An investigation of the role of Notch3 activity in the regulation of adult skeletal muscle stem cell behaviour

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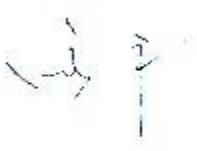

Siew Hui Low

Abstract

Adult skeletal muscle contains a population of tissue-specific stem cells (satellite cells) that are essential for effective growth, repair and regeneration. Normally maintained in a state of metabolic and proliferative quiescence, satellite cells become activated in response to growth stimuli or damage and proliferate to provide progeny that differentiate to augment, repair or generate new functional muscle fibres. In addition to producing differentiated cells, satellite cells also self-renew to maintain or replenish the stem cell compartment retain regenerative capacity. The precise mechanism by which this occurs remains uncertain, although several recent studies have implicated the Notch signalling pathway, an evolutionarily conserved system involved in determining cell fate behaviour and fate in a wide range of developing and adult tissues.

This thesis presents an investigation of the role of Notch signalling, specifically Notch3 activity, in the regulation of the adult skeletal muscle stem cell phenotype using an *in vitro* model of satellite cell self-renewal during regeneration. When mouse skeletal muscle precursor cells are induced to differentiate, most form myotubes (immature muscle fibres), whereas a small minority within the same culture adopt a reversible, quiescent, undifferentiated phenotype comparable to that of a satellite cell. This phenotype was shown to be dependent on the expression of the Notch3 receptor and its activation by a specific ligand, Delta-like 4, which is expressed by the nascent myotubes. Furthermore, studies in which isolated reserve cells were reactivated from the quiescent state revealed that Notch3 is rapidly inactivated and subsequently down-regulation prior to re-entry into the cell cycle, consistent with a role in maintaining the stem cell phenotype.

Together, data presented in this thesis suggest that satellite cells can be recruited for differentiation at different stages of activation and how once activated, muscle precursor cells could be sequestered back into a quiescent state during regeneration.

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Abbreviations

ACBD3	acyl-CoA binding domain containing 3
ADAM	A disintegrin and metalloprotease
ANK	cdc10/ankyrin repeat domain
APS	ammonium persulphate
bHLH	basic helix-loop-helix
BM	bone marrow
BMP	bone morphogenetic protein
bp	base pairs
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
Ca2+	calcium ion
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
cdk	cyclin dependent kinase
cDNA	complementary DNA
CEE	chick embryo extract
C. elegans	*Caenorhabditis elegans*
CIAP	calf intestinal alkaline phosphatase
CIR	CBF1-interacting corepressor
cKO	conditional knockout
CoA	coactivator
CoR	corepressor
CMV	cytomegalovirus
CO2	carbon dioxide
CSL	acronym for CBF1, Suppressor of Hairless, Lag1
C-terminal	carboxy-terminal
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
dH2O	distilled water
Dll1	Delta-like1
Dll3	Delta-like3

Dll4	Delta-like4
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulphoxide
dn	dominant negative
DNA	deoxyribonucleic acid
DNER	Delta/Notch-like EGF-related receptor
dNTPs	deoxynucleotide triphosphates
DOC	deoxycholate
DSL	acronym for Delta, Serrate, lag2
Dtx	Deltex
E11.5	embryonic day 11.5 post-coitum
ECD	extracellular domain
E. coli	*Escherichia coli*
EDL	*extensor digitorum longus* muscle
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor-like
eGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
E(spl)	enhancer of split
FCS	foetal calf serum
FGF	fibroblast growth factor
FT	fast twitch
g	G-force i.e. relative centrifugal force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G0 phase	gap 0 phase
G1 phase	gap 1 phase
G2 phase	gap 2 phase
GFP	green fluorescent protein
HA	haemagglutinin
HBSS	Hank's balanced salt solution
HDAC	histone deacetylase
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HES	Hairy Enhancer of Split
HEY	Hairy Enhancer of Split-related protein
HGF	hepatocyte growth factor
HI FCS	heat-inactivated foetal calf serum
HPRT	hypoxanthine guanine phosphoribosyl transferase
HRP	horse radish peroxidase
HS	horse serum
HSC	haematopoietic stem cell
IC	intracellular-coding domain
ICD	intracellular domain
IF	immunofluorescence
IP	immunoprecipitation
IRES	internal ribosome entry site
kb	kilobases
kDa	kilodaltons
Kul	Kuzbanian-like
LB	Luria Bertani
L-glut	L-glutamine
LNR	lin12/Notch repeat
LTR	long terminal repeat
MB	myoblasts
MCS	multiple cloning site
MESV	Murine Embryonic Stem Cell Virus
MyHC	myosin heavy chain
Mg2+	magnesium ion
Mib	Mind bomb
mig	acronym for pMSCV-IRES-eGFP
MNF	myocyte nuclear factor
MPCs	myogenic precursor cells
M phase	mitosis phase
MRF	myogenic regulatory factor
mRNA	messenger ribonucleic acid
MSCV	Murine Stem Cell Virus
mSP	muscle side population

MT	myotubes
Neur	Neuralised
NEXT	Notch Extracellular Truncation
NIC	Notch1 intracellular domain
NLS	nuclear localisation signal
NRR	negative regulatory region
NT	N-terminal
N-terminal	amino-terminal
oligo	oligonucleotide
PFA	paraformaldehyde
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with Tween 20
PCAF	P300/CBP-associated factor
PCMV	PCC4-cell-passaged myeloproliferative sarcoma virus
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiosus*
PGK	phosphoglycerate kinase
pofut1	protein *O*-fucosyltransferase 1
poly-A	poly-adenine
poly-T	poly-thymidine
PPGK	phosphoglycerate kinase promoter
PTB	phosphotyrosine binding
Puror	puromycin resistance
p value	probability value
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative real-time polymerase chain reaction
RC	reserve cells
RE/AC	repression/activation
RIP	regulated intramembrane proteolysis
RIPA buffer	radioimmunoprecipitation buffer
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA

RT-PCR	reverse transcription polymerase chain reaction
S1	site 1
S2	site 2
S3	site 3
SAP30	Sin3A-associated protein 30kDa
S-DMEM	supplemented DMEM
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel eletrophoresis
Sel1h	(Suppressor of lin-12) 1 homologue
Shh	Sonic hedgehog
SKIP	ski-interacting protein
SMRT	silencing mediator for retinoid or thyroid-hormone receptors
SP	side population
ST	slow twitch
S phase	synthesis phase
Su(dx)	Suppressor of Deltex
Su(H)	Suppressor of Hairless
SV40	simian virus 40
T0	Time 0 (i.e. 0 hours)
TACE	TNF- α- converting enzyme
TAD	transactivation domain
Taq	*Thermus aquaticus*
TBE	Tris-borate/EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TE	trypsin/EDTA
TE buffer	Tris-EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
TGF-β	transforming growth factor- β
Th1	T helper cell type 1
Th2	T helper cell type 2
TNF-α	tumor necrosis factor-α
VCAM1	vascular cell adhesion molecule 1

VSMC	vascular smooth muscle cells
-W	width
WB	Western blotting

Chapter 1: Introduction

1.1 An overview of adult mammalian skeletal muscle structure and function

Skeletal muscle is required for several essential physiological processes including maintenance of posture, locomotion, breathing and whole body metabolism. It is the most abundant tissue in adult mammals, constituting about one-third to one-half of the total body mass (Collins *et al*., 2005; Sloper *et al.*, 1978; Zierath & Hawley, 2004). The muscle fibre is the functional cellular unit of skeletal muscle, a syncytium containing many post-mitotic nuclei (myonuclei) that form during development by the fusion of muscle precursor cells (myoblasts).

Mature muscle fibres are highly specialised for the primary function of force generation and contain a registered array of myofibrils each composed of an "end-to end" series of contractile sarcomeres.

Sarcomeres are the contractile units of striated muscle, consisting predominantly of myosin-rich thick filaments and actin-rich thin filaments. Each sarcomere is demarcated by Z-discs, into which thin filaments are anchored by α-actinin: the thin filaments are directional and at each end of the sarcomere, the "plus" end is associated with the Z-disc with the "minus" end orientated towards the middle of the sarcomere. The bidirectional thick filaments are maintained in the centre of the sarcomere by titin molecules and in the region of overlap, each thick filament can interact with five adjacent thin filaments via cross-bridges, the N-terminal domains of the type II skeletal muscle myosin heavy chains which function as motor proteins with both actin binding and ATPase activities. During contraction, cross-bridges attach to the thin filaments and utilise energy from ATP- hydrolysis to undergo rotational power strokes that pull the thin filaments (and therefore the Z-discs) towards the centre of the sarcomere, before detaching and then reattaching to another binding site towards the "plus" end of the thin filament. The cross-bridges

cycle independently, generating force by increasing the overlap between the thick and thin filament and shortening the sarcomere (Huxley, 1957; Huxley, 2000).

Each muscle fibre is surrounded by a layer of connective tissue (endomysium), groups of muscle fibres, termed fascicles are surrounded by a perimysium and these are clustered into bundles to form a skeletal muscle, enveloped by the epimysium (Figure 1.1). Through their attachment to the skeleton via myotendinous junctions, myofibre contraction is transformed into movement.

When a myofibre matures, it is contacted by a single motor neuron and expresses distinctive molecules (e.g. myosin heavy chain) for contractile function. An individual muscle fibres contractile properties depends on its muscle fibre composition, including the slow-contracting/fatigue-resistant (slow twitch, ST) fibres and fast-contracting/non-fatigue-resistant (fast twitch, FT) fibres.

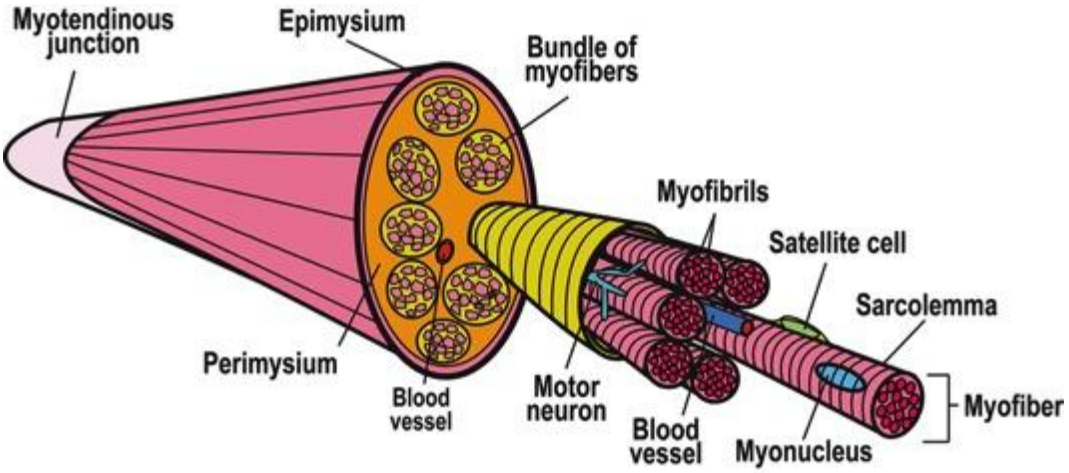


Figure 1.1 Structure of skeletal muscle
Skeletal muscle is formed hierarchical bundles of structures: bundles of myofibres contained by the perimysium, which is in turn contained by the epimysium. Myofibres cross the entire muscle lengthwise and attach to the bone through myotendinous junctions at both ends. Myofibres are multinucleated single cells made up of an array of stacked myofibrils. The myofibrils consist of thick and thin filaments organized into a contractile unit called a sarcomere. The myofibres are surrounded by a basal lamina. A population of quiescent muscle progenitor cells (satellite cells) are located underneath the basal lamina. A rich network of blood

vessels and nerves weaves between and within the myofibres. (Diagram taken from Scime *et al*., 2009).

1.2 Skeletal muscle development

Skeletal muscle originates from embryonic somites. The formation of committed skeletal muscle precursor cells in the myotome is stimulated by paracrine factors from the surrounding tissue (dorsal ectoderm, neural tube, and notochord), that induce the expression of MyoD (Maroto *et al*., 1997; Tajbakhsh *et al*., 1997). The precursor cells migrate to form specific muscle groups: for example, the deep back muscles are formed from cells migrated from the medial portion of somite, whilst the body and the limb muscles are formed from the lateral somites (Bischoff, 1994; Hauschka, 1994).

In the lateral somites, surrounding environment factors induce the transcription factor Pax3 that turns on the muscle-specific transcription factors, Myf5 and MyoD, although in the medial somites, MyoD is induced through an alternative pathway. Both MyoD (Davis *et al*., 1987) and Myf5 (Braun *at al*., 1989) are members of the basic helix-loop-helix (bHLH) family and myogenic regulatory factors (MRFs) that bind to specific sites on DNA and activate muscle-specific genes. MRFs are also called the MyoD family, consisting of MyoD (Tapscott *et al*., 1988), Myf-5 (Braun *et al*., 1989), myogenin (Wright et al., 1989), and MRF4 (Rhodes and Konieczny, 1989). Furthermore, MyoD also directly activates its own expression. The proliferative myotomal cells that express MyoD or Myf5 are termed myoblasts and eventually fuse and form multinucleated myotubes. Myoblasts proliferate without differentiating until specific growth factors are depleted. The myoblasts then exit the cell cycle and secrete fibronectin which is bound to the extracellular matrix by α5β1 integrin (Menko & Boettiger, 1987; Boettiger *et al*., 1995). Once aligned into chains, the myoblasts fuse to form myotubes, a process mediated by a group of metalloproteinases called meltrins (Yagami-Hiromasa *et al*., 1995). The primary myotubes formed by the fusion of embryonic myoblasts are thought to serve as a framework on which foetal myoblasts proliferate before fusing to form secondary myofibres during fetal development (Kelly & Zacks, 1969). During embryogenesis,

each of the bHLH MRFs (MyoD, Myf5, myogenin and MRF4) is expressed in a distinct temporal and spatial distribution pattern, relecting their unique roles in muscle development. Studies of knockout mice have demonstrated the critical roles of the MRFs in skeletal myogenesis in the embryo, yet reveal some degree of functional redundancy in the control of the skeletal myogenic development. In single *MyoD* or *Myf5* knockout mice, skeletal muscle develops with no apparent abnormalities (Rudnicki *et al*., 1992; Braun *et al*., 1992). However, double *MyoD*/*Myf5* knockout mice die shortly after birth with a complete absence of skeletal muscle (Rudnicki *et al*., 1993), implying that MyoD and Myf5 can substitute each other's activities. In contrast, single knockout mice lacking *myogenin* die soon after birth with a severe reduction in skeletal muscle bulk (Hasty *et al*., 1993). Furthermore, the muscle tissue that is formed consists of mononucleated, undifferentiated myoblasts consistent with an essential role for myogenin in terminal skeletal muscle differentiation that cannot be compensated for by other MRFs (Nabeshima *et al*., 1993). Initial studies reported that single *MRF4* knockout mice are viable and fertile, suggesting that other MRFs can compensate for the developmental functions of MRF4, although the muscles contained high levels of myogenin, implicating MRF4 in the postnatal down-regulation that normally occurs in skeletal muscle (Zhang *et al*., 1995). However, subsequent studies have shown that when MRF4 is compromised, skeletal muscle does not develop in the *MyoD*/*Mfy5* double knockout mouse (Kassar-Duchossoy *et al*., 2004), revealing a crucial role for MRF4 during early muscle development. Together, these studies have established a definite relationship between the MRFs with Myf5/MRF4 acting upstream of MyoD to direct the development of embryonic cells into myogenic lineage and myogenin acting downstream to induce terminal myoblast differentiation.

1.1.1 Studying satellite cells

Using enzymatic digestion, myofibres could be isolated from muscles together with satellite cells beneath the basal lamina (Beauchamp *et al*., 2000; Bischoff, 1975; Bischoff, 1986; Konigsberg *et al*., 1975; Rosenblat *et al*., 1995; Yablonka –Reuveni

and Rivera, 1994; Zammit *et al*., 2004). Satellite cells are mononucleated cells that reside between muscle fibres and basal lamina, and thus can be isolated together with single muscle fibers. They are responsible of assuming post-natal growth of muscle fibres, by adding new myonuclei to maintain the nuclear domains functional, but they are also responsible for nuclear turnover and, more importantly, muscle fibre regeneration after trauma in the adult (reviewed in Morgan and Partridge 2003)

There are two ways these isolated fibres can be studied; when cultured normally the satellite cells start to proliferate, differentiate into satellite-derived myoblasts, migrate off the isolated fibres onto their substrate and fuse to produce multinucleated myotubes (Zammit *et al*., 2006a); if they are maintained in a suspension culture (Zammit *et al*., 2004), they will similarly become activated, proliferate and differentiate while still being exposed to signals from the myofibres (Beauchamp *et al*., 2000). The latter allows the fate of the satellite cells to be studied without selection bias (Zammit *et al*., 2006a) and without interference from potential exogenous sources of myogenic cells such as connective tissue and vasculature (Ferrari *et al*., 1998; LaBarge and Blau, 2002; Tamaki *et al*., 2002).

In this thesis, satellite cell-derived myoblasts and the mouse myogenic cell line C2C12 (Yaffe and Saxel, 1977) were used to further investigate the role of Notch signalling. C2C12 cells have been chosen as they are much more manipulable in tissue culture than primary cells (Morgan *et al*., 1992). Furthermore, they are well-charecterised and have been used extensively in muscle cell differentiation studies (Cossin *et al*., 2002; Nofziger *et al*., 1999; Shawber *et al*., 1996).

The C2C12 cell line was subcloned from the C2 line, which was derived from the thigh muscle of an adult female C3H mouse after a crush injury (Yaffe and Saxel, 1977; Blau *et al*., 1983). They are able to differentiate rapidly and forming extensive contractile myotubes producing characteristic muscle proteins. In the presence of a high concentration of serum, C2C12 cells proliferate as undifferentiated myoblasts (Yoshida *et al*., 1998). Serum depletion induces differentiation in 40-60% of cells withdraw from the cell cycle and fuse to form myotubes.

These cultures are maintained as proliferating myoblasts in high serum growth media. However, differentiation can be induced by serum depletion, giving rise to multinucleated myotubes. Not all of the myoblasts differentiate and a small proportion exits the cell cycle and remains as mononucleate MyoD-ve reserve cells. The reserve cells are quiescent mononucleated and undifferentiated cells. They retain the ability to be activated and proliferate after which they can be induced to differentiate (Kitzmann *et al*., 1998; Lindon *et al*., 1998; Yoshida *et al*., 1998); leading again to a new mixed population of myotubes and reserve cells. The reserve cells have been found to express M-cadherin, Myf-5 and CD34 (Beauchamp *et al*., 2000).

This system is therefore a potentially useful in vitro model of satellite cells and may be used as a model of both myogenic differentiation and satellite cell specification.

1.3 Skeletal muscle Skeletal muscle regeneration

Normal adult skeletal muscle has low turnover of the postmitotic myonuclei (Decary *et al*., 1997). Daily wear and tear only produces a slow turnover of skeletal muscle fibres and it is estimated that no more than 1-2% of myonuclei are replaced every week in normal adult rat muscle (Schmalbruch & Lewis, 2000). Nonetheless, adult skeletal muscle retains the capability to complete a rapid and extensive regeneration in response to severe muscle damage. Two main phases characterise adult skeletal muscle regeneration: a degenerative phase and a regenerative phase (Charge & Rudnicki, 2004). When triggered by the disruption of myofibres, adult skeletal muscle enters the degenerative phase, which is accompanied by activation of inflammatory cells and myogenic cells. This is then followed by the regenerative phase of muscle repair, which begins with the activation and proliferation of undifferentiated muscle precursor cells.

1.3.1 Skeletal muscle regeneration capability depends on satellite cells

The regenerative capability of skeletal muscle is attributed to satellite cells, a relatively small population of adult stem cells occupying a specific niche of adult skeletal muscle. Each satellite cell is closely associated with an adult myofibre (see Fig. 1.1), residing in an interstitial layer between the sarcolemma and basal lamina (Mauro, 1961). After birth, 20-30% of the sub-laminar muscle nuclei in neonatal hind limb skeletal muscle are satellite cells (Bischoff, 1994). Satellite cells are generally in a non-proliferative, mitotically quiescent state in normal adult muscles (Schultz *et al*., 1978). However, satellite cells become activated in response to growth signals or myotrauma, start to express a sequence of MRFs myogenic markers and begin to proliferate and produce myoblasts, which ultimately differentiate and fuse to repair existing myofibres or form new fibres (Grounds and McGeachie, 1987; Rantanen *et al*., 1995). The regenerated tissue eventually becomes indistinguishable both morphologically and functionally from undamaged skeletal muscle (Charge & Rudnicki, 2004).

1.4 Identification of satellite cells

1.4.1 Satellite cell morphology

Adult skeletal muscle satellite cells are different from both the embryonic and foetal myoblast populations that are responsible for developmental myogenesis in all organisms analysed to date, including human (Cossu *et al.,* 1985), mouse (Cossu & Molinaro, 1987), birds (Hartley *et al.* 1992 and Feldman & Stockdale, 1992) and reptiles (Kahn & Simpson, 1974). Quiescent satellite cells are primarily identified by their morphological characteristics. They are located between the plasma membrane of the muscle fibre and the basement membrane within the basal lamina surrounding the myofibres. The major morphological features of satellite cells include a relatively high nuclear-to-cytoplasmic ratio, lack of organelles, small nucleus and large amount of nuclear heterochromatin (Schultz & McCormick, 1994). After activation, satellite cells lose some of these characteristics and they become easier to be identified as “swellings” on the myofibre (Schultz & McCormick, 1994).

1.4.2 Satellite cell molecular markers

A number of molecular markers have been identified which have helped the identification of satellite cells by immunostaining at the level of light microscopy. Some of these markers, such as MyoD (Davies et al., 1987; Tapscott et al., 1988), are expressed by both satellite cells and myoblasts (Zammit *et al*., 2004), whilst others are restricted to one or more phases of the myogenic pathway. For example, myocyte nuclear factor-β (MNF-β) normally expressed by quiescent satellite cells are down-regulated during activation, whereas MNF-α is expressed by proliferating myoblasts (Hawke & Garry, 2001). The most common and effective identification markers for satellite cells are M-cadherin and Pax7. M-cadherin, a calcium-dependent homotypic cell adhesion molecules, is not present on all quiescent satellite cells but its expression is upregulated during activation (Cornelison & Wold, 1997). The transcription factor Pax7, expressed by both quiescent and activated satellite cells and by myoblasts, is down regulated during differentiation. Pax3, a paralogue of Pax7 is also expressed by both quiescent and activated satellite cells. Studies of skeletal muscle from the *Pax7* knockout mouse initially suggested that Pax7 is an essential factor in the specification of the satellite cell population (Seale *et al*., 2000). Indeed, *in vitro* studies have shown that when satellite cell progeny are induced to differentiate, a proportion retain Pax7 expression and enter a state of undifferentiated quiescence, in a process akin to reconstitution of the satellite cell pool (Zammit *et al*., 2004; Olguin & Olwin, 2004). However, a later study revealed that satellite cells are initially present in *Pax7* null mice, but are progressively lost during the early postnatal development in both Pax3 +ve and Pax3 –ve muscle suggesting that Pax7 has an apoptotic function that cannot not be substituted by Pax3 (Oustanina *et al*., 2004 and Relaix *et al*., 2006). When Pax7 is not present, growth and regeneration is greatly compromised, leading to a rapid of loss of myofibres (Kuang *et al*., 2006). Recently, Lepper *et al*. (2009) showed that Pax7 is required for the maintenance of the satellite cell population in limb skeletal muscle in the immediate postnatal period but not beyond.

Several other satellite cell markers are less useful as their expression is not restricted to myogenic cells. For example, Cornelison *et al*. (1997) identified the tyrosine kinase receptor c-Met as a quiescent satellite cell marker and it has also been shown that most quiescent satellite cells express CD34, a hematopoietic stem cells marker (Beauchamp *et al*., 2000). Several cell adhesion molecules present on satellite cells, such as vascular adhesion molecule-1 (VCAM-1) and neural cell adhesion molecule (NCAM) have also been identified. VCAM-1 is widely expressed during embryogenesis, but it is present only in quiescent satellite cells in adult muscle (reviewed in Hawke & Garry, 2001; Covault, & Sanes, 1986), whereas NCAM is expressed in proliferating satellite cells and newly form myotubes but not in mature myotubes (Rosen *et al*., 1992).

During development, mammalian myogenesis occurs in two phases termed primary and secondary myogenesis. First, the primary myoblasts fuse to form primary myotubes that express the integrin receptor α*4β1*, but not VCAM-1. During secondary myogenesis, a second generation of α*4β1*-negative/VCAM-1-positive myoblasts interact with primary myotubes via α*4β1*, arrange themselves along the primary myotubes and fuse to form secondary myotubes. Most adult muscle fibres are derived from such secondary myotubes (Rosen *et al*., 1992; Jesse *et al*., 1998).

1.5 Dynamics of Satellite cells distribution

Satellite cells are present in all skeletal muscles but their distributions vary between species and even among different muscle fibre types within the same species. For example, in the mouse, there are more satellite cells in the soleus muscle than in the extensor digitorum longus (EDL) muscle, whilst more satellite cells are found in slow muscle fibres than the fast muscle fibres within the same muscle (Gibson & Schultz, 1982).

The underlying regulatory mechanism for satellite cell distribution remains largely unknown. In addition, the size of the satellite cell pool also varies with age, for example, the percentage of satellite cells relative to myonuclei was shown to slowly

decline with age in mouse soleus and EDL muscles. On the other hand, between 1 and 12 months, the absolute number of satellite cells increases significantly, in contrast to a significant decline in EDL muscle (Gibson & Schultz, 1983). During postnatal growth, satellite cells proliferate and fuse with growing muscle fibres, dramatically increasing the number of myonuclei associated with each fibre (Bischoff, 1994); some also form new satellite cells (reviewed in Morgan and Partridge, 2003). Yet the number of satellite cells rapidly declines with age to approximately 5% two months postnatally (Bischoff, 1994). Once sexual maturity is reached, it has been suggested that satellite cell numbers continue to decrease, albeit not as dramatically (reviewed in Chargé and Rudnicki, 2004). Adult skeletal muscle uses a mechanism that compensates for the turnover of terminally differentiated cells to maintain tissue homeostasis.

The latter proportion remains relatively constant throughout adulthood (Bischoff, 1994), but eventually declines at a slow rate (Bockhold *et al*., 1998; Gibson & Schultz, 1983; Schultz & Lipton, 1982). This continuous decline in the number of satellite cells has been proposed as a contributory factor in the decline in muscle bulk and regenerative ability observed with advancing age (Collins *et al*., 2005). However, the true extent of the variation in satellite cell number due to age remains unclear because of contradictory observations by various studies which have reported a decline, maintenance or even increase with age (Gibson & Schultz, 1983; Schultz & Lipton, 1982; Nnodim, 2000; Roth *et al*., 2000). Intriguingly, it has been reported that the number of Pax7-expressing satellites (i.e. the phenotype associated with self-renewal) declines with age, but that their intrinsic myogenic capacity does diminish (Shefer *et al*., 2006, Collins *et al*., 2007).

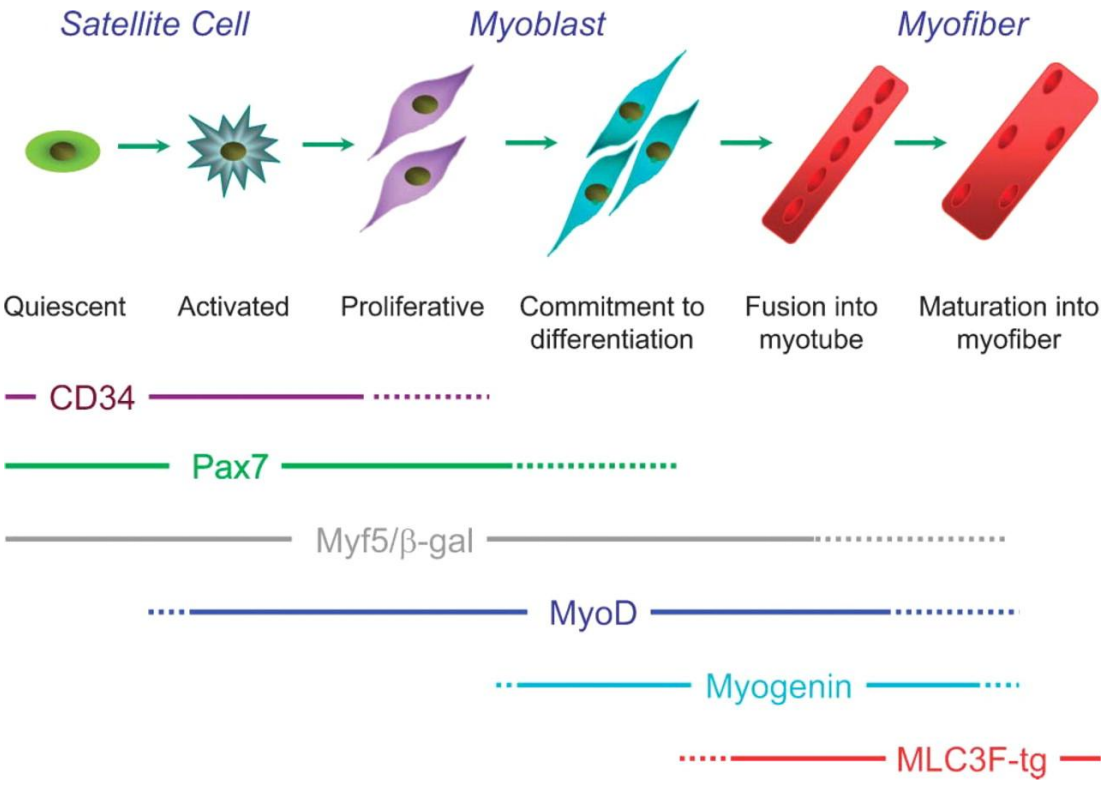


Figure 1.2 Schematic of markers during myogenesis
CD34, Pax7, and Myf5 are expressed in quiescent satellite cells. **Once** satellite cells are **activated** (MyoD upregulate)**,** and produces myoblasts **that further proliferate, before committing to differentiation** (Myogenin) **and fusing to form myotubes which then mature into myofibre (Diagram taken from reviewed in Zammit PS et al., 2006a).**

1.6 Molecular regulation of satellite cell proliferation and differentiation

1.6.1 Pax proteins

Several studies have suggested that Pax proteins play an important role in the regulation of adult myogenesis. Pax3 levels are regulated by mono-ubiquitination and proteasomal degradation during satellite cell activation, a process as shown by the inhibition of myogenic differentiation by sustained expression of mutant Pax3 (Boutet *et al*., 2007). On the other hand, although Pax7 is normally down regulated during myogenesis, sustained expression of Pax7 only delays the onset of myogenin expression without preventing myogenic differentiation and fusion. The presence of Pax7 is compatible with myogenic differentiation and its down-regulation is not a prerequisite for this process. Furthermore, the constitutive expression of Pax7 in

Pax7-null C2C12 sub-clones increases the proportion of cells expressing MyoD, suggesting that Pax7 can act upstream of MyoD (Zammit *et al*., 2006). This conflicts with the earlier studies in which the over-expression of Pax7 was found to suppress MyoD and prevent differentiation (Olguin & Olwin, 2004). In a subsequent study, it was shown that myogenin directly influences Pax7 expression and it was postulated to regulate Pax7 down-regulation (Olguin *et al*., 2007). When MyoD activity is enhanced under certain conditions, myogenin is up regulated and the presence of ectopic Pax7 does not prevent terminal differentiation, suggesting that Pax7 is incapable of preventing muscle differentiation once myogenin accumulates (Olguin & Olwin, 2004).

1.6.2 Myogenic Regulatory Factors (MRFs)

MyoD is required for skeletal muscle differentiation (Sabourin *et al*., 1999; Cornelison *et al*., 2000), whereas Myf5 is required to regulate satellite cell and myoblast proliferation and homeostasis (Gayraud-Morel *et al*., 2007; Ustanina *et al*., 2007). Quiescent satellite cells do not express detectable levels of MRFs, but activation is accompanied by the rapid up-regulation of Myf5 and MyoD (Grounds *et al*., 1992; Fuchtbauer & Westphal, 1992; Smith *et al.,* 1994; Yablonka-Reuveni & Riveria, 1994; Rantanen *et al.,* 1995; Cornelison & Wold, 1997; Cornelison *et al.,* 2000; Conboy & Rando, 2002; Zammit *et al.,* 2002). Myf5 up-regulation occurs within 12h of activation, before the onset of cell division and is immediately followed by a period of co-expression of Myf5 and MyoD (Cooper *et al*., 1999; Cornelison *et al*., 2000; Cornelison & Wold, 1997; Smith *et al*., 1994).

MyoD plays an important role in satellite cell differentiation. Megeney *et al* (1996) reported that *MyoD*$^{-/-}$ mice show reduced regenerative capacity with larger than normal population of MPC and a reduction of regenerated myotubes. In addition, the expression of M-cadherin was reduced significantly whilst expression of Insulin-like Growth Factor I (IGF-1) was increased significantly (Sabourin *et al*., 1999). M-cadherin is required for myoblast differentiation (Irintchev *et al*., 1994; Zeschnigk *et al*., 1995), whereas IGF-I promotes myoblast proliferation and inhibits

differentiation (Engert *et al*., 1996; Quinn & Roh, 1993). In contrast, in normal satellite cells MyoD serves to up-regulate M-cadherin and down-regulate IGF-I, thereby promoting myogenic differentiation. Together, these data suggest that MyoD plays an important role in satellite cell differentiation but are not sufficient to identify the exact downstream targets of MyoD that are important in the regenerative process.

There is evidence to suggest that Myf5 plays a distinct role from MyoD by promoting satellite cell self-renewal (Kuang *et al*., 2007). Muscle regeneration was found to be perturbed in adult *Myf5*$^{-/-}$ mice in that muscle fibre hypertrophy was observed whilst the satellite cell numbers were not significantly changed, although others have reported impaired proliferation *in vitro* (Gayraud-Morel *et al*., 2007).

MRF4 is required for myoblast specification in embryonic muscle progenitors. When MRF4 expression is disrupted, skeletal muscle does not develop in *Myf5*:*MyoD* double-null mice (Kassar-Duchossoy *et al*., 2004). However, in a later study using a *MRF4* knock-in mouse, expression of MRF4 was only observed during differentiation and was not up-regulated in *Myf5* null satellite cells, suggesting that Myf5 and MyoD are the only MRFs acting in the early stages of satellite cells activation (Gayrould-Morel *et al*., 2007).

1.6.3 Fusion of myogenic precursor cells (MPCs)

MPCs initiate terminal differentiation after the proliferative phase through the up-regulation of myogenin and MRF4 expression. They subsequently withdraw from the cell cycle, activate muscle specific proteins and initiate fusion to repair damaged muscle fibres. M-cadherin has been suggested as a molecule responsible for myoblast fusion during embryonic myogenesis and muscle regeneration (Moore & Walsh, 1993; Zeschnigk *et al*., 1995).

1.7 Alternative sources of myogenic regeneration

The satellite cell population may not be the sole contributing factor in the renewal of the satellite cell pool. Multipotent stem cells have been isolated from various adult tissues and shown to differentiate into multiple lineages both *in vitro* and *in vivo*. Such cells with the potential to differentiate into the myogenic lineage have been isolated from bone marrow (Bittner *et al*. 1999; Ferrari *et al*. 1998; Gussoni *et al*., 1999; LaBarge & Blau, 2002), adult somatic muscle (Asakura *et al*., 2002; Gussoni *et al*. 1999; Jankowski *et al*., 2002; Qu Petersen *et al*., 2002; Torrente *et al*., 2001) and various mesenchymal tissues (Young *et al*., 2001a; Young *et al*., 2001b).

Transplantation experiments have further suggested that non-muscle stem cells can participate in muscle regeneration. Ferrari *et al.* (1998) transplanted genetically marked bone marrow from transgenic mice into immunodeficient host mice either intramuscularly or intravenously and identified bone marrow-derived myogenic cells in the host musculature. It was suggested that these donor-derived cells are recruited through long-range signals, migrate into the damaged muscle from the circulation and take part in the regeneration process, Noteably, the differentiation timeline of bone marrow derived cells was different from that of committed adult myogenic precursors in that bone marrow-derived cells could only be detected in regenerating fibres after 2 weeks-post transplantation.

Subsequent studies reported that only a specific subpopulation of bone marrow cells, termed the side population (SP), was able to generate myofibre nuclei, illustrated by the ability to restore dystrophin expression in regenerating *mdx* mouse muscle (Gussoni *et al.*, 1999; Jackson *et al.,* 1999). Bone marrow-derived cells (BMDC) have also been shown to be able to contribute to the satellite cell pool (LaBarge & Blau 2002). In this study, mice were grafted with GFP+ve BMDC and the endogenous satellite cells ablated from the muscle stem cell niche following irradiation-induced damage. GFP+ve cells were observed in the satellite cell position and when triggered by exercise-induced damage, were able to self-renew and participate in muscle regeneration, giving rise to proliferative myoblasts and the expression of skeletal muscle specific proteins both *in vivo* and *in vitro*.

It has also been reported that adult skeletal muscle contains a resident muscle side population (mSP), identified by a similar Hoechst staining technique to that used to isolate bone marrow SP and can repopulate the ablated hematopoietic niche of lethally irradiated mice after intravenous injection (Gussoni *et al.*, 1999; Asakura *et al.*, 2002). The mSP is distinct from satellite cells as it does not express the satellite cell markers Myf5, Pax7 or desmin (Asakura *et al.*, 2002). Furthermore, mSP isolated from the muscles of the *Pax7* -/- mice lacking satellite cells were unable to undergo myogenesis but had significantly increased hematopoietic potential *in vitro*, suggesting that mSP may contain satellite cell progenitors (Seale *et al.,* 2000). The mSP can be further classified into phenotypically distinct subpopulations with 92% expressing Sca-1 and 16% positive for CD45, a haematopoietic lineage marker (Asakura *et al.,* 2002). *In vitro*, 9% of CD45+ve and 5% of CD45-ve mSP underwent myogenic conversion when co-cultured with myoblasts, although the CD45-ve mSP appeared to have greater myogenic potential after intramuscular injection (McKinney-Freeman *et al.,* 2002). In a later study, the CD45+ve/Sca-1+ve mSP cells isolated from uninjured muscle were found to have no myogenic potential, whereas a large fraction of the CD45+ve/Sca-1+ve cells isolated from regenerating muscle underwent myogenic differentiation *in vitro* or when co-cultured with Wnt protein expressing cells (Polesskaya *et al,.* 2003).

Both Camargo *et al.* (2003) and Corbel *et al* (2003) transplanted mice with single hematopoietic stem cells (HSCs). It was shown that CD45+ve, **but not CD45-ve HSCs could generate muscle when irradiated mice following regeration.** However, there was no evidence of HSC-derived muscle precursors suggesting that the CD45+ve cells had become incorporated during regeneration **without preceding myogenic conversion**. It was therefore hypothesized that CD45+ve cells that reside in muscle can give rise to myogenic precursors, but that HSC cannot (Camargo *et al.,* 2003; reviewed in Rudnicki*,* 2003).

Other studies have also identified cells with myogenic potential both within or associated with the vascular system. For example, a subpopulation of circulating human *blood–derived* cells expressing the hematopoietic stem cell marker AC133 also express myogenic markers and can undergo myogenesis when co-cultured with

myogenic cells or Wnt-expressing cells *in vitro* or when delivered directly into the musclse of transgenic *scid*/*mdx* mice or through the arterial circulation (Torrente *et al.,* 2004). Pericytes, present in adult skeletal muscle but phenotypically distinct from satellite cells, have also been shown to have myogenic potential, possibly equal to that of satellite cells (Dellavalle *et al*., 2007). These adult cells, derived from blood vessels, express the pericyte markers NG2 proteoglycan and alkaline phosphatase (ALP), but not endothelial markers. Dellavalle *et al*. (2007) showed that pericyte-derived cells could efficiently colonise dystrophic mouse muscle and generate skeletal muscle fibres after transplantation. Strikingly, this myogenic differentiation is approximately one order of magnitude higher than other types of stem cells.

1.8 Satellite cell self-renewal

In adult skeletal muscle, the satellite cell pool has an inherent capacity for self-renewal as shown by the fact that the population of quiescent adult satellite cells remains largely constant even after numerous cycles of degeneration and regeneration (Gibson & Schultz, 1983; Schultz *et al.,* 1985). Evidence of satellite cell self-renewal was obtained by transplanting purified populations of satellite cells into irradiated host mice, which showed that these cells can both regenerate new myofibres and repopulate host muscle with new satellite cells. This demonstrated that satellite cells are able to self-renew and replenish their niche and generate progeny capable of efficient regeneration (Collins *et al.,* 2005; Montarras *et al.,* 2005; reviewed in Le Grand, & Rudnicki, 2007). Fate tracing experiments have further suggested that not all satellite cells are equivalent in this respect with 80% dividing rapidly and contributing myonuclei to growing fibres, whilst the remaining 20% divide more slowly and have been suggested to be responsible for replenishing the satellite cell pool (Schultz, 1996; Gussoni *et al.,* 1997; Gross & Morgan, 1999; Heslop *et al.,* 2001). These observations are compatible with the view that only a small proportion of satellite cells have true stem cell potential, specifically in terms of the capacity for self-renewal (Baroffio *et al.,* 1995; Baroffio *et al.,* 1996; Beauchamp *et al.,* 1999; Schultz, 1996; Yoshida *et al.,* 1998).

Also consistent with this view, are two alternative (but not necessarily mutually exclusive) models for satellite cell self-renewal. In the 'stochastic' model, the progeny of each activated satellite cell are generated by symmetric divisions and, after a period of proliferation, one or more of the progeny return to quiescence while the remainder differentiate. A minority of the progeny then exit the cell cycle and return to a quiescence state, presumably due to extrinsic cues, thereby renewing the satellite cell niche (Zammit *et al.,* 2004; Olguin & Olwin, 2004). In an alternate model, the activated satellite cell divides asymmetrically and generates two distinguishable daughter cells: one daughter cell then divides symmetrically to generate myoblasts that progress along to the myogenic lineage for muscle regeneration, whereas the other daughter exits the cell cycle and returns to quiescence, maintaining the satellite cell pool (Dhawan & Rando, 2005; Chargé & Rudnicki, 2004). Evidence supporting the second model was obtained by the lineage tracing experiments of Kuang *et al.* (2007). These studies suggested that the Pax7+ve satellite cell pool contains a subpopulation of 'true' stem cells that never express Myf5 and that are capable of dividing asymmetrically to generate a Myf-ve daughter cell that replenishes the niche and a Myf5+ve daughter cell that is committed to proliferate symmetrically and undergo myogenesis. Furthermore, prospective isolation and transplantation of both types of daughter cells suggested that the Myf5-ve population was far more effective in its ability to contribute to the satellite cell pool compared with Myf5+ve cells which were restricted to muscle fibre regeneration (Kuang *et al.,* 2007).

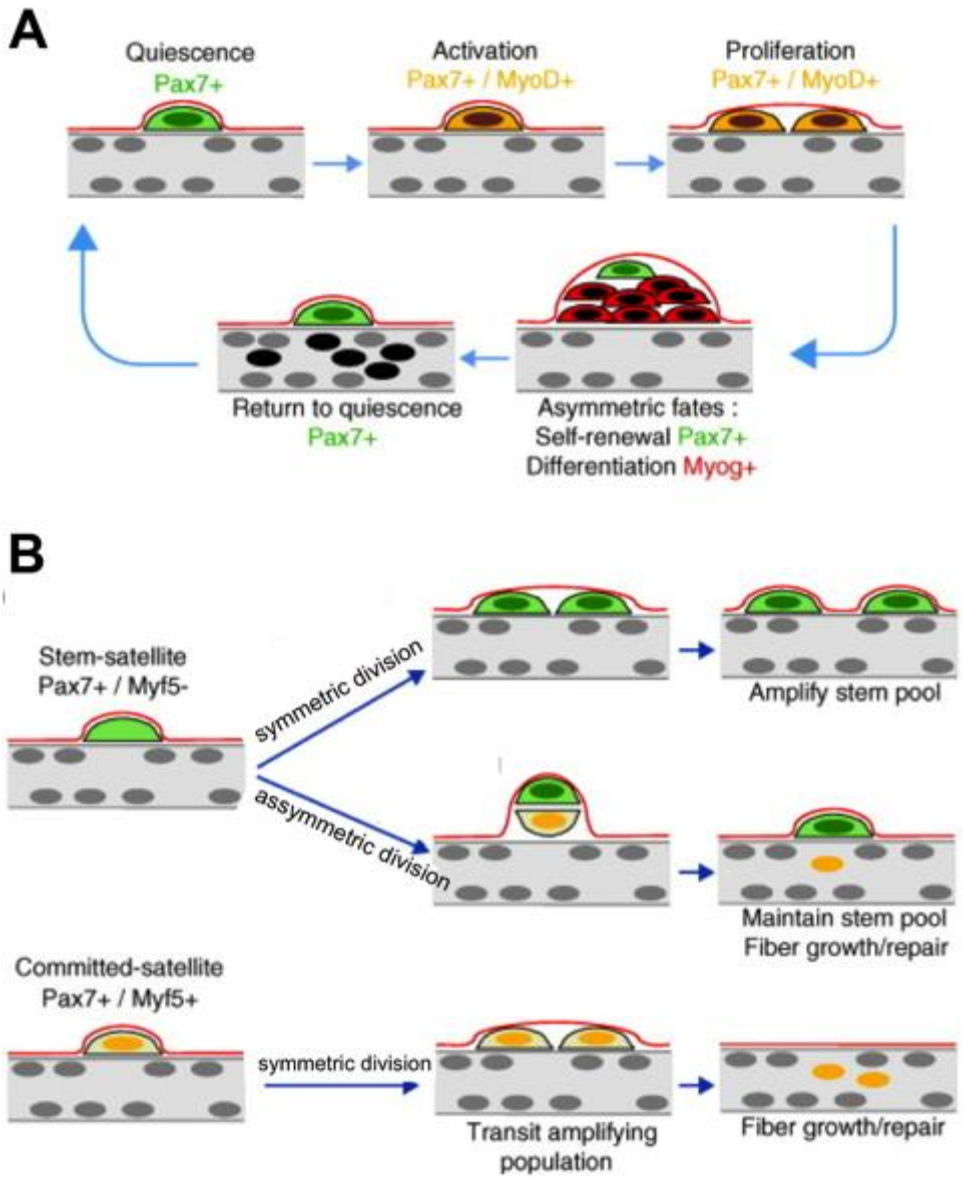


Figure 1.3 Two models of satellite cell self-renewal.
A. In this model, injuring the muscle causes the quiescent (Pax7+) satellite cells to enter an activation and proliferation (Pax7+/MyoD+) stage. The majority of the cells from this transitory proliferating population undergo terminal differentiation. The remaining cells return to a Pax7+/Myf5− stage thereby renewing the quiescent satelite cell pool. B. In a second model, Pax7+/Myf5− satellite 'stem' cells co-exist with Pax7+/Myf5+ 'committed' satellite cells. Pax7-only cells undergo symmetric or assymetric cell division to amplify or maintain, respectively, the stem cell pool. Committed Pax7+/Myf5+ satellite cells, probably through symemtric cell division, preferentially undergo terminal differentiation. (Diagram taken from Reviewed in Relaix and Marcelle, 2009).

1.9 The role of Notch signalling in satellite cell self-renewal

Several lines of evidence suggest that Notch signalling may be involved in the regulation of satellite cell behaviour, particularly in terms of proliferation and cell fate decisions. Indeed, the observation that when differentiation begins, satellite cell progeny can adopt divergent cell fates either to terminally differentiate or to return to quiescence (Zammit *et al.,* 2004; Beauchamp *et al.,* 2000), is consistent with some form of the 'lateral inhibition' through to cell-cell signalling is involved. Given the established roles of the Notch signalling pathway, a conserved cell-to-cell pathway that regulates cell proliferation, cell fate determination and asymmetric cell divisions during embryogenesis (Artavanis-Tsaknas *et al.,* 1999), it is highly probable that Notch signalling may play an important role in the maintenance of the satellite cell pool.

1.10 Notch signalling overview

The development of multicellular organisms requires spatially and temporally coordinated cell behaviour (Artavanis-Tsakonas *et al.,* 1999; Schweisguth, 2004). Regulation of cell fate, proliferation, growth, migration, survival, apoptosis, differentiation and morphogenesis involves cell-cell communication. The Notch signalling pathway is an evolutionarily conserved mechanism that facilitates short-range communication between cells. Under most circumstances, physical contacts between cells are required for the transmission of Notch signals for the selection of pre-existing developmental programs. Thus, Notch signalling, through regulating the activation of differentiation programs, can promote or suppress cell proliferation and cell death. Notch signalling is critical throughout development and in adult organisms from nematodes to humans, for tissue repair, regeneration and the maintenance of the capacity for self-renewal. Indeed, the Notch signalling pathway is crucial for neurogenesis (Louvi & Artavanis-Tsakonas, 2006), vasculogenesis, angiogenesis (Krebs *et al.,* 2000), somite formation (Artavanis-Taskonas *et al.,* 1999; Conboy & Rando, 2002; Gridley, 2007; McGrew & Pourquie, 1993; Weinmaster, 1998; Pourquie, 1999) and lymphogenesis (Kondo *et al.,* 2001).

It has also been shown to play a role during postnatal myogenesis (Conboy & Rando, 2002; Conboy *et al*., 2003; Conboy *et al*., 2005). A distinguishing feature of Notch signalling is the mechanism of signal transduction. This mechanism sets it apart from other conserved signalling pathways and relies on the ability of a ligand to bring about intramembrane receptor proteolysis, which is followed by receptor activation with the release of an active intracellular Notch receptor fragment into the cytoplasm of the recipient cell. After proteolytic release from the membrane, the active Notch intracellular domain (NICD) translocates to the nucleus and associates with a DNA binding protein to form a transcription complex. This transcription complex then activates downstream target genes. This "canonical" transduction pathway is used in most Notch-dependent processes. In addition, the amplitude and duration of the Notch activity can be regulated at various points in the pathway depending on the cellular context.

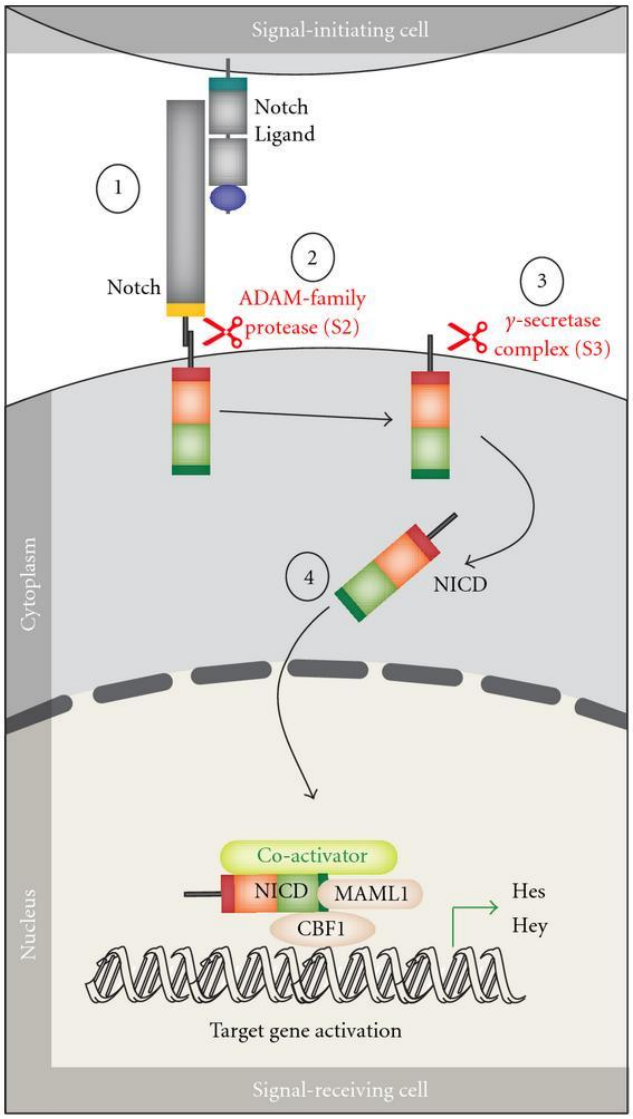


Figure 1.4 Notch signalling pathway
Notch signalling is initiated by a Notch ligand binding to a Notch receptor (1). This binding initiates two subsequent proteolytic cleavages of the Notch receptor by the ADAM protease (2) and the γ-secretase, respectively (3). The Notch IntraCellular Domain (NICD) is thereby released (4) and translocates to the nucleus and activates the downstream target genes including HES/HEY. (Diagram taken from reviewed in Ersvaer *et al*., 2011).

1.11 Modes of Notch signalling

Notch signalling functions via two distinct modes: lateral inhibition (Lewis, 1996) and inductive signalling (Haines *et al*., 2003).

1.11.1 Lateral inhibition

During lateral inhibition, Notch signalling inhibits all but one among a group of equipotent cells from adopting a particular fate during development. Initially, cells with equal developmental potential both send and receive Notch signals. Later, a small variation of Notch ligand expression occurs in some cells either through a stochastic event or by other unknown mechanisms, resulting in a rapid amplification of the difference by a transcriptional feedback mechanism. The presence of a cell with relatively high levels of surface Notch ligand causes an increased level of activation of Notch signal in the neighbouring cells, thereby inhibits these neighbouring cells from choosing the same cell fate. The selection of *Drosophila* neural precursors and *C. elegans* vulva development are classical examples of lateral inhibition (Greenwald, 1998; Parks *et al.,* 1997).

1.11.2 Inductive signalling

Inductive signalling typically occurs between non-equipotent cell populations when one group of cells signal to a distinct adjacent group of cells to promote a new cell fate along the interface between the two populations. Instead of repressing a given cell fate among equipotent cells, inductive Notch signalling instructs cells to adopt cell fate as a result of cell-cell interactions at the boundary between the cell populations. This is exemplified by Drosophila wing development and vertebrate somitogenesis (Conlon *et al*., 1995; Couso *et al*., 1995). In mammals, examples of cell fate decisions of bipotential precursor regulated by Notch signalling include T lymphoid versus B lymphoid cells, pancreatic exocrine versus endocrine, and arteries versus veins (Apelqvist *et al*., 1999; Pui *et al*., 1999; Lawson *et al*., 2001).

1.12 Notch receptor and ligand structure

Notch was identified by T. H. Morgan (Mohr, 1919) and named from the phenotype of mutant flies with notched wings. Artavanis-Tsakonas *et al.* (1983) cloned the *Notch* locus and showed that it encodes a single pass transmembrane protein (Wharton *et al*., 1985). *Drosophila* has a single Notch receptor whereas *C. elegans*

has two redundant Notch receptors. Mammals possess four Notch receptors namely Notch 1-4 and five Notch ligands namely Delta-like-1, -3, -4 and Jagged-1, -2.

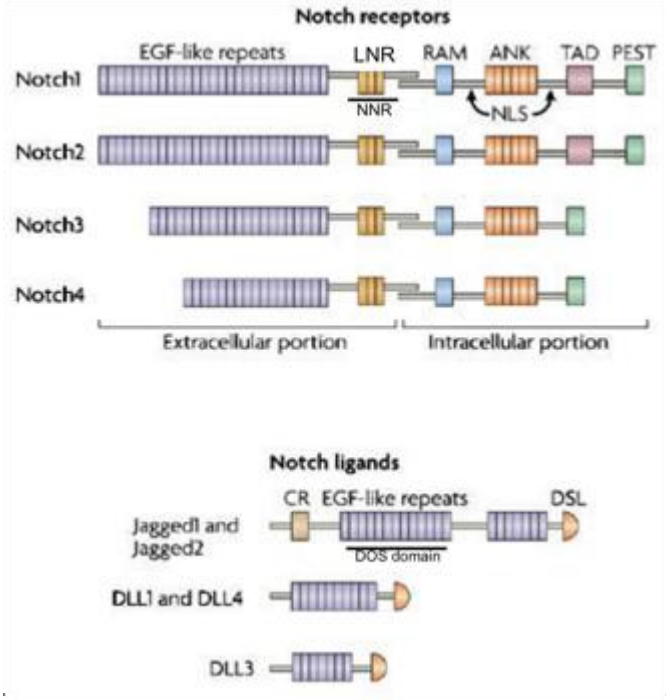


Figure 1.5 Structure of the vertebrate Notch receptors and ligands
(Diagram taken and adpated from Osborne & Minter, 2007)

The Extracellular Domain (ECD) is composed of between 36 (in *Drosophila* and some vertebrate Notch members) and 10 (in *C.elegans*) epidermal growthfactor-like (EGF) repeats, two extracellular sites of proteolytic processing and two conserved cysteine residues and three lin-12/Notch repeats (LNRs) (Schweisguth, 2004). The Intracellular Domain (ICD) comprises of a conserved PEST sequences, the RAM23 domain, six cdc10/ankyrin repeats (ANK), valine residue and three nuclear localisation signals (NLS) that sandwich the ANK region (Fleming, 1998; Shimizu et al., 2000; Mumm and Kopan, 2000).

In *Drosophila*, EGF repeats 11 and 12 in the ECD are necessary and sufficient for mediating cellular aggregation with ligand-expressing cells in tissue culture (Rebay etal., 1991). Repeats 11 and 12 are conserved in all vertebrate homologues and are needed for making productive interactions with ligands presented by nearby cells

(Fleming, 1998). Some of the EGF repeats bind calcium ions and play a significant role in determining the affinity and structure of Notch within the ligand binding (Cordle *et al*., 2008b) which has the ability to influence signalling efficiency.

A Negative Regulatory Region (NRR) follows the EGF repeats and plays a significant role in preventing receptor activation when ligands are not present. The majority of the Notch proteins get cleaved at site 1 (S1) which is located within a shapeless loop protruding from the heterodimerization sphere, with the help of a protease resembling furin (Blaumueller *et al.,* 1997). Fragments undergoing cleavage then are joined together by non-covalent associations between the C and N regions found at the terminal ends of the heterodimerization domain. Notch receptor ends have the C-terminal stop translocation signal consisting of arginine/lysine residues 3-4. The RBP-J Association Module (RAM) domain forms an intracellular binding module made up of 12 to 20 of the total amino acids and focussed on a tryptophan-any (WxP) design amino acid-proline sequence. A formless linker comprising the nuclear localization arrangement binds the RAM sphere to the 7 ankyrin repeats within the cdc10/ankyrin repeats (ANK) domain. A nuclear localization bipartite arrangement and a loosely defined evolutionary divergent transactivation sphere follow the ANK domain. The C terminus of the receptor consists of a conserved proline/glutamic acid/serine/threonine-rich (PEST) arrangement which that harbour degradation signals known as degrons that regulate the stability of the Notch IntraCellular Domain (NICD).

Notch ligands belong to the DSL family, named after the invertebrate ligands, Delta and Serrate from *Drosophila* and lag2 from *C. elegans* (Fleming, 1998; reviewed in Weinmaster, 1997). The majority of Notch ligands are type I transmembrane proteins featuring three associated structural regions, namely an N-terminal DSL motif, special tandem EGF repeats known as the DOS domain (Komatsu, 2008), and EGF-type repeats. The DSL ligands are classified by the absence or presence of the cysteine-rich spheres and the DOS domains. Both the DOS and DSL domains participate in binding to the Notch receptor.

1.13 Notch receptor activation

Activation of Notch receptors is facilitated by a series of sequential proteolytic events (Bray, 2006) and the major regulatory event is ectodomain shedding. Notch activation is triggered by the binding of a transmembrane DSL ligand with the EGF-repeat domain specific to a Notch ectodomain (Fehon *et al*., 1990; Weinmaster, 1997), inducing a proteolytic flow known as Regulated Intra-membrane Proteolysis (RIP). The ectodomain is released by an ADAM- facilitated and ligand-induced Notch cleavage which is a vital regulatory point within the transduction of the Notch signal (Brou *et al*., 2000; Mumm & Kopan, 2000). A site lying between Val-1711 and Ala-1710 provides the S2 cleavage site for the metallo-proteases and is located twelve amino acids ahead of the transmembrane domain, deeply buried in the NRR enveloping the heterodimerization domain and the LNR modules. S2 cleavage is essential for subsequent cleavage at S3 by γ-secretase (Kopan & Goate, 2000). The addition of monensin which disrupts the transport of the vesicles from the trans-Golgi network to the surface of the cell, blocks S3 and S2 (Schroeter *et al.,* 1998), but not S1cleavage (Blaumueller *et al.,* 1997). These findings are consistent with a proteolytic cascade in which Notch pathway is activated when Notch Extracellular Truncation (NEXT) is resented on the cell surface and converted to NICD. The main function of the NNR is to prevent proteolysis of the Notch when ligands are absent. Indeed, studies have shown that if a viral integration (Girard *et al*., 1996) or point mutation (Weng, 2004) disrupts the NRR, "leaky" Notch signalling can result in acute T cell lymphoblastic leukaemia in both humans and mice and mutation of the LNR domain leads to the Notch phenotype activation in *C. elegans* (Greenwald *et al*., 1990).

Notch ectodomain shedding results in a membrane-tethered intermediate known as NEXT (Wolfe & Kopan, 2004). γ-Secretase is an Intramembrane CLeavIng Protease (I-CLiPs) that progressively cleaves the NEXT in the transmembrane domain beginning in proximity to the S3 site and terminating somewhere in the centre of the S4 site transmembrane domain. After γ –secretase cleavage, the NICD can freely translocate to the nucleus to interact with the protein CSL (an acronym

for CBF1, Suppressor of Hairless, Lag1) and bind to DNA via the RAM domain. The NICD ANK domain interacts with CSL enabling recruitment of the Mastermind/Lag-3 co-activator, which in turn recruits the mediator transcription activation complex MED, thereby activating expression of downstream target genes (Kovall & Kovall, 2008).

Since receptor activation is the result of the interactions between Notch receptors and DSL ligands found at the cell surface (Brou *et al*., 2000), it is conceivable that Notch activation could be mediated by ligand expressed by the neighbouring cell or on the same cell. However, it has been demonstrated that S3 and S2 proteolytic cleavages are made when the receptor-ligand interactions take place in *trans*, presumably because S2 cleavage occurs in the plasma membrane (Mumm *et al*., 2000).

It has also been shown that the protein Mind bomb (Mib), previously thought to be involved in protein degradation, also has a primary role in protein trafficking by promoting internalization of the DSL ligand Delta which increases the efficiency with which Delta activates Notch (Itoh *et al.,* 2003). Mib1 is required for Notch ligand endocytosis and is thought to interact with and regulates all DSL Notch ligands (Koo *et al*., 2005). Indeed, ligand endocytosis is critically important for Notch activation as it is required for separation of the NECD from the NICD, consistent with the pan-Notch defects observed in *Mib1* –/– mouse embryos (Parks *et al*., 2000).

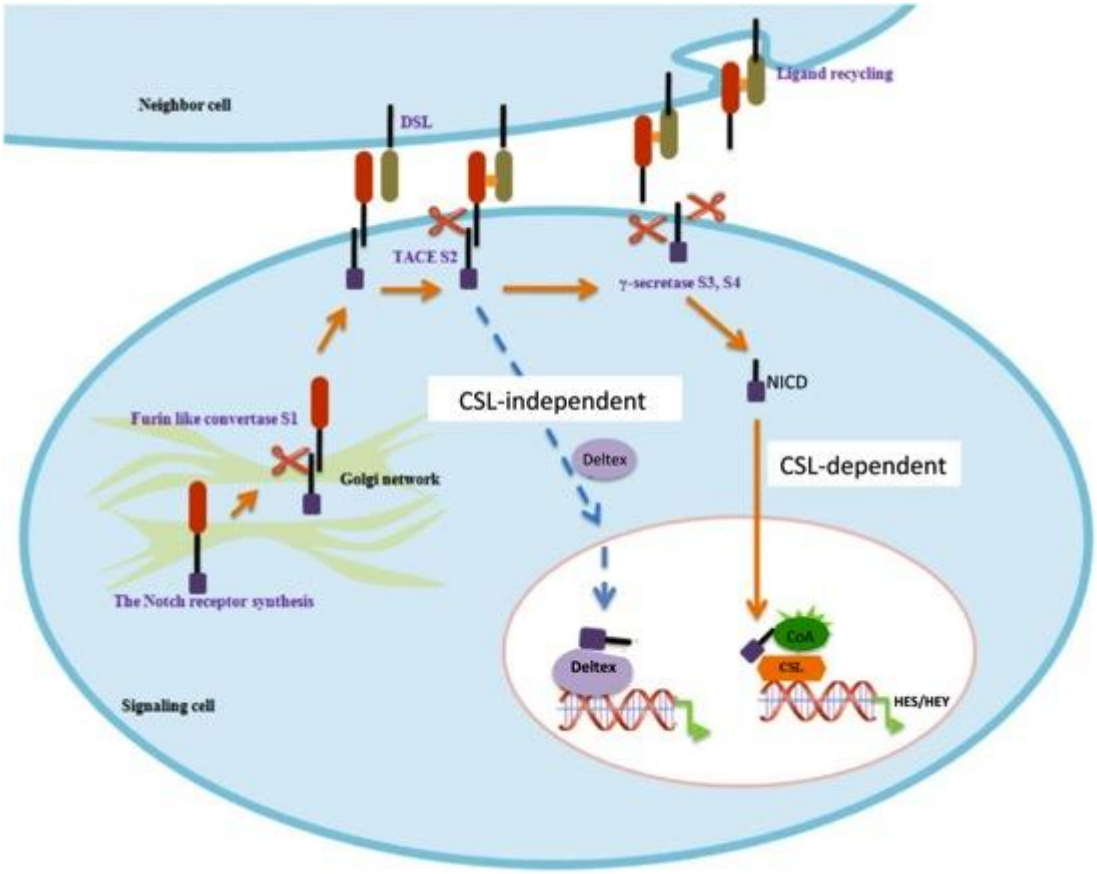


Figure 1.6 Notch signalling
Notch receptors are synthesised as full-length precursor proteins that are cleaved by a furin-like convertase in the Golgi apparatus before transported to the cell surface where they reside as heterodimers. Interaction of extracellular domain of a heterodimeric Notch receptor with the extracellular domain of DSL ligands (e.g. Delta-like or Jagged) on the adjacent cell, leads to a cascade of proteolytic cleavages. The second cleavage is mediated by ADAM/TACE/Kuzbanian family allowing to release the receptor ectodomain. The third cleavage mediated by the γ-secretase activity of presenilins which releases the active Notch ICD (NICD) into the cell. The NICD translocates into the nucleus and binds to the transcription factor CSL, which displaces the corepressor (CoR) complex and recruits coactivators (CoA), leading to transcriptional activation of downstream target genes (e.g. *Hes* and *Hey*). Downstream target genes can be activated via the CSL-independent Notch signalling pathway. Deltex molecules activated the heterodimeric Notch receptor in a ligand-independent manner and activates its gene targets independently of CSL.
(Diagram taken and adapted from Sassi N *et. al*., 2011).

1.14 Regulation of Notch receptor-ligand interactions

Temporal and spatial restriction of receptor or ligand expression is potentially the simplest way to regulating availability. Different receptors and ligands can have distinct (but often overlapping) expression patterns during development and may by regulated by distinct pathways (Wu & Bresnick, 2007). However, differences in receptor and ligand expression patterns are not sufficient to explain the differences observed in signalling activities and several post-translational modifications and regulation of trafficking are thought to play crucial roles in controlling controls receptor ligand availability and the productive interactions between them.

1.14.1 Notch receptor and ligand trafficking and endocytosis

Trafficking and endocytosis of DSL ligands has an important role to play in triggering the Notch signalling activity (Borgne, 2006). Ligand endocytosis is enhanced by mono-ubiquitination, facilitated by the E3 ubiquitin ligases Mind bomb and Neuralized. Endocytosis is followed by a poorly characterized process, which results in the presentation of active ligand on the cell surface. The current models of ligand modification involve ligand recycling, post-translational modification clustering in particular membrane micro-domains (Borgne, 2006; Nichols *et al*., 2007a). Members of the *Bearded* protein family regulate *Neuralized* activity in a negative manner and minimize the efficiency of Notch activation via Delta (Bardin, & Schweisguth, 2006) and are referred to as Notch targeting genes (Lai *et al.,* 2000). The negative role of *Bearded* proteins is modulated by micro RNAs that target *Bearded* mRNAs and the E (spl) mRNA, reducing their half life and mitigating their impact on *Neuralized* (Lai *et al*., 2005; Stark *et al*., 2003). MicroRNAs also play a key role in regulating the expression of Delta itself (Kwon *et al*., 2005).

Various mechanisms also control the turnover of Notch receptors at the cell surface, thereby regulating their availability for ligand binding. For instance, the E3 ubiquitin ligases Cbl, Su (Dx)/Itch, Nedd4 and Deltex can direct Notch receptor trafficking towards recycling or lysosomal degradation pathways, regulating receptor half-life (Bray, 2006). AP2/Numb-associated Kinase (NAK) and the AP2 component α-adaptin together with Numb, promote Notch degradation in the

daughters of dividing cells following asymmetrical division. Deltex family members are thought to be involved in ligand-independent Notch signalling (Kishi et al., 2001). Notch signaling may be autonomously activated by Deltex proteins (Matsuno et al., 1995).

Although Numb acts as a Notch antagonist in the majority of the systems, it can act as a Notch synergist in certain specific contexts, (Range *et al.,* 2008). In cases when the lateral Notch signalling takes place among resting cells, Numb is inactive. In vertebrates, studies have suggested that Numb is unable to associate with the protein ACBD3, which is trapped within the Golgi and that this physical separation makes Numb functionally inactive in resting cells (Zhou *et al.,* 2007). Golgi fragmentation occurring during mitosis allows formation of an ACBD3/Numb complex, which activates Numb to increase Notch activity by an unidentified mechanism.

1.14.2 Regulation of Notch-Ligand interaction by glycosylation

Notch receptors are large glycoproteins and gloycosylation, facilitated by Fringe proteins is critical for normal Notch signalling (Okajima *et al*., 2003). Glycosylation of Notch receptors involves the modification of their EGF repeats by two basic types of O-glycosylation through O-glucose ond O-fucose (Haines, 2003). When the synthesis of O-fucose is disrupted or the addition/removal of O-fucose glycan binding sites on Notch receptors occurs it lead to serious defects in Notch signalling in mammals and Drosophia (Stanley, 2007). Drosophila and mammalian Fringe proteins possess a fucose-specific 1,3 N-acetylglucosaminyltransferase activity (i.e. glycosyltransferase activity) that catalyses the addition of N-acetylglucosamine to O-linked fucose residues in the EGF repeats of Notch receptors, initiating their elongation (Brückner *et al*., 2000; Moloney *et al*., 2000; Moran *et al*., 1999). Such modification of the Notch ligand binding domain regulates ligand binding and thus receptor activation. Drosophila Fringe enhances Delta-Notch binding and inhibits Serrate-Notch binding (dependent of O-fucosylation of EGF repeat 12 of Notch (Lei *et al*., 2003)) (Brückner *et al*., 2000; Okajima *et al*., 2003). Indeed, Drosophila and

mammalian Fringe proteins, through the regulation of ligand-receptor activation, modulate the formation of compartment borders in the developing embryo (Evrard *et al*., 1998; Johnston *et al*., 1997; Moran *et al*., 1999; Shimizu *et al*., 2001; Zhang and Gridley, 1998).

1.15 Downstream target genes of Notch signalling

The main target genes activated by Notch signalling in mammals belong to the *Hes* (Hairy Enhancer of Split) and *Hey* (Hes-related protein) families (reviewed in Iso *et al*., 2003). They act as Notch effectors through negative regulation of downstream targets such as tissue-specific transcription factors. Seven members of Hes gene family (HES1-7) have been identified in mamals. They belong to the basic helix-loop-helix (bHLH) family of transcription factors and functions as transcriptional repressor that influence cell proliferation and differentation in embrogenesis and myogenesis. There are three conserved domains in Hes genens that impart transcriptional functions: the bHLH domain, the Orange domain and the WRPW motif (Kageyama *et al.,* 2007).

Three members of Hey gene family (HEY-1, -2, -L) have been identified in mammals. They shared similar structural elements as Hes family: the bHLH and the Orange domain. Although there is high level on sequence conservation within the family, there is less conservation amongst the two families. HES has a proline residue in the basic region and this is the most remarkable difference that distinguishes HES from HEY, which has a glycine at the corresponding position. All HES members share the C-terminal tetrapeptide WRPW motif, whereas the HEY family has YRPW or its variant. In addition, HEY family has an additional conserved region C-terminal to the tetrapeptide motif.

This divergence in structure may account for the fact of the distinct transcriptional mechanisms mediated by the two families. Hes proteins repress transcription by at least two mechanisms: active and passive repression. Active repression depends on the WRPW domain, interacting with co-repressor TLE/Groucho. TLE/Groucho recruits HDACs, which alter the chromatin structure thereby reducing the

accessibility of transcription mechanism. Hes can also repress via protein sequestration (passive repression) whereby a non-DNA-binding heterodimer was formed by, for example, Hes1 and another bHLH factor, E47. Thus reduce the availably of E47 to bind to its normal partner and forming functional heterodimer (e.g. MyoD-E47 and Mash1-E47) (reviewed in Iso *et al.*, 2003b). Thirdly, the Orange terminal may mediate repression via direct recruitment of some co-repressor or the regulation of WRPW-mediated repression.

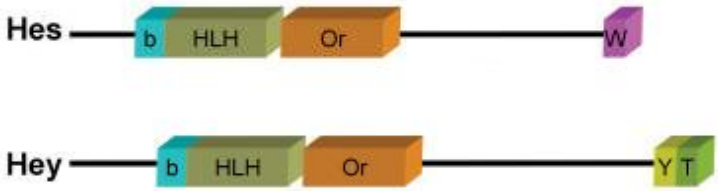


Figure 1.7 Domain organisation of Hes and Hey proteins.
(Diagram taken from reviewed in Fischer and Gessler, 2007).

On the other hand, Groucho/TLE is not a critical component of repression by HEY. The bHLH domain of HEY alone is sufficient for recruitment of co-repressor complex consists of N-CoR, mSin3A and HDAC1 (Iso *et al*, 2001).

Activation of Notch signalling in satellite cells promote their self-renewal via upregulation of Pax7, and maintain them in an undifferentiated stage by transcriptional repression of MyoD and myogenin via the Hes and Hey family of transcriptional repressors (Wen et al., 2012). Notch targets Hey1 transcriptionally represses key myogenic genes, including myogenin and Mef2c, preventing differentiation of muscle progenitors into multinucleated myotubes (Buas et al., 2010).

Fukada *et al.* (2007) identified HeyL as one of the most highly expressed genes in adult quiescent satellite cells. In a follow up study, both Hey1 and HeyL protein were similarly found to be simultaneously expressed in quiescent satellite cells, but

HeyL was not detected in activated and proliferating satellite cells whilst Hey1 was detected in activated and proliferating satellite cells cultured for 3 days. On the other hand, Hey2 was not expressed in quiescent satellite cells and myoblasts (Fukada *et al.* 2011).

Mourikis *et al.* (2012) showed that the conditional loss of RBP-J in quiescent satellite cells provokes their spontaneous differentiation and contributing to myoblasts, eventually leading to severe depletion of stem cell pool and compromised muscle regeneration. They noted Hes1 is only slightly induced when Notch signalling is activated but it is repressed when Notch pathway is inhibited. With absence of detectable RBP-J proteins, HeyL, Hey1, Hes1, MyoD and Pax7 transcripts were significantly downregulated, in contrast, Myogenin transcript level increased many folds. After the RBP-J depletion, the number of Pax7 cells in resting muscle suffered continuous decline and muscle regeneration failed at 32 days post RBP-J depletion for the conditional knockout (cKO) mice, indicating prolong blocking of Notch signalling resulting in the depletion of satellite cells.

Buas *et al*. (2009b) argued that the induction of all downstream targets of MyoD should be compromised if Hey1 repress intrinsic MyoD transcriptional activity. But it was found that co-expression of Hey1 with MyoD inhibited only one (cadherin-15) of the two normally expressed markers (cadherin-15 and myogenin). This specifies inhibitory effects of Hey1 on myogenesis suggested Hey1 exerts promoter-specific repressing, rather than targeting MyoD protein. Furthermore, Buas *et al*. (2009b) also showed that Hey1 is recruited to the promoter regions of myogenin and Mef2C and the expression of Hey1 in C2C12 myoblasts correlates with the reduced association of MyoD to these promoters. Thus strongly indicated Hey1 inhibit myogenesis through repressing the expression of key myogenic targets.

Nonetheless, Notch signalling may also influences myogenesis through incorporating other signalling pathways. One alternate signalling pathway in which Bone morphogentic protein (BMP) signalling induces the proliferation of satellite cells and also blocking their premature differentiation (Ono et al., 2011). In particular, BMP4/SMAD1 signalling promotes Notch-dependent activation of Hes1

and Hey1 to inhibit differentiation of satellite cells and C2C12 myoblasts (Dahlqvist et al., 2003).

1.16 Cell cycle regulation by Notch signalling

Several lines of evidence proposed that the Notch signalling pathway is possibly involved in the cell cycle (Cao *et al*., 2003). This is certainly important with respect to the maintenance of the satellite cell population in which some proliferating myoblasts exit the cell cycle and return to a quiescent state. Strong evidence has been shown that Notch activation promotes survival and inhibits apoptosis (Deftos *et al*., 1998; Jehn *et al*., 1999; Shelly *et al*., 1999; Tan-Pertel *et al*., 2000; Tsai *et al*., 2000; Rangarajan *et al*., 2001). Yet, under certain conditions apoptosis in monocytes could be triggered by Dll1-mediated Notch activation, whereas apoptosis in B-cells in triggered by constitutively active Notch 1 (Morimura *et al*., 2000). Notch1 was reported to delay S phase entry and reduce proliferation in endothelial cells through downregulation of p21 expression (Noseda *et al*., 2005). In another study, Notch1 was found to modulate the timing of G1-S progression through p21 and p27 downregulation thus promoting their degradation (Sarmento *et al*., 2005). Similarly Notch1 affects the apoptotic inhibitor Bcl-xL and the cell cycle via p21 and p27 (Jang *et al*.,2004 ; Campos *et al*., 2002) showed that a permanent state of cell cycling was induced in post-confluent vascular smooth muscle by activation of the Notch3 receptor. The failure to up-regulate p27 is associated to this lack of cell cycle arrest.

Cao *et al*. (2003) observed that p21 and p27 were both up-regulated when C2C12 was induced to differentiate, with p21 more abundantly expressed in myotubes while p27 was more abundantly expressed in reserve cells. Overexpression of the metalloprotease disintegrin ADAM12 in C2C12 was found to up regulate p27 and leads to cell cycle arrest at the G0 or G1 phase. During differentiation of C2C12 myoblasts, Adam12 and p27 may be committed during the generation of reserve cells as no myogenin or p21 was detected in ADAM12 overexpressing cells (Cao *et al*., 2003).

The induction of p21 is essential to irreversible cell cycle withdrawal that precedes differentiation (Halevy *et al*., 1995; SB Parker *et al*., 1995; Skapek *et al*., 1995; Wang & Walsh 1996; Walsh & Perlman 1997). Furthermore, a correlation was found between the induction of p21 and the appearance of an apoptosis-resistant phenotype during myogenesis. During differentiation, forced expression of p21 inhibits apoptosis (Wang & Walsh 1996). Therefore, p21 and p27 appear to participate in myogenesis. Notch could potentially regulate myogenesis through regulating the cell cycle via both p21 and p27, thereby inducing myoblast terminal differentiation or their returning to a quiescent state.

1.17 The role of Notch signalling during myogenic regulation

The mammalian Notch 1, 2, 3 and 4 receptors share a number of common characteristics (Beatus & Lendahl, 1998). The extra-cellular regions contain tandem repeat epidermal growth factor-like motifs and a family-specific Lin Notch Repeat (LNR) region (Wharton *et al.,* 1985a; Wharton *et al*., 1985b) (**Error! Reference ource not found.**). However the extent to which they perform different functions remains unclear.

Mice made homozygous for loss-of-function alleles of *Notch1* die between embryonic day 10 and 12, and no homozygous embryos can be recovered past embryonic day 12 (Huppert *et al.*, 2000). Mutation of human *Notch1* is associated with early developmental defect in the aortic valve (Garg *et al.,* 2005) and has been found to be an important prognostic marker in T-Cell Acute Lymphoblastic Leukaemia (Zhu *et al.*, 2006). An inactivating mutation in the ankyrin repeats of the *Notch2* gene induces early embryonic lethality by day 10.5 in mice, indicating that Notch2 also plays an essential role in development (Hamada *et al.,* 1999). Mutation of *Notch3* can result in CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy). In contrast to Notch1- and Notch2-null mice, homozygous Notch3-deficient mice develop normally and are fertile. Furthermore, whereas Notch1 and Notch2 are thought to share common functions, the Notch3 intracellular domain acts as a repressor by blocking the ability of the Notch1 intracellular domain to activate through the *Hes-1* and *Hes-5* promoters

(Beatus *et al.,* 1999). These observations support the hypothesis that different Notch receptors may perform different physiological and developmental functions.

In skeletal muscle, regulation of Notch signalling is involved in somitogenesis, muscle development, and the proliferation and cell fate determination of muscle stems cells during regeneration. During each of these processes, the spatial and temporal control of Notch signalling is essential for proper tissue formation (Luo *et al*., 2005). However, the role of these Notch proteins is not yet fully understood.

A number of studies have suggested that Notch signalling may be involved in regulating the satellite cells of adult skeletal muscle during muscle regulation (Conboy & Rando, 2002; Luo *et al.,* 2005; Kitzmann *et al.,* 2006) and also during embryonic development (Schuster-Gossler *et al.,* 2007; Vasyutina *et al*., 2007).

Evidence has been accumulating which demonstrates that Notch signalling could function as a switch that activates satellite cells into the differentiation pathway or maintains them in an immature stem cell-like stage by preventing their differentiation into myofibres (Nofziger *et al.,* 1999; Conboy & Rando, 2002; Conboy *et al.,* 2003; Shubert, 2004) when Notch activity is repressed or maintained respectively (Kitzmann *et al.,* 2006). Activation of Notch 1 appears to promote the proliferation of satellite cells (Numb-/Pax3+/Desmin-/Myf5-/MyoD-), whereas attenuation of Notch 1 by its antagonist Numb causes cells to exit from cell cycle, express myogenic regulatory factors (MRFs) and commit the progenitor cells to amyoblast cell fate (Numb+/Pax3-/Desmin+/My5+) and their myogenic differentiation (Conboy & Rando, 2002).

Adult satellite cells express Notch1, Notch2, and Notch3 (Kuang *et al*., 2007, Conboy *et al*., 2002, Fukada *et al*., 2007, Mourikis *et al*., 2012). Among them, Notch1 is targeted by all four Numb isoforms, Notch2 is variably repressed and Notch3 is not a target of any Numb protein during myogenesis (Beres *et al*., 2011). Furthermore, various structural and functional differences between Notch3 and Notch1/Notch2 have been reported (Bellavia *et al*., 2008).

Although several studies have demonstrated that Notch signalling (particularly Notch1) plays a critical role in muscle regeneration through promoting proliferation and self-renewal of satellite cells, much remains to be determined, especially regarding the role of Notch3 during muscle regeneration.

Mourikis *et al.* (2012) noted Notch activity declined as cells progressed from stem to committed cells, which is linked to simultaneous decreases in Notch receptor expression, primarily in Notch3. This paper demonstrated that disruption of the Notch Canonical pathway leads to severe depletion of satellite cell pools (Mourikis *et al.,* 2012). Although Notch signalling activity had been proposed as being involved in skeletal muscle homeostasis and satellite cell renewal, and the role of Notch1 has been described in this process, less is known about Notch3 precise involvement and how it participates to satellite cell self-renewal in their niche. The hypothesis raised in this thesis, and further developed in the next paragraph, is that Notch1 and Notch3 plays distinct and specific roles in the process of generating quiescent satellite cells located finally in their proper niche. The experimental studies conducted during the completion of this thesis aimed at testing this hypothesis and deciohering which precise role Notch1 and Notch3 have during the general process of generating a regenerating fibre with its satellite cells located under the basal lamina, and which ligand may be proposed as active during this process.

1.18 Aims

The aim of this project is to investigate the role of Notch3 activity in the regulation of adult skeletal muscle stem cell behaviour.

Here, mouse myogenic cell line C2C12 (Yaffe and Saxel, 1977) and satellite cell-derived myoblasts were used to further investigate the role of Notch3. These cultures are maintained as proliferating myoblasts, but can be induced to undergo differentiation into multinucleated myotubes. Not all of the myoblasts differentiate and a proportion exits the cell cycle and remains as mononucleate MyoD-ve reserve cells. Reserve cells express Myf-5 and CD34 (Beauchamp *et al*., 2000) retain the

ability to be reactivated, after which they proliferate and can then be induced to differentiate, leading again to a new mixed population of myotubes and reserve cells (Kitzmann *et al*., 1998; Lindon *et al*., 1998; Yoshida *et al*., 1998). This system is therefore a potentially useful in vitro model of satellite cells and may be used as a model of both myogenic differentiation and satellite cell specification. Using this system the expression of Notch receptors and ligands during myogenesis and their distribution following differentiation will be investigated.

In order to assess the hypothesis rised above, the following specific aims were defined:

1. Which Notch receptor is involved during muscle regeneration and for generating satellite cells in a proper location? To examine the role of Notch receptors and ligands by using stable transfection with full length and constitutively active Notch receptors and ligands; shRNA knockdown, RT-PCR and western blotting to analyse the role (temporal and spatial) of individual notch receptors and ligands in the decision process when skeletal muscle precursor cells are induced to differentiate.

2. Which ligand of Notch receptors is involved in the process of generating satellite cells in their proper niche? Investigate the potential significance of interaction between Notch3 receptor and Notch ligands in re-establishing and maintaining the stem cell pool during regeneration.

3. What is the specific role of Notch3 as compared to that described for Notch1 in the generation of satellite cells and their location in niches between basal lamina and regenerated muscle fibres? Investigate the downstream consequences of Notch3 activation in terms of potential interaction with other Notch components (particularly Notch1) and regulation of Notch target genes, using transcriptional profiling.

Chapter 2: Materials and Methods

2.1 Cell Lines

The murine and human derived cell lines used in this thesis are described below:

2.1.1 C2C12 cells

The skeletal muscle cell line C2C12 was subcloned from the C2 line, which was derived from the thigh muscle of an adult female C3H mouse after a crush injury (Yaffe and Saxel, 1977; Blau *et al*., 1983).

2.1.2 HEK 293T cells

Human embryonic kidney (HEK) 293 cells are an epithelial cell line derived from the embryonic human kidney. 293 cells were generated by the transformation of normal HEK cells with sheared adenovirus type 5 DNA (Graham *et al*., 1977; Louis *et al*., 1997). The HEK293T cell line stably and constitutively expresses the SV40 (simian virus 40) large T antigen.

2.1.3 NIH 3T3 cells

The mouse fibroblast cell line NIH-3T3 was established in the 1960s by George Todaro and Howard Green from disaggregated tissue of an embryonic Swiss mouse (Green H and Todaro GJ, 1967). These cells are highly contact inhibited, and are sensitive to sarcoma virus focus formation and leukaemia virus propagation (Jainchill *et al*., 1969; Andersson *et al*., 1979).

2.2 Cell culture

Tissue culture grade plastic surfaces (Nunc) were used to maintain HEK293T, 3T3 fibroblasts and C2C12. However several additional steps were necessary prior to seeding C2C12 and primary myoblast cell cultures. Initially, Permanox (plastic) 8-well Lab-Tek chamber slides (Scientific Laboratory Services; SLS) were pre-coated with growth factor-reduced Matrigel (BD Biosciences). Matrigel stock solution (1mg/ml) was stored at -20°C, thawed on ice to prevent gelling of the components

and diluted 1:10 in cold DMEM. The plastic was pre-coated with diluted Matrigel, excess liquid was removed and slides were incubated at 37°C in a humidified atmosphere with 5% carbon dioxide (CO_2) for 30 minutes, to facilitate setting of the Matrigel prior to the addition of cells.

2.2.1 Growth conditions

All cell cultures were grown at 37°C in a humidified atmosphere, with 5% CO_2. All media and solutions were pre-warmed to 37°C prior to addition to cell cultures.

2.2.2 Conditions for cell proliferation

2.2.2.1 C2C12 cell proliferation

Proliferating myoblasts were maintained in growth medium, comprising DMEM containing 4mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin.10% and foetal calf serum (FCS; PAA Laboratories),

2.2.2.2 HEK 293T cell proliferation

Cells were maintained in growth medium that comprising DMEM supplemented with 4mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 10% foetal calf serum (FCS; PAA Laboratories),

2.2.2.3 NIH 3T3 cell proliferation

Cells were maintained in growth medium that comprising DMEM supplemented with 4mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 10% foetal calf serum (FCS; PAA Laboratories),

2.2.2.4 Satellite cell-derived myoblasts

Proliferating primary myoblasts were maintained in growth medium, comprising DMEM containing 20% foetal calf serum (FCS; PAA Laboratories), 10% Horse serum, 1% chick embryo extract (CEE; PAA laboratories), 4mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin.

2.2.3 Subculture of cells

2.2.3.1 Subculture of C2C12 cells

Myoblasts were maintained at a low density in growth medium to prevent terminal differentiation. When myogenic cell cultures reached approximately 50% confluence, cells were replated at a lower density. Growth medium was removed and they were briefly washed in a small volume of trypsin/EDTA (TE) solution. After 5 min incubation at 37°, cells began to detach. The detachment of the adherent cells was monitored under the microscope. Once detached, cells were resuspended in DMEM (same volume as TE solution added previously) and centrifuge at 350g for ten minutes. Pellet was resuspended in an appropriate volume of growth medium. A viable cell count was performed and cells were replated at a lower density, around $6.5x10^3$ cells per cm^2.

2.2.3.2 Subculture of HEK 293T cells

At approximately 70% confluence, the HEK 293T cell cultures were replated at a lower density. To remove growth medium without dislodging the cells, a small volume as TE was added and the culture vessel was gently tapped to detach cells from the surface. The detachment of the adherent cells was monitored under the microscope. Once detached, cells were resuspended in DMEM (same volume of TE solution added previously) and centrifuge at 350g for ten minutes. Pellet was resuspended in an appropriate volume of growth medium. A viable cell count was performed and cells were replated at around $5.5x10^3$ cells per cm^2.

2.2.3.3 Subculture of NIH 3T3 cells

Cells were replated at a lower density when NIH 3T3 cell cultures reached approximately 60% confluence, to remove growth medium without dislodging the cells, a small volume of TE was added and the culture vessel was gently tapped to detach cells from the surface. The detachment of the adherent cells was monitored under the microscope. Once detached, cells were resuspended in DMEM (same volume as TE solution added previously) and centrifuge at 350g for ten minutes. Pellet was resuspended in an appropriate volume of growth medium. A viable cell count was performed and cells were replated at around $5.5x10^3$ cells per cm^2.

2.2.4 Induction of differentiation of C2C12 cells

C2C12 cells were plated at a confluency of approximately 60%. Before differentiation was induced, the cultures were propagated in growth medium for 24 hours. Growth medium was removed and the cells washed once the following day with pre-warmed PBS, then switched to pre-warmed differentiation medium (DMEM supplemented with 5% Horse serum, 4mM L-glut, 100U/ml penicillin and 100µg/ml streptomycin). Cultures were propagated for 5 days unless otherwise stated.

2.3 Frozen cell storage

Subcultures of cells were trypsinised and counted. 5x105 cells was resuspended in freezing medium, consisting of growth medium mixed with 10% dimethyl sulphoxide (DMSO), and aliquotted into 1ml cryovials (Nunc). Cryovials were placed in a 'Mr. Frosty' freezing container (Nalgene) containing isopropyl alcohol which provides a -1°C per minute cooling rate, and stored in a -80°C freezer. The next day the cryotubes were transferred to a liquid nitrogen storage tank for long-term storage.

2.4 Determination of cell viability and proliferation

To determine if the treatments and stimuli used affected the cells, cell viability assays were commenced in Notch3 knockdown or overexpress cultures and growth factor treated cells. The Caspase-Glo 3/7 and CellTiter 96® AQUEOUS One Solution Cell Proliferation Assay were used to check the response of the cells to different agents.

2.4.1 CellTiter 96® AQUEOUS One Solution Cell Proliferation Assay

Cell proliferation was determined by the CellTiter 96® AQueous one solution cell proliferation assay (Promega, UK). This is a colorimetric based assay for determining the number of viable cells in proliferation. The assay involves the bio-reduction of

the tetrazolium compound "*3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium*", to a coloured formazan product, facilitated by dehydrogenase enzymes found in metabolically active cells.

$1.5x10^3$ C2C12 cells were seed in a 96-well plate and were propagated in growth medium for 24 hours. Cell number was determined by a colorimetric assay using the CellTiter 96® AQueous One Solution cell proliferation assay (Promega). 20µL of CellTiter 96® AQueous one solution reagent were added into each well of a 96 well-plate containing 100µL of sample. The plate was incubated for 2 hours at 37°C in a humidified atmosphere with 5% CO2. Spectrophotometric analysis of cell proliferation was determined at 490nm using a 96-Microplate reader (Biochrom).

2.4.2 Caspase 3/7 activity assay

To investigate cell survival on shRNA Notch3 knockdown or Notch3 overexpress cultures, 1.5x103 cells of C2C12 myoblasts, shRNA Notch3 knockdown and Notch3/mig transfected C2C12 were plated into a 96-well white walled plate. Caspase-3/7 activity was analysed after 48 hour in differentiation medium (when Notch3 level upregulated).

For the control purpose, blank reaction (differentiation medium only) also included. After 48 hours the cells were subjected to Caspase 3/7 activities measurement with Caspase3/7-Glo assay kit (Promega). Briefly, the plates containing cells were removed from the incubator and allowed to equilibrate to room temperature for 30 minutes. 100µl of Caspase-Glo reagent was added to each well, the content of well was gently mixed with a plate shaker at 300–500 rpm for 30 seconds. The plate was then incubated at room temperature for 2 hours. The luminescence of each sample was measured in a Veritas™ Microplate Luminometer (Promega) with parameters of 1 minute lag time and 0.5 second/well read time. The final values for the activity were calculated by subtracting the background luminescence using the blank values.

2.5 Separation of differentiated myotubes and undifferentiated reserve cells by partial trypsinisation

2.5.1 Solutions used for separation

All media and solutions were sterile when purchased or sterilised using a 0.2µM filter (Sartorius), and they were pre-warmed to 37°C.

DMEM containing 4mM L-glut, 100U/ml penicillin, 100µg/ml streptomycin

PBS

Sterile PBS (GIBCO) was Ca2+/Mg2+-free and at pH7.4.

Trypsin

0.15% trypsin (GIBCO) was prepared in PBS.

Protein extraction buffer

1% SDS (sodium dodecyl sulphate)

1X protease inhibitor cocktail (for general use; Sigma (P2714)

2.5.2 Separation of myotubes and reserve cells for subsequent RNA or protein isolation

C2C12 cells seeded in T75cm^2 tissue culture flasks (Nunc) were induced to differentiate (section 2.2.4). After 5 days, cultures were separated into differentiated myotubes and undifferentiated reserve cells. Differentiation medium was removed; cells were washed with 8ml PBS and then incubated in 0.01% trypsin (Sigma) in PBS approximately one minute. The detached myotubes were retained into suspension and poured into a 25ml universal tube. The adherent cells rinsed again with PBS and then subjected to a series of 3 one-minute incubations in 0.01% trypsin (with intervening PBS washes) to remove residual myoblasts and myocytes. Three washings fractions were released into suspension and poured into a 25ml universal tube. Finally 10ml of differentiation medium were added to the remaining reserve cells in the flasks and incubate in the incubator for about one hour.

2.5.2.1 RNA isolation from myotubes and reserve cells

Myotubes fraction and washing fractions were centrifuged at 350g for ten minutes. The supernatant was aspirated then 1ml TRIzol® reagent (Invitrogen) was added to the pellet. Cells were triturated using an RNase-free pipette tip then each fraction transferred into an individual RNase-free Eppendorf tube. RNA was purified according to the manufacturer's protocol.

Medium was removed from the reserve cells and they were washed twice with differentiation medium to remove dead cells. TRIzol® reagent (1ml) was added to the flask and cells were scraped from the surface using a sterile scraper (Sarstedt), triturated and transferred to an RNase-free Eppendorf tube. RNA was purified according to the manufacturer's protocol.

2.5.2.2 Protein extraction from myotubes and reserve cells

Following trypsinisation, after myotubes and washings fractions had been transferred into a 25ml universal tube, the culture was rinsed twice with 5ml differentiation medium. Myotubes and washing fractions were centrifuged at 350g for ten minutes. Each pellet was resuspended in 20ml DMEM and centrifuged again, to wash the pellet and remove serum. Each fraction pellet was resuspended in 300µl protein extraction buffer and transferred in individual Eppendorf tube and stored at -80°C.

Differentiation medium was added to the dish containing reserve cells and it was returned to the incubator for an hour, and then rinsed twice with 5ml DMEM to remove serum. Protein extraction buffer (300µl) was added to the flask and cells were scraped from the surface using a sterile scraper, triturated, transferred to an Eppendorf tube and stored at -80°C for subsequent processing.

2.6 Mice

C57BL/10 mice were maintained in the Biological Services Unit of the Kings College, University of London. Mice were used for single fibre isolation and were approximately 8-12 weeks old.

2.7 Single fibre preparation

2.7.1 Solutions for single fibre preparation

Collagenase solution

0.2% collagenase type I, DMEM, 4mM L-glut, 100U/ml penicillin and 100µg/ml streptomycin. Four millilitres of collagenase solution was used to digest one muscle.

5% BSA solution

5% bovine serum albumin (BSA) was prepared in PBS and heat-inactivated at 56°C for 30 minutes.

DMEM, 4mM L-glut, 100U/ml penicillin and 100µg/ml streptomycin

2.7.2 Single fibre isolation

Mice were culled by cervical dislocation. Single muscle fibres were isolated from *extensor digitorum longus* (EDL) muscles (one in each hind-limb) as described by Rosenblatt *et al.* (1995). Muscles were digested in collagenase solution for 90 minutes in a shaking waterbath at 35°C. Heat- polished glass Pasteur pipettes were also pre-coated with 5% BSA solution. Petri dishes (Marathon) were pre-coated with 5% BSA solution, to prevent the attachment of myofibres. DMEM containing 4mM L-glut, 100U/ml penicillin and 100µg/ml streptomycin was added to the pre-coated dishes and incubated for 30 minutes at 37°C and 5% CO_2.

After 90 min digestion, individual muscles were transferred into separate dishes containing DMEM with 4mM L-glut, 100U/ml penicillin and 100µg/ml streptomycin and incubated for 30 min at 37°C and 5% CO_2 incubator. Muscles were triturated using the heat-polished glass pipettes to disaggregate into individual fibres. Single fibres were transferred into dishes containing fresh DMEM containing 4mM L-glut, 100U/ml penicillin and 100µg/ml streptomycin and kept at 37°C in a humidified atmosphere with 5% CO_2 then proceed to section 2.6.3.

2.7.3 Isolation and culture of satellite cell derived-myoblasts

Fibres were plated at 10 fibres per well in a Matrigel coated 6-well plate for 4 days. After the 5 day of culture, fibres were removed and cells were trypsinised and plated into Matrigel-coated Lab-tek-8 well-chamber slide at 30% cell density. The transfection of plasmids into primary satellite cell-derived myoblasts was performed 24hrs after the plating.

2.8 Plasmids

Cloning protocols see section 2.12.

2.8.1 eGFP-NIC/puro vector

The eGFP-NIC/puro vector was recloned by Dr Josephine Sales by inserting the eGFP-NIC fusion protein fragment into pMSCVpuro. The original EGFP-NIC plasmid encoded an enhanced green fluorescent protein (eGFP)-NIC fusion protein was a kind gift from Dr K Tezuka of Gifu University, Japan (Tezuka *et al*., 2002).

2.8.2 2pMSCV-IRES-eGFP ('mig')

mig was an acronym for the pMSCV-IRES-eGFP vector, a gift from Dr P S Zammit (Kings College, London, UK). The pMSCVpuro vector was modified to replace the puromycin selection gene with an internal ribosome entry site (IRES) and enhanced green fluorescent protein (eGFP)(Zammit *et al*., 2006b).

2.8.3 Numb/mig

The Numb/mig was reconstructed by Dr Josephine Sales by inserting the 3.3 kb Numb cDNA fragment (which was excised from Numb/pBluescript plasmid) into mig vector. The Numb/pBluescriptwas a kind gift from Dr W Zhong of Yale University, USA.

2.8.4 Notch3/mig

The Notch3/mig was reconstructed by Dr Josephine Sales. The N3IC-HA fragment was excised from the Notch3IC-HA/pcDNA3 and inserted into mig vector. The

Notch3IC-HA/pcDNA3 was originally from Dr I Screpanti (University “La Sapienza”, Rome, Italy).

2.8.5 MigR1

MigR1 was a gift from Dr M Bevan (University of Washington, USA). The eGFP-expressing retroviral vector, MigR1, was constructed by three-way ligation of a 5kb MSCV2.2 vector (Hawley *et al*., 1994), a 0.5kb IRES fragment from pCITE and a 0.8kb eGFP fragment (Pear *et al*., 1998). Target genes were cloned into the MCS of MigR1. This encoded a bi-cistronic transcript for the gene of interest and eGFP, separated by an IRES.

2.8.6 Jagged1

The Jagged1/MigR1vector was provided by Dr M Bevan. The Jagged1/MigR1 plasmid was released from the pMSCVpuro vector using EcoRI restriction enzymes and the Jagged1 insert was re-cloned into the CIAP-treated linearised pCI-neomycin vector.

2.8.7 Dll4

The Dll4 /MigR1 provided by Dr A Freitas (Pasteur Institute, France) was released from the pMSCVpuro vector using XhoI/EcoRI restriction enzymes and the Delta-like4 insert was re-cloned into the CIAP-treated linearised pCI-neomycin vector.

2.8.8 Dll1

The Dll1/MigR1 plasmid, provided by Dr A Freitas (Pasteur Institute, France) was released from the pMSCVpuro vector using Bgl II and Xho I restriction enzymes and the Delta-like1 insert was re-cloned into the CIAP-treated linearised pCI-neomycin vector.

2.8.9 mHey1

mHey1 was provided by Dr Eric Olson (University of Texas, USA)

A BamHI–MfuI partial-digestion fragment of mHey1 was cloned into the pGL3 basic luciferase vector, which lacks a promoter (Osamu Nakagawa *et al*., 2000)

2.8.10 mHey2

mHey2 was provided by Dr Eric Olson (University of Taxes). EcoRI–NruI fragment of mHey2 was cloned into the pGL3 basic luciferase vector, which lacks a promoter (Osamu Nakagawa *et al*., 2000)

2.8.11 mHeyL

mHeyL was also a kind gift from Dr Eric Olson (University of Taxes). A BamHI–BamHI fragment of mHeyL was cloned into the pGL3 basic luciferase vector, which lacks a promoter (Osamu Nakagawa *et al*., 2000)

2.8.12 pHes1-luc

Hes1-luc was a generous gift from Dr Ryoichiro Kageyama (University of Kyoto, Japan). The -460 to +46 promoter fragment of Hes1 gene was cloned into the pGV-B Vector (Nishimura *et al*., 1998).

2.8.13 Hes1

The Hes1 expression vector was also provided by Ryoichiro Kageyama (University of Kyoto, Japan). Full-length eDNA fragments of Hes1 was subcloned into the eukaryotic expression vector containing the CMV promoter, pCI-vector (Nishimura *et al*., 1998).

2.8.14 pHes5-luc

Hes5-luc was also a kind gift from Dr Ryoichiro Kageyama (University of Kyoto, Japan). A SmaI (-800) to SacI(+73) promoter fragment of Hes5 was cloned into pGV-B Vector (Nishimura *et al*., 1998).

2.8.15 Hes5

The Hes5 expression plasmid was also provided by Dr Ryoichiro Kageyama (University of Kyoto, Japan). Full-length eDNA fragments of Hes5 was subcloned into the eukaryotic expression vector containing the CMV promoter pSV-CMV.

2.8.16 pHes7-luc

The Hes7-luc was also provided by Dr Ryoichiro Kageyama (University of Kyoto, Japan). A HincII (-926) to BspEI (+70) promoter fragment of Hes7 was cloned into the pGL3-Basic vector (Nishimura *et al*., 1998).

2.8.17 Hes7

The Hes7 expression plasmid was provided by Dr Ryoichiro Kageyama (University of Kyoto, Japan). Full-length eDNA fragments of Hes7 was subcloned into the eukaryotic expression vector containing the cytomegalovirus promoter, pCI-vector (Hiromi Hirata *et al*., 2004).

2.8.18 pSV-β-Galactosidase

The pSV-β-Galactosidase Control Vector (Promega) is a positive control vector for monitoring transfection efficiencies of mammalian cells. The SV40 early promoter and enhancer drive transcription of the lacZ gene, which encodes the β-galactosidase enzyme. The pSV-β-Galactosidase Control Vector can be transfected individually or co-transfected with DNA of interest.

2.8.19 pmaxFP-Green-N

pmaxFP-Green-N vector is an eukaryotic (mammalian) expression vector encoding green fluorescent protein maxFP-Green obtained from Amaxa Biosystems. The SV40 early promoter provides neomycin resistance gene expression to select stably transfected eukaryotic cells using G418.

2.8.20 shRNA plasmids

Four shRNA plasmids (P1-P4) designed to knockdown expression of mouse Notch3 were obtained from SuperArray Bioscience Corporation, together with a control shRNA plasmid containing an irrelevant scrambled sequence (P5).

The shRNA sequences were as follows:

P1: GGCTGTCTTTCTACTCATCAT

P2: TCAGAGTGCTGCTGACTACTT

P3: TCCCAGCGAGCATCCTTATTT

P4: GTCTGCAAGGACAGAGTCAAT

All shRNA plasmids also encoded GFP and for stable transfection, were linearised with the restriction enzyme *Sca1*. The neomycin resistance plasmid pCINeo was obtained from Promega and was linearised with *DraII.*

2.9 Transfection

2.9.1 Preparation of transfection complexes

For each 10cm dish, the transfection complex consisted of:

A: 5µg DNA into OptiMEM I (final volume of 250ml)

B: 10µl Lipofectamine 2000 (Invitrogen) into 240ml OptiMEM

This was prepared in OptimMEM I medium (GIBCO) to give a final volume of 500µl, according to the description below.

First, the appropriate volume of Optimem I medium was placed in a sterile cryovial. To avoid adversely affecting transfection efficiency, Lipofectamine was added directly to the medium. The tube was flicked gently to mix the contents and incubated at room temperature for five minutes. Plasmid DNA was added, the tube was flicked to mix and then the complex was incubated for a further 20 minutes at room temperature. The dish was gently swirled while 500µl transfection complex was added in a drop-wise manner. The dish was incubated at 37°C in a humidified atmosphere with 5% CO2.

2.9.2 Transient Transfection of HEK 293T cells

HEK 293T cells were seeded at a density of $5.3x10^3$ cells per cm^2 in a 10cm propagated for 24-48 hours in growth medium. Growth medium was removed and transfected with transfection solution describe above. Cells were returned to the incubator for four hours, to allow the DNA to infect the cells and transmit the target gene into them. The supernatant was removed and replaced with fresh growth medium. Transfected cells were incubated for about 48 hours.

2.9.3 Transient transfection of C2C12 cells

C2C12 were seeded at a density of $8x10^2$ C2C12 cells per cm^2 and propagated for 24 hours in growth medium. Growth medium was removed and cells were briefly rinsed with OptiMEM I transfection complexes then added to the cells. Cells were returned to the incubator for four hours, to allow the DNA to transfect the cells and transmit the target gene into them. The supernatant was removed and cells were washed with growth medium. Fresh growth medium was added and the transfected cells were incubated for approximately 48 hours.

2.9.4 Stable Transfection of C2C12 (shRNA Notch3 knockdown)

Cultures of C212 myoblasts were grown to 60-70% confluence in growth medium and co-transfected one of the *ScaI* linearised shRNA plasmids (5μg) together with pClNeo linearised with *DraII* (0.5μg) at a ratio of 10:1, using Lipofectamine 2000 (Invitrogen) diluted in OptiMEM. Growth medium was removed and cells were briefly rinsed with OptiMEM I transfection complexes then added to the cells. Cells were returned to the incubator for four hours, to allow the DNA to transfect the cells and transmit the target gene into them. The supernatant was removed and cells were washed with growth medium. Fresh growth medium was added and the transfected cells were incubated for approximately 48 hours. After 48 hours in growth medium, 600 μg/ml Geneticin (G418; GIBCO) was added to the growth medium and the cells were selected for at least 10 days before. The concentration of G418 was chosen as the minimum required to kill untransfected cells within 6 days and was added to all transfected cultures.

2.9.5 Stable Transfection of NIH 3T3

Cultures of 3T3 fibroblasts were grown to 60-70% confluence in growth medium. Growth medium was removed and. cells were briefly rinsed with OptiMEM I, transfection complexes then added to the cells. Cells were returned to the incubator for four hours, to allow the DNA to transfect the cells and transmit the target gene into them. The supernatant was removed and cells were washed with growth medium. Fresh growth medium was added and the transfected cells were incubated for approximately 48 hours. After 48 hours in growth medium, 600 μg/ml Geneticin (G418; GIBCO) was added to the growth medium and the cells were

selected for at least 10 days before. The concentration of G418 was chosen as the minimum required to kill untransfected cells within 6 days and was added to all transfected cultures.

2.9.6 Transient transfection of satellite cell-derived myoblasts

Cultures of primary myoblasts were grown to 60% confluence in growth medium overnight. Primary cultures growth medium (20% FCS; 1% CEE; 10% HS) was removed and cells were briefly rinsed with OptiMEM I transfection complexes then added to the cells. Cells were returned to the incubator for four hours, to allow the DNA to transfect the cells and transmit the target gene into them. The supernatant was removed and cells were washed with growth medium. Fresh primary culture growth medium (20% FCS; 1% CEE; 10% HS) was added and the transfected cells were incubated for approximately 48 hours.

2.10 Co-culture of ligand-expressing 3T3 cells with reserve cells

Co-culture of Reserve Cells with DSL-Ligand-expressing 3T3 cells full-length murine Dll4 and Dll1 (de la Coste and Freitas, 2006) and Jagged 1 (Lehar *et al*., 2005) cDNAs were each cloned into pCI-neo (Promega) and transfected into 3T3 fibroblasts using Lipofectamine 2000. Transfected cells were selected by using G-418 and expression of the relevant ligand confirmed by RT-PCR and Western blotting (no endogenous expression of Dll1, Dll4 or Jagged 1 was detected).

For co-culture, 3T3 fibroblasts were grown to confluence in LAB-TEK® PermanoxTM 8-well chamber slides (for analysis by immunofluorescent staining) or 10cm-diameter dishes (for Western blot analysis) prior to the addition of reserve cells. C2C12 myoblasts were transfected with pmaxFP-Green-N (Amaxa) using Lipofectamine 2000 and transfectants selected with G- 418. Cultures of GFP+ve myoblasts were allowed to differentiate for 5 days; the reserve cells were isolated and then applied to the fibroblast monolayers (4000 reserve cells/well; 2 x 105 reserve cells/dish) an approximate ratio of 1:3 (reserve cell: fibroblast). Co-cultures were maintained for 24 hour in growth medium prior to analysis. Co-cultures

maintained in chamber slides were pulsed with BrdU (section 2.15) for 1 hour and analysed by immunostaining; co-cultures in dishes were used to produce protein and RNA for Western blotting and RT-PCR.

2.11 Transfections and luciferase assays

C2C12 myoblasts and primary myoblasts were co-transfected one of the reporter plasmid together with β -galactosidase plasmid at a ratio of 10:1 according to the Lipofectamine 2000 protocol (see section 2.9).

For luciferase assays, cells were seeded at a density of $1.5X10^3$cells per well in 96-well white walled plates and transfected with a total of 200 to 300 ng DNA. Cultures were maintained for 1 day in growth medium post-transfection and then switched to differentiation medium for 24 hours prior to the assays. Reporter activity was assayed using the Dual-Light system (Applied Biosystems) and was normalised to β-galactosidase activity to control for transfection efficiency variation among different wells according to the manufacturer's instructions. Luminescent signal was quantified by the Veritas™ Microplate Luminometer (Promega). All reporter assays shown in this study are based on data averaged from at least three independent transfections.

2.12 Cloning

2.12.1 Amplification of plasmids

2.12.1.1 Transformation of the plasmid into bacteria

Plasmids received were either sent in solution in a tube, or dehydrated on a piece of filter paper. Plasmids sent in solution could be used directly for transformation. However, those on paper required rehydration. The paper was first soaked in 1X TE (Tris-EDTA) buffer and incubated at room temperature for one hour. Chemically-competent XL1 Blue *E. coli* cells (Stratagene) were transformed using the solution

containing plasmid DNA as per the manufacturer's instructions. Transformed bacterial cells were grown overnight on LB (Luria Bertani) agar plates containing 100µg/µl ampicillin appropriate for selection of colonies in the specific plasmid.

2.12.1.2 Amplification and purification of the plasmid

A cooled sterile inoculating loop (SLS) was used to scrape colonies from the plate and transfer them into a sterile flask. The sterile flask was filled with 200ml LB broth containing the appropriate antibiotic, which has the same final concentration as used in the LB agar. Colonies were then grown overnight at 37°C in a shaking incubator. Bacteria were harvested and the QIAGEN Plasmid Purification Maxi Kit was used to purify the plasmid as per the manufacturer's protocol.

2.12.2 Restriction digestion of plasmids

Restriction digestion of plasmids to isolate a specific the DNA fragment or to linearise a vector in preparation for ligation was carried out using the restriction endonucleases specified in section 2.7.1. Between 2-5µg plasmid DNA was digested in a 50µl reaction, using restriction endonucleases with 1X reaction buffer, 3mM spermidine hydrochloride and 1X BSA if required according to the manufacturer's instructions.

2.12.3 Filling in 5' overhangs to form blunt ends

Isolated DNA fragments were blunt-ended prior to ligation into the appropriate vector was specified in section 2.7.1. Restriction digestions (50µl reactions) were treated with Klenow, a proteolytic product of *E. coli* DNA polymerase I, which fills in 5' overhangs to form blunt ends (Sambrook *et al*., 1989). One micro litre of Klenow and 1.65µl 1mM dNTPs (deoxynucleotide triphosphates; Invitrogen) was added. The reaction buffer was already incorporated in the digestion reaction and therefore no additional buffer was required. This reaction was incubated for a duration of 15 minutes at room temperature and the blunt-ended DNA was subsequently purified.

2.12.4 CIAP-treatment of linearised vectors

Calf intestinal alkaline phosphatase (CIAP; Promega) catalyses the hydrolysis of 5' phosphate groups in DNA and RNA. Treatment with CIAP is required to prevent

recircularisation and religation by removing phosphate groups from both 5' termini in all linearised vectors whether blunt- or sticky-ended.

2.12.4.1 CIAP-treatment of linearised vectors with blunt-ends

The restriction digestion reaction (50µl), containing linearised plasmid DNA, was mixed with 1µl CIAP, 6µl 10X CIAP reaction buffer (Promega) and 2µl dH2O. The reaction was incubated at 37°C for 15 minutes and then at 56°C for a further 15 minutes. Another 1µl CIAP was added to the reaction and the above incubation protocol was repeated. The higher temperature potentiates blunt end-accessibility (Perbal, 1988). Subsequently the CIAP-treated linearised DNA was purified.

2.12.4.2 CIAP-treatment of linearised vectors with sticky-ends

The restriction digestion reaction (50µl) was mixed with 1µl CIAP, 6µl 10X CIAP reaction buffer and 2µl dH2O, and incubated at 37°C for 30 minutes. Another 1µl CIAP was added to the reaction and the incubation process was repeated. CIAP-treated linearised DNA was then purified (section 2.8.5).

2.12.5 Gel purification

Gel purification is needed to remove enzymes in Klenow-treated blunt-ended DNA fragments, restriction-digested DNA fragments with sticky ends and CIAP-treated linearised DNA. An agarose gel (0.75%) was prepared and samples were run on the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions.

2.12.6 Ligation

T4 DNA ligase is the most commonly used DNA ligase which catalyses the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA (Sambrook *et al*., 1989). Therefore, this enzyme was used to join purified DNA fragments of interest (either 'sticky' or blunt ends). Purified samples were run on an agarose gel (0.75%) beside a low mass DNA ladder (Invitrogen), to approximately quantify the DNA. In general, the following reagents materials were used: Ten micro litre ligation reactions were prepared, containing 1µl 10X T4 DNA ligase reaction buffer, 1µl recombinant T4 DNA ligase (400 units of

enzyme) and variable quantities of linearised vector DNA, insert DNA and dH2O. The ligation mix was incubated overnight for approximately 16 hours, at 16°C, and then stored at -20°C until it was required for transformation into bacteria.

2.12.7 Transformation of ligated plasmids

According to the manufacturer's instructions, Plasmid DNA from ligation reactions (5µl) was used to transform XL1 Blue subcloning grade chemically-competent *E. coli* cells (Stratagene), Transformed bacterial cells were grown overnight on LB agar plates containing ampicillin, to select for colonies containing the plasmid of interest.

2.12.8 Amplification of correctly ligated plasmids

2.12.8.1 Amplification of plasmid DNA from individual colonies

Twelve colonies were selected individually using sterile tips and transferred into sterile tubes (Fisher), each filled with 3ml LB containing the appropriate antibiotic at the same final concentration as used in the LB agar. Colonies were grown overnight at 37°C in a shaking incubator. Bacteria were harvested and the QIAGEN Plasmid Purification Maxi Kit was used to purify the plasmid. Approximately 1.5ml bacterial cell suspension was transferred into an Eppendorf tube and centrifuged at 14,000g for 30 seconds at 4°C to pellet the bacteria. The supernatant was discarded followed by adding 100µl P1 buffer to the pellet and cells were thoroughly resuspended by vortexing. A 100µl P2 buffer was added to lyse cells. The samples were mixed by several inversions then incubated at room temperature for five minutes, which ensured that nuclei did not lyse. To neutralise the alkaline P2 buffer, 100µl cold P3 buffer (stored at 4°C) was added and mixed by several inversions. The tube was centrifuged at 14,000g for five minutes at 4°C. The supernatant which contained plasmid DNA was removed. It was then transferred into a new Eppendorf tube with 750µl cold absolute ethanol (stored at -20°C) to remove salt from the DNA. This was centrifuged at 14,000g for five minutes at 4°C, the supernatant was discarded and the pellet was washed with 1ml cold 70% ethanol (stored at -20°C). The pellet, containing purified plasmid DNA, was air-dried and resuspended in 30µl 1X TE buffer.

2.12.8.2 Confirmation of plasmid DNA sequences and plasmid amplification

To determine whether the vector contained the inserted DNA and if the inserted fragment was the correct size and orientation. Purified plasmid DNA isolated from individual transformed bacterial colonies was analysed by several diagnostic restriction digestion reactions. A colony containing correctly-ligated plasmid DNA was then chosen for amplification. 200µl of this bacterial cell suspension was transferred into a sterile flask filled with 200ml LB broth containing the appropriate antibiotic. Bacteria were grown overnight at 37°C in a shaking incubator, harvested and the QIAGEN Plasmid Purification Maxi Kit was used to purify the plasmid as per the manufacturer's protocol.

2.13 Fixation of C2C12 cell cultures or primary cultures

4% Paraformaldehyde (PFA) was prepared in PBS and aliquots were stored at -20°C. PFA was thawed and pre-warmed at 37°C prior to use.

2.13.1 Fixation of C2C12 cell cultures

Medium was removed from cell cultures in 8-well chamber slides. 200µl/well of PFA was added and cultures were incubated at room temperature for 5-10 minutes. PFA was removed and cultures were washed twice with PBS. PBS was added and cultures were either immunostained immediately, or stored at 4°C with the slides covered in Parafilm to prevent evaporation.

2.13.2 Fixation of primary cultures

Primary cultures were cultured in 8-well chamber slides. Approximately half of the medium (100µl) was removed from each well to fix the cultures, 200µl PFA was added and slides were incubated at room temperature for ten minutes. PFA was removed and cultures were rinsed twice with PBS. PBS was added and cultures were immunostained immediately, or slides were covered with Parafilm and prior to storage at 4°C.

2.14 Immunostaining C2C12 cell cultures or primary cultures

2.14.1 Solutions used for immunostaining

0.1% Triton

0.1% Triton X-100 was prepared in PBS.

5% HS

5% horse serum (HS; GIBCO) was diluted in PBS.

0.025% PBS-T

0.025% Tween-20 was diluted in PBS.

2.14.2 Immunostaining of C2C12 cell cultures or primary cultures

Fixed cells were permeabilised in 0.1% Triton for 10 min. Triton was removed and cultures were rinsed twice with PBS. Cells were blocked in 20% serum (DAKO) diluted with PBS for 30 minutes, to prevent non-specific antibody-binding. The serum used for blocking was from the same species that the secondary or tertiary antibody was raised in. Primary antibodies (see Table 2.1) were diluted in 5% HS and incubate overnight at 4°C. Slides were covered with Parafilm to prevent evaporation. Cultures were rinsed three times with 0.025% PBS-T after primary antibodies were removed. Fixed cell cultures were incubated with secondary antibodies (Table 2.2) diluted in 5% HS for 60 min at room temperature. Secondary antibodies were removed and cultures were washed three times with 0.025% PBS-T. Excess liquid was tipped off slides, cultures were mounted in DakoCytomation fluorescent mounting medium (DAKO) containing 100ng/ml 4',6-diamidino-2-phenylindole (DAPI) and a glass cover slip (SLS) was added.

2.15 BrdU pulsing, fixation and immunostaining of C2C12 cells

Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) is a synthetic nucleoside, which is an analogue of thymidine and commonly used to identify cell proliferation

(Zhang and McLennan, 1999). A BrdU-specific antibody was used to detect incorporated BrdU and hence indicate cells that were actively replicating their DNA. The DNA was denatured using acid to allow the antibody to bind effectively.

2.15.1 Solutions BrdU

The 4mM stock solution was stored in aliquots at -20°C and thawed at room temperature for use.

DMEM

DMEM was pre-warmed to 37°C prior to use.

PFA

4% PFA was prepared in PBS and aliquots were stored at -20^{o}C. PFA was thawed and pre-warmed at 37°C prior to use.

PBS

PBS was pre-warmed to 37°C before use.

70% ethanol

70% ethanol (Fisher)

0.5% BSA

0.5% BSA was prepared in PBS.

2M hydrochloric acid

Concentrated hydrochloric acid (BDH) was diluted using PBS.

0.1M borate buffer

0.1M sodium borate required heating to dissolve in dH2O. The solution was altered to pH8.5 using hydrochloric acid.

5% HS

5% HS was diluted in PBS.

0.025% PBS-T

0.025% Tween-20 was diluted in PBS.

0.1% Triton

0.1% Triton X-100 was diluted in PBS.

2.15.2 BrdU-pulsing and fixation of C2C12 cell cultures or Co-cultures

BrdU was added to cell cultures in 8-well chamber slides, at a final concentration of 40µM in the existing medium. Cultures were returned to the incubator for two hours to allow BrdU incorporation. The medium was later removed from the each well and cells were washed with PBS. Cells were then fixed with 4% PFA for 10 minutes and rinsed twice with PBS. PBS was added and cultures were immunostained immediately, or Parafilm® was used to cover the slides prior to storage at 4°C.

2.15.3 Immunodetection of BrdU incorporated into C2C12 cell cultures

Prior procced to the BrdU-detection protocol, cultures were co-stained to identify expression of another protein in addition to BrdU if they were first immunostained for that protein. Cultures were firstly incubated in 70% ethanol for 10 min then washed with 0.5% BSA and incubated with 2M hydrochloric acid for 15 min. Followed by 2 min wash with in 0.1M borate buffer. Cells were then permeabilise by Triton for 10 min and followed by two rinses with PBS. A 20% goat serum (DAKO) diluted in PBS blocking solution was added for 30 minutes. Cultures were incubated (covered in Parafilm® to prevent evaporation) overnight at 4°C with monoclonal rat anti-BrdU antibody (Abcam) diluted 1:500 in 5% HS. The primary antibody was removed and cultures were rinsed three times with 0.025% PBS-T. Alexa Fluor 488- or 594 conjugated goat anti-rat secondary antibody (Molecular Probes, Invitrogen) diluted 1:200 in 5% HS, was added for one hour at room temperature (at dark). Antibodies were removed and cultures were washed three times with 0.025% PBS-T. Cultures were mounted and observed as described in section 2.14.2.

2.16 Lysenin treatment of C2C12 cell cultures

Lysenin, a protein isolated from the earthworm *Eisenia foetida* (Sekizawa *et al*., 1997) can be used to detect Sphingomyelin. Sphingomyelin is an integral lipid component of mammalian cell membranes. Reserve cells express high levels of sphingomyelin in their plasma membranes to bind lysenin, but not proliferating myoblasts and differentiated myotubes (Nagata *et al*., 2006).

2.16.1 Solutions

2% BSA

2% BSA was diluted in PBS.

Lysenin

1µg/ml lysenin was prepared in 2% BSA.

2.16.2 Protocol for lysenin treatment of cell cultures

Fixed cell cultures were blocked with 2% BSA for 30-60 minutes, then incubate with lysenin for one hour. Cultures were then co-immunostained for lysenin and another antibody, as described in section 2.14.2.

Antibody **Supplier (Catalogue number)**	**Application**	**Dilution**
monoclonal rat anti-BrdU antibody (BU1/75 (ICR1)) Abcam (ab6326)	IF	1/500
monoclonal mouse anti-MyoD antibody (5.8A) Dako (M3512)	IF	1/100
monoclonal mouse anti-myosin heavy chain (MyHC) antibody Developmental Studies Hybridoma Bank (DSHB) (MF20)	IF WB	1/1500 1/3000
monoclonal mouse anti-Pax7 antibody DSHB (PAX7)	IF	1/100
monoclonal mouse anti-®-tubulin antibody (DM 1A) Sigma (T9026)	WB	1/5000
monoclonal mouse anti-cyclinA antibody (CY- A1) Sigma (C4710)	WB	1/2500
polyclonal rabbit anti-lysenin antibody (020- 521031) Peptide Institute, Inc. (14802-v)	IF	1/2000
polyclonal rabbit anti-GFP antibody Molecular Probes, Invitrogen (A11122)	IF	1/500
monoclonal mouse anti-GFP antibody Abcam (ab1218)	IF	1/500

polyclonal rabbit anti-p27 (C19) antibody Santa Cruz Biotechnology, Inc. (sc-528)	IF	1/250
polyclonal rabbit anti-p21 (M19) antibody Santa Cruz Biotechnology, Inc. (sc-471)	IF	1/250
monoclonal rat anti-Notch1 antibody DSHB (bTAN20)	IF	1/100
polyclonal goat anti-mouse Notch1 (M20) antibody Santa Cruz Biotechnology, Inc. (sc-6015)	WB	1/200
polyclonal rabbit anti-activated mouse Notch1 antibody Abcam (Ab8925)	WB	1/200
polyclonal goat anti-mouse Notch2 (M20) antibody Santa Cruz Biotechnology, Inc. (sc-7423)	WB	1/200
polyclonal goat anti-mouse Notch3 (M20) antibody Santa Cruz Biotechnology, Inc. (sc-7424)	WB	1/200
polyclonal goat anti-Jagged1 (C20) antibody Santa Cruz Biotechnology, Inc. (sc-6011)	WB	1/200
polyclonal goat anti-Dll4 (C20) antibody Santa Cruz Biotechnology, Inc. (sc-18640)	WB	1/200

Table 2.1 Primary antibodies used for immunofluorescence (IF) and Western blotting (WB)

Antibody **Supplier (Catalogue number)**	**Application**	**Dilution**
Biotinylated rabbit anti-rat antibody Dako (E0468)	IF WB	1/500 1/1000
Biotinylated rabbit anti-goat antibody Vector Laboratories, Inc. (BA-5000)	WB	1/1000
Streptavidin-HRP Invitrogen (43-4323)	WB	1/4000
HRP-conjugated goat anti-mouse antibody Dako (P0447)	WB	1/3000
HRP-conjugated swine anti-rabbit antibody Dako (P0217)	WB	1/3000
Alexa Fluor® 488-conjugated goat anti-rat antibody Molecular Probes, Invitrogen (A11006)	IF	1/400
Alexa Fluor® 594-conjugated goat anti-rat antibody Molecular Probes, Invitrogen (A11007)	IF	1/400
Alexa Fluor® 594-conjugated goat anti-mouse antibody Molecular Probes, Invitrogen (A11005)	IF	1/400
Alexa Fluor ®488-conjugated goat anti-mouse antibody Molecular Probes, Invitrogen (A11001)	IF	1/400
Alexa Fluor® 488-conjugated goat anti-rabbit antibody Molecular Probes, Invitrogen (A11008)	IF	1/400

Alexa Fluor® 594-conjugated goat anti-rabbit antibody Molecular Probes, Invitrogen (A11012)	IF	1/400
Alexa Fluor® 594-conjugated donkey anti-mouse antibody Molecular Probes, Invitrogen (A21203)	IF	1/400
Alexa Fluor® 488-conjugated donkey anti-mouse antibody Molecular Probes, Invitrogen (A21202)	IF	1/400
Alexa Fluor 488-conjugated donkey anti-goat antibody Molecular Probes, Invitrogen (A11055)	IF	1/400
Alexa Fluor® 594-conjugated donkey anti-goat antibody Molecular Probes, Invitrogen (A11058)	IF	1/400
Alexa Fluor® 594-conjugated donkey anti-rabbit antibody Molecular Probes, Invitrogen (A21207)	IF	1/400
Alexa Fluor® 488-conjugated donkey anti-rabbit antibody Molecular Probes, Invitrogen (A21206)	IF	1/400

Table 2.2 Secondary and tertiary antibodies used for immunofluorescence (IF) and Western blotting (WB)

2.17 RNA isolation from C2C12 cell cultures

C2C12 cell cultures were grown in either T75cm^2 tissue culture flasks or 10cm^2 petri dishes in preparation for RNA isolation. RNA was extracted from proliferating myoblasts, differentiated cultures and separated myotube and reserve cell samples (sections 2.2 and 2.5) for RT-PCR and Quantitative RT-PCR analysis. One millilitre of TRIzol® reagent was added to each T75cm^2 flask 10cm^2 Petri dishes and the cells were scraped, triturated and transferred to an RNase-free Eppendorf tube. Samples were stored at -80°C. Total RNA was isolated using TRIzol® reagent (Invitrogen) based on manufacturer's instructions.

2.18 RT-PCR (reverse transcription-PCR) analysis of C2C12 cell culture transcripts

2.18.1 One step RT-PCR (Qiagen)

Total RNA (from myoblasts, unseparated differentiated cultures, myotubes or reserve cells; section 2.2.2) were used to analysed RT-PCRs using a One-Step kit (Qiagen) with appropriate primers (

Table 2.3), according to the manufacturer's protocol.

2.18.2 Quantitative RT- PCR

Two micrograms of total RNA (from myoblasts, unseparated differentiated cultures, myotubes or reserve cells; section 2.12.3) were reverse transcribed in 40µl reactions, using Superscript reverse transcriptase with an oligo dT12-18 primer (both Invitrogen), according to the manufacturer's instructions.

Real-time RT-PCR was performed by means of the Rotor-Gene 6000 Real-time PCR system (Corbett Life Science). In each reaction, one micro litre of the resultant cDNA was diluted 20-fold with nuclease-free water and amplified using the Quantace SYBR Green QPCR Master mix, 300nM of each primer according to the protocol provided by the manufacturer.

After initial denaturation for 10 minutes at 95°C, thermal cycling was performed for 40 cycles of with steps of 95°C for 15 seconds, and annealing/extension at 60°C for 1 min with the fluorescence being read at the end of each cycle.

The analysis was performed with rotor-gene 6000 series software, version 1.7.4. The obtained values were within the linear range of a standard curve and were normalised to yield the same amount of glyceraldehyde phosphate dehydrogenase (GAPDH) messenger RNA (mRNA).

2.18.3 Sample preparation and running the gel

A 1.5% agarose gel was prepared in a large tank. RT-PCR products were combined with 10X DNA loading buffer, so that a final buffer concentration of 1X was obtained, and loaded onto the gel with a molecular weight marker. At this point an image of the gel was taken in order to determine the location of the molecular weight marker bands, which were used to confirm the size of the target transcripts.

Gene	Forward and Reverse Primer Sequences	Annealing Temp. (°C)	Product Size (bp)	Cycle #
HPRT[1]	5' GCTGGTGAAAAGGACCTCTC 3' 5' CACAGGACTAGAACACCTGC 3'	55	249	20[a]
GAPDH	5' CCTCGTCCCGTAGACAAAAT 3' 5' AATCTCCACTTTGCCACTGC 3'	55	102	40[b]
CD34 [2]	5' CCAGGGTATCTGCCTGGAAC 3' 5' GCTGGAGTTTGCTGGGAAGT 3'	55	218	20[a] 40[b]
MyoD [2]	5' CGGCGGCAGAATGGCTACGA 3' 5' GAGGGGCGGCGTCGGGAGAC 3'	63	253	20[a] 40[b]
Myogenin [1]	5' TTGCTCAGCTCCCTCAACCA 3' 5' TGGGCTGGGTGTTAGCCTTA 3'	59	445	28[a]
Notch1[1]	5' TGGACGCCGCTGTGAGTCA 3' 5' TGGGCCCGAGATGCATGTA 3'	59	202	20[a] 40[b]
Notch2[1]	5' GTGCCTGAATGCTGGAACG 3' 5' CTGCCGCCTTACAGGACAC 3'	57	227	20[a] 40[b]
Notch3[1]	5' CCTGGATGCTGGGGCGGACAC 3' 5' CGGCATGGCTGGCGATGAGCT 3'	63	218	20[a] 40[b]
Delta-like1[1]	5' ACCTACGGCAGTGCTGTCAC 3' 5' ATGGAGGGCTTCAATGATCA 3'	57	153	25[a], 40[b]
Delta-like4[1]	5' GCTCAAAAACACAAACCAGA 3' 5' CAAATGGCTGATATTCGACA 3'	54	225	25[a] 40[b]
Jagged1[1]	5' CGAGAGCAACCCCTGTAAAA 3' 5' CTTCGTCACACTGGCTGTCA 3'	55	236	20[a] 40[b]
Jagged2[1]	5' AGCCTGACCAATACCTCTG 3' 5' GCACACTCATCAATGTCGA 3'	56	190	20[a] 40[b]
Numb[1]	5' GCGTAAACAGAAGCGGGAGA 3' 5' GAGAGGTGGGAGAGGATGGG 3'	56	233	20[a] 40[b]
Hes1	5' GGTGCTGATAACAGCGGAAT 3' 5' TGGAATCCTTCACGCAAAA 3'	59	145	40[b]
Hes2	5' TGGAGTCCAGAGCAAGTTGA 3' 5' AGCAGCGGCTTTAGGTTCTT 3'	58	106	40[b]
Hes5	5' CTCGCTAATCGCCTCCAG 3' 5' CAGCTTCAGCTGCTCTATGC 3'	58	113	40[b]

Hes7	5’ CGGAGGAGCAATGGTCAC 3’ 5’ ATCTCCGCTTTCTCCAGCTT 3’	59	110	40[b]
Hey1	5’ GGGAAAGGGATGGTTGAGTT 3’ 5’ GGGTGAGCTCTCTTCATGGT 3’	58	114	40[b]
Hey2	5’ GTTCCGCTAGGCGACAGTAG 3’ 5’ CCACGTCGATGGTCTCGT 3’	58	100	40[b]
HeyL	5’ CATCGATGTGGGTCAAGAGA 3’ 5’ CAATCGTCGCAATTCAGAAA 3’	58	120	40[b]

Table 2.3 PCR primer sets

All PCR primers were obtained from Sigma (Genosys) and were designed using Primer3 Input (version 0.4.0), except: 1 (Dr Josephine Sales), 2 (Beauchamp *et al*., 2000). [a] One step RT-PCR, [b] Quantitative RT-PCR.

2.18.4 Analysis of RNA concentration, integrity and purity

Total RNA was isolated from proliferating myoblasts, differentiated myotubes and undifferentiated reserve cells, using TRIzol® reagent (Invitrogen), as described in section 2.12.3. In order to confirm the integrity of the RNA, one micro litre of each sample was run on a 1.4% agarose gel. The RNA was then quantified using a spectrophotometer and the optical density of the samples was recorded at wavelengths of "230, "260 and "280. The "260 reading was used to determine the total RNA concentration, which was greater than 11ng/µl per sample, meeting the SuperArray instruction requirements. The "260:"280 ratio was greater than two showing that there was no protein contamination. The "260: "230 ratio was calculated to be greater than 1.7, which confirms that there was no salt or guanidine contamination.

2.19 Analysis of C2C12 cell culture proteins

2.19.1 Solutions

10% SDS was prepared in sterile dH_2O and diluted to make other solutions.

Protein extraction buffer

Described in section 2.4.1.

RIPA buffer

150mM NaCl, 1% Igepal (CA-630); 0.5% deoxycholate (DOC); 0.1% SDS 50mM Tris,; 1X protease inhibitor cocktail (for general use; Sigma). This was prepared in sterile dH2O and kept on ice.

Resolving gel

25% of 1.5M Tris-HCl (pH8.8), 0.1% SDS and 10% acrylamide/bis acrylamide. This stock solution was prepared in sterile dH2O and stored at 4^{o}C for up to one month. 0.05% ammonium persulphate (APS) and 0.05% TEMED (N, N, N', N'-tetramethylethylenediamine) were added immediately prior to pouring the gel.

Stacking gel

25% of 0.5M TrisHCl (pH6.8), 1% SDS, 4% acrylamide/bis acrylamide, 0.05% of APS and 0.1% of TEMED.

Water-saturated butanol

One millilitre of dH2O was added to 5ml n-butanol (BDH) and the solution was shaken vigorously.

5X sample buffer

12.5% of 0.5M TrisHCl (pH6.8), 10% glycerol; 2% SDS; 5% β-mercaptoethanol and 0.05% bromophenol blue. This was prepared in sterile dH2O.

5X running buffer

15g Trizma Base 72g glycine 5g SDS This was prepared to give a final volume of one litre in dH2O, kept as a stock solution at room temperature, and diluted to 1X with dH2O prior to use.

Transfer buffer

3g Trizma Base and 14.4g of glycine. This was freshly prepared with dH2O to a volume of 800ml, 200ml methanol was added, and it was pre-cooled to 4°C.

10X TBS (Tris-buffered saline)

25ml of 1M TrisHCl (pH7.5); 8g NaCl ;0.2g KCl. This was prepared to give a final volume of 100ml in dH2O, kept as a stock solution at room temperature and diluted as appropriate for use.

TBS-T

0.2% Tween-20

1X TBS

This was prepared in dH2O.

Blocking solution

5% Marvel milk (Cadbury)

This was prepared in TBS-T.

Stripping buffer

100mM β-mercaptoethanol

2% SDS

62.5mM of TrisHCl

This was freshly prepared in dH2O prior to use.

2.19.2 Protein extraction and quantification

Protein was extracted from proliferating myoblasts (cultured for 24 hours), unseparated differentiated cultures, myotubes and reserve cells (sections 2.2 and 2.5). Cultures or samples were rinsed with DMEM and between 300µl and 1ml of ice- cold protein extraction buffer or RIPA buffer was added. Cells were scraped with a sterile scraper (Sarstedt) if required, triturated, transferred to an Eppendorf tube and stored at -80°C so that they could be processed when needed. Samples were thawed on ice and Bradford reagent (Bio-Rad) was used to determine the protein concentration as per the manufacturer's instructions. BSA standards were used to create a curve to quantify protein.

2.19.3 Western blot analysis of protein samples

2.19.3.1 SDS-polyacrylamide gel electrophoresis

For protein separation, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Bio-Rad Mini-PROTEAN® Cell Kit as per the manufacturer's instructions. Spacer and short glass plates were cleaned using sterile dH2O and methanol and clamped together in a casting frame so there was a 0.75mm space between them. The gel cassette assembly was secured using a casting stand before gel casting. Resolving gel was poured into the space between the plates until it was 75%-full and a layer of water-saturated butanol was poured on the top. It took the resolving gel roughly 45 minutes to polymerize. The butanol was subsequently removed and the gel surface was rinsed with dH2O. Stacking gel was poured above the resolving gel until the cassette was full and a comb was placed on top. The gel polymerised after approximately 30 minutes. Then the combs were removed.

Samples were prepared in dH_2O so that they contained an equal quantity of protein, between 10 and 25µg, and their final volumes were equal. A final 1X concentration was obtained by adding 5X sample buffer. When investigating intracellular proteins, the samples were denatured by applying heat (100^oC) for a duration of three minutes.

Two gel cassettes were inserted into the electrode assembly, with a central chamber between them, and then placed in a mini-tank. The central chamber was filled with 1X running buffer and a smaller quantity was added to the external chambers. Samples were loaded into the wells beside molecular weight markers and electrophoresed at 90 volts until the markers reached the bottom of the resolving gel.

However, separation of NotchICD protein was achieved by using NuPage 4-12% gradient Bis-Tris pre-cast polyacrylamide gels (Invitrogen) in a XCELL II Mini gel apparatus (Novex) with 1 x Nupage MES SDS running buffer (Invitrogen) according to manufacturer's instructions. Samples were mixed with 4x NuPage Sample Buffer (Invitrogen) containing β-mercaptoethanol before boiling for five minutes. The

Novex® Sharp Pre-Stained Protein Standard (Invitrogen) was used as a molecular weight marker.

2.19.3.2 Western blotting and hybridization

Gels were blotted onto nitrocellulose membranes (Hybond ECL, GE LifeSciences) after electrophoresis by using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) or XCell II Blot Module (Invitrogen), according to the manufacturer's instructions. Transfer took place at 120mA for approximately 2 hours. The pre-cast NuPage gels were transfered at 20 V constant voltage for 40 minutes.

Membranes were rinsed in dH2O and air-dried. An orbital shaker was used during all washing, blocking and hybridisation stages. Each wash cycle took ten minutes. Membranes were washed three times in TBS-T and then incubated in blocking solution for one hour to prevent non-specific antibody-binding. Primary antibodies (

Table 2.1) were diluted in blocking solution, applied to the membranes and incubated at 4^{o}C overnight. Membranes were washed three times with blocking solution. Secondary antibodies (

Table 2.2) were diluted in blocking solution and applied to membranes for one hour at room temperature, followed by tertiary antibodies if required (

Table 2.2). Membranes were washed once with diluted 250mM NaCl in TBS-T, twice with TBS-T and then once more for at least an hour with TBS-T. The ECL kit (Pierce) was used to detect immunoblotted proteins as per the manufacturer's protocol and BIOMAX film (Kodak).

2.19.3.3 Stripping Western blots

To detect expression of a second protein, membranes could be stripped once and re-probed. The membrane was submerged in stripping buffer and incubated at 50^{o}C for 30 minutes, with occasional agitation. It was washed twice in TBS-T and then exposed using the ECL Plus# kit with Hyperfilm to confirm that antibodies were

removed. The membrane was incubated for one hour in blocking solution and immunodetection continued as described in section 2.19.3.2.

2.20 Statistical analysis

Data was analysed using SPSS 17.0 Integrated Student Version and graphs were drawn using Microsoft Excel (version 14.0.0). Independent-samples t-tests were carried out to determine statistical significance.

2.21 Solution recipes

10X TBE (Tris-borate/EDTA) buffer

89mM Tris

89mM boric acid

3mM EDTA

This was prepared in dH2O, adjusted to pH8.3 and diluted with dH2O to make 1X TBE buffer.

0.2M sodium phosphate buffer

0.12M Na2HPO4

0.8M NaH2PO4

This was prepared in dH2O, adjusted to pH7 and treated to make it RNase-free. It was diluted as appropriate to produce other solutions.

10mM sodium phosphate buffer

0.2M sodium phosphate buffer was diluted as appropriate with RNase-free dH2O.

20X SSC buffer

3M NaCl 0.3M Na3citrate.2H2O This was prepared in dH2O, adjusted to pH7 and treated to make it RNase-free. This was diluted as appropriate to make other solutions.

10% SDS

10% SDS was diluted in sterile dH2O and was a constituent of several buffers.

2% SDS

10% SDS was diluted in RNase-free dH2O so it was at a final concentration of 2% .

RNA loading buffer

50% glycerol

5% 20X SSC buffer

0.1% bromophenol blue

This was prepared in RNase-free dH2O and autoclaved.

10X DNA loading buffer

50% glycerol

0.25% bromophenol blue

This was prepared in dH2O and sterilised using a 0.2µM filter (Sartorius).

10X TE (Tris-EDTA) buffer

100mM Tris

10mM EDTA

This was prepared in dH2O, adjusted to pH7.6 and autoclaved. It was diluted in sterile dH2O to make 1X TE buffer.

1M acetic acid

17.4M glacial acetic acid was diluted to the appropriate concentration using sterile dH2O.

Methylene blue solution

0.2% methylene blue

0.4M sodium acetate 0.4M acetic acid

This was prepared in sterile dH2O.

Stripping buffer

5% SDS was prepared by diluting 10% SDS in sterile dH2O.

DEPC (diethyl pyrocarbonate)-treated water

1 ml DEPC

1 litre dH2O water

Mixed overnight then autoclaved.

Agarose

0.5 g UltraPure agarose (Invitrogen)

50 ml 1 x TBE buffer Boiled until agarose is dissolved.

1 µl 10 mg/ml ethidium bromide per 50 ml Agarose.

Matrigel

1ml Matrigel

9ml DMEM

Bottom of plate is covered, excess is removed. Plate kept at 37^{o}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 dH2O

Protein loading buffer

3 parts 4 x NuPage sample buffer

1 part b-mercaptoethanol

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

1M dithiothreitol (DTT)

1.542 g dithiothreitol

Distilled water to 10 ml

Disburse into 500 ml aliquots and store at -20^{o}C

5 M NaCl Stock

292.2 g NaCl

Distilled water to 1 litre

Store at room temperature

Chapter 3: Expression of Notch receptors and ligands during differentiation of C2C12 cultures

3.1 Introduction

C2C12 cell culture and differentiation

The aim of the experiments in this chapter was to investigate expression of Notch signalling pathway components during myogenesis in C2C12 cell line. Phase-contrast images of the stages analysed during myogenesis are shown in Figure 3.1 C2C12 cultures were maintained as proliferating myoblasts in growth medium for 24 hours, then switched to differentiation medium and induced to undergo differentiation into multinucleated myotubes as described in material and methods, section 2.2.4. C2C12 cultures were isolated for examination from day 0 after the induction of differentiation, over a total period of 5 days. Fusion of myoblasts into myotubes was first observed after day 2 and continued over the next three days. Many large, multinucleated myotubes were observed after 5 days in culture (Figure 3.1).

Significantly, when C2C12 cell cultures are induced to differentiate, not all of the myoblasts differentiate; a proportion exits the cell cycle and persists as reversibly quiescent, mononucleated MyoD-ve, reserve cells (Yoshida *et al*., 1998), comparable to the way that some satellite cell progeny withdraw from differentiation when maintained on the surface of individual myofibres in suspension (Zammit *et al*., 2004). Reserve cells express Myf-5 and CD34 (Beauchamp *et al*., 2000) and retain the ability to be reactivated, after which they proliferate and can then be induced to differentiate, leading again to a new mixed population of myotubes and reserve cells (Kitzmann *et al*., 1998; Lindon *et al*., 1998; Yoshida *et al*., 1998). These characteristics make reserve cells a useful model of quiescent satellite cells. This system is therefore a potentially useful in vitro model of satellite cells and may be used as a model of both myogenic differentiation and satellite cell specification.

Since Notch signalling is implicated in determining cell fate in a range of developing and adult tissues including skeletal muscle (reviewed in Artavanis-Tsakonas *et al*., 1999), differences in the expression and/or distribution of Notch pathway components in C2C12 cultures may result in the specification of distinct fates. To further investigate the expression of components in differentiated cultures, undifferentiated reserve cells and differentiated myotubes were separated by partial trypsinisation to investigate the expression of Notch pathway components in cells that had adopted these alternate fates.

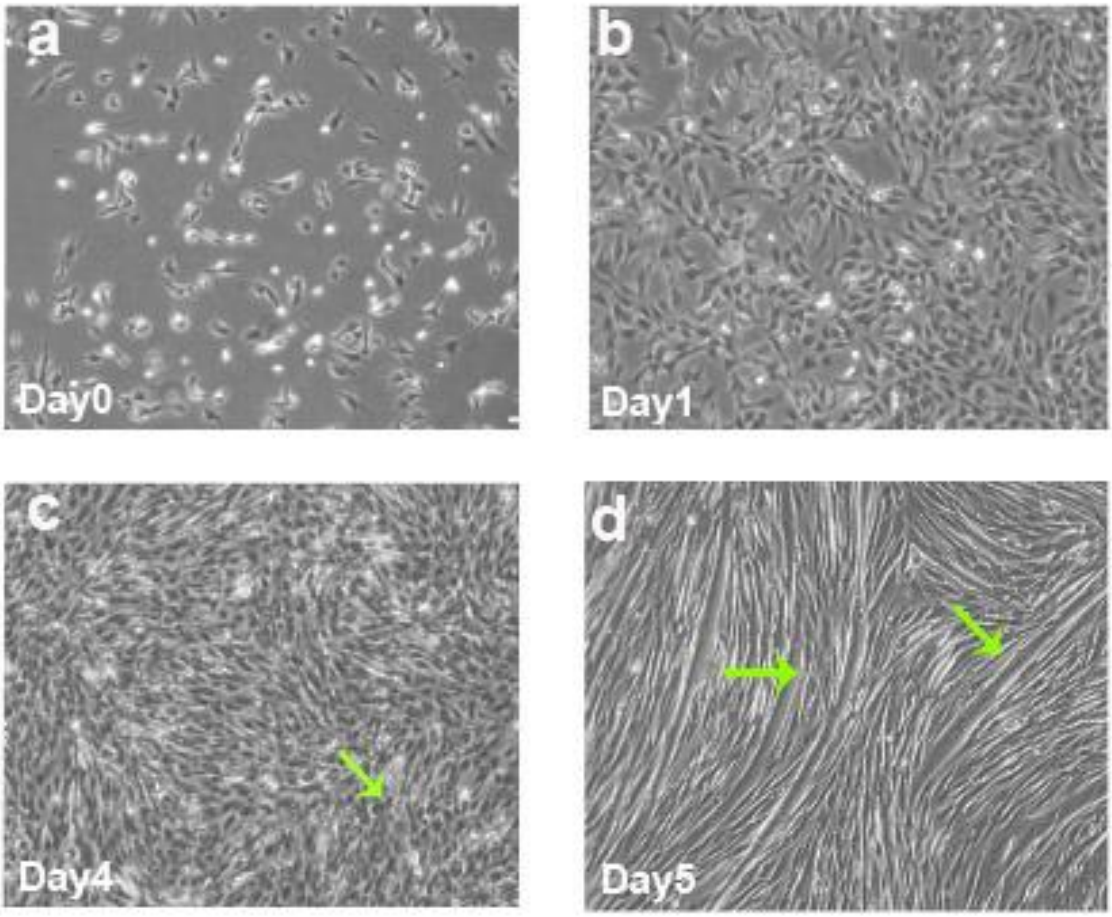


Figure 3.1 Phase-contrast images showing the morphology of proliferating and differentiated C2C12 cells.
a. Proliferating myoblasts (Day 0) were maintained in a growth medium. Differentiation was induced by serum-reduction and cultures were allowed to differentiate for 5 days. During the first 24 hours after differentiation was induced the cells underwent at least one further round of division and then withdrew from cell cycle (a). After 2 days morphological differentiation (i.e. myotubes formation) was first visible (arrow in c). Many large, multinucleated myotubes were visible by day 5 (arrows in d). (Bar: 20µM.)

3.2 Analysis of Notch receptor and ligand transcript expression during myogenesis in C2C12 cultures

To investigate expression of Notch signalling components during myogenic differentiation, cultures of C2C12 myoblasts were switched to low serum medium and allowed to differentiate for 5 days. Cultures were harvested at 24-hour intervals and total RNA was extracted for analysis by reverse transcription-polymerase chain reaction (RT-PCR).

Figure 3.2 shows that the expression of MyoD was present in proliferating myoblasts throughout differentiation. Myogenin was almost undetectable in proliferating myoblasts but increased once differentiation had begun, coinciding with myotubes formation from Day 2 onwards. Notch1, Notch2 and Notch3 transcripts were all present in proliferating myoblasts (albeit at relatively low levels). The Notch receptor transcripts were all up-regulated after 24 hours of differentiation and remained at similar levels for the subsequent 4 days. Notch4 receptor was not detected in any of the samples (data not shown).

Figure 3.2 also shows that Delta-like1 (DL1) transcript was expressed at a low level in proliferating myoblasts, but was upregulated when differentiation was induced. Delta-like 4 (DL4) was not detected in proliferating cultures, but was upregulated 1 day after differentiation was induced. Delta-like 3 (DL3) was absent from C2C12 cells (data not shown). Jagged1 and Jagged2 transcripts were expressed in proliferating myoblasts and throughout differentiation.

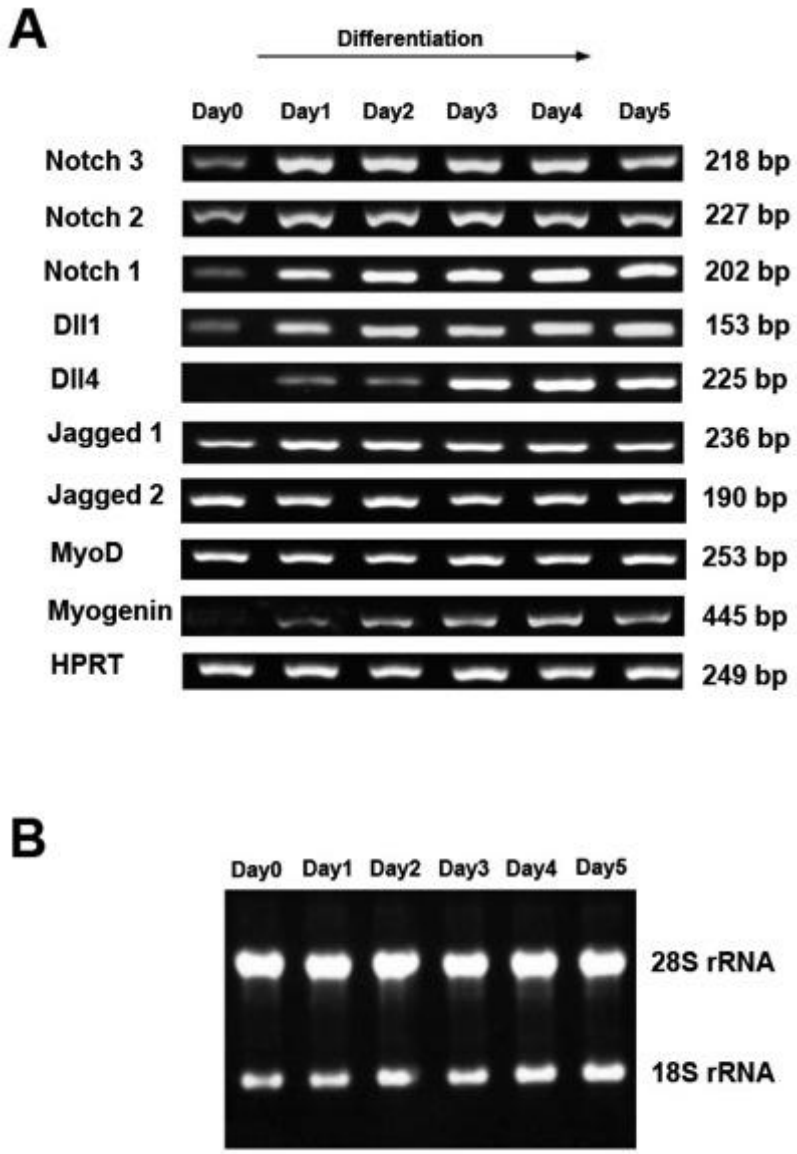


Figure 3.2 RT-PCR analysis of Notch receptor and ligand expression during differentiation of C2C12 cultures.

Total RNA was extracted from replicate C2C12 cultures immediately before inducing differentiation (Day 0) and at 24 hour intervals after induction (Day 1- Day 5). (A) RT-PCR analyses of Notch receptors (Notch1-4) and ligands (Dll1, 3 and 4; Jagged 1 and 2). Notch4 and Dll3 were not detectable in any of the samples (data not shown). Expression of the myogenic regulatory factors MyoD and Myogenin are shown as markers of differentiation; Hypoxanthine guanine phosphoribosyl transferase (HPRT) is presented as an RT-PCR control. Reaction products were run on 1.5% agarose gels and visualized with ethidium bromide with the corresponding product sizes shown on the right. Primer sequences, RT-PCR conditions and cycle numbers are provided in the Materials and Methods (section 2.18). (B) 1µg of each total RNA sample run on a 1.2% agarose gel and visualised with ethidium bromide to confirm sample integrity. n=3

3.3 Analysis of Notch receptor and ligand protein expression during myogenesis in C2C12 cultures

To examine the expression of Notch signalling components at the protein level, whole cell lysates were prepared from myoblasts and cultures differentiated for up to 5 days. Protein was extracted at 24 hour intervals and analysed by Western blotting (see 2.19).

Notch receptor antibodies used for Western blot analysis of the Notch receptors were specifically directed against the intracellular domain (ICD) of the protein. The membrane-bound form of each receptor has a higher molecular weight (around 230kDa) than the cleaved (about 120kDa), active (around 100kDa), intracellular forms that translocates to the nucleus, since upon cleavage a small portion of the receptor is lost extracellularly (Okochi *et al*., 2002). Since the ICD-specific antibodies will detect the receptor before and after cleavage it is, therefore, possible to determine the activation status from the size of the bands detected (i.e. an active band will be approximately 10kDa smaller than the inactive membrane-bound form). **Error! Reference source not found.** shows that the expression of skeletal uscle-specific myosin heavy chain (MyHC) was progressively up-regulated with time, thereby confirming the kinetics of myogenic differentiation. When protein extracts from proliferating myoblasts were probed with antibodies specific to Notch1, Notch2 or Notch3 respectively, two predominant bands were detected. In each case, as stated above, the larger molecular weight band corresponds to the membrane-tethered, inactive form of the receptor, while the smaller molecular weight band is the activated, cleaved form. These results show that proliferating myoblasts contained the membrane-bound and activated forms of Notch1, Notch2 and Notch3, although Notch3 was present at much lower levels than the other two receptors. The level of expression of Notch1 was maintained throughout differentiation: Figure 3.3 shows that cleaved, active Notch1 was expressed throughout myogenic differentiation, but was slightly up-regulated after 48 hours (Day 3). In contrast, the active form of Notch2 was highly expressed in myoblasts, but decreased during early differentiation and was no longer detectable by day 3. Notch3 transcript was barely detected at Day 0 and Western blot analysis showed

that the full-length (inactive) Notch3 protein was predominant until Day 2/3 of differentiation. Although expression of the active form was upregulated at the initiation of differentiation, it became more prominant at Day 3, coincident with myotube formation. In all cases, Western blot data were consistent with the observed transcript expression (Figure 3.2).

Notch receptor ligand, Dll1 gradually increased from Day0 to Day5. Interestingly, Dll4 was undetectable in proliferating cultures (Day 0), but was up-regulated between Day 2 and Day 3 as myotubes began to form. Jagged1 protein was highly expressed in proliferating myoblasts and throughout differentiation. Jagged1 expression increased as differentiation proceeded. A correlation is found between protein and transcript expression of Jagged1 and Dll4 (Figure 3.2), as Jagged1 was present throughout myogenic differentiation, while Dll4 was only expressed after 24-48 hours.

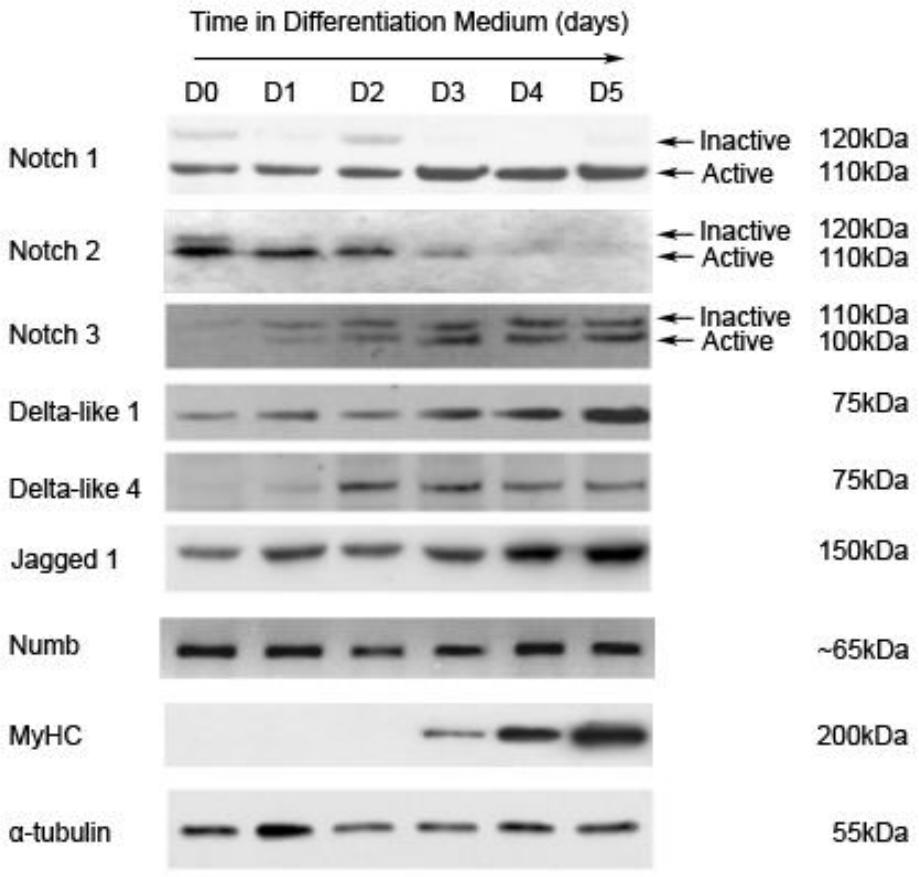


Figure 3.3 Western blot analysis of Notch receptor and ligand expression during differentiation of C2C12 cultures
Protein was extracted from C2C12 cultures immediately before inducing differentiation (Day 0) and at intervals after induction (Day 1- Day 5). Western blot analyses the expression of Notch receptors (Notch1-3), ligands (Dll1, Dll4 and Jagged 1) and Numb. Notch receptor and ligand expressions and activities were activated and detected after initiation of myotubes formation. Myosin heavy chain (MyHC) and α-Tubulin expression were used as differentiation and loading control respectively. n=3

3.4 Analysis of Notch receptor and ligand transcript expression in separated myotubes and reserve cells

Data presented in sections 3.2 and 3.4 shows that Notch signalling components are expressed in differentiated cultures, which contain both differentiated myotubes and quiescent reserve cells (Yoshida *et al*., 1998). To investigate the distribution of Notch signalling components in differentiated cultures, C2C12 cultures were allowed to differentiate for 5 days and then separated by a modified method of

partial trypsinisation originally described by Carnac et al. (1992), which selectively detaches the myotubes releasing them in suspension whilst the reserve cells remain attached. This technique was modified to include three additional washes using PBS (see Materials and Methonds section 2.5)

3.4.1 Separation of myotube and reserve cell fractions in differentiated C2C12 cultures

The phase contrast pictures shown in Figure 3.4 show the culture before and after the removal and collection of the myotubes with the reserve cells (d) remained attached. After 5 days of differentiation, cultures were separated into myotube and reserve cell fractions by partial trypsinisation. Briefly, cultures were rinsed with PBS and then incubated in 0.01% trypsin (Sigma) in PBS until the detachment of myotubes from the subscribe was observed. The detached myotubes were retained and the adherent cells rinsed again with PBS and then subjected to a series of 3 one-minute incubations in 0.01% trypsin (with intervening PBS washes) to remove residual myoblasts and myocytes (as described in Materials and Methods). Total RNA was isolated from each fraction (myotubes, washings and remaining reserve cells) and the purity of the fractions was confirmed by RT-PCR (Figure 3.5 and Figure 3.6).

Expression of CD34, myogenin and MyoD were used as markers of reserve cells, differentiated cells and myoblasts/early myotubes, respectively *(*Zammit *et al*., 2006). CD34 is a transmembrane sialomucin, expressed by haematopoietic stem cells (HSC) and progenitors and quiescent satellite cells (Beauchamp *et al*., 2000). CD34 is involved in the maintenance of quiescence and coexpression of CD34, Myf5 and M-cadherin defines the majority of quiescent satellite cells (Beauchamp *et al*., 2000). The satellite cell compartment is heterogeneous, as not all cells express these markers, which may result in divergent cell fates (Beauchamp *et al*., 2000). Thus CD34 is both a marker of satellite cells and of a population of muscle-derived stem cells.

MyoD, Myogenin and MRF are transcription factors markers that facilitate terminal differentiation of myoblasts into myocytes (Cornelison and Wold, 1997; Megeney *et al*., 1996; Yablonka-Reuveni and Rivera, 1994).

The results showed that CD34 was exclusively expressed in the reserve cell fraction and Myogenin and MyoD were more abundant in the myotube fraction as expected.

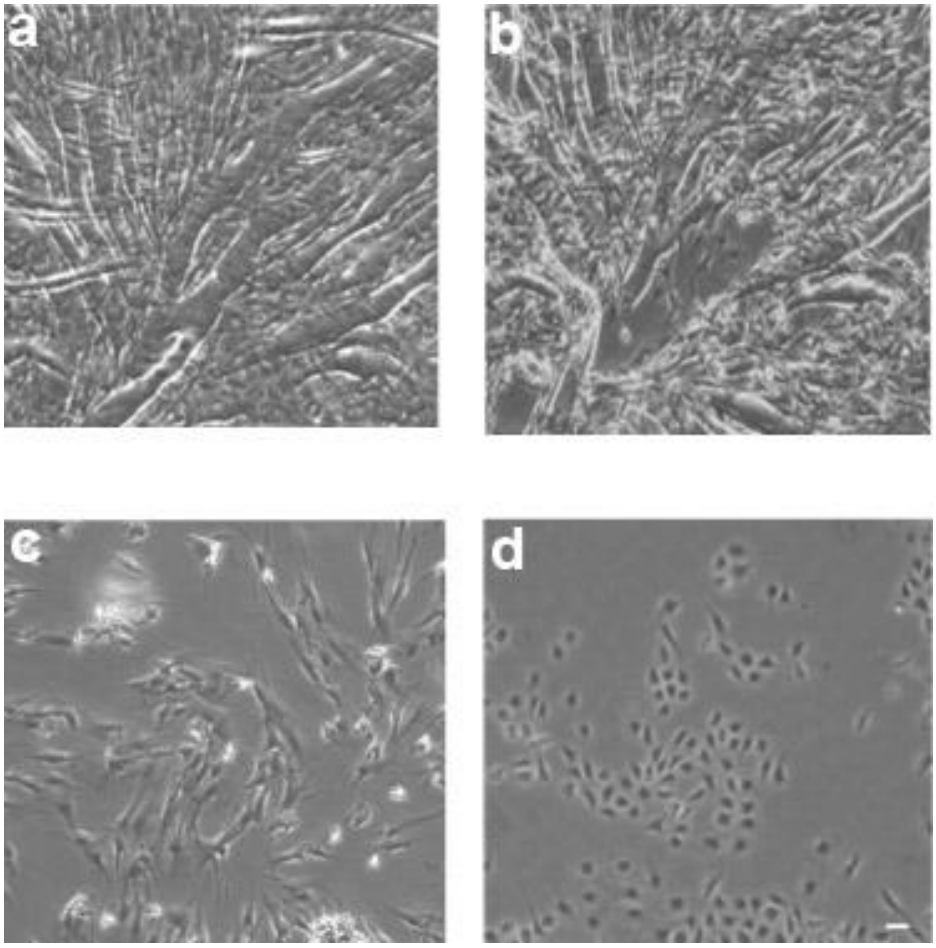


Figure 3.4 C2C12 myotube and reserve cell separation
C2C12 myoblasts were grown for 24 hours and then induced to differentiation. (a). Unseparated culture after 5 days of differentiation. During the initial brief trypsinisation, the myotubes detach (b) leaving undifferentiated single cells and myocytes (c). A further three washes with trypsin were used to remove the myocytes, leaving a pure reserve cell population (d). (Bar: 20µM.)

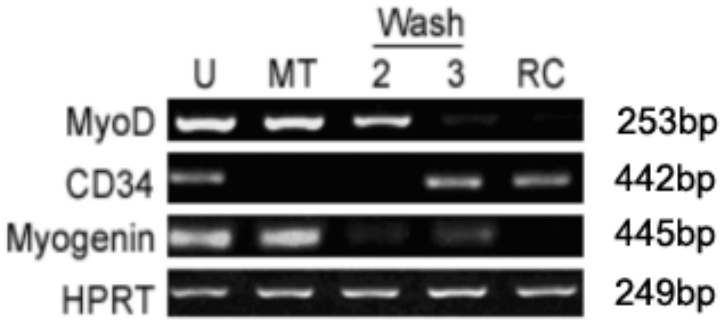


Figure 3.5 Validation of the partial trypsination protocol
C2C12 myoblasts were differentiated for 5 days. Total RNA was extracted from unseparated differentiated C2C12 cultures (U), myotubes (MT), washings (2, 3) and reserve cells (RC) by partial trypsinisation. RT-PCR analyses the efficiency of the modified partial trypsination protocol. CD34, MyoD and Myogenin were used to demonstrate efficient purification of the myotube (MT) and reserve cell (RC) fractions. HPRT is presented as an RT-PCR control. Reaction products were run on 1.5% agarose gels and visualized with ethidium bromide with the corresponding product sizes shown on the right. Primer sequences, RT-PCR conditions and cycle numbers are provided in the Materials and Methods (section 2.18). n=3

According to Figure 3.5, RT-PCR of total RNA from unseparated cultures, myotube, reserve cell fractions and two intermediate washes showed that the myotube fraction contained MyoD and myogenin transcripts, whilst the reserve cell fraction expressed only CD34, both consistent with the expected phenotypes. Cells detached by intermediate washes expressed MyoD and/or myogenin, presumably myoblasts and differentiated mononucleated myocytes. Therefore separation was successful in highly enriching each cell-type with no contamination between fractions.

3.4.2 Distribution of Notch receptor and ligand transcript expression in differentiated C2C12 cultures

Having validated the cell separation method, the distribution of Notch receptors and ligands between myotubes and reserve cells was investigated. Notch1 transcript was expressed in both myotubes and reserve cells, but was highly enriched in the myotubes fraction, whilst Notch 2 and Notch 3 were highly expressed in reserve cells only. Dll1 expression was relatively low in reserve cells

but high in myotubes. Dll4 was exclusively expressed in myotubes fraction only whereas Jagged1 transcripts were highly expressed in reserve cells only.

RT-PCR analysis of unseparated cultures, myotube and reserve cell RNA is shown in Figure 3.6. The analysis revealed the expected enrichment of MyoD in myotubes, is consistent with up-regulation of MyoD expression during myotube formation (Kitzmann *et al*., 1998). CD34, predicted from (Beauchamp *et al*., 2000), was highly elevated in reserve cells as expected.

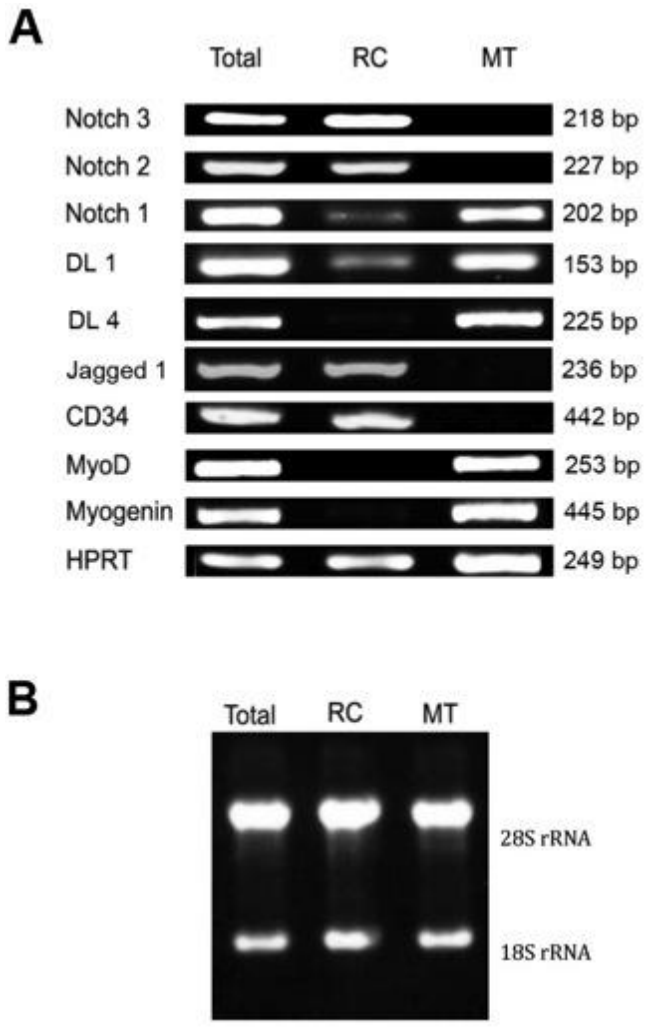


Figure 3.6 RT-PCR analysis of Notch receptor and ligand expression in separated myotubes and reserve cells

C2C12 myoblasts were differentiated for 5 days. Total RNA was extracted from unseparated differentiated C2C12 cultures (Total), myotubes (MT) and reserve cells (RC) by partial trypsinisation. (A). RT-PCR analyses of Notch receptors (Notch1-3)

and ligands (Dll1, Dll4 and Jagged 1). MyoD, Myogenin and CD34 were used to demonstrate efficient purification of the two fractions. HPRT is presented as an RT-PCR control. Reaction products were run on 1.5% agarose gels and visualized with ethidium bromide with the corresponding product sizes shown on the right. Primer sequences, RT-PCR conditions and cycle numbers are provided in the Materials and Methods (section 2.18). (B) 1µg of each total RNA sample run on a 1.2% agarose gel and visualised with ethidium bromide to confirm sample integrity. n=3

3.4.3 Distribution of Notch receptor and ligand protein expression in differentiated C2C12 cultures

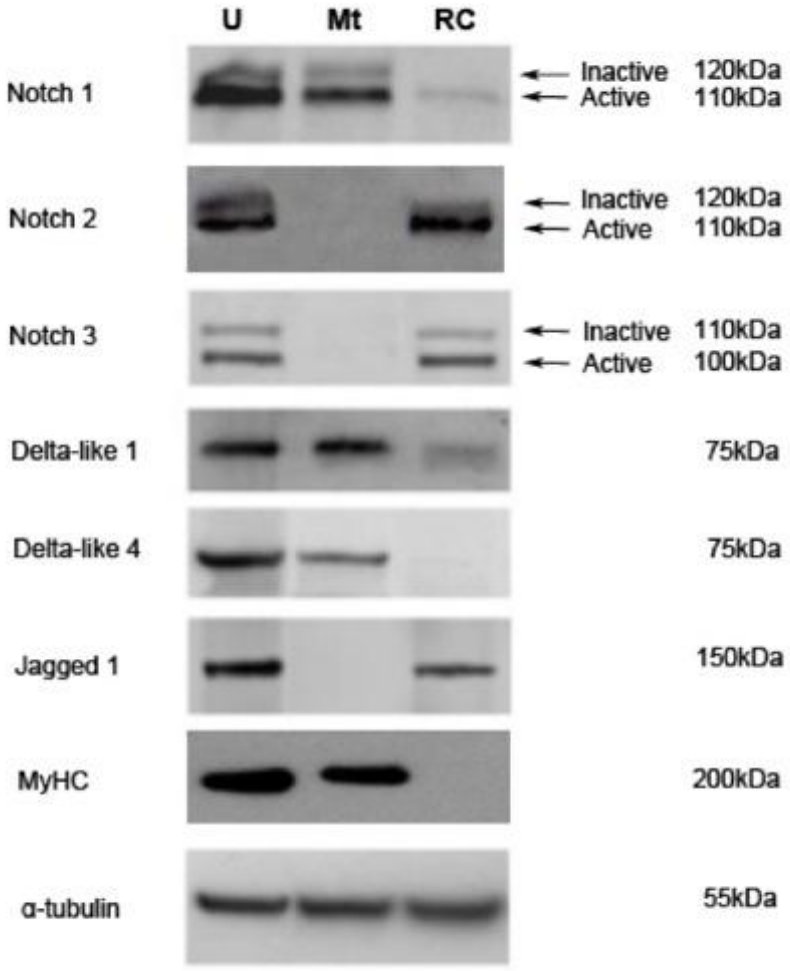


Figure 3.7 Western blot analysis of distribution of Notch receptors and ligands in separated myotubes and reserve cells
C2C12 myoblasts were differentiated for 5 days. Protein was extracted from unseparated differentiated C2C12 cultures (Total), myotubes (MT) and reserve cells (RC) by partial trypsinisation. Western blot analyses the expression of Notch receptors (Notch1-3), ligands (Dll1, Dll4 and Jagged 1). Notch receptor and ligand expressions and activities were activated and detected in myotubes or reserve cells

fraction. Myosin heavy chain (MyHC) and α-Tubulin expression were used as differentiation and loading control respectively. n=3

Protein extracts from myotubes and reserve cells were analysed by Western blotting (Figure 3.7). Using antibodies directed against of Notch1, Notch2 or Notch3 ICDs, allowing the determination of their activated status.

Figure 3.7 shows a very faint band of activated Notch1 was detected in the reserve cells whereas high levels of activated Notch1 were detected in the myotubes. When blots were probed with antibodies specific for Notch2 or Notch3 ICD, two bands were detected in the reserve cell fraction only. In both cases, the larger molecular weight band corresponds to the membrane-bound inactive form of the receptor, while the smaller molecular weight band is the activated, cleaved form of the receptor. Reserve cells contained the membrane-bound and activated forms of Notch2 and Notch3. Transcript Figure 3.6 shows only Notch1 in myotubes fraction, no Notch2 or Notch3. This is completely consistent with the protein data.

Western blot analysis showed that Dll1 was expressed at a low level in reserve cells was highly expressed in myotubes, consistent with observed the expression of its transcript reflecting the expression of its transcript (Figure 3.6A). The observed expression of Dll4 and Jagged1 proteins were also consistent with transcript expression with Dll4 was expressed only in the myotube fraction whilst Jagged1 protein was highly expressed in reserve cells only. This shows that following differentiation, Dll4 and Dll1 are expressed by myotubes, whereas Jagged1 is restricted to the reserve cell fraction.

3.5 Discussion

The aim of the experiments performed during this thesis was to decipher the role of the Notch signalling pathway and of the different receptors and ligands during myogenic differentiation using C2C12 cultures. The results demonstrated that myogenic differentiation is accompanied by changes in the expression profiles of

the Notch components, and that differential expression of receptors and ligands in were present at different stages of differentiation in the cellular model tested, i.e. C2C12.This is consistent with Notch signalling playing a role in the cell fate decisions during myogenesis, as initially hypothesized.

3.5.1 Proliferating myoblasts

Notch receptor expression was analysed both at the transcript and protein level. Notch1, Notch2 and Notch3 (but not Notch4) were expressed in proliferating myoblasts, although the level of Notch3 expressed was very low compared with Notch1 and Notch2. Therefore, whilst un-cleaved Notch3 was present in proliferating (and differentiating, see below) muscle cells, activated Notch3 was present from Day 1 only.

Since active Notch1 and Notch2 are expressed in proliferating myoblasts it suggests that they may play a role in maintaining a proliferative state as previously shown both in development and during adult tissue regeneration (Conboy *et al*., 2003; Conboy and Rando, 2002; Schuster-Gossler *et al*., 2007; Vasyutina *et al*., 2007). Active Notch3 is undetectable during proliferation; it is thought to perform functions distinct from Notch1 and Notch2 (Beatus *et al*., 1999; Beatus *et al.*, 2001). Although the active Notch3 ICD can bind to RBPJK, it is a poor transcriptional activator (Kadesch, 2000); indeed Notch3 can act as a transcriptional repressor by competing with the Notch1 ICD and blocking Notch1 ICD's ability to bind to a common co-activator, PCAF present in limited amount or by competing with Notch1 ICD and preventing it from binding to RBPJK and activating target genes (Beatus *et al*., 1999). Furthermore, the Notch2 ICD may be inhibited by Notch3 (Beres *et al*., 2006). Although, conversely Notch2 ICD may negatively regulate Notch1 and Notch3 (Shimizu *et al*., 2002), presumably via competitive binding.

	myoblasts	myotubes	reserve cells
Notch1	+	+	+/-
Notch2	+	-	-
Notch3	-	-	+
Delta-like1	+	+	-
Delta-like 4	-	+	-
Jagged 1	+	-	+

Table 3.1 Expression levels of Notch receptors and ligands in myoblasts, myotubes and reserve cells fraction

The activation of the various Notch receptors in proliferating myoblasts requires interaction with ligands on the surface of adjacent cells (Parks *et al*., 2000; Conboy and Rando, 2002; Ross and Kadesch, 2004). Both RT-PCR and Western blot analysis showed that Dll1 was expressed by proliferating myoblasts. In contrast, Dll4 was undetectable in proliferating cultures by RT-PCR and Western blot in proliferating myoblasts. RT-PCR showed that Jagged1 and Jagged2 transcripts were expressed at a high level in proliferating myoblasts. In addition, Western blot analysis confirmed the elevated expression of Jagged1 in myoblasts. It is likely, therefore, that Dll1 and Jagged1 (and probably Jagged2) expressed on the cell surface activates transmembrane Notch receptors (Notch1 and Notch2) on adjacent myoblasts, which signal via the RBPJK-dependent pathway to transactivate target genes. It has also been shown that activated Notch can autonomously induce Jagged1 activity (Ross and Kadesch, 2004), such that active Notch receptors and Jagged1 can be expressed simultaneously within a cell. Induced Jagged1 has no autocrine effect on Notch signalling, but can promote signalling in adjacent naive cells (Ross and Kadesch, 2004). Therefore, proliferating myoblasts may exist as a homogeneous

population where all cells express Jagged1, and possibly Jagged2 together with active Notch receptors.

3.5.2 Expression of Notch receptors and ligands during muscle cell differentiation.

The induction of differentiation in C2C12 cultures led to changes in the expression levels of the Notch receptors and ligands analysed. These changes were most apparent between 24 and 48 hours, coincident with the onset of morphological differentiation. This suggests that Notch signalling may potentially be involved in cell fate decisions made at this time: specifically concerning the decisions of the cells to become myotubes or maintained as reserve cells. This is directly connected to the initial hypothesis: Notch signalling is involved in the decision that leads to the generation of precursors, i.e. satellite cells, and in the process that leads to their proper location in the niche between basal lamina and differentiated muscle fibres.

Expression of activated Notch1 remained relatively constant throughout myogenic differentiation, although it was noticeably upregulated after 48 hours. In differentiated cultures, membrane-bound and active Notch1 were present at a highly elevated level in myotubes, with much lower level of expression in reserve cells. Previous studies (Kitzmann et. al., 2006; Sun et. al., 2008) have reported active Notch1 in quiescent reserve cells, but this is probably due to the separation technique used. The original method used to separate myotubes and reserve cells as described by Carnac and co-workers (Carnac et. al., 1992) was modified by the addition of controlled washes to obtain purer reserve cell fractions (Figure 3.5). RT-PCR analysis of cells removed by these washes showed them to express MyoD and/or Myogenin, suggesting that they are either myoblast or differentiated mononucleated myocytes: such contaminating cell types express Notch1 and would therefore provide a false positive result.

Active Notch2 was expressed initially but then began to disappear 48 hours after differentiation was induced. However, expression of the membrane-tethered form of Notch2 was maintained exclusively in reserve cells. The apparent disappearance of Notch2 from unseparated differentiated cultures may be explained because the reserve cells contribute only a small proportion of the total protein in a differentiated culture (myotubes contain a large amount of contractile proteins), so the signal is diluted and hence is not detected. In contrast, Notch1 was expressed in both fractions and was, therefore, detectable.

Active Notch3 was detectable sooner after the initiation of differentiation, reaching a maximum after 48-72 hours during myotube formation and was localised entirely in reserve cells. Notch3 (active/inactive form) was detected in unseparated cultures throughout myogenic differentiation, even though it was only expressed in the reserve cells. This suggests a particularly high level of Notch3 expression in reserve cells (compared with Notch2), indicating Notch3 could potentially be the dominant Notch receptor in these cells.

Since active Notch1 (very low level), Notch2 and particularly Notch3 are expressed in reserve cells it is possible that Notch signalling plays a role in reserve cell specification.

Active Notch1 may inhibit differentiation of a subset of cells (Nofziger *et al*., 1999; Shawber *et al*., 1996), ensuring that they are maintained as reserve cells. The marked up-regulation of Notch3 when cell fate decision is being made and its maintenance in the reserve cells strongly suggests that Notch3 plays a crucial role in the specification and maintenance of the stem cell phenotype within a differentiating culture. High expression of the active Notch3 ICD may prevent the Notch1 (Beatus *et al*., 1999) and Notch2 ICDs (Beres *et al*., 2006) from binding to RBPJK and/or a common coactivator, preventing transactivation of their downstream target genes and any unbound active Notch3 ICD may bind to RBPJK, and act as a weak transcriptional activator or possibly even a repressor of a specific cohort of target genes (Beatus *et al*., 1999; Kadesch, 2000). The outcomes of the

interacting regulatory mechanisms could result in the expression of a particular set of target genes and hence specify the maintenance of reserve cells.

Activation of the Notch receptors in reserve cells would require interaction between transmembrane Notch receptors with ligands on the surface of adjacent cells (reviewed in Artavanis-Tsakonas *et al*., 1999), or autonomous activation by Dtx proteins (Kishi *et al*., 2001). Both Dll1 and Dll4 expression are up-regulated upon the induction of differentiation. Dll1 was expressed in both myotubes (more abundantly) and reserve cells, but Dll4 was expressed exclusively in myotubes.

Dll4 was undetectable in proliferating cultures (Day 0), but was up-regulated between Day 2 and Day 3 as myotubes began to form. The pattern of Dll4 expression during myogenesis is coincidentally consistent with Notch 3 receptor.

In contrast, Jagged1 and Jagged2 were expressed throughout differentiation. However, Western blot analysis demonstrated a gradual increase in Jagged1 protein expression, in contrasts to the increasing expression of the Delta-like ligands. Following differentiation, Jagged1 was expressed in reserve cells presumably at a high level, since it was observed in unseparated cultures where the signal is diluted by the presence of myotube proteins. Therefore, it is possible that transmembrane Jagged1 on reserve cells could activate transmembrane Notch receptors on adjacent cells, which signal via the RBPJK-dependent pathway to transactivate target genes that specify those cells as reserve cells. Hence, Jagged1 recruit more reserve cells.

Numb could prevent NICD from translocation to the nucleus by interacting with the membrane-tethered form of Notch, thus inhibiting Notch signalling (McGill and McGlade, 2003). Specifically ubiquitin-mediated degradation and receptor endocytosis have been proposed as potential mechanisms for Numb-dependent Notch downregulation (Berdnik *et al*., 2002; McGill and McGlade, 2003).

Notch/Numb signalling pathways may regulate myogenic cell fate decisions (Conboy and Rando, 2002; Halevy *et al*., 2004). It is well-established that Notch signalling

inhibits MyoD expression and therefore differentiation (Kopan *et al*., 1994). This is consistent with the hypothesis that inhibition of Notch1 signalling as mediated by an up-regulation of the antagonist, Numb, may allow exit from a progenitor stage into differentiation, whilst constitutively high levels of Notch1 signalling maintain cells in the intermediate progenitor stage. It has therefore been suggested that the balance between Notch1 and Numb may control cellular homeostatic and cell fate decision. (Conboy and Rando, 2002).

Western blot analysis revealed that the inhibitory molecule Numb is expressed in proliferating myoblasts and was maintained throughout differentiation for 5 days. Notch1 is targeted by all four mammalian Numb isoforms ((65, 66, 71 and 72kDa (Dho *et al*., 1999)), Notch2 is variably repressed and Notch3 is not a target of any of the Numb proteins (Beres *et al*., 2006). Hence, in proliferating myoblasts, Numb could inhibit signalling of Notch1 and Notch2.

Results presented in this chapter have revealed the differential expression of Notch receptors and ligands during and myogenic differentiation and the differences in expression profiles in cells that adopt different fates. These changes were mostly observed after 24 to 48 hours, coincident with the onset of morphological differentiation, which suggests that the expression levels of the Notch signalling components may be involved in determining cell fate. Different receptor/ligand profiles are expressed in proliferating myoblasts, differentiated myotubes and undifferentiated reserve cells. These differences may underlie the distinct outcomes: regulating both the proliferation of muscle precursors through interaction with other myoblasts and, through interaction with differentiated cells, the specification of reserve cells. Most significantly, active Notch3 was expressed exclusively in the reserve cell fraction but was completely absent from the myotube fraction. The marked upregulation of Notch3 when the cell fate decision is being made and is maintenance in the reserve cells strongly suggests that Notch3 may play a crucial role in the specification and maintenance of the stem cell phenotype within a differentiating culture. Furthermore, the Notch ligand, Dll4 is up-regulated at approximately the same time as Notch3 during skeletal muscle cell differentiation, which leads to the hypothesis that the appearance of differentiated

cells during muscle regeneration may direct other as yet “undecided” cells into a stem cell fate, thereby re-establishing the stem cell pool.

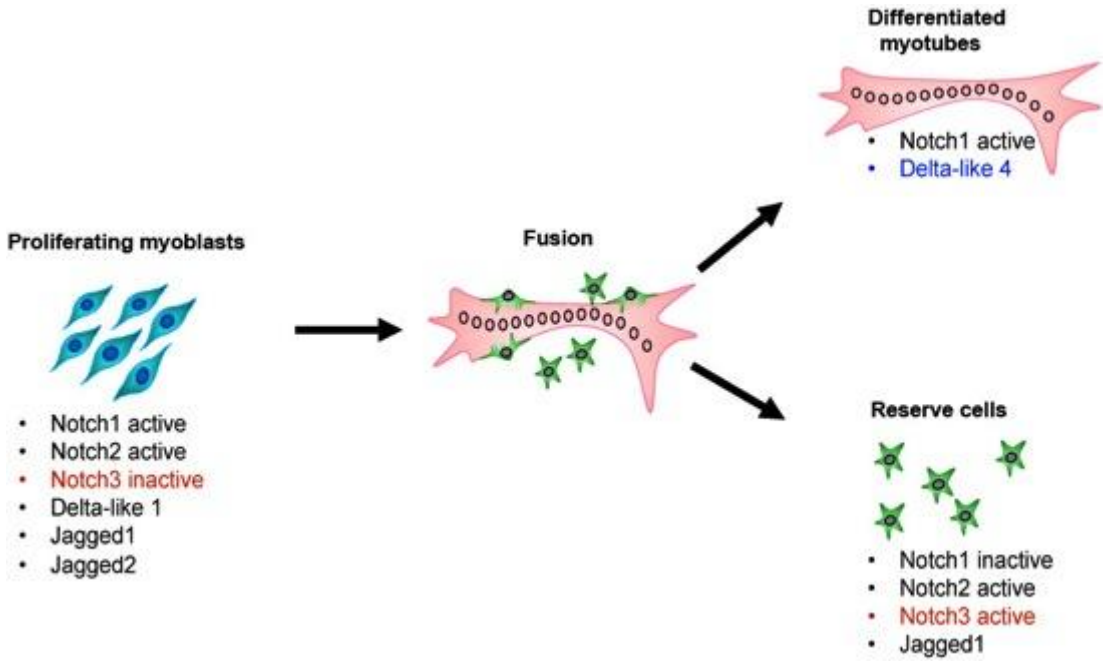


Figure 3.8 Possible model for Notch3 receptor function during myogenic differentiation.
Notch3 is activated after differentiation is induced in proliferating myoblast cultures (Notch3–ve, Notch1+ve, Notch2+ve, Dll1+). Cell fate is determined by the presence or absence of Notch3 activation by its ligand. In the presence of ligand in the adjacent nascent cells (possibly Dll4), Notch3 is activated and the cell is specified as a reserve cell.

Based on the results obtained, a simple model of the role of Notch3 in the specification of reserve cells is speculated as shown in Figure 3.8. After differentiation is induced in the activated myoblasts (Notch3–ve, Notch1+ve, Notch2+ve, Dll1+ve and Jagged1+), Notch3 is activated. Unfused single cells in differentiated cultures could either remain in the cell cycle or exit to become an undifferentiated reserve cell. For Notch3 to have an effect on myoblast fate decisions, it must interact with a Notch ligand. One possible explanation is that such

a ligand may be expressed on newly formed myotubes that can interact with remaining undifferentiated cells: one possible candidate is DL4.

Chapter 4: The effects of shRNA-mediated knockdown of Notch3 expression on the behaviour of skeletal muscle precursor cells

4.1 Introduction

Results presented in Chapter 3 show that Notch3 becomes up-regulated and active when skeletal muscle precursor cells are induced to differentiate and is subsequently maintained exclusively in reserve cells and not in myotubes. Thus, Notch3 is active when the decision is made either to commit to differentiation or to adopt a quiescent reserve cell fate. However, this is not enough to ascertain that Notch3 plays an active role in this decision, and functional studies must be carried out to further investigate the original hypothesis on the role of Notch3 in the establishment of quiescent satellite cells in their niche. The aim of this chapter was to investigate whether Notch3 activity is required for reserve cell specification by investigating the effects of inhibiting expression on the behaviour and differentiation of skeletal muscle precursor cells. The hypothesis was that if Notch3 is required, reduced levels of expression and therefore activity should promote differentiation and enhance myotube formation.

4.2 shRNA-mediated knockdown of Notch3 expression

Four SureSilencing™ shRNA/GFP plasmids designed to knockdown the expression of mouse Notch3 (P1-P4) and a control plasmid encoding an irrelevant scrambled sequence (P5), were obtained from SA Biosciences (sequences provided in Materials and Methods, section 2.8.20). To determine which plasmid was the most effective,

stably-transfected C2C12 cultures were generated by co-transfection with one of the above plasmids, together with a Neomycin resistance plasmid (pC1Neo) followed by selection in G418 (Materials and Methods, section 2.9.4). Cultures were allowed to differentiate for 48 hours to allow maximal Notch3 expression and then total RNA and protein were extracted for analysis by RT-PCR and Western blotting, respectively (Figure 4.1A and B). The results show that transfection with P3 markedly reduced the levels of both Notch3 transcript and protein, whereas P1, P2 and P4 had no significant effect compared with the control plasmid, P5. Importantly, Western blotting analysis showed the expression of the related Notch1 receptor was unaffected by the shRNA sequence encoded by P3 (Figure 4.1C). The G418-selected, uncloned cultures expressing P3 were therefore chosen for further analysis, together with equivalent cultures expressing the control shRNA plasmid, P5.

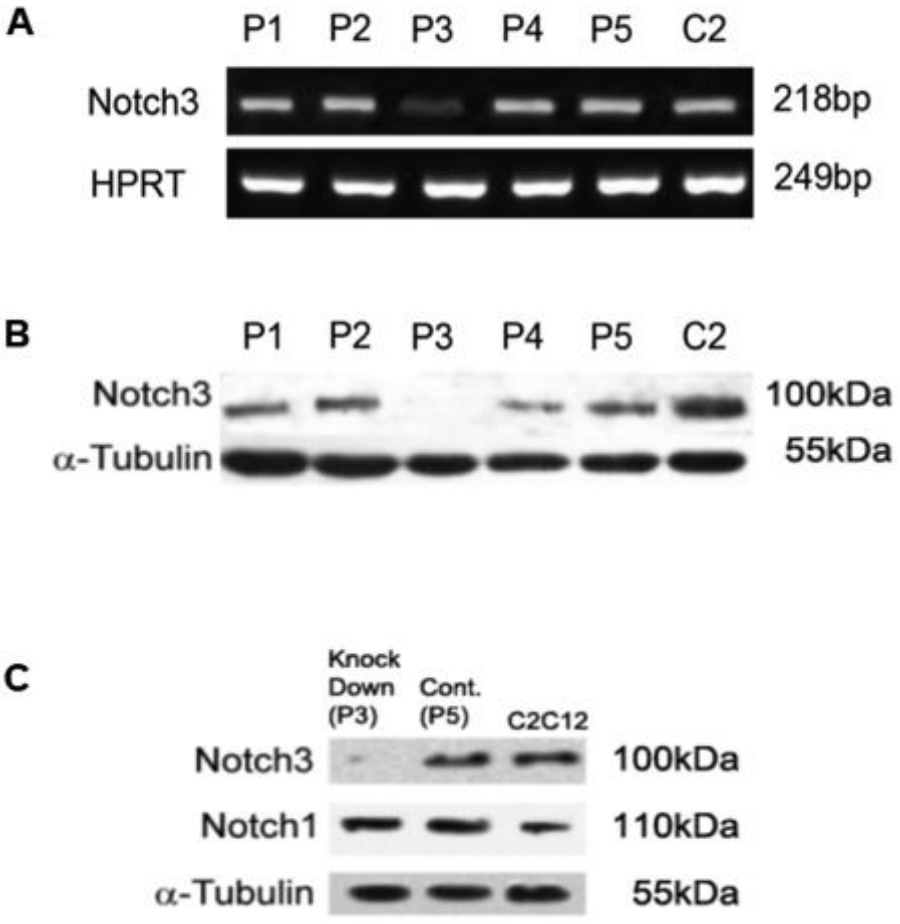


Figure 4.1 shRNA-mediated knockdown of Notch3 expression in C2C12 cultures
Proliferating C2C12 myoblasts were co-transfected with SureSilencing™ shRNA/GFP plasmids containing either potential shRNA knockdown sequences (P1-P4) or an irrelevant scrambled control sequence (P5), together with the Neomycin-resistance plasmid, pC1Neo. After selection with G418, cultures were transferred to differentiation medium for 48 hours to induce maximal levels of endogenous Notch3 expression as observed in untransfected C2C12 cells. (A) RT-PCR analysis of Notch3 transcript expression (HPRT) is shown as a control. (B) Western blot analysis of Notch3 protein levels. The membrane was probed with an antibody raised against the Notch3 ICD (M-20; Santa Cruz). (C) Western blot analysis of Notch1 protein levels in cultures transfected with P3 or P5, using an antibody raised against the activated Notch1CD (Abcam). α- Tubulin was used as a loading control for Western blot analyses: the sizes of the RT-PCR products and protein bands were determined by the use of appropriate markers and are indicated on the left. n=3

4.3 Effects of Notch3 knockdown on the behaviour of C2C12 cultures

4.3.1 Proliferation of C2C12 myoblasts

The effects of Notch3 knockdown on myoblast proliferation were assessed using C2C12 cultures stably expressing either the P3 (knockdown) or P5 (shRNA control) plasmids, compared with untransfected C2C12 myoblasts. Myoblast cultures were seeded in 96 well plates at a density of 1000 cells/well and after 24 hours, the number of cells was determined using a colorimetric assay (Materials and Methods, section 2.4). The results shown in Figure 4.2 show that knockdown of Notch3 had no effect on myoblast proliferation, consistent with the fact that Notch3 is present at very low levels and is inactive in proliferating cultures (Chapter 3, section 3.3).

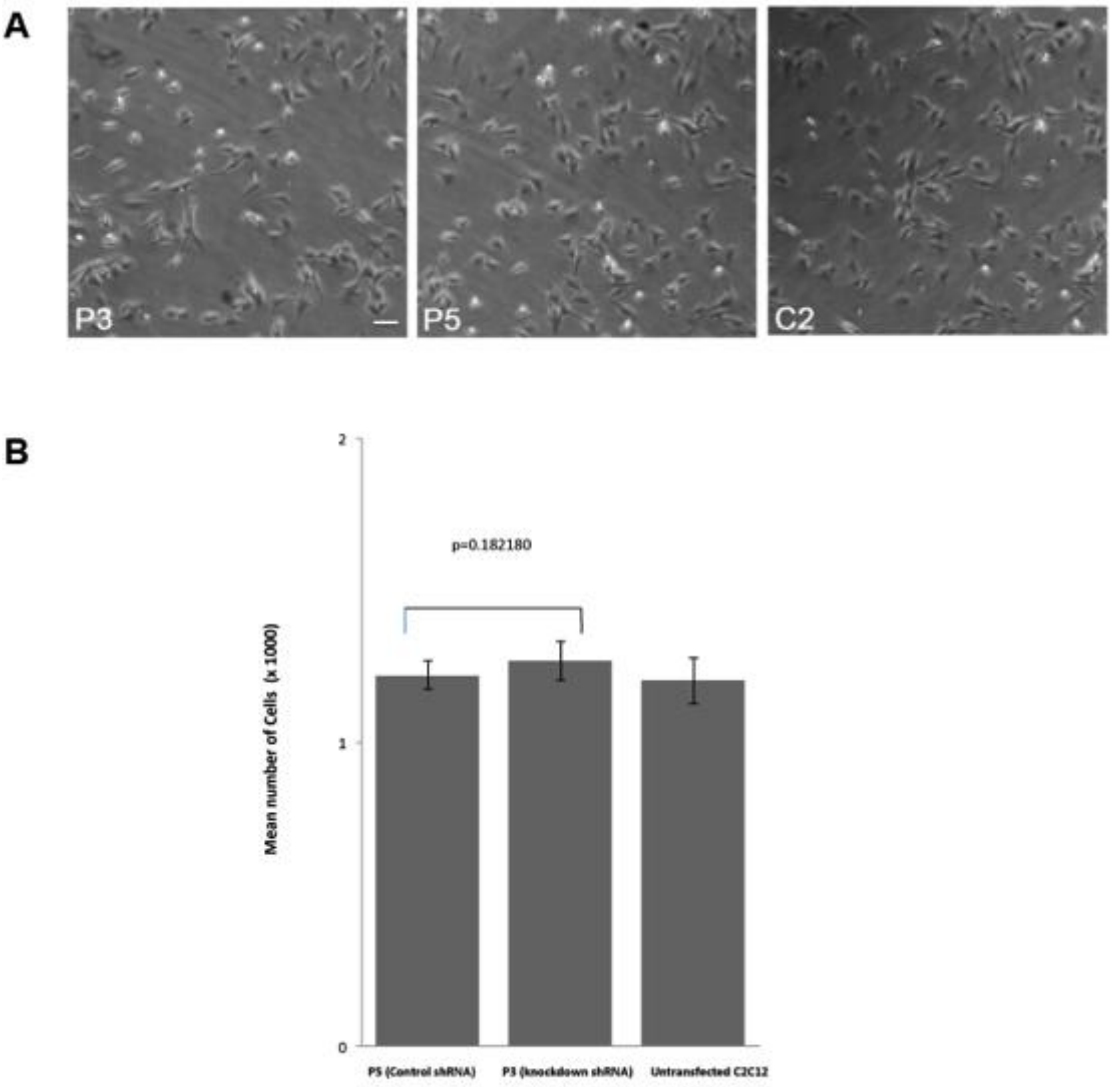


Figure 4.2 Effects of shRNA-mediated knockdown of Notch3 on C2C12 myoblast proliferation.

Proliferating C2C12 myoblasts, either untransfected, transfected with P3 (knockdown) or with P5 (control shRNA) were seeded at the same density (1000 cells/well) in 96 well plates and cultured for 24 hours as proliferating myoblasts in growth medium containing high levels of serum. (A) Representative phase contrast images of proliferating cultures (scale bar = 20µm). (B) Histogram showing cell number as determined using the CellTiter 96 Aqueous One Solution colorimetric cell proliferation assay (Promega). Each column shows the mean of three wells (± 1 SD) and when compared using a Student's t-test, there were no significant differences between the cultures.

4.3.2 Differentiation of C2C12 cultures

To investigate the effects of shRNA-mediated inhibition of Notch3 on myogenic differentiation, C2C12 cells transfected with either P3 (knockdown) or P5 (shRNA control) were transferred to and maintained in low serum-containing differentiation medium (Materials and Methods, section 2.2.4, section 2.9.4)). Although inhibition of Notch3 expression had no effect on proliferation in growth medium, it appeared that after 48 hours in differentiation medium, P3-transfected cultures contained more cells than untransfected or P5-transfected cultures, an observation that was confirmed using the cell proliferation assay (Figure 4.3). In untransfected cultures, Notch3 activity is upregulated by 48 hours after differentiation is induced (Chapter 3.3), during which time the myoblasts withdraw from the cell cycle and initiate a program of myogenic differentiation. Cultures in which Notch3 expression was inhibited by shRNA proliferated normally in growth medium, but when induced to differentiate, the absence of Notch3 appeared to increase or prolong proliferation (Figure 4.4), resulting in an increased number of subjectively rounder and smaller cells.

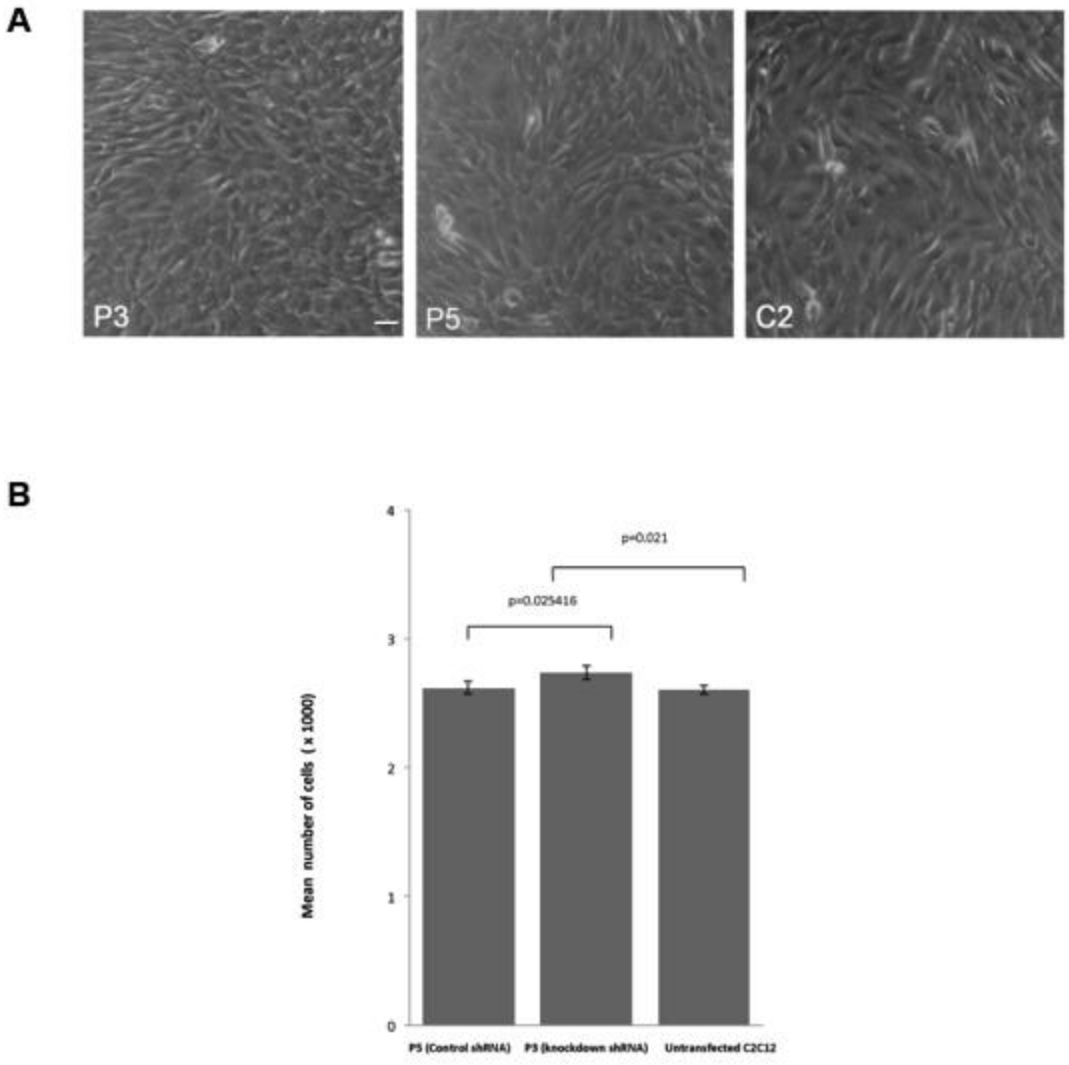


Figure 4.3 Effects of shRNA-mediated knockdown of Notch3 on cell proliferation in C2C12 cultures induced to differentiate

C2C12 myoblasts, either untransfected, transfected with P3 (Notch3 knockdown shRNA) or with P5 (control shRNA) were seeded at the same density (1000 cells/well) in 96 well plates, cultured for 24 hours in growth medium and then for a further 48 hours in differentiation medium containing low levels of serum. (A) Representative phase contrast images of cultures (scale bar = 20µm). (B) Histogram showing cell number determined using the CellTiter 96 Aqueous One Solution colorimetric cell proliferation assay (Promega). Each column shows the mean of three wells (± 1 SD) and when compared using a Student's t-test, cultures transfected with P3 contained significantly more cells than either those transfected P5 plasmid (p=0.025) or untransfected controls (p=0.021).

When skeletal muscle cultures are induced to differentiate, levels of the myogenic regulatory factor MyoD increase in cells destined to differentiate whereas those

that adopt the reserve cell fate become MyoD-ve (Yoshida *et al* 1998). To investigate the effect of Notch3 inhibition on MyoD expression, C2C12 cultures transfected with either P3 (knockdown) or P5 (shRNA control) were seeded into 8-well chamber slides (1000 cells/well), cultured for 24 hours in growth medium and then for a further 48 hours in differentiation medium. The cultures were then fixed and co-immunostained for MyoD and GFP (Materials and Methods, section 2.14.2). Figure 4.4 shows that in the absence of Notch3 (P3), the percentage of cells expressing MyoD was significantly increased (p=0.0001) compared with control cultures, indicating that Notch3 inhibits MyoD expression.

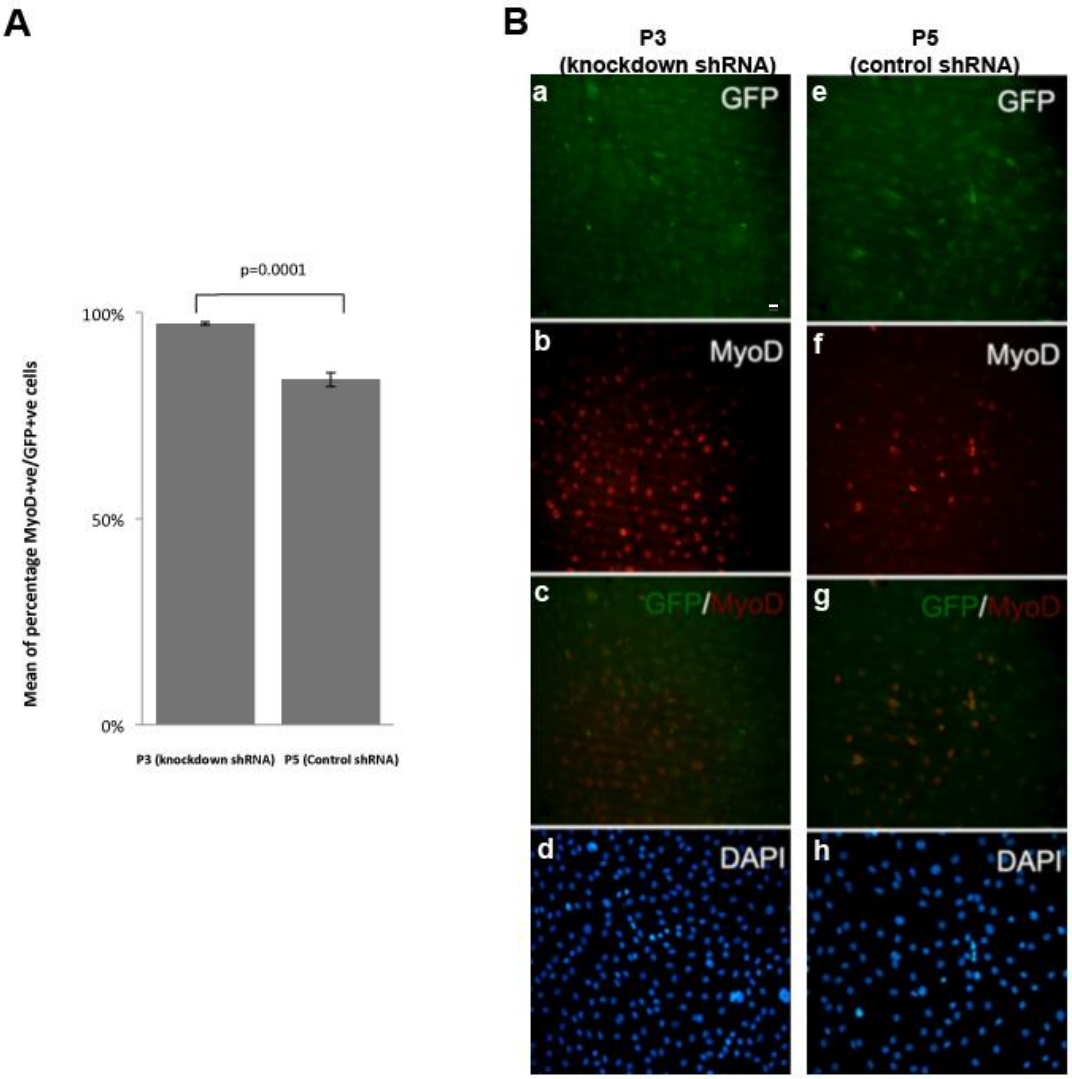


Figure 4.4 Effects of shRNA-mediated knockdown of Notch3 on MyoD expression in C2C12 cultures after 48 hours of differentiation.
C2C12 myoblasts expressing the shRNA plasmids P3 (Notch3 knockdown) or P5 (control) were seeded into 8-well chamber slides, cultured in growth medium for 24 hours and then induced to differentiate. After 48 hours, cultures were fixed and co-

immunostained for anti-MyoD antibody (Dako 5.8A); red) and anti-GFP antibody (A Molecular Probes; green) and counterstained with the nuclear stain, DAPI, to reveal all nuclei. (A) Histogram showing the mean percentage of GFP+ve cells expressing MyoD. For each column, three separate cultures were analysed (at least 200 cells/culture) and the mean value is shown ± 1SD. When compared using a Student's t-test, the percentage of GFP+/MyoD+ve cells was statistically greater in the P3-transfected cultures compared with the control ($p<0.0001$). (B) Representative fluorescent microscopy images showing cultures stained for MyoD (a, e) and GFP (b, f); also shown are overlaid images (c, g) and DAPI staining of the same cultures (scale bar = 20µm).

To investigate the effects of Notch3 knockdown on later stages of myogenic differentiation, P3-transfected (knockdown), P5-transfected (shRNA control) and untransfected C2C12 cultures were established as above and maintained for several days in differentiation medium. After 4 days, all cultures contained large numbers of differentiated, multinucleated myotubes, the number and size of which continued to increase during the following 2 days (Figure 4.5). Even after 4 days of differentiation, the Notch3 knockdown cultures appeared to contain relatively more myotubes and fewer mononucleated single cells than either control cultures (Figure 4.5A) and this shift in proportion appeared more marked at 6 days (Figure 4.5B).

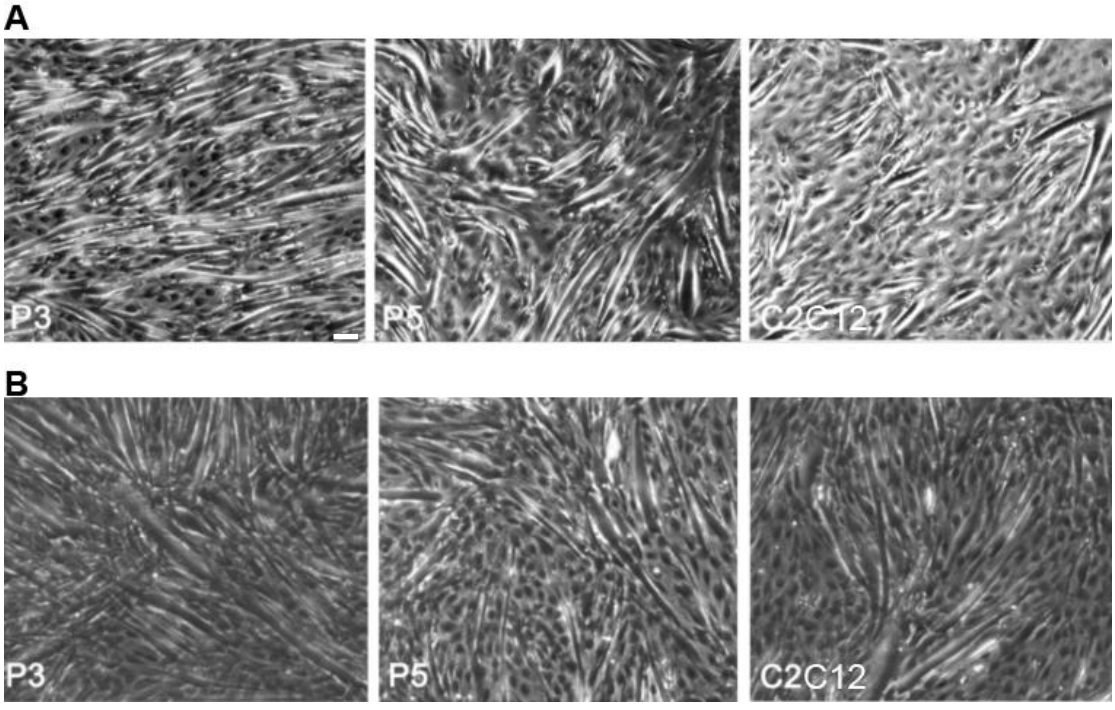


Figure 4.5 Effects of Notch3 knockdown on the morphology of differentiated C2C12 cultures.
Untransfected C2C12 myoblasts and myoblasts transfected with shRNA plasmids P3 (Notch3 knockdown) or P5 (shRNA control) were cultured in growth medium for 24 hours and then induced to differentiate. Phase contrast images of cultures were captured after 4 days (A) and 6 days (B) in differentiation medium (scale bar = 20µm). n=5

To further investigate the effect of Notch3 knockdown on differentiation, P3-transfected (knockdown) and P5-transfected (shRNA control) myoblasts were seeded into 8-well chamber slides (1000 cells/chamber), cultured for 24 hours in growth medium and then switched to differentiation medium. After 5 days, the cultures were fixed and co-immunostained with antibodies against skeletal muscle myosin heavy chain (MyHC) and GFP and counterstained with DAPI to reveal the nuclei (Figure 4.6A). To determine the fusion index (i.e. the proportion of cells that had differentiated), the percentage of total nuclei present in MyHC+ve cells, both multinucleated myotubes and mononucleated myocytes, were counted. The mean percentage of nuclei in MyHC+ cells was significantly higher in the Notch3 knockdown cultures (P3) compared with the shRNA control (P5) ($p=0.002$) (Figure 4.6B). Conversely, the mean percentage of GFP+ve undifferentiated cells (MyHC-ve) was significantly lower in knockdown cultures compared with shRNA controls ($p=0.0004$) (Figure 4.6C). As mentioned previously, the presence of GFP-ve cells in the G418-selected cultures was assumed to be due to the silencing or elimination of the shRNA plasmid in cells that had retained the Neomycin-resistance plasmid. Therefore, the decreased proportion of GFP+ve cells in the undifferentiated population in the P3-transfected cultures suggests that a large proportion of the cells that had not differentiated were not expressing the shRNA sequence and therefore, were probably Notch3+ve.

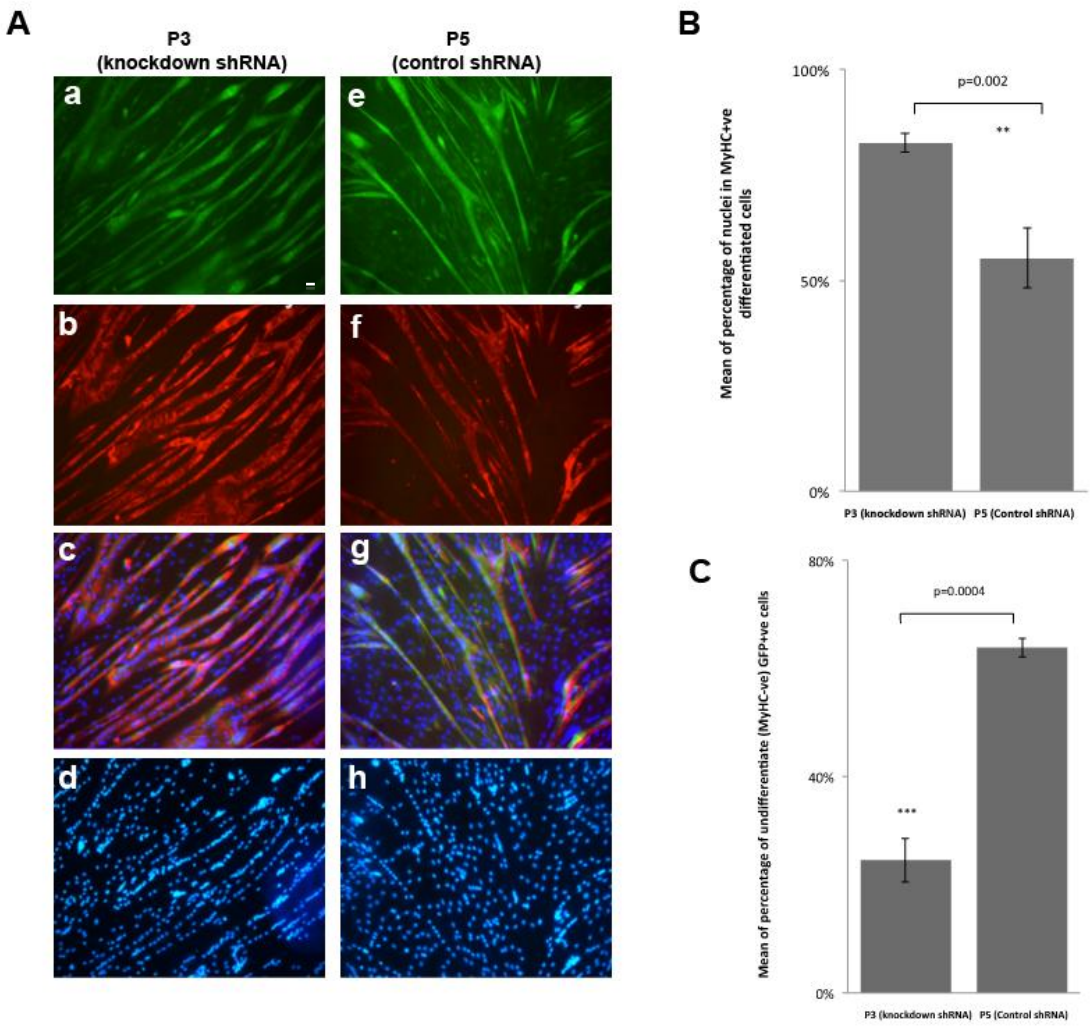


Figure 4.6 Effects of shRNA-mediated knockdown of Notch3 on the differentiation of C2C12 cultures.

(A) C2C12 myoblasts transfected with shRNA Notch3 knockdown plasmid (P3) or an shRNA control plasmid (P5) were allowed to differentiate for 5 days, fixed and co-immunostained with antibodies against skeletal muscle myosin heavy chain (MyHC) a marker of differentiation (MF20; red) and GFP (Molecular Probes; green): DAPI was used as a counterstain to reveal all nuclei (blue) (scale bar = 20µm). Histograms showng (B) the mean percentage of nuclei in differentiated (MyHC+ve) cells and (C) the mean percentage of undifferentiated cells (MyHC-ve) expressing GFP. For (A) and (B), 3 replicate cultures were analysed and at least 200 cells were counted per culture. The mean percentage of the 3 cultures are shown (± 1 SEM) and were compared using a Student's t-test: ** denotes a significant difference in the mean percentage of nuclei in MyHC+ve cells (p=0.002); *** indicates a significant difference in the mean percentage of undifferentiated, GFP+ve cells (p=0.0004).

4.3.3 Apoptosis of C2C12 cultures

The results presented above show that shRNA-mediated knockdown of Notch3 expression in differentiating C2C12 cultures results in an increase in the proportion of cells expressing MyoD followed by an increase in the proportion of cells that differentiate and a decrease in the proportion that persist as undifferentiated reserve cells.

It has been shown that when C2C12 cultures are induced to differentiate, the myoblasts can adopt one of three fates: whilst the majority undergo terminal differentiation to form myotubes, the remainder either persist as undifferentiated, quiescent reserve cells, or undergo apoptosis (Cao *et al*., 2003). Results presented above show that knockdown of Notch3 did not affect myoblast proliferation: indeed, proliferation appeared to be prolonged or increased during the period immediately after differentiation was induced. It is therefore unlikely that the reduction in the number of undifferentiated single cells observed in differentiated Notch3 knockdown cultures is due to an effect on proliferation. However, another possible reason for this reduction could be an increase in the level of apoptosis amongst undifferentiated cells as it has been previously suggested that Notch3 signaling may be a critical determinant of cell survival (Wang *et al*., 2002, Konishi *et al* 2010, Nwabo Kamdje *et al*., 2011).

To investigate potential effects on apoptosis, cultures of untransfected, P3-transfected (knockdown) and P5-transfected (shRNA control) C2C12 myoblasts were seeded in 96-well plates at the same density (1000 cells/well), maintained in growth medium for 24 hours and then transferred to differentiation medium. After 48 hours, Caspase 3/7 activity was measured using the Caspase3/7-Glo assay kit (Promega: Materials and Methods, section 2.4.2). A 48 hour time point was chosen as this is when Notch3 activity is highest following the induction of differentiation (Chapter 3, section 3.3). Caspases are a group of cysteine proteases that are the central effectors of apoptosis (Fesik and Shi, 2001), such that the level of caspase 3/7 activity is an accepted indicator of the level of apoptosis.

After 48 hours of differentiation, the mean level of caspase 3/7 activity was significantly higher in Notch3 knockdown cultures (P3) compared with either shRNA control (P5) or untransfected cultures (Figure 4.7).

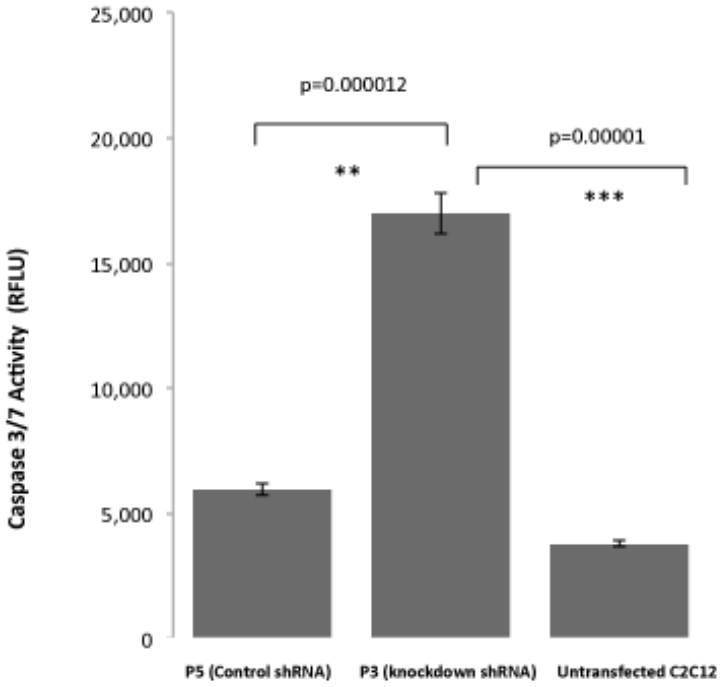


Figure 4.7 Effect of shRNA-mediated knockdown of Notch3 expression on apoptosis in differentiating C2C12 cultures
Untransfected (C2C12), P3-transfected (shRNA-mediated Notch3 knockdown) and P5-transfected (shRNA control) C2C12 cultures were seeded into 96-well plates, maintained in growth medium for 24 hours and then induced to differentiate. After 48 hours in differentiation medium, caspase 3/7 activity was measured using a Caspase3/7-Glo assay kit (Promega). Each column shows the mean activity from three wells, ± 1 SD, which were compared using a Student's t-test. * Denotes a significant difference in the mean caspase 3/7 activity in Notch3 knockdown cultures compared with shRNA control cultures (P5) (p=0.000012): *** denotes a significant difference compared with untransfected cells (C2C12) (p=0.00001).

These data show that the levels of caspase 3/7 activity in the shRNA –mediated Notch3 knockdown cultures were approximately 3.5 times greater than in either of the controls, suggesting that once differentiation is induced, Notch3 activity is required to prevent apoptosis, presumably of cells that have yet to differentiate or would have become reserve cells.

4.4 Effects of Notch3 knockdown and overexpression on the behaviour of mouse primary skeletal muscle cultures

Previous results in this chapter were obtained using the mouse skeletal muscle cell line C2C12 which can be propagated indefinitely and therefore selected for stable expression following transfection. However, this abnormal proliferative capacity, together with the ability to form tumours in vivo (Morgan *et al*., 2002), mean that results obtained with this line require further validation, particularly as Notch3 has also been implicated in tumourigenesis in other systems (Bellavia *et al*., 2002; Dang *et al*., 2006; Park *et al*., 2006; Yamaguchi et l., 2008; Serafin *et al*, 2011).

In order to further investigate the effects of Notch3 on the behaviour of skeletal muscle cell precursors, the shRNA Notch3 knockdown (P3) and shRNA control P5) plasmids described above were used to transiently transfect cultures of primary skeletal muscle cultures derived from mouse satellite cells. In the following experiments, single myofibres were obtained by collagenase-digestion of 8 week-old, C57BL/10 (*i.e. strain*) mouse EDL muscles and the satellite cells isolated by trypsinisation as described in Materials and Methods (section 2.7.2 and 2.7.3). The pooled satellite cells were cultured for 72 hours, and the satellite cell-derived myoblasts were then seeded into Matrigel-coated, 8-well chamber slides at 3000 cells/chamber. Cultures were transfected 24 hours after plating (Materials and Methods, section 2.9) and after a further 24 hours, cultures were induced to differentiate and were maintained for 48 hours prior to fixation and analysis. (Materials and Methods, section 2.2). In addition to shRNA-mediated knockdown of Notch3, the effects of overexpression of constitutively-active Notch3ICD were investigated by transfection with pMSCV-Notch3ICD-IRES-eGFP (pNotch3/MIG) compared with the empty control vector, pMSCV-IRES-eGFP (pMIG) (Materials and Methods, section 2.8). Both of these vectors were modified from the retroviral backbone pMSCV-puro (Clontech) as described in Zammit *et al* (2006) and although originally designed for retroviral packaging, were used as plasmids.

4.4.1 Effects of shRNA-mediated knockdown and overexpression of Notch3 on mouse primary skeletal muscle culture differentiation

To test the effects of modulating Notch3 activity on the ability of primary skeletal muscle myoblasts to differentiate, proliferating cultures of satellite cell-derived myoblasts were established as above and transfected with plasmids encoding either the shRNA Notch3 knockdown sequence (P3), the shRNA control sequence (p5), Notch3ICD (pNotch3/MIG) or the corresponding control, pMIG mig. After 48 hours of differentiation, cultures were co-immunostained for skeletal muscle MyHC and GFP and counterstained with DAPI. To determine the extent of differentiation, the mean percentages of total nuclei present in MyHC+ve cells (myotubes or mononucleated myocytes) were counted. Figure 4.8B shows that the mean percentages of nuclei in differentiated cells were similar following transfection with the control plasmids (P5 and pMIG). However, knockdown of Notch3 activity (P3) resulted in a significant increase in the fusion index (p0.0001) compared with the shRNA control (P5), whereas expression of active Notch3ICD significantly inhibited differentiation compared with the pMIG control (p=0.002). When the undifferentiated, MyHC-ve cell populations were examined, there was a significantly lower mean percentage of GFP+ve cells in the Notch3 knockdown cultures compared with the shRNA control and a significantly higher mean proportion of GFP+ve cells in the Notch3ICD transfected cultures compared with the pMIG control (in both cases, p=0.0003). All of these findings are consistent with the effects of shRNA-mediated knockdown of Notch3 activity on the differentiation of C2C12 cultures (Section 4.3.2), and suggest that in the knockdown cultures, a higher proportion of the remaining cells had formed or become incorporated into myotubes and that of those that did not differentiate, most had not been transfected and were presumably, Notch3+ve. Where NotchICD was overexpressed, differentiation was inhibited and the undifferentiated population was predominantly GFP+ve and therefore expressing Notch3ICD.

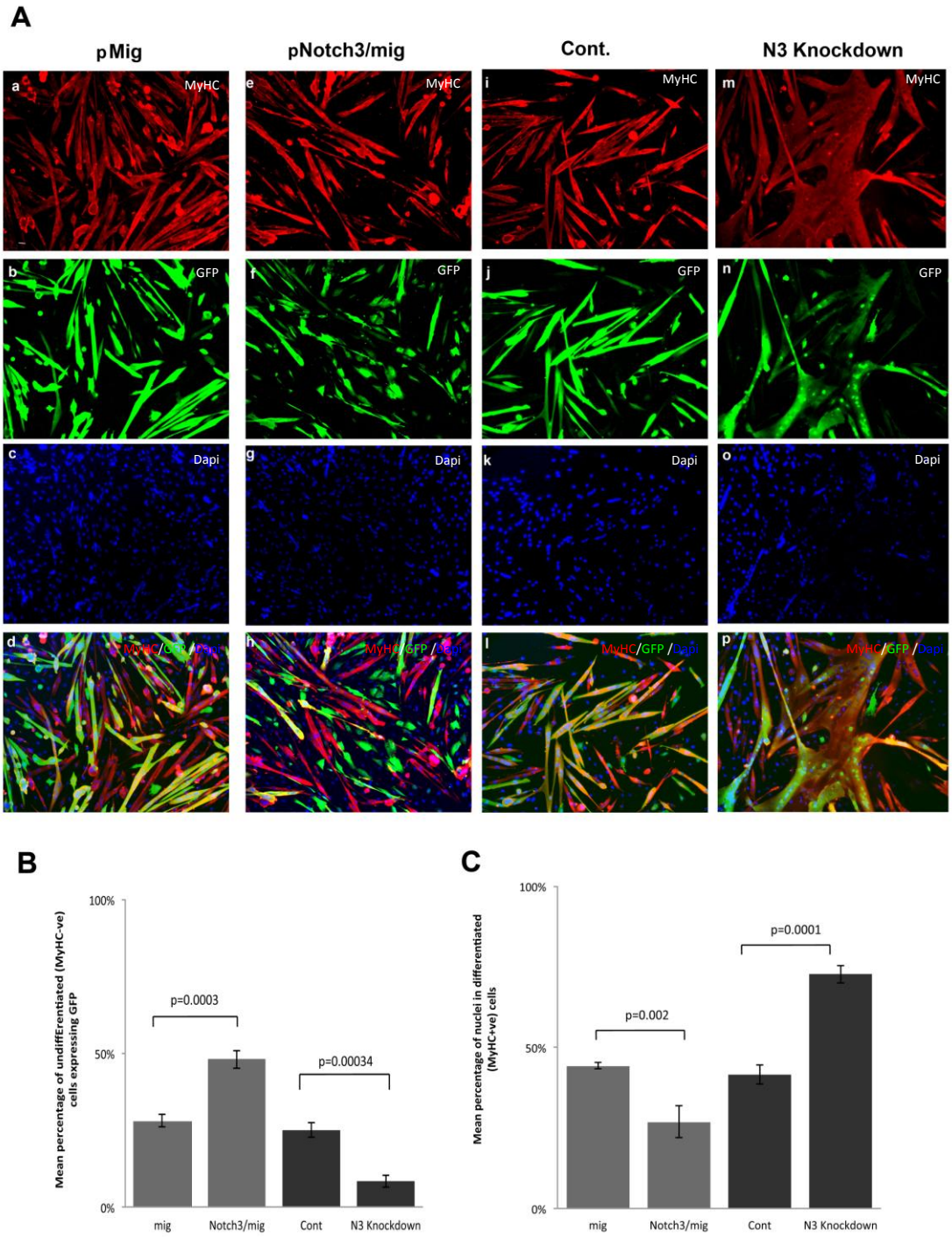


Figure 4.8 Effects of modulating Notch3 activity on the differentiated of mouse primary skeletal muscle cultures

Cultures of primary mouse satellite cell-derived myoblasts were established, transfected with either P3 (shRNA Notch3 knockdown), P5 (shRNA control), pNotch3/MIG (constitively-active Notch3ICD) or pMIG (control) and allowed to differentiate for 48 hours. (A) Fluorescence microscopy images of representative cultures fixed and co-immunostained for the expression of skeletal muscle MyHC (MF20 (DSHB); red) and GFP (Molecular Probes; green), counterstained with DAPI (blue) to reveal all nuclei. Scale bar = 20µm. (B) Histogram showing the mean percentages of nuclei in differentiated (MyHC+ve) cells. (C) Histogram showing the mean percentages of GFP+ve (i.e. transfected) undifferentiated, MyHC-ve cells. In

both (B) and (C), each column represents the mean of three cultures (at least 200 nuclei from each).

The fluorescence microscopy images shown in Figure 4.8A suggest that the myotubes formed in the Notch3 knockdown cultures were much larger than in either of the control cultures. To further investigate this observation, myotube size was determined by measuring the average width of a random sample of GFP+ve myotubes. Briefly, mouse primary skeletal muscle cultures were prepared, transfected, allowed to differentiate for 48 hours and co-immunostained for MyHC and GFP, all as described above. Five random areas were selected from 8-well chambers and mean the widths of individual myotubes (10 per field) were calculated from 10 measurements taken along the length of the cell. The data are presented in Figure 4.9 and confirm that the mean width of the myotubes in the shRNA-mediated Notch3 knockdown cultures (P3) was significantly greater than in the shRNA controls (P5) (p=0.000014). Conversely, the Notch3ICD-transfected cultures were less well-differentiated (Figure 4.8) and the average myotube width was smaller than in the pMIG-transfected controls.

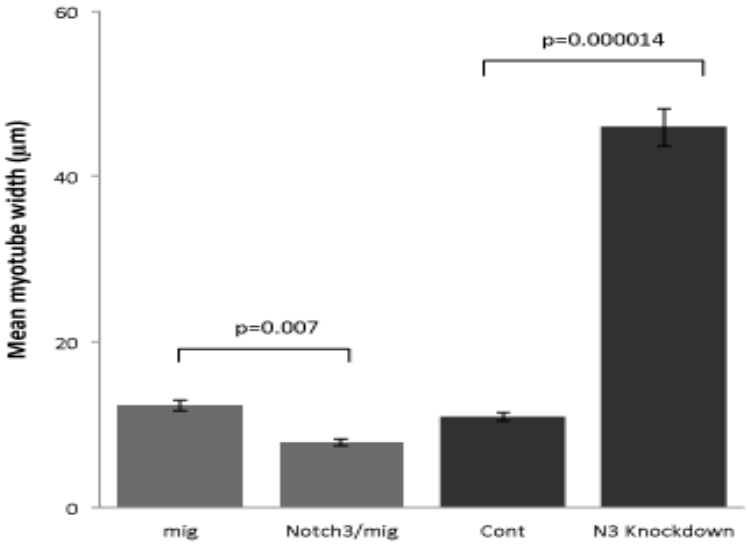


Figure 4.9 Effects of modulating Notch3 activity on myotube width in differentiated mouse primary skeletal muscle cultures

Cultures of primary mouse satellite cell-derived myoblasts were established, transfected with either P3 (shRNA Notch3 knockdown), P5 (shRNA control), pNotch3/MIG (constitively-active Notch3ICD) or pMIG (control) and allowed to differentiate for 48 hours. After co-immunostaining for skeletal muscle MyHC and GFP, the mean width of individual GFP+ve myotubes was calculated from 10 measurements taken along the length of the cell. For each condition, 3 cultures were analysed, involving measurement of 10 myotubes from each of 5 randomly selected fields.

The results presented in Figure 4.9 confirm the subjective observation that therefore inhibition of Notch3 activity increases myoblast fusion and promotes myotube hypertrophy in primary skeletal.

4.4.2 Effects of shRNA-mediated knockdown and overexpression of Notch3 on MyoD expression in differentiated mouse primary skeletal muscle cultures

To investigate the effects of modulating Notch3 activity on MyoD expression, mouse primary skeletal muscle cultures were prepared as described above and transfected with either P3 (shRNA knockdown), P5 (shRNA control), pNotch3ICD/MIG (Notch3ICD) or pMIG (Control). After 24 hours of differentiation, the cultures were fixed and co-immunostained for MyoD and GFP.

Figure 4.10 shows that when the GFP+ve populations were analysed (i.e transfected), the mean percentage of MyoD+ve cells was significantly higher in the P3-transfected (shRNA knockdown) compared with the P5-transfected (shRNA control) controls ($p=0.00001$). Conversely, cultures transfected with constitutively active Notch3ICD (pNotch3/MIG) contained a significantly lower mean percentage of MyoD+ve cells ($P=0.0000001$) than those transfected with the relevant control (pMIG). These results show that as in C2C12 cultures, (Figure 4.4), knockdown of Notch3 activity results in increased MyoD expression and further, that overexpression of Notch3 strongly inhibits MyoD expression.

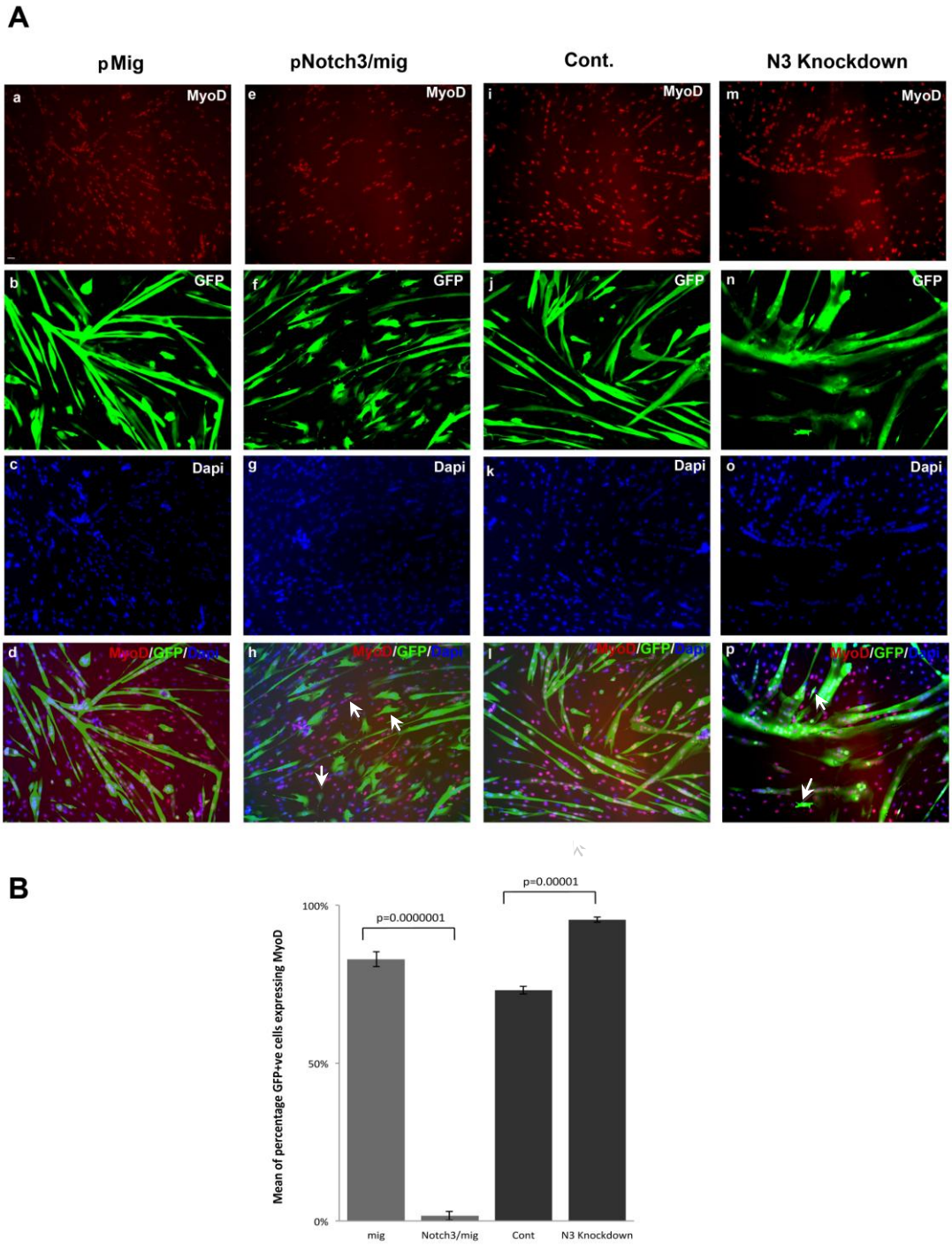


Figure 4.10 Effects of modulating Notch3 activity on MyoD expression in differentiated mouse primary skeletal muscle cultures

Cultures of primary mouse satellite cell-derived myoblasts were established, transfected with either P3 (shRNA Notch3 knockdown), P5 (shRNA control), pNotch3/MIG (constitutively-active Notch3ICD) or pMIG (control) and allowed to differentiate for 48 hours. (A) Fluorescence microscopy images of representative cultures fixed and co-immunostained for the expression of MyoD (Dako 5.8A; red) and GFP (Molecular Probes; green), counterstained with DAPI (blue) to reveal all nuclei. Scale bar = 20µm. (B) Histogram showing the mean percentages of transfected cells (i.e. GFP+ve) expressing MyoD. For each column, three cultures were analysed and at least 200 cells were counted from each.

4.4.3 Effects of shRNA-mediated knockdown and overexpression of Notch3 on Pax7 expression in differentiated mouse primary skeletal muscle cultures

Pax7 is a paired box transcription factor that is expressed by quiescent satellite cells, maintained during proliferation and down-regulated during myogenesis such that in a differentiated culture, Pax7 marks cells that have adopted an undifferentiated, reserve cell fate (Zammit *et al*., 2004, 2006; Halevy *et al*., 2004; Nagata *et al*., 2006). Although the C2C12 cell line used in earlier studies expressed little (if any) Pax7, primary skeletal muscle cultures established, transfected and differentiated as described above were co-immunostained for Pax7 and GFP to directly investigate the effects of modulating Notch3 activity on the number of single cells (Figure 4.9). The results clearly show that shRNA-mediated inhibition of Notch3 activity (P3) resulted in a significant decrease in the number of Pax7+ve cells compared with cultures transfected with the shRNA-control (P5) ($p=0.00004$): in contrast, a significantly greater percentage of cells expressing Pax7 were present in cultures transfected with constitutively-active Notch3ICD (pNotch3/MIG) compared with the relevant control plasmid, pMIG ($p=0.0007$). These data are consistent with and extend the findings presented in Figure 4.8 by showing that in the absence of normal levels of Notch3 activity, few (if any) myoblasts are able to adopt and maintain the Pax7+ve/MyoD-ve phenotype characteristic of a reserve cell fate (Nagata *et al*., 2006), whereas overexpression of Notch3 activity inhibits MyoD expression and increases the proportion of Pax7+ve cells that persist in differentiated cultures.

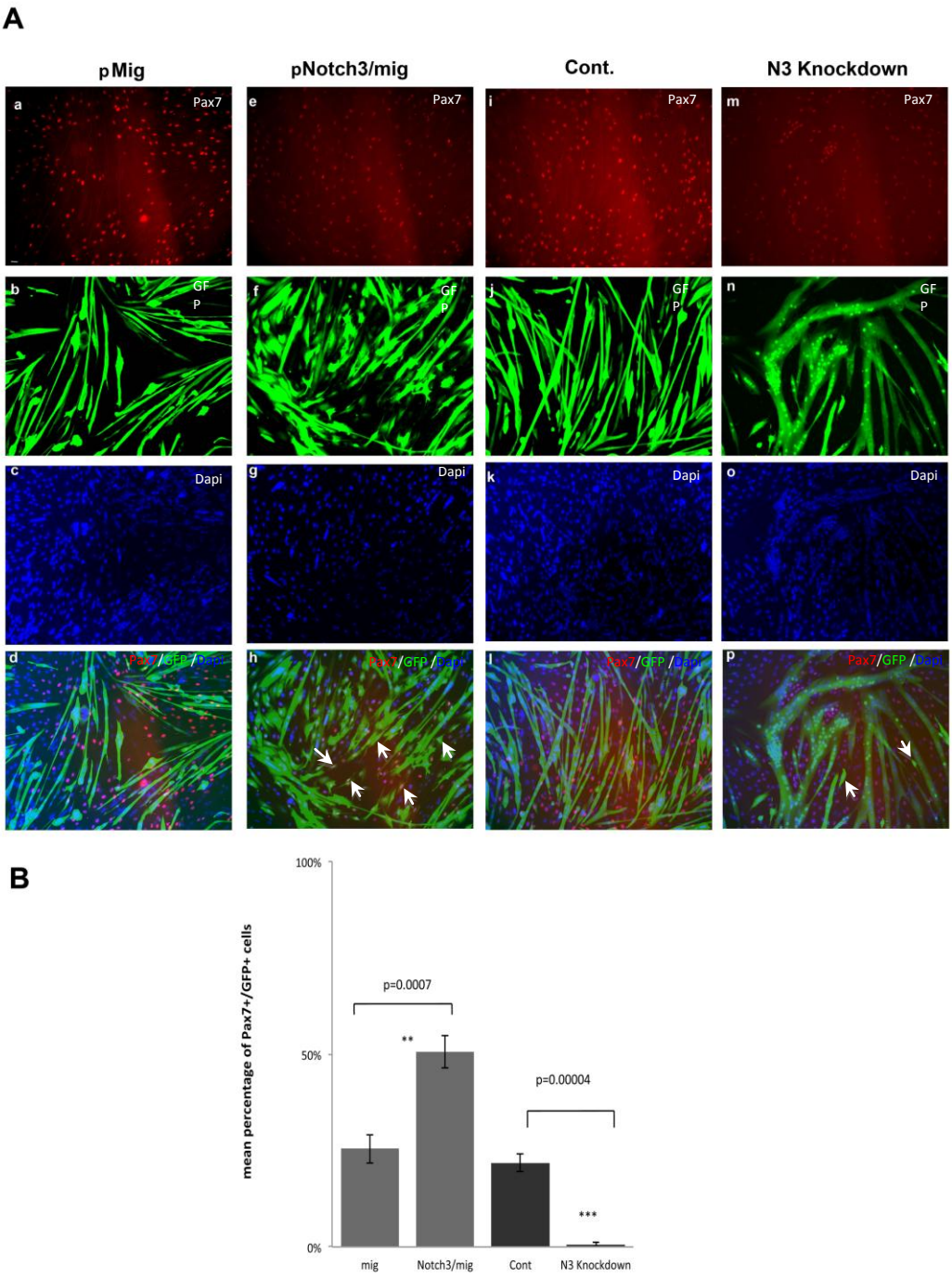


Figure 4.11 Effect of modulating Notch3 activity on Pax7 expression in differentiated primary skeletal muscle cultures.

Cultures of primary mouse satellite cell-derived myoblasts were established, transfected with either P3 (shRNA Notch3 knockdown), P5 (shRNA control), pNotch3/MIG (constitutively-active Notch3ICD) or pMIG (control) and allowed to differentiate for 48 hours. (A) Fluorescence microscopy images of representative cultures fixed and co-immunostained for the expression of Pax7 (DSHB; red) and GFP (Molecular Probes; green), counterstained with DAPI (blue) to reveal all nuclei. Scale bar = 20µm. (B) Histogram showing the mean percentages of transfected cells (i.e. GFP+ve) expressing Pax7. For each column, three cultures were analysed and at least 200 cells were counted from each.

4.5 Discussion

In chapter 3, inactive Notch3 was present during proliferation, whereas active Notch 3 was first detected at the time when the decision to become a myotube or reserve cell is being made. By Western blot analysis, active Notch 3 was found to be highly expressed by reserve cells, but was completely absent from the myotube fraction. These findings strongly suggest that Notch3 may play a crucial role in the specification and maintenance of the stem cell phenotype within a differentiating culture.

The hypothesis was that if Notch3 is required for self-renewal of the stem cell compartment, reduced levels of expression and therefore activity should promote differentiation and enhance myotube formation. Since Notch3 was expressed in myogenic cultures at the onset of differentiation and maintained in those cells that adopted a quiescent reserve cell fate, a lack of Notch3 would reduce the number of reserve cells in a differentiated culture. The shRNA was used to knockdown expression of Notch3 in C2C12 and primary cultures, immediately prior to initiating differentiation.

Reduced expression of Notch3 by shRNA plasmid had no apparent effect on C2C12 myoblast proliferation as demonstrated by proliferation assay. This is consistent with the fact active Notch3 is not present in proliferating cultures.

When the density of cultures reaches confluency, the repression of Notch3 appeared to cause a decrease in the size of C2C12 cells. From the phase contrast images, the morphology of cells in Notch3 knockdown cultures appear rounder and smaller. As Notch3 alters the actin cytoskeletal dynamics in VSMC, so there is an increase in actin fibres and steady-state levels of polymerised actin (Domenga *et al*., 2004). It is possible in C2C12 cells Notch3 may increase cell-size by altering actin dynamics, which may enhance cell motility and migration (Chakravarti *et al*., 2005). Therefore by inhibiting Notch3 expression the size of cells decreased.

MyoD is one of the four myogenic regulatory factors (MRFs) that are essential for myogenensis. MyoD is undetectable in the majority of quiescent satellite cells (Grounds *et al*., 1992). As satellite cells become activated in response to injury, they will express MyoD (Cooper *et al*., 1999). At earlier time-points following induction of differentiation, MyoD expression appeared to be elevated in cultures with reduced Notch3 expression. These observations are consistent with the hypothesis that loss of Notch3 leads to a decrease in the number of reserve cells such that almost all myogenic precursors that would have remained undifferentiated, instead, commit to differentiation. This suggests that Notch3 may inhibit myogenic differentiation by diminishing MyoD, hypothetically facilitated through either the RBP-JK-dependent or RBP-JK-independent pathway (Kopan *et al*., 1994; Shawber *et al*., 1996; Kuroda *et al*., 1999; Nofziger *et al*., 1999).

Repressed Notch3 accelerated the myotubes formation during differentiation. When differentiation was induced, the cultures, in which expression of Notch3 was reduced or absent, appeared to contain a greater number of larger myotubes compared to control and untransfected cultures. The myogenic differentiation process was significantly sped up in Notch3 knockdown C2C12 cultures. As a result, these cultures have a massive decrease in the number of undifferentiated reserve cells.

Wang *et al*. (2002) postulated that Notch3 signalling might be a critical determinant of VSMC survival. Notch3 may play an anti-apoptosis role. An apoptosis assay confirmed that loss of Notch3 leads to 3.5 times higher rate of apoptosis indicated by increased in Caspase 3/7 activity. Nevertheless, even with the higher rate of apoptosis, the lost of Notch3 still resulted in an increased fusion index characterised by higher average numbers of nuclei in myotubes.

Work by Cao and colleagues (2003) revealed that p27 appears to be involved in the regulation of myogenic differentiation, as it is highly upregulated when C2C12 myoblast differentiation is induced. p27 is found highly expressed in reserve cells, it induces and maintains a quiescent state in cells (Miskimins *et al*., 2001; Olashaw and Pledger, 2002). Since Notch3 highly upregulated p27 expression, which induces

cells to enter a quiescent state, it is plausible that suppressed Notch3 also suppressed level of p27 hence leading to loss of quiescent reserve cells population during myogenesis. On the other hand, in the apoptosis assay activity of Caspase3/7 was elevated in Notch3 knockdown cultures. This is consistent with the increased caspase3/7 activity coupled with the down-regulation of p27 in nuclei (Hashimoto *et al*. 2008).

In the primary cultures section, satellite cell-derived myoblasts were used to compare the Notch3 knockdown effect observed in C2C12 cells. Notch3 knockdown had no significant effect on proliferating primary myoblasts, but when differentiation was induced, Notch3 knockdown primary cultures produced changes that were similar to those observed when Notch3 was knocked down in C2C12. Cell population increased and in differentiated cultures the MyoD+ cells increased significantly. These results again consistent with the fact active Notch3 is not present in proliferating C2C12 cultures in the earlier findings.

When differentiation was induced, the effect of Notch3 on differentiation becomes apparent. Notch3 knockdown cultures significantly accelerated the myogenic differentiation process. As expected, knockdown Notch3 cultures had more MyoD+ cells after differentiation was induced consistent with the C2C12 results. Knockdown Notch3 upregulates MyoD expression, reflecting that Notch3 may inhibit myogenic differentiation by downregulating MyoD, presumably facilitated via either the RBP-JK-dependent or RBP-J-independent pathway (Kopan *et al*., 1994; Shawber *et al*., 1996; Kuroda *et al*., 1999; Nofziger *et al*., 1999). Active Notch3 was found expressed in reserve cells exclusively; reserve cells are in fact known to downregulate their expression of MyoD (Yoshida *et al*., 1998). It is possible that upon the induction of differentiation, a lack of Notch3 in the cultures upregulates MyoD expression and enhances differentiation, as a consequence loss of reserve cells population in Notch3 knockdown cultures.

Knockdown Notch3 in both C2C12 and primary cultures studies suggested that Notch3 inhibits differentiation. Since Notch3 also down-regulates Caspase 3/7 and highly upregulates p27, which induces cells to enter a quiescent state, it is likely

that endogenous Notch3 expression predominates in reserve cells, inhibiting their differentiation and ensuring that the cells become quiescent.

As mentioned earlier section, high levels of active Notch3 in reserve cells caused downregulation of MyoD expression and inhibition of differentiation as well as upregulation of p27 expression, which causes the cells to reversibly withdraw from the cell cycle and become quiescent. This is consistent with the high-level expression of the cdk inhibitor p27 observed in Notch3-expressing cells, which should induce cells to enter G0 phase (Cao *et al*., 2003).

Notch inhibition by its antagonist numb causes proliferating myoblasts to commit to terminal differentiation (Conboy and Rando, 2002). The inhibition of notch by overexpressing numb or by use of a γ-secretase inhibitor resulted in an enhanced fusion index of the myoblasts and increased differentiation (Kitzmann *et al*., 2006).

In differentiated cultures, repressed Notch3 showed a large increase in the number of nuclei in differentiated cells, as well as an increase in the width of myotubes. This is consistence to the results inNotch3 knockdown C2 cell line. The fusion index in the Notch3 knockdown cultures was 2 fold higher than controls. By estimate the width of myotube, suppressed Notch3 expression resulted 4 fold wider than controls. This suggested that suppressed Notch3 leading to myotubes hypertrophy.

Cultures of differentiated Notch3 knockdown clones have larger myotubes and fewer reserve cells again consistence with C2C12 Notch3 knockdown results. This observation has led to the hypothesis that inhibition of Notch3 enabling the recruitment of more reserve cells into the differentiation pathway leads to hypertrophy.

Analysis of the myotube and reserve cell populations in chapter 3 revealed that in C2C12 cells the level of Notch3 was significantly higher exclusively in the reserve cell population. This same pattern of expression was preserved in the Notch3 overexpression clones in primary cultures. Expression levels of Pax7+ cells increased approximately 25% more than controls in the differentiated cultures. Figure 4.11

proposed that overexpression of Notch3 keep a population of myoblasts in a slowly proliferating non-dividing state that is capable of giving rise to Pax7+ reserve cells.

Constitutively active Notch3 increased the number of Pax7+ cells, which withdrawed from cell cycle and become quiescent, and decrease the number of cells that express MyoD and differentiate. Thus endogenously active Notch3 may inhibit the differentiation of a subset of progeny by preventing MyoD expression, ensuring that they remain undifferentiated and quiescent and hence maintain their expression of Pax7. This is consistent with their effects during C2C12 differentiation, where Notch3 inhibit differentiation and reduce MyoD expression.

Differentiated primary cultures formed a heterogeneous population of differentiated myotubes and undifferentiated reserve cells, comparable with differentiated C2C12 cell cultures. Overexpressed constitutively active Notch3 was capable of inhibiting myogenic differentiation and resulted in downregulation of both MyoD and MyHC.

In proliferating primary myoblasts, constitutively active Notch3 completely downregulated MyoD expression and completed inhibited differentiation when it was induced. It is possible that upon the induction of differentiation, endogenous Notch3 remains active in a subset of cells, where it downregulates MyoD expression and inhibits differentiation, to ensure that those reserve cells remain undifferentiated. Reserve cells are in fact known to downregulate their expression of MyoD (Yoshida *et al*., 1998). Suggesting that in reserve cells where active Notch3 is highly expressed, it may act as the predominant receptor to inhibit myogenic differentiation.

Together, the results obtained from both Notch3 knockdown and overexpression in C2C12 or primary cultures suggests Notch3 may play an important role in the specification of cells that are maintained in a quiescent stem cell state and may therefore be involved in replenishment of the satellite cell pool.

For Notch3 to have an effect on myoblast fate decisions, it must interact with a Notch ligand. One possible explanation is that such a ligand may be expressed on newly formed myotubes that can interact with remaining undifferentiated cells: one possible candidate is DL4. When the expression of Notch ligands was investigated in the culture system used above, DL1 1 was expressed throughout differentiation and in differentiated cultures and was present in both myotube and reserve cell fractions. In contrast, DL4 is not expressed until myotubes started to form differentiated cultures, DL4 is only expressed by differentiated cells and not in the reserve cell fraction, the opposite pattern to Notch3. Previously, DL4 was found to be expressed in arterial endothelium (Shutter *et al*., 2000), but has not been reported in skeletal muscle cells.

Chapter 5: Notch3 regulation during C2C12 reserve cell activation

5.1 Introduction

The aims of the experiments presented in this chapter were to investigate which of these Notch DSL ligands could be responsible for maintaining Notch3 activity and therefore the quiescent, undifferentiated reserve cell phenotype.

When a skeletal muscle culture is induced to differentiate, myoblasts either withdraw permanently from the cell cycle and initiate terminal myogenesis, enter a reversible quiescent state (Kitzmann *et al*., 1998; Yoshida *et al*., 1998) or undergo apoptosis (Dee *et al*., 2002). Results presented in the previous chapters implicate Notch3 activity in determining myoblast cell fate. Specifically, in the absence of Notch3 activity, the vast majority of myoblasts differentiate whilst few adopt the reverse cell fate. This alteration of the normal balance of cell fates is accompanied by an increase in apoptosis, although it has yet to be determined whether this reflects a requirement for Notch3 in the survival of nascent reserve cells or is due to a failure to specify reserve cell fate. In a differentiated culture, Notch3 activity is restricted to the reserve cell population and overexpression of Notch3 inhibits differentiation markedly increasing the proportion of reserve cells.

Notch receptor activation requires interaction with DSL ligands (Introduction, sections 1.12 and 1.13). Since Notch3 is active in reserve cells present in differentiated cultures of the clonally-derived cell line C2C12, the DSL ligand(s) responsible must be expressed within the culture. Results presented in Chapter 3 show that DL1 1, Jagged 1 and Jagged 2 are present on proliferating myoblasts and that following differentiation, DL1 1 and DL4 are expressed by myotubes whilst reserve cells are Jagged 1+ve/Jagged 2+ve (section 3.2, 3.3).

5.2 Regulation of Notch3 and Notch1 receptor activity during C2C12 reserve cell activation

Previous studies have shown that reserve cells can be isolated from differentiated skeletal muscle cultures and activated to enter the cell cycle by exposure to mitogen-rich growth medium (Yoshida *et al*., 1998). If Notch3 activity is responsible for maintaining the reserve cell phenotype, this implies that this must be overcome, inhibited or lost during the activation process.

To investigate Notch3 expression during activation, C2C12 myoblasts were seeded into 10cm-diameter Petri dishes at $5x10^5$cells/dish and after 24 hours in growth medium, were transferred to differentiation medium (Materials and Methods, section 2.2.4). After 4 days of differentiation, reserve cells were isolated by partial trypsinisation as described in the Materials and Methods (section 2.5.2), pooled, replated and reactivated by culturing in serum-rich growth medium.

5.2.1 Time course of C2C12 reserve cell activation

To investigate the timing of re-entry into the cell cycle following transfer to growth medium, reserve cells were seeded into 8-well chamber slides at 2500 cells/chamber. After 1, 3, 6, 12 and 24 hours, cultures (three per time point) were pulsed with BrdU for 1 hour, washed, fixed and then immunostained for BrdU-incorporation to determine the percentage of cells that had entered S-phase during the pulse. Figure 5.1 shows that only approximately 7% of the reserve cells incorporated BrdU during the first hour and that the vast majority of cells entered S-phase 12-24 hours after being returned to growth medium.

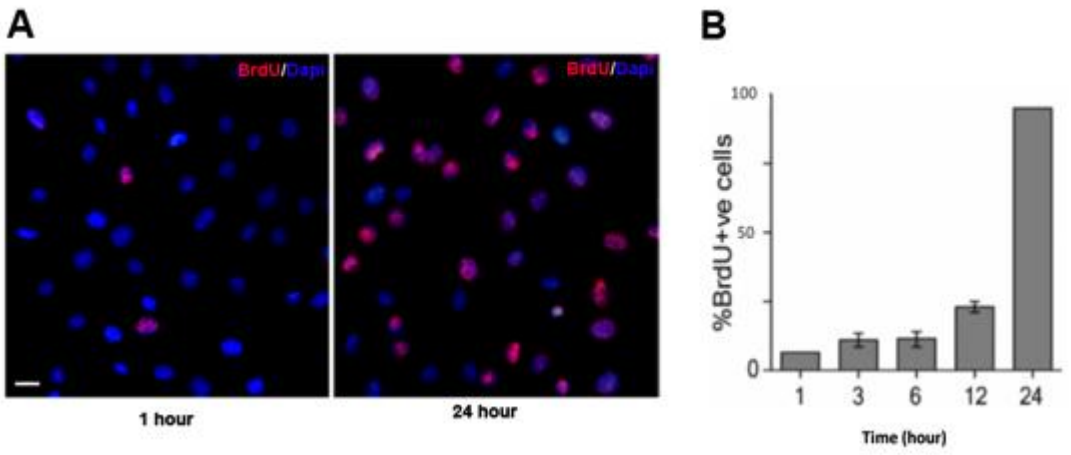


Figure 5.1 Time course of C2C12 reserve cell activation determined by BrdU incorporation

Quiescent reserve cells were isolated from C2C12 cultures after 4 days of differentiation and seeded into 8-well chamber slides at 2500 cells/chamber. After 1 hour, 3 hours, 6 hours, 12 hours and 24 hours in growth medium, cultures were pulsed for 1 hour with BrdU, fixed and immunostained with an antibody against BrdU (BU1/75, Abcam) and counterstained with DAPI to reveal all nuclei. (A) Representative cultures 1 and 24 hours after transfer to growth medium, immunostained for BrdU (BU1/75; red) and counterstained with DAPI (blue) (scale bar = 10µm). (B) Histogram showing the mean percentages of cells that had incorporated BrdU during the pulse. Three cultures were analysed at each time point with at least 100 cells counted per culture: mean values are shown ± SEM.

5.2.2 Changes in Notch3 and Notch1 expression during C2C12 reserve cell activation

The above data show that once returned to serum-rich growth medium, C2C12 reserve cells re-enter the cell cycle with the majority of cells entering S-phase after 12-24 hours and experiments were therefore carried out to investigate the expression of Notch3 and Notch1 during this activation process. Reserve cells isolated from differentiated C2C12 cultures as described above, seeded into 10cm-diameter Petri dishes at 10^5 cells/dish and maintained in growth medium. Individual cultures were taken after 0, 0.5, 0.75, 1.5, 3, 6, 12 and 24 hours of reactivation and total RNA and protein were extracted (Materials and Methods, sections 2.5.2.1 and 0) for analysis by RT- PCR and Western blotting, respectively. Figure 5.2 shows RT-PCR analysis of the expression of Notch3, Notch1 and MyoD transcripts during

activation. RT-PCR was carried out as described in the Materials and Methods (section 2.18) using the primers and conditions listed in section 2.18 (

Table 2.3).

The results show that as expected, freshly isolated reserve cells (0 hours) expressed Notch3, a very low level of Notch1 and no MyoD transcript. However, between 0.75 and 1.5 hours, Notch3 transcription was down-regulated, accompanied by an increase in the level of Notch1 and the appearance of MyoD mRNA. After 24 hours, by which time the cells had entered S-phase (Figure 5.1), Notch3 transcript was almost undetectable whilst both Notch1 and MyoD were clearly expressed.

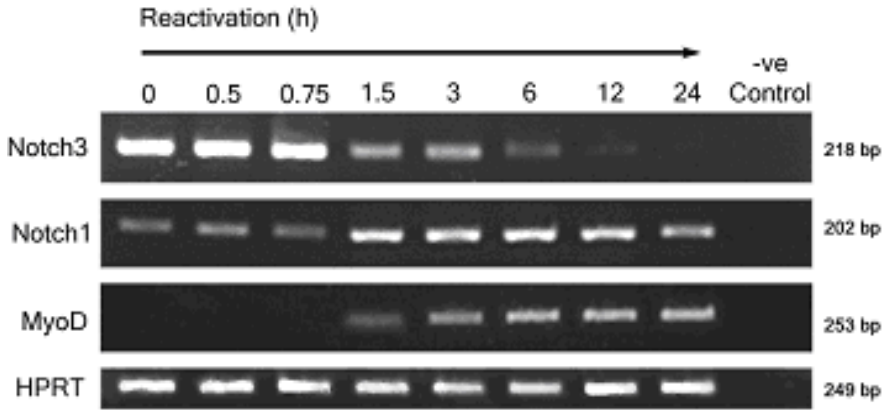


Figure 5.2 Expression of Notch3 and Notch1 transcripts during activation of C2C12 reserve cells
Quiescent C2C12 reserve cells were isolated and activated in serum-rich growth medium. Total RNA was extracted from cultures at the time points indicated and subjected to RT-PCR analysis for the presence of Notch3, Notch1 and MyoD transcripts. Reaction products were run on 1.5% agarose gels/TBE gels and stained with ethidium bromide. HPRT was used as a loading control and for the negative control reactions, RNA was replaced by water. Predicted product sizes are shown on the right and were consistent with appropriate DNA marker ladders. n=3

Western blotting analysis of protein extracted from a series of cultures taken at the same time points during C2C12 reserve cell activation were consistent with the RT-PCR results shown above and further, revealed changes in activities of the translated receptors (Figure 5.3). An antibody raised against the Notch3ICD was

used to distinguish between membrane-associated receptor ("inactive") and "active" ICD released from the membrane by S2/S3 cleavage (Introduction, section 1.13). The results show that during the first 1.5 hours, both active and inactive Notch3 was present although by 3 hours, active Notch3ICD could no longer be detected and there was evidence of the presence of unprocessed protein (i.e. pre-S1 cleavage). In contrast, Notch1 was first detected after 1.5 hours using an antibody specific for the active Notch1ICD and remained active at all subsequent time points.

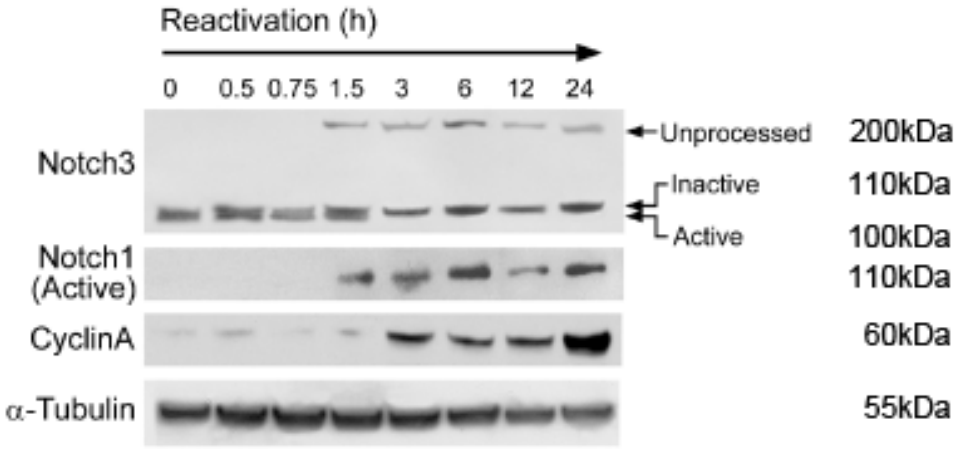


Figure 5.3 Expression of Notch3 and Notch1 proteins during C2C12 reserve cell activation
Quiescent C2C12 reserve cells were isolated and activated in serum-rich growth medium. Protein was extracted from cultures at the time points indicated, separated by SDS-PAGE on 10% acylamide gels and transferred onto nylon membrane. Notch3 was detected using an antibody raised against Notch3ICD (M-20, Santa Cruz) and Notch1 with an antibody specific for the active ICD (Ab8925, Abcam). The blot was also probed for cyclinA (CY- A1, Sigma) as a marker of re-entry into the S-phase cell cycle and α-tubulin as a loading control (Sigma, DM1A). Molecular masses were estimated from appropriate SDS-PAGE markers and are shown on the right. For Notch3, "unprocessed" corresponds to the receptor before S1 cleavage, "inactive" to membrane-associated receptor and "active to the ICD released by S2/S3 cleavage (Introduction, section 1.13). n=3

Results presented in Figure 5.2 and Figure 5.3 reveal a rapid switch in Notch receptor activity initiated approximately 1 hour after exposure to serum-rich medium. The data confirm that Notch3 is expressed and active in quiescent reserve

cells, whilst any Notch1 is inactive, consistent with the results presented in Chapter 3 (section 3.3). By 1.5 hours however, Notch3 was shown to be down-regulated at the level of transcription and any remaining receptor was inactive: in contrast, transcription of Notch1 increased and the translated receptor was active. Significantly, these events were accompanied by increased expression of MyoD and re-entry into the cell cycle as determined by the presence of cyclinA. Again, these findings are consistent with earlier results suggesting that Notch3 activity inhibits both MyoD expression and proliferation (Chapter 4, section 4.4.2) and the known pro-proliferative effects of Notch1 activity on skeletal muscle precursors .

5.3 Effects of exposure to specific DSL ligands on C2C12 reserve cell activation

In mammals, Notch signalling can be mediated by interactions between any of four receptors and five DSL ligands (Introduction, section 1.12). Evidence suggests that different receptor/ligand combinations can result in different outcomes including divergent cell fates reported in myeloid (Neves *et al*., 2006), T-cell (Amsen *et al*., 2004; Lehar *et al*., 2005; Rutz *et al*., 2005) and prosensory precursor cells (Brooker *et al*., 2006). To identify which DSL ligand(s) could be responsible for maintaining Notch3 activity in reserve cells within a differentiated culture, co-culture experiments were carried to investigate the effects of individual DSL ligands on reserve cell reactivation.

5.3.1 Generation of 3T3 fibroblast lines expressing individual DSL ligands

Full-length cDNAs encoding murine Delta-like 1 and 4 ((de La Coste *et al*., 2005) and Jagged1 (Lehar *et al*., 2005) were each cloned into the neomycin-resistance gene-containing expression plasmid, pC1-Neo (Promega) (Materials and Methods, section 2.12). Individual 3T3 fibroblast cultures were transfected with these plasmids and selected with G418 for 2 weeks (Materials and Methods, section 2.9.4). To confirm expression of the relevant ligands, total RNA was extracted from stable, uncloned cultures and analysed by RT-PCR (Materials and Methods, section 2.18). Details of primers and RT-PCR conditions are provided in

Table 2.3.

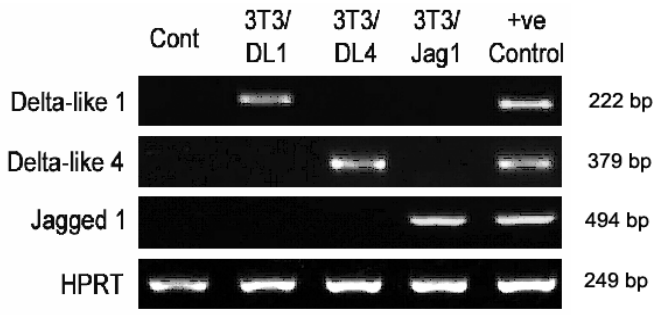


Figure 5.4 RT-PCR analysis of DSL ligand expression in transfected 3T3 fibroblast cultures

G418-resistant cultures of mouse 3T3 fibroblasts were generated by transfection with a neomycin-resistance plasmid (pC1-Neo) containing full-length cDNA encoding either DL1 1, DL4 or Jagged1(3T3/DL1, 3T3/DL4 and 3T3/Jag1, respectively). Total RNA was isolated and subjected to RT-PCR analysis for the expression of the three DSL ligands. Reaction products were run on 1.5% agarose/TBE gels and stained with ethidium bromide. "Cont." refers to untransfected control 3T3 fibroblasts cells, the "+ve Control" was total mouse embryo and HPRT was used as a control for the starting amount of total RNA. Predicted product sizes are shown on the right and were consistent with appropriate DNA marker ladders. n=3

Figure 5.4 shows that following selection, each of the 3T3 cultures expressed the transfected cDNA. Importantly, untransfected 3T3 fibroblasts did not contain detectable levels of transcript for any of the three DSL ligands examined and the results confirm that each of the transfected cultures expressed only one ligand, consistent with the transfected cDNA.

5.3.2 Effects of exposure to individual DSL ligands on C2C12 reserve cell activation

To investigate the effects of individual DSL ligands on the activation of reserve cells, C2C12 reserve cells were isolated and then co-cultured in growth medium with the 3T3 fibroblast lines described above. GFP+ve reserve cells were used in order to distinguish them from the fibroblasts in the co-cultures. C2C12 myoblasts were transfected with the GFP reporter plasmid pmaxFP-Green-n (Lonza) and selected with G418 to produce stable, GFP+ve cultures (Materials and Methods, section

2.9.4). Cultures of GFP+ve myoblasts were allowed to differentiate for 5 days and the reserve cells were isolated as described in the Materials and Methods (section 2.5). Control, untransfected 3T3 fibroblasts and G418-selected cultures of 3T3 fibroblasts expressing murine DL1, DL4 or Jagged1 (3T3, 3T3/DL1, 3T3/DL4 and 3T3/Jag1, respectively) were seeded into 8-well chamber slides and grown to confluency. The medium was removed from the fibroblast cultures and replaced with a suspension of C2C12 reserve cells in growth medium: 4 x 10^3 reserve cells were added to each co-culture, giving an approximate reserve cell:fibroblast ratio of 1:3. After 23 hours, co-cultures were pulsed with BrdU for 1 hour (Materials and Methods, section 2.15), washed, fixed and co-immunostained for GFP and BrdU to determine the proportion of reserve cells (identified by GFP expression) that had entered S-phase (identified by the incorporation of BrdU).

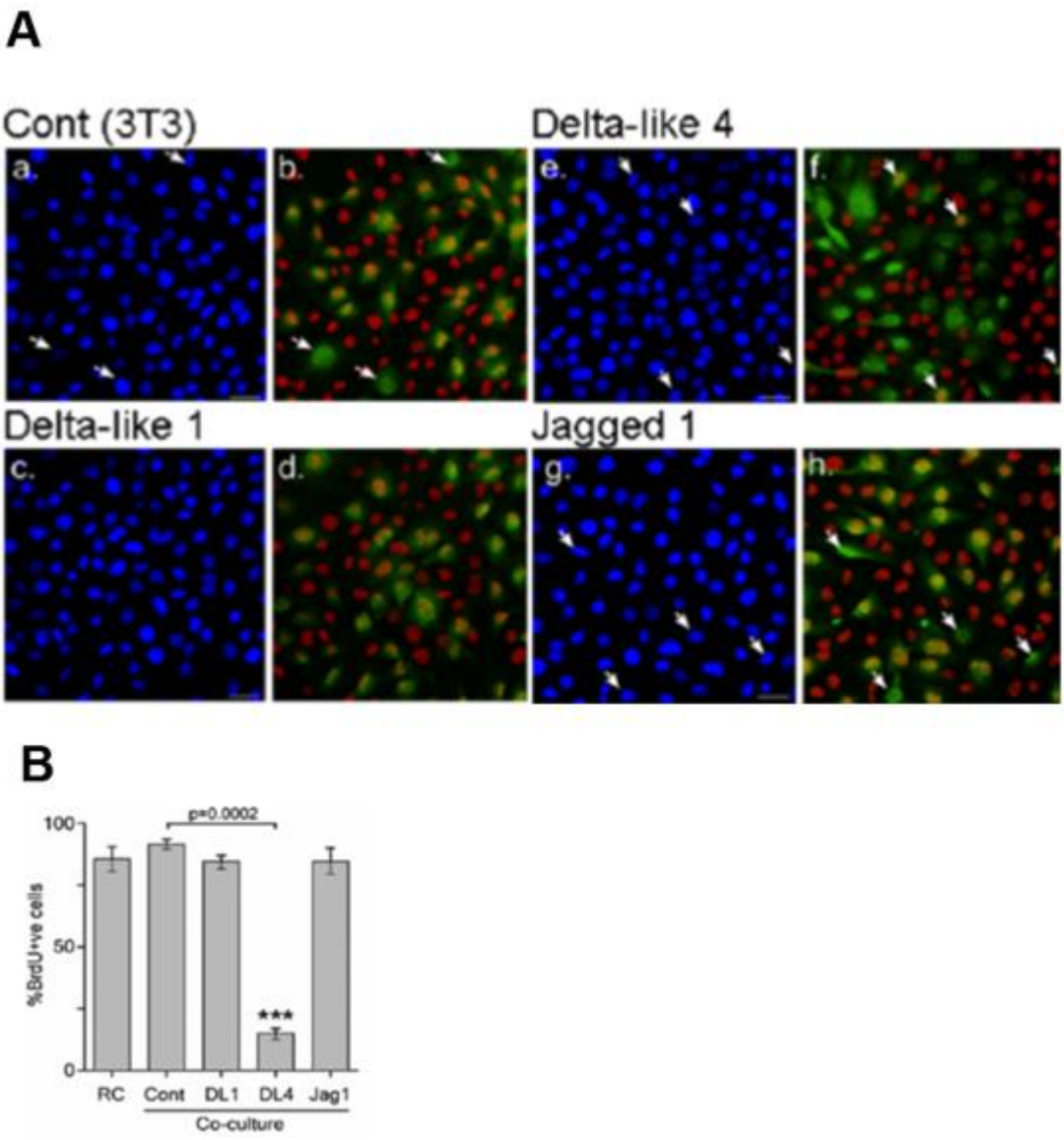


Figure 5.5 Effects of exposure to DSL ligands on re-entry of C2C12 reserve cells into the cell cycle.

Quiescent GFP+ve reserve cells were isolated from C2C12 cultures after 5 days of differentiation and co-cultured in serum-rich growth medium with confluent cultures of 3T3 fibroblasts either untransfected (control), or expressing DL1, DL4 or Jagged1 (3T3/DL1, 3T3/DL4 or 3T3/Jag1, respectively). Co-cultures were carried out in 8-well chamber slides with a reserve cell:fibroblast ratio of approximately 1:3. After 23 hours, the co-cultures were pulsed for 1 hour with BrdU, fixed, co-immunostained stained for GFP (Molecular probe; green) and BrdU (BU1/75; red) and counterstained with DAPI (blue) to reveal all nuclei. (A) Representative images of reserve cells co-cultured with untransfected 3T3 cells (a, b) and with fibroblasts expressing DL1 (c, d), DL4 (e, f) or Jagged1 (g, h). In a, b, g and h, arrows indicate GFP+ve/BrdU-ve reserve cells: in e and f, arrowheads indicate examples of GFP+ve/BrdU+ cells (scale bar = 20µm). (B) Histogram showing the mean percentage of GFP+ve cells that had incorporated BrdU during the 1 hour pulse. "RC" refers to reserve cells cultured alone and each column represents the mean of 3 cultures ± SEM (at least 200 GFP+ve cells were counted per culture). The mean

values were compared using a Student's t-test: *** denotes a statistically significant difference (p=0.0002) between reserve cells co-cultured with 3T3 cells expressing DL4 compared with cells co-cultured with control 3T3 fibroblasts.

BrdU incorporation occurs during S-phase and therefore reflects the proportion of cells that had re-entered the cell cycle and were actively synthesizing DNA during the pulse: i.e. in the above experiments, cells that were no longer in quiescence. This was complemented by directly determining the number of cells that retained reserve cell status after 24 hours of co-culture by incubation with lysenin, an earthworm protein that binds specifically to sphingomyelin, present at high levels in the plasma membrane of quiescent satellite cells and reserve cells (Nagata *et al*., 2006). GFP+ve C2C12 reserve cells were co-cultured for 24 hours with 3T3, 3T3/DL1, 3T3/DL4 and 3T3/Jag1 fibroblasts as above, fixed and incubated with lysenin. The cultures were then co-immunostained for GFP and bound lysenin (Materials and Methods, section 2.16) to determine the proportion of reserve cells that remained quiescent after 24 hours in growth medium.

After 24 hours in serum-rich growth medium, co-cultures were pulsed with BrdU for 1 hour to establish the proportion of GFP+ve cells that had initiated proliferation (Figure 5.5), or immunostained for lysenin-binding to determine the identify GFP+ve cells that had maintained the reserve cell phenotype (Nagata et. al.; 2006) (Figure 5.6).

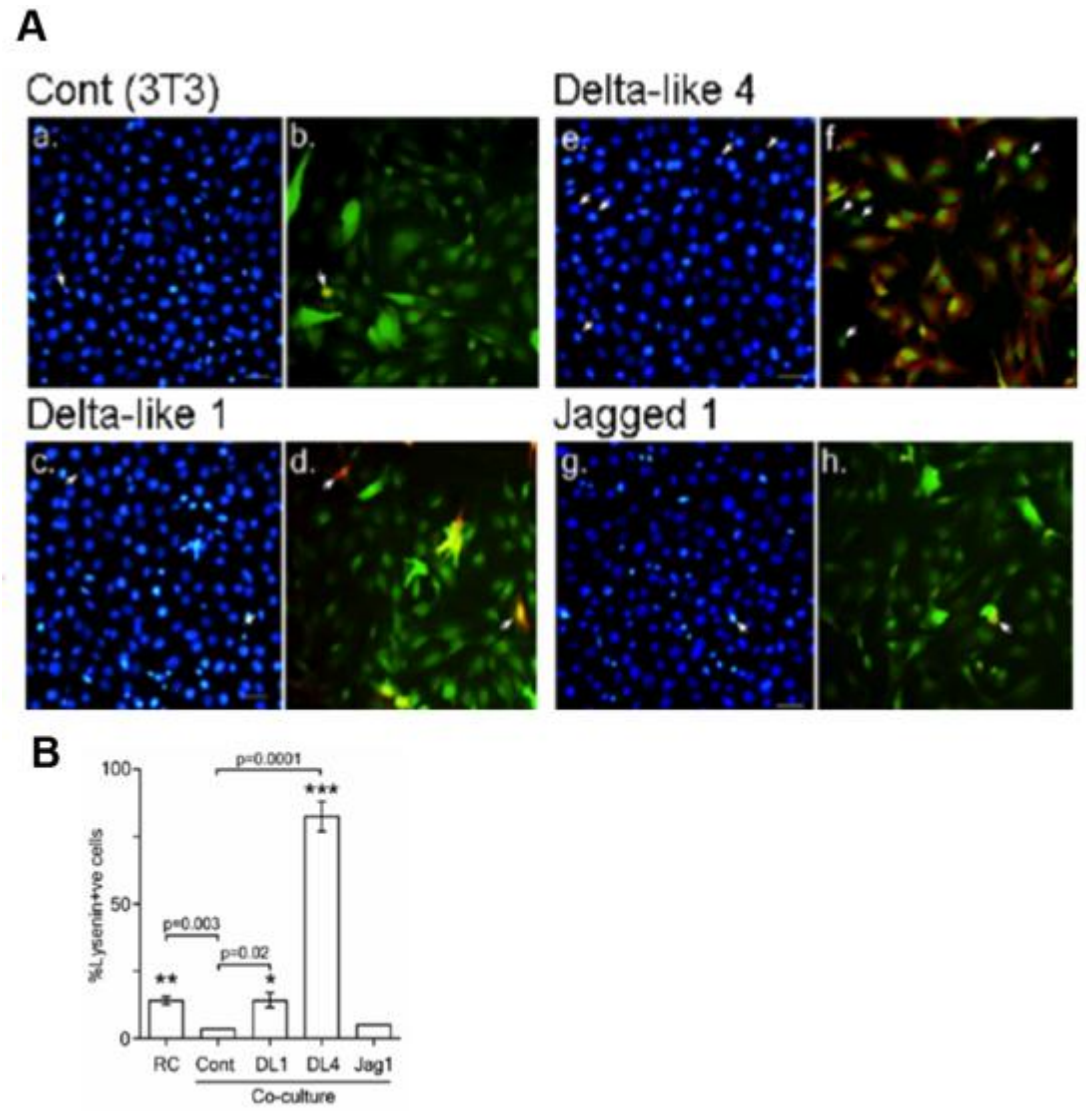


Figure 5.6 Effects of exposure to DSL ligands on maintaining C2C12 reserve cell quiescence

Quiescent GFP+ve reserve cells were isolated from C2C12 cultures after 5 days of differentiation and co-cultured in serum-rich growth medium with confluent cultures of 3T3 fibroblasts either untransfected (control), or expressing DL1, DL4 or Jagged1 (3T3/DL1, 3T3/DL4 or 3T3/Jag1, respectively). Co-cultures were carried out in 8-well chamber slides with a reserve cell:fibroblast ratio of approximately 1:3. After 24 hours, the co-cultures were fixed, incubated with lysenin and then co-immunostained for bound lysenin (rabbit anti-lysenin polyclonal antibody, Peptide Institute Inc.; red) and GFP (Molecular Probes; green) and counterstained with DAPI (blue) to reveal all nuclei. (A) Representative images of reserve cells co-cultured with untransfected 3T3 cells (a, b) and with fibroblasts expressing DL1 (c, d), DL4 (e, f) or Jagged1 (g, h). In a-d, g and h, arrows indicate GFP+ve/lysenin+ve reserve cells: in e and f, arrowheads indicate examples of GFP+ve/lysenin-ve cells (scale bar = 20µm). (B) Histogram showing the mean percentage of GFP+ve cells that bound lysenin. "RC" refers to reserve cells cultured alone and each column represents the mean of 3 cultures ± SEM (at least 200 GFP+ve cells were counted per culture). The mean values were compared to the control co-cultures (with untransfected 3T3

cells) using a Student's t-test: statistical differences are indicated by asterisks and p values.

Results presented in Figure 5.5 and Figure 5.6 show that when cultured alone, 85% of reserve cells were in S-phase during the BrdU pulse 23-24 hours after transfer to serum-rich growth medium, with 14% still in quiescence (as determined by lysenin binding) after 24 hours, consistent with data presented in Figure 5.1. Co-culture with control, untransfected 3T3 fibroblasts resulted in a small increase in the proportion of reactivated reserve cells, with 91% BrdU+ve and only 3% lysenin+ve cells after 24 hours. When compared with the control co-cultures, the presence of fibroblasts expressing either Jagged1 or DL1 had no effect on reserve cell activation, although the latter did result in a small but significant increase (from a mean of 3% to a mean of 14%) in the proportion of lysenin+ve cells present after 24 hours. In contrast, co-culture with 3T3 fibroblasts expressing DL4 inhibited reserve cell reactivation with only 15% entering S-phase and 82% remaining lysenin+ve after 24 hours. Thus, using both BrdU incorporation as a negative marker and lysenin binding as a positive indicator of reserve cell quiescence, these results show that exposure to DL4, but not DL1 or Jagged1, is sufficient to prevent C2C12 reserve cells from exiting the quiescent state and re-entering the cell cycle for at least 24 hours after stimulation with serum-rich medium.

5.4 Effects of exposure to specific DSL ligands on Notch3 and Notch1 regulation during C2C12 reserve cell activation

Data presented in Chapter 4 implicate Notch3 activity in maintaining the quiescent reserve cell phenotype, which is consistent with results presented above (section 5.2.2) showing that when reserve cells are activated by transfer to serum-rich medium, *Notch3* transcription is rapidly down-regulated and any remaining receptor becomes inactive prior to up-regulation of *MyoD* expression and entry into the cell cycle. That co-culture with fibroblasts expressing DL4 (but not DL1 or Jagged1) is sufficient to maintain the reserve cell phenotype in conditions that

normal promote activation, suggests that this ligand may interact specifically with Notch3 in this context. To test this hypothesis, the expression and regulation of Notch3 and Notch1 were investigated following co-culture with DSL ligand-expressing 3T3 fibroblasts.

Confluent cultures of control 3T3 fibroblasts, 3T3/DL1, 3T3/DL4 and 3T3/Jag1 fibroblasts were established in 10cm-diameter Petri dishes. Quiescent GFP+ve reserve cells were isolated and 2 x 10^5 cells were added to each fibroblast culture in a total volume of 10ml of growth medium, to give an approximate reserve cell:fibroblast ratio of 1:3. After 24 hours, total RNA and protein were extracted from replicate co-cultures (Materials and Methods, section 2.17 and 2.18) for analysis by RT-PCR and Western blotting, respectively.

Figure 5.7A shows RT-PCR analysis for the presence of Notch3, Notch1 and MyoD transcripts in reserve cell/3T3 fibroblast co-cultures. The results show that after 24 hours in growth medium, cultures of reserve cells and co-cultures of reserve cells with either control fibroblasts or fibroblasts expressing DL1 or Jagged1, contained similar low levels of Notch3 and apparently much higher levels of Notch1 transcript: in all cases, MyoD transcript was clearly present. Furthermore, Western blot analysis with anti-Notch3 antibody produced a single band corresponding to the membrane-associated form, suggesting that the remaining receptor was inactive: in contrast, probing with an anti-Notch1 antibody identified two bands, revealing the presence of both the membrane-associated and S2/S3-cleaved active ICD (Figure 5.6B). These results are consistent with the switch in Notch receptor activity shown in Figure 5.2 and Figure 5.3, suggesting that after 24 hours, the reserve cells had become activated reserve cell when cultured alone or in co-culture. Significantly, when quiescent reserve cells were co-cultured with 3T3 fibroblasts expressing DL4, Notch3 was maintained, both at the level of transcript and receptor activation, revealed by the detection of a lower molecular mass band corresponding to the presence of cleaved, active ICD (Figure 5.7). Furthermore, the increase in the level of Notch1 transcript observed in the other co-cultures was inhibited, active Notch1ICD was not detected by Western blotting and little or no MyoD transcript was present.

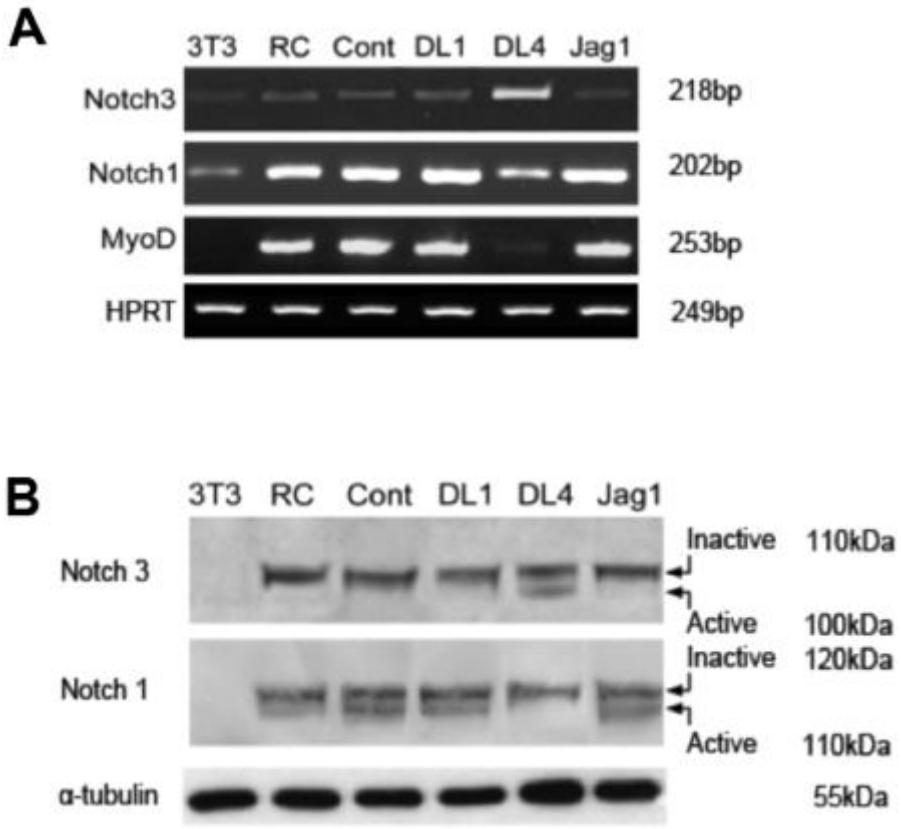


Figure 5.7 Effects of specific DSL ligands on Notch1 and Notch3 regulation during C2C12 reserve cell activation

Quiescent reserve cells were isolated from C2C12 cultures after 5 days of differentiation and co-cultured in serum-rich growth medium with confluent cultures of 3T3 fibroblasts either untransfected (control), or expressing DL1, DL4 or Jagged1 (3T3/DL1, 3T3/DL4 or 3T3/Jag1, respectively) with a reserve cell:fibroblast ratio of approximately 1:3. (A) RT-PCR analysis of total RNA extracted after 24 hours of co-culture for the presence of Notch3, Notch1 and MyoD transcripts. Reaction products were run on 1.5% agarose gels/TBE gels and stained with ethidium bromide. HPRT was used as a loading control. The predicted product sizes are shown on the right and were consistent with appropriate DNA marker ladders. (B) Western blot analysis of protein extracts prepared after 24 hours of co-culture. The membrane was probed for Notch3 (M-20; Santa Cruz) and Notch1 using antibodies raised against the ICD (M-20; Santa Cruz). In both cases, the upper band ("Inactive") corresponds to membrane-associated receptor; the lower band ("Active") to the active ICD released from the membrane by S2/S3 cleavage. α-tubulin was used as a loading control and approximate molecular masses estimated from a molecular mass ladder are shown on the right. In both (A) and (B), "3T3" refers to cultures of untransfected 3T3 fibroblasts cultured alone and "RC" to cultures of C2C12 reserve cells cultured alone. n=3

Together, these results show that exposure to DL4 inhibits the activation of quiescent reserve cells and subsequent entry into the cell cycle. This is associated with maintained Notch3 activity and suppression of Notch1 activity and MyoD up-regulation that normally accompany activation.

5.5 Discussion

The general hypothesis of this thesis is that Notch signalling is involved in the generation of satellite cells in the proper location. The aims of this chapter were to investigate which of Notch DSL ligands is responsible for maintaining Notch3 activity in quiescent cells. Previous studies have shown that activation of the Notch1 receptor promotes proliferation and prevents differentiation of skeletal muscle precursor cells, both during development and adult tissue regeneration (Conboy & Rando, 2002; Conboy *et al*., 2003). Results presented in the preceding two chapters show that reserve cells, a minority population that remains in state of undifferentiated quiescence within a differentiating skeletal muscle culture, express active Notch3. Furthermore, Notch3 activity is required for cells to adopt this fate as without it, cultures differentiate normally but contain few, if any reserve cells. If Notch 3 is required to establish and maintain a quiescent reserve cell phenotype and Notch1 is drives proliferation, this would predict that there must be some form of reciprocal regulation of the activities of the two receptors when cells are stimulated to exit the quiescent state and re-enter the cell cycle. The experiments presented in this chapter were therefore designed to investigate the expression and activities of Notch3 and Notch1 during the reactivation of reserve cells.

Reserve cells were purified by partial trypsinisation of fully differentiated C2C12 cultures and as observed previously, were found to express active Notch3 receptor (as demonstrated by the presence of cleaved Notch3 ICD), but no detectable Notch1 protein. In order to investigate Notch regulation during the transition from quiescence to proliferation, isolated reserve cells were transferred to serum-rich growth medium to stimulate entry into the cell cycle. Although the majority of reserve cells re-entered S-phase after 12-24 hours, the predicted reciprocal

regulation of *Notch3* and *Notch1* transcription were apparent as early as 90 minutes after exposure to growth medium. At the mRNA level, Notch3 was markedly down-regulated 45-90 minutes post-stimulation and was virtually undetectable by 12 hours: in contrast, Notch1 transcript was upregulated during the 45-90 minute period and maintained throughout re-entry into the cell cycle. Western blotting confirmed this switch but also showed that although Notch3 protein remained, there was no evidence of activity (i.e. cleaved ICD) after 3 hours, coincident with the appearance of Notch1 receptor activity. Significantly, this switch in Notch receptor activities has occurred immediately appearance of MyoD transcript and the increase in expression of cyclin A associated with cell cycle progression.

The canonical Notch signalling pathway involves interaction between a Notch receptor expressed by the signal-receiving cell and a DSL ligand on a signalling cell, the outcome of which in terms of the behaviour or fate of the signal-receiving cells depends upon both ligand and context (Rutz *et al*., 2005; de La Coste & Freitas, 2006). Data presented in an earlier chapter showed that in the context of the model system used here, DL1 is expressed by myoblasts and differentiated myotubes, Jagged1 and Jagged2 by myoblasts and reserve cells and DL4 is present on nascent myotubes (DL3 was not detected at any stage during differentiation). Based on the assumption that any one of these ligands could be responsible for Notch3 activity in reserve cells, experiments were carried out to investigate the effects of exposure to each of these ligands in a cellular context. Using a co-culture system in which quiescent reserve cells were cultured with 3T3 fibroblasts (which do not express any endogenous DSL ligands) transfected and selected to stably express individual ligands, it was possible to test the effects of the presence of each on reserve cell activation. This approach was chosen as previous studies have shown that soluble, as opposed to membrane-bound ligands can act in a dominant negative manner (Hukriede *et al*., 1997; Sun & Artavanis-Tsakonas, 1996; Sun & Artavanis-Tsakonas, 1997) and that ligand stabilisation is essential for Notch activation (Varnum-Finney *et al*., 2000).

Co-culture with 3T3 fibroblasts expressing either DL1 or Jagged1 had no effect on reserve cell activation compared with cells cultured with control untransfected

fibroblasts. This not only suggests that these ligands are unable to activate Notch3 when presented by another cell type, but also that cell autonomous interactions or reserve cell-reserve cell interactions involving the endogenous ligands do not maintain quiescence. In marked contrast, exposure to DL4 maintained Notch3 activity, prevented upregulation of MyoD expression and inhibited cells from re-entering the cell cycle, even after 24 hours in serum-rich growth medium.

These findings suggest that DL4 may be responsible for the activation of Notch3 in reserve cells and suggest a mechanism for their recruitment and maintenance. Results presented in previous chapters show that Notch3 is upregulated at the onset of differentiation and that DL4 is only expressed by newly-formed myotubes. Notch3/DL4 interaction can therefore only occur when myotubes first appear in the culture and can interact with Notch3+ve myoblasts, resulting in the Notch3 activity-mediated establishment and maintenance of quiescent state in a spatially limited population of undifferentiated myoblasts. Previous studies (reviewed in Hoffmann and Iruela-Arispe, 2007) of blood vessel development and maintenance have also suggested the importance of Notch3/DL4 interaction in specifying and maintaining cell fate, albeit between different cell type.

Chapter 6: Analysis of Notch3 target genes activities in skeletal muscle cultures

6.1 Introduction

Results presented in previous chapters suggest that the activation of Notch3 by the ligand DL4 may be responsible for the establishment and maintenance of the quiescent, undifferentiated reserve cells during the differentiation of skeletal muscle cultures, an *in vitro* model of the recruitment of satellite cells during regeneration.

The canonical Notch signalling pathway involves intranuclear interaction between NICD and the DNA-binding protein RBPJκ and in turn results in the transcription of target genes, the best characterised of which are members of the *Hes* (hairy and enhancer of split) and *Hey* (hairy/enhancer-of-split related with YRPW motif) gene families (reviewed in Iso *et al.,* 2003b; Introduction Section 1.16).

Experiments described in this chapter were designed to investigate the effects of Notch3 activity in terms of activation and repression of the *Hes* and *Hey* genes, in the context of mouse skeletal muscle myoblasts.

The aims of this chapter were to characterise the profile of target genes expressed in reserve cells and to determine how this is related to the up-regulation of Notch3 activity in that population. Experiments were then carried out to investigate whether overexpression of individual *Hes* genes can compensate for the lack of Notch3 signalling in cultures of shRNA Notch3 knockdown cells, to determine whether the observed ability of Notch3 to reduce apoptosis and promote the sequestration of reserve cells during differentiation can be ascribed to the expression or inhibition of specific target genes.

6.2 Quantitative RT-PCR analysis of Notch signalling pathway target genes in differentiated C2C12 cultures

Results presented in previous chapters show that the expression of Notch receptors and DSL ligands change as C2C12 cultures undergo differentiation and that the main stages of the myogenic lineage (proliferating myoblasts, differentiated myotubes and quiescent reserve cells) are associated with distinct expression profiles. To investigate the expression of the canonical Notch signalling pathway target genes (Hey1, Hey2, HeyL and Hes1, Hes5, Hes7 (reviewed in Iso *et al*., 2003b)) in these phenotypically-distinct populations as determined by their particular receptor/ligand combination, quantitative RT-PCR analysis was carried out using RNA extracted from C2C12 myoblasts, myotubes and reserve cells.

C2C12 myoblasts were seeded into five 10 cm-diameter Petri dishes (5 x10^5 cells per dish) in growth medium. After 24 hours, total RNA was extracted from 2 dishes of proliferating myoblasts using Trizol (Section 2.17): the remaining cultures were transferred to and maintained in differentiation medium. After 5 days, total RNA was extracted from myotube and reserve cell fractions, isolated from the differentiated cultures as described in Section 2.5.2.1). Quantitative RT-PCR analyses were carried out as described in Section 2.18.2, using the primers and cycling parameters presented in

Table 2.3.

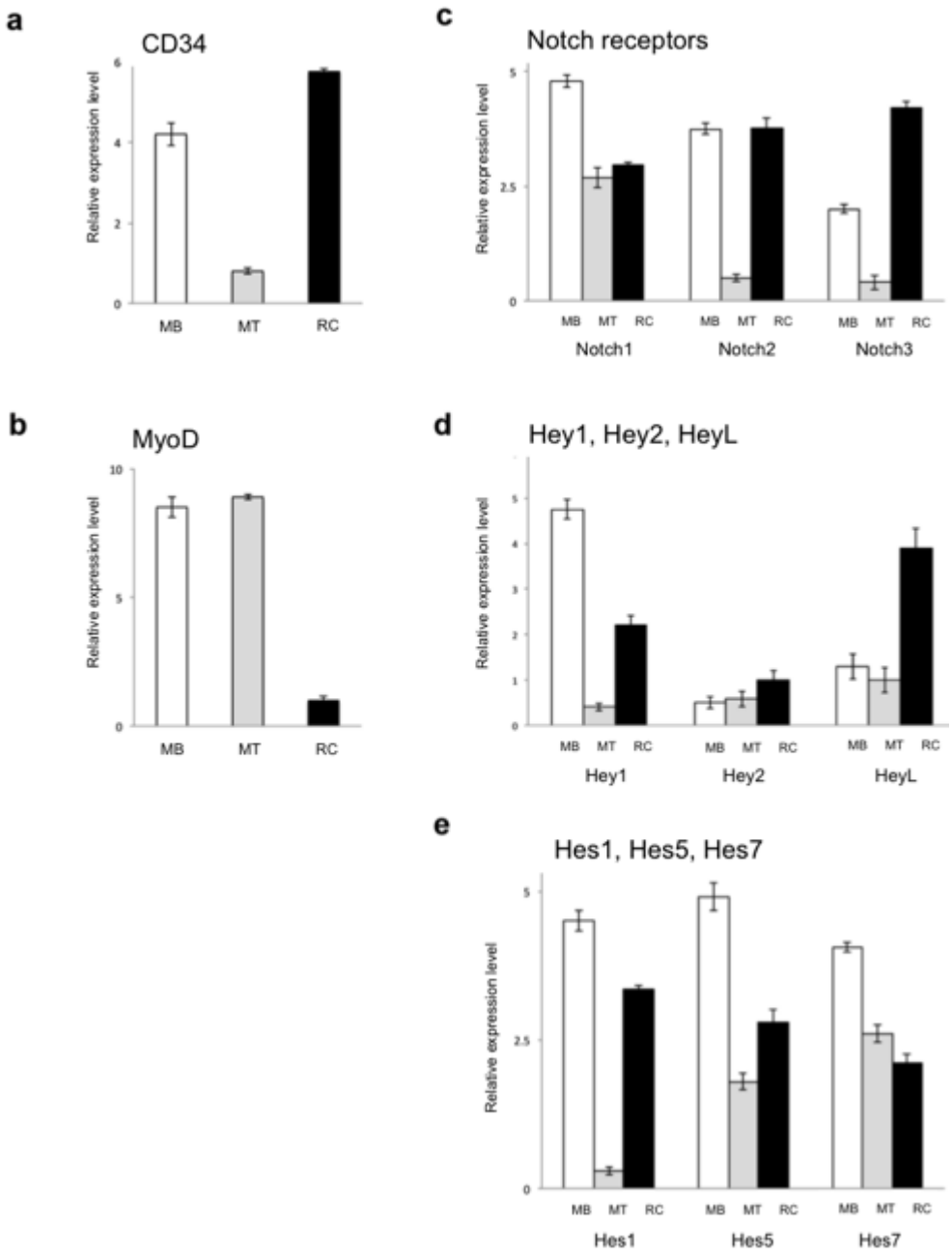


Figure 6.1 Expression of Notch receptors and canonical Notch signalling pathway target genes in proliferating myoblasts, myotubes and reserve cells
Total RNA was extracted from C2C12 myoblasts (MB), myotubes (MT) and reserve cells (RC). MT and RC fractions were obtained by partial trypsinisation of cultures after 5 days of differentiation. Expression levels were determined by quantitative RT-PCR and are presented as the mean ± SD from three replicate samples, relative to the expression of 18S ribosomal RNA. CD34 (a) and MyoD (b) were used to confirm efficient purification of the two fractions. (c) shows the relative expression of the receptors Notch1, Notch2, and Notch3. (d) and (e) show the relative expression of the Notch target genes Hey1, Hey2, HeyL and Hes1, Hes5 and Hes7, respectively.

The relative levels of expression of CD34 and MyoD (Figure 6.1a, b) are consistent with those expected from populations of proliferating myoblasts, myotubes and reserve cells. CD34 is marker of reserve cells, is expressed at lower levels in proliferating cultures and is absent from myotubes (Beauchamp *et al*., 2000), whereas MyoD is expressed by proliferating myoblasts (although variable according to position in the cell cycle), up-regulated during myotube formation, but absent from reserve cells (Kitzmann *et al*., 1998, Yoshida *et al*., 1998).

Figure 6.1c shows that Notch1 was highly expressed in proliferating myoblasts and although present, was detected at much lower levels following differentiation in both myotube and reserve cell fractions. Notch2 was expressed at similar levels in both myoblasts and reserve cells, but was down-regulated in differentiated myotubes. Notch3 was expressed by proliferating myoblasts but in differentiated cultures, was relatively highly expressed in reserve cells but down-regulated in myotubes. These data are consistent with the RT-PCR and Western blot results presented in Chapter 3 and together (assuming that each population is homogenous) suggest that proliferating myoblasts are $Notch1^{++}/Notch2^{++}/Notch3^{+}$ whilst in differentiated cultures, myotubes are $Notch1^{+}/Notch2^{-}/Notch3^{-}$ whereas reserve cells are $Notch1^{+}/Notch2^{+}/Notch3^{++}$. This suggests a role for Notch3 in generating reserve cells, which may represent quiescent stellite cells in the experimental system used here. Notch3 could then be implicated in the identity of quiescent satellite cells and in their future fate as muscle stem cells located in their niche.

Quantitative RT-PCR analyses of the same RNA samples (Figure 6.1d, e) suggested that that the Notch target genes *Hey1*, *Hes1*, *Hes5 and Hes7* are all highly expressed in proliferating myoblasts (Figure 6.1d, e). In differentiated cultures, cells that had adopted different fates expressed different combinations of Notch target genes compared with proliferating myoblasts. Differentiation to form myotubes was accompanied by a marked down-regulation of Hey1 and Hes1, whilst Hes5 and Hes7 were still expressed, but at lower levels. In contrast, reserve cells continued to

express Hey1, Hes1, Hes5 and Hes7, again at lower levels, but also showed marked up-regulation of *HeyL*. In all of the C2C12 samples assayed, expression of Hey2 was extremely low, consistent with previous findings (Reviewed in Iso *et al*., 2003b).

6.3 Effects of Notch1 and Notch3 activity on canonical Notch signalling pathway target gene expression

Results presented in the previous section show that in differentiated C2C12 cultures, cells that have undergone terminal myogenic differentiation express different combinations of Notch receptors and downstream canonical Notch signalling pathway targets. To investigate whether the expression of specific target genes could be related to the activity of particular Notch receptors, experiments were carried out to examine the effects of constitutively active Notch1ICD and Notch3ICD on target gene promoter activity using a series of luciferase reporter constructs.

C2C12 and C57BL/10 mouse primary myoblasts were seeded at a density of $1.5X10^3$cells per well in 96-well white walled plates and co-transfected with one of the reporter plasmids together with β -galactosidase plasmid at a ratio of 10:1 according to the Lipofectamine 2000 protocol (see section 2.9). Cultures were maintained for 1 day in growth medium post-transfection and then switched to differentiation medium for 24-48 hours prior to the assays. Reporter activity was assayed using the Dual-Light system (Applied Biosystems) and was normalised to β-galactosidase activity to control for transfection efficiency variation among different wells according to the manufacturer's instructions (see Section 2.11). All reporter assays shown are based on data averaged from at least three independent transfections.

6.3.1 Effects of Notch1ICD and Notch3ICD on *Hey* gene promoter activity

Overexpression of Notch1ICD increased both *Hey1* and *Hey2* promoter activity in both C2C12 and primary myoblasts, consistent with previous findings *(Nakagawa et al*., 2000), whereas Notch3ICD had no effect on either *Hey1* or *Hey2* promoter

activity in either system (Figure 6.2a,b, d, e). In contrast, Notch1ICD slightly decreased *HeyL* promoter activity in both primary myoblasts and C2C12, whereas overexpression of Notch3ICD increased activity in both C2C12 and mouse primary myoblasts (Figure 6.2c,f).

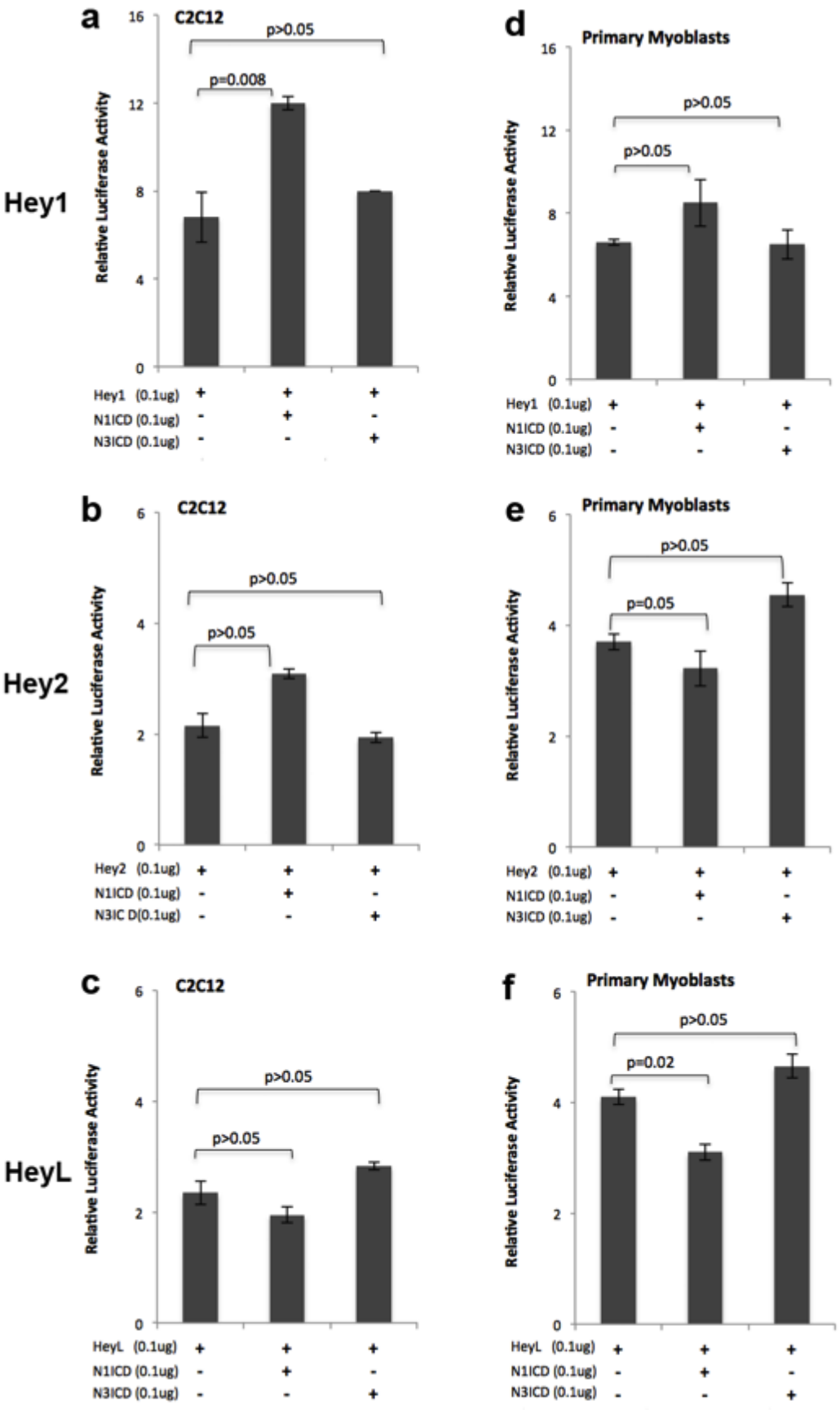


Figure 6.2 Effects of overexpression of constitutively active Notch1 and Notch3 on *Hey* gene promoter activity in skeletal muscle myoblasts

C2C12 and C57BL/10 mouse primary proliferating myoblasts were transfected with *Hey1* (a and d), *Hey2* (b and e) or *HeyL* (c and f) promoter/ luciferase reporter constructs with or without plasmids encoding Notch1ICD or Notch3ICD. Beneath each histogram bar, + denotes the presence, - the absence of each plasmid. A plasmid encoding β-galactosidase (30ng) was included in all transfections and luciferase activity was divided by β-galactosidase activity to normalise for transfection efficiency. All relative luciferase activities are presented as mean values ± SEM of at least three independent experiments, each performed in triplicate and when compared using a Student's t-test.

6.3.2 Effects of Notch1ICD and Notch3ICD on *Hes* gene promoter activity

The effects of Notch1ICD and Notch3ICD on *Hes1, Hes5* and *Hes7* promoter activity were determined using luciferase reporter constructs in C2C12 and C57BL/10 mouse primary myoblasts as described above for the *Hey* genes.

As shown in Figure 6.3, overexpression of Notch1ICD transactivated each of three *Hes* genes promoters tested in both C2C12 and mouse primary myoblasts, as shown by increased levels of luciferase activity compared with cultures transfected with the equivalent promoter/luciferase reporter construct alone. Overexpression of Notch3ICD was also found to increase *Hes5* and *Hes7* promoter activity, although the extent of transactivation differed from that of the Notch1ICD. In both C2C12 and mouse primary myoblasts, co-transfection with Notch1ICD resulted in much a greater increase in activity of both the *Hes1* and *Hes7* promoter constructs compared with co-transfection with the Notch3ICD (Figure 6.3a,d and Figure 6.3c,f, respectively). In marked contrast, the Notch3ICD was much more effective in transactivating the *Hes5* promoter construct than Notch1ICD (Figure 6.3b,e). This is in accordance with the initial hypothesis that Notch signalling, i.e. here both Notch1 and Notch3, are activated during muscle cell differentiation and trigger the activity of the target genes Hes 1, 5 and 7.

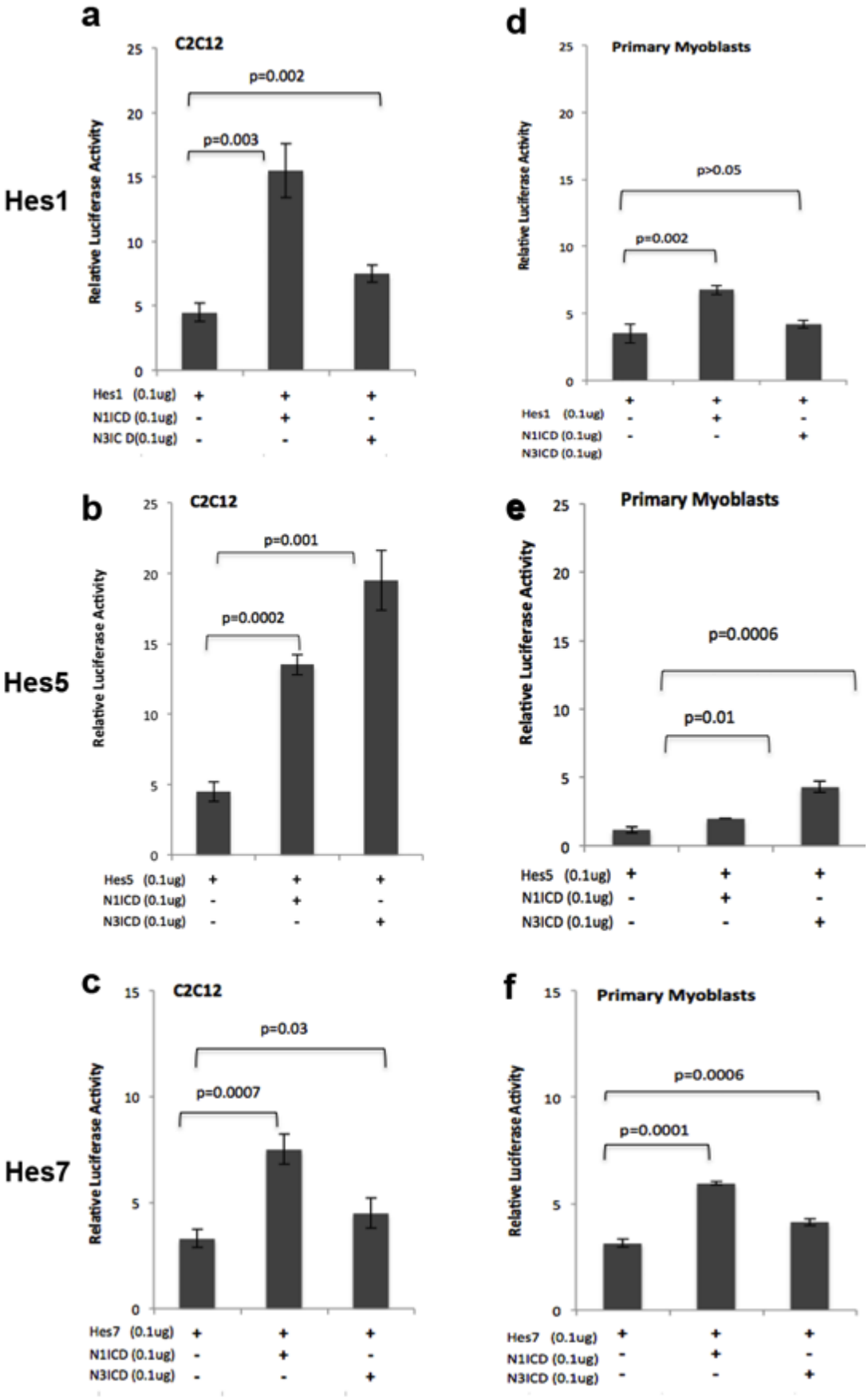


Figure 6.3 Effects of overexpression of constitutively active Notch1 and Notch3 on *Hes* gene promoter activity in skeletal muscle myoblasts

C2C12 and C57BL/10 mouse primary myoblasts were transfected with *Hes1* (a and d), *Hes5* (b and e) or *Hes7* (c and f) promoter/ luciferase reporter constructs with or without plasmids encoding Notch1ICD or Notch3ICD. Beneath each histogram bar, + denotes the presence, - the absence of each plasmid. A plasmid encoding β-galactosidase (30 ng) was included in all transfections and luciferase activity was divided by β-galactosidase activity to normalise for transfection efficiency. All relative luciferase activities are presented as mean values ± SEM of at least three independent experiments, each performed in triplicate and when compared using a Student's t-test.

The data presented in Figure 6.2 and Figure 6.3 suggest that in the cellular context of mouse skeletal muscle myoblasts, Notch1 and Notch3 activities may have differing effects on the expression of the canonical Notch signalling pathway target genes. For *Hey1*, *Hey2, Hes1*, and co-transfection with constitutively active Notch1ICD increased promoter activity, whereas the Notch3ICD caused little or no transactivation. In contrast, the Notch3ICD was significantly a more powerful transactivator of the *Hes5* promoter than the Notch1ICD and in the case of the *HeyL* promoter*;* Notch3ICD appeared to cause a limited increase in activity whilst Notch1ICD was significantly slightly repressive.

6.4 Regulation of Notch3 activity in the regulation of canonical Notch signalling pathway target gene expression

Comparing results from 6.2 and 6.3, significantly the most prominent variations in the levels of activation for each gene were those of Hes1, Hes5 and Hes7 in C2C12 and primary myoblasts (Figure 6.2 and Figure 6.3). The up-regulation or the down-regulation of these genes was found to be very similar between C2C12 and primary myoblasts. This suggests that the initial hypothesis of implication of several genes from the Notch family, i.e. here Notch1 and Notch3, in the generation of a cell fate during differenciation, may be justified through then activation of downstream targets? Therefore, the aims of this section were to further characterise the regulatory region of *Hes* in C2C12 cells and Notch3 knockdown cultures.

According to the results obtained in the previous chapter, Notch3 was implicated as a potentially important receptor in determining alternate cell fates upon the induction of differentiation in C2C12 cell cultures. When Notch3 in C2C12 cultures was inhibited, the size of the cells appeared to be smaller and rounder. In addition, MyoD expression appeared to be elevated in cultures with reduced Notch3 expression during differentiation (during which Notch3 was normally up-regulated). Despite the higher rate of apoptosis, the loss of Notch3 still resulted in increased fusion index characterised by higher average number of nuclei in myotubes in C2C12 cultures. In section 6.3, Notch3 was found to greatly enhance the activation of Hes5 in C2C12, which expressed Notch3 intrinsically. Hence we are interested to find out what is the implication of the absence of Notch3 to the regulation of *Hes* family. We also analysed and compared the regulation of *Hes* using shRNA Notch3 knockdown cultures.

As shown in Figure 6.4, Notch1 ICD was increased the luciferase activity of Hes1, Hes5 in the shRNA Notch3 knockdown cultures; in particular, there was a 3-fold increase in both Hes1 and Hes5 activity, although the increase in Hes7 luciferase activity was only marginal when compared to those of Hes1 and Hes5. While Notch3 IC was able to increase the luciferase activity of all three Hes genes in C2C12 cultures, it only managed to increase the activity of Hes1 and Hes5, but there was a significant reduction of the Hes7 activation. Furthermore, in C2C12, the increased activation of Hes5 due to Notch3 IC was significantly greater than the increased activation of Hes5 due to Notch1 IC, the activation of Hes5 in shRNA Notch3 knockdown cultures was completely opposite.

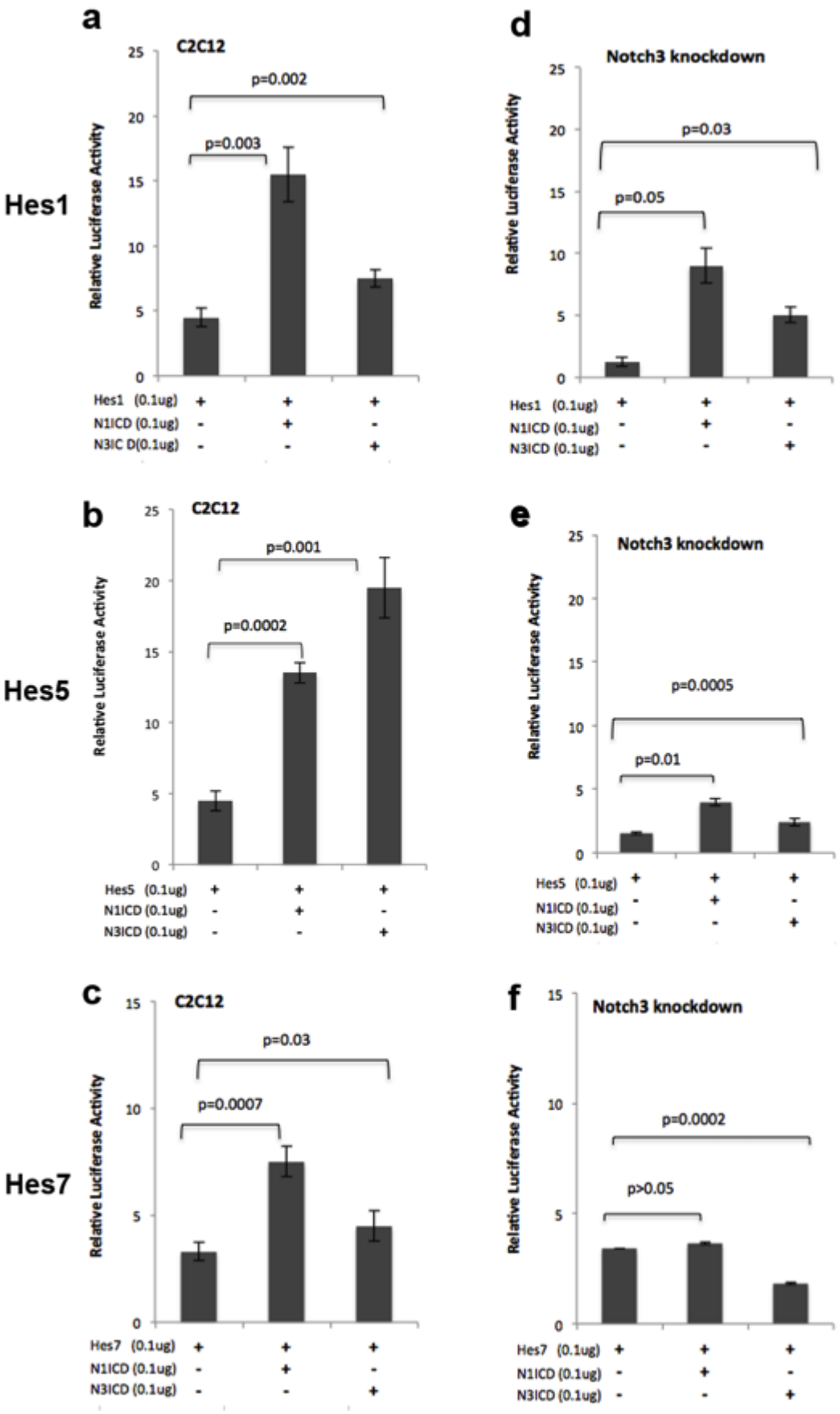


Figure 6.4 Effects of Notch1IC and Notch3IC on the Hes1, -5 and -7 promoter

Hes1, -5, -7 luciferase constructs were transfected into C2C12 myoblasts (a, b, c) or shRNA Notch3 knockdown culture (d, e, f) with or without Notch1 IC and Notch3IC constructs. β-Galactosidase was used to normalise for transfection efficiency. Luciferase activities shown with a standard error are the average of at least three independent experiments performed in triplicate, which were compared using a Student's t-test.

We next analysed the effects of Numb on Notch signalling in the myogenic cell line C2C12, in which differentiation to myotubes can be blocked by Notch signalling (Dahlqvist *et al*., 2003; Gustafsson *et al*., 2005).

As Numb inhibits Notch1, it would be expected that the magnitude of the increase in luciferase activity for Hes genes due to Notch1 IC would reduce. Indeed, as shown in Figure 6.5, the increase in Hes1 luciferase activity was significantly lower in both C2C12 and shRNA Notch3 knockdown cells when Numb was transfected. However, contrary to what was expected, there were 2-fold increase in the luciferase activity of Hes5 and Hes7 when Numb was transfected with Notch1 IC in C2C12. The luciferase activity of Hes5 and Hes7 was consistently increased when Numb was transfected with Notch3 IC in both C2C12 and shRNA Notch3 knockdown cultures.

Numb appears to consistently enhance the ability of Notch3 IC to up-regulate all three Hes genes in the context of shRNA Notch3 knockdown culture. Numb was also able to consistently to inhibit Notch1 IC's ability to up-regulate all three Hes genes in the shRNA Notch3 knockdown culture, but it was only capable of inhibiting Notch1 IC ability to up-regulate Hes1 activation in C2C12. The intrinsic expression of Notch3 in the C2C12 may have contributed to the increase of Hes5 and Hes7 activation when Numb was transfected, and this increase due to Numb-Notch3 interaction may have compensate and masked the reduction of Hes5 and Hes7 activity resulting from the inhibition of Notch1 by Numb.

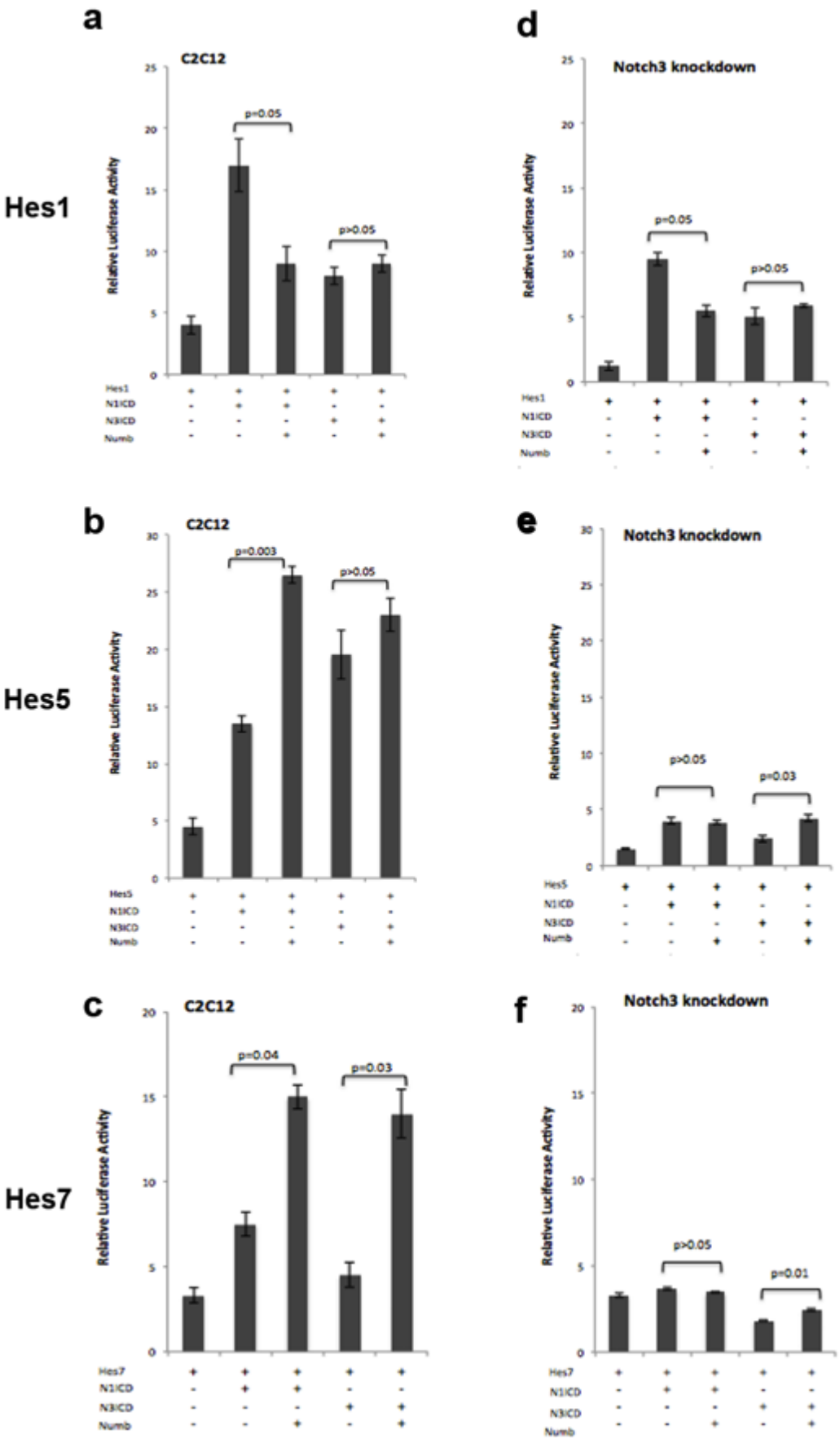


Figure 6.5 Effects of Numb on the Hes1, -5 and -7 activation in Notch

Hes1, -5, -7 luciferase constructs were transfected into C2C12 myoblasts (a, b, c) and shRNA Notch3 knockdown culture (d, e, f) with or without Notch1 IC and Notch3IC constructs, co-cultured with combinations of Notch- and Jagged-expressing cells. β-Galactosidase was used to normalise for transfection efficiency. Luciferase activities shown with a standard error are the average of at least three independent experiments performed in triplicate which were compared using a Student's t-test.

6.5 Investigation of the role of Notch3 in the regulation of *Hes* genes during skeletal muscle differentiation *in vitro*

The initial hypothesis of this experimental work was that Notch receptors may be directly involved in the decision that generates satellite cells in their proper niche. The aims of this section were to investigate the role of Notch3 in the regulation of Notch-responsive *Hes* gene activity and the potential involvement of these targets in controlling cell fate (i.e. differentiation to form myotubes or sequestration as reserve cells) during differentiation.

Evidence presented in previous chapters implicates Notch3 activity in the establishment and maintenance of a quiescent, stem cell-like reserve cell phenotype during skeletal muscle differentiation *in vitro* (Chapter 4 and Chapter 5). Compared with proliferating myoblasts, reserve cells show reduced levels of expression of *Hes1*, *Hes5* and *Hes7* which may reflect different relative levels of Notch receptor expression (Figure 6.1).

To examine the role of Notch3, quantitative RT-PCR was used to investigate the patterns of expression of *Hes1*, *Hes5* and *Hes7* during the early stages of differentiation were compared using wild-type C2C12 and the stable shRNA Notch3-knockdown line described in Chapter 4.

Replicate cultures of wild-type and shRNA Notch3-knockdown C2C12 myoblasts were seeded into 10 cm-diameter Petri dishes (5 x10^5 cells per dish) and maintained for 24 hours in growth medium, before switching to differentiation medium. At 24 hour intervals, three cultures were harvested and total RNA was extracted as

described in section 2.17. Quantitative RT-PCR analyses were carried out as described in Section 2.5.2.1, using the primers and cycling parameters presented in Table 2.3.

6.5.1 Comparison of the expression of *Hes1* following the induction of myogenic differentiation in wild-type and shRNA Notch3-knockdown C2C12 cultures

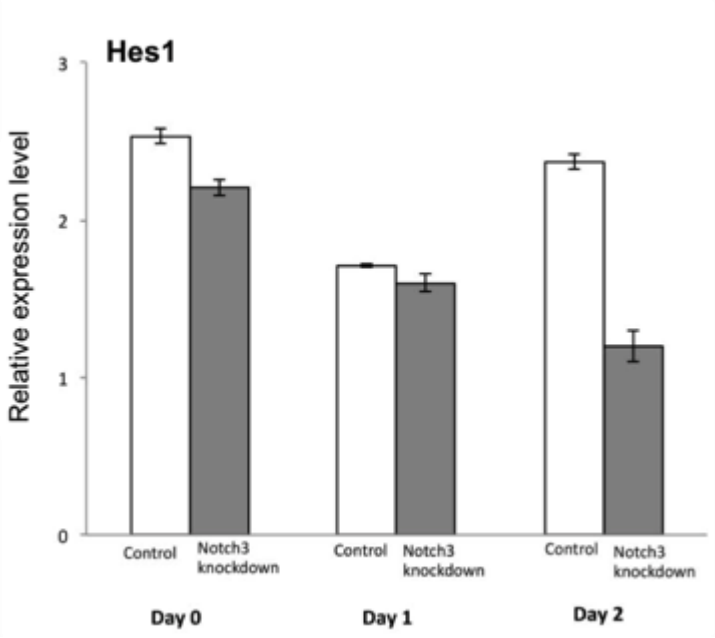


Figure 6.6 Quantitative RT-PCR analysis of *Hes1* expression during the onset of differentiation of wild-type and shRNA Notch3-knockdown C2C12 myogenic cultures
Total RNA was extracted from replicate wild-type C2C12 cultures (Control, open bars) and shRNA Notch3 knockdown C2C12 cultures (Notch3 knockdown, shaded bars) at 24-hour intervals after the induction of differentiation was induced. Levels of Hes1 transcript were determined by quantitative RT-PCR and are presented as the mean ± SD from three replicate samples, relative to the expression of 18S ribosomal RNA.

Results presented in Chapter 3 (section 3.3) show that in wild-type C2C12 cultures, Notch3 is up-regulated 24-48 hours after differentiation is induced. Figure 6.6 shows that during this period, the level of Hes1 transcript initially decreased in wild-

type C2C12 cells and then increased at 48 hours. However, in the shRNA Notch3 knockdown cultures, the increase in expression at 48 hours was not observed. This is consistent with the observation that following differentiation, *Hes1* was much more highly expressed in reserve cells than in myotubes (Figure 6.1) as the knockdown cultures contain few, if any, reserve cells.

6.5.2 Comparison of the expression of *Hes5* following the induction of myogenic differentiation in wild-type and shRNA Notch3-knockdown C2C12 cultures

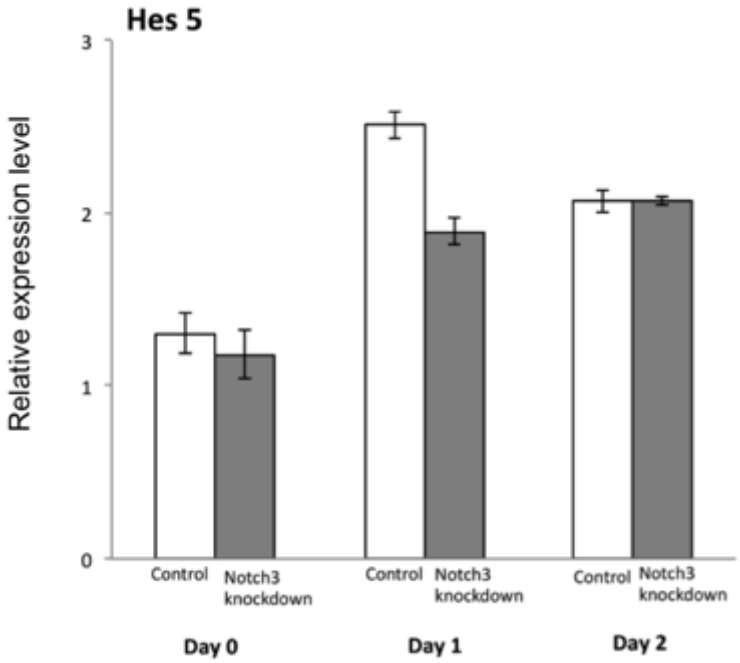


Figure 6.7 Quantitative RT-PCR analysis of *Hes5* expression during the onset of differentiation of wild-type and shRNA Notch3-knockdown C2C12 myogenic cultures
Total RNA was extracted from replicate wild-type C2C12 cultures (Control, open bars) and shRNA Notch3 knockdown C2C12 cultures (Notch3 knockdown, shaded bars) at 24-hour intervals after the induction of differentiation was induced. Levels of Hes5 transcript were determined by quantitative RT-PCR and are presented as the mean ± SD from three replicate samples, relative to the expression of 18S ribosomal RNA.

During the onset of differentiation, the levels of Hes5 transcript were found to increase in both the wild-type and the shRNA Notch3 knockdown C2C12 cultures

(Figure 6.7). That the same pattern of expression was observed both in both cell lines suggests that this increase is independent of changes in Notch3 expression.

6.5.3 Comparison of the expression of *Hes7* following the induction of myogenic differentiation in wild-type and shRNA Notch3-knockdown C2C12 cultures

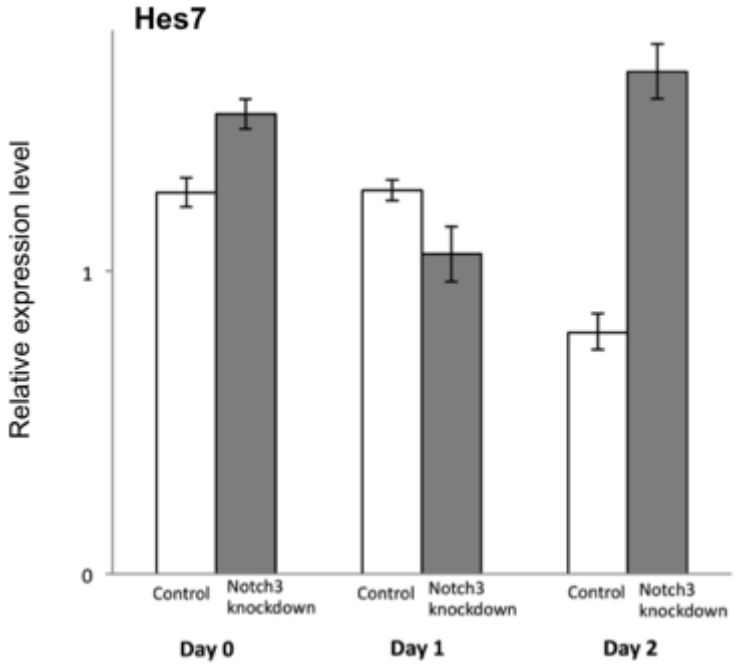


Figure 6.8 Quantitative RT-PCR analysis of *Hes7* expression during the onset of differentiation of wild-type and shRNA Notch3-knockdown C2C12 myogenic cultures
Total RNA was extracted from replicate wild-type C2C12 cultures (Control, open bars) and shRNA Notch3 knockdown C2C12 cultures (Notch3 knockdown, shaded bars) at 24-hour intervals after the induction of differentiation was induced. Levels of Hes7 transcript were determined by quantitative RT-PCR and are presented as the mean ± SD from three replicate samples, relative to the expression of 18S ribosomal RNA.

Figure 6.8 shows that in wild-type C2C12 cultures, the level of Hes7 transcript was decreased 48 hours after differentiation was induced: in contrast, the level of expression increased after 48 hours in the shRNA Notch3 knockdown cultures.

6.6 The effects of overexpression of *Hes* genes on the differentiation of C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Results presented in Chapter 5 suggest that Notch3 activity is involved in the determination of cell fate during myogenic differentiation: specifically, in establishment and maintenance of the quiescent, undifferentiated reserve cell population. In the absence of Notch3, the initial stages of differentiation are accompanied by increased levels of apoptosis and once fully differentiated, the cultures consisted of myotubes containing a higher average number of myonuclei and a markedly reduced number of reserve cells compared with wild-type cells. Data presented above (Figure 6.6 and Figure 6.8) show that during the first 48 hours after differentiation was induced, the time when Notch3 expression is up-regulated, the overall level of *Hes1* transcript was maintained whereas *Hes7* expression decreased compared with proliferating myoblasts. In the absence of Notch3, the level of Hes1 transcript was decreased whereas *Hes7* expression was greater compared with the controls.

It is possible that Notch3 promotes *Hes1* and inhibits *Hes7* expression and that inappropriate expression of one or both is incompatible with the reserve cell phenotype. Alternatively, the lack of Notch3 may prevent cells from adopting the reserve cell fate which normally results in the subsequent overall maintained levels of *Hes1* and down-regulation of *Hes7* in the cultures. To further investigate these possibilities, the behaviour of differentiating shRNA Notch3 knockdown and shRNA control C2C12 cultures were compared following transfection with expression vectors encoding *Hes1*, *Hes5* or *Hes7*.

6.6.1 Effects of *Hes* gene overexpression on myotube formation in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

If the lack of reserve cells observed in differentiated shRNA Notch3 knockdown cultures is due to the lack of Notch3 activity-mediated up-regulation of *Hes* activity, overexpression of the downstream target would be expected to reverse this effect

in knockdown cultures and possibly increase the number of reserve cells in Notch3+ve cultures.

To investigate this possibility, shRNA Notch3 knockdown and shRNA control C2C12 cultures were transfected with *Hes1, Hes5* or *Hes7* expression plasmids (section 2.8) using Lipofectamine 2000 (section 2.9.1) and seeded into Permanox 8-well Lab-Tek chamber slides at 10^3 cells/chamber in growth medium. After 24 hours, the medium was switched to differentiation medium and the cultures allowed to differentiate for 96 hours before they were fixed and stained for skeletal muscle myosin heavy chain (MyHC) expression, (as described in section 2.14). The numbers of nuclei in MyHC+ve cells were counted to determine the fusion index (i.e. the proportion of nuclei in differentiated cells). The expression of GFP+ve and MyHC-ve undifferentiated cells were counted to determine the percentage of undifferentiated cells expressing GFP in transfected cultures.

6.6.1.1 Effects of transfection with *Hes1*

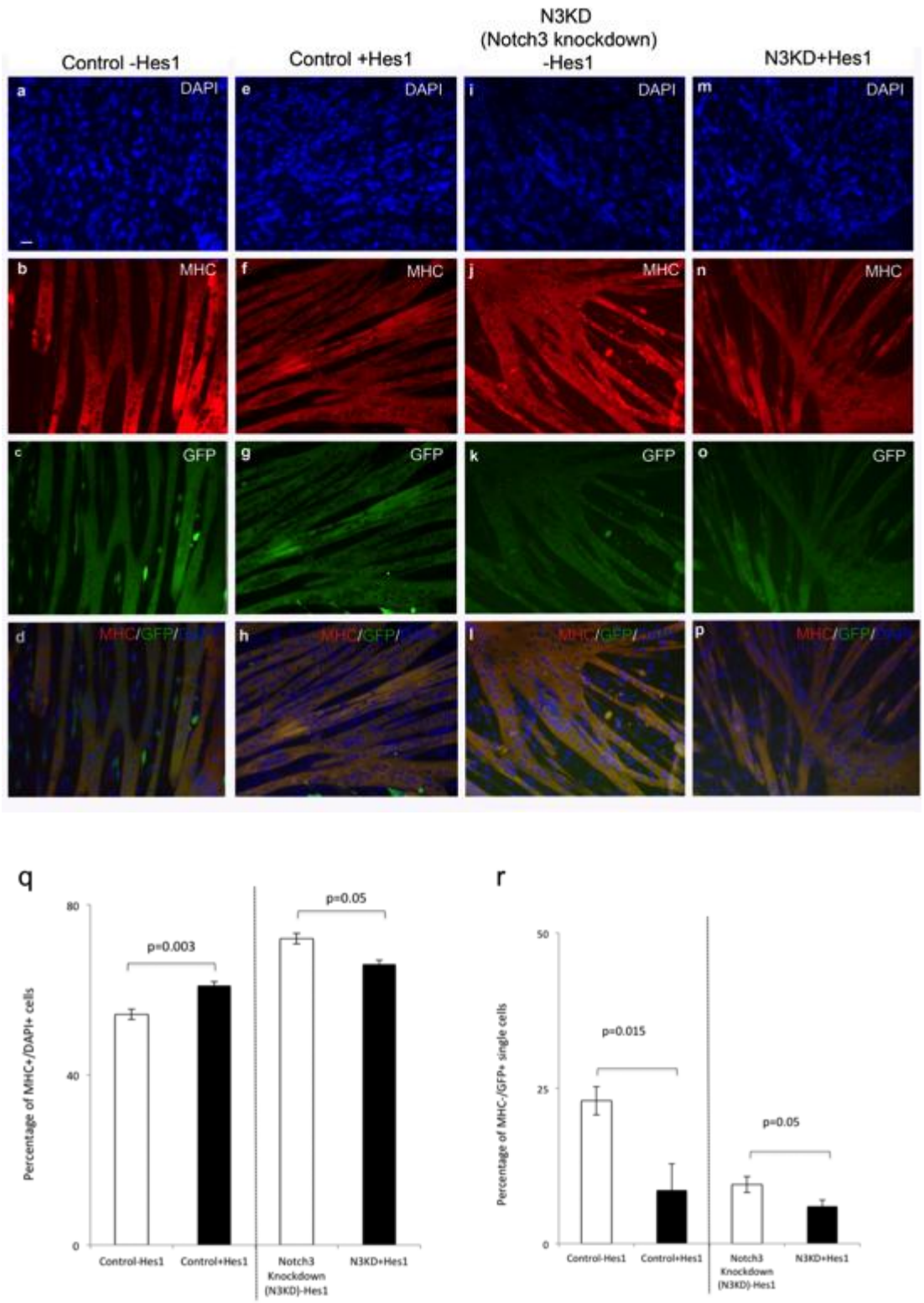


Figure 6.9 Effects of *Hes1* overexpression on myotube formation in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown)

were transfected with a *Hes1* expression construct. Twenty-four hours post-transfection, the cultures were allowed to differentiate for 96 hours fixed and then co-immunostained with anti-GFP and anti-MyHC antibodies. Representative images of untransfected control (a-d), *Hes1*-transfected control (e-h), untransfected Notch3 knockdown (i-l) and *Hes1*-transfected Notch3 knockdown (m-p) are shown stained with DAPI to reveal all nuclei (blue; a, e, i and m) and for MyHC (red; b, f, j and n) and GFP (green; c, g, k and o) expression. d, h, l and p are overlay images all three stains. (Scale bar = 20μM) To determine the number of nuclei in MyHC+ve cells or undifferentiated (MyHC-ve)/GFP+ve single cells, at least 200 nuclei were counted in randomly selected fields (q and r). The histogram bars represent the mean ± 1 SD of three replicate cultures which were compared using a Student's t-test.

Consistent with previous data (section 4.4), the percentage of nuclei in MyHC+ve cells was greater in the shRNA Notch3 knockdown compared to the control cultures (Figure 6.9) due to the reduction in the number of cells that adopt the undifferentiated reserve cell fate. When control cultures were transfected with the *Hes1* expression plasmid, a slight increase but significant ($p=0.0003$) in the level of differentiation was observed whereas in the shRNA Notch3 cultures, the level decreased ($p=0.05$), reflecting the persistence of a larger number of undifferentiated, mononucleated cells. The effect of Hes1 expression was statistically significant when analysed using a Student's t-test.

When the undifferentiated, GFP+ve and MyHC-ve cell populations were examined, there was a significantly lower mean percentage of GFP+ve/MyHC-ve cells in both control cultures transfected with Hes1 ($p=0.015$) and shRNA Notch3 cultures transfected with Hes1 expression plasmid ($p=0.05$).

6.6.1.2 Effects of transfection with *Hes5*

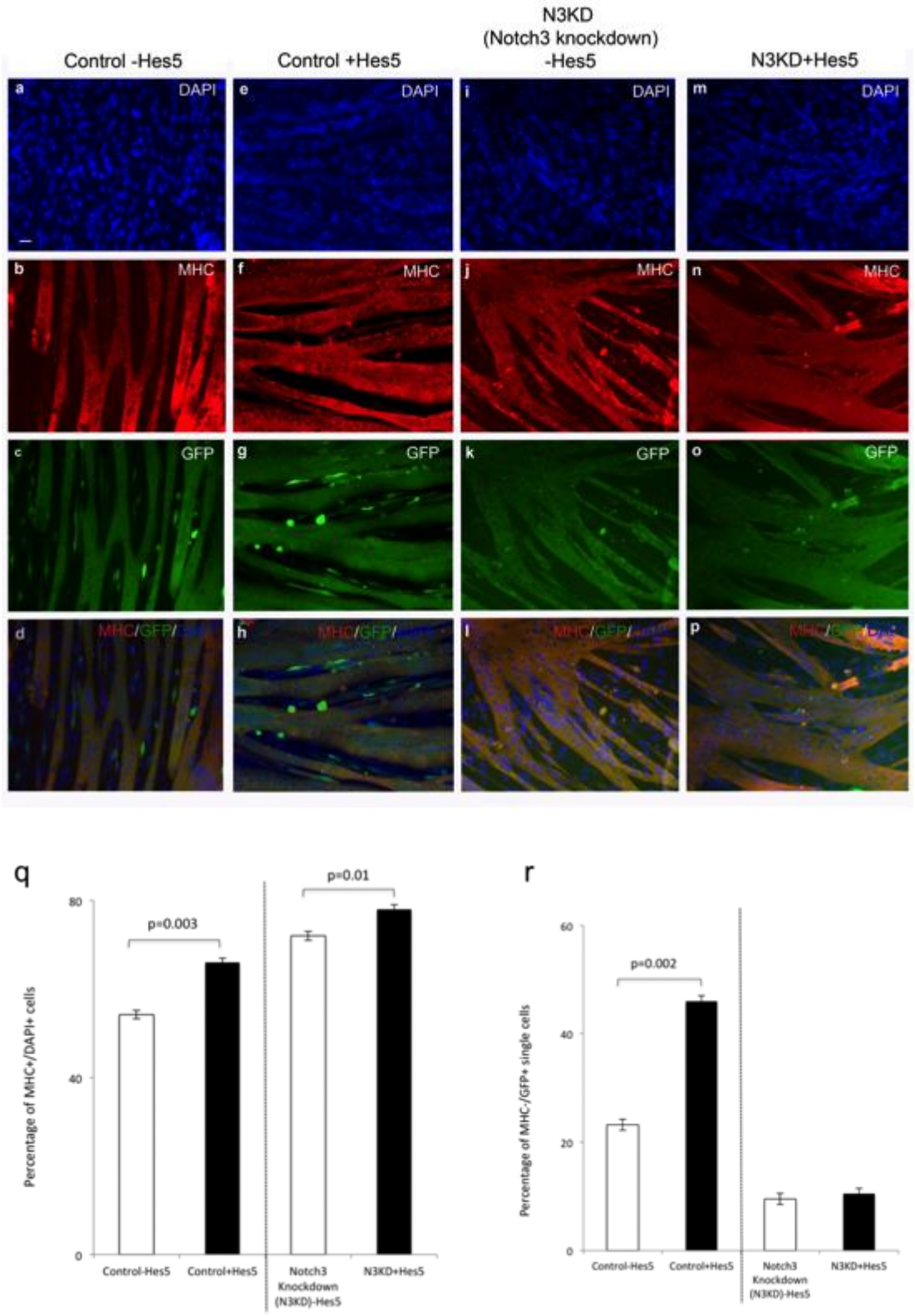


Figure 6.10 Effects of *Hes5* overexpression on the myotube formation in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes5 e*xpression construct. Twenty-four hours post-transfection, the cultures were allowed to differentiate for 96 hours fixed and then co-immunostained with anti-GFP and anti-MyHC antibodies. Representative images of untransfected control (a-d), *Hes5*-transfected control (e-h), untransfected Notch3 knockdown (i-l) and *Hes5*-transfected Notch3 knockdown (m-p) are shown stained with DAPI to reveal all nuclei (blue; a, e, i and m) and for MyHC (red; b, f, j and n) and GFP (green; c, g, k and o) expression. d, h, l and p are overlay images all three stains. (Scale bar =20µM). To determine the number of nuclei in MyHC+ve cells or undifferentiated (MyHC-ve)/GFP+ve single cells, at least 200 nuclei were counted in randomly selected fields (q and r). The histogram bars represent the mean ± 1 SD of three replicate cultures which were compared using a Student's t-test.

Data presented in Figure 6.10 show that overexpression of Hes5 in shRNA Notch3 knockdown C2C12 cells resulted in a slight but significantly increased in the fusion index (i.e. the number of nuclei in MyHC+ve cells) following differentiation, also transfection with the same plasmid produced a significant increase ($p=0.03$) in the fusion index of differentiated control C1C12 cells.

When the undifferentiated GFP+ve/MyHC-ve cell populations were examined, there was a significantly lower mean percentage of GFP+ve/MyHC-ve cells in the shRNA control cultures compared with *Hes5*-transfected control ($p=0.002$). However shRNA Notch3 knockdown and Hes5-transfected shRNA Notch3 knockdown cultures show no difference in percentage of GFP+ve/MyHC-ve cells.

6.6.1.3 Effects of transfection with *Hes7*

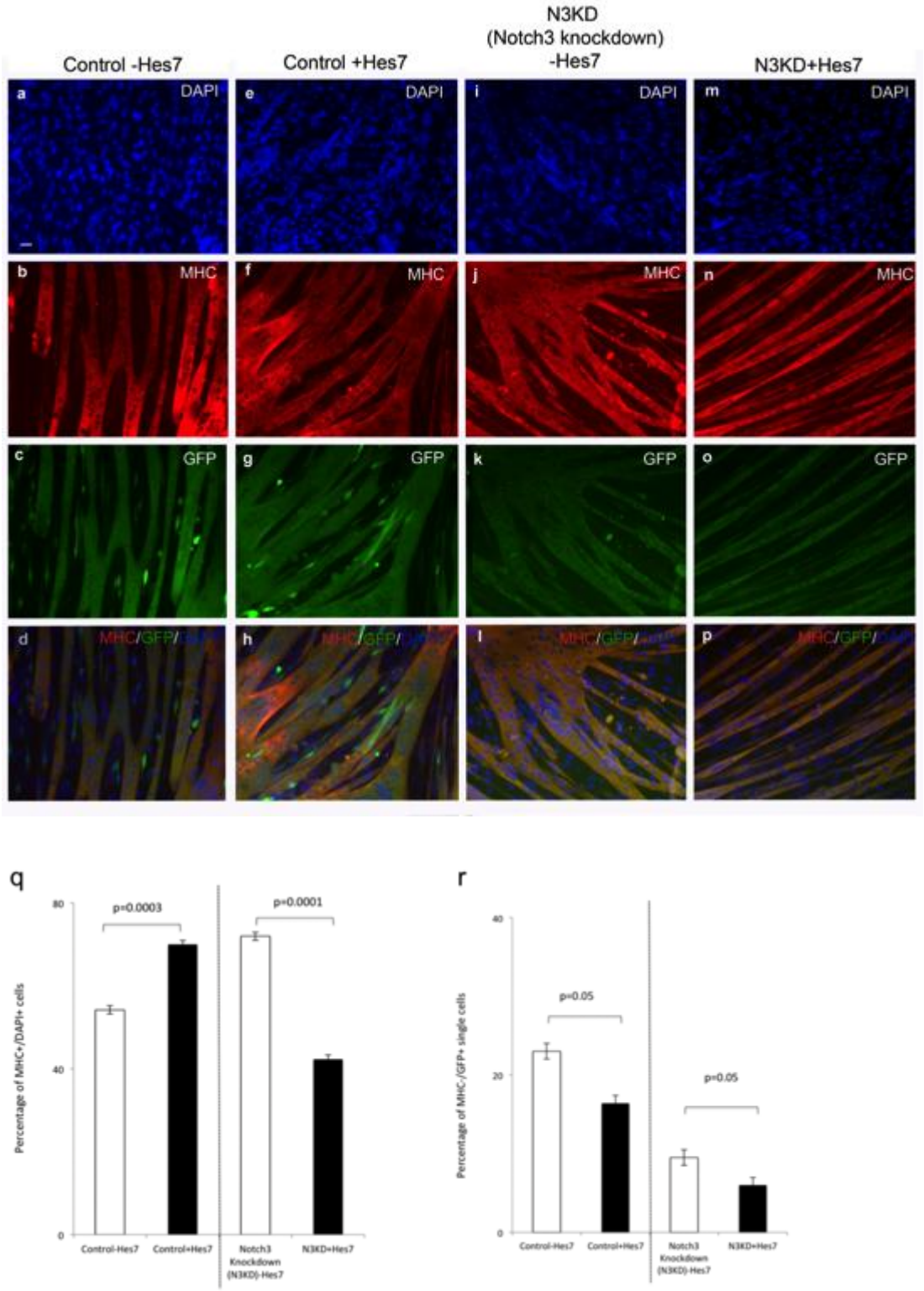


Figure 6.11 Effects of *Hes7* overexpression on myotube formation in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes7*expression construct. Twenty-four hours post-

transfection, the cultures were allowed to differentiate for 96 hours fixed and then co-immunostained with anti-GFP and anti-MyHC antibodies. Representative images of untransfected control (a-d), *Hes7*-transfected control (e-h), untransfected Notch3 knockdown (i-l) and *Hes7*-transfected Notch3 knockdown (m-p) are shown stained with DAPI to reveal all nuclei (blue; a, e, i and m) and for MyHC (red; b, f, j and n) and GFP (green; c, g, k and o) expression. d, h, l and p are overlay images all three stains. (Scale bar = 20μM). To determine the number of nuclei in MyHC+ve cells or undifferentiated (MyHC-ve)/GFP+ve single cells, at least 200 nuclei were counted in randomly selected fields (q and r). The histogram bars represent the mean ± 1 SD of three replicate cultures, which were compared using a Student's t-test.

Figure 6.11 shows that transfection of shRNA Notch3 knockdown cultures with Hes7 prior to the induction of differentiation resulted in a significant decrease in the percentage of nuclei in MyHC+ve cells compared with untransfected knockdown cultures (p=0.0001). However, the opposite was observed in control cultures where overexpression of Hes7 resulted in an increased fusion index (p=0.0003).

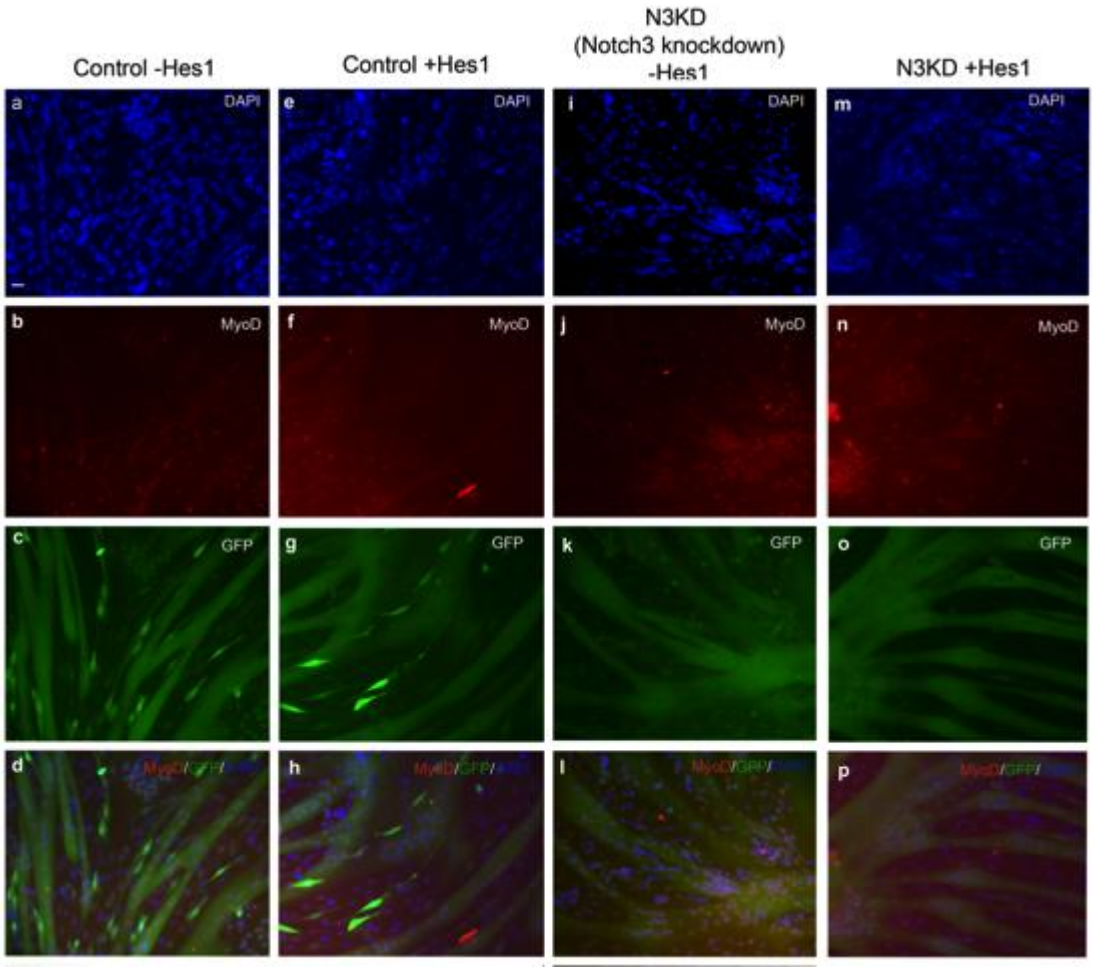
Overexpression of Hes7 caused significantly reduced luciferase activity in both control and Notch3 knockdown cells. A possible explanation is that Hes7 activity has different effects on the behaviour of residual mononucleated cells in differentiated cultures. Hes7 activity may promote the differentiation of reserve cells (consistent with the inhibition of Hes7 promoter activity by overexpression of Notch3 (Figure 6.4)) leading to an increase in the fusion indices of control cultures, but inhibit the differentiation of Notch3-ve myoblasts.

6.6.2 Effects of *Hes* gene overexpression on MyoD expression in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

The initial hypothesis involved a potential role of Notch genes in the cell fate towards self-renewing of the stem cell compartment. Since Notch genes activate Hes genes, we wanted to investigate the relationship between Hes genes activation and MyoD expression, as a marker of activated satellite cells absent of the self-renewing population. To investigate the effects of *Hes* genes on MyoD expression, shRNA Notch3 knockdown and shRNA control C2C12 cultures were transfected with *Hes1, Hes5* or *Hes7* expression plasmids (section 2.8) using Lipofectamine 2000 (section 2.9.1) and seeded into Permanox 8-well Lab-Tek chamber slides at 10^3

cells/chamber in growth medium. 24 Hours after transfection, the medium was switched to differentiation medium and the cultures allowed to differentiate for 96 hours before they were fixed and stained for MyoD expression, as described in section 2.14).

6.6.2.1 Effects of transfection with *Hes1*



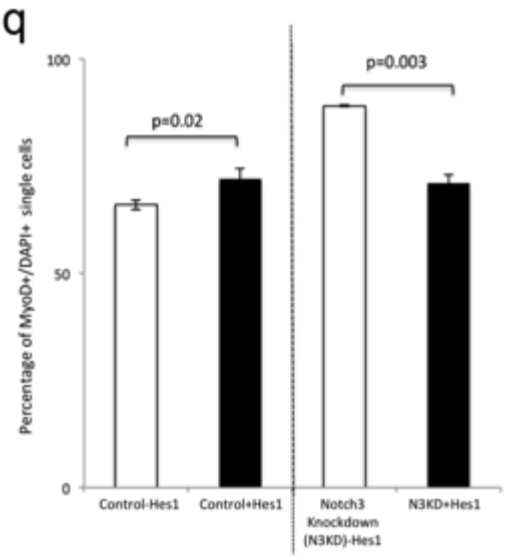


Figure 6.12 Effects of *Hes1* overexpression on MyoD expression in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes1* expression construct. Twenty-four hours post-transfection, the cultures were allowed to differentiate for 96 hours fixed and then co-immunostained with anti-GFP and anti-MyoD antibodies. Representative images of untransfected control (a-d), *Hes1*-transfected control (e-h), untransfected Notch3 knockdown (i-l) and *Hes1*-transfected Notch3 knockdown (m-p) are shown stained with DAPI to reveal all nuclei (blue; a, e, i and m) and for MyoD (red; b, f, j and n) and GFP (green; c, g, k and o) expression. d, h, l and p are overlay images all three stains. (Scale bar = 20µM). To determine the percentage of MyoD+ve cells, at least 200 nuclei were counted in randomly selected fields (q). The histogram bars represent the mean ± 1 SD of three replicate cultures were compared using a Student's t-test.

Figure 6.12 shows Hes1 overexpression resulted in a significant increase in the percentage of MyoD cells in the control cultures, whereas transfection of the Notch3 knockdown cultures with same plasmid resulted in a significant decrease MyoD expression to the same level as observed in the control, Notch3+ve cultures. This suggests that the expression of Hes1 results in an increase in the number of MyoD-ve cells, presumably reserve cells, within the differentiated cultures.

6.6.2.2 Effects of transfection with *Hes5*

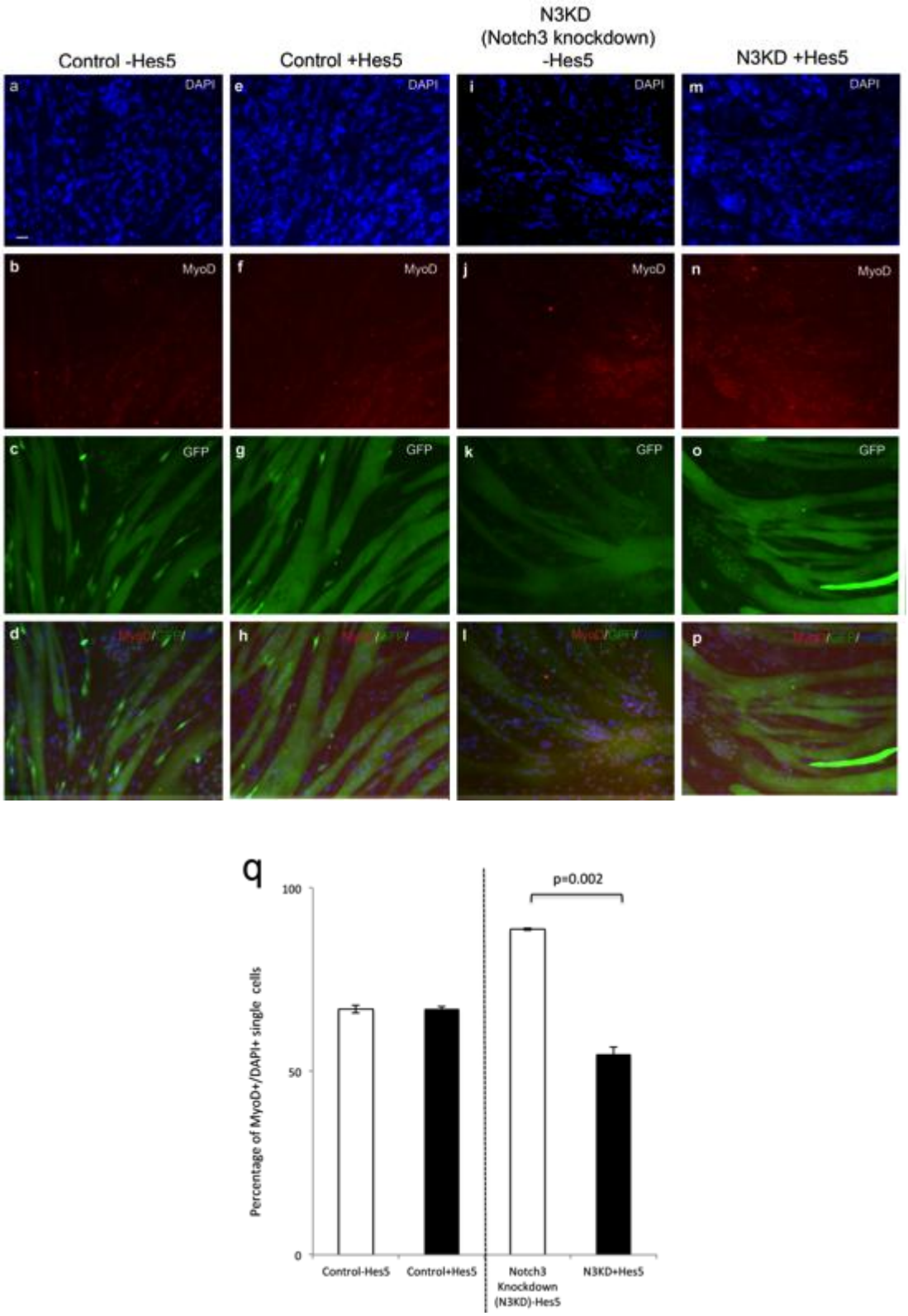


Figure 6.13 Effects of *Hes5* overexpression on MyoD expression in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes5* expression construct. Twenty-four hours post-

transfection, the cultures were allowed to differentiate for 96 hours fixed and then co-immunostained with anti-GFP and anti-MyoD antibodies. Representative images of untransfected control (a-d), *Hes5*-transfected control (e-h), untransfected Notch3 knockdown (i-l) and *Hes5*-transfected Notch3 knockdown (m-p) are shown stained with DAPI to reveal all nuclei (blue; a, e, i and m) and for MyoD (red; b, f, j and n) and GFP (green; c, g, k and o) expression. d, h, l and p are overlay images all three stains. (Scale bar = 20μM). To determine the percentage of MyoD+ve cells, at least 200 nuclei were counted in randomly selected fields (q). The histogram bars represent the mean ± 1 SD of three replicate cultures.

Overexpression of *Hes5* had no significant effect on MyoD expression in the control C2C12 cultures, but resulted in a significant decrease in the proportion of MyoD+ve cells in equivalent Notch3 knockdown cultures (Figure 6.13). As was observed with the *Hes1*, transfection with the Hes5 construct reduced the level of MyoD expression in the knockdown cultures to that observed in the control, Notch3+ve cells. There was no significant difference in MyoD expression in Hes5 transfected shRNA control cultures when compared with the shRNA control (Figure 6.14). However, there was a significant reduction ($p=0.002 < 0.05$) in the MyoD expression in the Hes5 transfected shRNA Notch3 knockdown cultures when compared with the shRNA Notch3 knockdown cultures.

6.6.2.3 Effects of transfection with *Hes7*

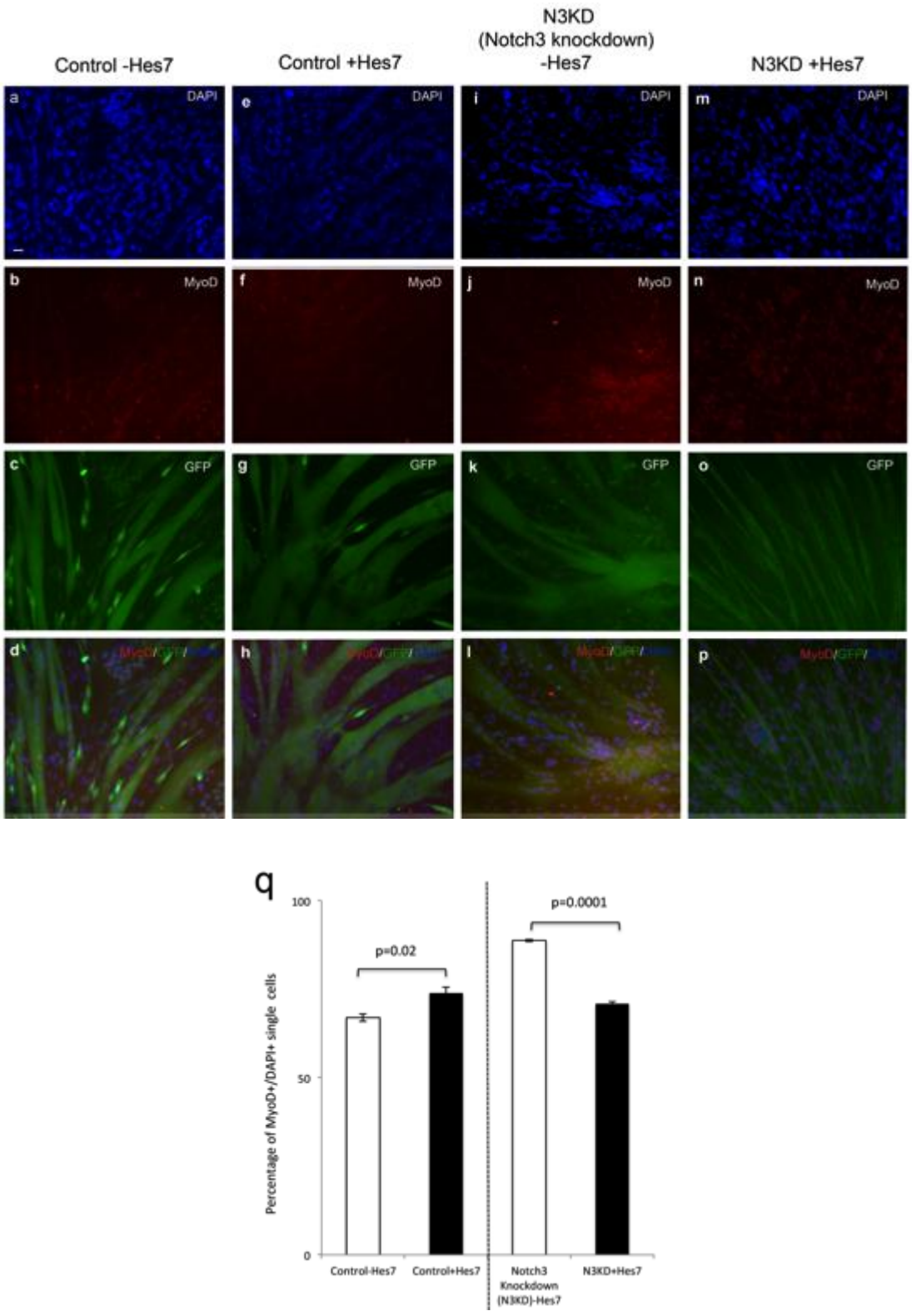


Figure 6.14 Effects of *Hes7* overexpression on MyoD expression in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes7* expression construct. Twenty-four hours post-transfection, the cultures were allowed to differentiate for 96 hours fixed and then

co-immunostained with anti-GFP and anti-MyoD antibodies. Representative images of untransfected control (a-d), *Hes7*-transfected control (e-h), untransfected Notch3 knockdown (i-l) and *Hes7*-transfected Notch3 knockdown (m-p) are shown stained with DAPI to reveal all nuclei (blue; a, e, i and m) and for MyoD (red; b, f, j and n) and GFP (green; c, g, k and o) expression. d, h, l and p are overlay images all three stains. (Scale bar = 20μM). To determine the percentage of MyoD+ve cells, at least 200 nuclei were counted in randomly selected fields (q). The histogram bars represent the mean ± 1 SD of three replicate cultures.

Figure 6.14 shows that transfection with *Hes7* resulted in a slight but significant increase in the percentage of MyoD+ve cells in the control cultures (p=0.02), and a significant decrease in the percentage of MyD+ve cells in the shRNA Notch3 knockdown cultures to similar levels to those observed in the untransfected controls (p=0.0001).

6.6.3 Effects of *Hes* gene overexpression on cyclin dependent kinase inhibitors p21 and p27 expression in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

The main aim of this section was to investigate the effects of *Hes* gene expression on p21 and p27, involved during the differentiation of myogenic cultures, in C2C12 and Notch3 knockdown cells. As Notch signalling has an effect on Hes gene expression, this further tests the hypothesis that Notch receptors have a direct effect on cell fate during differentiation.

Results presented in Section 4.4 suggest Notch3 activity may be involved in the regulation of cell cycle progression. As transfection with a constitutively active form of Notch3 was found to inhibit myoblast proliferation, but enhance proliferation when differentiation was induced. It has previously been suggested that Notch signalling may regulate the cell cycle by inducing changes in the expression of the cdk inhibitors p21 and p27 (Cao *et al*., 2003). p21 and p27 appear to be involved in the regulation of myogenic differentiation, as they are highly upregulated when C2C12 myoblast differentiation is induced (Cao *et al*., 2003).

p21 expression is a prerequisite for irreversible cell cycle withdrawal that precedes differentiation (Cao et al., 2003), and it was not expressed in proliferating myoblasts. p27 induces and maintains a quiescent state in cells (Medema *et al*., 2000; Miskimins *et al*., 2001; Olashaw and Pledger, 2002) and is normally expressed in reversibly quiescence cells (i.e. reserve cells).

To study the effects of *Hes* gene expression on p21 and p27 during the differentiation of myogenic cultures, shRNA Notch3 knockdown and shRNA control C2C12 cultures were transfected with *Hes1, Hes5* or *Hes7* expression plasmids (section 2.8) using Lipofectamine 2000 (section 2.9.1) and seeded into Permanox 8-well Lab-Tek chamber slides at 10^3 cells/chamber in growth medium. 24 Hours after transfection, the medium was switched to differentiation medium and the cultures allowed to differentiate for 96 hours before they were fixed and stained for p21 or p27 expression, as described in section 2.14).

6.6.3.1 Effects of transfection with *Hes1* on p21 expression

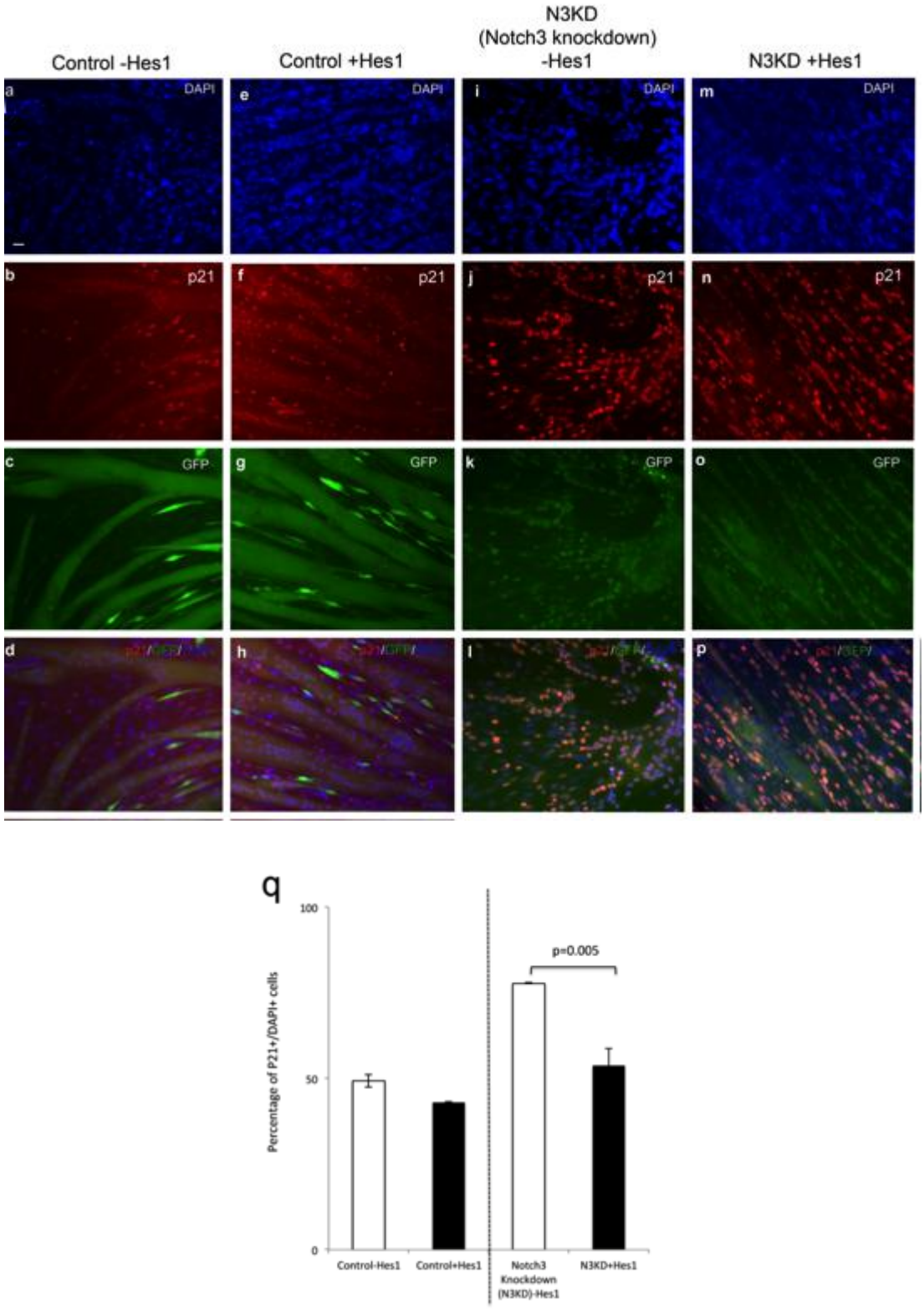


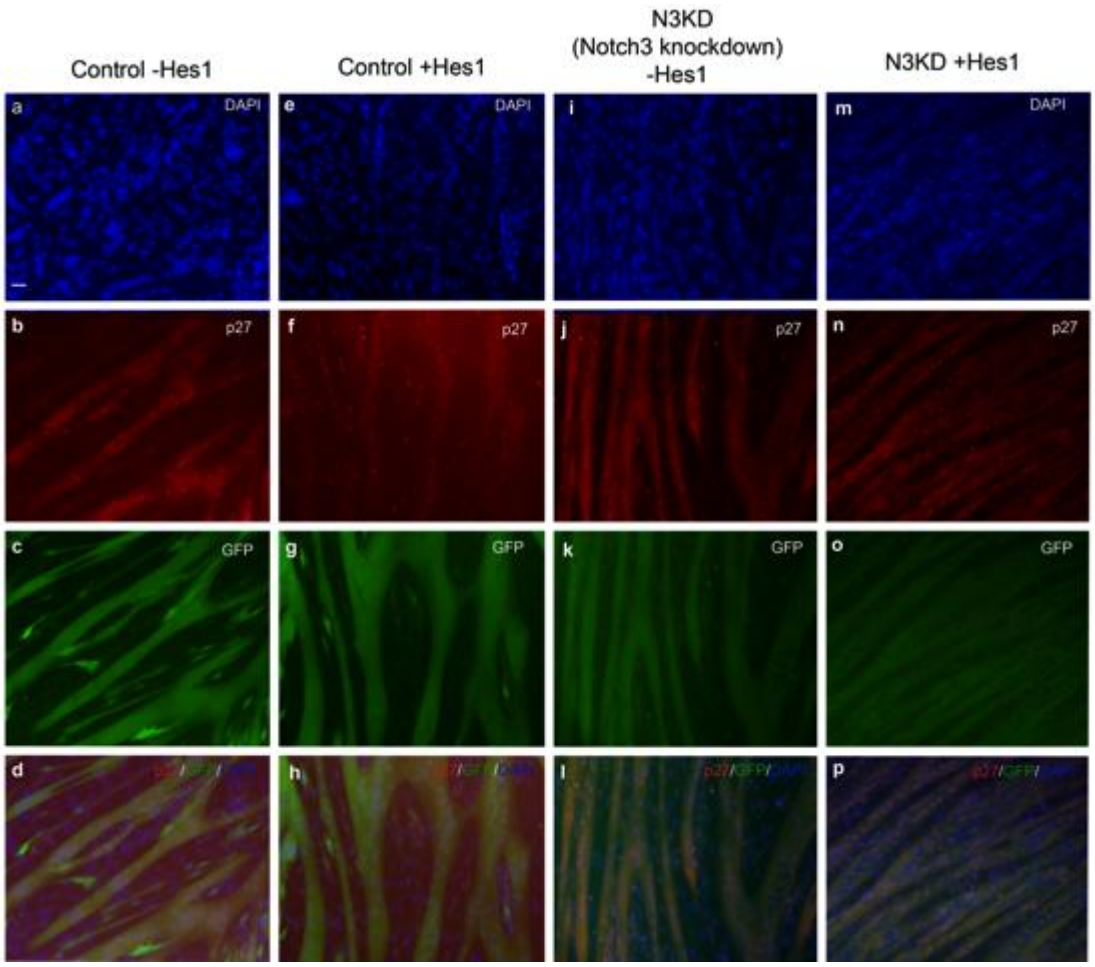
Figure 6.15 Effects of *Hes1* overexpression on p21 expression in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes1*expression construct. Twenty-four hours post-

transfection, the cultures were allowed to differentiate for 96 hours fixed and then co-immunostained with anti-GFP and anti-p21 antibodies. Representative images of untransfected control (a-d), *Hes1*-transfected control (e-h), untransfected Notch3 knockdown (i-l) and *Hes1*-transfected Notch3 knockdown (m-p) are shown stained with DAPI to reveal all nuclei (blue; a, e, i and m) and for p21 (red; b, f, j and n) and GFP (green; c, g, k and o) expression. d, h, l and p are overlay images all three stains. (Scale bar = 20μM). To determine the percentage of p21 cells, at least 200 nuclei were counted in randomly selected fields (q). The histogram bars represent the mean ± 1 SD of three replicate cultures. Statistical comparisons were carried out by Student's t-test.

Overexpression of Hes decreased the percentage of p21+ve cells in the differentiated control cultures. *Hes1*-transfected Notch3 knockdown cultures contained significantly fewer p21+ve cells compared with the equivalent untransfected control cultures (p=0.005).

6.6.3.2 Effects of transfection with *Hes1* on p27 expression



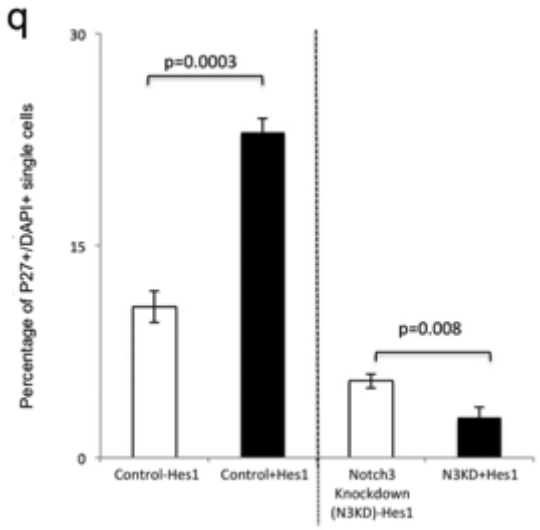


Figure 6.16 Effects of *Hes1* overexpression on p27 expression in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes1*expression construct. Twenty-four hours post-transfection, the cultures were allowed to differentiate for 96 hours fixed and then co-immunostained with anti-GFP and anti-p27 antibodies. Representative images of untransfected control (a-d), *Hes1*-transfected control (e-h), untransfected Notch3 knockdown (i-l) and *Hes1*-transfected Notch3 knockdown (m-p) are shown stained with DAPI to reveal all nuclei (blue; a, e, i and m) and for p27 (red; b, f, j and n) and GFP (green; c, g, k and o) expression. d, h, l and p are overlay images all three stains. (Scale bar = 20μM). To determine the percentage of undifferentiated p27+ve cells, at least 200 nuclei were counted in randomly selected fields (q). The histogram bars represent the mean ± 1 SD of three replicate cultures. Statistical comparisons were carried out by Student's t-test.

Figure 6.16 shows that the untransfected Notch3 knockdown cultures contained fewer p27+cells compared to the untransfected controls, consistent with the previously observed reduction in the number of reserve cells present following differentiation. Overexpression of *Hes1* resulted in a significant increase in the proportion of p27+ve cells in the control cultures (p=0.0003). Counting p27+ cells in mononucleated cell excluding myotubes, then the mean percentage of p27+ cells of the Hes1 transfected shRNA Notch3 knockdown control cultures is significantly less than that of shRNA Notch3 knockdown control cultures (p=0.008).

6.6.3.3 Effects of transfection with *Hes5* on p21 expression

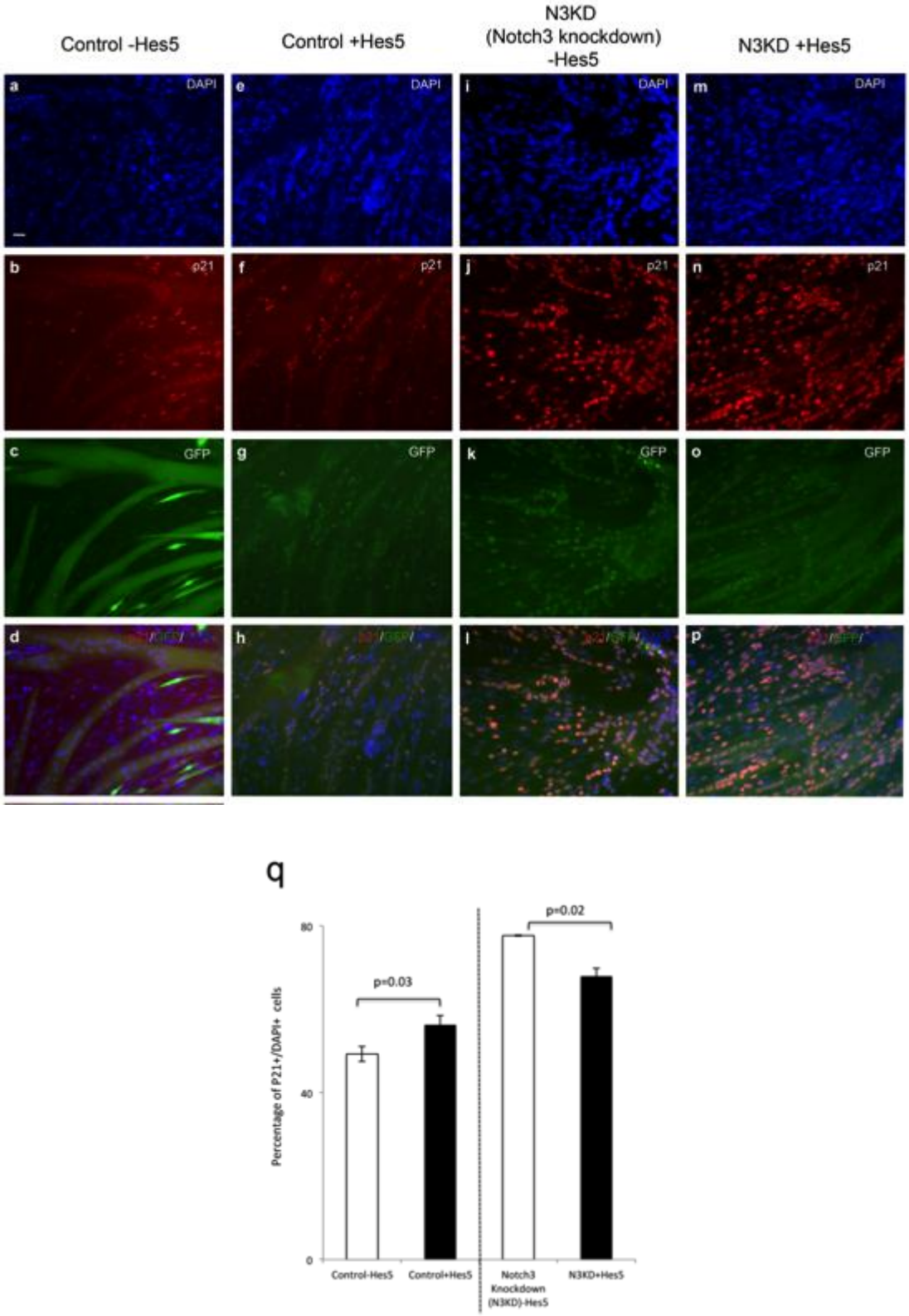


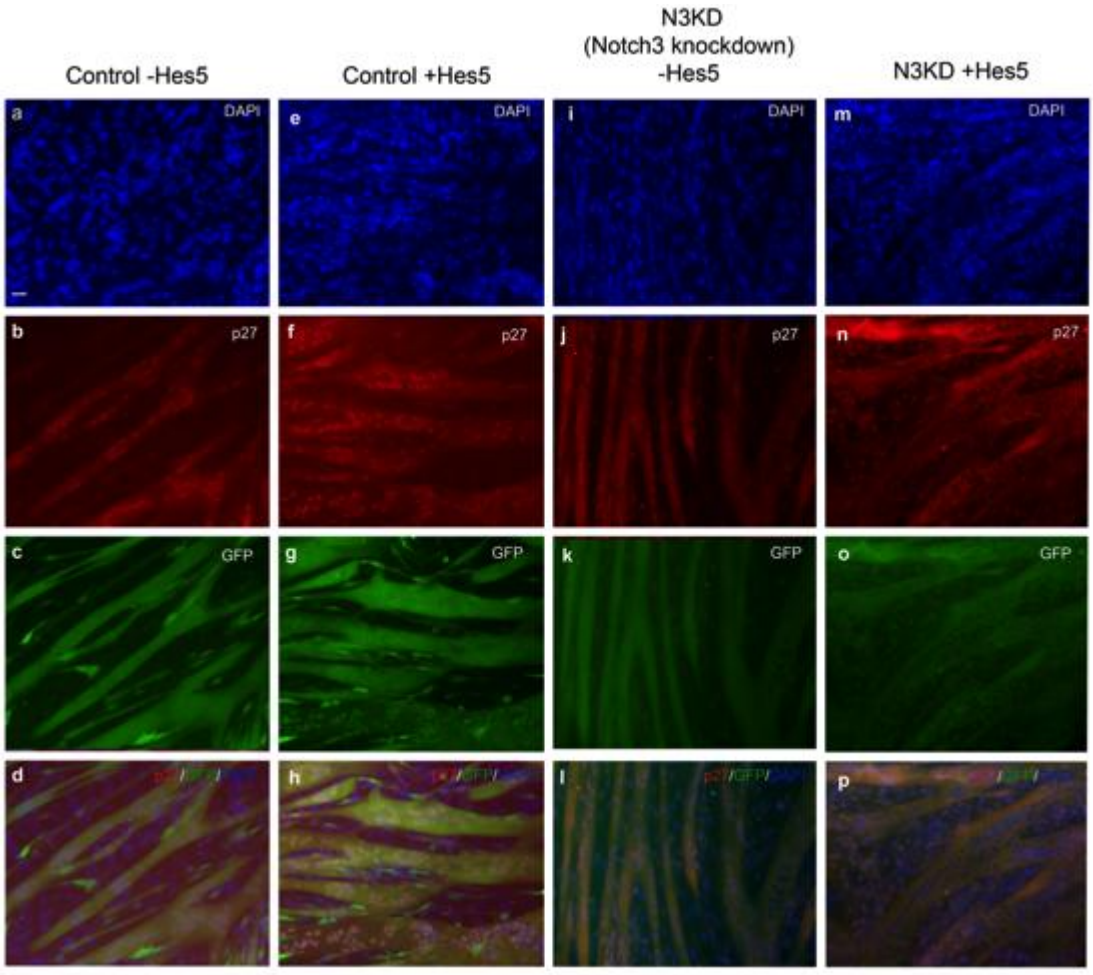
Figure 6.17 Effects of *Hes5* overexpression on p21 expression in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes5*expression construct. Twenty-four hours post-

transfection, the cultures were allowed to differentiate for 96 hours fixed and then co-immunostained with anti-GFP and anti-p21 antibodies. Representative images of untransfected control (a-d), *Hes5*-transfected control (e-h), untransfected Notch3 knockdown (i-l) and *Hes5*-transfected Notch3 knockdown (m-p) are shown stained with DAPI to reveal all nuclei (blue; a, e, i and m) and for p21 (red; b, f, j and n) and GFP (green; c, g, k and o) expression. d, h, l and p are overlay images all three stains. (Scale bar = 20μM)
To determine the percentage of p21 cells, at least 200 nuclei were counted in randomly selected fields (q). The histogram bars represent the mean ± 1 SD of three replicate cultures. Statistical comparisons were carried out by Student's t-test.

Transfection with the *Hes5* expression plasmid resulted in a significant increase in the percentage of p21+ve cells in the control cultures (p=0.03), but significantly decreased the proportion in the Notch3 knockdown cultures (p=0.02 (Figure 6.17)).

6.6.3.4 Effects of transfection with *Hes5* on p27 expression



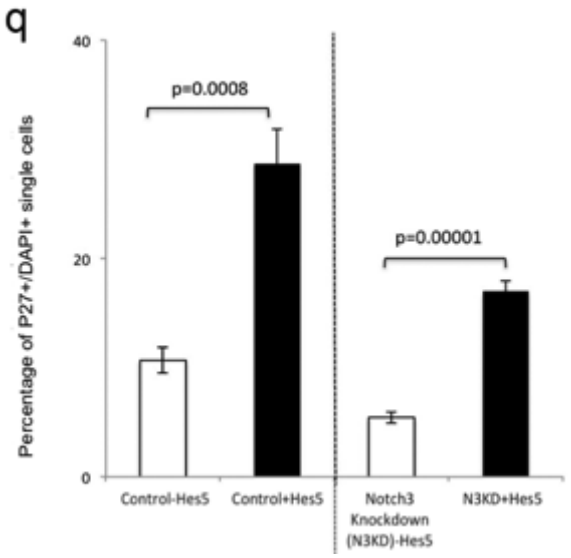


Figure 6.18 Effects of *Hes5* overexpression on p27 expression in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes5*expression construct. Twenty-four hours post-transfection, the cultures were allowed to differentiate for 96 hours fixed and then co-immunostained with anti-GFP and anti-p27 antibodies. Representative images of untransfected control (a-d), *Hes5*-transfected control (e-h), untransfected Notch3 knockdown (i-l) and *Hes5*-transfected Notch3 knockdown (m-p) are shown stained with DAPI to reveal all nuclei (blue; a, e, i and m) and for p27 (red; b, f, j and n) and GFP (green; c, g, k and o) expression. d, h, l and p are overlay images all three stains. (Scale bar = 20μM). To determine the percentage of undifferentiated p27+ve cells, at least 200 nuclei were counted in randomly selected fields (q). The histogram bars represent the mean ± 1 SD of three replicate cultures. Statistical comparisons were carried out by Student's t-test.

As was observed with the *Hes1* plasmid, overexpression of Hes5 in control cultures resulted in a significant increase in the percentage of p27+ve cells in the single cell population remaining after differentiation (Figure 6.18). However, in contrast to Hes1, overexpression of Hes5 also significantly increased the percentage of p27+ve single cells in the Notch3 knockdown cultures to greater levels than in the untransfected control cultures. This suggests that Hes5 may be involved in establishing a reversibly quiescent reserve cell phenotype and that during differentiation, Notch3 activity may promote *Hes5* expression as shown by the effect of the Notch3ICD on *Hes5* promoter activity (refer back to the promoter results (Figure 6.3))

6.6.3.5 Effects of transfection with *Hes7* on p21 expression

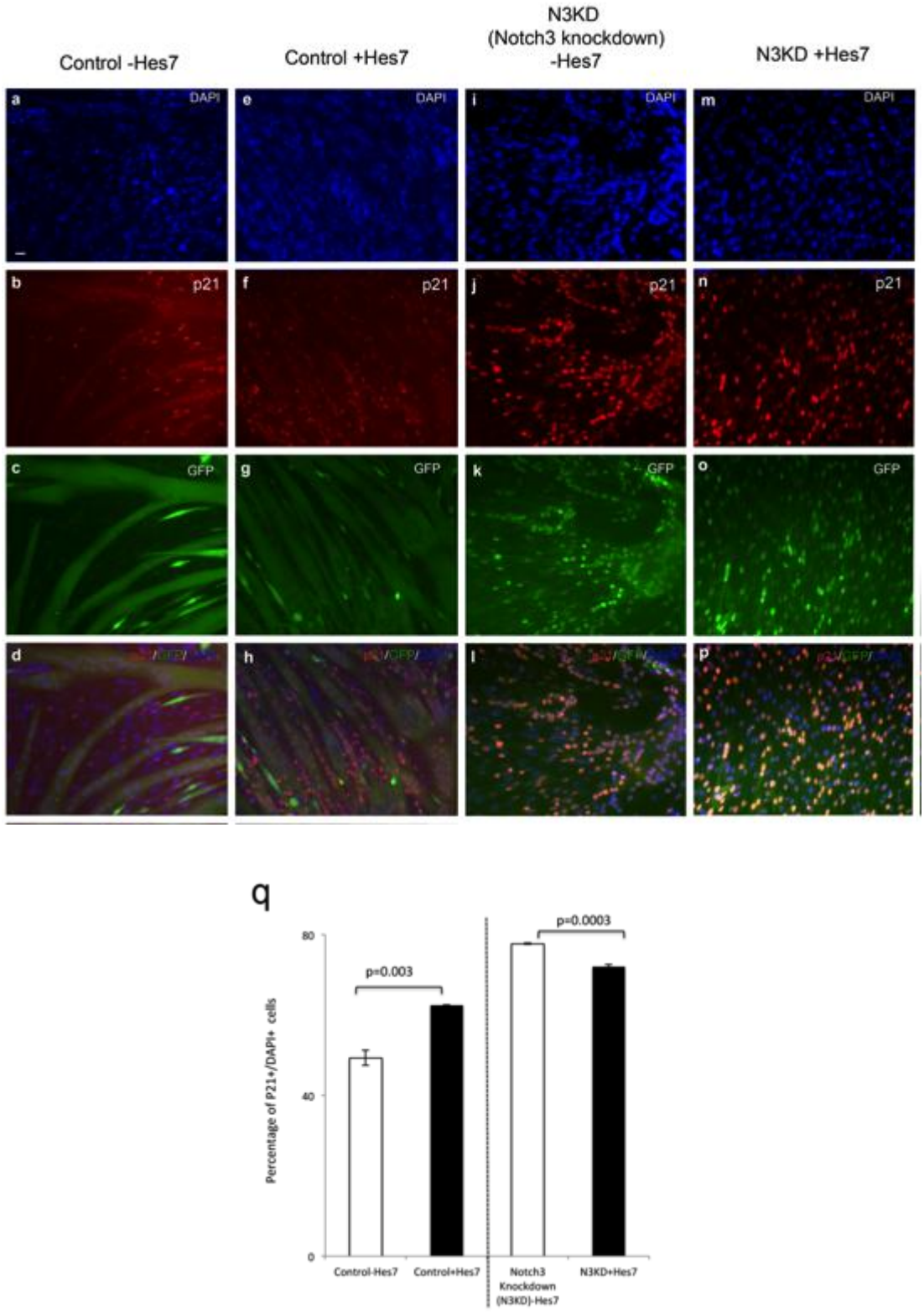


Figure 6.19 Effects of *Hes7* overexpression on p21 expression in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes7*expression construct. Twenty-four hours post-

transfection, the cultures were allowed to differentiate for 96 hours fixed and then co-immunostained with anti-GFP and anti-p21 antibodies. Representative images of untransfected control (a-d), *Hes7*-transfected control (e-h), untransfected Notch3 knockdown (i-l) and *Hes7*-transfected Notch3 knockdown (m-p) are shown stained with DAPI to reveal all nuclei (blue; a, e, i and m) and for p21 (red; b, f, j and n) and GFP (green; c, g, k and o) expression. d, h, l and p are overlay images all three stains. (Scale bar = 20μM). To determine the percentage of p21 cells, at least 200 nuclei were counted in randomly selected fields (q). The histogram bars represent the mean ± 1 SD of three replicate cultures. Statistical comparisons were carried out by Student's t-test.

Figure 6.19 shows that overexpression of Hes7 in control cultures resulted in a significant increase in the percentage of cells positive for p21 ($p=0.003$). Together with the observed increases in the myotube formation and MyoD expression (Figure 6.11 and Figure 6.14, respectively), this suggests that Hes7 may be associated with differentiation. However, Hes7 overexpression significantly decreased the percentage of p21+ve cells in Notch3 knockdown cultures ($p=0.0003$).

6.6.3.6 Effects of transfection with *Hes7* on p27 expression

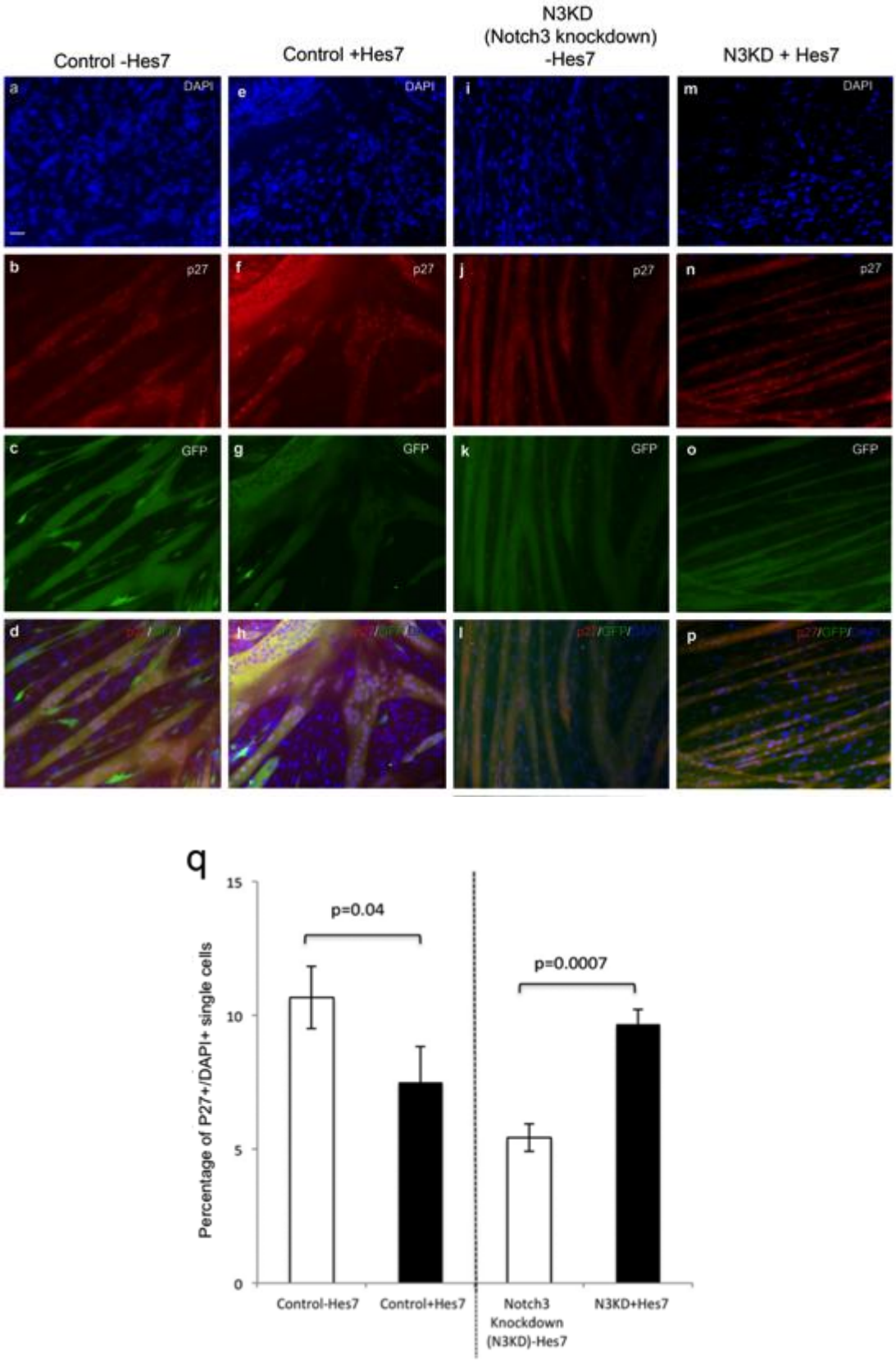


Figure 6.20 Effects of *Hes7* overexpression on p27 expression in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown)

were transfected with a *Hes7*expression construct. Twenty-four hours post-transfection, the cultures were allowed to differentiate for 96 hours fixed and then co-immunostained with anti-GFP and anti-p27 antibodies. Representative images of untransfected control (a-d), *Hes7*-transfected control (e-h), untransfected Notch3 knockdown (i-l) and *Hes7*-transfected Notch3 knockdown (m-p) are shown stained with DAPI to reveal all nuclei (blue; a, e, i and m) and for p27 (red; b, f, j and n) and GFP (green; c, g, k and o) expression. d, h, l and p are overlay images all three stains. (Scale bar = 20μM). To determine the percentage of undifferentiated p27+ve cells, at least 200 nuclei were counted in randomly selected fields (q). The histogram bars represent the mean ± 1 SD of three replicate cultures. Statistical comparisons were carried out by Student's t-test.

Data presented in Figure 6.20 show that in control cultures, overexpression of Hes7 resulted in a significant decrease in the percentage of p27+ve cells amongst the undifferentiated single cell population (p=0.04). Together with the p21 data, this suggests that Hes7 may promote differentiation whilst reducing the number of residual, quiescent reserve cells. In the Notch3 knockdown cultures however, the findings were reversed as Hes7 significantly increased the percentage of p27+ve single cells whilst decreasing p21 expression (p=0.0007).

6.6.4 Effect of *Hes* gene overexpression on apoptosis in C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures

Previous studies have suggested that Notch3 activity may be a critical determinant of vascular smooth muscle cell survival (Wang *et al*., 2002). Consistent with this, is the observation that caspase3/7 activity, a marker of apoptosis (Fesik & Shi, 2001), was more than three times greater in shRNA Notch3 knockdown cultures compared with shRNA control than and wild-type C2C12 and cultures (section 4.3.3).

Our results concerning down-expression of Notch3 raises the hypothesis that increased apoptosis caused by a lack of Notch3-mediated Hes expression may play a role in the consequences of Notch3 inhibition. To asses this hypothesis, shRNA Notch3 knockdown and shRNA control C2C12 cultures were transfected with either the *Hes1, Hes5 or Hes7* expression plasmid (section 2.7), and then seeded in white-

walled 96 well plates at 1500 cells/well maintained in growth medium for 24 hours and then transferred to differentiation medium. After 48 hours, caspase 3/7 activities were measured using the Caspase3/7-Glo Assay Kit (Promega) as described in section 2.4.2. A 48 hour time point was chosen as this is when Notch3 activity is highest following the induction of differentiation (Chapter 3, Section 3.3). Caspases are a group of cysteine proteases that are the central effectors of apoptosis (Fesik and Shi, 2001), such that the level of caspase 3/7 activity is an accepted indicator of the level of apoptosis.

6.6.4.1 Effects of transfection with *Hes1* on apoptosis

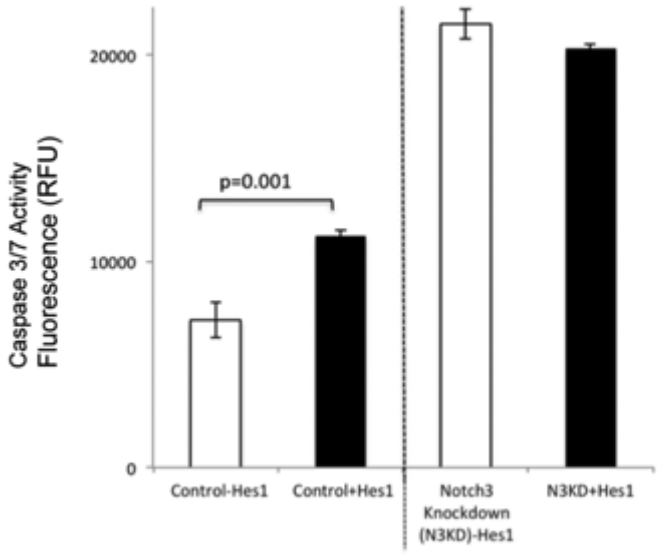


Figure 6.21 Effects of *Hes1* overexpression on apoptosis in differentiating C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures
Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes1* expression plasmid and induced to differentiate for 48 hours. Caspase 3/7 activities were measured and the results are presented as the mean value from three cultures ± 1 SD, which were compared using a Student's t-test. The columns represent untransfected cultures; the solid columns are cultures transfected to overexpress Hes1.

Data presented in Figure 6.7 confirm that after 48 in differentiation medium, Notch3 knockdown C2C12 cultures express greater levels of caspase 3/7 activity than control cultures, indicative of a greater level of apoptosis. Overexpression of Hes1, which is downstream of Notch signalling, failed to compensate for the lack of Notch3 in terms of preventing apoptosis and in the case of the control cells, even increased the level of caspase activity, thus suggesting that overexpression of known downstreal target genes cannot completely compensate for Notch3 knockdown.

Effects of transfection with *Hes5* on apoptosis

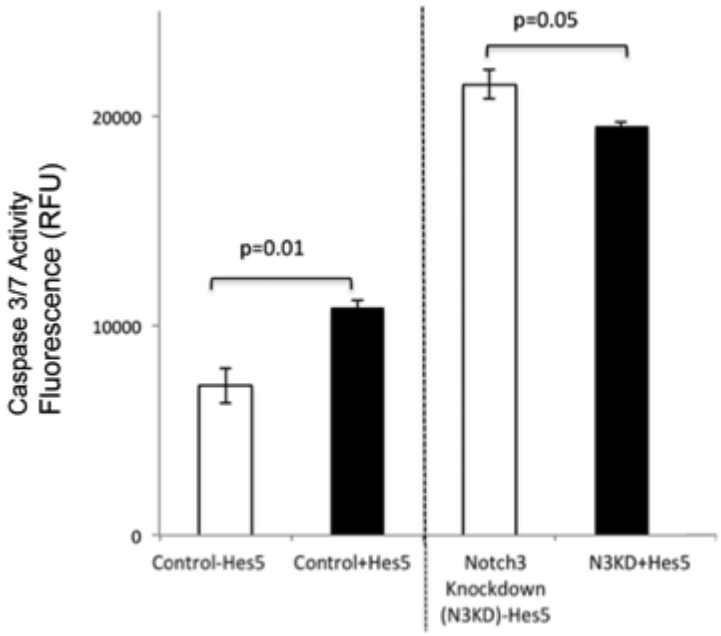


Figure 6.22 Effects of *Hes5* overexpression on apoptosis in differentiating C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures
Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes5* expression plasmid and induced to differentiate for 48 hours. Caspase 3/7 activities were measured and the results are presented as the mean value from three cultures ± 1 SD. The columns represent untransfected cultures; the solid columns are cultures transfected to overexpress Hes5. Statistical comparisons were carried out by Student's t-test.

Figure 6.22 shows that overexpression of Hes5 resulted in a significant increase in Caspase3/7 activity (and therefore apoptosis) in control cultures. In Notch3 knockdown cultures, *Hes5* transfection resulted in a significant reduction compared with the Notch3 knockdown control cultures, although the overall level was still considerably greater than in the non-knockdown cultures.

6.6.5 Effects of transfection with *Hes7* on apoptosis

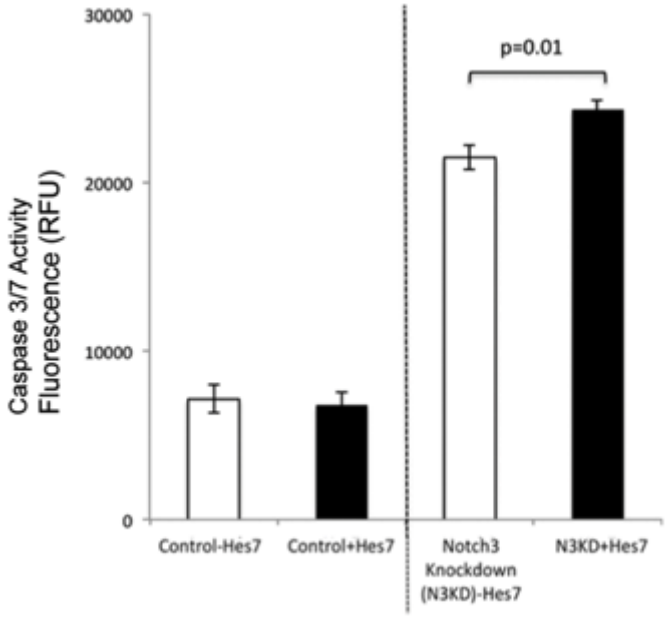


Figure 6.23 Effects of Hes7 overexpression on apoptosis in differentiating C2C12 and shRNA Notch3 knockdown C2C12 myogenic cultures
Stable lines of C2C12 myoblasts expressing either an irrelevant control shRNA/eGFP plasmid (Control) or shRNA Notch3 knockdown/eGFP plasmid (Notch3 knockdown) were transfected with a *Hes7* expression plasmid and induced to differentiate for 48 hours. Caspase 3/7 activities were measured and the results are presented as the mean value from three cultures ± 1 SD. The columns represent untransfected cultures; the solid columns are cultures transfected to overexpress Hes7. Statistical comparisons were carried out by Student's t-test.

Transfection of control C2C12 cultures with the *Hes7* expression plasmid had no effect on Caspase3/7 activity when compared with the untransfected control cultures (Figure 6.23). When Notch3 knockdown cultures were transfected

however, expression of Hes7 resulted in a small but significant increase compared with the untransfected Notch3 knockdown control cultures.

6.7 Discussion

	Control vs Control+Hes			N3KD vs N3KD+ Hes		
	Hes1	Hes5	Hes7	Hes1	Hes5	Hes7
Myotube formation	↑	↑	↑	↓	↑	↓
MyoD Expression	↑	–	↑	↓	↓	↓
p21	–	↑	↑	↓	↓	↓
p27	↑	↑	↓	↓	↑	↑
Apoptosis	↑	↑	–	–	↓	↑

Table 6.1 Summary of the effects of overexpression of Hes1, Hes5 and Hes7 in shRNA control and shRNA Notch3 cultures

Our initlal hypothesis is that members of the Notch receptor family play a role in the cell fate and self-renewing during muscle cell differentiation. We observed a differential expression of Notch1 and Notch3, which is consistent with this hypothesis, although the activation of downstream targets needed to be confirmed through Hes gene family activation. Chapter 6 revealed that Notch target genes Hey1, Hes1, Hes5 and Hes7 are all highly expressed in proliferating myoblasts. Notch1 and Notch2 were expressed at high levels in myoblasts. Myotube fractions only express Notch1 but not Notch2 or Notch3. Differentiation to form myotubes was accompanied by a marked down-regulation of Hey1 and Hes1, whilst Hes5 and Hes7 were still expressed, but at lower levels. The down-regulation of Hey1/Hes1 could be due to loss of Notch2 and or changes in Numb.

Hey1, Hes1, Hes5 and Hes7 were expressed in reserve cell fractions at lower levels, but there was significantly up-regulation of HeyL. Reserve cells expressed high level of Notch3. The result presented here is consistent with the observed HeyL highly expressed in quiescent satellite cells (Notch3IC+) but not in activated/proliferating satellite cells (Notch1+) or myofibres (Fukada et al., 2011; Mourikis et al., 2012).

Hey1 is also expressed in quiescent satellite cells. However, in contrast to HeyL, a weak Hey1 signal was detected in activated/proliferating satellite cells (Fukada et al., 2011).

As Numb inhibits Notch1 (Beres et al., 2011), it would be expected that the magnitude of the increase in luciferase activity for Hes genes due to Notch1 IC would be reduced. However, conflicting to what was expected in Hes1/Hey1, the luciferase activity of Hes5 and Hes7 were increased when Numb was transfected with Notch1 IC in C2C12. Numb was able to consistently reduced the luciferase activity of Hes1, Hes5 and Hes7 when transfected with Notch1 IC in shRNA.

Numb appears to consistently enhance the ability of Notch3 IC to up-regulate all three Hes genes in the context of shRNA Notch3 knockdown cultures. Numb was also able to consistently inhibit Notch1 IC's ability to up-regulate all three Hes genes in the shRNA Notch3 knockdown culture, but it was only capable of inhibiting Notch1 IC's ability to up-regulate Hes1 activation in C2C12. Beres et al. (2011) reported Notch1 and Notch2 are inhibited by numb but not Notch3 activity. The intrinsic expression of Notch3 in the C2C12 may have contributed to the increase of Hes5 and Hes7 activation when Numb was transfected, and this increase due to Numb-Notch3 interaction may have compensated and masked the reduction of Hes5 and Hes7 activity resulting from the inhibition of Notch1 by Numb.

Overexpression of Notch1ICD increased both Hey1 and Hey2 promoter activity in both C2C12, consistent with previous findings (Nakagawa et al., 2000), whereas Notch3ICD had no effect on either Hey1 or Hey2 promoter activity in either system. In contrast, Notch1ICD slightly decreased HeyL promoter activity, whereas overexpression of Notch3ICD increased activity in both C2C12 and mouse primary myoblasts.

Hes1 transfected shRNA control cultures shows a slight increase in the level of differentiation and MyoD+ cells. While in the shRNA Notch3 cultures, the level of both MyHC+ and MyoD+ cells decreased, reflecting the persistence of a larger number of undifferentiated, mononucleated cells. Overexpression of Hes1

decreased the percentage of p21+ populations in the differentiated control cultures. Untransfected Notch3 knockdown cultures contained fewer p27+cells compared to the untransfected controls, consistent with the previously observed reduction in the number of reserve cells present following differentiation. Overexpression of Hes1 resulted in a significant increase in the proportion of p27+ve cells in the control cultures, but significantly decreased the percentage in the Notch3 knockdown cultures.

Overexpression of Hes5 in shRNA Notch3 knockdown C2C12 cells resulted in a small, but significant increase in the fusion index following differentiation, while transfection with the same plasmid produced a significant larger increase in the fusion index of differentiated control C1C12 cells. shRNA control cultures show lower undifferentiated cells compared with Hes5-transfected control. But shRNA Notch3 knockdown and Hes5-transfected shRNA Notch3 knockdown cultures show no difference in percentage of undifferentiated cells. This is different from the previous finding (Beatus et al., 1999), in which Notch3 IC was found to repress Hes5 in vitro and in vivo. Different genes are active in different kinds of cells. Therefore this is very like due to the difference between the mouse muscle cell line being used in our study and the JEG cells and COS-7 cell line used by Beatus and coworkers.

Furthermore, overexpression of Hes5 resulted in a significant increase in the percentage of p21+ and p27+ cells in the transfected control cultures, but significantly decreased the p21+ proportion in the Notch3 knockdown cultures. However, in contrast to Hes1, overexpression of Hes5 also significantly increased the percentage of p27+ve single cells in the Notch3 knockdown cultures to greater levels than in the untransfected control cultures. This suggests that Hes5 may be involved in establishing a reversibly quiescent reserve cell phenotype and that during differentiation. Notch3 activity may promote Hes5 expression as shown by the effect of the Notch3ICD on Hes5

Hes7 transfected shRNA Notch3 knockdown cultures resulted in a significant decrease in MyHC+ve cells compared with untransfected knockdown cultures. The contrary was observed in control cultures where overexpression of Hes7 resulted in

an increased fusion index and MyoD+ cell populations. When the undifferentiated cell populations were examined, there was a significantly lower GFP+ve/MyHC-ve cells in the both shRNA control and shRNA Notch3 knockdown cultures compared with both Hes7-transfected cultures.

In control cultures, overexpression of Hes7 resulted in a significant decrease in the percentage of p27+ve cells amongst the undifferentiated single cell population. Together with the p21 data, this suggests that Hes7 may promote differentiation whilst reducing the number of residual, quiescent reserve cells. In the Notch3 knockdown cultures however, the findings were reversed as Hes7 significantly increased the percentage of p27+ve single cells whilst decreasing p21 expression.

The findings in this chapter suggested Hes1 prevents differentiation, but is probably responding to Notch1/Notch2 rather than Notch3. As during differention, Numb upregulates and inhibits Notch1/2 expression and therefore prevents cells from undergoing differentiation. This means most cells remain proliferating (Notch1/2+), whereas Notch3 has no effect on Numb upregulated. Hes7 appears to “favour” differentiation and may be inhibited by Notch3. Hes5 is probably the most interesting and may be related to Notch3 and reserve cells.

The main challenge is that while both the shRNA control and the knockdown cultures express GFP marker, none of the Notch target gene plasmids have a marker (i.e. mCherry) to examine the transfection efficiency when they are transfected into shRNA (GFP+) cells. Target genes expressing mCherry or other markers except GFP would facilitate data quantification. Besides, the data is based on transient transfection and so any effect may appear minor.

Chapter 7: General discussion and a potential model for the recruitment of satellite cells during skeletal muscle regeneration

Myofibre homeostasis, growth and repair require adult satellite cells, which are distributed throughout the tissue, each within an individual niche consisting of an underlying myofibre membrane, surrounding extracellular matrix and overlying basal lamina. Normally quiescent, satellite cells become activated in response to exercise or damage and proliferate to generate a pool of precursors (myoblasts) that differentiate into new myonuclei within existing or nascent myofibres (Charge & Rudnicki, 2004; Kuang *et al*., 2008). In addition to producing differentiated cells, satellite cells also self-renew to maintain or replenish the stem cell compartment retain regenerative capacity (Collins *et al*., 2005; Montarras *et al*., 2005; Sacco *et al*., 2008). The precise mechanism by which this occurs remains unclear, although numerous studies have implicated the Notch signalling pathway involved in determining cell fate behaviour and fate in a wide range of developing and adult tissues (Conboy & Rando, 2002; Conboy *et al*., 2005; Luo *et al*., 2005; Kitzmann *et al.,* 2006). We hypothesize that Notch signalling is indeed involved in the decision concerning cell fate between differentiation and self-renewing, and that several members of the Notch family receptors may be involved in different stages of this decision.

Since Notch1 role has been investigated in the generation of quiescent precursors, the aim of this project was to investigate the role of Notch3 activity in the regulation of adult skeletal muscle stem cell behaviour. Although Notch3 is known to be expressed by quiescent satellite cells (Fukada *et al*., 2007; Kuang *et al.,* 2007; Mourikis *et al*., 2012) and a recent study has shown that skeletal muscle regeneration is compromised in mice lacking the receptor (Kitamoto & Hanaoka, 2010), its precise function remains unclear.

Many of the studies presented in this thesis were carried out an *in vitro* model of satellite cell specification during regeneration. Cultures of the murine myogenic cell line C2C12 (Yaffe and Saxel, 1977; Blau et al., 1983) and primary satellite cell-derived cultures were maintained as proliferating myoblasts in growth media containing high levels of serum and as required, were induced to differentiate by transferring to low serum-containing differentiation medium. After a few days, the cultures contained large numbers of differentiated, post-mitotic myotubes formed by myoblast fusion. Significantly, not all of the myoblasts underwent differentiate with a proportion exiting the cell cycle and persisting as quiescent, undifferentiated precursors, termed "reserve cells" (Yoshida *et al*., 1998). These reserve cells share many phenotypic characteristics with quiescent satellite cells such as the presence of Pax7 and CD34, the lack of expression of MRFs and high levels of membrane sphingomyelin (demonstrated by the binding of lysenin) (Beauchamp *et al*., 2000; Nagata *et al.,* 2006). Furthermore, quiescent reserve cells can be reactivated to enter the cell cycle by the addition of growth medium and their progeny again induced to differentiate, leading again to a new mixed population of myotubes and reserve cells (Kitzmann *et al.*, 1998; Lindon *et al.,* 1998; Yoshida *et al.,* 1998). That apparently equivalent cells (C2C12 is a clonally-derived cell line) can adopt different fates within the same culture suggests that extrinsic factors can cause cells to adopt a satellite cell phenotype within in an environment where most cells undergo terminal differentiation.

The model outlined above was used to investigate the expression of Notch receptors and ligands during myogenesis and reserve cell formation. Both Notch1 and Notch2 were found to be expressed by proliferating myoblasts and were maintained throughout differentiation from myoblasts to myotubes. This is consistent with a role for Notch in driving the proliferation of myogenic precursor cells, during both development (Vasyutina *et al*., 2007) and postnatal regeneration (Conboy & Rando, 2002). In both cases, DL1 appears to be the prominent DSL ligand responsible for Notch activation (Conboy *et al*., 2005, Schuster-Gossler *et al.*, 2007). Notch3 was also shown to be present in proliferating cultures and throughout differentiation although in contrast to both Notch1 and Notch2 where the active

form was present, the Notch3 present during proliferation was inactive and the active receptor was first detected at the onset of differentiation, the time when the cell fate decision (i.e. myotube or reserve cell) was being made. Significantly, analysis of cultures separated into differentiated myotubes and undifferentiated, quiescent reserve cell fractions, revealed that Notch1 was expressed in both fractions (although at much higher levels in myotubes), Notch2 was expressed in both, whereas Notch3 activity was entirely restricted to reserve cells. This further supports that validity of the reserve cell model as this expression profile matches that reported for quiescent satellite cells (Fukada *et al*., 2007; Kuang *et al.,* 2007; Mourikis *et al*., 2012).

The temporal expression and distribution of the receptor suggested that Notch3 may be involved in regulating satellite cell behaviour, possibly in the specification of the quiescent, undifferentiated phenotype investigate. In cultures where Notch3 activity was inhibited by shRNA-mediated knockdown, myoblast proliferation was unaffected consistent with the observation that Notch3 activity was undetectable prior to the initiation of differentiation. However, following differentiation, cultures in which the expression of Notch3 was reduced or absent contained a greater number of larger myotubes and a vastly reduced number of reserve cells compared with equivalent controls. These results suggest that Notch3 activity is required for cells to adopt the satellite cell-like, reserve cell phenotype. This hypothesis was further supported by the effects of overexpression of constitutively active Notch3 ICD which was found to inhibit myoblast proliferation, to increase the number of quiescent, undifferentiated Pax7+ve cells whilst inhibiting MyoD expression and differentiation. Again, these findings are consistent with Notch3 activity inhibiting the differentiation myogenic precursor cells by preventing MyoD expression, ensuring that they remain undifferentiated and quiescent and hence maintain of Pax7 expression.

It is interesting to note that in contrast to the above findings many studies of other systems have concluded that Notch3 activity is associated with increased proliferation and disregulated activity has been linked to tumorigenicty in several contexts. For example, Notch3 has been implicated in neoplasia, including T cell

leukemogenesis (Bellavia *et al*., 2002) and several epithelial malignancies (Dang *et al*., 2000; Park *et al*., 2006; Dang *et al*., 2006). Although dispensable for normal embryonic growth (Krebs *et al.,* 2003), Notch3 may have a role in regulating terminal differentiation (Lardelli *et al*., 1996; Apelqvist *et al*., 1999; Dang *et al.,* 2003) cell cycle progression (Campos *et al*., 2002) and apoptosis (Giovannini *et al*., 2009; Wang *et al*., 2002). In the vascular system, Notch3 is involved in maintaining the phenotypic stability of adult arterial smooth muscle cells (Wang *et al*., 2008) and several defined mutations in the human Notch3 receptor result in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) due to disruption of normal vascular smooth muscle identity and function (Chabriat *et al*., 2009).

However, the outcome of signaling through a specific Notch receptor is both context and ligand dependent and can have different results in different situations (Rutz *et al.,* 2005; de La Coste & Freitas *et al.,* 2006; Benedito *et al.,* 2009). The anti-proliferative effect of Notch3 activity in skeletal muscle precursors was also supported by the observed changes in Notch receptor activity during reserve cell reactivation. When transferred to growth medium, reserve cells were shown to upregulate MyoD and re-enter the cell cycle. Significantly, this was preceded by a switch in Notch receptor activity: within 15 minutes Notch3 transcription was down-regulated and by 90 minutes the remaining receptor was inactive, whilst Notch1 transcription increased and receptor activity was detectable within the same timeframe, all prior to the increase in *MyoD* transcription.

For Notch3 to have an effect on myoblast fate decisions, its activity must be triggered by interaction with an appropriate ligands. One possible explanation for the timing of Notch3 activation was that the relevant ligand was not present within the cultures prior to the initiation of differentiation. The temporal expression patterns of the DSL ligands during differentiation suggested DL4 as a potential candidate. In contrast to DL1which was expressed throughout differentiation, DL4 was only expressed on newly formed myotubes that could potentially interact with remaining undifferentiated cells, DL4 activating Notch3 and imposing a quiescent, reserve cell phenotype. Although DL4 expression has not been previously reported

in skeletal muscle cells, it is expressed by arterial endothelium and is thought to instruct cell fate during vasculogenesis by interaction with Notch3 (Shutter *et al*., 2000) . Indeed, when reserve cells were co-cultured with fibroblasts expressing a range of DSL ligands, only exposure to DL4 was able to maintain the reserve cell phenotype by preventing activation and re-entry into the cell cycle.

Overall, the results presented in this thesis suggest that Notch3 activity resulting from a interaction with a specific DSL ligand DL4 is sufficient to impose and maintain an undifferentiated, stem cell-like phenotype in skeletal muscle myoblasts. One possible mechanism for the recruitment of satellite cells during regeneration is therefore that as myotubes form, their expression of DL4 activates Notch3 on neighbouring, as yet undifferentiated precursor cells and imposes a quiescent, undifferentiated phenotype. Thus, it could be that it is the restablishment of the satellite cell niche during regeneration that recruits a new cohort of stem cells from a homogeneous population of proliferating myoblasts.

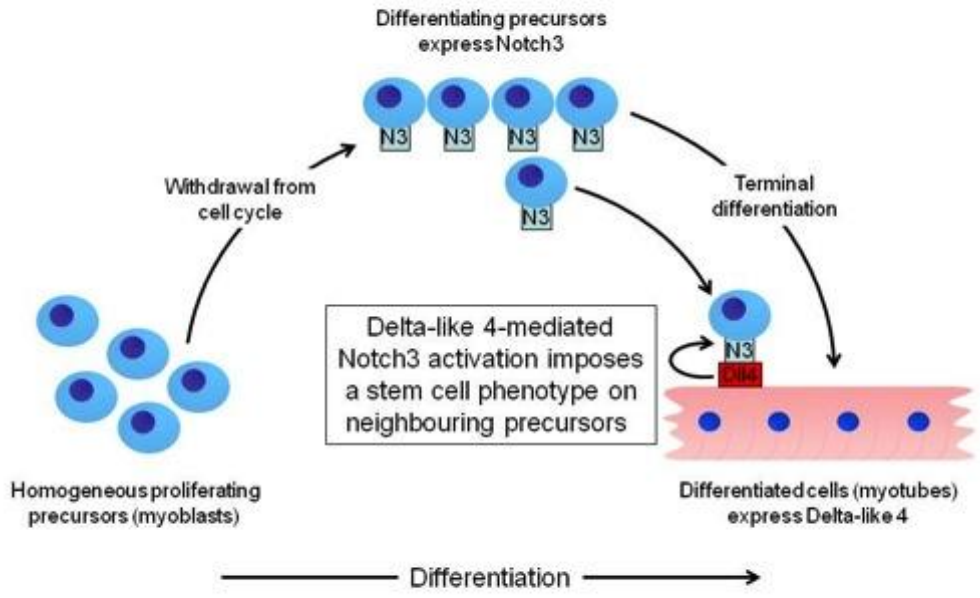


Figure 7.1 Model for a role of Notch3 in establishing a quiescent, undifferentiated phenotype in the context of myogenic differentiation

Notch3 becomes activated after differentiation is induced in proliferating myoblasts (Notch3–ve). Unfused single cells in differentiated cultures could either remain in the cell cycle or exit to become an undifferentiated reserve cell. Nascent myotubes expressing DL4 are sufficient to maintain active Notch3 in the quiescent cell population and inhibit re-entry into the cell cycle. Thereby recruiting them to replenish the stem cell compartment during the asynchronous process of muscle regeneration**.**

Recent studies of the mouse spermatogenetic system have suggested that different mechanisms may operate to maintain the stem cell compartment under different conditions: during normal turnover the niche retains a stem cell population (actual stem cells) that self-renew and give rise to transit amplifying progenitors, whereas if the niche is emptied, progenitors (potential stem cells) can be recruited back to the niche to assume the function of self-renewal (Nakagawa *et al*., 2007). There is evidence to suggest that there may be similar flexibility in the skeletal muscle stem cell lineage as studies of isolated muscle fibre preparations have shown that proliferating satellite cell progeny, defined as committed progenitors by the expression of *MyoD*, retain the ability to withdraw from the cell cycle, down-regulate MRF expression and reassume a Pax7+ve/MyoD-ve phenotype characteristic of a quiescent satellite cell (Zammit *et al*., 2004). In addition, transplanted myoblasts retain the ability to contribute to the satellite cell pool *in vivo* even after removal from the niche and extensive expansion *in vitro* (Cousins *et al*., 2004, Ehrhardt *et al*., 2007, Heslop *et al.,* 2001).

It has been proposed that the regenerative compartment of adult skeletal muscle is strictly hierarchical, based on a small population of true stem cells that express Pax7, but no MRFs (Kuang *et al.,* 2007). In this model, the true stem cells undergo niche-orientated asymmetric cell divisions giving rise to a new stem cell and a daughter fated to generate progeny for differentiation. However, although such a system could operate during skeletal muscle turnover and growth, it is difficult to apply to situations where the satellite cell niche is compromised such as following free muscle transplantation where complete regeneration is still accompanied by recruitment of an appropriate satellite cell cohort (Schultz *et al*., 1984). Notch-mediated stem cell recruitment during niche restoration provides an alternative mechanism that could operate following extensive damage and suggests that there

is more flexibility in the satellite cell lineage than implicit in a unidirectional stem cell to committed progenitor model. Indeed, this would be consistent with previous reports suggesting that differentiated muscle cells can establish and maintain quiescence in myoblasts that are otherwise capable of both proliferation and differentiation (Bischoff *et al*., 1969, Bischoff *et al*., 1990).

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