THE EFFECT OF WNT ISOFORMS ON MYOGENESIS

RHYS STEWART MCCOLL
BSc Honours Biochemistry

Submitted in fulfilment of the academic requirements for the degree of Master of Science in the

School of Life Sciences

University of KwaZulu-Natal

Pietermaritzburg

January 2014

As the candidate's supervisor I have approved this thesis/dissertation for submission.

Signed: _____ Name: Dr. C. U. Niesler Date: _____

ABSTRACT

Satellite cells are muscle stem cells that are responsible for the growth and repair of skeletal muscle tissue. Satellite cells typically exist in a quiescent state in their niche between the sarcolemma and basal lamina. In response to muscle tissue injury, activated satellite cells, otherwise known as myoblasts, migrate to the site of injury where they proliferate and subsequently differentiate and fuse to repair damaged myofibers. The success of muscle growth and repair is highly dependent on the speed and degree to which these myoblasts migrate, proliferate and differentiate. This overall process, referred to as myogenesis, is largely controlled by the myogenic regulatory factors, a group of basic helix-loop-helix transcription factors including MyoD, Myf5, myogenin and Mrf4. It has recently been found that the Wnt family of secreted signalling proteins are highly involved in the regulation of developmental processes such as myogenesis. Wnt proteins are a family of 21 highly-conserved, secreted, cysteine-rich signalling molecules which are found in all multi-cellular organisms. Wnt signalling is highly versatile and is initiated by the binding of extracellular Wnt to cell-surface Frizzled receptors (Fz). It is highly dependent on both the Wnt isoform and Fz type and may initiate one of three known signalling pathways. Wnt3A and Wnt7A are of particular interest as they have previously been linked with myogenesis. C2C12 myoblasts over-expressing Wnt3A have been seen to have reduced levels of motility and terminal differentiation. Wnt7A is suspected to maintain a healthy satellite cell pool by regulating self-renewal; injection of recombinant Wnt7A into mouse leg muscle resulted in increased satellite cell numbers. *In vitro* Wnt studies have typically involved the treatment of mouse cells with conditioned medium containing Wnt, often at unknown concentrations. In our study we wished to test the effects of known concentrations of recombinant Wnt3A and Wnt7A on mouse C2C12 and donor-derived human skeletal muscle myoblasts (HSkM) *in vitro*. Wnt3A and Wnt7A were seen to increase the rate of C2C12 migration in a dose dependent manner. HSkM cells treated with 10 ng/ml Wnt3A also displayed increased motility. Neither Wnt3A nor Wnt7A were seen to have any significant effects on the proliferation of C2C12 or HSkM cells. Wnt3A (10ng/ml and 100 ng/ml) but not Wnt7A was seen to decrease C2C12 terminal differentiation as measured by expression of myosin heavy chain (MyHC). Subsequent

confocal microscopy revealed that Wnt3A significantly reduced the percentage of MyoD^{+} C2C12 nuclei during differentiation. A reduction in nuclear MyoD would support the observed impaired commitment to differentiation. However, donor-derived human skeletal muscle myoblasts treated with 10 ng/ml Wnt3A were not seen to have significantly reduced nuclear MyoD levels or terminal differentiation; the reason for this is unclear but may relate to a number of factors including the concentration of Wnt, Fz and co-receptor profiles and the presence of specific extracellular matrix and serum factors. These studies provide new insight into the role of Wnts in myogenesis and lay the foundation for future work on Wnt3A and Wnt7A.

PREFACE

The experimental work described in this dissertation was carried out in the Discipline of Biochemistry, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, from January 2012 to December 2013, under the supervision of Dr. Carola. U. Niesler.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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DECLARATION 1 - PLAGIARISM

I, Rhys Stewart McColl, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

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ACKNOWLEDGEMENTS

Dr Carola Niesler, your great knowledge of cell biology has been indispensible in the completion of this thesis. You have always been patient with me and it is apparent to everyone that you put your all into producing the best post-grad students possible. It has been a great pleasure to work in Lab39.

Dr Kyle Goetsch, without you, Nick and I would have been lost in the lab without your instruction. You always had time to show us a new technique or trick to make life in the lab easier. I am grateful for your help that has seen me through these last two years.

Dr Celia Snyman, you have always been an example of persistence and determination. Thank you for all your invaluable advice and assistance

Thanks to all the lab mates. Nick Walker, thanks for all the help with confocal microscopy and for all the entertainment in and outside of the lab. Wanani Sibanda, you are the friendliest person I know and it has always been a great pleasure to chat. Trish Kahamba, your work ethic has been inspiring to all.

Thanks to Prof. Coetzer and Prof. Goldring for continuing to ensure the success and reputation of the biochemistry department.

Thanks to Robyn, Pat, Natalie, Tanya, Charmaine and all the admin staff for keeping the department running so smoothly. Without you guys, there would be chaos.

To mom and dad. Thanks for all the support during my varsity career, both emotional and financial. Evan though it may have seemed like I would never finish, you have always supported me in my choices and I am eternally grateful. Thanks also to Wendy and Evan for their continued support of the favourite child. Love you all.

To the NRF and UKZN, I greatly appreciate the financial support that I have received over my post-graduate degree. Finally, to all of those I have failed to mention by name, I thank you.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ABBREVIATIONS

βTrCP9	β-transducin repeat-containing protein 9
APC	adenomatous polyposis coli
ATP	adenosine triphosphate
BSA	bovine serum albumin
CD34	cluster of differentiation (34)
CD45	cluster of differentiation (45)
CK1	Casein Kinase-1
CRD	cysteine rich domain
DMEM	Dulbecco's Modified Eagle Serum
DM	differentiation media
Dsh	dishevelled protein
ECM	extracellular matrix
ECL	enhanced chemiluminescence
FCS	fetal calf serum
FGF	fibroblast growth factor
Fz	frizzled receptor
GAPDH	glyceraldehyde-3-Phosphate Dehydrogenase
GM	growth media
GSK-3β	glycogen synthase kinase-3β
HGF	hepatocyte growth factor
HRPO	horseradish peroxidase
HS	horse serum
IGF	insulin-like growth factor
IL-6	interleukin-6
JNK	jun N-terminal kinase
Kny	knypek
LIF	leukocyte inhibitory factor
LRP	low-density lipoprotein receptor related proteins
MT1-MMP	membrane type-1 matrix metalloproteinase
MMP-2	matrix metalloproteinase-2
MRF(s)	myogenic regulatory factor 5
Myf5	myogenic factor 5
MyoD	myoblast determination protein
MyHC	myosin heavy chain
NF-AT	nuclear factor of activated T-cells
Pax7	paired-box protein 7
PBS	phosphate buffered saline
PCP	planar cell polarity pathway
PDGF	platelet-derived growth factor
ROCK	rho-associated, coiled-coil containing protein kinase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sFRP	secreted Frizzled-Related Protein
Stbm	strabismus
TCF/LEF	T-cell factor/lymphoid enhancer factor
TGF-β	transforming growth factor-β
UV	ultra-violet

CHAPTER ONE

LITERATURE REVIEW

1.1 Adult Skeletal Muscle Tissue

Adult mammalian skeletal muscle tissue contains a large population of nuclei which are found within the myofiber structure (Cornelison and Wold, 1997; Charge and Rudnicki 2004). It has been shown that these myonuclei are terminally differentiated following myoblast fusion to myotubes (Hawke and Geary, 2001; Le Grand and Rudnicki, 2007). Therefore, these post-mitotic myonuclei are unable to proliferate and facilitate the growth and repair of existing muscle tissue. A population of precursor cells, known as satellite cells, exist within skeletal muscle tissue and are charged with the growth and repair of muscle fibers (Hawke and Geary, 2001; Le Grand and Rudnicki, 2007). The ability of these cells to differentiate and form new myofibers, suggests that they are part of the adult stem cell family.

1.1.1 Satellite cells – Identification

Satellite cells were first identified in frog leg muscle (Mauro, 1961). Initially these cells could only be identified by their morphological characteristics and location within the muscle tissue. They were seen to lie in a quiescent state in a niche between the sarcolemma and basal lamina along all muscle fibers (Mauro, 1961; Charge and Rudnicki 2004) (Figure 1.1). Skeletal muscle satellite cells display a relatively high nucleus-to-cytoplasm ratio and contain very few organelles (Hawke and Garry, 2001). The satellite cell nuclei are also of a smaller size than the myonuclei of the myofibers (Hawke and Garry, 2001). An increase in the amount of heterochromatin can be found within these cells when compared to terminally differentiated myonuclei (Charge and Rudnicki, 2004, Schultz and McCormick, 1994). The morphological-based identification process is however slow and tedious; a number of satellite cell markers have subsequently been identified. Satellite cells are *C*D45^{-} (a signalling molecule involved with cell growth and differentiation) and are positive for M-cadherin (cell adhesion molecule), Myf5 (myogenic regulatory factor), saliomucin CD34 (cell-adhesion molecule) as well as Pax3 and Pax7 (myogenic transcription factors) (Irintchev *et al.,* 1994; Beauchamp *et al.,* 2000; Sherwood *et al.,*2004; Kassar-Duchossoy *et al.,* 2005; Relaix *et al.,* 2005). The Pax7 transcription factor is

the most commonly used marker of satellite cells in skeletal muscle tissue (Tajbakhsh *et al.* 1997; Beauchamp *et al.* 2000; Shefer *et al.* 2006) (Figure 1.1).

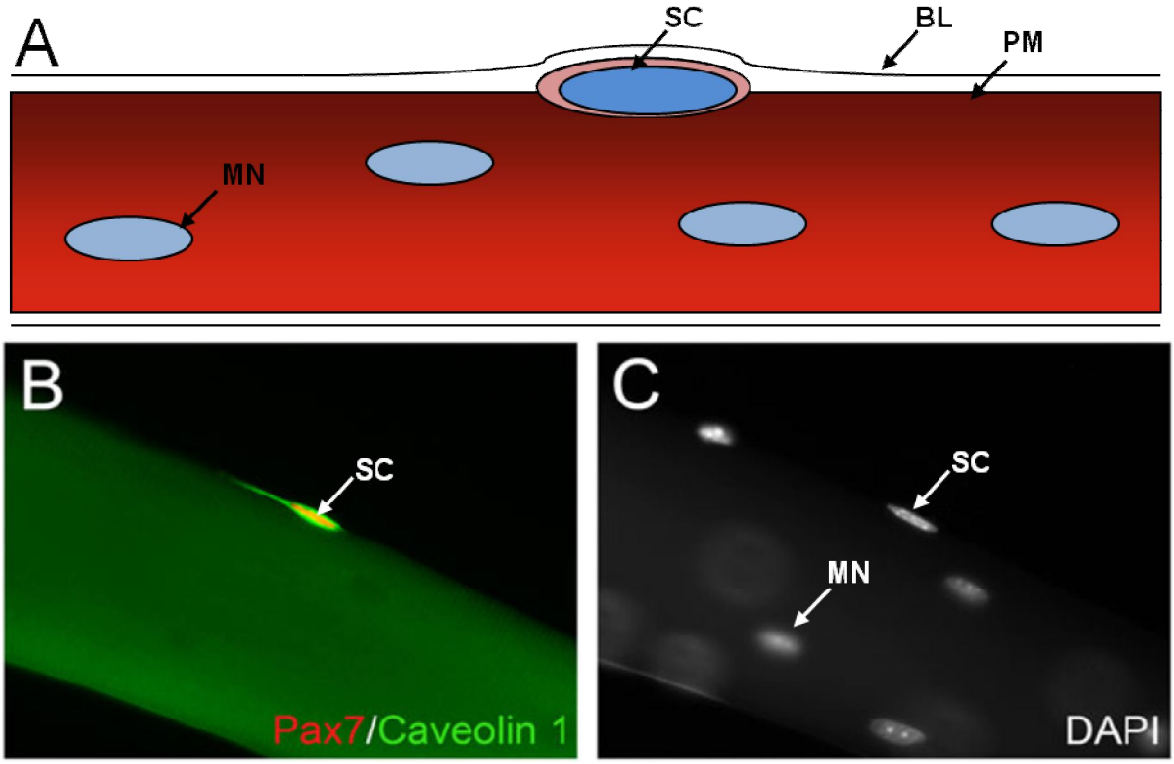

Figure 1.1 Localization of satellite cells within adult skeletal muscle. Skeletal muscle satellite cells occupy a unique niche within the muscle tissue. A) A longitudinal view of muscle tissue showing a satellite cell (SC) between the basal lamina (BL) and the plasma membrane (PM) of the myofiber structure. Post-mitotic myonuclei (MN) lie within the muscle fiber structure. B) A murine satellite cell labelled with anti-Pax7 antibodies (red-nuclei) can be seen along the muscle fiber which also stains positive for the structural protein caveolin-1 (green).C) DAPI nuclear staining of the muscle tissue reveals both the satellite cells and post-mitotic myonuclei within the muscle fiber. Constructed using references: Mauro, 1961; Charge and Rudnicki, 2004; Relaix and Zammit, 2012.

1.1.2 Satellite cells – The Myogenic Process

Satellite cells typically reside in a dormant/quiescent state and are activated by stress as a result of strain or trauma to the muscle tissue (Charge and Rudnicki, 2004). Activated cells derived from satellite cells, known as myogenic precursor cells or skeletal myoblasts, then migrate to the site of the muscle trauma where they undergo numerous rounds of replication before differentiation and fusion to produce multinucleated myofibers (Le Grand and Rudnicki, 2007; Burdzinska *et al.* 2008). During this process of myogenesis there is an up-regulation of myogenic regulatory factors (MRFs) such as MyoD, Myf5, myogenin and Mrf4 (Fuchtbauer and Westphal 1992; Burattini *et al.*,2004; Holterman and Rudnicki 2005) (Figure 1.2). These transcription factors facilitate the expression of structural proteins such a myosin heavy chain

(MyHC) and consequently terminal differentiation (Zammit *et al.*,2004; Kuang *et al.*,2006; Le Grand and Rudnicki, 2007). MyoD and Myf5 are vital for myogenic commitment of satellite cells while myogenin and Mrf4 are required for terminal differentiation and fusion (Seale and Rudnicki, 2000). Skeletal myoblasts that do not undergo fusion return to quiescence to replenish the satellite cell population (Kuang *et al.*, 2007) (Figure 1.2). This process is vital to maintain the population of satellite cells (Cornelison and Wold, 1997; Tajbaksh, 2005). Both Pax3 and Pax7 have been shown to be major regulators of satellite cell self-renewal and differentiation (Kuang *et al.*,2006). The up-regulated expression of Pax7 acts to maintain the quiescent state of satellite cells, whereas down-regulation is accompanied by cellular differentiation (Cornelison and Wold, 1997; Tajbaksh, 2005) (Figure 1.2). It has recently been documented that the expression of the various MRFs and ultimately the processes of satellite cell migration, proliferation and differentiation during myogenesis is largely controlled by Wnt signalling proteins (Brack *et al.*, 2008; Bentzinger *et al.*, 2012).

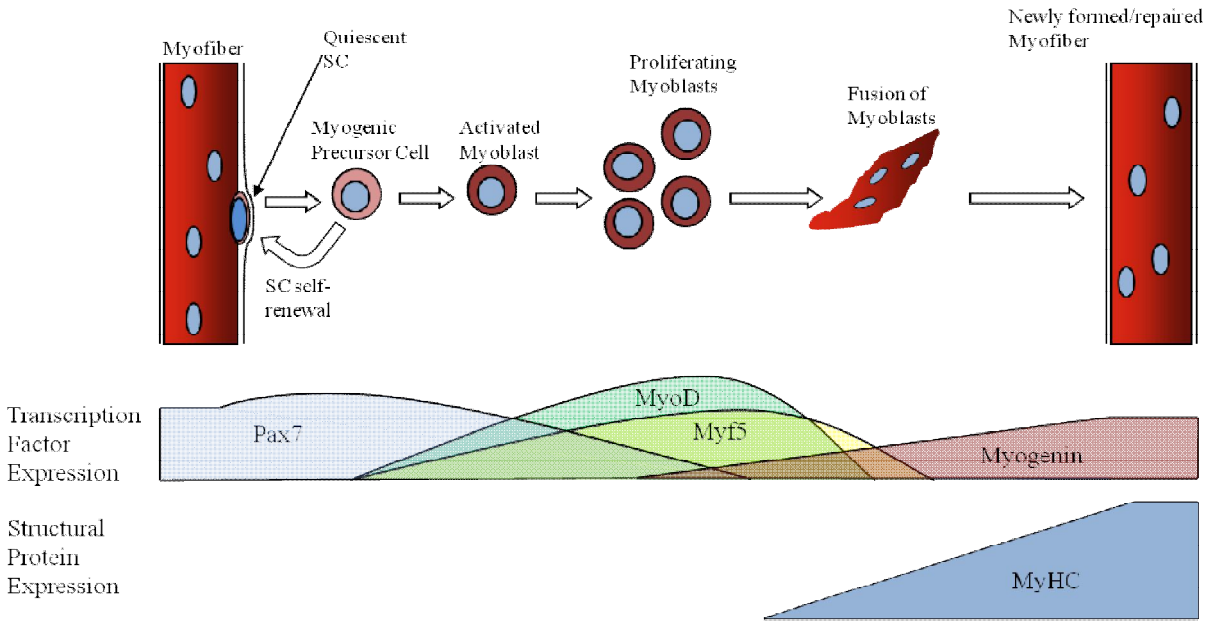

Figure 1.2 Protein expression during satellite cell activation, proliferation and differentiation. Pax7 is expressed by quiescent satellite cells and at reduced levels in activated satellite cells. An increase in Pax7 expression causes activated satellite cells to return to a quiescent state. MyoD and Myf5 are expressed as satellite cells become activated, start to proliferate and differentiate. Myogenin is up-regulated during differentiation and fusion of myoblasts. The structural protein, MyHC is produced during the formation of new myofibers. Constructed using references: Cornelison and Wold, 1997; Zammit *et al.*, 2004; Holterman and Rudnicki 2005.

1.2 Stages of Myogenesis

1.2.1 Activation

The initial activation of satellite cells leads to the reduced expression of Pax7 and the increased expression of Myf5 and MyoD (Cornelison and Wold, 1997, Tajbakhsh, 2005). This activation is accompanied by the ubiquitylation and breakdown of Pax3, which has been linked with cell commitment and the progression of myogenesis (Boutet *et al.*, 2007). Activated satellite cells, termed myoblasts, then move to the site of injury.

1.2.2 Migration

Cellular migration is essential for the development, growth and repair of tissues. In muscle tissue, myoblasts are able to move along the myofiber to the site of an injury, replicate, align and then fuse and repair the damaged tissue. Chemokines released by injured and surrounding myoblasts play an important role in controlling migration (Griffin *et al.*,2010). These chemokines include fibroblast growth factor (FGF), hepatocyte growth factor (HGF), platelet-derived growth (PDGF) and interleukin-4 (Robertson *et al.*,1993; Bischoff, 1997; Lafreniere *et al.*,2006). In addition to the various chemokines, myoblast migration is largely dependent on a group of cell surface proteins known as integrins (Friedl and Brocker, 2000). Integrins are the most prominent and well characterized trans-membrane receptors involved with the linkage of contractile actin and extracellular matrix (ECM) ligands during cell migration (Hynes, 2002). Integrins are αβ heterodimers consisting of a large, cell surface ECM binding domain and relatively short cytoplasmic tail which is linked to the actin cytoskeleton (Hynes, 2002). The areas at which the cell is anchored to the ECM via integrin binding are known as focal adhesions. Activated Rho-associated, coiled-coil containing protein kinase 2 (ROCK2) helps to modulate the formation of these focal adhesion points while the membrane associated cytoskeletal protein vinculin physically links integrins to the actin cytoskeleton of the cell (Bakolitsa *et al.*,2004; Gallant *et al.*,2005; Burridge *et al.*,1990) (Figure1.3). The initial stage of migration involves the formation of hand-like structures called lamellipodia which are formed by the distortion of the leading edge of the cell (Figure 1.3). The formation and extension of the lamellipodia involves actin polymerization which is regulated by members of the integrin family as well as cell surface proteoglycans (Humphries *et al.*,2006; Huttenlocher and Horwitz, 2011). The lamellipodia then

reach forward in the direction of movement and adhere to the ECM via integrin-based adhesion points (Friedl and Wolf, 2010; Huttenlocher and Horwitz, 2011). Actin myofibers within the main body of the cell then contract as integrins at the trailing edge of the cell detach from the ECM, and the cell pulls itself forward. Cellular actin fibers contain contractile myosin-motors which are regulated by the GTPase family. Rho/ROCK signalling is thought to mediate the retraction of the trailing edge and possibly cell detachment (Webb *et al.*, 2004). Integrins from the detached trailing end undergo endocytosis and are recycled to the leading edge of the cell (Huttenlocher and Horwitz, 2011). During forward movement, the cell will adapt morphologically to the ECM in an effort to take the path of least resistance. In addition, myoblasts are able to degrade the ECM, allowing for easier movement towards the site of injury. This degradation involves the proteolysis of ECM factors by cell-surface serine and metallo-proteinases. These include membrane type-1 matrix metalloproteinase (MT1-MMP) and matrix metalloproteinase-2 (MMP-2)which break down any ECM structures blocking forward migration (Ohuchi *et al.,* 1997).The specificity of integrin binding is determined by the extracellular domain which allows for the binding to ECM factors such as fibronectin, collagen and laminin (Friedl and Brocker, 2000; Huttenlocher and Horwitz, 2011). Myoblast cells tend to utilize integrin-dependent migration. The variability in the extracellular domain of integrins means that motility dynamics are determined both by a cell's integrin profile as well as ECM components (Huttenlocher and Horwitz, 2011). Alternatively, during integrin-independent migration, the extracellular matrix is left relatively unchanged as the cell moves quickly over the ECM using only minor adhesion contacts (Friedl and Brocker, 2000).

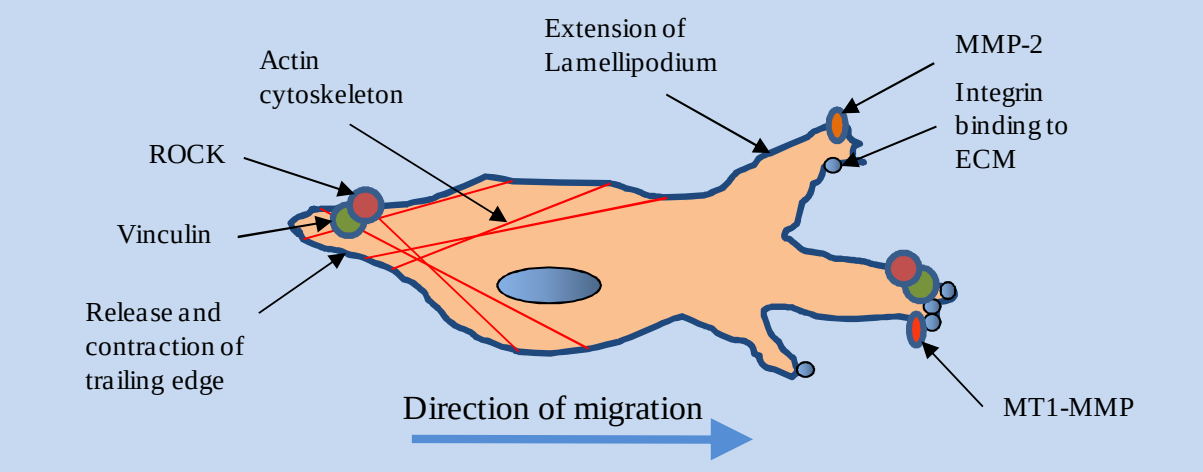

Figure 1.3 Integrin-dependent migration of skeletal muscle myoblasts. Integrins accumulate at the leading edge of the cell, followed by the extension of lamellipodia and attachment to the ECM. Actin fibers within the main cell-body contract as the trailing edge of the cell detaches from the ECM, pulling the cell forward. ROCK2 mediates the detachment of the trailing edge of the cell as well as formation of new focal adhesion sites. The cytoskeletal protein Vinculin links integrins at the focal adhesion points to cytoskeletal actin. Breakdown of ECM components by the proteinases MT1-MMP and MMP-2 allows for unimpeded movement through the matrix. Constructed using references: Ohuchi *et al.*, 1997; Humphries *et al.*, 2006; Huttenlocher and Horwitz, 2011.

1.2.3 Proliferation

The proliferation of activated myoblasts at the site of an injury is largely regulated by cytokines/growth factors that are secreted by macrophages and the myoblasts themselves (Hawke and Geary, 2001; Vitello *et al.*, 2004). Macrophages are drawn to the site of injury where they envelope tissue debris and secrete growth factors which encourage proliferation of myoblasts (Hawke and Geary, 2001; Vitello *et al.*,2004). These growth factors include insulin-like growth factor-1 (IGF-I), IGF-II, fibroblast growth factor (FGF), hepatoctye growth factor (HGF), leucocyte inhibitory factor (LIF), interleukin-6 (IL-6) and the transforming growth factor-β (TGF-β) (Table 1.1). These chemokines in turn activate the expression of the myogenic regulatory factors (MRFs). An increase in the expression of the MRF, Myf5 has been shown increase the rate of myoblast proliferation (Cornelison and Wold, 1997; Tajbakhsh, 2005). The return of activated satellite cells to a quiescent state has been associated with a reduction inMyf5 and MyoD expression and an increase in Pax7 expression (Cornelison and Wold, 1997; Tajbakhsh, 2005). This process of self-renewal is vital for the upkeep of the satellite cell population. Conditions such as muscular dystrophy are characterized by a dwindling of the satellite cell pool.

Table 1.1 The effect of secreted growth factors on myoblast proliferation and differentiation

Factor/s	Secreted by	Effect	Reference
IGF-I, IGF-II	macrophages, myoblasts	proliferation ↑ differentiation ↑	Hawke and Geary, 2001; Vitello *et al.*,2004
FGF	macrophages, myoblasts	proliferation ↑ differentiation ↓	Hawke and Geary, 2001; Vitello *et al.*,2004
HGF	macrophages, myoblasts	proliferation ↑ differentiation ↓	Hawke and Geary, 2001; Vitello *et al.*,2004
TGF-β	macrophages, myoblasts	proliferation ↑ differentiation ↓	Hawke and Geary, 2001; Vitello *et al.*,2004
IL-6	macrophages	proliferation ↑	Hawke and Geary, 2001
LIF	macrophages	proliferation ↑	Hawke and Geary, 2001

1.2.4 Differentiation and fusion

Differentiation of skeletal muscle myoblasts is a highly controlled process which involves; 1) the expression of muscle-specific regulatory factors, 2) exit from the cell cycle and 3) expression of contractile muscle proteins (Moran *et al.,* 2002). During the first step of differentiation, there is an up-regulation of the expression of the four MRFs; MyoD, myogenin, Mrf4 and Myf5 (Fuchtbauer and Westphal 1992; Moran *et al.,* 2002; Holterman and Rudnicki 2005). These muscle-specific MRFs interact with themyocyte enhancer factor 2 (MEF2) family of MADS box transcription factors and initiate production of muscle structural proteins via the E-box and MEF2 promoter regions (Moran *et al.,* 2002). The second step of differentiation involves the irreversible withdrawal of myoblasts from the cell cycle (Moran *et al.,* 2002; Joulia *et al,* 2003). This progression through the cell-cycle is regulated by the activation and inhibition of phosphoproteins by cyclin-complexed kinases (Moran *et al.,* 2002). These cyclin-kinase complexes are in turn controlled by cyclin-dependent kinase inhibitors (CK1) (Moran *et al.,* 2002). Contractile motor proteins such as myosin are produced during the third step of differentiation. Myosins consist of smaller subunits, including myosin heavy chain (MyHC) and give skeletal muscle its contractile ability (Weiss and Leinwand, 1996; Maccatrozzo *et al.,* 2007). Successful progression through differentiation leads to the fusion and formation of multi-nucleated myotubes and thus the growth and repair of the muscle tissue.

1.2.4.1 Myf5 and MyoD

Myf5 and MyoD are both members of the basic helix-loop-helix (bHLH) family of myogenic transcription factors (Joulia *et al,* 2003; Holterman and Rudnicki 2005). As with all members of the MRF family, Myf5 and MyoD form heterodimers with ubiquitous bHLH E-proteins (Sartorelli and Caretti, 2005). Formation of these heterodimers is required for DNA-binding and activation of target genes(Sartorelli and Caretti, 2005). Gene knockout experiments performed in mice suggest that MyoD initiates myoblast lineage commitment by up-regulating myogenin and Mrf4 expression, whereas Myf5 up-regulates myoblast proliferation (Le Grand and Rudnicki, 2007, Ustanina *et al.,* 2007). Further gene knockout studies have also revealed the functional redundancy that exists within the MRF signalling pathways (Rudnicki and Jaenisch, 1995; Kablar *et al.,* 1997; Chanoine *et al.,* 2004). Mice lacking a functional MyoD gene have been observed to have no physical abnormalities, but express four-fold higher levels of Myf5 (Megeney *et al.,*1996). It is suspected that Myf5 is able to compensate in part for the absence of MyoD. Despite this compensation, the rate of differentiation in MyoD-knockout myoblasts is decreased. Mice lacking a functional Myf5 gene show a four-fold increase in MyoD expression and no physical abnormalities (Megeney *et al.,*1996). However, mice lacking both the MyoD and Myf5 genes have severe abnormalities and lack myoblasts (Ustanina *et al.,* 2007).Since MyoD and Myf5 are expressed relatively early in the differentiation process, these two MRFs are commonly used to investigate the onset/activation of differentiation. Following MyoD andMyf5 expression, Myogenin and Mrf4 are up-regulated, leading to terminal myoblast differentiation and fusion into myofibers (Charge and Rudnicki, 2004). It is apparent that the expression of MyoD and Myf5 is essential for rapid myogenesis.

1.2.4.2 MyHC

Myosin is a eukaryotic, adenosine triphosphate (ATP) driven motor protein which interacts with actin to produce cellular movement such as muscle contraction (Weiss and Leinwand, 1996). Myosin is typically found as a hexameric protein and is made up of two 200kDa myosin heavy chain (MyHC) subunits and two pairs of 17-23 kDa, non-identical light chain subunits (MyLC) (Rayment *et al.,* 1993; Weiss and Leinwand, 1996; Maccatrozzo *et al.,* 2007) (Figure 1.4). It is the myosin heavy chain (MyHC) subunits that give myosin its contractile and filament-forming abilities, making MyHC the quintessential skeletal muscle marker (Weiss and Leinwand, 1996;

Maccatrozzo *et al.*,2007; Quintin *et al.*, 2008). The MyHC subunit can further be divided into two domains; a globular, N-terminal domain/head, responsible for contraction, and an alpha-helical coiled-coil carboxyl domain which allows for filament formation (Weiss and Leinwand, 1996; Maccatrozzo *et al.*,2007) (Figure 1.4).The two non-identical light chain subunits are referred to as the 'essential' and 'regulatory' light chains (MyLC-1 and MyLC-2 respectively) and bind to the neck region of the MyHC subunit (Rayment *et al.*,1993). The regulatory light chain (MyLC-2) is actively involved during muscle contraction where it is phosphorylated by myosin light chain kinase (MyLCK), resulting in a conformational change of the globular head and contraction (Morano *et al.*,1997). The exact role of the non-phosphorylatable essential light chain (MyLC-1) is not known, but it is suspected that the subunit contributes to the stability of the myosin structure (Morano *et al.*,1997). Nine mammalian isoforms of MyHC have been identified in striated cardiac and skeletal muscle fibers and show a high level of interspecies identity (Berg *et al.*,2001; Maccatrozzo *et al.*,2007).

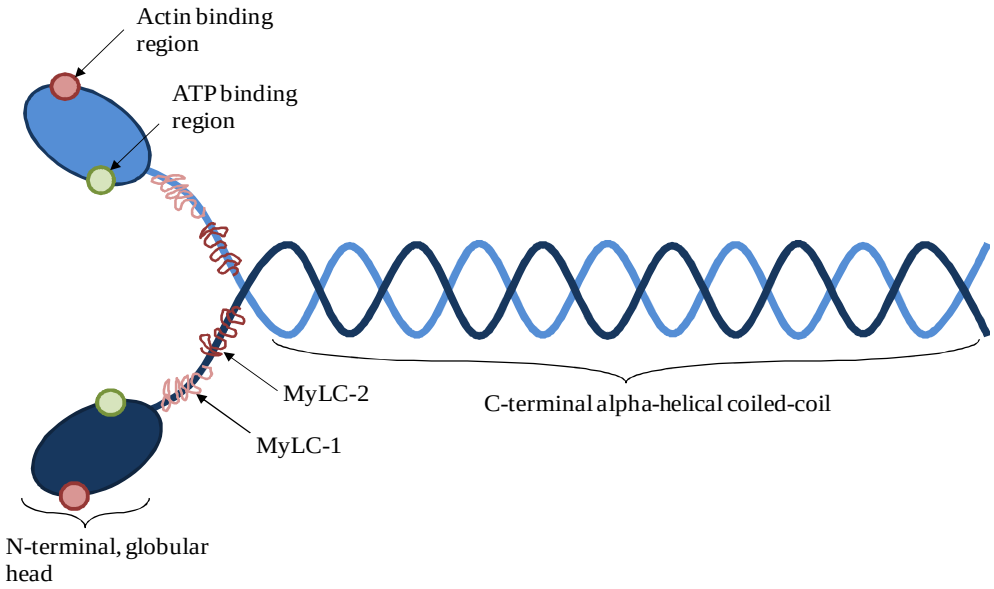

Figure 1.4 Myosin structural motifs. The hexameric myosin motor protein consists of two heavy (MyHC) and four light (MyLC) myosin chain subunits. The two heavy chain subunits contain an N-terminal, globular head, involved with contraction, and a filament-forming C-terminal, alpha-helical coiled-coil domain. Two non-identical MyLC subunits referred to as the 'essential' and 'regulatory' myosin light chains (MyLC-1 and MyLC-2 respectively), are bound to the neck of each MyHC subunit. The MyLC subunits are involved with contraction and maintaining the structural stability of the myosin protein. Constructed using references: Weiss and Leinwand, 1996; Maccatrozzo *et al.*,2007.

Being a motor protein, MyHC is expressed only in differentiated myoblasts. For this reason, MyHC can be used to determine the degree to which a population of myoblast cells has been terminally differentiated.

1.3 Wnt Signalling proteins

Wnt proteins are a family of at least 21 highly-conserved, secreted, cysteine-rich, lipid-modified, glycoproteins that are involved in a multitude of developmental and physiological processes (George-Weinstein *et al.*,1998; Cadigan and Liu, 2006; Maiese *et al.*, 2008). Wnt proteins are found in all multi-cellular organisms and act to regulate numerous stages of development, from patterning of the embryo and production of tissues and organs, to the regulation of cell migration, axon guidance and polarity (He *et al.*,2004;Cadigan and Liu, 2006; Endo *et al.*,2005; Nusse, 2008). In recent years it has been suggested that the Wnt proteins are important regulatory factors in migration, proliferation and lineage specification in a number of cell types (Tanaka *et al.*,2011, Bentzinger *et al.*, 2012).Wnt proteins interact with the seven-pass transmembrane receptor Frizzled (Fz) on the surface of cells and activate the expression of various transcription factors which in turn regulate processes including myogenesis (Peifer and Polakis, 2000; Huelsken and Behrens, 2002; Cadigan *et al.*, 2005; Janda *et al.*, 2012).

1.3.1 Wnt structure

Members of the Wnt gene family are characterized by their similarity to the *Drosophila Wingless* (Wg) and mouse Int-1 genes (Wodarz and Nusse, 1998). The genes coding for Wnt proteins are highly conserved and code for proteins of between 350 and 400 residues (Wodarz and Nusse, 1998; Janda *et al.*, 2012). Little is known about the structure of the Wnt proteins due to their highly insoluble nature and domains in their primary sequences not being clearly related to any known protein folds (Wodarz and Nusse, 1998; Janda *et al.*,2012). Their hydrophobic nature is due to the addition of palmitate and/or palmitoleic acid to a region of conserved cysteine residues found in all Wnt proteins (Willert *et al.*,2003; Takada *et al.*,2006). Despite difficulties in obtaining crystallized Wnt protein for crystallography, it is known that all Wnts share the features of a secreted protein i.e. a signal peptide, numerous potential N-glycosylation sites and a region of conserved cysteine residues (±22 residues) most likely involved in disulphide bond formation (Wodarz and Nusse, 1998). Glycosylation of secreted proteins such as Wnts ensures

their stability in the extracellular environment (Sola and Griebenow, 2010). It is thought that acylation of Wnts allows for intracellular trafficking as well as full activity of the secreted proteins (Janda *et al.*, 2012). A total of 19 human Wnt proteins have been characterized; they share 27-83% amino-acid sequence identity (Miller, 2002). Sequencing of Wnt genes has shown a high level of gene conservation between species (Table 1.2). This high level of conservation suggests that Wnts are vital signalling molecules and have similar functions and mechanisms of action between different species.

Table 1.2 Interspecies similarity of Wnt3A and Wnt7A protein sequences

Organisms Compared	Protein	Percentage identity (%)
Human-Mouse	Wnt3A	97
Human-Rhesus monkey	Wnt3A	99
Human-Chicken	Wnt3A	87
Human-Zebrafish	Wnt3A	83
Human-Mouse	Wnt7A	99
Human-Rhesus monkey	Wnt7A	98
Human-Chicken	Wnt7A	93
Human-Zebrafish	Wnt7A	85

Protein sequence alignment was done using the ClustalW alignment software with sequences obtained from the NCBI protein databank (see Appendix II).

1.4 Wnt signalling

There are three different Wnt signalling pathways; 1) the well studied and understood canonical/β-catenin dependent pathway, and the poorly understood non-canonical/β-catenin independent pathways which include the 2) Ca2+ and 3) planar cell polarity (PCP) pathways (Huelsken and Behrens, 2002; Nusse, 2003; Packard *et al.*, 2003; Laeremans *et al.*, 2010; Tanaka *et al.*,2011). Each of these pathways involves the binding of Wnt to Frizzled receptors (Fz) on the cell-surface leading to the activation and expression of target genes (Huelsken and Behrens, 2002). It has recently been established that certain Wnt proteins, including Wnt3A and Wnt7A, play an important role in myogenic differentiation and specification by regulation of MRFs such as MyoD and Myf5 (Maiese *et al.*, 2008).

1.4.1 Frizzled receptor

The seven-pass transmembrane receptor Frizzled (Fz) is believed to be involved in almost all Wnt signalling (He *et al.*,2004; Huang and Klein, 2004). Wnt lipid groups bind directly to the N-

terminal linked, cysteine rich domain (CRD) on the cell-membrane-associated Frizzled (Fz) receptor (Huelsken and Behrens, 2002; Janda *et al.*,2012). Fz receptors are exclusive to the cell-membrane and are often found coupled to heterotrimeric G proteins (Huang and Klein, 2004; Malbon, 2004; Janda *et al.*, 2012). A wide range of Fz isoforms and the numerous Wnt isoforms makes this type of signalling extremely versatile. The frequency of these receptors on the cell surface has been shown to be a major rate-limiting factor in response to extracellular ligand (Mukai *et al.*, 2010). Hela cells have been seen to control the frequency of these receptors via a cycle of ubiquitylation and deubiquitylation, which is controlled in part by the deubiquitylating enzyme UBPY/USP8 (Mukai *et al.*, 2010). Ubiquitylation of Fz results in endocytosis which is then followed by recycling of the receptors to the cell surface or transportation to the lysosome for destruction (Mukai *et al.*, 2010). Deubiquitylation triggers the recycling of Fz to the cell membrane.

1.4.1.1 Frizzled structure

Frizzled proteins range in length from around 500 to 700 amino acids and contain an extracellular, N-terminal-linked cysteine rich domain (CRD), a membrane anchored region consisting of seven hydrophobic domains and an intracellular signalling region (Huang and Klein, 2004) (Figure1.5). Knowledge of the structural conformation of Fz is limited and is based on the unbound cysteine rich domain (CRD) of Fz8 as well as the secreted CRD antagonist, secreted Frizzled-Related Protein (sFRP) (Janda *et al.*,2012). Fz8-CRD and sFRP are ~160 residues in length and consist largely of β-helical structures (Janda *et al.*,2012). The CRD contains 10 conserved cysteine residues, most likely forming structurally stabilizing disulphide bonds (Huang and Klein, 2004). Seven hydrophobic domains have been identified and lie between the CRD and intracellular C-terminus and are thought to form transmembrane α-helices (Huang and Klein, 2004) (Figure 1.5). Sequence analysis of the ten identified human Fz has shown that the receptors fall into four main clusters; Fz1, Fz2 and Fz7 which share 75% amino acid identity, Fz5 and Fz8 with 70% identity, Fz4, Fz9 and Fz10 with 65% identity, and Fz3 and Fz6 which share 50% identity (Fredriksson *et al.*,2003). A single cell may contain one or more types of Fz. This Fz profile determines how specific Wnts affect the cell.

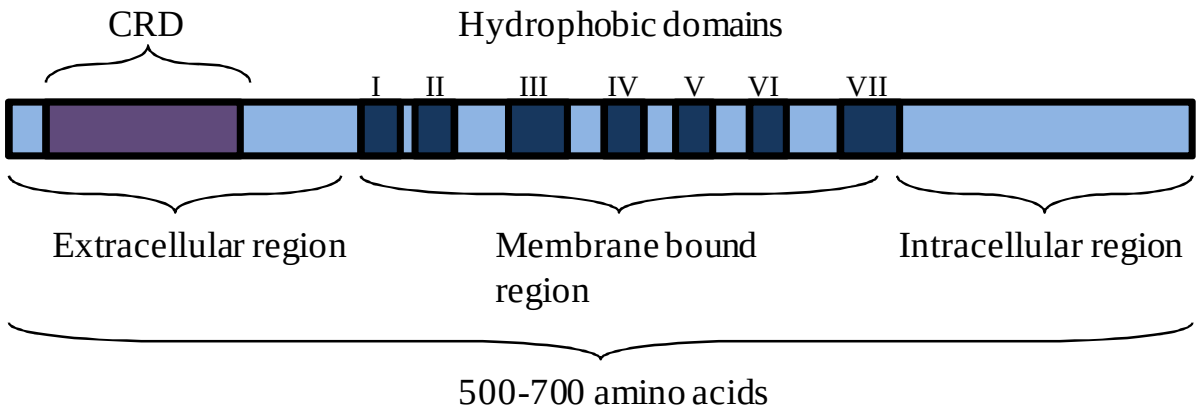

Figure 1.5 Proposed Frizzled protein motifs. Extracellular Wnt binds to the cysteine rich domain (CRD) of the frizzled receptor (Fz). The seven hydrophobic regions within the receptor structure form transmembrane α-helices (I-VII). Wnt binding causes structural change to the intracellular signalling region which in turn leads to activation of relevant transcription factors. Constructed using reference: Huang and Klein, 2004

1.4.2 Canonical signalling – β-Catenin dependent pathway

The Wnt-mediated canonical/β-catenin dependent pathway requires the interaction of Wnt proteins with Fz coupled to the Low-density Lipoprotein receptor related proteins 5 and 6 (Lrp5/6) (Peifer and Polakis, 2000; He *et al.*,2004; Cadigan *et al.,* 2005) (Figure 1.6).In the absence of Wnt, the signalling protein Dishevelled (Dsh) is inactive, resulting in the formation of the β-catenin destruction complex (Jin *et al.,* 2008). This complex is made up of axin, glycogen synthase kinase-3β (GSK-3β), Casein Kinase-1 (CK1) and adenomatous polyposis coli (APC) (Huelsken and Behrens, 2002; Jin *et al.,* 2008).β-catenin is phosphorylated by CK1 at serine 45 which then allows for the phosphorylation of serine/threonine residues 41, 37 and 33 by GSK-3β (Huelsken and Behrens, 2002). Phosphorylation of β-catenin targets it for ubiquitylation by β-transducin repeat-containing protein 9 (βTrCP9) (Huelsken and Behrens, 2002; Huang and Klein, 2004). Ubiquitylated β-catenin is broken down by the large protease/proteosome, which is modulated by the multi-subunit serine/threonine phosphatase, PP2A (Huelsken and Behrens, 2002; Huang and Klein, 2004). As a result, Wnt target genes are not transcribed.

When Wnt is present, it binds to the Frizzled receptor which then activates cellular Dishevelled protein (Dsh) and prevents the formation of a β-catenin destruction complex and subsequent phosphorylation of β-catenin (Hinoi *et al.,* 2000; Peifer and Polakis, 2000; Salic *et al.,* 2000) (Figure 1.6). When Dishevelled protein (Dsh) is active, it is thought to inhibit the formation of the destruction complex by displacing glycogen synthase kinase-3β (GSK-3β) from axin,

replacing it with GBP/Frat-1 (Huelsken and Behrens, 2002).If β-catenin breakdown is inhibited, the protein accumulates in the cytosol and ultimately moves into the nucleus (Peifer and Polakis, 2000). β-catenin then complexes with T-cell factor/lymphoid enhancer factor (TCF/LEF) within the nucleus resulting in the expression of Wnt target genes (Huelsken and Behrens, 2002; Tanaka *et al.*,2011).

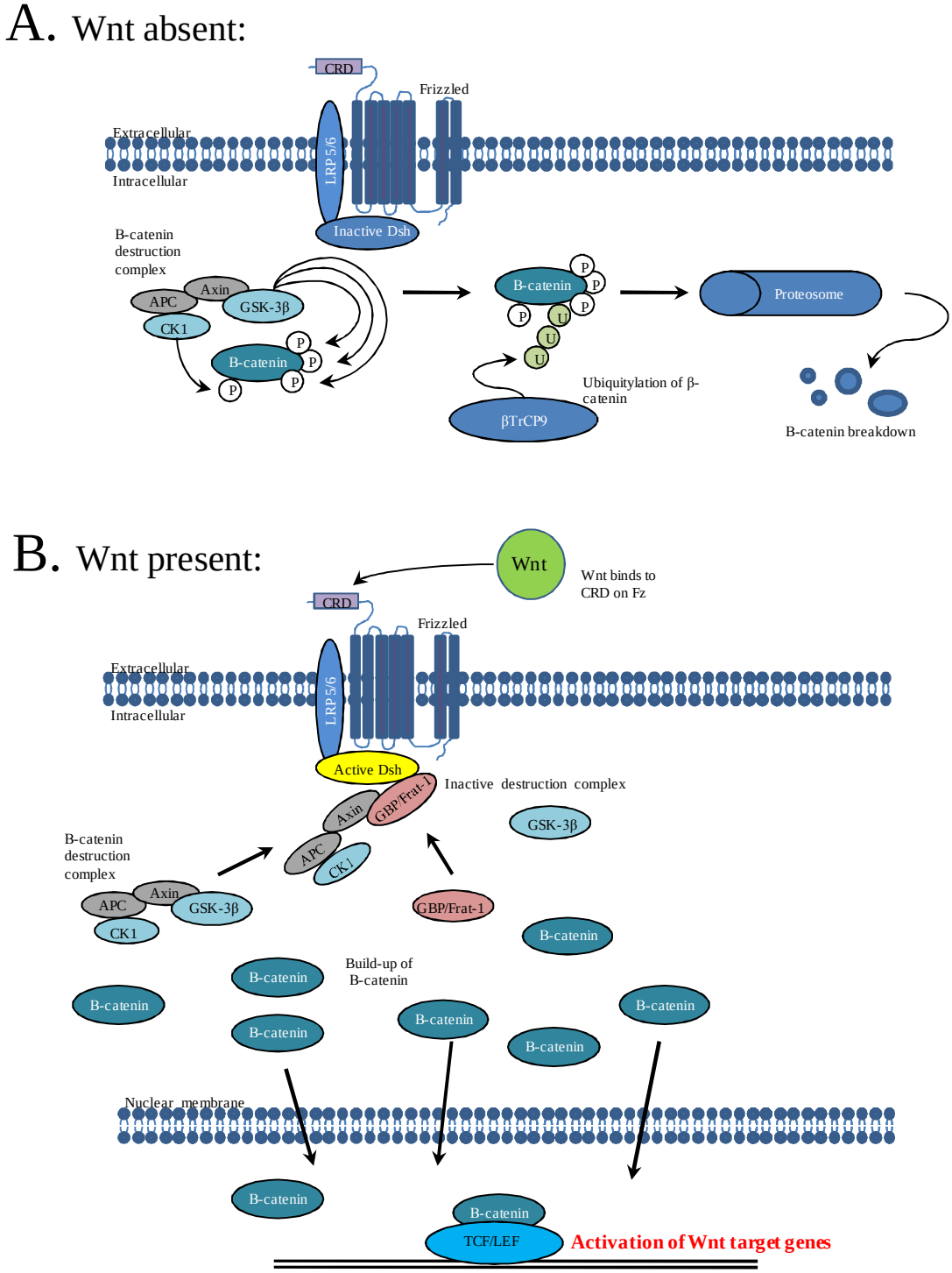

Figure 1.6 Canonical/β-catenin dependent Wnt signalling pathway. A) In the absence of Wnt proteins, Dsh remains inactive, maintaining cytosolic levels of the β-catenin destruction complex. Cytosolic β-catenin is phosphorylated by CK1 and GSK-3β on the destruction complex resulting in the ubiquitylation of β-catenin by β-transducin repeat-containing protein and its subsequent breakdown by cytosolic proteosomes. With the breakdown of β-catenin, no Wnt target genes are expressed. B) Extracellular Wnts bind to the Frizzled receptor on the cell surface, initiating the canonical/β-catenin dependent signalling pathway. Dsh is activated and prevents the formation of the β-catenin destruction complex which in turn leads to a build-up of β-catenin in the cytoplasm. β-catenin then translocates into the nucleus, complexing with TCF/LEF, activating expression of target genes. Constructed using references: Huelsken and Behrens, 2002; Huang and Klein, 2004; Tanaka *et al.*,2011

1.4.3 Non-canonical signalling

1.4.3.1 Planar Cell Polarity pathway

The poorly understood Planar Cell Polarity (PCP) pathway is thought to play a pivotal role in cell migration, acting primarily through the activity of Dsh, the small GTPase Rho and Rho-associated kinase (ROCK) (Huelsken and Behrens, 2002; Cadigan *et al.*,2005;Endo *et al.*,2005; Tanaka *et al,* 2011) (Figure 1.7). The PCP pathway branches from the canonical pathway in terms of the function of the Dsh (Huelsken and Behrens, 2002). Wnt binds to an uncoupled Fz (in the absence of LRP co-receptor) resulting in the activation of GTPase Rho by Dsh which in turn activates jun N-terminal kinase (JNK) (Huelsken and Behrens, 2002). Dsh is connected to downstream effectors such as RhoA and Rho-associated kinase (ROCK) via the signalling proteinDaam1 (Huelsken and Behrens, 2002). The activation of jun N-terminal kinase (JNK) causes cytoskeletal re-organization and the coordinated polarization of cells, which is required for processes such as migration (Cadigan and Nusse, 1997; Huelsken and Behrens, 2002; Cadigan *et al.*,2005). This re-organization can also be directly modulated by the activation of Rho-associated kinase (ROCK) (Lin, 2010; Huelsken and Behrens, 2002). Although not fully understood, the PCP pathway is known to involve the proteoglycan knypek (Kny), and the PDZ molecule strabismus (Stbm).

1.4.3.2 Calcium dependent pathway

The Wnt/Ca2+ pathway is mediated largely by the build-up of intra-cellular calcium (Huelsken and Behrens, 2002; Tanaka *et al.*,2011). This pathway involves the binding of Wnt to Fz and subsequent activation of phospholipase C (PLC) and Protein Kinase C (PKC) via G-proteins (Huelsken and Behrens, 2002; Tanaka *et al.* 2011) (Figure 1.7). Activation of Phospholipase C (PLC) leads to the accumulation of cytosolic calcium, activating the phosphatase calcineurin which in turn dephosphorylates the transcription factor NF-AT (Huelsken and Behrens, 2002). The activated NF-AT moves into and accumulates in the nucleus and regulates the expression of Ca2+ pathway genes as well as inhibiting the canonical/β-catenin dependent pathway (Huelsken and Behrens, 2002).

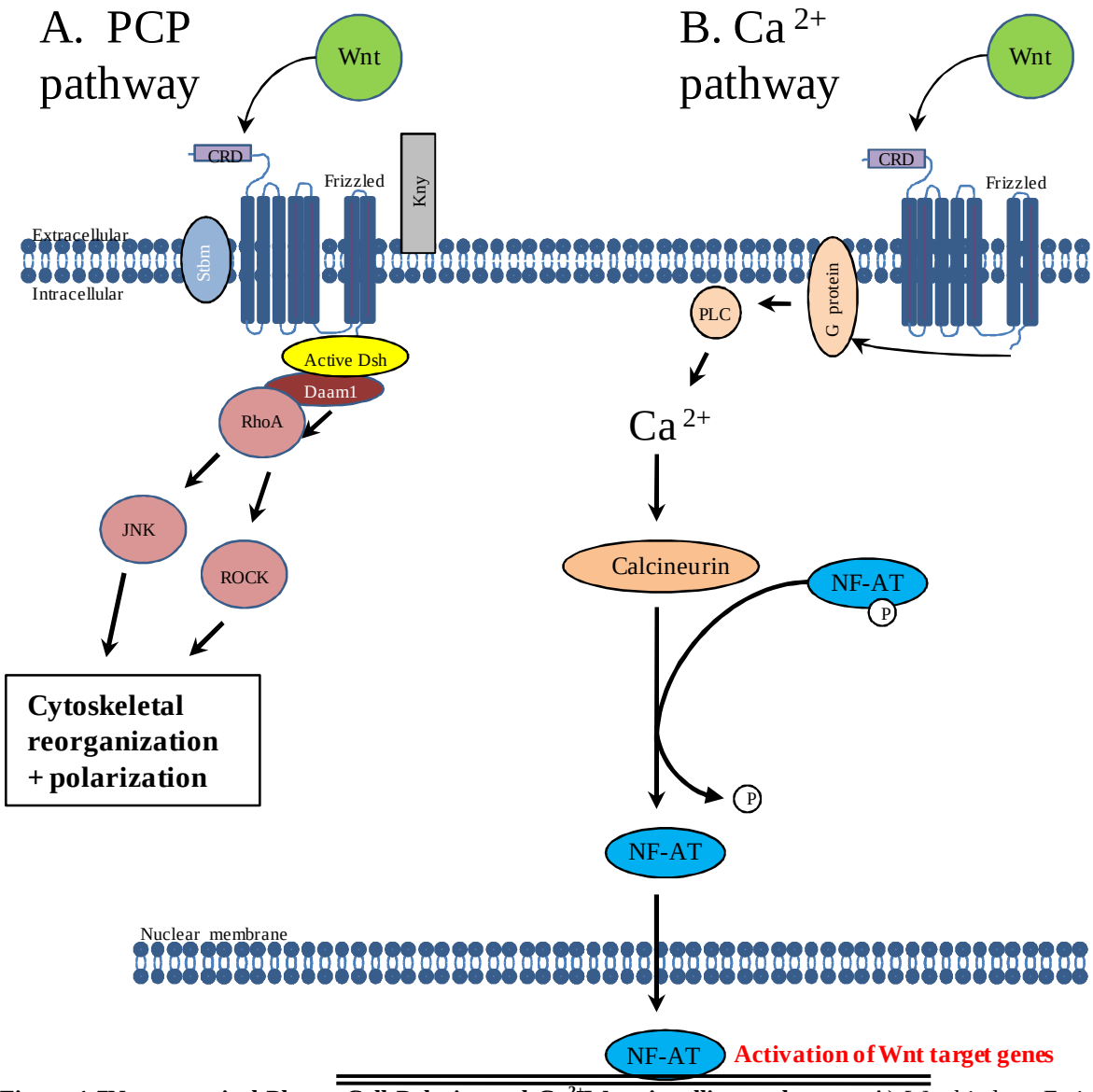

Figure 1.7Non-canonical Planar Cell Polarity and Ca^{2+}Wnt signalling pathways. A) Wnt binds to Fz in the absence of a LRP co-receptor, this activates Dsh to signal via Daam1 and activate the small GTPase RhoA which in turn activates JNK and ROCK resulting in cytoskeletal reorganization and polarization. The membrane bound Stbm and Kny proteins are thought to somehow be involved in the PCP pathway. B) Binding of Wnt causes the G protein-mediated activation of phospholipase C (PLC) resulting in a build-up of cytosolic Ca^{2+} ions. Elevated Ca^{2+} levels increase the activity of phosphatase calcineurin which then phosphorylates the transcription factor NF-AT. The active NF-AT moves into and accumulates in the nucleus and pathway specific genes are expressed. Constructed using references: Huelsken and Behrens, 2002; Cadigan *et al.*,2005; Endo *et al.*,2005; Tanaka *et al,* 2011

1.4.4 Wnt-Fz interactions

The functional specification of Wnt proteins is difficult to establish since Wnts are able to engage various isoforms of Fz, resulting in a range of effects (Janda *et al.*,2012). Each Fz is also able to interact with multiple Wnt types (Janda *et al.*,2012). This pleiotropy means that Wnt proteins can have a wide range of effects on different cells types due to variability in Fz receptor profile. Depending on the isoform of Fz, Wnts are able to act via the β-catenin dependent, PCP or Ca^{2+} pathways (Cheng *et al.,* 2008). For example, Wnt5a is known to signal via the canonical pathway in the presence of the receptor Frizzled 4 (Fz4) (Mikels and Nusse, 2006). In the absence of Fz4, Wnt5A is able to signal via the non-canonical pathways, depending once again on the Fz receptor to which it is bound (Mikels and Nusse, 2006). In addition to this, the signalling pathway initiated can be affected by the concentration of Wnt. For instance, low concentrations of Wnt3A (10 ng/ml) have been shown to engage Wnt/Ca^{2+} signalling in human articular chondrocytes whereas higher concentrations (100-200 ng/ml) activate the β-catenin-dependent pathway (Kestler and Kuhl, 2011; Nalesso *et al*, 2011).

1.4.5 Inhibition of Wnts

Wnt signalling can be inhibited by a number of secreted factors. Wnt inhibitors include Cerberus (Cer) and the Frizzled related protein B (FrzB) which bind to Wnt, blocking interaction with cell-surface Fz (Hsieh, *et al.,* 1999; Huelsken and Behrens, 2002). The Dickkopf family of secreted proteins are known to be involved with embryonic development through the inhibition of Wnt signalling. Dickkopf-related protein-1 (Dkk1) antagonises canonical Wnt signalling by binding to and blocking the LRP5/6 co-receptor (Hsieh, *et al.,* 1999; Huelsken and Behrens, 2002).In conjunction with the transmembrane protein Kremen, Dickkopf may also prevent LRP endocytosis and coupling to Fz (Hsieh, *et al.,* 1999; Huelsken and Behrens, 2002; Mao *et al.,* 2002; Semenov *et al.,* 2005). The Wnt target gene *naked* (*Nkd*) acts as a Wnt antagonist by binding to cytosolic Dsh and preventing the build-up of cytosolic β-catenin, thus preventing canonical Wnt signalling (Huelsken and Behrens, 2002). Dysregulation of Wnt signalling has been linked to a number of hereditary diseases, malformed embryos and cancers (Janda *et al.*,2012).

1.4.6 Wnt signalling in myogenesis

The role of Wnt proteins during embryogenesis has been well documented. For example, Wnt1 and Wnt3A are known to induce myogenesis in the myotome of the differentiating somite (Petropoulos and Skerjanc 2002; Ridgeway *et al.*,2000). However, focus has shifted towards identifying the roles of Wnts in the formation of adult tissues such as skeletal muscle. This research has been confounded however by the variability in Wnt-Fz interactions. Nevertheless, it has been suggested that proliferation and migration are largely regulated via the two non-canonical signalling pathways whereas myoblast commitment and differentiation is initiated by the canonical, β-catenin dependent pathway (Tanaka *et al,* 2011; von Maltzahn *et al.*,2012). Research on Wnts in adult tissue is on-going with a great deal of interest being shown in the Wnt proteins Wnt3A and Wnt7A which have both been previously linked embryogenesis and more recently, myogenesis.

1.4.7 Wnt3A

Wnt3A is one of the most widely studied Wnt proteins with numerous publications linking it to embryogenesis, more specifically to cell polarization and embryo patterning (Ridgeway *et al.*,2000). Wnt3A knockout studies carried out in mice have revealed severe deformities and abnormalities in developing embryos. Embryos lacking Wnt3A displayed highly impaired cellular patterning and polarization resulting in paraxial mesoderm and laterality defects, loss of the tailbud as well as improper vertebral patterning (van Amerongen and Berns, 2007). Less is known however about the role of Wnt3A in adult tissues. It is believed that Wnt3A plays an important role in developmental processes such as myogenesis. In terms of cell migration, exposure to elevated levels of Wnt3A has been seen to reduce the motility of C2C12 myoblasts(Tanaka *et al.*,2011) but increase the motility of Chinese hamster ovary cells (Endo *et al.*,2005). Recently, it has been noted that Wnt3A inhibits the differentiation of mouse C2C12 cells via the up-regulation of Id3 (inhibitor of differentiation 3) while inducing myofibroblast differentiation (into smooth muscle) via the up-regulation of TGF-ß signalling through SMAD2 in a ß-catenin-dependent manner(Cathy *et al.*,2011; Zhang *et al.*,2012). Furthermore, studies carried out on P19 embryonic carcinoma stem cells revealed that Wnt3A promotes myogenesis by the up-regulation of MyoD and Myogenin (Ridgeway *et al.,* 2000). However, injection of Wnt3A (10 µl of 100 ng/ml) into regenerating mouse muscle has been shown impair healing

(Brack *et al.*, 2008). These seemingly contradicting findings may be due to differing Fz receptor profiles on the different cell types. Some of the effects of Wnt3A are summarized in table 1.3.

Table 1.3 The multiple effects of Wnt3A on stem/precursor cells

Process	Effect	Cell type	Reference
Migration	↓	Myoblasts	Tanaka *et al.*,2011
	↑	Chinese hamster ovary cells (CHO)	Endo *et al.*,2005
	↑	Myofibroblasts	Cathy *et al.*,2011
	↑	Rat bone marrow mesenchymal stem cells	Chang *et al.* 2007
Proliferation	↑	Rat bone marrow mesenchymal stem cells	Chang *et al.* 2007
Differentiation	↓	Myoblasts	Zhang *et al.*,2012
	↑	Myofibroblasts (into smooth muscle)	Cathy *et al.*,2011
	↑	P19 embryonic carcenoma stem cells (smooth muscle)	Ridgeway *et al.*, 2000
	↑	Rat bone marrow mesenchymal stem cells	Chang *et al.* 2007
	↑	Cardiomyocytes	Nakamura *et al.*,2003

In addition, *in vivo* experiments have revealed some important information about Wnt3A. Parabiotic studies, linking the circulatory system of old and young mice, have revealed that serum factors within the circulatory system of young mice are able to rejuvenate the regenerative capacity of satellite cells in skeletal muscle of old mice (Conboy *et al.* 2005). Conversely satellite cells in young mice showed a reduced myogenic differentiation capability and trans-differentiated to acquire a fibrogenic lineage when exposed to the serum from old mice (Brack *et al.*, 2007). This myogenic-to-fibrogenic conversion was reflected by an increased collagen deposition further promoting the fibrotic environment. Further analysis revealed the Wnt pathway to be a pivotal mediator of the observed, reversible, phenotypic change. Specifically, the inhibition of the Wnt pathway by addition of sFRP3 (secreted Frizzled Related Protein 3) to the serum from aged mice reduced the myogenic-to-fibrogenic conversion, whereas addition of Wnt3A to "young serum" increased this conversion. It is suspected that the changing Wnt expression profile associated with ageing contributed to reduced myogenesis.

1.4.8 Wnt7A

Compared to Wnt3A, Wnt7A has been the centre of fewer scientific studies. It is known however that Wnt7A plays an important role in the regulation of cell number. Wnt7Aover-expression*in vivo*(via the electroporation of a CMV-Wnt7A expression plasmid into mouse leg muscle) has been observed to enhance muscle regeneration by increasing both the number and proportion of mouse satellite cells in relation to activated myoblasts(Le Grand *et al.*,2009). Muscle lacking Wnt7A exhibited a marked decrease in satellite cell number following

regeneration (Le Grand *et al.*,2009). This is most likely due to impaired replenishment of the quiescent satellite cell pool. It is suspected that Wnt7A interacts with Fz7, and signals through the planar cell polarity pathway to control the homeostatic level of satellite stem cells and hence helps regulates the regenerative potential of muscle tissue (Le Grand *et al.*,2009; Bentzinger *et al.*, 2012; von Maltzahn *et al.*,2012). In addition, Wnt7A is able to stimulate the expression of the muscle-specific transcription factor, myogenin, in marrow-derived multipotent adult stem cell populations (Belema Bedada *et al.*,2005)**.** This signalling is thought to be via the planar cell polarity pathway and does not affect the growth of myoblasts or their differentiation (Bentzinger *et al.*, 2012). No data is available in terms of the effect of Wnt7A on myoblast migration. The various effects of Wnt7A are summarized below (Table 1.4).

Table 1.4 The multiple effects of Wnt7A on stem/precursor cells

Process	Effect	Cell type	Reference
Proliferation	↑	Mouse skeletal muscle satellite cells	Le Grand *et al.*,2009
	-	Myoblasts	Bentzinger *et al.*, 2012
Differentiation	-	Myoblasts	Bentzinger *et al.*, 2012
	↑	Marrow-derived multipotent adult stem cells (myogenesis)	Belema Bedada *et al.*,2005

1.5 Myogenic cell lines

In vitro primary culture myoblasts have successfully been used in research and have provided much data relating to myogenesis. However, using primary culture myoblasts *in vitro* has a number of draw-backs, including the difficulty in isolating pure cultures, the high risk of bacterial and fungal contamination during isolation and a limited life-span in culture due to senescence. For this reason, a large portion of research involving myoblast culture makes use of 'immortalized' myogenic cell lines. One of the most commonly used cell lines in research is the mouse C2C12 myoblast line. The C2 cell line was first cultured from the thigh muscle of C3H mice, 3 days after crush injury to the muscle tissue (Yaffe and Saxel, 1977). Subsequently, the C2C12 cell line was produced by sub-cloning of C2 cells, the clone which showed the highest degree of differentiation (Blau *et al.*, 1985). Mutations in the C2C12 myoblast genetic make-up allows for prolonged survival in culture and lead to the term 'immortalized cell line' (Cornelison, 2008). These mutations have not been entirely characterized and are not fully understood. C2C12 myoblasts show rapid proliferation and differentiation under culture conditions and have a greatly increased lifespan when compared to primary culture. The myoblasts can be cultured

over many passages and can be cryopreserved for use at a later stage. Despite all these advantages, genetic mutations in C2C12 myoblasts result in a cell-line that is not identical to primary culture or myoblasts found *in vivo*. For this reason, experiments carried out on immortalized cell lines such as C2C12 myoblasts should be replicated in primary culture and *in vivo*.

1.6 Medical importance

The effective healing and growth of adult skeletal muscle relies heavily on a healthy population of correctly functioning satellite cells. The importance of these satellite cells is evident in people or animals with improper cell functionality. For example, individuals suffering from Duchene muscular dystrophy are unable to maintain a healthy population of quiescent satellite cells (Vitello *et al.*,2004, von Maltzahn *et al.*, 2012). Numerous rounds of degeneration and regeneration of muscle tissue in these individuals results in a depletion of satellite cell number. This then leads to muscle wasting/dystrophy characterized by the substitution of muscle with fatty-fibrous tissue (Vitello *et al.*,2004). For this reason, satellite cells and indeed all stem-type cells have drawn much interest from the medical world. Gene therapy involving myogenic factors/initiators (such as MyoD and Myf5) as well as direct injection of myoblasts can potentially be used to treat animals or people suffering from severe muscle injuries or genetic disorders. In much the same way that people suffering from diabetes require recombinant insulin, recombinant myogenic factors and signalling proteins such as Wnts could be used to treat muscle diseases such as muscular dystrophy or cystic fibrosis.

Treatment of diseases via the transplantation of myoblasts or the addition recombinant myogenic factors has great potential in terms of wound healing. However, a number of problems have arisen, especially with regards to transplantation of myoblasts. These difficulties include the rejection of the donor myoblasts, decreased migration and wound penetration of the transplanted myoblasts and the high expiry rate of injected myoblasts (Guerette *et al.*,1997).Rejection of donor myoblasts is largely due to the once overlooked expression of the major histocompatibility complex (MHC) on the cell surface. This problem can be overcome by injecting an individual's own myoblasts that have been cultured *in vivo*. This process requires the harvesting of myoblasts from the muscle tissue, expansion of the cell number *in vivo* and the reinjection of the myoblasts. It is therefore vital to encourage maximum cell proliferation *in vivo* in order to obtain

sufficient myoblasts for transplantation and wound healing. Potential proliferation-encouraging factors such as Wnt7A could be used to increase the yield of transplantable myoblasts. Obtaining sufficient numbers of myoblasts to facilitate wound healing via transplantation is one of the obstacles for this therapy. Advances in gene therapy may also be utilized at this time in order to correct any detrimental genetic mutations that the myoblasts may have.

Transplanted myoblasts also show reduced migration; this may be due to the cells becoming stuck within the interstitial tissue surrounding the damaged myofibers (Rando *et al.*,1995). Increased integrin-dependent migration of myoblasts could help sufficient cell numbers to reach the wound area. This would need to be accompanied by an increased breakdown of the migration-impeding ECM by proteinases such as MMP-2 and MT1-MMP.Treatment of *in* vivo myoblasts with the MMP activating factor, basic fibroblast growth factor (bFGF) prior to injection has been shown to increase wound healing (Kinoshita *et al.*, 1995). This is thought to be as a result of increased myoblast motility due to a greater level of ECM breakdown by the activated proteinases.

Another problem with myoblast transplantation is the high level of cell death among the newly transplanted cells. This has been attributed to immune responses following the transplantation. One way in which to address this problem is to ensure maximum myoblast proliferation once transplanted. The myoblasts can be treated with specific growth factors prior to injection, encouraging increased proliferation once *in situ*. The problem with this approach is that most proliferation encouraging factors also act to down-regulate differentiation. This is not ideal as this then decreases fusion and thus muscle healing. Research is therefore on-going to find ways to encourage such proliferation without preventing fusion.

The Wnt family of signalling proteins appear to be highly involved in the process of myogenesis. Of particular interest are Wnt3A and Wnt7A which have been shown to affect muscle stem cell migration, proliferation and differentiation. Although some work has been carried out regarding Wnt3A and Wnt7A in terms of myogenesis, these studies relied on Wnt-producing cell-lines. As a result of using a Wnt-producing cell-line, the actual concentration of Wnt in the culture medium is not always known or stated.

The aim of the current study was therefore to analyse the effect of a range of concentrations of recombinant Wnt3A and Wnt7A on 1) the proliferation and migration as well as 2) the differentiation of mouse C2C12 and donor-derived human skeletal muscle myoblasts.

CHAPTER TWO

THE EFFECT OF WNT3A AND WNT7A ON PROLIFERATION AND MIGRATION OF MOUSE C2C12 AND DONOR-DERIVED HUMAN MYOBLASTS

Satellite cells are skeletal muscle stem cells tasked with the growth and repair of muscle tissue. Damage to muscle tissue initiates myogenesis via the activation of satellite cells. Activated satellite cells, referred to as myoblasts, migrate to the site of injury where they proliferate, differentiate and fuse to repair the tissue. The speed at which myoblasts migrate and proliferate has a major effect on the success of healing. Diseases such as Duchene's muscular dystrophy are characterised by muscle wasting and the build-up of fibrotic tissue as a result of improper maintenance of the satellite cell pool. The Wnt family of secreted proteins are suspected to play an important role in the regulation of cell migration and proliferation. Of particular interest are Wnt3A and Wnt7A which have both been linked with myogenesis. Wnt3A-expressing C2C12 cells have been shown to have reduced motility; however treatment with recombinant Wnt3A is known to increase migration in Chinese hamster ovary cells and rat-derived fibroblast and mesenchymal stem cells *in vitro*. Injection of 10µgWnt7Ainto mouse leg muscle appears to increase the proportion of quiescent satellite cells. Previous studies involving Wnts have mostly involved an over-expressing cell line and/or Wnt-containing conditioned medium. In our study we wished to evaluate the effect of *known* concentrations of recombinant Wnt3A and Wnt7A on the migration and proliferation of mouse C2C12 and HSkM myoblasts *in vitro*. Using an *in vitro* wound assay, we established that Wnt3A and Wnt7A at 10 ng/ml and 100 ng/ml promoted wound closure in injured C2C12 myoblasts at 7 hours post wounding. HSkM myoblasts treated with 10 ng/ml Wnt3A also displayed a significant increase in motility, confirming the pro-migratory effect of Wnt3A. Neither Wnt3A nor Wnt7A were seen to have any significant effect on C2C12 or HSkM proliferation. Wnt signalling appears to be highly dependent on experimental conditions.

2.1 Introduction

Satellite cells are resident, quiescent skeletal muscle stem cells, characterized by their anatomical location between the sarcolemma and myofiber plasma membrane along muscle fibers and by their expression of proteins such as Pax7, M-cadherin, c-Met and CD34 (Mauro, 1961; Irintchev *et al.*, 1994; Beauchamp *et al.,* 2000; Charge and Rudnicki 2004; Sherwood *et al.,* 2004). When growth or repair of muscle tissue is required, the satellite cells exit quiescence as activated myoblasts, migrate to the site of growth/injury, proliferate and then differentiate and fuse to form new myotubes or repair damaged muscle (Le Grand and Rudnicki, 2007; Burdzinska *et al.* 2008). This overall process is referred to as myogenesis (Le Grand and Rudnicki, 2007; Burdzinska *et al*. 2008; Griffin *et al.,* 2010). A proportion of the myoblasts must return to a quiescent state in order to maintain the population of satellite cells within the tissue (Le Grand and Rudnicki, 2007). Myoblasts that undergo differentiation and fusion become terminally differentiated and are unable to re-enter mitosis (Hawke and Geary, 2001; Le Grand and Rudnicki, 2007). For this reason, a healthy population of satellite cells is required to facilitate the healthy growth and repair of muscle tissue. When an injury has occurred, the success of healing is largely determined by the rate at which the activated myoblasts reach the site of injury, proliferate and degree to which they differentiate.

Effective migration of myoblasts is vital for wound healing. Controlled migration ensures that a sufficient number of myoblasts reach the injury site and that the cells are able to line-up and form cell-cell adhesions required for differentiation and fusion (Kang *et al.,* 2004; Griffin *et al.,* 2010). With every serious injury, the regenerative potential of the muscle tissue is reduced (Charge and Rudnicki, 2004). This is in part due to the deposition of extracellular matrix (ECM) factors such as collagen I, resulting in scar tissue as well as the reduction in the number of local satellite cells. Loss of skeletal muscle mass, either due to old age or the recessive, X-linked Duchenne muscular dystrophy gene is referred to as sarcopenia and is associated with a reduction in satellite cell activity and number (Solomon and Bouloux, 2006).Duchenne muscular dystrophy is caused by a mutation in the dystrophin gene. Dystrophin is an anchor-type protein vital for keeping muscle cells intact by tethering structural proteins within the cells to proteins on the outer surface of the cell membrane (Sussman, 2002). The reduced stability of the muscle tissue means that wear and tear to the tissue is more severe. This stimulates excessive satellite

cell activation; as a result, the satellite cell population becomes diminished and is unable to cope with the rate of tissue damage.

The Wnt family of secreted proteins consists of 21 highly-conserved, cysteine-rich, lipid-modified glycoproteins which interact with members the seven-pass transmembrane receptor Frizzled (Fz) family (George-Weinstein *et al.,* 1998; Cadigan and Liu, 2006; Maiese *et al.,* 2008).Wnt proteins may initiate different signalling pathways, depending on both the Wnt type and the Fz profile (Cheng *et al.,* 2008). Signalling may be via the canonical/β-catenin dependent, or via one of the two non-canonical pathways, these being; the planar cell polarity (PCP) or the Ca2+ dependent pathways (Huelsken and Behrens, 2002; Nusse, 2003; Packard *et al.,* 2003; Laeremans *et al.,* 2010; Tanaka *et al.,* 2011). It has been suggested that proliferation and migration are largely regulated via the two non-canonical signalling pathways whereas myoblast commitment and differentiation is initiated by the canonical, β-catenin dependent pathway (Tanaka *et al,* 2011; von Maltzahn *et al.,* 2012).

Wnt3A is one of the most widely studied Wnt proteins with numerous publications highlighting its importance in cell polarization and tissue patterning during embryogenesis (Ridgeway *et al.,* 2000). Embryos lacking Wnt3A displayed highly impaired cellular patterning and polarization resulting in paraxial mesoderm and laterality defects, loss of the tailbud as well as improper vertebral patterning (van Amerongen and Berns, 2007). In adult tissue, Wnt3A has been shown to play an important role in myogenesis. *In vitro* studies have shown that Wnt3A-containing conditioned medium inhibits the differentiation of C2C12 myoblasts (Cathy *et al.,* 2011; Zhang *et al.,* 2012). This inhibition is thought to be as a result of Wnt3Asignalling via the canonical pathway and up-regulating the expression of Id3 (inhibitor of differentiation 3)(Cathy *et al.,* 2011; Zhang *et al.,* 2012). It has also been noted that elevated levels of Wnt3A in injured mouse muscle leads to impaired muscle repair and an increased deposition of fibrous connective tissue (Brack *et al.,*2007; von Maltzahn *et al.,* 2012). This impaired healing and build-up of connective tissue is typically associated with the regeneration of 'aged' muscle. In terms of cell migration, exposure to elevated levels of Wnt3A has been seen to increase the motility of Chinese hamster ovary cells as well as rat-derived fibroblast and bone marrow mesenchymal stem cells (Endo *et al.,* 2005; Shang *et al.,* 2007, Schlessinger *et al.,* 2009). It is suspected that Wnt3A is able to activate the Rho-associated kinase via the Dishevelled signalling protein which in turn stimulates

cell migration through the regulation of actin/myosin contractions (Schlessinger *et al.,* 2009). Unexpectedly, over-expression of Wnt3A by C2C12 myoblasts has been seen to reduce migration (Tanaka *et al.,* 2011). This reduced motility may be as a result of constant and prolonged exposure to Wnt3A as opposed to an acute and controlled exposure in response to injury. Little research has been conducted in terms of Wnt3A and proliferation. Studies have shown however that Wnt3A-containing conditioned medium increases the proliferation of rat bone marrow mesenchymal stem cells via non-canonical signalling (Chang *et al.,* 2007).

Wnt7A plays an important role in the regulation of cell number. Injection of Wnt7A has been observed to enhance the regeneration of mouse leg muscle by expanding and maintaining the satellite cell pool (Le Grand *et al.,* 2009; von Maltzahn *et al.,*2012).Muscle lacking Wnt7A on the other hand exhibited a marked decrease in satellite cell number following regeneration. This is most likely due to impaired replenishment of the quiescent satellite cell pool. It is suspected that Wnt7A interacts with Fz7, and signals through the planar cell polarity pathway to control the homeostatic level of satellite stem cells and hence helps regulates the regenerative potential of muscle tissue (Le Grand *et al.,* 2009; Bentzinger *et al.,* 2012; von Maltzahn *et al.,* 2012). No published data is available in terms of the effect of Wnt7A on myoblast migration.

Most Wnt studies have involved an over-expressing cell line which secretes Wnt protein into the surrounding medium. Myoblasts are either maintained in co-culture with the Wnt producing line or treated with harvested conditioned media. Such Wnt-containing conditioned media may contain up to a maximum of 200 ng/ml of Wnt (Willert *et al.,* 2003). In most studies however, the concentration of Wnt produced is not mentioned and this makes it difficult to correlate any effect to a specific Wnt concentration. In addition, cells which are grown in co-culture with Wnt-producing lines are constantly exposed to the signalling protein. It is possible that these prolonged exposures change the Fz receptor profile; furthermore these conditions do not mimic the change in *in vivo* Wnt expression levels that occur during wound healing. In the current study we analyzed the effect of specific doses of recombinant Wnt3A and Wnt7A on the proliferation and migration of both murine (C2C12) and human skeletal muscle myoblasts.

2.2 Materials and Methods

All chemicals used in the study were of an analytical grade. Cell culturing was performed under sterile conditions in a level II lamina flow hood (ESCO class II BSC). All cells were incubated in an Innova® CO-170 CO2-incubator (New Brunswick) at 37°C, 5% CO2.

2.2.1 Cell culture

Cells

Mouse C2C12, and donor-derived human skeletal muscle (HSkM) myoblasts were used in the study. C2C12 cells are an immortalized myoblast cell line capable of migration, proliferation, differentiation and fusion into myotubes. The C2C12 cells used in the study were kindly donated by the Cape Heart Centre, University of Cape Town. Passages ranged from P12 to P18. HSkM myoblasts were obtained from Lonza (cat.CC-2561, USA) and used at passage P3.

Culture media

C2C12 myoblasts were maintained in growth media (GM) consisting of Dulbecco's Modified Eagle's Medium (with 2% L-glutamine) (Sigma, cat.D5648-1L, USA) supplemented with 10% (v/v) fetal bovine serum (Biowest, cat.51810-500, USA), 3.7g/l sodium bicarbonate (Merck, cat.103025, USA) and 1% (v/v) Penicillin/Streptomycin (Lonza, cat.DE17-602E, RSA).

The HSkM cells were cultured in a 50/50 mixture of the C2C12 growth medium (GM) and HamsF10 nutrient broth (Sigma-Aldrich, cat.N6908, USA). This 50/50 media mix was also supplemented with 2.5 ng/ml human fibroblast growth factor (hFGF) (Sigma-Aldrich, cat.F0291, USA) with the fetal bovine serum concentration adjusted to 20%.

Thawing and culturing of myoblasts

Both C2C12 and HSkM cells were received as frozen stocks stored in cryopreservation tubes. When required, cells were thawed by gentle heating in a 37°C water bath for 1 minute. The semi-frozen cell suspension was then transferred into a T75 tissue culture flask (Nest Biotech, cat.0926B) containing 10 ml of the required medium (GM for C2C12 cells, 50/50 mix for HSkM cells). Exhausted culture medium was replaced with fresh medium every 48 hours during cell proliferation within the T75 flasks. Cells proliferating in the T75 culture flasks were passaged before reaching 80% confluence in order to prevent any undesired differentiation. Cells were

passaged by removing the medium, washing with phosphate buffered saline solution (PBS) and incubation with 2 ml trypsin (Cambrex, cat.17-161E) at 37°C for 3 minutes. After 3 minutes, the trypsin activity was neutralized by adding an equal volume of growth medium. The passaged cells were then divided into additional T75 flasks for future use or plated for experimental use.

2.2.2 Recombinant Wnt proteins

Recombinant murine Wnt3A (Peprotech, cat.315-20) and recombinant human Wnt7A (R and D Systems, cat.3008-WN/CF) were re-suspended in sterile PBS at a concentration of 100 µg/ml. At the start of our experimental work, these were the available species of recombinant Wnt3A and Wnt7A. These solutions were stored at -20°C. For both the migration and proliferation experiments, C2C12 myoblasts were treated with culture medium containing Wnt at a concentration of 0 ng/ml, 1 ng/ml, 10 ng/ml or 100 ng/ml. Due to the low proliferation rate of the HSkM cells, only the Wnt type and dose which showed the greatest effect on the C2C12 cell line was tested in human cells.

2.2.3 Analysis of cell migration in response to Wnt3A and Wnt7A

Cell migration was analysed using an *in vitro* wound assay (Goetsch *et al.*, 2011). A total of 30 000 cells (C2C12 or HSkM) were plated into the wells of a plastic, 24-well culture dish(TPP, cat.Z707791**,** USA) containing 500µl culture medium per well. The cells were allowed to reach 80% confluence after which they were 'wounded' using a plastic gel-loading tip. The culture medium was removed and the cells washed with PBS. Fresh culture medium containing either Wnt3A or Wnt7A was then added to the wells. Photographs of the wound areas were taken at 0, 3, 5 and 7 hours post-wounding using an Olympus CKX41 microscope(40x magnification) fitted with a Moticam 2300 3.0 megapixel camera. The wound areas of the images were determined by tracing along the border of the wound using the Motic2.0 image analysis software. The percentage wound closure at each time point was calculated using the formula: [(wound area at 0 hour - wound area at n hour) ÷ wound area at 0 hour] x 100. The rates of wound closure were calculated by plotting line graphs for the 0-3 hour, 3-5 hour and 5-7 hour time periods. The gradients of these individual line plots were used as the rate of wound closure i.e. percentage wound closure (%)/time (hours).

2.2.4 Analysis of cell proliferation in response to Wnt3A and Wnt7A

Cell proliferation was determined by way of cell counts. A total of 100 000 (C2C12) or 30 000 (HSkM) myoblasts were plated into the wells of 6-well plates (Thermo Scientific, catYO-01930-21, USA) containing growth medium and allowed to settle for 4 hours. After the cells had adhered to the plates, the media was removed and 2 ml of Wnt-containing culture medium added. The myoblasts were incubated for 24 (C2C12) or 48 hours (HSkM), trypsinized and counted using a Biorad TC-20 automated cell counter.

2.2.5 Statistical analysis

All data are represented as mean ± standard error of the mean (SEM) for each data set shown. The paired Student's t-test was performed for parametric data and paired Mann-Whitney U test for non-parametric data. All statistical tests were performed using the Genstat 12th edition statistical software (VSN International). Significance was set at $p < 0.05$.

2.3 Results

2.3.1 Wnt3A increases the migration of C2C12 myoblasts

In order to test the effect of Wnt proteins on migration, C2C12 cells cultured in 24-well plates were scratched in order to simulate a wound (Abbi and Guan, 2002; Goetsch *et al.*, 2011). The myoblasts were then treated with Wnt-containing culture media and phase contrast images taken at 0, 3, 5 and 7 hours post injury (Figure 2.1).

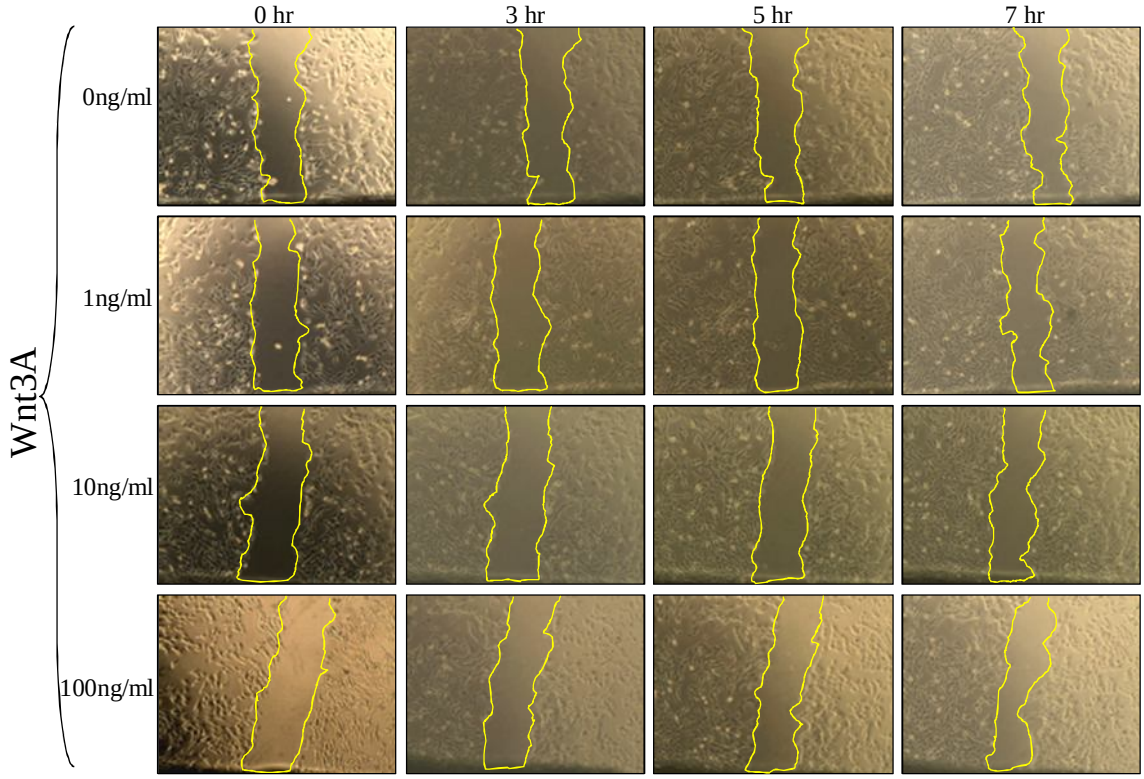

Figure 2.1 C2C12 myoblast wound areas in response to Wnt3A. Phase contrast images of wound areas taken at 0, 3, 5 and 7 hours post wounding. Wnt3A concentrations are shown on the left. Yellow borders outline wound areas. 40X magnification.

Calculation of percentage wound closure revealed that Wnt3A (10 ng/ml and 100 ng/ml) marginally increased C2C12 myoblast migration over the 7 hour time period (Figure 2.2A). Wnt3A at 10 ng/ml significantly increased migration from 18.9% (control) to 22.8% (10 ng/ml) after 5 hours of treatment (Figure 2.2C). After 7 hours, both the 10 ng/ml and 100 ng/mlWnt3A treatments had significantly increased migration from 29.1% (control) to 36.3% (10 ng/ml) and 35.4% (100 ng/ml) (Figure 2.2D). Unexpectedly, migration was slightly inhibited in response to 1 ng/mlWnt3A treatment at the 1 hour time point, dropping from 10.2% (control) to 8.9%

closure ($p<0.05$) (Figure 2.2B). Calculation of the rate of wound closure revealed that Wnt3A (1 ng/ml) significantly decreased the rate of closure during early wound repair control ($p<0.05$; Figure 2.2E). However, between 3 and 7 hours, the rate of wound closure was significantly increased after treatment with 10 ng/ml Wnt3A ($p<0.05$, Figure 2.2F and Figure 2.2G).

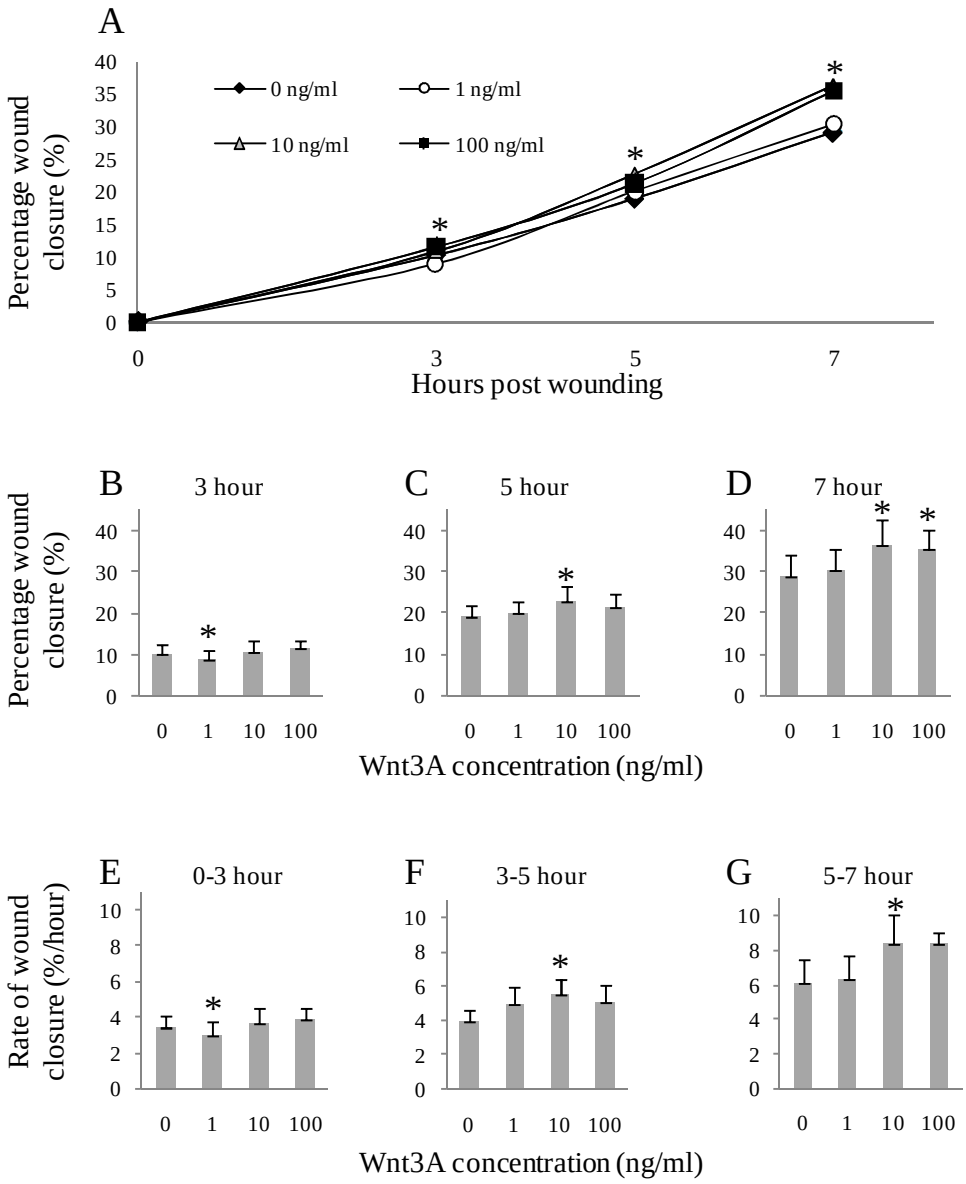

Figure 2.2 C2C12 myoblast wound repair in response to Wnt3A. Myoblast migration in response to Wnt3A was assessed using an *in vitro* wound healing assay. A)The percentage wound closure was determined at 3, 5 and 7 hours post wounding using the Motic 2.0 image analysis software. Panels B, C and D show the significant results and SEM values for the individual time points that cannot be clearly represented in panel A. B) Wound closure 3 hours post-wounding. C) Wound closure 5 hours post-wounding. D) Wound closure 7 hours post-wounding. E) Rate of wound closure, 0-3 hour. F) Rate of wound closure, 3-5 hour. G) Rate of wound closure, 5-7 hour. Data are expressed as mean + SEM; n=6. *$p<0.05$ (compared to 0 ng/ml).

2.3.2 Wnt7A increases the migration of C2C12 myoblasts

Migration analysis was carried out on C2C12 myoblasts treated with Wnt7A (0, 1, 10, 100 ng/ml). Phase contrast images were taken at 0, 3, 5 and 7-hours post wounding (Figure 2.3). As with the Wnt3A treated cells, no obvious differences in wound closure were observed in control versus Wnt7A treated cells (Figure 2.2).

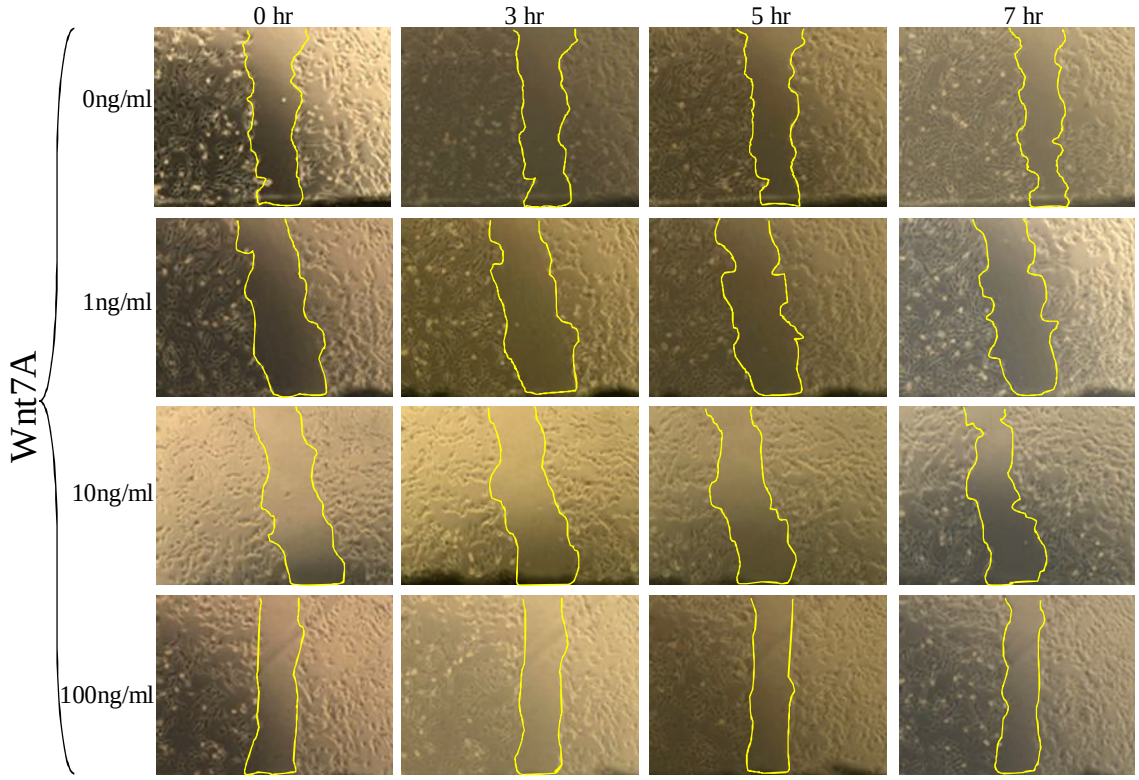

Figure 2.3 C2C12 myoblast wound areas in response to Wnt7A. Phase contrast images of wound areas taken at 0, 3, 5 and 7 hours post wounding. Wnt7A concentrations are shown on the left. Yellow borders outline wound areas. 40X magnification.

Calculation of percentage wound closure revealed that Wnt7A significantly increased wound closure at 5 and 7 hours post wounding, but not at the 3 hour time point (Figure 2.2A and Figure 2.2B). Wnt7A (10 ng/ml) increased the percentage wound closure from 18.9% to 23.7% at 5 hours post wounding, a 4.8% increase relative to control ($p<0.05$; Figure 2.4C). After 7 hours, the higher concentrations were seen to significantly increase migration from 29.1% (control) to 34.9% (10 ng/ml) and 33% (100 ng/ml) (Figure 2.4D). The plots for the rates of closure mirrored those of total wound closures. The rates of migration revealed that unlike Wnt3A, Wnt7A had no effect on the velocity of migration during early *in vitro* repair (0-3 hour).

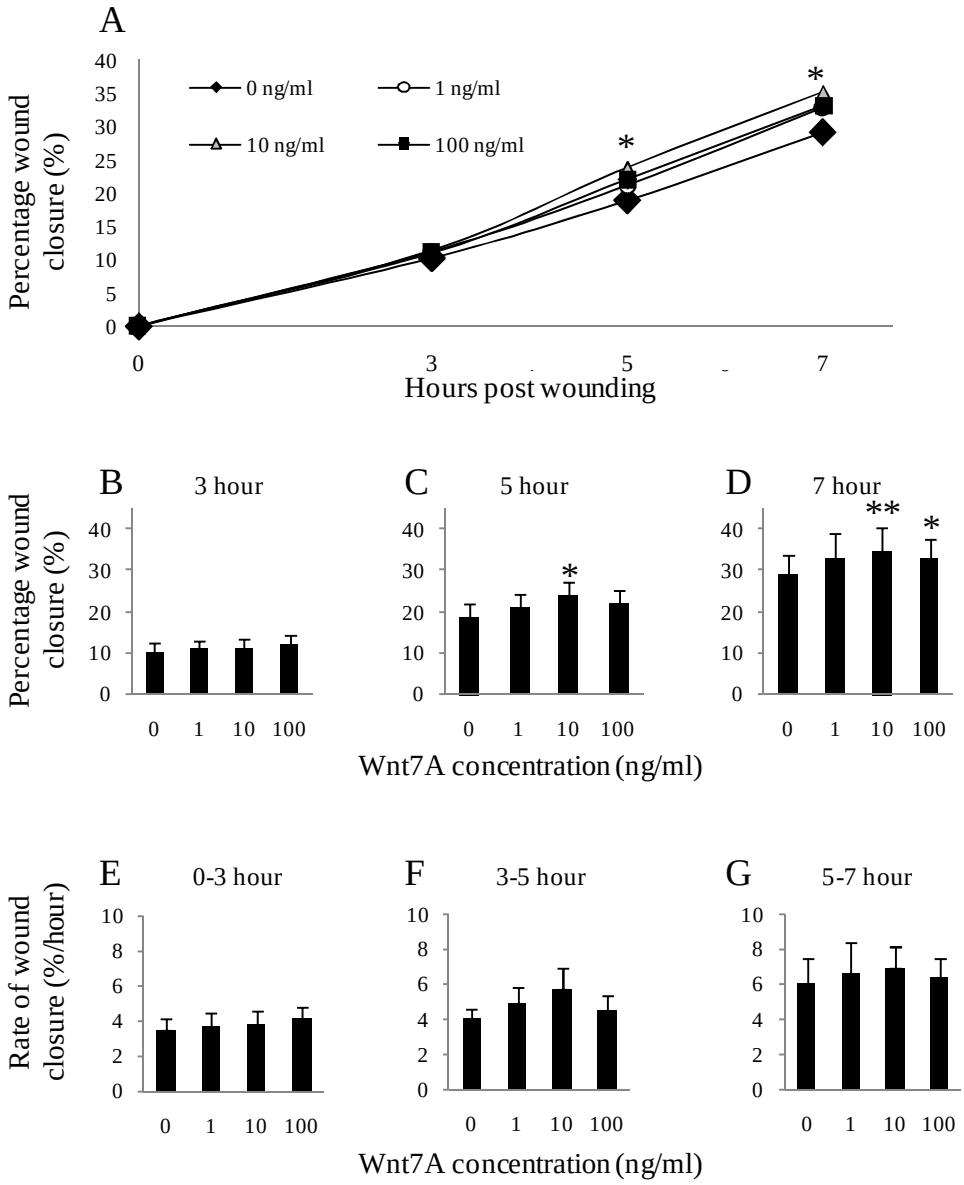

Figure 2.4 C2C12 myoblast wound repair in response to Wnt7A. Myoblast migration in response to Wnt7A was assessed using an *in vitro* wound healing assay. A) The percentage wound closure was determined at 3, 5 and 7 hours post wounding using the Motic 2.0 image analysis software. Panels B, C and D show the significant results and SEM values for the individual time points that cannot be clearly represented in panel A. B) Wound closure 3 hours post-wounding. C) Wound closure 5 hours post-wounding. D) Wound closure 7 hours post-wounding. E) Rate of wound closure, 0-3 hour. F) Rate of wound closure, 3-5 hour. G) Rate of wound closure, 5-7 hour. Data are expressed as mean + SEM; n=6. *p<0.05 (compared to 0 ng/ml).

2.3.3 Wnt3A increases the migration of HSkM myoblasts

Subsequent to C2C12 migration analysis, HSkM myoblasts were 'wounded' and treated with Wnt protein. Due to the difficulty in culturing sufficient numbers of HSkM myoblasts, only the Wnt concentration which showed the greatest effect on C2C12 migration was evaluated. The 10 ng/mlWnt3A treatment was selected (7.2% increase in wound closure after 7 hours) ($p<0.05$). Phase contrast microscopy images of migrating HSkM cells treated with Wnt3A were captured at 0, 3, 5 and 7 hours post-wounding; HSkM myoblasts appeared to close the wound area faster than the C2C12 cells (Figure 2.5).

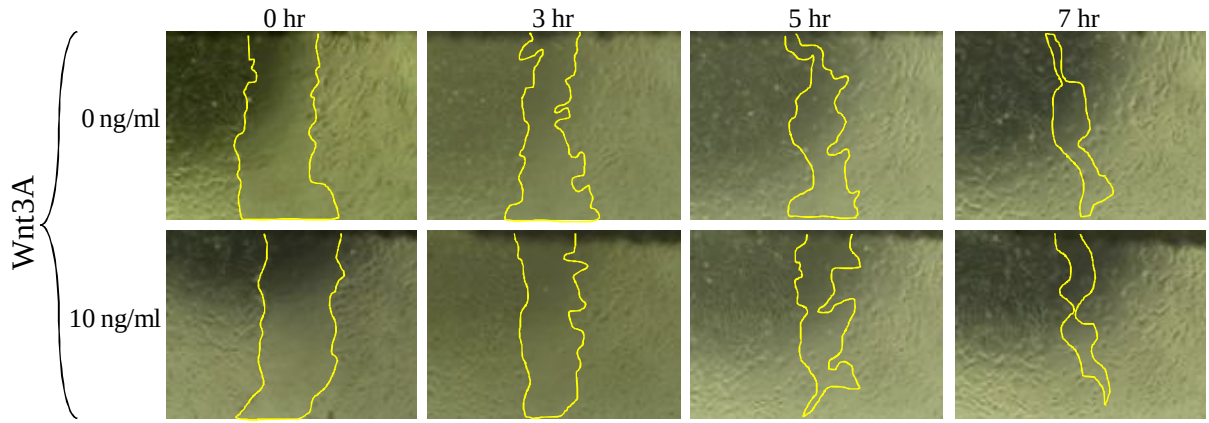

Figure 2.5 HSkM myoblast wound areas in response to 10 ng/ml Wnt3A. Phase contrast images of wound areas taken at 0, 3, 5 and 7 hours post wounding. Wnt3A concentrations are shown on the left. Yellow borders outline wound areas. 40X magnification.

Evaluation of the wound areas revealed that Wnt3A significantly increased wound closure in HSkM cells at 5 and 7hours but not 3 hours post-wounding(Figure 2.6). After 5 hours, wound closure had increased significantly from 52.9% (control) to 61.1% (10 ng/ml) ($p<0.05$; Figure 2.6B). Incubation with Wnt3A for 7 hours resulted in a significant increase in wound closure from 71.6% (control) to 80.9% (10 ng/ml) ($p<0.05$; Figure 2.6C). It was also confirmed that the HSkM myoblasts were quicker to close the wound area than the C2C12 cell line. The control C2C12 myoblasts reached a total of 29.1% wound closure after 7 hours while the HSkM myoblasts reached a figure of 71.6% closure. Although not significant, it seemed that Wnt3Ahad a greater initial effect on HSkM migration when compared to C2C12 cells. Between 0-3 hours,10 ng/mlWnt3A increased the rate of HSkM migration from 10.5% (%closure/hour; control) to 13.6% (%closure/hour). After this initial increase, the rate of migration seemed to level out at around 10% (%closure/hour) both for the control and Wnt3A treatment. This was

not the case in the C2C12 cells. In response to Wnt3A, the C2C12 myoblasts appeared to have largely unaffected migration during the initial stages of the wound assay (0-3 hours), but significantly increased migration during the latter stages (between 3 and 7 hours post wounding).

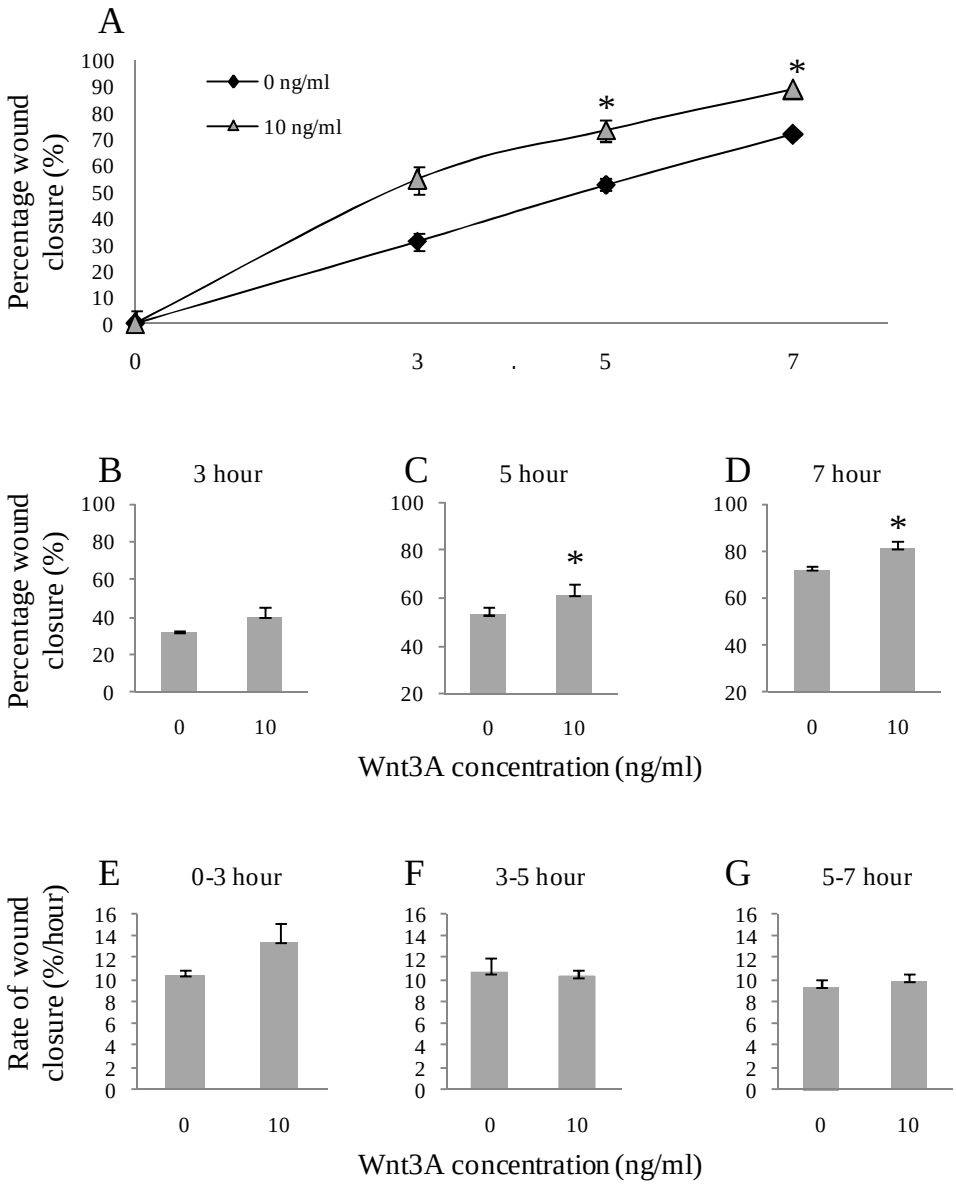

Figure 2.6 HSkM myoblast wound repair in response to 10 ng/ml Wnt3A. Myoblast migration in response to Wnt3A was assessed using an *in vitro* wound healing assay. A) The percentage wound closure was determined at 3, 5 and 7 hours post wounding using the Motic 2.0 image analysis software. Panels B, C and D show the significant results and SEM values for the individual time points that cannot be represented in panel A. B) Wound closure 3 hours post-wounding. C) Wound closure 5 hours post-wounding. D) Wound closure 7 hours post-wounding. E) Rate of wound closure, 0-3 hour. F) Rate of wound closure, 3-5 hour. G) Rate of wound closure, 5-7 hour. Data are expressed as mean + SEM; n=6. *p<0.05 (compared to 0 ng/ml).

2.3.4 Wnt3A has no significant effect on the proliferation of C2C12 myoblasts

In order to analyse the effect of Wnt3A on myoblast proliferation, 100 000 C2C12 myoblasts were seeded in GM into the wells of a 6-well plate and allowed to settle over a 4 hour period. The media was removed and Wnt3A-containing media added. The myoblasts were cultured for 24-hours and cell counts performed using a Biorad TC-20 automated cell-counter. It was observed that the cells increased in number by 100%. However, none of the Wnt3A concentrations (1 ng/ml, 10 ng/ml and 100 ng/ml) had any significant effect on the proliferation of the C2C12 myoblasts (Figure 2.7)

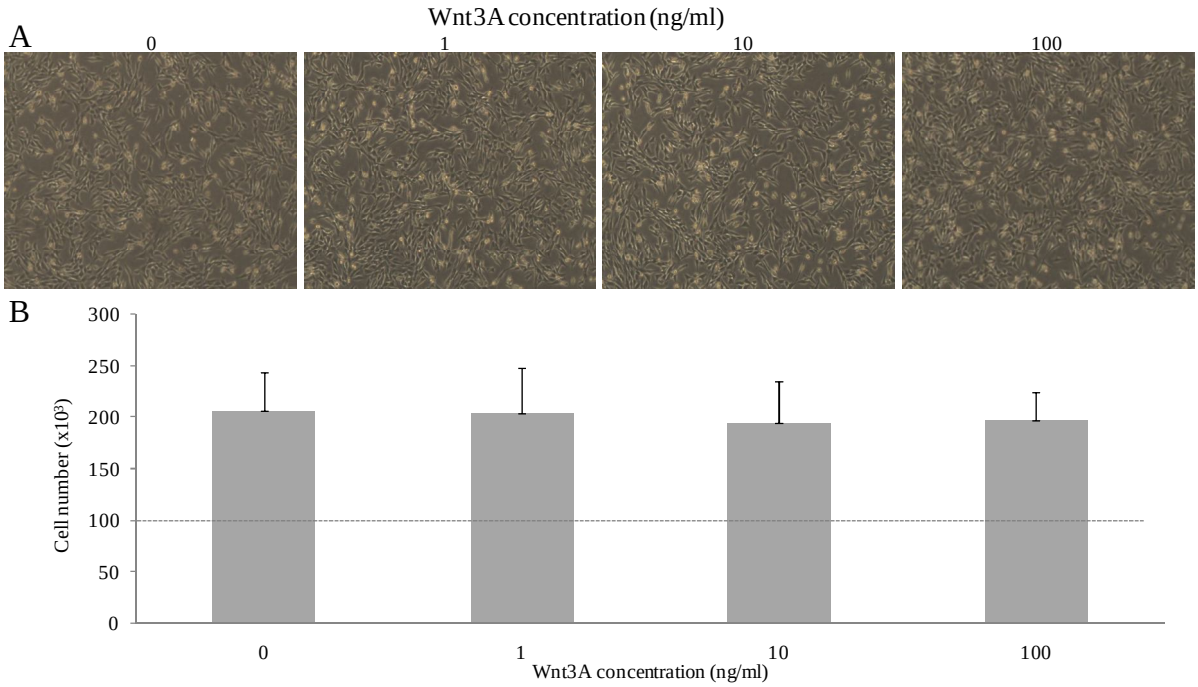

Figure 2.7 Effect of Wnt3A on C2C12 myoblast proliferation. The proliferation of C2C12 myoblasts in response to Wnt3A was evaluated using cell counts obtained by a Biorad TC-20 automated cell-counter. A total of $100x10^3$ C2C12 myoblasts were seeded and treated with Wnt3A at 0 ng/ml, 1 ng/ml, 10 ng/ml or 100 ng/ml for 24 hours. A) Phase contrast images of proliferating cells were taken 24 hours after treatment with Wnt3A. B) Cell numbers after 24 hours were calculated with the initial number of seeded cells represented by the dotted line. Data are expressed as mean + SEM; n=4. *p<0.05 (compared to 0 ng/ml).

2.3.5 Wnt7A has no significant effect on the proliferation of C2C12 myoblasts

In order to determine the effect of Wnt7A on C2C12 proliferation, myoblasts were incubated withWnt7A over a 24-hour period. The Wnt7A treatments and subsequent cell counts were carried out as described in section 2.3.4. Although Wnt7A (10 ng/ml and 100 ng/ml) tended to

reduce the increase in cell number when compared to control, this effect was not significant (Figure 2.8).

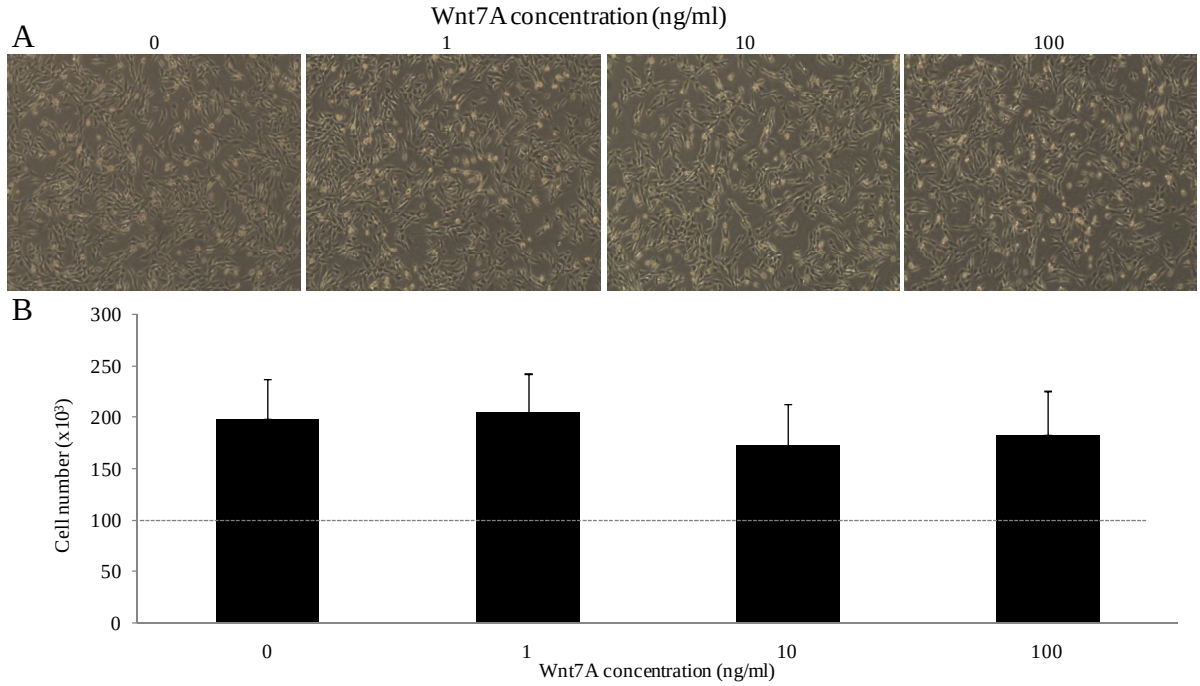

Figure 2.8 Effect of Wnt7A on C2C12 myoblast proliferation. The proliferation of C2C12 myoblasts in response to Wnt7A was evaluated using cell counts obtained by a Biorad TC-20 automated cell-counter. A total of 100x10^{3}C2C12 myoblasts were seeded and treated with Wnt7A at 0 ng/ml, 1 ng/ml, 10 ng/ml or 100 ng/ml for 24 hours. A) Phase contrast images of proliferating cells were taken 24 hours after treatment with Wnt7A. B) Cell numbers after 24 hours were calculated with the initial number of seeded cells represented by the dotted line. Data are expressed as mean + SEM; n=4. *p<0.05 (compared to 0 ng/ml).

2.3.6 Wnt7A has no effect on the proliferation of HSkM myoblasts

Although not significant, it appeared that Wnt7A at the 10 ng/ml and 100 ng/ml concentrations had a slightly inhibitory effect on C2C12 myoblast proliferation. In addition, Wnt7A has previously been linked to the regulation of satellite cell quiescence (Le Grand *et al.*, 2009; Bentzinger *et al.*, 2012; von Maltzahn *et al.*, 2012). As a result, we decided to investigate whether 100 ng/ml Wnt7A affects HSkM myoblast proliferation. A total of 30 000 HSkM myoblasts were plated into the wells of a 6-well plate, allowed to settle and subsequently treated with Wnt7A-containing GM. Due to their relatively slow doubling time, the HSkM cells were incubated for 48 hours before cell counts were performed. Analysis of the HSkM cell numbers revealed that Wnt7Aat 100 ng/ml had no significant effect on proliferation (Figure 2.9).

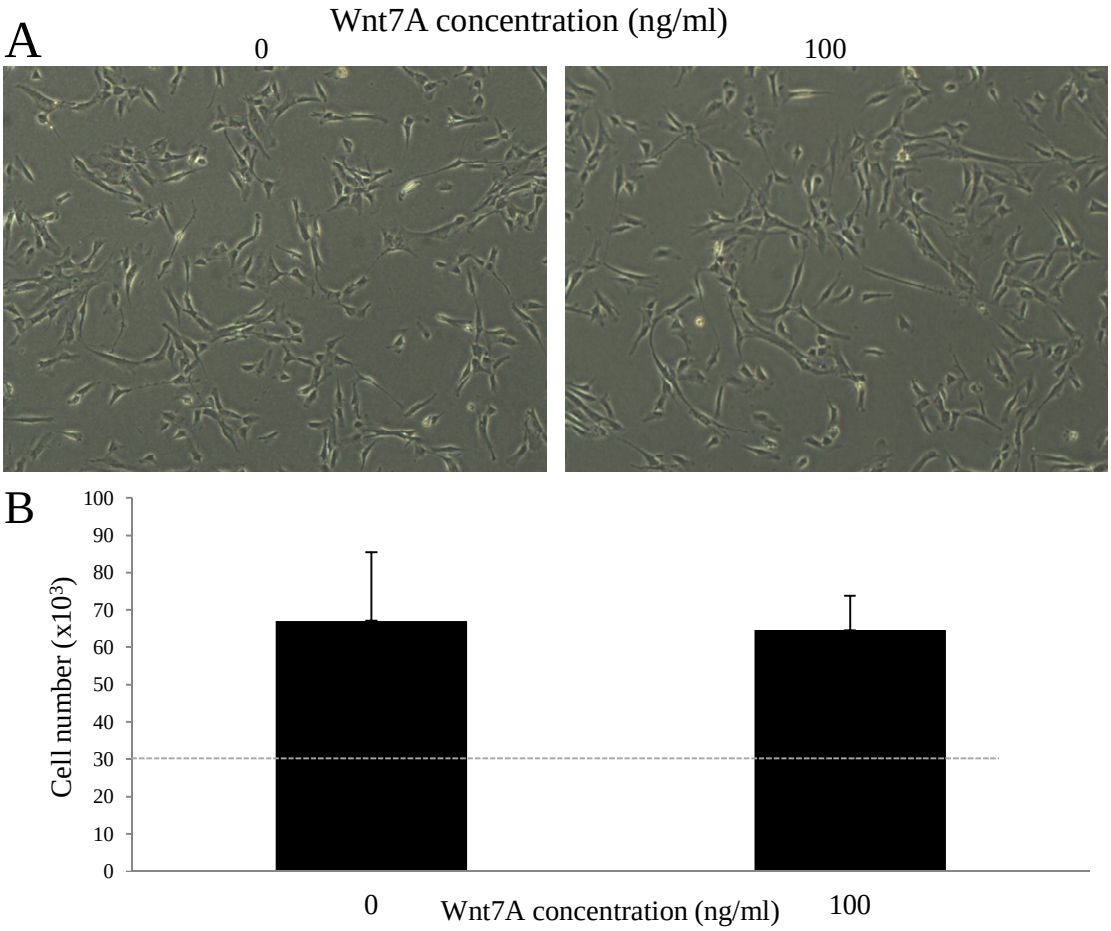

Figure 2.9 Effect of 100 ng/ml Wnt7A on HSkM myoblast proliferation. The proliferation of HSkM myoblasts in response to Wnt7A was evaluated using cell counts obtained by a Biorad TC-20 automated cell-counter. A total of $30x10^3$HSkM myoblasts were seeded and treated with Wnt7A at 0 ng/ml or 100 ng/ml for 48 hours. A) Phase contrast images of proliferating cells were taken 48 hours after treatment with Wnt7A. B) Cell numbers after 48 hours were calculated with the initial number of seeded cells represented by the dotted line. Data are expressed as mean + SEM; n=3. *$p<0.05$ (compared to 0 ng/ml).

2.4 Discussion

The Wnt family of secreted, signalling proteins are known to regulate embryonic patterning and tissue development. In addition to embryogenesis, the Wnt proteins are involved in adult tissue development. It is thought that Wnts help regulate the migration and proliferation of tissue progenitor cells during processes such as myogenesis. The rate at which muscle progenitor cells reach the site of injury and the degree to which they proliferate can greatly affect the success of healing. In this study, we attempted to define the *in vitro* effects of Wnt3A and Wnt7A on both the migration and proliferation of mouse and human skeletal muscle myoblasts.

The migration of myoblast cells was evaluated using an *in vitro* wound healing assay. Myoblasts were cultured until 80% confluent, wounded and treated with Wnt-containing growth medium. Phase contrast microscopy images were taken over a 7 hour period and the percentage wound closure calculated. The results of these treatments are summarized in Table 2.1.

Table 2.1 Effect of Wnt3A and Wnt7A on C2C12 migration (n=6, *p<0.05)

Wnt Concentration(ng/ml)	**Total Wound Closure (% closure)**		
Wnt3A	**3 hour treatment**	**5 hour treatment**	**7 hour treatment**
0	10.2	18.9	29.1
1	8.93*	20.0	30.2
10	10.8	22.7*	36.3*
100	11.5	21.3	35.4*
Wnt7A			
0	10.2	18.9	29.1
1	11.2	21.1	32.8
10	11.3	23.7*	34.9*
100	12.2	21.9	33.0*

Surprisingly, Wnt3A at 1 ng/ml had a slight but significant ($p<0.05$) inhibitory effect on C2C12 migration at the 3 hour time point.Wnt3A at 10 ng/ml and 100 ng/ml significantly increased the rate of C2C12 myoblast migration after 5-7 hours of treatment ($p<0.05$). It is possible that a cell's response to Wnt is dependent on both the concentration of the signalling molecule and the length of exposure. The increase in motility at the higher Wnt3A concentrations seems to contradict the study conducted by Tanaka *et al* (2011). Using an expression plasmid, Tanaka and his associates produced a line of Wnt3A over-expressing C2C12 myoblasts (Wnt3A-C2C12). These cells displayed reduced motility in a 2D wound assay (after 16 hours). Motility was equated to the number of cells that had moved into a 2D wound area during the assay. The

contradiction of results is not fully understood and may have a number of reasons. Firstly, Tanaka *et* al (2011) made use of a 16-hour 2D wound assay. Since the doubling time of myoblasts is regularly around 12-14 hours, wound closure due to cell proliferation may have occurred. A 7-hour 2D wound assay was utilized in our study in order to minimize undesired proliferation. The high flexibility of Wnt-Fz interactions may be a possible cause of the contradicting results. Different receptor profiles on the two populations of C2C12 cells would result in two different signalling pathways being activated. mRNA analysis of Wnt, Fz and LRP5/6 expression is required for a more complete overview of the signalling pathways.

In the study carried out by Tanaka *et al* (2011), Wnt3A-producing C2C12 cells were cultured in medium for 48 hours before wounding. Since Wnt production is heavily reliant on the presence of certain serum factors, it is conceivable that during the 48 hour incubation, some of these factors would have been depleted (Chakkalakal and Brack, 2012). Serum factors required for migration may have been among those depleted leading to the decrease in migration. It has been observed that a number of serum factors are able to bind to Fz and can affect the level of Wnt signalling (Chakkalakal and Brack, 2012). For example, the complement C1q, which is found in elevated levels in "aged" serum, has recently been shown to bind to Fz and encourages canonical Wnt signalling in satellite cells (Naito *et al.*, 2012). Therefore, serum factors could directly and indirectly affect Wnt signalling. The C2C12 myoblasts used by Tanaka *et al* (2011) over-expressed Wnt3A; it is possible that the prolonged exposure to Wnt3A altered the Fz receptor profile of the myoblasts or activated alternate signalling pathways. There is little support for this however as Mukai *et al*. (2010) found no evidence that extracellular Wnt levels influence Fz ubiquitylation–deubiquitylation in Hela cells. No publications could be found regarding adult skeletal myoblasts and their Fz receptor profiles in response to Wnt. Finally, the concentration of Wnt3A secreted by the C2C12 myoblasts was not stated in the study by Tanaka *et al* (2011). This again makes it difficult to directly compare these findings to our own. It is possible that Wnt3Aacts in a strictly dose-dependent manner, inhibiting migration at low concentrations and enhancing it at higher concentrations. This may explain the observed inhibition of migration by 1 ng/mlWnt3A (after 3 hours) and the increased migration caused by the 10 ng/ml and 100 ng/ml concentrations (at 5 and 7 hours post wounding). If the Wnt3A-C2C12 myoblasts produced relatively low levels of Wnt3A, this may explain the reduced migration. This seems unlikely however as Wnt producing cell lines typically produce larger amounts of protein. For example,

conditioned medium produced by the commonly usedLWnt3Afibroblast line (mouse-derived) typically contains between 100 ng/ml and 200 ng/ml of Wnt3A (Willert *et al.*, 2003).

Exposure to Wnt7A was seen to significantly increase the migration of C2C12 myoblasts at both 10 ng/ml and 100 ng/ml. Wnt7A has previously been shown to activate the planar cell polarity pathway (PCP) in satellite cells via the Fz7 receptor (Le Grand *et al.*, 2009). The PCP pathway results in cytoskeletal re-organization and the coordinated polarization of cells, which is required for migration (Cadigan and Nusse, 1997; Huelsken and Behrens, 2002; Cadigan *et al.*, 2005). It is conceivable that Wnt7A, signalling via the PCP pathway, results in cell polarization and cytoskeletal reorganization, thus increasing the migration of C2C12 myoblasts. In our research, we were unable to find any previous publications regarding Wnt7A and myoblast migration; this suggests that our findings may be novel.

The greatest effect on C2C12 migration was stimulated by 10 ng/mlWnt3A, 7 hours post injury. For this reason, the 10 ng/mlWnt3A concentration was chosen for the HSkM myoblast wound assay. Our data indicated that between the 3 and 7 hour time points, there was a significant increase in HSkM migration in response to Wnt3A. The calculated wound closures are summarized in Table 2.2.

Table 2.2 Effect of Wnt3A on HSkM migration

Wnt3A Concentration (ng/ml)	**Total Wound Closure (% closure, n=4, *p<0.05)**		
	3 hour treatment	**5 hour treatment**	**7 hour treatment**
0	31.5	52.9	71.6
10	40.5	61.1*	80.9*

By comparing the values in Tables 2.1 and 2.2, it is apparent that the HSkM myoblast wound closure values are substantially larger than those than those for the C2C12 cell line. The variance in wound closure is most likely due to the different donor species (mouse vs. human) as well as inherent differences between primary culture and an immortalized cell line. Despite this, it is obvious that Wnt3A increases the rate of wound closure for both cell types. This was suspected as Wnt proteins show a high level of interspecies relatedness. A BLAST alignment of mouse and human Wnt3A revealed a 97% sequence identity (Appendix II) and although recombinant mouse Wnt3A was used in this study, the high level of sequence identity suggests

that using human Wnt3A would likely yield the same results. Once again, our findings appear to be novel as no previous studies could be found regarding Wnt3A and HSkM migration.

Proliferation of C2C12 cells in response to Wnt protein was evaluated by culturing myoblasts in Wnt-containing medium for 24 hours, followed by analysis of cell numbers. The resulting cell counts revealed that neither Wnt3A nor Wnt7A had any significant effect on the proliferation of C2C12 myoblasts. This lack of an effect was unexpected as both Wnt3A and Wnt7A have been previously linked with the regulation of cell numbers. Wnt3A is known to increase the proliferation of rat bone marrow mesenchymal stem cells *in vitro* (Chang *et al.,* 2007). Wnt7A on the other hand has previously been shown to encourage the expansion of the satellite cell pool *in vivo* (Le Grand *et al.,* 2009, von Maltzahn *et al.,* 2012). In our study, C2C12 myoblasts cultured *in vitro* were treated with GM containing recombinant Wnt7A. It is possible that serum and extracellular matrix factors present *in vivo* cause Wnt7A to signal via a different pathway when compared to Wnt7A*in vitro*. A study by Bentzinger *et al.* (2013) found that it is Wnt7A in conjunction with fibronectin that regulates cell number. The Frizzled receptor Fz7 and the membrane bound Syndecan-4 protein were seen to form a co-receptor complex that binds to fibronectin and stimulate the ability of Wnt7A to expand the satellite cell pool (Bentzinger *et al.,* 2013). Since fibronectin was not present in our *in vitro* study, this may be why the *in vivo* and *in vitro* results differ. The relationship between fibronectin, Wnt7A and myoblast proliferation was only noted after completion of our experimental work. In future work we intend to include proliferation studies involving Wnt7A and myoblasts grown on ECM factors such as fibronectin.

To determine the effect of Wnts on human myoblast proliferation,Wnt7A was selected as it has previously been shown to be important in terms of satellite cell pool maintenance. HSkM myoblasts treated with 100 ng/ml recombinantWnt7A did not show any significant change in cell number when compared to the control. This result was not entirely unexpected as Wnt7A had no significant effect on C2C12 cell number. BLAST analysis of human and mouse Wnt7A revealed a 99% sequence identity (Appendix II).

In conclusion, it would seem that any results pertaining to Wnt signalling are highly dependent on conditions such concentration, serum factors, the cell's Fz profiles and potentially ECM factors. In our study, C2C12 and HSkM cells cultured in monolayer and treated with recombinant Wnt3A generally showed an increase in mobility. This was evident at 10 ng/ml and

100 ng/mlWnt3A. However, Wnt3A appeared to have no significant effect on the proliferation of either C2C12 or HSkM myoblasts. Although Wnt3A at higher doses was seen to increase the level of both C2C12 and HSkM migration, its use in regenerative therapy may be limited as injection of Wnt3A into injured mouse muscle has been shown to inhibit myogenesis (Brack *et al.,* 2008). This reduced healing however has been attributed to an inhibition of differentiation and it is therefore possible that at the right concentration, injected Wnt3A could increase the number myoblasts to reach the site of injury prior to differentiation.Wnt7A was also shown to increase migration at both 10 and 100 ng/ml. This result appears to be novel. Wnt7A however had no effect on proliferation. This was unexpected as Wnt7A has been shown to increase proliferation in the presence of fibronectin (Bentzinger *et al.,*2013). Future migration and proliferation work will include the culturing of myoblasts on various ECM factors in the present of Wnt protein. It is apparent that our study is one of the first to determine the dose dependent effects of Wnt3A and Wnt7A on myoblast migration and proliferation.

CHAPTER THREE
THE EFFECT OF WNT3A AND WNT7A ON THE DIFFERENTIATION OF MOUSE C2C12 AND HUMAN PRIMARY CULTURE MYOBLASTS

Activated skeletal muscle stem cells, known as myoblasts, facilitate the growth and repair of muscle tissue. These satellite cells are activated by injury to the muscle tissue after which they move to the site of damage and initiate repair. The degree to which these stem cells are able to differentiate and fuse to form myotubes has great bearing on the success of healing. The process of differentiation is heavily controlled by a group of helix-loop-helix transcription factors known as the myogenic regulatory factors (MRFs). This group includes MyoD, Myf5, myogenin and Mrf4. It is suspected that these myogenic regulatory factors, and indeed the entire process of myogenesis, are in part controlled by the activity of the Wnt family of secreted signalling proteins. The Wnt family is made up of 21 highly conserved, cysteine rich signalling molecules which are known to regulate tissue development. Classically, Wnt proteins have been linked with embryogenesis, but recently a number have been shown to play a role adult tissue development and maintenance. Wnt3A-expressing C2C12 myoblasts have been previously shown to display reduced differentiation. It is suspected that this inhibition is caused by an up-regulation of the inhibitor of differentiation 3 (Id3). No research publications were found regarding the effect of Wnt7A on myoblast differentiation; however Wnt7A has been heavily linked with maintenance of the satellite cell pool and could possible regulate the production of myotubes in a direct manner. In the current study we aimed to test the effect of known concentrations of recombinant Wnt3A and Wnt7A on the differentiation of mouse C2C12 and donor-derived human skeletal muscle (HSkM) myoblasts *in vitro*. Expression of the contractile muscle protein, myosin heavy chain (MyHC), was used to measure terminal differentiation. Only Wnt3A had a detrimental effect on C2C12 differentiation. Wnt7A appeared to have a minimal effect, possibly increasing differentiation slightly. Nuclear MyoD levels were analysed using confocal microscopy in order to determine the level of commitment to differentiation. Wnt3A (10 ng/ml and 100 ng/ml) was seen to reduce the percentage of MyoD positive C2C12 nuclei.

3.1 Introduction

Injury or trauma to skeletal muscle tissue results in the activation of resident, quiescent stem cells known as satellite cells (Beauchamp *et al.,* 2000; Charge and Rudnicki 2004; Sherwood *et al.,*2004). Activated satellite cells, referred to as myoblasts, migrate to the site of injury where they proliferate, differentiate, and finally fuse to form new fibers to repair damaged muscle (Le Grand and Rudnicki, 2007; Burdzinska *et al*. 2008). The terminal differentiation of these myoblasts is required for fusion so that repair of fibers can take place; it is therefore apparent that the success of healing post-injury is directly affected by the degree of differentiation. The differentiation of skeletal muscle myoblasts is a highly controlled, three step process which involves; a) the expression of muscle-specific regulatory factors, b) exit from the cell cycle and c) expression of contractile muscle proteins (Moran *et al.,*2002).

During the initial step of differentiation, there is an up-regulation of the four myogenic regulatory factors (MRFs). These muscle specific regulatory factors are MyoD, myogenin, Mrf4 and Myf5 (Moran *et al.,* 2002).The second step of differentiation is characterized by the irreversible withdrawal of myoblasts from the cell cycle (Moran *et al.,*2002; Joulia *et al,* 2003).Gene knockout experiments carried out in mice suggest that MyoD initiates myoblast lineage commitment by the up-regulation of myogenin and Mrf4 (Le Grand and Rudnicki, 2007, Ustanina *et al.,* 2007). Studies have also revealed a degree of functional redundancy between Myf5 and MyoD. Mice lacking a functional MyoD gene have no physical abnormalities, but express four-fold higher levels of Myf5 (Megeney *et al.,*1996). Similarly mice lacking a functional Myf5 gene show a four-fold increase in MyoD expression and no physical abnormalities (Megeney *et al.,*1996). Although primary involved with proliferation, it is suspected that Myf5 is able to compensate in part for the absence of MyoD. Despite this compensation, the rate of differentiation of MyoD knockout myoblasts is decreased. MyoD is therefore commonly used as a marker of myoblast commitment. Following up-regulation of MyoD and Myf5 expression, increased levels of myogenin and Mrf4 initiate the third step of differentiation, which involves terminal differentiation and fusion into myofibers (Charge and Rudnicki, 2004). During this third step, contractile motor proteins such as myosin are produced (Weiss and Leinwand, 1996; Maccatrozzo *et al.,*2007). Myosins consist of smaller subunits which include the quintessential muscle marker, myosin heavy chain (MyHC) (Maccatrozzo *et*

al.,2007). It is the myosin heavy chain (MyHC) subunits that give myosin its contractile and filament-forming abilities (Weiss and Leinwand, 1996; Quintin *et al.,* 2008). Terminally differentiated myoblasts fuse to form multi-nucleated myotubes (Weiss and Leinwand, 1996; Moran *et al.,* 2002; Maccatrozzo *et al.*,2007). It has recently been determined that the MRFs involved during myogenesis are regulated by the signalling of the Wnt family of secreted proteins (Brack *et al.*,2008; Bentzinger *et al.,* 2012).

Wnt signalling proteins are a family of highly, conserved, cysteine-rich and lipid-modified glycoproteins (George-Weinstein *et al.*,1998; Cadigan and Liu, 2006; Maiese *et al.,* 2008).Wnt signalling involves the binding of extracellular Wnt to the receptor Frizzled (Fz) and leads to the activation of Wnt target genes (Peifer and Polakis, 2000; Huelsken and Behrens, 2002; Janda *et al.,* 2012).It is thought that myoblast commitment and differentiation is initiated by the β-catenin dependent Wnt signalling pathway (Tanaka *et al,* 2011; von Maltzahn *et al.*,2012). In light of these findings, much attention had been placed on Wnt signalling during muscle growth and healing.

Wnt3A is one of the most widely studied Wnt proteins with a number of publications outlining its importance during embryogenesis. Wnt3A knockout studies carried out in mice have resulted in severely deformed embryos with highly impaired cellular patterning and polarization resulting in defects to the paraxial mesoderm, loss of the tailbud and incorrect vertebral patterning (van Amerongen and Berns, 2007). Wnt3A has also been observed to have an effect of adult tissue cells. *In vitro* studies suggest that Wnt3A-conditioned medium reduces the level on terminal differentiation of C2C12 myoblasts (Cathy *et al.*,2011; Zhang *et al.*,2012). It is suspected that Wnt3A, signalling via the β-catenin dependent pathway, up-regulates the expression of the inhibitor of differentiation 3 (Id3) and results in reduced differentiation (Cathy *et al.*,2011; Zhang *et al.*,2012). Inhibitors of differentiation are a family of regulatory proteins which contain a helix-loop-helix (HLH) domain but lack a basic domain (Liu *et al.*,2002; Pammer *et al.*,2004). These proteins form non-functional heterodimers with basic helix-loop-helix (bHLH) transcription factors such as MyoD and myogenin, preventing DNA binding and inhibiting differentiation (Liu *et al.*,2002). Over-expression of the Id proteins Id1, Id2 and Id3 have been shown to result in decreased differentiation in a number of cell types (Langlands *et al.,* 1997; Liu *et al.,* 2002). Reduced healing has been reported in injured mouse leg muscle tissue in

whichWnt3A is overexpressed (via the electroporation of a CMV-Wnt7A expression plasmid into mouse leg muscle) (von Maltzahn *et al.* 2012). However, the conclusion reached by von Malzahn *et al*(2012) was that Wnt3A promotes premature myoblast differentiation via canonical signalling, leading to a depletion of the satellite cell pool and impaired healing. It is also possible that the impaired healing was due to inhibited differentiation rather than premature differentiation as suggested by Zhang *et al.* (2012). Furthermore, excessWnt3A at the site of injury lead to an increased deposition of fibrous connective tissue (largely collagen), which is an unfavourable effect typically associated with the regeneration of 'aged' muscle (von Maltzahn *et al.*, 2012). It is apparent that further research into Wnt3A is required to fully understand its role during myogenesis and mechanism of action.

In vivo studies carried out in mice have shown that over-expression of Wnt7A enhances muscle regeneration, increasing both the number and proportion of satellite cells relative to activated myoblasts (Le Grand *et al.*,2009; von Maltzahn *et al.*,2012). Wnt7A has been shown to interact with the Fz7 receptor, activating the planar cell polarity pathway (PCP) and thereby control satellite cell number (Le Grand *et al.*,2009; Bentzinger *et al.*, 2012; von Maltzahn *et al.*,2012). In addition to maintaining the satellite cell pool, Wnt7A has been shown to increase myotube size and weight *in vivo*. A study in which of Wnt7A (10μg) was injected into injured mouse leg muscle resulted in thicker and heavier muscle (25% increased wet eight) with a 1.2 fold greater contractile force relative to untreated muscle (von Maltzahn *et al.*,2012). In addition, exposure to elevated levels of Wnt7A stimulates the expression of the muscle-specific transcription factor, myogenin, in marrow-derived multipotent adult stem cell populations (Belema Bedada *et al.*,2005). It is therefore conceivable that Wnt7A may increase the differentiation of skeletal muscle myoblasts via the up-regulation of myogenin.

In most published *in vitro* studies, cells have been exposed to Wnt-containing conditioned medium. Conditioned medium may contain varying concentrations of Wnt protein depending on the serum factors present, the Wnt type and the cell line producing the protein. For this reason it is often difficult to correlate observed effects to specific Wnt concentration. In addition, Wnt expressing cell lines will produce a number of non-Wnt proteins that may affect any response to the produced Wnt. For example, 3T3 fibroblasts are commonly used to produce Wnt protein. However, these cells will also secrete factors such as fibroblast growth factor (FGF) and ECM

factors such as fibronectin which could potentially alter Wnt signalling. In the current study we analyzed the effect of specific concentrations of recombinant Wnt3A and Wnt7A on the differentiation of both murine (C2C12) and human skeletal muscle myoblasts.

3.2 Materials and Methods

All chemicals used in the study were of an analytical grade. Cell culturing was performed under sterile conditions in a level II lamina flow hood (ESCO class II BSC). All cells were incubated in an Innova® CO-170 CO2-incubator (New Brunswick) at 37°C, 5% CO2.

3.2.1 Cell culture

Cells

Mouse C2C12, and donor-derived human skeletal muscle (HSkM) myoblasts were used in the study. C2C12 cells are an immortalized myoblast cell line capable of migration, proliferation, differentiation and fusion into myotubes. The C2C12 cells used in the study were kindly donated by the Cape Heart Centre, University of Cape Town. Passages ranged from P12 to P18. HSkM myoblasts were obtained from Lonza (cat.CC-2561, USA) and used at passage P3.

Culture media

C2C12 myoblasts were maintained in growth media (GM) consisting of Dulbecco's Modified Eagle's Medium (with 2% L-glutamine) (Sigma, cat.D5648-1L, USA) supplemented with 10% (v/v) fetal bovine serum (Biowest, cat.51810-500, USA), 3.7g/l sodium bicarbonate (Merck, cat.103025, USA) and 1% (v/v) Penicillin/Streptomycin (Lonza, cat.DE17-602E, RSA).

The HSkM cells were cultured in a 50/50 mixture of the C2C12 growth medium (GM) and HamsF10 nutrient broth (Sigma-Aldrich, cat.N6908, USA). This 50/50 media mix was also supplemented with 2.5 ng/ml human fibroblast growth factor (hFGF) (Sigma-Aldrich, cat.F0291, USA) with the fetal bovine serum concentration adjusted to 20%.

Thawing and culturing of myoblasts

Both C2C12 and HSkM cells were received as frozen stocks stored in cryopreservation tubes. When required, cells were thawed by gentle heating in a 37°C water bath for 1 minute. The semi-frozen cell suspension was then transferred into a T75 tissue culture flask (Nest Biotech, cat.0926B) containing 10 ml of the required medium (GM for C2C12 cells, 50/50 mix for HSkM cells). Exhausted culture medium was replaced with fresh medium every 48 hours during cell proliferation within the T75 flasks. Cells proliferating in the T75 culture flasks were passaged before reaching 80% confluence in order to prevent any undesired differentiation. Cells were

passaged by removing the medium, washing with phosphate buffered saline solution (PBS) and incubation with 2 ml trypsin (Cambrex, cat.17-161E) at 37°C for 3 minutes. After 3 minutes, the trypsin activity was neutralized by adding an equal volume of growth medium. The passaged cells were then divided into additional T75 flasks for future use or plated for experimental use.

3.2.2 Recombinant Wnt proteins

Recombinant murine Wnt3A (Peprotech, cat.315-20) and recombinant human Wnt7A (R and D Systems, cat.3008-WN/CF) were re-suspended in sterile PBS at a concentration of 100 µg/ml. At the start of our experimental work, these were the available species of recombinant Wnt3A and Wnt7A. These solutions were stored at -20°C. For both the migration and proliferation experiments, C2C12 myoblasts were treated with culture medium containing Wnt at a concentration of 0 ng/ml, 1 ng/ml, 10 ng/ml or 100 ng/ml. Due to the low proliferation rate of the HSkM cells, only the Wnt type and dose which showed the greatest effect on the C2C12 cell line was tested in human cells.

3.2.3Analysis of MyoD positive nuclei

Sterile glass coverslips were placed into the wells of a plastic 24-well culture plate (TPP, cat. Z707791, USA). A total of 30 000 C2C12 or HSkM myoblasts in 500 µl growth medium (GM) were plated onto each coverslip. Myoblasts were cultured to 80% confluence before the GM was removed and replaced with Wnt-containing differentiation medium (DM). C2C12 myoblasts were cultured in differentiation medium (DM) containing either Wnt3A or Wnt7A at a concentration of 0 ng/ml, 1 ng/ml, 10 ng/ml or 100 ng/ml. HSkM myoblasts can only be used until passage 4, after which time they senesce. Due to this limitation, only the Wnt treatment which showed the greatest effect on C2C12 myoblasts was used. Confocal microscopy was used to determine the percentage of MyoD positive nuclei after 24 or 72 hours incubation with Wnt. After incubation with Wnt, the myoblasts were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 minutes. Following fixing, the cells were washed with PBS and blocked using 5% donkey serum for 1 hour. The myoblasts were then incubated with a rabbit polyclonal anti-MyoD primary antibody (1/100, Santa Cruz, cat. sc-760, USA) for 2 hours at room temperature. Coverslips were then washed with PBS (4 x 5 minutes washes) and incubated with a fluorophore-linked donkey anti-rabbit IgY secondary antibody (1/1000, Abcam, cat.ab96919, UK) for 1 hour at room temperature. The myoblasts were then washed with PBS (2

x 5 minutes) and treated with 10 µg/ml Hoechst 33342 nuclear stain (Sigma-Aldrich; USA) for 5 minutes. The cells were again washed with PBS (3 x 5 minutes) and mounted on glass slides using moviol. Labelled myoblasts were viewed using a Zeiss LSM 710 confocal microscope. For each Wnt concentration, a total of 5 fields of view containing at least 20 cells were analysed. This was repeated for each experiment (n = 4). The percentage MyoD positive nuclei were calculated using the formula: 100 x (MyoD positive nuclei/total nuclei).

3.2.4 Analysis of morphology

The morphology of differentiating myoblasts treated with Wnt protein was analysed using both phase contrast and confocal microscopy. C2C12 and HSkM myoblasts were cultured to 80% confluence on glass coverslips in 24-well plates and subsequently treated with Wnt-containing differentiation medium for a total of 5 days. Exhausted differentiation medium was removed after 72 hours and fresh Wnt-containing medium added. Phase contrast images were taken using an Olympus CKX41 microscope fitted with a Moticam 2300 3.0 megapixel camera (40x magnification). After 5 days of differentiation, the myoblasts were fixed using 4% paraformaldehyde (20 minutes), washed with PBS (2 x 5 minutes) and blocked using 5% donkey serum (1 hour).The myoblasts' actin cytoskeletal structures were then labelled by treating with phalloidin (fluorophore linked) for 1 hour at room temperature. The cells were then washed with PBS (2 x 5 minutes), treated with 10 µg/ml Hoechst 33342 nuclear stain (5 minutes). The coverslips underwent a final PBS wash (2 x 5 minutes) and were mounted on glass slides using moviol. The labelled myoblasts were viewed using a Zeiss LSM 710 confocal microscope.

3.2.5 Analysis of MyHC expression

SDS-PAGE

Protein lysates were prepared according to Appendix IV. SDS-PAGE was carried out following the Laemmli protocol (Laemmli, 1970) and is outlined in Appendix IV. All gels were run in a bench-top electrophoresis unit (Biorad: Mini-PROTEIN 3 Cell, USA) and consisted of a 4.5% stacking and 12.5% running gels. A total of 20 µg protein lysate was typically added to each of the wells. Before loading, lysates were mixed with an equal volume of reducing sample treatment buffer as well as 1µl of bromophenol blue (Saarchem, cat.1437500CB, RSA) and boiled for 90 seconds. The PeqGold protein marker V (Peqlab, cat27-2210, Germany) was used

to determine protein band size. Once run, gels were either prepared for Western blotting or stained with the Coomassie G-250.

Western blotting

Western blotting was carried out according to the protocol outlined in Appendix V. SDS-PAGE proteins were transferred to nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences, UK) using a standard bench-top Western blotting apparatus (Omni Page, Cleaver Scientific Ltd, UK). Blots were transferred at 100 mA for 16 hours at 4 °C. Ponceau S. staining was used to determine protein transferral to the nitrocellulose membrane. Membranes were blocked using 5% milk powder and treated with primary and HRPO-linked secondary antibodies as outlined in Appendix V. Antibody dilutions are outlined in Appendix III.

3.2.5 Statistical analysis

All data are represented as Mean ± standard error of the means (SEM) for each data set shown. The paired Student's *t*-test was performed for parametric data and paired Mann-Whitney *U* test for non-parametric data. All statistical tests were performed using the Genstat 12th edition

3.3 Results

3.3.1 Wnt3A inhibits terminal differentiation of C2C12 myoblasts

C2C12 myoblasts were cultured on glass coverslips in 6-well plates until 80% confluence was reached. GM was then discarded, the cells washed with PBS and DM containing Wnt3A (0 ng/ml, 1 ng/ml, 10 ng/ml or 100 ng/ml) added. The myoblasts were differentiated for a total of 5 days with media (including Wnt3A) being replaced every 72 hours. Although neither the half-life of Wnt3A or Wnt7A are published, these proteins are highly stable due to their glycosylated nature (Sola and Griebenow, 2010). Replacement of Wnts was therefore synchronised with media changes. Phase contrast microscopy revealed that an increase in Wnt3A concentration resulted in a decrease in the number of myotubes formed (Figure 3.1A). Myotube formation was also visualized by labelling the actin cytoskeleton with Phalliodin. Fewer myotubes expressing actin were seen in the Wnt3A treated monolayers (Figure 3.1B). The total expression of myosin heavy chain (MyHC) was also evaluated in response to Wnt3A (Figure 3.1C). Wnt3A decreased MyHC expression in a dose-dependent manner. Relative to the control, the 1 ng/ml, 10 ng/ml and 100 ng/mlWnt3A treatments resulted in a 39%, 88% and 94% decrease in MyHC levels respectively (Figure 3.1D).

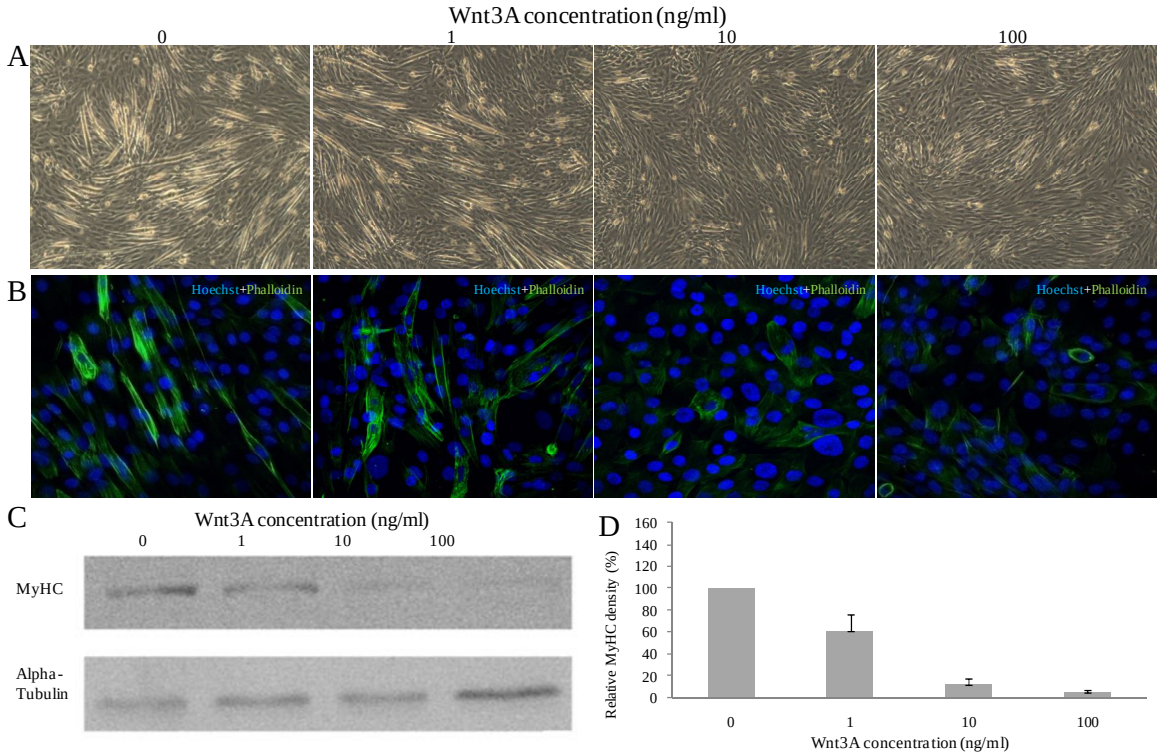

Figure 3.1 Differentiation of C2C12 myoblasts in response to Wnt3A. A) Phase contrast images of differentiating myoblasts were taken after 5 days incubation with Wnt3A at 0 ng/ml, 1 ng/ml, 10 ng/ml or 100 ng/ml. B) The actin cytoskeletal structure was labelled with phalloidin (green) and the myonuclei with Hoechst (blue). C) Western blots to determine MyHC expression were carried out on lysates prepared from day 5 differentiated myoblasts. Alpha-tubulin was used as the internal loading control. D) Densitometry was performed on the blots using the *ImageJ*, image analysis software and MyHC levels relative to alpha-tubulin and the untreated control calculated, Data are expressed as mean + SEM; n=3. *p<0.05 (compared to 0 ng/ml).

3.3.2 Wnt3A reduces the percentage MyoD positive nuclei in C2C12 myoblasts

C2C12 myoblasts were then labelled for MyoD to help establish the mechanism of Wnt3A-inhibited differentiation. The myoblasts were cultured to 80% confluence on glass coverslips and subsequently differentiated for 24 or 72 hours in medium containing Wnt3A at0 ng/ml, 1 ng/ml, 10 ng/ml or 100 ng/ml. The myoblasts were then fixed with 4% paraformaldehyde, blocked with 5% donkey serum and incubated with anti-MyoD primary antibody. A secondary antibody excited at 488nm was used to visualize primary bound anti-MyoD antibody and nuclei stained with Hoechst. Images were taken using a Zeiss LSM 710 confocal microscope (Figure 3.2A). The percent of MyoD positive nuclei was then calculated for each treatment (Figure 3.2B).After 24 hours, both 10 ng/ml and 100 ng/mlWnt3A had significantly ($p<0.05$, compared to 0 ng/ml) decreased the percentage MyoD positive nuclei from 5.6% (control) to 3.5%. Although not found to be significant, Wnt3A also appeared to decrease the percent of MyoD positive nuclei after 72 hours (Figure 3.2B). While the control displayed a total of 14% MyoD positive nuclei, 1 ng/ml, 10 ng/ml and 100 ng/mlWnt3A treatments resulted in 6%, 5.4% and 4.8% MyoD positive nuclei respectively at 72 hours.

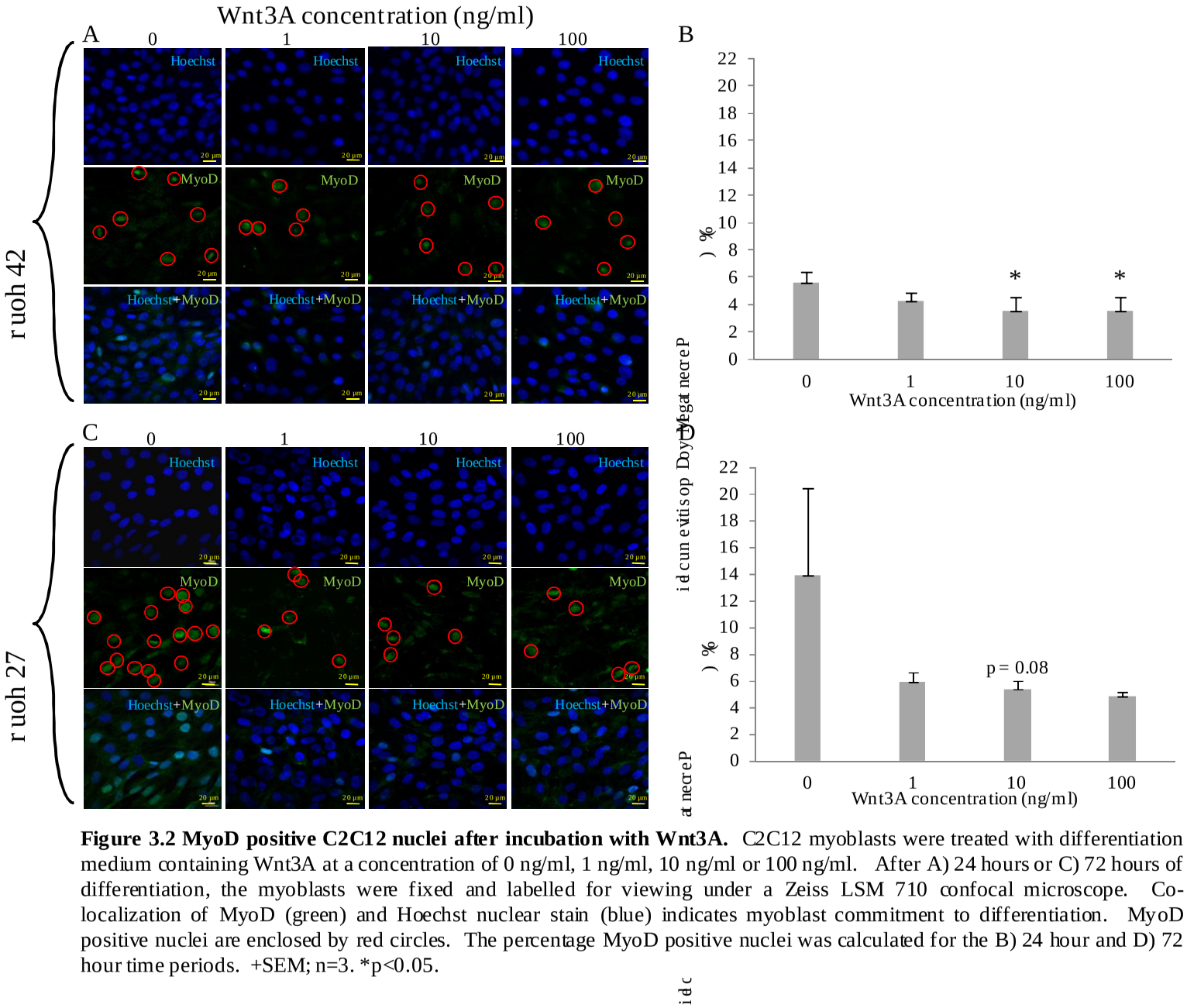

Figure 3.2 MyoD positive C2C12 nuclei after incubation with Wnt3A. C2C12 myoblasts were treated with differentiation medium containing Wnt3A at a concentration of 0 ng/ml, 1 ng/ml, 10 ng/ml or 100 ng/ml. After A) 24 hours or C) 72 hours of differentiation, the myoblasts were fixed and labelled for viewing under a Zeiss LSM 710 confocal microscope. Co-localization of MyoD (green) and Hoechst nuclear stain (blue) indicates myoblast commitment to differentiation. MyoD positive nuclei are enclosed by red circles. The percentage MyoD positive nuclei was calculated for the B) 24 hour and D) 72 hour time periods. +SEM; n=3. *p<0.05.

3.3.3 Wnt7A does not significantly affect C2C12 myoblast differentiation

C2C12 myoblasts were differentiated in DM containing Wnt7A (0 ng/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml) over a 5 day period. Phase contrast images of myoblasts revealed no overt difference in myotube formation between the treated and untreated cells (Figure 3.3A). Furthermore, actin labelling revealed little or no change in cytoskeletal structure in response to Wnt7A (Figure 3.3B). Expression of MyHC remained relatively constant in response to Wnt7A when compared to control (Figure 3.3C and Figure 3.3D).

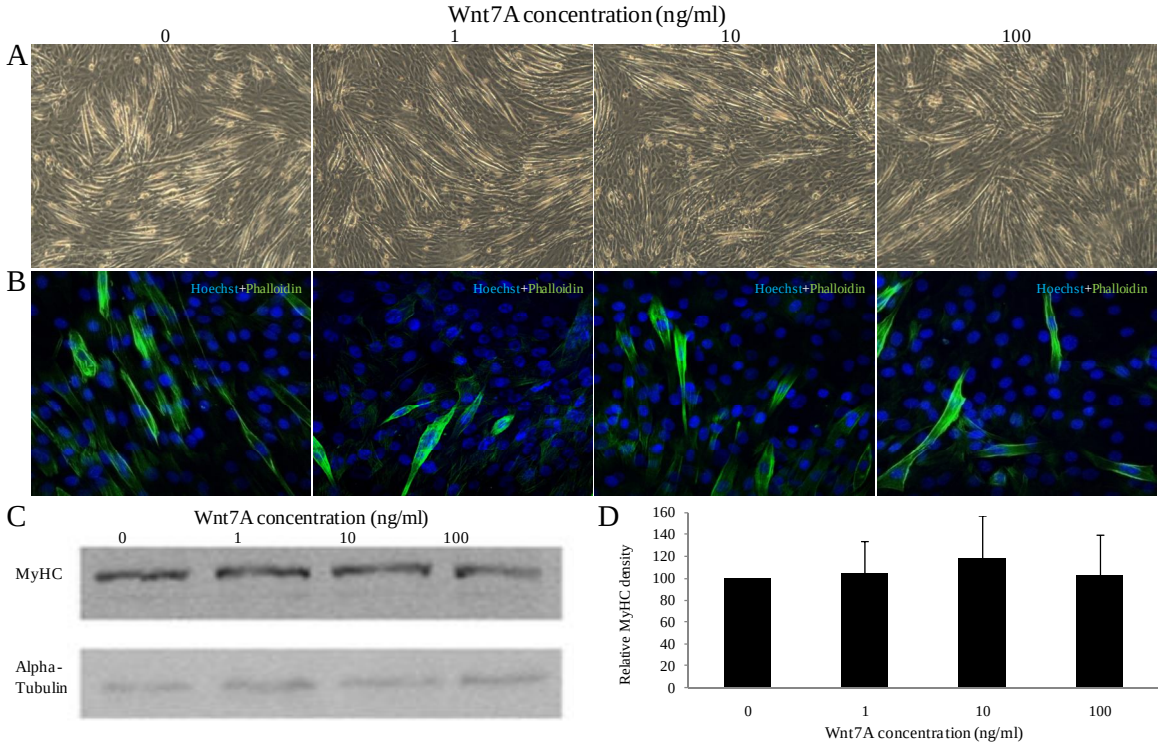

Figure 3.3 Differentiation of C2C12 myoblasts in response to Wnt7A. A) Phase contrast images of differentiating myoblasts were taken after 5 days incubation with Wnt7A at 0 ng/ml, 1 ng/ml, 10 ng/ml or 100 ng/ml. B) The actin cytoskeletal structure was labelled with phalloidin (green) and the myonuclei with Hoechst (blue). C) Western blots to determine MyHC expression were carried out on lysates prepared from day 5 differentiated myoblasts. Alpha-tubulin was used as the internal loading control. D) Densitometry was performed on the blots using the *ImageJ*, image analysis software and MyHC levels relative to alpha-tubulin and the untreated control calculated, Data are expressed as mean + SEM; n=3. *p<0.05 (compared to 0 ng/ml).

3.3.4 Wnt7A reduces the percentage MyoD positive nuclei in C2C12 myoblasts

After 24 hours, C2C12 myoblasts treated with 1 ng/mlWnt7A showed a significant ($p<0.05$) decrease in the number of MyoD positive nuclei, dropping from 6% (control) to 4% positive nuclei (Figure 3.4B). Neither the 10 nor 100 ng/mlWnt7A treatments appeared to significantly decrease nuclear MyoD expression (Figure 3.4B).After 72 hours of differentiation, 100 ng/ml Wnt7A lowered the percentage of MyoD positive C2C12 nuclei from 14% (control) to 5.65% ($p<0.05$, compared to 0 ng/ml). Neither the 1 ng/ml nor 10 ng/ml Wnt7A concentrations significantly decreased the percent of MyoD positive nuclei (Figure 3.4D).

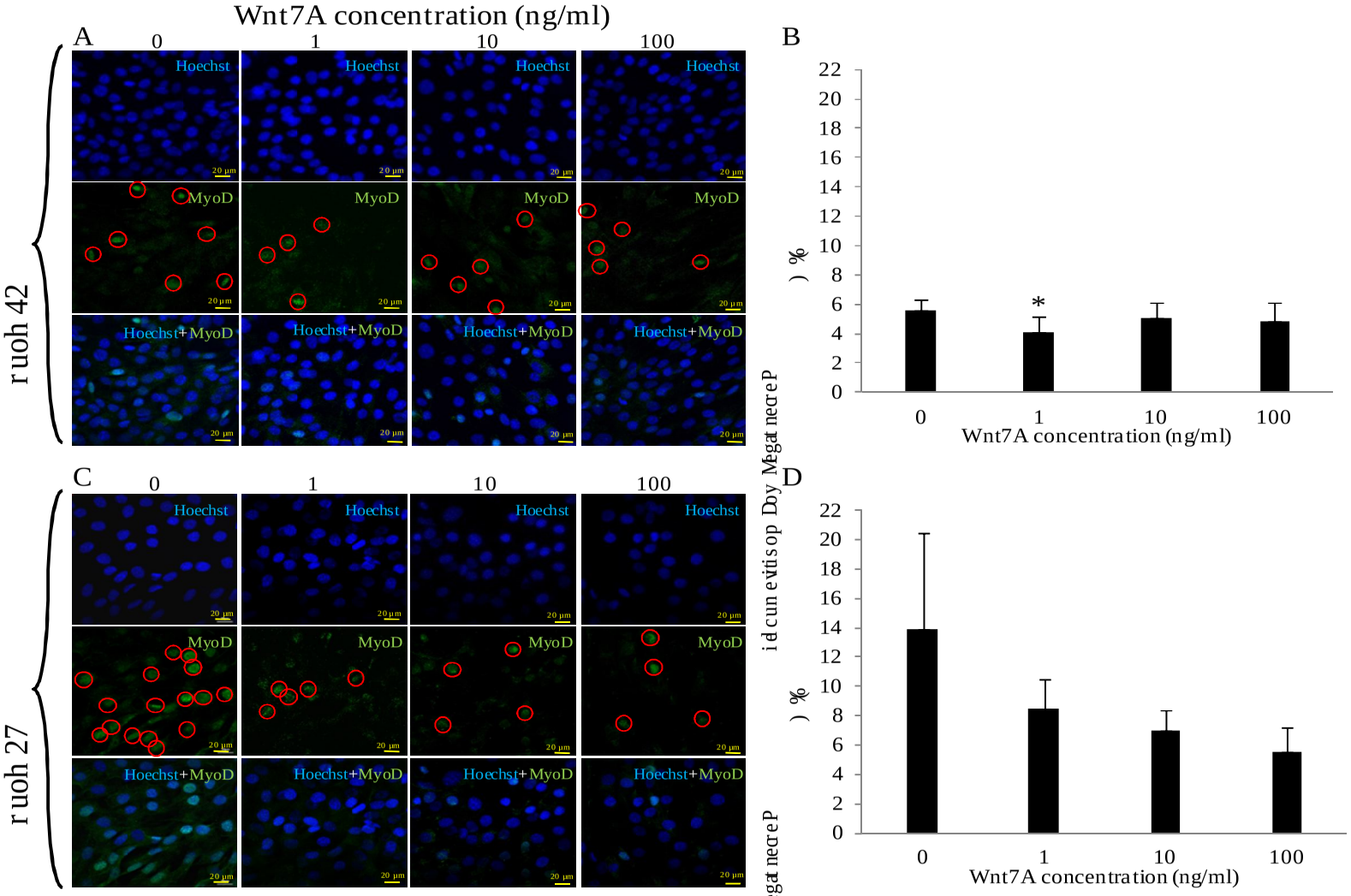

Figure 3.4 MyoD positive C2C12 nuclei after incubation with Wnt7A. C2C12 myoblasts were treated with differentiation medium containing Wnt7A at a concentration of 0 ng/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml. After A) 24 hours or C) 72 hours of differentiation the myoblasts were fixed and labelled for viewing under a Zeiss LSM 710 confocal microscope. Co-localization of MyoD (green) and Hoechst nuclear stain (blue) indicates myoblast commitment to differentiation. MyoD positive nuclei are enclosed by red circles. The percentage MyoD positive nuclei was calculated for the B) 24 hour and D) 72 hour time points. +SEM. *$p<0.05$.

3.3.5 Wnt3A does not significantly decrease terminal differentiation of HSkM myoblasts

To determine whether Wnt3A has a similar effect on donor-derived human skeletal myoblasts, HSkM cells were treated with differentiation medium containing Wnt3A (10 ng/ml) for 5 days. Media was replaced after 72 hours of differentiation. Phase contrast microscopy and analysis of the actin cytoskeleton revealed little difference in myotube formation between Wnt-treated myoblasts and the control (Figure 3.5a and Figure 3.5B). Western blot analysis of MyHC revealed an 18% drop in expression relative to control; however, this result was not seen to be statistically significant (Figure 3.5D).

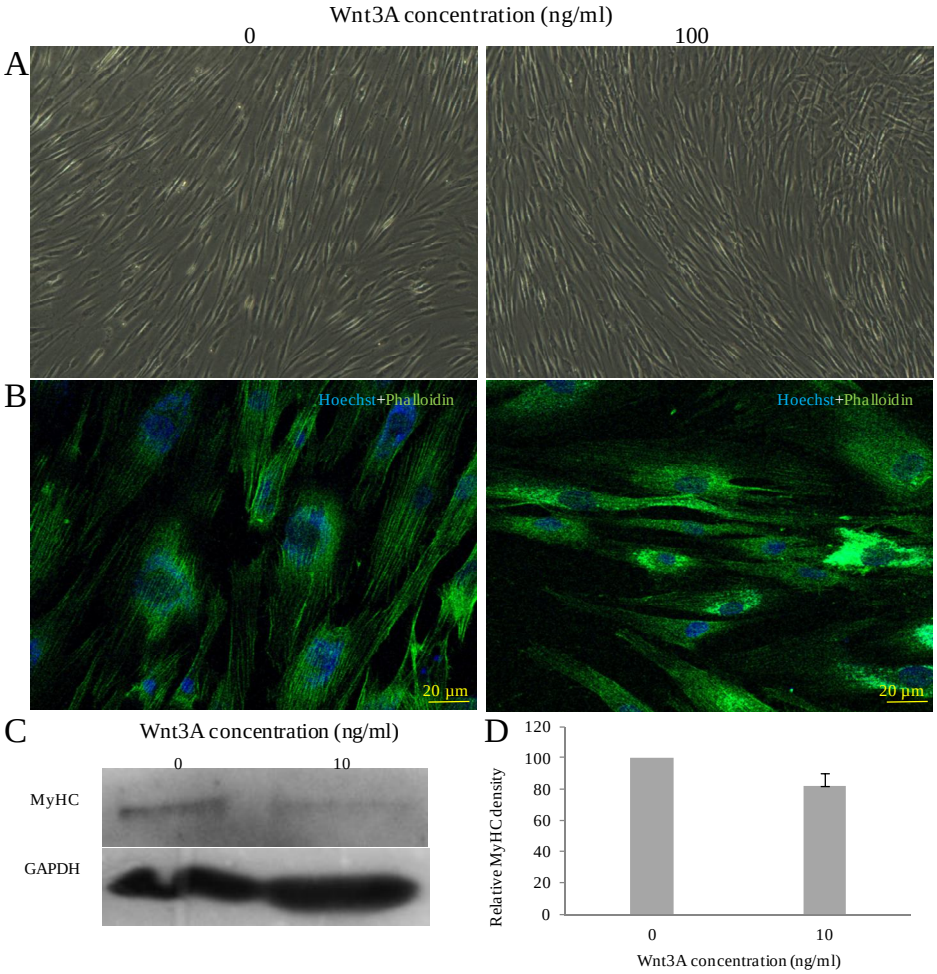

Figure 3.5 Differentiation of HSkM myoblasts in response to 10 ng/ml Wnt3A. A) Phase contrast images of differentiating myoblasts were taken after 5 days incubation with Wnt3A at 0 ng/ml or 10 ng/ml. B) The actin cytoskeletal structure was labelled with phalloidin (green) and the myonuclei with Hoechst (blue). C) Western blots to determine MyHC expression were carried out on lysates prepared from day 5 differentiated myoblasts. GAPDH was used as the internal loading control. D) Densitometry was performed on the blots using the *ImageJ*, image analysis software and MyHC levels relative to GAPDH and the untreated control calculated, Data are expressed as mean +SEM; n=3. *$p<0.05$ (compared to 0 ng/ml).

3.3.6 Wnt3A does not significantly decrease the percent of MyoD positive HSkM nuclei

The greatest effect on C2C12 nuclear MyoD was caused by Wnt3A at 10 ng/ml and 100 ng/ml. Both these concentrations appeared to have similar effects and caused a greater decrease in the percent of MyoD positive cells when compared Wnt7A. HSkM myoblasts were therefore differentiated in medium containing 0 ng/ml or 10 ng/mlWnt3A for 24 or 72 hours and MyoD positive nuclei counted. Although10 ng/mlWnt3A reduced the percentage of MyoD positive nuclei from 9.1% (control) to 4.8% (10 ng/ml; Figure 3.6B) after 24 hours, this result was not found to be significant. Similarly, Wnt3A had no significant effect on nuclear MyoD levels after 72 hours of differentiation (Figure 3.6D). After 72 hours of differentiation the control myoblasts displayed 17.2% MyoD positive nuclei while the Wnt3A-treated cells had 15.8% MyoD positive nuclei (Figure 3.6D).

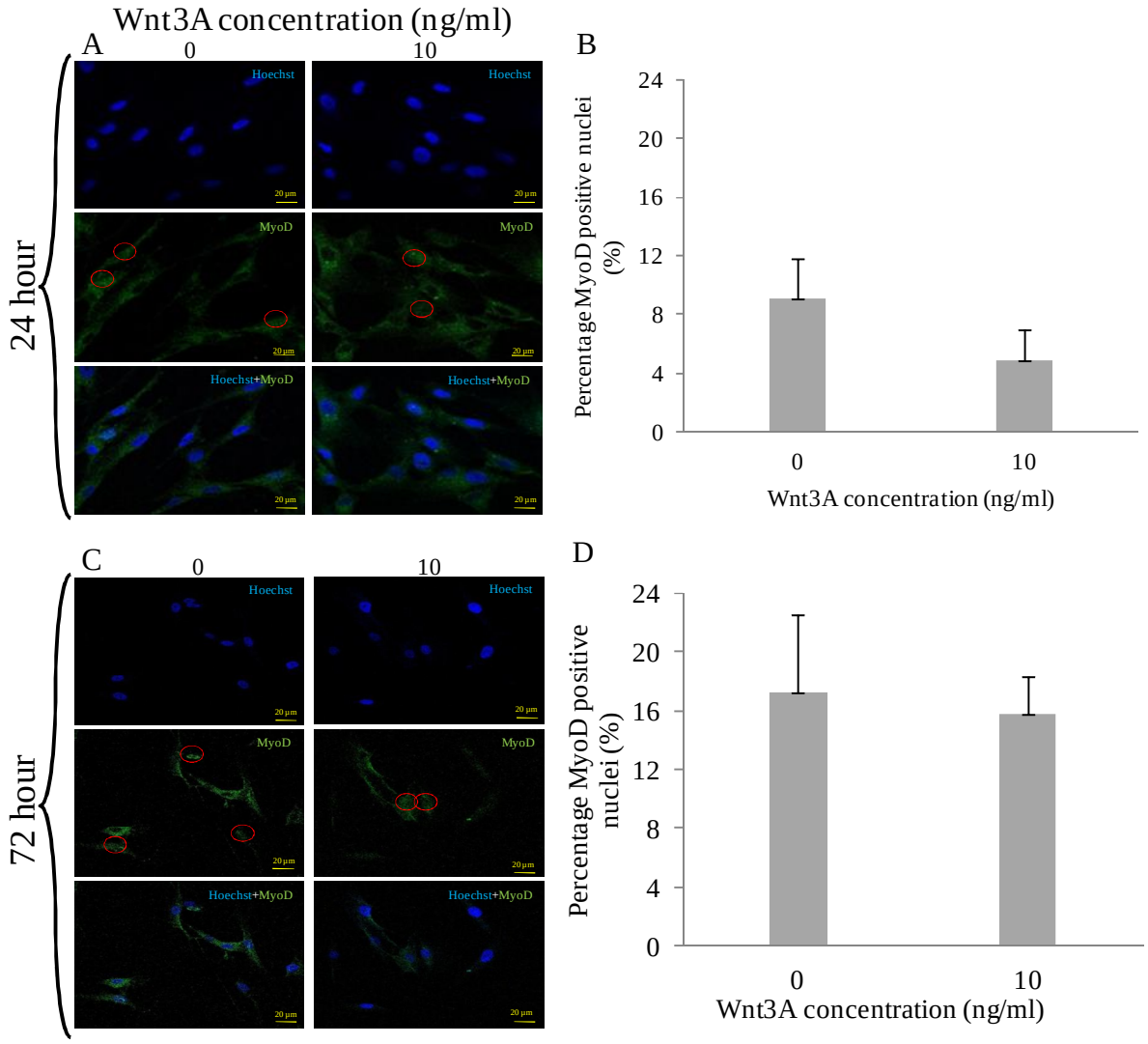

Figure 3.6 MyoD positive HSkM nuclei after incubation with Wnt3A. HSkM myoblasts were treated with differentiation medium containing Wnt3A at 0 ng/ml or 10 ng/ml. After A) 24 hours or C) 72 hours of differentiation, the myoblasts were fixed and labelled for viewing under a Zeiss LSM 710 confocal microscope. Co-localization of MyoD (green) and the Hoechst nuclear stain (blue) indicates myoblast commitment to differentiation. MyoD positive nuclei are enclosed by red circles. The percent of MyoD positive nuclei was calculated for the B) 24 hour and D) 72 hour time points. Data are expressed as mean + SEM; n=3. *p<0.05 (compared to 0 ng/ml).

3.4 Discussion

The successful growth and repair of muscle tissue relies heavily on the effective differentiation and fusion of myoblast cells. A number of signalling molecules and factors regulate this process and include the myogenic regulatory factor MyoD (Moran *et al.*, 2002; Holterman and Rudnicki 2005). MyoD is a basic helix-loop-helix transcription factor which is up-regulated during the initial stages of differentiation and accumulates in the nucleus, initiating the expression of essential muscle proteins. One such protein is the MyHC subunit which makes up part of the contractile myosin protein. In our study we wished to determine the effect of Wnt3A and Wnt7A on the differentiation of mouse C2C12 and human donor-derived myoblasts. Expression of nuclear MyoD was used to verify the onset of differentiation while MyHC Western blots were used to determine the degree of differentiation.

Terminal differentiation of myoblasts was determined using phase contrast microscopy (myotube alignment and formation), confocal microscopy (expression of actin during differentiation) and Western blotting (MyHC expression). C2C12 myoblasts were differentiated in Wnt-containing DM for a total of 5 days. By examination of phase contrast images, (Figure 3.1A and Figure 3.3B), it was apparent that Wnt3A decreased the number of myotubes produced. With each increasing concentration of Wnt3A, there was a further decrease in myotube formation. Wnt7Ahowever appeared to have no effect on myotube formation (Figure 3.3A). These results were supported by confocal microscopy images of the actin cytoskeletal structure of the myoblasts (Figure 3.1B and 3.3B). Myoblasts treated with Wnt3A were seen to have less cytoskeletal actin. Wnt7A also appeared to have no effect on actin formation. Western blots determined that the MyHC levels of cells treated with Wnt3A decreased with each increasing concentration of Wnt (Figure 3.5D). This trend was not seen in the Wnt7A treated cells (Figure 3.6D). MyHC levels are summarized in Table 3.1 and are displayed as a percentage of the control (normalized).

Table 3.1 Relative MyHC levels of Wnt treated C2C12 cells

Wnt concentration (ng/ml)	Relative MyHC levels (normalized to alpha-tubulin and untreated control; n=3, *p<0.05)
Wnt3A	
0	100
1	61
10	12
100	6
Wnt7A	
0	100
1	104
10	118
100	102

Nuclear MyoD in differentiating C2C12 myoblasts was analysed to try determine a mechanism of effect of Wnt signalling. C2C12 myoblasts were differentiated in Wnt-containing medium for 24 or 72-hours and viewed using a LSM confocal microscope to determine the percentage MyoD positive nuclei. A summary of these results can be seen in Table 3.2.

Table 3.2 Percent of MyoD positive C2C12 nuclei in response to Wnt3A or Wnt7A

Wnt concentration (ng/ml)	Percent MyoD positive nuclei (% positive, n=3, *p<0.05)	
Wnt3A	**24-hour**	**72-hour**
0	5.6	13.9
1	4.2	5.9
10	3.6*	5.4
100	3.6*	4.8
Wnt7A		
0	5.6	13.9
1	4.1*	8.5
10	5.1	7.0
100	4.8	5.6

Our results suggest that both Wnt3A and Wnt7A decrease the percentage MyoD positive nuclei during the early stages of C2C12 differentiation with this effect being most pronounced in the Wnt3A-treated cells. The reduction in response to Wnt7A was unexpected as a reduction in the percent of MyoD positive nuclei would logically be accompanied by inhibited differentiation. However, only Wnt3a was seen to inhibit this process.

Wnt3A was seen therefore to reduce both the percentage MyoD positive nuclei and the degree of terminal differentiation of C2C12 myoblasts. It is likely that Wnt3A signalling down-regulates nuclear MyoD expression which in turn decreases differentiation. AlthoughWnt7A (1 ng/ml) was also seen to reduce the percent of MyoD positive nuclei, the effect was not translated into a decrease in terminal differentiation. A possible explanation for this could be the functional redundancy between MyoD and Myf5. Myf5 is able to compensate in part for the absence of MyoD. This has been observed in mice lacking a functional MyoD gene which produce four-fold higher levels of Myf5, but have no physical abnormalities (Megeney *et al.*,1996). Similarly, mice lacking a functional MyoD gene displayed four-fold higher levels of Myf5. Elimination of both the MyoD and Myf5 genes resulted in severe abnormalities and no myoblasts being formed (Ustanina *et al.*, 2007). It is possible that Wnt3A inhibits the expression of both MyoD and Myf5, thus inhibiting differentiation. Wnt7A on the other hand may inhibit MyoD and not Myf5 and therefore had no effect on differentiation. In our research, Myf5 Western blots were run, but were unsatisfactory. It is suspected that weak, non-specific binding of the antibody resulted in poor blots. In future work we intend to use mRNA analysis to determine Myf5 expression levels. No other publications have been found that investigate the effect of Wnt3A or Wnt7A on MyoD or Myf5 expression during myoblast differentiation.

Inhibitors of differentiation (Id1, Id2 and Id3) form heterodimers with bHLH transcription factors such as MyoD, Myf5 and Myogenin, preventing DNA binding and myoblast differentiation (Liu *et al.*,2002; Pammer *et al.*,2004). It has previously been reported that Wnt3A inhibits the differentiation of C2C12 myoblasts by the up-regulation of the inhibitor of differentiation 3 (Id3) (Cathy *et al.*,2011; Zhang *et al.*,2012). Id3 is known to form heterodimers with MyoD which prevents the expression of muscle specific proteins such as myogenin and MyHC (Liu *et al.*,2002). It is likely that Wnt3A inhibits the production of MyoD and up-regulates the expression of Id3 in C2C12 myoblasts, thus inhibiting differentiation. Wnt7A has not been shown to up-regulate Id3 and may simply have an inhibitory effect on MyoD as demonstrated in our results.

During the course of this study we noticed the relatively low percentage of MyoD positive nuclei in the control C2C12 nuclei when compared to other published works. No contamination of the cells was observed; however it is possible that during cell-stock preparation, C2C12 myoblasts

were cultured post-confluence (above 90% confluence). Culturing these cells post-confluence is known to reduce their myogenic capacity. This may have resulted in the lower levels of nuclear MyoD. Being the only available batch of C2C12 myoblasts at the time, these cells were used throughout the MyoD testing. Despite these slightly lower levels of MyoD, the myoblasts were still able to differentiate, albeit with a lower efficiency.

Wnt3A was seen to have the greatest effect on C2C12 differentiation, with 10 ng/ml and 100 ng/ml concentrations reducing differentiation to similar levels. In the interest of preserving Wnt and optimising the usage of HSkM myoblasts, these human derived cells were treated with 10 ng/mlWnt3A. Although Wnt3A appeared to reduce MyoD positive HSkM nuclei after 24 hours of differentiation (Table 3.3), this effect was not significant. Furthermore, the effect was lost after 72 hours (Table 3.3). It was noted that the HSkM cells did not adhere to the glass coverslips as strongly as the C2C12 myoblasts. This accounts for the lower number of cells visible in the confocal images as more cells had been washed away during the preparation steps.

Table 3.3 Percent MyoD positive HSkM nuclei in response to Wnt3A

Wnt3A concentration (ng/ml)	**Percent MyoD positive nuclei (% positive, n=3)**	
	24-hour	**72-hour**
0	9.1	17.2
10	4.8	15.8

Interestingly after 24 hours, the percent of MyoD positive HSkM nuclei had dropped 46% when compared to control (C2C12 cells displayed a 24% drop after the same treatment). After 72 hours however, the percent of MyoD positive HSkM nuclei had dropped by only 8% in response to Wnt3A. This result suggested that HSkM differentiation may not be as severely affected as that for C2C12 myoblasts which saw a 61% drop in MyoD positive nuclei relative to the control. These C2C12 cells displayed severely impaired differentiation with an 88% drop in MyHC levels. The Wnt3A-treated HSkM cells only displayed an18% drop in MyHC levels after 5 days of differentiation. In addition, both phase contrast microscopy and confocal microscopy revealed little morphological change in the HSkM cells. Wnt3A was expected to have a greater effect on HSkM differentiation judging by the severe inhibition of C2C12 myotube formation, and the fact that human and mouse Wnt3A share ≈98% identity. It is possible that the difference in response

is due to varying Fz profiles between the cell types or the fact that only one Wnt3A concentration was used. Future HSkM work would need to include a wider range of Wnt concentrations.

In summary, Wnt3A but not Wnt7A caused a severe decrease in C2C12 myoblast differentiation. This could partly be explained by lowered expression of MyoD, however other factors such as Id3 and Myf5 are likely to play a part. Human myoblasts did not respond as overtly to Wnt3A as did the mouse C2C12 myoblasts. This could be as a result of Fz receptor profiles or a dose-dependent effect.

CHAPTER FOUR
DISCUSSION

Skeletal muscle myoblasts are the quintessential muscle stem cells and are responsible for the growth and repair of muscle tissue. The success of muscle growth and repair is determined largely by a) the velocity at which the myoblasts reach the site of injury, b) the rate at which the myoblasts proliferate within the wound area and c) the degree to which the cells differentiate and fuse to form/repair the muscle tissue. It has recently been established that the Wnt family of secreted proteins play an important role in tissue development. In our study we wished to evaluate the effects of recombinantWnt3A and Wnt7A on the migration, proliferation and differentiation of murine C2C12 and human donor-derived skeletal muscle myoblasts *in vitro*. The main findings of our research are summarized in Tables 4.1 and 4.2. All values are expressed as a decrease or increase relative to untreated control.

Table 4.1 The effects of Wnt3A and Wnt7A on the migration, proliferation and differentiation of C2C12 myoblasts (relative to control, *p<0.05)

Wnt concentration (ng/ml)	Wound closure			Proliferation	MyoD positive nuclei		MyHC levels
	3 hour	5 hour	7 hour		24 hour	72 hour	
Wnt3A							
1	↓12.5%*	↑5.8%,	↑3.8 %	↑1.4%	↓24.0%	↓41.9%	↓39.2%
10	↑5.9%	↑20.1%*	↑24.8%*	↓7.1%	↓39.2%*	↓47.3%	↓88.3%
100	↑12.7%,	↑12.7%	↑21.6%*	↓1.4%	↓38.0%*	↓48.5%	↓94.3%
Wnt7A							
1	↑9.8%	↑11.6%	↑12.7%	↑2.6%	↓28.5%*	↓25.5%	↑4.1%
10	↑10.8%	↑25.4%*	↑19.9%*	↓14.3%	↓9.4%	↓34.9%	↑18.4%
100	↑19.6%	↑15.9%	↑13.4%*	↓10.2%	↓15.0%	↓53.8%	↑1.7%

Table 4.2 The effects of Wnt3A and Wnt7A on the migration, proliferation and differentiation of HSkM myoblasts (relative to control, *p<0.05)

Wnt concentration (ng/ml)	Wound closure			Proliferation	MyoD positive nuclei		MyHC levels
	3 hour	5 hour	7 hour	48 hour	24 hour	72 hour	
Wnt3A							
10	↑28.3%	↑15.6%*	↑12.9 %*	-	↓32.2%	↓8.5%	↓18%
Wnt7A							
100	-	-	-	↓3.7%	-	-	-

By examination of the C2C12 wound closure values, it is apparent that Wnt3A increased the rate of myoblast migration. Confusingly, the only decrease in migration was seen at the 3 hour time point for the 1 ng/ml concentration. By 5 hours however, the rate of wound closure had increased beyond that of the control. It is possible that the cells react to Wnt3A in a dose dependent manner and the initial low concentration of Wnt3A causes a slight delay in myoblast migration. Of all the Wnt3A concentrations, the 10 ng/ml treatment had the greatest pro-migratory effect followed by 100 ng/ml. Our findings were seen to contradict those of Tanaka *et al.* (2011) who showed the migration of Wnt3A-expressing C2C12 myoblasts to be reduced. In our study, C2C12 myoblasts were treated with Wnt protein after 'wounding' and were given 7 hours to migrate. We wished to simulate an up-regulation of Wnt in response to an injury rather than having an over-expressing cell line that would constantly be exposed to the signalling protein. Constant exposure to Wnt could quite possibly result in the myoblasts changing their Fz receptor profile as has been shown in HELA cells (Mukai *et al.*,2010). HELA cells are able to recycle Fz in response to extracellular Wnt levels and thus limit the rate of Wnt binding and signalling. Furthermore, the concentration at which Wnt3A was secreted by Tanaka's myoblasts (Tanaka *et al.*,2011) was not stipulated and further complicates direct comparison with our findings.Wnt3A (10 ng/ml) was also seen to increase the migration of HSkM cells. When compared to the C2C12 results, it would seem that the HSkM cells responded quicker to Wnt3A with a larger 3 hour closure value. On the other hand, Wnt3A seemed to have an increasingly pro-migratory effect on the C2C12 cells over the 7 hour period. This result appears to be novel as no publications could be found involving Wnt and HSkM migration.

In addition to Wnt3A, Wnt7A was seen to increase the migration of C2C12 cells. Wnt7A concentrations tested (10 ng/ml and 100 ng/ml) significantly increased the percent wound closure at the 5 and 7 hour time points. As with Wnt3A, the 10 ng/mlWnt7A concentration was seen to have the greatest effect on migration. Once again, these findings appear to be novel. This result may be of particular interest as Wnt7A is already being studied as a potential treatment for Duchene's muscular dystrophy as it has been found to expand the satellite cell pool *in vivo* (Le Grand *et al.*,2009, von Maltzahn *et al.*,2012).

Neither Wnt3A nor Wnt7A were seen to have any significant effect on the proliferation of C2C12 or HSkM myoblasts. Although not significant, the most noticeable effects were caused

by 10 ng/ml and 100 ng/mlWnt7A treatments which caused a 14.3% and 10.2% decrease in C2C12 cell number relative to control respectively. Wnt7A at 100 ng/ml was also seen to decrease the proliferation of HSkM myoblasts (3.7% decrease).These proliferation findings were unexpected as Wnt7A has been linked with the maintenance of a healthy satellite cell population. Studies carried out by Bentzinger *et al.* (2013) and von Maltzahn *et al.*,(2012) have shown Wnt7A to increase satellite cell number *in vivo.* It has been suggested by Bentzinger *et al.* (2013) that it is in fact Wnt7A in conjunction with fibronectin that regulates cell number. The absence of fibronectin in our i*n vitro* study may account for the differing results.

In terms of differentiation, both Wnt3A and Wnt7A were seen to decrease the percentage of MyoD positive C2C12 nuclei. Wnt3A and Wnt7A concentrations (1 ng/ml, 10 ng/ml and 100 ng/ml) reduced this percentage after 24 and 72 hours of treatment, however, only Wnt3A was observed to inhibit differentiation. Differentiation was measured using Western blots sensitive for the muscle specific contractile protein MyHC. Wnt3A at 1 ng/ml, 10 ng/ml and 100 ng/ml was seen to drastically reduce C2C12 differentiation resulting in a 39%, 88% and 94% reduction in MyHC levels relative to control respectively (after 5 days of differentiation). This was expected as previous studies have shown Wnt3A to reduce C2C12 myoblast differentiation via the up-regulation of the inhibitor of differentiation 3 (Id3) (Cathy *et al.*,2011; Zhang *et al.*,2012). Id3 is known to form heterodimers with MyoD, inhibiting differentiation (Cathy *et al.*,2011; Zhang *et al.*,2012).Wnt3A (10 ng/ml) also decreased the percent of MyoD positive HSkM nuclei. This reduction however was not as drastic as that seen for the C2C12 cells. HSkM myoblasts treated with 10 ng/ml Wnt3A did not display significantly altered myotube formation (using Western blots and phase contrast microscopy).

Despite reducing the percentage of MyoD positive C2C12 nuclei, Wnt7A did not decrease terminal differentiation. In fact, Wnt7A may encourage terminal differentiation with the 10 ng/ml treatment increasing MyHC levels by 18% relative to control. These increases were minimal however and were not seen to be significant ($p<0.05$). It is suspected that the functional redundancy between MyoD and Myf5 may be a reason for the relatively unchanged MyHC levels in the Wnt7A treated cells, despite the decrease in nuclear MyoD. In the absence of MyoD, levels of Myf5 in primary culture murine myoblasts have been shown to increase 5-fold, leaving differentiation relatively unaffected (Megeney *et al.*,1996). It is possible that Wnt7A

affects MyoD while having no effect of Myf5 and thus allows for successful differentiation. Wnt3A on the other hand may inhibit the expression of Myf5 and up-regulate Id3 resulting in impaired differentiation. It is also possible that Wnt3A has no effect on Myf5 at all and inhibition is attributed to Id3. Myf5 Western blots were unsuccessful and so the levels of the MRF cannot be commented on. In future work we wish to evaluate levels of Myf5 using mRNA analysis. The effect of Wnt7A on Id proteins has not been documented and it is possible that this Wnt has no effect on Id expression and thus no inhibitory effect.

Wnt signalling is extremely complex with the rate and type of Wnt signalling being highly dependent on a number of factors. These factors include the type and concentration of Wnt, Fz and co-receptor profile as well as ECM and serum factors. Our research and indeed most *in vitro* experimentation cannot always accurately mimic the *in vivo* environment. Although our *in vitro* findings should not be overlooked, future work should include factors to mimic the *in vivo* environment more closely and hopefully understand the role of the Wnt proteins in myogenesis more fully. Testing could potentially include incubating of myoblasts with Wnt while cultured on different ECM factors in order to test Wnt-ECM interactions. In this way we can explore the therapeutic potential of these versatile proteins.

APPENDIX I: CONFERENCE ATTENDANCE

A1.1 University of KwaZulu-Natal Postgraduate Research Day 2012, Faculty of Science & Agriculture

EFFECT OF WNT3A AND WNT7A ON MYOBLAST MIGRATION AND DIFFERENTIATION

Rhys McColl

206507208@stu.ukzn.ac.za

School of Life Sciences

Supervised by Dr. Carola Niesler

Skeletal muscle myoblasts are responsible for the maintenance, growth and repair of muscle fibers. Injury to muscle tissue stimulates the migration of myoblasts to the wound area followed by proliferation, differentiation and fusion with the damaged myofibers. Wnt secreted proteins are a family of highly conserved, cysteine-rich, lipid-modified, glycoproteins that are found in all multi-cellular animals. They are typically associated with regulating embryogenesis, cell migration and more recently, myogenesis. Wnt3A has previously been shown to increasethe migration of Chinese hamster ovary cells (CHO) [1] and to inhibit the differentiation of C2C12 cells [2]. Wnt7A is able to stimulate the expression of myogenin in marrow-derived multipotent adult stem cell populations [3] while little is known in terms of its effect on myoblasts. In this study, the effects of Wnt3A and Wnt7A on the migration and differentiation of both murine C2C12 myoblasts and human primary-culture myoblasts were evaluated. Migration was analysed using an *in vitro* wound healing assay whereas differentiation was assessed via Myosin Heavy Chain (MyHC) expression. In C2C12 myoblasts, migration was significantly increased following incubation with Wnt3A (10 and 100 ng/ml) or Wnt7A (10 ng/ml). Differentiation of these myoblasts was observedmorphologically to decrease in response to Wnt3A, but not Wnt7A (specifically MyHC expression). Levels of MyHC at day 5 of differentiation decreased progressively in C2C12s treated with increasing concentrations of Wnt3A (1-100 ng/ml). Wnt7A did not decrease MHC expression irrespective of the dose tested. The effects of these Wnt proteins on the human skeletal muscle myoblasts are currently being evaluated.

References

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[2] Zhang, L., Shi, S., Zhang, J., Zhou, F., ten Dijke, P. (2012) Wnt/β-catenin signaling changes C2C12 myoblast proliferation and differentiation by inducing Id3 expression., *Biochemical and Biophysical Research Communications419*, 83-88.

[3] Bedada, F. B., Technau, A., Ebelt, H., Schulze, M., and Braun, T. (2005) Activation of Myogenic Differentiation Pathways in Adult Bone Marrow-Derived Stem Cells, *Molecular and Cellular Biology25*, 9509-9519.

APPENDIX II: BASIC LOCAL ALIGNMENT SEARCH TOOL

All protein alignments were carried out using the NCBI Basic Local Alignment Search Tool (BLAST).

A2.1 Alignment of human and mouse Wnt3A

Query: Mouse Wnt3A – Protein databank accession number: P27467.1

Subject: Human Wnt3A – Protein databank accession number: NP_149122

Score	Expect	Method	Identities	Positives	Gaps
709 bits(1831)	0.0	Compositional matrix adjust.	338/352(96%)	344/352(97%)	0/352(0%)

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Query  1    MAPLGYLLVLCSLKQALGSYPIWWSLAVGPQYSSLSTQPILCASIPGLVPKQLRFCRNYV  60
            MAPLGY L+LCSLKQALGSYPIWWSLAVGPQYSSL +QPILCASIPGLVPKQLRFCRNYV
Sbjct  1    MAPLGYFLLLCSLKQALGSYPIWWSLAVGPQYSSLGSQPILCASIPGLVPKQLRFCRNYV  60

Query  61   EIMPSVAEGVKAGIQECQHQFRGRRWNCTTVSNSLAIFGPVLDKATRESAFVHAIASAGV  120
            EIMPSVAEG+K GIQECQHQFRGRRWNCTTV +SLAIFGPVLDKATRESAFVHAIASAGV
Sbjct  61   EIMPSVAEGIKIGIQECQHQFRGRRWNCTTVHDSLAIFGPVLDKATRESAFVHAIASAGV  120

Query  121  AFAVTRSCAEGSAAICGCSSRLQGSPGEGWKWGGCSEDIEFGGMVSREFADARENRPDAR  180
            AFAVTRSCAEG+AAICGCSSR QGSPG+GWKWGGCSEDIEFGGMVSREFADARENRPDAR
Sbjct  121  AFAVTRSCAEGTAAICGCSSRHQGSPGKGWKWGGCSEDIEFGGMVSREFADARENRPDAR  180

Query  181  SAMNRHNNEAGRQAIASHMHLKCKCHGLSGSCEVKTCWWSQPDFRTIGDFLKDKYDSASE  240
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Sbjct  301  SHGIDGCDLLCCGRGHNARAERRREKCRCVFHWCCYVSCQECTRVYDVHTCK  352
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A2.2 Alignment of human and mouse Wnt7A

Query: Mouse Wnt7A – Protein databank accession number: NP_033553.2

Subject: Human Wnt7A – Protein databank accession number: NP_004616.2

Score	Expect	Method	Identities	Positives	Gaps
725 bits(1872)	0.0	Compositional matrix adjust.	347/349(99%)	348/349(99%)	0/349(0%)

```
Query  1    MNRKARRCLGHLFLSLGMVYLRIGGFSSVVALGASIICNKIPGLAPRQRAICQSRPDAII  60
            M RKARRCLGHLFLSLG+VYLRIGGFSSVVALGASIICNKIPGLAPRQRAICQSRPDAII
Sbjct  1    MTRKARRCLGHLFLSLGIVYLRIGGFSSVVALGASIICNKIPGLAPRQRAICQSRPDAII  60

Query  61   VIGEGSQMGLDECQFQFRNGRWNCSALGERTVFGKELKVGSREAAFTYAIIAAGVAHAIT  120
            VIGEGSQMGLDECQFQFRNGRWNCSALGERTVFGKELKVGSREAAFTYAIIAAGVAHAIT
Sbjct  61   VIGEGSQMGLDECQFQFRNGRWNCSALGERTVFGKELKVGSREAAFTYAIIAAGVAHAIT  120

Query  121  AACTQGNLSDCGCDKEKQGQYHRDEGWKWGGCSADIRYGIGFAKVFVDAREIKQNARTLM  180
            AACTQGNLSDCGCDKEKQGQYHRDEGWKWGGCSADIRYGIGFAKVFVDAREIKQNARTLM
Sbjct  121  AACTQGNLSDCGCDKEKQGQYHRDEGWKWGGCSADIRYGIGFAKVFVDAREIKQNARTLM  180

Query  181  NLHNNEAGRKILEENMKLECKCHGVSGSCTTKTCWTTLPQFRELGYVLKDKYNEAVHVEP  240
            NLHNNEAGRKILEENMKLECKCHGVSGSCTTKTCWTTLPQFRELGYVLKDKYNEAVHVEP
Sbjct  181  NLHNNEAGRKILEENMKLECKCHGVSGSCTTKTCWTTLPQFRELGYVLKDKYNEAVHVEP  240

Query  241  VRASRNKRPTFLKIKKPLSYRKPMDTDLVYIEKSPNYCEEDPVTGSVGTQGRACNKTAPQ  300
            VRASRNKRPTFLKIKKPLSYRKPMDTDLVYIEKSPNYCEEDPVTGSVGTQGRACNKTAPQ
Sbjct  241  VRASRNKRPTFLKIKKPLSYRKPMDTDLVYIEKSPNYCEEDPVTGSVGTQGRACNKTAPQ  300

Query  301  ASGCDLMCCGRGYNTHQYARVWQCNCKFHWCCYVKCNTCSERTEMYTCK  349
            ASGCDLMCCGRGYNTHQYARVWQCNCKFHWCCYVKCNTCSERTEMYTCK
Sbjct  301  ASGCDLMCCGRGYNTHQYARVWQCNCKFHWCCYVKCNTCSERTEMYTCK  349
```

APPENDIX III: ANTIBODY DILUTIONS

A3.1 Confocal microscopy and Western blot antibody dilutions

Table A3.1 Antibody dilutions

Technique	Primary antibody dilution	Secondary antibody dilution
Confocal Microscopy	Mouse monoclonal anti-Vinculin (SIGMA, cat.129K4849, USA) 1/1000	Donkey anti-mouse Dylight 488 (Jackson Immunoresearch, cat.94650, USA) 1/1000
	Goat polyclonal anti-Rock-2 (Santa Cruz, cat.J2209) 1/500	Donkey anti-goat Cy5 596nm (Jackson Immunoresearch, cat.81817, USA) 1/4000
	Rabbit polyclonal anti-MyoD (Santa Cruz, cat.sc-760, USA) 1/100	Donkey anti-rabbit Dylight 488 (Abcam, cat.ab96919, UK) 1/1000
Western blot	Mouse monoclonal anti-MyHC (DSHB MF-20, USA) 1/200	Donkey anti-mouse IgG (Abcam, cat.ab96857, UK) 1/4000
	Rabbit polyclonal anti-GAPDH (Cell Signalling, cat.21185, USA) 1/4000	Donkey anti-rabbit IgG (Abcam,cat.ab96919, UK) 1/4000
	Mouse monoclonal anti-α-tubulin (Santa Cruz, cat.sc-5286, USA) 1/400	Donkey anti-mouse IgG (Abcam, cat.ab96857, UK) 1/4000

APPENDIX IV: PROTEIN LYSATE PREPARATION AND SDS-PAGE

A4.1 Lysate preparation

Cells cultured in 6-well plates were detached by treatment with 1 ml trypsin (5 minutes) and transferred to 15ml falcon tubes. The activity of trypsin was neutralized by the addition of 1ml GM to each falcon tube. The tubes were then spun in a bench-top centrifuge (MRC; Polychem Supplies) at 100g x 5 minutes. The supernatant was discarded and the pellet re-suspended in 1 ml PBS. The cells were then spun again at 100g x 5 minutes. This PBS re-suspension and centrifugation was repeated two further times. After the final centrifugation step, the pellets were re-suspended in 45µl RIPA buffer (Sigma, cat.R0278) containing 1µl protease inhibitor cocktail (Sigma, cat.P8340) and placed on ice for 1 hour. The cells were then sonicated at setting 13 (VirSonic60, Polychem supplies) and separated into 15µl and stored at -20°C in 15 µl aliquots.

A4.2 SDS-PAGE

A4.2.1 Bradford assay

A total of 1µl lysate sample was added to 99µl of PBS and 900µl Bradford reagent (SIGMA, cat.B6916, USA). The solutions were mixed thoroughly, given 2 minutes to develop and then transferred into plastic cuvettes (Optima Scientific, cat.2711110). Each concentration was set in triplicate. Absorbance was measured at 595nm (Ultrospec II E, LKB Biochrom) and the protein concentrations calculated using a standard calibration curve (Figure A5.1). The standard curve was developed using bovine serum albumin (Roche, cat.10735086001).

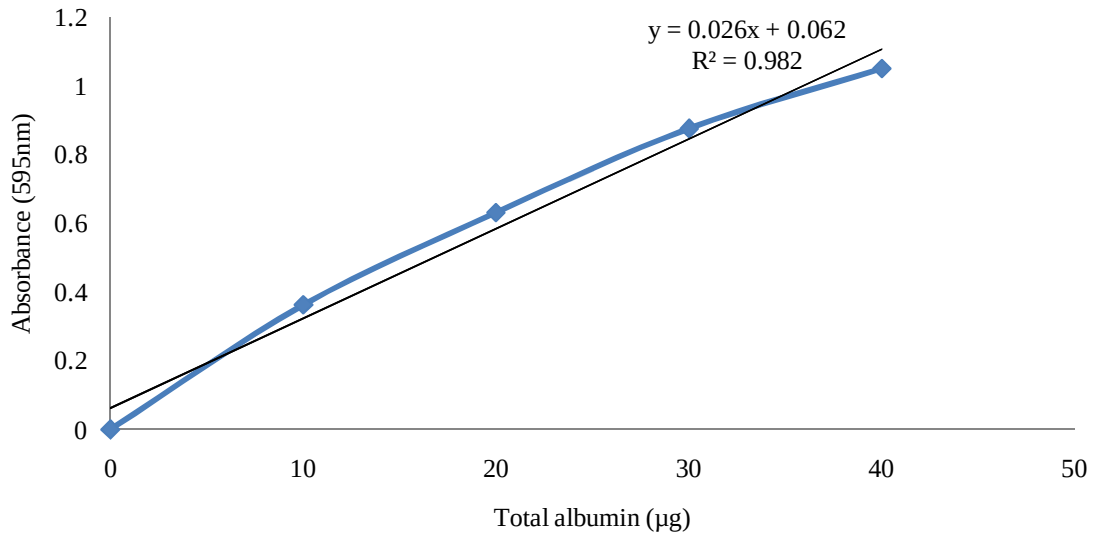

Figure A4.1 Bradford protein standard curve. Bovine serum albumin was diluted to a concentration of 1mg/ml using PBS. A range of bovine serum protein (0-40µg) was added to 900µl of Bradford reagent and made up to 1 ml using PBS. Solutions were vortexed and allowed to develop for 5 minutes. Absorbance readings were taken in at 595nm.

A4.2.2 SDS-PAGE reagents

Monomer solution (30% (m/v) acrylamide, 2.7% (m/v) Bis-acrylamide): Acrylamide (73 g) and Bis-acrylamide (2 g) were made up to 250ml in distilled water, filtered though Whatmans number 2 filter paper and stored in an amber glass bottle at 4°C.

4 x Running gel buffer (1.5M Tris-HCL, pH 8.8): Tris (45.37g) was dissolved in 200ml distilled water, titrated to pH 8.8 with HCL and made up to 250ml. Filtered through Whatmans number 2 filter paper and stored in a plastic bottle at 4°C.

4 x Stacking gel buffer (500 mM Tris-HCL, pH 6.8): Tris (3g) was dissolved in 50ml distilled water and titrated to pH 6.8 with HCL. The buffer was stored in a plastic bottle at 4°C.

SDS stock solution (10% m/v): SDS (5g) was dissolved in 50ml distilled water and filtered through Whatman's No.1 filter paper and stored in a glass bottle at room temperature.

Ammonium persulfate initiator (10% m/v): Ammonium persulfate (0.1g) was dissolved in 1ml distilled water. Initiator was prepared fresh before use and stored at room temperature for no longer than 2-3 hours.

Reducing sample treatment buffer (125mM Tris-HCl, pH 6.8): 4ml of 10% SDS stock solution, 2.5ml of 4x stacking gel buffer and 1ml of 2-mercaptoethanol were mixed together, titrated to pH 6.8 and made up to 10ml with distilled water. The treatment buffer was stored in a plastic container at room temperature.

Tank buffer(250mM Tris-HCl, pH 8.3): Tris (3g), glycine (14.4g) and 10ml of 10% SDS stock solution were mixed into 900ml of distilled water, titrated to pH 8.3 and made up to 1000ml.

All gels consisted of a 4% (m/v) polyacrylamide stacking gel and 12.5% (m/v) polyacrylamide according to Laemmeli's protocol (Laemmli, 1970). Volumes of the various components in each gel are given in Table A4.3.

Table A4.1 Stacking and running gel composition

Reagents	Stacking gel (4%)	Running gel (12.5%)
Monomer Solution	0.94ml	6.25ml
4x Running Gel Buffer	-	3.75ml
4x Stacking Gel Buffer	1.75ml	-
10% SDS Stock Solution	70µl	150µl
Distilled Water	4.3ml	4.75ml
Initiator	35µl	75µl
TEMED	15µl	7.5µl

APPENDIX V: WESTERN BLOT

A5.1 Reagents

*Blotting buffer:*Tris (6.05g) and glycine (14.4g) were dissolved in 700ml distilled water followed by the addition of 200ml methanol. The solution was titrated to pH 8.8 and made up to 1000ml.1ml of 10% (m/v) SDS was added and the solution. Blotting buffer was made up fresh before use.

Ponceau: Ponceau S. (0.1g)(SIGMA, cat.P3504, USA) was dissolved in 1ml of 100% (v/v) acetic acid and made up to 100ml with distilled water. The Ponceau S stain was stored in a plastic bottle at room temperature.

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