MOLECULAR AND CELLULAR REGULATION OF STEM CELLS DURING MUSCLE REGENERATION

by

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ABSTRACT

Skeletal muscle is among the few adult tissues with the capacity to regenerate after injury. This is due to the resident stem cells within the muscle, the satellite cells. In the absence of injury, these stem cells remain quiescent and reside within their niche beneath the basement membrane adjacent to the myofiber. Upon muscle injury, satellite cells are activated and will proliferate, self-renew, and differentiate into transiently amplifying myoblasts, which also self renew and give rise to differentiating myocytes. These myocytes will fuse to themselves and to the injured myofibers to repair muscle damage. While the cellular processes of muscle regeneration are understood, many questions remain. Despite similarities in their expression patterns and function, many characteristics are dissimilar between developmental myogenic precursors and satellite cells. Chapter 2 of this dissertation reviews what is known about the unique properties of these closely related cells, and Chapter 3 of this dissertation directly tests the requirement of satellite cells during muscle regeneration. In addition to the myogenic cells, many nonmuscle cells are involved in the process of muscle regeneration. Chapter 3 of this dissertation also shows that connective tissue fibroblasts prevent premature differentiation of satellite cells and are an important component of the satellite cell niche. One signaling pathway shown to regulate stem cells in other tissue contexts is the Wnt/β-catenin pathway. Multiple studies describe the role of Wnt/β-catenin signaling in muscle regeneration; however, there is no consensus as to the

functional role of this signaling pathway in adult myogenesis. In Chapter 4 of this dissertation, the requirement for Wnt/β-catenin in the satellite cells and their progeny is tested *in vivo*. Surprisingly, despite evidence that the Wnt/β-catenin signaling pathway is active in myogenic cells during regeneration, satellite cells and their progeny do not require β-catenin. Chapter 4 also discusses our results that show that extension of Wnt/β-catenin signaling prolongs the time myogenic cells spend in the myoblast phase of regeneration. This dissertation demonstrates the importance of the connective tissue fibroblasts and also critically tests the function of the Wnt/β-catenin signaling pathway in muscle regeneration.

This dissertation is dedicated to my family, my parents

and sister, as well as my lab family.

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CHAPTER 1

INTRODUCTION

Phases of myogenesis

Myogenesis proceeds in successive, distinct, but overlapping phases (Biressi et al., 2007a; Stockdale, 1992). Each phase involves specification of myoblasts, which express the myogenic regulatory factors (MRFs) *Myf5*, *MyoD*, and/or *Mrf4*; differentiation of committed mononuclear myocytes, which express myogenin (MyoG); fusion of myocytes into multinucleate myofibers; and finally maturation of myofibers, including the expression of different myosin heavy chain (MyHC) isoforms giving myofibers different speeds of contraction. During embryonic myogenesis [embryonic day (E)10.5-E12.5], embryonic myoblasts differentiate into primary myofibers to establish the initial pattern of axial and limb muscles. In axial muscles, embryonic myogenesis includes both the formation of the primary myotome and muscle derived from the initial translocating dermomyotomal cells. During fetal myogenesis (E14.5-E17.5), fetal myoblasts give rise to secondary myofibers, resulting in muscle growth and fiber type maturation. Neonatal myogenesis occurs during postnatal growth [postnatal day (P)0-P21]. During this rapid postnatal growth, progenitors that lie beneath the basal lamina contribute to muscle growth, and the final MyHC composition is patterned. Adult myogenesis occurs after muscle injury and is mediated by satellite cells, the adult muscle

stem cell. In response to muscle injury, quiescent satellite cells become activated, proliferate, and differentiate into myoblasts that contribute to the regenerating myofibers, repairing muscle damage. The phases of myogenesis were initially recognized and classified based on the distinctive *in vitro* characteristics of embryonic, fetal, and adult myoblasts. These classes of myoblasts differ in their appearance, media requirements, response to extrinsic signaling molecules, drug sensitivity, and morphology of the myofibers they generate (Biressi et al., 2007a; Stockdale, 1992). *In vivo*, the primary, secondary, and adult myofibers express different MyHCs and muscle enzymes (Gunning and Hardeman, 1991; Wigmore and Evans, 2002). More recently, microarray studies and genetic analyses of the *Pax3/7* and *Myf5/MyoD/Mrf4* families of transcription factors have revealed that different classes of myoblasts express different genes and have different genetic requirements for myogenic specification (Biressi et al., 2007b; Kassar-Duchossoy et al., 2004; Kassar-Duchossoy et al., 2005; Relaix et al., 2006). Together these data strongly argue that embryonic, fetal, and adult myoblasts are distinct populations of myogenic cells. The differences between progenitors at these different phases of myogenesis will be discussed in depth in Chapter 2 of this dissertation.

Satellite cells

Satellite cells are the adult muscle progenitors responsible for muscle regeneration. These cells were identified and named due to their unusual location beneath the basal lamina just peripheral to the myofiber membrane (Mauro, 1961). Since their initial discovery 50 years ago several molecular markers for satellite cells have been identified. One of the most robust and specific markers is the paired box transcription

factor Pax7. Pax7 is expressed by myogenic progenitors during development (Relaix et al., 2004). At birth, progenitors located beneath the basal lamina also express Pax7. These Pax7+ cells contribute to postnatal muscle growth and become the satellite cells within the adult muscle. Pax7 is expressed in quiescent, activated, and proliferating satellite cells (Seale et al., 2000). Normally the satellite cells are quiescent; however, in response to muscle injury these cells become activated and begin to proliferate. A subset of these cells will go back to repopulate the niche, while others will continue to proliferate and differentiate into myoblasts, which express MRFs such as MyoD. The myoblasts give rise to MyoG+ myocytes that fuse to injured myofibers or to each other to repair muscle damage (as reviewed in Hawke and Garry, 2001). Using transplantation experiments, several studies have shown that satellite cells are capable of regenerating muscle, and when single isolated satellite cells are transplanted into muscle they can to give rise to 21,000 – 84,000 cells, which is the equivalent of 14-17 doublings (Collins et al., 2005; Sacco et al., 2008). Therefore, satellite cells are sufficient to regenerate muscle. Satellite cells have been the presumed mediator of muscle regeneration; however, recently it has been shown that other populations of cells such as mesangioblasts and PW1+/Pax7- interstitial cells also have the ability to contribute to muscle regeneration (Mitchell et al., 2010; Sampaolesi et al., 2003). Despite the multitude of studies showing that satellite cells are sufficient to regenerate muscle, their necessity has never explicitly tested. In Chapter 3 of this dissertation, I will discuss my work showing that satellite cells are indispensible for muscle regeneration and are the muscle stem cell responsible for regeneration.

Nonmyogenic cell types involved in muscle regeneration

The complicated process of muscle regeneration involves the coordination of multiple cell types within the muscle tissue that respond to injury and regulate the myogenic cells as well as perform other functions necessary for efficient regeneration. For example, there are resident macrophages within the interstitium of the muscle. Upon injury, resident macrophages are activated and circulating monocytes are recruited from the blood to the site of injury to clear myofiber debris. During regeneration, macrophages also send signals to satellite cells directing cell migration, proliferation, and differentiation (as reviewed in Tidball and Villalta, 2010). Another tissue within the muscle, the connective tissue, plays an important role in muscle structure and organization and is also a vital component of the regeneration process. In addition to transmitting the muscle contractile force to tendon and bone, the connective tissue surrounds and hierarchically organizes the muscle into single fibers, fascicles, and whole anatomical muscles. The connective tissue consists of both the extracellular matrix proteins such as collagens, laminins, and fibronectin as well as the connective tissue fibroblasts that secrete this matrix (Kuhl et al., 1982; Lipton, 1977). Following injury, there is a transient increase in extracellular matrix called fibrosis. This fibrosis prevents further injury to the muscle, creates a scaffold for the regenerating muscle, and provides a substrate for migration and cell-cell interactions between satellite cells themselves and with other cell populations (Grounds, 2008; Huard et al., 2002). Regulation of fibrosis is critical since overproduction or lack of resolution of this process is a common and detrimental feature of muscle diseases and/or deficient regeneration. Despite the clinical relevance of fibrosis, little is known about the connective tissue fibroblasts that secrete

this matrix. I explicitly test the functional requirement of connective tissue fibroblasts during muscle regeneration in Chapter 3 of this dissertation.

The connective tissue fibroblasts of the limb originate from the lateral plate mesoderm and express Tcf4, a member of the TCF/LEF family of transcription factors (Kardon et al., 2003). During development, the Tcf4+ connective tissue fibroblasts are in close proximity to and regulate the maturation of muscle (Kardon et al., 2003; Mathew et al., 2011). In Chapter 3 of this dissertation I will discuss my work showing that Tcf4 is a specific marker for the connective tissue fibroblasts in the adult. I will also show that during regeneration the connective tissue fibroblasts prevent the premature differentiation of satellite cells to allow for accumulation of an adequate pool of progenitors to efficiently regenerate the muscle.

Wnt signaling in myogenesis

Wnt signaling is an important and conserved signaling pathway that is important for both development and regeneration in many systems. Wnt proteins are lipid modified, secreted ligands that bind to the Frizzled (Fz) and LRP5/6 transmembrane receptors to mediate signaling (as reviewed in Clevers and Nusse, 2012). Wnt/β-catenin pathway signaling is regulated by the level of β-catenin protein in the cytoplasm. In the absence of Wnt ligand, β-catenin is phosphorylated and targeted for proteasomal degradation by the destruction complex. This complex consists of the core components GSK3β, APC, and Axin. The binding of Wnt ligand to the Fz/LRP co-receptors results in interactions between LRP, Dishevelled (Dsh), and members of the destruction complex, preventing the degradation of β-catenin. β-catenin accumulates in the cytoplasm and translocates to

the nucleus where it binds to TCF/LEF transcription factors and activates transcription of target genes. In addition to the Wnt/β-catenin pathway, Wnt ligands can also bind Fz receptors to activate the Planar Cell Polarity (PCP) pathway as well as the Wnt Ca^{2+} pathway (Veeman et al., 2003). Although some Wnt ligands, such as Wnt3a, are thought to exclusively activate the β-catenin dependent pathway, most Wnts ligands are more promiscuous and can activate multiple Wnt pathways in a context dependent manner (Chien et al., 2009).

The different phases of myogenesis have different requirements for Wnt/β-catenin signaling. Wnt/β-catenin is required during axial myogenesis as dermomyotome formation is disrupted in *Wnt1*$^{-/-}$*; Wnt3*$^{-/-}$ mice (Ikeya and Takada, 1998; Linker et al., 2003). During limb development, β-catenin is required for delamination of myogenic progenitors from the dermomyotome and migration into the limb; however, once in the limb, embryonic progenitors do not require β-catenin for myoblast specification or myofiber differentiation. β-catenin does determine the number of fetal myogenic progenitors and the number and fiber type of differentiated myofibers (Hutcheson et al., 2009). These results demonstrate that embryonic and fetal myogenesis in the limb are developmentally distinct and have different cell-autonomous requirements for β-catenin.

Despite many studies examining Wnt/β-catenin signaling in muscle regeneration there is no clear consensus for the function of this pathway, and this is due to several experimental factors. Many studies use the immortalized C2C12 cell line (Bernardi et al., 2011; Gavard et al., 2004; Goichberg et al., 2001; Han et al., 2011; Kim et al., 2008; Pansters et al., 2011; Tanaka et al., 2011), and although this cell line was originally derived from satellite cells, their expression profile is very similar to fetal myoblasts

(Biressi et al., 2007a). Many studies examine the expression pattern of Wnt pathway components in whole muscle, and while this is *in vivo*, it is not specific to the myogenic cells, as other cells within the muscle tissue are likely responding to Wnt/β-catenin signaling (Abiola et al., 2009; Armstrong and Esser, 2005; Aschenbach et al., 2006; Brack et al., 2008; Kim et al., 2006; Le Grand et al., 2009; Polesskaya et al., 2003). The best evidence supporting the role of Wnt/β-catenin signaling in muscle regeneration is also plagued by similar problems. The injection or electroporation of Wnt3a into regenerating muscle increases fibrosis and decreases fiber size (Brack et al., 2008; Le Grand et al., 2009); however, it is unknown whether these effects are specific to myogenic cells. Therefore, we decided to rigorously test the role of Wnt/β-catenin signaling in myogenic cells during muscle regeneration. In Chapter 4 of this dissertation, I will discuss our results that show Wnt/β-catenin signaling is active but not required for muscle regeneration, and that constitutively active β-catenin in satellite cells delays myoblast differentiation and extends the time required for muscles to regenerate.

In whole, this dissertation determines the requirement for both satellite cells and connective tissue fibroblasts during muscle regeneration. It also tests the necessity of Wnt/β-catenin signaling in satellite cells for stem cell function and muscle regeneration, and determines the consequence of continued β-catenin signaling during muscle regeneration.

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CHAPTER 2

ORIGIN OF VERTEBRATE LIMB MUSCLE: THE ROLE OF PROGENITOR AND MYOBLAST POPULATIONS

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CHAPTER ONE

Origin of Vertebrate Limb Muscle: The Role of Progenitor and Myoblast Populations

Malea Murphy *and* Gabrielle Kardon

Contents

Abstract

Muscle development, growth, and regeneration take place throughout vertebrate life. In amniotes, myogenesis takes place in four successive, temporally distinct, although overlapping phases. Understanding how embryonic, fetal, neonatal, and adult muscle are formed from muscle progenitors and committed myoblasts is an area of active research. In this review we examine recent expression, genetic loss-of-function, and genetic lineage studies that have been conducted in the mouse, with a particular focus on limb myogenesis. We synthesize these studies to present a current model of how embryonic, fetal, neonatal, and adult muscle are formed in the limb.

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1. Introduction

Muscle development, growth, and regeneration take place throughout vertebrate life. In amniotes, myogenesis takes place in successive, temporally distinct, although overlapping phases. Muscle produced during each of these phases is morphologically and functionally different, fulfilling different needs of the animal (reviewed in Biressi *et al.*, 2007a; Stockdale, 1992). Of intense interest is understanding how these different phases of muscle arise. Because differentiated muscle is postmitotic, muscle is generated from myogenic progenitors and committed myoblasts, which proliferate and differentiate to form muscle. Therefore, research has focused on identifying myogenic progenitors and myoblasts and their developmental origin, defining the relationship between different progenitor populations and myoblasts, and determining how these progenitors and myoblasts give rise to different phases of muscle. In this review, we will give an overview of recent expression, genetic loss-of-function, and genetic lineage studies that have been conducted in mouse, with particular focus on limb myogenesis, and synthesize these studies to present a current model of how different populations of progenitors and myoblasts give rise to muscle throughout vertebrate life.

2. Myogenesis Overview

In vertebrates, all axial and limb skeletal muscle derives from progenitors originating in the somites (Emerson and Hauschka, 2004). These progenitors arise from the dorsal portion of the somite, the dermomyotome. The limb muscle originates from limb-level somites, and cells delaminate from the ventrolateral lip of the dermomyotome and migrate into the limb, by embryonic day (E) 10.5 (in forelimb, slightly later in hindlimb). Once in the limb, these cells proliferate and give rise to two types of cells: muscle or endothelial (Hutcheson *et al.*, 2009; Kardon *et al.*, 2002). Thus, the fate of these progenitors only becomes decided once they are in the limb. Those cells destined for a muscle fate then undergo the process of myogenesis. During myogenesis, the progenitors become specified and determined as myoblasts, which in turn differentiate into postmitotic mononuclear myocytes, and these myocytes fuse to one another to form multinucleated myofibers (Emerson and Hauschka, 2004).

Myogenic progenitors, myoblasts, myocytes, and myofibers critically express either Pax or myogenic regulatory factor (MRF) transcription factors. A multitude of studies have shown that progenitors in the somites and in the limb express the paired domain transcription factors Pax3 and Pax7 (reviewed in Buckingham, 2007). Subsequently, determined

myoblasts, myocytes, and myofibers in the somite and in the limb express members of the MRF family of bHLH transcription factors. The MRFs consist of four proteins: Myf5, MyoD, Mrf4 (Myf6), and Myogenin. These factors were originally identified by their *in vitro* ability to convert 10T1/2 fibroblasts to a myogenic fate (Weintraub *et al.*, 1991). Myf5, MyoD, and Mrf4 are expressed in myoblasts (Biressi *et al.*, 2007b; Kassar-Duchossoy *et al.*, 2005; Ontell *et al.*, 1993a,b; Ott *et al.*, 1991; Sassoon *et al.*, 1989), while Myogenin is expressed in myocytes (Ontell *et al.*, 1993a,b; Sassoon *et al.*, 1989). In addition, MyoD, Mrf4, and Myogenin are all expressed in the myonuclei of differentiated myofibers (Bober *et al.*, 1991; Hinterberger *et al.*, 1991; Ontell *et al.*, 1993a,b; Sassoon *et al.*, 1989; Voytik *et al.*, 1993). Identification of these molecular markers of the different stages of myogenic cells has been essential for reconstructing how myogenesis occurs.

In amniotes, there are four successive phases of myogenesis (Biressi *et al.*, 2007a; Stockdale, 1992). In the limb, embryonic myogenesis occurs between E10.5 and E12.5 in mouse and establishes the basic muscle pattern. Fetal (E14.5–P0; P, postnatal day) and neonatal (P0–P21) myogenesis are critical for muscle growth and maturation. Adult myogenesis (after P21) is necessary for postnatal growth and repair of damaged muscle. Each one of these phases involves proliferation of progenitors, determination and commitment of progenitors to myoblasts, differentiation of myocytes, and fusion of myocytes into multinucleate myofibers. The progenitors in embryonic and fetal muscle are mononuclear cells lying interstitial to the myofibers. After birth, the neonatal and adult progenitors adopt a unique anatomical position and lie in between the plasmalemma and basement membrane of the adult myofibers and thus are termed satellite cells (Mauro, 1961). During embryonic myogenesis, embryonic myoblasts differentiate into primary fibers, while during fetal myogenesis fetal myoblasts both fuse to primary fibers and fuse to one another to make secondary myofibers. During fetal and neonatal myogenesis, myofiber growth occurs by a rapid increase in myonuclear number, while in the adult myofiber hypertrophy can occur in the absence of myonuclear addition (White *et al.*, 2010).

Embryonic, fetal, and adult myoblasts and myofibers are distinctive. The different myoblast populations were initially identified based on their *in vitro* characteristics. Embryonic, fetal, and adult myoblasts differ in culture in their appearance, media requirements, response to extrinsic signaling molecules, drug sensitivity, and morphology of myofibers they generate (summarized in Table 1.1; Biressi *et al.*, 2007a; Stockdale, 1992). Recent microarray studies also demonstrate that embryonic and fetal myoblasts differ substantially in their expression of transcription factors, cell surface receptors, and extracellular matrix proteins (Biressi *et al.*, 2007b). It presently is unclear whether neonatal myoblasts differ substantially from fetal myoblasts. Differentiated primary, secondary, and adult myofibers also differ, primarily in their expression of muscle contractile proteins, including

Table 1.1 Summary of characteristics of embryonic, fetal, and adult myoblasts and myofibers

	Culture appearance and clonogenicity	Signaling molecule response	Drug sensitivity	Myofiber morphology in culture
Embryonic myoblasts	Elongated, prone to differentiate and form small colonies, do not spontaneously contract in culture	Differentiation insensitive to TGFβ-1 or BMP4	Differentiation insensitive to phorbol esters (TPA), sensitive to merocynine 540	Mononucleated myofibers or myofibers with few nuclei
Fetal myoblasts	Triangular, proliferate (to limited extent) in response to growth factors, spontaneously contract in culture	Differentiation blocked by TGFβ-1 and BMP4	Differentiation sensitive to phorbol esters (TPA)	Large, multinucleated myofibers
Satellite cells/ Adult myoblasts	Round, clonogenic, but undergo senescence after a limited number of passages, spontaneously contract in culture	Differentiation blocked by TGFβ-1 and BMP4	Differentiation sensitive to phorbol esters (TPA)	Large, multinucleated myofibers

All from Biressi *et al.* (2007b) or review of Biressi *et al.* (2007a).

	MyHCemb	MyHCperi	MyHCI	MyHCIIa	MyHCIIx	MyHCIIb
Embryonic myofibers	+	−	+	−	−	−
Fetal myofibers	+	+	+/−	+/−	+/−	+/−
Adult myofibers	−	−	−	+	+	+

Derived from Agbulut *et al.* (2003), Gunning and Hardeman (1991), Lu *et al.* (1999), Rubinstein and Kelly (2004), and Schiaffino and Reggiani (1996).

isoforms of myosin heavy chain (MyHC), myosin light chain, troponin, and tropomyosin, as well as metabolic enzymes (MyHC differences are summarized in Table 1.1; Agbulut *et al.*, 2003; Biressi *et al.*, 2007b; Gunning and Hardeman, 1991; Lu *et al.*, 1999; Rubinstein and Kelly, 2004; Schiaffino and Reggiani, 1996).

The finding that myogenesis occurs in successive phases and that embryonic, fetal, neonatal, and adult muscle are distinctive raises the question of how these different types of muscle arise. Potentially, these muscle types arise from different progenitors or alternatively from different myoblasts. Another possibility is that the differences in muscle arise during the process of differentiation of myoblasts into myocytes and myofibers. In addition, there is the overlying question of whether differences arise because of intrinsic changes in the myogenic cells or whether changes in the extrinsic environment are regulating myogenic cells.

Five theoretical, simplistic models could explain how these different types of muscle arise (Fig. 1.1). In these models, we have combined fetal and neonatal muscle into one group. (While embryonic and adult muscle are clearly distinct, the distinction between fetal and neonatal muscle is not so clear. Other than birth of the animal, fetal and neonatal muscle appear not to be discrete, but rather to be a gradually changing population of myogenic cells). In the first theoretical model, three different progenitor populations give rise to three distinct myoblast populations and these myoblasts, in turn, give rise to the different types of muscle. In this model, all differences in muscle could simply reflect initial intrinsic heterogeneities in the original progenitor populations, and it will be critical to understand the mechanisms that generate different types of progenitors. A second model is that all muscle derives from a progenitor population that changes over time to give rise to three different populations of myoblasts, and these different myoblast populations give rise to different types of muscle. In this model, the interesting question is understanding what intrinsic or extrinsic factors regulate changes in the progenitor population. In the third model there is a single invariant progenitor population which gives rise to three initially similar myoblast populations. These myoblast populations change over time such that they give rise to different muscle types. In this scenario, understanding the intrinsic or extrinsic factors that lead to differences in myoblasts will be important. In the fourth model, there is a single invariant progenitor population which gives rise to an initial myoblast population. This initial myoblast population both gives rise to embryonic muscle and gives rise to a successive series of myoblast populations. These gradually differing myoblasts then give rise to different types of muscle. Here, differences in muscle arise entirely from differences in the myoblast populations, and so it will be critical to ascertain the intrinsic and extrinsic factors altering the myoblasts. In the final model, a single invariant progenitor population gives rise to a single myoblast population. Subsequently, in the process of myoblast

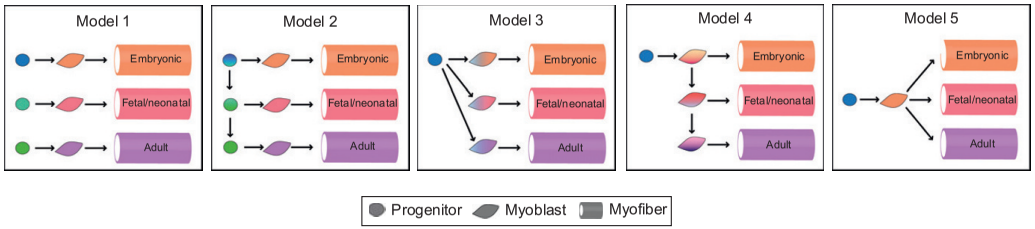

Figure 1.1 Five theoretical models describing derivation of embryonic, fetal/neonatal, and adult limb muscle in mouse.

differentiation differences arise so that different muscle types are generated. However, this final model is unlikely to be correct because, as described above, it is well established that different myoblast populations are present and identifiable. It should be noted that a common component of all of these models is the assumption, currently made by most muscle researchers, that progenitors give rise to myoblasts and that myoblasts give rise to differentiated muscle and that this progression is irreversible. In all likelihood, myogenesis is considerably more complex than these five models. We present these models simply as a starting point to evaluate current data.

In this review, we will discuss what is known about the Pax3/7 and MRF family of transcription factors and how these data allow us to construct a model of muscle development. We focus on Pax3 and 7 and the MRFs because these both mark different myogenic populations and are functionally critical for myogenesis. We will limit our discussion to studies conducted in mouse, largely because of the availability of genetic tools available to conduct lineage, cell ablation, and conditional mutagenesis experiments (Hutcheson and Kardon, 2009). In addition, we will concentrate on myogenesis in the limb because all phase of myogenesis—embryonic, fetal/neonatal, and adult—have been studied in the limb. For discussions of myogenic progenitors in other model organisms, such as chick and zebrafish, and in the head and trunk, we refer the reader to several excellent recent reviews (Buckingham and Vincent, 2009; Kang and Krauss, 2010; Otto *et al.*, 2009; Relaix and Marcelle, 2009; Tajbakhsh, 2009)

3. Expression Analyses of Pax3/7 and MRF Transcription Factors

Multiple expression studies have established that Pax3 and Pax7 label muscle progenitors (summarized in Table 1.2). Both Pax3 and Pax7 are initially expressed in the somites. Pax3 is first expressed (beginning at E8) in the presomitic mesoderm as somites form, but is progressively restricted, first to the dermomyotome and later to dorsomedial and ventrolateral dermomyotomal lips (Bober *et al.*, 1994; Goulding *et al.*, 1994; Horst *et al.*, 2006; Schubert *et al.*, 2001; Tajbakhsh and Buckingham, 2000). Pax7 expression initiates later (beginning at E9) in the somites and is expressed in the dermomyotome, with highest levels in the central region of the dermomyotome (Horst *et al.*, 2006; Jostes *et al.*, 1990; Kassar-Duchossoy *et al.*, 2005; Relaix *et al.*, 2004). In the limb, Pax3+ progenitors are transiently present between E10.5 and E12.5 (Bober *et al.*, 1994). Although Pax3 is generally not expressed in association with muscle after E12.5, some adult satellite cells have been reported to express Pax3 (Conboy and Rando, 2002; Relaix *et al.*, 2006). Unlike Pax3 (and unlike

Table 1.2 Summary of Pax3, Pax7, Myf5, MyoD, Myogenin, and Mrf4 expression in embryonic, fetal/neonatal, and adult progenitors, myoblasts, and myofibers

	Pax3	Pax7	Myf5	MyoD	Mrf4	Myogenin
Embryonic progenitors	+ (reviewed in Buckingham, 2007; Relaix *et al.*, 2004)	+ (reviewed in Buckingham, 2007; Relaix *et al.*, 2004)	–	–	–	–
Embryonic myoblasts/ myocytes	–	–	+ (myoblasts) (Biressi *et al.*, 2007a,b; Kassar-Duchossoy *et al.*, 2005; Ott *et al.*, 1991)	+ (myoblasts) (Ontell *et al.*, 1993a; Sassoon *et al.*, 1989)	–/?	+ (myocytes) (Ontell *et al.*, 1993a; Sassoon *et al.*, 1989)
Embryonic myofibers	–	–	–	+ (Ontell *et al.*, 1993a; Sassoon *et al.*, 1989)	– (Bober *et al.*, 1991)	+ (Ontell *et al.*, 1993a; Sassoon *et al.*, 1989)
Fetal progenitors	–	+ (reviewed in Buckingham, 2007; Relaix *et al.*, 2004)	–	–	–	–
Fetal myoblasts/ myocytes	–	–	+ (myoblasts) (Biressi *et al.*, 2007a,b; Kassar-Duchossoy *et al.*, 2005)	+ (myoblasts) (Ontell *et al.*, 1993b)	–	+ (myocytes) (Ontell *et al.*, 1993b)

Fetal myofibers	−	−	−	+ (Ontell *et al.*, 1993b)	+ (Bober *et al.*, 1991)	+ (Ontell *et al.*, 1993b)
Adult progenitors	+/− (Conboy and Rando, 2002; Relaix *et al.*, 2006)	+ (Seale *et al.*, 2000)	+/− (Beauchamp *et al.*, 2000; Cornelison and Wold, 1997; Kuang *et al.*, 2007)	− (Cornelison and Wold, 1997; Kanisicak *et al.*, 2009; Yablonka-Reuveni *et al.*, 1999)	− (Cornelison and Wold, 1997; Gayraud-Morel *et al.*, 2007)	− (Cornelison and Wold, 1997)
Adult myoblasts/ myocytes	−	−	+ (myoblasts) (Cornelison and Wold, 1997; Kuang *et al.*, 2007)	+ (myoblasts) (Cornelison and Wold, 1997; Kanisicak *et al.*, 2009; Yablonka-Reuveni and Rivera, 1994)	+/− (Cornelison and Wold, 1997; Gayraud-Morel *et al.*, 2007)	+ (myocytes) (Cornelison and Wold, 1997; Yablonka-Reuveni and Rivera, 1994)
Adult myofibers	−	−	− (Hinterberger *et al.*, 1991; Voytik *et al.*, 1993)	+ (Hinterberger *et al.*, 1991; Kanisicak *et al.*, 2009; Voytik *et al.*, 1993)	++ (Gayraud-Morel *et al.*, 2007; Haldar *et al.*, 2008; Hinterberger *et al.*, 1991; Voytik *et al.*, 1993)	+ (Hinterberger *et al.*, 1991; Voytik *et al.*, 1993)

in the chick), Pax7 is not expressed in progenitors in the limb until E11.5 and then continues to be expressed in fetal and neonatal muscle (Relaix *et al.*, 2004). In the adult, Pax7 labels all satellite cells (Seale *et al.*, 2000). Much of this analysis of Pax3 and Pax7 expression has been based on RNA *in situ* hybridization and immunofluorescence. In addition, a variety of reporter alleles (both "knock-ins" and transgenes) have been developed to genetically mark Pax3+ and Pax7+ cells: $Pax3^{IRESnLacZ}$, $Pax3^{GFP}$, $Pax7^{LacZ}$, $Pax7^{nGFP}$, $Pax7^{nLacZ}$ (Mansouri *et al.*, 1996; Relaix *et al.*, 2003, 2005; Sambasivan *et al.*, 2009). These alleles have been extremely useful, as they can increase the sensitivity of detection of Pax3+ and Pax7+ cells. Nevertheless, these reporters should be used with care because, as has been often noted, the stability of the of reporter does necessarily not match the stability of the endogenous protein. For instance, the Pax3 protein is tightly regulated by ubiquitination and proteasomal degradation (Boutet *et al.*, 2007), and it has been shown that the GFP from the $Pax3^{GFP}$ allele is expressed similarly to Pax3, but perdures longer than the endogenous Pax3 protein (Relaix *et al.*, 2004).

The MRFs are expressed in myoblasts, myocytes, and myofibers in different phases of limb myogenesis (summarized in Table 1.2). Myf5, MyoD, Mrf4, and Myogenin are all first expressed in somitic cells (Bober *et al.*, 1991; Ott *et al.*, 1991; Sassoon *et al.*, 1989; Tajbakhsh and Buckingham, 2000). However, somitic cells migrating into the limb do not initially express the MRFs (Tajbakhsh and Buckingham, 1994). Myf5 and MyoD are the earliest MRFs expressed in the developing limb. Myf5 is expressed at E10.5 in embryonic myoblasts and continues to be expressed in fetal and adult myoblasts (Biressi *et al.*, 2007b; Cornelison and Wold, 1997; Kassar-Duchossoy *et al.*, 2005; Kuang *et al.*, 2007; Ott *et al.*, 1991). Myf5 is also expressed in many, but not all adult quiescent satellite cells (Beauchamp *et al.*, 2000; Cornelison and Wold, 1997; Kuang *et al.*, 2007). Unlike the other MRFs, Myf5 expression is limited to myoblasts (or adult progenitors), as it is downregulated in differentiated myogenic cells. MyoD also begins to be expressed in the limb at E10.5 in embryonic myoblasts and myofibers (Ontell *et al.*, 1993a; Sassoon *et al.*, 1989), and subsequently is also expressed in fetal and adult myoblasts and myofibers (Cornelison and Wold, 1997; Hinterberger *et al.*, 1991; Kanisicak *et al.*, 2009; Ontell *et al.*, 1993b; Voytik *et al.*, 1993; Yablonka-Reuveni and Rivera, 1994). Unlike Myf5, MyoD rarely appears to be expressed in quiescent satellite cells (Cornelison and Wold, 1997; Yablonka-Reuveni and Rivera, 1994; Zammit *et al.*, 2002). Myogenin is expressed in the limb by E11.5 (Ontell *et al.*, 1993a; Sassoon *et al.*, 1989) and is primarily found in differentiated myocytes and myofibers of embryonic, fetal, and adult muscle (Cornelison and Wold, 1997; Hinterberger *et al.*, 1991; Ontell *et al.*, 1993a,b; Sassoon *et al.*, 1989; Voytik *et al.*, 1993; Yablonka-Reuveni and Rivera, 1994). Mrf4 is the last MRF to be expressed in the limb. It is first expressed in the limb at E13.5, with stronger expression in fetal myofibers by E16.5, and continues to be expressed as the predominant MRF in adult myofibers (Bober *et al.*, 1991; Gayraud-Morel *et al.*, 2007; Haldar *et al.*, 2008; Hinterberger *et al.*, 1991; Voytik *et al.*, 1993).

Similar to Pax3 and Pax7, expression analyses of the MRFs have been facilitated by the generation of reporter alleles *Myf5*^{nLacz}, *Myf5*$^{GFP\text{-}P}$, and *Mrf4*$^{nLacZ\text{-}P}$ (Kassar-Duchossoy *et al.*, 2004; Tajbakhsh *et al.*, 1996). These "knock-in" alleles have allowed for increased sensitivity in tracking Myf5+ and Mrf4+ cells. However, these alleles must be used with caution as Myf5 and Mrf4 are genetically linked, and the reporter constructs disrupt the expression of the linked gene to varying degrees (Kassar-Duchossoy *et al.*, 2004).

These expression studies are important both for establishing which myogenic populations are labeled by Pax3, Pax7, and MRF genes and also for describing the temporal–spatial relationship between the expression of these transcription factors and the cell populations they label. Most significantly, these studies are critical for generating testable hypotheses about gene function and cell lineage relationships. In terms of gene function, the expression of Pax3 and Pax7 in progenitors suggests that these genes are important for specification or maintenance of progenitors. The expression of MyoD and Myf5 in myoblasts suggests that these MRFs may be critical for myoblast determination. Finally, the expression of MyoD, Myogenin, and Mrf4 in myocytes or myofibers suggests that these MRFs play a role in differentiation. Thus gene expression studies strongly implicate Pax and MRF as playing roles in myogenesis and are a good starting point for designing appropriate functional experiments. However, as will be described in the following section, gene expression does not necessarily indicate critical gene function. For instance, Pax7 is strongly expressed in adult satellite cells, but is not functionally important for muscle regeneration by satellite cells (Lepper *et al.*, 2009). In terms of lineage, the finding that Pax3 is expressed before Pax7 in muscle progenitors in the limb suggests that Pax3+ cells may give rise to Pax7+ cells. In addition, the demonstration that MRFs are expressed after Pax3 also suggests that Pax3+ cells give rise to MRF+ myoblasts. However, gene expression data is not sufficient to allow us to reconstruct cell lineage. For instance, because Pax3 is only transiently expressed in progenitors, but not in myoblasts or differentiated myogenic cells, it is impossible to trace the fate of these Pax3+ progenitors. Conversely, continuity of gene expression, for example, the expression of MyoD in both myoblasts and myofibers, does not necessarily indicate continuity of cell lineage because new cells may initiate gene expression *de novo* while other cells may downregulate gene expression.

4. Functional Analysis of Pax3/7 and MRF Transcription Factors

Mouse genetic loss-of-function studies not only demonstrate that Pax3 is required for limb myogenesis, but also indicate that Pax3+ progenitors are essential to generate all the myogenic cells in the limb (Table 1.3). Pax3 function has been studied for over 50 years because of

Table 1.3 Summary of phenotypes with loss of function in mouse of Pax3, Pax7, Myf5, MyoD, Myogenin, Mrf4, and combinations of Pax3, Pax7, and MRFs

	Pax3 (Lepper *et al.*, 2009; Relaix *et al.*, 2004 and references therein)	Pax7 (Kuang *et al.*, 2006; Lepper *et al.*, 2009; Oustanina *et al.*, 2004; Relaix *et al.*, 2006; Seale *et al.*, 2000)	Pax3/Pax7 (Relaix *et al.*, 2005)	Pax3/Myf5/Mrf4 (Tajbakhsh *et al.*, 1997)	Myf5 (Gayraud-Morel *et al.*, 2007; Kassar-Duchossoy *et al.*, 2004)	MyoD (Gayraud-Morel *et al.*, 2007; Kablar *et al.*, 1997; Megeney *et al.*, 1996; Rudnicki *et al.*, 1992; White *et al.*, 2000; Yablonka-Reuveni *et al.*, 1999)	Mrf4 (Zhang *et al.*, 1995)	Myogenin or Myogenin/Myf5 or Myogenin/MyoD or Myogenin/Mrf4 (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993; Rawls *et al.*, 1995, 1998; Venuti *et al.*, 1995)	Myf5/MyoD (Kassar-Duchossoy *et al.*, 2004; Kassar-Duchossoy *et al.*, 2005)	Myf5/Mrf4 (Braun and Arnold, 1995; Kassar-Duchossoy *et al.*, 2004; Tajbakhsh *et al.*, 1997)	MyoD/Mrf4 (Rawls *et al.*, 1998)	Myf5/MyoD/Mrf4 (Kassar-Duchossoy *et al.*, 2004; Rudnicki *et al.*, 1993)	MyoD/Mrf4/Myogenin (Valdez *et al.*, 2000)
Axial	Defects in somite segmentation, epaxial and hypaxial dermomyotome, trunk muscle	No phenotype observed	Only form primary myotome. No embryonic or fetal axial muscle	Defective primary myotome. No embryonic or fetal axial muscle	Delay of primary myotome formation, lack of some epaxial muscles in adult	Normal primary myotome and epaxial muscles, delay in hypaxial muscles	No phenotype observed	Embryonic axial muscle normal, no MyHCperi+ fetal axial muscle	Delay of primary myotome, embryonic axial muscle at E12.5, no fetal axial muscle	Delayed primary myotome, lack of some epaxial muscle	Embryonic axial muscle normal, no axial fetal muscle	No myotome or axial muscle	Only Myf5+ myoblasts, no myofibers
Embryonic Limb E11.5–E14.5	No limb muscle due to defects in delamination, migration, maintenance of limb progenitors	No phenotype observed	No limb muscle (see Pax3 phenotype)	No limb muscle (see Pax3 phenotype)	No phenotype observed	2.5 day delay in limb myogenesis, no limb muscle until E13.5	No phenotype observed	Normal embryonic limb myoblasts and MyHCemb+ myofibers	No limb muscle at E12.5 (MyoD phenotype), a few myofibers at E14.5	No phenotype observed	Normal embryonic limb myoblasts and myofibers	No limb muscle	Not explicitly tested

Fetal Limb E14.5–E18.5	No limb muscle (see above)	No phenotype observed	No limb muscle (see Pax3 phenotype)	No limb muscle (see Pax3 phenotype)	No phenotype observed	No phenotype observed	No phenotype observed	No MyHCperi+ fetal limb myofibers, a few residual myofibers, myoblasts present	Few myofibers at E14.5, no fetal myofibers by birth	No phenotype observed	Myoblasts present, but few residual myofibers	No limb muscle	No differentiated myofibers, no MyHCemb
Neonatal Limb P0–P21	Dead	Defects in satellite cell survival, proliferation, and differentiation (as tested by conditional deletion)	Dead	Dead	No phenotype observed	No phenotype observed	No phenotype observed	Few residual myofibers, perinatal death	No muscle at birth, perinatal death	No phenotype observed, perinatal death	Few residual myofibers, perinatal death	No limb muscle, perinatal death	No differentiated myofibers, no MyHCemb, perinatal death
Adult/ regeneration	Dead (Pax3 null mice). Not required (as tested by conditional deletion)	No effect on adult muscle regeneration (as tested by conditional deletion)	Dead	Dead	Impaired regeneration with delayed differentiation, fiber hypertrophy, increased fat and fibrosis	Delayed and impaired regeneration with increased number of satellite cells and fewer differentiated myofibers	Not explicitly tested	Dead	Dead	Dead	Dead	Dead	Dead

It should be noted that some *Myf5* null and *Mrf4* null alleles affected the expression of Mrf4 and Myf5, respectively. Thus, for instance, the $Myf5^{nlacZ/nlacZ}$ mice, originally described as *Myf5* null mice (Tajbakhsh *et al.*, 1997), are also null for *Mrf4* (Kassar-Duchossoy *et al.*, 2004). Only the $Myf5^{tm1Rud}$ allele leaves *Mrf4* intact (Kassar-Duchossoy *et al.*, 2004). In this table, phenotypes described for both *Myf5* null and compound *Myf5* and *MyoD* null are based on $Myf5^{tm1Rud}$ mice. Similarly, while three *Mrf4* null alleles were generated (Braun and Arnold, 1995; Patapoutian *et al.*, 1995; Zhang *et al.*, 1995), only one *Mrf4* null allele leaves *Myf5* intact (Olson *et al.*, 1996; Zhang *et al.*, 1995). The phenotype described here for the *Mrf4* null is based on this allele from the Olson lab (Zhang *et al.*, 1995).

the availability of a naturally occurring functional null allele of Pax3, the Splotch mutant (Auerbach, 1954; Epstein *et al.*, 1993). In *Pax3*^{Sp} Splotch mutants (which generally die by E14.5), as well as other splotch mutants such as *Pax3*^{SpD} (which die at E18.5), no embryonic or fetal muscle forms in the limb (Bober *et al.*, 1994; Franz *et al.*, 1993; Goulding *et al.*, 1994; Vogan *et al.*, 1993). There is a complete lack of myoblasts, myocytes, and myofibers, as indicated by the lack of expression of MRFs and muscle contractile proteins. Functional Pax3 is required for multiple aspects of somite development and limb myogenesis. In the somite, Pax3 regulates somite segmentation and formation of the dorsomedial and ventrolateral dermomyotome (Relaix *et al.*, 2004; Schubert *et al.*, 2001; Tajbakhsh and Buckingham, 2000). For limb myogenesis, Pax3 is required for maintenance of the ventrolateral somitic precursors, delamination (via activation of Met expression) from the somite of limb myogenic progenitors, migration of progenitors into the limb, and maintenance of progenitor proliferation (Relaix *et al.*, 2004). Interestingly, in the adult conditional deletion of *Pax3* in satellite cells revealed that, despite observed expression of Pax3 in satellite cells of some muscles (Relaix *et al.*, 2006), Pax3 is not required for muscle regeneration (Lepper *et al.*, 2009). Together these data show that Pax3 is required for embryonic myogenesis in the limb, but is not subsequently required in the adult. Whether Pax3 is required for fetal limb myogenesis has not been explicitly tested. These functional data also elucidate the nature of the progenitors which give rise to limb muscle. The complete absence of muscle in the limb in *Pax3* mutants, in combination with the early transient (E10.5–E12.5) expression of Pax3 in limb muscle progenitors, suggests that these early Pax3+ progenitors (present up to E12.5) give rise to all embryonic and fetal myoblasts, myocytes, and myofibers in the limb. This suggests that our theoretical Model 1, in which multiple distinct progenitors give rise to different myoblasts and myofibers, is unlikely to be correct. Instead Models 2–4 (or some variant of them), in which all muscle ultimately derives from one initial progenitor population, are more likely representations of limb myogenesis.

Functional analysis of Pax7 has established that Pax7 regulates neonatal progenitors and also reveals that there are at least two genetically distinct populations of progenitors (Table 1.3). Analysis of *Pax7* loss-of-function alleles has been complicated. Although no muscle phenotypes were initially recognized in null *Pax7*$^{LacZ/LacZ}$ (Mansouri *et al.*, 1996), subsequent analysis suggested that no satellite cells were specified in the absence of Pax7 (Seale *et al.*, 2000). Then a series of papers (Kuang *et al.*, 2006; Oustanina *et al.*, 2004; Relaix *et al.*, 2006) determined that, in fact, satellite cells were present in *Pax7* null mice. However, Pax7 was found to be critical for maintenance, proliferation, and function of satellite cells. More recently, conditional

deletion of *Pax7* in satellite cells, via a tamoxifen-inducible $Pax7^{CreERT2}$ allele and a $Pax7^{fl}$ allele, has surprisingly shown that Pax7 is not required after P21 (the end of neonatal myogenesis) for effective muscle regeneration (Lepper *et al.*, 2009). However, consistent with the previous studies (Kuang *et al.*, 2006; Oustanina *et al.*, 2004; Relaix *et al.*, 2006), conditional deletion of *Pax7* between P0 and P21 did show a requirement for Pax7 in neonatal satellite cells for proper proliferation and myogenic differentiation (Lepper *et al.*, 2009). Thus, this study demonstrates that Pax7 is dispensable in the adult, but required in neonatal satellite cells for their maintenance, proliferation, and differentiation. Prior to birth, myogenesis appears not to require Pax7, as gross muscle morphology is normal (Oustanina *et al.*, 2004; Seale *et al.*, 2000). However, the reduced number of satellite cells just after birth (Oustanina *et al.*, 2004; Relaix *et al.*, 2006) suggests that proliferation and/or maintenance of fetal progenitors may be functionally dependent on Pax7. In total, these functional studies reveal that there are at least two populations of progenitors: Pax7-functionally dependent neonatal satellite cells and Pax7-functionally independent adult satellite cells. Thus, a model of myogenesis in which there is only one invariant progenitor population (as seen in Models 3, 4, and 5) is unlikely to be correct.

Compound mutants of *Myf5*, *MyoD*, and *Mrf4* demonstrate that embryonic and fetal myoblasts have different genetic requirements for their determination (Table 1.3). Over the past 20 years, multiple loss-of-function alleles of all four MRFs have been generated and allowed for detailed characterization of their function. However, analysis of Myf5 and Mrf4 function has been complicated because these two genes are genetically linked, and so many of the original *Myf5* and *Mrf4* loss-of-function alleles also affected the expression of the neighboring gene (see discussion in Kassar-Duchossoy *et al.*, 2004; Olson *et al.*, 1996). Single loss-of-function mutants of *Myf5* or *Mrf4* (in which genetically linked Mrf4 and Myf5 expression remain intact) show no defects in embryonic or fetal limb myogenesis (Kassar-Duchossoy *et al.*, 2004; Zhang *et al.*, 1995), and *MyoD* mutants have only a minor phenotype, a 2–2.5 day delay in embryonic limb myogenesis (Kablar *et al.*, 1997; Rudnicki *et al.*, 1992). Compound *Myf5* and *Mrf4* null mutants have normal embryonic and fetal limb muscle (Braun and Arnold, 1995; Kassar-Duchossoy *et al.*, 2004; Tajbakhsh *et al.*, 1997). Compound *MyoD* and *Mrf4* mutants (in which *Myf5* expression remains intact) have normal embryonic myoblasts and myofibers (but with a 2 day delay in development, reflecting the *MyoD* null phenotype) and fetal myoblasts (although fetal myofibers are absent, see below; Rawls *et al.*, 1998). Compound *Myf5* and *MyoD* loss-of-function mutants (in which *Mrf4* expression is intact) contain no fetal myoblasts or myofibers. However, a few residual embryonic myofibers are present and therefore

indicate the presence of some embryonic myoblasts (Kassar-Duchossoy *et al.*, 2004). In triple *Myf5*, *MyoD*, and *Mrf4* loss-of-function mutants, no embryonic or fetal myoblasts or myofibers are present (Kassar-Duchossoy *et al.*, 2004; Rudnicki *et al.*, 1993). Together these genetic data indicate that embryonic myoblasts require Myf5, MyoD, or Mrf4 for their determination, although these MRFs differ somewhat in their function. MyoD can most efficiently determine embryonic myoblasts, as embryonic myogenesis is normal in compound *Myf5* and *Mrf4* mutants. While Myf5 can determine embryonic myoblasts, the inability of Myf5 to act as a differentiation factor leads to a delay in limb myogenesis in compound *MyoD* and *Mrf4* mutants. Mrf4 can only poorly substitute for Myf5 or MyoD as a determination factor, and so in the absence of *Myf5* and *MyoD*, limb embryonic myogenesis is only partially rescued by *Mrf4*. Unlike embryonic myoblasts, fetal myoblasts require either Myf5 or MyoD for their determination, and Mrf4 is not able to rescue this function. These data argue that embryonic and fetal myoblasts have different genetic requirements for their determination and therefore concurs with previous culture data showing that embryonic and fetal myoblasts are distinct. The presence of at least two classes of myoblasts therefore excludes Model 5, in which one myoblast population gives rise to different types of myofibers, and argues in favor of Models 1–4, in which multiple myoblast populations are important for generating different types of myofibers. It is likely that embryonic, fetal, and adult myoblasts are distinct populations. However, the genetic requirements of adult myoblasts has not been completely tested. Loss of either *Myf5* or *MyoD* leads to delayed or impaired muscle regeneration (Gayraud-Morel *et al.*, 2007; Megeney *et al.*, 1996; White *et al.*, 2000; Yablonka-Reuveni *et al.*, 1999). The role of Mrf4 in regeneration has not been explicitly tested, although the lack of Mrf4 expression in adult myoblasts suggests Mrf4 may not be required (Gayraud-Morel *et al.*, 2007). To test whether Myf5, MyoD, or Mrf4 may be acting redundantly in the adult will require conditional deletion of these MRFs in adult progenitors since compound mutants die at birth.

Compound mutants of *MyoD*, *Mrf4*, and *Myogenin* reveal that embryonic and fetal myoblasts have different genetic requirements for their differentiation (Table 1.3). Loss of *Mrf4* results in no muscle phenotype in the limbs, while loss of *MyoD* results in only a delay in embryonic limb myogenesis (Kablar *et al.*, 1997; Rudnicki *et al.*, 1992; Zhang *et al.*, 1995). Formation of embryonic myofibers (MyHCemb+) is largely unaffected with loss of *Myogenin* (although myosin levels appear lower and myofibers are less organized); however, differentiation of fetal myofibers (MyHCperi+) is completely impaired (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993; Venuti *et al.*, 1995). This lack of fetal muscle is due to a defect

in differentiation *in vivo*; myoblasts are still present in *Myogenin* mutant limbs and can differentiate *in vitro* (Nabeshima *et al.*, 1993). A similar phenotype is seen in compound *Myogenin/MyoD*, *Myogenin/Mrf4*, *Myogenin/Myf5*, and *Mrf4/MyoD* null mutants. In all of these mutants, embryonic muscle differentiates, but fetal muscle does not (Rawls *et al.*, 1995, 1998; Valdez *et al.*, 2000). Also, myoblasts from these compound mutants are present and *in vitro* can differentiate. In triple *Myogenin/Mrf4/MyoD* animals, no embryonic or fetal myofibers differentiate and myoblasts from these animals cannot differentiate *in vitro* (Valdez *et al.*, 2000). Together these genetic data argue that differentiation of embryonic myofibers requires Myogenin, MyoD, or Mrf4. Myf5, which is not normally expressed in differentiating myogenic cells, is not sufficient to support myofiber differentiation. The genetic requirement of fetal myofiber differentiation is more stringent and requires Myogenin *and* either Mrf4 or MyoD. Thus, the differentiation of embryonic and fetal myofibers has different genetic requirements and argues that the embryonic and fetal myoblasts (from which the myofibers derive) are genetically different. Therefore, these data support Models 1–4, in which different embryonic and fetal myoblast populations are important for the generation of embryonic and fetal myofibers.

5. Cre-Mediated Lineage and Ablation Analyses of PAX3, PAX7, and MRF+ Cells

Cre-mediated lineage analysis in mice has provided the most direct method to test the lineage relationship of progenitors and myoblasts giving rise to embryonic, fetal, neonatal, and adult muscle. These lineage studies have been enabled by the development of *Cre/loxP* technology (Branda and Dymecki, 2004; Hutcheson and Kardon, 2009). To genetically label and manipulate different populations of muscle progenitors or myoblasts, Cre lines have been created in which Cre is placed under the control of the promoter/enhancers sequences of *Pax3/7* or MRFs. Several strategies have been used to create these Cre lines. For $Pax3^{Cre}$, $Myf5^{Cre}$, and $MyoD^{Cre}$ lines, *Cre* has been placed into the ATG of the endogenous locus (Engleka *et al.*, 2005; Kanisicak *et al.*, 2009; Tallquist *et al.*, 2000). For $Pax7^{Cre}$, $Mrf4^{Cre}$, and another $Myf5^{Cre}$ line an *IRESCre* cassette was placed at the transcriptional stop (Haldar *et al.*, 2008; Keller *et al.*, 2004). $Myogenin^{Cre}$ was created as a transgene, by placing *Cre* under the control of a 1.5 kb Myogenin promoter and a 1 kb MEF2C enhancer (Li *et al.*, 2005). Recently, tamoxifen-inducible *Cre* alleles have also been created, and these *CreERT2* alleles allow for temporal control of labeling and manipulation because

Cre-mediated recombination only occurs after the delivery of tamoxifen. A tamoxifen-inducible *Pax7*^{CreERT2} allele has been created by placing a *CreERT2* cassette into the ATG of *Pax*7 (Lepper and Fan, 2010; Lepper *et al.*, 2009). For each of these alleles, the ability to label and manipulate the appropriate cell requires that the Cre be faithfully expressed wherever the endogenous gene is expressed. Placing the *Cre* or *CreERT2* cassette at the endogenous ATG is the most likely strategy for ensuring that Cre expression recapitulates endogenous gene expression. However, these alleles are all "knockin/knockout" alleles in which the *Cre* disrupts expression of the targeted genes. If there is any potential issue of haplo-insufficiency, such a targeting strategy may be problematic. For the *Pax3*^{Cre}, *Myf5*^{Cre}, and *MyoD*^{Cre} lines, haplo-insufficiency has not been found to be an issue. For *Cre* alleles generated by targeted *IRESCre* to the stop or by transgenics, the fidelity of the *Cre* needs to be carefully verified. The advantage of such Cre lines, of course, is that the endogenous gene remains intact.

To follow the genetic lineage of the Pax3+, Pax7+, or MRF+ cells, these Cre lines have been crossed to various *Cre*-responsive reporter mice. In the reporter mice, reporters such as *LacZ* or *YFP* are placed under the control a ubiquitous promoter. In the absence of *Cre*, these reporters are not expressed because of the presence of a strong transcriptional stop cassette flanked by *loxP* sites, while the presence of *Cre* causes recombination of the *loxP* sites and the permanent expression of the reporter. Therefore, in mice containing both the *Cre* and the reporter, cells expressing the *Cre* and their progeny permanently express the reporter, thus allowing the fate of Pax3+, Pax7+, or MRF+ cells to be followed. The number of cells genetically labeled in response to Cre can be dramatically affected by the reporter lines used, and the utility of each reporter must be verified for each tissue and age of animal being tested. The *R26R*^{LacZ} and *R26*^{RYFP} reporters (Soriano, 1999; Srinivas *et al.*, 2001) are commonly used with good success in the embryo to label myogenic cells. In the adult, the endogenous *R26R* locus may not be sufficient to drive high levels of reporter expression, and so reporters such as *R26R*^{mTmG} (Muzumdar *et al.*, 2007) or *R26R*^{NZG} (Yamamoto *et al.*, 2009) in which a CMV β-actin promoter additionally drives reporter expression, may be necessary.

The *Cre/loxP* system can also be used to test the requirement of particular cell populations for myogenesis, by crossing *Cre* lines with *Cre*-responsive ablater lines (Hutcheson and Kardon, 2009). In these ablater lines, *Cre* activates the expression of cell-death-inducing toxins, such as *diptheria toxin* (Brockschnieder *et al.*, 2006; Wu *et al.*, 2006). The lack of receptor for diphteria toxin in mice and the expression of only the diptheria toxin fragment A (DTA, which cannot be transferred to other cells without the diptheria toxin fragment B) ensures that only cells expressing Cre, and therefore DTA, will be cell-autonomously killed. Analogous to gene loss-of-function experiments, cell ablation experiments enable the researcher to test the *necessity* of particular genetically labeled progenitors and myoblasts for myogenesis.

The expression and functional studies of Pax3 strongly suggested that Pax3+ progenitors give rise to all embryonic, fetal, neonatal, and adult muscle. Particularly because Pax3 is only transiently expressed in progenitors in the early limb bud, tracing the lineage of Pax3+ progenitors required that the cells be genetically labeled via $Pax3^{Cre}$. These Pax3 lineage studies reveal that Pax3+ cells entering the limb are initially bipotential and able to give rise to both endothelial cells and muscle (Hutcheson *et al.*, 2009; Table 1.4). Moreover, Pax3+ cells give rise to all embryonic, fetal, and adult myoblasts and myofibers (Engleka *et al.*, 2005; Hutcheson *et al.*, 2009; Schienda *et al.*, 2006). Thus, these early Pax3+ progenitors give rise to all limb muscle and exclude Model 1 of limb myogenesis, in which multiple distinct progenitors give rise to embryonic, fetal, neonatal, and adult myofibers. Of course, it is formally possible that the Pax3+ cells migrating into the limb are a heterogeneous population in which subpopulations give rise to embryonic, fetal, and adult myoblasts (and so Model 1 might be correct). However, to test this possibility, early markers of these subpopulations would be required. The necessity of Pax3+ progenitors is demonstrated by the lack of any embryonic or fetal muscle when these cells are genetically ablated (Hutcheson *et al.*, 2009). Although not formally demonstrated (because of the P0 death of $Pax3^{Cre/+};R26R^{DTA}$ mice), it is likely that the Pax3+ progenitors are also required for the formation of all adult limb muscle. In addition, these lineage studies demonstrated that all Pax7+ progenitors in the embryo and Pax7+ satellite cells in the adult are derived from the Pax3+ progenitors (Hutcheson *et al.*, 2009; Schienda *et al.*, 2006). This finding thus supports Model 2 of limb myogenesis, in which an initial progenitor population gives rise to other progenitor populations.

Genetic lineage studies of Pax7+ progenitors have established that, unlike Pax3+ progenitors, Pax7+ progenitors in the limb are restricted to a myogenic fate (Hutcheson *et al.*, 2009; Lepper and Fan, 2010). Consistent with the later expression of Pax7 (beginning at E11.5), Pax7+ progenitors do not give rise to embryonic muscle, but do give rise to all fetal and adult myoblasts and myofibers in the limb (Hutcheson *et al.*, 2009; Lepper and Fan, 2010). Pax7+ cells labeled in the early limb (via tamoxifen delivery to E11.5 $Pax7^{CreERT2/+};R26R^{LacZ/+}$ mice) also give rise to Pax7+ adult satellite cells, although it is unclear whether these labeled cells directly become satellite cells or whether their progeny give rise to satellite cells (Lepper and Fan, 2010). The loss of fetal limb muscle when Pax7+ cells are genetically ablated demonstrates that these Pax7+ progenitors are required for fetal myogenesis in the limb (Hutcheson *et al.*, 2009). The test of whether Pax7+ progenitors are *necessary* for adult myogenesis awaits the generation of $Pax7^{CreERT2/+};R26R^{DTA/+}$ mice, in which Pax7+ progenitors are genetically ablated after birth.

Recent lineage analyses of Myf5+ and MyoD+ cells have unexpectedly revealed that two populations of myoblasts may give rise to muscle (Table 1.4).

Table 1.4 Summary of genetic lineage and ablation studies in mouse

	Pax3Cre lineage	Pax3Cre-mediated ablation	Pax7Cre lineage	Pax7CreERT2 lineage	Pax7Cre-mediated ablation	Myf5Cre lineage	Myf5Cre-mediated ablation	MyoDCre lineage	MyogeninCre lineage	MyogeninCre-mediated ablation	Mrf4Cre lineage	Mrf4Cre-mediated ablation
References	Engleka *et al.* (2005), Hutcheson *et al.* (2009), Schienda *et al.* (2006)	Hutcheson *et al.* (2009)	Hutcheson *et al.* (2009)	Lepper and Fan (2010)	Hutcheson *et al.* (2009)	Gensch *et al.* (2008), Haldar *et al.* (2008), Kuang *et al.* (2007)	Gensch *et al.* (2008), Haldar *et al.* (2008)	Kanisicak *et al.* (2009)	Gensch *et al.* (2008), Li *et al.* (2005)	Gensch *et al.* (2008)	Haldar *et al.* (2008)	Haldar *et al.* (2008)
Embryonic progenitors	+	None			Present	Not analyzed	Present	Not analyzed	Not analyzed in limb	Present	Not analyzed in limb	Not analyzed in limb
Embryonic myoblasts		None			Present	+	Present	+	Not analyzed in limb	Present	Not analyzed in limb	Not analyzed in limb
Embryonic myofibers		None			Present		Present	+	+	None	Not analyzed in limb	Not analyzed in limb
Fetal/Neonatal progenitors		None	+	★	None	Not analyzed	Present	Not analyzed	Not analyzed	Not analyzed	Not analyzed	Not analyzed
Fetal/Neonatal myoblasts		None			None	Not analyzed	Present	+ (neonatal)	Not analyzed	Not analyzed	Not analyzed	Not analyzed
Fetal/Neonatal myofibers		None			None		Present	Not analyzed	+	None	Not analyzed	None
Adult progenitors	+/−	None	+		(Dead)	+/−	(dead)		Not analyzed	(Dead)	Not analyzed	(Dead)
Adult myoblasts		None			(Dead)		(Dead)	+	Not analyzed	(Dead)	Not analyzed	(Dead)
Adult myofibers		None			(Dead)		(Dead)	+	Not analyzed	(Dead)	+	(Dead)

"+" shows cells actively transcribing the gene of interest (e.g., transcribing Pax3).
Gray boxes denote progenitors, myoblasts, and myofibers entirely derived from the genetically labeled cell population (e.g., Pax3+ cells). Hatched boxes show progenitors, myoblasts, and myofibers where only some of the cells are derived from the genetically labeled cell population. Star denotes timing of tamoxifen delivery in *Pax7*^{CreERT2} mice.

Analysis of Myf5 lineage, using two different *Myf5*^{Cre} lines, shows that Myf5+ cells are not restricted to a muscle fate, as cells in the axial skeleton and ribs are derived from Myf5+ cells (Gensch *et al.*, 2008; Haldar *et al.*, 2008). This likely reflects early transient expression of Myf5 in the presomitic mesoderm. In contrast, MyoD+ cells appear to be restricted to a muscle fate (Kanisicak *et al.*, 2009). Interestingly, analysis of the Myf5 lineage shows that Myf5+ cells give rise to many, but not all embryonic, fetal, and adult myofibers (Gensch *et al.*, 2008; Haldar *et al.*, 2008). The distribution of Myf5-derived myofibers appears to be stochastic, as epaxial and hypaxial, slow and fast, and different anatomical muscles are randomly Myf5-derived. Unlike Myf5, analysis of MyoD lineage reveals that MyoD+ cells give rise to all embryonic and adult myofibers (fetal myofibers were not explicitly examined; Kanisicak *et al.*, 2009). Consistent with these lineage studies, ablation of Myf5+ cells did not lead to any dramatic defects in embryonic or fetal muscle (the *Myf5*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice die at birth from rib defects), as presumably Myf5- myoblasts compensated for the loss of Myf5+ myoblasts (Gensch *et al.*, 2008; Haldar *et al.*, 2008). Ablation of the MyoD lineage has not yet been published, but based on the lineage studies a complete loss of muscle would be expected. Together, these lineage and ablation studies argue that there are at least two populations of myoblasts, one Myf5-dependent and one Myf5-independent, thus excluding Model 5, in which only one myoblast population generates all limb muscle. It is not yet clear whether there may, in fact, be three populations of myoblasts: Myf5+MyoD−, Myf5+MyoD+, and Myf5−MyoD+. The finding that all muscle is MyoD-derived would suggest that there are no myoblasts that are Myf5+MyoD−. However, because MyoD is strongly expressed in embryonic and fetal myofibers, the finding that all muscle is YFP+ in *MyoD*$^{Cre/+}$;*R26R*$^{YFP/+}$ mice may simply reflect MyoD expression in all myofibers, and not MyoD expression in all myoblasts. Another question yet to be resolved is whether multiple myoblast populations are present during embryonic, fetal, and neonatal myogenesis.

Analysis of the Myf5 and MyoD lineages has also revealed interesting insights about adult satellite cells. The great majority of quiescent satellite cells have been shown to be YFP labeled in *Myf5*$^{Cre/+}$;*R26R*$^{YFP/+}$ mice (Kuang *et al.*, 2007). Given that most quiescent satellite cells express Myf5 (Beauchamp *et al.*, 2000; Cornelison and Wold, 1997), it is likely that the Myf5 lineage in satellite cells is simply reflecting active Myf5 transcription in satellite cells. However, the finding that all quiescent satellite cells are YFP labeled in *MyoD*$^{Cre/+}$;*R26R*$^{YFP/+}$ mice was quite surprising (Kanisicak *et al.*, 2009). Multiple studies have shown that quiescent satellite cells do not express MyoD, although activated satellite cells do express MyoD (Cornelison and Wold, 1997; Yablonka-Reuveni and Rivera, 1994). Thus, the finding that quiescent satellite cells are YFP+ in *MyoD*$^{Cre/+}$;*R26R*$^{YFP/+}$ mice suggests that all quiescent satellite cells are derived from previously activated, MyoD+ satellite cells (as suggested by Zammit *et al.*,

2004). Alternatively, all quiescent satellite cells may be derived from MyoD+ myoblasts. To definitively test whether satellite cells indeed are derived from MyoD+ myoblasts, $MyoD^{CreERT2/+}$;$R26R^{YFP/+}$ mice will need to be induced with tamoxifen in the embryo or fetus, before satellite cells are present. It will also be interesting to test using $Myf5^{CreERT2/+}$; $R26R^{YFP/+}$ mice whether Myf5+ myoblasts in the embryo or fetus give rise to satellite cells. Such a finding that MyoD+ or Myf5+ myoblasts give rise to satellite cells would profoundly change current models of myogenesis (excluding all five Models presented) because this would demonstrate that myoblasts can return to a more progenitor-like state.

Lineage analysis using $Myogenin^{Cre}$ and $Mrf4^{Cre}$ mice demonstrates that by birth all myofibers have expressed both Myogenin and Mrf4 (Gensch *et al.*, 2008; Haldar *et al.*, 2008; Li *et al.*, 2005; Table 1.4). A closer examination of the Myogenin lineage reveals that all embryonic and fetal muscle has derived from Myogenin+ myocyctes and/or myofibers (Gensch *et al.*, 2008; Li *et al.*, 2005). It would be worthwhile to similarly determine to what extent embryonic muscle has expressed Mrf4 since expression studies have found Mrf4 to be expressed in at least some embryonic limb muscle (Hinterberger *et al.*, 1991). Consistent with the finding that all fetal muscle has expressed Myogenin and Mrf4, ablation of Myogenin+ or Mrf4+ cells leads to a complete loss of all muscle by birth (Gensch *et al.*, 2008; Haldar *et al.*, 2008).

6. Molecular Signals Distinguishing Between Different Phases of Myogenesis

Layered on top of these expression, functional, and lineage studies concentrating on Pax3, Pax7, and MRFs are functional studies demonstrating that embryonic, fetal, and adult myogenic cells show differential sensitivity to signaling molecules. Recent microarray studies demonstrated that members of the Notch, FGF, and PDGF signaling pathways are differentially expressed in embryonic versus fetal myoblasts (Biressi *et al.*, 2007b). In addition, fetal myoblasts show upregulation of components of the TGFβ and BMP signaling pathways compared to embryonic myoblasts (Biressi *et al.*, 2007b). Such findings are consistent with *in vitro* studies demonstrating that embryonic myoblast differentiation is insensitive to treatment with TGFβ or BMP, while fetal myoblast differentiation is blocked in the presence of TGFβ or BMP (Biressi *et al.*, 2007b; Cusella-De Angelis *et al.*, 1994). Interestingly, studies examining adult myogenesis also demonstrate that BMP signaling is active in activated satellite cells and proliferating myoblasts (Ono *et al.*, 2010). Furthermore, inhibition of BMP signaling results in an increase in differentiated myocytes at the expense of

proliferating myoblasts *in vitro* and smaller diameter regenerating myofibers *in vivo* (Ono *et al.*, 2010). Therefore, in mouse TGFβ and BMP signaling appear to have no effect on embryonic myoblasts, whereas they inhibit differentiation of both fetal and adult myoblasts. Thus, with respect to TGFβ and BMP signaling, fetal and adult myoblasts behave similarly. It is interesting to note that in the chick limb BMP signaling has also been shown to differentially regulate embryonic versus fetal and adult myogenesis, although BMP effects were different from those found in the mouse (Wang *et al.*, 2010).

The Wnt/β-catenin pathway also differentially regulates embryonic versus fetal and adult myogenesis. The role of β-catenin in embryonic and fetal myogenesis was tested by conditionally inactivating or activating β-catenin in embryonic muscle via $Pax3^{Cre}$ or in fetal muscle via $Pax7^{Cre}$ (Hutcheson *et al.*, 2009). After myogenic cells enter the limb, embryonic myogenic cells were found to be insensitive to perturbations in β-catenin. However, during fetal myogenesis β-catenin critically determines the number of Pax7+ progenitors and the number and fiber type of myofibers. β-catenin has also been found to positively regulate the number of Pax7+ satellite cells in the adult (Otto *et al.*, 2008; Perez-Ruiz *et al.*, 2008; but see Brack *et al.*, 2008). Thus similar to the findings for TGFβ and BMP signaling, embryonic myogenesis is insensitive to β-catenin signaling, while fetal and adult myogenesis is regulated by β-catenin.

These studies demonstrate that during embryonic myogenesis Pax3+ progenitors are insensitive to TGFβ, BMP, and Wnt/β-catenin signaling. Yet during fetal and adult myogenesis, TGFβ, BMP, and Wnt/β-catenin signaling are important for positively regulating and maintaining the population of Pax7+ progenitors. During development, postmitotic myofibers must differentiate, while proliferating progenitors must be maintained for growth. Therefore, in the same environment some progenitors must differentiate, while others must continue to proliferate. It has been hypothesized that embryonic, fetal, and adult progenitors and/or myoblasts are intrinsically different so that these cells will respond differently to similar environmental signals (Biressi *et al.*, 2007a,b). Thus, differential sensitivity to TGFβ, BMP, and Wnt/β-catenin signaling may be a molecular mechanism to allow embryonic progenitors to differentiate, but maintain a fetal and adult progenitor population.

The above examples demonstrate that embryonic, fetal, and adult myogenesis are differentially regulated by different signaling pathways. Until recently, what signals regulate the transitions from embryonic to fetal, neonatal, and adult myogenesis have been unknown. The expression of Pax7 in progenitors demarcates progenitors as being fetal/neonatal/adult progenitors, as opposed to Pax3+ embryonic progenitors. Now elegant *in vitro* and *in vivo* studies demonstrate that that the transcription Nfix is expressed in fetal and not embryonic myoblasts, and Pax7 directly binds and

activates the expression Nfix (Messina *et al.*, 2010). Moreover, Nfix is critical for regulating the transition from embryonic to fetal myogenesis. Nfix both represses genes highly expressed in embryonic muscle, such as MyHCI, and activates the expression of fetal-specific genes, such as α7-integrin, β-enolase, muscle creatine kinase, and muscle sarcomeric proteins. Thus Nfix functions as an intrinsic transcriptional switch which mediates the transition from embryonic to fetal myogenesis. Recent studies have also demonstrated that extrinsic signals from the connective tissue niche, within which muscle resides, are also important for regulating muscle maturation (Mathew *et al.*, 2011). The connective tissue promotes the switch from the fetal to adult muscle by repressing developmental isoforms of myosin and promoting formation of large, multinucleate myofibers. Determining the full range of intrinsic and extrinsic factors that regulate the transitions from embryonic to fetal, neonatal, and adult myogenesis will be important areas for future research.

7. Current Model of Myogenesis

From these expression, functional, and lineage studies, a current model of myogenesis in the limb emerges that is a variant of our theoretical Models 2 and 4 (Fig. 1.2). Embryonic, fetal, neonatal, and adult muscle derive from three related, but distinct populations of progenitors. From the somite, Pax3+ progenitors migrate into the limb and are bipotential, giving rise to either endothelial cells or muscle. Myogenic Pax3+ cells require Pax3 function for their delamination from the somites, migration, and maintenance. Pax3+ cells give rise to and are required for embryonic myogenesis. In addition, Pax3+ cells give rise to Pax7+ progenitors. In turn, these Pax3-derived, Pax7+ progenitors give rise to and are required for fetal myogenesis. These Pax7+ progenitors also appear to give rise to neonatal muscle, but whether the fetal and neonatal progenitors are exactly the same population is unclear. Unlike fetal Pax7+ progenitors, neonatal Pax7+ progenitors may reside underneath the basal lamina of myofibers, similar to satellite cells. Also, while it has been shown that neonatal Pax7+ cells require Pax7 for their maintenance and proper function, it has not been explicitly tested whether fetal Pax7+ cells require Pax7. Adult muscle derives from Pax7+ progenitors, satellite cells, which reside under the myofiber basal lamina. Unlike Pax7+ neonatal progenitors, Pax7+ satellite cells do not require Pax7 for their maintenance and function. Also, the great majority of quiescent Pax7+ satellite cells express Myf5. Pax7+ satellite cells are likely to directly derive from fetal or neonatal Pax7+ progenitors. However, the finding that all quiescent Pax7+ satellite cells have expressed MyoD in their lineage suggests that satellite cells may derive indirectly from

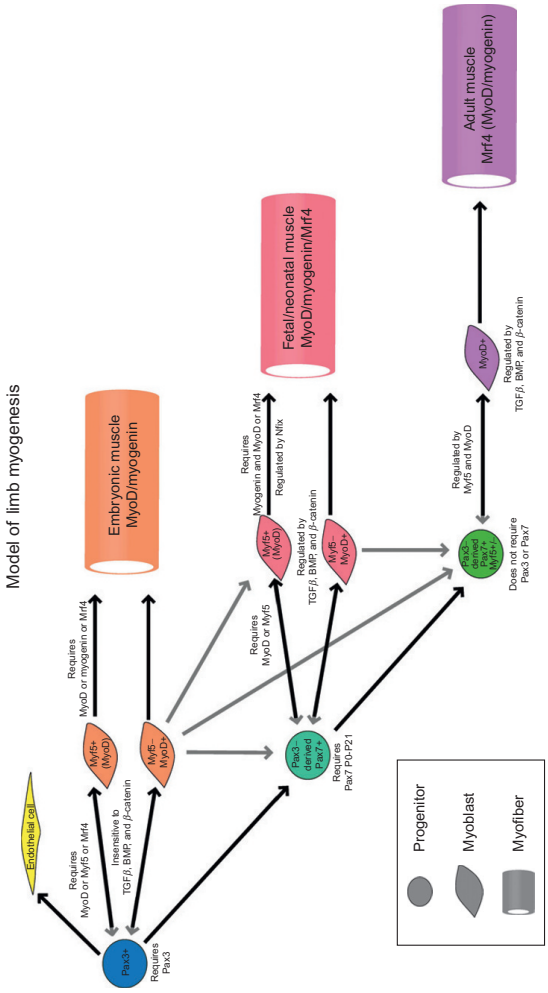

Figure 1.2 Summary of current model of embryonic, fetal/neonatal, and adult limb myogenesis in the mouse.

Pax7+ fetal or neonatal myogenic progenitors via MyoD+ (or potentially Myf5+) myoblasts (gray arrows in Fig. 1.2). Also, some Pax7+ satellite cells may derive from adult myoblasts, generated by activated Pax7+ satellite cells. Both scenarios would suggest that the progression from progenitor to myoblasts may not be irreversible, and myoblasts may give rise to Pax7+ progenitors.

There are multiple distinct populations of myoblasts that give rise to embryonic, fetal/neonatal, and adult muscle. Embryonic myoblasts are distinct from fetal/neonatal myoblasts. Embryonic limb myoblasts require either MyoD, Myf5, or Mrf4 for their determination, while fetal myoblasts require either MyoD or Myf5 (Mrf4 cannot support fetal myoblasts). Adult myoblast function is regulated by Myf5 and MyoD, but whether Myf5 and MyoD are required has not been formally tested. Within embryonic and fetal myoblasts there appear to be at least two subpopulations, Myf5-independent and Myf5-dependent. Differentiation of embryonic and fetal myoblasts into differentiated myocytes and myofibers is differentially regulated by MRFs and signaling. Embryonic myoblasts require either MyoD, Myogenin, or Mrf4 for their differentiation, while fetal myoblasts require Myogenin and Mrf4 or MyoD. Also, while embryonic myogenesis is insensitive to TGFβ, BMP, and β-catenin signaling, fetal myogenesis is regulated by these signaling pathways. The expression of Nfix within fetal myoblasts is critical for their differentiation into fetal myofibers. Once differentiated, embryonic, fetal/neonatal, and adult myofibers express different combinations of MRFs, muscle contractile proteins (including MyHC isoforms), and metabolic enzymes.

From this model, it is clear that amniote myogenesis is complex. Multiple related, although distinct progenitor and myoblast populations give rise to embryonic, fetal, neonatal, and adult muscle. In the future, it will be important to resolve the relationships between myogenic progenitors and myoblasts and definitively answer whether myoblasts ever give rise to progenitors. Also, the extrinsic cell populations and molecular signals differentially regulating the different phases of myogenesis are largely unknown. Finally, a critical question is the identification of the intrinsic and extrinsic factors that maintain the populations of myogenic progenitors, particularly in the embryo and fetus where progenitors reside alongside actively differentiating myogenic cells.

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CHAPTER 3

SATELLITE CELLS, CONNECTIVE TISSUE FIBROBLASTS AND THEIR INTERACTIONS ARE CRUCIAL FOR MUSCLE REGENERATION

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Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration

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SUMMARY

Muscle regeneration requires the coordinated interaction of multiple cell types. Satellite cells have been implicated as the primary stem cell responsible for regenerating muscle, yet the necessity of these cells for regeneration has not been tested. Connective tissue fibroblasts also are likely to play a role in regeneration, as connective tissue fibrosis is a hallmark of regenerating muscle. However, the lack of molecular markers for these fibroblasts has precluded an investigation of their role. Using Tcf4, a newly identified fibroblast marker, and Pax7, a satellite cell marker, we found that after injury satellite cells and fibroblasts rapidly proliferate in close proximity to one another. To test the role of satellite cells and fibroblasts in muscle regeneration in vivo, we created *Pax7*^{CreERT2} and *Tcf4*^{CreERT2} mice and crossed these to *R26R*^{DTA} mice to genetically ablate satellite cells and fibroblasts. Ablation of satellite cells resulted in a complete loss of regenerated muscle, as well as misregulation of fibroblasts and a dramatic increase in connective tissue. Ablation of fibroblasts altered the dynamics of satellite cells, leading to premature satellite cell differentiation, depletion of the early pool of satellite cells, and smaller regenerated myofibers. Thus, we provide direct, genetic evidence that satellite cells are required for muscle regeneration and also identify resident fibroblasts as a novel and vital component of the niche regulating satellite cell expansion during regeneration. Furthermore, we demonstrate that reciprocal interactions between fibroblasts and satellite cells contribute significantly to efficient, effective muscle regeneration.

KEY WORDS: Muscle regeneration, Satellite cells, Connective tissue, Fibrosis, Tcf4, Tcf7L2, Pax7, Mouse

INTRODUCTION

Adult vertebrate muscle has a remarkable capacity for regeneration. Because myonuclei within muscle fibers are post-mitotic, regeneration must be mediated by myogenic progenitors or stem cells. To efficiently regenerate an appropriately sized muscle, these cells proliferate, differentiate into muscle, and also replenish themselves. How proliferation versus differentiation of stem cells is carefully balanced is poorly understood, but is known to involve extrinsic signals, including signals from non-muscle cells (Cornelison, 2008). Here, we examine in vivo the role of satellite cells, the cells proposed to be the primary stem cells of adult muscle; muscle connective tissue (MCT) fibroblasts, a cell population in abundance in regenerating muscle, the function of which is largely unexplored; and their interactions during muscle regeneration.

Muscle satellite cells were first identified by their unique anatomical position between the sarcolemma and basement membrane of myofibers and were hypothesized to be the stem cell responsible for adult vertebrate muscle regeneration (Mauro, 1961). Subsequently, satellite cells were found to express the transcription factor Pax7 (Chen et al., 2006; Seale et al., 2000). Functionally, Pax7 is required prior to postnatal day (P) 21 in mice for maintenance of satellite cells (Kuang et al., 2006; Oustanina et al., 2004; Relaix et al., 2006; Seale et al., 2000), but afterwards is not required for regeneration (Lepper et al., 2009). Genetic lineage studies using *Pax7*^{CreER} mice have established that Pax7^{+} cells give rise to regenerated muscle after injury (Lepper et al., 2009; Lepper and Fan, 2010; Shea et al., 2010). Furthermore, transplanted satellite cells can regenerate myofibers in vivo, as first shown by Collins and colleagues (Collins et al., 2005) and subsequently by others (Cerletti et al., 2008; Kuang et al., 2007; Montarras et al., 2005; Sacco et al., 2008). Satellite cells are suggested to be crucial for muscle regeneration because manipulations of important signaling pathways alter both satellite cells and muscle regeneration (for reviews, see Kang and Krauss, 2010; Kuang et al., 2008). However, despite this wealth of studies (Kang and Krauss, 2010; Kuang et al., 2008), surprisingly, the necessity of satellite cells has not been explicitly tested. Furthermore, the finding of other stem cells able to regenerate muscle [e.g. mesoangioblasts and PW1^{+}/Pax7^{-} interstitial cells (PICs)] (Kuang et al., 2008; Mitchell et al., 2010), has called into question the requirement of satellite cells for regeneration.

Satellite cells are regulated by their surrounding niche, which includes multiple cell types. Endothelial and inflammatory cells, particularly macrophages, regulate satellite cell proliferation and differentiation (Christov et al., 2007; Cornelison, 2008; Robertson et al., 1993; Tidball and Villalta, 2010). Another population likely to be an integral component of muscle regeneration is the MCT fibroblasts. Connective tissue fibrosis, an increase in extracellular matrix (ECM), is characteristic of regenerating muscle, as well as many other regenerating tissues (Cornelison, 2008; Goetsch et al., 2003; Huard et al., 2002; Serrano and Munoz-Canoves, 2010; Tomasek et al., 2002; Verrecchia and Mauviel, 2007). This fibrotic ECM is synthesized largely by fibroblasts resident in the MCT (Alexakis et al., 2007; Bailey et al., 1979; Kuhl et al., 1982; Lipton, 1977; Sanderson et al., 1986; Sasse et al., 1981; Zou et al., 2008). However, study of MCT fibroblasts has been hindered by the lack of robust markers for these cells. Recently, we have identified the transcription factor Tcf4 (transcription factor 7-like 2, Tcf7L2) as an excellent marker of MCT fibroblasts (Mathew et al., 2011). Tcf4^{+} fibroblasts are derived developmentally from the lateral plate

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mesoderm and are a separate lineage from somitically derived myogenic cells (Kardon et al., 2003). During development, Tcf4$^+$ fibroblasts are closely associated with developing muscle and regulate muscle fiber type and maturation (Kardon et al., 2003; Mathew et al., 2011). The observation that MCT fibrosis is characteristic of regenerating muscle and our recent finding that fibroblasts regulate muscle development together suggest that Tcf4$^+$ MCT fibroblasts might be important regulators of muscle regeneration.

In this study, we examine in vivo the role of satellite cells, MCT fibroblasts and their interactions in muscle regeneration. Using mouse genetics to genetically label and ablate Pax7$^+$ satellite cells and Tcf4$^+$ MCT fibroblasts, we demonstrate the necessity of satellite cells for muscle regeneration and the regulation of satellite cells by nearby MCT fibroblasts. Importantly, we also demonstrate that reciprocal interactions between satellite cells and fibroblasts ensure efficient and effective muscle regeneration.

MATERIALS AND METHODS

Mice

We generated the *Pax7^{CreERT2}* targeting vector using published methods (Wu et al., 2008). In brief, we recombineered a 7.4 kb fragment of mouse *Pax7* from a BAC library (CHORI clone RP24-128A11) into the pStart plasmid and introduced an *IRES-CreERT2-FRT-Neo-FRT* cassette (from D. Kopinke and L. C. Murtaugh, University of Utah) 8 bp after the endogenous stop codon. The targeting vector was electroporated into G4 ES cells and selected with G418 and FIAU. Twenty-nine of 148 clones exhibited homologous recombination upon Southern blotting with a probe outside the 5′ homology arm. Appropriate recombination was confirmed by a 3′ probe for 28/29 of these, and one of these clones was used to generate chimeras. Subsequent genotyping was performed using the following primers: Forward 1: 5′-GCTGCTGTTGATTACCTGGC-3′; Reverse 1: 5′-GCACTGAGACAGGACCG-3′; and Reverse 2: 5′-CAAAAGACGGCAATATGGTG-3′. PCR products: wild-type, 417 bp; CreERT2, 235 bp. *Neo* was removed by crossing *Pax7$^{tCreERT2+neo}$* mice with *R26R^{Flpe}* mice (Farley et al., 2000). *Tcf4^{CreERT2}* mice were generated (in collaboration with M. L. Angus-Hill and M. R. Capecchi, University of Utah) by replacing the translated part of exon 1 of *Tcf4* by a *CreERT2* cassette (Feil et al., 1997) and GCSF polyA using published methods (Wu et al., 2008). Details of *R26R^{lacZ}*, *R26R^{YFP}*, *R26R^{mTmG}*, *R26R^{DTA}* and *Polr2a^{nlacZ}* mice have been published (Haldar et al., 2008; Muzumdar et al., 2007; Soriano, 1999; Srinivas et al., 2001; Wu et al., 2006). For all experiments, mice were bred onto a C57/Bl6J background and used at 6-8 weeks of age.

Muscle injury, tamoxifen and EdU delivery

Injury was induced by injecting 25 μl of 1.2% $BaCl_2$ or 25 μl of 10 mM cardiotoxin in normal saline into the right tibialis anterior (TA) muscle. The left TA served as the uninjured control. Each dose of tamoxifen was 10 mg in corn oil delivered via gavage (Park et al., 2008). For EdU labeling, mice received 100 μg 5-ethynyl-2′-deoxyuridine (EdU; Invitrogen) in PBS by intraperitoneal injection 8 hours before harvest. All animal protocols were Institutional Animal Care and Use Committee (IACUC) approved.

Immunofluorescence, histology and microscopy

For section immunofluorescence, flash-frozen or OCT-embedded (GFP$^+$) muscles were sectioned at 10 μm, fixed for 5 minutes in 4% paraformaldehyde (PFA), washed in PBS and then, if needed, subjected to antigen retrieval, which consists of heating slides in citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate in H_2O) in a 2100 PickCell Retriever followed by quenching for 5 minutes in 3% H_2O_2. Cells were fixed for 20 minutes in 4% PFA. Tissue sections and cells were blocked for 30-60 minutes in 5% serum or 0.5% TNB blocking reagent (PerkinElmer) in PBS, incubated overnight at 4°C in primary antibody, washed in PBS, incubated for 2 hours at room temperature in secondary antibody, washed in PBS, then, when needed, incubated for 3 hours in Vector ABC, washed in PBS and labeled for 10 minutes with PerkinElmer TSA Fluorescein or TSA Cy3. Afterwards, slides were washed in PBS, and EdU labeled using the Invitrogen Click-iT Kit per manufacturer instructions. Slides were then washed in PBS, post-fixed for 5 minutes in 4% PFA and mounted with Fluoromount-G (SouthernBiotech) with 2 mg/ml Hoechst. For Sirius Red staining, flash-frozen sections were fixed for 1 hour at 56°C in Bouin's fixative, washed in water, stained for 1 hour in Master*Tech Picro Sirius Red, washed in 0.5% acetic acid, dehydrated, equilibrated with xylene and mounted using Permount (Kiernan, 1990). Nile Blue staining was carried out according to published methods (Kiernan, 1990). Cells and Sirius Red- and Nile Blue-stained sections were imaged in bright-field on a Zeiss Axioplan2 microscope. Immunofluorescent sections were imaged on a Nikon AR1 confocal microscope. Each confocal image is a composite of maximum projections, derived from stacks of optical sections.

For section immunofluorescence, the following antibodies were used and required antigen retrieval: Pax7 [2.4 μg/ml, mouse IgG1, PAX7, Developmental Studies Hybridoma Bank (DSHB)], MyoD (4 μg/ml, mouse IgG1, Sc-32758, Santa Cruz Biotechnology), MyHCemb (3 μg/ml, mouse IgG1, F1.652, DSHB), MyHCl (1.5 μg/ml, mouse IgG1, M8421, Sigma), MyHCperi+II (10 μg/ml, mouse IgG1, M4276, Sigma), laminin (2.5 μg/ml, rabbit polyclonal, L9393, Sigma), Tcf4 (10 μg/ml, mouse IgG2a, 05-511, Millipore; or 0.7 μg/ml, rabbit monoclonal, 2569, Cell Signaling) and phospho-histone H3 (5 μg/ml, rabbit polyclonal, 06-570, Millipore). The following antibodies were used on sections and did not require antigen retrieval: PDGFRα (5 μg/ml, goat polyclonal, AF1062, R&D Systems), F4/80 (2 μg/ml, rat IgG2a, 14-4801, eBioscience) and GFP (20 μg/ml, chick polyclonal, GFP-1020, Aves Labs).

For immunofluorescence of isolated myofibers or fibroblasts, the following antibodies were used, did not require antigen retrieval and were visualized with fluorophore-conjugated secondary antibodies (e.g. 488 or 594 goat anti-mouse IgG2A): Syndecan 4 (1:1500, chick polyclonal, gift of D. D. Cornelison, University of Missouri), CD34 (10 μg/ml, rat IgG2a, 14-0341, eBioscience) and aSMA (5.2 μg/ml, mouse IgG2a, A2547, Sigma), as well as GFP, PDGFRα, Tcf4 (see above).

Cell culture

For myofiber preparations, TAs were digested with 400 U/ml Collagenase I (Worthington) for 90 minutes at 37°C, washed in PBS, fixed for 5 minutes in 4% PFA, washed in PBS and mounted onto slides before being processed for immunofluorescence. Five days post-injury (dpi) with $BaCl_2$, MCT fibroblasts were isolated from TAs by digestion with 2000 U/ml Collagenase I for 60 minutes at 37°C. Cells were filtered, spun at 2500 rpm (1258 ***g***) for 20 minutes and plated (25,000 cells/cm^2) on 6-cm plastic dishes containing gelatin-coated coverslips for 2 hours at 37°C in F12 Hams with 10% FBS. Cells in the supernatant were discarded and 24-48 hours later adherent cells were harvested.

Semi-quantitative PCR

For semi-quantitative PCR, fibroblasts were isolated by pre-plating cells from limb muscles of neonatal wild-type mice and myoblasts isolated by fluorescence-activated cell sorting (FACS) of yellow fluorescent protein (YFP)$^+$ cells from limb muscles of neonatal *Pax7$^{Cre/+}$;R26R$^{YFP/+}$* mice as described by Mathew et al. (Mathew et al., 2011). Total RNA was extracted using the TissueLyser II and Qiagen RNeasy Lipid Tissue Mini Kit and reverse transcribed with Invitrogen Superscript III. Equal amounts of RNA were amplified by 34 cycles of PCR using primers for *Gapdh* (5′-GCACCACCAACTGCTTAGC-3′; 5′-GCCGTATTCATTGTCATACC-3′), *Tcf4* (5′-GGAGGAGAAGAACTCGGAAAA-3′; 5′-AGGTAGGG-GCTCGTCAGGT-3′) and *Col6a3* (5′-ACAAATGCCCTTGCTGCTAC-3′; 5′-ATCGCCCACAATGCCCAGAA-3′).

Quantification and statistics

The number of Pax7$^+$, MyoD$^+$ or Tcf4$^+$ nuclei was determined using the ImageJ Analyze Particles function. Co-labeling of Pax7, MyoD or Tcf4 with phosphohistone-H3 (PHH3) or EdU was determined by additive image overlay in ImageJ. For MyHCemb (embryonic myosin heavy chain) or MyHCtotal (total myosin heavy chain), the total number of MyHCemb$^+$ or MyHCtotal$^+$ pixels was counted. For quantification of MCT extracellular matrix, Sirius Red$^+$ area was quantified by selecting red pixels in Adobe Photoshop, deleting all non-red pixels, converting the resulting image to a

binary image and counting Red$^+$ pixels using the ImageJ Analyze Particles function. For each variable, counts of two to three sections across the entire TA were averaged for three to five individuals of each genotype per time point and analyzed using Student's two-tailed *t*-test.

RESULTS

Tcf4 is highly expressed in MCT fibroblasts during adult muscle regeneration

During development and in the adult, fibroblasts in the MCT endomysium, perimysium and epimysium strongly express Tcf4 (Mathew et al., 2011). To determine whether Tcf4$^+$ MCT fibroblasts are present during muscle regeneration in the adult, we examined the tibialis anterior (TA) muscle of wild-type mice five days post injury (dpi) by $BaCl_2$. $BaCl_2$ causes myofiber hypercontraction and death, but does not affect the surrounding mononuclear populations, such as the satellite cells and fibroblasts (Caldwell et al., 1990; Hansen et al., 1984). In TAs at 5 dpi, there is an abundance of Sirius Red$^+$ MCT surrounding regenerating myofibers (Fig. 1B). Within these MCT regions and outside the laminin$^+$ regenerating myofibers, cells strongly expressing Tcf4 were detected by immunofluorescence (Fig. 1A,C). Because Tcf4$^+$ cells do not co-label with Pax7, MyoD or F4/80 (Fig. 1E,F), they are unlikely to be myogenic progenitors, myoblasts or macrophages. Furthermore, many of these Tcf4$^+$ cells co-label with PDGFRα (platelet-derived growth factor receptor alpha), a receptor expressed on MCT fibroblasts (Fig. 1D) (Joe et al., 2010; Olson and Soriano, 2009; Uezumi et al., 2010).

We characterized Tcf4$^+$ cells further by isolating and growing MCT fibroblasts in culture. MCT fibroblasts were isolated by plating cells freshly dissociated from adult TAs (at 5 dpi by $BaCl_2$) on plastic culture dishes. After two hours, myogenic cells, which do not readily adhere to plastic (Richler and Yaffe, 1970), were discarded and adherent cells were washed and grown for 24-48 hours. The adherent cells were highly enriched with fibroblasts (identifiable morphologically by their pseudopodia and large, round nuclei), and these fibroblasts were Tcf4$^+$ (Fig. 1G,H). In addition, Tcf4$^+$ fibroblasts were PDGFRα$^+$ and αSMA$^+$ (alpha smooth muscle actin, another marker of fibroblasts) (Tomasek et al., 2002) (Fig. 1G,H). MCT fibroblasts have been shown previously to synthesize high levels of ECM and to uniquely synthesize collagen VI, whereas myogenic cells do not synthesize this collagen (Zou et al., 2008). We found by semi-quantitative PCR that fibroblasts, isolated via pre-plating, express Tcf4 and collagen VI whereas myoblasts, isolated by FACS of YFP$^+$ cells from *Pax7$^{Cre/+}$;R26R$^{YFP/+}$* mice (in which myogenic precursors and their descendents are labeled) (Hutcheson et al., 2009) do not (Fig. 1I). In summary, we show that during muscle regeneration MCT fibroblasts express Tcf4, and that Tcf4$^+$ cells are neither myogenic cells nor macrophages.

Pax7$^+$ satellite cells and Tcf4$^+$ fibroblasts rapidly expand in close proximity to one another after muscle injury

To assess the potential role of satellite cells and MCT fibroblasts during muscle regeneration, we characterized the temporal-spatial relationship between Pax7$^+$ satellite cells and Tcf4$^+$ fibroblasts during normal regeneration after $BaCl_2$ injury in wild-type mice. Similar to the findings of others (d'Albis et al., 1988), we observed that regenerating myofibers begin to express the developmental myosin heavy chain isoform MyHCemb at 3 dpi, express peak levels at 5 dpi, and by 14 dpi the mature, regenerated myofibers no longer express MyHCemb, but only mature MyHCI/II isoforms

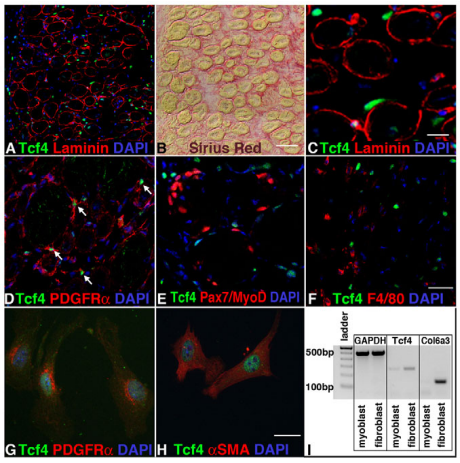

Fig. 1. Tcf4 is highly expressed in muscle connective tissue (MCT) fibroblasts during muscle regeneration. (**A-C**) Tcf4$^+$ cells at 5 dpi ($BaCl_2$) are interstitial to regenerating laminin$^+$ myofibers within Sirius Red$^+$ MCT. A and B show adjacent sections. (**D-F**) In muscle at 5 dpi, some Tcf4$^+$ cells are PDGFRα$^+$ (D, arrows), and all are Pax7$^-$ and MyoD$^-$ (E) and F4/80$^-$ (F). (**G**,**H**) MCT fibroblasts isolated from TAs at 5 dpi are Tcf4$^+$, PDGFRα$^+$ (G) and αSMA$^+$ (H). (**I**) Semi-quantitative PCR shows that neonatal MCT fibroblasts, but not myoblasts, express Tcf4 and Col6a3. Scale bars: in B, 50 μm for A,B; in C, 12.5 μm; in F, 25 μm for D-F; in H, 10 μm for G,H.

(Fig. 2A-G,CC; data not shown). An increase in MCT ECM is also characteristic of muscle regeneration (Huard et al., 2002). By quantifying the amount of Sirius Red$^+$ MCT (Dubowitz and Sewry, 2007), we found that MCT increases to peak levels at 3 dpi, in close proximity to the regenerating myofibers, and then gradually resolves to near-normal amounts by 21 dpi (Fig. 2V-BB,FF). Concentrated in the region of regenerating myofibers, Pax7$^+$ satellite cells rapidly proliferated within 1 dpi (Fig. 2GG) and expanded from 25 cells/mm^2 at 1 dpi to 180 Pax7$^+$ cells/mm^2 by 5 dpi (Fig. 2H-N,DD). Satellite cells either differentiate into myoblasts and myofibers (which downregulate Pax7) or return to quiescence as Pax7$^+$ satellite cells lying under the basement membrane of myofibers (Seale et al., 2000). By 28 dpi, the number of quiescent Pax7$^+$ satellite cells returned to normal, uninjured levels (Fig. 2DD; data not shown). We also found that 3 dpi Tcf4$^+$ fibroblasts rapidly proliferate (Fig. 2HH) and increase from 165 cells/mm^2 at 1 dpi to peak levels of 650 Tcf4$^+$cells/mm^2 at 5 dpi (Fig. 2O-U,EE). These Tcf4$^+$ fibroblasts were in close proximity to satellite cells and regenerating myofibers and lay within the Sirius Red$^+$ MCT (Fig. 2D,K,R,Y). Concomitant with the decrease in MCT, the number of Tcf4$^+$ fibroblasts decreased to normal, uninjured levels by 28 dpi (Fig. 2U,EE; data not shown). Thus, we show for the first time that during muscle regeneration MCT fibroblasts rapidly expand in regions of regenerating myofibers and MCT fibrosis, and in close association with satellite cells. The close temporal and spatial relationship between satellite cells and MCT fibroblasts suggests that interactions between these two cell types might be important for regeneration.

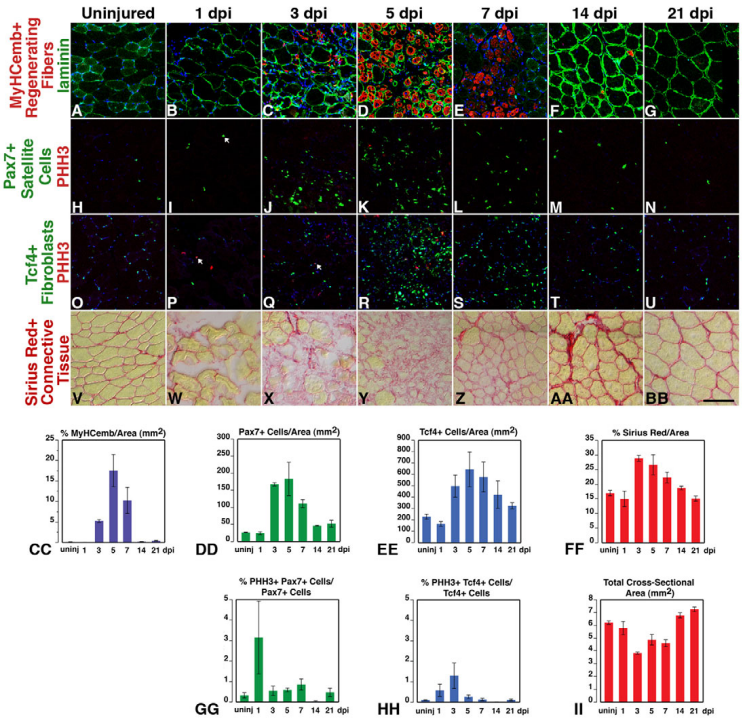

Fig. 2. After $BaCl_2$ injury, Pax7$^+$ satellite cells and Tcf4$^+$ fibroblasts expand rapidly in close proximity to one another and regenerating myofibers. (**A-G**,**CC**) MyHCemb$^+$ regenerating myofibers. (**H-N**,**DD**,**GG**) Pax7$^+$ satellite cells. (**O-U**,**EE**,**HH**) Tcf4$^+$ muscle connective tissue (MCT) fibroblasts. (**V-BB**,**FF**) Sirius Red$^+$ MCT. Arrows in I, P and Q label a few of the PHH3$^+$ Pax7$^+$ or PHH3$^+$Tcf4$^+$ cells. (**II**) Tibialis anterior (TA) cross-sectional area measured on Sirius Red-stained sections 30-40 μm from TA origin. At each time point, adjacent sections are shown. Scale bar: 100 μm for A-BB. For all graphs, mean ± s.e.m. are plotted.

Pax7^{CreERT2} mice allow for efficient manipulation of satellite cells

To genetically manipulate satellite cells in the adult, we created tamoxifen-inducible *Pax7^{CreERT2}* mice by homologous recombination. In these mice, an *iresCreERT2* cassette (Feil et al., 1997) is inserted just after the endogenous termination codon, maintaining the 3′UTR and the endogenous poly(A) sequence (Fig. 3A). To test the efficiency of Cre-mediated recombination in Pax7$^+$ satellite cells, we crossed *Pax7^{CreERT2}* to *R26R^{lacZ}*, *R26R^{YFP}*, *R26R^{mTmG}* or *Polr2a^{nlacZ}* reporter mice, which express β-galactosidase, cytoplasmic YFP, or membrane-bound GFP in response to Cre (Haldar et al., 2008; Muzumdar et al., 2007; Soriano, 1999; Srinivas et al., 2001). In the absence of tamoxifen, no *Pax7$^{CreERT2/+}$;R26R$^{reporter/+}$* mice ever expressed any of the reporters. We tested whether, in the presence of tamoxifen, *Pax7^{CreERT2}* mice genetically labeled Pax7$^+$ cells by harvesting uninjured TAs from adult *Pax7$^{CreERT2/+}$;R26R$^{mTmG/+}$* mice one day after three daily tamoxifen doses. In TA sections, nearly all Pax7$^+$ satellite cells lying under the laminin$^+$ basement membrane of myofibers were GFP$^+$ (Fig. 3F-I). In addition, no other cells (e.g. myonuclei or interstitial cells, such as fibroblasts) other than sublaminar Pax7$^+$ cells, were GFP$^+$ (Fig. 3J,K-N). To quantify the efficiency of Cre-mediated recombination in satellite cells, we isolated single myofibers from uninjured TAs of *Pax7$^{CreERT2/+}$;R26R$^{YFP/+}$* mice, one day after five daily tamoxifen doses. Ninety-five percent of all Pax7$^+$ satellite cells were YFP$^+$ (n=764). In addition, we verified that YFP$^+$ cells on the myofibers were satellite cells, as YFP$^+$ cells were co-labeled with Syndecan4 and CD34, two other satellite cell markers (Fig. 3C,D) (Beauchamp et al., 2000; Cornelison et al., 2001). We also confirmed that Pax7$^+$ cells give rise to regenerated muscle by injuring TAs from *Pax7$^{CreERT2/+}$;Polr2a$^{nlacZ/+}$* mice (with five tamoxifen doses) and then staining 14 dpi TAs for β-galactosidase in section or in whole mount (Fig. 3O and Fig. 5B). As expected, all regenerated myofibers were β-galactosidase$^+$.

Pax7^{CreERT2} mice were designed to preserve endogenous Pax7 expression and function. To test whether Pax7 expression and function were intact, we generated *Pax7$^{CreERT2/CreERT2}$;R26R$^{YFP/+}$* mice. YFP$^+$ CD34$^+$ satellite cells were present on myofibers

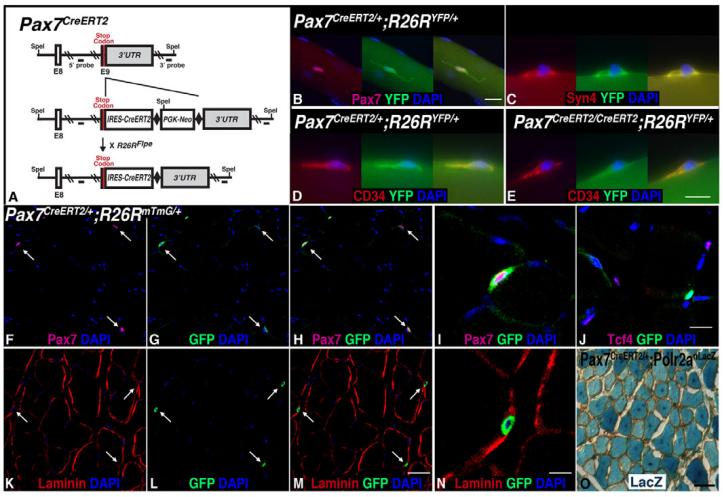

Fig. 3. *Pax7*^{CreERT2} mice efficiently label satellite cells. (**A**) *Pax7*^{CreERT2} targeting strategy. (**B-D**) In myofibers isolated from uninjured tibialis anterior muscles (TAs) from *Pax7*$^{CreERT2/+}$;*R26R*$^{YFP/+}$ mice one day after five daily tamoxifen doses, 95% of Pax7^{+} satellite cells are YFP^{+} (B) and YFP^{+} cells are Syndecan4^{+} (C) and CD34^{+} (D). (**E**) In fibers isolated from uninjured TAs from *Pax7*$^{CreERT2/CreERT2}$;*R26R*$^{YFP/+}$ mice one day after five tamoxifen doses, normal numbers of CD34^{+} satellite cells are present. (**F-O**) In cryosections of uninjured TAs from *Pax7*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice one day after three tamoxifen doses, nearly all Pax7^{+} cells are GFP^{+} (F-I), lie within laminin^{+} myofiber basement membrane (K-N), and are Tcf4^{-} (J). Tomato in Cre-myofibers was quenched by antigen retrieval. Arrows indicate Pax7^{+} satellite cells. (O) At 14 dpi, all regenerating myofibers are β-galactosidase^{+} in *Pax7*$^{CreERT2/+}$;*Polr2a*$^{nlacZ/+}$. Scale bars: in B, 20 μm; in E, 10 μm for C-E; in M, 25 μm for F-H,K-M; in J, 12.5 μm; in N, 6.25 μm for I,N; in O, 50 μm.

isolated from uninjured TAs of *Pax7*$^{CreERT2/CreERT2}$;*R26R*$^{YFP/+}$ mice (1 day after five daily tamoxifen doses; Fig. 3E). In addition, we counted equivalent numbers of Pax7^{+} cells in cryosections of uninjured and injured TAs at 5 dpi ($BaCl_2$) from *Pax7*$^{CreERT2/+}$ and *Pax7*$^{CreERT2/CreERT2}$ mice (data not shown). As the loss of Pax7 prior to P21 has been found to impair satellite cell proliferation and maintenance (Kuang et al., 2006; Lepper et al., 2009; Oustanina et al., 2004; Relaix et al., 2006), the finding of equivalent numbers of Pax7^{+} cells in 6-week-old *Pax7*$^{CreERT2/+}$ and *Pax7*$^{CreERT2/CreERT2}$ mice confirms that Pax7 function is not compromised. Thus, our data demonstrate that *Pax7*^{CreERT2} mice allow for high efficiency Cre-mediated genetic manipulation of satellite cells, without compromising Pax7 expression or function.

Ablation of Pax7^{+} satellite cells leads to a complete loss of muscle regeneration, misregulation of Tcf4^{+} fibroblasts and increased MCT fibrosis

Satellite cells have been proposed to be the primary stem cell responsible for regenerating muscle. To test whether satellite cells are necessary for regeneration, we genetically ablated Pax7^{+} satellite cells using *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. In these mice, Cre activates expression of diphtheria toxin A (DTA) (Wu et al., 2006) and kills Pax7^{+} satellite cells specifically in response to tamoxifen. We found that in the injured TAs of *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice after five tamoxifen doses (strategy in Fig. 4A-I) and at 5 dpi ($BaCl_2$), 91% of Pax7^{+} cells were ablated (*P=0.02, *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice, n=6; Fig. 4A-C). Satellite cell ablation was also confirmed by analyzing the number of Pax7^{+} or Syndecan4^{+} satellite cells (per mm of myofiber length) on isolated uninjured myofibers from *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ and *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice 30 days after five tamoxifen doses. In this assay, 83-84% of satellite cells were ablated (*P=8×10^{-5} Pax7^{+} cells, n=149 myofibers and three mice or *P=1×10^{-7} Syn4^{+} cells, n=172 myofibers and three mice; see Fig. S1A-D in the supplementary material).

Satellite cell ablation severely impaired muscle regeneration at 5 dpi, resulting in an 89% reduction in MyHCemb^{+} regenerating myofibers (*P=0.02; Fig. 4D-F) compared with *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice (also with five tamoxifen doses). In addition to impairing muscle, satellite cell ablation affected the expansion of Tcf4^{+} fibroblasts, as their numbers were reduced by 52% (P=0.09; Fig. 4G-I).

Muscle regeneration was dramatically impaired at 28 dpi by satellite cell ablation (but with no other apparent effects on mouse survival or behavior). In *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (Fig. 4J-V), only a few small, clonal patches of Pax7^{+} cells remained (which presumably escaped Cre-mediated DTA ablation, data not shown). TAs were entirely fibrotic with no visible muscle (except for adjacent uninjured extensor digitorum longus, EDL, muscles) in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice, compared with fully regenerated muscles in *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice (Fig. 4S,T). Rather than the normal hypertrophy seen after injury (Fig. 2U,V), the weight of injured TAs (normalized to left uninjured TAs) was reduced by 38% (*P=0.01, n=6), and the cross-sectional area reduced by 60% (*P=0.02; Fig. 4L,O,R) in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice. In sections through the entire TA, few myosin^{+} (MyHCI and II) myofibers were present in the injured

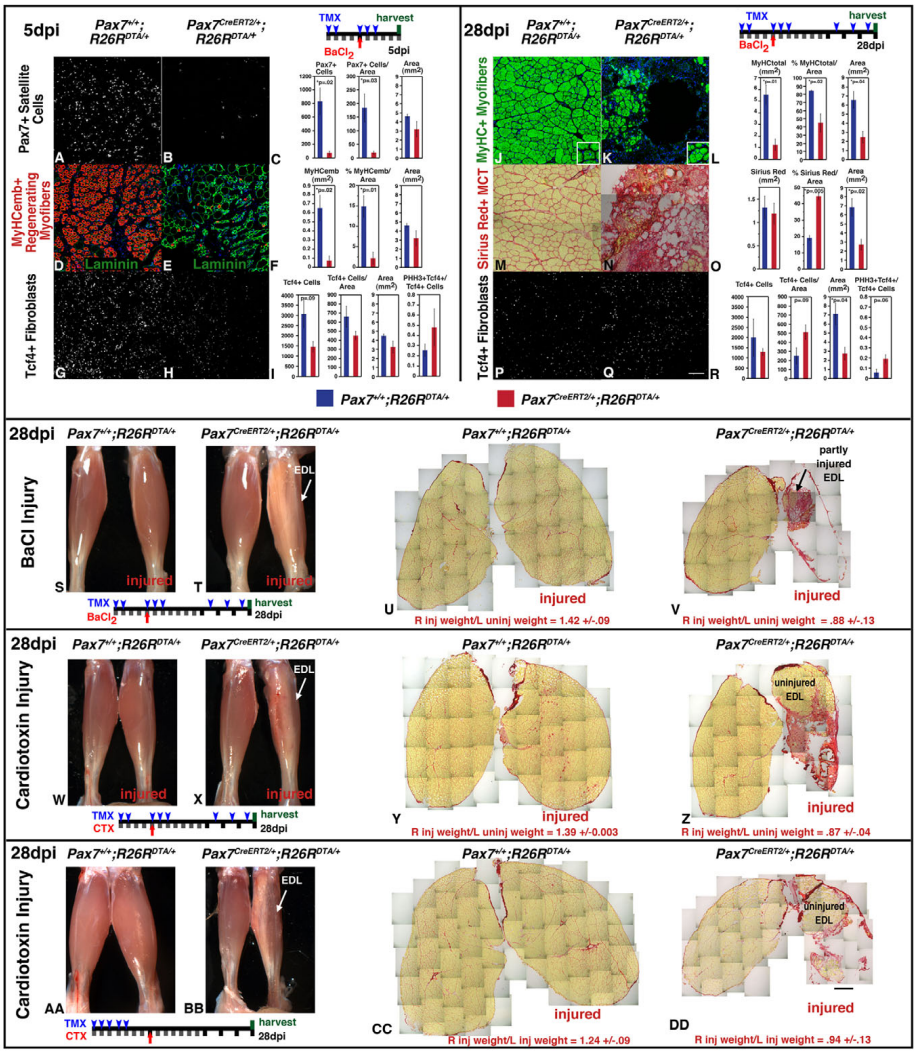

Fig. 4. Ablation of Pax7⁺ satellite cells leads to complete loss of muscle regeneration. (**A-I**) At 5 dpi, 91% of Pax7⁺ cells are ablated (A-C), resulting in fewer MyHCemb⁺ regenerating myofibers (D-F) and Tcf4⁺ fibroblasts (G-I) in *Pax7$^{CreERT2/+}$;R26R$^{DTA/+}$* mice. (**J-R**) At 28 dpi, tibialis anterior (TA) cross-sectional area (L,O,R) and MyHCemb⁺ regenerated myofibers (J-L) are reduced, whereas the proportion of Sirius Red⁺ MCT (M-O), and the density of Tcf4⁺ fibroblasts (P-R) is increased in *Pax7$^{CreERT2/+}$;R26R$^{DTA/+}$* mice. Insets in J and K show residual, incompletely injured myofibers with peripheral nuclei in *Pax7$^{CreERT2/+}$;R26R$^{DTA/+}$* mice compared with regenerated myofibers with centralized nuclei in *Pax7$^{+/+}$;R26R$^{DTA/+}$*. (**S-Z**) At 28 dpi, {$BaCl_2$ or cardiotoxin (CTX)}, injured TAs are completely fibrotic or edemic in *Pax7$^{CreERT2/+}$;R26R$^{DTA/+}$* mice, in whole mount (S,T,W,X) and Sirius Red-stained cross-sections (U,V,Y,Z). (**AA-DD**) Ablation of satellite cells prior to CTX injury leads to loss of regenerated muscle. In all tamoxifen/injury strategy schema, gray bars represent one day and black bars one week, tamoxifen (TMX) administration is indicated by blue arrowheads and $BaCl_2$ or CTX application is indicated by red arrows. Whole mount images have been flipped so the injured limb (R) is on the right. TA weights include attached extensor digitorum longus (EDL). Scale bars: in Q, 100 μm for A,B,D,E,G,H,J,K,M,N,P,Q; in DD, 500 μm for U,V,Y,Z,CC,DD. For all graphs, mean ± s.e.m. are plotted.

region (77% reduction in MyHCI/II area, **P*=0.01; Fig. 4J-L,U-V). The few myofibers in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice after $BaCl_2$ injury were residual, incompletely injured, small myofibers with peripheral nuclei, whereas myofibers in *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice were regenerated myofibers with characteristic centralized nuclei (insets in Fig. 4J,K). In *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice, muscle was replaced by a 2.3-fold increase in Sirius Red$^+$ MCT (Sirius Red$^+$ MCT area/total area, **P*=0.0002; Fig. 4M-O,U,V), and the number of Tcf4$^+$ fibroblasts/cross-sectional area was increased twofold (*P*=0.09; Fig. 4P-R).

We also tested the requirement of Pax7$^+$ satellite cells for muscle regeneration after injury by cardiotoxin (CTX), a protein kinase C inhibitor that causes cellular apoptosis (Gayraud-Morel et al., 2007; Sinha-Hikim et al., 2007). Twenty-eight days post-injury, muscle had not regenerated in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (Fig. 4W-Z). TA weight was reduced by 37% (**P*=0.0003, *n*=6). Muscle was largely replaced by Sirius Red$^+$ MCT (Fig. 4Y,Z) and increased Nile Red$^+$ adipose tissue (see Fig. S1M,N in the supplementary material). To test whether regeneration ever recovered, we harvested cardiotoxin-injured muscles at 56 dpi. Injured TAs still did not regenerate with ablation of satellite cells, and, in fact, the weight of TAs was even more reduced, by 56% (**P*=4×10^{-6}, *n*=6), in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice (see Fig. S1E-H in the supplementary material).

Other cell populations have also been proposed as stem cells important for muscle regeneration. Some of these populations, such as PICs (Mitchell et al., 2010), initially do not express Pax7, but later express Pax7 during their differentiation into muscle. Our analysis of *Pax7*$^{CreERT2/+}$;*R26R*^{mTmG} mice found that in the absence of injury, delivery of tamoxifen caused Cre-mediated recombination only in satellite cells residing beneath the basal lamina of myofibers. Delivery of tamoxifen after injury in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice could potentially cause Cre-mediated ablation of other stem cells, which after injury express Pax7. To exclude potential ablation of these other cells that express Pax7 after injury, we repeated CTX injury experiments with *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ and *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice, but with five tamoxifen doses prior to injury. Similar to our other experiments, there was a complete absence of regenerated muscle, the weight of injured TAs was reduced by 24% (*P*=0.06, *n*=7), and muscle was replaced by Sirius Red$^+$ MCT in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (Fig. 4AA-DD). To determine further whether other stem cell populations might regenerate muscle, we ablated satellite cells, injured muscles via CTX, then at 28 dpi re-injured muscles and assessed regeneration 28 days later. Again, muscle was unable to regenerate, and the weight of injured TAs was reduced by 44% (**P*=0.002, *n*=16; see Fig. S1I-L in the supplementary material) in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice.

We conclude that genetic ablation of Pax7$^+$ satellite cells resulted in a complete loss of regenerated muscle, demonstrating that satellite cells are required for regeneration. Furthermore, ablation of satellite cells led to misregulation of Tcf4$^+$ fibroblasts and a dramatic increase in MCT, revealing that satellite cells also have an effect on MCT fibroblasts and fibrosis.

Tcf4^{CreERT2} mice allow for manipulation of connective tissue fibroblasts

To genetically manipulate MCT fibroblasts in the adult, we created tamoxifen-inducible *Tcf4*^{CreERT2} mice. Previously, we generated *Tcf4*^{GFPCre} mice and showed that these mice allow for genetic manipulation of MCT fibroblasts (Mathew et al., 2011). To produce a tamoxifen-inducible version, we replaced the translated part of exon 1 and its splice donor with a *CreERT2* cassette placed at the endogenous *Tcf4* start codon (Fig. 5A). This substitution of exon 1 with *CreERT2* resulted in a 'knock-in/knockout allele' such that *Tcf4* is not expressed from the *Tcf4*^{CreERT2} allele. Similar to *Tcf4*^{GFPCre} and other *Tcf4* alleles (Korinek et al., 1998; Mathew et al., 2011), heterozygous *Tcf4*$^{CreERT2/+}$ mice are phenotypically normal and have normal muscle regeneration (data not shown).

We characterized *Tcf4*^{CreERT2} mice by crossing them to *R26R*^{lacZ} or *R26R*^{mTmG} reporter mice. TAs at 5 dpi ($BaCl_2$) were harvested from adult *Tcf4*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice after five tamoxifen doses (tamoxifen scheme in Fig. 6M-X; no reporter was ever expressed in the absence of tamoxifen), cryosectioned and immunolabeled for Tcf4 and GFP. Membrane-bound GFP$^+$ cells were Tcf4$^+$, exhibited a fibroblast morphology, were found interstitial to the regenerating laminin$^+$ myofibers, and did not co-label with the F4/80 macrophage marker (Fig. 5C-K). To characterize further the GFP$^+$ cells, we isolated fibroblasts by pre-plating cells from 5-dpi TAs of *Tcf4*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice after five tamoxifen doses. GFP$^+$ cells exhibited a fibroblast morphology; co-labeled with Tcf4 and the fibroblast markers αSMA and PDGFRα; and did not express the myogenic markers Pax7 or MyoD (Fig. 5L-O). Quantification of the efficiency of the Tcf4^{CreERT2} allele was problematic using *Tcf4*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice as we found that high levels of GFP expressed from the *R26R*^{mTmG} allele were toxic to fibroblasts in culture (data not shown). Based on the efficiency of ablation of fibroblasts in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (see next section), we estimate that the *Tcf4*^{CreERT2} allele causes Cre-mediated recombination in ~40% of MCT fibroblasts. To establish that *Tcf4*^{CreERT2} mice never caused Cre-mediated recombination in myogenic cells, we injured the TAs of *Pax7*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ and *Tcf4*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ mice that had been given five tamoxifen doses, and harvested TAs at 14 dpi. TAs were collagenase-treated to loosen myofibers (and this treatment also removed interstitial MCT fibroblasts) and processed for β-galactosidase staining. Although all myofibers were β-galactosidase$^+$ in the injured TAs (and some myofibers were β-galactosidase$^+$ in uninjured TAs) from *Pax7*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ mice, myofibers were never β-galactosidase$^+$ in TAs from *Tcf4*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ mice (*n*=10; Fig. 5B). This indicates that in *Tcf4*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ mice Cre-mediated recombination never occurs in satellite cells, myoblasts or myofibers, despite high numbers of these cells during muscle regeneration. Altogether, our data show that *Tcf4*^{CreERT2} mice allow for genetic manipulation of 40% of MCT fibroblasts and do not cause Cre-mediated recombination in satellite cells or macrophages, two other major populations present in regenerating muscle.

Ablation of Tcf4$^+$ MCT fibroblasts alters the expansion of satellite cells and impairs muscle regeneration

To test the potential function of Tcf4$^+$ fibroblasts during muscle regeneration, we ablated fibroblasts during regeneration using *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. We first assessed the rate of fibroblast ablation by quantifying the decrease in Tcf4$^+$ cells in injured TAs at 5 dpi ($BaCl_2$) of *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice with five tamoxifen doses (strategy in Fig. 6M-X) and found a 67% decrease in Tcf4$^+$ cells (**P*=9×10^{-5}, *n*=10). Potentially, this decrease in Tcf4$^+$ cells could partially reflect the loss of one allele of *Tcf4* in *Tcf4*$^{CreERT2/+}$ mice (as *Cre* replaces one *Tcf4* allele) and, thus, our decreased ability to detect Tcf4$^+$ cells. To test this, we quantified the numbers of Tcf4$^+$ cells in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice with no tamoxifen and found an apparent 25% decrease in Tcf4$^+$ cells. As

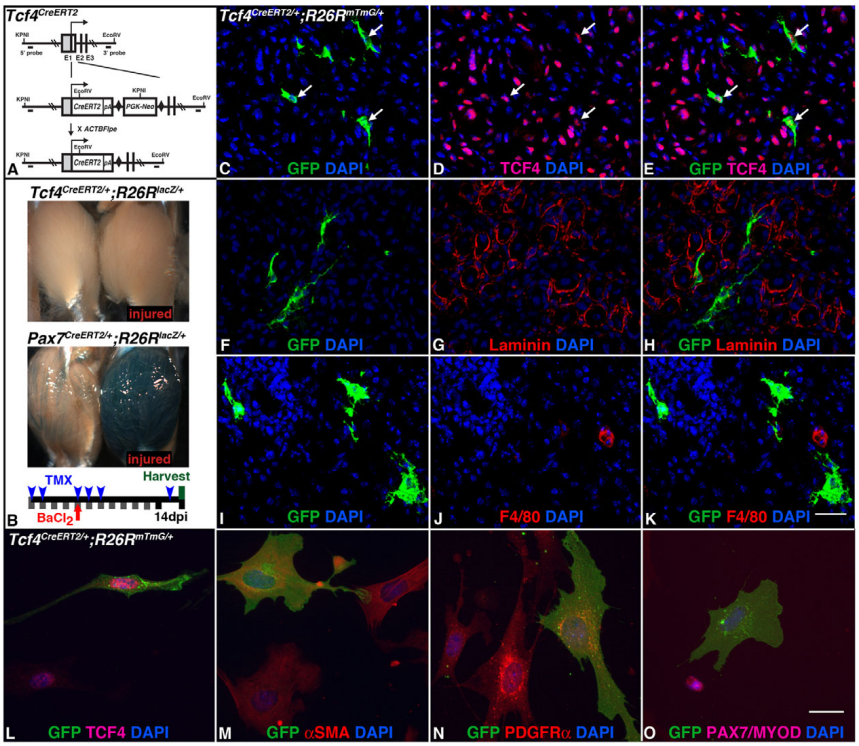

Fig. 5. *Tcf4*^{CreERT2} mice label muscle connective tissue (MCT) fibroblasts. (**A**) *Tcf4*^{CreERT2} targeting strategy. (**B**) At 14 dpi, several myofibers in uninjured tibialis anterior muscles (TAs) and all myofibers in injured TAs of *Pax7*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ mice are β-galactosidase^{+}, but in TAs of *Tcf4*$^{CreERT2/+}$;*R26R*$^{lacZ/+}$ mice no myofibers are β-galactosidase^{+}. (**C-K**) In cryosections of TAs at 5 dpi ($BaCl_2$) from *Tcf4*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice (five tamoxifen doses), Tcf4^{+} fibroblasts are GFP^{+} (F-K), lie in between laminin^{+} regenerating myofibers (F-H) and are not F4/80^{+} (I-K). (**L-N**) MCT fibroblasts isolated from TAs at 5 dpi ($BaCl_2$) from *Tcf4*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice (five tamoxifen doses) are GFP^{+} (L-N), Tcf4^{+} (L), αSMA^{+} (M) and PDGFRα^{+} (N). (**O**) In preparations of all mononuclear cells from the same *Tcf4*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice as shown in L-N, GFP^{+} cells are Pax7^{-} and MyoD^{-}. Scale bars: in K, 25 μm for C-K; in O, 10 μm for L-O.

we have not detected decreased numbers of fibroblasts isolated and cultured from *Tcf4*$^{CreERT2/+}$ versus *Tcf4*$^{+/+}$mice, this decrease in Tcf4^{+} cells probably results from fibroblasts expressing Tcf4 at levels below the limit of immunofluorescent detection. From these data, we then calculate the ablation rate of Tcf4^{+} cells to be 42%; 67% decrease in Tcf4^{+} cells in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice (five tamoxifen doses) minus the apparent 25% decrease in Tcf4^{+} cells in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice (no tamoxifen).

Ablation of 42% of fibroblasts strongly affected satellite cell dynamics during muscle regeneration. At 5 dpi, fibroblast ablation led to a 51% reduction in Pax7^{+} cells in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice (*P=0.002; Fig. 6M-O). This reduction in satellite cells was not due to an effect of Tcf4 haploinsufficiency, as levels of Pax7^{+} cells were equivalent at 5 dpi in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice in the absence of tamoxifen (data not shown). With ablation of fibroblasts, the reduction in Pax7^{+} cells was not due to reduced proliferation of Pax7^{+} cells (Fig. 6M-O). In addition to reduced numbers of Pax7^{+} cells, there was a 79% reduction in MyoD^{+} cells (P=0.06; Fig. 6P-R), indicating a decrease in activated MyoD^{+}Pax7^{+} satellite cells and/or MyoD^{+} differentiating myoblasts (see Cornelison and Wold, 1997; Yablonka-Reuveni and Rivera, 1994). In addition, there was a 31% decrease in MyHCemb^{+} regenerating myofibers (P=0.10; Fig. 6S-U). These results indicate that genetically reducing the numbers of fibroblasts negatively affected the initial muscle regenerative process.

To determine the mechanism underlying this impairment in muscle regeneration, we examined *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice at 3 dpi. With five tamoxifen doses (strategy in Fig. 5A-L), there was a 19% reduction in the number of Tcf4^{+} fibroblasts (calculated as above, but high variance precluded statistical significance, n=10; Fig. 6J-L) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. This ablation of

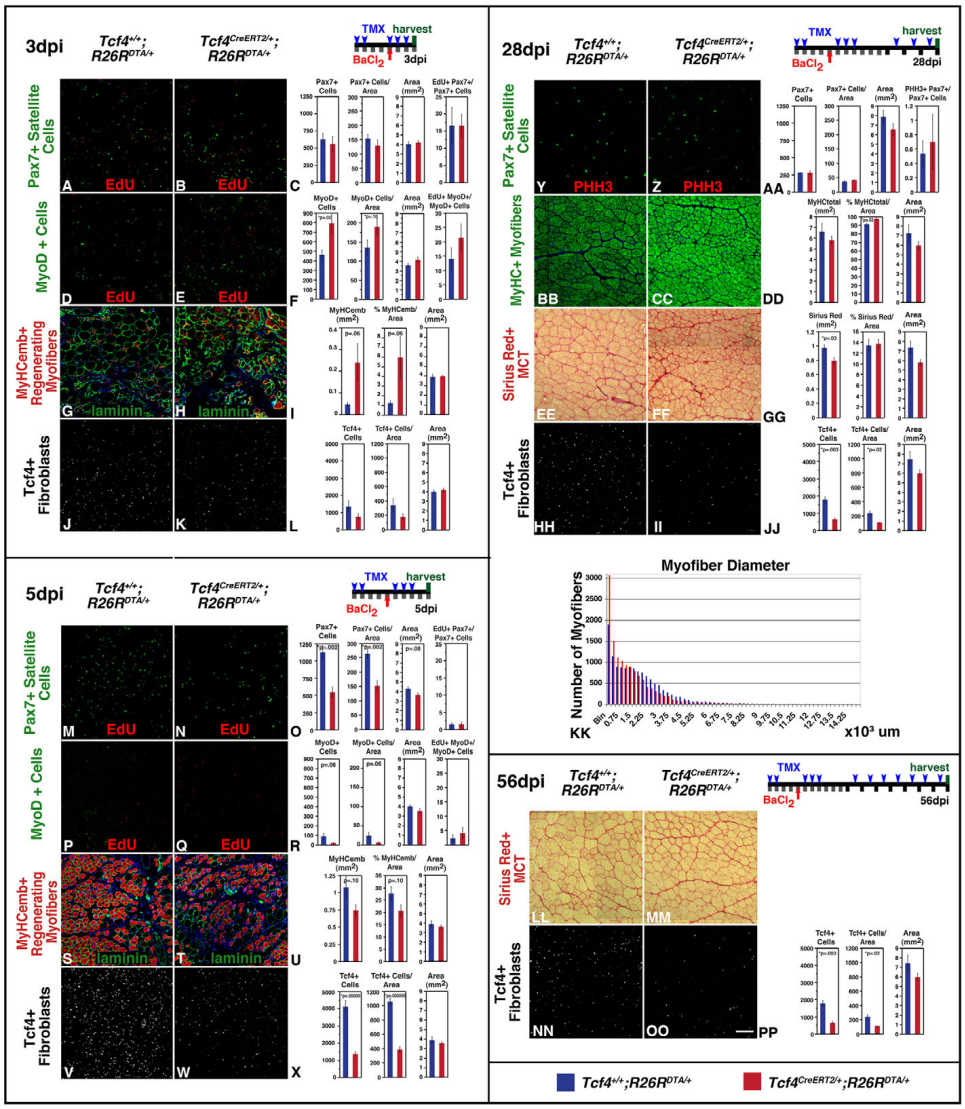

Fig. 6. During muscle regeneration, ablation of Tcf4⁺ fibroblasts leads to premature satellite cell differentiation and smaller regenerated myofibers. (**A-L**) At 3 dpi, Tcf4⁺ cells are reduced (J-L), with no change in Pax7⁺ cells (A-C), but with an increase in MyoD⁺ progenitors/myoblasts (D-F) and MyHCemb⁺ regenerating myofibers (G-I) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. (**M-X**) At 5 dpi, 42% of Tcf4⁺ cells were calculated to be ablated (V-X), resulting in fewer Pax7⁺ cells (M-O), MyoD⁺ progenitors/myoblasts (P-R) and MyHCemb⁺ regenerating myofibers (S-U) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. (**Y-KK**) At 28 dpi, despite Tcf4⁺ fibroblast ablation (HH-JJ), Pax7⁺ cells recover (Y-AA), muscle largely regenerates (BB-GG), but diameter of myofibers is reduced (BB,CC,EE,FF,KK) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ versus *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice. (**LL-PP**) By 56 dpi, regenerated muscle is recovered in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. Scale bars: in OO,100 μm for all photomicrographs. For all graphs, mean ± s.e.m. are plotted.

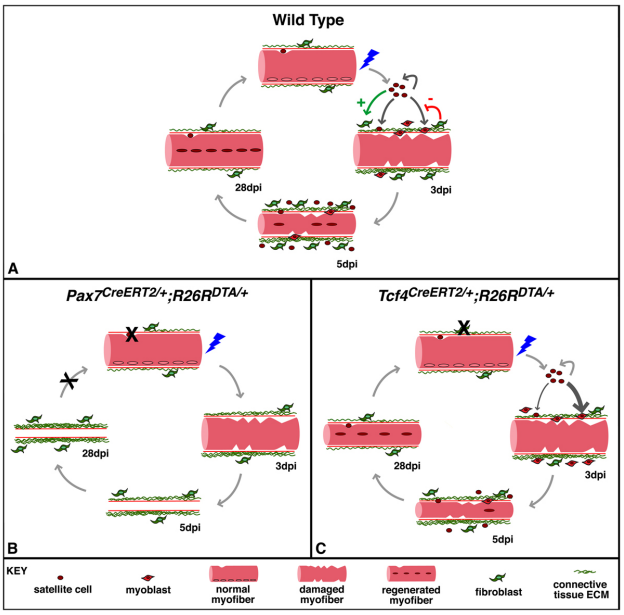

Fig. 7. Model of the role of Pax7$^+$ satellite cells and Tcf4$^+$ muscle connective tissue (MCT) fibroblasts and their interactions during muscle regeneration.
(**A-C**) Summary of cells and their interactions after injury during normal regeneration (A), with ablation of satellite cells (B) and with partial ablation of MCT fibroblasts (C). (A) During normal regeneration, satellite cells and fibroblasts rapidly proliferate. Satellite cells are absolutely required for normal regeneration of myofibers. Reciprocal interactions between satellite cells and fibroblasts are also important for regeneration. (B) With ablation of satellite cells, no regeneration of myofibers occurs and muscle is largely replaced by MCT and fibroblasts. (C) Partial ablation of fibroblasts leads to premature differentiation of satellite cells and results in smaller muscles with smaller myofibers at 28 dpi, when muscle regeneration is normally complete.

fibroblasts did not affect the number or proliferative status of Pax7$^+$ satellite cells (Fig. 6A-C). However, the number of MyoD$^+$ cells was increased 1.7-fold (*P=0.02; Fig. 6D-F) and the area of regenerating myofibers was increased fivefold (P=0.06; Fig. 6G-I) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice, indicating that Pax7$^+$ satellite cells had prematurely activated and begun differentiating with ablation of fibroblasts. Altogether, these data indicate that ablation of fibroblasts led to premature activation and differentiation of Pax7$^+$ satellite cells at 3 dpi and resulted in depletion of the pool of satellite cells and MyoD$^+$ cells and a consequent reduction in regenerating myofibers by 5 dpi.

We tested the consequences of this early impairment in muscle regeneration by examining injured TAs from *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice at 28 dpi. Tcf4$^+$ fibroblasts continued to be ablated (38% reduction, calculated as above, *P=0.003, n=6; Fig. 6HH-JJ) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (tamoxifen strategy, Fig. 6Y-JJ). Injured TAs had regenerated, but were somewhat smaller in cross-sectional area (21% reduction, P=0.10; Fig. 6AA,DD,GG,JJ) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. The number of Pax7$^+$ satellite cells had recovered in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice to levels similar to those found in *Tcf4*$^{+/+}$;*R26R*$^{DTA/+}$ mice (Fig. 6Y-AA). Myofibers had regenerated, although the ratio of MyHC$^+$ myofibers to Sirius Red$^+$ MCT was skewed; there was a 7% increase in the proportion of MyHC (*P=0.02; Fig. 6BB-DD) and a 19% decrease in the amount of Sirius Red$^+$ MCT (*P=0.03; Fig. 6EE-GG). Most striking was the finding that the diameter of the regenerated myofibers was smaller, with a higher frequency of smaller myofibers in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (Fig. 6BB-GG,KK). By 56 dpi, with continued ablation of Tcf4$^+$ fibroblasts (Fig. 6LL-PP), injured TAs continued to be slightly smaller in cross-sectional area (27% reduction, but high variance precluded statistical significance, n=6; Fig. 6PP) in *Tcf4*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice. However, the diameter of myofibers was equivalent to that found in control mice (Fig. 6LL-MM).

In summary, genetic ablation of a significant number of Tcf4$^+$ MCT fibroblasts caused Pax7$^+$ cells to differentiate prematurely and thus impaired the early expansion of Pax7$^+$ cells. This alteration in early satellite cell dynamics resulted in smaller muscles with smaller diameter myofibers at 28 dpi, when muscle regeneration is normally complete.

DISCUSSION

Muscle regeneration requires the coordinated interaction of multiple cell types. Our in vivo immunofluorescent, lineage and ablation studies demonstrate that satellite cells, fibroblasts and their interactions are crucial for muscle regeneration (summarized in Fig. 7).

A multitude of studies have implicated satellite cells as the primary stem cell responsible for muscle regeneration. However, the necessity of satellite cells has not been demonstrated previously, and the existence of other stem cells capable of regenerating muscle suggests that multiple types of stem cells might contribute to normal regeneration. To test the role of satellite cells during muscle regeneration, we generated *Pax7*^{CreERT2} mice. *Pax7*^{CreERT2} mice allow for efficient genetic labeling and manipulation of satellite cells without compromising Pax7 function, enabling ‘clean’ manipulation of satellite cells without any potential functional consequence of reduced Pax7 expression or function (Kuang et al., 2006; Lepper et al., 2009; Oustanina et al., 2004; Relaix et al., 2004; Seale et al., 2000). Using these

Pax7^{CreERT2} mice, we show that all regenerated muscle derives from Pax7$^+$ satellite cells and, most significantly, ablation of satellite cells led to a complete and persistent loss of muscle regeneration. Although Pax7 is expressed in the neural crest, we have not seen neural crest-derived glial cells labeled in our lineage experiments or obvious glial defects with ablation of Pax7-derived cells. As *Pax7*^{CreERT2} only labels satellite cells prior to injury (and not myonuclei or other potential myogenic progenitors) and ablation of these satellite cells prior to injury resulted in a complete loss of regenerated muscle, we can conclude that Pax7$^+$ satellite cells are absolutely required for normal muscle regeneration. It should be noted that our experiments do not formally rule out that other stem cells can contribute to muscle regeneration. For instance, other stem cells might contribute to muscle regeneration, but only in the presence of satellite cells (e.g. PICs present in juvenile and regenerating muscle) (Mitchell et al., 2010). In addition, other stem cells might not contribute to normal endogenous muscle regeneration, but could have important therapeutic potential as these cells, when transplanted, are able to regenerate muscle (e.g. mesoangioblasts) (Sampaolesi et al., 2003). Nevertheless, our experiments establish that during normal regeneration, Pax7$^+$ satellite cells are the endogenous stem cell population responsible for regenerating muscle.

Our experiments identify for the first time that MCT fibroblasts are a major cellular component of regenerating muscle. Although transient MCT fibrosis has long been recognized as a feature of regenerating muscle, no previous studies have examined the dynamics of the MCT fibroblasts. We previously showed that Tcf4 is highly expressed in MCT fibroblasts associated with developing, neonatal and adult muscle (Mathew et al., 2011). Here, we demonstrate that Tcf4$^+$ fibroblasts rapidly expand and are present in high numbers in the MCT of regenerating muscle. Recently, two groups have identified by FACS analysis cells from muscle which are PDGFRα^+ and non-myogenic (Joe et al., 2010; Uezumi et al., 2010). In culture, these cells differentiate into fibroblasts and adipocytes and when transplanted into muscle after glycerol injection can contribute to ectopic fat in skeletal muscle. The finding that both fibro-adipogenic cells and Tcf4$^+$ fibroblasts express PDGFRα suggests that these populations might be overlapping. Future experiments will explicitly compare the relationship between Tcf4$^+$ fibroblasts and fibro-adipogenic cells.

Our analysis begins to elucidate the role of Tcf4$^+$ MCT fibroblasts in the transient MCT fibrosis characteristic of muscle regeneration. During regeneration, increased MCT maintains the structural and functional integrity of regenerating muscle (Kaariainen et al., 2000), orients forming myofibers (Sanes, 2004), and sequesters and presents growth factors necessary for satellite cells (Cornelison, 2008). However, excessive ECM during regeneration can impede mechanical function and hinder muscle regeneration (Huard et al., 2002; Sato et al., 2003). Thus, MCT fibrosis must be precisely regulated during regeneration. We found that during normal regeneration Tcf4$^+$ fibroblasts rapidly expand within the MCT, concomitant with the increase in MCT, and later decrease in number, as MCT diminished. This spatial-temporal correlation between the numbers of Tcf4$^+$ fibroblasts and MCT fibrosis was apparent not only during normal regeneration, but also in our ablation experiments. Modest ablation of Tcf4$^+$ fibroblasts led to an effect on fibrosis, with somewhat less MCT and proportionately more muscle at 28 dpi. More striking was the finding that the substantial MCT fibrosis seen with ablation of satellite cells was associated with an increase in the overall density of fibroblasts. Together, these data suggest that Tcf4$^+$ fibroblasts are likely to be an important source of the increased ECM found during regeneration.

Most importantly, our experiments demonstrate that during regeneration Tcf4$^+$ MCT fibroblasts are a significant component of the cellular niche regulating satellite cell-mediated regeneration. During normal muscle regeneration, Pax7$^+$ satellite cells and Tcf4$^+$ fibroblasts rapidly expand in close proximity after injury. Ablation of fibroblasts resulted in premature satellite cell differentiation at 3 dpi, followed by depletion of the satellite cell pool, differentiating myoblasts and regenerating myofibers at 5 dpi, and resulted in smaller muscles and smaller diameter myofibers at 28 dpi, when muscle regeneration is normally complete. Thus, during regeneration, fibroblasts regulate the expansion of satellite cells by preventing their premature differentiation. These results complement our previous findings that during development Tcf4$^+$ fibroblasts promote muscle maturation. In particular, we found in vitro that myoblasts cultured in the presence of Tcf4$^+$ fibroblasts formed larger, more multinucleated myofibers than myoblasts cultured alone (Mathew et al., 2011). In addition, other experiments have shown in vitro that fibroblasts are a source of trophic signals for myogenic cells (Cooper et al., 2004; Joe et al., 2010; Kusner et al., 2010; Melone et al., 2000). However, this is the first demonstration in vivo that fibroblasts regulate muscle regeneration.

Our studies also reveal that fibroblasts are dynamically regulated by satellite cells. Ablation of satellite cells leads to a decrease in the number of Tcf4$^+$ fibroblasts at 5 dpi, but an increase in the overall density of fibroblasts at 28 dpi. Thus, early during the regenerative process, satellite cells and fibroblasts reciprocally and positively regulate the expansion of each other. Subsequently, during the late phases of regeneration the number of fibroblasts is not positively regulated by satellite cells, but is likely to be negatively regulated by satellite cells and other myogenic cells.

Altogether, our experiments demonstrate that satellite cells, fibroblasts and their interactions are crucial for muscle regeneration and suggest the following model of cell interactions during muscle regeneration (Fig. 7A). During regeneration, satellite cells proliferate, differentiate into myofibers, as well as self-renew. Satellite cells are absolutely required for muscle regeneration. Fibroblasts also rapidly proliferate in close association with satellite cells, regenerating myofibers and within the MCT. During the early phase of regeneration, fibroblasts ensure the expansion of satellite cells by preventing their premature differentiation. In turn, satellite cells positively regulate the number of fibroblasts. This positive feedback between satellite cells and fibroblasts ensures efficient and effective muscle repair. Later during the regenerative process, the number of fibroblasts are negatively regulated to prevent excessive MCT fibrosis.

Thus, fibroblast-satellite cell interactions are dynamic. Early reciprocal, positive fibroblast-satellite interactions promote regeneration. Later, negative regulation of fibroblasts, potentially via myogenic cells, inhibits excessive fibrosis. The dynamic nature of these interactions suggest that therapeutic treatments to reduce fibrosis during regeneration, after muscle injury or in the context of muscle diseases will need to be carefully monitored in order to avoid interfering with the early pro-regenerative interaction between MCT fibroblasts and satellite cells. As connective tissue fibrosis and expansion of resident fibroblasts are characteristic of many regenerating tissues (Tomasek et al., 2002; Verrecchia and Mauviel, 2007), dynamic interactions between fibroblasts and stem cells might be important not only for muscle, but for the regeneration of other tissues.

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Competing interests statement
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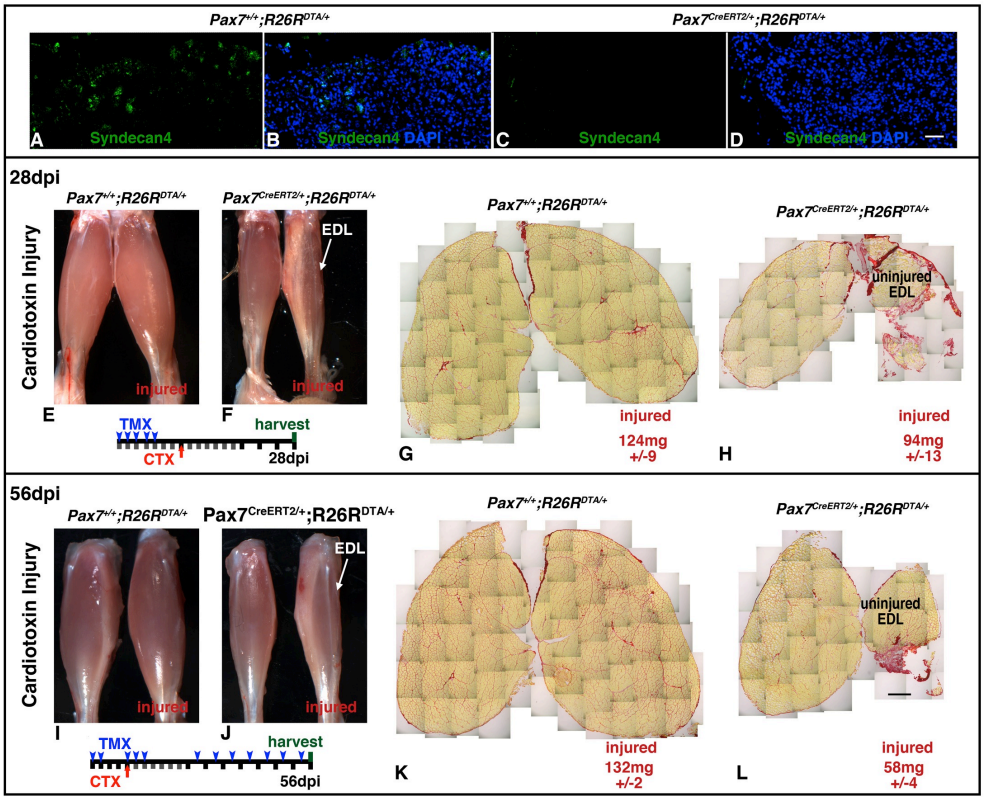
Figure S1: *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice efficiently ablate satellite cells and result in long-term loss of regenerated myofibers. (A-D) In injured TA muscle from mice 5 dpi ($BaCl_2$ injury) Syndecan4+ satellite cells are present in *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice, but no Syndecan4+ cells are present in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (both genotypes with 5 tamoxifen doses; see schematic of strategy, each grey bar represents one day and each black bar represents one week). (E-H) Ablation of Pax7+ satellite cells prior to injury, via 5 tamoxifen doses prior to injury, leads to a complete loss of regenerated myofibers 28dpi (cardiotoxin injury) in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice, as shown in whole mount (F) and Sirius Red stained cross-sections through TAs (H), while muscle regeneration is complete in *Pax7*$^{+/+}$;*R26R*$^{DTA/+}$ mice (E, G). (I-L) Ablation of Pax7+ satellite cells in *Pax7*$^{CreERT2/+}$;*R26R*$^{DTA/+}$ mice (still results in a complete loss of regenerated myofibers 56dpi (cardiotoxin injury), as shown in whole mount (I-J) and Sirius Red stained cross-sections through TAs (K-L). D scale bar = 50um, A-D; L scale bar = 500um G-H, K-L.

Table S1: Antibodies used in this study

Murphy Figure S1



Supplemental Table: Antibodies used in study

Antibody	Type	Source	Product Number	Working Concentration	Na Citrate Antigen Retrieval (sections)	Secondary and Amplification used in section	Secondary used in culture
Pax7	Mouse IgG1	DSHB	PAX7	2.4μg/ml	no	biotin goat anti-mouse IgG1, ABC, TSA	Alexa 594 goat anti-mouse IgG1
Syndecan 4	Chick polyclonal	gift DD Cornelison		1:1500	no	Dylight 488 donkey anti-chick	Cy3 donkey anti-chick
CD34	Rat IgG2a	EBioscience	14-0341	10μg/ml	no		Cy3 goat anti-rat
MyoD	Mouse IgG1	Santa Cruz Biotechnology	Sc-32758 (5.8A)	4μg/ml	no	biotin goat anti-mouse IgG1, ABC, TSA	Alexa 594 goat anti-mouse IgG1
MyHC embryonic	Mouse IgG1	DSHB	F1.652	3μg/ml	yes	biotin goat anti-mouse IgG1, ABC, TSA	
MyHCI	Mouse IgG1	Sigma	M8421 (NOQ7.5.4D)	1.5μg/ml	yes	biotin goat anti-mouse IgG1, ABC, TSA	
MyHC Peri+II	Mouse IgG1	Sigma	M4276 (MY-32)	10μg/ml	works with or without	biotin goat anti-mouse IgG1, ABC, TSA	
Laminin	Rabbit polyclonal	Sigma	L-9393	2.5μg/ml	yes	biotin goat anti-rabbit and streptavidin conj. Dylight 488 or Cy2	
Tcf4	Mouse IgG2a	Millipore	05-511 (Clone 6H5-3)	10μg/ml	yes	biotin goat anti-mouse IgG2a, ABC, TSA	
Tcf4	Rabbit monoclonal	Cell Signaling	2569 (C48H11)	0.7μg/ml	yes	biotin goat anti-rabbit, ABC, TSA OR streptavidin conj. Dylight 488	biotin goat anti-rabbit, streptavidin conj. Cy3
PDGFRα	Goat polyclonal	R&D Systems	AF1062	5μg/ml	no	Dylight 594 donkey anti-goat	
αSMA	Mouse IgG2a	Sigma	A 2547 (Clone 1A4)	5.2μg/ml	no	Alexa 488 goat anti-mouse IgG1	
F4/80	Rat IgG2a	eBioscience	14-4801	2μg/ml	no	Dylight 594 goat anti-rat	
GFP	Chick polyclonal	Aves Labs	GFP-1020	20μg/ml	no	Dylight 488 donkey anti-chick	goat anti-chick Cy2
Phospho-histone H3	Rabbit polyclonal	Millipore	06-570	5μg/ml	yes	biotin goat anti-rabbit and streptavidin conj. Cy3	

CHAPTER 4

WNT/β-CATENIN SIGNALING IS ACTIVE, BUT NOT REQUIRED FOR STEM CELL FUNCTION DURING MUSCLE REGENERATION

Abstract

Wnt/β-catenin signaling is a critical regulator of adult stem cells in multiple tissues. Active Wnt/β-catenin signaling is clearly required for proliferation of intestinal, stomach, and hair follicle stem cells, although its role in adult hematopoietic stem cells is more controversial. A multitude of studies have implicated Wnt/β-catenin as an important regulator of the adult muscle stem cells, known as satellite cells, during muscle regeneration. In addition, we have previously demonstrated that β-catenin is required for fetal myogenesis. However, *in vivo* genetic studies manipulating signaling specifically within satellite cells during regeneration are lacking. Our analysis of satellite cells, transit-amplifying myoblasts, differentiating myocytes, and regenerated myofibers during regeneration reveals that Wnt/β-catenin signaling is transiently active, predominantly in myoblasts, 1-3 days postinjury. However, conditional genetic deletion of β-catenin within satellite cells and their derivatives demonstrates that β-catenin is *not* required for satellite cells to activate, proliferate, regenerate muscle, or return to their niche, even after multiple rounds of injury. Instead, down-regulation of transiently activated β-catenin is important for promoting an efficient regenerative response. Thus, despite clear activation

of Wnt/β-catenin signaling within myogenic cells, β-catenin is not required for adult muscle regeneration. Hence, muscle progenitors are differentially sensitive to Wnt signaling; while fetal progenitors require β-catenin, adult satellite cells do not.

Introduction

Skeletal muscle has extraordinary regenerative ability despite being post mitotic. This capacity for regeneration is due to the satellite cells, the resident muscle stem cells. Satellite cells are so named because of their unique niche adjacent to the myofiber membrane beneath the basal lamina. Satellite cells have the classical attributes of adult stem cells: they reside in a well-defined niche, give rise to transiently amplifying myoblasts, and self-renew. Additionally we, and others, have shown that muscle regeneration after injury is entirely dependent upon the satellite cells (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). Thus deciphering the molecular signals that regulate satellite cell activation, differentiation, and self-renewal is critical for understanding muscle regeneration.

How satellite cells regenerate muscle is well-characterized at a cellular level (reviewed in (Shi and Garry, 2006; Tedesco et al., 2010). All satellite cells express the transcription factor Pax7 and under normal conditions quiescently reside within their niche. In response to muscle injury, they become activated, leave the niche, proliferate, and express the myogenic regulatory factor MyoD. A subset of satellite cells return to the niche to self-renew. The MyoD+ cells are a transiently amplifying population of cells called myoblasts. Myoblasts differentiate into postmitotic myocytes, that express the myogenic regulatory factor myogenin (MyoG), and these myocytes in turn fuse to injured

myofibers to repair the damage. Regenerating myofibers express the developmental isoform of skeletal muscle MyHC MyHCembryonic (MyHCemb). The slow (MyHCI) and fast (MyHCII) isoforms of MyHC replace MyHCemb as the myofibers mature. Regenerated myofibers can be identified by their centrally localized nuclei for several months after injury. Although the cellular processes involved in muscle regeneration are well characterized, the molecular control of these processes is less elucidated.

In the search to identify molecular regulators of satellite cell-mediated muscle regeneration, many studies have focused on signaling pathways involved in muscle development as well as regulation of other tissue-specific stem cells. One obvious candidate is the Wnt/β-catenin signaling pathway. Wnts are secreted ligands involved in cell-cell signaling in many developmental and adult contexts (Clevers and Nusse, 2012). In the absence of Wnt ligand, β-catenin is phosphorylated by the APC/Axin destruction complex and degraded. When Wnt proteins bind to the Fz/LRP receptors, β-catenin is no longer degraded, it accumulates and translocates to the nucleus where it activates transcription of target genes.

Wnt/β-catenin is essential for myogenesis within the somite as well as within fetal limb muscle development (Hutcheson et al., 2009). Additionally, the Wnt/β-catenin signaling pathway is essential for the regulation of stem cells such as intestinal and hair follicle stem cells (reviewed in Schuijers and Clevers, 2012). Therefore many studies have examined the role of Wnt/β-catenin in muscle regeneration (recently reviewed in Tsivitse, 2010; von Maltzahn et al., 2012). Much work has gone into determining the expression pattern of Wnt pathway components in adult myogenesis *in vitro* and *in vivo* (Abiola et al., 2009; Armstrong and Esser, 2005; Aschenbach et al., 2006; Bernardi et al.,

2011; Brack et al., 2008; Brack et al., 2009; Goichberg et al., 2001; Han et al., 2011; Kim et al., 2008; Kramerova et al., 2006; Le Grand et al., 2009; Nastasi et al., 2004; Otto et al., 2008; Polesskaya et al., 2003; Tanaka et al., 2011; Zhao and Hoffman, 2004). Nevertheless, it is unclear in which cells and when Wnt/β-catenin signaling is important *in vivo* during muscle regeneration. Also, surprisingly the cell-autonomous role of β-catenin within satellite cells and their derivatives has not been explicitly tested.

In this study we determined when during muscle regeneration and in which myogenic cells Wnt/β-catenin signaling is active. Then using genetic conditional loss and gain of function studies in mouse, we specifically tested the cell autonomous functional role of β-catenin in satellite cells and their derivatives during muscle regeneration. We find that Wnt/β-catenin signaling is transiently active in myoblasts during the early stages of muscle regeneration. However, despite activation of signaling, β-catenin is not functionally required within satellite cells or their derivatives for muscle regeneration. Instead, early down-regulation of β-catenin signaling in myoblasts and myocytes is important for efficient muscle regeneration.

Results

Wnt/β-catenin signaling is transiently active in myogenic cells after injury

There is ample evidence that Wnt/β-catenin signaling is active within muscle tissue during regeneration, as pathway components that change their phosphorylation state, intracellular localization, or levels are expressed in regenerating muscle *in vivo* and in myogenic cell culture (Armstrong and Esser, 2005; Aschenbach et al., 2006;

Goichberg et al., 2001; Kim et al., 2006; Otto et al., 2008; Polesskaya et al., 2003). Additionally, expression of downstream transcriptional targets and Wnt/β-catenin reporter activity increase in regenerating muscle (Bernardi et al., 2011; Brack et al., 2008; Naito et al., 2012; Tanaka et al., 2011). However, the reception of Wnt/β-catenin signaling has not been explicitly quantified specifically within the myogenic lineage *in vivo* at different times after injury. We wanted to determine whether Wnt/β-catenin signaling is active within the myogenic cells *in vivo* and whether reception of signal changes over time after injury. To do this we utilized the *TCF/Lef:H2B-GFP*^{Tg} reporter, which has 6 TCF/Lef DNA binding sites before a minimal promoter to drive expression of H2B-GFP (Ferrer-Vaquer et al., 2010). In these mice, cells that are responding to Wnt/β-catenin signaling express nuclear-localized GFP. To determine the percent of myogenic cells responding to Wnt/β-catenin signaling, we performed FACS analysis as previously published (Joe et al., 2010); CD31-CD45-Sca1-α-7integrin+ cells were identified as myogenic cells and include activated satellite cells, myoblasts, and potentially myocytes. Mononuclear cells were isolated from TAs, either uninjured or injured with $BaCl_2$, of *TCF/Lef:H2B-GFP*^{Tg} mice and the percentage of GFP+ myogenic cells were determined by FACS analysis. In uninjured muscle only 1.9% of myogenic cells are GFP+, and so few cells are responding to Wnt/β-catenin signaling (Fig. 4.1A). However, at 1 dpi 32.7% of the myogenic cells are GFP+ (Fig. 4.1B), but by 3 dpi the percentage of GFP+ myogenic cells decreased to 8.5% (Fig. 4.1C). To determine which myogenic cells are responding to Wnt/β-catenin, we analyzed sections of TAs from *TCF/Lef:H2B-GFP*$^{Tg/+}$ mice at different days post injury. Immunofluorescence of Laminin and GFP of uninjured muscle show that GFP+ cells lie within the interstitial

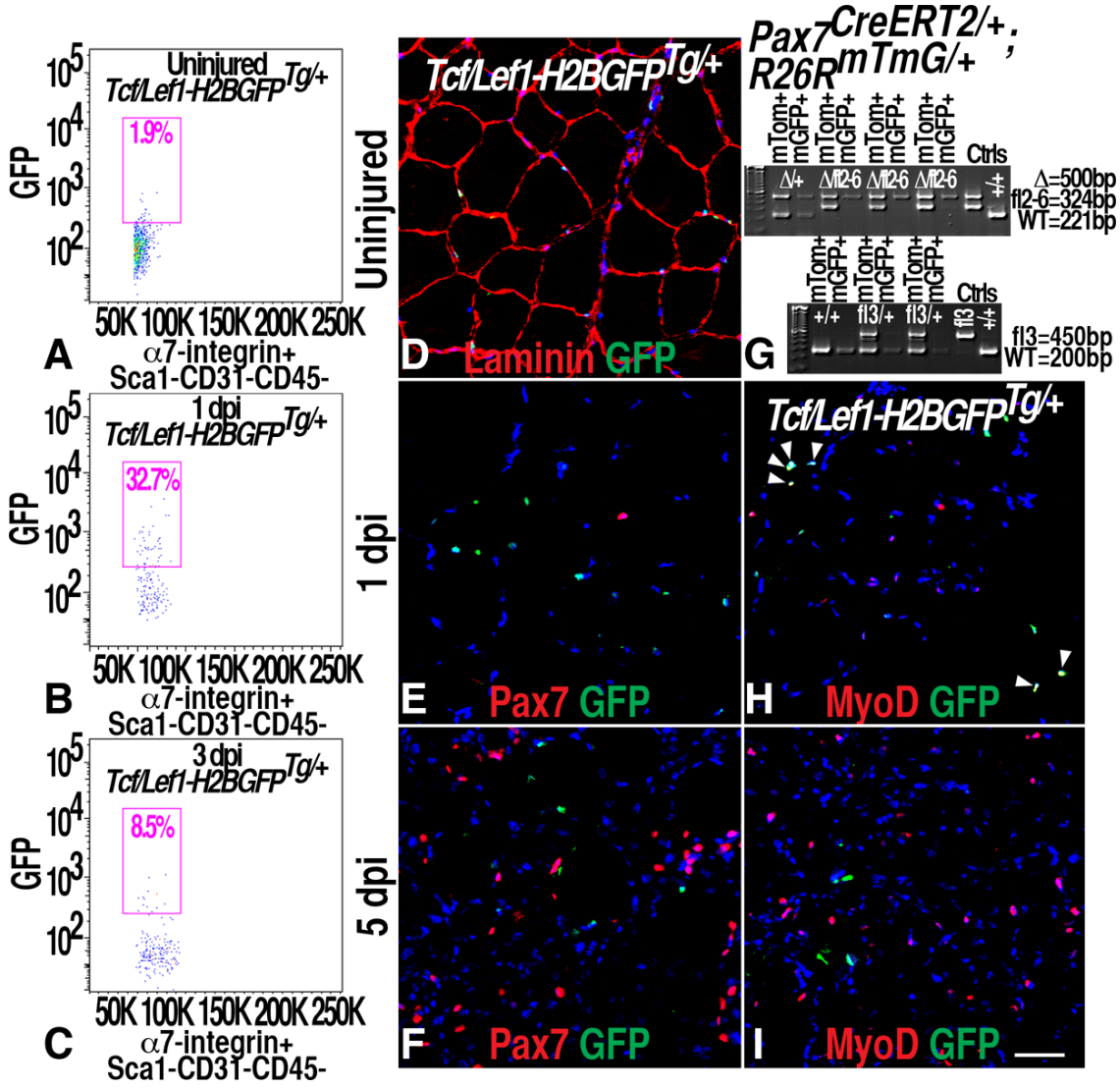


Fig. 4.1 Wnt/β-catenin is transiently active in myogenic cells after injury. (A-C) FACS analysis of *TCF/Lef:H2B-GFP*^{Tg} GFP+, α-7integrin+, CD31-, and CD45- myogenic cells at 0, 1, and 3 dpi. (D) Uninjured muscle stained with Laminin and GFP. (E,F) Pax7+ satellite cells and GFP at 1 and 5 dpi. (H,I) MyoD+ myoblasts and GFP at 1 and 5 dpi. Arrows indicate double labeling. Genomic PCR of sorted GFP+ and Tom+ cells. Scale bar = 120μm

space as well as underneath the basal lamina (Fig. 4.1D). The GFP+ cells beneath the basal lamina are myonuclei because immunofluorescent labeling of GFP and Pax7 in uninjured muscle does not show colabeling (data not shown). At 1 dpi immunofluorescence of Pax7, MyoD, and GFP revealed that Pax7+ cells did not express GFP; however, a subset of the MyoD+ cells are GFP+ (arrowheads Fig. 4.1H,E). We did not see any labeling of MyoG+ cells at 1 dpi (data not shown). Because we do not see colabeling of Pax7 and GFP, this suggests that the MyoD+ cells that express GFP are myoblasts that have already down regulated Pax7 and will differentiate. At 5 dpi no Pax7+, MyoD+, or MyoG+ cells were colabeled with GFP (Fig. 4.1F,I and data not shown). This, in conjunction with the FACS analysis at 3 dpi, suggests that the window for myogenic cells to respond to Wnt/β-catenin signaling has passed by 5 dpi. These results show that Wnt/β-catenin signaling is active in myogenic cells during regeneration, but occurs only during an early and transient period and is most prominent in MyoD+ myoblasts.

β-catenin not required for satellite cells to regenerate muscle or to return to niche

Our analysis of the *TCF/Lef:H2b-GFP* reporter finds that Wnt/β-catenin signaling is active within the myogenic cells during muscle regeneration. The precise role of Wnt/β-catenin signaling has been unclear, as there is conflicting evidence supporting roles in proliferation as well as differentiation of satellite cells during regeneration (Brack et al., 2008; Otto et al., 2008). However, none of these previous studies tested *in vivo* the cell autonomous role of β-catenin in satellite cells during muscle regeneration. Therefore,

to test this we conditionally deleted β-catenin specifically in satellite cells and analyzed the effects on muscle regeneration.

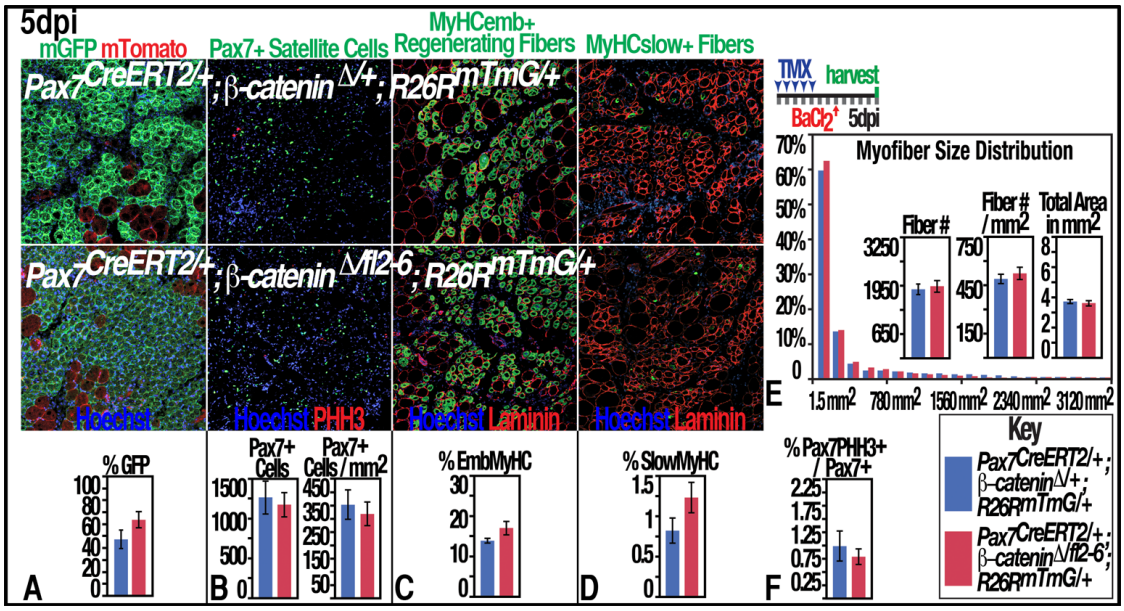
To genetically delete β-catenin in satellite cells, we used *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/fl2-6}$; R26R$^{mTmG/+}$* mice. We have previously shown that *Pax7^{CreERT2}* mice, after delivery of tamoxifen (TMX), specifically and efficiently (94% recombination) label satellite cells (Murphy et al., 2011). The β-catenin loss of function allele, *β-catenin^{fl2-6}*, creates a functional null following Cre mediated deletion of exons 2 through 6 (Brault et al., 2001). The fate of recombined cells was tracked via the *R26R^{mTmG}* reporter, which ubiquitously expresses membrane bound Tomato (mTomato) until Cre mediated recombination excises the mTomato, resulting in expression of membrane bound GFP (mGFP) (Muzumdar et al., 2007). To ensure that complete deletion of β-catenin required only one recombination event, we analyzed *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/fl2-6}$; R26R$^{mTmG/+}$* mice, and to control for any possible heterozygous phenotype they were compared to *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/+}$; R26R$^{mTmG/+}$* mice. Satellite cells are the only cells that express Pax7 in uninjured muscle (Murphy et al., 2011). Therefore, by delivering TMX before injury, in control *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/+}$; R26R$^{mTmG/+}$* mice all satellite cells and their progeny will express mGFP and be heterozygous for β-catenin, while in mutant *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/fl2-6}$; R26R$^{mTmG/+}$* mice all satellite cells and their progeny express mGFP and should be null for β-catenin. To confirm that expression of mGFP faithfully reflected recombination in the β-catenin locus, we isolated, via FACS, GFP+ and Tomato+ cells from muscle tissue of *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/+}$; R26R$^{mTmG/+}$* and *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/fl2-6}$; R26R$^{mTmG/+}$* mice given 5 doses of TMX, injured with $BaCl_2$, and harvested 5 (dpi). We then performed genomic PCR to identify wild type

(WT), *β-catenin* $^{fl2-6}$, and *β-catenin* $^{\Delta 2-6}$ alleles. As expected, we found that from *Pax7* $^{CreERT2/+}$*;β-catenin* $^{\Delta/+}$*;R26R* $^{mTmG/+}$ mice (n=2) GFP+ and Tomato+ cells were positive for both the WT and *β-catenin* $^{fl2-6}$ alleles (Fig. 4.1G). In contrast, from *Pax7* $^{CreERT2/+}$*; β-catenin* $^{\Delta/fl2-6}$*; R26R* $^{mTmG/+}$ mice (n=3) Tomato+ cells contained both the fl2-6 and Δ2-6 alleles, whereas the GFP+ cells contained only the Δ2-6 allele (Fig. 4.1G). Therefore, after TMX delivery to *Pax7* $^{CreERT2/+}$*; β-catenin* $^{\Delta/fl2-6}$*; R26R* $^{mTmG/+}$ mice, mGFP expression reflects a complete loss of β-catenin in Pax7+ satellite cell derived myogenic cells.

Having established that delivery of TMX to *Pax7* $^{CreERT2/+}$*; β-catenin* $^{\Delta/fl2-6}$*; R26R* $^{mTmG/+}$ mice results in deletion of β-catenin in satellite cells, we tested whether satellite cells require β-catenin to regenerate muscle after injury. After 5 consecutive daily doses of TMX, we injured muscle by injecting $BaCl_2$ into the right tibialis anterior (TA) muscle, and the left TA served as an uninjured control. $BaCl_2$ injury induces a stereotyped pattern of muscle regeneration, with the peak of satellite cells and myofiber regeneration occurring 5 dpi and regeneration complete by 28 dpi (Murphy et al., 2011). Thus we harvested muscle from *Pax7* $^{CreERT2/+}$*; β-catenin* $^{\Delta/fl2-6}$*; R26R* $^{mTmG/+}$ mice and *Pax7* $^{CreERT2/+}$*; β-catenin* $^{\Delta/+}$*; R26R* $^{mTmG/+}$ littermate controls at 5 and 28 dpi to assess the consequence of deletion of β-catenin in satellite cells at both the peak of muscle regeneration and when regeneration should be complete. At 5 dpi, comparison of *Pax7* $^{CreERT2/+}$*; β-catenin* $^{\Delta/fl2-6}$*; R26R* $^{mTmG/+}$ and *Pax7* $^{CreERT2/+}$*; β-catenin* $^{\Delta/+}$*; R26R* $^{mTmG/+}$ mice reveals that there is no difference in the number or proliferation (as shown by co-labeling with Pax7 and Phosphohistone H3) of Pax7+ satellite cells (Fig. 4.2B). Satellite cells give rise to MyoD+ cells, which include activated Pax7+MyoD+ satellite cells and

Pax7- MyoD+ myoblasts, that proliferate and differentiate into muscle, and the peak number of MyoD+ cells after $BaCl_2$ injury occurs at 3 dpi (Murphy et al., 2011, and unpublished results). Comparison of mutant and control mice at 3 dpi shows that loss of β-catenin did not affect either the number or proliferation of MyoD+ cells (Fig. 4.2O). Regenerating myofibers transiently express MyHCembryonic (MyHCemb), an immature form of MyHC that is replaced by slow and fast isoforms as the nascent muscle fibers mature. We found no difference in the amount of MyHCemb expression between the *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/fl2-6}$; R26R$^{mTmG/+}$* and *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/+}$; R26R$^{mTmG/+}$* mice, indicating that the number of regenerating myofibers at 5dpi did not differ with loss of β-catenin (Fig. 4.2C). In addition, the number and cross sectional area of fibers, as well as the total cross sectional area of the muscle, did not differ at 5 dpi (Fig. 4.2E). At 28 dpi, there continues to be no difference in the total cross sectional area of the muscle, although there is a slight shift in the distribution of individual fiber size towards larger fibers (Fig. 4.2K). Overall, our results indicate that loss of β-catenin in the satellite cells does not impair muscle regeneration.

The *Pax7$^{CreERT2/+}$* Cre driver is very efficient (94% recombination), however just a few transplanted satellite cells have the capacity to generate 25,000 new myonuclei (Collins et al., 2005). To determine whether a few, nonrecombined escapers retaining one allele of β-catenin could outcompete β-catenin null satellite cells, we examined the contribution of the β-catenin null satellite cells to the regenerating muscle. We compared the amount of GFP expression between *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/fl2-6}$; R26R$^{mTmG/+}$* and *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/+}$; R26R$^{mTmG/+}$* mice as a representation of the contribution of the recombined satellite cells to the muscle and saw no difference at either 5 or 28 dpi (Fig.


5dpi
mGFP mTomato
Pax7+ Satellite Cells
MyHCemb+ Regenerating Fibers
MyHCslow+ Fibers
Pax7CreERT2/+;β-catenin Δ/+;R26RmTmG/+
Pax7CreERT2/+;β-catenin Δ/fl2-6;R26RmTmG/+
Hoechst
Hoechst PHH3
Hoechst Laminin
TMX
harvest
BaCl2
5dpi
Myofiber Size Distribution
Fiber #
Fiber # / mm2
Total Area in mm2
1.5mm2 780mm2 1560mm2 2340mm2 3120mm2
% GFP
Pax7+ Cells
Pax7+ Cells / mm2
% EmbMyHC
% SlowMyHC
% Pax7PHH3+ / Pax7+
Key
Pax7CreERT2/+;β-catenin Δ/+;R26RmTmG/+
Pax7CreERT2/+;β-catenin Δ/fl2-6;R26RmTmG/+
A
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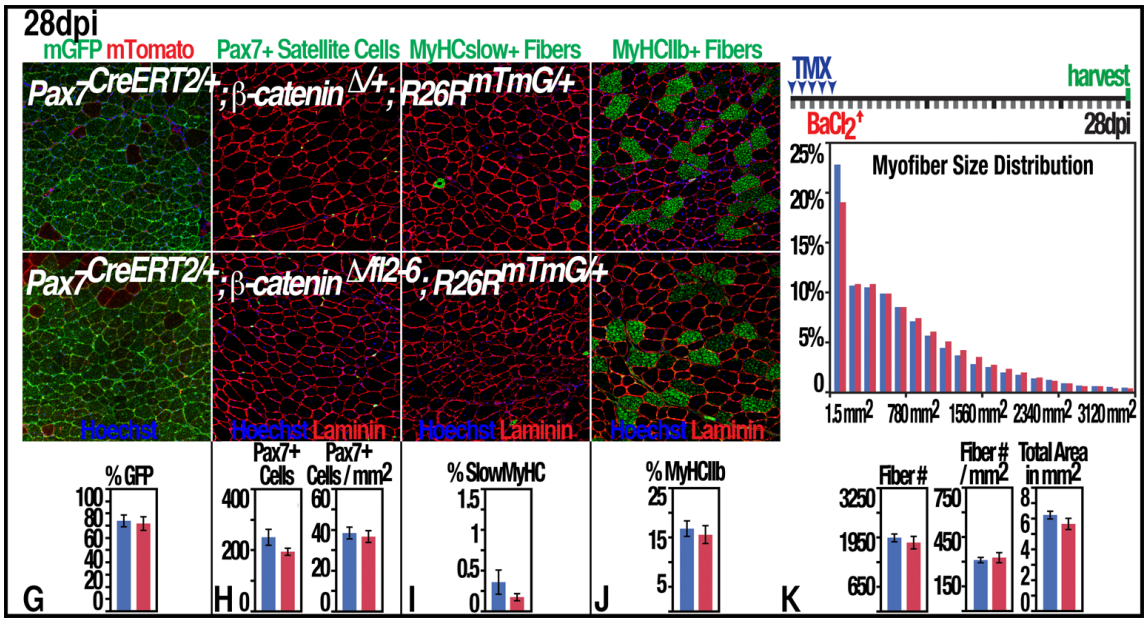
28dpi
mGFP mTomato
Pax7+ Satellite Cells
MyHCslow+ Fibers
MyHCIIb+ Fibers
Pax7CreERT2/+;β-catenin Δ/+;R26RmTmG/+
Pax7CreERT2/+;β-catenin Δ/fl2-6;R26RmTmG/+
Hoechst
Hoechst Laminin
TMX
harvest
BaCl2
28dpi
Myofiber Size Distribution
1.5mm2 780mm2 1560mm2 2340mm2 3120mm2
% GFP
Pax7+ Cells
Pax7+ Cells / mm2
% SlowMyHC
% MyHCIIb
Fiber #
Fiber # / mm2
Total Area in mm2
G
H
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K

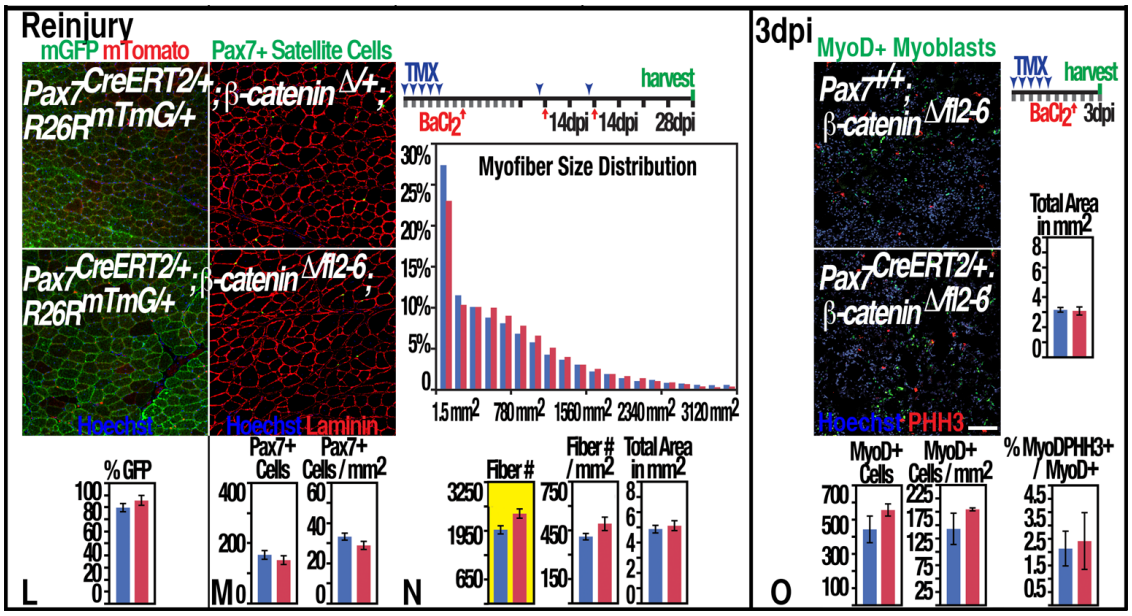


Fig. 4.2 β-catenin is not required for satellite cells to regenerate muscle or return to their niche (A-F) At 5 dpi there is no difference between mutant and wild type in the amount of GFP expression (A), Number of Pax7+ Satellite cells or their proliferation (B,F), Regenerating fibers (C) or MyHCslow expression (D) Or any difference in muscle of fiber size (E). (G-K) At 28 dpi there is no difference between mutant and wild type in above parameters as well as MyHCIIb (J) (L-N)Even after 3 rounds of injury and regeneration there is no difference in Satellite cell contribution of number. (O)At 3 dpi the myoblasts are not affected. Yellow denotes significance scale bar = 100μm

4.2A, G, 4.3A). Therefore, loss of β-catenin does not affect the ability of satellite cells to contribute to regenerated muscle.

Our results demonstrate that loss of β-catenin does not affect satellite cell activation, proliferation, or differentiation during muscle regeneration. Another important function of stem cells is the ability to self-renew and return to their niche. To determine whether loss of β-catenin inhibits self-renewal of satellite cells during regeneration, we compared the number of Pax7+ satellite cells between $Pax7^{CreERT2/+}$; $\beta\text{-}catenin^{\Delta/fl2\text{-}6}$; $R26R^{mTmG/+}$ and $Pax7^{CreERT2/+}$; $\beta\text{-}catenin^{\Delta/+}$; $R26R^{mTmG/+}$ mice at 28 dpi. However, neither the number nor location of satellite cells within their niche beneath the Laminin+ basal lamina of the muscle differed (Fig. 4.2H), indicating that β-catenin is not required for satellite cell self-renewal.

Potentially, the function of β-catenin in satellite cells may only be uncovered after multiple rounds of regeneration. To test this possibility, after TMX induced inactivation of β catenin in satellite cells, we successively injured the TA muscle and allowed it to regenerate three times (see strategy in Fig. 4.2L-N). Even after repeated rounds of regeneration, we detected no difference between $Pax7^{CreERT2/+}$; $\beta\text{-}catenin^{\Delta/fl2\text{-}6}$; $R26R^{mTmG/+}$ and $Pax7^{CreERT2/+}$; $\beta\text{-}catenin^{\Delta/+}$; $R26R^{mTmG/+}$ mice in either the amount of GFP expression or the number of Pax7+ satellite cells within the niche (Fig. 4.2L,M). We did observe with loss of β-catenin a shift in the fiber size distribution toward larger fibers (as we saw at 28dpi, after a single injury) and more fibers, but no significant change in the fiber density or overall muscle size (Fig. 4.2N). Therefore, loss of β-catenin in satellite cells somewhat altered the size and number of regenerated fibers, but overall did not affect muscle regeneration or satellite cell self-renewal. β-catenin also has been

implicated in the determination of fiber type. During fetal myogenesis, β-catenin positively regulates the differentiation of MyHCI+ slow myofibers, as shown by conditional loss and gain of β-catenin in Pax7+ progenitors (Hutcheson et al., 2009). Additionally, it has been shown in satellite cell derived C2C12 cells that expression of MyHCIIb is directly regulated by β-catenin binding, via Tcf/Lef, to the MyHCIIb promoter (Shanely et al., 2009). We tested *in vivo* whether β-catenin is required cell autonomously for determination of fiber type during muscle regeneration. Examination of *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/fl26}$; R26R$^{mTmG/+}$* and *Pax7$^{CreERT2/+}$; β-catenin$^{Δ/+}$; R26R$^{mTmG/+}$* mice at 5 and 28 dpi showed no difference in the expression of either MyHCI or MyHCIIb (Fig. 4.2D,I). Therefore, *in vivo* determination of slow MyHCI+ and fast MyHCIIb+ myofibers after regeneration does not require β-catenin.

In summary, deletion of β-catenin in the satellite cell population did not affect their ability to contribute to muscle regeneration or return to their niche. Even after multiple injuries, β-catenin is not required for satellite cell activation, proliferation, differentiation, or self-renewal.

Activation of β-catenin in satellite cells alters kinetics of myoblast differentiation, resulting in prolonged regeneration

Our genetic experiments clearly demonstrate that β-catenin is not required in satellite cells or their derivatives for muscle regeneration. However, our analysis of the *TCF/Lef:H2BGFP$^{Tg/+}$* reporter showed that Wnt/β-catenin signaling is transiently active in myogenic cells during muscle regeneration (Fig. 4.1B,C). Together these experiments suggest that while activation of Wnt/β-catenin signaling is not required, once activated,

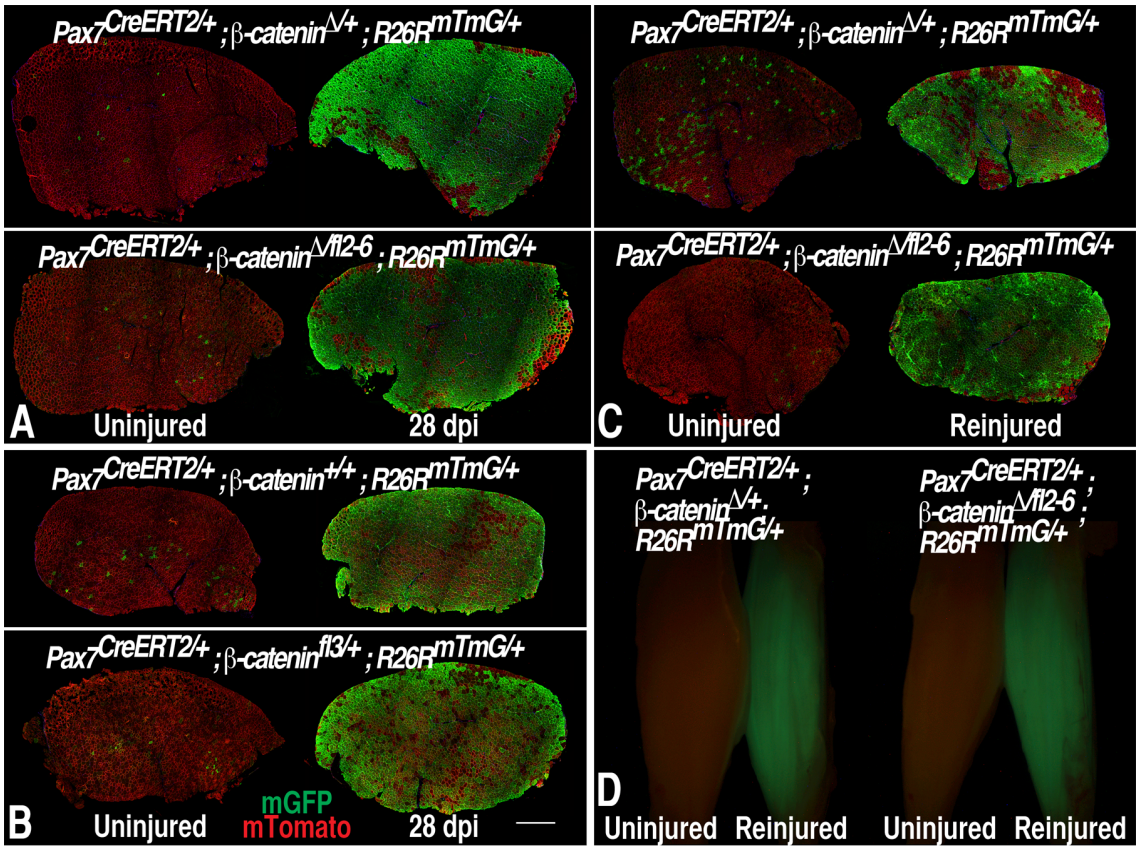


Fig.4.3 β-catenin loss or gain in satellite cells does not affect contribution to muscle
For all images contralateral control shown to left and cross sections through entire TA and EDL and whole mount images of reinjury (D) Loss of β catenin (A) 28 dpi, (B) Gain of function (C,D) Reinjury Scale bar = 100μm

prompt down-regulation of Wnt/β-catenin signaling may be necessary for proper muscle regeneration. To test this, we constitutively activated β-catenin in satellite cells and their derivatives and assayed for its affect on muscle regeneration.

To constitutively activate β-catenin in satellite cells and their derivatives, we used *Pax7*$^{CreERT2/+}$*; β-catenin*$^{fl3/+}$*; R26R*$^{mTmG/+}$ mice. In the *β-catenin*^{fl3} allele, the presence of Cre results in the deletion of exon 3 and the formation of a stabilized, constitutively active form of β-catenin (Harada et al., 1999). To activate β-catenin in the satellite cells, we again used *Pax7*^{CreERT2} Cre, and to track recombination we used the *R26R*^{mTmG} reporter (Murphy et al., 2011; Muzumdar et al., 2007). In *Pax7*$^{CreERT2/+}$*; β-catenin*$^{fl3/+}$*; R26R*$^{mTmG/+}$ mice, after delivery of TMX, the satellite cells will express stabilized, activated β-catenin and mGFP. We again confirmed that expression of mGFP reflects recombination in the β-catenin locus by isolating, via FACS, GFP+ and Tomato+ cells from muscle tissue of *Pax7*$^{CreERT2/+}$*; β-catenin*$^{fl3/+}$*; R26R*$^{mTmG/+}$ and *Pax7*$^{CreERT2/+}$*; β-catenin*$^{+/+}$*; R26R*$^{mTmG/+}$ mice given 5 doses of TMX, injured with $BaCl_2$, and harvested 5 dpi. We then performed genomic PCR to identify WT and *β-catenin*^{fl3} alleles. As expected in *Pax7*$^{CreERT2/+}$*; β-catenin*$^{+/+}$*; R26R*$^{mTmG/+}$ mice, both GFP+ and Tomato+ cells were positive only for the WT allele (Fig. 4.1G). In *Pax7*$^{CreERT2/+}$*; β-catenin*$^{fl3/+}$*; R26R*$^{mTmG/+}$ mice (n=2), Tomato+ cells express both the *β-catenin*^{fl3} and WT alleles, whereas GFP+ cells express only the WT allele because the primer binding sites for the *β-catenin*^{fl3} allele had been deleted by recombination (Fig. 4.1G). Therefore, after TMX delivery to *Pax7*$^{CreERT2/+}$*; β-catenin*$^{fl3/+}$*; R26R*$^{mTmG/+}$ mice mGFP expression reflects recombination of exon 3 and constitutive activation of β-catenin in Pax7+ satellite cells and their derivatives.

Constitutive activation of β-catenin in fetal myogenic progenitors during development results in an increase in the number of Pax7+ cells at birth (Hutcheson et al., 2009). In addition, activated Pax7+ satellite cells express nuclear β-catenin *in vitro* (Otto et al., 2008). Therefore, we tested whether constitutive activation of β-catenin in satellite cells would affect satellite cell number or proliferation after injury. We delivered 5 daily doses of TMX and injured the right TA by injecting $BaCl_2$, leaving the left TA as the uninjured control. TAs were harvested at 5 dpi, when the number of Pax7+ cells and regenerating myofibers peaks, and at 28 and 60 dpi when regeneration is ordinarily complete. Comparison of $Pax7^{CreERT2/+}$; $\beta\text{-catenin}^{fl3/+}$; $R26R^{mTmG/+}$ mice to $Pax7^{CreERT2/+}$; $\beta\text{-catenin}^{+/+}$; $R26R^{mTmG/+}$ littermate controls showed that there was no difference in the number or proliferation of Pax7+ satellite cells (Fig. 4.4B,H). There continued to be no difference in the number of satellite cells at 28 dpi (Fig. 4.4J), although at 60 dpi there was a slight, but not significant, decrease in the number of satellite cells in $Pax7^{CreERT2/+}$; $\beta\text{-catenin}^{fl3/+}$; $R26R^{mTmG/+}$ as compared to $Pax7^{CreERT2/+}$; $\beta\text{-catenin}^{+/+}$; $R26R^{mTmG/+}$ mice ($p=0.09$, Fig. 4.4Q). Overall, we found that constitutive activation of β-catenin in the satellite cells does not significantly alter the number or proliferation of Pax7+ satellite cells during muscle regeneration or their return to the niche.

Previous studies have suggested that activation of Wnt/β-catenin signaling, by addition of Wnt3a, during muscle regeneration leads to premature differentiation, resulting in a decrease in fiber size, but increase in number of fibers (Brack et al., 2008; Le Grand et al., 2009). We tested whether constitutive activation of β-catenin specifically in the satellite cells would disrupt the normal progression of myogenesis during regeneration. Upon muscle injury, satellite cells proliferate, give rise to MyoD+ cells

(which include activated satellite cells and myoblasts) that proliferate and differentiate into myogenin+ (MyoG+) myocytes, which in turn fuse and differentiate into myofibers that transiently express MyHCemb. Normally, the number of MyoD+ cells peaks at approximately 140 MyoD+ cells/mm^2 at 3dpi, declines to 35 at 5 dpi, and no MyoD+ cells are present at 28 dpi (Fig. 4.2O, 4.4C, Murphy et al., 2011, and data not shown). At 5 dpi there was a 79% increase in the number of MyoD+ cells in *Pax7$^{CreERT2/+}$; β-catenin$^{fl3/+}$; R26R$^{mTmG/+}$* compared with control *Pax7$^{CreERT2/+}$; β-catenin$^{+/+}$; R26R$^{mTmG/+}$* mice (75 versus 42 MyoD+ cells/mm^2, p=0.023), although there was no difference in proliferation of these cells (Fig. 4.4C,H). There was no difference in either the number or proliferation of MyoG+ myocytes (Fig. 4.4D,H), but there was a decrease in MyHCemb expression (although not significant, p=0.102, Fig. 4.4E) and 44% fewer myofibers (356 versus 636 myofibers/mm^2, p=0.033, Fig. 4.4G). The increased number of MyoD+ cells, without an increase in proliferation of Pax7+ or MyoD+ cells, suggests that continued β-catenin signaling extends the time that myogenic cells remain as Pax7+MyoD+ activated satellite cells or Pax7- MyoD+ myoblasts, and consequently results in fewer regenerated myofibers at 5dpi. The lack of difference in the number of MyoG+ myocytes may be due to the high variance in these data. It may also reflect an inflection point, in which in *Pax7$^{CreERT2/+}$; β-catenin$^{fl3/+}$; R26R$^{mTmG/+}$* mice the number of MyoG+ cells are increasing, but in the *Pax7$^{CreERT2/+}$; β-catenin$^{+/+}$; R26R$^{mTmG/+}$* mice they are decreasing as they differentiate and fuse into myofibers.

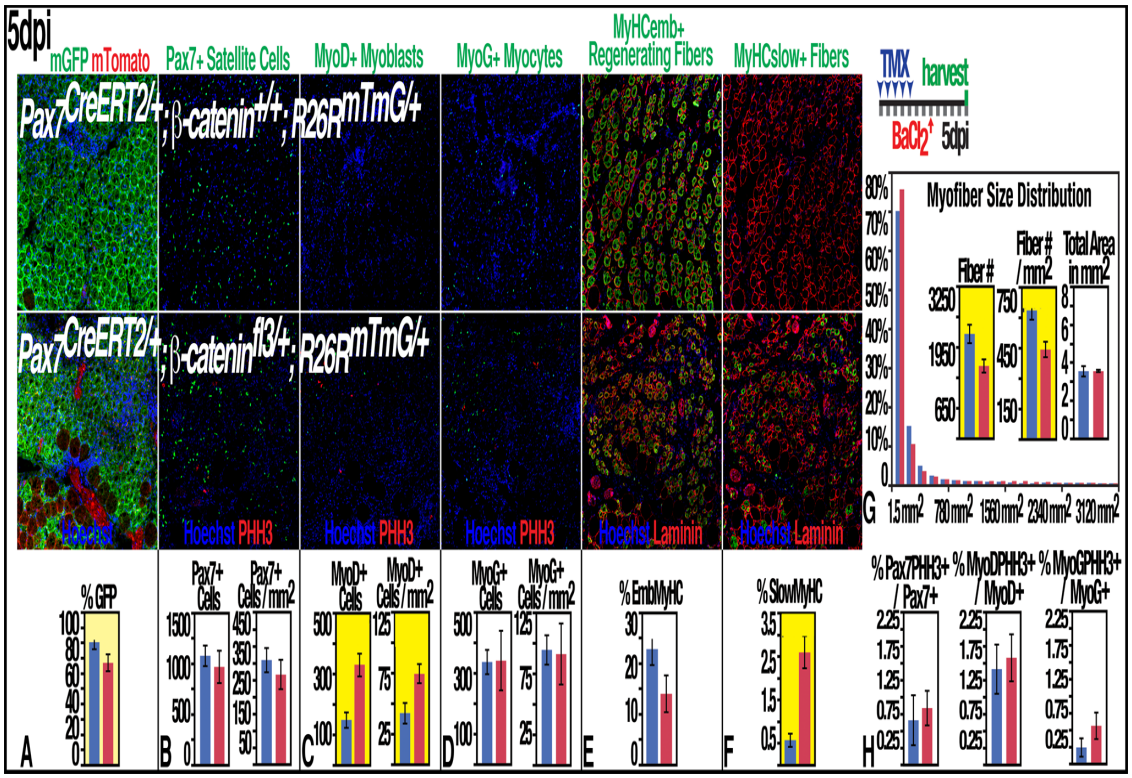
To test if continued activation of β-catenin signaling would block myofiber differentiation, we examined TAs at 28 dpi when regeneration is normally complete. No MyoD was expressed in either *Pax7$^{CreERT2/+}$; β-catenin$^{fl3/+}$; R26R$^{mTmG/+}$* or *Pax7$^{CreERT2/+}$;*

β-catenin$^{+/+}$*; R26R*$^{mTmG/+}$ mice (data not shown), and so the MyoD+ cells present at 5 dpi do not remain in an undifferentiated state. At 28 dpi in control *Pax7*$^{CreERT2/+}$*; β-catenin*$^{+/+}$*; R26R*$^{mTmG/+}$ mice there are normally few MyoG+ myocytes, but in *Pax7*$^{CreERT2/+}$*; β-catenin*$^{fl3/+}$*; R26R*$^{mTmG/+}$ mice there is a 6.8-fold increase in number of MyoG+ cells (21.8 versus 3.2 MyoG+ cells/mm^2, p=0.0082, Fig. 4.4K). There was also a 3.2 fold increase in MyHCemb expression (17.6% versus 5.4% MyHCemb/mm^2, p=0.009, Fig. 4.4L). The total number of fibers is unchanged, but there is a shift in the distribution of fiber sizes to more small fibers and fewer large fibers (Fig. 4.4O). The TAs of *Pax7*$^{CreERT2/+}$*; β-catenin*$^{fl3/+}$*; R26R*$^{mTmG/+}$ mice also have a larger total cross sectional area (6.6 versus 5.7 mm^2, p=0.052), and this increase is partly due to an increase in Sirius Red+ muscle connective tissue (12% versus 9.7% Sirius Red+/ mm^2, p=0.087) (Fig. 4.4O,N). In total, these data show that at 28 dpi, when muscle regeneration is normally complete, constitutive activation of β-catenin leads to a prolonged regenerative response (continued presence of MyoG+ myocytes and MyHCemb+ regenerating myofibers, and smaller regenerated myofibers) and an increase in connective tissue fibrosis. We examined the TAs even later, at 60 dpi, to test whether constitutive activation of β-catenin had long term effects on muscle regeneration. At 60 dpi there are more MyoG+ myocytes in *Pax7*$^{CreERT2/+}$*; β-catenin*$^{fl3/+}$*; R26R*$^{mTmG/+}$ compared to *Pax7*$^{CreERT2/+}$*; βcatenin*$^{+/+}$*; R26R*$^{mTmG/+}$ mice (3.3 versus 1.2 MyoG+ cells/mm^2, p=0.056) (Fig. 4.4R), although there is no expression of MyHCemb in either genotype (data not shown). Neither the number of myofibers nor the total muscle cross sectional area differs, but there is still a shift in fiber distribution toward smaller fibers (Fig. 4.4T). Therefore, continued activation of β-catenin at 5dpi results in an extension of the myoblast phase,

leading to fewer regenerated myofibers. Subsequently, 28dpi is an extension of the myocyte phase, and consequently, regenerated myofibers are smaller and muscle regeneration continues well past the normal time required for muscle regeneration.

Potentially, constitutive activation of β-catenin could prevent myogenic cells from regenerating muscle, and the apparent extension of the regenerative response could result from a small population of nonrecombined "escaper" cells regenerating muscle at a delayed rate. To test this, we compared GFP expression (which reflects expression of the *β-catenin^{fl3}* allele) in *Pax7$^{CreERT2/+}$; β-catenin$^{fl3/+}$; R26R$^{mTmG/+}$* and *Pax7$^{CreERT2/+}$; β-catenin$^{+/+}$; R26R$^{mTmG/+}$* mice. At 5 dpi, there is less GFP expression in *Pax7$^{CreERT2/+}$; β-catenin$^{fl3/+}$; R26R$^{mTmG/+}$* mice, but this likely reflects the decrease in regenerated fibers (Fig. 4.4A,G). However, at 28 and 60 dpi, there is no difference in GFP expression between the *Pax7$^{CreERT2/+}$; β-catenin$^{fl3/+}$; R26R$^{mTmG/+}$* and *Pax7$^{CreERT2/+}$; β-catenin$^{+/+}$; R26R$^{mTmG/+}$* mice (Fig. 4.3B, 4.4I,P). While constitutive activation of β-catenin prolongs the regenerative response, ultimately expression of constitutive β-catenin does not prevent myogenic cells from regenerating muscle.

Our previous studies found that constitutive activation of β-catenin in fetal myogenic progenitors converts all myofibers to slow, MyHCI+ myofibers (Hutcheson et al., 2009). We tested whether constitutive activation of β-catenin in satellite cells would have a similar effect on regenerated myofibers. We compared the amount of MyHCI expression between *Pax7$^{CreERT2/+}$; β-catenin$^{fl3/+}$; R26R$^{mTmG/+}$* and *Pax7$^{CreERT2/+}$; β-catenin$^{+/+}$; R26R$^{mTmG/+}$* mice at 5 dpi and found a 4-fold increase in MyHCI expression (2.3 versus 0.57 MyHCI+/mm^2, p=0.0004 Fig. 4.4F). At 28 and 60dpi, TAs from *Pax7$^{CreERT2/+}$; β-catenin$^{fl3/+}$; R26R$^{mTmG/+}$* mice continue to have a 3 to 4 fold increase in


5dpi
mGFP mTomato
Pax7+ Satellite Cells
MyoD+ Myoblasts
MyoG+ Myocytes
MyHCemb+ Regenerating Fibers
MyHCslow+ Fibers
Pax7CreERT2/+;β-catenin+/+;R26RmTmG/+
Pax7CreERT2/+;β-cateninfl3/+;R26RmTmG/+
TMX
harvest
BaCl2
5dpi
Myofiber Size Distribution
Fiber #
Fiber # / mm2
Total Area in mm2
Hoechst
Hoechst PHH3
Hoechst Laminin
% GFP
Pax7+ Cells
Pax7+ Cells / mm2
MyoD+ Cells
MyoD+ Cells / mm2
MyoG+ Cells
MyoG+ Cells / mm2
% EmbMyHC
% SlowMyHC
% Pax7PHH3+ / Pax7+
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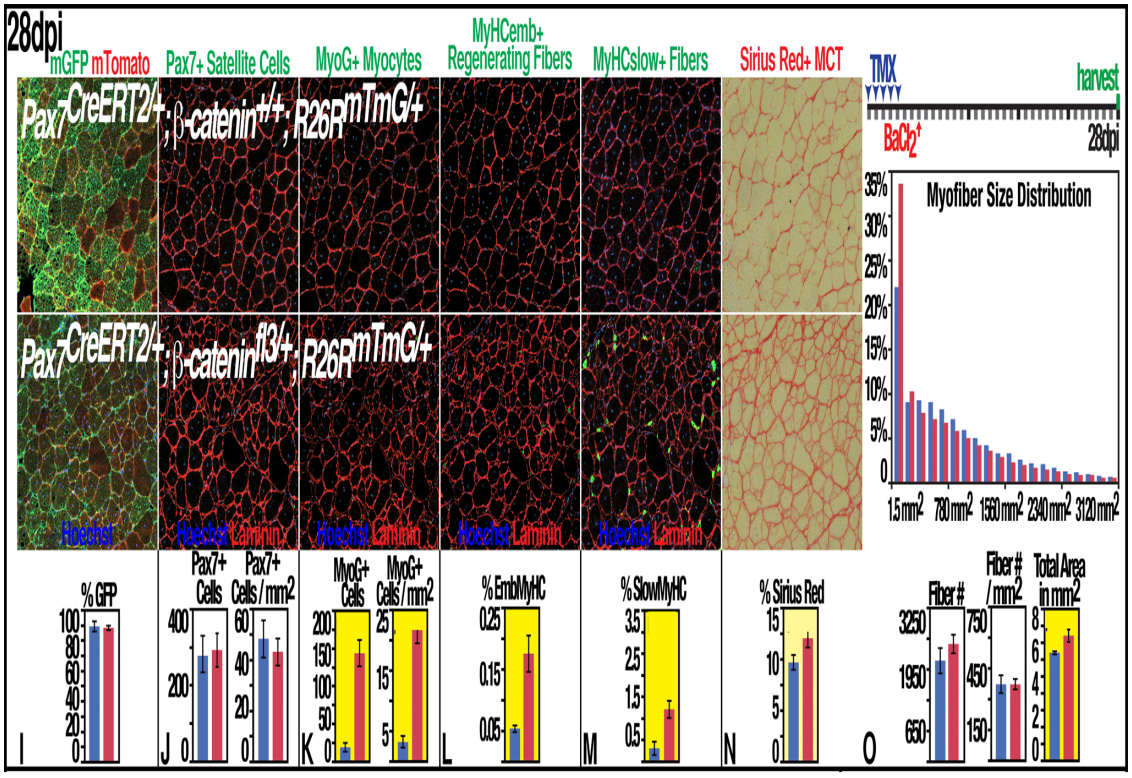
28dpi
mGFP mTomato
Pax7+ Satellite Cells
MyoG+ Myocytes
MyHCemb+ Regenerating Fibers
MyHCslow+ Fibers
Sirius Red+ MCT
Pax7CreERT2/+;β-catenin+/+;R26RmTmG/+
Pax7CreERT2/+;β-cateninfl3/+;R26RmTmG/+
TMX
harvest
BaCl2
28dpi
Myofiber Size Distribution
Hoechst
Hoechst Laminin
% GFP
Pax7+ Cells
Pax7+ Cells / mm2
MyoG+ Cells
MyoG+ Cells / mm2
% EmbMyHC
% SlowMyHC
% Sirius Red
Fiber #
Fiber # / mm2
Total Area in mm2
I
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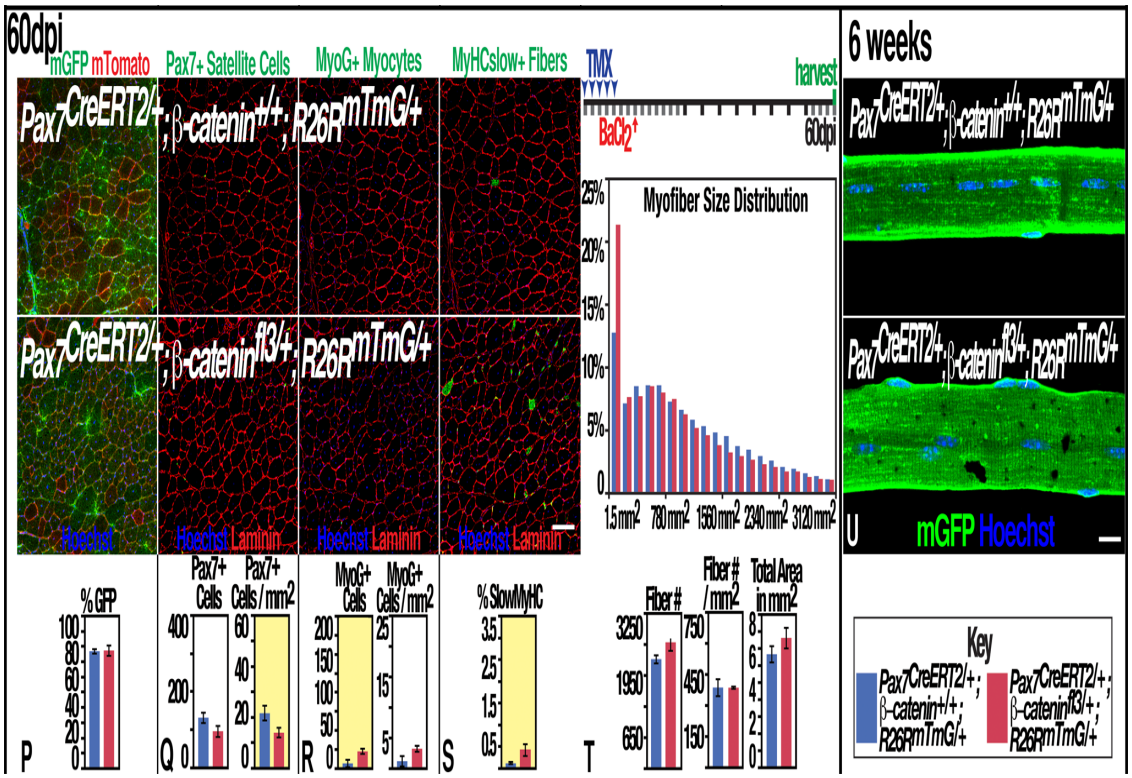

Fig. 4.4 Constitutive activation of β-catenin in satellite cells alters the kinetics of myoblast differentiation, resulting in a prolonged regenerative response
Prolonged myoblast phase at 5dpi(A-H) as sown by continued MyoD expression resulting in smaller fibers (G) This leads to extended regeneration at 28dpi (I-O) but it has mostly resolved by 60dpi (P-T) There are no structural defects when in mutants (U) Yellow denotes significance (S) Scale bar = 100μm, (U) scale bar = 25μm

MyHCI expression (1.2% versus 0.30% at 28dpi, p=0.02, and 0.43% versus 0.12% at 60dpi, p=0.089, Fig. 4.4 M,S). Interestingly, while only small percent of the total area is MyHCI+ in the *Pax7$^{CreERT2/+}$; β-catenin$^{fl3/+}$; R26R$^{mTmG/+}$* mice at 28 and 60 dpi, 80 to 90% of the total area is GFP+ (Fig. 4.4 I,P). Therefore, β-catenin positively regulates the expression of MyHCI, but unlike during fetal myogenesis, β-catenin is not sufficient to convert all myofibers to a slow, MyHCI+ fiber type.

β-catenin is also a member of the adherens junction complex and localizes to the membrane of muscle fibers (Nastasi et al., 2004). Mutations in *Calpain3* result in an accumulation of β-catenin at the myofiber membrane, causing limb girdle muscular dystrophy type 2A in humans (Kramerova et al., 2006). The mouse model of *Calpain3* mutations shows disrupted sarcomere structure and a defect in myoblasts fusion. Thus potentially constitutive activation of β-catenin might cause structural defects in the muscle. We isolated myofibers from *Pax7$^{CreERT2/+}$; β-catenin$^{fl3/+}$; R26R$^{mTmG/+}$* and *Pax7$^{CreERT2/+}$; β-catenin$^{+/+}$; R26R$^{mTmG/+}$* mice 6 weeks after injury and analyzed regenerated GFP+ fibers for sarcomere structure and did not observe any defects (n=3 mutant mice and 3 control mice, Fig. 4.4U). Hence, we find activated β-catenin does not result in myofiber structural defects.

In summary, constitutive activation of β-catenin in the satellite cells and their derivatives affects the timing of the regenerative response to muscle injury. Neither the number nor proliferation of satellite cells was altered, but MyoD+ cells, MyoG+ myocytes, MyHCemb+ regenerating myofibers are present for an extended period. This extension in the regenerative response consequently results in a shift toward smaller myofibers. β-catenin also positively regulates MyHCI expression, but is not sufficient to

convert myofibers to a slow fiber type. While constitutive activation of β-catenin did alter the kinetics of muscle regeneration, it did not prevent satellite cells from giving rise to regenerated muscle, nor did it disrupt sarcomere structure.

Discussion

Wnt/β-catenin signaling is transiently active early in regenerating myogenic cells

We have shown by *Tcf/Lef:H2BGFP*^{Tg} reporter activity that Wnt/β-catenin signaling is activated immediately following injury (1 dpi) in myogenic cells. However, this activation is transient, as reporter expression is reduced by 3 to 5 dpi. Also, Wnt/β-catenin signaling does not appear to be active in Pax7+ satellite cells, but is most prominent in MyoD+ myoblasts. Many studies have investigated the expression of Wnt/β-catenin signaling components during muscle regeneration and have seen evidence for active signaling during muscle regeneration (Abiola et al., 2009; Armstrong and Esser, 2005; Aschenbach et al., 2006; Bernardi et al., 2011; Brack et al., 2008; Brack et al., 2009; Goichberg et al., 2001; Han et al., 2011; Le Grand et al., 2009; Otto et al., 2008; Polesskaya et al., 2003; Tanaka et al., 2011). These studies strongly support that Wnt/β-catenin signaling is active during muscle regeneration (Model Fig. 4.5B). However the strategies used were not specific for Wnt/β-catenin signaling within the myogenic population; did not measure signaling over time; or they were performed *in-vitro*. In addition to expression analysis, other studies have used the TOPgal Wnt/β-catenin reporter activity to demonstrate active signaling *in vivo* 2 and 5 dpi after muscle injury, but assessment of activity specifically within the myogenic cells over time was not

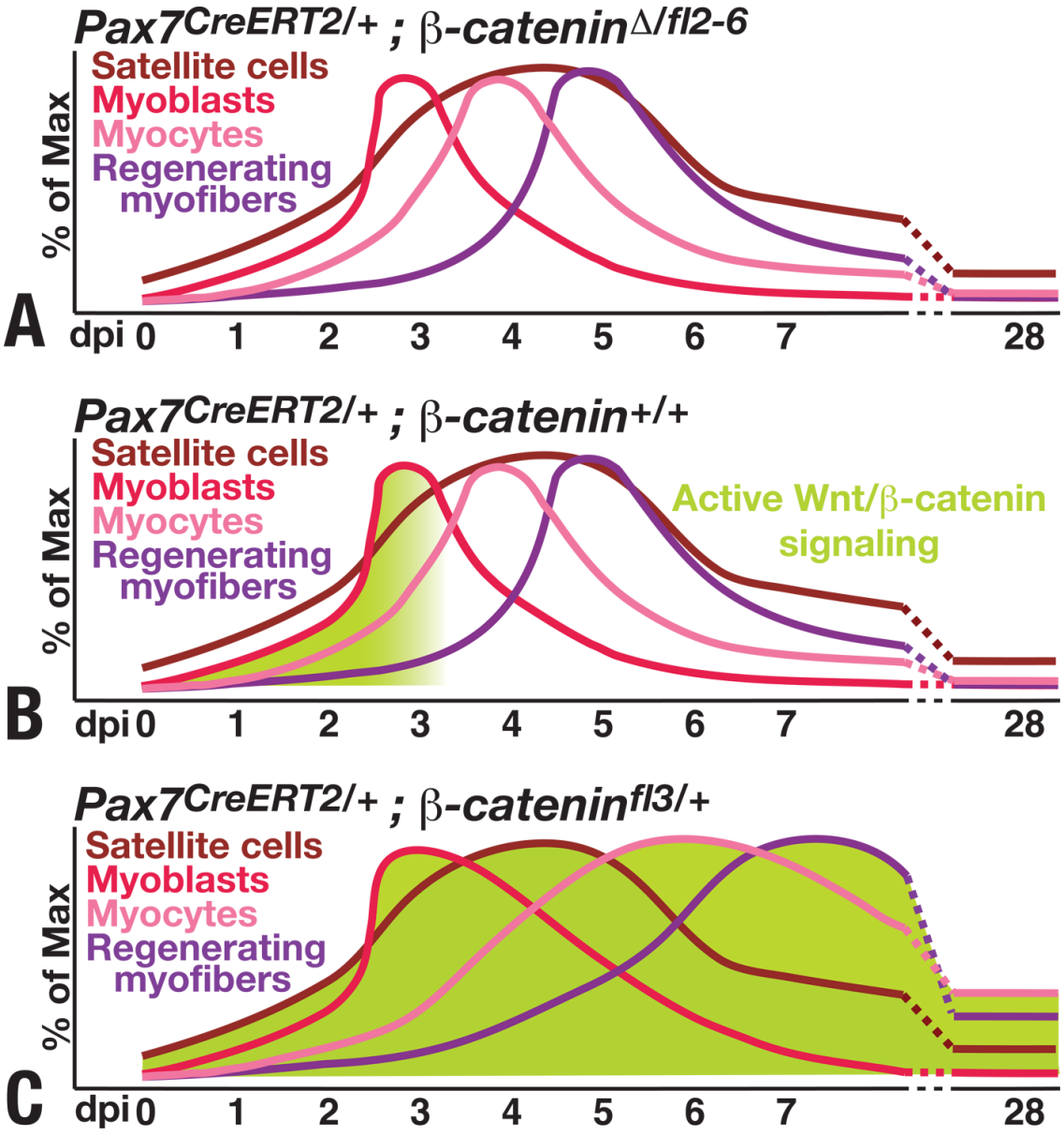

Fig. 4.5 Model of Wnt/β-catenin in muscle regeneration (A)β-catenin loss does not affect satellite cell lineage. (C) Constitutive activation of β-catenin expands myogenesis (B) Wnt/β-catenin signaling is active transiently during the first few days of regeneration.

performed *in vivo* (Brack et al., 2008; Naito et al., 2012). Our *in vivo* analysis of Wnt/β-catenin signaling specifically within the myogenic cells demonstrates that signaling is primarily active in myoblasts, not satellite cells, and is only transiently active during the first days of the regenerative process.

β-catenin is not required in satellite cells to regenerate muscle or return to niche

Despite evidence from this and a multitude of previous studies showing that Wnt/β-catenin signaling is active during muscle regeneration, we discovered that β-catenin is not required within the satellite cell lineage for regeneration. Deletion of β-catenin within the satellite cells and their progeny does not affect activation, proliferation, differentiation, or fusion to repair damaged myofibers (Model Fig. 4.5A). β-catenin deficient satellite cells were also able to return to their niche, as well as reactivate and regenerate muscle even after multiple rounds of injury. These results are surprising because previous *in vitro* studies suggest that loss of Wnt/β-catenin signaling would result in several phenotypes, including less myofiber differentiation (Brack et al., 2008; Brack et al., 2009; Descamps et al., 2008; Kim et al., 2008) more differentiation (Gavard et al., 2004; Tanaka et al., 2011), or decreased proliferation of satellite cells (Otto et al., 2008). Additionally, *in vivo* data suggests that loss of β-catenin would result in smaller muscle fibers (Armstrong et al., 2006; Brack et al., 2008; Brack et al., 2009). The fact that our results differ from previous work presumably reflects differences in experimental design, as experiments that are not specific to the myogenic lineage would also affect other cell types responding to Wnt/β-catenin signaling during muscle regeneration.

Interestingly, when β-catenin is deleted within the muscle fibers during development using $HSACre^{Tg}$ mice there were no defects in muscle size, organization, or structural integrity, although β-catenin is required within the muscle for proper neuromuscular junction formation (Li et al., 2008). Similarly, we did not observe any obvious defects in regeneration when β-catenin was deleted in adult myofibers using TMX inducible $HSA^{CreERT2}$ mice (data not shown) (Schuler et al., 2005).

Activation of β-catenin in satellite cells alters kinetics of myoblast differentiation, resulting in prolonged regeneration

We have shown that β-catenin is not required in the myogenic cells for muscle regeneration, however there is active Wnt/β-catenin signaling within these cells. Wnt/β-catenin signaling is only transiently active within the myogenic cells during regeneration; therefore, constitutive activation of β-catenin in satellite cells extends the time window of myogenic cells responding to Wnt signal. When β-catenin is constitutively active in the satellite cells, we saw no direct effect upon the satellite cells themselves in number, proliferation, or ability to return to the niche. Therefore the satellite cells themselves are insensitive to manipulations of β-catenin. This is in contrast to work by others showing that activation of Wnt/β-catenin signaling by exposure to Wnt3a, adenoviral-β-catenin, or compliment C1q either increased or decreased proliferation of satellite cells (Brack et al., 2007; Kim et al., 2006; Naito et al., 2012; Otto et al., 2008). However, in the myoblast progeny of those satellite cells we see that at 5 dpi there is an extension of the myoblast phase at the expense of myofiber formation. The extension of the myoblast phase is not indefinite as by 28 dpi there are no more MyoD+ cells, but the initial extension of the

myoblast phase results in a successive extension of the myocyte phase. There is also continued expression of MyHCembryonic because these myocytes are still fusing to form new muscle. We interpret these results as an extension and not just a delay because the size of the whole muscle is increased despite the shift in fiber size distribution toward smaller fibers at 28 dpi (Fig. 4.5C). By 60 dpi, regeneration is mainly complete, so constitutive activation of β-catenin does not prevent differentiation but does greatly extend the time the muscle is in a state of regeneration. We also see that at 60 dpi the fiber size distribution is still shifted toward smaller fibers. Smaller fiber size was observed in other studies in which Wnt/β-catenin signaling was experimentally activated during muscle regeneration *in vivo* (Brack et al., 2008; Le Grand et al., 2009). However the authors determined that this was due to premature differentiation as they delivered single doses of Wnt3a, which based on our results, might have a very different effect compared to constitutive activation of β-catenin. Several studies have exogenously activated Wnt/β-catenin signaling either by delivery of Wnt3a or LiCl to myogenic cells *in vitro* and proposed that Wnt/β-catenin promotes differentiation and fusion (Abiola et al., 2009; Bernardi et al., 2011; Brack et al., 2008; Pansters et al., 2011) as well as prevents differentiation (Gavard et al., 2004; Tanaka et al., 2011). Our conclusions are based upon analysis of the different phases of myogenesis over an extended period of time of continued activation of β-catenin specifically in the myogenic lineage. *In vitro* experiments are not easily amenable to all phases of regeneration, and *in vivo* non-genetic activation of the pathway also affects other populations of cells within the muscle tissue sensitive to Wnt/β-catenin signaling.

Activation of β-catenin in myogenic lineage extends MRFs expression and does not disrupt muscle structure

Our results show that constitutive activation of β-catenin results in an expansion of MyoD expression. Interestingly, there is evidence *in vitro* that MyoD and β-catenin will directly bind to enhance MyoD transcriptional activity during myogenic differentiation (Kim et al., 2008). It is possible that the time extension of β-catenin availability extends the time MyoD has enhanced transcriptional activity, in turn extending the myoblast phase of regeneration.

Constitutive activation of β-catenin also results in continued expression of MyoG. β-catenin is also an integral member of the adherens junction complex, and MyoG expression has been shown to be downstream of adherens junction formation *in vitro* (Gavard et al., 2004; Goichberg et al., 2001). However, there is evidence that excess accumulation of β-catenin at the myofiber membrane disrupts the structural integrity of muscle. Mutations in *Calpain3* result in limb girdle muscular dystrophy type 2A, as *Calpain3* functions to regulate the amount of m-cadherin and β-catenin localized to the membrane (Kramerova et al., 2006). Additionally, loss of the muscle specific E3-ligase Ozz, which targets membrane β-catenin for degradation, results in an accumulation of β-catenin at the membrane that disrupts the sarcomere structure (Nastasi et al., 2004). Constitutive activation of β-catenin in the satellite cells could result in an accumulation of β-catenin at the membrane of regenerated fibers disrupting sarcomere structure; however we observed no disruption in the sarcomere structure in regenerated fibers. In line with this, other studies that activated β-catenin in either the myogenic lineage or the myofibers

themselves during development found no disruption in muscle structure (Liu et al., 2012; Wu et al., 2012).

Adult and fetal myogenic progenitors have different sensitivity to Wnt/β-catenin

Previously, our lab established that during development β-catenin is required for fetal myogenesis. Loss of β-catenin in the fetal myogenic progenitors results in fewer myofibers and less MyHCI expression. Constitutive activation of β-catenin results in disorganized myofibers, increased number of Pax7+ progenitors, and increased MyHCI expression (Hutcheson et al., 2009). Our results clearly show a fundamental difference between myogenic progenitors in the fetus and the adult in their sensitivity to Wnt/β-catenin signaling. Although required in the fetus, β-catenin is not required in the adult for either myofiber number or MyHCI expression. Activation of β-catenin does not affect satellite cell numbers or myofiber organization in the adult, but does result in an increase in MyHCI expression. It is not clear whether β-catenin regulation of MyHCI is direct or the increase in MyHCI is a reflection of more immature myofibers, as all myofibers express MyHCI during development (Schiaffino and Reggiani, 1996). We have shown using similar genetic tools and methods of analysis that adult stem cells do not always behave similarly to their developmental counterparts.

Satellite cells are analogous to hematopoietic stem cells

There are striking similarities between satellite cells and hematopoietic stem cells (HSCs) in relation to Wnt/β-catenin signaling. Wnt/β-catenin signaling is generally

thought to provide instructional cues to HSCs (as reviewed in Rossi et al., 2012). Like myogenic progenitors, HSCs require β-catenin during development. If β-catenin is deleted from the aorta-gonad-mesonephros, HSCs do not differentiate from the endothelial precursors (Ruiz-Herguido et al., 2012). However, in the adult β-catenin is dispensable for HSC function (Cobas et al., 2004). Therefore, like myogenic progenitors hematopoietic progenitors in the embryo and the adult differ in their requirement for Wnt/β-catenin signaling. Interestingly, when β-catenin is constitutively activated in the adult HSCs, terminal differentiation into all of the hematopoietic lineages is blocked (Kirstetter et al., 2006; Scheller et al., 2006). In adult myogenesis, constitutively activated β-catenin prolongs differentiation but does not prevent it, but the similarities between HSCs and satellite cells are evident. In summary, β-catenin is active but not required with in the satellite cells during muscle regeneration; however, continuous β-catenin signaling prolongs the regenerative response.

Methods

Mice

All mouse lines used were previously published. We reported the generation of the *Pax7*^{CreERT2} Cre driver previously and they are available from The Jackson Laboratory as stock number 017763 (Murphy et al., 2011). The reporters used were the *R26R*^{mTmG} (Muzumdar et al., 2007) and *Tcf/Lef1-H2B/eGFP*^{Tg} (Ferrer-Vaquer et al., 2010). Conditional alleles included β-catenin loss of function, *β-catenin*$^{\Delta/fl2\text{-}6}$ (Brault et al., 2001), and constitutive gain of function, *β-catenin*$^{fl3/+}$ (Harada et al., 1999). For all experiments, mice were bred onto a C57/Bl6J background and were 6 to 8 weeks old.

Muscle injury and tamoxifen delivery

Injury was induced by injecting 25ul of 1.2% $BaCl_2$ in normal saline into the right tibialis anterior (TA) muscle. Left TA served as the uninjured control. Each dose of tamoxifen was 10mg in corn oil delivered via gavage.

Immunofluorescence, histology, and microscopy

For section immunofluorescence, flash-frozen muscles were immediately sectioned at 10um. Slides designated for GFP staining were immediately fixed in 20% paraformaldehyde (PFA) at 4^{o}C overnight. For all other staining, sections were fixed 5 min. in 4% PFA. Sections were washed in PBS and then if needed (see Table 4.1), subjected to antigen retrieval, consisting of heating slides in citrate buffer (1.8mM citric acid, 8.2 mM sodium citrate in H_20) in a 2100 PickCell Retriever, and quenched for 5 min. in 3% H_2O_2. Tissue sections were blocked 30-60 min. in 5% serum or 0.5% TNB blocking reagent (PerkinElmer) in PBS, incubated overnight at 4^{o}C in 1^{o} antibody, washed in PBS, incubated 2 hr. at room temperature in 2^{o} antibody, washed in PBS, when needed (see Table 4.1) incubated 3 hr. in Vector ABC, washed in PBS and labeled 10 min with PerkinElmer TSA Fluorescein or TSA Cy3. Slides were then washed in PBS, post fixed 5 min. in 4% PFA and mounted with Fluoromount-G (SouthernBiotech) with 2mg/ml Hoechst. Antibodies are listed in Table 4.1. For Sirius Red staining, flash frozen sections were fixed 1 hr. at 56^{o}C in Bouin's fixative, washed in water, stained 1 hr. in Master*Tech Picro Sirius Red, washed in 0.5% acetic acid, dehydrated, equilibrated with xylene, and mounted in Permount (Kiernan, 1990). Sirius Red sections were imaged in

bright-field on a Zeiss Axioplan2 microscope. Immunofluorescent sections were imaged on a Nikon AR1 confocal microscope. Each confocal image is a composite of maximum projections, derived from stacks of optical sections.

Fiber preparations

42 dpi TAs were digested with 400U/ml Collagenase I (Worthington) 50 min. 37^{o}C, washed in PBS, fixed 5 min. in 4% PFA, washed in PBS, and mounted onto slides, and processed for immunofluorescence.

Quantification and statistics

The number of Pax7+, MyoD+, or MyoG+ nuclei was determined using Image J Analyze Particles function. Co-labeling of Pax7, MyoD, or MyoG with PHH3 was determined by additive image overlay in ImageJ. For GFP, MyHCemb, MyHCslow, or MyHCIIb the total number of positive pixels was counted. For quantification of ECM, Sirius Red+ area was quantified by selecting red pixels in Adobe Photoshop, deleting all non-red pixels, converting resulting image to a binary image and counting Red+ pixels using Image J Analyze Particles function. For each variable, counts of two sections across the entire TA were averaged for three to six individuals of each genotype per time point and analyzed using a Student's two-tailed T test. On all bar charts, mean +/- 1 SEM shown. Fiber distribution was determined using a program to be described in a later paper.

Genomic and semiquantitative PCR and FACS

For genomic PCR, GFP+ and Tomato+ cells from were isolated from both left and right TAs at 5dpi from *Pax7*$^{CreERT2/+}$*; R26R*$^{mTmG/+}$*; β-catenin*$^{+/+, fl3/+, \Delta/+,}$ and $^{\Delta/fl2\text{-}6}$ mice. Muscles were stripped of tendons and placed in 3ml Hamm's F12 with 60ul of DNAseI (Roche). Tissue was manually minced using forceps, and then digested with the addition of 1000 U/ml Collagenase I (Worthington) for 30 min. at 37°C, triturated and incubated an additional 30 min. Then cells were passed through 70-mm and 40-mm filters, washed with an additional 7ml of F12, and then spun at 1500 rpm (1258 ***g)*** for 15 min. Pelleted cells were then resuspended in FACS buffer (1% BSA; 1%PBS) and sorted based on high GFP and low Tomato expression (GFP+) or high tomato and low GFP expression (Tomato+) on a FACS AriaII (BD Biosciences). After collection, cells were spun down again for 15 min. 1500rpm, and DNA was isolated by HOT SHOT and the PCR genotyping was performed as previously published in (Brault et al., 2001; Harada et al., 1999). For semiquantitative PCR, myogenic cells were isolated similar to methods described above. After resuspension, cells were incubated the following antibodies for 1 hr. (CD31-eFluor450; CD45-eFluor450; Scal-PE-Cy7; α7integrin-APC; see Table 4.1 for concentrations) similar to methods described previously by Yi et al., 2011. Cells were washed with 15ml FACS buffer, spun at 1500 rpm for 15 min., and re-suspended for FACS sorting. Total RNA was extracted using Qiagen RNeasy Mini and Micro kit, depending on the number of cells collected. cDNA was reverse transcribed using Invitrogen Superscript III. Equal amounts of RNA were amplified by 34 cycles of PCR using primers for GAPDH (5'-gcaccaccaactgcttagc-3'; 5'-gccgtattcattgtcatacc-3'), and Axin2 (5'-aagagaagcgacccagtcaa-3'; 5' ctgcgatgcatctctctctg-3').

Table 4.1
Antibodies used in study

Antibody	Type	Source	Product Number	Working Conc.	Antigen Retrieval	Secondary and Amplification
Pax7	Mouse IgG1	DSHB	PAX7	2.4μg/ml	yes	biotin conj. mouse IgG1 secondary, ABC, TSA
MyoD	Mouse IgG1	Santa Cruz Biotechnology	Sc-32758 (5.8A)	4μg/ml	yes	biotin conj. mouse IgG1 secondary, ABC, TSA
MyoG	Mouse IgG1	Santa Cruz Biotechnology	Sc-12732 (F5D)	4μg/ml	yes	biotin conj. mouse IgG1 secondary, ABC, TSA
MyHC embryonic	Mouse IgG1	DSHB	F1.652	3μg/ml	yes	biotin conj. mouse IgG1 secondary, ABC, TSA
MyHC Slow	Mouse IgG1	Sigma	M8421 (NOQ7.5.4D)	1.5μg/ml	yes	biotin conj. mouse IgG1 secondary, ABC, TSA
MyHCIIb	Mouse IgG1	DSHB	BF-F3	12.6μg/ml	yes	biotin conj. mouse IgG1 secondary, ABC, TSA
Laminin	Rabbit polyclonal	Sigma	L-9393	2.5μg/ml	yes	biotin conj. rabbit secondary and streptavidin Dylight 488 or 594
GFP	Chick polyclonal	Aves Labs	GFP-1020	20μg/ml	no	Dylight 488 goat anti chick
Phospho-histone H3	Rabbit Polyclonal	Millipore	06-570	5μg/ml	yes	biotin conj. rabbit secondary and streptavidin Dylight 488 or 594
α7 integrin – 649	RatIgG2b	Ablab (CANANDA)	Clone R2F2	0.5μg/ml	n/a	n/a
Sca1 – PECy7	RatIgG2a	eBioscience	25-5981	0.05μg/ml	n/a	n/a
CD31 – eFluor450	RatIgG2a	eBioscience	48-0311	1.0μg/ml	n/a	n/a
CD45 – eFluor450	RatIgG2b	eBioscience	48-0451	2.0μg/ml	n/a	n/a

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Myogenesis requires the coordinated interactions of several cell types and multiple signaling pathways. During muscle regeneration in the adult, myogenic progenitor cells must produce an adequate number of cells, differentiate, and incorporate themselves into the muscle pattern established during development. These processes are intrinsically linked and require both temporal and spatial regulation. The focus of this dissertation is to determine the role of two cell populations, satellite cells and connective tissue fibroblasts, as well as the Wnt/β-catenin signaling pathway during muscle regeneration.

Previous characterization of connective tissue fibroblasts during muscle regeneration had been limited by the lack of molecular markers. Recently, our lab discovered the first molecular marker of connective tissue fibroblasts. *Tcf4* (*Tcf7L2*), a member of the Tcf/Lef family of transcription factors and the most downstream effector of the Wnt/β-catenin signaling pathway, is specifically expressed in muscle connective tissue fibroblasts (Kardon et al., 2003) Tcf4 labels a population of cells in the lateral plate mesoderm that form a pre-pattern of muscle prior to, and independent of, the migration of myogenic cells into the limb during development. Subsequently, we have shown that Tcf4+ cells within the lateral plate mesoderm give rise to the connective tissue fibroblasts

present within the limb at birth, and that Tcf4 is continually expressed by the connective tissue fibroblasts in adult muscle (Mathew et al., 2011). Evidence suggests that these cells are playing a role in muscle regeneration (Cornelison, 2008; Huard et al., 2002; Joe et al., 2010; Mann et al., 2011; Serrano et al., 2011), and we chose to investigate the spatial and temporal expression pattern of the Tcf4+ connective tissue fibroblasts in relation to satellite cells, myofiber regeneration, and fibrosis during muscle regeneration. We discovered that Tcf4+ connective tissue fibroblasts greatly expand during muscle regeneration. This increase occurs at the same time and in the same area as satellite cells, myofiber regeneration, and fibrosis. This dramatic increase in the number of cells during a critical window suggests a functional role for these cells during muscle regeneration. In order to genetically manipulate the connective tissue fibroblasts in the adult and avoid any developmental defects, our lab, in collaboration with the Cappechi lab, created a TMX inducible allele of *Tcf4*. *Tcf4*^{CreERT2} specifically labels the connective tissue fibroblasts in muscle; however, at about 40% recombination, the efficiency is relatively low. To determine the role of the connective tissue fibroblasts, we crossed the *Tcf4*^{CreERT2} mice to the *R26R*^{DTA} mice, which expresses diphtheria toxin-α after Cre mediated recombination, resulting in cell autonomous ablation of connective tissue fibroblasts. When connective tissue fibroblasts are ablated, satellite cells prematurely differentiate resulting in smaller myofibers after regeneration. These results show that connective tissue fibroblasts are required for muscle regeneration and function to prevent the premature differentiation of the satellite cells, which allows for an adequate pool of progenitors to accumulate and efficiently regenerate the muscle. This is the first direct

evidence demonstrating that connective tissue fibroblasts are required for muscle regeneration.

Connective tissue fibroblasts are playing a functional role in muscle regeneration, but the molecular mechanisms by which these cells interact with satellite cells has yet to be determined. One possible candidate for is the TGFβ signaling pathway. Unfortunately, the recombination efficiency of the *Tcf4*^{CreERT2} may hinder interpretation of genetic loss of function experiments. Therefore, identification of a more efficient molecular marker for connective tissue fibroblasts is of great interest to our lab. Performing a microarray experiment comparing expression between isolated connective tissue fibroblasts at various time points after injury would certainly aid in identification of a molecular marker as well as possible candidates for cell-cell signaling between connective tissue fibroblasts and satellite cells. Since satellite cells prematurely differentiate when 40% of the connective tissue fibroblasts are deleted, a microarray comparing gene expression in satellite cells from *Tcf4*$^{CreERT2/+}$*; R26R*$^{+/+}$ and *Tcf4*$^{CreERT2/+}$*; R26R*$^{DTA/+}$ mice might indicate what signaling pathways are disrupted. I anticipate that future work on connective tissue fibroblasts during regeneration from the Kardon lab will further elucidate the mechanisms by which these cells regulate muscle regeneration.

Satellite cells are known to be sufficient for muscle regeneration; however, their requirement had not been determined. In light of recent work identifying other non-satellite cell populations capable of contributing to muscle regeneration, we decided to explicitly test the requirement of satellite cells in muscle regeneration (Mitchell et al., 2010; Sampaolesi et al., 2003). To test this, we used a similar strategy as detailed above. We created, in collaboration with the Cappechi lab, a TMX inducible allele of *Pax7*,

Pax7^{CreERT2}, and confirmed that this allele specifically, and only following delivery of TMX, induces recombination in satellite cells. We crossed *Pax7*$^{CreERT2/+}$ mice to *R26R*$^{DTA/+}$ mice and delivered TMX to both *Pax7*$^{+/+}$; *R26R*$^{DTA/+}$ and *Pax7*$^{CreERT2/+}$; *R26R*$^{DTA/+}$ mice prior to injury. When satellite cells are ablated, muscle fibers do not regenerate, the number of connective tissue fibroblasts is decreased, and fibrosis is increased. Therefore, we have shown that satellite cells are the endogenous muscle stem cell responsible for muscle regeneration and that they positively regulate the connective tissue fibroblasts during regeneration.

The generation of the *Pax7*^{CreERT2} allele allows for the genetic manipulation of the satellite cells and can be used to ask questions specifically regarding myogenesis or more broadly about the general adult stem cell properties and requirements. From the previous results, one important outstanding question is the identity of the molecular signal from satellite cells that positively regulates the connective tissue fibroblasts. Also, there are many questions regarding the requirement for satellite cells during normal homeostasis in the adult or aged mouse. A common condition associated with aging is a loss in muscle mass as well as an inability to rebuild muscle mass. Research into the mechanisms of muscle aging will be aided by our ability to specifically manipulate the satellite cells using the *Pax7*^{CreERT2} allele.

Multiple studies have looked at the role of Wnt/β-catenin signaling during muscle regeneration; however, the function of this pathway remains controversial. Much of this controversy stems from a lack of uniformity in experimental design, differences in the behavior of cells *in vitro* and *in vivo*, and that components of the Wnt pathway also signal in a noncanonical manner. Although the expression of Wnt/β-catenin pathway

components has been extensively studied, we wanted to confirm in our injury model ($BaCl_2$ injection) that Wnt/β-catenin signaling was active within the myogenic lineage after injury. We used *TCFLef:H2B-GFP*^{Tg} reporter mice that express nuclear localized GFP in response to Wnt/β-catenin signaling (Ferrer-Vaquer et al., 2010). By FACS analysis we showed that myogenic cells are responding to Wnt/β-catenin signaling early in the regeneration process followed by a decrease later in regeneration. We sought to directly test the requirement for β-catenin specifically within the myogenic lineage without the complication of developmental defects. We crossed *Pax7*^{CreERT2} mice to *β-catenin*$^{fl2-6}$ mice, which harbor a conditional loss of function allele that deletes exons two through six by Cre mediated recombination and creates a functional null (Brault et al., 2001). In the *Pax7*$^{CreERT2/+}$; *β-catenin*$^{\Delta/fl2-6}$ mice, β-catenin is deleted in satellite cells after TMX delivery. We saw no defects in muscle regeneration, nor did we see any difference in satellite cell number or inability to return to the niche after injury. Additionally, when we performed multiple rounds of injury, we saw no effects on satellite cells or muscle regeneration. Therefore, β-catenin is not required in satellite cells or their progeny for activation, proliferation, differentiation, or for their return to the niche. If loss of β-catenin made satellite cells slightly less efficient at regenerating muscle than their wild type counterparts, we might expect that the few satellite cells that did not undergo recombination would have contributed a greater number of fibers to the regenerated muscle. To address this concern, we followed recombination using *R26R*^{mTmG} reporter mice, which express membrane bound Tomato in the absence of recombination and membrane bound GFP subsequent to recombination. We did not see any difference in the contribution of recombined cells between *Pax7*$^{CreERT2/+}$; *β-catenin*$^{\Delta/fl2-6}$ and

Pax7$^{CreERT2/+}$; *β-catenin*$^{Δ/+}$ mice; although, the high level of recombination may make subtle differences difficult to detect. Alternatively, we could deliver a lower dose of TMX to *Pax7*$^{CreERT2/+}$; *β-catenin*$^{Δ/fl2\text{-}6}$; *R26R*$^{mTmG/+}$ and *Pax7*$^{CreERT2/+}$; *β-catenin*$^{Δ/+}$; *R26R*$^{mTmG/+}$ mice and determine whether the contribution of recombined GFP+ cells differs between mutant and control mice. Then, by the classic standards of stem cell function serial transplantation (analogous to the re-injury model) and competition (low dose TMX) we could say that satellite cells do not require β-catenin. These results are surprising given the expression of the Wnt/β-catenin reporter in the myogenic cells after injury and should caution against the interpretation that expression implies function.

Although these results show that β-catenin is absolutely not required within the myogenic lineage for muscle regeneration, we know from preliminary experiments in collaboration with Richard Lang that loss of Wnt signaling in macrophages results in a decrease in satellite cell number at both 3 and 5 dpi. Therefore, Wnt signaling from macrophages is either noncanonical or the Wnt/β-catenin signal is being received by a non-myogenic cell type within the muscle that effects the satellite cell population. The connective tissue fibroblasts are a likely candidate for this role. The expansion of the Tcf4+ connective tissue fibroblasts during regeneration corresponds to the time when macrophages go through the M1 to M2 transition, switching from pro-inflammatory and phagocytic to anti-inflammatory and signal secreting (Tidball and Villalta, 2010). It will be interesting to pick apart these direct interactions and determine how they are modulating muscle regeneration.

The *TCF/Lef:H2B-GFP*^{Tg} reporter data show that Wnt/β-catenin signaling is down regulated soon after injury. We wanted to determine the effect of continued β-

catenin signaling on satellite cells and muscle regeneration overall. We activated β-catenin in satellite cells by crossing *Pax7*^{CreERT2} mice to mice harboring the gain of function *β-catenin*^{fl3} allele. In these mice, exon three of β-catenin is deleted upon Cre mediated recombination, which is required for phosphorylation and degradation of β-catenin (Harada et al., 1999). When we constitutively activated β-catenin in satellite cells, we saw no effect on the satellite cell number or their ability to return to their niche after injury. However, constitutively active β-catenin signaling in the myoblast progeny of satellite cells resulted in a delay of myofiber formation at 5 dpi and an extension of MyoG and MyHCembryonic expression long after the normal period of regeneration. Therefore, it is important to downregulate Wnt/β-catenin signaling to prevent prolonged muscle regeneration. Interestingly, we observed a significant increase in the expression of MyHCslow when β-catenin is not degraded. This correlates with the phenotypes observed during development when β-catenin is constitutively active in the fetal myogenic population even though the other phenotypes, including disrupted fiber orientation and increased Pax7+ cells, are not recapitulated in the adult (Hutcheson et al., 2009). Our results provide an example in which the information provided by a molecular signal is transduced by shutting off the signal.

The satellite cells are not susceptible to constitutive activation of β-catenin, but the MyoD+ myoblasts are affected. Even though there was an extension in the timing of MyoG expression, it is not clear whether this is a direct effect of β-catenin expression in the myocytes or if it is an indirect consequence of the earlier effect on the myoblasts. It would be interesting to use a TMX inducible MyoG Cre to activate β-catenin only in the myocytes to address this question. It is intriguing that no adult cancer is associated with

skeletal muscle even though there is a stem cell population. Possibly, one reason for this is that the satellite cells are impervious to either loss or overexpression of Wnt/β-catenin signaling.

Scientists have been observing vertebrate muscle regeneration for nearly 150 years (Carlson, 1973). There have been many reports and reviews describing the cellular and molecular processes involved in muscle regeneration (Charge and Rudnicki, 2004; Hawke and Garry, 2001; Shi and Garry, 2006; Zammit et al., 2006). Despite the extent of previous work, our examination of the normal time course of events during muscle regeneration suggests further elucidation is needed. In our regeneration model, the peak number of Pax7+ satellite cells occurs at 5 dpi. This is the same time as the peak of MyHCembryonic expression. This is counterintuitive if the Pax7+ cells are giving rise to nascent myofibers. The expectation would be that the number of satellite cells should be decreasing as the myofibers are forming. It is possible that our time course was not performed at a fine enough scale to accurately portray each peaks. Therefore, a finer scale time course including 1, 2, 3, 4, 5, 6, 7, 14, 21, and 28 dpi may provide more insight. However, the number of MyoD+ myoblasts, which by all accounts are the immediate progeny of the satellite cells, peak at 3 dpi, which is earlier than satellite cells. Also of note, the number of MyoD+ myoblasts at their peak is at least 2-fold less than the peak number of satellite cells. This leaves an average of 500 Pax7+ cells in one 10μm section in excess. Most reports suggest that the self-renewal of satellite cells occurs early; however, the timing has not been specifically tested or reported in the literature. Regardless, it is difficult to imagine that all of those cells would return to the niche since in uninjured muscle there are only about 200 satellite cells in one cross section. We have

never detected any significant level of apoptosis after injury; although, TUNEL staining may not be optimized for muscle tissue or apoptosis may not be the mode of cell death. It would be interesting to include both MyoD+ myoblasts and MyoG+ myocytes into the refined time course to better understand the relationships between the myogenic cells *in vivo*. Crossing $Pax7^{CreERT2}$ mice to mice with a nuclear reporter and giving single doses of TMX at various time points after injury could also be used to discern the significance of these observations.

Another question that has largely been unaddressed *in vivo* is the mechanism and cellular processes involved in myogenic cell fusion to muscle. With the advancements in live, *in vivo* confocal microscopy and the development of stable and strongly expressed fluorescent reporters, it may be possible to use $Pax7^{CreERT2}$ mice to visualize muscle cell fusion in the endogenous environment.

Additionally, we noticed when examining the normal myogenic regeneration exhibited in wild type controls from the β-catenin loss and gain of function experiments that the expression of MyHCslow appears to be upregulated after injury. This phenomena, although mentioned in a few reports, has not been specifically characterized and may represent an interesting similarity to embryonic muscle development as all muscles initially express MyHCslow.

This thesis has definitively shown that satellite cells and connective tissue fibroblasts are required for muscle regeneration and that Wnt/β-catenin signaling is not required but must be turned off for precise muscle regeneration. However, it has raised many questions as well, and I expect that future efforts will greatly expand our understanding of the cell-cell interactions occurring during muscle regeneration.

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APPENDIX

REVIEW OF WNT SIGNALING IN MUSCLE REGENERATION

A whole host of studies have examined the expression of Wnts and downstream components of both the β-catenin dependent and independent Wnt pathways during muscle regeneration. Secreted Frizzled-related proteins (sFRPs) modulate Wnt signaling by binding to Wnt ligands to either prevent binding to receptors or expand the range of signal by preventing interactions with the extracellular matrix, reviewed in (Mii and Taira, 2011). Both C2C12 cells and primary myogenic cells in culture express sFRPs (Polesskaya et al., 2003; Tanaka et al., 2011). Additionally, in whole muscle isolates, transcription of sFRPs increases following injury (Le Grand et al., 2009; Polesskaya et al., 2003). The expression of sFRPs suggests that Wnt signaling may be active and require modulation for appropriate regeneration.

Myogenic cells express the receptors and intracellular components required to transduce Wnt signaling. Lrp5 and 6 are up-regulated in C2C12 cells following the switch to differentiation media, and the Fzd receptors 2 and 8 peak in expression early at day 2 in differentiation media while Fzd 1, 4, and 5 peak 2 days later (Tanaka et al., 2011). Fzd7 is localized to satellite cells on isolated myofibers and this expression increases after 2 days in culture (Le Grand et al., 2009). FACs isolated myogenic cells from myofiber explants have increased expression of Fzd1 and 2 after 4 days in culture

(Brack et al., 2008). Whole muscle shows increased expression of Fzds three days after injury as well as during hypertrophy (Armstrong and Esser, 2005; Le Grand et al., 2009) Dsh protein is also up-regulated after exercise and hypertrophy (Armstrong and Esser, 2005; Aschenbach et al., 2006). Therefore, myogenic cells express the receptors necessary for reception of Wnt signals.

Expression of multiple Wnt ligands has been demonstrated both *in vitro* and *in vivo*. Both C2C12 cells and primary myoblasts have been shown to express the Wnt ligands Wnt4, 5b, 9a, 10b, and 11, as well as R-spondin (Rspn) 2 and 3. Rspn proteins have also been shown to bind to Fzd/LRP receptors and activate β-catenin signaling. However, C2C12s also express Wnt10a, 2b, and 5 whereas primary myogenic cells express Wnt1, 3a, 5a, 7a, and Rspo1 (Abiola et al., 2009; Bernardi et al., 2011; Brack et al., 2008; Han et al., 2011; Otto et al., 2008; Polesskaya et al., 2003; Tanaka et al., 2011). Additionally, whole muscle has been analyzed for expression of Wnt ligands, and in addition to all of the Wnts found in *in vitro* myogenic cells (except Wnt3a) Wnt2, 7b, 8b, 10a, and 16a were also expressed (Abiola et al., 2009; Le Grand et al., 2009; Polesskaya et al., 2003). The expression of these proteins not found in purely myogenic cells probably reflects expression of Wnts by other cell types residing in muscle such as connective tissue fibroblasts and macrophages. These results show that adult myogenic cells have the capacity for autocrine Wnt signaling because they express the receptors and downstream components necessary to respond to Wnt signal as well as various Wnt ligands. This does not preclude the possibility that other cell types send paracrine Wnt signals to myogenic cells. These other cell types may even be modulating the signals by expressing Wnt ligands that are not expressed by the myogenic cells.

Expression analysis of the downstream components that undergo phosphorylation, a change in intracellular localization, or the expression of known transcriptional targets suggests that the Wnt/β-catenin signaling pathway is active during regeneration. Phosphorylated GSK3β, which indicates it is no longer participating in the degradation of β-catenin, is increased in muscle after exercise (Aschenbach et al., 2006). Many studies have examined the phosphorylation state as well as the intracellular localization of β-catenin protein because it is the central mediator of the Wnt/ β-catenin signaling pathway. β-catenin localization in C2 cells is dynamic, localizing to the nucleus in proliferation media, to the adherens junctions in differentiating myocytes, and diffusely within the cytoplasm in myotubes (Goichberg et al., 2001). Immunofluorescence for β-catenin in satellite cells residing on isolated myofibers show nuclear localization after 24hrs in culture and co-localize with BrdU. Therefore, there is active Wnt/β-catenin signaling during the proliferative phase of satellite cells. Nuclear localization decreases as these cells differentiate and up-regulate Myogenin (Otto et al., 2008). The nuclear protein fraction isolated from hypertrophied muscle shows an increase in β-catenin and muscle that has been strenuously exercised shows an increase in activated, de-phosphorylated β-catenin protein (Armstrong and Esser, 2005; Aschenbach et al., 2006). After injury there is an increase in total β-catenin protein levels in whole muscle (Kim et al., 2006; Polesskaya et al., 2003). Immunofluorescence on sections of regenerating muscle from both mouse and humans show dephosphorylated β-catenin positive nuclei under the basement membrane, and in humans this co-localizes with the proliferation mark Ki-67 (Otto et al., 2008). Co-localization of nuclear β-catenin with proliferation markers

suggests that active Wnt/β-catenin signaling is concurrant with proliferation in progenitor cells.

Several studies have looked at the expression or up-regulation of known Wnt signaling target genes to indicate active signaling. In C2C12 cells there is evidence for both up-regulation and down-regulation of Wnt target genes when these cells are induced to differentiate by switching to low serum media. Wnt/β-catenin targets c-Myc, CyclinD1, and Fosl1 show decreased expression, whereas Axin2 and Tcf4 are up-regulated (Bernardi et al., 2011; Tanaka et al., 2011). FACs isolated cells from myofiber explants cultured for 4 days up-regulate Axin2 (Brack et al., 2008). Hypertrophied muscle has increased expression of c-Myc, CyclinD1, Pitx2, and Lef-1, and Axin2 is up-regulated following injury or exercise (Armstrong and Esser, 2005; Aschenbach et al., 2006; Brack et al., 2008). Immunofluorescence of hypertrophied muscle shows nuclear c-Myc expression in cells under the basement membrane and within the interstitial space (Armstrong and Esser, 2005). These results show that Wnt/β-catenin signaling is active with the muscle tissue during regeneration.

In addition to Wnt/β-catenin targets, β-catenin independent targets are also up-regulated in myogenic cells. The noncanonical transcriptional targets Tle-2 and CyclinD3 are up regulated in C2C12 cells after switching to differentiation media (Tanaka et al., 2011). On freshly isolated myofibers Vangl2, an effector of the PCP pathway, is not expressed by satellite cells. However after 2 days in culture, Vangl2 can be detected by immunofluorescence in the Pax7+ progenitor cells. Other than the correlation of proliferation marks with β-catenin in satellite cells, the expression of Wnt pathway components does not provide insight into the actual function of Wnt signaling in

myogenic cells. It does however show that the Wnt/β-catenin pathway is active during muscle regeneration as well as the β-catenin independent Wnt pathway.

Wnt/β-catenin signaling could have different transcriptional outputs because of different available binding sites and/or binding partners, and therefore function differently during the different stages of myogenic differentiation. Evidence for this comes from work by (Kim et al., 2008) showing that MyoD specifically binds to β-catenin to promote transcription of target genes at the myotube stage in C2C12 cells while in the myoblast stage MyoD binds to E2a proteins, such as E47, to bind to Ebox elements on transcriptional targets. Interestingly, it has been shown that on muscle fibers isolated from MyoD null mice there is a delay of activation of the progenitor cells residing on the fiber, but proliferation eventually reaches the level of the wild type fiber-associated cells. This correlates to the delay and subsequent restoration of nuclear localization of β-catenin in these cells (Macharia et al., 2010). While β-catenin may bind to TCF/LEFs in satellite cells to promote proliferation it appears that in more differentiated cells it might interact with MyoD to promote differentiation.

The effort to understand the function of Wnt signaling in muscle regeneration has focused mostly on manipulating Wnt signaling either by overexpression or repression of various components of the pathway in cell culture systems or non-cell type specific techniques *in vivo*. Multiple studies have investigated the role of specific Wnt ligands on muscle regeneration. However, many of these studies have shown conflicting data. Wnt4 has been shown to activate the TOPflash β-catenin signaling reporter assay in primary myogenic cells, however another study saw that not only did Wnt4 not activate the TOPflash reporter in C2C12 cells, but when Wnt4 was expressed with Wnt3a it could

repress the reporter activity seen with Wnt3a alone (Bernardi et al., 2011; Tanaka et al., 2011). It is not clear whether Wnt4 is acting through the β-catenin dependent pathway during regeneration; however, both studies show that Wnt4 promotes differentiation in cultured myogenic cells. Additionally, when isolated myofibers are co-cultured with cells secreting Wnt4 there is a decrease in proliferation of fiber associated progenitor cells (Otto et al., 2008). The function of Wnt3a has also been investigated in muscle regeneration, presumably because it is the prototypical β-catenin dependent Wnt ligand. Despite the fact that C2C12 cells express Wnt3a, it has not been detected *in vivo* during muscle regeneration. When isolated myofibers are cultured with Wnt3a expressing cells the fiber associated progenitor cells display increased proliferation (Otto et al., 2008). When C2C12 cells are treated with adenoviral Wnt3a they have decreased expression of differentiated muscle proteins such as troponin (Tanaka et al., 2011). However, when recombinant Wnt3a is added to isolated myofibers one group saw an increase in Desmin staining 2 days after treatment, suggesting that Wnt3a is promoting differentiation (Brack et al., 2008). Additionally, when Wnt3a is either injected or electroporated into regenerating muscle it promotes premature differentiation as seen by an early increase in regenerated fiber size but slightly later the fiber size is decreased compared to controls. This premature differentiation ultimately results in a decrease in final fiber caliber, an increase in fiber number, and more extracellular matrix (Brack et al., 2008; Brack et al., 2007; Le Grand et al., 2009). Wnt10b appears to be involved in preventing differentiation because primary muscle cells from Wnt10b-/- mice fuse rapidly and after injury of Wnt10b-/- mice, mononuclear interstitial cells show an increase in Myogenin expression (Vertino et al., 2005). Don't know what to say about (Abiola et al., 2009). Although Wnt

7a can act through the β-catenin dependent pathway in other systems, it appears to act independently of β-catenin in muscle regeneration. Wnt7a promotes self-renewal of satellite cells through Vangl2 and the PCP pathway. Wnt7a-/- mice have 18% fewer satellite cells before injury and after injury they have 36% fewer satellite cells without any obvious myogenesis defects (Le Grand et al., 2009). It also appears that Wnt7a is acting through PI(3)K/TOR pathway to promote hypertrophy of myofibers (von Maltzahn et al., 2012). Treating primary myogenic cells with Rspn2 results in an increase in Myf5 transcript levels (Han et al., 2011). Manipulation of Wnt ligands appears to have an effect upon regeneration; however, there does not seem to be a consensus as to whether some Wnts are acting through β-catenin as well as what the ultimate function of these proteins is. This may be due to the fact that many Wnts appear to be expressed in regenerating muscle and may act together to modulate the signal. It has been shown that β-catenin dependent and β-catenin independent pathway Wnts can modulate each other's ability to bind receptors in other systems (Bryja et al., 2009; Topol et al., 2003). Therefore, in these experiments, increasing one Wnt ligand could activate downstream targets of that Wnt but also repress other Wnt signals.

sFRPs are secreted Fzd related proteins that can bind to Wnt ligands and either block their binding to receptors or increase the diffusion of the ligand (Mii and Taira, 2011). Continuously adding sFRPs to C2C12 cells or primary myogenic cells cultured in differentiation media for 8 days results in decreased cell fusion, smaller myotubes, and fewer myotubes as well as a decrease in MRF expression. This effect is sensitive to timing because if sFRPs are given Day1-3, Day1-5, or Day3-8 there was no effect (Descamps et al., 2008). When sFRPs were injected directly into the muscle at 2 days

post injury there was no effect; however, when injected at 3.5 days postinjury, sFRPs induce decreased fiber size and number (Brack et al., 2008). Therefore, there is a narrow time frame during which sFRPs can prevent differentiation.

β-catenin dependent Wnt signaling focuses on the destruction or accumulation of β-catenin, therefore by experimentally manipulating the amount of β-catenin the pathway can be activated or repressed. There are chemicals that have been found to prevent the degradation of β-catenin such as LiCl or BIO. When these are added to C2C12 cells or primary myogenic cells in culture there is an increase in myotube area, but when LiCl is added to C2 cells there is a decrease in myogenin expression (Bernardi et al., 2011; Gavard et al., 2004). When adenoviral β-catenin is injected into a model of ischemic muscle injury total proliferation is increased; however, this was not a myogenic cell specific effect (Kim et al., 2006). Small molecule inhibitors of β-catenin added to C2C12 cells promote differentiation, shown as an increase in troponin expression in myotubes (Tanaka, Terada et al. 2011). When EGCG, a chemical that induces β-catenin breakdown, is added to cultured isolated myofibers the fiber-associated progenitors proliferate less (Otto et al., 2008). Knockdown of expression of β-catenin by siRNA in C2 cells results in an increase in myogenin expression; however, when β-catenin siRNA is added to C2C12 cells there is impaired regeneration, although how that was determined was not explicitly shown (Gavard et al., 2004; Kim et al., 2008).

There are well-characterized genetic tools available in mouse to genetically manipulate β-catenin *in vivo*. The β-catenin loss of function allele *β-catenin*$^{fl2\text{-}6}$ has LoxP sites flanking exons 2-6 in the β-catenin locus, creating a functional null following Cre-mediated recombination (Brault et al., 2001). When adenoviral Cre is injected into the

muscle of *β-catenin*$^{fl2\text{-}6/fl2\text{-}6}$ mice undergoing hypertrophy the Cre infected fibers are smaller than adjacent uninfected fibers (Armstrong et al., 2006). BCL9 and BCL9-2 are β-catenin co-activators, and when the conditional null alleles *BCL9*$^{fl/fl}$ and *BCL9-2*$^{fl/fl}$ are crossed to the *Myf5*^{cre} driver BCL9/9-2 are deleted in the myogenic lineage and in ~95% of the adult satellite cells. When *Myf5*^{cre}*; BCL9*$^{fl/fl}$; *BCL9-2*$^{fl/fl}$ mice are injured there are fewer and smaller caliber regenerated myofibers at 4 and 6dpi and this effect was not due to proliferation of satellite cells (Brack et al., 2009). Interestingly, it was shown that treatment of C2C12 cells with a dominant negative version a TCF/LEF, in which the β-catenin binding site is mutated preventing β-catenin from activating TCF/LEF target gene expression, had no effect on C2C12 cells (Kim et al., 2008). The evidence suggests that β-catenin is important for myogenesis and the lack of phenotype when TCF/LEFs no longer bind β-catenin may reflect an overriding function of β-catenin as a co-activator for MyoD in myoblasts. Alternatively, because β-catenin binds to the cytoplasmic tail of cadherin as well as a-catenin to link cadherin to the actin cytoskeleton, it is an important member of the adherens junction complex. It has been shown that there is convergence of the Wnt/β-catenin signaling pathway and the adherens junction complex through β-catenin, reviewed in (Nelson and Nusse, 2004). It is possible that the Wnt signaling pathway may be utilized to regulate the level of β-catenin not just to activate transcriptional targets but to regulate the availability of β-catenin for adherens junctions. Myogenin expression and differentiation have been shown to be downstream of adherens junctions in C2 cells (Goichberg et al., 2001). M-cadherin is required for myoblast fusion (Charrasse et al., 2006). When p120, another catenin that binds to cadherin at the membrane to maintain the structural integrity of the junction complex, is knocked down

by siRNA in C2C12 cells they have decreased differentiation (Gavard et al., 2004). However when β-catenin is mutated at the α-catenin binding site there is no effect on differentiation (Kim et al., 2008). The Calpain3 protein regulates the amount of M-cadherin and β-catenin localized to the membrane by degrading β-catenin. In primary culture of myogenic cells isolated from mice null for Calpain3 there is an increase in M-cadherin and β-catenin localized to the membrane of myocytes. This results in an increase in fusion but a subsequent block in differentiation (Kramerova et al., 2006). Ozz is a muscle specific E3 ubiquitin ligase that acts on β-catenin to promote proteasomal degradation. Mice null for Ozz show an increase in β-catenin localization to the membrane of myofibers as well as a disruption of myofibril organization and increased splitting (Nastasi et al., 2004). Mutations in both Calpain3 and Ozz have been shown to cause myopathies in humans. In addition to myogenic proliferation and differentiation as well as muscle structure and function, β-catenin expression in muscle has been shown to be involved in setting up appropriate neuromuscular junctions (NMJ) in the diaphragm. One group interested in NMJ formation took advantage of a Cre-responsive activating allele of β-catenin. This allele of β-catenin, $\beta\text{-}catenin^{fl3}$, deletes the phosphorylation site necessary for proteasomal degradation of β-catenin after Cre-mediated recombination (Harada et al., 1999). When the $\beta\text{-}catenin^{fl3}$ allele is crossed to the *MyoCre* (a transgenic line in which Cre is driven by the myogenin promoter and MEF2c enhancer) β-catenin is not degraded in the myofibers. The authors saw that there is aberrant localization and defassiculation of the Phrenic nerve and the diaphragm myofibers have smaller diameters, but the sarcomere structure is normal and there is no sign of degradation or regeneration (Liu et al., 2012). When β-catenin is deleted in the myofibers by crossing

the *β-catenin*$^{fl2\text{-}6}$ allele to the transgenic *HSACre* line, which expresses Cre in the mature myofibers, there are disrupted NMJ in the diaphragm. However, there is no affect on the muscle structure, size, or organization (Li et al., 2008). These results show that muscle is sensitive to over-accumulation of β-catenin and therefore the regulation of β-catenin levels either dependently or independently of Wnt signaling is essential for myocyte fusion and muscle function.

Although there are a multitude of studies examining the role of Wnts and β-catenin in muscle regeneration very little consensus has been made concerning the ultimate function of these proteins during muscle regeneration. This is due to both biological and experimental factors. Muscle regeneration is a complicated process orchestrated by a multitude of cells and signals that must all cooperate to achieve fully functional, structurally appropriate myofibers. Using cell culture to simplify the experimental system to ask direct questions about the function of specific Wnt pathway components is a relevant and useful endeavor. However, there are caveats to these experiments that should be considered when interpreting the data. Transformed myogenic cell lines are a useful tool because they are easier to use, will proliferate indefinitely, and can be developmentally synced by switching from proliferation media to differentiation media. These properties are necessary for experiments requiring large amounts of protein for biochemistry or reporter assays, but transformation has induced genetic changes. Although the C2C12 cell line was originally isolated from mouse satellite cells, they express CollagenVI, which is not expressed by primary myogenic cells (Zou et al., 2008). Therefore, care must be taken when interpreting results from this cell line because their expression profile and behavior may not reflect a purely myogenic character. Primary

myogenic cells have not undergone the genetic changes associated with transformation, but just by taking these cells out of their normal environment and putting them into a dish their behavior is affected. The tension of the substrate primary myoblasts as well as C2C12 cells are grown on in cell culture can affect adhesion, proliferation, and differentiation (Boontheekul et al., 2007). Even when experiments are conducted *in vivo* analysis of the effects of experimental manipulation can be complicated. Addition or knockdown of proteins in a non-cell type specific manner could have unknown consequences on cell types other that the myogenic population such as connective tissue fibroblasts or macrophages which both have been shown to be involved in directing muscle regeneration (Murphy et al., 2011; Tidball and Villalta, 2010). To directly determine the role of β-catenin in muscle regeneration I have specifically deleted and prevented degradation of β-catenin within the satellite cells and will discuss my results in chapter 4 of this thesis.

For muscle regeneration to happen *in vivo* multiple processes within the myogenic lineage must occur. The satellite cells must activate, proliferate, self-renew, and differentiate into myoblasts. These myoblasts must proliferate and differentiate into myocytes that then fuse to either themselves or damaged myofibers. Once the myofibers are formed they must grow to the correct size while organizing sarcomere structure, express the correct MyHC profile for the needs of the muscle, as well as communicate to the system that they have reached the necessary myonuclear number. Therefore, multiple factors are influencing the ultimate outcome of an appropriately sized myofibers. Many studies use myofiber size as a readout of functional regeneration. A smaller myofiber certainly reflects a defect in regeneration, but that alone does not elucidate which process

is affected: proliferation, differentiation, fusion, or hypertrophy or whether the defect occurred in the progenitor, myoblast, myocytes, or myofibers themselves.

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