MyoD*-deficient myoblasts, suggestive of specific roles in muscle differentiation downstream of MyoD. In particular, an unknown gene similar to *Arachidonate 5'-Lipoxygenase* (5-LOX) (dp3-5) was expressed in proliferating and differentiating wildtype and C2C12 myoblasts, but notably absent in $MyoD^{-/-}$ cells (Figure 3C). Inhibition of 5-LOX enzyme activity in wildtype myoblasts did not interfere with differentiation (not shown), suggesting that 5-LOX inhibitors do not affect dp3-5. Taken together, several genes with previously undescribed roles in myogenesis were identified in wildtype myoblasts.

The ability for *MyoD*-deficient cells to maintain their proliferative activity two months after transplantation in wildtype muscle (Figure 1) suggested that their gene expression profile would more closely resemble that of satellite cells *in vivo*. Importantly, the identification of known satellite cell specific markers including *VCAM1* (Figure 4, 6) and *Integrin-α7*, as genes expressed highly in *MyoD*-deficient myoblasts demonstrates that $MyoD^{-/-}$ cells express satellite cell specific mRNAs that are low or absent in wildtype myoblasts. Moreover, transcripts for *IgSF4* and *Neuritin*, genes not expressed in wildtype myoblasts, were detected in cells within uninjured skeletal muscle (Figure 6). Furthermore, the frequency of cells expressing these markers and their overall

expression levels were markedly increased in regenerating *mdx* muscles, which undergo repeated cycles of degeneration and regeneration. These data are consistent with the specific expression of these genes in the muscle satellite cell lineage. Interestingly, several other genes expressed at high levels in *MyoD*$^{-/-}$ myoblasts including *Hoxc10*, *TcRβ*, *Klra18*, and *G0S2* were activated during muscle regeneration (Figure 6 I, J). These results indicate a specific induction of these markers during muscle regeneration and suggest that MyoD-:Myf5+ myogenic progenitors have different activities in regenerating muscle.

IgSF4 is a recently identified member of the immunoglobulin protein superfamily (IgSF) of proteins with substantial homology to *Synaptic cell adhesion molecule* which functions at neuronal synapses (Biederer et al., 2002). IgSF proteins represent a diverse group of proteins characterized by the Ig homology domain that regulate a number of 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). Interestingly, Neuritin induces neurite outgrowth in primary neuron cultures. The function of these two 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; Peterson et al., 1994). *Hoxc10* is also induced early during the regeneration of Axolotl forelimbs coincident with the appearance of the undifferentiated blastema cells,

suggesting a specific role for Hoxc10 in de-differentiation (Carlson et al., 2001). The expression of *Hoxc10* in undifferentiated amphibian limb progenitors is consistent with its expression in "less committed" 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* are induced upon activation prior to their coexpression in committed myogenic precursors (Cornelison and Wold, 1997). Analysis of regenerating muscle *in* indicated that 50% of activated satellite cells coexpress MyoD and Myf5, 30% express MyoD alone and 20% express Myf5 alone 3 hours postinjury (Cooper et al., 1999). Our current study is consistent with these findings and supports a specific role for myogenic cells expressing Myf5 but not MyoD during muscle regeneration. Moreover, the activation of *Myf5nLacZ* in quiescent muscle satellite cells (Beauchamp et al., 2000) suggests that *Myf5* expression is associated with "less committed" progenitors (i.e. satellite cells) that possess multipotent differentiation plasticity (Asakura et al., 2001; Wada et al., 2002). Taken together, cultured *MyoD*$^{-/-}$ myoblasts are genetically similar to satellite cells and provide a convenient model to elucidate genetic networks activated in this adult progenitor compartment.

Interestingly several cDNAs expressed specifically in *MyoD*$^{-/-}$ myoblasts are also expressed in hematopoietic or endothelial cells including *Vascular cell adhesion molecule-1* (*VCAM1*), *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 II). This result implies a close developmental relationship between satellite cells and hematopoietic/endothelial lineages that has been previously proposed (Bianco and Cossu, 1999; De Angelis et al., 1999; Ordahl, 1999). The identification of myogenic clones similar to satellite cell-derived myoblasts in explants of dorsal aorta from midgestation mouse embryos has challenged the notion that satellite cells are derived from somitic progenitors (De Angelis et al., 1999). The expression of hematopoietic and endothelial markers by satellite cells and myogenic precursors is consistent with a non-somitic origin of these cells.

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, other techniques including representational difference analysis remain powerful approaches to identify and clone differentially expressed genes as demonstrated in this study. 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 immediately used for expression studies and screening of cDNA or genomic libraries.

In summary, investigation of the phenotype of *MyoD*$^{-/-}$ cells *in vivo* demonstrates their capacity to incorporate into skeletal muscle while maintaining their potential as myogenic progenitors. Moreover, the identification of mRNAs expressed in *MyoD*$^{-/-}$ cells has revealed a number of novel genes that are also expressed by satellite cells *in vivo*. Functional and genetic analyses of the genes identified in this survey will be important for elucidating the mechanisms activated in different myogenic cell populations during muscle repair. Finally, this study suggests that approaches to transiently downregulate *MyoD* expression in myogenic stem cells or precursor cells may improve the success of myoblast transplantation therapy.

## 2.5 Materials and Methods

### Cell cultures

Primary myoblast cultures were prepared from adult (6-8 week old) *MyoD*$^{-/-}$ (Rudnicki et al., 1992) and Balb/c (Jackson Laboratories) control animals using established procedures (Rando and Blau, 1994). Primary low passage (<p6) myoblast cultures derived from multiple (>3) animals were pooled for use in the gene expression studies to control for biological variability and to maintain their primary characteristics. Desmin immunoreactivity confirmed that cell cultures were >98% pure. 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. 5'-Lipoxygenase activity was inhibited in differentiating wildtype myoblasts by treatment with 20 μM AA-861 (Sigma) or 2.5 μM Nordihydroguaiaretic acid (NDGA) (Sigma).

### Myoblast Transplantation

*MyoD*$^{-/-}$ or wildtype myoblasts were stably transfected with a *PGK-nLacZ* cassette (nuclear localized LacZ) using Lipofectamine reagent (Invitrogen). Wildtype myoblasts were also prepared from heterozygous *Myf5nLacZ* mice (Tajbakhsh et al., 1996) for

transplantation experiments. Muscle regeneration was induced in tibialis anterior (TA) muscles of 6-8 week old Scid/bg recipients (Jackson Labs) by intramuscular injection of 25 µl of 10 µM cardiotoxin (ctx) (Latoxan). $5x10^5$ *MyoD*$^{-/-}$*:PGK-LacZ* or wildtype myoblasts were injected directly into damaged TA muscles 2 days after administration of ctx using a 29 gauge insulin syringe. Two months after cell injections, TA muscles were flash frozen for preparation of cryosections. X-Gal immunohistochemistry was performed as previously described (Kablar et al., 1997). Myoblast cultures were initiated from transplanted TA muscles as described above.

**Representational Difference Analysis**

Representational difference analysis of cDNAs was performed essentially as described previously (Hubank and Schatz, 1994). Briefly, double stranded cDNA was digested with the four-cutter DpnII (New England Biolabs) and ligated with R-Bgl-24 adaptors. PCR was used to amplify the cDNA pools prior to subtractive hybridizations. R-Bgl-24 adaptors were subsequently removed from the cDNA pools, with new J-Bgl-24 adaptors ligated only to "tester" cDNA pools. For subtractive hybridizations, wildtype myoblast cDNA "tester" was subtracted against MEF cDNA at 1:100 and 1:400 to yield DP1 and DP2 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 DP1 and DP2 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 wildtype and *MyoD*$^{-/-}$ myoblasts (as described above) for array analysis. Hindlimb muscles from three 6-8 week-old mice were used for each isolate to control for biological variability between animals. Biotin-labeled cRNA was obtained for each replicate, fragmented, and hybridized to Murine Genome U74A version 1 chips (Affymetrix) at the Montreal Genome Center. Primary data and comparison analysis was done using Affymetrix Microarray Suite 4.0 and Excel spreadsheets. Only genes with >2 fold changes in each of two pair wise comparisons were subjected to further analysis.

**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). PolyA+ mRNAs were prepared using following two rounds of selection with oligo d(T) cellulose (Amersham Bioscience). The quantity and quality of mRNAs was verified by spectrometry, gel electrophoresis and northern analysis. Double stranded cDNAs were generated from mRNA samples using the Universal RiboClone cDNA synthesis kit as per manufacturer's instructions (Promega). The yield of double stranded cDNA obtained was determined by radioactive monitoring of first and second strand synthesis reactions. $^{32}$P-dCTP labeled cDNAs were electophoresed through denaturing alkaline agarose to verify the quality of cDNA preparations.

**Expression Analysis**

Northern blot studies were performed according to standard techniques using random-primed $^{32}$P-dCTP radiolabelled RDA products as probes (Maniatis et al., 1982). 15 μg of total RNA from various tissues and cell lines were 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 wildtype, or 3-week-old *mdx* mice 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) and 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 cyrosections using goat anti-VCAM1 antibody (Santa-Cruz), followed by secondary staining with a biotin conjugated secondary antibody (Zymed). Immunohistochemistry on cultured cells was performed by fixation with 4% PFA for 5 minutes, followed by permeabilization with 0.5% Triton X-100 for 5 minutes. Cells were incubated with primary antibodies against Desmin (DAKO), Pax7 (Developmental Studies Hybridoma Bank), Myf5 (Santa Cruz), β-Gal (Molecular Probes), and VCAM (Santa-Cruz). Secondary detection was performed with FITC or Rhodamine conjugated antibodies (Chemicon).

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# Chapter 3

## Pax7 is required for the specification of muscle satellite cells

**Preface:**

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I performed all of the experiments described in this chapter, assembled the data and figures, and wrote the manuscript. Considerable advice and intellectual focus for the study were given by my supervisor, Dr. M.A. Rudnicki. He also made a significant contribution to the final version of the manuscript. Luc A. Sabourin provided expert technical advice with the experiments and had significant intellectual input into the project. Adele Girgis-Gabardo provided technical assistance in establishing and expanding primary myoblast cultures for the RDA experiment. Drs. Ahmed Mansouri and Peter Gruss generously provided the *Pax7*-deficient mice.

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# Pax7 Is Required for the Specification of Myogenic Satellite Cells

**Patrick Seale,*‖ Luc A. Sabourin,† Adele Girgis-Gabardo,† Ahmed Mansouri,‡ Peter Gruss,‡ and Michael A. Rudnicki*†§‖**
*Departments of Biology and Pathology
†Institute for Molecular Biology and Biotechnology
McMaster University
1280 Main Street West
Hamilton, Ontario
Canada L8S 4K1
‡Department of Molecular Cell Biology
Max Planck Institute for Biophysical Chemistry
Am Fassberg
37077 Gottingen
Germany

## Summary

**The paired box transcription factor *Pax7* was isolated by representational difference analysis as a gene specifically expressed in cultured satellite cell-derived myoblasts. In situ hybridization revealed that *Pax7* was also expressed in satellite cells residing in adult muscle. Cell culture and electron microscopic analysis revealed a complete absence of satellite cells in *Pax7*$^{-/-}$ skeletal muscle. Surprisingly, fluorescence-activated cell sorting analysis indicated that the proportion of muscle-derived stem cells was unaffected. Importantly, stem cells from *Pax7*$^{-/-}$ muscle displayed almost a 10-fold increase in their ability to form hematopoietic colonies. These results demonstrate that satellite cells and muscle-derived stem cells represent distinct cell populations. Together these studies suggest that induction of Pax7 in muscle-derived stem cells induces satellite cell specification by restricting alternate developmental programs.**

## Introduction

Muscle satellite cells represent a distinct lineage of myogenic progenitors responsible for the postnatal growth, repair, and maintenance of skeletal muscle (reviewed in Seale and Rudnicki, 2000). At birth, satellite cells account for ~30% of sublaminar muscle nuclei in mice followed by a decrease to <5% in a 2-month-old adult (Bischoff, 1994). This decline in satellite cell nuclei reflects the fusion of satellite cells during the postnatal growth of skeletal muscle (Gibson and Schultz, 1983). Satellite cells were originally defined on the basis of their unique position in mature skeletal muscle and are closely juxtaposed to the surface of myofibers such that the basal lamina surrounding the satellite cell and its associated myofiber is continuous (Bischoff, 1994).

In mice >2 months of age, satellite cells in resting skeletal muscle are mitotically quiescent and are activated in response to diverse stimuli, including stretching, exercise, injury, and electrical stimulation (Schultz et al., 1985; Appell et al., 1988; Rosenblatt et al., 1994; reviewed in Bischoff, 1994). The descendents of activated satellite cells, called myogenic precursor cells, undergo multiple rounds of cell division before fusion with new or existing myofibers. The total number of quiescent satellite cells in adult muscle remains constant over repeated cycles of degeneration and regeneration, suggesting that the steady-state satellite cell population is maintained by self-renewal (Gibson and Schultz, 1983; Schultz and Jaryszak, 1985; Morlet et al., 1989). Therefore, satellite cells have been suggested to form a population of monopotential stem cells that are distinct from their daughter myogenic precursor cells as defined by biological and biochemical criteria (Grounds and Yablonka-Reuveni, 1993; Bischoff, 1994).

Satellite cells clearly represent the progenitors of the myogenic cells that give rise to the majority of the nuclei within adult skeletal muscle. However, recent studies have identified a population of pluripotential stem cells, also called side-population (SP) cells, in adult skeletal muscle. Muscle-derived SP cells are readily isolated by fluorescence-activated cell sorting (FACS) on the basis of Hoechst dye exclusion (Gussoni et al., 1999; Jackson et al., 1999). Purified SP cells derived from muscle exhibit the capacity to differentiate into all major blood lineages after tail vein injection into lethally irradiated mice (Jackson et al., 1999). Of particular significance is the observation that transplanted SP cells isolated from bone marrow or muscle actively participate in myogenic regeneration. However, only muscle-derived SP cells appear to give rise to myogenic satellite cells (Gussoni et al., 1999). In addition, SP cells convert to desmin-expressing myoblasts after exposure to appropriate cell culture conditions (Gussoni et al., 1999). However, whether SP cells are equivalent to satellite cells, are progenitors for satellite cells, or represent an entirely independent cell population has remained unclear.

The gene expression profile of quiescent satellite cells and their activated progeny is largely unknown. Quiescent satellite cells express the *c-Met* receptor (receptor for hepatocyte growth factor) and M-cadherin protein (Irintchev et al., 1994; Cornelison and Wold, 1997). Activated satellite cells upregulate *MyoD* or *Myf5* before entering S-phase (Cornelison and Wold, 1997). Proliferating myogenic precursor cells, the daughter cells of satellite cells, express desmin, Myf5, MyoD, and other myoblast specific markers (George-Weinstein et al., 1993; Cornelison and Wold, 1997). Nevertheless, the paucity of cell-lineage specific markers has been a significant impediment to understanding the relationship between satellite cells and their progeny.

Our poor understanding of molecular events responsible for satellite cell development and activation indicated the use of a PCR-based subtractive hybridization approach (Hubank and Schatz, 1994) to identify tissue-specific genes expressed in the satellite cell myogenic lineage. Results from this analysis identified several myoblast-specific genes potentially involved in satellite cell function. *Pax7* was selected for further analysis based

§To whom correspondence should be addressed (e-mail: mrudnicki@ottawahospital.on.ca).
‖Present address: Ottawa Hospital Research Institute, 501 Smyth Road, Ottawa, Ontario, Canada K1H 8L6.

on the established role of the closely related Pax3 protein in regulating the developmental program of embryonic myoblasts (Maroto et al., 1997; Tajbakhsh et al., 1997). In this article, we demonstrate a unique requirement for Pax7 in the specification of myogenic satellite cells.

## Results

### Identification of Genes Expressed in Satellite Cell-Derived Myoblasts

To gain insight into the developmental program responsible for the differentiation and activation of skeletal muscle satellite cells, representational difference analysis (RDA) of cDNAs was employed to identify genes expressed specifically in satellite cell-derived myoblasts (Hubank and Schatz, 1994). This analysis resulted in the identification of 17 distinct products corresponding to 12 known and 5 potentially novel genes by searching GenBank (NCBI) using the FASTA program (unpublished data). RDA clone *dp3–7* encoded a fragment from within the *Pax7* mRNA. Pax7 is a member of the paired-box family of transcription factors that play important regulatory roles in the development of diverse cell lineages (Mansouri, 1999). Therefore, a full-length 4.3-kb *Pax7* cDNA was isolated from an adult mouse skeletal muscle cDNA library (Clontech) to facilitate further analyses (NCBI accession number AF254422).

### *Pax7* is Specifically Expressed in Proliferating Myoblasts

Detailed expression analysis of the distribution of *Pax7* mRNA was conducted using Northern blot analysis (Figure 1). These analyses demonstrated that *Pax7* was expressed exclusively in proliferating primary myoblasts, with comparable levels of expression in both wild-type and *Myo*$^{-/-}$ cultures (Figure 1A). However, *Pax7* mRNA was downregulated after myogenic differentiation (Figure 1A). Furthermore, *Pax7* was not expressed at detectable levels in a variety of nonmuscle cell lines (Figure 1B). Rather, *Pax7* was strictly expressed in myogenic cells, including low levels in proliferating C2C12 mouse myoblasts, which is a continuous cell line originally derived from satellite cells (Figure 1B). In addition, *Pax7* mRNA was not detectable in 20 μg of total RNA from several adult mouse tissue samples (Figure 1C). Analysis of poly(A)$^+$ RNA from select mouse tissues revealed expression of *Pax7* at low levels only in adult skeletal muscle (not shown). Therefore, in adult mice, *Pax7* expression appears specific to the satellite cell myogenic lineage.

### *Pax7* Is Expressed in Satellite Cells

To localize *Pax7* mRNA in skeletal muscle, we performed in situ hybridization on fresh frozen sections of tibialis anterior and gastrocnemius muscles from wild-type (Balb/c), *MyoD*$^{-/-}$, *mdx*, and compound mutant *mdxMyoD*$^{-/-}$ animals. Interestingly, *Pax7* mRNA was associated with a subset of nuclei in discrete peripheral locations within undamaged wild-type (Figures 2A and 2C) and *MyoD*$^{-/-}$ (not shown) skeletal muscle. Propidium iodide (PI) staining was used to identify all nuclei within skeletal muscle, thereby allowing for the enumeration of *Pax7*-positive cells (Figures 2B, 2D, and 2F). The in situ hybridization was repeated on muscle sections from

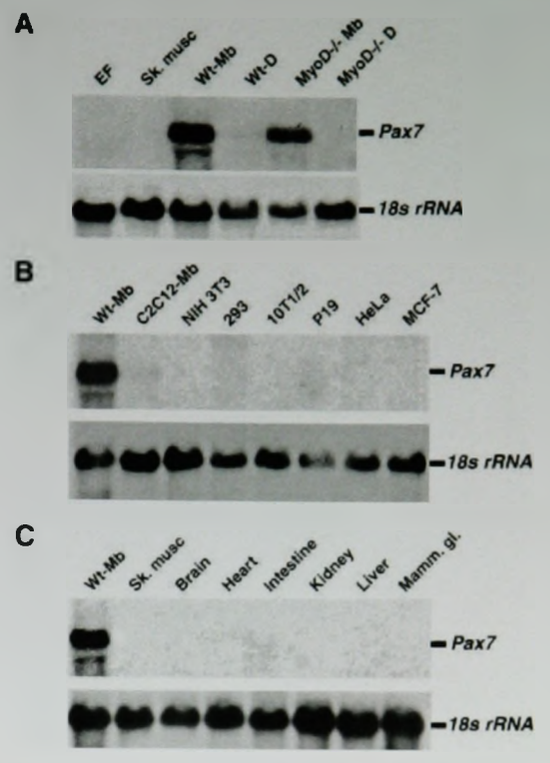


Figure 1. *Pax7* Is Expressed Specifically in Proliferating Myoblasts

(A) *Pax7* was expressed at high levels in proliferating wild-type myoblasts (Wt-Mb) and *MyoD*-deficient cells (MyoD$^{-/-}$ Mb) cells and downregulated in response to differentiation conditions (Wt-D and MyoD$^{-/-}$ D).

(B) Expression of *Pax7* was specific to myogenic cells, with low levels detected in C2C12 myoblasts.

(C) *Pax7* was not detected in RNA from a panel of tissues.

three independent mice by using three separate sequences as anti-sense cRNA probes to verify the expression patterns described. Approximately 5% of muscle nuclei (including satellite cell nuclei and myonuclei) were associated with *Pax7* expression in adult wild-type muscle. By contrast, the number of *Pax7*-positive cells increased to 22% in *MyoD*$^{-/-}$ muscle. The increased expression of *Pax7* in *MyoD*$^{-/-}$ muscle strongly supports the notion that *Pax7* is expressed in satellite cells, because previous work has revealed that *MyoD*-deficient muscle contains increased numbers of satellite cells (Megeney et al., 1996). At high magnification (200×), *Pax7* appeared to be expressed in cells residing beneath the basal lamina of wild-type muscle fibers in positions characteristic for quiescent satellite cells (Figure 2C).

To determine whether *Pax7* was upregulated in regenerating skeletal muscle, we analyzed 3-week-old *mdx* and compound mutant *mdxMyoD*$^{-/-}$ skeletal muscle by in situ hybridization. Lack of dystrophin protein causes *mdx* muscle to undergo repeated cycles of muscle degeneration and regeneration (Sicinski et al., 1989). As predicted, given the high levels of expression in cultured satellite cell-derived myoblasts, *Pax7* was widely expressed in regenerating areas of *mdx* and *mdxMyoD*$^{-/-}$ skeletal muscle (Figure 2E). Centrally located nuclei within muscle fibers of *mdx* (Figure 2E), *MyoD*$^{-/-}$ (not shown), and *mdxMyoD*$^{-/-}$ (not shown) muscle were also associated with *Pax7* expression, suggesting that recently activated and fusing myogenic precursors express *Pax7*. Lastly, a similar distribution of immunoreactive nuclei was observed in muscle sections stained with

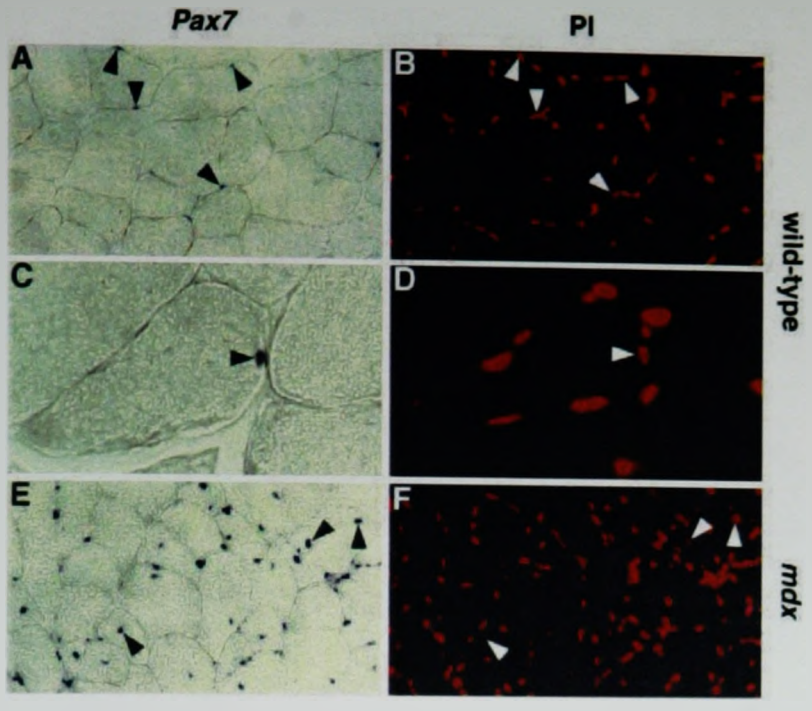


Figure 2. Expression of *Pax7* in Muscle Satellite Cells

(A) In situ hybridization revealed that *Pax7* mRNA was expressed at a frequency and location consistent with specific expression in satellite cells and myogenic precursor cells.
(B) *Pax7* expression was associated with PI-positive nuclei (40× magnification).
(C and D) High magnification (200×) of *Pax7*-expressing cell in wild-type muscle was characteristic of a satellite cell residing beneath the basal lamina.
(E and F) Increased numbers of cells expressed *Pax7* in regenerating *mdx* muscle (40×). Black and white arrowheads indicate cells stained positive for *Pax7* mRNA and PI-positive nuclei, respectively.

anti-Pax7 antibody (Developmental Studies Hybridoma Bank). Taken together, these results support the notion that *Pax7* is expressed within the satellite cell lineage. Therefore, these results raise the hypothesis that Pax7 is required for the ontogeny or function of muscle satellite cells.

### Skeletal Muscle Deficiency in Pax7 Mutant Animals

To evaluate possible roles for Pax7 in the formation or function of satellite cells, we examined skeletal muscle from mice carrying a targeted null mutation in *Pax7* (Mansouri et al., 1996b). Mice deficient for *Pax7* express muscle-specific markers, including *MyoD* and *Myf5*, in a normal spatial and temporal pattern within the developing myotome (Mansouri et al., 1996b). However, *Pax7*$^{-/-}$ mice were significantly smaller than their wild-type and heterozygous counterparts (Figure 3A). The body weight of *Pax7*$^{-/-}$ mice at 7 days of age was 50% reduced in comparison with wild-type littermates ($n = 20$). This weight differential increased with age such that at 2 weeks of age, mutant animals were ~33% the weight of wild-type littermates. As previously reported, *Pax7* mutant animals failed to thrive and usually died within 2 weeks after birth (Mansouri et al., 1996b). In addition, we observed that mutant mice exhibited muscle weakness characterized by an abnormal gait and splayed hind limbs (not shown). Light microscopic analysis of hematoxylin-eosin (HE)-stained lower hind limb skeletal muscle (below the knee) of 1-week-old wild-type (Figure 3B) and *Pax7*$^{-/-}$ (Figure 3C) animals revealed a 1.5-fold reduced diameter of *Pax7* mutant fibers ($n = 100$ fibers). However, the overall organization of muscle fibers was not affected. Moreover, the diaphragms of 7-day-old *Pax7*$^{-/-}$ mice (Figure 3E) were notably thinner than those of their wild-type littermates (Figure 3D). Therefore, the markedly decreased muscle mass and reduced fiber caliber of *Pax7* mutant muscle suggested that the postnatal growth phase of skeletal muscle normally mediated by satellite cells was deficient in the absence of Pax7.

### Absence of Satellite Cell-Derived Myoblasts from *Pax7*$^{-/-}$ Muscle

To gain insight into satellite cell function in *Pax7* mutant mice, we cultured primary cells directly from the muscle of 7- to 10-day-old wild-type mice and *Pax7*$^{-/-}$ littermates in five independent experiments. After 2 days in culture, many bursts of satellite cell-derived myoblasts were readily identified in wild-type primary cultures on the basis of morphological criteria (Figure 4A) and immunocytochemistry by using both anti-desmin and anti-c-Met antibodies that mark satellite cell-derived myoblasts (Figures 4B–4E). Strikingly, no myoblasts were identified in mutant cultures, which instead were uniformly composed of fibroblasts and adipocytes, as identified by morphological and immunochemical criteria (Figures 4F–4J).

To further investigate whether myogenic cells were present in postnatal *Pax7* mutant muscle, individual muscle fibers from 7- to 10-day-old wild-type mice and *Pax7*$^{-/-}$ littermates were isolated in five independent experiments and cultured in methylcellulose stem-cell medium. Methylcellulose stem-cell medium readily promotes the activation, migration, and proliferation of satellite cells associated with muscle fibers (A. Asakura and M.A.R., unpublished observation). After 48 and 72 hr in culture, satellite cells associated with wild-type fibers generated distinct bursts of desmin-expressing myogenic cells. By contrast, *Pax7* mutant muscle fibers did not give rise to any mononuclear cells. After 2 weeks in culture, large colonies of fully contractile myosin heavy chain (MHC)-expressing myotubes were present in cultures of wild-type but not *Pax7*$^{-/-}$ fibers (not shown). Therefore, these results suggest that satellite cells do not exist or, alternately, that they fail to proliferate in the absence of Pax7.

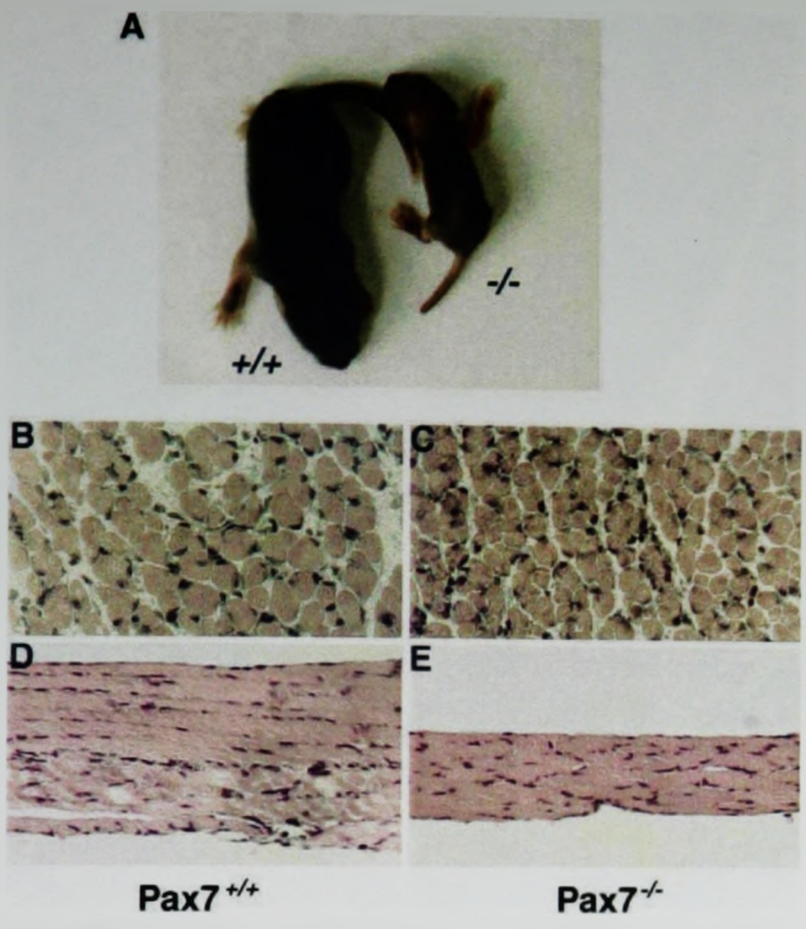


Figure 3. Skeletal Muscle Deficiency in *Pax7*$^{-/-}$ Mice

(A) Seven-day-old *Pax7* mutant animals were approximately one-half the weight of wild-type animals and had splayed hind limbs and an abnormal gait.

(B and C) HE-stained tibialis anterior muscle sections (40×) revealed a normal histological appearance of *Pax7* mutant muscle (C), but fiber diameter was reduced 1.5-fold compared with that of wild-type muscle (B).

(D and E) The diaphragm of a mutant animal (E) shown here in cross-section was significantly thinner than that in wild-type animals (D) (40×).

**Complete Ablation of Satellite Cells in *Pax7*$^{-/-}$ Muscle**

To determine whether or not satellite cells were present in mutant animals, we used transmission electron microscopy (TEM) to analyze skeletal muscle from wild-type and *Pax7*$^{-/-}$ mice. Biopsies from gastrocnemius muscle of three 7- to 10-day-old wild-type mice and mutant littermates were analyzed by TEM. For each sample, 100 peripheral sublaminar nuclei were analyzed and identified as either satellite cell or myofiber nuclei. Criteria for the identification of satellite cells consisted of the following: a plasma membrane separating the satellite cell from its adjacent muscle fiber, an overlying basal lamina continuous with the satellite cell and associated fiber, and the characteristic heterochromatic appearance of the nucleus (reviewed in Bischoff, 1994).

Satellite cells were readily identified in wild-type muscle and comprised 25% of peripheral sublaminar nuclei (n = 300) (Figures 5A–5D). By contrast, satellite cells could not be identified in >300 sublaminar nuclei examined from mutant muscles (Figures 5E and 5F).

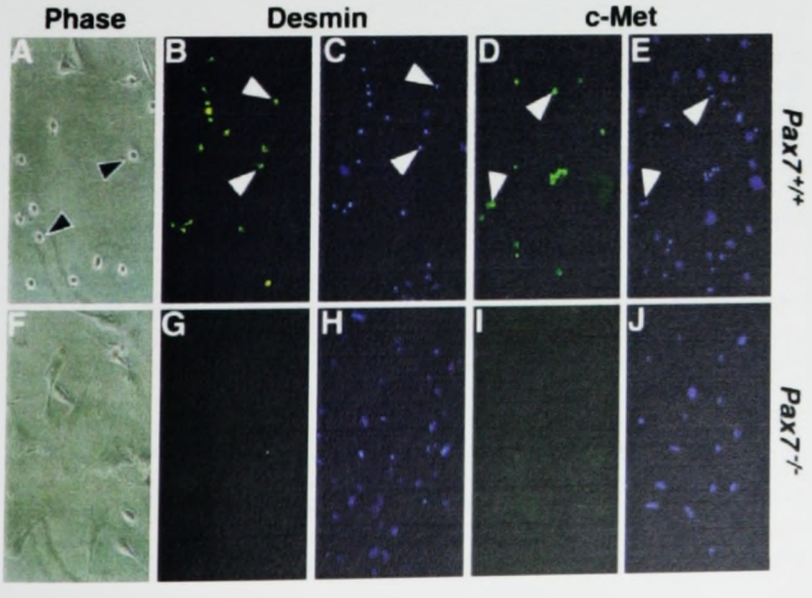


Figure 4. Absence of Myoblasts in Cultures Derived from *Pax7*$^{-/-}$ Muscle

Primary cell cultures were analyzed by phase microscopy (A and F) and by immunocytochemistry with anti-desmin (B and G) and anti-c-Met (D and I) antibodies. Cells stained with antibodies were counterstained with Hoechst 33342 (C, E, H, and J) to show all nuclei. Black arrowheads indicate satellite cell-derived myoblasts; white arrowheads indicate immunoreactive cells and corresponding nuclei.

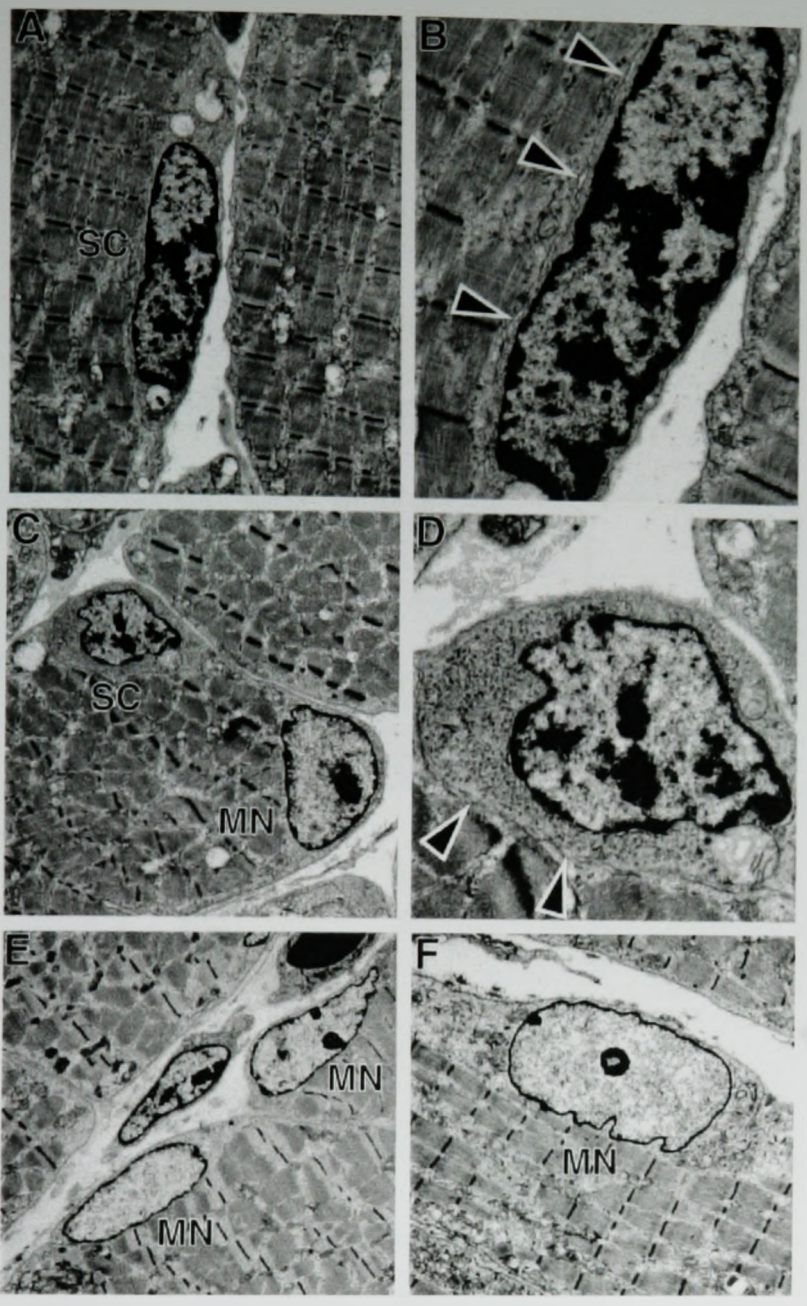


Figure 5. Complete Ablation of Satellite Cells in *Pax7*$^{-/-}$ Muscle

Transmission electron micrographs of 7- to 10-day-old *Pax7*$^{+/+}$ (A through D) and *Pax7*$^{-/-}$ (E and F) muscle.

(A and C) Satellite cells (SC) are readily identified in *Pax7*$^{+/+}$ muscle (7500×).

(B and D) High magnification of satellite cells clearly revealed the plasma membrane (black arrowheads) separating the satellite cell from its adjacent myofiber, the continuous basal lamina surrounding the satellite cell and myofiber, and the heterochromatic appearance of the nucleus (20,000×).

(E and F) Myonuclei (fiber nuclei) (MN) but not satellite cells were present in *Pax7* mutant muscles. Other ultrastructural differences were not detected.

Furthermore, satellite cells were not found in mutant muscle from E18 embryos (18 days post-coitum; not shown). Therefore, in the absence of Pax7, complete ablation of muscle satellite cells was observed. The failure of muscle satellite cells to form in *Pax7*$^{-/-}$ muscle thus unequivocally establishes an essential role for Pax7 in the ontogeny of the satellite cell lineage.

### Muscle-Derived SP Cells are Present in *Pax7* Mutant Muscle

To investigate the relationship between satellite cells and muscle-derived pluripotent stem cells, we performed FACS analysis of cells isolated from wild-type and *Pax7*$^{-/-}$ muscle. Recent work has identified a population of pluripotent stem cells (also called side-population [SP] cells) in skeletal muscle as defined by Hoechst 33342 dye exclusion (Gussoni et al., 1999; Jackson et al., 1999). Cell suspensions isolated directly from 1-week-old skeletal muscle were stained with Hoechst dye in the presence or absence of verapamil. The SP cell population is sensitive to verapamil, which is thought to prevent dye efflux through the inhibition of mdr (multidrug resistant)-like proteins (Goodell et al., 1996, 1997). On the basis of results from three independent trials with six 7- to 10-day-old *Pax7*$^{-/-}$ and wild-type animals, the proportion of muscle SP cells was unaffected by the absence of Pax7 (Figures 6A–6D). The relative proportion of SP cells in wild-type (1.8%) (Figure 6A) versus Pax7 mutant (1.5%) (Figure 6C) muscle did not differ significantly. Taken together, these data indicate that muscle satellite cells are either a population distinct from muscle SP cells or, alternately, represent only a small subpopulation of muscle SP cells.

### Stem Cells Derived From *Pax7*$^{-/-}$ Exhibit Markedly Increased Hematopoietic Potential

To characterize the differentiation potential of *Pax7*-deficient stem cells, we assayed dissociated muscle

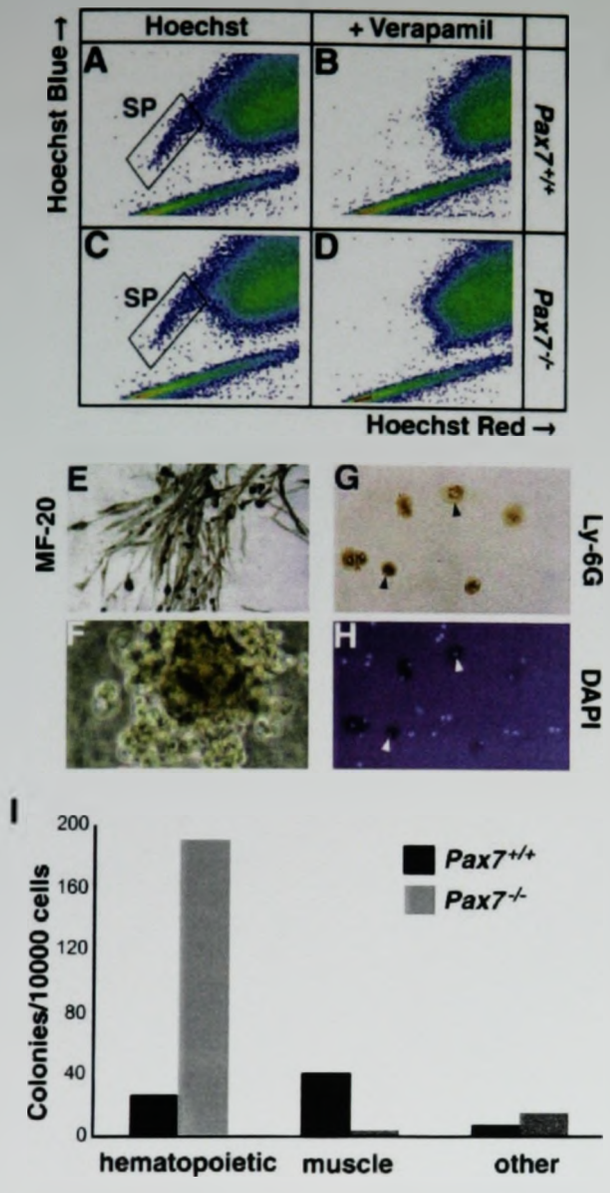


Figure 6. Enhanced Hematopoietic Potential of *Pax7*$^{-/-}$ Muscle–Derived Plunpotent Stem Cells

(A through D) FACS analysis of Hoechst-stained muscle-derived cells demonstrated approximately equal numbers of verapamil-sensitive SP cells in both *Pax7*$^{+/+}$ (A and B) and *Pax7*$^{-/-}$ (C and D) muscles.

(E) MHC-positive muscle colonies predominate in stem-cell medium/methylcellulose cultures of *Pax7*$^{+/+}$ muscle cells.

(F through H) *Pax7*$^{-/-}$ muscle cells have increased hematopoietic potential (F) and generate granulocyte and monocyte colonies verified by Ly-6G immunoreactivity (G and H).

(I) Colony-forming assay of muscle cells cultured in stem-cell medium/methylcellulose over a period of 2 weeks demonstrated almost a 10-fold increase in hematopoietic potential of *Pax7* mutant stem cells. Other cells represent both fibroblasts and adipocytes.

cells from 7- to 10-day-old *Pax7*$^{-/-}$ and wild-type animals for colony formation in methylcellulose stem-cell medium, which allows the growth of muscle as well as hematopoietic colonies (A. Asakura and M. A. R., unpublished data). Seven independent experiments were analyzed in which 10,000 cells from both wild-type and *Pax7*$^{-/-}$ muscle were cultured. Hematopoietic colonies included granulocytic and monocytic cells and were present in both wild-type and mutant cultures on the basis of immunoreactivity with Ly-6G (Figures 6G and 6H) and Integrin $\alpha_M$ chain (not shown). Ly-6G is a cell surface antigen, which is expressed exclusively in granulocyte and monocyte lineages (Fleming et al., 1993). Integrin $\alpha_M$ chain, also known as MAC-1, is expressed on granulocytes, macrophages, and natural killer cells (Leenen et al., 1994). Wild-type cultures were predominantly composed of contractile muscle colonies reactive with antibody to MHC (Figure 6E). By contrast, *Pax7*$^{-/-}$ cultures exhibited a markedly increased potential for hematopoietic differentiation (Figure 6F) and generated ~10-fold the number of hematopoietic colonies compared with wild-type cultures (Figure 6I). To rule out the possibility that the presence of differentiating muscle cells was inhibiting hematopoietic differentiation in wild-type cultures, we analyzed mixed cultures of *Pax7*$^{-/-}$ and wild-type cells. Results from these experiments showed that hematopoietic colony formation was not adversely affected by differentiating myocytes (not shown).

The colony-forming assays summarized in Figure 6I depict the average number of hematopoietic, skeletal myocyte, and other (e.g., fibroblast and adipocyte) colonies from seven independent isolations performed in triplicate. Therefore, stem cells isolated from muscle lacking *Pax7* exhibited a strongly increased propensity toward hematopoietic differentiation and were incapable of forming adult myoblasts. Importantly, highly purified SP cells from wild-type muscle convert to myoblasts under the appropriate culture conditions (Gussoni et al., 1999). Taken together, these results suggest the hypothesis that induction of *Pax7* in pluripotent muscle-derived stem cells directs the specification of satellite cells through restriction of developmental potential (Figure 7).

## Discussion

*Pax7* was molecularly cloned by RDA in a screen designed to identify genes specifically expressed in the muscle satellite cell lineage. On the basis of Northern blot analysis, expression of *Pax7* was confined to proliferating myoblasts and was strongly downregulated during terminal differentiation (Figure 1). In situ hybridization studies revealed the apparent localization of *Pax7* mRNA to satellite cells and their daughter myogenic precursor cells (Figure 2). Analysis of postnatal skeletal muscle from *Pax7*$^{-/-}$ animals revealed a complete absence of myogenic satellite cells as determined by primary cell culture and TEM (Figures 4 and 5). However, FACS analysis indicated that the proportion of SP cells was unaffected by the absence of Pax7 (Figure 6). Importantly, cells cultured from wild-type muscle efficiently gave rise to MHC-expressing myocyte colonies, whereas *Pax7*$^{-/-}$ muscle cells were unable to form myoblasts but exhibited a 10-fold increase in hematopoietic potential (Figure 6). Taken together, these data implicate Pax7 in the specification of myogenic satellite cells from uncommitted progenitors in skeletal muscle (Figure 7).

Pluripotential muscle-derived stem cells purified by FACS exhibit the capacity to efficiently reconstitute the marrow compartment and appear to give rise to muscle satellite cells after intravenous injections in mice (Gussoni et al., 1999; Jackson et al., 1999). Moreover, purified

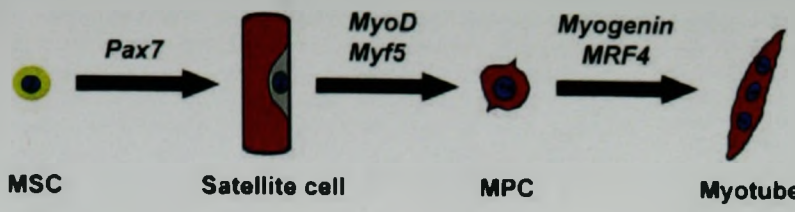


Figure 7. Role for Pax7 in the Specification of Satellite Cells

Muscle-derived pluripotent stem cells primarily give rise to myoblasts when cultured in stem-cell medium. By contrast, *Pax7*$^{-/-}$ muscle stem cells exhibit almost a 10-fold increase in propensity toward hematopoietic differentiation and are incapable of forming adult myoblasts. These data therefore implicate Pax7 in regulating the specification of adult muscle satellite cells by restricting the fate of pluripotent stem cells. Taken together, these experiments suggest the following hypothesis. Pluripotent stem cells (MSC) within muscle represent the progenitors of sublaminar satellite cells that are specified following induction of *Pax7*. Satellite cells are subsequently activated in response to physiological stimuli to generate daughter myogenic precursor cells (MPC) before terminal differentiation into new or previously existing fibers.

muscle-derived SP cells convert to desmin-expressing myoblasts in response to appropriate cell culture conditions (Gussoni et al., 1999). On the basis of our results demonstrating normal numbers of SP cells and the complete absence of satellite cells in *Pax7* mutant muscle, it is clear that SP cells and satellite cells represent distinct cell populations. Furthermore, the absence of muscle differentiation and the ~10-fold increased hematopoietic potential of *Pax7*$^{-/-}$ muscle-derived stem cells suggest the hypothesis that the progenitors for satellite cells are present within the muscle SP fraction. According to this model, upregulation of *Pax7* in muscle-derived pluripotent stem cells induces satellite cell specification by restricting alternate developmental programs (Figure 7). Alternately, muscle SP cells and satellite cells may represent independent cell populations. Inherent differences in the compositions of the SP fractions of wild-type and *Pax7*$^{-/-}$ muscle could explain the increased hematopoietic differentiation in mutant cultures.

Pax7 belongs to a family of genes that encode paired-box-containing transcription factors involved in the control of developmental processes (Jostes et al., 1990; Schafer et al., 1994). Different members of the Pax-family of transcription factors appear to regulate the development and differentiation of diverse cell lineages during embryogenesis (Noll, 1993; Strachan and Read, 1994; Mansouri et al., 1996a, 1996b, 1999). *Pax7* and the closely related *Pax3* gene belong to a paralogous subgroup of Pax genes based on similar protein structures and partially overlapping expression patterns during mouse embryogenesis (Jostes et al., 1990; Goulding et al., 1991). Interestingly, the *Pax3* gene plays an essential role in regulating the developmental program of embryonic myoblasts (Maroto et al., 1997; Tajbakhsh et al., 1997).

Pax7 and Pax3 proteins both bind identical sequence-specific DNA elements, which suggests that they regulate similar sets of target genes (Schafer et al., 1994). Furthermore, increased expression and gain-of-function mutations in both Pax3 and Pax7 are associated with the development of alveolar rhabdomyosarcomas, indicating that both molecules regulate similar activities in myogenic cells (Bennicelli et al., 1999). However, *Pax7* but not *Pax3* is expressed in adult human primary myoblasts (Schafer et al., 1994). Interestingly, differential expression of alternately spliced Pax7 transcripts correlates with muscle regenerative efficiency in different strains of mice (Kay et al., 1995, 1998). Although the Pax3 and Pax7 proteins are structurally similar, their different spatial-temporal patterns of expression suggest that they regulate myogenesis in distinct cell types during development.

*Splotch* (*Sp*) mice, lacking a functional *Pax3* gene, do not survive to term and fail to form limb muscles as the result of impaired migration of *Pax3*-expressing cells originating from the somite (Daston et al., 1996; Tremblay et al., 1998; reviewed in Borycki and Emerson, 1997). Compound mutant *Sp/Myf5*$^{-/-}$ mice do not express *MyoD* in their somites, suggesting that Myf5 and Pax3 function upstream of MyoD in myogenic determination (Tajbakhsh et al., 1997). Moreover, forced expression of *Pax3* induces *MyoD* expression and subsequent myogenesis in nonmuscle tissues from avian embryos (Maroto et al., 1997). However, ectopic expression of *Pax3* in C2C12 myoblasts efficiently inhibits myogenic differentiation (Epstein et al., 1995). In addition, co-expression of *MyoD* and *Pax3* is not observed in the mouse myotome (Williams and Ordahl, 1994). Therefore, Pax3 was suggested to function as an indirect upstream factor, which induced migration or other cellular changes to facilitate subsequent induction of *MyoD* transcription (reviewed in Borycki and Emerson, 1997).

Recent work suggests that Pax3 functions together with the Six1 transcription factor and the transcriptional co-regulators Eya2 and Dach2 to regulate the proliferation of pre-muscle masses in the somite (Heanue et al., 1999). *Pax3* and *Dach2* are co-expressed in the somite and appear to participate in a positive regulatory feedback loop. Ectopic expression experiments reveal that combinations of either Dach2 and Pax3 or Eya2 and Six1 synergize to induce myogenesis (reviewed in Relaix and Buckingham, 1999). It is interesting to speculate that an analogous regulatory network functions together with Pax7 in the specification of adult satellite cells.

Several genes have been suggested to represent direct targets for transcriptional regulation by Pax family members. Pax3 is believed to regulate *c-Met* transcription required to mediate the migration of somitic limb muscle precursors (Epstein et al., 1996). The c-Met receptor is expressed in quiescent satellite cells and is thought to activate satellite cells in response to hepatocyte growth factor (Allen et al., 1995; Cornelison and Wold, 1997). Thus, Pax7 may control the activation and migration of satellite cell precursors as a function of c-Met activity.

Regulatory elements in the neural cell adhesion molecule (NCAM) promoter are responsive to four Pax proteins, including Pax3 and Pax7 (Holst et al., 1997). NCAM is a member of the immunoglobulin superfamily of transmembrane proteins and has been implicated in the migration and differentiation of neural crest cells (Cunningham et al., 1987). Downregulation of NCAM in the developing neural crest of Pax7 mutant animals may be implicated in the dysgenesis of neural crest derivatives reported previously (Mansouri et al., 1996b). NCAM is

also expressed in activated satellite cells and myogenic precursor cells during muscle regeneration (Hurko and Walsh, 1983; Bischoff, 1994). NCAM may thus represent an important Pax7 target gene in satellite cells. Identification of transcriptional targets for Pax7 in the satellite cell lineage will be important for elucidating the mechanisms responsible for satellite cell specification, self-renewal, and activation.

Our experiments demonstrate that *Pax7*$^{-/-}$ muscle-derived pluripotent stem cells display almost an order of magnitude increase in hematopoietic potential (Figure 6I). Therefore, induction of *Pax7* in muscle-derived pluripotent stem cells appears to induce myogenic specification by restricting alternate developmental programs (Figure 7). This lineage-restricting function of Pax7 appears analogous to the role of Pax5 in regulating B cell development. Pro-B cells lacking Pax5 abnormally give rise to mature T cells expressing $\alpha/\beta$-T cell receptors. Pax5 thus suppresses alternate lineage choices of B cell progenitors in a cell-autonomous manner (Nutt et al., 1999; Rolink et al., 1999).

Muscle satellite cells and embryonic muscle are believed to be derived from distinct progenitors during development (reviewed in Seale and Rudnicki, 2000). Indeed, the presence of grossly normal skeletal muscle in *Pax7*$^{-/-}$ mice, which completely lack satellite cells, underscores the assertion that embryonic myoblasts and myogenic satellite cells develop independently. The continued presence of muscle SP cells in *Pax7*$^{-/-}$ mice has important implications for the origin of satellite cells and the mechanism responsible for their self-renewal. SP cells may form a reservoir of satellite cell progenitors, which differentiate into myogenic satellite cells during the latter stages of embryonic muscle development and persist in adult skeletal muscle to maintain steady-state numbers of satellite cells. In addition, it remains possible that satellite cells themselves undergo self-renewal or that myogenic precursor cells de-differentiate to contribute to the satellite cell population.

Recent work suggests that satellite cells are derived from endothelial precursors associated with the embryonic vasculature (De Angelis et al., 1999). Therefore, an interesting possibility is that progenitors associated with the embryonic vasculature either directly or indirectly give rise to satellite cells at embryonic times, which reflects the vascularization of the tissue. Moreover, putative vasculature-associated precursors may continue to give rise to pluripotent stem cells in adult muscle.

The pluripotent nature of adult stem cells isolated from diverse tissues raises the possibility of combined gene and stem-cell therapy for a variety of degenerative diseases, including muscular dystrophy. For example, ectopic expression of *Pax7* and *dystrophin* in pluripotential stem cells may result in the generation of high numbers of pre-satellite cells that would efficiently contribute to the damaged muscle of Duchenne patients. Indeed, ectopic expression of different members of the Pax gene family may direct the development of pluripotential stem cells into a range of discrete cell lineages. Detailed analysis of the potential of muscle stem cells ectopically expressing developmental control genes will elucidate the utility of such an approach.

## Experimental Procedures

### Molecular Cloning of Pax7 and Expression Analysis

RDA was performed as described by Hubank and Schatz (1994). Satellite cell-derived myoblast cDNA was subtracted twice against mouse embryonic fibroblast (MEF) cDNA (1:100; 1:400) and once against skeletal muscle cDNA (1:400) to generate the final difference products. The full-length mouse cDNA for *Pax7* was isolated by screening an adult mouse skeletal muscle library (Clontech) using the RDA clone as a probe (Maniatis et al., 1982).

Total RNA was extracted as previously described (Chomczynski and Sacchi, 1987). Northern blot analysis of 20 μg of total RNA from tissue or cell cultures was performed according to Maniatis et al. (1982). In situ hybridization for *Pax7* mRNA was performed as described by Braissant and Wahli (1998). Sections were counterstained with 100 μg/mL PI (Sigma) in PBS for 10 min at room temperature. Three different Pax7 sequences from the full-length cDNA were used as cRNA probes: Pax7-Sal1, nts 150–1600; dp3–7, nts 4200–4700; and Pax7-Cla1, nts 515–1500.

### Myoblast and Stem-Cell Culture

Primary muscle cultures were isolated as in Sabourin et al. (1999). Primary MEFs were isolated from 13.5-day-old Balb/c mouse embryos (Robertson, 1987). Single muscle fibers were isolated from hind limb skeletal muscles, as described by Cornelison and Wold (1997). Individual fibers were cultured in methocult GF M3434 containing 15% FBS, 1% BSA, 10 $^{-4}$M 2-mercaptoethanol, 10 μg/mL pancreatic insulin, 200 μg/mL transferrin, 50 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL IL-6, and 3 units/mL EPO (Stem Cell Technologies) from 48 hr to 10 days.

For hematopoietic colony-forming assays, cell suspensions were derived from skeletal muscle by digestion in 0.4% collagenase Type A (Roche)/DMEM for 1.5 hr at 37° C, filtered (74 μm; Costar Netwell), and resuspended at 100 cells/μl in 10% horse serum/DMEM. Approximately 10,000 cells were cultured in 3 ml of methocult (Stem Cell Technologies) for 14 days.

### FACS

Hoechst staining and FACS analysis were performed essentially as described previously (Goodell et al., 1996). FACS was performed on a Becton-Dickinson FacStar flow cytometer equipped with dual lasers. Hoechst dye was excited at 350 nm, and its fluorescence was measured at two wavelengths using a 424BP44 filter (blue emission) and a 650LP filter (red emission). A 640 DMSP mirror was used to separate wavelengths.

### Immunocytochemistry and Electron Microscopy

Primary cell cultures or colonies picked from methocult medium were fixed and stained as described elsewhere (Sabourin et al., 1999) using anti-c-Met SP260 (Santa Cruz), anti-desmin DE-U-10 (DAKO), anti-mouse Ly-6G (clone RB6-8C5) (Pharmingen), anti-mouse integrin $\alpha_M$ (M1/70) (Pharmingen), and MF20 mAb (anti-MHC).

Gastrocnemius muscle was prepared for TEM by overnight fixation at 4 C in 2% gluteraldehyde/0.1 M cacodylate (pH 7.4) and processed using standard procedures as described by Kablar (1995). Randomly chosen fields were viewed with a Jeol 1200EX Biosystem TEM. Diaphragm and tibialis anterior muscles were prepared for HE staining as described by Bancroft and Stevens (1990).

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**References**


Allen, R.E., Sheehan, S.M., Taylor, R.G., Kendall, T.L., and Rice, G.M. (1995). Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. J. Cell. Physiol. *165*, 307–312.

Appell, H.J., Forsberg, S., and Hollmann, W. (1988). Satellite cell activation in human skeletal muscle after training: evidence for muscle fiber neoformation. Int. J. Sports Med. *9*, 297–299.

Bancroft, J.D., and Stevens, A. (1990). Theory and Practice of Histological Techniques, Third Edition (Edinburgh and New York: Churchill Livingstone).

Bennicelli, J.L., Advani, S., Schafer, B.W., and Barr, F.G. (1999). PAX3 and PAX7 exhibit conserved *cis*-acting transcription repression domains and utilize a common gain of function mechanism in alveolar rhabdomyosarcoma. Oncogene *18*, 4348–4356.

Bischoff, R. (1994). The satellite cell and muscle regeneration. In Myogenesis, A.G. Engel and C. Franszini-Armstrong, eds. (New York: McGraw-Hill), pp. 97–118.

Borycki, A.G., and Emerson, C.P. (1997). Muscle determination: another key player in myogenesis? Curr. Biol. *7*, R620–R623.

Braissant, O., and Wahli, W. (1998). Differential expression of peroxisome proliferator-activated receptor-alpha, -beta, and -gamma during rat embryonic development. Endocrinology *139*, 2748–2754.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156–159.

Cornelison, D.D., and Wold, B.J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev. Biol. *191*, 270–283.

Cunningham, B.A., Hemperly, J.J., Murray, B.A., Prediger, E.A., Brackenbury, R., and Edelman, G.M. (1987). Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. Science *236*, 799–806.

Daston, G., Lamar, E., Olivier, M., and Goulding, M. (1996). Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. Development *122*, 1017–1027.

De Angelis, L., Berghella, L., Coletta, M., Lattanzi, L., and Zanchi, M., Cusella-De Angelis, M.G., Ponzetto, C., and Cossu, G. (1999). Skeletal myogenic progenitors originating from embryonic dorsal aorta coexpress endothelial and myogenic markers and contribute to postnatal muscle growth and regeneration. J. Cell Biol. *147*, 869–878.

Epstein, J.A., Lam, P., Jepeal, L., Maas, R.L., and Shapiro, D.N. (1995). Pax3 inhibits myogenic differentiation of cultured myoblast cells. J. Biol. Chem. *270*, 11719–11722.

Epstein, J.A., Shapiro, D.N., Cheng, J., Lam, P.Y., and Maas, R.L. (1996). Pax3 modulates expression of the c-Met receptor during limb muscle development. Proc. Natl. Acad. Sci. USA *93*, 4213–4218.

Fleming, T.J., Fleming, M.L., and Malek, T.R. (1993). Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6–8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. J. Immunol. *151*, 2399–2408.

George-Weinstein, M., Foster, R.F., Gerhart, J.V., and Kaufman, S.J. (1993). In vitro and in vivo expression of alpha 7 integrin and desmin define the primary and secondary myogenic lineages. Dev. Biol. *156*, 209–229.

Gibson, M.C., and Schultz, E. (1983). Age-related differences in absolute numbers of skeletal muscle satellite cells. Muscle Nerve 6, 574–580.

Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., and Mulligan, R.C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J. Exp. Med. *183*, 1797–1806.

Goodell, M.A., Rosenzweig, M., Kim, H., Marks, D.F., DeMaria, M., Paradis, G., Grupp, S.A., Sieff, C.A., Mulligan, R.C., and Johnson, R.P. (1997). Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat. Med. *3*, 1337–1345.

Goulding, M.D., Chalepakis, G., Deutsch, U., Erselius, J.R., and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. EMBO J. *10*, 1135–1147.

Grounds, M.D., and Yablonka-Reuveni, Z. (1993). Molecular and cell biology of skeletal muscle regeneration. Mol. Cell. Biol. Hum. Dis. *3*, 210–256.

Gussoni, E., Soneoka, Y., Strickland, C.D., Buzney, E.A., Khan, M.K., Flint, A.F., Kunkel, L.M., and Mulligan, R.C. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature *401*, 390–394.

Heanue, T.A., Reshef, R., Davis, R.J., Mardon, G., Oliver, G., Tomarev, S., Lassar, A.B., and Tabin, C.J. (1999). Synergistic regulation of vertebrate muscle development by Dach2, Eya2, and Six1, homologs of genes required for Drosophila eye formation. Genes Dev. *13*, 3231–3243.

Holst, B.D., Wang, Y., Jones, F.S., and Edelman, G.M. (1997). A binding site for Pax proteins regulates expression of the gene for the neural cell adhesion molecule in the embryonic spinal cord. Proc. Natl. Acad. Sci. USA *94*, 1465–1470.

Hubank, M., and Schatz, D.G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res. *22*, 5640–5648.

Hurko, O., and Walsh, F.S. (1983). Human fetal muscle-specific antigen is restricted to regenerating myofibers in diseased adult muscle. Neurology *33*, 737–743.

Irintchev, A., Zeschnigk, M., Starzinski-Powitz, A., and Wernig, A. (1994). Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. Dev. Dyn. *199*, 326–337.

Jackson, K.A., Mi, T., and Goodell, M.A. (1999). Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proc. Natl. Acad. Sci. USA *96*, 14482–14486.

Jostes, B., Walther, C., and Gruss, P. (1990). The murine paired box gene, *Pax7*, is expressed specifically during the development of the nervous and muscular system. Mech. Dev. *33*, 27–37.

Kablar, B. (1995). Structural study on the appearance of innervation in the stomach of mouse and rat embryos. Tissue Cell *27*, 309–315.

Kay, P.H., Mitchell, C.A., Akkan, A., and Papadimitriou, J.M. (1995). Association of an unusual form of a *Pax7*-like gene with increased efficiency of skeletal muscle regeneration. Gene *163*, 171–177.

Kay, P.H., Harmon, D., Fletcher, S., Robertson, T., Ziman, M., and Papadimitriou, J.M. (1998). Pax7 includes two polymorphic homeoboxes which contain rearrangements associated with differences in the ability to regenerate damaged skeletal muscle in adult mice. Int J. Biochem. Cell Biol. *30*, 261–269.

Leenen, P.J., de Bruijn, M.F., Voerman, J.S., Campbell, P.A., and van Ewijk, W. (1994). Markers of mouse macrophage development detected by monoclonal antibodies. J. Immunol. Methods *174*, 5–19.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Mansouri, A., Hallonet, M., and Gruss, P. (1996a). Pax genes and their roles in cell differentiation and development. Curr. Opin. Cell Biol. *8*, 851–857.

Mansouri, A., Stoykova, A., Torres, M., and Gruss, P. (1996b). Dysgenesis of cephalic neural crest derivatives in Pax7–/– mutant mice. Development *122*, 831–838.

Mansouri, A., Goudreau, G., and Gruss, P. (1999). Pax genes and their role in organogenesis. Cancer Res. *59*, 1707s–1710s.

Maroto, M., Reshef, R., Munsterberg, A.E., Koester, S., Goulding, M., and Lassar, A.B. (1997). Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. Cell *89*, 139–148.

Megeney, L.A., Kablar, B., Garrett, K., Anderson, J.E., and Rudnicki, M.A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev. *10*, 1173–1183.

Morlet, K., Grounds, M.D., and McGeachie, J.K. (1989). Muscle precursor replication after repeated regeneration of skeletal muscle in mice. Anat. Embryol. *180*, 471–478.

Noll, M. (1993). Evolution and role of Pax genes. Curr. Opin. Genet Dev. *3*, 595–605.

Nutt, S.L., Heavey, B., Rolink, A.G., and Busslinger, M. (1999). Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature *401*, 556–562.

Relaix, F., and Buckingham, M. (1999). From insect eye to vertebrate muscle: redeployment of a regulatory network. Genes Dev. *13*, 3171–3178.

Robertson, E.J. (1987). Embryo-derived stem cell lines. In Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E.J. Robertson, ed. (Oxford: IRL Press Ltd.), pp. 71–112.

Rolink, A.G., Nutt, S.L., Melchers, F., and Busslinger, M. (1999). Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors. Nature *401*, 603–606.

Rosenblatt, J.D., Yong, D., and Parry, D.J. (1994). Satellite cell activity is required for hypertrophy of overloaded adult rat muscle. Muscle Nerve *17*, 608–613.

Sabourin, L.A., Girgis-Gabardo, A., Seale, P., Asakura, A., and Rudnicki, M.A. (1999). Reduced differentiation potential of primary MyoD–/– myogenic cells derived from adult skeletal muscle. J. Cell Biol. *144*, 631–643.

Schafer, B.W., Czerny, T., Bernasconi, M., Genini, M., and Busslinger, M. (1994). Molecular cloning and characterization of a human PAX-7 cDNA expressed in normal and neoplastic myocytes. Nucleic Acids Res. *22*, 4574–4582.

Schultz, E., and Jaryszak, D.L. (1985). Effects of skeletal muscle regeneration on the proliferation potential of satellite cells. Mech. Ageing Dev. *30*, 63–72.

Schultz, E., Jaryszak, D.L., and Valliere, C.R. (1985). Response of satellite cells to focal skeletal muscle injury. Muscle Nerve *8*, 217–222.

Seale, P., and Rudnicki, M.A. (2000). A new look at the origin, function, and "stem-cell" status of muscle satellite cells. Dev. Biol. *218*, 115–124.

Sicinski, P., Geng, Y., Ryder-Cook, A.S., Barnard, E.A., Darlison, M.G., and Barnard, P.J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science *244*, 1578–1580.

Strachan, T., and Read, A.P. (1994). PAX genes. Curr. Opin. Genet. Dev. *4*, 427–438.

Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell *89*, 127–138.

Tremblay, P., Dietrich, S., Mericskay, M., Schubert, F.R., Li, Z., and Paulin, D. (1998). A crucial role for Pax3 in the development of the hypaxial musculature and the long-range migration of muscle precursors. Dev. Biol. *203*, 49–61.

Williams, B.A., and Ordahl, C.P. (1994). Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. Development *120*, 785–796.

# Chapter 4

## Identification of a novel population of myogenic progenitors in *Pax7*-deficient muscle

**Preface:**

The results presented in this chapter were prepared as a manuscript to be submitted for publication. Dr. Sophie Chargé and I collaborated on all aspects of this study. I outbred the *Pax7*$^{-/-}$ mice into different genetic backgrounds to enhance their survival into adults. Cardiotoxin injury experiments were performed collaboratively by Dr. Chargé and myself. Dr. Chargé performed the morphometric characterization of regenerating muscles presented in Figures 1, 2, 3C, did the crush-injury experiments (Figure 4) and the Pax3/MyoD double staining (Figure 8). I performed the immunohistochemical analysis of regenerating muscle (Figure 3), the transplant studies (Figure 5), the immunohistochemical analysis of single muscle fibers (Figure 6) and the isolation of myogenic cells from whole muscle (Figures 7 and 8). The article was cowritten by Dr. Chargé and myself. Considerable advice and intellectual contributions were provided by Dr. M.A. Rudnicki.

# Identification of a novel population of myogenic progenitors in *Pax7*-deficient muscle

Sophie B.P. Chargé[1,2], Patrick Seale[1,2,3] and Michael A. Rudnicki[2, 3, 4]

[1]Authors contributed equally to this work

[2]Ottawa Health Research Institute
Molecular Medicine Program
501 Smyth Road,
Ottawa, Ontario
Canada, K1H 8L6
Tel: 613-739-6740
Fax: 613-737-8803

[3]Department of Biology
McMaster University
Hamilton, Ontario
Canada, L8S 4K1

[4]Correspondence should be addressed to M.A.R.
E-mail: mrudnicki@ohri.ca

Running Title: Myogenesis in adult *Pax7*$^{-/-}$ mice

Key words: Pax7, Pax3, skeletal muscle, regeneration, myoblast, adult stem cell

## 4.1 Abstract

Activation of muscle satellite cells is associated with the growth and regeneration of skeletal muscle. The paired-domain transcription factor, Pax7 is expressed in quiescent satellite cells and is required for satellite cell specification. In this study we report a severe deficit in the regenerative capacity of *Pax7*-deficient muscle. Adult *Pax7*$^{-/-}$ mice displayed an aggravated muscle wasting phenotype characterized by spinal kyphosis, loss of fast myofibers and calcification. Acute injury induced by cardiotoxin or crush-injury resulted in a profound regeneration deficit characterized by rare regenerated myofibers, extensive calcification and a marked increase in the deposition of adipose and fibrotic tissues. The recovery of small numbers of myoblasts from whole muscle homogenates but absence of myogenic cells from cultured single muscle fibers indicated the presence of myogenic progenitors other than satellite cells in *Pax7*$^{-/-}$ muscle. Significantly, the *Pax7*-deficient myoblasts express Pax3 and MyoD but are not able to effect functional muscle regeneration *in vivo*.

## 4.2 Introduction

Skeletal muscle is a stable tissue, which normally undergoes relatively low nuclear turnover (Schmalbruch and Lewis, 2000). However, upon injury, skeletal muscle has the remarkable ability to induce a rapid and extensive regeneration process to prevent loss of muscle mass (reviewed by Seale and Rudnicki, 2002; Charge and Rudnicki, 2003). The regenerative capacity of skeletal muscle has been principally attributed to the muscle satellite cell population. The activation of muscle satellite cells generates proliferative myogenic precursor cells (mpcs), which differentiate to repair damaged fibers or fuse with one another to form new fibers (reviewed by Charge and Rudnicki, 2003). At the molecular level, satellite cell activation following injury is reminiscent of embryonic myogenesis. In particular, the expression profile and function of the myogenic regulatory factors (MRFs) have been established (Bhagwati et al., 1996; Cooper et al., 1999; Cornelison and Wold, 1997; Smith et al., 1994; reviewed by Charge and Rudnicki, 2003). However, early events in muscle regeneration and in particular the specification of adult myogenic progenitors are less well understood.

The anatomical definition of the adult muscle satellite cell describes it being closely associated with the myofiber, localized between the basal lamina and the myofiber plasma membrane (Mauro, 1961). Although few molecular markers for quiescent satellite cells have been identified, Pax7, a paired-box transcription factor, is specifically expressed in quiescent and newly activated satellite cells. More importantly, the absence of satellite cells in young *Pax7*$^{-/-}$ skeletal muscle demonstrates a requirement for Pax7 in the ontogeny of satellite cells (Chapter 3; Seale et al., 2000). *Pax7*$^{-/-}$ mice

appear normal at birth but fail to thrive and subsequently die at 2-3 weeks from unknown causes (Mansouri et al., 1996; Seale et al., 2000). The runted phenotype of *Pax7*$^{-/-}$ animals is in part due to a decrease in skeletal muscle mass resulting from reduced myofiber size. The decreased myofiber size is attributable to the lack of satellite cell fusion during the postnatal growth phase of myofibers (Seale et al., 2000). However, the normal appearance of postnatal *Pax7*-deficient muscle indicates that embryonic and fetal myogenesis is unaffected and that the requirement for Pax7 is limited to the satellite cell lineage.

Although satellite cells have a well-defined function during muscle regeneration, the isolation of independent stem cell populations with myogenic capacity from various adult tissues suggests alternative cell sources for muscle repair. For example, bone marrow derived cells participate in muscle repair following intravenous transplantation (Bittner et al., 1999; Ferrari et al., 1998; Gussoni et al., 1999; LaBarge and Blau, 2002). In addition, muscle-derived stem cells isolated on the basis of Hoechst dye exclusion as side-population cells (muSP) display myogenic differentiation potential following intramuscular or intravenous injection (Asakura et al., 2002; Gussoni et al., 1999). *In vitro* data suggest that muSP cells can undergo myogenic conversion in the absence of *Pax7* (Asakura et al., 2002). Furthermore, depletion of the satellite cell pool by muscle irradiation prior to bone marrow transplantation potentiates the myogenic commitment of marrow cells during exercise-induced regeneration (LaBarge and Blau, 2002). Together, these data suggest that adult progenitor populations distinct from satellite cells are capable of skeletal muscle differentiation during regeneration.

In this study, we have investigated regeneration in *Pax7*$^{-/-}$ muscle to determine the extent to which Pax7-independent myogenesis contributes to the repair process. Our experiments demonstrate that the Pax7-dependent satellite cell pool mediates the majority of muscle regeneration and indicates that a novel population of Pax3-expressing myogenic progenitors is present in adult muscle.

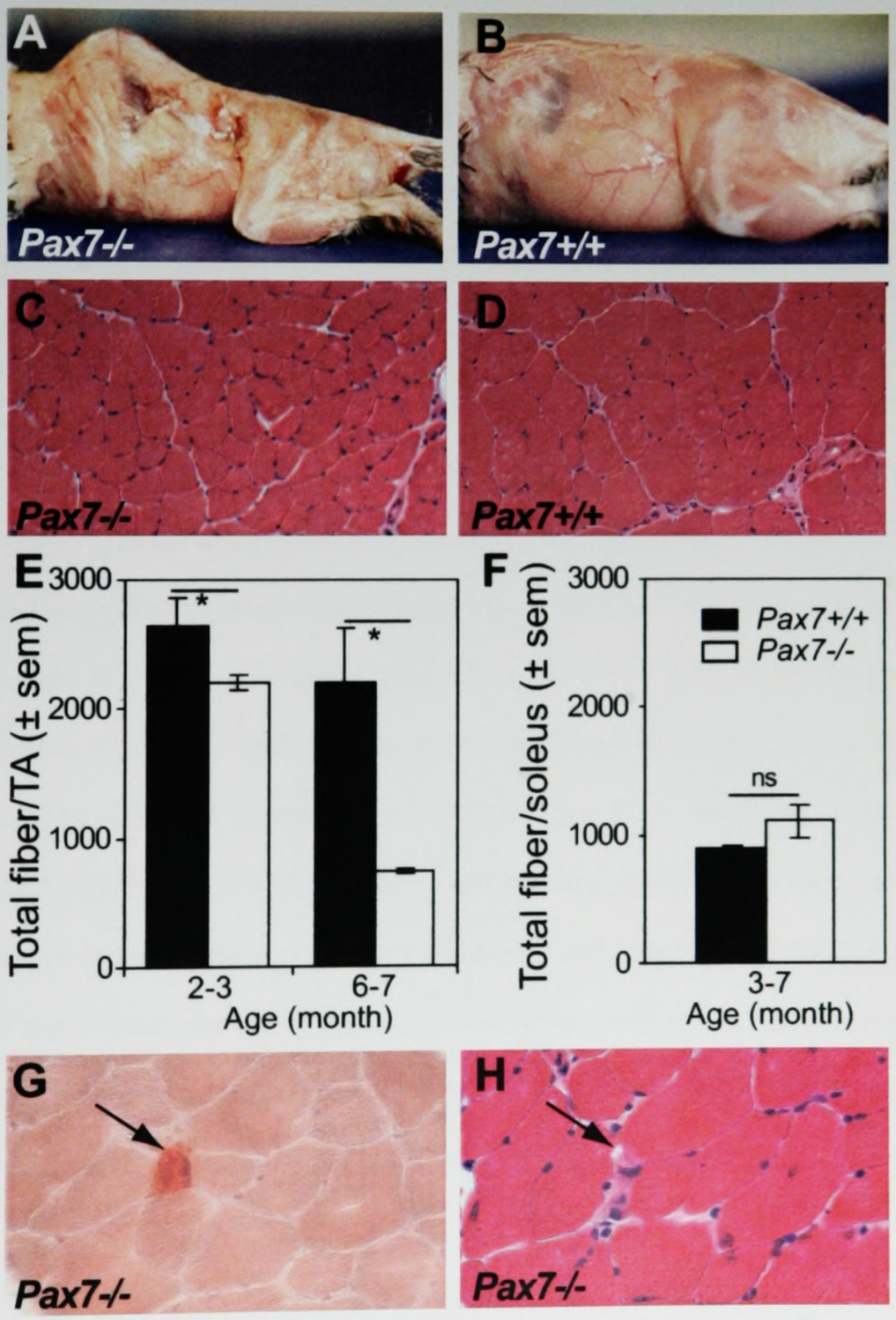
## 4.3 Results

### 4.3.1 *Pax7*$^{-/-}$ muscle fibers do not regenerate during muscle ageing

Viable adult *Pax7*$^{-/-}$ mice were generated by outcrossing the original knock-out animals (Mansouri et al., 1996) into the SV129 genetic background. To investigate the ability of *Pax7*$^{-/-}$ mice to undergo muscle regeneration in response to a low but chronic level of muscle degeneration, we studied the ageing of skeletal muscle in these animals. By 6 months of age, *Pax7*$^{-/-}$ mice displayed prominent kyphosis (curvature of the spinal column) typical of extensive muscle wasting and a hallmark of ageing (Figure 1A, B) (Deconinck et al., 1997; Grady et al., 1997; Megeney et al., 1996). *Pax7*$^{-/-}$ muscles form normally during embryonic and fetal development but fail to grow postnatally as evidenced by the decreased girth of individual fibers (Chapter 3; Seale et al., 2000) (Figure 1C, D). The wasting muscle phenotype was accompanied by a significant decrease in Tibialis Anterior (TA) myofiber number apparent at 2-3 months of age (Figure 1E). Specifically, the number of TA fibers in 6-7 month old mice was decreased to 1/3 of that observed in wildtype littermates (Figure 1E). The decreased myofiber number was due to a specific loss of fast IIb fibers as determined by fiber typing analysis

**Figure 1 Accelerated muscle wasting in adult *Pax7*$^{-/-}$ mice**

By 6 months of age, *Pax7*$^{-/-}$ mice developed a curvature of the spine characteristic of extensive muscle wasting (**A**) compared to wildtype littermates (**B**). TA muscle cross-sections stained with H&E revealed normal skeletal muscle architecture in adult *Pax7*$^{-/-}$ mice (**C**) compared to wildtype littermates (**D**). Total fiber number per midbelly TA cross-section was significantly reduced in *Pax7*$^{-/-}$ mice compared to wildtype littermates (n= 4, 5 for 2-4 months and n= 2, 2 for 6-7 months for *Pax7*$^{+/+}$ and *Pax7*$^{-/-}$, respectively) (**E**). Total fiber number was not affected in *Pax7*$^{-/-}$ mice compared to wildtype littermates (n= 2 for each genotype) (**F**). Small calcium deposits (arrow) stained with Alizarin Red (**G**) were present in between *Pax7*$^{-/-}$ myofibers which otherwise appear normal on a spaced serial cross-section stained with H&E (**H**).


A
Pax7-/-
B
Pax7+/+
C
Pax7-/-
D
Pax7+/+
E
Total fiber/TA (± sem)
3000
2000
1000
0
*
*
2-3
6-7
Age (month)
F
Total fiber/soleus (± sem)
3000
2000
1000
0
Pax7+/+
Pax7-/-
ns
3-7
Age (month)
G
Pax7-/-
H
Pax7-/-

(data not shown). Furthermore, normal fiber numbers in the slow Soleus muscle of adult *Pax7*$^{-/-}$ mice supports the conclusion that muscle wasting resulted from a specific loss of fibers from predominantly fast muscles (Figure 1F). Together these results demonstrate that muscle wasting due to ageing is accelerated in the absence of Pax7.

The phenotype of adult *Pax7*-deficient muscle suggests that an active regeneration process does not replace the chronic loss of myofibers during ageing. In support of this hypothesis, the number of newly regenerated myofibers with characteristic centrally located nuclei, remained insignificant in adult *Pax7*$^{-/-}$ TA muscles (5±1 and 4±1 centrally nucleated fibers/TA cross-section for adult *Pax7*$^{-/-}$ (n= 8) and wildtype littermates (n= 7), respectively). The presence of calcium deposits in some adult *Pax7*$^{-/-}$ TA muscles further suggested an abnormal regenerative process (Figure 1G, H). *Pax7*$^{-/-}$ muscle fibers did not display signs of extensive damage (Figure 1C, D); they were resistant to Evans Blue dye incorporation (data not shown) and serum creatine kinase levels were normal at all ages studied (216 U/L ±92 (n= 2) and 579 U/L ±179 (n= 6) in *Pax7*$^{-/-}$ and 328 U/L ±206 (n= 2) and 1148 U/L ±500 (n= 6) in *Pax7*$^{+/+}$ at P3 and in adults, respectively). Thus, the absence of extensive muscle damage in newborn and adult *Pax7*$^{-/-}$ muscle suggests that abnormal muscle regeneration during ageing is not due to an early exhaustion of regeneration capacity. Rather, it supports the hypothesis that the loss of *Pax7*$^{-/-}$ myofibers is due to chronic muscle degeneration which is left unrepaired.

4.3.2 The regeneration of *Pax7*$^{-/-}$ muscle is severely compromised following cardiotoxin-induced injury

To assess the regenerative capacity of *Pax7*-deficient muscle in response to acute muscle degeneration, focal injuries were induced by injection of cardiotoxin (ctx) directly into the TA muscle. Analysis of TA muscles at 10 days and 1 month after ctx injection demonstrated the absolute requirement for Pax7 in inducing an efficient muscle regeneration process. At 10 days and 1 month post-ctx injection, wildtype TA muscles regained a normal muscle architecture characterized by numerous centrally nucleated, regenerated myofibers (Figure 2A, B and Table I) without appreciable deposition of calcium, adipose or fibrotic tissues (Figure 2A, D). In sharp contrast, *Pax7*$^{-/-}$ TA muscles displayed a severe regeneration deficit, with only rare centrally nucleated myofibers observed at 10 days (9 ±6 fibers (n= 3)) and 1 month (61 ±50 fibers (n= 4)) post-ctx injection within the entire TA cross-section (Figure 2E, F; I, J; M, N; and Table I). The low number of *Pax7*$^{-/-}$ centrally nucleated myofibers did not grow and remained significantly smaller than wildtype regenerated fibers even 1-month after injury (Figure 2B compare to 2F, J, N; arrows).

By 1 month postinjury, *Pax7*$^{-/-}$ TA muscle had been replaced by extensive deposition of calcium (Figure 2H, L, P), adipose tissue (Figure 2F, J; arrowhead) or fibrotic tissue (Figure 2O; arrowhead). This almost complete absence of repair in adult *Pax7*$^{-/-}$ TA muscle is the most striking regeneration deficit reported in any mouse model to date. These results indicate that Pax7 is absolutely required for functional skeletal muscle regeneration.

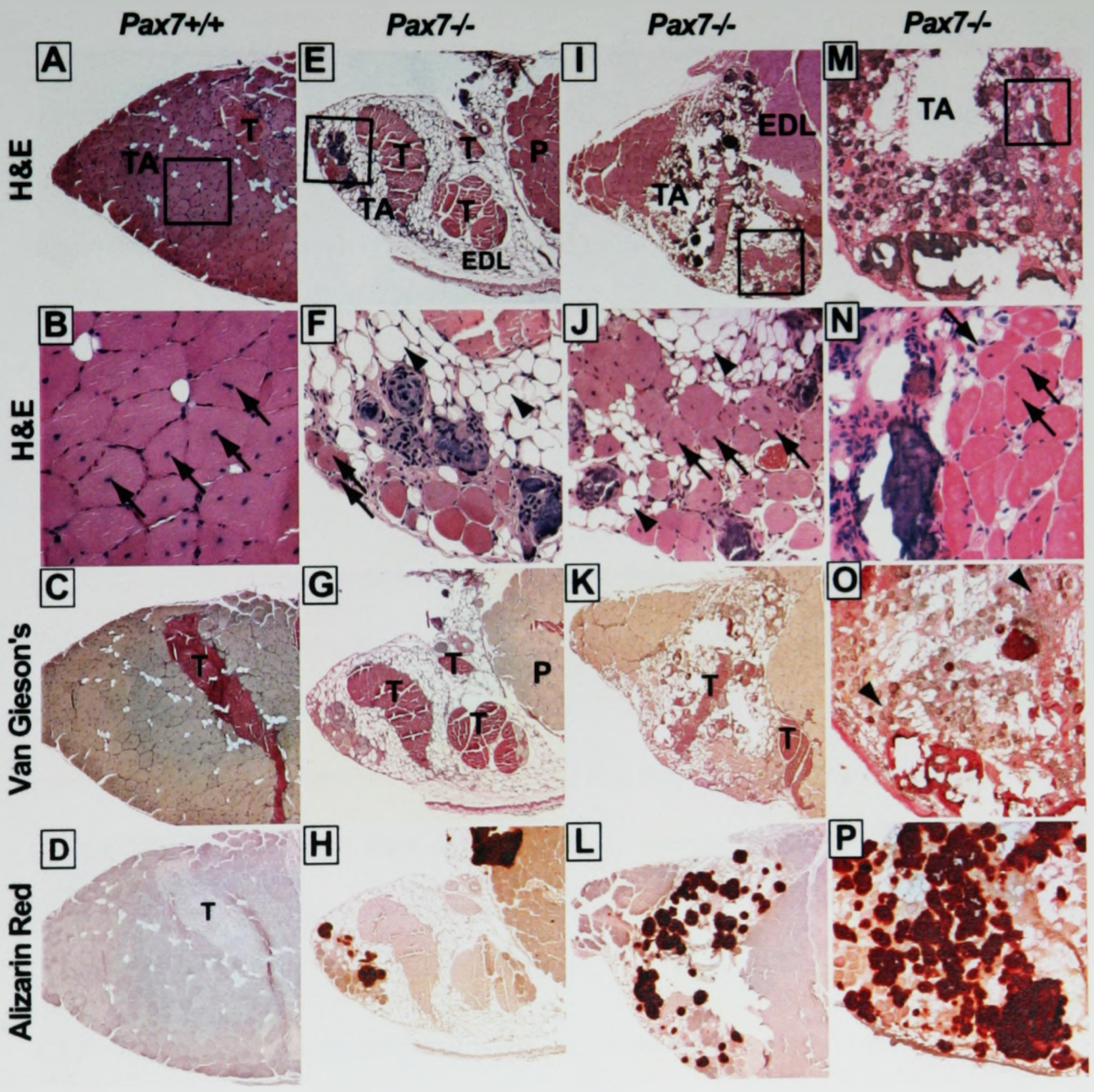
**Table I: Regenerating myofibers in ctx-injured adult TA muscles**

| | Centrally nucleated fiber number/ TA cross-section ± s.e.m. (n) | |
|---|---|---|
| Genotype | 10 days post-ctx | 1 month post-ctx |
| *Pax7*$^{+/+}$ | >700 (2) | >700 (3) |
| *Pax7*$^{-/-}$ | 9 ± 6** (3) | 61 ± 50** (4) |

Numbers for *Pax7*$^{+/+}$ are estimated. Numbers for *Pax7*$^{-/-}$ are from at least 2 TA cross-sections per sample. **, P <0.01.

**Figure 2** **Replacement of *Pax7*$^{-/-}$ myofibers by adipose tissue, fibrotic tissue or calcium deposits in muscle during regeneration**

Lower hindlimb cross-sections from adult *Pax7*$^{+/+}$ (**A-D**) or *Pax7*$^{-/-}$ (**E-H**; **I-L**; **M-P**) mice 1 month after ctx injection into the TA muscle. Sections were stained with H&E to show overall architecture of the muscle (**A**, **E**, **I**, **M**), with Van Gieson's staining for collagen in pink (**C**, **G**, **K**, **O**), with Alizarin Red staining for calcium deposits in red (**D**, **H**, **L**, **P**). **B**, **F**, **J**, **N** are higher magnifications of **A**, **E**, **I**, **M**, respectively. Regenerating *Pax7*$^{+/+}$ TA muscles contained large centrally nucleated regenerated myofibers (**B**; arrows), without extensive calcium deposition, adipogenesis or fibrogenesis (**A-D**). In contrast, *Pax7*$^{-/-}$ TA muscles displayed a severe regeneration deficit (**E**, **I**, **M**) with only rare small centrally nucleated myofibers present (**F**, **J**, **N**; arrows). The injured muscle was replaced by extensive calcium deposition (**H**, **L**, **P**), adipogenesis (**F**, **J**; arrow head) or fibrogenesis (**O**; arrow head). TA: Tibialis anterior, EDL: Extensor digitorum longus, P: Plantaris, T: tendon.


Pax7+/+
Pax7-/-
Pax7-/-
Pax7-/-
A
E
I
M
H&E
T
TA
T
T
P
TA
T
EDL
EDL
TA
TA
B
F
J
N
H&E
C
G
K
O
Van Gieson's
T
T
P
T
T
T
T
D
H
L
P
Alizarin Red
T

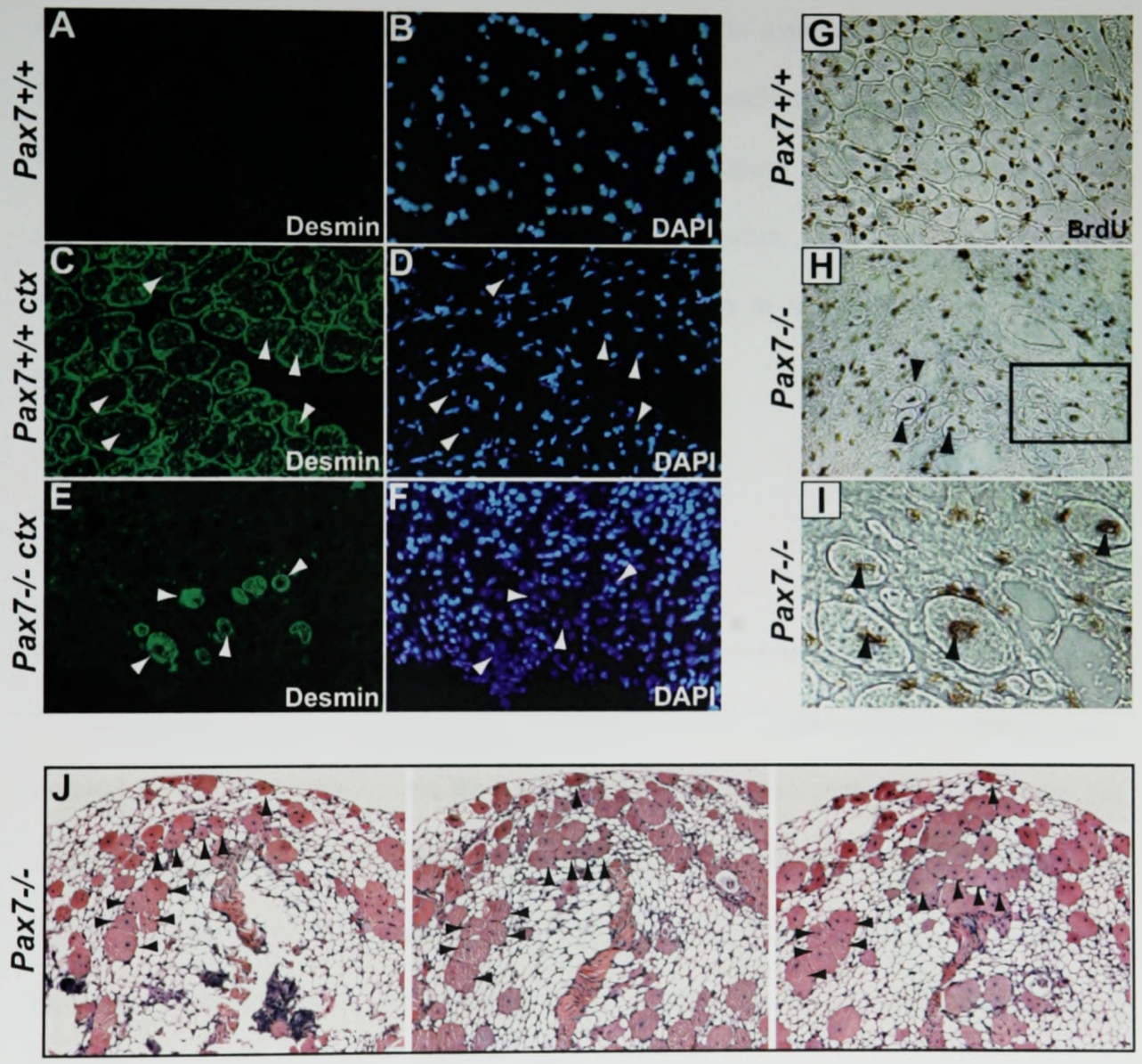
Nevertheless, the identification of small numbers of centrally nucleated myofibers in *Pax7*$^{-/-}$ muscle at 10 days and 1 month after injury suggested a limited capacity for regeneration. Regenerated fibers at 10 days and 1 month after administration of ctx normally express embryonic Myosin Heavy Chain (Emb-MyHC) and high levels of Desmin compared to uninjured fibers (Figure 3C, D compare to 3A, B). Importantly, the small number of centrally nucleated *Pax7*$^{-/-}$ myofibers at 10 days following ctx injury was immunoreactive for Desmin (Figure 3E, F) and Emb-MyHC (data not shown) confirming their newly regenerated state. In addition, BrdU, injected after administration of ctx was detected in central nuclei within regenerated *Pax7*$^{+/+}$ and *Pax7*$^{-/-}$ fibers at 10 days postinjury, indicating that proliferating cells had differentiated and fused into myofibers (Figure 3G, I). Consistent with the regenerative deficit of *Pax7*$^{-/-}$ muscle, only 3.5% of the BrdU labeled nuclei were found within muscle fibers, compared to the 45% of BrdU+ nuclei localized in regenerated *Pax7*$^{+/+}$ fibers. Finally, analysis of spaced serial cross-sections demonstrated that regenerated *Pax7*$^{-/-}$ myofibers were multinucleated and extended over several hundred μm suggesting that they were had been formed *de novo* (Figure 3J). Thus, by these three criteria, centrally nucleated *Pax7*$^{-/-}$ myofibers observed after acute muscle injury are newly regenerated.

### 4.3.3 *Pax7*$^{-/-}$ muscle does not functionally regenerate in response to crush-injury

Although ctx is commonly used to study muscle regeneration, its non-specific toxicity to mononuclear cells and muscle fibers induces extensive degeneration. Therefore, we also employed an alternate and milder model of muscle regeneration

**Figure 3 Centrally nucleated *Pax7*$^{-/-}$ fibers are newly regenerated**

Myofibers in uninjured muscle were not centrally nucleated and did not express elevated levels of Desmin (**A-B**). Regenerated TA myofibers from *Pax7*$^{+/+}$ (**C-D**) and *Pax7*$^{-/-}$ (**E-F**) muscle 10 days post-ctx injection expressed high levels of Desmin and contained centrally positioned nuclei (arrowheads point to Desmin-positive regenerated fibers and corresponding DAPI-stained central nuclei). Central myonuclei in *Pax7*$^{+/+}$ (**G**) and *Pax7*$^{-/-}$ muscle (**H-I**) are BrdU positive (arrowheads) following multiple BrdU injections during the regenerative process. Boxed area from (H) is magnified in (**I**). (**J**) Myofibers with central myonuclei (arrows) in *Pax7*$^{-/-}$ TA muscle 1 month post-ctx injection can be followed along several hundred micrometers as determined on spaced serial cross-sections stained for H&E (distances between each section = 100 μm).


A
B
Pax7+/+
Desmin
DAPI
C
D
Pax7+/+ ctx
Desmin
DAPI
E
F
Pax7-/- ctx
Desmin
DAPI
G
Pax7+/+
BrdU
H
Pax7-/-
I
Pax7-/-
J
Pax7-/-

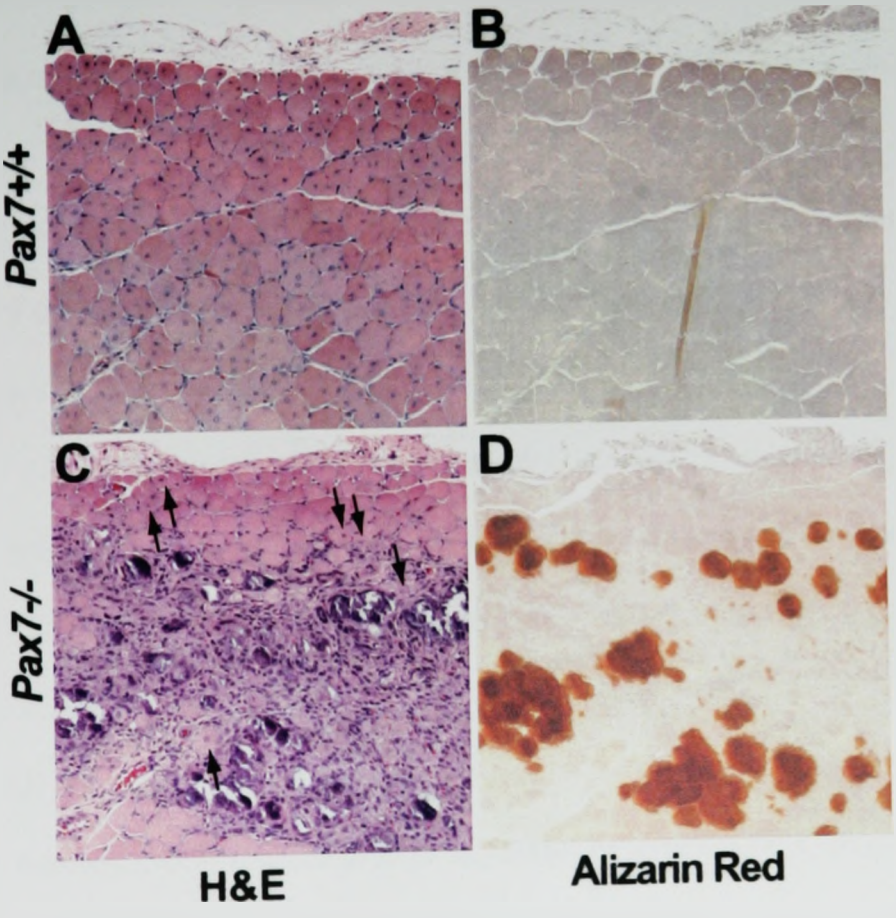
induced by crush-injury. As with ctx injury and aging, *Pax7*-deficient muscles displayed a dramatic impairment in regenerative capacity after crush-injury. Specifically, 10 days after injury, large calcium deposits and extensive fibrosis were apparent in damaged $Pax7^{-/-}$ muscle (n= 4). By contrast, TA muscles from sex matched, wildtype littermates (n= 3) were fully regenerated with large centrally nucleated myofibers (Figure 4). Interestingly, in contrast to ctx-induced injury, no adipose tissue differentiation was observed in crush injured $Pax7^{-/-}$ TA muscles. Together, these data demonstrate a requirement for Pax7 for efficient muscle regeneration in response to both mild and severe muscle damage.

4.3.4 Cell autonomous deficit in muscle regeneration

To eliminate the possibility that the absence of Pax7 in the muscle tissue environment may impair regeneration, wildtype cells were transplanted into regenerating *Pax7*-deficient muscles. Muscle SP cells (muSP) ($2x10^4$) and primary satellite cell-derived myoblasts ($5x10^5$) prepared from adult *ROSA26* transgenic mice, in which the *LacZ* reporter gene is constitutively expressed (Zambrowicz et al., 1997), were transplanted directly into regenerating *Pax7*-deficient TA muscles 2 days post-ctx injection. Both exogenous *ROSA26* myoblasts (Figure 5A, B) and muSP cells (Figure 5C, D) were able to functionally differentiate into Desmin-positive centrally nucleated fibers 14 days after injury (n= 2). The efficiency of engraftment and myogenic differentiation of transplanted *ROSA26* cells in these experiments was qualitatively similar in $Pax7^{+/+}$ and $Pax7^{-/-}$ muscles. These data demonstrate that wildtype myogenic

**Figure 4 Regeneration deficit in *Pax7*$^{-/-}$ muscle after crush-injury**

TA cross-sections from 1 month old *Pax7*$^{+/+}$ (**A-B**) and *Pax7*$^{-/-}$ littermate (**C-D**) 10 days after crush-injury. H&E staining of *Pax7*$^{+/+}$ TA revealed an efficient muscle regeneration characterized by numerous centrally nucleated myofibers (**A**). In contrast, only rare centrally nucleated myofibers were found in *Pax7*$^{-/-}$ TA (**C**, arrows). Instead damaged TA muscles were infiltrated by inflammatory cells and replaced by fibrosis and large calcium deposits (**D**).


A
B
Pax7+/+
C
D
Pax7-/-
H&E
Alizarin Red

cells are capable of muscle differentiation in *Pax7*$^{-/-}$ muscle, confirming that the regeneration deficit in *Pax7*$^{-/-}$ muscle is cell autonomous resulting from the absence of sufficient numbers of myoblasts.

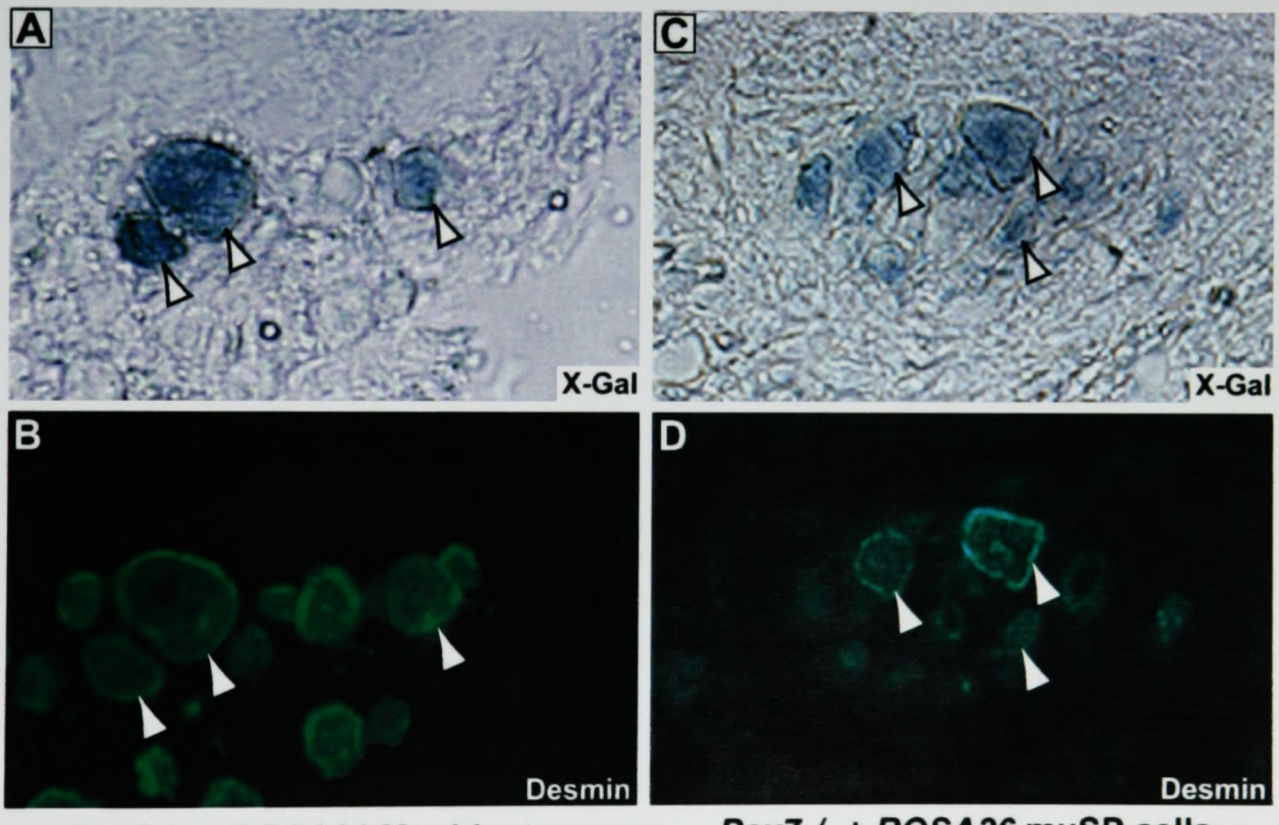
### 4.3.5 Absence of satellite cells in *Pax7*-deficient adult mice

Skeletal muscle regeneration is thought to be accomplished primarily through the activation of muscle satellite cells (Charge and Rudnicki, 2003; Seale, 2002). In previous analyses of *Pax7*-deficient muscle, we reported an absence of myogenic satellite cells in postnatal muscles. However, the newly formed myofibers in regenerating *Pax7*$^{-/-}$ muscle suggested that adult myogenic progenitors were present in adult *Pax7*-deficient mice. To identify and enumerate satellite cells in adult *Pax7*$^{-/-}$ mice, single muscle fibers from the extensor digitorum longus (EDL) muscle were isolated (Rosenblatt et al., 1995). All satellite cells associated with freshly isolated wildtype muscle fibers (time 0) coexpressed Pax7 and Syndecan4 (Figure 6A, B) or Pax7 and CD34 (not shown) suggesting that Pax7 is expressed in all quiescent satellite cells in EDL muscle.

All wildtype muscle fibers analyzed at time 0 for the expression of satellite cell specific markers CD34 (Beauchamp et al., 2000) or Syndecan4 (Cornelison et al., 2001) were associated with at least one satellite cell. Specifically, 8.7 ±1.9 Syndecan4-positive cells/fiber (Figure 6C, D) and 3.9 ±1.8 CD34-positive cells/fiber (Figure 6G, H) were detected in adult (6-8 week old) wildtype fibers (n= 16 fibers from 3 different mice and n= 35 fibers from 2 different mice for Syndecan-4 and CD34, respectively). By contrast, Syndecan4- (Figure 6E, F) or CD34-expressing cells (Figure 6I, J) were not associated

**Figure 5 Wildtype cells undergo myogenic differentiation in *Pax7*$^{-/-}$ muscles**

Exogenous primary myoblasts (**A-B**) or muscle side-population (muSP) cells (**C-D**) from *ROSA26* mice (constitutively expressing LacZ) formed X-Gal-positive myofibers in *Pax7*$^{-/-}$ muscle by day 14 after ctx injection (**A, C**). Expression of Desmin in newly regenerated fibers formed from ROSA26 myoblasts (**B**) or muSP cells (**D**) confirmed the capacity for wildtype cells to undergo myogenesis in a *Pax7*-deficient environment.




*Pax7-/-* + *ROSA26* Myoblasts

*Pax7-/-* + *ROSA26* muSP cells

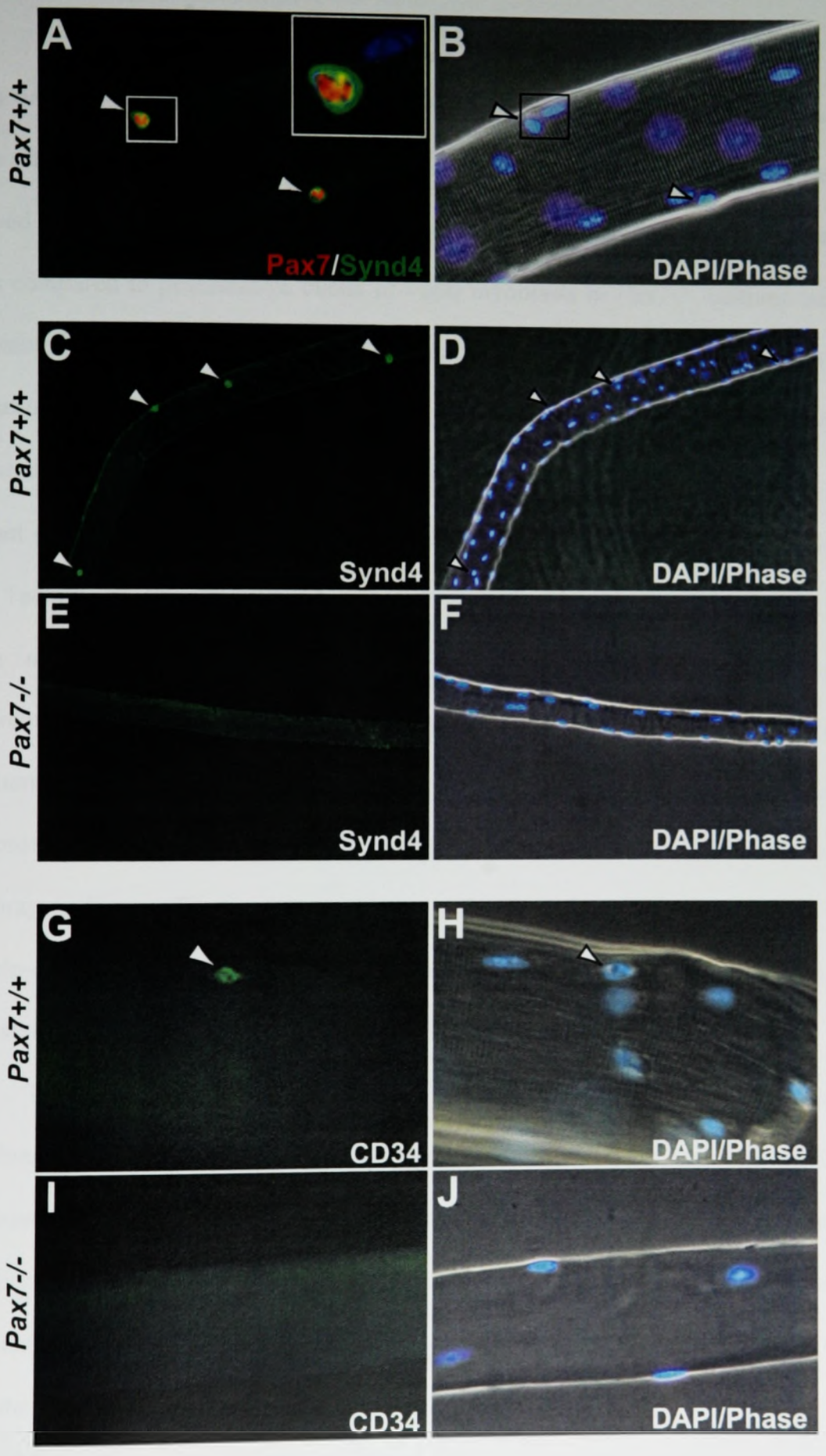
with any *Pax7*$^{-/-}$ myofibers (n= 56 fibers from 3 different mice and n= 65 fibers from 2 different mice for Syndecan4 and CD34, respectively). In support of these findings, only 9% of *Pax7*$^{-/-}$ myofiber cultures (n= 76 fibers from 3 mice) yielded any mononuclear cells after several days in growth medium (average of 1.85 ±0.34 cells/fiber). Importantly, the mononuclear cells associated with *Pax7*$^{-/-}$ fibers were negative for myoblast specific markers including MyoD, Myf5 and Desmin (data not shown). By contrast, all *Pax7*$^{+/+}$ fibers (n= 60 fibers from 3 mice) gave rise to an average of 11.8 ±0.97 cells/fiber that readily gave rise to proliferative bursts of MyoD-expressing myoblasts. Together, these data confirm the absence of a myogenic cell population closely associated with adult *Pax7*$^{-/-}$ EDL muscle fibers classically defined as muscle satellite cells.

### 4.3.6 *Pax7*$^{-/-}$ muscle contains low numbers of MyoD-expressing myogenic cells

To determine whether myogenic cells could be isolated from *Pax7*$^{-/-}$ muscle, single cell suspensions were prepared from hindlimb muscles and analyzed after 15 hrs in growth conditions or after an additional 3 days in differentiation medium. Interestingly, freshly isolated cell preparations from hindlimb *Pax7*$^{-/-}$ skeletal muscle yielded some MyoD (Figure 7A-D) or Myf5 (not shown) expressing myogenic cells. Although present, *Pax7*$^{-/-}$ myogenic cells were recovered at an extremely low frequency (~1/150) compared with wildtype muscle (363,012 ±34,247 (n= 2) and 2,961 ±1,096 (n= 4) MyoD-positive cells and 242,132 ±5,843 (n= 2) and 1,277 ±578 (n= 3) Myf5-positive cells per gram of muscle in *Pax7*$^{+/+}$ and *Pax7*$^{-/-}$ mice, respectively). The frequency of MyoD-positive

**Figure 6 Absence of satellite cells associated with *Pax7*$^{-/-}$ myofibers**

**(A-B)** Pax7 (Rhodamine, red) and Syndecan-4 (FITC, green) proteins are present in quiescent satellite cells associated with freshly isolated EDL myofibers. All *Pax7*$^{+/+}$ fibers contained Syndecan4 positive cells **(C-D)** and CD34 expressing cells **(G-H)**, whereas no Syndecan4 **(E-F)** or CD34 **(I-J)** positive cells were associated with *Pax7*$^{-/-}$ fibers. Arrowheads show satellite cells and corresponding nuclei.

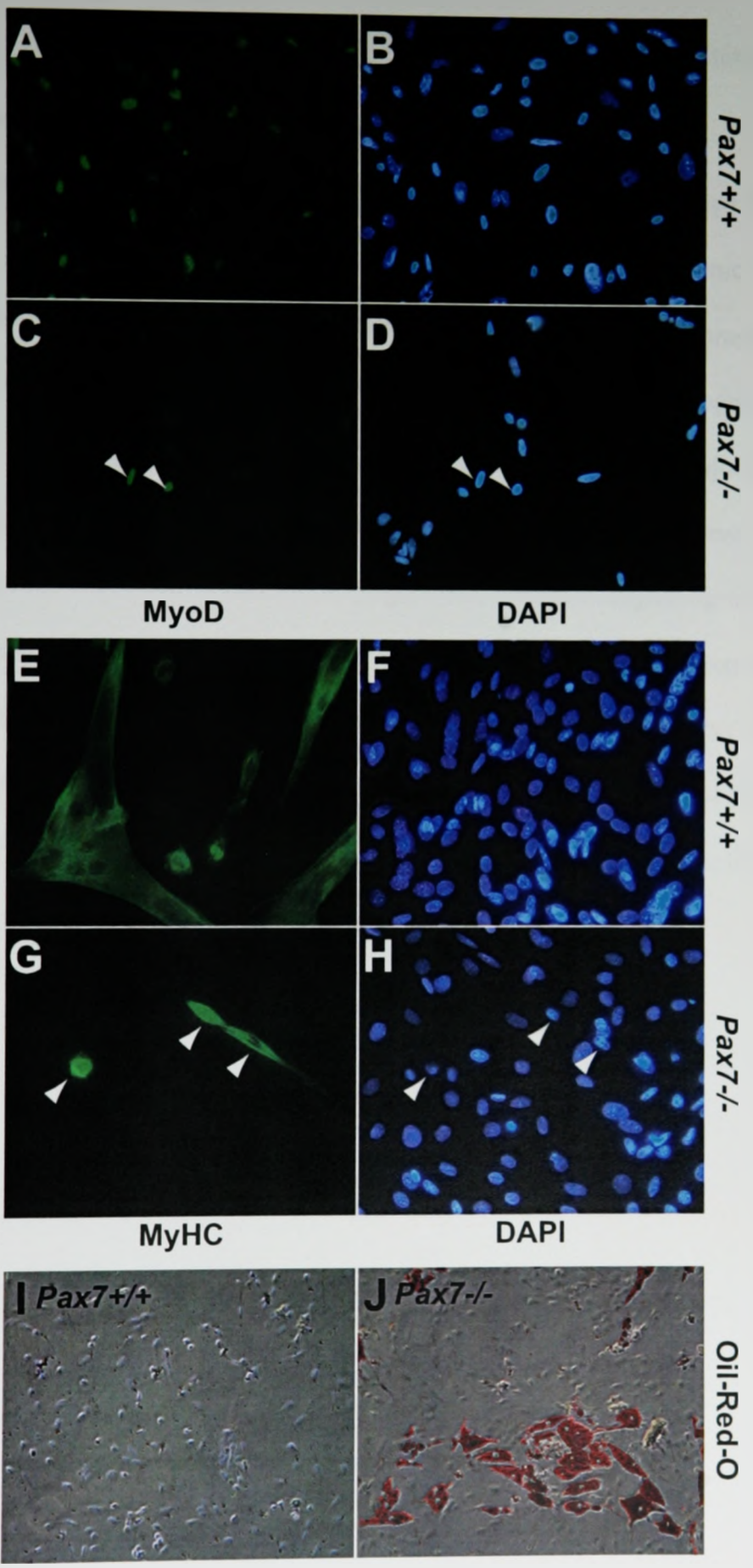

A
B
Pax7+/+
Pax7/Synd4
DAPI/Phase
C
D
Pax7+/+
Synd4
DAPI/Phase
E
F
Pax7-/-
Synd4
DAPI/Phase
G
H
Pax7+/+
CD34
DAPI/Phase
I
J
Pax7-/-
CD34
DAPI/Phase

cells was not increased in cultures prepared from regenerating *Pax7*$^{-/-}$ TA muscle 3-4 days after ctx injection (data not shown). Following plating at clonal density, *Pax7*$^{-/-}$ cells formed small colonies of 6-20 MyoD-positive/Desmin-positive myoblasts after 10-20 days compared to proliferative bursts of >500 myoblasts in *Pax7*$^{+/+}$ cultures isolated at the same time (data not shown). The inability for *Pax7*$^{-/-}$ cells to expand in culture suggests a proliferative deficit under standard myoblast growth conditions. Even when cultured in the presence of high concentrations of growth factors (5% chick embryo extract or 50 ng/ml Stem Cell Growth Factor/1 µg/ml Insulin, Methocult M3434 (Stem Cell Technologies) or on Matrigel coated dishes), *Pax7*$^{-/-}$ myogenic cells did not expand (data not shown). Nevertheless, *Pax7*$^{-/-}$ myogenic cells underwent myogenic differentiation and expressed MyHC (Figure 7G, H) after stimulation in differentiation medium for 3 days. *Pax7*$^{-/-}$ myogenic cells were not specific to limb muscle as demonstrated by the presence of MyoD-positive cells in cultures isolated from the diaphragm (Figure 7E, F). In contrast to wildtype cultures, freshly isolated *Pax7*$^{-/-}$ muscle-derived cells were markedly enriched in adipocytes as revealed by Oil-Red-O lipid staining following 24 hrs in growth media (Figure 7K, L).

Together these results indicate that low numbers of *Pax7*-deficient myogenic cells are obtained from whole skeletal muscle suspensions (diaphragm and limb muscle) but not from single fiber cultures. This alternative anatomical location together with their inability to grow under standard myoblast growth conditions strongly suggests that *Pax7*-deficient myoblasts represent a novel myogenic progenitor population that is distinct from the satellite cell compartment.

**Figure 7 Identification of a Pax7-independent myogenic cell population**

Cell suspensions from whole hindlimb muscles of *Pax7*$^{+/+}$ mice yielded large numbers of MyoD-positive cells after 15 hours in growth conditions (**A-B**). By contrast, only rare rare MyoD-positive cells (arrowhead) were observed in similar preparations from *Pax7*-deficient muscles (**C-D**). Myogenic cells obtained from both *Pax7*$^{+/+}$ (**E-F**) and *Pax7*$^{-/-}$ (**G-H**) suspensions were capable of fusion, terminal differentiation and upregulation of late differentiation markers including MyHC following 3 days in differentiation media. Large numbers of adipogenic cells, stained with Oil-Red-O, were present in *Pax7*$^{-/-}$ cultures (**J**) but not in wildtype preparations (**I**) after 24 hrs in growth media.

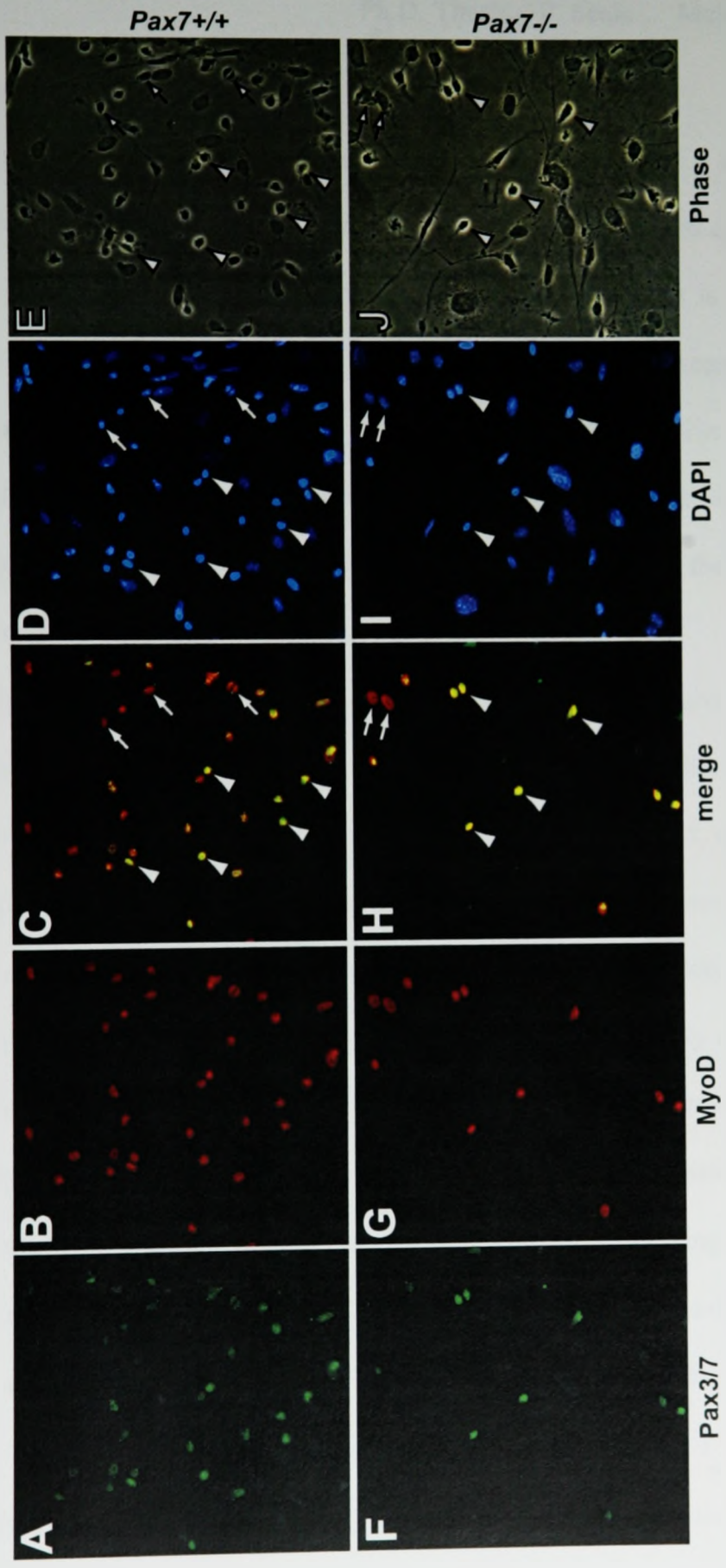

A
B
C
D
Pax7+/+
Pax7-/-
MyoD
DAPI
E
F
G
H
Pax7+/+
Pax7-/-
MyHC
DAPI
I Pax7+/+
J Pax7-/-
Oil-Red-O

### 4.3.7 Pax3 and MyoD are coexpressed in *Pax7*$^{-/-}$ myogenic cells

The Pax7-independent specification of myogenic cells suggested the hypothesis that the paralogous Pax3 protein was involved in their myogenic commitment. Immunohistochemical analysis of cell cultures from limb and diaphragm muscle (as described above) was performed using an anti-Pax3 antibody that recognizes both Pax3 and Pax7. In wildtype diaphragm cultures (Figure 8 A-E), the majority of MyoD-expressing cells also expressed Pax3 (Figure 8C, arrowheads). However a substantial number of cells expressed MyoD alone (Figure 8C, arrows), suggesting that Pax3/7 had already been downregulated. Interestingly, the vast majority of MyoD-expressing cells in cultures derived from *Pax7*$^{-/-}$ muscle (Figure 8 F-J) coexpressed Pax3 (Figure 8H, arrowheads). As with wildtype cultures, cells expressing MyoD but not Pax3 were also detected (Figure 8H, arrows). These results suggest that Pax3 specifies a distinct population of myogenic progenitors in adult *Pax7*$^{-/-}$ muscle.

**Figure 8 Pax3 and MyoD are coexpressed in *Pax7*$^{-/-}$ myogenic cells**

Cell suspensions obtained from *Pax7*$^{+/+}$ skeletal muscle (**A-E**) revealed that Pax3 or Pax7 and MyoD were coexpressed in a large proportion of cells (**C**, arrowheads). The majority of MyoD expressing cells in cultures derived from *Pax7*$^{-/-}$ muscle (**F-J**) coexpressed Pax3 (**H**, arrowheads). Cells expressing MyoD but not Pax3/7 were also detected in wildtype (**C**, arrows) and *Pax7*$^{-/-}$ cultures (**H**, arrows).


Pax7+/+
Pax7-/-
A
B
C
D
E
F
G
H
I
J
Pax3/7
MyoD
merge
DAPI
Phase

## 4.4 Discussion

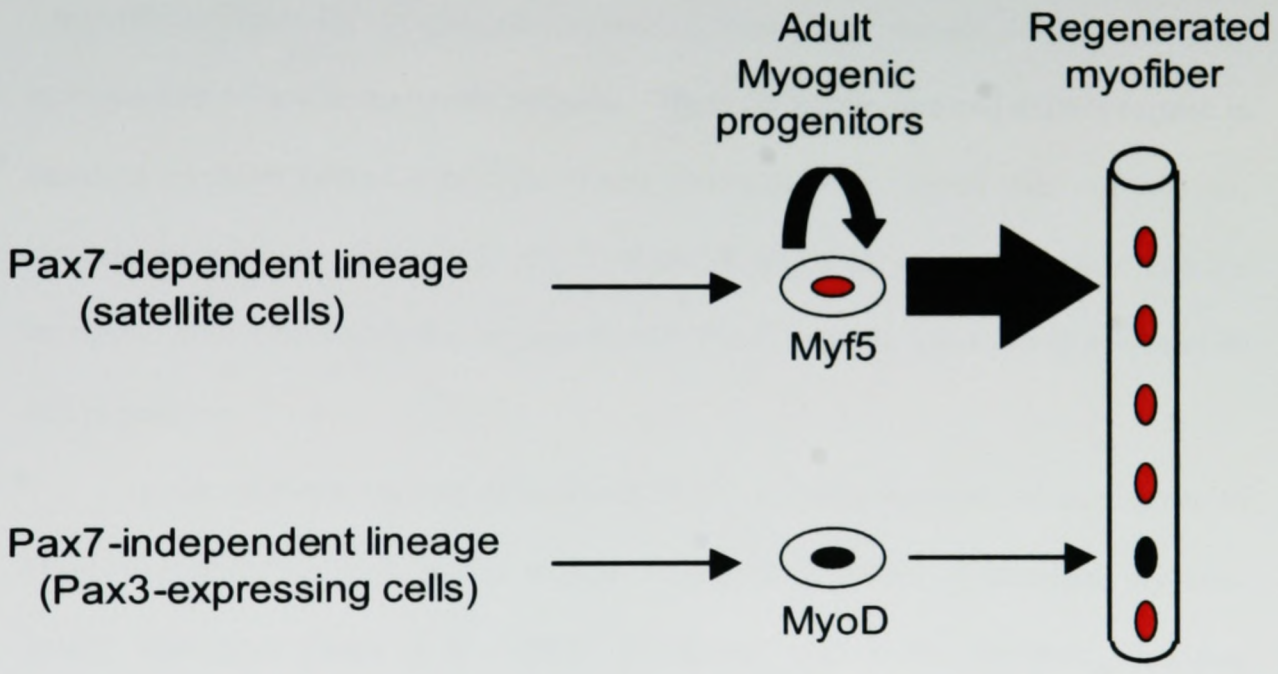
Our analysis of *Pax7*-deficient adult mice clearly establishes a requirement for Pax7 in skeletal muscle regeneration. Importantly, these experiments demonstrate that Pax7 is not only required for the development of satellite cells, but is also absolutely required for all postnatal myogenesis. Furthermore, our results suggest that Pax3-expressing myogenic progenitors derived independently from the satellite cell lineage are present in adult muscle. Taken together, these experiments have identified a novel adult myogenic progenitor population that appears to depend on Pax3 rather than Pax7 for their myogenic specification.

Using three independent models of muscle injury, our results demonstrate a profound regeneration deficit in adult *Pax7*$^{-/-}$ mice. In the case of mild, chronic muscle degeneration during normal ageing, fast *Pax7*$^{-/-}$ muscle fibers are lost, accompanied by calcium deposition in the wasting muscle (Figure 1). However, in response to more acute damage following crush or ctx-induced injuries, *Pax7*$^{-/-}$ muscle is rapidly replaced by an inflammatory site, large calcium deposits and adipose tissue with only rare regenerated myofibers being formed (Figure 2 and 4). These results demonstrate that a *Pax7*-dependent myogenic cell lineage is absolutely required in adult myogenesis (Figure 9).

The rare regenerating myofibers in *Pax7*$^{-/-}$ TA muscles following ctx injection or crush-injury suggest the presence of Pax7-independent myogenic progenitors. Although, the origin and complete characterization of these myogenic cells remain to be determined, using different isolation and culture procedures we were able to unequivocally distinguish them from the satellite cell population. Indeed, adult muscle

**Figure 9 Pax3 and Pax7 dependent cell lineages in adult muscle**

We demonstrate that *Pax7*-dependent cells are required for effective adult skeletal muscle regeneration whereas Pax7-independent, Pax3-expressing myogenic cells do not contribute significantly to this process. The Pax7-expressing cell population comprises the adult muscle satellite cell population and therefore satellite cells are likely the main contributor to adult skeletal muscle regeneration. We hypothesize that adult stem cells upregulate Pax7 and either directly fuse to regenerating myotubes and contribute to the satellite cell pool during adult myogenesis.


Adult
Myogenic
progenitors
Regenerated
myofiber
Pax7-dependent lineage
(satellite cells)
Myf5
Pax7-independent lineage
(Pax3-expressing cells)
MyoD

satellite cells are, by definition, closely associated with myofibers, wedged in between the basal lamina and the fiber plasmalemma (Bischoff, 1994). Single myofiber isolation, by way of a mild collagenase digestion and mechanical trituration, allows the specific isolation of muscle fibers with their associated satellite cells (Rosenblatt et al., 1995). We were able to repeatedly isolate wildtype EDL myofibers with the correct number of satellite cells (Zammit et al., 2002). By contrast, satellite cells were not detected on *Pax7*$^{-/-}$ myofibers (Figure 6). Single cell suspensions from *Pax7*$^{-/-}$ muscle did however yield mononuclear cells with myogenic potential. These cells were rare and did not expand in standard myoblast culture conditions. These observations combined with our previous electron microscopic studies (Chapter 3; Seale et al., 2000) strongly suggest that the myogenic cells responsible for regeneration in *Pax7*$^{-/-}$ muscle are a novel non-satellite cell population.

In our previous analysis of newborn *Pax7*$^{-/-}$ animals, we reported an absence of MyoD-expressing cells in whole muscle homogenates grown in standard myoblast growth conditions (Seale et al., 2000). We cannot exclude the possibility that low numbers of myogenic cells were overgrown by other cell types including adipocytes and fibroblasts in these mixed cultures thereby precluding their detection. However, we did report low numbers of MyHC expressing myocytes following long-term culture of low-density suspensions in methylcellulose medium. The small number of myogenic cells may have been residual fetal myoblasts in 7-day-old muscle or may have represented Pax3-expressing adult myogenic cells as described in the current study.

*Pax7*-deficient adult myogenic cells did not expand using a variety of culture

conditions suggesting an intrinsic proliferation deficit. The inability for *Pax7*-deficient muscles to regenerate may reflect the inefficient expansion of Pax3-dependent myogenic progenitors. Interestingly, *Pax7*$^{-/-}$ myogenic cells appeared to express lower levels of Myf5 (data not shown). Significantly, several studies have noted a role for Myf5 in promoting myoblast proliferation. For example homozygous *Myf5nLacZ* (e.g. *Myf5*-deficient) embryos possess significantly reduced numbers of LacZ-expressing myogenic progenitors (Tajbakhsh et al., 1996). Myf5 is also preferentially expressed in proliferating avian myoblasts, whereas MyoD appears to be upregulated in differentiating cells within the developing wing (Delfini et al., 2000). These observations are consistent with the proliferation deficit of *Myf5*$^{-/-}$ myoblasts (Montarras et al., 2000) and the markedly increased rate of proliferation in *MyoD*$^{-/-}$ myoblasts that express high levels of Myf5 (Sabourin et al., 1999). Taken together, these findings support the notion that adult Pax3+:MyoD+ myogenic cells do not undergo expansion because they do not express sufficient levels of Myf5. Adult progenitors that express Pax3 and MyoD may thus possess an increased propensity to differentiate directly without undergoing Myf5-dependent expansion. These results thus implicate Myf5 as being important for proliferation during adult myogenesis, with MyoD acting downstream to promote cell-cycle withdrawal and differentiation.

During embryonic myogenesis, it has been established that Pax3 functions upstream of MyoD in the myogenic specification of stem cells (Bendall et al., 1999; Heanue et al., 1999; Maroto et al., 1997; Ridgeway and Skerjanc, 2001; Tajbakhsh et al., 1997). Therefore, Pax7 may be analogously required for the myogenic specification of

adult stem cells and their subsequent proliferation via a Myf5-dependent pathway. Functional analysis of Pax3 and Pax7 DNA binding targets will be required to elucidate mechanisms involved in myogenic specification downstream of Pax3 or Pax7 during embryonic and adult myogenesis respectively.

A recent report analyzing the expression of *Pax3* from a *Pax3nLacZ* knock-in reporter mouse suggested that *Pax3* might also be expressed in adult muscle (Buckingham et al., 2003). Interestingly, although *Pax3* appears to be expressed in some adult progenitors, its expression does not compensate for the absence of Pax7. The inability for Pax3 to compensate for the absence of Pax7 in these cells is of particular interest, given the high degree of homology, and similar *in vitro* DNA binding activities of these proteins (Bennicelli et al., 1999; Schafer et al., 1994). Further studies characterizing the expression kinetics and differential activity of Pax3 and Pax7 in adult muscle cells are needed to resolve this question.

The role of these novel Pax3-expressing myogenic cells identified in *Pax7*-deficient muscle is unclear. It is possible that it is a population specific to the *Pax7*-deficient muscles. However, more interesting is the possibility that these progenitors are also present in wildtype muscle but have not been defined due to their low abundance and growth deficit *in vitro*. This novel cellular population may represent myogenic progenitors which under normal circumstances upregulate *Pax7*, and give rise to muscle satellite cells. However, this seems unlikely since Pax genes have been demonstrated to function upstream of the MRFs. Another possibility is that this adult myogenic population is distinct from the adult satellite cell population, possibly representing a

et al., 1999; LaBarge and Blau, 2002). A recent report by LaBarge and Blau suggests that BM-derived cells not only contribute to regenerating myofibers but also to the muscle satellite cell pool (LaBarge and Blau, 2002). However, experiments in which local irradiation was used to ablate progenitor populations in skeletal muscle results in a long-term deficit in regenerative capacity (Heslop et al., 2000; Pagel and Partridge, 1999; Wakeford et al., 1991). This result suggests that circulating blood cells are unlikely to participate at physiologically significant levels to muscle regeneration. Furthermore, experiments by Wagers et al. demonstrate that purified hematopoietic stem cells do not differentiate as skeletal muscle in a transplant model (Wagers et al., 2002). Moreover, *in vitro* studies suggest that resident satellite cells possess sufficient proliferative capacity to induce the regeneration of the muscle they reside in, assuming all satellite cells survive the injury (Zammit et al., 2002). The present study indirectly supports the hypothesis that non-resident stem cells are not required during normal regeneration. In summary, the extent to which marrow cells contribute to the regeneration of muscle remains to be resolved including the identity and phenotype of the marrow fraction that contains this activity. In our study, it is possible that the low level of regeneration observed in the absence of satellite cells is derived from the bone marrow. Experiments in which marked *Pax7*-deficient marrow is introduced into wildtype recipients will indicate whether bone marrow cells require the upregulation of *Pax7* to differentiate as muscle.

Adipose tissue differentiation has been reported in several models of acute muscle regeneration such as in the *mdx* mouse (Pastoret and Sebille, 1995) and in some human muscle diseases including muscular dystrophy (Lin et al., 1969) and mitochondrial

myopathy (DiMauro et al., 1980). Similarly, ectopic bone formation in skeletal muscle has been described in human diseases (Shore et al., 2000). However, the origin of these mesenchymal progenitors is unknown. Recent data have demonstrated the potential for adult muscle satellite cells to differentiate into osteogenic or adipogenic cells *in vitro* (Asakura et al., 2001; Wada et al., 2002). Therefore, it has been postulated that aberrant activation of satellite cells during muscle regeneration may lead to reversal of lineage commitment at the expense of effective muscle regeneration. Our observations that adipogenic and osteogenic tissue formation is markedly increased in the absence of muscle satellite cells following muscle damage suggest that cells other than satellite cells are involved in these ectopic cellular differentiations.

## 4.5 Materials and methods

### Animals

Mice carrying a targeted null mutation in *Pax7* (hereafter referred to as *Pax7*$^{-/-}$) generously provided by Dr. Peter Gruss (Mansouri et al., 1996) were outbred into the SV129 background to increase survival at the University of Ottawa Barrier facility. *ROSA26* transgenic mice were obtained from the Jackson Laboratory (Zambrowicz et al., 1997). Mouse serum was prepared by coagulation and centrifugation of blood samples, stored frozen and assayed by the Department of Biochemistry at the Children Hospital of Eastern Ontario.

### Cardiotoxin and crush-induced regeneration

Two to seven month-old *Pax7*$^{-/-}$ and wildtype littermates were anesthetized with Halothane gas. 25 µl of 10 µM cardiotoxin (ctx) (Latoxan, France) was injected into the midbelly of the TA muscle, using a 29 G 1/2 insulin syringe. Mice were sacrificed at 10 days or 1 month post-ctx injection. For crush injuries, a small incision was made at the TA level and muscle was crushed using large forceps prior to suturing of the wound. Mice were sacrificed at 10 days postinjury. For cell proliferation assays, 30 mg/kg of 5-bromo-deoxyuridine (BrdU, Sigma) was injected intraperitoneally on day 4, 6, 7 and 8 after ctx injection. To assess myofiber damage, mice were injected intraperitoneally with 100 mg/kg body weight of Evans Blue dye in saline 12 hr prior to sacrifice.

**Histology and immunocytochemistry**

Total fiber number was determined on uninjured contralateral legs as follows. TA muscle was isolated, cut at midbelly, embedded in OCT (Tissue-Tek)/20% sucrose in liquid nitrogen or fixed in 4% paraformaldehyde prior to embedding in paraffin. 10 µm cryosections or 4 µm paraffin sections were stained with Hematoxylin and Eosin (H&E), pictures were taken and fibers were counted using NIH Image software. For ctx-injured muscle, whole lower hind legs were frozen or paraffin embedded as above. Cross-sections were stained with H&E, Alizarin Red S (calcification), or Van Gieson's stain (fibrosis). Regenerating fibers were identified by immunostaining with an antibody specific to Desmin (DAKO) or to embryonic fast MyHC (F1.652, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, USA) as described (Hughes et al., 1993). Fiber types were identified with monoclonal antibodies recognizing fast type IIb MyHC (BF-F3, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) and type IIa MyHC (A4.74, DSHB) as described (Hughes et al., 1993). BrdU detection was performed using the BrdU *in-situ* dectection kit (BD Biosciences). Sections were analyzed on an axioplan 2 Zeiss microscope. Central myonuclei in regenerating and control muscles were counted on at least 2 independent cross-sections of the entire TA muscle per mouse analyzed.

**Cell cultures**

Extensor Digitorum Longus (EDL) muscle fibers were isolated by collagenase digestion as described previously (Rosenblatt et al., 1995). Fibers were either plated on

matrigel coated dishes for culture in growth media and subsequent differentiation as described (Charge et al., 2002) or directly immunostained for satellite cell markers. For total myoblast isolation, hindlimb or diaphragm muscles were homogenized in collagenase/dispase and myogenic cells were recovered as described previously (Megeney et al., 1996). Myogenic cultures were maintained in growth medium consisting of Ham's F-10 medium (Invitrogen) supplemented with 20% FCS, and 2.5 ng/ml bFGF. Differentiation was induced by switching cultures to DMEM medium (Invitrogen) supplemented with 5% Horse Serum. For immunohistochemical analysis, single fibers or cell cultures were fixed with 4% paraformaldehyde (or 1% paraformaldehyde for Pax3 staining), cells were permebealized in 0.3% Triton-X/PBS and non-specific antigens were blocked in 5% horse serum/PBS (or 5% goat serum/PBS for Pax3 staining). Cells were reacted with primary antibodies as follows: Desmin (DAKO); CD34 (BD Pharmingen); MyoD (1/200, 5.8A, BD Pharmingen); all MyHC (1/10, MF-20, Developmental Studies Hybridoma Bank (DSHB)); Myf5 (C-20, Santa-Cruz); Pax7 (1/10, DSHB); Pax3 (1/500, Geneka); and Syndecan4 (gift of B. Olwin, (Cornelison et al., 2001)). Secondary detection was performed using Fluorescein or Rhodamine conjugated antibodies (Chemicon). Cells were reacted with the nuclear dye DAPI before mounting and analysis on an axioplan 2 Zeiss microscope.

**Cell transplantation**

Muscle-derived side-population (muSP) cells were obtained from hindlimb muscle suspensions by staining with 5 μg/ml Hoechst 33342 dye as described previously

(Asakura et al., 2002). Cells were fractionated using a MoFlo cell-sorter (DAKO-Cytomation). $2x10^4$ muSP cells or $5x10^5$ cultured primary satellite cell derived myoblasts (isolated as described above) from *ROSA26* muscle were injected into the TA muscle of 6 week old $Pax7^{+/+}$ or $Pax7^{-/-}$ littermates 2 days after injection of ctx. Mice were sacrificed 14 days after ctx injection to analyze myogenic contribution of transplanted cells. Implanted LacZ-expressing cells were detected using whole-mount X-Gal reaction as described previously (Asakura et al., 2002), prior to preparation of serial cryosections for immunohistochemistry as described above.

**Statistical analysis**

Errors quoted are standard error of the mean (sem) throughout. Data were analyzed using unpaired *t* tests. * and ** indicate significance at $P<0.05$ and $P<0.01$ throughout.

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# Chapter 5

## Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration

**Preface:**

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My role in this work was the initial identification and characterization of the $CD45^+$:$Sca1^+$ adult stem cells (Figure 1) which formed the basis for the Wnt experiments. I also developed the myoblast coculture procedure for inducing the myogenic specification of adult stem cells (Asakura et al., 2002 and Figure 2). Dr. Anna Polesskaya performed all the experiments exploring the role of Wnt signaling in the myogenic recruitment of this adult stem cell population (Figures 3-6). I helped Dr. Polesskaya set-up and analyze the *in vivo* regeneration experiments (Figure 6). Finally, I assembled the data for the article, prepared the figures and wrote the manuscript with substantial input from Dr. M.A. Rudnicki. Considerable advice and intellectual focus for the study were also given by Dr. M.A. Rudnicki.

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# Wnt Signaling Induces the Myogenic Specification of Resident CD45+ Adult Stem Cells during Muscle Regeneration

**Anna Polesskaya,[1,3,4] Patrick Seale,[1,2,3] and Michael A. Rudnicki[1,2,*]**
[1]Ottawa Health Research Institute
Molecular Medicine Program
501 Smyth Road
Ottawa, Ontario
Canada, K1H 8L6
[2]Department of Biology
McMaster University
Hamilton, Ontario
Canada, L8S 4K1

## Summary

**The observation that CD45+ stem cells injected into the circulation participate in muscle regeneration raised the question of whether CD45+ stem cells resident in muscle play a physiological role during regeneration. We found that CD45+ cells cultured from uninjured muscle were uniformly nonmyogenic. However, CD45+ cells purified from regenerating muscle readily gave rise to determined myoblasts. The number of CD45+ cells in muscle rapidly expanded following injury, and a high proportion entered the cell cycle. Investigation of candidate pathways involved in embryonic myogenesis revealed that Wnt signaling was sufficient to induce the myogenic specification of muscle-derived CD45+ stem cells. Moreover, injection of the Wnt antagonists sFRP2/3 into regenerating muscle markedly reduced CD45+ stem cell proliferation and myogenic specification. Our data therefore suggest that mobilization of resident CD45+ stem cells is an important factor in regeneration after injury and highlight the Wnt pathway as a potential therapeutic target for degenerative neuromuscular disease.**

## Introduction

Adult skeletal muscle possesses a remarkable ability to regenerate following injury. New myofibers formed during repair are thought to arise solely from the muscle satellite cell lineage. Satellite cells are specialized myogenic stem cells, which reside beneath the basal lamina of mature myofibers (Bischoff, 1994; Seale and Rudnicki, 2000). In response to diverse stimuli, including stretching, exercise, injury, and electrical stimulation, activated satellite cells give rise to myogenic precursor cells (mpcs) that proliferate prior to terminal differentiation and fusion into myofibers (reviewed by Seale and Rudnicki, 2000). The persistence of satellite cells in muscle subjected to repeated cycles of degeneration and regeneration has been interpreted to suggest that satellite cells are maintained by self-renewal (Bischoff, 1994).

*Correspondence: mrudnicki@ohri.ca
[3]These authors contributed equally to this work.
[4]Present address: CNRS, UPR 9079, 7 rue Guy Moquet, 94800, Villejuif, France.

Recent studies have identified novel populations of adult stem cells in skeletal muscle. For example, side-population (SP) cells isolated from muscle tissue on the basis of Hoechst dye exclusion reconstitute the blood system of irradiated mice following bone marrow transplantation (BMT) (Gussoni et al., 1999; Jackson et al., 1999). Furthermore, muscle-derived SP cells participate in the regeneration of skeletal muscle and give rise to satellite cells (Asakura et al., 2002; Gussoni et al., 1999). In vitro, muscle SP cells readily form hematopoietic colonies, but do not spontaneously differentiate as myogenic cells (Asakura et al., 2002). Notably, a percentage of muscle SP cells commit to muscle following coculture with satellite cell-derived myoblasts (Asakura et al., 2002).

Various cell surface markers have also been employed to purify adult stem cell populations from skeletal muscle, including c-kit, Sca1, CD34, and CD45 (Dell'Agnola et al., 2002; Lee et al., 2000; Mahmud et al., 2002; McKinney-Freeman et al., 2002; Romero-Ramos et al., 2002; Tamaki et al., 2002; Torrente et al., 2001). The cell surface antigen Sca1, used routinely in purification protocols for hematopoietic stem cells, is expressed on over 80% of muscle SP cells. By contrast, the hematopoietic-restricted lineage marker CD45 is expressed on about 16% of SP cells (Asakura et al., 2002). Importantly, Sca1 and CD45 are not expressed on muscle satellite cells (Asakura et al., 2002). Almost all muscle-derived hematopoietic progenitor and in vivo blood reconstitution activity is derived from CD45+ cells (Asakura et al., 2002; McKinney-Freeman et al., 2002). By contrast, muscle-derived CD45+ cells purified from uninjured muscle are uniformly nonmyogenic in vitro and do not form muscle efficiently in vivo (McKinney-Freeman et al., 2002). In this study, we investigated the myogenicity of resident CD45+:Sca1+ cells in response to muscle damage.

Coculture and in vivo injection experiments indicate that CD45+ SP as well as CD45+ SP cells possess myogenic potential (Asakura et al., 2002; McKinney-Freeman et al., 2002). Furthermore, the potential of bone marrow-derived cells to give rise to satellite cells has recently been documented in transplanted recipients (LaBarge and Blau, 2002). In this study, we investigated the hypothesis that molecular cues implicated in embryonic muscle development function analogously in adult tissue to induce the myogenic commitment of stem cells.

In the developing embryo, skeletal muscle of the trunk and limbs arises from somites (Christ and Ordahl, 1995). Somitic muscle precursors are specified in response to proteins secreted from the neural tube and notochord. These signals culminate in the expression of Pax3 and the myogenic determination factors Myf5 and MyoD. Sonic hedgehog (Shh), secreted by the notochord, and several proteins of the Wnt family (wingless), secreted by the dorsal neural tube (Wnt1, Wnt3) and surface ectoderm (Wnt4, Wnt6, Wnt7a), are required for induction of embryonic myogenesis (Munsterberg et al., 1995; Tajbakhsh et al., 1998).

The Wnt family of genes encodes for over twenty cysteine-rich secreted glycoproteins that act by binding to

Frizzled (Fzd) receptors on target cells. Binding of Wnt to Fzd activates Disheveled (Dvl), leading to the inactivation of Glycogen synthase kinase-3β (GSK-3β), a cytoplasmic serine-threonine kinase. The GSK-3β target β-catenin is stabilized, translocates to the nucleus, and activates TCF (T cell factor)-dependent transcription on specific promoters (reviewed by Dierick and Bejsovec, 1999; Wodarz and Nusse, 1998). Wnt signaling directs cell fate determination in various tissues, including kidney (Labus et al., 1998; Vainio and Uusitalo, 2000), CNS (Patapoutian and Reichardt, 2000), hematopoietic (Van Den Berg et al., 1998), and skeletal muscle (Cossu and Borello, 1999). Moreover, Wnt signaling is implicated in postnatal wound healing and tissue regeneration in zebrafish and hydra (Hobmayer et al., 2000; Labus et al., 1998; Poss et al., 2000).

Wnt proteins initiate myogenesis in explants of mouse paraxial mesoderm by activating expression of Myf5 and MyoD (Tajbakhsh et al., 1998). Myogenesis in presomitic mesoderm and early somites is inhibited by the Wnt antagonist soluble Frizzled-related protein 3 (sFRP3/Frzb1) (Borello et al., 1999). Therefore, Wnt signaling appears to be necessary and, in some instances, sufficient to induce and maintain the myogenic program in embryonic precursor cells.

This study demonstrates a biological role for endogenous CD45$^+$ muscle stem cells in maintaining tissue integrity by participating in regeneration. Moreover, these experiments establish that Wnt signaling is the mechanism by which CD45$^+$ adult stem cells are induced to undergo myogenic specification. Importantly, our study suggests that targeting the Wnt pathway represents a promising therapeutic approach for the treatment of neuromuscular degenerative disease.

## Results

### Myogenic Commitment of CD45$^+$:Sca1$^+$ Cells during Muscle Regeneration

Cells expressing the pan-hematopoietic marker CD45 and the stem cell marker Stem Cell Antigen-1 (Sca1) were purified from uninjured tibialis anterior (TA) muscle and at varying time points after cardiotoxin (ctx) induced regeneration (Figure 1A). The proportion of CD45- and Sca1-expressing cells increased by an average of 10-fold during regeneration (n = 6) (Figure 1A). Interestingly, selective incorporation of BrdU into CD45$^-$:Sca1$^+$ (60% of BrdU+ cells) and CD45$^+$:Sca1$^+$ cells (18% of BrdU+ cells) at 4 days post ctx injection suggested that these cells undergo extensive proliferation during regeneration (Figure 1B). These observations demonstrate that muscle cells expressing CD45 and Sca1 are activated and proliferate in response to muscle damage.

To specifically identify cells that had entered the myogenic program, muscle regeneration was induced in heterozygous *Myf5nLacZ* animals in which the bacterial *LacZ* gene is expressed from the *Myf5* gene locus. In these reporter mice, expression of LacZ faithfully recapitulates the expression pattern of the endogenous *Myf5* gene and is rapidly induced following myogenic commitment (Tajbakhsh and Buckingham, 1995). CD45$^+$:Sca1$^+$ cells were fractionated from uninjured and regenerating muscle 4 days after ctx injection and immediately used to prepare cytospins.

Importantly, CD45$^+$:Sca1$^+$ and CD45$^-$:Sca1$^+$ cells purified from uninjured muscle were always *Myf5nLacZ* negative and never gave rise to determined muscle cells in vitro (n = 6) (data not shown). Strikingly, however, 7.2% ± 2.6% of CD45$^+$:Sca1$^{high}$ (n = 6) (see Figure 1A for sorting gate) and 3.8% ± 1.8% of CD45$^-$:Sca1$^-$ (n = 3) cells from regenerating muscles 4 days postinjury coexpressed *Myf5nLacZ* (Figure 1C). A similar proportion of CD45$^+$:Sca1$^{high}$ cells purified from regenerating muscle (4 days post injury) expressed MyoD (Figure 1D), the muscle-specific intermediate filament protein Desmin (Figure 1D), and the satellite-cell-specific Pax7 protein (data not shown). Furthermore, CD45$^+$:Sca1$^+$ cells fractionated from regenerating muscle differentiated to MHC-expressing myocytes following culture in differentiation medium (Figure 1D). The complete absence of *Myf5nLacZ* expression in CD45$^+$:Sca1$^-$ cells isolated throughout regeneration indicated the specific activation of myogenesis in CD45$^+$:Sca1$^+$ and CD45$^-$:Sca1$^+$ cells. Similar results were obtained in experiments on cells that had been sorted twice.

We also examined the impact of ctx on the numbers of myogenic progenitors present within injured muscle. Interestingly, 18 hr post ctx injection, the number of *Myf5nLacZ*+ cells was reduced by approximately 30-fold relative to uninjured muscle ($1.18 \times 10^3 \pm 1 \times 10^3$ compared to $4.1 \times 10^4 \pm 1.6 \times 10^4$ *Myf5nLacZ*+ cells/g tissue) (Figure 1E). This observation was not due to ctx-induced *Myf5* promoter silencing, since colony-forming assays of whole muscle cells produced a similar decline in MyoD$^+$ and Desmin$^+$ myogenic cells 18 hr after ctx injection.

To determine the relative myogenic contribution of CD45$^+$ and Sca1$^+$ cells during regeneration, we calculated the numbers of *Myf5nLacZ*-expressing cells derived from the various muscle fractions. Our analysis (n > 3 for each time point) revealed that CD45$^-$:Sca1$^{high}$, CD45$^-$:Sca1$^+$, and CD45$^+$:Sca1$^-$ gave rise to an average of $1.54 \times 10^5$, $3.9 \times 10^5$, and $2 \times 10^3$ *Myf5nLacZ*+ cells/g tissue, respectively, 4 days post ctx injection (Figure 1F). These numbers represent average values compiled from independent experiments in which fractionated populations from *Myf5nLacZ* muscle were used to prepare cytospins and X-gal-expressing cells enumerated. Notably, committed myogenic progenitors (CD45$^-$:Sca1$^+$ cells) accounted for $6.0 \times 10^6$ *Myf5nLacZ*+ cells/g tissue by 4 days postinjury. The apparent toxicity of ctx on satellite cells thus raises the question of whether the resident satellite cell population does indeed represent the only source of myogenic progenitors following ctx-induced muscle injury.

Taken together, these experiments document the capacity for muscle-derived CD45$^+$ and Sca1$^+$ cells to undergo myogenic specification in response to muscle damage. Importantly, this observation demonstrates that nonsatellite-cell-derived progenitors participate in normal repair processes.

### Myogenic Commitment of CD45$^+$:Sca1$^+$ Cells Induced by Coculture with Myoblasts or Exposure to Lithium

As stated previously, CD45$^+$:Sca1$^+$ cells purified from uninjured skeletal muscle do not form myogenic cells

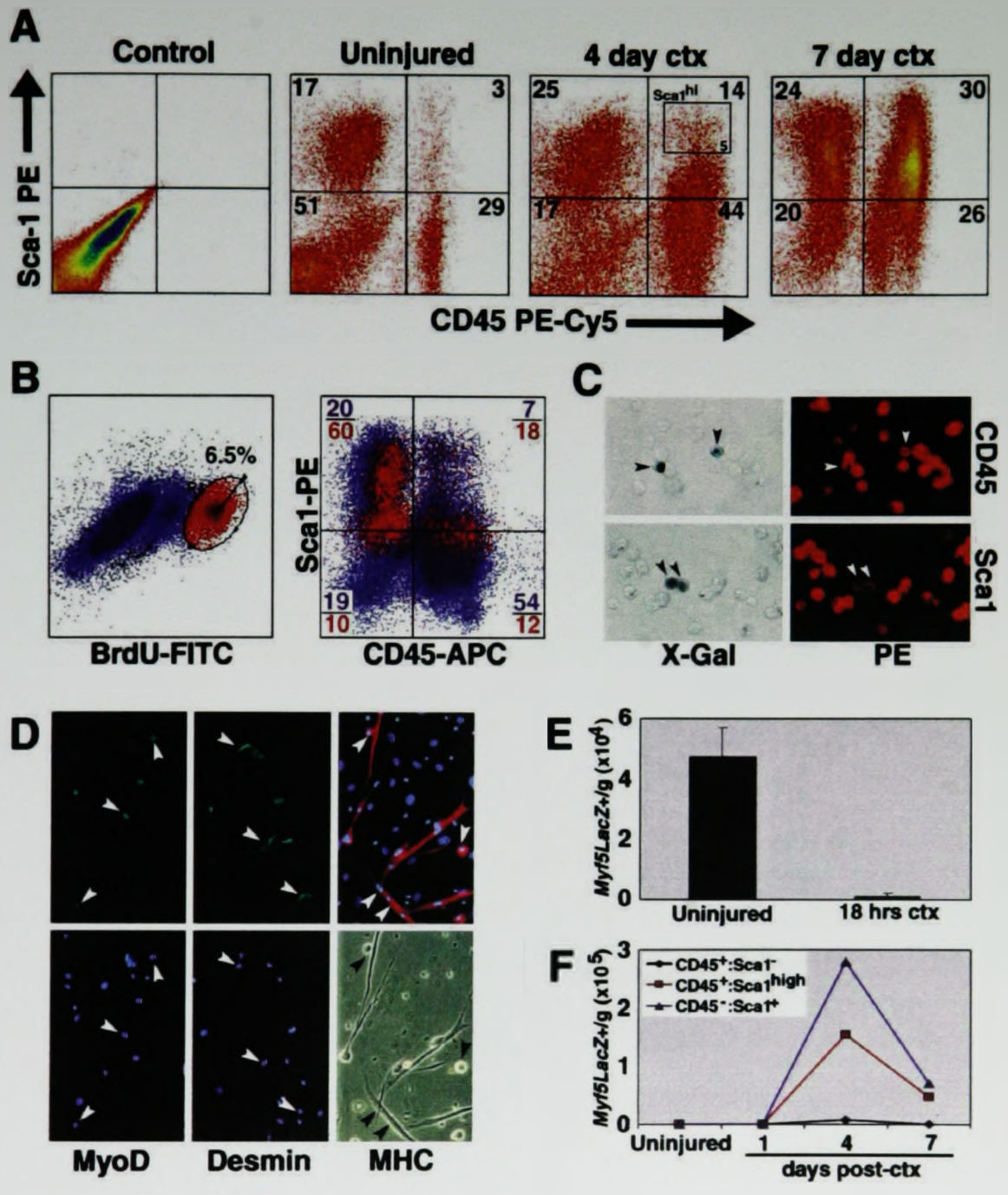


Figure 1. Myogenic Recruitment of CD45$^+$:Sca1$^+$ Cells in Regenerating Muscle

(A) Flow cytometric analysis of skeletal muscle-derived cells demonstrated that the proportion of cells expressing the hematopoietic markers CD45 and Sca1 increased dramatically in regenerating muscle (4 and 7 days after cardiotoxin [ctx] injection). The gating for CD45$^+$:Sca1$^{high}$ cells is shown for the 4 day time point.

(B) In vivo cell proliferation experiments indicated that 60% and 18% of BrdU$^+$ cells were CD45$^-$:Sca1$^-$ and CD45$^+$:Sca1$^+$, respectively, at 4 days postinjury (red = BrdU$^+$ cells, blue = total cells).

(C) ~7%–10% of CD45$^+$:Sca1$^{high}$ cells purified from regenerating (4 days), but not uninjured, *Myf5nLacZ* skeletal muscle coexpressed CD45, Sca1, and *Myf5nLacZ* as detected by X-Gal reaction.

(D) Fractionated CD45$^+$:Sca1$^{high}$ cells gave rise to MyoD- and Desmin-expressing skeletal muscle cells in culture. Moreover, a similar proportion of CD45$^+$:Sca1$^{high}$ cells differentiated to Myosin Heavy Chain (MHC)-expressing myotubes.

(E) The number of *Myf5nLacZ*$^+$ satellite cells was ~30-fold lower 18 hr after cardiotoxin injection compared to uninjured muscle.

(F) Quantitative analysis indicated that the CD45$^+$:Sca1$^{high}$ (red) and CD45$^-$:Sca1$^+$ (blue) fractions gave rise to an average of $1.54 \times 10^5$ and $3.9 \times 10^5$ myogenic cells, respectively, while the CD45$^+$:Sca1$^-$ (black) fraction contained negligible myogenic activity.

spontaneously (data not shown and Asakura et al., 2002; McKinney-Freeman et al., 2002). However, in coculture with primary myoblasts, 0.5% ± 0.03% of input CD45$^+$:Sca1$^+$ muscle cells from EGFP transgenic mice formed mononuclear, MHC-expressing myocytes (Figure 2A, ctrl). This frequency of myogenic differentiation

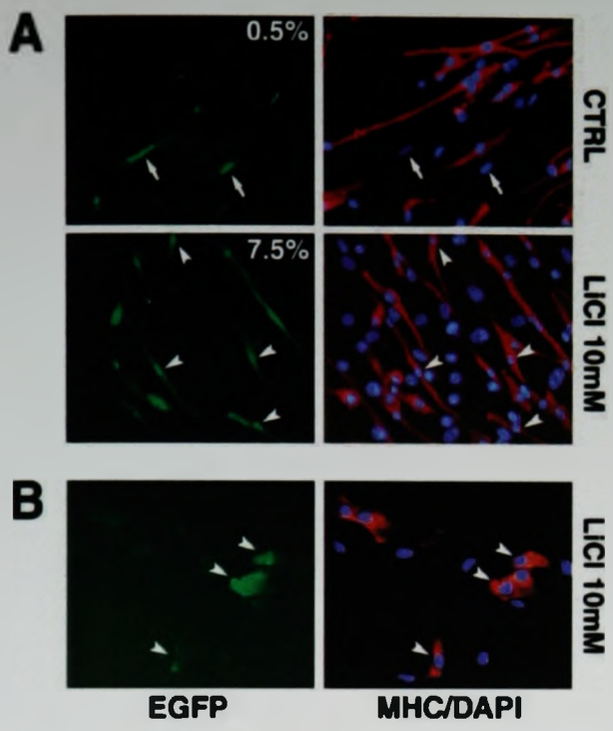


Figure 2. Myogenic Commitment of CD45$^+$:Sca1$^+$ Cells Induced by Coculture with Myoblasts or Exposure to Lithium

(A) EGFP-expressing CD45$^+$:Sca1$^{high}$ cells cocultured with primary myoblasts gave rise to mononuclear MHC-expressing myocytes at a frequency of 0.5%. In cocultures supplemented with 10 mM LiCl, 7.5% of EGFP+:CD45$^+$:Sca1$^{high}$ cells formed MHC-expressing myocytes (arrowheads). Arrows indicate EGFP+, nonmyogenic cells.
(B) CD45$^+$:Sca1$^{high}$ cells cultivated alone in differentiation medium supplemented with 10 mM LiCl formed, MHC-expressing myocytes (arrowheads).

is an underestimate of the actual efficiency, since EGFP is only detectable in up to 50% of muscle cells from these transgenic mice and the plating efficiency of CD45$^+$:Sca1$^+$ cells is low (discussed later). The complete absence of myogenic cells observed in CD45$^+$:Sca1$^+$ fractions cultured alone (n = 6) ruled out any possible contamination of cocultures with myoblasts due to sorting.

The Wnt signaling pathway is activated by lithium through inhibition of GSK-3β, and stabilization of β-catenin in treated cells (Hedgepeth et al., 1997). Therefore, to investigate whether the Wnt signaling pathway was involved in this phenomenon, cocultures of CD45$^+$:Sca1$^+$ muscle cells and primary myoblasts were exposed to 10 mM LiCl. Strikingly, treatment of cocultures with 10 mM LiCl resulted in a 15-fold increase in the frequency of GFP+ MHC-expressing myocytes to 7.5% (n = 3) of input cells (Figure 2A). Moreover, CD45$^+$:Sca1$^+$ cells cultured without myoblasts in LiCl-containing differentiation medium underwent myogenic differentiation as evidenced by MHC expression (Figure 2B). In growth conditions, however, LiCl induced rapid death of myogenic cells within 48 hr, precluding further analysis of these cultures. In summary, these results suggested that activation of the Wnt signaling pathway induced myogenic specification in CD45$^+$:Sca1$^+$ cells isolated from adult skeletal muscle.

We also tested whether Sonic hedgehog (Shh) could stimulate myogenesis in CD45$^+$:Sca1$^+$ muscle cells. Addition of 10 or 100 ng/ml of recombinant Shh to CD45$^+$:Sca1$^+$ cells alone or in coculture with myoblasts did not influence their myogenic differentiation efficiency. However, a 3- to 4-fold increase in CD45$^+$:Sca1$^+$ cell survival was observed following exposure to 100 ng/ml Shh (data not shown). Our results thus implicate Wnts, but not Shh, in the myogenic specification of CD45$^+$:Sca1$^+$ cells.

### Induction of Wnt and sFRP Expression in Regenerating Muscle

The expression kinetics of genes in the Wnt signaling cascade was analyzed by semiquantitative RT-PCR during skeletal muscle regeneration. The mRNAs for *Wnts5a*, *5b*, *7a*, and *7b* were induced in regenerating muscle (4 days postinjury), whereas *Wnt4* was strongly downregulated (Figure 3A). To determine whether upregulation of *Wnts* at the mRNA level corresponded to increased protein expression, Western blot analysis of Wnt5a protein was performed. Wnt5a was strongly expressed from day 2 to day 10 of regeneration in two independent experiments (data not shown). *Wnt1* and *Wnt3a* mRNAs were not expressed in any of the samples analyzed (data not shown). We also observed a strong but late induction of *sFRPs 1*, *2*, and *3*, but not of *sFRP4* (Figure 3A). Fzds were not highly expressed in total muscle, and there was no induction during regeneration (data not shown).

In addition, we analyzed Affymetrix array experiments on regenerating mouse gastrocnemius muscle performed at the CNMC Microarray Center. Gene expression was assayed in uninjured muscle (control) and at 12 hr, 1 day, 4 days, and 10 days post ctx injection with four independent replicates performed for each time point (publicly available at http://microarray.cnmcresearch.org). Only genes that showed >2-fold expression changes between control and experimental samples (regenerating) following four possible pairwise comparisons were studied further. Our analysis of these data confirmed that *Wnts 5a*, *5b*, *7a*, and *7b* were upregulated as early as 24 hr postinjury, with high levels of expression maintained through the 10 day regeneration time course (data not shown). *sFRPs*, by contrast, were upregulated late in regeneration from day 4 to day 10 postinjury. Specifically, sFRP1, 2, and 4 were upregulated 7.3 ± 1.2-, 4.9 ± 0.3-, and 7.4 ± 4.1-fold, respectively, at 10 days of regeneration relative to uninjured muscle (average of four pairwise comparisons). In summary, our gene expression studies suggest roles for *Wnts 5a*, *5b*, *7a*, and *7b* in regenerating muscle.

### Wnt and sFRP Expression in Myoblasts, Myotubes, and Isolated Muscle Fibers

Given the capacity for CD45$^+$:Sca1$^+$ cells to undergo myogenic conversion in coculture with primary myoblasts (Figure 2), we examined myoblasts, myotubes, and myofibers for the expression of Wnts and sFRPs. Importantly, *Wnts 5a* and *5b* were expressed in proliferating myoblasts, but not in differentiated myotubes. By contrast, *Wnt7a* was expressed in myotubes, but not in myoblasts (Figure 3B). Interestingly, all three *Wnts* were expressed in isolated single muscle fibers. However,

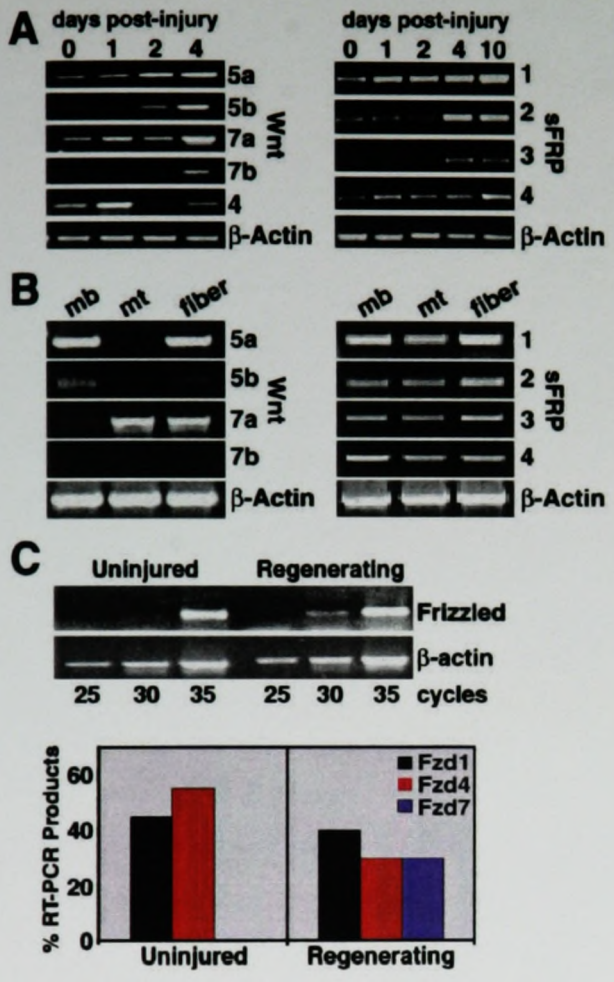


Figure 3. Upregulation of Wnts and sFRPs in Regenerating Muscle

(A) RT-PCR analysis indicated increased expression of *Wnts 5a, 5b, 7a,* and *7b* in regenerating TA muscle. By contrast, expression of *Wnt4* was downregulated following injury. Increased expression of *sFRPs 1, 2, 3,* but not *sFRP4,* was observed in regenerating muscle.

(B) RT-PCR studies showed that *Wnts 5a* and *5b* were expressed in muscle fibers (fiber) and proliferating myoblasts (mb); *Wnt7a* is primarily expressed in fibers and cultured myotubes (mt) (3 days of differentiation in culture). *sFRPs 1-4* were expressed in myoblasts, myotubes, and fibers.

(C) Wnt receptors, *Fzd 1,4* and *Fzd 1,4,7* were expressed in purified CD45$^+$:Sca1$^-$ cells from uninjured and regenerating muscle (4 days postinjury), respectively.

mRNA for *Wnt7b* was not detected in any sample. Lastly, *sFRPs 1–4* were also expressed in myoblasts, myotubes, and muscle fibers (Figure 3B). These results therefore suggest the hypothesis that expression of Wnt5a and Wnt5b in myoblasts induces the myogenic commitment of CD45$^-$ adult stem cells in our coculture experiments. Moreover, these data suggest that combined signaling by Wnts 5a, 5b, and 7a secreted by myofibers and myoblasts in regenerating muscle are responsible for the myogenic commitment of adult muscle-derived stem cells.

### CD45$^+$:Sca1$^+$ Cells Express *Frizzled-1, -4,* and *-7*

If CD45$^+$:Sca1$^+$ cells represent the target for Wnts during muscle regeneration, we predicted that CD45$^+$:Sca1$^+$ cells would express the Wnt-receptor *Frizzled* (*Fzd*).

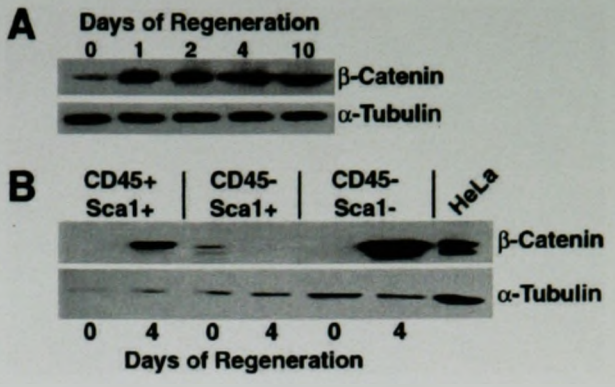


Figure 4. CD45$^+$:Sca1$^+$ Cells Upregulate β-Catenin during Regeneration

(A) Western blot analysis revealed increased levels of β-catenin protein in extracts of regenerating muscle.

(B) High levels of β-catenin protein were observed in fractionated CD45$^+$:Sca1$^+$ and CD45$^-$:Sca1$^-$ cells from regenerating (4 days postinjury), but not uninjured, skeletal muscle.

Therefore, CD45$^+$:Sca1$^+$ cells were isolated from resting and regenerating TA muscle and examined for expression of *Fzds*. RT-PCR for *Fzds* was performed with fully degenerate primers followed by cloning and sequencing of PCR products. CD45$^+$:Sca1$^+$ cells from resting muscle were observed to express *Fzd 1* and *4*. By 4 days after ctx injection, CD45$^+$:Sca1$^+$ cells upregulated *Fzd* expression overall and additionally expressed *Fzd7* (Figure 3C). Importantly, the observed upregulation in the expression of *Fzd* mRNAs was specific to the CD45$^+$:Sca1$^+$ population as no change in *Fzd* mRNA expression was observed in RNA isolated from resting and regenerating total TA muscle (data not shown).

### CD45$^+$:Sca1$^+$ Cells Upregulate β-Catenin during Muscle Regeneration

To determine whether Wnt signaling was activated in regenerating muscle, Western blot analysis was employed to detect β-catenin. Stabilization and nuclear accumulation of β-catenin is the hallmark for activation of the canonical Wnt pathway in responder cells (Pandur et al., 2002). β-catenin was strongly upregulated in extracts from regenerating TA muscle relative to uninjured muscle (Figure 4A). Importantly, expression of β-catenin protein was induced to high levels in CD45$^+$:Sca1$^+$ cells after muscle injury (Figure 4B). By contrast, CD45$^-$:Sca1$^-$ cells did not express detectable levels of β-catenin. In regenerating muscle, the CD45$^-$:Sca1$^-$ population, composed almost exclusively of myoblasts (our unpublished data), also expressed high levels of β-catenin. These data support the hypothesis that CD45$^+$:Sca1$^+$ cells respond to Wnt signaling via the canonical Wnt signaling pathway in regenerating TA muscle.

### Ectopic Wnts Induce Myogenic Commitment of CD45$^+$:Sca1$^+$ Cells

To investigate whether Wnts were sufficient to induce myogenic conversion of CD45$^+$:Sca1$^+$ cells, a panel of stable cell lines was established that expressed recombinant HA-tagged Wnt proteins. Following coculture with AtT-20 cells that expressed ectopic Wnts 5a, 5b,

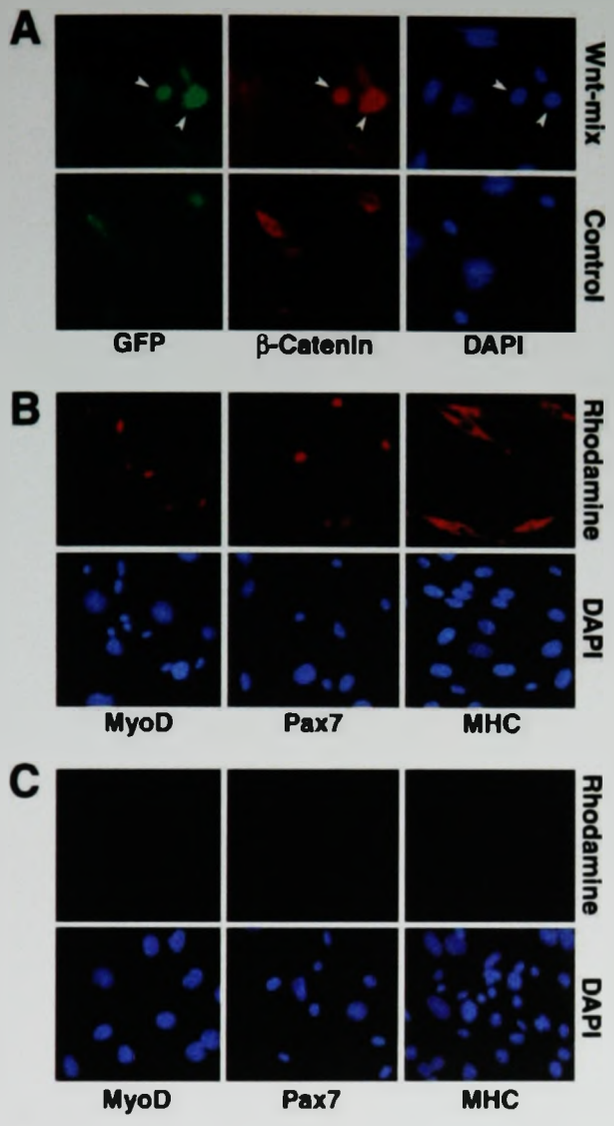


Figure 5. Ectopic Wnts Induce Myogenic Commitment of CD45$^+$:Sca1$^+$ Cells

(A) EGFP-expressing CD45$^+$:Sca1$^-$ cells from uninjured muscle displayed nuclear and/or cytoplasmic accumulation of β-catenin protein (arrowheads) after 3 days of coculture with cell lines expressing Wnts 5a, 5b, 7a, and 7b (Wnt mix). By contrast, expression of β-catenin was confined to the plasma membrane in CD45$^+$:Sca1$^+$ cells cocultured with cells transfected with empty pLNCX vector (control).

(B) CD45$^+$:Sca1$^-$ cells expressed MyoD and Pax7 following 3 days of coculture with Wnt 5a-, 5b-, 7a-, and 7b-expressing cells. CD45$^+$:Sca1$^+$ cells differentiated as MHC-expressing myocytes following exposure of cocultures to low-mitogen conditions.

(C) CD45$^+$:Sca1$^+$ cells did not initiate expression of MyoD, Pax7, or MHC in coculture with cell lines stably transfected with control empty vector (pLNCX).

7a, and 7b (Wnt mix), EGFP-expressing CD45$^+$:Sca1$^+$ cells displayed cytoplasmic and/or nuclear localization of β-catenin (arrowheads) consistent with activation of Wnt signaling in these cells (Figure 5A). By contrast, CD45$^+$:Sca1$^-$ cells cocultured with AtT-20 cells stably transfected with empty vector did not accumulate cytoplasmic or nuclear β-catenin (Figure 5A).

In growth conditions, CD45$^+$:Sca1$^+$ cells cocultured with Wnt cell lines initiated expression of the myogenic determination protein MyoD and the satellite cell marker Pax7 (Figure 5B). In addition, MHC-positive myocytes were observed after the cultures were switched to differentiation conditions for 48 hr (Figure 5B). By contrast, CD45$^+$:Sca1$^+$ cells cocultured with control, non-Wnt-expressing AtT-20 cells did not express any myogenic markers (Figure 5C). Thus, signaling by a mixture of Wnts 5a, 5b, 7a, and 7b led to myogenic commitment of CD45$^+$:Sca1$^+$ cells in vitro. Individual Wnt-expressing cell lines induced myogenic commitment of CD45$^+$:Sca1$^+$ cells but at a lower efficiency (data not shown).

The plating efficiency of CD45$^+$:Sca1$^+$ cells was reproducibly 2%–4% of input cells, about the same plating efficiency as observed with newly isolated primary myoblasts. After 3 days of culture, 2%–4% of the number of input EGFP+ CD45$^+$:Sca1$^+$ cells were present after coculture with Wnt-expressing AtT-20 cells. Importantly, over 90% of surviving CD45$^+$:Sca1$^-$ cells converted to the myogenic lineage. Taken together, these experiments unequivocally demonstrate that Wnt signaling activates the myogenic recruitment of CD45$^-$:Sca1$^+$ cells isolated from uninjured muscle.

### Injected sFRPs Severely Reduce the Myogenic Recruitment of CD45$^+$:Sca1$^+$ Cells during Regeneration

To evaluate the relevance of Wnt signaling as an effecter of muscle regeneration in vivo, we injected recombinant Wnt antagonists sFRP2 and 3 (100 ng of each) on a daily basis into regenerating muscles of *Myf5nlacZ* mice. Three control animal groups were employed to assess possible extraneous effects. One group (uninjured control) was not injected with ctx and did not receive subsequent sFRP injections. The second group received an initial injection of PBS rather than ctx, followed by daily sFRP injections. The final group was injected with ctx to induce regeneration, followed by daily injection of PBS in the place of sFRPs.

Flow cytometric analysis of muscle cells demonstrated that the increase in the CD45$^+$:Sca1$^{high}$ cells observed 4 days following injury (Figure 6B) was reduced about 4-fold by daily injections of sFRPs 2 and 3 (Figure 6C). Furthermore, the reduced numbers of CD45$^+$:Sca1$^{high}$ cells did not result from a concomitant decrease in total numbers of mononuclear cells. Importantly, injection of sFRPs into uninjured muscle did not induce regeneration or produce any morphological changes in the TA muscle (Figure 6A and data not shown).

The proportion of CD45$^+$:Sca1$^+$ cells expressing *Myf5nLacZ* was examined at 5 days following daily sFRP injections. Similar to our previous experiments (Figure 1), 6.71% ± 1.44% of CD45$^+$:Sca1$^{high}$ cells obtained directly from regenerating muscle 4 days after injury expressed *Myf5nLacZ* (n = 3) (Figure 6D). Importantly, *Myf5nLacZ* was not expressed in CD45$^+$:Sca1$^+$ cells isolated from uninjured muscle (Figure 6D). Strikingly, daily injection of sFRP 2 and 3 into regenerating muscle resulted in about a 6-fold reduction in the numbers of *Myf5nLacZ*-expressing cells in the CD45$^+$:Sca1$^+$ fraction (Figure 6D). Thus, inhibition of Wnt signaling markedly reduced the myogenic recruitment of CD45$^+$:Sca1$^+$ cells in vivo.

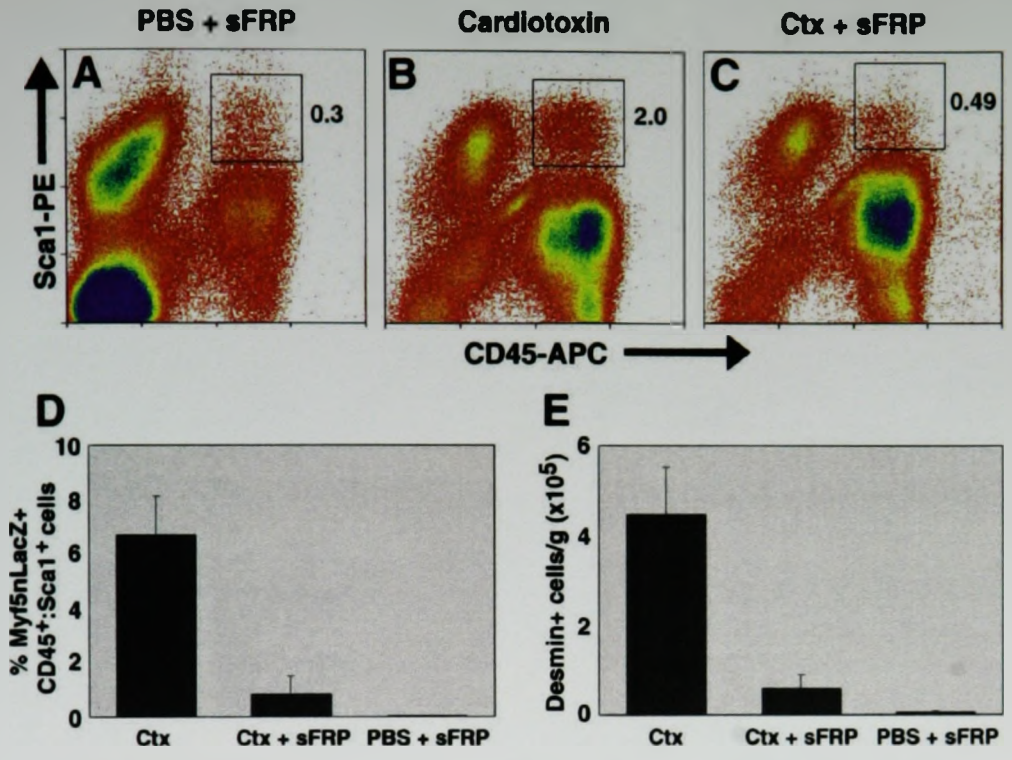


Figure 6. Injection of sFRPs Markedly Decreases Recruitment CD45$^+$:Sca1$^+$ Cells during Regeneration

(A–C) Flow cytometric analysis for CD45 and Sca1 revealed a decreased proportion of CD45$^+$:Sca1$^{high}$ cells in regenerating muscle treated daily with sFRPs 2 and 3 (C), compared to regenerating muscle injected with PBS (B). Injection of sFRPs into uninjured muscle (A) did not induce regeneration or influence the proportion of CD45$^+$ and Sca1$^+$ cells.

(D) The proportion of CD45$^+$:Sca1$^{high}$ cells that coexpressed *Myf5nLacZ* was reduced by 6-fold, following treatment of regenerating muscle with sFRPs 2 and 3.

(E) A 7-fold reduction in the number of Desmin-expressing myoblasts was recovered from regenerating muscle treated with sFRPs.

To further characterize the effect of sFRPs on muscle regeneration, we analyzed the recovery of myogenic cells in the total pool of mononuclear cells from muscle 4 days postinjury. $1 \times 10^4$ cells from the three experimental groups were plated in each well and analyzed 24 hr later for expression of Desmin, a marker specific to skeletal muscle cells (Figure 6E). We found that daily sFRP injection produced about a 7-fold decrease in the number of mononuclear Desmin-expressing myoblasts in 4 day regenerating TA muscle relative to PBS-injected regenerating muscle ($4.47 \times 10^5 \pm 1 \times 10^5$ compared to $6.03 \times 10^4 \pm 3.03 \times 10^4$ cells/gram tissue) (Figure 6E). Taken together, these results strongly implicate Wnt signaling in the commitment of muscle stem cells to the myogenic lineage during regeneration.

## Discussion

CD45 is considered a lineage-restricted pan-hematopoietic marker that is not expressed on satellite cells (Asakura et al., 2002) or on any other nonhematopoietic cell types (reviewed by Penninger et al., 2001; Sasaki et al., 2001). Following experimentally induced injury in adult skeletal muscle, a 10-fold expansion in the numbers of cells coexpressing the cell surface markers CD45 and Sca1 was observed (Figure 1A). CD45$^+$:Sca1$^-$ cells isolated from uninjured muscle displayed no myogenic potential whatsoever in our experiments and in previous analyses (data not shown; Asakura et al., 2002; McKinney-Freeman et al., 2002). By contrast, a high proportion of CD45$^+$:Sca1$^+$ cells isolated from regenerating muscle readily underwent myogenic differentiation in vitro (Figures 1C and 1D). These results provide direct evidence that CD45$^+$ adult stem cells have a normal physiological role for tissue regeneration in vivo.

The observed upregulation of *Wnts* in regenerating muscle suggested a role for Wnt signaling in muscle regeneration (Figure 3A). Stimulation of the Wnt signaling cascade with lithium in freshly isolated CD45$^+$:Sca1$^+$ cells was sufficient to induce muscle specification (Figure 2). Moreover, myogenic commitment of CD45$^+$:Sca1$^+$ cells was induced by coculture with cells ectopically expressing Wnt proteins (Figure 5). The stabilization of nuclear β-catenin specifically in CD45$^+$:Sca1$^+$ cells isolated from regenerating muscle is consistent with the hypothesis that these cells are targeted for Wnt-induced myogenesis in vivo (Figure 4). Moreover, the marked reduction in numbers of myogenic CD45$^+$:Sca1$^+$ cells in injured muscle treated with sFRPs demonstrated a functional requirement for Wnt in the myogenic specification of these cells (Figure 6). Taken together, these findings unequivocally implicate Wnt signaling in the myogenic recruitment of adult muscle stem cells (see Figure 7).

Enriched populations of adult stem cells were first isolated from skeletal muscles as side-population (SP) cells on the basis of Hoechst dye exclusion (Gussoni et al., 1999). The capacity for muscle-derived cells to en-

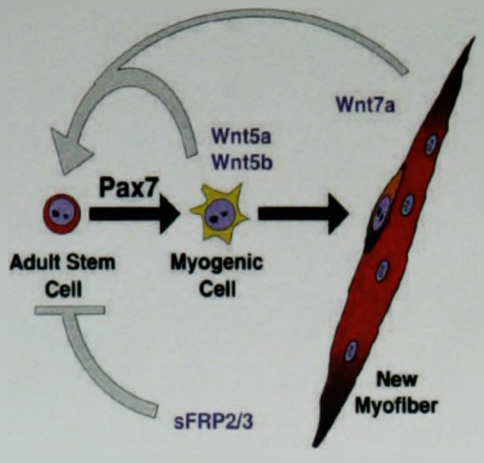


Figure 7. Role for Wnt Signaling in Myogenic Recruitment of Adult Stem Cells

Our experiments suggest the hypothesis that Wnt signals secreted from damaged fibers, resident myoblasts, and possibly other cell types in injured muscle induces the activation of myogenic transcription factors and commitment of stem cells to muscle precursors. Wnt signaling may converge on activation of the *Pax7* gene to induce myogenic specification. Following repair, secretion of Wnt antagonists, sFRPs 2 and 3, blocks Wnt signals, thereby interrupting the myogenic recruitment of stem cells.

graft bone marrow and reconstitute all blood cell lineages in irradiated mice suggested the existence of a multipotent stem cell compartment in skeletal muscle (Jackson et al., 1999). Moreover, muscle SP cells participate in skeletal myogenesis and give rise to myogenic stem cells following intravenous or intramuscular injection (Asakura et al., 2002; Gussoni et al., 1999). It is unclear, however, whether muscle SP cells with hematopoietic activity are also capable of muscle differentiation or if the SP compartment contains distinct muscle- and blood-forming cells. Recent studies have demonstrated that the hematopoietic activity in uninjured skeletal muscle is derived from cells expressing CD45 (Asakura et al., 2002; McKinney-Freeman et al., 2002). Previously, we and others have noted that cultured CD45$^+$ cells from uninjured muscle did not enter the myogenic differentiation program (our unpublished data; Asakura et al., 2002; McKinney-Freeman et al., 2002). Importantly, in the current study, we demonstrate that endogenous CD45$^+$ cells are recruited into the myogenic lineage in response to muscle injury.

Several other groups have characterized cells derived from adult skeletal muscle that exhibit stem cell properties (Qu-Petersen et al., 2002; Tamaki et al., 2002; Torrente et al., 2001). For instance, clonal populations of muscle cells isolated on the basis of adherence properties display multipotent myogenic, neurogenic, and endothelial differentiation plasticity (Qu-Petersen et al., 2002). These cultured muscle cells express CD34 and Sca1 but are uniformly CD45 and c-kit negative. A similar study demonstrated myogenic, endothelial and adipogenic differentiation from cloned CD34$^+$:CD45$^-$ muscle cells (Tamaki et al., 2002). There has, however, been no consensus on the interrelationships between these various populations. One difficulty in comparing such populations is that their derivation as continuous cultured clonal cell lines or even as short-term cultures invariably alters their molecular phenotype and differentiation potential (Tang et al., 2001). For example, CD45$^-$:Sca1$^+$ muscle cells cultured in defined medium containing IL-6 and SCF acquire hematopoietic competence and CD45 expression (Howell et al., 2002). Moreover, we have observed that CD45$^+$:Sca1$^+$ cells purified from regenerating muscle lose expression of CD45 after 2 days in myoblast growth conditions (data not shown). Whether different muscle cell populations with varying degrees of myogenic potential are related remains unknown, and further study will be required to elucidate potential relationships.

The purpose of our study was to identify molecular mechanisms by which nonmyogenic cells are recruited to the myogenic lineage under conditions of physiological stress. For this reason, we investigated the myogenic potential of cells expressing the hematopoietic restricted CD45 antigen. The expression of CD45 on cells that commit to the myogenic lineage in regenerating muscle suggests a hematopoietic origin for these cells (Figure 1). Importantly, CD45 is not expressed in quiescent satellite cells, activated satellite cells on muscle fibers, or in satellite cell derived myoblasts (data not shown and Asakura et al., 2002). Furthermore, expression of Sca1 in muscle is associated with vascular structures and is not expressed in the satellite cell lineage (Asakura et al., 2002). Therefore, we are confident that CD45$^+$:Sca1$^+$ cells represent a source of progenitor cells distinct from muscle satellite cells.

The number of myogenic progenitors directly derived from CD45$^+$:Sca1$^+$ cells in regenerating muscle at 4 days postinjury is low in comparison to myoblasts obtained from the CD45$^-$:Sca1$^-$ fraction. Presumably, this low frequency reflects the transitional state of CD45$^+$:Sca1$^+$/*Myf5nLacZ*$^+$ cells, which rapidly give rise to proliferating CD45$^-$:Sca1$^-$ myoblasts indistinguishable from satellite-cell-derived muscle precursors. Consistent with this notion, CD45 expression is quickly lost on myogenic cells derived from the CD45$^+$:Sca1$^+$ fraction in vitro. This observation suggests that the hematopoietic properties of CD45$^+$:Sca1$^+$ are lost, as these cells adopt a myogenic fate in response to Wnt signals. In the future, clonal marking will be required to establish whether single CD45$^+$:Sca1$^+$ cells are indeed multipotent stem cells capable of hematopoietic and myogenic commitment.

Satellite cells are believed to be the sole source of myogenic progenitors and thus entirely responsible for muscle regeneration following ctx-induced injury. However, we observed an ~30-fold decrease in the total number of satellite cells 18 hr after ctx injection, suggesting that satellite cells were likely killed by exposure to ctx. Notably, by 4 days, the total number of myogenic progenitors in a regenerating TA muscle reached ~$3.9 \times 10^5$. This expansion of myogenic progenitors appears to exceed the capacity of the TA muscle, given the starting number of satellite cells and the fact that satellite cells do not migrate between muscle groups (Bischoff, 1994; Schultz et al., 1986). Taken together, our data therefore suggests that CD45$^+$ stem cells resident in muscle play a major role in effecting muscle regeneration.

One potential source for the CD45$^+$ cells is the circula-

tion. However, several lines of evidence argue that the stem cells responsible for muscle regeneration following ctx-induced injury are resident within the tissue and do not migrate in significant numbers from the circulation in nonirradiated animals. First, high-dose local irradiation of limbs results in a long-term deficit in muscle growth and regeneration, suggesting that circulating stem cells do not normally restore muscle stem cell pools (Heslop et al., 2000; Pagel and Partridge, 1999; Wakeford et al., 1991). Second, transplanted muscles do not efficiently incorporate host nuclei following injury and regeneration (Schultz et al., 1986). Third, the numbers of $CD45^-Sca1^+$ cells undergo a 10-fold expansion possibly mediated by cell division in situ as evidenced by BrdU incorporation (Figure 1B). Together, these experiments demonstrate that circulating stem cells do not normally contribute to stem cell pools within damaged muscle. Therefore, $CD45^-$ stem cells appear to represent a stable and resident population of cells within muscle tissue.

Several upstream signaling pathways have been implicated as positively or negatively regulating myogenic specification during embryonic development. For example, Shh expressed in the floor plate and the notochord and Wnt family members expressed in the dorsal neural tube combinatorially activate myogenesis in the somite (Munsterberg et al., 1995; Schmidt et al., 2000). Wnts directly stimulate myogenesis in the somite whereas Shh is believed to direct myogenesis through activation of Noggin expression in the dorsal somite. Noggin subsequently inhibits the negative activity of lateral plate-derived BMP4 on myogenesis (Hirsinger et al., 1997; Marcelle et al., 1997; Reshef et al., 1998). Notch signaling pathways also appear to exert negative effects on myogenesis (Kopan et al., 1994; Shawber et al., 1996). In explants of paraxial mesoderm from mouse embryos, Wnt1 preferentially activates *Myf5*, whereas Wnt7a preferentially activates *MyoD* (Tajbakhsh et al., 1998). Our study suggests a role for Wnt signaling in regenerating skeletal muscle analogous to its role in embryonic myogenesis.

The molecular mechanisms that function downstream of Wnt to induce myogenesis in $CD45^-$:$Sca1^+$ cells are unclear. Our results demonstrate specific upregulation of β-catenin protein in these cells. However, the identification of specific downstream target genes regulating muscle determination in these cells will require future studies. It is clear from our experiments that exposure of $CD45^-$:$Sca1^-$ cells to Wnt proteins enhanced their survival in culture. From this result, it is conceivable that the Wnt-dependent specification of stem cells operates by promoting the selective survival of cells with myogenic potential. In fact, selective survival of myogenic progenitors has not been ruled out as a mechanism for the Wnt-dependent specification of embryonic muscle lineages. To distinguish between survival and instructive cues as mechanisms for Wnt-induced myogenesis, it will be necessary to pinpoint direct transcriptional targets of the Wnt signal transduction cascade in stem cells. Interestingly, TCF/LEF binding sites are contained in the Pax7 promoter, suggesting that direct activation of Pax7 by Wnt signaling may induce myogenesis in stem cells (Figure 7).

Soluble Wnt antagonists, sFRPs, are expressed in many tissue types and are upregulated during development or regeneration (Levin et al., 2001; Leyns et al., 1997). sFRPs block Wnt signaling through direct binding (Lescher et al., 1998). Similar to Wnt-Fzd interaction, sFRPs do not seem to have strict specificity for distinct Wnts. For example, sFRP1 was shown to block Wnt4 activity in kidney development (Davies and Fisher, 2002), Wnt7a in the cerebellum (Hall et al., 2000), and Wnt1 in epithelial cells (Dennis et al., 1999). Chick sFRP3 was suggested to antagonize Wnt1, 3a, 5a, 7a, and 8c during embryogenesis (Baranski et al., 2000).

In developing muscle, sFRP3/frzb1 abolishes myogenesis in mouse somites by specifically downregulating Myf5 (Borello et al., 1999). In *Xenopus* embryos, overexpression of sFRP3/Frzb1 suppresses MyoD and leads to abnormal head and trunk formation (Leyns et al., 1997). Taken together, these observations suggest a developmental role for sFRPs in antagonizing Wnt-activation of myogenesis. In our study, we found that *sFRPs* were upregulated in the latter stages of muscle regeneration (Figure 3A). We hypothesize that late expression of sFRPs inhibits the Wnt-dependent recruitment of myogenic progenitors after regeneration is complete (Figure 7). Moreover, the reduced myogenic specification of stem cells observed in regenerating muscle injected with sFRPs demonstrates a functional role for Wnts in adult stem cell differentiation and highlights the importance in timing of sFRP expression. Importantly, abrogation of Wnt signaling in regenerating muscle markedly reduced the pool of muscle progenitors in vivo, revealing the essential role of Wnts for regenerative myogenesis.

In conclusion, endogenous adult stem cells resident in muscle expressing classical hematopoietic markers participate in muscle regeneration. This result demonstrates that nonsatellite-cell-derived myogenic progenitors have a physiological role in muscle regeneration and tissue homeostasis. A central question to the study of adult stem cells has been the identity of signals regulating their differentiation into specialized cell types. In this report, we have established a role for Wnts in the myogenic commitment of adult stem cells during skeletal muscle regeneration. Our data demonstrate that Wnts are necessary and sufficient for the myogenic specification of adult stem cells. Further studies are required to define the precise molecular targets for Wnts in stem cell populations. The ability of injected sFRP proteins to attenuate the myogenic differentiation of $CD45^-$ stem cells unequivocally underscores the clinical potential for modulating Wnt signaling in muscle tissue. Clearly, modulation of the Wnt pathway for the treatment of neuromuscular diseases represents an important therapeutic possibility that must now be actively explored.

## Experimental Procedures

### Cell Sorting

Mononuclear cells were obtained from hind limb muscles of β-*actin-EGFP* transgenic mice (Hadjantonakis et al., 1998) or *Myf5nLacZ* transgenic mice (Tajbakhsh and Buckingham, 1995). Muscle cells were recovered as described previously (Megeney et al., 1996). Mononuclear cells were washed twice with DMEM supplemented with 5% FBS and suspended at a concentration of 2–3 × $10^6$ cells/

ml. Staining was performed for 30–45 min on ice using the following antibodies: CD45-APC, clone 30-F11 or CD45.2-FITC (clone 104), Sca1-PE, clone D7 (BD Pharmingen). Alternatively, we used CD45-biotin, clone 30-F11, followed by 10 min incubation with Streptavidin tri-color conjugate (Caltag Labs). Primary antibodies were diluted at 1:200, and Streptavidin tri-color conjugate was diluted 1:1000. After two washes with DMEM at 4°C, cells were separated on a MoFlo cytometer (DakoCytomation) equipped with three lasers. Sort gates were strictly defined based on isotype control stained cells and single antibody staining. Dead cells and debris were excluded by gating on forward and side scatter profiles. Sorting was performed using single cell mode to achieve the highest possible purity. The purity of sorted populations was routinely >98%.

For direct analysis of sorted cell populations, cells were washed and suspended in phosphate-buffered saline (PBS) and cytospun onto silanized slides (DAKO). X-gal staining was performed as described previously (Kablar et al., 1997).

### Cell Culture and Stable Cell Lines

Primary myoblasts were isolated from hind limb muscle of 3-week-old Balb/c mice and maintained in HAM's F-10 medium (Invitrogen) supplemented with 20% FBS and 2.5 ng/ml bFGF (Invitrogen). Single muscle fibers were prepared from the extensor digitorum longus muscle as described previously (Rosenblatt et al., 1995). AtT-20, BOSC 23, C3H10T1/2, and Cos1 cells were obtained from the ATCC and maintained in DMEM supplemented with 10% FBS. Stable cell lines expressing HA-Wnt proteins were derived as described previously (Shimizu et al., 1997). Expression of HA-Wnts was confirmed by Western blot analysis with anti-HA antibody (HA-7, Sigma).

### Coculture Experiments and Immunohistochemistry

For coculture experiments, primary myoblasts or Wnt-expressing cells were mixed with purified $CD45^-$:$Sca1^+$ cells at a ratio of 1:1 and seeded on collagen-coated 2-well Permanox chamber slides (Lab-Tek). The density was $2 \times 10^4$ cells/chamber for coculture in growth conditions and $4 \times 10^4$ cells/chamber for differentiation experiments. The cocultures were maintained in HAM's F-10 medium, supplemented with 20% FBS for 3 days, and switched to DMEM/5% horse serum for differentiation experiments. For the $Li^+$ or Shh conversion experiments, LiCl (Sigma) at 10 mM or Shh-N (R&D Systems) at 10 or 100 ng/ml was added to the differentiation media. For immunohistochemical analysis, cells were fixed with 2% PFA for 15 min at room temperature, permeabilized with 0.05% Triton X-100 for 15 min, blocked with 1%BSA/5% HS in PBS, and stained for 2 hr, room temperature, with the following antibodies: MyoD, clone 5.8A (BD Pharmingen); Myosin Heavy Chain, clone MF-20 (Developmental Studies Hybridoma Bank [DSHB]); Pax7 (DSHB); or β-catenin (BD Transduction Laboratories). Fluorescein- or Rhodamine-conjugated antibodies (Chemicon) were used for secondary detection. Cover slides were mounted and analyzed using a Zeiss Axioscop fluorescent microscope.

### RT-PCR, Cloning, and Sequencing

Total RNA was extracted using RNAeasy kits (Qiagen), according to manufacturer's instructions. For analysis of Frizzled gene expression, RT-PCR was performed with fully degenerate primers corresponding to conserved *frizzled* sequences YPERPIIF and WWVILS LTW, as previously described (Malik and Shivdasani, 2000). The products were cloned into the TOPO-PCRII vector (Invitrogen) and sequenced. RT-PCR analysis of Wnt mRNAs was performed using the GeneAmp PCR Core kit (Perkin-Elmer). The following primers were used: Wnt1 (5′-ACGTACAGTGGCCGCCTG-3′; 5′-ACGCGCGT GTGCGTGCAGTT-3′; 203 bp); Wnt3a (5′-GGAGATGGTGGTAGAG AAA-3′; 5′-ATAGACACGTGTGCACTC-3′; 322 bp); Wnt4 (5′-AGC CCCCGTTCGTGCCTGCGGTCC-3′; 5′-ACTCCACCCGCATGTGTG TCA-3′; 607 bp); Wnt5a (5′-AATGGCTTTGGCCACGTTTTT-3′; 5′-TGGATTCGTTCCCTTT-3′; 541 bp); Wnt5b (5′-AGTGCAGAGACC GGAGATGTTC-3′; 5′-GGCAAAGTTCTTCTCACGC-3′; 459 bp); Wnt7a (5′-AGCGCGGCGCTGCCTGGGCC-3′; 5′-CTTCAGAAAGGT GGGCCGCTTGTTT-3′; 752 bp); Wnt7b (5′-CCGCACCTCGCCGGGG GCCGAC-3′; 5′-GTCGGCCCCCGGCGAGGTGCGG-3′; 180 bp); sFRP1 (5′-CGCCCGTCTGTCTGGACCG-3′; 5′-CTCGCTTGCACAG AGATGT-3′; 257 bp); sFRP2 (5′-TTCGGCCAGCCCGACTTCTCC-3′; 5′-TAGGTCGTCGAGACAGACAGGGG-3′; 234 bp); sFRP3 (5′-ATTT TCCTATGGATTCAAGTACTG-3′; 5′-TTGACTTTCTTACCAAGCCGA TCCTT-3′; 396 bp); sFRP4 (5′-TGGATAGACATCACACCAGA TAT-3′; 5′-CCTGAAGCCTCTCTTCCCA-3′; 423 bp).

### Cardiotoxin-Induced Regeneration

5- to 8-week-old mice were anesthetized with Halothane gas. 25 μl of 10 μM cardiotoxin (Latoxan) was injected directly into the TA muscle using a 29 G 1/2 insulin syringe. For cell proliferation assays, 0.3 mg/kg of 5-bromo-deoxyuridine (BrdU, Sigma) was injected intraperitoneally 90 min prior to sacrificing animals. Cells that had incorporated BrdU were detected by flow cytometry using a FITC conjugated anti-BrdU antibody (BD Pharmingen). For sFRP experiments, 100 ng of recombinant sFRP 2 and 3 (R&D Systems) were injected into regenerating TA muscle. Control animals received injections of equal volumes of PBS. For analysis of total TA cell populations, $1 \times 10^4$ mononuclear cells were plated on collagen-coated chamber slides overnight and then stained with anti-Desmin antibody (DAKO) at 1:200. Donkey anti-mouse FITC (Chemicon) at 1:500 was used for secondary detection.

### Western Blot Analyses

Uninjured and regenerating TA muscles were flash frozen in liquid nitrogen, crushed, and lysed in extraction buffer (50 mM Tris-HCl [pH 7.4], 0.1% Triton X-100, 5 mM EDTA, 250 mM NaCl, 50 mM NaF) including protease inhibitors (Complet, Roche). The extracts were normalized for protein content using Bio-Rad dye. 50 μg of lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose filters. Filters were probed with antibodies to Wnt5a, 1:200 (AF645, R&D Systems); β-catenin, 1:250 (BD Transduction Laboratories); and α-tubulin, 1:2000 (T 9026, Sigma). Secondary detection was performed with horseradish peroxidase-conjugated antibodies (BioRad). Protein expression was visualized using the ECL Plus kit (Amersham).

### Affymetrix Expression Profiling of Regenerating Muscle

Gene expression profiling and data analysis of regenerating mouse gastrocnemius muscle was performed at the CNMC Research Center, as described by Zhao et al. (2002). Briefly, gastrocnemius muscles were injected with 100 μl of 10 mM cardiotoxin (ctx) (Calbiochem). RNA was prepared from four individual muscles at time 0 (no injection), 12 hr, 1 day, 2 days, and 10 days after ctx injection. Biotin-labeled cRNA was obtained for each replicate, fragmented, and hybridized to Murine Genome U74A version 1 chips (Affymetrix). Primary data and comparison analysis was done using Affymetrix Microarray Suite 4.0 as described previously (Chen et al., 2000). Lists of genes with >2 fold changes after four possible pairwise comparisons between control and experimental samples were made available as Excel spreadsheets on the CNMC website (http://microarray.cnmcresearch.org).

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**References**


Asakura, A., Seale, P., Girgis-Gabardo, A., and Rudnicki, M.A. (2002). Myogenic specification of side population cells in skeletal muscle. J. Cell Biol. *159*, 123–134.

Baranski, M., Berdougo, E., Sandler, J.S., Darnell, D.K., and Burrus, L.W. (2000). The dynamic expression pattern of frzb-1 suggests multiple roles in chick development. Dev. Biol. *217*, 25–41.

Bischoff, R. (1994). The satellite cell and muscle regeneration. In Myogenesis, A.G. Engel, and C. Franszini-Armstrong, eds. (New York: McGraw-Hill), pp. 97–118.

Borello, U., Coletta, M., Tajbakhsh, S., Leyns, L., De Robertis, E.M., Buckingham, M., and Cossu, G. (1999). Transplacental delivery of the Wnt antagonist Frzb1 inhibits development of caudal paraxial mesoderm and skeletal myogenesis in mouse embryos. Development *126*, 4247–4255.

Chen, Y.W., Zhao, P., Borup, R., and Hoffman, E.P. (2000). Expression profiling in the muscular dystrophies: identification of novel aspects of molecular pathophysiology. J. Cell Biol. *151*, 1321–1336.

Christ, B., and Ordahl, C.P. (1995). Early stages of chick somite development. Anat. Embryol. (Berl.) *191*, 381–396.

Cossu, G., and Borello, U. (1999). Wnt signaling and the activation of myogenesis in mammals. EMBO J. *18*, 6867–6872.

Davies, J.A., and Fisher, C.E. (2002). Genes and proteins in renal development. Exp. Nephrol. *10*, 102–113.

Dell'Agnola, C., Rabascio, C., Mancuso, P., Capillo, M., Pruneri, G., Gobbi, A., Minucci, S., Ronzoni, S., Volorio, S., Calabrese, L., et al. (2002). In vitro and in vivo hematopoietic potential of human stem cells residing in muscle tissue. Exp. Hematol. *30*, 905–914.

Dennis, S., Aikawa, M., Szeto, W., d'Amore, P.A., and Papkoff, J. (1999). A secreted frizzled related protein, FrzA, selectively associates with Wnt-1 protein and regulates wnt-1 signaling. J. Cell Sci. *112*, 3815–3820.

Dierick, H., and Bejsovec, A. (1999). Cellular mechanisms of wingless/Wnt signal transduction. Curr. Top. Dev. Biol. *43*, 153–190.

Gussoni, E., Soneoka, Y., Strickland, C.D., Buzney, E.A., Khan, M.K., Flint, A.F., Kunkel, L.M., and Mulligan, R.C. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature *401*, 390–394.

Hadjantonakis, A.K., Gertsenstein, M., Ikawa, M., Okabe, M., and Nagy, A. (1998). Generating green fluorescent mice by germline transmission of green fluorescent ES cells. Mech. Dev. *76*, 79–90.

Hall, A.C., Lucas, F.R., and Salinas, P.C. (2000). Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. Cell *100*, 525–535.

Hedgepeth, C.M., Conrad, L.J., Zhang, J., Huang, H.C., Lee, V.M., and Klein, P.S. (1997). Activation of the Wnt signaling pathway: a molecular mechanism for lithium action. Dev. Biol. *185*, 82–91.

Heslop, L., Morgan, J.E., and Partridge, T.A. (2000). Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. J. Cell Sci. *113*, 2299–2308.

Hirsinger, E., Duprez, D., Jouve, C., Malapert, P., Cooke, J., and Pourquie, O. (1997). Noggin acts downstream of Wnt and Sonic Hedgehog to antagonize BMP4 in avian somite patterning. Development *124*, 4605–4614.

Hobmayer, B., Rentzsch, F., Kuhn, K., Happel, C.M., von Laue, C.C., Snyder, P., Rothbacher, U., and Holstein, T.W. (2000). WNT signalling molecules act in axis formation in the diploblastic metazoan Hydra. Nature *407*, 186–189.

Howell, J.C., Yoder, M.C., and Srour, E.F. (2002). Hematopoietic potential of murine skeletal muscle-derived CD45(-)Sca-1(+)c-kit(-) cells. Exp. Hematol. *30*, 915–924.

Jackson, K.A., Mi, T., and Goodell, M.A. (1999). Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proc. Natl. Acad. Sci. USA *96*, 14482–14486.

Kablar, B., Krastel, K., Ying, C., Asakura, A., Tapscott, S.J., and Rudnicki, M.A. (1997). MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle. Development *124*, 4729–4738.

Kopan, R., Nye, J.S., and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. Development *120*, 2385–2396.

LaBarge, M.A., and Blau, H.M. (2002). Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. Cell *111*, 589–601.

Labus, M.B., Stirk, C.M., Thompson, W.D., and Melvin, W.T. (1998). Expression of Wnt genes in early wound healing. Wound Repair Regen. *6*, 58–64.

Lee, J.Y., Qu-Petersen, Z., Cao, B., Kimura, S., Jankowski, R., Cummins, J., Usas, A., Gates, C., Robbins, P., Wernig, A., and Huard, J. (2000). Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. J. Cell Biol. *150*, 1085–1100.

Lescher, B., Haenig, B., and Kispert, A. (1998). sFRP-2 is a target of the Wnt-4 signaling pathway in the developing metanephric kidney. Dev. Dyn. *213*, 440–451.

Levin, J.M., El Andalousi, R.A., Dainat, J., Reyne, Y., and Bacou, F. (2001). SFRP2 expression in rabbit myogenic progenitor cells and in adult skeletal muscles. J. Muscle Res. Cell Motil. *22*, 361–369.

Leyns, L., Bouwmeester, T., Kim, S.H., Piccolo, S., and De Robertis, E.M. (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. Cell *88*, 747–756.

Mahmud, N., Weiss, P., Li, F., and Hoffman, R. (2002). Primate skeletal muscle contains cells capable of sustaining in vitro hematopoiesis. Exp. Hematol. *30*, 925–936.

Malik, T.H., and Shivdasani, R.A. (2000). Structure and expression of a novel frizzled gene isolated from the developing mouse gut. Biochem. J. *349*, 829–834.

Marcelle, C., Stark, M.R., and Bronner-Fraser, M. (1997). Coordinate actions of BMPs, Wnts, Shh and noggin mediate patterning of the dorsal somite. Development *124*, 3955–3963.

McKinney-Freeman, S.L., Jackson, K.A., Camargo, F.D., Ferrari, G., Mavilio, F., and Goodell, M.A. (2002). Muscle-derived hematopoietic stem cells are hematopoietic in origin. Proc. Natl. Acad. Sci. USA *99*, 1341–1346.

Megeney, L.A., Kablar, B., Garrett, K., Anderson, J.E., and Rudnicki, M.A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev. *10*, 1173–1183.

Munsterberg, A.E., Kitajewski, J., Bumcrot, D.A., McMahon, A.P., and Lassar, A.B. (1995). Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. Genes Dev. *9*, 2911–2922.

Pagel, C.N., and Partridge, T.A. (1999). Covert persistence of mdx mouse myopathy is revealed by acute and chronic effects of irradiation. J. Neurol. Sci. *164*, 103–116.

Pandur, P., Maurus, D., and Kuhl, M. (2002). Increasingly complex: new players enter the Wnt signaling network. Bioessays *24*, 881–884.

Patapoutian, A., and Reichardt, L.F. (2000). Roles of Wnt proteins in neural development and maintenance. Curr. Opin. Neurobiol. *10*, 392–399.

Penninger, J.M., Irie-Sasaki, J., Sasaki, T., and Oliveira-dos-Santos, A.J. (2001). CD45: new jobs for an old acquaintance. Nat. Immunol. *2*, 389–396.

Poss, K.D., Shen, J., and Keating, M.T. (2000). Induction of lef1 during zebrafish fin regeneration. Dev. Dyn. *219*, 282–286.

Qu-Petersen, Z., Deasy, B., Jankowski, R., Ikezawa, M., Cummins, J., Pruchnic, R., Mytinger, J., Cao, B., Gates, C., Wernig, A., and Huard, J. (2002). Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J. Cell Biol. *157*, 851–864.

Reshef, R., Maroto, M., and Lassar, A.B. (1998). Regulation of dorsal somitic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression. Genes Dev. *12*, 290–303.

Romero-Ramos, M., Vourc'h, P., Young, H.E., Lucas, P.A., Wu, Y., Chivatakarn, O., Zaman, R., Dunkelman, N., el-Kalay, M.A., and Chesselet, M.F. (2002). Neuronal differentiation of stem cells isolated from adult muscle. J Neurosci Res *69*, 894–907.

Rosenblatt, J.D., Lunt, A.I., Parry, D.J., and Partridge, T.A. (1995).

Culturing satellite cells from living single muscle fiber explants. In Vitro Cell. Dev. Biol. Anim. *31*, 773–779.

Sasaki, T., Sasaki-Irie, J., and Penninger, J.M. (2001). New insights into the transmembrane protein tyrosine phosphatase CD45. Int. J. Biochem. Cell Biol. *33*, 1041–1046.

Schmidt, M., Tanaka, M., and Munsterberg, A. (2000). Expression of (beta)-catenin in the developing chick myotome is regulated by myogenic signals. Development *127*, 4105–4113.

Schultz, E., Jaryszak, D.L., Gibson, M.C., and Albright, D.J. (1986). Absence of exogenous satellite cell contribution to regeneration of frozen skeletal muscle. J. Muscle Res. Cell Motil. *7*, 361–367.

Seale, P., and Rudnicki, M.A. (2000). A new look at the origin, function, and "stem-cell" status of muscle satellite cells. Dev. Biol. *218*, 115–124.

Shawber, C., Nofziger, D., Hsieh, J.J., Lindsell, C., Bogler, O., Hayward, D., and Weinmaster, G. (1996). Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. Development *122*, 3765–3773.

Shimizu, H., Julius, M.A., Giarre, M., Zheng, Z., Brown, A.M., and Kitajewski, J. (1997). Transformation by Wnt family proteins correlates with regulation of beta-catenin. Cell Growth Differ. *8*, 1349–1358.

Tajbakhsh, S., and Buckingham, M.E. (1995). Lineage restriction of the myogenic conversion factor myf-5 in the brain. Development *121*, 4077–4083.

Tajbakhsh, S., Borello, U., Vivarelli, E., Kelly, R., Papkoff, J., Duprez, D., Buckingham, M., and Cossu, G. (1998). Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. Development *125*, 4155–4162.

Tamaki, T., Akatsuka, A., Ando, K., Nakamura, Y., Matsuzawa, H., Hotta, T., Roy, R.R., and Edgerton, V.R. (2002). Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle. J. Cell Biol. *157*, 571–577.

Tang, D.G., Tokumoto, Y.M., Apperly, J.A., Lloyd, A.C., and Raff, M.C. (2001). Lack of replicative senescence in cultured rat oligodendrocyte precursor cells. Science *291*, 868–871.

Torrente, Y., Tremblay, J.P., Pisati, F., Belicchi, M., Rossi, B., Sironi, M., Fortunato, F., El Fahime, M., D'Angelo, M.G., Caron, N.J., et al. (2001). Intraarterial injection of muscle-derived CD34(+)Sca-1(+) stem cells restores dystrophin in mdx mice. J. Cell Biol. *152*, 335–348.

Vainio, S.J., and Uusitalo, M.S. (2000). A road to kidney tubules via the Wnt pathway. Pediatr. Nephrol. *15*, 151–156.

Van Den Berg, D.J., Sharma, A.K., Bruno, E., and Hoffman, R. (1998). Role of members of the Wnt gene family in human hematopoiesis. Blood *92*, 3189–3202.

Wakeford, S., Watt, D.J., and Partridge, T.A. (1991). X-irradiation improves mdx mouse muscle as a model of myofiber loss in DMD. Muscle Nerve *14*, 42–50.

Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. Annu. Rev. Cell Dev. Biol. *14*, 59–88.

Zhao, P., Iezzi, S., Carver, E., Dressman, D., Gridley, T., Sartorelli, V., and Hoffman, E.P. (2002). Slug is a novel downstream target of MyoD. Temporal profiling in muscle regeneration. J. Biol. Chem. *277*, 30091–30101.

# Chapter 6

## Pax7 is necessary and sufficient for myogenic commitment in CD45$^{+}$ adult stem cells

**Preface:**

The results presented in this chapter were prepared as a manuscript to be submitted for publication. I performed most of the experiments, prepared the figures and wrote the manuscript. Jeff Ishibashi helped to establish the Pax7 retroviral expression systems and performed the RT-PCR studies to detect endogenous Pax7 transcripts in retrovirally transduced cells (Figure 4B). Dr. Anthony Scimé helped to rescue the Pax7-adenovirus in 293 cells. Considerable advice and intellectual contributions as well as significant input into the final manuscript were provided by Dr. M.A. Rudnicki.

# Pax7 is necessary and sufficient for myogenic commitment in CD45$^+$ adult stem cells

Patrick Seale[1,2], Jeff Ishibashi[2], Anthony Scimè[2] and Michael A. Rudnicki[1,2,3]

[1]Department of Biology
McMaster University
Hamilton, Ontario
Canada, L8S 4K1

[2]Ottawa Health Research Institute
Molecular Medicine Program
501 Smyth Road,
Ottawa, Ontario
Canada, K1H 8L6
Tel: 613-739-6740
Fax: 613-737-8803

[3]Correspondence should be addressed to M.A.R.

E-mail: mrudnicki@ohri.ca

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## 6.1 Abstract

Endogenous $CD45^+$ adult stem cells in muscle tissue undergo myogenesis in response to acute injury. Significantly, $CD45^+$:$Sca1^+$ cells purified from regenerating wildtype muscle expressed Pax7 and gave rise to skeletal myoblasts. By contrast, $CD45^+$:$Sca1^+$ cells from regenerating *Pax7*-deficient muscle did not undergo myogenic commitment. Retroviral expression of Pax7 was sufficient to induce myogenesis in $CD45^+$:$Sca1^+$ cells purified from uninjured muscle. Specifically, cultures infected with Pax7 but not with control virus gave rise to bursts of myoblasts expressing high levels of Myf5 and Sca1 which differentiated as myogenin and Myosin Heavy Chain expressing myocytes. Moreover, $CD45^+$:$Sca1^+$ cells ectopically expressing Pax7 differentiated *in vivo*, readily forming dystrophin-positive fibers after transplantation into *mdx* muscle. Moreover, intramuscular injection of Adenovirus-Pax7 significantly improved the regeneration of *Pax7*-deficient muscle. Altogether, these results demonstrate that muscle-derived stem cells require Pax7 for their myogenic commitment and suggest a role for Pax7 in gene therapy based approaches to treat degenerative muscle disease.

## 6.2 Introduction

Skeletal muscle possesses a remarkable capacity to regenerate in response to acute or chronic muscle injuries. The satellite cell lineage is a muscle-specific stem cell compartment that until recently was thought to give rise to all skeletal myoblasts during regeneration (reviewed by Seale and Rudnicki, 2002; Hawke and Garry, 2001). Satellite cells are located beneath the basal lamina of mature fibers and are activated in response to trauma. Activated satellite cells give rise to committed myogenic precursor cells that express MyoD and Myf5 and proliferate in regenerating muscle prior to undergoing differentiation and fusion (Bischoff, 1994; Cooper et al., 1999; Schultz et al., 1985). The paired-box transcription factor, Pax7 is expressed in satellite cells and is required for the development of this lineage of myogenic progenitors (Chapter 3; Seale et al., 2000). Furthermore, adult *Pax7*-deficient limb muscles do not contain satellite cells and fail to regenerate following chronic or acute injury (Chapter 4).

The identification of adult stem cells in muscle and marrow has challenged the notion that satellite cells are solely responsible for adult myogenesis (reviewed by Seale et al., 2001). For example, bone marrow derived cells participate in muscle repair following intravenous transplantation (Bittner et al., 1999; Ferrari et al., 1998; Gussoni et al., 1999; LaBarge and Blau, 2002). In addition, muscle-derived side-population cells (muSP) and $CD34^+$:$Sca1^+$ cells muscle stem cells differentiate into muscle following systemic delivery (Gussoni et al., 1999; Torrente et al., 2001). In addition, clonally derived stem cells from muscle reconstitute hemaotopoietic lineages in irradiated

recipients yet retain their myogenic differentiation potential after long term engraftment (Cao et al., 2003).

Recently, LaBarge and Blau reported that stably engrafted GFP-marked marrow cells gave rise to satellite cells in mice undergoing extensive exercise (LaBarge and Blau, 2002). This observation suggests that adult stem cells differentiate into specialized cell types by first giving rise to tissue-specific somatic stem cells such as satellite cells in muscle. The capacity for marrow derived adult stem cells to engraft the muscle satellite cell niche has important therapeutic implications for delivering a renewable source of myoblasts to dystrophic or cachexic muscle. Taken together, these studies document the capacity for adult stem cells to undergo myogenesis during regenerative responses and are consistent with the notion that adult stem cells can replenish the satellite cell pool in adult muscle.

To assess the contribution of endogenous muscle-derived stem cell populations in regenerating muscle, we analyzed the myogenicity of $CD45^+$ cells derived from uninjured and regenerating muscle (Chapter 5). CD45 is considered a pan-hematopoietic cell marker that is not expressed in satellite cells, myoblasts or any other non-hematopoietic tissues (reviewed by Sasaki et al., 2001 and Penninger et al., 2001). CD45-expressing cells from uninjured muscle do not give rise to skeletal myoblasts *in vitro* (McKinney-Freeman et al., 2002; Polesskaya et al., 2003). However, muscle injury induced the myogenic commitment of resident $CD45^+$:$Sca1^+$ cells (Chapter 5). Importantly, the myogenic differentiation of $CD45^+$ cells from uninjured muscle is recapitulated by *in vitro* exposure to Wnt proteins. These observations indicate that activation of the Wnt-

signaling pathway in endogenous adult stem cells induces their myogenic specification and subsequent contribution to normal repair processes.

Pax7 and its paralog Pax3 are expressed in the developing somitic muscle anlagen (Goulding et al., 1994; Jostes et al., 1990; reviewed by Parker et al., 2003). Embryonic and fetal myogenesis proceed normally in the absence of Pax7 (Mansouri et al., 1996; Seale et al., 2000). However, the absence of satellite cells in postnatal *Pax7*$^{-/-}$ muscles emphasizes a distinct requirement for Pax7 in satellite cell ontogeny (Seale et al., 2000). By contrast, Pax3 is required for the formation of diaphragm and limb muscles (Bober et al., 1994b; Daston et al., 1996; Tremblay et al., 1998). Elegant genetic studies revealed that Pax3 functions upstream of MyoD in the development of embryonic body muscles (Tajbakhsh et al., 1997). Furthermore, ectopic expression of Pax3 is sufficient to induce skeletal myogenesis in embryonic tissues (Bendall et al., 1999; Heanue et al., 1999; Maroto et al., 1997) and P19 embryonal carcinoma cells (Ridgeway and Skerjanc, 2001). In addition, an activated form of Pax3 in which the transactivation domain of Forkhead (FKHR) is fused to the DNA binding region of Pax3, activates expression of MyoD, myogenin, and Six1 (Khan et al., 1999). Taken together, these data indicate an essential role for Pax3 in myogenic specification upstream of the myogenic regulatory factors (MRFs).

In the current study we demonstrate that Pax7 is necessary for the myogenic commitment of $CD45^{+}$:$Sca1^{+}$ cells during regeneration. Moreover, ectopic expression of Pax7 was sufficient to induce myogenesis in $CD45^{+}$:$Sca1^{+}$ cells isolated from uninjured tissue. Together with the profound regeneration deficit in *Pax7*$^{-/-}$ muscle (Chapter 4),

these observations illustrate a requirement for Pax7 in the myogenic specification of adult stem cells. Strategies to activate Pax7 endogenously or its ectopic expression in stem cells may thus be a viable approach to generate a renewable source of muscle progenitors for clinical applications.

## 6.3 Results

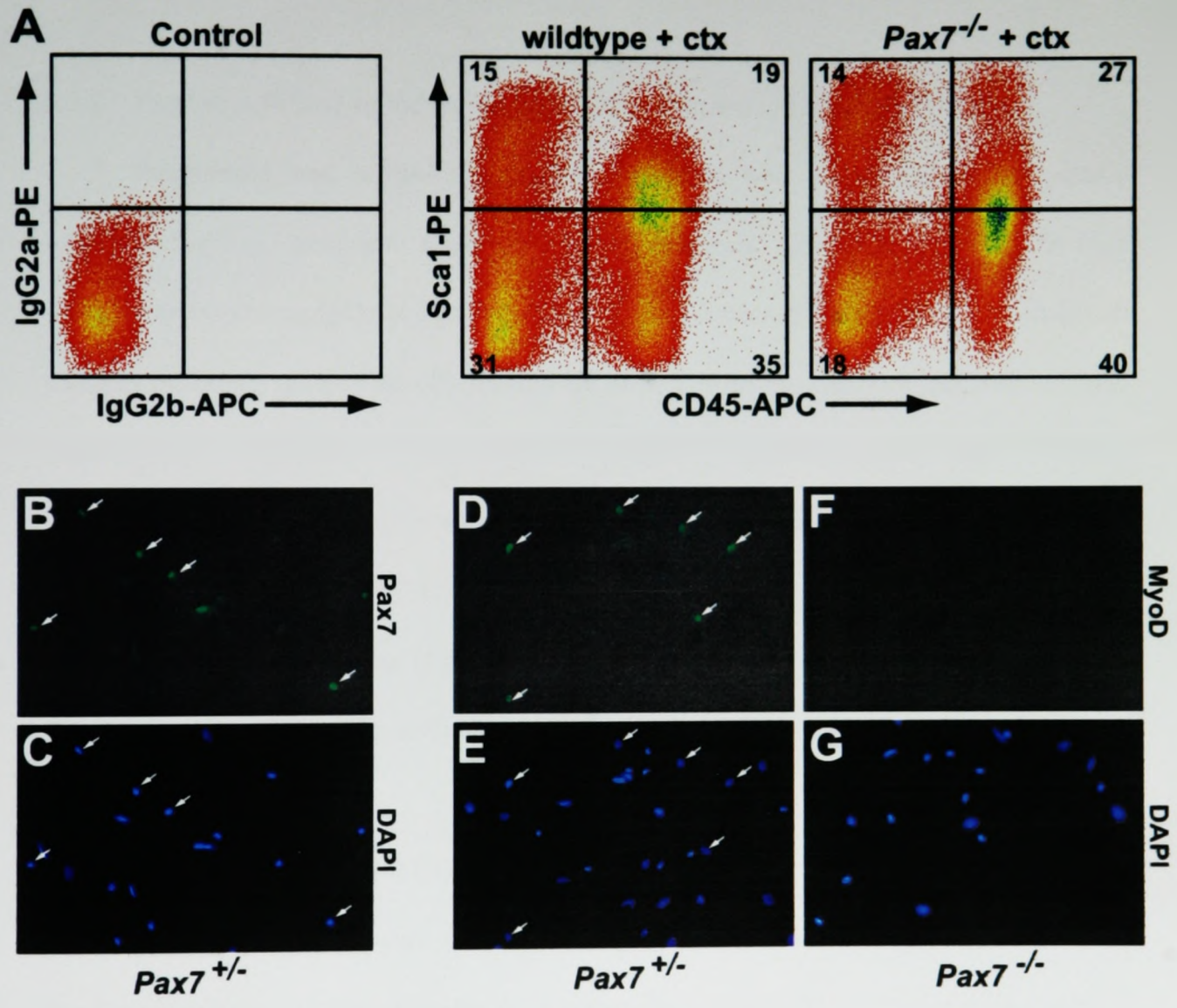
### 6.3.1 Pax7 is required for the myogenicity of $CD45^+$:$Sca1^+$ cells during regeneration

In a previous report, we documented the capacity for endogenous $CD45^+$:$Sca1^+$ muscle derived cells to undergo myogenesis during regeneration (Chapter 5). To determine whether Pax7 is required for the myogenic specification of this adult stem cell population, we analyzed the myogenic potential of $CD45^+$:$Sca1^+$ cells in regenerating *Pax7*$^{-/-}$ muscle. Flow cytometric analysis of mononuclear cells isolated from 4-6 week old *Pax7*$^{-/-}$ and wildtype littermates 4 days after administration of cardiotoxin (ctx) revealed an increased proportion of $CD45^+$:$Sca1^+$ cells in *Pax7*$^{-/-}$ muscle relative to wildtype (27+/-1.4% compared to 19+/-3.5%; p=0.006; n=3) (Figure 1A).

Importantly, Pax7 was expressed in ~10% of freshly isolated $CD45^+$:$Sca1^+$ cells from regenerating *Pax7*$^{+/-}$ (Figure 1B, C) muscle. Moreover, Desmin (not shown) and MyoD-expressing (Figure 1D, E) cells were readily detected in the $CD45^+$:$Sca1^+$ population from regenerating *Pax7*$^{+/-}$ muscle. By contrast, $CD45^+$:$Sca1^+$ cells from regenerating *Pax7*$^{-/-}$ muscle did not give rise to any MyoD-expressing myogenic cells (n=3 isolations with cells from 2 mice pooled for each isolation) (Figure 1F, G). All of the MyoD-expressing *Pax7*$^{-/-}$ myoblasts were $CD45^-$ and $Sca1^-$. These results demonstrate

**Figure 1 Pax7 is required for the myogenic specification of $CD45^+$:$Sca1^+$ cells**

(**A**) Flow cytometric analysis of cell suspensions derived from regenerating wildtype and *Pax7*$^{-/-}$ muscle (4 days after cardiotoxin (ctx) injection) showed an increased proportion of $CD45^+$ cells in *Pax7*$^{-/-}$ samples. (**B**, **C**) Pax7 was expressed in ~10% of $CD45^+$:$Sca1^+$ cells purified from regenerating *Pax7*$^{+/-}$ muscle. (**D-G**) MyoD was induced in $CD45^+$:$Sca1^+$ cells from regenerating *Pax7*$^{+/-}$ muscle (**D**, **E**) but was not expressed in $CD45^+$:$Sca1^+$ cells from regenerating *Pax7*$^{-/-}$ muscle (**F**,**G**).


A
Control
wildtype + ctx
Pax7-/- + ctx
IgG2a-PE
IgG2b-APC
Sca1-PE
CD45-APC
15
19
31
35
14
27
18
40
B
Pax7
C
DAPI
Pax7 +/-
D
F
MyoD
E
G
DAPI
Pax7 +/-
Pax7 -/-

that the myogenic differentiation of $CD45^+$:$Sca1^+$ cells require Pax7. Therefore, the low numbers of Pax3-expressing skeletal myoblasts recovered in *Pax7*$^{-/-}$ muscle suspensions (Chapter 4) are not derived from CD45 or Sca1 positive cells.

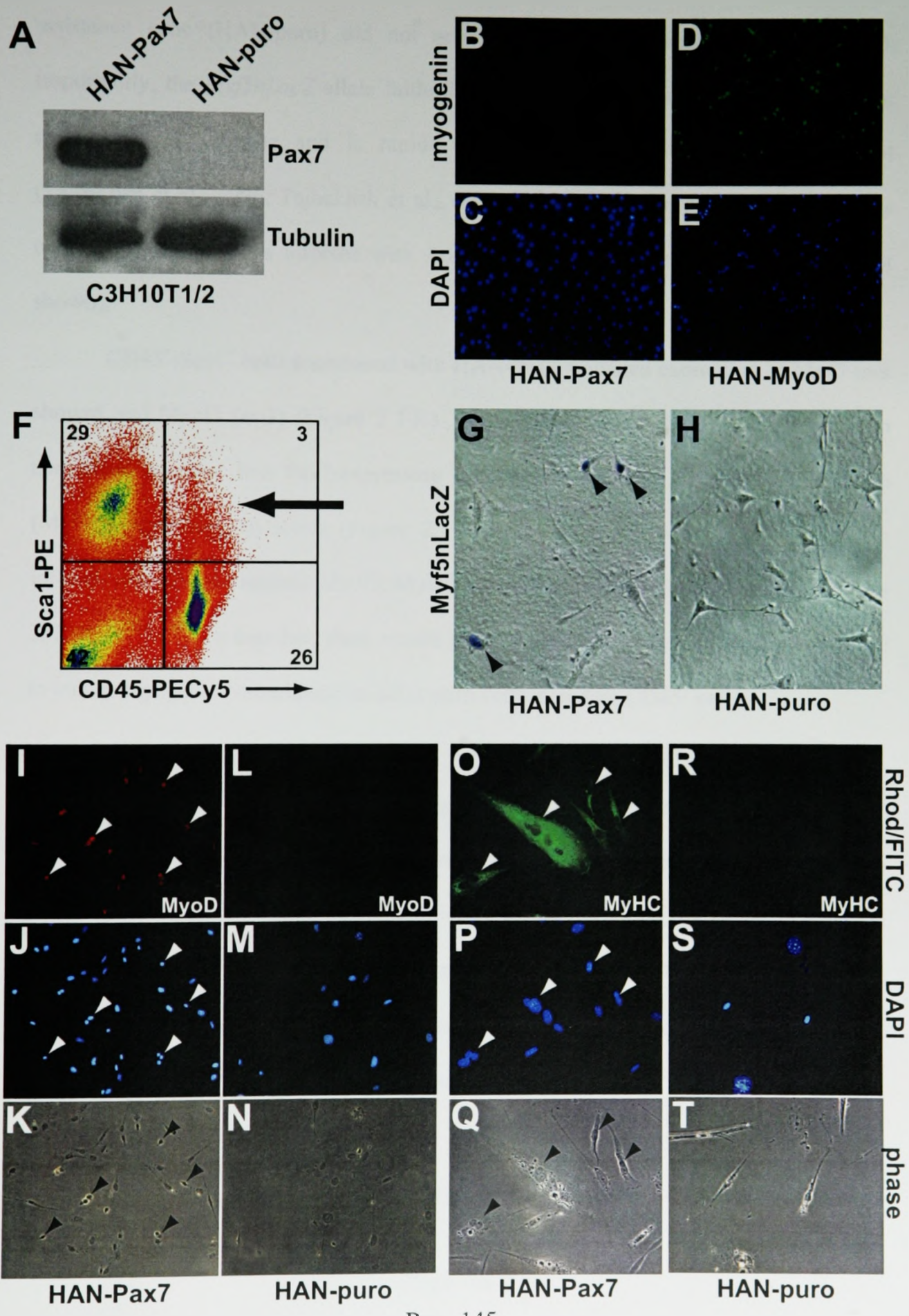
### 6.3.2 Pax7 is sufficient to induce myogenic commitment in $CD45^+$:$Sca1^+$ cells

Adenoviral and retroviral expression systems were developed to ectopically introduce the Pax7 gene into adult stem cell populations. Pax7 was expressed to high levels from retrovirus (pHAN-Pax7) in C3H10T1/2 fibroblasts (Figure 2A) and other cell cultures including primary myoblasts (not shown). Ectopic and stable expression of Pax7 did not activate myogenesis in C3H10T1/2 fibroblasts. Specifically, Pax7 did not induce MyoD (not shown), Myf5 (not shown) or myogenin expression (Figure 2B, C) whereas retroviral expression of MyoD efficiently converted C3H10T1/2 cells to myogenin-expressing skeletal myocytes (Figure 2D, E). These results indicate that Pax7, like Pax3 (Maroto et al., 1997) is not sufficient to induce the myogenic differentiation program in mesenchymal progenitors.

To determine whether Pax7 is sufficient to activate the myogenic program in adult $CD45^+$ progenitors, cells were fractionated from uninjured muscle and transduced with Pax7-expressing retrovirus. Similar to previous data, about 3% of mononuclear cells in uninjured muscle coexpressed CD45 and Sca1 (Figure 2F). Strikingly, transduction of $CD45^+$:$Sca1^+$ cells from *Myf5nLacZ* reporter mice with HAN-Pax7 retrovirus specifically induced Myf5nLacZ expression and myogenesis in about 50% of infected cells (Figure 2G). By contrast, transduction of cells with control retrovirus expressing the puromycin

**Figure 2 Pax7 induces myogenic commitment in CD45$^+$:Sca1$^+$ cells**

(**A**) Westen blot analysis with anti-Pax7 antibody confirmed high levels of ectopic Pax7 in C3H10T1/2 cells infected with retrovirus-Pax7 (HAN-Pax7) but not with control virus expressing a puromycin resistance marker (HAN-puro). (**B-E**) HAN-Pax7 did not induce expression of myogenin or myogenic specification in C3H10T1/2 cells (**B**, **C**). By contrast, MyoD virus (HAN-MyoD) efficiently converted C3H10T1/2 cells to myogenin expressing myocytes (**D**, **E**). (**F**) Flow cytometric analysis of cell suspensions derived from uninjured wildtype showed that ~3% of cells coexpressed CD45 and Sca1. **(G, H)** HAN-Pax7 (**G**) but not HAN-puro (**H**) induced Myf5nLacZ expression in CD45$^+$:Sca1$^+$ cells. HAN-Pax7 (**I-K**) but not HAN-puro (**L-N**) activated expression of MyoD in CD45$^+$:Sca1$^+$ cells from uninjured muscle. (**O-Q**) HAN-Pax7 infected CD45$^+$:Sca1$^+$ cultures differentiated into Myosin Heavy Chain expressing myocytes under differentiation conditions, whereas HAN-puro infected cells did not undergo myogenic differentiation (**R-T**). DAPI staining (blue) was used to visualize all nuclei.


A
HAN-Pax7
HAN-puro
Pax7
Tubulin
C3H10T1/2
B
D
myogenin
C
E
DAPI
HAN-Pax7
HAN-MyoD
F
29
3
26
Sca1-PE
CD45-PECy5
G
H
Myf5nLacZ
HAN-Pax7
HAN-puro
I
L
O
R
MyoD
MyoD
MyHC
MyHC
Rhod/FITC
J
M
P
S
DAPI
K
N
Q
T
phase
HAN-Pax7
HAN-puro
HAN-Pax7
HAN-puro

resistance gene (HAN-puro) did not activate Myf5nLacZ expression (Figure 2H). Importantly, the *Myf5nLacZ* allele faithfully recapitulates the expression pattern of the endogenous *Myf5* gene and is rapidly induced following myogenic commitment (Tajbakhsh et al., 1996; Tajbakhsh et al., 1997). Similar activation of *Myf5nLacZ* was observed in muSP cells infected with Adenovirus-Pax7 but not Adenovirus-GFP (not shown).

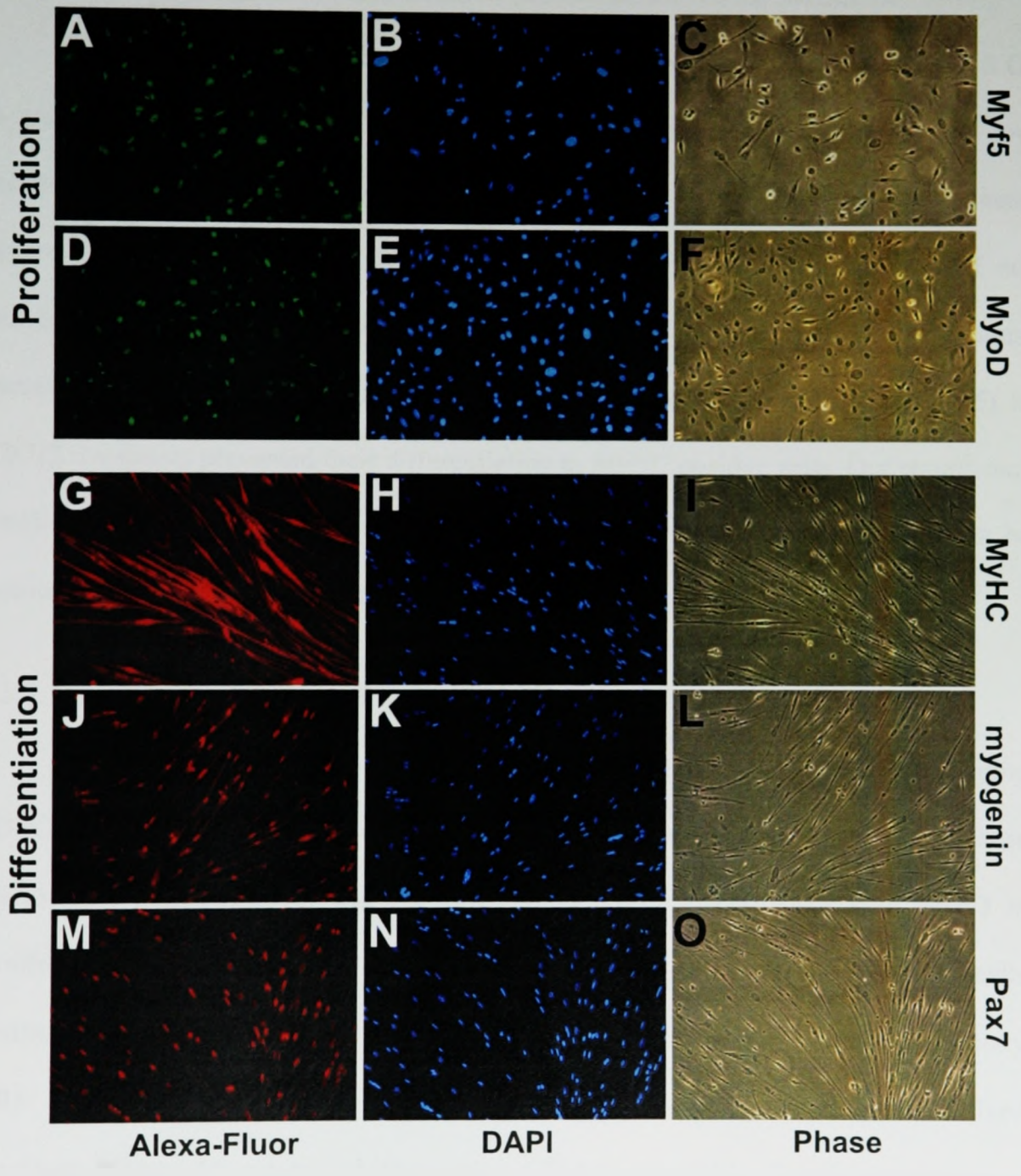
$CD45^+$:$Sca1^+$ cells transduced with HAN-Pax7 activated expression of Myf5 (not shown) and MyoD (n=3) (Figure 2 I-K). Exposure of these cultures to differentiation conditions revealed that Pax7-expressing cells differentiated to Myosin Heavy Chain (MyHC)-expressing myocytes (Figure 2, O-Q). By contrast, control HAN-puromycin infected cells did not express MyoD, MyHC or undergo myogenic differentiation (Figure 2, L-N, R-T). Taken together, these results provide direct evidence that Pax7 is sufficient to induce myogenic commitment in adult stem cells including $CD45^+$ cells.

### 6.3.3 $CD45^+$:$Sca1^+$ cells stably expressing Pax7 are bonified myogenic progenitors

$CD45^+$:$Sca1^+$ cells expressing retroviral Pax7 were stably selected using the puromycin resistance marker expressed from the retroviral construct, hereafter called CDSC-Pax7 cells (n=4 independent isolates analyzed). CDSC-Pax7 cells displayed a stellate, fibroblastic morphology reminiscent of C2C12 myoblasts compared to the round, refractile appearance of primary myoblasts derived from satellite cells. Importantly, proliferating CDSC-Pax7 cells expressed high levels of the myogenic determination factor, Myf5 (Figure 3 A-C) as well as MyoD (Figure 3 D-F). Moreover, CDSC-Pax7

**Figure 3 $CD45^+$:$Sca1^+$ cells expressing Pax7 give rise to myoblasts**

Myf5 (**A-C**) and MyoD (**D-F**) protein (green) are expressed in proliferating CDSC-Pax7 cells. Stimulation of CDSC cultures in low mitogen medium induced the formation of multinucleated myotubes and expression of differentiation markers including MyHC (red) (**G-I**) and myogenin (red) (**J-L**). Sustained expression of Pax7 (red) (**M-O**) in these cultures did not interfere with their differentiation. DAPI staining (blue) was used to visualize all nuclei.


A
B
C
Myf5
Proliferation
D
E
F
MyoD
G
H
I
MyHC
Differentiation
J
K
L
myogenin
M
N
O
Pax7
Alexa-Fluor
DAPI
Phase

cells cycled approximately 3 times faster than satellite cell myoblasts isolated at the same time (not shown) and have been maintained as primary cultures for over 3 months and after numerous passages in culture.

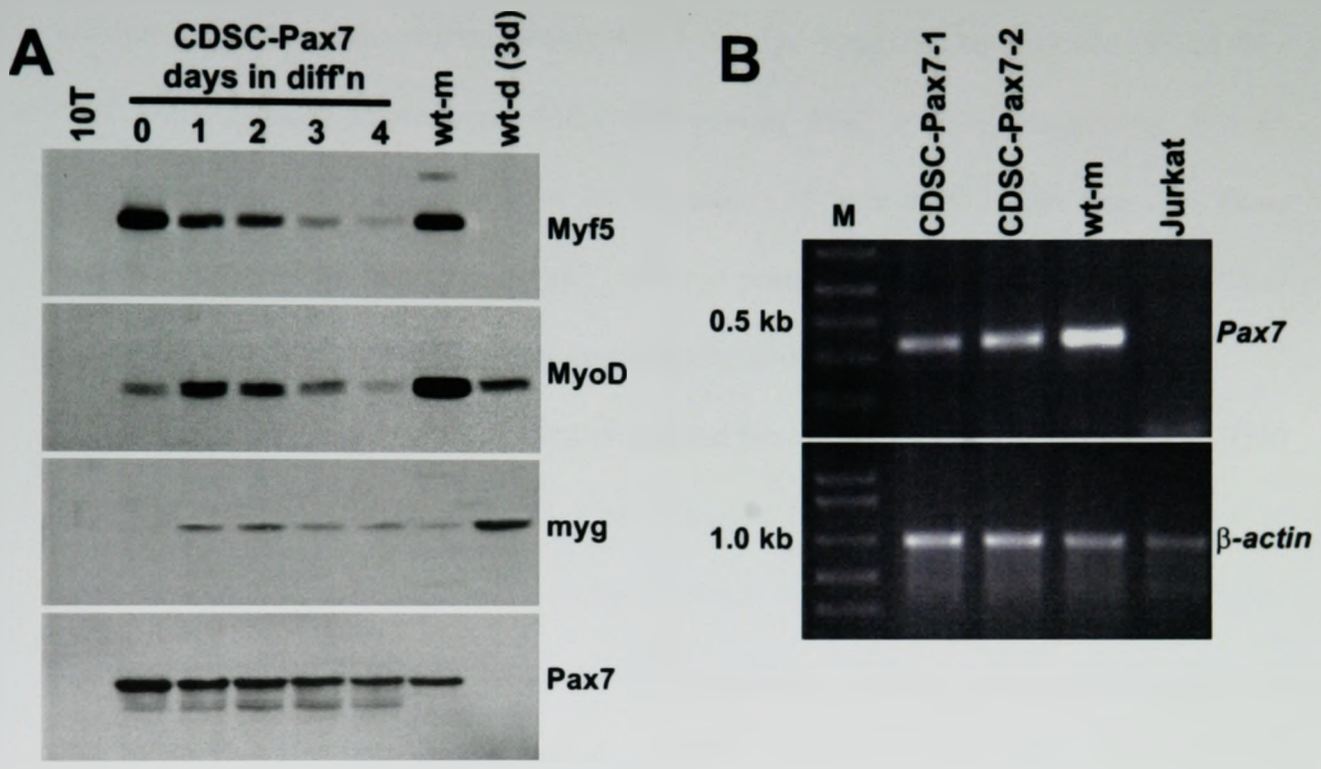
Importantly, CDSC-Pax7 cultures efficiently differentiated as MyHC (Figure 3 G-I) and myogenin -expressing multinucleated myotubes following exposure to low mitogen containing medium (Figure 3 J-L). These results demonstrate that the continued expression of Pax7, normally downregulated upon myogenic differentiation, did not interfere with cell-cycle arrest and differentiation (Figure 3 M-O). By contrast overexpression of Pax7 (not shown) or Pax3 (not shown and Epstein et al., 1995) in C2C12 myoblasts prevented their differentiation to MyHC positive cells. Our results thus confirm that CDSC-Pax7 cells represent a stable myogenic cell population that can be maintained as proliferating myoblasts and differentiate appropriately *in vitro*.

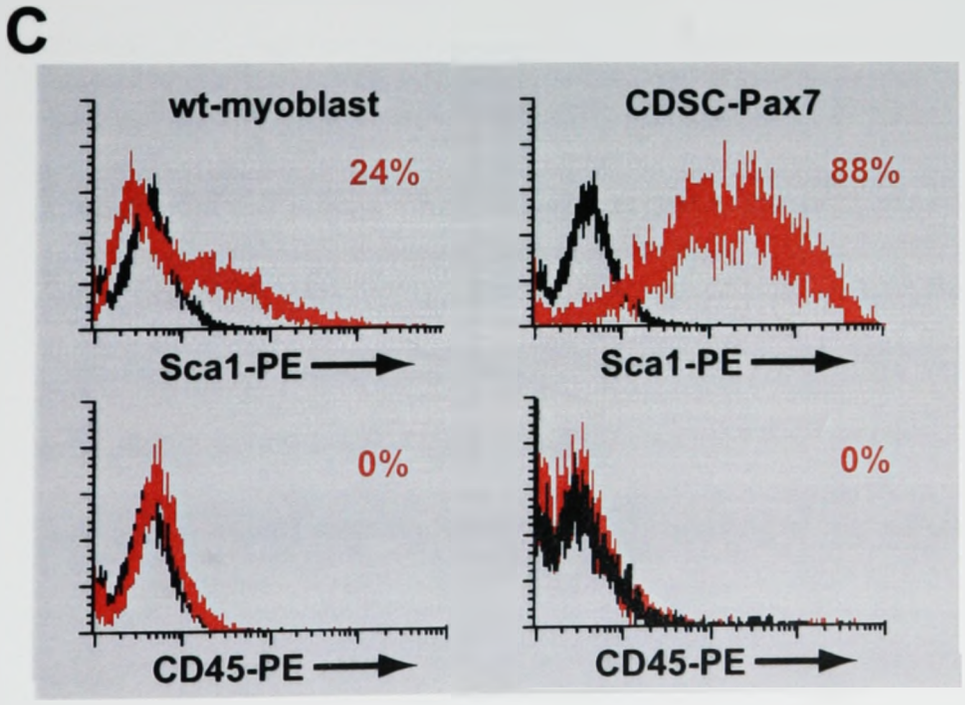
### 6.3.4 CDSC-Pax7 cells express high levels of Myf5 and Sca1

The expression pattern of myogenic factors in proliferating and differentiating CDSC-Pax7 cell lines (n=2) was analyzed by Western blot analysis. Interestingly, these analyses showed that Myf5 was expressed at markedly higher levels than MyoD in proliferating CDSC-Pax7 cells (Figure 4A- day 0). Moreover, CDSC-Pax7 cells continued to express Myf5 protein during their differentiation. This pattern of primary MRF expression contrasts the expression profile exhibited by satellite cell-derived myoblasts (Figure 4A- wt-m), which expressed high levels of MyoD and lower levels of Myf5 and subsequently downregulated Myf5 upon differentiation (wt-d). MyoD was transiently upregulated in CDSC-Pax7 cells as they entered their differentiation program

**Figure 4 CDSC-Pax7 cells express high levels of Myf5 and Sca1**

(**A**) Western blot analysis of CDSC-Pax7 cells in proliferation conditions (day 0) and during differentiation (days 1-4) revealed high levels of Myf5 expression and low levels of MyoD. Myogenin (myg) was upregulated during the differentiation of CDSC-Pax7 and satellite cell-derived myoblasts (wt-d). Note the sustained expression of Pax7 during the differentiation of CDSC-Pax7 cells. C3H10T1/2 (10T) lysate was used as a negative control. (**B**) RT-PCR analysis indicated that CDSC-Pax7 cells (2 different lines) upregulated the endogenous *Pax7* mRNA. Satellite cell derived myoblasts (wt-m) and Jurkat cells were used as positive and negative controls respectively in this experiment. (**C**) Flow cytometry indicated that CDSC-Pax7 cells lost expression of CD45 but retained high levels of Sca1. About 24% of satellite cell derived myoblasts (wt-myoblasts) expressed low levels of Sca1.


A
CDSC-Pax7
days in diff'n
10T
0 1 2 3 4
wt-m
wt-d (3d)
Myf5
MyoD
myg
Pax7
B
M
CDSC-Pax7-1
CDSC-Pax7-2
wt-m
Jurkat
0.5 kb
Pax7
1.0 kb
β-actin


C
wt-myoblast
24%
Sca1-PE
0%
CD45-PE
CDSC-Pax7
88%
Sca1-PE
0%
CD45-PE

(Figure 4A- day 1, 2). Myogenin (myg) was similarly upregulated during the differentiation of CDSC-Pax7 cells, albeit at lower levels compared with differentiating satellite cell-derived myoblasts (wt-d). Interestingly, myogenic commitment in CDSC-Pax7 cells induced expression of the endogenous Pax7 mRNA, suggesting that an autoregulatory mechanism regulates its expression (Figure 4B). Taken together, these analyses demonstrate that CDSC-Pax7 cells and primary satellite cell-derived myoblasts express a distinct profile of myogenic proteins *in vitro*.

CDSC-Pax7 cells were originally derived from cells expressing cell surface CD45 and Sca1 proteins. Flow cytometry was employed to determine whether expression of these markers was maintained after stable selection *in vitro*. These experiments revealed that CDSC-Pax7 cells continued to express high levels of Sca1 (~90% of cells showed intense staining) but had lost expression of CD45 in culture (Figure 4C). Interestingly, a proportion of primary satellite cell derived myoblasts displayed low levels of Sca1 staining, that has not been detected previously by immunohistochemistry. Sca1 levels were not increased in satellite cell-derived myoblasts overexpressing Pax7, demonstrating that CDSC-Pax7 cells did not likely arise from a small number of committed myoblasts fractionated with the $CD45^{+}$:$Sca1^{+}$ cells (not shown). These data suggest that CDSC-Pax7 cells lost their hematopoietic properties including expression of CD45 during their differentiation into the myogenic lineage.

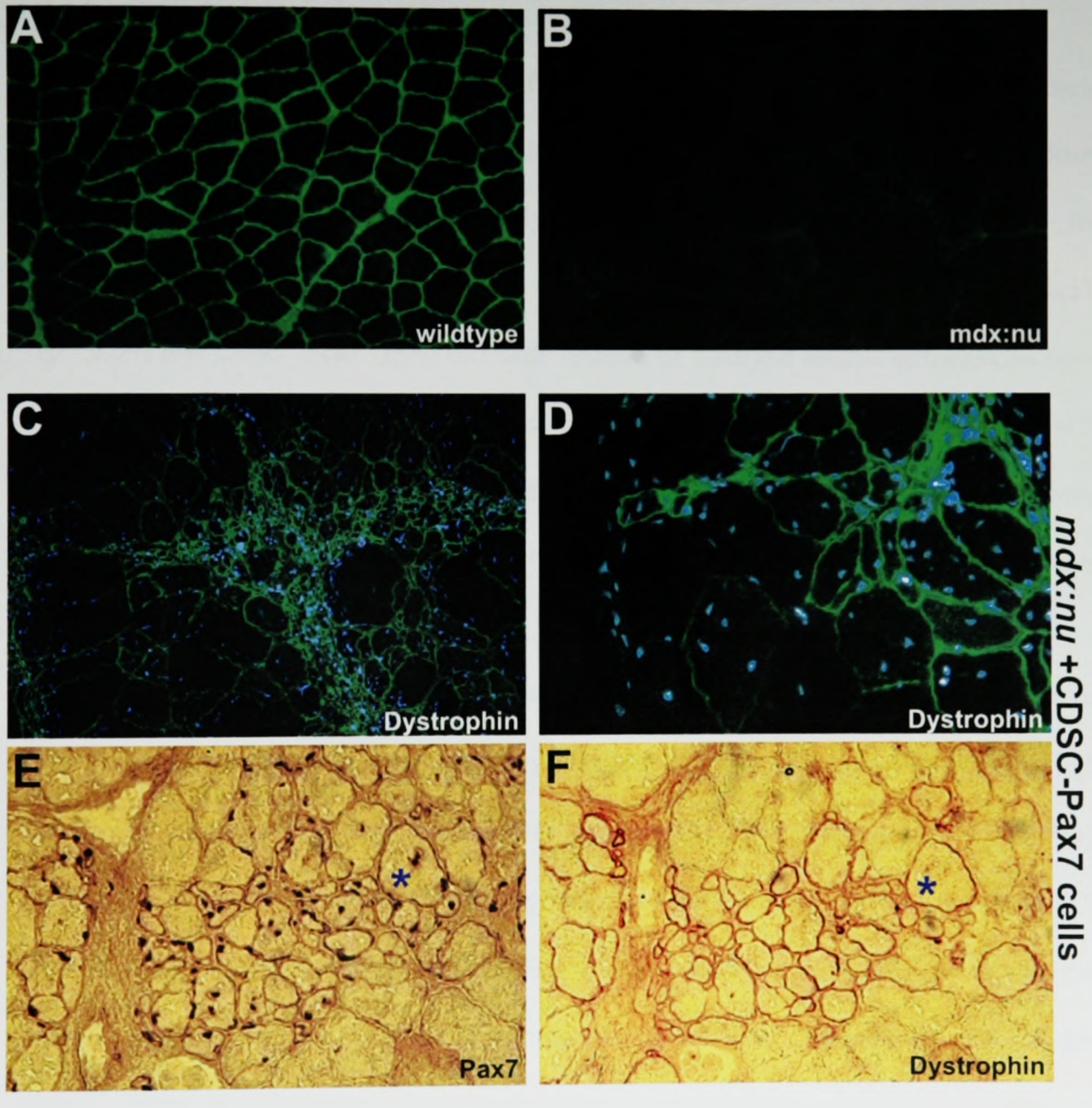
Preliminary transcriptional profiling analysis using Affymetrix arrays confirmed the markedly increased levels of Sca1 (31 fold) and Myf5 (6 fold) gene expression in CDSC-Pax7 cells relative to primary satellite cell myoblasts.

### 6.3.5 CDSC-Pax7 cells differentiate *in vivo*

To establish whether CDSC-Pax7 cells were capable of integrating and differentiating as myofibers *in vivo*, intramuscular transplantation studies were performed in dystrophic muscle. Specifically, $1x10^5$ CDSC-Pax7 cells were injected into the tibialis anterior muscle (TA) of 4-6 week old *mdx:nude* mice. *Mdx* mice carry a point mutation in the *dystophin* gene and are a mouse model for Duchenne muscular dystrophy (Bulfield et al., 1984; Sicinski et al., 1989). The presence of the *mdx* mutation in *nude* immunodeficient mice that lack a functional immune response provides a convenient model to assess the efficacy of myoblast or stem cell transplantation (Blaveri et al., 1999; Partridge et al., 1989). As expected, dystrophin was localized at the myofiber sarcolemma in wildtype muscle (Figure 5A) and was absent in *mdx:nu* skeletal muscle (Figure 5B). Two months after transplantation of CDSC-Pax7 cells, TA muscles were processed for immunohistochemical detection of dystrophin, and Pax7. These experiments revealed that CDSC-Pax7 cells differentiated efficiently *in vivo*, readily forming large numbers of dystrophin-expressing myofibers in *dystrophin*-deficient host muscle (Figure 5C, D). Pax7 protein was downregulated immediately following differentiation, and its expression was not observed within regenerated myofibers (not shown). Therefore, the expression of Pax7 protein in centrally positioned nuclei within dystrophin$^+$ regenerated fibers confirmed the donor cell origin of these fibers (Figure 5E, F). These results document the capacity for CDSC-Pax7 cells to differentiate

**Figure 5 CDSC-Pax7 cells efficiently regenerate dystrophic muscle**

(**A**) Wildtype muscle expressed dystrophin at the plasmalemma of all myofibers. (**B**) Dystrophin protein was not detected in muscle sections from *dystrophin*-deficient *mdx:nude* mice (*mdx:nu*). (**C-F**) CDSC-Pax7 cells differentiated *in vivo* after transplantation, readily forming large numbers of dystrophin expressing myofibers (green) in *mdx:nude* muscle (**C**, **D**). The sustained expression of Pax7 protein in central nuclei of regenerated fibers (**E**, red staining) confirmed the donor origin of dystrophin-positive myofibers in serial sections (**F**, red staining).


A
wildtype
B
mdx:nu
C
Dystrophin
D
Dystrophin
mdx:nu +CDSC-Pax7 cells
E
Pax7
F
Dystrophin

appropriately *in vivo* and efficiently contribute to the repair of dystrophic muscle despite constitutive Pax7 expression.

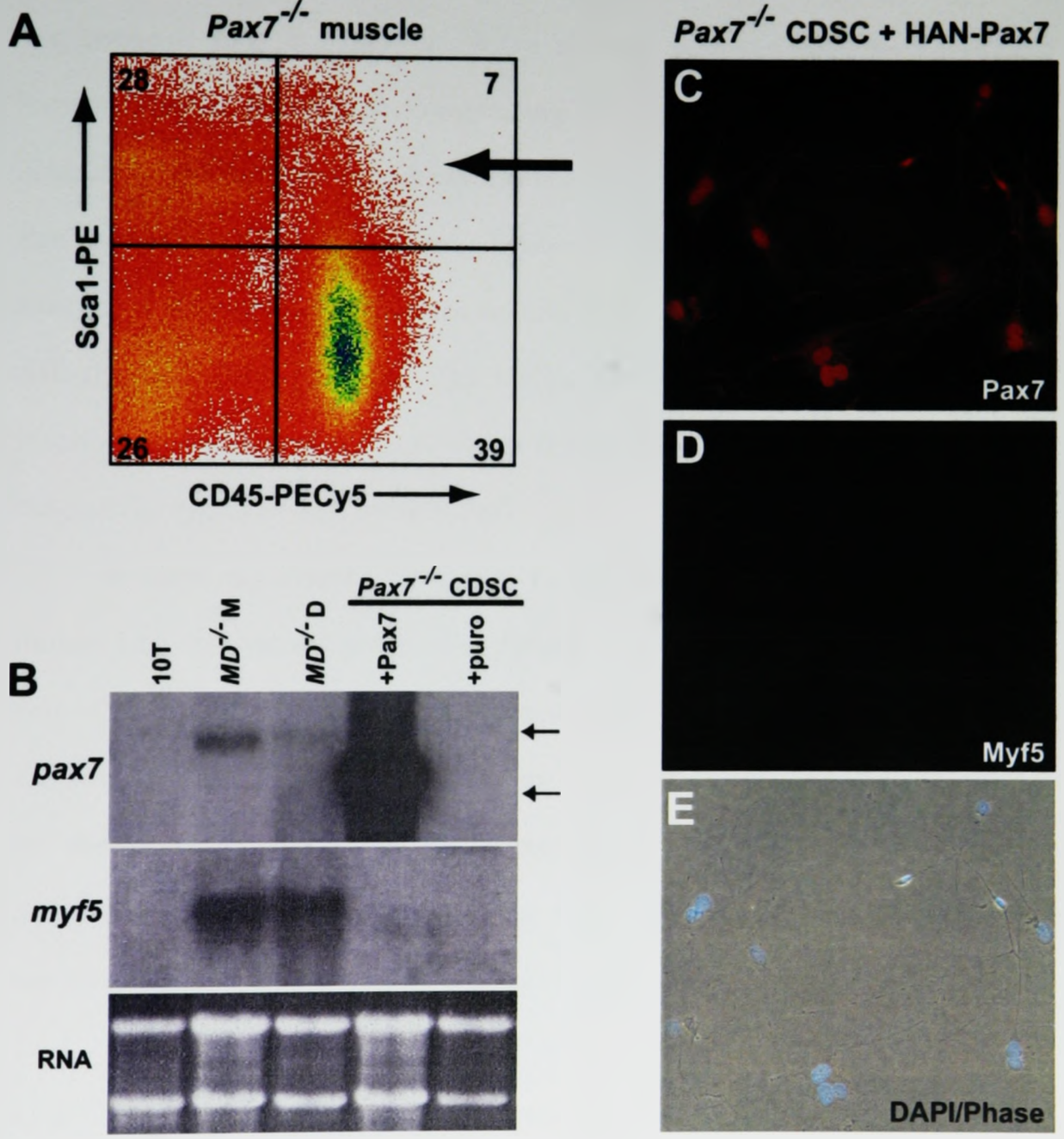
### 6.3.6 Pax7 does not induce myogenesis in CD45$^+$:Sca1$^+$ cells from *Pax7*$^{-/-}$ muscle

The myogenic specification of CD45$^+$:Sca1$^+$ cells from wildtype muscle suggested that Pax7 would similarly induce myogenesis in the same cell fraction from *Pax7*$^{-/-}$ muscle. Flow cytometric analysis of *Pax7*$^{-/-}$ muscle cell suspensions for expression of CD45 and Sca1 showed a significantly higher proportion of CD45$^+$:Sca1$^-$ ($p= 0.04$) and CD45$^+$:Sca1$^+$ ($p=0.02$) cells relative to wildtype muscle suspensions (n=4 independent experiments) (Figure 6A compare to Figure 2F). Specifically, 39+/-4% versus 26+/-5% of cells were CD45$^+$:Sca1$^-$ and 7+/-1% versus 3.4+/-1.3% of cells were CD45$^+$:Sca1$^+$ cells in muscle suspensions from *Pax7*$^{-/-}$ and wildtype littermates respectively.

Transduction of *Pax7*$^{-/-}$ CD45$^+$:Sca1$^+$ cells with Pax7 retrovirus did not induce expression of *Myf5* mRNA as assessed by northern blot hybridization (Figure 6B). High levels of retroviral *Pax7* transcript (lower band on Pax7 blot) was produced in Pax7 infected cultures. Furthermore, despite high levels of Pax7 protein expression (Figure 6C) in retrovirally transduced *Pax7*$^{-/-}$ CD45$^+$:Sca1$^+$ cells, Myf5 (Figure 6 C-E) or MyoD (not shown) proteins were not detected immunochemically. These experiments indicate that *Pax7*$^{-/-}$ CD45$^+$:Sca1$^+$ cells do not commit to the myogenic lineage in response to ectopic expression of Pax7. Taken together, these results suggest important intrinsic differences between wildtype and *Pax7*-deficient populations of CD45$^+$:Sca1$^+$ cells.

**Figure 6 Pax7 does not induce myogenesis in *Pax7*-deficient CD45$^+$:Sca1$^+$ cells**

(**A**) Flow cytometric analysis of muscle cell suspensions from uninjured *Pax7*$^{-/-}$ muscle revealed an increased proportion of CD45$^+$ cells (compare to Figure 2F). (**B**) Northern analysis shows that *MyoD*$^{-/-}$ satellite cell derived myoblasts (*MD*$^{-/-}$ M) and differentiating cells (*MD*$^{-/-}$ D) express endogenous *Pax7* (upper arrow, Pax7 blot) and *Myf5* transcripts. *Pax7*$^{-/-}$ CD45$^+$:Sca1$^+$ cells (CDSC) transduced with HAN-Pax7 (+Pax7) or HAN-puro (+puro) did not initiate expression of *Myf5* mRNA. The retroviral transcript producing Pax7 is smaller than the endogenous *Pax7* mRNA (e.g. lower arrow). (**C-E**) Ectopic expression of Pax7 (red) (**C**) in *Pax7*$^{-/-}$ CDSC cells did not induce Myf5 protein expression (**D**). DAPI staining (blue) was used to visualize all nuclei.


A
Pax7-/- muscle
28
7
26
39
Sca1-PE
CD45-PECy5
B
10T
MD-/- M
MD-/- D
Pax7-/- CDSC
+Pax7
+puro
pax7
myf5
RNA
Pax7-/- CDSC + HAN-Pax7
C
Pax7
D
Myf5
E
DAPI/Phase

### 6.3.7 Pax7 promotes myogenic commitment in *Pax7*-deficient CD45$^-$:Sca1$^-$ cells

In cell suspensions from uninjured muscle, satellite cells and their daughter myogenic precursors are uniformly CD45$^-$ and Sca1$^-$. In *Pax7*$^{-/-}$ muscles, the extremely rare myogenic cells in muscle tissue are similarly separated with the CD45$^-$:Sca1$^-$ fraction. However, MyoD or Myf5-expressing *Pax7*$^{-/-}$ myoblasts do not survive in culture (Chapter 4). Interestingly, ectopic expression of Pax7 in cultured CD45$^-$:Sca1$^-$ cells from *Pax7*$^{-/-}$ muscle resulted in the upregulation of Myf5 protein (n=3) (Figure 7 A-C). Analysis of HAN-puromycin infected cultures at this time did not reveal any myogenic cells (Figure 7 D-F). Importantly, all Myf5-expressing myoblasts (Figure 7 G-I) and MyHC-expressing differentiated myotubes (Figure 7 J-L) in Pax7 infected CD45$^-$:Sca1$^-$ cultures also expressed high levels of Pax7.

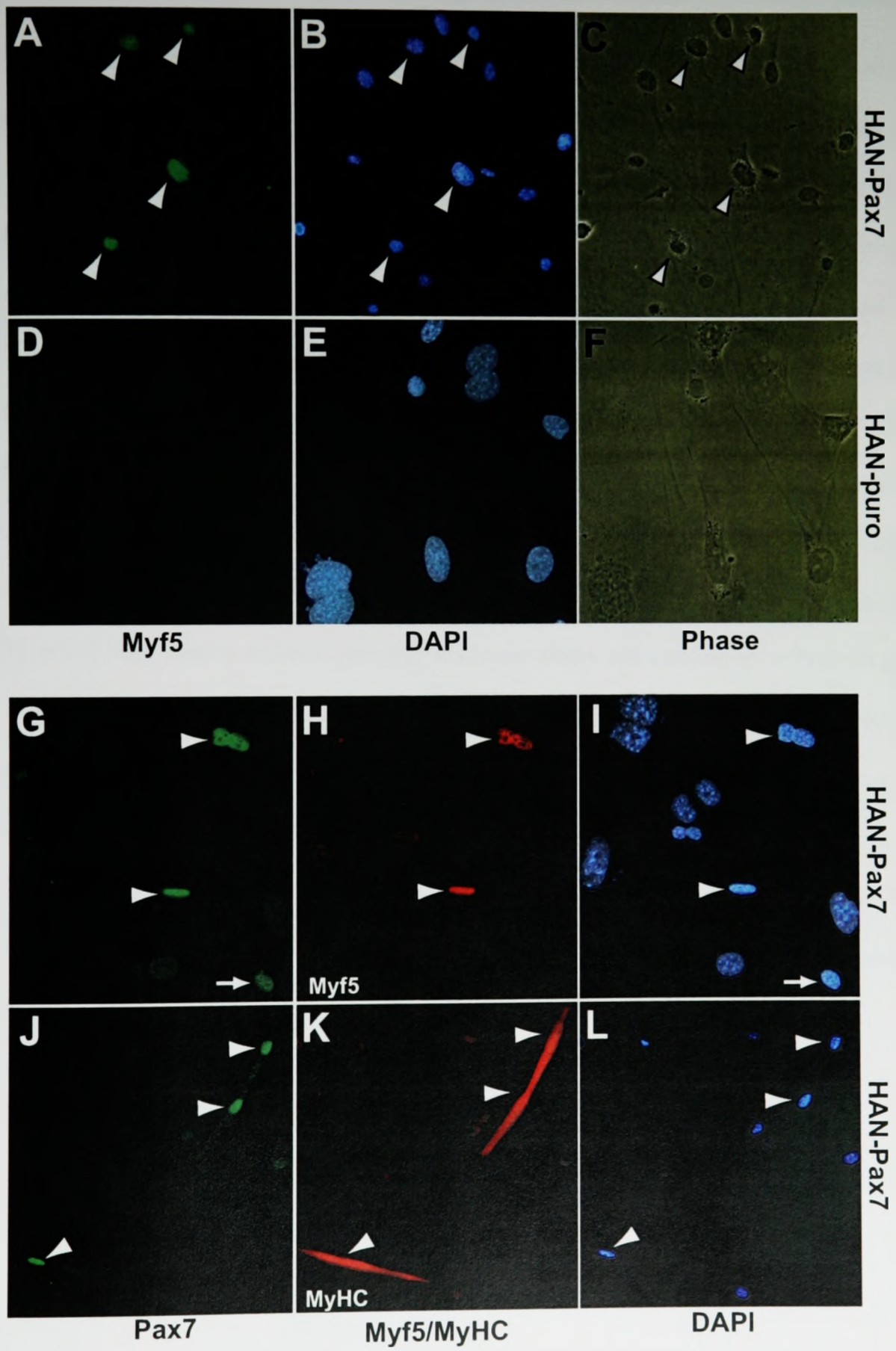
In these experiments we cannot formally exclude the possibility that Pax7 increased the survival and proliferative capacity of committed myoblasts present at the time of isolation. However, given the extremely low number of myogenic cells initially recovered in culture (see Chapter 4), the low rate of myoblast infectivity (~5-10%) and the absence of any Myf5 or MyoD-expressing cells after selection for puromycin resistance, our results strongly suggest that Pax7 induces myogenic specification in a non-myoblast cell type that is CD45 and Sca1 negative.

### 6.3.8 Adenoviral expression of Pax7 enhances regeneration in *Pax7*-deficient muscle

To investigate whether Pax7 was sufficient to stimulate myogenesis *in vivo*, adenovirus was used to ectopically express Pax7 in damaged *Pax7*$^{-/-}$ muscle. $1x10^8$

**Figure 7 Pax7 promotes myogenesis in CD45$^-$:Sca1$^-$ cells from *Pax7*$^{-/-}$ muscle**

(**A-C**) Ectopic expression of Pax7 (HAN-Pax7) induced Myf5 expression (green) and myogenic commitment in CD45$^-$:Sca1$^-$ cells from *Pax7*$^{-/-}$ muscle. (**D-F**) By contrast, Myf5 expressing cells were completely absent from HAN-puro infected cultures. (**G-L**) CD45$^-$:Sca1$^-$ cells from *Pax7*$^{-/-}$ muscle expressed Myf5 (red) (**H**) and MyHC (red) (**K**) only in cells that also expressed Pax7 protein (**G**, **J**). Arrowheads indicate cells coexpressing Pax7 and Myf5/MyHC. Arrow in G, I depicts a Pax7+, Myf5- cell.


A
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Myf5
DAPI
Phase
G
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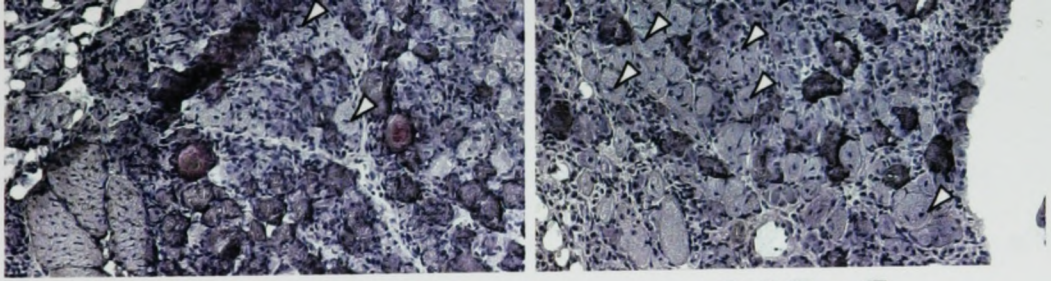
I
HAN-Pax7
Myf5
J
K
L
HAN-Pax7
MyHC
Pax7
Myf5/MyHC
DAPI

adenoviral particles expressing either Pax7 (Ad-Pax7) or the bacterial *β-galactosidase* gene (LacZ) (Ad-LacZ) were injected directly into injured TA muscles of 4-6 week old *Pax7*$^{-/-}$ animals 2 days after administration of ctx (n=4). Immunohistochemisty for Pax7 in adenovirus infected muscles demonstrated a high level of Pax7 expression mostly in mononuclear cells (Figure 8B). Presumably, the adenovirus is lost or downregulated in differentiating cells that have undergone multiple rounds of proliferation. Furthermore, X-gal staining of whole TA muscles infected with Ad-LacZ revealed widespread viral integration within the muscle (Figure 8 C, D). To assess the effect of Pax7 expression in damaged tissue, TA muscles were analyzed and scored for regeneration 12 days after infection by enumerating the numbers of regenerated fibers with centrally located nuclei. The newly regenerated status of centrally nucleated fibers was confirmed by Desmin and embryonic MyHC immunoreactivity (not shown). Ad-Pax7 induced a markedly enhanced regenerative response relative to Ad-LacZ in *Pax7*$^{-/-}$ muscle. Pax7 infected muscle displayed a notable improvement in overall structure and tissue integrity and possessed a 3.9 fold increase in the number of regenerated myofibers (n=4) (Figure 8 E-G). These results establish the presence of adult stem/progenitor cells in *Pax7*$^{-/-}$ muscle that remain capable of Pax7-dependent myogenesis *in vivo*.

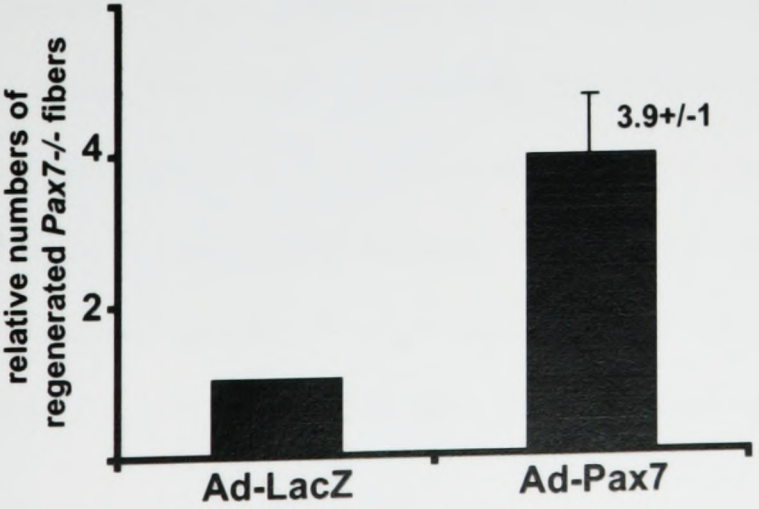
**Figure 8 Adenovirus-Pax7 markedly enhances regeneration *in vivo***

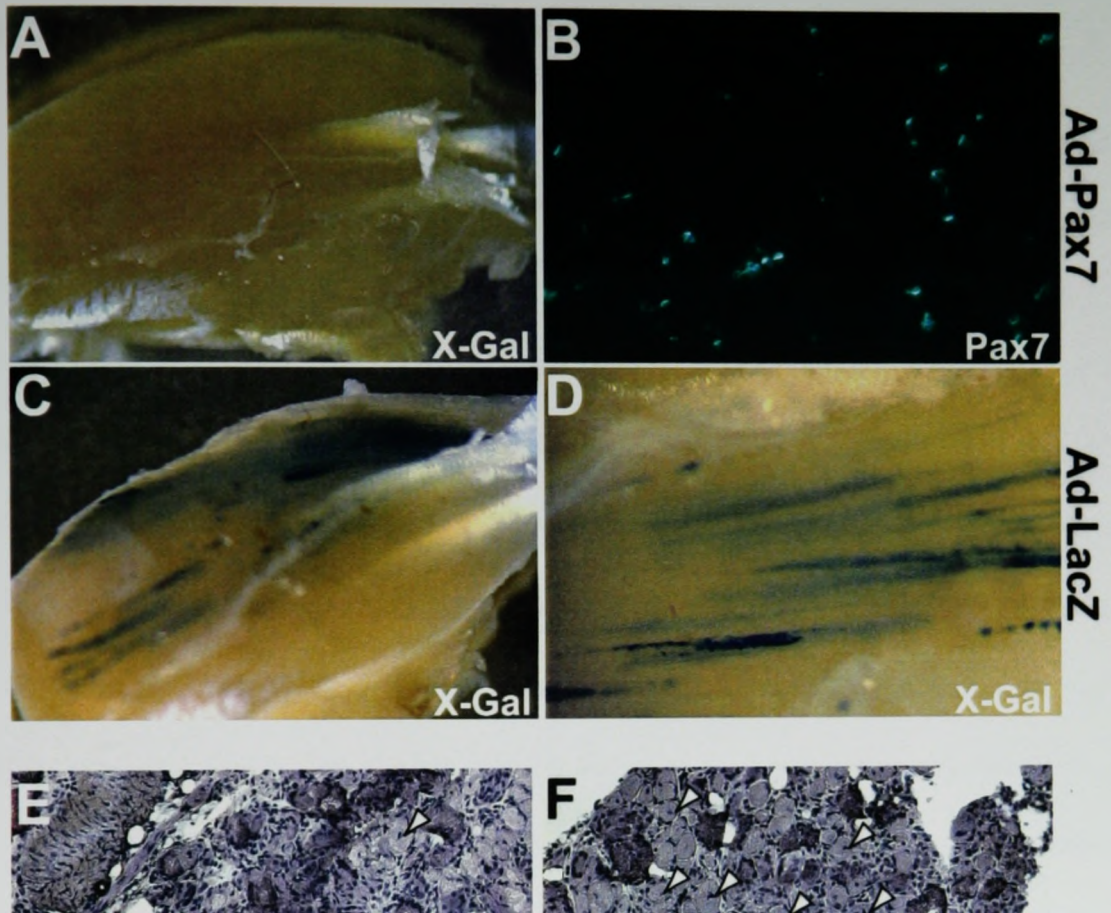
(**A**, **B**) Infection of muscle tissue with Adenovirus expressing Pax7 (Ad-Pax7) resulted in high level of Pax7 protein expression as demonstrated by immunohistochemistry with anti-Pax7 antibody (**B**). (**C**, **D**) Infection of muscle tissue with control Adenovirus-LacZ (Ad-LacZ) revealed widespread distribution of LacZ expression throughout the tibialis anterior (TA) muscle. (**E**, **F**) Infection of cardiotoxin damaged $Pax7^{-/-}$ muscles with Ad-Pax7 resulted in markedly improved muscle integrity and a significantly increased number of centrally nucleated regenerated fibers (**F**, arrowheads) relative to muscles treated with Ad-LacZ (**E**). (**F**) On average, Ad-Pax7 infection resulted in a 3.9+/-1 fold increase in the number of regenerated $Pax7^{-/-}$ myofibers.



Ad-LacZ Ad-Pax7

G




A
X-Gal
B
Pax7
Ad-Pax7
C
X-Gal
D
X-Gal
Ad-LacZ
E
F

## 6.4 Discussion

Endogenous $CD45^+$:$Sca1^+$ muscle derived stem cells undergo myogenic commitment in response to Wnt proteins in damaged muscle and appear to play an important role in the regeneration process (Chapter 5; Polesskaya et al., 2003). In this article, we demonstrate a requirement for Pax7 in the myogenic specification of CD45+ adult stem cells. Specifically, the absence of myogenic differentiation in $CD45^+$:$Sca1^+$ cells isolated from regenerating *Pax7*$^{-/-}$ muscle indicates that induction of Pax7 in these cells is necessary for their myogenic commitment (Figure 1). In addition, Pax7 induced myogenesis in "unstimulated" $CD45^+$:$Sca1^+$ cells from uninjured muscle, forming highly proliferative myoblasts that readily differentiated as multinucleated myotubes (Figures 2 and 3). Moreover, $CD45^+$:$Sca1^+$ cells ectopically expressing Pax7 (CDSC-Pax7) differentiated *in vivo*, efficiently contributing to the regeneration of dystrophic muscle (Figure 5). Expression of Pax7 *in vivo* using an adenovirus vector also enhanced myogenesis in regenerating *Pax7*$^{-/-}$ muscle. Taken together, these experiments demonstrate that Pax7 is sufficient to induce myogenic specification in adult stem cells. In addition, these results highlight the possibility of designing strategies to upregulate or ectopically express Pax7 in stem cells to treat muscle wasting diseases.

Several groups have recently reported the identification of adult stem cells in different tissues. The capacity for bone marrow-derived cells to undergo myogenesis and participate in muscle regeneration suggested the potential to deliver stem cells systemically to damaged muscles (Bittner et al., 1999; Corti et al., 2002; Ferrari et al., 1998; Gussoni et al., 1999; LaBarge and Blau, 2002). However the yield of donor cell

derived fibers after systemic cell transplantation is still too low to achieve clinical benefit (Ferrari et al., 2001; reviewed by Partridge, 2003). Clearly, the identification of signaling molecules and stimuli that promote the mobilization and activation of endogenous stem cells is needed to improve the efficiency of this phenomenon.

The absence of regeneration in muscle subjected to high doses of local irradiation (Heslop et al., 2000; Pagel and Partridge, 1999; Wakeford et al., 1991) strongly suggests that the stem cells responsible for regeneration are resident to muscle tissue and do not migrate in substantial numbers from the circulation. Nevertheless, it is possible that marrow derived cells home and take up residence in muscle tissue in a continuous manner. Interestingly, marrow cells have been demonstrated to give rise to functional satellite cells (LaBarge and Blau, 2002), suggesting that the adult satellite cell pool can be replenished from upstream stem cell compartments.

Several studies have documented the existence of adult stem cell populations distinct from satellite cells resident in skeletal muscle tissue (Asakura et al., 2002; Cao et al., 2003; Gussoni et al., 1999; Jackson et al., 1999; McKinney-Freeman et al., 2002; Qu-Petersen et al., 2002; Torrente et al., 2001). The developmental origin of these different populations and their biological relevance for maintaining tissue integrity and contributing to regeneration has remained unclear (reviewed by Partridge, 2003). Recently, we showed that endogenous $CD45^+$ muscle-derived cells are recruited to the myogenic lineage during regeneration (Chapter 5; Polesskaya et al., 2003). These results offer compelling evidence that adult stem cells resident to the tissue play a physiological role in adult myogenesis. Importantly, investigation of candidate signaling pathways in

regenerating muscle revealed that Wnts were sufficient to induce the myogenic activation of $CD45^+$ progenitors. In the current work we further demonstrate that induction of Pax7 is required for the myogenic specification of $CD45^+$ progenitors downstream of Wnt signals. Taken together, these observations suggest the hypothesis that Pax7 functions as a direct transcriptional target of the β-Catenin complex in Wnt stimulated adult stem cells.

This Pax7-dependent pathway operating in adult muscle stem cells appears analogous to the function of Pax3 in embryonic muscle precursors. In the early embryo, Pax3 is expressed in the presomitic mesoderm and immature epithelial somites prior to the onset of muscle specific gene expression (Goulding et al., 1994; Williams and Ordahl, 1994). Moreover, Pax3 has been demonstrated to function genetically upstream of MyoD in the formation of trunk and body wall muscle (Tajbakhsh et al., 1997). Consistent with a direct role for Pax3 in myogenic induction, ectopic Pax3 activates expression of MyoD in embryonic tissues (Bendall et al., 1999; Heanue et al., 1999; Maroto et al., 1997). Similarly, Pax3 is necessary and sufficient for MyoD activation in embryonal P19 cells (Ridgeway and Skerjanc, 2001). However, Pax3 also regulates cell survival in the presomitic mesoderm in areas that do not express Pax7 (Borycki et al., 1999) suggesting an indirect mechanism by which Pax3 may act genetically upstream of MyoD. The work of Borycki et al. suggests that selective survival of Pax3-expressing somitic progenitors is essential for myogenic specification. Our experiments in adult muscle do not rule out the possibility that Pax7 promotes the survival of CD45+ progenitors that are competent to

give rise to myogenic cells. Characterization of the downstream targets of Pax7 in CD45+ adult progenitors is required to directly address this issue.

In explanted embryonic tissues, signals from the floor plate and neural tube are required for induction of the MRFs (Cossu et al., 1996a; Munsterberg and Lassar, 1995; Pourquie et al., 1995; Pourquie et al., 1996). In particular, Wnt7a activates expression of MyoD in explanted paraxial mesoderm from 10.5 day old mouse embryos (Tajbakhsh et al., 1998). The requirement for Pax3 expression in a subset of somitic muscle precursors prior to the onset to MyoD expression suggests that Wnt signals may activate Pax3 and indirectly promote MRF expression (Borycki et al., 1999). An analogous requirement for Pax7 in the myogenic commitment of adult $CD45^+$ progenitors (Figure 1) strongly suggests a conserved hierarchy whereby Wnt signaling activates Pax3 or Pax7 expression upstream of the MRFs in somitic and adult muscle stem cells. In support of this hypothesis is the observed loss of Pax3 expression in P19 mesodermal precursors engineered to express a dominant negative form of the Wnt effector protein, β-Catenin (Petropoulos and Skerjanc, 2002). Further studies investigating the transcriptional targets of activated β-Catenin in stem cells undergoing myogenic commitment will reveal whether Wnts directly activate Pax3 or Pax7 gene expression.

A confounding result in our experiments was the inability for Pax7 to induce myogenesis in $CD45^+$:$Sca1^+$ cells recovered from *Pax7*$^{-/-}$ muscle (Figure 6). Several possible explanations may account for this observation. First, $CD45^+$:$Sca1^+$ muscle cells represent a heterogeneous cell population, as evidenced by their nonuniform response to stimuli such as myoblast coculture, Wnt proteins and ectopic expression of Pax7 (results

herein and Chapter 5). The profound growth deficit in *Pax7*$^{-/-}$ muscles (Chapter 4; Seale et al., 2000) suggests an altered microenvironment and cellular composition within the muscle. Previous analysis of muscle suspensions from *Pax7*$^{-/-}$ mice revealed a significantly increased number of hematopoietic progenitors and adipogenic cells (Chapters 3 & 4). We also observed altered proportions of CD45 and Sca1 expressing cells in uninjured (Figure 6A compared to Figure 2F) and regenerating muscle (Figure 1A). Therefore, the putative stem cell subpopulation that coexpresses CD45 and Sca1 may have been exhausted prematurely during postnatal muscle formation. It is also conceivable that a reduced proportion of stem cells in the *Pax7*$^{-/-}$ CD45$^{+}$:Sca1$^{+}$ muscle fractions were not detected in our assay due to a low efficiency of retroviral transduction (~10% of surviving CD45$^{+}$:Sca1$^{+}$ cells with GFP virus). Clearly, the identification of additional markers expressed by adult muscle-derived stem cells will be vital to the design of future studies.

Alternatively, adult stem cells may require additional signals to undergo myogenesis in response to Pax7. These inductive cues may be absent in *Pax7*$^{-/-}$ muscle due to an altered cellular milieu. Our experiments also revealed that the endogenous *Pax7* gene is upregulated during the myogenic specification of CD45$^{+}$:Sca1$^{+}$ cells (Figure 4B). Therefore, endogenous gene activity, possibly through the regulated expression of different isoforms (Kay and Ziman, 1999; Ziman and Kay, 1998), may be essential to the stability of myogenic commitment in stem cells. Future experiments addressing the functional differences between CD45$^{+}$:Sca1$^{+}$ cells in wildtype and *Pax7*-deficient muscle

provides a unique opportunity to gain a more complete understanding of the function and role of these cells during postnatal muscle development.

Although $CD45^+$ cells from *Pax7*$^{-/-}$ muscle were apparently unable to undergo myogenesis, ectopic Pax7 induced expression of Myf5 and myogenic specification in *Pax7*-deficient $CD45^-$:$Sca1^-$ cells (Figure 7). In these experiments, it is possible that Pax7 promoted the survival or expansion of committed myoblasts, however the absence of any myogenic cells in HAN-puro infected cultures after 10 days is strongly suggestive of *de novo* myogenesis in an undefined cell type. Moreover, Adenovirus-Pax7 significantly increased the *in vivo* regenerative capacity of *Pax7*$^{-/-}$ muscle (Figure 8). Taken together, these results confirm the presence of *Pax7*$^{-/-}$ muscle progenitors that require the activity of Pax7 to generate sufficient numbers of myoblasts for effective regeneration. Further studies will be required to molecularly characterize the responsive cells and their developmental relationship to other muscle stem cell populations.

The dominant expression of Myf5 in Pax7 infected $CD45^+$:$Sca1^+$ cells (CDSC-Pax7) (Figure 4A) suggests a paradigm wherein Pax7 preferentially activates Myf5. Specifically, *Myf5* mRNA is expressed at 6 fold higher levels in CDSC-Pax7 cells relative to primary satellite cell derived myoblasts (Affymetrix; data not shown). Interestingly, Pax3 has been specifically implicated in myogenesis upstream of MyoD (Tajbakhsh et al., 1997), suggesting that Pax3 and Pax7 specify distinct myogenic lineages through the preferential activation of MyoD and Myf5 respectively.

Several experimental observations have noted a role for Myf5 in promoting myoblast proliferation. For example homozygous *Myf5nLacZ*, (e.g. *Myf5*-deficient)

embryos display significantly reduced numbers of LacZ-expressing myogenic progenitors (Tajbakhsh et al., 1996). Moreover, in avian embryos, Myf5 is preferentially expressed in proliferating myoblasts, whereas MyoD appears to be upregulated in differentiating cells (Delfini et al., 2000). Furthermore, *Myf5*$^{-/-}$ satellite cell-derived myoblasts display a profound proliferation deficit (Montarras et al., 2000). The increased growth rate of CDSC-Pax7 cells is reminiscent of *MyoD*$^{-/-}$ myoblasts that also express elevated levels of Myf5 (Sabourin et al., 1999). Intriguingly, the rare myogenic cells recovered from adult *Pax7*$^{-/-}$ muscles express Pax3 and MyoD but fail to proliferate in culture (Chapter 4). Taken together, these observations raise the possibility that Pax7 activates expression of Myf5 to promote adult myoblast expansion whereas Pax3 preferentially induces MyoD. The activity of *MyoD* and *Myf5* regulatory elements in response to Pax3 and Pax7 will be the ultimate test of this hypothesis.

The requirement for Pax7 in the specification of muscle satellite cells (Chapter 3) and its induction during the myogenic recruitment of CD45$^{+}$ adult stem cells suggests a developmental relationship between CD45$^{+}$ adult muscle stem cells and satellite cells. We speculate that CD45$^{+}$:Sca1$^{+}$ cells can give rise to satellite cells by a Pax7-dependent mechanism in response to Wnt signals. This hypothesis is supported from the studies of LaBarge and Blau demonstrating that adult marrow stem cells first give rise to satellite cells before differentiating into muscle fibers (LaBarge and Blau, 2002). In the absence of *Pax7*, adult muscle-derived stem cells may preferentially undergo hematopoietic differentiation as suggested by the increased hematopoietic progenitor activity in *Pax7*$^{-/-}$ muscle (Chapter 3). Clonal studies assessing the plasticity of CD45$^{+}$:Sca1$^{+}$ cells are

required to determine whether these cells are indeed capable of both myogenic and hematopoietic differentiation. Interestingly, clonally derived stem cells from muscle reconstitute all the hematopoietic lineages and still retain their myogenic differentiation potential after stable and long-term marrow engraftment (Cao et al., 2003). Altogether, these experiments suggest that adult stem cell populations play an important physiological role in muscle tissue and may provide a renewable source for satellite cells.

In conclusion, our work establishes that Pax7 is necessary and sufficient for the myogenic specification of adult stem cells resident to muscle tissue. The proliferative and stable characteristics of primary CDSC-Pax7 cells and their efficient engraftment into dystrophic muscle suggests that approaches to deliver Pax7 to stem cells or upregulate its expression may be of therapeutic value for the treatment of degenerative muscle disease.

## 6.5 Materials and methods

### Mice

Mice carrying a targeted null mutation in *Pax7* (hereafter referred to as $Pax7^{-/-}$) were generously provided by Dr. Peter Gruss (Mansouri et al., 1996) and outbred into the SV129 background to increase survival as described before (Chapter 4). $Pax7^{+/+}$ or $Pax7^{+/-}$ littermates obtained from $Pax7^{+/-}$ intercrosses were used as wildtype mice in this study unless otherwise specified. *Myf5nLacZ* mice were generated and provided by Dr. S. Tajbakhsh (Tajbakhsh et al., 1996). *Mdx* mice were obtained from Jackson Labs. *Mdx:nu* mice were provided by Dr. T.A. Partridge (see Blaveri et al., 1999).

### Cell sorting

Mononuclear cells were recovered from uninjured hindlimb muscles or from ctx damaged TA muscles of $Pax7^{-/-}$, $Pax7^{+/-}$ and $Pax7^{-/-}$ mice. Muscle cells were recovered as described previously (Megeney et al., 1996). Mononuclear cells were washed twice with ice-cold DMEM supplemented with 5% FBS, passed through 30 μm filters (Miltenyi Biotec) and suspended at a concentration of 2-3 x $10^6$ cells/ml. Staining was performed for 30 min on ice using the antibodies: CD45-APC (30-F11), CD45.2-FITC (104), Sca1-PE or FITC, (D7) all from BD Pharmingen and CD45-TC (30-F11) from Caltag. Primary antibodies were diluted in cell suspensions at 1:200. After two washes with cold PBS supplemented with 2% FBS, cells were separated on a MoFlo cytometer (DakoCytomation), equipped with 3 lasers. Sort gates were strictly defined based on isotype control stained cells and single antibody staining. Dead cells and debris were

excluded by gating on forward and side scatter profiles. Sorting was performed using single cell mode to achieve the highest possible purity. The purity of sorted populations was routinely >98%.

**Retroviral and adenoviral gene expression**

Retrovirus was produced according to the 3-plasmid HIT system with plasmids pHIT60, pHIT456, and pHAN-puro as described elsewhere (Soneoka et al., 1995). pHIT60 encodes the MLV retroviral gag-pol, pHIT456 expresses an amphotrophic envelope protein and pHAN-puro is an expression vector with a hybrid CMV-5' LTR promoter driving production of the retroviral transcript. Pax7 or MyoD is translated from this full transcript, whereas the puromycin resistance marker is expressed following retroviral integration from a shorter transcript produced by the SV40 early promoter located 3' to the multiple cloning site. Transient cotransfection of all three plasmids into 293FT cells (Invitrogen) by calcium phosphate method (Graham and van der Eb, 1973) routinely produced viral titres between $10^6$ and $10^7$ cfu per ml/supernatant as assayed by puromycin selection. pHAN-EGFP or empty pHAN-puro was used to produce puromycin-resistant virus for controls. Retrovirus-containing medium was harvested after 48 hours and filtered through a 0.45um filter (Millipore).

Purified $CD45^+$:$Sca1^+$ (CDSC) or $CD45^-$:$Sca1^-$ cells were spun down, counted, and 20-50, 000 cells were then cultured overnight on collagen coated 4 well chamber slides in HAM's F10 medium (Invitrogen) supplemented with 20% FBS, antibiotics and 10 ng/ml stem cell factor (R&D systems). The following day, cells were incubated for 6

hr with retrovirus at a 1:1 ratio (complete medium: retrovirus supernatant) with the addition of 8 μg/ml polybrene (hexadimethrine bromide; Sigma). After infection, cells were rinsed twice with PBS and all cells were replated in myoblast growth medium (as described below). After 48 hours, infected pools were selected in 1 μg/ml puromycin (Sigma) to establish stable CDSC-Pax7 lines. CDSC cells infected with puro displayed enlarged spread out processes, did not proliferate and usually died within 5-7 days after infection. C3H10T1/2 cells were incubated overnight with MyoD, Pax7 or puro virus and 8 μg/ml polybrene.

Adenovirus (type V) was prepared using the Ad-Max adenovirus creation kit that uses frt/FLP technology for the recombination (Microbix Biosystems). Briefly, mouse Pax7 was cloned into pDC516 downstream of the murine CMV promoter. pDC516-Pax7 was cotransfected with the adenovirus genome plasmid, pBHGfrtΔE1,E3FLP, into HEK 293 cells by calcium phosphate method (Graham and van der Eb, 1973) to rescue recombinant adenovirus. Agarose overlays were used to identify plaques (cell lysis) produced by infectious virus. Restriction digest analysis of adenovirus DNA isolated from cored plaques confirmed the correct viral genome structure. Western analysis of infected C3H10T1/2 cultures with Pax7 antibody confirmed high level Pax7 expression. Virus was propagated during 3 rounds of infection in 293 cells prior to scaling up for large scale purification. Adenovirus was purified in CsCl gradients by centrifugation, dialyzed against sterile PBS and frozen down in 15% glycerol at -80°C. Titres of purified adenovirus were determined by plaque assays on 293 cells and were always above $10^{10}$ pfu/ml.

**Western blot analyses**

Cell cultures were lysed in RIPA extraction buffer (50mM Tris-HCl pH 7.4, 1% Nonidet P-40, 0.5% NaDeoxycholate, 0.1% Sodium-dodecyl-sulphate, 5 mM EDTA, 150 mM NaCl, 50 mM NaF) supplemented with protease inhibitors (Complet, Roche). The extracts were normalized for protein content using Bio-Rad dye. 40 μg of lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose filters. Filters were probed with antibodies to Pax7 (Developmental Studies Hybridoma Bank [DSHB]); Myf5, 1:1000 (C-20, Santa-Cruz Biotechnology), MyoD, 1:1000 (C-20, Santa-Cruz Biotechnology), myogenin (F5D, DSHB), and α-tubulin, 1:2000 (T 9026, Sigma). Secondary detection was performed with horseradish peroxidase-conjugated antibodies (BioRad). Protein expression was visualized using the ECL Plus kit (Amersham).

**Cardiotoxin-induced regeneration and *in vivo* adenovirus infections**

Four to six week old *Pax7*$^{-/-}$ and wildtype littermates were anesthetized with Halothane gas. 25 μl of 10 μM cardiotoxin (ctx) (Latoxan, France) was injected into the midbelly of the TA muscle, using a 29½ G insulin syringe. Mice were sacrificed at 4 days or two weeks after ctx injection for regeneration assays. For adenovirus infections, 25 μl of sterile PBS containing $10^8$ particles of purified adenovius-Pax7 or -LacZ was injected 2 days after ctx injection into damaged TA muscles with a 29½ G insulin syringe. All procedures with adenovirus were performed under a laminar flow hood and

animals were regularly monitored for adverse reaction. Animals were sacrificed 14 days after ctx injections for analysis of muscle regeneration.

**Histology and immunocytochemistry**

For analysis of regeneration and enumeration of regenerated myofibers, TA muscles were isolated, embedded in OCT (Tissue-Tek)/20% sucrose and immediately frozen in liquid nitrogen. 10 μm cryosections (cross-sections) from the TA midbelly at the site of ctx injection were stained with Hematoxylin and Eosin (H&E). Regenerating fibers were further identified by immunostaining with an antibody specific to Desmin, 1:200 (D33, DAKO) or to embryonic fast MyHC (F1.652, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, USA) followed by secondary detection with anti-mouse FITC conjugated antibody, 1:200 (Chemicon). Sections were analyzed on an axioplan 2 Zeiss microscope. Central myonuclei in regenerating muscles were counted on at least 2 independent cross-sections of the entire TA muscle per mouse analyzed.

**Cell cultures**

Primary satellite cell-derived myoblasts were established from purified $CD45^-$ :$Sca1^-$ fractions of hindlimb muscle of 4-6 week old *Pax7*+/+ or *Pax7*+/- mice. Myoblasts and CDSC-Pax7 cells were maintained in HAM's F-10 medium (Invitrogen) supplemented with 20% FBS, and 2.5 ng/ml bFGF (Invitrogen) on collagen coated dishes. CDSC-Pax7 cells and primary satellite cell derived myoblasts were induced to

differentiate for 1-3 days in DMEM supplemented with 5% Horse-serum. *MyoD*$^{-/-}$ myoblasts used as a control sample in Northern blot analysis were derived in previous studies (Sabourin et al., 1999). C3H10T1/2 and HEK 293 cells were obtained from the ATCC and maintained in DMEM supplemented with 10% FBS.

For immunohistochemical analysis, cell cultures were fixed with 4% paraformaldehyde, non-specific antigens were blocked in 5% horse serum/PBS and cells were reacted with primary antibodies as follows: Desmin, 1:200 (DAKO); MyoD, 1:200 (5.8A, BD Pharmingen); all MyHC (MF-20, DSHB); Myf5, 1:1000 (C-20, Santa-Cruz Biotechnology); Pax7 (DSHB) and myogenin (F5D, DSHB). Secondary detection was performed using Fluorescein or Rhodamine conjugated antibodies, 1:200 (Chemicon). *Myf5nLacZ* expression was detected by X-Gal reaction as described previously (Polesskaya et al., 2003). Cells were reacted with the nuclear dye DAPI (Sigma) before mounting and analysis on an axioplan 2 Zeiss microscope.

**Cell Transplantation**

Primary CDSC-Pax7 cells cultured in myoblast conditions were trypsinized, washed twice with PBS and suspended at $5x10^5$ cells/25 µl in sterile PBS for cell transplantation. Cells were injected directly into the TA midbelly of 4-6 week old *mdx:nude* mice. Mice were sacrificed 2 months after cell injections to analyze the myogenic contribution of transplanted cells. Myofibers generated from transplanted cells were detected in *dystrophin*-deficient host muscles by immunostaining with an antibody

specific to dystrophin, 1:500 (Sigma) and Pax7 (DSHB) followed by secondary detection with FITC or peroxidase conjugated anti-mouse antibodies (Chemicon).

**RT-PCR and Northern analysis**

Total RNA was extracted using RNAeasy kits (Qiagen), according to manufacturer's instructions. RT-PCR analysis for endogenous *Pax7* mRNA was performed using the GeneAmp PCR Core kit (Perkin-Elmer). RT-PCR using 1 µg of total RNA was conducted as per manufacturer's instructions with the following modifications. cDNA synthesis was extended for 1 hour at 42°C, and 5 µl of the first-strand RT product was used for PCR amplification. PCR conditions for *Pax7* were 94°C-5 min; 35 cycles of (94°C-45 sec; 56°C-45 sec; 72°C-45 sec); 72'C-7min. The PCR primers span intron 8 of the *Pax7* gene (Pax7-exon8-fwd 5' gct acc agt aca gcc agt atg 3' and Pax7-exon9-rev 5' gtc act aag cat ggg tag atg 3') and amplify sequence in the 3'-UTR of the gene that is not contained in the viral Pax7 expression cassette. RT-PCR products were analyzed by electrophoresis through a TAE-ethidium-agarose gel.

Northern blot studies were performed according to standard techniques using random-primed $^{32}$P-dCTP radiolabelled cDNA fragments as probes (Redi-prime, Amersham) (Maniatis et al., 1982) 15 µg of total RNA from various cell cultures was electrophoresed in denaturing-Formaldehyde gels and transferred to Hybond-N filters (Amersham Bioscience).

**Affymetrix expression profiling**

Gene expression profiling of primary CDSC-Pax7 and wildtype satellite cell-derived myoblasts purified from the same animals was performed at the Ottawa Genome Center. RNA was prepared from proliferating CDSC-Pax7 cells and satellite cell derived myoblasts using Qiagen Rneasy mini kits according to manufacturer's protocol. Biotin-labeled cRNA were synthesized, fragmented, and hybridized to Murine Genome U74Av2 chips (Affymetrix). Primary data and comparison analysis was performed using Affymetrix Microarray Suite 4.0.

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# Chapter 7

## Summary and conclusions

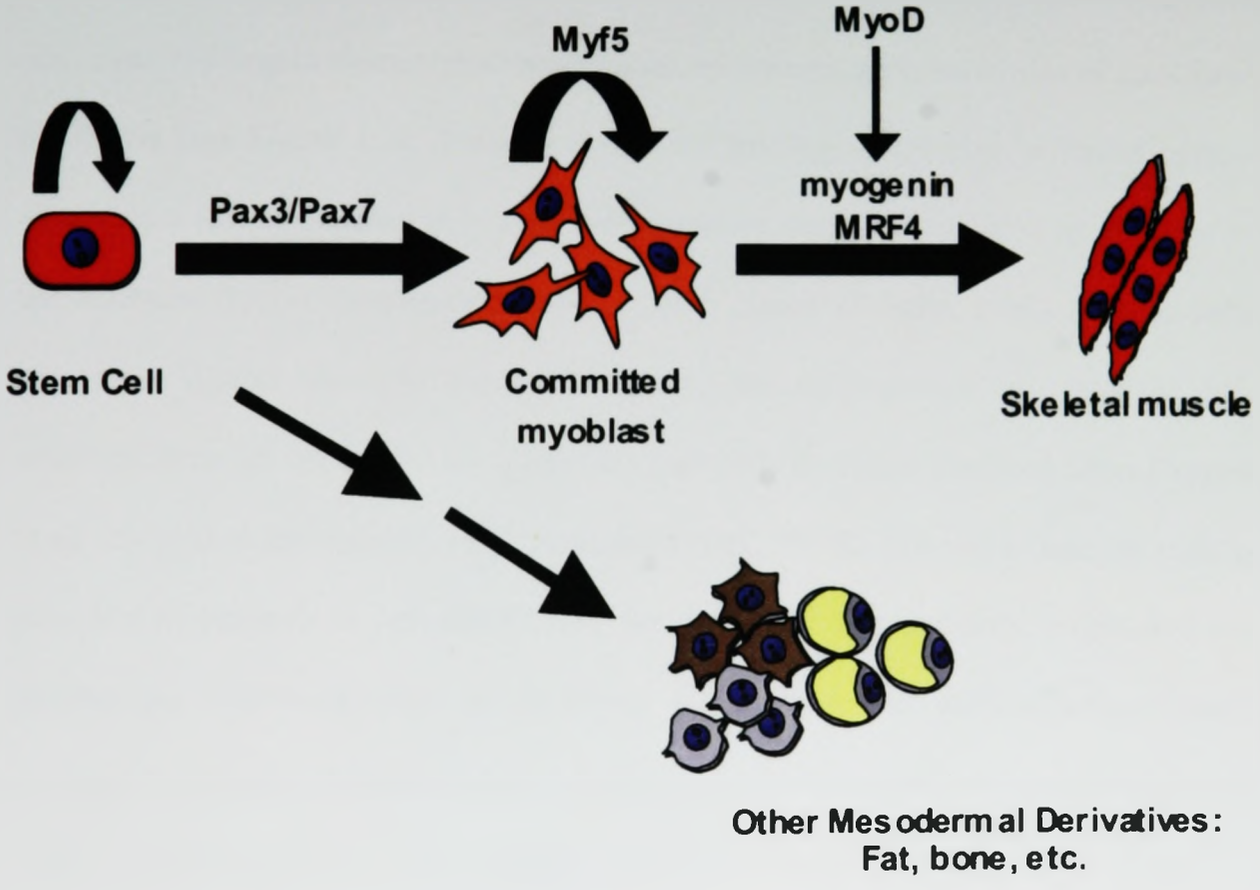
Skeletal muscle is an ideal model system to investigate molecular events involved in stem cell commitment, self-renewal and differentiation. Skeletal myogenesis involves at least two distinct developmental transitions. First, mesodermal stem cells commit to the myogenic lineage and give rise to proliferative myoblasts that express muscle-specific transcription factors such as MyoD and Myf5. Myoblasts then exit the cell-cycle, elongate and fuse with one another to form multinucleated myotubes (Figure 7.1). From many years of research, we have a sophisticated understanding of the basic mechanisms underlying terminal myocyte differentiation; however the biological processes involved in myogenic specification are less well defined. An interesting theme arising from recent studies is the observation that myogenic signaling pathways implicated in the specification of embryonic progenitors are similarly activated for the myogenic commitment of adult stem cell populations.

### 7.1 Postnatal myogenesis: growth and regeneration

A specialized and distinct lineage of progenitors called muscle satellite cells were traditionally thought to be the only myogenic cells with proliferative capacity in postnatal muscle. During the late stages of fetal development and after birth, muscle satellite cells are mitotically active, giving rise to committed myoblasts that fuse with existing fibers (Kelly, 1978; Moss and Leblond, 1971; Schultz, 1996). The number of fibers within each

**Figure 7.1 Myogenic specification and differentiation**

The formation of specialized muscle cells requires the initial commitment of mesodermal stem cells to committed myoblasts in a process that involves expression of Pax3 or Pax7. Committed myoblasts expressing Myf5 proliferate and upregulate MyoD at the onset of myogenic differentiation. Myogenin is required for the terminal differentiation of myoblasts to form multinucleated myotubes.


Myf5
MyoD
Pax3/Pax7
myogenin
MRF4
Stem Cell
Committed
myoblast
Skeletal muscle
Other Mesodermal Derivatives:
Fat, bone, etc.

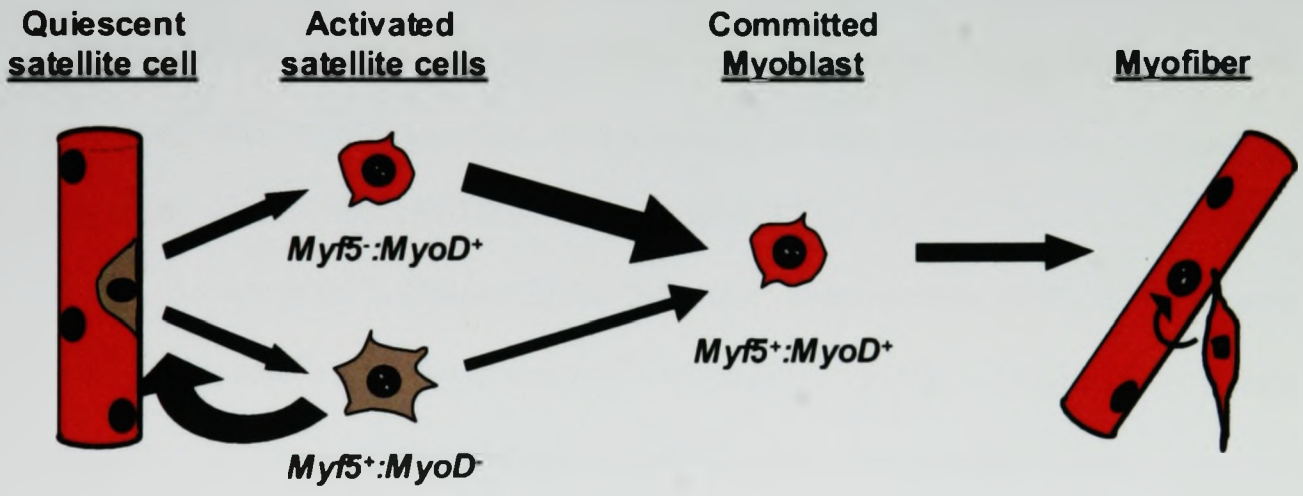
muscle group is already established around the time of birth prior to the peak activity of satellite cells (Ontell et al., 1988; Ontell and Kozeka, 1984a; Ontell and Kozeka, 1984b; Ross et al., 1987). Therefore, postnatal muscle growth occurs by a hypertrophic mechanism that involves satellite cell fusion. The proportion of satellite cells declines from about 30% of sublaminar muscle nuclei in newborn mice to less than 5% in adult muscle (Bischoff, 1994; Schultz, 1989; Schultz, 1996). In adult muscle, satellite cells are quiescent, residing in characteristic depressions underneath the basal lamina of associated myofibers (see Figure 1.3). Tritiated thymidine labeling of growing postnatal muscle identified a slowly dividing myogenic subpopulation that was suggested to give rise to the quiescent reserve satellite cells found in adult muscle (Schultz, 1996). Satellite cells respond to injuries caused by exercise, stretching, disease processes, etc., enter the cell-cycle and generate committed myogenic daughter cells to replace damaged fibers (Appell et al., 1988; Darr and Schultz, 1987; Rosenblatt et al., 1994). The role of satellite cells in postnatal myogenesis is well established, however their developmental origin, and the mechanisms regulating their commitment, self-renewal and differentiation remain unclear. In addition, it remains to be determined whether satellite cells behave in the same way during postnatal muscle growth as in regenerative myogenesis.

## 7.2 Identifying satellite specific markers

To develop our understanding of adult satellite cell function, the first goal of this research project was to identify genes expressed specifically in the satellite cell lineage. Ongoing analysis of *MyoD*$^{-/-}$ satellite cells suggested an increased capacity for self-

**Figure 7.2 Role for Myf5 in satellite cell self-renewal**

Quiescent satellite cells upregulate either *Myf5* or *MyoD* prior to entering a developmental stage in which both genes are expressed. The phenotype of *MyoD*$^{-/-}$ satellite cells suggests that activated satellite cells that express Myf5 but not MyoD (e.g. Myf5+:MyoD-) undergo preferential self-renewal to repopulate the satellite cell compartment. MyoD+:Myf5- cells undergo myogenic commitment and differentiation.


Quiescent satellite cell
Activated satellite cells
Committed Myoblast
Myofiber
Myf5⁻:MyoD⁺
Myf5⁺:MyoD⁺
Myf5⁺:MyoD⁻

renewal at the expense of myogenic commitment and differentiation (Cornelison et al., 2000; Megeney et al., 1996; Sabourin et al., 1999; Yablonka-Reuveni et al., 1999a). Interestingly, quiescent satellite cells on isolated myofibers express either *MyoD* or *Myf5* upon activation (Cornelison and Wold, 1997). Further experiments demonstrated the presence of three distinct subsets of myogenic precursor cells *in vivo* that expressed MyoD, Myf5 or both factors (Cooper et al., 1999). Taken together, these studies suggested the hypothesis that satellite cells that express either Myf5 or MyoD are functionally distinct. Together these studies suggested a model in which MyoD-:Myf5+ activated satellite cells preferentially self-renew, whereas MyoD+:Myf5- cells undergo myogenic commitment and differentiation (Figure 7.2).

As described in Chapter 2, the "primitive" characteristics of *MyoD*$^{-/-}$ cells were exploited to identify genes expressed by satellite cells. Notably, our analysis uncovered a number of known satellite cell markers as well as many novel genes that were expressed by satellite cells. The expression of satellite cell specific genes in *MyoD*$^{-/-}$ cells supports the hypothesis that *MyoD*$^{-/-}$ cells model a transient cell type intermediate between a quiescent satellite cell and a determined myoblast. Furthermore, the expression of genes specific to other lineages such as *Neuritin/Nestin* (neurons) and hematopoietic markers such as *IgSF4*, and *T-cell-receptor-β* (see Table II-Chapter 2 for full description of genes) suggests that *MyoD*$^{-/-}$ cells are less committed to the myogenic pathway compared to their wildtype counterparts. Interestingly, transplanted *MyoD*$^{-/-}$ cells appear to give rise to satellite cells *in vivo* (Chapter 1, Figure 1; B. Kablar and M.A. Rudnicki, unpublished data), further demonstrating the utility of *MyoD*$^{-/-}$ cells as an *in vitro* model to study

genetic pathways operating in satellite cells. Future experiments characterizing the function of genes expressed by *MyoD*$^{-/-}$ cells identified in this work will be extremely valuable for elucidating mechanisms that regulate satellite cell activation and self-renewal.

### 7.3 A hematopoietic/endothelial origin for satellite cells

The expression of many genes in *MyoD*$^{-/-}$ cells that are also expressed by hematopoietic and endothelial cells such as *VCAM1*, *PlGF*, *Cadherin6*, *E25*, *Laminin-α5*, etc. (see Table II, Chapter 2 for complete listing and references) suggests a developmental relationship between satellite cells and hematopoietic/endothelial lineages. The developmental origin of satellite cells remains a matter of contention. Early studies with quail-chick chimeras concluded that satellite cells were derived from the somite (Armand et al., 1983). However a study by DeAngelis et al. challenged this widely held view and suggested embryonic blood vessels as the source of satellite cells (De Angelis et al., 1999). Specifically, the isolation of myogenic precursors resembling satellite cells in embryonic dorsal aorta explants but not in somitic explants suggests that satellite cells are derived from stem cells associated with blood vessels. Moreover, satellite cell like clones were recovered from the limbs of *c-met* null embryos that do not contain somite derived muscle. Based on this study, the authors developed the hypothesis that satellite cells are formed from vessel-derived cells as vascular structures migrate into muscle forming areas. Interestingly, further characterization of this cell population, now termed meso-

angioblasts, revealed that they possess multipotent mesenchymal differentiation plasticity (Minasi et al., 2002).

With the identification of novel satellite cell markers such as Pax7 (discussed later), IgSF4, and Hoxc10, it will be of interest to examine their expression during the late stages of embryonic development. The expression pattern of these genes may prove extremely useful for investigating the developmental origin of satellite cells.

Transcriptional profiling of wildtype satellite cell-derived myoblasts was also performed to identify genes that define the identity of committed myogenic precursors (see Chapter 2). Expression analysis identified only three genes in this screen that were not upregulated during myogenic differentiation, *Pb99*, *Asb2* and *Pax7* (see Chapter 2, Table 1). The expression patterns of these genes were specific to myogenic cells and muscle tissue implying an important role in establishing or maintaining the myoblastic phenotype.

### 7.4 Pax7 is required for the development of the satellite cell lineage

Pax7 is a member of the paired-box family of developmental control transcription factors that includes nine members, each regulating the development of specific cell lineages (Figure 7.3). Strikingly, mutations in five of the nine *Pax* genes are associated with human diseases illustrating the essential role of *Pax* genes in normal development (reviewed by Chi and Epstein, 2002). Pax proteins are categorized into four subclasses based on their primary protein structure. Pax7 and the closely related Pax3 proteins contain two separate DNA binding domains- the paired-box and homeodomain- as well as a conserved octapeptide sequence (Figure 7.3). Pax7 and Pax3 bind identical DNA

**Figure 7.3 Summary of the mammalian *Pax* gene family**

The nine Pax genes are divided into four subgroups based on their primary protein structure. The paired-box domain, octapeptide (OP) and homeodomain are shown. The percentages indicate the level of amino-acid sequence identity between individual proteins in each subgroup. The naturally occurring or targeted mouse mutations are indicated together with human conditions resulting from loss of function mutations in *Pax* genes. Pax genes are implicated in the development of diverse cell lineages. Pax3 deficiency (splotch) results in neural tube defects, craniofacial and skeletal abnormalities and the absence of hypaxial limb muscles (Epstein et al., 1991; Franz et al., 1993; Tassabehji et al., 1994; Tremblay et al., 1998). *Pax7*$^{-/-}$ mice display defective neural crest derivatives (Mansouri et al., 1996) and lack muscle satellite cells (work described herein). Pax4 is required during pancreas development (Sosa-Pineda et al., 1997). Pax6 is required for the development of several tissues including eyes, central nervous system (CNS) and endocrine glands (reviewed by Simpson and Price, 2002). Pax2 plays a role in kidney, eye, ear and brain development (Keller et al., 1994; Torres et al., 1995). Pax5 is required for B-cell development (Urbanek et al., 1994). Pax8 is required for thyroid development (Macchia et al., 1998; Mansouri et al., 1998). Pax1 is required for the formation of the sclerotomal structures including the vertebral column, sternum and scapula (Wilm et al., 1998). Pax9 is involved in the development of teeth and pharyngeal pouch derivatives (Peters et al., 1998; Stockton et al., 2000). (Figure was adapted from (Underhill, 2000)).

| | Mutants | | |
|---|---|---|---|
| | **Mouse** | **Human** | **Developmental role** |
| Pax3 Pax7 | *Splotch* | Waardenburg | Neural tube/muscle |
| | Pax7 ko | | Neural crest/satellite cells |
| Pax4 Pax6 | Pax4 ko | | Pancreas development |
| | *Sey* | Aniridia | Eye specification, CNS, pancreas |
| Pax2 Pax5 Pax8 | *Kdr*, | Renal coloboma | Kidney, eye, ear, brain |
| | Pax5 ko | | B-cell development |
| | Pax8 ko | Hypothyroidism | Thyroid development |
| Pax1 Pax9 | *Undulated* | | vertebrae, scapula, sternum |
| | Pax9 ko | Oligodontia | Teeth, pharyngeal pouch derivatives |

target sequences suggesting that they regulate similar sets of target genes (Schafer et al., 1994). Furthermore, gain of function mutations in both Pax3 and Pax7 are associated with the development of alveolar rhabdomyosarcomas, indicating that both molecules regulate similar activities in muscle cells (Bennicelli et al., 1999).

Pax7 was specifically expressed in cultured myoblasts, as well as quiescent and activated muscle satellite cells *in vivo* (Chapter 3, 4). Importantly, the absence of muscle satellite cells in *Pax7*$^{-/-}$ muscles (Chapter 3, 4) reveals a novel role for Pax7 in the specification of adult myogenic progenitors. The normal development of embryonic and fetal muscle in the absence of Pax7 reinforces the assertion that embryonic and adult muscle progenitors develop independently. Moreover, Pax7 is an extremely specific marker for satellite cells in adult muscle and has become the "gold-standard" for identifying satellite cells. As such, the development of transgenic mice expressing GFP from the *Pax7* locus will provide a powerful research tool to rapidly purify Pax7 expressing myogenic cells by flow cytometry. A Pax7 reporter mouse may also serve to define the embryonic precursors that lie upstream of satellite cells.

### 7.5 Impaired regeneration in *Pax7*-deficient muscle

The increased survival of outbred *Pax7*$^{-/-}$ animals allowed a more detailed analysis of the adult muscle phenotype. As demonstrated in chapter 4, adult *Pax7*$^{-/-}$ displayed severe spinal kyphosis due to weakened back muscles (Figure 1, Chapter 4). Moreover, *Pax7*$^{-/-}$ muscle fibers were lost as a function of normal wear and tear with increasing age. In addition, *Pax7*-deficient muscles were incapable of functional regeneration following experimentally induced cardiotoxin or crush-injury (Figures 2 and

3, Chapter 4). These experiments confirm the essential role of satellite cells in the maintenance and regeneration of muscle tissue.

The recovery of small numbers of myogenic progenitors from *Pax7*$^{-/-}$ muscle that coexpressed Pax3 and MyoD suggests that these cells represent a separate Pax7-independent adult myogenic lineage (Figure 7, Chapter 4). Alternatively, Pax3 expressing cells may normally upregulate Pax7 during regenerative responses. Interestingly, Buckingham and coworkers have demonstrated that nLacZ is expressed from the *Pax3* gene locus in satellite cells of specific muscles (Buckingham et al., 2003). Further experiments are required to address the origin and characteristics of these Pax3 expressing cells. The inability for Pax3 to compensate for the absence of Pax7 in these cells is of particular interest, given the high degree of homology, and similar *in vitro* DNA binding activities of these proteins (Bennicelli et al., 1999; Schafer et al., 1994). The identification of Pax3 and Pax7 target genes in myogenic cells will be important for elucidating their respective roles.

## 7.6 The myogenic potential of adult stem cells

The presence of normal numbers of muSP cells in *Pax7*$^{-/-}$ muscles demonstrates that muSP cells and satellite cells represent distinct entities in adult muscle (Figure 6, Chapter 3). Further studies demonstrated that muSP cells require cell-cell contact with myoblasts to undergo myogenic differentiation (Asakura et al., 2002). The robust hematopoietic progenitor activity in *Pax7*$^{-/-}$ muscle suspensions suggested the hypothesis that Pax7 functions to restrict the development of alternative lineages in adult stem cells.

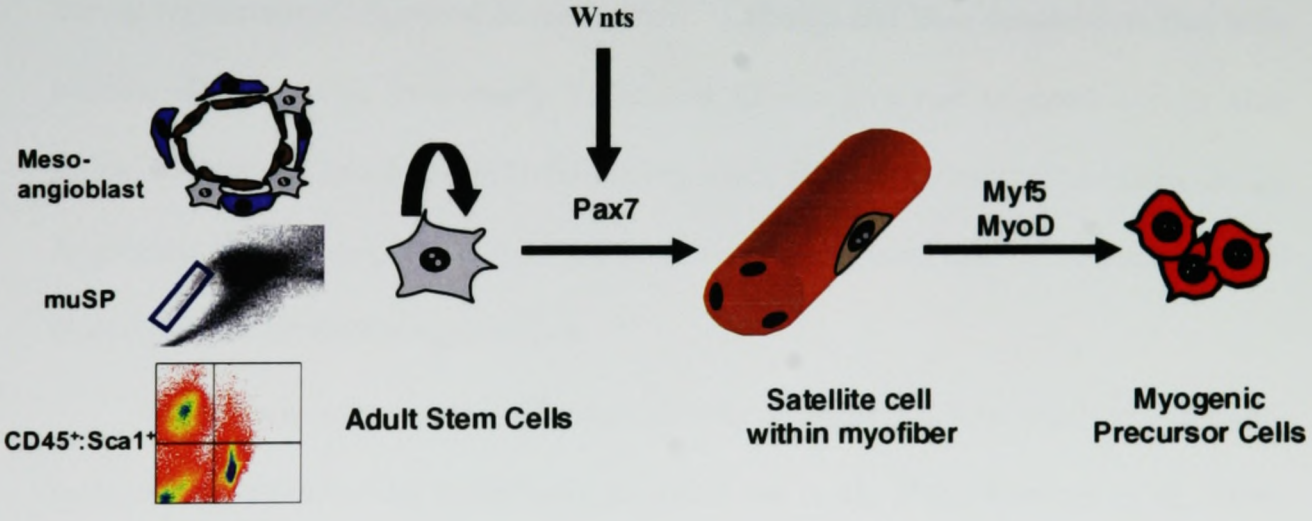
Analogous mechanisms direct the specification and reinforce the Pax5-dependent commitment of B-lymphocytes (Nutt et al., 1999). By this model, induction of Pax7 in adult stem cells is required for the myogenic specification of adult stem cell populations.

Notably, the ectopic expression of Pax7 was sufficient to induce the myogenic specification of $CD45^+$ progenitors and muSP derived from skeletal muscle (Figure 2, Chapter 6). Moreover, Pax7 was required for the myogenic differentiation of $CD45^+$ cells in regenerating muscle (Figure 1, Chapter 6). These results are consistent with the hypothesis that adult stem cells give rise to satellite cells upon upregulation of Pax7 (Figure 7.4). The role of Pax7 in committing adult stem cells to the myogenic lineage appears analogous to the function of Pax3 in embryonic progenitors (Maroto et al., 1997). Significantly, several Pax genes appear to activate complex developmental programs upon ectopic expression in specific cell types. For example, ectopic expression of Pax4 induces pancreatic β-cell differentiation in embryonic stem cell cultures (Blyszczuk et al., 2003) and ectopic Pax6 is sufficient to induce eye specification in certain embryonic tissues (Desplan, 1997).

The myogenic activation of $CD45^+$ cells in regenerating muscle in response to Wnt signals (Figure 1, Chapter 5) demonstrates a previously unrecognized role for endogenous stem cells in adult myogenesis. The expression of the hematopoietic restricted CD45 marker on these progenitors suggests that these cells change their fate from a hematopoietic-type cell to muscle in response to Wnt (Chapter 5) and expression of Pax7 (Chapter 6). Of particular significance is the finding that signaling pathways previously demonstrated to induce embryonic muscle development (Cossu and Borello,

**Figure 7.4 Wnts activate Pax7-dependent myogenesis in adult stem cells**

Adult stem cells, for example meso-angioblasts, muSP cells or fractionated $CD45^{+}$:$Sca1^{+}$ cells, upregulate Pax7 in response to Wnt signaling. Pax7 induces the myogenic commitment of adult stem cells which are then hypothesized to give rise to muscle satellite cells before generating proliferating myoblasts that express Myf5 and MyoD.


Wnts
Meso-
angioblast
muSP
CD45+:Sca1+
Pax7
Adult Stem Cells
Satellite cell
within myofiber
Myf5
MyoD
Myogenic
Precursor Cells

1999; Cossu et al., 1996a; Tajbakhsh et al., 1998; Tajbakhsh and Cossu, 1997) are also activated during adult muscle repair (Chapter 6). Importantly, these results also suggest that pharmacological upregulation of Wnt signaling may be clinically viable for treating cachexia and neuromuscular disease.

The absence of regeneration in muscles subjected to high doses of local radiation argues that the stem cells responsible for muscle repair reside within the tissue (Heslop et al., 2000). However, it is possible that $CD45^+$ adult stem cells that give rise to muscle during regeneration originated in the marrow. LaBarge and Blau demonstrate that bone marrow derived cells, presumably expressing CD45, give rise to satellite cells after stable, long term marrow reconstitution in irradiated recipients (LaBarge and Blau, 2002). Regardless of their origin, these experiments establish a novel role for non-satellite cell derived stem cells during regeneration.

muSP and marrow derived adult stem cells appear to form satellite cells after systemic or intramuscular transplantation (Asakura et al., 2002; Gussoni et al., 1999; LaBarge and Blau, 2002) indicating that under some circumstances adult stem cell populations can be integrated into muscle as satellite cells. The satellite cell forming activity of adult stem cells however appears to be extremely inefficient and it is possible that transplanted donor cells only sporadically give rise to satellite cells. It will also be important to demonstrate that the formation of satellite cells does not occur as a result of cell fusion (Wang et al., 2003; Ying et al., 2002). A more compelling physiological demonstration of this phenomenon is thus awaited. In principle, if marrow derived cells give rise to satellite cells as part of normal development, newborn mice reconstituted with

GFP or LacZ marked marrow should yield a high proportion of labeled myoblasts and myofibers over a relatively short interval.

Fate mapping experiments using Cre-loxP technology is a powerful approach to address many of these unanswered questions. The generation of mice expressing Cre-recombinase from promoters activated in different adult stem cell populations could be employed for these analyses. For example CD45-cre mice could be interbred with LacZ reporter mice to mark all progeny arising from CD45 (e.g. Cre-recombinase) expressing cells. If $CD45^+$ adult stem cells provide a significant input to the satellite cell compartment in adult muscle, LacZ staining should be observed in most adult muscle fibers. The appearance of LacZ expression in muscle only after acute injuries would indicate that $CD45^+$ adult stem cells constitute a reserve stem cell population awakened only in response to extreme damage.

### 7.7 Satellite cell self renewal

The stability of satellite cell numbers in old muscle subjected to repeated cycles of degeneration and regeneration has been interpreted to indicate that satellite cells possess an intrinsic capacity for self-renewal divisions (Bischoff, 1994). The mechanism for the maintenance of satellite cell numbers into old age remains unclear but may, under some circumstances, involve the myogenic specification of upstream adult stem cells. Alternatively, differential activation of Myf5 and MyoD may be implicated in balancing self-renewal versus differentiation in activated satellite cells as discussed previously. A central issue to the biology of satellite cells is whether they are mostly derived during

fetal development or whether other adult stem cells function normally to replenish this compartment.

Interestingly, a recent study demonstrates that satellite cells express the machinery that is used in the *drosophila* and mammalian central nervous system for asymmetric cell divisions (Dooley et al., 2003; Rath et al., 2002; Shen et al., 2002). Specifically, Conboy et al. report that satellite cells segregate Numb protein differentially in activated progeny (Conboy and Rando, 2002). Their studies suggest that activated Notch1 is associated with the generation of self-renewing cells that express Pax3 but not Numb, Desmin, Myf5 or MyoD. By contrast, inhibition of Notch promotes myogenic commitment as evidenced by expression of Numb, Desmin and Myf5. Further experiments are required to determine whether Numb+ and Numb- satellite cell progeny exhibit functional differences *in vivo*. Taken together, the mechanism responsible for maintenance of satellite cell numbers in adult muscle remains unclear and may involve a combination of the strategies discussed here.

### 7.8 Pax3 and Pax7 regulate independent muscle lineages

The high degree of sequence homology between Pax3 and Pax7 (Figure 7.3) and their similar DNA binding activity, suggests overlapping functions for these proteins in myogenesis. However, a number of experimental observations suggest that Pax3 and Pax7 specify distinct myogenic cell lineages via the preferential activation of MyoD and Myf5 respectively.

First of all, the absence of body muscles in compound mutant *Splotch:Myf5*$^{-/-}$ embryos places Pax3 together with Myf5 in a genetic hierarchy upstream of *MyoD*. In addition, the ectopic expression of Pax3 in mesodermal tissue activates expression of *MyoD* (Bendall et al., 1999; Heanue et al., 1999; Maroto et al., 1997). By contrast, in our experiments ectopic expression of Pax7 in CD45$^{+}$ progenitors or muSP cells preferentially activates *Myf5* (Figure 7.5A). In addition, myoblasts recovered from *Pax7*$^{-/-}$ muscles appear to express Pax3 and MyoD (Figure 7, Chapter 4). Additional anecdotal evidence supporting an interaction between Pax7 and Myf5 is based on the expression of *Myf5nLacZ* in quiescent satellite cells that also express Pax7.

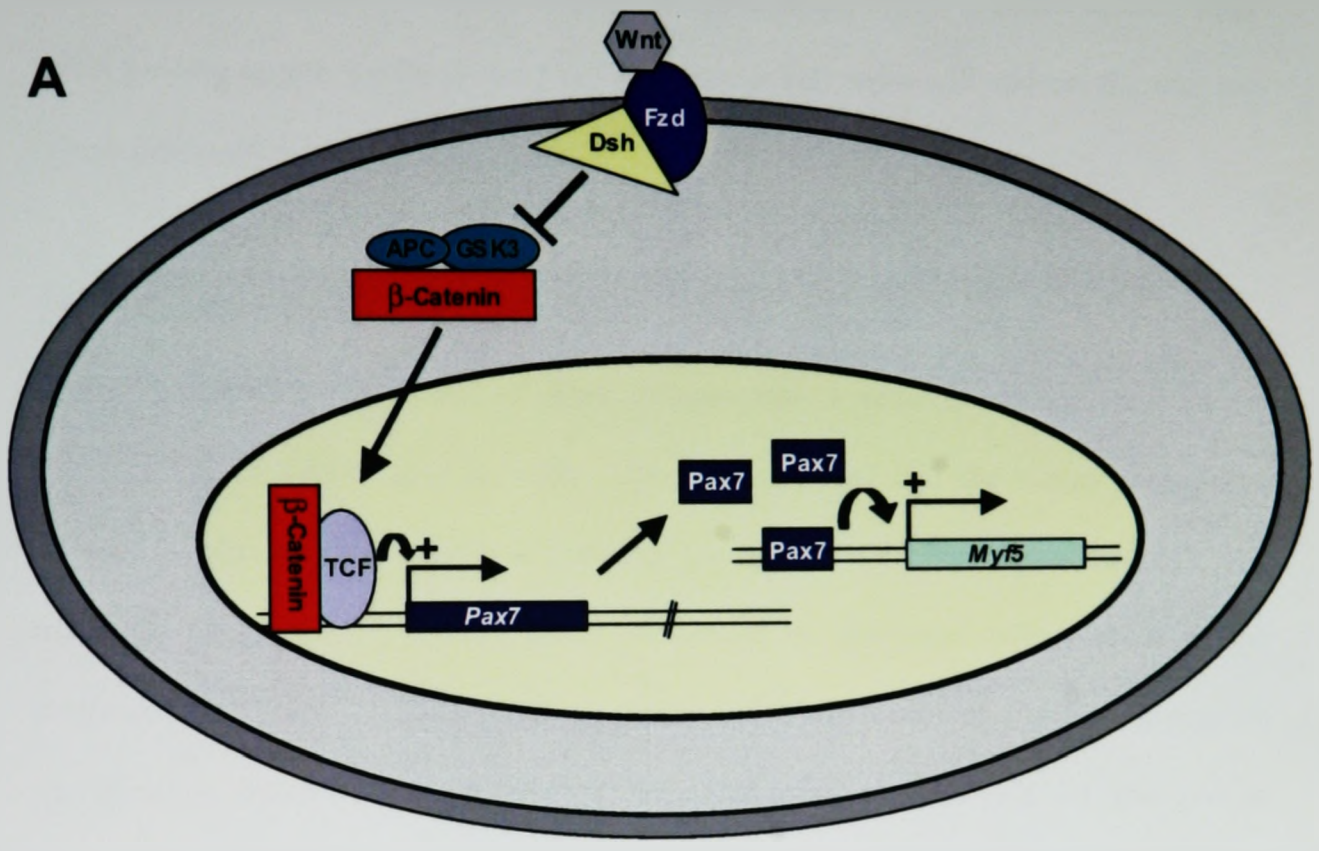
Several studies suggest a role for Myf5 in promoting myoblast proliferation. For example homozygous *Myf5nLacZ*, (e.g. *Myf5*-deficient) embryos display significantly reduced numbers of LacZ-expressing myogenic progenitors (Tajbakhsh et al., 1996). Moreover, Myf5 is preferentially expressed in proliferating myoblasts, whereas MyoD appears to be upregulated in differentiating cells within developing avian muscle (Delfini et al., 2000). These results are consistent with the proliferation deficit in *Myf5*$^{-/-}$ myoblasts (Montarras et al., 2000) and the markedly increased rate of proliferation in *MyoD*$^{-/-}$ myoblasts (Sabourin et al., 1999) and CDSC-Pax7 cells (Chapter 6). These results thus implicate Myf5 as being important for proliferation during adult myogenesis, with MyoD acting downstream to promote cell-cycle withdrawal and differentiation.

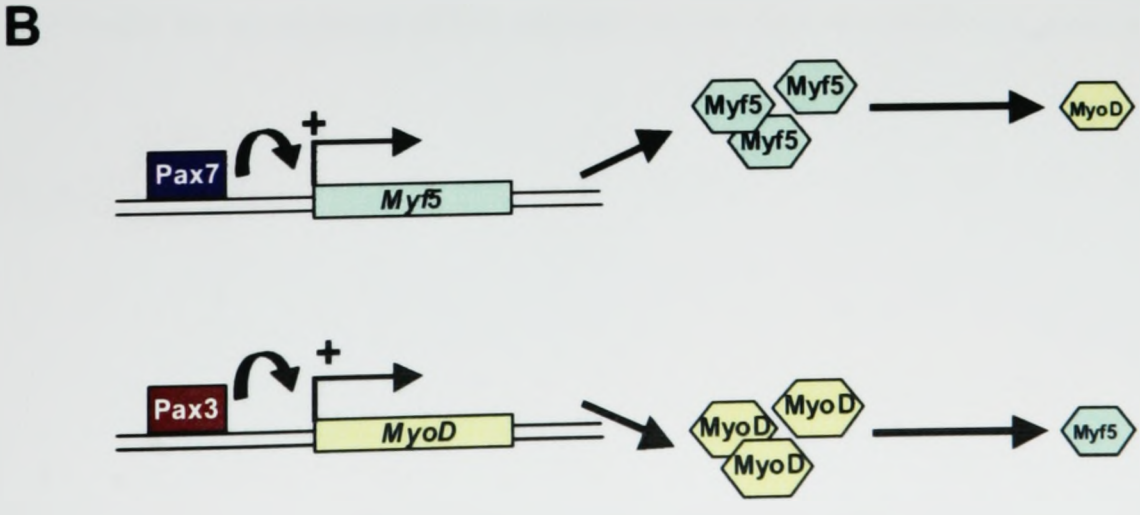
Taken together, these findings support a simple model in which Pax7 directly activates *Myf5* whereas Pax3 activates *MyoD* (Figure 7.5B). Alternatively Pax3 and Pax7 may indirectly activate MyoD and Myf5 respectively. Notably, experiments by Boryicki

**Figure 7.5 Model for myogenic commitment by Pax3 and Pax7**

(**A**) Adult stem cells such as $CD45^{+}$:$Sca1^{+}$ cells undergo myogenic specification in response to Wnt stimulation. In this model, Wnt binds to the Frizzled (Fzd) receptor and activates Disheveled (Dsh) which subsequently inactivates GSK-3β. The GSK-3β substrate, β-Catenin is stabilized and translocates to the nucleus where it associates with T-Cell-Factor (TCF). β-Catenin/TCF transcriptional complexes bind DNA and activate target genes. We speculate that Pax7 may represent a direct target gene of β-Catenin/TCF in Wnt-stimulated adult stem cells. Pax7 protein may then directly activate expression of Myf5 to induce the myogenic commitment of adult stem cells.

(**B**) Based on experimental observations, we propose that Pax7 induces myogenesis by preferentially activating the expression of Myf5. Myf5 can subsequently induce MyoD expression as demonstrated in genetic studies (Tajbakhsh et al., 1997). By contrast, Pax3 activates myogenesis via regulation of MyoD expression.


A
Wnt
Fzd
Dsh
APC
GSK3
β-Catenin
β-Catenin
TCF
Pax7
Pax7
Pax7
Pax7
Myf5


B
Pax7
Myf5
Myf5
Myf5
Myf5
MyoD
Pax3
MyoD
MyoD
MyoD
MyoD
Myf5

et al. support an indirect role for Pax3 in promoting the selective survival of progenitors that subsequently go on to express MyoD (Borycki et al., 1999). Identification of direct DNA binding targets for Pax3 and Pax7 in different cell types will address the accuracy of this proposed model.

## 7.9 Concluding remarks

In summary, this body of work demonstrates a requirement for Pax7 in the specification of muscle satellite cells. This discovery has laid the groundwork for a number of downstream studies addressing the function of Pax7 in stem cells and myogenic progenitors. Several issues remain to be addressed with respect to the molecular pathways operating both upstream and downstream of Pax7 in myogenic specification. In addition, these studies have established a novel role for endogenous adult stem cells in the regeneration of skeletal muscle. Importantly, a more comprehensive understanding of the mechanisms regulating the behavior of these cells may lead to the development of new therapies to treat degenerative muscle disease.

## References

Adams, G. R., and Haddad, F. (1996). The relationships among IGF-1, DNA content, and protein accumulation during skeletal muscle hypertrophy. J Appl Physiol *81*, 2509-2516.

Adams, G. R., and McCue, S. A. (1998). Localized infusion of IGF-I results in skeletal muscle hypertrophy in rats. J Appl Physiol *84*, 1716-1722.

Allen, R. E., Sheehan, S. M., Taylor, R. G., Kendall, T. L., and Rice, G. M. (1995). Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. J Cell Physiol *165*, 307-312.

Amthor, H., Christ, B., and Patel, K. (1999). A molecular mechanism enabling continuous embryonic muscle growth - a balance between proliferation and differentiation. Development *126*, 1041-1053.

Anderson, J. E. (2000). A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells. Mol Biol Cell *11*, 1859-1874.

Anderson, J. E., Mitchell, C. M., McGeachie, J. K., and Grounds, M. D. (1995). The time course of basic fibroblast growth factor expression in crush- injured skeletal muscles of SJL/J and BALB/c mice. Exp Cell Res *216*, 325-334.

Appell, H. J., Forsberg, S., and Hollmann, W. (1988). Satellite cell activation in human skeletal muscle after training: evidence for muscle fiber neoformation. Int J Sports Med *9*, 297-299.

Armand, O., Boutineau, A. M., Mauger, A., Pautou, M. P., and Kieny, M. (1983). Origin of satellite cells in avian skeletal muscles. Arch Anat Microsc Morphol Exp *72*, 163-181.

Asakura, A., Komaki, M., and Rudnicki, M. (2001). Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation *68*, 245-253.

Asakura, A., and Rudnicki, M. A. (2002). Cellular and molecular mechanisms regulating skeletal muscle development. In Mouse Development (Academic Press), pp. 253-278.

Asakura, A., Seale, P., Girgis-Gabardo, A., and Rudnicki, M. A. (2002). Myogenic specification of side population cells in skeletal muscle. J Cell Biol *159*, 123-134.

Austin, L., Bower, J., Kurek, J., and Vakakis, N. (1992). Effects of leukaemia inhibitory factor and other cytokines on murine and human myoblast proliferation. J Neurol Sci *112*, 185-191.

Austin, L., Bower, J. J., Bennett, T. M., Lynch, G. S., Kapsa, R., White, J. D., Barnard, W., Gregorevic, P., and Byrne, E. (2000). Leukemia inhibitory factor ameliorates muscle fiber degeneration in the mdx mouse. Muscle Nerve *23*, 1700-1705.

Bachner, D., Ahrens, M., Schroder, D., Hoffmann, A., Lauber, J., Betat, N., Steinert, P., Flohe, L., and Gross, G. (1998). Bmp-2 downstream targets in mesenchymal development identified by subtractive cloning from recombinant mesenchymal progenitors (C3H10T1/2). Dev Dyn *213*, 398-411.

Baroffio, A., Hamann, M., Bernheim, L., Bochaton-Piallat, M. L., Gabbiani, G., and Bader, C. R. (1996). Identification of self-renewing myoblasts in the progeny of single human muscle satellite cells. Differentiation *60*, 47-57.

Bartolomei, M. S., Webber, A. L., Brunkow, M. E., and Tilghman, S. M. (1993). Epigenetic mechanisms underlying the imprinting of the mouse H19 gene. Genes Dev *7*, 1663-1673.

Barton-Davis, E. R., Shoturma, D. I., Musaro, A., Rosenthal, N., and Sweeney, H. L. (1998). Viral mediated expression of insulin-like growth factor I blocks the aging-related loss of skeletal muscle function. Proc Natl Acad Sci U S A *95*, 15603-15607.

Beauchamp, J. R., Heslop, L., Yu, D. S., Tajbakhsh, S., Kelly, R. G., Wernig, A., Buckingham, M. E., Partridge, T. A., and Zammit, P. S. (2000). Expression of CD34 and myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J Cell Biol *151*, 1221-1234.

Beauchamp, J. R., Morgan, J. E., Pagel, C. N., and Partridge, T. A. (1999). Dynamics of myoblast transplantation reveal a discrete minority of precursors with stem cell-like properties as the myogenic source. J Cell Biol *144*, 1113-1122.

Bendall, A. J., Ding, J., Hu, G., Shen, M. M., and Abate-Shen, C. (1999). Msx1 antagonizes the myogenic activity of Pax3 in migrating limb muscle precursors. Development *126*, 4965-4976.

Benito, M., Valverde, A. M., and Lorenzo, M. (1996). IGF-I: a mitogen also involved in differentiation processes in mammalian cells. Int J Biochem Cell Biol *28*, 499-510.

Bennicelli, J. L., Advani, S., Schafer, B. W., and Barr, F. G. (1999). PAX3 and PAX7 exhibit conserved cis-acting transcription repression domains and utilize a common gain of function mechanism in alveolar rhabdomyosarcoma. Oncogene *18*, 4348-4356.

Bianco, P., and Cossu, G. (1999). Uno, nessuno e centomila: searching for the identity of mesodermal progenitors. Exp Cell Res *251*, 257-263.

Biederer, T., Sara, Y., Mozhayeva, M., Atasoy, D., Liu, X., Kavalali, E. T., and Sudhof, T. C. (2002). SynCAM, a synaptic adhesion molecule that drives synapse assembly. Science *297*, 1525-1531.

Birchmeier, C., and Gherardi, E. (1998). Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. Trends Cell Biol *8*, 404-410.

Birnboim, H. C. (1988). Rapid extraction of high molecular weight RNA from cultured cells and granulocytes for Northern analysis. Nucleic Acids Res *16*, 1487-1497.

Bischoff, R. (1990). Interaction between satellite cells and skeletal muscle fibers. Development *109*, 943-952.

Bischoff, R. (1994). The satellite cell and muscle regeneration. In Myogenesis, A. G. Engel, and C. Franszini-Armstrong, eds. (New York, McGraw-Hill), pp. 97-118.

Bischoff, R. (1997). Chemotaxis of skeletal muscle satellite cells. Dev Dyn *208*, 505-515.

Bittner, R. E., Schofer, C., Weipoltshammer, K., Ivanova, S., Streubel, B., Hauser, E., Freilinger, M., Hoger, H., Elbe-Burger, A., and Wachtler, F. (1999). Recruitment of bone marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. Anat Embryol *199*, 391-396.

Blanco-Bose, W. E., Yao, C. C., Kramer, R. H., and Blau, H. M. (2001). Purification of mouse primary myoblasts based on alpha 7 integrin expression. Exp Cell Res *265*, 212-220.

Blau, H. M., Brazelton, T. R., and Weimann, J. M. (2001). The Evolving Concept Review of a Stem Cell: Entity or Function? Cell *105*, 829-841.

Blau, H. M., and Springer, M. L. (1995). Muscle-mediated gene therapy. N Engl J Med *333*, 1554-1556.

Blau, H. M., Webster, C., and Pavlath, G. K. (1983). Defective myoblasts identified in Duchenne muscular dystrophy. Proc Natl Acad Sci U S A *80*, 4856-4860.

Blaveri, K., Heslop, L., Yu, D. S., Rosenblatt, J. D., Gross, J. G., Partridge, T. A., and Morgan, J. E. (1999). Patterns of repair of dystrophic mouse muscle: studies on isolated fibers. Dev Dyn *216*, 244-256.

Blyszczuk, P., Czyz, J., Kania, G., Wagner, M., Roll, U., St-Onge, L., and Wobus, A. M. (2003). Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. Proc Natl Acad Sci U S A *100*, 998-1003.

Bober, E., Brand-Saberi, B., Ebensperger, C., Wilting, J., Balling, R., Paterson, B. M., Arnold, H. H., and Christ, B. (1994a). Initial steps of myogenesis in somites are independent of influence from axial structures. Development *120*, 3073-3082.

Bober, E., Franz, T., Arnold, H. H., Gruss, P., and Tremblay, P. (1994b). Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. Development *120*, 603-612.

Boengler, K., Pipp, F., Fernandez, B., Richter, A., Schaper, W., and Deindl, E. (2003). The ankyrin repeat containing SOCS box protein 5: a novel protein associated with arteriogenesis. Biochem Biophys Res Commun *302*, 17-22.

Bonilla, E., Samitt, C. E., Miranda, A. F., Hays, A. P., Salviati, G., DiMauro, S., Kunkel, L. M., Hoffman, E. P., and Rowland, L. P. (1988). Duchenne muscular dystrophy: deficiency of dystrophin at the muscle cell surface. Cell *54*, 447-452.

Borycki, A. G., and Emerson, C. P. (1997). Muscle determination: another key player in myogenesis? Curr Biol *7*, R620-623.

Borycki, A. G., Li, J., Jin, F., Emerson, C. P., and Epstein, J. A. (1999). Pax3 functions in cell survival and in pax7 regulation. Development *126*, 1665-1674.

Braissant, O., and Wahli, W. (1998). A simplified in situ hybridization protocol using non-radioactively labelled probes to detect abundant and rare mRNAs on tissue sections. Biochemica *1*, 10-16.

Braun, T., and Arnold, H. H. (1995). Inactivation of Myf-6 and Myf-5 genes in mice leads to alterations in skeletal muscle development. Embo J *14*, 1176-1186.

Braun, T., Bober, E., Winter, B., Rosenthal, N., and Arnold, H. H. (1990). Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. Embo J *9*, 821-831.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., and Arnold, H. H. (1989). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. Embo J *8*, 701-709.

Bredt, D. S. (1999). Knocking signalling out of the dystrophin complex [news]. Nat Cell Biol *1*, E89-91.

Brenman, J. E., Chao, D. S., Xia, H., Aldape, K., and Bredt, D. S. (1995). Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. Cell *82*, 743-752.

Brooks, S. V., and Faulkner, J. A. (1994). Skeletal muscle weakness in old age: underlying mechanisms. Med Sci Sports Exerc *26*, 432-439.

Buckingham, M. (1994). Muscle differentiation. Which myogenic factors make muscle? Curr Biol *4*, 61-63.

Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Montarras, D., Rocancourt, D., and Relaix, F. (2003). The formation of skeletal muscle: from somite to limb. J Anat *202*, 59-68.

Bulfield, G., Siller, W. G., Wight, P. A., and Moore, K. J. (1984). X chromosome-linked muscular dystrophy (mdx) in the mouse. Proc Natl Acad Sci U S A *81*, 1189-1192.

Cantini, M., and Carraro, U. (1995). Macrophage-released factor stimulates selectively myogenic cells in primary muscle culture. J Neuropathol Exp Neurol *54*, 121-128.

Cantini, M., Massimino, M. L., Bruson, A., Catani, C., Dalla Libera, L., and Carraro, U. (1994). Macrophages regulate proliferation and differentiation of satellite cells. Biochem Biophys Res Commun *202*, 1688-1696.

Cao, B., Zheng, B., Jankowski, R. J., Kimura, S., Ikezawa, M., Deasy, B., Cummins, J., Epperly, M., Qu-Petersen, Z., and Huard, J. (2003). Muscle stem cells differentiate into haematopoietic lineages but retain myogenic potential. Nat Cell Biol.

Carlson, B. M. (1995). Factors influencing the repair and adaptation of muscles in aged individuals: satellite cells and innervation. J Gerontol A Biol Sci Med Sci *50 Spec No*, 96-100.

Carlson, M. R., Komine, Y., Bryant, S. V., and Gardiner, D. M. (2001). Expression of Hoxb13 and Hoxc10 in developing and regenerating Axolotl limbs and tails. Dev Biol *229*, 396-406.

Carlsson, L., Li, Z., Paulin, D., and Thornell, L. E. (1999). Nestin is expressed during development and in myotendinous and neuromuscular junctions in wild type and desmin knock-out mice. Exp Cell Res *251*, 213-223.

Carnac, G., Fajas, L., L'Honore, A., Sardet, C., Lamb, N. J., and Fernandez, A. (2000). The retinoblastoma-like protein p130 is involved in the determination of reserve cells in differentiating myoblasts. Curr Biol *10*, 543-546.

Chakravarthy, M. V., Davis, B. S., and Booth, F. W. (2000). IGF-I restores satellite cell proliferative potential in immobilized old skeletal muscle. J Appl Physiol *89*, 1365-1379.

Chan, P. Y., and Takei, F. (1989). Molecular cloning and characterization of a novel murine T cell surface antigen, YE1/48. J Immunol *142*, 1727-1736.

Charge, S. B., Brack, A. S., and Hughes, S. M. (2002). Aging-related satellite cell differentiation defect occurs prematurely after Ski-induced muscle hypertrophy. Am J Physiol Cell Physiol *283*, C1228-1241.

Chargé, S. B. P., Seale, P., and Rudnicki, M. A. (2003). Identification of a novel population of myogenic cells in Pax7-deficient muscle. J Cell Biol. Submitted.

Charge, S.B., and M.A. Rudnicki. 2003. Cellular and molecular regulation of muscle regeneration. *Physiological reviews*. in press.

Chi, N., and Epstein, J. A. (2002). Getting your Pax straight: Pax proteins in development and disease. Trends Genet *18*, 41-47.

Christ, B., and Ordahl, C. P. (1995). Early stages of chick somite development. Anat Embryol (Berl) *191*, 381-396.

Cifuentes-Diaz, C., Nicolet, M., Goudou, D., Rieger, F., and Mege, R. M. (1993). N-cadherin and N-CAM-mediated adhesion in development and regeneration of skeletal muscle. Neuromuscul Disord *3*, 361-365.

Coletti, D., Yang, E., Marazzi, G., and Sassoon, D. (2002). TNFalpha inhibits skeletal myogenesis through a PW1-dependent pathway by recruitment of caspase pathways. Embo J *21*, 631-642.

Conboy, I. M., and Rando, T. A. (2002). The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell *3*, 397-409.

Cooper, R. N., Tajbakhsh, S., Mouly, V., Cossu, G., Buckingham, M., and Butler-Browne, G. S. (1999). In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. J Cell Sci *112*, 2895-2901.

Cornelison, D. D., Filla, M. S., Stanley, H. M., Rapraeger, A. C., and Olwin, B. B. (2001). Syndecan-3 and syndecan-4 specifically mark skeletal muscle satellite cells and

are implicated in satellite cell maintenance and muscle regeneration. Dev Biol *239*, 79-94.

Cornelison, D. D., Olwin, B. B., Rudnicki, M. A., and Wold, B. J. (2000). MyoD(-/-) satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. Dev Biol *224*, 122-137.

Cornelison, D. D., and Wold, B. J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev Biol *191*, 270-283.

Corti, S., Strazzer, S., Del Bo, R., Salani, S., Bossolasco, P., Fortunato, F., Locatelli, F., Soligo, D., Moggio, M., Ciscato, P., *et al.* (2002). A subpopulation of murine bone marrow cells fully differentiates along the myogenic pathway and participates in muscle repair in the mdx dystrophic mouse. Exp Cell Res *277*, 74-85.

Cossu, G., and Borello, U. (1999). Wnt signaling and the activation of myogenesis in mammals. Embo J *18*, 6867-6872.

Cossu, G., Cicinelli, P., Fieri, C., Coletta, M., and Molinaro, M. (1985). Emergence of TPA-resistant 'satellite' cells during muscle histogenesis of human limb. Exp Cell Res *160*, 403-411.

Cossu, G., Kelly, R., Tajbakhsh, S., Di Donna, S., Vivarelli, E., and Buckingham, M. (1996a). Activation of different myogenic pathways: myf-5 is induced by the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm. Development *122*, 429-437.

Cossu, G., and Mavilio, F. (2000). Myogenic stem cells for the therapy of primary myopathies: wishful thinking or therapeutic perspective? J Clin Invest *105*, 1669-1674.

Cossu, G., Tajbakhsh, S., and Buckingham, M. (1996b). How is myogenesis initiated in the embryo? Trends Genet *12*, 218-223.

Darr, K. C., and Schultz, E. (1987). Exercise-induced satellite cell activation in growing and mature skeletal muscle. J Appl Physiol *63*, 1816-1821.

Daston, G., Lamar, E., Olivier, M., and Goulding, M. (1996). Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. Development *122*, 1017-1027.

Davis, R. L., Weintraub, H., and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell *51*, 987-1000.

De Angelis, L., Berghella, L., Coletta, M., Lattanzi, L., Zanchi, M., Cusella-De Angelis, M. G., Ponzetto, C., and Cossu, G. (1999). Skeletal myogenic progenitors originating from embryonic dorsal aorta coexpress endothelial and myogenic markers and contribute to postnatal muscle growth and regeneration [see comments]. J Cell Biol *147*, 869-878.

Decary, S., Mouly, V., Hamida, C. B., Sautet, A., Barbet, J. P., and Butler-Browne, G. S. (1997). Replicative potential and telomere length in human skeletal muscle: implications for satellite cell-mediated gene therapy. Hum Gene Ther *8*, 1429-1438.

Deconinck, A. E., Rafael, J. A., Skinner, J. A., Brown, S. C., Potter, A. C., Metzinger, L., Watt, D. J., Dickson, J. G., Tinsley, J. M., and Davies, K. E. (1997). Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. Cell *90*, 717-727.

Deleersnijder, W., Hong, G., Cortvrindt, R., Poirier, C., Tylzanowski, P., Pittois, K., Van Marck, E., and Merregaert, J. (1996). Isolation of markers for chondro-osteogenic differentiation using cDNA library subtraction. Molecular cloning and characterization of a gene belonging to a novel multigene family of integral membrane proteins. J Biol Chem *271*, 19475-19482.

Delfini, M., Hirsinger, E., Pourquie, O., and Duprez, D. (2000). Delta 1-activated notch inhibits muscle differentiation without affecting Myf5 and Pax3 expression in chick limb myogenesis. Development *127*, 5213-5224.

Deng, Y., and Wu, X. (2000). Peg3/Pw1 promotes p53-mediated apoptosis by inducing Bax translocation from cytosol to mitochondria. Proc Natl Acad Sci U S A *97*, 12050-12055.

Desplan, C. (1997). Eye development: governed by a dictator or a junta? Cell *91*, 861-864.

Dietrich, S., Abou-Rebyeh, F., Brohmann, H., Bladt, F., Sonnenberg-Riethmacher, E., Yamaai, T., Lumsden, A., Brand-Saberi, B., and Birchmeier, C. (1999). The role of SF/HGF and c-Met in the development of skeletal muscle. Development *126*, 1621-1629.

DiMauro, S., Mendell, J. R., Sahenk, Z., Bachman, D., Scarpa, A., Scofield, R. M., and Reiner, C. (1980). Fatal infantile mitochondrial myopathy and renal dysfunction due to cytochrome-c-oxidase deficiency. Neurology *30*, 795-804.

Dodson, M. V., and Allen, R. E. (1987). Interaction of multiplication stimulating activity/rat insulin-like growth factor II with skeletal muscle satellite cells during aging. Mech Ageing Dev *39*, 121-128.

Dooley, C. M., James, J., Jane McGlade, C., and Ahmad, I. (2003). Involvement of numb in vertebrate retinal development: evidence for multiple roles of numb in neural differentiation and maturation. J Neurobiol *54*, 313-325.

Doumit, M. E., Cook, D. R., and Merkel, R. A. (1993). Fibroblast growth factor, epidermal growth factor, insulin-like growth factors, and platelet-derived growth factor-BB stimulate proliferation of clonally derived porcine myogenic satellite cells. J Cell Physiol *157*, 326-332.

Duclos, F., Straub, V., Moore, S. A., Venzke, D. P., Hrstka, R. F., Crosbie, R. H., Durbeej, M., Lebakken, C. S., Ettinger, A. J., van der Meulen, J., *et al.* (1998).

Progressive muscular dystrophy in alpha-sarcoglycan-deficient mice. J Cell Biol *142*, 1461-1471.

Dzierzak, E. (1999). Embryonic beginnings of definitive hematopoietic stem cells. Ann N Y Acad Sci *872*, 256-262; discussion 262-254.

Edwall, D., Schalling, M., Jennische, E., and Norstedt, G. (1989). Induction of insulin-like growth factor I messenger ribonucleic acid during regeneration of rat skeletal muscle. Endocrinology *124*, 820-825.

Epstein, D. J., Vekemans, M., and Gros, P. (1991). Splotch (Sp2H), a mutation affecting development of the mouse neural tube, shows a deletion within the paired homeodomain of Pax-3. Cell *67*, 767-774.

Epstein, J. A., Lam, P., Jepeal, L., Maas, R. L., and Shapiro, D. N. (1995). Pax3 inhibits myogenic differentiation of cultured myoblast cells. J Biol Chem *270*, 11719-11722.

Epstein, J. A., Shapiro, D. N., Cheng, J., Lam, P. Y., and Maas, R. L. (1996). Pax3 modulates expression of the c-Met receptor during limb muscle development. Proc Natl Acad Sci U S A *93*, 4213-4218.

Ervasti, J. M., Ohlendieck, K., Kahl, S. D., Gaver, M. G., and Campbell, K. P. (1990). Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. Nature *345*, 315-319.

Faulkner, J. A., Brooks, S. V., and Zerba, E. (1995). Muscle atrophy and weakness with aging: contraction-induced injury as an underlying mechanism. J Gerontol A Biol Sci Med Sci *50 Spec No*, 124-129.

Feldman, J. L., and Stockdale, F. E. (1992). Temporal appearance of satellite cells during myogenesis. Dev Biol *153*, 217-226.

Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G., and Mavilio, F. (1998). Muscle regeneration by bone marrow-derived myogenic progenitors. Science *279*, 1528-1530.

Ferrari, G., Stornaiuolo, A., and Mavilio, F. (2001). Failure to correct murine muscular dystrophy. Nature *411*, 1014-1015.

Fiore, F., Sebille, A., and Birnbaum, D. (2000). Skeletal muscle regeneration is not impaired in Fgf6 -/- mutant mice. Biochem Biophys Res Commun *272*, 138-143.

Floss, T., Arnold, H. H., and Braun, T. (1997). A role for FGF-6 in skeletal muscle regeneration. Genes Dev *11*, 2040-2051.

Franz, T., Kothary, R., Surani, M. A., Halata, Z., and Grim, M. (1993). The Splotch mutation interferes with muscle development in the limbs. Anat Embryol (Berl) *187*, 153-160.

Gal-Levi, R., Leshem, Y., Aoki, S., Nakamura, T., and Halevy, O. (1998). Hepatocyte growth factor plays a dual role in regulating skeletal muscle satellite cell proliferation and differentiation. Biochim Biophys Acta *1402*, 39-51.

Galli, R., Borello, U., Gritti, A., Minasi, M. G., Bjornson, C., Coletta, M., Mora, M., De Angelis, M. G., Fiocco, R., Cossu, G., and Vescovi, A. L. (2000). Skeletal myogenic potential of human and mouse neural stem cells. Nat Neurosci *3*, 986-991.

Garry, D. J., Yang, Q., Bassel-Duby, R., and Williams, R. S. (1997). Persistent expression of MNF identifies myogenic stem cells in postnatal muscles. Dev Biol *188*, 280-294.

Gibson, M. C., and Schultz, E. (1983). Age-related differences in absolute numbers of skeletal muscle satellite cells. Muscle Nerve *6*, 574-580.

Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., and Mulligan, R. C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med *183*, 1797-1806.

Goodell, M. A., Rosenzweig, M., Kim, H., Marks, D. F., DeMaria, M., Paradis, G., Grupp, S. A., Sieff, C. A., Mulligan, R. C., and Johnson, R. P. (1997). Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med *3*, 1337-1345.

Goulding, M., Lumsden, A., and Paquette, A. J. (1994). Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. Development *120*, 957-971.

Grady, R. M., Teng, H., Nichol, M. C., Cunningham, J. C., Wilkinson, R. S., and Sanes, J. R. (1997). Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. Cell *90*, 729-738.

Graham, F. L., and van der Eb, A. J. (1973). Transformation of rat cells by DNA of human adenovirus 5. Virology *54*, 536-539.

Grounds, M. D. (1998). Age-associated changes in the response of skeletal muscle cells to exercise and regeneration. Ann N Y Acad Sci *854*, 78-91.

Grounds, M. D., and Yablonka-Reuveni, Z. (1993). Molecular and cell biology of skeletal muscle regeneration. Mol Cell Biol Hum Dis Ser *3*, 210-256.

Gu, Y., Sorokin, L., Durbeej, M., Hjalt, T., Jonsson, J. I., and Ekblom, M. (1999). Characterization of bone marrow laminins and identification of alpha5-containing laminins as adhesive proteins for multipotent hematopoietic FDCP-Mix cells. Blood *93*, 2533-2542.

Gussoni, E., Blau, H. M., and Kunkel, L. M. (1997). The fate of individual myoblasts after transplantation into muscles of DMD patients. Nat Med *3*, 970-977.

Gussoni, E., Pavlath, G. K., Lanctot, A. M., Sharma, K. R., Miller, R. G., Steinman, L., and Blau, H. M. (1992). Normal dystrophin transcripts detected in Duchenne muscular dystrophy patients after myoblast transplantation. Nature *356*, 435-438.

Gussoni, E., Soneoka, Y., Strickland, C. D., Buzney, E. A., Khan, M. K., Flint, A. F., Kunkel, L. M., and Mulligan, R. C. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature *401*, 390-394.

Hartley, R. S., Bandman, E., and Yablonka-Reuveni, Z. (1992). Skeletal muscle satellite cells appear during late chicken embryogenesis. Dev Biol *153*, 206-216.

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N., and Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature *364*, 501-506.

Hauschka, S. D. (1994). The embryonic origin of muscle. In Myology, A. G. Engel, and C. Franzini-Armstrong, eds. (New-York, McGraw-Hill), pp. 3-73.

Hawke, T. J., and Garry, D. J. (2001). Myogenic satellite cells: physiology to molecular biology. J Appl Physiol *91*, 534-551.

Heanue, T. A., Reshef, R., Davis, R. J., Mardon, G., Oliver, G., Tomarev, S., Lassar, A. B., and Tabin, C. J. (1999). Synergistic regulation of vertebrate muscle development by Dach2, Eya2, and Six1, homologs of genes required for Drosophila eye formation. Genes Dev *13*, 3231-3243.

Heslop, L., Morgan, J. E., and Partridge, T. A. (2000). Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. J Cell Sci *113*, 2299-2308.

Hu, E., Tontonoz, P., and Spiegelman, B. M. (1995). Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. Proc Natl Acad Sci U S A *92*, 9856-9860.

Hu, J. S., Olson, E. N., and Kingston, R. E. (1992). HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic regulatory factors. Mol Cell Biol *12*, 1031-1042.

Hubank, M., and Schatz, D. G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res *22*, 5640-5648.

Hughes, S. M., and Blau, H. M. (1990). Migration of myoblasts across basal lamina during skeletal muscle development. Nature *345*, 350-353.

Hughes, S. M., Cho, M., Karsch-Mizrachi, I., Travis, M., Silberstein, L., Leinwand, L. A., and Blau, H. M. (1993). Three slow myosin heavy chains sequentially expressed in developing mammalian skeletal muscle. Developmental Biology *158*, 183-199.

Irintchev, A., Zeschnigk, M., Starzinski-Powitz, A., and Wernig, A. (1994). Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. Dev Dyn *199*, 326-337.

Jackson, K. A., Mi, T., and Goodell, M. A. (1999). Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proc Natl Acad Sci U S A *96*, 14482-14486.

Jesse, T. L., LaChance, R., Iademarco, M. F., and Dean, D. C. (1998). Interferon regulatory factor-2 is a transcriptional activator in muscle where It regulates expression of vascular cell adhesion molecule-1. J Cell Biol *140*, 1265-1276.

Johnson, M. D., Wu, X., Aithmitti, N., and Morrison, R. S. (2002). Peg3/Pw1 is a mediator between p53 and Bax in DNA damage-induced neuronal death. J Biol Chem *277*, 23000-23007.

Johnson, S. E., and Allen, R. E. (1993). Proliferating cell nuclear antigen (PCNA) is expressed in activated rat skeletal muscle satellite cells. J Cell Physiol *154*, 39-43.

Jostes, B., Walther, C., and Gruss, P. (1990). The murine paired box gene, Pax7, is expressed specifically during the development of the nervous and muscular system. Mech Dev *33*, 27-37.

Kablar, B., Krastel, K., Ying, C., Asakura, A., Tapscott, S. J., and Rudnicki, M. A. (1997). MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle. Development *124*, 4729-4738.

Kablar, B., Krastel, K., Ying, C., Tapscott, S. J., Goldhamer, D. J., and Rudnicki, M. A. (1999). Myogenic determination occurs independently in somites and limb buds. Dev Biol *206*, 219-231.

Kardon, G., Heanue, T. A., and Tabin, C. J. (2002). Pax3 and Dach2 positive regulation in the developing somite. Dev Dyn *224*, 350-355.

Kaushal, S., Schneider, J. W., Nadal-Ginard, B., and Mahdavi, V. (1994). Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD. Science *266*, 1236-1240.

Kawai, T., Matsumoto, M., Takeda, K., Sanjo, H., and Akira, S. (1998). ZIP kinase, a novel serine/threonine kinase which mediates apoptosis. Mol Cell Biol *18*, 1642-1651.

Kay, P. H., and Ziman, M. R. (1999). Alternate Pax7 paired box transcripts which include a trinucleotide or a hexanucleotide are generated by use of alternate 3' intronic splice sites which are not utilized in the ancestral homologue. Gene *230*, 55-60.

Keller, S. A., Jones, J. M., Boyle, A., Barrow, L. L., Killen, P. D., Green, D. G., Kapousta, N. V., Hitchcock, P. F., Swank, R. T., and Meisler, M. H. (1994). Kidney and retinal defects (Krd), a transgene-induced mutation with a deletion of mouse chromosome 19 that includes the Pax2 locus. Genomics *23*, 309-320.

Kelly, A. M. (1978). Satellite cells and myofiber growth in the rat soleus and extensor digitorum longus muscles. Dev Biol *65*, 1-10.

Khan, J., Bittner, M. L., Saal, L. H., Teichmann, U., Azorsa, D. O., Gooden, G. C., Pavan, W. J., Trent, J. M., and Meltzer, P. S. (1999). cDNA microarrays detect activation of a myogenic transcription program by the PAX3-FKHR fusion oncogene. Proc Natl Acad Sci U S A *96*, 13264-13269.

Kiefer, J. C., and Hauschka, S. D. (2001). Myf-5 is transiently expressed in nonmuscle mesoderm and exhibits dynamic regional changes within the presegmented mesoderm and somites I-IV. Dev Biol *232*, 77-90.

Kimura, H., Ohtomo, T., Yamaguchi, M., Ishii, A., and Sugimoto, K. (1996). Mouse MCM proteins: complex formation and transportation to the nucleus. Genes Cells *1*, 977-993.

Kurek, J. B., Bower, J. J., Romanella, M., Koentgen, F., Murphy, M., and Austin, L. (1997). The role of leukemia inhibitory factor in skeletal muscle regeneration. Muscle Nerve *20*, 815-822.

Kurek, J. B., Nouri, S., Kannourakis, G., Murphy, M., and Austin, L. (1996). Leukemia inhibitory factor and interleukin-6 are produced by diseased and regenerating skeletal muscle. Muscle Nerve *19*, 1291-1301.

Kuroda, T. S., Fukuda, M., Ariga, H., and Mikoshiba, K. (2002). The Slp homology domain of synaptotagmin-like proteins 1-4 and Slac2 functions as a novel Rab27A binding domain. J Biol Chem *277*, 9212-9218.

Kuschel, R., Yablonka-Reuveni, Z., and Bornemann, A. (1999). Satellite cells on isolated myofibers from normal and denervated adult rat muscle. J Histochem Cytochem *47*, 1375-1384.

LaBarge, M. A., and Blau, H. M. (2002). Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. Cell *111*, 589-601.

Laclef, C., Hamard, G., Demignon, J., Souil, E., Houbron, C., and Maire, P. (2003). Altered myogenesis in Six1-deficient mice. Development *130*, 2239-2252.

Lagutina, I., Conway, S. J., Sublett, J., and Grosveld, G. C. (2002). Pax3-FKHR knock-in mice show developmental aberrations but do not develop tumors. Mol Cell Biol *22*, 7204-7216.

Lamberts, S. W., van den Beld, A. W., and van der Lely, A. J. (1997). The endocrinology of aging [see comments]. Science *278*, 419-424.

Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., and Weintraub, H. (1991). Functional activity of myogenic HLH proteins requires hetero- oligomerization with E12/E47-like proteins in vivo. Cell *66*, 305-315.

Lawlor, M. A., Feng, X., Everding, D. R., Sieger, K., Stewart, C. E., and Rotwein, P. (2000). Dual control of muscle cell survival by distinct growth factor- regulated signaling pathways. Mol Cell Biol *20*, 3256-3265.

Lawlor, M. A., and Rotwein, P. (2000a). Coordinate control of muscle cell survival by distinct insulin-like growth factor activated signaling pathways. J Cell Biol *151*, 1131-1140.

Lawlor, M. A., and Rotwein, P. (2000b). Insulin-like growth factor-mediated muscle cell survival: central roles for akt and cyclin-dependent kinase inhibitor p21. Mol Cell Biol *20*, 8983-8995.

Lee, J. Y., Qu-Petersen, Z., Cao, B., Kimura, S., Jankowski, R., Cummins, J., Usas, A., Gates, C., Robbins, P., Wernig, A., and Huard, J. (2000). Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. J Cell Biol *150*, 1085-1100.

Leibovitch, M. P., Solhonne, B., Guillier, M., Verrelle, P., Leibovitch, S. A., and Verelle, P. (1995). Direct relationship between the expression of tumor suppressor H19 mRNA and c-mos proto-oncogene during myogenesis. Oncogene *10*, 251-260.

Lescaudron, L., Peltekian, E., Fontaine-Perus, J., Paulin, D., Zampieri, M., Garcia, L., and Parrish, E. (1999). Blood borne macrophages are essential for the triggering of muscle regeneration following muscle transplant. Neuromuscul Disord *9*, 72-80.

Leshem, Y., Spicer, D. B., Gal-Levi, R., and Halevy, O. (2000). Hepatocyte growth factor (HGF) inhibits skeletal muscle cell differentiation: a role for the bHLH protein twist and the cdk inhibitor p27. J Cell Physiol *184*, 101-109.

Lin, C. H., Hudson, A. J., and Strickland, K. P. (1969). Fatty acid metabolism in dystrophic muscle in vitro. Life Sci *8*, 21-26.

Lin, J., Wu, H., Tarr, P. T., Zhang, C. Y., Wu, Z., Boss, O., Michael, L. F., Puigserver, P., Isotani, E., Olson, E. N., *et al.* (2002). Transcriptional co-activator PGC-1 alpha drives the formation of slow- twitch muscle fibres. Nature *418*, 797-801.

Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J., and Efstratiadis, A. (1993). Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell *75*, 59-72.

Liu, L., Vachon, P. H., Kuang, W., Xu, H., Wewer, U. M., Kylsten, P., and Engvall, E. (1997). Mouse adhalin: primary structure and expression during late stages of muscle differentiation in vitro. Biochem Biophys Res Commun *235*, 227-235.

Lluis, F., Roma, J., Suelves, M., Parra, M., Aniorte, G., Gallardo, E., Illa, I., Rodriguez, L., Hughes, S. M., Carmeliet, P., *et al.* (2001). Urokinase-dependent plasminogen activation is required for efficient skeletal muscle regeneration in vivo. Blood *97*, 1703-1711.

Luttun, A., Tjwa, M., and Carmeliet, P. (2002). Placental growth factor (PlGF) and its receptor Flt-1 (VEGFR-1): novel therapeutic targets for angiogenic disorders. Ann N Y Acad Sci *979*, 80-93.

Macchia, P. E., Lapi, P., Krude, H., Pirro, M. T., Missero, C., Chiovato, L., Souabni, A., Baserga, M., Tassi, V., Pinchera, A., *et al.* (1998). PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis. Nat Genet *19*, 83-86.

Magnaghi, P., Roberts, C., Lorain, S., Lipinski, M., and Scambler, P. J. (1998). HIRA, a mammalian homologue of Saccharomyces cerevisiae transcriptional co-repressors, interacts with Pax3. Nat Genet *20*, 74-77.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular cloning : a laboratory manual (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory).

Mansouri, A., Chowdhury, K., and Gruss, P. (1998). Follicular cells of the thyroid gland require Pax8 gene function. Nat Genet *19*, 87-90.

Mansouri, A., Stoykova, A., Torres, M., and Gruss, P. (1996). Dysgenesis of cephalic neural crest derivatives in Pax7-/- mutant mice. Development *122*, 831-838.

Maroto, M., Reshef, R., Munsterberg, A. E., Koester, S., Goulding, M., and Lassar, A. B. (1997). Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. Cell *89*, 139-148.

Marthiens, V., Padilla, F., Lambert, M., and Mege, R. M. (2002). Complementary expression and regulation of cadherins 6 and 11 during specific steps of motoneuron differentiation. Mol Cell Neurosci *20*, 458-475.

Mason, I. J. (1994). The ins and outs of fibroblast growth factors. Cell *78*, 547-552.

Matsumura, F., and Yamashiro, S. (1993). Caldesmon. Curr Opin Cell Biol *5*, 70-76.

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol *9*, 493-495.

Mbalaviele, G., Nishimura, R., Myoi, A., Niewolna, M., Reddy, S. V., Chen, D., Feng, J., Roodman, D., Mundy, G. R., and Yoneda, T. (1998). Cadherin-6 mediates the heterotypic interactions between the hemopoietic osteoclast cell lineage and stromal cells in a murine model of osteoclast differentiation. J Cell Biol *141*, 1467-1476.

McGeachie, J. K. (1985). The fate of proliferating cells in skeletal muscle after denervation or tenotomy: an autoradiographic study. Neuroscience *15*, 499-506.

McGeachie, J. K. (1989). Sustained cell proliferation in denervated skeletal muscle of mice. Cell Tissue Res *257*, 455-457.

McGeachie, J. K., and Grounds, M. D. (1995). Retarded myogenic cell replication in regenerating skeletal muscles of old mice: an autoradiographic study in young and old BALBc and SJL/J mice. Cell Tissue Res *280*, 277-282.

McGeachie, J. K., Grounds, M. D., Partridge, T. A., and Morgan, J. E. (1993). Age-related changes in replication of myogenic cells in mdx mice: quantitative autoradiographic studies. J Neurol Sci *119*, 169-179.

McKinney-Freeman, S. L., Jackson, K. A., Camargo, F. D., Ferrari, G., Mavilio, F., and Goodell, M. A. (2002). Muscle-derived hematopoietic stem cells are hematopoietic in origin. Proc Natl Acad Sci U S A *99*, 1341-1346.

Megeney, L. A., Kablar, B., Garrett, K., Anderson, J. E., and Rudnicki, M. A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev *10*, 1173-1183.

Megeney, L. A., and Rudnicki, M. A. (1995). Determination versus differentiation and the MyoD family of transcription factors. Biochem Cell Biol *73*, 723-732.

Mennerich, D., and Braun, T. (2001). Activation of myogenesis by the homeobox gene Lbx1 requires cell proliferation. Embo J *20*, 7174-7183.

Mennerich, D., Schafer, K., and Braun, T. (1998). Pax-3 is necessary but not sufficient for lbx1 expression in myogenic precursor cells of the limb. Mech Dev *73*, 147-158. bin/cas/tree/store/mod/cas_sub/browse/browse.cgi?year=1998&volume=1973&iss ue=1992&aid=1896.

Merly, F., Lescaudron, L., Rouaud, T., Crossin, F., and Gardahaut, M. F. (1999). Macrophages enhance muscle satellite cell proliferation and delay their differentiation. Muscle Nerve *22*, 724-732.

Miller, K. J., Thaloor, D., Matteson, S., and Pavlath, G. K. (2000). Hepatocyte growth factor affects satellite cell activation and differentiation in regenerating skeletal muscle. Am J Physiol Cell Physiol *278*, C174-181.

Minasi, M. G., Riminucci, M., De Angelis, L., Borello, U., Berarducci, B., Innocenzi, A., Caprioli, A., Sirabella, D., Baiocchi, M., De Maria, R., *et al.* (2002). The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. Development *129*, 2773-2783.

Miner, J. H., and Wold, B. (1990). Herculin, a fourth member of the MyoD family of myogenic regulatory genes. Proc Natl Acad Sci U S A *87*, 1089-1093.

Mitchell, C. A., McGeachie, J. K., and Grounds, M. D. (1996). The exogenous administration of basic fibroblast growth factor to regenerating skeletal muscle in mice does not enhance the process of regeneration. Growth Factors *13*, 37-55.

Molkentin, J. D., Black, B. L., Martin, J. F., and Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell *83*, 1125-1136.

Molkentin, J. D., and Olson, E. N. (1996). Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. Proc Natl Acad Sci U S A *93*, 9366-9373.

Montarras, D., Lindon, C., Pinset, C., and Domeyne, P. (2000). Cultured myf5 null and myoD null muscle precursor cells display distinct growth defects. Biol Cell *92*, 565-572.

Moss, F. P., and Leblond, C. P. (1971). Satellite cells as the source of nuclei in muscles of growing rats. Anat Rec *170*, 421-435.

Munsterberg, A. E., and Lassar, A. B. (1995). Combinatorial signals from the neural tube, floor plate and notochord induce myogenic bHLH gene expression in the somite. Development *121*, 651-660.

Murgia, C., Blaikie, P., Kim, N., Dans, M., Petrie, H. T., and Giancotti, F. G. (1998). Cell cycle and adhesion defects in mice carrying a targeted deletion of the integrin beta4 cytoplasmic domain. Embo J *17*, 3940-3951.

Musaro, A., McCullagh, K. J., Naya, F. J., Olson, E. N., and Rosenthal, N. (1999). IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. Nature *400*, 581-585.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., and Nonaka, I. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature *364*, 532-535.

Naeve, G. S., Ramakrishnan, M., Kramer, R., Hevroni, D., Citri, Y., and Theill, L. E. (1997). Neuritin: a gene induced by neural activity and neurotrophins that promotes neuritogenesis. Proc Natl Acad Sci U S A *94*, 2648-2653.

Naidu, P. S., Ludolph, D. C., To, R. Q., Hinterberger, T. J., and Konieczny, S. F. (1995). Myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenesis. Mol Cell Biol *15*, 2707-2718.

Naya, F. J., Wu, C., Richardson, J. A., Overbeek, P., and Olson, E. N. (1999). Transcriptional activity of MEF2 during mouse embryogenesis monitored with a MEF2-dependent transgene. Development *126*, 2045-2052.

Naya, F. S., and Olson, E. (1999). MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. Curr Opin Cell Biol *11*, 683-688.

Nnodim, J. O. (2000). Satellite cell numbers in senile rat levator ani muscle. Mech Ageing Dev *112*, 99-111.

Nutt, S. L., Heavey, B., Rolink, A. G., and Busslinger, M. (1999). Commitment to the B-lymphoid lineage depends on the transcription factor Pax5 [see comments]. Nature *401*, 556-562.

Odelberg, S. J., Kollhoff, A., and Keating, M. T. (2000). Dedifferentiation of Mammalian Myotubes Induced by msx1. Cell *103*, 1099-1109.

Olson, E. N. (1993). Regulation of muscle transcription by the MyoD family. The heart of the matter. Circ Res *72*, 1-6.

Olson, E. N., Arnold, H. H., Rigby, P. W., and Wold, B. J. (1996). Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. Cell *85*, 1-4.

Ontell, M. (1981). Muscle fiber necrosis in murine dystrophy. Muscle Nerve *4*, 204-213.

Ontell, M. (1986). Muscular dystrophy and muscle regeneration. Hum Pathol *17*, 673-682.

Ontell, M., Feng, K. C., Klueber, K., Dunn, R. F., and Taylor, F. (1984). Myosatellite cells, growth, and regeneration in murine dystrophic muscle: a quantitative study. Anat Rec *208*, 159-174.

Ontell, M., Hughes, D., and Bourke, D. (1988). Morphometric analysis of the developing mouse soleus muscle. Am J Anat *181*, 279-288.

Ontell, M., and Kozeka, K. (1984a). The organogenesis of murine striated muscle: a cytoarchitectural study. Am J Anat *171*, 133-148.

Ontell, M., and Kozeka, K. (1984b). Organogenesis of the mouse extensor digitorum logus muscle: a quantitative study. Am J Anat *171*, 149-161.

Ordahl, C. P. (1999). Myogenic shape-shifters [comment]. J Cell Biol *147*, 695-698.

Ordahl, C. P., and Le Douarin, N. M. (1992). Two myogenic lineages within the developing somite. Development *114*, 339-353.

Ordahl, C. P., and Williams, B. A. (1998). Knowing chops from chuck: roasting myoD redundancy. Bioessays *20*, 357-362.

Orimo, S., Hiyamuta, E., Arahata, K., and Sugita, H. (1991). Analysis of inflammatory cells and complement C3 in bupivacaine-induced myonecrosis. Muscle Nerve *14*, 515-520.

Ozawa, E., Noguchi, S., Mizuno, Y., Hagiwara, Y., and Yoshida, M. (1998). From dystrophinopathy to sarcoglycanopathy: evolution of a concept of muscular dystrophy. Muscle Nerve *21*, 421-438.

Pagel, C. N., and Partridge, T. A. (1999). Covert persistence of mdx mouse myopathy is revealed by acute and chronic effects of irradiation. J Neurol Sci *164*, 103-116.

Papadakis, M. A., Grady, D., Black, D., Tierney, M. J., Gooding, G. A., Schambelan, M., and Grunfeld, C. (1996). Growth hormone replacement in healthy older men improves body composition but not functional ability [see comments]. Ann Intern Med *124*, 708-716.

Pardanaud, L., and Dieterlen-Lievre, F. (1999). Manipulation of the angiopoietic/hemangiopoietic commitment in the avian embryo. Development *126*, 617-627.

Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L. M., Catala, M., and Dieterlen-Lievre, F. (1996). Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. Development *122*, 1363-1371.

Park, S. K., Lee, H. S., and Lee, S. T. (1996). Characterization of the human full-length PTK7 cDNA encoding a receptor protein tyrosine kinase-like molecule closely related to chick KLG. J Biochem (Tokyo) *119*, 235-239.

Parker, M. H., Seale, P., and Rudnicki, M. A. (2003). Looking Back to the Embryo: Transcriptional Networks in Adult Myogenesis. Nat Reviews Gen 4:497-507.

Partridge, T. (2000). The current status of myoblast transfer. Neurol Sci *21*, S939-942.

Partridge, T., Lu, Q. L., Morris, G., and Hoffman, E. (1998). Is myoblast transplantation effective? [letter]. Nat Med *4*, 1208-1209.

Partridge, T. A. (2003). Stem cell route to neuromuscular therapies. Muscle Nerve *27*, 133-141.

Partridge, T. A., Morgan, J. E., Coulton, G. R., Hoffman, E. P., and Kunkel, L. M. (1989). Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. Nature *337*, 176-179.

Pastoret, C., and Sebille, A. (1995). mdx mice show progressive weakness and muscle deterioration with age. J Neurol Sci *129*, 97-105.

Patapoutian, A., Yoon, J. K., Miner, J. H., Wang, S., Stark, K., and Wold, B. (1995). Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. Development *121*, 3347-3358.

Penninger, J. M., Irie-Sasaki, J., Sasaki, T., and Oliveira-dos-Santos, A. J. (2001). CD45: new jobs for an old acquaintance. Nat Immunol *2*, 389-396.

Peters, H., Neubuser, A., Kratochwil, K., and Balling, R. (1998). Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. Genes Dev *12*, 2735-2747.

Peterson, R. L., Jacobs, D. F., and Awgulewitsch, A. (1992). Hox-3.6: isolation and characterization of a new murine homeobox gene located in the 5' region of the Hox-3 cluster. Mech Dev *37*, 151-166.

Peterson, R. L., Papenbrock, T., Davda, M. M., and Awgulewitsch, A. (1994). The murine Hoxc cluster contains five neighboring AbdB-related Hox genes that show unique spatially coordinated expression in posterior embryonic subregions. Mech Dev *47*, 253-260.

Petropoulos, H., and Skerjanc, I. S. (2002). Beta -catenin is essential and sufficient for skeletal myogenesis in p19 cells. J Biol Chem *277*, 15393-15399.

Polesskaya, A., Seale, P., and Rudnicki, M. A. (2003). Wnt Signaling Induces the Myogenic Specification of Resident CD45+ Adult Stem Cells during Muscle Regeneration. Cell *113*, 841-852.

Pourquie, O., Coltey, M., Breant, C., and Le Douarin, N. M. (1995). Control of somite patterning by signals from the lateral plate. Proc Natl Acad Sci U S A *92*, 3219-3223.

Pourquie, O., Fan, C. M., Coltey, M., Hirsinger, E., Watanabe, Y., Breant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M., and Le Douarin, N. M. (1996). Lateral and axial signals involved in avian somite patterning: a role for BMP4. Cell *84*, 461-471.

Powell-Braxton, L., Hollingshead, P., Giltinan, D., Pitts-Meek, S., and Stewart, T. (1993a). Inactivation of the IGF-I gene in mice results in perinatal lethality. Ann N Y Acad Sci *692*, 300-301.

Powell-Braxton, L., Hollingshead, P., Warburton, C., Dowd, M., Pitts-Meek, S., Dalton, D., Gillett, N., and Stewart, T. A. (1993b). IGF-I is required for normal embryonic growth in mice. Genes Dev *7*, 2609-2617.

Pownall, M. E., Gustafsson, M. K., and Emerson, C. P., Jr. (2002). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu Rev Cell Dev Biol *18*, 747-783.

Pownall, M. E., Strunk, K. E., and Emerson, C. P., Jr. (1996). Notochord signals control the transcriptional cascade of myogenic bHLH genes in somites of quail embryos. Development *122*, 1475-1488.

Puri, P. L., and Sartorelli, V. (2000). Regulation of muscle regulatory factors by DNA-binding, interacting proteins, and post-transcriptional modifications. J Cell Physiol *185*, 155-173.

Qu, Z., Balkir, L., van Deutekom, J. C., Robbins, P. D., Pruchnic, R., and Huard, J. (1998). Development of approaches to improve cell survival in myoblast transfer therapy. J Cell Biol *142*, 1257-1267.

Qu-Petersen, Z., Deasy, B., Jankowski, R., Ikezawa, M., Cummins, J., Pruchnic, R., Mytinger, J., Cao, B., Gates, C., Wernig, A., and Huard, J. (2002). Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J Cell Biol *157*, 851-864.

Rando, T. A., and Blau, H. M. (1994). Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. J Cell Biol *125*, 1275-1287.

Rath, P., Lin, S., Udolph, G., Cai, Y., Yang, X., and Chia, W. (2002). Inscuteable-independent apicobasally oriented asymmetric divisions in the Drosophila embryonic CNS. EMBO Rep *3*, 660-665.

Reimann, J., Irintchev, A., and Wernig, A. (2000). Regenerative capacity and the number of satellite cells in soleus muscles of normal and mdx mice. Neuromuscul Disord *10*, 276-282.

Relaix, F., Wei, X., Li, W., Pan, J., Lin, Y., Bowtell, D. D., Sassoon, D. A., and Wu, X. (2000). Pw1/Peg3 is a potential cell death mediator and cooperates with Siah1a in p53-mediated apoptosis. Proc Natl Acad Sci U S A *97*, 2105-2110.

Relaix, F., Wei, X. J., Wu, X., and Sassoon, D. A. (1998). Peg3/Pw1 is an imprinted gene involved in the TNF-NFkappaB signal transduction pathway. Nat Genet *18*, 287-291.

Relaix, F., Weng, X., Marazzi, G., Yang, E., Copeland, N., Jenkins, N., Spence, S. E., and Sassoon, D. (1996). Pw1, a novel zinc finger gene implicated in the myogenic and neuronal lineages. Dev Biol *177*, 383-396.

Reshef, R., Maroto, M., and Lassar, A. B. (1998). Regulation of dorsal somitic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression. Genes Dev *12*, 290-303.

Ridgeway, A. G., Petropoulos, H., Wilton, S., and Skerjanc, I. S. (2000a). Wnt signaling regulates the function of MyoD and myogenin. J Biol Chem *275*, 32398-32405.

Ridgeway, A. G., and Skerjanc, I. S. (2001). Pax3 is essential for skeletal myogenesis and the expression of Six1 and Eya2. J Biol Chem *276*, 19033-19039.

Ridgeway, A. G., Wilton, S., and Skerjanc, I. S. (2000b). Myocyte enhancer factor 2C and myogenin upregulate each other's expression and induce the development of skeletal muscle in P19 cells. J Biol Chem *275*, 41-46.

Ringelmann, B., Roder, C., Hallmann, R., Maley, M., Davies, M., Grounds, M., and Sorokin, L. (1999). Expression of laminin alpha1, alpha2, alpha4, and alpha5 chains, fibronectin, and tenascin-C in skeletal muscle of dystrophic 129ReJ dy/dy mice. Exp Cell Res *246*, 165-182.

Robertson, E. J. (1987). Embryo-derived stem cells. In Teratomas and embryonic stem cells: a practical approach, E. J. Robertson, ed. (Oxford, United Kingdom, IRL Press), pp. 71-112.

Rosen, G. D., Sanes, J. R., LaChance, R., Cunningham, J. M., Roman, J., and Dean, D. C. (1992). Roles for the integrin VLA-4 and its counter receptor VCAM-1 in myogenesis. Cell *69*, 1107-1119.

Rosenblatt, J. D., Lunt, A. I., Parry, D. J., and Partridge, T. A. (1995). Culturing satellite cells from living single muscle fiber explants. In Vitro Cell Dev Biol *31*, 773-779.

Rosenblatt, J. D., Yong, D., and Parry, D. J. (1994). Satellite cell activity is required for hypertrophy of overloaded adult rat muscle. Muscle Nerve *17*, 608-613.

Ross, J. J., Duxson, M. J., and Harris, A. J. (1987). Formation of primary and secondary myotubes in rat lumbrical muscles. Development *100*, 383-394.

Rougon, G., and Hobert, O. (2003). New Insights into the Diversity and Function of Neuronal Immunoglobulin Superfamily Molecules. Annu Rev Neurosci.

Rudnicki, M. A., Braun, T., Hinuma, S., and Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell *71*, 383-390.

Rudnicki, M. A., and Jaenisch, R. (1995). The MyoD family of transcription factors and skeletal myogenesis. Bioessays *17*, 203-209.

Rudnicki, M. A., Schnegelsberg, P. N., Stead, R. H., Braun, T., Arnold, H. H., and Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. Cell *75*, 1351-1359.

Sabourin, L. A., Girgis-Gabardo, A., Seale, P., Asakura, A., and Rudnicki, M. A. (1999). Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. J Cell Biol *144*, 631-643.

Sasaki, T., Sasaki-Irie, J., and Penninger, J. M. (2001). New insights into the transmembrane protein tyrosine phosphatase CD45. Int J Biochem Cell Biol *33*, 1041-1046.

Schafer, B. W., Czerny, T., Bernasconi, M., Genini, M., and Busslinger, M. (1994). Molecular cloning and characterization of a human PAX-7 cDNA expressed in normal and neoplastic myocytes. Nucleic Acids Res *22*, 4574-4582.

Schmalbruch, H., and Lewis, D. M. (2000). Dynamics of nuclei of muscle fibers and connective tissue cells in normal and denervated rat muscles. Muscle Nerve *23*, 617-626.

Schultz, E. (1976). Fine structure of satellite cells in growing skeletal muscle. Am J Anat *147*, 49-70.

Schultz, E. (1989). Satellite cell behavior during skeletal muscle growth and regeneration. Med Sci Sports Exerc *21*, S181-186.

Schultz, E. (1996). Satellite cell proliferative compartments in growing skeletal muscles. Dev Biol *175*, 84-94.

Schultz, E., and Jaryszak, D. L. (1985). Effects of skeletal muscle regeneration on the proliferation potential of satellite cells. Mech Ageing Dev *30*, 63-72.

Schultz, E., Jaryszak, D. L., and Valliere, C. R. (1985). Response of satellite cells to focal skeletal muscle injury. Muscle Nerve *8*, 217-222.

Schultz, E., and Lipton, B. H. (1982). Skeletal muscle satellite cells: changes in proliferation potential as a function of age. Mech Ageing Dev *20*, 377-383.

Seale, P., Asakura, A., and Rudnicki, M. A. (2001). The potential of muscle stem cells. Dev Cell *1*, 333-342.

Seale, P., and Rudnicki, M. A. (2000). A new look at the origin, function, and "stem-cell" status of muscle satellite cells. Dev Biol *218*, 115-124.

Seale, P., and Rudnicki, M. A. (2002). Adult Skeletal Muscle Growth and Regeneration. In Stem Cells: A Cellular Fountain of Youth, M. P. Mattson, and G. Van Zant, eds. (New York, Elsevier), pp. 177-200.

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., and Rudnicki, M. A. (2000). Pax7 is required for the specification of myogenic satellite cells. Cell *102*, 777-786.

Semsarian, C., Wu, M. J., Ju, Y. K., Marciniec, T., Yeoh, T., Allen, D. G., Harvey, R. P., and Graham, R. M. (1999). Skeletal muscle hypertrophy is mediated by a Ca2+-dependent calcineurin signalling pathway. Nature *400*, 576-581.

Sheehan, S. M., and Allen, R. E. (1999). Skeletal muscle satellite cell proliferation in response to members of the fibroblast growth factor family and hepatocyte growth factor. J Cell Physiol *181*, 499-506.

Sheehan, S. M., Tatsumi, R., Temm-Grove, C. J., and Allen, R. E. (2000). HGF is an autocrine growth factor for skeletal muscle satellite cells in vitro. Muscle Nerve *23*, 239-245.

Shen, Q., Zhong, W., Jan, Y. N., and Temple, S. (2002). Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. Development *129*, 4843-4853.

Shibasaki, F., Price, E. R., Milan, D., and McKeon, F. (1996). Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. Nature *382*, 370-373.

Shore, E. M., Glaser, D. L., and Gannon, F. H. (2000). Osteogenic induction in hereditary disorders of heterotopic ossification. Clin Orthop, 303-316.

Sicinski, P., Geng, Y., Ryder-Cook, A. S., Barnard, E. A., Darlison, M. G., and Barnard, P. J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science *244*, 1578-1580.

Simpson, T. I., and Price, D. J. (2002). Pax6; a pleiotropic player in development. Bioessays *24*, 1041-1051.

Sleckman, B. P., Khan, W. N., Xu, W., Bassing, C. H., Malynn, B. A., Copeland, N. G., Bardon, C. G., Breit, T. M., Davidson, L., Oltz, E. M., *et al.* (2000). Cloning and functional characterization of the early-lymphocyte-specific Pb99 gene. Mol Cell Biol *20*, 4405-4410.

Smith, C. K., 2nd, Janney, M. J., and Allen, R. E. (1994). Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. J Cell Physiol *159*, 379-385.

Smith, J., and Schofield, P. N. (1997). Stable integration of an mdx skeletal muscle cell line into dystrophic (mdx) skeletal muscle: evidence for stem cell status. Cell Growth Differ *8*, 927-934.

Soneoka, Y., Cannon, P. M., Ramsdale, E. E., Griffiths, J. C., Romano, G., Kingsman, S. M., and Kingsman, A. J. (1995). A transient three-plasmid expression system for the production of high titer retroviral vectors. Nucleic Acids Res *23*, 628-633.

Sorokin, L. M., Pausch, F., Frieser, M., Kroger, S., Ohage, E., and Deutzmann, R. (1997). Developmental regulation of the laminin alpha5 chain suggests a role in epithelial and endothelial cell maturation. Dev Biol *189*, 285-300.

Sosa-Pineda, B., Chowdhury, K., Torres, M., Oliver, G., and Gruss, P. (1997). The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. Nature *386*, 399-402.

Spitz, F., Demignon, J., Porteu, A., Kahn, A., Concordet, J. P., Daegelen, D., and Maire, P. (1998). Expression of myogenin during embryogenesis is controlled by Six/sine oculis homeoproteins through a conserved MEF3 binding site. Proc Natl Acad Sci U S A *95*, 14220-14225.

Stockton, D. W., Das, P., Goldenberg, M., D'Souza, R. N., and Patel, P. I. (2000). Mutation of PAX9 is associated with oligodontia. Nat Genet *24*, 18-19.

Suelves, M., Lopez-Alemany, R., Lluis, F., Aniorte, G., Serrano, E., Parra, M., Carmeliet, P., and Munoz-Canoves, P. (2002). Plasmin activity is required for myogenesis in vitro and skeletal muscle regeneration in vivo. Blood *99*, 2835-2844.

Sykes, D. E., and Weiser, M. M. (1995). Rat intestinal crypt-cell replication factor with homology to early S-phase proteins required for cell division. Gene *163*, 243-247.

Taguchi, A., Wanaka, A., Mori, T., Matsumoto, K., Imai, Y., Tagaki, T., and Tohyama, M. (1996). Molecular cloning of novel leucine-rich repeat proteins and their expression in the developing mouse nervous system. Brain Res Mol Brain Res *35*, 31-40.

Tajbakhsh, S., Bober, E., Babinet, C., Pournin, S., Arnold, H., and Buckingham, M. (1996). Gene targeting the myf-5 locus with nlacZ reveals expression of this myogenic factor in mature skeletal muscle fibres as well as early embryonic muscle. Dev Dyn *206*, 291-300.

Tajbakhsh, S., Borello, U., Vivarelli, E., Kelly, R., Papkoff, J., Duprez, D., Buckingham, M., and Cossu, G. (1998). Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. Development *125*, 4155-4162.

Tajbakhsh, S., and Cossu, G. (1997). Establishing myogenic identity during somitogenesis. Curr Opin Genet Dev *7*, 634-641.

Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax- 3 and Myf-5 act upstream of MyoD. Cell *89*, 127-138.

Tassabehji, M., Newton, V. E., and Read, A. P. (1994). Waardenburg syndrome type 2 caused by mutations in the human microphthalmia (MITF) gene. Nat Genet *8*, 251-255.

Tatsumi, R., Anderson, J. E., Nevoret, C. J., Halevy, O., and Allen, R. E. (1998). HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. Dev Biol *194*, 114-128.

Teboul, L., Gaillard, D., Staccini, L., Inadera, H., Amri, E. Z., and Grimaldi, P. A. (1995). Thiazolidinediones and fatty acids convert myogenic cells into adipose- like cells. J Biol Chem *270*, 28183-28187.

Tidball, J. G. (1995). Inflammatory cell response to acute muscle injury. Med Sci Sports Exerc *27*, 1022-1032.

Torrente, Y., Tremblay, J. P., Pisati, F., Belicchi, M., Rossi, B., Sironi, M., Fortunato, F., El Fahime, M., D'Angelo, M. G., Caron, N. J., *et al.* (2001). Intraarterial injection of muscle-derived CD34(+)Sca-1(+) stem cells restores dystrophin in mdx mice. J Cell Biol *152*, 335-348.

Torres, M., Gomez-Pardo, E., Dressler, G. R., and Gruss, P. (1995). Pax-2 controls multiple steps of urogenital development. Development *121*, 4057-4065.

Tremblay, J. P., Malouin, F., Roy, R., Huard, J., Bouchard, J. P., Satoh, A., and Richards, C. L. (1993). Results of a triple blind clinical study of myoblast transplantations without immunosuppressive treatment in young boys with Duchenne muscular dystrophy. Cell Transplant *2*, 99-112.

Tremblay, P., Dietrich, S., Mericskay, M., Schubert, F. R., Li, Z., and Paulin, D. (1998). A crucial role for Pax3 in the development of the hypaxial musculature and the long-range migration of muscle precursors. Dev Biol *203*, 49-61.

Tzankoff, S. P., and Norris, A. H. (1977). Effect of muscle mass decrease on age-related BMR changes. J Appl Physiol *43*, 1001-1006.

Underhill, D. A. (2000). Genetic and biochemical diversity in the Pax gene family. Biochem Cell Biol *78*, 629-638.

Urbanek, P., Wang, Z. Q., Fetka, I., Wagner, E. F., and Busslinger, M. (1994). Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. Cell *79*, 901-912.

Vaittinen, S., Lukka, R., Sahlgren, C., Hurme, T., Rantanen, J., Lendahl, U., Eriksson, J. E., and Kalimo, H. (2001). The expression of intermediate filament protein nestin as related to vimentin and desmin in regenerating skeletal muscle. J Neuropathol Exp Neurol *60*, 588-597.

van Essen, A. J., Busch, H. F., te Meerman, G. J., and ten Kate, L. P. (1992). Birth and population prevalence of Duchenne muscular dystrophy in The Netherlands. Hum Genet *88*, 258-266.

Vilquin, J. T., Kinoshita, I., Roy, B., Goulet, M., Engvall, E., Tome, F., Fardeau, M., and Tremblay, J. P. (1996). Partial laminin alpha2 chain restoration in alpha2 chain-deficient dy/dy mouse by primary muscle cell culture transplantation. J Cell Biol *133*, 185-197.

Wada, M. R., Inagawa-Ogashiwa, M., Shimizu, S., Yasumoto, S., and Hashimoto, N. (2002). Generation of different fates from multipotent muscle stem cells. Development *129*, 2987-2995.

Wagers, A. J., Sherwood, R. I., Christensen, J. L., and Weissman, I. L. (2002). Little evidence for developmental plasticity of adult hematopoietic stem cells. Science *297*, 2256-2259.

Wakeford, S., Watt, D. J., and Partridge, T. A. (1991). X-irradiation improves mdx mouse muscle as a model of myofiber loss in DMD. Muscle Nerve *14*, 42-50.

Wang, X., Willenbring, H., Akkari, Y., Torimaru, Y., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., Olson, S., and Grompe, M. (2003). Cell fusion is the principal source of bone marrow-derived hepatocytes. Nature *422*, 897-901.

Watt, D. J., Morgan, J. E., Clifford, M. A., and Partridge, T. A. (1987). The movement of muscle precursor cells between adjacent regenerating muscles in the mouse. Anat Embryol *175*, 527-536.

Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S., and et al. (1991a). The myoD gene family: nodal point during specification of the muscle cell lineage. Science *251*, 761-766.

Weintraub, H., Dwarki, V. J., Verma, I., Davis, R., Hollenberg, S., Snider, L., Lassar, A., and Tapscott, S. J. (1991b). Muscle-specific transcriptional activation by MyoD. Genes Dev *5*, 1377-1386.

Williams, B. A., and Ordahl, C. P. (1994). Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. Development *120*, 785-796.

Williams, B. A., and Ordahl, C. P. (1997). Emergence of determined myotome precursor cells in the somite. Development *124*, 4983-4997.

Wilm, B., Dahl, E., Peters, H., Balling, R., and Imai, K. (1998). Targeted disruption of Pax1 defines its null phenotype and proves haploinsufficiency. Proc Natl Acad Sci U S A *95*, 8692-8697.

Wolf, G. (2001). The uncoupling proteins UCP2 and UCP3 in skeletal muscle. Nutr Rev *59*, 56-57.

Wright, W. E. (1985). Myoblast senescence in muscular dystrophy. Exp Cell Res *157*, 343-354.

Yablonka-Reuveni, Z., and Rivera, A. J. (1994). Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. Dev Biol *164*, 588-603.

Yablonka-Reuveni, Z., Rudnicki, M. A., Rivera, A. J., Primig, M., Anderson, J. E., and Natanson, P. (1999a). The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. Dev Biol *210*, 440-455.

Yablonka-Reuveni, Z., Seger, R., and Rivera, A. J. (1999b). Fibroblast growth factor promotes recruitment of skeletal muscle satellite cells in young and old rats. J Histochem Cytochem *47*, 23-42.

Yamaguchi, A., Taniguchi, M., Hori, O., Ogawa, S., Tojo, N., Matsuoka, N., Miyake, S., Kasai, K., Sugimoto, H., Tamatani, M., *et al.* (2002). Peg3/Pw1 is involved in p53-mediated cell death pathway in brain ischemia/hypoxia. J Biol Chem *277*, 623-629.

Yang, J. T., Rando, T. A., Mohler, W. A., Rayburn, H., Blau, H. M., and Hynes, R. O. (1996a). Genetic analysis of alpha 4 integrin functions in the development of mouse skeletal muscle. J Cell Biol *135*, 829-835.

Yang, X. M., Vogan, K., Gros, P., and Park, M. (1996b). Expression of the met receptor tyrosine kinase in muscle progenitor cells in somites and limbs is absent in Splotch mice. Development *122*, 2163-2171.

Ying, Q. L., Nichols, J., Evans, E. P., and Smith, A. G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545-548.

Yoshida, N., Yoshida, S., Koishi, K., Masuda, K., and Nabeshima, Y. (1998). Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. J Cell Sci *111*, 769-779.

Zambrowicz, B. P., Imamoto, A., Fiering, S., Herzenberg, L. A., Kerr, W. G., and Soriano, P. (1997). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. Proc Natl Acad Sci U S A *94*, 3789-3794.

Zammit, P. S., Heslop, L., Hudon, V., Rosenblatt, J. D., Tajbakhsh, S., Buckingham, M. E., Beauchamp, J. R., and Partridge, T. A. (2002). Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. Exp Cell Res *281*, 39-49.

Zeschnigk, M., Kozian, D., Kuch, C., Schmoll, M., and Starzinski-Powitz, A. (1995). Involvement of M-cadherin in terminal differentiation of skeletal muscle cells. J Cell Sci *108*, 2973-2981.

Zhang, W., Behringer, R. R., and Olson, E. N. (1995). Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. Genes Dev *9*, 1388-1399.

Ziman, M. R., and Kay, P. H. (1998). Differential expression of four alternate Pax7 paired box transcripts is influenced by organ- and strain-specific factors in adult mice. Gene *217*, 77-81.