THE ROLE OF MMP-14 AND MMP-2 IN MEDIATING MYOBLAST FUSION

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

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Abstract

Satellite cells are muscle precursor cells that have the ability to self-renew, proliferate and differentiate into myoblasts that eventually elongate and fuse to form myotubes which are vital for regeneration and repair of muscle. Satellite cells reside in a niche, between the sarcolemma of the muscle fiber and the basal lamina, which consists of mostly collagen IV, proteoglycans and laminin. Matrigel is a gelatinous protein mixture that consists primarily of collagen IV and laminin and therefore resembles the basal lamina. Matrix Metalloproteinases (MMPs) are zinc endopeptidases, proteolytic peptidases which break peptide bonds within their substrates. MMP-14 (membrane bound) also known as membrane-type 1 matrix metalloproteinase (MT1-MMP) is one of the major matrix metalloproteinases (MMPs) involved in muscle repair and regeneration, together with MMP-2 (secreted). MMP-2 is a secreted gelatinase A, which is activated by MMP-14. MMP-2 is also known to be activated by nitric oxide (NO), therefore allowing active MMP-2 to release growth factors such as Hepatocyte Growth Factor (HGF) from the extracellular matrix (ECM). There are two forms of MMP-2, intracellular MMP-2 and extracellular (secreted) MMP-2. Secreted MMP-2 contains a peptide signal that helps direct it outside the cell, while intracellular MMP-2 lacks this feature and is therefore retained within the cell. Intracellular MMP-2 activity is known to be a major cause of muscular atrophy. Secreted MMP-2 is known to degrade ECM components, facilitating satellite cell mobility and release of growth factors such as HGF, aiding in muscle regeneration. MMP-2 can cleave collagen IV due to the presence of a fibronectin-like domain within its catalytic domain; this is not the case with MMP-14. MMP-14 and MMP-2 together degrade collagens, fibronectin, laminin-2/4 and other adhesion molecules. This clears the path for the myoblast to align and fuse to form myotubes which then finally align to form mature muscle fibers. The levels of MMP-14 and MMP-2 must be regulated; low levels can cause muscular dystrophy. The current study analysed expression levels, activity and role of MMP-14 and MMP-2 in C2C12 myoblast differentiation. C2C12 myoblasts first proliferated (Day 0), then aligned and elongated (Days 1-2) and then finally fused into myotubes (Days 3-5) during differentiation. MMP-14 and MMP-2 protein levels were high during the elongation period and also during fusion of C2C12 myoblasts. MMP-14 was localised at the focal adhesions, where actin filaments terminate during myoblast proliferation and fusion.

Inhibition of MMPs using BB94 (10 μM) was observed to significantly reduce C2C12 myoblasts fusion. Secreted MMP-2 seems to play a vital role in the C2C12 differentiation, as activity was seen during myogenesis; when neutralised with an antibody, an 18% decrease in fusion was observed. Matrigel promoted an increase of MMP-2 expression within the cell during fusion (day 5 of differentiation), while no intracellular MMP-2 protein was observed at day 2 of differentiation. Levels of secreted MMP-2 increased significantly from day 2 to day 5 of differentiation; however, the presence of Matrigel significantly reduced levels of secreted MMP-2 detected in conditioned media at day 5 compared to uncoated conditions. The decrease is, in part, due to the fact that MMP-2 was found to bind to Matrigel. In conclusion, MMP-14 and MMP-2 play an important role in C2C12 myoblast elongation and fusion. This study provides further insight into the role of MMPs in myogenesis and lays the foundation for future work.

PREFACE

The experimental work described in this dissertation was carried out in the Discipline of Biochemistry, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, from January 2015 to June 2016, under the supervision of Dr. Carola. U. Niesler and Dr Celia Snyman.

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

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DECLARATION 1 - PLAGIARISM

I, Mthokozisi Siphesihle Nkosi, declare that:

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Acknowledgements

I would like to acknowledge first and foremost the kindness of my Lord and saver for guiding me throughout this year. Without his loving mercy's, I would not seize to exist, as scripture states, I can do all things through Christ who strengthens me. This study forms; part of my testimony on how great my God is, as I dedicate the study to the community of Newcastle and my super awesome mother, Nomthandazo M Vilakazi.

Secondly to my mentors and supervisors, Dr C Niesler and Dr C Snyman, for all the motivational words, financial strengthening, patience, encouragements, and equipment of skills, without them I would be totally lost. They have imparted to me their valuable wisdom, pushed me to seek greatness. I pray that God would continue to bless them and their families.

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ABBREVIATIONS

3D	three-dimensional
BSA	bovine serum albumin
CD44	cluster of differentiation (44)
CM	condition media
DMEM	Dulbecco's Modified Eagle Serum
DM	differentiation media
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
ECL	enhanced chemiluminescence
FCS	fetal calf serum
FGF	fibroblast growth factor
GAPDH	glyceraldehyde-3-Phosphate Dehydrogenase
GM	growth media
HGF	hepatocyte growth factor
HRPO	horseradish peroxidase
HS	horse serum
ICC	immunocytochemistry
IGF	insulin-like growth factor
IL-6	interleukin-6
MAPK	mitogen-activated protein kinase
MMPs	matrix metalloproteinases
MT1-MMP	membrane type-1 matrix metalloproteinase
MMP-14	matrix metalloproteinase-14
MMP-2	matrix metalloproteinase-2
MRF(s)	myogenic regulatory factors
Myf5	myogenic factor 5
MyoD	myoblast determination protein
MyoG	myogenin
MyHC	myosin heavy chain
NF-AT	nuclear factor of activated T-cells
Pax7	paired-box protein 7
PBS	phosphate buffered saline
PenStrep	penicillin-streptomycin
PDGF	platelet-derived growth factor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TGF-β	transforming growth factor-β
TBST	tris buffered saline with tween

Chapter 1:
Literature review

1.1. **Skeletal muscle regeneration and repair**

Skeletal muscle is an essential component of the human body; it is required for body movement, breathing and postural behaviour (Ohtake et al., 2006). Extreme activity or injury may lead to muscle damage; therefore a cellular repair system is needed for the regeneration of this tissue (De Bari et al., 2003). Satellite cells are muscle stem cells which, following a severe muscle injury, are activated to myoblast precursors and assist in regeneration (Grounds, 2014). Satellite cells are located in a niche environment between the basal lamina and sarcolemma of the myofiber (Mauro, 1961, Muir et al., 1965, Charge and Rudnicki, 2004, Pannérec et al., 2012). They were originally identified in the rear limb muscle of frogs (Mauro, 1961); subsequent studies were carried out to isolate and characterize these cells in mice and fruit bats (Muir et al., 1965). Satellite cells are spindle-shaped (elongated body) with small nuclei and reduced organelle content in comparison to other mammalian somatic cells (Charge and Rudnicki, 2004). C2C12 cells represent an immortal myoblast cell line derived from satellite cells that were isolated from the thigh muscle a of C3H mouse which had been subjected to a crush injury (Yaffe and Saxel, 1977). C2C12 cells differentiate into myocytes and myotubes under appropriate culture conditions and are therefore commonly used as an *in vitro* model of skeletal muscle myogenesis. Extracellular matrix (ECM) components and growth factors within the satellite cell niche keep them quiescent until they are needed for tissue repair and regeneration (Mauro, 1961, Allen et al., 1995, Grefte et al., 2012, Sanes, 2003).

Muscle regeneration is a process during which new muscle tissue is generated following a myonecrotic event, where inflammatory cells assist in activating quiescent satellite cells (Grounds, 2014). Inflammation is crucial for complete muscle regeneration; when inflammation is prevented or delayed, the removal of necrotic tissue will be impaired leading to incomplete muscle repair (Grounds, 2014). Inflammatory cells such as neutrophils, macrophages, mast cells and lymphocytes secrete essential enzymes and cytokines that assist in the removal of necrotic tissues around the wounded area, allowing for new muscle regeneration (Grounds, 2014). ECM degradation is very important during muscle regeneration

and therefore matrix metalloproteinases (MMPs) play a vital role in the degradation of various ECM components (Lluri and Jaworski, 2005b, Ohtake et al., 2006, Vu and Werb, 2000).

1.1.1. **Myogenesis**

The sequential activation of intrinsic muscle-specific transcription factors known as MRFs (Myogenic regulatory factors) forms the basis of myogenesis; these MRFs include MyoD, MyoG (myogenin), Mrf4 and Myf5 (Enwere et al., 2014). Myogenesis includes stages of satellite cell activation to myoblasts, proliferation, differentiation and fusion (Enwere et al., 2014, Bentzinger et al., 2012) (Figure 1.1). During embryogenesis, most embryonic progenitors give rise to embryonic skeletal muscle; while the rest give rise to satellite cells which act as a stem cell reservoir in post-natal tissue (Figure 1.1). Paired-box (Pax) protein 3 is a regulator of embryonic muscle development, and is expressed by embryonic progenitors prior to their specification into satellite cells (Bentzinger et al., 2012, Relaix et al., 2006). Pax 7 is known to play a role in self-renewal pathways that maintains post-natal satellite cell population, and also prevents premature differentiation by negatively regulating myogenin (Le Grand and Rudnicki, 2007, Relaix et al., 2006, Bentzinger et al., 2012).

Following activation, the expression of Pax7 is gradually lost, while Myf5 and MyoD expression increases (Enwere et al., 2014, Bentzinger et al., 2012) (Figure 1.1). Myf5 is known to regulate satellite cell activation and proliferation (Charge and Rudnicki, 2004, Enwere et al., 2014, Zhu and Miller, 1997). While MyoD is known to promote differentiation by activating myogenin, which in turns upregulates the production of myosin heavy chain (MyHC), down-regulation of Pax7 is crucial for myogenin expression (Charge and Rudnicki, 2004, Enwere et al., 2014). Mrf4 and myogenin are both required for successful terminal differentiation (Zhu and Miller, 1997).

During final stages of fusion, many aligned elongated myoblasts fuse with one another to form multi-nucleated myotubes (Figure 1.1). Fusion occurs in two stages; early fusion is when mono-nucleated cells proliferate and fuse, producing multinucleated myotubes. The second fusion step occurs when additional myoblasts fuse to existing myotubes to form larger myotubes which finally align to form muscle fibers. These stages are distinguishable by their cellular morphologies and gene expression patterns (Ohtake et al., 2006, Enwere et al., 2014, Charge and Rudnicki, 2004).

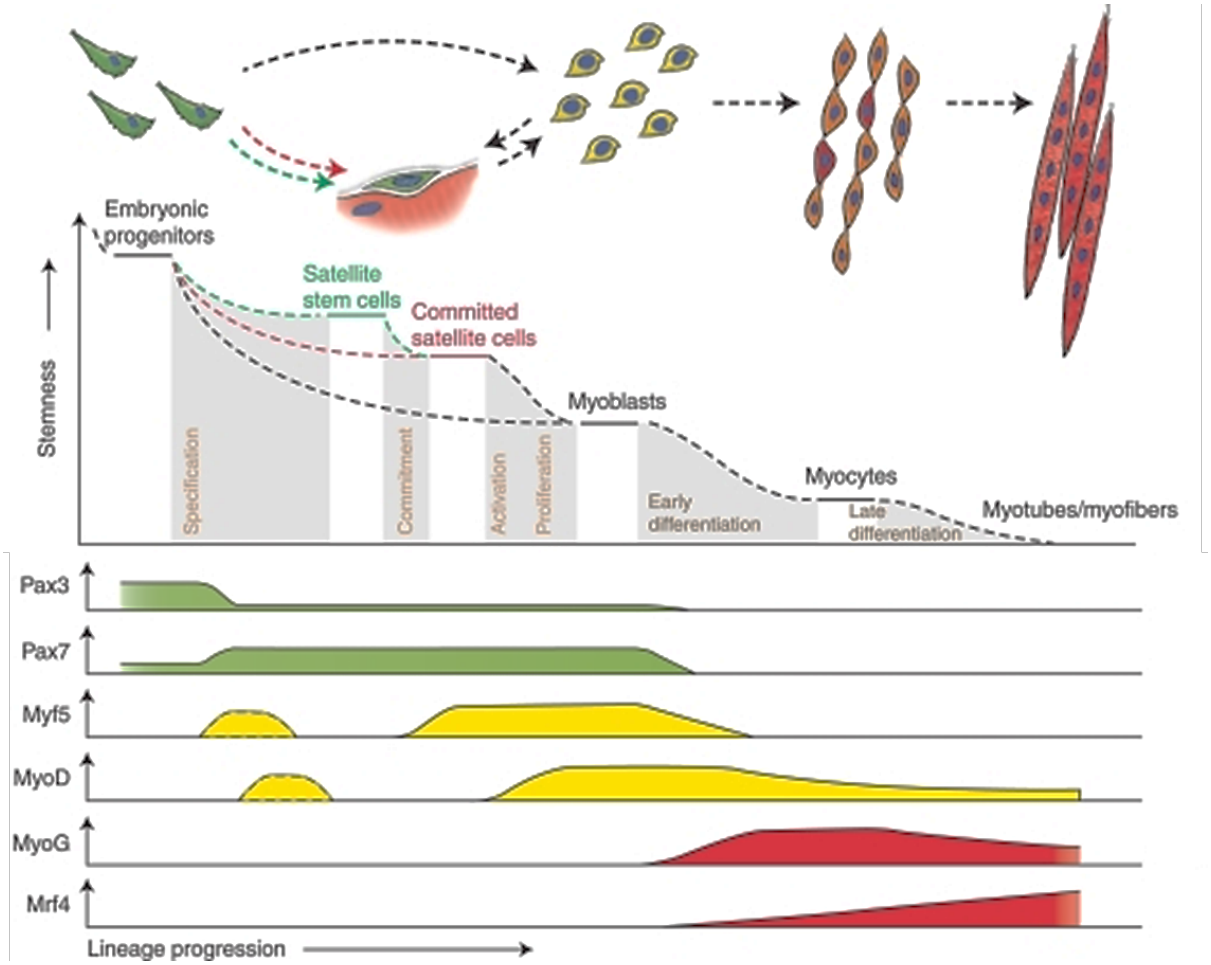


Figure 1.1: **Satellite cells and mammalian skeletal myogenesis**. Schematic diagram that shows the journey of embryonic progenitors to give rise to satellite cells which can form myotubes/myofibers. Embryonic progenitors contains high levels of paired-box (Pax) protein 3; the cells will undergo a period of specification where Pax 3 levels drop while Pax 7 levels increases, giving rise to satellite cells. Levels of transcription factors such as myogenic differentiation 1 (MyoD), Pax 7, myogenin (MyoG), Myf5 and muscle regulatory factor 4 (Mrf4) change as myogenesis progresses. Myoblasts align and fuse during late differentiation to form myotubes/myofibers. Figure is taken from and edited using Bentzinger et al., 2012.

1.1.2. **Regulation of myogenesis during regeneration**

Skeletal muscle myofibers are surrounded by an extracellular matrix (ECM) which provides tissue structure support, and also regulates many cellular processes, such as activation proliferation, differentiation and fusion of satellite cells (Ohtake et al., 2006, Bernal et al., 2005). The ECM consists of proteins, proteoglycans, and polysaccharides (Figure 1.2 A); the ratio of these components differs with each tissue type. The reticular lamina consists mainly of collagen I, Collagen VI, fibronectin and decorin (Thomas et al., 2015). The basal lamina of the

skeletal muscle is composed primarily of triple-helical collagen IV and laminin (Sanes, 2003). Perlecan help connect collagen VI to collagen IV in the basal lamina, where nidogen connects collagen IV to laminins that interacts with other proteins such as dystroglycan complex (Figure 1.2 A). Collagen IV and laminin from the basal lamina interact with satellite cells via integrins assisting in maintaining their quiescence (Thomas et al., 2015, Sanes, 2003). The basement membrane is surrounded by interstitial connective tissue which is mostly composed of collagens but also contains components like fibronectin, integrins, tenascins, laminins, and proteoglycans (Kjaer, 2004, Thomas et al., 2015) (Figure 1.2). Growth factors are found within the basal lamina and intramuscular connective tissue (Booth, 2006, Allen et al., 1995). They activate quiescent stem cells, and regulate proliferation and aspects of migration and terminal differentiation (Allen et al., 1995). Key growth factors that regulate myogenesis include Hepatocyte Growth Factor (HGF), Fibroblast Growth Factor (FGF), Transforming Growth Factor beta (TGF-β), Insulin-like Growth Factor (IGFs) and Platelet-derived Growth Factors (PDGFs) (Kawada et al., 2009, Allen et al., 1995, Booth, 2006). Growth factors reside within the ECM and will undergo activation via the help of proteases, such as serine proteases and MMPs (Figure 1.2 B) (Allen et al., 1995, Thomas et al., 2015, Filippin et al., 2011b, Filippin et al., 2011a, Mann et al., 2011). Hepatocyte Growth Factor (HGF) is a major factor known to facilitate activation of satellite cells to myoblasts (Allen et al., 1995); levels of expression of growth factors such as HGF therefore play a vital role in myogenesis (Allen et al., 1995, Walker et al., 2015).

There are cell signalling molecules that plays an major role in myogenesis, such as canonical (classical) NF-kB activity that increases during myogenic activation and proliferation (Enwere et al., 2014). A cytokine known as TNF-α-like weak inducer of apoptosis (TWEAK), plays a vital role in controlling the levels of NF-kB with the help of cellular inhibitor of apoptosis 1 (cIAP1) (Enwere et al., 2014). After the proliferation stage, TWEAK will signal the increase of NF-kB to stop myoblasts from dividing and start aligning to fuse and form a mature muscle fiber bundle (Enwere et al., 2014).

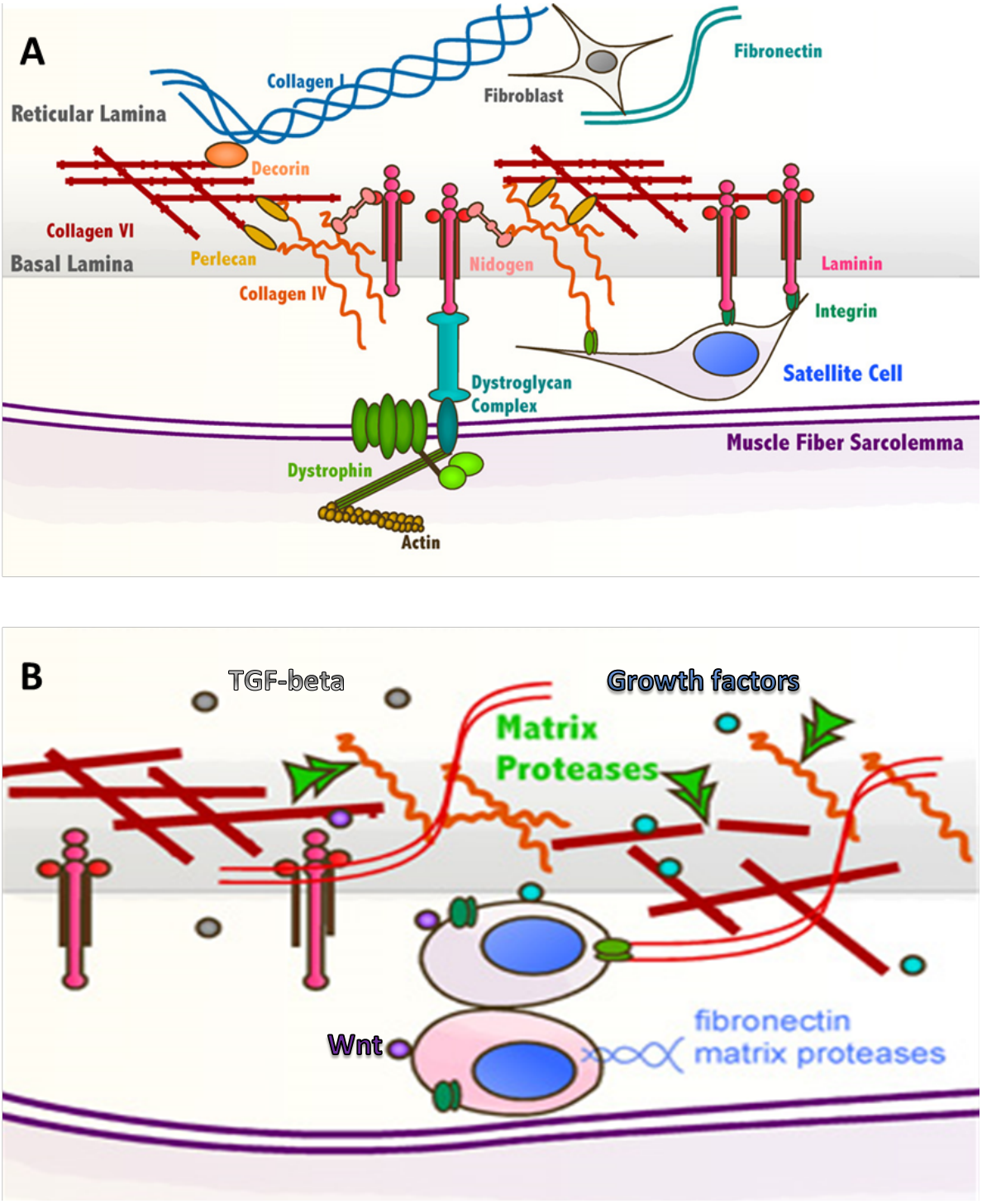


Figure 1.2: **A schematic diagram of the niche environment of a satellite cell**. **A)** Niche environment of quiescent satellite cell showing ECM components such as collagens, fibronectin, laminins, integrins, proteoglycans and other proteins such as dystrophin and actin. **B)** Activated niche environment showing active MMPs release growth factors (light blue circles) and activate satellite cells. Figure is taken from and edited using Thomas et al., 2015.

1.2. **MMPs**

MMPs (Matrix Metalloproteinases) are a family of zinc endopeptidases that can selectively digest individual components of the extracellular matrix (ECM) (Chen and Li, 2009). They were first discovered in tadpoles and have been studied intensely since then, particularly in the context of cancer (Maskos, 2005, Gomis-Ruth et al., 1997). There are twenty-five MMP family members; 6 are membrane-type (MT) MMPs and the rest are secreted (Visse and Nagase, 2003, Maskos, 2005). In skeletal muscle, MMPs control homeostasis and maintain myofiber function by regulating skeletal muscle cell migration, differentiation and regeneration (Chen and Li, 2009, Davis et al., 2013). MMP-14 (membrane bound) and MMP-2 (secreted) are thought to be particularly important in skeletal muscle; knockout studies in mice have revealed muscular dysfunction as part of their phenotype (Table 1.1).

MMP	Phenotype	References
MMP-14 (also known as MT1-MMP)	Premature death, due to **skeletal defects and dwarfism**. Phenotype normal at birth but develop multiple abnormalities (defect in remodelling of the connective tissue, increased bone resorption and defective secondary ossification centres) and die by 3-12 weeks. Defects in lung and submandibular gland and angiogenesis defects.	(Holmbeck et al., 1999, Löffek et al., 2011)
MMP-2	No overt phenotype and **reduced body size**. Decreased bone mineralisation is observed, joint erosion, defects in osteoblast and osteoclast growth. A decrease in primary ductal invasion in the mammary gland. A reduced neovasculation and tumour progression.	(Itoh et al., 1998, Löffek et al., 2011)
MMP-14/MMP-2 combined	The mice died immediately after birth with respiratory failure, abnormal vessels and **immature muscle fibers**.	(Oh et al., 2004, Löffek et al., 2011)

Table 1.1: **Phenotype of MMP-14 and MMP-2 single and double knockout mice**

MMP activity is regulated by specific tissue inhibitor of metalloproteinases (TIMPs) that bind MMPs in a 1:1 stoichiometry (Visse and Nagase, 2003). Four members of the TIMP family (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) have been identified (Chen and Li, 2009). TIMP-2

controls MMP-14 activity in muscle and facilitates maturation of secreted proMMP-2 (Visse and Nagase, 2003, Ohtake et al., 2006). Together MMP-14 and MMP-2 can degrade almost all ECM components (Table 1.2); with MMP-9, they are central enzymes in regulation adaptation of skeletal muscle (Davis et al., 2013).

Name & location	Substrates	References
MMP-2 **Secreted**	Collagens (type I, II, III, IV, V, VII, X, and XI), gelatin, elastin, fibronectin, vitronectin, laminin, entactin, tenascin, SPARC, aggrecan, link protein, galectin-3, versican, decorin, myelin, basic protein.	(Visse and Nagase, 2003, Seiki, 2003, Osenkowski et al., 2004, Ohtake et al., 2006, Nagase et al., 2006, Botos et al., 1996, Murphy, 1995)
MMP-14 **Membrane- bound**	Collagens (type I, II, and III), gelatin, fibronectin, tenascin, proMMP-2, vitronectin, laminins, entactin, aggrecan, E-cadherin, dermatin sulphate proteoglycan, perlecan, CD44, perlecan.	(Visse and Nagase, 2003, Seiki, 2003, Osenkowski et al., 2004, Ohtake et al., 2006, Nagase et al., 2006, Botos et al., 1996, Murphy, 1995)

Table 1.2: **MMP-2 and MMP-14 substrates**

1.2.1. **MMP-14 and MMP-2**

MMP-14 and MMP-2 are similar in structure with exception of a trans-membrane, cytoplasmic and furin-cleavage domain seen only in MMP-14 and a triple fibronectin type II repeat in the catalytic domain of MMP-2 (Figure 1.3) (Murphy and Crabbe, 1995, Visse and Nagase, 2003, Anita and Murphy, 1998). Mature MMP-14 and MMP-2 are ~54-58 kDa and 64 kDa respectively (Nagase et al., 2006, Murphy and Crabbe, 1995). The signal sequence, a short peptide sequence located at the N-terminus of MMP-14 and MMP-2, directs MMP-14 and MMP-2 towards the plasma membrane. There are two MMP-2 forms produced, intracellular MMP-2 and extracellular (secreted) MMP-2, where intracellular MMP-2 is produced without the signal peptide while secreted MMP-2 contains a signal peptide that directs MMP-2 outside the cells (Liu, 2011). A pro-peptide of about 80 amino acids blocks the catalytic domain and must be removed for activation of both MMP-14 and MMP-2. A furin consensus sequence (Arg108-Arg109-Lys110-Arg111) is found between the pro-peptide and catalytic domains on MMP-14 (not MMP-2), which, together with the cysteine switch, helps activate MMP-14 during pro-peptide cleavage (Nagase et al., 2006, Visse and Nagase, 2003). This is followed

by a catalytic metalloproteinase domain of about 170 amino acids, a linker peptide of variable lengths (known as a 'hinge region') and a hemopexin domain of about 200 amino acids (Nagase et al., 2006).

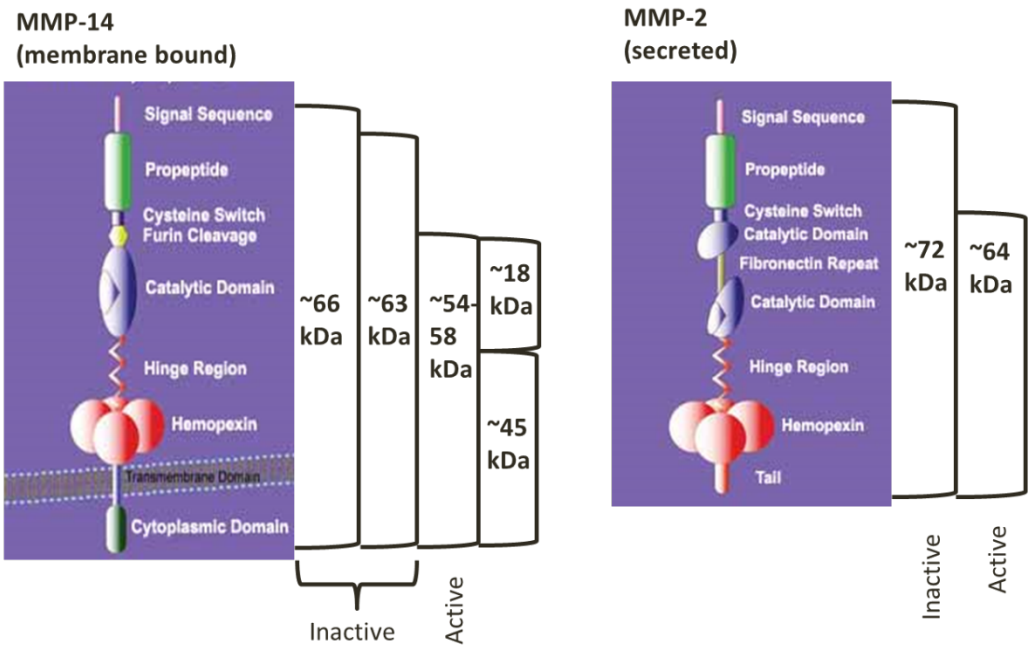


Figure 1.3: **A schematic diagram of the structure of MMP-14 and MMP-2**. Structural components and different processed forms of MMP-14 and MMP-2 are shown. The structural components consist of a cytoplasmic domain, trans-membrane domain (hydrophobic residues), and extracellular region containing hemopexin, hinge region, catalytic domain, cysteine switch, pro-peptide and signal sequence. MMP-2 lacks the trans-membrane domain, furin cleavage domain and cytoplasmic domain while MMP-14 lacks a fibronectin repeat. Figures are taken and edited using sigmaaldrich.com and Remacle et al., 2006, Anita and Murphy, 1998.

The catalytic domain of all MMPs is similar (Maskos, 2005). The active site zinc is bound in the sequence HELGHXXGXXH by three histidines residues, together with a 'catalytic' Glutamine and a water molecule at the active site cleft (Figure 1.4a). The water molecule can only enter the active site once the pro-domain has been cleaved; the water helps in peptide hydrolysis. The positioning of the S1' pocket serves to accommodate hydrophobic residues. The different sizes of the S1' pocket (allowing different sized substrates) and the lack of either Leu193 or Tyr193 leads to the different substrate specificity of MMPs (Nagase, 2001, Nagase et al., 2006). The triple-repeat of fibronectin type II domain on MMP-2 allows it to bind with collagen IV, while MMP-14 lacks this feature (Figure 1.3). Therefore MMP-2 can degrade collagen IV, while MMP-14 cannot. The catalytic domain contains three calcium-binding sites; calcium ions help in stabilizing the complex (Iyer et al., 2006, Maskos, 2005).

The reaction mechanism is as follows: the carbonyl group of the scissile peptide bond, which is facing the catalytic zinc, is strongly polarized (Figure 1.4b). The water molecule that is bound to the zinc is activated by the glutamate and it is oriented to attack the electrophilic carbonyl carbon. The tetrahedral intermediate is stabilized by the zinc ion. One water molecule is shuttled via the glutamine carboxylate to the amino group of the scissile bond that is probably stabilized by the carbonyl group of the first alanine by the 'edge strand'. The peptide bond is cleaved and a proton is transferred to the amino group; the two product fragments leave the active site (Figure 1.4b).

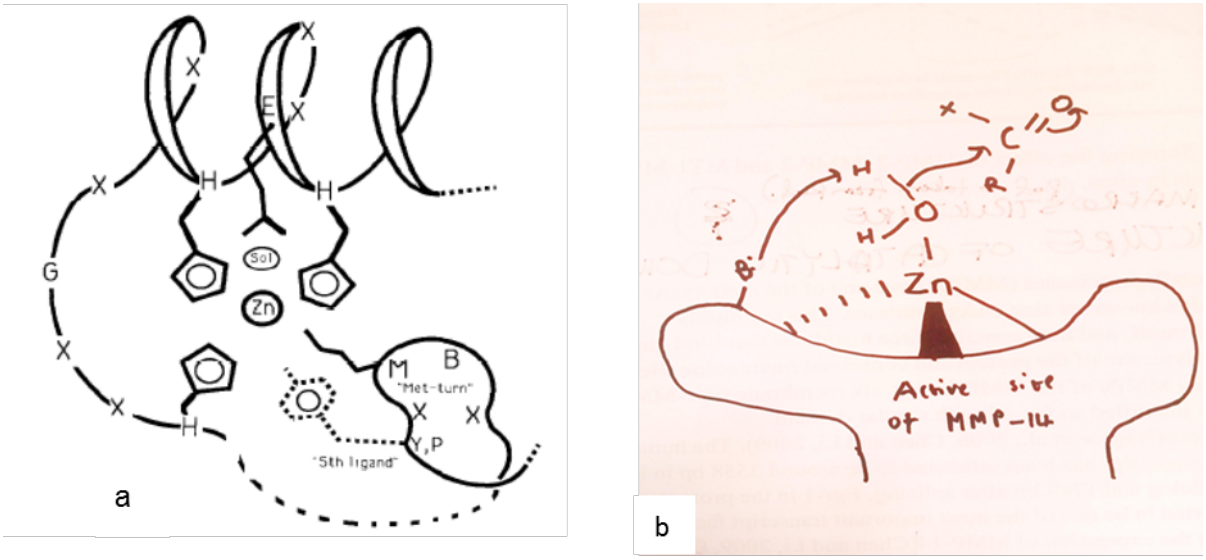


Figure 1.4: **a) Schematic representation of zinc environment in the "active" site of MMP-14 and b) schematic diagram for the reactions of MMP-14 in binding of substrate.** The zinc signatures, Met-turn are shown by a continuous line while variable features by a dashed chain trace. The three histidines (H) residues serve as zinc ligands and glutamic acid (E) residue helps in polarizing of a water molecule involved in nucleophilic attack of incoming scissile peptide bond. Figure is taken from and edited using Bode et al., 1993.

The hinge region (15-65 amino acids) is an elongated peptide segment that is in close contact with the catalytic and the hemopexin-like domain (Maskos, 2005). It is represented as a spring which allows flexibility of the MMP-14 (Figure 3). The hinge-region is important for the stability of the enzyme and the degradation of complex substrates such as fibrillar collagen and for activation of proMMP-14 which require the concerted action of the catalytic and the hemopexin domain (Visse and Nagase, 2003, Osenkowski et al., 2004, Nagase et al., 2006).

The hemopexin-like (also known as PEX) domain is the largest domain; it contains an additional loop structure (eight amino acids) on the outermost strand of each blade that is responsible for its high substrate specificity (Ndinguri et al., 2012, Zarrabi et al., 2011). The PEX domain is organized in four β-sheets (blades). The arrangements of the four blades form a four-bladed propeller with a central channel. The structure of the PEX domain is stabilized via a double sulphide bond and central calcium ion. The four bladed beta-propeller structures provide a suitable flat surface for protein-protein interactions. In the collagenase superfamily, the PEX domain is important for the ability to process triple-helical collagen like collagen I (Maskos, 2005). The catalytic domain alone can't process collagen, but is able to cleave gelatin-like peptides (Maskos, 2005). The strands of each twisted sheet are linked in a W-like topology with the first strands forming the central pore and also defining the direction of the channel. The PEX domain is very important in interactions with other molecules such as TIMPs, but also in dimerization. Dimerization occurs via the PEX domains just after activation via initial cleavage.

The trans-membrane domain consists of mainly hydrophobic amino acid residues that insert into the cellular plasma membrane. The cytoplasmic domain is composed of mainly hydrophilic residues which extend from the trans-membrane domain. The cytoplasmic tail has been reported to play a role in the formation of oligomers and the localisation of the enzyme near substrates such as CD44 (Mori et al., 2013, Seiki, 2003).

The lack of a trans-membrane domain and the presence of the signal peptide allows MMP-2 to be secreted and not membrane bound like MMP-14 (Thomas et al., 2015, Nagase et al., 2006, Davis et al., 2013, Anita and Murphy, 1998).

1.2.1.1. **Processed forms of MMP-14 and MMP-2**

MMP-14 can exist in 5 processed forms (Figure 1.3) (Remacle et al., 2006, Snyman and Niesler, 2015). The zymogen (inactive) form containing all the domains is a ~ 66 kDa protein (Remacle et al., 2006, Golubkov et al., 2007). Partial processing results in a ~63 kDa intermediate (Remacle et al., 2006, Hofmann et al., 2000) that lacks the signal domain, but still has a furin site. Secreted MMP-2 before it is processed will contain a signal peptide that directs MMP-2 outside the cell, but intracellular MMP-2 lacks this signal peptide therefore retaining it within the cell. Further processing results in a mature 54-58 kDa active form, by furin-cleavage (by cysteine switch) removing the pro-peptide (Remacle et al., 2006, Golubkov et al.,

2007). This can be further auto-catalytically cleaved to an inactive form of ~45 kDa (mature MMP-14 without catalytic domain) and a soluble form of ~18 kDa (catalytic domain) (Remacle et al., 2006).

1.2.1.2. **MMP-14 matures proMMP-2**

The mature active form of MMP-14 is responsible for the activation of pro-MMP-2 (~72 kDa) by cleavage of the pro-domain to active MMP-2 (~64 kDa) (Figure 1.3). To facilitate this, TIMP-2 binds to one of the MMP-14 catalytic domains (Figure 1.5). ProMMP-2 then binds to the unoccupied catalytic domain of a second MMP-14 and also binds to C-terminal domain of TIMP-2 through its PEX domain. MMP-14 form dimers on the cell surface through interaction of the PEX domains. The proMMP-2 forms a tight complex with TIMP-2 though PEX domain interactions with the non-inhibitory C-terminal domain of the TIMP. The complex formation is essential for proMMP-2 activation by MMP-14 on the cell surface. The complex binds to an active MMP-14 via the free N-terminal MMP inhibitory domain of TIMP-2, which orientates the pro-peptide of proMMP-2 to an adjacent active MMP-14 (Nagase et al., 2006). MMP-14 then cleaves the bait region of proMMP-2, partly activating it. The final step occurs when MMP-2 dissociates from the membrane and is fully activated by intermolecular processing (Visse and Nagase, 2003). MMP-2 and MMP-14 are together able to cleave collagens, fibronectin, laminin-2/4 and other adhesion molecules (Seiki, 2003, Ohtake et al., 2006, Wernig et al., 2004). This cleavage allows myoblasts to migrate, align and fuse to form myotubes and also assists in release of growth factors such as HGF from the ECM (Thomas et al., 2015, Ohtake et al., 2006, Allen et al., 1995, Hara et al., 2012).

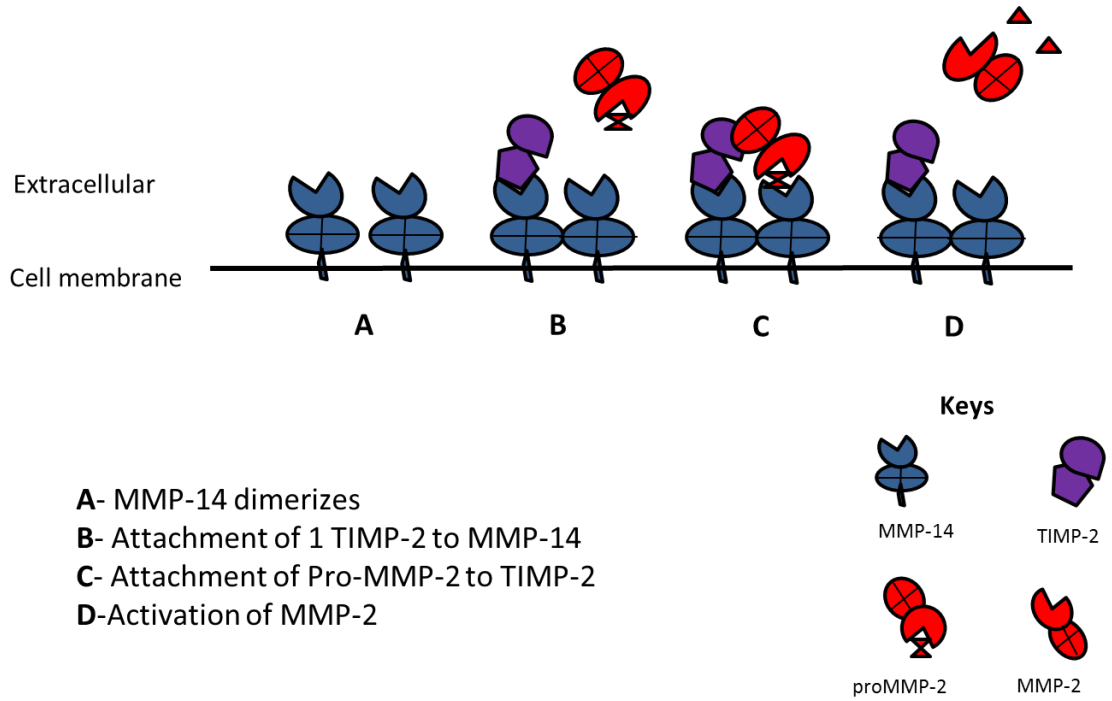


Figure 1.5: **A schematic diagram showing activation of proMMP-2 by MMP-14 and TIMP-2.** Figure was constructed using Visse and Nagase, 2003.

1.2.1.3. **Inhibitors of MMPs**

Batimastat (BB94), an angiogenesis inhibitor, inhibits MMPs by inserting one of its rings within the MMP specificity site (Botos et al., 1996). IC_{50} values for inhibition of MMP 1-3 and MMP 7-9 shown in Table 1.3 (Botos et al., 1996).

MMPS	IC_{50} (~ nM)	References
MMP-1	3	(Botos et al., 1996, Hidalgo and Eckhardt, 2001)
MMP-2	4	
MMP-3	20	
MMP-7	6	
MMP-8	10	
MMP-9	1	

Table 1.3: **BB94 IC50 for MMPs**

Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and 1,10 Phenanthroline are metal chelators, which can also inhibit MMPs by chelating calcium and zinc (Bencini and Lippolis, 2010, Přibil, 2013). EDTA falls within the aminopolycarboxylic acid family; it chelates metal ions via its two amines and four carboxylates adopting a stable octahedral geometry complex with metal ion (Přibil, 2013) (Figure 1.6). However EDTA is a non-specific inhibitor of metalloproteases, capable of influencing/binding essential ions needed for other enzyme activity and cell survival (Osenkowski et al., 2004, Harayama et al., 1999, Murphy, 1995). 1,10 Phenanthroline, similar to 2,2`-bipyridine, is a heterocyclic organic compound that can chelate metal ions (Bencini and Lippolis, 2010) (Figure 1.6). 1,10 Phenanthroline has an higher afinity for zinc ion then Ca^{2+}, making it more specific to MMPs in comparison to EDTA (Bencini and Lippolis, 2010).

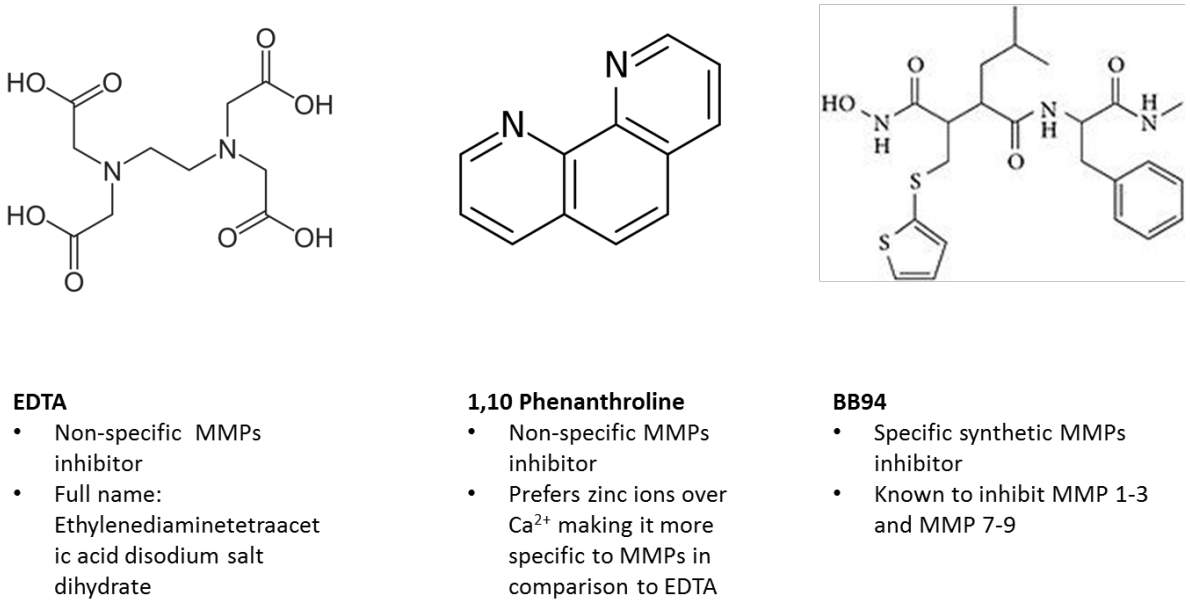


Figure 1.6: **MMP inhibitors.** Structures of MMP inhibitors (EDTA, 1,10 Phenanthroline and BB94) and their characteristics are shown. Figure is taken from and edited using Wikipedia, Bencini and Lippolis, 2010, Přibil, 2013, Osenkowski et al., 2004, Murphy, 1995, Botos et al., 1996, Harayama et al., 1999.

1.3. MMP-14 and MMP-2 in skeletal muscle regeneration

MMPs and TIMPs play important roles in skeletal muscle-derived myoblast migration and differentiation *in vitro* (Ohtake et al., 2006, Lluri and Jaworski, 2005a, Thomas et al., 2015). MMP inhibition by BB94 decreases myoblast fusion, emphasizing the importance of these proteases in skeletal muscle myogenesis (Lluri and Jaworski, 2005b, Ohtake et al., 2006). MMP-2 and MMP-14 knock-out studies in mice have also highlighted the importance of regulating both MMP-14 and MMP-2 levels, where knockout of MMP-14 (Table 1.1) leads

skeletal defects, dwarfism and premature death (Holmbeck et al., 1999, Itoh et al., 1998). In the absence of MMP-2 and MMP-14, cultured embryonic cells fail to form myotubes efficiently further supporting a role for MMP-2 and MMP-14 in successful myotube formation (Chen and Li, 2009).

Collagens are glycine-rich structures comprising of three polypeptide α-chains in a triple helix structure; they interact with cells primarily via receptors known as integrins (Kjaer, 2004). Collagen I is the most abundent of all collagens, contributing the major component of interstitial ECM that surrounds skelatal muscle fibers (Kjaer, 2004). In muscle, collagen IV forms a major component of the basal lamina interacting with laminins-2/4 and non-collagenous glycoproteins such as entactin/nidogen (Kjaer, 2004, Sanes, 2003). Collagen I is degraded by both MMP-14 and MMP-2, whereas collagen IV is degraded by MMP-2 not MMP-14 (Table 1.2).

Fibronectin is a glycoprotein that occurs in three different forms: soluble dimers, insoluble fibronectin fibrils and cell surface fibronectin oligomers. Fibronectin binds to collagens, integins and tenascin (Kjaer, 2004, Hocking et al., 2008) (Figure 1.2). During muscle regeneration, tenascins and fibronectins are produced by fibroblasts and deposited at the site of damage (Hocking et al., 2008). Fibronectin is degraded by both MMP-14 and MMP-2 (Table 1.2).

Laminins are found mainly in the basal lamina and consists of multiple heterodimers of α, β and γ chains (Kjaer, 2004). Laminins -2 and -4 (LM-211 and LM-221) are isoforms found in skeletal muscle and are located mainly at neuromuscular junctions (Grounds et al., 2005, Sanes, 2003). Laminins bind to the ECM components such as perlecan, proteoglycan and collagen IV in the basement membrane, connecting the myofiber to the basement membrane (Kjaer, 2004, Sanes, 2003). Both laminin isoforms are degraded by MMP-14 and MMP-2 (Ohtake et al., 2006).

Matrigel is a gelatinous protein mixture produced by Engelbreth-Holm-Swarm (EHS) mouse sarcoma that resembles the basal lamina as it is composed primarily of collagen IV and laminin; it has been shown to promote muscle differentiation (Grefte et al., 2012). Matrigel is degraded by secreted MMP-2 (Kuwahara et al., 2012).

TIMP-2, MMP-14 and MMP-2 are essential for myogenesis (Figure 1.7) (Ohtake et al., 2006, Lluri and Jaworski, 2005b). The effect of TIMP-2 on MMP-14 and MMP-2 is highly dose-

dependent with low TIMP-2 levels promoting MMP-14 activation of pro-MMP-2, but higher levels inhibiting MMP-14 activity by occupying MMP-14 catalytic domain and therefore preventing pro-MMP-2 activation (Chen and Li, 2009, Apte et al., 1997, Ohtake et al., 2006). During the elongation stage of myogenesis, MMP-14 degrades collagen, laminin and fibronectin thus allowing the myoblast to migrate and fuse to form myotubes (Ohtake et al., 2006) (Figure 1.1 and 1.7). MMP- 14 processes laminin-2 and 4, cadherin, fibronectin and collagen I *in vivo* and *in vitro* and appears to regulate both ECM-based and cell to cell adhesion (Ohtake et al., 2006, Seiki, 2003). A deficiency of MMP-14 and MMP-2 results in incomplete muscle tissue formation which leads to muscular dystrophy (Ohtake et al., 2006, Lluri and Jaworski, 2005a, Holmbeck et al., 1999, Oh et al., 2004).

MMP-14 protein as well as MMP-2 and TIMP-2 mRNA have been previously analysed during myoblast differentiation (Ohtake et al., 2006, Lluri and Jaworski, 2005b) (Figure 1.7). MMP-14 protein expression was shown to reach maximum levels within 24 hours of the initiation of differentiation, while MMP-2 mRNA took at least 72 hours to reach its highest levels. Levels of TIMP-2 mRNA was at its lowest at 24 hours, but reaches maximum levels during the fusion period of differentiation (Ohtake et al., 2006). Levels of MMP-14, MMP-2 and TIMP-2 decreased following fusion (Figure 1.7). This study provided some insight into the temporal expression of these factors; however, the expression level of secreted MMP-2 was not explored.

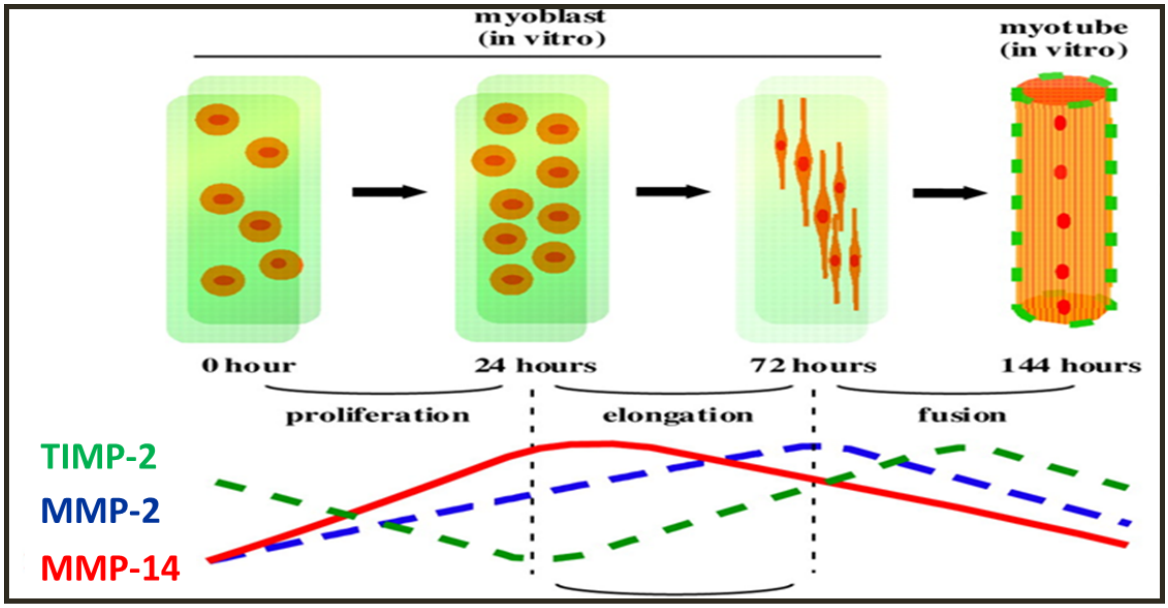


Figure 1.7: **Profile of TIMP-2 (mRNA), MMP-2 (mRNA) and MMP-14 (protein) expression during myogenesis**. Three distinct stages of myoblast differentiation are shown; Proliferation, elongation and fusion. Levels of MMP-14 and MMP-2 are high while TIMP-2 levels are very low during the elongation stages; after which levels of TIMP-2 start to increase, while MMP-2 and MMP-14 decrease. Figure taken and edited using Ohtake et al., 2006.

Extracellular (secreted) MMP-2 is essential during repair and regeneration of necrotic damaged muscle whereas intracellular MMP-2 plays a pivotal role in muscular atrophy (Liu, 2011). Muscular atrophy occurs when muscle wastes due to decreased muscle protein synthesis and increased muscle degradation (Liu, 2011). A previous study suggested one of the major causes of muscular atrophy is due to intracellular MMP-2 digesting some essential intracellular matrix proteins (Liu, 2011). A study showed that MMP-2 knockout mice show decreased muscular atrophy, highlighting the importance of developing a selective MMP-2 inhibitor as a potential treatment for muscular atrophy (Liu, 2011, Liu et al., 2010).

HGF plays a major role in activating and assisting satellite cells in the muscular repair and regeneration (Allen et al., 1995, Hara et al., 2012). Active secreted MMP-2 is known to activate and release HGF (Mann et al., 2011, Filippin et al., 2011b, Filippin et al., 2011a, Hara et al., 2012). Previous studies reveal that mechanical stress induced to satellite cells leads to a cascade of signal transduction pathways including; calcium-calmodulin formation and subsequent nitric oxide (NO) radical production by NO synthase (Figure 1.8) (Hara et al., 2012, Filippin et al., 2011a, Filippin et al., 2011b). This assists in MMP-2 activation, which in turn releases HGF from the ECM, and allows HGF to interact with the c-Met receptor that induces gene expression (Figure 1.8). The calcium-calmodulin formation happens in three steps. Step one occurs after mechanical perturbation (due to stretching or injury) where proton ions enter the cell via the MechanoSensitive Cation channel (MSC channel). Step two is the release of stored calcium ions from the sarcoplasmic reticulum and distribution on the cell surface. Step three is the activation of the Long-lasting-type Voltage-gated Calcium-ion channel (L-VGC channel) by depolarization of protons, which mediates the influx of extracellular calcium ions to form calcium-calmodulin formation (Figure 1.8). This highlights that MMP-14 and MMP-2 do not just function in degrading ECM factors, but also possible help in releasing essential growth factors such as HGF that assist myoblast fusion.

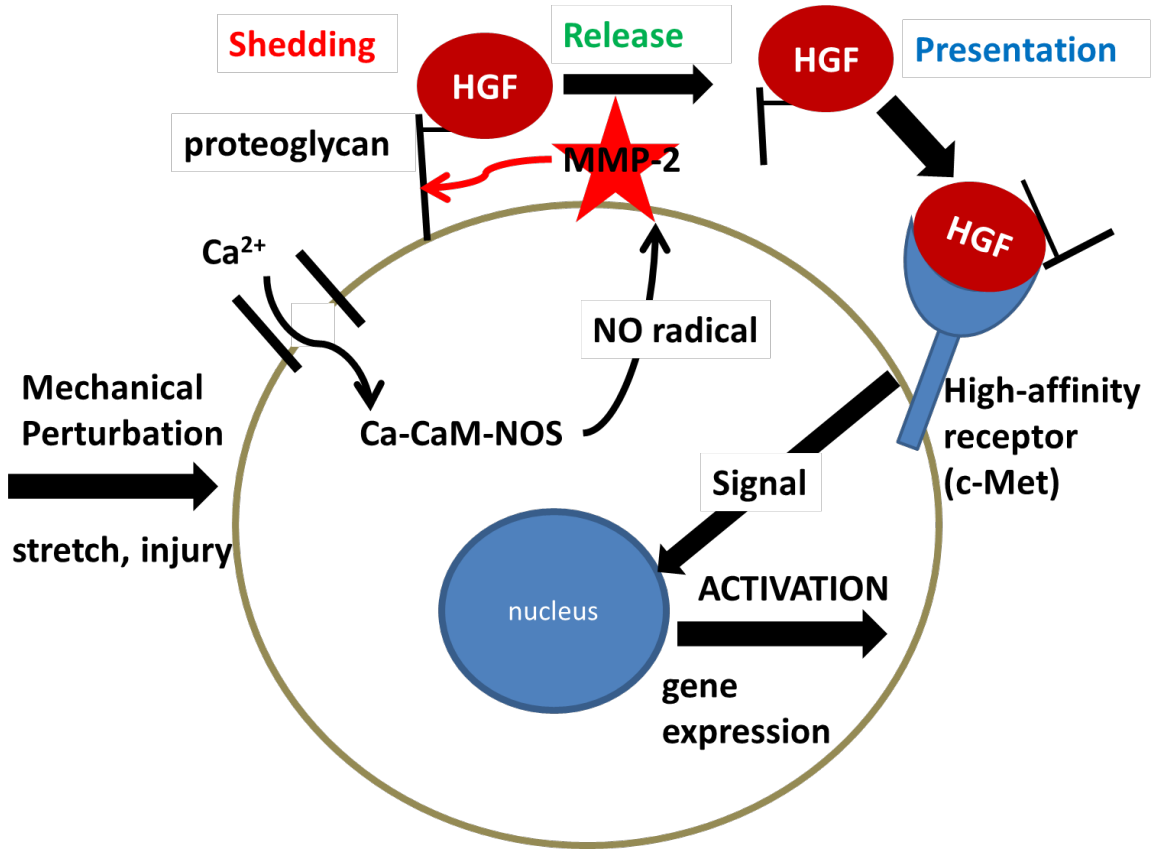


Figure 1.8: **Schematic diagram showing how MMP-2 helps activate and release HGF to assist in muscular repair and regeneration**. Schematic diagram showing signalling transduction following mechanical stress that leads to calcium-calmodulin formation, nitric oxide (NO) radical production by NO synthase, MMP-2 activation, release of HGF from the extracellular matrix, and presentation of HGF to the receptor c-Met to induce gene expression. Figure constructed using Hara et al., 2012.

1.4. Summary and Aims

MMPs are zinc endopeptidases that can digest specific ECM factors to regulate ECM-based cell adhesion which allows for myoblasts to elongate and fuse to form myotubes and subsequently mature muscle fibers. MMP-14 matures proMMP-2 with the help of TIMP-2. Unlike MMP-14, MMP-2 can digest collagen IV, because it contains a fibronectin-like domain within its catalytic domain that recognises collagen IV. MMP-2 is also critical for the release of growth factors such as HGF from the extracellular matrix. A greater understanding of the role of MMP-14/MMP-2 in myogenesis is required, particularly in the presence of relevant ECM factors, as this will provide insight into the mechanism by which optimal skeletal muscle

regeneration can be achieved in injured and diseased tissue. Therefore the aim of the study is to:

a) Study the protein expression and activity of MMP-14 and MMP-2 during myoblast differentiation
b) Establish whether Matrigel (mimicking components of the basal lamina) affects MMP-2 expression during myogenesis

Chapter 2:
General materials and methods

2.1. **C2C12 murine skeletal muscle myoblast culture**

Cell culture work was carried out under sterile conditions in an Airstream® level II biological safety cabinet (ESCO class II BSC). All cells were incubated in a humidified Innova® CO-170 CO_2-incubator (New Brunswick) at 37°C, 5% CO_2.

C2C12 myoblasts represent a cell-line that was isolated from the thigh of C3H mice following injury and then further sub-cloned (Yaffe and Saxel, 1977, Blau et al., 1983); these cells have progenitor-like characteristics and spontaneously differentiate following serum removal. C2C12 myoblasts (ATCC, cat.CRC-1772, USA) were cultured until ~70% confluence in growth media (GM) containing Dulbecco's Modified Eagle's Medium (containing 2% L-glutamine) (Sigma, cat.D5648-1L, USA) supplemented with 10% (v/v) Fetal Calf Serum (FCS) (Biowest, cat.51810-500, USA), 3.7g/L sodium bicarbonate (Merck, cat.103025, USA) and 1% (v/v) Penicillin/Streptomycin (Lonza, cat.DE17-602E, Switzerland). C2C12 cells were passaged by incubation with 1 mL trypsin (Lonza, cat.CC-5012, Switzerland). Media was changed to differentiation media (DM) containing DMEM with 2% L-Glutamine, supplemented with 2% (v/v) horse serum (HS) (Invitrogen, cat.16050-130, USA) and 2% PenStrep to induce differentiation.

2.2. **Lysate preparation**

Cells were cultured in either growth media or in differentiation media for up to 5 days. Day 0 (D0) represents the proliferation period, whereas days 1-5 represent differentiation where fusion was observed between day 3 and 5. At each time point, conditioned media was harvested and stored at -20ºC. Cells were also trypsinzed, centrifuged, re-suspended in 200µL RIPA buffer (Sigma, cat.R0278) containing 2 µL protease inhibitor cocktail (Sigma, cat.P8340) and placed on ice for 1 h. The cells were then sonicated at setting 14 (VirSonic60, Polychem Supplies) and stored aliquots at -20°C. The protein concentrations were determined using the Bradford method (Bradford, 1976). All experiments were repeated at least 4 times.

2.3. **Concentration of protein in conditioned media**

The conditioned media (Days 1-5) was precipitated using 99.9% cold ethanol centrifugation as previously described (Bouloumié et al., 2001), with minor modification. A 1:1 ratio of sample:cold ethanol (total volume 10 mL) was added to a 15 mL centrifuge tubes and incubated on ice for 1.5 hours and then centrifuged in a bench-top centrifuge (MRC; Polychem Supplies) at 100 g for 2 minutes. The supernatant was discarded, while the pellet was re-suspended in 1 mL running gel buffer (from SDS-PAGE protocol Section 2.5.1) and kept on ice. The concentration of samples was determined using the Bradford method (Bradford, 1976).

2.4. **Bradford protein assay**

10 µL of sample was added to 90 µL of PBS and then mixed with 900 µL of Bradford reagent (Sigma, cat.B6916, USA). The mixture was transferred into a 1 mL plastic micro-cuvette (Optima Scientific, cat.2711110) and absorbance measured at A_{595} with a spectrophotometer (Bio mate^3/Thermo Electron Corporation). Protein concentrations were calculated from a linear equation (x=((y+0.0264)/0.003)) obtained from the calibration curve (Figure 2.1). Bovine serum albumin (BSA) standards were used to obtain a calibration curve (Figure 2.1). Measurements were performed in triplicate.

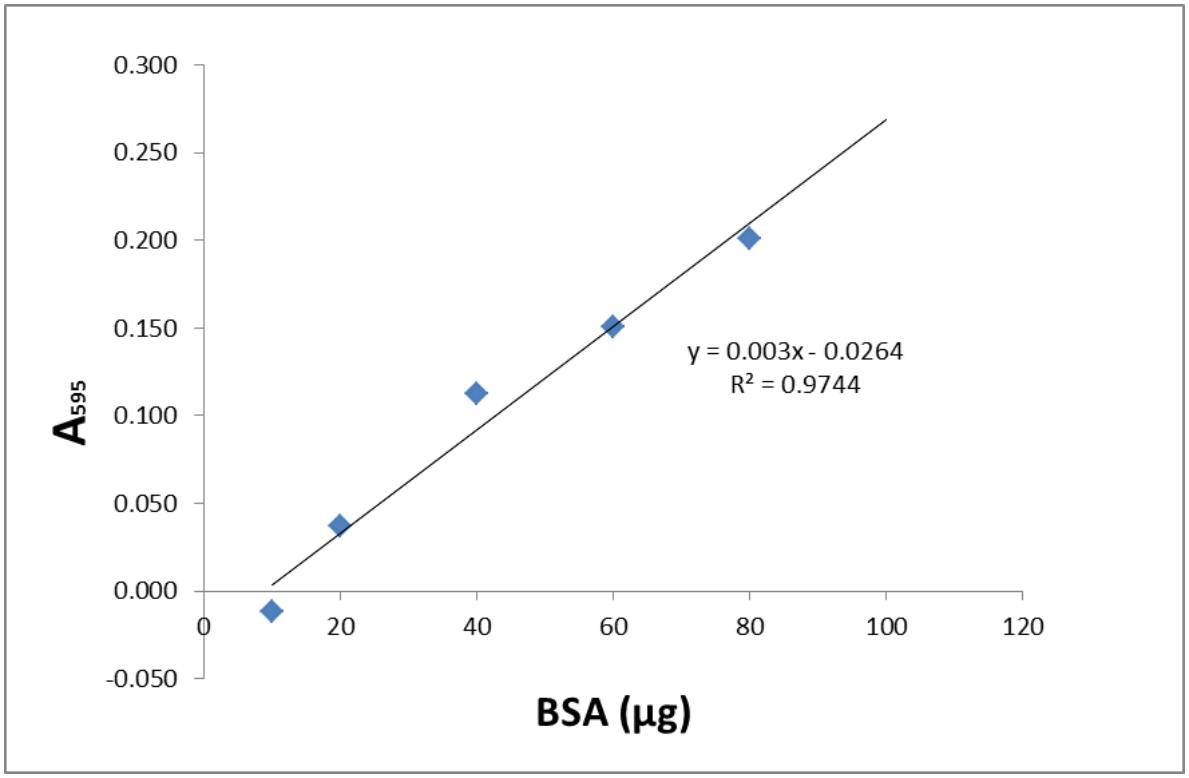


Figure 2.1: **Bradford protein calibration curve**. Bovine serum albumin (BSA) standards were prepared (0-80 µg) and mixed with 900 µL Bradford reagent and made up to 1 mL with PBS. Absorbance was read at A_{595}, (N=3); a blank was included. The absorbance readings were plotted against the BSA standards to generate a standard curve.

2.5. **SDS-PAGE and zymogram reagent and procedure**

2.5.1. **Reagents**

Solution A – Monomer solution [30 % (w/v) acrylamide, 2.7 % (w/v) Bis-acrylamide]: Acrylamide (58.4 g) and Bis-acrylamide (1.6 g) were dissolved and made up to 200 mL with distilled water than filtered and then refrigerated.

Solution B- 4 x Running Gel Buffer (1.5 M Tris-HCl, pH 8.8): Tris (36.3 g) was dissolved in 150 mL of distilled water, adjusted to pH 8.8 with HCl and made up to 200 mL with distilled water than filtered and then refrigerated.

Solution C- 4 x Running Gel Buffer (500 mM Tris-HCl, pH 6.8): Tris (3.0 g) was dissolved in 30 mL of distilled water, adjusted to pH 6.8 with HCl and made up to 50 mL with distilled water than filtered and then refrigerated.

Solution D- SDS Stock [10 % (w/v) SDS]: Sodium dodecyl (laury) sulfate (20 g) is dissolved in 200 mL of distilled water with gentle heating.

Solution E- Initiator [10 % (w/v) Ammonium persulfate]: Ammonium persulfate (0.1 g) was made up to 10 mL and stored in fridge.

Solution F- Stock Tank Buffer [25 mM Tris-HCl, 192 mM Glycine, 0.1 % (w/v) SDS, pH 8.3]: Tris (3 g) and glycine (14.4 g) were dissolved and made up to 1 L with distilled water. 500 µL of solution D [10 % (m/v) SDS] is added just before use.

Solution H- Reducing Treatment Buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]: Buffer C (2.5 mL), 10% SDS (4 mL) (solution D), glycerol (2 mL) and 2-mercaptoethanol (1 mL) were made up to 10 mL with distilled water.

Solution I- Non-reducing treatment Buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, pH 6.8]: Buffer C (2.5mL), 10% SDS (4 mL) (solution D) and glycerol (2 mL) were made up to 10 mL with distilled water.

Stain stock solution for SDS-PAGE [1 % (w/v) Coomassie blue R-250]: Coomassie blue R-250 (1 g) was dissolved in 100 mL of distilled water by magnetic stirring for 1 h at room temperature and then filtered.

Staining solution [0.125% (w/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]: Stain Stock (62.5 mL) was mixed with methanol (250 mL) and acetic acid (50 mL), and made up to 500 mL with distilled water.

Destaining solution I [50% (v/v) methanol, 10 % (v/v) acetic acid]: Methanol (500 mL) was mixed with acetic acid (100 mL) and made up to 1 L with distilled water.

Destaining solution II [7% (v/v) acetic acid, 5% (v/v) methanol]: Acetic acid (70 mL) was mixed with methanol (50 mL), and made up to 1 L with distilled water.

Stock gelatin solution [1% (m/v) gelatin in running gel buffer]: Gelatin (0.5 g) was dissolved in running gel buffer (5 mL) with heating in microwave. Solution made fresh every time.

Renaturation solution [2.5% (v/v) Triton-X 100]: Triton-X (6.25 mL) was diluted to 250mL with distilled water.

Enzyme activity incubation buffer [50 mM Tris-HCl, 0.15 M $NaCl_2$, 10 mM $CaCl_2$, pH 7.6]: Tris base (6.057 g), $NaCl_2$ (8.766 g), NaN_3 (0.5 g) and $CaCl_2$*2H2O (1.47 g) were dissolved

in 850 mL distilled water. The pH was adjusted to 7.6 with 0.1 M HCl and the volume made up to 1 L.

Zymography staining solution [0.2% (m/v) Coomassie Brilliant Blue R-250]: Coomassie Brilliant Blue R-250 (0.2 g) was dissolved in 50 ml distilled water and 50 mL methanol by stirring at room temperature and filtered. Stored in dark amber bottle. Stained gel for 4 h no destaining needed.

2.5.2. **Procedure**

SDS-PAGE: was carried out as per Laemmli protocol (Laemmli, 1970), where gels consisted of a 4.5 % (m/v) polyacrylamide stacking gel and 12% (m/v) polyacrylamide running gel. Volumes of the SDS-PAGE reagents combinations are listed in Table 2.1.

Reagents	**Stacking gel (4%)**	**Running gel (12.5%)**
Solution A	0.94 mL	6.25 mL
Solution B	-	3.75 mL (2.25)*
***1% (m/v) gelatin**	-	1.5 mL
Solution C	1.75 mL	-
Solution D	70 µL	150 µL
Solution E	35 µL	75 µL
Distilled water	4.3 mL	4.75 mL
TEMED	15 µL	7.5 µL

Table 2.1: **Volumes of SDS-PAGE and Zymogram reagents combination**

Zymogram: the procedure for SDS-PAGE was modified from that described in (Heussen and Dowdle, 1980), in that 0.1% (m/v) gelatin was incorporated into the gel, to allow the detection of proteinases. This was carried out by adding 1% (m/v) gelatin in running gel buffer (1.5 mL), to running gel buffer (2.25 mL) and the rest of the solution for casting a 12.5% gel, all at 37^{o}C, and pouring the gel as quickly as possible. The gel was overlaid with the overlay solution for substrate gels, and allowed to set. The SDS-PAGE was carried out normal. After electrophoresis, the running gel was soaked in two changes of 2.5% (v/v) Triton X-100 (50 mL) over 1 h at RT. Following this, the gel was incubated in the pH 7.4 assay buffer, for 24 h or 48 h at 37^{o}C. It was then stained in 0.2% (m/v) Coomassie Brilliant Blue R-250 solution for 1 h, and destained in several changes of methanol:acetic acid: distilled water (30:10:60). The presence of proteolytically active components in the gel was indicated by clear bands in the gel after staining, due to the digestion of gelatin.

2.6. **Western blot**

2.6.1. **Reagents**

Blotting buffer: Tris (24.2 g), glycine (57.6 g) and SDS (4 g) were dissolved in 3200 mL of distilled water and methanol (800 mL) was added. The volume was made up to 4000 mL in a large beaker, the exact volume not being critical.

Wash Solution: Tris Tween buffered saline (TTBS) – [20mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5]: NaCl (16 g), KCl (0.4 g) and Tris base (6 g) were dissolved in 1600 mL of distilled water than adjusted pH to 7.5 and bring volume to 2 L with distilled water. Finally add 1 mL Tween 20 (cat.# 170-6531) to 2 L TBS solution to make 0.05% TTBS.

Ponceau S stain [0.1% in 15 (v/v) acetic acid]: Ponceau S (0.2 g) and acetic acid (2 mL) was made up to 200 mL with distilled water.

Phosphate buffered saline (PBS): NaCl (14 g), KCl (0.4), Na2HPO4 (2.88 g) and KH2PO4 (0.24 g) were dissolved in 1600 ml of distilled water than adjusted pH to 7.5 and bring volume to 2 L with distilled water.

Lysis buffer (compare to RIPA buffer) [50 mM Tris-HCl, 150 mM NaCl, 0.1 % SDS pH 8.0]: Tris (0.12 g), SDS (0.10 g) and NaCl (0.174 g) were dissolved in 16 mL of distilled water than adjusted pH to 8.0 and bring volume to 20 mL with distilled water.

Blocking solution: 5% non-fat dry milk in TTBS: Mix 60 ml of TTBS with 3 g of non-fat dry milk (Cat. # 170-6404); this is enough for one 8.5 x 13.5 cm Criterion^{TM} blot. Alternatively, 3% bovine serum albumin (BSA) can also be used as a blocking solution.

2.6.2. **Procedure**

Following SDS-PAGE, usually on duplicate gels, one gel was stained to show the total protein pattern or used for quantification (equal loading proofs), while the other was used for blotting. Nitrocellulose was cut to a suitable size and, to avoid entrapment of air, carefully floated onto blotting buffer, before being totally immersed. The immersed nitrocellulose was sandwiched, with the gel lying squarely on top of it, between 3 pieces of Whatman No. 4 filter paper and two pieces of Scotchbrite foam, also totally immersed in blotting buffer. The whole apparatus was immersed in a tank of cold water, kept at 8^{o}C by a refrigerated circulator. The apparatus was connected to a power supply so that the nitrocellulose was on the anodal side of the gel, and blotting was effected for 16 h at 200 mA. The buffer was stirred with a magnetic stirrer throughout the process to ensure even distribution of cooling. After 16 h, the sandwich was removed and the filter paper was peeled off the gel. The gel was carefully removed, and stained to assess the efficiency of the blotting. Stained with Ponceau S for at least 30 min then washed twice with distilled water, appropriate pencil marking were made on the nitrocellulose paper and photos were taken to prove equal loading. The nitrocellulose paper was cut according to desired size.

The nitrocellulose sheet was removed from the filter paper and air dried for about 1.5 h. The nitrocellulose strip was blocked for 1 h with 5% (m/v) non-fat milk powder in TBS, washed in TBS (3 x 5 min) and incubated for 2 h with primary antibody in 0.5% BSA-TBS (Table 2.2). Following washing in TBS (2 x 5 min), it was incubated in HRPO-linked secondary antibody (Table 2.2) in 0.5% BSA-TBS for 1 h, and again washed in TBS (3 x 5 min). It was immersed in substrate solution and reacted in dark until bands were clearly evident against a lightly-stained background. Finally, the strip was removed from the substrate solution, and washed in distilled water and dried between filter paper. This last step ensured good preservation of the bands before photography.

2.7. **Antibody dilutions**

The optimized antibody dilution used for confocal and Western blot studies are shown in Table 2.2.

Technique	Primary antibody dilution	Secondary antibody dilution
Confocal Microscopy	Rabbit monoclonal anti-MMP-2 (Abcam, cat. ab92536, UK) 1/400 Mouse monoclonal anti-MMP-14 (Abcam, cat.ab78738, UK) 1:300	Donkey anti-rabbit Dylight 488 (Abcam, cat.ab96919, UK) 1/1000 Donkey anti-mouse Alexa Flour 488 (Jackson Immunoresearch, cat.016-540-084, USA) 1/2000
Western blot	Rabbit polyclonal anti-GAPDH (Cell Signalling, cat.21185, USA) 1/4000 Rabbit monoclonal anti-MMP-2 (Abcam, cat. ab92536, UK) 1/1000 Mouse monoclonal anti-MMP-14 (Abcam, cat.ab78738, UK) 1:1000	HRPO-linked rabbit anti-mouse secondary antibody (Dako, cat.P0260, SA) 1:4000 HRPO-linked goat anti-rabbit secondary antibody (Dako, cat.P0448, SA) 1:6000

Table 2.2: **Antibody dilutions for confocal microscopy and Western blots**

2.8. **Preparation of BB94 stock**

BB94 (Abcam, cat. ab142087, UK) (10 mM) stock was prepared by dissolving in DMSO (SIGMA, cat. D2650, USA). BB94 stock was further diluted in differentiation media to 0.1 mM final concentration and sterilized using 0.2 µm filters. Stocks stored until further usage at 4 °C.

2.9. **Preparation of Ladd's stain**

Ladd's stain (KAPLOW and Ladd, 1965) also known as Ladd multiple stain is a combined nuclear and cytoplasm stain. It stains various tissue elements in different shades allowing better viewing of contents within tissue using bright-field microscopy. Colleagues in the lab discovered that it specifically stains actin of differentiated C2C12 myotubes, this allows us to easily distinguish between myotubes and myoblasts. The Ladd's stain was prepared as follow, 0.365g of toluidine blue (SIGMA, cat. 89640-5G, USA) and 0.135g of basic fuchsin (SIGMA, cat. 47860-25G, USA) was dissolved in 30% ethanol and made up to 50 ml. The mix was stirred with a stirrer bar for at least an hour than filtered through filter paper and stored 50 ml falcon tube at room temperature.

Chapter 3:

Analysis of MMP-14 and MMP-2 in C2C12 myoblast differentiation

Abstract

Satellite cells are muscle precursor cells that have the ability to self-renew, proliferate, differentiate and fuse into myotubes which are vital for regeneration and repair of muscle. MMPs are zinc endopeptidases known to cleave a variety of extracellular matrix factors and hence play an important role in skeletal muscle repair. MMP-14 is membrane-bound, and is also known as membrane-type 1 matrix metalloproteinase (MT1-MMP). It is one of the major matrix metalloproteinases (MMPs), whereas MMP-2 is a secreted gelatinase A but may also occur intracellular. MMP-2 is known to be activated by MMP-14. MMP-14 and MMP-2 degrade a range of proteins including laminins 2/4, fibronectins and collagens I and IV which forms bulk of muscle fibers basement membrane. MMP-2 and MMP-14 knock-out studies in mice have highlighted the importance of regulating both MMP-14 and MMP-2 levels, where knockout of MMP-14 leads to skeletal defects, dwarfism and premature death. In this study we explored the expression levels, activity and role of MMP-14 and secreted MMP-2 during C2C12 myoblast differentiation. C2C12 myoblasts first proliferated (Day 0), then aligned and elongated (Days 1-2) and finally fused into myotubes (Days 3-5) during differentiation. MMP-14 and MMP-2 protein levels were high during the elongation period and also during fusion of C2C12 myoblast differentiation. MMP-14 in proliferating and fused myoblasts was localised at the focal adhesions where actin filaments terminate. Inhibition of MMPs using BB94 (10 µM) was observed to significantly reduce C2C12 myoblast fusion. Secreted MMP-2 seems to play a vital role in the C2C12 differentiation, as activity was seen during myogenesis; when inhibited with neutralizing antibodies an 18% decrease in fusion was observed. In conclusion, MMP-14 and MMP-2 play an important role in C2C12 myoblast differentiation, with MMP-2 specifically important during fusion.

3.1. **Introduction**

Skeletal muscle is an essential component of the human body, and is crucial for movement, breathing and posture (Ohtake et al., 2006, Enwere et al., 2014). Muscle satellite cells are a population of stem cells found between the sarcolemma and basement membrane of the muscle fiber (Thomas et al., 2015). Under basal conditions these satellite cells remain quiescent, but following necrotic injury they are activated to become myoblasts, which proliferate, differentiate and fuse to form new multinucleated muscle fibers (Enwere et al., 2014, Grounds, 2014). The muscle fibers will further align together to form muscle tissue and facilitate repair. This process of myogenesis is regulated by a family of transcription factors including myogenic differentiation 1 (MyoD), myogenin and muscle regulatory factor 4/5 (Mrf4/5) which control the expression of muscle specific proteins such as muscle creatine kinase (MCK) and myosin heavy chain (MyHC) (Thomas et al., 2015, Enwere et al., 2014, Zhu and Miller, 1997).

Matrix Metalloproteinases (MMPs) are zinc endopeptidases capable of cleaving components of the extracellular matrix (ECM) such as laminin and collagen IV; these are major components of the basement membrane of muscle fibers (Chen and Li, 2009, Thomas et al., 2015). To date, twenty-five members have been identified, of which six are membrane-bound and nineteen secreted. MMP-14 is known as membrane-type 1 matrix metalloproteinase (MT1-MMP), whereas MMP-2 is a secreted gelatinase. Together they are able to degrade almost all ECM components in the body (Visse and Nagase, 2003, Murphy and Crabbe, 1995, Snyman and Niesler, 2015, Kajita et al., 2001). MMP- 14 processes laminin-2 and 4, cadherin, fibronectin and collagen I *in vivo* and *in vitro* and appears to regulate both ECM-based and cell-to-cell adhesion (Ohtake et al., 2006, Seiki, 2003). MMP activity is modified by specific Tissue Inhibitor of Metalloproteinases (TIMPs) that bind MMPs in a 1:1 stoichiometry (Visse and Nagase, 2003). TIMP-2 is specifically found in muscle and controls MMP-14 activity, and facilitates maturation of proMMP-2 and secretion of active MMP-2 (Ohtake et al., 2006). Previous studies showed that MMP inhibition by BB94, a commercially available inhibitor, decreased myoblast fusion, emphasizing the importance of these proteases in skeletal muscle myogenesis (Lluri and Jaworski, 2005b, Ohtake et al., 2006). Levels of TIMP-2 and MMP-14 are critical for MMP-2 activation (Ohtake et al., 2006). Low TIMP-2 levels are associated with MMP-14 mediated activation of pro-MMP-2; but higher levels of TIMP-2 block activity of MMP-14 and therefore preventing pro-MMP-2 activation (Ohtake et al., 2006, Chen and Li, 2009, Apte et al., 1997).

MMP-14 knock-out studies in mice have highlighted the importance of MMP-14; knockout of MMP-14 leads to premature death, skeletal defects and dwarfism (Holmbeck et al., 1999, Itoh et al., 1998). Cultured embryonic cells failed to form myotubes efficiently in the absence of MMP-2 and MMP-14, indicating that these two MMPs are crucial for successful myotube formation (Chen and Li, 2009). A deficiency of MMP-14 causes self-destruction of muscle tissue which leads to muscular dystrophy (Ohtake et al., 2006, Lluri and Jaworski, 2005a).

MMP-14 and MMP-2 are therefore important during myogenesis, however, their roles require further investigation. The current study aimed to determine the localisation, expression, activity and role of MMP-14 and MMP-2 during C2C12 myoblast differentiation.

3.2. **Materials and methods**

C2C12 myoblasts were cultured in growth media (GM; DMEM with 10% Fetal Calf Serum (FCS)) to reach 70 % confluence. The cells were than washed with PBS and differentiated in differentiation media (DM; DMEM with 2% Horse Serum (HS)) for a period of five days. Cell lysates (containing MMP-14 protein), and conditioned media (containing secreted MMP-2) were harvested for further analysis. Conditioned media was further concentrated using 99.9 % ethanol (Section 2.3).

3.2.1. **Analysis of cellular MMP-14**

3.2.1.1. **MMP-14 localization**

C2C12 cells were cultured in 24 well plates containing glass coverslips and differentiated for 5 days. At days 0, 1, 2, 3, and 5 cells were washed with PBS (3 x 5 minutes), fixed with 4% paraformaldehyde (20 minutes), washed with PBS (3 x 5 minutes) and blocked using 5% (v/v) donkey serum (Sigma, cat.D9663, USA) for 30 minutes at room temperature. The slides were then incubated with mouse monoclonal anti-MMP-14 (Abcam, cat.ab78738, UK, 1:300 dilution) overnight @ 4^{o}C, followed by fluorescent-tagged secondary antibodies for 1 hour with: Alexa Fluor 488 anti-mouse (Jackson, cat.016-540-084, USA, 1:2000). The actin cytoskeletal structures were then labelled with phalloidin (1:1000 dilution) for 1 hour at room temperature, followed by incubation with 10 µg/ml Hoechst 33342 nuclear stain (Sigma, cat.B2661, USA, 1:2000 dilution) for 5 minutes. The coverslips underwent a final set of PBS

washes (10 x 5 minutes) and were mounted on glass slides using 2 µl moviol. The labelled cells were viewed using a Zeiss LSM 710 confocal microscope.

3.2.1.2. **Expression levels of MMP-14**

SDS-PAGE was carried out as per Laemmli protocol (Laemmli, 1970). All gels were run using a bench-top electrophoresis unit (Hoefer: Dual Gel Caster SE 245, USA) and consisted of a 4.5% stacking and 12% running gels. A total of ~30 µg protein lysate was added to each of the wells. Before loading, samples were mixed with an equal volume of reducing sample treatment buffer as well as 1 µL of bromophenol blue (Saarchem, cat.1437500CB, RSA) and boiled for 5 minutes. A prestained protein ladder (Thermo Scientific, cat. #26619, SA) was used to determine protein band size. Once run, gels were either prepared for Western blotting or stained with the Coomassie R-250.

To determine the expression levels of MMP-14, a Western blot was carried out by transfer of proteins from SDS-PAGE to a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences). The blot was carried out in a cold room (~4°C) for 16 hours at 200 mA. The blot was stained with Ponceau S. to show protein transfer and then blocked overnight with 5% (w/v) fat-free milk powder in TTBS with 0.05% Tween 20. The membrane was incubated with either mouse monoclonal anti-MMP-14 (Abcam, cat.ab78738, UK, 1:1000 dilution); the internal loading control was rabbit monoclonal anti-GAPDH (Cell Signalling, cat.2118, USA, 1:4000 dilution). The membrane were washed with TTBS and incubated in secondary antibody for 2 hours in room temperature: HRPO-linked rabbit anti-mouse secondary antibody (Dako, cat.P0260, SA, 1:4000 dilution) or HRPO-linked goat anti-rabbit secondary antibody (Dako, cat.P0448, SA, 1:6000 dilution). The membranes were then washed with TTBS (10 x 5 minutes) and antibody-antigen complexes were detected using an enhanced chemiluminescence (ECL) kit (Immun-Star™ Western C™ Chemiluminescent Kit, BioRad, USA). Images of protein expression were obtained using the SYNGENE G: Box Chemi-XR5 machine fitted with 5.0 megapixel camera (plugged to PC with (plugged to PC with GeneSys (http://www.syngene.com/genesys) programme). An image analysis program known as Image J (https://imagej.nih.gov/ij/) was used to calculate the mean grey value (mean grey value is the median value of pixels in the image or selection) of the blots; these values were normalised to the internal control.

3.2.2. **Analysis of secreted MMP-2 in conditioned media**

3.2.2.1. **Dot blot**

To determine whether MMP-2 is found in FCS, a 2 µl dot of concentrated growth media (GM; containing 10% FCS), differentiation media (DM; containing 2% HS) and day 2 conditioned media (CM) were pipetted on a nitrocellulose paper, blocked with 5% (w/v) fat-free milk powder and then followed normal Western blot procedures (Section 2.6).

3.2.2.2. **Analysis of secreted MMP-2 levels and activity in conditioned media**

The concentrated proteins (days 1-5) and concentrated differentiation media (which served as a negative control) were analysed via Western blots (Section 2.6 and 3.2.1.2).

To show MMP-2 activity in samples collected at days 1-5 of differentiation, a zymogram was carried out (Section 2.5). A recombinant active human MMP-2 full length protein was used (Abcam, cat.ab81550, UK) as a positive control for MMP-2 action. MMP-2 was separated on a 12 % non-reducing SDS-polyacrylamide gel containing copolymerised 0.1% (m/v) gelatin. The SDS-PAGE is renatured upon removal of SDS by incubation of the gel in Triton X-100 (Heussen and Dowdle, 1980). A gelatinase incubation buffer at pH 7.6 was used at 37°C to promote activity of MMP-2, incubated over 2 days (Lluri and Jaworski, 2005b). To confirm inhibition of MMP-2 by BB94, one gelatin gel was incubated in gelatinase incubation buffer containing 10 µM BB94. The gel was then stained with Coomassie R-250 for 1 hour and analysed using the SYNGENE G: Box Chemi-XR5 fitted with 5.0 megapixel camera.

3.2.3. **MMP inhibition**

Cells were cultured to 70% confluence and then differentiated for 5 days (media change every 48 hours) in the absence or presence of BB94 (1 and 10 µM) and DMSO (1/1000 dilution) control. The DMSO control was included as BB94 was dissolved in DMSO. At day 5, cells were fixed with 70 % ethanol for 10 minutes and stained with 6 % Ladd's stain (KAPLOW and Ladd, 1965). Images (5 field of view per experiment) were taken using an Olympus CKX41 inverted light microscope and a Motic camera (20x objective). Fusion indexes were calculated (total number of nuclei in a myotubes per field of view divided by the total number of nuclei within the same field of view multiplied by 100) (Micheli et al., 2011).

3.2.4. **MMP-2 neutralization**

MMP-2 neutralization was done in a similar way as Section 3.2.3 with minor modification. Anti-MMP-2 rabbit monoclonal primary antibody (Abcam, cat. ab92536, UK) was incorporated into the differentiation media to a final concentration of 5 µg/ml (1/400 dilution) (Shen et al., 2010), the media was changed every 48 hours.

3.3.**Results**

3.3.1. **Baseline C2C12 myoblast differentiation**

In order to determine the expression of MMP-14 during myogenesis, C2C12 cells were differentiated for a period of 5 days (Figure 3.1). It was observed that during the proliferation stage (Day 0), cells were mono-nucleated with a star-shaped structure (white arrows), while during the elongation stage (Days 1-2) they align and elongate longitudinally in close proximity (green arrows) to one another to facilitate fusion into multinucleated myotubes (red arrows) between days 3-5 of differentiation (Figure 3.1).

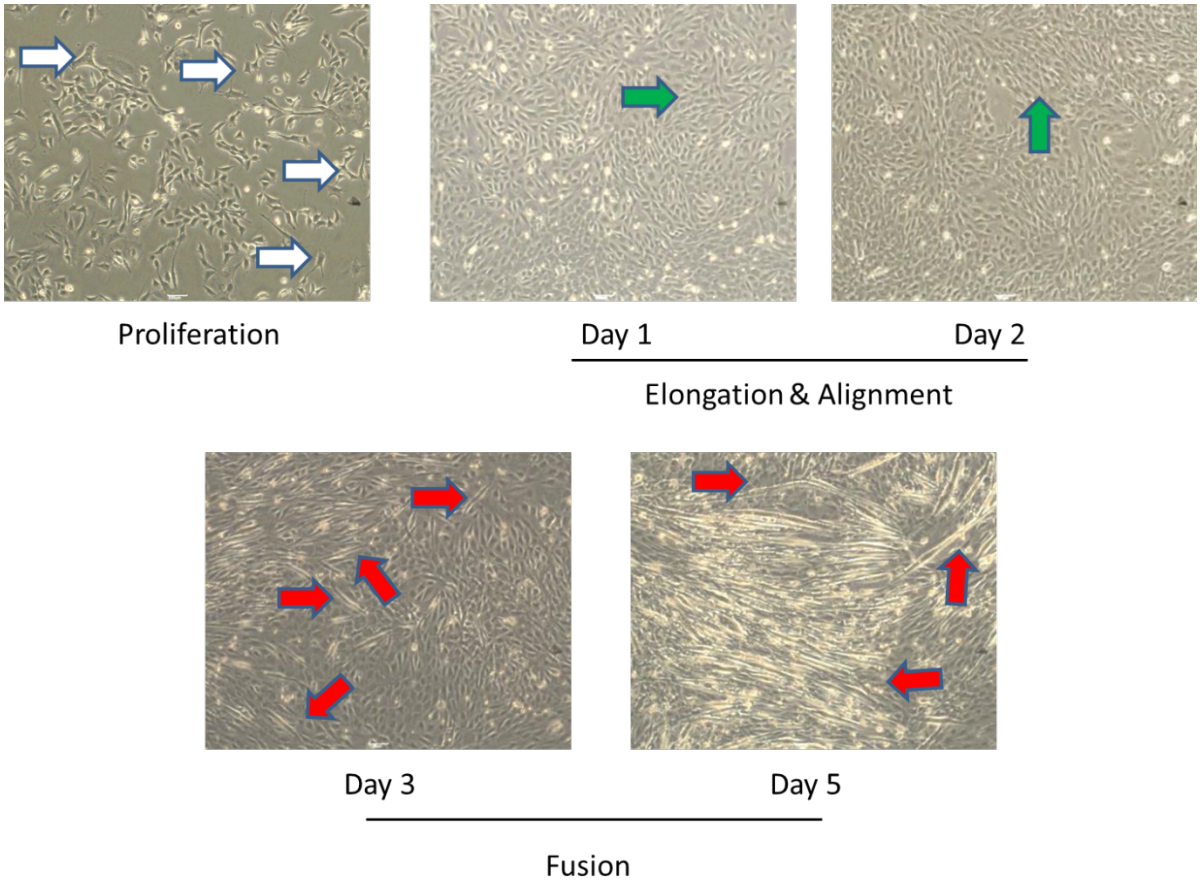


Figure 3.1: **Differentiation of C2C12 myoblasts**. Brightfield images (40X Magnification) of differentiating C2C12 myoblasts at days 0-5. Day 0 represents the proliferation stage; days 1-2 represented C2C12 myoblast elongation and alignment; days 3-5 represents fusion. White arrows indicate mono-nucleated star-shaped myoblasts, green arrows indicate the alignment and elongation of myoblast and red arrows indicate the actual fusion of myoblasts to form multi-nucleated myotubes.

In order to analyse the localisation of MMP-14 during these stages of myogenesis, C1C12 cells were plated on glass coverslips and immunostained for MMP-14; the actin cytoskeleton and nuclei were stained as described in Section 3.2.1.1 (Figure 3.2).

Analysis by confocal microscopy revealed that under proliferative conditions, MMP-14 (green) is localised to regions where actin filaments (red) terminates at focal adhesions (yellow arrows). In response to differentiation cues, MMP-14 becomes distributed throughout the cytoplasm and does not concentrate in focal adhesions during elongation and initial fusion (days 1-3). By day 5, when fusion is fully underway, MMP-14 is localised more extensively on the cell membrane (Figure 3.2).

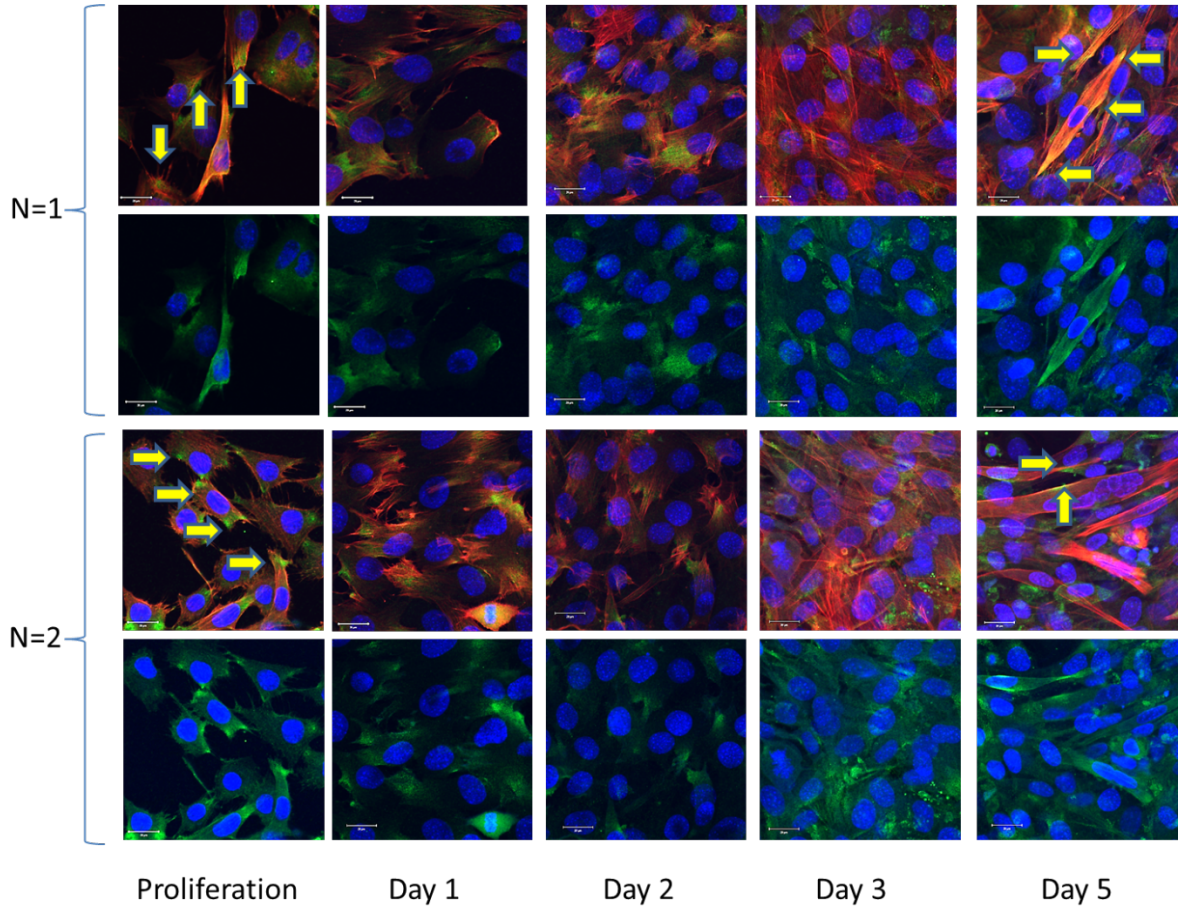


Figure 3.2: **Localisation of MMP-14 during C2C12 myoblast differentiation**. Cells cultured in growth media (D0) and then subjected to differentiation media for 5 days to locate MMP-14 (green) within the cells via confocal analysis (630X Magnification). Stress fibers (actin) are stained with phalloidin (red); nuclei are stained with Hoechst (blue). Yellow arrows: focal adhesions where actin filaments terminate. Two representative experiment (N=1 and N=2) are shown. Top row of each experiment show nucleus (blue), actin (red) and MMP-14 (green) while bottom row of each experiment show only MMP-14 and nucleus.

To determine the levels of MMP-14 expressed within the cell, proteins harvested from cell lysates were separated by SDS-PAGE; MMP-14 as well as GAPDH (internal control) were then detected by Western blotting (as described in Section 3.2.1.2).

MMP-14 was detected in proliferating myoblasts (Day 0) (Figure 3.3). Following induction of differentiation, protein expression was observed to increase by ~40% by day 3, however, this effect was not significant (Figure 3.3). By day 5 of differentiation, normalized MMP-14 expression levels were similar to those observed under proliferative conditions (Figure 3.3).

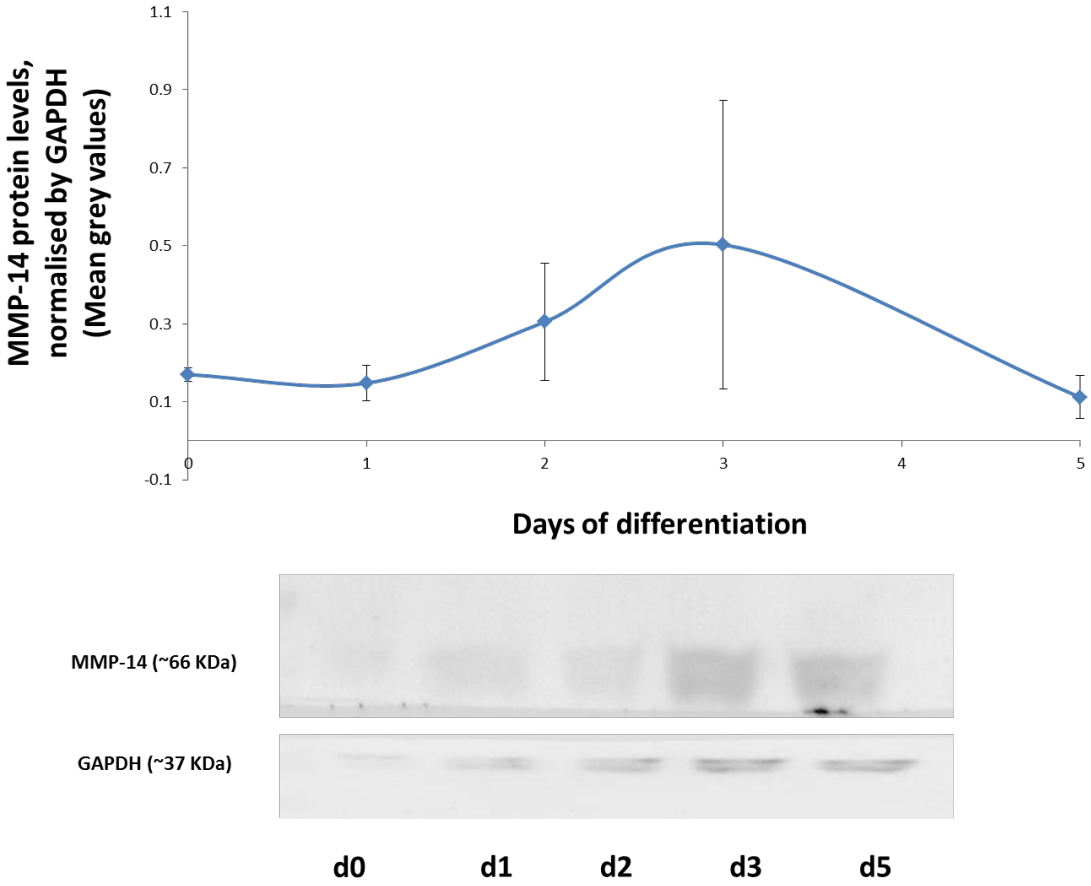


Figure 3.3: **Expression of MMP-14 in C2C12 cell lysate during differentiation**. Western blot analysis of expressed protein levels of MMP-14 at day 0 (proliferation) and days 1-5 (differentiation). GAPDH was used as an internal control. The MMP-14 mean grey values were first normalised and plotted. Densitometry was performed on the blots using an image analysis software known as Image J. Data are expressed as mean and SEM; n=3, *p<0.05.

3.3.2. Protein profile and activity of secreted MMP-2 during C2C12 myoblast differentiation

Previous research carried out by Ohtake et al (2006) analysed the mRNA levels of MMP-2. We were however interested in determining the levels of secreted MMP-2 protein in the conditioned media of differentiating myoblasts. C2C12 myoblasts were cultured in differentiation media (as described in Section 2.2) and harvested at days 1, 2, 3 and 5; conditioned differentiation media was used as a control. Conditioned media was then concentrated using 99.9 % cold ethanol and protein concentration determined by Bradford to ensure equal loading. Following SDS-PAGE and Western blot analysis, levels of secreted MMP-2 within the conditioned media were assessed using Image J software (https://imagej.nih.gov/ij/) as described in (Section 2.3.2 and 2.4.3). Nitrocellulose (NC) membranes were stained with Ponceau S to demonstrate transfer of protein and equal loading

of protein; proof of equal loading is a challenge when dealing with conditioned media as well as data analysis (Figure 3.4).

MMP-2 levels fluctuated during the initial stages of differentiation (Days 1-3); however, interestingly, levels were seen to increase at day 5 of C2C12 myoblast differentiation (Figure 3.4). The increase in MMP-2 at day 5 could be related/linked to the higher MMP-14 expression at day 3 as MMP-14 is known to assist in MMP-2 activation (Figure 3.2).

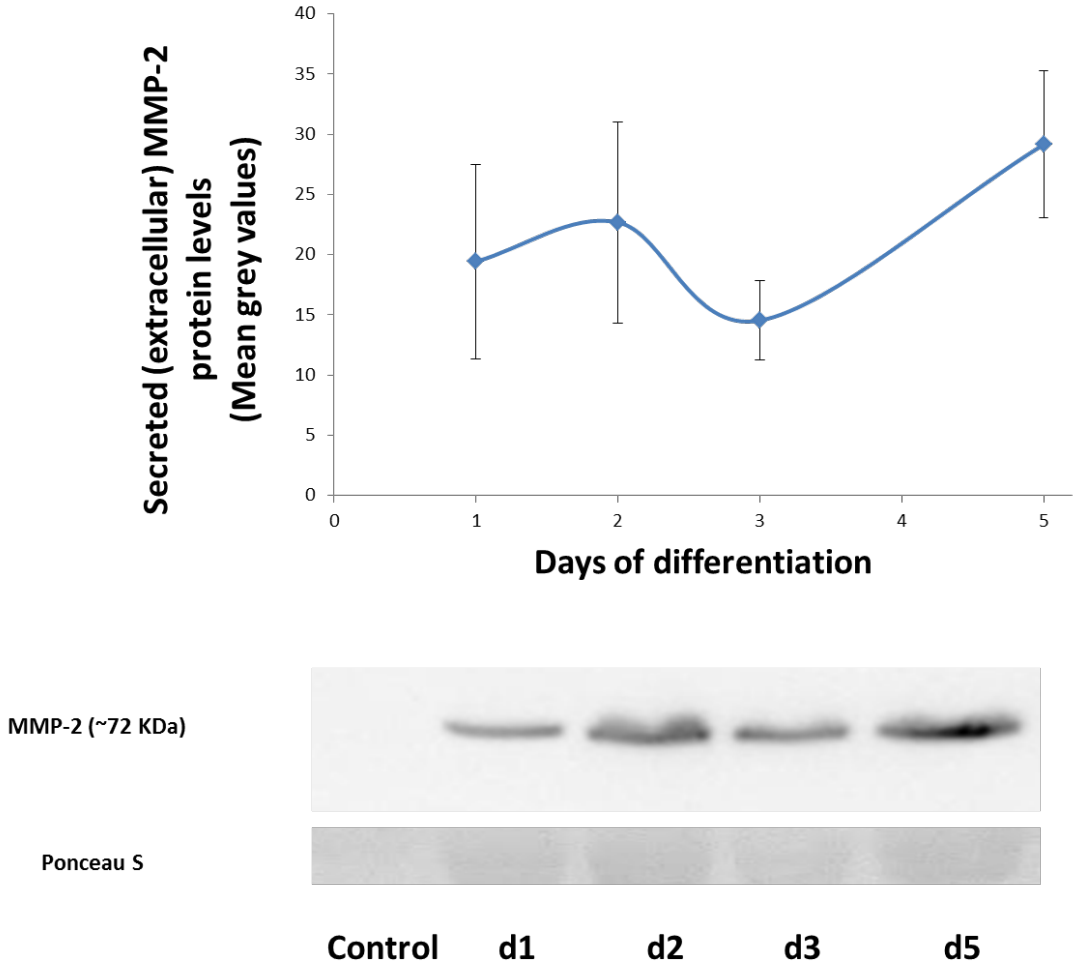


Figure 3.4: **Expression of secreted MMP-2 protein during C2C12 myoblast differentiation**. Western blot analysis of the expression levels of secreted MMP-2 at day 0 (DM control), conditioned media at d1-d5 of differentiation, Ponceau S was used as a loading control. Densitometry was performed on the blots using an image analysis software known as Image J. Data are expressed as mean and SEM; n=4, *p<0.05.

We next wished to determine whether the MMP-2 detected in the conditioned media was active. Recombinant active MMP-2 (0.5 µg) was used as a positive control while DM (concentrated) was used as a negative control in the zymogram. Conditioned media (60 µg) from days 1, 2, 3 and 5 was separated on a 12 % gelatin non-reducing SDS-PAGE gel (Figure

3.5). MMP-2 activity was observed at all days of C2C12 myoblast differentiation (Figure 3.5). DM control showed no activity indicating that the secreted MMP-2 responsible for the activity (Figure 3.5). Recombinant active MMP-2 confirmed correct size of MMP-2 in conditioned media. The secreted MMP-2 is therefore active during myogenesis of C2C12 cells.

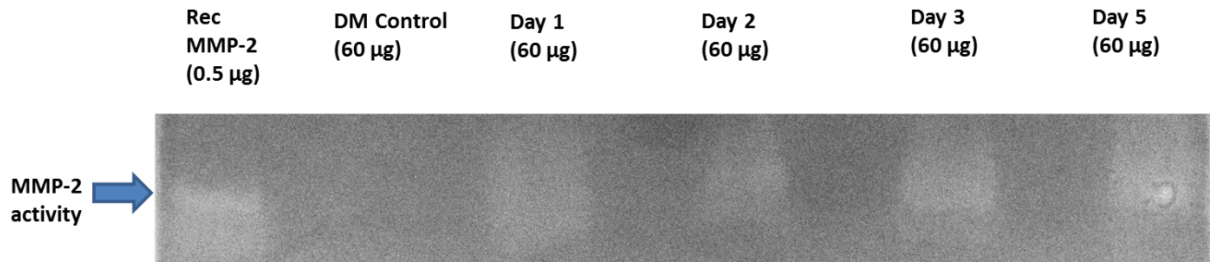


Figure 3.5: **MMP-2 secreted during differentiation is active**. Concentrated conditioned media (Days 1, 2, 3 and 5), differentiation media (negative control) and recombinant active MMP-2 (positive control) were separated on a 12 % non-reduced SDS-PAGE gelatin gel. MMP-2 activity was observed during C2C12 myoblast differentiation.

C2C12 proliferation was carried out in growth media containing 10% FCS. However, FCS has previously been reported to contain active MMP-2. To confirm this, we carried out a dot blot to determine whether MMP-2 is present in growth media (containing 10% FCS). Differentiation media (containing 2% HS) was also tested. Day 2 conditioned media was used as a positive control. As expected, concentrated GM contained detectable MMP-2 levels, whereas DM did not (Figure 3.6). Indeed, MMP-2 levels seemed higher in GM than CM (Figure 3.6). Secreted MMP-2 levels were therefore not further analysed under proliferative conditions; MMP-2 detected in conditioned media during differentiation could be regarded as having been secreted by myoblasts.

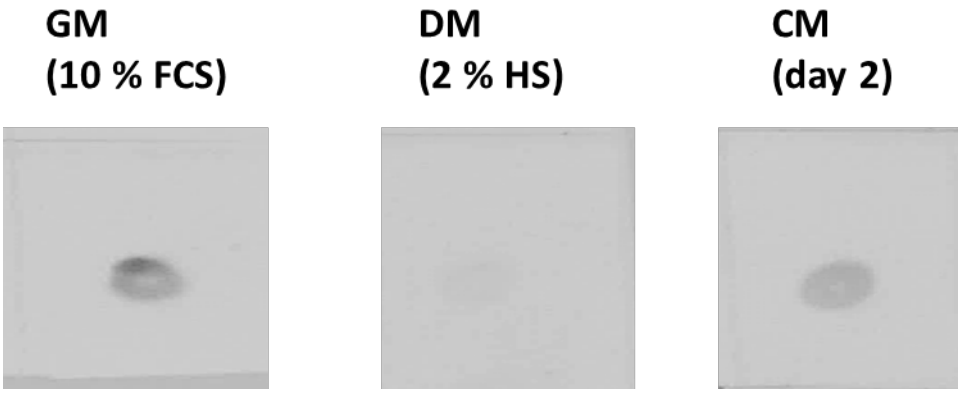


Figure 3.6: **Dot blot to determine if MMP-2 is found in culture media**. Determination of MMP-2 in growth media (GM) with 10% FCS, differentiation media (DM) with 2% HS and day 2 conditioned media (positive control). It is observed that GM contains proMMP-2 that can interfere with the results, while DM does not. This means DM is ideal to use to determine the amount of MMP-2 protein secreted by C2C12 myoblast during differentiation.

3.3.3. **MMPs are important for fusion**

BB94 is a commercially available broad spectrum synthetic MMP inhibitor (Botos et al., 1996). To establish the importance of MMPs during myoblast fusion, we differentiated C2C12 cells for five days in the presence or absence of BB94 (1 and 10 μM); a DMSO (1/1000) control was included as BB94 had been reconstituted in DMSO. Fusion was determined following LADD staining and microscopy (as described in Section 3.2.3).

Analysis of differentiated cells at day 5 revealed substantial fusion under control conditions and in the presence of 1 μM BB94; however, following inhibition with 10 μM BB94, fewer myotubes were observed (Figure 3.7 A). Calculation of fusion indices revealed that, under control conditions (in the absence or presence of DMSO), myoblasts reached fusion indexes of 54 ± 3% (DM control) or 56 ± 2% (DMSO control) by day 5. Fusion remained at high levels in the presence of 1 μM BB94 (50 ± 3%), however, it was significantly decreased to 27 ± 5% ($p<0.05$) in the presence of 10 μM BB94 compared to the DMSO control and 1 μM BB94 (Figure 3.7 B). This confirmed the importance of MMP activity for successful fusion.

To confirm inhibition of MMP-2 by BB94, two gelatin gels were prepared as (Section 3.2.2.2); recombinant active MMP-2 (0.5 μg) activity was analysed in both gels. One of the gels was incubated in buffer that contained 10 μM BB94. MMP-2 activity was not observed in this gel; however in the gel incubated in the absence of the inhibitor (control), activity was observed (Figure 3.7 C). This confirms that BB94 inhibited MMP-2 activity.

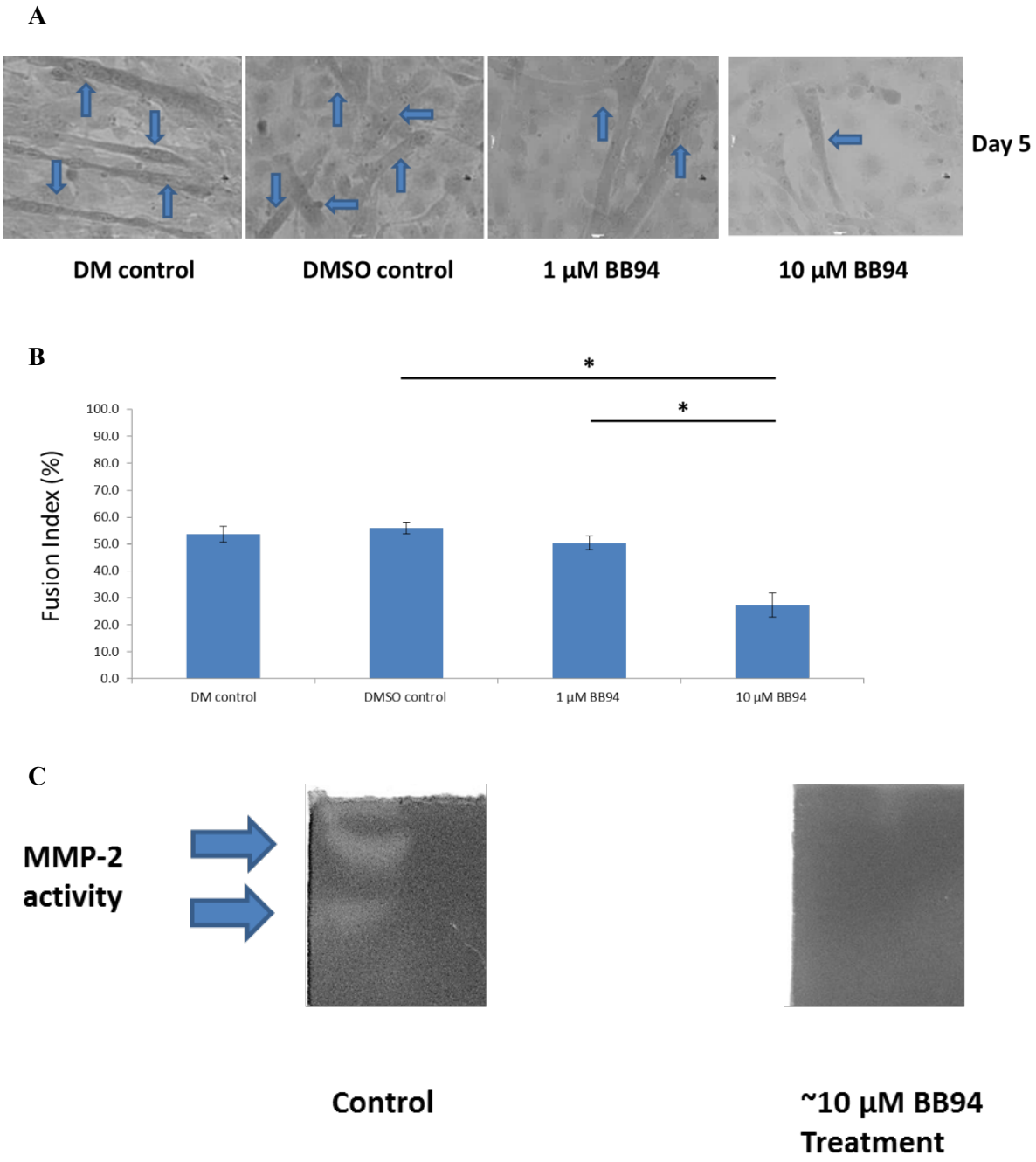


Figure 3.7: **The effect of BB94 on C2C12 myoblast differentiation**. C2C12 myoblasts were cultured to ~70% confluence in growth media, then treated with differentiation media (DM control), 1/1000 DMS0 dilution in DM (DMSO control) or BB94 (1 or 10 µM) in DM for the period of 5 days. Media change was carried out every 48 hours. Conditioned media was harvested for further analysis, and cells fixed with 70 % ethanol and stained with 6 % Ladd's stain. **A)** Bright-field photos (200X Magnification), where blue arrows show fused myoblasts. **B)** Fusion index at day 5. N=4, all figures represent mean ± SEM,* = $p<0.05$. **C)** MMP-2 activity inhibition by 10 µM BB94 viewed on a zymogram with recombinant active MMP-2 protein loaded.

3.3.4. **MMP-2 is crucial for C2C12 myoblast fusion**

Given that active MMP-2 is secreted by differentiating C2C12 myoblasts, and MMPs are crucial for fusion, we next wished to determine whether the secreted active MMP-2 (Figure 3.5) is responsible for successful fusion. C2C12 cells were differentiated in the presence or absence of monoclonal anti-MMP-2 antibody (1/400 dilution: 5 µg/ml); media was changed every 48 hours. The presence of monoclonal anti-MMP-2 antibody decreased C2C12 myoblast fusion from 52 ± 5% to 34 ± 4%, an 18 % decrease from control (Figure 3.8). MMP-2 is therefore, at least in part, responsible for successful myoblast fusion.

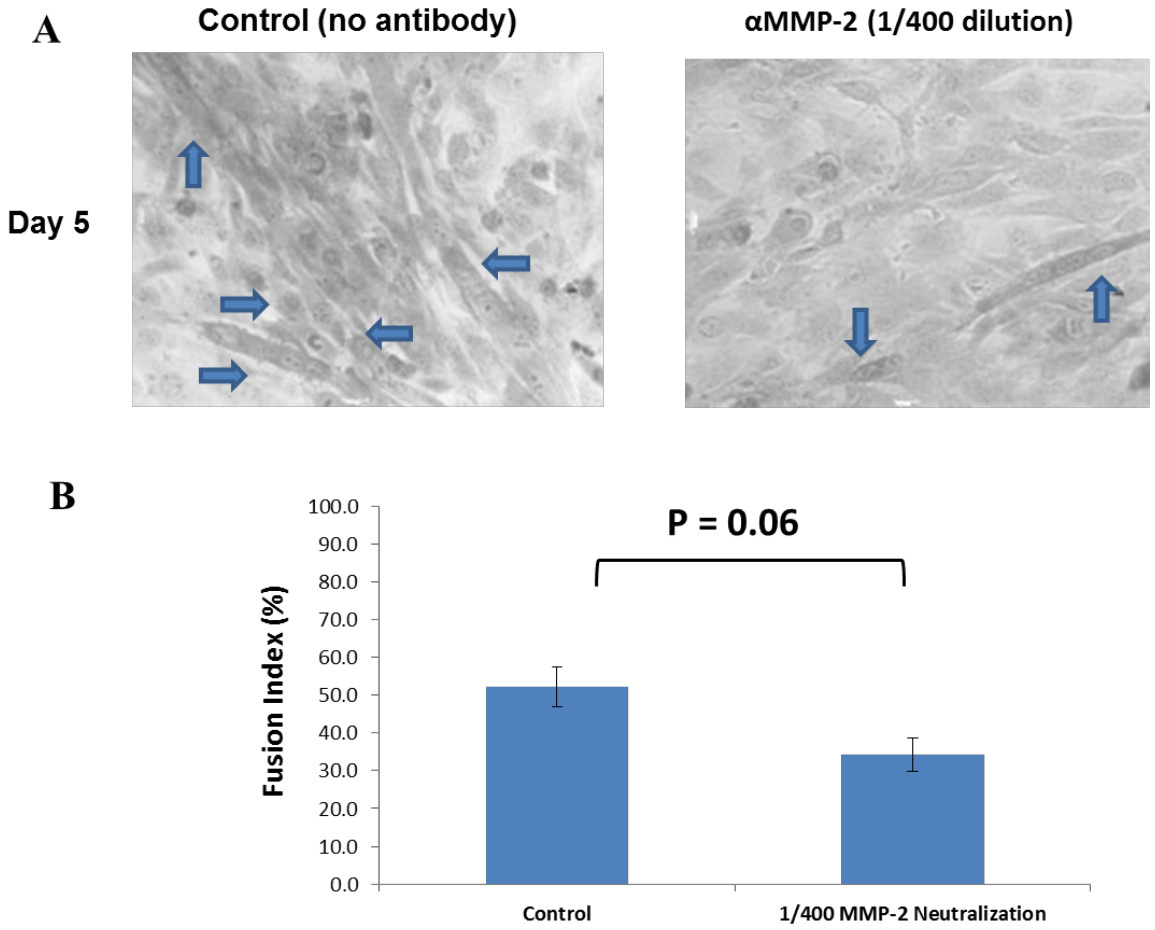


Figure 3.8: **The effect of MMP-2 neutralization on C2C12 myoblast differentiation**. C2C12 myoblasts were grown to 70% confluence in growth media, then treated with differentiation media (control) in the presence or absence of 1/400 monoclonal anti-MMP-2 antibody for a period of 5 days. Cells were then fixed with 70 % ethanol and stained with 6 % Ladd's stain. **A)** Bright-field photos taken at day 5 (200X Magnification) where blue arrows show fused myoblasts. **B)** Fusion index at day 5. N=4, all figures represent mean ± SEM,*$p<0.05$.

3.4. **Discussion**

Successful C2C12 myoblast differentiation was observed, where myoblasts first proliferate (Day 0), then align and elongate in close proximity to one another (Day 1-2) and finally form myotubes at days 3-5 (Figure 3.1).

Confocal microscopy revealed that MMP-14 is expressed by proliferating myoblasts (Day 0), mostly localised at focal adhesion where actin filaments terminate in myoblasts during early and late differentiation (Figure 3.2). The location of MMP-14 at the outer membrane is crucial to understand its role in degrading ECM factors during the elongation period of C2C12 myoblast myogenesis (Ohtake et al., 2006), where actin fibers 'end' at focal adhesions – perhaps this is where the ECM must be digested to allow migration. MMP-14 is also important for activating secreted proMMP-2 into active MMP-2 which can assist MMP-14 in degrading ECM factors as MMP-14 cannot degrade collagen IV, while MMP-2 can (Ohtake et al., 2006, Murphy and Crabbe, 1995, Nagase, 2001). The reason why MMP-14 cannot degrade collagen IV, is due to the lack of a triple repeat of fibronectin type II domain within its catalytic domain which can recognise and bind collagen IV while MMP-2 has this feature (Murphy and Crabbe, 1995, Nagase, 2001).

Figure 3.3 showed a similar profile to recent studies (Ohtake et al., 2006). Minor differences were found where the levels of MMP-14 expressed in the cell were low during day 0. Ohtake et al., (2006) observed high levels of MMP-14 during elongation period of differentiation; this observation was similar to our results where high MMP-14 levels seen during elongation period and reaching maximum during initial fusion of C2C12 differentiation (Day 3) (Figure 3.3). This suggests that MMP-14 is essential during the elongation period, where MMP-14 degrades ECM factors allowing the myoblasts to migrate, align, elongate and fuse into myotubes; our results suggests that indeed MMP-14 is important during the elongation period, but also important during initial fusion of C2C12 myoblasts. Previous studies stressed the importance of the different levels of MMP-14, MMP-2 and TIMP-2 in myoblast myogenesis (Ohtake et al., 2006, Lluri and Jaworski, 2005a, Lluri and Jaworski, 2005b). The different profile of MMP-14 expression/levels during proliferation and differentiation highlights the potential importance of MMP-14 during, proliferation, migration and fusion of C2C12 myoblasts.

The MMP-2 protein levels profile also followed a similar profile to secreted mRNA profile explored by Ohtake et al., (2006), with minor differences where mRNA levels showed maximum peak at day 3 and not day 5. This makes sense because mRNA levels will increase

prior to proteins levels. Intracellular MMP-2 levels were previously found to be constant during C2C12 myoblast myogenesis (Lluri and Jaworski, 2005b). This may be because most of the MMP-2 protein is secreted. MMP-14 protein expression was maximum at day 3 (Figure 3.3) while secreted MMP-2 protein levels were increasing at day 5 (Figure 3.4); this may suggest that MMP-14 is activating proMMP-2 to active MMP-2. Figure 3.5 shows that secreted MMP-2 is active during differentiation of C2C12 myoblasts. MMP-2 activity was observed as early as day 1; this would explain why we can observe MMP-14 protein levels in proliferating myoblasts (Figure 3.3). Unfortunately, we couldn't analyse secreted MMP-2 protein levels of proliferating (Day 0) myoblasts due to the presence of MMP-2 in FCS (Figure 3.6). In future we could use serum-free media like F-12 to further analyse MMP-2 secreted by proliferating myoblasts. Our results illustrate that MMP-14 and MMP-2 are not just important during the elongation period as predicted by Ohtake et al., (2006), but also crucial during fusion.

Figure 3.7 showed how important MMPs are in C2C12 myoblast fusion. Ohtake et al., (2006) carried out a similar study were they inhibited C2C12 myoblast fusion by BB94 (10 μM), and they concluded that inhibition of MMPs during the elongation step of myogenesis leads to a decrease in myotubes formation. The possible reason is that during the elongation step of C2C12 myoblast myogenesis, MMPs such as MMP-14 and MMP-2 are needed to cleave ECM components to allow the cells to move into close proximity to one another and align prior to fusing with one another to form myotubes. Figure 3.7 C, showed that BB94 can indeed inhibit MMP-2 activity, stressing importance of MMP-2 activity during C2C12 myoblasts fusion. This confirms that MMPs play a vital role in C2C12 myoblast fusion.

The activity of the secreted MMP-2 is seen all throughout C2C12 myoblast differentiation (Figure 3.5), where it was further noted that BB94 can inhibit MMP-2 activity meaning that possibly MMP-2 is important in C2C12 myoblast differentiation. The importance of MMP-2 was further confirmed by Figure 3.8, where it was observed that by neutralizing MMP-2 activity by using a MMP-2 monoclonal antibody, fusion decreased by ~18% in comparison to the control. This tended towards significance ($p=0.006$), suggesting that MMP-2 may not work alone. The prohibitive cost of the experiment meant that we only neutralized every 48 hours; it would be beneficial in the future to neutralize every 24 hours. We also need to include an isotype control for MMP-2 to ensure MMP-2 antibody specificity. Another experiment we could explore in the future is to neutralise MMP-14 activity and harvest the conditioned media to see if there is any decrease of secreted MMP-2 levels seen; this would confirm a direct relationship between MMP-14 and MMP-2.

In conclusion, we have shown that MMP-14 and MMP-2 do not only play a role during the elongation period of myogenesis, but are also essential during fusion. We next wished to investigate whether Matrigel affects MMP-2 expression and role in myogenesis.

Chapter 4:

Effect of Matrigel on MMP-2 expression and activity

Abstract

Satellite cells reside in a niche between the sarcolemma of the muscle fiber and the basal lamina; the basal lamina consists of mostly collagen IV, proteoglycans and laminin. Matrigel is a gelatinous protein mixture that consists primarily of collagen IV and laminin and therefore resembles the basal lamina. MMPs such as MMP-2, are known to degrade ECM components, facilitating satellite cell mobility and release of growth factors such as HGF, aiding in muscle regeneration. Secreted MMP-2 is specifically known to process components of the basal lamina, such as collagen IV and laminin. In this study we explored the role of Matrigel on MMP-2 expression and secretion during C2C12 myoblast differentiation. C2C12 myoblasts were differentiated on Matrigel for a period of 5 day; cell lysates and conditioned media were harvested and analysed at day 2 and 5. Interaction of MMP-2 with Matrigel was also investigated. Matrigel promoted an increase of MMP-2 expression within the cell during day 5 of fusion, while no intracellular MMP-2 protein was observed at day 2 of differentiation. Levels of secreted MMP-2 increased significantly from day 2 to day 5 of differentiation; under control conditions, however, the presence of Matrigel significantly reduced levels of secreted MMP-2 detected in conditioned media at day 5 compared to uncoated conditions. The decrease may be in part due to the fact that MMP-2 was found to bind to Matrigel. In conclusion, levels of secreted MMP-2 in conditioned media of differentiating C2C12 myoblasts is significantly decreased at day 5 compared to uncoated conditions; this is not due to a decrease in intracellular MMP-2 levels at this time point, but may be due to the ability of Matrigel to bind and sequested MMP-2. A direct role for MMP-2 in myoblast fusion still requires investigation.

4.1. **Introduction**

Skeletal muscle myofibers are surrounded by an extracellular matrix (ECM) which helps in tissue structure support, and also regulates many cellular processes (Davis et al., 2013, Ohtake et al., 2006, Thomas et al., 2015). The ECM consists of different types of proteins, proteoglycans, and polysaccharides. The ratio of these components differs with each tissue type (Thomas et al., 2015). The intramuscular connective tissue is mostly composed of collagens, but also contains components like fibronectin, integrins, tenascins, laminins, and proteoglycans (Thomas et al., 2015, Kjaer, 2004). Collagen I is the most abundant of all collagens in the ECM that surrounds skeletal muscle fibers (Kjaer, 2004). Collagen IV forms a major component of the basement membrane and interacts with proteoglycans and laminins (Thomas et al., 2015, Sanes, 2003). The niche environment, which keeps satellite cells quiescent, consists of ECM factors such as collagen IV and laminins and low amounts of growth factors such as Hepatocyte Growth Factor (HGF) (Bentzinger et al., 2012, Allen et al., 1995). When a satellite cell is activated it often moves through this ECM prior to differentiation and fusion with target myofibers (Thomas et al., 2015).

MMPs are able to degrade ECM components and other adhesion molecules, releasing satellite cells from their niche (Thomas et al., 2015) and growth factors (such as HGF) from the ECM, thereby aiding in muscle repair and regeneration (Ohtake et al., 2006, Thomas et al., 2015, Allen et al., 1995). Collagen IV, a major component of the basal lamina, is degraded by MMP-2, but not MMP-14. This is due to the triple-repeat of fibronectin type II domain on MMP-2 catalytic domain that allows additional specificity in MMP-2 to bind with collagen IV; MMP-14 lacks this feature (Anita and Murphy, 1998, Nagase, 2001, Visse and Nagase, 2003).

Matrigel is a gelatinous protein mixture produced by Engelbreth-Holm-Swarm (EHS) mouse sarcoma; it resembles the basal lamina as it is composed primarily of collagen IV and laminins; it has been shown to improve myoblast differentiation *in vitro* (Grefte et al., 2012). A study by Goetsch, (2012) also revealed that Matrigel significantly improved C2C12 myoblast migration. MMP-2 is not just essential during repair and regeneration of necrotic damaged muscle, but also plays a pivotal role in muscular atrophy (Liu, 2011). A previous study done by Liu, (2011), suggested a major cause of muscular atrophy to be the proteolytic activity of intracellular MMP-2. In the previous chapter we showed that secreted MMP-2 plays a vital role in C2C12 myogenesis. In the current chapter, we aimed to determine whether Matrigel affects MMP-2 expression by myoblasts.

4.2. **Materials and methods**

4.2.1. **Effect of Matrigel on myoblast myogenesis**

Cold 10.1 mg/mL Matrigel solution (BD Bioscience, cat.CN-354230, USA) was diluted to a desired concentration of 120 µg/mL with DMEM, this was kept on ice. T75 flasks were coated with 5 mL Matrigel (120 µg/mL) overnight at 37^{o}C, where the flasks were washed with DMEM before C2C12 myoblasts were cultured. Uncoated flasks served as the control, where C2C12 myoblasts were differentiated for a period of 5 days with differentiation media, and cell lysates and conditioned media were harvested at day 2 and 5 for further analysis.

4.2.2. **Effect of Matrigel on MMP-2 expression**

The cell lysate harvested (Section 4.2.1.1) were analysed for MMP-2 protein levels via Western blots and GAPDH was used as an internal control. The blots were analysed using the Image J program, where the data was normalised by GAPDH data and plotted (N=4) with mean ± SEM. The conditioned media was also analysed in a similar fashion as above for secreted MMP-2 protein levels. The loading control was a Ponceau S stain.

4.2.3. **Does secreted MMP-2 bind to Matrigel components?**

Glass slides were coated with Matrigel (120 µg/mL) overnight, after which the glass slides were washed with DMEM and incubated with day 2 conditioned media for 25 minutes. We then washed the glass slides with PBS (3 times) and slides underwent immunocytochemical staining with rabbit monoclonal anti-MMP-2 antibody (1/400 dilution) (green) (Section 3.2.1.1) and viewed with Zeiss LSM 710 confocal microscope.

4.3. **Results**

4.3.1. **Matrigel promotes C2C12 myoblast differentiation**

Matrigel is a gelatinous protein mixture composed primarily of collagen IV and laminin, therefore resembling the basal lamina (Grefte et al., 2012). To better understand how MMP-2 help facilitate C2C12 myoblast fusion, it was crucial to mimic the *in vivo* environment where cells/tissues are constantly surrounded by ECM factors. To determine the effect of Matrigel on myogenesis, C2C12 myoblasts were differentiated for a period of 5 days in uncoated or coated

(120 µg/mL Matrigel) flasks (Figure 4.1). Matrigel improved alignment and elongation of C2C12 myoblast at day 2 in comparison to the control (Figure 4.1). Myoblast fusion was improved by day 5 with more myotubes observed in Matrigel-coated flasks in comparison to control (Figure 4.1; red arrows), myotubes were bigger in size in comparison to the control.

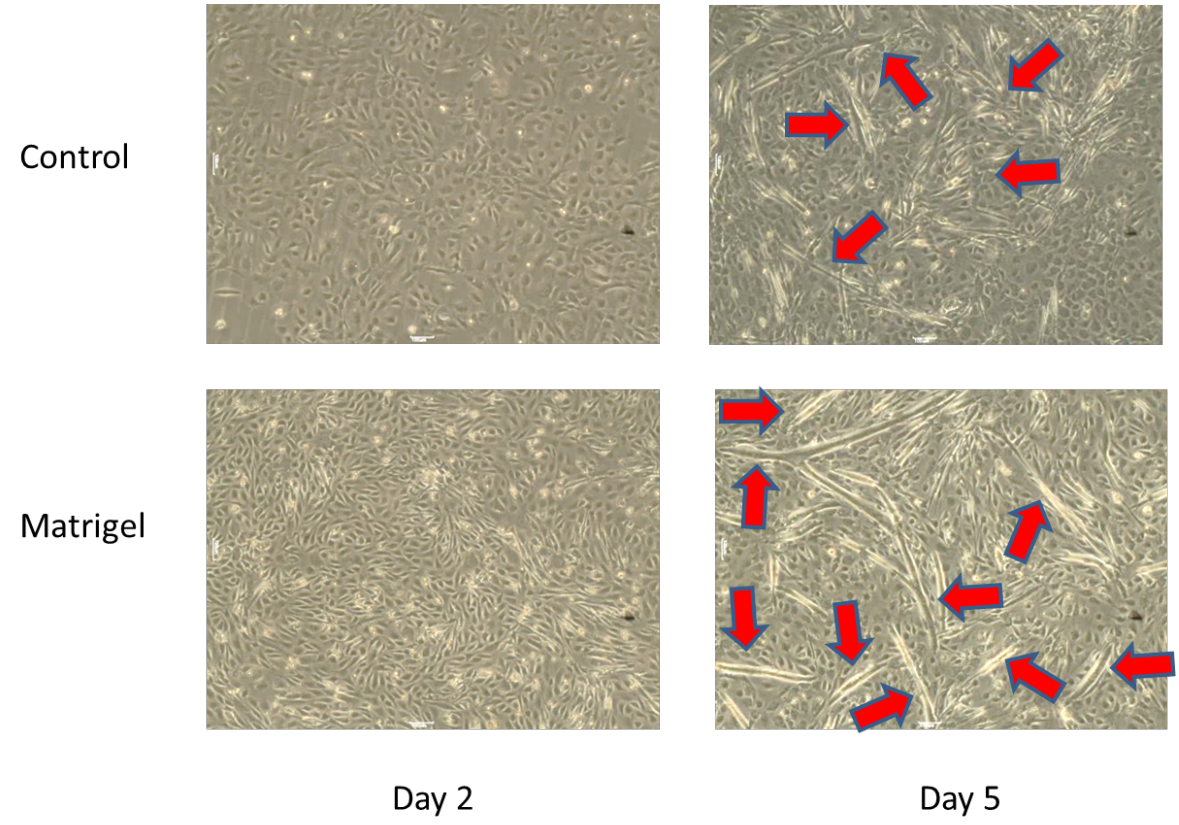


Figure 4.1: **Effect of Matrigel on C2C12 myoblasts differentiation**. Bright-field images (40X Magnification) of C2C12 myoblast differentiation at days 2 and 5 cultured in the presence or absence of Matrigel. Red arrows indicating C2C12 fusion day 5 for Matrigel-coated and uncoated (Control) conditions.

4.3.2. **Effect of Matrigel on secretion of MMP-2 protein into the conditioned media**

We next wished to determine whether Matrigel alters the secretion of MMP-2 by myoblasts. C2C12 myoblasts were therefore cultured in the presence or absence of Matrigel and conditioned media harvested and concentrated as described in Section 4.2.1. Samples were analysed via SDS PAGE and Western blot.

MMP-2 protein levels detected in the conditioned media at day 2 were similar in the presence or absence of Matrigel (Figure 4.2). However, unlike cells differentiated in the absence of Matrigel (where an increase in MMP-2 was detected in the conditioned media by day 5), cells cultured on Matrigel were observed to contain significantly lower levels of MMP-2 in their conditioned media at day 5 of differentiation than control (Figure 4.2). We therefore next

analysed the levels of intracellular MMP-2 in differentiating C2C12 myoblasts under the same conditions.

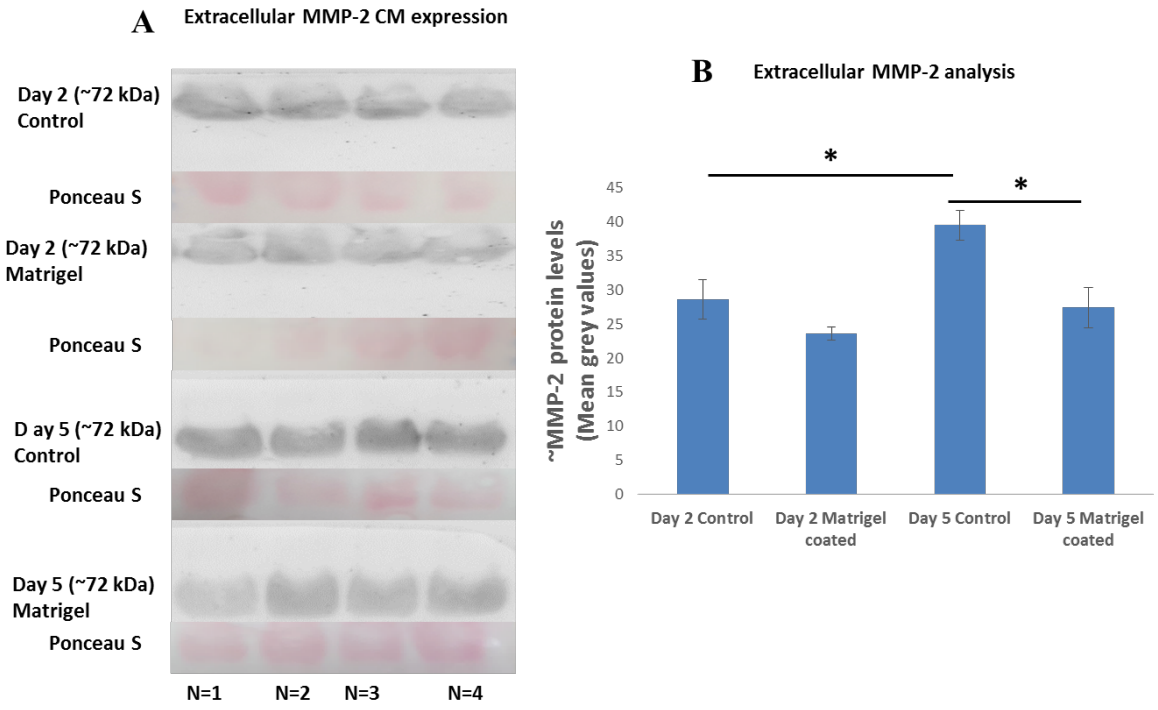


Figure 4.2: **Effect of Matrigel on extracellular (secreted) MMP-2 protein levels**. C2C12 myoblasts cultured in Matrigel coated flask and uncoated (Control) subjected to differentiation for 5 days, where conditioned media were harvested at days 2 and 5 for MMP-2 protein level analysis. **A)** Western blot analysis of the protein levels of MMP-2 secreted at days 2 and 5 of differentiation showing the blots with MMP-2 and loading control (Ponceau S) where the blots were further analysed using Image J. **B)** The MMP-2 mean grey values were plotted. N=4, all figures represent mean ± SEM, *p<0.05.

4.3.3. **Effect of Matrigel on intracellular MMP-2 expression**

In order to determine whether Matrigel prevents an increase in intracellular MMP-2 expression, cells were differentiated in the presence or absence of Matrigel and lysates harvested at days 2 and 5 of differentiation. Samples were then subjected to SDS PAGE, Western Blot and quantitative analysis using Image J. MMP-2 data was normalised to GAPDH expression (Figure 4.3).

Intracellular MMP-2 protein in differentiating C2C12 cells were undetectable at day 2 for both Matrigel-coated and un-coated conditions (Figure 4.3). By day 5, MMP-2 intracellular protein expression was detectable in both Matrigel and control cells, with higher levels evident in cells cultured on the Matrigel, however this was not significant (Figure 4.3). Taken together with Figure 4.2, this data suggests firstly that most MMP-2 is secreted during early differentiation (Day 2) and secondly that Matrigel does not negatively or significantly affect the expression of intracellular MMP-2 during late differentiation (Day 5) (Figure 4.3).

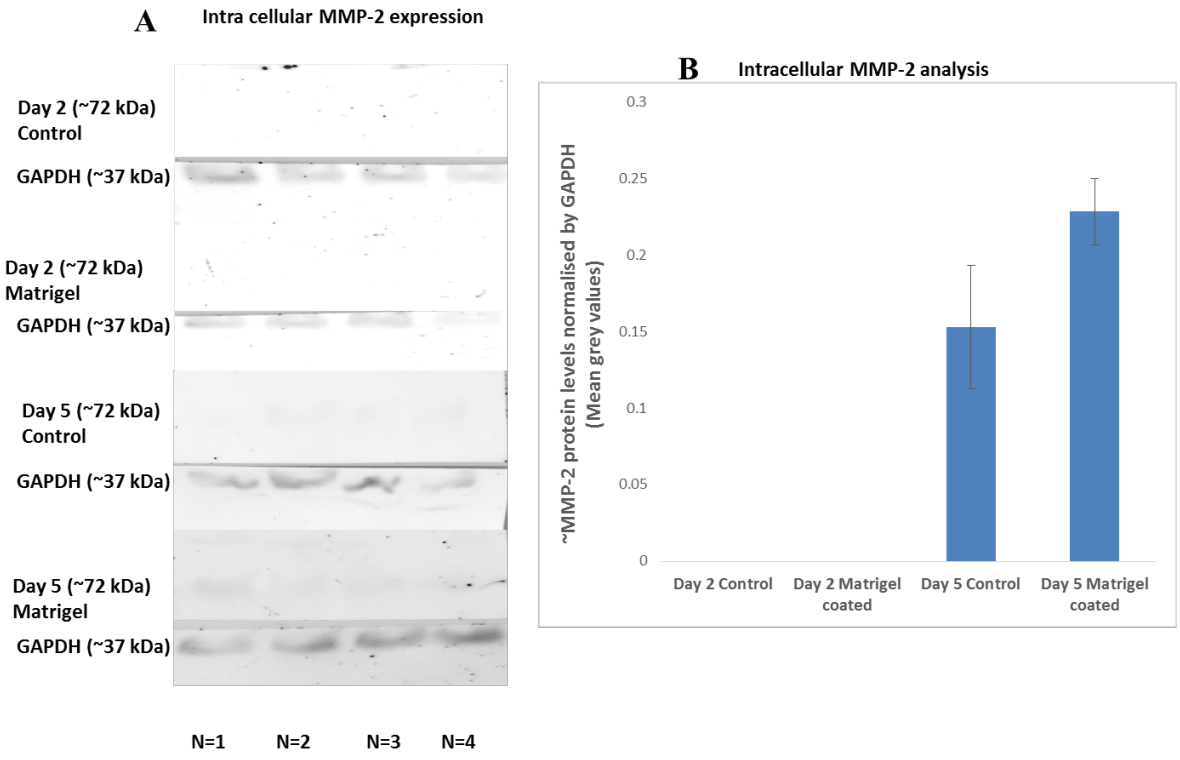


Figure 4.3: **Effect of Matrigel on intracellular MMP-2 protein expression**. C2C12 myoblasts cultured either in the presence or absence of Matrigel were differentiated for 5 days and cell lysates harvested at days 2 and 5. **A)** SDS PAGE and Western blot analysis of lysates was the carried out to analyse the protein expression of MMP-2. **B)** All data was normalised to GAPDH, the internal control and plotted. N=4, all data represents mean ± SEM, *p<0.05.

4.3.4. **Secreted MMP-2 binds to Matrigel components**

The extracellular matrix is known to bind many proteins (Grefte et al., 2012). Given the perceived decrease in secreted MMP-2 in the conditioned media of cells cultured on Matrigel, we sought to determine whether Matrigel itself may bind or sequester the secreted MMP-2, thereby explaining, at least in part, the decrease in MMP-2 seen in Figure 4.2. Glass slides were coated overnight with either 0 or 120 µg/mL Matrigel; the slides were then incubated with conditioned media (250 µL; day 2; 60 µg/mL) for 25 minutes at 37°C after which they were

washed, fixed, blocked and immunocytochemically stained with mouse anti-MMP-2 antibody (green) and viewed under confocal microscopy (Figure 4.4). Secreted MMP-2 (green arrows) in the conditioned media was observed to bind to Matrigel, but was not observed on the uncoated slide (Figure 4.4). Therefore it is possible that at least a portion of the secreted MMP-2 was bound to Matrigel, accounting for the observed decrease in secreted levels in cells cultured on the matrix factor (Figure 4.2).

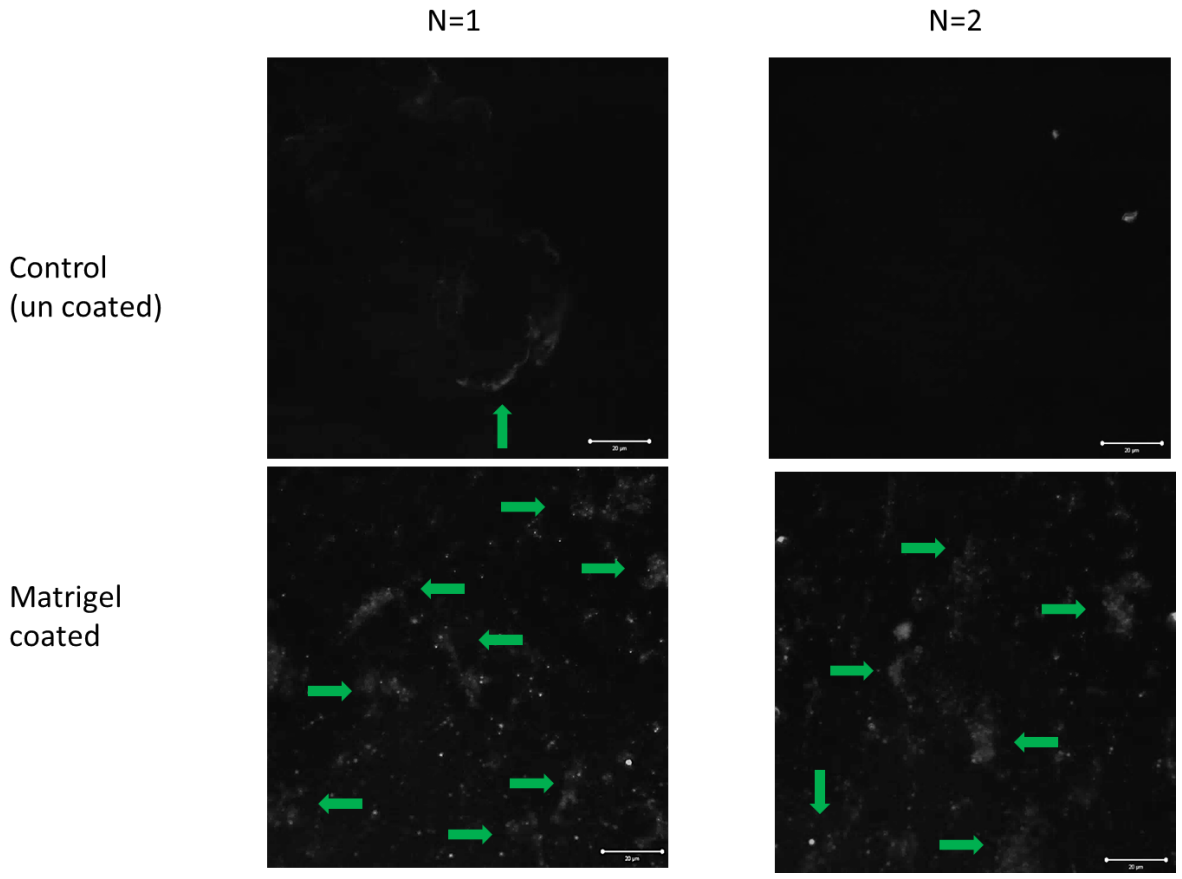


Figure 4.4: **Secreted MMP-2 proteins interact with Matrigel**. Glass slides, either coated with Matrigel or left uncoated (control), were incubated for 30 minutes with day 2 conditioned media. Slides were then washed and immunostained to locate MMP-2 via confocal analysis (630X Magnification). Green arrows indicate positive MMP-2 staining. MMP-2 was detected on slides coated with Matrigel, but not uncoated slides.

4.4. **Discussion**

In the previous chapter we observed that secreted MMP-2 plays a vital role in C2C12 fusion. Matrigel, an extracellular matrix comprised primarily of collagen IV and laminin, is known to increase C2C12 myoblast fusion (Grefte et al., 2012); this was confirmed in Figure 4.1. Given the ability of MMP-2 to proteolytically cleave collagen IV, we next investigated whether the presence of Matrigel alters MMP-2 expression and secretion by C2C12 myoblasts. At day 2 of differentiation, extracellular (secreted) MMP-2 was detected in the conditioned media;

however there was no significant difference between uncoated and Matrigel-coated conditions (Figure 4.2). Intracellular MMP-2 protein expression (Figure 4.3) was undetectable for both Matrigel-coated and uncoated conditions, at this time-point, suggesting that all expressed MMP-2 had been secreted from the myoblasts.

By day 5, we detected significantly higher MMP-2 protein in the media of myoblasts cultured on uncoated wells in comparison to Matrigel-coated wells (Figure 4.2). Levels of MMP-2 in the conditioned media of cells cultured on Matrigel were in fact significantly lower than those cultured in the absence of Matrigel; this despite the fact that intracellular MMP-2 expression was not decreased at day 5 under coated vs. uncoated conditions. At day 2 of differentiation, elongation and myoblast alignment is underway; Ohtake et al., (2006) hinted that it is at this stage where secreted MMP-2 plays a pivotal role in preparing myoblasts for fusion. We show higher expression of MMP-2 at day 5 than day 2 (irrespective of the presence or absence of Matrigel), suggesting a role for MMP-2 in later fusion as well.

The lower MMP-2 secretion at day 5 under Matrigel versus uncoated conditions was puzzling, as Matrigel is known to promote fusion. Further investigation detected some secreted MMP-2 bound to Matrigel, explaining at least in part, the lower MMP-2 levels detected in the conditioned media of cells cultured on Matrigel. MMP-2 has a fibronectin-like domain in its catalytic domain that can interact with collagen IV (Murphy and Crabbe, 1995, Nagase, 2001). Figure 4.4 demonstrates that some of the secreted MMP-2 protein is bound to Matrigel; this may assist degradation of collagen IV by MMP-2 by ensuring proximity of the enzyme for its substrate. A question that remains to be answered is whether this secreted MMP-2 is responsible (at least in part) for the increased fusion observed in response to Matrigel. Extracellular (secreted) MMP-2 may play a role in differentiation via its release of HGF, which in turn stimulates activation of gene expression (Figure 1.8).

In summary, MMP-2 and Matrigel seems to play an important role during C2C12 myoblast, elongation and fusion, where the presence of Matrigel did not significantly affect intracellular MMP-2 expression, but decreased significantly the amount of secreted MMP-2 found in the conditioned media. This effect is part, due to Matrigel components binding to MMP-2, as secreted MMP-2 is known to be the major processor of Matrigel. In future we would like to explore the relationship of HGF and MMP-2 on C2C12 myoblast fusion as previous research has shown that MMP-2 is able to help release HGF from ECM factors.

Chapter 5:
General Discussion

Undifferentiated mouse C2C12 myoblast cells are capable of proliferating, differentiating and fusing into myotubes, making an ideal *in vitro* model for skeletal muscle repair and regeneration studies. Growth factors, such as HGF, are known to be one of the major factors required for successful repair and regeneration (Allen et al., 1995, Walker et al., 2015). Skeletal muscle repair and regeneration requires the activity of MMPs, in order to release growth factors from the extracellular matrix (ECM) and facilitate satellite cell activation to aid in skeletal muscle repair and regeneration (Thomas et al., 2015). MMP-2, which is activated by MMP-14 and TIMP-2 (Ohtake et al., 2006), or via nitric oxide (NO) radicals (Hara et al., 2012, Filippin et al., 2011a, Filippin et al., 2011b), is known to release HGF from the ECM, therefore aiding in muscle repair and regeneration. Previous studies (Ohtake et al., 2006, Liu, 2011, Lluri and Jaworski, 2005b), have shown that MMP-14 and MMP-2 play an important role in myogenesis; this was also evident in our studies.

Our main findings were as follows:

- MMP-14 expression was maximum at day 3
- MMP-14 was mostly localised to the focal adhesion at day 5
- Secreted MMP-2 (levels and activity) was maximum at day 5
- Secreted MMP-2 is essential during C2C12 myoblast fusion
- Matrigel increased intracellular MMP-2 expression at day 5
- Secreted MMP-2 can bind to Matrigel

Our results show that MMPs, especially MMP-2 activity is not just important during C2C12 myoblast elongation but also during the fusion stage. During the elongation stage, MMP-14 and MMP-2 assist in degrading ECM components allowing cells to migrate, align and fuse to form myotubes (Ohtake et al., 2006). It is not clear how exactly MMP-2 facilitates myoblast fusion, but it may be due to its ability to release HGF from the extracellular matrix, which would then facilitate satellite cell activation and differentiation (Figure 1.8 and 5.1). In this respect, our observation that secreted MMP-2 in the conditioned media can bind to Matrigel components may suggest that the extracellular matrix can act as a repository for these proteases as well as growth factors. Collagen IV makes up the majority of the basal lamina and is

degraded by MMP-2, but not MMP-14 (Nagase, 2001). This is due to the triple-repeat of fibronectin type II domain on MMP-2 catalytic domain that allows additional specificity in MMP-2 to bind with collagen IV and degrade it; MMP-14 lacks this feature (Anita and Murphy, 1998, Nagase, 2001, Visse and Nagase, 2003).

Recent studies have indicated that there are two forms of MMP-2, namely intracellular MMP-2 and extracellular MMP-2; the main structural difference between them is that extracellular MMP-2 contains a signal peptide that helps direct it to the outside of the cell while intracellular MMP-2 lacks this feature and is therefore retained within the cell (Liu, 2011) (Figure 5.1). Intracellular MMP-2 helps in degrading intracellular structural components within the cell that assist in cell structure and cell communication such as actin filaments (Liu, 2011), while extracellular (secreted) MMP-2 activated by NO radical (Hara et al., 2012, Filippin et al., 2011a) or MMP-14/TIMP-2 complex (Ohtake et al., 2006) can then further degrade extracellular matrix components allowing cells to migrate, align and fuse to form multinucleated myotubes; it can also help in releasing growth factors such as HGF that aid in muscle repair and regeneration (Figure 5.1). Intracellular MMP-2 has been reported to play a pivotal role in muscular atrophy (Liu, 2011). Specifically, intracellular MMP-2 can digest some essential intracellular components; transcription factors such as Activator Protein (AP-1) and Nuclear Factor of Activated T-cells (NFAT) lead to the expression and activation of intracellular MMP-2 during muscular atrophy (Liu, 2011) (Figure 5.1). Furthermore, MMP-2 knockout mice demonstrate a decrease in muscular atrophy in comparison to MMP-9 and MMP-13 knockout mice, further supporting a role for this protease in muscle atrophy (Liu et al., 2010, Liu, 2011).

Our results showed that Matrigel had no significant influence to the expression of intracellular MMP-2, however the presence of Matrigel did have an effect on the amount of free MMP-2 secreted to the conditioned media, where some of the secreted MMP-2 was bound to Matrigel components. At day 2 levels of intracellular MMP-2 was undetectable, but the levels increased during day 5 for both the Matrigel-coated and uncoated experiment. From approximately day 5 this is where myotubes cells express intracellular matrices such as MyHC, which is very crucial during muscle maturation and muscle contractions. Does the muscle cell produce intracellular MMP-2 in order to regulate intracellular proteins such as MyHC??? Much of the research has focused on extracellular MMP-2 where it showed much importance in particular to muscle repair and regeneration, but the role of intracellular MMP-2 during muscle maturation still requires attention.

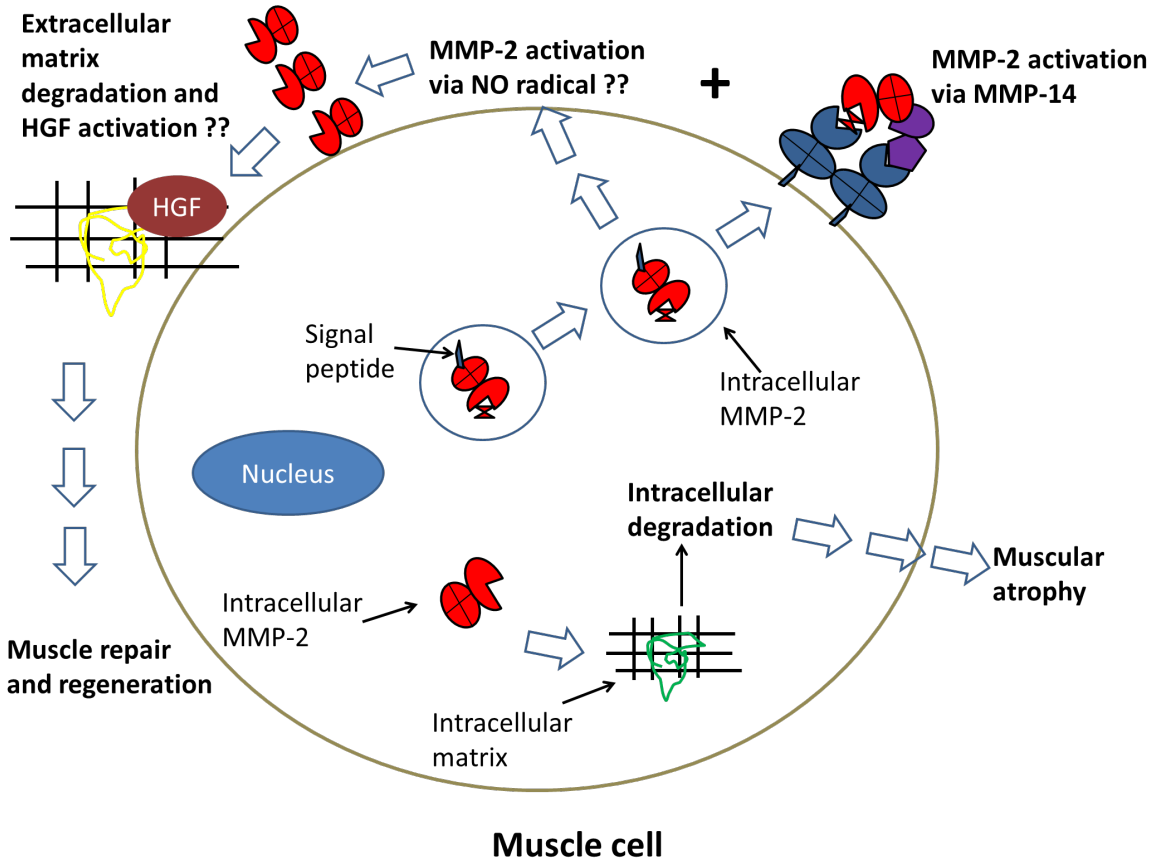
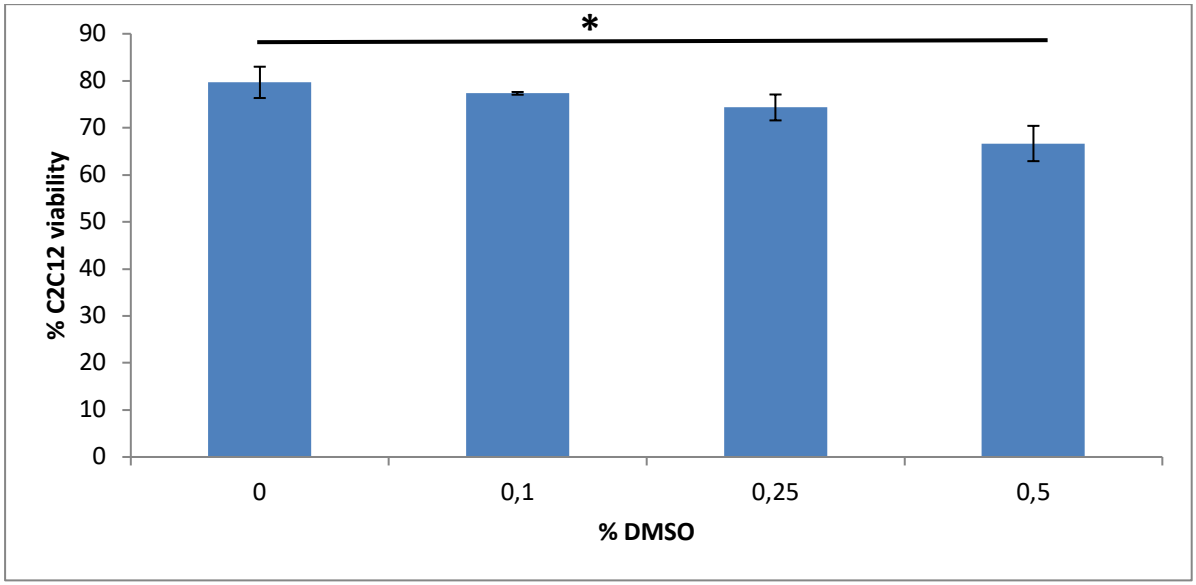


Figure 5.1: **Schematic diagram to show the role of MMP-2 in muscular atrophy, repair and regeneration**. A schematic diagram showing the role of intracellular MMP-2 during muscular atrophy and also the path of extracellular MMP-2 during muscle repair and regeneration. Extracellular MMP-2 contains a signal peptide that helps direct it outside the cell where it can either be activated by MMP-14/TIMP-2 complex or nitric oxide (NO) radical; active MMP-2 can degrade extracellular matrix (ECM) aiding in muscle repair and regeneration. Intracellular MMP-2 processes intracellular matrix therefore leading to muscle waste (Muscular atrophy). Figure constructed using Ohtake et al., 2006, Hara et al., 2012, Liu, 2011, Filippin et al., 2011a, Filippin et al., 2011b, Allen et al., 1995.

In conclusion, MMP-14 and MMP-2 are important during C2C12 myoblast myogenesis; our results showed that specific inhibition of MMP-2 decreases fusion. MMP-14 is a critical activator of MMP-2; MMP-2 in turn is known to activate HGF which is one of the mechanisms by which muscle regeneration is facilitated. Secreted MMP-2 can degrade Matrigel; our results also showed that secreted MMP-2 also binds to Matrigel components, suggesting that the ECM can act as a repository for proteases as well as growth factors. MMP-2 occurs as an extracellular and intracellular proteins; the differential role of these two MMP-2 forms in myogenesis requires further exploration, especially as intracellular MMP-2 activity is linked to muscular atrophy.

Future work would therefore explore the role of MMP-14 and MMP-2 (intracellular versus extracellular) on human myoblast differentiation; specifically a direct link between these proteases and fusion will be further explored in the presence of extracellular matrix factors known to be present in skeletal muscle tissue. Furthermore, an analysis of relationship of MMP-2, HGF release and myoblast elongation versus fusion also requires attention. Finally our study will be extended to include 3D bioengineered skeletal muscle tissue (already generated within the laboratory) to explore the role of MMP-2 during muscle regeneration in a system which mimics the *in vivo* environment more closely.

Appendix I: Supplementary figure



Supplementary Figure 1: **Effect of Dimethyl Sulfoxide (DMSO) on C2C12 cell viability.** Cells were cultured with different dilutions of DMSO in growth media (0-0.5% DMSO) over two days and viability determent using 5 µL trypan blue. This is after cells are trypsinized and solution neutralised by DMEM was mixed 1:1 ratio sample:trypan blue (total 10 µL) and then analysed using automated BioRad TC-20 Cell Counter. Addition of 0.5% DMSO, but not 0.1% or 0.25% DMSO resulted in a significant decrease in cell viability. Data includes ± SEM and TTEST *p>0.05.

Appendix II: Conference attendance and presentations

International conference: Indian Ocean Rim Muscle Colloquium (IORMC) 2016, Stellenbosch

MMP-14 and MMP-2 in C2C12 differentiation

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Supervised by Dr Carola Niesler and Dr Celia Snyman

Introduction: MMPs are a family of zinc endopeptidases which bind to and cleave a range of extracellular matrix (ECM) components. MMP-14 is a membrane-bound collagenase, whereas MMP-2 is a secreted gelatinase, which is activated by MMP-14. Remodelling of the ECM is particularly crucial following skeletal muscle necrosis when satellite cells are activated to facilitate myogenesis. Studies suggest that MMP-14 and MMP-2 may be important in mediating this regenerative process.

Aim: To investigate the expression, activity and role of MMP-2 and MMP-14 during myoblast fusion in the presence of extracellular matrix components such as collagen IV.

Methods: To study secreted MMP-2 conditioned media was harvested and concentrated. Activity and levels of cellular MMP-14 and secreted MMP-2 were then assessed via Zymograms and Western blots.

Results: Analysis of MMP-2 expression revealed an increase in secretion as early as day 1 of differentiation. MMP-14 was also expressed early and localised more predominantly to the outer extremities of the cell where fusion would occur. The MMP inhibitor, BB94, reduced myoblast fusion supporting the premise that MMP's are crucial for myogenesis.

Conclusion: MMP-14 is expressed and MMP-2 is secreted during differentiation and fusion of C2C12 myoblasts.

Local conference: Postgraduate Collage Research Day, Collage of Agriculture, Engineering and Science UKZN, 2015

Analysis of MMP-14 and MMP-2 in C2C12 differentiation

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Supervised by Dr Carola Niesler and Dr Celia Snyman

Satellite cells are muscle precursors that have the ability to self-renew, proliferate and differentiate into myoblasts that eventually elongate and fuse to form myotubes which are vital for regeneration and repair of muscle. MMP-14, also known as membrane-type 1 matrix metalloproteinase (MT1-MMP), is one of the major matrix metalloproteinases (MMPs) involved in muscle differentiation together with MMP-2 and TIMP-2. MMP-2 is a secreted gelatinase A, which is activated by MMP-14. MMPs are zinc endopeptidases, a proteolytic peptidase which breaks substrate peptide bonds within the molecule. Hemopexin domain of both MMP-14 and MMP-2 is essential in substrate recognition, where it contains 4 blades stabilised by a calcium ion. MMP-2 can cleave collagen IV due to the presence of a fibronectin-like domain within its catalytic domain which is not the case with MMP-14. MMP-14 and MMP-2 degrades collagen, fibronectin, laminin-2/4 and other adhesion molecules by cleavage. This clears the path for the myoblast to elongate and fuse to form myotubes which than finally align to form mature muscle fiber. The location and the exact manner of activity of MMP-14 and MMP-2 in muscle differentiation is still under research while the majority of the literature is based on cancer. The levels of MMP-14 must be constantly regulated because low levels can cause muscular dystrophy. EDTA and 1,10 Phenanthroline are metal chelators that inhibit MMPs (non-specifically) by chelating both calcium ions and zinc ions. BB-94 is synthetic MMPs inhibitor which inhibits MMPs by inserting one of its rings in the catalytic domain. Intracellular calcium level regulation is vital in myoblast differentiation. M cadherin (M CAD) is a calcium-depended cell-cell adhesion protein also relevant during the fusion process of differentiating myotubes. The study reviews the role of MMP-14, MMP-2, M-CAD and intracellular calcium levels in muscle differentiation.

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