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The Effect of Pax3 Over-expression on Myoblast Function

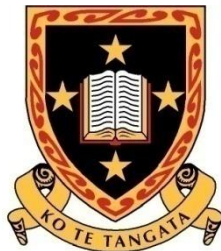
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Abstract

The paired-box transcription factor 3 (Pax3), is a powerful myogenic regulatory factor during embryo-myogenesis. The expression of Pax3 is involved in determining somitic cell fate, resulting in the formation of the first differentiated muscle, the myotome. In addition, Pax3 expression in embryonic hypaxial precursor cells prevents premature differentiation, whilst promoting cellular migration and thus ensuring the proper development of limb muscles. Subsequent muscle growth in the developing embryo is provided by myogenic precursor cells marked by the expression of Pax3 and its paralogue, Pax7. Following foetal development, these precursor cells establish the distinct lineage of myogenic stem cells referred to as satellite cells, responsible for post-natal development and regeneration. However, satellite cells are not the only population of cells that generate myogenic precursor cells in adult muscle for regeneration and growth. Recently, it has been shown that in adult muscle, Pax3 is expressed in a population of novel, interstitial cells, and is up-regulated upon muscle injury.

To determine the role played by Pax3 in post-natal muscle development and regeneration, three Pax3 over-expressing myoblast (C2C12) cell lines were generated and experimentally compared with control cells. Four facets of myoblast function were assessed: proliferation, differentiation, survival and migration.

The proliferation assay showed that Pax3 over-expression significantly ($P < 0.001$) slowed C2C12 myoblast proliferation, Pax3 over-expressing clones only reached 63% of the proliferative rate of control C2C12 cells. Furthermore, this study clearly showed that over-expression of Pax3 significantly ($P < 0.01$) delayed the differentiation of C2C12 myoblasts. Analysis showed that following 72 h of low serum induced differentiation 52% of the control C2C12 cell population had fused to form myofibres, in comparison only 16-32% of the myoblast population that over-expressed Pax3 had fused to form myofibres. Taken together, these data suggest that following satellite cell activation, Pax3 expression could be maintained in precursor cells to expand the myogenic population, as their myogenic commitment is delayed by Pax3 expression and this would facilitate increased cell cycling, albeit at a reduced rate. Furthermore, a low

percentage of fusion strongly indicates a higher proportion of cells that are renewing the quiescent satellite cell population.

Furthermore, over-expression of Pax3 also conferred a dose-dependent anti-apoptotic function to C2C12 myoblasts. Myoblasts that over-expressed a low level of Pax3 were more resistant to apoptosis in comparison to control C2C12 cells and to myoblasts that over-expressed Pax3 at higher level. The anti-apoptotic function conferred by Pax3 expression would come into effect within the Pax3⁺ self-renewing satellite cell lineage.

Finally, Pax3 over-expression was shown to increase the migratory ability of C2C12 myoblasts. In a chemotaxis assay, there was a strong indication that an intermediate over-expression of Pax3 increased myoblast sensitivity to chemotactic signals, priming cells for migration. The delay in differentiation also exhibited by Pax3 over-expression may function to allow satellite cell-derived progenitors to migrate to sites of injury, therefore enhancing skeletal muscle regenerative capacity.

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List of Abbreviations

Δ	Delta - change
3'	3 prime
5'	5 prime
α	alpha
ANOVA	analysis of variance
ATCC	American Type Culture Collection
β	beta
bHLH	basic helix-loop-helix
BLAST	Basic Local Alignment Search Tool
BMP	bone morphogenetic protein
bp	base pair(s)
BSA	bovine serum albumin
$^{\circ}\text{C}$	degrees Celsius
C2C12	Murine myoblast cell line
CDK	cyclin-dependent kinase
cDNA	complimentary DNA
CEE	chicken embryo extract
$c\lambda$	carrageen lambda
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dox	doxycycline
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
EBs	embryoid bodies
E	embryonic day
ES	embryonic stem cells
FBS	foetal bovine serum

FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
g	gravity
g	gram(s)
G0, 1	phase of the cell cycle
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
HLH	helix-loop-helix
hr	hour(s)
HRP	horseradish peroxidase
HS	horse serum
ICC	immunocytochemistry
IgG	immunoglobulin G
IGF	insulin-like growth factor
IPTG	isopropyl thio- β -galactoside
kDa	kilo Dalton(s)
L	litres
LacZ	β -galactosidase reporter gene
LMP	low melting point
M	molar, moles per litre
μ	micro (10^{-6})
μ l	microlitre
μ M	micromolar
mA	milliamp(s)
mM	millimolar
mdx	muscle dystrophy mouse phenotype
MyHC	myosin heavy chain
min	minute(s)
MOPS	3-(N-morpholine)-propane-sulfonic acid
MQ	milli Q
MRF	myogenic regulatory factor
mRNA	messenger RNA
MSTN	myostatin
n	nano (10^{-9})

NSS	normal sheep serum
NCID	Notch intracellular domain
OD	optical density
ORF	open reading frame
P	probability
PAGE	polyacrylamide gel electrophoresis
Pax	paired-box
PBS	phosphate buffer saline
PBS-T	phosphate buffer saline with Tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
PVP	polyvinylpyrrolidone
Q	glutamine
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
S	synthesis phase of the cell cycle
Sca-1	stem cell antigen-1
SDS	sodium dodecyl sulphate
s	second(s)
SEM	standard error of the mean
SF	ligand scatter factor
Smad	mammalian homolog to mothers against decapentaplegic
TA	<i>tibialis anterior</i>
TAE	tris acetate EDTA
<i>Taq</i>	DNA polymerase from <i>Thermus aquaticus</i>
TBS	tris buffered saline
TBS-T	TBS with Tween 20
TGF- β	transforming growth factor- β
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	units of enzyme
UTP	uridine triphosphate
UV	ultraviolet

X-gal

X-5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

Chapter One: Literature Review

1.0 Introduction

The following introduction contains a literature review on relevant past and current research on skeletal muscle. The paired-box transcription factor 3 (Pax3) is initially introduced and its involvement in embryonic myogenesis. Satellite cell function during post-natal growth and regeneration is then addressed; this includes a summary on skeletal muscle wasting and stem cell therapy. The aims and objectives of this study follows, stating what knowledge gaps exist and how this study seeks to fill some of those knowledge gaps.

1.1 Paired-box transcription factor 3

The Pax3 gene contains a 1437 bp cDNA open reading frame that is translated into a 479-amino acid protein (NM_008781), with a molecular weight of 56 kDa. Murine Pax3 expression is first detected at embryonic day (E) 8.5, corresponding to its importance in transcriptional regulation, which is fundamental in embryo patterning thus ensuring the correct morphological development of embryos (Goulding *et al.*, 1991). Pax3 belongs to the Pax protein family, which includes a number of transcription factors that are indispensable for correct spatiotemporal gene expression during mammalian embryology (Stuart *et al.*, 1994). Each Pax family member contains a 128-amino acid paired-box that binds as a monomer to a consensus of Pax binding sequences (Treisman *et al.*, 1991). Pax3 and a few other Pax proteins, including Pax7, also contain a 60-amino acid paired-type homeodomain; this binds as a dimer to specific recognition motifs that contain TAAT palindrome sequences. The initial binding of one homeodomain was found to synergistically increase the affinity of a second homeodomain binding by 300-fold (Wilson *et al.*, 1993). Pax3 also contains an additional octapeptide sequence, which is conserved but has an unknown function (Stuart *et al.*, 1994). The Pax3 protein can be functionally divided, whereby the N-terminal region is responsible for inhibiting transcription, whereas the C-terminus binds to basal promoters for transcriptional activation: this was found to be dosage

dependent (Chalepakis *et al.*, 1994). It is possible that Pax3 deals with dosage dependent gene expression by altering its binding characteristics via alternative splicing. Two isoforms of Pax3, termed Q⁻ and Q⁺, were found to be naturally transcribed. A cotransfection assay containing a reporter plasmid demonstrated that the isoforms had differential binding to specific DNA sequences that required the use of both sub-domains. The difference between the two isoforms is the presence (Q⁺) or absence (Q⁻) of a single glutamine residue within the linker region that joins the two sub-domains of the Pax3 protein (Vogan *et al.*, 1996).

As the effect of Pax3 expression is dosage dependent, alterations in its spatiotemporal expression can result in abnormalities (Chalepakis *et al.*, 1994). There are a number of characterised mutations in Pax3, caused by point mutations, splice-site mutations, and chromosomal deletion that causes Waardenburg syndrome in humans. There are similar mutations seen in mouse Pax3, resulting in *Spotch* mouse featuring pigmentation disturbances like those witnessed in Waardenburg patients. The case of both conditions is attributed to the mutated gene affecting the migration of cells from the neural crest and thus affecting somitogenesis (Tassabehji *et al.*, 1994). There are a number of conditions implicated with Waardenburg syndrome including deafness, with the severity of the condition depending on the severity of the mutation affecting Pax3 expression. There is also a disorder that affects musculoskeletal development, known as Klein-Waardenburg syndrome; patients share many of the same characteristics as Waardenburg patients (Hoth *et al.*, 1993; Underhill, 2000).

Pax3 was also found to be involved in myogenic tumours known as Rhabdomyosarcomas (RMS), a common malignancy in children (Kurmasheva *et al.*, 2005). The majority of Alveolar Rhabdomyosarcomas (ARMS) are caused by a mutation that fuses the Pax3 allele to the forkhead gene (Pax3-FKHR), which has up to 100 times the transcriptional capacity of the wild-type Pax3 gene (Bennicelli *et al.*, 1995; Bennicelli *et al.*, 1996). These tumours are established as they are prevented from committing to the myogenic lineage and thus remain in the cell cycle as a proliferative population of myogenic progenitors (Kurmasheva *et al.*, 2005), marked with the expression of MyoD (Dias *et al.*, 1990). Pax3 expression also offers apoptotic resistance to these tumour cells via its interaction with the anti-apoptotic protein BCL-XL (Margue *et al.*, 2000). It is thought that the origin of ARMS is mesenchyme stem cells that have failed to commit to a

lineage, which matches evidence of ARMS frequency of occurrence in areas scarce of skeletal muscle (Anderson *et al.*, 1999). In corroboration of this hypothesis, it has also been shown that Pax3 over-expression can direct the differentiation of multipotent mesenchyme stem cells into a myogenic lineage (Gang *et al.*, 2008).

Pax3 misexpression is also involved in embryonic Rhabdomyosarcomas (ERMS), however in this case hypermethylation of CpG islands upstream of Pax3 is considered to be the cause (Kurmasheva *et al.*, 2005). Hypermethylation is linked with the suppression of gene expression, which allows the outside environment (epigenetic effects) to regulate development. This regulation occurs during normal muscle development where Pax3 expression and thus MyoD activation and myogenic initiation are inversely correlated to upstream CpG island methylation. Analysed ERMS samples showed a mean of 52% methylation in upstream Pax3 targets in comparison to normal muscle samples which only had a mean of 18% methylation (Kurmasheva *et al.*, 2005). This demonstrates that ERMS cells through CpG methylation can cause the deregulation of the Pax3 myogenic program thus resulting in tumour formation. The lower level of Pax3 expression in ERMS can result in inadequate activation of downstream targets affecting terminal differentiation and promoting sustained cell cycling (Relaix *et al.*, 2004). The two tumour sub-types that are caused by the misregulation of Pax3 expression do so by two different methods, over-expression and under-expression, demonstrating the importance of Pax3 regulation and its level of expression (Kurmasheva *et al.*, 2005).

1.2 Embryonic myogenesis

Myogenesis, the formation of skeletal muscle, is a highly complex and regulated process that requires the timely expression of a cascade of regulatory transcription factors that direct myogenic cell fate. The paired-box transcription factor Pax3, is one of the main upstream regulators of this myogenic cascade during primary myogenesis (Maroto *et al.*, 1997). Activation of myogenesis achieved by the expression of the transcriptional networks results in the expression of the basic helix-loop-helix (bHLH) domain-containing myogenic regulatory factors (MRFs). These factors, which include myogenic factor 5

(Myf5), myogenic differentiation 1 (MyoD), muscle regulatory factor 4 (MRF4) and myogenin, are critical for myogenic progression (Pownall *et al.*, 2002; Buckingham, 2006).

1.2.1 Primary myogenesis

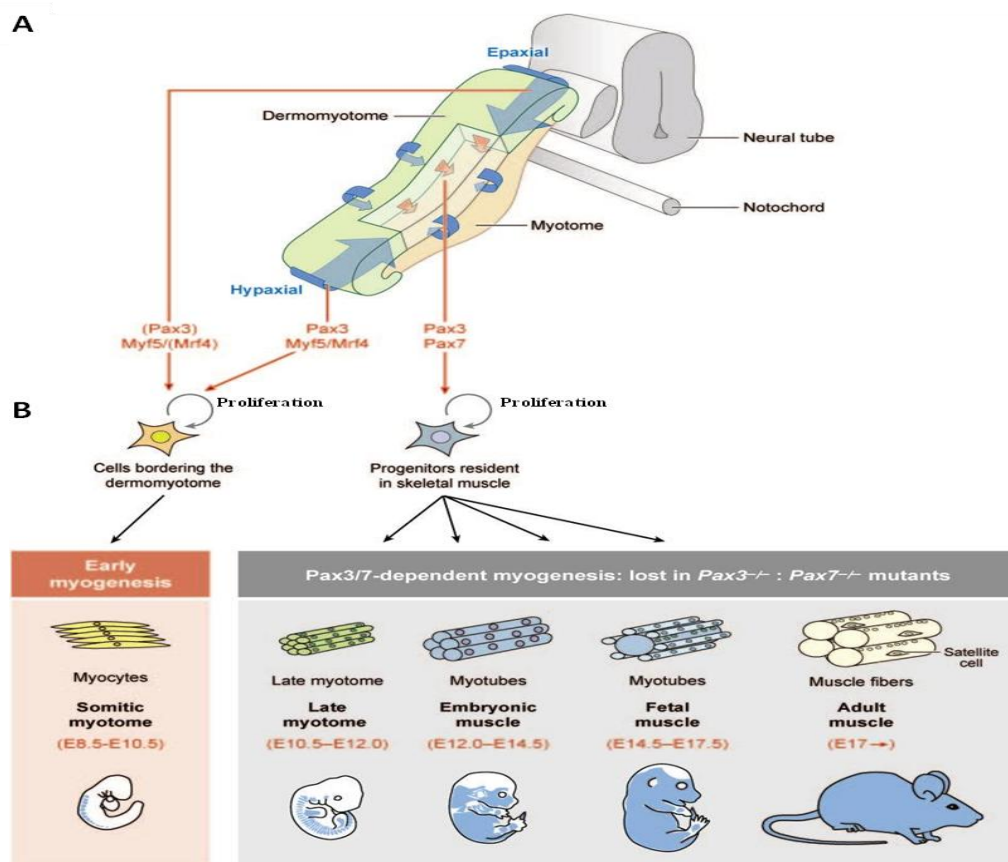
There are three main phases involved in myogenesis; the primary phase is the formation of the first differentiated muscle, the myotome, which occurs during somitogenesis. Somites form in successive pairs from segments of paraxial mesoderm following an anterior-posterior progression on either side of the neural tube and notochord (Christ & Ordahl, 1995). This is followed by the somites differentiating into ventral and dorsal sections, known as the sclerotome and dermomyotome respectively (Cossu *et al.*, 1996b). Initially somites are spheres of epithelial cells that will express paired-box transcription factors Pax3 and Pax7; however as somites mature Pax3 expression is modulated (Williams & Ordahl, 1994). This results in the somites disaggregating into a ventral mesenchyme, which gives rise to cartilage for the vertebrae column and ribs (Brand-Saberi & Christ, 1999). The dermomyotome, which gives rise to the formation of body and limb skeletal muscle, will continue to express Pax3 and retain its epithelial structure (Williams & Ordahl, 1994).

Pax3-expressing myogenic progenitor cells at this stage are now restricted to the epithelium of the dermomyotome, myogenesis is initiated with these cells expressing Myf5 and MRF4, resulting in cellular delamination and myotome formation under the dermomyotome. The myotome does not form if these specific MRFs are not expressed (Gros *et al.*, 2004; Kalcheim & Ben-Yair, 2005), as these factors are necessary to specify myogenic commitment of the myogenic progenitor cells (Kassar-Duchossoy *et al.*, 2004). Pax3 is detected in the forming myotome during E8.5-E10.5; the number of Pax3-expressing cells increases as the epithelial structure of the dermomyotome is lost. These cells also come to express Pax7, with 87% of myotomal cells becoming Pax3⁺/Pax7⁺, although interestingly co-expression of other myogenic determination genes in this population of cells was not detected (Relaix *et al.*, 2005). The remaining approximately 10% of myotomal cells that only express Pax3 activate the myogenic program via Myf5 expression; consequently these cells are responsible for the myogenic

differentiation of the myotome (Relaix *et al.*, 2005). Lineage-ablation studies have shown that myogenic specification via Myf5 expression during myotome formation can be compensated for by the expression of MyoD. These studies concluded that the absence of Myf5-expressing progenitors will result in the proliferative expansion of a compensatory Myf5-independent, MyoD-expressing myoblast lineage. The presence of two different redundantly acting myogenic lineages ensures the proper formation of the myotome (Gensch *et al.*, 2008; Haldar *et al.*, 2008). Downstream of Myf5 and MyoD is myogenin, which is responsible for the terminal differentiation of committed myoblasts. Myogenin-null mice, although producing the usual myoblast population, lack terminal differentiation, resulting in perinatal death (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). Thus, the correct spatiotemporal expression of the MRFs during myotome formation results in committed, terminally differentiated myoblasts fusing to form multinucleated primary myotubes; this is known as primary myogenesis. This initial muscle structure grows through secondary myogenesis, where the existing myotubes act as a template for secondary myoblasts to fuse with. The resulting secondary myotubes subsequently fuse to form myofibres and thus produce the functional muscle (Gullberg *et al.*, 1998). The formation of the myotome during murine embryonic development is represented in Figure 1.1.

As the myotome forms, Pax3 expression is further adjusted as the dermomyotome/myotome delineates into epaxial and hypaxial domains (Goulding *et al.*, 1994). This modulation of Pax3 expression is caused by up-regulation of miR-27, which interferes with Pax3 expression and allows for myogenic differentiation of progenitor cells (Crist *et al.*, 2009). The epaxial domain gives rise to back musculature, while the hypaxial domain gives rise to the muscle of the rest of the body and limbs (Brand-Saberi *et al.*, 1996). Interestingly, smooth muscle and skeletal muscle arise from the same clonal origin, that is, the Pax3 progenitors present in the central epithelium of the dermomyotome (Esner *et al.*, 2006). During delineation the signalling factor N-cadherin controls Pax3 progenitor cell fate; initially it is expressed in both cell types, progenitors of smooth and skeletal muscle, to conserve the integrity of the epithelium. However, as the structure delineates and the epithelium breaks down, N-cadherin is only expressed in apical cells entering the myotome, thus promoting a terminally differentiated myogenic program (Cinnamon *et al.*, 2006). Within the epaxial

domain, Pax3 expression is down-regulated as the transcriptional activators of MyoD are turned on for myogenic differentiation. However, Pax3 expression is continued in the hypaxial domain as MyoD is repressed, allowing for limb muscle precursor cell migration. This was observed in studies performed on homozygous *Spotch* mutant mice, which fail to develop limb muscles as the Pax3 protein is not synthesised (Bober *et al.*, 1994; Goulding *et al.*, 1994; Williams & Ordahl, 1994), which also results in increased apoptosis of hypaxial progenitor cells (Dickman *et al.*, 1999).



A Buckingham M, Relaix F. 2007. *Annu. Rev. Cell Dev. Biol.* 23:645-73

Figure 1.1 Myotome formation during early embryo-myogenesis.

(A) Pax3-positive myogenic progenitor cells delaminating from the epithelial dermomyotome (green) initiate myogenesis, developing the underlying myotome (blue arrows). As the epithelial structure of the dermomyotome is lost, a second wave of progenitor cells, positive for both Pax3 and Pax7 (red arrows), enter the myotome. (B) Schematic representation of the muscle derivatives that result from each myogenic progenitor population and the time during murine embryonic development when each event occurs.

1.2.2 Limb myogenesis

Myogenic progenitor cells of the limb arise from the limb bud level of the hypaxial domain. They migrate to dorsal and ventral positions where the muscle masses will form, which is highly regulated by a number of homeobox genes (Christ & Ordahl, 1995; Tajbakhsh & Buckingham, 2000). Similar to the Pax3-null condition, embryos null for the tyrosine kinase receptor c-met and its ligand scatter factor/hepatocyte growth factor (SF/HGF) result in cell migration defects producing similar muscle phenotypes (Yang *et al.*, 1996). The combined expression of c-met and SF/HGF is required for dermomyotome cellular delamination as well as having implications in guiding migration (Birchmeier & Brohmann, 2000), with HGF expression possibly being involved in lamellipodia formation (Salerno *et al.*, 2009). Pax3 regulates the expression of these genes, with their expression being severely compromised in the absence of Pax3 (Epstein *et al.*, 1996; Birchmeier & Brohmann, 2000). An additional factor, Lbx1 is expressed in limb muscle progenitor cells prior to and during migration, playing a major role in ensuring the correct long-range migration of cells (Buckingham, 2001). This was confirmed by Lbx1-null mice embryos, which exhibited forelimb muscle abnormalities and a lack of hindlimb muscle, with no evidence to indicate premature differentiation or inefficient delamination (Brohmann *et al.*, 2000; Gross *et al.*, 2000).

Once the progenitor cells reach their target sites, the myogenic process is coordinated by several MRFs. Initially, the myogenic determination gene Myf5 is expressed, which in the hypaxial somite and limb buds has been shown to be directly activated by Pax3 (Bajard *et al.*, 2006). The Pax3/Myf5 interplay causes the precursor cells to form into myoblasts and undergo extensive proliferation before the skeletal muscle forms (Delfini *et al.*, 2000). Proliferating cells were not seen to express MyoD, and MyoD was not activated until post-mitotic cells activated the differentiation program, suggesting that Pax3 and Myf5 are genetically upstream of MyoD (Tajbakhsh *et al.*, 1997; Bajard *et al.*, 2006). Differentiation can be delayed by the expression of the homeo-box factor Msx1, which promotes proliferation by keeping myoblasts dividing (Houzelstein *et al.*, 1999). Following proliferation, the myoblasts differentiate in response to the

expression of myogenic differentiation factors, including myogenin and Mef2 (Buckingham *et al.*, 2003).

1.2.3 Embryonic myogenic signalling pathways

As mentioned previously, Pax3 is one of the main upstream genes that regulate lineage specification during primary myogenesis, being globally expressed prior to myogenic commitment (Williams & Ordahl, 1994). Protein activity from members of the family sine oculis homeobox (SIX) and eyes absent homologue (EYA) are responsible for regulating Pax3 expression, as well as independently binding to MRF regulatory sequences. Expression of SIX1 and SIX4, in conjunction with its cofactors EYA1 and EYA2, induces Pax3 expression and can bind in parallel to the hypaxial enhancer element to initiate Myf5 and Mrf4 expression (Grifone *et al.*, 2007). Whereas, in the epaxial domain, the SIX protein family controls MRF4 expression independently in addition to enhancing MyoD and myogenin expression, thus promoting terminal differentiation (Grifone *et al.*, 2005).

The development of the dermomyotome and the specification of myogenic precursor cells are also influenced by signalling molecules produced by tissues neighbouring the paraxial mesoderm (Cossu *et al.*, 1996a; Brand-Saberi, 2005). Epaxial muscle specification requires activating cues from axial structures such as the neural tube and notochord. For example, it was found that the neural tube acts as a positive activator of myogenesis through the Myf-5-dependent pathway (Cossu *et al.*, 1996a). When myogenesis has been initiated the notochord secretes the signalling molecule Sonic hedgehog (SHH), which maintains Myf5 expression by binding to the early epaxial enhancer, a Myf5 regulatory sequence (Gustafsson *et al.*, 2002). The neural tube and surface ectoderm are also responsible for Wnt signalling; a complex network of proteins involved in regulating embryonic development, producing Wnt1 and Wnt7a respectively, which preferentially induce myogenesis via Myf5 and MyoD, respectively, through the β -catenin pathway (Tajbakhsh *et al.*, 1998). In the epaxial domain in the absence of Myf5 and MRF4, MyoD is still expressed, as shown by Myf5/MRF4 double-mutant studies (Tajbakhsh *et al.*, 1997). However, if protein kinase C (PKC) inhibitor is present, MyoD expression is blocked, indicating that Wnt signalling is dependent

on the PKC-pathway. When a more constitutively active form of Pax3 was expressed, the Pax3-FKHR gene, MyoD expression was not affected, suggesting that Wnt signalling acts on Pax3 induction (Brunelli *et al.*, 2007). The myogenic induction pathways mentioned above are illustrated in Figure 1.2.

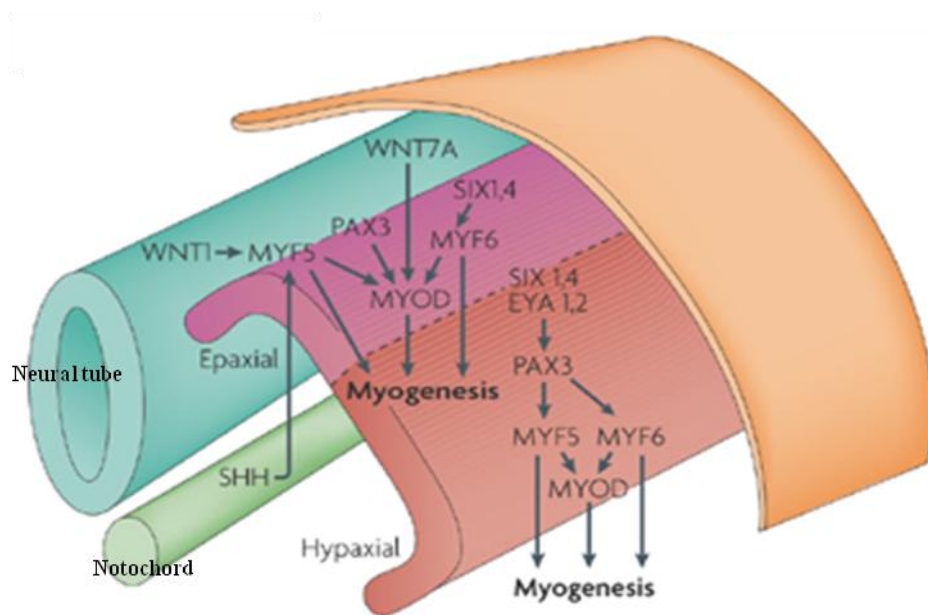


Figure 1.2 *Murine embryonic myogenic induction signalling pathways.*

In the epaxial domain myogenesis can be activated via numerous pathways, WNT1 released from the dorsal neural tube activates Myf5 expression, this expression is maintained by SHH, which is released from the notochord. In turn, Myf5, Wnt7A and Pax3 can directly activate MyoD initiating myogenesis. MRF4, referred to as Myf6 in the illustration, is dependent on the SIX protein family members, SIX1 and SIX4, and can also activate myogenesis via MyoD. In the hypaxial domain myogenesis is under Pax3 control, MyoD expression relies on Myf5 and Mrf4 expression, which is directly activated by Pax3. Pax3 induction requires expression of the SIX protein family members, SIX1 and SIX4 and their cofactors eyes absent 1 homologue (EYA1) and EYA2. Modified from Bryson-Richardson & Currie, 2008.

On the other hand, axial structures are not required for the determination of hypaxial muscle precursor cells as they are required to migrate to the limb buds and body wall before differentiating to form muscle (Cossu *et al.*, 1996a). Nevertheless hypaxial muscles do require signals from the surface ectoderm and lateral mesoderm to negatively regulate myogenic induction, allowing for the correct specification of migrating progenitor cells (Gullberg *et al.*, 1998). *In vitro* studies performed by Cossu *et al.* (1996a) showed that the dorsal ectoderm also induces myogenic differentiation through a MyoD-dependent pathway, yet *in vivo*, the dorsal ectoderm initially does not express any member of the MyoD family. However, if the lateral plate is mechanically separated from the paraxial mesoderm within a chick embryo then MyoD expression is seen in the hypaxial domain where it is not normally observed (Pourquie *et al.*, 1995), thus indicating that the lateral plate releases signals that are responsible for repressing myogenic differentiation of hypaxial muscle precursor cells. It was found that cells expressing bone morphogenic protein-4 (BMP-4) can replace the inhibitory action of components expressed by the lateral plate mesoderm (Pourquie *et al.*, 1996). This implicates BMP-4 as a possible negative signal secreted by the lateral plate mesoderm that requires derepression for myogenesis to proceed. Fibroblast growth factors (FGFs) are also known to be secreted during myotome formation, resulting in a proliferative non-differentiated population (Haub & Goldfarb, 1991). Both BMP-4 and FGFs are thought to be regulated in a derepression fashion, resulting from Notch/Delta signalling or from antagonist Noggin action that activates a repressed myogenic programme (Yun & Wold, 1996; Tajbakhsh & Cossu, 1997).

1.2.3 Secondary myogenesis

In the following sections, Pax gene expression, in the context of myogenesis, refers to Pax3 and Pax7 gene expression. Following somite patterning, myotome formation and hypaxial precursor muscle migration, there is a period of exceptional muscle growth, known as the second myogenic phase (Bryson-Richardson & Currie, 2008). It was originally thought that a highly proliferative, non-differentiated population of cells provided the myonuclei for muscle growth. In 2005, a population of myogenic precursor cells were

discovered that co-express Pax3 and Pax7; these cells originate from the early dermomyotome during somite maturation and epithelial disintegration (Kassar-Duchossoy *et al.*, 2005; Relaix *et al.*, 2005). These highly proliferative cells are distinct from myogenic cells and were also found in the limb buds, originating from the hypaxial domain of the dermomyotome. Generally these cells remain highly proliferative as they don't express any specific muscle markers, and are considered somitic stem cells (Zammit, 2008). However, they can become incorporated into muscle fibres by activating Myf5 and MyoD expression (Ben-Yair & Kalcheim, 2005; Gros *et al.*, 2005; Kassar-Duchossoy *et al.*, 2005; Relaix *et al.*, 2005). Thus, these cells provide a reservoir of myogenic precursor cells that maintain muscle growth throughout foetal development. This was confirmed by Pax3 and Pax7 double mutant studies, which resulted in only skeletal muscle from the early myotome forming, failing to provide muscle mass growth for secondary myogenesis (Relaix *et al.*, 2005). Without Pax expression, these cells failed to activate myogenic determination genes, thus adopting other cell fates as the myogenic programme is not activated (Borycki *et al.*, 1999). Derivatives of the ventral somite, bone and cartilage, as well as adipose tissue, are default pathways in the absence of myogenic commitment (Rudnicki *et al.*, 1993). Also, as both Pax3 and Pax7 play a role in ensuring cell survival, the majority of cells that don't assume other cell fates will apoptose (Borycki *et al.*, 1999; Kassar-Duchossoy *et al.*, 2005).

These Pax-positive cells that provide muscle growth during foetal development and are found in all developing muscle masses will establish themselves as satellite cells in the peri-natal period. These cells have stem cell-like properties and are responsible for post-natal growth and regeneration (Gros *et al.*, 2005) and in a quiescent state will take up position under the basal lamina that forms around muscle fibres (Buckingham, 2007). The Pax-positive derived satellite cells account for over 90% of satellite cells present at birth, while the remainder is accounted for by stem cells in adjacent structures that are recruited following injury (Sampaolesi *et al.*, 2003). The Pax-positive cells that satellite cells originate from also contribute to production of a heterogeneous population of muscle stem cells. This depends on whether a distinct population of Pax-expressing cells (approximately 80%) express Myf5 during peri-natal development before re-entering the quiescent state (Beauchamp *et al.*, 2000; Day

et al., 2007), as *Myf5* is a direct target of *Pax3* (Bajard *et al.*, 2006). As these cells are capable of self-renewal via asymmetric division, there are two resulting populations of cells, $Pax^+/Myf5^-$ or $Pax^+/Myf5^+$. The two populations result in different capabilities, as $Pax^+/Myf5^-$ exhibit more stem-like properties and are more inclined to maintain satellite cell numbers via asymmetric cell division. Whereas $Pax^+/Myf5^+$ cells have properties similar to committed progenitors disposed towards terminal differentiation but are less efficient at regenerating muscle (Kuang *et al.*, 2007).

There are many signalling pathways involved in stem cell maintenance, and determination of whether specific stem cells are pushed into a program of differentiation or maintained as stem cells. During embryo-myogenesis, the family of fibroblast growth factors (FGF) have been indicated to regulate *Pax*-positive stem cell fate, especially *Fgfr4* (Lagha *et al.*, 2008a). FGF signalling is involved in regulating myogenesis, with consequent effects of promoting the proliferation (von Scheven *et al.*, 2006) and differentiation of myoblasts (Hammond *et al.*, 2007). Specific *Fgfr4* expression is detected at myogenic sites from actively dividing myoblasts (Stark *et al.*, 1991), with down-regulation of expression (Edom-Vovard *et al.*, 2001) or expression of dominant-negative *Fgfr4* (Marics *et al.*, 2002) resulting in preventing the expression of myogenic genes *Myf5*, *MyoD* and *MyHC*. However, knocking out the *Fgfr4* gene does not result in lethality; myogenesis proceeds normally in the embryo, indicating that other members of the FGF family can act as redundant receptors (Weinstein *et al.*, 1998). Chromatin immunoprecipitation experiments were used to demonstrate that *Pax3* lies genetically upstream of *Fgfr4* and controls its expression during myogenesis via the 3' regulatory element of *FgFr4*. In the differentiating progeny of *Pax*-positive cells, in which *Pax3* is down-regulated, *Fgfr4* signalling is further modulated and controlled by *MyoD*, which binds to an E-box within the 3' regulatory region. In addition, in a myogenic context, *Pax3* also controls the expression of *sprouty1*, involved in the *Fgfr4* signalling pathway. This led Lagha *et al* (2008a) to propose that *Pax3*, via *Fgfr4* signalling, controls the myogenic cell fate of *Pax*-positive expressing cells. Interfering with *Fgfr4* signalling by either up-regulating *sprouty1* or interrupting signalling with *Fgf8* expression, a potential *Fgfr4* ligand (MacArthur *et al.*, 1995), results in preventing differentiation and increasing the stem cell pool of $Pax^+/Myf5^-$ cells (Hammond *et al.*, 2007).

During primary myogenesis, Pax3 plays the predominant role of transcriptional regulator of myogenic progression (Tajbakhsh *et al.*, 1997). As development proceeds into the late neo-natal/early peri-natal stages, the Pax3/Pax7 positive cells that become satellite cells result in a hierarchy shift towards Pax7 expression. Although dispensable during early myogenesis, Pax7 expression is a requisite for satellite cell maintenance (Seale *et al.*, 2000; McFarlane *et al.*, 2006). In addition, in adult muscle satellite cells the anti-apoptotic function acquired by Pax7 expression cannot be compensated for by Pax3 (Relaix *et al.*, 2006). Illustrated in Figure 1.3 is the lineage progression of the Pax-positive somatic stem cells that are responsible for the secondary phase of myogenic growth and development and for establishing the satellite cell pool.

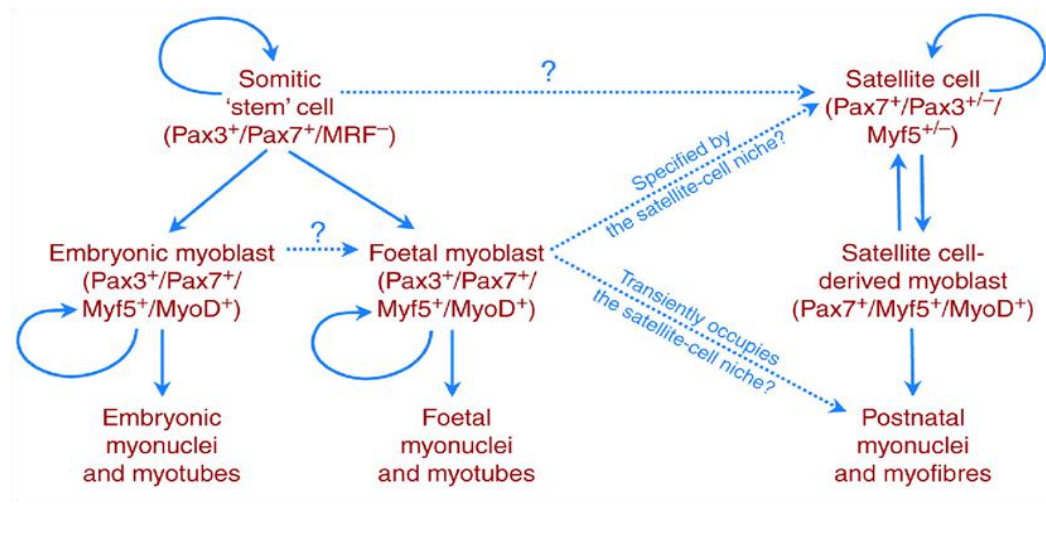


Figure 1.3 Somatic stem cell lineage progression during myogenic development.

The self-renewing population of myogenic precursor cells marked with Pax3 and Pax7 expression establish the somatic stem cell pool. These cells can expand into a proliferative population of myogenic progenitors via the expression of Myf5 and MyoD during embryonic and foetal development, providing the myonuclei for growth of myotubes. Somatic stem cells may become incorporated under the basal lamina during development and foetal myoblasts can become niche specified, resulting in the satellite cell population. The renewing population of satellite cells provides the myonuclei for post-natal growth and development and during the early stages of post-natal growth, foetal myoblasts that occupy the satellite cell niche, aid in providing myonuclei (Zammit, 2008).

1.3 Post-natal myogenesis

The last myogenic phase comprises maintenance and growth of post-natal muscle. As muscle cells are incapable of division, the nuclei for growth, regeneration and repair are acquired from satellite cells. First discovered in 1961 (Mauro, 1961), these cells are now accepted as being muscle stem cells, as once activated they are capable of providing a proliferating and differentiating population of progenitors that maintain cell numbers via self-renewal (Collins *et al.*, 2005). In early post-natal mouse muscle, 30-35% of the sublaminal nuclei are contributed by satellite cells. This number drops to only 1-4% by adulthood as the satellite cells are only required to provide the myonuclei for growth and regeneration (Allbrook *et al.*, 1971). As in primary myogenesis, post-natal myogenesis follows a highly orchestrated genetic program that initiates either repression or induction depending on cell-cycle progression and external cues (Charge & Rudnicki, 2004; Zammit *et al.*, 2006b). Recently, *in vivo* research using a murine model has indicated that the dependency of myogenic progenitor cells on Pax-regulation during skeletal muscle development and regeneration is only maintained until juvenile development, at which stage post-natal muscle regeneration proceeds in the absence of Pax-regulation (Lepper *et al.*, 2009). To the contrary, reviewed below are numerous studies, which indicate both Pax3 and Pax7 regulation of post-natal regeneration processes.

1.3.1 Satellite cell physiology

As the Pax-positive cells of secondary myogenesis form into quiescent satellite cells, Pax7 becomes the main satellite cell marker (Halevy *et al.*, 2004). Pax3 in comparison is down-regulated, with transient expression only seen during the activation of the proliferating progeny of satellite cells in most muscles (Conboy & Rando, 2002; Day *et al.*, 2007). On the contrary, Pax3 expression is also detected in quiescent satellite cells in certain muscle types such as the diaphragm and a subset of the trunk and limb muscles (Relaix *et al.*, 2006). This was confirmed by using a (Pax3)GFP⁺ mouse line to isolate satellite cells from skeletal muscle, using flow cytometry. The (Pax3)GFP⁺ mouse line showed localised spatial expression as quiescent Pax3 positive satellite cells. Flow

cytometry led to the isolation of a homogenous Pax7⁺, CD34⁺, CD45⁻, Sca1⁻ cell population that show regenerative capacity when grafted into the *tibialis anterior* muscle of immunodeficient *mdx* (dystrophin-deficient) mice (Montarras *et al.*, 2005).

Stem cell antigen-1 (Sca1) was first identified as an antigen that allowed for bone marrow stem cell purification (Spangrude *et al.*, 1988). This membrane protein, which is not initially expressed by satellite cells, is expressed by a subset of satellite cell-derived myoblasts, with differentiation inducing a marked up-regulation of expression (Shen *et al.*, 2003). This results in a heterogeneous myoblast population: Sca1⁻ myoblasts are highly proliferative and inclined to differentiate, providing myotubes for growth, whereas, Sca1⁺ myoblasts maintain cells in a non-differentiated, slowly proliferating state that could be involved in satellite cell renewal (Mitchell *et al.*, 2005). On the other hand, CD34 is initially expressed by satellite cells and was found to be a marker of satellite cell quiescence (Beauchamp *et al.*, 2000). A member of the sialomucin family of surface molecules, CD34 has identified functions involved in haematopoietic cell differentiation and cell-cell adhesion (Krause *et al.*, 1996). The quiescent satellite cells that express CD34 were also found to express Myf5, indicating that CD34 marks satellite cells that are committed to the myogenic lineage (Beauchamp *et al.*, 2000). Furthermore, CD34⁺ satellite cell-derived myoblasts, in comparison to CD34⁻ myoblasts, had a higher capacity to replace dystrophin in *mdx* mice (Jankowski *et al.*, 2002). These two markers, Sca1 and CD34, can possibly be used to define and separate the two satellite cell lineages based on their myogenic regenerative capacity or ability to self-renew (Jankowski *et al.*, 2002). Occupy

Other genes that satellite cells express include the tyrosine kinase receptor, c-met. Present in both quiescent and activated satellite cells as well as derived mono-nucleated muscle cells (Cornelison & Wold, 1997) c-met is thought to be involved in mediating satellite cell activation (Allen *et al.*, 1995). N-cadherin and M-cadherin are cell-adhesion proteins that allow communication with the underlying myofibres (Irintchev *et al.*, 1994). M-cadherin was found to be expressed in 94% of cells between the basal lamina and the underlying myofibre (Sajko *et al.*, 2004). Underlying myofibres are part of a unique niche that surrounds satellite cells and occupation of this niche is used to identify satellite cells, as their molecular signature is hard to distinguish from that of myoblasts

(Yablonka-Reuveni, 1995). Furthermore, this niche is an important regulator of satellite cells, providing a dynamic micro-environment that can either maintain quiescence or provide cues for activation (Zammit, 2008). The importance of the niche was shown by transplantation experiments where intact myofibres containing as little as seven satellite cells were transplanted into radiation-ablated muscle. The myofibre niche provided the cues for these satellite cells to be self-sufficient, generating thousands of myonuclei for growth as well as repopulating the original host muscle stem cell population (Collins *et al.*, 2005). The niche contains diffusible factors, such as hepatocyte growth factor (HGF), which is present in the overlying basal lamina in both pro and active form. Proteolytic cleavage provides an abundance of pro-HGF, which is released to activate satellite cells when a muscle is stretched or injured (Tatsumi & Allen, 2004). Furthermore, Notch signalling, which responds to Notch ligands that the underlying myofibres present during growth or injury, is also involved in satellite cell activation (Conboy *et al.*, 2003). In primary myoblast cultures, Notch signalling has been shown to activate transient Pax3 expression, which is detected upon satellite cell activation (Conboy & Rando, 2002). Interestingly, it is actually degeneration in the satellite cell niche, rather than of the actual stem cell, that is responsible for loss of regenerative capacity in old muscle (Conboy *et al.*, 2005). Deregulation can be caused by insufficient presentation of delta ligands for Notch signalling (Conboy *et al.*, 2003). Research found that exposure of aged satellite cells to the systemic factors present in serum from young cells rejuvenated the lost regenerative capacity of the aged satellite cells (Conboy *et al.*, 2005).

1.3.2 Satellite cell regulation

Quiescent satellite cells will reside under the basal lamina of the muscle fibre until the skeletal muscle stretches or is injured; this subsequently activates the quiescent cells (Zammit *et al.*, 2006a). Release of active HGF ligand during injury interacts with the active c-met receptor expressed by satellite cells, inciting their activation. This release is mediated as it is proportional to muscle damage (Tatsumi *et al.*, 1998). Active HGF was found to be the first molecule to induce DNA synthesis in satellite cells when compared to an array of growth factors, and thus initiates the cell cycle (Gal-Levi *et al.*, 1998). Experimental exogenous

injection of purified pro-HGF confirmed the role of HGF, as it activated quiescent satellite cells in undamaged rat muscle (Tatsumi *et al.*, 1998), with a dosage-dependent increase amplifying the proliferative rate of progenitor cells (Gal-Levi *et al.*, 1998). Also, as part of satellite cell activation, HGF expression results in the down-regulation of caveolin-1, a marker for satellite cell quiescence (Volonte *et al.*, 2005). However, HGF would seem to be temporally regulated; although exogenous injection into already regenerating muscle increases the active satellite cell pool, the regenerative potential did not increase. The subsequent reduction in regenerative capacity comes from inhibition of differentiation by HGF (Miller *et al.*, 2000). This inhibition of differentiation was confirmed by ectopic expression of HGF inhibiting the expression of MRFs such as MyoD, myogenin and MyHC, with ectopic expression of MyoD reversing this inhibitory effect (Gal-Levi *et al.*, 1998). Curiously, HGF and c-met, which are genetically downstream of Pax3, exhibit a similar effect of inhibiting myogenic differentiation during embryomyogenesis to allow for somatic limb migration (Lagha *et al.*, 2008b). In support, studies have shown that over-expression of Pax3 does inhibit the differentiation of C2C12 cultured myoblasts (Epstein *et al.*, 1995) as well as inhibiting the differentiation of satellite cell-derived myoblasts (Collins *et al.*, 2009).

HGF is not the only molecule to play a role in satellite cell activation, as there are many other candidates, such as the insulin-like growth factors (IGF)-I and -II (Goldspink, 2002). IGF-I activates satellite cells and causes hypertrophy by stimulating myoblast proliferation and differentiation (Florini *et al.*, 1996), whereas, IGF-II affects differentiation in a dose-dependent manner. At low concentrations IGF-II stimulates differentiation, however, as it is up-regulated, differentiation is inhibited (Florini *et al.*, 1996). In addition, the temporal regulation of IGF-II is important: if it is expressed initially after injury then there is a delay in regeneration. In comparison, if it is expressed during fibre enlargement then it enhances late regeneration (Kirk *et al.*, 2003). There are also two splice variants of the IGF-I gene, both of which are specifically expressed by injured or stretched muscles. The splice variant known as “mechanosensitive autocrine growth factor” (MGF) acts as the initial satellite cell activator, and is expressed directly after injury to activate and enhance proliferation. The second splice variant, IGF-IEa, which is more similar to its parent protein, is responsible

for maintaining protein synthesis during late regeneration to finish restoration (Yang & Goldspink, 2002; Hill & Goldspink, 2003).

The FGF family, which is involved in regulating progression and fate of embryo-myogenic stem cells, may also play a role during post-natal myogenesis. Within the embryo, Pax3-regulated sprouty1/Fgfr4 interplay suggests that sprouty1 prevents premature differentiation by negatively regulating Fgfr4 signaling (Hammond *et al.*, 2007), as mentioned in Section 1.3.4. A similar expression profile was discovered for satellite cells, as quiescent satellite cells express sprouty1 and Fgfr4, however following activation only Fgfr4 was expressed (Fukada *et al.*, 2007). The down-regulation of sprouty1 during satellite cell activation coincides with the global expression of MyoD, a sign of satellite cell entry into the myogenic program (Kastner *et al.*, 2000). Fgfr4 signalling is not only seen in activating satellite cells but also in regenerating muscle following injury (Zhao & Hoffman, 2004), with Fgfr4-null mice demonstrating defects in muscle regeneration. During *in vivo* muscle regeneration, Fgfr4 signalling was highly expressed during myoblast withdrawal from the cell-cycle and during the subsequent fusion to form multinucleated myotubes (Zhao *et al.*, 2006). In comparison to embryonic muscle, the regenerating muscle of Fgfr4-null mice has a much more evident phenotype showing reduced efficiency in myogenic terminal differentiation. Interestingly, after only one round of degeneration/regeneration, Fgfr4-null mouse muscle was calcified, as well as being replaced by adipose fat. These processes have been documented occurring in chronic inflammatory states and in human dystrophic muscle, however, only after years of degeneration and regeneration (Zhao *et al.*, 2006). Furthermore, the transcriptional pathway for Fgfr4 signalling during post-natal muscle regeneration was found to be under the control of MyoD. MyoD activates a transcriptional enhancer factor Tead2 which in turn induces Fgfr4 expression via binding to an M-CAT motif in the Fgfr4 promoter region (Zhao *et al.*, 2006). The importance of Fgfr4 signalling during post-natal myogenesis has been demonstrated, with absence of Fgfr4 expression resulting in myogenic cells seeking alternative paths of differentiation, undermining muscle regeneration.

Where satellite cell-derived myogenic cells require FGF signalling for efficient terminal differentiation, Notch signalling on the other hand prevents premature differentiation. Notch signals through Delta ligands that bind to their

corresponding Notch receptors, resulting in the release of the Notch intracellular domain (NICD) via proteolytic cleavage. The NICD relocates to the nucleus where the transcription factor RBPJ mediates its signal for the activation of genes that prevent uncontrolled differentiation, thus regulating proliferation and cell fate (Vasyutina *et al.*, 2007a). The importance of Notch signalling and its mediator RBPJ was shown by a conditional RBPJ knockout in somatic cells, which resulted in the incorrect temporal expression of myogenic differentiating genes. Furthermore, it resulted in the depletion of the Pax-positive derived satellite cell pool, thus severely affecting future development of muscle tissue. This underlines the importance of RBPJ-mediated Notch signalling in maintaining and developing the satellite cell pool (Vasyutina *et al.*, 2007b). In post-natal myogenesis the Notch-1 receptor is expressed by satellite cells during activation, with continued expression promoting proliferation and preventing differentiation (Conboy & Rando, 2002). To allow for myogenic differentiation of the proliferating progeny, Numb (an inhibitor of Notch) is expressed which prevents the nuclear translocation of NICD and thus interrupts Notch signalling, thus permitting myogenic commitment (Nofziger *et al.*, 1999). It has been indicated that Notch signalling is involved in ensuring satellite cell self-renewal facilitated by the asymmetric distribution of Numb during cell division. This could result in daughter cells having divergent fates, either becoming a terminally-differentiated myogenic cell or self-renewing to maintain satellite cell numbers (Conboy & Rando, 2002; Shinin *et al.*, 2006). This was further confirmed by inhibiting the proteolytic cleavage of Notch receptors via DAPT, a γ -secretase-inhibitor, creating a differentiating phenotype marked by the repression of Pax7 (Kuang *et al.*, 2007). There is no evidence at this time to indicate that Notch signalling is regulated by the Pax genes and thus this pathway must be modulated by extrinsic myogenic controls. These extrinsic factors also seem to control the expression of members of the Transforming growth factor-beta (TGF- β) superfamily, which have roles in regulating myogenic progression (Lagha *et al.*, 2008b).

The first member of this family, named TGF- β , which the TGF- β superfamily of proteins was named after, was shown in a dose-dependent manner to inhibit differentiation and reduce proliferation of satellite cells, with the inhibitory effect being reversible (Allen & Boxhorn, 1987). The experiment was then repeated in the presence and absence of FGF and IGF-I, to test the effect of

these mitogens in regulating myogenic progression of satellite cells. It was found that the addition of these specific mitogens can restore the proliferative potential of satellite cells treated with TGF- β , however differentiation inhibition was maintained. This indicates that during post-natal myogenesis the expression of TGF- β prevents premature differentiation, with the combined expression of mitogens inducing the proliferative expansion of satellite cells. Furthermore, by altering the concentrations of the three different factors (TGF- β , IGF-I and FGF) the myogenic progression of satellite cells *in vitro* can be controlled and maintained throughout proliferation, differentiation or quiescence (Allen & Boxhorn, 1989).

Myostatin (Mstn) is a member of the TGF- β superfamily and is a negative regulator of myogenesis. Its role is confirmed by its own naturally occurring mutation that results in a double-muscling phenotype, as seen in the Belgian Blue and Piedmontese cattle (Kambadur *et al.*, 1997; McPherron *et al.*, 1997). Myostatin is initially expressed as a 52 kDa precursor protein that is proteolytically cleaved and secreted by myoblasts as a 26 kDa mature peptide (Thomas *et al.*, 2000; Arnold, 2001). The addition of this mature peptide to C2C12 myoblasts *in vitro* results in inhibition of proliferation via arrest of cell cycle progression at the G1-phase. This is caused by Mstn up-regulating p21, inhibiting cyclin-CDK activity and increasing the amount of hypophosphorylated Rb protein, which in turn is responsible for cell cycle arrest. Thus, the excessive hyperplasia seen in Belgian Blue and Piedmontese cattle is a result of unregulated myoblast proliferation (Thomas *et al.*, 2000). In an additional study, Mstn was also shown to inhibit the differentiation of C2C12 myoblasts. Transcriptional analysis of C2C12 cells in low serum medium treated with Mstn showed reduced levels of the myogenic determination genes and p21, thus preventing myogenic differentiation. However, this resulted in increased phosphorylation levels of Smad3, a protein involved in the TGF- β signalling cascade (Zhang *et al.*, 1998), as well as amplified Smad3 association with MyoD, indicating that Mstn inhibits differentiation by reducing MyoD activity. This was confirmed by using a dominant-negative Smad3-expressing construct, which recovered MyoD activity. Therefore, Mstn can be seen as a powerful regulator that controls the amount of hyperplasia and hypertrophy that occurs during post-natal growth by controlling the myogenic progression of myoblasts (Langley *et al.*, 2002).

The up-regulation of p21 caused by the expression of Mstn, as seen in C2C12 cells, also occurs in satellite cells, preventing their progression from the G1- to S-phase in the cell cycle. This blocks satellite cell activation and thus the expression of Mstn promotes a quiescent state. Also, using CD34 as a marker, immunohistochemical analysis demonstrated that Mstn-null mouse muscle fibres had an increased number of satellite cells per unit length in comparison to wildtype. This could be due to the increased proliferative rate procured in the absence of Mstn, with delayed myogenin expression allowing for a boost in self-renewal (McCroskery *et al.*, 2003). The role of Mstn in regulating satellite cell activity was further investigated by comparing wildtype and Mstn-null mice treated with notexin (a potent neurotoxin obtained from snake venom). Two days after injury, in areas of regeneration, Mstn-null mice showed a dramatic increase in the number of migrating satellite cells and the number undergoing myogenic commitment via MyoD expression. In addition, increased numbers of macrophages were found in notexin treated Mstn-null mice, with an increased inflammatory response reducing the amount of inflamed tissue. In the absence of Mstn within regenerated muscle there was evidence to indicate a more complete healing with less fibrosis (McCroskery *et al.*, 2005). The negative regulation of satellite cell function exhibited by Mstn has been shown to act via a negative regulation of Pax7 expression. This in turn both affects the activation of MyoD expression as well as the ability of satellite cells to withdraw from the cell cycle to renew the stem cell population. Consequently, if Mstn expression is blocked, a Pax7 over-expressing phenotype occurs which amplifies the self-renewing satellite cell lineage (McFarlane *et al.*, 2006).

1.3.3 Satellite cell function

The expression of the Pax genes maintains satellite cells in a quiescent state as these genes repress MyoD. In response to growth or injury, growth factors from the surrounding extracellular environment activate satellite cells (Zammit *et al.*, 2006b). Intermediate progenitor cells are marked by Pax3 expression, which represses continued myogenic progression (Conboy & Rando, 2002). Pax3 is regulated by ubiquitination, which results in proteasomal degradation of Pax3, allowing intermediate progenitors to develop into myogenic progenitors.

Interestingly, this proteasomal degradation pathway does not affect the Pax7 protein, as its expression is maintained (Boutet *et al.*, 2007). Pax3 expression can also be fine tuned by miRNA interference. Prior to satellite cell progenitor differentiation, miR-27 is up-regulated to negatively regulate Pax3 protein synthesis (Crist *et al.*, 2009). Subsequently, MyoD will be expressed in the corresponding proliferating progeny as well as increased Myf5 expression, which is already activated in the majority of satellite cells. Pax7 has been shown to modulate Myf5 expression by interacting with the methyltransferase complex resulting in chromatin modifications and transcriptional activation (McKinnell *et al.*, 2008). Up-regulated Myf5 transcription leads to the down-regulation of Pax7, and activation of myogenin, marking cellular differentiation. This is followed by cell-cycle withdrawal and myoblast fusion with pre-existing multinucleated myotubes, providing hypertrophy for regeneration. Also, differentiating myoblasts can fuse together to form new myotubes, supplying hyperplasia for growth (Hawke & Garry, 2001; Halevy *et al.*, 2004; Zammit *et al.*, 2006b). Alternatively, following proliferation, satellite cell-derived myoblasts can change their cell fate by down-regulating MyoD while continuing to express Pax7, leading to cell-cycle withdraw. Using this alternative path, a small sub-population of satellite cell progeny can revert to their quiescent satellite cell status and thus maintain satellite cell numbers (Zammit *et al.*, 2004).

MyoD expression can be prolonged into and during differentiation if the progeny are growing within a rich, high mitogen media, as myoblasts still have proliferative potential during the early phases of differentiation (Yablonka-Reuveni & Paterson, 2001; Shefer *et al.*, 2006). Post-translational modifications, repressor proteins and epigenetics controlling DNA accessibility all regulate MyoD and can increase the proliferative period before differentiation is induced (Berkes *et al.*, 2004). Pax3/Pax7 is thought to regulate MyoD expression. This was shown by using cultured satellite cells expressing dominant-negative forms of Pax3 and Pax7, resulting in a Myf5-directed differentiation pathway. Differentiation was marked by a delay in myogenin expression, due to the consequent down-regulation of MyoD (Relaix *et al.*, 2006; Zammit *et al.*, 2006b). Furthermore, when dominant-negative forms of Pax3 and Pax7 were expressed within the Myf5 negative satellite cell line mentioned above (Kuang *et al.*, 2007) there was no differentiation, indicative that the Pax genes regulate myogenesis

through MyoD (Relaix *et al.*, 2006). As described above and in Section 1.3.2, the progression of satellite cells and their function in regenerating post-natal muscle is a highly regulated process that results in introducing population heterogeneity (Figure 1.4) (Buckingham & Montarras, 2008).

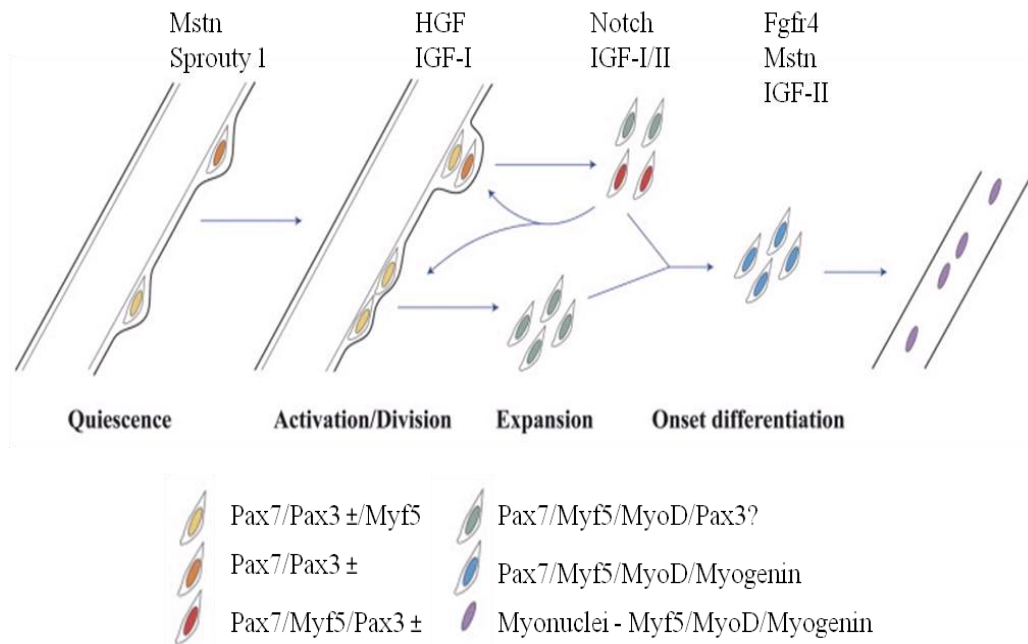


Figure 1.4 Regulation and gene expression during satellite cell function.

Expression of Mstn and sprouty1 maintains satellite cells in a quiescence state, injury results in growth factors such as HGF and IGF-I activating the cell cycle. Satellite cell progeny expansion involves both asymmetric (red nuclei) and symmetric division (green nuclei), depending on the expression of myogenic genes Myf5 or MyoD, respectively. A number of regulatory molecules regulate myogenic progenitor cell progression into myoblasts, with the onset of differentiation being marked with the down-regulation of Pax expression and up-regulation of myogenin expression. Modified from Buckingham & Montarras, 2008).

1.3.4 Satellite cell self-renewal

The ability to replenish the quiescent satellite cell pool is a requirement fulfilled by satellite cells, meaning they fit the stem cell definition. Following the lineage progression from satellite cells to differentiating satellite cell-derived myoblasts involves cell multiplication via symmetric division and self-renewal through asymmetric division (Shinin *et al.*, 2009). DNA tracking during the S-phase demonstrated that satellite cell division can be asymmetrical, resulting in both quiescent satellite cells and myonuclei for growing muscle (Moss & Leblond, 1971). Asymmetric division within a satellite cell sub-population has been witnessed to co-segregate all template DNA to one sister cell which is biased to self-renewal. This is known as the ‘immortal strand hypothesis’, it is presumably to prevent accumulation of new mutations within the self-renewing stem cell population (Shinin *et al.*, 2006). Alternatively, co-segregation of DNA could be due to epigenetic differences between template and new DNA strands, and it is this fact that facilitates differential gene expression and divergent cell fates within sister cells (Lansdorp, 2007). Either way, the co-segregation of template DNA coincides with the sister cell that continues to express Pax7 as well as co-segregating the Notch inhibitor Numb, promoting self-renewal (Shinin *et al.*, 2006).

Functional evidence for satellite cell self-renewal comes from cell labelling studies using neo-natal myoblasts from Myf5nlacZ⁺ mice. These myoblasts express a nuclear localising LacZ gene that shows β -galactosidase (β -gal) activity in each of their donor cells. Following transplantation into post-natal skeletal muscle, positive β -gal staining was detected in the host muscle as well as within the satellite cell niche. Isolation and culturing of these cells confirmed them as functional satellite cells that give rise to myoblasts (Heslop *et al.*, 2001). More recently this was re-confirmed: transplanting a single Pax7⁺/Luciferase⁺ satellite cell into injured mouse muscle resulted in extensive proliferation that contributed to the muscle fibre as well as to mono-nucleated luciferase-expressing stem cells (Sacco *et al.*, 2008). Both experiments demonstrate that activated satellite cells contribute both to repair and to renewal of the myogenic stem cell population (Heslop *et al.*, 2001; Sacco *et al.*, 2008), resulting in the conclusion

that the ability to self-renew is autonomous to each muscle satellite cell (Sacco *et al.*, 2008).

In growing or regenerating muscle, asymmetric division gives rise both to satellite cells that are disposed to differentiation following fast but limited symmetrical replication, as well as to a slowly proliferating population of cells that return to G0 between cell cycles and later via symmetric division produce larger amounts of myogenic cells than stem cells (Schultz, 1996). In this way a small sub-population of satellite cells during activation and replication always maintain the myogenic stem cell pool, and thus the majority of myonuclei acquired during post-natal myogenesis originate from this pool (Zammit, 2008). The rate at which satellite cells proliferate also plays a part in determining their fusion capability. The fast-dividing satellite cell population is biased toward fusing with pre-existing myofibres (hypertrophy), whereas the slowly dividing population responsible for self-renewal predominantly fuses together (hyperplasia) (Rouger *et al.*, 2004).

The heterogeneity seen in the satellite cell population is also seen in C2C12 myoblasts that are induced to differentiate, resulting in a population of cells committed to terminal differentiation and a population of cells that withdraw from the cell cycle. If these latter reserve cells are isolated and re-cultured to induce differentiation, this once again results in two populations of cells, a strong indication that C2C12 cells are a good *in vitro* model of satellite cell renewal (Yoshida *et al.*, 1998). In addition, C2C12 cells also show the same bias as satellite cells in respect to fusion and proliferation rate (Baroffio *et al.*, 1996). However, when using C2C12 cells to study satellite cells the natural *in vivo* environment is absent and it has been shown that the satellite cell niche regulates many facets of satellite cell function. For example, asymmetric division and thus satellite cell renewal is prejudiced towards apical-basal division in regard to the orientation of satellite cells within their niche. Therefore, satellite cells that divide in the plane of the myofibre instead of perpendicular to it, are inclined to divide symmetrically (Kuang *et al.*, 2007). The effect of an absent satellite cell niche was demonstrated by grafting isolated satellite cells into the *tibialis anterior* muscle of immunodeficient *mdx* mice before and after culturing. The regenerative capacity of the isolated satellite cells declined considerably if they were cultured before grafting. Isolated cells that were expanded in culture were shown to have inferior

proliferation and rapid differentiation (Montarras *et al.*, 2005). The satellite cells in culture seem to lack self-renewal signals and thus with declining asymmetric division, cells gradually become a continuum of satellite cell-derived myogenic progenitor cells expressing MyoD. This resulted in the loss of their stem cell-like capabilities as they become a homogenous population of myogenic progenitors with decreased regenerative capability.

To summarise the self-renewal process, following satellite cell activation and during proliferation, satellite cells progeny can take alternative paths and maintain cell numbers via self-renewal. Initially satellite cell activation leads to the global expression of MyoD, an eventual decline in Pax7 expression and terminal differentiation (Yablonka-Reuveni & Rivera, 1994; Zammit *et al.*, 2004). Following replication, an alternative cell fate for satellite cell-derived myoblasts results in the down-regulation of MyoD, and maintained Pax7 expression, leading to cell cycle withdraw (Zammit *et al.*, 2004). This was further confirmed by Pax7⁺/MyoD⁻ satellite cell-derived myoblasts re-expressing quiescence markers such as the nestin transgene (Day *et al.*, 2007), and sphingomyelin (Nagata *et al.*, 2006). Cell cycle withdrawal is facilitated by p21 expression, which if it is synchronised with Myf5 expression denotes myoblasts that are returning to quiescence, in comparison to p21 expression synchronisation with MyoD expression denoting a differentiating myoblast (Kitzmann & Fernandez, 2001).

The renewing Pax7⁺/MyoD⁻ satellite cell lineage was further investigated using MyoD⁻ null satellite cell-derived myoblasts. This resulted in amplification of stem cell characteristics and of the renewal process, when grafted into regenerating muscle. Microarray analysis revealed that in comparison to wildtype, MyoD-null myoblasts down-regulated the myogenic determination genes and up-regulated anti-apoptotic genes, corresponding to increased survival (Asakura *et al.*, 2007). Following the myogenic progression of MyoD-null myoblasts during muscle regeneration revealed that differentiation does proceed but it is substantially delayed due to an extended proliferative period (White *et al.*, 2000). This indicates that in the absence of MyoD, a Myf5-directed differentiation pathway proceeds, which has been shown to be marked by a delay in myogenin expression (Zammit *et al.*, 2006b), with an apparent bias towards quiescence (Asakura *et al.*, 2007). It has also been witnessed that following satellite cell division, one cell can maintain MyoD expression and commit to terminal

differentiation, whereas the other daughter cell can down-regulate MyoD and act to replenish the satellite cell pool (Zammit *et al.*, 2004). As MyoD expression can be modified epigenetically (Berkes *et al.*, 2004), the above observation strongly indicates that asymmetric division is indeed regulated by chromosomal epigenetic differences, which facilitates the difference in gene expression between the two lineages (Lansdorp, 2007).

As mentioned previously, Pax7 is the main satellite cell marker, with Pax7-null studies further confirming its importance in satellite cell physiology. Juvenile Pax7-null mice contain a considerable number of satellite cells (Oustanina *et al.*, 2004), possibly specified by the Pax7 paralogue Pax3, which has been seen to share functional redundancies (Mansouri, 1998). However, as post-natal development progresses these numbers decline, resulting in inefficient regeneration, therefore demonstrating the heavy involvement of Pax7 in self-renewal, as well as its role as a requisite for satellite cell survival (Oustanina *et al.*, 2004). In the absence of Pax7 expression within Pax3-expressing muscle, satellite cells were still increasingly lost due to apoptosis, validating the necessity for Pax7 in satellite cell survival (Relaix *et al.*, 2006). Also, over-expression of Pax7 directs proliferating myoblasts to withdraw from the cell cycle by repressing MyoD expression, thus promoting the self-renewal pathway (Olguin & Olwin, 2004; McFarlane *et al.*, 2006). This inhibition of myogenesis was also seen during an *in vitro* study on human satellite cells, where maintained Pax7 expression prevented satellite cell-derived myoblasts from differentiating (Pawlikowski *et al.*, 2009). The presence of satellite cells both in Pax7-null juvenile and Pax7-null adult skeletal muscle does suggest however that Pax7 is not exclusively involved in satellite cell specification (Oustanina *et al.*, 2004). Interestingly, in a different study, Pax7-null mice were devoid of functional satellite cells, however, there was a separate Pax3-expressing lineage. These cells were defined as myogenic as they co-expressed MyoD following injury, resulting in regenerated myofibres (Kuang *et al.*, 2006). Instead of being located in the normal satellite cell niche, the Pax3-positive lineage were found in the interstitial muscle environment: possibly they are residue pre-satellite cells that require Pax7 for specification. However, the presence of the Pax3-expressing lineage in both Pax7-null and wildtype mice suggests that these cells represent a novel myogenic lineage (Kuang *et al.*, 2006).

1.4 Skeletal muscle wasting

There are a number of skeletal muscle diseases that result in loss of body mass and muscle atrophy. Cachexia, a progressive skeletal muscle wasting syndrome, is associated with several disease states, such as cancer, aids, and diabetes. The cachexia metabolic response causes specific loss of adipose tissue (Argiles *et al.*, 2003) and skeletal muscle mass (Tisdale, 1991). The catabolic response of cachexia results in degradation of skeletal muscle proteins, releasing amino acids in response to infection and illness, resulting in muscle atrophy (Hasselgren & Fischer, 2001).

Muscle atrophy can also be caused by age-related degeneration of muscle mass, with distinct decline in ability to regenerate; a condition known as sarcopenia (Rosenberg, 1997). These weaker muscles are more susceptible to stress and injury and have an increased recovery time (Doherty, 2003). It is thought that the gradual failure of satellite cells to regenerate aged muscle fibres is caused by the diminished levels of myogenic activation signals and growth factors (Conboy *et al.*, 2005). With an increase in the aging population, sarcopenia is becoming more common, increasing the need for a treatment to maintain the quality of life in the elderly. Individuals with muscular atrophy suffer many complications, such as loss of mobility, fragile bones, susceptibility to infection and general loss of health. Consequently skeletal muscle atrophy can be a serious life threatening condition, illustrating the importance of finding an effective therapeutic cure (Giordano *et al.*, 2003; Argiles *et al.*, 2005).

Muscular dystrophy disorders, in addition to cachexia and sarcopenia, cause muscular atrophy and muscle weakness: a group of inherited disorders that can affect the developing embryo as well as post-natal development (Emery, 1998). Duchenne muscular dystrophy is the most common disorder, carried on the X-chromosome and thus normally occurring with males. At childhood the disorder causes progressive muscle wasting, which by adolescence has caused considerable weakness, leading to premature death (Emery, 2002).

1.5 Stem cell therapy

Stem cell therapy is based on treating damaged or diseased tissue through application with embryonic stem (ES) cells. These multipotent cells have a large potential in self-renewal and differentiation, giving rise to continuous generations of cells that can aid in regeneration (Darabi *et al.*, 2008b). Cultures of ES cells when induced to differentiate will form embryoid bodies (EB), multi-cellular structures consisting of all three embryonic layers (Kubo *et al.*, 2004). Continued growth and differentiation results in the EB establishing a variety of specialised cell types (Doetschman *et al.*, 1985), including skeletal muscle (Rohwedel *et al.*, 1994). The main issue preventing the therapeutical use of these cells is the ability to purify the correct population of tissue-specific, embryonic-derived cells from the differentiating ES cell mass (Darabi & Perlingeiro, 2008).

These EB grown in culture lack certain embryonic skeletal muscle structures, indicating that specific myogenic inductive signals are lacking, resulting in an altered spatial-temporal gene expression and incorrect patterning of the paraxial mesoderm (Darabi *et al.*, 2008a). Pax3 has been shown to be a strong transcriptional regulator involved in initiating myogenesis in the early paraxial mesoderm (Williams & Ordahl, 1994). Pax-positive cells residing in the epithelium of the dermomyotome during embryogenesis can give rise to multiple lineages (Kardon *et al.*, 2002; Ben-Yair & Kalcheim, 2005), and in the absence of Pax3 expression, cells that would form into myogenic progenitors will differentiate into alternative tissue such as bone and cartilage (Relaix *et al.*, 2005) and adipose tissue (Rudnicki *et al.*, 1993). Furthermore, expression of Pax3 has been shown to direct mesenchymal stem cells to differentiate into a myogenic lineage (Gang *et al.*, 2008), suggestive that Pax3 could be used to alter ES cell transcription and specify a myogenic lineage (Darabi & Perlingeiro, 2008). This theory of lineage-specific reprogramming was tested by generating a doxycycline (dox) inducible Pax3 over-expressing ES cell line. This experiment was successful in inducing MRFs expression and generating ES cell-derived myogenic progenitors. However, *in vivo* injection of these cells into injured *tibialis anterior* (TA) mouse muscle resulted in tumour formation (Darabi *et al.*, 2008a). The tumour was defined as a teratoma, which contains undifferentiated cells of ectodermal and mesodermal origin (Darabi *et al.*, 2008b). Specific paraxial

mesoderm markers, which were up-regulated following Pax3 induction and for specified differentiated paraxial mesoderm tissue, were used to purify the ES cell-derived myogenic progenitors from the undifferentiated ES cell mass. Subsequent *in vivo* injection into *mdx* mice and immunodeficient mice resulted in the restoration of dystrophin expression and myosin heavy chain expression, respectively. The purified Pax3 lineage reprogrammed ES cells were successful in providing substantial engraftment of myogenic cells, without the formation of teratomas (Darabi *et al.*, 2008a), suggestive that stem cell therapy might be used in future therapy of muscular dystrophy disorders (Darabi *et al.*, 2008b).

1.6 Aims and Objectives

Myofibre nuclei are post-mitotic and incapable of re-entering the cell cycle. Therefore, skeletal muscle growth and regeneration requires a source of myogenic progenitors, other than the aforementioned cells. Satellite cells are a distinct lineage of quiescent myogenic progenitor stem cells that reside under the basal lamina that is present around muscle fibres. When required, satellite cells are activated to proliferate and differentiate into myoblasts that contribute to increasing muscle mass. The importance of Pax3 during embryonic myogenesis and tumorigenesis is well documented:

- Pax3 and proliferation: Pax3-FKHR expression results in the proliferative expansion of uncommitted myogenic precursor cells (Kurmasheva *et al.*, 2005). Furthermore, the proliferative potential of embryonic limb muscle progenitor cells decreases if Pax3 is replaced by Pax7 (Relaix *et al.*, 2004).
- Pax3 and differentiation: Pax3-FKHR expression maintains myogenic precursor cells in the cell cycle preventing myogenic commitment and thus inhibiting differentiation (Kurmasheva *et al.*, 2005). Pax3 expression also prevents pre-mature differentiation of hypaxial progenitor cells during limb myogenesis (Bober *et al.*, 1994).
- Pax3 and survival: Tumour cells expressing Pax3-FKHR have increased resistance to apoptosis (Kurmasheva *et al.*, 2005), facilitated through Pax3 interaction with the anti-apoptotic protein BCL-XL (Margue *et al.*, 2000).
- Pax3 and migration: Pax3 promotes the migration of hypaxial progenitor cells during embryonic myogenesis (Bober *et al.*, 1994), whether Pax3 plays a similar role in the migration of satellite cell-derived progenitors remains to be defined.
- Pax3 and myogenic gene expression: During embryonic-myogenesis Pax3 is in direct or indirect control over the myogenic regulatory factors Myf5 (Bajard *et al.*, 2006) and MyoD (Tajbakhsh *et al.*, 1997), respectively. The active post-natal Pax3 protein synthesis regulation pathways indicate a similar transcriptional power in post-natal muscle (Boutet *et al.*, 2007; Crist *et al.*, 2009).

The aim of this study was to elucidate the role(s) of the Pax3 gene in the development and regeneration of post-natal muscle. Although the role of Pax3 during embryonic skeletal muscle development and tumorigenesis is well researched, recent evidence cautions against inferring the function of post-natal muscle from embryonic studies (Lepper *et al.*, 2009). Furthermore, this same study suggested that Pax3 and Pax7 are not required for post-natal myogenesis (Lepper *et al.*, 2009). However, there is evidence that suggests an active post-natal transcriptional role for Pax3. Pax3 expression is highly regulated during post-natal growth, by either miRNA interference (Crist *et al.*, 2009) or proteasomal degradation (Boutet *et al.*, 2007). This illustrates the importance of maintaining the correct spatial-temporal gene expression of Pax3.

In this study the role of Pax3 in post-natal myogenesis was investigated via a gain of function assay by generating Pax3 over-expressing myoblast (C2C12 cells) cell lines. C2C12 cells have been shown to have similar population dynamics, proliferation and fusion rates as satellite cell-derived myoblasts, proving it to be a good *in vitro* model of post-natal myogenesis (Baroffio *et al.*, 1996; Yoshida *et al.*, 1998). The effect of Pax3 during proliferation, differentiation, survival, migration and myogenic gene expression were analysed with Pax3 over-expressing myoblasts to determine whether Pax3 plays an analogous role during post-natal myogenesis as observed during embryonic myogenesis.

Chapter Two: Materials and Methods

2.1 Materials

2.1.1 PCR oligonucleotide primers

All primers were custom synthesised by either Sigma or Invitrogen. Primer sequences are listed in Table 1. Oligonucleotides were re-suspended in 100 µl of MilliQ water and stored at -20°C. Working primer solutions for PCR were diluted to 10 µm with MilliQ water and also stored at -20°C. Invitrogen synthesised Pax3 primers were used in standard PCR amplification: these were also designed for mRNA quantification using semi-quantitative and quantitative PCR analysis.

Table 1 *PCR oligonucleotide primers*

Gene		Primer Sequence (5' TO 3')	Product Size (bp)	Use
Pax3	Fwd	GGATCCATGACCACGTGGCCGGCGC	1437	Standard PCR
	Rev	GCGGCCGCCTAGAACGTCCAAGGCTTAC		
Pax3	Fwd	GCCTCAGACCGACTATGCTC	234	RT-PCR
	Rev	GCGGCCGCCTAGAACGTCCAAGGCTTAC		
Myf5	Fwd	TGCCATCCGCTACATTGAGAG	353	RT-PCR
	Rev	CCGGGGTAGCAGGCTGTGAGTTG		
p21	Fwd	TCCAGGAGGCCCGAGAACG	428	RT-PCR
	Rev	GCTAAGGCCGAAGATGGGGAAGAG		
Tubulin	Fwd	GCTTCTTGTTTTCCACAG	450	RT-PCR
	Rev	TGCTGAGAAAGCCTACC		
H3.3A	Fwd	GGCTCGTACAAAGCAGACTGCC	225	RT-PCR
	Rev	GCAATTTCTCGACCAGACG		

2.1.2 Common Solutions

Common solutions used in this study are listed in the Appendix, and were prepared exactly as described by Ausubel *et al.* (1987) and Sambrook *et al.* (1989)

2.1.3 Enzymes

Enzymes used in this study are listed below in Table 2. Buffers for enzymatic reactions were always supplied with their respective enzyme and were used according to manufacturer's instructions.

Table 2 Enzymes

Enzyme	Source
<i>Taq</i> DNA polymerase	Roche
FastStart <i>Taq</i> DNA polymerase	Roche
T4 DNA ligase	Invitrogen
Rnase H	Invitrogen
Superscript II Reverse Transcriptase	Invitrogen
BamH1	Invitrogen
Not1	Invitrogen
EcoR1	Invitrogen
Apa1	Invitrogen

2.1.4 Antibodies

Antibodies listed in Table 3 were used for either immunocytochemistry or Western blot analyses.

Table 3 Antibodies

Primary Antibodies			
Antibody	Source	Description	Use
Pax3	DSHB*	Monoclonal mouse	Western
p21	Becton Dickinson	Monoclonal mouse	Western
MyoD	Becton Dickinson	Monoclonal mouse	Western
Myf5	Santa Cruz (Global)	Polyclonal rabbit	Western
Pax7	DSHB*	Monoclonal mouse	Western
Myogenin	Santa Cruz (Global)	Polyclonal rabbit	Western
Tubulin	Sigma Aldrich	Monoclonal mouse	Western
GapDH	Rsearch Diagnostics	Monoclonal mouse	Western
MyHC	DSHB*	Mouse	ICC
* DSHB - Developmental Studies Hybridoma Bank			
Secondary Antibodies			
Antibody	Source	Description	Use
P0447	Med Bio, Dako	Polyclonal goat anti-mouse	Western
P0448	Med Bio, Dako	Polyclonal goat anti-rabbit	Western
RPN1001V1	Amersham	Biotin sheep	ICC

2.1.5 Mammalian cell lines

C2C12 murine myoblasts (Yaffe & Saxel, 1977) sourced from the American Type Culture Collection (ATCC) were used for all cell culture experiments in these studies.

2.2 Methods

The initial methods in this chapter describe the standard molecular biology techniques used (Sections 2.2.1 – 2.2.6.7). Sections 2.2.7 and 2.2.8 specifically detail the use of these techniques in generating a Pax3-pcDNA3 vector, which was then transfected into C2C12 cells and clonal cell lines selected. The remaining sections describe the methods for functional assays to determine proliferation, differentiation, migration and apoptosis (Sections 2.2.9 to 2.2.15) of the Pax3 over-expressing cell lines and control cells. These techniques were used to determine whether Pax3 over-expression altered myoblast function *in vitro*.

2.2.1 Polymerase Chain Reaction (PCR)

2.2.1.1 Reverse transcription PCR

First strand cDNA synthesis for generation of cDNA templates for PCR was prepared using SuperScript First Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. Two micrograms of total RNA was used to prepare all cDNA samples using 1 µl of Oligo(dT)₁₂₋₁₈ primers, 1 µl of 10 mM dNTPs, made up to 10 µl with DEPC-treated water. This mixture was incubated for 5 min at 65°C followed by 1 min on ice. Nine microlitres of reaction mix containing 10 × RT buffer, 25 mM MgCl₂, 0.1 M DTT and RNaseOUT was added to the previous RNA mix and incubated at 42°C for 2 min. Reverse transcription was subsequently initiated with the addition of 1 µl of Superscript II reverse transcriptase and incubated for 50 min at 42°C. The reaction was terminated at 70°C for 15 min. Non-specific RNA was degraded following incubation with 1 µl RNaseH at 37°C for 20 min and stored at -20°C.

2.2.1.2 PCR amplification

PCR amplifications were carried out using *Taq* DNA polymerase in a 50 μ l reaction mix that contained 1 \times PCR buffer, 0.2 μ M of both forward and reverse primers, 0.2 mM of dNTPs and 2 μ l of template. Thermocycling for PCR reactions were carried out in a Hybaid MBS 0.5S PCR system (Hybaid, Ashford, United Kingdom). Pax3 was the only gene amplified via routine PCR, the PCR conditions used were: 90°C for 30 s, 58°C for 1 min and 72°C for 1 min, repeated for 35 cycles. The reaction was completed with a final elongation step of 72°C for 5 min followed by a 4°C cooling period. Negative controls were also set-up containing 2 μ l of MilliQ water instead of template DNA to check for contamination. Standardisation of PCR amplification was performed on cDNA templates derived from *tibialis anterior* muscle of a 3 week old *mdx* mouse and a Myostatin-null mouse.

2.2.1.3 Semi-quantitative PCR

Semi-quantification of cDNA templates were performed using the same materials and method outlined in Section 2.2.1.2. To determine the cycle number range that represents the linear increase in amplification, cDNA samples were amplified with 20, 25, 30 and 35 cycles of PCR. The resulting amplicons were run on 1% agarose gels stained with ethidium bromide as outlined in Section 2.2.2.1, for size determination and quantified on a GS-800 calibrated densitometer (Bio-Rad). The cycle number that allowed for the best quantification over the range of cDNA templates to be measured was chosen to be used during experimental work.

2.2.1.4 Quantitative PCR (Real-Time PCR)

Real-Time quantification of cDNA templates were carried out using the LightCycler® FastStart DNA MasterPlus SYBR Green kit (Roche) according to the manufacturer's instructions. Reactions were performed in 20 μ l capillaries and amplified using the LightCycler 2.0 Carousel-based PCR system (Roche Applied Sciences, Nonnenwald 2, 82372 Penzberg, Germany). Real-Time PCR MasterMix

was prepared as required: the MasterMix contained the FastStart *Taq* DNA polymerase, reaction buffer, MgCl₂, SYBR Green I dye and dNTP mix.

Capillaries were placed in a pre-cooled capillary lead-block, with additional capillaries added for negative control and calibration samples. Reaction mixes were made up consisting of 4 µl of MilliQ water, 0.5 µl of both sense and antisense primers and 2 µl of MasterMix for each sample to be quantified and then aliquoted into the top of each capillary. Three microlitres of cDNA template were added to the reaction mix in the capillaries and mixed by pipeting. Capillaries were placed in a centrifugation carousel and centrifuged at $700 \times g$ (3000 rpm) for 5 s in the LC Carousel Centrifuge 2.0 (Roche). The capillaries were transferred to the LightCycler 2.0 machine, for amplification and quantification.

A protocol of pre-incubation and denaturation, quantitative amplification, melting curve and cooling was followed under the control of the LightCycler software 4.0. All Real-Time PCR analyses completed for these studies used the following cycling conditions for quantitative amplification, 95°C for 5 s, 60°C for 10 s and 72°C for 10 s, for 45 cycles. Melting curves followed amplification, following 60 s at 65°C the temperature was increased at a rate of 0.2°C/sec for product identification.

For quantification, standard curves were generated for each primer set using serial dilutions of cDNA template derived from actively growing, Pax3 stable, over-expressing C2C12 cells and non-transfected C2C12 cells. Calibration standards were subsequently amplified alongside samples to standardise each run. To determine that the melting curve temperature matched the correct product size of amplification, following initial amplification of each primer set, capillaries were placed in 1.7 ml tubes and centrifuged at $2000 \times g$ for 2 min. The amplification products were then run on a 1% agarose gel for size determination, as outlined in Section 2.2.2.1.

2.2.2 Gel electrophoresis

2.2.2.1 DNA electrophoresis

Agarose gels (Invitrogen) were prepared appropriate to the size of the DNA fragments to be separated, ranging between 0.6 - 1.2%. Gels were made up with 1 × TAE, which was also used as the running buffer, and cast in gel boxes (Invitrogen), with 300 ng/ml of ethidium bromide added for DNA visualisation. Prior to loading, DNA samples were mixed with 10 × DNA loading dye. A 1 Kb+ DNA ladder (Invitrogen) was loaded alongside samples for size determination. Electrophoresis was carried out at 70-120 V. DNA was visualised under ultraviolet (UV) light (312 nm) using a Gel Doc System (Bio-Rad Laboratories, Hercules, CA) and photographed.

2.2.2.2 RNA electrophoresis

RNA was fractionated by electrophoresis on formaldehyde/agarose gels and 18s and 28s ribosomal bands were examined to verify the integrity of extracted RNA. RNA gels were prepared containing 1% agarose dissolved in DEPC-treated water, then 1 × MOPS and 0.66 M formaldehyde was added. Gels were cast in Invitrogen RNA gel boxes and run with 1 × MOPS buffer. Initially, RNA samples were quantified using the NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies Inc., Wilmington, DE, USA). Prior to loading, 2 µg of RNA was mixed with an equal amount of RNA loading dye, containing ethidium bromide, which was heated to 65°C for 5 min. The desired separation of RNA bands was obtained with electrophoresis proceeding at 70 V. RNA bands were visualised and photographed using the Gel Doc system (Bio-Rad). The densities of 18s and 28s ribosomal bands were measured on a GS-800 calibrated densitometer (Bio-Rad) to verify accurate quantification and assess integrity of the RNA.

2.2.2.3 Protein electrophoresis

To estimate the concentration of protein samples to be separated via electrophoresis, a Bradford assay was used (Bradford, 1976). The Protein Assay Dye Reagent (Bio-Rad) was diluted to 1:5 using MilliQ water. Bovine serum albumin (BSA) was used to prepare the standards, ranging in concentration from 0 to 10 mg/ml. The standards and samples were made up to a volume of 100 μ l, with the samples containing 1 μ l of protein. The assay reaction mixture contained 1.2 ml of dye with 100 μ L of sample or standard and mixed by inversion. The absorbance was measured at 595 nm using a Helios UV spectrophotometer (Thermo Spectronic, Cambridge, U.K) and protein concentrations were estimated from the BSA standard curve.

Protein electrophoresis was carried out on pre-cast NuPage 4-12% gradient Bis-Tris SDS polyacrylamide gels (Invitrogen), using 1 \times NuPage MES SDS running buffer in Novex Mini Cells (Invitrogen). Following protein estimation and prior to loading, 15 μ g of each protein sample was boiled for 5 min in the presence of a 1:4 β -mercaptoethanol:4 \times loading dye. Protein samples were loaded alongside a SeeBlue Plus 2 pre-stained standard (Invitrogen) for size determination. Electrophoresis was performed at a constant current of 50 mA until the desired level of separation was obtained.

2.2.3 Enzymatic Reactions

2.2.3.1 DNA purification

The Promega Wizard DNA purification system was used to purify PCR amplicons for cloning. PCR products (50 μ l) with loading dye was run on a 0.8% low melt agarose gel (Mercury) at 70 V (see Section 2.2.2.1) then specific fragments corresponding to expected sizes were excised from the gel under UV light using a razor blade. Each excised agarose fragment was heated to 65°C in a 1.7 ml eppendorf tube for 5 min. 1 ml of Wizard purification resin was mixed with the melted gel and extracted DNA. Using a syringe the resin/DNA was purified from the agarose by passing through a mini-column and then washing twice in 2 ml 80% isopropanol. The purified resin/DNA was collected by centrifuging the

mini-columns at $10,000 \times g$ for 2 min; purified DNA was eluted following a 1 min incubation period with MilliQ water and briefly centrifuging at $10,000 \times g$.

2.2.3.2 Restriction endonuclease digestion

Restriction endonuclease digests were performed at 37°C for 1-2 h using the appropriate $10 \times$ buffer for the specific restriction enzyme used. 10-20 U of enzyme were used for each digest depending on the DNA concentration. Following restriction digests, fragment size was verified by gel electrophoresis (see Section 2.2.2.1) and specific fragments were purified using the wizard kit (see Section 2.2.3.1).

2.2.3.3 DNA Ligation

Restriction endonuclease digestions on DNA inserts or cloning vectors (see Section 2.2.3.2) were used to generate complementary 3' and 5' extensions for cloning. Ligation reactions used 1 U of T4 DNA ligase to clone a 3-fold molar excess of purified DNA inserts (see Section 2.2.3.1) into 25-50 ng of linearised cloning vector, using the respective $10 \times$ ligation buffer. Ligation reactions were performed overnight at 4°C .

2.2.4 Transformation and growth of bacteria

2.2.4.1 Transformation of competent cells

Transformation of *E.coli* strain DH5 α (Invitrogen) and Top10 (Invitrogen) cells were performed following ligation reactions (see Section 2.2.3.3). Ligation mix (4 μl) was added to a 50 μl aliquot of competent cells, which was incubated on ice for 30 min. The transformation mixture was then heat shocked at 42°C for 45 s and subsequently placed on ice for a further 2 min. 450 μl of SOC LB medium (Invitrogen) was then added to the mixture and incubated in a shaking incubator at 37°C for 1 h. During this time, using aseptic techniques, 100 μl of 20 mg/ml X-gal and 2.5 μl of 200 mg/ml IPTG were spread onto ampicillin (50 $\mu\text{g}/\text{ml}$) agar plates. Following the incubation period, 200 μl of the transformation

was also spread onto the same agar plates. The plates were incubated overnight at 37°C.

2.2.4.2 Culturing of Bacteria

Positive colonies that contained the cloning vector following transformation (see Section 2.2.4.1) or obtained from glycerol stocks, were seeded in 5 ml LB broth containing 50 µg/ml ampicillin. Cultures were grown in 50 ml plastic tubes (Nalge Nunc International) for small scale culture and in 1 L flasks for large scale culture, at 37°C in a shaking incubator (250 rpm) overnight.

2.2.5 Plasmid DNA extraction

2.2.5.1 Miniprep plasmid DNA extraction

Following overnight growth of Miniprep cultures (see Section 2.2.4.2), cells were lysed and DNA extracted and purified exactly as described by the manufacturer's protocol (Qiagen, QIAprep spin Miniprep system). Briefly, bacteria were harvested via centrifugation at 3500 rpm for 5 min. Buffer P1 (250 µl) followed by buffer P2 (250 µl) were added to the bacterial pellet. Each sample was inverted several times and left to incubate for 5 min. Buffer P3 (350 µl) was subsequently added and mixed as previously. The supernatant containing plasmid DNA was obtained via centrifugation at 20,000 × *g* for 1 min and removed to a QIAprep spin column, where centrifugation was repeated. Buffer PE (750 µl) was used to wash the spin column, which was centrifuged twice to remove any remaining buffer. MilliQ water (50 µl) was used to elute the plasmid DNA from the spin column, which was collected by centrifugation following a 1 min incubation period.

2.2.5.2 Maxiprep plasmid DNA extraction

Following overnight growth of Maxiprep cultures (see Section 2.2.4.2) cells were lysed and DNA extracted and purified exactly as described by the manufacturer's protocol (Qiagen, plasmid Maxi kit). Cells were harvested from

250 ml culture via centrifugation at $6000 \times g$ for 15 min. The pellet was re-suspended with sequential addition of 10 ml buffer P1, followed by mixing with buffer P2 and buffer P3. Re-suspended cultures were incubated on ice for 20 min, followed by centrifugation at $20,000 \times g$ for 30 min and $20,000 g$ for 15 min. The supernatant containing plasmid DNA was purified by passing the supernatant through a Qiagen column by gravity flow. Retained plasmid DNA was washed twice with buffer QC and eluted in the elution buffer. Isopropanol was used to precipitate purified plasmid DNA, which was collected via centrifugation at $20,000 \times g$ for 10 min. The resulting pellet was washed twice with 70% ethanol and left to air dry. MilliQ water was used to re-suspend plasmid DNA, with the volume determined according to the size of the pellet.

2.2.6 Mammalian Cell Culture

2.2.6.1 Culturing of C2C12 myoblasts

C2C12 cells were maintained in DMEM proliferation medium (Life Technologies, Grand Island, NY, USA) containing penicillin (1×10^5 IU/I) and streptomycin (100 mg/l, Sigma Cell Culture Ltd, St Louis, MO, USA) and 10% foetal bovine serum (FBS) (Invitrogen), buffered with NaHCO_3 (41.9 mM/l, Sigma) and gaseous CO_2 . Phenol red (7.22 mM/l, Sigma) was used as a pH indicator. Unless otherwise stated, cells were grown and manipulated on 10 cm plates (BD Falcon Biosciences Discovery Labware, Bedford, MA) and incubated at an atmosphere of 37°C , 5% CO_2 .

2.2.6.2 Differentiation of C2C12 myoblasts

To induce differentiation of C2C12 cells, medium was switched to differentiation medium, which was exactly as described for the proliferation medium except that 2% horse serum (HS) replaced 10% FBS.

2.2.6.3 Trypsinisation and passage of C2C12 myoblasts

To passage C2C12 cells, the medium was removed and cells were washed twice with phosphate buffered saline (PBS). To detach cells from plate 5 ml of 1 × trypsin was added for 30 s at room temperature to washed cells, following which 4.5 ml of the 1 × trypsin was removed and then the plate was incubated at 37°C for a further 10 min. For a routine media change, cells were re-suspended in 3 ml proliferation media and added to a fresh plate containing 7 ml of proliferation media, while gently swirling. Alternatively, if specific cell densities were required, re-suspended cells were quantified using a haemocytometer and diluted in the appropriate volume of proliferation media. Freshly plated cells were incubated overnight.

2.2.6.4 Transfection of murine C2C12 myoblasts

C2C12 cells were grown as described in Section 2.2.6.1, until 70-80% confluent. Lipofectamine 2000 (Invitrogen) was used for transfection of DNA plasmids according to the manufacturer's instructions. In separate tubes, 800 µl of serum-free DMEM was added to each of 12.5 µg of construct DNA (tube 1) and 40 µl of Lipofectamine 2000 (tube 2). The contents of tube 2 was added drop-wise to tube 1 while aeration was applied to tube 1 using an auto-pipette, followed by incubation at room temperature for 20 min. The resulting transfection mix was gently mixed with 8.4 ml of proliferation medium then pipetted onto a 10 cm plate of pre-washed C2C12 cells. Transfected cells were incubated overnight. For a transient transfection, a media change with proliferation media was performed the following day and transfected cells were incubated until they reached 70-80% confluence. Protein was then collected using the protocol outline in Section 2.2.6.7.

2.2.6.5 Selection of stable transfected constructs in C2C12 myoblasts

C2C12 myoblasts were transfected following the protocol described in Section 2.2.6.4. Following overnight incubation, transfected cells were passaged (see Section 2.2.6.3) at three separate clonal densities on 10 cm plates (1000

cells/plate, 2000 cells/plate and 3000 cells/plate) and incubated for 24 h. Transfection of plasmid DNA into C2C12 cells conferred geneticin antibiotic resistance allowing DMEM containing geneticin (0.6 mg/ml) to be used to select for stably transfected cells. Initially geneticin was added with fresh proliferation medium and incubated for 4 d, after which proliferation medium containing geneticin was routinely replaced as required. Transfected cells were grown until over 75% of the colonies filled the 400 × magnification field of view of a compound microscope. Colonies were initially selected by circling the entire colony on the bottom of the plate with a fine tip pen. Medium was removed from the plate and by gently using a 10 µl pipette individual colonies were scraped and removed with 2 µl of media. Each of the transfected colonies were transferred to 24 well plates and grown in 6 ml of proliferation media containing geneticin. When each colony reached sub-confluence (70-80%) they were passaged and transferred to a 6 well plate in 4 ml of proliferation media with geneticin, where they were maintained as triplicate cell lines.

2.2.6.6 Harvesting RNA from C2C12 myoblasts

Proliferation medium was removed from cells grown on 6 well plates; the cells were washed twice with 4 ml PBS, and a 1 ml pipette was used to ensure complete removal of medium and wash. To lyse cells, 1 ml of Trizol reagent (Invitrogen) was added to cells and repeatedly rinsed over the well. Trizol lysates were collected in 1.7 ml tubes. Trizol lysates were incubated for 5 min at room temperature, and then 0.2 ml of chloroform was added per 1 ml of Trizol. Samples were shaken vigorously for 15 s and incubated at room temperature for 3 min, then phases were separated via centrifugation at 12,000 × *g* for 15 min. All centrifugation during RNA isolation was performed at 2-8°C. The top aqueous layer containing total RNA was carefully collected without disturbing lower layers and transferred to a fresh 1.7 ml tube. RNA was precipitated using 0.5 ml isopropanol for every 1 ml of Trizol originally used and incubated at room temperature for 10 min. The RNA precipitate was pelleted by centrifugation at 12,000 × *g* for 10 min. Supernatant was removed and the RNA pellet washed with 1 ml 75% ethanol, followed by vortexing and centrifugation at 7,500 × *g* for 5 min. Following the wash, the supernatant was removed and the pellet was air-

dried for 5 min. Total RNA was re-suspended in DEPC-treated water, the volume depending on the size of the pellet, and incubated at 55°C for 5-10 min until pellets were re-suspended. Total RNA was stored at -80°C until cDNA was generated (see Section 2.2.1.1).

2.2.6.7 Harvesting protein from C2C12 myoblasts

Proliferation medium was removed from cells grown on 6 well plates; the cells were washed twice with 4 ml PBS, and a 1 ml pipette was used to ensure complete removal of medium and wash. Cells were scraped off the plate surface using 100 µl of protein lysis buffer and transferred to a 1.7 ml tube. Cells were passed through a 25 gauge needle ten times to further lyse cells. Cells were centrifuged at 13,000 rpm for 2 min, to pellet cellular debris; protein extract was collected in the supernatant and stored at -20°C until further analysis (see Section 2.2.2.3). Cells grown on 10 cm plates were washed in 10 ml volumes of PBS and harvested in 200 µl protein lysis buffer.

2.2.7 Generation of Pax3-pcDNA3 over-expressing construct

The 1437 bp murine *Pax3* cDNA sequence (NM_008781.3) was amplified using standard PCR (see Section 2.2.1.2). Pax3 PCR products were run on a 0.8% agarose gel to determine size (see Section 2.2.2.1), and correct sized amplicons were wizard purified and eluted in MilliQ water (see Section 2.2.3.1). Purified Pax3 PCR products were subsequently ligated into the multiple cloning region of a pGEMTeasy vector (Promega) (see Section 2.2.3.3), which was chosen for initial cloning due to its ease of ligation with PCR products. Following ligation, the pGEMTeasy cloning vector containing the Pax3 insert was transformed into Top10 cells (see Section 2.2.4.1). Plates containing transformed cells were incubated overnight at 37°C, and blue/white screening was used to identify colonies containing the Pax3-pGEMTeasy cloning vector. The pGEMTeasy vector contains a Lac operon that encodes for β-galactosidase enzyme that uses X-gal as a substrate and IPTG as an inducer. Successful ligation of Pax3 DNA will interrupt the Lac operon preventing X-gal metabolism and therefore these colonies remain white. Positive white colonies were selected and cultured

overnight according to Section 2.2.4.2. Purified cloning vector containing the Pax3 insert was obtained from the cultured cells using the Miniprep plasmid DNA extraction kit (see Section 2.2.5.1). To further confirm presence of the Pax3 insert within the pGEMTeasy vector an endonuclease restriction digest was carried out (see Section 2.2.3.2). Apa1 restriction enzyme was used, which cuts at two sites within the Pax3-pGEMTeasy vector: once within Pax3 at 14 bp and once again in pGEMTeasy at 1127 bp, resulting in two fragments, a 1165 bp and a 3285 bp length of DNA. Miniprep digests were run on a 0.6% agarose gel; presence of the two specific DNA fragments confirmed the presence of the insert within pGEMTeasy.

The pcDNA3 expression vector (Invitrogen) was selected to over-express Pax3, which was prepared for directional cloning using an endonuclease restriction digest with BamH1 and EcoR1 enzymes (see Section 2.2.3.2). The Pax3 insert was excised from the pGEMTeasy cloning vector using the same BamH1/EcoR1 restriction digest and thus resulting in complimentary 5' and 3' ends. The Pax3 insert was sub-cloned into the linearised pcDNA3 expression vector (see Section 2.2.3.3) and subsequently transformed into competent Top10 cells (see Section 2.2.4.1). Transformed colonies were used as templates for Pax3 PCR amplification using Pax3 primers (see Section 2.2.1.2). Positive colonies determined via colony PCR were cultured overnight (see Section 2.2.4.2). The cloned Pax3-pcDNA3 over-expression vector was subsequently harvested using Maxiprep plasmid DNA extraction kit (see Section 2.2.5.2). A diagnostic digest was used to further confirm the presence of the Pax3 insert, and the restriction endonuclease enzyme used was Apa1 (see Section 2.2.3.2). The Apa1 enzyme cleaves once within the Pax3 sequence and once within the pcDNA3 vector, thus in the presence of the Pax3 insert, two fragments would result from the digest; 368 bp and 6487 bp. Also, an EcoR1/BamH1 restriction digest was repeated, validating Pax3 insert presence by releasing the 1437 bp insert.

2.2.8 Generation of Pax3-pcDNA3 over-expressing C2C12 cell line

The Pax3-pcDNA3 over-expression construct (see Section 2.2.7) and an empty pcDNA3 vector were transfected into mammalian C2C12 cells according to Section 2.2.6.4. The pcDNA3 over-expression vector is driven by a CMV promoter and contains cassettes for geneticin and ampicillin resistance. These attributes were used to select for stably transfected C2C12 colonies, according to the protocol in Section 2.2.6.5. Protein lysates (see Section 2.2.6.7) and RNA (see Section 2.2.6.6) were harvested from stably transfected clones and analysed for Pax3 expression using western blot analysis (2.2.11), semi-quantitative PCR (see Section 2.2.1.3) and quantitative PCR (see Section 2.2.1.4).

2.2.9 Methylene blue proliferation assay

Prior to assay, C2C12 cells and Pax3 transfected C2C12 clones were grown in DMEM medium as described in Section 2.2.6.1. Cell proliferation was assessed in uncoated 96-well Nunc microtitre plates (Roskilde, Denmark). C2C12 cultures were seeded at 1000 cells/well in proliferation media; each assay contained eight replicates of each cell line. After a 24 h attachment period, medium was replaced with fresh proliferation medium. Cells were fixed following incubation periods of 0, 24, 48, 72 and 96 h. Proliferation was then assessed using the colourimetric end point assay described by Oliver *et al.* (1989). Briefly, proliferation medium was removed and cells washed once with PBS then fixed for 30 min in 10% formol saline. The fixed cells were then stained for 30 min with methylene blue (Merck) in 0.01 M borate buffer (pH 8.5, Sigma). Four sequential washes with borate buffer removed excess stain. Methylene blue was eluted by the addition of 100 μ l of 1:1 (v/v) ethanol and 0.1 M HCl. The plates were then gently shaken for 30 seconds and the absorbance was read at 655 nm for each well by a microplate photometer (BioRad model 3550 microplate reader, BioRad, Hercules, CA, USA). Absorbance at 655 nm is linearly proportional to cell numbers.

2.2.10 Differentiation Assay

Prior to assay, C2C12 cells and Pax3 transfected C2C12 clones were grown in proliferation medium (see Section 2.2.6.1). Cells were subsequently seeded at a density of 20,000 cells/cm². For analysis of protein and RNA, cells were seeded on 10 cm plates. For Myosin Heavy Chain (MyHC) immunocytochemistry, cells were seeded on Thermanox coverslips (Nunc) inside the wells of 24 well plates (Nunc) and on Microtest™ 96 well microtitre black walled clear bottom assay plates (Optilux™), for non-fluorescent and fluorescent analysis, respectively. Following a 24 h attachment period, C2C12 myoblasts were induced to differentiate with the removal of proliferation medium and addition of differentiation medium (see Section 2.2.6.1). Plates were incubated for up to 96 h, with cells fixed for staining (see Section 2.2.12) or harvested for protein (see Section 2.2.6.7) and RNA (see Section 2.2.6.6) at their respective times.

2.2.11 Western blot analysis

Protein samples collected from C2C12 cells and Pax3 transfected C2C12 clones (see Section 2.2.6.7) were separated on a protein gel as described in Section 2.2.2.3. The protein gel was washed with MilliQ water and transferred to a Nitrocellulose membrane (Invitrogen) by electroblotting using the Invitrogen iBlot™ machine. The transferred membranes were stained in Ponceau for 5 min, to verify gel loading accuracy and transfer integrity. Ponceau stain was removed through washing with TBST buffer, followed by blocking to prevent non-specific binding of antibodies. Membranes were either blocked in 5% skim milk in TBST overnight at 4°C or in 0.3% BSA block for 1 h at room temperature. The membranes were subsequently probed with the primary antibody, which was diluted in the same solution that was used to block the respective membrane. Membranes were subsequently washed 5 times with TBST for 5 min, followed by incubation with the respective secondary antibody. The secondary antibodies used were conjugated to Horseradish Peroxidase (Dako) which used Western Lightning (PerkinElmer) Western Blot Chemiluminescence Reagent for detection.

Chemiluminescence was performed on the membranes following a final wash with TBST.

Specific primary and secondary antibody dilutions and incubation times for each western blot analysis used are described in Table 4. For more detailed description of antibodies refer to Section 2.1.4.

Table 4 Western blot protocols

Gene	Primary Dil.	Incubation	Secondary Anti.	Secondary Dil.	Incubation
MyoD	1:10,000	O/N 4°C	PO447	1:5,000	1 hr RT
p21	1:400	3 hr RT	PO447	1:2,000	1 hr RT
Myogenin	1:400	3 hr RT	PO448	1:2,000	1 hr RT
Pax7	1:500	3 hr RT	PO447	1:2,000	1 hr RT
Pax3	1:500	3 hr RT	PO447	1:2,000	1 hr RT
Myf5	1:400	3 hr RT	PO448	1:2,000	1 hr RT
Tubulin	1:10,000	1 hr RT	PO447	1:10,000	1 hr RT
GapDH	1:20,000	1 hr RT	PO447	1:20,000	1 hr RT

2.2.12 Immunostaining

2.2.12.1 MyHC immunocytochemistry

Two MyHC immunocytochemistry experiments were performed using differentiated C2C12 cell lines, on two different types of plates as described in Section 2.2.10. Non-fluorescent visualisation of MyHC immunostaining performed on coverslips, used washing volumes of 1 ml and antibody dilution volumes of 200 µl. Fluorescent visualisation of MyHC immunostaining performed on 96 well microtitre plates, used washing volumes of 200 µl and antibody dilution volume of 100 µl.

Myoblast cultures were washed with PBS buffer before being fixed with 70% ethanol:formaldehyde:glacial acetic acid (20:2:1) for 30 s, and subsequently washed in PBS three times. Cell membranes were then permeabilized in PBS+0.1% Triton X-100 at room temperature for 60 min. Cells were washed with 1 × TBST on a shaking platform at 50-100 rpm at room temperature for 5 min prior to blocking with 0.35% carrageenan (Cλ, Sigma, C3889 λ-Carrageenan Type IV) in PBS plus 10% normal sheep serum (NSS) for 60 min at room temperature.

Blocking solution was then replaced with the primary antibody, 1:200 dilution of mouse anti-MyHC (MF20; DSHB), in C λ containing 5% NSS, and incubated overnight at 4°C. Cells were then washed with 1 \times TBST three times on a shaking platform at 50-100 rpm at room temperature for 5 min. The secondary antibody, 1:300 dilution of biotin sheep anti-mouse (RPN1001VI, Amersham), in C λ containing 5% NSS was added for 60 min at room temperature. Cells were washed in TBST as before and incubated with tertiary antibody, which depended on the type of nuclear counter-staining to be used.

Fluorescent assessment of differentiation performed on 96 well Optilux™ microtitre plates used a conjugated fluorophor: 1:400 dilution of Alexa Fluor 488 (Invitrogen) in C λ containing 5% NSS for 60 min at room temperature. Cells were washed with TBS as previously described, and nuclei were counter-stained with DAPI at a 1:1000 dilution in PBS for 5 min at room temperature. Cells were washed in TBST twice, as previously described, and stored at 4°C prior to visualisation. MyHC-immunostaining and nuclei counter-staining were visualised on a Leica DMI 6000B Fluorescence and Light Microscope (Leica Microsystems, Heidelberg, Germany), using Leica AF6000 fluorescence system software at 200 \times magnification. To visualise Alexa Fluor 488 immunostained MyHC, an excitation filter of 495 nm and emission filter of 519 nm were used. To visualise DAPI nuclei stain, an excitation filter of 358 nm and emission filter of 461 nm were used. Eight random images were acquired from each of the differentiated cell lines, Image-Pro Plus computer software was used to analyse the images, which automatically counted the total number of nuclei. Myotube nuclei were manually counted from each image; the fusion index was determined by dividing the number of myotube nuclei by the total number of nuclei.

Non-fluorescent assessment of differentiation performed on Thermanox coverslips used a DAB chromogen (3,3'-diaminobenzidine tetrachloride, Sigma D-5637): a 1:300 dilution of Steptavidin biotinylated horseradish peroxidase complex (RPN1051V, Amersham) in C λ containing 5% NSS for 60 min at room temperature. Cells were washed in TBS as previously described, and developed with DAB, freshly prepared prior to use. Cells were incubated at room temperature for 2-3 min, and the development of a brown precipitate where the primary MyHC antibody had bound was monitored under a microscope. The reaction was stopped with the removal of the chromogen reagent and cells were

washed twice in TBS. Coverslips were transferred to a coverslip rack were MyHC-immunostained cultures were counterstained with Gills haematoxylin, dehydrated and mounted (see Section 2.2.12.2).

2.2.12.2 Haematoxylin cell staining and Visualisation

Non-fluorescent MyHC-immunostained cultures on coverslips (see Section 2.2.12.1) were incubated in 1:1 Gills haematoxylin for 2-4 min and rinsed in running tap water, followed by three washes in Scott's water for 2 min before counterstaining with 1% eosin for 2 min. The cells were then rinsed in running tap water before being dehydrated with sequential washes of increasing concentration of ethanol ($2 \times 70\%$, $2 \times$ absolute ethanol) for 5 min, followed by two washes in xylene for 5 min. The coverslips were subsequently mounted onto slides, stained cells face down, with DPX solution (BDH) and left to dry for 48 h.

Immunostained cellular images were acquired using a Leica CTR 6500 microscope (Leica Microsystems, Heidelberg, Germany) with $200 \times$ magnification. The Leica Microsystems application suite generated a tiling array of each coverslip to be analysed (500 images per coverslip). Microsoft Excel was used to generate four random numbers; these numbers corresponding to the image number in the tiling array, which were checked to ascertain the images selected were full and uncompromised. Non-fluorescent analyses of differentiating C2C12 and Pax3 over-expressing C2C12 clones were performed on coverslips as triplicates, with the corresponding 4 images from each coverslip being analysed. The four selected images from the original 500 images from each coverslip were analysed using Image-Pro Plus computer software; however, myotube nuclei number and the total nuclei number were manually counted to determine the fusion index.

2.2.13 Apoptosis assay

A fluorimetric homogeneous caspase assay (Roche) was used to determine caspase activity from C2C12 cells, when induced to apoptose by incubation in serum free medium in comparison to treatment with proliferation medium (10% FBS). Prior to assay, control and Pax3 over-expressing clones were grown as

described in Section 2.2.6.1, subsequently cells were seeded at a density of 40,000 cells/well on Microtest™ 96 well microtitre black walled clear bottom assay plates (Optilux™) and incubated for 16 h. Cells were washed three times with PBS before addition of treatment media, either serum free or 10% FBS (basal apoptosis level), and incubated for 8 h. Two wells were also set-up as reagent blank media only, which contained no cells, and two wells were left empty for positive controls. Working solutions for the apoptosis kit are unstable and were prepared immediately before use: the caspase substrate working solution was diluted 1:10 with incubation buffer and positive control lysates were also diluted 1:10 with incubation buffer. Following the 8 h incubation time, 100 µl of diluted positive control lysate was added to the positive control wells, followed by 100 µl of diluted substrate working solution being added to each well in the assay. Plates were wrapped in aluminium foil and incubated for a further 1-2 h at 37°C. Free R110 levels, which are proportional to the amount of activated caspases present, were fluorimetrically measured following 5 s of shaking, using an excitation filter of 499 nm and emission filter of 521 nm on a Bio-Strategy Synergy 2 multi-mode plate reader (BioTek Instruments, Winooski, US) using Gen5™ software (BioTek).

2.2.14 Migration assay

A migration assay was performed on control and Pax3 over-expressing C2C12 clones. The chemo-attractant media used was DMEM containing 2% HS plus 5% CEE (optimal) or DMEM containing only 5% CEE (intermediate) or 2% HS (suboptimal), a negative control containing only DMEM was also tested. Chemotaxis was assessed using cell culture inserts containing polyethylene terephthalate 0.8 µm membranes (BD Biosciences, San Jose, CA).

Prior to assay, insert membranes were treated with 1% Matrigel in DMEM for 30 min and left to dry. The above four test media treatments were aliquoted in 900 µl volumes, into a 24-well plate, the inserts were then placed inside these wells. Seventy-five thousand cells were aliquoted in a 350 µl volume into each insert and the plates were incubated for 7 h at 37°C. After incubation, cells remaining on the top of the membrane, which had not migrated, were removed with pre-wet swabs. A scalpel blade was used to cut the membrane from the

insert, which was then fixed in 70% ethanol:formaldehyde:glacial acetic acid (20:2:1) for 30 s. The membranes were washed in PBS before being stained in Gill's haematoxylin for 2 min and subsequently washed twice in Scott's water for 1 min. The membranes were wet-mounted onto slides and viewed using an Olympus BX50 Microscope (Olympus Optical Co., Germany) with attached camera. Images were taken at 100 × magnification, using Spot Basic software (Spot software, Diagnostic Instruments Inc.).

2.2.15 Statistical methods

Data obtained from multiple wells for Pax3 quantification, proliferation, apoptosis and both differentiation assays, were pooled to give a population mean (\pm SEM), and data from multiple experiments had the mean of the mean (\pm SEM) calculated. A one-way analysis of variance (ANOVA) was performed on pooled means to determine the level of variance, if the ANOVA analysis was significant than comparison between individual means were made. A Post-hoc T-test was used to determine the significant level of difference between control cells and Pax3 over-expressing clones.

For the migration assay, cell counts were averaged for each membrane and then averaged again for the total three membranes. A two-way ANOVA was performed to determine whether the differences among the four cell lines were caused by the three different treatments and not random variation. The two-way ANOVA was performed on log transformed cell counts; comparisons with either control or optimal treatment were performed using the Dunnetts test.

All statistical analyses were performed using the Genstat (version 11) computer software.

3.0 Results

3.1 Stable over-expression of Pax3

3.1.1 Generation of Pax3-pcDNA3 over-expressing construct

The paired box transcription factor 3, Pax3 is a powerful myogenic transcriptional controller during embryo-myogenesis (Maroto *et al.*, 1997). However, the role of Pax3 in post-natal myogenesis is yet to be fully determined. To ascertain whether Pax3 plays an active transcriptional role in post-natal myogenesis, or if it acts as a marker for myogenic stemness, stable over-expressing Pax3-pcDNA3 C2C12 cell lines were generated (see Section 2.2.8). The Pax3 1437 bp cDNA (NM_008781) open reading frame was successfully amplified from a 3 week old *mdx* mouse mRNA template and a Myostatin-null mouse mRNA template (Figure 3.1a). Pax3 PCR amplicons from both templates were subsequently cloned into the multiple cloning site of the pGEMTeasy cloning vector. Blue/White screening produced a combination of white and blue colonies, of which the positive white colonies were selected. Pax3-pGEMTeasy DNA was harvested and purified, and an ApaI diagnostic digestion was performed to confirm the presence of the Pax3 insert in the pGEMTeasy cloning vector (Figure 3.1b). Following an EcoRI/BamHI restriction digestion the Pax3 insert was sub-cloned into the pcDNA3 over-expression vector. Following transformation into competent cells, colony PCR was successful in selecting positive colonies through amplification of the Pax3 insert. An ApaI diagnostic digestion further confirmed successful sub-cloning into pcDNA3, as did an EcoRI/BamHI digestion, which released the 1437 bp Pax3 insert (Figure 3.1c).

The Pax3 insert in the pcDNA3 vector was sequenced and compared to Pax3 sequence (NM_008781). Results revealed that all clones contained one common mutation in the Pax3 insert, resulting in an amino acid change of glycine being substituted with aspartic acid. This was determined to be a real change, as various templates showed the same substitution at amino acid position 419 in the coding sequence. This was positively confirmed in the electropherogram as resulting from a single nucleotide change of adenine for guanine. The Pax3 sequence (NM_008781) that was used to design Pax3 primers coded for a

transcriptional variant of Pax3. Protein sequences from the Pax3 transcriptional variant (NM_008781) and murine Pax3 complete coding sequence (CDS) (BC048699) were aligned (Figure 3.1d). Both sequences were identical except for the single amino acid substitution at position 140 in the protein sequence and the terminal six amino acids. Due to the presence of the single amino acid substitution in multiple templates, including the Pax3 CDS, it was determined that this mutation was neutral and naturally occurring. As a result, the Pax3-pcDNA3 construct, which had the insert originally amplified from the 3 week old *mdx* mouse template, was used in all of the experiments reported herein. Recently, the National Centre for Biotechnology Information (NCBI) has released an updated version of the Pax3 transcriptional variant (NM_008781.4). This Pax3 sequence (NM_008781.4) was identical to the Pax3 insert that was cloned into pcDNA3, supporting the decision to use this sequence as the Pax3 insert.

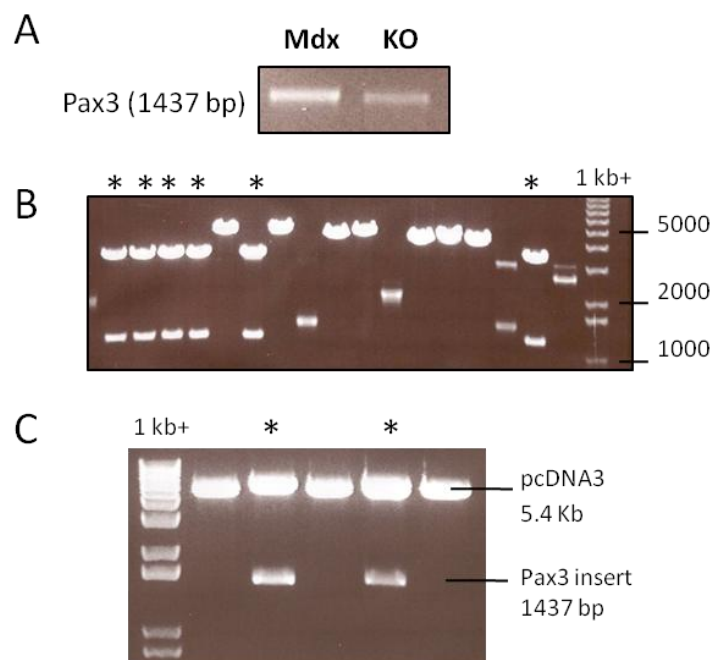


Figure 3.1 *Generation of over-expressing Pax3-pcDNA3 construct.* (A) PCR amplification of the 1437 bp Pax3 coding sequence. (B) Apal diagnostic digest on Pax3-pGEMTeasy minipreps, positive restriction digest contains two DNA fragments; 1165 bp and 3285 bp. (C) EcoR1/BamH1 restriction digest on Pax3-pcDNA3 minipreps, releasing the 1437 bp Pax3 insert. (B,C) * denotes positive clones.

D

			1	50
Pax3	BC	(1)	MTTLGAVPRMMRPGPGQNYPRSGFPLEVSTPLGQGRVNQLGGVFINGRP	
Pax3	clone	(1)	MTTLGAVPRMMRPGPGQNYPRSGFPLEVSTPLGQGRVNQLGGVFINGRP	
Pax3	NM	(1)	MTTLGAVPRMMRPGPGQNYPRSGFPLEVSTPLGQGRVNQLGGVFINGRP	
			51	100
Pax3	BC	(51)	LPNHIRHKIVEMAHHGIRPCVISRQLRVSHGCVSKILCRYQETGSIRPGA	
Pax3	clone	(51)	LPNHIRHKIVEMAHHGIRPCVISRQLRVSHGCVSKILCRYQETGSIRPGA	
Pax3	NM	(51)	LPNHIRHKIVEMAHHGIRPCVISRQLRVSHGCVSKILCRYQETGSIRPGA	
			101	150
Pax3	BC	(101)	IGGSKPKQVTPDVEKKIEEYKRENPGMFSWEIRDKLLKDAVCDRNTVPS	
Pax3	clone	(101)	IGGSKPKQVTPDVEKKIEEYKRENPGMFSWEIRDKLLKDAVCDRNTVPS	
Pax3	NM	(101)	IGGSKPKQVTPDVEKKIEEYKRENPGMFSWEIRDKLLKAVCDRNTVPS	
			151	200
Pax3	BC	(151)	VSSISRILRSKFGKGESEADLERKEAESEKKAKHSIDGILSERASAPQ	
Pax3	clone	(151)	VSSISRILRSKFGKGESEADLERKEAESEKKAKHSIDGILSERASAPQ	
Pax3	NM	(151)	VSSISRILRSKFGKGESEADLERKEAESEKKAKHSIDGILSERASAPQ	
			201	250
Pax3	BC	(201)	SDEGSDIDSEFDLPLKRRQRRSRTTFTAEQLEELERAFERTHYDITYTRE	
Pax3	clone	(201)	SDEGSDIDSEFDLPLKRRQRRSRTTFTAEQLEELERAFERTHYDITYTRE	
Pax3	NM	(201)	SDEGSDIDSEFDLPLKRRQRRSRTTFTAEQLEELERAFERTHYDITYTRE	
			251	300
Pax3	BC	(251)	ELAQRACLTEARVQVWFNSRRARWRKQAGANQLMAFNHLIPGGFPPTAMP	
Pax3	clone	(251)	ELAQRACLTEARVQVWFNSRRARWRKQAGANQLMAFNHLIPGGFPPTAMP	
Pax3	NM	(251)	ELAQRACLTEARVQVWFNSRRARWRKQAGANQLMAFNHLIPGGFPPTAMP	
			301	350
Pax3	BC	(301)	TLPTYQLSETSYQPTSIPQAVSDPSSTVHRPQPLPPTVHQSTIPSNADS	
Pax3	clone	(301)	TLPTYQLSETSYQPTSIPQAVSDPSSTVHRPQPLPPTVHQSTIPSNADS	
Pax3	NM	(301)	TLPTYQLSETSYQPTSIPQAVSDPSSTVHRPQPLPPTVHQSTIPSNADS	
			351	400
Pax3	BC	(351)	SSAYCLPSTRHGFSSYTD SFVPPSGPSNPMNPTIGNGLSPQVMGLLTNHG	
Pax3	clone	(351)	SSAYCLPSTRHGFSSYTD SFVPPSGPSNPMNPTIGNGLSPQVMGLLTNHG	
Pax3	NM	(351)	SSAYCLPSTRHGFSSYTD SFVPPSGPSNPMNPTIGNGLSPQVMGLLTNHG	
			401	450
Pax3	BC	(401)	GVPHPQTDYALSPLTGGLEPTTTVSASCSQRLEHMKNVDSLPTSQPYCF	
Pax3	clone	(401)	GVPHPQTDYALSPLTGGLEPTTTVSASCSQRLEHMKNVDSLPTSQPYCF	
Pax3	NM	(401)	GVPHPQTDYALSPLTGGLEPTTTVSASCSQRLEHMKNVDSLPTSQPYCF	
			451	484
Pax3	BC	(451)	PTYSTAGYSMDPVTGYQYGQYQGS AFHYLKPDI A	
Pax3	clone	(451)	PTYSTAGYSMDPVTGYQYGQYQGS KPWTF -----	
Pax3	NM	(451)	PTYSTAGYSMDPVTGYQYGQYQGS KPWTF -----	

Figure 3.1 Generation of over-expressing Pax3-pcDNA3 construct continued. (D) Comparison of Pax3 protein sequence between Pax3 insert in pcDNA3, Pax3 transcriptional variant 1 (NM) and murine pax3 complete coding sequence (BC).

3.1.2 Generation of stable Pax3 over-expressing cell lines

Prior to committing to the generation of stable cell lines over-expressing Pax3, as it is a lengthy procedure, C2C12 cells were transiently transfected (see Materials and Methods Section 2.2.6.4) to generate samples in which to optimise Pax3 western blots and determine antibody specificity. Western blot analysis of actively growing C2C12 cells transiently transfected with either Pax3-pcDNA3 or empty pcDNA3 vector (negative control) showed that the Pax3 over-expressing construct produced 1.53 times more Pax3 protein than the control. Protein isolated from the transiently transfected cell lines, a Pax7 stable over-expressing cell line, and a non-transfected C2C12 control cell line were used to determine cross-reactivity between Pax3 and Pax7 primary antibodies. The results indicated limited cross-reactivity between anti-Pax3 and anti-Pax7 antibodies and their paralogues Pax7 and Pax3, respectively (Figure 3.2). There is a possible cross-reaction, as the 56 kDa Pax3 protein and the lower 54 kDa Pax7 isoform migrated to a similar extent on an SDS-polyacrylamide gel. However, as Pax7 expression was not detected in the transient Pax3 over-expressing cells, the cross-reactivity was considered inconsequential.

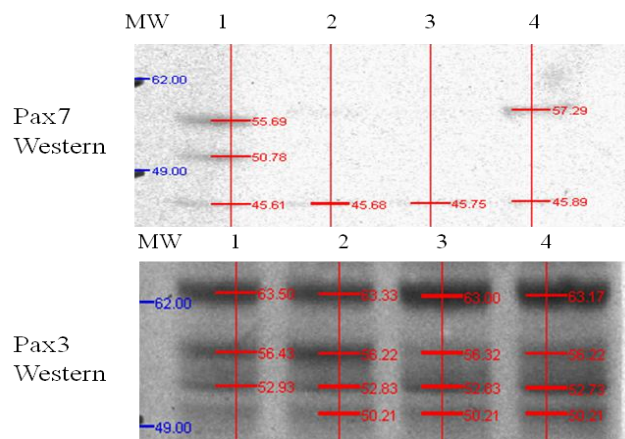


Figure 3.2 Pax3 and Pax7 western blot analysis. Pax7 (54 and 57 kDa) and Pax3 (56 kDa) western blot analysis on C2C12 cells stably over-expressing Pax7 (lane 1), C2C12 cells transiently transfected with either Pax3-pcDNA3 (lane 2) or empty pcDNA3 vector (lane 3) and non-transfected C2C12 cells (lane 4), SeeBlue Plus 2 pre-stained standard was used to determine molecular weights (kDa) of detected bands.

Stable transfection of Pax3-pcDNA3 into C2C12 cells, using geneticin to select for neomycin resistance and genome integration (see Materials and Methods Section 2.2.6.5) resulted in clones that over-expressed Pax3 to a greater extent than the transiently transfected cells. A total of 49 Pax3 over-expressing stable clones were isolated, as well as a stably transfected empty pcDNA3 vector control. All 49 stable transfectants were analysed for Pax3 protein expression by western blot analysis. Ponceau staining indicated uneven sample loading in four lanes. This result was confirmed following GAPDH analysis; therefore these clones (clones 17, 32, 34, and 48) were excluded from further analysis. Pax3 protein expression levels were normalised to GAPDH expression for the remaining 45 clones (Table 5). GAPDH protein expression levels varied considerably between the stable clonal cell lines (Table 5). In subsequent assays, it was observed that tubulin expression was more consistent among samples and therefore tubulin expression was used for western blot normalisation in the remaining experiments described herein.

Table 5 Pax3 protein expression, normalised to GAPDH, in stably transfected C2C12 clones. Pax3 and GAPDH protein levels were assessed by Western blot and densitometry of protein extracts isolated from 49 Pax3 stable over-expressing clones and empty vector control

Sample	Pax3 Density	GapDH density	Normalised Pax3 expression
pcDNA3	16.04	282.1	0.057
Pax3 Clone 1	26.5	473.9	0.056
Pax3 Clone 2	39.1	361.1	0.108
Pax3 Clone 3	48.7	363.6	0.134
Pax3 Clone 4	40.8	437.3	0.093
Pax3 Clone 5	31.9	399.2	0.080
Pax3 Clone 6	55.1	427.2	0.129
Pax3 Clone 7	36.9	409.3	0.090
Pax3 Clone 8	55.4	404.2	0.137
Pax3 Clone 9	44.2	398.1	0.111
Pax3 Clone 10	33.1	326.7	0.101
Pax3 Clone 11	76.6	270.2	0.283
Pax3 Clone 12	31.2	337.5	0.092
Pax3 Clone 13	41.3	365.1	0.113
Pax3 Clone 14	60.0	433.0	0.139
Pax3 Clone 15	29.6	495.2	0.060
Pax3 Clone 16	64.9	408.0	0.159
Pax3 Clone 18	38.1	367.0	0.104
Pax3 Clone 19	59.1	424.1	0.139
Pax3 Clone 20	61.5	389.0	0.158
Pax3 Clone 21	183.1	274.7	0.667
Pax3 Clone 22	94.3	257.9	0.366
Pax3 Clone 23	84.1	279.4	0.301
Pax3 Clone 24	97.8	414.0	0.236
Pax3 Clone 25	91.9	372.1	0.247
Pax3 Clone 26	72.7	336.5	0.216
Pax3 Clone 27	88.0	207.1	0.425
Pax3 Clone 28	81.9	251.3	0.326
Pax3 Clone 29	199.0	309.0	0.644
Pax3 Clone 30	98.2	270.7	0.363
Pax3 Clone 31	128.3	304.4	0.421
Pax3 Clone 33	111.7	215.5	0.518
Pax3 Clone 35	88.3	136.5	0.647
Pax3 Clone 36	156.3	144.5	1.082
Pax3 Clone 37	78.1	201.4	0.388
Pax3 Clone 38	61.2	193.8	0.316
Pax3 Clone 39	63.4	145.6	0.435
Pax3 Clone 40	89.2	156.7	0.569
Pax3 Clone 41	141.8	239.0	0.593
Pax3 Clone 42	117.8	147.9	0.796
Pax3 Clone 43	147.7	146.6	1.008
Pax3 Clone 44	103.4	200.2	0.516
Pax3 Clone 45	72.4	147.6	0.491
Pax3 Clone 46	27.5	101.5	0.271
Pax3 Clone 47	61.2	221.9	0.276
Pax3 Clone 49	82.0	166.0	0.494

A subset of 9 Pax3 over-expressing clones that represented the range, low through to high, of Pax3 protein expression were further analysed using semi-quantitative PCR. PCR amplification was carried out for 25 and 18 cycles, for Pax3 and tubulin respectively; the cycle number chosen equated with linear amplification of the product (a 234 bp Pax3 fragment and a 450 bp tubulin fragment). Pax3 normalised to tubulin expression (Figure 3.3) illustrates that Pax3 RNA:protein ratios were inconsistent among the Pax3 over-expressing clones. Three clones were chosen for further functional analysis: clones 3, 29, and 43. Clone 43 was chosen as it exhibited a strong over-expression of Pax3 protein as well as having a high Pax3 mRNA profile. Clone 29 was selected as it displayed a similar Pax3 RNA:protein ratio as clone 43 but absolute levels of expression were intermediate in comparison. This provided the opportunity to investigate whether varying levels of Pax3 expression had varying effects on myogenic potential. Clone 3 was selected as it displayed the highest over-expression of Pax3 mRNA, but with low protein expression.

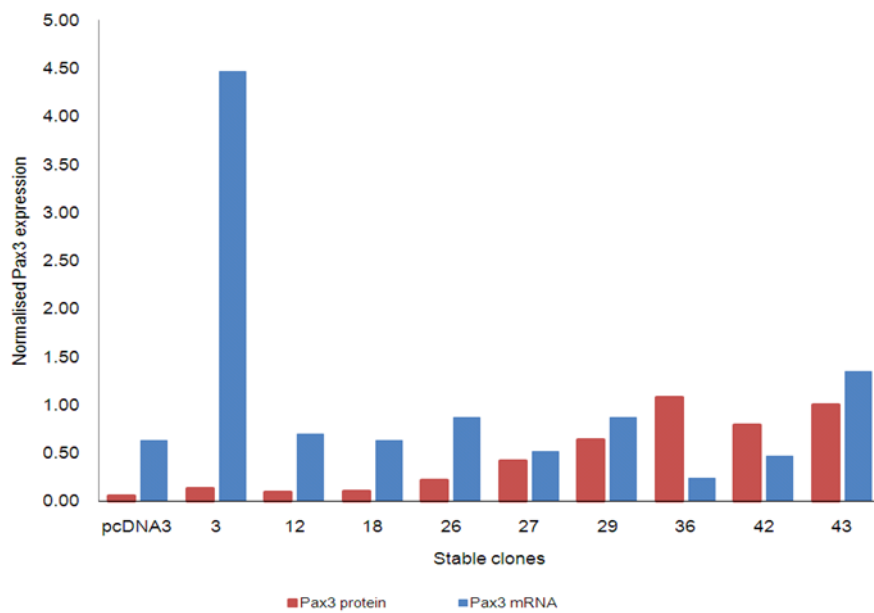


Figure 3.3 Comparison of Pax3 expression levels in stably transfected Pax3 over-expression clones. GAPDH normalised Pax3 protein and tubulin normalised Pax3 mRNA expression from western blot and semi-quantitative RT-PCR respectively, for nine stable Pax3 over-expressing clones and a stable empty pcDNA3 vector control.

3.1.3 Characterisation of selected Pax3 clones

Pax3 and Pax7 western blot analyses were performed in three independent experiments to obtain an accurate protein expression characterisation of the three selected clones, 3, 29 and 43, in comparison to control cell-lines, pcDNA3 and C2C12 (Figure 3.4a). Densitometric analysis of the Pax3 Western blots normalised to tubulin (Figure 3.4b) shows that the two control cell lines had a similar level of Pax3 expression. Pax3 over-expressing clones expressed significantly higher levels of Pax3 protein: clone 3 expressed Pax3 3.4-fold higher ($P < 0.001$), clone 29 expressed Pax3 2.2-fold higher ($P < 0.01$) and clone 43 expressed Pax3 4.1-fold higher ($P < 0.001$). Pax7 western blot analysis showed that only control C2C12 and pcDNA3 cell lines expressed a detectable level of Pax7 protein (Figure 3.4a).

Real-Time PCR quantification of Pax3 mRNA was also performed on the same cell-lines analysed by Western blot. Histone 3.3A mRNA levels were used for normalisation of Pax3 expression. The graph in Figure 3.4b illustrates Pax3 protein and mRNA expression for each of the cell lines. Absolute levels of Pax3 mRNA differed between this characterisation of the Pax3 clones quantified by Real-Time PCR from the analysis performed using semi-quantitative PCR (Figure 3.3); however quantitative Real Time PCR is more accurate than semi-quantitative PCR. In addition, the trend of protein expression is also different between the two experiments. The earlier Pax3 protein quantification via Western blot (Figure 3.3) was a singular experiment, whereas this later analysis was performed 3 times and thus provided results that could be analysed statistically. Furthermore, GAPDH protein expression was used to normalise earlier Pax3 Western blot analysis, whereas tubulin protein expression was used for normalisation of this later Pax3 protein quantification as it proved to be a more reliable method. Therefore, the characterisation of the selected Pax3 over-expressing clones and control cells is based on the later experimental results. It is also worth noting that this later quantification of Pax3 mRNA and protein is analysed relative to the level of Pax3 expression in control cells, in that C2C12 cells are assigned the arbitrary expression value of one.

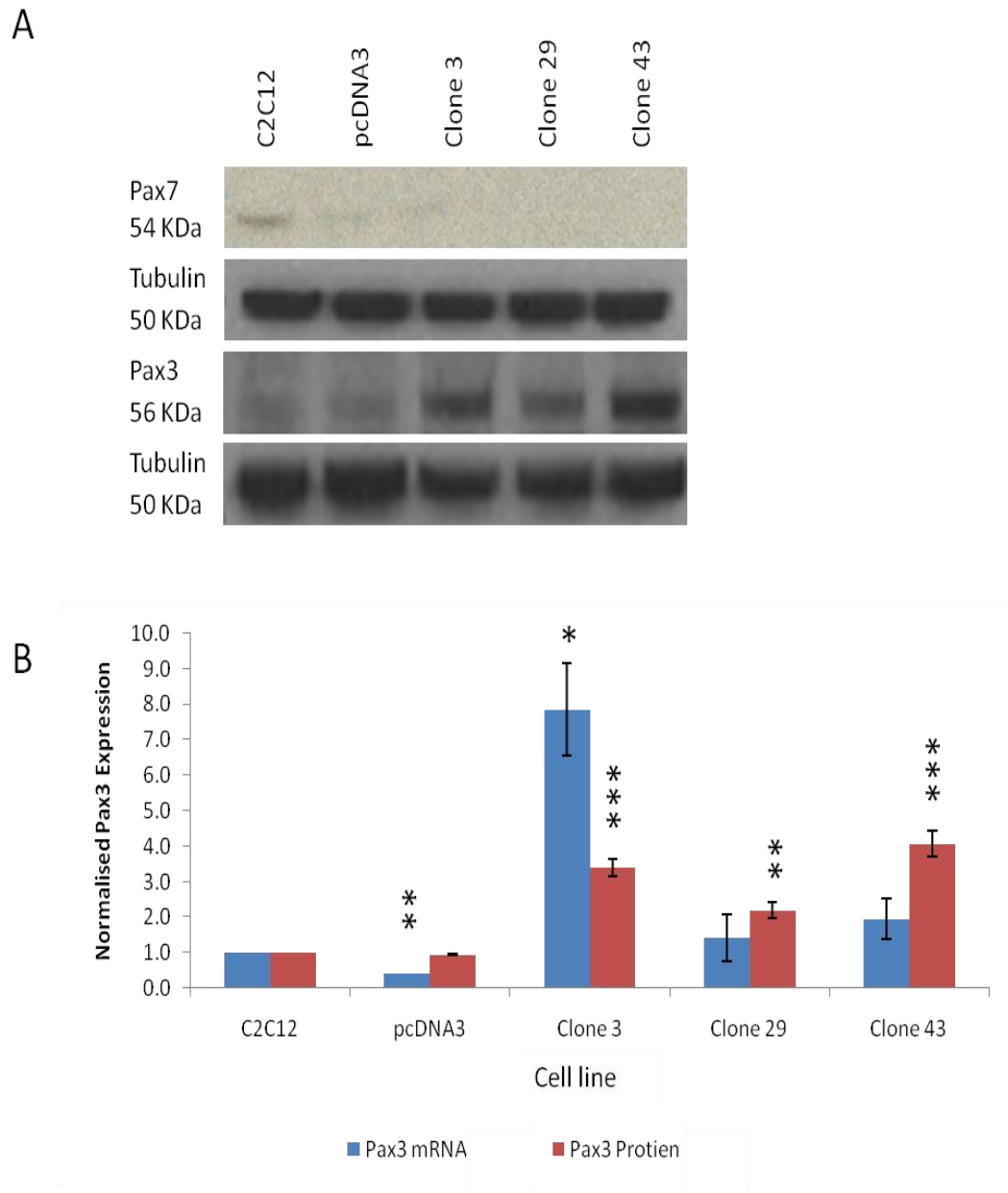


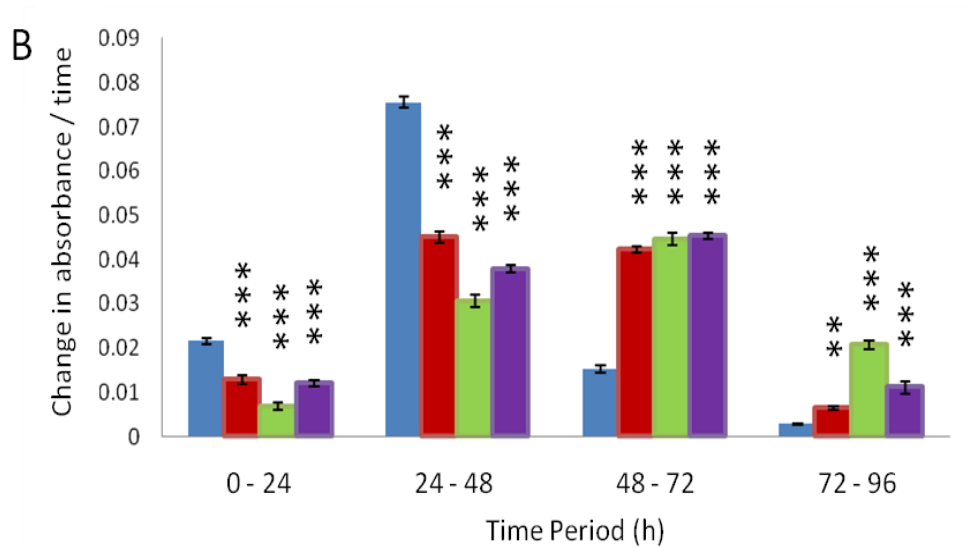
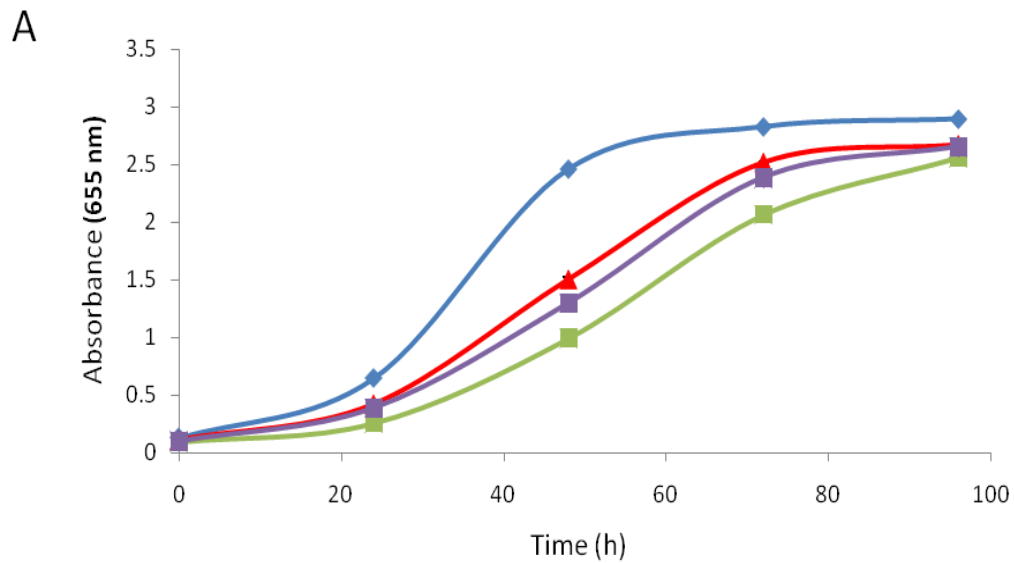
Figure 3.4 *Characterisation of stable over-expressing Pax3 clones.* (A) Western blots showing Pax3 and Pax7 protein expression collected from actively growing Pax3 over-expressing stable cell lines (Clone 3, 29 and 43) and C2C12 control lysates. (B) Control cell and Pax3 over-expressing clones mean (\pm SEM) Pax3 expression from densitometric analysis of Pax3 western blot results ($n=3$), normalised to tubulin and Pax3 Real-Time PCR analysis ($n=2$), normalised to histone 3.3A mRNA transcription. A one-way ANOVA was performed to establish the level of variance followed by a Post-hoc T-test to determine statistical differences, indicated as, $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) compared to C2C12.

3.2 Functional Analysis

To assess the role of Pax3 in post-natal myogenesis, the three selected Pax3 clones (3, 29, and 43) were assessed in four biological assays: proliferation, differentiation, migration and apoptosis. A control cell line, non-transfected C2C12 cells, was also analysed for experimental comparison.

3.2.1 Pax3 over-expression slows the proliferation of myoblasts

The methylene blue proliferation assay was used to assess and compare the growth rates of the three over-expressing Pax3 clones with each other and to control cells. An altered growth rate due to sustained Pax3 expression, would strongly suggest that Pax3 has transcriptional regulatory control over myogenic cell cycling. On four separate occasions, a proliferation assay was performed with freshly grown Pax3 over-expressing clones and control cells. In decreasing order the proliferation assay showed the growth rate as C2C12 > Clone 3 > Clone 43 > Clone 29 (Figure 3.5). In the initial 48 h of the proliferation assay, prior to control cells reaching confluence, all three Pax3 over-expressing clones exhibited significantly slower proliferation rates (Figure 3.5). Once control cells reached confluence, all three Pax3 over-expressing clones proliferated significantly faster in comparison to control cells for the remaining 48 h (Figure 3.5b). However, throughout the proliferation assay, control cells had significantly the highest overall growth rate, illustrated in the gradient of the growth curves when comparing among Pax3 over-expressing clones and control cells (Figure 3.5a). This data strongly suggest that sustained Pax3 over-expression inhibits C2C12 myoblast proliferation. Furthermore, this trend was also clearly observable in the difference existing between the levels of cell density among the cell lines in all cell culture experiments performed prior to experimental set-up



Legend



Figure 3.5 *Stable over-expression of Pax3 slows myoblast proliferation.* (A) Methylene blue assay assessing mean (\pm SEM) proliferation of control C2C12 cells in comparison to Pax3 over-expressing stable cell lines (Clone 3, 29 and 43) over a 96 h time period. (B) Comparison of mean (\pm SEM) growth rates over 4 time periods for the same methylene blue assay shown in (A). A one-way ANOVA was performed to establish the level of variance followed by a Post-hoc T-test to determine statistical differences, indicated as, $P < 0.01$ (**) and $P < 0.001$ (***) compared to C2C12. (A,B) Absorbance (655 nm) is directly proportional to cell number, $n=8$ replicates.

3.2.2 Pax3 over-expression delays the differentiation of myoblasts

3.2.2.1 Non-Fluorescent differentiation assay

In order to determine whether Pax3 influences post-natal myogenic differentiation, as it does in embryonic limb myogenesis (Bober *et al.*, 1994; Goulding *et al.*, 1994; Williams & Ordahl, 1994), and also whether Pax3 induces a quiescent, self-renewal pathway, the Pax3 cell lines and control cells were exposed to low serum conditions, which are known to induce C2C12 cells to differentiate (Blau *et al.*, 1985). In this non-fluorescent assay, differentiation was determined by DAB chromogen stained MyHC and cell numbers were obtained via Gills hematoxylin staining, and counted manually (see Materials and Methods Section 2.2.12.2). Microscope images of three time points; 12 h, 48 h, and 72 h, for each cell line (Figure 3.6a) clearly illustrates the delay in differentiation exhibited by all Pax3 over-expressing clones in comparison to control cells. The fusion index for each cell line for the 48 h and 72 h time points was calculated manually by dividing the number of myotube nuclei (myotubes are defined as having a minimum of three nuclei) by the total number of nuclei. At both the 48 h and 72 h time points the control cells had a significantly higher rate of differentiation (Figure 3.6b), reaching 52% fusion at 72 h, in comparison to 32% fusion for both clone 3 and clone 43 and only 16% fusion for clone 29. The decreasing ability of these cell lines to differentiate follows the same trend observed in the proliferation assay: C2C12 > Clone 3 > Clone 43 > Clone 29.

At the 12 h time point very few myotubes had formed for both Pax3 over-expressing clones and control cells, however there was clearly an initiation of differentiation, observed as mono-nucleated cells expressing the MyHC structural protein. Therefore, the proportion of MyHC positive cells was determined instead of the fusion index. All three Pax3 clones at 12 h had a higher proportion of MyHC expressing cells in comparison to control, with clone 3 ($P < 0.01$) being significantly higher (Figure 3.6c).

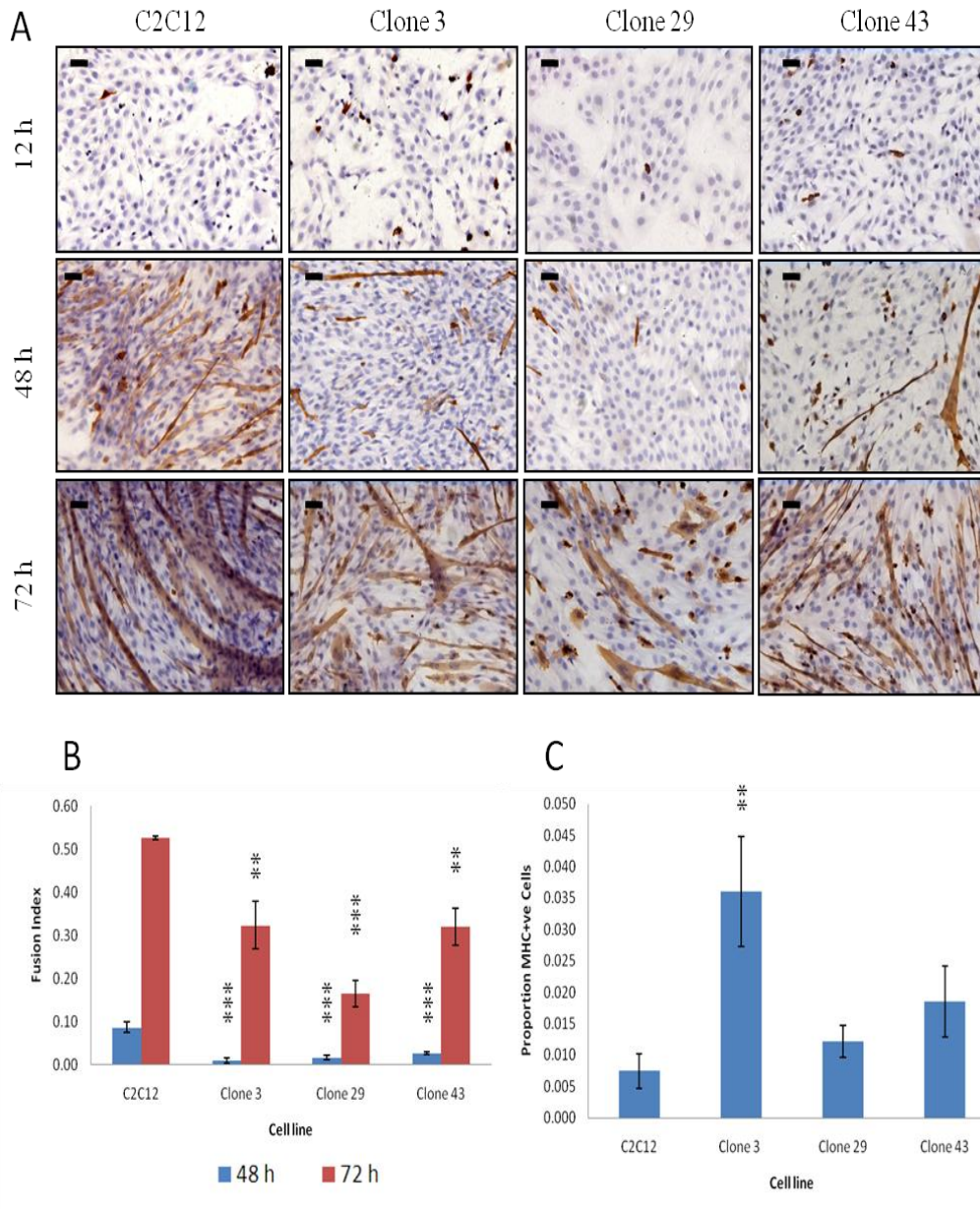


Figure 3.6 Stable over-expression of Pax3 delays terminal myogenic differentiation. (A) Representative images of Pax3 stable over-expressing cell lines in comparison to C2C12 control cells during induced differentiation, stained with Gills haematoxylin (purple nuclei) and DAB chromogen (brown myotubes). Scale bar represents 50 μm . (B) Mean fusion index (mean myotube nuclei/mean total nuclei) for 48 h and 72 h of differentiation \pm SEM. (C) The mean proportion of MyHC expressing cells at 12 h of differentiation \pm SEM. (B,C) Mean (\pm SEM) were calculated from 4 random images for each of three coverslips per time point for the stable Pax3 over-expressing clones (Clone 3, 29 and 43) in comparison to the C2C12 control cells. A one-way ANOVA was performed to establish the level of variance followed by a Post-hoc T-test to determine statistical differences, indicated as, $P < 0.01$ (**) and $P < 0.001$ (***) compared to C2C12.

3.2.2.2 Fluorescent differentiation assay

A fluorescent differentiation assay was performed using a fluorescent MyHC stain for myotubes and DAPI staining for nuclei (see Materials and Methods Section 2.2.12.1). This assay differs from the non-fluorescent differentiation assay as it uses a 96 well format instead of 24 well coverslips. Furthermore, total cell counts were performed using the Image-Pro Plus computer software. This assay is still under experimental optimisation, and it is suspected that the automated cell counting program, which estimates the total number of DAPI stained nuclei is inaccurate when distinguishing single cells at the late time points, when clustering occurs.

Two time points were tested: 48 h and 72 h, which demonstrated similar, but less statistically significant results, than the non-fluorescent differentiation assay. Following 48 h of differentiation, the fluorescent differentiation assay indicated no significant difference in the fusion index among Pax3 over-expressing clones and control cells. However, following 72 h of differentiation, all three Pax3 over-expressing clones had a significant delay in differentiation in comparison to control cells (Figure 3.7b).

The proportion of MyHC positive cells was also determined for each time point (Figure 3.7c), and at the 72 h time point this analysis results in eliminating the significant level of difference between Pax3 over-expressing clone 3 and control cells, originally seen in the fusion index analysis. Comparison of fusion index data to the proportion of MyHC positive cells suggests that Pax3 over-expression could alter the ability of myoblasts to form mono- and bi-nucleated myocytes (hyperplasia) in contrast to the ability to fuse to pre-existing myotubes (hypertrophy).

Overall, this fluorescent differentiation experiment, although showing minor changes in differentiation trends in comparison to the non-fluorescent differentiation experiment, it does indicate a similar conclusion that over-expression of Pax3 delays myogenic differentiation. This can be clearly seen in the fluorescent microscope images at the 72 h time point (Figure 3.7a).

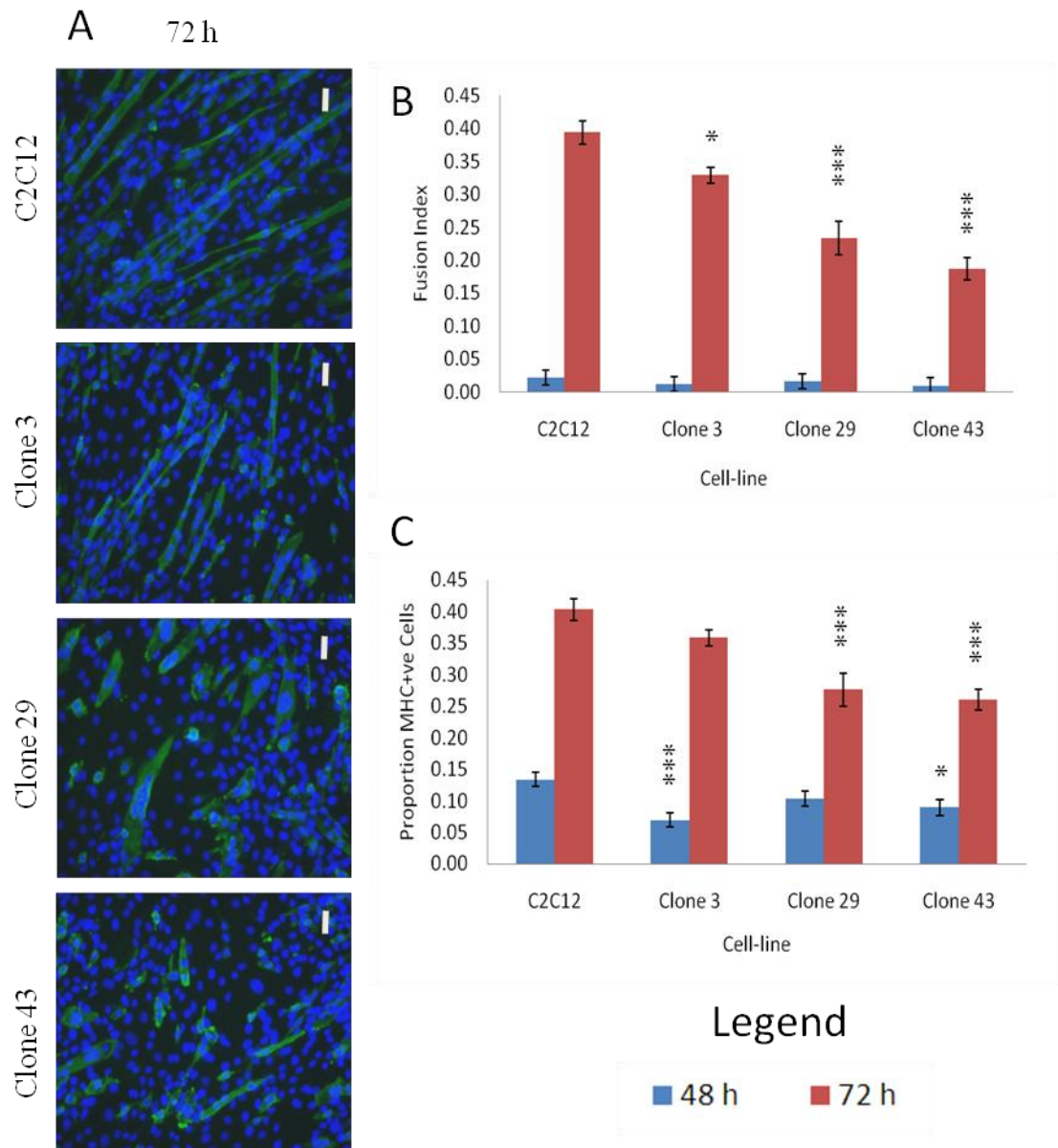


Figure 3.7 *Stable over-expression of Pax3 delays terminal myogenic differentiation, fluorescent assay.* (A) Representative fluorescent images of Pax3 stable over-expressing cell lines in comparison to C2C12 control cells during induced differentiation, stained with DAPI (blue nuclei) and streptavidin Alexa Flour 488 (green myotubes). Scale bar represents 50 pixels. (B) Fusion index (mean myotube nuclei/mean total nuclei) for 48 h and 72 h of differentiation \pm SEM. (C) The mean proportion of myosin heavy chain expressing cells at 48 h and 72 h of differentiation \pm SEM. (B,C) Mean \pm SEM were calculated from 8 random images for the 3 stable Pax3 over-expressing clones in comparison to the C2C12 control cells during induced differentiation. A one-way ANOVA was performed to establish the level of variance followed by a Post-hoc T-test to determine statistical differences, indicated as, $P < 0.05$ (*) and $P < 0.001$ (***) compared to C2C12.

3.2.3 Pax3 over-expression alters C2C12 cell survival

As previously mentioned, during embryonic myogenesis Pax3 is known to have an anti-apoptotic effect in the Pax3/Pax7 positive pre-satellite cells (Relaix *et al.*, 2006). To determine the anti-apoptotic effect conferred by over-expression of Pax3, if any, apoptosis assays were performed. As discussed in the materials and methods (see Section 2.2.13), a caspase assay fluorimetrically quantified caspase activity from the Pax3 clones and C2C12 control following 8 h of serum starvation in comparison to cells grown in the presence of 10% FBS. Four assays were performed in triplicate for each cell line; two assays were completed on the same day with the same cell cultures, confirming assay repeatability. A representative caspase assay is shown (Figure 3.8a), with caspase activity being proportional to the level of programmed cell death (apoptosis). At basal level of apoptosis, when the cells were grown in the presence of 10% FBS, control cells had significantly higher susceptibility to apoptosis in comparison to clone 3 ($P < 0.001$), clone 29 ($P < 0.01$) and clone 43 ($P < 0.001$). Following serum deprivation, clone 3 ($P < 0.001$) and clone 29 ($P < 0.001$) remained significantly less susceptible to apoptosis in comparison to control cells. However, clone 43 ($P < 0.001$) displayed a significantly higher level of apoptosis in comparison to control cells when deprived of serum.

The percentage increase in caspase activity from basal levels to levels induced by serum starvation was calculated to normalise results to account for cell density differences among the four cell lines. The mean percentage change represents the average of four assays (Figure 3.8b). This measure indicates the susceptibility of cells to serum starvation induced apoptosis. The trend showed that both clone 3 and clone 43 were more susceptible to cell death in comparison to control cells when serum starved, however, only clone 43 was statistically significantly different ($P < 0.01$) to control cells. On the other hand, clone 29 had a smaller mean percentage change in cell death following serum starvation in comparison to control cells, however the level of difference was not statistically significant.

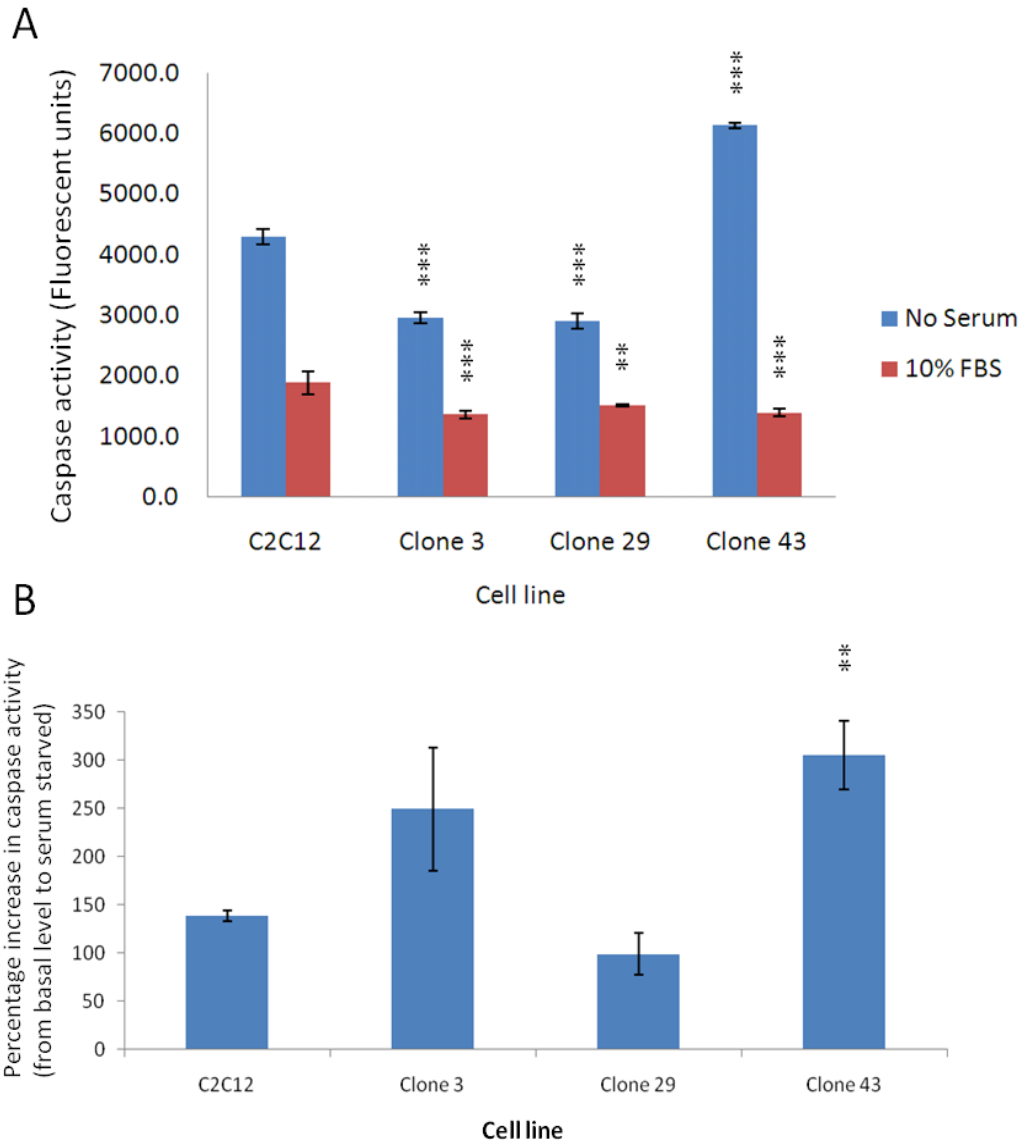


Figure 3.8 *Stable over-expression of Pax3 alters C2C12 cell survival.* (A) A representative apoptosis assay comparing caspase activity between Pax3 clones and control C2C12 cells following 8 h of either serum withdrawal or 10% FBS, mean \pm SEM calculated from 3 replicates. (B) The percentage increase in caspase activity from basal levels to levels induced by serum starvation for Pax3 clones and C2C12 control cells, mean \pm SEM were calculated from 4 individual fluorescent apoptosis assays which contained 3 replicates of each cell line. (A,B) A one-way ANOVA was performed to establish the level of variance followed by a Post-hoc T-test to determine statistical differences, indicated as, $P < 0.01$ (**) and $P < 0.001$ (***) compared to C2C12.

3.2.4 Pax3 over-expression promotes C2C12 migration

In response to growth or injury, satellite cells activate and migrate to sites of regeneration (Zammit *et al.*, 2006a). To determine whether Pax3 is involved in regulating the migration of myogenic cells, a migration assay was performed on the Pax3 over-expressing clones and control cells. Three positive control media were tested containing chemo-attractants: 5% CEE and 2% HS (optimum); 5% CEE (intermediate); 2% HS (sub-optimum). Serum-free DMEM was used as the negative control medium. The ability of each cell line to migrate across a porous membrane in response to the above treatments was assessed. Three membranes were tested per treatment per cell line, with migrated cells being counted on four representative fields per membrane. Clustering of migrated cells affected the ability to obtain representative fields on certain membranes, especially in the control cells. This resulted in larger standard errors, which reduced statistical significance among Pax3 clones and the control cells.

Cell counts were log transformed to normalise variance and a two-way ANOVA was performed to compare migration among Pax3 over-expressing cell lines and control cells for the three treatments (Figure 3.9a). Both clone 3 and clone 29 consistently had a higher average migration for all three treatments in comparison to control cells and clone 43, indicating that Pax3 over-expression may promote migration. Furthermore, to determine whether Pax3 over-expression increased the chemotactic response and sensitivity to chemotactic signals, the percentage drop in migration from optimal treatment to intermediate (5% CEE) and sub-optimal (2% HS) is illustrated (Figure 3.9b). The migration of all three Pax3 over-expressing clones was less affected than that of the control cells by the absence of 2% HS in the intermediate treatment, as they were able to migrate almost as well even with a reduced chemotactic signal. However, when the chemotactic signal was reduced to a sub-optimal level (only 2% HS), the Pax3 over-expressing clones behaved similar to control cells. Unfortunately, *statistically* there were no significant differences among the four cell lines within the two analyses described above. Although this experiment did not provide statistically significant results, there was a strong indication that an intermediate over-expression of Pax3 (clone 3 and 29) primed cells for migration and increased their sensitivity to chemotactic signals. In this experiment the bottom of the

membrane inserts was not coated with $1 \times$ Matrigel (a membrane matrix), this omission may have caused the clustering of migrated cells and consequently higher standard errors. To confirm the trends suggested by these data it is recommended the experiment is repeated with Matrigel applied to the bottom of the membrane inserts.

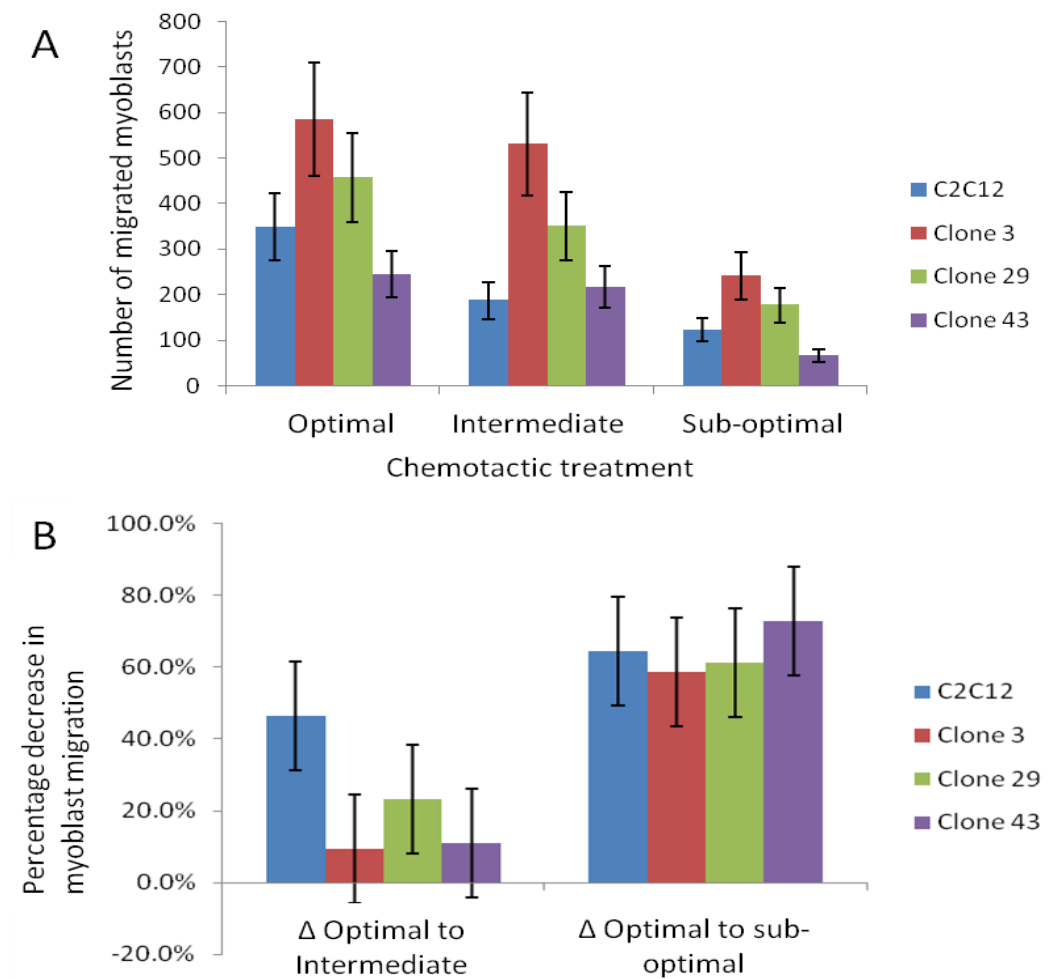


Figure 3.9 *Stable over-expression of Pax3 alters C2C12 cell migration.* (A) Comparison of the number of migrated cells among Pax3 stable over-expressing clones and C2C12 control cells, mean \pm SEM were calculated from 4 representative microscope images from each of three membranes per cell line per treatment. (B) Comparison of the percentage decrease in cell migration from optimal treatment (5% CEE and 2% HS) to that of intermediate (5% CEE) and sub-optimal (2% HS) treatments among Pax3 stable over-expressing clones and C2C12 control cells. (A,B) A two-way ANOVA was performed on log transformed cell counts, the Dunnetts test was used to determine significant differences.

3.3 Temporal gene expression analysis during myogenic differentiation

3.3.1 Analysis of myogenic transcription during differentiation

There are two pathways a myogenic progenitor can take during differentiation, quiescence or fusion, and the specific expression or repression of myogenic determination genes determines which pathway is taken (Zammit *et al.*, 2006b). To determine if over-expression of Pax3 alters temporal myogenic gene expression during differentiation, RNA samples were collected every 24 h from Pax3 clones and control C2C12 cells throughout a 96 h period of induced differentiation and analysed by Real-Time PCR. Analysis was carried out using primers designed to amplify Pax3, p21, and Myf5, Histone 3.3A was used to normalise for differences in template concentration. Following Real-Time PCR, correct amplicon size was verified on agarose gels, which matched to the correct fragment length of Pax3 (234 bp), p21 (428 bp) and Myf5 (353 bp), results are not shown.

Due to time constraints, analysis of only one differentiation assay for the four cell lines was completed, thus this experiment acts only as an indicator of possible alterations in gene transcription caused by the over-expression of Pax3. However, each cDNA sample was analysed twice for each cell line, this provided a means of assessing the accuracy of each gene transcription analysed via Real-Time PCR. This is shown as mean normalised expression \pm SEM on the transcription profiles in Figure 3.10.

The four cell lines displayed the same profile in Pax3 mRNA expression levels as indicated in Section 3.1.3, with all three Pax3 over-expressing clones expressing consistently higher Pax3 mRNA in comparison to control cells (Figure 3.10). Furthermore, both clone 3 and clone 29 up-regulated Myf5 and p21 transcripts in comparison to control cells. Whereas clone 43, initially displayed the same up-regulation in transcription as clone 3 and 29, but at later stages of differentiation expressed Myf5 and p21 transcripts at a similar level to control cells (Figure 3.10).

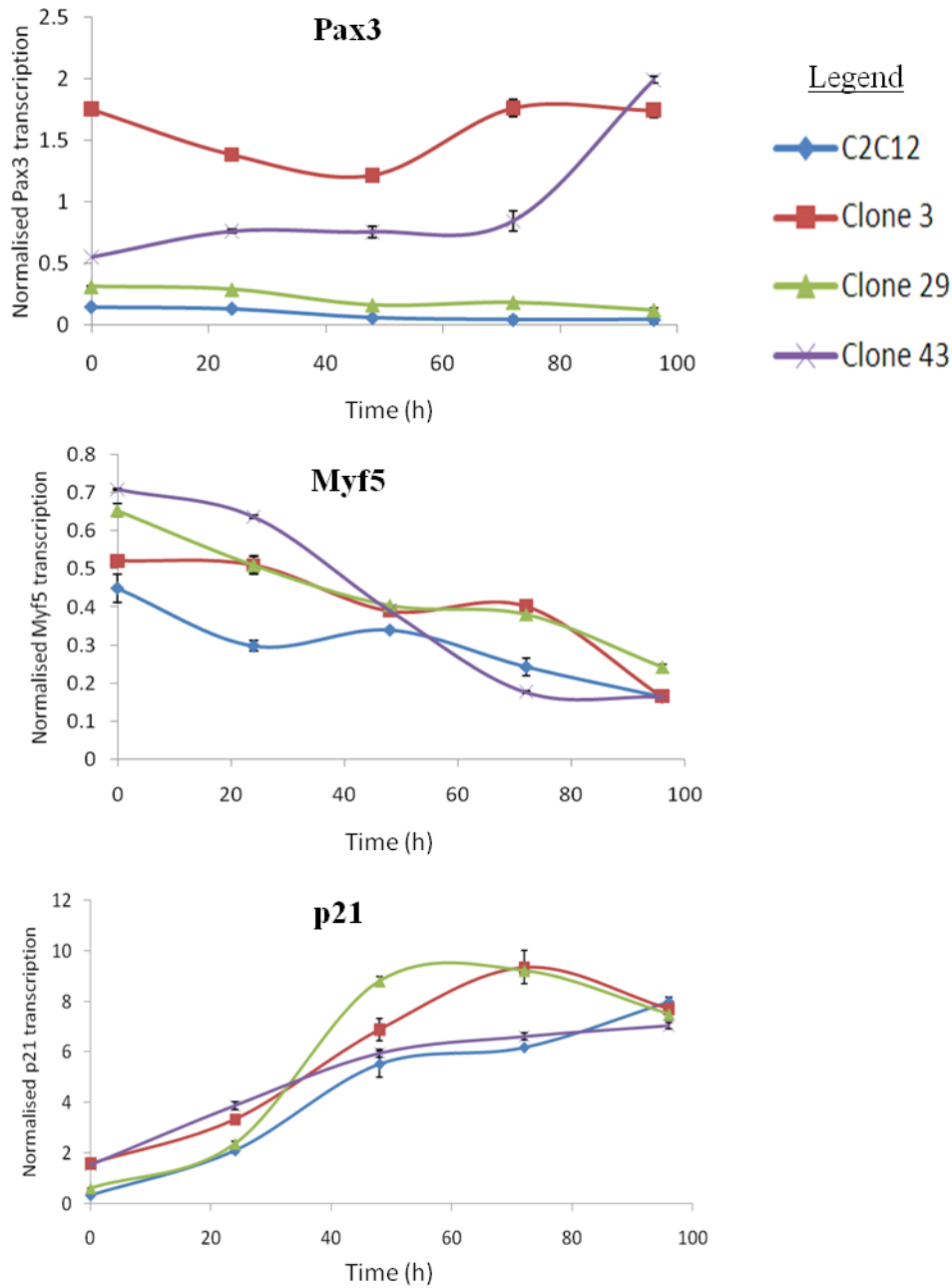


Figure 3.10 *The effect of Pax3 over-expression on myf5 and p21 transcription during differentiation.* Comparison of Real-Time PCR quantification of a 234 bp Pax3 fragment, 353 bp myf5 fragment, and a 428 bp p21 fragment between Pax3 stable overexpressing clones and C2C12 control cells during 96 h of differentiation, histone 3.3A was analysed for normalisation. Mean \pm SEM were calculated from 2 replicates of the same cDNA samples.

3.3.2 Protein expression analysis during myogenic differentiation

In addition to collecting RNA samples, cell lysate samples were also collected every 24 h from Pax3 clones and control C2C12 cells through a period of 96 h of induced differentiation and analysed by Western blot. The myogenic genes analysed were Pax3, p21, Myf5, MyoD and myogenin; tubulin was used to normalise for differences in gel loading (Figure 3.11). However, as previously mentioned in Section 3.3.1, analysis of only one differentiation assay for the four cell lines was completed. Nonetheless, control cell lysates were analysed each time with one of the three Pax3 over-expressing clones, this provided a means of assessing the accuracy of each gene expression analysed via Western blot. This is shown as mean normalised expression \pm SEM on the C2C12 expression profiles in Figure 3.11b.

The Pax3 western blot analysis showed that Pax3 clone 43 during the course of differentiation was the strongest Pax3 protein expresser, as previously demonstrated in Section 3.1.3. The remaining two Pax3 clones, 3 and 29, although generally expressing more Pax3 protein than control cells, did not exhibit the constant level of Pax3 expression seen in clone 43 (Figure 3.11b); this could be due to an active Pax3 protein synthesis regulation pathway. All three Pax3 over-expressing clones demonstrated elevated levels of Myf5 expression at time 0, in comparison to control cells; these levels remained consistently higher in the Pax3 over-expressing clones during the course of differentiation. Interestingly, the level of Myf5 expressed by each cell line paralleled Pax3 protein expression (Figure 3.11b). MyoD, an important myogenic regulatory factor, showed similar elevated levels of expression at time 0 for the Pax3 over-expressing clones 3 and 43 in comparison to control cells, while clone 29 displayed a similar level of MyoD expression as control cells. Curiously, the peak in MyoD expression for all three Pax3 over-expressing clones coincides with peaks in both Myf5 and Pax3 expression (Figure 3.11b). Furthermore, there was a clear up-regulation of p21 expression for Pax3 over-expressing clones 3 and 29, with peaks in p21 expression synchronizing with peaks in expression of the myogenic regulatory factor genes, MyoD and Myf5 (Figure 3.11b). Intriguingly, myogenin expression profiles for the Pax3 over-expressing clones show an increasing level of expression as differentiation proceeds, however, unlike clones 3 and 43, the level

of myogenin expressed by clone 29 dropped following the peak in p21 expression. In comparison, control cells exhibited a relevantly constant level of myogenin expression (Figure 3.11b).

Furthermore, to illustrate the synchronization of p21 expression with either Myf5 or MyoD, the expression profiles are graphed for each of the Pax3 over-expressing clones and control cells (Figure 3.12). Pax3 over-expressing clone 3 and 29 both demonstrate a defined synchronization of p21 expression with Myf5 expression. In comparison, the control cells and clone 43 show a synchronization of p21 expression with both Myf5 and MyoD. However, clone 43 consistently expresses more Myf5 than control cells. These results require confirmation with replicate experiments.

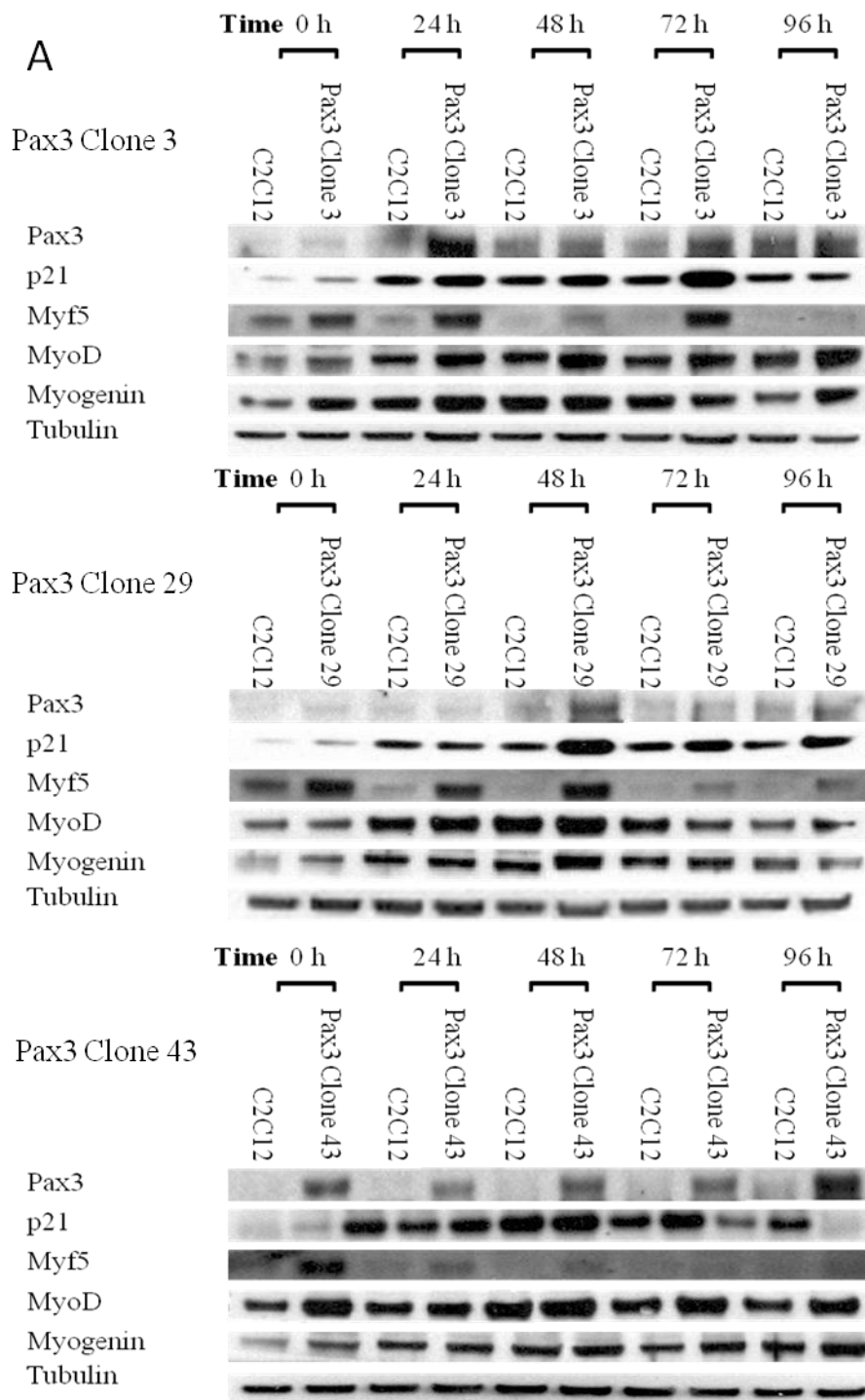


Figure 3.11 *Myogenic temporal gene expression analysis during differentiation.* (A) Pax3, p21, Myf5, MyoD, and myogenin western blot analysis on cell lysates from Pax3 stable over-expressing clones and C2C12 control cells during 96 h of differentiation, tubulin was analysed for normalisation.

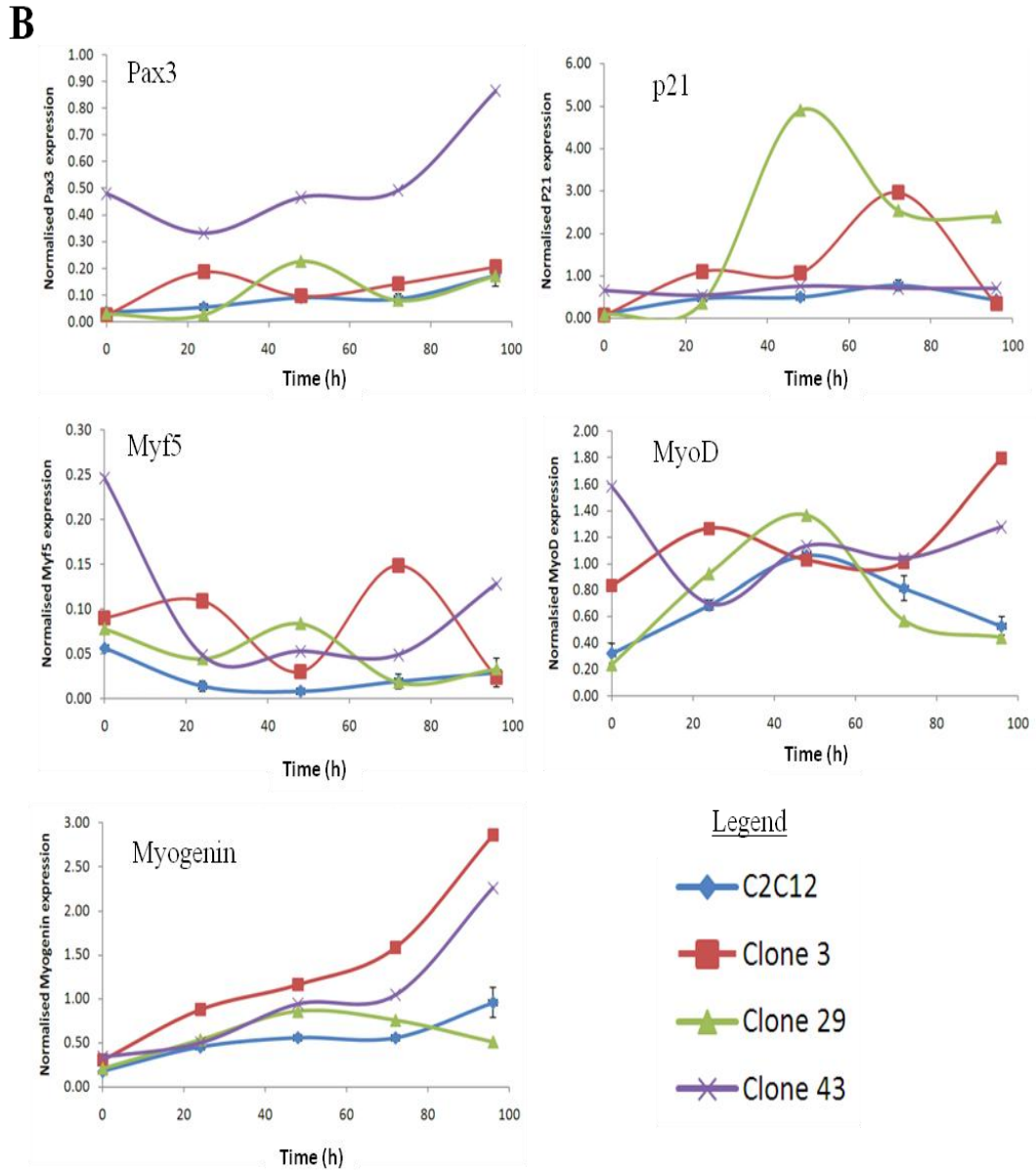


Figure 3.11 *Myogenic temporal gene expression analysis during differentiation continued.* (B) Densitometric analysis of tubulin normalised Pax3, p21, Myf5, MyoD, and myogenin western blots for Pax3 stable over-expressing clones and C2C12 control cells. Mean \pm SEM for C2C12 control cells were calculated from 3 western blots of the same cell lysate samples, represented in (A).

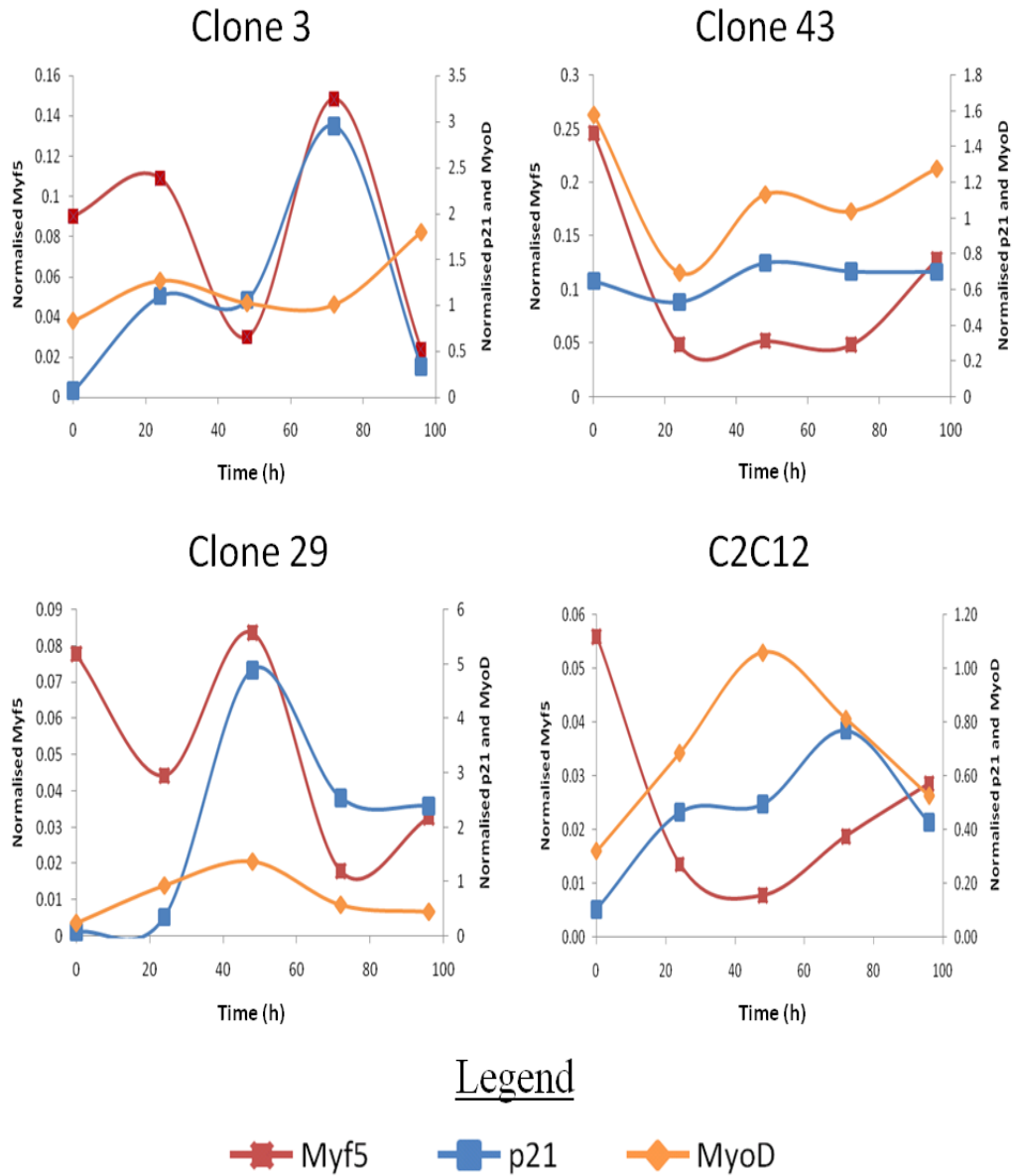


Figure 3.12 *The effect of Pax3 over-expression on Myf5 or MyoD synchronization with p21 expression.* p21, Myf5 and MyoD western blot profiles from cell lysates from Pax3 stable over-expressing clones and C2C12 control cells during 96 h of differentiation, tubulin was analysed for normalisation.

Chapter Four: Discussion

4.1 Overview

Currently, there are inconsistencies in the literature about the involvement of Pax3 in post-natal myogenesis, especially due to its varied spatiotemporal expression in post-natal muscle. While most reviews acknowledge that Pax3 is expressed by quiescent satellite cells and is also expressed transiently during activation, it is the level of expression that creates difficulties in determining the role of Pax3 in post-natal myogenesis, as it varies depending on the muscle type (Buckingham, 2007; Lagha *et al.*, 2008b; Yablonka-Reuveni *et al.*, 2008). As a result, the majority of research has focused on the paralogue of Pax3, Pax7, which seems to play a major role in satellite cell physiology. As Pax3 and Pax7 are both members of the Pax family of genes, they share similar characteristics in their binding to DNA, as described in various studies showing overlapping roles for these two genes (Mansouri, 1998; Relaix *et al.*, 2004). In the absence of Pax7, regeneration in post-natal muscle progresses, albeit with less efficiency. In this scenario Pax3 has been shown to be actively transcribed (Oustanina *et al.*, 2004). In addition, a novel Pax3-expressing myogenic population of cells was recently described: it is able to regenerate damaged muscle both in wildtype and Pax7-null mice (Kuang *et al.*, 2006). These findings show some compensatory mechanisms for Pax3 and Pax7; this was also observed in this study, which showed similar results to experiments that over-expressed Pax7 in C2C12 myoblasts (McFarlane *et al.*, 2006).

Pax3 is heavily involved during embryo-myogenesis, where its expression regulates the myogenic transcriptional cascade of somite-derived myoblasts (Williams & Ordahl, 1994). Furthermore, Pax3 misregulation has been shown to be detrimental during post-natal development, with under-expression or over-expression causing myogenic tumours, known as rhabdomyosarcomas (RMS) (Kurmasheva *et al.*, 2005). This strongly suggests that Pax3 has some regulatory control during adult skeletal myogenesis. Pax3 over-expressing C2C12 myoblast cell lines were used in this study to investigate the regulatory role played by Pax3 during post-natal myogenesis. The stable over-expression of Pax3 in actively

growing C2C12 cells did not induce the expression of its paralogue, Pax7, whereas actively growing control cells exhibited transient expression levels for both Pax3 and Pax7 protein.

4.2 Pax3 and Proliferation

Previous evidence has suggested that Pax3 promotes the proliferation of myogenic progenitor cells. For example, the increased transcriptional capacity of the Pax3-FKHR translocation gene product results in unspecified myogenic expansion of progenitor cells, which causes the formation of RMS tumours (Kurmasheva *et al.*, 2005). Furthermore, the proliferative potential of embryonic limb muscle progenitors decreases if Pax3 is replaced by Pax7 (Relaix *et al.*, 2004). Interestingly, Notch1 signalling promotes the proliferation of Pax3-positive satellite cells during activation (Conboy & Rando, 2002). More recent experimental data also confirms this theory, as constitutive retroviral expression of Pax3 was shown to increase the proliferation of C2C12 cells, while expression of a dominant-negative form of Pax3 slows cell-division (Collins *et al.*, 2009). It was also observed that altering Pax3 expression had an effect on cell morphology, with Pax3 over-expression causing a decrease in cell size (Collins *et al.*, 2009).

On the other hand, constitutive Pax3 over-expression in the three Pax3 clones in these experiments strongly suggests that over-expression of Pax3 slows C2C12 cell division. The methylene blue proliferation assay showed that the control cells reached a significantly higher proliferation rate during exponential growth in comparison to all three Pax3 over-expressing clones (Figure 3.5a). One limitation of this assay is that cellular methylene blue uptake can be affected by cell size (Collins *et al.*, 2009) and thus the Pax3 over-expressing clones could absorb less dye, which could be interpreted as slower proliferation. However, there was no observable difference in cell size in the over-expressing Pax3 clones in comparison to control cells, as seen in Figure 3.6a. In addition, the large significant difference witnessed in the growth rates between C2C12 control cells and Pax3 clones suggests that this experimental limitation is inconsequential. Moreover, the data from the methylene blue assay was always confirmed during cell culture work, as C2C12 control cells always had the highest cell density

following seeding and during culture preparation prior to setting up the biological assays.

The contradictory results from two different experiments, retroviral Pax3 over-expression (Collins *et al.*, 2009) and stably-integrated Pax3-pcDNA3 over-expression, suggests that the level and length of Pax3 expression has an effect on myogenic growth. In the absence of Pax3, via expression of a dominant-negative form of Pax3, proliferation is slowed (Collins *et al.*, 2009). This demonstrates that Pax3 is required for progenitor cell amplification (Conboy & Rando, 2002), as it is required in post-natal muscle for satellite cell activation (Relaix *et al.*, 2006). However, the affect of retroviral over-expression of Pax3 on myogenic proliferation was only assessed at one time point at 72 h (Collins *et al.*, 2009). According to the methylene blue proliferation assay, the growth curve of control C2C12 cells has plateaued by 72 h, at which stage Pax3 over-expressing cells are still in a linear amplification phase. Furthermore, only actively dividing cells will take up the retroviral Pax3 vector, which could have the effect of selecting for a colony of cells that are fast dividers. These studies demonstrate that constitutive Pax3 over-expression slows the proliferation of C2C12 cells. Furthermore, Pax3 over-expression has been shown to inhibit myogenic differentiation (Epstein *et al.*, 1995). Therefore I propose that Pax3 over-expression, whilst slowing cell division, will also enhance the ability of myogenic progenitors to expand the myogenic population; as their myogenic commitment is inhibited, these cells will be maintained in the cell cycle. This is observed during the formation of RMS (Kurmasheva *et al.*, 2005).

4.3 Pax3 and Differentiation

In post-natal muscle, Pax3 is transiently expressed during the activation of satellite cells (Day *et al.*, 2007), as well as being expressed in quiescent satellite cells in specific muscles, such as the majority of forelimb muscles (Relaix *et al.*, 2006). The expression of Pax3 during satellite cell activation results in an intermediate proliferative population of progenitors that are prevented from activating the myogenic program (Conboy & Rando, 2002). This has been shown in a number of studies, where Pax3 expression prevents initiation of differentiation: during limb embryo-myogenesis (Bober *et al.*, 1994; Hammond *et*

al., 2007) and during the formation of RMS tumours (Kurmasheva *et al.*, 2005). Furthermore, *in vitro* over-expression of Pax3 was found to inhibit C2C12 differentiation (Epstein *et al.*, 1995), as well as delaying the differentiation of satellite cell-derived myoblasts (Collins *et al.*, 2009). I obtained similar results during MyHC immunostaining of the Pax3 over-expressing clones and control, which indicated that over-expression of Pax3 delayed the differentiation of C2C12 cells, determined by the fusion index (Figure 3.6b and Figure 3.7a). This delay in differentiation varied depending on the level of Pax3 over-expression; with Pax3 clone 29 (the lowest Pax3 protein over-expresser studied) significantly delaying the differentiation of C2C12 cells the most. However, there was no complete inhibition of myogenic progression, as MyHC expression (an indication of terminal differentiation) was detected, at varying levels, in all Pax3 clones after only 12 h in differentiation medium (Figure 3.6a and Figure 3.7c).

It is worthwhile noting that the fluorescent assay, performed on 96 well microtitre plates, produced less significant differences in the fusion index between Pax3 over-expressing clones and control cells, in comparison to the non-fluorescent assay, which was performed on Thermanox coverslips. This could be caused by the fact that the Optilux™ 96 well microtitre plates used in the fluorescent assay, were found to promote the differentiation of myogenic cells independently (unpublished data, Developmental Biology Group, AgResearch), resulting in an additional exogenous effect influencing cellular differentiation. Moreover, as described in the methods and results, the protocol used in the non-fluorescent assay is more accurate as it has a larger sample size and uses manual counting. In comparison, the protocol used for the fluorescent assay, which is still under optimisation, uses an automated cell counting program that is affected by cell clustering. Thus, results from the non-fluorescent assay were considered more significant and indicative of the effect of Pax3 over-expression on C2C12 myogenic differentiation.

As determined by the fusion index, all Pax3 clones significantly delayed the differentiation of C2C12 cells. However, this significant difference is reduced when assessing only the proportion of MyHC-positive cells instead of the fusion index, for the same assay. Curiously, at the 12 h time point, the Pax3 over-expression clones showed a higher proportion of MyHC-positive cells in comparison to control cells. All three Pax3 clones demonstrated the ability to form

mono- and bi-nucleated myocytes at a similar or faster rate to control C2C12 cells, resulting in growth by hyperplasia (Figure 3.6c and Figure 3.7b). The subsequent fusion to form myotubes (hypertrophy) is where the delay in differentiation was observed during Pax3 over-expression. This hypothesis was confirmed by data that showed that the rate of satellite cell proliferation determines satellite cell fusion capability; a fast-dividing population is biased towards fusing with pre-existing myofibres, whereas a slowly dividing population predominantly fuses together to form new fibres (Rouger *et al.*, 2004), with the Pax3 over-expressing clones mimicking the slow-dividing phenotype.

Alternatively, a number of regulation pathways, such as ubiquitination resulting in Pax3 proteasomal degradation (Boutet *et al.*, 2007) or miRNA interference (Crist *et al.*, 2009) could modulate Pax3 expression levels within the over-expressing Pax3 clones, allowing for terminal differentiation. Clone 3, which over-expressed Pax3 mRNA the most, but only produced an intermediate level of Pax3 protein, could be a candidate for such regulation (Figure 3.4). Pax3 clone 3 also had the highest proportion of MyHC-positive cells at 12 h, indicating myogenic progression (Figure 3.6c). During the course of differentiation, the possible effect of either proteasomal degradation or miRNA interference can also be seen when comparing the Pax3 protein profiles (by Western blot) of Pax3 clones and control cells to the Pax3 mRNA profiles (by Real-Time PCR) of Pax3 clones and control cells (Figure 3.12a). Due to post-transcriptional and post-translational regulation pathways, it was considered that the proteome of the tested cell lines were of more functional importance than mRNA profiles, as protein is the final gene product that has a functional effect.

There are also a number of regulatory pathways that are active during embryonic muscle development under Pax3 control, that mediate myogenic progenitor cell progression in post-natal muscle. In embryonic muscle, Pax3 mediates Sprouty1 expression, which in turn negatively regulates Fgfr4 and terminal differentiation (Hammond *et al.*, 2007). The importance of Fgfr4 signalling during post-natal myogenesis has also been demonstrated, with the absence of Fgfr4 expression resulting in myogenic cells seeking alternative fates, thus undermining muscle regeneration (Zhao *et al.*, 2006; Fukada *et al.*, 2007). Following satellite cell activation, Sprouty1 is down-regulated, a possible consequent of Pax3 down-regulation. Subsequently, the global expression of

MyoD and the up-regulation of Fgfr4 expression marks satellite cell progenitor entry into the myogenic programme (Kastner *et al.*, 2000). It is possible that Sprouty1, which has not shown to be under direct Pax3 control in post-natal muscle, is down-regulated independently of Pax3, thereby allowing for Fgfr4 signalling and the myogenic progression of Pax3 over-expression clones.

A similar situation is observed in the HGF/c-met interplay. In post-natal muscle both genes are involved in the activation of satellite cells (Tatsumi *et al.*, 1998). From an array of growth factors, exogenous HGF was found to initiate the proliferation of progenitor cells most efficiently (Gal-Levi *et al.*, 1998) whilst inhibiting their terminal differentiation (Miller *et al.*, 2000). In embryomyogenesis, HGF and c-met are downstream Pax3 targets, and exhibit a similar effect in the repression of differentiation of myogenic progenitors (Lagha *et al.*, 2008b). Within the *in vitro* analysis, the lack of a natural *in vivo* niche, and thus the lack of exogenous HGF signalling, could prevent the delay in differentiation attributed by Pax3 over-expression. Interestingly, retroviral constitutive expression of Pax3 was shown to delay the myogenic differentiation of satellite cell-derived myoblasts to a larger extent when the experiment was performed within the myofibre niche as compared to *in vitro* culture (Collins *et al.*, 2009). Myogenin expression, indicating commitment to myogenic differentiation, is expressed by myogenic progenitor cells as the Pax genes, specifically Pax7, are down-regulated (Halevy *et al.*, 2004; Zammit *et al.*, 2004). However, in the Pax3 over-expressing clones, as the myogenin profiles show (Figure 3.10b), the cells are capable of initiating differentiation. This indicates either that Pax3 over-expression doesn't completely inhibit differentiation or that a regulatory pathway, which could be Sprouty1/Fgfr4 or HGF/c-met interplay, is interfering with Pax3 over-expression and promoting terminal myogenic differentiation. Retroviral over-expression of Pax3 in C2C12 cells and satellite cell-derived myoblasts showed that differentiation does proceed but with a delayed phenotype. Furthermore, retroviral expression of a dominant-negative form of Pax3 resulted in inhibition of differentiation, suggesting that activation of Pax3 gene targets are required for the initiation of myogenic differentiation (Collins *et al.*, 2009).

C2C12 cells have been shown to behave in a similar manner to satellite cells when induced to differentiate; both cell types produce a heterogenic population of cells that are either committed to differentiation or to the renewal of

the myogenic progenitor population (Yoshida *et al.*, 1998). Asymmetric division leads to population heterogeneity, resulting in fast-dividing progenitors that have limited proliferation prior to differentiation, and slow-dividing progenitors that renew the stem cell population and later, through symmetric division, enhance the myogenic population (Schultz, 1996). The Pax3 clones, in comparison to C2C12 cells, display a significantly lower fusion index, indicating a population of cells that is more biased to self-renewal than to differentiation. I propose that over-expression of Pax3 in C2C12 cells alters population heterogeneity in favour of the slowly-dividing self-renewing population of cells. This is also matched by data on proliferation, which shows that Pax3 over-expression significantly slows the proliferation of cells, for all clones. Furthermore, the Pax3 clones, as mentioned above, showed bias towards the formation of mono- and bi-nucleated myocytes instead of fusing to form myofibres in comparison to C2C12 cells.

Gene expression analysis performed on differentiating Pax3 clones and C2C12 cells also supported the above proposed hypothesis (Figure 3.10b). At the intermediate progenitor stage of post-natal muscle regeneration, once Pax3 expression becomes down-regulated, MyoD expression marks entry into the myogenic program with co-expression of Pax7 (Zammit *et al.*, 2004). Myf5 is already expressed by the majority of satellite cell progeny (Zammit *et al.*, 2006b), however, Myf5 has been shown to be regulated by Pax3 (Bajard *et al.*, 2006). This was confirmed in the Pax3 over-expressing cell lines which were found to express Myf5 to a higher extent than control C2C12 cells, with Myf5 expression profiles generally matching Pax3 expression profiles, as determined via Western blot. Both Myf5 and Pax3 are genetically upstream of MyoD (Bajard *et al.*, 2006), and in post-natal muscle, expression of a dominant-negative form of Pax3 and Pax7 results in repression of MyoD expression (Relaix *et al.*, 2006). However, the effect of Pax3 over-expression on MyoD expression is difficult to determine, as the expression profiles are similar to those of control cells, as previously shown by Epstein *et al.* (1995). However, MyoD did peak at a higher level in all three Pax3 clones in comparison to C2C12 cells, which has also been previously demonstrated (Collins *et al.*, 2009). This pattern of expression matches hypaxial precursor cell gene expression, where these precursor cells require expression of both Pax3 and Myf5 to induce MyoD expression (Tajbakhsh *et al.*, 1997), suggesting that Pax3 has indirect control of MyoD and that maintained Pax3

expression will facilitate MyoD expression as it causes up-regulation of Myf5. In addition, this could also place Pax3 in indirect control over Fgfr4 signalling, as Fgfr4 signalling has been shown to be transcriptionally regulated by MyoD during post-natal myogenesis (Zhao *et al.*, 2006).

Interestingly, during the course of differentiation, p21 was generally up-regulated in the Pax3 over-expressing clones in comparison to control cells. This could explain the consequent slowing of proliferation observed with Pax3 over-expression, as p21 caused down-regulation of cell cycle activators, preventing cell cycle transition (McFarlane *et al.*, 2006). The synchronisation of p21 up-regulation (cell cycle withdrawal) with high Myf5 and low MyoD expression indicates quiescent, non-differentiating myoblasts, whereas the opposite expression pattern, high MyoD and low Myf5 expression, occurs in differentiating myoblasts (Kitzmann & Fernandez, 2001). Interestingly, in the Pax3 over-expressing clones, at 48 h of differentiation, clone 29 demonstrated synchronisation of p21 expression with Myf5 expression (Figure 3.11), indicating an early withdrawal of progenitor cells from the cell cycle to renew the myogenic population. Pax3 clone 3 showed a similar p21 and Myf5 expression profile, but peaking at the later time point of 72 h (Figure 3.11). On the contrary, Pax3 clone 43 did not show a similarly defined expression profile, however, the gradual incline in p21 expression exhibited as differentiation proceeded was matched with a gradual incline in both Myf5 and MyoD expression (Figure 3.11). This is corroborated by the fusion index results, which suggest that clone 29 delays myogenic differentiation more efficiently than clones 3 and 43. The expression profile of the control cells on the other hand, demonstrated that p21 expression synchronised mainly with MyoD expression (Figure 3.11). As it has been determined that proliferating cultures become heterogenic in gene expression, the high MyoD, low Myf5 expression profile of control cells suggests that the control cell line had a higher proportion of differentiating myoblasts that divide symmetrically. In rat post-natal muscle regeneration, 80% of progenitor cells divided symmetrically and 20% divided asymmetrically (Schultz, 1996). In the Pax3 over-expressing clones, up-regulation of Myf5 expression in comparison to control cells, in synchronisation with a peak for p21 expression, suggests an alteration to this proportional division and indicates that a larger proportion of quiescent myoblasts divide asymmetrically. However, there was still a proportion

of cells that were fast-dividing and capable of differentiation; as the myogenin expression profiles of the Pax3 clones showed (Figure 3.10b), myogenesis still proceeded, contrary to previous research (Epstein *et al.*, 1995).

The differences in results based on Pax3 over-expression in the same cell type between this study and Epstein *et al.* (1995), could be due to differences in expression levels of Pax3. The Pax3-FKHR was used by Epstein *et al.* (1995), which has 100 times the transcriptional efficiency of Pax3 in comparison to wildtype Pax3 (Bennicelli *et al.*, 1995), as used in this study, which could be responsible for the complete inhibition of myotube formation observed by Epstein *et al.* (1995). Although contradictory, both sets of experimental data are mimicked during naturally occurring *in vivo* circumstances. For example, complete inhibition of differentiation is witnessed during expression of the naturally occurring mutated Pax3-FKHR gene, resulting in the generation of myogenic tumours consisting of undifferentiated myogenic progenitor cells (Kurmasheva *et al.*, 2005). In addition, maintained wildtype Pax3 expression in the hypaxial domain during embryo-myogenesis is shown to delay differentiation, as seen in the Pax3 over-expression clones. Furthermore, primary myocytes are formed in the absence of Pax7 expression via a Myf5-directed differentiation pathway (Buckingham & Relaix, 2007), which is also possibly occurring in the Pax3 over-expressing clones. This strongly suggests that the level of Pax3 expression has direct influence on myogenic precursor cell fate.

Research performed by Lepper *et al.* (2009) showed that stem cells display age-dependent changes in their genetic requirement, when comparing embryonic and post-natal stem cell biology. Interestingly, the *in vitro* experiments performed in this study, on a post-natal murine cell line, displayed a similar phenotype and genotype to embryonic hypaxial precursor cells, which are positive for both Pax3 and Myf5. Furthermore, Collins *et al.* (2009) has demonstrated that Pax3 expression is required for the differentiation of satellite cell-derived myoblasts in an *in vitro* study, whereas, the *in vivo* study performed by Lepper *et al.* (2009), strongly suggested that post-natal muscle regeneration and growth proceeds in the absence of Pax3 and Pax7 regulation. Putting inconsistencies aside, taken together this data would suggest that the satellite cell niche plays an important regulatory role in satellite cell function, which may bypass the need for Pax3 or Pax7 expression during post-natal muscle progression.

4.4 Pax3 and Survival

During embryo-myogenesis, muscle progenitor survival in the dermomyotome and in the developing myotome is supported by the anti-apoptotic function of Pax3. In the developing hypaxial somite the anti-apoptotic role of Pax3 becomes a requisite for cell survival (Bajard *et al.*, 2006). This is observed in naturally-occurring *Spotch* mutant mice (Pax3-null), which exhibit increased apoptosis in the thoracic muscles (Dickman *et al.*, 1999). Confirmation of Pax3 anti-apoptotic function in embryonic tissue was shown using anti-sense Pax3 oligonucleotides to disrupt Pax3 expression, resulting in down-regulation of MyoD and increased programmed cell death (apoptosis). Some rescue effect was observed, when Pax7 was up-regulated in response to the absence of Pax3, also resulting in the down-regulation of MyoD (Borycki *et al.*, 1999).

The Pax-positive myogenic stem cell population that develops into quiescent satellite cells requires both Pax3 and Pax7 expression, not only for myogenic specification but also for cell survival. In the absence of both Pax3 and Pax7 in muscle progenitor cells, extensive apoptosis takes place if cells don't assume other cell fates (Kassar-Duchossoy *et al.*, 2005). Thus, Pax3 and Pax7 are both necessary for the survival of myogenic cells during embryonic and pre-natal development. However, during post-natal development, as illustrated by juvenile Pax7-null mice, considerable cell death is observed in the satellite cell population (Oustanina *et al.*, 2004). This was confirmed in Pax3-expressing muscle, where in the absence of Pax7 expression; satellite cells were still increasingly lost due to apoptosis, validating the necessity for Pax7 in satellite cell survival (Relaix *et al.*, 2006). An experiment that knocked-out MyoD illustrated that the Pax3- and Pax7-positive self-renewing lineage of myogenic cells are increasingly resistant to apoptosis. In comparison to wild-type myoblasts, MyoD-null myoblasts down-regulate myogenic determination genes and up-regulate anti-apoptotic genes, corresponding to increased cell survival (Asakura *et al.*, 2007). Quiescent satellite cells transiently express Pax3 and are negative for MyoD expression (Relaix *et al.*, 2006), thus reflecting the above situation, which suggests that Pax3 also plays an anti-apoptotic role during post-natal myogenesis, maintaining the satellite cell population.

Pax3 over-expression through fusion with the Forkhead gene (Pax3-FKHR) (Bennicelli *et al.*, 1995) results in an oncogenic effect provided by its anti-apoptotic function, which results in resistance to cell death and to the formation of myogenic tumours (RMS). The anti-apoptotic function of Pax3 is obtained through its interaction with the anti-apoptotic gene BCL-XL. Ectopic expression of either Pax3 or Pax3-FKHR in primary human myoblasts and fibroblasts induces BCL-XL expression (Margue *et al.*, 2000). Interestingly, the less transcriptionally active form of Pax3, in comparison to Pax3-FKHR, induced higher levels of BCL-XL expression, suggesting that lower levels of Pax3 result in a larger up-regulation of BCL-XL and therefore increased cell survival (Bennicelli *et al.*, 1996; Margue *et al.*, 2000).

The above described data match the results reported in this study, which indicated that the lowest Pax3 over-expressing clone, clone 29, had consistently lower activation of caspase activity with treatment in both 10% FBS and with no serum (Figure 3.8a), and therefore a lower level of apoptosis. Furthermore, clone 29 had the overall lowest percentage increase in cell death caused by serum starvation in comparison to the other Pax3 over-expressing cells and control cells (Figure 3.8b). By comparison, clone 3 and clone 43, both the strongest Pax3 over-expressers, exhibited the highest percentage activation of caspase activity in comparison to control cells following serum starvation (Figure 3.8b). In addition, clone 43, the highest Pax3 over-expresser, exhibited significantly higher cell death above that of control cells when serum starved (Figure 3.8a). Moreover, the level of survival exhibited by the individual clones also seems to be directly related to the level of myogenic commitment displayed by each clone, determined by the level of MyoD and myogenin expression. During the course of differentiation, Pax3 over-expressing clone 29 generally showed a lower level of MyoD and myogenin expression in comparison to control cells and Pax3 over-expression clones 3 and 43, while exhibiting the highest resistance to apoptosis.

As both Pax3 and Pax7 offer an anti-apoptotic function to myogenic cells, the control C2C12 cells used in the apoptosis assay were analysed for the level of Pax3 and Pax7 expression. Western blot analysis indicated transient expression levels for both Pax3 and Pax7 for the control cells (Figure 3.2), whereas the same Western blot expression analysis on the Pax3 clones indicated no detectable expression of Pax7. Thus, the results suggest that the transient expression of both

Pax3 and Pax7 offers a larger survival effect than the over-expression of Pax3 alone. However, as shown by clone 29, a specifically low level of Pax3 expression, above that of a transient expression level, provides a significantly higher survival than transient expression of both Pax3 and Pax7. Taken together, the data suggest that, Pax3 has an anti-apoptotic function, most likely mediated through its target gene BCL-XL in a dosage dependent manner (Margue *et al.*, 2000), and that in post-natal muscle this anti-apoptotic effect of Pax3 is increased if co-expressed with Pax7.

4.5 Pax3 and Migration

During embryo-myogenesis Pax3, through its targets c-met and SF/HGF, is vital for the delamination and migration of myogenic progenitor cells to sites of limb myogenesis (Epstein *et al.*, 1996; Birchmeier & Brohmann, 2000). During post-natal muscle regeneration, satellite cells are activated and migrate to sites of injury, but the role played by Pax3 during this process remains to be determined. It is known that satellite cells and their proliferating progeny express the c-met receptor following activation (Cornelison & Wold, 1997). In addition, the surrounding basal lamina satellite cell niche contains diffusible HGF growth factor, which is released in active form following injury (Tatsumi & Allen, 2004). It has been postulated that HGF could be involved in lamellipodia formation, implicating its involvement in cell migration (Kawamura *et al.*, 2004; Salerno *et al.*, 2009). Furthermore, Notch signalling, activated in response to growth and injury, up-regulates transient Pax3 expression (Conboy & Rando, 2002), which could be involved in mediating satellite cell progenitor migration to sites of injury.

The chemotactic assay used to analyse the migrational abilities of the cells indicated that a specific level of Pax3 over-expression may enhance the migration of C2C12 myoblasts in comparison to control C2C12 cells. This difference in migrational ability between Pax3 over-expressing clones and control cells was amplified when fewer chemoattractants were used within a treatment, such as when only 5% CEE was used in comparison to the combination of both 5% CEE and 2% HS (see Section 3.2.4). The level of Pax3 expression could have an effect on migrational ability, as Pax3 clone 43 (expressing the most Pax3 protein) migrated less than control cells (Figure 3.9). On the other hand, this could be due

to an increased sensitivity to apoptosis (demonstrated by Pax3 clone 43) as determined by the apoptosis assay performed (Figure 3.8), as cells are kept in the absence of serum on the upper chamber during the migration assay. However, Pax3 clone 3 also demonstrated a higher susceptibility to apoptosis in comparison to control cells, whilst still exhibiting the best chemotactic ability. This suggests that a biphasic relationship exists between Pax3 expression and migration, similar to the one existing for the level of Pax3 expression and survival.

The over-expression of Pax3 in two clones (clone 3 and clone 29) resulted in an increase in migration for the majority of the treatments in comparison to control cells, suggesting that Pax3 plays an active transcriptional role in myoblast migration. Even though this experiment did not provide results that were statistically significant, it did suggest that an intermediate over-expression of Pax3 (clones 3 and 29) in comparison to transient expression (control cells) or large over-expression (clone 43) of Pax3 prepared cells for migration and increased their sensitivity to chemotactic signals. Therefore, it is not unreasonable to consider that, in post-natal muscle, Pax3 could mediate satellite cell activation by regulating the HGF and c-met signalling pathways, and also by mediating the inhibition of myogenic differentiation to promote the migration of satellite cell-derived progeny to sites of injury.

4.6 Concluding Remarks

Recent *in vivo* research has investigated the roles played by Pax3 and Pax7 in skeletal muscle formation and regeneration. As previously indicated, Pax3 is the master myogenic transcription factor during embryo-myogenesis, and Pax7 acts as the major myogenic transcriptional factor during peri-natal and juvenile muscle development. However, inactivation of Pax3 and Pax7 expression during post-natal development results in normal regeneration processes proceeding, indicating that adult satellite cells do not require either Pax3 or Pax7 for post-natal myogenic specification (Lepper *et al.*, 2009). To the contrary, Pax3 expression has been detected in specific post-natal muscles, such as the diaphragm, trunk and limb muscles; expressed in quiescent satellite cells and transiently during satellite cell activation (Relaix *et al.*, 2006). Isolated satellite cells from these muscle types showed regenerative capacity when grafted into the *tibialis anterior* muscle of

immunodeficient *mdx* mice (Montarras *et al.*, 2005). Expression of Pax3 in these specific muscle types indicates that Pax3 is either involved in the muscle regeneration process or acts as a muscle-specific stem cell marker. However, dominant-negative expression constructs of Pax3 have indicated an active transcriptional role for Pax3, as the construct prevented C2C12 cells, as well as satellite cell-derived myoblasts, from initiating the myogenic program (Collins *et al.*, 2009). Similarly, Pax7 has also been proven to be important in post-natal myogenesis, for precursor cell specification, and for satellite cell self-renewal (McFarlane *et al.*, 2006; Relaix *et al.*, 2006), as Pax7-null studies have shown that regeneration is severely compromised in the absence of Pax7 (Oustanina *et al.*, 2004). However, regeneration has been shown to proceed, facilitated by a novel Pax3-expressing lineage present in the interstitial muscle environment in both Pax7-null and wildtype muscle (Kuang *et al.*, 2006). The regenerative ability of this novel Pax3 lineage does result in reduced myogenic differentiation in the absence of Pax7, indicating that co-expression of Pax7 enhances muscle stem cell specification (Seale *et al.*, 2004; Kuang *et al.*, 2006). In this novel, interstitial Pax3-expressing myogenic lineage it was found that Pax3-positive precursor cells can generate up to 5% of the MyoD-positive cells in wildtype muscle (Kuang *et al.*, 2006).

The results from this study indicate that over-expression of Pax3 in a post-natal mouse cell line model activates a myogenic precursor state, as witnessed during transient Pax3 expression in satellite cells (Conboy & Rando, 2002). Continued Pax3 expression would maintain cells in the precursor state, suggesting that *in vivo* this may occur to promote the migration of satellite cell-derived precursor cells to distal sites of injury. The hyperplasia observed during differentiation of the Pax3 over-expressing clones is possibly induced by a Myf5-directed differentiation pathway, as observed in embryo-myogenesis during primary differentiation (Buckingham & Relaix, 2007). As myogenic specification is not completely reliant on Pax7 (Kuang *et al.*, 2006), maintained Pax3 expression can result in myogenic specification via MyoD expression, although at a reduced rate. In the Pax3 over-expressing clones, this smaller proportion of committed progenitors, obtained via MyoD expression provides myogenic growth via hypertrophy, and is responsible for the myotubes formed during low serum induced differentiation. I propose that for efficient hypertrophy, Pax7 expression

is required and that at this stage during *in vivo* myogenic growth Pax3 would have been down-regulated to prevent further delay in myogenic differentiation. Whereas with sustained expression of only Pax3, which will result in a small proportion of cells that express MyoD, a similar delay in differentiation is observed in MyoD-null myoblasts (White *et al.*, 2000). Thus, if Pax3 expression is maintained, this could result in an increase in the propensity of cells that enter the G(0)-phase of the cell cycle between mitotic division, increasing the proportion of cells that divide asymmetrically and express Myf5. Therefore, in comparison to control cells, Pax3 over-expression delays the differentiation phenotype, as its over-expression increases the proportion of cells that self-renew. The satellite cell progeny that renews the myogenic stem cell population will be negative for MyoD expression, which has been shown to result in an up-regulation of anti-apoptotic genes (Asakura *et al.*, 2007), resulting in increased cell survival. The delay in differentiation observed with the over-expression of Pax3 could also be caused by the slower rate of cell division, and thus an extended proliferation period is required to allow the cells to reach confluence.

Population heterogeneity could be introduced by the proteasomal Pax3 degradation pathway via ubiquitination (Boutet *et al.*, 2007), or miRNA interference via miR-27b (Crist *et al.*, 2009), resulting in the small subset of cells that express MyoD. Research into both pathways of Pax3 regulation suggest a role for Pax3 in post-natal myogenesis, as interference of either pathway results in satellite cell-derived myoblasts continuing to proliferate as differentiation is delayed, due to the continued expression of Pax3 (Boutet *et al.*, 2007; Crist *et al.*, 2009). There is considerable evidence to indicate a role for Pax3 in post-natal myogenesis, such as aberrant Pax3 expression resulting in numerous abnormal muscle conditions (Chalepakis *et al.*, 1994; Underhill, 2000) or myogenic tumours (Bennicelli *et al.*, 1995; Kurmasheva *et al.*, 2005), indicating the importance of the correct spatial-temporal Pax3 protein expression in post-natal myogenesis. The lack of *in vivo* research confirming the role of Pax3 during regeneration could be made more difficult by the specific localised expression of Pax3 and the proteasomal degradation pathway that would eliminate any trace of Pax3 expression. In addition, as the majority of the experiments performed show, the effects of Pax3 expression is dosage dependent, demonstrating threshold effects, similar to those seen with other Pax proteins in the literature (Buckingham, 2007).

The naturally-occurring level of Pax3 expression detected during post-natal myogenesis has been described as transient, demonstrating its strength as a myogenic transcriptional regulator.

As there is conflicting research on the involvement of Pax3 in post-natal myogenesis, more research is required to determine its role. An *in vivo* analysis of distal skeletal muscles following injury could possibly emphasize the action of Pax3, as its expression is maintained to allow for precursor cell migration. In this situation, satellite cells might replicate the hypaxial Pax3-positive precursor population during embryo-myogenesis. In addition, research into regulation pathways involved with mediation of post-natal progression would be informative, such as sprouty1/Fgfr4 or HGF/c-met interplay, as both pathways have been previously shown to be under Pax3 control. If Pax3 is actively involved in the post-natal regeneration process, its transcriptional power and myogenic potential are questions that offer a possible strategy for muscle atrophy and dystrophy therapy. Over-expression of Pax3 could provide a population of myogenic progenitors that are biased to replenish the myogenic stem cell pool, while providing hyperplasia for myogenic growth. Furthermore, this therapeutic strategy has the advantage of two naturally-occurring protein synthesis regulation pathways that could be used to mediate Pax3 expression levels.

Appendix

Common Solutions:

Agarose

0.5 g UltraPure agarose (Invitrogen)

50 ml 1 x TAE buffer

Boiled until agarose is dissolved.

1 μ l 10 mg/ml ethidium bromide per 50 ml Agarose.

Denaturing RNA agarose gel

0.6 g agarose

36 ml DEPC treated water

Boil to dissolve agarose then cooled to $\sim 60^{\circ}\text{C}$

In fume hood add:

5 ml 10 x MOPS

9 ml formaldehyde

DEPC (diethyl pyrocarbonate)-treated water

2 ml DEPC

2 l MilliQ water

Mixed overnight then autoclaved.

DNA 1kb+ Ladder

90 μ l 10 x DNA loading dye

810 μ l MilliQ water

100 μ l 1 μ g/ml 1kb+ ladder

DNA Loading Dye (10 x)

10 ml 50% glycerol

2 ml 50 x TAE

Pinch of bromophenol blue

Eosin (1% solution)

10 g Eosin Y
1 l MilliQ water
2.0 ml acetic acid (5% aqueous)
1 crystal of thymol

Gill's Haematoxylin

4.0 g Haematoxylin
0.4 g sodium iodate
35.2 g aluminium sulphate
710 ml MilliQ water
250 ml ethylene glycol
40 ml glacial acetic acid

Matrigel

200 µl Matrigel
1800 µl DMEM
Bottom of plate is covered, excess is removed. Plate kept at 37°C to allow Matrigel to set.

MOPS (10 x) Solution (3-[N-Morpholino]propane-sulfonic acid)

41.8% MOPS powder
50 mM sodium acetate
10 mM EDTA

PBS (Phosphate Buffered Saline)

1 PBS tablet (Oxoid)
100 ml MilliQ water

PBS (10x)

1 PBS tablet
10 ml MilliQ water

Ponceau S stain

0.1% Ponceau S

0.1% Acetic acid

Make up to desired volume with MilliQ water

Protein loading buffer

3 parts 4 x NuPage sample buffer

1 part β -mercaptoethanol

Protein lysis buffer

50 mM Tris (pH 7.5)

250 mM sodium chloride

5 mM EDTA

0.1% NP-40

1 x Protease inhibitor

RNA Loading Dye (2 x)

2 ml MOPS (10 x)

2 ml deionised formaldehyde

5 ml deionised formamide

40 μ l of 10% bromophenol blue

1 ml glycerol

100 μ l of 0.5 M EDTA (pH 8.0)

40 μ l of 10 mg/ml ethidium bromide

RNA running buffer

1 part 10 x MOPS

9 parts DEPC water

Scott's tap-water

2.0 g sodium bicarbonate

20.0 g magnesium sulphate

1 l MilliQ water

1 crystal of thymol

TAE (Tris-acetate EDTA) for DNA gels (50 x)

242 g Tris (base)

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (ph 8.0)

Made up to 1 l with MilliQ water

TBS (Tris buffered saline)

50 ml 1 M Tris

30 ml 5 M Sodium chloride

Made up to 1 l with MilliQ water

TBS-T (Tris buffered saline + Tween-20)

999 ml TBS

1 ml Tween-20

Western blocking buffer

0.15 g BSA

0.5 g PVP

0.5 g PEG

50 ml TBS-T

20:2:1 fixative

20 parts 70% ethanol

2 parts 40% formaldehyde

1 part glacial acetic acid

75% ethanol

75 ml 100% ethanol

25 ml DEPC-treated water

0.35% Carageenen-lambda

0.175 g Carageenen from seaweed (Sigma)

50 ml PBS

1X SDS running buffer

50 ml 20X NuPAGE™ MES SDS Running Buffer (Invitrogen)

950 ml dH₂O

10X Trypsin

2.5% trypsin in PBS

Bacterial growth media

LB media

Bacteria were grown in LB Broth (Lennox L Broth) which was prepared by adding 20 g LB Broth base to 1 L H₂O, then autoclaved and stored at 4°C.

LB plates

Bacterial colonies were grown on LB Agar (Lennox L Agar) plates. LB Agar plates were prepared by adding 32 g LB Agar to 1 L H₂O, followed by autoclaving. Molten LB Agar (20 ml) was poured into Petri dishes (10 cm; Nunc) and left to set before storing at 4°C.

Cell culture media

Na HCO₃ Stock solution

22 g NaHCO₃

500 ml H₂O

0.32 ml 0.5% phenol red

DMEM

50 ml 2× DMEM

8 ml HCO₃⁻

1 ml penicillin G (200 U/ml) and streptomycin (200 µg/ml)

41 ml H₂O

Proliferation medium

10 ml FBS

90 ml DMEM

Differentiation medium

2 ml HS

98 ml DMEM

References

- Allbrook DB, Han MF & Hellmuth AE. (1971). Population of muscle satellite cells in relation to age and mitotic activity. *Pathology* **3**, 223-243.
- Allen RE & Boxhorn LK. (1987). Inhibition of skeletal muscle satellite cell differentiation by transforming growth factor-beta. *J Cell Physiol* **133**, 567-572.
- Allen RE & Boxhorn LK. (1989). Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I, and fibroblast growth factor. *J Cell Physiol* **138**, 311-315.
- Allen RE, Sheehan SM, Taylor RG, Kendall TL & Rice GM. (1995). Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. *J Cell Physiol* **165**, 307-312.
- Anderson J, Gordon A, Pritchard-Jones K & Shipley J. (1999). Genes, chromosomes, and rhabdomyosarcoma. *Genes, chromosomes & cancer* **26**, 275-285.
- Argiles JM, Busquets S, Felipe A & Lopez-Soriano FJ. (2005). Molecular mechanisms involved in muscle wasting in cancer and ageing: cachexia versus sarcopenia. *Int J Biochem Cell Biol* **37**, 1084-1104.
- Argiles JM, Moore-Carrasco R, Fuster G, Busquets S & Lopez-Soriano FJ. (2003). Cancer cachexia: the molecular mechanisms. *Int J Biochem Cell Biol* **35**, 405-409.
- Arnold H, Della-Fera, M. A., and Baile, C. A. (2001). Review of myostatin history, physiology and applications. *LifeXY* **1**, 1014-1022.
- Asakura A, Hirai H, Kablar B, Morita S, Ishibashi J, Piras BA, Christ AJ, Verma M, Vineretsky KA & Rudnicki MA. (2007). Increased survival of muscle stem cells lacking the MyoD gene after transplantation into regenerating skeletal muscle. *Proc Natl Acad Sci U S A* **104**, 16552-16557.
- Ausubel FA, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA & Struhl K. (1987). *Current Protocols in Molecular Biology*. Wiley Interscience, New York.
- Bajard L, Relaix F, Lagha M, Rocancourt D, Daubas P & Buckingham ME. (2006). A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. *Genes Dev* **20**, 2450-2464.

- Baroffio A, Hamann M, Bernheim L, Bochaton-Piallat ML, Gabbiani G & Bader CR. (1996). Identification of self-renewing myoblasts in the progeny of single human muscle satellite cells. *Differentiation* **60**, 47-57.
- Beauchamp JR, Heslop L, Yu DS, Tajbakhsh S, Kelly RG, Wernig A, Buckingham ME, Partridge TA & Zammit PS. (2000). Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *J Cell Biol* **151**, 1221-1234.
- Ben-Yair R & Kalcheim C. (2005). Lineage analysis of the avian dermomyotome sheet reveals the existence of single cells with both dermal and muscle progenitor fates. *Development* **132**, 689-701.
- Bennicelli JL, Edwards RH & Barr FG. (1996). Mechanism for transcriptional gain of function resulting from chromosomal translocation in alveolar rhabdomyosarcoma. *Proc Natl Acad Sci U S A* **93**, 5455-5459.
- Bennicelli JL, Fredericks WJ, Wilson RB, Rauscher FJ, 3rd & Barr FG. (1995). Wild type PAX3 protein and the PAX3-FKHR fusion protein of alveolar rhabdomyosarcoma contain potent, structurally distinct transcriptional activation domains. *Oncogene* **11**, 119-130.
- Berkes CA, Bergstrom DA, Penn BH, Seaver KJ, Knoepfler PS & Tapscott SJ. (2004). Pbx marks genes for activation by MyoD indicating a role for a homeodomain protein in establishing myogenic potential. *Mol Cell* **14**, 465-477.
- Birchmeier C & Brohmann H. (2000). Genes that control the development of migrating muscle precursor cells. *Curr Opin Cell Biol* **12**, 725-730.
- Blau HM, Pavlath GK, Hardeman EC, Chiu CP, Silberstein L, Webster SG, Miller SC & Webster C. (1985). Plasticity of the differentiated state. *Science* **230**, 758-766.
- Bober E, Franz T, Arnold HH, Gruss P & Tremblay P. (1994). Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development* **120**, 603-612.
- Borycki AG, Li J, Jin F, Emerson CP & Epstein JA. (1999). Pax3 functions in cell survival and in pax7 regulation. *Development* **126**, 1665-1674.
- Boutet SC, Disatnik MH, Chan LS, Iori K & Rando TA. (2007). Regulation of Pax3 by proteasomal degradation of monoubiquitinated protein in skeletal muscle progenitors. *Cell* **130**, 349-362.
- Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-254.

- Brand-Saberi B. (2005). Genetic and epigenetic control of skeletal muscle development. *Ann Anat* **187**, 199-207.
- Brand-Saberi B & Christ B. (1999). Genetic and epigenetic control of muscle development in vertebrates. *Cell Tissue Res* **296**, 199-212.
- Brand-Saberi B, Wilting J, Ebensperger C & Christ B. (1996). The formation of somite compartments in the avian embryo. *Int J Dev Biol* **40**, 411-420.
- Brohmann H, Jagla K & Birchmeier C. (2000). The role of Lbx1 in migration of muscle precursor cells. *Development* **127**, 437-445.
- Brunelli S, Relaix F, Baesso S, Buckingham M & Cossu G. (2007). Beta catenin-independent activation of MyoD in presomitic mesoderm requires PKC and depends on Pax3 transcriptional activity. *Dev Biol* **304**, 604-614.
- Bryson-Richardson RJ & Currie PD. (2008). The genetics of vertebrate myogenesis. *Nature reviews* **9**, 632-646.
- Buckingham M. (2001). Skeletal muscle formation in vertebrates. *Current opinion in genetics & development* **11**, 440-448.
- Buckingham M. (2006). Myogenic progenitor cells and skeletal myogenesis in vertebrates. *Current opinion in genetics & development* **16**, 525-532.
- Buckingham M. (2007). Skeletal muscle progenitor cells and the role of Pax genes. *Comptes rendus biologies* **330**, 530-533.
- Buckingham M, Bajard L, Chang T, Daubas P, Hadchouel J, Meilhac S, Montarras D, Rocancourt D & Relaix F. (2003). The formation of skeletal muscle: from somite to limb. *J Anat* **202**, 59-68.
- Buckingham M & Montarras D. (2008). Skeletal muscle stem cells. *Current opinion in genetics & development* **18**, 330-336.
- Buckingham M & Relaix F. (2007). The role of Pax genes in the development of tissues and organs: Pax3 and Pax7 regulate muscle progenitor cell functions. *Annu Rev Cell Dev Biol* **23**, 645-673.
- Chalepakis G, Jones FS, Edelman GM & Gruss P. (1994). Pax-3 contains domains for transcription activation and transcription inhibition. *Proc Natl Acad Sci U S A* **91**, 12745-12749.
- Charge S & Rudnicki M. (2004). Cellular and molecular regulation of muscle regeneration. *Physiol Rev* **84**, 209-238.
- Christ B & Ordahl CP. (1995). Early stages of chick somite development. *Anat Embryol (Berl)* **191**, 381-396.

- Cinnamon Y, Ben-Yair R & Kalcheim C. (2006). Differential effects of N-cadherin-mediated adhesion on the development of myotomal waves. *Development* **133**, 1101-1112.
- Collins CA, Gnocchi VF, White RB, Boldrin L, Perez-Ruiz A, Relaix F, Morgan JE & Zammit PS. (2009). Integrated functions of Pax3 and Pax7 in the regulation of proliferation, cell size and myogenic differentiation. *PloS one* **4**, e4475.
- Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA & Morgan JE. (2005). Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell* **122**, 289-301.
- Conboy IM, Conboy MJ, Smythe GM & Rando TA. (2003). Notch-mediated restoration of regenerative potential to aged muscle. *Science* **302**, 1575-1577.
- Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL & Rando TA. (2005). Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* **433**, 760-764.
- Conboy IM & Rando TA. (2002). The regulation of notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Dev Cell* **3**, 397-409.
- Cornelison DD & Wold BJ. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev Biol* **191**, 270-283.
- Cossu G, Kelly R, Tajbakhsh S, Di Donna S, Vivarelli E & Buckingham M. (1996a). Activation of different myogenic pathways: myf-5 is induced by the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm. *Development* **122**, 429-437.
- Cossu G, Tajbakhsh S & Buckingham M. (1996b). How is myogenesis initiated in the embryo? *Trends Genet* **12**, 218-223.
- Crist CG, Montarras D, Pallafacchina G, Rocancourt D, Cumano A, Conway SJ & Buckingham M. (2009). Muscle stem cell behavior is modified by microRNA-27 regulation of Pax3 expression. *Proc Natl Acad Sci U S A* **106**, 13383-13387.
- Darabi R, Gehlbach K, Bachoo RM, Kamath S, Osawa M, Kamm KE, Kyba M & Perlingeiro RC. (2008a). Functional skeletal muscle regeneration from differentiating embryonic stem cells. *Nat Med* **14**, 134-143.
- Darabi R & Perlingeiro RC. (2008). Lineage-specific reprogramming as a strategy for cell therapy. *Cell Cycle* **7**, 1732-1737.

- Darabi R, Santos FN & Perlingeiro RC. (2008b). The therapeutic potential of embryonic and adult stem cells for skeletal muscle regeneration. *Stem cell reviews* **4**, 217-225.
- Day K, Shefer G, Richardson JB, Enikolopov G & Yablonka-Reuveni Z. (2007). Nestin-GFP reporter expression defines the quiescent state of skeletal muscle satellite cells. *Dev Biol* **304**, 246-259.
- Delfini MC, Hirsinger E, Pourquie O & Duprez D. (2000). Delta 1-activated notch inhibits muscle differentiation without affecting Myf5 and Pax3 expression in chick limb myogenesis. *Development* **127**, 5213-5224.
- Dias P, Parham DM, Shapiro DN, Webber BL & Houghton PJ. (1990). Myogenic regulatory protein (MyoD1) expression in childhood solid tumors: diagnostic utility in rhabdomyosarcoma. *Am J Pathol* **137**, 1283-1291.
- Dickman ED, Rogers R & Conway SJ. (1999). Abnormal skeletogenesis occurs coincident with increased apoptosis in the Splotch (Sp2H) mutant: putative roles for Pax3 and PDGFRalpha in rib patterning. *Anat Rec* **255**, 353-361.
- Doetschman TC, Eistetter H, Katz M, Schmidt W & Kemler R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *Journal of embryology and experimental morphology* **87**, 27-45.
- Doherty TJ. (2003). Invited review: Aging and sarcopenia. *J Appl Physiol* **95**, 1717-1727.
- Edom-Vovard F, Bonnin MA & Duprez D. (2001). Misexpression of Fgf-4 in the chick limb inhibits myogenesis by down-regulating Fkrl expression. *Dev Biol* **233**, 56-71.
- Emery AE. (1998). The muscular dystrophies. *Bmj* **317**, 991-995.
- Emery AE. (2002). The muscular dystrophies. *Lancet* **359**, 687-695.
- Epstein JA, Lam P, Jepeal L, Maas RL & Shapiro DN. (1995). Pax3 inhibits myogenic differentiation of cultured myoblast cells. *J Biol Chem* **270**, 11719-11722.
- Epstein JA, Shapiro DN, Cheng J, Lam PY & Maas RL. (1996). Pax3 modulates expression of the c-Met receptor during limb muscle development. *Proc Natl Acad Sci U S A* **93**, 4213-4218.
- Esner M, Meilhac SM, Relaix F, Nicolas JF, Cossu G & Buckingham ME. (2006). Smooth muscle of the dorsal aorta shares a common clonal origin with skeletal muscle of the myotome. *Development* **133**, 737-749.
- Florini JR, Ewton DZ & Coolican SA. (1996). Growth hormone and the insulin-like growth factor system in myogenesis. *Endocr Rev* **17**, 481-517.

- Fukada S, Uezumi A, Ikemoto M, Masuda S, Segawa M, Tanimura N, Yamamoto H, Miyagoe-Suzuki Y & Takeda S. (2007). Molecular signature of quiescent satellite cells in adult skeletal muscle. *Stem Cells* **25**, 2448-2459.
- Gal-Levi R, Leshem Y, Aoki S, Nakamura T & Halevy O. (1998). Hepatocyte growth factor plays a dual role in regulating skeletal muscle satellite cell proliferation and differentiation. *Biochim Biophys Acta* **1402**, 39-51.
- Gang EJ, Bosnakovski D, Simsek T, To K & Perlingeiro RC. (2008). Pax3 activation promotes the differentiation of mesenchymal stem cells toward the myogenic lineage. *Exp Cell Res* **314**, 1721-1733.
- Gensch N, Borchardt T, Schneider A, Riethmacher D & Braun T. (2008). Different autonomous myogenic cell populations revealed by ablation of Myf5-expressing cells during mouse embryogenesis. *Development* **135**, 1597-1604.
- Giordano A, Calvani M, Petillo O, Carteni M, Melone MRAB & Peluso G. (2003). Skeletal muscle metabolism in physiology and in cancer disease. *Journal of Cellular Biochemistry* **90**, 170-186.
- Goldspink G. (2002). Gene expression in skeletal muscle. *Biochem Soc Trans* **30**, 285-290.
- Goulding M, Lumsden A & Paquette AJ. (1994). Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. *Development* **120**, 957-971.
- Goulding MD, Chalepakis G, Deutsch U, Erselius JR & Gruss P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *Embo J* **10**, 1135-1147.
- Grifone R, Demignon J, Giordani J, Niro C, Souil E, Bertin F, Laclef C, Xu PX & Maire P. (2007). Eya1 and Eya2 proteins are required for hypaxial somitic myogenesis in the mouse embryo. *Dev Biol* **302**, 602-616.
- Grifone R, Demignon J, Houbron C, Souil E, Niro C, Seller MJ, Hamard G & Maire P. (2005). Six1 and Six4 homeoproteins are required for Pax3 and Mrf expression during myogenesis in the mouse embryo. *Development* **132**, 2235-2249.
- Gros J, Manceau M, Thome V & Marcelle C. (2005). A common somitic origin for embryonic muscle progenitors and satellite cells. *Nature* **435**, 954-958.
- Gros J, Scaal M & Marcelle C. (2004). A two-step mechanism for myotome formation in chick. *Dev Cell* **6**, 875-882.

- Gross MK, Moran-Rivard L, Velasquez T, Nakatsu MN, Jagla K & Goulding M. (2000). Lbx1 is required for muscle precursor migration along a lateral pathway into the limb. *Development* **127**, 413-424.
- Gullberg D, Velling T, Lohikangas L & Tiger CF. (1998). Integrins during muscle development and in muscular dystrophies. *Front Biosci* **3**, D1039-1050.
- Gustafsson MK, Pan H, Pinney DF, Liu Y, Lewandowski A, Epstein DJ & Emerson CP, Jr. (2002). Myf5 is a direct target of long-range Shh signaling and Gli regulation for muscle specification. *Genes Dev* **16**, 114-126.
- Haldar M, Karan G, Tvrđik P & Capecchi MR. (2008). Two cell lineages, myf5 and myf5-independent, participate in mouse skeletal myogenesis. *Dev Cell* **14**, 437-445.
- Halevy O, Piastun Y, Allouh MZ, Rosser BW, Rinkevich Y, Reshef R, Rozenboim I, Wleklinski-Lee M & Yablonka-Reuveni Z. (2004). Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal. *Dev Dyn* **231**, 489-502.
- Hammond CL, Hinits Y, Osborn DP, Minchin JE, Tettamanti G & Hughes SM. (2007). Signals and myogenic regulatory factors restrict pax3 and pax7 expression to dermomyotome-like tissue in zebrafish. *Dev Biol* **302**, 504-521.
- Hasselgren PO & Fischer JE. (2001). Muscle cachexia: current concepts of intracellular mechanisms and molecular regulation. *Annals of surgery* **233**, 9-17.
- Hasty P, Bradley A, Morris JH, Edmondson DG, Venuti JM, Olson EN & Klein WH. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene [see comments]. *Nature* **364**, 501-506.
- Haub O & Goldfarb M. (1991). Expression of the fibroblast growth factor-5 gene in the mouse embryo. *Development* **112**, 397-406.
- Hawke TJ & Garry DJ. (2001). Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol* **91**, 534-551.
- Heslop L, Beauchamp JR, Tajbakhsh S, Buckingham ME, Partridge TA & Zammit PS. (2001). Transplanted primary neonatal myoblasts can give rise to functional satellite cells as identified using the Myf5nlacZl⁺ mouse. *Gene Ther* **8**, 778-783.
- Hill M & Goldspink G. (2003). Expression and splicing of the insulin-like growth factor gene in rodent muscle is associated with muscle satellite (stem) cell activation following local tissue damage. *J Physiol* **549**, 409-418.

- Hoth CF, Milunsky A, Lipsky N, Sheffer R, Clarren SK & Baldwin CT. (1993). Mutations in the paired domain of the human PAX3 gene cause Klein-Waardenburg syndrome (WS-III) as well as Waardenburg syndrome type I (WS-I). *Am J Hum Genet* **52**, 455-462.
- Houzelstein D, Auda-Boucher G, Cheraud Y, Rouaud T, Blanc I, Tajbakhsh S, Buckingham ME, Fontaine-Perus J & Robert B. (1999). The homeobox gene *Msx1* is expressed in a subset of somites, and in muscle progenitor cells migrating into the forelimb. *Development* **126**, 2689-2701.
- Irintchev A, Zeschnigk M, Starzinski-Powitz A & Wernig A. (1994). Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. *Dev Dyn* **199**, 326-337.
- Jankowski RJ, Deasy BM, Cao B, Gates C & Huard J. (2002). The role of CD34 expression and cellular fusion in the regeneration capacity of myogenic progenitor cells. *J Cell Sci* **115**, 4361-4374.
- Kalcheim C & Ben-Yair R. (2005). Cell rearrangements during development of the somite and its derivatives. *Current opinion in genetics & development* **15**, 371-380.
- Kambadur R, Sharma M, Smith TP & Bass JJ. (1997). Mutations in myostatin (GDF8) in double-musled Belgian Blue and Piedmontese cattle. *Genome Res* **7**, 910-916.
- Kardon G, Campbell JK & Tabin CJ. (2002). Local extrinsic signals determine muscle and endothelial cell fate and patterning in the vertebrate limb. *Dev Cell* **3**, 533-545.
- Kassar-Duchossoy L, Gayraud-Morel B, Gomes D, Rocancourt D, Buckingham M, Shinin V & Tajbakhsh S. (2004). *Mrf4* determines skeletal muscle identity in *Myf5:Myod* double-mutant mice. *Nature* **431**, 466-471.
- Kassar-Duchossoy L, Giacone E, Gayraud-Morel B, Jory A, Gomes D & Tajbakhsh S. (2005). *Pax3/Pax7* mark a novel population of primitive myogenic cells during development. *Genes Dev* **19**, 1426-1431.
- Kastner S, Elias MC, Rivera AJ & Yablonka-Reuveni Z. (2000). Gene expression patterns of the fibroblast growth factors and their receptors during myogenesis of rat satellite cells. *J Histochem Cytochem* **48**, 1079-1096.
- Kawamura K, Takano K, Suetsugu S, Kurisu S, Yamazaki D, Miki H, Takenawa T & Endo T. (2004). N-WASP and WAVE2 acting downstream of phosphatidylinositol 3-kinase are required for myogenic cell migration induced by hepatocyte growth factor. *J Biol Chem* **279**, 54862-54871.
- Kirk S, Oldham J, Jeanplong F & Bass J. (2003). Insulin-like growth factor-II delays early but enhances late regeneration of skeletal muscle. *J Histochem Cytochem* **51**, 1611-1620.

- Kitzmann M & Fernandez A. (2001). Crosstalk between cell cycle regulators and the myogenic factor MyoD in skeletal myoblasts. *Cell Mol Life Sci* **58**, 571-579.
- Krause DS, Fackler MJ, Civin CI & May WS. (1996). CD34: structure, biology, and clinical utility. *Blood* **87**, 1-13.
- Kuang S, Charge SB, Seale P, Huh M & Rudnicki MA. (2006). Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. *J Cell Biol* **172**, 103-113.
- Kuang S, Kuroda K, Le Grand F & Rudnicki MA. (2007). Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell* **129**, 999-1010.
- Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ & Keller G. (2004). Development of definitive endoderm from embryonic stem cells in culture. *Development* **131**, 1651-1662.
- Kurmasheva RT, Peterson CA, Parham DM, Chen B, McDonald RE & Cooney CA. (2005). Upstream CpG island methylation of the PAX3 gene in human rhabdomyosarcomas. *Pediatric blood & cancer* **44**, 328-337.
- Lagha M, Kormish JD, Rocancourt D, Manceau M, Epstein JA, Zaret KS, Relaix F & Buckingham ME. (2008a). Pax3 regulation of FGF signaling affects the progression of embryonic progenitor cells into the myogenic program. *Genes Dev* **22**, 1828-1837.
- Lagha M, Sato T, Bajard L, Daubas P, Esner M, Montarras D, Relaix F & Buckingham M. (2008b). Regulation of Skeletal Muscle Stem Cell Behavior by Pax3 and Pax7. *Cold Spring Harbor symposia on quantitative biology*.
- Langley B, Thomas M, Bishop A, Sharma M, Gilmour S & Kambadur R. (2002). Myostatin Inhibits Myoblast Differentiation by Down-regulating MyoD Expression. *J Biol Chem* **277**, 49831-49840.
- Lansdorp PM. (2007). Immortal strands? Give me a break. *Cell* **129**, 1244-1247.
- Lepper C, Conway SJ & Fan CM. (2009). Adult satellite cells and embryonic muscle progenitors have distinct genetic requirements. *Nature* **460**, 627-631.
- MacArthur CA, Lawshe A, Xu J, Santos-Ocampo S, Heikinheimo M, Chellaiah AT & Ornitz DM. (1995). FGF-8 isoforms activate receptor splice forms that are expressed in mesenchymal regions of mouse development. *Development* **121**, 3603-3613.
- Mansouri A. (1998). The role of Pax3 and Pax7 in development and cancer. *Crit Rev Oncog* **9**, 141-149.

- Margue CM, Bernasconi M, Barr FG & Schafer BW. (2000). Transcriptional modulation of the anti-apoptotic protein BCL-XL by the paired box transcription factors PAX3 and PAX3/FKHR. *Oncogene* **19**, 2921-2929.
- Marics I, Padilla F, Guillemot JF, Scaal M & Marcelle C. (2002). FGFR4 signaling is a necessary step in limb muscle differentiation. *Development* **129**, 4559-4569.
- Maroto M, Reshef R, Munsterberg AE, Koester S, Goulding M & Lassar AB. (1997). Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. *Cell* **89**, 139-148.
- Mauro A. (1961). Satellite cell of skeletal fibers. *Journal of Biophysical & Biochemistry Cytology* **9**, 493-498.
- McCroskery S, Thomas M, Maxwell L, Sharma M & Kambadur R. (2003). Myostatin negatively regulates satellite cell activation and self-renewal. *J Cell Biol* **162**, 1135-1147.
- McCroskery S, Thomas M, Platt L, Hennebry A, Nishimura T, McLeay L, Sharma M & Kambadur R. (2005). Improved muscle healing through enhanced regeneration and reduced fibrosis in myostatin-null mice. *J Cell Sci* **118**, 3531-3541.
- McFarlane C, Hennebry A, Thomas M, Sharma M & Kambadur R. (2006). Myostatin signals through Pax7 to regulate post-natal myogenesis. In *Frontiers in Myogenesis, Society for Muscle Biology Pine Mountain, Georgia, U.S.A.*
- McKinnell IW, Ishibashi J, Le Grand F, Punch VG, Addicks GC, Greenblatt JF, Dilworth FJ & Rudnicki MA. (2008). Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex. *Nat Cell Biol* **10**, 77-84.
- McPherron AC, Lawler AM & Lee SJ. (1997). Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387**, 83-90.
- Miller KJ, Thaloor D, Matteson S & Pavlath GK. (2000). Hepatocyte growth factor affects satellite cell activation and differentiation in regenerating skeletal muscle. *Am J Physiol Cell Physiol* **278**, C174-181.
- Mitchell PO, Mills T, O'Connor RS, Graubert T, Dzierzak E & Pavlath GK. (2005). Sca-1 negatively regulates proliferation and differentiation of muscle cells. *Dev Biol* **283**, 240-252.
- Montarras D, Morgan J, Collins C, Relaix F, Zaffran S, Cumano A, Partridge T & Buckingham M. (2005). Direct isolation of satellite cells for skeletal muscle regeneration. *Science* **309**, 2064-2067.

- Moss FP & Leblond CP. (1971). Satellite cells as the source of nuclei in muscles of growing rats. *Anat Rec* **170**, 421-435.
- Nabeshima Y, Hanaoka K, Hayasaka M, Esumi E, Li S & Nonaka I. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect [see comments]. *Nature* **364**, 532-535.
- Nagata Y, Kobayashi H, Umeda M, Ohta N, Kawashima S, Zammit PS & Matsuda R. (2006). Sphingomyelin levels in the plasma membrane correlate with the activation state of muscle satellite cells. *J Histochem Cytochem* **54**, 375-384.
- Nofziger D, Miyamoto A, Lyons KM & Weinmaster G. (1999). Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. *Development* **126**, 1689-1702.
- Olguin HC & Olwin BB. (2004). Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. *Dev Biol* **275**, 375-388.
- Oliver MH, Harrison NK, Bishop JE, Cole PJ & Laurent GJ. (1989). A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. *J Cell Sci* **92**, 513-518.
- Oustanina S, Hause G & Braun T. (2004). Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. *Embo J* **23**, 3430-3439.
- Pawlikowski B, Lee L, Zuo J & Kramer RH. (2009). Analysis of human muscle stem cells reveals a differentiation-resistant progenitor cell population expressing Pax7 capable of self-renewal. *Dev Dyn* **238**, 138-149.
- Pourquie O, Coltey M, Breant C & Le Douarin NM. (1995). Control of somite patterning by signals from the lateral plate. *Proc Natl Acad Sci U S A* **92**, 3219-3223.
- Pourquie O, Fan CM, Coltey M, Hirsinger E, Watanabe Y, Breant C, Francis-West P, Brickell P, Tessier-Lavigne M & Le Douarin NM. (1996). Lateral and axial signals involved in avian somite patterning: a role for BMP4. *Cell* **84**, 461-471.
- Pownall ME, Gustafsson MK & Emerson CP, Jr. (2002). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annu Rev Cell Dev Biol* **18**, 747-783.
- Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, Mansouri A, Cumano A & Buckingham M. (2006). Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J Cell Biol* **172**, 91-102.

- Relaix F, Rocancourt D, Mansouri A & Buckingham M. (2004). Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes Dev* **18**, 1088-1105.
- Relaix F, Rocancourt D, Mansouri A & Buckingham M. (2005). A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature* **435**, 948-953.
- Rohwedel J, Maltsev V, Bober E, Arnold HH, Hescheler J & Wobus AM. (1994). Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. *Dev Biol* **164**, 87-101.
- Rosenberg IH. (1997). Sarcopenia: origins and clinical relevance. *The Journal of nutrition* **127**, 990S-991S.
- Rouger K, Brault M, Daval N, Leroux I, Guigand L, Lesoeur J, Fernandez B & Chérel Y. (2004). Muscle satellite cell heterogeneity: in vitro and in vivo evidences for populations that fuse differently. *Cell Tissue Res* **317**, 319-326.
- Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold HH & Jaenisch R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351-1359.
- Sacco A, Doyonnas R, Kraft P, Vitorovic S & Blau HM. (2008). Self-renewal and expansion of single transplanted muscle stem cells. *Nature* **456**, 502-506.
- Sajko S, Kubinova L, Cvetko E, Kreft M, Wernig A & Erzen I. (2004). Frequency of M-cadherin-stained satellite cells declines in human muscles during aging. *J Histochem Cytochem* **52**, 179-185.
- Salerno MS, Dyer K, Bracegirdle J, Platt L, Thomas M, Siriatt V, Kambadur R & Sharma M. (2009). Akirin1 (Mighty), a novel promyogenic factor regulates muscle regeneration and cell chemotaxis. *Exp Cell Res* **315**, 2012-2021.
- Sambrook J, Fritsch EF & Maniatis T. (1989). *Molecular cloning: A laboratory manual, 2nd ed.* Cold Spring Harbour Laboratory Press, N.Y.
- Sampaolesi M, Torrente Y, Innocenzi A, Tonlorenzi R, D'Antona G, Pellegrino MA, Barresi R, Bresolin N, De Angelis MG, Campbell KP, Bottinelli R & Cossu G. (2003). Cell therapy of alpha-sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblasts. *Science* **301**, 487-492.
- Schultz E. (1996). Satellite cell proliferative compartments in growing skeletal muscles. *Dev Biol* **175**, 84-94.

- Seale P, Ishibashi J, Scime A & Rudnicki MA. (2004). Pax7 is necessary and sufficient for the myogenic specification of CD45⁺:Sca1⁺ stem cells from injured muscle. *PLoS Biol* **2**, E130.
- Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P & Rudnicki MA. (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell* **102**, 777-786.
- Shefer G, Van de Mark DP, Richardson JB & Yablonka-Reuveni Z. (2006). Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. *Dev Biol* **294**, 50-66.
- Shen X, Collier JM, Hlaing M, Zhang L, Delshad EH, Bristow J & Bernstein HS. (2003). Genome-wide examination of myoblast cell cycle withdrawal during differentiation. *Dev Dyn* **226**, 128-138.
- Shinin V, Gayraud-Morel B, Gomes D & Tajbakhsh S. (2006). Asymmetric division and cosegregation of template DNA strands in adult muscle satellite cells. *Nat Cell Biol* **8**, 677-687.
- Shinin V, Gayraud-Morel B & Tajbakhsh S. (2009). Template DNA-strand cosegregation and asymmetric cell division in skeletal muscle stem cells. *Methods in molecular biology (Clifton, NJ)* **482**, 295-317.
- Spangrude GJ, Heimfeld S & Weissman IL. (1988). Purification and characterization of mouse hematopoietic stem cells. *Science* **241**, 58-62.
- Stark KL, McMahon JA & McMahon AP. (1991). FGFR-4, a new member of the fibroblast growth factor receptor family, expressed in the definitive endoderm and skeletal muscle lineages of the mouse. *Development* **113**, 641-651.
- Stuart ET, Kiuoussi C & Gruss P. (1994). Mammalian Pax genes. *Annual review of genetics* **28**, 219-236.
- Tajbakhsh S, Borello U, Vivarelli E, Kelly R, Papkoff J, Duprez D, Buckingham M & Cossu G. (1998). Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. *Development* **125**, 4155-4162.
- Tajbakhsh S & Buckingham M. (2000). The birth of muscle progenitor cells in the mouse: spatiotemporal considerations. *Curr Top Dev Biol* **48**, 225-268.
- Tajbakhsh S & Cossu G. (1997). Establishing myogenic identity during somitogenesis. *Current opinion in genetics & development* **7**, 634-641.
- Tajbakhsh S, Rocancourt D, Cossu G & Buckingham M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. *Cell* **89**, 127-138.

- Tassabehji M, Newton VE, Leverton K, Turnbull K, Seemanova E, Kunze J, Sperling K, Strachan T & Read AP. (1994). PAX3 gene structure and mutations: close analogies between Waardenburg syndrome and the *Plotch* mouse. *Hum Mol Genet* **3**, 1069-1074.
- Tatsumi R & Allen RE. (2004). Active hepatocyte growth factor is present in skeletal muscle extracellular matrix. *Muscle Nerve* **30**, 654-658.
- Tatsumi R, Anderson JE, Nevoret CJ, Halevy O & Allen RE. (1998). HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev Biol* **194**, 114-128.
- Thomas M, Langley B, Berry C, Sharma M, Kirk S, Bass J & Kambadur R. (2000). Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. *J Biol Chem* **275**, 40235-40243.
- Tisdale MJ. (1991). Cancer cachexia. *British journal of cancer* **63**, 337-342.
- Treisman J, Harris E & Desplan C. (1991). The paired box encodes a second DNA-binding domain in the paired homeo domain protein. *Genes Dev* **5**, 594-604.
- Underhill DA. (2000). Genetic and biochemical diversity in the Pax gene family. *Biochemistry and cell biology = Biochimie et biologie cellulaire* **78**, 629-638.
- Vasyutina E, Lenhard DC & Birchmeier C. (2007a). Notch function in myogenesis. *Cell Cycle* **6**, 1451-1454.
- Vasyutina E, Lenhard DC, Wende H, Erdmann B, Epstein JA & Birchmeier C. (2007b). RBP-J (Rbpsi) is essential to maintain muscle progenitor cells and to generate satellite cells. *Proc Natl Acad Sci U S A* **104**, 4443-4448.
- Vogan KJ, Underhill DA & Gros P. (1996). An alternative splicing event in the Pax-3 paired domain identifies the linker region as a key determinant of paired domain DNA-binding activity. *Mol Cell Biol* **16**, 6677-6686.
- Volonte D, Liu Y & Galbiati F. (2005). The modulation of caveolin-1 expression controls satellite cell activation during muscle repair. *Faseb J* **19**, 237-239.
- von Scheven G, Alvares LE, Mootosamy RC & Dietrich S. (2006). Neural tube derived signals and Fgf8 act antagonistically to specify eye versus mandibular arch muscles. *Development* **133**, 2731-2745.
- Weinstein M, Xu X, Ohyama K & Deng CX. (1998). FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. *Development* **125**, 3615-3623.

- White JD, Scaffidi A, Davies M, McGeachie J, Rudnicki MA & Grounds MD. (2000). Myotube formation is delayed but not prevented in MyoD-deficient skeletal muscle: studies in regenerating whole muscle grafts of adult mice. *J Histochem Cytochem* **48**, 1531-1544.
- Williams BA & Ordahl CP. (1994). Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* **120**, 785-796.
- Wilson D, Sheng G, Lecuit T, Dostatni N & Desplan C. (1993). Cooperative dimerization of paired class homeo domains on DNA. *Genes Dev* **7**, 2120-2134.
- Yablonka-Reuveni Z. (1995). Development and postnatal regulation of adult myoblasts. *Microscopy research and technique* **30**, 366-380.
- Yablonka-Reuveni Z, Day K, Vine A & Shefer G. (2008). Defining the transcriptional signature of skeletal muscle stem cells. *Journal of animal science* **86**, E207-216.
- Yablonka-Reuveni Z & Paterson BM. (2001). MyoD and myogenin expression patterns in cultures of fetal and adult chicken myoblasts. *J Histochem Cytochem* **49**, 455-462.
- Yablonka-Reuveni Z & Rivera AJ. (1994). Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev Biol* **164**, 588-603.
- Yaffe D & Saxel O. (1977). Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* **270**, 725-727.
- Yang SY & Goldspink G. (2002). Different roles of the IGF-I Ec peptide (MGF) and mature IGF-I in myoblast proliferation and differentiation. *FEBS Lett* **522**, 156-160.
- Yang XM, Vogan K, Gros P & Park M. (1996). Expression of the met receptor tyrosine kinase in muscle progenitor cells in somites and limbs is absent in Splotch mice. *Development* **122**, 2163-2171.
- Yoshida N, Yoshida S, Koishi K, Masuda K & Nabeshima Y. (1998). Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. *J Cell Sci* **111**, 769-779.
- Yun K & Wold B. (1996). Skeletal muscle determination and differentiation: story of a core regulatory network and its context. *Curr Opin Cell Biol* **8**, 877-889.
- Zammit PS. (2008). All muscle satellite cells are equal, but are some more equal than others? *J Cell Sci* **121**, 2975-2982.

- Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA & Beauchamp JR. (2004). Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? *J Cell Biol* **166**, 347-357.
- Zammit PS, Partridge TA & Yablonka-Reuveni Z. (2006a). The skeletal muscle satellite cell: the stem cell that came in from the cold. *J Histochem Cytochem* **54**, 1177-1191.
- Zammit PS, Relaix F, Nagata Y, Ruiz AP, Collins CA, Partridge TA & Beauchamp JR. (2006b). Pax7 and myogenic progression in skeletal muscle satellite cells. *J Cell Sci* **119**, 1824-1832.
- Zhang Y, Feng XH & Derynck R. (1998). Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. *Nature* **394**, 909-913.
- Zhao P, Caretti G, Mitchell S, McKeehan WL, Boskey AL, Pachman LM, Sartorelli V & Hoffman EP. (2006). Fgfr4 is required for effective muscle regeneration in vivo. Delineation of a MyoD-Tead2-Fgfr4 transcriptional pathway. *J Biol Chem* **281**, 429-438.
- Zhao P & Hoffman EP. (2004). Embryonic myogenesis pathways in muscle regeneration. *Dev Dyn* **229**, 380-392.