HEPATOCYTE GROWTH FACTOR REGULATES MYOGENESIS OF MOUSE AND HUMAN SKELETAL MYOBLASTS

By

Trish. R. Kahamba
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School of Life Sciences

Department of Biochemistry

University of KwaZulu-Natal

Pietermaritzburg campus

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

Signed:  Name: Dr. C. U. Niesler Date: 26/8/2013

ABSTRACT

Satellite cells are quiescent skeletal muscle specific stem cells that are activated in response to injury to aid in muscle repair and regeneration. The interaction of hepatocyte growth factor (HGF) with these cells is crucial for their activation. However, to date, research on the effect of HGF on skeletal muscle satellite cells has yielded conflicting data. Clarity is therefore required as to its effect on downstream myogenic processes. Furthermore, mouse and rat cell lines and primary culture have been widely used for *in vitro* studies to investigate the effect of HGF on skeletal muscle physiology and disease; very few studies have been carried out in primary cultured human skeletal myoblasts. As a result, we aimed to investigate and compare the effect of HGF (2, 10 and 50 ng/ml) on mouse C2C12 myoblast versus primary culture human skeletal myoblast (HSkM) proliferation, migration and differentiation. Proliferation was assessed via both cell counts and crystal violet assay, while migration was investigated using the scratch assay. Differentiation was determined via analysis of expression patterns of transcription factors implicated in myogenic commitment (i.e. Pax7, MyoD) as well expression of the structural protein Myosin Heavy Chain (MyHC). We demonstrate a dose-dependent effect of HGF on myoblast proliferation whereby an increase in proliferation was detected in response to 2 ng/ml HGF, whilst 10 ng/ml HGF resulted in a reduction in proliferation capacity of both C2C12 and HSkM myoblasts. Interestingly, the reduction in proliferation in response to 10 ng/ml HGF was accompanied by a down-regulation in Pax7 expression during differentiation of both mouse and human myoblasts. HGF also affected myoblast migration and differentiation in a dose-dependent manner that was inversely proportional to proliferation. HGF (10 ng/ml) stimulated an increase in myogenic commitment and terminal differentiation of C2C12 and HSkM myoblasts as reflected by the increased percentage MyoD positive cells, improved fusion and greater MyHC expression. C2C12 myoblast migration was also stimulated at this HGF concentration, but reduced in response to the lower HGF (2 ng/ml) dose. The decrease in proliferation following incubation with 10 ng/ml HGF, allows cells to exit proliferation into either a mode of migration or differentiation. Our data confirms the importance of HGF during myogenesis and highlights the sensitivity of satellite cells to changing HGF concentration. This has implications in the regulation of skeletal muscle wound repair.

Keywords: Hepatocyte growth factor, C2C12 myoblasts, Human primary cultures myoblasts, myogenesis, proliferation, migration, differentiation.

PREFACE

The project described in this dissertation was implemented in the College of Agriculture, Engineering and Science, Department of Biochemistry, School of Life Science, University of KwaZulu-Natal, Pietermaritzburg Campus, from January 2013 to August 2013, under the supervision of Dr. C. U. Niesler.

These studies denote 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: PLAGARISM

I, Trish. R. Kahamba, declare that:

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DECLARATION 2 – PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Kahamba, T.R., Goetsch, K.P., Niesler, C.U. (2013). Hepatocyte growth factor (HGF) promotes C2C12 and Human skeletal myoblast proliferation and differentiation in a dose-dependent manner, *Muscle and Nerve*. [**Contributed original data, analysis, conceptualisation of ideas and key experiments and interpretation for the majority of the paper]. SUBMITTED**

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Declaration Publications FHDR 22/05/08 Approved

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ABBREVIATIONS

c-met	Gene for HGF receptor
c-Met	HGF receptor
DMEM	Dulbecco's Modified Eagle Media
ECM	extracellular matrix
Erk	extracellular signal-regulated kinases
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
Gab1	Grb2- associated adaptor protein chain
Grb2	growth factor receptor-bound protein 2
HAI-1	HGFA inhibitor type 1
HAI-2	HGFA inhibitor type 2
HGF	hepatocyte growth factor
HGFA	HGF activator
HL	hairpin loop
HS	horse serum
HS	heparan sulphate
HSkM	Human skeletal myoblast
HSPGs	heparan sulphate proteoglycans

ICC	immunocytochemistry
IGF	insulin-like growth factor
IL-1	interlukin-1
IPT	immunoglobulin-plexin transcription
K1-K4	kringle domains 1-4
LM	laminin
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor 2
MMP	matrix metalloproteinase
MRF(s)	myogenic regulatory factor(s)
Myf5	myogenic factor 5
MyHC	myosin heavy chain
MyoD	myogenic differentiation factor
Pax3	pair homeodomain box 3
Pax7	pair homeodomain box 7
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PI3K	phosphatidylinositol 3-kinase

p38	Mitogen activated protein kinase
PSI	plexin-semaphorin-integrins
Raf	v-raf murine sarcoma viral oncogene homolog kinases
Ras	rat sarcoma viral oncogene homology
ROCK	Rho-associated protein kinase
Sema	semaphorin domain
Shp2	SRC homology protein tyrosine phosphatase 2
SDS	sodium dodecyl sulfate
SPH	serine protease homology
STAT3	signal transducer and activator of transcription 3
TNF-α	tumor necrosis factor-α
TGF-β	transforming growth factor-β
tPA	tissue type plasminogen activator
TTPS	type II transmembrane serine proteases
uPA	urokinase-type plasminogen activator

CHAPTER 1

LITERATURE REVIEW

1.1 SKELETAL MUSCLE REGENERATION

Skeletal muscle is the most abundant tissue of the body and is essential for locomotion, metabolism and production of heat (Anderson, 2006). It is composed of muscle fibres (termed myofiber) and contractile units that are bound together by connective tissues (Baj et al., 2005). Most importantly, skeletal muscle has the ability to regenerate after injury or disease such as muscle dystrophy. The generation process of new muscle, called myogenesis, is facilitated by resident stem cells termed satellite cells (Tedesco et al., 2010) (Figure 1.1), which represent up to 12% of nuclei in adult skeletal muscle (Baj et al., 2005).

Satellite cells were discovered in 1961, between the basal lamina and sarcolemma of muscle fibres (Mauro, 1961). They have since emerged as a model of postnatal stem cells both *in vivo* and *in vitro* and are also of particular relevance in the treatment of inherited muscle diseases (Baj et al., 2005). Furthermore, they are not only involved in muscle regeneration, but also in myogenesis required for skeletal muscle growth, maintenance, and homeostasis (Boldrin et al., 2010). These cells are a source of committed, proliferative muscle precursors (termed myoblasts) in normal muscle and represent a reserve of cells for myogenesis and regeneration (Anderson, 2006).

Satellite cells exist in a mitotically and a metabolically quiescent state and show limited transcription activity (Boldrin et al., 2010). When physiologically required (e.g. following injury, stress or growth), they become activated and enter into the cell cycle, proliferate and migrate to the site of injury (O'Reilly et al., 2008). These cells then differentiate and fuse with adjacent myofibers or form new fibers thereby participating in the regeneration process (Boldrin et al., 2010) (Figure 1.1). The response of satellite cells is largely influenced by the nature of the injury (e.g. chemical, ischemic, and traumatic) (Baj et al., 2005), changes in the composition of the extracellular matrix (ECM) (Boldrin et al., 2010) and secretion of growth factors (Anderson, 2006).

The ECM is a non-cellular structure that surrounds and supports all mammalian tissues and organs (Alberts et al., 2002). It provides vital physical scaffolding for the cells it surrounds and initiates biochemical and biomechanical signals that are required for tissue differentiation, morphogenesis and homeostasis. The ECM is also responsible for regulation of cell behaviour through (a) interaction of its matrix molecules with each other (e.g. fibronectin and collagen 1), (b) cell-

extracellular matrix signal transduction pathways (e.g. integrin signalling pathways) and (c) interaction with growth factors (e.g. HGF with heparin sulphate) (Bosman and Stamenkovic, 2003).

Growth factors are essential for myogenesis and skeletal muscle regeneration. They play a crucial role in the activation of satellite cells from quiescence and also regulate myogenic processes such as proliferation, migration, differentiation and myotube fusion during skeletal muscle repair and regeneration. The growth factors which contribute the most to these processes are Fibroblast Growth factor (FGF), Transforming Growth Factor β (TGF-β), Insulin-like Growth Factor (IGF), Platelet Derived Growth factor (PDGF) and Hepatocyte Growth Factor (HGF) families (Gal-Levi et al., 1998b, O'Reilly et al., 2008, Yamada et al., 2010a, Miller et al., 2000).

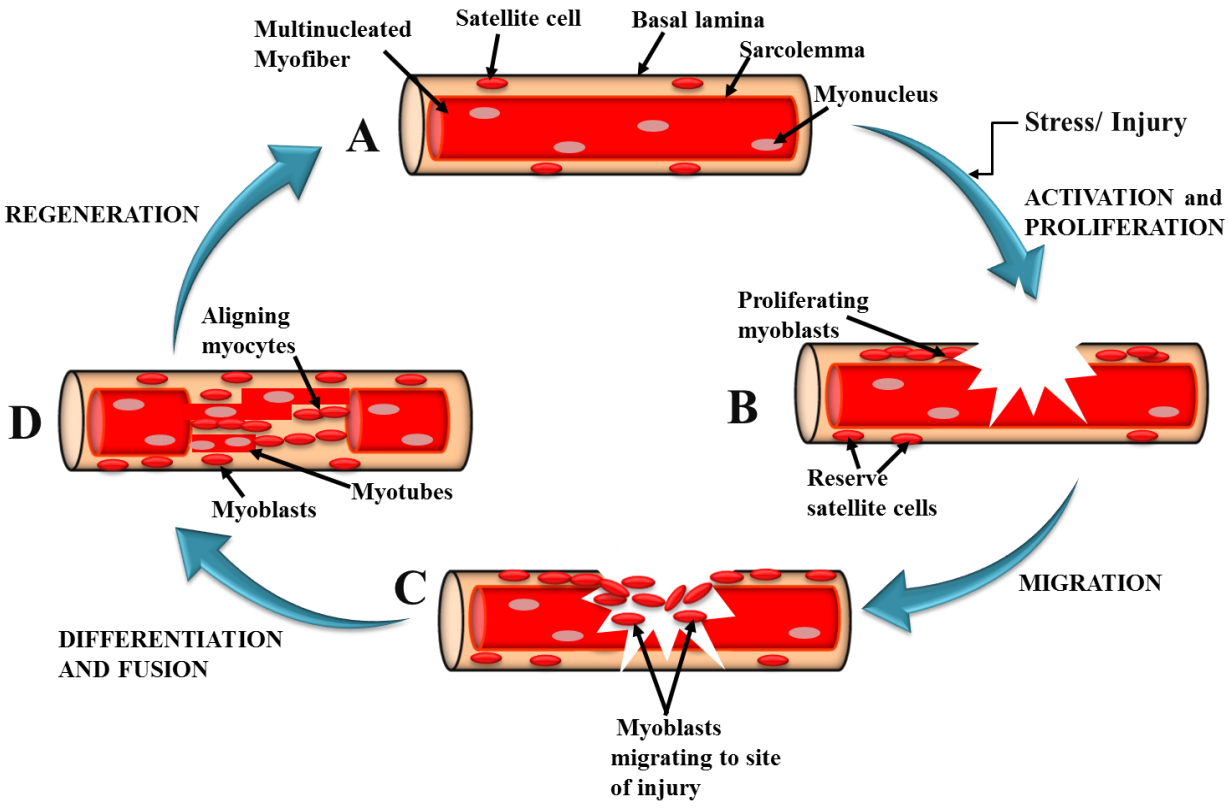

Figure 1.1: Schematic diagram of the processes involved in skeletal muscle regeneration after mild injury. When skeletal muscle is stressed or injured, satellite cells are activated from quiescence (**A**) and proliferate to generate precursors termed myoblasts (**B**). These myoblasts migrate to the site of injury (**C**), differentiate, align, and fuse to replace the injured fibre (**D**). Diagram compiled from the following references: Anderson, 2006; Baj et al., 2005; Boldrin et al., 2010; Tedesco et al., 2010.

1.1.1 Fibroblast Growth Factor (FGF)

Fibroblast Growth Factor (FGF) refers to a large family of related polypeptide growth factors that comprise five members; FGF-1, 2, 5, 6 and 7 (Karalaki et al., 2009). FGF is secreted by fibroblasts and satellite cells and are released in high concentrations during inflammation after tissue disruption. FGF induce activation and proliferation of satellite cells to provide sufficient cells for regeneration. FGF also promote migration of distant satellite cells to site of injury (Husmann et al., 1996) and inhibit differentiation through the negative regulation of MyoD and myogenin expression (Jones et al., 2005). FGF-1 and 2 stimulate satellite cell proliferation and inhibit differentiation. FGF-2 induces entry of satellite cells into the cell cycle, but does not accelerate the transition from proliferation to differentiation (Kastner et al., 2000). Expression of FGF-6 is stimulated after skeletal muscle injury and induces satellite cell morphological changes, alters their adhesion and ability to differentiate (Floss et al., 1997). FGF-7 regulates myoblast division and differentiation in developing myotubes and is expressed during embryonic limb muscle development (Husmann et al., 1996).

The functions of FGF are mediated through binding to the FGF receptor (FGFR), a transmembrane tyrosine kinase receptor. Four distinct FGFR genes have been discovered, (FGFR 1-4) and of these FGFR1 and 4 are the most prominent in satellite cells (Floss et al., 1997) . The expression of these receptors is up-regulated during skeletal muscle repair and regeneration, however, FGFR-1 regulates satellite cell proliferation whilst FGFR-4 regulates differentiation to aid in proper and effective repair (Kastner et al., 2000).

1.1.2 Transforming growth factor-beta (TGF-β)

Transforming growth factor-beta (TGF-β) is a member of a family of dimeric polypeptide growth factors and exists as three isoforms: TGF-β1, TGF-β2, and TGF-β3 (Droguett et al., 2006). TGF-β is secreted by nearly every cell in the body and regulates embryonic development as well as postnatal cell proliferation, migration, differentiation and wound healing (Husmann et al., 1996). Upon injury, TGF-β is released by platelets at the site of injury and it induces the production of more TGF-β by satellite cells; however, excessive production and deposition of TGF-β can lead to fibrosis (McLennan and Koishi, 2002). The common characteristic of all TGF-β isoforms is their ability to bind to and modulate the function of ECM proteins such as biglycan (Liu et al., 2004). TGF-β is also stored in the ECM until activated by signals such as wound healing (Karalaki et al.,

2009). TGF-β regulates ECM reorganization throughout muscle regeneration and is responsible for the reconstruction of basal lamina and ECM that surrounds the damaged myofibers and activated satellite cells (Husmann et al., 1996).

1.1.3 Insulin-like growth factor (IGF)

The insulin-like growth factor (IGF) comprises two isoforms, namely IGF-I and IGF-II (Karalaki et al., 2009, Philippou et al., 2007). IGF-I is known to induce skeletal muscle hypertrophy by increasing the size of myotubes, promoting DNA and protein synthesis as well as inhibiting protein degradation (Pelosi et al., 2007). During skeletal muscle regeneration, IGF-I promotes satellite cell proliferation by up-regulating the expression of intracellular mediators such as cyclin-D and is also able to stimulate differentiation by inducing myogenin gene expression (Bark et al., 1998). Moreover, IGF-I promotes satellite cell survival during early phases of differentiation. Following injury, IGF-I also modulates the inflammatory response by down-regulating pro-inflammatory cytokines and reducing fibrosis (Bark et al., 1998);(Hill and Goldspink, 2003).

The role of IGF-II in skeletal muscle repair is not well defined; however, it has been shown to be involved in myotube formation (Philippou et al., 2007). Expression of IGF-I and II occurs at different stages of muscle regeneration, but IGF-I expression precedes that of IGF-II (Hayashi et al., 2004).

1.1.4 Platelet derived growth factor (PDGF)

Platelet derived growth factor (PDGF) was first identified in human platelets as a factor that promoted growth. PDGF has three isoforms: PDGF-AA, -AB and –BB. Following injury, PDGF-BB is released from the injured myofibers, degranulating platelets and infiltrating activated macrophages; it stimulates satellite cell migration. It also enhances satellite cell proliferation, but inhibits their differentiation (Husmann et al., 1996); (Karalaki et al., 2009).

1.1.5 Other factors in skeletal muscle regeneration

In order to reach the site of injury during repair, myoblasts have to migrate through the extracellular matrix (ECM) (Boonen and Post, 2008). Matrix metalloproteinases (MMPs) play a pivotal role in the degradation of ECM components, which not only allows migration of the myoblasts, but also results in the release of and cellular exposure to cytokines (e.g. HGF, FGF) that regulate myoblast proliferation and differentiation (Torrente et al., 2000). MMP2 and 9 in particular, are secreted by satellite cells and their expression levels are increased during skeletal muscle injury and regeneration (Koskinen et al., 2002). They are secreted into the ECM as inactive precursors which are activated following cleavage by proteinases and plasmin (Torrente et al., 2000). When active they degrade basal lamina components (Brennan et al., 2004).

The basal lamina is a specialized sheet of connective tissue that surrounds myofibers (Alberts et al., 2002) (Figure 1.1). Integrity of the basal lamina is essential to keep the satellite cells in their niche; defects in the basal lamina allow the cells to move throughout the muscle leading to loss of the reserve population of these cells (Brennan et al., 2004). The most important constituents of the basal lamina are collagen IV, laminin 2 (LM-211) and proteoglycans such as decorin. Laminin and collagen IV form an intricate structure with multiple binding sites for proteoglycans and integrins (Boonen and Post, 2008).

Integrins comprise a large family of cell surface receptors composed of an α- and β-subunit (Kovanen, 2002). After binding to the appropriate ligand, integrins act as signal transducers, inducing migration, cell shape change and cell-cell interactions (Brzoska et al., 2006). Integrin α7 is the main isoform located in skeletal muscle and it binds myofibers to laminin and is up-regulated during regeneration (Kaariainen et al., 2001). Integrin α3 is also located on quiescent satellite cells and is known to bind to laminin. The interaction of integrin α3 and laminin is essential for myoblast migration and differentiation during regeneration (Brzoska et al., 2006).

Laminin refers to a family of large disulphide-linked heterotrimeric glycoproteins (400-900 kDa) that contain one α, β and γ subunit and are represent the major component of the basal lamina (Siler et al., 2000). The laminin trimers are characterized according to the composition of the α, β and γ chains and to date fifteen laminin isoforms have been identified. Of these isoforms, only laminin-2 (LM-211) is specific for skeletal muscle (Gawlik and Durbeej, 2011). LM-211 consists of α2, β1 and γ1 proteins (Ehrig et al., 1990). In addition, laminin-deficient mice completely lack the basal lamina leading to a decrease in the satellite cell pool. During skeletal muscle regeneration, myoblast

numbers decrease due to diminished proliferation rates and increased apoptosis. Absence of the laminin α2 chain results in muscular dystrophy in humans and mice (Boonen and Post, 2008).

Collagens are important fibrous and structural proteins within the ECM and basal lamina. They constitute up to 30% of the total protein mass of a multicellular animal. Over time they form cumulative intermolecular cross-links leading to stiffness and reduced function in aged skeletal muscles (Avery and Bailey, 2005). Seven collagen isoforms have been identified, including collagen I, II, III, IV, V, VI and VII; which are all composed of three polypeptide α-chains coiled into a triple helix structure. However, only collagen IV is specific to the basal lamina of myofibers and is known to interact with laminin and several proteoglycans (Frantz et al., 2010).

Proteoglycans are a family of glycoproteins located mainly in connective tissue and contain subunits of glycosaminoglycans. The proteoglycan family is composed of several proteins including decorin, hyaluronan and syndecans. These proteins occupy a part of the ECM within tissues and participate in the molecular events that regulate cell proliferation and adhesion. Decorin is known to be involved in myoblast differentiation and migration, in part by modulating the biological activities of growth factors such as TGF-β (Droguett et al., 2006). Hyaluronan is found in high concentrations on the myoblast cell surface and stimulates myogenesis by enhancing differentiation and influencing migration via its interaction with cell surface receptors such as CD44 (Mylona et al., 2006). Quiescent satellite cells express syndecan-3 and -4, which are transmembrane heparan sulphate proteoglycans that are up-regulated upon satellite cell activation. In syndecan-3 knockout mice, satellite cells express low levels of c-Met and contain an abnormally disorganized basal lamina. When muscle damage is induced in these animal models, scar tissue formation is prominent. In addition, isolated satellite cells differentiate abnormally, with fusion of the myoblasts into syncytia instead of myofibers (Cornelison et al., 2004);(Rauch et al., 2005).

Skeletal muscle repair and regeneration is therefore regulated by numerous factors, one factor not yet discussed is hepatocyte growth factor (HGF), a key regulator of satellite cell activity during myogenesis and muscle regeneration (Cornelison et al., 2004). It is expressed not only in regenerating muscle, but also in uninjured muscle tissue; it is processed and released upon injury, when it promotes the activation and proliferation of satellite cells for muscle repair (Cornelison and Wold, 1997, Bischoff, 1997). The ability of HGF to stimulate mitogenesis, morphogenesis, satellite cell migration and growth gives it a central role in skeletal muscle regeneration (Karalaki et al., 2009).

1.2 HEPTACTOCYTE GROWTH FACTOR (HGF) STRUCTURE AND FUNCTION

Hepatocyte growth factor (HGF) was initially discovered in 1984 as an important agent that stimulated the growth of hepatocytes after rat liver damage (Michalopoulos et al., 1984); hence this factor was given the name hepatocyte growth factor (HGF). In 1989 it was isolated from human plasma (Zarnegar and Michalopoulos, 1989) and since then its role has been studied in various animals and tissues (Schmidt et al., 1995); (Gal-Levi et al., 1998a, Gal-Levi et al., 1998b); (Zeng et al., 2002).

1.2.1 Structural and Biochemical Properties of HGF

HGF, alternatively known as scatter factor, is a multidomain glycoprotein synthesized and secreted mainly by mesenchymal cells including fibroblasts, endothelial cells, vascular smooth muscle cells and satellite cells (Birchmeier and Gherardi, 1998). Upon synthesis, HGF is stored in the extracellular matrix (ECM) as a biologically inactive pro-HGF single-chain precursor (approximately 90kDa) (Grierson et al., 2000). However, in response to stimuli such as skeletal muscle injury, pro-HGF is activated to a heterodimeric form via proteolytic cleavage by serine proteases at Arg494-Val495 (Tatsumi and Allen, 2004). In the activated form, HGF exists as a disulfide-linked heterodimer which contains an α (60kDa) and β-chain (30kDa). The α-chain, which is also the heavy chain, consists of an amino-terminal hairpin loop domain (HL) and four kringle domains (K1-K4); whilst the β-chain (light chain) consists of a serine protease homology (SPH) domain (Grierson et al., 2000) (Figure 1.2). Although HGF contains a SPH domain, it lacks any enzymatic (proteolytic) activity due to replacement of two of the three amino acids necessary for the catalytic triad (histidine and serine instead of glutamine and tyrosine, respectively) (Strain, 1993) (Figure 1.2).

The HL region of HGF contains clusters of amino acids that are necessary for sufficient binding of HGF to its receptor. The kringle domains (K1, K2, K3 and K4) play a pivotal role in the activation of the HGF receptor and are important for the mitogenic activity of HGF. The SPH domain contributes additional binding sites which are essential for efficient HGF receptor activation (Holmes et al., 2007) (Figure 1.2)

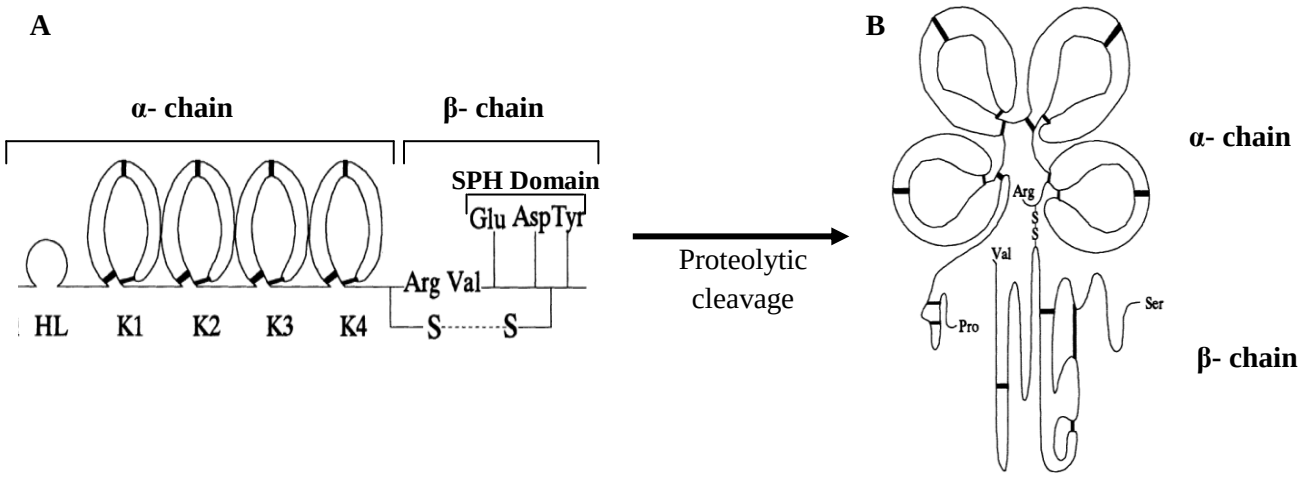

Figure 1.2: HGF schematic structure. **A)** pro-HGF single chain precursor. **B)** HGF in its activated form. The four kringle domains (K) and HL domain are on the α-chain, and the SPH domain is on the β-chain. Proteolytic cleavage of pro-HGF into its active form is important for activity. Modified from (Grierson et al., 2000).

HGF is highly conserved across mammals. There is a 99% amino acid similarity between human and gorilla, and 91% between human and mouse. However, homology deviates in birds, with only 75% amino acid similarity between human and chicken (Table 1.1).

Table 1.1: HGF homology across different species

Species		Maximum amino acid identity
Human	Gorilla, Chimpanzee	99%
Human	Rhesus monkey	98%
Human	Hedgehog, Sheep, Rabbit	93%
Human	Guinea pig	91%
Human	Mouse	91%
Human	Rodents	88%
Human	Chickens	75%

Information retrieved from the Protein data base accessed on National Centre for Biotechnology Information (MCBI) website utilising the Basic Local Alignment Search Tool (BLAST) on 31/06/13

1.2.2 Activators of HGF

Activators of HGF include enzymes, which are either serine proteases of blood plasma origin or transmembrane serine proteases. Both classes are involved in HGF activation (Sisson et al., 2009).

The proteases of blood plasma origin include urokinase-type plasminogen activator (uPA), tissue type plasminogen activator (tPA) and HGF activator (HGFA). Both uPA and tPA are known to function in blood clotting and ECM degradation, and are also known to activate HGF. These proteases are therefore ideal candidates for HGF-activating enzymes in injured tissues including skeletal muscle (Miyazawa, 2010). The HGF amino acid sequence is 40% similar to plasminogen (a proenzyme and substrate of uPA/tPA which facilitates the lysis of fibrin blood clots); uPA and tPA proteolytically cleave the single inactive HGF to its active heterodimeric form (Sisson et al., 2009). HGFA is located in the serum and plasma in an inactive or active form. The inactive precursor can be activated by thrombin via limited proteolysis in injured tissue. HGFA activation of HGF is more effective compared to uPA and tPA (Grierson et al., 2000).

Both matripase and hepsin are members of the family of type II transmembrane serine proteases (TTPS), which are known to activate pro-HGF (Kirchhofer et al., 2005) with comparable efficiency to that of HGFA (Miyazawa, 2010). These enzymes are localized on the cell surface in order to interact with HGF and other ECM components; they cleave pro-HGF at Arg thereby converting it to its active form (Kirchhofer et al., 2005) (Figure 1.2).

1.2.3 Cellular regulation of HGF levels

Regulation of HGF levels is essential as abnormal activation and overexpression of this cytokine leads to *c-met* gene amplification and mutations which result in the development of a variety of malignancies (Sattler et al., 2011). Regulation of HGF production and availability is very important and is attributed to several factors including two important HGFA inhibitors; HGFA inhibitor type 1 (HAI-1) and type 2 (HAI-2) (both which are members of the Kunitz-type family of serine proteases) (Birchmeier and Gherardi, 1998). These competitive inhibitors are important physiological regulators of serine protease activity particularly hepsin and HGFA. In normal muscle, they bind to the active sites of the proteases thus preventing the interaction of HGF with its activators. However, during injury when HGF activation is essential, the inhibitors detach from the proteases and in order to initiate proper serine protease-HGF binding. To allow subsequent terminal

differentiation of myoblasts during muscle regeneration, HAI-1 and HAI-2 are up-regulated leading to down-regulation of active HGF (Shia et al., 2005).

The expression of the HGF gene is restricted to mesenchymal cells including satellite cells and fibroblasts (Seki et al., 1991). HGF gene expression is up-regulated in response to tumor-necrosis factor (TNF-α), interleukins (IL-1α and IL-1β) and is down-regulated by growth factors such as transforming growth factor-β (TGF-β) (Shia et al., 2005).

1.2.4 HGF receptors: Structure and Function

The HGF receptors exist in high and low affinity forms, namely c-Met and heparan sulphate proteoglycans (HSPGs), respectively. The α-chain of HGF heterodimer contains a high affinity receptor binding site for c-Met while the β-chain contains a low affinity receptor binding site for HSPGs (Basilico et al., 2008) (Figure 1.3).

c-Met is a protooncogene product that is expressed as a transmembrane protein with tyrosine kinase activity in its cytoplasmic region (Grierson et al., 2000). This receptor is a 190 kDa glycoprotein, which can be cleaved into an α chain (50 kDa) and β chain (140 kDa). The α chain and a section of the β chain are linked by a disulfide bond and located on the cell surface, whilst the COOH terminal portion of β chain is located in the cytoplasm of the cell and contains the tyrosine kinase domain (Trusolino et al., 2010). The extracellular portion of c-Met consists of three domains: the semaphorin (Sema) domain that includes the whole of the α and part of the β chain; the plexin-semaphorin-integrins (PSI) domain and four immunoglobulin-plexin transcription (IPT) domains (Basilico et al., 2008) (Figure 1.3). The Sema domain serves as a receptor recognition site, while the IPT domain is responsible for high affinity binding of c-Met to α-chain of HGF. The PSI domain functions as a link to orientate the extracellular portion of c-Met for effective and proper HGF binding (Kozlov et al., 2004). The cytoplasmic region is composed of three portions: a juxtamembrane sequence (down-regulates kinase activity upon phosphorylation); a catalytic region (responsible for activation of kinase activity following phosphorylation) and a carboxyl-terminal multifunctional docking site (important for downstream signalling) (Trusolino et al., 2010) (Figure 1.3). The binding of HGF with the extracellular domains of c-Met (Figure 1.3) results in conformational changes within the domains. These conformational changes are a signal for autophosphorylation of the cytoplasmic domains and activation of downstream pathways such as MAPK and PI3K involved in cell motility, proliferation, survival and differentiation (Grierson et

al., 2000). HGF-c-Met binding is therefore important during embryogenesis and adult skeletal muscle regeneration (Basilico et al., 2008).

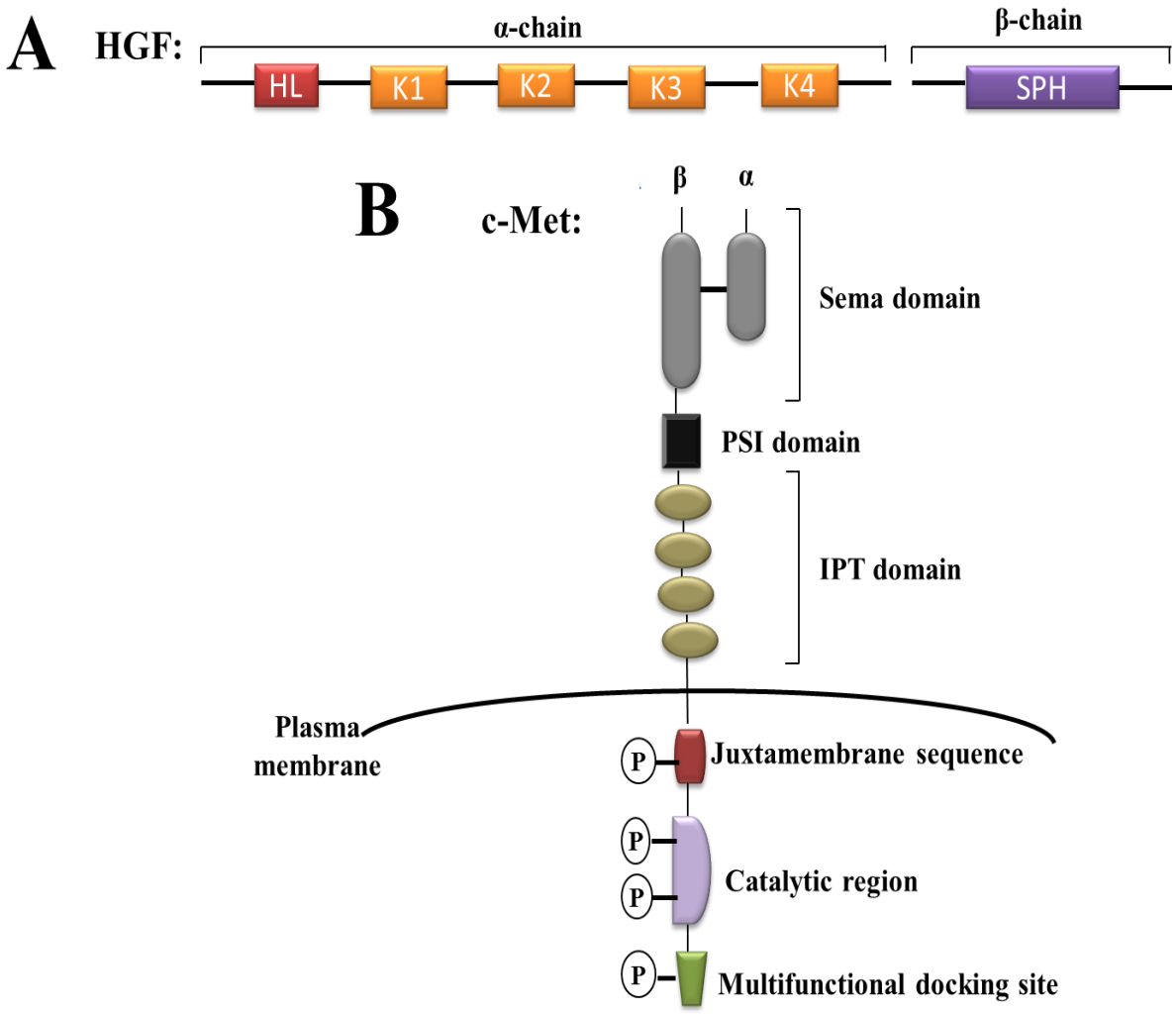

Figure 1.3: Domain structure of HGF and c-Met. Both HGF and c-Met consist of α and β chains, which are used for binding of the two proteins during signal transduction. The c-Met extracellular portion is composed of three domains (Sema, the plexin-semaphorin-integrins (PSI) and immunoglobulin-plexin transcription (IPT)); the intracellular portion consists of a Juxtamembrane sequence, catalytic region and a multifunctional docking site in the C-terminal region. Diagram compiled from the following references: Basilico et al., 2008 and Kozlov et al., 2004.

HSPGs are a family of cell surface proteoglycans which include syndecans and glypicans, located within the ECM (Dreyfuss et al., 2009). HSPGs function as scaffold structures that non-covalently bind and interact with a wide variety of growth factors including HGF and FGF. HSPGs are involved in cellular signalling either as receptors or co-receptors, they are known to be low affinity HGF co-receptors (Chirgadze et al., 1999).

The ability of HSPGs to bind to HGF is facilitated by their low degree of sulphation; their ligand-binding sites are located within distinct sulphated domains formed by complex, cell-specific, chemical modifications of the heparan sulphate (HS) disaccharide repeat (Dreyfuss et al., 2009). Within the β-chain of HGF, the SPH domain is identified as a site important for HSPGs binding (Grierson et al., 2000) (Figure 1.3). Low affinity binding of HGF to HSPGs stimulates activation of phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, signalling routes that regulate cell survival and proliferation, respectively (Derksen et al., 2002) (Figure 1.4). HSPGs promote dimerization of HGF, thereby stimulating c-Met activity and cross-linking to HGF. They also regulate HGF affinity for c-Met via induced conformational changes of HGF and protect HGF from proteolytic degradation (Birchmeier and Gherardi, 1998).

Interestingly, it has been reported that HGF mediates colocalization of c-Met and HSPGs and the complex formed between HGF, c-Met and HSPGs bring essential intracellular signalling molecules such as adaptor proteins: growth factor receptor-bound protein 2 (Grb2) and Grb2-associated adaptor protein (Gab1) into proximity which facilitate c-Met activation (Derksen et al., 2002). HSPGs localize HGF to satellite cells and ECM components within the microenvironment and are also essential for the formation of a chemotactic gradient which is important for satellite cell migration (van der Voort et al., 2000).

1.2.5 HGF Signalling

Co-operation between HGF and c-Met domains is essential for multifunctional biological activities of HGF (Basilico et al., 2008) (Figure 1.4). Following HGF binding, c-Met is activated via receptor dimerization, internalized by clathrin-mediated endocytosis and recruited into early endosomes. c-Met then travels along the microtubule network to late intracellular compartments; this is followed by trans-phosphorylation of the catalytic domain and phosphorylation of the multifunctional docking site (Grierson et al., 2000) (Figure 1.3). The docking sites are essential for attachment of a variety of adaptor proteins and direct kinase substrates including the growth factor receptor-bound protein 2 (Grb2), Grb2-associated adaptor protein (Gab1), SRC homology protein tyrosine phosphatase 2 (Shp2), PI3K and signal transducer and activator of transcription 3 (STAT3). This leads to the activation of downstream pathways which include mitogen-activated protein kinase (MAPK), PI3K/AKT and STAT3 that mediate c-Met-dependent satellite cell proliferation, migration, survival and differentiation (Birchmeier and Gherardi, 1998) (Figure 1.4).

Ras (rat sarcoma viral oncogene homology) binds to Grb2 leading to indirect activation of Raf (v-raf murine sarcoma viral oncogene homolog) kinases (Figure 1.4). This subsequently leads to activation of the MAPK cascade which results in successive activation of different protein kinases including extracellular signal-regulated kinases (Erk1 and Erk2) and p38. These downstream elements are responsible for activation of the cell cycle (leading to cell proliferation) and alterations in cytoskeletal functions that control cell migration (Grierson et al., 2000). Binding of Shp2 to Gab1 also results in the activation of the MAPK cascade and stimulation of satellite cell migration. Disruption of Shp2/Gab1 interaction has been reported to lead to defective myoblast migratory patterns (Li et al 2009).

PI3K can bind either directly to c-Met or indirectly via Gab1, and signals through AKT to regulate cell survival and resistance to apoptosis via degradation of pro-apoptotic protein p53 (Birchmeier et al., 2003) (Figure 1.4). The direct binding of STAT3 to c-Met results in STAT3 phosphorylation, dimerization and translocation into the cell nuclei. This leads to regulation of genes implicated in cell proliferation and differentiation (Zhang et al., 2002) (Figure 1.4).

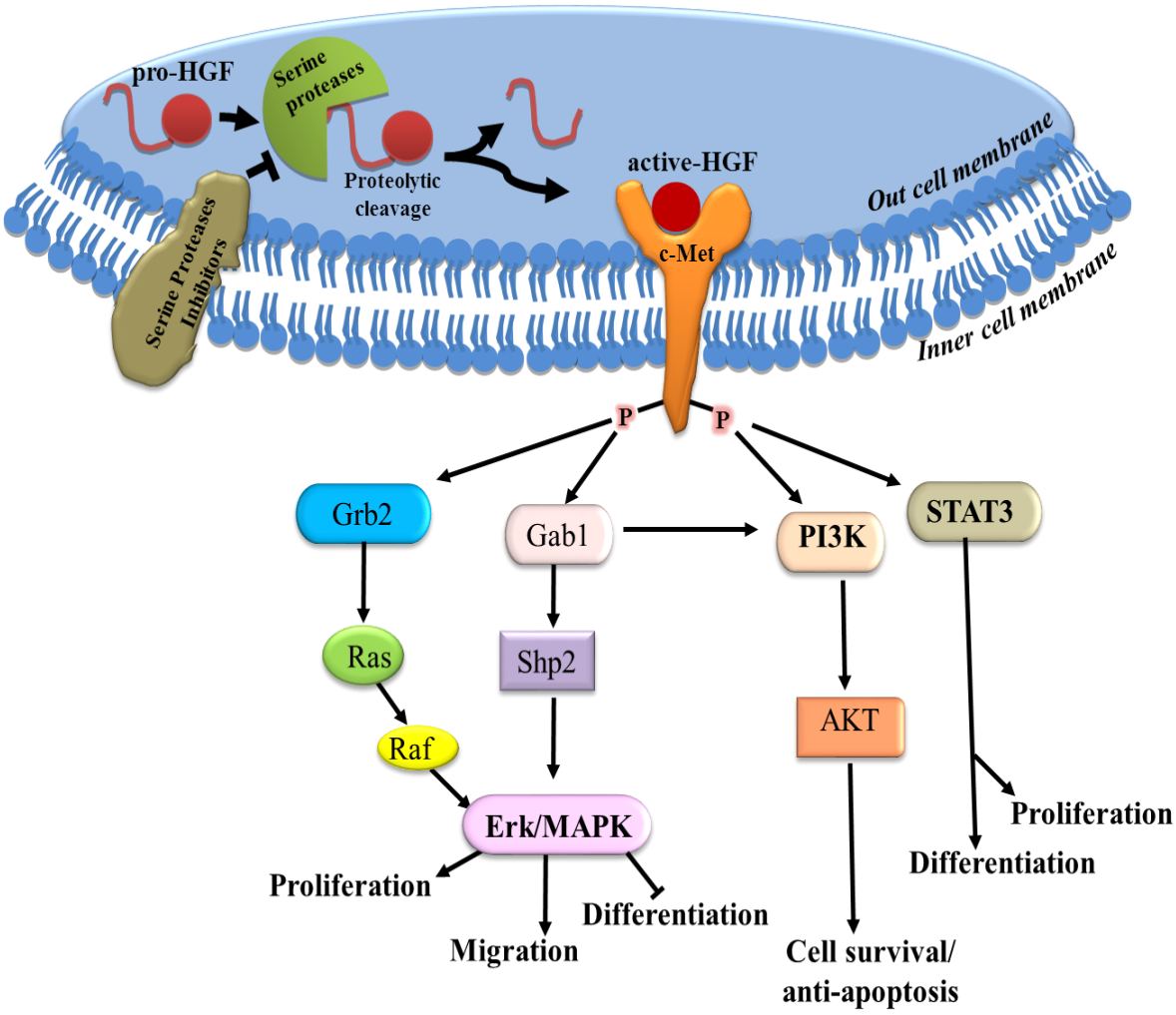

Figure 1.4: HGF signalling in satellite cells. Phosphorylation of multifunctional docking sites results in recruitment of the adaptor proteins Grb2 and Gab1 as well as effector molecules PI3K and STAT3. PI3K can bind either directly to c-Met or indirectly via Gab1 which leads to the regulation of genes implicated in cell proliferation and differentiation. Activation of MAPK results in stimulation of cell proliferation, migration and inhibition of differentiation. HGF also promotes cell survival via PI3K signalling and can regulate cell proliferation and differentiation via STAT3 signalling. Diagram compiled from the following references: Zhang et al., 2002; Grierson et al., 2000; Birchmeier and Gherardi, 1998; Li et al 2009.

1.3 HGF BIOLOGICAL ACTIVITY

HGF has been termed a pleiotrophic growth factor due to its ability to promote a wide range of biological activities in numerous target cells (Grierson et al., 2000); (Yamada et al., 2010b). Some of these biological and behavioural effects promoted by HGF *in vitro* and *in vivo* are listed in Table 1.2 below.

Table 1.2: Processes and cells targeted by HGF

Process	Target cells	Reference
Proliferation	Satellite cells, keratinocytes, hepatocytes cells	(Miller et al., 2000); (Moorby et al., 1995)
Differentiation	Satellite cells, mammary epithelial cells	(Tatsumi et al., 2002); (Rosen et al., 1990)
Cytoskeletal changes	Satellite cells, hepatocyte cells	(Ben-Ze'ev et al., 1990); (Pagan et al., 1997)
Migration	Satellite cells, MDCK cells	(Grierson et al., 2000); (Stoker, 1989)
Mitogenesis	Satellite cells, hepatocyte cells, keratinocytes	(Gal-Levi et al., 1998b); (Russell et al., 1984)
Anti-apoptosis	Myoblasts, bronchial and alveolar epithelial cells	(Umeda et al., 2006)
Activation	Satellite cells	(Gal-Levi et al., 1998b); (O'Reilly et al., 2008) (Yamada et al., 2010a)
Change in morphology	MDCK, kidney cells	(Dowrick et al., 1993)

1.3.1 Role of HGF in skeletal muscle development during embryogenesis

HGF and *c-met* genes are expressed throughout embryogenesis particularly during early organogenesis and gastrulation (Andermarcher et al., 1996). During early organogenesis, skeletal muscle develops from migratory and non-migratory muscle precursor cells that are produced by the lateral lip of the dermomyotome. Mice that lack c-Met and HGF die during the second part of gestation and lack migratory myogenic muscle precursor cells (Dietrich et al., 1999). During gastrulation, the expression of HGF and c-Met coincide; they are initially expressed in the endoderm and mesoderm and later in the node and notochord. During this phase, HGF regulates detachment of specific myogenic muscle precursor cells and subsequent migration from the somites into limb bud and diaphragm (Andermarcher et al., 1996); (van der Voort et al., 2000). The migration of these myoblasts is an important process in the development of skeletal muscle during embryogenesis and is therefore largely regulated by HGF. The absence of HGF and c-Met during embryogenesis prevents the development of limb muscle, shoulder muscle, and the diaphragm (Birchmeier and Gherardi, 1998); (Andermarcher et al., 1996).

Interestingly, the *c-met* promoter contains a binding site for Pax3; in other words this transcription factor initiates gene expression of the HGF receptor (Epstein et al., 1996). Pax3 can therefore regulate the release of myoblasts by activating *c-met* genes during skeletal muscle development (Dietrich et al., 1999).

1.3.2 Role of HGF in satellite cell activation during adult skeletal muscle repair

Satellite cell activation is central to skeletal muscle repair and is triggered primarily by HGF following skeletal muscle injury (Le Grand, 2007). Extrinsic mechanical stretch of the myofibers can trigger a number of intracellular signals, including nitric oxide synthesis that results in HGF activation. As previously discussed, HGF induces pro-myogenic mitogen activated protein kinase (MAPK) signalling cascades via c-Met (Karalaki et al., 2009). The p38α/β MAPK functions as a molecular switch to activate quiescent satellite cells (Jones et al., 2005).

Satellite cell activation requires up-regulation of muscle transcription factors and muscle-specific genes (Karalaki et al., 2009). Quiescent satellite cells exhibit limited gene expression and protein synthesis (Le Grand, 2007). Paired and homeodomain box 7 gene (Pax7) is a member of a large family of transcription factors involved in cell determination during embryogenesis in mammals (Yablonka-Reuveni and Rivera, 1994). The Pax proteins are categorized by several conserved elements; two DNA binding domains, a paired domain and a homeodomain (Le Grand, 2007). Pax3 is expressed by resident satellite cells in restricted muscle types, such as the diaphragm. Although both Pax3 and Pax7 are expressed during muscle development, Pax3 is the major transcription factor in embryonic muscle development, while Pax7 is essential in postnatal adult muscle (Yablonka-Reuveni et al., 2008). Pax7 is expressed by both quiescent and activated satellite cells and is also important for specification, survival and self-renewal of satellite cells and activation of myogenic genes (Le Grand, 2007). Studies by Gill *et al*., have demonstrated that expression of Pax7 during satellite cell activation and proliferation is largely regulated by HGF (Gill et al., 2010).

Following activation, a portion of the satellite cell population will leave its niche and move to the injured site where it will increase the expression of Pax7, as well as myogenic regulatory factors (MRFs): myogenic differentiation factor (MyoD), myogenic factor 5 (Myf5) and MRF-4; whilst a portion of the population remains in the niche where they continually self-renew (Hawke and Garry, 2001). Quiescent satellite cells do not express MyoD, myogenin or myocyte enhancer factor 2 (MEF2) or other known markers of terminal differentiation (Le Grand, 2007) (Figure 1.5).

MRFs are involved in myogenic fate determination during early embryogenesis and adult skeletal muscle (Yablonka-Reuveni et al., 2008). When fully activated, satellite cells express MyoD. This promotes progression to differentiation (Karalaki et al., 2009).

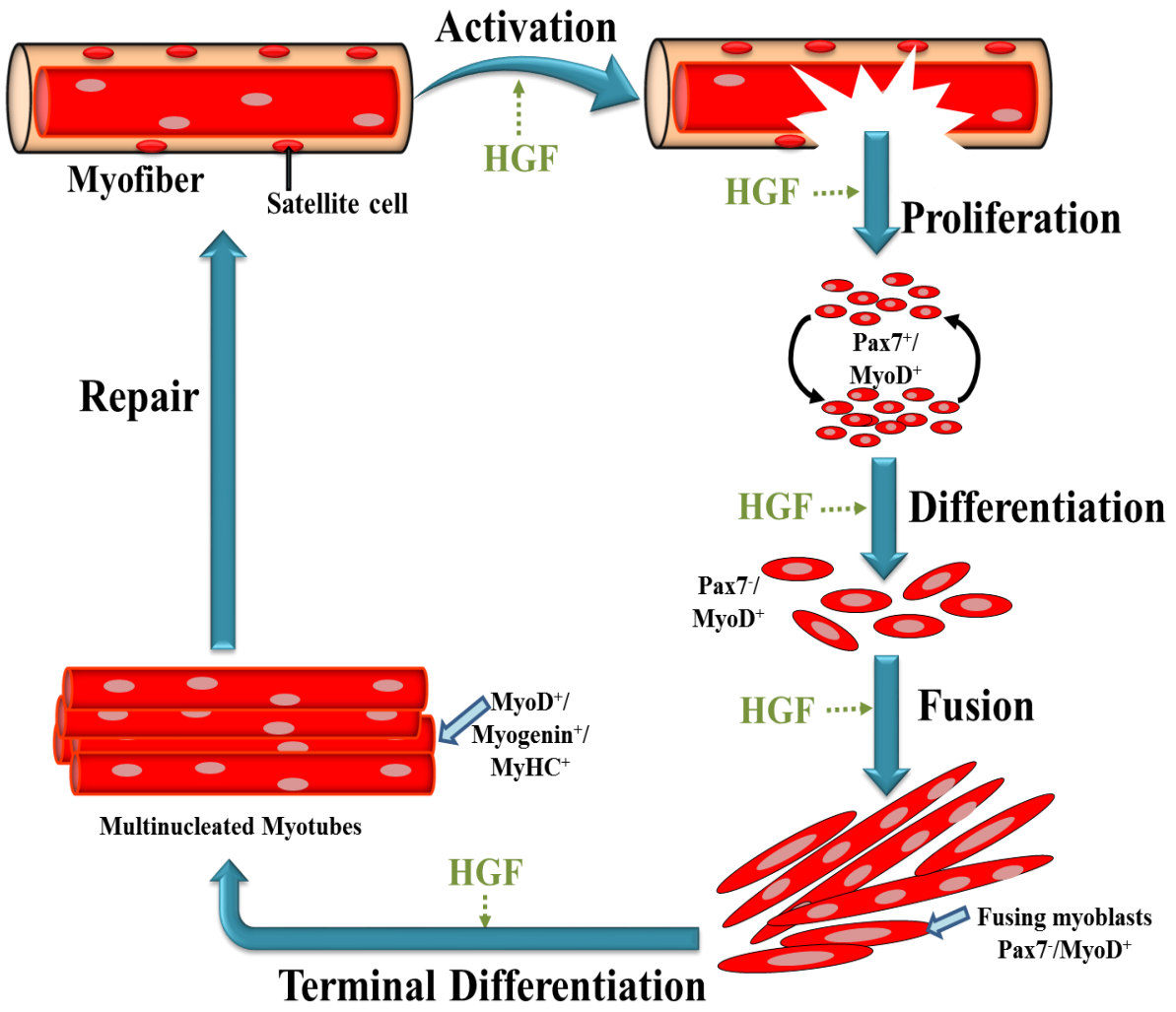

Figure 1.5: Role of HGF in satellite cell activation in adult skeletal muscle. Satellite cell activation, proliferation and differentiation is largely regulated by HGF (shown in green) and is based on the expression patterns of Pax7, MyoD, myogenin and MyHC. During proliferation, myoblasts co-express Pax7 and MyoD. Upon commitment to differentiation, the cells down-regulate Pax7 and express MyoD, myogenin and MyHC. Diagram complied from the following references: Karalaki et al., 2009; Le Grand, 2007; Yablonka-Reuveni et al., 2008; Hawke and Garry, 2001.

1.3.3 Role of HGF during satellite cell proliferation during myogenesis

Satellite cell proliferation is one of the key processes involved in myogenesis. Throughout embryogenesis, and in the adult skeletal muscle, the ability of satellite cells to proliferate is regulated by growth factors such as HGF and FGF (O'Reilly et al., 2008). During embryogenesis, cell proliferation is the primary means by which cell mass increases. Adult skeletal muscle generally express a variety of factors that act to maintain the proliferation status of satellite cells, these include transmembrane receptors (e.g. c-Met), intracellular signalling molecules and transcription factors (e.g. Pax7, MyoD (Figure 1.5)). However, in adult skeletal muscle proliferation becomes restricted as the capacity of the satellite cells to divide becomes exhausted as a result of progressive shortening of their telomere during each cell cycle (Zammit et al., 2006a).

To date, *in vitro* studies of the role of HGF during satellite cell proliferation have yielded contradictory findings. Proliferation studies in C2C12 myoblasts have indicated promotion of cell growth by HGF (10 and 50 ng/ml) (Anastasi, 1997). These findings correlated with similar studies in rat skeletal myoblasts, where the rate of cell proliferation was shown to increase upon addition of HGF (3 and 30 ng/ml) (O'Blenes et al., 2010). In contrast, Yamada *et al*. demonstrated that high levels of HGF (over 10 ng/ml) suppress the proliferative activity of rat satellite cells (Yamada et al., 2010a). Gal-Levi *et al*. came to similar conclusions using the chicken skeletal muscle cells and C2 cells. They found a reduction in activation of quiescent satellite cells and decreased growth upon exposure to exogenous HGF (20 and 50 ng/ml) (Gal-Levi et al., 1998b) (Table 1.3).

1.3.4 Role of HGF during satellite cell migration

During early tissue formation, satellite cells proliferate and are capable of extensive migration (Andermarcher et al., 1996) (Figure 1.6). Migration of satellite cells occurs widely during both regeneration of skeletal muscle following injury and myogenesis for the purpose of muscle maintenance (Phillips et al., 1987). It has been reported that satellite cells migrate in the following ways: migration within a gradient of chemoattractants (e.g. in response to HGF, TGF-β) and migration of cells to injury site (during wound healing) (Firtel and Chung, 2000).

Muscle trauma results in an increased release of HGF and FGF, that stimulate chemotaxis of satellite cells from surrounding niches (Watt et al., 1994). Focal crush injury at one end of a muscle results in the HGF-mediated activation and movement of satellite cells beneath the basal lamina from distant uninjured tissue toward the crush site (Bischoff, 1994). However, if the injury stimulus is perpendicular to the direction of the myofibers, some cells are able to cross the basal lamina of uninjured myofibers and migrate transversely through the muscle toward the injury site (Schultz et al., 1988).

A migrating cell is usually polarized and exhibits cell projections such as filopodia (cylindrical, thin, needle-like projections protruding from the cell surface), lamellipodia (flat, broad, sheet-like structures at the leading edge of cells) at the front, and large focal adhesion complexes at the back (Figure 1.6). The typical Rho GTPases (Cdc42, Rac1 and RhoA), play a crucial role in controlling cell polarity and regulating different aspects of cytoskeletal dynamics. Cdc42 regulates the direction of migration, Rac induces membrane projection at the front of the cell through stimulation of actin polymerization and integrin adhesion complexes, and Rho promotes actin contraction in the cell body (Mayor and Carmona-Fontaine, 2010) (Figure 1.6). Vinculin is a commonly expressed actin filament binding protein that localizes and concentrates in some sites of focal adhesions of cultured myoblasts (Le Clainche and Carlier, 2008). The focal adhesion sites and the role of vinculin in them are of particular interest for several reasons: focal adhesions are the main sites of cell adherence and they are located at the termini of stress fibres (Siegel et al., 2011). Vinculin expression levels have been shown to be higher in migrating cells, as it is mainly involved in the regulation of focal adhesions of migrating cells (Moon et al., 2004).

Two main pathways that are implicated in cell migration in response to HGF are Ras/MAPK and PI3K. Activation of these pathways results in cytoskeletal reorganization which is essential for cell motility and the formation of focal adhesions. These changes in actin organizations in satellite cells include extension of lamellipodia and formation of filopodia (Warn et al., 1993); (Grierson et al.,

2000) (Figure 1.6). Once activated to migrate, satellite cells can move into the injury site and repair the wound (Taher et al., 2002).

Migration of satellite cells isolated from the rat hind leg muscle has been shown to be stimulated by HGF (1-50 ng/ml) and inhibited at higher concentrations (100 ng/ml) (Bischoff, 1997). However, findings by Miller *et al*. in mouse skeletal muscle cells revealed that HGF increased cell migration at 6.25 ng/ml, but decreased in response to 50 ng/ml (Miller et al., 2000) (Table 1.3). Similarly, Suzuki *et al*., reported that exposure of C2C12 to exogenous HGF (15 ng/ml) increased migratory capacity of the cells, but 50 ng/ml HGF decreased migration (Suzuki et al., 2000).

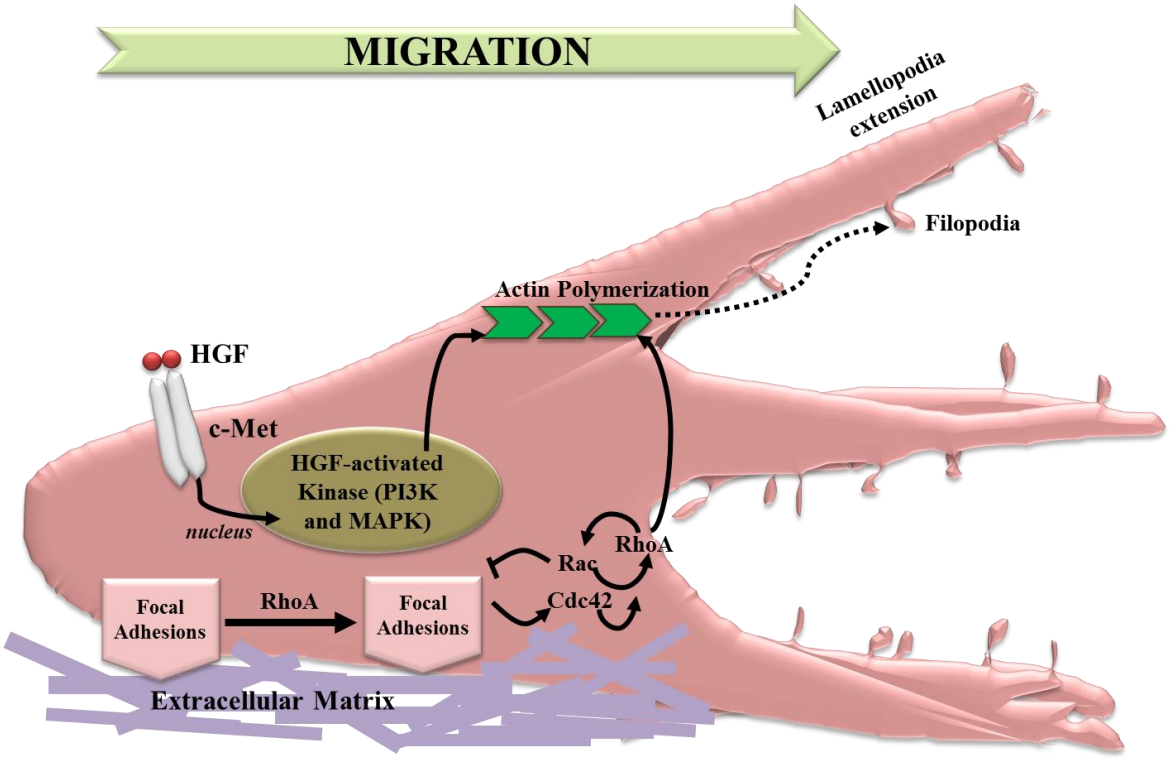

Figure 1.6: Mechanism of HGF-mediated satellite cell migration. HGF-c-Met binding results in autophosphorylation of c-Met leading to the activation of PI3K and MAPK which in turn regulate actin polymerization, filopodia and lamellopodia extension. A migrating cell needs to perform a coordinated series of steps to move starting with formation of filopodia, followed by lamellopodia extension and traction of the cells at the focal adhesion. Cdc42 regulates the direction of migration, Rac induces membrane projection at the front of the cell through stimulation of actin polymerization and integrin adhesion complexes, and Rho promotes actin contraction in the cell body. Diagram complied from the following references: Bischoff, 1994; Schultz et al., 1988; Mayor and Carmona-Fontaine, 2010; Warn et al., 1993.

1.3.5 Role of HGF during muscle differentiation

Following injury, skeletal muscle undergoes a process of degeneration and regeneration steps. These include proteolytic modifications of damaged myofibers and phagocytosis of debris by neutrophils, followed by necrotic changes during which damaged myofibers are removed by macrophages. Disruption of the basal lamina and plasma membrane releases HGF which then activates satellite cells to start proliferating and they ultimately differentiate into myotubes and myofibers. However, if the injury ruptures the basal lamina, fibroblasts migrate into the wound and deposition of collagen I occurs (fibrotic tissue); this prevents optimal muscle healing. It has been observed that TGF-β enhances the proliferation of fibroblasts and promotes formation of scar tissue, thereby impairing the healing process (Li et al., 2007; Shi and Garry, 2006). (*See Section 1.1.2*)

Similar to repair, regeneration requires the controlled up-regulation of MRFs (Myf5, myogenin, MyoD and myogenin) (Zammit et al., 2006b) (Figure 1.5). Myf5 is the earliest transcription factor to be expressed during myogenesis and is able to activate genes required for myogenic commitment. MyoD is a key transcription factor for myogenic differentiation and is implicated in specification of myogenic lineage. Expression of myogenin marks the beginning of terminal myogenic differentiation together with a variety of regulatory and structural muscle genes of skeletal muscle myocytes (eg myosin heavy chain, MyHC). Myogenin expression results in down-regulation of MyoD; and is also associated with fusion of myoblasts to new or existing myofibers leading to regeneration of the injured muscle (Le Grand, 2007).

HGF has been shown to inhibit differentiation of myoblasts via the down-regulation of MyoD, MRF-4 and myogenin (Charge and Rudnicki, 2004); (Halevy et al., 2004); (Leshem et al., 2000); (Rosen et al., 1990). This inhibitory activity of HGF on differentiation is through regulation of p27 and Twist protein. P27 is a cyclin-dependent kinase inhibitor, whereas Twist is an inhibitor of MyoD expression and therefore an inhibitor of the progression to terminal differentiation. When in the proliferative phase, satellite cell levels of p27 increase, but upon exit from cell cycle, cells down-regulate Twist expression and undergo differentiation. HGF induces Twist protein expression and down-regulates p27 levels; this supports the inhibitory effect of HGF on myogenic progenitor cells differentiation (Leshem et al., 2000).

Exogenous administration of HGF (5 ng/ml) to turkey satellite cells and embryonic myoblasts inhibits their differentiation while exhibiting variation in its ability to stimulate proliferation (Zeng et al., 2002). MyHC protein levels decrease when C2 satellite cells are exposed to increasing levels of HGF (0-50 ng/ml); suggesting an inhibition or delay in differentiation (Gal-Levi et al., 1998b). In addition, binding of HGF (50 ng/ml) to c-Met *in vitro*, has been shown to result in the silencing of MyoD and myogenin expression, inhibition of synthesis of MyHC as well as myotube formation of C2C12 myoblasts (Anastasi et al., 1997).

Table 1.3: Summary of HGF studies and main findings

	HGF concentrations	Cell lines	Effect	Reference
PROLIFERATION	2.5, 5, 10, 50, 100, 500 ng/ml	Rat satellite cells	>20ng/ml ↓	(Yamada et al., 2010a)
	0.3, 3, 30 ng/ml	Rat skeletal myoblasts	↑	(O'Blenes et al., 2010)
	10, 50 ng/ml	C2C12 myoblasts	↑	(Anastasi et al., 1997)
	2,20, 50 ng/ml	Chicken skeletal muscle cells & C2 cells	>20ng/ml ↓	(Gal-Levi et al., 1998)
MIGRATION	6.25, 50 ng/ml	Mouse skeletal muscle cells	↑	(Miller et al., 2000)
	0.1, 1, 6 nM	C2C12 myoblasts	↑	(Bandow et al., 2004)
	50 ng/ml	C2 myoblasts	↑	(Kim et al., 2011)
DIFFERENTIATION	2,20,50 ng/ml	Chicken skeletal muscle satellite cell & C2 cell lines	↓	(Gal-Levi et al., 1998)
	0, 0.1, 6 nM	C2C12 cells	↓	(Bandow et al., 2004)

Key: ↑ indicates an increase in HGF effect and ↓ indicates a decrease in HGF effect

1.4 AIMS

We have highlighted the role of HGF in regulating numerous key satellite cell functions. HGF has been shown to regulate proliferation, migration and differentiation of satellite cells during embryogenesis, wound repair and regeneration, but some results have been conflicting making it difficult to make overall deductions.

A better understanding of the effects of HGF on myogenic processes (proliferation, migration and differentiation) could aid our understanding of the process of myogenesis. Thus the present study was designed with the following objectives:

1. To investigate the role of different doses of exogenous HGF on proliferation, migration and differentiation of C2C12 mouse myoblasts versus primary cultured human skeletal myoblasts.
2. To utilize protein expression techniques to determine the molecular mechanisms underlying the effect of HGF on these processes.

CHAPTER 2

THE EFFECT OF HEPATOCYTE GROWTH FACTOR (HGF) ON C2C12 MYOBLAST PROLIFERATION, MIGRATION AND DIFFERENTIATION

ABSTRACT

Satellite cells are mononucleated skeletal muscle specific cells that are located between the basal lamina and sarcolemma of myofibers. These cells are crucial for skeletal muscle regeneration in the event of injury or growth and their activation is largely regulated by HGF. To date, studies of the role of HGF during myogenesis have yielded conflicting and contradictory results making it difficult to make overall deductions. In this study, we investigated the effect of three different concentrations of HGF (2, 10 and 50 ng/ml) on C2C12 myoblast proliferation, migration and differentiation. Cell counts were used to determine proliferation while migration was analysed using the scratch assay. To determine myogenic commitment, nuclear $Pax7^{+}/MyoD^{+}$ expression was analysed, while terminal differentiation was assessed through total MyHC expression analysis. Vinculin localisation and expression was also examined to elucidate potential mechanisms underlying effects seen on proliferation and migration. Our results support widely documented *in vitro* evidence that HGF stimulates proliferation and regulates migration and differentiation. We show that treatment of C2C12 cells with a low HGF concentration (2 ng/ml) promotes proliferation and decreases migration; under differentiation conditions, commitment to myogenesis and subsequent terminal differentiation were also decreased. In contrast, treatment of C2C12 cells with a five-fold higher HGF concentration (10 ng/ml) promotes C2C12 migration (via down-regulation of vinculin) and differentiation but decreases proliferation. These findings indicate the importance of HGF in skeletal muscle regeneration and highlight the sensitivity of satellite cells to changing HGF levels.

Keywords: Hepatocyte Growth Factor, C2C12 myoblasts, proliferation, migration, differentiation

2.1. INTRODUCTION

In response to skeletal muscle injury satellite cells are activated and enter the cell cycle. The activated satellite cells (termed myoblasts) undergo a series of proliferation steps (Halevy et al., 2004), after which they withdraw from the cell cycle, align, differentiate and fuse into myofibers (Schmalbruch, 2006). The activation of satellite cells and subsequent expansion of the myoblast population is regulated by growth factors, such as fibroblast growth factor (FGF), insulin growth factor-1 (IGF-1) and hepatocyte growth factor (HGF) (Gal-Levi et al., 1998b); (O'Reilly et al., 2008); (Yamada et al., 2010a); (Yamada et al., 2010b) (O'Blenes et al., 2010); (Miller et al., 2000). HGF is sequestered in the ECM of skeletal muscle and stored in an inactive form; however, upon injury it is released and consequently activates the satellite cell via receptor signalling mechanisms (Birchmeier and Gherardi, 1998). This growth factor has central roles in wound healing and tissue regeneration and regulates cell survival, motility and proliferation (Cornelison et al., 2004).

The proliferation rate of satellite cells and any other cell types depends on: 1) the rate of cell division, 2) the portion of the cell population undergoing cell division, 3) regulation by growth factors (Pardee, 1989). The production of HGF is elevated in muscle after damage, followed by a direct increase in satellite cell division and proliferation; suggesting a stimulatory effect of HGF on proliferation (O'Reilly et al., 2008). Some proliferation studies in C2C12 myoblasts indicated promotion of cell growth by HGF (at 10 and 50 ng/ml) (Anastasi, 1997). These findings correlated with similar studies in rat skeletal myoblast, where the rate of cell proliferation was shown to increase upon addition of HGF (at 3 and 30 ng/ml) (O'Blenes et al., 2010). In contrast, Yamada *et al*. demonstrated that high levels of HGF (over 10 ng/ml) suppress the proliferative activity of rat satellite cells (Yamada et al., 2010a). Gal-Levi *et al*. came to similar conclusions using the chicken skeletal muscle cells and C2 cells. They found a reduction in activation of quiescent satellite cells and a decrease in their growth upon exposure to exogenous HGF (20 and 50 ng/ml) (Gal-Levi et al., 1998).

Growth factor regulated changes in the actin cytoskeleton of satellite cells is essential for the maintenance of various cellular activities including proliferation and cytokinesis (Liu et al., 2008); (Khurana et al., 2002). One of the main cytoskeletal proteins with relevant implications is vinculin. Vinculin is a 117 kDa cytoskeletal adaptor protein located in the focal adhesions with central roles in cell contact regulation (Ezzell et al., 1997). Genetic inactivation of vinculin leads to paralysis and abnormalities in muscle structure and to embryonic lethality in mice (Marg et al., 2010). The absence of vinculin in satellite cells results in reduction of cell adhesion via the inhibition of focal

adhesion assembly and prevention of actin polymerization (a necessary step in proliferation) (Ezzell et al., 1997). Furthermore, the changes in vinculin organization and expression in response to HGF is a necessary step in satellite cell progression through the cell cycle, suggesting a possible regulation of proliferation (Ben-Ze'ev et al., 1990). Studies performed on vinculin-null cells and animals indicate that besides modulating cell adhesion, this protein also affects some aspects of satellite cell migration (Siegel et al., 2011).

Migration of satellite cells is a crucial step in skeletal muscle repair, regeneration and development (Ben-Ze'ev et al., 1990). Satellite cells in their migratory phase express high levels of vinculin (Le Clainche and Carlier, 2008) and upon exposure to HGF myoblasts show very rapid changes in morphology and cytoskeletal organization which is associated with changes in vinculin expression (Ben-Ze'ev et al., 1990). In rat muscle, exogenous HGF (10 ng/ml) resulted in increased migration and up-regulation of vinculin expression (Siegel et al., 2011); (Grierson et al., 2000). Vinculin influences the extension of lamellopodia during cell migration (Ezzell et al., 1997) and regulates satellite cell migration via ERK signalling pathway by controlling the accessibility of adaptor protein, paxillin for focal adhesion kinase (FAK) interaction (Subauste et al., 2004).

The MAPK/ERK signalling pathway is one of the downstream pathways activated when HGF binds to c-Met and is known to regulate satellite cell migration (Grierson et al., 2000). HGF is reported to act as a chemo-attractant of myoblasts and to increase their migration both *in vitro* and *in vivo* (Tatsumi et al., 2002); (Tatsumi and Allen, 2004). HGF null mutant mice completely lack muscle in the limb, supporting an important role of HGF in regulation of migration of precursor limb cells from the dermomyotome to their final location where they differentiate to form skeletal muscle (Bladt et al., 1995). Migration of satellite cells isolated from the rat hind leg muscle is stimulated by HGF (1-10 ng/ml) and is inhibited at higher HGF concentrations (100 ng/ml) (Bischoff, 1997). Similar results have been produced by Miller *et al*. in mouse skeletal muscle cells where HGF increased cell migration at 6.25 ng/ml, but decreased it at 50 ng/ml (Miller et al., 2000). HGF stimulated (0.1, 1 and 6 ng/ml) C2C12 cell expression of MMP-9 has been reported to be mediated through activation of PI3K, thereby increasing their migration capacity (Bandow et al., 2004).

Besides stimulating skeletal muscle precursor cell proliferation and migration, HGF is also implicated in regulating their differentiation during myogenesis (Li et al., 2008). Skeletal muscle differentiation is the final step of myogenesis regulated by several myogenic regulatory factors (MRFs) including MyoD, Myf-5, MRF-4 and myogenin. MRFs are proteins expressed in cells committed to differentiate and, together with Pax7, are implicated in specification of the myogenic lineage (McFarlane et al., 2008); (Buckingham et al., 2006); (Seale et al., 2004). Pax7 is expressed in quiescent satellite cells and is not only necessary for specification, but maintenance, survival and self-renewal of satellite cells (Kuang et al., 2006); (Seale et al., 2004); (Zammit et al., 2006b). Following activation and proliferation, satellite cells initially co-express Pax7 and MyoD (McFarlane et al., 2008), but subsequently down-regulate Pax7 in order to allow the induction of myogenin and Myosin Heavy Chain (MyHC) during terminal differentiation (Knapp et al., 2006).

Accumulating evidence shows that HGF influences differentiation of myoblasts via the down-regulation of MyoD, Myf-5, MRF-4 and myogenin (Charge and Rudnicki, 2004); (Halevy et al., 2004); (Rosen et al., 1990). Leshem *et al*. showed that the inhibiting effects of HGF on differentiation is through co-ordinated regulation of p27 (a cyclin-dependent kinase) and Twist protein (a basic helix-loop-helix transcription factor) (Leshem et al., 2000). Exogenous administration of HGF (5 ng/ml) to turkey satellite cells and embryonic myoblasts inhibit their differentiation while exhibiting variation in its ability to stimulate proliferation (Zeng et al., 2002). MyHC protein levels decrease when satellite cells are exposed to increasing levels of HGF (0-50 ng/ml); suggesting an inhibition or delay in differentiation (Gal-Levi et al., 1998b).

Previous work on HGF has focused on proliferation in combination with either migration or differentiation; in our study we aimed to investigate the effect of HGF on all three processes (proliferation, migration and differentiation) in C2C12 myoblasts. According to the literature, a wide range of HGF doses (0.1-100 ng/ml) have been used in numerous cell lines, using various analysis methods, culture periods and culture protocols; inconsistencies were therefore reported. In this study, we investigated the effect of three different concentrations of HGF (2, 10 and 50 ng/ml) on proliferation, migration and differentiation. The effect of HGF on protein expression levels of vinculin, Pax7, MyoD and MyHC was also assessed.

2.2. EXPERIMENTAL PROCEDURE

2.2.1. Cell culture

Murine C2C12 cells were donated by the Cape Heart Centre (University of Cape Town). This immortalized cell line was originally derived from satellite cells from the thigh muscle of female mice and are adherent in culture. They can be maintained in an undifferentiated state if cultured and maintained in sub-confluent state in growth media; however, in differentiation media, they differentiate first into myocytes and after several days in media they form myotubes (Yaffe and Saxel, 1977). C2C12 cells have myogenic properties and were used for the experiments to analyse proliferation, migration and differentiation. Passages ranging from 11-20 were used.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Scientific Laboratories, USA, cat.SH30243.01) supplemented with 10% (v/v) fetal bovine serum (Biowest, USA, cat.S181H-500), 2% (v/v) L-glutamine (Lonza, USA, cat.BE17-605E) and 2% (v/v) Penicillin-Streptomycin (Lonza, USA, cat.DE17-602E). This growth media was used for both proliferation and migration assays. For differentiation studies, cells were cultured to 70-80% confluence then changed from growth media to DMEM supplemented with 2% (v/v) horse serum (Biowest, USA, cat.S090H-100). Human HGF (PeproTech, USA, cat.100-39) was used at a final concentration of 2, 10 and 50 ng/ml. C2C12 cells were incubated at 37^{o}C in a humidified atmosphere at 5% CO_2. All experiments were carried out under sterile conditions in a Class II Biohazard Safety Cabinet.

2.2.2. Determination of C2C12 myoblast proliferation:

2.2.2.1. Crystal violet assay

Crystal violet assay was conducted as previously described by Gillies et al., (Gillies *et al*., 1986). Briefly, to analyse the effect of HGF on proliferation, a standard curve was first generated (*see Appendix E*) then 3 x 10^4 cells/well were seeded in 6-well plates containing growth media and incubated at 37^{o}C, 5% CO_2 for 24 h. The media was changed to growth media containing 0, 2, 10, 50 ng/ml HGF and cells were incubated for a further 24 h. Cells were then stained with crystal violet solution (Sigma, Germany, cat.C-3886) and absorbance determined at 595 nm using an ELISA microplate reader (Optima, USA).

2.2.2.2. Cell count assay

Cells (5 x 10^4) were seeded in T25 tissue culture flasks (Nunc, USA, cat.65232) containing growth media and incubated at 37^{o}C, 5% CO_2 for 24 h. HGF (0, 2, 10 and 50 ng/ml) was then added to the flasks and incubated for a further 24 h. Thereafter, cells were trypsinised for 5 min and a small sample (20 µl) was removed and transferred onto a Hemocytometer counting chamber where cells were carefully counted using a brightfield microscope (10 X magnification).

2.2.3. Cell viability test

Cells were plated at 3 x 10^4 per T25 flask and incubated at 37^{o}C, 5% CO_2 for 24 h. The growth media was replaced with normal growth media, growth media containing HGF (10 ng/ml) or serum-free growth media (as a positive control) (Niesler et al., 2007), and cells incubated for a further 24 h. Cell were stained with trypan blue (Biorad, USA, cat.145-0013) and viability determined using an automated TC-20 cell counter (Bio-Rad, USA, serial.508BR1910).

2.2.4. Migration: Scratch assay

C2C12 myoblasts were plated at 5 x 10^4 cells/well on coverslips in 24-well culture plates (Cell Star, USA, cat.66210) and allowed to reach 80% confluence. Cells were scratched as described previously (Goetsch and Niesler, 2011) using a sterile loading tip and fresh growth media containing 0, 2, 10 or 50 ng/ml HGF was added. The rate of wound closure was observed using the Olympus CKX41 brightfield microscope at 0, 3, 5 and 7 h and images were taken at the different time points with *Motic* 3 megapixel camera. The edges of the wound were traced using *Motic 2.0* image analysis software which measured the area of the wound. Percentage wound closure was then calculated using the following equation:

$$\% \text{ Wound closure} = \frac{\text{Area of wound at 0 h- Area of wound x h}}{\text{Area of wound at 0 h}} \times 100$$

2.2.5. Immunocytochemistry (Proliferation and Migration)

Proliferating and migrating cells were fixed in 4% paraformaldehyde for 15 min, blocked with 5% donkey serum (Biowest, USA, cat.S2170-100) for 30 min. Differentiating cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma, Germany, cat.T9284) for 20 min and blocked with 5% donkey serum for 30 min. Primary antibodies were added overnight and dilutions used were as follows: monoclonal mouse anti-vinculin antibody (Sigma, USA, cat.V9131; 1:2000), monoclonal goat anti-Pax7 antibody (Santa Cruz, USA, cat.sc-7748; 1:100), rabbit polyclonal anti-MyoD (Santa Cruz, USA, cat.sc-760; 1:100), monoclonal mouse MF-20 (Developmental Hybridoma Studies, USA, cat.M29; 1:100). DyLight 488-conjugated AffiniPure donkey anti-rabbit lgG (Jackson ImmunoResearch, USA, cat.CN-711-485-152), DyLight 549-conjugated AffiniPure donkey anti-goat lgG (Jackson ImmunoResearch, USA, cat.CN-703-505-003) and DyLight 594-conjugated AffiniPure donkey anti-mouse lgG secondary antibodies (Jackson ImmunoResearch, USA, cat.CN-715-515-151) were used at 1:1000 and incubated for 1 h. Hoechst nuclei stain (Sigma, Germany, cat.B2261; 10 µg/ml stock; 1:2000) was added to all the cells for 5 min. Moviol was used as the mounting agent. All images were captured using the Zeiss 710LSM confocal microscope.

2.2.6. Percentage MyoD and Pax7 expressing myoblasts and Differentiation index

Five random fields of view of differentiated cells (> 30 cells/field of view) were captured using the Zeiss 710LSM confocal microscope and analysed with ImageJ software (http://rsbweb.nih.gov/ij/) or Zen 2009 software. The percentage MyoD and Pax7 expressing myoblasts, as well as the differentiation index were calculated using the following formulae:

$$\%\ \text{MyoD+ cells} = \frac{\text{Number of nuclei staining positive for MyoD}}{\text{Total number of nuclei}} \times 100$$

$$\%\ \text{Pax7+ cells} = \frac{\text{Number of nuclei staining positive for Pax7}}{\text{Total number of nuclei}} \times 100$$

$$\%\ \text{Differentiation index} = \frac{\text{Number of MyHC positive cells (myocytes/myotubes)}}{\text{Total number of nuclei}} \times 100$$

2.2.7. Immunocytochemistry (Differentiation)

Cells were differentiated for 5 days then fixed in 4% paraformaldehyde containing 0.1% Triton X-100 (Sigma, Germany, cat.T9284) for 20 min, blocked with 5% donkey serum for 30 min. Phalloidin stain (Sigma, USA, cat.P1951; 1:16 000) was added for 45 min. Hoechst nuclei stain (Sigma, Germany, cat.B2261; 10 µg/ml stock; 1:2000) was added to all the cells for 5 min. Five random fields of view were captured using the Zeiss 710LSM confocal microscope. ImageJ software was utilized to determine formation of myotubes. The fusion index for myotube formation was calculated using the following formula:

$$\% \text{ Fusion index} = \frac{\text{Number of nuclei per myotube (two or more nuclei)}}{\text{Total number of nuclei}} \times 100$$

2.2.8. Western blotting

We performed electrophoretic separation of proteins from C2C12 lysates prepared on day 0 (in growth media), day 5 (in differentiation media) of control and HGF (2, 10, 50 ng/ml) treated cultures. Proteins (30 µg) were loaded onto 12.5% Tris/Glycine reducing SDS-PAGE and transferred onto nitrocellulose membrane (Life Sciences, South Africa, cat.P/N 66485) for Western Blot. Primary antibodies and dilutions used were as follows: monoclonal mouse anti-vinculin antibody (Sigma, USA, cat.V9131; 1:2000); mouse anti-MyHC (MF-20) (Developmental Studies Hybridoma Bank, USA, cat.M29; 1:100); mouse monoclonal α-tubulin (Santa Cruz, USA, cat.sc-5286; 1:50). Anti-mouse (Dako, Denmark, cat.P-0260) or anti-rabbit (Dako, Denmark, cat.P-0448) HRP conjugated secondary antibodies were used at 1:8000 and 1:4000, respectively. (Incubation times in primary and secondary antibodies were 2 h and 1 h, respectively). HRP activity was visualized using Enhanced Chemiluminescence (ECL) (Bio-Rad, USA, cat.170-570) and GeneSys Image Acquisition software (Vacutec, South Africa). Densitometry analysis was done using the Versa Doc Imaging System (Bio-Rad, USA) and a computer program called Quantity One and ImageJ.

2.2.9. Statistics

All statistical analysis was performed with either a Student's t-test for parametric data or a Mann-Whitney-U test for non-parametric data, with values of $p<0.05$ considered statistically significant. The results from each experiment were expressed as Mean ± standard error of the mean (SEM). All experiments were completed in duplicate with each experiment repeated 3-9 times (n=3-9).

2.3. RESULTS

This study investigated the effect of HGF on C2C12 myoblast proliferation, migration and differentiation. In all experiments; 0, 2, 10 and 50 ng/ml HGF was tested, as these represented the range of doses used in literature. Proliferation was investigated via two quantitative methods namely, manual cell counts and the crystal violet assay. Following proliferation, we focused on analysing the effect of HGF on C2C12 myoblast migration, using a scratch assay. Finally we investigated the role of HGF in C2C12 myoblast differentiation via analysis of myotube formation and expression of transcription factors specific for differentiation ($Pax7^+/MyoD^+$) and the structural protein MyHC.

2.3.1. HGF affects C2C12 myoblast proliferation in a dose-dependent manner

Study of the proliferative capacity of myoblasts aids in the understanding of the functionality of the cells (Anastasi, 1997). Therefore, to assess the proliferative capacity of C2C12 myoblasts upon exposure to 0, 2, 10 and 50 ng/ml HGF, we used two quantitative assays, namely manual cell counts and the crystal violet assay. Brightfield analysis revealed that the confluence of the cells was lowest at 10 ng/ml HGF compared to untreated control, 2 and 50 ng/ml HGF (Figure 2.1A), suggesting that C2C12 myoblast proliferation capacity was down-regulated in response to 10 ng/ml HGF.

Quantification revealed a dose-dependent effect of HGF on C2C12 cell number in both assays. Cells treated with 2 ng/ml HGF showed a significant increase in cell number ($2.27 \pm 0.146 \times 10^5$ cells) compared to the untreated control ($1.74 \pm 0.189 \times 10^5$ cells, $p<0.01$). This increase was also significant compared to 50 ng/ml HGF ($1.84 \pm 0.186 \times 10^5$ cells, $p<0.01$). A significant decrease in cell number ($1.15 \pm 0.145 \times 10^5$ cells) was demonstrated when cells were treated with 10 ng/ml HGF compared to control ($p<0.01$). This effect was also significant compared to cells treated with 2 ng/ml and 50 ng/ml HGF ($p<0.01$) (Figure 2.1B).

Utilisation of the crystal violet assay confirmed the dose-dependent effect of HGF on C2C12 cell number. A small non-significant increase in cell number at 2 ng/ml HGF ($7.87 \pm 1.08 \times 10^4$ cells) was detected compared to the untreated control ($7.41 \pm 1.20 \times 10^4$ cells). However, HGF (10 ng/ml) resulted in a significant decrease in cell number ($5.79 \pm 0.572 \times 10^4$ cells) compared to both untreated control and 2 ng/ml HGF ($p<0.05$). At 50 ng/ml, cell numbers approached control values ($7.02 \pm 0.236 \times 10^4$ cells) and were significantly increased compared to 10 ng/ml HGF ($p<0.05$) (Figure 2.1C).

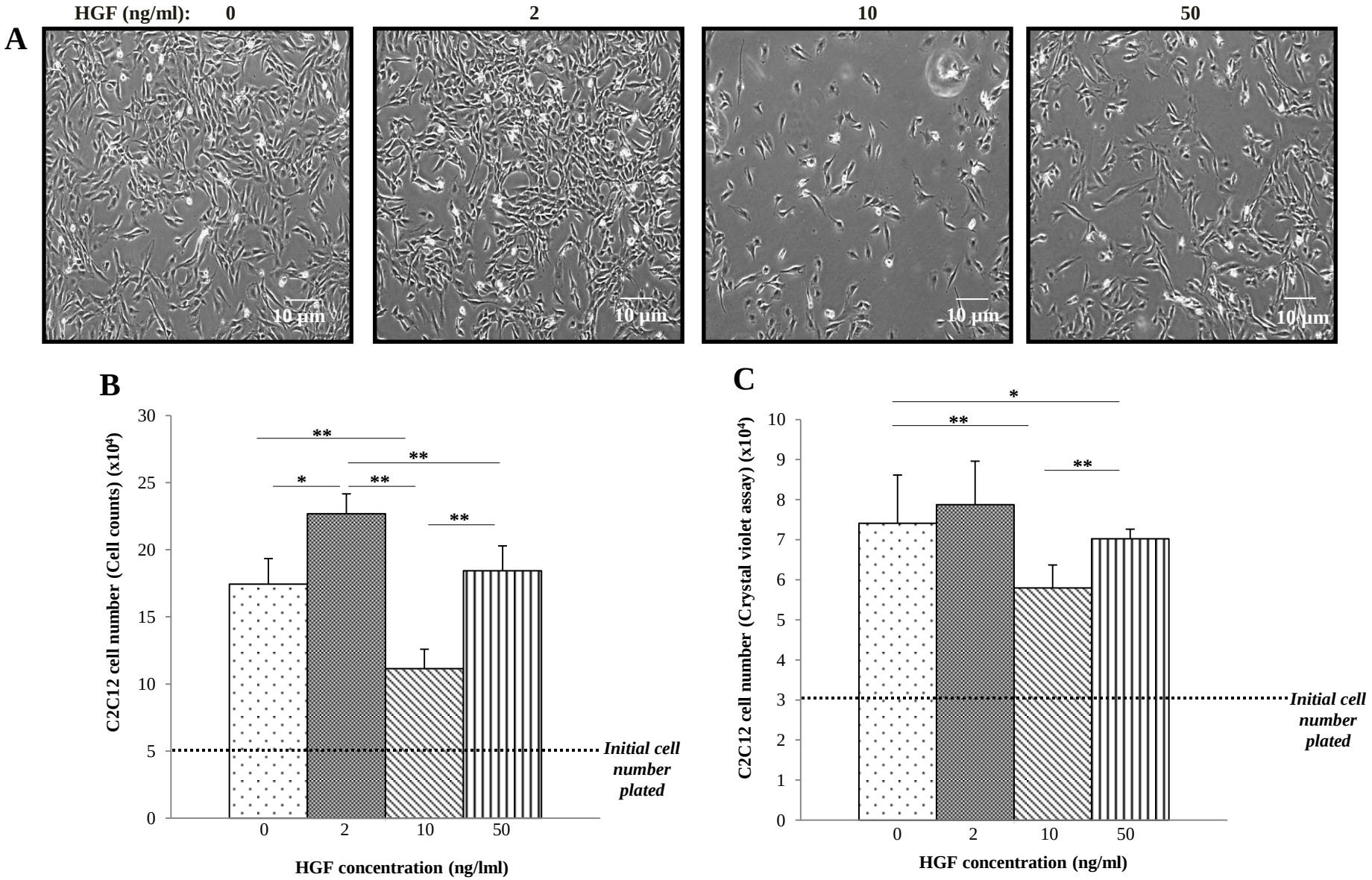

Figure 2.1: Proliferation of C2C12 myoblasts in response to HGF. Equal numbers of cells were plated and cultured for 24 h under proliferation conditions, followed by treatment with HGF for a further 24 h. **A**) Phase contrast images indicate the confluence levels of C2C12 myoblasts under different HGF concentrations (0, 2, 10 or 50 ng/ml). Images were captured using *Motic* 2.0 camera at 40 X magnification. Scale bar = 10 µm. **B**) Analysis of proliferation via cell counts. **C**) Analysis of proliferation via crystal violet assay. ** $p<0.01$, *$p<0.05$. All data is mean ± SEM. n=6.

To confirm that the decrease in proliferation observed at 10 ng/ml was not due to apoptosis, we performed a live-dead assay i.e. Trypan blue assay (Figure 2.2). Images of cells cultured in the absence of serum (positive control) revealed fewer cells per field of view; however, both control and HGF-treated (10 ng/ml) cells appeared to have similar cell numbers (Figure 2.2A). Trypan blue viability demonstrated a significant decrease in viability in serum free conditions (61.3 ± 4.41%) compared to control (89 ± 4.58, $p<0.05$) (Figure 2.2B). However, in response to HGF no significant difference in percentage dead cells (13 ± 1.68) was seen compared to control (11 ± 5.57) (Figure 2.2B). Together, the trypan blue and morphological assessments suggest that the effect of HGF (10 ng/ml) on cell number is not due to apoptosis.

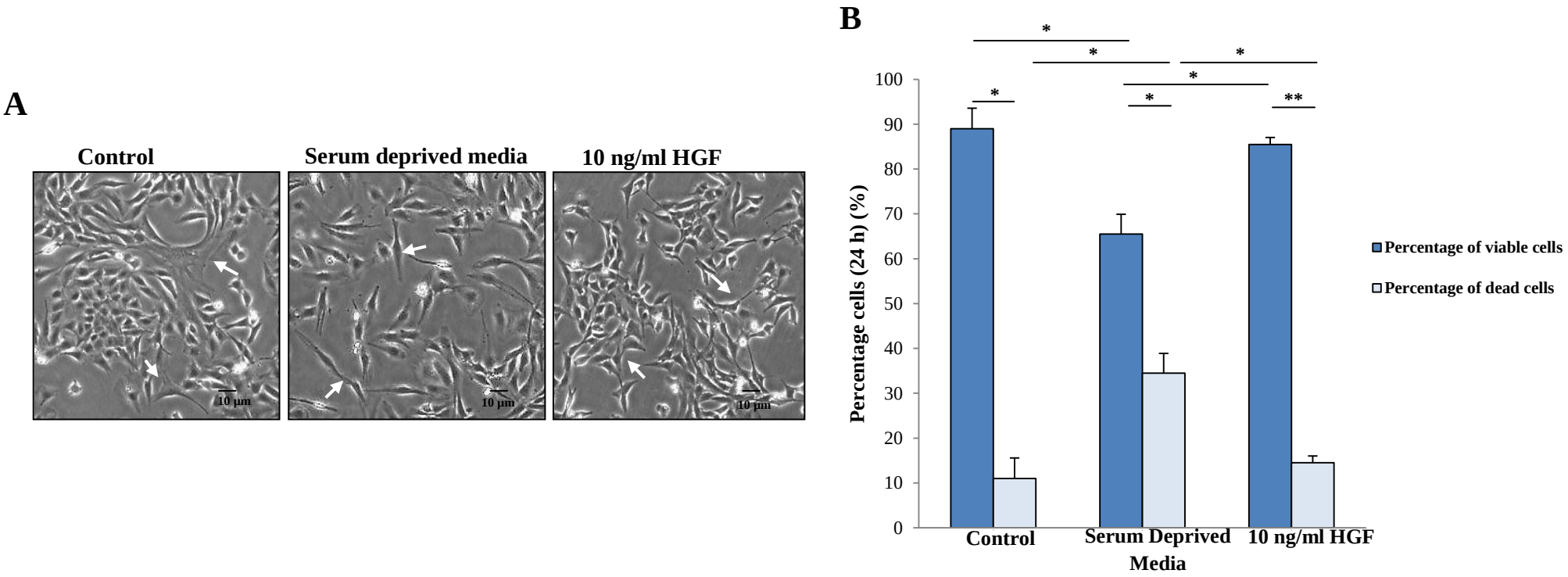

Figure 2.2: Trypan blue assay of C2C12 cells under different growth media conditions. C2C12 myoblasts were cultured in normal growth media for 24 h followed by replacement with either growth media, serum-deprived or growth media containing 10 ng/ml HGF for a further 24 h. The arrows indicate distinct morphologies observed under different conditions. **A)** Phase contrast images of C2C12 cells under different culture conditions captured using *Motic* 2.0 camera at 40 X magnification. Scale bar = 10 µm. **B**) Trypan blue viability test under different growth media conditions. *$p<0.05$; **$p< 0.01$. All data is mean ± SEM, n=3

2.3.2. HGF affects vinculin expression in proliferating C2C12 myoblasts in a dose-dependent manner.

Given the decreased C2C12 proliferation observed in response to 10 ng/ml HGF, examination of the expression of vinculin in these cells would aid in understanding the mechanism via which HGF affects proliferation. HGF-treated C2C12 lysates were electrophoretically separated, and transferred onto nitrocellulose membrane, followed by probing with anti-vinculin antibody. Cells treated with 10 ng/ml HGF had the most prominent vinculin expression levels compared to the untreated control, 2 ng/ml and 50 ng/ml HGF (Figure 2.3A). Densitometry analysis revealed an insignificant decrease in vinculin expression in response to 2 ng/ml HGF (0.693 ± 0.237) compared to untreated control (0.958 ± 0.18), and an increase in vinculin expression in response to 10 ng/ml HGF (1.13 ± 0.07) compared to untreated control. A significant increase in vinculin expression was observed in response to 10 ng/ml compared to 2 ng/ml HGF ($p<0.05$) (Figure 2.3B). Since changes in vinculin expression in response to HGF are a necessary step in satellite cell progression through the cell cycle, this data suggests a possible regulation of myoblast proliferation by HGF through vinculin expression which contributes to the dose-dependent effect of HGF on C2C12 myoblast proliferation.

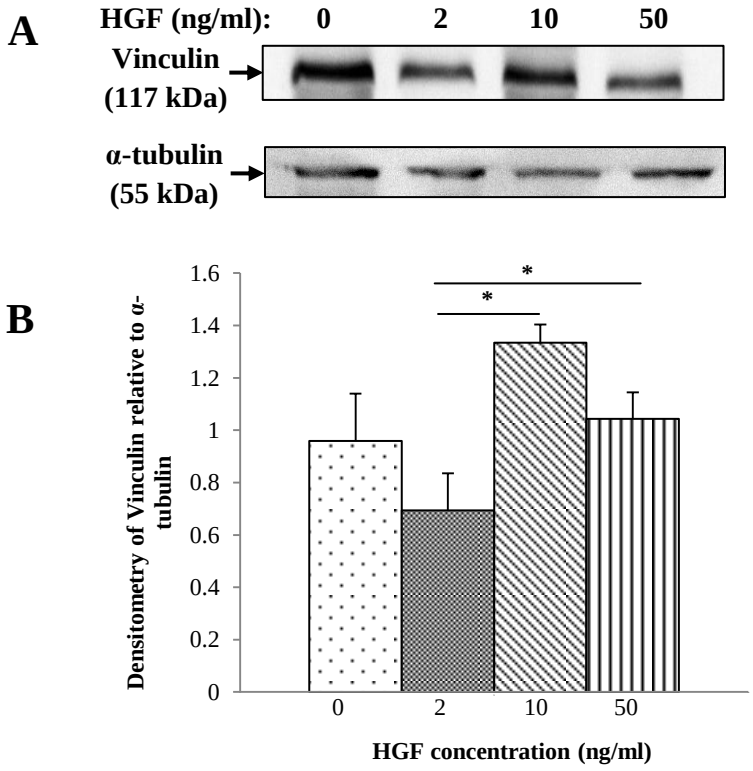

Figure 2.3: Vinculin expression in proliferating C2C12 myoblasts treated with 2, 10, 50 ng/ml HGF. A) Band intensities of vinculin observed in response to 0, 2, 10 and 50 ng/ml HGF. **B**) Densitometry analysis of western blot using imageJ software. All data is mean ± SEM. n=3**.** * $p<0.05$

2.3.3. HGF regulates the percentage wound closure of C2C12 myoblasts

To test the effect of HGF on C2C12 myoblast migration, we utilized the scratch assay (Goetsch and Niesler, 2011) and calculated the percentage wound closure and the rate of migration over a 7 h period. HGF (10 ng/ml and 50 ng/ml) was observed to decrease the wound size, suggesting increased migration (Figure 2.4A).

Following 3 h post-wounding, there was a significant increase in percentage wound closure in response to 10 ng/ml HGF (29 ± 3.98%) compared to 2 ng/ml HGF (17.6 ± 3.22%, $p<0.05$). Following 5 h, there was a significant increase in percentage wound closure at 10 ng/ml HGF (51 ± 2.13%) compared to untreated control (36.4 ± 3.71%, $p<0.01$). This increase was also significant compared to 2 ng/ml (31.1 ± 4.65%, $p<0.01$) and 50 ng/ml HGF (39.3 ± 3.22%, $p<0.05$). Finally, after 7 h, HGF (10 ng/ml) significantly increased migration (65.8 ± 3.67%) compared to untreated control (50.2 ± 3.12%, $p<0.05$) and 2 ng/ml HGF (43.4 ± 4.83%, $p<0.01$). At 50 ng/ml, HGF did not significantly change migration compared to untreated control (Figure 2.4B).

The rate of migration over the 7 h period (Figure 2.4C) was determined by calculating gradients from the percentage wound closure graph (Figure 2.4B). HGF significantly increased the rate of migration in response to 10 ng/ml (8.718 ± 0.609%/h, $p<0.05$) and decreased the rate of migration at 2 ng/ml HGF (6.016 ± 1.098%/h, $p<0.05$) compared to untreated control (7.194 ± 0.638%/h). HGF (50 ng/ml) did not significantly change rate of wound closure in comparison to untreated control, however, a significant increase in migration rate was seen compared to 2 ng/ml HGF (8.219 ± 0.54%/h, $p<0.05$) (Figure 2.4C). Interestingly, the graphical analysis of the effect of HGF on the rate of migration (Figure 2.4C) resembled a similar trend as the vinculin expression data (Figure 2.3B), where both graphs showed an increase in vinculin expression and rate of migration in response to 10 ng/ml and a decrease in response to 2 ng/ml HGF.

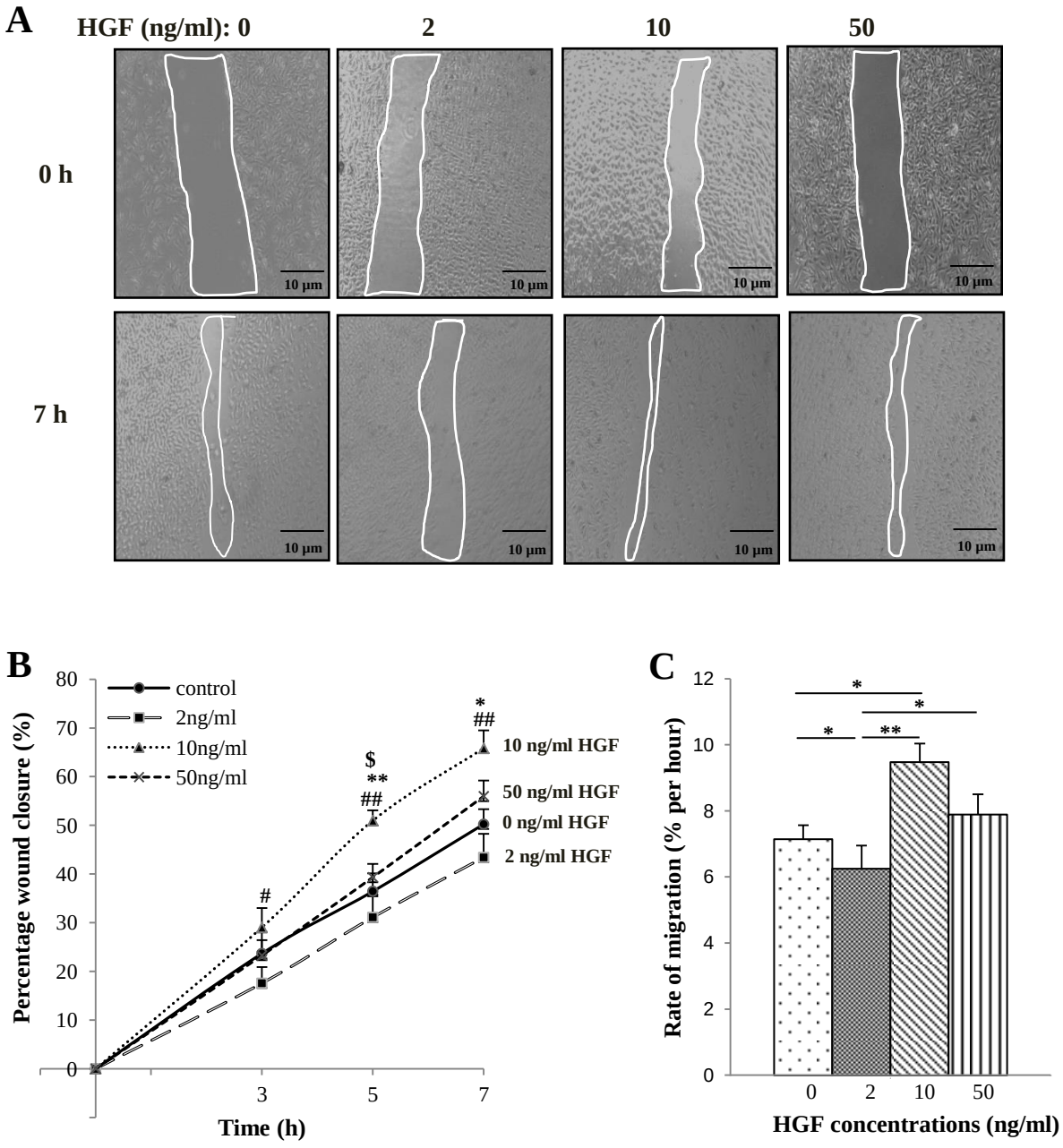

Figure 2.4: HGF increases migration of C2C12 myoblasts. A) Phase contrast images of wound closure in C2C12 myoblasts following incubation with different doses of HGF (40 X magnification). A scratch assay was performed and cells treated with 0, 2, 10 or 50 ng/ml HGF. The white lines within the images highlight the scratch front. Images at 0 and 7 h are shown. Scale bar = 10 µm. **B**) Graph of percentage wound closure over 7 h. ** = *p<0.01*, * = *p<0.05*. **C**) Graph of rate of migration generated from the gradients of Figure 2.4 B. All data is mean ± SEM. # indicates significant difference between 2 and 10 ng/ml HGF; * indicates a statistical significance between 0 and 10 ng/ml HGF; $ indicates a statistical significance between 10 ng/ml and 50 ng/ml HGF. **/## = *p<0.01*, */#/$ = *p<0.05*. n=9

2.3.4. HGF dose-dependently regulates vinculin intensity in migrating C2C12 myoblasts

Here we sought to further determine the mechanism underlying the effect of HGF on C2C12 myoblast migration. Using immunocytochemistry, we assessed vinculin intensity levels in cells at the leading front following 7 h incubation with 0, 2, 10 and 50 ng/ml HGF. Confocal imaging revealed that all the cells showed vinculin labelling. Increased vinculin levels were observed in response to 10 ng/ml HGF compared to untreated control, where cells formed large vinculin positive plaques at their leading fronts. However, cells treated with 2 ng/ml HGF expressed vinculin at similar levels to untreated control (Figure 2.5A).

Quantification of vinculin intensity revealed that the effect of HGF followed a hyperbolic shape. A significant increase in vinculin intensity level in cells treated with 10 ng/ml HGF (0.764 ± 0.277) and 50 ng/ml HGF (0.575 ± 0.158) compared to untreated control (0.246 ± 0.157, $p<0.01$) and 2 ng/ml HGF (0.387 ± 0.237, $p<0.01$). The vinculin intensity levels at 2 ng/ml HGF was not different to untreated control (Figure 2.5B). These findings highlight that HGF dose-dependently regulates vinculin expression in migrating C2C12 myoblasts; this may contribute to the regulation of the migratory capacity of these cells.

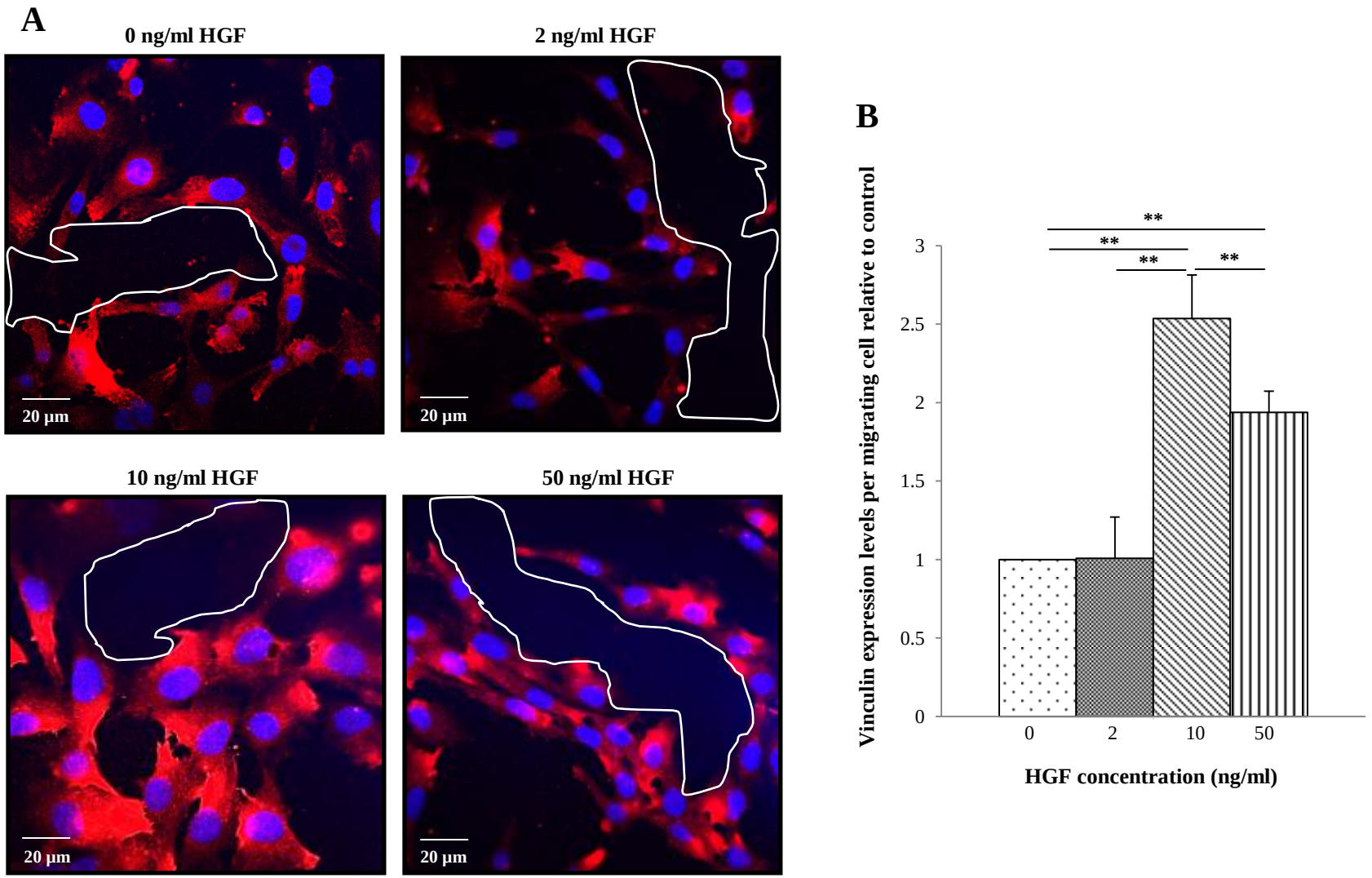

Figure 2.5: Vinculin expression in HGF-treated migrating C2C12 myoblasts. A) Cells were scratched and allowed to migrate for 7 h followed by fixation, immunocytochemistry and analysis using the Zeiss Confocal microscope at 40 X magnification. Scale bar = 20 µm. Red indicates vinculin whilst the blue indicates the cell nuclei stained with Hoechst. The white lines within the images highlight the scratch front. **B**) Vinculin expression levels in migrating cells were assessed relative to control using ImageJ. All data is mean ± SEM. n=3**.** ** *p<0.01*.

2.3.5. Pax7 expression levels in differentiating C2C12 cells are regulated by exogenous HGF

We next examined whether HGF induces dose-dependent changes in the expression of Pax7 transcription factors. Pax7 is implicated in specification of myogenic linage and its down-regulation is necessary for differentiation to occur (McFarlane et al., 2008). Cells were cultured to 80% confluence then switched to differentiation media containing 0, 2, 10 and 50 ng/ml HGF and cultured for 3 days; Pax7 expression was then analysed by confocal microscopy on day 1, 2 and 3.

Pax7 expression was observed to be maintained longer in response to 2 ng/ml HGF compared to untreated control. In contrast, in response to 10 ng/ml HGF, Pax7 was down-regulated earlier than untreated control (Figure 2.6).

Pax7 expression on day one post-differentiation increased in response to 2 ng/ml HGF (61.4 ± 16.6%) relative to untreated control cells (40.8 ± 10.2%), but this was not statistically significant (Figure 2.7). On day 2, a reduction in Pax7 expression was observed in response to 10 ng/ml HGF compared to untreated control levels (Figure 2.7); this decrease was significant (22.4 ± 0.88%) compared to untreated control (35.8 ± 5.75%, $p<0.05$) and 50 ng/ml HGF (28.4 ± 1.35%, $p<0.01$). Cells treated with HGF (2 ng/ml) showed higher Pax7 levels (43 ± 6.79%) compared to untreated control. On day 3, Pax7 expression was almost undetectable in untreated control (3.66 ± 2.16%) as well as cells treated with 10 ng/ml HGF (2.79 ± 1.5%) and 50 ng/ml HGF (2.16 ± 0.71%) cells. However, HGF (2 ng/ml) showed greater (maintained) Pax7 expression (13.6 ± 2.88%, $p<0.01$) compared to untreated control ($p<0.01$).

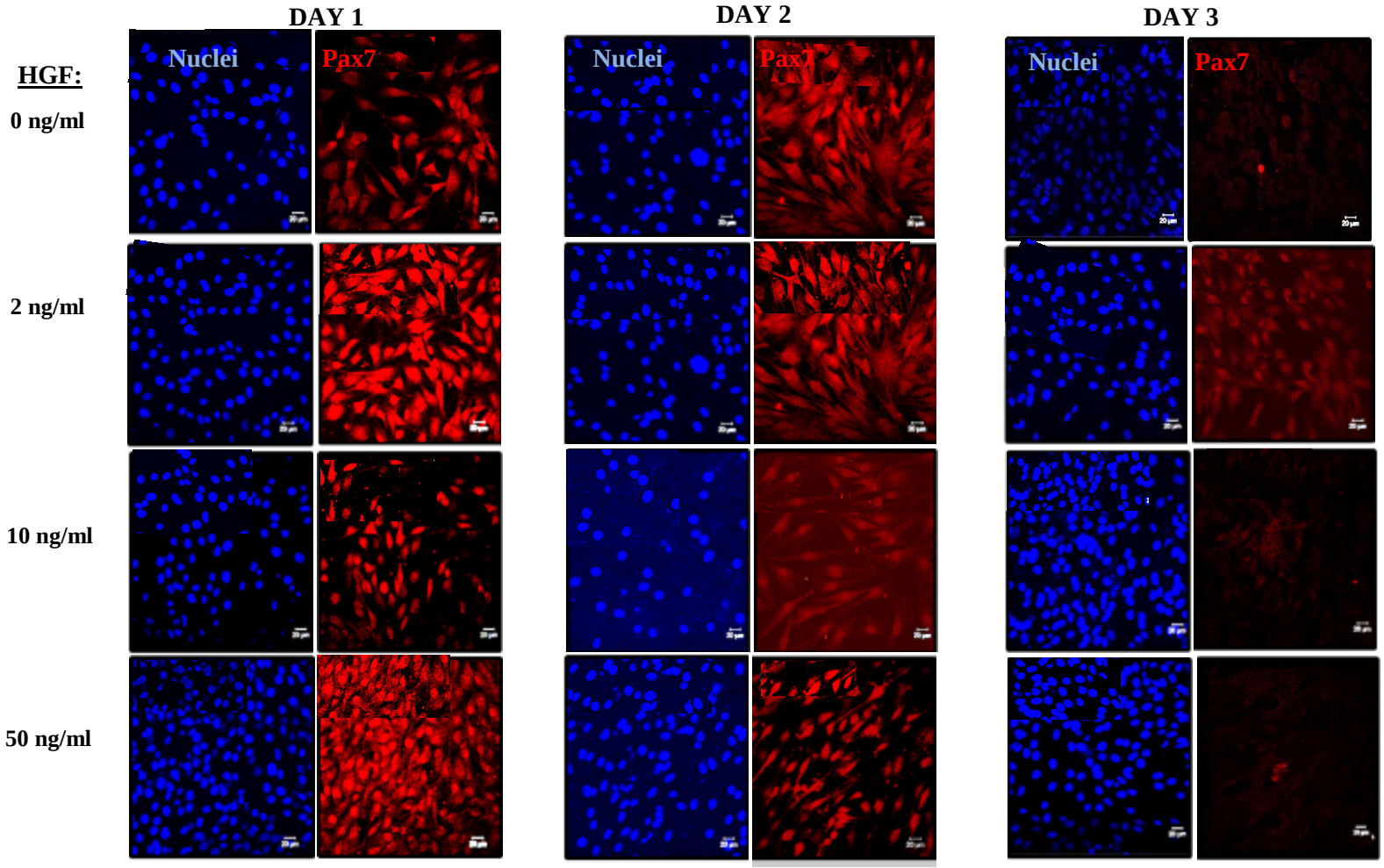

Figure 2.6: Pax7 expression of C2C12 myoblasts in response to HGF on day 1, 2 and 3 post differentiation. Cells were allowed to differentiate for 1-3 days followed by fixation, immunocytochemistry at then analysis using the Zeiss confocal microscope. Scale bar = 20 µm. Blue indicates the Hoechst nuclear stain, red indicate Pax7.

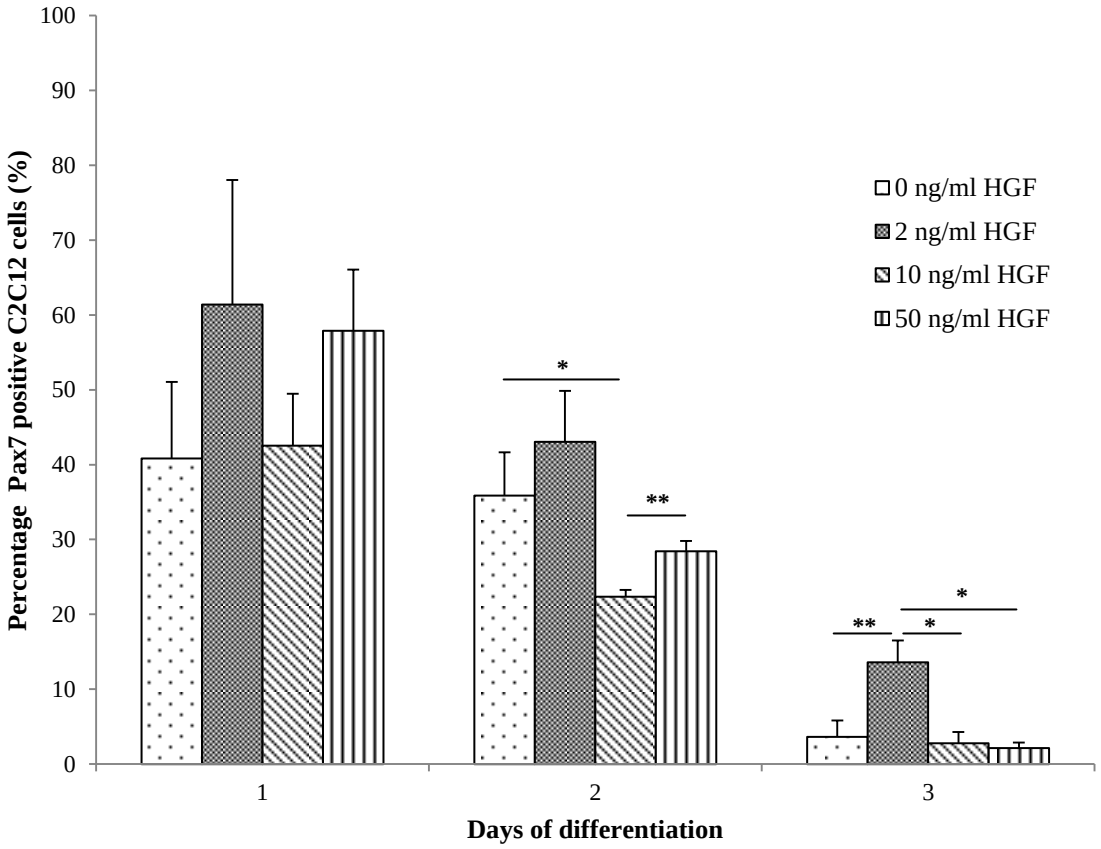

Figure 2.7: Percentage Pax7^{+} differentiating C2C12 cells in response to HGF. Cells were cultured in growth media until they were 80% confluent. Differentiation media was then added and cells were fixed with 4% paraformaldehyde at day 1, 2, 3 and then probed with anti-Pax7 antibody. Five random fields of view were observed for each treatment (from Figure 2.6) and quantified using ImageJ. ** *p<0.01*, **p<0.05*. All data is mean ± SEM. n=3.

2.3.6. MyoD expression levels in differentiating C2C12 cells are regulated by exogenous HGF

MyoD is a transcription factor expressed in cells committed to differentiation (McFarlane et al., 2008); (Buckingham et al., 2006); (Seale et al., 2004). Cells were cultured to 80% confluence then switched to differentiation media containing 0, 2, 10 and 50 ng/ml HGF and cultured for 3 days; MyoD expression was analysed by confocal microscopy at day 1, 2 and 3.

At day 1 of differentiation a notable increase in nuclear MyoD expression was observed in response to 10 ng/ml HGF compared to untreated control cells. On day 2, cells exposed to 10 ng/ml HGF continued to show higher MyoD expression compared to untreated control cells, but expression was observed to decline by day 3. In response to 2 ng/ml HGF, cells appeared to display lower intensity of MyoD compared to control (Figure 2.8).

Quantification of MyoD expression showed that there was a significant decrease in percentage MyoD expressing cells in response to 2 ng/ml HGF (24.5 ± 1.65%) compared to untreated control (32.6 ± 3.33%, $p<0.01$). At 10 ng/ml we found a significant increase in MyoD positive cells compared to untreated control ($p<0.01$). Cells treated with 50 ng/ml HGF also showed a significant increase in the percentage of MyoD expression ($p<0.01$) compared to untreated control. On day 2 an overall increase in percentage MyoD expressing cells was observed under all conditions compared to day 1. The greatest percentage was seen in response to 10 ng/ml HGF (69.9 ± 6.08%), and this increase in MyoD was significant compared to 2 ng/ml HGF ($p<0.01$) and 50 ng/ml HGF ($p<0.05$). On day 3 a decrease in MyoD expression was demonstrated compared to expression levels observed on day 1 and 2. A significant change in percentage MyoD expressing cells was demonstrated in response to 2 ng/ml HGF (30.3 ± 2.23%) compared to 50 ng/ml HGF (38.3 ± 7.27%, $p<0.05$) (Figure 2.9).

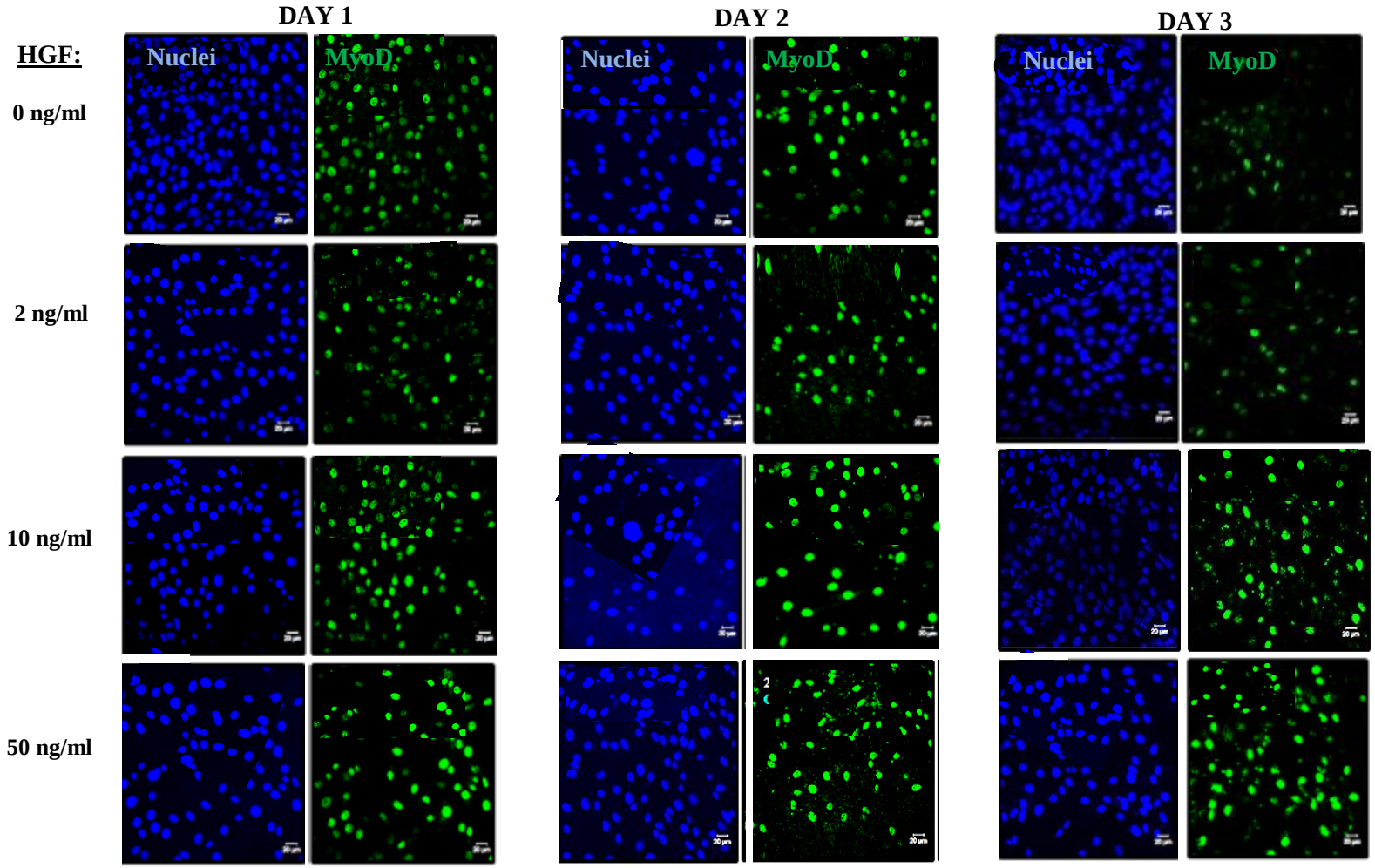

Figure 2.8: MyoD expression of C2C12 myoblasts in response to HGF on day 1, 2 and 3 post differentiation. Cells were allowed to differentiate for 1-3 days followed by fixation, immunocytochemistry at then analysis using the Zeiss confocal microscope. Scale bar = 20 µm. Blue indicates the Hoechst nuclear stain, green indicates MyoD.

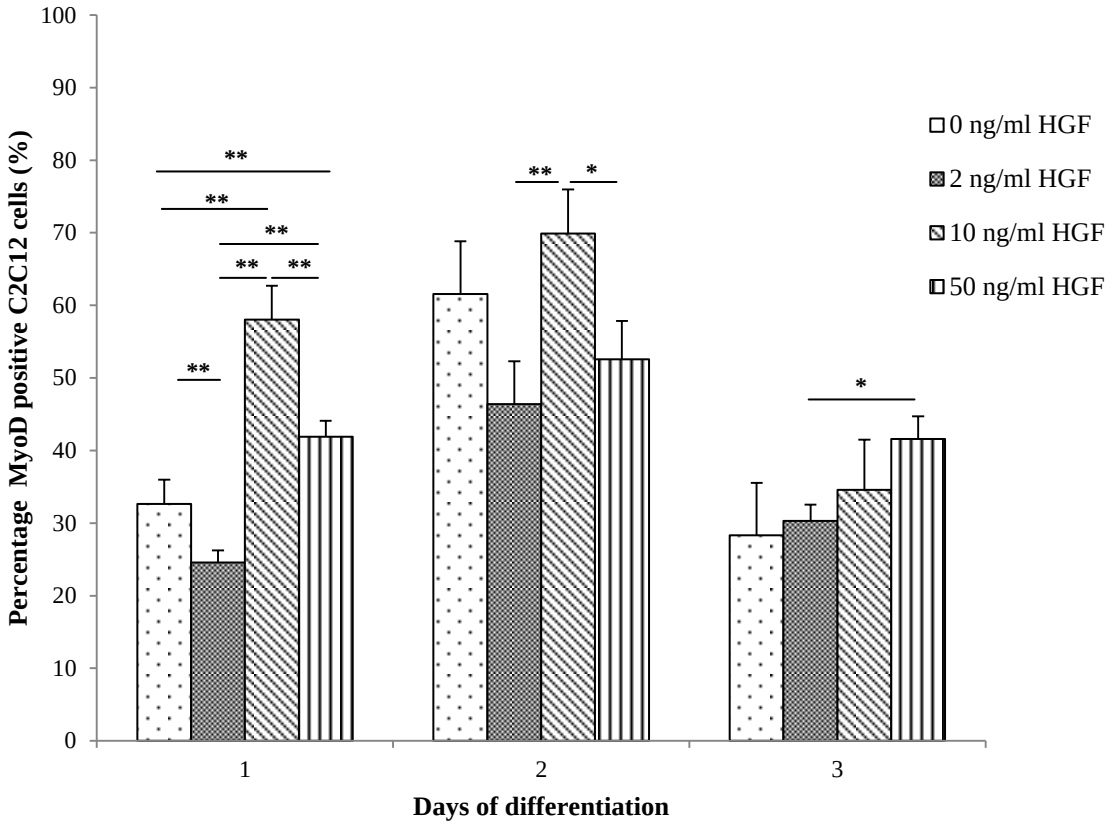

Figure 2.9: Percentage MyoD positive differentiating cells in response to HGF. Cells were cultured in growth media until they were 80% confluent. Differentiation media was then added and cells were fixed with 4% paraformaldehyde at day 1, 2, 3 and then probed with anti-MyoD antibody. Five random fields of view were observed for each treatment, followed by quantification using ImageJ. ** *p<0.01*, **p<0.05*. All data is mean ± SEM. n=3.

2.3.7. HGF affects C2C12 myoblast differentiation in a dose-dependent manner

The process of differentiation includes fusion of individual myoblasts to form myocytes and eventually multinucleated myotubes. We therefore monitored this process in the presence of exogenous HGF at 0, 2, 10 and 50 ng/ml. Brightfield images revealed that cells cultured in 10 ng/ml HGF formed longer myotubes compared to those cultured in 0, 2 and 50 ng/ml HGF (Figure 2.10A), suggesting that C2C12 differentiation capacity was up-regulated in response to10 ng/ml HGF. The differentiation of C2C12 cells into myotubes was then confirmed by staining with Phalloidin (Figure 2.10B) and immunocytochemistry with myosin heavy chain (MyHC) (Figure 2.10C). Both Phalloidin and MyHC staining confirmed an increase in myotubes in response to 10 ng/ml HGF and a decrease in response to 2 ng/ml HGF (Figure 2.10B and C).

Assessment of the differentiation index demonstrated a significant increase in differentiation in response to 10 ng/ml HGF (53.7 ± 3.84%) compared to control (40.7 ± 4.77%, $p<0.05$). This increase in differentiation was also significant compared to 2 ng/ml HGF (30.3 ± 2.86%, $p<0.01$) and 50 ng/ml HGF (38.4 ± 4.77%, $p<0.01$). A decrease in differentiation was detected at 2 ng/ml compared to untreated control ($p<0.05$), 10 ng/ml HGF ($p<0.01$) and 50 ng/ml HGF ($p<0.05$) (Figure 2.11A).

Determination of the fusion index confirmed a significant increase in fusion in response to 10 ng/ml HGF (47.7 ± 7.58%) compared to untreated control (35.9 ± 3.25%, $p<0.05$), 2 ng/ml HGF (29.4 ± 1.63%, $p<0.01$) and 50 ng/ml HGF (32.6 ± 4.39%, $p<0.01$). A decrease in fusion was shown at 2 ng/ml HGF compared to untreated control ($p<0.01$). Cells treated with 50 ng/ml HGF also revealed a significant decrease in fusion index ($p<0.01$) compared to 10 ng/ml HGF (<0.01), but displayed a slight increase compared to 2 ng/ml (Figure 2.11).

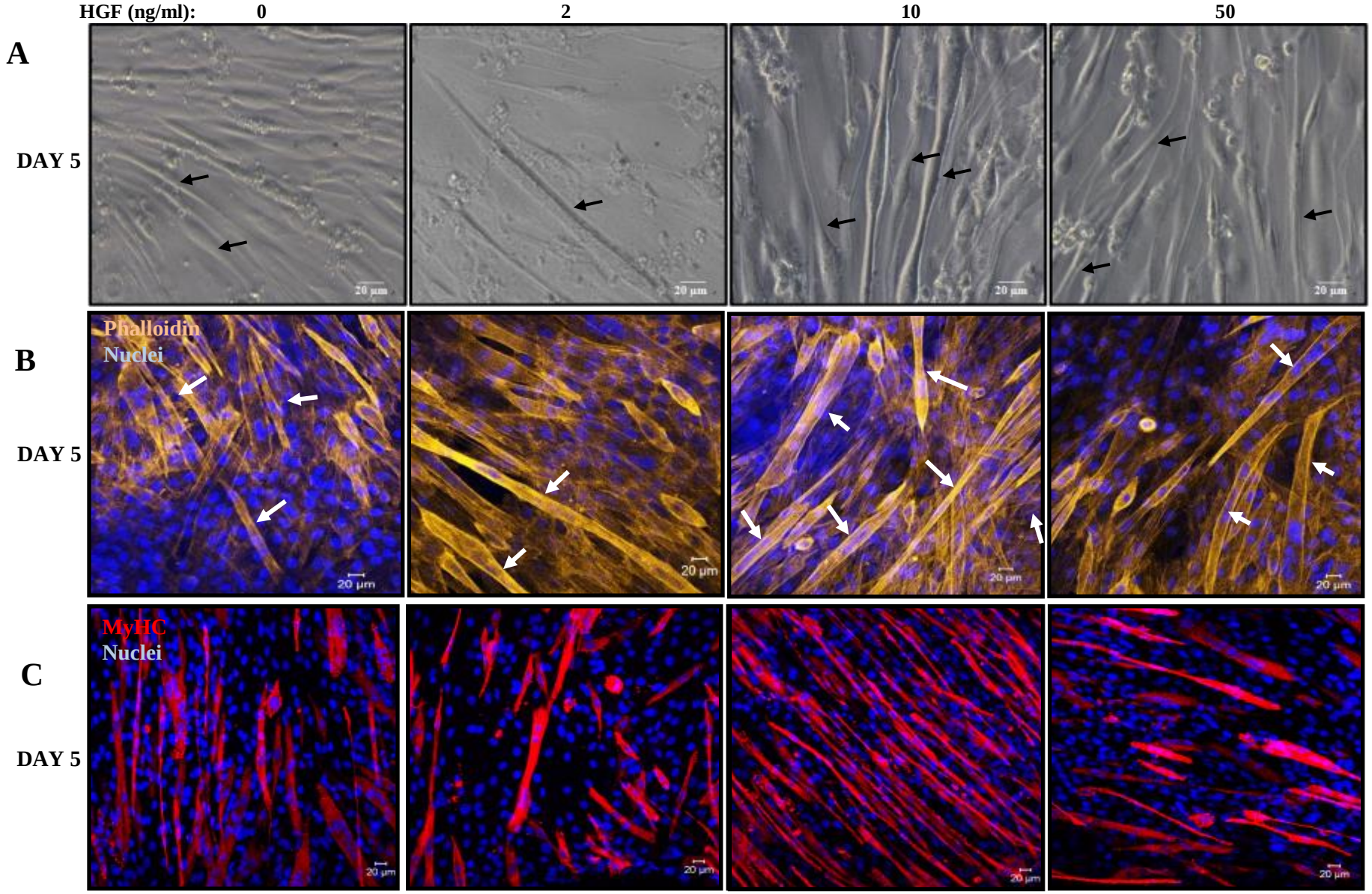

Figure 2.10: Myotube formation in differentiating C2C12 myoblasts treated with 0, 2, 10 or 50 ng/ml HGF. Cells were allowed to differentiate for 5 days followed by fixation, staining with Phalloidin-TRITC or immunocytochemistry with MyHC and analysis using the Zeiss Confocal microscope at 40 X magnification. Scale bar = 20 µm. **A**) Phase contrast images of differentiating cells at day 5. **B**) Confocal images of myotubes, blue indicates the Hoechst nuclear stain, orange indicates Phalloidin. **C**) Confocal images of myotubes, blue indicates the Hoechst nuclear stain, red indicates MyHC staining myotubes; the arrows indicate myotubes.

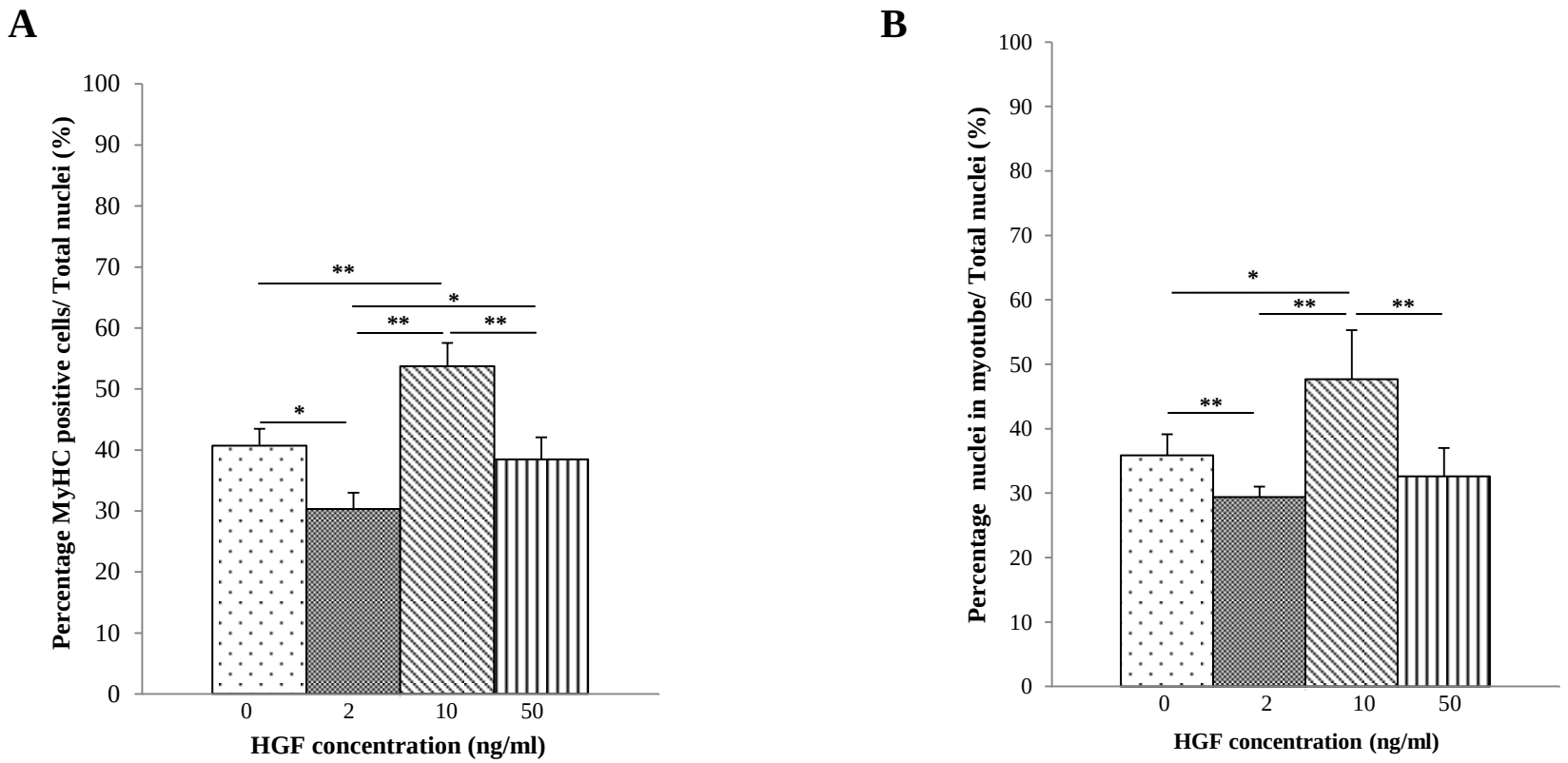

Figure 2.11: Differentiation and Fusion index of C2C12 myoblasts following treatment with 0, 2, 10 or 50 ng/ml HGF. A) The differentiation index (i.e. percentage MyHC positive cells (myocytes and myotubes)) was analysed for each of the HGF treatments using ImageJ software. **B**) The fusion index (i.e. percentage myotubes formed, two or more nuclei) was analysed for each of the HGF treatments using ImageJ software. ** $p<0.01$, *$p<0.05$. All data is mean ± SEM. n=3.

2.3.8. Expression of Myosin Heavy Chain (MyHC) during terminal differentiation is regulated by HGF

Given the increased C2C12 differentiation and fusion observed in response to 10 ng/ml HGF (Figure 2.10 and 2.11), we aimed to verify these observations via western blot analysis. HGF-treated C2C12 lysates were electrophoretically separated, and transferred onto nitrocellulose membrane, followed by probing with anti-MyHC antibody. The density of immunoreactive MyHC band was strongly prominent in response to 10 ng/ml HGF compared to the untreated control, 2 and 50 ng/ml HGF. Cells treated with 50 ng/ml HGF demonstrated less MyHC expression compared to untreated control and 2 ng/ml HGF (Figure 2.12A).

Band intensities were quantified relative to control using densitometry. Results revealed a significant increase in MyHC expression at 10 ng/ml HGF (1.49 ± 0.06) compared to untreated control ($p<0.05$) (Figure 2.12B). This data confirms the regulatory effect of HGF on terminal myoblast differentiation.

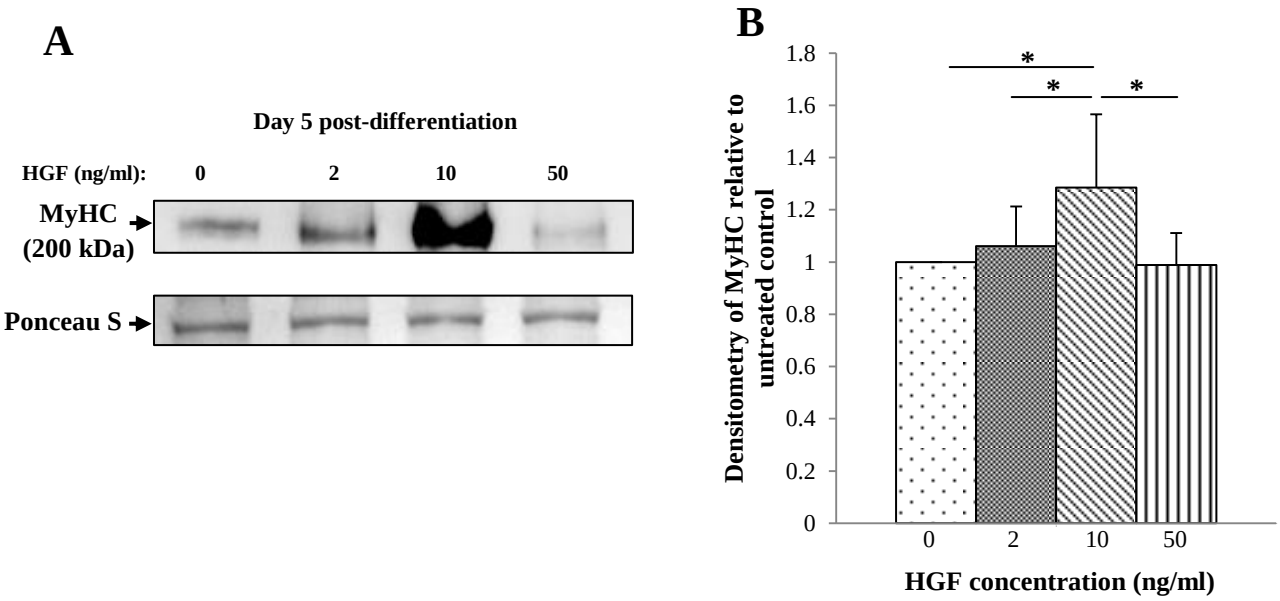

Figure 2.12: Myosin heavy chain (MyHC) expression in differentiating C2C12 myoblast in response to HGF. Cells were allowed to differentiate for 5 days; lysates were harvested and run on SDS-PAGE followed by western blot analysis. **A)** Band intensities observed for MyHC on day 5 lysates**. B)** Corresponding densitometry analysis of MyHC expression relative to untreated control**.** *$p<0.05$. All data is mean ± SEM. n=4.

2.4 2DISCUSSION

Satellite cells are quiescent skeletal muscle cells that play a pivotal role in maintenance, myogenesis and repair of the skeletal muscle (Boldrin et al., 2010). Quiescent and activated satellite cells express Pax7; however, upon the onset of differentiation, they down-regulate Pax7 and up-regulate MyoD and myogenin (Olguin and Pisconti, 2012). Satellite cells are activated by different environmental cues including growth factors, such as FGF and HGF. HGF has been shown to be involved not only in satellite cell activation, but also in proliferation, migration, and differentiation and is a key regulator of skeletal muscle regeneration (Tatsumi and Allen, 2004).

Satellite cell proliferation is an important process in skeletal muscle repair and regeneration, however, *in vitro* the effect of HGF on proliferation is highly dose-dependent (Yamada et al., 2010a, Gal-Levi et al., 1998). Yamada *et al*. demonstrated that high levels of HGF (over 10 ng/ml) suppress the proliferative activity of rat satellite cells (Yamada et al., 2010a). Gal-Levi *et al*. came to similar conclusions using the chicken skeletal muscle cells and C2 cells. They found a reduction in activation of quiescent satellite cells and decreased growth upon exposure to exogenous HGF (20 and 50 ng/ml) (Gal-Levi et al., 1998). Our data confirms that there is a dose-dependent effect of HGF on C2C12 myoblast proliferation. Our cell count and crystal violet assay showed similar trends in terms of the dose-dependent effect of HGF on proliferation. In both assays, HGF (2 ng/ml) stimulated proliferation, however, 10 ng/ml HGF resulted in a significant decrease in proliferation compared to the untreated control. The decrease in cell number in response to 10 ng/ml is unlikely to be due to cell death; hence we speculate that somewhere between 2 and 10 ng/ml HGF there might be a threshhold dose that supresses proliferation.

HGF has been shown to regulate satellite cell proliferation via two independent pathways i.e. Ras/Raf pathway and Stat3 pathway (Birchmeier et al., 2003, Kim et al., 2011). Based on the increase and decrease in proliferation in response to 2 ng/ml and 10 ng/ml HGF, respectively, we suspect involvement of these two pathways that contributes to this dose-dependent effect (Birchmeier et al., 2003, Kim et al., 2011). Other studies have shown that HGF receptor (c-Met) coupling to MAPK/ERK pathway via the adaptor, Grb2, plays a role in stimulating proliferation (Leshem et al., 2002). In accordance with these findings, we suspect a similar mechanism at lower HGF dose (2 ng/ml) where an increase in proliferation is observed. Short-lived activation of MAPK in C2 cell line was demonstrated to stimulate proliferation whilst sustained activation leads to differentiation (Gredinger et al., 1998). We therefore suggest that HGF (2 ng/ml) might have

induced this short-lived activation of MAPK which resulted in increased proliferation and decreased differentiation.

Analysis of the percentage wound closure further indicated that HGF (10 ng/ml) (while decreasing proliferation), stimulated migration. HGF is known to promote satellite cell migration to the site of injury via activation of the Ras/Raf pathway or MAPK/ERK cascade (Charge and Rudnicki, 2004). The smaller decrease in response to 2 ng/ml HGF may be a reflection of the increased proliferation observed at this concentration.

We next examined the effect of HGF on the localization and expression of scaffold protein, vinculin. Vinculin is a ubiquitously expressed actin filament binding protein that localizes in focal adhesions; is known to be actively involved in myoblast proliferation and migration (Le Clainche and Carlier, 2008). Vinculin expression levels have been shown to be higher in migrating cells (Moon et al., 2004). Vinculin image quantification and vinculin protein expression analysis (Western blot) both reviewed that cells treated with 10 ng/ml HGF showed high vinculin expression levels, which correlated with both an increased percentage in wound closure and a decrease in proliferation. Since cells treated with 50 ng/ml HGF showed no change in the rate of migration and increased proliferation, we speculate that increased proliferation may not necessarily correlate to migration as is commonly thought in literature, however, this requires further investigation. Our results therefore indicate that HGF increases vinculin expression and increased vinculin expression in turn promotes migration, but decreases proliferation of C2C12 myoblasts in a dose-dependent manner, an important mechanism that is essential during *in vivo* wound healing.

Myoblast differentiation is essential during skeletal muscle repair and formation and is detected by the up-regulation of MRFs such as MyoD and down-regulation of Pax7 (Gal-Levi et al., 1998a, Gal-Levi et al., 1998b). To examine the effect of HGF on myoblast differentiation, we firstly examined expression of MyoD and Pax7 over 3 days of differentiation. Our data suggests that MyoD promotes myoblast progression to terminal differentiation when cells are exposed to 10 ng/ml HGF. At this concentration, the cells showed significantly increased percentage MyoD cells at day 1. Furthermore, a remarkably reduced Pax7 expression, a necessary step for cells to exit proliferation and enter the differentiation phase (Knapp et al., 2006); (Gal-Levi et al., 1998a, Gal-Levi et al., 1998b) was observed at day 2. The early and high expression levels of MyoD at 10 ng/ml indicated early promotion of C2C12 myoblast entry into differentiation at this HGF concentration. Also, Pax7 decrease at day 2 suggests ability to enter differentiation. These results

correlate with other findings that MyoD expression is induced in satellite cells in response to HGF treatments (15 ng/ml) in rat soleus muscle (Ishido and Kasuga, 2012).

Maintenance of Pax7 expression is essential for self-renewal (Gill et al., 2010) but delays differentiation (Zammit et al., 2006b). We observed that cells treated with 2 ng/ml showed maintenance of Pax7 expression throughout day 1, 2 and 3 of differentiation; this suggests that HGF at this concentration can delay differentiation in a portion of the cell population by promoting Pax7 expression. Zammit *et al*., reported that expression of Pax7 delays onset of differentiation through delayed expression of myogenin (Zammit et al., 2006a). Consistent with these findings, Olguin *et al*., 2012 showed that expression of Pax7 in adult primary myoblasts down regulates MyoD and prevents myogenin induction leading to delayed differentiation (Olguin and Pisconti, 2012). We therefore suspect that the delayed differentiation we observed in response to HGF (2 ng/ml) might be through delayed induction of myogenin; however, this requires further investigation.

To confirm the effect of HGF on terminal differentiation we assessed myotube formation after 5 days of differentiation. Previous studies have shown that the addition of exogenous HGF to satellite cells dose-dependently inhibits the expression of MyHC, which is expressed late in muscle differentiation (Miller et al., 2000, Bandow et al., 2004). In our study the HGF concentrations that increased proliferation (i.e. 2 ng/ml) showed less stimulatory effect on terminal differentiation as compared to 10 ng/ml HGF, which had the highest effect on promoting differentiation and decreased proliferation. HGF is known to inhibit myoblast differentiation into myofibers via its inhibitory effect on the activity of MyoD and myogenin (Gal-Levi et al., 1998a, Bandow et al., 2004, Gal-Levi et al., 1998b). Other studies have shown that HGF receptor coupling to MAPK/ERK pathway via the adaptor, Grb2, plays a role in inhibiting myoblast differentiation and stimulating proliferation (Leshem et al 2002). Additionally, *in vitro* studies suggest that the binding of HGF to its receptor c-Met dose-dependently results in the silencing of MyoD and myogenin gene expression and inhibits the synthesis of muscle specific structural protein, MyHC as well as myotube formation (Anastasi, 1997). The increased differentiation and fusion index as well as elevated MyHC expression observed in response to HGF (10 ng/ml) correlated well with the increase in percentage MyoD cells observed.

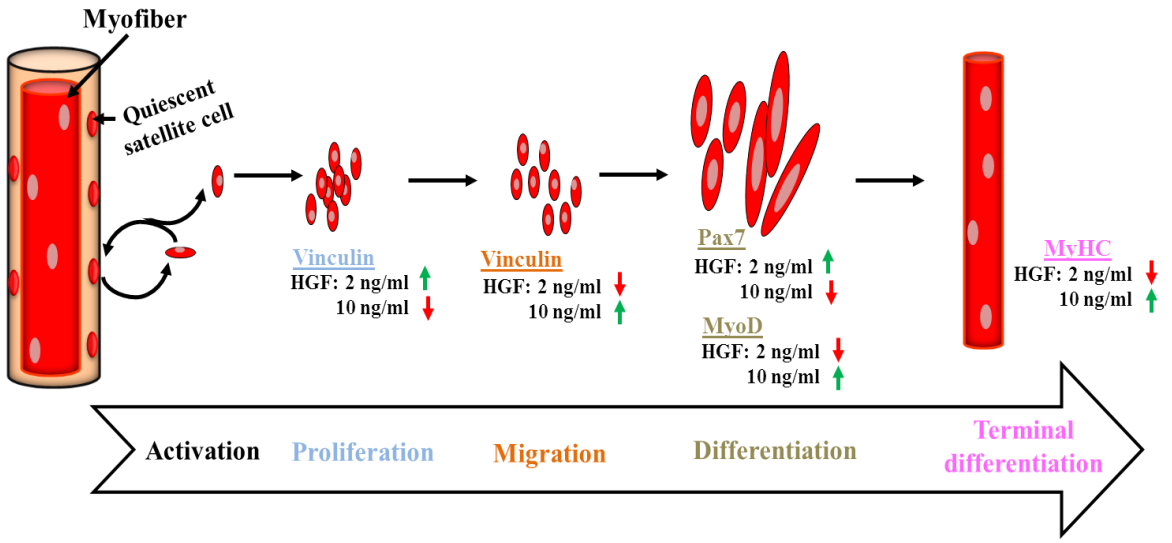

Figure 2.13: Summary of findings of the effect of HGF on C2C12 myoblast proliferation, migration and differentiation. Myoblast proliferation was elevated and reduced in response to 2 ng/ml and 10 ng/ml HGF, respectively, via vinculin expression analysis. Migrations studies revealed that HGF stimulated and reduced the migratory activity of C2C12 myoblasts in an opposite effect to proliferation. During both early and terminal differentiation, HGF (2 ng/ml) up-regulated Pax7 expression and down-regulated MyoD and MyHC expression. Opposite effects were observed in response to 10 ng/ml HGF; there was down-regulation of Pax7 expression, up-regulation of MyoD and MyHC expression. ↑Indicate increase, ↓indicate decrease

In summary, we have demonstrated dose-dependent effects of HGF on C2C12 myoblast proliferation, migration and differentiation and provide evidence that HGF participates in all these myogenic processes. C2C12 myoblast proliferation rates are increased or decreased by HGF at 2 ng/ml and 10 ng/ml respectively. The decrease in proliferation at 10 ng/ml HGF which shows an up-regulation of vinculin expression level correlates with the initiation of cell migration, revealed by the increase in migration at 10 ng/ml HGF. Importantly, HGF causes divergent effects on migration as well as differentiation. HGF (2 ng/ml) resulted in increased proliferation, but registered a decrease in both migration and differentiation. A dose-dependent effect of HGF on differentiation was observed, with 10 ng/ml showing increased MyoD and MyHC expression accompanied by down-regulation in Pax7. However, most of the dose-dependent effects of HGF on myogenesis did not follow the classical hyperbolic shape. We conclude that the ability of HGF to alter activity of the myogenic processes is supportive of its role in skeletal muscle regeneration, providing further evidence that HGF plays a key role in regulation of wound repair and regeneration and is a potential drug target for muscle disease.

CHAPTER 3

THE EFFECT OF HEPATOCYTE GROWTH FACTOR (HGF) ON HUMAN SKELETAL MYOBLAST PROLIFERATION AND DIFFERENTIATION

ABSTRACT

Hepatocyte growth factor (HGF), a pleiotrophic cytokine has been previously reported to dose-dependently regulate mouse myoblast proliferation, migration and differentiation, but the fact whether HGF has the same effect on primary cultured human skeletal myoblasts (HSkM) is not known. In this context, the objective of this study was to investigate the effect of HGF (2 and 10 ng/ml) on HSkM myoblast proliferation and differentiation. Cell counts were used to determine proliferation while immunocytochemistry for analysis of nuclei $Pax7^{+}/MyoD^{+}$ expression was used to determine myogenic commitment. Terminal differentiation was assessed through total MyHC expression analysis. We demonstrate that treatment of HSkM with a low HGF concentration (2 ng/ml) promotes proliferation and decrease differentiation. In contrast, treatment of HSkM cells with a higher HGF concentration (10 ng/ml) decreases proliferation, but promotes differentiation. These findings confirm previous work in C2C12 myoblasts. Since HSkM cells represent a more accurate *in vitro* model, these findings highlight the importance of HGF in the skeletal muscle and further underscore the role of HGF as a central regulator of myogenesis.

Keywords: Hepatocyte Growth Factor, primary cultured human skeletal myoblasts, proliferation, differentiation

3.1. INTRODUCTION

The immortalized C2C12 cell line is a valuable tool for the identification of mechanisms related to myogenic processes; however, this model does not necessarily represent the complexity of skeletal muscle physiology (Peault et al., 2007). In most cases, the mutations found in mouse cell lines are very different to those in human cells; for example, a mutation in the dystrophin gene reflects as a mild pathological phenotype in mice, but in humans it can cause an advanced and fatal disease (Mamchaoui et al., 2011). As such, use of human primary culture often represents a more accurate *in vitro* model (Martin et al., 2009).

Primary culture of human skeletal myoblasts (HSkM) allows analysis of the sequential processes during skeletal muscle regeneration, signal transduction and metabolism (Martin et al., 2009); (Mamchaoui et al., 2011). Furthermore, these cells are candidates for transplantation therapies to treat diseases such as muscular dystrophies or severe muscle trauma (Peault et al., 2007). They serve as a powerful tool for investigating pathological mechanisms (including those associated with muscle) and for developing cell-based biotherapies (Mamchaoui et al., 2011).

In skeletal muscle, the balance between myogenic precursor proliferation and differentiation is influenced by growth factors including FGF and HGF. HGF elicits several signalling cascades, mainly through c-Met receptor phosphorylation which terminates in the nucleus with the activation of specific transcription factors (Pax7, Myf5, MyoD and myogenin) (Goel and Dey, 2002). Findings from *in vitro* experimentation and animal models suggest that HGF is a major inducer of satellite cell activation and proliferation (Morgan and Partridge, 2003, Gal-Levi et al., 1998b). Similar observations were demonstrated by us in chapter 2. Human studies by O'Reilly *et al*. demonstrated activation of satellite cells in human skeletal muscle associated with increased HGF as early as 24 h post-exercise (O'Reilly et al., 2008).

Differentiation and fusion of myoblasts is critical to the development of mature multinucleated skeletal muscle myotubes, and during these processes, the myoblasts exit the cell cycle and their mitotic activity ceases (Charge and Rudnicki, 2004). HGF has been reported to impair differentiation in animal models (Gal-Levi et al., 1998b). O'Reilly *et al*. reported for the first time that in the human skeletal muscle, down-regulation of active HGF is a necessary step for differentiation to occur (O'Reilly et al., 2008). However, not much more is known regarding the effect of HGF on human primary cultured skeletal myoblasts and this requires further investigation.

Previously, we established a dose-dependent effect of HGF on C2C12 myoblast proliferation and differentiation (*see Chapter 2*). To date, most HGF studies in skeletal muscle regeneration and repair have focused mainly on mouse and rat primary culture and immortalised cell lines. The present study was therefore designed to investigate the role of HGF on human primary cultured skeletal myoblast proliferation and differentiation. Human recombinant HGF was tested at 2 and 10 ng/ml as these were the concentrations that revealed significant differences in C2C12 mouse myoblast studies (*see Chapter 2*). Furthermore, we investigated the underlying molecular mechanisms involved via transcription factor expression analysis.

3.2. EXPERIMENTAL PROCEDURE

3.2.1. Cell culture

Human primary cultured myoblasts (HSkM) were purchased from Lonza, USA (cat.CC-2561). The cells were isolated from the upper arm or leg muscle tissue of normal donors. They were contained in a Clonetics® Skeletal Muscle System composed of optimized growth media. The cells were cultured in Skeletal Muscle Cell Medium BulletKit® (Lonza, USA, cat.CC-3160) during passage 1 and 2. From passage 3 HSkM were cultured in human myoblast growth media which consisted of HAMS-F10 (Life Technologies, South Africa, cat.31550-015) supplemented with 10% (v/v) fetal bovine serum (Biowest, USA, cat.S181H-500), 2% (v/v) L-glutamine (Lonza, USA, cat.BE17-605E) and 2% (v/v) Penicillin-Streptomycin (Lonza, USA, cat.DE17-602E) and 2.5 ng/ml FGF (Promega, USA, cat. G507A). For differentiation studies, monolayers of HSkM cells were expanded until they were 80% confluent then switched from growth media to differentiation media (HAMS-F10 supplemented with 2% (v/v) horse serum) (Biowest, USA, cat.S090H-100). Human HGF (PeproTech, USA, cat.100-39) was used at a final concentration of 2 and 10 ng/ml to assess effect on proliferation and differentiation. HSkM cells were incubated at 37^{o}C in a humidified atmosphere at 5% CO_2. All experiments were carried out under sterile conditions in a Class II Biohazard Safety Cabinet. Passages 2-3 were used for all experiments.

3.2.2. Proliferation Assay (TC-20 Automated Counter)

Cells were grown to initial densities of 5 x 10^4 cells/well in 6 well tissue culture plate containing growth media and incubated at 37^{o}C, 5% CO_2 for 24 h. HGF (0, 2 and10 ng/ml) was then added to cells and they were incubated for a further 24 h. Thereafter, cells were trypsinised for 5 min and a small sample (10 µl) was removed and transferred onto a counting slide (Bio-rad, USA, cat.145-0011). The slide was inserted into a TC-20™ Automated cell counter (Biorad, USA, serial.508BR1910) and cells numbers were automatically determined.

3.2.3. Immunocytochemistry

Differentiated cells were fixed in 4% paraformaldehyde containing 0.1% Triton X-100 (Sigma, Germany, cat.T9284) for 20 min and blocked with 5% donkey serum (Biowest, USA, cat.S2170-100) for 30 min. Primary antibodies were added overnight and dilutions used were as follows: monoclonal goat anti-Pax7 antibody (Santa Cruz, USA, sc-7748; 1:500), rabbit polyclonal anti-MyoD (Santa Cruz, USA, cat.sc-760; 1:100), monoclonal mouse MF-20 (Developmental Hybridomal Studies, USA, cat.MF29; 1:100). DyLight 488-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch, USA, cat.CN-711-485-152), DyLight 488-conjugated AffiniPure donkey anti-mouse lgG (Jackson ImmunoResearch, USA, cat.CN-715-485-151) and DyLight 594-conjugated AffiniPure donkey anti-goat lgG, secondary antibodies (Jackson ImmunoResearch, USA, cat.CN-715-515-151) were used at 1:1000 to detect appropriate primary antibodies and incubated for 1 h. Hoechst nuclei stain (Sigma, Germany,cat.B2261; 10 µg/ml stock, 1:2000) was added to all the cells for 5 min. Moviol was used as the mounting agent. All images were captured using the Zeiss 710LSM confocal microscope (Germany).

3.2.4. Quantification of MyoD and Pax7^{+} myoblasts

Five random fields of view of differentiated cells (> 30 cells/ field) were captured using the Zeiss 710LSM confocal microscope and analysed with ImageJ software (http://rsbweb.nih.gov/ij/). The percentage MyoD and Pax7 expressing myoblasts was calculated using the following formulae:

$$\%\ \text{MyoD positive cells} = \frac{\text{Number of nuclei staining positive for MyoD}}{\text{Total number of nuclei}} \times 100$$

$$\%\ \text{Pax7+ cells} = \frac{\text{Number of nuclei staining positive for Pax7}}{\text{Total number of nuclei}} \times 100$$

3.2.5. Fusion and Differentiation Indices

Fusion and differentiation indices were analysed as described previously (Micheli et al., 2011). Five random fields of view of fused myoblasts stained with myosin heavy chain (MyHC) were captured using the Zeiss 710LSM confocal microscope. ImageJ software was utilized to determine myotube formation. The fusion and differentiation indices for myotube formation were then calculated using the following formulae:

$$\text{Differentiation index} = \frac{\text{Number of MyHC positive cells (myocytes and myotubes)}}{\text{Total number of nuclei}} \times 100$$

$$\text{Fusion index} = \frac{\text{Number of nuclei per MyHC positive myotube}}{\text{Total number of nuclei}} \times 100$$

3.2.6. Statistics

All statistical analysis was performed with either a Student's t-test for parametric data or a Mann-Whitney-U test for non-parametric data, with values of $p<0.05$ considered significant. The results from each experiment were expressed as Mean ± standard error of the mean (SEM). All experiments were completed in duplicate with each experiment repeated 3-4 times (n=3-4).

3.3. RESULTS

3.3.1. HGF regulates HSkM myoblast proliferation in a dose-dependent manner

Proliferation of HSkM myoblasts was assessed using an automated cell counter which allowed quantitative determination of cell number. Morphological analysis revealed that following 24 and 48 h, HGF (10 ng/ml) resulted in a lower cell confluence compared to untreated control and 2 ng/ml HGF (Figure 3.1A); this suggested that HSkM proliferation capacity was down-regulated in response to 10 ng/ml HGF. Quantitative analysis confirmed this observation. The initial cell number plated was 5×10^4 cells. Following 24 h, untreated control cells had doubled their population to $1.02 \pm 0.055 \times 10^5$ cells, whereas those treated with 10 ng/ml HGF had only increased in number to $0.784 \pm 0.959 \times 10^5$ cells, a significant decrease compared to untreated control ($p<0.05$). Conversely, cells cultured in 2 ng/ml HGF had increased their number to $1.38 \pm 0.039 \times 10^5$ cells. This increase was significant compared to untreated control ($p<0.05$) and 10 ng/ml HGF ($p<0.05$).

A similar trend was observed following 48 h of culture with 2 ng/ml HGF significantly increasing the proliferation ability of HSkM, whereas 10 ng/ml HGF did not.

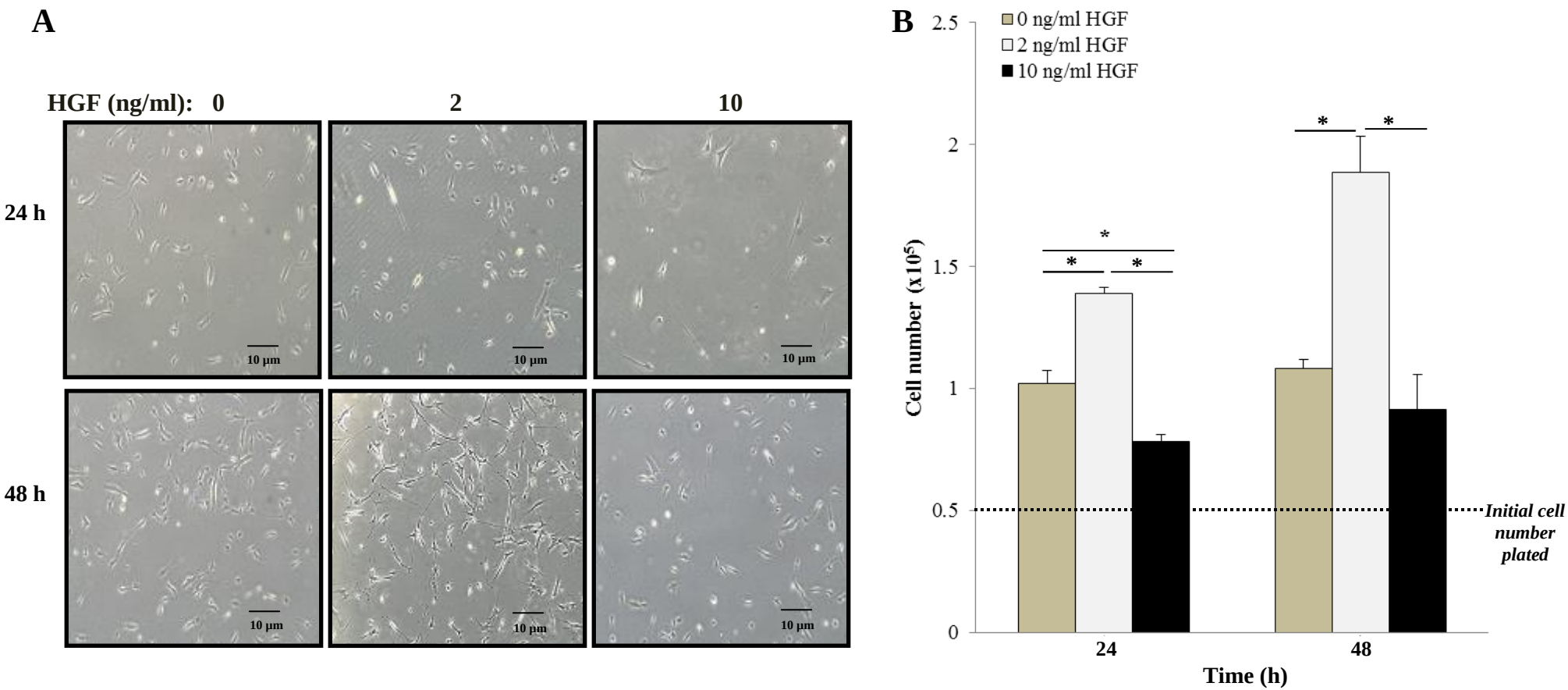

Figure 3.1: Proliferation assay of HSkM myoblasts in response to HGF. Equal numbers of cells (5 x 10^4) were plated and cultured for 24 h, followed by treatment with HGF (0, 2 and 10 ng/ml) for either 24 or 48 h. **A**) The phase contrast images showing the confluence of HSkM cells in response to HGF concentrations. Images were acquired with *Motic* 2.0 camera at 40 X magnification. Scale bar = 10µm. **B**) Proliferation was then analysed via cell counts. HGF was observed to affect proliferation in a dose-dependent manner. *$p<0.05$. All data is mean ± SEM. n=3.

3.3.2. HGF stimulates Pax7 expression levels in differentiating HSkM cells.

Exit of satellite cells from the cell cycle and entry to differentiation requires the down-regulation of Pax7 and subsequent increase in MyoD expression (Gal-Levi et al., 1998b). As such, our first step was to assess the expression levels of these transcription factors upon treatment of differentiating cells with HGF (2 and 10 ng/ml) which would aid our understanding of the role HGF plays in myoblast differentiation.

Morphological assessment revealed maximum Pax7 expression at day 1 and 2 which decreased at day 3 and 5 of differentiation. Images of cells following incubation with HGF (10 ng/ml) revealed a down-regulation of Pax7 at day 3 compared to untreated control and those incubated with 2 ng/ml. By day 5 of differentiation, cells treated with 10 ng/ml HGF were largely devoid of Pax7 (Figure 3.2).

The expression patterns of Pax7 in HSkM were quantified and examined for the distribution of the $Pax7^+$ subgroup within mononucleated cell populations. On day 1 of differentiation, Pax7 expression was significantly up-regulated by cells treated with 2 ng/ml HGF (51 ± 1.69%) relative to untreated control cells (43.9 ± 2.07%, $p<0.05$) (Figure 3.3). However, percentage $Pax7^+$ cells treated 10 ng/ml HGF were similar to untreated control (Figure 3.3). The highest Pax7 expression was observed at day 2 post-differentiation in cells treated with 2 ng/ml HGF. Cells treated with 10 ng/ml HGF revealed a significant decrease in percentage of $Pax7^+$ cells (38.2 ± 3.21%) compared to untreated control (52.4 ± 3.46%, $p<0.05$) and 2 ng/ml HGF (53.9 ± 5.14%, $p<0.05$) (Figure 3.3).

By day 3, both untreated control cells and those exposed to 10 ng/ml HGF showed a lower percentage of $Pax7^+$ cells compared to levels observed on day 1 and 2. In response to 2 ng/ml HGF, the percentage $Pax7^+$ cells were maintained at levels significantly higher than untreated control and 10 ng/ml HGF ($p<0.05$) (Figure 3.3).

On day 5 of differentiation, a significant decline in percentage of $Pax7^+$ cells was identified in response to 10 ng/ml HGF (4.7 ± 0.216%) compared to untreated control (12.1 ± 0.665%, $p<0.05$) and 2 ng/ml HGF (27 ± 2.84%, $p<0.05$). Although percentage $Pax7^+$ cells in response to 2 ng/ml HGF were decreased by 16% in comparison to day 3, they were still significantly higher than untreated control (Figure 3.3). Maintenance of Pax7 expression by cell treated with 2 ng/ml HGF suggests delayed differentiation, whilst the decreased expression of Pax7 observed in response to 10 ng/ml HGF suggests induced differentiation.

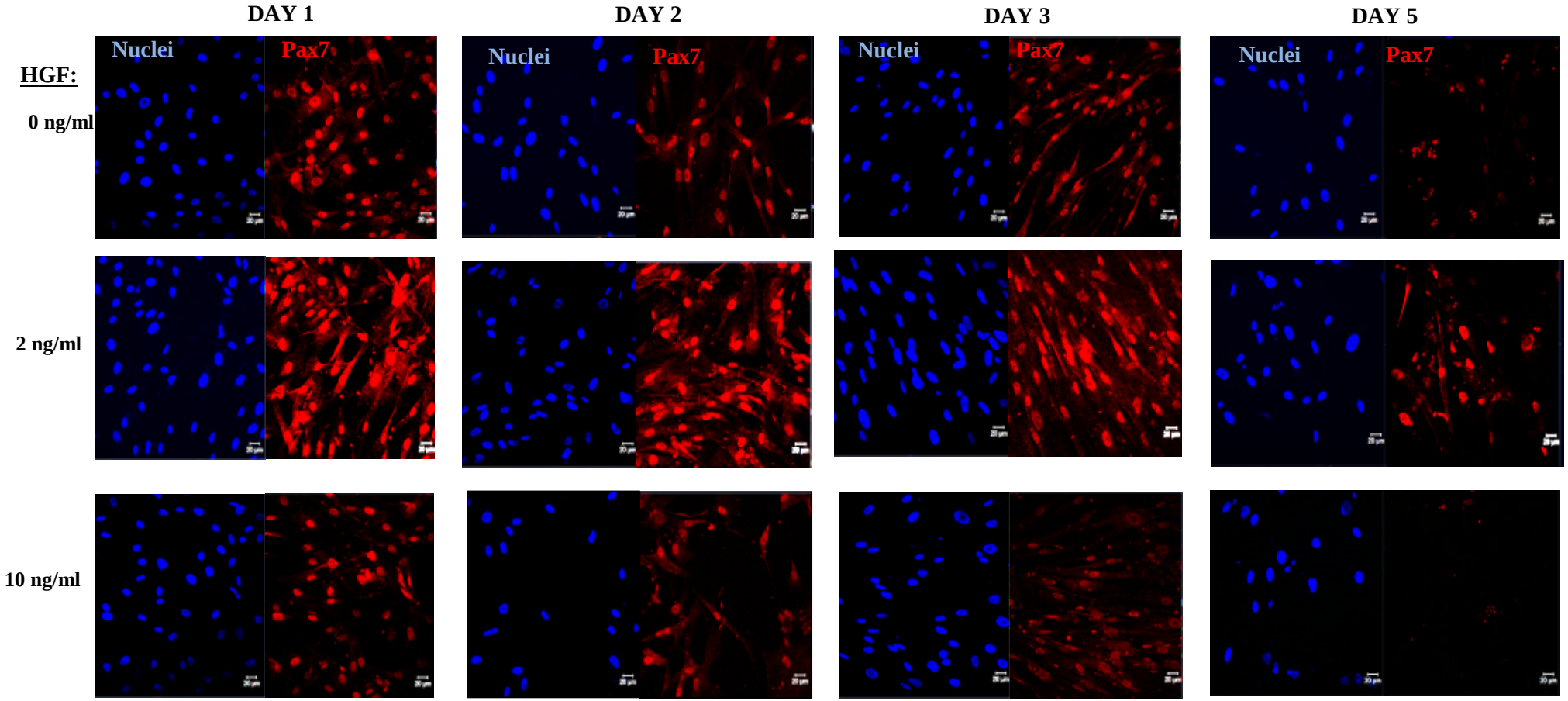

Figure 3.2: Pax7 expression in differentiated HSkM myoblasts in response to HGF. Cells were allowed to differentiate for 1-5 days followed by fixation, immunocytochemistry and then analysis using the Zeiss confocal microscope, 40 X magnification. Scale bar = 20µm. Blue indicates the Hoechst nuclear stain and red indicates Pax7.

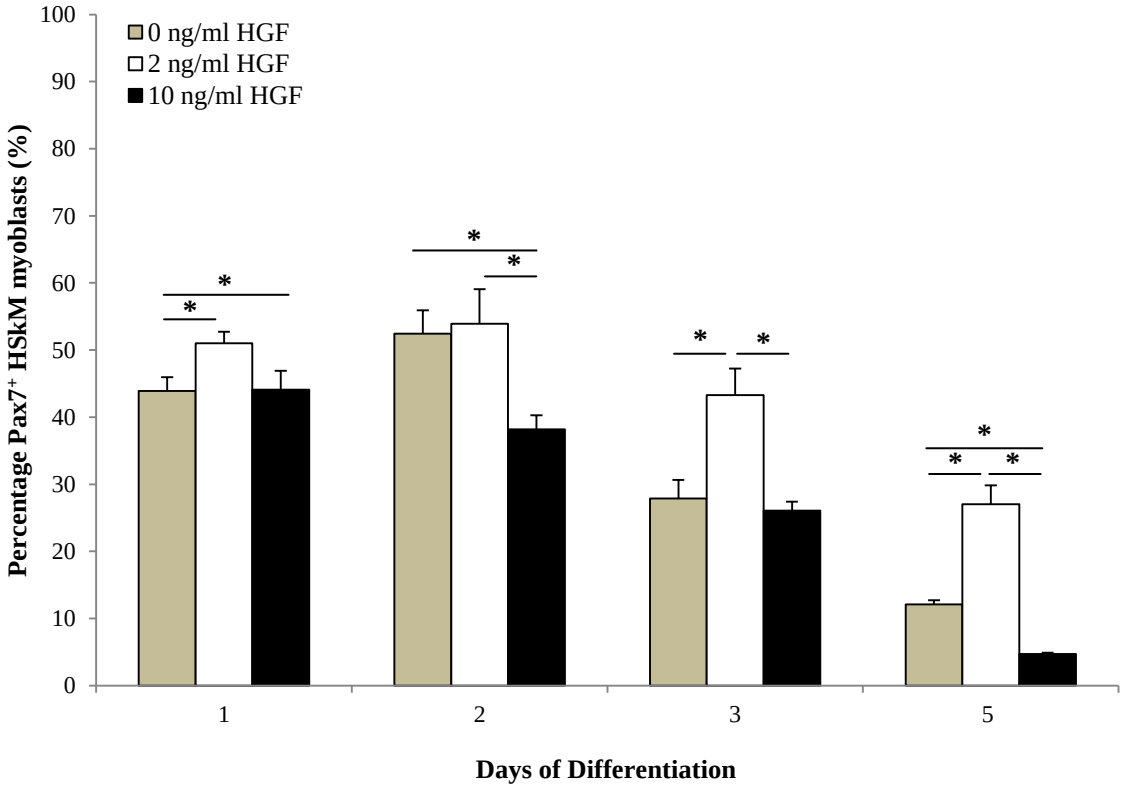

Figure 3.3: Percentage Pax7$^+$ cells in response to HGF during differentiation. Cells were allowed to differentiate for 1-5 days in the presence or absence of HGF followed by fixation, immunocytochemistry at then analysis using the Zeiss confocal microscope. Five random fields of view (from Figure 3.2) were observed for each treatment and quantified using Image J. *$p<0.05$. All data is mean ± SEM. n=4.

3.3.3. HGF increases the number of MyoD expressing HSkM cells

The expression patterns of MyoD in differentiating human myoblasts following incubation with HGF were assessed. Morphological analysis revealed fewer MyoD positive cells in response to 2 ng/ml HGF at day 1, 2 and 3 of differentiation (Figure 3.4).

Quantitative analysis of MyoD expression on day 1 demonstrated that cells treated with 2 ng/ml HGF revealed 6% and 13% decrease in MyoD positive cells compared to untreated control ($p<0.05$) and those treated with 10 ng/ml HGF ($p<0.05$). The percentage MyoD positive cells increased significantly in response to 10 ng/ml HGF (39 ± 1.56%) compared to untreated control (32.2 ± 3.24%, $p<0.05$) (Figure 3.5).

MyoD expression peaked at day 2 across all treatments, however, the greatest increase was identified in cells exposed to 10 ng/ml HGF (62.2 ± 1.28%) compared to untreated control (52.4 ± 0.952%, $p<0.05$) and those exposed to 2 ng/ml HGF (48.2 ± 2.23%, $p<0.05$). This increase was 23% more than the MyoD expression levels that were observed on day 1 (Figure 3.5).

On day 3 of differentiation, 10 ng/ml HGF resulted in a small, but significant decline in MyoD positive cells (33.1 ± 2.15%) compared to untreated control (35.9 ± 0.952%, $p<0.05$) and 2 ng/ml HGF (45 ± 3.13%, $p<0.05$). This decline was 29% less than the expression that we had observed on day 2. The smallest decline in percentage of MyoD positive cells was observed with cell treated with 2 ng/ml HGF compared to untreated control ($p<0.05$) (Figure 3.5).

On day 5 of differentiation, exposure to 10 ng/ml HGF resulted in a significant decrease in percentage MyoD positive cells (16.2 ± 0.712%) compared to untreated control (22.4 ± 1.18%, $p<0.05$) and 2 ng/ml HGF (25 ± 0.989%, $p<0.05$). This decrease was 46% less than peak levels observed on day 2. In contrast, cells exposed to 2 ng/ml HGF showed a 23% decrease in MyoD positive cells compared to peak levels observed on day 2 (Figure 3.5).

Overall, exposure of HSkM to 2 ng/ml HGF resulted in increased percentage $Pax7^{+}$ cells and decreased percentage MyoD positive cells, suggesting delayed differentiation due to increased proliferation. In contrast, incubation of HSkM in 10 ng/ml HGF, revealed a decrease in percentage $Pax7^{+}$ cells which was accompanied by increased percentage MyoD positive cells, indicating early commitment to differentiation (Figure 3.5).

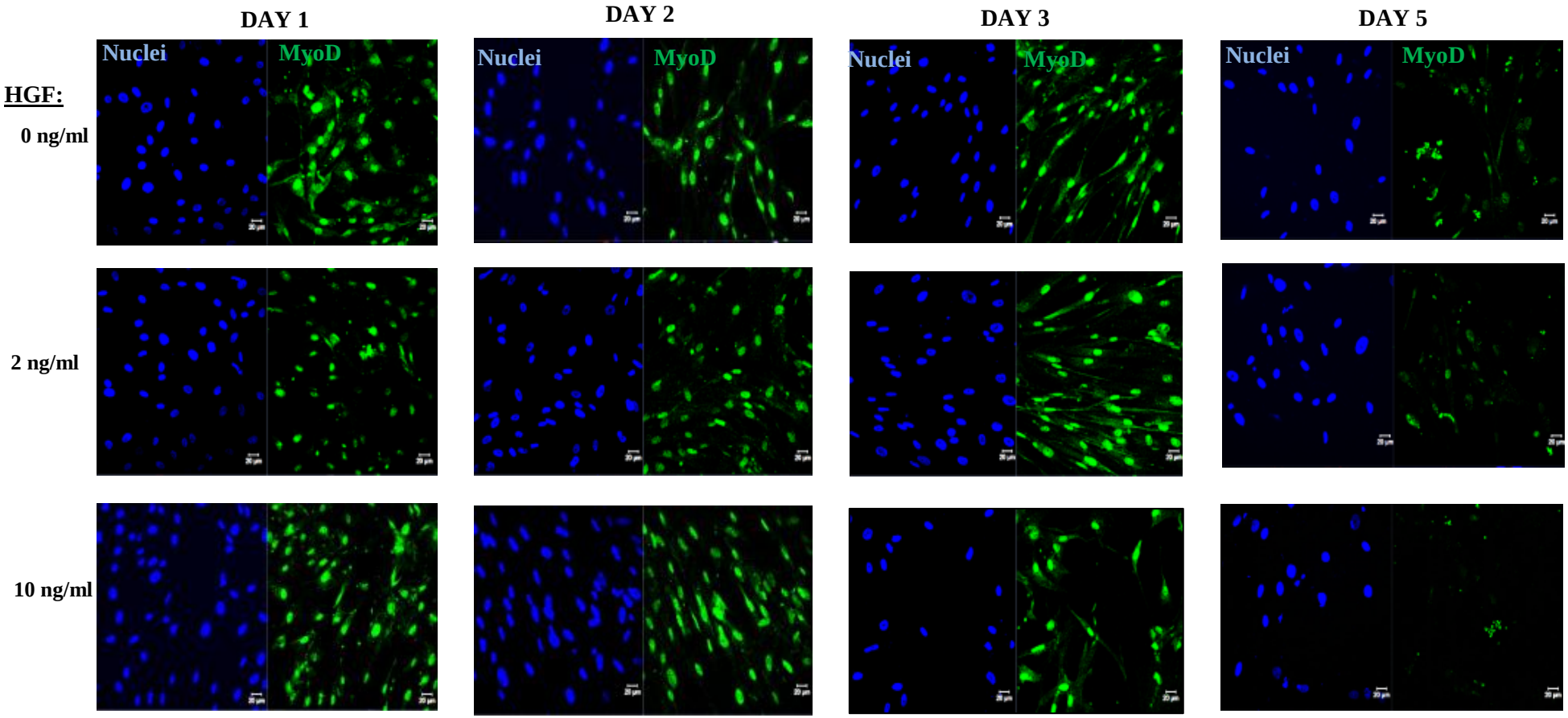

Figure 3.4: MyoD expression in differentiating HSkM myoblasts in response to HGF. Cells were allowed to differentiate for 1, 2, 3 and 5 days followed by fixation, immunocytochemistry and then analysis using the Zeiss confocal microscope 40 X magnifications. Scale bar = 20µm. Blue indicates the Hoechst nuclear stain; green indicates MyoD.

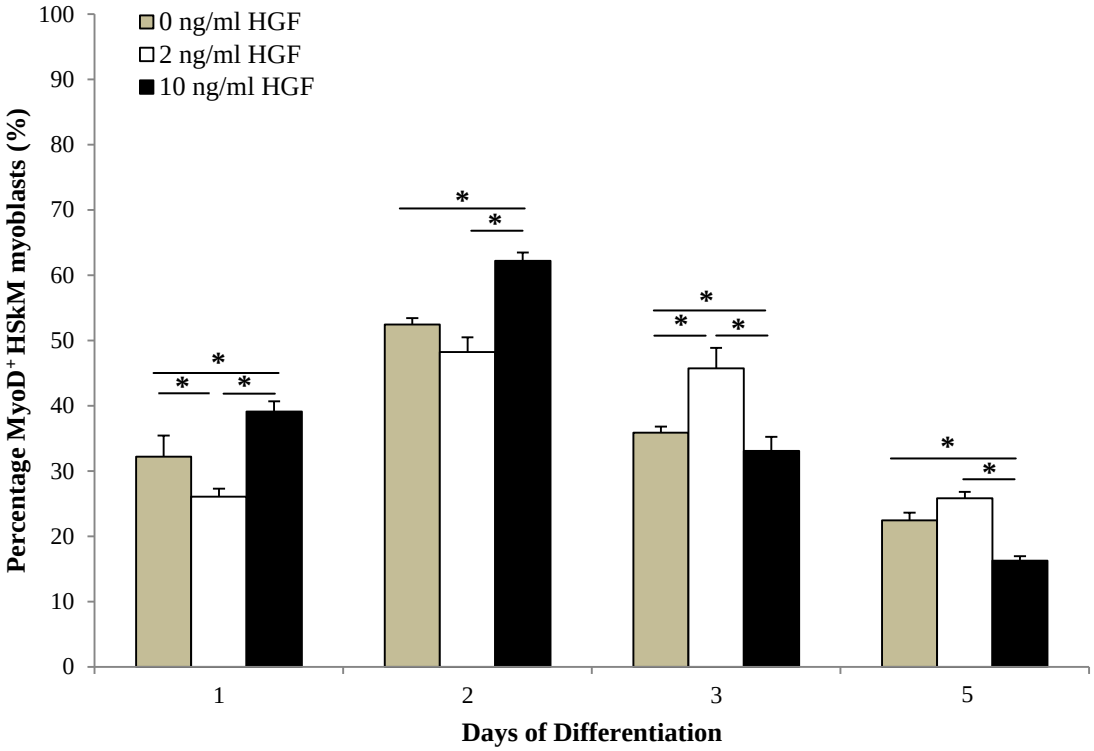

Figure 3.5: Percentage MyoD$^+$ cells in response to HGF during differentiation. HSkM cells were allowed to differentiate for 1-5 days followed by fixation, immunocytochemistry at then analysis using the Zeiss confocal microscope. Five random fields of view (from Figure 3.4) were observed for each treatment and quantified by Image J. *$p<0.05$. All data is mean ± SEM. n=4.

3.3.4. HGF promotes differentiation and myotube formation

Myoblast differentiation is a crucial step during skeletal muscle regeneration and is characterised by a permanent exit from cell cycle and up-regulation of myogenin and myosin heavy chain (MyHC). The differentiation of HSkM cells into myotubes was analysed by immunocytochemistry.

Under brightfield microscopy, we observed that cells cultured in differentiation media containing 10 ng/ml HGF differentiated more compared to untreated control; whilst a decrease was observed in response to 2 ng/ml HGF (Figure 3.6A). This observation was confirmed by confocal microscopy of differentiated cells labelled with a mouse monoclonal anti-MyHC antibody. Cells incubated with HGF (10 ng/ml) revealed a large number of MyHC positive myotubes which were long and striated compared to the untreated control. However, in response to 2 ng/ml HGF, fewer myotubes were formed and they demonstrated random alignment compared to untreated control (Figure 3.6B).

HSkM myoblasts first form mononucleated MyHC positive myocytes prior to fusion. We therefore quantified a differentiation index, calculated by identifying the number of MyHC positive cells as a percentage of the total nuclei per field of view. HGF at a concentration of 10 ng/ml significantly increased the differentiation index by 17.6% (61.2 ± 2.27%) compared to control (43.6 ± 4.54%, $p<0.01$). HGF (2 ng/ml) significantly decreased differentiation by 28% compared to 10 ng/ml HGF ($p<0.01$). This decrease was 10.4% significantly less compared to untreated control ($p<0.01$), at (Figure 3.7A).

We then calculated a fusion index, measured as a percentage nuclei detected in MyHC-labelled myotubes (containing two or more nuclei per tube). The fusion index analysis showed a similar trend to that observed in differentiation index analysis. A significant increase in the fusion index was displayed in response to 10 ng/ml HGF (52.6 ± 1.96%) compared to untreated control (30.8 ± 2.41%, $p<0.01$), whereas 2 ng/ml HGF decreased fusion to 24.6 ± 1.23%, compared to untreated control ($p<0.01$) (Figure 3.7B).

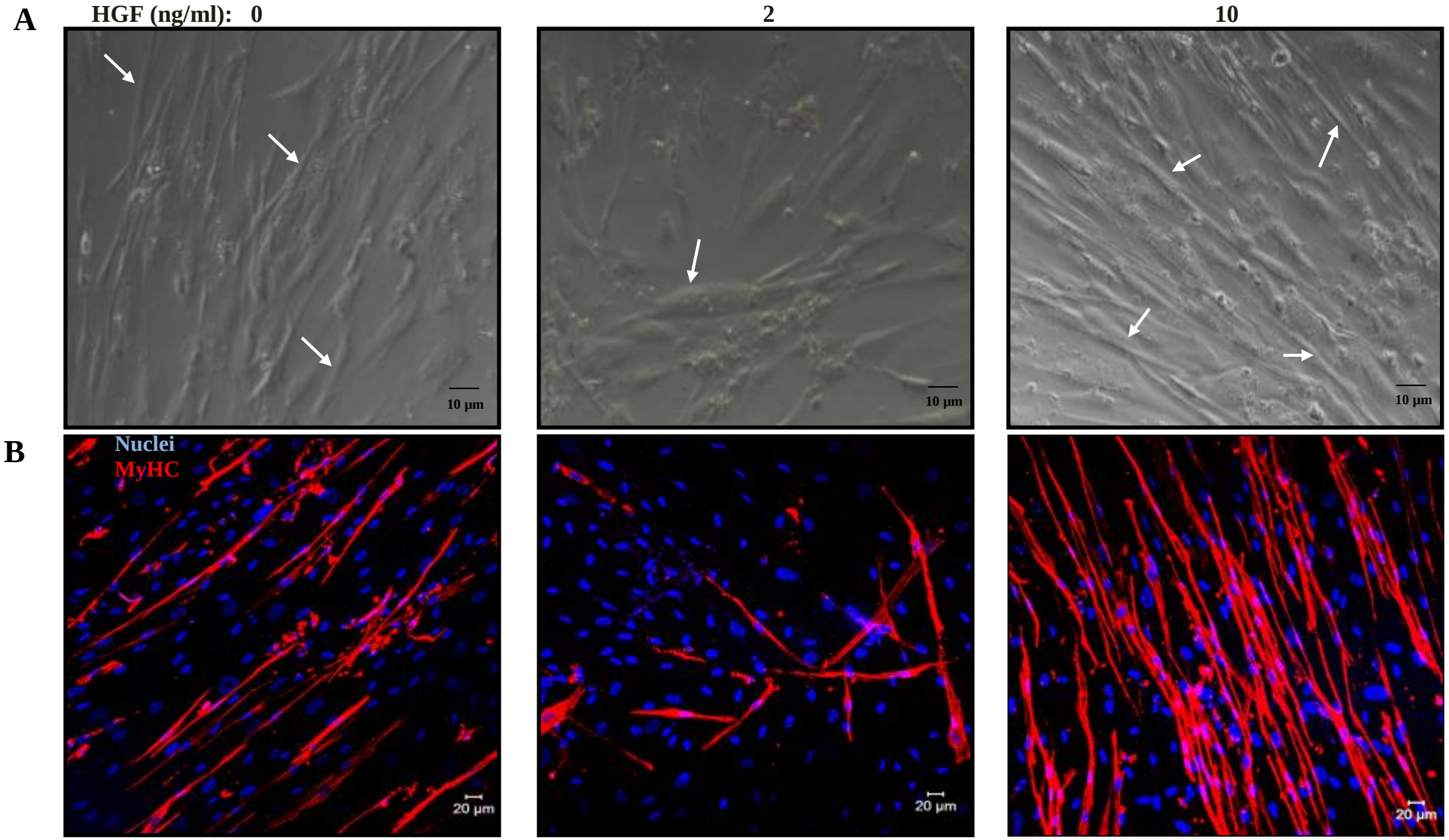

Figure 3.6: Myotube formation in differentiated HSkM myotubes treated with HGF. A) Phase contrast images of myotubes formed after day 7 of differentiation captured using the *Motic 2.0* camera, 10 X maginification. The arrows indicate the myotubes. Scale bar = 10 µm. **B**) Immunocytochemistry was carried out using MF20, an antibody directed against sarcomeric myosin (red) at day 7 of differentiation. Specific antibody labelling was visualized using DyLight 488 secondary antibody (red). Nuclei were visualized with Hoechst (blue) using the Zeiss Confocal microscope at 25 X magnification. Scale bar = 20 µm.

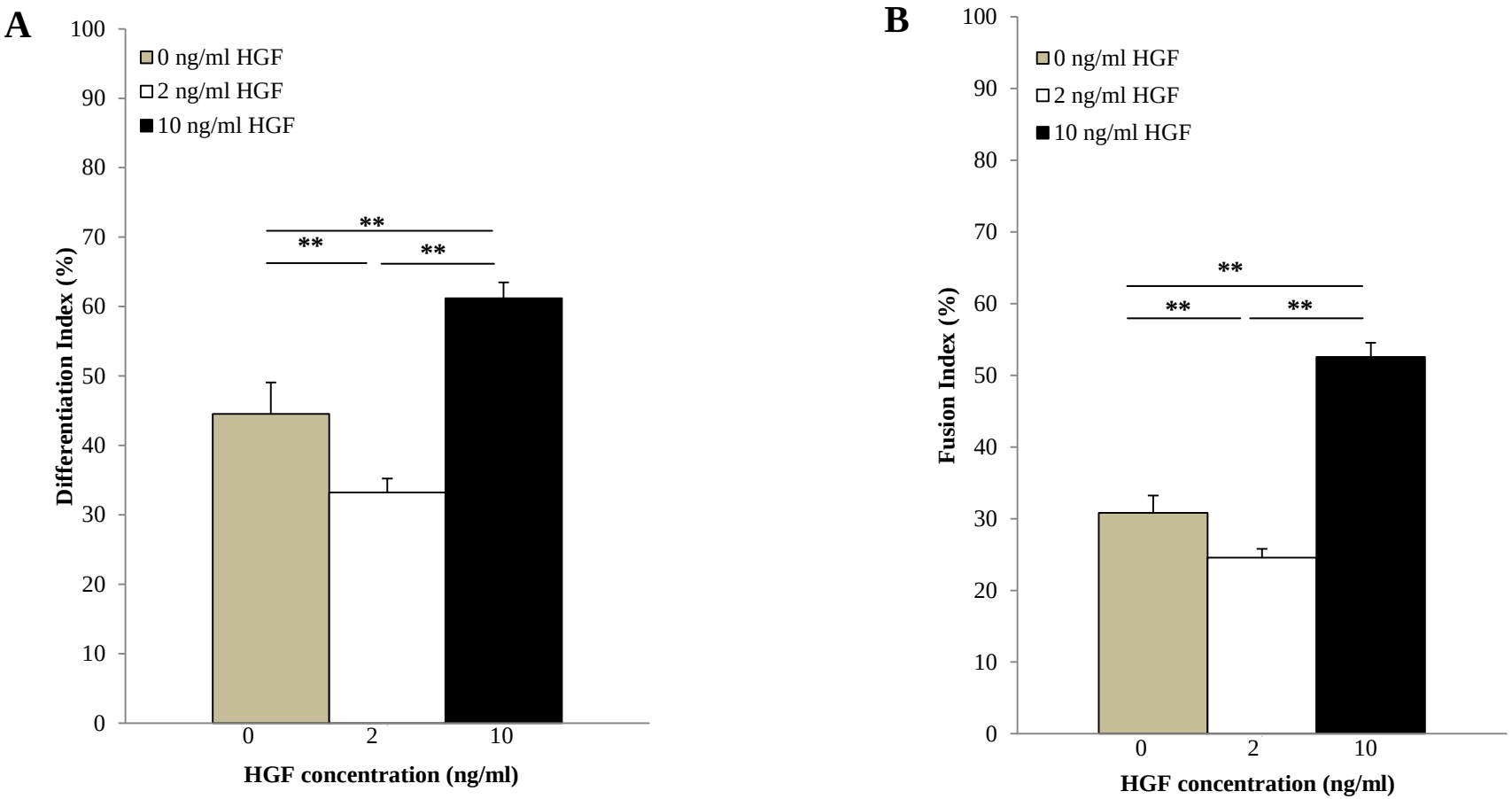

Figure 3.7: Differentiation and Fusion indices of HSkM myoblasts (day 7 of differentiation) following incubation with 0, 2 or 10 ng/ml HGF. A) Differentiation index of HSkM (day 7 of differentiation). Differentiation index was calculated by identifying the number of cells staining positive for MyHC as a percentage of the total nuclei per field of view using ImageJ software. **B**) The fusion index (i.e. percentage myotubes formed) was analysed for each of the HGF treatments using ImageJ software. Fusion index was measured as a percentage of the number of nuclei detected in MyHC-labelled myotubes (two or more). ** $p<0.01$, *$p<0.05$. All data is mean ± SEM. n=3.

3.4. DISCUSSION

Primary cultured human skeletal myoblasts provide additional benefits in research compared to immortalised cell lines; primary cultures have been shown to maintain the metabolic characteristics of skeletal muscle and thus mimic the actual skeletal muscle physiology more closely (Peault et al., 2007). However, these cells have finite lifespan and undergo cellular senescence following successive divisions (Mamchaoui et al., 2011); (Martin et al., 2009).

The use of transplantation therapies to treat diseases such as muscular dystrophies or severe muscle trauma requires proliferation and myoblast expansion (Peault et al., 2007). Hence investigating and understanding the HSkM myoblast proliferative capacity *in vitro* was of particular interest in this study. Findings from animal models suggest that HGF is a major inducer of satellite cell activation and proliferation (Morgan and Partridge, 2003, Gal-Levi et al., 1998b). In our study, the proliferative capacity of HSkM myoblasts was significantly enhanced by HGF (2 ng/ml), but decreased by 10 ng/ml HGF; this suggests a dose-dependent effect on proliferation in response to HGF. These findings are similar to the results observed previously in C2C12 myoblasts.

Myoblast differentiation is essential during skeletal muscle regeneration and is initiated by partial or complete restriction of cell proliferation as well as the up-regulation of MyoD, down-regulation of the Pax7 transcription factor (Olguin and Pisconti, 2012). In this study, we demonstrate that incubation of HSkM in HGF (2 ng/ml) increased the percentage Pax7 expressing cells, but decreased MyoD expression when assayed over 3 days in differentiation media. Notably, Pax7 expression is maintained up to day 5 of differentiation, suggesting a delay in differentiation in these cultures. Contrary to this, treatment of HSkM with HGF (10 ng/ml) decreased the percentage Pax7 expressing cells, but increased percentage MyoD expressing cells and by day 5 of differentiation, the cells were largely devoid of Pax7, suggesting early entry into the differentiation phase. *In vivo* studies on c-Met receptor upon administration of HGF (50 ng/ml) in mice have been shown to result in the silencing of MyoD expression (Anastasi et al., 1997). In addition, *in vivo* c-Met levels have also been shown to be regulated during differentiation after direct injection of HGF (6.25 and 15 ng/ml) into tibialis anterior muscle (Miller et al., 2000). Therefore, the decrease in MyoD expression i.e. decreased differentiation in response to HGF (2 ng/ml) could stem from such a decrease in receptor levels. So far, however, the pathways involved in the paracrine signalling triggered by HGF in HSkM remain largely elusive.

Human studies by O'Reilly *et al*. demonstrated that upon activation of satellite cells in human skeletal muscle by HGF, the cells begin to up-regulate HGF inhibitors (HAI-1 and HAI-2; *see chapter 1, section 1.2.3*) so that proliferating myoblasts can be driven towards differentiation (O'Reilly et al., 2008). We suggest that the increase and decrease in MyoD and Pax7 expression, respectively, might be due to increased expression of HGF inhibitors at 10 ng/ml HGF, which assist the cells to differentiate faster compared to those treated with 2 ng/ml HGF and untreated control, however, this requires further investigation.

We further investigated the effect of HGF on HSkM myoblast terminal differentiation via expression of MyHC. Previous studies have highlighted that cell fusion is accompanied by activation of muscle structural genes including MyHC (Airey et al., 1991); (Felsenfeld et al., 1991); (Gredinger et al., 1998). Similar studies have shown that the addition of exogenous HGF to satellite cells dose-dependently inhibits the expression of MyHC, which is expressed late in muscle differentiation (Miller et al., 2000, Bandow et al., 2004). In our study we observed a similar trend on the effect of HGF on differentiation.Our data suggest that in HSkM myoblasts, HGF (10 ng/ml) enhanced myoblast terminal differentiation and fusion, which was shown by increased MyHC expression and percentage fusion levels. However, there was a significant decrease in MyHC expression in response to 2 ng/ml HGF, suggesting HGF-induced delay of terminal differentiation at this particular dose. Myogenin and MRF4 have been implicated as essential MRFs for terminal differentiation. Down-regulation of myogenin during terminal differentiation allows re-entry of myoblasts into the cell cycle via down-regulation of MyHC (Mastroyiannopoulos et al., 2012). These findings open the possibility of down-regulatory effect of HGF (2 ng/ml) on myogenin expression, since a decreased MyHC expression was observed. This, however, requires further investigation.

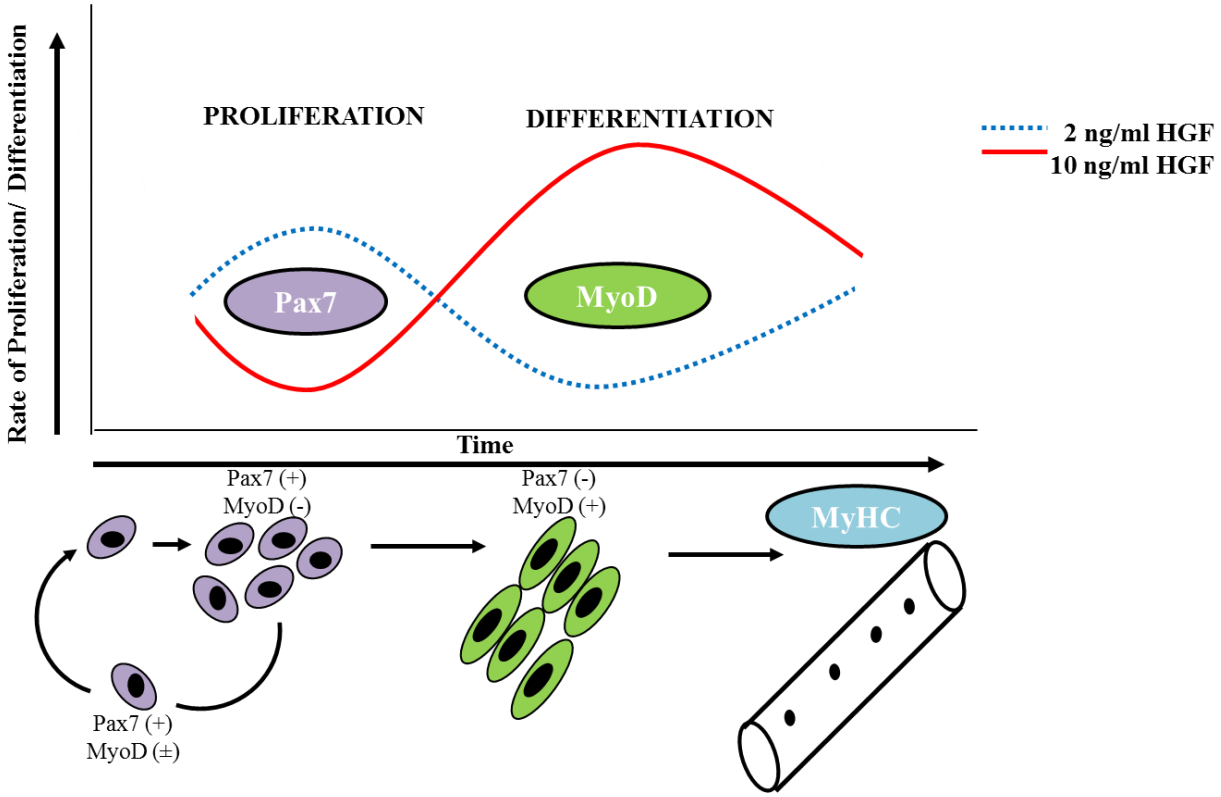

Figure 3.8: Schematic representation of the molecular events regulating HSkM myoblast proliferation and differentiation in the presence of HGF. Myoblast proliferation was elevated at 2 ng/ml HGF and reduced at 10 ng/ml HGF. Under differentiation condition, Pax7 was maintained and less MyoD and MyHC was expressed, suggesting delayed differentiation at 2 ng/ml HGF. Opposite effects were observed at 10 ng/ml HGF; under proliferative condition there was suppressed proliferation accompanied with down-regulation of Pax7 and up-regulation of MyoD expression. During differentiation, Pax7 was largely down-regulated and MyHC was greatly expressed suggesting early activation of differentiation in response to 10 ng/ml HGF.

In summary, our data confirm a dose-dependent effect of HGF on human myoblast proliferation and differentiation. Since the onset of differentiation results in partial or complete termination of proliferation, HGF (10 ng/ml) supresses proliferation, but increases early entry into differentiation. However, a lower HGF concentration (2 ng/ml) results in the opposite effect. Commitment to differentiation of myoblasts exposed to HGF (10 ng/ml) is accompanied by elevated expression levels of MyoD and down-regulation of Pax7. Our findings therefore highlight that the effect of HGF on myogenesis is highly dose-dependent and confirms previous work in C2C12 mouse myoblasts.

CHAPTER 4

GENERAL DISCUSSION

Following injury or stress, skeletal muscle is able to regenerate to create new myofibers. Regeneration is initiated by satellite cells, quiescent skeletal muscle-specific stem cells, localised between the basal lamina and sarcolemma (Dietrich et al., 1999). However, the activity of satellite cells during regeneration requires activation of these cells from quiescence, a process facilitated by growth factors including HGF (Bischoff, 1994). Upon activation, satellite cells proliferate into myoblasts, migrate to site of injury where they differentiate and fuse into myocytes and form myotubes to replace the damaged myofibers (Tedesco et al., 2010). HGF is known to play a key role not only in satellite cell activation, but also in the regulation of proliferation, migration and differentiation; however, the exact effects of HGF on these processes are not well defined in present literature. Therefore, in this current study we investigated and compared the effect of HGF on mouse versus human myoblast, proliferation, migration and differentiation. A summary of the main findings is presented in Table 4.1.

C2C12 cells represent an immortalized cell line which was originally derived from satellite cells from the thigh muscle of female mice. They possess myogenic properties and differentiate first into myocytes and after several days in media they form myotubes (Yaffe and Saxel, 1977). Although the immortalized C2C12 cell line is a valuable tool for the identification of mechanisms related with myogenic processes: activation, proliferation, migration or differentiation; such models do not necessarily represent the complexity of skeletal muscle physiology (Peault et al., 2007). In most cases, the mutations implemented in these cell lines remain in a mouse context, and there are frequently major variances between humans and mice (Mamchaoui et al., 2011). As such, primary culture of human cells may be a more physiologically-relevant model and circumvent some of the restrictions linked to cell lines (Martin et al., 2009).

Table 4.1: Summary of findings from chapters 2 and 3

Myogenic process	Analysis Method	C2C12 murine myoblasts		Human skeletal myoblasts	
		2 ng/ml HGF	10 ng/ml HGF	2 ng/ml HGF	10 ng/ml HGF
Proliferation	Cell counts	↑	↓	↑	↓
	Western Blot (Vinculin expression)	↓	↑	N/D	N/D
Migration	Scratch assay	↑	↓	N/D	N/D
	ICC (Vinculin expression)	↑	↓	N/D	N/D
Differentiation	ICC (%Pax7^{+})	↑	↓	↑	↓
	ICC (%MyoD^{+})	↓	↑	↓	↑
	ICC (Fusion)	↓	↑	↓	↑
	Western Blot (MyHC expression)	↓	↑	N/D	N/D

Key: ***ND = Not Done,***

↑ ***= increase of myogenic process***

↓ ***= decrease of myogenic process.***

Satellite cell proliferation is an important process in skeletal muscle repair and regeneration, however, *in vitro* the effect of HGF on proliferation is highly dose-dependent (Villena and Brandan, 2004, Yamada et al., 2010a). This is supportive to our results which also reflected a dose-dependent effect of HGF on myoblast proliferation. Proliferation studies in C2C12 myoblasts revealed a 4.5 fold increase (relative to number plated) in myoblast number 24 h after exposure to exogenous HGF (2 ng/ml). However, when these studies were carried out in HSkM myoblasts, a 1.5 fold increase was registered in the presence of HGF (2 ng/ml) after 24 h, and a further 3.8 fold increase in cell number was attained after 48 h of culture. Proliferation is known to be more responsive to environmental regulation, such as low oxygen culture conditions, in mice compared to human cells (Martin et al., 2009). Furthermore, human myoblasts have relatively short proliferative lifespan *in vivo* and their proliferative capacity *in vitro* is poor compared to C2C12 myoblasts (Mamchaoui et al., 2011). This may explain the lower cell numbers observed with HSkM myoblasts compared to C2C12 myoblast numbers in the presence of exogenous HGF. However, in response to 10 ng/ml HGF, a significant decrease in proliferation in both C2C12 and HSkM myoblasts was seen. From these findings, we suggest that HGF (10 ng/ml) might have a threshhold dose that supresses proliferation and begins promoting other processes e.g. differentiation or migration.

Leshem *et al.* demonstrated that HGF receptor coupling to MAPK pathway via the adaptor, Grb2, is involved in the stimulation of proliferation and inhibition of myoblast differentiation (Leshem et al., 2002). Other studies have revealed cross-talk between the MAPK and PI3K pathways downstream of HGF (Yu et al., 2001). Additionally, it has been reported that inhibition of PI3K in C2 myoblasts increases cell proliferation, suggesting that activation of this pathway might cause a decrease in proliferation (Zeng et al., 2002). We suspect that the MAPK pathway contributes to the increase in proliferation in response to 2 ng/ml HGF, while the PI3K pathway initiates a decrease in proliferation at higher HGF concentration. Apart from MAPK and PI3K, STAT3 signalling pathway has also been implicated in the regulation of myoblast proliferation. This pathway is another molecular event that promotes C2C12 myoblast proliferation and inhibits differentiation by repressing MyoD expression (Kami and Senba, 2002); (Sun et al., 2007); (Wang et al., 2008). Given the increase in proliferation observed when both C2C12 and HSkM myoblasts were exposed to HGF (2 ng/ml), we propose an implication of STAT3 pathway during this effect of HGF. Low affinity binding of HGF to HSPGs stimulates activation of MAPK pathways signalling routes that regulate cell proliferation (Derksen et al., 2002); this could be the reason for the dose-dependent effects, c-Met versus HSPGs as signalling receptors. This however, requires further investigation.

Myoblast differentiation is essential during skeletal muscle repair and is regulated through the expression of MRFs including MyoD and transcription factors including Pax7. This process is also characterised by partial or complete restriction of cell proliferation i.e. cell cycle exit (Olguin and Pisconti, 2012). During differentiation, MyoD expression was stimulated in response to 10 ng/ml HGF followed by a down-regulation of Pax7. This is indicative of termination of proliferation in the cell population and commitment to differentiation. Furthermore, in adult muscle, activation of PI3K is necessary initiation of differentiation (Leshem et al., 2002). Therefore, when PI3K activation is above a certain threshold, differentiation is stimulated, as observed in our study. It has also been suggested that overexpression of an activated form of Ras prevents the differentiation of C2C12 myoblasts (Lassar et al., 1989); (Brennan et al., 1990). A separate study suggested that binding of c-Met to Grb2 supresses p38 activation, hence contributing to the inhibitory effect of HGF on skeletal muscle differentiation (Leshem et al., 2002). As a result, further work to elucidate the activity of the MAPK and PI3K pathways in response to HGF would be prudent.

HGF has been reported to regulate the differentiation of the chicken skeletal muscle satellite cells and C2 myogenic cell line by controlling the expression levels of MyoD (Anastasi, 1997); (Gal-Levi et al., 1998b); (Leshem et al., 2000). Exposure of C2 myoblasts to exogenous HGF (10 ng/ml) demonstrated suppression of Pax7 and induction of MyoD, activities that were shown to be essential for myoblasts to complete the cell cycle and enter the differentiation process (Yamane et al., 2004); (Gill et al., 2010). These results are consistent with our findings; exogenous HGF (10 ng/ml) stimulated both C2C12 and HSkM myoblast to enter the differentiation process by supressing Pax7 expression. Moreover, work by Yoshida *et al*., showed that down-regulation of MyoD induces self-renewal and quiescence of C2C12 myoblasts (Yoshida et al., 1998), hence we propose that the down-regulation of MyoD expression at 2 ng/ml indicates that HGF delays differentiation and promotes proliferation. In C2C12 myoblasts, MyoD expression was largely down regulated by day 3 of differentiation but in HSkM myoblasts, expression of this protein was down regulated by day 5 of culture. Interestingly, both C2C12 and HSkM myoblasts revealed a peak in MyoD expression by day 2 of differentiation, followed by a decrease at day 3. As early as day 3, C2C12 myoblasts were showing alignment indicative of entry into terminal differentiation. This was confirmed at day 5 when these cells formed myotubes. In contrast, the HSkM myoblasts showed alignment by day 5 and fusion and myotube formation was only observed at day 7 of differentiation. In all these observations, we noted that the effect of HGF on differentiation was highly dose-dependent. Since, MyoD expression was elevated at 10 ng/ml HGF compared to 2 ng/ml HGF, we expected increased MyHC expression at 10 ng/ml compared to 2 ng/ml HGF, as MyoD has been reported to induce the expression of other muscle specific proteins such as MyHC and

alpha-actin (Burattini et al., 2004). We observed that higher doses of HGF (10 ng/ml) resulted in increased MyHC expression associated with a 48% fusion index in C2C12 myoblasts and a 52.6% was observed in HSkM myoblasts. A lower HGF concentration (2 ng/ml) resulted in lower MyHC expression associated with a 29.4% fusion index in C2C12 myoblasts and 24.6% in HSkM myoblasts. These data highlight that although HGF differentially affects myoblast differentiation in a dose-dependent manner, the absolute effects are essentially the same in both HSkM and C2C12 myoblasts.

Analysis of the percentage wound closure indicated that HGF (10 ng/ml) while decreasing proliferation, stimulated migration. The lower HGF dose (2 ng/ml) that increased proliferation, on the other hand, resulted in reduced wound closure. Vinculin is a ubiquitously expressed actin filament binding protein that localizes in focal adhesions and is known to be actively involved in myoblast migration (Le Clainche and Carlier, 2008). Vinculin expression levels have been shown to be higher in migrating cells, as it is mainly involved in the regulation of focal adhesions of migrating cells (Moon et al., 2004). Cells treated with 10 ng/ml HGF revealed higher vinculin expression levels which correlated with an increased percentage wound closure. Our results therefore indicate that HGF affects vinculin expression in migrating C2C12 myoblasts in a dose-dependent manner. For future studies, the examination of migration of primary cultured human myoblasts in the presence of HGF is essential to confirm C2C12 results. The ability of HGF to either negatively or positively alter migratory capabilities via vinculin expression of satellite cells is supportive of its function in skeletal muscle repair (Bischoff, 1997, Yamada et al., 2010b).

Taken together, our results demonstrate the central role that HGF plays during all the myogenic processes involved in the stages of skeletal muscle regeneration in both mouse C2C12 myoblast lines and primary cultured human myoblasts. HGF stimulates myoblast expansion and growth by means of mitogenic activities. Although the effects of HGF are highly dose-dependent, the extent of the effects on proliferation and differentiation are differential and dependent on the cell type. In an injury scenario, our data suggests that at lower HGF concentrations (2 ng/ml); proliferation is stimulated to increase cell population to initiate beginning of repair. Increasing HGF concentration (10 ng/ml) (later during injury) accelerates the transition of myoblasts from proliferation to differentiation to promote alignment, fusion and repair. We therefore conclude that the modulation of myogenic processes (proliferation and differentiation) by HGF indicate the importance of this cytokine in skeletal muscle regeneration and is a potential drug target for muscle disease. We hope that further studies in primary cultured human myoblasts may lead to the clarification of clinical and diagnostic roles of HGF for improved muscle repair.

APPENDIX A:

BLAST SEQUENCE ALIGNMENT OF HUMAN AND MURINE HGF

hepatocyte growth factor [Mus musculus]

Sequence ID: emb|CAA51054.1| **Length:** 748 **Number of Matches:** 1

Range 1: 22 to 748 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
1366 bits(3535)	0.0	Compositional matrix adjust.	660/727(91%)	699/727(96%)	2/727(0%)

```
Mouse   1    MWVTKLLPALLLQHVLLHLLLLPIAIPYAEGQRKRRNTIHEFKKSAKTTLIKIDPALKIK  60
             MW TKLLP LLLQHVLLHLLLL +AIPYAEGQ+KRRNT+HEFKKSAKTTL K DP LKIK
Human   22   MWGTKLLPVLLLQHVLLHLLLLHVAIPYAEGQKKRRNTLHEFKKSAKTTLTKEDPLLKIK  81

Mouse   61   TKKVNTADQCANRCTRNKGLPFTCKAFVFDKARKQCLWFPFNSMSSGVKKEFGHEFDLYE  120
             TKKVN+AD+CANRC RN+G  FTCKAFVFDK+RK+C W+PFNSMSSGVKK FGHEFDLYE
Human   82   TKKVNSADECANRCIRNRGFTFTCKAFVFDKSRKRCYWYPFNSMSSGVKKGFGHEFDLYE  141

Mouse   121  NKDYIRNCIIGKGRSYKGTVSITKSGIKCQPWSSMIPHEHSFLPSSYRGKDLQENYCRNP  180
             NKDYIRNCIIGKG SYKGTVSITKSGIKCQPW+SMIPHEHSFLPSSYRGKDLQENYCRNP
Human   142  NKDYIRNCIIGKGGSYKGTVSITKSGIKCQPWNSMIPHEHSFLPSSYRGKDLQENYCRNP  201

Mouse   181  RGEEGGPWCFTSNPEVRYEVCDIPQCSEVECMTCNGESYRGLMDHTESGKICQRWDHQTP  240
             RGEEGGPWCFTSNPEVRYEVCDIPQCSEVECMTCNGESYRG MDHTESGK CQRWD QTP
Human   202  RGEEGGPWCFTSNPEVRYEVCDIPQCSEVECMTCNGESYRGPMDHTESGKTCQRWDQQTP  261

Mouse   241  HRHKFLPERYPDKGFDDNYCRNPDGQPRPWCYTLDPHTRWEYCAIKTCADNTMNDTDVPL  300
             HRHKFLPERYPDKGFDDNYCRNPDG+PRPWCYTLDP T WEYCAIKTCA + +N+TDVP+
Human   262  HRHKFLPERYPDKGFDDNYCRNPDGKPRPWCYTLDPDTPWEYCAIKTCAHSAVNETDVPM  321

Mouse   301  ETTECIQGQGEGYRGTVNTIWNGIPCQRWDSQYPHEHDMTPENFKCKDLRENYCRNPDGS  360
             ETTECIQGQGEGYRGT NTIWNGIPCQRWDSQYPH+HD+TPENFKCKDLRENYCRNPDG+
Human   322  ETTECIQGQGEGYRGTSNTIWNGIPCQRWDSQYPHKHDITPENFKCKDLRENYCRNPDGA  381

Mouse   361  ESPWCFTTDPNIRVGYCSQIPNCDMSHGQDCYRGNGKNYMGNLSQTRSGLTCSMWDKNME  420
             ESPWCFTTDPNIRVGYCSQIP CD+S GQDCYRGNGKNYMGNLS+TRSGLTCSMWDKNME
Human   382  ESPWCFTTDPNIRVGYCSQIPKCDVSSGQDCYRGNGKNYMGNLSKTRSGLTCSMWDKNME  441

Mouse   421  DLHRHIFWEPDASKLNENYCRNPDDDAHGPWCYTGNPLIPWDYCPISRCEGDTTPTIVNL  480
             DLHRHIFWEPDASKLN+NYCRNPDDDAHGPWCYTGNPLIPWDYCPISRCEGDTTPTIVNL
Human   442  DLHRHIFWEPDASKLNKNYCRNPDDDAHGPWCYTGNPLIPWDYCPISRCEGDTTPTIVNL  501

Mouse   481  DHPVISCAKTKQLRVVNGIPTRTNIGWMVSLRYRNKHICGGSLIKESWVLTARQCFPSR-  539
             DHPVISCAKTKQLRVVNGIPT+T +GWMVSL+YRNKHICGGSLIKESWVLTARQCFP+R
Human   502  DHPVISCAKTKQLRVVNGIPTQTTVGWMVSLKYRNKHICGGSLIKESWVLTARQCFPARN  561

Mouse   540  -DLKDYEAWLGIHDVHGRGDEKCKQVLNVSQLVYGPEGSDLVLMKLARPAVLDDFVSTID  598
              DLKDYEAWLGIHDVH RG+EK KQ+LN+SQLVYGPEGSDLVL+KLARPA+LD+FVSTID
Human   562  KDLKDYEAWLGIHDVHERGEEKHKQILNISQLVYGPEGSDLVLLKLARPAILDNFVSTID  621

Mouse   599  LPNYGCTIPEKTSCSVYGWGYTGLINYDGLLRVAHLYIMGNEKCSQHHRGKVTLNESEIC  658
             LP+YGCTIPEKT+CS+YGWGYTGLIN DGLLRVAHLYIMGNEKCSQHH+GKVTLNESE+C
Human   622  LPSYGCTIPEKTTCSIYGWGYTGLINADGLLRVAHLYIMGNEKCSQHHQGKVTLNESELC  681

Mouse   659  AGAEKIGSGPCEGDYGGPLVCEQHKMRMVLGVIVPGRGCAIPNRPGIFVRVAYYAKWIHK  718
             AGAEKIGSGPCEGDYGGPL+CEQHKMRMVLGVIVPGRGCAIPNRPGIFVRVAYYAKWIHK
Human   682  AGAEKIGSGPCEGDYGGPLICEQHKMRMVLGVIVPGRGCAIPNRPGIFVRVAYYAKWIHK  741

Mouse   719  IILTYKV  725
             +ILTYK+
Human   742  VILTYKL  748
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Figure A: Blast sequence alignment of Human and Mouse (Mus musculus) HGF retrieved from National Centre for Biotechnology (NCBI) Protein database using the Basic Local Alignment Search Tool (BLAST) on 31/06/13. The results show 91% amino acid similarities between mice and humans, which indicates that HGF is highly conserved between the two species.

APPENDIX B:

CELL CULTURE REAGENTS

C2C12 Myoblast Growth Media (10% v/v FCS): A solution of 430 ml Dulbecco's Modified Eagle Serum (DMEM) (Thermo Scientific Laboratories, USA, cat.SH30243.01), 10 ml L-glutamine (2% v/v) (Lonza, USA, cat.BE17-605E), 10 ml PenStrep (2% v/v) (Lonza, USA, cat.DE17-602E), and 50 ml fetal calf serum (10% v/v) (Biowest, USA, cat.S181H-500) was made up and filter sterilized. The media was aliquoted into 50 ml falcon tubes and stored at 4^{o}C.

C2C12 Myoblast Differentiation Media (2 % v/v HS): A solution of 475 ml DMEM (Thermo Scientific Laboratories, USA, cat.SH30243.01), 10 ml L-glutamine (2 % v/v) (Lonza, USA, cat.BE17-605E), 10 ml PenStrep (2% v/v) (Lonza, USA, cat.DE17-602E) and 5 ml horse serum (HS, 1% v/v) (Biowest, USA, cat.S090H-100) was made up and filter sterilized. The media was aliquoted into 50 ml falcon tubes and stored at 4^{o}C.

Skeletal Muscle Cell Medium BulletKit® (Lonza, USA, cat.CC3161): The bulletkit is bought already made-up by the manufacturer. It is composed of 500 ml of Skeletal Muscle Cell Basal Medium and the following growth supplements: 0.5 ml hEGF, 5 ml insulin, 5 ml Fetuin, 0.5 ml Dexamethasone, 0.5 ml GA-1000 and 5 ml BSA. The media was aliquoted into 50 ml falcon tubes and stored at 4^{o}C.

Human Myoblast Growth Media (10% v/v FCS): A solution of 152 ml HAMS-F10 (Life Technologies, South Africa, cat.31550-015), 4 ml L-glutamine (2% v/v) (Lonza, USA, cat.BE17-605E), 4 ml PenStrep (2% v/v) (Lonza, USA, cat.DE17-602E), 2.5 ng/ml FGF (Promega, USA, cat.G507A) and 40 ml fetal calf serum (20 % v/v) (Biowest, USA, cat.S181H-500) was made up and filter sterilized. The media was aliquoted into 50 ml falcon tubes and stored at 4^{o}C.

Human Myoblast Differentiation Media (2 % HS): A solution of 188 ml HAMS-F10 (Life Technologies, South Africa, cat.31550-015), 4 ml L-glutamine (2% v/v) (Lonza, USA, cat.BE17-605E), 4 ml PenStrep (2% v/v) (Lonza, USA, cat.DE17-602E) and 4 ml horse serum (HS, 2% v/v) (Biowest, USA, cat.S090H-100) was made up and filter sterilized. The media was aliquoted into 50 ml falcon tubes and stored at 4^{o}C.

Hepatocyte Growth Factor (HGF) preparation: Human HGF (PeproTech, USA; cat.100-39) was made up to a stock concentration of 10 µg/ml in sterile PBS and was stored in 50 µl aliquots at -20^{o}C. When required, the stock solution was serially diluted in growth or

differentiation media to final concentrations of 2, 10 and 50 ng/ml and was used to assess proliferation, migration and differentiation.

Sterile Phosphate buffered saline (PBS; pH 7.2): 16 g NaCl, 0.4 g KCl, 2.3 g Na_2HPO_4 and 0.4 g KH_2PO_4 were dissolved in 1.6 liters distilled water and stirred with a magnetic stirrer until dissolved. The pH was adjusted to pH 7.2 with HCl. Separately, 0.264 g $CaCl_2$ was dissolved in 200 ml distilled water and 0.2 g $MgCl_2.6H_2O$ was dissolved in 200 ml distilled water. All three solutions were autoclaved and then added together once they had cooled to make up sterile PBS for cell culture.

Sterile Trypsin: Since C2C12 cells are adherent cells, trypsin (Highveld, USA, cat.CN3796) was used to lift them up during experiments. The trypsin contained 0.125% Trypsin, 0.1% Versene EDTA and Ca^{2+} & Mg^{2+} free PBS. The trypsin was stored in 50 ml aliquots at 4^oC and was always warmed in a 37^oC water bath before use.

APPENDIX C:

CRYOPRESERVATION (LIQUID NITROGEN STORAGE), THAWING, PASSAGING, AND DIFFERENTIATION

Cells were stored in cryotubes (Nunc, USA, cat.65232) containing growth media supplemented with 10% v/v DMSO in 1 ml aliquots of approximately 1 x 10^6 cell in liquid nitrogen (-196^{o}C). For experimentation, cells were thawed for one minute in a water bath at 37^{o}C, and then instantly transferred to a T25 flask tissue culture flask (Nunc, USA, cat.156367) containing 4 ml of warm growth media.

When cells had reached ~70% confluence, they were split 1:4 and moved to a different T75 flask. To passage, cell media within a T75 tissue culture flask (Nest, USA, cat.0926B) was discarded and cells were washed with warm sterile PBS. After discarding the PBS, 2 ml of trypsin was then added to the flask and incubated at 37^{o}C for 3-5 min. Equal volume of growth media was added to cells to neutralize the trypsin. The cells were then split into new T75 flasks containing warm growth media and cultured for future use, or counted and plated out for experiments.

Differentiation was performed in 24-well tissue culture plates (Cell Star, USA, cat.662160) on 12 mm round coverslips (Lasec, USA, cat.0111520). Initially, the cells were cultured in growth media, until they were 80% confluent. The growth media was then replaced with differentiation media. Cells were incubated at 37^{o}C in a humidified atmosphere at 5% CO_2 for either 1-5 days (C212 cells) or 1-7 days (HSkM cells), with media being changed every two days.

APPENDIX D:

DETERMINATION OF CELL CULTURE CONTAMINATION

Culture contaminants were determined either by confocal or brightfield microscopy as described previously (Stacey, 2011). Briefly, for confocal microscopy, 1x 10^4 cells were plated out in 24 well plates on coverslips and incubated for 24 h, thereafter they were fixed with 4% paraformaldehyde for 15 min and stained with Hoechst (1: 2000) for 5 min. After several washes with PBS, coverslips were mounted with 4 µl Moviol onto microscope slides and viewed with the Zeiss confocal microscope. The presence of blue string-like structures around the nuclei indicated the presence of mycoplasma. For brightfield microscopy, 5 x 10^4 cells were plated out in T25 tissue culture flasks and incubated for 24 h; pictures were then taken with the *Motic* 3 megapixel camera at 40 X magnification. Black, rod-like shaped motile particles indicated bacteria contamination, which was associated with suppressed cell growth and increased cell death. Budding, shiny structures within cell cultures (visible for a few hours, however, after several hours the culture media became turbid) indicated the presence yeast infection (Stacey, 2011).

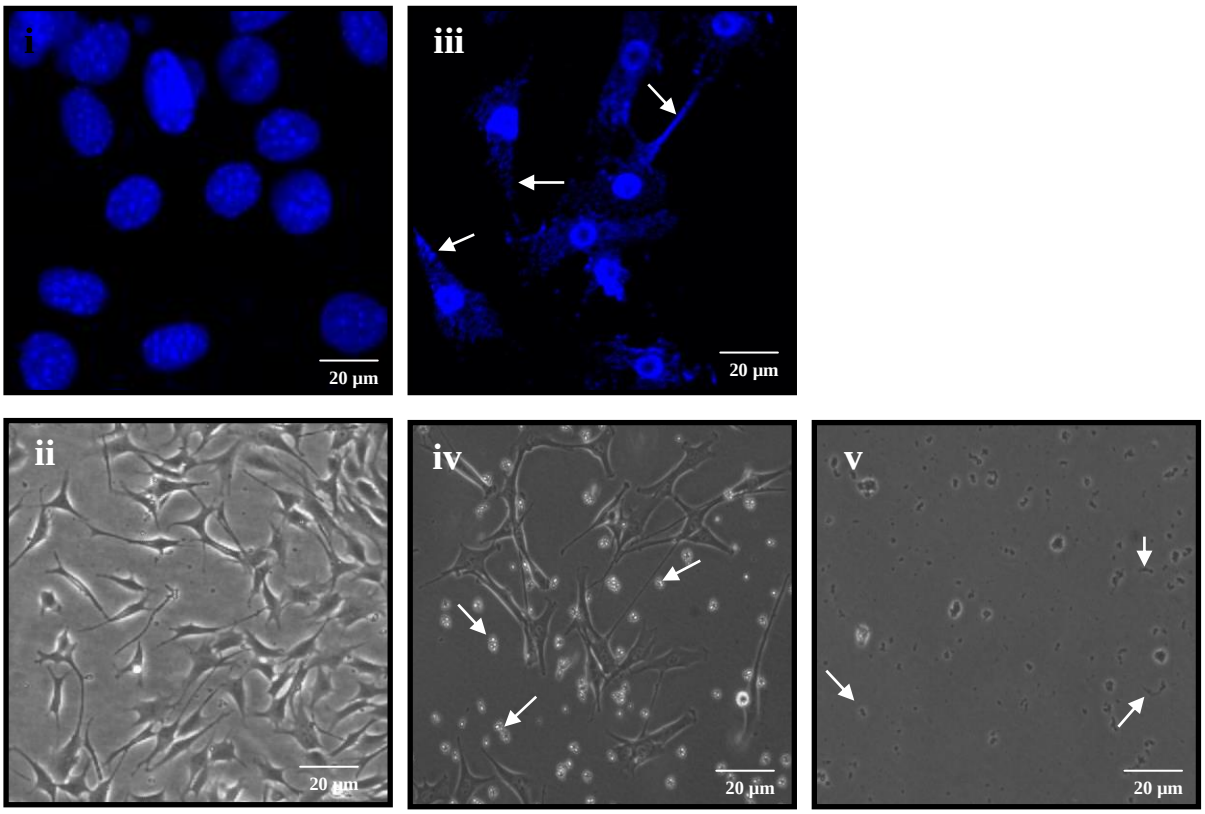

Figure D: Confocal and bright-field Images of different types of contamination. **i)** Confocal images of non-contaminated C2C12 cells. **ii)** Phase contrast images non-contaminated C2C12 cells. **iii**) C2C12 cell nuclei and mycoplasma stained brightly under confocal microscopy allowing clean culture (**i**) to be easily distinguished from infected culture (**iii**). **iv)** Budding yeast cells within cell culture indicating the presence of yeast infection. **v)** Intense bacterial-infected C2C12cells. Arrows indicate contamination.

APPENDIX E:

CRYSTAL VIOLET STANDARD CURVE

Reagents

0.2% Crystal violet dye: 0.2 g of crystal violet powder (Sigma, Germany, cat.C-3886) was dissolved in 100 ml methanol. The solution was stored in a plastic bottle at room temperature.

Method

Cells were plated out at initial densities of 10 000, 20 000, 40 000 and 80 000 cells in six well plates containing growth media and incubated at 37^{o}C, 5% CO_2 for 24 h. Media was removed and cells were stained with crystal violet dye for 10 min. The wells were then washed with distilled water and 1% sodium dodecyl sulphate (SDS) was then added to solubilize the cells. 100 µl of cell solutions were then transferred to a 96 well ELISA plate (this was done in triplicate). Using FluoStar Optima microplate reader machine (BMG, Labtech, USA), absorbance readings were obtained and a standard curve generated. The linear equation generated from the curve was then used to calculate the cell numbers during subsequent proliferation experiments.

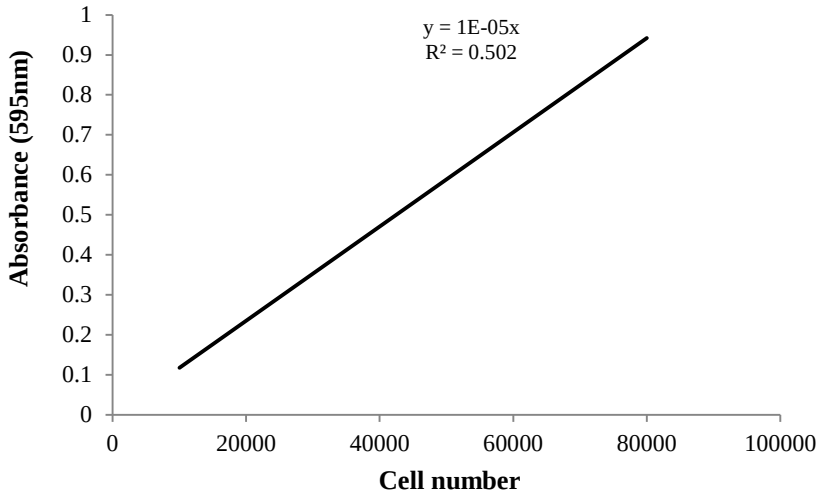

Figure E: Crystal violet assay standard curve. The graph illustrates a linear relationship between crystal violet absorbance and cell number.

APPENDIX F:

ANTIBODY OPTIMIZATION FOR IMMUNOCYTOCHEMISTRY

To determine the best antibody concentrations to use during subsequent immunocytochemistry experiments, a series of antibody dilutions were used. Initially, ~3 x 10^4 cells were plated out on coverslips in 24 well tissue culture plates (in duplicates) and cultured for 24 h. Media was removed, cells were washed with PBS then fixed for 15 min with 4% paraformaldehyde. This was followed by several washes of the cells with PBS then blocking with 5% donkey serum for 30 min and incubation with serially-diluted antibody solutions overnight at 4^{o}C. Serially diluted secondary antibody solutions were then added after several washes of the cells with PBS. Hoechst was then added at a final concentration of 1:2000 and several washes were carried out. Finally coverslips were mounted on microscope slides with Moviol then viewed with the Zeiss confocal microscope. The dilution range that revealed moderate brightness was then applied during subsequent immunocychemistry experiments.

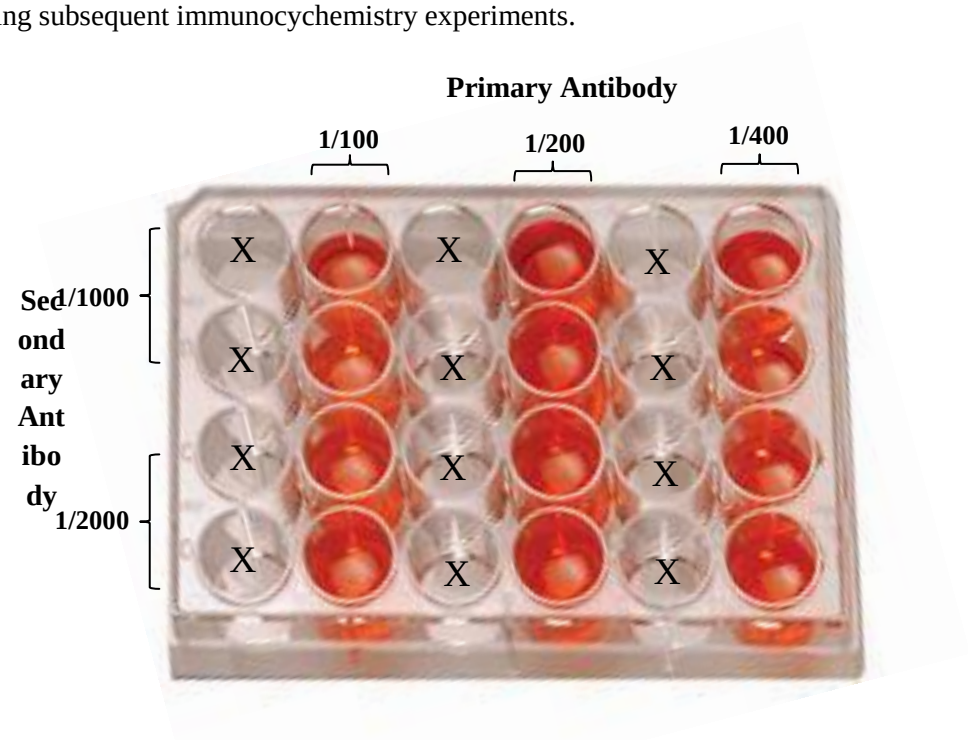

Figure F: Antibody optimization for immunocytochemistry: hypothetical layout of primary and secondary antibody dilutions. X indicates empty wells. Primary antibody dilutions were carried out in duplicate.

APPENDIX G:

OPTIMIZATION OF ANTIBODIES FOR WESTERN BLOTS: DOT BLOTS

Reagents

Immuno Star™ Western C™ Kit: The kit (Biorad, USA, cat.170-5070) contained Lumino/Ehancer solution and peroxidase solution, which was made up in a 1:1 ratio prior to immediate use.

Method

Dot blots were performed to determine the best antibody concentrations to use during subsequent western blot experiments. On a small piece of nitrocellulose, small circles were drawn in pencil and 1µl of cell lysates was placed in the circle and allowed to air dry. Non-fat milk (5% (m/v)) made up in Tween-Tris Buffer Saline (TTBS) (*Appendix L*) was then used to block for 3 h. (*NB: gloves should be worn at all times when handling protein lysates and nitrocellulose*). Different ranges of primary antibodies were made up in TTBS in serial dilutions and added for 2 h. The papers were washed several times with TTBS on a rocker. Different ranges of the secondary antibody also made up in TTBS were the applied for 1 h and several washes were done using TTBS. (*NB: Each primary and secondary antibody has its own recommended dilution determined from literature and data sheets (Appendix H*)). Immuno Star™ Western C™ Kit was added to the nitrocellulose paper which had been placed between two pieces of transparent film. Enhanced Chemiluminescence was used for development of dots on light film (Kodak Biomax Light film; Sigma, Germany) in the dark room with no white light. The light film was exposed to the nitrocellulose paper for 30 sec -10 min depending on primary antibody. After exposure, the film was first placed in developer ((1:3 dilution in water) Structirix G128, Agfa, USA) until dots were formed, then secondly in fixative solution ((1:4 dilution in water) G333c Rapid fixer, Agfa, USA), finally in water. The dilution range that showed maximal binding of both primary and secondary antibodies without non-specific binding was selected.

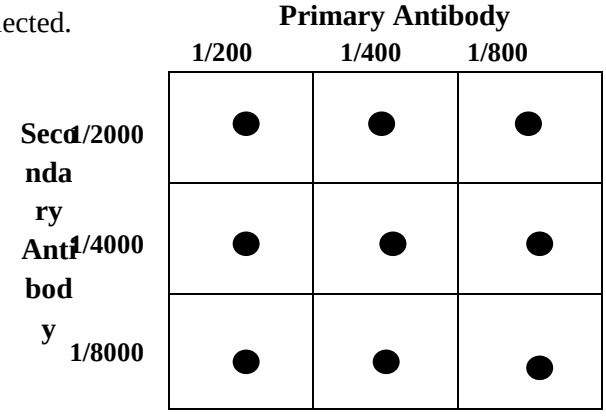

Figure G: Antibody optimization for western blot: hypothetical layout of primary and secondary antibody dilutions.

APPENDIX H:

ANTIBODY DILUTIONS AND STAINS FOR WESTERN BLOT ANALYSIS AND CONFOCAL MICROSCOPY

Table H.1: Primary antibody dilutions used for western blot analysis and immunocytochemistry

Antibody	Host species	Molecular weight (kDa) of protein	Application	Dilution factor	Company
Pax3/7	Goat monoclonal	~57	ECL ICC	1/100	Santa Cruz, USA, sc-7748
Vinculin	Mouse monoclonal	~117	ECL ICC	1/2000	Sigma, Germany, V9131
MyoD	Rabbit polyclonal	~45	ECL ICC	1/100	Santa Cruz, USA, sc-760
MyHC (MF-20s)	Mouse monoclonal	~200	ECL ICC	1/200	Developmental Studies Hybridoma Bank, USA, MF29
α-tubulin	Mouse monoclonal	~52-55	ECL	1/50	Santa Cruz, USA, sc-5286

Table H.2: Secondary antibody dilutions used for western blot analysis and immunocytochemistry (ICC) as well as immunohistochemical stains

Antibody	Application	Dilution factor	Company
Polyclonal rabbit anti-mouse Immunoglobulins/ HRP	ECL	1/8000	Dako, USA, P-0260
Polyclonal goat anti- rabbit Immunoglobulins/ HRP	ECL	1/4000	Dako, USA, P-0448
Hoechst 33342	Nuclear Stain	1/2000	Sigma, Germany, B2261
Phalloidin-TRITC	Cytoskeletal stain	1/16 000	Sigma, Germany, P1951
DyLight 488-conjugated AffiniPure donkey anti-rabbit lgG	ICC	1/1000	Jackson ImmunoResearch, USA, cat.CN-711-485-152
DyLight 488 and 594-conjugated AffiniPure donkey anti-mouse lgG	ICC	1/1000	Jackson ImmunoResearch, USA, cat.CN-715-485-151
DyLight 594-conjugated AffiniPure donkey anti-mouse lgG	ICC	1/1000	Jackson ImmunoResearch, USA, cat.CN-715-515-151
DyLight 549-conjugated AffiniPure donkey anti-goat lgG	ICC	1/1000	Jackson ImmunoResearch, USA, cat.CN-705-505-003

APPENDIX I:

CELL LYSATE PREPARATION

Reagents

RIPA Buffer (pH 8): RIPA buffer (Sigma, Germany, cat.R0278) consists of; 50 mM Tris-HCl with 150 mM NaCl, 1% Igepa CA-630 (NP-40), 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS). The buffer was stored at 4^{o}C.

Protease Inhibitor Cocktail (PIC): The PIC (Sigma, Germany, cat.P8340) comprises of the following enzymes; pepstain A, leupeptin hemisulfate, AEBSF- [4-(2-Aminoethyl)benzenesufonyl fluoride hydrochloride], aprotinin, bestatin hydrochloride and E-64-[N-(trans-Epoxysuccinyl)-L-leucine 4- guanidinobutylamide]. PIC was aliquoted into 20 µl eppendorfs and stored at -20^{o}C.

Method

Cells were harvested after trypsinizing for 3-5 min and centrifuged at 12 000g for 5 min on a bench top centrifuge (MRC, Polychem Supplies, USA). The supernatant was discarded and then the cell pellet was resuspended in 100 µl RIPA and 1 µl of PIC and lysed while incubated on ice for 1 h. The cell suspensions were then sonicated using Ultrasonic cleaner (Shalam Laboratory Suppliers) and the protein concentration determined using the equation obtained from Bradford Protein Assay (*Appendix J*). The suspensions were aliquoted into small volumes (20 µl) and stored at -20^{o}C.

APPENDIX J:

BRADFORD PROTEIN ASSAY

Reagents

Bradford Reagent: The reagent (Sigma, USA, cat.B 6916) contains Brilliant blue G in phosphoric acid and methanol. The reagent was stored at 4^{o}C. A standard curve was created using bovine serum albumin (BSA) (Roche Diagnostic, USA, cat.70117254) stock solution (1 mg/ml).

Method

A solution of PBS, 1 mg/ml Bovine Serum Albumin stock and Bradford reagent (Sigma-Aldrich, USA; cat.081M4362) was made up as follows: 0, 5, 10, 15, 20, 25, 30 µl BSA stock was aliquoted into 1 ml plastic micro-cuvettes (Lasec, cat.PLPS112117), PBS was added in decreasing amounts of 100, 95, 85, 80, 75, 70 µl and a constant volume of 900 µl Bradford reagent was added (this was done in replicates of five to get an accurate assessment of protein concentrations). The absorbance values of the solutions were measured at 595 nm and the average absorbance calculated and used to generate the standard curve (Figure J). Thereafter, 5 µl of cell lysate was added to 95 µl PBS buffer and 900 µl Bradford reagent. Absorbance was read at 595 nm. Protein concentrations were calculated using the linear equation generated from the standard curve.

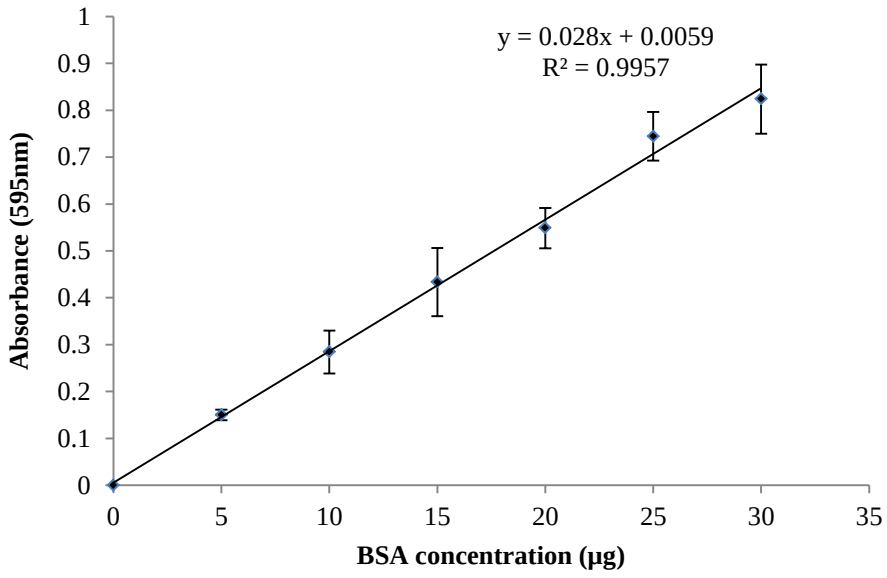

Figure J: Bradford protein assay standard curve. The graph shows a linear relationship between absorbance of BSA protein solution at 595 nm and BSA concentration. The graph shows the standard deviations of each reading.

APPENDIX K:

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL (SDS-PAGE)

Reagents

Acrylamide/Bis-acrylamide monomer stock solution: 73 g acrylamide (30% m/v) and 2 g bis-acrylamide were dissolved in 250 ml distilled water. The solution was filter through Whatman No 1 filter paper and stored at 4^{o}C in a brown glass bottle.

4 x running gel buffer (1.5M Tris-HCl, pH 8.8): 72.72 g Tris was dissolved in 400 ml distilled water. The pH was adjusted to pH 8.8 with HCl. The solution was stored at 4^{o}C.

4 x stacking gel buffer (500 mM Tris-HCl, pH 6.8): 3 g Tris was dissolved in 50 ml distilled water. The pH was adjusted to pH 6.8 with HCl. This solution pH was checked every time before running the gel and stored at 4^{o}C.

SDS stock solution (10% w/v SDS): 5 g SDS was dissolved in 50 ml distilled water. The solution was stored in a glass bottle at room temperature.

Ammonium persulfate initiator solution: 0.1 g ammonium persulfate was dissolved in 1 ml distilled water. This initiator was made up fresh every time before use.

2 x Reducing sample treatment buffer (125 mM Tris-HCl, pH 6.8): 2.5 ml of 4 x stacking gel buffer, 4 ml SDS stock solution, 2 ml glycerol and 1 ml 2-mercaptoethanol were made up to 10 ml with distilled water and adjusted to pH 6.8. This solution was stored in a plastic container at room temperature for not more than 2 weeks.

Electrode (tank) buffer (250 mM Tris-HCl, pH 8.3): 0.75 g Tris, 3.6 g glycine and 2.5 ml SDS stock solution were made up to 250 ml distilled water and titrated to pH 8.3. The solution was stored in a plastic container at 4^{o}C.

Coomassie R-250 stain: 1.25 g Coomassie brilliant blue R-250, 225 ml methanol, 50 ml glycerol, 200 ml distilled water were mixed together and made up to 500 ml with distilled water. The solution was stored in a plastic container at room temperature.

Destain solution 1 (50% (v/v) methanol, 10% (v/v) acetic acid): 500 ml methanol, 100 ml acetic acid were measured and made up to 1 litre with distilled water. The solution was stored in a plastic container at room temperature.

Destain solution 2 (70% (v/v) methanol, 5% (v/v) acetic acid): 50 ml methanol, 70 ml acetic acid were measured and made up to 1 litre with distilled water. The solution was stored in a plastic container at room temperature.

Coomassie Binding Blue G-250 stain dye: 0.2 g Coomassie G-250, 2.4 ml phosphoric acid, 50 g aluminium sulfate, 100 ml ethanol (100%) and 876 ml distilled water were combined, stirred and stored in a plastic container at room temperature. (*NB: This stain does not require destaining*).

Method

Laemmli system was used for SDS-PAGE (Laemmli, 1970) with several amendments. The Bio-Rad electrophoresis unit (Bio-Rad Mini-PROTEAN 3 Cell) was used for running all gels. Stacking gel (12.5%) was prepared according to Table K and the gels were cast into space between two glass plates to a depth of ~3 cm from the top of the glass plate. The gels were immediately overlayed with distilled water to allow for even polymerization. Once the gels were set (evidenced by the appearance of the interface between gel solution and water), the water was removed. Stacking gel solution was then poured in, up to the top of the glass plate and a 10 well comb inserted to form the sample application wells. Once the gels had set, the wells were rinsed with distilled water. The Bradford protein assay was used to determine protein (30 ug) concentration (*Appendix J*); each sample was added to an equal volume of reducing treatment buffer. Samples were immersed in boiling water for 2-3 min (to denature the proteins) and allowed to cool on ice. (*NB: All protein lysates must be kept on ice to avoid protein degradation*). Tank buffer was poured into the inner and outer electrode compartments, samples and 3 µl PeqGold protein marker V (Peqlab, USA, cat.27-2210) were then loaded on the gels. The gel unit was connected to a power pack and gels were then run at room temperature at a constant current of 18 mA/gel (for ~ 1 h 30 min) until the bromophenol blue tracker dye was 0.5 cm from the bottom of the gel unit. The gels were removed from the glass plates and loaded for western blot analysis. After western blot, the gels were stained with Coomassie binding blue G-250 to determine total protein transfer.

Table K: Preparation of 12.5% running gel and stacking gel for SDS-PAGE

Reagent	**12.5% running gel**	**Stacking gel**
Acrylamide/Bis-acrylamide monomer stock solution	6.25 ml	940 µl
4 x running gel buffer	3.75 ml	-
4 x stacking gel buffer	-	1.75 ml
SDS stock solution	150 µl	70 µl
Ammonium persulfate initiator solution	75 µl	35 µl
distilled water	4.75 ml	4.3 ml
TEMED	7.5 µl	15 µl

APPENDIX L:

WESTERN BLOT

Reagents

Blotting Buffer: 30.325 g Tris and 72 g Glycine were dissolved in 4.5 litres distilled water, followed by addition of 1 litre methanol. The solution was made up to 5 litres with distilled water, 10% (m/v) SDS was added to the buffer before use. The buffer was stored in a plastic container at 4^{o}C.

Tween-Tris Buffer Saline (TTBS; 20 mM Tris, 150 mM NaCl, 0.05% Tween 20) pH 7.5: 8 g NaCl, 0.2 g KCl, 3 g Tris base were weighed out and dissolved in 800 ml distilled water. The pH was titrated to pH 7.5 with HCl and volume was bought to 1 litre with distilled water. 0.5 ml Tween (Sigma, Germany, cat.P1379) was added to the solution and the buffer was stored in a plastic container at 4^{o}C.

Ponceau S. stain: 0.1 g Ponceau S (Fluka, USA, cat.81460) was dissolved in 1 ml acetic acid (100%) and made up to final volume of 100 ml distilled water. The solution was adjusted to pH 6 with HCl, and then stored in a plastic bottle at 4^{o}C.

Method

After running the SDS-PAGE gel (*Appendix K*), western blot analysis was performed. The gel was placed within a blotting sandwich made up as follows: 1 x sponge, 2 x blotting paper, nitrocellulose membrane (Life Sciences, South Africa, cat.P/N 66485), gel, 2 x blotting paper, and sponge. This was carried out in blotting buffer, and air bubbles were removed. The sandwich was transferred into a western blot cassette and placed into the western blot apparatus (Cleaver Scientific Ltd, USA) containing blotting buffer. Since proteins are negatively charged, the gel was placed on the positive terminal and the nitrocellulose on the positive terminal of the apparatus. Protein transfer was performed in the fridge (4^{o}C) at 200 mA for 16 h.

The nitrocellulose was then placed in Ponceau S stain for detection of proteins. The nitrocellulose was washed with distilled water and an image of the membrane was captured using a Versa Doc imaging system (Biorad, USA). The gel was placed in Coomassie G-250 stain overnight, to determine total transfer. The nitrocellulose membrane was blocked with 5% (m/v) non-fat milk powder made up in TTBS for 2 h on a shaker. Thereafter, the nitrocellulose was cut at molecular weights of the antibodies that were going to be probed for and placed in the appropriate antibody dilution made up in TTBS overnight at 4^{o}C. This was followed with a wash step using TTBS (4 x 8 min), and the addition of appropriate secondary antibodies made

up in TTBS for 1 h on shaker with vigorous agitation. The nitrocellulose was washed (6 x 8 min) in TTBS.

Immuno Star™ Western C™ Kit (*Appendix G*) was added to the nitrocellulose membrane which had been placed between two pieces of transparent film. The film was first cleaned with 70% ethanol and dried prior to use. Enhanced Chemiluminescence (ECL) technique was applied for the development of bands on light film (Kodak Biomax Light film; Sigma, USA) in the dark room with no white light. The light film was exposed to the nitrocellulose paper for 30 sec -10 min depending on primary antibody. After exposure, the film was first placed in developer ((1:3 dilution in water) Structirix G128, Agfa, USA) until dots were formed were performed, then secondly in fixative solution ((1:4 dilution in water) G333c Rapid fixer, Agfa, USA), finally in water. The film was allowed to dry and an image of it was taken using VersaDoc Imaging System (Bio-Rad, USA).

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