

Expression and Putative Functions of Fibroblast Growth Factor 10 (Fgf10) in Developing and Adult Skeletal Muscles

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Abstract

Although the genetic regulation of vertebrate myogenesis during early development has been well studied, the intrinsic mechanisms controlling muscle fiber-type specification and maturation are much less understood. Fibroblast growth factors (*Fgfs*) are potent regulators of myoblast proliferation and differentiation *in vitro*. However, their *in vivo* functions in myogenesis have yet to be fully elucidated.

This study was prompted by the discovery of *Fgf10* expression in adult mouse skeletal muscles. Using an *Fgf10*-nLacZ reporter line, I set out to characterize this novel expression pattern in order to learn more about *Fgf10*'s putative function/s. I have shown that intriguingly *Fgf10* is only expressed in a subset of adult muscles, which are predominantly fast-twitch. The expression begins during late embryonic development, but is subsequently conserved and restricted to the same muscles throughout adult life. Moreover, within each positive muscle, only a subset of myofibers expresses *Fgf10*.

Immunolabeling with Pax7 antibodies showed that a subpopulation of *Fgf10*-expressing myonuclei expresses this satellite stem cell marker. To delineate the function of *Fgf10*, I examined the limbs of newborn *Pax3-Cre::Fgf10(flox/-)* double transgenic mice, and I discovered that myofiber growth was impaired in *Fgf10*-expressing muscles. Moreover, the distal myofiber organization was severely disorganized in certain muscles, indicating defective attachment to the bone. This suggested that *Fgf10* might also be regulating tendon development.

Collectively, these studies suggest that *Fgf10* plays diverse roles in the developing musculoskeletal system, including the formation and maturation of fast fibers, and also in tendon development. *Fgf10* may also play a role in muscle regeneration in response to injury, however this

remains to be tested in physiological challenge and regeneration paradigms *in vivo*.

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Chapter 1

Introduction

1.1 Overview of Vertebrate Myogenesis

Myogenesis is the process of skeletal muscle formation during embryonic and adult development. Structurally, skeletal muscle is a highly organized tissue with dynamic plasticity properties, which render it capable of adapting to different functional requirements and states of injury. Its remarkable ability to regenerate after injury is due to a small population of satellite cells (muscle stem cells), which are programmed during embryogenesis and persist into adulthood. Under normal conditions, they remain in a quiescent state until they are activated upon injury. Following this event, they undergo myogenesis in a manner similar to embryonic myogenesis, rapidly proliferating into myoblasts before committing to differentiation and fusing into myotubes. Critically, satellite cell function has been shown to decline with age, which can lead to impaired muscle regeneration during later life. Together, a greater understanding of the molecular basis for myogenesis and the mechanisms regulating muscle repair, will lead to significant advances in therapeutic treatments for diseases afflicting skeletal muscle, including atrophy, myopathies and muscular dystrophies.

1.1.1 Somitogenesis

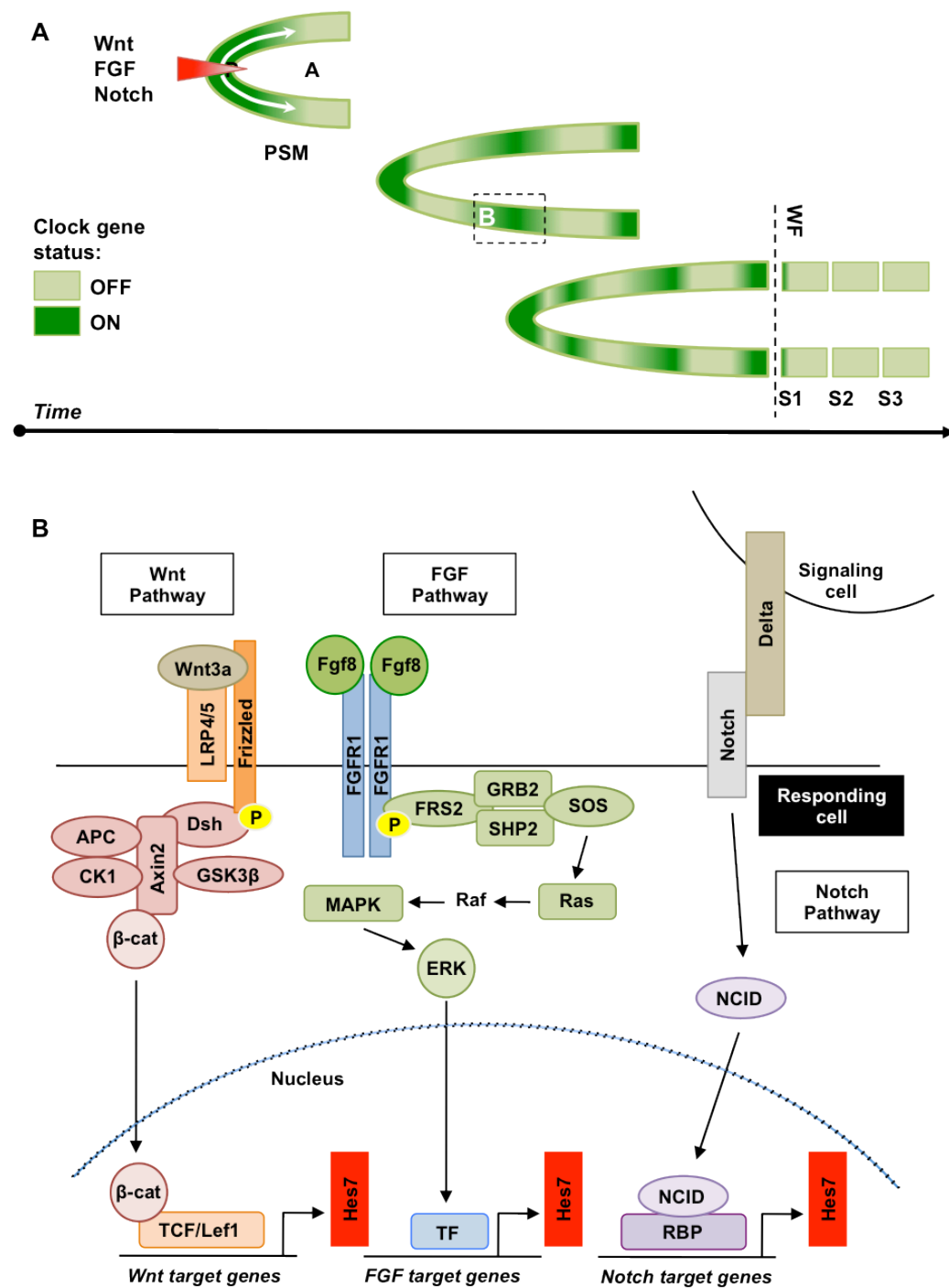
In all vertebrate species, the segmentation process during embryogenesis is central to the body plan as it confers movement and rigidity to the body. Segmentation is achieved through somitogenesis, which generates somites, and these will eventually give rise to the vertebrae and bones, skeletal muscle, and also some of the dermis (Dequeant and Pourquie, 2008). During this process, the unsegmented presomitic (or paraxial) mesoderm (PSM) segments into symmetrical pairs of epithelial blocks (the somites) along the anterior-to-posterior axis of the embryo (Maroto et al., 2012)

During embryogenesis, the site of gastrulation (or the blastopore) is one of the first morphological landmarks to develop. Also called the primitive streak in some vertebrates such as in chick, it forms early on in development at the midline of the embryo. Gastrulation itself is the migration of cells towards the blastopore or primitive streak, their subsequent infiltration through the structure, and the ultimate generation of the three germ layers: endoderm, mesoderm and ectoderm, which collectively give rise to all embryonic tissues (Maroto et al., 2012). In chick and mouse, a population of progenitor cells for the presomitic mesoderm is specified within the primitive streak. These divide rapidly to give rise to cells of the posterior PSM and express the PSM-specific marker *Tbx6* (T-box 6) (Cambray and Wilson, 2007; Dequeant and Pourquie, 2008; McGrew et al., 2008).

A key inherent property of PSM is the ability of individual cells to switch gene expression 'on' and 'off' in a synchronized manner, producing a wave-like effect of gene expression from the posterior to the anterior end of the PSM. Somites bud off at the anterior end of two parallel strips of PSM, which lie either side of the notochord. At regular intervals (e.g. every 2 hours in mouse), cells at the anterior end of PSM undergo changes in their adhesive and migratory properties, eventually merging together to form an epithelial unit of cells known as the 'somite'. Somites are formed in an anterior-to-posterior sequence. As the body axis lengthens, budding of cells from the anterior region of the PSM is compensated for by the addition of cells at the posterior end (Stickney et al., 2000; Stockdale et al., 2000). A variety of genes are expressed in the PSM in cycles corresponding to the time taken for a somite to form. This dynamic timing of gene expression is referred to as the 'segmentation clock' (Figure. 1.1A). The 'clock' genes include the bHLH transcription factors *Hairy1* and *Hairy2* in chick, and *hairy* and *enhancer of split 1* (*Hes1*) and *Hes7* in mouse. *Fgf8* is also expressed in a cyclical manner,

such that its transcription levels are highest at the posterior end of the PSM, generating an FGF8 gradient, which decreases in the anterior direction. Cells are arranged in a loose mesenchymal structure where the FGF8 gradient is highest in the posterior PSM. In contrast, cells in the anterior PSM, where FGF8 signalling is less active, are more compact and begin to undergo epithelialization (Dubrulle et al., 2001). The border separating these two regions of FGF8 signaling is known as the 'determination' or 'wave' front. Reduced FGF8 signaling at the wavefront is thought to be sufficient to enable cells in the PSM to proceed with segmentation. In the 'clock and wavefront' model, originally proposed by Cooke and Zeeman (1976), the presence of a longitudinal positional information gradient along the anterior-to-posterior (A-P) axis interacts with the segmentation clock to establish a set time when a cell will undergo a change of state (e.g. change in movement or adhesiveness) (Cooke and Zeeman, 1976). In this way, it is highly likely that the longitudinal FGF8 gradient (wavefront) integrates with the segmentation clock to initiate the change of state required for segmentation (Baker et al., 2006). Components of the Notch and Wnt signaling pathways have also been shown to cycle through the PSM in mouse with a similar rhythm to somite formation (Figure 1.1B) (Aulehla et al., 2003; Dequeant et al., 2006; Ishikawa et al., 2004; Niwa et al., 2007; Pourquie, 2011). However, the degree of crosstalk between these pathways, and indeed the mechanisms by which the timings of the molecular oscillations are maintained, remain unclear (Maroto et al., 2012; Palmeirim et al., 1997; Pourquie, 2011).

Figure 1.1: Regulation of somitogenesis by the ‘segmentation clock’. (A) The pre-somitic mesoderm (PSM) contains cells which inherently switch gene expression ON and OFF to produce somites. This produces synchronized oscillations of gene expression in the posterior (P) to anterior (A) direction. Wnt, FGF and Notch signaling components appear to cycle through the PSM at the same rate as the oscillations, however the timing of these is still unclear. As the oscillations approach the wave front (WF) they slow down, causing the somites (S1-S3) to form in pairs from the anterior PSM. (B) Wnt, FGF and Notch signaling components activate the expression of the segmentation-specific genes *Hes7* (hairy/enhancer of split related gene) in the PSM. These components are also expressed in a cyclical fashion corresponding to the timing of somite formation. There is a degree of cross-talk between the pathways but this is not completely understood (adapted from Maroto. M., 2012).



1.1.2 Morphogenetic patterning of somites

The somite matures and differentiates as soon as it is generated. A newly-formed somite is composed of two cell populations, with different morphological traits and developmental fates. These cells are arranged in a ball-like structure (Figure 1.2). The cells in the outer structure are arranged in a single columnar epithelial layer. This layer encases a cavity containing a population of loose mesenchymal cells, called the somitocoele cells. As the somite matures, the density of the core somitocoele cells increases, whereas the density of the cells in the epithelial wall is unaltered (Mittapalli et al., 2005). It has been found that some cells in the epithelial layer undergo an epithelial-to-mesenchymal transition to contribute to the somitocoele, however a reciprocal transition is yet to be observed (Wong et al., 1993).

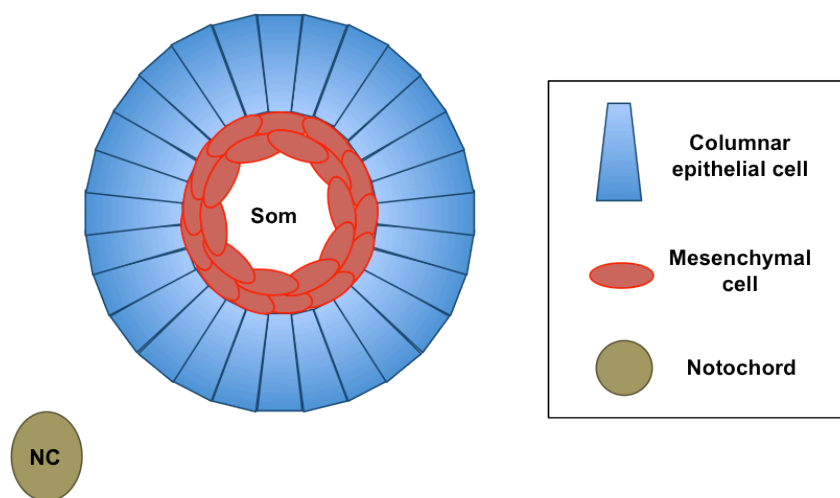


Figure 1.2: Structure of newly-formed somite. Columnar epithelial cells form a spherical structure, encasing a cavity with the somitocoele (mesenchymal) cell population. Somitocoele cells increase in number during somite maturation. The ventral region will later de-epithelialize.

Somite maturation is subsequently characterized by signals from surrounding tissues which regulate the specification and differentiation of somites into its three main derivatives: the sclerotome, dermatome and myotome, Figure 1.3 (Maroto et al., 2012). The ventral region of the somite de-epithelializes to form the sclerotome, controlled by regulatory signals from the notochord such as SHH and Noggin. In this way, somitocoele cells retain their mesenchymal properties throughout somite maturation up to sclerotome development (Mittapalli et al., 2005). This somitic compartment harbours skeletal and cartilage progenitors which go on to form the vertebrae and ribs (Huang et al., 2000). The most dorsal somitic region remains epithelial and differentiates into the dermamyotome, which contains muscle progenitor cells (MPCs) and will also later give rise to dermis. Skeletal muscles of the trunk and limbs derive from this somitic compartment (Bentzinger et al., 2012). MPCs located in the dorso-medial and ventro-lateral lips (DML and VLL) of the dermamyotome migrate ventrally to form the earliest muscle mass, the myotome (Deries et al., 2010). The central portion of the dermomyotome subsequently intercalates into the myotome. Epaxial regions of the dermomyotome and myotome give rise to dorsal muscles, whereas lateral trunk and limb muscles are derived from hypaxial regions. At the limb bud level, the ventral portion of the dermomyotome undergoes an epithelio-mesenchymal (EMT) transition which enables myoblasts to migrate into the limb bud mesenchyme, whilst retaining their ability to divide so that they can proliferate upon arrival at their destination within the limb (Venters et al., 2004). Ventral elongation of the dermomyotome and myotome gives rise to body wall muscles. Muscles located within extremities, in addition to the diaphragm, are formed from MPCs which have migrated some distance after de-laminating from the DML and VLL of the dermomyotome at limb level (Bentzinger et al., 2012; Cinnamon et al., 1999; Parker et al., 2003; Vasyutina and Birchmeier, 2006). Most

head muscles (jaw, eye and facial) are derived from the pharyngeal and prechordal head mesoderm; the genetic regulation of these muscle groups differs to those of the trunk and limbs (Mootoosamy and Dietrich, 2002; Shih et al., 2008). Epaxial and hypaxial muscles of the neck, the pharyngeal and laryngeal muscles, and tongue musculature, however, are derived from the occipital 'somites' (Mootoosamy and Dietrich, 2002). These are the most cranial somites and were secondarily incorporated into the head during evolution (Gans and Northcutt, 1983). Following the identification of the bHLH transcription factor *Scleraxis* (*Scx*), a marker for both tendon progenitors and mature tendons, a fourth somitic domain was identified and named the 'syndetome.' FGF signaling from the myotome induces *Scx* expression in cells located in the dorsal sclerotome, and the interaction between the two domains generates the syndetome (Brent et al., 2003; Smith et al., 2005).

Myogenic commitment within the somite is highly dependent on extrinsic signals, see Figure 1.3. These include members of the *Wnt*, *hedgehog* (HH), Notch and bone morphogenetic protein (BMP) families. Wnt ligands bind cellular Frizzled (Fzd) receptors and exert their function through either canonical activation of β -catenin or through alternative non-canonical pathways (Komiya and Habas, 2008). Wnt1 and Wnt3, which emanate from the dorsal region of the neural tube, and Wnt4, Wnt6 and Wnt7a, which are secreted from surface ectoderm, are particularly important to patterning of the dermomyotome, the myotome, and subsequent stages of myogenesis (Parr et al., 1993). In mutant mice lacking *Wnt1* and *Wnt3* expression, the dermomyotome does not develop correctly and expression of *Pax3* and *Myf5* (myogenic transcription factors) is also markedly reduced (Ikeya and Takada, 1998). Variations in Frizzled receptor expression in different regions of the somite also leads to differential regulation of myogenic transcription factors. For example, Wnt7a binds Fzd7 in the hypaxial region of the somite to induce *MyoD*

expression via a non-canonical, β -catenin-independent pathway. Wnt1 has been shown to signal canonically via Fzd1/Fzd6 in the epaxial domain of the somite to regulate *Myf5* expression (Borello et al., 2006; Brunelli et al., 2007).

Sonic hedgehog (*Shh*), the prototypic member of the hedgehog (HH) family, also stimulates expression of myogenic genes. *Shh* is secreted by the developing floor plate and notochord, and binds the Patched receptor, which stimulates the release of *smoothed*. *Smoothed* stimulates downstream signaling pathways which prompt the nuclear translocation of GLI transcription factors to activate *Shh* target genes (Chen et al., 2013). In *Shh* knockout (*Shh*^{-/-}) mice, the sclerotome was smaller and *Myf5* expression levels in the dermomyotome were reduced (Chiang et al., 1996). In zebrafish, removal of *Shh* expression stimulates proliferation of muscle progenitor cells. However, they are unable to fully differentiate into myofibers (Hammond et al., 2007). Conversely ectopic *Shh* expression in chick inhibits *Pax3* expression in the dermomyotome (Johnson et al., 1994). These findings indicate that a finely-regulated balance in *Shh* signaling is required to ensure MPCs can differentiate fully into muscle.

Notch signaling has been shown to inhibit myogenic differentiation (Buas and Kadesch, 2010). Notch receptors recognise the ligands Delta and Jagged, presented to them by neighbouring cells. Upon receptor activation, the intracellular domain of the Notch receptor (NCID) is cleaved by the metalloproteinase TACE and γ -secretases which enables its translocation to the nucleus, wherein it binds the transcription factor *RBP-J* to activate Notch target genes. In *RBP-J* conditional mutant mice, a wide domain of MPCs differentiated in an uncontrolled manner, as observed by expression of the myogenic markers *MyoD* and *myogenin*. This indicates that Notch signaling prevents premature differentiation of MPCs and maintains a progenitor population (Vasyutina et al., 2007).

Similarly to Notch, BMPs in the lateral plate also inhibit myogenic differentiation (Bentzinger et al., 2012). Part of the transforming growth factor-beta (TGF- β) superfamily, BMPs can bind one of two receptors, BMPR1 and BMPR2, or a combination of both, to activate different downstream targets. Binding to BMPR1 facilitates phosphorylation of Smad1, Smad5 or Smad8 (known as R-Smad.) A heterotrimeric complex of two R-Smads and a common Smad4 translocates to the nucleus to activate target genes in the canonical BMP pathway. An alternative pathway can also be induced via TGF- β -activated MAP kinases (Zhang and Li, 2005). In a manner similar to Notch, BMP has been found to delay expression of *Myf5* and *MyoD* in some populations of MPCs, thereby maintaining them in an undifferentiated state (Hirsinger et al., 1997). It has been suggested that there is a degree of 'cross-talk' between the Wnt and BMP signaling pathways, given that these proteins can sometimes share similar spatial or temporal expression patterns. However, because the cross-talk can have either synergistic or antagonistic effects depending on the biological context, its mechanism in myogenesis remains to be elucidated (Itasaki and Hoppler, 2010).

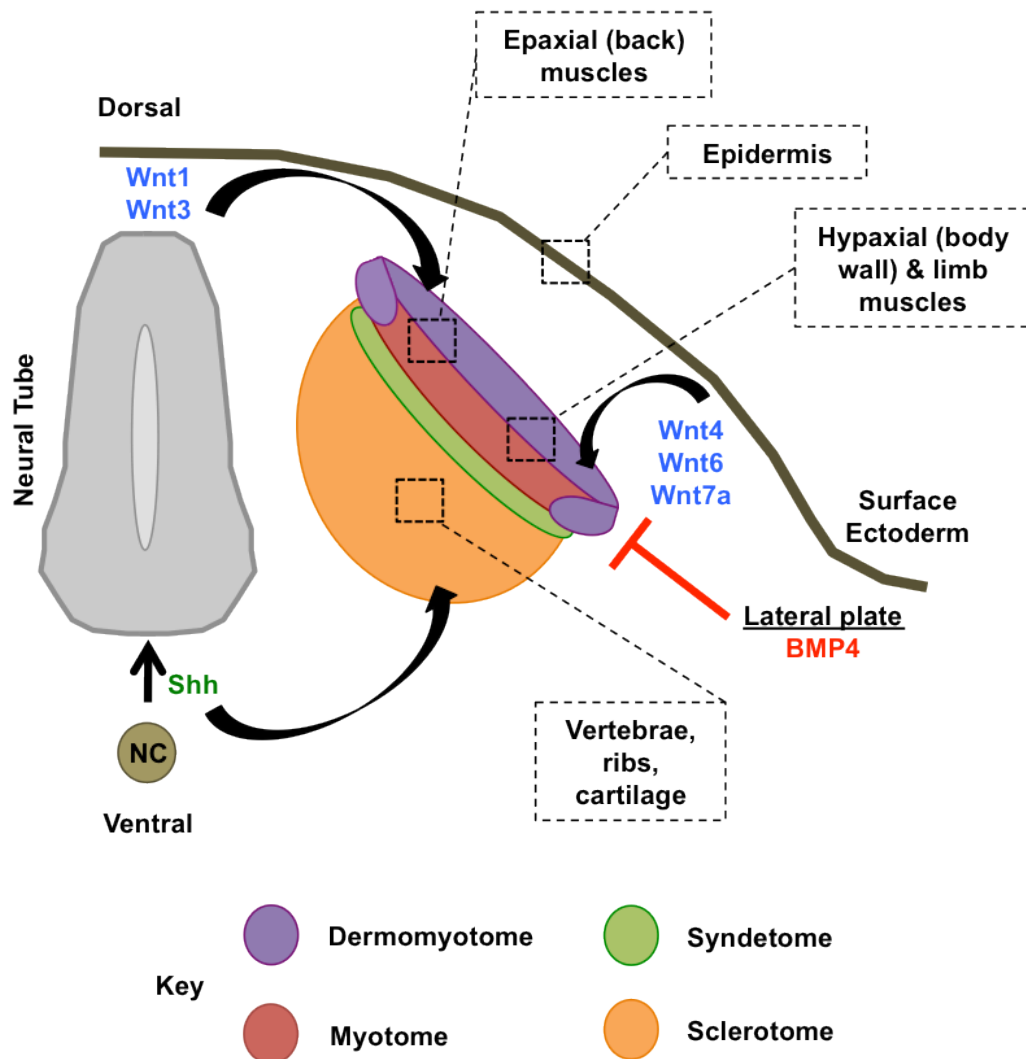
Transverse Section

Figure 1.3: Extrinsic patterning of the somite. Wnt signals from the dorsal neural tube (*Wnt1/Wnt3*) and surface ectoderm (*Wnt4/Wnt6/Wnt7a*) pattern the epaxial and hypaxial dermomyotome respectively. The epaxial region gives rise to muscles of the back, whereas muscles of the body wall and limbs originate in the hypaxial domain. The surface ectoderm will also eventually produce the dermis. Shh signals from the notochord (NC) and floor plate to pattern the ventral sclerotome, which harbours skeletal progenitors for the vertebrae, ribs and cartilage. BMP signals from the lateral plate mesoderm inhibit myogenic differentiation in the myotome and dermomyotome (adapted from Maroto. M., 2012).

1.1.3 Genetic regulatory networks in myogenesis

In addition to the extrinsic signals determining myogenic cell fate during somite patterning, a complex hierarchy of intrinsic interactions also regulate myogenesis.

1.1.3.1 Myogenic regulatory factors (MRFs)

Before they became known as the myogenic regulatory factors (MRFs), the basic helix-loop-helix (bHLH) genes *Myf5*, *MyoD*, *Myogenin* and *Mrf4* (also called *Myf6* or *herculin*) had demonstrated an ability to induce myogenic characteristics in other cell types such as fibroblasts (Braun et al., 1990; Davis et al., 1987; Edmondson and Olson, 1989; Miner and Wold, 1990; Sassoon et al., 1989). The collective term was coined after the factors were found to be highly conserved at the protein level, in addition to their high levels of expression in skeletal muscle (Rudnicki and Jaenisch, 1995). The MRFs have overlapping but distinct expression patterns, and activate muscle-specific gene transcription by binding E-box DNA sequences located within these genes (Rawls et al., 1998; Rescan, 2001). *Myf5* is the first MRF to be expressed in the developing mouse embryo, with a high level of expression in the somite at E8 which is significantly reduced by E14 (Ott et al., 1991). *Myogenin* is expressed soon after *Myf5* at E8.5, and is subsequently expressed throughout embryonic development. *MyoD* expression begins slightly later at E10.5 (Sassoon et al., 1989). *MRF4* has been shown to be transiently expressed between E10-11, it is then upregulated at E16 and continues to be expressed after birth (Bober et al., 1991). In the limb bud, the onset of MRF expression is slightly delayed. *Myf5* is first transiently upregulated between E10-12. Both *MyoD* and *myogenin* are expressed together after E11. *MRF4* expression remains unchanged and is active from E16 onwards (Bober et al., 1991; Ott et al., 1991; Sassoon et al., 1989). Interestingly, *Myf5* knockout (*Myf5*^{-/-}) and *MyoD* knockout (*MyoD*^{-/-}) mice

do not show a skeletal muscle phenotype, neither morphological nor physiological. However, compensatory mechanisms appear to come into play in both mutants. For example, *MyoD* mutants display a significant upregulation of *Myf5* (Rudnicki et al., 1992). In the *Myf5* mutants, the expression of other MRFs appeared delayed, which could render them capable of inducing the formation of normal skeletal muscle system later on. Additionally, the migratory capacity of MPCs is not compromised in *Myf5* mutant mice, which suggests that the determination and migration of limb MPCs is not solely dependent upon *Myf5* (Braun et al., 1994; Braun et al., 1992). Together, these observations suggested that *Myf5* and *MyoD* are capable of functionally substituting for one another. Indeed this seems evident, as in *Myf5/MyoD* double knockout (*Myf5*^{-/-}/*MyoD*^{-/-}) mice, skeletal muscle fails to develop completely (Rudnicki et al., 1993). These findings supported an early model for myogenesis which relies on two waves of myogenic differentiation, the first being *Myf5*-dependent, and the second *MyoD*-dependent. The molecular mechanism behind the functional redundancy of *Myf5* and *MyoD* during myotome formation has been explored using lineage-ablation experiments in mice. These experiments sought to distinguish between two proposed models of functional redundancy. The first relied on the use of distinct *Myf5*- or *MyoD*-dependent cell lineages, which could interchangeably compensate for the loss of the other. Alternatively, both *Myf5* and *MyoD* could act redundantly within the same progenitor cell population in order to give rise to the myotome. By conditionally ablating *Myf5*-expressing cells through expression of the diphtheria toxin, and combining this approach with lineage tracing, it was first revealed that *Myf5* is only expressed in a subset of developing myoblasts. Moreover, when *Myf5* expression was removed, myogenesis was rescued by a *Myf5*-independent cell population. Further still, an expansion of the *MyoD*-expressing cell population was observed at the same time. The presence of this *MyoD*-

expressing, *Myf5*-independent cell population appears to form the basis of the functional redundancy between the two genes (Gensch et al., 2008; Haldar et al., 2008). Similarly, a reduction in MYOD levels in terminally differentiated myotubes also leads to an increase in *Myf5* expression, most likely to compensate for the reduction in *MyoD* (Mastroiannopoulos et al., 2012).

In *MRF4* knockout (*MRF4*^{-/-}) mice, skeletal muscle develops normally but this is accompanied by a significant upregulation in myogenin, which likely compensates in its absence (Rawls et al., 1998). *Myogenin* expression is specific to muscle differentiation and in its absence, skeletal muscle is formed but it is significantly diminished, resulting in fewer myofibers (Hasty et al., 1993; Venuti et al., 1995). The function of *MRF4* was investigated by combining its inactivation with that of *myogenin* or *MyoD*. In *MRF4/myogenin* (*MRF4*^{-/-}/*myogenin*^{-/-}) double mutant mice, the numbers of residual myofibers which developed were comparable to those in *myogenin*^{-/-} mutants. However, in *MRF4/MyoD* (*MRF4*^{-/-}/*MyoD*^{-/-}) mutant mice, although myogenin is expressed, the muscles are severely compromised which indicates myogenin is unable to compensate in the absence of these genes *in vivo* (Rawls et al., 1998). Within this genetic hierarchy *Myf5* and *MRF4* act upstream of *MyoD* and *myogenin* to specify myoblasts, whereas the latter two genes regulate their differentiation.

1.1.3.2 *Pax3/Pax7*

The *Pax* genes are paired-box transcription factors known to regulate many developmental processes during embryogenesis (reviewed by Buckingham and Relaix., 2007). The closely-related factors *Pax3* and *Pax7* are well characterised in myogenesis. *Pax3* is expressed in the presomitic mesoderm, subsequently in the somite, and finally throughout the dermomyotome where it is particularly enriched in the DML and VLLs. In contrast to *Pax3*, *Pax7* expression is restricted to the central

dermomyotome (Sato et al., 2010). *Pax3* mutant mice (or *Spotch* mutants which have impaired *Pax3* function) display somitic defects and show ablation of both hypaxial and epaxial regions of the dermomyotome, resulting in a disorganized and underdeveloped myotome and also trunk muscle defects (Tremblay et al., 1998). In addition, limb muscles are also absent in *Spotch* mutants, indicating that *Pax3* expression is crucial to the migration of MPCs (Relaix et al., 2004). Due to the lack of limb muscles in these mice, a study was carried out on mice heterozygous for *Pax3* which display a milder mutant phenotype. The hypaxial somite is partially rescued in these animals, in addition to its derivatives which include some hindlimb muscles, and *Myf5* expression was also attenuated in these regions. Furthermore, a -145 base pair (bp) regulatory sequence was also identified 5' to the *Myf5* gene. This sequence can bind *Pax3* and as a result *Pax3* acts genetically upstream of *Myf5* (Bajard et al., 2006). Activation of *Myf5* in the epaxial domain relies on an enhancer situated at -5.5kb from the gene. As in the hypaxial domain, *Pax3* also activates *Myf5* via another dermomyotomal target: *Dmrt2*, which is able to bind the enhancer (Sato et al., 2010).

As previously mentioned, epithelial cells in the central region of the dermomyotome express *Pax7*, and they also co-express *Pax3* (Messina and Cossu, 2009). Although, unlike in *Pax3*^{-/-} mice, *Pax7* mutants (*Pax7*^{-/-}) do not display an embryonic muscle phenotype (Mansouri et al., 1996). In *Pax3/Pax7* double knockout mice, however, only the primary myotome is formed and subsequent myogenic development is halted (Relaix et al., 2005). This finding led to the suggestion that *Pax3/Pax7*-positive MPCs give rise to cells of the myogenic lineage. However, *Pax7* alone appears dispensable in embryonic myogenesis (Messina and Cossu, 2009). The function of *Pax7* in adult myogenesis has been widely explored and its essential role in the maintenance of satellite cells will be discussed later

(Lepper et al., 2011; Oustanina et al., 2004; Relaix et al., 2006; Sambasivan et al., 2011b; von Maltzahn et al., 2013).

1.1.3.3 *Six1/Six4*

First identified in *Drosophila*, *sine oculis*-related homeobox 1 (*Six1*) and *Six4* transcription factors are generally considered the pinnacle of the myogenic regulatory network. The *Six* genes act in synergy with *eyes absent* (*Eya*) genes to activate *Pax3* expression in the hypaxial region (VLL) of the dermomyotome, which gives rise to hypaxial MPCs. In *Six1/Six4* double knockout (*Six1*^{-/-}/*Six4*^{-/-}) mice, *Pax3* expression in the dermomyotome is abrogated and as a result, they present a severe muscle phenotype. Lack of *Pax3* expression in the double knockout mice results in reduced levels of myogenic regulatory factor (MRF) expression, which diminishes some ventral muscles and hypaxial muscles at trunk level. Similarly, although the proliferative capacity of MPCs within the dermomyotome is not affected in these mutants, they do not migrate correctly and as a result lose their myogenic identity, before undergoing apoptosis. Failure of the precursor cells to migrate into the limb bud leads to complete abrogation of limb muscles and some ventral muscles. *Six* and *Eya* genes are activated independently and interact with each other biochemically to induce *Pax3* expression in the dermomyotome (Grifone et al., 2007; Grifone et al., 2005).

1.1.3.4 *Summary of genetic regulation of trunk muscles*

Pax3 is expressed in skeletal MPCs which are located in the dermomyotome. Epaxial and hypaxial-derived muscles have different requirements for *Pax3*, as shown in *Pax3* ablation studies. *Pax7* is also expressed in the somite around the same time as *Pax3* but its function in initial myoblast specification is redundant. *Pax3* expression in the dermomyotome is regulated by *Six* and *Eya* genes, and *Six1* and *Six4* regulate hypaxial expression in particular. *Paraxis*, another bHLH

transcription factor, has also been shown to act upstream of *Pax3* in the epaxial somite. In *Paraxis* knockout (*Paraxis*^{-/-}) mice, although somites do not develop and therefore *Pax3* expression is absent, some underdeveloped skeletal muscle does form. Analysis of *Myf5/Paraxis* double knockout (*Myf5*^{-/-}/*Paraxis*^{-/-}) mice also revealed that *Paraxis* regulates *MyoD* in the epaxial somite (Burgess et al., 1995; Wilson-Rawls et al., 1999). *Myf5* is under the direct regulation of *Pax3*, and its expression is closely followed by that of *MRF4*. *MyoD* and *Myogenin* are genetically downstream of *Myf5* and *MRF4* (Figure 1.4).

1.1.3.5 Summary of genetic regulation of limb muscles

In the developing limb bud, *Pax3*-expressing MPCs begin migrating into the forelimb at E9.25, and have finished migrating into the hindlimb by E11. Migratory cells do not express *Pax7*, and they are guided into the limb bud by the production of scatter factor (SF). Migratory myoblasts express the SF receptor c-Met, and the presence of SF in the limb bud mesenchyme directs their movement (Birchmeier and Brohmann, 2000). In addition to *Pax3*, migrating myoblasts also express *Lbx1* and deleting this homeobox gene leads to a complete loss of all hindlimb and some forelimb muscles, highlighting the importance of this gene to migration (Birchmeier and Brohmann, 2000). *Meox2* is also expressed in the presomitic mesoderm and by migrating myoblasts. In its absence, a subset of limb muscles fails to develop. Interestingly, in *Meox2* mutant (*Meox2*^{-/-}) mice, *Pax3* and *Myf5* are downregulated but *MyoD* appears unaffected (Mankoo et al., 1999). Once migratory myoblasts reach their destination in the limb bud, they begin to express the MRFs. Here, *Myf5* is directly regulated by *Six1* and *Six4*, unlike in the dermomyotome (Giordani et al., 2007).

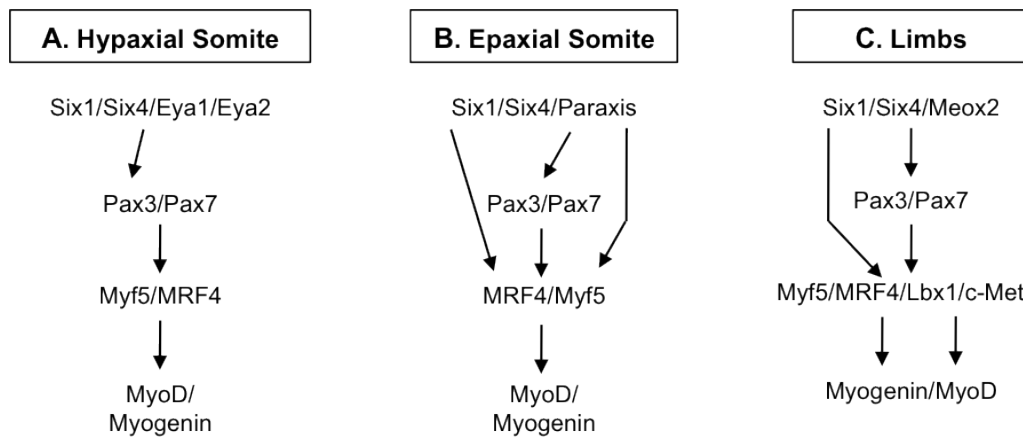


Figure 1.4: Genetic regulatory networks of myogenesis in the somites and limbs. (A) In the hypaxial region of the somite, *Six1* and *Six4* regulate *Pax3* expression in synergy with *Eya1* and *Eya2*. *Pax7* is present but its function is redundant in embryonic myogenesis. (B) In the epaxial somite, *Pax3* is under the control of *Paraxis*, which also regulates *Myf5*. The *Six* genes are still active but they directly induce MRF4 expression. (C) In the developing limb, *Meox2* also induces *Pax3* expression in addition to the *Six* genes (adapted from Bismuth. K., 2010).

1.1.4 Vertebrate myogenesis occurs in three temporally-distinct, but spatially-overlapping phases.

For many years, the developing mouse limb bud was used as a model for studying myogenesis. A key finding was that myogenesis takes place in two successive phases during embryogenesis, see Figure 1.5. These phases were identified by two distinct populations of myofibers which formed at two developmental time-points. The first set, termed ‘primary myofibers’, could be identified from E8.5. The second set, which were found to align with primary myofibers were slightly smaller, and started to develop around E14, coinciding with the onset of fetal development. Based on these findings, the two phases were named “embryonic” and “fetal” myogenesis, or “primary” and “secondary” respectively. Accordingly, the myoblasts recruited in these phases are also termed “embryonic” and “fetal” myoblasts. Importantly, primary and

secondary myofibrils were found to express different myosin isoforms, indicating that they derive from separate cell lineages (Miller, 1992; Rudnicki et al., 1993). The precise timings of these phases have since been elucidated, with embryonic myogenesis taking place between E10.5-12.5, followed by fetal myogenesis at E14.5-17.5 (Biressi et al., 2007a). It is also now known that during fetal myogenesis, fetal myoblasts fuse to primary myofibers and to each other to create secondary myofibers (Hutcheson et al., 2009). Fetal myogenesis can therefore be further characterized by growth and maturation of the myofiber network, and the start of muscle innervation by motor neurons (Messina and Cossu, 2009). At the end of fetal myogenesis, satellite cells can be morphologically identified as they adopt their unique position between the basal lamina and sarcolemma of the myofiber (Biressi et al., 2007a). The third wave of myogenesis, termed post-natal and adult myogenesis, is characterized by dramatic physiological changes which occur after birth. Muscle regeneration in the adult is mediated by satellite cells (Hutcheson et al., 2009).

Embryonic, fetal and adult myoblasts were identified as distinct classes after they showed different behavioral traits *in vitro* (Biressi et al., 2007b). It rapidly became of intense interest to find out whether these different classes also derive from distinct myogenic progenitor populations. As discussed already, *Pax3* is essential for limb MPC migration. When its expression is removed in conjunction with that of *Pax7*, no axial or limb muscles are produced, although the primary myotome does form. Removal of *Pax7* alone does not result in a defective muscle phenotype. These findings revealed that *Pax3*⁺/*Pax7*⁺ somitic cells are the common progenitor population for myoblasts. However, since both genes are downregulated during myogenesis, lineage-tracing studies were carried out to assess the relative contributions of *Pax3*⁺ and *Pax7*⁺ cells (Hutcheson et al., 2009). Interestingly, both give rise to embryonic

myoblasts. By the start of fetal myogenesis, *Pax3* has been downregulated but a subset of *Pax3*-derived (now expressing *Pax7*) fetal myoblasts facilitates this phase. The study also revealed that although embryonic myoblasts (and primary myofibers) derive from *Pax3*^{+ve}*Pax7*^{-ve} cells, they had never expressed *Pax7*. Furthermore, in the absence of *Pax7*, the number of fetal myofibers formed is decreased, indicating that *Pax7* expression is essential to their differentiation. *Pax3*^{+ve}*Pax7*^{-ve} somitic cells can give rise to endothelial cells and embryonic myoblasts. A subset of *Pax3*⁺ cells also give rise to *Pax7*⁺ cells, which subsequently give rise to fetal myoblasts. A small proportion of these *Pax7*-expressing cells are then retained into adult myogenesis as satellite cells, their role to be discussed further in section 1.3.

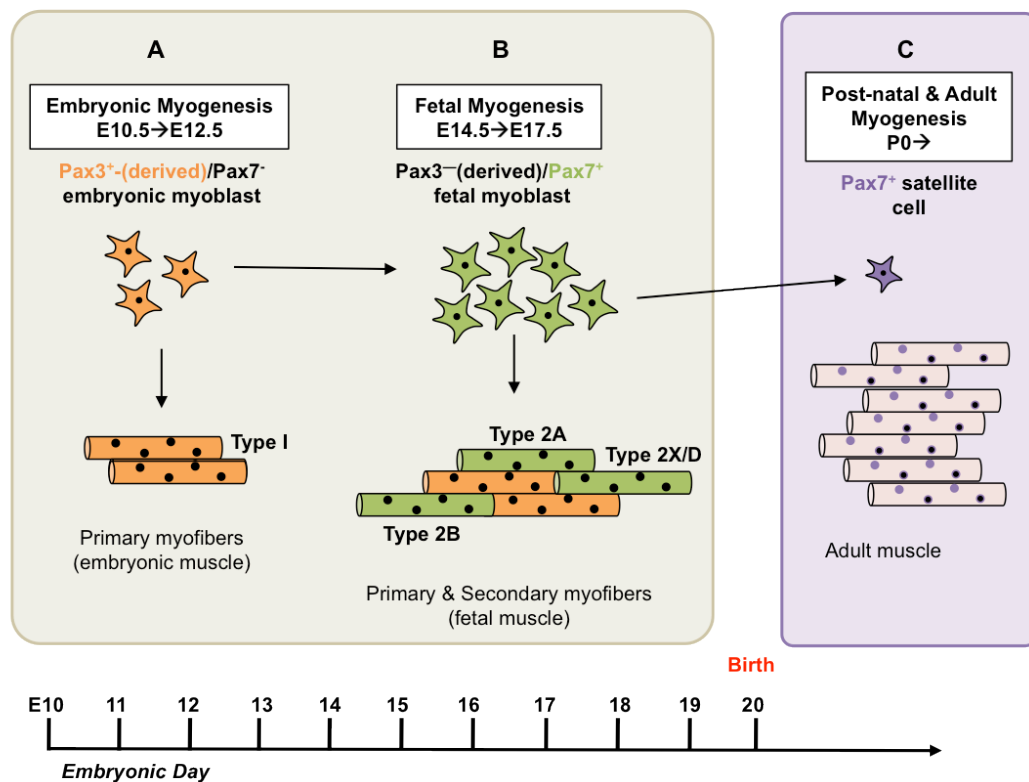


Figure 1.5: Embryonic, fetal and adult myogenesis occur in temporally-distinct but successive phases. (A) Embryonic (or primary) myogenesis takes place between E10-E12.5. *Pax3*-derived embryonic myoblasts fuse into primary myofibers, which adopt a slow (Type I) phenotype after birth. (B) Fetal (or secondary) myogenesis occurs from E14.5-E17.5. *Pax7*-positive (*Pax3*-derived) fetal myoblasts fuse into secondary myofibers, which populate the primary myofiber network, facilitating muscle hypertrophy. After birth, a small number of *Pax7*-expressing myoblasts are programmed into satellite cells and persist into adulthood to mediate further muscle growth or muscle repair upon injury (adapted from Messina and Cossu., 2009).

1.2 Skeletal Muscle Structure and Physiology

Skeletal muscles form an integral part of the musculoskeletal system. In coordination with the skeletal system, nervous system, tendons and other connective tissues, they facilitate a range of activities from low intensity (e.g. posture, standing) to high intensity (e.g. running or

jumping). They are referred to as striated owing to their jagged-striped morphology when visualized microscopically. These striations are due to the structured organization of contractile units called sarcomeres, which are composed of interacting protein filaments and facilitate muscle contraction. The sarcomeres are arranged as long cylindrical cells composed of nuclei and mitochondria, and together they make up muscle fibers or myofibers. Individual myofibers are encased in a thin connective tissue layer called the endomysium. Several myofibers are grouped into parallel bundles also called fascicles, which are in turn ensheathed in a second layer called the perimysium. Clusters of fascicles comprise muscle itself, encased in an epimysium layer, and tethered to the skeleton at the extremities via tendons, see Figure 1.6A.

1.2.1 Skeletal muscle ultrastructure

1.2.1.1 The myosin superfamily

The structural and functional features of individual muscle fibers are tailored to their specialized function, however their ability to respond to the requirements of the nervous system is determined by the molecular motors which convert chemical energy into work. The proteins driving skeletal muscle contraction belong to class II myosins (which also includes motors of smooth muscle and cardiac contraction, and some non-muscle myosins). The myosin molecules expressed in cardiac and skeletal muscles are known as sarcomeric myosins as they make up the supramolecular contractile unit of muscle: the sarcomere (Reggiani et al., 2000). Myosin is composed of two elongated chains (myosin heavy chains (MyHC)), which have a globular domain or 'head' at the amino (NH_2 -) terminus of the filament, see Figure 1.6C. The globular domain is composed of over 800 amino acids and contains binding sites for both ATP and actin. ATP is hydrolysed in this domain to generate the force behind contraction. The carboxyl (COOH -) ends of these chains associate

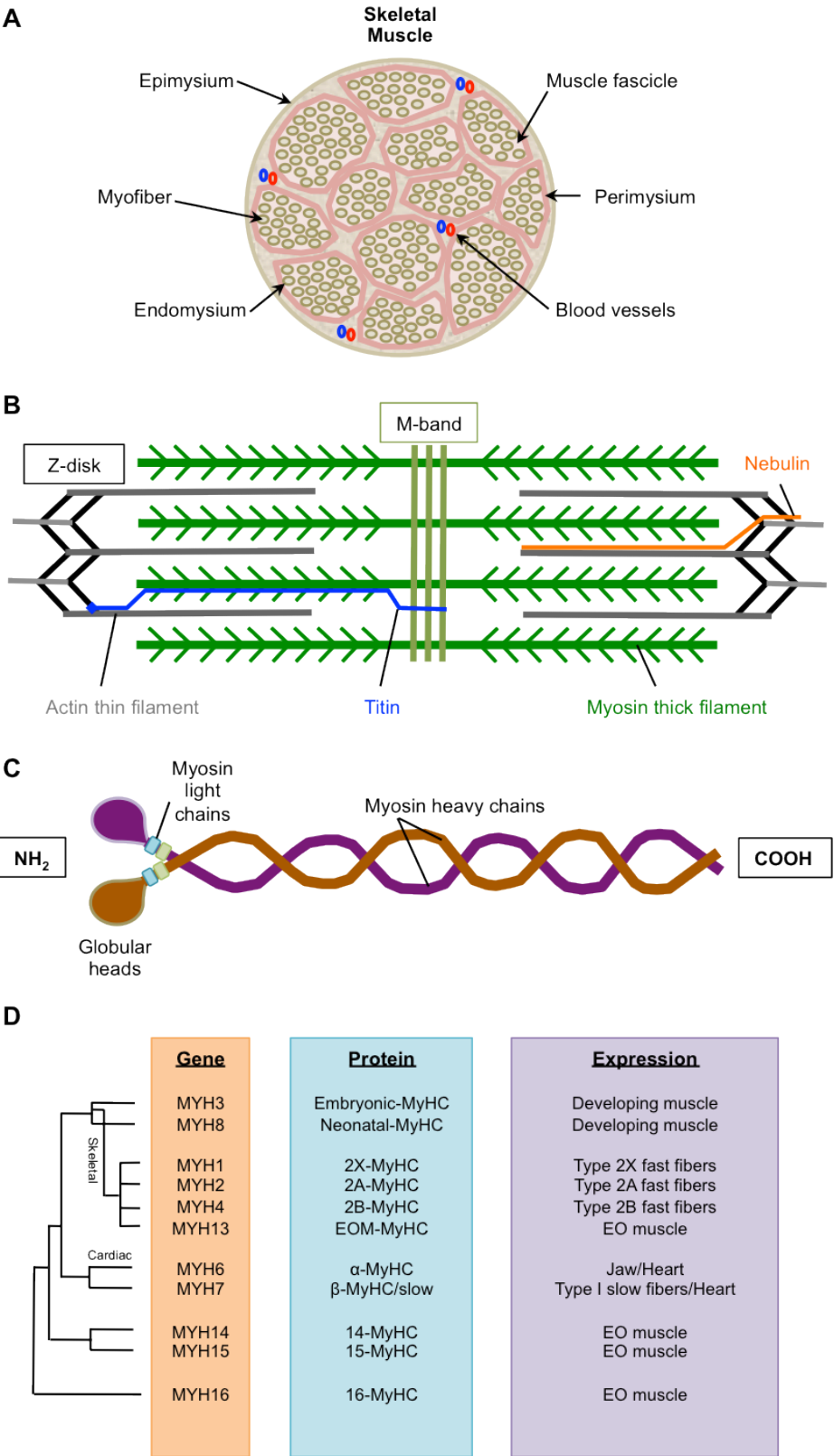
to form an α -helical coiled coil or elongated filament which stretches for more than 1,000 amino acids in length. At the COOH- end of the globular domain, there are two IQ domains positioned at this junction. These bind two myosin light chains (MyLC), one each from the MyLC1 (alkali) and MyLC2 (regulatory) families respectively. MyHCs are assembled in such a way within the sarcomere that the globular heads create a “thick” filament which is critical to contraction (Fike and Kaplowitz, 1994; Schiaffino and Reggiani, 2011). There are multiple isoforms of both MyHCs and MyLCs which give them a huge diversity and thus make up the myosin superfamily. The mammalian genome contains 11 sarcomeric MyHC genes, which encode different MyHC isoforms and were highly conserved throughout vertebrate evolution (Figure 1.6D). Most of these genes are grouped into two clusters. The first contains the cardiac muscle-specific MyHC- α (encoded by *MYH6*) and MyHC-1 (encoded by *MYH7*). The second contains the three isoforms expressed in adult skeletal muscle (MyHC-2A, -2X and -2B, encoded by *MYH2*, *MYH1* and *MYH4* respectively), the two developmental isoforms expressed in embryonic (MyHC-emb; *MYH3*) and neonatal (MyHC-neo; *MYH8*) muscles, and finally an isoform unique to extraocular muscles (MyHC-EO; *MYH13*). The six MyHC genes, which are expressed predominantly in skeletal muscle (MyHC-1; 2A; 2B; 2X; emb; neo), are conserved on chromosome 11 in mice. Their expression is regulated by the MRF transcription factor family, and components of the calcineurin pathway. In addition to inducing expression of the sarcomeric genes, members of the MRF family can also upregulate upstream regulatory proteins which can give rise to myogenic conversion. Critically, the MRFs (bHLH proteins) also bind E-box motifs found in the promoters and enhancers of muscle-specific genes, which also regulates their expression (Beylkin et al., 2006; da Costa et al., 2007).

1.2.1.2 Structure of the sarcomere

The different components of the sarcomere are detailed in Figure 1.6B. The sarcomere is fundamental to contraction because of its ability to shorten quickly, and switch ON and OFF, properties both facilitated by its highly organized structure. It is composed of a multitude of proteins which interact to provide contractile, structural and regulatory support (Engel, 2004). MyHC filaments self-assemble within myofibers to form “thick” filaments which are essential to force generation. Thick filaments are packed into parallel sections with their globular heads at a bipolar orientation, and positioned at the center of the sarcomere (Ono, 2010). “Thin” filaments are interdigitated in a hexagonal arrangement around thick filaments and are composed of actin monomers, associated with tropomyosin and troponin molecules (Marston and Redwood, 2003). Tropomyosin filaments run the length of thin filaments and form a complex with troponin molecules. A conformational change in this complex facilitates muscle contraction by enabling intracellular calcium (Ca^{2+}) uptake (Huang et al., 1999). The mechanism of contraction will be discussed in section 1.2.2.2. Essentially, when the sarcomere is activated, the thick filaments move away from one another. Filaments in the M-band, which runs down the center of the sarcomere, ensures that tension in the activated state is uniformly distributed, and also that the thick filaments remain tightly-packed (Agarkova et al., 2003). M-band filaments are composed of two large structural protein: myomesin and M-protein (Obermann et al., 1996). The third most abundant protein in the sarcomere is Titin (or Connectin) and this forms a third filament system which run parallel to the thick and thin filaments (Fulton and Isaacs, 1991). These long, elastic filaments also help to center the thick and thin filaments, and link them to the Z-line (Soeno et al., 1999). Another abundant protein in the sarcomere is nebulin. Nebulin’s function within the sarcomere is gradually being elucidated. It regulates thin filament length,

acts as a bridge between myofibrils, and in addition to its structural role, could be involved in the regulation of muscle contraction (Ottenheijm et al., 2012).

Figure 1.6: Muscle ultrastructure and the myosin superfamily. (A) Muscle structure. Myofibers are arranged into fascicles, surrounded by the perimysium. Fascicles are bundled together to make up the bulk of the muscle, ensheathed in an epimysium. This is tethered to the bone via tendons (adapted from Yin et al., 2013). (B) Sarcomere structure. Myosin thick filaments are interdigitated with actin thin filaments to make up the sarcomere. Filaments within the M-band are composed of myomesin and M-protein. These ensure tension is spread evenly across the sarcomere during contraction. They also help to link the thin filaments to the Z-disk. Nebulin and titin also add structural integrity to the sarcomere (adapted from Schiaffino and Reggiani., 2011). (C) Structure of myosin protein. The carboxyl tails (COOH-) of the myosin heavy chain (MyHC) molecules associate to form an α -helix. A globular head at the amino end (NH₂-) of the protein contains ATP-binding sites. The neck region of the molecule (at the COOH- end of the globular domain) contains two IQ sites which bind myosin light chains (MyLC) (adapted from Hutagalung et al., 2002). (D) Mammalian myosin genes, proteins and sites of expression. Spacing and length of branches in phylogenetic tree do not represent actual sizes. MYH14 is expressed at the transcript level in both slow and extraocular (EO) muscle, but the protein is only detected in EO muscles (adapted from Rossi et al., 2010).



1.2.1 Skeletal muscle fiber-type heterogeneity

1.2.1.2 *Extrafusal fibers*

The earliest paper published remarking on functional properties of different muscles was by Dorothy Needham in 1926. She discussed that the colour of skeletal muscle reflects its myoglobin content, and this gives indications as to its fast or slow contractile properties. Slow-twitch muscles, which are specialized for endurance activities, are oxidative in nature and contain huge quantities of myoglobin, which gives the muscle a red appearance. Conversely fast-twitch muscles, which are designed for short bursts of energy, rely on glycolytic metabolism and as such do not contain as much myoglobin, making them appear white. Since 1926, dramatic advances in the techniques used to identify myosin isoforms have helped to elucidate single myosin isoforms (“pure”) or combinations of two or more isoforms, also known as “hybrid” fibers. These techniques include immunohistochemistry using MyHC-specific antibodies, electrophoresis to analyse MyHC isoforms, and biochemical analyses of myofibrillar adenosine triphosphatase (mATPase) (Pette and Staron, 2000). Hybrid fibers are believed to link pure fibers together, which further adds to myofiber diversity.

We now know that there are four major myosin isoforms expressed in adult skeletal muscle. These are collectively referred to as extrafusal fibers because they are innervated by α -motor neurons (MNs), to be discussed later. The major fiber types are: slow Type I (MyHCI) and three fast fiber types - Type 2A (MyHCIIa), Type 2B (MyHCIIb) and Type 2D (MyHCIIId). The latter is believed to be the same as Type 2X (MyHCIIx) and so the two are frequently denoted as Type 2X/D. A host of hybrid fibers have been identified from combination of these four major types (Pette and Staron, 2000).

Analysis of mATPase was particularly instrumental in the identification of Type 2 fibers, which both predominate in fast-twitch muscles as opposed to Type 1 fibers (Brooke and Kaiser, 1970; Guth and Samaha, 1969). Further to this, in cat muscle Type 2A fibers were found to be more fatigue-resistant in comparison to Type 2B fibers, rendering them closer to Type 1 fibers in biochemical nature (Burke et al., 1971). However given that they also contain a large number of glycolytic enzymes, Type 2A and Type 2B fibers can also be respectively known as fast-twitch oxidative and fast-twitch glycolytic fibers (Peter et al., 1972). Identification of the third fiber-type, Type 2X/D, occurred some years later after the characterization of Type 2A and 2B. This fiber-type was found to share common traits with Type 2B MyHC and all Type 2 MyHC. However, since it did not express epitopes unique to both Type 2A and 2B MyHC, it was identified as a novel isoform. This was confirmed further still when its functional properties were found to be intermediate between Type I and Type 2B MyHC (Schiaffino et al., 1989). Although there had been experimental evidence for an additional Type 2D protein, it was subsequently revealed that the Type 2X isoform is encoded by a distinct muscle fiber-type specific gene, and is therefore not the product of post-transcriptional regulation of the MyHC genes (DeNardi et al., 1993).

1.2.1.3 Intrafusal fibers

The muscle spindle refers to units of muscle fibers that are embedded within extrafusal myofibers, and are scattered throughout skeletal muscle. This neuromuscular spindle is a fusiform-shaped capsule containing very narrow intrafusal fibers. In contrast to the much larger extrafusal fibers which facilitate contraction, intrafusal fibers within the muscle spindle act as stretch receptors. They measure the rate and change in length of the spindle, and relay this information back to the central nervous system (CNS) via afferent motor neurons. Intrafusal fibers

are innervated by γ -motor neurons (Kirkpatrick et al., 2008). There are two sizes of intrafusal fiber. The first is a small, single chain of nuclei termed 'nuclear chain fiber'. The second type is larger accommodating several small nuclei, called 'nuclear bag fiber'.

Intrafusal fibers in the adult are thought to be developmental myosin isoforms which have persisted into adulthood. Extrafusal fibers, which are also derived from these isoforms, rapidly mature after birth into fast or slow-twitch myofibers due to afferent skeletal innervation.

1.2.1.4 Skeletal muscle innervation

The body can be stimulated to move voluntarily or involuntarily. Involuntary movements include contraction of heart muscle and smooth muscles in the gut, which do not require conscious brain activity. In contrast, voluntary muscle contraction occurs as a result of conscious brain activity. The motor cortex in the brain is largely responsible for the body's movements. Motor control can be classified as either higher or lower-order depending on whether the movement initiated is active or passive (voluntary or involuntary). Most lower-order motor control does not require a cortical command, and can be initiated by sensory inputs into the spinal cord alone, such as the spinal reflex (Ghosh and Haggard, 2014). In vertebrate locomotion, the spinal reflex arc facilitates contraction of muscles (effector organs) in response to sensory inputs, relayed via the CNS. The arc is therefore composed of two key elements: afferent MNs, which relay sensory input and efferent MNs, which generate a response to this input i.e. movement in this case. The cell bodies of afferent MNs are located within the dorsal root ganglia (situated outside the spinal cord) and they synapse onto interneurons within the cord or directly onto MNs. Sensory information from the spinal cord is then relayed along MNs originating in the ventral horns of the cord, to innervate muscle fibers. Muscle stretching results from the stretch reflex (Figure 1.7A). In an

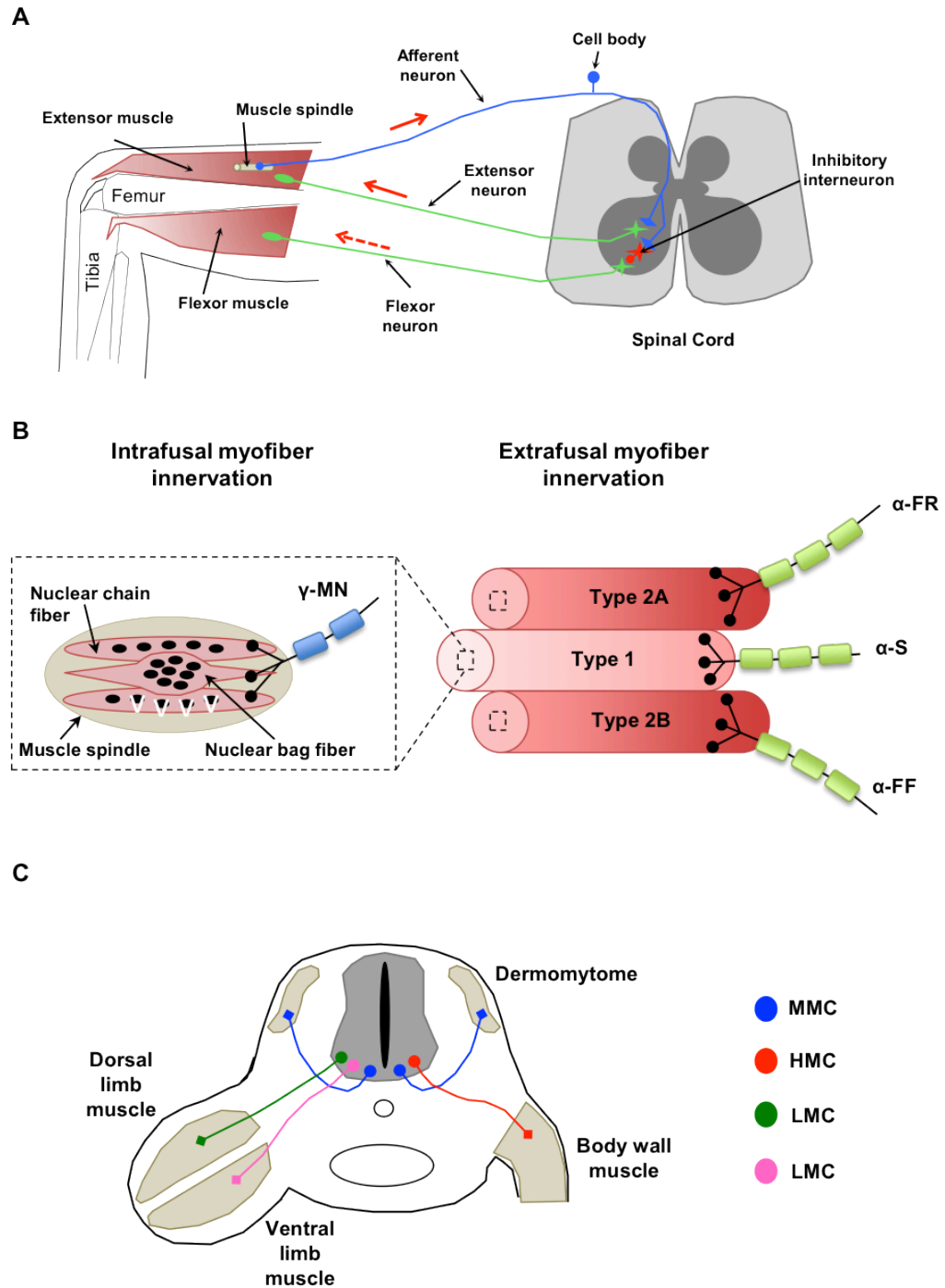
extensor muscle (which causes stretch), information from the afferent MN is relayed directly onto a MN within the spinal cord via an excitatory synapse. This innervates the muscle unit within the muscle to stimulate stretch. To complement this, information from the same afferent neuron is relayed onto an inhibitory interneuron within the spinal cord. This prevents information being relayed along motor neurons innervating flexor muscles which antagonize the stretch motion (Ishikawa and Komi, 2007).

As previously mentioned, extrafusal and intrafusal myofibers are innervated by α - and γ -MNs respectively (Figure 1.7B). A third class of MN, β -MN have also been identified to innervate both classes of muscle fiber. Just as extrafusal fibers predominate in muscle, α -MNs are also the most numerous and are classified according to the specific fiber-type they innervate, ie. slow-twitch fatigue-resistant (S) (Type I MyHC), fast-twitch fatigue-resistant (FR) (Type 2A MyHC) and fast-twitch fatigable (FF) (Type 2B MyHC), see Figure 1.7B (Burke et al., 1973). The collective term for the motor neuron and the number of myofibers it innervates is the 'motor unit', and muscles range in the number of motor units they contain depending on their size. This region is also referred to as the neuromuscular junction (NMJ) (Burke et al., 1973). There is also size diversity amongst the α -MN subtypes, with S MNs typically being narrower than FF and FR MNs. These diameter differences have implications for the firing rate of nerve impulses between the subtypes. S α -MNs require a lower current to begin firing because they have a lower action potential threshold in comparison to fast α -MNs. This explains why fast α -MNs are employed for short bursts of contractile activity such as jumping, whereas prolonged activities such as standing rely on S α -MNs (Traub, 1977). Differences in size between α - and γ -MNs also reflect their different functions. As extrafusal myofibers are wider in diameter, so too are the α -MNs which possess larger cell bodies, and the dendritic processes which branch off of these cell bodies are also more complex since they innervate

multiple myofibers. γ -MNs are narrower in diameter, their dendritic processes are much simpler and they display shorter conduction velocities (Shneider et al., 2009).

The different neurons discussed so far are grouped during development so that their functions are coordinated. They are segregated into motor columns located along the rostro-caudal axis of the neural tube. Neurons in the medial motor column (MMC) innervate epaxial muscles of the dorsal body, whilst hypaxial motor column (HMC) motor neurons innervate hypaxial muscles located in the ventral body wall. Finally lateral motor column (LMC) motor neurons project to the limb muscles (Figure 1.7C) (Bonanomi and Pfaff, 2010; Dasen and Jessell, 2009).

Figure 1.7: The stretch reflex and myofiber innervation. (A) Stretch Reflex schematic. Signals from the muscle spindle in an extensor muscle are relayed along the afferent motor neuron into the central nervous system. This neuron synapses onto an extensor motor neuron to stimulate stretch within the same muscle. At the same time, the afferent motor neuron synapses onto an inhibitor interneuron, which prevents the signal being relayed to an antagonist flexor muscle, thereby facilitating stretch (adapted from Clarac et al., 2000). (B) Extrafusal vs. Intrafusal myofiber innervation. Extrafusal and intrafusal myofibers are innervated by α - and γ -motor neurons (MNs) respectively. α -MNs are subdivided into fast-twitch fatigue-resistant (FR), slow-twitch fatigue-resistant (S) and fast-twitch fatigable (FF) depending on the type of extrafusal myofiber they are innervating (adapted from Kanning et al., 2010). (C) Diversification of motor neuron subtypes. The medial motor column (MMC) spans rostrocaudal levels and projects motor neurons onto axial muscles. The hypaxial motor column (HMC) contains MNs which innervate body wall muscles. The lateral motor column (LMC) is divided into lateral and medial subgroups, innervating dorsal and ventral limb muscles respectively (adapted from Bonanomi et al., 2010).



1.2.1.5 The importance of functionally-diverse fiber types

Although the extrafusal fiber types discussed so far are the most prevalent in skeletal muscles of the trunk and limbs, other muscles of the body have a functional requirement for specialized myosin isoforms. These include masticatory muscles of the jaw, and extraocular muscles (EOM), which control eye and eyelid movements. Jaw muscles are unique because they contain a different composition of myosin isoforms to meet the functional demands of eating habit variations within different mammals. They are also derived from a different myogenic lineage compared to those of the trunk and limbs. In rodents for example, jaw muscles are composed of both fast and slow myofibers as seen in the limbs. In contrast, jaw muscles in sheep and cows are predominantly slow. Further analysis of the composition of jaw muscles led to the identification of a novel “masticatory” MyHC isoform which is jaw-specific in several mammals. This isoform is unique because its ATP-ase activity levels are much higher compared to limb muscle myosin isoforms. As a result, the contraction time of masticatory myofibers is half that of those in the trunk and limbs (Hoh, 2002). A unique myosin isoform, encoded by *MYH15* is exclusively expressed in EOM from P7 onwards. Functionally, MyHC15 is a slow-type myofiber and its post-natal upregulation corresponds to maturation of the visual system (Rossi et al., 2010).

1.2.2 Mechanism of muscle contraction

Understanding the mechanism of muscle contraction has prompted numerous studies and is represented in Figure 1.8 (Geeves and Holmes, 1999; Holmes and Geeves, 2000; Kraft et al., 2005). The mechanism of contraction centers around the “power-stroke” or “sliding filament” model, whereby the thick myosin and thin actin filaments are arranged in an initial conformation. This conformation changes state to move the thin filament

along the thick filament in a power-stroke motion. This is powered by ATP hydrolysis which depends on Ca^{2+} uptake (Geeves and Holmes, 1999). This movement also forms the basis of the sliding filament theory. The thin filament proteins troponin and tropomyosin mediate this movement. Troponin binds intracellular Ca^{2+} , and this stimulates the tropomyosin filaments to shift along the thin filament, effectively opening myosin-binding sites on the thin filament. This enables myosin-actin cycling and therefore contraction (Galinska-Rakoczy et al., 2008).

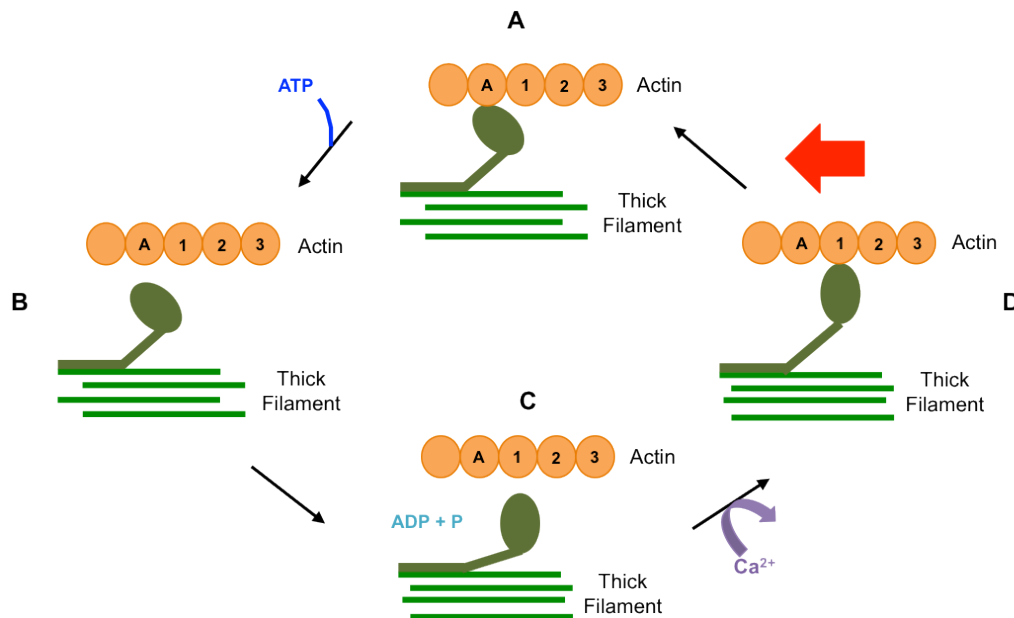


Figure 1.8: The sliding filament model. (A) Rigor state. In the absence of ATP, the globular head of the myosin thick filament binds Actin (A). (B) Release. ATP binds myosin and releases it from actin. (C) ATP hydrolysis. When ATP is hydrolysed it the myosin head re-orientates itself to face the next actin molecule along (1). (D) Calcium (Ca^{2+}) enters the cycle and removes inactivation of the actin-binding site so that the myosin head can bind once more, generating an overall stroke movement to the 'left' (adapted from King et al., 2004).

1.2.3 Fiber-type re-modeling during post-natal development and in the adult

As discussed in section 1.1.4, primary and secondary myofibers, formed during embryonic and fetal myogenesis, express different MyHC isoforms. It was found in mammals that primary myofibers express embryonic (fast) and Type I (slow) MyHC, the latter predominating. Therefore, primary myofibers are programmed for a slow phenotype. Secondary myofibers however express both embryonic and neonatal (the developmental isoforms, neonatal is also fast) MyHC, and do not express Type I MyHC. Therefore secondary myofibers are programmed for a fast phenotype (Biressi et al., 2007b). Expression of Type I MyHC in primary fibers is innervation-independent (Condon et al., 1990b). The majority of primary myofibers retain expression of Type I MyHC. Similarly, secondary myofibers retain their fast phenotype, and since they are more numerous within developing muscle, the number of fast fibers increases rapidly in all muscles (Condon et al., 1990a). Mature Type 2 (fast) myofibers are detectable 5 days after birth in mice, and this change in myosin isoform expression correlates with physiological changes that occur after birth. These include an increase in neuromuscular activity, maturation of the neuromuscular junction and changes in hormone levels (notably thyroid hormone). To meet these new functional demands, skeletal myofibers mature further and adapt their MyHC content during post-natal development (Agbulut et al., 2003).

1.2.3.1 Fiber-type switching

In the adult, skeletal muscle has the capacity to re-model itself in response to different types of activity. Due to the complexity of exercise, the adaptive changes are specific to the length, duration and type of physical activity (Widegren et al., 2001). The two key types of physical activity which cause re-modelling are endurance and strength activities.

The former requires fatigue-resistant muscles, and this phenotype can be achieved through repetitive activities (e.g. running). On the other hand, strength activities require muscles to overcome resistance, and this is mediated by an increase in the number of myofibers (hypertrophy) (Egan and Zierath, 2013). Changes in nervous innervation or hormonal levels can also induce fiber-type switching in adult muscle. For example, cross reinnervation studies in rats revealed that fast and slow muscles can adapt their metabolic properties when stimulated with slow and fast motor neurons respectively (Hoh, 1975). Fast-to-slow fiber-type switching occurs in the sequential order: 2B→2X→2A→1, and is usually induced by low-frequency electrical stimulation or muscle overloading. A slow-to-fast fiber type switch occurs in the reverse order, and is conversely induced by high-frequency stimulation or muscle unloading (Schiaffino and Reggiani, 2011; Termin et al., 1989).

1.3 Adult Myogenesis

Skeletal muscle is largely a stable and post-mitotic tissue. During adult life, it is subjected to the wear and tear of daily activity, which can result in minor injuries. Upon activation of a small number of satellite cells, these can be repaired through the formation of new myotubes, thereby maintaining homeostasis without provoking a more dramatic response. In contrast, when severe muscle trauma occurs, a greater number of satellite cells are activated and several mechanisms come into play, including inflammatory responses, to ensure their sustained proliferation and subsequent differentiation is maintained. In the context of the latter, regeneration will be discussed in greater detail.

1.3.1 Muscle regeneration

As in most tissues, a hallmark of muscle repair is activation of the innate immune system, which prompts an influx of inflammatory cells to the site of injury.

In skeletal muscle, the principal cell types constituting this response are macrophages and monocytes. These cells receive guidance signals from damaged blood vessels in the area, secrete cytokines and clear necrotic debris from damaged myofibers through phagocytosis (Tidball, 2005). Interestingly, two subclasses of macrophages have been identified to mediate this response at different points of regeneration. In addition to their classical role in the innate immune response, macrophages also play an active role in tissue repair. This is due to their ability to adopt different phenotypes depending on the surrounding microenvironment. Macrophages are divided into two classes depending on their activation and specific function. The 'M1' macrophages are activated during the inflammatory phase and initiate phagocytosis. 'M2' macrophages accumulate at the site of injury following removal of necrotic tissue, and subsequently participate in the repair and remodeling process (Cote et al., 2013). M2 macrophages can be further subdivided into three subgroups: M2a (activated by interleukin-4 and -13), M2b (activated by immune complexes or toll-like receptors) or M2c (activated by interleukin-10). According to their phenotype, macrophages are able to promote muscle injury or stimulate its repair (Villalta et al., 2009). The mechanism of M1 and M2 macrophage accumulation following muscle injury remains elusive. It is currently thought that accumulation of M1 macrophages is the result of the infiltration and differentiation of a particular subset of monocytes. In contrast, it is thought that M2 macrophage accumulation is a result of either circulating M2 precursors, proliferation of M2 macrophages or the differentiation of monocyte-derived M1 macrophages into M2 macrophages (Arnold et al., 2007; Jenkins et al., 2011; Nahrendorf et al., 2007)

In addition to macrophages, T-lymphocytes can also give rise to cell-types which contribute to muscle repair. These Th1 and Th2 cells display different cytokine profiles, which act to either promote or inhibit

tissue fibrosis, Th1 cells have been shown to secrete anti-fibrotic cytokines such as tumour necrosis factor-alpha (TNF- α), whereas Th2 cells produce the pro-fibrotic cytokines Il-4 and Il-5. The delicate balance in activation between the two classes ensures optimal muscle repair (Wynn, 2008). Fibroblasts also play a critical role in extracellular matrix (ECM) remodelling after injury. To do this, they secrete collagen, and whilst they account for most collagen synthesis post-injury, it has also been shown that satellite cells can produce significant levels of type-I collagen (Alexakis et al., 2007). Other growth factors which play an important role in the prevention of fibrosis include myostatin, TGF- β , connective tissue growth factor (CTGF) and platelet-derived growth factor (PDGF) (Bonner, 2004; Heydemann et al., 2009; Li et al., 2008; Vial et al., 2008).

1.3.2 Satellite cell origins

After their discovery in 1961, satellite cells were first convincingly established as the regenerative cell-type of skeletal muscle in the early 1970s, as evidenced through their ability to incorporate radiolabeled thymidine during proliferation assays. This demonstrated that they were able to break quiescence, and were therefore capable of generating myoblasts and fusing into new myofibers (Moss and Leblond, 1971). The term 'satellite' refers to their peripheral location on myofibers, specifically found between the plasma and basement membranes of the myofiber (Murphy et al., 2011). Whilst they are mainly associated with extrafusal myofibers, satellite cells have also been identified on intrafusal fibers, however much less is known about this muscle spindle-associated group (Kirkpatrick et al., 2010). In their normal quiescent state, satellite cells express a host of different markers, including integrin $\alpha 7 \beta 1$, CD34, c-Met, vascular cell adhesion molecule (VCAM) and the chemokine C-X-C motif receptor 4 (CXCR4), reviewed in (Kuang and Rudnicki, 2008).

The exact embryological origins of satellite cells have, until recently, remained obscure. In early experiments using quail-chick chimeras, it was suggested that, like trunk and limb muscles, satellite cells also originate within the somite. Briefly, somites from donor quail embryos were transplanted into host chick embryos. It was found that donor cells from quail somites formed part of the chick limb and helped to give rise to both satellite cells and terminally differentiated myofibers. These findings suggested shared somitic origins for all myogenic cell lineages, however the progenitor cell types and their development remained unknown (Armand et al., 1983). Advances in mouse genetics manipulation (particularly *Pax3* and *Pax7* knock-in reporter alleles). combined with cell-labelling techniques, have helped to elucidate that satellite cells originate in the dermomyotome. This somitic compartment is marked by expression of *Pax3* and *Pax7* in the embryonic progenitors, and therefore they are the major source adult satellite cells (Gros et al., 2005; Relaix et al., 2005). There is also increasing evidence that some satellite cells may have alternative origins. Most notably, several studies have shown that nonsatellite cells are capable of differentiating into satellite cells and replenishing the satellite cell niche. For example, pericytes have been shown to differentiate into satellite cells post-natally (Dellavalle et al., 2011). However, the extent to which this occurs remains to be elucidated. There is also a growing suggestion that mammalian adult satellite cells could be derived from dedifferentiation of fetal or adult myofibers, as this occurs in newt amphibian. However whether this occurs in mammalian myofibers requires further investigation (Brockes and Kumar, 2002).

Further functional studies have revealed that during post-natal development (P0-P21), removal of *Pax7* expression severely compromises muscle growth and regeneration (Kuang et al., 2006; Relaix and Zammit, 2012). The requirement for *Pax7* expression for satellite cell function in adult muscle has been subject to debate in recent years. In a

study by Lepper *et al.*, it was suggested that *Pax7* expression was dispensable in adult life. After inducing deletion of *Pax7* in satellite cells during the first three weeks of life, via tamoxifen, they claimed that satellite cell number and muscle regeneration were not impaired (Lepper *et al.*, 2009; Lepper and Fan, 2010). However, these findings were questioned in a later study when it was found that continuous, as opposed to temporary, tamoxifen administration (via intraperitoneal injections followed by tamoxifen-supplemented chow diet) fully removed *Pax7* expression. Ablation of *Pax7* was found to give rise to severe regeneration defects, and as such contested the findings of Lepper *et al.* (von Maltzahn *et al.*, 2013). Interestingly, of the reduced number of myogenic cells capable of regeneration in *Pax7*^{-/-} muscles, nearly all of these have been shown to express *Pax3*, and are exclusively found in the muscle interstitial space, not within the satellite cell niche itself. This suggests a potential role for a new myogenic lineage, which is anatomically distinct to *Pax7*-expressing satellite cells, yet the precise role of these *Pax3*⁺ interstitial remains to be elucidated (Kuang *et al.*, 2006).

In addition to embryological origin, satellite cells also display heterogeneity depending on a series of other factors (Biressi and Rando, 2010). The functional diversity of different muscle groups is reflected in resident satellite cells which have different gene expression profiles. For example, satellite cells in extraocular muscles (EOM) display elevated levels of molecular markers such as *Pitx2* and *Tcf21* (Sambasivan *et al.*, 2009). Furthermore, not all satellite cells have the capacity to express markers of their muscle of origin when grown in culture or engrafted into different muscles, for example EOM satellite cells (Sambasivan *et al.*, 2009). However, contradictory findings suggest that satellite cells do retain a muscle-specific gene expression pattern and are subsequently able to give rise to specific myosin isoforms when engrafted into host muscles (Hoh and Hughes, 1988). There is also evidence to suggest that

satellite cells associated with either fast or slow-type extrafusal fibers display different functional characteristics (Kalhovde et al., 2005). Together, this evidence also suggests that satellite cells are heterogeneous, a topic which is currently provoking debate. It has been proposed that satellite cells are made up of 'true' stem cells in addition to myogenic progenitors. The development of markers specific to satellite 'stem' cell is being investigated to help clarify this (Kuang et al., 2007).

1.3.3 Satellite cell activation and differentiation

Upon muscle injury, satellite cells become activated and emerge quiescence to re-enter the cell cycle and proliferate. To do this, they activate the p38 α / β MAPK pathway (Troy et al., 2012). Proliferating satellite cells rapidly upregulate the MRF *MyoD* to become transit-amplifying cells or myoblasts (Cooper et al., 1999; Wen et al., 2012). *Pax7* is downregulated and the myoblasts initiate expression of the remaining MRFs to become fully committed myocytes, marked by expression of *myogenin* (Figure 1.9) (Zammit et al., 2006b). At this point, the myocytes stop proliferating and either fuse together to form new myotubes, or fuse with pre-existing, damaged, myotubes (Siegel et al., 2011). Crucially, a fraction of satellite cells do not terminally differentiate, and this is essential to replenishment of the satellite cell pool (Troy et al., 2012).

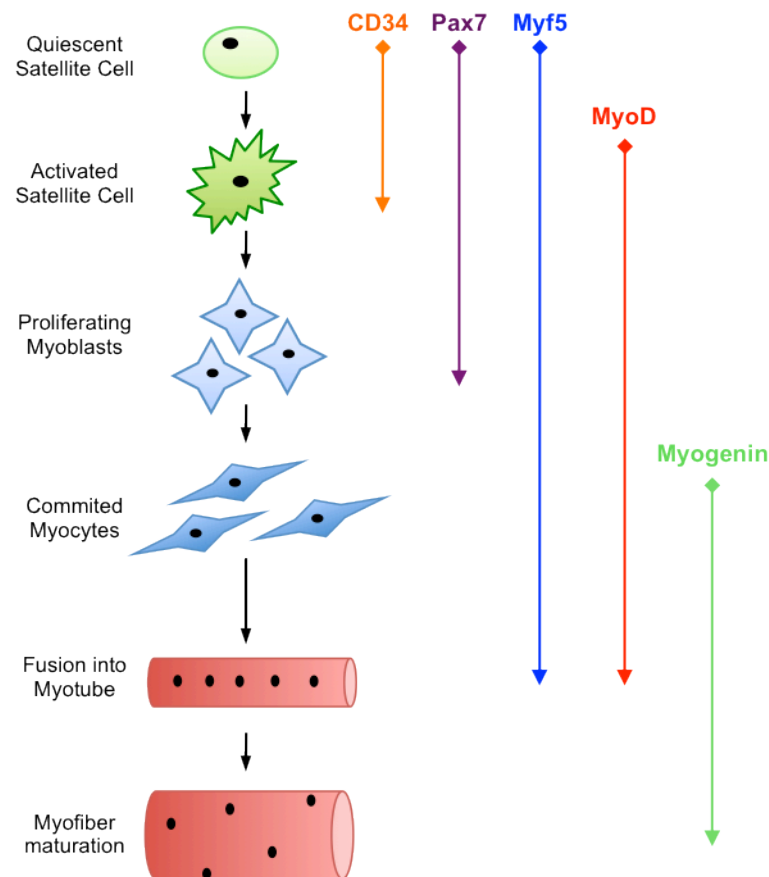


Figure 1.9: Satellite cell activation and differentiation. CD34 is a marker of quiescent and activated satellite cells, as is Pax7, which is also expressed by proliferating myoblasts. Myf5 is also expressed by these cells and can be detected in early myotubes. MyoD is also expressed from the start of activation through to differentiation into myotubes. Similarly, myogenin is upregulated during differentiation and myofiber maturation (adapted from Zammit et al., 2006a).

1.3.4 Satellite cell self-renewal

There are two key mechanisms of satellite cell division, which facilitate either proliferation or self-renewal. Symmetric division occurs in the planar orientation and gives rise to two identical daughter cells, which will have similar fates, and thus forms the basis of proliferation.

Asymmetric division occurs in an apical-basal orientation to give rise to two non-identical daughter cells. At the basal surface one of these will retain stem cell characteristics, and the second at the apical surface will go on to differentiate into muscle. The Par complex (composed of Partitioning Defective-3 and -6, and protein kinase C) is inherited by only one daughter cell, and when it activates the p38 α / β MAPK pathway, it ensures the daughter cells adopt diverging fates (Troy et al., 2012). In this way, the stem cell pool is maintained homeostatically. Understandably, erratic divisions can have deleterious effects in that too many symmetric divisions can lead to over proliferation (e.g. tumour growth). Similarly, mistimed asymmetric divisions can lead to premature organ development (Egger et al., 2011). Within their niche (the local environment and cellular components in direct contact with satellite cells), satellite cells are arranged in an orientation which lends itself to asymmetric self-renewal, see Figure 1.10 (Bentzinger et al., 2013). The basal lamina covers the basal side of the satellite cell, whilst the apical side lies directly next to the myofiber. The basal surface of the satellite cell expresses the laminin receptor $\alpha_5\beta_1$ integrin, and the apical surface expresses the cell adhesion molecule M-Cadherin (Burkin and Kaufman, 1999; Irintchev et al., 1994). Several signaling pathways have been shown to regulate asymmetric division of satellite cells, including Notch, Wnt and BMP (Kuang et al., 2007).

In addition, it has also been shown that satellite cells can also divide symmetrically. The choice between the two mechanisms is largely dependent on the mitotic spindle orientation relative to the myofiber's longitudinal axis (Kuang et al., 2007). Symmetric self-renewal may indeed be favoured to replenish and increase numbers of satellite cells in a disease state or after injury. Furthermore, it has been shown that the satellite cell niche factor, *Wnt7a*, also promotes symmetric cell division during muscle regeneration. In this way, the niche itself can dictate the

mode of satellite cell self-renewal (Morrison and Kimble, 2006). In other tissues, neuronal stem cells exhibit asymmetric cell division, whereas mammalian male germline stem cells and haematopoietic stem cells divide symmetrically (Wu et al., 2009; Zhong and Chia, 2008)

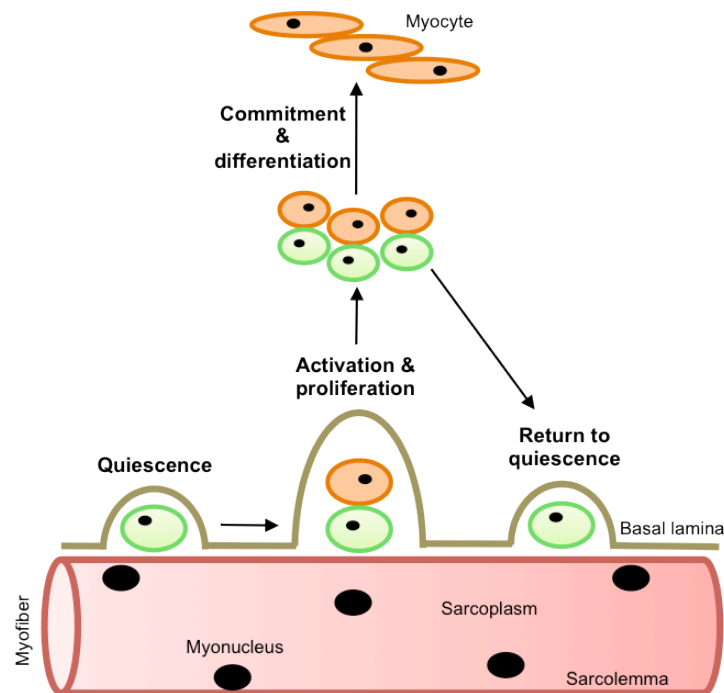


Figure 1.10: Satellite cell division and self-renewal. Upon injury, satellite cells are activated to proliferate. To achieve this they divide asymmetrically to generate two non-identical daughter cells. One of these daughter cells retains stem-cell characteristics to renew the stem-cell pool, and will return to quiescence. The other daughter cell will go on to commit to the myogenic lineage, and later differentiate to form new myofibers or fuse to existing, damaged ones (adapted from Yin et al., 2013).

1.3.5 The satellite cell niche and the local microenvironment

The stem cell niche specifically refers to the structures and cell-types surrounding the stem cell which are crucial to its maintenance and regulation (Morrison and Spradling, 2008). Their plasticity characteristics, and ability to maintain tissue homeostasis under adverse physiological or pathological conditions, are reflected by the functional properties of the niche itself (Voog and Jones, 2010).

The principal component of the satellite cell niche is the myofiber itself, as this is directly associated with the satellite cell (Figure 1.11). Studies have shown the myofiber secretes SDF-1, the ligand for the CXCR4 receptor expressed by satellite cells, and this signaling interaction promotes satellite cell migration (Ratajczak et al., 2003). The ECM components of the myofiber also influence the behaviour of satellite cells. These include laminin, the main constituent of the apical sarcolemma, and type IV collagen which makes up the basal lamina (Woodley et al., 1983). Expression of $\alpha_v\beta_1$ integrin in the basal lamina is critical to satellite cell anchorage. Satellite cells express cell-surface proteoglycan receptors which recruit various types of growth factors including hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF), in their inactive precursor state. Harboring these factors within the niche is thought to facilitate efficient responses to muscle injury, since the factors can be rapidly converted into active forms by surrounding cells (Xi, 2009). The wider microenvironment surrounding the niche also plays an important role in satellite cell maintenance and muscle regeneration. This predominantly includes interstitial cells, and also the blood vessels and motor neurons associated with the myofiber. Muscle interstitial cells include connective tissue fibroblasts which make up the stroma between the basal lamina and epimysium of the myofiber, and also adipocytes and fibrocytes which are derived from fibrocyte/adipocyte progenitor cells (FAPs) (Bi and Kuang, 2012; Yin et al., 2013). A recent study showed that

FAPs do not have myogenic potential, and remain quiescent in healthy muscle. During muscle regeneration, however, they proliferate rapidly to create an environment conducive to differentiation of myogenic progenitors (Joe et al., 2010). Another class of interstitial cell was recently identified, telocytes also exist in the satellite cell niche and secrete VEGF suggesting they could stimulate vasculogenesis, thereby enhancing satellite cell self-renewal (Deasy et al., 2009). Motoneuron innervation is also essential to satellite cell maintenance, as sustained denervation has been shown to render satellite cells vulnerable to apoptosis, resulting in significantly reduced numbers of satellite cells in denervated muscles (Jejurikar et al., 2002). VEGF has also been shown to promote myofiber regeneration after injury, and is under investigation as a potential therapeutic option for different muscle disorders (Arsic et al., 2004).

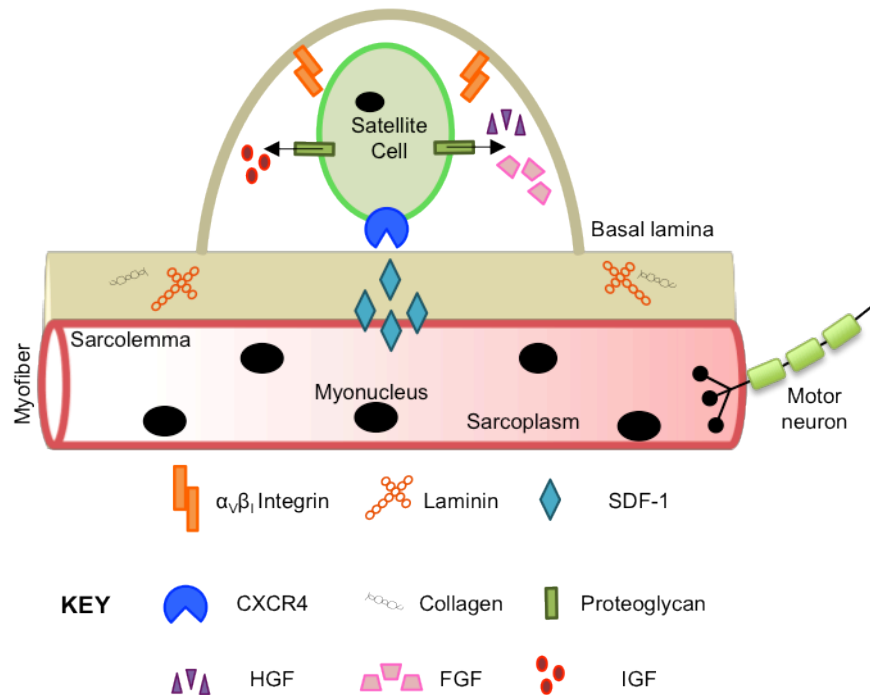


Figure 1.11: The satellite cell niche. Schematic of the immediate niche environment. The satellite cell recruits inactive precursors of hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF) in order to efficiently respond to injury. The satellite cell also displays the CXCR4 receptor which binds SDF-1 secreted by the myofiber, which assists in its migration to sites of injury (adapted from Yin et al., 2013).

1.3.4.1 Non-satellite cell contribution to muscle regeneration

Whilst satellite cells mediate muscle regeneration in the overwhelming majority, there is evidence for resident non-myogenic cell populations, and stem cells in the circulatory system, which can also contribute to muscle regeneration. A subset of interstitial cells expressing *PW1*, a participant in the p53 cell stress pathway, have been shown to be as numerous as satellite cells during juvenile growth and development, and they also decline in number around P21. Although *PW1*-expressing cells (or PICs) do not express *Pax7*, they proliferate rapidly during regeneration. Interestingly, they were also shown to have not derived

from the satellite cell lineage (Mitchell et al., 2010). Pericytes (also called perivascular or mural cells) have also been implicated in regenerating skeletal muscle. They appear capable of generating satellite cells and can also fuse with developing myofibers (Cappellari and Cossu, 2013; Dellavalle et al., 2011). Mesoangioblasts, vessel-associated stem cells derived from the dorsal aorta, have also been shown to regenerate skeletal muscle in dystrophic mice. This was believed to be due to their ability to infiltrate the capillary system (Minasi et al., 2002; Sampaolesi et al., 2003). Further examples of non-satellite cell contributors include muscle side population (SP) cells (rare multipotent stem cells that can play a role in myogenesis) and muscle-derived stem cells (MDSCs, potential satellite cell precursors which are not myogenic lineage-restricted) (Huard et al., 2003; Penton et al., 2013).

1.3.6 Pathogenesis of muscular disorders

Skeletal muscle disorders comprise a range of conditions which are characterized by progressive atrophy (muscle wasting) or degeneration. These include heritable myopathies such as Duchenne muscular dystrophy (DMD), and sarcopenia (age-related muscle loss) (Shi and Garry, 2006). DMD is caused by mutations in the dystrophin gene, leading to abnormalities in dystrophin protein which is expressed in smooth, skeletal and cardiac muscle. Dystrophin is a rod-shaped protein located underneath the sarcolemma of the myofiber, linking its cytoskeletal elements to the membrane (Rando, 2001). As an X-linked recessive disease, it affects approximately 1:3500 males. Patients with DMD present delayed walking during the juvenile period, and are typically wheelchair-bound in their early teens. A recent study has shown that numbers of satellite cells in dystrophic muscle appear higher, even in advanced stages of the disease. This would indicate that regeneration in dystrophic muscle is impaired not by a lack of satellite cells, but rather

their inability to promote regeneration or differentiation (Kottlors and Kirschner, 2010; Shi et al., 2013).

Reduced muscle function in later life often results from progressive loss of muscle mass and strength, which is also hampered by the fact that skeletal muscle regeneration appears to slow down with ageing (Grounds, 1998; Vandervoot and Symons, 2001). A hallmark of sarcopenia is a reduction in myofiber size and number, particularly Type 2 myofibers (Verdijk et al., 2007). It is widely believed the alterations in satellite cell function with age are due to both intrinsic changes within the cells themselves and extrinsic changes within the surrounding niche. Intrinsically, it has been suggested that because satellite cells endure long periods of quiescence during adult life, they are subjected to oxidative and inflammatory stresses, which inevitably will be detrimental to their functionality (Bohr, 2002). Such effects have already been observed in other stem cell types (Ito et al., 2004; Renault et al., 2009), and may well render satellite cells susceptible to cellular senescence (Jang et al., 2011). Extrinsically, downregulated levels of Notch signaling in aged satellite cells is believed to contribute to their deterioration (Conboy et al., 2003). Furthermore, aged muscles have been shown to express elevated levels of TGF- β , whose downstream target phospho-Smad3 (pSmad3) antagonizes Notch, resulting in impaired regenerative capacity within satellite cells (Carlson et al., 2008). The influence of systemic factors on aged satellite cells has been examined using parabiotic pairings (a shared circulatory system) between young and old mice, also known as the heterochronic parabiosis model. In this way, old mice are exposed to factors in young serum. Crucially, these experiments have revealed that exposing satellite cells from old mice to the younger circulatory system restores the activation of Notch signalling. Furthermore, their proliferation is enhanced and therefore their regenerative capacity is also restored. These findings would suggest that the age-related decline in satellite cell activity can be

altered extrinsically by a younger systemic environment (Conboy et al., 2005).

1.4 Tendon Development

Tendons are connective tissues that transmit muscle-generated forces to the skeleton via specialized structures called the enthesis and myotendinous junction. They act as anchors for muscle but are also flexible to enable movement. Since muscles have different functions, tendons are also diverse in structure to accommodate these (Schweitzer et al., 2010). Tendon development is closely related to that of muscle. The presence of the myotome is essential to the proper formation of tendon progenitor cells (TNPs). Studies in chick revealed Fibroblast Growth Factor (FGF) signaling from the myotome induces *Sc/x* expression in dorsal sclerotomal cells, i.e. TNPs (Brent and Tabin, 2004; Smith et al., 2005). TNPs are induced in slightly different ways depending on their location within the embryo. Trunk tendons arise from the compartmentalisation of *Sc/x*-expressing cells in the somite. In contrast, limb TNPs are physically mixed with MPCs in the limb bud itself (Tozer and Duprez, 2005). In the developing autopod, the most distal region of the limb bud where the digits form, *Sc/x*-expressing TNPs are found underneath the ectoderm where they associate with skeletal elements of the digits. In mouse, TNP induction takes place between E9 and E12.5 (Pryce et al., 2009; Schweitzer et al., 2001). Following induction, around E12.5, all TNPs throughout the body organise into clusters between developing muscle and skeleton, a process induced by TGF β signaling. By E13.5, the progenitors differentiate into distinct tendons. In some cases, this is dependent upon *Sc/x* expression (Pryce et al., 2009; Schweitzer et al., 2010).

1.4.1 Tendon physiology

Tenoblasts (immature tenocytes) mature into more elongated tenocytes (tendon cells), which have a lower metabolic activity than their predecessors. Together they account for 90-95% of cell types in tendons. Tenocytes primarily produce collagen type I to maintain the extracellular matrix (ECM), and both tenocytes and tenoblasts reside between collagen fibres along the tendon's long axis. The enthesis can be characterised by four zones: dense tendon, fibrocartilage, mineralized fibrocartilage and bone itself (Sharma and Maffulli, 2005).

1.4.1.1 Myotendinous junction

The myotendinous junction (MTJ) is the specialized interface between skeletal muscle and tendon, and is the main region of force transmission. It is predominantly composed of the actin filaments found at the end of muscles (protruding from the last Z-line) connected to surrounding proteins of the sarcolemma, basement membrane and the collagen fibrils of the tendons themselves (Charvet et al., 2012). Different molecules are enriched at both the muscle and tendon side of the MTJ, including type IV collagen and laminin, and type I collagen and tenascin respectively (Aumailley and Smyth, 1998; Chiquet and Fambrough, 1984). Expression of collagen type XXII also appears to be restricted to the MTJ (Koch et al., 2004). Although tendon and muscle progenitors develop independently from one another, they are closely associated throughout, with tendon progenitors condensing into mature tendon under the influence of migrating muscle progenitors (Kardon, 1998). This would suggest that signals emanating from muscle progenitors influence tendon development. In mouse, FGFs appear to play a role in this cross-talk, notably *Fgf-4*, *-6* and *-8* (Edom-Vovard et al., 2001a; Edom-Vovard et al., 2001b; Tozer and Duprez, 2005).

1.5 The Role of Fibroblast Growth Factors (*Fgfs*) in Vertebrate Myogenesis

1.5.1 Fibroblast growth factors

The FGF family regulates a wide range of developmental and homeostatic processes. FGFs were originally isolated as growth factors for fibroblasts, with acidic and basic FGF (*Fgf-1* and *Fgf-2* respectively) the first to be characterized (Baird and Klagsbrun, 1991; Burgess and Maciag, 1989). Following this, a further seven FGFs were identified. Using homology-based sequence searches, a total of 23 family members have been characterized to date in mice and humans. Given their similar chromosomal locations, it is thought that *Fgf-19* is the human ortholog of mouse *Fgf-15* (Kumar and Chapman, 2012). In total therefore, the FGF family comprises 22 members in both species (Itoh, 2007). The polypeptides range in size from 17-34 kDa, and their amino acid sequences are well conserved across different species (Ornitz and Itoh, 2001). Due to their sequence similarity and modes of action, the family is divided into seven subfamilies: *Fgf-1, 2; Fgf-4, 5, 6; Fgf-3, 7, 10, 22; Fgf-8, 17, 18; Fgf-9, 16, 20; Fgf-15, 19, 20, 21* and *Fgf-11, 12, 13* and *14* (Itoh and Ornitz, 2011). Generally, FGFs can function in either an endocrine, paracrine or intracrine manner. *Fgfs-11* to *14* are considered the intracrine subfamily because they function intracellularly, they are not secreted out of the cell and do not interact with FGF receptors (FGFRs) (Itoh and Ornitz, 2011). It is believed that these FGFs can translocate into the nucleus and exert their activity through an intracrine signalling pathway, binding to nuclear receptors and directly activating gene transcription (Halper, 2010).

1.5.2 Fibroblast growth factor receptors (*Fgfrs*) and FGF signaling

Four FGFR genes (*Fgfr1-Fgfr4*) have been characterized. A typical FGF receptor is composed an intracellular domain harbouring tyrosine

kinase residues, three extracellular immunoglobulin (Ig)-like domains and a transmembrane domain. FGFRs1-3 exist as different isoforms, due to alternative splicing of the Ig-like III domain (Figure 1.12) (Beenken and Mohammadi, 2009; Hajihosseini et al., 2001). Exon IIIa encodes the first half of this domain, and this is subsequently spliced to either exon IIIb or IIIc, and then to the transmembrane domain-encoding exon. Isoform expression is tissue specific. IIIc isoform is usually expressed in mesenchymal tissues, and IIIb is expressed in epithelial tissues (Seth et al., 2008; Turner and Grose, 2010). Crucially, alternative splicing of the receptors also generates ligand-binding specificity, such that ligands produced in either an epithelial or mesenchymal tissue tend to activate receptor isoforms derived from the neighbouring tissue. This is not true for all ligands, however, as FGF-1 appears to bind to both IIIb and IIIc isoforms (Beenken and Mohammadi, 2009). FGFR4 does not have isoforms since there is only a single variant of its Ig-III like domain (Powers et al., 2000).

Paracrine FGFs (*Fgf-1, 2; Fgf-4, 5, 6; Fgf-3, 7, 10, 22; Fgf-8, 17, 18* and *Fgf-9, 16, 20*) contain a heparan sulfate proteoglycan (HSPG) binding site (HBS) permitting them to bind FGFRs in an HSPG-dependent manner. The HSPG incorporates parts of the β 1- β 2 loop and β 10- β 12 region, creating a positively charged surface, therefore increasing their affinity. In contrast, endocrine FGFs (*Fgf-19, 21* and *23*) have reduced affinity for FGFRs owing to reduced HSPG binding, which enhances their diffusion. To improve their affinity, endocrine FGFs depend on β -klotho proteins to assist in receptor binding (Beenken and Mohammadi, 2009).

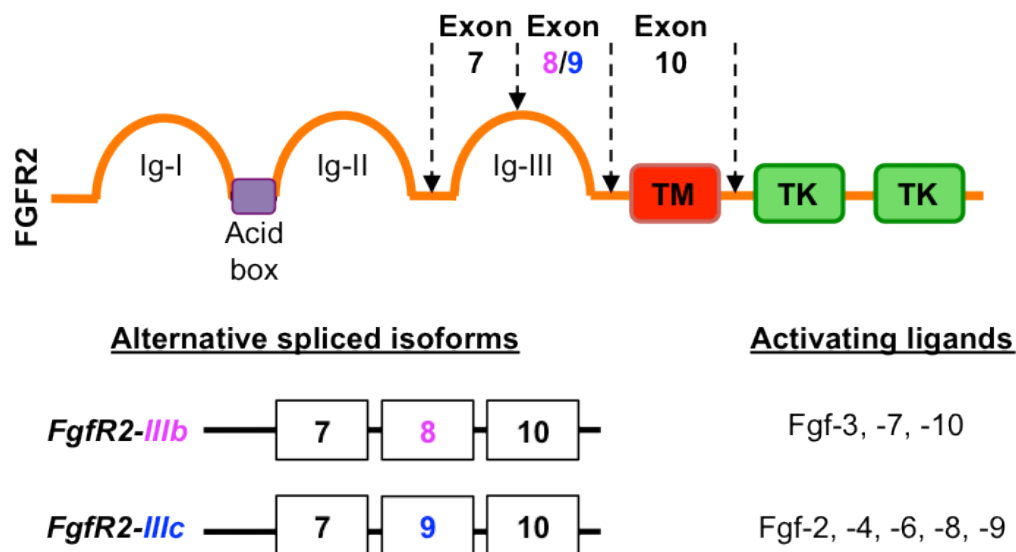


Figure 1.12. FGFR splicing and specificity. Schematic structure of FgfR2, emphasizing the third immunoglobulin-like III (Ig-III) domain. Alternative splicing of exons 8 or 9 in this region gives rise to *IIIb* and *IIIc* isoforms respectively. The main ligands which activate these isoforms are also listed. TM, transmembrane; TK, tyrosine kinase (adapted from Hajihosseini et al., 2001).

The receptor is activated by the binding of two FGF ligands to the Ig II and Ig III regions of the extracellular domain, via the HSPG domain, provoking the receptor to homodimerize (Lemmon and Schlessinger, 2010; Thisse and Thisse, 2005). This induces transphosphorylation of the tyrosine kinase residues, which can then recruit docking proteins or adaptor domains (e.g. src homology-2) to stimulate downstream phosphorylation and subsequently signal transduction pathways, (Figure 1.13) (Dailey et al., 2005). The most frequently activated pathway is the Ras/MAPK pathway, with its downstream effectors *Pea3* and *Erm* stimulating proliferation and differentiation (Dorey and Amaya, 2010). Alternatively, the *Akt* or *protein kinase C* (PKC) pathways are activated to regulate cell survival, migration and morphology (Dailey et al., 2005; Dorey and Amaya, 2010). FGF signaling levels can be regulated at multiple

checkpoints, mainly by the *Sprouty* proteins, which act as negative feedback regulators. Other attenuators of FGF signaling include *XFLRT3*, *Map kinase phosphatase 3* (MKP3) and *Sef* (Furthauer et al., 2002; Hajihosseini, 2008).

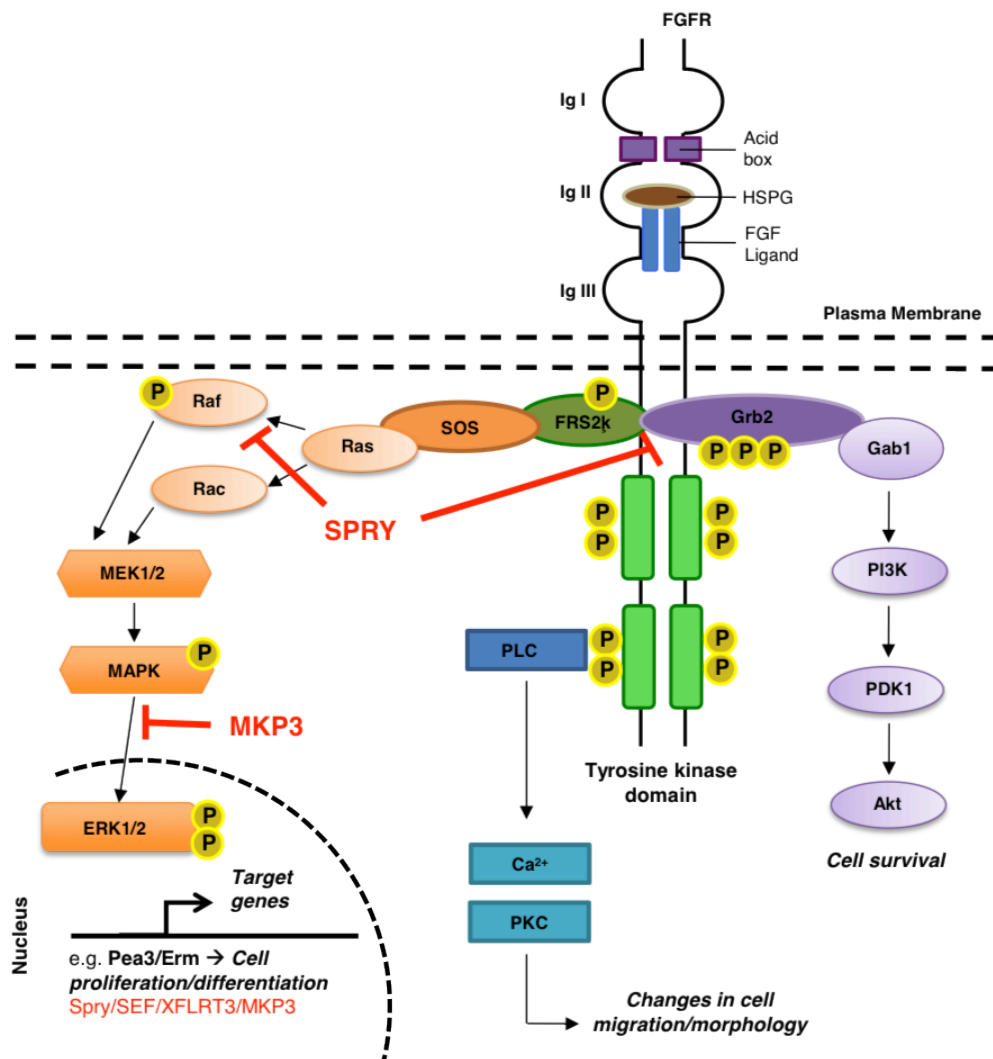


Figure 1.13: FGF signaling pathway. Upon ligand binding to the Ig-II and Ig-III domains of the extracellular portion, the FGFR homodimerizes, stabilised by heparan sulfate proteoglycan (HSPG) sequences. Dimerization induces transautophosphorylation of the tryrosine kinase residues, which in turn stimulates either the Ras/MAPK, PLCγ or Akt pathways to promote a range of downstream cellular effects. FGF signaling can be attenuated via expression of the inhibitors *Sprouty* (*Spry*), *Sef*, *XLRT3* and *MKP3* (adapted from Dorey and Amaya., 2010).

1.5.3 Expression and function of *Fgfs* in myogenesis

Many of the early findings on the potential role for *Fgfs* in myogenesis were borne out of *in vitro* studies. FGF1 and FGF2 were found to repress terminal differentiation in culture, with FGF2 being the most potent mitogen out of the pair (Clegg et al., 1987). It was also established that different *Fgfs* were expressed by proliferating myoblasts (*Fgfs*-1, 2, 6 and 7) in comparison to differentiated myotubes (*Fgfs*-5 and 7) (Brunetti and Goldfine, 1990; Hannon et al., 1996; Lathrop et al., 1985; Seed and Hauschka, 1988). These findings suggested FGFs were potent regulators of myoblast proliferation and differentiation *in vitro*. Several *Fgfs* were also found to be expressed during myogenesis *in vivo*, however early investigations into the functions of these genes were hampered due to their mutations causing embryonic lethality, therefore preventing their function in muscle from being analyzed (Feldman et al., 1995). However, with the advance in conditional gene inactivation technology, the potential regulatory mechanisms of *Fgfs* in myogenesis are gradually being elucidated.

Fgf-1 mRNA was found to be transiently expressed in developing mouse skeletal muscle from E12.5-15.5, however its function during embryogenesis remains unclear (Zhang et al., 2001). Interestingly, *Fgf1* is upregulated in muscle biopsies isolated from patients with fascioscapulohumeral muscular dystrophy (FSHMD) (Saito et al., 2000). This is a relatively common myopathy with a unique epigenetic etiology, which is linked to the distal end of chromosome 4q (van der Maarel et al., 2012). It is now known that FGF1 induction in differentiating myoblasts and regenerating muscles is due to an interaction between the *Fgf1* promoter (in skeletal muscles) and an internal ribosomal entry site (IRES), via a transcriptional-translational coupling mechanism (Conte et al., 2009). It is possible that *Fgf1* mediates regeneration in this disease state, potentially via satellite cell activation.

During embryogenesis *Fgf2* is expressed by migrating myoblasts (Webb et al., 1997). Like its family member *Fgf1*, *Fgf2* is also expressed in *mdx* mouse models of Duchenne Muscular Dystrophy, suggesting again that *Fgf2* could play a role in muscle regeneration under disease conditions (Bikfalvi et al., 1997). Indeed, a recent study found that *Fgf2* induces calcium signaling in satellite cells which stimulates their activation (Liu and Schneider, 2014).

The *Fgf-4/5/6* subfamily comprises some of the more characterized *Fgf* family members in myogenesis. Transient expression of *Fgf4* in the developing somite appears to coincide with expression of *MyoD*, suggesting a potential regulatory link between the two genes (Drucker and Goldfarb, 1993; Niswander and Martin, 1992). *Fgf4* expression was also identified at myofiber extremities in axial and limb muscles in the developing chick embryo. Furthermore, overexpression of *Fgf4* in chick limb mesenchyme leads to upregulation of FGF signaling modulators (*Pea3*, *Sprouty1*, *Sprouty2*), although this upregulation was later found to not be limited to limb muscles (Eloy-Trinquet et al., 2009). *Fgf4* overexpression also induces a decrease in the number of differentiated myofibers alongside a decrease in *MyoD* expression (Edom-Vovard et al., 2001b). Similarly, *Fgf5* overexpression has the same effect on myofiber differentiation and *MyoD* expression, which suggests that both *Fgf4* and 5 could regulate *MyoD* expression during myofiber formation (Yaylaoglu et al., 2005). *Fgf6* displays a unique expression pattern because it is restricted to the skeletal muscle lineage. However, early studies attempting to elucidate its function using knockout (*Fgf6*^{-/-}) mice were inconclusive as they contradicted each other as to the impact of this mutation on muscle regeneration, and whether or not it is impaired in *Fgf6*-deficient muscles (Fiore et al., 1997; Floss et al., 1997). More recently, it was shown that injecting recombinant FGF6 into injured muscles of *Fgf6*-deficient mice appears to accelerate myotube formation, thereby rescuing

the effects of injury. This acceleration also coincided with an increase in calcineurin expression, which is involved in the specification of slow fiber-types (Armand et al., 2005; Parsons et al., 2003). These findings potentially implicate *Fgf6* in slow-fiber specification.

Fgf8 is also thought to play a role in myogenesis because it is expressed in the central region of the myotome as the somite matures (Stolte et al., 2002). It is also thought to function with *Fgf4* to regulate muscle-tendon interactions in the developing limb, however a more thorough functional analysis has yet to be carried out (Eloy-Trinquet et al., 2009).

Various FGF signaling components are also involved in myogenesis, with *FgfR1* and *FgfR4* the main receptors expressed in skeletal muscle. FGFR1 signaling is thought to facilitate myoblast proliferation and migration, as downregulation of the pathway stimulates terminal differentiation of myoblasts (Itoh et al., 1996; Olwin et al., 1994a). An interesting function for FgfR1 in protection against atrophy (decrease in muscle mass) has also been investigated. In hindlimb suspension models, which induce atrophy, FGFR1 is enriched by a subset of myofibers, conferring protection against this degenerative process (Eash et al., 2007). *FgfR4* expression in the developing myotome is now known to be induced directly by *Pax3*. When FGFR4 signaling is inhibited, limb muscles fail to develop and expression of the MRFs is severely impaired, suggesting FGFR4 signaling acts upstream of this network (Marics et al., 2002). In the light of this, regeneration after injury is significantly impaired in *FgfR4*-deficient muscles (Zhao et al., 2006). *Spry1* has also been implicated in muscle regeneration. It is expressed in quiescent *Pax7*-expressing satellite cells, and is thought to maintain them in a quiescent state. In its absence, the number of *Pax7*⁺ satellite cells is reduced and the stem cell pool is diminished (Shea et al., 2010). A more recent study has also shown that *Spry1* is robustly expressed by satellite cells, and this

robustness weakens with age, which can then lead to depletion of these cells and an inability to self-renew (Chakkalakal et al., 2012).

1.6 Fibroblast Growth Factor 10 (*Fgf10*)

Fgf10 was the tenth FGF identified after homology-based PCR (polymerase chain reaction) was used to generate cDNA from rat embryos. The cDNA was shown to encode a protein sharing a conserved amino acid (approx. 120) core and around 60% sequence identity with FGF-3 and FGF-7. The sequence also displayed a serine-rich domain at the amino-terminus (Yamasaki et al., 1996). The human homolog for FGF-10 was found to share 96% sequence homology with rat FGF-10. The same hydrophobic, serine-rich sequence was also identified at the amino-terminus and was thought to be a signal sequence (Emoto et al., 1997). The mouse homolog of FGF-10 was also characterized and showed similar expressions to its rat counterpart, with high abundance in embryos and lungs, and limited expression in the heart and brain (Hajihosseini et al., 2008; Tagashira et al., 1997).

1.6.1 *Fgf10* knockouts

The first *Fgf10* knockout (*Fgf10*^{-/-}) line was generated by inserting a PGK-*neo* cassette, in the reverse orientation, into exon 1 of the coding sequence (Figure 1.14). Exon 1 was elected because it contained the translation start site and the potential signal peptide.

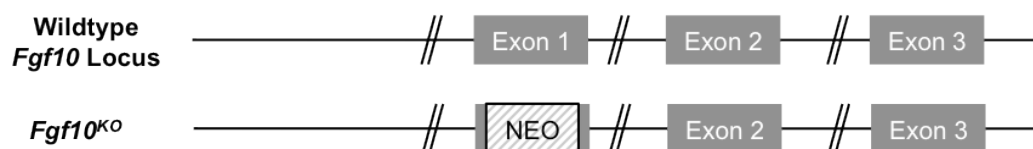


Figure 1.14: Schematic representation of *Fgf10* knockout line. The wildtype *Fgf10* locus is composed of a promoter and three coding exons. A neomycin cassette is inserted into exon 1 to create an *Fgf10* null allele.

Interestingly, *Fgf10*^{-/-} mice show perinatal lethality and further examination of these pups revealed a complete absence of both forelimbs and hindlimbs, whilst all other structures remained intact. There are two key signaling centres which regulate vertebrate limb development: the apical ectodermal ridge (AER) and zone of polarizing activity (ZPA). Both regions govern the anterior-to-posterior and proximal-to-distal outgrowth of the limb, by secreting signals in a feedback loop which maintains this outgrowth. The AER is a thickened region of the ectoderm which forms at the dorsal-ventral boundary of the distal limb bud. Removing the AER results in truncated limb development, however this can be rescued by replacing the AER with an FGF bead. Specifically, FGF8 has been shown to maintain cells in an undifferentiated, proliferative state so as to drive outgrowth of the limb. SHH signals from the ZPA can activate *Fgf* expression in the AER, thereby promoting a signaling feedback loop (Mariani et al., 2008; Pownall and Isaacs, 2010). *Fgf10* is expressed in the limb bud mesenchyme, and also signals reciprocally with *Fgf8* in the AER. *Fgf10* expression is initiated by both *Tbx4* and *Tbx5* in the hindlimb and forelimb respectively. Once this FGF positive feedback-signalling loop is established, it is self-maintaining and no longer under the control of the T-box genes (Duboc and Logan, 2011).

Fgf10 heterozygous (*Fgf10*^{+/-}) mice develop normally, suggesting complete abrogation of *Fgf10* expression is detrimental to limb bud development. *Fgf10*^{-/-} mice also lacked lungs, although development of the trachea and surrounding structures appeared unaffected (Min et al., 1998). FGF10 binds the FGFR2IIIb receptor with high affinity, and mice lacking FGFR2IIIb (FGFR2IIIb^{-/-}) also lack lungs and limbs. Additional phenotypic effects of *Fgf10* knockout mice include ablation of the thyroid, pituitary and salivary glands. Kidneys, digestive organs, teeth and hair follicles also displayed minor defects (De Moerlooze et al., 2000; Ohuchi et al., 2000).

In humans, mutations in *Fgf10* expression manifest themselves as lacrimo-auriculo-dento-digital or LADD syndrome, and also aplasia of the lacrimal and salivary glands or ALSG syndrome. LADD syndrome, believed to be originally termed Levy-Hollister syndrome based on clinical observations, is characterized by hypoplastic (underdeveloped) salivary glands, cup-shaped ears and hearing abnormalities, and digital and dental defects. It is an autosomal dominant congenital disorder. ALSG is also autosomal-dominant and characterized by xerophthalmia (inability to produce tears), xerostomia (dryness in the mouth) and also dental defects (Milunsky et al., 2006). *Fgf10* haploinsufficiency has been implicated in both diseases (Entesarian et al., 2005; Shams et al., 2007). Specifically, two missense mutations have been implicated in human LADD syndrome. The first is an R80S (arginine to serine) substitution in FGF10. The arginine located at position 80 is known to interact with the D3 region of the Fgfr2IIIb receptor. It is thought that this likely impairs the ligand-receptor interaction, which therefore leads to insufficient signal transduction. The second mutation is a *de novo* one, with a glycine to glutamic acid substitution at position 138 in FGF10. This highly conserved amino acid residue is of predicted functional importance, and this mutation also leads to FGF10 haploinsufficiency (Entesarian et al., 2007). Mutations in the FGFR2IIIb receptor may also confer a dominant negative effect on normal signaling which also contributes to LADD syndrome (Shams et al., 2007).

1.6.2 Roles of *Fgf10* in tissue development

Expression studies and knockout investigations have identified potential roles for *Fgf10* in the development of a variety of organs. In lung development, FGF10-FGFR2IIIb signaling mediates branching morphogenesis of the distal epithelium, which eventually gives rise to the branching lobes of the lung (Bellusci et al., 1997; Lu et al., 2005; Park et

al., 1998). In a manner similar to lung development, FGF10 signaling also appears to maintain the stomach epithelial progenitor niche, and their subsequent morphogenesis and differentiation. It has also been shown that *Fgf10* controls gastric gland specification (Nyeng et al., 2007). Similarly, *Fgf10* also maintains pancreatic epithelial cells in a progenitor state, and its ectopic expression leads to increased proliferation and an inability to differentiate. Conversely, when FGF10 signaling is removed, pancreatic progenitors fail to proliferate (Norgaard et al., 2003). FGF10 can also help support hepatoblast proliferation and survival in liver development (Berg et al., 2007). Interestingly, *Fgf10* is also expressed in white adipose tissue (WAT) (Yamasaki et al., 1999). Perturbed FGF10 signaling was found to prevent preadipocyte differentiation. Similarly, disruptions to the gene itself led to a reduced amount of WAT produced (Sakaue et al., 2002). Studies have also proposed that *Fgf10* is necessary for the maintenance of the stem cell compartment in mouse incisors (Harada et al., 2002; Yokohama-Tamaki et al., 2006). Erk is believed to be activated in this signaling pathway to stimulate proliferation in the dental epithelium (Cho et al., 2009). In the developing vertebrate limb, the T-box transcription factor *Tbx5* (under the control of β -catenin signaling) is expressed in the lateral plate mesoderm and regulates *Fgf10* in this region to promote limb outgrowth. Removal of *Tbx5* leads to a loss of *Fgf10* expression and loss of forelimbs (Ng et al., 2002). Furthermore, it was found that *Tbx5* binding sites are located within the *Fgf10* promoter (Rallis et al., 2003).

Owing to the perinatal lethality of *Fgf10*^{-/-} mice, the regulatory role of this gene in adult tissues has remained elusive. In 2001, a reporter strain was developed which utilized an *Mlc1v-nlacZ-24* transgene inserted 114Kb upstream of exon 1 of the *Fgf10* gene (Figure 1.15). LacZ, as indicated by production of the protein β -galactosidase (β -gal), was found

to be expressed in an extremely similar pattern to *Fgf10*, thereby recapitulating *Fgf10* expression.



Figure 1.15: Schematic representation of the *Fgf10*-nLacZ line. A nuclear-targeted LacZ construct is inserted downstream of the promoter and upstream of exon 1.

β -gal can be detected using antibodies or X-gal staining solution, as discussed in Chapter 2. Furthermore, the high stability of the β -gal protein renders it useful for short-term lineage tracing. It also creates an *Fgf10* hypomorph, as evidenced by *Fgf10*^{LacZ/-} mice (inheriting one copy of *Fgf10*^{LacZ} and one copy of *Fgf10* null alleles), which display phenotypes consistent with reduced *Fgf10* expression such as impaired lung branching (El Agha et al., 2012; Kelly et al., 2001). In 2008, a more comprehensive analysis of *Fgf10*-expressing cells in the developing, post-natal and adult brain was carried out using the *Fgf10*^{nLacZ} reporter mouse. Between E14.5-15.5, lacZ expression was observed in the developing hypothalamus, in addition to some brain stem nuclei and spinal cord neurons. By E18.5, this expression had expanded to the cerebellum and dorsal midbrain. These domains of *Fgf10* expression expand during post-natal development and persist into adulthood. At the adult ages surveyed, lacZ expression could also be found in the amygdala, hippocampus, neocortex and olfactory bulbs (Hajihosseini et al., 2008). More recently, a new transgenic line has been developed to explore *Fgf10* function. The *Fgf10*^{Cre-ERT2} (*Fgf10*^{iCre}) knock-in line was generated by inserting a Cre-

ERT2-IRES-YFP cassette into exon 1 of the *Fgf10* gene (in frame with the ATG) and will be discussed in greater detail in Chapter 2 (El Agha et al., 2012).

1.6.3 Evidence for *Fgf10* in post-natal and adult skeletal muscles

Using the *Fgf10*^{nLacZ} reporter line, a subset of skeletal muscles have been found to express β -gal, indicating *Fgf10* expression, at a variety of post-natal and adult ages. Interestingly, within these muscles, the myofiber population is heterogeneous for β -gal expression. Furthermore, the myonuclei population within a β -gal-expressing myofiber is also heterogeneous for β -gal. This would suggest that β -gal-expressing myonuclei are derived from a subpopulation of *Fgf10*-expressing myogenic progenitors.

1.7 Aims

In the light of this novel expression pattern for *Fgf10* in post-natal and adult skeletal muscles, several lines of investigation were pursued to attempt to elucidate a role for this gene in muscle development. The main aims of this project, and the principal methodologies used to achieve them, are outlined as follows:-

1. To investigate the timing and pattern of *Fgf10* expression in developing, post-natal and adult skeletal muscles.
 - X-gal staining in *Fgf10*^{nLacZ} reporter mice to survey *Fgf10* expression (as indicated by β -gal) at a range of embryonic, post-natal and adult ages.
 - Immunohistochemistry for fiber-type markers and the β -gal reporter to see if *Fgf10*-expressing myonuclei are restricted to a particular fiber-type.
2. To investigate the function of *Fgf10* in myogenesis by removing its expression from myogenic progenitor cells.
 - Using Cre/LoxP technology to specifically delete *Fgf10* expression from *Pax3*-expressing myogenic progenitor cells, and characterizing resulting muscle phenotypes after *Fgf10* deletion, with a particular focus on muscles of the limbs.
 - Immunohistochemistry for fiber-type markers to see if the proportions of different fiber-types within *Fgf10*-expressing muscles are altered after deletion of *Fgf10*.
3. To investigate the effects of *Fgf10* over-expression in a skeletal muscle cell line *in vitro*.
 - Generation of an *Fgf10* over-expressing vector.

- Setting up transient transfections into C2C12 mouse myoblast cells to observe the effects of Fgf10 over-expression on their differentiation into myotubes.

Chapter 2

Materials and Methods

2.1 Transgenic Animals

Unless otherwise stated, all transgenic animals were bred as heterozygotes and maintained on a mixed genetic background, in accordance with local regulations for the use of transgenic animals, and a Home Office license issued to Dr Mohammed Hajihosseini.

2.1.1 *Fgf10*-LacZ; *Fgf10*-floxed; *Fgf10*-CreERT2 and *Fgf10* KO

A variety of mice were used in this study and schematics of the allelic modifications are presented in Figure 2.1. The *Fgf10*-LacZ (*Fgf10*^{nLacZ/+}) reporter strain contains a LacZ cDNA (*Mlcv1v-nLacZ-24* transgene) with a nuclear localization signal. After mapping, it was found to have been inserted upstream of exon 1 in the *Fgf10* gene, but downstream of the promotor, which has not yet been characterized for *Fgf10* (Figure 2.1B). The pattern of LacZ expression was shown to closely replicate that of *Fgf10*. The transgene generates two separate transcripts (not a fusion protein) and this, coupled with the stability of the β -galactosidase protein, permits transient lineage tracing of *Fgf10*-expressing cells. Importantly, insertion of the transgene reduces *Fgf10* expression slightly, thereby creating a hypomorphic phenotype for this gene. This line has previously been used for lineage-tracing investigations in the developing heart and lungs (Kelly et al., 2001; Maillieux et al., 2005). The line is usually maintained on a heterozygous background, however reporter mice are also viable as homozygotes (*Fgf10*^{nLacZ/nLacZ}), but they are seemingly infertile (Maillieux et al., 2005).

Mice containing a conditional allele for *Fgf10* were generated by inserting loxP sequences either side of exon 2 in the *Fgf10* gene (Figure 2.1C) (Abler et al., 2009). To achieve this, first *Fgf10* genomic DNA-containing phages were isolated carrying a 10Kb fragment of *Fgf10* with a loxP sequence situated within the intron, 0.85Kb downstream of exon 2. Second, targeting vectors were generated, containing a second loxP

sequence located in an intron 0.3Kb upstream of exon 2. Following linearization and electroporation into R1-45 embryonic stem (ES) cells, and homologous recombination between the genome and vectors, these were introduced into C57Bl/6 embryos. Observation of the F1 generation revealed the targeted *Fgf10* allele (*Fgf10^{fllox}*) was hypomorphic. Furthermore, mice homozygous for exon 2 deletion within *Fgf10* (*Fgf10^{fllox/fllox}*), after crossing with a Cre deleter strain, showed no differences when compared to established *Fgf10* knockout animals (Abler et al., 2009; Urness et al., 2010).

The *Fgf10*-Cre^{ERT2} (*Fgf10^{Cre-ERT2/+}* or *Fgf10^{iCre/+}*) knock-in line was generated by inserting a Cre-ERT2-IRES-YFP construct into part of exon 1 of the *Fgf10* gene, in frame with the start codon (Figure 2.1D). The construct contains cDNA for tamoxifen-inducible Cre recombinase (Cre-ERT2 gene) and a yellow fluorescent protein (YFP) reporter gene, separated by an independent ribosomal entry site (IRES). The tamoxifen-inducible system is dependent upon the nuclear localization of ligand-binding domains within the estrogen receptor, which occurs after ligand-binding. Cre recombinase is fused to mutated ligand-binding domains, which are no longer capable of binding estrogen but can still bind its antagonist tamoxifen. Upon tamoxifen induction, the Cre recombinase protein can translocate to the nucleus wherein it excises loxP-flanked DNA sequences, thereby generating a lineage or cell-type-specific conditional knockout (Nagy, 2000). Cre recombinase is therefore produced in all *Fgf10*-expressing cells, thus Cre-mediated recombination occurs in a lineage-specific manner. Insertion of the construct leads to heterozygous loss of *Fgf10* function. This renders the allele non-functional thereby creating an *Fgf10* null allele. As a result, *Fgf10^{CreERT2}* homozygotes are not viable. In addition, *Fgf10* and *Cre* expression levels do not correlate directly, with *Cre* levels consistently lower than those of *Fgf10*. This inefficient expression is believed to be due to regulatory elements within

intron 1, which maintain *Fgf10* expression. Furthermore, although *Fgf10*-expressing cells are exclusively labeled upon tamoxifen administration, constitutive expression of the reporter is induced which is independent of the *Fgf10* promoter (El Agha et al., 2012).

To disrupt its function, the *Fgf10* knockout allele was generated by inserting a neomycin cassette (PGK-*neo*) in reverse orientation into exon 1, thereby preventing transcription (Figure 2.1E). Homozygous knockouts die at birth, whereas heterozygotes develop normally despite reduced *Fgf10* expression levels (Min et al., 1998).

2.1.2 ROSA26-Tomato

ROSA26 is a ubiquitously expressed endogenous locus, which is widely used in conjunction with reporter proteins to facilitate visualization of Cre-mediated recombination. Tandem dimer tomato (tdTomato) is a red fluorescent protein (RFP) variant, which displays enhanced brightness and photostability when excited under a 554nm laser. The tomato reporter cDNA construct is inserted into the ROSA26 locus, containing a stop codon flanked by loxP sites upstream of the reporter (Figure 2.1F). Upon Cre-mediated recombination, excision of the neomycin cassette permits tomato expression. Importantly, homozygous ROSA26 knock-in mice are also viable (Muzumdar et al., 2007; Shaner et al., 2004; Soriano, 1999). Mice sourced from The Jackson Laboratory (www.jax.org).

2.1.3 Pax3-Cre

The *Pax3*-Cre (P3Pro-Cre) transgene relies on the proximal 1.6Kb region of the mouse *Pax3* promoter to drive Cre recombinase expression, rendering it constitutively active in *Pax3*-expressing cells (Figure 2.1G). Within the skeletal muscle lineage, this transgene displays a rostro-caudal gradient of expression, and is restricted to all caudal muscles (muscles posterior to the diaphragm) (Jarad and Miner, 2009; Li et al., 2000). Mice

sourced from Prof. Thomas Braun (Max-Planck Institute), via Prof. Andrea Münsterberg (UEA).

2.1.4 Scleraxis-GFP

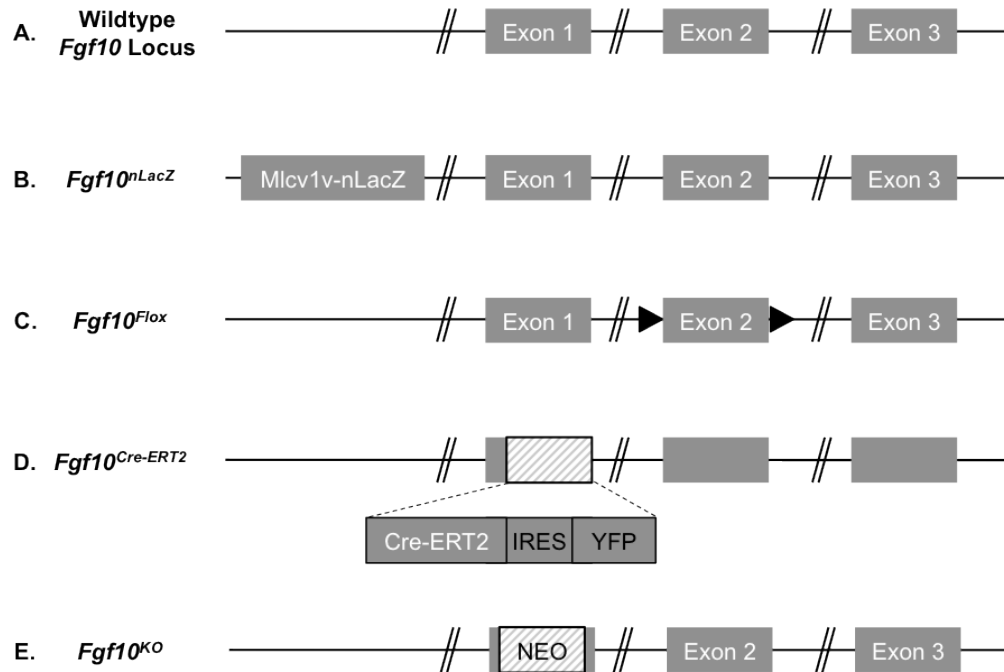
The bHLH transcription factor *Scleraxis* (*Scx*) is expressed specifically in developing tendons and their progenitors, and ligaments (Schweitzer et al., 2001). The open reading frame (ORF) of the *green fluorescent protein* (*GFP*) gene was inserted into exon 1 of the *Scx* gene, in frame with the start codon and replacing most of the exon (Figure 2.1I). Design of the constructs ensured the transgene transcripts included exon 2 of the *Scx* gene. GFP was shown to be robustly expressed in developing tendons. In addition, *Scx*-GFP homozygotes were found to be viable and did not display phenotypic differences (Pryce et al., 2007).

2.1.5 Breeding and Genotyping

After weaning, tail biopsies were obtained from transgenic litters. These were digested in tail digest buffer (1M Tris, 0.5M EDTA, 5.0M NaCl, 2mg/mL SDS diluted in double-distilled H₂O) containing 7µg/mL Proteinase K (diluted from 20mg/mL stock) overnight, at 55°C. The next day, samples were centrifuged at 13,000rpm for 10 minutes to remove undigested tissue and fur, and genomic DNA was precipitated with an equal volume of isopropanol. DNA was spooled out and re-suspended in 30% TE buffer in H₂O (3mM Tris-BASE, 0.3mM EDTA). 1µL DNA was used for genotyping using the Expand Long Template PCR System© (Roche Applied Science, Mannheim, Germany). Primer sequences and PCR cycles used during genotyping are listed in Table 2.1 and 2.2 respectively.

Figure 2.1: Schematic representations of the transgenic lines used. (A) The wildtype *Fgf10* locus contains a promoter and three coding exons, the promoter region has yet to be characterized. (B) In the *Fgf10*^{nLacZ} reporter line, a nuclear LacZ construct is inserted downstream of the promoter and upstream of exon 1. (C) In the *Fgf10*^{Flox} line, exon 2 is flanked by loxP sequences, which mediate conditional inactivation upon Cre-mediated recombination. (D) In the *Fgf10*^{Cre-ERT2} line, a Cre-ERT2-IRES-YFP construct is inserted into part of exon 1, giving rise to both Cre-ERT2 and YFP products instead of FGF10. (E) In the *Fgf10* knockout line, a neomycin cassette is inserted into exon 1 of the gene, creating an *Fgf10* null allele. (F) The *ROSA26*-tomato reporter contains a floxed neomycin cassette in front of Tomato cDNA, controlled by the *ROSA26* promoter. (G) The *Pax3*-Cre transgene utilizes the proximal 1.6Kb region of the *Pax3* promoter to drive Cre recombinase expression. (H) Scxg12 is an 11Kb genomic clone from the *Scx* locus which was used to design the Scx-GFP construct. (I) The open reading frame (ORF) of green fluorescent protein (GFP) was subcloned at the start codon of exon 1 to replace most of it. Grey boxes represent coding regions, white boxes represent untranslated regions (UTRs).

Fgf10 Lines



Reporter Line



Pax3 Line



Scleraxis-GFP Line

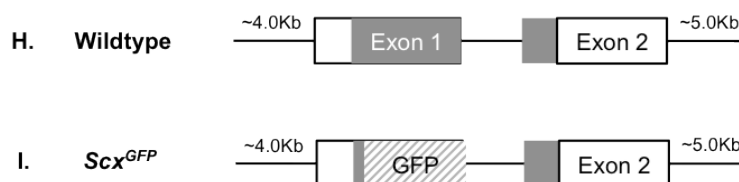


Table 2.1 – Primer pairs to detect genotypes.

Allele	Primer Sequences (5'-3')	Product size (Kb)	PCR Cycle Name
Fgf10 ^{LacZ}	GCA TCG AGC TGG GTA ATA AGC GTT GGC AAT	LacZ: 0.8	Fgf10-LacZ
	GAC ACC GAC ACA ACT GGT AAT GGT AGC GAC		
	CGA GTG GAG CAT GTA CTT CCG TGT CCT GAA	WT: 0.5	
	TCC CTA CCC AGT CAC AGT CAC AGC TGC ATA		
Fgf10 ^{Flox}	GAG GCA GGA TAA CCA GTA TCT GG	Floxed: 1.8	R2-IIIc floxed
	GAA ATT GCA GAG ATT GCA AAG GAA GC	WT: 1.2	
Fgf10 ^{Cre-ERT2}	AGC AGG TCT TAC CCT TCC AGT ATG TTC C	Cre: 0.55	Fgf10- CreERT2
	TCC ATG AGT GAA CGA ACC TGG TCG		
	AGC AGG TCT TAC CCT TCC AGT ATG TTC C	WT: 0.3	
	CTC CTT GGA GGT GAT TGT AGC TCC G		
Fgf10 ^{KO}	CAC CAA AGA ACG GAG CCG GTT G ACT CTT TGG CCT CTA TCT AG	KO: 0.9	Fgf10 KO
Tomato*	CTG TTC CTG TAC GGC ATG G	Tomato: 0.2	Fgf10 KO
	GGC ATT AAA GCA GCG TAT CC		
	AAG GGA GCT GCA GTG GAG TA	WT: 0.3	
	CCG AAA ATC TGT GGG AAG TC		
Scleraxis ^{GFP} *	CCT GCT GGA GTT CGT GAC CGC GAG GGG TAG TGG CAC ATC AGC	GFP: 0.5	Fgf10 KO

**Fluorescent tomato and GFP reporters can also be identified under UV illumination.*

Note: Genotyping of Pax3^{CRE} allele will be discussed in greater detail in Chapter 5.

Table 2.2 – PCR Cycles used during genotyping.

Name of Cycle	Cycle Stage	Temperature (°C)	Length (min)
Fgf10-LacZ	Denaturation	94	2
	Annealing x 15 cycles	94	0.5
		62	0.75
		68	1
		94	0.5
	Extension (+40s increment) x 17 cycles	62	0.5
		68	1
		Final Elongation	68
Hold		6	15
R2-Illc floxed	Denaturation	94	2
	Annealing x 10 cycles	94	0.5
		61	0.5
		68	3.2
		94	0.5
	Extension (+20s increment) x 16 cycles	61	0.5
		68	3.2
		Final Elongation	68
Hold		5	15
Fgf10-CreERT2	Denaturation	94	2
	Annealing x 10 cycles	94	0.5
		61	0.5
		68	2.67
		94	0.5
	Extension (+40s increment) x 17 cycles	61	0.5
		68	2.67
		Final Elongation	68
Hold		6	15
Fgf10-KO	Denaturation	94	2
	Annealing x 15 cycles	94	0.5
		60	0.5
		68	1.17
		94	0.5
	Extension (+20s increment) x 17 cycles	60	0.5
		68	1.17
		Final Elongation	68
Hold		6	15

2.2 Animal Treatments

2.2.1 Time mating

Males and females were housed overnight, and females were checked every morning for vaginal plugs. The day of vaginal plug observation was noted as E0.5.

2.2.2 BrdU administration

5-Bromo-2'-deoxyuridine (BrdU; Sigma Aldrich, Gillingham, UK) was administered continuously in drinking water as a 1mg/mL solution containing 0.25mg/mL glucose, to sweeten the taste. Fresh BrdU solution was supplied every 72 hours for 21 days, in drinking bottles protected from light.

2.2.3 Tamoxifen administration

Tamoxifen (Altronin Spezialfutter, Lage, Germany) was administered in two ways. The first was via *ad libitum* supply of food pellets supplemented with 0.4g tamoxifen citrate per Kg dry weight. The second was via intraperitoneal injection of a 20mg/mL solution prepared in 10% EtOH/corn oil, mixed for several hours at 55°C, at 80-100mg/Kg bodyweight.

2.3 Tissue Processing

Animals to be used for immunohistochemistry were sacrificed by cervical dislocation. Freshly-dissected muscles were fresh-frozen in liquid nitrogen-cooled iso-pentane and stored at -80°C until cryostat sectioning. In some cases muscles were embedded in OCT mounting medium (Agar Scientific, Essex, UK) and stored at -80°C until use.

For newborn tissue isolation, pups were decapitated and the skin was removed from the limbs. The limbs were then detached from the trunk and either fresh-frozen or embedded in OCT and stored at -80°C, to be sectioned later.

For embryonic tissue isolation, time-mated mothers were sacrificed by CO₂ asphyxiation followed by cervical dislocation. For embryos younger than embryonic day 16 (E16), embryos were fixed briefly in 4% PFA for 10-15 minutes, taken through two PBS washes (15 minutes each) and dehydrated through a graded ethanol series: 30%, 50%, 70%, 90% into absolute ethanol (10-40 minutes per round) for storage at 4°C ahead of preparation for vibratome sectioning. For E16 embryos and older, as much of the skin as possible was removed from the limbs without damaging the underlying musculature. The limbs were detached from the trunk and mounted in either the transverse or longitudinal plane in OCT and stored at -80°C until use.

2.3.1 X-gal staining

Animals to be used for X-gal staining were sacrificed by CO₂ asphyxiation and transcardially perfused with 4% paraformaldehyde (PFA, pH7.4, prepared in PBS). After removal of skin, superficial fascia, and internal organs, they were post-fixed in 0.5% glutaraldehyde::2% PFA solution for 45 minutes at room temperature in preparation for X-gal staining. For wholemount X-gal staining, bodies were washed out of post-fix with PBS twice (15 minutes each) and incubated in 0.5µg/mL X-gal (from a 20mg/mL stock dissolved in DMF) diluted into pre-warmed X-gal staining buffer (2mM MgCl₂/5mM K₄Fe(CN)₆/5mM K₃Fe(CN)₆), overnight in a rotating hybrid oven at 37°C.

2.3.2 Succinate dehydrogenase (SDH) stain

Remove frozen cryostat sections from -80°C storage, and place slides into a coplin jar containing freshly-made SDH working solution (1-methoxyphenazine methosulphate [8.4mg], nitroblue tetrazolium (NBT) [30.7mg], disodium succinate stock [0.24M], azide/EDTA/PO₄ buffer stock [10mL], ddH₂O; buffer stock components: sodium azide [12.2mg, disodium EDTA [465.3mg], NaH₂PO₄.H₂O [448.5mg], Na₂HPO₄.7H₂O [5.83g],

ddH₂O, pH adjusted to 7.6). Incubate sections for 5-20 minutes depending on the size of the section. Stop enzymatic reaction by dipping slides once into ddH₂O, and leave to air-dry for 10 minutes in the dark before mounting.

2.3.3 Muscle digestion and fiber isolation

Immediately after sacrifice by cervical dislocation, muscles were dissected tendon-to-tendon and incubated in 0.2% Collagenase I in DMEM 1 (Dulbecco's Modified Eagle Medium) at 37°C for 90 minutes (for DMEM varieties please refer to Table 2.5). Digested muscles were then transferred to 9cm petri dishes (previously coated in 5% Bovine Serum Albumin (BSA)) filled with DMEM 1 for 30 minutes. The muscle bulk was disrupted with a wide BSA-rinsed, round-edged Pasteur pipette to separate the muscle fibers. Healthy, viable fibers were then selected using a narrower BSA-rinsed, round-edged Pasteur pipette and transferred to a fresh DMEM 1-filled petri dish. The healthiest fibers were then selected and transferred to an eppendorf tube containing 4% PFA for fixation at room temperature for 10 minutes. After three 5-minute washes in PBS, the fibers were incubated in X-gal staining solution for 3 hours.

2.3.4 Wholemout staining for Aceytlcholinesterase activity

Entire muscle fiber bundles, immobilized on inert parafilm rods, were fixed in 10% neutral formalin solution buffered with 0.2M acetate for 3 hours at 4°C. After several rinses in dH₂O, muscles were incubated in a solution containing: 2.5% copper sulphate/3.7% glycine/0.2M acetate buffer and the supernatant collected from centrifuging 15mg acetylthiocholine iodide/2.5% copper sulphate added drop-wise/dH₂O at 6g (254rpm) for 15 minutes. After further washes in dH₂O, muscle fiber bundles were stained with 1% ammonium polysulfide for 5 minutes. Muscles were then washed in dH₂O for 3 hours and cleared in glycerol for a further 3 hours. Muscles were either immobilized onto 5% agarose

plates for imaging in wholemount or embedded in 3% agar in preparation for vibratome sectioning. Acetylcholinesterase catalyzes the conversion of the thiocholine ester into thiocholine. This is captured by the copper ions to give a colourless copper thiocholine precipitate. Ammonium polysulfide then converts the colourless copper thiocholine into brown copper sulfide. The brown precipitate is therefore produced directly at the site of acetylcholinesterase activity (Kobayashi et al., 1994).

In some cases, the muscle fiber bundles were incubated with X-gal staining solution prior to staining for acetylcholinesterase.

2.4 Tissue Sectioning

2.4.1 Cryostat

Prior to sectioning, frozen muscles or entire limbs were mounted onto a cryostat chuck with a small amount of OCT. Muscles/limbs embedded in OCT blocks were affixed in the same manner. For sectioning in the transverse plane, individual muscles were cut in the distal-to-proximal direction. Similarly entire limbs were mounted in the same way. For sectioning in the longitudinal plane, limbs were cut in the anterior-to-posterior direction. 12-20µm sections were obtained on a Leica® HM560 freezing microtome, and collected serially at four sections per slide, with every fifth section collected on a separate slide.

Cryostat sections were collected on TESPA (3-aminopropyl-triethoxysilane)-coated slides (Sigma Aldrich, Gillingham, UK). To coat, slides were dipped in acetone for 1 minute, then 4% TESPA in acetone for 30s, followed by quick dips in acetone, fresh acetone and finally dH₂O. The slides were left to dry overnight in a 37°C oven.

2.4.2 Vibratome

In preparation for sectioning, individual muscles or embryos were serially rehydrated through a graded ethanol series (90%, 70%, 50%,

30%). The tissue was transferred to 3% agar in dH₂O for 30 minutes at 80°C and agitated throughout before being blocked in the required orientation. Embryos were embedded to be sectioned in the dorsal-to-ventral direction and individual muscles were embedded to be sectioned in the distal-to-proximal direction. 60-80µm thick sections were obtained using a Leica© 1200 vibrating microtome and stored serially in PBS-filled 48-well plates until use.

2.5 Immunohistochemistry

2.5.1 Antigen retrieval

In some cases, prior to immunolabeling, sections were incubated in 10mM citrate buffer (pH 6.0) containing 0.05% Tween-20 for 10 minutes at room temperature. The sections were then transferred into fresh 10mM citrate/0.05% Tween-20 buffer (pre-warmed to 70°C) and left for a further 15 minutes at 70°C (inverting every 5 minutes to allow ample penetration.) The sections were then left to cool to room temperature and washed twice in PBS (10 minutes per wash.)

2.5.2 Immunolabeling of cryostat sections

For details of antibodies used on tissue sections and their dilutions, please refer to Tables 2.3 and 2.4. Upon thawing tissue sections were left to air dry for 15-20 minutes. In a humidified chamber, the sections were fixed in 1% PFA in PBS for 10 minutes and subsequently washed in PBS three times (10 minutes each.) For fiber-type subtype specific antibodies, sections were fixed again in 100% methanol (MeOH) for 8 minutes at -20°C, followed by three more 10-minute washes in PBS. For pan fiber-type antibodies, sections were taken straight to blocking. Non-specific binding sites were blocked with 10% Normal Goat Serum (NGS) (Invitrogen, Paisley, UK) diluted in PBST (PBS containing 0.5% Tween-20) for 1 hour at room temperature. Following blocking, sections were

incubated with primary antibodies (Table 2.3) diluted in PBST overnight at 4°C. The next day, sections were washed 3 times with PBST, each wash lasting 10 minutes. Sections were then incubated with the relevant secondary antibodies (Table 2.4) diluted in PBST for 1 hour at room temperature. These were conjugated to either Alexa-488 or -568 or -Biotin if signal amplification was required. In the case of the latter, sections were washed again 3 times in PBST (10 minutes per wash) and the sections were incubated with Streptavidin conjugated to either Cy2 or TexasRed for 1 hour at room temperature. After incubation with the relevant secondary or tertiary antibodies, the sections were washed 3 times with PBS (5 minutes per wash) and counterstained with 5µg/mL Hoechst in PBS for 10 minutes, before mounting in Vectashield (Vector Laboratories, Peterborough, UK). Coverslips were sealed with clear nail enamel.

2.5.2.1 Haematoxylin and eosin staining

Tissue slides were arranged in a staining rack and dipped into Mayer's Haemalum solution (Merck Millipore, Darmstadt, Germany) for 3-10 minutes. Slides were rinsed under running tap water for 20 minutes (min.) and transferred to 0.1% Eosin (Merck Millipore) solution for 5-12 minutes. After another rinse under running tap water for 10 minutes (max.) slides were dehydrated via quick dips through an increasing ethanol (EtOH) concentration series: 70%; 80%; 95%; 100%; 100% (fresh.) Slides were placed into Xylene (or Clerene substitute) solution for 7 minutes, and subsequently transferred into fresh Xylene (or Clerene) solution for a further 7 minutes. Slides were mounted with DPX-mounting medium.

2.5.3 Immunolabeling of vibratome sections

Immunolabeling of vibratome sections was carried out in small glass vials. If required, antigen retrieval was carried out before blocking. Non-specific binding sites were blocked with 20% NGS and 1% Triton X-100 in

PBS, for 2 hours at room temperature. The sections were then incubated with primary antibodies diluted in PBS containing 0.2% NGS and 0.1% Triton X-100 overnight at 4°C. The next day, sections were washed 5 times (1 hour per wash) with 0.2% NGS and 0.1% Triton-X100 in PBS at room temperature. Sections were then incubated with secondary antibodies (as described in section 2.5.2.1) overnight at 4°C. In the case of Biotin amplification, sections were washed 5 times (1 hour per wash) in 0.2% NGS and 0.1% Triton X-100 in PBS and incubated overnight at 4°C on the third day with the relevant Streptavidin complexes. Before mounting, sections were rinsed 6 times (30 minutes per wash) in PBS and incubated with 5µg/mL Hoechst in PBS for 10 minutes. Coverslips were sealed with clear nail enamel.

On some X-gal stained sections, peroxidase labeling was carried out. Prior to blocking, sections were treated with 6% Hydrogen peroxide (H₂O₂) in PBS for 1.5 hours at room temperature to bleach endogenous peroxidases. Sections were taken through the immunolabeling procedure outlined above and incubated with HRP-conjugated secondary antibodies on day 2. After the last PBS wash, sections were washed three times in 0.05M Tris-HCL (pH 8.0) with each wash lasting 20 minutes. The staining was then visualized with 3,3' diaminobezidine (DAB, Vector Laboratories.) The sections were mounted with a glycerol-based mounting medium (10% gelatin in dH₂O mixed 1:1 with glycerol.) For details of antibodies used on tissue sections and their dilutions, please refer to Tables 2.3 and 2.4.

2.5.3.1 Haematoxylin and eosin staining

Vibratome sections were transferred to a PBS-filled 24-well plate. After two 5 minute washes in 75% EtOH, sections were stained with Mayer's Haemalum solution for 20s only. Immediately after two 30s rinses in 75% EtOH, sections were mounted onto gelatin-coated slides. Mounted slides were arranged into Coplar jars and dehydrated through 90% then 100%

EtOH at 10 minutes each, followed by Histoclear solution for 10 minutes. Slides were mounted with Depex mounting medium.

Table 2.3 - Primary Antibodies

Target	Host	Manufacturer	Catalogue Number	Fixation	Dilution
Actin	Ms	Abcam	ab3280	n/a	1:1000*
β-gal	Ms	Cell Signaling Technology	2372S	4% PFA	1:1000**
β-gal	Rb	Millipore	AB1211	4% PFA	1:1000**
β-gal	Ck	Abcam	Ab9361	1% PFA/ 4% PFA	1:1000**; 1:250- 1:750
Embryonic myosin	Ms	Gift	A4.1025	1% PFA/ 4% PFA	1:40; 1:60**
HA epitope	Ms	Cell Signaling Technology	2367S	4% PFA	1:500- 1:1000*; 1:500a
Laminin α2	Rt	Abcam	ab11576	1% PFA	1:400
Myosin	Ms	DSHB	MF.20	1% PFA/ 4% PFA	1:100
Pax3	Ms	DSHB	Pax3	4% PFA	1:200**
Pax7	Ms	DSHB	Pax7	1% PFA	1:80
Pax7	Ms	R&D Systems	MAB1675	1% PFA	1:80
PHH3 (Phospho-histone 3)	Rb	Cell Signaling Technology	9701	4% PFA	1:50
Tenascin C	Rb	Abcam	ab108930	1% PFA	1:250***
Type 1 MyHC (MHCs)	Ms	Leica Biosystems	NCL-MHCs	1% PFA/ MeOH	1:40
Type 2 MyHC (MHCf)	Ms	Leica Biosystems	NCL-MHCf	1% PFA/ MeOH	1:60
Type 1 MyHC	Ms	DSHB	A4.840	1% PFA/ MeOH	1:40
Type 2A MyHC	Ms	DSHB	A4.74	1% PFA/ MeOH	1:40
Type 2B MyHC	Ms	DSHB	BF.F3	1% PFA/ MeOH	1:40

Host species abbreviations: Chicken (Ck); Mouse (Ms); Rabbit (Rb); Rat (Rt)

Unless otherwise stated, all dilutions apply to immunolabeling of cryostat sections.

*Dilutions for Western Blot.

**Dilutions for vibratome sections.

***Tissue sections were blocked in 5% Bovine Serum Albumin (BSA) in PBST as opposed to NGS blocking agent.

a - Dilution for immunocytochemistry.

Table 2.4 - Secondary Antibodies

Host	Target	Conjugation	Manufacturer	Catalogue Number	Dilution
Gt	Ms	AlexaFluor®488	Invitrogen	A-11001	1:1000
Gt	Ms	AlexaFluor®568	Invitrogen	A-11004	1:1000
Gt	Ms IgG ₁	AlexaFluor®488	Invitrogen	A-21121	1:200
Gt	Ms IgM	AlexaFluor®488	Invitrogen	A-21042	1:200
Gt	Ms IgG _{2a}	Biotin	Jackson Labs	115-065-206	1:300
Gt	Ms IgG ₁	Biotin	Jackson Labs	115-065-205	1:300
Gt	Ms IgG	Biotin	Jackson Labs	115-066-146	1:300
Gt	Rb	AlexaFluor®488	Invitrogen	A-11008	1:1000
Gt	Rb	AlexaFluor®568	Invitrogen	A-11011	1:1000
Gt	Rb	HRP	Sigma Aldrich	A-9169	1:1000
Gt	Ms	HRP	Sigma Aldrich	A-4416	1:1000
Gt	Rt	488	Invitrogen	A-11006	1:1000
Gt	Ck	AlexaFluor®568	Invitrogen	A-11041	1:500*; 1:1000
-	F-actin	AlexaFluor®488 Phalloidin	Invitrogen	A12379	1:200
N/A		Streptavidin-Cy2	Jackson Labs	016-220-084	1:800
N/A		Streptavidin-Texas Red	Jackson Labs	016-070-084	1:800

2.6 Cell Culture

2.6.1 Cell lines

Four cell lines were used during the course of the investigations. These were C2C12 cells, ARPE-19 cells, MEF cells and MIMCD-3 cells and their media requirements are summarized in Table 2.5.

C2C12 cells (a gift from Dr Peter Zammit, King's College London) are derived from a mouse myoblast cell line originally established from cultured thigh muscle myoblasts. They comprise a pure myoblast population that proliferate rapidly and can be induced to differentiate into myotubes upon serum-starvation (Blau et al., 1985; Grabowska et al., 2011; Yaffe and Saxel, 1977). Proliferating C2C12s were maintained in DMEM-2, differentiation into myotubes was achieved by culturing the myoblasts in DMEM-3. ARPE-19 cells are human retinal pigment

epithelial (RPE) cells The cell line was established from a primary RPE culture giving rise to a uniform population of highly proliferative epithelial cells (Dunn et al., 1996). These cells were cultured in DMEM-4. MIMCD-3 cells are inner medullary collecting duct epithelial cells, originally derived from an SV40 transgenic mouse (Rauchman et al., 1993). MIMCD-3 cells were cultured in DMEM-5. Both lines kindly donated by Dr Deborah Goldspink (UEA). Mouse embryonic fibroblasts (MEFs) were maintained in DMEM-6 and kindly donated by Dr Matthew Whelband (UEA).

All cells were maintained in T75cm² flasks, and incubated at 37°C and 5%CO₂. They were passaged as required with 0.25% Trypsin-EDTA (1X) (Invitrogen).

Table 2.5a - Varieties of Dulbecco's Modified Eagle Medium (DMEM) used in cell culture.

DMEM	Manufacturer	Catalogue Number	Formulation	Supplements
1	Invitrogen	10938-025	- High glucose (4.5g/L) - Non Essential Amino Acids (NEAA) - No glutatmine - Phenol red	D (2%); E
2	Invitrogen	10938-025	- High glucose (4.5g/L) - Non Essential Amino Acids (NEAA) - No glutatmine - Phenol red	A; D; E
3	Invitrogen	10938-025	- High glucose (4.5g/L) - Non Essential Amino Acids (NEAA) - No glutatmine - Phenol red	B; D; E
4	Invitrogen	31330-038	- High glucose (4.5g/L) - Non Essential Amino Acids (NEAA) - Ham's F-12 - HEPES - L-glutatmine - Phenol red	E; F
5	Invitrogen	31331-028	- High glucose (4.5g/L) - Non Essential Amino Acids (NEAA) - Glutamax - Phenol red	A
6	Invitrogen	10938-025	- High glucose (4.5g/L) - Non Essential Amino Acids (NEAA) - No glutatmine - Phenol red	A

Table 2.5b: Supplements for DMEM varieties.			
Supplement	Percentage (per 500mL DMEM)	Manufacturer	Catalogue Number
A. Fetal Bovine Serum (FBS) Heat inactivated	10%	GE Healthcare	A15-104
B. Horse serum (HS) Heat inactivated	2%	Invitrogen	26050-070
C. Newborn Calf Serum (NCS) Heat inactivated	10%	Invitrogen	26010-074
D. L-Glutamine (L-Glut) [200mM]	1% or 2%	Invitrogen	25030-024
E. Penicillin-Streptomycin (P/S) [10,000U/L]	1%	Invitrogen	15140-122
F. Gentamicin [50mg/mL]	0.001%	Sigma Aldrich	G1397

2.6.2 Coverslip coating

In some instances, cells were seeded onto 13mm glass coverslips in 24-well plates in order to facilitate easier visualization of fluorescently labeled cells. To improve cell adherence to this surface, the coverslips were coated aseptically in a poly-D-lysine (PDL) solution. PDL (Sigma Aldrich) stock solution (2mg/mL) was diluted 1:100 into sterile water and applied to single coverslips in 24-well plates. The plates were then transferred to the incubator for 2 hours before the PDL solution was removed and the plates were left to air-dry (lids removed) in a Tissue Culture hood.

2.6.3 Immunocytochemistry

In preparation for immunolabeling, media was removed from the well plates and the cells were rinsed in PBS several times. Following this, cells were fixed in 4% PFA in PBS for 10 minutes at room temperature.

After a 5-minute PBS rinse, the cells were permeabilised in 1% NP-40 in PBS for another 5 minutes, and this was followed by another 5-minute PBS rinse. Cells were blocked in PBS containing 10% NGS for an hour at room temperature before incubation with primary antibodies, diluted in 0.2% NGS in PBS, overnight at 4°C. The next day, cells underwent three 5-minute PBS washes. This was followed by a 1 hour incubation with fluorescently-conjugated secondary antibodies diluted in 0.2% NGS in PBS at room temperature. After another couple of 5-minute rinses in PBS, cells were incubated with 5µg/mL Hoechst in PBS for 10 minutes, before mounting in Vectashield. The edges of the coverslips were sealed with clear nail enamel.

2.6.4 X-gal stain

After media was removed from the cells and they were rinsed thoroughly in PBS, they were fixed in 0.5% glutaraldehyde (Sigma Aldrich) in PBS for 15 minutes at room temperature. After further PBS rinses, the cells were incubated in X-gal staining solution at 37°C until β-gal expressing cells turned blue (approximately 1-2 hours).

2.7 Microscopy

Fluorescently-labelled sections or cells were imaged on a Zeiss© Axio Imager.M2, and images were acquired and processed using Axiovision© 3.8 software. Wholemount tissue was imaged using an upright microscope, as were X-gal stained cells or tissues with a differential interference contrast (DIC) filter. Fluorescently labeled, intact tissues were visualized using a Zeiss© SteREO Lumar.V12, at low magnification. Cultured cells were imaged using an Axiovert 40 CFL. Composite images were put together using Adobe Photoshop©. All cell counts and measurements were made using ImageJ© software.

2.8 Reverse-Transcriptase PCR (RT-PCR) reaction

2.8.1 RNA extraction and DNase treatment

To extract RNA from cells, whole T75cm² flasks were trypsinized with 0.25% Trypsin (Invitrogen) and the cell suspension was spun down at 800rpm for 5 minutes. The cell pellet was re-suspended in 0.5-1mL TRI-reagent (Sigma Aldrich) and 110µL chloroform was added to the solution. The sample was shaken vigorously for 15s and incubated on ice for 3 minutes. The resulting mixture was then centrifuged at 12,000rpm for 15 minutes at 4°C to separate the RNA, DNA and protein-containing phases. The upper aqueous phase was then transferred to a fresh eppendorf tube and 100µL (per 500µL TRI-reagent) 95% EtOH in ddH₂O was added to it and mixed gently. The total volume was then transferred to spin columns, part of the SV Total RNA Isolation System© (Promega, Mannheim, Germany). The columns were centrifuged for 1 minute at 12,000rpm at 4°C. A fresh DNase mix (40µL Yellow Core buffer; 5µL 0.09M MnCl₂; 5µL DNase I) was applied to each column and incubated for 15 minutes at room temperature. 200µL SV DNase STOP solution was then added to the columns and they were subsequently centrifuged at 12,000rpm for 1 minute at 4°C. After addition of 250µL SV RNA Wash solution, the columns were centrifuged again at 13,500rpm for 2 minutes at 4°C. RNA was eluted in 20-50µL nuclease-free H₂O.

To isolate RNA from cells for qRT-PCR assays, T75cm² flasks were trypsinized with 0.25% Trypsin (Invitrogen) and the cell suspension was spun down at 800rpm for 5 minutes. The cell pellet was re-suspended in 600µL Buffer RLT from the RNeasy Plus Mini Kit© (Qiagen, Manchester, UK), and RNA was extracted as per the manufacturer's protocol. RNA was eluted in 40µL nuclease-free H₂O.

All tissue used for qRT-PCR analyses was snap-frozen after dissection. Total RNA was isolated from these muscles using the RNeasy

Fibrous Tissue Mini Kit© (Qiagen) and eluted in 50µL nuclease-free H₂O. RNA purity and concentration was assessed using a Nanodrop spectrophotometer.

2.8.2 RT-PCR

Two-step RT-PCR was carried out using Illustra Ready-To-Go RT-PCR beads (GE Healthcare©) according to manufacturer's instructions, using 1µg template RNA. Please refer to Table 2.6 for the RT-PCR cycles, and Tables 2.7 and 2.8 for the sequences of the primers used to detect FGF ligands and FGF receptors respectively. Primers used to amplify Fgfs1-8 are as those published in (Kastner et al., 2000; Ozawa et al., 1997). Primers used to amplify Fgfs9-21 and -23 are as published in (Hajihosseini and Heath, 2002). Primer sequences for Fgf-22 are as published in (Zhong et al., 2006), and the sequences for β-actin are as published in (Wylie and Chantler, 2001). Samples were resolved on 1.5% agarose in TBE gels and visualized under UV illumination.

Table 2.6 – Cycles used during RT-PCR					
Reverse Transcription			Polymerase Chain Reaction		
Temperature (°C)	Length (min)	Number of cycles	Temperature (°C)	Length (min)	Number of cycles
42	15	1	95	0.5	35
95	5		56	0.5	
			72	1	

Table 2.7 – Primer pairs to detect Fgf ligands.		
Gene	Primer sequences (5'-3')	Product size (bp)
Fgf-3	GAT GGG CCT GAT CTG GCT TCT GCT	573
	CAC CAT CTC ATG GTC CTT GTG GCC	
Fgf-7	CAA TCT ACA ATT CAC AGA	622
	AGC CCC TTT TGA TTT AAG	
Fgf-10	CTC TTT TTG GTG TCT TCG TTC CC	228
	CGC TGA CCT TGC CGT TCT TCT C	
Fgf-22	CTA TGG GTC GCG GGT CTA C	171
	AGT TGG TGC CGT CGT GTC CGT	
β-actin	TGT GAT GGT GGG AAT GGG TCA G	514
	TTT GAT GTC ACG CAC GAT TTC C	

Table 2.8 – Primer pairs to detect FGF receptor isoforms.		
Gene	Primer sequences (5'-3')	Product size (bp)
FGFR1-IIIb	GCA GGG CTG CCT GCC AAC GAG ACA GTG	300
	GGT CTG GTG CAG TGA GCC ACG CAG ACT G	
FGFR1-IIIc	GCA GGG CTG CCT GCC AAC GAG ACA GTG	306
	GAA CGG TCA ACC ATG CAG AGT GAT GG	
FGFR2-IIIb	CCC ATC CTC CAA GCT GGA CTG CCT	317
	CTG TTT GGG CAG GAC AGT GAG CCA	
FGFR2-IIIc	CCC ATC CTC CAA GCT GGA CTG CCT	310
	CAG AAC TGT CAA CAA TGC AGA GTG	
FGFR3-IIIb	GAC AGA CAC ACG GAT GTG CTG GA	350
	GTG AAC ACG CAG CAA AAG GCT TT	
FGFR3-IIIc	GAC AGA CAC ACG GAT GTG CTG GA	348
	AGC ACC ACC AGC CAC GCA GAG	
FGFR4	TAC AGC TAT CTC CTG GAT GTG CTG	195
	GAA ACC GTC GGC GCC GAA GCT GCT	

2.9 Western Blot

2.9.1 Protein extraction and sample preparation

To extract protein from cells in T75cm² flasks or 6-well plates, the flasks/plates were placed on ice and the media was removed. After three rinses in ice-cold PBS, the cells were lysed in ice-cold RIPA lysis buffer

(50mM Tris-HCL (pH 7.4); 150mM NaCl; 2.5mM MgCl₂; 1% (v/v) NP-40; 0.05% (v/v) Triton X-100 and 0.5% (w/v) sodium deoxycholate) containing a freshly-added protease inhibitor cocktail (Thermo Fisher Scientific, Hemel Hempstead, UK), diluted 1:100. The lysate was then centrifuged at 13,000rpm for 15 minutes at 4°C and the supernatant transferred to a fresh eppendorf tube. After an additional spin to ensure full clearing of the lysate, protein concentration was measured using the Pierce BCA Protein Assay Kit© (Thermo Fisher Scientific) in a 96-well plate, according to Manufacturer's protocol.

To extract protein from entire muscles, tissue samples (previously snap-frozen in liquid nitrogen) were thawed on ice in 600µL-1mL ice-cold RIPA lysis buffer with freshly-added protease inhibitors. The samples were transferred to round-bottomed 2mL snap-lid tubes and a sterile stainless steel ball bearing was added each tube. The samples were then homogenized in a Qiagen© TissueLyser II for 2 minutes or until the bulk of the muscle had visibly been broken down. The lysates were centrifuged at 12,000rpm for 10 minutes at 4°C and the supernatant transferred to a fresh eppendorf tube. To ensure an even more thorough homogenization, the supernatant was sonicated at 15V in 30s pulses (and placed on ice between pulses to keep samples as cold as possible) for a maximum of 5 minutes. Protein concentration was measured using the Pierce® BCA Protein Assay Kit as outlined above.

20-45µg protein lysate was mixed with 5X Lamelli buffer (625mM TRIS-HCL (pH 6.8); 2% (w/v) SDS; 10% (v/v) glycerol; bromophenol blue) and ddH₂O to a final volume of 40µL. All samples were denatured by boiling at 95°C for 2 minutes prior to loading.

2.9.2 SDS-PAGE and protein transfer

1mm-thick PolyAcrylamide Gel Electrophoresis (PAGE) gels were prepared using Mini-PROTEAN® glass plates and casting frames (BioRad, Hemel Hempstead, UK). Running gels were prepared at 8-10% with 40% Acrylamide (BioRad), running gel buffer (1.5M Tris-HCL (pH 8.8); 0.4% (w/v) SDS in ddH₂O), ddH₂O and the polymerizing agents ammonium persulfate (AMPS; Sigma Aldrich) and *N,N,N',N'*-Tetramethylethylenediamine or TEMED (Sigma Aldrich). 5% stacking gels were prepared with 40% acrylamide, stacking gel buffer (0.5M Tris-HCL (pH6.8); 0.4% (w/v) SDS in ddH₂O), ddH₂O, AMPS and TEMED. AMPS was freshly prepared as a 10% (w/v) solution in ddH₂O for each gel. The quantities of these reagents are outlined in Table 2.9.

Table 2.9 – Volumes of components of running and stacking gels for Western Blotting.		
Running Gel	8%	10%
40% Acrylamide	3.6mL	4.5mL
Running Gel Buffer	4.5mL	4.5mL
ddH ₂ O	9.9mL	9.0mL
10% AMPS	108μL	108μL
TEMED	21μL	21μL
Stacking Gel	5%	
40% Acrylamide	1mL	
Stacking Gel Buffer	2mL	
ddH ₂ O	5mL	
10% AMPS	72μL	
TEMED	14μL	

All components of the running gel were combined, except for AMPS and TEMED, which were added immediately prior to setting. Upon addition of these agents, the top of the gel was levelled off with ddH₂O and allowed to set. The water was then poured away and any excess carefully removed with filter paper. The stacking gel was prepared in exactly the same way with the addition of a comb. The set gels were then placed in a Mini-

PROTEAN® Tetra cell and the tank was filled with reducing migration buffer (0.25M Tris-BASE; 1.92M Glycine; 1% (w/v) SDS in 5L ddH₂O), diluted 1:10 from a 10X stock. Protein samples were loaded alongside 10µL of PageRuler Plus Prestained Protein Ladder® (Thermo Fisher Scientific) using a micro-syringe, and run at 200V at room temperature for 40 minutes.

During gel running, the reagents required for protein transfer were prepared. Pieces of extra thick blot paper and 0.45µm nitrocellulose membrane (BioRad) were cut to size and soaked in transfer buffer (0.048 Tris-BASE; 0.039M Glycine; 0.0375% (w/v) SDS; 20% MeOH in 1L ddH₂O) in square petri dishes. After running, the PAGE gels were gently removed from the glass plates and the stacking gel was carefully removed with a sharp blade. These were also soaked in transfer buffer for a minimum of 10 minutes. To assemble the transfer stack, a piece of filter paper was dried very briefly and the nitrocellulose membrane was placed directly on top of it. The gel was then placed on top of the membrane and this was covered with a second piece of filter paper. Extra care was taken to carefully roll out any air bubbles at every step of this assembly to maximise protein transfer efficiency. The transfer stack was then placed in a semi-dry transfer cell (BioRad) and run at 15V for 35 minutes.

2.9.3 Immunoblotting (Western blotting)

After transfer, the membrane was blocked in PBST solution containing 10% (w/v) milk powder in square petri dishes, for 2 hours at room temperature. Following blocking, the membrane was incubated in primary antibody solution (diluted in PBST with 0.5% (w/v) milk), either in a square petri dish or in a clear plastic bag depending on antibody dilution, overnight at 4°C.

The next day, the membrane was allowed to return to room temperature before several washes in PBST with 0.5% milk for 1 hour.

The membrane was then incubated with HRP-conjugated secondary antibodies diluted in the same solution for 1 hour at room temperature. Following this, the membrane was washed again in PBST with 0.5% milk for 30 minutes.

2.9.4 ECL detection

The ECL reagents comprised two solutions to facilitate visualization of the protein bands. 45 μ L of coumaric acid (15mg/mL prepared in DMSO; Sigma Aldrich) and 100 μ L of luminol (44mg/mL prepared in DMSO; Sigma Aldrich) were added to 10mL of 100mM Tris-HCL to make ECL solution A. 6 μ L of 30% hydrogen peroxide (H₂O₂) was added to the same amount to make ECL solution B. Prior to detection, solutions A and B were mixed and added to the membrane to incubate for 1 minute. The membrane was then exposed to hyperfilm (GE Healthcare) for varying lengths of time depending on quality of detection, before development.

In some cases, the membranes were stripped and re-blotted. This was achieved by adding a re-blot solution (10X ReBlot Plus Strong Antibody Stripping Solution (Merck Millipore) diluted 1:10 into ddH₂O) to the membrane for 10 minutes. After three 5-minute washes in PBST, the membrane was incubated with another primary antibody.

2.10 RNA *in situ* hybridization

In preparation for *in situ* hybridization, tissues were isolated under RNase-free conditions and fixed overnight in 4% PFA at 4°C. Tissues were then blocked in paraffin and 7 μ m-thick sections were cut on a microtome. Briefly, sections were de-waxed in Xylene, dehydrated in 100% EtOH, and subsequently rehydrated. Sections were then fixed in 4% PFA for 5 minutes. Sections were prehybridized in a pre-hybridization solution (50% formamide; 5 x SSC; 1 x Denhardt's solution, 0.1% Tween-20, DEPC-water) for 2 hours at 60-65°C in a humidified chamber. Following a wash in 0.1M Triethanolamine (+0.25% acetic anhydride),

35S[UTP]-labelled riboprobes antisense to *Fgf10* (584bp) and *FgfR2-IIIb* (161bp) were applied to the sections, and left to incubate in a humidified chamber overnight at 60-65°C. The next day, the probes were washed off and the sections were dehydrated, and coated in photographic emulsion. Signal was left to develop in the dark for 2-3 weeks. Silver granules were visualized under dark field, and overlay images were processed in Adobe Photoshop®.

2.11 qRT-PCR

2.11.1 Reverse Transcription

For each RNA sample, 500ng of RNA was transcribed. The desired volume of RNA was made up to 17µL with nuclease-free H₂O and 1µL Random Primers (0.25µg/µL) and heated to 70°C for 10 minutes to anneal the primers. The annealed RNA was immediately plunged into ice, and 8µL master mix containing M-MLV Reverse Transcriptase (200U/µL; Promega) 5X M-MLV Buffer (Promega); and 100mM dNTP mix (BioLine) was added to each reaction ahead of primer extension, to give a total volume of 25µL. Please refer to Table 2.10 for quantities of each reagent per reaction. For PCR cycles used during reverse transcription, please refer to Table 2.11. Following primer extension, the cDNA samples were diluted to 0.5ng/µL in nuclease-free H₂O ahead of TaqMan qRT-PCR.

Table 2.10 – Volumes of reagents used per reaction during reverse transcription.	
Reagent	Volume per reaction (µL)
5X M-MLV buffer	5
M-MLV	0.5
125X dNTPs	0.2
Nuclease-free H ₂ O	1.3
<i>Total</i>	7

Table 2.11 - Cycles used during reverse transcription.					
Primer Annealing			Primer Extension		
Temperature (°C)	Length (min)	Number of Cycles	Temperature (°C)	Length (min)	Number of Cycles
70	10	1	42	60	1
			70	10	

2.11.2 qRT-PCR Assay

Quantitative PCR was carried out using TaqMan reagents and the Standard Curve method. The standard curve was made up of six increments: 20ng, 10ng, 5ng, 2.5ng, 1.25ng, 0.625ng. 10µL of diluted cDNA (5ng total) from each sample was loaded into a MicroAmp® Fast Optical 96-well plate (Invitrogen), in duplicate. Similarly 10µL of Standard Curve dilutions were loaded in duplicate. An additional 'no template control' was loaded for each gene assayed, using 10µL of nuclease-free water. Primers and probes for *Fgf10*, *Myh3* and *Myh8* were designed using the Universal Probe Library (Roche Applied Science) for the qRT-PCR assays. *18S*, *β-actin* and *Gapdh* were used as reference genes. Primer sequences to detect *Fgf10* are as published in *El Agha et al., 2012*, those for *Myh8* (neonatal myosin) as in (Evans et al., 2008), and those for *18S* as detailed in (Corps et al., 2006). Details of the primer sequences and UPL probes used can be found in Table 2.12. Primers and probes were prepared as separate mastermixes using FastStart Universal Probe mastermix© (Roche Applied Science) for each gene assayed and 10µL of primer/probe mix was added to each well. A separate primer/probe mix from Primer Design© was used to detect *Gapdh* and *β-actin*. Please refer to Table 2.13 for the volumes of reagents used in the mastermixes. All qRT-PCR assays were run on an Applied Biosystems 7500 Fast Real-Time PCR machine. The results were analyzed using the $\Delta\Delta C_T$ relative quantification method.

Table 2.12 – Primer sequences and compatible UPL probes used during qRT-PCR assays.

Gene	Primer Sequences (5'-3')	UPL Probe
<i>Fgf10</i>	ATG ACT GTT GAC ATC AGA CTC CTT	63
	CAC TGT TCA GCC TTT TGA GGA	
<i>Myh8</i>	ACA CAT CTT GCA GAG GAA GG	41
	TAA CCC AGA GAG GCA AGT G	
<i>18S</i>	Primer Sequences (5'-3')	Probe Sequence
	GCC GCT AGA GGT GAA	FAM- ACCGGCGCAAGACGGACCAG- TAMRA
	ATT CTT G	
	CAT TCT TGG CAA ATG CTT	
	TCG	

Table 2.13 – Volumes of reagents used per reaction in qRT-PCR assays.

Reagent	Fgf10, Myh8, 18S	Reagent	GAPDH
Mastermix	10	Mastermix	10
Primer FOR	0.5	Primer/Probe mix	1
Primer REV	0.5		
Probe	0.25		
Nuclease-free H ₂ O	3.75	Nuclease-free H ₂ O	4
<i>Total</i>	15	<i>Total</i>	15

2.12 Skeletal staining with Alcian Blue and Alizarin Red

In preparation for skeletal staining, the skin was gently peeled away from the bodies of newborn pups to expose the underlying tissue. Extra care was taken around the limbs to preserve the digits. As many of the internal organs as possible were also removed. To stain for cartilage, an Alcian blue solution was prepared (80mL of 95% EtOH; 20mL glacial acetic acid and 15mg Alcian blue crystals in 100mL ddH₂O). Bodies were incubated in Alcian blue in glass vials for 6-8 hours at room temperature. During this, and in all subsequent steps, the glass vials were placed on a Stuart© Roller Mixer SRT6D and rolled gently. The Alcian blue solution was then poured off and the bodies underwent several 1-hour rinses in 95% EtOH in ddH₂O. The bodies were then placed in 1% (w/v) KOH in ddH₂O overnight. The next day, bodies were rinsed a few times with 0.5% KOH. They were then placed into fresh 0.5% KOH containing 50µL Alizarin Red (from a 15mg/mL stock in ddH₂O; Sigma Aldrich) overnight.

The following day, Alizarin red solution was poured away and excess stain was removed with several washes in 0.5% KOH. Once the background/excess stain had faded, bodies were switched into a 0.25% KOH/20% (v/v) glycerol for long-term storage.

2.13 Generation of an Fgf10-HA tagged overexpression vector.

Fgf10-HA construct design, generation and verification were kindly carried out by Marta Mikolajczak.

2.13.1 Linearization of the pmCherry-N1 vector.

To subclone the Fgf10-HA tagged construct into the pmCherry-N1 vector, the mCherry coding sequence was first excised from the vector (Figure 2.2). The multiple cloning site (MCS) in pmCherry-N1 is located between the cytomegalovirus immediate early promoter ($P_{CMV\ IE}$) and the start of the mCherry coding sequence. To excise this coding sequence from the vector, *EcoRI* and *NotI* restriction endonucleases (New England BioLabs, UK) were utilized. The *EcoRI* restriction site is located within the MCS, and *NotI* is located towards the end of the mCherry coding sequence. Treatment of pmCherry-N1 with these restriction endonucleases, overnight at 37°C, generated 'sticky ends' which would later facilitate ligation of the Fgf10-HA construct. After excision of the mCherry coding sequence, the linearized vector (from hereon referred to as pN1) was treated with Calf-intestinal alkaline phosphatase (CIAP; Sigma Aldrich), for 1 hour at 37°C as per manufacturer's instructions, to prevent its re-ligation. pN1 vector DNA was subsequently run on a TAE-agarose gel, the corresponding band excised and purified from the gel using a GeneClean kit (MP Bio) as per manufacturer's instructions.

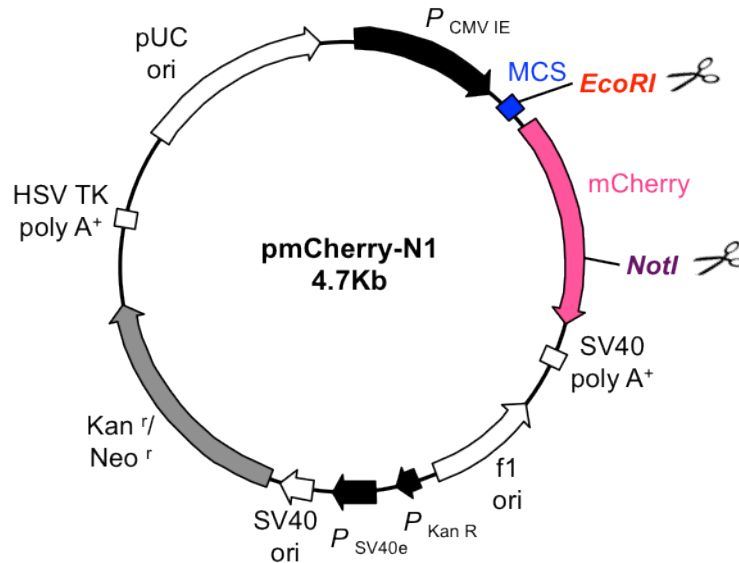


Figure 2.2: Restriction map of the pmCherry-N1 mammalian vector. The mammalian expression vector contains the mutant fluorescent protein mCherry. **mCherry** coding sequence was excised from the vector using *EcoRI* and *NotI* restriction endonucleases. The vector also contains a neomycin-resistance cassette (Neo^r) consisting of an SV40 early promoter (P_{SV40e}), kanamycin/neomycin resistance gene, and polyadenylation signals from the herpes simplex virus thymidine kinase (HSVTK) gene. The bacterial promoter ($P_{\text{Kan R}}$) confers resistance in *E.coli*. SV40 and pUC origins in the vector also permit its propagation within mammalian and bacterial cells respectively. Adapted from Clontech Laboratories Inc Vector Information.

2.13.2 Generation of the Fgf10-HA tagged construct.

Rat Fgf10 cDNA was amplified by Expand Long Template PCR (Roche Applied Science) from a pGEM-T vector (Promega, UK) containing this sequence (Figure 2.3), kindly supplied by Dr Saverio Bellusci (Justus-Liebig University Giessen, Germany).

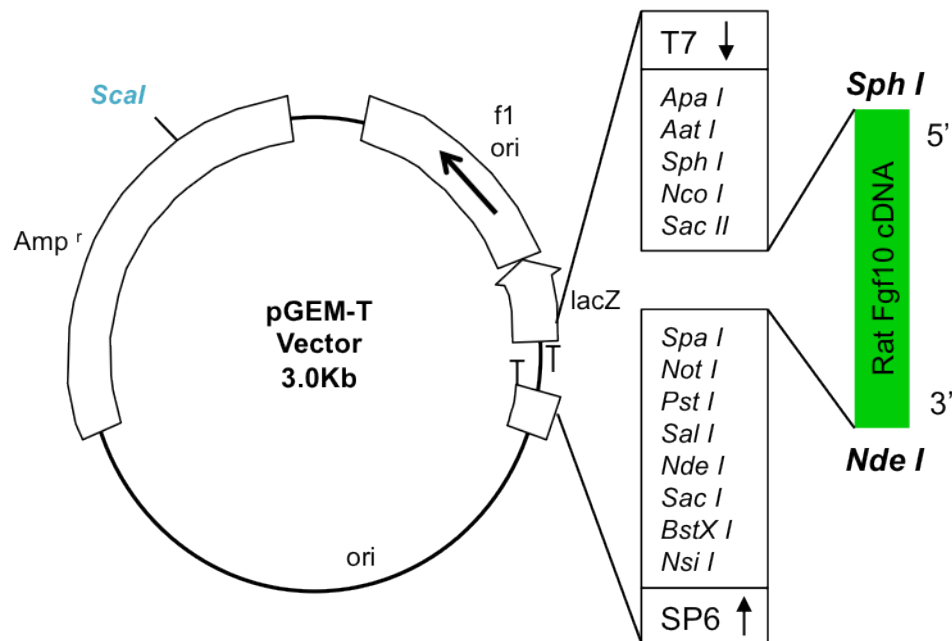


Figure 2.3: Restriction map of pGEM-T vector containing full-length rat Fgf10 cDNA sequence. The rat Fgf10 cDNA sequence is inserted into the pGEM-T vector at *Sph I* and *Nde I* restriction sites. The vector confers resistance to ampicillin. Map adapted from Promega Technical Manual.

In order to create a stable fusion construct, the HA tag was fused at the C-terminus of the Fgf10 coding sequence, joined by a short linker region encoding two small, neutral amino acids, alanine and guanine (Figure 2.4A). To generate the construct, primers were designed to amplify the Fgf10 coding sequence with the HA tag fused at the C-terminus, see Table 2.14. The forwards primer (P1) contained the *EcoRI* restriction site followed by the start of the Fgf10 coding sequence. The reverse primer (P2) contained the end of the Fgf10 coding sequence, short of its stop codon, followed by the linker region and the HA tag coding sequence. The reverse primer also incorporated the *NotI* restriction site. The size of the rFgf10-HA construct is 707bp, which is almost the same size as the excised mCherry sequence. After amplification by PCR, the product was precipitated and digested with *EcoRI* and *NotI* to generate

complementary 'sticky ends' to pN1. The construct DNA was then purified using the GeneClean kit (MP Bio).

A



Table 2.14: Primer sequences used to generate and validate Fgf10-HA tag fusion construct.

Primer	Sequence 5'-3'	T _m (°C)	GC %
P1	GCTTCGAATTCAGATGTGGAAATGGATACTGACACATTG	62	38
P2	GTCAAGCGGCGCTTAAGCGTAATCTGGAACATCGTATG GGTAACCAGCTGAGTGGACCACCATGGGGAGGAAGTG	75	59
P3	GCC TTA AGA TAC ATT GAT GAG TTT GG	72	38
P4	GCC TAC AAC TGC AAC ATC AAG TTG G	74	48

Figure 2.4: Design of the Fgf10-HA tag fusion construct. (A) The HA tag is fused to the C-terminus of the rat Fgf10 coding sequence, joined by a short linker region composed of alanine and guanine residues. The forwards primer (P1) comprises of the *EcoRI* restriction site upstream of the start of the Fgf10 coding sequence. The reverse primer (P2) contains the HA-tag at the C-terminus of the Fgf10 coding sequence, joined by alanine and guanine residues, with a *NotI* restriction site downstream of the HA-tag. A third primer (P3) binds within the pN1 vector itself and was used to validate insertion of the Fgf10-HA construct. P4 binds within the mCherry sequence and was also used to confirm excision of this sequence. Melting temperatures (T_m) and %G-C content of the primer sequences are also displayed. Primers designed by Marta Mikolajczak.

2.13.3 Subcloning the Fgf10-HA construct into the pN1 vector.

Cleaned up vectors and constructs were then incubated with T4 DNA Ligase overnight at room temperature (Sigma Aldrich). The newly-ligated vector was then transformed into competent DH5α *E.coli* cells.

Transformation was carried out by mixing 3-5 μ L ligated DNA with 50 μ L competent cells, leaving on ice for 30mins, then heat-shocking the bacteria for 45s at 42°C. Heat-shocked bacteria were then incubated for 1 hour at 37°C in LB media (without antibiotic), before being plated onto selective kanamycin-containing LB-agar plates, and left overnight at 37°C. Surviving colonies were picked the next day and grown in LB media overnight at 37°C. Plasmid DNA (from hereon referred to as pF10-HA) was then extracted using HiSpeed Mini or Midi Prep Kits as per manufacturer's instructions (Qiagen). DNA concentration was determined using a Nanodrop or spectrophotometer.

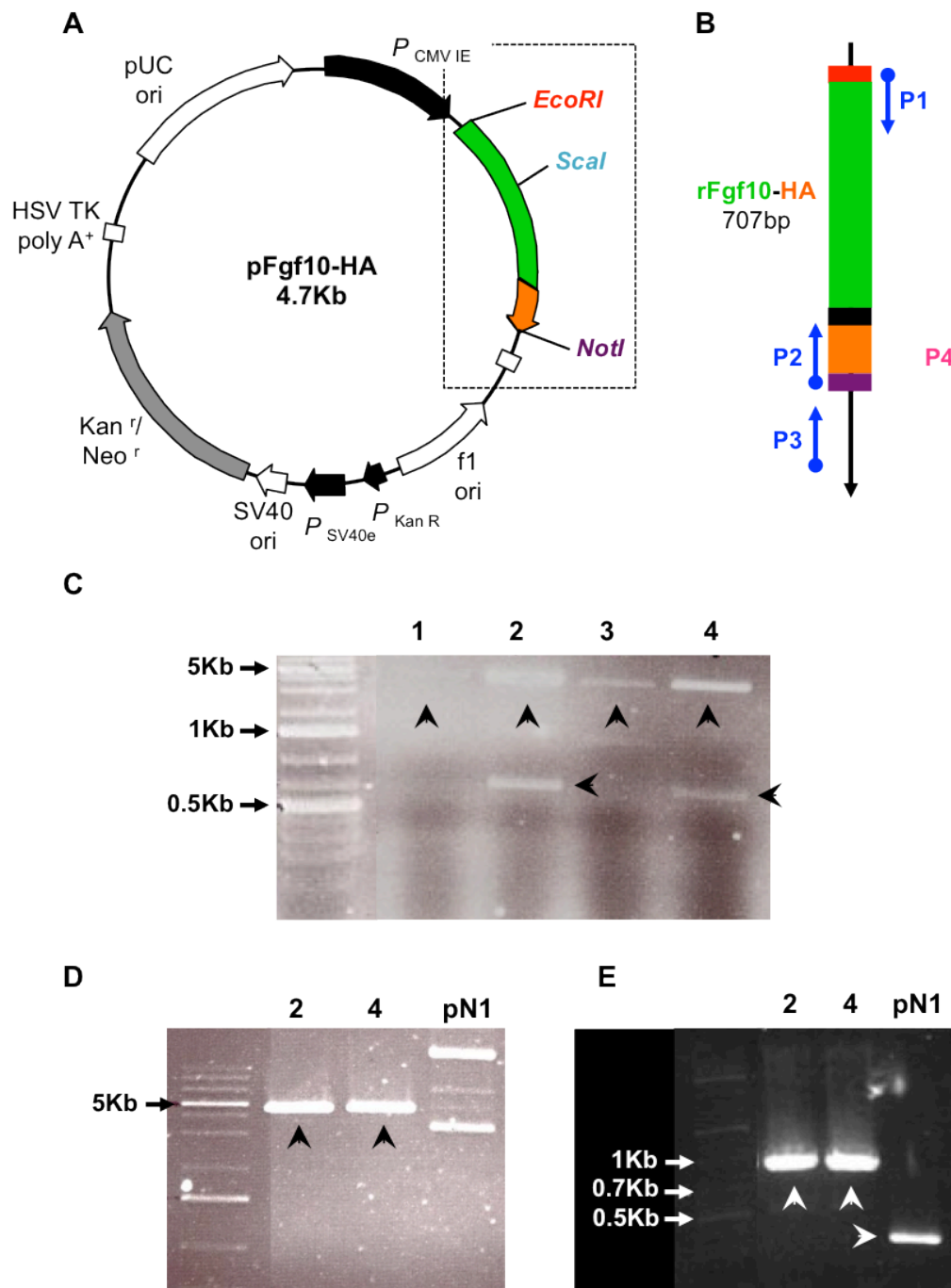
2.13.4 Verification of cloned construct.

To confirm that the Fgf10-HA construct had inserted into the pN1 vector (Figure 2.5A), digestion with restriction enzymes and diagnostic PCRs were carried out. Digestion with *EcoRI* and *NotI* recovered at least two colonies containing the Fgf10-HA insert, evident as a 700bp product (Figure 2.5C), with a band of 4Kb for the rest of the vector. To further verify this, *ScaI* restriction enzyme was used as the Fgf10 coding sequence contains a unique site for this endonuclease. Incubation with this enzyme successfully linearized the vector to give a band of approx. 4.7Kb (Figure 2.5D). In the negative pN1 control (lane 3), which does not contain the construct, this band is not seen. Presence of the construct was also confirmed by PCR. Using P1 and P3, which bind within the insert and on the vector (Figure 2.5B), a band of approximately 940bp is given. Using P3 and P4, which bind within mCherry sequence and the vector, a smaller band of approximately 370bp is produced, which should not be seen if mCherry has been successfully excised and replaced with the construct. Indeed, in both positive samples, the 940bp band corresponding to the insert is detected, but the smaller band

corresponding to the mCherry fragment is not seen. However in the negative pN1 control the opposite is found (Figure 2.5E).

The pF10-HA was found not to contain any mutations after Sanger sequencing was carried out. Sequencing information provided by Marta Mikolajczak (Figure 6.6).

Figure 2.5: Schematic of pF10-HA restriction map and diagnostic digest and PCR results to confirm presence of construct. (A) An HA-epitope has been fused to the C-terminus of the Fgf10 coding sequence and subcloned into a pN1 vector, lacking the mCherry coding sequence. (B) Primer sequence locations to confirm presence of F10-HA insert. P4 binds to the mCherry sequence and was also used in diagnostic PCRs. (C) Digestion of four vector samples with *EcoRI* and *NotI* revealed the insert (707bp band) in samples 2 and 4. (D) Digestion of pF10-HA with *ScaI* in samples 2 and 4 successfully linearized the vector (4.7Kb band). The pN1 vector could not be linearized with this enzyme (lane 3). (E) Using P1 and P3 primers, as outlined in Table 6.3, a fragment of 940bp can be seen for samples 2 and 4. Incorporation of P4 primer, which binds within mCherry, gives a fragment of 370bp. This band can be seen in the pN1 negative control, but not in samples 2 and 4. Gel images in panels C, D and E provided by Marta Mikolajczak.



2.14 Statistical Analysis

All statistical analyses were carried out using Graphpad Prism 6® software. Assuming non-Gaussian distribution of expression data, the unpaired, non-parametric Student's t- or Mann-Whitney test (comparing two groups), or Kruskal-Wallis (comparing three or more groups) tests were used where applicable.

Chapter 3

Expression and localization of *Fgf10*-expressing cells in developing, post-natal and adult skeletal muscles

3.1 Introduction

Fgf10 expression can be detected in several ways, however each method presents a unique set of limitations. At the mRNA level, *in situ* hybridization for *Fgf10* gives robust expression patterns, but these do not necessarily mean the protein is being expressed. There are a number of commercially available antibodies for *Fgf10* (Santa Cruz®, Abcam®, R&D Systems®). These can detect over-expressed FGF within cells, however they are not so reliable for detecting endogenous levels, as judged by immunohistochemistry and western blotting analyses (Kosman et al., 2007). This lack of specificity in immunohistochemical analyses has also been observed in other studies (Memarzadeh et al., 2007)(Mohammad. K. Hajihosseini, unpublished results).

To overcome this issue, a number of reporter strains have been developed to detect *Fgf10*, including the *Fgf10^{nLacZ}* reporter line (Kelly et al., 2001). With this line, the lacZ mRNA transcript is expressed separately, and the β -galactosidase (β -gal) protein product is extremely stable. Using this reporter line is good for detecting *Fgf10*-expressing cells. However, because β -gal is durable, it is also expressed in the descendents of these cells. Thus, this should be taken into consideration when analyzing the timing of *Fgf10* expression.

The genetic hierarchies governing embryonic and fetal myogenesis have been relatively well characterized. However, the mechanisms regulating fiber-type specification and their subsequent maturation during adult life are not completely understood. This chapter will characterize some interesting expression patterns for *Fgf10* in postnatal and adult muscles, indicating potential novel functions for this gene in myogenesis.

3.1.1 Aims

Using the *Fgf10^{nLacZ}* line, novel expression patterns for *Fgf10* in postnatal and adult skeletal muscles were examined. The timing of *Fgf10*

expression in myogenesis during embryonic development was also explored.

3.2 Results

3.2.1 *Fgf10* is expressed in a subset of postnatal and adult skeletal muscles

I investigated *Fgf10* expression in all muscles, including craniofacial muscles, and those of the forelimb and hindlimb. The striking pattern of β -gal (blue) expression was consistently reproduced in a subset of muscles at different adult ages. Moreover, the myofiber populations within these muscles were heterogeneous for β -gal expression. The muscles were designated a score according to their relative proportions of β -gal-expressing myofibers, as judged by eye. These results can be seen in Table 3.1. Within the myofibers themselves, the colour intensity of the β -gal protein in the myonuclei, as revealed by X-gal staining, was also taken into consideration. Some myofibers appeared to be weakly-stained in comparison to others. These strong-to-faint variations in β -gal intensity could reflect current expression in *Fgf10*-expressing cells, or prior expression in their descendants.

3.2.1.1 *Fgf10* expression in craniofacial and neck muscles

Among the craniofacial muscles surveyed, the *digastricus* displays a unique pattern of β -gal expression, as it appears to be solely composed of β -gal-expressing myofibers (Figure 3.1C). The *masseter* muscle also contains a subpopulation of β -gal-expressing myofibers, randomly distributed throughout the muscle bulk (Figure 3.1D). In the neck, the *sternomastoideus* (Figure 3.1E, F) also contains a high proportion of β -gal-positive (β -gal^{+ve}) myofibers. Conversely the *sternohyoideus*, which sits directly underneath the *sternomastoideus*, contains fewer β -gal^{+ve} myofibers (Figure 3.1E, G). Adjacent to the *sternohyoideus*, the posterior belly of the *digastricus* is also solely composed of β -gal^{+ve} myofibers

(Figure 3.1E). Together, these muscles serve to open and close the jaw through head-neck flexion and extension respectively (Haggman-Henrikson et al., 2013). The *temporalis* and *levator labii superioris* muscles also contain a high proportion of β -gal^{+ve} myofibers (data not shown). These muscles function to close the jaw and elevate the lips respectively (Cox et al., 2012; Ishida et al., 2010).

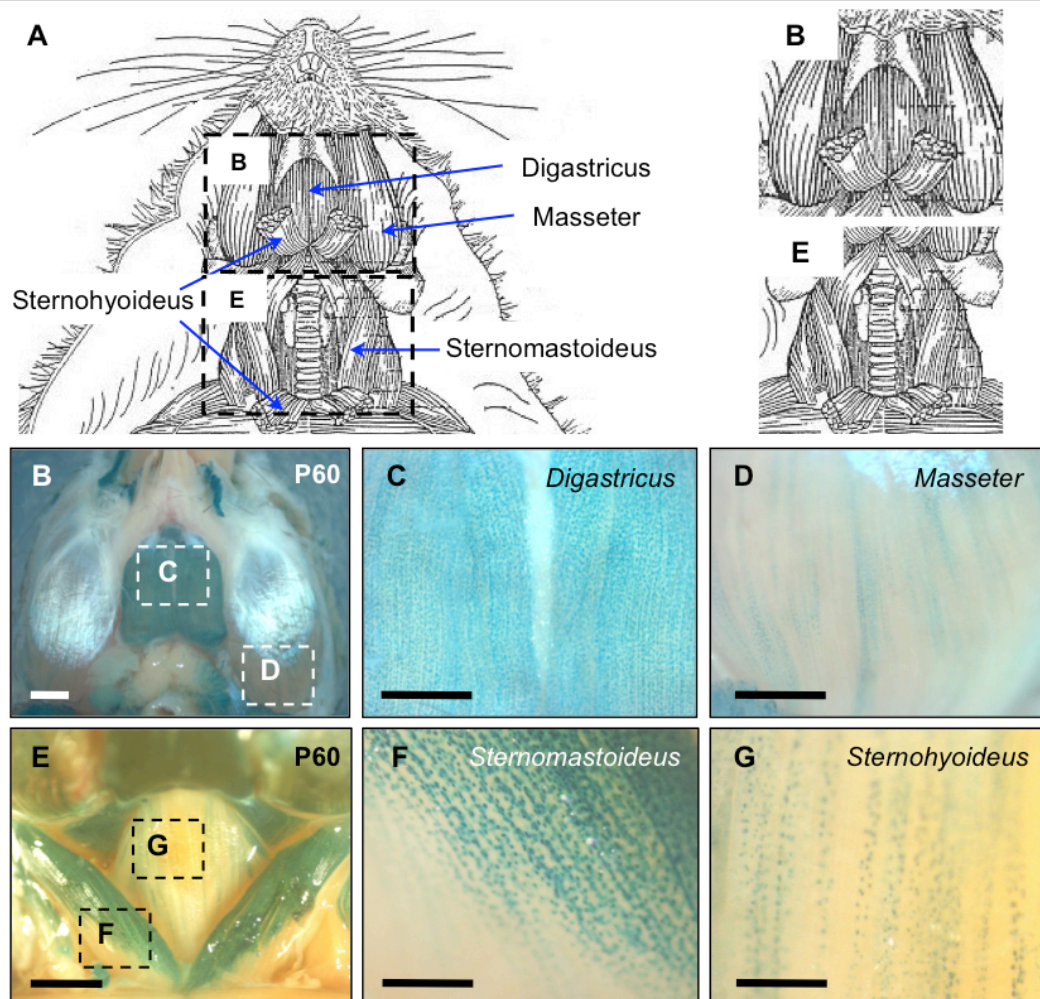


Figure 3.1: *Fgf10* expression in skeletal muscles of the head and neck at P60. (A) Schematic representation of ventral view of head and neck muscles. (B) Schematic and X-gal wholemount ventral view of jaw muscles. (E) Schematic and X-gal wholemount ventral view of neck muscles. The *digastricus* is densely populated with LacZ⁺ (β-gal⁺) myofibers (C). The *sternomastoideus* also contains a large proportion of β-gal⁺ myofibers (F). Lower proportions are found in the *masseter* and *sternohyoideus* (D, G). Scale bars: 0.25cm (B, E); 500μm (C, D) and 250μm (F, G). All muscle schematics herein adapted from (Greene, 1955).

3.2.1.2 *Fgf10* expression in forelimb and shoulder muscles

The *acromiotrapezius* and *spinodeltoideus* muscles of the shoulder contain varying numbers of β -gal^{+ve} myofibers. At its insertion into the humerus, underneath the *spinodeltoideus*, all myofibers of the *acromiotrapezius* appear β -gal^{+ve}. However, at the lateral end of the muscle, β -gal^{+ve} myonuclei appear to trail off along the myofibers (Figure 3.2E). In the *spinodeltoideus* muscle, fewer β -gal^{+ve} myofibers are interspersed within the muscle (Figure 3.2G). The *acromiotrapezius* is a large thin muscle which is part of the *trapezius* muscle group and is involved in movements of the scapula. The *spinodeltoideus* functions in conjunction with the *acromiotrapezius* to act on the glenohumeral joint (shoulder joint) (Tosolini et al., 2013). The *teres major*, another scapulohumeral muscle, also contains a very high proportion of β -gal^{+ve} myofibers. Acting in synergy with the *latissimus dorsi*, a broad muscle of the back which also contains a small proportion of β -gal^{+ve} myofibers (data not shown), it medially rotates, adducts and extends the forelimb (Iamsaard et al., 2012). In the upper forelimb, the medial head of the *triceps brachii* contains a small proportion of β -gal^{+ve} myofibers, and these appear localized at the distal end of the muscle (Figure 3.2F). The three proximal heads of the *triceps brachii* (Figure 3.2C) unite to act on the elbow joint (Tosolini et al., 2013). In the lower forelimb, the *flexor carpi ulnaris* contains a small number of β -gal^{+ve} myofibers (Figure 3.2F), this muscle flexes the wrist (Tosolini et al., 2013). The *pectoralis major*, a large triangular muscle covering the upper thorax, contains a relatively high proportion of β -gal^{+ve} myofibers (Figure 3.2J). This muscle plays a critical role in shoulder adduction and flexion of the humerus (Manske and Prohaska, 2007).

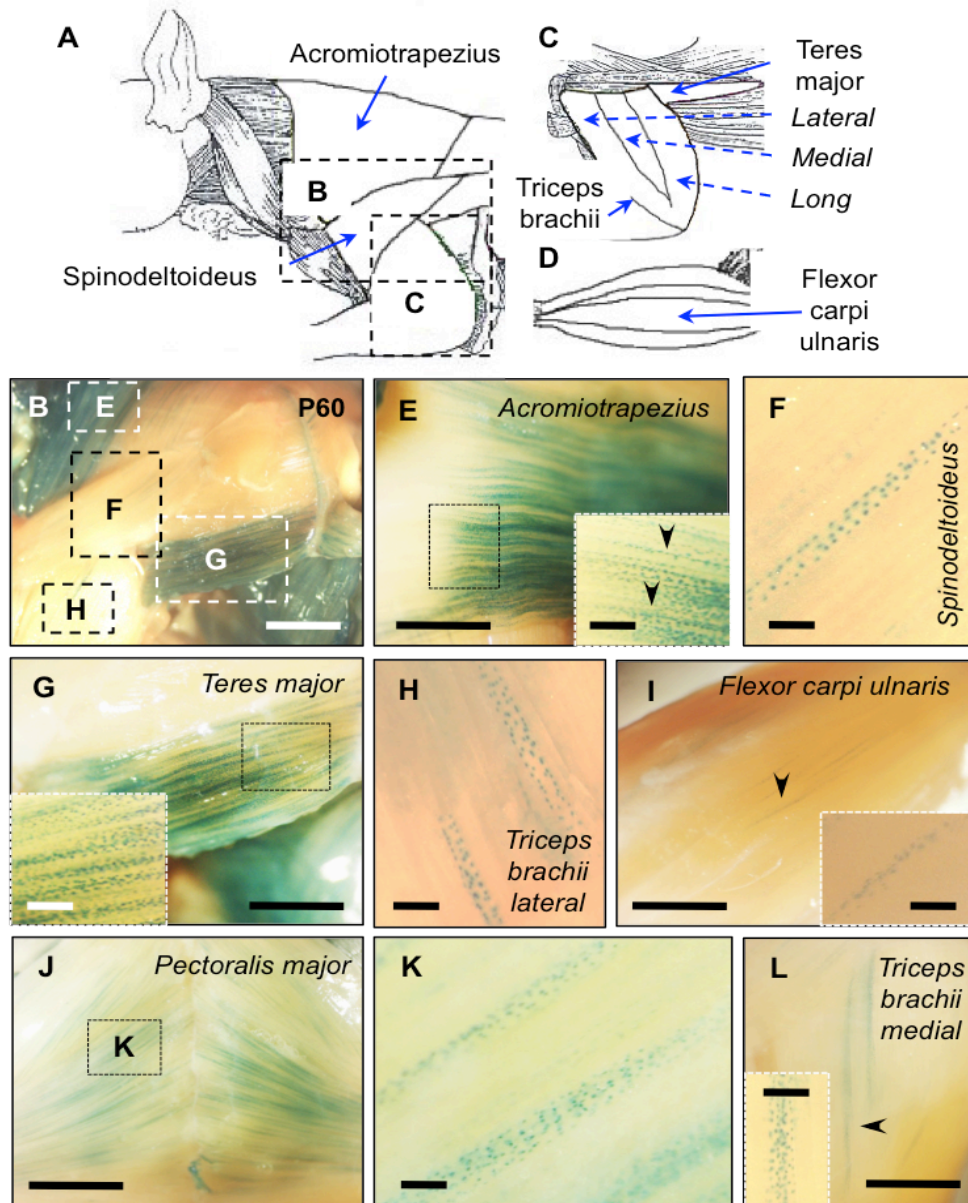


Figure 3.2: *Fgf10* expression in skeletal muscles of the shoulder and forelimb at P60. (A, C, D) Schematic of lateral view of superficial shoulder, upper and lower forelimb muscles respectively. (B) X-gal wholemount lateral view of shoulder and upper forelimb muscles. β -gal⁺ve myofibers in the *acromiotrapezius* (E), *teres major* (G), *flexor carpi ulnaris* (I), medial and lateral heads of the *triceps brachii* (H, L), *pectoralis major* (J, K) and *spinodeltoideus* (F). Scale bars: 0.25cm (B); 500μm (E, G, I, J, L); 125μm (F, H, K); 150μm (inset: E, G, I, L).

3.2.1.3 *Fgf10* expression in intercostal and back muscles

Two of the deep muscles of the thoracic wall, the *longissimus thoracis* and *spinalis thoracis* are densely packed with β -gal^{+ve} myofibers (Figure 3.3B, C). These muscles comprise two out of three *erector spinae* muscles which extend the vertebral column (Delp et al., 2001). In the lumbar region, the *quadratus lumborum* contains several β -gal^{+ve} myofibers whilst there are fewer present in the adjacent *psoas major* (Figure 3.3E, F). The precise function of the *quadratus lumborum* remains to be elucidated yet is widely accepted to cause lateral flexion as it lies parallel to the spine (Phillips et al., 2008). Likewise the exact function of the *psoas major* remains unclear; whilst it is largely considered one of the main hip joint flexors, its other functions are not completely understood (Sajko and Stuber, 2009). Interestingly, the intercostal muscles display a dorso-ventral gradient of β -gal expression (Figure 3.3G).

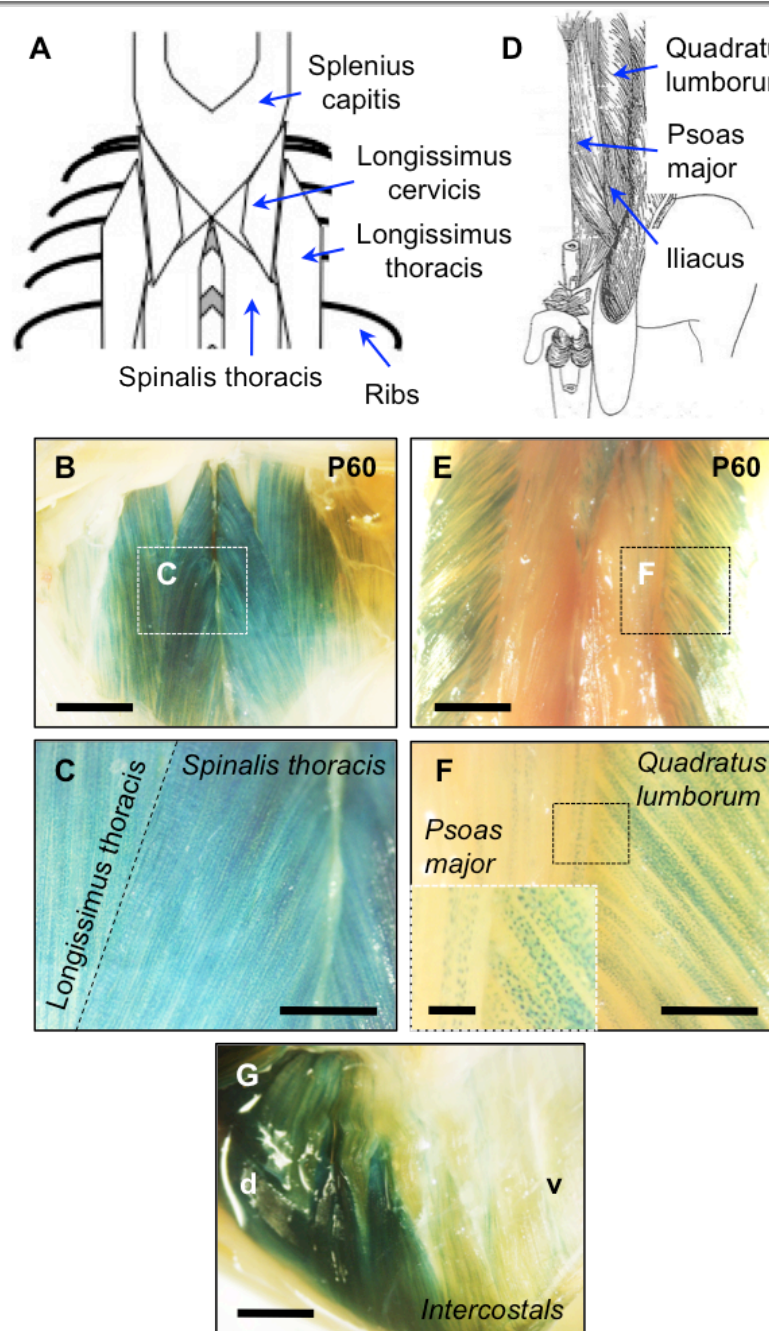


Figure 3.3: *Fgf10* expression in skeletal muscles of the back and intercostals at P60. (A, D) Schematic of dorsal view of upper back muscles and muscles of the lumbar region, respectively. β -gal⁺ myofibers in the *longissimus thoracis* and *spinalis thoracis* (B, C), *quadratus lumborum*, *psoas major* (E, F). (G) Lateral view of dorso-ventral β -gal expression gradient in intercostal muscles. Scale bars: 0.25cm (B, E); 500 μ m (C, F); 125 μ m (inset: F); 1cm (G).

3.2.1.4 *Fgf10* expression in hindlimb muscles

In the hindlimb, two muscles contain roughly the same proportion of β -gal^{+ve} myofibers, the *biceps femoris* (Figure 3.4B, C) and the *tibialis anterior* (Figure 3.4E, F). The *biceps femoris* is one of three hamstring muscles and is comprised of both a long and short head. It extends to the posterior aspect of both the hip and knee joints (Dahmane et al., 2006). The *tibialis anterior* is part of the dorsal flexor complex (Hesselink et al., 2002). The *tensor fasciae latae*, *gluteus maximus* and *semitendinosus* also contained β -gal^{+ve} myofibers, however in much smaller numbers in comparison to the *biceps femoris* and *tibialis anterior* (data not shown).

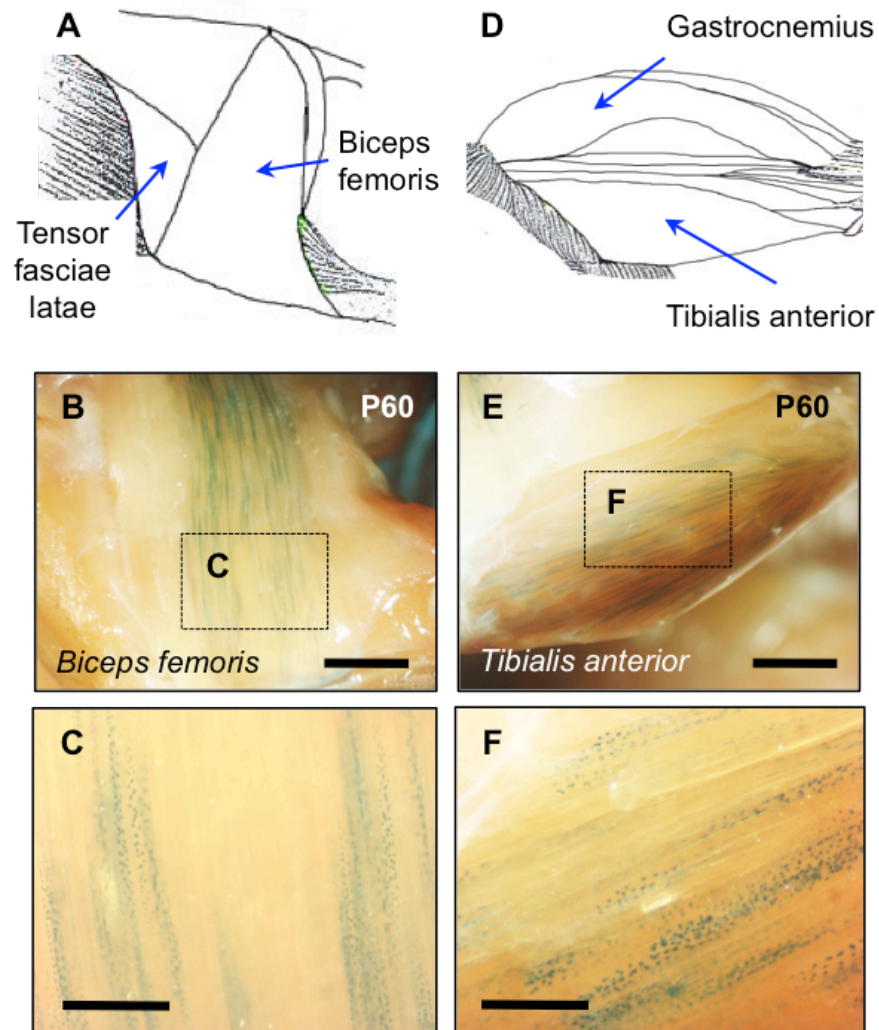


Figure 3.4: *Fgf10* expression in skeletal muscles of the hindlimb at P60.

(A) Schematic of lateral view of upper hindlimb. (D) Schematic of lateral view of lower hindlimb. The *biceps femoris* (B, C) and *tibialis anterior* (E, F) are both heterogeneous for β -gal expression. Scale bars: 0.25cm (B, E); 125 μ m (C, F).

3.2.2 The pattern of β -gal expression in adult skeletal muscles is established during post-natal development.

The pattern of β -gal expression was highly consistent across the adult ages examined. Given the longevity of the β -gal protein, the survey was extended to include younger ages in order to verify at what point the pattern is established post-natally. In mice, the first three weeks after birth is a period of rapid growth, characterized by a huge increase in skeletal muscle facilitated by myofiber hypertrophy (increase in size). Satellite cells generate the new myonuclei which enable this process (White et al., 2010).

Fgf10 expression was investigated in skeletal muscles at P22, around the time of weaning, and was found to strikingly resemble the expression patterns seen at older adult ages. In addition to the densely-populated *digastricus* (Figure 3.5B), the *masseter* also appeared to contain a higher proportion of β -gal^{+ve} myofibers in comparison to older ages (Figure 3.5C). The *acromiotrapezius* (Figure 3.5D) and *spinalis thoracis* (Figure 3.5H) also displayed a similar abundance in β -gal^{+ve} myofibers as seen at older ages. The *spinodeltoideus* (Figure 3.5F), *teres major* (Figure 3.5G), *quadratus lumborum* (Figure 3.5I), *biceps femoris* (Figure 3.5J) and *tibialis anterior* (Figure 3.5L) also contained β -gal^{+ve} myofibers in similar proportions to their adult counterparts. A small number of β -gal^{+ve} myofibers were seen in the *tensor fasciae latae* (Figure 3.5K). In addition, the *clavotrapezius* (Figure 3.5E) had a very similar expression pattern to the *acromiotrapezius*, with fewer β -gal^{+ve} myofibers visible in the *spinotrapezius* which lies in between them.

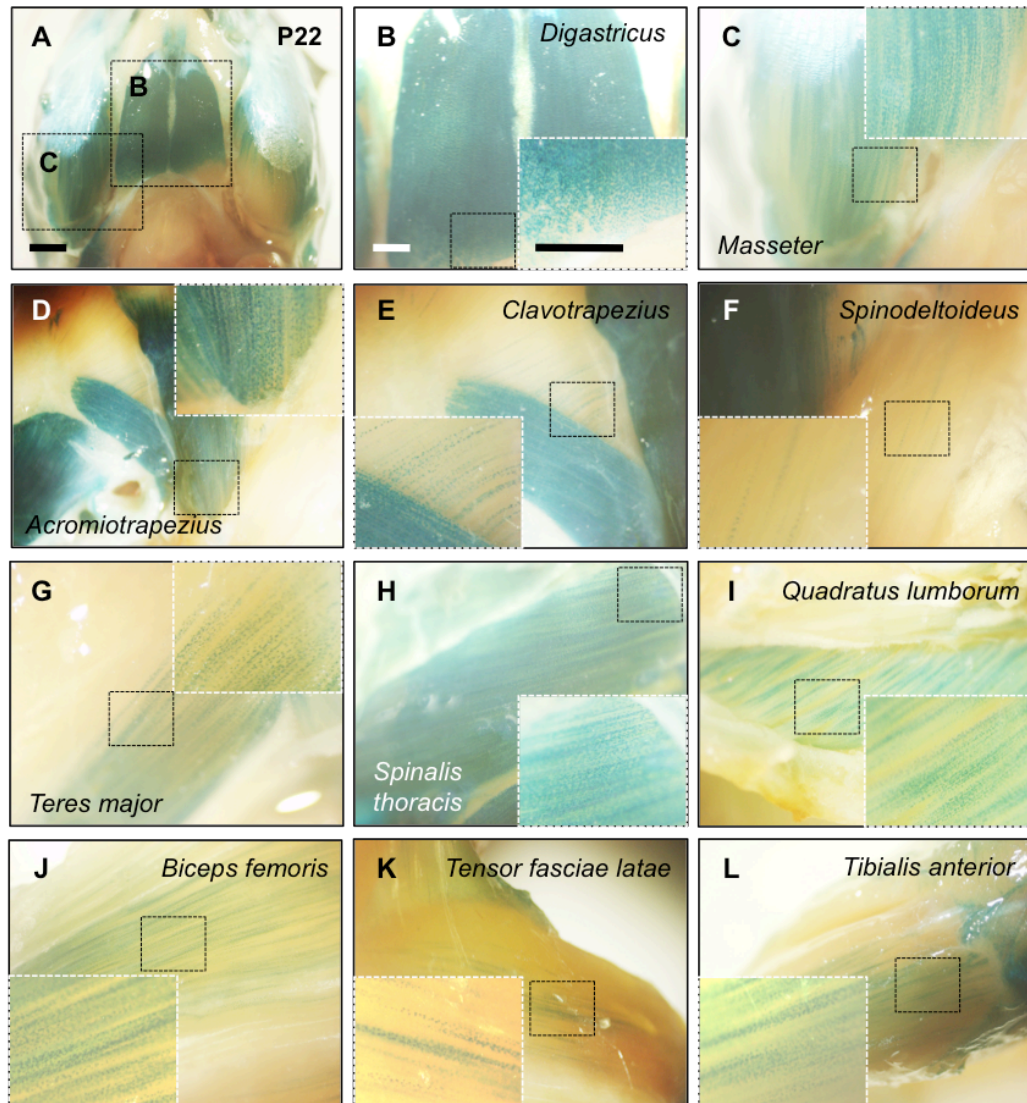


Figure 3.5: *Fgf10* expression in skeletal muscles at P22.

(A) Dorsal view of *Fgf10* expression in jaw muscles. High proportions of β -gal-expressing myofibers present in the *digastricus* (B), *masseter* (C), *acromiotrapezius* (D), *clavotrapezius* (E) and *spinalis thoracis* (H). The numbers of β -gal-expressing myofibers seen in the *spinodeltoideus* (F), *teres major* (G), *quadratus lumborum* (I), *biceps femoris* (J), *tensor fasciae latae* (K) and *tibialis anterior* (L) also closely resembles the proportions seen at older ages. Scale bars: 0.25cm (A-L); 500 μ m (inset: B-L).

In the light of the well-established expression patterns seen at P22 and at older ages, the survey was extended further to incorporate even younger animals, to see if these patterns have already been set up at birth. In newborn *Fgf10*^{nLacZ} pups (P0), it is evident that *Fgf10* is already highly expressed in some muscles, whilst the remaining muscles, which are β -gal^{+ve} at older ages, are faintly-stained which indicates that *Fgf10* is being upregulated (Figure 3.6). The *acromiotrapezius* and *teres major* show high levels of *Fgf10* expression (Figure 3.6C, D). The *triceps brachii* also contains clearly-defined β -gal^{+ve} myofibers at this age (Figure 3.6E). However, in the *biceps femoris* and *tibialis anterior*, β -gal expression appears hazy within the myofibers (Figure 3.6B, F). Although less defined, it is present nonetheless, suggesting *Fgf10* is expressed at lower levels at this time-point in these hindlimb muscles, perhaps as the myofibers are slightly less mature than those in the forelimb muscles (Martin, 1990).

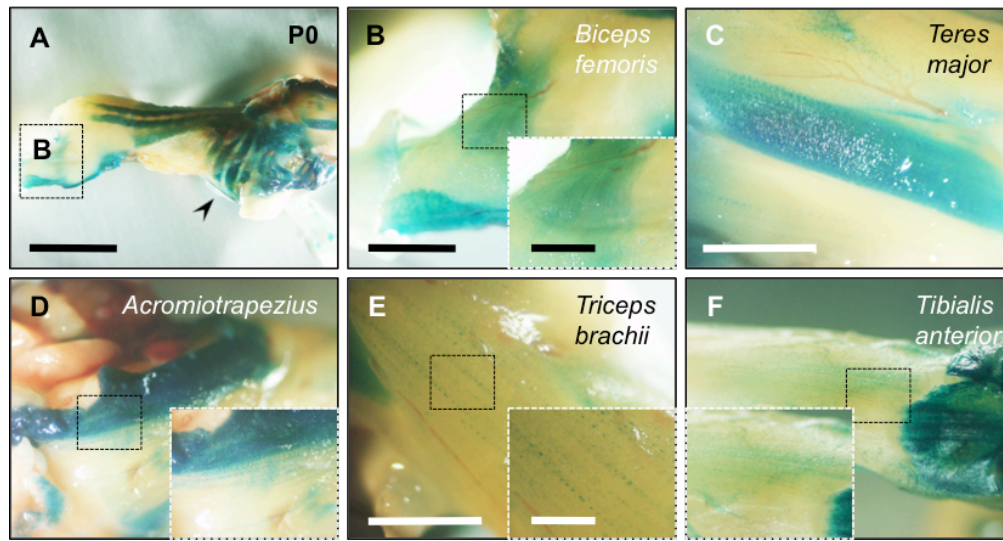


Figure 3.6: *Fgf10* expression in skeletal muscles at birth. (A) Sagittal view of an X-gal stained *Fgf10*^{nLacZ} pup. High levels of β -gal expression visible in muscles of the intercostal region (indicated by arrowhead). The *teres major* and *acromiotrapezius* display significant upregulation of β -gal (C, D). Defined β -gal⁺ myofibers also seen in the *triceps brachii* (E). *Biceps femoris* (B) and *tibialis anterior* (F) display lower levels of β -gal expression. Scale bars: 1.25cm (A); 0.19cm (B, D, F); 0.125cm (C, E); 950 μ m (inset: B, D, F); 313 μ m (inset: E).

The results of the X-gal wholemount survey are summarized in Tables 3.1a, b. The muscles were designated a score, ranging from '+' to '+++' to indicate low to high proportions of β -gal⁺ myofibers. A low score implied $\leq 25\%$ myofibers were β -gal⁺, a mid score implied 25-75% of myofibers were β -gal⁺, and a high score indicated $\geq 75\%$ of myofibers were β -gal⁺, as judged by eye. Given the specific expression pattern for *Fgf10* in the muscles surveyed, the predominant fiber-type in each muscle is also listed. Interestingly, *Fgf10* appears to be expressed in muscles predominantly composed of fast (Type 2) myofibers.

To verify that β -gal⁺ cells were indeed expressed in post-natal developing muscles, longitudinal sections through the hindlimbs of *Fgf10*^{nLacZ} pups aged P4 were immunolabelled with anti- β -gal and anti-

sarcomeric myosin antibodies (Figure 3.7). β -gal-expressing myonuclei are visible within the developing *biceps femoris* of the hindlimb (Figure 3.7B, C). Further immunostaining with anti-laminin $\alpha 2$ or anti-dystrophin antibodies would also confirm that β -gal is expressed within the myofiber proper.

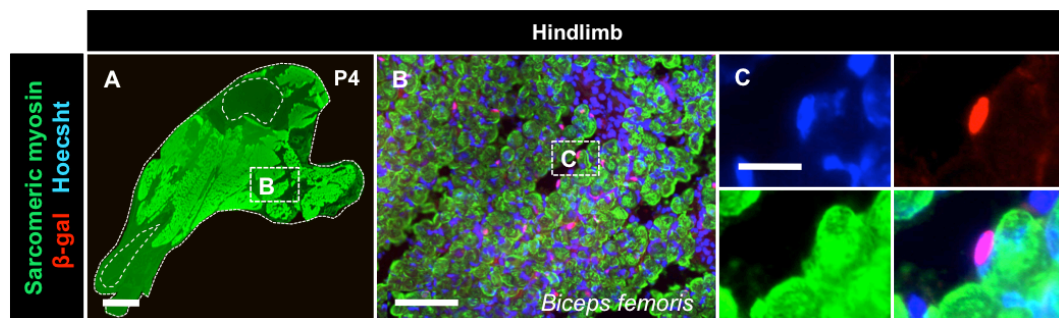


Figure 3.7: *Fgf10*-expressing cells co-localize within developing muscle groups in the hindlimb at P4. (B, C) β -gal-expressing myonuclei are incorporated into developing myofibers of the *biceps femoris*. Primary antibodies: Ms- α -sarcomeric myosin; Ck- α - β -gal. Secondary antibodies: Gt- α -Ms AlexaFluor488; Gt- α -Ck AlexaFluor 568; Hoescht counterstain. Scale bars: 100 μ m (A, B); 50 μ m

Table 3.1a: *Fgf10* expression in jaw, neck, shoulder and forelimb skeletal muscles throughout adult development.

Muscle	Post-natal		Adult				Main fiber type	Reference
	P4 (n=4)	P22 (n=4)	P60 (n=4)	P120 (n=3)	P180	P350		
<i>Digastricus</i>	-	+++	+++	+++	+++	+++	Type 2B	(Sano et al., 2007)
<i>Masseter</i>	-	+++	++	++	++	++	Type 2X	(Sano et al., 2007)
<i>Sternomastoideus</i>	-	++	++	++	++	++	Type 2D/B	(Guido et al., 2010)
<i>Sternohyoideus</i>	-	+	+	+	+	+	Type 2B/2X	(O'Connell et al., 2013)
<i>Acromiotrapezius</i>	++	+++	+++	+++	+++	+++	Type 2A	(Prado et al., 2005)
<i>Latissimus dorsi</i>	-	-	-	-	-	-	Type 2B	(Gillott et al., 1994)
<i>Pectoralis major</i>	-	++	++	++	++	++	Type 2	(DiMario and Stockdale, 1997)
<i>Spinodeltoideus</i>	-	+	+	+	+	+	Unknown	-
<i>Spinotrapezius</i>	-	-	-	-	-	-	Type I	(Delp and Duan, 1996)
<i>Teres major</i>	++	+++	+++	+++	+++	+++	Type 2	(Lovering and Russ, 2008)
<i>Biceps brachii</i>	-	-	-	-	-	-	Type 2B	(Fuentes et al., 1998)
<i>Flexor carpi ulnaris</i>	-	++	+	+	+	+	Type 2	(McIntosh et al., 1985)
<i>Triceps brachii (lateral, long)</i>	-	+	+	+	+	+	Type 2B	(Fuentes et al., 1998)
<i>Triceps brachii (medial)</i>	-	++	++	++	++	++	Type 2B	(Fuentes et al., 1998)

Table 3.1b: *Fgf10* expression back and hindlimb skeletal muscles throughout adult development.

Muscle	Post-natal		Adult				Main fiber type	Reference
	P4 (n=4)	P22 (n=4)	P60 (n=4)	P120 (n=3)	P120- P180 (n=4)	P180- P350 (n=4)		
<i>Longissimus thoracis</i>	+	+++	+++	+++	+++	+++	Type 2	(Granlund et al., 2011)
<i>Psoas major</i>	-	+	++	++	++	++	Type 2B	(Sano et al., 2007)
<i>Quadratus lumborum</i>	-	++	++	++	++	++	Type 2	(Schilling et al., 2005)
<i>Spinalis thoracis</i>	+	+++	+++	+++	+++	+++	Type 2B	(MacArthur et al., 2008)
<i>Biceps femoris</i>	+	++	++	++	++	++	Type 2B	(Moore et al., 2014)
<i>Extensor digitorum longus</i>	-	-	-	-	-	-	Type 2B	(Augusto et al., 2004)
<i>Gastrocnemius</i>	-	-	-	-	-	-	Type 2B	(Augusto et al., 2004)
<i>Gluteus maximus</i>	-	-	-	++	+	-	Type 2A	(Manttari and Jarvilehto, 2005)
<i>Quadratus femoris</i>	-	-	-	++	-	-	Unknown	-
<i>Semimembranosus</i>	+	+	++	++	++	++	Type 2B	(Ecob-Prince et al., 1986)
<i>Semitendinosus</i>	-	-	-	-	-	-	Type 2B	(Roy et al., 1984)
<i>Soleus</i>	-	-	-	-	-	-	Type 1	(Augusto et al., 2004)
<i>Tensor fasciae latae</i>	-	+	+	++	++	+	Type 2	(Okada et al., 2009)
<i>Tibialis anterior</i>	+	++	++	++	++	++	Type 2B	(Augusto et al., 2004)

3.2.3 Muscle fiber bundles are heterogeneous for *Fgf10* expression.

In order to better visualize the heterogeneity for *Fgf10* expression within muscles, X-gal stained *biceps femoris* and *tibialis anterior* muscles were sectioned in both the longitudinal and transverse plane and counterstained. In addition to the mixed population of myofibers throughout the muscle, it can also be seen that not all myonuclei in a β -gal-expressing myofiber are β -gal⁺. Moreover, some myonuclei are a more intense blue colour than others (Figure 3.8D).

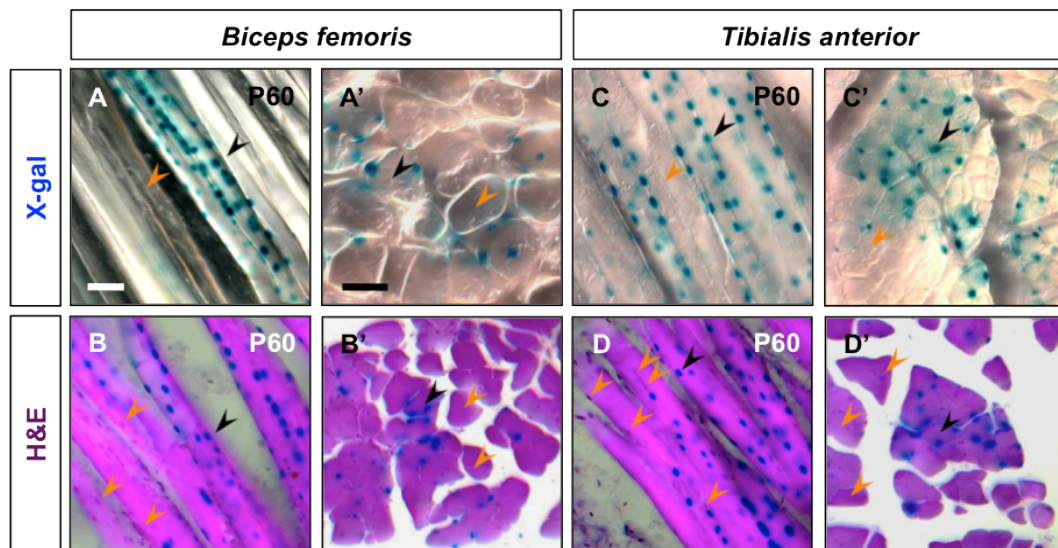


Figure 3.8: Heterogeneity of the myofiber population within the *biceps femoris* and *tibialis anterior*. Upper panel: X-gal stained tissue. Lower panel: X-gal stained tissue, counterstained with haematoxylin to reveal β -gal⁺ myonuclei in myofiber. Orange arrowheads indicate β -gal^{-ve} myonuclei and black arrowheads indicate β -gal⁺ myofibers. In both the longitudinal (A, B, C, D) and transverse (A', B', C', D') planes β -gal-expressing myofibers sit directly next to non- β -gal-expressing myofibers. The myonuclei population within a myofiber can also be heterogeneous for *Fgf10* expression, as indicated by the arrowheads in D. Scale bars: 50 μ m (A, A').

In sections, a subset of myofibers expresses β -gal, although it could be argued that this results from penetration issues with the X-gal staining solution. To explore this further, myofibers were isolated from digested *biceps femoris* and *tibialis anterior* muscles, and incubated in X-gal solution. It became clear β -gal is indeed expressed by a subset of myofibers, thereby confirming that the variation in number of β -gal-expressing myofibers within X-gal stained muscle sections is not caused by a lack of solution penetration. Sections only give a limited view of the myofibers, however their isolation allows for clearer observation of β -gal expression along the length of the fiber, the results of which are quite interesting (Figure 3.9). A trail of β -gal-expressing cells is visible in many of the fibers (Figure 3.9A). The most intensely-stained nuclei are often seen in the centre of the fiber (Figure 3.9C, K, H) and these are flanked by cells expressing less β -gal (Figure 3.9D, J, L). From these results it could be suggested that the weakly-stained myonuclei are descendents of the strongly-stained ones. It is also important to acknowledge however, that viability of the isolated myofibers was not verified during this experiment. It is potentially possible therefore that the lack of β -gal expression at the end of the myofibers may be due to hypercontraction, a process which occurs during myofiber death (Pasut et al., 2013).

To further explore the intensity differences amongst β -gal⁺ myonuclei, cryostat sections of *tibialis anterior* muscle were immunolabelled with anti- β -gal antibodies and the numbers of faintly- and intensely-stained β -gal⁺ myonuclei were quantified in four regions across the section (anterior, lateral, posterior, medial) at three different levels within the muscle (distal, middle and proximal) (Figure 3.10).

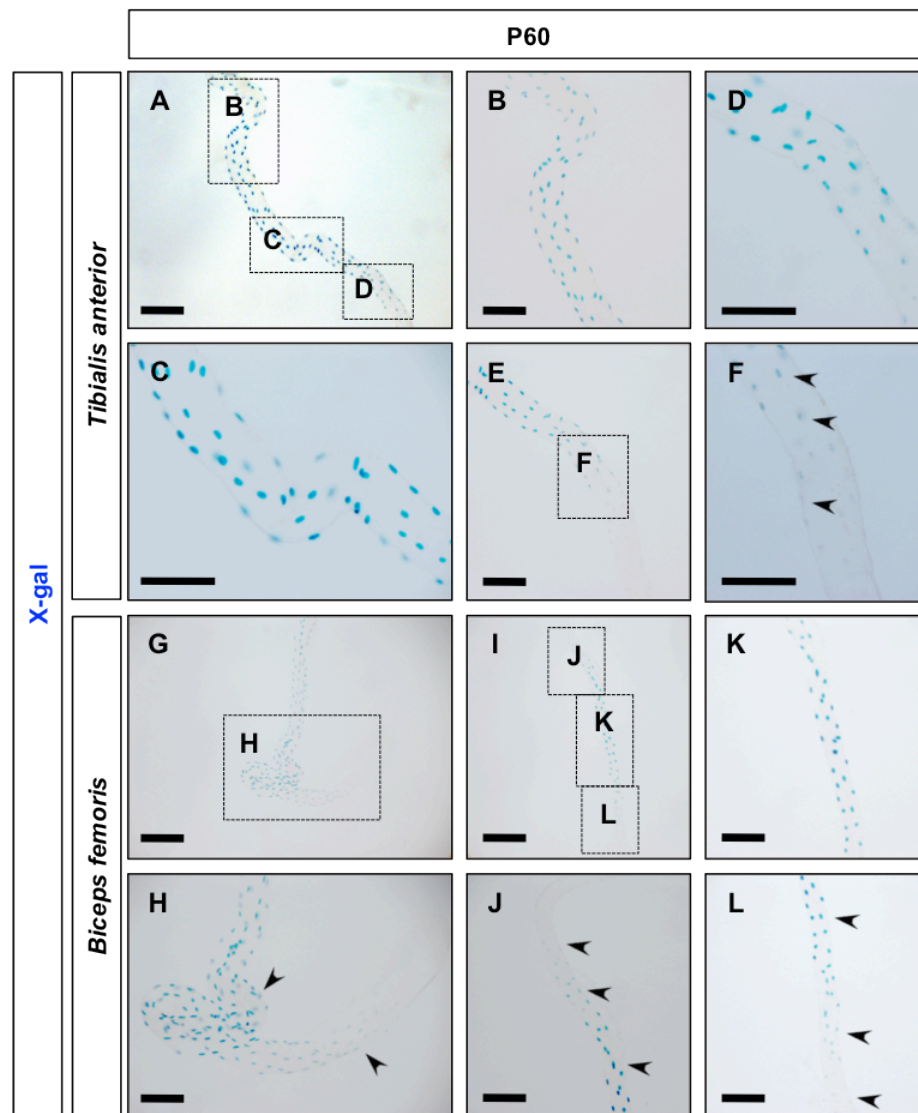


Figure 3.9: β -gal expression in isolated myofibers. Intensely-stained β -gal-expressing cells often populate the center of the myofiber (C, H, K). β -gal expression appears to trail off towards the extremities of the myofibers, with a clear demarcation of the gradual decrease of β -gal expression in these regions (F, H, J, L). Scale bars: 200 μ m (A, G); 100 μ m (B-F; H-L).

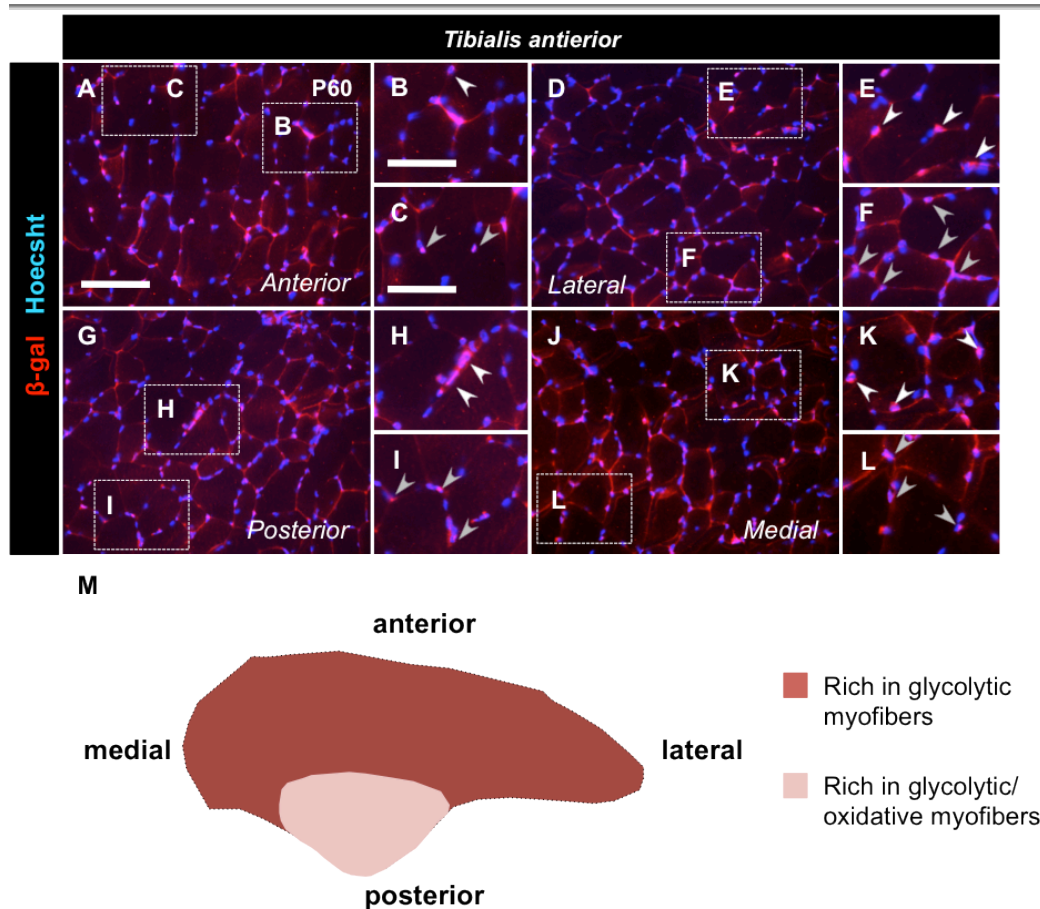


Figure 3.10: Distribution of intense vs. faint β -gal⁺ myonuclei in the *tibialis anterior* at P60. (A, D, G, J) Differences in intensity of β -gal-expressing myonuclei can be seen throughout the *tibialis anterior*, with intensely-stained (indicated by white arrowheads; B, E, H, K) and faintly-stained (indicated by grey arrowheads; C, F, I, L) myonuclei appearing evenly distributed across the regions of the sections. Faint β -gal⁺ myonuclei are consistently more numerous than intense ones, with no significant difference across the planar regions throughout the muscle (data not shown). Primary antibody: Ck- α - β -gal. Secondary antibody: Gt- α -Ck AlexaFluor568; Hoechst counterstain. (M) Schematic transverse view through *tibialis anterior* at mid-belly level. Myofiber heterogeneity is present in the muscle. Scale bars: 100 μ m (A, D, G, J); 50 μ m (B, C, E, F, H, I, K, L).

The distribution of faint and intense β -gal⁺ myonuclei appeared fairly even across the different regions (anterior, lateral, posterior, medial) of the

muscle sections and this did not change throughout the length of the muscle (Figure 3.10A-L). The proportion of faint β -gal⁺ myonuclei consistently outnumbered that of the intense β -gal⁺ myonuclei, and again there were no significant regional differences in these proportions (data not shown). It is also important to note that myofiber regionalisation exists within this muscle (Figure 3.10M). In the transverse plane, the posterior region of the muscle is enriched with oxidative/glycolytic (mixture of Type 2A and sporadic Type 1) myofibers. This central core of oxidative/glycolytic enrichment occupies approximately two-thirds of the muscle length, in a disto-proximal direction. The outer (medial, anterior, lateral) regions of the muscle, are predominantly composed of glycolytic (Type 2B) myofibers (Nicklas et al., 2012). Based on this information, it would also be interesting to carry out a more thorough analysis of β -gal distribution in the two key regions (glycolytic and oxidative/glycolytic) of the muscle, at more regular intervals along its length, in order to optimally take into account the myofiber heterogeneity.

3.2.3.1 *Fgf10* and its receptor can be detected by *in situ* hybridization in adult skeletal muscle.

To investigate whether the conserved patterns of β -gal expression seen throughout adult development are due to longevity of the protein or active *Fgf10* expression, RNA *in situ* hybridization was carried out on P60 *tibialis anterior* sections using *Fgf10* and *FgfR2IIIb*-specific ³⁵S[UTP]-labelled riboprobes. *In situs* were kindly carried out by Dr Ritva Rice at the Institute of Biotechnology, Helsinki, Finland. As a negative control, ³⁵S[UTP]-labelled sense riboprobes for both ligand and receptor were also incubated on adjacent sections. This probe did not bind and therefore no signal was detected (data not shown). The *Fgf10* signal appears enriched across the anterior-to-medial regions of the muscle, and in the surrounding areas the signal is much weaker (Figure 3.11B) which could mirror the

differences in β -gal expression seen throughout the muscle. In areas where the *in situ* signal appears strongest, *Fgf10* expression seems perinuclear (Figure 3.11C). This could indicate that in myofibers themselves, the RNA transcript may localize with the myonuclear membrane. Other domains of the muscle, particularly laterally and posteriorly, do not appear to express *Fgf10* (Figure 3.11D).

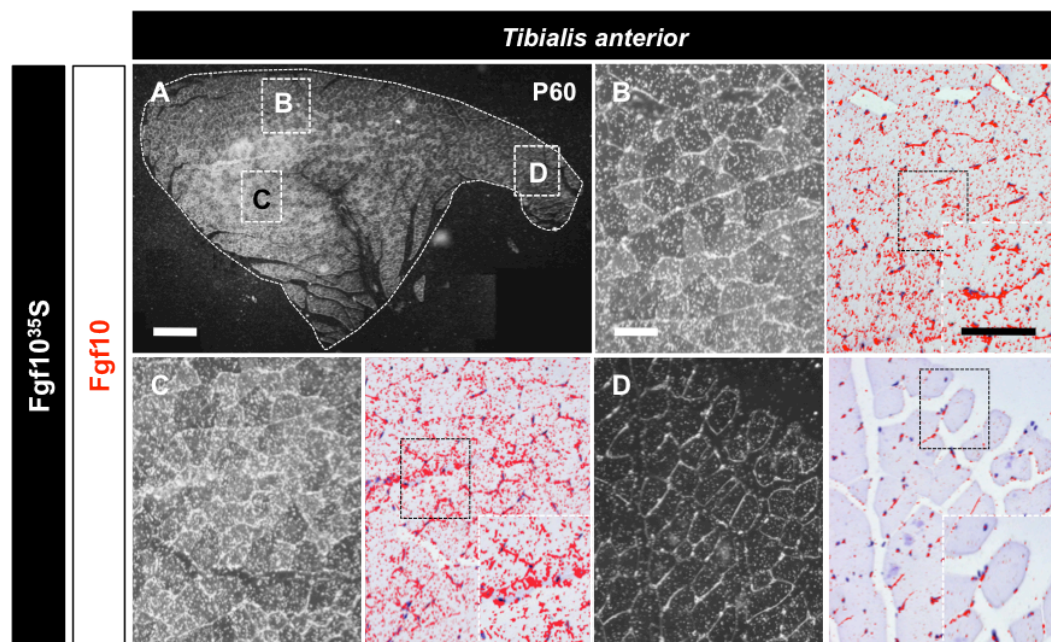


Figure 3.11: *Fgf10*-expressing myonuclei are clustered within the anterior-medial regions of the *tibialis anterior* muscle at P60. Signal obtained from *in situ* hybridization (dark field) with *Fgf10*-specific riboprobe is presented in (A, B, C, D). *In situ* signal has been false-colored to red in adjacent image panels. (B) Heterogeneity of *Fgf10* expression can be seen across the muscle, with regions containing a stronger *Fgf10* signal residing next to those with a weaker signal. (C) In enriched *Fgf10* expression domains, the signal is perinuclear and appears to form ‘tails’ of expression connected to neighboring myonuclei. (D) In lateral regions there is very little or no positive signal for *Fgf10*. Scale bars: 100 μ m (A); 25 μ m (B, C, D – insets).

The distribution of the main receptor for *Fgf10*, *FgfR2-IIIb*, was also investigated by *in situ* hybridization. Interestingly, this was also found to

be expressed in a heterogeneous manner throughout the *tibialis anterior* at P60 (Figure 3.12). *FgfR2IIIb*-expressing cells were also localized to the anterior-medial regions of the muscle (Figure 3.12B). Expression of the receptor also appeared nuclear, as was observed for *Fgf10* (Figure 3.12C). The mRNA expression patterns for *Fgf10* could indicate that *Fgf10* is expressed in adult skeletal muscle, however repeating the in situ on a muscle negative for *Fgf10* expression (such as the gastrocnemius) will substantiate this further.

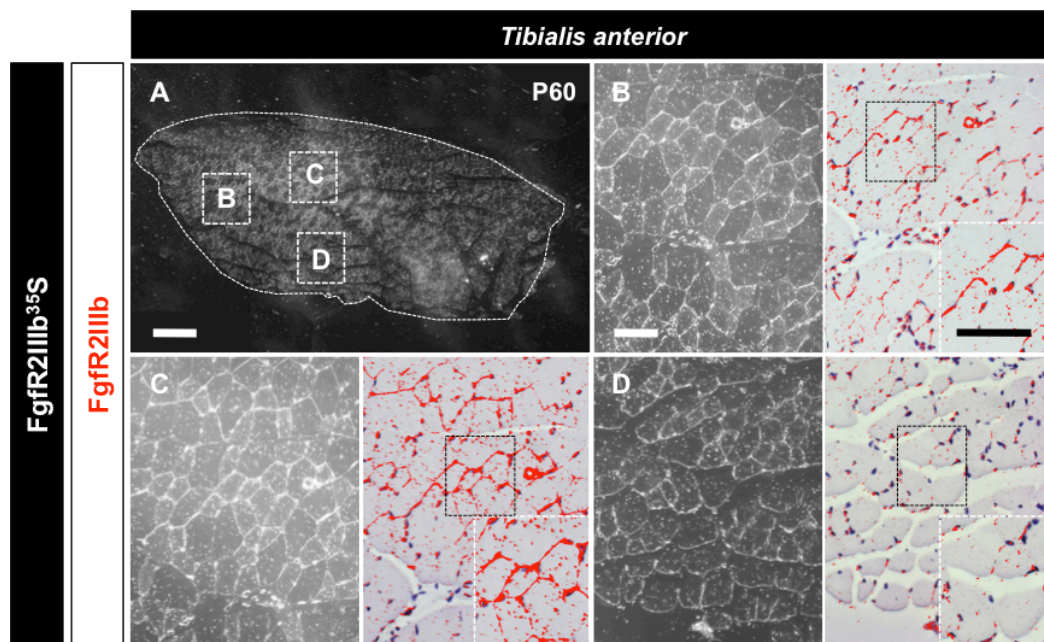


Figure 3.12: *FgfR2IIIb*-expressing myonuclei are expressed throughout the anterior-medial regions of the *tibialis anterior* muscle at P60. Signal obtained from in situ hybridization (dark field) with *FgfR2IIIb*-specific riboprobe is presented in (A, B, C, D). In situ signal has been false-colored to red in adjacent image panels. (B) *FgfR2IIIb* is also heterogeneously expressed across the muscle, with various regions containing a stronger *FgfR2IIIb* signal than others. (C) In areas of strong expression, the *FgfR2IIIb* signal is nuclear and displays similar ‘tails’ of expression as seen with *Fgf10* (D) In lateral regions there is very little or no positive signal for *FgfR2IIIb*. Scale bars: 100µm (A); 50µm (B, C, D); 100µm (insets – B, C, D).

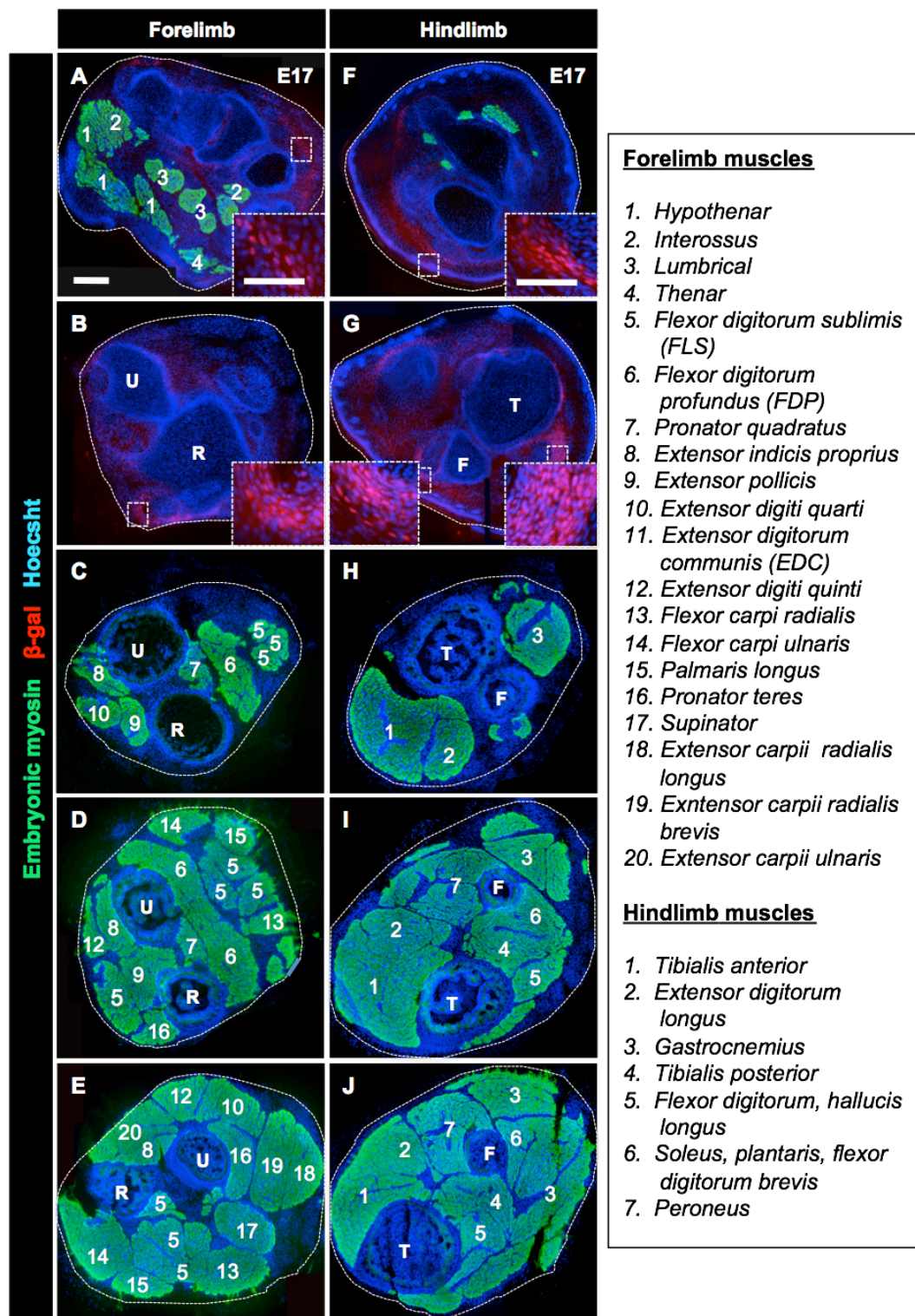
3.2.4 *Fgf10* expression is potentially upregulated in developing muscles around birth.

Fgf10 expression appears to be upregulated in muscle around birth and during the first few weeks of life. Its expression was investigated at various embryonic stages in order to determine the earliest time-point it is expressed in developing muscle.

At E17, towards the end of fetal (secondary) myogenesis when the muscle anlagen begins to undergo hypertrophy (Biressi et al., 2013), β -gal was found not to be expressed in developing limb muscles (Figure 3.13). Serial sections (distal-to-proximal) through both the forelimb and hindlimb were immunolabelled with β -gal and embryonic myosin to investigate co-localisation between *Fgf10* and developing myofibers respectively. β -gal expression could be seen in the distal regions of the limbs, and appeared to closely associate with developing tendons (Figure 3.13A, B, F, G). However, in the proximal limb sections β -gal was not yet expressed within embryonic myofibers (Figure 3.13C-E, H-J).

To confirm whether β -gal⁺ cells were co-localising with developing tendons, distal hindlimb sections with immunolabelled with β -gal and tenascin C, a marker of developing and differentiated tendon (Schweitzer et al., 2001). At this level, β -gal⁺ cell populations appear to either encircle or associate with three major tendons of the hindlimb, the tendon of the *tibialis posterior* (Figure 3.14B, F), *flexor digitorum brevis* (Figure 3.14C, G), and *flexor hallucis* (Figure 3.14D) (Peters et al., 1998). Several β -gal⁺ cells co-express the tendon marker tenascin C at the periphery of these tendons, suggesting that they are differentiating into this cell-type.

Figure 3.13: *Fgf10* is not yet expressed by developing muscles at E17. In the distal regions of the forelimb (A, B) and hindlimb (F, G), β -gal⁺ cell populations appear to associate with tendons, however they are not found within the muscle groups (C-E, H-J). Section outline demarcated by white dashed line. Forelimb muscles in C-E adapted from (Watson et al., 2009). Hindlimb muscles in H-J adapted from (Inanlou et al., 2003). Primary antibodies: Ms- α -embryonic myosin, Ck- α - β -gal. Secondary antibodies: Gt- α -Ms AlexaFluor488, Gt- α -Ck AlexaFluor568, Hoechst counterstain. All scale bars: 100 μ m.



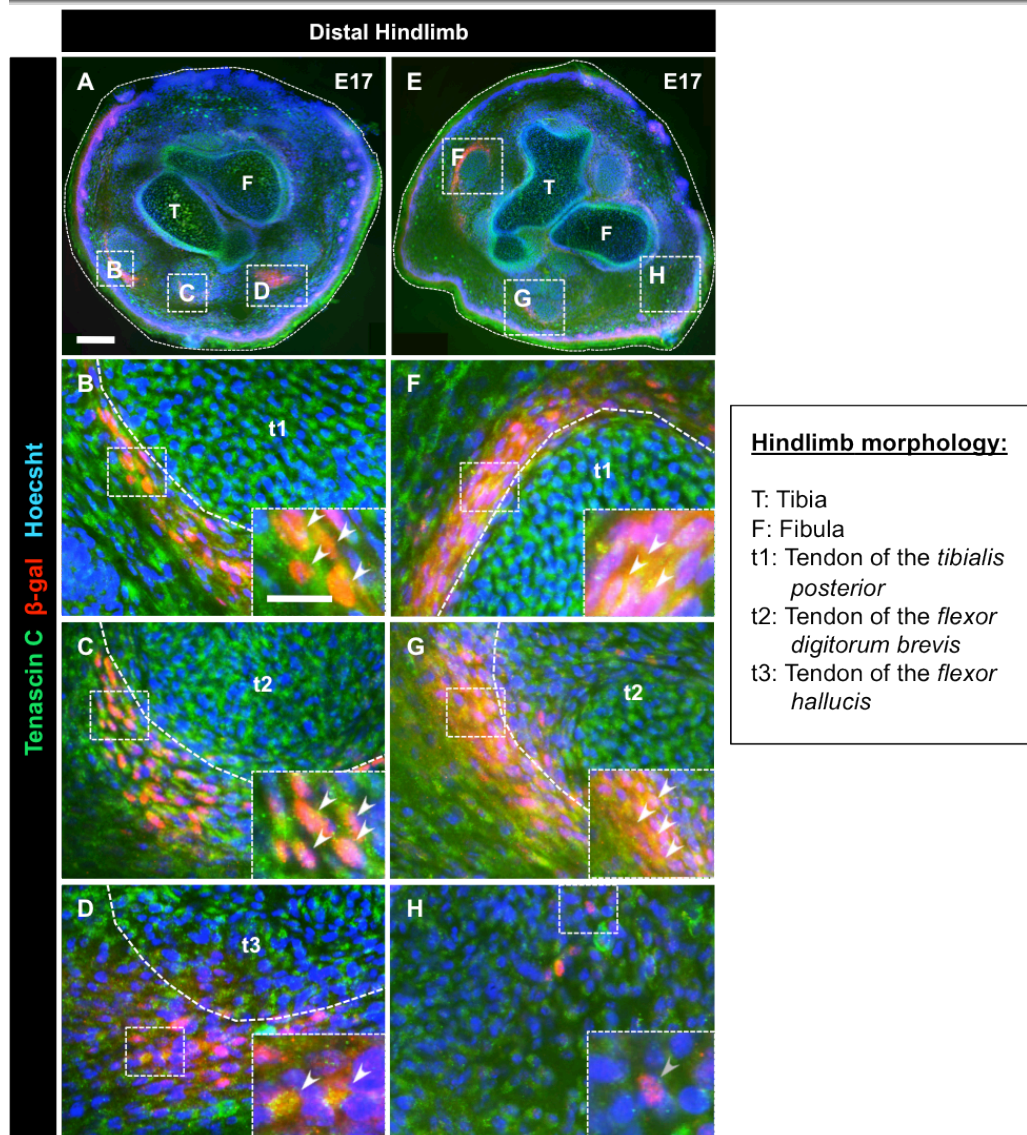
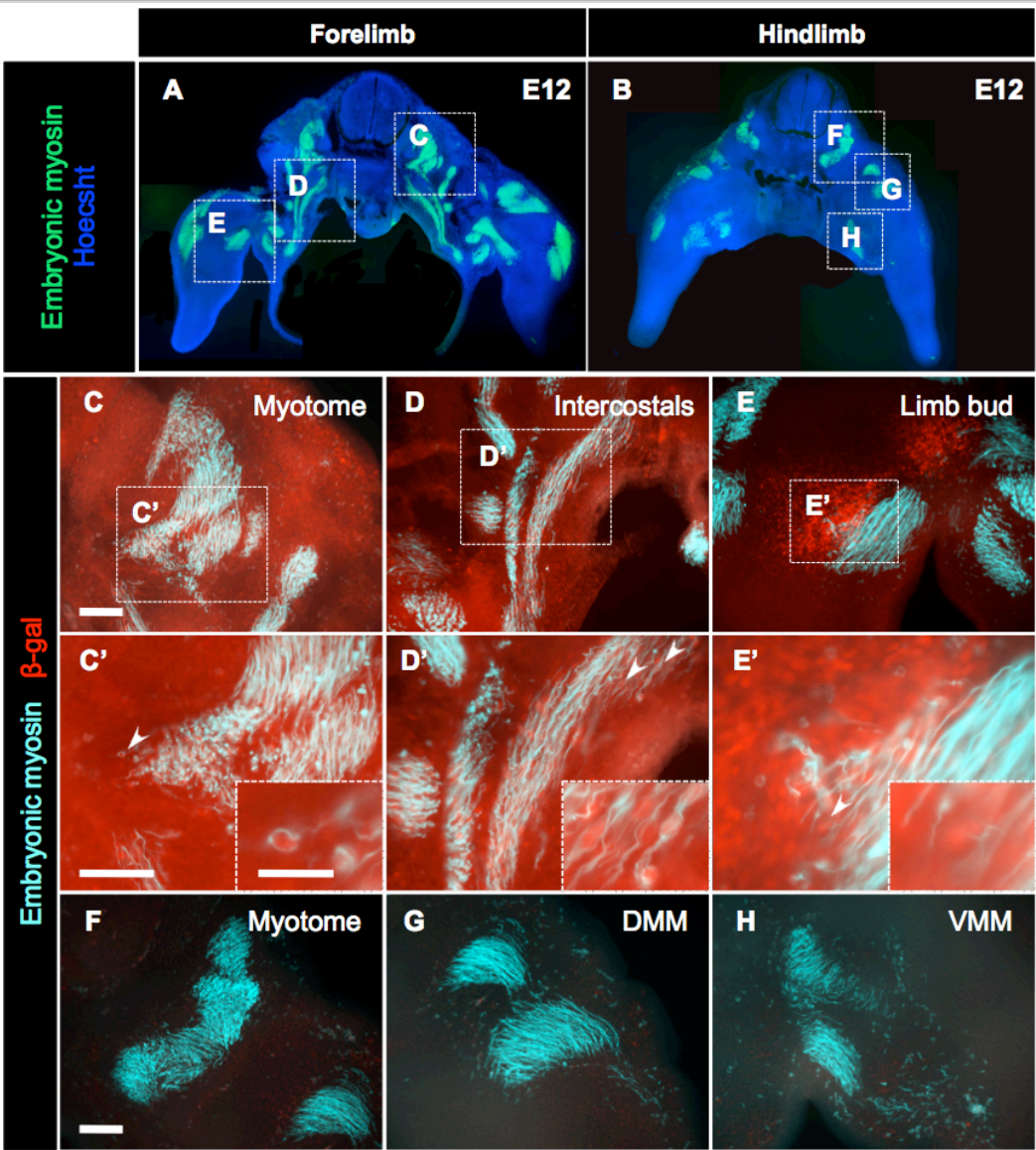


Figure 3.14: *Fgf10*-expressing cells may give rise to a subgroup of tendons during embryonic development. In the distal hindlimb, β -gal^{+ve} cell populations display a targeted directionality towards three of the major tendons in the hindlimb: the *tibialis posterior* (B, F), *flexor digitorum brevis* (C, G) and *flexor hallucis* (D). In addition, several, but not all, β -gal^{+ve} cells also co-localise with the tendon marker *tenascin C* which suggests they are in the process of differentiating into tendon (indicated by white arrowheads). However, not all β -gal^{+ve} cells express the tendon marker (H, indicated by grey arrowhead). Primary antibodies: Rb- α -Tenascin C, Ck- α - β -gal. Secondary antibodies: Gt- α -Rb AlexaFluor488, Gt- α -Ck AlexaFluor568, Hoecsht counterstain. Scale bars: 100 μ m (A); 50 μ m (insets).

At E12, β -gal-expressing cells can be seen within the regions corresponding to the developing myotome, and also in the early intercostal muscles and finally within the forelimb itself (Figure 3.15C-E). In the myotome area, β -gal^{+ve} cells co-localise with embryonic myosin in the region corresponding to migrating cells from the ventrolateral lip (VLL) of the dermomyotome, suggesting that a proportion are myoblasts (Figure 3.15C'). In the trunk region, β -gal^{+ve} cells also appear to co-localise with developing myofibers (Figure 3.15D'). Finally, within the forelimb bud itself, the β -gal^{+ve} cell population is closely associated with developing myofibers but the two markers are not yet co-localising (Figure 3.15E'). Conversely, in the hindlimb at E12, there are no β -gal^{+ve} cells associated with either the myotome or dorsal muscle masses (DMM) or ventral muscle masses (VMM).

At E9, β -gal^{+ve} cell populations were also detected within the developing forelimb bud and in regions which may correspond to the somite (Figure 3.16A). In the somite, the anatomical location of these cell populations may correspond to myotomal cells (Figure 3.16B, C.) However, the orientation of the section is limiting and without further co-immunolabeling with an anti-*Pax3* antibody (to label myotomal cells), the identity of the β -gal^{+ve} cells cannot be confirmed. Previous attempts to use an anti-*Pax3* antibody were unsuccessful. In the developing hindlimb no β -gal^{+ve} cells were detected.

Figure 3.15: *Fgf10*-expressing cells co-localise with developing myofibers at forelimb level but not at hindlimb level at E12. (A, B) Location of developing myofibers in the forelimb and hindlimb. In the forelimb, β -gal-expressing cells co-localise with myoblasts in the myotomal region (C, C') and also with trunk myofibers (D, D'). In the limb bud however β -gal^{+ve} cells and embryonic myofibers do not co-localise (E, E'). In the hindlimb, there are no β -gal^{+ve} cells in the myotomal region, dorsal muscle masses (DMM) or ventral muscles masses (VMM). Embryonic myosin signal false-coloured to cyan blue. Primary antibodies: Ms- α -embryonic myosin; Ck- α - β -gal. Secondary antibodies: Gt- α -Ms AlexaFluor488; Gt- α -Ck AlexaFluor 568; Hoescht counterstain. Scale bars: 100 μ m (C-H); 50 μ m (insets, C'-E').



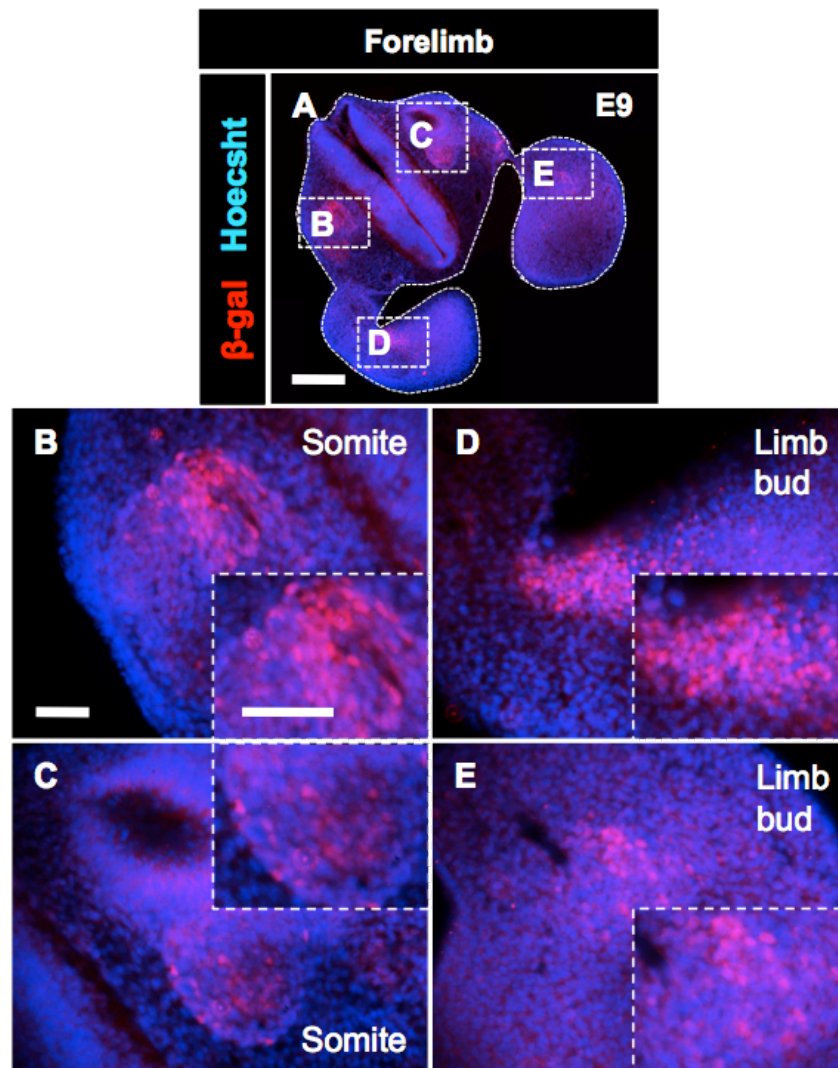


Figure 3.16: *Fgf10*-expressing cells are present within the somite and developing forelimb bud at E9. (B, C) β -gal⁺ cells appear to localize with the myotomal compartment of the developing somite. (D, E) Distinct populations of β -gal⁺ cells also arise within the forelimb bud at this age. Primary antibody: Ck- α - β -gal. Secondary antibody: Gt- α -Ck AlexaFluor 568; Hoechst counterstain. Scale bars: 100 μ m (A); 50 μ m (B-E); 100 μ m (insets, B-E).

3.3 Discussion

Using *Fgf10*^{nLacZ} reporter mice, a striking and completely novel pattern of *Fgf10* expression has been observed in a subgroup of post-natal and adult skeletal muscles. The aim of this chapter was to identify which muscles express *Fgf10* in post-natal and adult life, and to also elucidate the timing of *Fgf10* expression during embryonic muscle development. This was achieved by detecting the LacZ product, β -galactosidase (β -gal) either enzymatically with X-gal substrate in wholemount, which gives a blue-coloured precipitate, or alternatively using antibodies against β -gal.

Interestingly, β -gal was mostly expressed by the same cohort of muscles in adult *Fgf10*^{nLacZ} mice. Within these muscles, the myofiber population was heterogeneous for β -gal expression. Moreover, the myonuclei populations within β -gal^{+ve} myofibers also displayed heterogeneity for β -gal. The majority of β -gal^{+ve} myonuclei were a faint blue colour, whilst the remainder were more intensely-stained. As β -gal is a bacterial protein, it is considerably more stable than endogenous mammalian proteins. This reporter line has previously been used to transiently lineage-trace *Fgf10*-expressing cells in other tissues *in vivo* (Kelly et al., 2001; Mailleux et al., 2005). It is possible that the faint β -gal^{+ve} myonuclei indicate prior *Fgf10* expression, and those which are more intensely stained are still expressing *Fgf10*. A similar paradigm has also been observed in the adult mammalian brain. In the pons (part of the brainstem), subsets of neurons were found to express *Fgf10*, however the intensity of the lacZ/ β -gal expression also varied between neurons (Figure 3.17A) (Hajihosseini et al., 2008). In this respect, the β -gal expression patterns observed in the brainstem mirror those seen in skeletal muscle (Figure 3.17B). RNA *in situ* hybridization for *Fgf10* was performed on *tibialis anterior* sections, a muscle which consistently contains a significant proportion of β -gal-expressing myofibers in the adult. The *in situ* revealed a potential signal for *Fgf10* in a subpopulation of myonuclei at P60 (Figure

3.17C, D). This heterogeneity is similar to β -gal expression and could indicate that the *Fgf10* gene is active in adult skeletal muscle at this age. However, further in situ analysis is required with more negative control muscle samples to substantiate this.

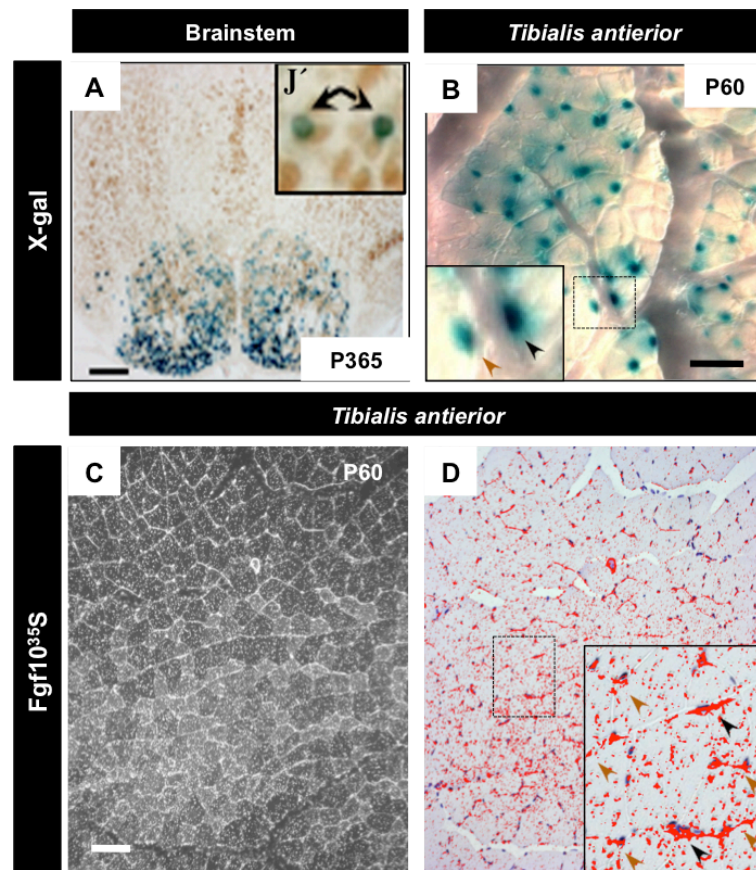


Figure 3.17: Variations in intensity of β -gal protein, using the *Fgf10*^{nlacZ} reporter line, are reflected by different levels of *Fgf10* expression. *LacZ* acts as a reporter for *Fgf10* expression. Its product, β -galactosidase, can be detected by X-gal staining. (A, B) Variations in intensity of β -gal expression can be seen in both X-gal stained adult brain and muscle tissue, with strong expression in some nuclei (black arrowheads) and faint expression in others (brown arrowheads). Note larger dots of β -gal expression are likely due to slight variations in section thickness. (C) *Fgf10* mRNA *in situ* signal also differs in intensity in skeletal muscle, seen more clearly in the overlay image (D, inset). Scale bars: 250 μ m (A); 50 μ m (B, C, D). Panel A adapted from Hajhosseini et al., 2008.

The relative proportions of β -gal^{+ve} myofibers differed substantially, and also appeared conserved, between muscles. For example, the *digastricus* (jaw), *teres major* (upper forelimb), *spinalis thoracis* and *longissimus thoracis* (back) were consistently densely populated with β -gal^{+ve} myofibers. Conversely, moderate levels of β -gal expression were observed in the *masseter* (jaw), *sternohyoideus* (neck), *pectoralis major* (upper thorax), *triceps brachii* (upper forelimb), *quadratus lumborum* and *psoas major* (lumbar region), and *biceps femoris* and *tibialis anterior* (hindlimb). Meanwhile few β -gal^{+ve} myofibers were consistently seen in the *flexor carpii ulnaris* and *biceps brachii* (forelimb), *latissimus dorsi* (shoulder), *gluteus maximus* and *tensor fasciae latae* (hindlimb). The majority of craniofacial muscles, as opposed to limb muscles, are derived from cranial mesoderm, which is composed of prechordal and pharyngeal mesoderm. Limb and trunk muscles on the other hand derive from presomitic mesoderm, which segments into somites, unlike cranial mesoderm. Conversely tongue muscles and some neck muscles are derived from anterior somites (Sambasivan et al., 2011a). Since *Fgf10* is expressed by muscles derived from both cranial and presomitic mesoderm, it seems unlikely that embryological origin determines its expression.

The fiber-type compositions have already been elucidated for many of these muscles in mice. It appears *Fgf10* is predominantly expressed by fast-twitch muscles, mainly composed of Type 2 fibers. However, *Fgf10* is not expressed in all fast muscles, as evidenced by the absence of *Fgf10* expression in the *gastrocnemius* and *extensor digitorum longus* in the hindlimb (Table 3.1). There are three adult Type 2 fiber isoforms: 2A (fast-twitch oxidative), 2B (fast-twitch glycolytic) and 2X/D (fast-twitch, mixed oxidative-glycolytic) myofibers. It is possible that *Fgf10* expression is enriched in a particular fiber subtype, which could explain why it is not expressed in all fast muscles since the fiber-type compositions vary significantly. This would suggest that *Fgf10* expression is linked directly to

the function of the muscle it is expressed in, and perhaps the varying proportions of $\beta\text{-gal}^{+ve}$ myofibers reflect a different functional need for them between the muscles. In the jaw for example, the *digastricus*, which contains a significant proportion of $\beta\text{-gal}^{+ve}$ myofibers, serves to open the jaw by depressing the mandible. In contrast, the *masseter* contains fewer $\beta\text{-gal}^{+ve}$ myofibers, and antagonizes the action of the *digastricus* to close the jaw. Similarly, the *sternomastoideus* in the neck helps to elevate the hyoid bone and it contains a significant proportion of $\beta\text{-gal}^{+ve}$ myofibers. Its antagonist, the *sternohyoideus*, also contained a smaller proportion of $\beta\text{-gal}^{+ve}$ myofibers like the *masseter* (Haggman-Henrikson et al., 2013). In the hindlimb, the *tibialis anterior* also contains a moderate proportion of $\beta\text{-gal}^{+ve}$ myofibers, and it predominantly acts to flex the lower leg. However, the *gastrocnemius* counteracts to extend the lower leg, and this muscle does not express *Fgf10* (Hesselink et al., 2002). It is possible that *Fgf10* expression may correlate with function in agonist/antagonist muscle pairs. However, the reasons for differences in numbers of $\beta\text{-gal}^{+ve}$ myofibers within these pairs remain unclear.

Fgf10 expression in skeletal muscle appears to be upregulated at around birth. In certain muscle groups of newborn mice, such as the *acromiotrapezius* and *teres major*, $\beta\text{-gal}$ was already highly expressed. In others however, including the *biceps femoris* and *tibialis anterior*, $\beta\text{-gal}$ expression was faint yet present nonetheless, indicating it was in the process of being upregulated. By P22, $\beta\text{-gal}$ expression patterns matched those seen at older ages, with the exceptions of higher numbers of $\beta\text{-gal}^{+ve}$ myofibers in the *masseter*, *clavotrapezius* (shoulder) and *tensor fasciae latae* (hindlimb). These first three weeks of life are critical to growth and maturation of muscles. In response to increasing hormone levels, maturation of excitation-contraction coupling mechanisms, and increased neuromuscular activity, muscles rapidly alter their developmental myosin isoform content to mature, adult isoforms (Agbulut et al., 2003). Muscles also grow through hypertrophy (increase in size)

during this period. The β -gal expression patterns at P0 and P22 indicate *Fgf10* is expressed in certain muscles of the forelimb at birth, and is subsequently upregulated in other muscle groups during post-natal development. Expression of *Fgf10* in the forelimb muscles could be attributed to an earlier functional requirement for these muscles around P0. The upregulation of *Fgf10* in the remaining muscles during this critical period suggests it might play a role in the hypertrophic mechanism, potentially by stimulating the maturation of fast fiber-types. To explore this further, it would be beneficial to conduct a qRT-PCR time course of *Fgf10* expression in these muscles on every other day (or every 7 days) during this period. This would help to validate whether the increase in β -gal expression within these muscles between P0-P22 is an accurate indication of *Fgf10* upregulation at birth.

At the embryonic stages investigated, β -gal-expressing cells were identified in the developing forelimb buds and also in the somites at E9 and E12. At E17, β -gal-expressing cells co-localised with the tendon marker tenascin C in three major tendons of the distal hindlimb. These findings suggest a potential dual function for *Fgf10* during embryonic muscle and tendon development. It is possible that the *Fgf10*-expressing cells localized to the somitic region between E9-E12 could constitute a subpopulation of *Pax3*-expressing myoblasts, and are therefore likely to contribute to primary myogenesis. This could be verified by performing co-immunolabelling with anti-*Pax3* and anti- β -gal antibodies. A second wave of *Fgf10*-expressing cells could also give rise to tendon later in development.

The patterning of individual limb muscles is poorly understood and studies so far have only focused on limb patterning as a whole. The T-box transcription factors *Tbx4* and *Tbx5* are expressed in the hindlimb and forelimb respectively, and their inactivation severely disrupts limb muscle and tendon patterning (Hasson et al., 2010; Ouimette et al., 2010; Saito et al., 2006; Szeto et al., 1999). *Tbx4* and *Tbx5* expression activates *Fgf10*

expression in the limb bud mesenchyme. However, once the limb is fully formed their activity is no longer required. A direct link between the regulation of *Fgf10* expression by *Tbx4/5* specifically within muscle has yet to be established (Hasson et al., 2007; Minguillon et al., 2005; Tzchori et al., 2009). Given the specific patterns and timing of β -gal expression within the muscles discussed, it seems more likely that a requirement for *Fgf10* is related to function of the muscle, rather than patterning of these muscles in embryonic development.

A significant feature of β -gal⁺ muscles is that they are predominantly composed of fast (Type II) fibers. It is therefore possible that *Fgf10* is involved in specification of fast fiber-types. Similarly, because it appears to be upregulated after birth in the majority of β -gal⁺ muscles, it is possible that *Fgf10* helps mediate muscle hypertrophy. Likewise *Fgf10* could play a role in muscle regeneration in the adult, so perhaps a subset of *Fgf10*-expressing cells is satellite cells. These hypotheses will be explored in the following Chapter.

Chapter 4

Putative function(s) of *Fgf10* in skeletal muscles *in vivo*

4.1 Introduction

The mechanisms controlling myoblast proliferation and migration have gradually been elucidated. However, our understanding of the molecular and cellular programming of distinct muscle fiber types is limited (Pin et al., 2002). Muscle fiber types fall into two classes depending on their specific expression of contractile protein isoforms and metabolic enzymes. The slow oxidative myofibers (Type 1) are fatigue-resistant, whilst the fast glycolytic myofibers (Type 2) are not fatigue-resistant and are employed during shorter, more intense activities. It still remains unclear how exactly primary and secondary myofibers are pre-determined to give rise to the various fiber subtypes.

Development of the neuromuscular junction (NMJ) is closely associated with that of myofibers. The NMJ is a highly specialised synapse which transfers signals from motor neurons to skeletal muscle fibers via the release of the neurotransmitter acetylcholine (ACh) in vertebrates. During primary myogenesis, muscle cells express acetylcholine receptors (AChRs) in an embryonic form. Upon contact with the muscle, the AChRs are induced to adopt an adult form through a shift in gene expression (Bernareggi et al., 2012). The mechanisms deployed by motor axons to induce postsynaptic differentiation at the NMJ are relatively well characterized. However, signals emanating from the myofibers themselves require further characterization. Agrin is a well-characterized neuronal factor which stabilizes the AChR clusters. To do this, it activates the receptor tyrosine kinase MuSK via the LDL receptor LRP4, and this trimeric complex is essential to the proper formation of the NMJ (Wu et al., 2012).

Whilst it is widely acknowledged that satellite cells contribute significantly towards post-natal muscle fiber growth (hypertrophy), maturation and repair, there is great debate surrounding the specific mechanisms employed by satellite cells in these processes (Hutcheson et

al., 2009; Kuang and Rudnicki, 2008; McCarthy et al., 2011; Zammit et al., 2006a). Resounding evidence for their role during post-natal muscle growth came from the *Pax7* knockout (*Pax7*^{-/-}) mouse, in which over 90% of satellite cells are deleted, and severely compromised post-natal muscle growth was observed. In addition, myofiber regeneration is also compromised in these animals (Oustanina et al., 2004). However, given that very few of these animals survive into adulthood, and the growing body of evidence that adult satellite cells differ intrinsically from their younger counterparts, the conclusions made about satellite cells in this model cannot be reliably extended to adult muscle hypertrophy (Lepper et al., 2009).

4.1.1 Aims

Fgf10 appears to be expressed in muscles which are mainly fast-twitch in nature. This could indicate a specific role for the gene in the specification or patterning of fast (Type 2) myofibers. Given the levels of its expression at postnatal and adult ages, it is also possible that *Fgf10* could play a role in muscle regeneration. Therefore, a subset of *Fgf10*-expressing cells could constitute a group of satellite cells. Taking these factors into consideration, the aims of this chapter are to address the following hypotheses:-

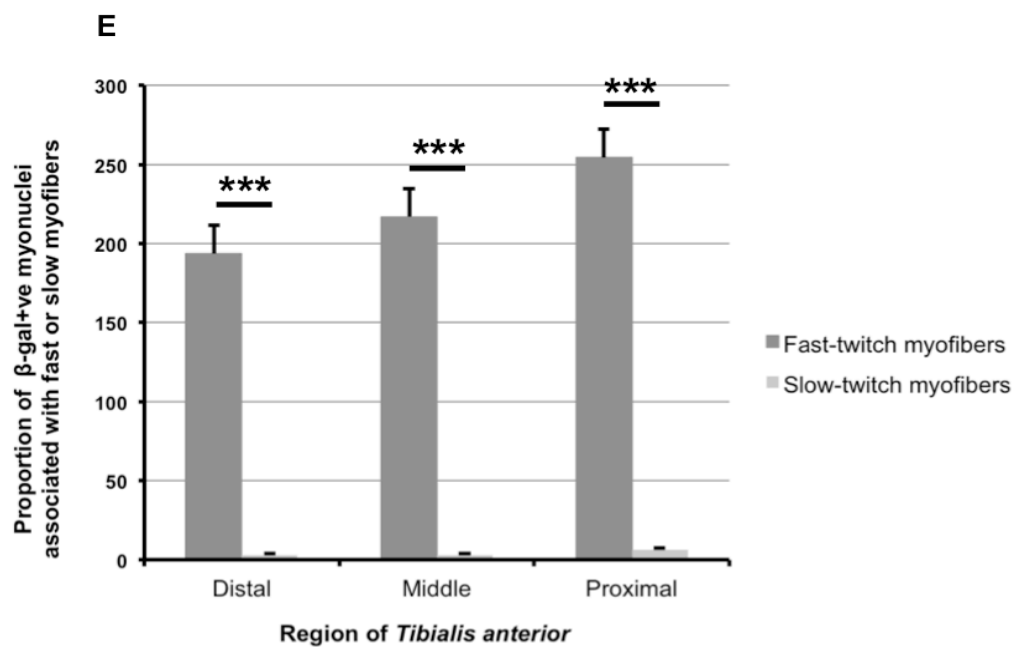
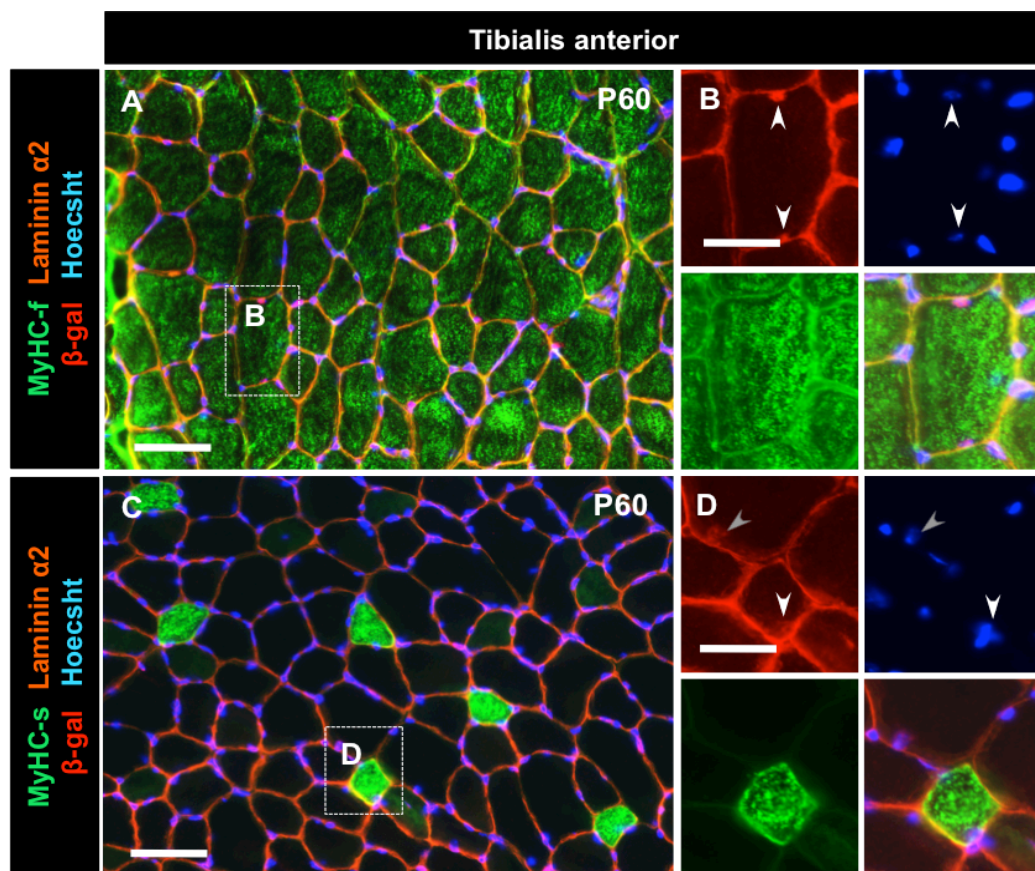
1. Is *Fgf10* expression enriched in a specific fast-twitch fiber subtype?
2. Does *Fgf10* expression correlate with the persistence of neonatal myosin in adult skeletal muscle?
3. Is *Fgf10* involved in establishing the neuromuscular junction (NMJ)?
4. Are a subset of *Fgf10*-expressing cells satellite cells?

4.2 Results

4.2.1 The majority of *Fgf10*-expressing myonuclei are found within type 2B myofibers.

In order to investigate whether fast- or slow-twitch fibers express *Fgf10*, transverse cryostat sections of P60 *tibialis anterior* muscle were immunolabelled with an anti- β -gal antibody, and pan-fast or slow myosin heavy chain (MyHC-f or MyHC-s) antibodies to visualize in which proportions the β -gal-expressing myonuclei co-localize with the two fiber-types (Figure 4.1). Again, four non-overlapping quadrants were imaged across the sections, under the x40 objective, and an average proportion of β -gal^{+ve} myonuclei associated with either fiber type was calculated, at each muscle level (distal, middle, proximal) surveyed. The *tibialis anterior* contains approximately 80% Type 2 (fast) fibers and 20% Type 1 (slow) fibers, and was selected for analysis because it is frequently used by muscle biologists and well characterized (Bloemberg and Quadrilatero, 2012). At the three levels surveyed, β -gal^{+ve} myonuclei co-localised with both fast (Figure 4.1A, B) and slow (Figure 4.1C, D) fiber markers. When quantified, 98% of β -gal^{+ve} myonuclei co-localised with the MyHC-f marker throughout the muscle. A Student's unpaired *t*-test was carried out and gave a *p*-value of 0.0002, deeming this difference statistically significant (Figure 4.1E). Laminin- α 2 and β -gal were detected with different AlexaFluor568® secondary antibodies, and their localization is distinguishable as they occupy different antigenic compartments. If this experiment were to be repeated, it would be worth immunolabelling adjacent sections with either the anti- β -gal or laminin- α 2 antibodies, in conjunction with the anti-MyHC antibodies. This would allow clearer visualization of β -gal, and the myofiber border on the adjacent section, so as to verify the co-localisation patterns seen.

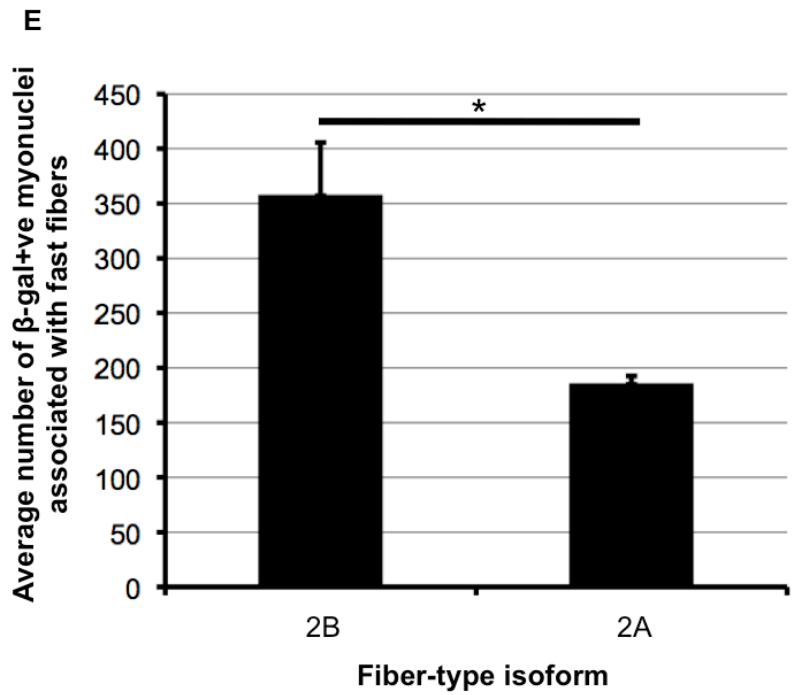
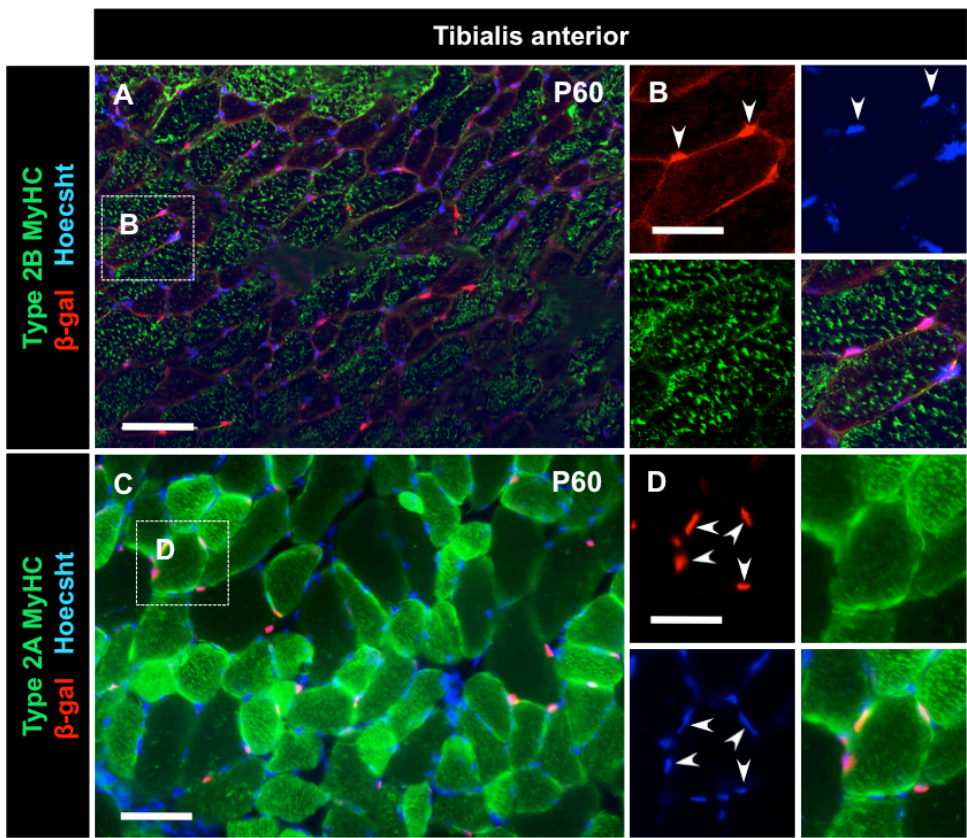
Figure 4.1: β -gal-expressing myonuclei co-localise with both fast and slow myofibers. The MyHC-f and MyHC-s antibodies give punctate cytoplasmic staining for all the type 2 fiber subtypes and type 1 fibers respectively. Laminin- $\alpha 2$ highlights the basal lamina of the myofibers; the cross-sectional area of type 2 myofibers is markedly bigger than type 1 myofibers. β -gal^{+ve} myonuclei (white arrowheads), can be found within fast (A, B) and slow (C, D) myofibers. β -gal^{+ve} myonuclei can also be seen in myofibers negative for MyHC-s (grey arrowheads) in (D). (E) The majority of β -gal^{+ve} myonuclei counted co-localise with fast fibers. Error bars represent standard error, n=3. *** $p \leq 0.0001$. Primary antibodies: Ms- α -MyHC-s; Ms- α -MyHC-f; Rt- α -Laminin $\alpha 2$; Ck- α - β -gal. Secondary antibodies: Gt- α -Ms AlexaFluor488; Gt- α -Rt AlexaFluor568; Gt- α -Ck AlexaFluor 568; Hoechst counterstain. Scale bars: 100 μ m (A, C); 50 μ m (B, D).



To determine which fast-fiber subtype β -gal^{+ve} myonuclei co-localised with, additional P60 *tibialis anterior* sections were taken from the three levels within the muscle, and immunolabelled with anti- β -gal antibodies and either type 2B- or 2A-specific MyHC antibodies (Figure 4.2A-D). It is worth noting that the image panels presented in Figure 4.2 are not representative of serial muscle sections, and therefore the type 2B and type 2A myofibers detected are not equally distributed.

Using the same quantification method as detailed on pg.168, 66% of β -gal^{+ve} myonuclei counted were found to co-localise with type 2B myofibers. Another Student's unpaired *t*-test revealed a *p*-value of 0.0231, indicating a statistically significant difference. Taken together, these results indicate that *Fgf10* expression is potentially preferentially expressed by type 2B myofibers. Future investigations would benefit from repeating this analysis and surveying a higher number of quadrants across the glycolytic and oxidative/glycolytic regions of the muscle (as described in Figure 3.10), so as to investigate the myofiber heterogeneity with greater accuracy. Similarly, it would be beneficial to repeat the double-immunolabelling experiment with anti- β -gal and anti-type 2X/D fiber antibodies, in order to observe whether β -gal associates with this third fast fiber subtype. An alternative approach to investigate β -gal expression in fast fiber subtypes would have been to stain for succinate dehydrogenase (SDH) activity, which is compatible with the X-gal staining procedure. However this method relies on recognizing colour intensities within the fibers which is not as specific as immunolabelling with antibodies.

Figure 4.2: β -gal-expressing myonuclei preferentially co-localise with type 2B myofibers. β -gal⁺ myonuclei, indicated by the white arrowheads, can be found within MyHC-2B (A, B) and MyHC-2A (C, D) fibers (non-serial sections displayed). (E) Approximately twice the number of β -gal⁺ myonuclei co-localise with type 2B myofibers. Error bars represent standard error, n=3. * $p \leq 0.05$. Primary antibodies: Ms- α -MyHC-2A; Ms- α -MyHC-2B; Ck- α - β -gal. Secondary antibodies: Gt- α -Ms AlexaFluor488; Gt- α -Ck AlexaFluor 568; Hoechst counterstain. Scale bars: 100 μ m (A, C); 50 μ m (B, D).



4.2.2 *Fgf10* expression does not correlate with persistence of neonatal myosin isoforms in post-natal and adult muscles.

An investigation was carried out to see if *Fgf10* expression correlated with the persistence of neonatal myosin isoforms. Of the six MyHC genes which are expressed in skeletal muscle, two are expressed during development. Embryonic MyHC (MyHC-emb) and neonatal (or perinatal/fetal) MyHC (MyHC-neo) are expressed sequentially, and their transcripts are detectable around E9.5 and E10.5 respectively. MyHC-emb expression peaks around E15, whereas MyHC-neo persists until approximately P7 (Beylkin et al., 2006; Lyons et al., 1990). Both isoforms are downregulated after birth and replaced with mature adult MyHC isoforms. However, the rate at which this occurs depends on the designated function of the muscle. For example, in slow-twitch muscles, developmental myosin isoforms are downregulated at a slower rate in comparison to fast-twitch muscles, which see a more rapid replacement with adult fast and slow MyHC isoforms (Agbulut et al., 2003).

Interestingly, in the murine *masseter*, developmental myosin isoforms persist during post-natal and adult development in comparison to other skeletal muscles of the trunk and limbs. MyHC-emb is present until around P14, whereas MyHC-neo can be detected up to three months after birth. The exact function for these isoforms in the post-natal *masseter* has yet to be elucidated (Agbulut et al., 2003; d'Albis et al., 1986). Similarly, studies on the adult human *digastricus* have revealed a small population of neonatal myosin fibers which also persist after birth (Wang et al., 2004). Given the high proportions of $\beta\text{-gal}^{+ve}$ myofibers which are present in both muscles throughout post-natal and adult development (see Table 3.1a), it is possible that *Fgf10* expression might correlate with neonatal myosin persistence in adult skeletal muscle. To explore this further, cohorts of muscles (both $\beta\text{-gal}^{+ve}$ and $\beta\text{-gal}^{-ve}$, as determined by the survey in Table 3.1) were isolated from wildtype mice at three different ages: young (P10),

adult (P45-50) and old (P95-100). Two animals were used per age bracket in order to have two biological replicas. After RNA extraction, qRT-PCR analysis was carried out to measure the levels of neonatal myosin (encoded by *Myh8*) in order to investigate whether MyHC-neo persists in *Fgf10*-expressing muscles post-natally. β -gal^{+ve} muscles included: *digastricus* (DIG), *masseter* (MASS), *teres major* (TM), *triceps brachii* (TB), *biceps femoris* (BF) and the *tibialis anterior* (TA). β -gal^{-ve} muscles included: *tensor fasciae latae* (TFL), *gastrocnemius* (GC), *extensor digitorum longus* (EDL) and *soleus* (SOL). At P10, certain muscles within this cohort (i.e. MASS, TB, TFL and BF) did not yield good-quality RNA and could not be carried through to further analyses. Therefore a reduced number of muscles were investigated at this age. All other muscle RNA concentrations ranged from 50-500ng/ μ L, and *Myh8* expression levels were normalized to both *18s* and *Gapdh* house keeping genes, as both have been validated as internal reference genes in mouse skeletal muscle (Coley et al., 2012; Piccioni et al., 2014).

At P10, low levels of *Myh8* were detected in the β -gal^{+ve} *teres major* and *tibialis anterior*, and also in the β -gal^{-ve} *gastrocnemius*, *extensor digitorum longus* and *soleus* (Figure 4.3). Interestingly, *Myh8* was not detected in the *digastricus* at P10. This may be because the C_T values for *18S* expression in one of the *digastricus* samples were not consistent with the rest of the P10 muscle samples, most likely due to a loading error prior to cDNA synthesis. This resulted in over-elevated *Myh8* levels when normalized to the much lower *18S* value, and for analysis purposes was omitted. That said, the *Myh8* level in the parallel P10 *digastricus* sample was very low, indicating that *Myh8* is not expressed in this muscle at this age. At P45-50 and P95-100, *Myh8* persists to a certain degree in the *masseter*, its levels decreasing in old muscle. *Myh8* was not detected in any of the other muscles screened. These data suggest that neonatal myosin persists in a range of skeletal muscles at young post-natal ages,

irrespective of *Fgf10* expression, and continues to be expressed uniquely in the *masseter* in the adult.

In order to validate whether the decrease in *Myh8* seen between P45-50 and P95-100 is significant, this experiment would need to be repeated so that statistical analysis can be performed. Fresh RNA samples, of good quality, could also be generated for the missing muscles at P10 and re-analyzed.

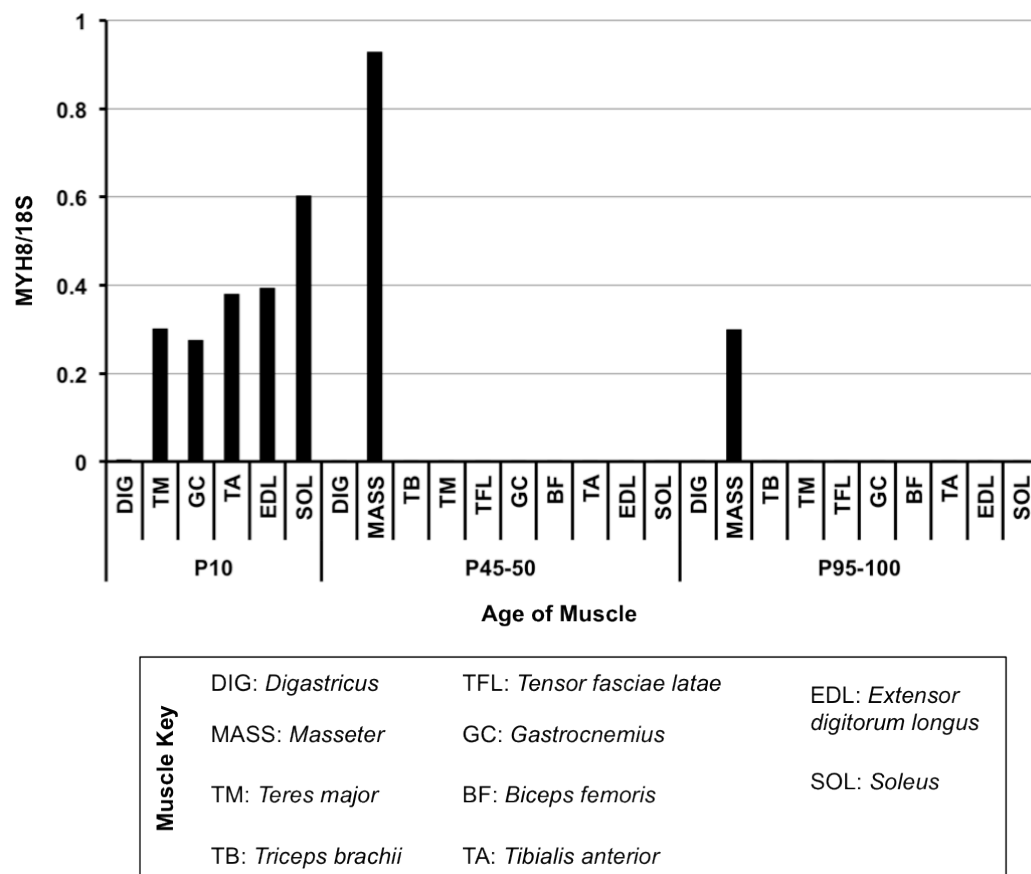


Figure 4.3: Neonatal myosin (*Myh8*) mRNA levels as determined by qRT-PCR in post-natal and adult muscles. *Myh8* can be detected at low levels in the *teres major*, *gastrocnemius*, *tibialis anterior*, *extensor digitorum longus* and *soleus* at P10. At P45-50 *Myh8* is detected in the *masseter*, and its levels decrease by P95-100. No statistical analysis carried out owing to n=2 for all muscle samples.

4.2.3 *Fgf10* expression does not appear to co-localize with the neuromuscular junction.

An investigation was also carried out to see if *Fgf10* may be involved in establishing the NMJ. At the NMJ, the presynaptic motor terminal harbors synaptic vesicles containing acetylcholine. This neurotransmitter is released onto the apposing postsynaptic myofiber membrane, which contains hundreds of acetylcholine receptors (AChRs). In order to dissect the mechanisms of NMJ assembly, understanding the accumulation of synaptic vesicles and the clustering of AChRs is of great interest (Sanes and Lichtman, 2001). It is currently understood that clustering and stabilization of AChRs are mediated by the activation of the muscle tyrosin kinase MuSK, which is in turn stimulated by the transmembrane protein Lrp4 and the heparan sulfate proteoglycan agrin, which is released by nerves (Li et al., 2011). It is also known that myopodia (muscle cell-generated filopodia) facilitate muscle-nerve contacts as they respond to agrin and therefore play a key role in NMJ assembly (Madhavan et al., 2006). Recent evidence has implicated FGF-2, which is secreted from muscle and stimulates axonal filopodia extensions towards the postsynaptic myofiber. Furthermore, FGFR1 signaling promotes axonal filopodia formation and FGF-2 knockdown in muscle reduces the establishment of the NMJ (Li et al., 2011).

An acetylcholinesterase (AChE) histochemical reaction reveals motor end plates (MEPs) at the NMJ, which look like black speckles running across the myofibers (Figure 4.4A-D). In vibratome sections, AChE can be seen more clearly clustering around certain myofibers (Figure 4.4E, F).

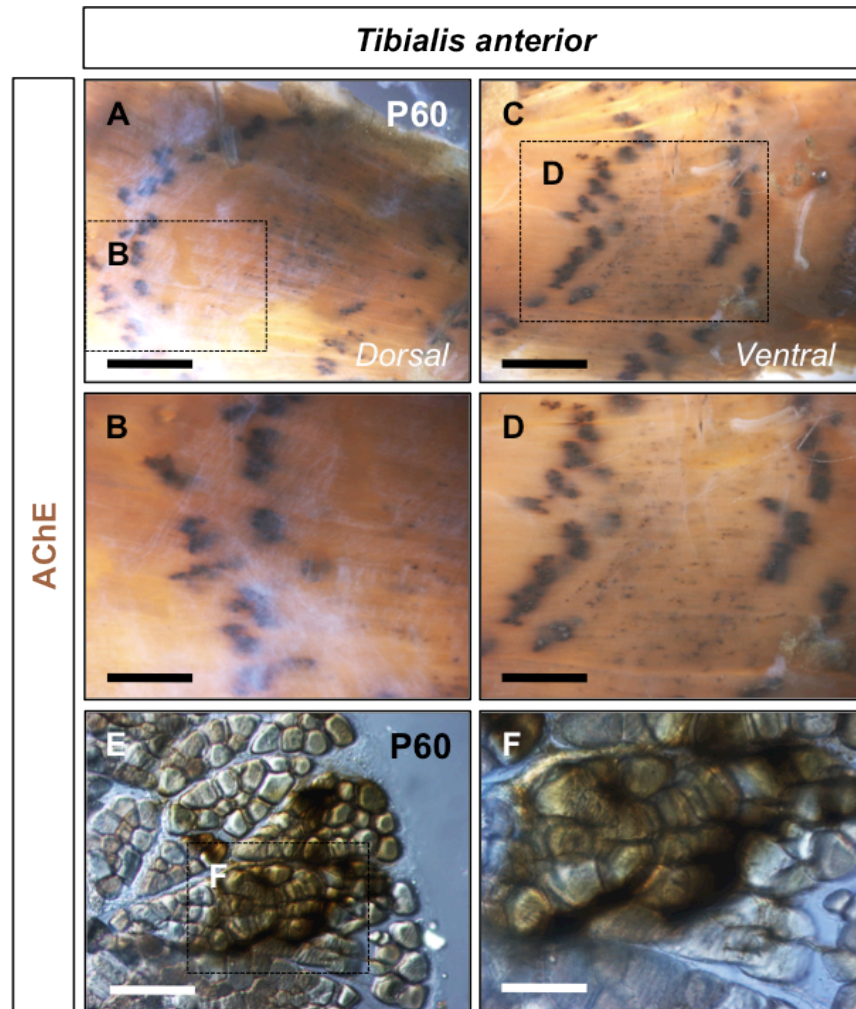


Figure 4.4: Location and span of motor end plate (MEP) regions in the mouse *tibialis anterior*. (A-D) After an acetylcholinesterase (AChE) histochemical reaction the MEPs are revealed as brown speckles on the dorsal and ventral surfaces of the muscle. (E, F) AChE clustering around myofibers can be visualized in vibratome sections. Scale bars: 20µm (A, E); 10µm (B, D, F) and 15µm (C).

To investigate whether *Fgf10* might be playing a role in NMJ assembly, X-gal stained *tibialis anterior* and *biceps femoris* muscles were also stained for AChE to see if the majority of β -gal-expressing myonuclei were located in close proximity to the NMJ (Figure 4.5). It became apparent that in both muscles β -gal⁺ myofibers are not uniquely

associated with MEPs, as the distribution of β -gal^{+ve} myofibers within the muscle does not correlate directly with MEP sites (Figure 4.5A, B). β -gal^{+ve} myofibers can be seen adjacent to β -gal^{-ve} myofibers within MEP regions (Figure 4.5C, D). In vibratome sections it also became clear that not all β -gal^{+ve} myonuclei are situated at the NMJ (Figure 4.5E, F).

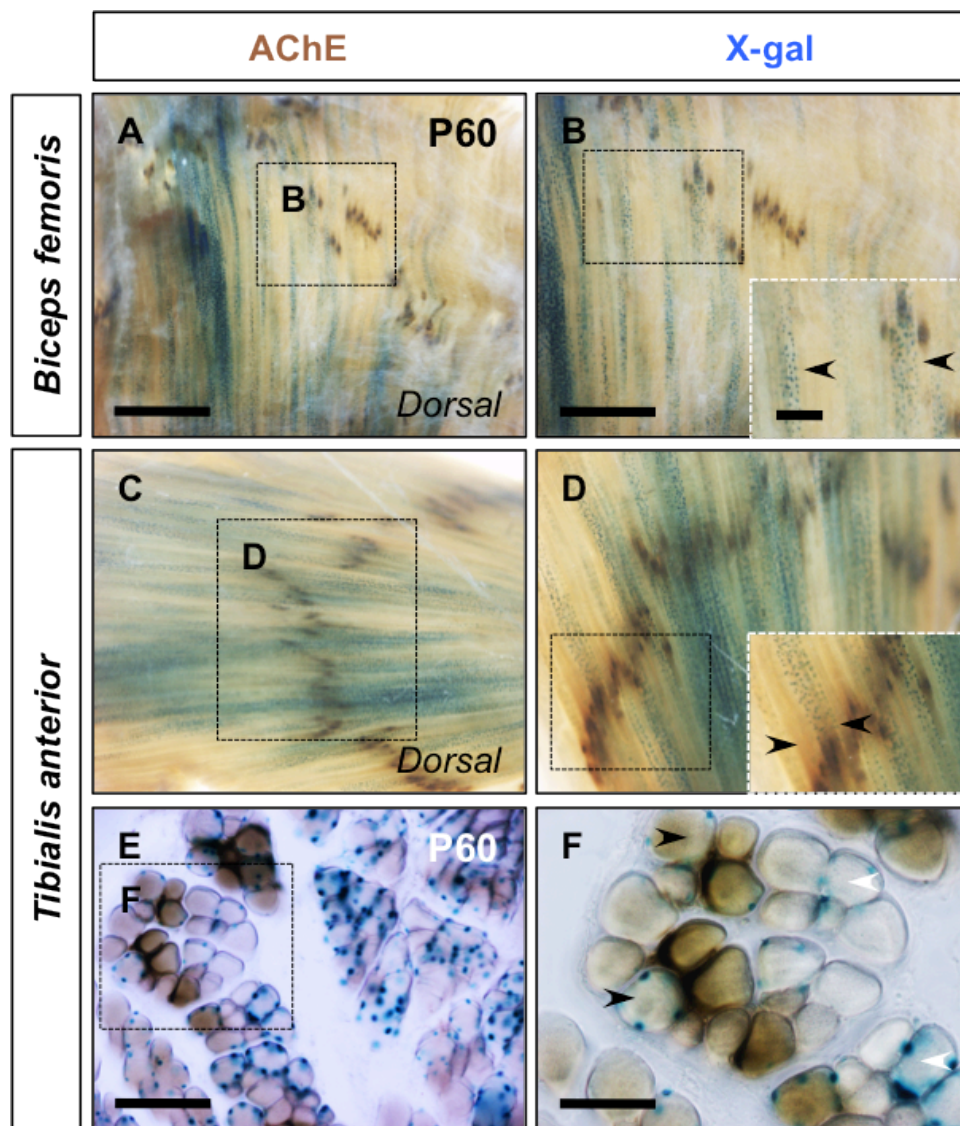


Figure 4.5: β -gal-expressing myonuclei are not clustered at the NMJ. (A-D) β -gal^{+ve} myonuclei run the length of the myofiber and are not localized to the NMJ. (E, F) The vast majority of β -gal^{+ve} myonuclei are not located near the NMJ, indicated by grey arrowheads. Scale bars: 15μm (A, C); 12μm (B, D – insets 6μm); 20μm (E) and 10μm (F).

4.2.4 A small fraction of *Fgf10*-expressing myonuclei express the satellite cell marker *Pax7*.

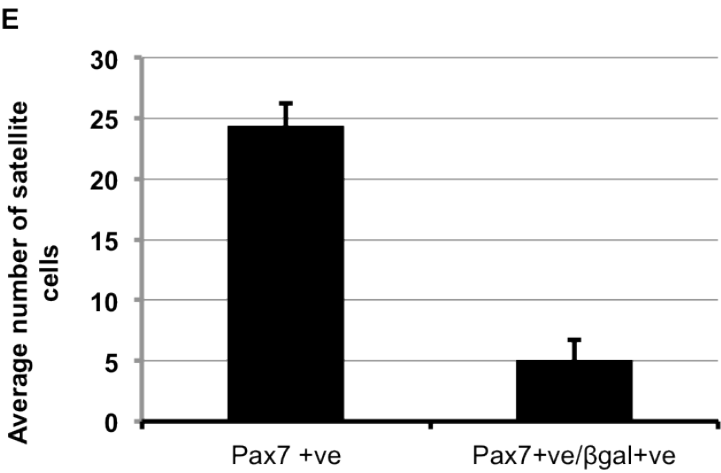
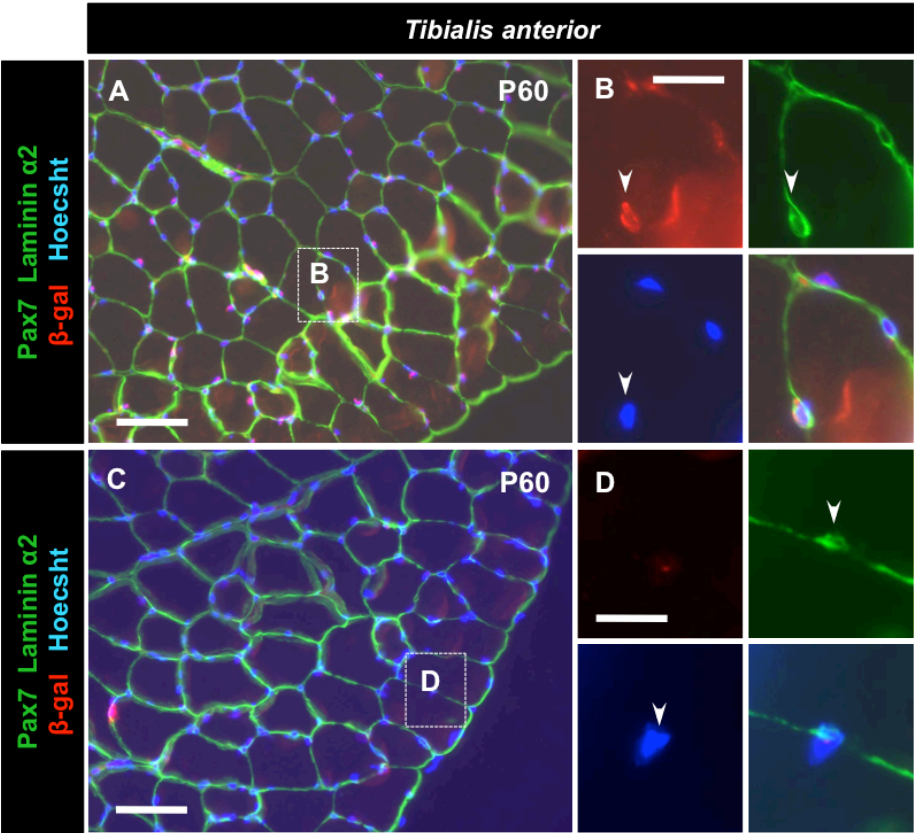
Satellite cells play a multifaceted role in skeletal muscle plasticity and remodelling. They are positioned between the sarcolemma and basement membrane of the myofiber (Murphy et al., 2011). Myonuclei within myofibers are post-mitotic, and therefore regeneration in adult muscle is mediated by myogenic progenitors or satellite cells. Otherwise quiescent, satellite cells can be stimulated to proliferate and differentiate into myoblasts in order to fuse to existing damaged myofibers or alternatively fuse into new ones. The satellite cell pool is continuously replenished, although their proliferative capacity and overall numbers gradually decline with age (Oustanina et al., 2004). Whilst they can be identified based on their anatomical location, they express a host of markers including: *Pax7*, α_7 and β_1 integrin, CD34 and NCAM (Beauchamp et al., 2000; Burkin et al., 2005; Covault and Sanes, 1986; Seale et al., 2000). It is increasingly acknowledged that satellite cells display heterogeneity between and within muscles, and as a result there is no single molecular marker specific to the population.

Satellite cells typically account for approximately 2-5% of total myonuclei in healthy adult muscle (Putman et al., 2001). Whilst the number of *Fgf10*-expressing myonuclei exceeds this, it is possible that a proportion of these myonuclei are, or previously descended from, satellite cells. The intensity differences in the blue β -gal colour are perhaps an indication of this also. To investigate this further, immunostaining for *Pax7* was carried out on adult *tibialis anterior* cryostat sections, in conjunction with laminin- α 2 in order to visualize the basal lamina and verify satellite cell identity. The same quantification method was used as detailed in section 4.2.1, surveying four different fields across the muscle at three regions throughout the muscle. An average was taken of the satellite cells counted in this manner. This analysis revealed that a small proportion of

β -gal-expressing myonuclei co-localise with *Pax7* (Figure 4.6A, B), however the majority do not (Figure 4.6C, D). When quantified, 21% of *Pax7*-expressing myonuclei were found to co-localise with anti- β -gal antibodies (Figure 4.6E). These data suggest a subset of *Fgf10*-expressing cells is satellite cells.

In order to explore this hypothesis further, it would be beneficial to repeat this immunolabelling experiment on isolated myofibers (enzymatically dissociated) from the *tibialis anterior* muscle. This would facilitate clearer visualization of the satellite cells.

Figure 4.6: A subset of *Pax7*-expressing satellite cells expresses β -gal. (A-D) Satellite cells can be identified by their anatomical location within the basal lamina, highlighted by laminin- α_2 expression, of myofibers. (E) Approximately 20% of *Pax7*^{+ve} myonuclei also express β -gal. Error bars represent standard error, n=3. Primary antibodies: Ms- α -*Pax7*; Rt- α -Laminin α_2 ; Ck- α - β -gal. Secondary antibodies: Gt- α -Ms AlexaFluor488; Gt- α -Rt AlexaFluor488; Gt- α -Ck AlexaFluor 568; Hoechst counterstain. Scale bars: 100 μ m (A, C); 50 μ m (B, D).



4.3 Discussion

Results in Chapter 3 showed that *Fgf10* is expressed in a subgroup of skeletal muscles during post-natal and adult development. Using an *Fgf10^{nLacZ}* reporter line, a specific expression pattern for β -gal was observed in a subgroup of muscles, and this pattern was recapitulated in the same muscle cohort at various ages. Furthermore, *Fgf10* expression was limited to a subpopulation of myofibers in varying numbers within these muscles. The myonuclei populations within these myofibers were also heterogeneous for *Fgf10* expression. It was subsequently shown that the muscles which express *Fgf10* have previously been characterised as fast muscles, and in several instances composed predominantly of type 2B myofibers. With this in mind, it was hypothesized that *Fgf10* could play a role in fast fiber specification. Given that the pattern of *Fgf10* expression is established around birth in some muscles, and is rapidly upregulated post-natally in others, it would appear that *Fgf10* expression might correlate with muscle hypertrophy. Since *Pax7*-expressing satellite cells are amongst the key mediators of this process, it was also hypothesized that a subgroup of *Fgf10*-expressing myonuclei might express this marker. The potential role for *Fgf10* in neuromuscular junction assembly and persistence of a developmental myosin isoform were also explored in this Chapter.

Skeletal myofibers are classified as either Type 1 (slow) or Type 2 (fast), and display differences in fatigability, contractility and metabolism. Whilst it is not completely understood how primary and secondary myofibers are pre-patterned during embryonic myogenesis to give rise to slow and fast fibers, the molecular mechanisms which establish and maintain both phenotypes are gradually being elucidated. Promoters of the slow phenotype include calcineurin phosphatase and CaM kinase signaling, which both activate the MEF2 transcription factor. A member of the mitogen-activated protein kinase (MAPK) family, extracellular signal-

regulated kinase 1/2 (ERK 1/2), is enriched in fast fibers compared to slow fibers (Shi et al., 2008). The transcription factor *Sox6* is also implicated in the fast phenotype as it suppresses slow myofiber-specific genes (An et al., 2013). To investigate the identity of the fiber types composed of β -gal-expressing myonuclei, sections of *tibialis anterior* were immunolabelled with anti- β -gal and fast or slow myosin heavy chain (MyHC) antibodies. β -gal⁺ myonuclei predominantly co-localized with type 2B myofibers, rather than type 2A myofibers (Figure 4.2E). Six1 proteins are also enriched in the myonuclei of adult mouse fast fibers (Grifone et al., 2004). In the embryo, myotomal *Six1/Six4* expression has also been shown to induce fast-type muscle specific genes (Niro et al., 2010). In addition, *Six1* and *Six4* directly induce *Fgf6* and *Fgf4* in the myotome, which subsequently induce *scleraxis* expression in the syndetome (Brent and Tabin, 2004). *Six1* has previously been shown to regulate *Fgf10* expression during otic development, therefore it is possible that FGF10 signaling is regulated in fast fibers by the *Six* genes (Zheng et al., 2003).

Adult fiber-type phenotype is also partly established by neuromuscular activity (Pette and Vrbova, 1985). The neuromuscular junction (NMJ) is formed around E14 in all muscles and matures postnatally. Receptors for ACh, the excitatory neurotransmitter in vertebrates, form clusters on myofibers during embryonic development in a process called 'prepatternning' which occurs before the first motoneurons make contact. In mice, aneural and neural AChR clusters can arise on the same myofiber during formation of the NMJ. Assembly of the NMJ is mediated by the agrin/Musk/Lrp4 complex, which stimulates AChR clustering to ensure the NMJ forms adequately (Wu et al., 2010). To explore whether *Fgf10* might also be involved in NMJ formation, *tibialis anterior* and *biceps femoris* (both β -gal⁺) muscles were incubated in X-gal staining solution (to reveal the β -gal⁺ myofibers) and subsequently stained for AChE, which hydrolyses ACh at the NMJ. The AChE stain

reveals the MEPs as brown dots, which traverse the myofibers in a wave-like orientation (Figure 4.6). β -gal⁺ myofibers do not appear to correlate uniquely with innervation sites, as myofibers which do not express β -gal are also innervated (Figure 4.7). It is possible however that *Fgf10* expression is stimulated upon innervation, and descendants of *Fgf10*-expressing myonuclei could disperse along the length of the myofiber away from the site of innervation.

Postnatal muscle development is characterized by several key events, including altered neuromuscular activity, increased hormone levels, excitation-contraction coupling and growth. During postnatal development, muscles rapidly adapt to meet the new functional demands through maturation of the developmental MyHCs to the adult (i.e. fast or slow) MyHCs (Agbulut et al., 2003; Mutungi et al., 2003). In the *masseter* and *digastricus*, neonatal myosin has been shown to persist for up to three months, the reasons for which are not completely understood. *Fgf10* is highly expressed in both the *masseter* and *digastricus* during post-natal and adult development. To investigate whether *Fgf10* expression correlates with MYH8 persistence in postnatal and adult development, *Myh8* mRNA levels were measured by qRT-PCR in muscles (both *Fgf10*-expressing and non *Fgf10*-expressing) at different ages to see if it was still present in older *Fgf10*-expressing muscles (Figure 4.3). *Myh8* was detected in all the muscles, apart from the *digastricus*, at P10. In the older cohort, *Myh8* persisted in the *masseter* alone. This suggests that *Fgf10* expression is not linked to persistence of developmental MyHCs, and properties unique to the *masseter* alone demand this isoform at older ages.

Skeletal muscle also grows rapidly during post-natal development; this is predominantly mediated by myofiber hypertrophy (increase in size of individual muscle fibers) (White et al., 2010). This cellular enlargement of myofibers can only occur if the rate of protein synthesis exceeds the

rate of degradation. Two key signaling pathways have been shown to regulate protein synthesis: the IGF-1-Akt-mTOR (positive) and the myostatin-Smad2/3 (negative) pathways. In addition, the proliferation and fusion of satellite cells, to facilitate an increase in myonuclei, is thought to contribute in part to overall myofiber growth. However, recent evidence suggests satellite cells are not the sole mediators in this process (Moss and Leblond, 1971; Pallafacchina et al., 2013; Schiaffino et al., 2013; White et al., 2010). To explore whether any β -gal-expressing cells could potentially be satellite cells, β -gal co-localization with the satellite cell marker *Pax7* was investigated in adult *tibialis anterior* muscle. A small fraction of β -gal⁺ myonuclei also expressed this nuclear transcription factor, and co-localization was seen in approximately 20% of *Pax7*-expressing cells (Figure 4.8A, B, E).

To gain a deeper insight into the potential functions of *Fgf10* in skeletal muscle *in vivo* a novel transgenic line was developed using Cre/LoxP technology. *Fgf10* expression was specifically deleted from *Pax3*-expressing muscle progenitors in this line, so that skeletal muscle phenotype in the absence of *Fgf10* could be explored. The generation of this line and subsequent analyses will be explained in Chapter 5.

Chapter 5

Investigation of *Fgf10*'s function(s) *in vivo*

5.1 Introduction

The advent of the Cre-loxP recombinase system in the 1980s has allowed significant progress in mouse genome modifications, by providing a tool to activate or inactivate gene function in order to explore the effects on downstream processes.

Cre is a 38kDa site-specific recombinase protein, encoded by the P1 bacteriophage, belonging to the enteric bacteria family (members include *Escherichia coli*) (Lobocka et al., 2004). It recognizes *loxP* sequences on DNA molecules and forms a DNA-protein complex at these sites, in order to facilitate recombination or synapsis (Hamilton and Abremski, 1984). *LoxP* sequences measure 34-bp, containing an 8-bp spacer region flanked by a pair of 13-bp palindromic repeats (Abremski and Hoess, 1984). Crucially, the location and orientation of these sequences determines the type of recombination, see Figure 5.1.

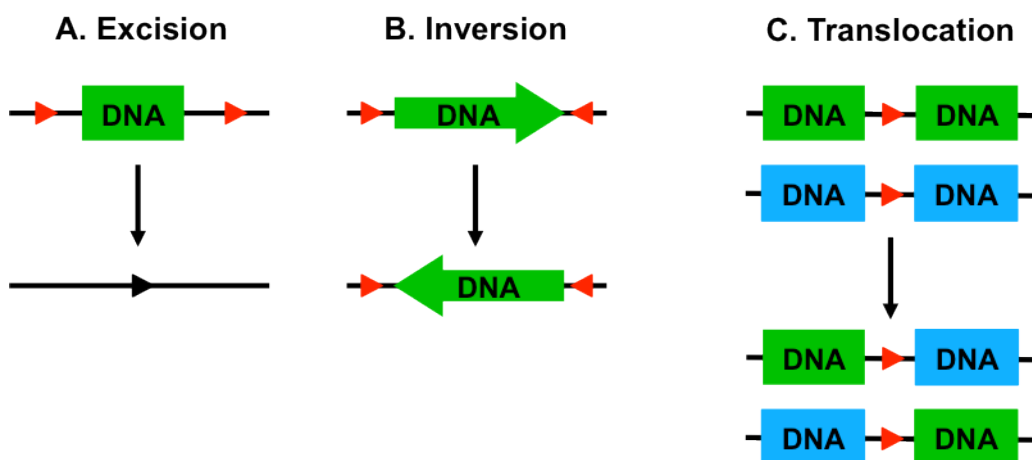


Figure 5.1: Cre-mediated recombination is determined by the location and orientation of the *loxP* sites. *LoxP* sequences (red triangles) are directional and can be inserted on the same (*cis*) or different (*trans*) DNA strands. (A) If *loxP* sequences flank a DNA segment in a *cis*-arrangement, in the same orientation, Cre recombinase excises the segment. (B) Inverted *loxP* sequences flanking a DNA segment in a *cis*-arrangement result in inversion of the segment. (C) If *loxP* sequences are in the same orientation in a *trans*-arrangement, recombination mediates translocation of the segment (adapted from Nagy., 2000).

Importantly, Cre-mediated recombination can be utilized to generate lineage or cell type-specific knockouts. This is achieved by inserting *loxP* sequences either side of a functionally critical region of the gene of interest ('floxed'), and breeding these transgenic animals with mice carrying a cell type-specific Cre recombinase transgene. In this way, the gene function is disrupted in a lineage-specific manner, whereas in all other cell types its function is intact (Nagy, 2000). This powerful adaptation of the technology facilitated a unique insight into the role of *Fgf10* specifically within the skeletal muscle lineage.

Classically, gene function is explored by removing its expression and observing the knockout phenotype. The *Fgf10* knockout allele was created by inserting a neomycin (PGK-neo) cassette in reverse orientation into exon 1, impeding translation of the gene. Homozygous *Fgf10* knockout (*Fgf10*^{-/-}) mice do not develop lungs or limbs, and die at birth (Min et al., 1998). Therefore, it is not possible to investigate the function of *Fgf10* in limb muscles in the knockout mouse, because the limbs do not develop. To circumvent this issue, a *Pax3*-Cre (P3Pro-Cre) transgenic line was obtained. The *Pax3* transcription factor is expressed during early development in the dorsal neural tube and somites, giving rise predominantly to myogenic progenitors but also a subpopulation of endothelial progenitor cells (Goupille et al., 2011). Mutations in its expression result in neural crest and hypaxial muscle development defects. Homozygous *Pax3* knockout (*Pax3*^{-/-}) (*Spotch*) mice display severe cardiac and muscle defects and die in utero as a result, implicating the transcription factor in cardiac neural crest cell migration (Li et al., 2000). The P3Pro-Cre transgene utilizes the proximal 1.6Kb region of the murine *Pax3* promoter to initiate expression of Cre recombinase, so that it is constitutively active within all *Pax3*-expressing cells (Li et al., 2000). The transgene displays an interesting expression pattern within the skeletal muscle lineage at post-natal and adult ages, as evidenced by use of a lacZ reporter. All muscles of the head, neck, forelimb and diaphragm

were found not to express the transgene (as shown by a lack of lacZ expression). The large pectoral and back muscles were also negative. However, below the diaphragm, all muscles (including those of the hindlimb) expressed the transgene. Further still, within the intercostal muscles, the most rostral muscles were negative for the transgene. The P3Pro-Cre transgene therefore displays a distinct rostrocaudal expression pattern, which also suggests the transgene could lack an element to direct expression in rostral muscles (Jarad and Miner, 2009).

In order to investigate the potential functions of *Fgf10* within the skeletal muscle lineage, double transgenic mice were generated from *Pax3*-Cre and *Fgf10*-floxed lines in order to remove *Fgf10* expression specifically within *Pax3*-expressing cells or myogenic progenitors. The overall aim of this Chapter is to characterize the *Fgf10* conditional knockout phenotype, in order to gain a better insight into the function of this gene within skeletal muscle.

5.1.1 Aims

Taking into consideration *Fgf10* expression within fast (Type 2) fiber types and a subpopulation of satellite cells, the aims of this chapter include the following hypotheses:-

1. Do the myofibers display any morphological abnormalities indicating impaired development?
2. Is fast-fiber type specification compromised in the *Fgf10* conditional knockout?
3. Is the number of satellite cells reduced in the *Fgf10* conditional knockout?

5.2 Results

5.2.1 Generation of an *Fgf10* cKO transgenic line.

To generate the *Pax3*-Cre::*Fgf10* conditional knockout (cKO) line, Breeding Scheme 1 was initially established (Figure 5.2). In addition to the *Pax3*^{Cre} allele, the male also carried an *Fgf10*-Cre^{ERT2} allele, which results in heterozygous loss of *Fgf10* function, therefore creating an *Fgf10* null allele. This is due to the tamoxifen-inducible Cre-ERT2 construct being inserted into part of exon 1 of the *Fgf10* gene (El Agha et al., 2012). The *Pax3*^{Cre}::*Fgf10*^{CreERT2} male was mated with a female carrying a floxed *Fgf10* allele (*Fgf10*^{flox}), and a tomato fluorescent reporter cDNA construct inserted into the ubiquitous ROSA26 locus, which contains a floxed STOP codon upstream of the reporter. Both copies of the *Fgf10* null (*Fgf10*-Cre^{ERT2}) and *Fgf10*^{flox} alleles are required in order for *Fgf10* expression to be removed in *Pax3*-expressing cells. Expression from the floxed allele is removed by Cre-mediated recombination under the control of the *Pax3* promoter. Recombination will also excise the STOP codon in the tomato reporter, thereby enabling its transcription.

The desired F1 genotypes from this double transgenic cross are listed in Figure 5.2. Using this cross, there is a 1:8 probability of obtaining an *Fgf10* cKO mouse, the probability of an *Fgf10* cKO mouse carrying a tomato reporter is reduced further still to 1:16.

Breeding Scheme 1



F0 $Pax3-Cre/+::Fgf10-Cre^{ERT2}/+$ $Fgf10-flox/+::Rosa-Tomato/+$

F1 desired genotypes:

1. $Pax3-Cre/+::Fgf10-Cre^{ERT2}/flox::Rosa-Tom/+$
2. $Pax3-Cre/+::Fgf10-Cre^{ERT2}/flox$

*Probability of obtaining conditional knockout with
Tomato reporter: 1 in 16*

Figure 5.2: Breeding Scheme 1 to generate *Fgf10* cKO mice. The male carries the $Pax3^{Cre}$ allele and *Fgf10* null ($Fgf10-Cre^{ERT2}$) allele. The female carries an $Fgf10^{flox}$ allele and is heterozygous for the ROSA26-Tomato reporter. To remove *Fgf10* expression from *Pax3*-expressing cells, copies of the *Fgf10* null and floxed alleles need to be inherited. There is a 1:16 chance of obtaining an *Fgf10* cKO also carrying a tomato reporter.

To verify that the *Fgf10* allele was being removed specifically within *Pax3*-expressing cells, different muscle samples were genotyped (Figure 5.3). These included the *triceps brachii* and *tibialis anterior* of the forelimb and hindlimb respectively, and the *masseter* of the head. The former two muscles are somite-, and therefore, *Pax3*-derived, whereas the masseter derives from the branchial arches and is not *Pax3*-derived. Samples were genotyped for the *Fgf10*-floxed allele (measuring 1.8Kb). If *Pax3*-Cre mediated excision of the $Fgf10^{flox}$ allele had occurred, a smaller PCR product of 500bp, corresponding to the excised segment, was also seen (Figure 5.3A). Therefore, by inference, the presence of the excised segment indicates that the $Pax3^{Cre}$ allele is also present (in some cases genotyping was carried out for $Pax3^{Cre}$). Following identification of the

excised floxed allele, samples were also genotyped for the *Fgf10*-Cre allele (data not shown).

Pax3^{Cre} mice develop a white spot on their bellies (Figure 5.4D), akin to *Spotch* mutants (Engleka et al., 2005). When comparing wildtype and *Fgf10* cKO animals, validation of *Pax3*-mediated Cre recombination can be seen in the expression of the tomato reporter (Figure 5.4). Cre recombination excises the STOP codon in the ROSA26 locus, upstream of the tomato reporter. Therefore, the fluorescent protein should be seen where *Pax3*-Cre is active, and also in the descending cells. As expected, in the *Pax3*^{Cre} animals there is expression of the tomato reporter in muscles of the trunk and limbs, which produces a vivid red colour (Figure 5.4E) in comparison to wildtype animals (Figure 5.4B). In the non-*Pax3*-derived head muscles, there is no tomato expression as seen by the markedly reduced colour (Figure 5.4F). In addition, the *Pax3*^{Cre} animals have a curved tail (Figure 5.4C) in comparison to their wildtype littermates (Figure 5.4A). This is likely due to a tendon defect in these animals. The images presented in Figure 5.4 were obtained using a hand-held camera, and as such are not entirely representative of the actual sizes of the animals, although care was taken to ensure accurate photos were taken. In future analyses it would be necessary to weigh the animals, in order to determine whether the *Pax3*^{Cre} animals are indeed hypertrophic (have increased muscle mass), as the current image in Figure 5.4E suggests. Similarly, the kyphosis (bend in back), which can be seen Figure 5.4E, is not a recurring feature in all *Pax3*^{Cre} animals.

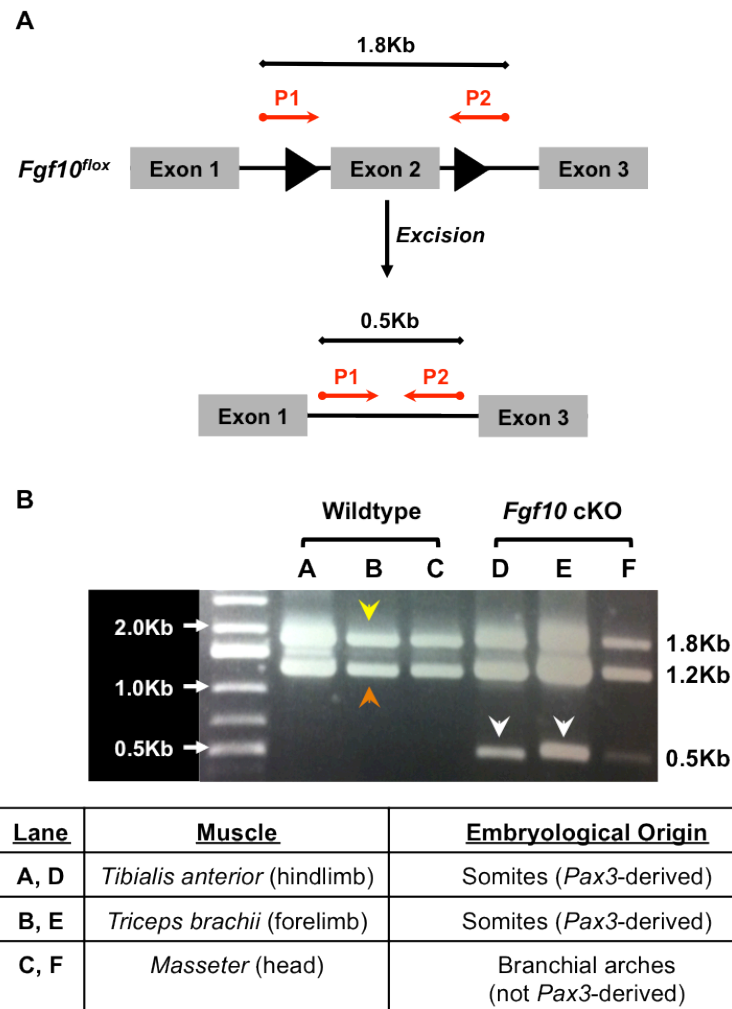


Figure 5.3: Genotyping result for muscle samples from a wildtype and *Fgf10* cKO mouse. (A) Primers P1 and P2 bind the floxed region of the *Fgf10*^{flox} allele to give a 1.8Kb band (highlighted by the yellow arrowhead on B). If *Cre*-mediated excision occurs, the excised segment can be detected at 500bp (white arrowheads). A second primer set (not shown) binds a wildtype region on the same strand measuring 1.2Kb (orange arrowhead). (B) In the wildtype muscle samples, the floxed and wildtype portions of the *Fgf10* allele can be detected, but an excision band is not seen. In the *Fgf10* cKO muscle samples, both the *Pax3*-derived *tibialis anterior* and *triceps brachii* show an excision band. A very faint excision band is also seen in the *masseter* sample, but this is likely due to a contaminating source of *Pax3*-containing material such as tendon.

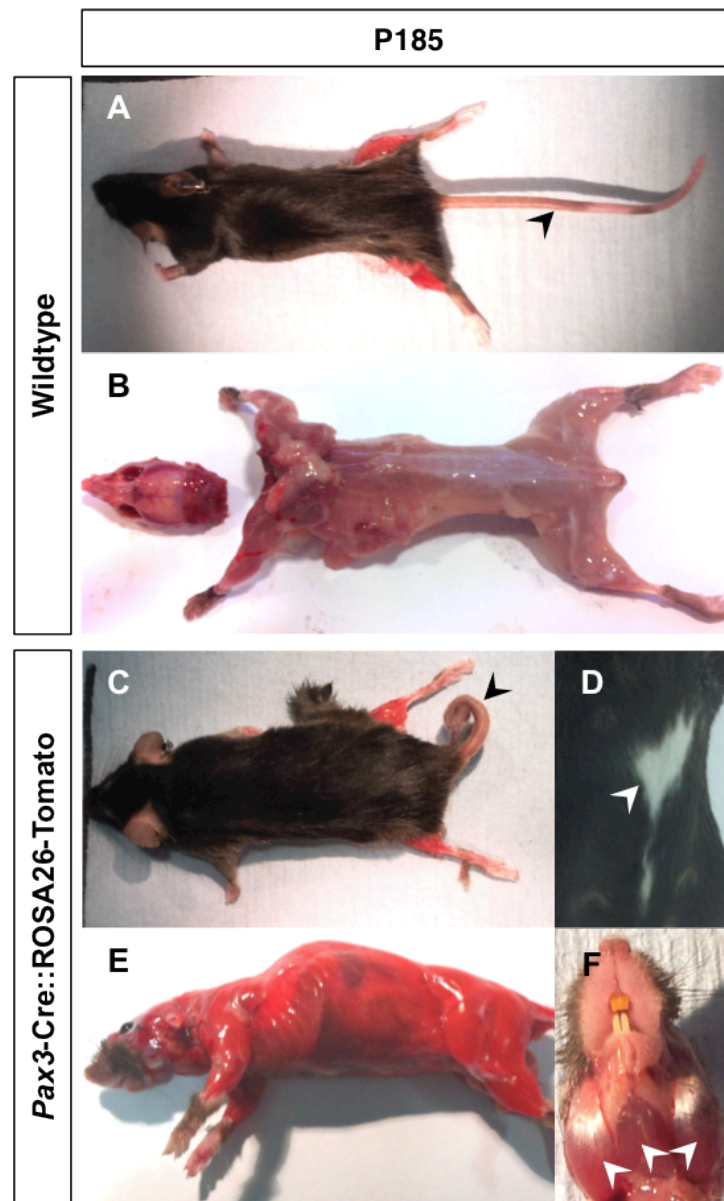


Figure 5.4: Phenotypic characteristics of *Pax3*^{CRE}::ROSA26-Tomato mutants at P185. *Pax3* heterozygotes have curved tails (C) in comparison to their wildtype littermates (A), they also develop white spots on their bellies (D). Use of the tomato reporter can be validated in the *Pax3* heterozygotes. In *Pax3*-derived muscles of the trunk and limbs, bright red tomato protein is visible (E). In contrast, in muscles of the head, which have a different embryological origin, the colour of the flesh is noticeably duller, indicating tomato protein is absent (F).

5.2.2 *Fgf10* cKO mice display perinatal lethality.

In order to increase the probability of obtaining cKO animals, Breeding Scheme 2 was established. This involved mating a homozygous *Fgf10*-floxed (*Fgf10*^{flox/flox}) mouse with a mouse carrying the *Pax3*^{Cre} allele and one copy of the *Fgf10* null (*Fgf10*^{CreERT2}) allele (Figure 5.5). The second scheme increases the chances of obtaining a conditional knockout to 1:4, and the other potential genotypes are listed in Figure 5.5.

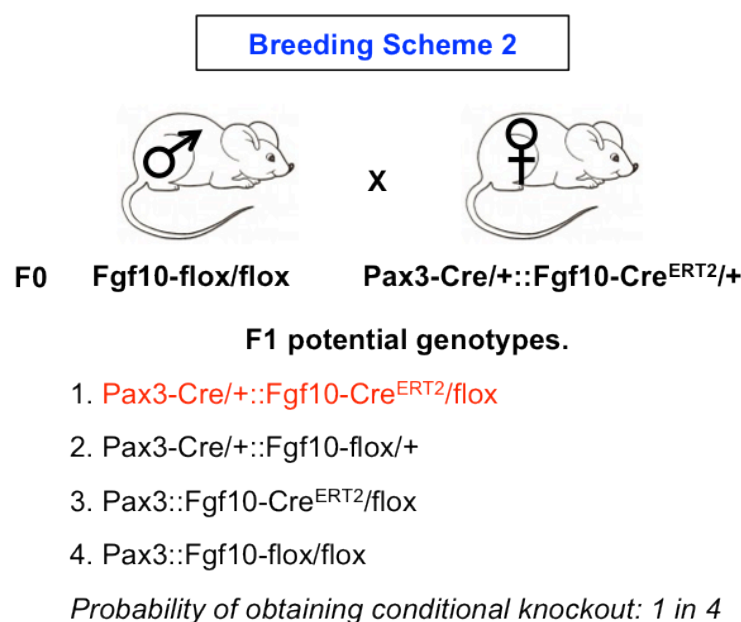


Figure 5.5: Breeding Scheme 2 to generate *Fgf10* cKO mice. A homozygous *Fgf10*-floxed (*Fgf10*^{flox/flox}) mouse is crossed with a mouse carrying the *Fgf10* null allele (i.e. heterozygous for *Fgf10* expression) and the *Pax3*^{Cre} allele. This scheme gives rise to four different allelic combinations, with the chances of obtaining a cKO animal increased to 1:4, in contrast to Breeding Scheme 1.

The *Fgf10* cKO mice were recovered in accordance with normal Mendelian ratios during embryonic development (data not shown). However, it became apparent that they were not viable for long, as the majority did not survive beyond P0, apart from one (Table 5.1). Specifically, *Fgf10* cKO mice die within the first few hours after birth, so all

tissue processing for these animals occurred within this time frame. Newborn litters were initially genotyped for the *Fgf10*-floxed allele (as described earlier) to identify in which pups recombination had occurred, and therefore which pups also carried the *Pax3*^{Cre} allele (Figure 5.6A). The litters were then genotyped for *Fgf10*^{CreERT2} to identify the conditional knockout animals (Figure 5.6B). The *Fgf10*^{CreERT2} allele gives a product size of 500bp and the corresponding wildtype region measures 300bp. Specific muscles from an *Fgf10* cKO and wildtype pup were then genotyped to verify the *Pax3*^{Cre}-mediated recombination (Figure 5.6C). For example, the excised floxed segment is detectable in the tail and *tibialis anterior* samples of Pup 1, and again in the masseter sample, likely due to contaminants (Figure 5.6i-iii). The excised floxed allele is not seen in Pup 2 (*Pax3*^{Cre} negative).

Table 5.1: Proportions of genotypes recovered in newborn and adult litters from Breeding Schemes 1 and 2.

Age	Number of Litters	Genotypes Recovered	
		Wildtype	<i>Pax3</i> ^{Cre} :: <i>Fgf10</i> ^{CreERT2/flox} (<i>Fgf10</i> cKO)
P0	5	21	10
P39-94	2	10	0
P186-335	3	14	1

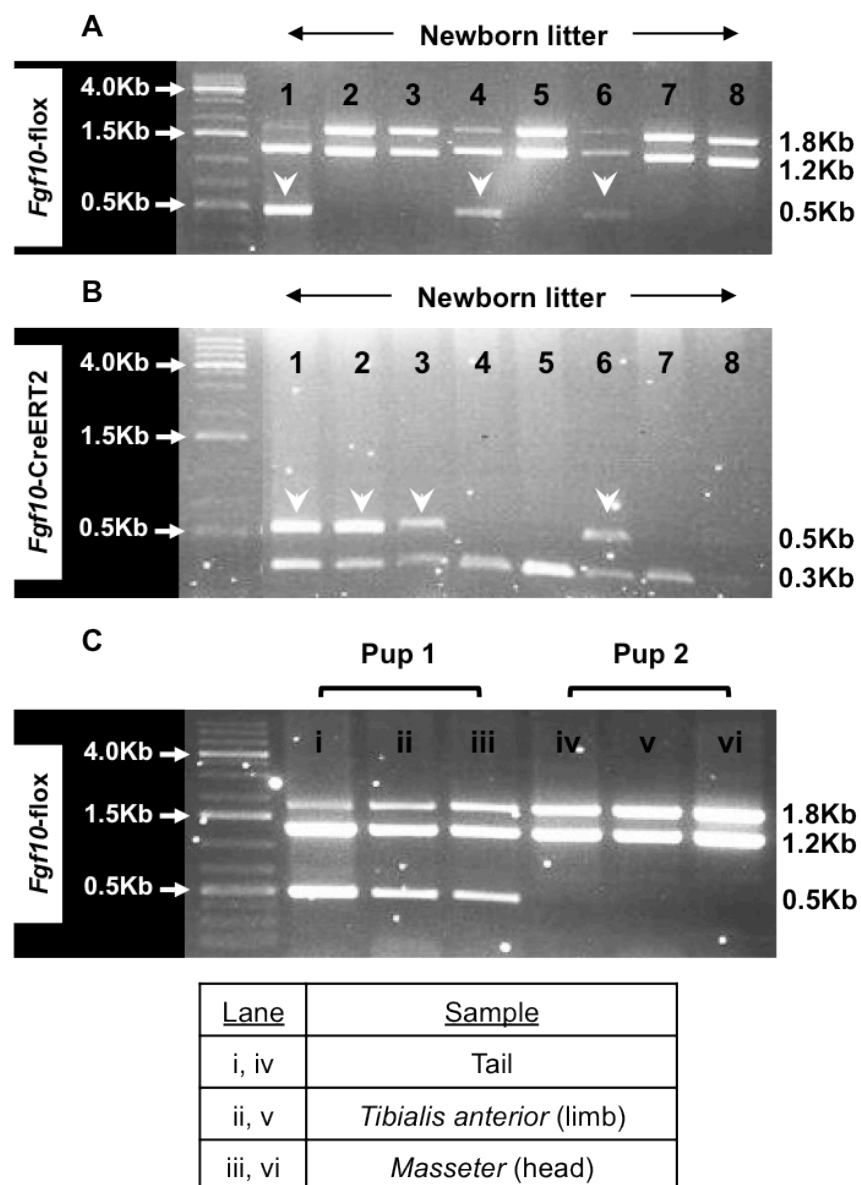


Figure 5.6: Genotyping results for a litter from Breeding Scheme 2.

(A) Pups are genotyped for the *Fgf10*^{flox} allele (1.8Kb). If the excised floxed band is detected (white arrowhead) then by inference that pup is *Pax3*^{Cre} positive. (B) Subsequently the pups are genotyped for the *Fgf10*^{CreERT2} allele (500bp – white arrowhead). (C) In muscle samples from an *Fgf10* cKO Pup 1, excision of the *Fgf10*-floxed allele is detectable in tail and *tibialis anterior* samples (i, ii), which is not the case for *Pax3*^{Cre}-negative Pup 2 (iv, v).

The *Fgf10* cKO pups display several phenotypic abnormalities. Firstly, they are slightly smaller than their wildtype littermates (Figure 5.7). They also appear to have difficulty breathing, visibly gasping with an increased delay between breaths. As a result, the colour of their flesh is noticeably grey in appearance. Whilst they are able to move, this movement is reduced and they also fail to develop a milk spot, suggesting they are not able to suckle.

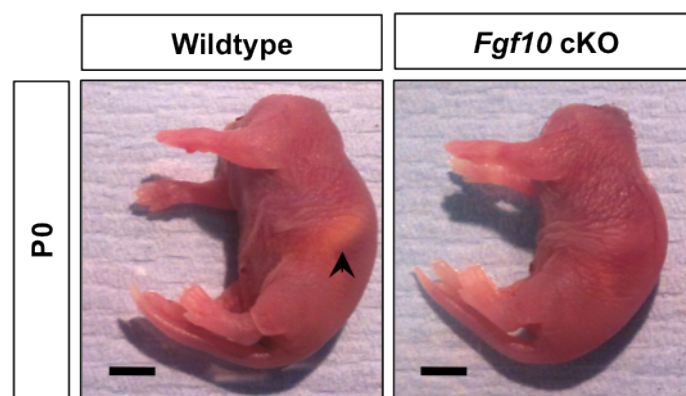


Figure 5.7: *Fgf10* cKO pups are smaller than their wildtype littermates. In addition to this, the pups also fail to develop a milk spot (indicated by the arrowhead), which implies they cannot suckle. Scale bars: 0.5cm.

We suspected that the breathing difficulties may be due to defects in the intercostal muscle structure. To explore this further, the rib cages were isolated from a wildtype and *Fgf10* cKO pup, and sagittal sections obtained in an anterior-to-posterior direction. The morphology of the intercostal muscles was revealed by haematoxylin and eosin staining (Figure 5.8). When compared, no striking differences were observed between the muscle groups. It is also possible that cardiac defects contributed to the perinatal lethality, given that *Pax3* is expressed in cardiac neural crest progenitors, and *Fgf10* has also been identified in the developing myocardium (Nelms et al., 2011; Vega-Hernandez et al., 2011). However, these potential defects were not explored in greater detail.

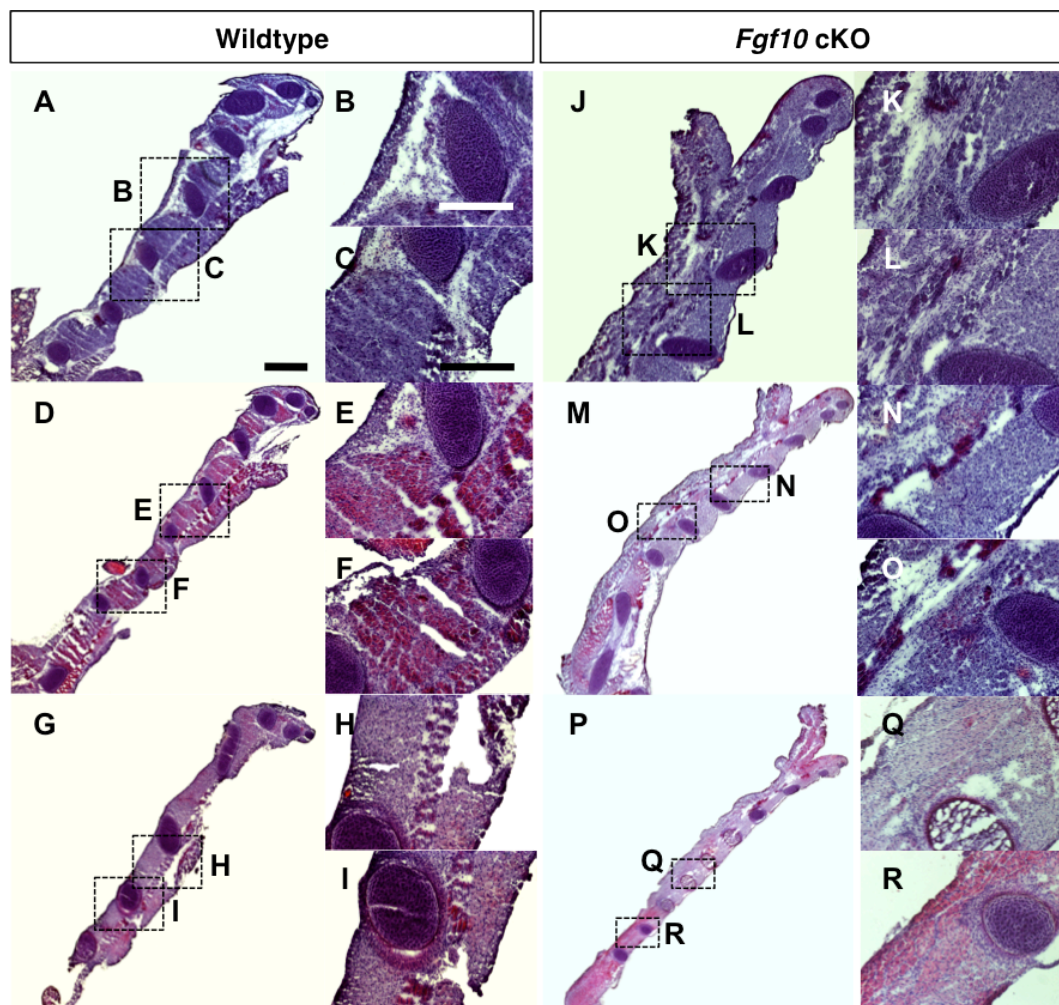


Figure 5.8: Sagittal view through the intercostal muscle structure of a wildtype and *Fgf10* cKO mouse. In the sagittal plane, the muscle density appears equal between the wildtype and *Fgf10* cKO mouse, at both the anterior (A, J) and posterior level (G, P). Haematoxylin and eosin counterstain. All scale bars: 100µm.

As a prototype, the *tibialis anterior* was isolated from the single *Fgf10* cKO mouse (P185) which survived into adulthood, and the composition of fast (Type 2) and slow (Type 1) myofibers was analyzed using MyHC-f and MyHC-s antibodies respectively, to see if removal of *Fgf10* expression had altered fiber-type specification (Figure 5.9A, B). *Tibialis anterior* muscles from ‘control’ littermates, ranked in order of reduction in *Fgf10* expression, were also analyzed in the same way (Figure 5.9C-H).

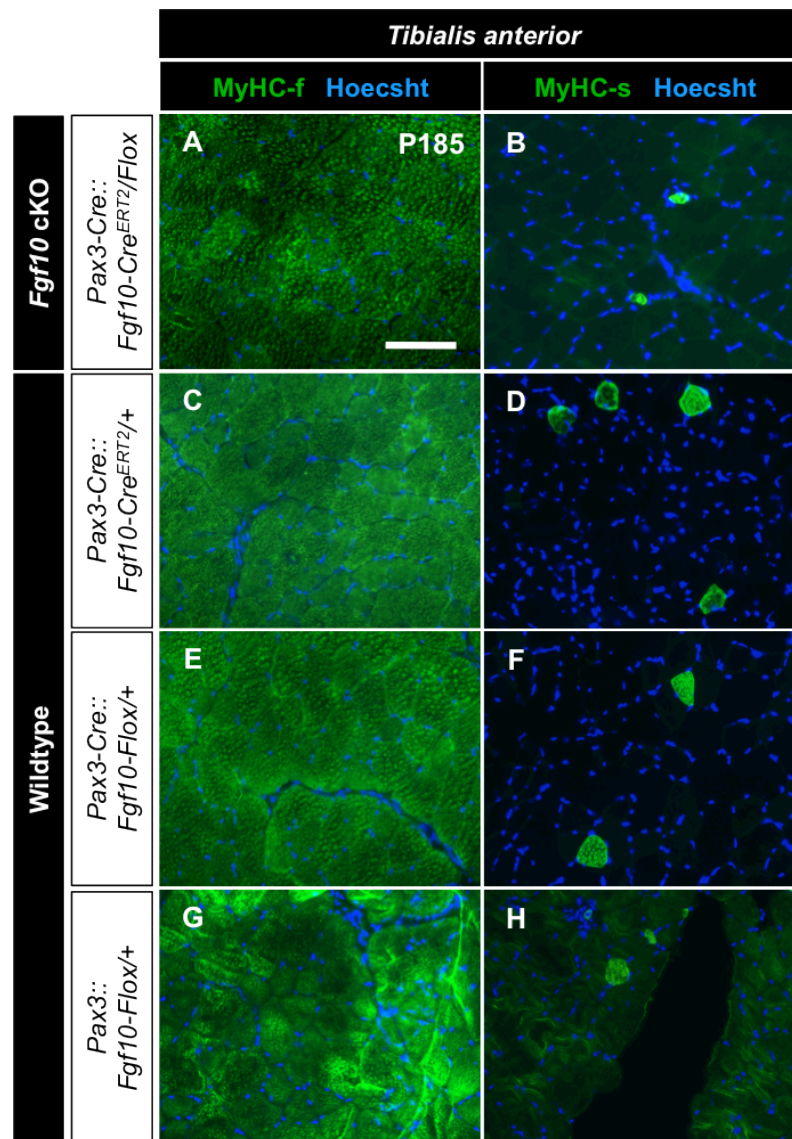


Figure 5.9: Fast and slow myofiber content in *tibialis anterior* muscles of *Fgf10* cKO and wildtype mice. MyHC-f and MyHC-s antibodies were used to detect fast and slow myofibers respectively. (A, B) When compared to the wildtype ‘control’ genotypes, which are ranked in order of % reduction of *Fgf10* expression (refer to Figure 5.5), the fiber-type composition in the cKO muscle does not appear significantly different to the control genotypes (C-H). Primary antibodies: Ms- α -MyHC-s; Ms- α -MyHC-f. Secondary antibody: Gt- α -Ms AlexaFluor488; Hoecsht counterstain. Scale bar: 100 μ m.

A range of sections was selected from different levels within the *tibialis anterior*, and the numbers of fast and slow myofibers were quantified in the cKO and control animals (Figure 5.10). Although two animals were analyzed for each control genotype, the availability of only one cKO animal meant that statistical analyses could not be carried out on these data. Nevertheless, it would appear that the numbers of fast or slow fibers are not significantly altered in the cKO.

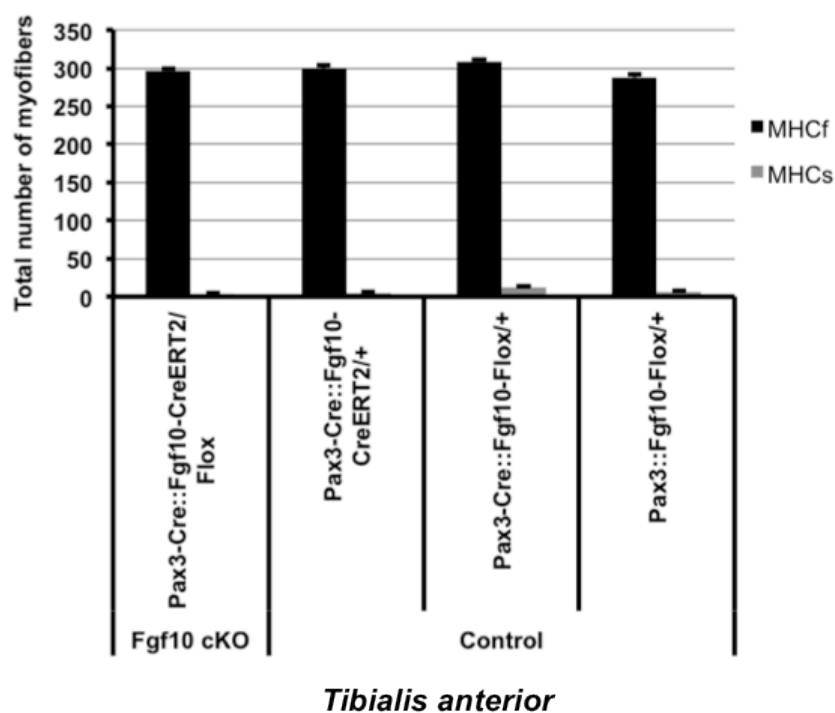


Figure 5.10: The number of fast and slow myofibers is not altered in the *tibialis anterior* of the *Fgf10* cKO. MyHC-f and MyHC-s antibodies were used to detect fast and slow myofibers respectively. The numbers of fast and slow myofibers in the cKO appear to mirror those seen in the control groups. For *Fgf10* cKO, n=1; for control groups n=2.

Given that subsequent *Fgf10* cKO mice were found to die perinatally, the survival of one of these animals into adulthood was attributed to genetic stochasticity, which permitted its survival. All further analyses were carried out tissue isolated from newborn pups.

5.2.3 A subset of hindlimb muscles of *Fgf10* cKO mice have disorganized myofibers

Muscle structure was analyzed in the lower forelimb (Figure 5.11) and hindlimb (Figure 5.12) of both wildtype and *Fgf10* cKO animals using haematoxylin and eosin (H&E) staining. In the forelimb, there does not appear to be any striking differences in the cKO, with all muscle groups appearing intact and organized.

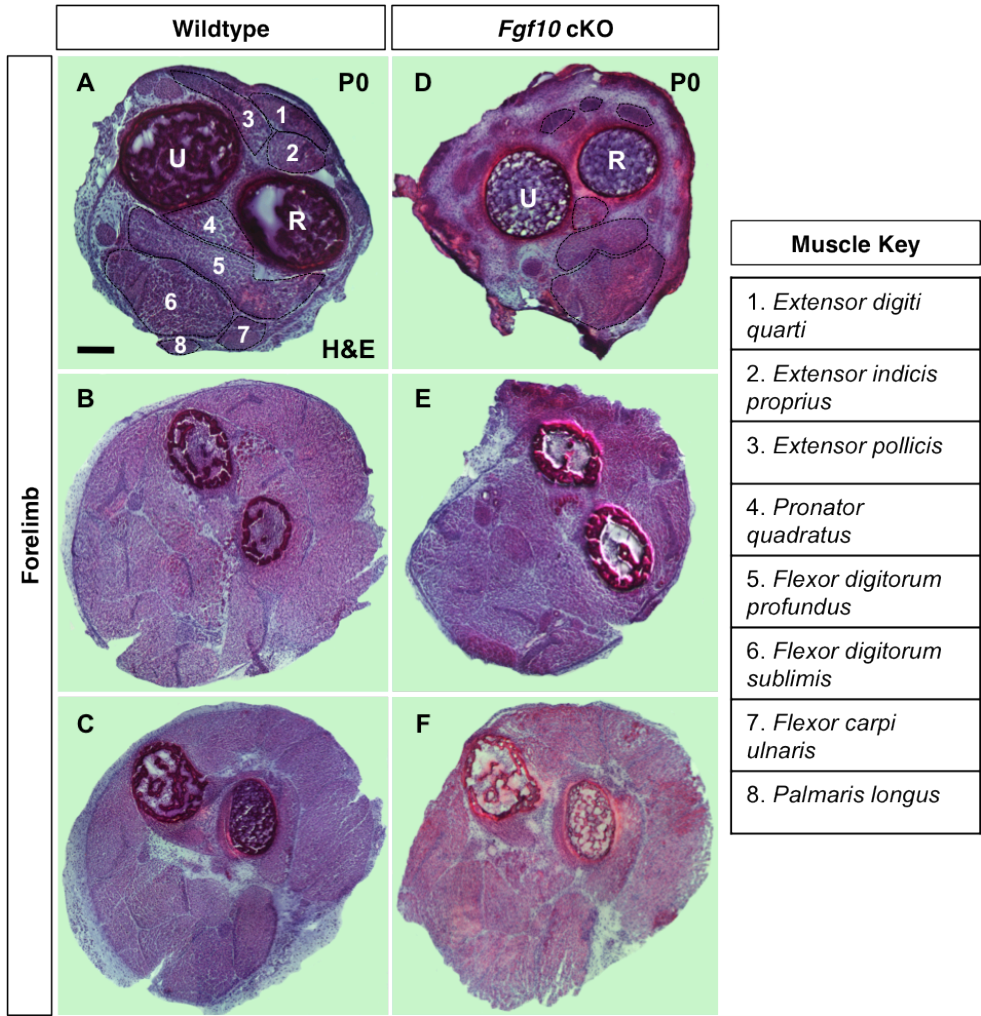


Figure 5.11: There are no differences in the organization of muscle structure in wildtype and *Fgf10* cKO forelimbs. Overall muscle organization in the forelimb appears unaffected after removal of *Fgf10* expression (D-F) compared to the wildtype (A-C). Abbreviations: Ulna (U), Radius (R), haematoxilyn and eosin (H&E) counterstain. Scale bar: 100µm.

Serial sections through the hindlimb were also cut disto-proximally and analyzed. In contrast to the forelimb, the myofibers of certain hindlimb muscle groups were highly disorganized. The tissue fragmentation visible in the wildtype hindlimb sections (Figure 5.12A, G) is due to artefacts of the cryostat sectioning technique. In the most distal sections surveyed (Figure 5.12J), indications of this disorganization can be seen in the region of the *tibialis posterior* and *flexor digitorum brevis* (Figure 5.12L). It becomes more striking in posterior sections (Figure 5.12M, P – highlighted by the dashed line), where the myofibers are hypercontracted, reduced in number and surrounded by vacuous spaces (Figure 5.12O, R). A similar disorganization is also seen in the *extensor digitorum longus* (Figure 5.12K, N, Q). However the effect is mildly severe in comparison, as more fully formed myofibers are present, yet they do not seem to have compacted efficiently in comparison to the wildtype (Figure 5.12B, E, H). Hypercontraction of the myofibers within the *tibialis posterior*, *soleus*, *plantaris* and *flexor digitorum brevis* suggests defective attachment at the tibia and fibula. The myofibers of these muscles appear to attach correctly in the most proximal regions of the hindlimb, suggesting that the distal tendons of these muscle groups are functioning incorrectly.

To investigate the morphology of the distal tendons of the affected muscles further, additional distal sections through the hindlimb were immunolabeled with a tenascin C antibody, a marker of tendons. As detailed in Chapter 3, an *Fgf10*-LacZ reporter mouse was used to analyze β -gal expression (product of *LacZ*, indicating *Fgf10* expression) in skeletal muscles. At E17, it was also noted that β -gal-expressing cells appeared to co-localise with tenascin C expression in developing tendons of the *tibialis posterior* and *flexor digitorum brevis*, suggesting that *Fgf10* is involved in their formation. Of the sections surveyed, some interesting differences were seen in the tendons of these muscles in the *Fgf10* cKO (Figure 5.13). In the cKO, both tendons of the *tibialis posterior* (t1) (Figure 5.13H) and the *flexor digitorum brevis* (t2) (Figure 5.13I) appear slightly smaller, in

comparison to the wildtype tendons at the same level in the hindlimb (Figure 5.13B, C). Streams of tenascin C-expressing cells can be seen approaching t1 (Figure 5.13B), and this stream appears wider in the cKO (Figure 5.13H), which suggests fewer of these cells have been incorporated into the tendon itself. In more proximal sections, both t1 and t2 are noticeably absent in the cKO (Figure 5.13K, L) in comparison to the wildtype (Figure 5.13E, F). Overall, the development of these tendons appears to have been arrested prematurely in the cKO. They are less well established (Figure 5.13J) and are noticeably smaller in size (Figure 5.13G), in comparison to the wildtype (Figure 5.13A, D). These observations may help to explain why the myofibers of these muscles appear hypercontracted in the cKO.

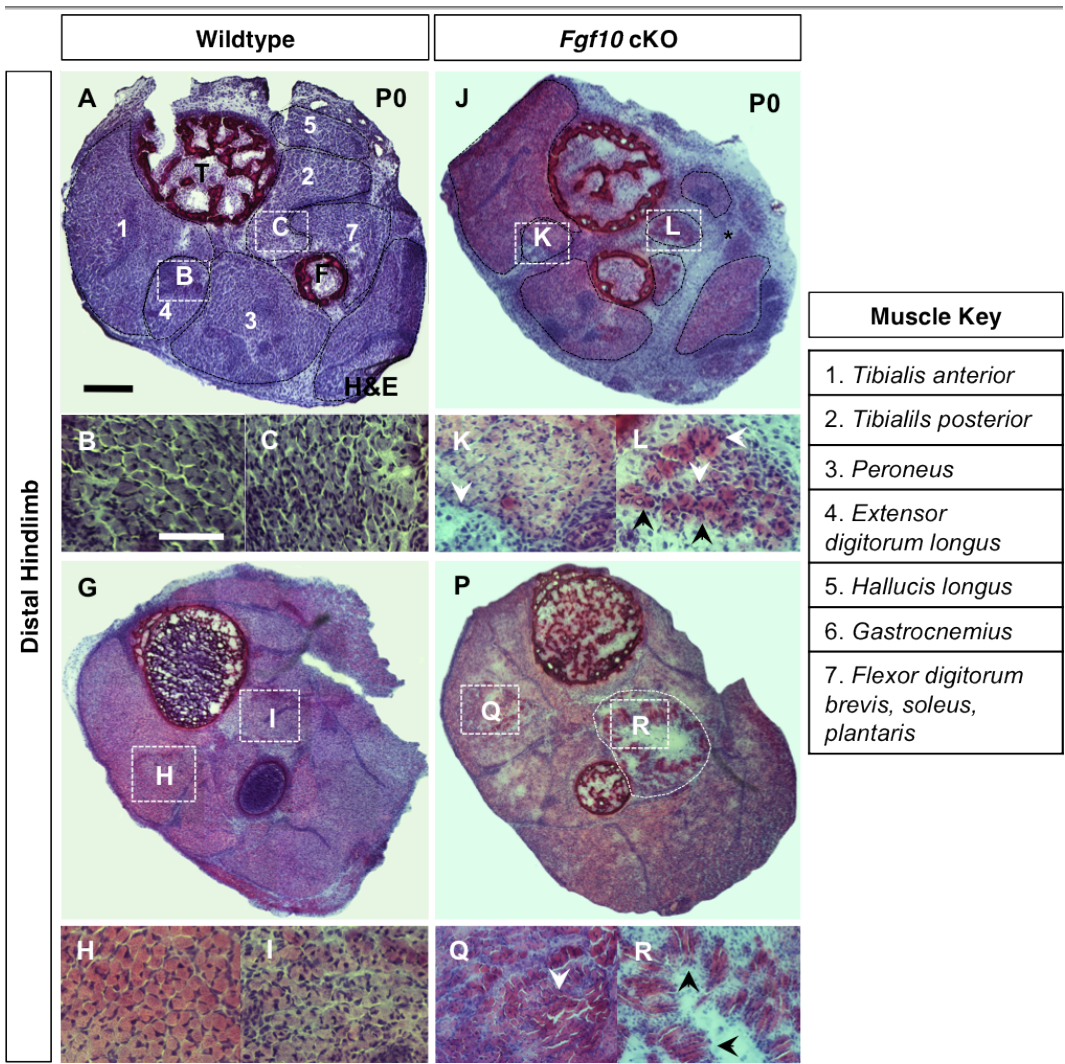


Figure 5.12: The morphology of *tibialis posterior*, *soleus*, *plantaris*, *flexor digitorum brevis* and *extensor digitorum longus* muscles is significantly altered in the *Fgf10* cKO hindlimb. Myofibers of the *tibialis posterior*, *soleus*, *plantaris*, *flexor digitorum brevis* appear highly disorganized and massively reduced in number (indicated by arrowheads) in the distal regions of the *Fgf10* cKO hindlimb (J, L, M, O, P, R) compared to the wildtype (A, C, D, F, G, I). Myofibers of the *extensor digitorum longus* also appear reduced in number and less compact in the cKO (K, N, Q) compared to the wildtype (B, E, H). Haematoxylin and eosin counterstain. All scale bars: 100µm.

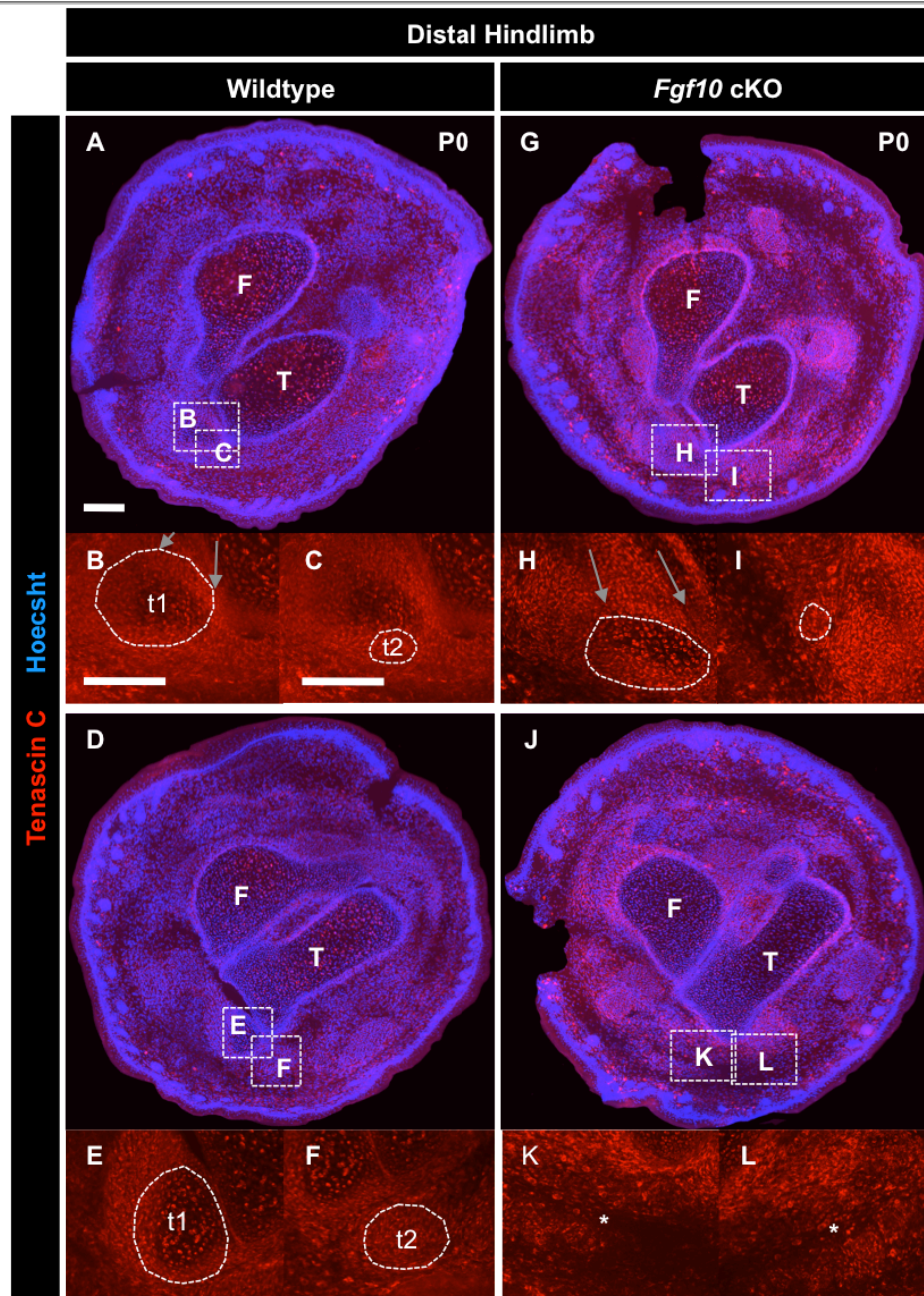


Figure 5.13: The morphology of tendons of the *tibialis posterior* and *flexor digitorum brevis* is significantly altered in the *Fgf10* cKO. Tendons of the *tibialis posterior* (t1) and *flexor digitorum brevis* (t2) appear reduced in size in the *Fgf10* cKO (H, I) in comparison to the wildtype (B, C). At a slightly more proximal level, t1 and t2 are absent in the cKO (K, L) in contrast to the wildtype (E, F). Abbreviations: T – Tibia, F – Fibula. Primary antibody: Rb- α -Tenascin C. Secondary antibody: Gt- α -Rb AlexaFluor568; Hoechst counterstain. Scale bars: 100µm (A); 50µm (B, C).

5.2.4 *Fgf10* cKO mice do not display skeletal defects.

Given the tendon defects presented by the *Fgf10* cKO mice in the distal regions of the hindlimb, the skeletal structure of these mice was also investigated to see if there were any differences which may have facilitated these defects (Figure 5.14, 5.15). This was achieved by using alizarin red and alcian blue staining, which stain ossified bone and cartilage respectively (Ovchinnikov, 2009). When comparing the overall structures, there are no striking differences between the cKO and wildtype (Figure 5.14A, G). The skull size of the cKO is noticeably smaller (Figure 5.14H) than the wildtype (Figure 5.14B). However, its overall structure is intact, with no major craniofacial bones absent. In the lower forelimb, the radius and ulna bones do not show any differences in the cKO (Figure 5.14K) in comparison to the wildtype (Figure 5.14E). This is also true for the humerus in the upper forelimb (Figure 5.14K', E'). Similarly in the hindlimb, the skeletal structure appears unaffected in the cKO. The distal regions of the tibia and fibula of the lower hindlimb look identical in the cKO (Figure 5.14J) compared to the wildtype (Figure 5.14F), and the femur bones also appear normal (Figure 5.14J' F'). The vertebral column also appears unaffected in the cKO (Figure 5.15A), with the same number of vertebrae seen in the wildtype (Figure 5.15B), and all of the ribs are intact. Together, these results suggest that there are no skeletal patterning abnormalities incurred in the cKO.

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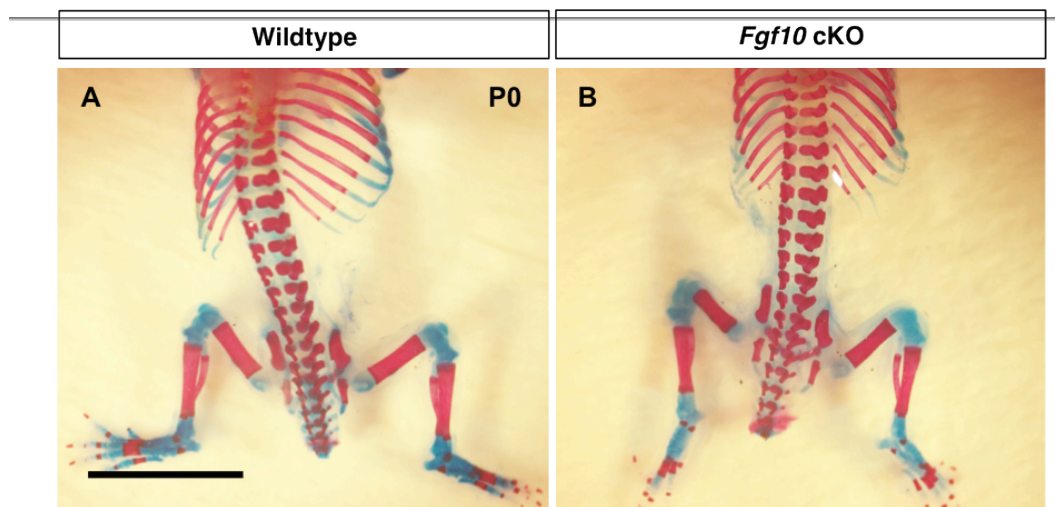


Figure 5.15: Development of the vertebral column is unaffected in the *Fgf10* cKO. In the cKO (B), there is the same number of vertebrae and ribs as seen in the wildtype (A). Scale bar: 0.75cm.

5.2.5 Myofiber number and diameter is altered in *tibialis anterior* muscles of *Fgf10* cKO mice.

To assess whether the number and diameter of myofibers was affected in muscles of cKO mice, hindlimb sections were immunolabelled with an anti-laminin $\alpha 2$ antibody (to visualize the myofiber border) and the *tibialis anterior* muscle was analyzed, as this contains a significant number of *Fgf10*-expressing myofibers (Figure 3.8). Four non-overlapping fields were processed using Image J software (NIH), and the average minimal Feret's diameter was measured, as presented in Figure 5.16. This method of quantification was repeated for the wildtype animals. Interestingly, the average number of myofibers was significantly increased in this muscle in the cKO (Figure 5.17A), based on a p -value of 0.032 after a Student's unpaired t -test. However the diameter of these myofibers was also significantly reduced (p -value 0.0036) (Figure 5.17B). These data suggest that when *Fgf10* expression is removed, the myofibers do not grow to their correct size as a result of inefficient growth of the myonuclear domain. This apparent failure of the individual myofibers to enlarge in

cross-sectional area suggests that the mechanisms regulating normal muscle hypertrophy during post-natal development are impaired when *Fgf10* expression is removed (Schiaffino et al., 2013). Alternatively, it is also possible that the presence of a greater number of myofibers is due to increased hyperplasia (muscle growth by increase in the number of myofibers). In mouse, postnatal muscle growth is predominantly facilitated by hypertrophy (Ontell et al., 1984). However, in the absence of *Fgf10* expression, perhaps compensatory hyperplasia is responsible for the increase in myofibers seen in the *tibialis anterior*.

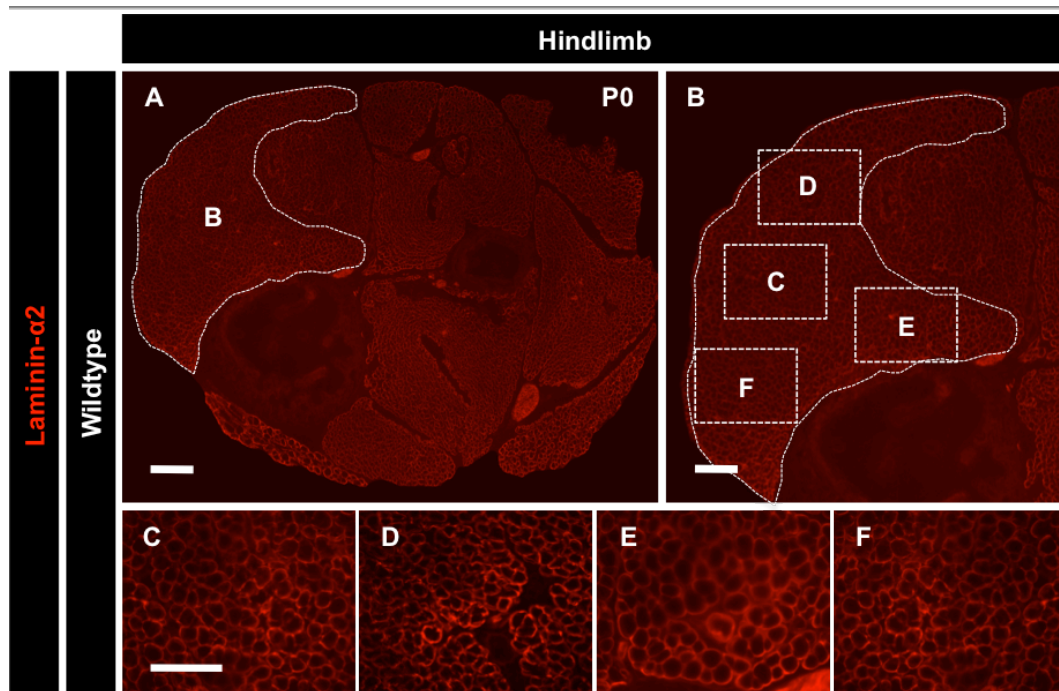


Figure 5.16: The average number and diameter of myofibers in the *tibialis anterior* of wildtype P0 animals were counted using an anti-laminin $\alpha 2$ antibody. (A) Myofiber border can be visualized using an anti-laminin $\alpha 2$ antibody (AlexaFluor568-conjugated secondary antibody). (B) Under the x40 objective, four non-overlapping quadrants (C-F) were imaged across the *tibialis anterior* (highlighted by dashed white line). ImageJ software was used to calculate myofiber diameter and number within each quadrant. The same analysis was performed on *Fgf10* cKO hindlimb sections (data not shown). Primary antibody: Rt- α -Laminin $\alpha 2$. Secondary antibody: Gt- α -Rt AlexaFluor568. Scale bar: 100 μ m (A); 75 μ m (B); 50 μ m (C-F).

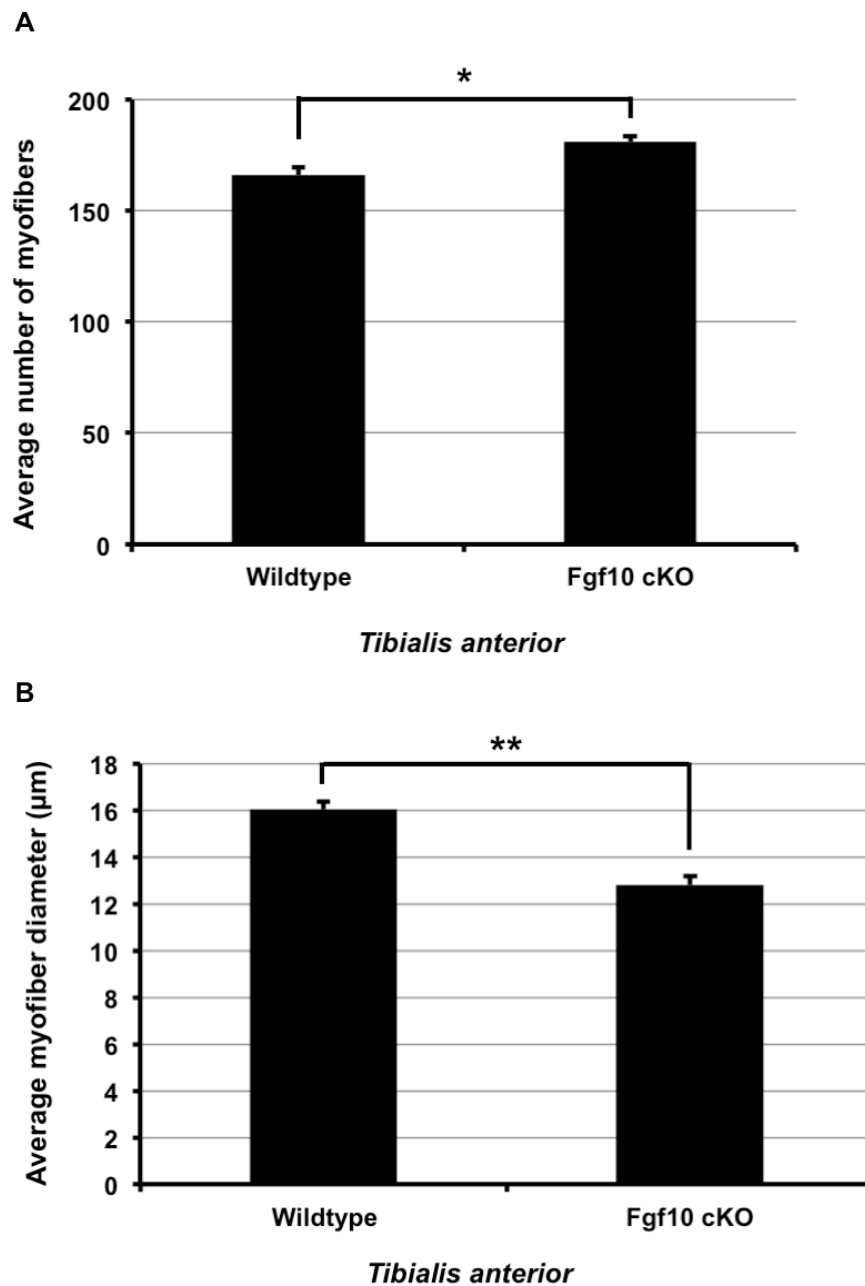


Figure 5.17: Myofiber number is increased but diameter is reduced in the *tibialis anterior* of *Fgf10* cKO mice. (A) The average number of myofibers in the *tibialis anterior* increases in the cKO. (B) Myofiber diameter is reduced in the *tibialis anterior* of the cKO. Error bars represent standard error, n=3. * $p \leq 0.05$, ** $p \leq 0.01$.

To assess whether the proliferation of myonuclei was affected in the *Fgf10* cKO, hindlimb sections from wildtype and cKO mice were immunolabelled with an anti-phosphohistone H3 (PHH3) antibody. PHH3 detects a serine residue in histone H3, which is phosphorylated during chromatin condensation of late G2/M phase of the cell cycle. It is therefore a mitosis marker and can be used to assess proliferation (Ladstein et al., 2012). The number of proliferating myonuclei was assessed in the *tibialis anterior* (where *Fgf10* is highly expressed) and also in the *tibialis posterior* (whose distal attachment is impaired in the cKO) to see if proliferation is impaired or elevated after removal of *Fgf10* expression (Figure 5.18). PHH3+ve myonuclei can be seen in both muscles in wildtype (Figure 5.18A-F) and cKO (Figure 5.18G-L) hindlimbs. When quantified, a Student's unpaired *t*-test revealed *p*-values of 0.3772 and 0.7327 for the *tibialis anterior* and *tibialis posterior* respectively. This confirmed there is no statistical difference between the numbers of proliferating myonuclei in either muscle in wildtype and cKO animals (Figure 5.18M).

Adjacent hindlimb sections were also immunolabelled with an anti-Caspase 3 antibody to assess whether myofibers in the distal region of the *tibialis posterior* were apoptotic (Sabine et al., 2012). No such fibers were detected (data not shown).

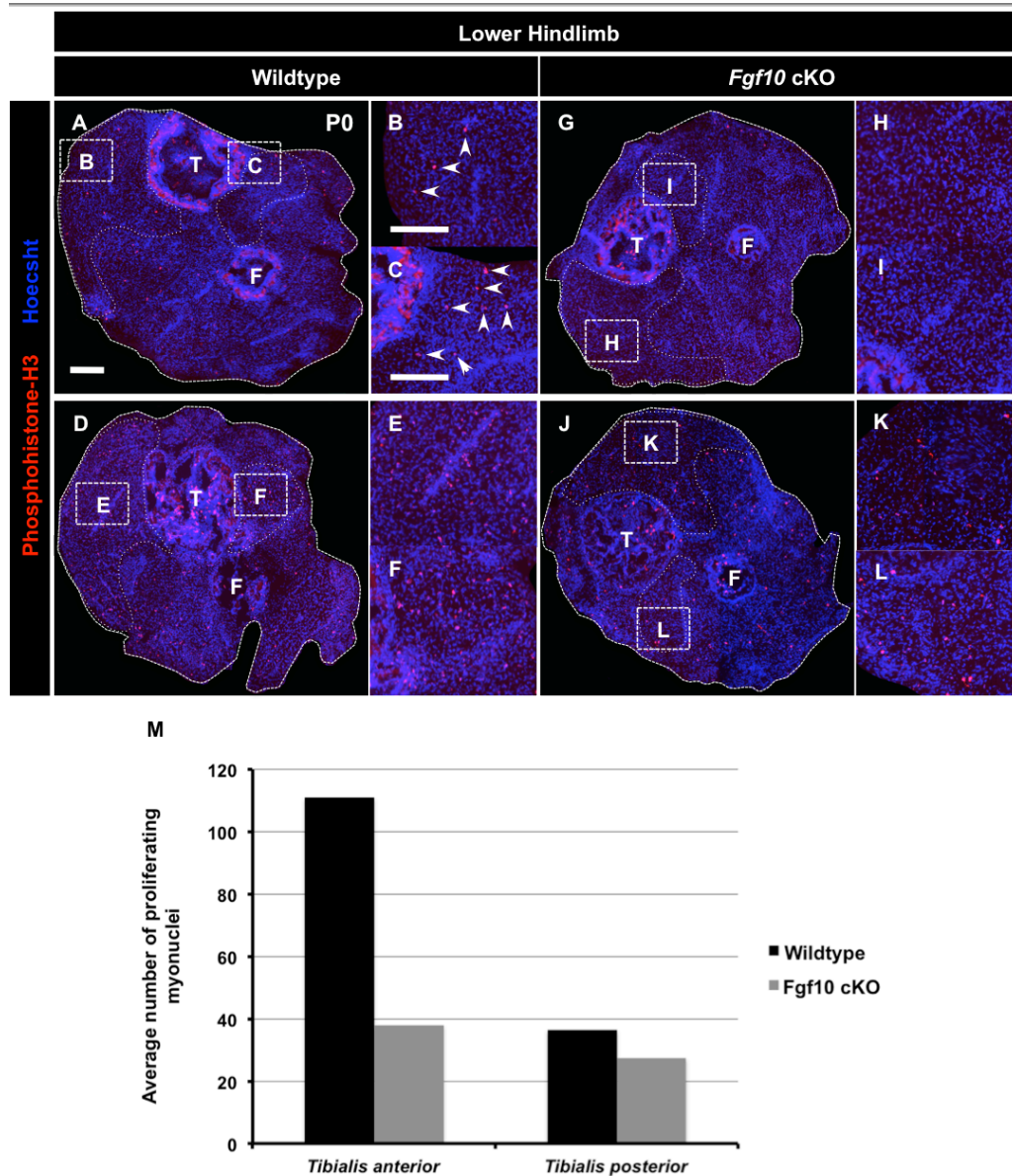


Figure 5.18: Myonuclear proliferation is unaffected in the *Fgf10* cKO.

Hindlimb sections were immunolabelled with an anti-phosphohistone H3 (PHH3) antibody to quantify the number of proliferating myonuclei (indicated by arrowheads) in *tibialis anterior* (B, E, H, K) and *tibialis posterior* (C, F, I, L) muscles. Abbreviations: Tibia (T), Fibula (F). (M) Average number of proliferating myonuclei in wildtype and *Fgf10* cKO muscles, n=2. Primary antibody: Rb- α -phospho-histone 3 (PHH3). Secondary antibody: Gt- α -Rb AlexaFluor568; Hoechst counterstain. Scale bars: 100 μ m (A); 45 μ m (B, C).

5.2.6 Fiber-type specification does not appear to be altered in *Fgf10* cKO mice.

In Chapter 3, it was also established through use of the *Fgf10*-lacZ reporter line that β -gal (*Fgf10*)-expressing myofibers were predominantly fast (type 2) myofibers, suggesting a potential role in fast fiber-type specification. To explore whether the proportions of fast and slow (type 1) myofibers were altered in muscles of the *Fgf10* cKO, disto-proximal sections through the lower limbs were immunolabelled with MyHC-f and MyHC-s antibodies, to detect all fast fiber subtypes and slow fibers respectively.

In the forelimb muscles, the fast fiber content in the cKO (Figure 5.19E-H) appears the same as in the wildtype (Figure 5.19A-D). Similarly there does not appear to be a difference in the slow fiber content in the cKO (Figure 5.20E-H) compared to the wildtype (Figure 5.20A-D).

Likewise in the hindlimb muscles, the fast fiber content in the cKO (Figure 5.21E-H) mirrors that seen in the wildtype (Figure 5.21A-D). There also does not appear to be any differences in slow fiber content in the cKO (Figure 5.22E-H) compared to the wildtype (Figure 5.22A-D). In the *tibialis anterior*, where *Fgf10* is expressed in a high proportion of fast fibers, the numbers of fast and slow myofibers were quantified. Four random, non-overlapping fields were assessed and the numbers of each fiber type were counted (Figure 5.23). No statistical difference was found between the number of slow myofibers in wildtype and cKO *tibialis anterior* muscle (p -value: 0.9343). However, the number of fast fibers was significantly reduced in the cKO muscle (p -value: 0.0055). Given that the number of myofibers in *tibialis anterior* increases in the cKO, it is possible that maturity of the fast-fiber phenotype is compromised in the cKO, which could explain the reduced number of fast fibers counted in this muscle.

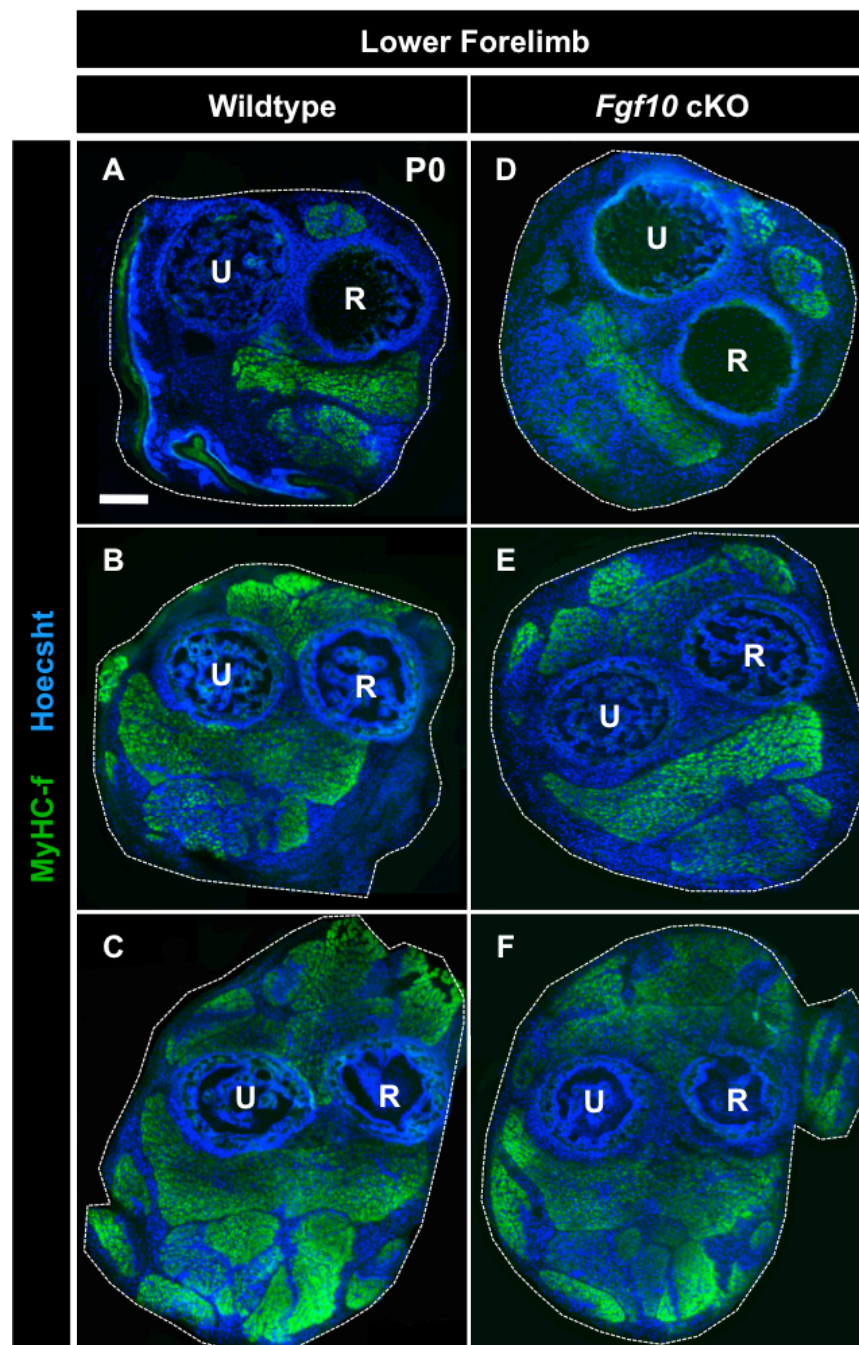


Figure 5.19: Composition of fast (Type 2) myofibers in wildtype and *Fgf10* cKO forelimb muscles. Fast fiber content appears unchanged in forelimb muscles of the cKO (D-F). Abbreviations: Ulna (U), Radius (R). Primary antibody: Ms- α -MyHC-f. Secondary antibody: Gt- α -Ms AlexaFluor488; Hoechst counterstain. Scale bar: 100 μ m.

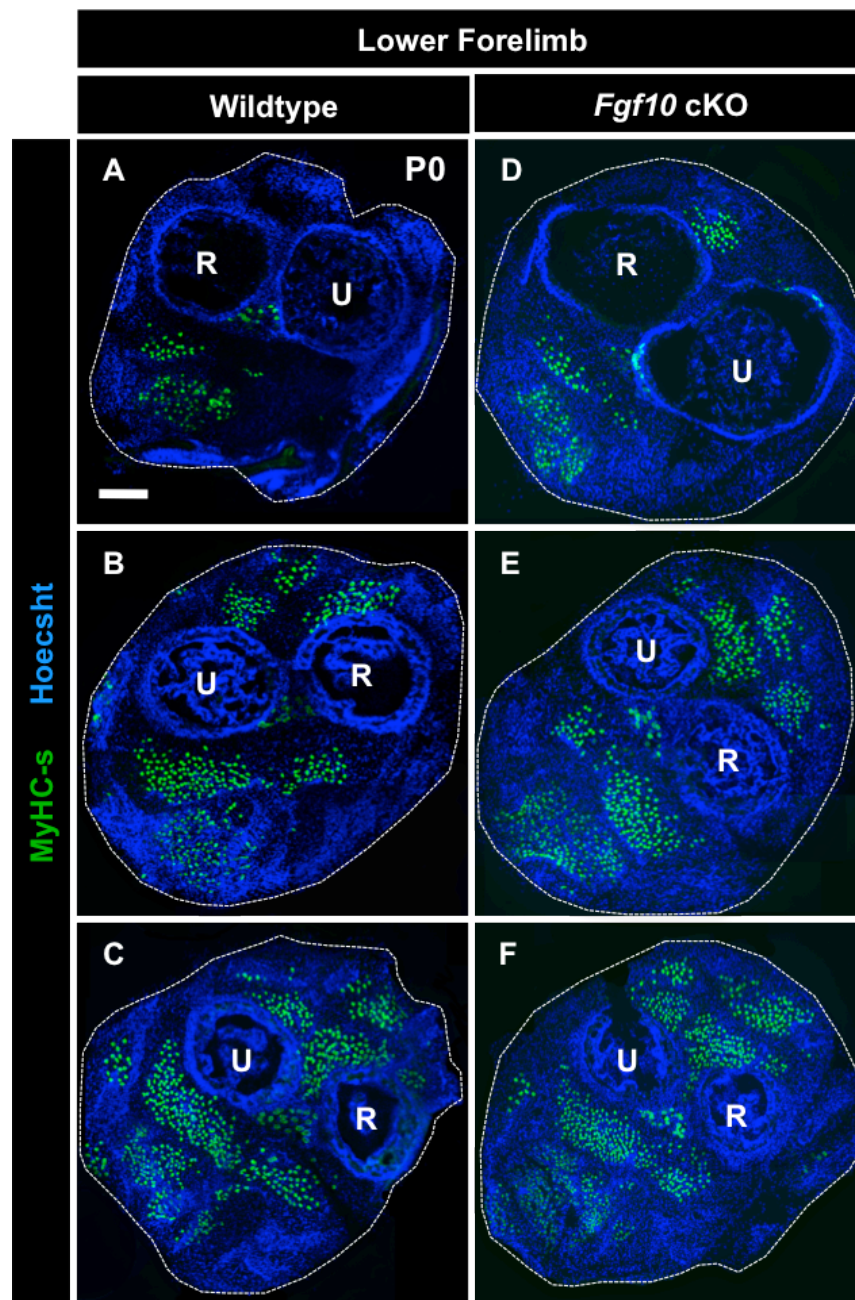


Figure 5.20: Slow (Type 1) myofiber content in wildtype and *Fgf10* cKO forelimb muscles. The number of slow fibers appears unchanged in forelimb muscles of the cKO (D-F). Abbreviations: Ulna (U), Radius (R). Primary antibody: Ms- α -MyHC-s. Secondary antibody: Gt- α -Ms AlexaFluor488; Hoecsht counterstain. Scale bar: 100 μ m.

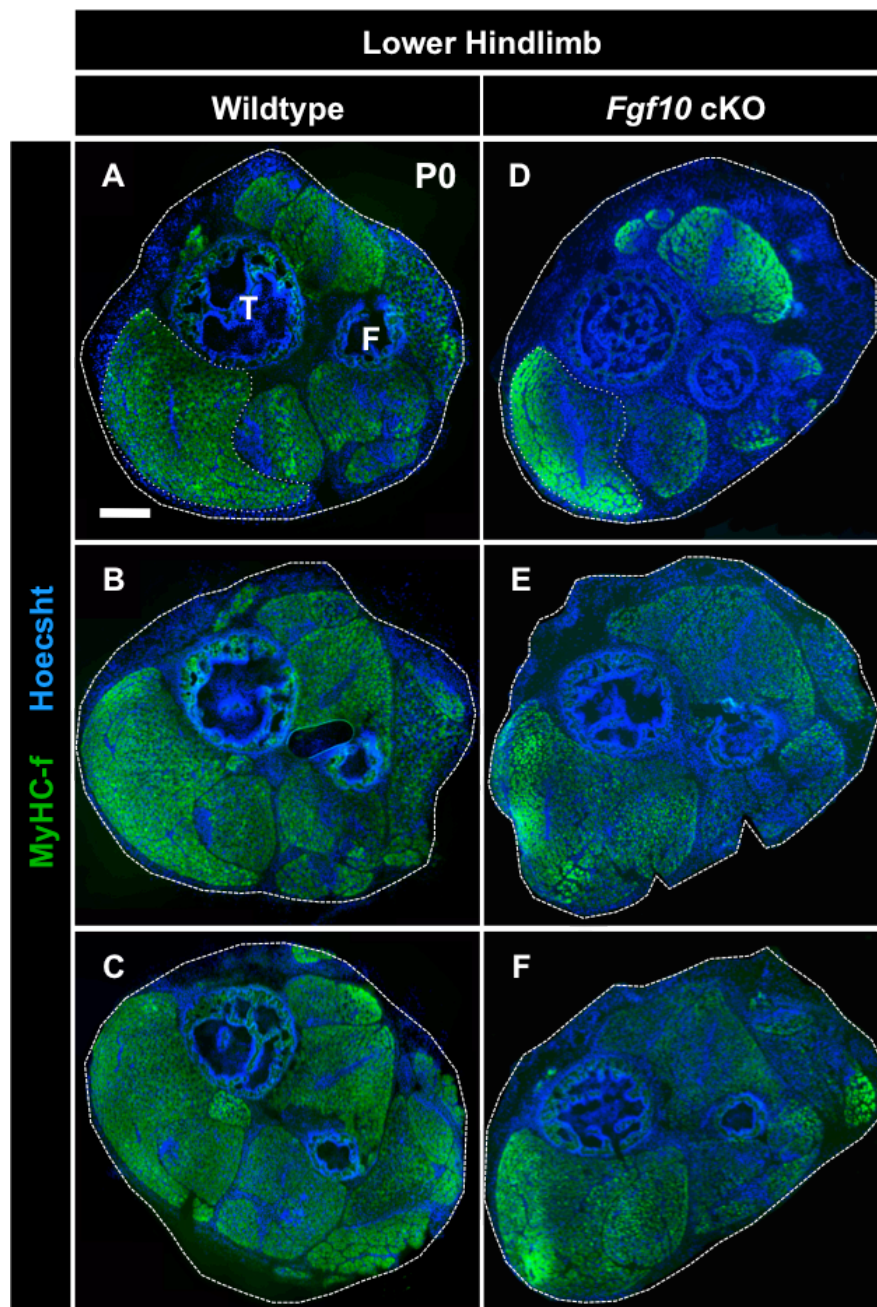


Figure 5.21: Fast myofiber content in wildtype and *Fgf10* cKO hindlimb muscles. The number of fast fibers appears unchanged in hindlimb muscles, including the *tibialis anterior* (outlined by dotted line), of the cKO (D-F). Abbreviations: Tibia (T), Fibula (F). Primary antibody: Ms- α -MyHC-f. Secondary antibody: Gt- α -Ms AlexaFluor488; Hoecsht counterstain. Scale bar: 100 μ m.

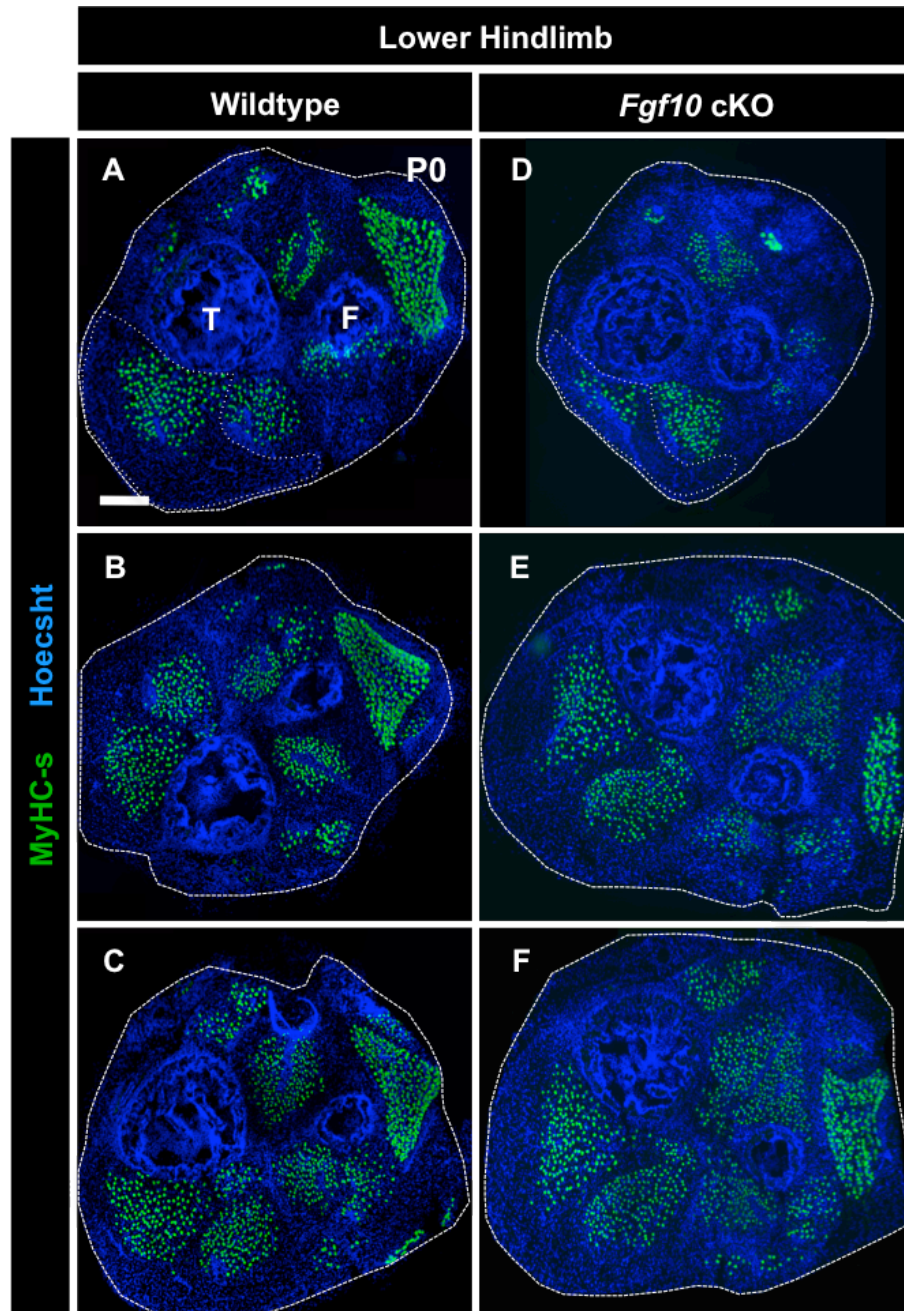


Figure 5.22: Slow myofiber content in wildtype and *Fgf10* cKO hindlimb muscles. The number of slow fibers also appears unchanged in hindlimb muscles of the cKO (D-F). Abbreviations: Tibia (T), Fibula (F). Primary antibody: Ms- α -MyHC-s. Secondary antibody: Gt- α -Ms AlexaFluor488; Hoechst counterstain. Scale bar: 100 μ m.

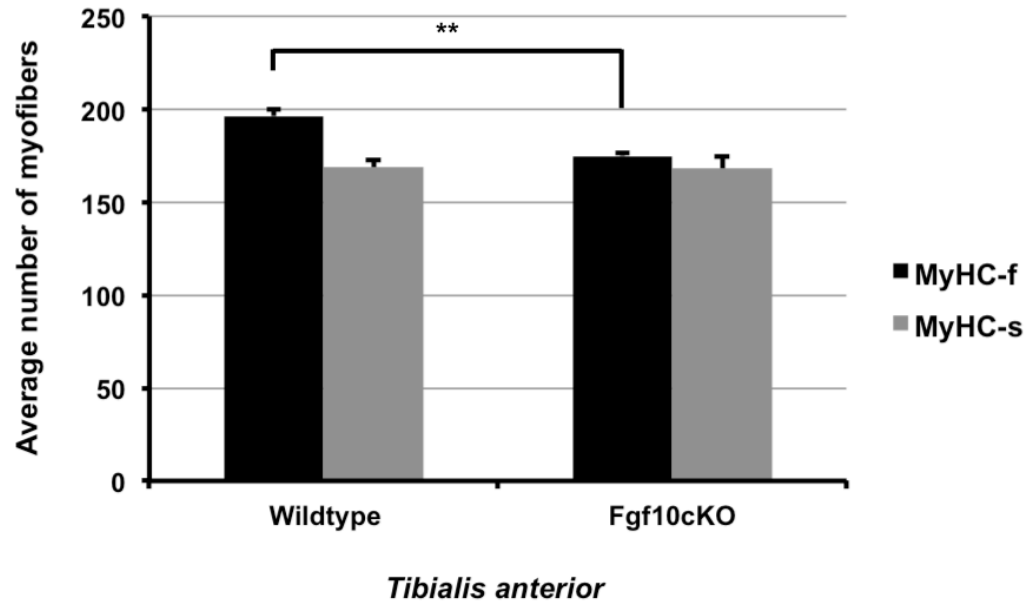
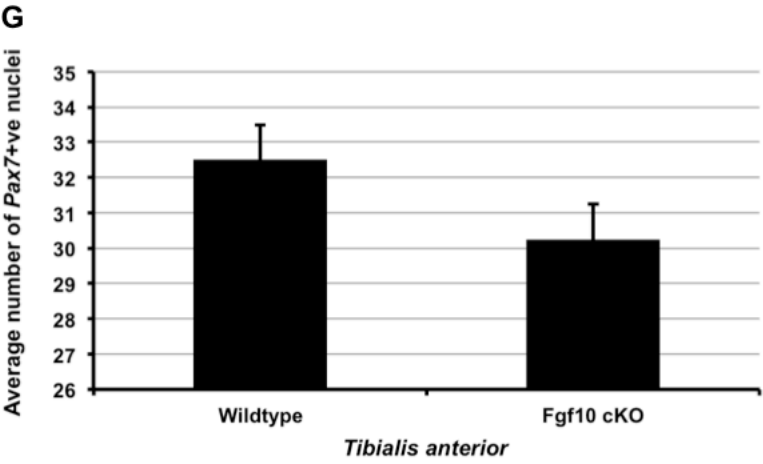
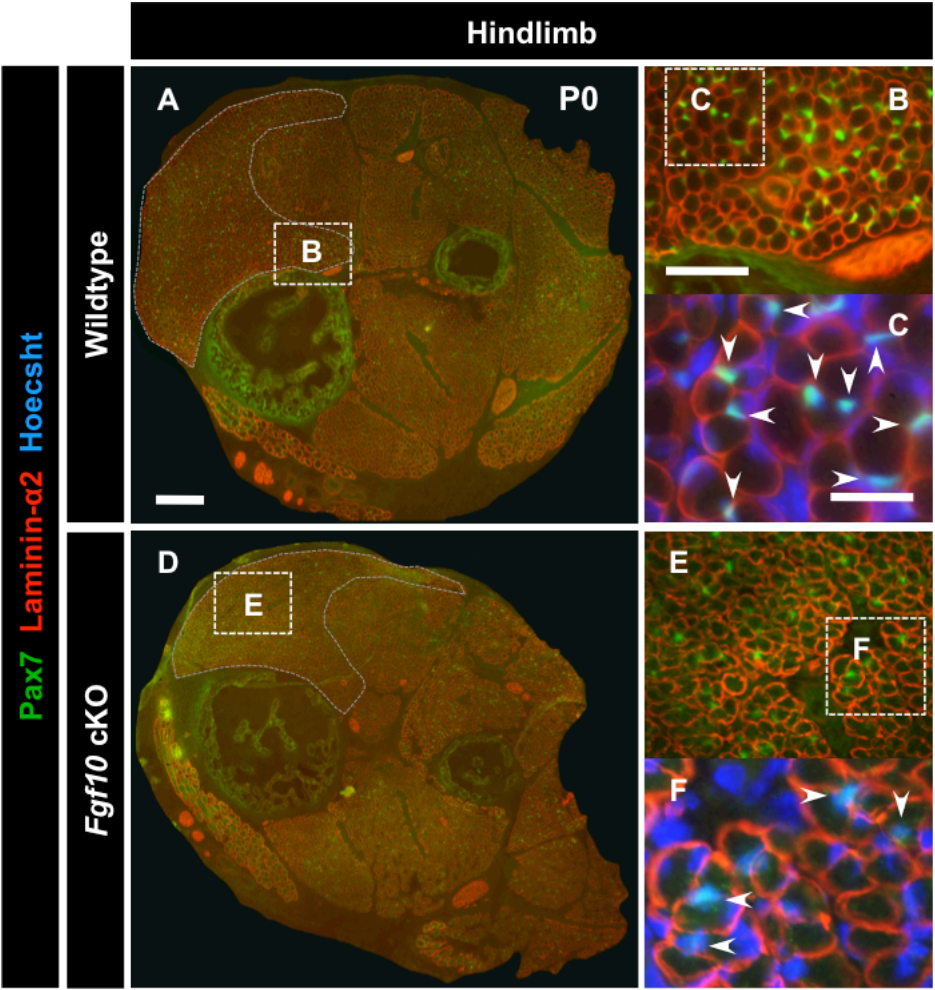


Figure 5.23: The number of fast fibers, but not slow fibers, is significantly reduced in the *tibialis anterior* of *Fgf10* cKO mice. The average number of slow (Type 1) myofibers is unchanged in wildtype and cKO *tibialis anterior* muscles. In contrast, the average number of fast (Type 2) myofibers is significantly decreased in the cKO. Error bars represent standard error, n=3. ** $p \leq 0.01$.

5.2.7 The number of satellite cells is not affected in *Fgf10* cKO mice.

In Chapter 4 it was discussed that a small proportion of *Fgf10*-expressing myonuclei co-localised with the satellite cell marker *Pax7*. This suggested that the majority of *Fgf10*-expressing myonuclei could be derived from *Pax7*-expressing satellite cells. Given the upregulation of *Fgf10* expression in skeletal muscle during early post-natal development, it is possible that *Fgf10* expression correlates with muscle hypertrophy (growth). To explore this further, hindlimb sections from *Fgf10* cKO mice were immunolabelled with anti-*Pax7* and anti-laminin $\alpha 2$ antibodies (to define the myofiber border). The total number of satellite cells was counted in four different regions of the *tibialis anterior* muscle (from 4 non-overlapping fields), and an average was taken. This average was then compared to the average number of satellite cells in the wildtype *tibialis anterior* (Figure 5.24A-F). Although the numbers of *Pax7*^{+ve} myonuclei were reduced in the cKO, this difference was found not to be statistically significant as a *p*-value of 0.7800 was obtained when an unpaired Student's *t*-test was carried out (Figure 5.24G).

Figure 5.24: Comparison of numbers of *Pax7*^{+ve} myonuclei in *tibialis anterior* of wildtype and *Fgf10* cKO mice. Satellite cells in the *tibialis anterior* (outlined by dotted line) were identified by expression of *Pax7* and their anatomical location confirmed with laminin- $\alpha 2$ immunostaining (C, F – indicated by arrowheads). (G) When quantified, the numbers of satellite cells in the cKO *tibialis anterior* were not significantly lower than those in the wildtype. Error bars represent standard error, n=3. Primary antibodies: Ms- α -*Pax7*; Rt- α -Laminin $\alpha 2$. Secondary antibodies: Gt- α -Ms AlexaFluor488; Gt- α -Rt AlexaFluor568; Hoechst counterstain. Scale bars: 100 μ m (A); 25 μ m (B); 10 μ m (C).



5.3 Discussion

In Chapters 3 and 4, it was discussed that *Fgf10* is expressed in a subgroup of skeletal muscles. The myofiber population within these muscles is heterogeneous for *Fgf10* expression, but it was shown that *Fgf10* is preferentially expressed in fast (Type 2) myofibers, and a small proportion of *Fgf10*-expressing myonuclei also co-localise with the satellite cell marker *Pax7*.

The function of *Fgf10* in skeletal muscle has yet to be elucidated because of limitations of knockout models. The *Fgf10* knockout (*Fgf10*^{-/-}) mutation is a perinatal lethal one, with knockout mice suffering from lung and limb agenesis, and colonic and cecal atresia (El Agha et al., 2012; Min et al., 1998). As a result, use of this model to study the function of *Fgf10* in limb muscles is not possible. To overcome this issue, compound transgenic mice were generated which relied on Cre-loxP technology to conditionally delete *Fgf10* expression in the skeletal muscle cell lineage. This made use of a *Pax3*-Cre (P3Pro-Cre) transgene, whereby the Cre recombinase protein is constitutively active in all *Pax3*-expressing cells, the majority of which are myogenic progenitor cells (Li et al., 2000). A breeding scheme was set up in order for *Fgf10* to be deleted in the *Pax3*-lineage, specifically through one null and one floxed copy of the allele (Figure 5.5). Cre-mediated recombination was validated through use of the tomato fluorescent reporter (mice sourced from The Jackson Laboratory).

A striking feature of the *Pax3*-Cre::*Fgf10*-conditional knockout (cKO) line is that deleting *Fgf10* expression from the *Pax3* lineage is also a perinatal lethal mutation. *Fgf10* cKO mice survived gestation and were recovered at their expected Mendelian ratios. However, they did not remain viable for long and died within a few hours after birth. *Fgf10* cKO mice displayed phenotypic features which distinguished them from their wildtype littermates. These mice were unable to suckle (lack of milk spot). They were also slightly smaller in size and had difficulty breathing. They

were also less mobile than their littermates (Figure 5.7). *Pax3* expression in the neural crest cell lineage has been shown to be vital to the development of cardiac progenitor cells in heart development, and endothelial cells which line pulmonary arteries and veins in lung development (Olaopa et al., 2011; She et al., 2012). It is therefore likely that the perinatal lethality was caused by either breathing or cardiac defects.

Lineage tracing analyses have shown that endothelial cells derive from *Pax3*-expressing somitic progenitors, with different extrinsic cues determining either myogenic or endothelial cell fate (Kardon et al., 2002; Messina and Cossu, 2009). It has also been previously shown that *Fgf10* knockout mice lack pulmonary arteries and veins, suggesting that removing *Fgf10* expression from the endothelial cell lineage impairs development of these vessels, which help to facilitate efficient gaseous exchange with the alveolar epithelium (Marguerie et al., 2006). It is possible that removing *Fgf10* expression from certain intercostal muscles impedes their function, and this could give rise to breathing difficulties. When intercostal muscle structure was investigated in the sagittal plane, there were no striking differences seen in the cKO (Figure 5.8). However, if intercostal muscle structure were investigated in a different plane of orientation, perhaps differences in morphology of the muscles would be seen. As previously alluded to, *Pax3* expression is also critical to early cardiac neural crest cell morphogenesis (Li et al., 2013; Olaopa et al., 2011). *Fgf10* is expressed in the pharyngeal mesoderm which gives rise to the arterial pole of the heart (Kelly et al., 2001). Removing *Fgf10* expression from this myocardial progenitor cell population, which originates from the cardiac neural crest, is also likely to lead to cardiac defects in the cKO (Torlopp et al., 2010). Although one cKO mouse did survive into adulthood, this was attributed to inefficient *Cre*-mediated recombination, which in itself is a stochastic event. The numbers of fast and slow fibers were quantified in the *tibialis anterior* of this cKO, in

addition to the 'wildtype' or control littermates, which express *Fgf10* to varying degrees (Figure 5.9). There does not appear to be a significant difference between the numbers of fast and slow fibers in the cKO, however, as only one was analyzed, definitive conclusions cannot be drawn from these data (Figure 5.10).

When observing muscle morphology in the cKO, muscles of the forelimb appear to develop normally (Figure 5.11). However, the *tibialis posterior*, *soleus*, *plantaris* and *flexor digitorum brevis* muscles in the hindlimb all contained disorganized and hypercontracted myofibers (Figure 5.12). This defect was only visible in the distal region of the hindlimb, suggesting that the myofibers had failed to attach to the tibia and fibula bones and therefore, the distal tendons for these muscles had failed to develop properly. Indeed, when distal regions of the cKO hindlimb were immunolabelled with a tendon marker (tenascin C) and compared to corresponding wildtype regions, the tendons of the *tibialis posterior* and *flexor digitorum brevis* appeared reduced in size and in the case of the latter, misplaced (Figure 5.13). In contrast, the bones in the hindlimb themselves, and the overall skeletal structure, were unaffected in the cKO (Figure 5.14, 5.15).

In the early stages of development, initiation of hindlimb budding is dependent on expression of *Pitx1*, which then stimulates expression of the T-box transcription factor *Tbx4*. *Tbx4* is responsible for establishing the *Fgf10-Fgf8* loop, which facilitates limb bud outgrowth (DeLaurier et al., 2006; Duboc and Logan, 2011). FGF signals emanate from the center of the myotome (a region of *Pax3* expression) to stimulate *Scleraxis* expression, and determine the position of the syndetome by inducing regions of the sclerotome adjacent to the myotome to adopt a tendon cell fate (Brent et al., 2003; Brent and Tabin, 2004; Smith et al., 2005). In the developing limb, *Fgf4* and *Fgf8* are expressed at the muscle-tendon boundary. Similarly, the downstream effector of FGF signaling, *Pea3*, and negative modulators of the pathway, *Sprouty1* and 2, are also enriched at

the myotendinous junction (Eloy-Trinquet et al., 2009). The smaller sizes of the *tibialis posterior* and *flexor digitorum brevis* tendons in the cKO suggest their development was arrested prematurely, which likely resulted from impaired migration, or a reduced number, of tendon progenitor cells. This may have been caused by a reduced FGF10 signal from *Pax3*-expressing cells in the myotome during early tendon cell specification. However, the reasons why these particular tendons are affected, specifically in the distal region of the hindlimb, remain unclear.

Analyses on the *tibialis anterior* muscle, which contains a high proportion of *Fgf10*-expressing myofibers, provided an interesting insight into how myofiber morphology is affected in the cKO. Firstly, the number of myofibers increased in this muscle in the cKO, but they were narrower in diameter than in the wildtype muscle (Figure 5.16). Secondly, the number of fast fibers was slightly reduced in the cKO, although the number of slow fibers remained unchanged (Figure 5.22). Finally, the number of *Pax7*-expressing satellite cells was also reduced, but not significantly, in the cKO (Figure 5.23).

Although the *tibialis anterior* in cKO mice displays an increased number of myofibers, their cross-sectional area is reduced. This would suggest that individual myofibers are failing to enlarge efficiently, and therefore hypertrophy of the muscle is impaired. Efficient hypertrophy can only occur when the rate of protein synthesis exceeds its degradation. The IGF-Akt-mTOR and myostatin-Smad2/3 pathways stimulate and repress protein synthesis respectively (Schiaffino et al., 2013). FGF can also activate the Akt-mTOR pathway which promotes protein synthesis, it is therefore possible that the reduction in *Fgf10* expression within this muscle is limiting the turnover of protein synthesis, hence they are reduced in size (Martelli et al., 2007). Muscle hypertrophy during early post-natal stages is also partly mediated by proliferation of satellite cells, which were originally believed to fuse to the growing myofiber and therefore contribute new myonuclei (Moss and Leblond, 1970; Schiaffino

et al., 1976). However, it is now widely acknowledged that skeletal myofibers are capable of robust hypertrophy independent of satellite cells (Lee et al., 2012; McCarthy et al., 2011). The decrease in *Pax7*⁺ myonuclei in the cKO *tibialis anterior* is therefore unlikely to have contributed to the reduced hypertrophy in this muscle. Observing the true impact of the decreased number in satellite cells might be possible if the muscle could be challenged under regenerative conditions. The decreased number of fast fibers seen in the cKO *tibialis anterior* could be attributed to delayed maturation of this fiber phenotype, on account of the impaired hypertrophy seen.

From the analyses in this Chapter, it would appear that *Fgf10* might play different roles within the *Pax3* lineage, in both tendon patterning during embryonic development, and subsequently in myofiber hypertrophy during post-natal development. It would be interesting to further investigate its potential role in tendon patterning by observing expression of another tendon marker, *Scleraxis*, in the regions of the hindlimb where tendon formation appears to be impaired.

Chapter 6

***In vitro* studies of *Fgf10* function**

6.1 Introduction

The classic genetic approach to study gene function is to carry out loss-, gain- or misexpression of gene function studies. Overexpression of individual genes is an important means of investigating their native role in biological processes, which otherwise may have been missed due to lethality or gene compensation in loss-of-function studies (Prelich, 2012). The foundations for using overexpression as a genetic tool were derived from studies in plants and bacteria, which explored phenotypes resulting from varying gene dosages (Birchler and Veitia, 2007; Showe and Onorato, 1978). These studies paved the way for manipulation of genetic material in larger organisms.

Establishing reliable methods to detect *Fgf10* expression is fundamental to elucidating its function. Although several transgenic reporter lines exist for *Fgf10*, generation of these modified alleles can render the mice hypomorphic for *Fgf10* (El Agha et al., 2012; Kelly et al., 2001). Haploinsufficiency for *Fgf10* expression has been attributed to abnormal phenotypes including hypoplastic salivary glands and aplastic lacrimal glands (Entesarian et al., 2005). These results indicate that levels of *Fgf10* are critical in development. Typically, FGF's mode of action centres around its secretion from a source cell, and its subsequent binding to a receptor on a target cell (Kosman et al., 2007). However, there is growing evidence to show that FGFs can also be detected within the nuclei of target cells. Proteins either passively diffuse into the nucleus if small in size, or require active transport if larger than 45kDa (Silver, 1991). In the case of the latter, active transportation is facilitated by the nuclear localization signal (NLS) within the protein binding to importins. The NLS-importin complex is recognized and allowed entry into the nucleus by the nuclear pore complex. FGFs-1, 2 and 3 have been shown to contain one or more NLSs, which facilitate this translocation into the nucleus (Claus et al., 2003; Kiefer and Dickson, 1995; Lin et al., 1996; Quarto et al., 1991; Sheng et al., 2004). NLS sequences are typically characterized by a

group of basic residues, which are recognized by the importins (Boulikas, 1993; Lange et al., 2007). These sequences can be either 'classical', wherein the basic residues are in sequence. Or, alternatively they can be 'bipartite', where the residues are separated along a stretch of the sequence (Robbins et al., 1991). Overall FGF10 is a basic protein, containing various short sequences of basic residues which could act as NLS motifs. One such motif has already been identified, as the sequence shared a high sequence similarity with the established NLS in FGF1 sequence, and was also situated away from the receptor-ligand interface (Kosman et al., 2007). A second potential NLS has also been identified (Marta Mikolajczak, unpublished findings).

C2C12 mouse myoblast cells are subclones of C2 myoblasts, originally isolated from thigh muscles of dystrophic adult mice, 70 hours after undergoing crush injury (Yaffe and Saxel, 1977). These cells were shown to proliferate when grown in high serum concentrations, and to express *Myf5* and *MyoD*. Upon serum starvation, the cells withdraw from the cell cycle and upregulate production of contractile proteins, prompting their fusion into myotubes and the expression of *myogenin* (Andres and Walsh, 1996; Blau et al., 1983; Guo et al., 1995; Halevy et al., 1995; Miller, 1990).

Using the immortalized C2C12 myogenic cell line, the principal aim of this chapter is to explore the effects of overexpressing *Fgf10* on the differentiation of myoblasts, using an HA-tagged overexpression vector.

6.1.1 Aims

This chapter will address the following hypotheses:-

1. Do C2C12 cells express *Fgf10* or its receptor, *FgfR2-IIIb*, endogenously?
2. What are the subcellular locations of FGF10 in C2C12 cells?
3. Does *Fgf10* overexpression accelerate the differentiation of myotubes?
4. Does mutation of the NLS in FGF10 affect myotube formation?

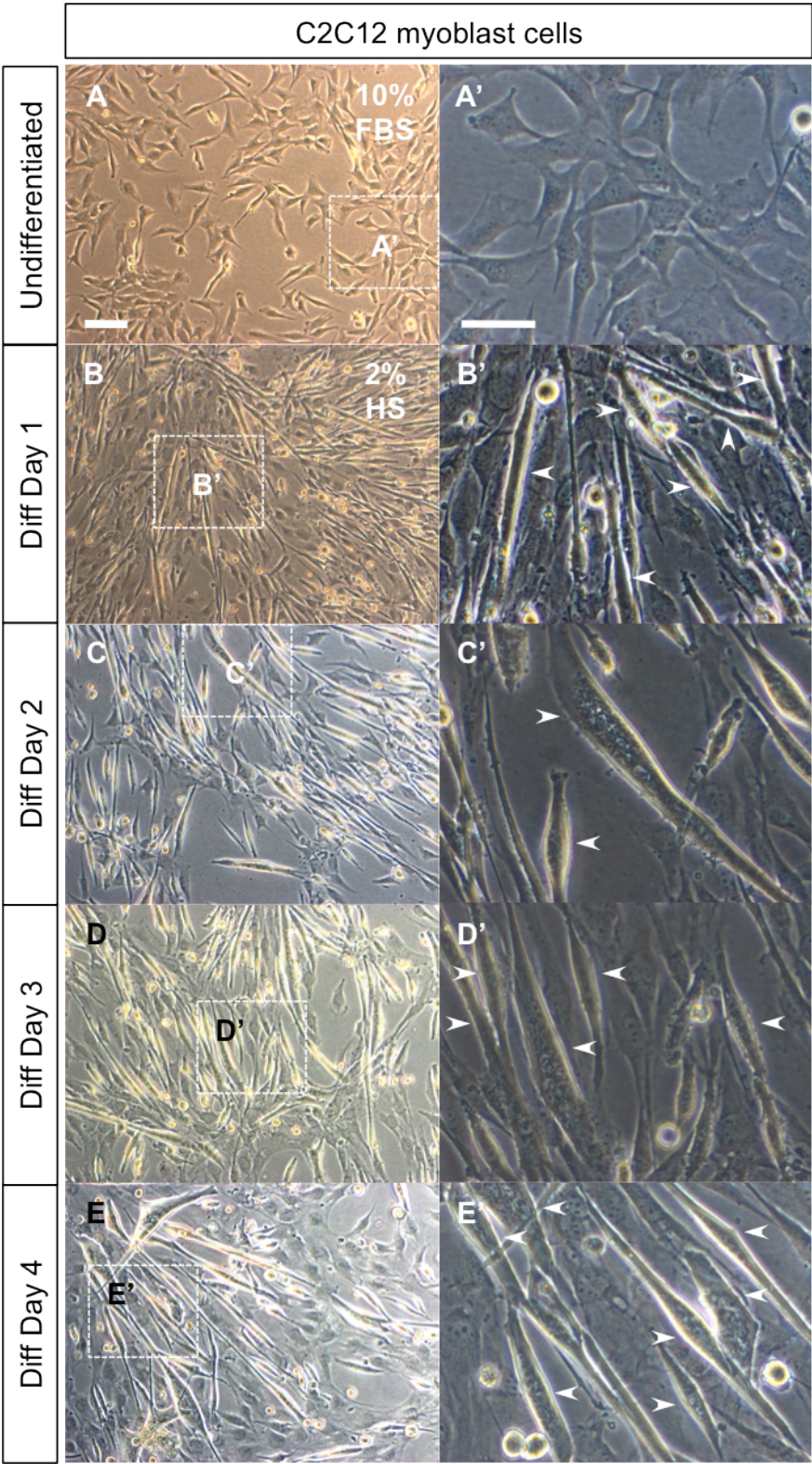
6.2 Results

6.2.1 C2C12 cell line as a model of myoblast differentiation.

C2C12 cells were maintained in an undifferentiated state in DMEM-2 (see Section 2.6.1) containing 10% fetal bovine serum (FBS). To induce differentiation and fusion into myotubes, the cells were cultured in DMEM-3, containing 2% horse serum (HS). For differentiation to occur efficiently, undifferentiated myoblasts were approximately 80-90% confluent. Differentiation takes place over 4-5 days, and elongation of the myocytes can be seen early on, with larger myotubes visible later (Figure 6.1).

Figure 6.1: Differentiation of C2C12 myoblast cells *in vitro*.

(A, A') Undifferentiated C2C12s are maintained in a proliferative state in DMEM containing 10% fetal bovine serum (FBS). (B-E') To induce differentiation, cells are switched to low-serum media containing 2% horse serum (HS). The course of differentiation takes around 4-5 days. (B, B') On differentiation day (Diff Day) 1 elongated myocytes can be seen which rapidly fuse to form myotubes. (E, E') By Diff Day 4, myotubes of varying sizes can be seen. All scale bars 100µm.



6.2.2 *Fgf10*, but not its receptor, is expressed in both myoblasts and myotubes.

To investigate if C2C12 myoblast cells express *Fgf10*, RT-PCR was used to semi-quantitatively detect *Fgf10* levels in both undifferentiated and differentiated C2C12s. *Fgf10* is expressed in myoblasts, and the levels of this gene appear to decrease during differentiation (Figure 6.2). FGF receptors were also screened in both samples. Interestingly, the receptor for *Fgf10*, *Fgfr2-IIIb*, is not expressed in either myoblasts or myotubes. *Fgfr2-IIIc*, with which FGF10 does not interact, is expressed at low levels in myoblasts, but this expression disappears during differentiation. Likewise *Fgfr1-IIIb* is expressed in myotubes but not in myoblasts. *Fgfr1-IIIc* and *Fgfr4* are both expressed in undifferentiated and differentiated states (Figure 6.3). The absence of the main receptor for *Fgf10*, FGFR2-IIIb, raises interesting questions regarding its mode of action in these cells. As a secreted ligand, FGFR2-IIIb is the main mediator of FGF10's paracrine effects during organogenesis (De Moerlooze et al., 2000; Hajihosseini et al., 2009; Zhang et al., 2006). However, since it is not expressed by C2C12s, this may imply an alternative mode of action is employed by FGF10. This is likely due to be an intracellular function, as there is growing evidence to suggest that FGF10 is directly targeted to the cell nucleus, as has previously been shown with its closely relative, FGF3 (Antoine et al., 2005; Kosman et al., 2007). In the brain, the hypothalamus also presents a similar paradigm. RNA *in situ* hybridization and RT-PCR both revealed that *Fgf10* is expressed by cells which line the ventral wall of this structure, called tanycytes. However, the *Fgfr2IIIb* receptor is absent in the hypothalamus. This indicates that FGF10 may exert its function in a receptor-independent mechanism, possibly involving nuclear targeting (Haan et al., 2013; Hajihosseini et al., 2008). FGF10 can also bind FGFR1-IIIb, although with a much lower affinity than FGFR2-IIIb (Cui and Li, 2013; Luo et al., 1998). *Fgfr1-IIIb* is expressed at low levels in

differentiated myotubes, so it is possible that FGF10 is signaling through this receptor, which is being upregulated during differentiation. Expression of the remaining *Fgf10* family members, *Fgf-3*, *-7* and *-22* was also examined in undifferentiated C2C12 myoblasts (Figure 6.4). *Fgf-7* was also found to be expressed in myoblasts, whereas *Fgf-3* and *Fgf-22* were not.

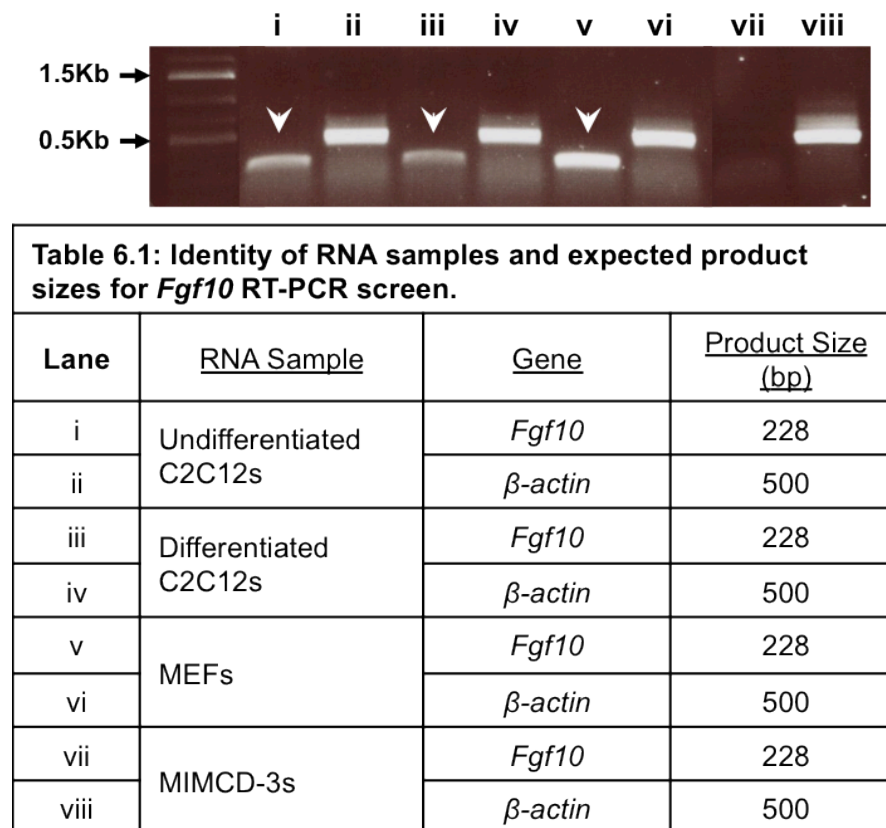


Figure 6.2: Expression profile of *Fgf10* in C2C12 myoblasts and myotubes by RT-PCR. Levels of *Fgf10* appear decreased in the differentiated myotube sample (iii) in comparison to undifferentiated myoblasts (i). RNA from mouse embryonic fibroblasts was used as a positive control for *Fgf10* expression (v). RNA from mouse epithelial inner medullary collecting duct (MIMCD-3) cells was used as a negative control for *Fgf10* expression (vii). β -actin was used as an RNA quality control for all samples (ii, iv, vi, viii).

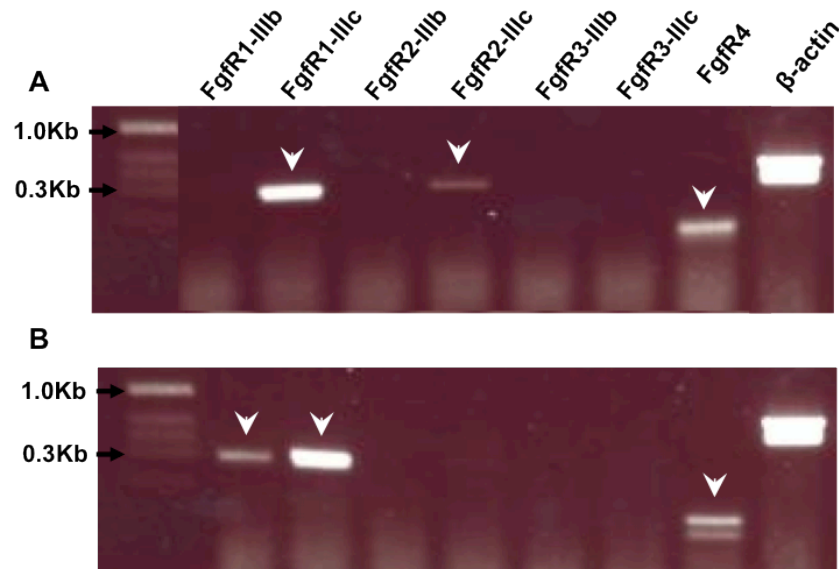


Table 6.2: Expected product sizes for RT-PCR screen of FGF receptors.

Gene	Product Size (bp)	Expression in myoblasts	Expression in myotubes
<i>FGFR1-IIIb</i>	300	-	+
<i>FGFR1-IIIc</i>	306	+++	+++
<i>FGFR2-IIIb</i>	317	-	-
<i>FGFR2-IIIc</i>	310	+	-
<i>FGFR3-IIIb</i>	350	-	-
<i>FGFR3-IIIc</i>	348	-	-
<i>FGFR4</i>	195	++	++
<i>β-actin</i>	500	+++	+++

Figure 6.3: Expression profile of *Fgf* receptors in C2C12 myoblasts and myotubes by RT-PCR. (A) In undifferentiated myoblasts, *FgfR1-IIIc* and *FgfR4* are highly expressed and *FgfR2-IIIc* is expressed at low levels. (B) In differentiated myotubes, *FgfR1-IIIc* and *FgfR4* continue to be highly expressed, however expression of *FgfR2-IIIc* is lost. *FgfR1-IIIb* is expressed at low levels. *β -actin* served as an RNA quality control.

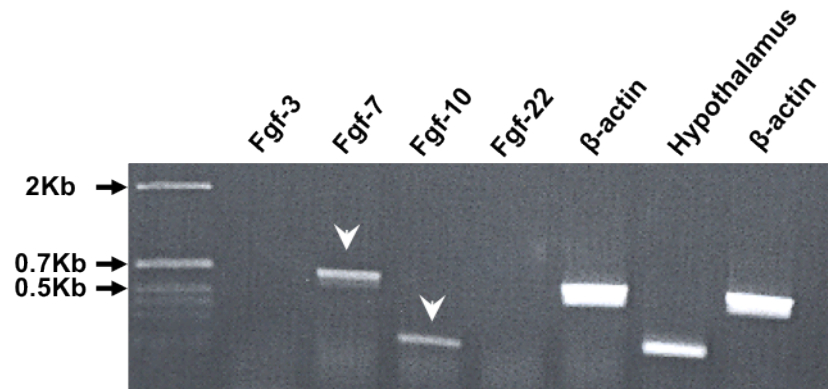


Table 6.3: Expected product sizes for RT-PCR screen of *Fgf10* family members.

<u>Gene</u>	<u>Product Size (bp)</u>	<u>Summary</u>
<i>Fgf3</i>	573	-
<i>Fgf7</i>	622	+
<i>Fgf10</i>	228	+
<i>Fgf22</i>	171	-
<i>β-actin</i>	500	+
<i>Fgf10</i> (Hypothalamus)	228	+
<i>β-actin</i>	500	+

Figure 6.4: Expression profile of *Fgf10* family members in C2C12 myoblasts by RT-PCR. *Fgf7* (ii) is expressed in addition to *Fgf10* (iii), whereas expression of *Fgf3* (i) and *Fgf22* (iv) is absent from these cells. RNA from the hypothalamus provided a positive control for *Fgf10* expression (vi). *β-actin* served as an RNA quality control (v, vii).

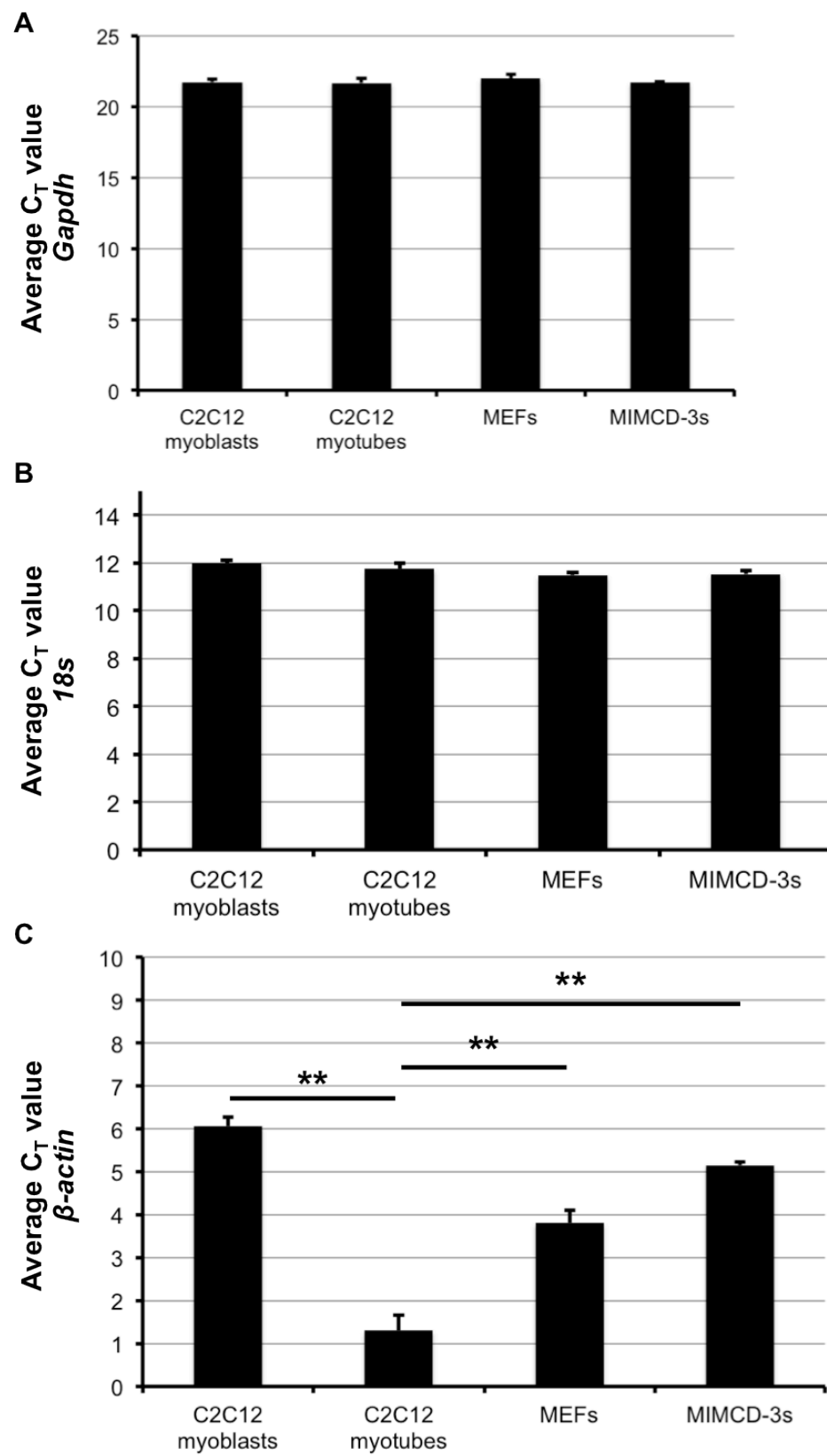
6.2.2.1 Quantification of *Fgf10* expression in C2C12 myoblasts and myotubes by qRT-PCR

500ng RNA from C2C12 myoblasts and myotubes, MEFs (positive control for *Fgf10* expression) and MIMCD-3 cells (negative control for *Fgf10* expression) was reverse transcribed as detailed in Section 2.11.1.

Initially, expression of *Gapdh*, *18S* and β -actin reference genes was investigated. Quantitative PCR was carried out as detailed in Section 2.11.2. Both *Gapdh* and *18S* were uniformly expressed across the cell samples (Figure 6.5A, B). However, β -actin expression was found to vary significantly between the samples (Figure 6.5C), based on the non-parametric Kruskal-Wallis test. As *18S* was more highly expressed than *Gapdh*, it was selected as the reference gene for this assay.

To quantify *Fgf10* expression, the cDNA volume loaded per reaction (i.e. in 10 μ L) was increased 4-fold to 20ng. The increments on the standard curve were also increased to 40ng, 20ng, 10ng, 5ng, 2.5ng and 1.25ng. RNA from MEFs and MIMCD-3s were used as positive and negative controls respectively. The results of this assay are summarized in the box plots in Figure 6.6. *Fgf10* was highly expressed in MEFs, whereas its expression in MIMCD-3s was negligible (data not shown). Although its levels in C2C12s were also low, *Fgf10* did appear to be downregulated during differentiation into myotubes. A non-parametric Mann-Whitney test confirmed this difference to be statistically significant ($p=0.0152$, Figure 6.6).

Figure 6.5: Comparison of average C_T values for *Gapdh*, *18S* and β -actin in C2C12 cells, MEFs and MIMCD-3 cells. (A, B) Both *Gapdh* and *18S* are uniformly expressed in the samples, with *18S* expression exceeding that of *Gapdh*. (C) β -actin is variably expressed in the cell lines. Error bars represent standard error, $n=6$. Kruskal-Wallis test gave $p=0.0019$.



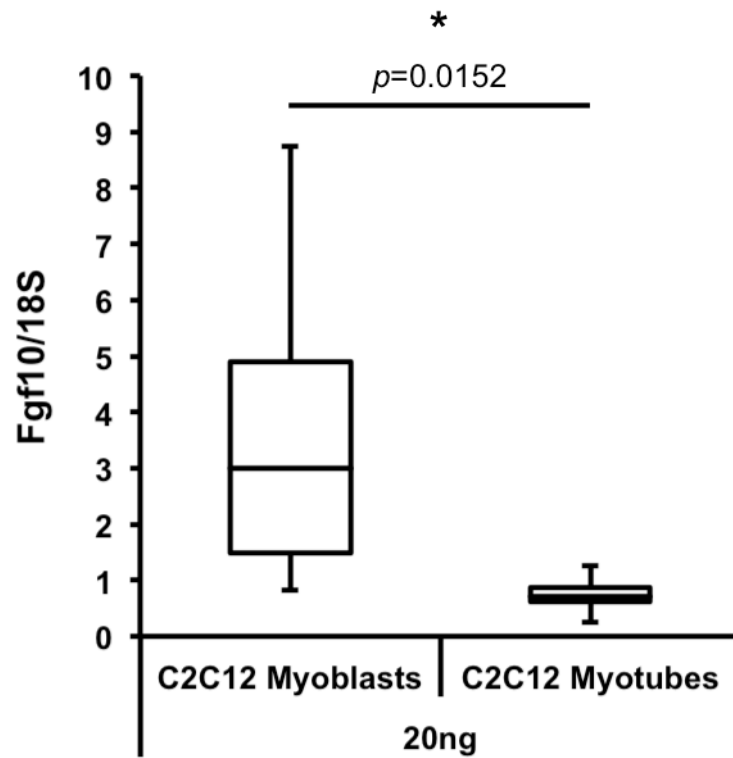


Figure 6.6: Box-plot analysis of *Fgf10* expression (normalized against *18S*) in C2C12 myoblasts and myotubes. Box plots show the minimum, 25th percentiles, median, 75th percentiles, and maximum for levels of *Fgf10*. Error bars represent standard error, n=6. Mann-Whitney test calculated statistical significance (* $p \leq 0.05$).

6.2.3 Transient transfections of pF10-HA into C2C12 cells.

As previously mentioned, commercial antibodies against FGF10 do not work well in immunohistochemistry, limiting our understanding of the subcellular localization of this protein, which would help to facilitate a greater understanding of its function. For this reason, FGF10 fusion reporter constructs were developed to visualize its localization. Initially, the pmCherry-C1 mammalian expression vector was used (Clontech Laboratories Inc.) This vector is designed to express a protein of interest fused to the C-terminus of the mutant fluorescent protein, mCherry. mCherry is a monomeric protein, derived from the tetrameric red fluorescent protein DsRed, which originated in red mushroom coral, *Discosoma sp* (Dietrich and Maiss, 2002; Shaner et al., 2004). Fgf10 cDNA was subcloned into pmCherry-C1 vector, however sequencing revealed that the construct contained mutations, and therefore this vector was not used in further analyses. Although mCherry is the most photostable of the red fluorophores, and functions as a monomer like FGF10, it is also a considerably large tag with a molecular weight of 29kDa (Shaner et al., 2005). FGF10 has a molecular weight of approximately 20kDa (Yamasaki et al., 1996). Attachment of the mCherry protein to this small, secreted ligand may obstruct its native localization and function, as has been shown previously with other fluorescent proteins (Miyawaki, 2011). To circumvent this problem, the influenza hemagglutinin (HA) epitope was fused to the Fgf10 cDNA, and subcloned into a pN1 vector (the pmCherry-N1 vector without the mCherry coding sequence). The HA epitope tag is regularly used in overexpression vectors owing to its small size (1.1kDa) which minimizes steric hindrance, and is therefore unlikely to interfere with the tertiary structure and function of the FGF10 protein (Lobbestael et al., 2010; Vieyres et al., 2013). The generation of this vector and its verification was carried out by Marta Mikolajczak, and is described in Section 2.14. Briefly, the HA coding sequence was fused to the C-terminus of the Fgf10 cDNA sequence. After excision of the

mCherry coding sequence, the Fgf10-HA construct was subcloned into pN1 (Figure 6.7). The pF10-HA vector was found not to carry any mutations after Sanger sequencing was carried out (Figure 6.8).

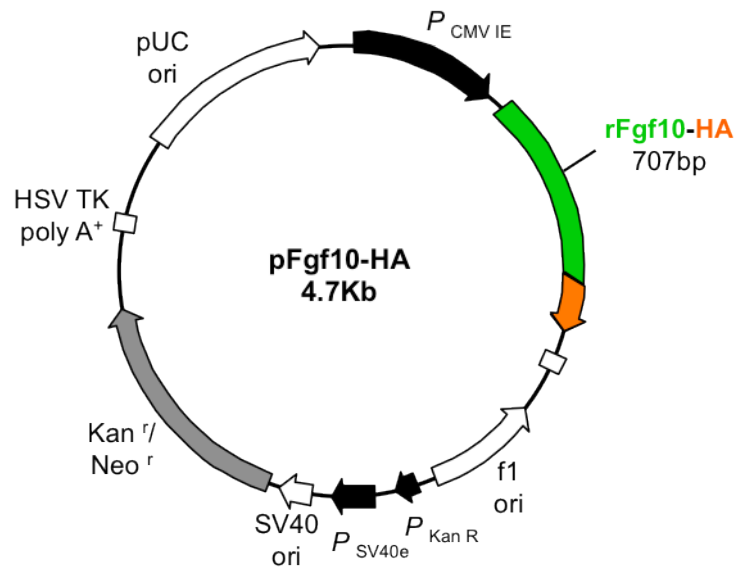


Figure 6.7: Schematic of the pF10-HA restriction map. An HA-tagged Fgf10 coding sequence was subcloned into pN1 mammalian vector.

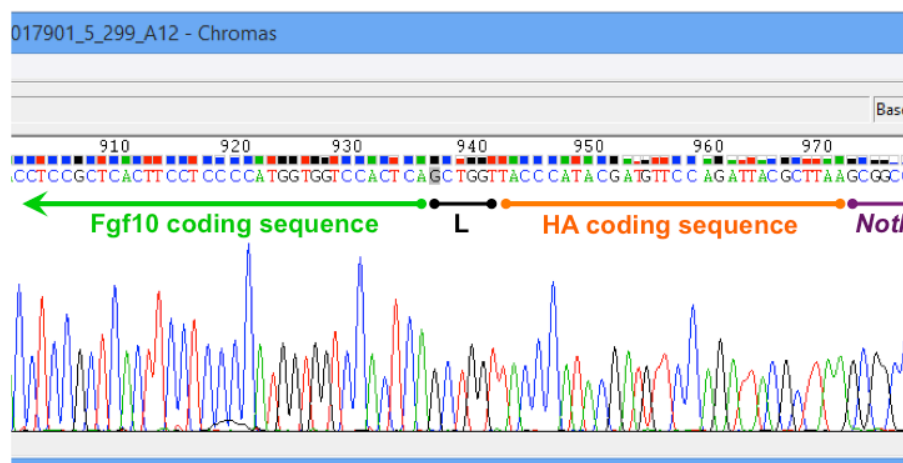


Figure 6.8: Snapshot of sequencing trace for Fgf10-HA construct. Sanger sequencing revealed there were no mutations in either the Fgf10 or HA coding sequences. Sequencing trace provided by Marta Mikolajczak.

In order to study the localization of FGF10, transient transfections of the pF10-HA overexpression vector were carried out. In contrast to stable transfections, transiently-transfected genetic material is not integrated into the genome of the host cell, and as a result is only expressed for a limited period of time. Surrounding environmental factors and the process of cell division can therefore reduce the amount of transfected material (Kim and Eberwine, 2010). FGF10 localization was either observed in undifferentiated myoblasts, 24 hours post-transfection, or in differentiated myotubes, which had been induced for 5 days from the day of transfection. Since the latter stimulates myoblasts to stop proliferating, transient transfections were considered optimal for both analyses. Various transfection reagents, including jetPRIME® (Polyplus transfection, France) and TurboFect® (ThermoScientific, UK), were sampled in preliminary studies to verify transfection efficiency in C2C12 cells. jetPRIME is a polymer-based, non-liposomal reagent which forms a cationic (positively-charged) complex with DNA. The complex enters the cell via endocytosis and absorbs acidity within the endosome, causing it to swell and rupture, therefore releasing the DNA into the cytoplasm (Nafissi et al., 2014; Sandbichler et al., 2013). TurboFect is also a cationic polymer-based transfection reagent (Oba and Tanaka, 2012).

24 hours prior to transfection, cells were seeded onto poly-D-lysine (PDL)-coated coverslips in 12-well plates, at a density of 18×10^4 in order to achieve 70-90% confluency on the day of transfection. Cells were transfected with varying amounts of DNA:transfection reagent, as per manufacturer's instructions. The cells were immunolabelled with an anti-HA antibody (see Table 2.3) the next day. Cells were transfected with either pF10-HA or pmCherry-N1 (abbreviated to pmN1) in order to compare efficiencies of the transfection reagents (Figure 6.9). Transfection efficiency was calculated as a percentage of transfected cells out of the total number of cells, screened from 5 non-overlapping fields under the x40 objective. Overall, transfection efficiency was around 10%

in C2C12 cells. Increasing amounts of DNA (1µg-3µg) in conjunction with increasing volumes of jetPRIME (2µL-6µL) appeared to have no effect on the transfection efficiency of the pF10-HA vector. Minimal proportional increases were observed with the pN1 vector. In contrast, TurboFect appeared to boost transfection of the pN1 vector to approximately 10%. Whilst the number of visible cells transfected with pF10-HA and TurboFect did not reflect this increase, likely the same proportion of cells were transfected with the construct originally, but did not proceed to express the fusion construct. Lipofectamine® (Invitrogen, UK) reagent was also trialled, but the transfection efficiency was similar to that of jetPRIME (data not shown). Overall, TurboFect appeared the optimal transfection reagent and was used for subsequent analyses.

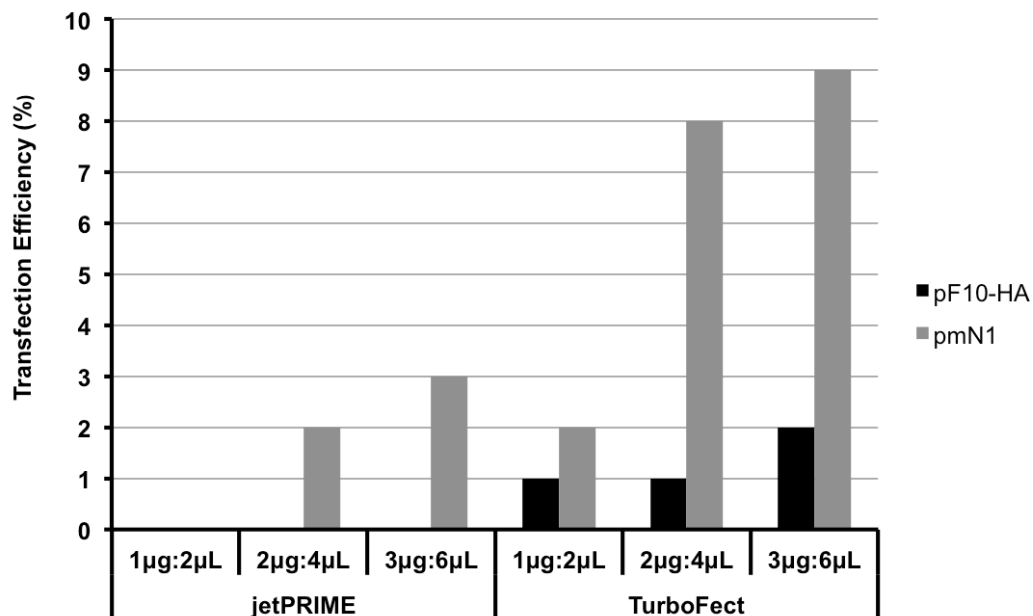


Figure 6.9: Comparing transfection efficiencies of jetPRIME® and TurboFect® in C2C12 cells. Cells were plated at 18×10^4 density 24 hours prior to transfection. Cells were transfected with 1µg, 2µg or 3µg pF10-HA or pN1 DNA, in conjunction with 2µL, 4µL or 6µL jetPRIME or TurboFect. Transfection efficiency was calculated as the percentage of transfected cells out of the total number of cells, screened from 5 different x40 fields. Efficiency of pF10-HA transfection with jetPRIME was negligible, and showed a very small increase to 2% and 3% with pN1. Efficiency of pN1 transfection with TurboFect was increased from 2% to 9% at higher volumes of the reagent. The number of cells transfected with pF10-HA and TurboFect also appeared low (around 2%) but this can likely be attributed to underproduction of the F10-HA construct, n=3.

6.2.4 HA-tagged FGF10 protein is found both within the nucleus and cytoplasm of C2C12 myoblasts.

FGF10-HA localization was observed 24 hours post-transfection, and was found within the nucleus and the cytoplasm, as indicated by anti-HA staining (Figure 6.10C-F). In some instances, FGF10-HA appeared confined to the cytoplasm alone (Figure 6.10A), and in other cells the majority of its expression was contained within the nucleus (Figure 6.10B). When quantified, around 40% showed FGF10-HA localization within the cytoplasm alone, and a further 40% showed FGF10-HA in the nucleus in addition to the cytoplasm (termed 'both'; Figure 6.11). The remaining 20% of cells contained FGF10-HA in the nucleus alone. An unpaired Student's *t*-test showed no statistical difference was found between the relative proportions of FGF10-HA localization, however its preferential expression in the cytoplasm is indicative of the fact that it is a secreted protein. Its retention in the cytoplasm in certain cells may be due to interacting partners preventing its translocation to the nucleus. Similarly, its expression within the nucleus, which appears contained within specific structures such as the nucleolus, is indicative of specific targeting to this organelle, most likely via an NLS sequence (Kosman et al., 2007).

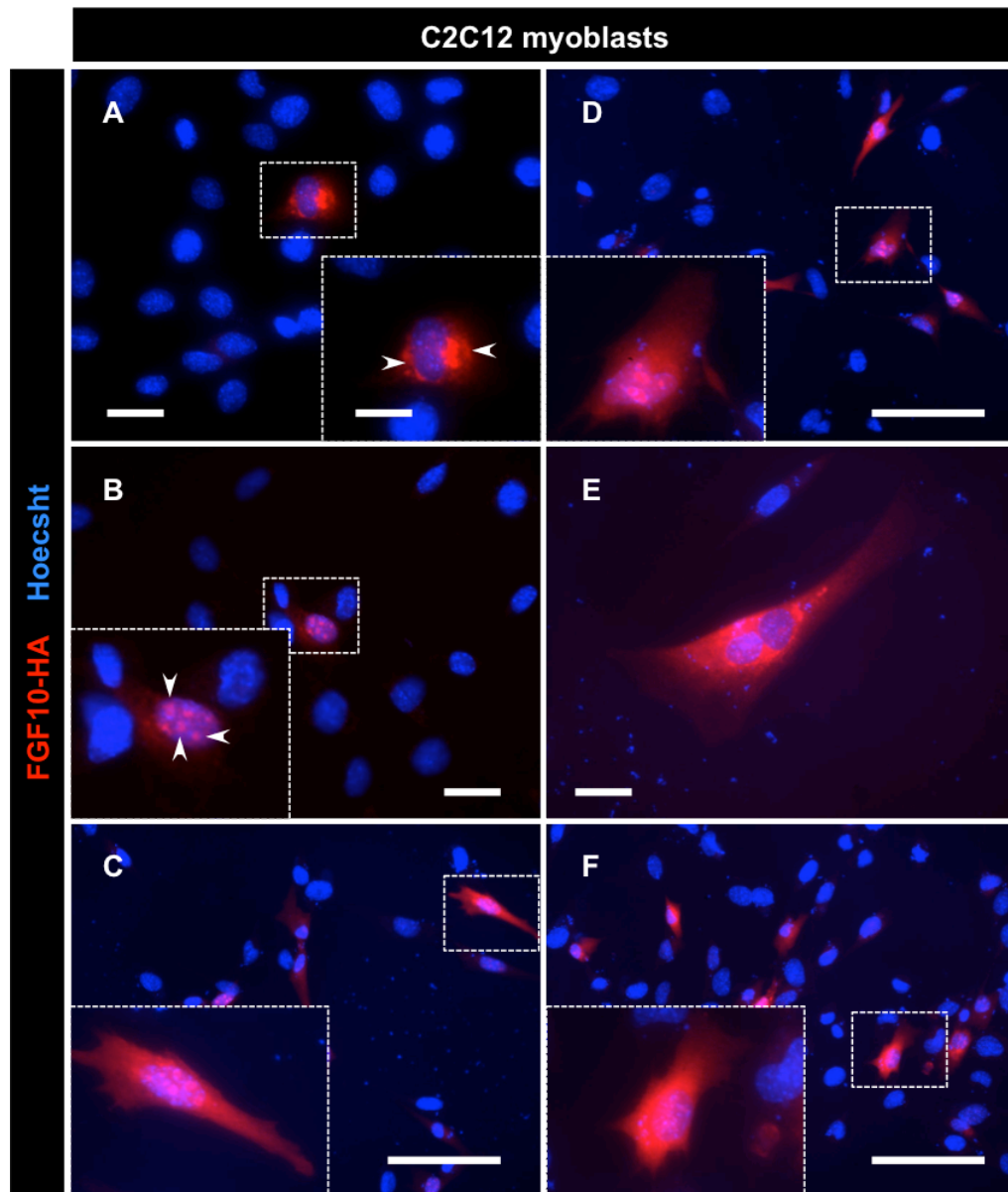


Figure 6.10: FGF10-HA localizes to the nucleus and cytoplasm of undifferentiated C2C12 myoblasts. (A) FGF10-HA is predominantly found exclusively within the cytoplasm (indicated by white arrowheads) or within the cytoplasm and the nucleus, specifically in structures resembling the nucleolus (C-F). (B) In some cells the majority of FGF10-HA appeared targeted to the nucleus. Primary antibody: Ms- α -HA. Secondary antibody: Gt- α -Ms AlexaFluor568; Hoechst counterstain. Scale bars: 50 μ m (A, B, E); 100 μ m (C, D, F); 20 μ m (all insets).

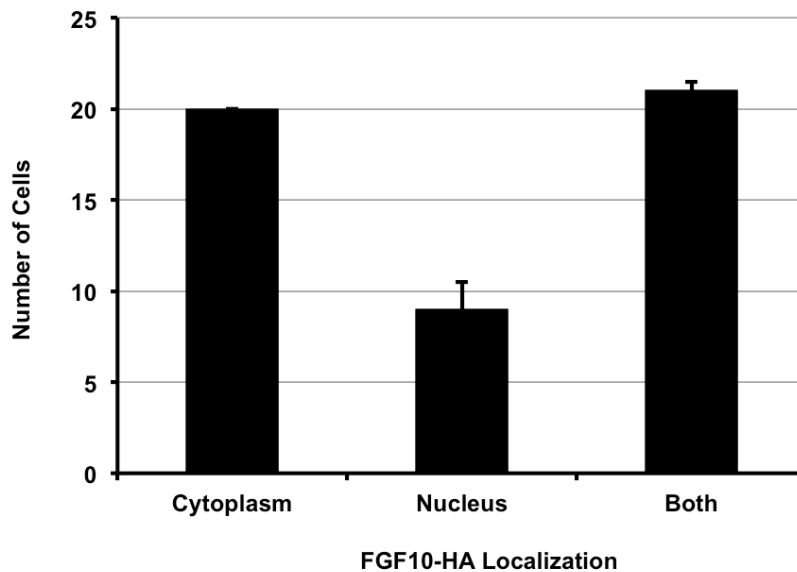


Figure 6.11: Proportions of transfected C2C12 myoblasts containing FGF10-HA in either the cytoplasm, or nucleus, or both. Around 40% of transfected cells contained FGF10-HA localized within the cytoplasm, or in the nucleus in addition to the cytoplasm (both). Approximately 20% of cells contained FGF10-HA predominantly in the nucleus. Unpaired Student's *t*-test revealed no statistical difference between these proportions.

6.2.5 C2C12 myoblast fusion is not altered by *Fgf10* overexpression.

FGF10-HA localization was observed in C2C12 myotubes on Differentiation Day 5 (D5), when most myotubes have fully-formed. Myotubes were visualized using an Alexa Fluor® 488-conjugated Phalloidin probe which binds filamentous actin (Neufeld et al., 2004). FGF10-HA can be seen at the periphery of the nuclei and within the nuclei themselves (Figure 6.12A). It can also be seen within the cytoplasm, and appears as puncta at high magnification (Figure 6.12A' inset). FGF10 could potentially act as a secretory signal between growing myotubes and myocytes which have not yet fused (Figure 6.12B, B'). In some cases, FGF10 appeared to shuttle between two populations of *Fgf10*-overexpressing myocytes, possibly to fuse into a single, larger myotubes (Figure 6.12C, C').

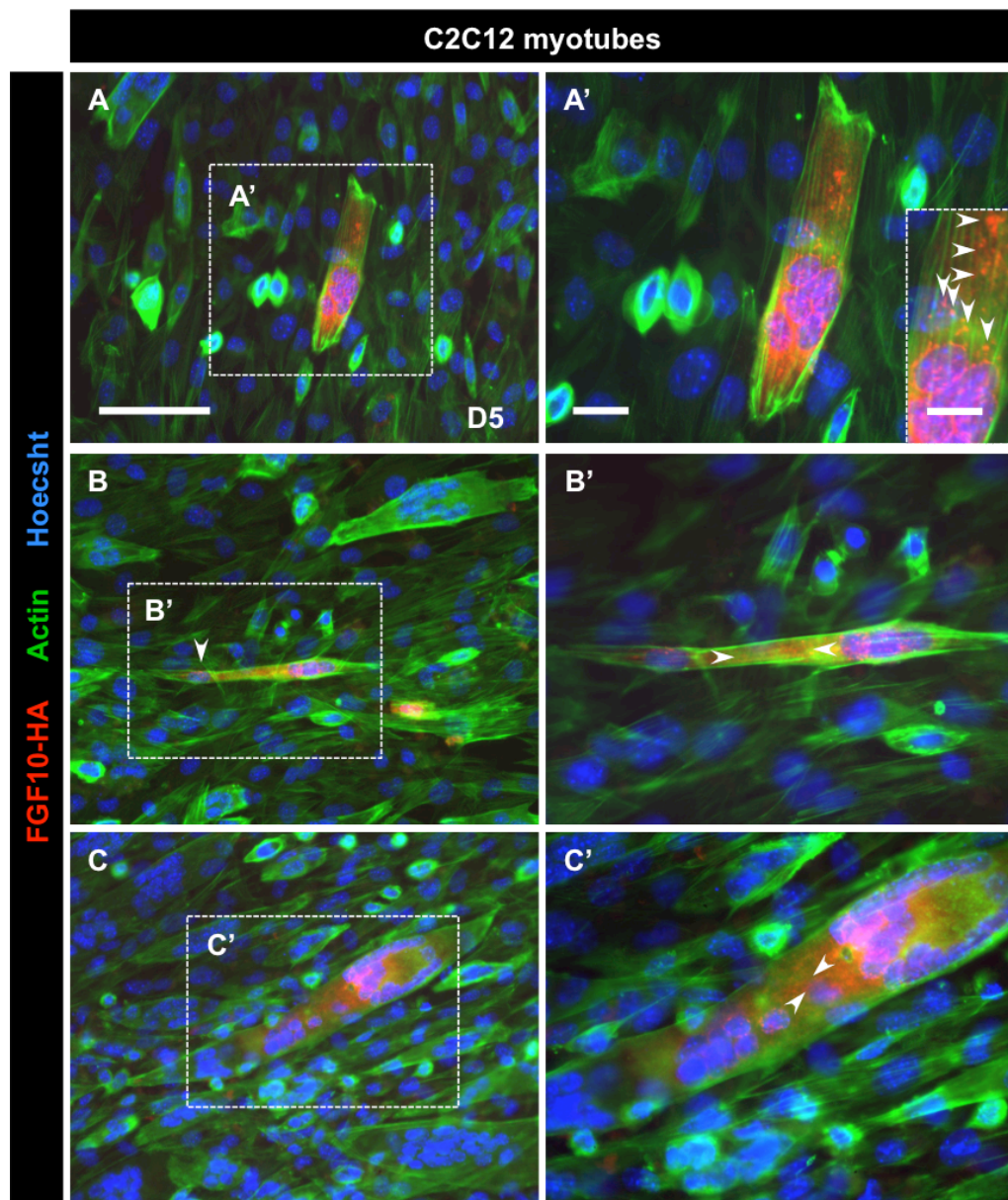


Figure 6.12: FGF10-HA localizes to the cytoplasm and nucleus in differentiated C2C12 myotubes. (A, A') FGF10-HA is located within the nuclei and at their peripheries. At high magnification, puncta are visible emanating away from the nucleus (see white arrowheads, inset). (B, B') FGF10-HA secretion throughout the myotube may recruit other myocytes to fuse. (C, C') FGF10-HA signaling between myotubes could play a role in the fusion process. Primary antibody: Ms- α -HA. Secondary antibody: Gt- α -Ms AlexaFluor568; Phalloidin-AlexaFluor388; Hoechst counterstain. Scale bars: 100 μ m (A); 50 μ m (A'); 20 μ m (A' – inset).

FGF10-HA shuttling between growing myotubes may play a role in the fusion process (Figure 6.13).

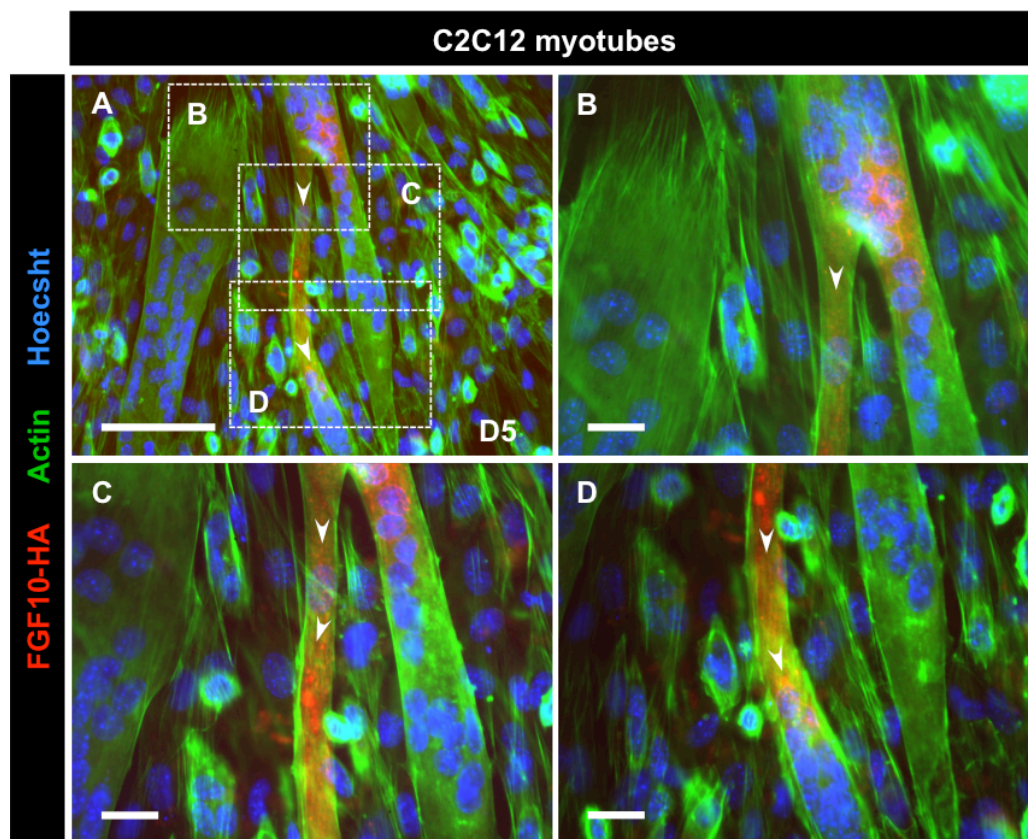


Figure 6.13: FGF10-HA is expressed in myotube extensions.

(A) FGF10-HA can be seen in appendages of the myotubes, and may enhance the fusion process by recruiting neighbouring myotubes via these extensions (B, C, D). Primary antibody: Ms- α -HA. Secondary antibody: Gt- α -Ms AlexaFluor568; Phalloidin-AlexaFluor388; Hoechst counterstain. Scale bars: 100 μ m (A); 50 μ m (B-D).

To measure myoblast fusion in *Fgf10*-overexpressing myotubes, the myoblast fusion index (MFI) was calculated by quantifying the percentage of myonuclei in myotubes where the HA tag was detected, that contained more than two nuclei, out of the total cell nuclei number. As a control, the MFI was also calculated for myoblasts transfected with the

pmCherry-N1 (pmN1) vector, wherein mCherry is produced but should have no effect. Whilst the FGF10-HA protein can be seen in specific localization patterns (Figure 6.12A), the mCherry protein is diffusely expressed throughout the myotube and does not localize to specific subcellular compartments (Figure 6.14B).

A Student's unpaired *t*-test revealed no significant difference in MFI values between *Fgf10*-overexpressing or control myotubes at D5. In addition, no significant difference was found in the lengths and widths of myotubes overexpressing *Fgf10* (Figure 6.15).

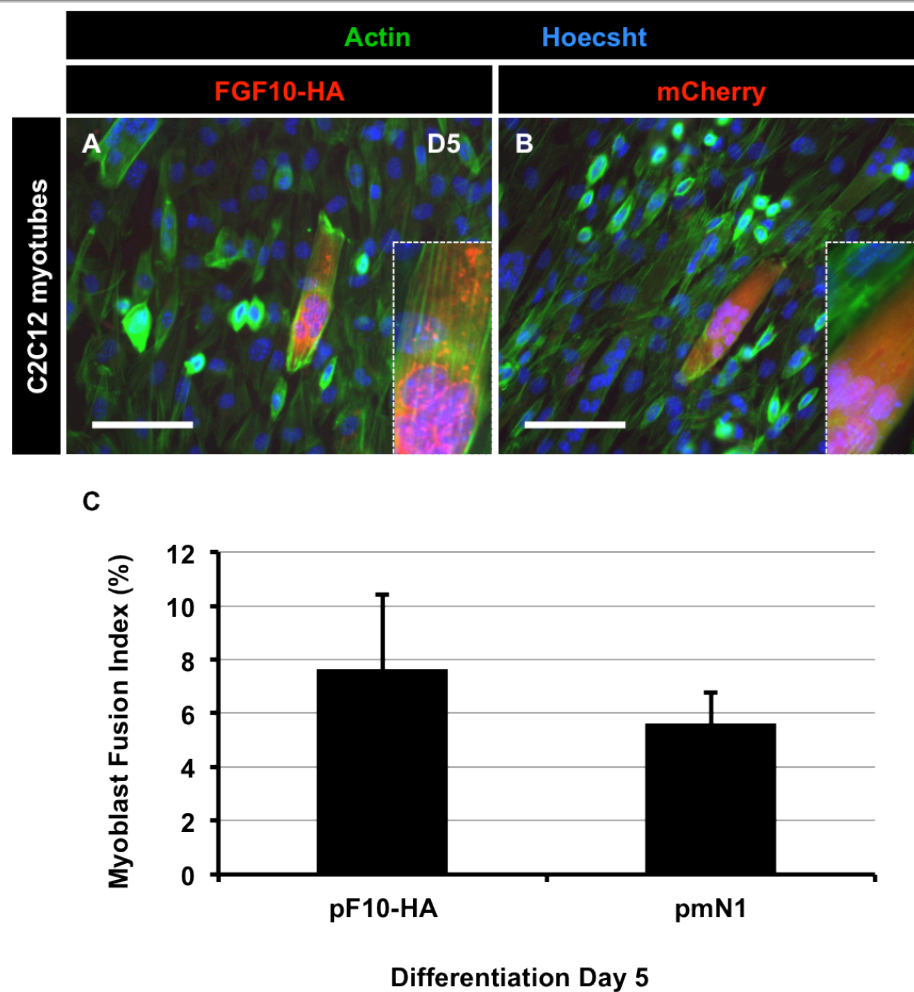


Figure 6.14: C2C12 myotube formation is not enhanced by *Fgf10* overexpression. (A) Expression of FGF10-HA is localized to specific structures within the myotube, unlike mCherry (B). Primary antibody: Ms- α -HA. Secondary antibody: Gt- α -Ms AlexaFluor568; Phalloidin-AlexaFluor388; Hoechst counterstain. Scale bars: 100 μ m. (C) There is no significant difference in myoblast fusion, as measured by the myoblast fusion index (MFI), between FGF10- or mCherry-expressing myotubes at Differentiation Day 5 (D5). Error bars represent standard error, n=3.

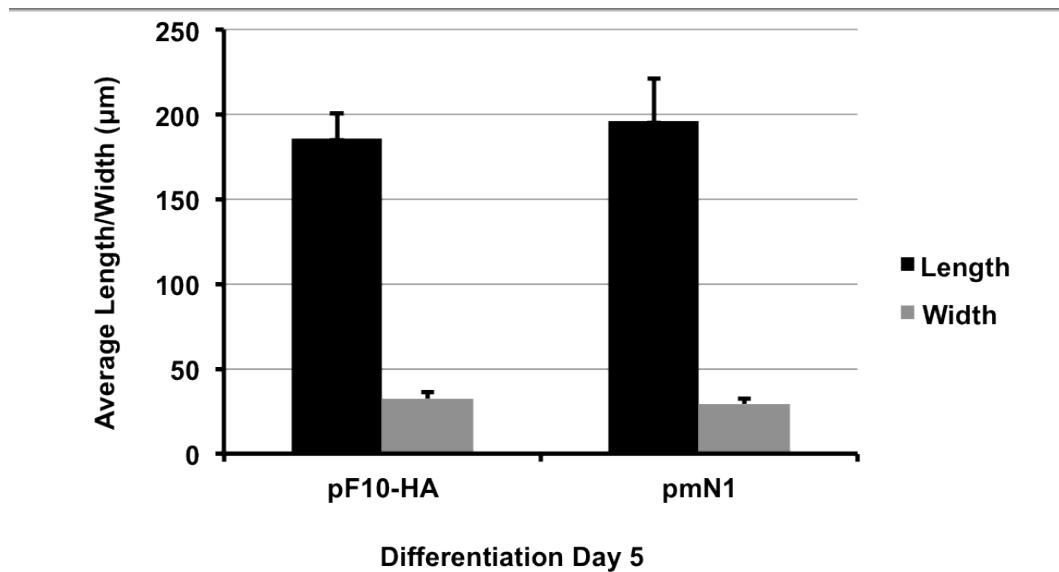


Figure 6.15: Myotube dimensions are not significantly altered by *Fgf10* overexpression. Measurements of the lengths and widths of myotubes expressing either *Fgf10* or mCherry showed no significant differences. Error bars represent standard error, n=3.

In order to examine myoblast fusion dynamics more closely, a differentiation assay was set up to measure the MFI at the early stages of differentiation. On Differentiation Days 1-4 (D1-4), the morphology of the differentiating myocytes was analysed to see if *Fgf10* overexpression accelerated this process and prompted early fusion. The MFI was compared for both pF10-HA and pmN1 populations on each day of the timecourse (Figure 6.16). On D1, no myotubes containing two or more nuclei were detected which expressed either *Fgf10* or mCherry. On D2, a very small number of nascent myotubes expressing *Fgf10* were detected (0.8%), containing no more than two nuclei. However, this increase was found to be insignificant. By D3, a sharp increase was seen in the numbers of *Fgf10*-expressing and control myotubes (6.7% and 6.0% respectively). This is to be expected since myoblast fusion typically occurs around Day 2 and Day 3 of C2C12 cell differentiation (Yamamoto et al., 2008). On D4, these numbers increased further still (13% and

12%). Although the MFI for *Fgf10*-expressing myotubes is slightly higher than for control myotubes on D3 and D4, this difference is not statistically significant.

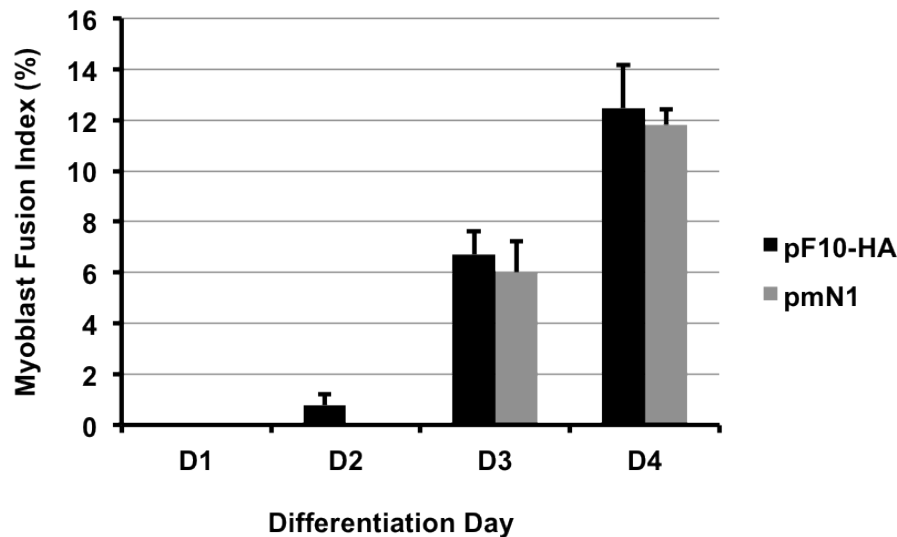


Figure 6.16: Myotube formation is not accelerated by *Fgf10* overexpression. Myotubes were not detected on D1. A small number of nascent *Fgf10*-overexpressing myotubes were detected (0.8%) on D2, but this was not significant. By D3, the number of myotubes expressing *Fgf10* (6.7%) and mCherry (6.0%) had increased, however the proportion of *Fgf10*-overexpressing myotubes was not significantly higher. At D4, the numbers of myotubes increased further still, to 13% and 12% respectively, but still this difference was not significant. Error bars represent standard error, n=3.

The dimensions of the differentiating myocytes were also measured to see if *Fgf10* overexpression was having an effect on their elongation, and therefore preparation for fusion. Interestingly, on D1 and D2, the average length of *Fgf10*-expressing myocytes was significantly shorter than control myocytes (p -value: 0.0024, 0.0041; Figure 6.17A). Similarly, they were also significantly narrower than control myocytes on these days (p -value: 0.0073, 0.0106; Figure 6.17B). By D3 and D4, there were no significant differences in length and width between *Fgf10*-expressing and

control myotubes. These data indicate that *Fgf10*-overexpressing myocytes are smaller overall than control myocytes prior to fusion on D3.

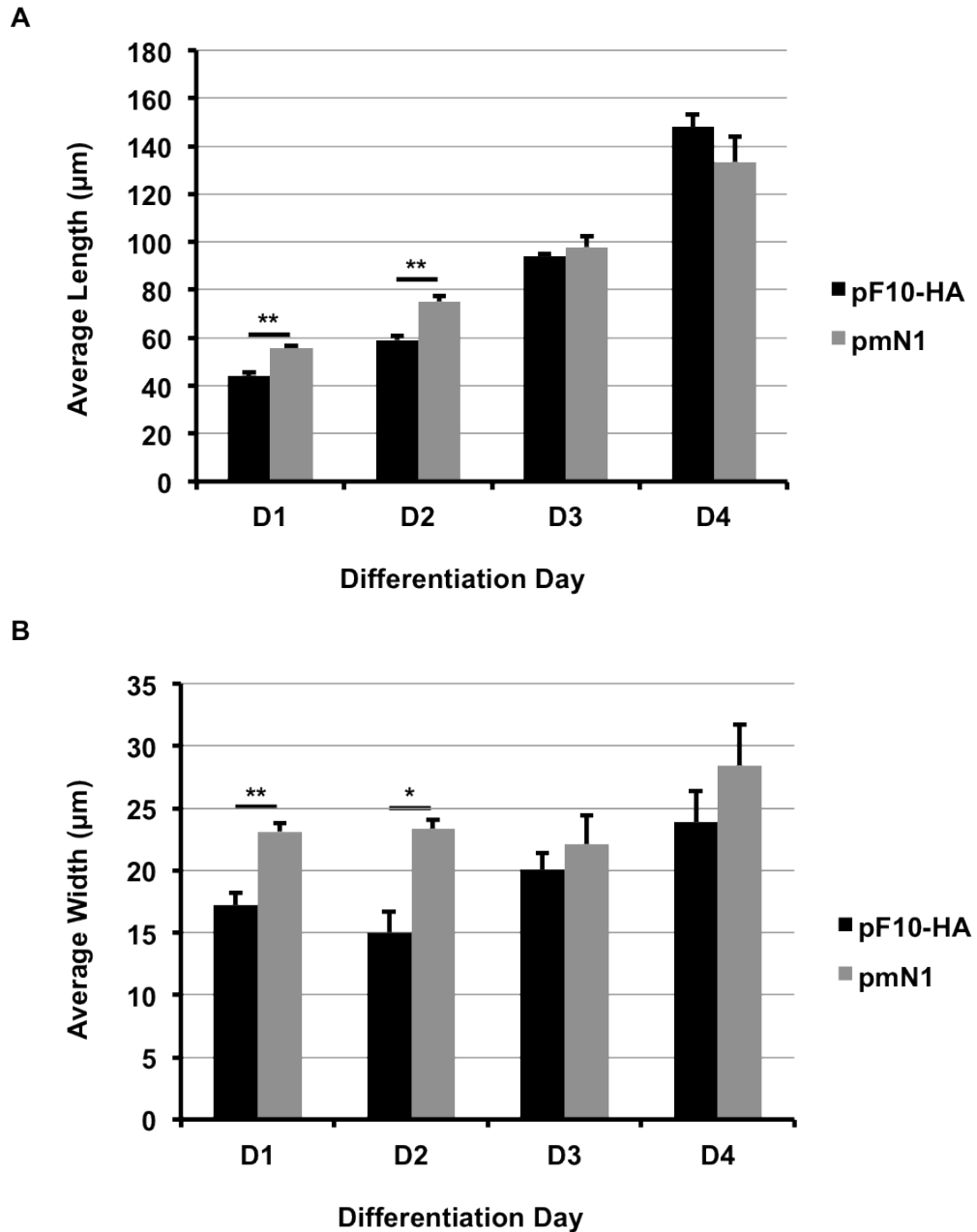


Figure 6.17: Average length and width of *Fgf10*-overexpressing myocytes is significantly lower than control myocytes on D1 and D2. *Fgf10*-overexpressing myocytes are smaller overall than control myocytes prior to fusion occurring. By D3 and D4, differences in length and width of *Fgf10*-overexpressing and control myotubes are no longer significant. Error bars represent standard error, $n=3$. * $p \leq 0.05$, ** $p \leq 0.01$.

6.2.6 A missense mutation in the FGF10 NLS prevents its translocation to the nucleus.

Fgf10 heterozygous mutations during development are believed to result in two disorders in humans. Aplasia of lacrimal and salivary glands (ALSG) and lacrimo-auriculo-dento-digital (LADD) syndrome are autosomal dominant disorders which share several phenotypic traits, the latter being the more severe condition of the two. These include underdeveloped lacrimal and salivary systems, digital and dental defects, and in some cases renal and respiratory abnormalities (Entesarian et al., 2005; Milunsky et al., 2006). The effects of these mutations have not yet been investigated. Aberrations in the *Fgf10* gene and abnormalities in the FGFR2 and FGFR3 sequences have all been linked to ALSG and LADD syndromes, leading to impaired or non-functional FGF10 signaling activity (Entesarian et al., 2007; Rohmann et al., 2006; Shams et al., 2007). However, one mutation, a glycine to glutamic acid substitution at position 138 (G138E) in human FGF10 sequence, was identified as a *de novo* missense mutation (not inherited) (Entesarian et al., 2007). This glycine residue is highly conserved and is located within the putative NLS motif identified by Kosman et al., 2007. It is possible that this missense mutation disrupts the nuclear function of FGF10.

To explore this further, site-directed mutagenesis was used to generate an *Fgf10* construct containing this glycine to glutamic acid mutation. Using the pGEM-T vector containing the rat *Fgf10* cDNA (Figure 2.3), specific primers were designed to incorporate the equivalent mutation at position 145 (G145E) on the rat *Fgf10* sequence. Briefly, the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) was used to amplify the entire vector sequence containing the incorporated G145E mutation. The methylated and hemi-methylated parental template DNA (pGEM-T with rat *Fgf10* cDNA sequence) was then digested with *Dpn I* restriction enzyme, leaving the mutated vector sequence behind to be transformed into XL-Gold ultracompetent cells.

Mutated DNA was then extracted by HiSpeed Midi Prep (Qiagen), and verified by Sanger sequencing. The mutated FGF10 construct also contained an HA-epitope tag as used in the pF10-HA vector, and was therefore detected by anti-HA immunolabeling. G145E vector was designed and generated by Marta Mikolajczak.

Since wildtype FGF10 appears to have a nuclear role in C2C12 cells, it was hypothesized that should the G145E mutation prevent its translocation to the nucleus, perhaps myoblast fusion is compromised. The G145E mutant construct was transfected into C2C12 myoblasts and expression was observed 24 hours later. Expression was also observed in myotubes at Differentiation Day 5 (D5). This missense mutation in the putative NLS motif did exclude FGF10 from the nuclei, rendering its expression cytoplasmic, as seen in both myoblasts (Figure 6.18A, B) and myotubes (Figure 6.18C, D). MFI was also measured at D5 for cells overexpressing G145E and the control pmN1 vector, and no significant difference was found in myoblast fusion (Figure 6.19A). Similarly the length and width of myotubes expressing G145E were compared to control myotubes and again, no significant difference was observed in their sizes at D5 (Figure 6.19B). However, this investigation was not as exhaustive as earlier experiments, and would therefore require a more extensive analysis.

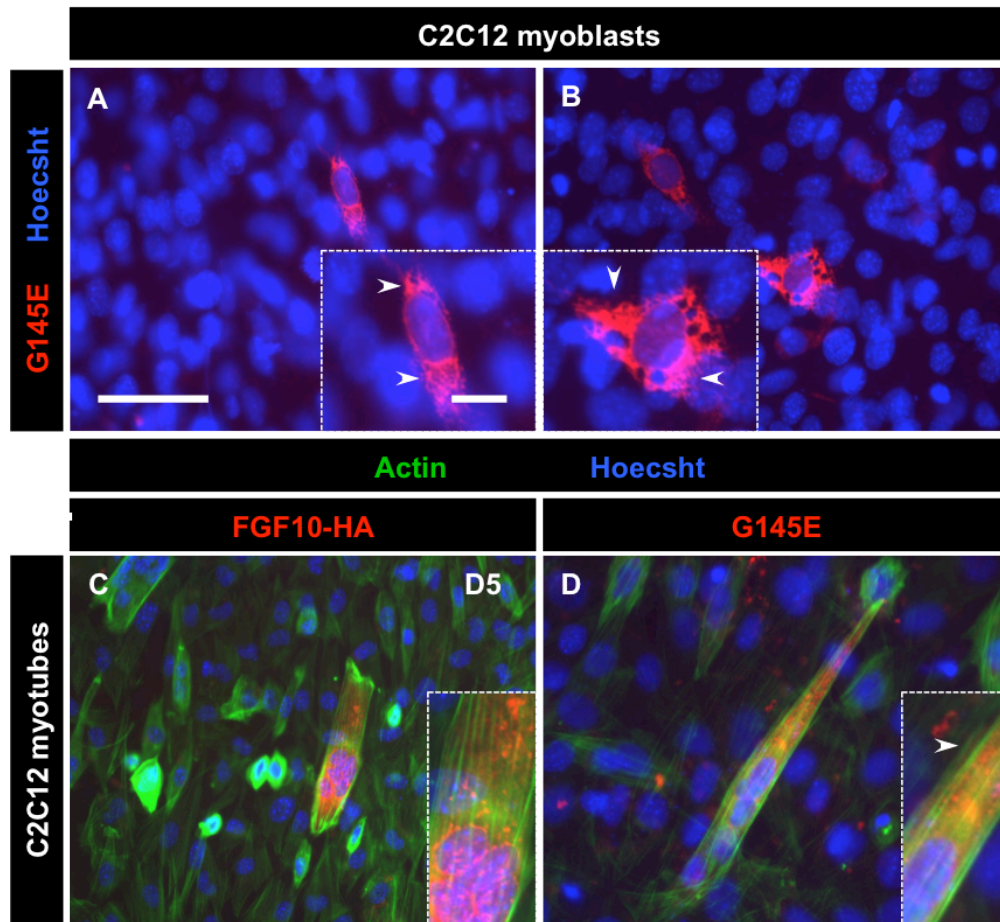


Figure 6.18: The human missense mutation G138E in a putative NLS motif of the FGF10 sequence, implicated in LADD syndrome, prevents FGF10 translocation to the nucleus. The highly conserved glycine residue was mutated to glutamic acid at position 145 in the rat Fgf10 sequence (G145E). This mutated construct also contained an HA epitope tag, detectable with antibodies. (A, B) G145E mutation confines FGF10 expression to the cytoplasm of C2C12 myoblasts (indicated by white arrowheads). (D) In mature myotubes, this exclusion from the nuclei is maintained, in contrast to wildtype FGF10 localization (C). Primary antibody: Ms- α -HA. Secondary antibody: Gt- α -Ms AlexaFluor568; Phalloidin-AlexaFluor388; Hoechst counterstain. Scale bars: 100 μ m (A); 20 μ m (A, inset).

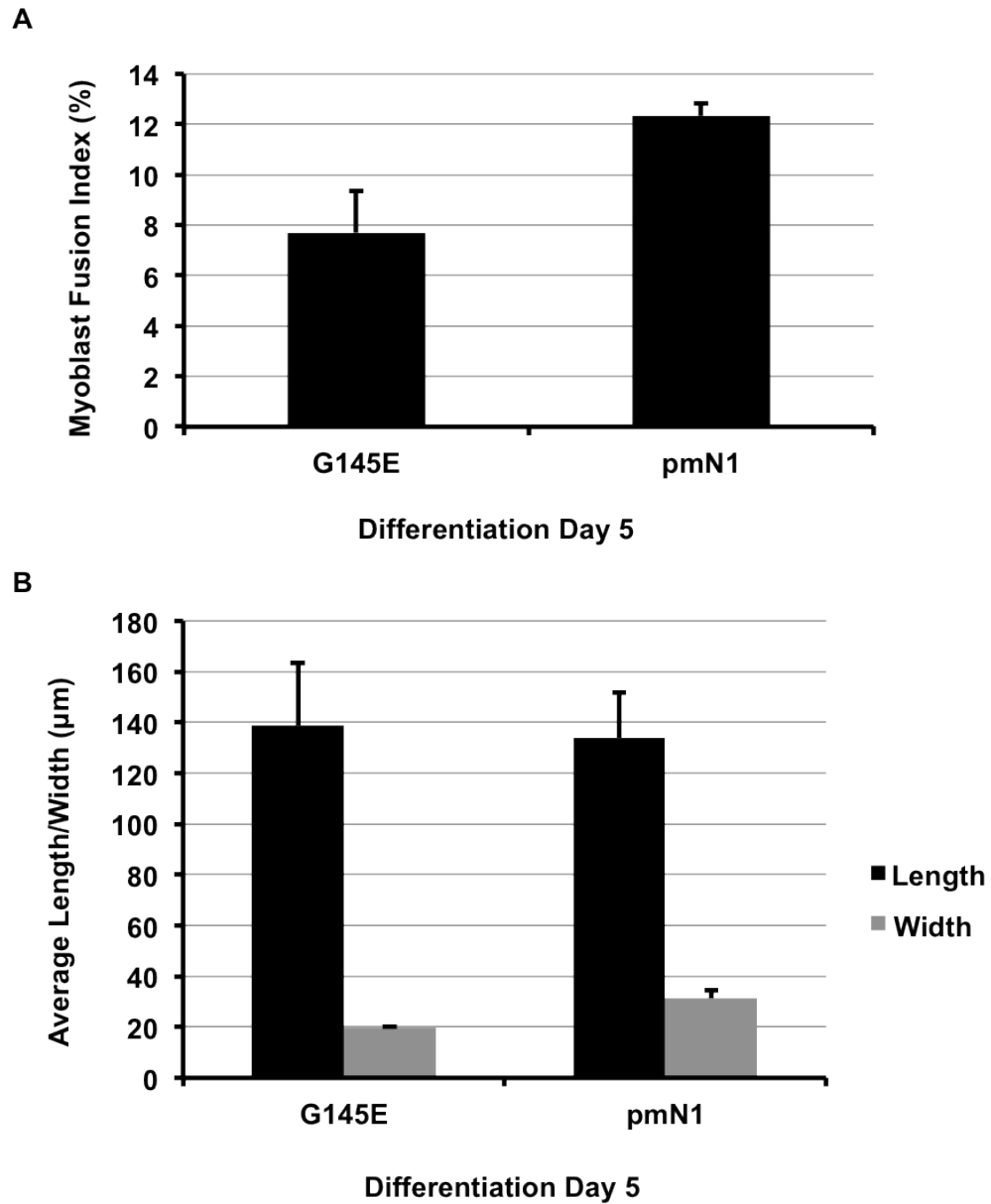


Figure 6.19: C2C12 myotube formation is not impaired with overexpression of mutated FGF10 G145E construct.

(A) Sequestration of FGF10 to the cytoplasm does not affect the MFI in comparison to control myotubes.

(B) Myotubes overexpressing G145E mutated Fgf10 construct show no significant differences in length or width compared to control myotubes. Error bars represent standard error, n=2.

6.3 Discussion

As a member of the fibroblast growth factor (FGF) family, *Fgf10* plays a crucial role in cell proliferation, differentiation, migration and survival (Berg et al., 2007; El Agha et al., 2014; Haan et al., 2013; Nyeng et al., 2008; Yokohama-Tamaki et al., 2006). In order to elucidate its function in skeletal muscle development, it was essential to first establish where it is expressed. Since commercial antibodies do not provide a reliable means of detecting *Fgf10*, significant progress has been made in the generation of two reporter lines: an *Fgf10*-LacZ reporter line and more recently, the *Fgf10*-CreERT2 line. However, the allelic modifications incurred in the generation of these lines reduce the levels of FGF10 produced, by 25% and 50% respectively (Hajihosseini. M.K., unpublished data) (El Agha et al., 2012; Kelly et al., 2001).

To further investigate the role of *Fgf10* in myogenesis, an *in vitro* model of myoblast differentiation was used. The immortalized C2C12 myoblast cell line permits the visualization of the differentiation of myoblasts into myocytes, and their subsequent elongation and fusion into differentiated myotubes upon serum starvation, over the course of 4-5 days (Yaffe and Saxel, 1977). *Fgf10* is endogenously expressed by C2C12 myoblasts, but it is significantly downregulated during differentiation, as found by quantitative RT-PCR (Figure 6.6). This would suggest its expression is not required for terminal differentiation. Interestingly, the receptor to which it binds with the highest affinity, FGFR2-IIIb, is absent in these cells. To investigate the effects of overexpressing *Fgf10* in C2C12 myoblasts, an overexpression vector was generated. This contained the full-length rat *Fgf10* cDNA sequence, without the stop codon, and an HA epitope tag fused at the C-terminus to facilitate detection with anti-HA antibodies. In this way, FGF10 localization could also be investigated in these cells. Visualization of FGF10-HA 24 hours after its transfection into myoblasts revealed that FGF10 localizes to the cytoplasm and the nucleus. In the nucleus, it has a punctate

expression pattern indicating that it potentially associates with the nucleoli. In differentiated myotubes, anti-HA immunolabeling revealed FGF10 is still present in the nucleus and can be seen at the nuclear membrane. Its secretion into the cytoplasm of the myotubes can also be seen, and in some cases it appears to shuttle between myotubes in close proximity to each other. This suggests a potential role in myocyte fusion to the maturing myotubes as they grow in size. Over the course of differentiation, myocytes overexpressing *Fgf10* do not form myotubes earlier than expected (around Day 3) but they are smaller in size suggesting their elongation in preparation for fusion is delayed. By the end of differentiation, *Fgf10*-overexpressing myotubes do not appear phenotypically different to control myotubes. Together, these findings suggest FGF10 is exerting its function in C2C12 cells via a receptor-independent mechanism, likely an intracellular one.

Most FGFs are small signaling molecules, which exert their function by binding with high affinity to one of the four known tyrosine kinase FGF receptors (FGFR1-4). Several paracrine FGFs have been found to have a dual role of signal transduction, as they are capable of reaching the cell cytosol and nucleus. To do this, exogenous FGF is first endocytosed, and then translocated into the cytosol before being imported into the nucleus. It is thought that vesicular transmembrane electrical potential is necessary for translocation, suggesting a membrane-derived component such as endosomes facilitates translocation of FGF (Malecki et al., 2004; Malecki et al., 2002).

FGF1, or acidic fibroblast growth factor (aFGF), is synthesized in the cytosol without a signal sequence for secretion. When exported, it forms a multiprotein complex including the S100A13 and p40 Syt1 proteins, which forms upon stress induction (Prudovsky et al., 2003). Translocation of FGF into the cell cytosol requires binding to FGFR, however it does not depend on tyrosine kinase receptor activity. For FGF1, this translocation is mediated by FGFR1 and FGFR4 and additional

intracellular proteins (Klingenberg et al., 2000; Sorensen et al., 2008; Wesche et al., 2006). FGF1 also contains two NLSs, a monopartite one at the N-terminus and a bipartite one at the C-terminus. For exogenous FGF1, nuclear import is dependent on both sequences. However, several conflicting reports exist surrounding the nuclear import of endogenously-synthesized FGF1 (Imamura et al., 1992; Wesche et al., 2005; Zhan et al., 1992). FGF1's export from the nucleus is mediated by phosphorylation by PKC δ and interaction with exportin 1 and the nuclear export sequence. Following this, it is rapidly degraded in the cytosol (Nilsen et al., 2007; Sletten et al., 2014; Wiedlocha et al., 2005). Most recently, it has been found that LRRC59 is critical to nuclear import of FGF1. Therefore, LRRC59 could facilitate an interaction between FGF1 and the nuclear import complex (Zhen et al., 2012).

FGF2 is also synthesized in the cytosol without a signal peptide. However, its secretion is constitutive and relies on heparan sulfate proteoglycans and phosphatidylinositol 4,5-bisphosphate on the cell surface (Nickel and Seedorf, 2008). FGF2 displays functional diversity because it is synthesized as multiple isoforms due to alternative usage of CUG translational start sites which sit upstream of the conventional methionine start codon (Prats et al., 1989). The 18kDa variant, designated the low molecular weight (LMW) isoform, is comprised of 155 amino acids and serves as the core sequence of the remaining, high molecular weight (HMW) isoforms. The isoforms have different subcellular locations (Arnaud et al., 1999; Bugler et al., 1991; Powell and Klagsbrun, 1991). LMW FGF2 is secreted, relying on the cytosolic protein Hsp90 for translocation across the endosomal membrane into the cytosol (Wesche et al., 2006). However, HMW FGF2 is sequestered in the nucleus and cytosol (Bugler et al., 1991; Renko et al., 1990). The HMW variants contain stretches of Glycine/Arginine (GR) repeats at the N-terminus that serve as an NLS (Dono et al., 1998; Quarto et al., 1991). Similarly LMW FGF-2 contains a bipartite NLS at the C-terminus, containing two arginine

residues, and this sequence is therefore common to the HMW FGF2 isoforms too (Claus et al., 2003). In the nucleus, LMW and HMW FGF2 are distributed differently. LMW FGF2 is found in the nucleoli and Cajal bodies in NIH3T3 or Schwann cells. HMW FGF2 however localizes at the periphery of the nucleoli and in the nucleoplasm (Arese et al., 1999; Claus et al., 2003).

Biosynthesis of FGF3 also involves use of CUG translational start sites to generate isoforms with extensions at the N-terminus, giving rise to multiple isoforms. A single CUG start site initiates FGF3 translation, and the isoforms are targeted to the nucleus and secretory pathway in equal measures. FGF3 contains three membrane-translocating sequences that facilitate its nuclear import. Two are situated at the C-terminus, and the N-terminus contains a bipartite NLS. Whilst the NLS is not essential to nuclear accumulation of FGF3, it is sufficient for recognition and binding by karyopherin- α 1 in the nuclear import machinery (Antoine et al., 1997; Kiefer et al., 1994). In the nucleus, FGF3 interacts with ribosomal protein S2 (rpS2), and the Nuclear FGF3-binding protein (NoBP). NoBP is essential to pre-rRNA processing and rpS2 forms a part of the ribosome small subunit. Taken together, these findings suggest nuclear FGF3 is a critical regulator of ribosomal biogenesis (Antoine et al., 2005; Reimers et al., 2001).

It is still unknown whether endogenous FGF10 can translocate into the nucleus, or what its nuclear function might be. When overexpressed in C2C12 cells, it can be seen in both the nucleus and the cytoplasm of myoblasts and myotubes, and it also appears to shuttle between fusing myotubes, suggesting it has a nuclear role in addition to a paracrine role. Studies *in vitro* have shown that multinucleated myotubes are formed in a specific order, starting with the differentiation of myoblasts into elongated myocytes. Small, nascent myotubes are formed originally from myocytes which have migrated, adhered and fused to one another. Nascent myotubes then grow in size and mature by fusing with other myotubes or

more myocytes (Abmayr and Pavlath, 2012). FGFs-1, -2 have largely been shown to promote myoblast proliferation and repress differentiation *in vitro*, whereas *in vivo* studies have revealed that FGF-4 promotes myogenic differentiation (Clegg et al., 1987; Lagha et al., 2008; Seed and Hauschka, 1988). During tongue myogenesis, FGF6 acts downstream of the TGF β /Smad4 signaling pathway to regulate myogenic differentiation and fusion (Han et al., 2012). In satellite cells, it has also been shown that *Sulf*-mediated repression of FGF signaling, mainly via FGFR4, disrupts their ability to differentiate and therefore form new myofibers (Langsdorf et al., 2007). Similarly when activity of the negative regulator of FGF signaling, *Sprouty2*, is impaired, myotubes fail to develop completely (de Alvaro et al., 2005). It is possible that FGF10 acts as a chemotactic signal which promotes myocyte differentiation and recruits myocytes or myotubes for fusion. In the nucleus, FGF10 could be directly targeting the networks controlling differentiation, or the synthesis of contractile proteins which are upregulated during differentiation. When a lacrimo-auriculo-dento-digital (LADD) syndrome-related missense mutation was introduced into the *Fgf10* sequence by site-directed mutagenesis, myotube formation was not compromised. However this is more than likely due to compensatory effects of endogenous FGF10.

To further the investigation into *Fgf10*'s function in myoblast differentiation, it would be interesting to carry out loss-of-function studies using siRNAs, or targeted genetic modifications with transcription activator-like effector nucleases (TALENs) (Gaj et al., 2013).

Chapter 7

Discussion

7.1 Discussion

Myogenesis, or the process of skeletal muscle formation, is regulated by cell intrinsic and extrinsic cues, which determine myogenic cell fate, and ensure 'proper' differentiation into functional muscles. Although significant progress has been made in elucidating the molecular basis for myogenesis, and the mechanisms underpinning muscle repair, the complexity of this process is far from being fully understood. Skeletal muscle function can be impaired by myopathic disease, and in the most severe cases can lead to irreversible loss of function. Such diseases include Duchenne muscular dystrophy, which has a considerably high prevalence worldwide and a somber prognosis, and as of yet no cure. Age-related loss of muscle mass and strength, as characterized by sarcopenia, is also more prevalent and having an impact on society. For this reason, it is imperative to further dissect the regulatory factors involved in myogenesis, and its dysregulation, so that effective treatment options can be devised (Romanick et al., 2013; Skuk, 2013).

In mammals, the fibroblast growth factor (FGF) family comprises 22 signaling molecules which are critical regulators of many developmental and homeostatic processes. *In vitro* studies revealed a potential role for FGFs in myoblast proliferation and differentiation, as both processes in cultured myoblasts appeared to be influenced by the presence of FGFs-1, 2, 4 and 5 (Clegg et al., 1987; Lathrop et al., 1985; Olwin and Rapraeger, 1992; Seed and Hauschka, 1988). Expression patterns for these FGFs, and additional family members, have also been characterized during skeletal muscle development *in vivo*. Transient expression of FGF-4, 5, 7 and 8 has been observed in the myotome (Fraidenraich et al., 2000; Huang et al., 2003; Mason et al., 1994). FGF-1, 2 and 5 have also been detected within differentiated myofibers (Eash et al., 2007; Hannon et al., 1996). FGFR1 and FGFR4 are the two main FGFRs expressed in adult skeletal muscle (Olwin et al., 1994b). Early studies to investigate the function of these factors and receptors found that mutations in these

genes resulted either in embryonic lethality, which prevented the function from being analyzed within muscle, or gave rise to no discernible phenotype in muscle development and maintenance (Feldman et al., 1995; Guo et al., 1996; Hebert et al., 1994; Yamaguchi and Rossant, 1995). More recently it has been shown that FGF-1 is upregulated during myoblast differentiation via a transcription-translation coupling mechanism; FGF-2 induces calcium signaling during satellite cell activation; and FGFs-4 and 8 regulate muscle-tendon interactions in the developing limb (Conte et al., 2009; Eloy-Trinquet et al., 2009; Liu and Schneider, 2014). In contrast to these factors, FGF-6 is considered the major ligand in skeletal muscle, as it is almost exclusively expressed in developing and adult skeletal muscle. This suggests it may have a role in the signaling networks regulating somite patterning, and also during regeneration in the adult (Grass et al., 1996; Zhao and Hoffman, 2004). *Fgf6* has been implicated in myoblast differentiation and fusion in the tongue via a Smad4-mediated TGF β signaling pathway. Myogenic differentiation and myoblast fusion were both impaired in *Smad4*-deficient myogenic cells, and this defect was partially rescued by exogenous FGF-6 (Han et al., 2012).

Fgf10 knockout mice lack lungs and limbs, and die at birth, thus elucidating the function of this gene in adult tissues has remained elusive (Min et al., 1998). A transgenic reporter line was developed which relied on insertion of a nuclear-targeted *lacZ* transgene upstream of *Fgf10* exon 1 (Kelly et al., 2001). *LacZ* expression, resulting in the production of β -galactosidase (β -gal) protein, can be detected by X-gal staining or anti- β -gal antibodies, and was found to faithfully recapitulate *Fgf10* expression (Kelly et al., 2001). Using this reporter line, we have determined a novel expression pattern for *Fgf10* in skeletal muscles during post-natal and adult development.

A striking pattern of β -gal expression, highlighted by X-gal staining, was seen in a specific subset of these muscles. Interestingly, this

expression pattern was consistently reproduced at different post-natal and adult ages. Intriguingly, within these muscles, the myofiber population was heterogeneous for *Fgf10* expression. Further to this, within the β -gal-expressing myofibers themselves, the intensity of β -gal expression in the myonuclei varied, which is potentially indicative of ongoing or a prior *Fgf10* expression. The results of the survey are summarized in Table 3.1 (Chapter 3). The *Fgf10*^{n_{lacZ}} reporter line has certain limitations in that the β -gal product is extremely stable. Therefore, although this line is beneficial for detecting *Fgf10*-expressing cells, the β -gal durability renders it unreliable when establishing timing of *Fgf10* expression. RNA *in situ* hybridization for *Fgf10* in muscle sections at P60 substantiated the patterns of β -gal expression observed, thereby confirming *Fgf10* is expressed in adult muscle.

Expression of β -gal in skeletal muscles at younger ages was also investigated. Crucially, the adult patterns of expression were already well established at 3 weeks of age, suggesting *Fgf10* is expressed early on. At embryonic day 12 (E12), β -gal^{+ve} co-localised with an embryonic myosin marker, which labels the early myofibers, in regions corresponding to the somite and intercostal muscles. β -gal^{+ve} cells were present in the developing forelimb, but did not yet co-localise with embryonic myosin. In the hindlimb, however, β -gal^{+ve} cells were not detected. At E17, limb muscles did not yet express *Fgf10*, however β -gal^{+ve} cells did appear to co-localise with the tendon marker Tenascin C in distal regions of the hindlimb. By P0, β -gal expression was significantly upregulated in some muscles (e.g. *teres major*), and looked to be increasing in the remaining muscles. Therefore, two populations of *Fgf10*-expressing cells could have dual roles in both embryonic and post-natal muscle development. Together, the localization and timing of *Fgf10* expression in skeletal muscle suggests putative functions for this gene in muscle fiber-type specification, hypertrophy, regeneration and tendon development, or a combination of these processes.

7.1.1 *Fgf10* could regulate fast fiber-type specification or maturation

Muscles such as the *digastricus* (jaw), *sternomastoideus* (neck), *acromiotrapezius* (shoulder), *teres major* (back), *spinalis thoracis*, and *longissimus thoracis* (thoracic wall) consistently contained high proportions of β -gal-expressing myofibers (Figure 7.1 – highlighted in red). Moderate proportions were seen in the *masseter* (jaw), *sternohyoideus* (neck), *pectoralis major* (upper thorax), *quadratus lumborum* (lumbar region), *biceps femoris* and *tibialis anterior* (hindlimb) (Figure 7.1 – highlighted in green). Finally, the *triceps brachii* and *flexor carpii ulnaris* (forelimb), *spinodeltoideus* (shoulder) and *psoas major* (lumbar region) contained very few β -gal-expressing myofibers. (Figure 7.1 – highlighted in orange). The intercostal muscles displayed a dorso-ventral gradient in *Fgf10* expression.

Given the consistent patterns of β -gal expression within the same subset of muscles at post-natal and adult ages, it is likely that common factors shared by these muscles underlie *Fgf10*'s expression. For example, do the muscles share a common embryological origin? Do they perform similar functions, and therefore share similar structural properties? *Fgf10* is expressed in both muscles of the head, which are derived from cranial mesoderm, and also in limb muscles, which are derived from presomitic mesoderm (Sambasivan et al., 2011a). Therefore, it is unlikely that embryological origin of these muscles is a major determining factor in *Fgf10* expression. The principal functions of the muscles are listed in Table 7.1. Muscles which contain a high proportion of β -gal-expressing myofibers (Figure 7.1 – in red) are largely involved in extension movements, whereas those which contain moderate or low proportions (Figure 7.1 – green and orange respectively) are predominantly involved in flexion movements. Collectively, β -gal-expressing muscles are also predominantly composed of fast-twitch (Type 2) myofibers, suggesting *Fgf10* may be involved in fast fiber specification.

Immunohistochemical analyses on the *tibialis anterior* revealed that *Fgf10*-expressing myonuclei do co-localise with fast fiber markers, and the majority are found within Type 2B fibers. Interestingly, when an *Fgf10* conditional knockout line was generated, wherein *Fgf10* expression was removed from *Pax3*-expressing myogenic progenitor cells by Cre/LoxP technology, the number of fast fibers in the *tibialis anterior* was significantly reduced, yet the number of slow fibers remained unchanged. However, the overall number of myofibers within this muscle had significantly increased, suggesting fewer fast fibers were detected owing to impaired maturation of the fast myosin isoforms, which could indicate a hypertrophic defect. This would suggest *Fgf10* regulates fast fiber maturation rather than specification.

Several factors are enriched in fast-twitch glycolytic fibers, which could help to elucidate why *Fgf10* is also expressed in a subset of fast fibers. These include Six1 and Eya1 proteins, which are found in the myonuclei of fast fibers (Grifone et al., 2004). During embryonic development it is thought that the *Six1/Six4* genes induce fast-type muscle specific genes in the myotome, thereby programming early myofibers for a fast phenotype (Niro et al., 2010). *Six1* has also been found to regulate *Fgf10* during development of auditory system, therefore expression of *Fgf10* in fast fibers may also be regulated by *Six1* (Zheng et al., 2003). Similarly insulin-like growth factor 1 (*Igf-1*) is also expressed by fast fibers (Song et al., 2005). IGF-1 is a major regulator of muscle growth, and stimulates the PI(3)K/Akt/mTOR pathway to facilitate hypertrophy post-natally (Rommel et al., 2001). Interactions between IGF-1 and FGFs have been reported previously in neuronal and bone cells, and it is thought that SRC homology 2 (SH2) domains in the receptors for both ligands could facilitate these interactions (Chen and Wang, 2014; Johnson-Farley et al., 2007; Liu et al., 2012). As *Fgf-10* and *Igf-1* are both upregulated post-natally in fast-twitch muscles, perhaps an interaction between the two is helping to mediate muscle hypertrophy.

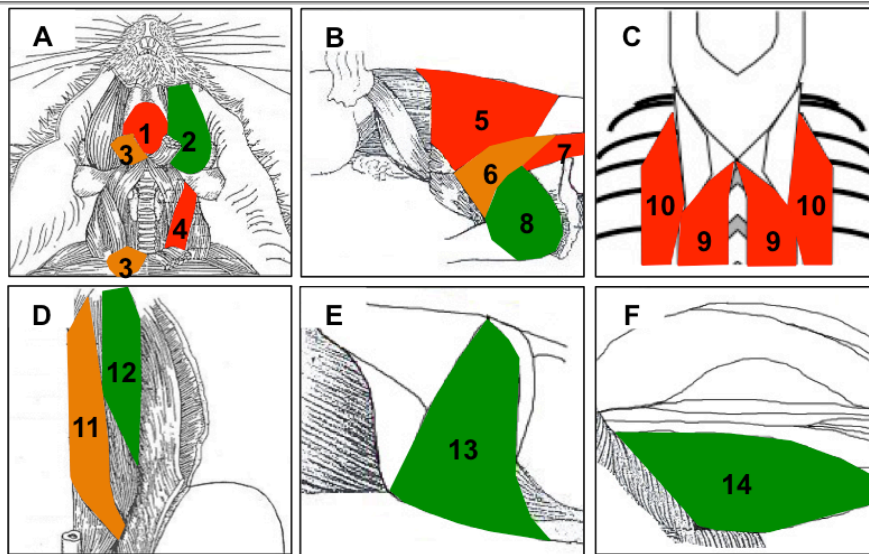


Table 7.1: Functions of *Fgf10*-expressing muscles.

No.	Location	Muscle	Function
1	Jaw and neck (A)	<i>Digastricus</i>	Jaw extensor
2		<i>Masseter</i>	Jaw flexor
3		<i>Sternohyoideus</i>	Jaw flexor
4		<i>Sternomastoideus</i>	Head rotator; neck flexor
5	Shoulder and forelimb (B)	<i>Acromiotrapezius</i>	Scapula rotator and flexor
6		<i>Spinodeltoideus</i>	Forelimb flexor and rotator
7		<i>Teres major</i>	Forelimb extensor
8		<i>Triceps brachii</i>	Lower forelimb extensor
9	Back (C, D)	<i>Spinalis thoracis</i>	Spine extensor
10		<i>Longissimus thoracis</i>	Spine extensor
11		<i>Psoas major</i>	Hip joint flexor
12		<i>Quadratus lumborum</i>	Spine flexor
13	Hindlimb (E, F)	<i>Biceps femoris</i>	Hindlimb flexor
14		<i>Tibialis anterior</i>	Ankle flexor

Figure 7.1: Regions of β -gal expression in postnatal and adult skeletal muscles. Muscles were colour-coded based on whether they contained high (red), moderate (green) or low (orange) numbers of β -gal expressing myofibers. Schematics adapted from (Greene, 1955).

7.1.2 *Fgf10* may have a role in muscle hypertrophy

As presented in Chapter 3, the levels of β -gal expression in certain muscles (*teres major*, *acromiotrapezius*, *intercostals*) were already high in newborn *Fgf10*^{nLacZ} pups. In other muscles which express *Fgf10* at later ages, such as the *biceps femoris* and *tibialis anterior* of the hindlimb, β -gal was expressed at reduced levels, suggesting *Fgf10* was in the process of being upregulated. At E17 however, *Fgf10* was not yet expressed by these hindlimb muscles. Together, these findings suggest that *Fgf10* expression in skeletal muscle is initiated towards the end of gestation, and is rapidly upregulated after birth. This time frame also corresponds to rapid changes in mouse development, notably a huge increase in muscle growth, primarily facilitated by hypertrophy, around the time of birth and throughout post-natal development (Russell, 2010). In the *Fgf10* cKO, the *tibialis anterior* revealed a potential hypertrophic defect. The average number of myofibers increased in this muscle but their diameter was reduced. These findings could implicate a switch from muscle hypertrophy to hyperplasia to facilitate growth, in the absence of *Fgf10*. Hypertrophy is characterized by enlargement of contractile elements and expansion of the extracellular matrix within the myofibers, whereas hyperplasia is an increase in the number of myofibers (Schoenfeld, 2010). The increase in myofibers observed in the *tibialis anterior* when *Fgf10* is removed could indicate hyperplasia as a compensatory mechanism for *Fgf10*-expressing muscles to achieve sufficient growth. Alternatively, both myofiber hypertrophy and hyperplasia may be acting to achieve this. In myostatin-deficient animals, skeletal muscle mass increases dramatically through a combination of myofiber hypertrophy and hyperplasia (Chisada et al., 2011). As myostatin normally acts as a negative regulator of muscle mass, perhaps removal of *Fgf10* expression leads to dysregulation of this critical gene.

The level of *Fgf10* expression in certain muscles at P0, more so than in others, likely reflects different functional requirements for these

muscles at this point in development. After birth, mice suckle milk from the mother during the first 3-4 weeks of life. Levels of β -gal were highest in the intercostal muscles, the *acromiotrapezius* and the *teres major*. The latter two muscles are involved in movement of the scapula and forelimb respectively (Iamsaard et al., 2012; Tosolini and Morris, 2012). It is possible that the levels of *Fgf10* expression in the intercostal muscles correspond to these muscles being used as the mouse breathes independently. Similarly, initial movements of the *acromiotrapezius* and *teres major* to enable suckling could potentially trigger *Fgf10* expression in these forelimb muscles. However, this theory does not support why *Fgf10* expression continues to persist at high levels in these muscles during later post-natal and adult life. Other important changes also occur during postnatal development, such as increased neuromuscular activity, maturation of excitation-contraction coupling and increased thyroid hormone levels, and these factors could all play a role in *Fgf10* upregulation after birth (Agbulut et al., 2003). However, these factors will affect all muscles, whereas *Fgf10* is only expressed in a subset. Compensatory hyperplasia has previously been shown to occur in muscles where the IGF1-Akt/PKB pathway is impaired, in order to facilitate muscle growth (Fernandez et al., 2002). Thus, it is possible that a perturbed interaction between FGF10 and IGF-1 could have resulted in hyperplasia in the *tibialis anterior* of the *Fgf10* cKO.

7.1.3 *Fgf10* may be involved in muscle regeneration

Although the number of *Fgf10*-expressing myonuclei in adult muscles far exceeds the number that would represent the satellite cell population, it is possible that a subset is satellite cells (Putman et al., 2001). Further still, the variations in β -gal intensity (strong vs faint) within the myonuclei could reflect *Fgf10*-expressing satellite cells and their descendants, respectively. In the adult *tibialis anterior*, 20% of *Pax7*-expressing satellite cells co-localised with anti- β -gal antibodies. In the

Fgf10 cKO, the number of satellite cells was reduced, but not significantly in the *tibialis anterior* (Chapter 5). This would suggest that *Fgf10* expression is not essential for satellite cell specification. Although β -gal expression is nuclear, FGF10 is a secreted protein and therefore could play a regulatory role in the satellite cell's immediate surroundings. The main structures of the niche are the basement membrane and the sarcolemma, and it confers protective properties on both the satellite cells by preventing their depletion, and also on the host myofiber during the rapid proliferation of the satellite cells (Scadden, 2006). A series of regulatory factors act on satellite cells within their niche to maintain their myogenic potential, which inevitably declines with age. These include TGF β , insulin-like growth factor 1 (IGF1), myostatin, Wnts and hepatocyte growth factor (HGF) (Bentzinger et al., 2010). Most recently, it was shown that in older muscle fibers the 'aged' niche expresses *Fgf-2*, causing satellite cells to emerge from quiescence but lose their self-renewal properties, thereby reducing their regenerative capacity. Disregulation of the negative modulator of FGF signaling, *Sprouty 1*, was believed to be responsible for this decline (Chakkalakal et al., 2012). It is possible that *Fgf10* expression within the satellite cells themselves plays an opposite role and acts to maintain quiescence. It would be interesting to see if the number of *Fgf10*-expressing *Pax7*⁺ satellite cells is altered after injury.

7.1.4 *Fgf10* could play a role in tendon development

At E17, β -gal-expressing cells were detected in the most distal regions of the developing hindlimb. Clusters of β -gal⁺ cells appeared to closely associate with developing tendons of the *tibialis posterior*, *flexor digitorum brevis* and *flexor hallucis*. Most, but not all, of the β -gal⁺ cells also expressed the tendon marker tenascin C, suggesting that these cell populations were differentiating into tendon (Chapter 3). Therefore, another hypothesis was that *Fgf10* could regulate tendon development. In the *Fgf10* cKO, myofiber organization in distal regions of the *tibialis*

posterior, *soleus*, *plantaris* and *flexor digitorum brevis* was severely compromised. The myofibers were hypercontracted, which suggested they had failed to attach at the tibia and fibula, indicating that their tendons were not functioning optimally. The *Fgf10* cKO did not present any abnormalities in skeletal structure itself. However, the tendons of the *tibialis posterior* and *flexor digitorum brevis* did appear significantly reduced in size and less well established in the *Fgf10* cKO, in comparison to the wildtype (Chapter 5).

The dynamics of tendon formation in the trunk and in the limb are inherently different. In the developing limb bud, *Scleraxis* (*Sclx*)-expressing tendon progenitor cells (TNPs) are mixed with the migrating myoblasts. Limb tendons themselves arise from the lateral plate mesoderm, from the endogenous progenitor population. At the axial level however, TNPs are compartmentalized in the syndetome of the developing somite (Schweitzer et al., 2010). FGF signaling is known to be one of the cues regulating early tendon development. In mice, FGF4 and FGF6 emanate from the center of the myotome and directly induce expression of the bHLH transcription factor *Sclx* to form the syndetome (Brent et al., 2003; Brent and Tabin, 2004; Brown et al., 2014; Edom-Vovard et al., 2002; Eloy-Trinquet et al., 2009; Schweitzer et al., 2001). TGF β signaling also robustly induces *Scx* expression in this domain (Pryce et al., 2009). FGF4, 8, and effectors of the signaling pathway *Pea3* and *Sprouty1, 4*, are also enriched at the muscle-tendon boundary during limb development (Eloy-Trinquet et al., 2009). It is possible that a proportion of the β -gal^{+ve} cells identified in the somitic area at E12 constituted tendon progenitors. At this developmental stage, tendon progenitors align between differentiating cartilage and muscle tissue, therefore *Fgf10* could play a role in regulating this alignment (Pryce et al., 2009). As of yet, no one has studied tendon development in mice bearing mutations which impede FGF signaling (Schweitzer et al., 2010). Whilst this largely reflects the redundancy in FGF ligands and their receptors,

perhaps findings from the *Fgf10* cKO line could be the first to indicate that targeted deletion of *Fgf10* has a specific impact on the other FGFs involved in tendon induction. This also begs the question, why are tendons of the *tibialis posterior* and *flexor digitorum brevis* specifically affected? These muscles share a common function in that they mediate plantar flexion of the ankle (Becerro de Bengoa Vallejo et al., 2008; Kohls-Gatzoulis et al., 2004). However, this function is shared by other muscles in the hindlimb, such as the *gastrocnemius*, which did not present a tendon defect. It is therefore likely that different tendon progenitors are heterogenous in their requirement for *Fgfs* expressed during patterning of the somite. Therefore, the effects seen on the morphology of these particular tendons potentially reflect a direct regulation by *Fgf10*.

7.2 Future Directions

A number of investigations could be carried out to further elucidate a role for *Fgf10* in skeletal muscle.

To further explore its role in fiber-type specification, it would be interesting to remove *Fgf10* expression from the *Sox6* cell lineage, which promotes fast fiber specification, to see if there is a switch to slow fibers in muscles which have been shown to express *Fgf10* (An et al., 2013). Similarly, there could be compensation by other fast fiber subtypes, such as Type 2X/D, which may be upregulated as *Fgf10* appears to be enriched in Type 2B myofibers. This investigation could be achieved by setting up a conditional knockout line, much like the *Fgf10* cKO line used in this study, which induces constitutive activation of Cre recombinase under the *Sox6* promoter in order to specifically inactivate *Fgf10* expression in this cell lineage. Furthermore, the use of an *Fgf10* inducible knockout line could be used to explore whether fiber-type specification is affected after removing *Fgf10* expression at post-natal ages. The *Fgf10*^{CreERT2::Fgf10}^{Flox} inducible knockout makes use of the fact that one *Fgf10* allele is knocked out by the presence of the *Cre* allele. The second *Fgf10* allele can then

be removed upon induction of *Cre*, usually via tamoxifen administration. Incorporating a Tomato reporter into this knockout would also enable *Fgf10*-deficient myofibers to be tracked (El Agha et al., 2012).

Muscle regeneration is characterized by three key phases: the inflammatory phase, satellite cell activation, proliferation and fusion, and finally maturation of new myofibers and muscle remodeling. Myofiber necrosis induces the inflammatory response, which is characterized by a two-wave influx of macrophages. The first wave phagocytoses damaged tissue, and the second wave release factors which promote myogenic precursor proliferation. Satellite cells then rapidly proliferate to give rise to new stem cells and committed myogenic precursors. These rapidly differentiate into new myofibers, which subsequently mature and grow (Ciciliot and Schiaffino, 2010). To further explore a role for *Fgf10* in muscle regeneration, a *Pax7^{CreERT2}::Fgf10^{flox/flox}* line could be generated which would enable the impact of removal of *Fgf10* expression in satellite cells to be explored in the adult (Lepper and Fan, 2012). As *Fgf10* was shown to be expressed by a subset of quiescent satellite cells (Chapter 5), it would also be fascinating to use a model of muscle injury (Souza and Gottfried, 2013). This would enable us to explore whether *Fgf10* is involved in satellite cell activation and proliferation during the regeneration process.

As *myogenin* is critical to myoblast fusion during myofiber formation, it may also be worth removing *Fgf10* expression from the *myogenin* cell lineage to see if *Fgf10* is a mediator of this fusion process (Londhe and Davie, 2011). This may also help to further elucidate its role in muscle hypertrophy.

Fgf10 also appears to be expressed by a subset of tendon progenitor cells (TNPs) around E17 (Chapter 3). It would be beneficial to further characterize *Fgf10* expression in TNP populations, potentially through the use of an *Fgf10^{CreERT2}::Scleraxis^{GFP}* transgenic line. *Scleraxis* is robustly expressed by TNPs and the use of the GFP reporter line has

been well characterized in elucidating the fate of these cells (Schweitzer et al., 2001). Together, this double transgenic line would enable *Fgf10* expression in TNP to be investigated during their proliferation and differentiation in the embryo.

Both *Fgf10* and its receptor, *FgfR2IIIb*, were shown to be expressed in adult muscle by RNA *in situ* hybridization. This suggests a paracrine signaling mechanism for FGF10 in muscle, and it may be interesting to explore whether the effectors (such as *Pea3*) or modulators (such as *Sprouty*) of the signaling pathway are also enriched within the myofibers themselves. The *FgfR2IIIb* knockout (*FgfR2IIIb*^{-/-}) mutation phenocopies the *Fgf10* knockout (De Moerloose et al., 2000). Therefore it would be worth investigating whether removing *FgfR2IIIb* expression from *Pax3*-expressing cells also gives rise to the same defects observed in the *Fgf10* cKO.

Finally, further *in vitro* studies could be carried out to elucidate potential binding partners for FGF10. In addition to the overexpression studies carried out in Chapter 6, which used an HA-epitope tagged FGF10 fusion construct to visualize FGF10 subcellular localization, it would also be beneficial to carry out siRNA-mediated knockdown of *Fgf10* in C2C12 myoblasts, in order to observe whether their differentiation is impaired (Dennstedt and Bryan, 2011). In culture, C2C12 myoblasts differentiate into myotubes which express fast myosin heavy chain isoforms (Cooper et al., 2004). Therefore, it may also be worthwhile to generate a stable *Fgf10* overexpression line, using primary mouse myoblasts as an alternative, to observe the effects of *Fgf10* overexpression on fiber-type differentiation. Further still, on the premise that *Fgf10* has an intracellular role, pull-down assays could be carried out to identify the proteins that FGF10 interacts with. In addition, FGF10 localizes to the cell nucleus, indicating a potential nuclear role (Chapter 6). Thus it could also be useful to carry out ChIP-Seq analyses in order to find out if FGF10 binds to any of the MRF genes, and therefore modulates the differentiation pathway.

7.3 Concluding Remarks

The investigations carried out in this study have begun to characterize the *Fgf10*-expressing lineage in skeletal muscle, suggesting putative functions in muscle fast fiber-type specification, regeneration, hypertrophy and, and also a second potential role in tendon development. The mechanisms regulating fiber-type specification are being elucidated. However, the complexity of these genetic networks are far from fully resolved. The need to elucidate these mechanisms has never been more pressing, particularly since myopathic conditions such as sarcopenia and Duchenne Muscular Dystrophy preferentially destroy fast-twitch fibers during the progression of the disease (Webster et al., 1988). *Fgf10* could play a role in the development and growth of fast fibers, both post-natally and in the adult. A greater understanding of its precise function in fast fibers could have a significant impact on the design of therapeutic treatments designed to combat the degeneration of these fibers.

List of Abbreviations

ALSG – aplasia of lacrimal and salivary glands
APC – adenomatous polyposis coli protein
ATP – adenosine triphosphate
bHLH – basic helix-loop-helix
BMP – bone morphogenetic protein
BrdU – 5'-bromo-2'-deoxyuridine
BSA – bovine serum albumin
c-Met – hepatocyte growth factor receptor
CD34 - Hematopoietic Progenitor Cell Antigen CD34
cDNA – complementary DNA
CK1 – casein kinase 1
CO₂ – carbon dioxide
COOH – carboxyl terminus
CRE – cre recombinase
CTGF – connective tissue growth factor
CXCR4 – C-X-C motif receptor 4
DAB – 3, 3' diaminobenzidine
DMD – Duchenne Muscular Dystrophy
DMEM – Dulbecco's Modified Eagle Medium
DMF – N, N-dimethylformamide
DML – dorsomedial lip
DNA – deoxyribonucleic acid
Dsh – dishevelled
E – embryonic day
ECM – extracellular matrix
EDTA - Ethylenediaminetetraacetic acid
EMT – epithelial-to-mesenchymal transition
EOM – extraocular muscle

ERK – extracellular signal-regulated kinase 1/2
ERT2 – oestrogen receptor t2
ES – embryonic stem
EtOH – ethanol
Eya – *eyes absent*
FAP – fibrocyte/adipocyte progenitor
FBS – fetal bovine serum
FF – fast-twitch fatigable
FGF – fibroblast growth factor
FGFR – fibroblast growth factor receptor
FR – fast-twitch fatigue-resistant
FRS2 – fibroblast growth factor receptor substrate 2
GFP – green fluorescent protein
GRB2 – Growth factor receptor-bound protein 2
GSK3 β – glycogen synthase kinase 3 beta
HBS - heparan sulfate proteoglycan binding site
HGF – hepatocyte growth factor
HH – hedgehog
HMC – hypaxial motor column
HS – horse serum
HSPG – heparan sulfate proteoglycan
Ig – immunoglobulin
IGF – insulin-like growth factor
IL4 – interleukin 4
IRES – internal ribosome entry site
K₃Fe(CN)₆ – potassium ferricyanide
K₄Fe(CN)₆ - potassium hexacyanoferrate(II) trihydrate
KO – knockout
L-Glut – l-glutamine
LADD – lacrimo-auriculo-dento-digital
Lef1 – lymphoid enhancer factor 1
LMC – lateral motor column

LRP4/5 – LDL related receptor 4/5
M-cadherin – cell adhesion molecule
MAPK – mitogen-activated protein kinase
mATPase – myofibrillar adenosine triphosphate
MDSC – muscle-derived stem cell
MEK 1/2 – mitogen activated protein kinase kinase
MeOH – methanol
Meox2 – mesenchyme homeobox 2
MgCl₂ - magnesium chloride
MKP3 – map kinase phosphatase 3
MMC – medial motor column
MN – motor neuron
MPC – myogenic progenitor cell
MRF – myogenic regulatory factor
MRF4 – myogenic factor 6 or herculin
MTJ – myotendinous junction
Myf5 – myogenic factor 5
MyHC – myosin heavy chain
MyLC – myosin light chain
MyoD – myoblast determination protein 1
NC – notochord
NCID – notch intracellular domain
NCS – newborn calf serum
NGS – normal goat serum
NMJ – neuromuscular junction
NP-40 - nonyl phenoxypolyethoxylethanol
OCT – optimum cutting temperature
ORF – open reading frame
P/S – penicillin streptomycin
Par – partitioning defective
Pax3 – paired box transcription factor 3
Pax7 - paired box transcription factor 7

PBS – phosphate buffered saline
PBST – phosphate buffered saline with Tween-20
PDGF – platelet-derived growth factor
PDK1 – phosphoinositide-dependent kinase-1
PDL – poly-D-lysine
PFA – paraformaldehyde
PI3K – phosphoinositide 3 kinase
PIC – PW1-expressing cell
PKC – protein kinase C
PLCY – phospholipase C gamma
PSM – presomitic mesoderm
RFP – red fluorescent protein
RIPA - radioimmunoprecipitation assay
RNA – ribonucleic acid
RT-PCR – reverse transcription polymerase chain reaction
S – slow-twitch fatigue resistant
SDF1 – stromal cell-derived factor 1
SDS – sodium dodecyl sulfate
SF – scatter factor
Shh – sonic hedgehog
SHP2 – Src homology region domain-containing phosphatase 2
SMAD – amalgam of ‘mothers against decapentaplegic’ (MAD) (*Drosophila*) and ‘small body size’ (SMA) (*C. elegans*)
SOS – Son of sevenless homolog
SP – side population
TACE - tumor necrosis factor- α -converting enzyme
TCF – t-cell factor
Tcf21 – transcription factor 21
TE – Tris-EDTA
TESPA - 3-Triethoxysilylpropylamine
TF – transcription factor
TGF β – transforming growth factor beta

Th1 – T-helper cell 1
TNF α – tumor necrosis factor alpha
TNPs – tendon progenitor cells
TX100 - polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether
UTR – untranslated region
VCAM – vascular cell adhesion molecule
VEGF – vascular endothelial growth factor
VLL – ventrolateral lip
WAT – white adipose tissue
WF – wavefront
Wnt – amalgam of ‘wingless’ (*Drosophila*) and ‘int-1’ (*mouse*)
X-gal - 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
YFP – yellow fluorescent protein
 β -cat – beta-catenin
 β -gal - beta galactosidase

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