EXTRACELLULAR MATRIX FACTORS INFLUENCE MYOBLAST ACTIVATION, DIFFERENTIATION AND FUSION

By

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

Signed: _____________ Name: Dr. C. U. Niesler Date: _____________________

ABSTRACT

Satellite cells are muscle stem cells that reside in a niche between the basal lamina and sarcolemma of mature muscle fibers. Upon muscle injury, these cells are activated to myoblasts that subsequently proliferate, migrate and differentiate into myotubes in order to facilitate repair. Extracellular matrix (ECM) and growth factors are known to regulate certain aspects of myogenesis however, a comprehensive study of the direct effects of niche ECM factors on C2C12 myogenesis has not previously been conducted and forms the core of this study.

We first examined the role of hepatocyte growth factor (HGF) on C2C12 myogenesis. HGF is known to initiate activation of quiescent satellite cells in the niche and regulate various aspects of differentiation. In this study, we determined that HGF has a dose-dependent dual role in C2C12 myogenesis. HGF (2 ng/ml) significantly promoted cell division, but reduced myogenic commitment and fusion. Conversely, 10 ng/ml HGF reduced proliferative capability, but increased differentiation. This is potentially regulated by changes in c-Met expression; analysis revealed significantly *decreased* c-Met expression in differentiating cells cultured with 2 ng/ml HGF, but *increased* expression in proliferating cells with 10 ng/ml HGF. Furthermore, investigation into the mechanisms by which HGF affects myogenesis, revealed that mitogen-activated protein kinase (MAPKs: ERK, JNK or p38K) and phosphatidylinositol-3-kinase (PI3K) inhibition abrogated the HGF-stimulated increase in cell number. Interestingly, PI3K and p38 kinase facilitated the negative effect of HGF on proliferation, while ERK inhibition abrogated the HGF-mediated decrease in differentiation.

Next, we analyzed the effect of the satellite cell niche ECM on C2C12 myogenesis. Collagen IV and laminin, the major components of the basal lamina, bind to and interact with satellite cells via integrins and other cell surface proteins such as the tetraspanin, CD9. Matrigel significantly increased terminal fusion but had no effect on $Pax7^+$ and $MyoD^+$ cell numbers. Collagen IV, the second largest constituent of Matrigel, was observed to significantly increase $MyoD^+$ cell numbers and terminal fusion without effecting percentage $Pax7^+$ cell numbers. Furthermore collagen IV stimulated an increase in CD9 expression on differentiating cells, such that cells cultured on collagen IV required higher levels of neutralizing anti-CD9 monoclonal antibodies to reduce fusion. These results indicate, for the

first time, that the interaction of collagen IV with CD9 is a critical mediator of skeletal muscle fusion and that the observed pro-myogenic effect is accompanied, on a molecular level, by an increase in the number of committed MyoD positive cells.

Extracellular matrix (ECM) and growth factors are known to have complex interactions that may modulate their activity *in vivo*. Lastly, in an attempt to more closely mimic *in vivo* conditions, murine C2C12 myoblasts were cultured on collagen IV in HGF-supplemented media followed by assessment of differentiation and proliferation. Collagen IV was not able to negate the negative effect of HGF (2 ng/ml) on fusion but was able to restore normal MyHC expression. Due to a collagen IV stimulated increase in CD9 expression in differentiating myoblasts, cells cultured on collagen IV required higher levels of neutralizing anti-CD9 monoclonal antibodies to reduce fusion; an effect not observed when cells were differentiated in the presence of HGF alone. HGF (10 ng/ml) treated samples we unable to demonstrate any fusion when CD9 was completely blocked suggesting that CD9 is a crucial co-factor in HGF (10 ng/ml)-induced fusion. These results show, for the first time, that collagen IV is able to modulate certain aspects of the dual role of HGF on myogenesis.

In summary, we identified a novel dose-dependent dual role of HGF in myogenesis and uncovered that these effects are mediated by changes in c-Met expression and downstream MAPK and PI3K signalling. We showed, for the first time, that collagen IV is able to positively mediate C2C12 differentiation via a CD9-dependent pathway. Lastly, we revealed that collagen IV is able to mediate the dose-dependent effects of HGF on C2C12 myogenesis.

PREFACE

The experimental work described in this dissertation was carried out in the Discipline of Biochemistry, School Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, from January 2012 to September 2015, under the supervision of Dr. C. U. Niesler.

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

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DECLARATION 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).

Walker NL, Kyle P. Goetsch*, Trish R. Kahamba*, Nicholas J. Woudberg and Carola U. Niesler[#]. Hepatocyte growth factor (HGF) modulates myoblast proliferation and differentiation in a dose-dependent manner. Growth Factors, 33, 229-41.

Walker NL and Niesler CU. Collagen IV promotes myoblast fusion in a CD9-dependent manner [Contributed all data and analysis of figures in the paper]. Submitted, Cell and Tissue Research; 30 November 2015.

Walker NL and Niesler CU. Collagen IV modulates the dose-dependent effect of HGF in C2C12 myogenesis. [Contributed all data and analysis of figures in the paper]. In preparation.

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ABBREVIATIONS

2D	two-Dimensional
3D	three-Dimensional
BMPs	bone morphogenetic proteins
BrdU	Bromodeoxyuridine
BSA	bovine serum albumin
BSC	biological safety cabinet
CD9	cluster of differentiation (9)
CD34	cluster of differentiation (34)
CD44	cluster of differentiation (44)
DDR	discoidin domain receptor
DM	differentiation media
DMEM	Dulbecco's Modified Eagle Serum
DMSO	Dimethyl sulfoxide
ECM	extracellular matrix
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Swarm
ERK	Extracellular signal-regulated kinases
FCS	fetal calf serum
FGF	fibroblast growth factor
FGF-2	fibroblast growth factor-2
GAPDH	glyceraldehyde-3-Phosphate Dehydrogenase
GDFs	growth differentiation factors
GM	growth media
HGF	hepatocyte growth factor
HS	horse serum
HRPO	horseradish peroxidase
ICC	Immunocytochemistry
IGF	Insulin-like growth factor
JNK	Jun N-terminal kinases
MAPK	mitogen-activated protein kinase
MRF(s)	myogenic regulatory factor(s)
MRF4	myogenic regulatory factor 5
Myf5	myogenic factor 5
MyoD	myoblast determination protein
MyHC	myosin heavy chain
NCAM	neural cell adhesion molecule

Pax3 paired-box protein 3
Pax7 paired-box protein 7
PBS phosphate buffered saline
PenStrep Penicillin-Streptomycin
PDGF platelet-derived growth factor
PI3K Phosphoinositide 3-kinase

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM structural equation modeling

TGF-β1 transforming growth factor-β1
TBST tris buffered saline with tween

UV ultra-violet

VEGF Vascular endothelial growth factor

CONFERENCE CONTRIBUTIONS

Walker N.L. & Niesler C.U. Effect of Growth and Extracellular Matrix Factors on Differentiation of Myoblasts. Presented at the Postgraduate Research Day, College of Science, Agriculture and Engineering, University of KwaZulu-Natal, Pietermaritzburg, South Africa; 2012.

Walker N.L. & Niesler C.U. Collagen IV is pro-myogenic and also regulates the dose-dependent effect of HGF on myogenesis. Presented at the Indian Ocean Rim Muscle Colloquium (IORMC), Singapore; 2013.

Walker N.L. & Niesler C.U. Collagen IV is pro-myogenic and also regulates the dose-dependent effect of HGF on myogenesis. Presented at SASBMB, South Africa; 2014.

Walker N.L. & Niesler C.U. The extracellular matrix modulates the effect of HGF on myogenesis. Presented at “Stem Cells: From basic research to bioprocessing”, London, UK; 2015.

Walker N.L. & Niesler C.U. The extracellular matrix modulates the effect of HGF on myogenesis. Presented at “Stem Cells in Drug Discovery”, Cambridge, UK; 2015.

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frustration. You have been an ear when I needed to talk and your love has been unconditional, thank you.

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction:

Myofibers (skeletal muscle cells) are post-mitotic and therefore unable to divide and repair damaged muscle themselves (Grounds *et al.*, 2002). However, a cellular repair system is present in adult muscle in the form of a stem/progenitor cell known as the satellite cell (De Bari *et al.*, 2003). Satellite cells reside in a niche between the basal lamina and plasma membrane of the adjacent myofiber (Mauro, 1961, Grefte *et al.*, 2012). They are positioned along the entire length of the muscle fiber and ensure the capability of muscle repair regardless of the site of injury (Muir *et al.*, 1965). Morphologically, satellite cells are fusiform in shape with a small nucleus and reduced organelle content (Charge and Rudnicki, 2004).

In healthy, uninjured muscle, satellite cells are metabolically inactive, a state known as quiescence. Upon muscle damage, they are activated from quiescence by hepatocyte growth factor (HGF) and begin to proliferate (Allen *et al.,* 1995). Activated satellite cells, also known as myoblasts, migrate along the basal lamina towards the site of injury where they differentiate and facilitate tissue repair (Allen *et al.,* 1995). The precise composition of the extracellular matrix (ECM) changes as the cell moves from its niche, along the basal lamina and into the wound. The two main protein components of the basal lamina are collagen IV and laminin (Boonen and Post, 2008). However, upon entering a fibrotic wound, myoblasts come into contact with high levels of collagen I, fibronectin and decorin (Vaz *et al.*, 2012). Previous research has highlighted the importance of the ECM and growth factors in regulating the activation, commitment, migration and differentiation of myoblasts (Borojevic, 1999, Goetsch *et al.*, 2011, Grefte *et al.*, 2012, Melo *et al.*, 1996, Osses and Brandan, 2002, Schenke-Layland *et al.*, 2007, Vaz, 2009) and a comprehensive assessment of the effects of HGF and the niche ECM on myogenesis forms the basis of this study.

1.2 Satellite cell characterization:

Satellite cells were first identified by Mauro (1961) in the hind limb muscles of frogs. Research by Muir *et al.* (1965) identified and further characterized these cells in skeletal muscle of mice and fruit bats. Electron microscopy allowed the visualization of mammalian satellite cells residing in their niche between the basal lamina and plasma membrane of a myofiber (Figure 1.1) (Muir *et al.*, 1965). Although satellite cells were suspected to play a role in skeletal muscle repair in 1961, conclusive proof of this was only shown some 17 years later. Snow (1978), using autoradiography, showed that satellite cells were essential for the regeneration of muscle in Sprague-Dawley rats. Here, satellite cells autoradiographically labeled with tritiated thymidine were transplanted into non-radioactive littermates. Following muscle damage, newly repaired myotubes appeared radioactively labeled thus proving that the satellite cells had differentiated into myotubes during fiber repair. In this study, activated satellite cells were also shown to be capable of myotube formation *in vitro* confirming their stem cell-like nature (Snow, 1978).

Satellite cells are not the only mononucleated cells found in skeletal muscle tissue (Figure 1.1). Pericytes are contractile cells that regulate capillary flow to the myofiber. They have recently been shown to retain a certain level of plasticity allowing them to differentiate into muscle cells and fibroblasts, and are able to assist in skeletal muscle regeneration (Dellavalle *et al.*, 2007).

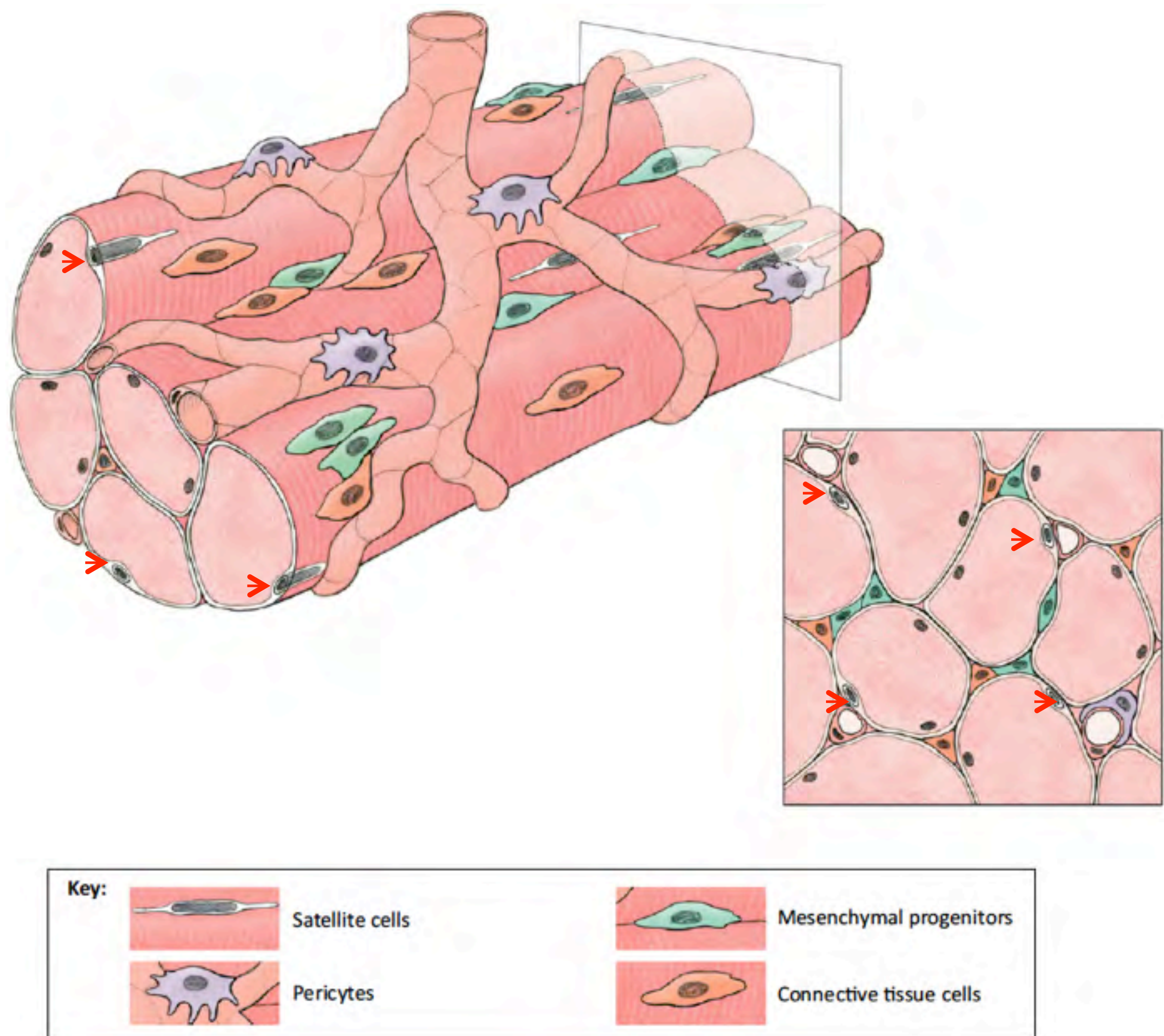


Figure 1.1: Satellite cell localization. Skeletal muscle is made up of densely packed, parallel myofibers. These fibers are multinucleated (containing myonuclei) and are surrounded by pericytes and satellite cells (red arrows indicate satellite cells). Blood vessels supply the fiber with nutrients, growth factors and circulating inflammatory cells in times of muscle injury. Mesenchymal progenitors and connective tissue cells are also found integrated within skeletal muscle tissue (Pannerec *et al.*, 2012).

Mesenchymal progenitors are capable of differentiating into a range of cell types, including adipocytes and muscle (Uezumi *et al.*, 2010). Within skeletal muscle, they secrete hepatocyte growth factor (HGF) which plays a vital role in the activation of satellite cells upon muscle injury (Catlow *et al.*, 2003). Connective tissue cells are one of the most abundant cell types found embedded in skeletal muscle; they form a sheath of connective tissue around each muscle fiber (Rowe, 1981). This not only protects the fiber, but also transfers tension between adjacent muscle fibers and supplies a element of elasticity to the muscle bundle (Trotter and Purslow, 1992). Unlike myofibers, satellite cells are mononuclear and express specific satellite cell proteins such as Pax7 and muscle regulatory factors (MRFs), which allow them to be identified in skeletal muscle tissue (Charge and Rudnicki, 2004)

1.3 Pax and Myogenic Regulatory Factors (MRFs):

Paired-box (Pax) proteins 3/7 and the myogenic regulatory factors (MRFs) are transcription factors specific to muscle cell lineages (Seale *et al.*, 2000). They play distinct, stage-specific roles during myogenesis and their use as molecular markers has allowed great insight into myogenesis. The expression of these transcription factors at different stages of muscle regeneration is outlined in Figure 1.2.

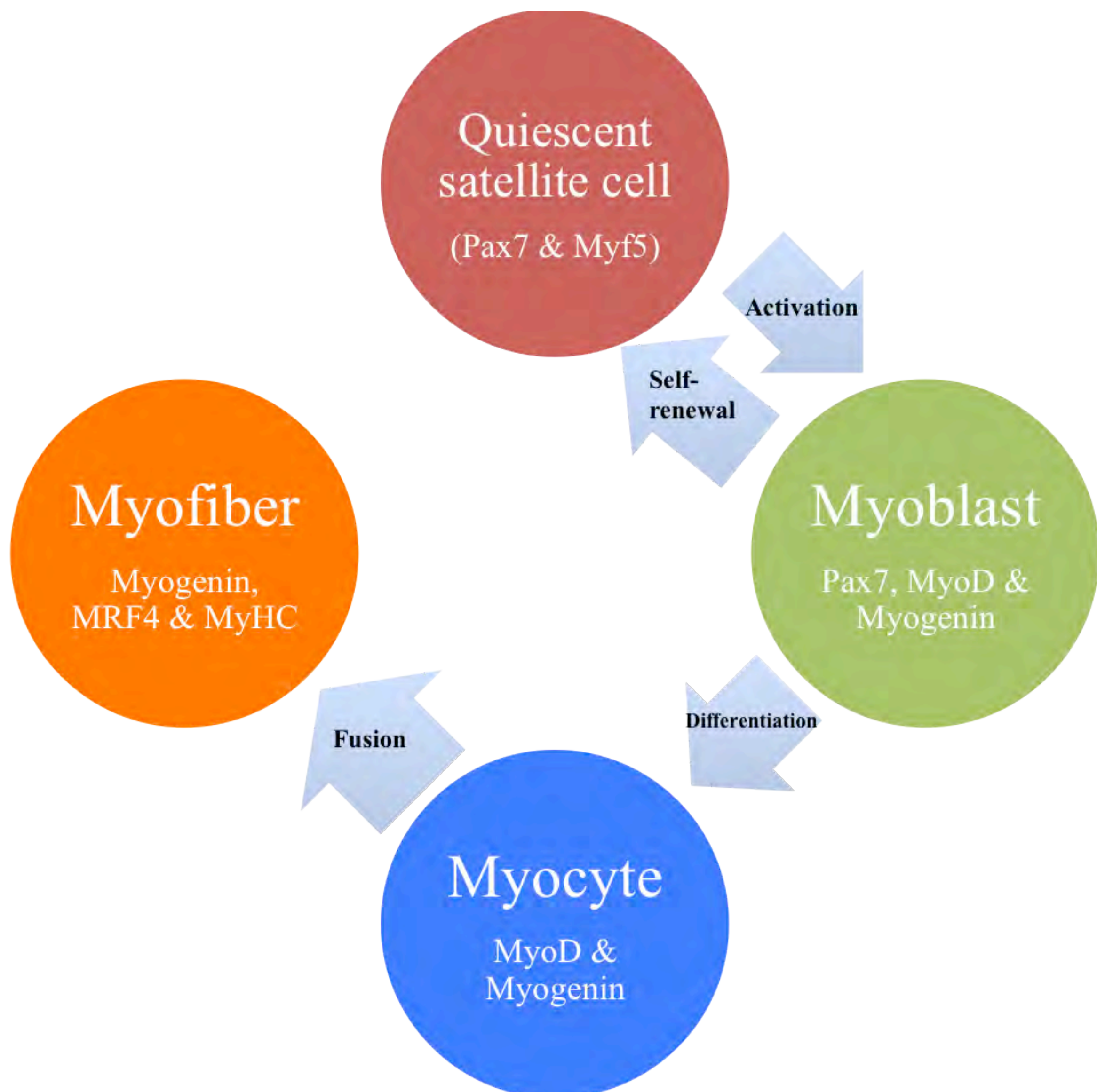


Figure 1.2: The progression from quiescent satellite cell to multinucleated myofiber showing the expression of transcription factors during this process. Quiescent satellite cells express Pax7 with low levels of Myf5. Myoblasts express the transcriptions factors Pax7, MyoD and myogenin. Differentiated myocytes down-regulate expression of Pax7, but continue to express MyoD and myogenin. Mature multinucleated muscle fibers express transcription factors myogenin and MRF4 as well as the structural protein Myosin Heavy Chain (MyHC) (Charge and Rudnicki, 2004, Cornelison and Wold, 1997, Fuchtbauer and Westphal, 1992, Gayraud-Morel *et al.*, 2007, Le Grand and Rudnicki, 2007, Ustanina *et al.*, 2007, Bentzinger *et al.*, 2012).

Pax3 is a major regulator of embryonic muscle development, however, its importance in postnatal skeletal muscle growth and repair is overshadowed by Pax7 (Young and Wagers, 2010). Once activated, proliferating myoblasts increase expression of the paired-box transcription factor Pax7 (Le Grand and Rudnicki, 2007). Pax7 is known to play a role in the self-renewal pathway that is responsible for maintaining the population of satellite cells in muscle. Pax7 is expressed in both quiescent and activated satellite cells, but is down-regulated prior to differentiation (Relaix *et al.*, 2006). After several proliferative cycles, myoblasts exit the cell cycle and enter a state of differentiation which allows subsequent fusion and myotube formation (Dhawan and Rando, 2005). If Pax7 expression is not downregulated, cells do not differentiate, but rather re-enter a quiescent state.

Following down-regulation of Pax7 in differentiating cells, MyoD expression increases, suggesting that Pax7 plays a role in self renewal via direct suppression of MyoD expression (Olguin and Olwin, 2004). MyoD expression is accompanied by up-regulation of Myf5, myogenin and myosin heavy chain (MyHC) expression, before subsequent fusion into multinucleated muscle fibers as reflected in Figure 1.2 (Le Grand and Rudnicki, 2007). Through various knockout studies it has been shown that MyoD is a crucial regulatory factor of post-natal myogenesis with knockouts unable to effectively differentiate satellite cells. Triple knockouts of myogenin, MRF4 and Myf5 also show a complete inability to perform successful myogenesis (Gayraud-Morel *et al.*, 2007). This indicates that the regulatory factors work in unison to ensure successful myogenesis.

Satellite cells express a number of cell surface proteins including M-cadherin, neural cell adhesion molecule (N-CAM), c-Met and CD9 (Table 1.1). c-Met and CD9 are of particular relevance to this study as HGF signals through c-Met and collagen IV is known to bind to CD9. These surface proteins will be discussed in more detail in the following sections.

Table 1.1: Common satellite cell proteins and their functions.

Markers	Expression	Function	References
Transcription Factors:			
Pax3	Q	Myogenic specification (embryonic)	(Horst *et al.*, 2006)
Pax7	Q/A	Myogenic specification (adult), self renewal	(Seale *et al.*, 2000)
MRFs:			
MyoD	A	Commitment to differentiation	(Cornelison *et al.*, 2000)
Myf5	A/D	Commitment to differentiation	(Ustanina *et al.*, 2007)
Myogenin	D	Differentiation, fusion	(Fuchtbauer and Westphal, 1992)
MRF4	D	Differentiation, fusion	(Bentzinger *et al.*, 2012)
Cell surface proteins:			
c-Met	Q/A	Receptor for HGF	(Cornelison and Wold, 1997)
CD9	Q/A	Role in integrin signaling, fusion	(Beauchamp *et al.*, 2000) (Tachibana and Hemler, 1999, Charrin *et al.*, 2013).
Structural proteins:			
MyHC	D	Terminal differentiation	(Bader *et al.*, 1982)

Abbreviations: Q: Quiescent; A: Activated; D: Differentiated; NCAM: neural cell adhesion molecule; Pax3/7: paired-box transcription factor 3/7; Myf5: myogenic factor 5; MyHC: myosin heavy chain.

C2C12 cells are an immortalized myoblast line used as a research model for myogenesis isolated from C3H mice following a crush injury experiment (Yaffe and Saxel, 1977). Upon stimulation to differentiate, C2C12 cells are observed to downregulate Pax7, upregulate MyoD and begin differentiation (Zammit *et al.*, 2006b). This is followed by the expression of the abovementioned MRFs and followed by fusion into actin and myosin positive myotubes (Burattini *et al.*, 2004, Olguin *et al.*, 2007). This pattern of expression of transcription factors as differentiation progresses closely mirrors that of primary culture myoblast differentiation and *in vivo* myogenesis (Olguin *et al.*, 2007). This outlines the value in quantifying C2C12 transcription factor and MRF levels as a primary step in understanding specific stages of myogenesis.

1.4 Skeletal muscle repair:

Adult skeletal muscle is considered an extremely stable tissue type. Schmalbruch and Lewis (2000) estimate that approximately 2% of mononuclei are replaced each week in healthy adult mice (Schmalbruch and Lewis, 2000). Skeletal muscle also possesses the ability to rapidly regenerate following injury.

Muscle tissue repair can be classified into two distinct stages, namely degradation and regeneration. Degeneration begins with necrosis and partial or full autolysis of the fiber ensues (Schmalbruch and Lewis, 2000). This degradation releases factors which act as chemotactic signals to inflammatory cells in the bloodstream. Neutrophils are present at the site of injury within 6 hours of injury followed by macrophages approximately 48 hours post-injury (Figure 1.3A) (Tidball, 1995). Although fibroblasts are recruited to the site of injury, deposition of large amounts of fibrotic scar tissue is not characteristic for these types of injuries (Schmalbruch and Lewis, 2000). The released factors from the damaged muscle fiber also stimulate activation of the regeneration process. This is characterized by mitotic cell division of satellite cells followed by migration into the injury site and later, fusion of myoblasts to existing myofibers or to one another forming new myotubes (Figure 1.3B) (Schmalbruch and Lewis, 2000). Late in the regeneration stage, remodeling occurs and myotubes increase in size due to hypertrophy. Following complete regeneration, repaired myofibers are morphologically indistinguishable from undamaged fibers and exhibit complete contractile functionality (Figure 1.3C) (Schmalbruch and Lewis, 2000).

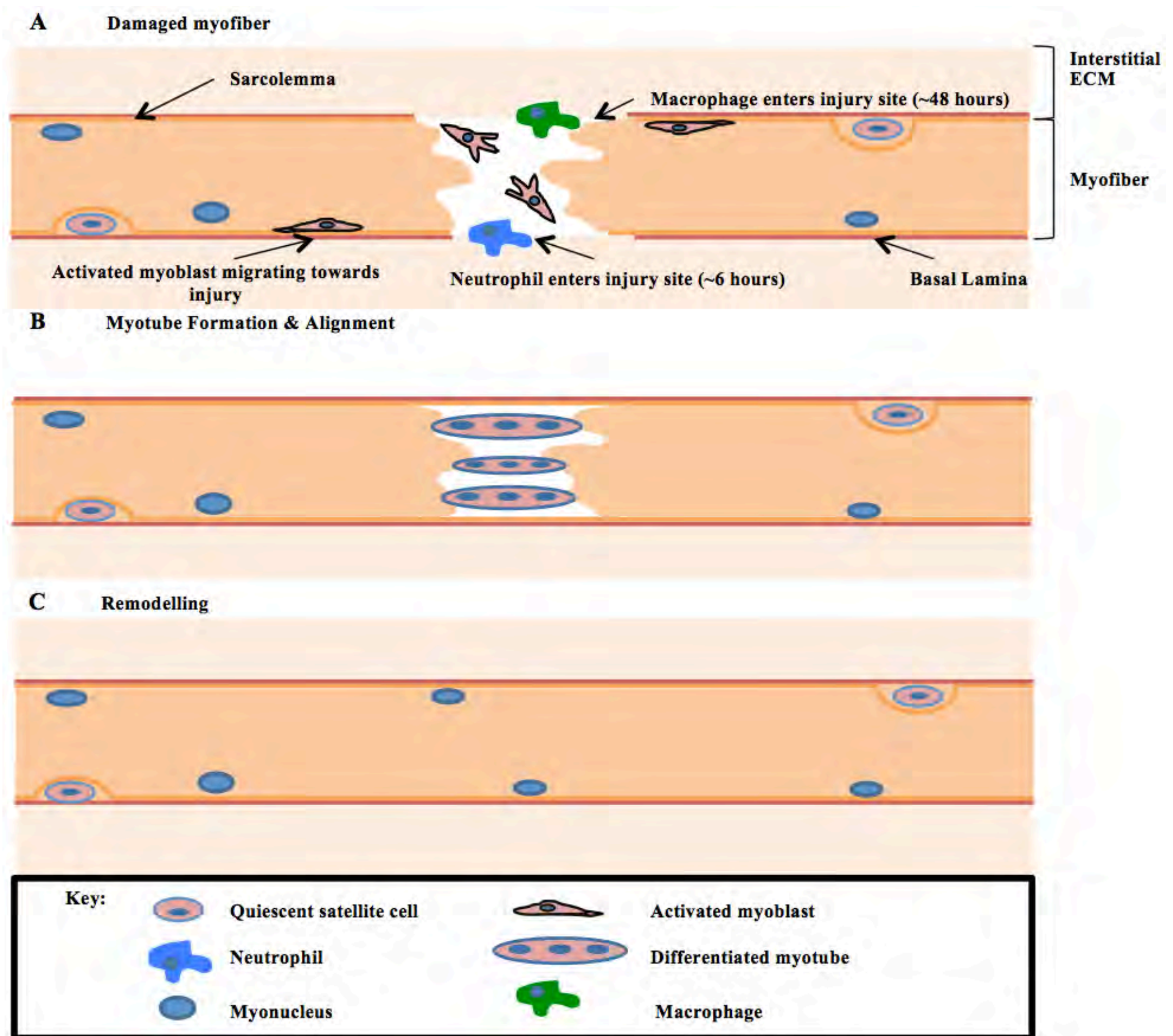


Figure 1.3: Normal muscle regeneration. Neutrophils and macrophages enter injury site. Activated myoblasts differentiate into multinucleated myotubes and fuse to existing myofibers thereby bridging the injury without scar tissue formation. Full muscle repair ensues (Constructed from (Jarvinen *et al.*, 2000, Occleston *et al.*, 2010, Tidball and Wehling-Henricks, 2007)).

However, in cases of extremely severe or frequent injury (Figure 1.4A), excessive ECM deposition by infiltrating fibroblasts may result in the formation of a fibrotic scar (Figure 1.4B) (Jarvinen *et al.*, 2000). The fibrotic scar, composed of ECM factors including fibronectin and collagen III (which is later remodeled to collagen I), prevents myoblasts from fusing and bridging the original muscle fiber (Figure 1.4C) (Tidball and Wehling-Henricks, 2007). This, in turn, results in a loss of contractile ability (Tidball and Wehling-Henricks, 2007). This type of scarring also occurs as a result of repetitive injuries characteristic of myopathies such as Duchenne muscular dystrophy, where increasing fibrosis causes transdifferentiation of myoblasts into fibroblasts (Grounds, 2014).

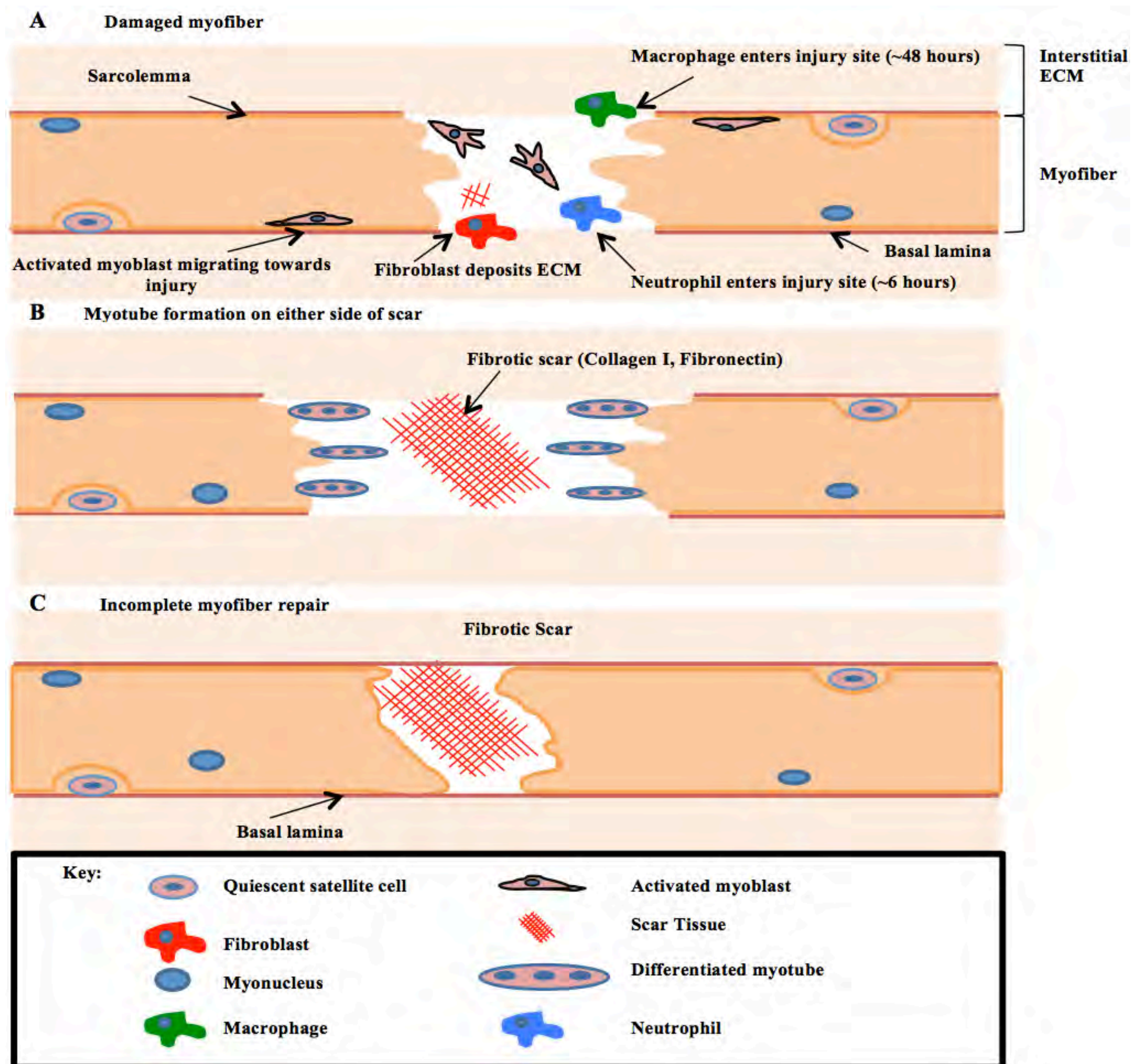


Figure 1.4: Scar tissue formation following repeated or extremely severe muscle injury. Fibroblasts enter the site of injury and deposit ECM proteins such as collagen I and fibronectin. Myotubes then attach to either side of the fibrotic scar and fuse with the existing muscle fibers. This results in fibrotic scar tissue formation and impeded muscle function (Constructed from (Jarvinen *et al.*, 2000, Occleston *et al.*, 2010, Tidball and Wehling-Henricks, 2007)).

1.5 Satellite cell niche:

The stem cell niche is defined as the local microenvironment that supports, maintains and regulates stem cell identity and function. In particular, the stem cell niche has been shown to regulate self-renewal via a mechanism known as asymmetric cell division (Kuang *et al.*, 2008). Asymmetric cell division is characterized by mitosis resulting in daughter cells of different cellular fates (Morrison and Kimble, 2006) as opposed to symmetric cell division which results in identical daughter cells. With regard to myogenesis, asymmetric cell division results in one daughter cell that will migrate to the site of injury and differentiate,

facilitating regeneration. The other daughter cell will return to the niche and re-enter quiescence (Kuang *et al.*, 2007). This process is extremely important in maintaining a population of satellite cells within the muscle and thus ensuring its regenerative potential.

The satellite cell niche is found along the surface of muscle fibers between the sarcolemma and the basal lamina. The satellite cell is in contact with the muscle fiber, components of the basal lamina and the vascular system that supplies the niche (Fuchs *et al.*, 2004). The muscle fiber has been shown to supply the satellite cell with mechanical, electrical, and chemical signals that play a role in the regulation of its function (Pallafacchina *et al.*, 2010). The basal lamina surrounding myofibers consists of various ECM components such as laminin-211, collagen IV and various proteoglycans such as syndecans, glypican-1, perlecan, decorin and biglycan. These factors contribute to maintaining quiescence of the satellite cells while still in their niche (Pallafacchina *et al.*, 2010). The vascular system supplies nutrients and oxygen to the muscle fiber. This network supplies extrinsic signals from the circulatory system, which along with factors released by macrophages and fibroblasts, regulate the quiescence, activation and proliferation of satellite cells (Pallafacchina *et al.*, 2010).

Growth and ECM factors of the satellite cell niche and wound (Table 1.2) regulate the process of muscle repair and regeneration (Allen *et al.*, 1995, Amento and Beck, 1991, Catlow *et al.*, 2003, De Bari *et al.*, 2003, Ewton *et al.*, 1988, Fuchs *et al.*, 2004, Goetsch *et al.*, 2011, Grinnell, 1984, Lehto *et al.*, 1985, Lin *et al.*, 2010, Podleski *et al.*, 1979, Schabort *et al.*, 2009, Schenke-Layland *et al.*, 2007, Schonherr *et al.*, 1995, Tatsumi *et al.*, 1998, Vaz, 2009, von der Mark and Ocalan, 1989, Yao *et al.*, 1996). An understanding of how these factors affect myogenesis may provide clues as how to improve the restoration of muscle function post-injury.

Table 1.2: Comparison of components found within the satellite cell niche and those found in a fibrotic wound.

Component	Stem cell niche	Fibrotic Wound	References
Fibronectin	Not expressed	Fibronectin	(Dourdin *et al.*, 1997)
Collagens	Col IV, VI	Col I, III	(Kjaer, 2004, Friedl *et al.*, 1998)
Laminin	Laminin 211, 411, 511	Not expressed	(Grounds *et al.*, 2005)
Proteoglycans	Biglycan, Syndecans	Decorin, Dermatan Sulfates	(Mochida *et al.*, 2009)
Growth Factors	HGF, TGF-β	TGF-β	(Amento and Beck, 1991) (Allen *et al.*, 1995)

1.6 Extracellular matrix and growth factors in the niche and wound:

The extracellular matrix (ECM) surrounding skeletal muscle was initially thought to act solely as a scaffold to support and maintain the structure of the tissue, but has since been shown to regulate many cellular processes (Grounds *et al.*, 2005). These include cell survival, activation, proliferation, migration and differentiation (Friedl and Brocker, 2000). A vast array of proteins, proteoglycans and polysaccharides form the lattice-like meshwork of the ECM (Wu *et al.*, 2005). Individual components of the ECM such as laminin, fibronectin, collagen I, tenacin and decorin have been shown to influence stem cell activation, migration and differentiation in various tissues (Lin *et al.*, 2010, Schenke-Layland *et al.*, 2007, Friedl and Brocker, 2000, Wehrle-Haller and Chiquet, 1993). Growth factors control many aspects of myogenesis including activation, proliferation, migration, differentiation and fusion of myoblasts into myotubes. In this respect, important growth factor families include the hepatocyte growth factor (HGF) family, the fibroblast growth factor (FGF) family and the transforming growth factor beta (TFG-β) family.

1.6.1 Fibronectin: Fibronectin has been shown to be crucial in embryogenesis with fibronectin knockout mice rarely developing beyond day 11 of embryogenesis (George *et al.*, 1993). This is due to the vital role that fibronectin plays in guiding the migration of various cell types during early embryogenesis (Darribere and Schwarzbauer, 2000). Fibronectin, in its insoluble form, is a glycoprotein that forms fibrils as components of the ECM. This protein exists in two other forms: cell surface fibronectin oligomers and a soluble dimeric form located in the blood (Grinnell, 1984). The insoluble form of fibronectin has been shown

to be able to bind various ECM components including collagen and tenacin (Hocking *et al.*, 2008). Along with tenacin, fibronectin is among the first ECM factors to be produced by fibroblasts in severely injured muscle tissue (Grinnell, 1984). Fibronectin forms multimeric cross-linked structures with fibrin which act as a scaffold for invading inflammatory cells and myoblasts (Dourdin *et al.*, 1997). Fibronectin has been shown to improve the myogenic differentiation of C2C12 cells *in vitro* (Table 1.3) (Garcia *et al.*, 1999, Lin *et al.*, 2010). Garcia *et al* (1999) showed that blocking with monoclonal antibodies against fibronectin itself, or the integrin with which it interacts (α5β1), abrogates the observed increase in C2C12 differentiation.

1.6.2 Collagens: Collagens are a family of structural proteins known to perform numerous functions *in vivo*. Fibrillar collagens include collagen I, II, III, IV, V, VI and XI and are all composed of three polypeptide α-chains which arrange into a triple helix (Ricard-Blum and Ruggiero, 2005). The basal lamina has been shown to be rich in collagen IV, which forms the basic scaffold into which laminin networks are integrated (Timpl and Brown, 1996). Collagen IV binds to cell surface receptors knows as integrins as well as tetraspanins (Leitinger and Hohenester, 2007). Integrins are the major transmembrane receptors involved in cell adhesion to the ECM (Humphries *et al.*, 2006).

Although little is known regarding the effect of collagen IV on myoblast differentiation, collagen IV is known to interact with the CD9 receptor (Castro-Sanchez *et al.*, 2010). This cell surface glycoprotein is expressed on the surface of C2C12 cells and has been shown to be vital in the normal development of skeletal muscle. CD9 expression is upregulated in the early stages of C2C12 differentiation and blocking CD9 using monoclonal antibodies substantially inhibits and delays conversion of C2C12 cells to elongated myotubes (Tachibana and Hemler, 1999, Charrin *et al.*, 2013). This suggests that collagen IV may positively regulate myogenesis via CD9. Knockout studies have shown that collagen IV is not essential for early embryo development, but is essential for the correct structural assembly of various basement membranes during late development (Poschl *et al.*, 2004). At day 10.5 to 11.5 lethality occurs in collagen IV null mice due to structural abnormalities in the basement membrane between parietal endoderm cells and trophoblast cells known as the Reichert's membrane. Collagen IV has been shown to increase the differentiation of human and mouse embryonic stem cell types into mesodermal cell lineages including hematopoietic,

endothelial, and smooth muscle cells (Table 1.3) (Ali *et al.*, 1998, Schenke-Layland *et al.*, 2007, Taru Sharma *et al.*, 2012).

Collagen I is the most abundant collagen subtype within mammalian tissues and is the final product of collagen remodeling during scar tissue formation (Ricard-Blum and Ruggiero, 2005). TGF-β promotes fibrosis by stimulating collagen I deposition by fibroblasts following severe muscle injury (Tidball and Wehling-Henricks, 2007). Additionally, the interstitial ECM surrounding skeletal muscle fibers has been shown to be rich in collagen I, which increases integrin $\alpha1\beta1$ expression on fibroblasts (Kjaer, 2004). Increased expression of integrin $\alpha1\beta1$ causes the clumping of fibroblasts on collagen fibers via a critical GFOGER (O denotes hydroxyproline) motif within the collagen's I domain (Emsley *et al.*, 2000). Collagen I also binds to discoidin domain receptor 1 and 2 (DDR1 and DDR2) (Leitinger and Hohenester, 2007). DDRs are receptor tyrosine kinases (RTKs) involved in the regulation of cell growth, differentiation and metabolism (Mohan *et al.*, 2001). Collagen I has been shown to inhibit the differentiation of rat primary culture satellite cells (Table 1.3) (Kjaer, 2004, Grefte *et al.*, 2012).

1.6.3 Laminins: Laminins, composed of multiple heterodimers consisting of α, β and γ polypeptide chains, are a major protein component of the basal lamina. They are key bridge molecules, connecting the myofiber to the basal lamina; their absence results in congenital muscle dystrophies (Grounds *et al.*, 2005). These conditions arise as, without the connection of the myofiber to the basal lamina, the contractile force generated by the myofiber cannot be transferred effectively to the interstitial connective tissue (Grounds *et al.*, 2005). Laminin-211, found around the sarcolemma of muscle fibers, binds to collagen IV in the basal lamina (Grounds *et al.*, 2005). Laminin-111 and laminin-211 (merosin) differ in their alpha domains, however laminin-111 has been shown to improve the repair of skeletal muscle in merosin deficient mice (Van Ry *et al.*, 2013). This suggests a similar functional nature of these two isoforms. Although the effect of laminin deficiency is well documented in congenital muscular dystrophies, its effect on myoblast differentiation remains a topic for debate. Laminin-111 has been shown by some to increase C2C12 differentiation (Grossi *et al.*, 2007) while others have found that laminin had no effect on C2C12 differentiation (Vaz, 2009) (Table 1.3).

1.6.4 Decorin: Decorin, a proteoglycan, is composed of a leucine-rich core protein containing 12 subunits with each subunit containing a 24 amino acid polypeptide (Mochida *et al.*, 2009). Decorin plays a vital role in collagen fibrillogenesis by binding to collagen and causing a delay in fibril assembly. This results in the reduction of the fibril diameter and ensures correct fibril assembly (Mochida *et al.*, 2009). Irregular collagen contours and a loosely packed collagen network are the result of decorin scarcity during myogenesis (Mochida *et al.*, 2009). Goetsch *et al.,* 2011 found that decorin increases collagen I-stimulated, but not fibronectin-stimulated migration of mouse myoblasts. Riquelme *et al.* (2001) found that by minimizing the expression of decorin using antisense mRNA, they enhanced terminal differentiation of C2C12 cells (Table 1.3). In contrast, recent studies have shown decorin to aid in the differentiation of C2C12 cells (Table 1.3) through the suppression of myostatin activity (Kishioka *et al.*, 2008). Li *et al* (2007) showed that decorin inhibits transforming growth factor TGF-β1 and can reduce the formation of fibrous scar tissue *in vivo*. This resulted in improved muscle healing after injury using decorin gene transfer (Li *et al.*, 2007). The knockout of decorin expression performed by Riquelme *et al.* (2001) may possibly have activated a compensatory mechanism that is responsible for their observed improvement in C2C12 terminal differentiation. The findings of Li *et al* (2007) were observed using an animal model as opposed to the C2C12 *in vitro* model used in the two other studies mentioned. These differences in the models used may contribute to the observed contrasting results.

1.6.5 Matrigel: Matrigel, as it is known by its trade name, is an exogenous mixture of mainly collagen IV, laminin-111 and various proteoglycans (Hughes *et al.*, 2010). These components closely mimic the ECM of the satellite cell niche, providing a valuable tool in the study of niche ECM factors. Matrigel is commonly utilized to improve the proliferative and myogenic potential of primary isolated myoblasts (Grefte *et al.*, 2012). Grefte *et al* showed that Matrigel did indeed improve the fusion of C2C12 cells *in vitro*. Melo *et al.* (1996) demonstrated that the ECM is essential for skeletal muscle differentiation by culturing C2C12 myoblasts in the presence or absence of Matrigel. Cells plated in the absence of Matrigel were unable to form myotubes by day 3 while those differentiated on Matrigel had fused at this time point. Interestingly, when cells were plated in the absence of Matrigel, the production of the differentiation-stage specific transcription factor myogenin was not prevented, however later muscle-specific gene products such as MyHC were not produced by day 3 of differentiation (Melo *et al.*, 1996, Osses and Brandan, 2002). This is

compelling evidence that the components of Matrigel speed up C2C12 terminal differentiation.

Table 1.3: The known effects of various ECM and growth factors on stem cell differentiation.

Component	[Concentration]	Effect on Differentiation	Cell type	References
Collagen I	[1.2 mg/ml]	↓	Rat primary muscle culture	(Grefte *et al.*, 2012)
Collagen IV	[10 µg/ml]	↑	Various embryonic stem cell types, neural progenitors	(Schenke-Layland *et al.*, 2007, Taru Sharma *et al.*, 2012, Ali *et al.*, 1998)
Fibronectin	[10 µg/ml, 5 µg/ml]	↑	C2C12 myoblasts, Human mesenchymal multipotent stem cells	(Garcia *et al.*, 1999, Lin *et al.*, 2010)
Laminin	[10 µg/ml]	↑	C2C12, rat primary muscle culture	(Lin *et al.*, 2010, Grossi *et al.*, 2007, Vaz, 2009, Foster *et al.*, 1987)
	[5 µg/ml]	No effect		
Decorin	Overexpressing transfected cells	↑	C2C12 myoblasts	(Kishioka *et al.*, 2008)
	Gene Silencing (antisense RNA)	↑	C2C12 myoblasts	(Riquelme *et al.*, 2001)
	Gene transfer	↑	CD cells	(Li *et al.*, 2007)
HGF	Overexpressing transfected cells	↓	C2, Mouse primary muscle culture	(Yamane *et al.*, 2004, Leshem *et al.*, 2000)
Matrigel	[1 mg/ml]	↑	Rat primary culture	(Grefte *et al.*, 2012)
TGF-β1		↓	C2C12 myoblasts	(Schabort *et al.*, 2009)
FGF-2	[0.1 mg/ml]	↑	*In vivo* mouse	(Armand *et al.*, 2006)

1.6.6 HGF: Hepatocyte growth factor (HGF) exists in two forms, pro-HGF and mature HGF. Pro-HGF is secreted by mesenchymal cells as a single chain and remains bound in the ECM in an inactive form. Upon muscle injury, it is cleaved and activated by a serine protease (Catlow *et al.*, 2003). Mature (active) HGF is dimeric and exists as a heavy α-chain (69 kDa) and light β-chain (34 kDa) heterodimer. This growth factor is known to play a role in development and regeneration of a range of tissues including the endothelium, kidney, lung and skeletal muscle (Nakamura and Mizuno, 2010). HGF has been shown to impede the differentiation of embryonic mouse tongue cells and increase the proliferation and migration of C2C12 myoblasts *in vitro* (Yamane *et al.*, 2004, Barbero *et al.*, 2001). With regards to

skeletal muscle, HGF is of vital importance in the initial stages of muscle regeneration (Matsumoto and Nakamura, 1997).

1.6.6.1 Stability:

In vivo studies investigating the effect of HGF on liver regeneration have shown that HGF has a blood half-life of approximately 3 minutes (Appasamy *et al.*, 1993, Ido *et al.*, 2004, Xue *et al.*, 2003). This is mainly due to HGF uptake by the liver and does not suggest that HGF is inherently unstable (Appasamy *et al.*, 1993). In fact, two studies have demonstrated that HGF is stable at a range of temperatures, pH's and ionic strengths (Nayeri *et al.*, 2002, Nayeri *et al.*, 2004). HGF has however been observed to degrade significantly over a 3 hour period when placed in culture flow environments. However, HGF levels remained at over 90% 3 hours post addition to static media at 37 °C and 5% CO_2 (Meneghello *et al.*, 2014).

1.6.6.2 c-Met receptor:

The c-Met receptor is a high affinity receptor for HGF. It exists as a 190 kDa transmembrane protein composed of an α-chain (50 kDa) and a β-chain (140 kDa) (Tam *et al.*, 2000). Upon binding of HGF to the c-Met receptor, Met kinase becomes active (Bottaro *et al.*, 1991). This results in 2 tyrosine residues (Tyr1349 and Tyr1356) in the carboxy-terminal tail becoming phosphorylated (Figure 1.5). These residues become docking sites for a range of adaptor proteins including phosphatidylinositol-3-kinase (PI3K), Grb2-associated adaptor protein (Gab1) and growth factor receptor-bound protein 2 (Grb2) (Figure 1.5). These pathways proceed to mediate Met-dependent cell proliferation, migration, survival and differentiation (Faria *et al.*, 2011). These effects will be further discussed in section 1.6.6.3. Blocking the binding of HGF to the c-Met receptor has been shown to prevent the activation of quiescent satellite cells in a crush muscle extract experiment, thus confirming the importance of this receptor in satellite cell activation (Tatsumi *et al.*, 1998).

Regulation of HGF signalling via c-Met can be achieved by a number of mechanisms. Firstly, HGF has been observed to be rapidly internalized and degraded in cells such as hepatocytes, and the density of the HGF:c-Met complex on the cell surface is observed to decrease within 30 minutes of exposure to elevated HGF levels (Naka *et al.*, 1993). c-Met also has a negative regulatory site, a tyrosine residue in its juxtamembrane domain which acts by recruiting the E3 ubiquitin-protein ligase, casitas B-lineage lymphoma (c-CBL) (Organ and Tsao, 2011).

This causes c-Met to be ubiquitinated and thus targeted for proteasome degradation. HGF signaling is also regulated by the ability of c-Met to bind a number of protein tyrosine phosphatases (PTPs). These phosphatases can control HGF signaling by modifying the kinase or binding domains of c-Met (Organ and Tsao, 2011). Lastly, phospholipase Cγ (PLCγ) can bind to c-Met and activate protein kinase C (PKC) which acts as a negative regulator of c-Met activity (Organ and Tsao, 2011).

1.6.6.3 Intracellular signaling:

Growth factor receptor-bound protein 2 (Grb2) and Grb2-associated adaptor protein (Gab1) transduce signals through mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinases (Erk1 and Erk2), Jun amino-terminal kinases (JNK1, JNK2 and JNK3) and p38 (Figure 1.5). These kinases promote cell proliferation and mediate cell migration while signaling through PI3K mediates cell survival and resistance to apoptosis in a wide range of cell types (Faria *et al.*, 2011). Proliferation rates are also regulated via signalling through SHP2, a tyrosine phosphatase that mediates MAPK activity downstream of Grb2 (Li *et al.*, 2009).

The PI3K pathway signals through Akt and has been shown to be important in proliferation, differentiation and survival of muscle stem cells (Ceci *et al.*, 2004, Guttridge, 2004). Akt has been observed to be vital in the IGF induced increase of myogenin expression in C2C12 cells (Xu and Wu, 2000). Inhibition of this pathway by LY294002 a potent, reversible inhibitor of phosphoinositide 3-kinases (PI3K), (Maira *et al.*, 2009)(Maira *et al.*, 2009) has been shown to interfere with myotube formation and the expression of muscle-specific proteins such as MyoD and MyHC (Jiang *et al.*, 1999).

Activation of the ERK pathway by myostatin has been shown to negatively regulate myogenesis in C2C12 cells, and this effect can be reversed with the addition of PD98059, a potent, selective inhibitor of ERK kinase (Figure 1.5) (Yang *et al.*, 2006).

Activation of the p38 pathway by 5 mM creatine has been shown to increase C2C12 fusion and MyHC expression *in vitro (Deldicque et al., 2007).* Inhibition of p38 by SB205380, a highly specific inhibitor of p38 MAPK, has been shown to inhibit sarcomeric myosin expression in human embryonal rhabdomyosarcoma cells, indicating a pivotal role for p38 during myogenesis (Mauro *et al.*, 2002). Inhibition using both PD98059 and SB205380

allowed Kook *et al.*, (2008) to determine that mechanical stretch-induced C2C12 proliferation was not due to an ERK related mechanism but solely due to p38. They also showed that 3 uM SB205380 prevented myogenin expression, suggesting that ERK activation was essential for efficient C2C12 differentiation. However, inhibition of p38 increased MyoD expression and myotube formation suggesting that a downregulation of activity of this kinase is necessary for myogenesis (Kook *et al.*, 2008).

Activation of jun N-terminal kinase (JNK) has been shown to induce growth arrest and differentiation in human embryonal rhabdomyosarcoma cells (Mauro *et al.*, 2002). JNK has been suggested to play a role in the inhibition of myogenesis of in L6 and C2 myoblasts via the cytoplasmic redistribution of Myf5 (Figure 1.5) (Tam *et al.*, 2000). Inhibition with SP600125, a highly specific and potent reversible inhibitor of JNK, has been shown to reduce the proliferative potential of primary culture mouse myoblasts (Shi *et al.*, 2013).

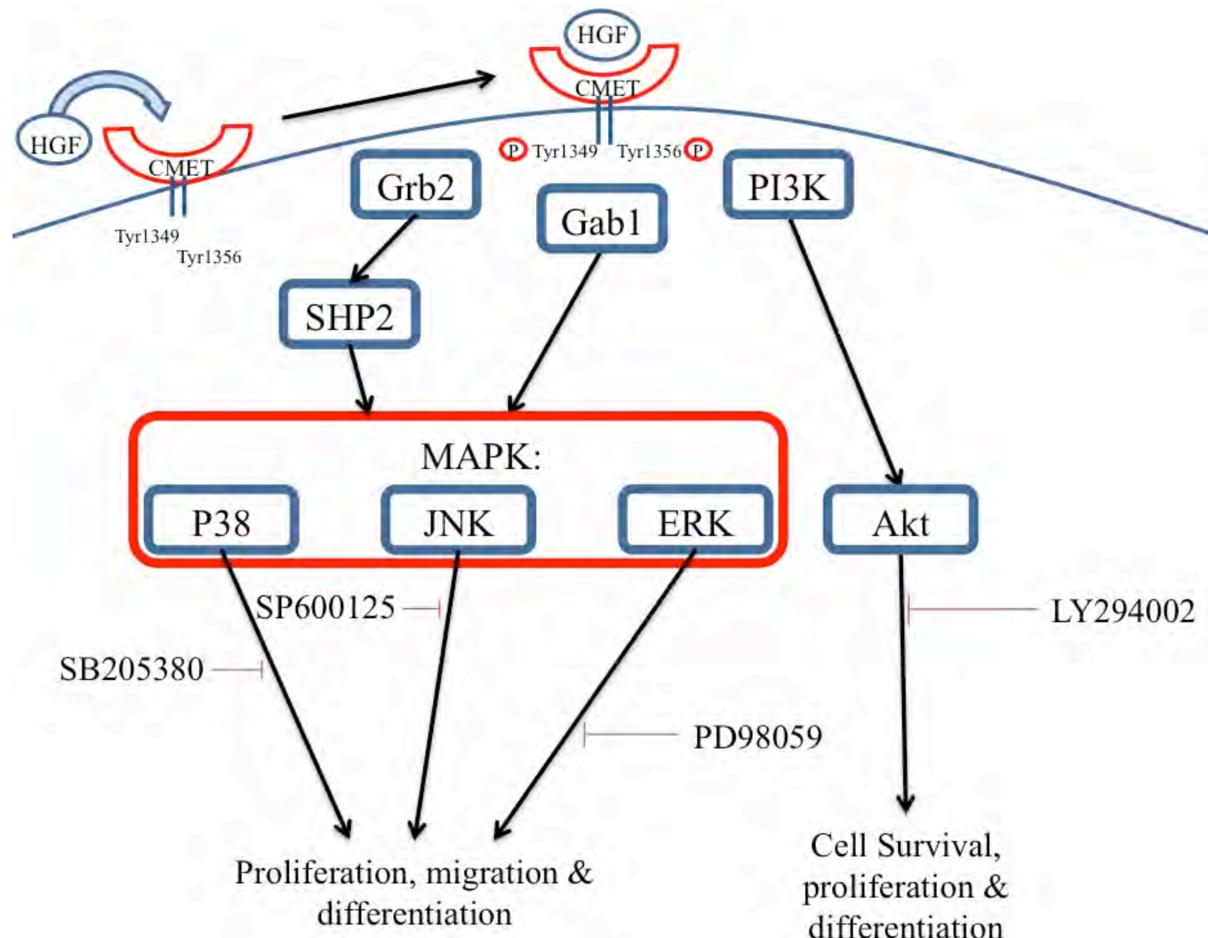


Figure 1.5: Schematic representation of c-Met activated signalling in response to HGF.

1.6.7 TGF-β: The transforming growth factor beta (TFG-β) superfamily of proteins consists of more than 40 members and includes TGF-β isoforms, bone morphogenetic

proteins (BMPs) and growth differentiation factors (GDFs) (Gumienny and Padgett, 2002). This group of proteins regulates numerous cellular processes such as growth, cell-cycle progression, apoptosis, and differentiation (Vilar *et al.*, 2006). TGF-β2 has been observed to suppress myogenic differentiation in the C2 myoblast cell line (Ewton *et al.*, 1988), as well as promote proliferation and inhibit myogenic differentiation in the C2C12 myoblast cell line (Schabort *et al.*, 2009). However, addition of TGF-β2 to primary myofiber cultures has resulted in a reduction of satellite cell numbers indicating the suppression of satellite cell proliferation in their natural niche (Bischoff, 1990). Much of the study of the TGF-βs and myoblast differentiation has shifted towards myostatin, a potent inhibitor of myogenesis (McCroskery *et al.*, 2003).

1.6.8 FGF: Fibroblast growth factor was first identified by its ability to significantly promote the proliferation of 3T3 fibroblasts (Armelin, 1973). FGF binds to a tyrosine kinase FGF receptor on the surface of cells (Montero *et al.*, 2001). FGF (5 ng/ml) was observed to increase the expression of differentiation-specific markers in C2 cells while 25 ng/ml induced a reduction in these markers and resulted in impaired cell fusion (Pizette *et al.*, 1996). FGF has been shown to be important in myogenesis and wound healing (Barrientos *et al.*, 2008). Satellite cells express the abovementioned tyrosine kinase FGF receptors, which suggests that FGF may play a role in skeletal muscle myogenesis (Armand *et al.*, 2006). Indeed, when recombinant FGF is injected into the muscle of injured mice, accelerated muscle repair is observed (Armand *et al.*, 2006). These results would suggest a key role for FGF in myogenesis, however its specific effect on the myogenic pathway is relatively unknown. Interactions between ECM factors and FGF have not yet been examined and may yield results that are more aligned with *in vivo* conditions.

Growth factors are therefore of vital importance in development, maintenance and repair of skeletal muscle. They have been shown to interact with specific protein components of the ECM and therefore their *in vitro* combination with ECM factors should more closely mimic *in vivo* conditions.

1.7 Extracellular matrix – growth factor interactions:

The study of the effect of extracellular matrix proteins on the behavior of cells has primarily been carried out using culture dishes coated with single ECM components. This approach has yielded many insightful results and we are beginning the understand the role of single ECM components and growth factors in modulating stem cell behavior (Ali *et al.*, 1998, Goetsch *et al.*, 2011, Grefte *et al.*, 2012, Grinnell, 1984, Kishioka *et al.*, 2008, Lin *et al.*, 2010, Podleski *et al.*, 1979, Schenke-Layland *et al.*, 2007, Yao *et al.*, 1996). However, ECM components are known to have complex interactions with each other and with growth factors and these interactions are integral to the influence that the ECM and growth factors have *in vivo* (Poschl *et al.*, 2004, Schonherr *et al.*, 1995).

ECM combination assays attempt to more closely simulate *in vivo* conditions by allowing these *de novo* ECM interactions to take place. Combined ECM experiments often show synergistic effects of the individual ECM components. For instance, fibronectin and laminin have each been shown to increase the differentiation of adult neural stem/progenitor cells and their combination enhanced this increase (Cooke *et al.*, 2010). Decorin has also been shown to modulate the positive effect that collagen I has on C2C12 migration, but was unable to modulate fibronectin-stimulated migration (Goetsch *et al.*, 2011). *In vivo* studies have shown that, in combination with Matrigel, HGF delays C2C12 myotube formation when transplanted into nude mice (Barbero *et al.*, 2001). These studies emphasize the importance of investigating ECM interactions and not simply single factors in isolation.

There are various mechanisms by which ECM-growth factor interactions can modulate cell behavior. The ECM can directly bind a growth factor, resulting in the sequestration of the growth factor and prevention of its presentation to its receptor; a mechanism or negative regulation (Hynes, 2009). This type of binding can also protect the growth factor from degradation and thus acts as a type of growth factor reservoir. In addition, direct binding can also facilitate successful presentation of the growth factor to its receptor. An example of this type of binding occurs when FGF-2 binds to heparan sulfate (Schultz and Wysocki, 2009). Crystal structure analysis revealed that heparan sulfate binds both FGF-2 and its receptor, facilitating subsequent signaling (Schlessinger *et al.*, 2000). It has been suggested that in addition to this, heparan sulfate also assists in the dimerization of two FGF receptor molecules, an important early step in receptor activation (Wilgus, 2012). Integrin-ECM

interactions can also lead to growth factor receptor activation, facilitating an indirect interaction between the ECM and growth factors. An example of this type of activation is when αVβ3 integrin, expressed on endothelial cells, binds to the glycoprotein vitronectin. This binding and integrin signaling enhances cellular responsiveness to Vascular Endothelial Growth Factor (VEGF) and can result in activation of VEGF receptor 2 (Wilgus, 2012).

Reversible binding of HGF to immobilized collagen IV has been reported and resulted in a collagen-HGF complex that was able to induce primary hepatocyte proliferation in a dose-dependent manner. Further investigation revealed that binding was due to specific peptide sequences unique to collagenous proteins suggesting a specific role for collagens in HGF bioavailability and activity (Schuppan *et al.*, 1998). However, very little is known about the effect of collagen IV in mediating HGF signaling in myoblasts.

The clinical significance of growth factor-ECM interactions has been suggested in the treatment of chronic wounds. Growth factor therapy is a promising technology, however, challenges with dosage regulation has suggested that an effective delivery system is lacking (Briquez *et al.*, 2015). Due to the recently realized importance of the ECM in regulating the bioavailability and presentation of growth factors, ECM-inspired delivery systems in the form of biodegradable ECM bandages, have been suggested for growth factor therapies. An example of such a system involves a biomatrix constructed with heparan sulfate-mimetic molecules to sequester heparan-binding growth factors such as FGF-2 (Liu *et al.*, 2007). In other studies, growth factor binding sites were identified in a range of ECM factors including collagens, fibronectin and vitronectin (Schuppan *et al.*, 1998, Martino and Hubbell, 2010, Upton *et al.*, 2008). The binding sites identified on fibronectin were described as "highly promiscuous" and bound to a wide range of growth factors including insulin-like growth factor binding-protein-3, FGF-2 and VEGF with a high affinity (Martino and Hubbell, 2010). Vitronectin was observed to bind IGF and EGF and this complex was observed to accelerate wound healing in non-healing ulcers in a human pilot study (Upton *et al.*, 2008).

1.8 3D skeletal muscle generation:

Future applications of 3D tissue generation technique may involve transplantation of engineered tissue to patients with muscle damage. One method to construct three-dimensional skeletal muscle involves the seeding of myoblasts onto a biodegradable scaffold

(Huang *et al.*, 2004). The scaffolds used in the successful generation of 3D muscle by Huang *et al.* (2004) resembled sponge and were made up of 50% poly-L-lactic acid and 50% polylactic-glycolic acid. The cells attach and grow on the scaffolds to create functional three-dimensional skeletal tissue (Levenberg *et al.*, 2005).

An early obstacle encountered in the first three-dimensional skeletal muscle models is that they were unable to outgrow the limitations of diffusion (Levenberg *et al.*, 2005). *In vivo* muscle development relies on a vascular system to nourish the tissue; this is absent in traditional *in vitro* muscle cultures (Levenberg *et al.*, 2005). However, in 2005, a team of researchers created vascularized skeletal muscle *in vitro*. The team co-incubated myoblasts, embryonic fibroblasts and endothelial cells on a scaffold similar to those described earlier and observed the *de novo* formation of simple vascularized muscle tissue (Levenberg *et al.*, 2005). This development will allow larger muscle to be grown and take researchers one step closer to both accurate models for muscle regeneration as well as autologous muscle transplants.

The second method of producing 3D tissues, including muscle, is using 3D bioprinters. This method may involve either printing a suitable scaffold from a biodegradable material or ECM component or by printing cells directly into a supportive gelatinous matrix, cell by cell, in progressive 3D layers (Jakab *et al.*, 2010). Once the basic shape of the tissue is achieved, the cells are stimulated to terminally differentiate (Norotte *et al.*, 2009). This technique, although in its infancy, is rapidly evolving with the advancement in cell and printer technology. Tissue generation using 3D bioprinters offers many important advantages to the abovementioned scaffold-seed method. There is a far greater degree of control: 3D bioprinting allows high-resolution control of cell distribution, density and even the precise placement of different cell types (Ventola, 2014). This control could be an extremely valuable tool in overcoming the abovementioned challenge to creating vascularized tissues, which will be essential if the *in vitro* production of complex organs is to be realized (Norotte *et al.*, 2009).

Research cited in this chapter outlines the ability of the ECM to influence myoblast behavior. The use of biologically relevant substrates and growth factor supplements may aid in the development of *in vitro* functional 3D muscle tissue.

1.9 Summary, objectives and aims:

In summary, extracellular matrix and growth factors are known to regulate many aspects of cell behavior including myogenesis. ECM factors physically interact with growth factors and may modify their action and bioavailability *in vivo*. By examining the influence that ECM factors have on growth factor-mediated satellite cell differentiation, we may be able to better understand muscle regeneration and the processes that impair or promote it.

The objective of this study was therefore to understand how the extracellular matrix of the satellite cell niche regulates the function of HGF in myogenesis.

To address this objective we aimed to:

First establish a baseline by investigating:

A) The effect of HGF on myogenesis.
B) The effect of collagen IV on myogenesis.

Second, determined how the ECM regulates the effect of HGF by investigating:

C) The effect of HGF on myogenesis in the presence of collagen IV.

CHAPTER 2

DOSE-DEPENDENT MODULATION OF MYOGENESIS BY HGF: IMPLICATIONS FOR C-MET EXPRESSION AND DOWNSTREAM SIGNALLING PATHWAYS

Hepatocyte growth factor (HGF) regulates satellite cell activation, proliferation and differentiation. We analysed the dose-dependent effects of HGF on myogenesis. Murine C2C12 myoblasts were treated with 0, 2 or 10 ng/ml HGF followed by assessment of proliferation and differentiation. HGF (2 ng/ml) significantly promoted cell division, but reduced myogenic commitment and fusion. Conversely, 10 ng/ml HGF reduced proliferative capability, but increased differentiation. c-Met expression analysis revealed significantly *decreased* expression in differentiating cells cultured with 2 ng/ml HGF, but *increased* expression in proliferating cells incubated with 10 ng/ml HGF. Mitogen-activated protein kinase (MAPKs: ERK, JNK or p38K) and phosphatidylinositol-3-kinase (PI3K) inhibition abrogated the HGF-stimulated increase in cell number. Interestingly, PI3K and p38 kinase facilitated the negative effect of HGF on proliferation, while ERK inhibition abrogated the HGF-mediated decrease in differentiation. Dose-dependent effects of HGF are mediated by changes in c-Met expression and downstream MAPK and PI3K signalling.

Includes data from:

WALKER, N., KAHAMBA, T., WOUDBERG, N., GOETSCH, K. & NIESLER, C. 2015. Dose-dependent modulation of myogenesis by HGF: implications for c-Met expression and downstream signalling pathways. Growth Factors, 33, 229-41.

2.1 Introduction

During skeletal muscle regeneration, the activation of satellite cells, their subsequent expansion and the terminal differentiation of the myoblast population is regulated by growth factors, such as fibroblast growth factor (FGF), insulin growth factor-1 (IGF-1), transforming growth factor beta (TGF-β) and hepatocyte growth factor (HGF) (Gal-Levi *et al.*, 1998, O'Reilly *et al.*, 2008, Yamada *et al.*, 2010, O'Blenes *et al.*, 2010, Miller *et al.*, 2000, Pownall and Isaacs, 2010). HGF, a heparin-binding protein, is sequestered in an inactive form in the extracellular matrix (ECM) of uninjured muscle fibers (Miller *et al.*, 2000); however, upon tissue injury it is cleaved and released to stimulate satellite cell activation (Birchmeier and Gherardi, 1998). HGF transduces its effects on satellite cells through specific interaction with the c-Met receptor (Humphrey *et al.*, 1995). c-Met is a transmembrane tyrosine kinase cell surface receptor consisting of a 145 kDa and 50 kDa β- and α-chain (Sonnenberg *et al.*, 1993). It has been shown to be essential during satellite cell activation, proliferation, migration and differentiation (Organ and Tsao, 2011).

Upon binding of HGF to the c-Met receptor, the kinase becomes active (Bottaro *et al.*, 1991). This results in the phosphorylation of two tyrosine residues (Tyr1349 and Tyr1356) in the carboxy-terminal tail of c-Met (Figure 1.5). The phosphorylated residues become docking sites for a range of adaptor proteins including phosphatidylinositol-3-kinase (PI3K), Grb2-associated adaptor protein (Gab1) and growth factor receptor-bound protein 2 (Grb2). These pathways proceed to mediate c-Met-dependent cell proliferation, migration, survival and differentiation (Faria *et al.*, 2011). Grb2 and Gab transduce signals through mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinases (ERK1 and ERK2), Jun amino-terminal kinases (JNK1, JNK2 and JNK3) and p38. It has also been shown that HGF regulates proliferation rates via signalling through SHP2, a protein tyrosine phosphatase that mediates MAPK activity; these proliferation rates were dependent on the HGF dose utilized (Li *et al.*, 2009, Chazaud, 2010). MAPKs are thought to be more intensively involved in regulating cell proliferation, differentiation and cell migration, while signaling through PI3K mediates cell survival and resistance to apoptosis (Faria *et al.*, 2011, Lluis *et al.*, 2006, Li *et al.*, 2000, Knight and Kothary, 2011, Keren *et al.*, 2005, Organ and Tsao, 2011).

Regulation of HGF:c-Met signaling can be achieved by a number of mechanisms. In hepatocytes, HGF-bound c-Met receptors are internalized and the c-Met cell surface concentration is observed to decrease within 30 minutes of exposure to elevated HGF levels (Naka *et al.*, 1993). HGF is subsequently degraded and released by the cell. HGF signaling is also regulated by a number of protein tyrosine phosphatases (PTPs), which bind to the cytoplasmic domain of c-Met. These phosphatases can control HGF signaling by modifying the intracellular kinase activity or binding domains of c-Met (Organ & Tsao 2011). Receptor protein tyrosine phosphatase beta (RPTP-β) dephosphorylates Tyr1356, while leukocyte common antigen-related molecule (LAR) counteracts Met auto-phosphorylation, which inhibits MAPK and PI3K activity. Similarly, CD148 prevents binding of signal transducers Gab1 and p120 catenin (Baldanzi and Graziani, 2014). Lastly, phospholipase Cγ (PLCγ) can bind to the carboxy-terminal tail of c-Met and activate protein kinase C (PKC), which acts as a negative regulator of c-Met activity (Organ and Tsao, 2011).

In vitro, it has been demonstrated that HGF activates rat primary culture satellite cells at concentrations as low as 2.5 ng/ml (Tatsumi *et al.*, 1998). Further studies in primary rat skeletal myoblasts and the C2C12 murine cell line have also demonstrated a promotion of proliferation in response to HGF at concentrations ranging from 3 ng/ml to 50 ng/ml (Anastasi *et al.*, 1997, O'Blenes *et al.*, 2010, Allen *et al.*, 1995). In contrast however, a separate study utilising rat satellite cells demonstrated a suppression of proliferation in response to HGF at concentrations greater than 10 ng/ml (Yamada *et al.*, 2010). A reduction in activation and proliferation was also observed in both chicken skeletal muscle and mouse C2 cells in response to addition of exogenous HGF at 20 and 50 ng/ml (Gal-Levi *et al.*, 1998). These conflicting results suggest potential dose-dependent sensitivity regarding the effect of HGF on cellular proliferation.

Skeletal myogenesis is regulated by several myogenic regulatory factors (MRFs) including MyoD, Myf-5, MRF-4 and myogenin (Gal-Levi *et al.*, 1998, Halevy and Cantley, 2004). MRFs are proteins expressed in cells committed to differentiate; together with Pax7, they are implicated in specification of the myogenic lineage (McFarlane *et al.*, 2008, Buckingham *et al.*, 2006, Seale *et al.*, 2004). Differentiation studies published to date suggest an inhibitory effect of HGF (2.5 – 50 ng/ml) on myoblast differentiation (Gal-Levi *et al.*, 1998, Halevy *et al.*, 2004, Zeng *et al.*, 2002, Leshem *et al.*, 2000, Yamane *et al.*, 2004). Accumulating evidence implies that HGF may influence myogenesis via its regulation of MyoD, Myf-5 and

myogenin (Charge and Rudnicki, 2004, Halevy *et al.*, 2004, Rosen *et al.*, 1990). In addition, some studies provide evidence of a role for p27 (a cyclin-dependent kinase) and Twist (a basic helix-loop-helix transcription factor) in the mediation of HGF on differentiation (Leshem *et al.*, 2000).

HGF therefore plays a central role during skeletal muscle myogenesis; however the effect of different doses on cellular activities is unclear and requires further examination. In the present study we culture the C2C12 murine cell line to examine the effects of different HGF concentrations on proliferation and differentiation. Furthermore, we investigate whether dose-dependent sensitivities are related to changes in the total protein expression of the c-Met receptor and utilise both MAPK and PI3K inhibitors in an attempt to understand the signaling mechanisms at play.

2.2 Materials and methods

2.2.1 Cell culture

The C2C12 murine cell line was donated by Prof Anna-Mart Engelbrecht (Department of Physiological Sciences, University of Stellenbosch, South Africa). Cells were cultured in Dulbecco's Modified Eagle's Medium (Highveld Biological, CN3193-9) supplemented with 10% (v/v) Fetal Bovine Serum (Biowest, S181H-500), 2% (v/v) L-glutamine (Lonza, BE17-605E) and 2% (v/v) Penicillin-Streptomycin (Lonza, DE17-602E). For differentiation studies, cells were cultured to a confluence of 70% after which media was changed to differentiation media (DMEM supplemented with 2% (v/v) horse serum) (Biowest, S090H-100).

2.2.2 HGF addition

Human HGF (PeproTech, cat.100-39) was used at a final concentration range of 0, 2 and 10 ng/ml and media changed every 48 hours during differentiation studies. Cells were incubated at 37°C in a humidified incubator at 5% CO2. All experiments were carried out under sterile conditions in a Class II Biohazard Safety Cabinet. Clustal W alignments were conducted to compare the amino acid sequences of HGF used in this study and those found in mice (*Mus Musculus*) (Appendix III). A score of 89.48% similarities was observed.

2.2.3 Inhibitors

PI3K Inhibitor (LY294002, final concentration: 2.5 µM, Santa Cruz, SC-201426), ERK inhibitor (PD98059, final concentration: 12.5 µM, Santa Cruz, SC-3532), p38 inhibitor (SB205380, final concentration: 5 µM, Santa Cruz, SC-3533) and JNK inhibitor (SP600125, final concentration: 5 µM, Santa Cruz, SC-200635) were reconstituted in DMSO (Sigma, D2650). Inhibitors were added to growth or differentiation media and replenished every 48 hours with media change. Final DMSO concentrations never exceeded 0.04% and were not found to be toxic to C2C12 cells (Appendix I, Supplementary Figure 1); this is in agreement with previous studies where DMSO was shown to be non-toxic in C2C12 cells at concentrations up to 0.1% (Moorwood *et al.*, 2011).

2.2.4 Cell counts

The cell counts were carried out as previously described (Taylor *et al.*, 2001). Briefly, C2C12 cells were seeded in T25 tissue culture flasks (100 000 cells) containing growth media and incubated at 37°C (5% CO_2) for 24 hours. HGF (0, 2, 10 ng/ml) was then added and cells incubated for a further 24 hours. Thereafter, cells were trypsinized and counted using the automated BioRad TC-20 Cell Counter; viability was also assessed using 10 µl trypan blue (BioRad, 145-0021).

2.2.5 Immunocytochemistry

Cells were differentiated to day 5 in the presence or absence of HGF (0, 2 or 10 ng/ml), fixed in 4% paraformaldehyde containing 0.1% Triton X-100 (Sigma, T9284) for 20 minutes and blocked with 5% donkey serum for 1 hour. For Myosin Heavy Chain (MyHC) detection, coverslips were incubated with a mouse monoclonal MF20 primary antibody (Developmental Studies Hybridoma Bank; dilution 1/200) overnight at 4°C. This was followed by 4 x 5 minute PBS wash steps prior to incubation with secondary antibody for 1 hour at room temperature. The secondary antibody was DyLight 594-conjugated AffiniPure donkey anti-mouse lgG secondary antibodies (Jackson ImmunoResearch, CN-715-485-151, 1/1000 dilution). Hoechst (Sigma, B2267; 10 mg/ml stock; 1/2000 dilution) was added to all the cells for detection of nuclei. Moviol was used as mounting agent. All images were captured using the Zeiss 710 LSM confocal microscope.

2.2.6 Fusion Index

The fusion index was calculated as described previously (Micheli *et al.*, 2011). Briefly, myoblasts were differentiated on coverslips in the presence or absence of HGF (2 ng/ml or 10 ng/ml) and fixed at day 5. Immunocytochemistry was carried out to detect the expression of MyHC in the differentiating cells. Five random fields of view of cells were captured using the Zeiss 710 LSM confocal microscope. ImageJ software was utilized to determine formation of myotubes. The fusion index was calculated as the number of nuclei within MyHC-labeled myotubes (two or more nuclei per myotube) divided by the total number of nuclei per field of view, multiplied by 100.

2.2.7 Western Blotting

Myoblasts were cultured in either growth or differentiation media in the presence or absence of HGF (2 ng/ml or 10 ng/ml). Proliferating cells were harvested at day 0 (in growth media for 24 hours), while differentiating cells were harvested at days 1, 2 and 5 of differentiation. During differentiation, media (supplemented with or without HGF) was changed every 2 days. Cell lysates were prepared in 100 µl RIPA buffer (Sigma-Aldrich, R0278) containing 1 µl Protease Inhibitor Cocktail (PIC) (Sigma-Aldrich, P8340) for 1 hour on ice, followed by centrifugation at 12 000 rpm for 5 minutes. Lysates were sonicated using the Ultrasonic cleaner (Shalam Laboratory Suppliers) and protein concentrations determined via the Bradford Assay (Bradford, 1976). Total protein (30 µg) was loaded onto 12.5% SDS Poly-acrylamide gel, and following separation, was transferred onto a Nitrocellulose membrane (Life Sciences, P/N 66485) using Western Blotting. The membrane was then incubated with primary antibodies overnight. Primary antibody dilutions: rabbit anti-c-Met 1:500 (Invitrogen, 182257); mouse anti-alpha-tubulin 1:1000 (Santa Cruz Biotechnology B-7, sc-5286); and mouse anti-MyHC 1:1000 (Developmental Hybridoma Bank, MF20-S). Secondary antibody dilutions: goat anti-rabbit (Dako PO448) 1:12000 for c-Met detection; rabbit anti-mouse (Dako PO260) 1:12000 for myogenin and alpha-tubulin detection and 1:20 000 for MyHC detection for 1 hour. HRP activity was visualized using Enhanced Chemiluminescence (ECL, BioRad, 170-5070) and GeneSys Image Acquisition software (Vacutec, South Africa). Densitometric analysis was carried out using the Versa Doc Imaging System and Quantity One 2.6 (Bio-Rad).

2.2.8 Statistical Analysis

The results from each experiment were expressed as a Mean ± SEM. The ANOVA statistical test was performed on all data followed by the Tukey's pairwise post hoc test on all qualifying data sets. Samples were considered to be statistically significant if they produced a p value of less that 0.05. The number of experimental repeats is specified in each figure legend.

2.3 Results

2.3.1 C2C12 myoblast differentiation is regulated by HGF

To determine the downstream effect of HGF on terminal myogenic differentiation, we first assessed C2C12 myotube fusion and sarcomeric myosin heavy chain (MyHC) expression at day 5 of differentiation.

Myotube formation occurred in response to all doses of HGF with the highest levels of densely packed, aligned myotubes observed in response to 10 ng/ml HGF (Figure 2.1A). Fewer MyHC-expressing myotubes were observed in response to 2 ng/ml HGF, along with reduced alignment and reduced MyHC expression (Figure 2.1A). Under control conditions, the fusion index for C2C12 was 42% (Figure 2.1B), in line with independently published studies (Velica and Bunce, 2011). Treatment with 10 ng/ml HGF significantly ($p<0.05$) increased tube formation by 12%; in contrast 2 ng/ml HGF significantly reduced tube formation by 17% in response to 2 ng/ml HGF compared to the control ($p<0.002$) (Figure 2.1B). These data was supported by a 30% increase in total MyHC expression in C2C12 cells treated with 10 ng/ml HGF when compared to control ($p<0.05$; Figure 2.1C). Furthermore, a 20% reduction in MyHC production was observed when C2C12 cells were treated with 2 ng/ml HGF during differentiation compared to control (Figure 2.1C).

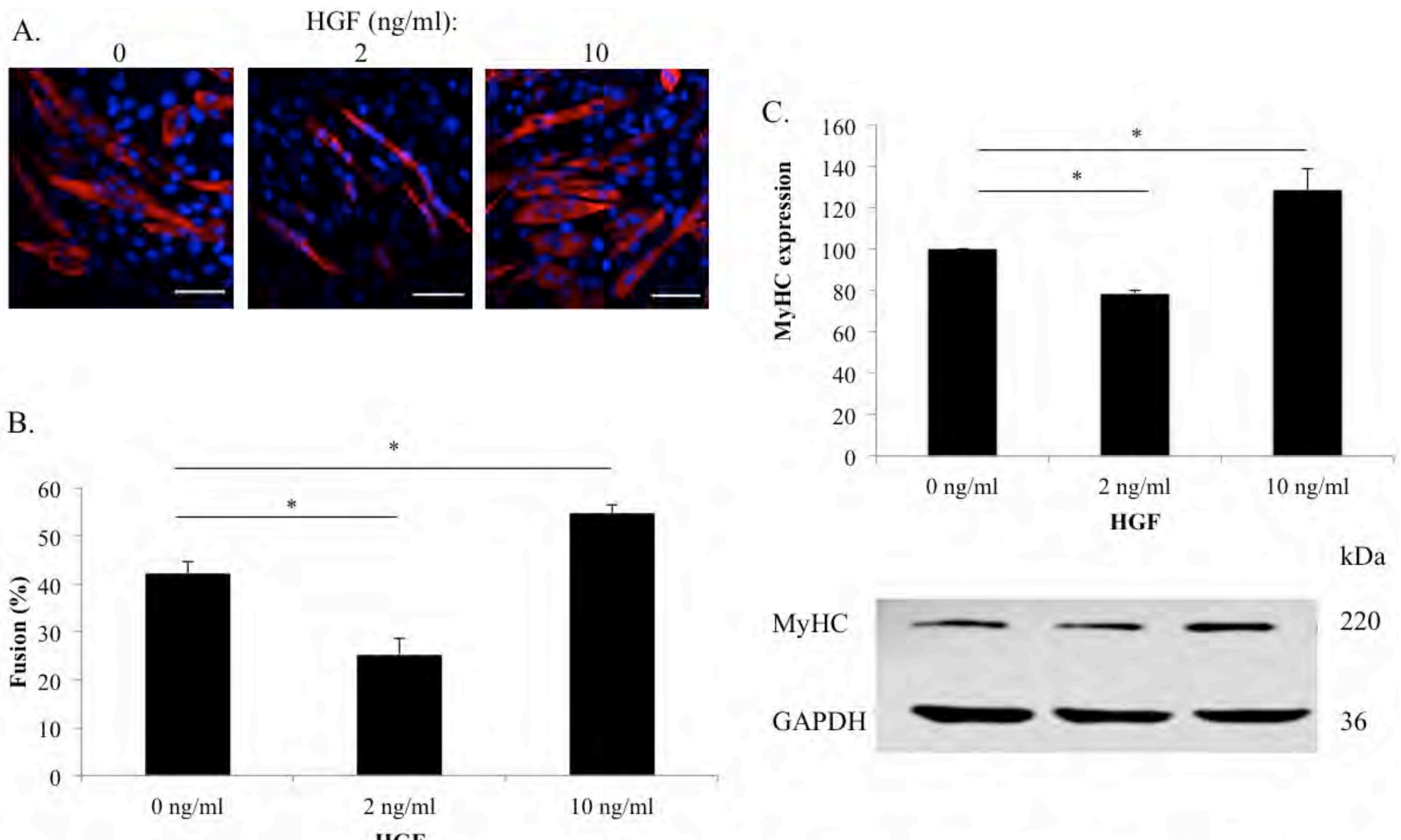


Figure 2.1: C2C12 myoblast differentiation in response to HGF. Myotube formation in differentiating C2C12 myoblasts was assessed at day 5 post-induction of differentiation. Cells were incubated with 0, 2 and 10 ng/ml HGF in differentiation media. **A**) Representative images of differentiated C2C12 myoblasts (day 5) showing MyHC expression (Red) and nuclei (Blue). **B**) Fusion index calculated as the percentage C2C12 myotube formation at day 5. **C**) Western blot and densitometric analysis of MyHC expression at day 5. GAPDH was used as a loading control and MyHC expression was calculated relative to GAPDH expression. Data represents 4 (B) and 3 (C) independent experiments. All immunofluorescence images were taken with the Zeiss 710 LSM confocal microscope with the 40X objective. Scale bar = 40 μm. Data is presented as mean ±

2.3.2 HGF regulates c-Met receptor expression during myoblast differentiation

In order to gain insight into the mechanism by which HGF may be mediating its effect on myogenesis, we analysed c-Met expression in differentiating C2C12 cells.

A correlation between c-Met expression and HGF concentration was observed. In response to 2 ng/ml, c-Met expression was significantly suppressed by 30% at day 1 of differentiation ($p<0.05$; Figure 2.2A). In contrast, cells treated with 10 ng/ml HGF demonstrated a 20% increase in c-Met expression at day 1 ($p<0.05$; Figure 2.2A). The significant decrease in c-Met expression in response to 2 ng/ml HGF was maintained through day 2 ($p<0.005$; Figure 2.2B), whereas the increase in response to 10 ng/ml HGF is lost ($p=0.484$; Figure 2.2B). By day 5 of differentiation, c-Met receptor expression levels had equalised and there was no significant difference between control and HGF treated cells (Figure 2.2C). This data suggests that the observed dose dependent effect of HGF on myoblast differentiation may be mediated, at least in part, by changes in c-Met receptor expression levels.

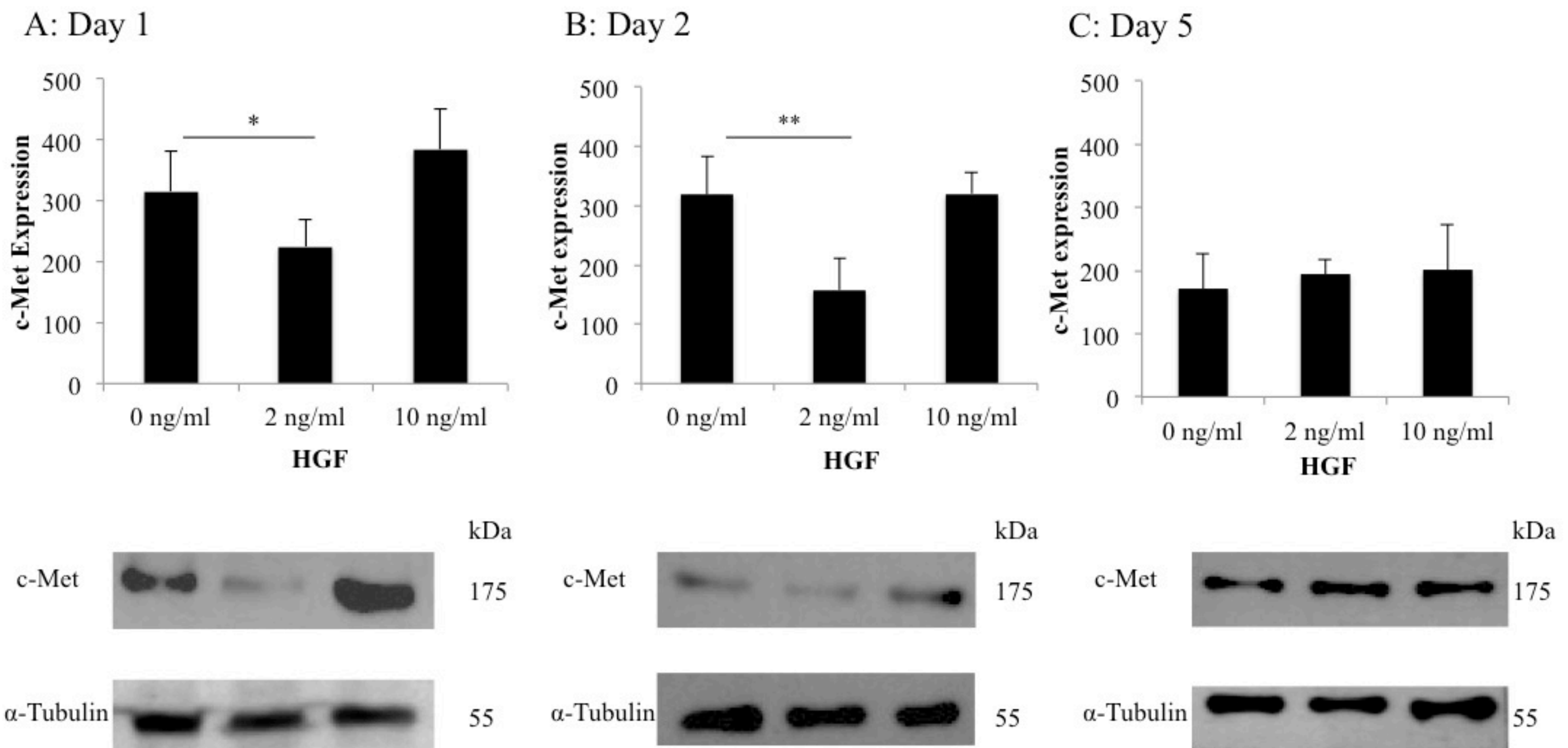


Figure 2.2: Effect of HGF on c-Met expression in differentiating C2C12 myoblasts. Western blot and densitometric analysis of c-Met expression levels in differentiating C2C12 myoblasts treated with 0, 2 or 10 ng/ml HGF. Media was changed every two days and cell lysates harvested at days 1 (**A**), 2 (**B**) and 5 (**C**) post-induction of differentiation. Alpha-tubulin was used as a loading control and c-Met expression was calculated relative to alpha-tubulin expression. Data represents 6 independent experiments. Images were assessed by Quantity One image analysis software. Data is presented as mean ± SEM. $^*p<0.05$ and $^{**}p<0.005$

2.3.3 Inhibition of ERK, modulates the dose-dependent effect of HGF on C2C12 myoblast differentiation

Intracellular pathways known to mediate HGF signalling include the MAPK's as well as PI3K. In an effort to begin to clarify the intracellular mechanisms underlying the observed dose-dependent response of differentiating myoblasts to HGF, we utilised the PI3K inhibitor (LY294002), p38 inhibitor (SB205380), JNK inhibitor (SP600125) and ERK inhibitor (PD98059).

LY294002, SB205380 and SP600125, but not PD98059, were observed to reduce C2C12 differentiation in the presence or absence of HGF (Figure 2.3). Myosin heavy chain (MyHC) expression and the presence of fused myotubes were greatly reduced in cells treated with these inhibitors regardless of HGF exposure (Figure 2.3). Interestingly, PD98059 was observed to abrogate the negative effect of 2 ng/ml HGF on MyHC expression, suggesting that the ERK signaling pathway may be responsible, at least in part, for the inhibitory effect of HGF on myoblast differentiation.

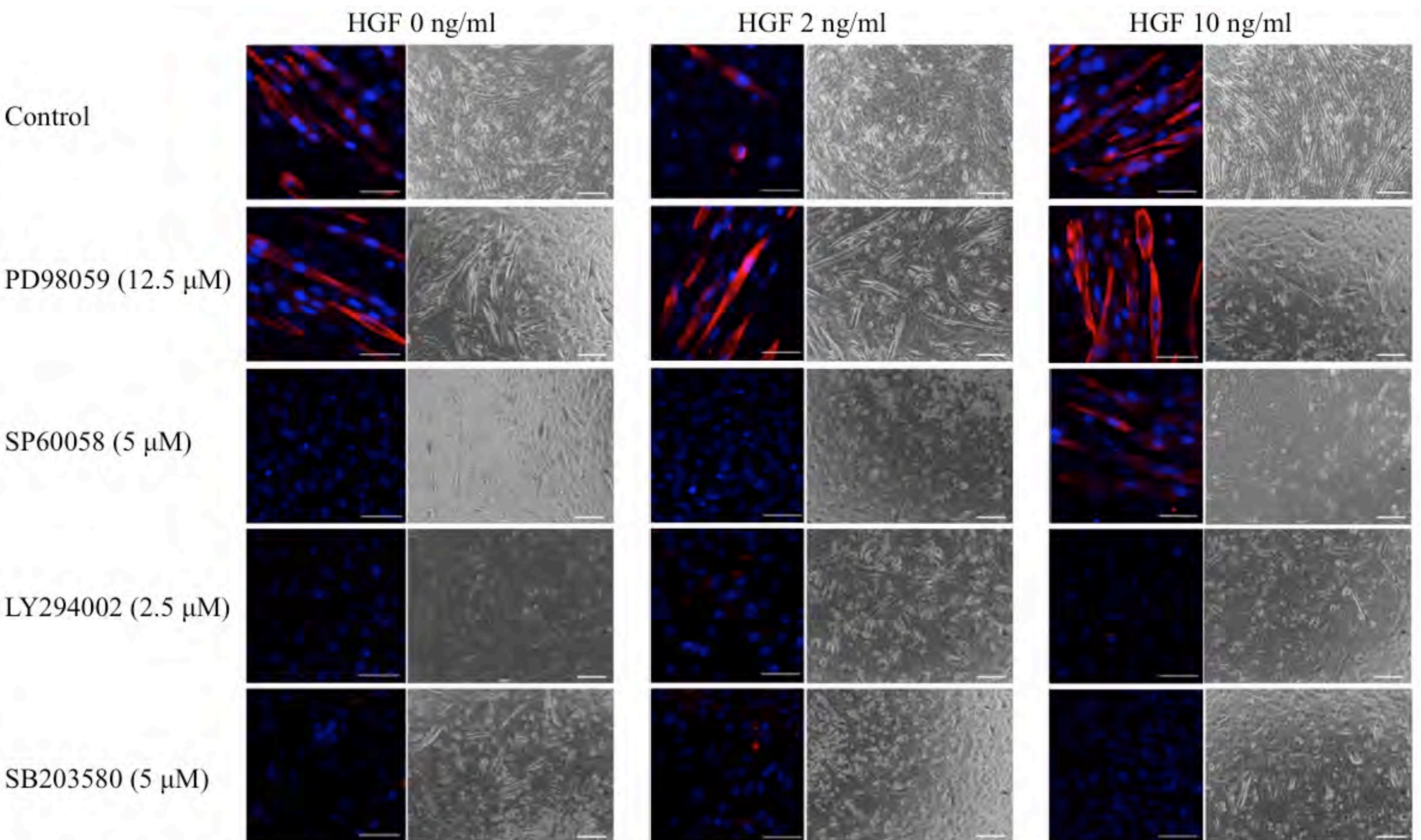


Figure 2.3: The role of MAPK's and PI3K in mediating the dose-dependent effect of HGF on differentiation. Differentiating C2C12 myoblasts were incubated with 0, 2 and 10 ng/ml HGF in the presence or absence of PD98059 (12.5 μM), SP600125 (5 μM), LY294002 (2.5 μM) or SB203580 (5 μM). Representative images were taken at day 5 of differentiation using confocal microscopy (left panel of each HGF set) and phase contrast microscopy (right panel of each HGF set). Confocal microscopy shows MyHC expression (Red) and nuclei (Blue); images were taken with a Zeiss 710 LSM confocal microscope using the 40X objective. Scale bar = 40 μm and 100 μm for confocal and brightfield images respectively.

2.3.4 HGF regulates myoblast proliferation in a dose-dependent manner

Next we tested whether the dose-dependent effect of HGF was restricted to differentiation, or whether HGF also played a key role within the myoblast proliferation cycle. Myoblasts were seeded at 100 000 cells per well and cultured in the presence or absence of 2 or 10 ng/ml HGF for 24 hours prior to cell counts. Compared with control, C2C12 myoblast numbers were significantly increased in response to 2 ng/ml HGF ($p<0.05$), but decreased following incubation with 10 ng/ml HGF ($p<0.05$) (Figure 2.4A). Cell counts revealed a significant 21% increase, from $3.9x10^5$ cells to $4.7x10^5$ cells (Figure 2.4A; $p<0.05$) in response to 2 ng/ml HGF compared to the untreated control. However, following incubation with 10 ng/ml HGF, a significant reduction in C2C12 myoblast numbers was observed ($p<0.05$) (Figure 2.4A). Cell counts revealed a significant 40% decrease; from $3.9x10^5$ cells to $2.4x10^5$ cells (Figure 4A; $p<0.05$) in response to 10 ng/ml HGF. Interestingly, cells incubated in the presence of HGF (2 ng/ml) showed a small, but significant increase in C2C12 cell viability from 84% (control) to 89% ($p<0.003$; Figure 2.4B). HGF at 10 ng/ml did however not significantly affect cell viability when compared to control (84%) (Figure 2.4B). It is therefore possible that at least part of the ability of HGF to promote cell growth is due to a pro-survival rather than a pro-proliferative mechanism. Analysis of c-Met expression levels in proliferating C2C12 cells revealed a significant increase in cells treated with 10 ng/ml HGF, but not 2 ng/ml HGF (Figure 4C). This suggests that the anti-proliferative effect of HGF could be mediated by an increase in c-Met receptor expression.

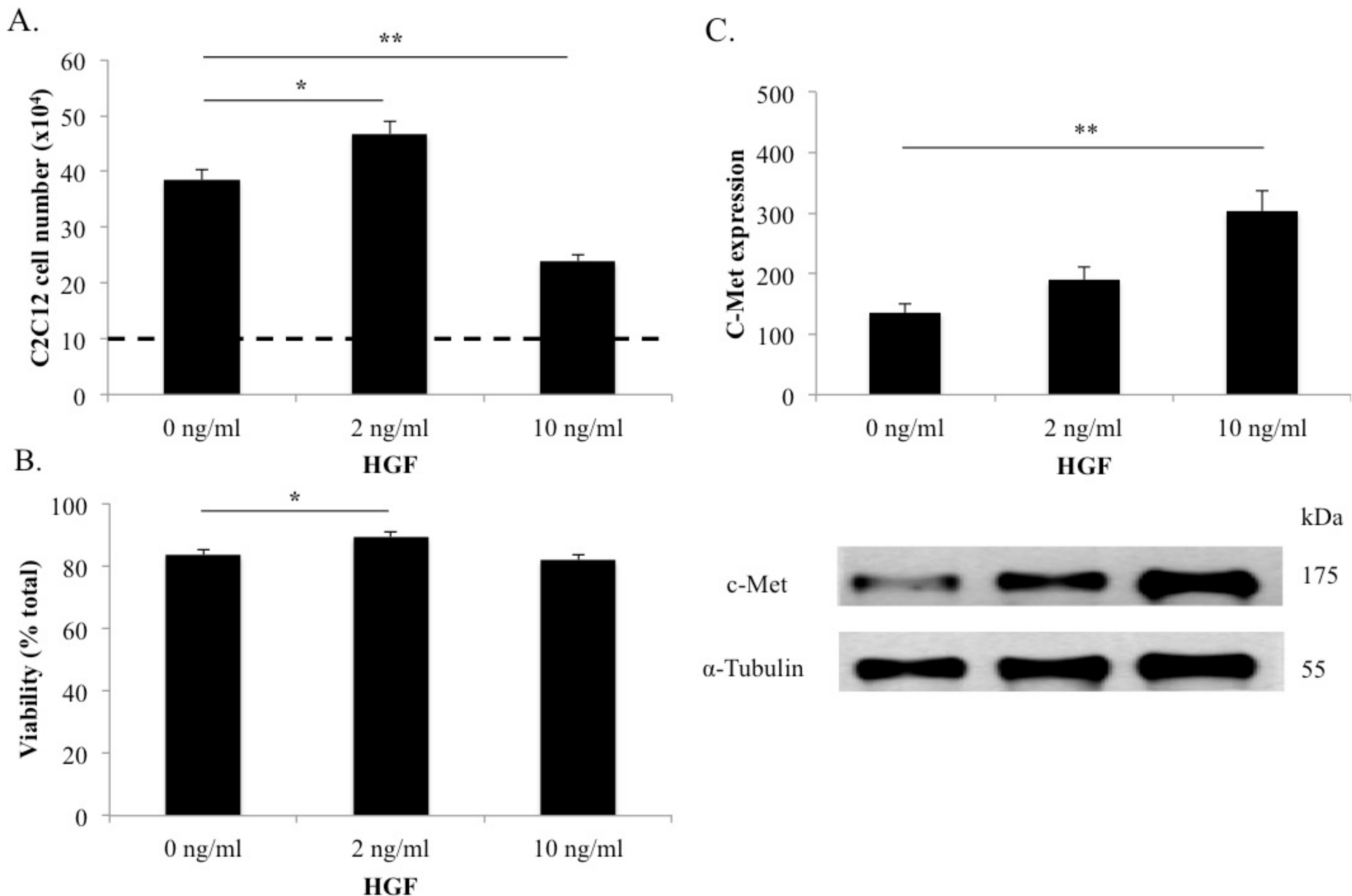


Figure 2.4: Effect of HGF on C2C12 myoblast cell number and c-Met expression. Cells were incubated for 24 h with growth media containing 0, 2 and 10 ng/ml HGF. C2C12 cell numbers (**A**: Total, **B**: Viable) were determined using the BioRad TC-20 Cell Counter. Dashed line represents initial cell number seeded (100 000 cells/flask). C) Western blot and densitometric analysis of c-Met expression levels in proliferating C2C12 myoblasts treated with 0, 2 or 10 ng/ml HGF. Alpha-tubulin was used as a loading control and c-Met expression was calculated relative to alpha-tubulin expression. Flasks were seeded with 10 x 10^4 cells/flask. Data represents 4 (A, B) and 3 (C) independent experimental repeats. Data is presented as mean ± SEM. *$p<0.05$ and **$p<0.005$.

2.3.5 ERK and JNK mediate pro-proliferative effects of HGF

To determine the role of MAPK and PI3K pathways in mediating the effect of HGF on proliferation, we next inhibited PI3K, ERK, p38 and JNK signaling pathways while incubating proliferating C2C12 cells with 0, 2 or 10 ng/ml HGF. PD98059 and SP600125 (ERK and JNK inhibitors respectively) significantly reduced the pro-proliferative effect of 2 ng/ml HGF ($p<0.005$) such that when treated with 2 ng/ml HGF and either PD98059 (12.5 µM) or SP600125 (5 µM), the ability of HGF to significantly increase C2C12 cell numbers was abolished (Figure 2.5A and B). This suggests that the pro-proliferative effect of 2 ng/ml may be facilitated, at least in part, by the ERK and JNK pathways; however, these pathways seem to play little role in mediating the anti-proliferative effect of 10 ng/ml HGF in C2C12 cells. Interestingly, inhibition of the JNK pathway also significantly ($p<0.05$) reduced cell viability in the presence of 2 ng/ml HGF (Figure 2.5D, $p<0.05$). Therefore, in addition to mediating myoblast proliferation, HGF may also act as a pro-survival factor via the JNK pathway (Figure 2.5D).

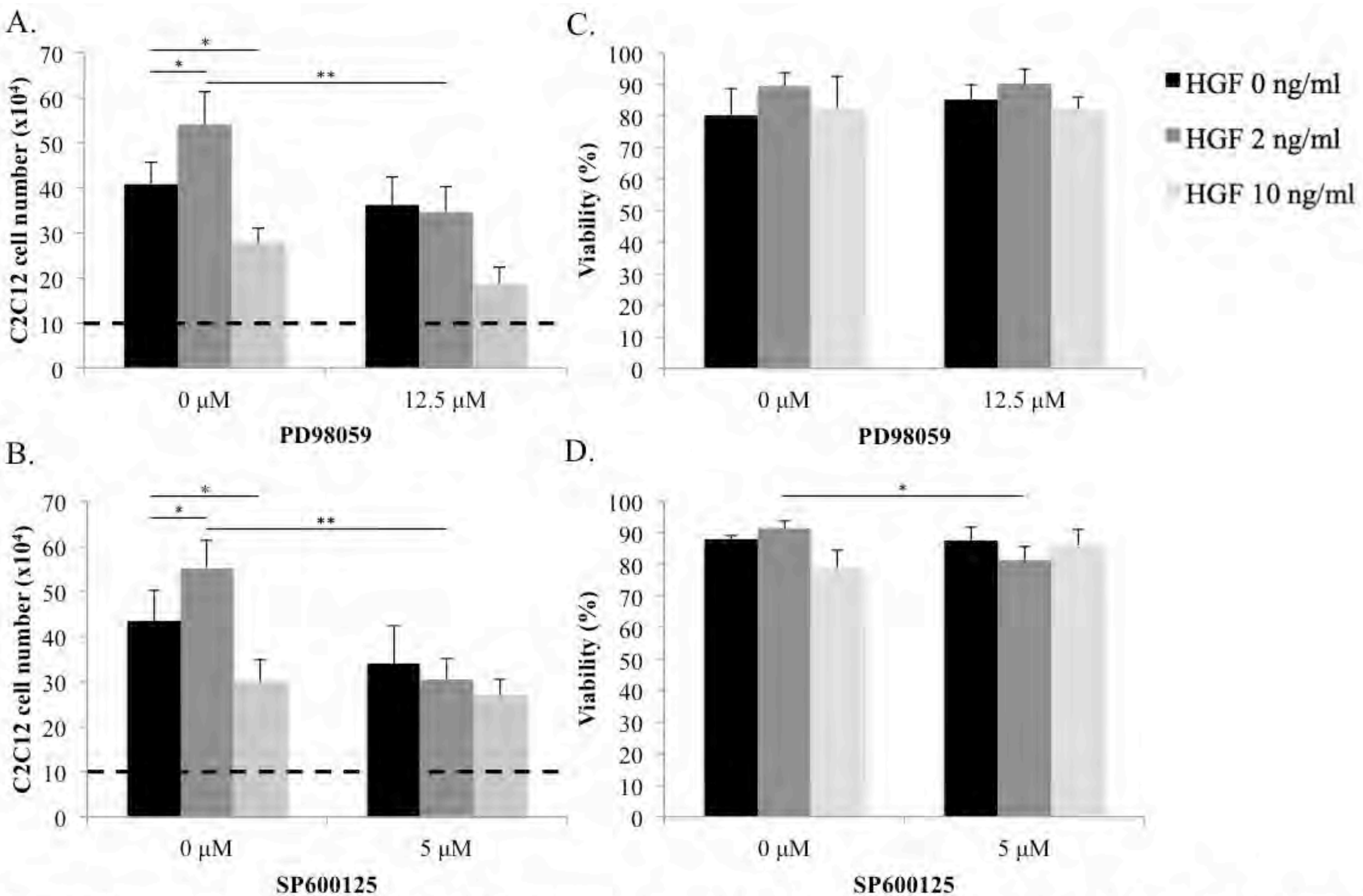


Figure 2.5: The role of ERK and JNK in mediating the effect of HGF on cell number. Proliferating C2C12 myoblasts were treated with 0, 2 or 10 ng/ml HGF as well as **A**) PD98059 (12.5 µM) or **B**) SP600125 (5 µM) and cell number determined at 24 h. In addition, the effect of **C**) PD98059 and **D**) SP600125 on cell viability was also assessed using Trypan Blue. C2C12 myoblasts (100 000 cells/flask) were initially seeded and analysis performed using the TC20 automated cell counter (Biorad) for 4 independent experimental repeats, each in duplicate. Dashed line represents initial cell number seeded. Data is presented as mean ± SEM. *$p<0.05$ and **$p<0.00.5$.

2.3.6 Inhibition of the PI3K and p38 pathways negates the effect of HGF on myoblast proliferation

LY294002 and SB203580 (PI3K and p38 inhibitors respectively) were observed to neutralize both the pro- and anti- proliferative effects of HGF (Figure 2.6A and B). LY294002 (2.5 μM) and SB203580 (5 μM) significantly reduced the pro-proliferative effect of 2 ng/ml HGF ($p<0.05$) such that, when treated with 2 ng/ml HGF and either LY294002 or SB203580, the ability of HGF to significantly increase C2C12 cell numbers was abolished (Figure 2.6A and B). LY294002 (2.5 μM) and SB203580 (5 μM) also significantly decreased the anti-proliferative effect of 10 ng/ml HGF (Figure 2.6A and B). Under these conditions, no significant change in cell viability was observed (Figure 2.6C and D).

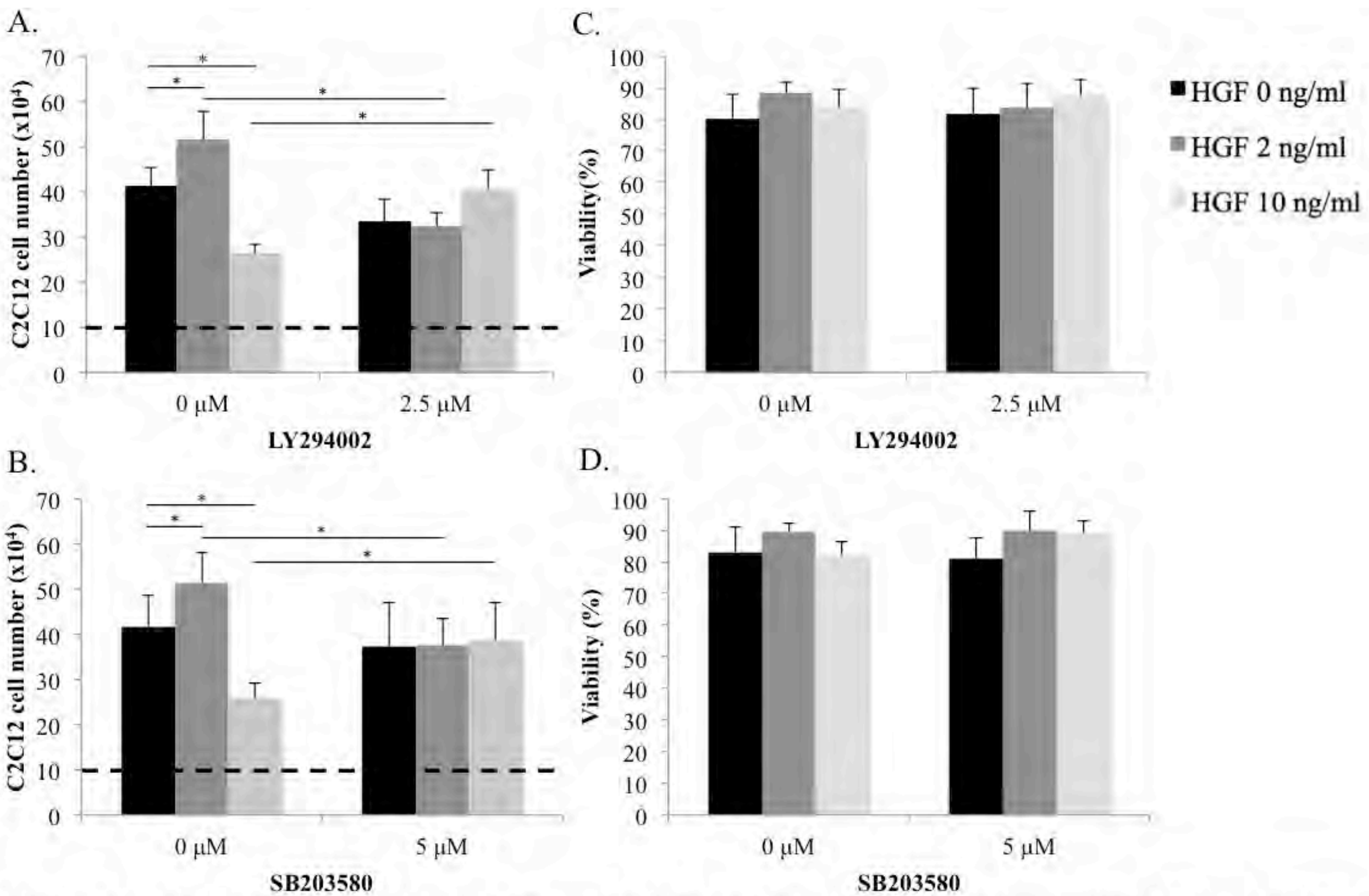


Figure 2.6: The role of PI3K and p38K in mediating the effect of HGF on cell number. Proliferating C2C12 myoblasts were treated with 0, 2 or 10 ng/ml HGF as well as **A**) LY294002 (2.5 µM), **B**) SB203580 (5 µM) and cell number determined at 24 h. In addition, the effect of **C**) LY294002 (2.5 µM) and **D**) SB203580 (5µM) on cell viability was assessed using Trypan Blue. C2C12 myoblasts (100 000 cells/flask) were initially seeded and analysis performed using the TC20 automated cell counter (Biorad) for 4 independent experimental repeats, each in duplicate. Dashed line represents initial cell number seeded. Data is presented as mean ± SEM. *$p<0.05$

2.4 Discussion

Satellite cell activation, proliferation and subsequent skeletal myoblast differentiation are critical for successful myogenesis following muscle injury in adult mammals. In this regard, the relationship between growth factors and myogenesis has been extensively investigated *in vitro* using primary culture myoblasts and myogenic cell lines (Nadal-Ginard, 1978, Florini *et al.*, 1996, Florini *et al.*, 1991, Coolican *et al.*, 1997, Linkhart *et al.*, 1981, Zanou and Gailly, 2013, Jimenez-Amilburu *et al.*, 2013). It is also well established that myogenesis in tissue culture is accompanied by an irreversible withdrawal from the cell cycle resulting in commitment of post-mitotic myoblasts to fusion and formation of multinucleated myotubes (Zammit *et al.*, 2006a). The local concentration of growth factors supplied endogenously (by interacting cells) or exogenously (recombinant protein addition) is therefore critical for maintaining cells in a proliferative state or, alternatively, inducing differentiation (Florini *et al.*, 1991). HGF, a key growth factor during myogenesis, has been shown to regulate satellite cell activation, proliferation and differentiation (Anastasi *et al.*, 1997, Gal-Levi *et al.*, 1998, Yamada *et al.*, 2010). However, several discrepancies have arisen with regard to the effect of particular HGF doses on myogenesis (O'Blenes *et al.*, 2010, Yamada *et al.*, 2010, Anastasi *et al.*, 1997, Gal-Levi *et al.*, 1998, Bandow *et al.*, 2004). This may be due to differences in the cell species used or in divergent extracellular environments, leading to changes in the type of intracellular signalling pathway activated. Furthermore, very few studies have investigated the expression of the c-Met receptor in response to HGF during myoblast proliferation and differentiation. In the present study we have investigated the effect of HGF on proliferation and differentiation in murine C2C12 myoblasts. Furthermore, we determined the expression of c-Met in response to HGF under these conditions and then attempted to understand the signaling mechanisms at play by selectively inhibiting specific signalling pathways downstream of c-Met.

In response to HGF we observed a dose-dependent effect in mouse C2C12 skeletal myoblasts. In essence, the lower HGF concentration (2 ng/ml) significantly increased cell number (promoting a proliferative response) while decreasing myogenic commitment and subsequent differentiation and fusion. In contrast, a five-fold higher concentration of HGF (10 ng/ml) had the opposite effect, with a decrease in cell number and an increase MyHC expression, resulting in increased fusion. This is the first study to investigate the dose-dependent effect of HGF on both proliferation and differentiation in mouse skeletal

myoblasts and demonstrates a complementary dose-dependent regulatory system coordinated by HGF.

The premise that HGF plays a dual role in regulating myogenic proliferation and differentiation is not new. HGF has been well documented in activating quiescent satellite cells to proliferate and differentiate (Miller *et al.*, 2000, Allen *et al.*, 1995, Tatsumi *et al.*, 1998). In a study by Yamada *et al*. (2010), satellite cells were observed to respond to high concentrations of HGF (10-500 ng/ml) by increasing myostatin protein expression and secretion; this was accompanied by a decreased cell proliferation and MyoD expression in primary cultures suggesting a re-entry into a quiescent state. Yamada *et al*. (2010) hypothesize that this re-entry into quiescence could be via the observed increase in myostatin expression. This was proposed as neutralising antibodies (against myostatin) allowed cells to re-express MyoD and myogenin, even when incubated with higher concentrations of HGF (Yamada *et al.*, 2010). In response to lower HGF concentration (2.5 ng/ml), BrdU incorporation was observed to increase, suggesting elevated proliferation; interestingly myogenin mRNA expression levels were reduced in response to both 2.5 ng/ml and 500 ng/ml HGF compared with control, suggesting decreased induction of differentiation. In our present study, we demonstrate a decrease in myoblast cell number in response to 10 ng/ml HGF and a decrease in Myosin Heavy Chain expression in response to 2 ng/ml HGF; this is consistent with the observations of Yamada *et al.* (2010). However, in addition, 10 ng/ml HGF was observed to increase terminal differentiation as seen by the increased Myosin Heavy Chain expression (day 5). This has not previously been demonstrated. Gal-Levi *et al.* (1998) observed a decrease in MyHC expression levels with increasing HGF concentrations in chicken satellite cells and a myogenic C2 cell line. This is not in agreement with our current study, however expression was determined over a 2 day period with HGF concentrations of 20 and 50 ng/ml, exceeding our highest HGF dose. It can therefore be hypothesized, that a temporal increase in extracellular concentrations of HGF (as would be experienced post-injury) is key in the modulation of satellite cell activation and subsequent differentiation. Together, these results suggest an intriguing dose-dependent regulatory effect of HGF on the balance between quiescence and activation as well as proliferation and differentiation.

HGF binds to the c-Met receptor, a membrane-bound, disulfide-linked heterodimer with an intracellular tyrosine kinase domain, found on both quiescent and activated satellite cells

(Giordano *et al.*, 1989, Cornelison and Wold, 1997). Results by Leshem *et al.* (2002) have shown that coupling of c-Met with Grb2 is required for inhibition of muscle differentiation mediated by HGF. This inhibition occurred only when Phosphatidylinositol 3-Kinase (PI3K) signalling downstream of c-Met was low, suggesting that increased coupling of PI3K to c-Met would lead to an up-regulation of muscle regulatory factors, such as MyoD, thereby promoting cell differentiation (Leshem *et al.*, 2002, Maina *et al.*, 2001). This supports our observed increase in c-Met expression levels at the onset of myogenic commitment (day 1) in response to 10 ng/ml HGF. Our findings suggest that subsequent dose-dependent regulation of differentiation by HGF is possibly correlated with total c-Met receptor level expression with an observed significant drop in c-Met receptor levels in samples incubated with 2 ng/ml HGF at day 1 and day 2. Although HGF is the dominant binding factor to c-Met, there are a multitude of signalling adaptors (Grb2, Gab) and cell surface co-receptors, such as CD44, ICAM-1 and several integrin's, which mediate biological responses unique to c-Met (Organ and Tsao, 2011). Studies in epithelial and cancer cells have shown that in this way, despite constant c-Met expression levels, HGF can elicit differential effects via downstream signalling mediators (Organ *et al.*, 2011, Hammond *et al.*, 2010).

Potential downstream pathways that have been suggested to mediate the dose-dependent effect of HGF on myogenesis include PI3K, p38, JNK and ERK (Halevy and Cantley, 2004). Furthermore, PI3K activity is required for HGF-induced MAPK activation, adding an additional layer of complexity (Halevy and Cantley, 2004). We attempted to glean a better understanding of the mechanisms involved in the dose-dependent effect of HGF on C2C12 proliferation and differentiation by utilizing specific inhibitors against some of the above-mentioned pathways.

We showed that the addition of LY294002 decreased C2C12 myoblast fusion, irrespective of HGF dose. Our findings were supported by Jiang *et al* (1999) and Sumitani *et al* (2002) who reported LY294002, and resulting inhibition of Akt, interfered with myotube formation and the expression of muscle-specific proteins such as MyoD and MyHC (Jiang *et al.*, 1999, Li *et al.*, 2000, Sumitani *et al.*, 2002). Furthermore, we showed that addition of 2.5 μM LY294002 to proliferating C2C12 cells treated with either 2 ng/ml HGF and 10 ng/ml HGF abrogated the effect of this growth factor on cell number. This result was expected in part due to the known involvement of the PI3K/AKT pathway in myoblast proliferation, survival and regulation of apoptosis (Mandl *et al.*, 2007).

We observed that the addition of PD98059 reduced the negative effect of 2ng/ml HGF, on differentiation, confirming that the ERK signaling pathway may mediate, at least in part, the inhibitory effect of HGF on myogenesis (Yang *et al.*, 2006). Our study also showed that PD98059 modulated the pro-proliferative effect of 2 ng/ml HGF on C2C12 myoblasts. IGF-1 has been observed to activate the ERK pathway and lead to increased proliferation of mouse primary culture myoblasts (Madhala-Levy *et al.*, 2012). This, together with our results strongly suggests that the pro-proliferative effect of 2 ng/ml HGF is regulated, at least in part, by the ERK pathway. It has also been demonstrated that PI3K activity is required for HGF-induced MAPK activation; this adds an additional layer of complexity and may explain why LY294002 is able to affect both pro- and anti-proliferative pathways in myoblasts (Halevy and Cantley, 2004).

Li *et al* (2000) had also previously shown that p38 inhibition prevents myogenic differentiation (Li *et al.*, 2000). Similarly, our results showed that SB205380 reduced myotube formation further supporting the notion that p38 is vital for effective C2C12 differentiation. However, as with LY294002, the inhibition was independent of HGF. In our proliferation studies, SB203580 was able to negate both the positive and negative effects of HGF on cell numbers. In support of our findings, Jones *et al* (2005) found that p38 is vital to the activation of primary culture satellite cells and their subsequent proliferation. Inhibition of p38 with SB203580 drove MM14 cells towards a quiescent-like state where they exit the cell cycle, but fail to differentiate (Jones *et al.*, 2005). These results underscore the importance of keeping in mind that changes in cell number are determined by numerous contributing factors, not proliferation alone.

We showed that inhibition of JNK with SP600125 abolished the pro-proliferative effect of 2 ng/ml HGF and prevented C2C12 fusion, regardless of HGF treatment. Interestingly, cell viability was reduced when JNK was inhibited with SP600125, further supporting JNKs' well-documented role in cell survival. These results suggest that not only does the inhibition of JNK mediate HGF-induced proliferation, but also that this effect is compounded by a reduction in cell viability. A schematic representation of our findings is shown in Figure 2.7.

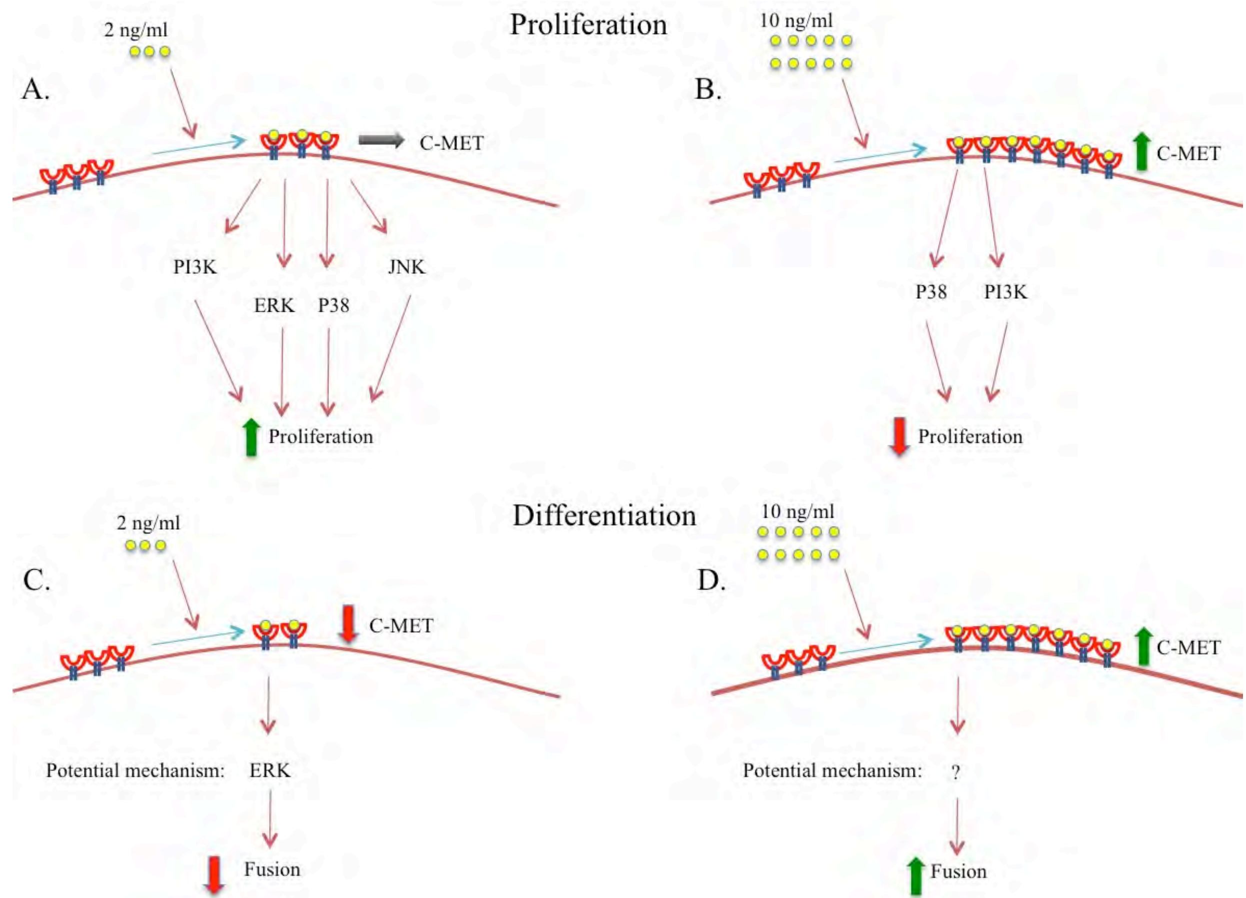


Figure 2.7: Schematic representation of our findings. A) 2 ng/ml HGF promotes proliferation in C2C12 myoblasts through the PI3K, JNK, ERK and p38 pathways. We have shown that inhibition of these pathways reverses the pro-proliferative effect of 2 ng/ml HGF on C2C12 cells. **B**) 10 ng/ml HGF inhibits proliferation in C2C12 myoblasts through the PI3K and p38 pathways. We have shown that inhibition of these pathways reverses the anti-proliferative effect of 10 ng/ml HGF on C2C12 cells. **C**) 2 ng/ml HGF inhibits fusion in C2C12 myoblasts. **D**) 10 ng/ml HGF promotes fusion in C2C12 myoblasts.

In conclusion, our findings support a role for HGF in regulating both myoblast cell number and terminal differentiation in a dose-dependent manner (Figure 2.7). However, we further suggest that not only does HGF regulate these processes, but that proliferation and differentiation in C2C12 mouse myoblasts can be either promoted *or* inhibited in response to changing exogenous HGF concentrations. Changes in c-Met receptor expression were noted in response to HGF and the dose-dependent effects of the growth factor on proliferation and differentiation could be correlated to expression levels of this receptor. Furthermore, our results suggest that P13K and p38 mediate the anti-proliferative effect of the higher dose HGF, whereas the ERK signalling pathway is, at least in part, responsible for the negative effect of the lower HGF dose on differentiation.

CHAPTER 3

COLLAGEN IV PROMOTES MYOBLAST FUSION IN A CD9-DEPENDENT MANNER

It is becoming increasingly apparent that the extracellular matrix (ECM) of skeletal muscle, in addition to providing structural support, provides regulatory cues to the resident satellite cell population and thereby modulates activation and subsequent myogenesis. Satellite cells reside between the basal lamina and sarcolemma where they interact with matrix factors of their niche. Collagen IV and laminin, the major components of the basal lamina, bind to and interact with satellite cells via integrin's and other cell surface proteins. Of particular interest to us is CD9, a tetraspanin transmembrane protein expressed on myoblasts and known to interact with collagen IV. In the current study we first analyzed the effect of Matrigel (an exogenous mixture of factors that simulates the basal lamina ECM), as well as its two main constituents, laminin and collagen IV, on fusion. We then analyzed the role of key myogenic transcription factors as well CD9 in an effort to understand the mechanism underlying the effects observed. Matrigel and collagen IV (the second largest constituent of Matrigel), but not laminin, significantly increased terminal fusion of C2C12 myoblasts. Collagen IV was observed to significantly increase percentage MyoD^{+} cells without affecting Pax7^{+} cell numbers. Furthermore collagen IV stimulated an increase in CD9 expression on differentiating cells such that cells cultured on collagen IV required higher levels of neutralizing anti-CD9 monoclonal antibodies to reduce fusion. These results identify the interaction of collagen IV with CD9 as a critical mediator of skeletal muscle myogenesis.

Includes data from:

Walker NL and Niesler CU. Collagen IV promotes myoblast fusion in a CD9-dependent manner [Contributed all data and analysis of figures in the paper]. Submitted, Matrix Biology; 30 November 2015.

3.1 Introduction

Satellite cells reside in quiescence in their niche between the basal lamina and the sarcolemma of a muscle fiber (Mauro, 1961). The extracellular matrix of the satellite cell niche is known to regulate stem cell self-renewal and differentiation *in vivo,* however, the mechanism by which it does so is unclear (Dellatore *et al.*, 2008). The importance of the niche ECM in stem cell behavior is outlined by the fact that disruption of its components results in defective regeneration in the majority of stem cell types in the body (Jones and Wagers, 2008). In addition to providing structural support to the niche, the ECM plays a vital role in mediating the availability of growth factors, thereby regulating stem cell quiescence and activation (Thomas *et al.*, 2015). The complexity of the *in vivo* niche makes it difficult to fully understand the roles of individual components, therefore individual ECM factor and simple combination studies are required. In this study we examine the role of collagen IV, the major collagen component of the basal lamina, on myoblast behavior.

Collagen IV is essential for basement membrane stability and serves as the main scaffold material of the niche ECM (Poschl *et al.*, 2004). Through molecular interactions with integrins, collagen IV is able to influence cell fate in developing and adult tissues (Khoshnoodi *et al.*, 2008). Embryoid body formation is the principle step in the differentiation of embryonic stem cells and it has been shown that collagen IV (10 µg/ml) supports this formation (Taru Sharma *et al.*, 2012). Schenke-Layland *et al* (2007) demonstrated that collagen IV was able to direct early embryonic stem cell differentiation towards smooth muscle lineages in both mouse and human embryonic stem cells (Schenke-Layland *et al.*, 2007). With regard to skeletal muscle, collagen IV is found exclusively in the basement membrane (Foidart *et al.*, 1981). Recent studies have shown that collagen IV is able to increase migration and viability in insulin like growth factor I (IGF-1) gene engineered C2C12 cells (Ito *et al*., 2015). Collagen IV also significantly increased fusion and the number of myotubes displaying striations in samples stimulated to express IGF-1, as well as increasing the displacement of electrical pulse stimulated myotubes (Ito *et al*., 2015). This suggests that collagen IV can enhance the contractile ability of myoblasts *in vitro.*

Laminins are a group of basal lamina proteins and are composed of a heavy α-chain and two light chains, β and γ. Laminin-211 (merosin) is the subtype found around the sarcolemma of muscle fibers and is known to bind to collagen IV in the basal lamina (Grounds *et al.*, 2005).

Laminin-211 deficiency is well documented in congenital muscle dystrophies (Guo *et al.*, 2003). Laminin-111, which differs from laminin-211 only in its alpha domain, has been shown to promote the migration of C2C12 cells *in vitro* (Yao *et al.*, 1996). However the effect of laminin-111 on the differentiation of C2C12 cells remains a topic of debate. Grossi *et al* (2007) utilized a magnetic bead stimulation model whereby beads were coated with 10 µg/ml laminin-111 and allowed to interact with differentiating C2C12 cells in a magnetic field. This method supplied both chemical stimulation via laminin interactions and mechanical stimulation via magnetic bead contact. They observed a 1.5 fold increase in MyoD and myogenin expression when compared to mechanical stimulation with uncoated beads. However, this study revealed that incubation of C2C12 cells on plates coated with 10 µg/ml laminin-111 without mechanical stimulation did not result in increased myotube formation (Grossi *et al.*, 2007). This suggests that, in a 2D environment, signaling through the laminin receptor alone is not enough to increase C2C12 differentiation. This is in contrast to Foster *et al* (1987) who showed that laminin-111 enhances skeletal myogenesis when rat hindlimb tissue was cultured on plates coated with 10 µg/ml laminin-111. Here, whole muscle fibers were isolated and injured, and improved wound healing was observed *in vitro*. It was however concluded that this effect was due to the selective promotion of myoblast proliferation by laminin rather than increased myogenic potential of the myoblast population (Foster *et al.*, 1987).

Matrigel, an exogenous mixture of proteins secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, closely mimics the components of the basal lamina. Matrigel is commonly utilized to improve the proliferative and myogenic potential of isolated primary culture myoblasts. Langen *et al* (2003) demonstrated that by merely reducing the media serum component and culturing C2C12 cells on Matrigel, significantly reduced C2C12 proliferation and improved myogenesis can be achieved when compared to protocols involving the use of horse serum on uncoated plates (Langen *et al.*, 2003). Furthermore, it has been shown to improve the fusion of C2C12 myoblasts into myotubes *in vitro* (Grefte *et al.*, 2012). Grefte *et al* (2012) differentiated primary culture myoblasts isolated from Sprague-Dawley rats on 1 mg/ml Matrigel and observed 35% increase in fusion when compared to non-treated controls after just three days. Fusion reached 50% by day 5 cultured on Matrigel (Grefte *et al.*, 2012).

CD9 is a member of the tetraspanin family of transmembrane proteins and is known to bind to collagen IV (Tachibana and Hemler, 1999). Tetraspanins, also known as the transmembrane 4 superfamily, are believed to act as a molecular scafford with a role in binding specific proteins to one another on the cell surface (Hemler, 2005). Blocking of CD9 using neutralizing monoclonal antibodies has been shown to delay the fusion of C2C12 cells into myotubes (Tachibana and Hemler, 1999). Furthermore, Charrin *et al* (2013) have shown that disruption of CD9 expression in knockout mice results in disorganized and abnormal muscle regeneration characterized by the formation of large dystrophic muscle fibers (Charrin *et al.*, 2013). This suggests that CD9 is vital for the efficient progress of myogenesis.

In the current study we determined whether the pro-myogenic effects of Matrigel can be ascribed to either one of its major components, collagen IV or laminin-111. We then examined potential mechanisms for the effects observed, notably by analyzing myogenic transcription factor and CD9 expression. Our findings suggest a novel role for CD9 in mediating the pro-myogenic effects of collagen IV on myoblasts.

3.2 Materials and methods:

All reagents were of an analytical grade and obtained from Sigma-Aldrich (USA), unless stated otherwise. All cell culture was performed under sterile conditions in a level II biological safety cabinet (ESCO Class II BSC).

3.2.1 Cell Culture

The C2C12 murine cell line was purchased from the American Type Culture Collection (ATTC, CRL-1772). Cells were cultured in Dulbecco's Modified Eagle's Medium (Highveld Biological, CN3193-9) supplemented with 10% (v/v) Fetal Bovine Serum (Biowest, S181H-500), 2% (v/v) L-glutamine (Lonza, BE17-605E) and 2% (v/v) Penicillin-Streptomycin (Lonza, DE17-602E). For differentiation studies, cells were cultured to a confluence of 70% after which media was changed to differentiation media (DMEM supplemented with 2% (v/v) horse serum) (Biowest, S090H-100).

3.2.2 Differentiation

Differentiation media consists of 485 ml DMEM, 10 ml PenStrep (2% v/v) and 5 ml horse serum (16050-130; Invitrogen; USA) filter sterilized prior to storage in 50 ml falcon tubes at 4°C. When cells reached approximately 70% confluence, the media was changed to differentiation media. Cells were maintained at 37 °C, 5% CO_2 in a humidified incubator.

3.2.3 ECM Coating

T25 culture flasks and 24 well plates containing glass coverslips were coated with 3 ml and 500 µl respectively with the solutions described below. All plates were incubated at 37 °C for 4 hours, before the protein solutions were removed and the plates allowed to air dry under U.V. light overnight. Plates and wells are washed with sterile PBS before use and stored at 4 °C for no longer than a month.

3.2.3.1 Collagen IV Coating

Collagen IV solution (Human; 0.3 mg; C6745; Sigma-Aldrich; USA) was diluted to a stock solution of 0.1 mg/ml with sterile PBS at 4 °C. This stock was then diluted further to the desired concentration range (12.5, 25 and 50 μg/ml) with sterile PBS.

3.2.3.2 Laminin-111 Coating

Laminin solution (Mouse; 1 mg; L2020; Sigma-Aldrich; USA) was diluted to a stock solution of 0.1 mg/ml with sterile PBS at 4 °C. This stock was then diluted further to the desired concentration range (6.25, 12.5 and 25 μg/ml) with sterile PBS.

3.2.3.3 Matrigel Coating

A cold 10.1 mg/ml Matrigel solution (CN-354230; BD Biosciences; USA) was diluted to a stock concentration of 0.6 mg/ml in double distilled water. This stock was then diluted further to the desired concentration range (30, 60 and 120 μg/ml) with sterile PBS. All dilutions and coating procedures were done on ice.

A Clustal W alignment was conducted to compare the amino acid sequences of human collagen IV used in this study and collagen IV found in mice (*Mus Musculus*) (Appendix III). A score of 79.2899 showed that the amino acid sequence of human collagen IV is highly similar to mouse collagen IV.

3.2.4 Immunocytochemistry and Confocal Microscopy

C2C12 cells were plated onto coverslips in a 24 well plate and allowed to reach 70% confluence in 500 μl growth media. Wells had previously been coated with the ECM factors as described earlier. HGF addition took place with initial plating out and at all media changes (day -1, 0 and 3). Differentiating (day 1, day 2 and day 5) cell samples were cultured in differentiating media for 24, 48 and 120 hours respectively. Coverslips were subsequently washed with PBS and fixed with 4% paraformaldehyde for 15 minutes. They were then washed with PBS and blocked using 5% donkey serum. Coverslips were then incubated with primary antibodies for 4 hours at room temperature: day 0/1: mouse monoclonal anti-Pax7, mouse monoclonal anti-CD9; day 2: rabbit polyclonal anti-MyoD; day 5: mouse monoclonal anti-MyHC, mouse monoclonal anti-CD9 (see table 3.1 for optimized dilutions). Coverslips

were then washed 4 x 8 minutes with PBS before being incubated with appropriate secondary antibody for 40 minutes (Table 3.1). These were washed 3 x 8 minutes with PBS before being treated with Hoechst 33342 nuclear stain (Stock: 10 mg/ml; 1/4000; Sigma-Aldrich; USA). Coverslips were then washed for a further 4 x 8 minutes with PBS before being mounted with Moviol and finally visualized using the Zeiss LSM 710 confocal microscope.

Table 3.1: Antibody concentrations (optimized) used in Confocal microscopy

	Primary antibody	Dilution	Secondary antibody	Dilution
Confocal Microscopy	Mouse monoclonal anti-Pax7 (ab55494; Abcam; UK)	1/500	Donkey anti-mouse Dylight 488 (715-485-151, Jackson ImmunoResearch)	1/1000
	Rabbit polyclonal anti-MyoD (sc-760, Santa Cruz; USA)	1/100	Donkey anti-rabbit Dylight 488 (711-455-152, Jackson ImmunoResearch)	1/1000
	Mouse monoclonal anti-MyHC (MF-20; DSHB; USA)	1/400	Donkey anti-mouse Dylight 494 (715-515-151, Jackson ImmunoResearch)	1/4000
	Mouse monoclonal anti-CD9 (BD 551808, USA)	1/400	Donkey anti-mouse Dylight 488 (715-485-151, Jackson ImmunoResearch)	1/1000

3.2.4.1 Assessment of percentage Pax7$^+$ and MyoD$^+$ cells

Cells shown to be expressing nuclear Pax7 or MyoD were counted and determined as a percent of total nuclei on proliferating (percentage Pax7$^+$), day 1 (percentage Pax7$^+$) and day 2 (percentage MyoD$^+$) differentiating cultures. Due to the fact that Pax7 is expressed in proliferating myoblasts and down-regulated following induction to differentiate, Pax7 assessment was not conducted beyond day 1 of differentiation (Zammit *et al.*, 2006b). Additionally, MyoD expression was examined at day 2 of differentiation as MyoD expression has previously been shown to peak at day 2 of differentiation in C2C12 myoblasts (Panda *et al.*, 2014). Images were examined using Zen software (Zeiss) allowing visualization of a single plane (either Pax7 or nuclear staining) and accurate quantification. Approximately 20 cells per image were analysed and a minimum of 5 fields of view (i.e. 100 cells/n), randomly selected, were used for n=1. Experiments were repeated n = 6-8 times.

3.2.4.2 Assessment of terminal differentiation via a fusion index

The fusion index was determined via quantitative analysis of confocal images of differentiated myotubes (day 5). Images were exported using Zen software (Zeiss) as a single plane with both the red channel (MyHC) and the blue channel (Hoechst). This allowed us to count the number

of nuclei in fused myotubes (containing 2 or more nuclei). This number was divided by the total number of nuclei in the field of view and multiplied by 100. A minimum of 4 fields of view, randomly selected, were used for n=1. Experiments were repeated n = 6-8 times.

3.2.5 Protein separation by SDS-PAGE

Lysate sample preparation is outlined in Appendix IV. The Laemmli protocol was used for SDS-PAGE (Laemmli, 1970) with several modifications outlined in Appendix IV. The BioRad electrophoresis unit (BioRad; Mini-PROTEIN 3 Cell; USA) was utilized for the running of all polyacrylamide gels. 10% or 12.5% gels were cast between two glass plates, as described in the Appendix IV, and allowed to solidify (~1 hour) before the stacking gel was added with a 10 fingered comb to create the lanes for sample loading. Once the stacking gel had set (~30 minutes) the comb was gently removed and lanes washed with distilled water. Electrode (tank) buffer was poured into the inner and outer electrode compartments before the prepared samples were loaded. The samples were prepared by adding an equal volume of reducing sample treatment buffer to the sample and boiling the solution for 2 minutes. Samples were then stored on ice until loaded. 1 µl bromophenol blue (1437500CB; Saarchem; RSA) was added to each sample so that the buffer front could be clearly discerned. 25 µg of protein per lane was added to the gel as determined by the Bradford Assay (Appendix IV). 3 µl of the PeqGold protein marker V (27-2210; Peqlab; Germany) was run in at least one lane of each gel for later determination of protein molecular weights. Gels were run at 4 °C with 18 mA per gel until the buffer front reached the bottom of the running gel. The gel was removed from between the glass plates and either stained with the Coomassie G-250 stain or processed for western blot analysis.

3.2.6 Western Blot analysis of CD9

Cells were differentiated on collagen IV coated and non-coated T25 culture flasks for up to 5 days. Proteins were separated in a 12% SDS-PAGE gel and transferred to nitrocellulose. The nitrocellulose was then blocked in 5% (m/v) low fat milk powder (made up in TBST) for 1 hour. Primary antibody (Mouse monoclonal anti-CD9, BD 551808, USA) was made up in TBST was then added to the nitrocellulose for 2 hours. GAPDH (Cell signalling, 2118) was used as a loading control. This was followed by wash steps with TBST (4 x 8 minutes). All

wash steps were performed with vigorous agitation. HRPO-linked secondary antibody (Rabbit anti-mouse IgG, Dako, P016102, USA) made up in TBST was then added for 1 hour. This was followed by washing with TBST (6 x 8 minutes).

Enhanced Chemiluminescence (ECL) technique was used for the development of the bands. Light film (Kodak BioMax light film; Z370398; Sigma-Aldrich; USA) was utilized. The substrate was made by adding peroxide and enhancer solutions in a 1:1 ratio (Immun-Star WesternC; 70-5070; Bio-Rad, USA). The substrate (1 ml) was added to the nitrocellulose for 2 minutes. Prior to development, the developer (Structurix G128; Agfa; 5TBN; Belgium) and the fixative (G333c Rapid Fixer; Agfa; EGCQT; Belgium) solutions were made up with water in a 1 in 4 dilution and a 1 in 3 dilution, respectively.

3.2.6.1 Densitometry

The bands that developed on the film were analyzed by use of computer software (Image J, http://imagej.nih.gov/ij/). Densitometric analysis was selected and the background was adjusted so that only the bands were visible for all experiments.

3.2.7 Monoclonal antibody blocking

C2C12 myoblasts (15 000 cells/well) were plated in collagen IV-coated and non-coated wells of a flat-bottom 96 well culture plate (Corning, CR/3596) and allowed to reach 70% confluence. Media was then replaced with diffferentiation media containing mouse monoclonal anti-CD9 (BD 551808, USA; 0, 1/1600, 1/800, 1/1600 dilution) and allowed to differentiate for 7 days. Media (containing various dilutions of mAbs) was replaced every 2 days.

3.2.8 Statistical analysis

The results from each experiment were expressed as a Mean ± SEM. The student’s t-test was performed on all data. Samples were considered to be statistically significant if they produced a *p* value of less that 0.05. The number of experimental repeats is specified in each figure legend.

3.3 Results

3.3.1 Matrigel increases terminal fusion of C2C12 myoblasts

Matrigel is a commercially available protein mixture that closely mimics the basal lamina. It is composed mainly of laminin-111 and collagen IV that together make up 90% of its total mass (Yu and Machesky, 2012). To determine the effects of Matrigel on C2C12 cell terminal differentiation, cells were cultured on Matrigel (0, 30, 60 and 120 µg/ml) and the effects on fusion determined by confocal microscopy. Matrigel (30 and 60 µg/ml) visibly increased differentiation (Figure 3.1A); calculation of percentage fusion at day 5 revealed a significant increase from 42 ± 3% (control) to 65 ± 3% (30 µg/ml, $p < 0.005$) and 66 ± 2% (60 µg/ml, $p < 0.005$) (Figure 3.1B). This confirms the ability of Matrigel to positively modulate terminal differentiation.

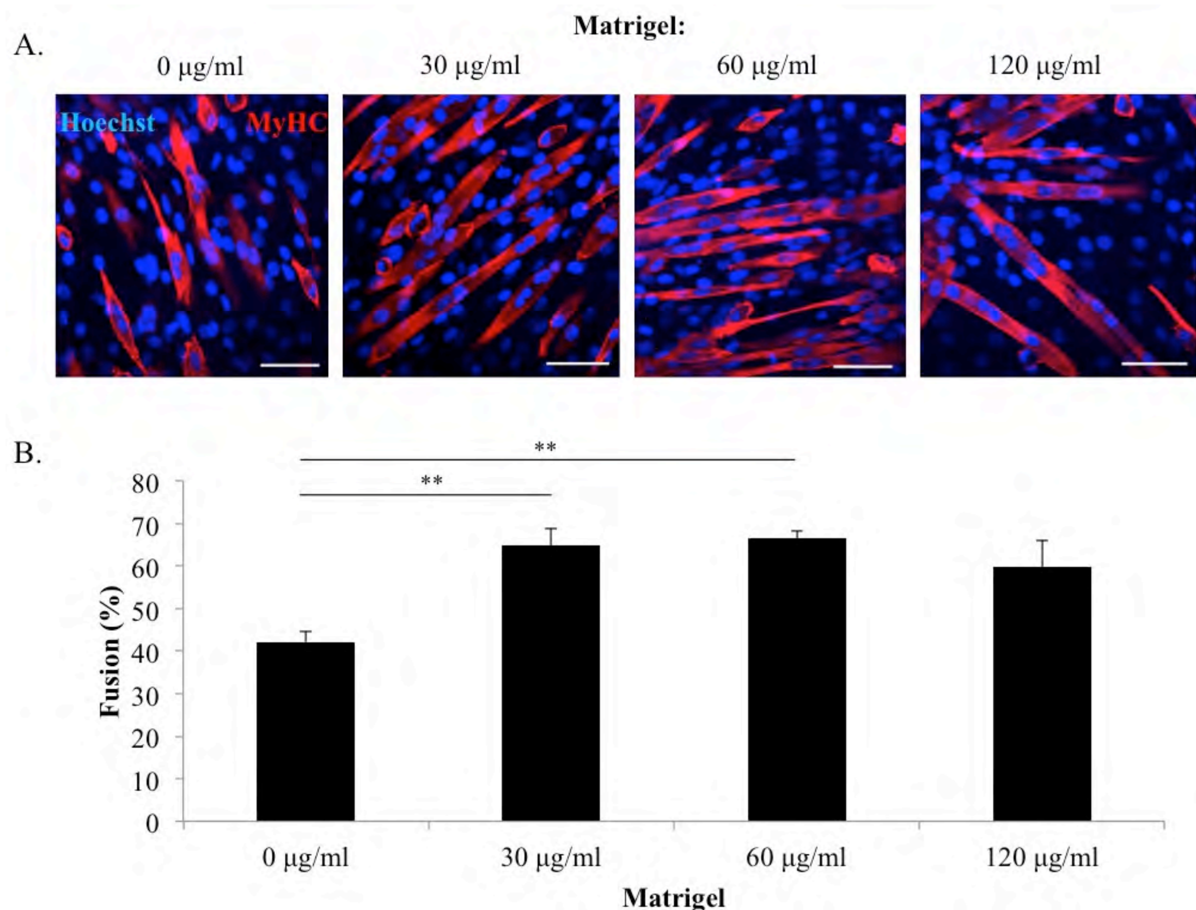


Figure 3.1: C2C12 myoblast differentiation in response to Matrigel. C2C12 myoblasts were incubated in growth media on Matrigel-coated glass coverslips (0, 30, 60 and 120 µg/ml) and allowed to reach 70% confluence. Cells were then induced to differentiate for 5 days prior to fixation. **A**) Representative images of differentiated C2C12 cells showing MyHC (red) and nuclei (blue). **B**) Fusion index calculated as percent C2C12 myotube formation at day 5 of differentiation. Scale bar represents 50 µm. N = 6, all figures represent mean ± SEM, * = p < 0.05.

3.3.2 C2C12 cells down-regulate Pax7 expression in response to differentiation cues

Activated, proliferating myoblasts express the paired-box transcription factors Pax7 and Pax3, as well as MyoD and myogenin (Le Grand and Rudnicki, 2007). Pax7 is known to play a role in the self-renewal pathway, which is responsible for ensuring a constant population of satellite cells in the tissue. It is expressed in both quiescent and activated satellite cells, but is down-regulated upon differentiation (Relaix *et al.*, 2006). Down-regulation of Pax7 results in a up-regulation in MyoD as cells begin to align for differentiation (Olguin *et al.*, 2007). A subsequent down-regulation of MyoD and up-regulation of myogenin and MyHC are the final molecular steps in differentiation of satellite cells into myotubes (Olguin *et al.*, 2007). By examining the expression of these proteins in a population of cells, deductions regarding their progression of differentiation can be made. Maintained Pax7 expression has been shown to inhibit differentiation by interfering with the expression of critical myogenic transcription factors such as MyoD and myogenin (Olguin *et al.*, 2007, Zammit *et al.*, 2006b). In our study, differentiation cues visibly reduced the number of Pax7$^+$ cells when compared to proliferating cells (Figure 3.2A). When quantified, under proliferative conditions 26 ± 5% of C2C12 myoblasts express nuclear Pax7 (Figure 3.2B). In response to differentiation media (24h) there was a significant decrease in the percentage Pax7$^+$ cells to 12 ± 4% ($p < 0.035$) (Figure 3.2B).

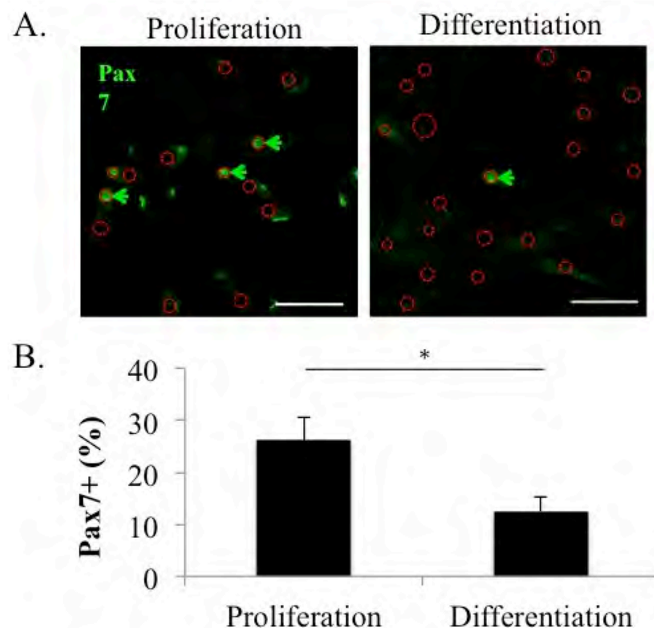


Figure 3.2: Percentage Pax7$^+$ cells in proliferating versus differentiating C2C12 cells. C2C12 myoblasts were plated onto glass coverslips in growth medium and allowed to reach 70% confluence. Cells were then either fixed for confocal microscopy or induced to differentiate for 1 day prior to fixation. Nuclear Pax7 expression was observed and expressed as a percentage of total cells. **A**) Representative images of C2C12 cells stained with anti-Pax7 antibodies under proliferative conditions and after day 1 of differentiation. **B**) Graph showing percentage Pax7$^+$ myoblasts under proliferative conditions and at day 1 of differentiation. Green arrows show Pax7$^+$ cells. Red outlined circles represent nuclei. Scale bar represents 20 µm. N = 24, all figures represent Mean ± SEM, * = p < 0.05.

3.3.3 Matrigel does not affect Pax7 expression in differentiating C2C12 myoblasts

To determine the effects of Matrigel on differentiating C2C12 Pax7 expression, C2C12 cells were cultured on Matrigel (0, 30, 60 and 120 µg/ml) and the effects on Pax7 expression determined by confocal microscopy. Pax7 expression appeared similar when Matrigel treated samples were compared to untreated controls (Figure 3.3A) and quantification revealed no significant changes in percentage $Pax7^{+}$ cells (Figure 3.3B). Percentage Pax7 levels remained between 13 and 18% ± 4% for all treatments.

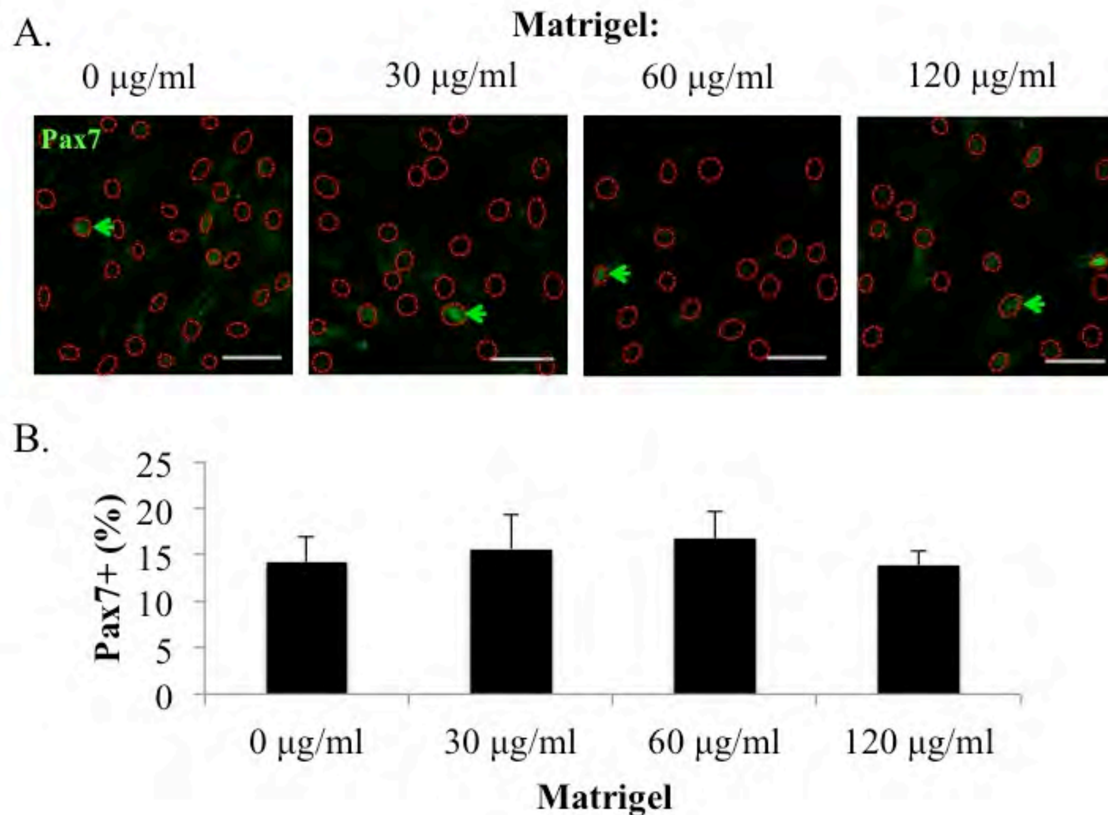


Figure 3.3: Effect of Matrigel on Pax7 expression of C2C12 myoblasts. C2C12 myoblasts were plated in growth media on Matrigel-coated glass coverslips (0, 30, 60 and 120 µg/ml) and allowed to reach 70% confluence. Cells were then induced to differentiate for 1 day prior to fixation. **A**) Representative images of C2C12 cells stained with anti-Pax7 antibodies. **B**) Graph showing percentage $Pax7^{+}$ myoblasts at day 1 of differentiation. Red outlined circles represent nuclei. Green arrow shows $Pax7^{+}$ cells. Scale bar represents 20 µm. N = 6, all figures represent mean ± SEM.

3.3.4 Matrigel does not affect MyoD expression in differentiating C2C12 myoblasts

To determine the effects of Matrigel on C2C12 commitment to differentiation, C2C12 cells were cultured on Matrigel (0, 30, 60 and 120 µg/ml) and the effects on MyoD expression determined by confocal microscopy. MyoD expression appeared visibly similar when Matrigel treated samples were compared to untreated controls (Figure 3.4A) and quantification revealed no significant changes in percentage MyoD $^{+}$ cells (Figure 3.4B). MyoD^{+} cell numbers remained between 45 and 51% ± 16% for all treatments.

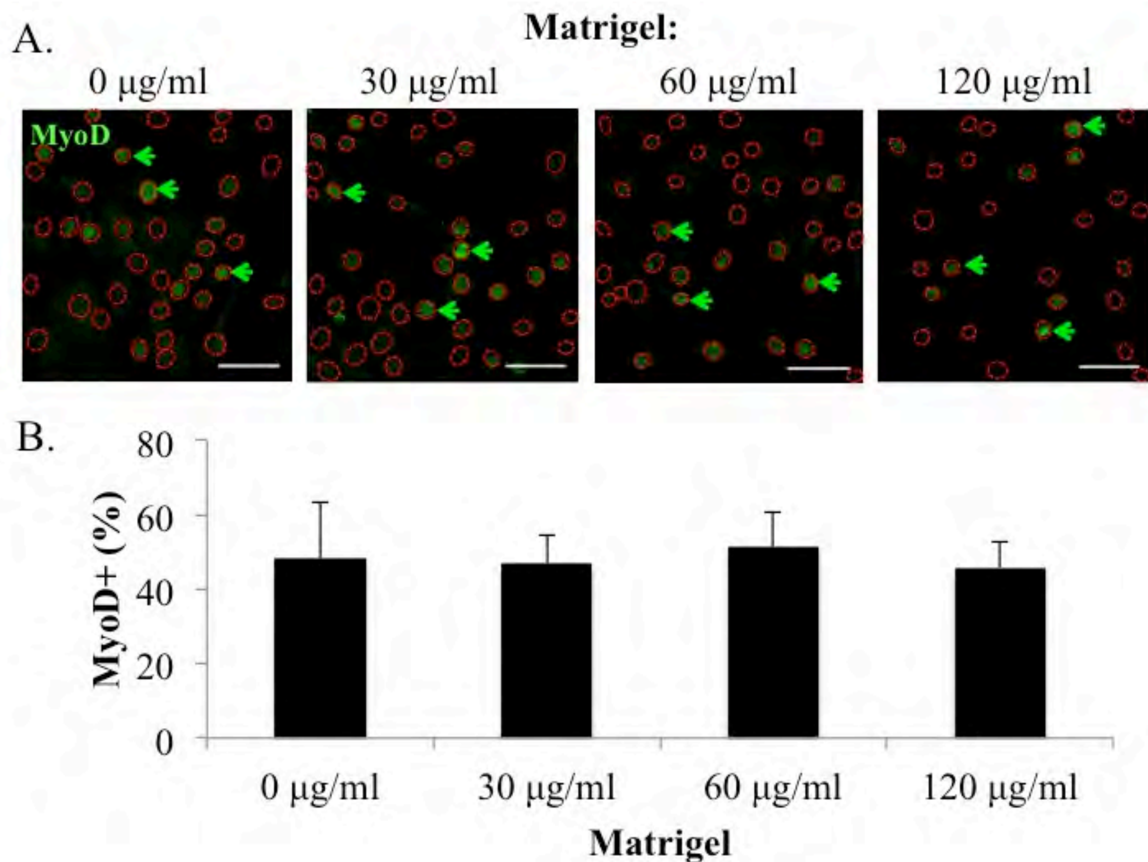


Figure 3.4: Effect of Matrigel on myogenic commitment of C2C12 myoblasts. C2C12 myoblasts were plated in growth media on Matrigel-coated glass coverslips (0, 30, 60 and 120 µg/ml) and allowed to reach 70% confluence. Cells were then induced to differentiate for 2 days prior to fixation. **A**) Representative images of C2C12 cells stained with anti-MyoD antibodies. **B**) Graph showing percentage MyoD^{+} myoblasts at day 2 of differentiation. Red outlined circles represent nuclei. Green arrow shows MyoD^{+} cells. Scale bar 50 µm. N = 6, all figures represent mean ± SEM.

3.3.5 Laminin does not affect fusion in differentiating C2C12 myoblasts

Matrigel's largest constituent, providing over 60% of its mass, is laminin (Haber *et al.*, 1988). To determine whether the effect of Matrigel on terminal differentiation was due to its high laminin content, we examined the effects of varying concentrations of laminin-111 (0, 6.25, 12.5 and 25 µg/ml) on C2C12 myoblasts. Laminin-111 did not visibly increase fusion levels (Figure 3.5A). Following quantification, no significant changes were observed in percentage fusion when C2C12 cells were cultured on laminin-111 (Figure 3.5B) when compared to control. Percentage fusion remained between 40 and 45% ± 10% for all treatments. This suggests that the effects observed in response to Matrigel (*section 3.3.1*) are not attributed to laminin-111.

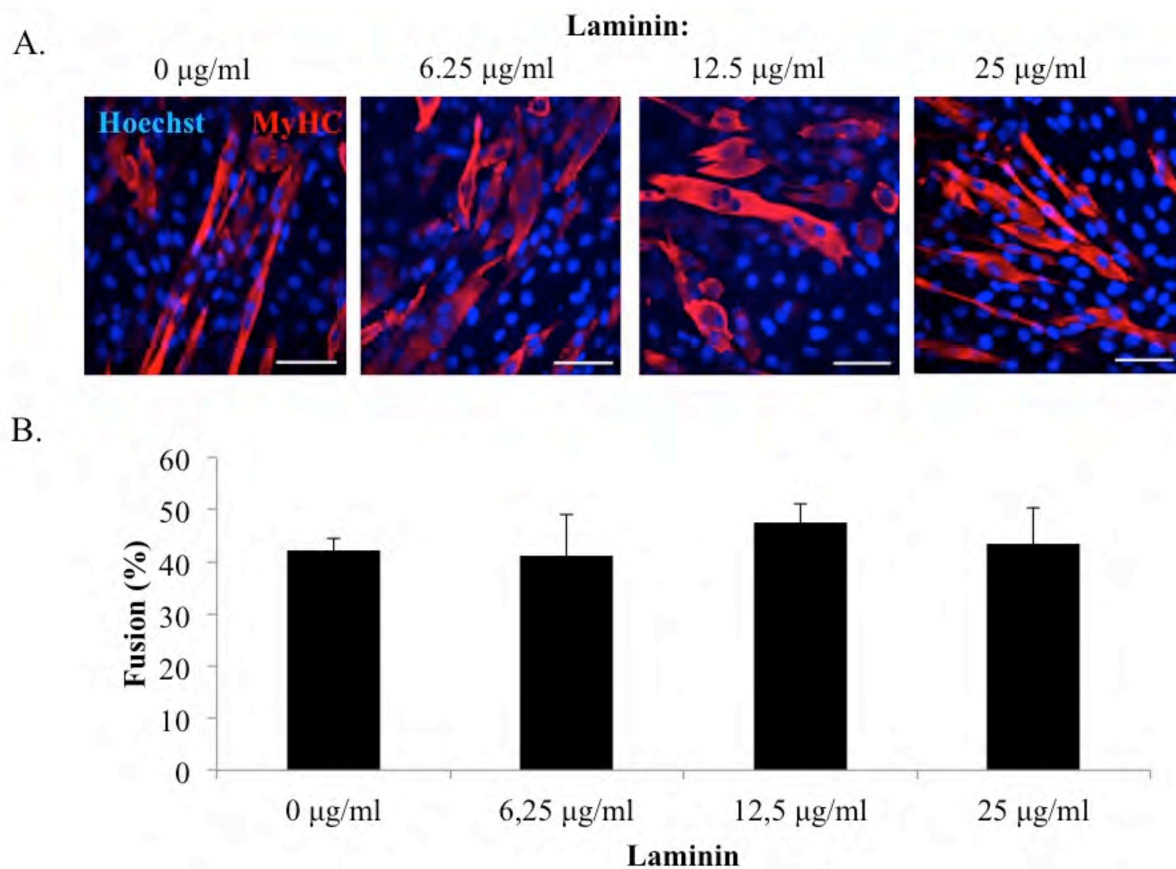


Figure 3.5: The effect of laminin-111 on the differentiation of C2C12 myoblasts. C2C12 myoblasts were plated in growth media on laminin-coated glass coverslips (0, 6.25, 12.5 and 25 µg/ml) and allowed to reach 70% confluence. Cells were then induced to differentiate for 5 days prior to fixation. **A**) Representative images of differentiated C2C12 cells showing MyHC (red) and nuclei (blue). **B**) Fusion index calculated as percentage C2C12 myotube formation at day 5 of differentiation. Scale bar represents 50 µm. N = 6, all figures represent mean ± SEM.

3.3.6 Collagen IV increases fusion of C2C12 myoblasts

Matrigel's second largest constituent, providing approximately 30% of its mass, is collagen IV (Haber *et al.*, 1988). To determine the effects of collagen IV, Matrigel's second largest constituent, on fusion, C2C12 cells were differentiated on varying concentrations of collagen IV (0, 12.5, 25 and 50 µg/ml) and fusion analysed by confocal microscopy. Collagen IV (25 µg/ml) visibly increased terminal differentiation at day 5 (Figure 3.6A) which translated to a significant increase in fusion from 42% ± 3% to 59 ± 6% ($p < 0.05$, Figure 3.6B). This suggests that the effect of Matrigel on myoblast fusion may be mediated, at least in part, by collagen IV.

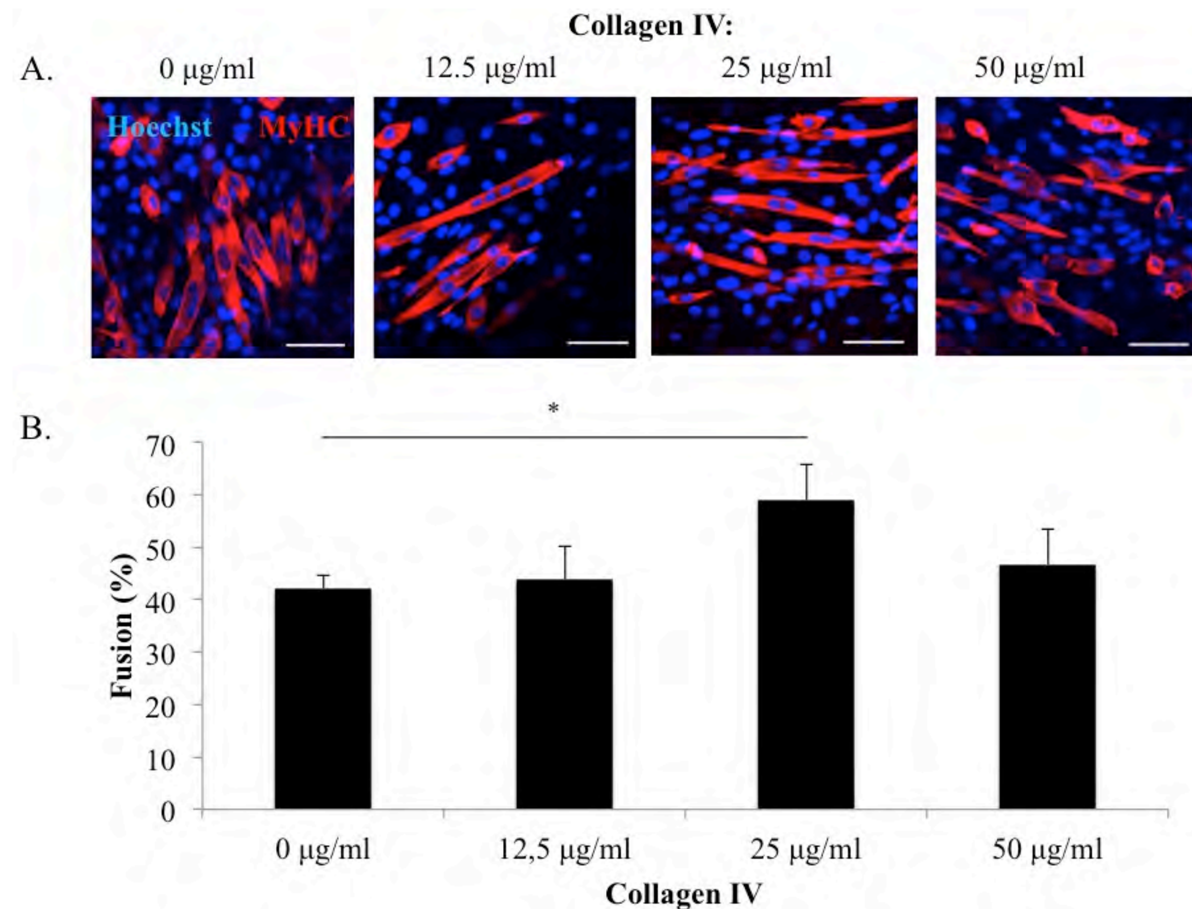


Figure 3.6: The effect of collagen IV on the differentiation of C2C12 myoblasts. C2C12 myoblasts were plated in growth media on collagen IV-coated glass coverslips (0, 12.5, 25 and 50 µg/ml) and allowed to reach 70% confluence. Cells were then induced to differentiate for 5 days prior to fixation. **A**) Representative images of differentiated C2C12 cells showing MyHC (red) and nuclei (blue). **B**) Fusion index calculated as percentage C2C12 myotube formation at day 5 of differentiation. Scale bar represents 50 µm. N = 6, all figures represent mean ± SEM, * = p < 0.05.

3.3.7 Collagen IV does not affect Pax7 expression in differentiating C2C12 myoblasts

To determine whether the effects of collagen IV on fusion (*section 3.3.5*) are mediated by changes in percentage Pax7$^+$ cells, C2C12 cells were differentiated on varying concentrations of collagen IV (0, 12.5, 25 and 50 µg/ml) and analysed by confocal microscopy. Pax7 expression appeared similar when collagen IV treated samples were compared to uncoated controls (Figure 3.7A) and quantification revealed no significant changes in percentage Pax7$^+$ cells (Figure 3.7B). Percentage Pax7 levels remained between 14 and 17% ± 3% for all treatments observed.

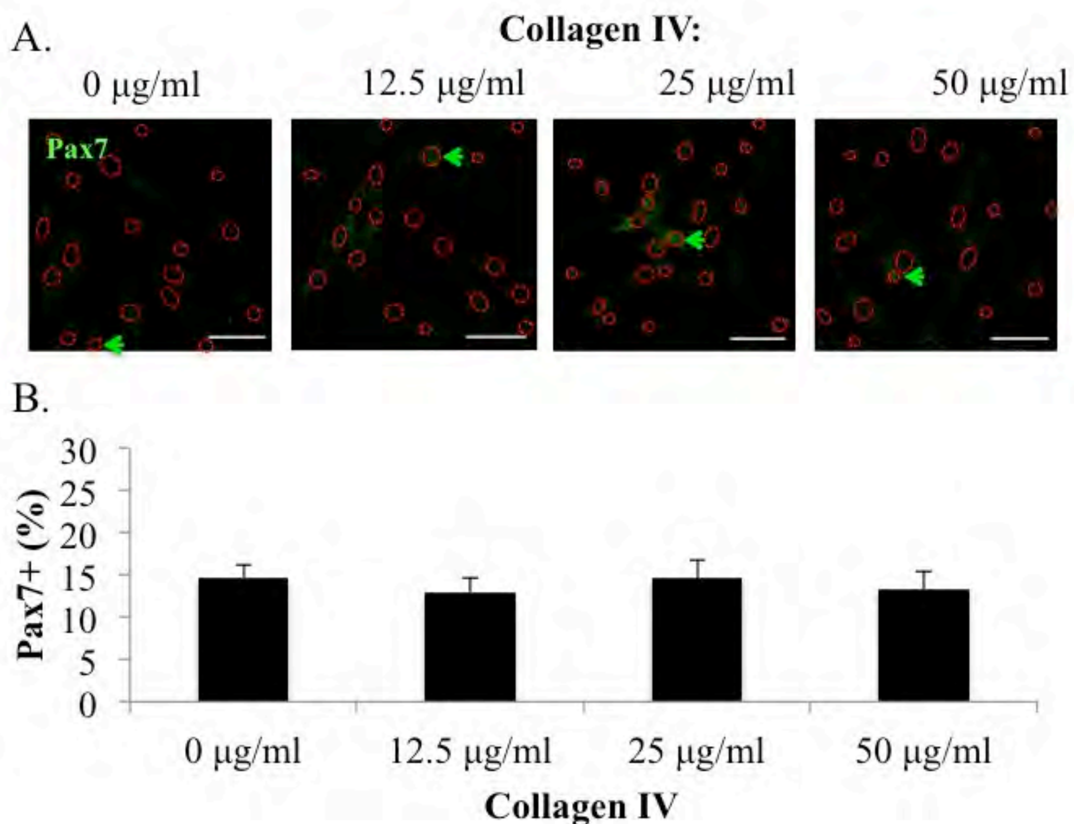


Figure 3.7: Effect of collagen IV on Pax7 expression of C2C12 myoblasts. C2C12 myoblasts were plated in growth media on collagen IV-coated glass coverslips (0, 12.5, 25 and 50 µg/ml) and allowed to reach 70% confluence. Cells were then induced to differentiate for 1 day prior to fixation. **A**) Representative images of C2C12 cells stained with anti-Pax7 antibodies. **B**) Graph showing percentage Pax7$^+$ myoblasts at day 1 of differentiation. Red outlined circles represent nuclei. Green arrow shows Pax7$^+$ cells. Scale bar represents 20 µm. N = 6, all figures represent mean ± SEM.

3.3.8 Collagen IV increases MyoD expression in differentiating C2C12 myoblasts

To determine whether the effects of collagen IV on fusion (*section 3.3.5* are mediated by changes in percentage MyoD$^+$ cells, C2C12 cells were differentiated on collagen IV (0, 12.5, 25 and 50 μg/ml) and analysed by confocal microscopy. Collagen IV (25 μg/ml) was observed to visibly increase the %MyoD$^+$ cells (Figure 3.8A). Quantitative analysis revealed that collagen IV (25 μg/ml) significantly increased %MyoD$^+$ cells from 48% ± 7% to 67% ± 4% MyoD$^+$ cells at day 2 ($p < 0.05$, Figure 3.8B).

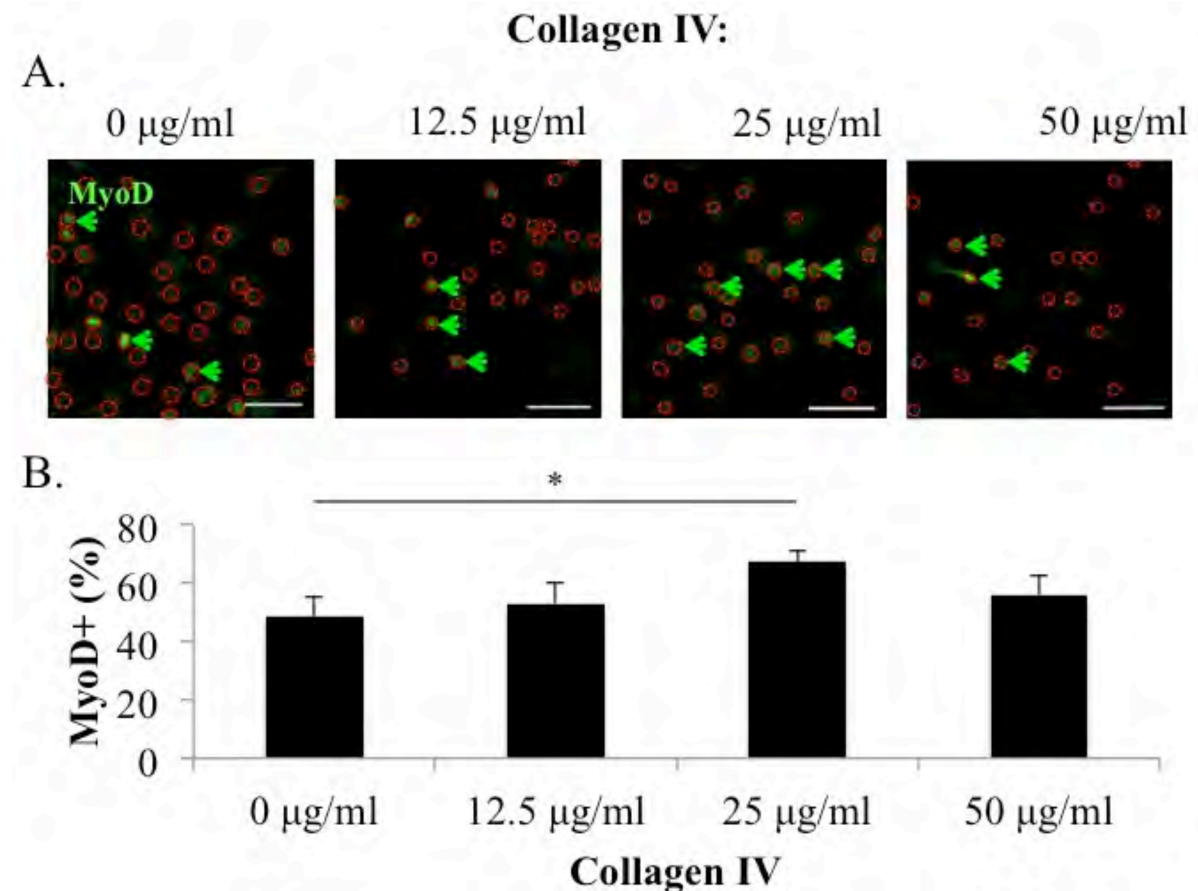


Figure 3.8: Effect of collagen IV on myogenic commitment of C2C12 myoblasts. C2C12 myoblasts were plated in growth media on collagen IV-coated glass coverslips (0, 12.5, 25 and 50 μg/ml) and allowed to reach 70% confluence. Cells were then induced to differentiate for 2 days prior to fixation. **A**) Representative images of C2C12 cells stained with anti-MyoD antibodies. **B**) Graph showing percentage MyoD$^+$ myoblasts at day 2 of differentiation. Red outlined circles represent nuclei. Green arrow shows MyoD$^+$ cells. Scale bar 50 μm. N = 6, all figures represent mean ± SEM, * = p < 0.05.

3.3.9 Collagen IV increases CD9 expression in differentiating C2C12 myoblasts

In order to gain insight into the mechanism by which collagen IV may be mediating its effect on myogenesis, we analysed CD9 expression in differentiating C2C12 cells cultured in the presence or absence of 25 µg/ml collagen IV. In response to collagen IV, CD9 expression was observed to visibly increase when cells were differentiated at both day 1 and day 5 (Figure 3.9A) Densitometric analysis of CD9 expression revealed that at day 1 of differentiation, CD9 expression increased in response to collagen IV by 93% from 100% to 193% relative to control ($p = 0.073$; Figure 3.9B). This increase was maintained and by day 5 of differentiation, CD9 expression levels were significantly higher in cells cultured on collagen IV (145%) compared to control (100%) ($p < 0.05$; Figure 3.9B).

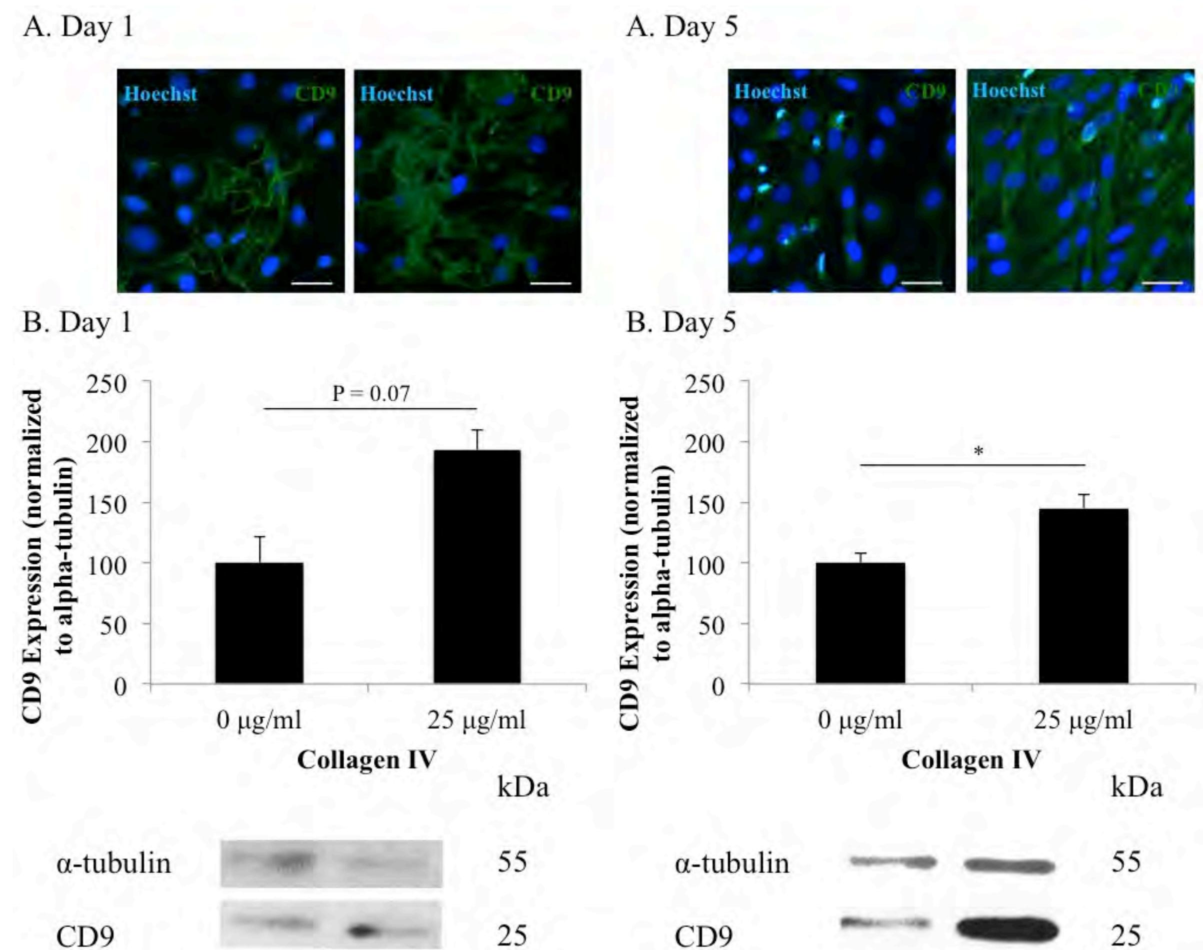


Figure 3.9: C2C12 CD9 expression in response to collagen IV and differentiation cues. C2C12 myoblasts were plated in growth media on collagen IV-coated plates or glass coverslips (0 and 25 µg/ml) and allowed to reach 70% confluence. Cells were then induced to differentiate for 1 or 5 days prior to fixation or lysate preparation. **A**) Representative images of C2C12 cells showing CD9 expression (white arrows) and nuclei. **B**) Graph showing CD9 expression levels (normalized to internal loading control) in C2C12 cells differentiated (day 1 and day 5) on 0 or 25 µg/ml collagen IV. Alpha-tubulin (55 kDa) was utilized as the loading control. Confocal images show CD9 expression and localization. Blots were assessed by ImageJ image analysis software. Scale bar represents 20 µm. Data represents mean ± SEM, * = $p < 0.05$; N=3.

3.3.10 CD9 mediates fusion in response to Collagen IV

To determine whether there is a direct relationship between the observed increased fusion and the up-regulated CD9 expression in response to collagen IV, we used monoclonal antibodies to block CD9 on differentiating C2C12 cells cultured in the presence and absence of collagen IV. Blocking CD9 using a monoclonal anti-CD9 antibody (at 10 μg/ml) has been previously shown to inhibit C2C12 fusion *in vitro* in the absence of collagen IV (Tachibana and Hemler, 1999). A range of monoclonal anti-CD9 antibody concentrations was utilized and fusion was analyzed at day 7. We observed that, at the highest dilution (1/1600; 0.313 μg/ml), fusion levels were similar to the paired control cultured in the absence of anti-CD9 antibody (Figure 3.10A,B). However, at a dilution of 1/800 (0.625 μg/ml), fusion in cells cultured in the absence of collagen IV was much lower (± 50% decrease) than in those cells differentiated in the presence of the matrix factor (Figure 3.10C). At a dilution of 1/400 (1.25 μg/ml), fusion was all but absent in cells cultured on plastic, while some fusion was still evident in cells cultured on collagen IV (Figure 3.10D). At a dilution of 1/200 (2.5 μg/ml) fusion was completely inhibited in collagen IV-coated and non-coated samples (Figure 3.10E).

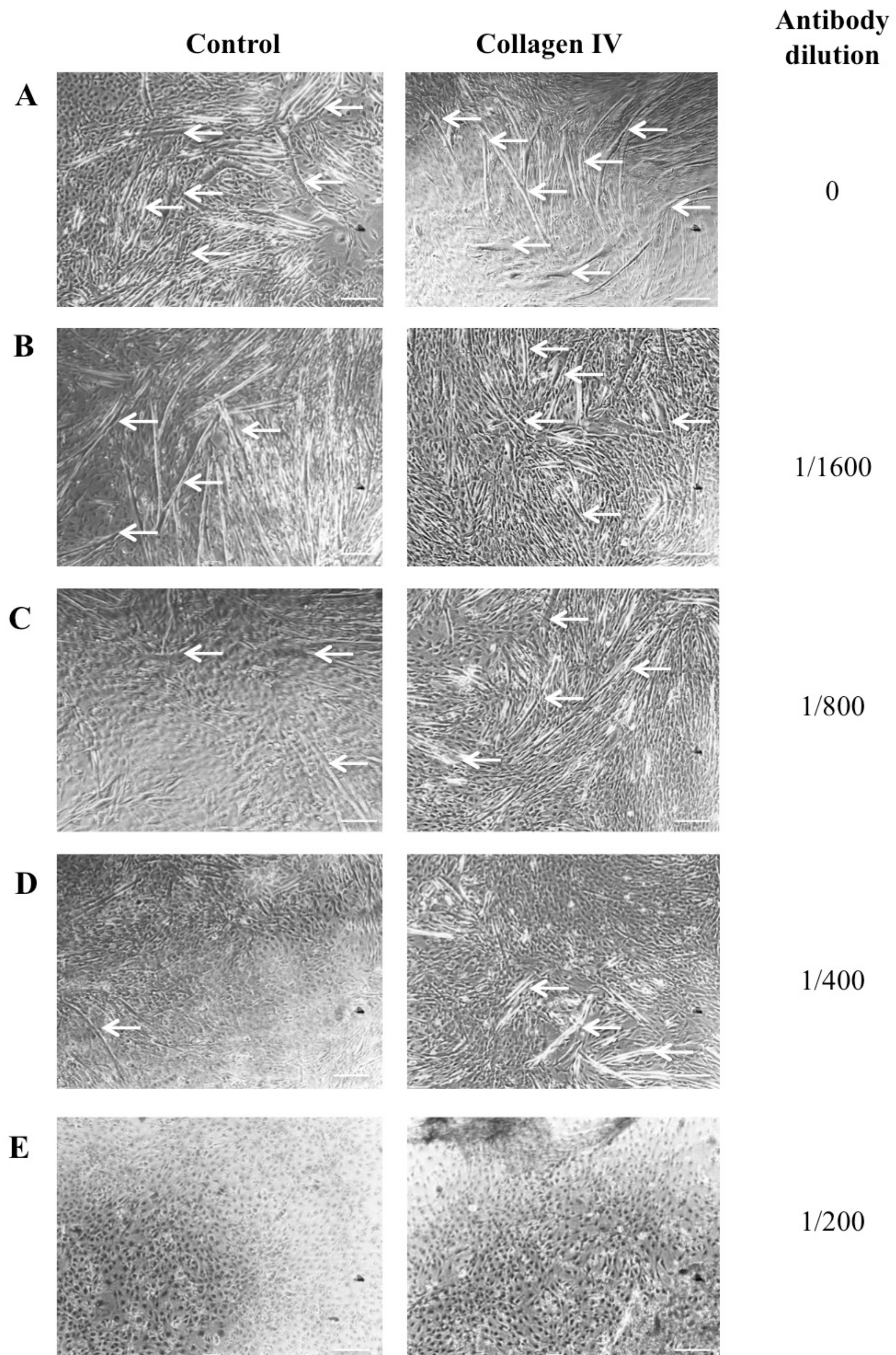


Figure 3.10: Collagen IV treated C2C12 myoblasts are more resistant to disruption of differentiation by blocking of CD9. C2C12 myoblasts were plated in growth media on 0 or 25 µg/ml collagen IV-coated wells of a 96 well plate and allowed to reach 70% confluence. Cells were then induced to differentiate for 7 days. **A-E:** Differentiating cells were incubated in the presence of varying dilutions of anti-CD9 monoclonal antibody as indicated. Representative images were taken at day 7 using phase contrast microscopy. Scale bar represents 200 µm.

3.4 Discussion

Satellite cells exist in a niche between the sarcolemma and basal lamina of skeletal muscle tissue (Grefte *et al.*, 2012, Mauro, 1961). The basal lamina of skeletal muscle is a complex mixture of ECM and growth factors, with major components being the proteins laminin and collagen IV (Pallafacchina *et al.*, 2010). In this study, we attempted to mimic aspects of the satellite cell niche environment by culturing C2C12 cells on exogenous ECM components found in the basal lamina. The effect of these factors on differentiation was assessed.

Matrigel significantly increased fusion of C2C12 cells at day 5 of differentiation, with 60 μg/ml having the greatest effect. This result was expected as Matrigel has previously been shown to increase the fusion of myoblasts (Grefte *et al.*, 2012). Matrigel, even in its growth factor reduced form, is an extremely complex mixture of proteins and growth factors (Yu and Machesky, 2012). In an effort to understand which component was responsible for the effect on fusion, we examined the effects of the main constituents of Matrigel, namely laminin (± 60%) and collagen IV (± 30%), on the differentiation and fusion of C2C12 cells.

Laminin (6.25, 12.5 and 25 μg/ml) did not significantly affect the terminal differentiation of C2C12 cells. This is in agreement with results from Grossi *et al* (2007) and leads us to believe that this constituent of the basal lamina does not contribute to the increased fusion seen in response to Matrigel. Collagen IV however, at a concentration of 25 μg/ml, was observed to significantly increase fusion at day 5 of differentiation. It was also able to significantly increase the percentage $MyoD^+$ cells when compared to untreated controls. Matrigel is composed of approximately 30% collagen IV, which translates to approximately 20 μg/ml of collagen IV in a 60 μg/ml Matrigel solution (Yu and Machesky, 2012). The observed increase in $MyoD^+$ cells at day 2 of differentiation suggests an increase in the pool of cells that have committed to differentiate. This finding is supported by the recent work of Ito *et al* (2015) who showed that collagen IV significantly promoted fusion and contractibility of C2C12 IGF-1 gene engineered myoblasts under electrical stimulation (Ito *et al.*, 2015). Therefore, the pro-myogenic effects of Matrigel may, in part, be mediated by collagen IV via a MyoD dependent pathway. The role of MyoD in the pathway could be confirmed by performing MyoD knock-down studies using RNAi. This would allow us to selectively reduce MyoD expression in the presence of collagen IV and quantify the effect of terminal fusion.

We next analysed the role of CD9 in mediating the collagen IV-stimulated increase in C2C12 fusion. The CD9 cell surface glycoprotein is found on C2C12 cells and has been shown to be vital in the normal development of skeletal muscle (Tachibana and Hemler, 1999, Charrin *et al.*, 2013). CD9 expression is also upregulated in the early stages of C2C12 differentiation and blocking CD9 using monoclonal antibodies substantially delays conversion of C2C12 cells to elongated myotubes (Tachibana and Hemler, 1999, Charrin *et al.*, 2013). Collagen IV could possibly be increasing downstream fusion of C2C12 myoblasts via an interaction with the CD9 cell surface receptor. We therefore analysed the expression of CD9 in differentiating C2C12 cells cultured on collagen IV. Our results indicate increased CD9 expression in C2C12 cells exposed to collagen IV. Although collagen IV has been observed to increase CD9 expression and induce cell migration through CD9 in MDA-MB-231 cells *in vivo*, this has not been previously shown in myoblasts (Castro-Sanchez *et al.*, 2010). The observed increase began at day 1 of differentiation and was maintained to significant levels by day 5 of differentiation. Furthermore, blocking of CD9 using a monoclonal antibody at a concentration of 1.25 μg/ml almost completely abrogated differentiation of C2C12 cells in the absence of collagen IV; collagen IV-treated samples however showed low levels of fusion in the presence of the same concentration of anti-CD9 antibodies. This may be due to an upregulation of cell surface CD9 when cultured on collagen IV and lack of sufficient anti-CD9 antibodies to block the increased number of CD9 receptors, leaving some CD9 molecules free to maintain a differentiation potential. This is supported by the observation that, at a higher antibody concentration (Figure 3.11) (2.5 μg/ml) fusion was completely blocked in cells cultured on collagen IV, whereas at a lower concentration (0.625 μg/ml) differentiation was restored to cells differentiated on collagen IV coated plates when compared to controls (Figure 3.11). Our findings are supported by the study of Tachibana and Hemler (1999); they observed that anti-CD9 antibodies inhibited C2C12 cell differentiation. This was not a non-specific effect, as use of an isotype control in the study did not similarly decrease differentiation (Tachibana and Hemler, 1999). These results support the premise that the increase in differentiation observed when C2C12 myoblasts are cultured on Matrigel is due to a collagen IV/CD9-dependent mechanism. It also emphasizes the regulatory importance of the basal lamina during skeletal muscle regeneration.

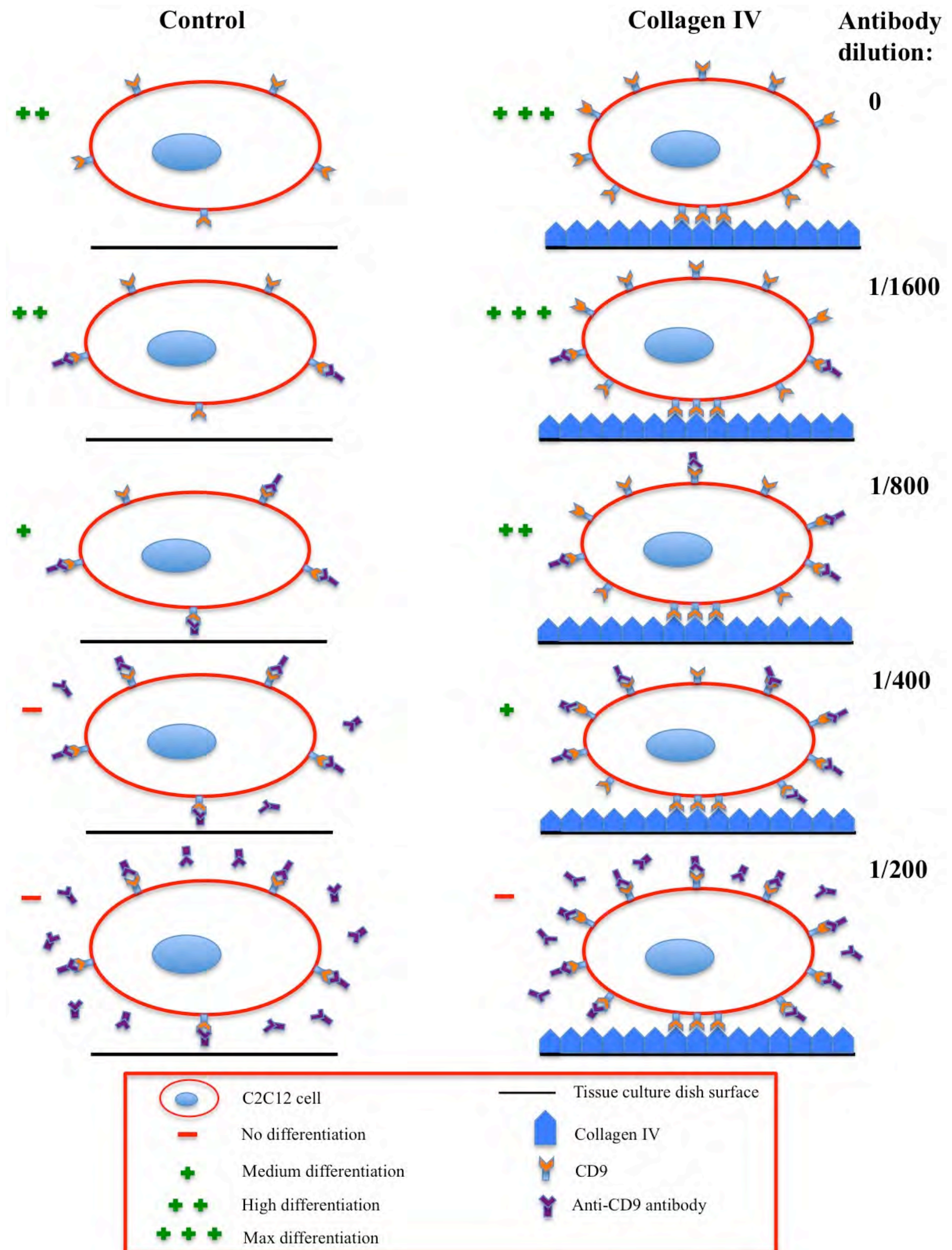


Figure 3.11: Schematic representation of CD9 blocking. The upregulation of CD9, when cells are cultured on collagen IV coated plates, makes them more "resistant" to blocking with anti-CD9 antibodies. At an antibody dilution of 1/1600, both collagen IV and control samples have free CD9 sites; differentiation is therefore observed in both. At 1/800 and 1/400, cells cultured on collagen IV have some CD9 sites free and this allows some differentiation to proceed. However, in control samples, CD9 expression is not upregulated and all endogenous sites are blocked resulting is disrupted differentiation. At a dilution of 1/200, the antibody is in excess and blocks all CD9 sites in the presence and absence of collagen IV, resulting in disrupted differentiation.

In summary, we have shown, for the first time, that the collagen IV-mediated increase in terminal differentiation of myoblasts is mediated, at least in part, by CD9. This suggests that extracellular matrix factors do not only signal via integrins and presents an additional mechanisms whereby they can communicate with the cells in the satellite cell niche.

CHAPTER 4

COLLAGEN IV MODULATES THE DOSE-DEPENDENT EFFECT OF HGF ON C2C12 MYOGENESIS

Extracellular matrix (ECM) and growth factors are known to have complex interactions that may modulate their activity *in vivo*. We have previously shown that HGF regulates myogenesis in a dose-dependent manner. Furthermore, we showed that components of the basal lamina, such as collagen IV, also regulate myogenesis. In the current study we aimed to determine whether collagen IV mediates the dose dependent effect of HGF on myogenesis. Murine C2C12 myoblasts were cultured on collagen IV, in the presence or absence of HGF, and differentiation and proliferation assessed. Collagen IV was not able to negate the negative effect of HGF (2 ng/ml) on fusion, but was able to restore normal MyHC expression. Collagen IV did not alter the pro-myogenic effect of 10 ng/ml HGF. Under proliferative conditions, collagen IV alone was unable to affect C2C12 cell numbers, cell viability or CD9 expression. Furthermore, collagen IV was observed to be unable to modulate the previously reported dose-dependent effects of HGF on C2C12 cell numbers. Due to a collagen IV stimulated increase in CD9 expression in differentiating myoblasts, cells cultured on collagen IV required higher levels of neutralizing anti-CD9 monoclonal antibodies to reduce fusion; an effect not observed when cells were differentiated in the presence of HGF alone. In HGF (10 ng/ml) treated samples, fusion was completely inhibited when CD9 was completely blocked suggesting that CD9 is a crucial co-factor in HGF (10 ng/ml)-induced fusion. These results show, for the first time, that despite the proliferative effect of low concentrations of HGF, collagen IV still promoted myogenic regulatory factor expression and early differentiation. The interactive effects of collagen IV and HGF are confirmed to be mediated by CD9, but there may be an upper limit to the effect on fusion.

4.1 Introduction

In vivo, the extracellular stimuli that a satellite cell is exposed to, is a lot more complex than our single ECM factor studies can simulate. In healthy, uninjured muscle, the satellite cell resides in quiescence in its collagen IV and laminin rich niche between the basal lamina and sarcolemma of the muscle fiber (Sanes, 2003). Upon injury, HGF stimulates the satellite cell to become active before migrating out of its niche towards the site of injury which is characterized by ECM factors such as fibronectin and collagen I. Therefore the cell is in constant contact with a multitude of external ECM components and the composition and concentration of the satellite cell ECM changes as the cell progresses through myogenesis during repair.

In the previous chapters we examined the effects of extracellular matrix proteins such as HGF, collagen IV and laminin on C2C12 differentiation and proliferation. We found that HGF (2 ng/ml) significantly promoted cell division, but reduced myogenic commitment and fusion. Conversely, 10 ng/ml HGF reduced proliferative capability, but increased differentiation. We then determined that collagen IV, and not laminin, positively regulated differentiation of C2C12 cells; cells cultured on 25 µg/ml collagen IV showed a significant increase in fusion at day 5 when compared to cells cultured on uncoated tissue culture plates. This was accompanied by an increase in CD9 expression; neutralizing antibody studies further suggested a potential role for CD9 in mediating the observed increase in fusion.

ECM and growth factors are known to have complex interactions with each other and with growth factors. Combined ECM experiments often show synergistic effects of the individual ECM components. For instance, fibronectin and laminin individually have been shown to increase the differentiation of adult neural stem cells and their combination enhances this increase (Cooke *et al.*, 2010). Decorin has been shown to modulate collagen I, but not fibronectin stimulated migration of C2C12 cells *in vitro* (Goetsch *et al.*, 2011). Furthermore, collagen I and decorin were shown to have a physical interaction via separate core protein-binding domains (Schonherr *et al.*, 1995). Previously, Collagen IV has been shown to interact with a range of ECM factors including laminin, fibronectin and decorin and is vital for basement membrane stability (Poschl *et al.*, 2004, Aumailley and Timpl, 1986, Charonis *et al.*, 1985, Leppert *et al.*, 2000). Interestingly, collagen IV has also been shown to bind HGF with a mild affinity (Schuppan *et al.*, 1998). Reversible binding of HGF to immobilized

collagen IV has been reported to result in a collagen-HGF complex that is able to induce primary hepatocyte proliferation in a dose-dependent manner. Further investigation revealed that binding was due to specific peptide sequences unique to collagenous proteins suggesting a specific role for collagens in HGF bioavailability and activity (Schuppan *et al.*, 1998). It therefore becomes important to investigate combinations of ECM and growth factors to mimic the *in vivo* environment more closely and thereby more fully understand how, together, these proteins modulate cell behavior. At this stage, very little is known about the effect of collagen IV in mediating HGF signaling in myoblasts.

In this study, the effects of collagen IV on the previously observed dose-dependent effect of HGF on C2C12 myogenesis was examined. Terminal differentiation and proliferation were evaluated and we attempt to uncover the mechanisms at play by examining transcription factor expression and the role of the tetraspanin CD9.

4.2 Materials and methods:

All reagents were of an analytical grade and obtained from Sigma-Aldrich (USA), unless stated otherwise. All cell culture was performed under sterile conditions in a level II biological safety cabinet (ESCO Class II BSC).

4.2.1 Cell Culture

The C2C12 murine cell line was purchased from the American Type Culture Collection (ATTC, CRL-1772). Cells were cultured in Dulbecco's Modified Eagle's Medium (Highveld Biological, CN3193-9) supplemented with 10% (v/v) Fetal Bovine Serum (Biowest, S181H-500), 2% (v/v) L-glutamine (Lonza, BE17-605E) and 2% (v/v) Penicillin-Streptomycin (Lonza, DE17-602E). For differentiation studies, cells were cultured to a confluence of 70% after which media was changed to differentiation media (DMEM supplemented with 2% (v/v) horse serum) (Biowest, S090H-100).

4.2.2 Differentiation

Differentiation media consists of 485 ml DMEM, 10 ml PenStrep (2% v/v) and 5 ml horse serum (16050-130; Invitrogen; USA) filter sterilized prior to storage in 50 ml falcon tubes at 4°C. When cells reached approximately 70% confluence, the media was changed to differentiation media. Cells were maintained at 37 °C, 5% CO_2 in a humidified incubator.

4.2.3 ECM Coating and HGF addition

4.2.3.1 Collagen IV Coating

Collagen IV (Human; 0.3 mg; C6745; Sigma-Aldrich; USA) was diluted to a stock solution of 0.1 mg/ml with sterile PBS containing 0.25% acetic acid at 4 °C. T25 culture flasks and 24 well plates containing glass coverslips were coated with 3 ml and 500 µl of a 25 µg/ml collagen IV solution respectively. All plates were incubated at 37 °C for 4 hours, before the protein solutions were removed and the plates allowed to air dry under U.V. light overnight. Plates and wells are washed with sterile PBS before use and stored at 4 °C for no longer than a month.

4.2.3.2 HGF addition

Active human HGF (PeproTech, cat.100-39) was used at a final concentration range of 0, 2 and 10 ng/ml and media changed every 48 hours during differentiation studies. Cells were incubated at 37°C in a humidified incubator at 5% CO2. All experiments were carried out under sterile conditions in a Class II Biohazard Safety Cabinet.

Clustal W alignments were conducted to compare the amino acid sequences of the ECM and growth factors used in this study and those found in mice (*Mus Musculus*) (Appendix III). Scores between 79.2899 and 88.4882 showed that proteins were highly conserved in all cases.

4.2.4 Immunocytochemistry and Confocal Microscopy

C2C12 cells were plated onto coverslips in a 24 well plate and allowed to reach 70% confluence in 500 µl growth media. Wells had previously been coated with the collagen IV as described earlier. HGF addition took place with initial plating out and at all media changes (day -1, 0 and 3). Differentiating (day 1, day 2 and day 5) cell samples were cultured in differentiating media for 24, 48 and 120 hours respectively. Coverslips were subsequently washed with PBS and fixed with 4% paraformaldehyde for 15 minutes. They were then washed with PBS and blocked using 5% donkey serum. Coverslips were then incubated with primary antibodies for 4 hours at room temperature: day 0: mouse monoclonal anti-CD9; day 2: rabbit polyclonal anti-MyoD; day 5: mouse monoclonal anti-MyHC, mouse monoclonal anti-CD9. Coverslips were then washed 4 x 8 minutes with PBS before being incubated with appropriate secondary antibody for 40 minutes (Table 4.1). These were washed 3 x 8 minutes with PBS before being treated with Hoechst 33342 nuclear stain (Stock: 10 mg/ml; 1/4000; Sigma-Aldrich; USA). Coverslips were then washed for a further 4 x 8 minutes with PBS before being mounted with Movial and finally visualized using the Zeiss LSM 710 confocal microscope.

Table 4.1: Antibody concentrations (optimized) used in Confocal microscopy

	Primary antibody	Dilution	Secondary antibody	Dilution
Confocal Microscopy	Rabbit polyclonal anti-MyoD (sc-760, Santa Cruz; USA)	1/100	Donkey anti-rabbit Dylight 488 (711-455-152, Jackson ImmunoResearch)	1/1000
	Mouse monoclonal anti-MyHC (MF-20; DSHB; USA)	1/400	Donkey anti-mouse Dylight 494 (715-515-151, Jackson ImmunoResearch)	1/4000
	Mouse monoclonal anti-CD9 (BD 551808, USA)	1/400	Donkey anti-mouse Dylight 488 (715-485-151, Jackson ImmunoResearch)	1/1000

4.2.4.1 Assessment of percentage MyoD$^+$ cells

Cells shown to be expressing nuclear MyoD were counted and determined as a percentage of total nuclei on day 2 (percentage MyoD$^+$) of differentiation. Images were examined using Zen software (Zeiss) allowing visualization of a single plane and accurate quantification. Approximately 20 cells per image were analysed and a minimum of 5 fields of view (i.e. 100 cells/n), randomly selected, were used for n=1. Experiments were repeated n = 6-8 times.

4.2.4.2 Assessment of terminal differentiation via a fusion index

The fusion index was determined via quantitative analysis of confocal images of differentiated myotubes (day 5). Images were exported using Zen software (Zeiss) as a single plane with both the red channel (MyHC) and the blue channel (Hoechst). This allowed us to count the number of nuclei in fused myotubes (containing 2 or more nuclei). This number was divided by the total number of nuclei in the field of view and multiplied by 100. A minimum of 4 fields of view, randomly selected, were used for n=1. Experiments were repeated n = 6-8 times.

4.2.5 Protein separation by SDS-PAGE

Lysate sample preparation is outlined in Appendix IV. The Laemmli protocol was used for SDS-PAGE (Laemmli, 1970) with several modifications outlined in Appendix IV. The BioRad electrophoresis unit (BioRad; Mini-PROTEIN 3 Cell; USA) was utilized for the running of all polyacrylamide gels. 10% or 12.5% gels were cast between two glass plates, as described in the Appendix IV, and allowed to solidify (~1 hour) before the stacking gel was added with a 10 fingered comb to create the lanes for sample loading. Once the stacking gel had set (~30 minutes) the comb was gently removed and lanes washed with distilled water.

Electrode (tank) buffer was poured into the inner and outer electrode compartments before the prepared samples were loaded. The samples were prepared by adding an equal volume of reducing sample treatment buffer to the sample and boiling the solution for 2 minutes. Samples were then stored on ice until loaded. 1 μl bromophenol blue (1437500CB; Saarchem; RSA) was added to each sample so that the buffer front could be clearly discerned. 25 μg of protein per lane was added to the gel as determined by the Bradford Assay (Appendix IV). 3 μl of the PeqGold protein marker V (27-2210; Peqlab; Germany) was run in at least one lane of each gel for later determination of protein molecular weights. Gels were run at 4 °C with 18 mA per gel until the buffer front reached the bottom of the running gel. The gel was removed from between the glass plates and either stained with the Coomassie G-250 stain or processed for western blot analysis.

4.2.6 Western Blot analysis

Gels were placed within a blotting sandwich made up of the following; a piece of nitrocellulose (Hybond-C Extra; Amersham Biosciences; UK) the same size as the gel which was place over the gel, two pieces of Whatman No.4 filter paper was placed on either side of the gel and nitrocellulose, followed by two pieces of Scotchbrite foam placed on either side of the filter paper. The sandwich was placed in a western blot cassette and loaded into the western blot apparatus (Omni Page; Cleaver Scientific Ltd; UK) containing blotting buffer. The nitrocellulose must be place on the positive side of the apparatus as the proteins in the gel are negatively charged. The blot was run in the fridge (4 °C) at 400 mA for 4 hours or 60 mA for 16 hours (Appendix V).

The nitrocellulose was then removed and the gel placed in Coomassie G-250 stain to determine whether total protein transfer had occurred. The nitrocellulose was placed in the Ponceau S. stain (2 minutes) and destained with TBST until clear bands were visible. This gave an indication of whether the protein transfer was performed correctly and equal loading had been achieved. The nitrocellulose was washed a further two times in TBST to remove the remaining Ponceau S. stain. The nitrocellulose was then blocked in 5% (m/v) low fat milk powder (made up in TBST) for 1 hour. Primary antibody (Table 4.2) made up in TBST was then added to the nitrocellulose for 2 hours. This was followed by wash steps with TBST (4 x 8 minutes). All wash steps were performed with vigorous agitation. HRPO-linked

secondary antibody made up in TBST was then added for 1 hour. This was followed by washing with TBST (6 x 8 minutes).

Table 4.2: Antibody concentrations used in Western blot

	Primary antibody	Dilution	Secondary antibody	Dilution
Western Blot	Mouse monoclonal anti-MyHC (MF-20; DSHB; USA)	1/400	Donkey anti-mouse IgG (ab96857, Abcam; UK)	1/4000
	Mouse monoclonal anti-CD9 (BD 551808, USA)	1/400	Donkey anti-mouse IgG (ab96857, Abcam; UK)	1/1000

Enhanced Chemiluminescence (ECL) technique was used for the development of the bands. All steps of ECL which involves the use of the light film (Kodak BioMax light film; Z370398; Sigma-Aldrich; USA) were performed in the dark room with no white light present. The substrate was made by adding peroxide and enhancer solutions in a 1:1 ratio (Immun-Star WesternC; 70-5070; Bio-Rad, USA). The substrate (1 ml) was added to the nitrocellulose for 2 minutes. The nitrocellulose was placed between two pieces of transparent film and the position of the molecular weight markers were indicated on the transparent film for easy identification in the dark room. Prior to development, the developer (Structurix G128; Agfa; 5TBN; Belgium) and the fixative (G333c Rapid Fixer; Agfa; EGCQT; Belgium) solutions were made up with water in a 1 in 4 dilution and a 1 in 3 dilution, respectively. A piece of light film was cut to the same size as the nitrocellulose and placed over the nitrocellulose located between the transparency sandwich. The light film was left on the nitrocellulose for up to 10 minutes depending on the antibodies being used. The film was placed in the developer solution until the bands developed (~5 minutes) and then placed in the fixative (2 minutes). Finally, the film was washed with water and allowed to dry.

4.2.6.1 Densitometry

The bands that developed on the film were analyzed by use of computer software (Image J, http://imagej.nih.gov/ij/). Densitometric analysis was selected and the background was adjusted so that only the bands were visible for all experiments.

3.2.7 Monoclonal antibody blocking

C2C12 myoblasts (15 000 cells/well) were plated in collagen IV-coated and non-coated wells of a flat-bottom 96 well culture plate (Corning, CR/3596) and allowed to reach 70% confluence. Media was then replaced with diffferentiation media containing mouse monoclonal anti-CD9 (BD 551808, USA; 0, 1/1600, 1/800, 1/1600 dilution) and allowed to differentiate for 7 days. Media (containing various dilutions of mAbs) was replaced every 2 days.

4.2.8 Statistical analysis

The results from each experiment were expressed as a Mean ± SEM. The student's t-test was performed on all data. Samples were considered to be statistically significant if they produced a p value of less that 0.05. The number of experimental repeats is specified in each figure legend.

4.3 Results

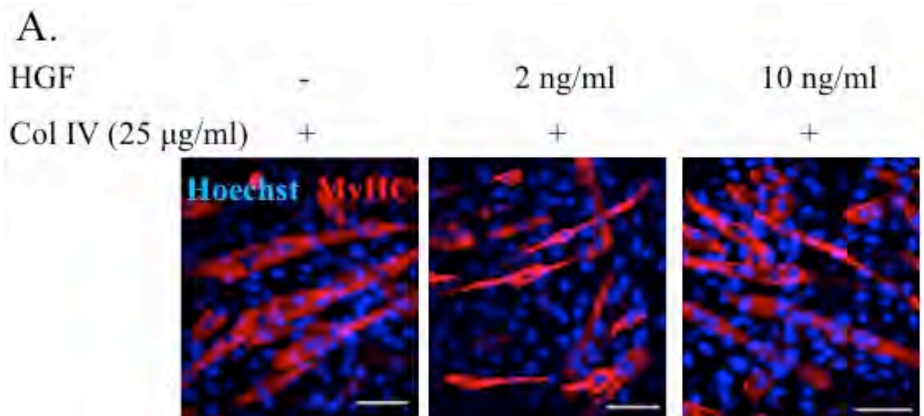
4.3.1 Collagen IV mediates HGF-modulated C2C12 differentiation

We previously reported that HGF (2 ng/ml) significantly inhibited fusion and MyHC expression in day 5 differentiating C2C12 cells (*Section 2.3.1*). To determine whether collagen IV modifies the effect of HGF on C2C12 fusion and MyHC expression, cells were cultured and differentiated on collagen IV (25 μg/ml) coated coverslips (for fusion index assessment) or flasks (for Western blotting) in media supplemented with HGF (0, 2 and 10 ng/ml). In the presence of collagen IV, HGF (2 ng/ml) was observed to still reduce the number of fused myotubes, whereas HGF (10 ng/ml) did not elicit the previously reported increase in myotube fusion (Figure 4.1A). Calculation of percentage fusion revealed that 2 ng/ml HGF significantly ($p < 0.05$) reduced terminal fusion at day 5 in cells cultured on collagen IV when compared to the collagen IV coated control (HGF 0 ng/ml) from 50 ± 3% to 38 ± 5% (Figure 4.1B). However, the addition of the HGF (10 ng/ml) to collagen IV did not significantly increase fusion when compared to collagen IV alone (Figure 4.1B). HGF, at either dose was however unable to significantly alter MyHC expression in the presence of collagen IV, when compared to cells cultured on collagen IV in the absence of HGF (Figure 4.1C). This suggests that although collagen IV can restore normal MyHC expression to C2C12 cells treated with 2 ng/ml HGF, it is unable to restore normal levels of fusion. Furthermore, collagen IV abolished the ability of HGF (10 ng/ml) to promote fusion or MyHC expression over and above that of collagen IV on its own (Figure 4.1B, C).

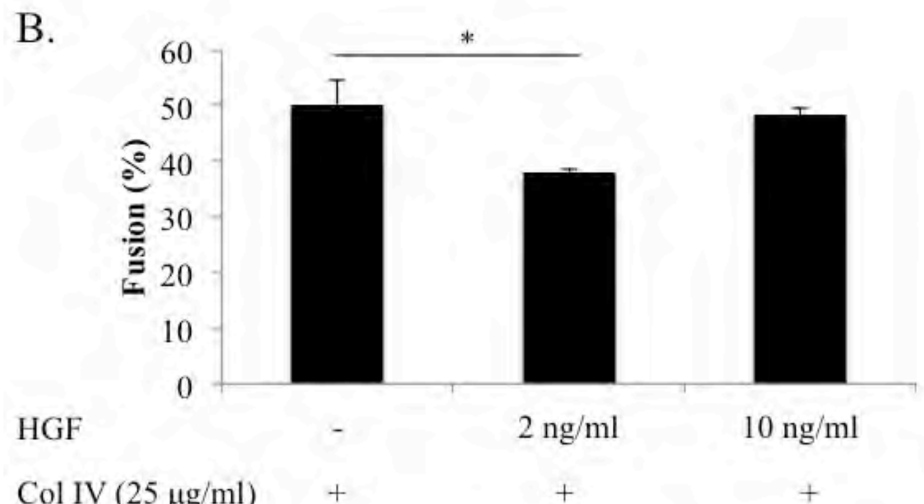
A.

HGF - 2 ng/ml 10 ng/ml

Col IV (25 μg/ml) + + +



B.



C.

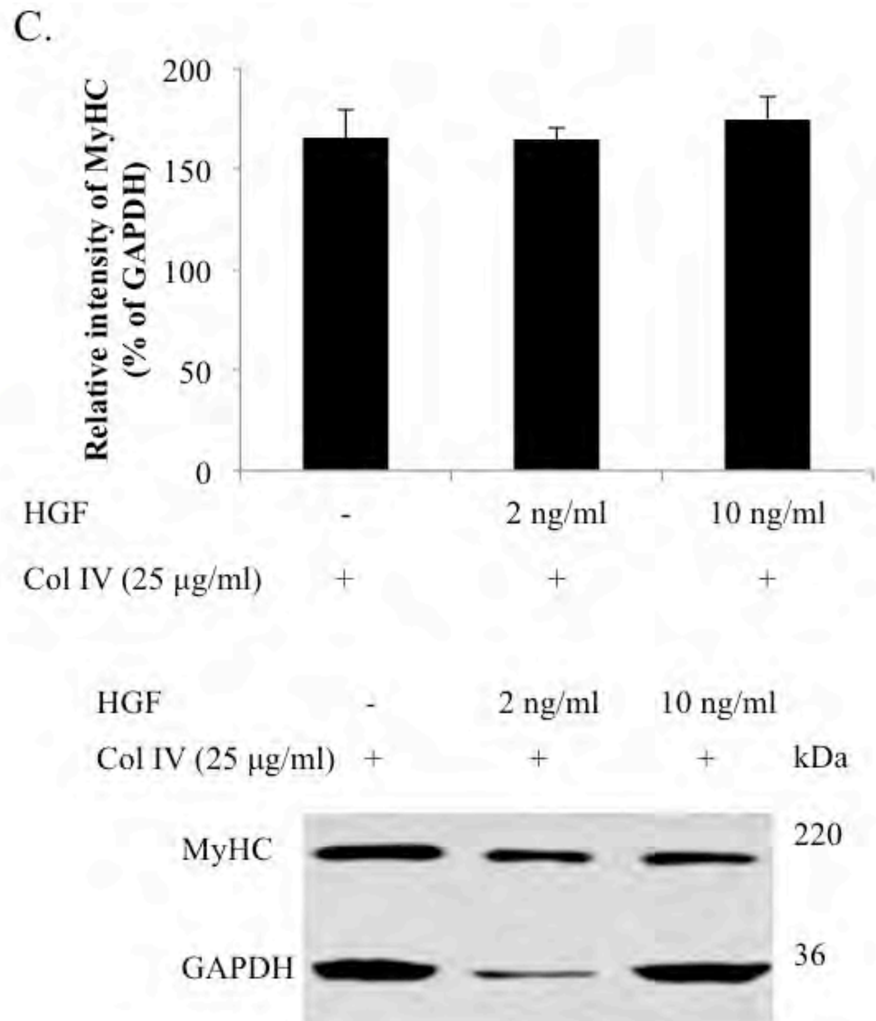
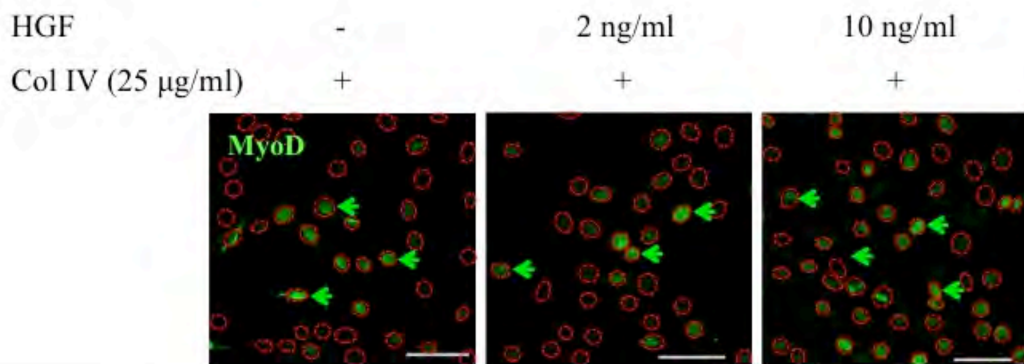


Figure 4.1: Collagen IV modulates HGF-mediated myogenesis. C2C12 cells were differentiated for 5 days on collagen IV-coated coverslips (25 μg/ml) in the presence or absence of HGF (2, 10 ng/ml). **A**) Representative images of differentiated C2C12 cells showing MyHC (red) and nuclei (blue). **B**) Fusion index calculated as percentage C2C12 myotube formation at day 5 of differentiation. **C**: Western blot and densitometric analysis of MyHC expression with GAPDH used as a loading control. MyHC expression was calculated relative to GAPDH expression. Scale bar = 50 μm. Data represents 6 (**A** and **B**) and 3 (**C**) independent experimental repeats. Data is presented as mean ± SEM. *$p<0.05$.

4.3.2 Collagen IV prevents the dose-dependent effect of HGF on MyoD expression

We previously reported (Kahamba, 2013), Walker *et al*., 2015) that HGF may mediate its dose-dependent effect via MyoD. To determine the effect of collagen IV on the HGF-mediated change in percentage $MyoD^+$ cells, myoblasts were cultured and induced to differentiate on collagen IV (25 μg/ml) coated plates in media supplemented with HGF (0, 2 and 10 ng/ml) for 2 days. HGF at either dose was unable to significantly alter percentage $MyoD^+$ cell numbers (HGF 2 ng/ml = 42 ± 6%; HGF 10 ng/ml = 44 ± 7%) in the presence of collagen IV, when compared to samples coated on collagen IV alone (46 ± 3%; Figure 4.2A, B). These results suggest that collagen IV (25 μg/ml) prevents HGF from significantly affecting the percentage $MyoD^+$ in differentiating C2C12 cells.



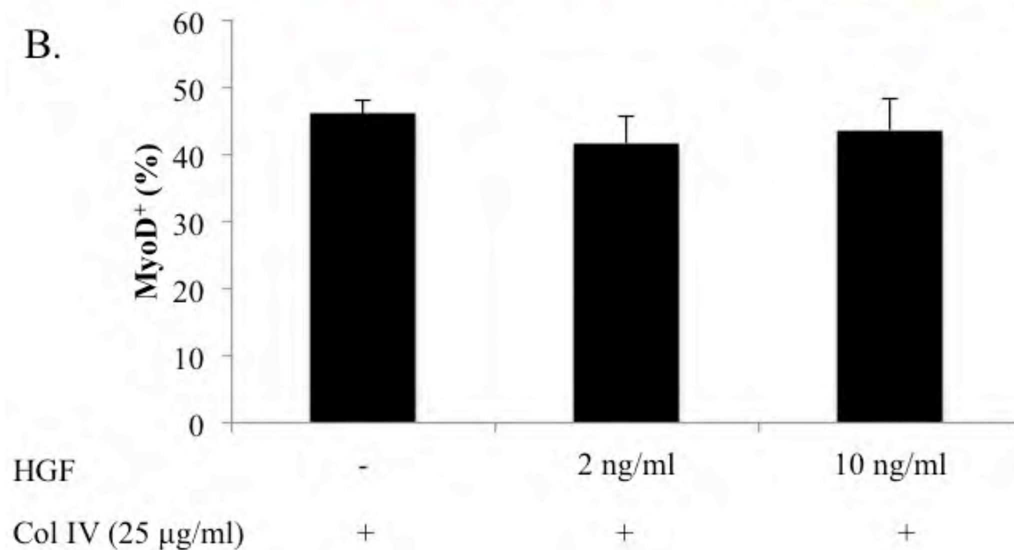


Figure 4.2: HGF, in the presence of collagen IV, does not modulate percentage $MyoD^+$ C2C12 myoblasts. C2C12 myoblasts were plated in HGF supplemented growth media on coated glass coverslips and allowed to reach 70% confluence. Cells were then induced to differentiate for 2 days prior to fixation and immunostaining for MyoD. **A**) Representative images showing $MyoD^+$ cells. **B**) Percentage $MyoD^+$ cells. Red outlined circles represent nuclei. Green arrow shows $MyoD^+$ cells. Scale bar = 20 μm. Data represents 6 independent experimental repeats. Data is presented as mean ± SEM.

4.3.3 HGF treated C2C12 cell differentiation is not more resistant to CD9 blocking than control samples

Blocking CD9 using an anti-CD9 monoclonal antibody has been shown to inhibit C2C12 fusion *in vitro* (Tachibana and Hemler, 1999). We showed in the previous chapter that C2C12 cells differentiated on collagen IV increase their CD9 expression and appear more resistant to the negative effect of CD9 neutralizing antibodies on fusion. In an effort to understand whether CD9 plays a role in mediating the negative effect of collagen IV on HGF (10 ng/ml)-mediated differentiation, we cultured cells on collagen IV in the presence or absence of HGF (10 ng/ml) as well as HGF (10 ng/ml) alone, and assessed the effect of anti-CD9 antibodies on differentiation. At a dilution of 1/1600 (0.313 μg/ml), fusion was similar to control conditions (no antibody) in all samples treated (Figure 4.3). However, at a concentration of 1/800 (0.625 μg/ml), a reduction in fusion was observed in all groups with a greater reduction in HGF samples (compared to collagen IV and collagen IV + HGF samples) (Figure 4.3). Interestingly, we observed that HGF-mediated fusion was completely abrogated at an antibody dilution of 1/400 (1.25 μg/ml) in cells cultured in the absence of collagen IV while cellular fusion, albeit in very low levels, was observed in the presence collagen IV and collagen IV + HGF samples (Figure 4.3).

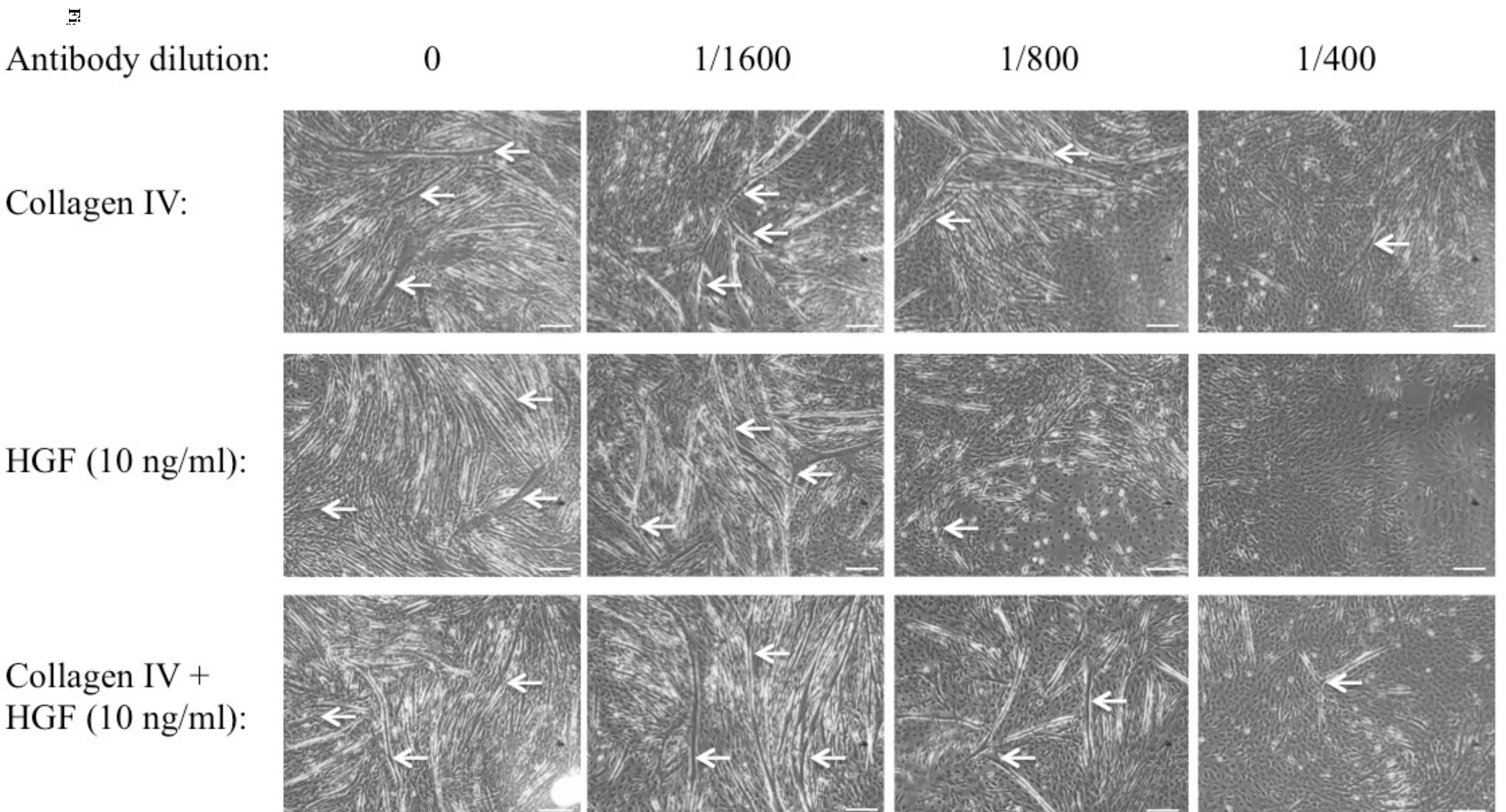


Figure 4.3: CD9 neutralizing antibodies decrease both HGF and collagen IV stimulated fusion. Differentiating C2C12 myoblasts, cultured on 25 μg/ml collagen IV in the presence or absence of 10 ng/ml HGF, were incubated in the presence of varying dilutions of anti-CD9 monoclonal antibody (as indicated). Representative images were taken at day 7 of differentiation using phase contrast microscopy. Scale bar represents 200 μm.

4.3.4 Collagen IV does not significantly affect C2C12 myoblast proliferation, viability or CD9 expression

Given the ability of collagen IV to modulate the effects of HGF on differentiation, we next tested whether collagen IV also plays a key role in regulating myoblast proliferation. C2C12 cells (10 x 10^4 cells/plate) were cultured on collagen IV (0, 25 µg/ml) coated plates and cell number and viability assessed after 24 hours. Cell number and viability were not significantly affected in response to collagen IV (Figure 4.4A and B). Cell numbers at 24 hours were 24,9 x 10^4 under control conditions and 25,3 x 10^4 when cultured on collagen IV. Confocal analysis revealed similar levels of CD9 expression (Figure 4.4C) and although CD9 expression was previously observed to increase in differentiating C2C12 cells (*Section 3.3.7*), no significant change in CD9 expression was observed in response to collagen IV under proliferative conditions (Figure 4.4D). These results suggest that differentiation cues may be required in conjunction with collagen IV exposure for this ECM factor to affect the behavior of C2C12 cells.

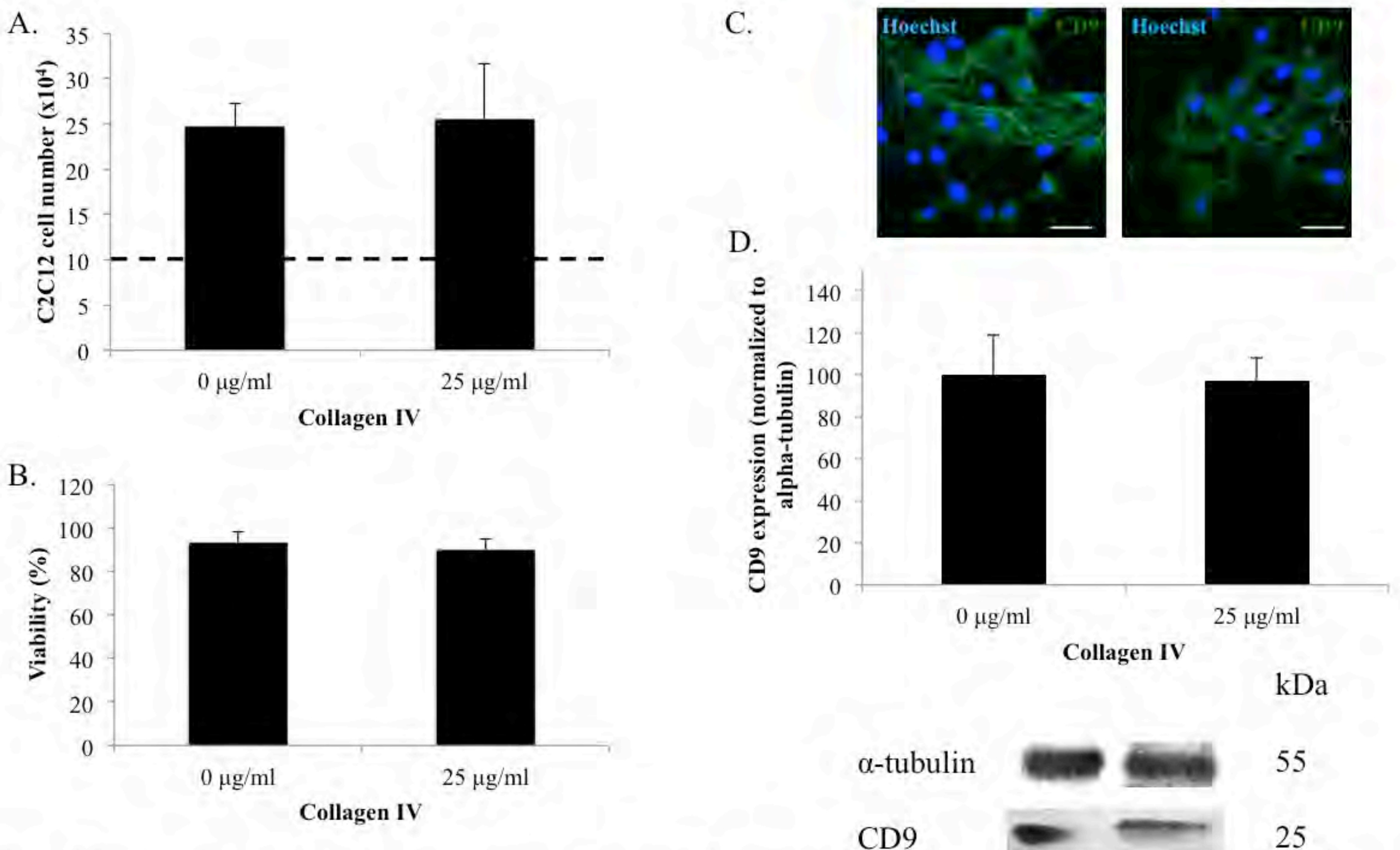


Figure 4.4: Effect of collagen IV on C2C12 myoblast cell number, viability and CD9 expression. C2C12 cell numbers (**A**: Total, **B**: Viable) were determined using the BioRad TC-20 cell counter and trypan blue. Dashed line represents initial cell number seeded, (10 x 10^4 cells/flask). Representative images showing CD9 expression (green) and nuclei (blue). **D**) Western blot and densitometric analysis of CD9 expression levels in proliferating C2C12 myoblasts cultured on 0 and 25 µg/ml collagen IV. Alpha-tubulin was used as a loading control and CD9 expression was calculated relative to alpha-tubulin expression. Data represents 3-4 independent experimental repeats. Data is presented as mean ± SEM. Scale bar represents 20 µm.

4.3.5 Collagen IV does not modulate the dose-dependent effect of HGF on proliferation

To determine whether collagen IV modifies the dose-dependent effect of HGF on C2C12 cell number, cells were cultured on collagen IV (25 µg/ml) in media supplemented with HGF (0, 2 and 10 ng/ml). In the presence of collagen IV, HGF (2 ng/ml) stimulated the characteristic increase in proliferation, whereas incubation with HGF (10 ng/ml) caused the expected reduction in cell number (Figure 4.5A). Cell counts revealed a significant 29% increase from 30 x 10^4 cells to 46 x 10^4 cells (Figure 4.5A; $p < 0.05$) in response to 2 ng/ml HGF in combination with 25 µg/ml collagen IV when compared to cell counts conducted on samples cultured in the absence of HGF. Following incubation with 10 ng/ml HGF in combination with 25 µg/ml collagen IV a significant 37% reduction in cell numbers was observed from 30 x 10^4 cells to 19 x 10^4 cells (Figure 4.5A; $p < 0.05$) when compared to cell counts conducted on samples cultured in the absence of HGF. The percentage viability was also not affected and remained at previously reported (*section 2.3.4*) levels of approximately 85-90% (Figure 4.5B).

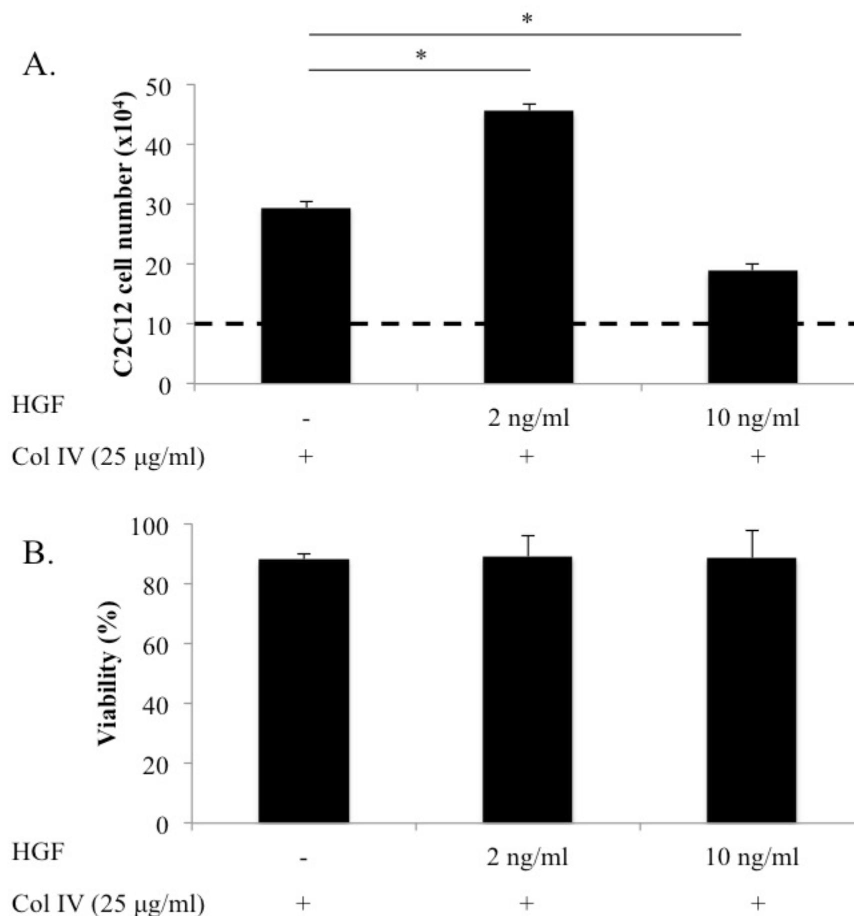


Figure 4.5: Collagen IV does not alter the dose-dependent effect of HGF on C2C12 proliferation. C2C12 myoblasts were plated in HGF supplemented growth media in coated flasks and allowed to reach 70% confluence. Cell numbers were determined using the BioRad TC-20 cell counter and trypan blue. **A**) Total cell numbers. **B**) Viable cell numbers. Dashed line represents initial cell number seeded, flasks were seeded with 10 x 10^4 cells/flask. Data represents 4 independent experimental repeats. Data is presented as mean ± SEM. *$p<0.05$.

4.4 Discussion

We previously reported that HGF (2 ng/ml) inhibited C2C12 MyHC expression and fusion, while 10 ng/ml HGF promoted MyHC expression and fusion (Walker *et al.*, 2015). Furthermore we subsequently showed that collagen IV, a major component of the basal lamina of the satellite cell niche, is able to promote myogenesis (Walker & Niesler, 2015; submitted). In the current chapter we determined whether collagen IV can modulate the previously reported dose-dependent effects of HGF on C2C12 myoblasts. Our results revealed that although collagen IV (25 µg/ml) did not negate the negative effect of HGF (2 ng/ml) on fusion, it did restore normal MyHC expression. This leads us to believe that HGF (2 ng/ml) may negatively regulate C2C12 differentiation via at least two mechanisms; 1) inhibition of cell-specific contractile protein expression (reversible by collagen IV) and 2) prevention of the later stages of fusion (not reversible by collagen IV exposure). On the other hand, in the presence of collagen IV (25 µg/ml), HGF (10 ng/ml) was not able to stimulate an increase in fusion and MyHC expression over and above collagen IV levels suggesting that maximum C2C12 *in vitro* fusion has been reached or that HGF and collagen IV mediate their pro-myogenic effects via a common pathway.

In order to begin delineating the role of CD9 in mediating collagen IV and HGF-mediated differentiation, CD9 neutralizing antibodies were used at a dilution range from 1/400 to 1/1600. Following blocking of CD9 using a monoclonal antibody at a dilution of 1/1600, normal fusion was observed in all samples treated. However, at a dilution of 1/800, collagen IV-treated samples were more "resistant" to the inhibitory effect of the CD9 neutralizing antibodies when compared to samples treated with HGF (10 ng/ml) alone. In addition to this, we observed that fusion was completely abrogated at a dilution of 1/400 for samples treated with HGF (10 ng/ml) while fusion, albeit in very low levels, is present in samples treated with collagen IV. This may be due to an upregulation of CD9 when cultured on collagen IV and lack of sufficient anti-CD9 antibodies to fully block this increase, leaving some CD9 molecules free to maintain a differentiation potential. Alternatively, the presence of fusion in collagen IV samples when treated with a dilution of 1/400 anti-CD9 antibodies may suggest that although all CD9 sited are blocked, collagen IV may also induce C2C12 differentiation via a CD9 independent pathway as well as increasing CD9 cell surface expression. In addition to CD9, collagen IV is known to bind to cell surface receptors knows as integrins (Leitinger and Hohenester, 2007). Integrins are the major transmembrane receptors involved in cell adhesion to the ECM

(Humphries *et al.*, 2006). Integrins interact with the ECM and assist in signaling and cell-fate determination, and it is through these interactions that the ECM is believed to influence myogenesis and could account for the increased increase in fusion (Hynes *et al.*, 2002). This however, requires further investigation.

Anti-CD9 antibodies were able to block HGF-stimulated fusion at high dilutions. This suggests that the previously documented pro-myogenic effects of HGF on fusion (Walker *et al.*, 2015) may be due to a CD9-dependent pathway, however it is necessary to detect the expression of CD9 in response to HGF. A lack of increased CD9 expression would explain the inhibition of fusion at higher antibody dilutions. This hypothesis is supported by findings that knocking down CD9 expression significantly inhibited the well-documented effects of VEGF and HGF on endothelial cell motility and invasion *in vitro* (Kamisasanuki *et al.*, 2011). Furthermore, *in vivo* studies show that knocking down CD9 expression with siRNA inhibited HGF- and VEGF-induced subconjunctival angiogenesis (Kamisasanuki *et al.*, 2011). Other studies reinforce a specific requirement for CD9 in HGF signaling; an M.S. study from Michigan State University determined that CD9 is required for CD82 suppression of HGF-induced Met activation (Spotts, 2010).

In response to collagen IV, C2C12 proliferation and viability were not observed to be affected; CD9 expression was also not significantly altered under proliferative conditions. It may be worthwhile to analyze CD9 expression levels following 48 hours of proliferative culture on collagen IV to determine whether extended incubation is required to stimulate changed in CD9 expression levels. HGF has been reported to demonstrate a dose-dependent effect on C2C12 proliferation. HGF (2 ng/ml) was observed to significantly increase C2C12 proliferation with 10 ng/ml HGF having the opposite effect and significantly inhibiting differentiation (Walker *et al.*, 2015). Combination studies revealed that collagen IV was unable to regulate the previously reported effects of HGF on proliferation of C2C12 myoblasts.

In summary, we demonstrated that collagen IV, along with being a positive modulator of myogenesis, can also mediate the dose-dependent effects of HGF on myogenesis. This demonstrates the ability for the ECM to affect the action of growth factors and highlights the need to consider ECM-growth factor interactions during *in vitro* studies.

CHAPTER 5: DISCUSSION

Undifferentiated C2C12 cells are a mouse myoblast cell line capable of differentiation and fusion into myotubes, making them an ideal model for skeletal muscle repair studies *in vitro*. We attempted to examine the effects of a range of ECM and growth factors on various aspects of myogenesis.

HGF is a growth factor that has been shown to be vital in the activation of satellite cells during early stages of myogenesis. We differentiated C2C12 cells in media supplemented with HGF and noted a dose dependent effect of this supplementation. At low concentrations (2 ng/ml) differentiation was impaired, but at higher concentrations (10 ng/ml) this effect was the opposite and fusion was in fact promoted. However, with regard to proliferation, 2 ng/ml HGF significantly increased cell numbers, while 10 ng/ml significantly reduced them when compared to untreated controls. This result suggests a dual mechanism of HGF on myoblasts during proliferation and differentiation. In an attempt to understand the mechanisms at play, we examined the expression levels of the c-Met receptor in response to treatment with HGF. Changes in c-Met receptor expression levels were noted in response to HGF and the dose dependent effects of the growth factor on proliferation and differentiation could be correlated directly to expression levels of this receptor. Grp2 and p85 are two distinct mediators of the effect that HGF can have on differentiation. Grp2 signals via the Ras pathway, which reduces myoblast differentiation and signaling via the p85 mediator results in signaling via the phosphatidylinositol 3-kinase (PI3K) pathway that induces cell differentiation (Leshem *et al.*, 2002). Although our results suggest that PI3K and p38 mediate the anti-proliferative effect of HGF at higher concentrations, the pro-proliferative mechanism is still unclear and may be related to changes in adaptor protein association (such as Grb2) at the cell membrane.

Matrigel, even in its growth-reduced form, is a complex mixture of over 1000 ECM proteins and growth factors (Hughes *et al.*, 2010, Kohen *et al.*, 2009). Matrigel was observed to increase terminal fusion in C2C12 myoblasts. However, it is difficult to interpret this result, as Matrigel is comprised of numerous proteins and growth factors. In an attempt to determine which portion of Matrigel was responsible for this result, we differentiated C2C12 cells on the major components of Matrigel, laminin and collagen IV. We used a subtype of laminin, laminin-111 in our studies, which is the major laminin component Matrigel

(Neubauer *et al.*, 2009). This laminin does however differ in its alpha domain from the laminin found in the skeletal muscle basal lamina, which is laminin-211 (Holmberg and Durbeej, 2013). We proceeded with laminin-111 because of its relevance to the earlier Matrigel study and due to the fact that laminin interacts with collagen through its beta domain, which is identical on both subtypes in this study (Kleinman *et al.*, 1987). We found that laminin-111 alone had no effect on C2C12 differentiation. It would however be important to investigate laminin-211 in similar studies in the future, as it is the subtype found in the basal lamina of skeletal muscle. We then differentiated C2C12 cells on collagen IV, the second largest constituent of Matrigel. Here, we observed that collagen IV increased fusion similarly to the Matrigel-induced fusion observed. Collagen IV also increased the percentage of $MyoD^+$ cells. This suggests that these cells were committed to differentiate and that fusion levels may have been more substantially increased by collagen IV over a longer time frame. This result led us to believe that the positive effect on fusion displayed by Matrigel was, at least in part, due to its second largest constituent, collagen IV.

Collagen IV has been shown to interact with the CD9 receptor; blocking the CD9 receptor on C2C12 cells has been shown to reduce their fusion *in vitro* (Tachibana and Hemler, 1999, Charrin *et al.*, 2013). Collagen IV may therefore interact with the CD9 receptor to increase myoblast fusion. Assessment of CD9 expression on C2C12 cells cultured in absence and presence of collagen IV exposed a possible mechanism for our findings; CD9 expression is elevated at day 1 and day 5 of differentiation in response to collagen IV. Blocking of CD9 (with monoclonal antibodies raised against CD9) further supported this finding with differentiation of C2C12 cells cultured on collagen IV more resistant to the inhibitory effects of these neutralizing antibodies. Assessment of MyoD at earlier time points (day 1), as well as myogenin, could lead to additional insight into the mechanism at play. Collagen IV was not observed to have a significant effect on C2C12 cell number, viability or CD9 expression under proliferative conditions.

In an attempt to more closely mimic *in vivo* conditions, we then supplemented C2C12 cells differentiating on collagen IV with HGF. Interestingly, collagen IV did not prevent lower concentrations of HGF (2 ng/ml) from inhibiting differentiation; however it was able to restore normal levels of MyHC, suggesting that 2 ng/ml HGF may be inhibiting the final stages of fusion, but not the earlier stages characteristic of myogenic progression. This was supported by the normal levels of MyoD in samples treated with collagen IV and HGF (day

1). Higher concentrations of HGF (10 ng/ml) did not increase collagen IV-induced fusion suggesting that these components do not have a synergistic or additive effect on myogenesis.

Blocking of CD9 with monoclonal antibodies raised against CD9 in samples treated with HGF (10 ng/ml) and cultured on collagen IV exposed the possibility that collagen IV may be producing its pro-myogenic effect on C2C12 cells by numerous mechanisms. This is suggested as although fusion was reduced in samples blocked with a dilution of 1/400 anti-CD9 antibodies, it was not completely abolished. This was in contrast to samples treated with HGF (10 ng/ml) where a dilution of 1/400 anti-CD9 antibodies completely abrogated all fusion. This suggests that CD9 activity is required for successful HGF signaling, which is in agreement with previously published results (Kamisasanuki *et al.*, 2011, Spotts, 2010). Analysis of CD9 expression levels in response to HGF is necessary before drawing further conclusions.

Interestingly, collagen IV was unable to modulate the dose-dependent effects of HGF on proliferation. This may be attributed to the fact that CD9 levels are not significantly elevated in proliferating C2C12 cells cultured on collagen IV suggesting a different mechanism at play under proliferative conditions.

In summary:

1. HGF has a dose-dependent dual role in myogenesis. Our results suggest that PI3K and p38 mediate the anti-proliferative effect of HGF at higher concentrations, however the pro-proliferative mechanism of 2 ng/ml HGF is still unclear.
2. Collagen IV promotes myogenesis in part through an increase in CD9 expression, however, multiple mechanisms may be at play.
3. Collagen IV is able to modulate the negative effect of HGF (2 ng/ml) on C2C12 MyHC expression, but is unable to restore adequate fusion.
4. Collagen IV does not to modulate the dose-dependent dual role of HGF on C2C12 proliferation.
5. HGF (10 ng/ml) requires active CD9 receptors to successfully promote C2C12 fusion.

Future work:

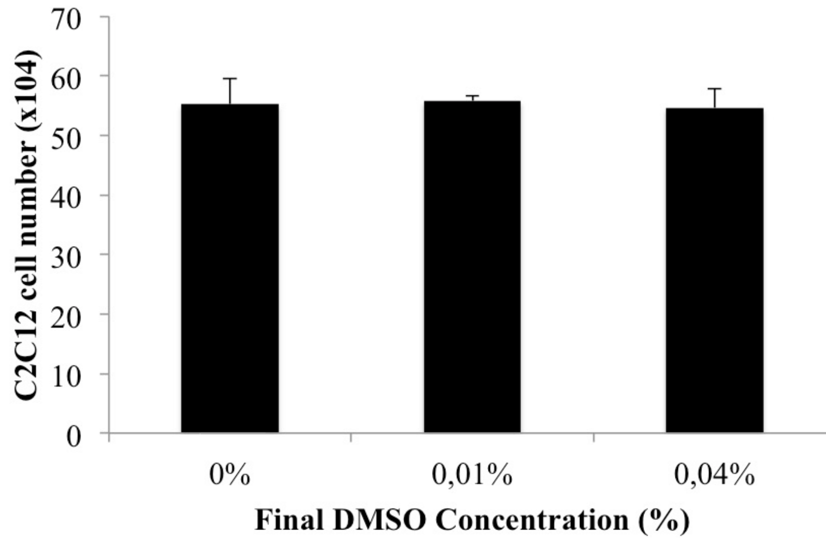
1. Analysis of fusion proteins such as kirrel, nephrin WAVE and nap1 would be valuable in understanding the effects of low doses of HGF on fusion (Kestila *et al.*, 1998, Richardson *et al.*, 2007, Strunkelnberg *et al.*, 2001).

2. Including analysis of the effect of collagen I and fibronectin (major components of fibrotic scar tissue found in extreme muscle injury) on myogenesis would further our understanding into the role of the ECM in fibrosis and wound repair.

3. Finally, although 2D monolayer cell culture studies provide tremendous insight into the role of ECM factors in myogenesis, investigations must be extended to 3D bioengineered tissue. This will provide information as to the role of the ECM factors on skeletal muscle repair in a system which more closely mimics the *in vivo* environment.

APPENDIX I: SUPPLEMENTARY FIGURE

Chapter 2:



Supplementary Figure 1: Effect of Dimethyl Sulfoxide (DMSO) on C2C12 cell viability. Viability of proliferating C2C12 myoblasts incubated in growth media containing either 0.04% or 0.01% DMSO was assessed at 24h. 100 000 C2C12 myoblasts were initially seeded and analysis performed using the TC20 automated cell counter (BioRad) for 3 independent experimental repeats, each in duplicate. Data is presented as mean ± SEM.

APPENDIX II: CONFERENCE ATTENDENCE AND PRESENTATIONS

Postgraduate Research Day, Faculty of Science & Agriculture, UKZN, 2012

EFFECT OF GROWTH AND EXTRACELLULAR MATRIX FACTORS ON DIFFERENTIATION OF MYOBLASTS

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Satellite cells are muscle stem cells that reside between the myolemma and the sarcolemma of mature muscle fibers. These cells are the means of cellular muscle repair and are activated to myoblasts upon muscle damage to proliferate and differentiate into myotubes. Differentiated myotubes fuse to existing muscle fibers with *in situ* necrotic muscle injury allowing full muscle repair. With shear-type injuries these myotubes fuse to fibrotic scar tissue in the wound and full muscle regeneration is not possible. ECM factors in the satellite cell niche maintain and regulate aspects of quiescence, activation, proliferation and differentiation. Following muscle injury, growth factors and interstitial extracellular matrix (ECM) factors direct the process of muscle regeneration and self-renewal. This process is controlled by a range of transcription factors and initiated by the transcription factor Pax7. Hepatocyte growth factor (HGF) initiates activation of quiescent satellite cells, but its downstream effect on differentiation is unknown. The activated myoblast will come into contact with interstitial extracellular matrix factors such as collagens, fibronectin, decorin and laminin. The effect of these ECM factors on C2C12 satellite cell activation and differentiation is relatively unknown. By differentiating C2C12 murine myoblasts in the presence of various ECM and growth factors we were able to assess the effect that these factors had on Pax7 nuclear localization and therefore commitment to differentiation. This study shows that collagen I and HGF stimulate the commitment to

differentiation of satellite cells via Pax7 activation. Decorin was observed to lower average Pax7 levels in the nucleus of proliferating and differentiating myoblasts. The addition of decorin is shown to negate the ability for collagen I to increase Pax7 nuclear localization. In conclusion, ECM factors have the ability to influence myogenesis. The use of ECM factors to induce rapid muscle healing *in vivo* requires further research.

COLLAGEN IV IS PRO-MYOGENIC AND ALSO REGULATES THE DOSE-DEPENDENT EFFECT OF HGF ON MYOGENESIS.

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Extracellular matrix (ECM) and growth factors are known to have complex interactions with one another which may modulate their activity *in vivo*. This supports the need to add combinations of ECM and growth factors during *in vitro* studies to more closely mimic *in vivo* conditions. Murine C2C12 myoblasts were cultured on collagen IV in HGF-supplemented media followed by assessment of differentiation and proliferation. Collagen IV was not able to negate the negative effect of HGF (2 ng/ml) on fusion but was able to restore normal MyHC expression. Collagen IV did not alter the pro-myogenic effect of 10 ng/ml HGF. Collagen IV restored normal MyoD expression at day 2 of differentiation when cells were exposed to HGF. In proliferative conditions, collagen IV alone was unable to effect C2C12 cell numbers, cell viability or CD9 expression. Furthermore, collagen IV was observed to be unable to modulate the previously reported dose-dependent effects of HGF on C2C12 cell numbers. These results show that collagen IV is able to modulate certain aspects of the dual role of HGF on myogenesis.

The South African Society for Biochemistry and Molecular Biology (SASBMB), Cape Town, South Africa, 2014

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"Stem Cells: From basic research to bioprocessing", London, UK; 2015

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“Stem Cells in Drug Discovery”, Cambridge, UK, 2015

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DOSE-DEPENDENT MODULATION OF MYOGENESIS BY HGF: IMPLICATIONS FOR C-MET EXPRESSION AND DOWNSTREAM SIGNALLING PATHWAYS

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Supervised by Dr Carola Niesler

Introduction

Hepatocyte growth factor (HGF) regulates satellite cell activation, proliferation and differentiation. HGF transduces its effects on satellite cells through specific interaction with the c-Met receptor.

Aim

We analysed the dose-dependent effects of HGF on C2C12 differentiation, proliferation and c-Met expression. We also attempted to understand the mechanisms at play by selectively inhibiting pathways downstream from c-Met.

Method

Murine C2C12 myoblasts were treated with 0, 2 or 10 ng/ml HGF followed by assessment of differentiation, proliferation and c-Met expression. Specific inhibitors of PI3K, ERK, JNK and p38K were utilized to determine pathway specificity.

Results

HGF (2 ng/ml) significantly promoted cell division, but reduced myogenic commitment and fusion. Conversely, 10 ng/ml HGF reduced proliferative capability, but increased differentiation. c-Met expression analysis revealed significantly *decreased* expression in differentiating cells cultured with 2 ng/ml HGF, but *increased* expression in proliferating cells with 10 ng/ml HGF. Mitogen-activated protein kinase (MAPKs: ERK, JNK or p38K) and phosphatidylinositol-3-kinase (PI3K) inhibition abrogated the HGF-stimulated increase

in cell number. Interestingly, PI3K and p38 kinase facilitated the negative effect of HGF on proliferation, while ERK inhibition abrogated the HGF-mediated decrease in differentiation.

Conclusion

Dose-dependent effects of HGF are mediated by changes in c-Met expression and downstream MAPK and PI3K signalling.

APPENDIX III: CLUSTAL W (1.81) MULTIPLE SEQUENCE ALIGNMENTS

A3.1 Collagen IV (Human vs. Mouse) Score 79.2899

CLUSTAL 2.1 Multiple Sequence Alignments Sequence type explicitly set to Protein Sequence format is Pearson Sequence 1: gi|1674441|gb|AAB19039.1| 1690 aa Sequence 2: gi|176866126|ref|NP_444415.2| 1691 aa Start of Pairwise alignments Aligning... Sequences (1:2) Aligned. Score: 79.2899 Guide tree file created: [clustalw.dnd] There are 1 groups Start of Multiple Alignment Aligning... Group 1: Sequences: 2 Score:28276 Alignment Score 9044 CLUSTAL-Alignment file created [clustalw.aln]

A3.2 HGF (Human vs. Mouse) Score 89.4882

Sequence type explicitly set to Protein Sequence format is Pearson Sequence 1: gi|337938|gb|AAA64297.1| 723 aa Sequence 2: gi|632774|gb|AAB31855.1| 728 aa Start of Pairwise alignments Aligning... Sequences (1:2) Aligned. Score: 89.4882 Guide tree file created: [clustalw.dnd] There are 1 groups Start of Multiple Alignment Aligning... Group 1: Sequences: 2 Score:11635 Alignment Score 4497 CLUSTAL-Alignment file created [clustalw.aln]

A3.3 Laminin 111 vs. Laminin 211 (Mouse) Score 33.8746

CLUSTAL 2.1 Multiple Sequence Alignments Sequence type explicitly set to Protein Sequence format is Pearson Sequence 1: gi|117647249|ref|NP_032507.2| 3118 aa Sequence 2: gi|148706391|gb|EDL38338.1| 3079 aa Start of Pairwise alignments Aligning... Sequences (1:2) Aligned. Score: 33.8746 Guide tree file created: [clustalw.dnd] There are 1 groups Start of Multiple Alignment Aligning... Group 1: Sequences: 2 Score:24218 Alignment Score 9334 CLUSTAL-Alignment file created [clustalw.aln]

APPENDIX IV: LYSATE PREPARATION, PROTEIN DETERMINATION AND SDS-PAGE

A4.1 Lysate Preparation

A4.1.1 Reagents

Protease Inhibitor Cocktail: The cocktail (Sigma, cat.P8340) consists of the following enzymes, AEBSF – [4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride], aprotinin, bestatin hydrochloride, E-64-[N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide], leupeptin hemisulfate salt and pepstatin A. The protease inhibitor was aliquoted into 20 µl Eppendorfs and stored at -20 °C.

RIPA Buffer (pH 8): The buffer (Sigma, cat.R0278) contains the following, 50 mM Tris-HCl with 150 mM sodium chloride, 1% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). The buffer was stored at 4 °C.

A4.1.2 Method

Cells were collected in a 15ml falcon via trypsinization and spun at 100 x g for 5 minutes on the bench top centrifuge (MRC; Polychem Supplies). The supernatant was carefully removed leaving the cell pellet behind. The cells were resuspended in 1 ml PBS and spun again at the same speed. This washing step was repeated three times. All subsequent steps were performed on ice. The cells were resuspended in 24 µl of RIPA buffer and 1 µl Protease Inhibitor Cocktail. It must be noted that these amounts are for cells from one well of a 6-well plate and must be adjusted to suit cell number. The cells were lysed for 1 hour on ice and were then sonicated at setting 13 (VirSonic 60, Polychem Supplies) until all cells were ruptured. Finally the samples were transferred into Eppendorfs (10µl aliquots) and stored at -20 °C.

A4.2 Bradford Assay

A4.2.1 Reagents

Coomassie Dye: 0.12 g Coomassie G-250 (Sigma, cat.B-0770) was dissolved in 5.7 ml perchloric acid (2% w/v) and made up to 200 ml with distilled water. The reagents were dissolved by stirring with a magnetic stirrer and were filtered through Whatman No.1 filter paper. The Coomassie dye was stored in a brown glass bottle at room temperature and kept

in the dark for up to 6 months. A calibration curve was created using a bovine serum albumin (BSA) stock solution (1 mg/ml) ranging from a 5-50% dilution.

A4.2.2 Method

5 µl of sample was added to 95 µl PBS buffer in a 2 ml Eppendorfs (Sigma, cat.T-7813) along with 900 µl Coomassie dye solution for 2 minutes. This mixture was then transferred to a 1 ml plastic micro-cuvette (Optima Scientific, cat.2711110) and read at A_{595} on a spectrometer (Ultrospec II E, LKB Biochrom). Protein concentrations were calculated from a linear equation generated from the calibration curve. All samples were run in triplicate to get an accurate assessment of protein concentrations.

Bovine serum albumin standards were used to construct a standard curve (Figure A5.1) as described by (Cazzolli *et al.*, 2001).

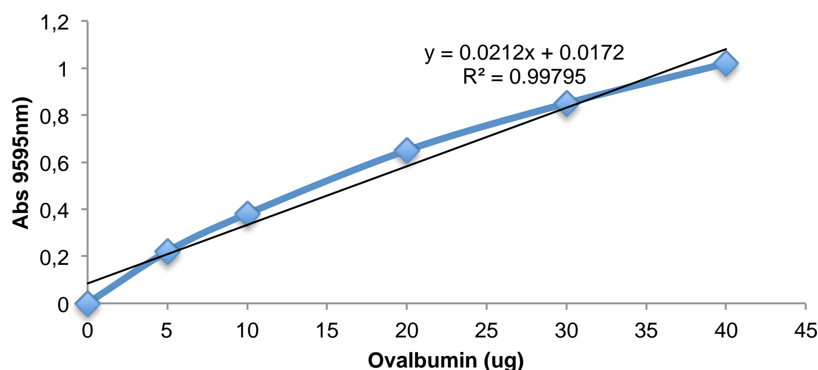

Figure A4.1: Bovine Serum Albumin (BSA) Bradford standard curve. Bovine serum albumin (BSA, Roche, 10735086001)) was used to construct a standard curve to determine the unknown concentrations of C2C12 cell lysates. BSA protein was made up in $d.H_20$ to a concentration of 1 mg/ml that served as a stock solution. A desired range (0 – 40 µg) of protein was added to Bradford reagent (900 µl), made to 1 ml with $d.H_20$ in Eppendorfs, vortexed and left to develop for 5 minutes. The protein-dye mixtures were transferred into plastic cuvettes and absorbance readings taken at 595 nm with a spectrophotometer. A linear regression formula was determined (**y = 0.0212x + 0.0172**) with a correlation coefficient of 0.99795. The regression formula was used to determine the protein concentrations of C2C12 lysates. To ensure accuracy, triplicates of each sample were tested.

A4.3 SDS-PAGE

A4.3.1 Reagents

Acrylamide/Bis-acrylamide monomer stock solution: 73 g acrylamide (30% m/v) and 2 g bis-acrylamide (2.67% m/v) were dissolved in 250 ml distilled water. The solution was filtered through Whatman No.1 filter paper and stored at 4 °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 titrated to pH 8.8 with HCl. The solution was filtered through Whatman No.1 filter paper and stored at 4 °C in a glass bottle.

4 x stacking gel buffer (500mM Tris-HCl, pH 6.8): 3 g Tris was dissolved in 50 ml distilled water. The pH was titrated to pH 6.8 with HCl. This solutions pH should be checked weekly. The solution was stored in a glass bottle at 4 °C.

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

Ammonium persulfate initiator solution: 0.1 g ammonium persulfate (10% m/v) was dissolved in 1 ml distilled water. This reagent was made up fresh prior to use.

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

Electrode (tank) buffer (250mM 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 °C.

Coomassie G-250 stain: 0.2 g Coomassie G-250, 24 ml phosphoric acid, 50 g aluminum sulfate, 100 ml ethanol were made up to 1 liter with distilled water. The solution was filtered with Whatman No.1 filter paper and stored in a plastic bottle at room temperature.

Table A4.3.1. Preparation of 10% and 12.5% Laemmli running and stacking gels for SDS-PAGE

Reagents	10%	12.5%	Stacking Gel
Monomer Solution	**5 ml**	**6.25 ml**	**0.94 ml**
4x Running Gel Buffer	**3.75 ml**	**3.75 ml**	**-**
4x Stacking Gel Buffer	**-**	**-**	**1.75 ml**
Distilled Water	**6 ml**	**4.75 ml**	**4.3 ml**
SDS Stock Solution	**150 µl**	**150 µl**	**70 µl**
Initiator Solution	**75 µl**	**75 µl**	**35 µl**
TEMED	**7.5 µl**	**7.5 µl**	**15 µl**

APPENDIX V: WESTERN BLOT AND DOT BLOT

A5.1 Western Blot

A5.1.1 Reagents

Blotting buffer: 27.23 g Tris and 64.8 g glycine were dissolved in 3.5 liters distilled water, followed by the addition of 900ml methanol. The solution was then made up to a final volume of 4.5 liters with distilled water. Prior to use 10% (m/v) SDS was added to the solution (i.e. 4.5 ml in 450 ml). Blotting buffer was used for 3 blotting runs before fresh blotting buffer was made again. Blotting buffer was stored in the fridge (4°C).

Tris buffer saline-Tween (TBST; 20mM Tris, 200mM NaCl, pH 7.4): 2.42g Tris and 11.69 g NaCl were dissolved in 950 ml distilled water. The pH was titrated to pH 7.4 with HCl and subsequently made up to 1 liter with distilled water. Finally, 500 µl Tween-20 (Sigma, cat.P1379) was added to the solution that was stored in the fridge (4 °C).

Ponceau S. stain: 0.1g Ponceau S. (Fluka, cat.81460) was dissolved in 1 ml acetic acid (100%) and made up to a final volume of 100 ml with distilled water. The solution was stored in a plastic bottle at room temperature.

A5.2 Dot Blot

Dot blot analysis was used to determine the optimal dilution of the primary and secondary antibodies for western blot analysis. A piece of nitrocellulose was marked into a grid with a pencil (Refer to figure 3.3). Note: gloves were worn at all times when dealing with protein samples. 1 µl of a protein sample was spotted into each square marked out on the nitrocellulose. The whole nitrocellulose piece was then blocked for 30 minutes, with 5% (m/v) non-fat milk powder made up in TBST. The nitrocellulose was cut into the marked squares that had been labeled with a predetermined antibody dilution range. Each antibody will have its own unique dilution range determined from the literature. Each square was placed into a separate well of a 12-well plate. The primary antibody dilutions were made up in TBST, and 500 µl of the specific dilution was added to each well for 2 hours. This was followed by washing steps with TBST (3 x 5 minutes). Secondary antibody dilutions were

made up in TBST and added to the well (500 µl) for 1 hour. The wells were then washed again with TBST (4 x 5 minutes). ECL was performed on the nitrocellulose squares. The dilution range showing optimal binding of both primary and secondary antibodies without non-specific binding was selected.

	Primary Antibody			
Secondary Antibody	1/250	1/500	1/1000	1/2000
1/2000	●	●	●	●
1/4000	●	●	●	●
1/8000	●	●	●	●

Figure A5.2.1: Dot blot layout for primary and secondary antibody optimizations.

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