CHAPTER ONE

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

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Contents

1. Introduction 2
2. Myogenesis Overview 2
3. Expression Analyses of Pax3/7 and MRF Transcription Factors 7
4. Functional Analysis of Pax3/7 and MRF Transcription Factors 11
5. Cre-Mediated Lineage and Ablation Analyses of PAX3, PAX7, and MRF+ Cells 17
6. Molecular Signals Distinguishing Between Different Phases of Myogenesis 22
7. Current Model of Myogenesis 24
Acknowledgments 26
References 27

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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Current Topics in Developmental Biology, Volume 96
ISSN 0070-2153, DOI: 10.1016/B978-0-12-385940-2.00001-2

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

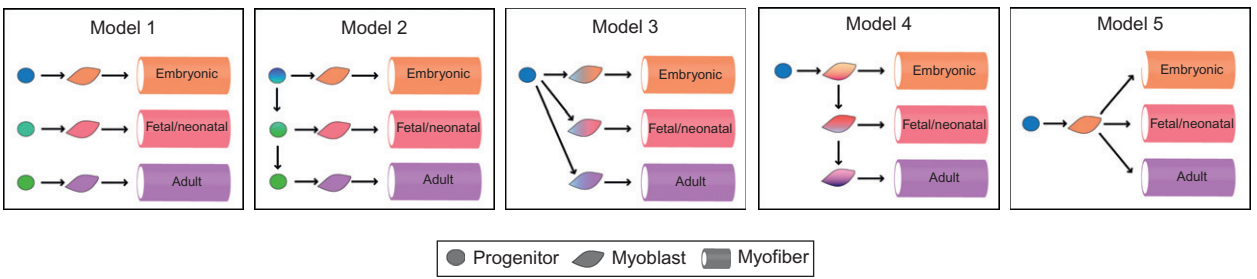

Figure 1.1 Five theoretical models describing derivation of embryonic, fetal/neonatal, and adult limb muscle in mouse.

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 *Myf4* (Kassar-Duchossoy *et al.*, 2004). Only the $Myf5^{lacP/lacP}$ 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 $Myf5^{lacP/lacP}$ mice. Similarly, while three *Myf4* null alleles were generated (Braun and Arnold, 1995; Patapoutian *et al.*, 1995; Zhang *et al.*, 1995), only one *Myf4* 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 *Pax7* (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

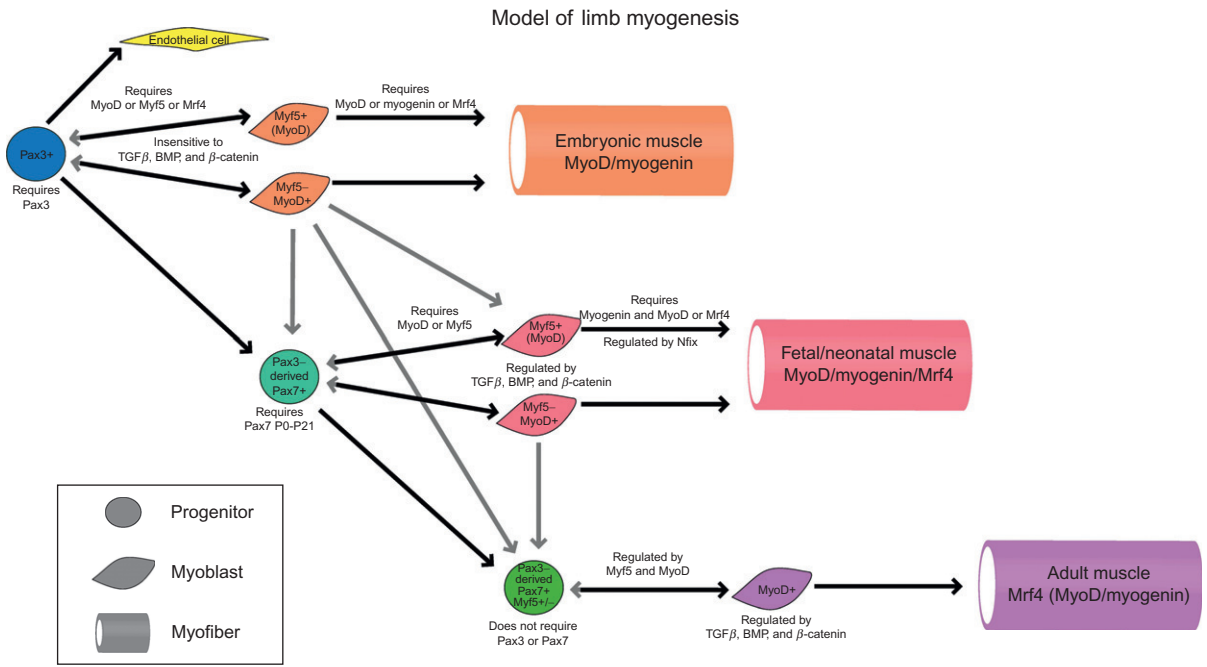

Figure 1.2 Summary of current model of embryonic, fetal/neonatal, and adult limb myogenesis in the mouse.

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.

ACKNOWLEDGMENTS

We thank D.D. Cornelison, D. Goldhamer, F. Relaix, and S. Tajbakhsh for discussion and review of the manuscript. We also thank members of the Kardon lab (particularly D.A. Hutcheson), S. Biressi, and G. Messina for many helpful discussions. Research in the Kardon lab is supported by the Pew Foundation, MDA, and NIH R01 HD053728.

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