Grape seed extract affects adhesion competence and maturation of primary isolated rat myoblasts after contusion injury

by
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Dissertation presented for the degree of Master in Physiology in the Faculty of Science at Stellenbosch University

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March 2013

DECLARATION

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Date: March 2013

ABSTRACT

Contusion injuries cause significant muscle damage, activating a series of cellular events. Satellite cells (SC), the key role players in muscle regeneration, are activated to proliferate and develop into mature myoblasts, which could fuse to form new myotubes or to repair damaged fibres. Evidence suggests that anti-oxidants, such as those found in grape seed extract (GSE), enhance repair, but their effect on SCs is still unclear.

This study aimed to harvest and culture primary rat myoblasts to investigate the effect of chronic *in vivo* GSE supplementation on SCs following a standardised crush injury.

Using a modified pre-plate technique, myoblasts were harvested from rat muscle and then compared with the immortal C2C12 cell line for proliferation and differentiation competence. Several media options were compared: i) DMEM with or without L-glutamine, ii) Ham's F10 or iii) DMEM with L-glutamine and Ham's F10 combined. Primary myoblasts proliferated and differentiated at a much slower rate than C2C12 cells. The combined media was selected for further use.

To investigate the effects of GSE on the recovery, rats were supplemented daily with GSE or placebo 14 days prior to a standardised mass-drop crush injury to the *gastrocnemius*. SCs were isolated and cultured from uninjured (NI, baseline) and from injured rats 4 hours (4h), 3 days (3d) or 14 days (14d) post-injury. Expression of myogenic proteins Pax7, M-cadherin, MyoD, CD56, desmin and CD34 was determined by flow cytometry. Myoblasts were sorted according to their CD56 and CD34 expression and three sub-sets were collected and re-cultured, namely $CD56^{+}/CD34^{-}$, $CD56^{-}/CD34^{+}$ and $CD56^{+}/CD34^{+}$. After 24 hours, sorted cells were stained for desmin expression.

Pax7, M-cadherin and MyoD were present in 100% of isolated cells from all groups confirming their myogenic SC identity. For all groups, desmin was expressed only in ~80% of SCs. Lower adhesion competency in GSE supplemented groups resulted in lower yield obtained for culturing. Expression of CD56 increased significantly 3d post-injury in the placebo group. In contrast, with GSE, CD56 already increased 4h post-injury and decreased again 3d post-injury. Although CD34 expression did not differ dramatically, expression pattern resembled that of CD56. Immunocytochemistry

revealed a range in morphology and desmin expression of sorted myoblasts. More myoblasts with high desmin expression were observed in the two $CD56^+$ sub-sets (irrespective of CD34 expression), indicating that CD56 is still expressed in more mature myoblasts.

Flow cytometry revealed a population of myoblasts expressing particularly high levels of desmin, primarily in the non-injured baseline GSE group. We hypothesise that this result is an indication of preparedness of myoblasts to respond earlier to injury, enabling quicker repair. This cell population with high desmin content is restored in skeletal muscle after repair (14d), only when supplemented with GSE.

In conclusion, GSE attenuated adhesion competence of primary myoblasts in culture, but resulted in earlier maturation of SCs, possibly due to baseline preparedness of myoblasts in uninjured muscle for a quick response. Both reduced adhesion competence and early progression of myoblasts could enhance wound healing in skeletal muscle.

OPSOMMING

Kneuswonde veroorsaak aansienlike skade aan skeletspier, wat ’n reeks sellulêre prosesse in werking stel. Satellietselle, die hoofrolspelers tydens spierregenerasie, vermenigvuldig en ontwikkel tot volwasse mioblaste, wat saamsmelt om nuwe spiervesels te vorm. Antioksidante, soos die wat in druiwepit-ekstrak voorkom, bespoedig herstel, maar hul uitwerking op satellietselle is steeds onduidelik.

Die doel van hierdie studie was om mioblaste uit rotspiere te isoleer en te kweek om die effek van langdurige *in vivo* aanvulling van druiwepit-ekstrak op satellietselle na ’n kneusbesering te bepaal.

‘n Aangepaste protokol is gebruik om primêre mioblaste te isoleer, wat daarna met C2C12 selle, ten opsigte van hul vermenigvuldigings- en differensiasievermoë vergelyk is. Verskeie groeimedia is gebruik: i) DMEM met of sonder L-glutamien, ii) Ham F10 en iii) ’n kombinasie van DMEM, L-glutamien en Ham F10. Primêre mioblaste het stadiger vermenigvuldig en gedifferensieer as C2C12 selle. Die gekombineerde medium is vir verdere gebruik gekies.

Om die uitwerking van druiwepit-ekstrak op spierherstel te ondersoek, is rotte vir 14 dae onderwerp aan daaglikse aanvullings van druiwepit-ekstrak of *placebo* voor ’n gestandardiseerde kneusbesering aan die *gastrocnemius*. Satellietselle is geïsoleer vanuit onbeseerde spier (basiskontrole) en vanuit beseerde spier 4 ure (4h), 3 dae (3d) en 14 dae (14d) na die besering. Die uitdrukking van spierverwante proteïene Pax7, M-cadherin, MyoD, CD56, desmin en CD34 is vasgestel met ‘n vloeisitometer. Mioblaste is daarna gesorteer op grond van hul CD56- en CD34-uitdrukking. Drie sub-groepe is versamel en verder gekweek, nl. $CD56^{+}/CD34^{-}$, $CD56^{-}/CD34^{+}$ en $CD56^{+}/CD34^{+}$. Na 24 uur is gesorteerde selle gekleur om desmin-uitdrukking te bepaal.

Pax7, M-cadherin en MyoD is deur 100% satellietselle in alle groepe uitgedruk, wat hul spierverwante identiteit bevestig, alhoewel slegs 80% selle in alle groepe desmin uitgedruk. Druiwepit-ekstrak het die vermoë van selle om aan plate te heg onderdruk, wat gelei het tot ’n laer opbrengs van mioblaste. Drie dae na die besering in die placebo groep het die CD56-uitdrukking beduidend toegeneem. In teenstelling hiermee het CD56-uitdrukking in die druiwepit-ekstrak groep 4 ure na die besering beduidend toegeneem en weer afgeneem na 3 dae. Hoewel daar nie sulke

dramatiese verskille was tussen groepe ten opsigte van CD34-uitdrukking nie, was daar ’n soortgelyke tendens as vir CD56-uitdrukking. Immunositochemie het ’n verskeidenheid van morfologieë en variërende desminvlakke in gesorteerde mioblaste blootgestel. In die twee $CD56^+$ groepe is meer mioblaste wat hoë desmin vlakke uitdruk gevind, wat aandui dat CD56 uitgedruk word deur meer volwasse mioblaste, ongeag van CD34-uitdrukking.

Tydens vloeisitometrie is ’n populasie selle wat hoë desminvlakke uitdruk, hoofsaaklik in die onbeseerde en 14d druiwepit-ekstrak groepe gevind. Dit is ’n aanduiding dat sommige mioblaste voorbereid is om na ‘n besering vinniger te reageer. Na die herstelproses word hierdie groep selle hernu in die teenwoordigheid van druiwepit-ekstrak-aanvulling.

Die resultate het gevolglik daartoe gelei dat druiwepit-ekstrak die hegtingsvemoë van mioblaste verlaag, maar dat die aanvulling *in vivo* tot vroeër ontwikkeling van mioblaste lei, waarskynlik deur satellietselle voor te berei vir ‘n vinnige respons na ’n besering. Beide die onderdrukking van aanhegting aan kultuurplate en die vroeë ontwikkeling van mioblaste, kan die herstel van die skeletspier verbeter.

ACKNOWLEDGEMENTS

My sincere gratitude goes to the following people who have made the completion of this thesis possible:

- My principle supervisor, Prof. Kathy Myburgh for her guidance, constant encouragement, and for always challenging me to strive for excellence.
- My husband, Adriaan, for his unconditional love, support and patience throughout the completion of my study.
- My parents whom have never failed to encourage me and gave me the inspiration to persevere. Also, for their financial support to allow me to study full-time again.
- Prof DuPont-Versteegden (Center for Muscle Biology, University of Kentucky, Lexington, USA) who shared with us her protocol for harvesting rat myoblasts.
- Dr. Carola Niesler (Department of Biochemistry, UKZN, Pietermaritzburg) for her invaluable advice with regards to many aspects of this thesis.
- Kyle Goetsch (Department of Biochemistry, UKZN, Pietermaritzburg) who taught me the basic principles of cell culture and how to troubleshoot effectively.
- The muscle research group for their input in the development of the study, for their support and help, specifically Filippo, Ashwin, Paul, Mari and Maritza.
- My fellow students in the Physiology department with whom I shared many insightful conversations and specifically to Delita, Danzil, Mark, Gina and Justin with whom I shared many hours in the laboratory.
- My line managers, Dr. B Loos and Prof. G Stevens, who allowed me time to complete this thesis and my colleague, Rozanne, for taking over many duties to allow me to focus on the completion of my thesis.
- To the NRF and the Harry Crossley bursary for funding.
- To the Heavenly Father for all the blessings that I received to enable me to complete this study.

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NOMENCLATURE

ADAM12	A disintegrin and metalloproteinase 12
ANOVA	Analysis of variance
AP-1	Activator protein-1
APO N	Apochromatic aberration correction, normal field
Bcl-2	B-cell lymphoma-2
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BP	Band pass
BSA	Bovine serum albumin
°C	Celsius (degrees)
Ca^{+}	Calcium
CCD	Charge coupled device
CD	Cluster of differentiation
CFU-F	Colony-forming unit-fibroblasts
CK	Creatine kinase
CO_2	Carbon dioxide
CXCR4	C-X-C chemokine receptor type 4
d	day
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl-sulphoxide
DNA	Deoxyribonucleic acid
DOMS	Delayed onset of muscle soreness
E-C-L	Entactin-collagen-laminin
EDL	*Extensor digitorum longus*
EGF	Epidermal growth factor
EHS	Engelbreth Holm Swarm

eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal regulated kinase
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	FGF-receptor
fMHC	Fetal myosin heavy chain
FMO	Fluorochromes minus one
FN	Fibronectin
FSP-1	Fibroblast-specific protein-1
g	grams
g	g-force / relative centrifugal force
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GDF-8	Growth and differentiation factor-8
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
GPX	Glutathione peroxidase
GSE	Grape seed extract
h	hour
HDAC4	Histone deacetylase 4
HeNe	Helium Neon
HGF	Hepatocyte growth factor
H_2O_2	Hydrogen peroxide
HSC	Hematopoietic stem cell
HSPGs	Heparin sulphate proteoglycans
I-κB	Inhibitor of kappa B
ICAM-1	Intercellular adhesion molecule-1

IFN-γ	Interferon-γ
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IL	Interleukin
iNOS	inducible nitric oxide synthase
JNK	c-Jun NH_2-terminal kinase
LFA-3	Leukocyte function associated antigen 3
LP	Long pass
m	milli-
	meter
MAPK	Mitogen-activated protein kinase
M-cad	M-cadherin
MDSC	Muscle derived stem cell
Mef2	Myocyte enhancer factor 2
MGF	Mechano growth factor
MHC	Myosin heavy chain
MMP-2	Matrix metalloproteinase 2
MNF	Myocyte nuclear factor
MRF	Myogenic regulatory factor
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
Mstn	Myostatin
MyoD	Myogenic differentiatioin-1
Myf5	Myogenic factor 5
n	nano
NADPH	Nicotinamide adenine dinucleotide phosphatase (reduced)
NCAM	Neural cell adhesion molecule

NF-κB	Nuclear factor kappa-B
NG2	Neural/Glial antigen 2
NI	Non-injured
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOD/SCID	Non-obese diabetic/severe combined immunodeficient
NOS	Nitric oxide synthase
NSAIAOD	Non-steroidal anti-inflammatory and anti-oxidant drugs
O_2	Oxygen
$ONOO^-$	Peroxynitrite
ONOOH	Peroxynitrous acid
ORAC	Oxygen radical absorbance capacity assay
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGFR6	Platelet derived growth factor receptor 6
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PIC	$PWI^+/Pax7^-$ interstitial cells
Pl	Placebo
P	Passage
PP	Pre-plate
PS	Pigskin gelatin
RIO	Reactive oxygen intermediate
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Sca-1	Stem cell antigen 1

α-SMA	Alpha smooth muscle actin
SMAD	Contraction of SMA and MAD
SOD	Superoxide dismutase
SP	Side population
TA	*Tibialis anterior*
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
trunc	Truncated
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGFR-2	VEGF receptor-2
VLA4	Very late antigen 4
UBG	Ultra-violet, blue, green
UV	Ultra-violet

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Chapter 1 Introduction

Skeletal muscle is a uniquely structured tissue, accommodated for force production and movement. It is therefore the tissue that is most damaged during sports activities and for high-performance athletes, a quick recovery is crucial.

Due to the nature of many contact sports, contusion injuries are common occurrences. These are injuries caused by blunt objects, characterised by haematoma, pain and a reduction in functional force production; this is in part due to the formation of scar tissue which often prevents full recovery [18]. A better understanding of the muscle regeneration process is vital.

The regenerative process in skeletal muscle has been studied in human subjects [81, 310] or using intervention protocols in animal models [49, 281]. Satellite cells are the main role player in the regeneration of intact myofibres [281]. It has become more and more clear that the satellite cell population, which is defined as the progenitor cells residing between the sarcolemma and the basal lamina of skeletal muscle, is a heterogeneous group of cells [21]. Satellite cells do not all progress through the series of events of activation, proliferation, differentiation and fusion in a homogenous manner. As a result, the expression of myogenic marker proteins does not occur in a simplistic time-dependent way [164, 61]. Therefore, it is sometimes easier to study satellite cell behaviour in culture. Indeed, cell culture is a well-accepted model to study the regulation of these cells and the formation of myotubes [40, 65].

Despite our knowledge of satellite cell progression *in vitro*, conflicting data from many laboratories regarding the temporal and spatial expression patterns of specific myogenic proteins on satellite cells *in vivo* leave many questions [232, 69, 92]. *In vivo*, many cells express different markers [88], even in small percentages [84], making it very challenging to use only these markers to identify satellite cells and myoblasts during days and weeks of regeneration.

In order to combine the study of skeletal muscle injury in an animal model with the analysis of myogenic cells, viable myoblasts have to be harvested and isolated from

these animals post-injury. Published myoblast isolation protocols are often not consistent [300, 214, 166], and therefore a specific protocol for the purpose of this study was optimised. Resulting isolated myoblasts were then utilised for further characterisation.

An advantage of isolating satellite cells at different time points after injury is that the satellite cells are already influenced, not only by the injury, but also changes in the niche. The micro-environment of satellite cells influences the up- or down-regulation of many myogenic proteins [145]. The two main contributors to changes in the micro-environment of the satellite cell are the inflammatory response to the injury [258] and oxidative stress [315]. These two factors do not act in isolation, but often have a combined effect within the satellite cell niche [266]. Free radicals and reactive oxygen species (ROS), which cause oxidative stress, are released by infiltrating inflammatory cells like neutrophils into a wound area [220]. There is evidence that reactive oxygen species break down structural proteins [204], including damage to myofibrillar proteins in skeletal muscle [86, 197].

In this study, the aim was to alter the presence of oxidative stress in the skeletal muscle and determine the effects on regenerating rat myoblasts after a standardised contusion injury. Grape seed extract (GSE) was shown previously to be a very effective anti-oxidant [11]. Grape seed derived proanthocyanidins have been shown to improve wound healing in several tissues [143, 203, 136]. GSE is known to prevent infiltration of neutrophils and the phagocytic macrophage species into a muscle wound site [143] and to improve free radicals scavenging thus preventing oxidative stress. GSE further enhances the production of nitric oxide [255], an important signalling molecule which causes vasodilation [77, 5]. Increased blood supply to the wound area would provide better access of distant muscle progenitors to the injury site [213].

In light of the above, the following literature review aimed to describe i) the current understanding of muscle regeneration, ii) the contribution of various myogenic cells to the repair of muscle tissue and iii) the markers used to identify the myogenic cell populations. Relevant injury models, especially the contusion injury and the role of oxidative stress and anti-oxidants in the repair and regeneration process, will be discussed.

The first experimental chapter (Chapter 3) of this study aimed to establish a technique to harvest and isolate myogenic cells from rat muscle and expand them in culture successfully. Numerous challenges presented themselves and after several adaptations to the initial protocol, contamination was eliminated and a sufficient yield was obtained for use in further analysis. Isolated myoblasts were compared with the immortal C2C12 cell line and several media options were explored.

The second experimental chapter (Chapter 4) of this study was designed to investigate rat muscle subjected to a contusion injury *in vivo* and the effect of chronic daily supplementation of an anti-oxidant, grape seed extract, on these responses. After the injury, myogenic cells were harvested and isolated from the tissue at various time points during the progress of regeneration. Cells were expanded in culture and analysed for morphological changes and expression of various relevant proteins.

Very little was known about the way in which grape seed extract affects satellite cells upon injury. The findings of the study contributes to a new understanding of the way in which GSE improved muscle injury repair as reported before [143, 203]. Chronic supplementation of GSE affects the muscle satellite cells in such a way that their progress through regeneration is enhanced.

Chapter 2 Literature Review

2.1 GENERAL INTRODUCTION

Skeletal muscle is a highly specialised differentiated tissue, consisting of bundles of multinucleated fibres with contractile properties. Day-to-day wear and tear inflicts small lesions to these cells, which elicit areas of active regeneration. About 1-2% of myonuclei are replaced weekly in normal adult rat muscle; whilst myonuclear turnover in denervated rat *soleus* and *extensor digitorum longus* (EDL) muscle is lower [249].

Skeletal muscle can easily be damaged during intensive physical activities or trauma such as lacerations, or more chronic conditions such as genetic dystrophies and myopathies. However, its remarkable ability to repair and regenerate rapidly prevents the long-term loss of functional capacity [49]. For muscle to function properly, not only do the contractile elements of myofibres need to be repaired, but motor neurons, blood vessels and extracellular connective tissue matrix need to be rebuilt, if any of these were jeopardised [49].

After muscle damage, an orchestrated set of cellular responses follows, involving a wide variety of factors. The exact progression of muscle regeneration depends on the type and magnitude of the trauma. However, muscle regeneration can generally be divided into two phases: the degenerative phase which goes hand in hand with the inflammatory response, and the regenerative phase during which the muscle tissue is built up and remodelled, sometimes with accompanying fibrosis.

Although a sequence of events has been characterised, the endogenous regulatory pathways are complex and the effects of interventions poorly understood.

2.1.1. Primary degenerative phase

The initial degenerative phase involves removal of debris and even necrosis if cells are severely damaged [49, 258]. When the myofibre sarcolemma is disrupted, its permeability increases, leading to the leakage of cytosolic muscle proteins, like creatine kinase (CK). The serum levels of these proteins rise and are often used as

an indirect determinant of the presence of muscle damage after mechanical stress and extensive physical exercise.

Sarcolemmal damage and subsequent disruption of the sarcoplasmic reticulum lead to excess calcium influx into the cytosol. This triggers various processes including activation of calcium-dependent adhesion molecules and protein breakdown through calpain activity. Calcium-dependent proteolysis plays a major role during the degenerative phase, leading to focal or total autolysis of the injured muscle tissue [49]. The broken or partially degraded myofibrils result in the build-up of debris, which is usually removed by phagocytic cells, like macrophages [229]. More detail on the inflammatory response and its role in this phase will follow in section 2.5.1.

2.1.2. Secondary regenerative phase

It is during the regenerative phase that muscle repair takes place. Although this phase may take more than two weeks to complete, it starts directly after injury with the activation and expansion of myogenic progenitor cells. This is mediated mainly by satellite cell proliferation and migration, both of which provide sufficient extra myonuclei for muscle repair [49, 111]. The newly formed myogenic progenitors can contribute to the repair process by fusing to existing fibres or with one another to form new myofibres.

Satellite cells, which originated embryonically from the somite, have the unique ability to express Pax7. Lepper *et al.* found that the elimination of $Pax7^+$ cells negated the regeneration process, implying that no other myogenic precursors were able to compensate adequately for the lack of $Pax7^+$ satellite cells under the conditions of their experiment [159].

Despite this finding, evidence exists that other non-satellite cells could participate in myogenesis. A number of cell types from the interstitium and the peripheral vascular tissues have been shown to have myogenic potential, but it is still unclear how much they contribute to the regeneration process [52]. These include various stem cells derived from bone marrow and the skeletal muscle itself.

As mentioned earlier, satellite cells are activated upon stimulation to increase in number by proliferation and migration to the injury site. Activated satellite cells can be identified by their upregulated expression of myogenic regulatory factors (MRFs)

Myf5 and MyoD. In this activated state they are no longer referred to as satellite cells, but myoblasts [69]. When myoblast number is sufficient these cells align and adhere, either to each other or to existing myofibres [122].

Some very distinct changes take place in the plasma membranes of participating myogenic precursor cells. Cell-substrate adhesion is enhanced by the loss of filopodia and stress fibres [71] and the increased expression of cell adhesion molecules, such as M-cadherin and neural cell adhesion molecule (NCAM or CD56) [122]. In order to elicit mature action potentials when myotubes appear, the voltage-dependent ion channel pattern on the sarcolemma must mature.

Before, during and just after fusion, expression of many cellular components of the participating cells changes. Cytoskeletal actin shows a distinct pattern with focal adhesion localisation in early differentiation [71] followed by high rates of protein synthesis in the new fibres. Characteristic muscle proteins, such as sarcomeric desmin, α-actin, heavy and light chain myosin, tropomyosin and troponin-C and –I are synthesised. The onset of expression of these muscle proteins is not simultaneous. The first muscle specific protein expressed is desmin, followed by α-actinin and titin, then muscle isoforms of actin and myosin to form myofibrils [311]. Regenerating fibres are characterised by expression of embryonic forms of myosin heavy chain (MHC).

These structural proteins need to undergo complex spatial organisation and the orientation of thick and thin filaments is essential for myofibrillar assembly and formation of sarcomeres, the functional unit of mature contractile skeletal muscle. Sarcomere formation is a complex process characterised by the assembly of the cytoskeletal framework before myofibrillar assembly, which are the key components for force production. The sarcomeres then have to be anchored to the sarcolemma for force transduction across many fibres and ultimately the whole muscle [27]. This occurs at the site of Z-discs, via structures called costameres, which contain proteins such as desmin and cytoskeletal γ-actin with its associated tropomyosin 4 (Tm4). These Tm4/γ-actin complexes have been shown to be involved in organisation during myogenesis [292], but desmin plays a major role in the higher level of sarcomere alignment, especially in regulating sarcomere numbers [160]. Although these proteins are associated with Z-discs in normal adult skeletal muscle, it has

been shown that desmin and the Tm4/γ-actin complexes form longitudinal structures similar to stress fibres during muscle regeneration, providing a scaffold for myofibrillogenesis [160, 292].

The histological analysis of regenerative muscle samples usually shows that newly formed muscle fibres have relatively small circumferences and centrally located myonuclei. When fusion is complete, intracellular proteins increase in organisation and density and myonuclei move to the periphery of the newly formed muscle fibre [49]. Established muscle fibres have nuclei found only on the periphery. A myonucleus from an adult rat muscle is reported to be 11-15 μm in diameter [121], similar to the reported size of human myonuclei (12 μm). Satellite cell nuclei are smaller, approximately 8-9 μm in diameter, although the satellite cell nuclei from dystrophic patients could reach up to 12 μm in length [296].

When muscle damage is severe, it is also necessary to repair extracellular structures associated with the skeletal muscle tissue. Repair of the extracellular matrix is evident after crush injury in mice and although the presence of a basal lamina is not a requirement for muscle regeneration, a pre-existing basal lamina improves muscle repair [103]. Crush injury also damage the vascular system in the injured site, so that angiogenesis also forms part of regeneration [98].

It is clear that muscle regeneration is a complex process involving changes on a cellular and tissue level to generate functional skeletal muscle. This thesis aimed to determine the effects of contusion injury on muscle regeneration at the cellular level, i.e. at the myoblast (or activated satellite cell) level. Due to the possible involvement of non-satellite cells as muscle progenitors during regeneration, a better understanding of these cell types and their contribution to muscle regeneration was sought. The following sections will focus on progenitor cells with myogenic capacity, especially satellite cells, followed by a discussion of some of the proteins that change as the biological functionality of myoblasts alter during regeneration. The proteins selected for discussion are those most relevant to the thesis.

2.2. PRECURSOR CELL POPULATIONS

Known sources of myogenic precursor cells include satellite cells, muscle-derived stem cells, muscle side population cells, pericytes and bone marrow-derived stem cells. Others not included in this review, since they are beyond the scope of the study, are mesangioblasts (derived from the mesoderm and found in the walls of blood vessels) [217], embryonic stem cells and adipocytes derived from brown fat [192, 175, 248].

2.2.1. Satellite cells

The key to our current understanding of muscle repair and regeneration has been the identification in the early 1960's of the primary role players in the high regenerative ability of adult skeletal muscle, the satellite cells [133]. The biology of satellite cells has been the main focus of a significant number of studies over the past 50 years and we have gained a great deal of insight in the cellular and molecular mechanisms at play during muscle repair [49].

2.2.1. i) Activation and proliferation

After muscle injury, quiescent satellite cells are activated to proliferate, differentiate and fuse with each other or to myofibres to repair and regenerate the damaged muscle [145, 313, 133]. Undifferentiated mono-nucleated quiescent satellite cells reside in indentations between the sarcolemma and the basal lamina at the periphery of the mature multi-nucleated myotube. These cells have a high nuclear-to-cytoplasmic ratio and the amount of nuclear heterochromatin is increased in comparison to myonuclei [111].

Activated satellite cells are usually characterised by the development of abundant rough endoplasmic reticulum, increased cytoplasmic volume, presence of fine granulation and microfilaments in the cytoplasm and a small number of rounded mitochondria [30]. An activated satellite cell appears as a swelling on the myofibre and cytoplasmic extensions appear on one or both poles of the cell. When these satellite cells express myogenic proteins they are termed myoblasts [111].

Mechanisms involved in satellite cell activation have not been clarified completely to date. Activation is usually limited to areas where myofibre damage is evident and depending on the severity of the trauma, could continue even 9-10 days after injury.

Mechanical disruption of the integrity between the sarcolemma and basal membrane will activate satellite cells [90]. Other role players in activation include cytokines released by inflammatory cells and growth factors, such as hepatocyte growth factor (HGF), insulin-like growth factor (IGF) and fibroblast growth factor (FGF) [90].

Hepatocyte growth factor (HGF) is viewed by some as the primary factor capable of activating quiescent satellite cells. The HGF receptor (c-met) is specifically expressed on quiescent satellite cells and upon muscle damage, there is an increase in HGF transcript and protein levels, in proportion to the degree of injury [111]. Although no reports could be found to indicate an increase in c-met expression after skeletal muscle injury, c-met mRNA is upregulated in the heart after myocardial infarction [284]. Also, c-met is co-localised with HGF on the satellite cell surface after crush-injury [277]. HGF is most active in the early stages of regeneration, as immunostaining intensity decreases with time after injury [49]. It has also been shown *in vitro* that exogenous HGF suppresses differentiation [96]. However, it would seem that there is some misinterpretation of the literature. When HGF is injected (50 ng) to mouse *tibialis anterior* muscle after a local freeze injury, differentiation is only reduced when HGF is administered in the early stages (day 0-3), but not at the later stages (day 4-6) [188]. This suggests that c-met is down-regulated first, a hypothesis supported by the decrease in c-met mRNA levels in primary myoblasts after 4 days in culture, despite high levels of HGF mRNA levels at the same time point [96].

IGF-1 is a more ubiquitous growth factor and its expression increases after ischaemia or myotoxin-induced injury. However, muscle has its own splice-variant of this growth factor, named mechano growth factor (MGF), which differs from the splice variant released from tissues into circulation, insulin-like growth factor-1Ea (IGF-1Ea). MGF is expressed much earlier than IGF-1Ea [114] and promotes proliferation of myoblasts, while blunting differentiation [306]. This suggests a complementary role to HGF. IGF-1Ea is expressed in much higher levels by injured muscle, but only after an initial delay post-injury, suggesting a role during differentiation [114]. Silencing of IGF-1Ea mRNA results in reduced differentiation capability [180]. This supports its importance in stimulating protein synthesis towards an increase in myoblast and myotube size during differentiation.

The role of the FGF family is multi-functional, possibly due to the availability of different fibroblast growth factor receptors on satellite cells (FGFR-1 and -4). A specific fibroblast growth factor, with the ability to bind to both these receptors [85], is basic fibroblast growth factor (bFGF or FGF-2). Neutralising this growth factor *in vivo* leads to a reduced number of regenerating myofibres and reduced size of regenerating myofibres [156]. When genetically modified myoblasts, which overexpress bFGF, were transplanted into skeletal muscle of Wistar rats, myoblast proliferation improved after crush injury [268]. This growth factor is a common supplement for primary myoblast cell cultures due to its effect on proliferation [25, 319, 231]. *In vitro*, bFGF has been shown to activate myoblast proliferation and an increase in bFGF results in supressed expression of myostatin, a negative regulator of muscle growth [168]. Although bFGF mRNA was still found to be present in primary myoblasts *in vitro* just prior to fusion and even in myotubes after *in vivo* injury [106], it has been shown that bFGF mRNA is downregulated during differentiation of both mouse Sol 8 and rat L6 myoblasts [199]. Basic FGF is a potent suppressor of myoblast differentiation [59], through the inhibition of myogenin, by inducing phosphorylation of a conserved site in the DNA-binding domain of this MRF [160].

Receptors for FGF and HGF are both trans-membrane with cytoplasmic tyrosine kinase domains, which auto-phosphorylate when the growth factor binds. This in turn activates downstream signalling pathways which are still poorly understood. Syndecan-3 and -4 are co-receptors for tyrosine kinases, and are expressed on quiescent satellite cells. The syndecans co-localise with c-met and FGFR-1 in satellite cells [67] and it has been shown that syndecan forms a ternary complex with bFGF and its receptor [55]. Although it is not yet determined if HGF and c-met interact with syndecan-3 or -4, it has been shown that syndecan-1 is involved in the clustering of HGF and c-met. Thus, there is a possibility that syndecan-3 or -4 on satellite cells act as co-activators of c-met [149].

Many other growth factors are involved in the regulation of satellite cells; these include platelet derived growth factor and endothelial derived growth factor [111]. Furthermore, numerous cytokines are also activation factors and play many different roles in the injured environment.

Macrophages are involved in the activation of myogenic cells, via the release of these cytokines. They stimulate proliferation of myoblasts specifically, as shown by co-culture studies of macrophages with primary rat myoblasts, or myoblasts grown in macrophage conditioned media [42, 43]. Although the conditioned media was associated with a lower expression of myogenin initially, the end result was an increased number of myotubes. When this macrophage conditioned media was injected into rat muscle after muscle ablation myogenesis was faster and muscle mass increased [43]. The factors secreted by macrophages, such as IGF-1 [171], IL-6 and IL-1 also stimulate autocrine secretion of IL-6 by myoblasts, which in turn stimulate their proliferation even further [44].

From many studies it is clear that cytokines act in various combinations as the signalling agents between all cells involved during inflammation. For example, TNF-α and IL-12 upregulate IFN-γ expression; this in turn activates the macrophages. Furthermore, in conjunction with TNF-α, IL-1β, (predominantly expressed by inflammatory cells) will stimulate fibroblasts to produce collagen. In response to TNF-α and IFN-γ, expression of the major histocompatibility complexes I and II respectively is increased in satellite cells, which induces the expression of adhesion molecules, such as ICAM-1. IFN-γ and TNF-α also up-regulate the release of cytokines, IL-6 and TGF-β from muscle cells, which are normally released constitutively only at low levels [205]. The pituitary gland is stimulated by TNF-α, IL-1, IL-6 and IFN-γ to produce glucocorticoid hormones, which will inhibit the production of these cytokines [62, 37]. For further discussion see section 2.5.1.

2.2.1. ii) Migration

For adequate muscle regeneration, sufficient numbers of satellite cells are required at the site of injury. In a non-proliferative quiescent state, satellite cells are wide-spread in the skeletal muscle tissue comprising a very small fraction of the total muscle nuclear number (1-6%) [45, 111]. Upon injury the proliferation of local satellite cells might not be adequate for optimal muscle regeneration, so myogenic cells from nearby viable myofibres would be important sources for additional myogenic cells.

The basal lamina plays an important role in the chemotaxis of these cells. If the basal lamina is left intact after limited damage to the muscle, precursor cells will

migrate under the basal lamina from an intact portion of the myofibre to the damaged location on the same myofibre. Laminin, the main constituent of the basal lamina, will enhance myoblast migration [57]. If the basal lamina is in some way disrupted, myogenic cells may need to migrate across interstitial spaces from healthy intact fibres to the damaged myofibre where they can participate in the repair process [111, 117].

A number of damaging conditions, such as ischemia, thermal injury, crush injury and myotoxins have been shown to stimulate satellite cell migration to the site of injury. This most likely occurs in response to concentration gradients of soluble factors known to be released by damaged muscle fibres [23], such as hepatocyte growth factor (HGF). HGF is released by the muscle extracellular matrix where it is bound to heparin sulphate proteoglycans (HSPGs), but recent evidence shows a rapid up-regulation of HGF in the spleen. Thus it can act as an endocrine or paracrine/autocrine regulator when it is released from storage sites or upregulated [49, 57, 36].

2.2.1. iii) Self-renewal of satellite cells

Not all satellite cells will form myotubes after activation. Satellite cells undergo asymmetric division to give rise to a majority of cells destined to differentiate into myotubes and a smaller portion (approximately 10%) that repopulate the satellite cell compartment. It is now widely accepted that at least some fraction of satellite cells self-renew after injury, to replenish the quiescent satellite cell pool [133, 146, 145, 313]. Upon engraftment, freshly isolated or fibre-associated satellite cells participate in muscle repair, but some also enter the satellite cell niche, therefore demonstrating differentiation and “self-renewal” capability [61, 46, 144, 196, 245]. Self-renewal is not the only mechanism for satellite cell niche replenishment. Less specialised progenitors/stem cells, from the skeletal muscle interstitial space or distant locations (i.e. bone marrow), may also repopulate the satellite cell niche to contribute to myogenesis during subsequent cycles of regeneration.

2.2.2. Muscle derived stem cells (MDSC)

Stem cells which normally reside in skeletal muscle are referred to as muscle derived stem cells (MDSC); this is therefore an umbrella term which describes a heterogeneous population [218]. These cells can be found under the basal lamina, in

the interstitial spaces and even in the peripheral vascular tissue. They have varying potential, including an ability to regenerate cardiac and skeletal muscle, bone and cartilage.

Because of the wide range of markers expressed by different muscle-derived stem cells, their origin is still unclear. There are suggestions that circulating stem cells may infiltrate tissues, but there is also much evidence that the stem cells primarily responsible for regeneration and repair in tissue are usually the resident stem cells in that specific tissue [218].

Due to the wide range of cell types which fall into this category, it is very difficult to determine their specific contribution to muscle regeneration. Some MDSCs have been reported to repopulate the satellite cell niche and participate in regeneration of myofibres at varying frequencies [125, 230]. Expression of membrane proteins such as CD45, Sca-1, CD31 and CD34 could reflect the capacity of different cells to become myogenic and their potential to proliferate for immediate involvement in regeneration or potential to repopulate the satellite cell niche (see Figure 2.1).

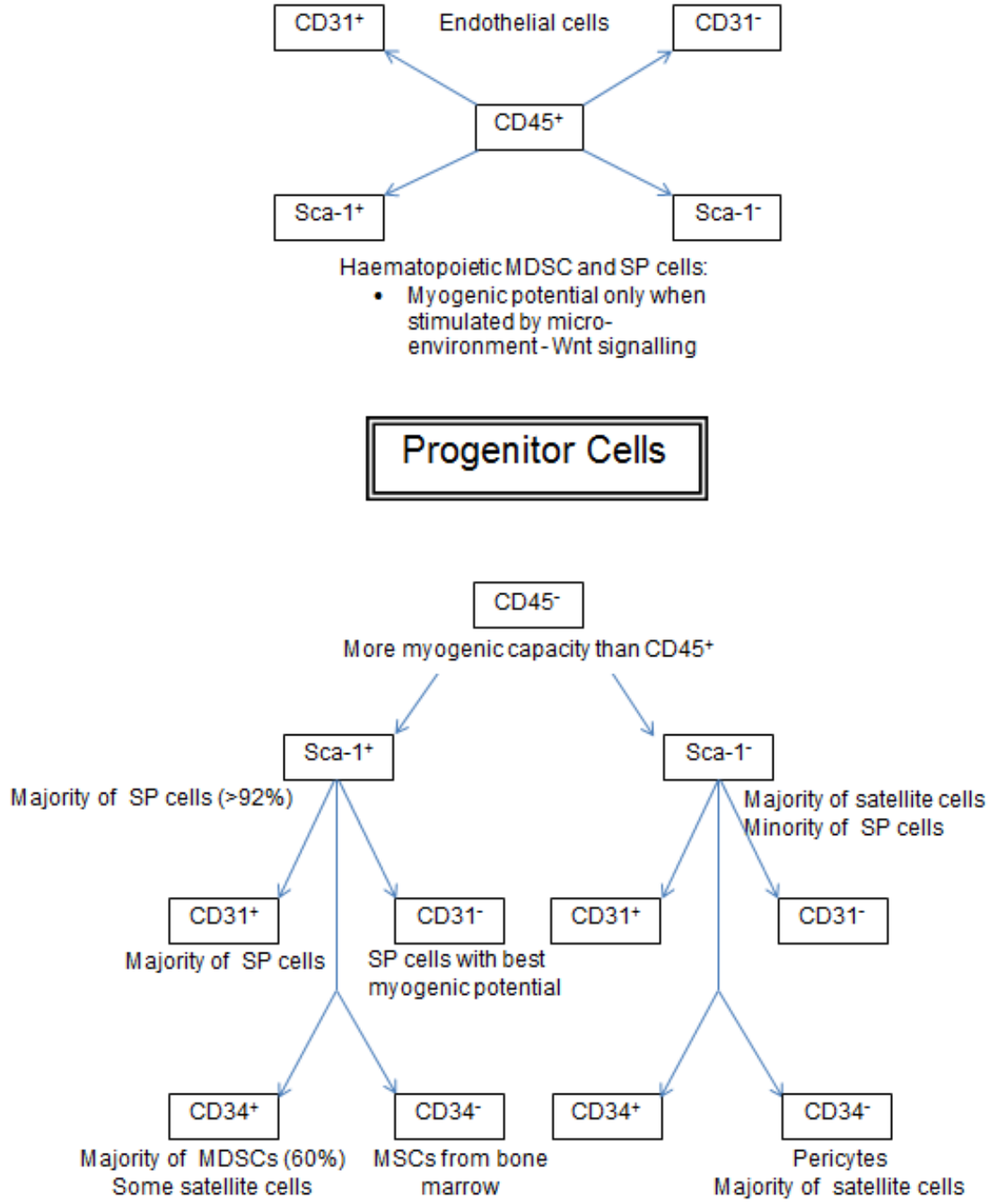


Figure 2.1. Schematic presentation of proteins expressed by progenitor cells found in skeletal muscle.

The myogenic cell subpopulations can be divided broadly into two groups, namely $CD45^+$ and $CD45^-$. The $CD45^-$ subpopulation has more myogenic potential, whereas $CD45^+$ is more hematopoietic and will only show myogenic capabilities under micro-

environmental influences such as Wnt signalling or when satellite cells are depleted [218] (see Figure 2.1).

MDSC cells are closely associated with the capillaries surrounding myofibres. Some MDSC cells are a subpopulation of pericytes or capillary endothelial cells, such as the myogenic-endothelial progenitor cells, which are $CD34^{+}/CD45^{-}$ and capable of regenerating muscle and vasculature [218]. Myo-endothelial cells on the myofibre periphery close to blood vessels have been shown to express CD34, CD144, CD56 and CD31 but not CD45 [217]. They can either be Sca^{+} or Sca^{-}, with approximately 60% $CD45^{-}/CD34^{+}/Sca\text{-}1^{+}$.

The potential myogenic capacity of the different MDSC populations is controversial. For example, $CD34^{+}$ cells from mouse skeletal muscle do not express myogenic markers at the time of sorting by flow cytometry, but have the ability to repopulate the *tibialis anterior* muscle of NOD/SCID mice, while $CD34^{-}$ could not [273]. The same group later reported that a small percentage of sorted $CD34^{-}$ cells express myogenic markers, with increasing expression after several days in culture [274]. Both populations therefore had the potential to develop into myotubes *in vitro*.

2.2.3. Skeletal muscle side population cells

Skeletal muscle has a population of cells that participate in myogenesis, but reside outside the traditional satellite cell niche. This population of pluripotent stem cells are called side population (SP) cells and the majority are $CD45^{-}$ [217]. They can be considered a muscle-derived stem cell but they are found in many tissues and show potential stem-like properties [10].

Apart from residing outside the basal lamina in skeletal muscle, SP cells can be distinguished from satellite cells due to the fact that the majority of these cells express Sca-1, they are present in $Pax7^{-/-}$ mouse skeletal muscle, and they can exclude Hoechst dye [252]. Their myogenic differentiation capacity does not rely on Pax7 expression [9]. Meeson *et al.* showed a distinctive transcription profile for skeletal muscle SP cells that was more similar to bone marrow SP cells than myoblasts [183]. SP cells do not develop into myocytes spontaneously *in vitro* after isolation [10], but co-cultures between these two cell-types have resulted in

increased Pax7 expression and the incorporation of SP cells into myotubes, thus relying heavily on “niche”-derived promyogenic stimulation.

Although most literature reports suggest that SP cells do not express myogenic proteins Myf5, Pax7 and desmin [211, 49], Kallestad *et al.* reported the expression of Pax7 and M-cadherin in a small percentage of these cells. A positive aspect of the study, which is a possible explanation for the contradicting results regarding myogenic expression, was that the analysis of muscle-derived progenitor cells was done directly *ex vivo*, to minimise culture condition effects on the cells. Furthermore, the variable percentages of myogenic protein expression found in their study could be explained by different muscle groups analysed (*medial recti extra ocular* muscles vs. *tibialis anterior*) and different animal models tested (rabbit vs. mouse) [132].

As mentioned earlier, a marker expressed in most (>92%) side population cells is stem cell antigen-1 (Sca-1), the marker which confirms their stem cell-like characteristic. In normal non-regenerating muscle a minor subpopulation is $CD31^-$ /$CD45^-$, while the majority of SP cells express Sca-1 and CD31, reflecting their endothelial-like phenotype [285].

The myogenic capacity of SP cells has been investigated using three main approaches: Firstly *in vivo* injury and subsequent investigation of the relative proportion of SP cells present in the area; secondly, isolation and differentiation *in vitro* and thirdly, isolating SP cells and re-introducing them *in vivo*.

Skeletal muscle SP cells increased significantly after cardiotoxin induced injury in adult mouse hindlimb [183]. These SP cells actively proliferated and expressed several myogenic regulatory genes and mesenchymal lineage markers. Intramuscular injections of SP cells into dystrophic muscle *in vivo* resulted in successful SP engraftment in approximately 9% of regenerating mouse fibres [105]. Tanaka *et al.* found a small population of isolated SP cells expressing satellite cell markers syndecan-4 and Pax7 (0.25%) and subsequently termed these satellite SP cells [276]. This small fraction of satellite SP cells demonstrated myogenic capacity *in vitro* and when transplanted into regenerating mouse muscle, repopulation of the satellite cell niche was highly efficient [276].

Uezumi *et al*. subdivided SP cells into three sub-fractions according to CD31 and CD45, namely $CD31^{+}/CD45^{-}$, $CD31^{-}/CD45^{-}$ and $CD31^{-}/CD45^{+}$ to investigate the myogenic capacity of SP cells identified according to above mentioned markers [285] (see Figure 2.1).

Of the different SP sub-fractions sorted by Uezumi *et al.*, the $CD31^{-}/CD45^{-}$ population showed the greatest myogenic potential and indeed differentiated into myofibres after intramuscular transplantation [285]. Co-transplantation of these $CD31^{-}/CD45^{-}$ side population (SP) cells and green fluorescent protein (GFP)-positive myoblasts into the *tibialis anterior* muscle of immuno-deficient NOD/SCID mice or dystrophin-deficient mdx mice, resulted in the formation of higher number and more widely spread GFP^{+} fibres compared to controls. Based on these results, it seems that the role of $CD31^{-}/CD45^{-}$ SP cells is to stimulate cell proliferation of myoblasts and promote migration of these myoblasts *in vivo* [201]. Alternatively it is possible that significant fusion of SP and GFP^{+} cells took place.

Nonetheless, the large percentage of non-myogenic SP cells indicates that the main role of SP cells might not be participation in myogenesis. Due to CD31 and Sca-1 co-expression their role may be vascularisation. In the blood vessels, specifically the micro-vascular walls of skeletal muscle, another type of cell capable of myogenesis resides. Unlike SP cells, these so-called pericytes express Pax7 and will be discussed next.

2.2.4. Pericytes

Typically, pericytes in culture have large, flat cell bodies (150 x 100 µm) with irregular shapes and long processes [194]. Originally pericytes were thought to be osteogenic precursor cells, because they participated in bone regeneration after injury and had the ability to differentiate into bone material *in vitro* and *in vivo* [218]. However, by transgenically labelling pericytes with Alkaline Phosphatase GreERT2, Dellavelle showed that they also fuse with myotubes *in vivo* in the absence of environmental manipulation, providing evidence that they contribute to myogenesis under natural conditions [75].

Pericytes do not express Sca-1, CD34 or CD45 [217]. Pericytes in skeletal muscle are $Pax7^{+}$ cells, but in contrast with satellite cells they are separated from the

basement membrane [36, 76]. However, the primary function of pericytes in skeletal muscle is still unclear. *In vitro* dye transfer experiments between isolated hamster pericytes and human umbilical vein endothelial cells (HUVEC) confirmed that coupling between pericytes and endothelial cells is possible. The pericytes were able to generate a hyperpolarising signal. This led to the conclusion that pericytes might, in response to exercise, generate signals conducted by the endothelial lining for vasodilation of blood vessels [194]. Indeed, they are sensitive to IL-8, a cytokine released during strenuous exercise.

Increased blood flow to an injury site would also increase the access of myogenic progenitors from other sources to the injury site. Such cells include circulating bone marrow cells, which also have shown myogenic capacity.

2.2.5. Bone marrow derived stem cells

Bone marrow is a complex tissue containing hematopoietic stem cells (the primary source of blood cells) and mesenchymal stem cells (MSC). MSCs, also called stromal cells, are a group of heterogeneous cells which appear fibroblastic. After isolation, MSCs are adherent to plastic and display a colony forming unit in culture (CFU-F). They have the potential to differentiate into various tissue lineages such as adipocytes, osteocytes, chondrocytes, tenocytes, myoblasts and neurons [224, 175]. Although mesenchymal stem cells are usually derived from bone marrow, they are also found in adipose tissue, muscle, skin and the periosteum.

The Mesenchymal and Tissue Stem Cell Committee of the International Society of Cellular Therapy (http://www.celltherapysociety.org/index.php) define mesenchymal stem cells as plastic adherent cells expressing CD90, CD105, CD44 and CD73, but they are $CD45^-$, $CD34^-$, $CD31^-$ and also do not express CD14, CD11b, CD79a or CD19. They must have the potential to differentiate into multiple cell lineages *in vivo* [184, 78].

Since bone marrow contains multipotent MSC and marrow-derived cells display satellite cell characteristics *in vitro*, it was proposed that these cells contribute to the skeletal muscle niche, a proposal confirmed via bone marrow transplantation in a study by Ferrari *et al.* in 1998 where genetically modified progenitor cells were

recruited from the transplanted bone marrow to the site of muscle injury and participated in repair [285, 175].

However, the majority of transplant studies report only 0.2-5% of total muscle fibres to be donor-derived after bone marrow transplantation, possibly because the rate of fusion is also very low [293]. Dreyfus *et al.* (2004) followed transplanted GFP^+ bone marrow-derived stem cells in irradiated adult mice and showed that after 1, 3 and 6 months GFP^+ cells were found in the satellite cell niche under the basal membrane of skeletal muscle, expressing muscle specific markers M-cadherin, Pax7 and NCAM [82]. Furthermore, bone marrow MSCs can contribute to muscle regeneration after injury for prolonged periods of time. Human bone marrow MSCs were injected into mouse *tibialis anterior* muscle after myotoxin injury. Four months after injection, approximately 5% heteromyofibres were observed, expressing genetic information from both the endogenous skeletal muscle and the transgenic donor cells. This supports previous findings that in co-cultures with myoblasts, MSCs will fuse with myoblasts to form myotubes [74].

The distinctions between muscle progenitor cells in the satellite cell niche and from other locations are not always clear. Differences can be seen in their behaviour *in vitro* or in the heterogeneity of the various membrane proteins that do not necessary identify a specific myogenic cell type. Bone marrow MSCs are more proliferative than satellite cells, but have a slower proliferation rate in comparison with MSCs derived from skeletal muscle tissue [184]. The authors did not fully characterise these MSCs, or sorted these into subtypes. Figure 2.1 is a schematic presentation of four of the major membrane proteins which may be expressed or absent in various myogenic populations or expressed in a specific myogenic population in different ratios.

In summary, progenitor cells are either $CD45^+$ or $CD45^-$. Haematopoietic and endothelial cells are usually associated with CD45 expression, while $CD45^-$ cells have more myogenic potential. Although SP cells express CD45, Sca-1 and CD31 in variable percentages, the majority express Sca-1 and CD31, but not CD45. However, $Sca\text{-}1^+/CD31^-/CD45^-$ SP cells are most myogenic. Variable CD34 expression by SP has been reported. Satellite cells do not express Sca-1, CD45 or CD31, but some fractions express CD34. The majority of $CD45^-$ MDSC expresses Sca-1 and CD34, while all pericytes do not express CD34.

Due to overlapping expression of membrane proteins between myogenic precursor cells, more research is required to fully understand the cells expressing them and how they may be influenced by *in vivo* manipulations aimed at improving regeneration. Although overlapping marker expression has been reported for the variety of non-satellite cell populations in skeletal muscle, the expression profile of satellite cells has been well defined. Satellite cells are still considered to be the main cell population responsible for muscle regeneration, the proteins expressed in satellite cells and how they are used to determine satellite cell activity and progression through regeneration will be discussed next.

2.3. BIOLOGICAL ROLES OF MYOGENIC SPECIFIC CELL IDENTIFIERS

Myogenic cells residing in the skeletal muscle niche, including satellite cells are often at different stages of specification or maturation. It has become crucial to distinguish between the different populations in order to truly understand their role during the process of muscle regeneration. Various markers have been identified; these are usually used in combination in order to identify satellite cells at different stages of myogenesis.

2.3.1. Pax7

One of the main satellite cell-specific transcription factors is Pax7, a key genetic regulator required for satellite cell maintenance in the postnatal period. Seale *et al*. identified this transcription factor in quiescent and proliferating satellite cells [252] and since then it has been used extensively to identify the satellite cell populations. The Pax-genes code for a family of paired-box transcription factors known to play important roles during embryonic development. The nine members of the family known in mammals (Pax1-9) can be divided into four subgroups with Pax3 and Pax7 grouped together and of specific relevance to myogenesis. Both play a role in embryonic development of skeletal muscle, but Pax3 is not expressed in all adult skeletal muscle, in contrast with Pax7 which is expressed in all satellite cells [36].

The mechanism by which Pax7 regulates the satellite cell population has been investigated intensively, because the specification and survival of satellite cells is believed to depend on Pax7. This hypothesis is supported by the finding that Pax7 null mice have a severe reduction of satellite cells in skeletal muscle [36] and

regenerative myogenesis is severely compromised in the absence of Pax7 [212, 252]. However, if the Pax7 gene is genetically inactivated after post-natal day 21 in mice, normal limb muscle regeneration does still take place. Even the inactivation of Pax3 would not hamper the regenerative capacity of skeletal muscle post-natally. These findings imply that there could be other regulators that compensate for the absence of these Pax transcription factors [159, 133].

There is a sequential activation process during myogenesis; with Pax7 activating a group of transcription factors called muscle regulatory factors (MRFs). Myf5 expression is upregulated first, which in turn activates MyoD and the other MRFs (Myogenin and MRF4). These MRFs are then responsible for the expression of muscle differentiation genes [36]. Although adult regeneration proceeds similarly to embryonic and fetal myogenesis, it is difficult to follow *in vivo*. For example, there is no clear consensus on when a satellite cell becomes a myoblast or whether Myf5 plays an essential role in myogenesis.

Some results argue against a simple sequential activation model. For example, there is a cross-inhibitory interaction between Pax7 and certain MRFs and this is independent of the transcriptional regulatory ability of Pax7 [36].

2.3.2. Myogenic regulatory factors

The four main MRF proteins are MyoD (myogenic differentiation 1), Myf5 (myogenic factor 5), myogenin and MRF4 (also known as myf6). They all have a basic helix-loop-helix (bHLH) structure and can bind to enhancer elements of genes encoding for proteins involved in terminal differentiation, which are required to generate the contractile properties of skeletal muscle [36, 111]. The four proteins are not expressed simultaneously, but follow a temporal expression profile during myogenesis, making them useful tools for establishing the state of the satellite cell or muscle precursor cell. Unfortunately this means that no single MRF can be used to identify the whole population of satellite cells [111].

When satellite cells enter the cell cycle, they will begin to express MyoD and/or Myf5. From gene knockout experiments it appears that Myf5 and MyoD play roles in determination of the myogenic cell fate and the formation of myoblasts during embryogenesis. Simultaneous inactivation results in a complete lack of skeletal

muscle formation, in contrast with relatively normal muscle development where only one is inactivated. They both act upstream of myogenin and MRF4 [36, 111]. When MyoD increases sufficiently, proliferating myoblasts exit the cell cycle to differentiate and become terminally differentiated myocytes. MyoD upregulates myogenin and MRF4 (the “late” MRF’s) and structural proteins like myosin heavy chain and desmin, or metabolic proteins such as muscle creatine kinase, increase [49]. Myogenin knockout mice show normal initiation of myogenesis, but defects in the differentiation of the myocytes into myofibres, which is also the case with MRF4 knockout mice [36].

MyoD is usually expressed in the satellite cell nuclei after stimuli such as exercise, injury and overload. Ishido *et al*. found that MyoD is expressed early after denervation and peaks on day 3, surprisingly in myonuclei. Lack of MyoD expression in satellite cells in this study suggests that denervation cannot be used as a model to understand activation and myogenesis of satellite cells. There is a possibility that MyoD could be in an inactivated state allowing continued satellite cell proliferation, with no stimuli for differentiation. This transcription factor is involved in the regulation of muscle specific genes, coding for myosin light chain, muscle creatine kinase, acetylcholine receptor, troponin I and desmin and these proteins are broken down rather than synthesised during denervation [121].

Myogenin is the only MRF for which there are no compensatory MRFs during embryonic muscle development. Its absence leads to severe deficiency of myoblast differentiation and death in myogenin-null mice within days after birth [110]. There seems to be a different function for myogenin during embryogenesis than postnatal myogenesis. When myogenin is conditionally deleted post-natally normal muscle development takes place with little phenotypical consequence for muscle formation, except smaller adult size. However, in this study, Myf5, MyoD and MRF4 were upregulated to a greater extent [182]. *In vitro* studies where myogenin was deleted from satellite cells, normal myotubes were formed. Prenatally, myogenin is probably involved in the gene expression of structural components of the Z-line, a critical anchor point for the basic contractile unit of the muscle fibre, called the sarcomere. In adult muscle, the genes dependent on myogenin encode for factors involved in

cell fusion or innervation or for membrane associated or secreted proteins, but not for structural or contractile proteins [182].

MRF4 is the myogenic regulatory factor expressed during the terminal stage of muscle regeneration [68], although some studies have found that MRF4 is briefly expressed as early as 12 hours after stretch injury. Embryonically MRF4 cannot be detected in limb buds or visceral arches where one would usually find Myf5, MyoD and myogenin. It is highly expressed post-natally, but in normal adult skeletal muscle neither the transcript nor protein can be detected [28]. In many studies it is the only MRF that is absent from satellite cells during the regeneration process, however it is up-regulated with Myf5 and myogenin mRNAs in myofibres subjected to stretch and/or low-frequency stimulation [1, 124]. It is therefore more responsive to stimuli requiring adaptation and would most likely not be useful in studies of myotoxins or crush injury.

2.3.3. Adhesion molecules

Engrafted myogenic cells with the ability to develop into myofibres [133] or to occupy the satellite cell niche, all express very specific cell surface proteins [196, 245, 46]. Myoblasts need to form linkages across their cell membrane for interaction between the extracellular environment and the intracellular cytoplasmic structures, and for adherence to existing myofibres.

After injury, satellite cell adhesion capability is a vital requirement for docking of migrating satellite cells to structures in the injured site, for proper alignment of satellite cells, fusion and the formation of myotubes [57, 51].

A group of cell adhesion molecules are responsible for these interactions [304] and can be divided into four major sub-groups, namely cadherins, integrins, selectins and the immunoglobulin super family [301].

2.3.3. i) M-cadherin

More than two decades ago, Donalies *et al.* isolated mRNA from C2 cells and myotubes and found an unidentified mRNA clone, sharing 60% homology with the known cadherins. It was named M-cadherin as it was first found in myogenic cells [79]. M-cadherin was considered to be specific to skeletal muscle cells, as fibroblasts did not express it [79]. Although it has since also been found in specialized

adherence junctions, called *contactus adherens*, in the granular cell layer of the cerebellar glomerulus [115], this trans-membrane protein is still considered a muscle specific marker of activated satellite cells involved in calcium dependent cell-cell adhesion. After ischaemia of adult muscle, M-cadherin was found to be upregulated in all activated satellite cells by day 3 [198], results which were supported by the study of Fuchtbauer and Westphal [92].

Although M-cadherin is involved in early muscle regeneration and has even been found on quiescent satellite cells from normal muscle [120, 198], it is upregulated after initiation of myotube formation and shows a similar expression pattern to some muscle specific proteins, like troponin T, myosins and muscle creatine kinase, suggesting its involvement in fusion and maturation of muscle fibres [79]. When myofibres are mature, they usually appear M-cadherin negative [71], indicating that the role of this protein is no longer required.

An *in vivo* study on M-cadherin-deficient mice showed that M-cadherin is not essential for the development and regeneration of skeletal muscle. These mice were perfectly capable of regenerating injured muscle in the absence of the M-cadherin mRNA and protein. This unexpected finding could be explained by the upregulation of N-cadherin, which could compensate for the lack of M-cadherin [115].

L6 cells, which do not express N-cadherin like most other mammalian cells, have been used to investigate the role of M-cadherin. When M-cadherin function is blocked, the potential of the L6 cells to fuse was significantly reduced. Troponin T was down-regulated at the protein level at the same time, suggesting that M–cadherin plays a role in organisation of the myofilament elements in the cytoplasm [316].

M-cadherin varies in localisation, depending on the phase of regeneration. In non-differentiating myoblasts, M-cadherin associated more with the actin filaments, but it was associated more with microtubules in differentiating myotubes [135]. This implies the possibility that M-cadherin might play different roles during different stages of myogenesis, such as migration of activated satellite cells to the injured site in early phases and alignment of myoblasts preceding the process of fusion. Both M-

cadherin and NCAM are usually localized on the side of the satellite cell that faces the myofibre.

2.3.3. ii) NCAM (CD56)

NCAM, the neural cell adhesion molecule, is also viewed as a good marker of activated satellite cells [30, 120]. It is expressed in both satellite cells and myofibres, which indicates it can play a role in the attachment between satellite cells and myofibres [111]. However, the protein is not present in mature uninjured fibres.

It is one of the more reliable markers of satellite cells, but is not limited to this cell type as it is also expressed by lymphocytes [217]. In a study by Irintchev *et al.*, the expression pattern of M-cadherin significantly differed from that of NCAM, with the latter expressed during later stages of muscle regeneration. This could be due to its role during innervation of the newly formed muscle fibre [120].

Capkovic *et al*. found NCAM to be present only in non-proliferating myoblasts which express myogenin and muscle creatine kinase, suggesting they are committed to differentiation [45]. NCAM overexpression leads to enhanced myogenic differentiation [122]. In contrast to M-cadherin, NCAM is calcium independent with different isoforms expressed on myoblasts (a transmembrane isoform) and myofibres (a glycosylphosphatidylinositol (GPI) anchored isoform). The shift from one isoform to the other may be a key event in the formation of the myotubes by myoblast fusion.

In summary, although M-cadherin and NCAM are both adhesion molecules, they probably have distinct regulatory roles, leading to a percentage of satellite cells that are $NCAM^-$/M-$cadherin^+$ [122].

2.3.3. iii) CD34

A proportion of myogenic cells in the skeletal muscle niche express the transmembrane protein CD34, along with classical myogenic markers MyoD, M-cadherin and myogenin [230, 17]. CD34 is traditionally associated with the identification of hematopoietic stem cells (HSC), but other cell types, including mast cells, eosinophils, hair follicle cells and even neurons have also been shown to be $CD34^+$ [207]. The specific roles of this protein are still unclear.

In HSC, CD34 is associated with increased proliferation and/or blocked differentiation, thereby maintaining the undifferentiated progenitor phenotype of these cells. CD34 has also been implicated in adhesive interactions between HSC and other bone marrow cells under certain conditions; specifically it has been shown to inhibit adhesion of HSC, mast cells and eosinophils to each other or to the epithelial vessel wall [207, 80]. This inhibitory effect on adhesion results in higher mobility of $CD34^+$ cells, explaining why several studies report increased migration associated with CD34 expression [2, 208, 24]. A recent report on CD34 knockout mice suggested that CD34 regulates the function and motility of satellite cells after acute and chronic injuries. Satellite cell proliferation was impaired and myogenic progression delayed, while migration of distant satellite cells to the injured area was reduced in the absence of CD34 [2].

The time-dependant requirement for migration and adhesion of satellite cells correlates with the temporal expression of two CD34 isoforms, a full-length protein incorporating a longer intracellular region and a shorter truncated isoform. The truncated CD34 isoform is expressed in quiescent muscle progenitor cells with a switch to the full length isoform upon cell activation. The splice variant of CD34 has been shown to act as a more potent inhibitor of adhesion than $CD34^{full}$ [208]. This is followed by complete transcriptional shutdown [17], which lifts the inhibitory effect and allows fusion to occur. $CD34_{trunc}$ mRNA is two- to three-fold more abundant than the $CD34_{full}$ mRNA in skeletal muscle cultures. However, the majority of satellite cells are $CD34^-$ *in vivo*.

The proportion of CD34-expressing cells reported in skeletal muscle is not consistent (see Table 2.1). This is probably due to the fact that the different studies harvested cells from different locations with different levels of commitment, but also from different animal models. The $CD34^+$ cells found in the interstitial spaces of skeletal muscle are $CD45^-$ with the potential to differentiate into endothelial cells, myogenic cells or adipocytes [274]. After isolation of adult mouse skeletal muscle satellite cells, Qu-Peterson *et al.* showed that 83% of early pre-plated cells were $CD34^+/CD45^-$ and expressed desmin [230] and Tamaki *et al*. reported 70-80% $CD34^+/CD45^-$ cells present in adult mouse muscle [274]. However, Ieronimakis *et al*. reported percentages ranging from 6-14% $CD34^+/CD45^-$ cells isolated from mouse,

depending on muscle type investigated [119]. Pisani *et al.* reported only 53% of human isolated satellite cells expressed CD34 [223], while only 10% of isolated rat satellite cells express CD34 [174]. These differences in CD34 expression between studies were probably not only due to the model used, but also due to passage number and isolation criteria for each respective study.

Table 2.1. Proportion of $CD34^+/CD45^-$ cells isolated from skeletal muscle reported in literature.

Animal model	Muscle source	% $CD34^+$	Reference
Mouse	Hindlimb	83	Qu-Peterson *et al.* [230]
Mouse	*Tibialis anterior, Extensor digitorum longus, soleus, plantaris, gastrocnemius, quadriceps femoris*	70-80	Tamaki *et al.* [274]
Mouse	Hindlimb, pectoral muscles, triceps	6-14	Ieronimakis *et al.* [119]
Human	Orthopedic and abdominal surgeries	53	Pisani *et al.* [223]
Rat	Hindlimb	10	Machida *et al.* [174]

The possibility that a completely separate population of $CD34^+$ cells with distinct functions exist has been proposed. One possibility is that $CD34^+$ cells are satellite cells responsible for self-renewal, as a result of asymmetric division of existing satellite cells [119, 230, 245]. These cells will retain more stem-like properties and due to the inhibitory effect of CD34 on adhesion, they would participate less in fusion. In support of this hypothesis, isolated $CD34^+$ mouse myoblasts had a significantly lower fusion index, compared to isolated $CD34^-$ myoblasts; furthermore a significantly higher number of $CD34^+$ cells remained mononuclear after 96 hours in differentiation media. In addition, remaining mononuclear myoblasts from the $CD34^-$ fraction, expressed CD34 after 4 days in differentiation media, indicating asymmetric division took place, possibly for satellite self-renewal [125].

Although CD34 is not a reliable marker of the complete myogenic progenitor population, its expression may be useful as an indicator of the fate and commitment of a specific satellite cell.

2.3.4. Other markers

Myogenic precursor cells also express a number of other proteins, which could be used to identify them. Cornelison and Wold identified c-met, as a marker of quiescent satellite cells [68], although c-met expression is also associated with epithelial and other mesodermal cell types [238]. C-met is the receptor for hepatocyte growth factor (HGF), which has been shown to be essential for migration of the myogenic precursor cells from the somite to the developing limb during embryogenesis. Due to the lack of myogenic precursor cells, c-met deficient embryos wouldn't form limb skeletal muscle [111]. After crush injury, c-met is localised with HGF, but by 4 days after injury, c-met expression is weak in myoblasts and myotubes, while HGF expression is still high in myoblasts. C-met is not expressed in myotubes [277].

Other markers for satellite cells in different stages of regeneration with functions ranging from activation, cell adhesion, migration and transcription factors include CXCR4 [219], Syndecan-3 and -4 [276, 286, 66, 67] myocyte nuclear factor (MNF or Foxk1) [111] and SM/C-2.6 [93].

Integrin-α7β1 is present on satellite cell membranes. This integrin complex binds selectively to laminin, but not to other extracellular components [262]. Although the β1-chain is also found on fibroblasts, the α7-chain is myoblast specific, so it can be used to sort isolated primary myogenic cell populations [25]. In C2C12 cultures α7B is expressed much earlier than α7A, with the latter correlating with myogenin expression [63]. However, Yao *et al.* did not find any significant differences between the two isoforms [308]. It may therefore not be as useful as M-cadherin and NCAM to track the time course of regeneration.

2.3.4. i) Desmin

Another useful identifier of myogenic precursor cells is the structural protein desmin. Many myoblast isolation studies confirm myogenic identity of the isolated population by desmin expression [53, 166, 257, 240, 154, 172].

During myogenesis, desmin is one of the earliest muscle-specific structural proteins to be expressed. Expression levels are low in proliferating myoblasts, but increase in differentiated myotubes [216]. In myoblasts, desmin is randomly organised or radially expressed, but after fusion, desmin accumulates and becomes more organised and localises at the level of the Z-discs [154]. Desmin is one of several intermediate filament proteins, including vimentin, nestin, lamins and cytokeratins [216], which form an intracellular network [56] to provide a three-dimensional scaffold in regenerating cells. When translation of desmin mRNA was inhibited in C2C12 cells, differentiation and fusion was completely blocked, indicating its role in the formation of myofibres [161]. As myogenesis progresses, desmin affects spatial organisation of sarcomeres, desmosomes, nuclei and other organelles such as mitochondria [243]. In desmin-null mice, mitochondria showed irregular localisation and altered respiratory capacity [189]. Anesti *et al.* suggested that desmin serves to halt mitochondrial trafficking in locations where these organelles finally reside [7]. This theory is supported by the fact that mitochondria are found in pairs at the Z-discs of sarcomeres [189] and successive Z-disks are connected by desmin, along the plasma membranes of skeletal muscle [243, 210].

Desmin continues to be expressed in normal adult skeletal muscle, unlike many of the other myogenic markers expressed during myogenesis. It plays a critical role in the maintenance of the mechanical and structural integrity of the contractile apparatus, by stabilising the sarcomeres, and is responsible for transmission of muscle contractile force between separate myofibrils and the sarcolemma [243, 29].

In the absence of desmin during development, skeletal muscle shows a mild and progressive fibrotic adaptation. This results in stiffer muscle, accumulation of collagen and increased gene expression of extracellular matrix proteins. In addition, an increased state of inflammation and incomplete regeneration persists, with increased numbers of inflammatory cells present [186]. There are also some sarcomeric ultrastructural changes, which lead to reduced active and passive muscle forces [6, 12]. Mice lacking desmin showed irregular organisation of myofibres with misaligned myofibrils, Z-disk streaming and mitochondria degeneration and disorganisation. Since anchorage of myofibrils to the sarcolemma was compromised,

transmission of muscle force was impaired. Thus, mice visibly lacked strength and fatigued quickly [216].

Desmin expression is also altered after muscle damage. It is rapidly lost after eccentric exercise, but not during isometric or concentric contractions [163]. This is probably due to membrane disruption during this type of exercise, which leads to increased calpain activity, which in turn leads to hydrolysis of the intermediate filament network. However, in another study desmin intensity was decreased after eccentric exercise, but western blot analysis showed no decrease in protein levels [15]. The desmin filaments probably disassembled, which resulted in decreased immunostaining, but not in a decrease in protein content.

In addition, Yu *et al.* showed with high-resolution immunohistochemistry, that desmin remodelling takes place after eccentric exercise in humans. Instead of locating only at Z-discs, single longitudinal desmin strands were evident, aligning next to the myofibres to link several Z-discs [311]. This is evidence that desmin plays a different role during regeneration after muscle damage compared to non-injured muscle. However, eccentric exercise does not cause severe muscle damage. Myotoxin injury in rats resulted in the total loss of desmin expression, confirmed by Western blot analysis [289], confirming that proteolysis of the intermediate filament network occurs in severe muscle damage. Regenerating myotubes showed intense desmin expression two days after toxin injection [289]. Creuzet *et al.* also found that desmin expression disappears in necrotic mouse myofibres after freeze lesions to the *pectoralis major* muscle, but satellite cells and newly formed myofibres in the injured area showed increased staining intensity already two or four days after injury respectively [69].

Due to these changes in organisation and expression levels during myogenesis in both myoblasts and newly formed myotubes, this intracellular protein is useful evidence for investigating effectiveness of the regeneration process, especially after skeletal muscle injury.

2.4. GENERAL INTRODUCTION TO MUSCLE INJURY MODELS

More than 90% of all sports-related muscle injuries are due to strain or contusion, while lacerations including the underlying skeletal muscle occur less frequently during sports. Muscle strains, the leading cause of muscle injury, are usually associated with sprinting and jumping, while contusion commonly occurs during contact sports such as American football, rugby, karate, judo and soccer [8, 18, 128, 127].

These acute injuries cause significant pain, discomfort and sometimes reduce physical ability for extended periods of time. The functional capacity of injured muscle seldom returns to pre-injury levels, due to scar tissue formation or so-called fibrosis. When incomplete recovery occurs, there is therefore a tendency for recurrent injuries [137, 138, 295].

To the elite and professional sportsmen, the risk of injury is of great importance, as the decision to withdraw from events or their submaximal performance could have dramatic financial and tactical repercussions [8]. Also, due to the recent development of health consciousness and physical fitness, more individuals are engaging in exercise and sports activities on a recreational level. This means that not only professional and amateur sportsmen are suffering from injuries, but also generally active individuals [153].

2.4.1. Studying injuries and the use of injury models

Many studies are dedicated to investigating exercise-induced muscle injury with delayed onset muscle soreness (DOMS). Due to ethical implications in human studies, animal models are required to study more traumatic causes such as contusion. More indirect causes of muscle damage include bacterial and viral infections, injuries due to vascular obstruction or traumatic vascular injury, neuropathic conditions and myopathies [18].

In studies on human subjects, investigators are unable to control contusion injuries sustained in the field [18]. Due to the nature of small animal laboratory research, one can control for many confounding variables, such as the impact load, the surface area of the impact and absence of laceration [19]. Also, if appropriate procedures are

followed to promote muscle damage, a controlled regeneration process can be expected [49].

Irrespective of the initiating mechanism of muscle damage, the recovery of an injured muscle follows a fairly constant pattern of healing [127, 295]. However, the kinetics and amplitude of the regeneration steps vary depending on the extent of the injury, the muscle injured and in terms of research, the animal model used [49].

Muscle injury models fall into one or more of the following categories: i) direct infliction of trauma by crushing or freezing the muscle, ii) the use of myotoxins [305], iii) denervation or devascularisation and iv) repeated bouts of intensive contractile stimulation, especially eccentric contractions [14]. In order to study abnormal regeneration associated with genetic defects, mice with spontaneous or artificial deregulation of specific genes are generally used [49].

Regardless of the mechanism of injury - ischaemia, myotoxin, stretch, electrical stimulation [114], long term denervation [30] and various chronic myopathies and dystrophy - they all trigger regeneration of skeletal muscle. For models such as muscular dystrophies, transient devascularisation or the injection of toxic substances [177] it is difficult to control the extent of damage; a contusion injury on the other hand can be standardised more easily [49].

2.4.2. Contusion injury

Muscle contusion injuries are most common in the muscle belly area of the upper arm and thigh. They occur repetitively in many contact sports, but also occur in non-athletes following simple falls and accidents [19]. The induction of mechanical injury is physiologically more relevant than the use of myotoxins in research [137]. Also, contusions are usually caused by a sudden blow to the muscle with a blunt, non-penetrating object and rarely disrupt the skin [153, 128, 127, 18, 137]. The extent of damage is influenced by the contractile status of the muscle at the time of impact. According to Crisco *et al.* [70], contracted muscle is able to absorb more energy during impact than relaxed muscle. This was confirmed by the study of Beiner *et al*., who explained that in contracted muscle, the force of the injury is distributed over the entire muscle belly, thereby protecting the muscle from more severe localised damage. In contrast, contusion of relaxed muscle exposes the underlying muscle

(closer to the bone) to the force of impact [18]. This type of injury is also called crush injury.

Several studies have been conducted to evaluate the immediate response to injury and subsequent long-term muscle regeneration [190]. The initial injured area is characterised by a mixture of muscle cells and collagen connective tissue due to the partial rupture of the muscle, as well as infiltrative blood cells due to capillary rupture [18, 19]. In contusion injuries, capillary rupture gives blood-borne inflammatory cells and cytokines direct access to the site of injury [258].

Histological changes in the crush injury site follow a distinct time course. Within one day of injury, evidence of necrosis of damaged myofibres is evident, with simultaneous infiltration of leukocytes and proliferation of myoblasts. By day three there is extensive phagocytosis of necrotic muscle tissue, and myoblasts begin forming new myotubes via fusion. This is followed by progressive replacement of damaged tissue with regularly aligned myotubes. Kasemkijwattana *et al.* developed a mouse contusion injury model and found muscle repair to take place rapidly within the first two weeks after injury [134], but Myburgh *et al*. showed incomplete recovery by day fourteen after a similar crush injury in rats [203]. Bunn *et al.* (2004) found that large crush injuries display a delayed inflammatory response when compared to low crush injuries [37], which could explain the reported differences in recovery time.

Since the middle of the twentieth century, researchers have been trying to develop a standardised muscle crush injury [19]. In the early 60's, a hammer device was used to crush muscle and bone of monkeys, a technique which was later modified to a spring loaded hammer [126] and then a reflex hammer [283]. Hemostat forceps which crush muscle through skin incisions has been used by a number of researchers [18]. The mass-drop injury model, introduced by Kvist *et al*. [148] and the drop-ball technique [137], involve single impact trauma to the muscle, by dropping a solid weight or ball on a flat impact surface from a certain height onto the specific muscle [258].

Early mobilisation after muscle trauma such as a contusion injury, leads to better recovery and muscle fibre regeneration in comparison with immobilisation. Khattak *et al*. used the drop ball technique to injure the *gastrocnemius* muscle of Sprague

Dawley rats, to evaluate regeneration in a mobilised versus immobilised group. Rats were sacrificed either 3 days or 21 days after injury. There was a significantly higher number of regenerating myofibres in the mobilised group after three days compared to the immobilised group, but a significant reduction of centrally nucleated myofibres in both groups in the following 18 days, suggesting the conversion to mature muscle cells [137].

2.5. PHYSIOLOGICAL FACTORS AFFECTING TISSUE INJURY AND REGEGENERATION

Skeletal muscle damage disrupts not only the muscle fibres, but the whole micro-environment of the muscle. Biologically active molecules are released into the extracellular space upon muscle injury [123]. Some of these promote the degradation and removal of cellular debris or are responsible for the progress of satellite cells from quiescence to proliferation. These stimulating molecules are released by the injured fibres or from the surrounding connective tissue or by invading cells [49]. For the purpose of this study, a brief description of the invading cells will be followed by a more detailed discussion of oxidative stress. Despite negative perceptions of inflammation, this complex process is required and essential for regeneration. Nonetheless, inflammation itself must be regulated [33].

2.5.1. The inflammatory response

One of the important regulatory processes following muscle damage is inflammation, which is responsible for the removal of cellular debris via phagocytosis, and for the release of signalling factors. These factors promote muscle precursor cell activation, attraction and growth, prevent the muscle cells from undergoing apoptosis and facilitate vascular and muscle fibre repair. The inflammatory response also consists of two phases, which occur in parallel with the two phases of muscle regeneration. During the first phase a pro-inflammatory environment persists, with a switch to an anti-inflammatory environment in the later phase [258].

The onset of inflammation is characterised by vasodilation and increased permeability of the capillary wall, which leads to increased plasma proteins and

invasion of phagocytic leukocytes into the damaged area [49, 258]. The increased number of non-muscle cells in the damaged site is the main histopathological characteristic of the early phase following muscle injury [49].

Inflammatory cells in the injured environment are activated by the release of growth factors and interleukins from the injured muscle and they generate chemotactic signals to attract circulating inflammatory cells like neutrophils. Neutrophils in turn release cytokines such as IL-1 and IL-8 to recruit macrophages to the site of injury [258]. The inflammatory cell type observed abundantly in the injured area within the first six hours post injury is the neutrophil. Neutrophil count peaks within 24 hours, but remains elevated above baseline until approximately 5 days post injury in severe cases. These neutrophils are responsible for phagocytosis of necrotic cellular debris caused by the auto digestion of muscle fibres. Large numbers of mature neutrophils are released from bone marrow in response to damage, and an increase in the production of new immature neutrophils increases their numbers even further [258].

Approximately 48 hours after the injury, macrophages invade the injured area to further phagocytose the cellular debris [49]. Macrophages can be divided into subtypes with different features and functions. There is evidence that macrophages can change from the pro-inflammatory to anti-inflammatory subtype, depending on the signals from the micro-environment. In the presence of pro-inflammatory cytokines TNF-α and IL-1β, macrophages of the M1 subtype will increase satellite cell proliferation [203]. Anti-inflammatory macrophages of the M2 subtype are not involved in phagocytosis, but rather enhance differentiation. They release cytokines and growth-related mediators to stimulate regeneration [258].

One of the negative effects of inflammation is the occurrence of secondary damage during the early phase of inflammation. This is caused by the increased release of free radicals during the first 24 hours after damage. Neutrophils are key role players, because they have been shown to generate free radicals [258]. In addition, the M1 macrophages can increase the release of reactive oxygen species which promote tissue destruction. This oxidative stress also has an effect on myoblasts after injury.

Skeletal muscle shows an adaptive response to ROS activity which is induced during contractions. The antioxidant enzymes, such as superoxide dismutase and catalase

are activated and stress kinases and heat shock proteins respond to increased ROS production during exercise [227].

2.5.2. Oxidative stress

Oxidative stress is a concept described as the disturbance in the pro-oxidant-antioxidant balance in favour of the first, or the disruption of redox signalling and control. Oxidative stress is now considered when there is i) an increase in the formation of radicals, ii) decrease in the anti-oxidant levels, iii) disturbance of the redox balance and iv) oxidative damage to cellular components. Markers in these four groups can be used to evaluate the oxidative stress levels in cells [227]. Damage to proteins, DNA, lipids and the extracellular matrix are common effects of oxidative stress.

During cellular metabolism there is a constant production of highly reactive molecules, most containing oxygen and/or nitrogen atoms, which will participate in redox reactions [54]. These are collectively termed reactive oxygen species (ROS) and are the main role players in oxidative stress. In skeletal muscle, ROS are produced mainly by the mitochondria during metabolism, but also by the sarcolemma, sarcoplasmic reticulum, transverse tubules and by oxidases in the cytosol [226].

2.5.2. i) Oxidant species

Free radicals are atoms/molecules which are capable of independent existence despite containing an unpaired electron, in contrast with most atoms with electrons associated in pairs. These free radicals are usually the products of redox reactions. Most reactive molecules either have a central oxygen or nitrogen, which leads to the terms reactive oxygen species (ROS) and reactive nitrogen species (RNS) respectively. These also include non-free radical species [227]. The reactive molecules superoxide, hydroxyl radicals and nitric oxide are the most abundant free radicals produced in cells.

Superoxide is an intermediate in many biochemical reactions, and is produced by immune and damaged cells. This negatively charged anion is membrane impermeable, although it has the ability to diffuse into cells after membrane disruption. Although it has a long half-life, it is relatively unreactive, but can convert

to many other, more reactive oxidants, such as hydrogen peroxide [227, 151]. Superoxide is usually formed when oxygen is not completely oxidised in the mitochondrial electron transport chain during aerobic respiration in muscle cells during exercise [83].

The dismutation of superoxide by the anti-oxidant enzyme forms hydrogen peroxide. Hydrogen peroxide can also be formed from urate and amino acid oxidases. This is also a stable oxidant with a long half-life, but unlike superoxide it is membrane permeable. Although it is a weak oxidising agent, it is cytotoxic and can inactivate some enzymes. It has little effect on DNA and lipids. In the presence of catalysts such as iron and copper it can participate in the Fenton and Haber-Weiss reactions, which form more reactive oxygen species, such as hydroxyl radicals [227, 151]. Hydroxyl radicals are highly reactive agents with strong oxidising potential, probably the most damaging free radical present in biological systems. They are not membrane permeable and damage molecules in their close proximity.

Hydrogen peroxide can also form hydrogen chlorite in the presence of myeloperoxidase, especially in neutrophils, which synthesise these agents for destruction of microbes. It causes damage, by oxidising thiols, lipids, ascorbate and NADPH. Another derivative, hyperchlorous acid is membrane permeable and will cause protein fragmentation and aggregation [227].

During the degenerative phase of muscle repair, muscle cells are lysed by neutrophil oxidative burst and a few days later by macrophages through a superoxide-independent mechanism. Damaged muscle cells influence the macrophages to increase nitric oxide (NO) production. This mechanism can also be enhanced by cytokines. This leads to a free radical burst and release of more reactive oxygen species [90].

Nitric oxide (NO) is an important role player during the healing process. It promotes repair in later phases, but also manages the balance between myogenesis and fibrosis [72]. However, if the amount of NO is too high, it may also have cytotoxic effects. As a weak reducing agent it can react with oxygen to form nitrogen dioxide or with superoxide to form peroxynitrite. This is a strong oxidising agent, which can cause DNA damage, depletion of thiol groups and nitration of proteins [227].

However, NO also reduces lysis by neutrophils and decreases superoxide concentration by forming less reactive species and by increasing their apoptosis and preventing their docking by inhibition of expression of adhesion molecules [90].

The enzyme responsible for NO synthesis is nitric oxide synthase (NOS) which occurs in three isotypes (eNOS, nNOS and iNOS). After skeletal muscle injury, increases in eNOS and iNOS mRNA transcript levels have been observed [318] with no change in nNOS [242]. The iNOS mRNA increased as early as 6 hours post-injury, indicating that the higher NO-levels observed after injury are mainly due to the activity of the iNOS isotype. iNOS is activated in the cytosol of infiltrating macrophages, but also in the muscle fibres themselves possibly explaining the multiple roles of NO [242, 317, 318, 89, 72].

Nitric oxide is indirectly involved in muscle regeneration by enhancing vasodilation and inhibiting fibrosis [72]. It is also directly involved in the activation of satellite cells in the first 24 hours after injury [294]. NO synthesis causes the release of HGF from the extracellular matrix where it is normally stored. This is probably via the mediation of matrix metalloproteinases. HGF binding to its receptor c-Met on satellite cells cause their activation [279, 90]. Increased iNOS levels correlate with increased MMP-2 expression in crush injured *gastrocnemius*, playing an important role in the signalling cascade required for proper muscle regeneration [89].

In summary, it seems that NO could delay wound repair during the early phases of healing, but promote the process during later phases. It is important to note that NO is cytoprotective at nano-molar concentrations, but become cytotoxic at the micro-molar level [72].

2.5.2. ii) The signalling role of ROS in skeletal muscle

Redox sensitive mechanisms play a vital role in normal muscle function [129]. For example H_2O_2 can react with a variety of different cellular molecules and activate more than one signalling pathway [226, 90].

Redox signalling usually controls gene expression by modifying the phosphorylation status of transcription activating factors, such as NF-κB, which is central in the inflammatory response. The NF-κB family of transcription factors regulate the expression of more than 150 genes. Increased ROS production initiates a sequence

of events which leads to degradation of IκB, the inhibitor of NF-κB, resulting in the liberation and nuclear translocation of NF-κB. There is some evidence that ROS also inhibit NF-κB transcription, diminishing the DNA activity of this transcription factor. Thus, the redox control of NF-κB is still unclear [226].

TGF-β1 has been described as a chemoattractant for phagocytes and inflammatory cells during the early phase of regeneration [49]. Upon binding of the TGF-β to its cell surface receptors, SMAD proteins are activated and translocate to the nucleus to trigger the expression of target genes [49, 185]. Smad3 is a transducer of TGF-β which leads to the inhibition of differentiation. This intracellular effector of TGF-β signalling represses the MyoD family of transcription factors [165, 167]. TGF-β also increases the expression of HDAC4, a key inhibitor of myoblast differentiation [298]. However, TGF-β1 through Smad3 inhibits Sca-1, which is an inhibitor of myogenic cell proliferation and differentiation. This might seem contradictory, but Sca-1 enhances turnover of the extracellular matrix and prevention of fibrosis through metalloproteinases (MMPs), therefore opposing the main role of TGF-β [170].

2.5.2. iii) Muscle injury and oxidative stress

Oxidative stress is present primarily during the destruction phase after injury. Auto-oxidation of myoglobin, which leaks into circulation upon muscle damage, is a possible source of reactive oxygen species. In patients with rhabdomyolysis, myoglobin accumulation in the renal tubules caused oxidative stress, and further lead to lipid peroxidation. The release of iron from the haeme group of myoglobin and haemoglobin is another possible candidate for forming free radicals in the injury site [225]. However, myoglobin acts as a $NO^{\bullet}$ scavenger under normoxic conditions which protects tissue against excessive NO. In contrast, under hypoxic conditions deoxygenated myoglobin is responsible for nitrite dependent $NO^{\bullet}$ production. This process has been demonstrated in perfused hearts and reduced oxidative damage after ischemia/reperfusion injury in the presence of added nitrite [112]. The role of myoglobin in the onset of oxidative stress is controversial, but can be explained by the different oxidation states of the molecule: ferrous, ferric and ferry, or cross-linked myoglobin [113]. It is unclear what the effect of myoglobin is during muscle injury and it likely depends on the extent of the injury and whether or not the blood supply is also disrupted.

It has been found that oxidative stress enhances neutrophil attraction, adhesion and migration. When xanthine oxidase is blocked, neutrophil infiltration is reduced, while neutrophil adhesion is increased by H_2O_2 [203]. The mobilised inflammatory cells, neutrophils and macrophages that infiltrate the injured area also contribute to the ROS at the injury site. Macrophages in particular produce ROS as part of the phagocytic mechanism to remove necrotic tissue [90].

An enzyme which is stimulated after injury is the NADPH oxidase, which leads to a respiratory burst, which in turn leads to the release of ROS from the mitochondria [220, 221]. Although ROS are an integral part of the recovery process after injury, ROS will damage non-injured muscle tissue in close proximity to the injured area as well [227]. When the degenerative phase makes way for the regenerative phase, ROS would hinder this process. Therefore strategies to reduce the damage caused to intact muscle fibres are still to be found.

Other conditions in skeletal muscle cause oxidative stress and these conditions are exploited in studies investigating ROS production. Myocardial ischemia-reperfusion injury is commonly used to study the effects of oxidative stress in cardiac tissue. In skeletal muscle, the method to induce ischemia and subsequent reperfusion is an acute bout of claudication. This causes significant oxidative stress [130]. These models are also used to investigate potential mechanisms that will reduce damage.

2.5.3. Anti-oxidant systems reduce oxidative stress

2.5.3. i) Endogenous anti-oxidants

There are a few inherent anti-oxidant strategies in most cells, providing cells with defence mechanisms against the effects of oxidative damage. These strategies are either enzymatic or non-enzymatic. Any substance with the capability of delaying or preventing the oxidation of a substrate can be considered an anti-oxidant. Some dietary constituents proved to be valuable anti-oxidant agents. They can be localised within various organelles such as mitochondria, or in the vascular and extracellular spaces [227]. The anti-oxidant enzymes are fibre-dependent and tend to be abundant in fibre types with high metabolic rate and mitochondria [227, 228]. Upregulation of antioxidant enzymes occurs after ROS-activation of the transcription factors NF-κB and activator protein-1 (AP-1) [227].

The anti-oxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase. These can either change reactive species into less active molecules, or prevent less active species from forming reactive species. They can also minimise the pro-oxidants, such as iron and copper via metal binding proteins [228].

SOD has three isoforms and needs copper-zinc or manganese as co-factors. In skeletal muscle, 15-35% of their activity is localised to the mitochondria, with the remaining 65-85% acting in the sarcoplasm. SOD is responsible for the removal of superoxide molecules. GPX has five isoforms and catalyses the reduction of H_2O_2 or organic hydroperoxide to water and alcohol, with reduced glutathione as substrate. It is considered an important intracellular anti-oxidant with a wide range of activity, mainly protecting against damage to membrane lipids, proteins and nucleic acids. Catalase, which is widely distributed in cells, will break down H_2O_2 with iron as co-factor to form H_2O and O_2 [228, 227].

The importance of the superoxide dismutase has been demonstrated by the missense mutations in the gene encoding for SOD1 identified in patients with amyotrophic lateral sclerosis. The accumulation of many reactive oxygen species produces oxidative stress, which is a major cause of the pathology of the disease [107].

Many studies have shown that mesenchymal stem cells have greater enzymatic and non-enzymatic antioxidant capacity than more differentiated counterparts, such as myoblasts. The superoxide dismutase and glutathione levels are elevated, which might explain the superior capacity of MSC to differentiate and survive post-transplantation. Usually, inflammation causes oxidative stress at the site of transplantation which could limit the survival capacity of transplanted cells. Thus, cell types with higher tolerance for oxidative stress, with higher ant-oxidant capacity, have a higher survival rate after transplantation [287].

Non-enzymatic anti-oxidants are usually low molecular weight agents with the capability of scavenging ROS species. These include glutathione, uric acid and bilirubin, as well as coenzyme Q10. Some dietary supplements also have the natural ability to scavenge free radicals [227]. The awareness of reactive oxygen species

and free radicals has led to the increased investigation into exogenous anti-oxidants which could enhance health and reduce the effects of oxidative damage in tissue. Vitamin E and C are the best known anti-oxidant agents, but carotenoids, especially β–carotenes, and vitamin A have also been shown to have free radical scavenging capabilities [227]. Other anti-oxidants, many of which are plant-derived compounds, would include triterpenoids and polyphenols. These compounds protect cells against inflammatory stress and can effectively reduce the pathology in many conditions [203].

2.5.3. ii) A potential treatment option: Grape seed extract

Grapes are excellent sources of antioxidants. Grape seed extract contains proteins and carbohydrates and abundant amounts of biologically active polyphenolic bioflavonoids, in the proanthocyanidine class (PCO). PCO is a high-molecular-weight polymer that combines as dimers, trimers or oligomers of catechin or epicatechin [54, 136].

Grape seed proanthocyanidin extract (GSE) has a wide spectrum of biological activities. Inflammation and oxidative stress are two major effects of injury that GSE may modify. GSE has not been investigated much in the context of skeletal muscle injury; therefore its effects in other models will be mentioned briefly. Its anti-inflammatory properties are some of its many beneficial effects. *In vivo* supplementation of proanthocyanidins extracted from blackcurrants reduced circulating TNF-α, IL-1β and leukocytes during carrageenan-induced paw oedema in rats, with no effect on IL-6. Pro-inflammatory cytokines induce synthesis of adhesion molecules that incorporate into the blood vessel wall. In systemic sclerosis patients, proanthocyanidins reduce ICAM-1, VCAM-1 and E-selectin, which could hamper inflammatory cell migration from the circulation to the interstitial space and prevent the release of pro-inflammatory cytokines [97]. Similar results have been observed in patients with collagen induced arthritis. Reduced synovial inflammation, cartilage and bone erosion coincides with a reduction in TNF-α, IL-17 and NF-κB [54].

In vitro effects of PCO are contradictory. It has been shown to have an effect on cell cycle and apoptosis regulatory genes such as bcl-2, p53 and c-myc. Evidence suggests that GSE could play an anti-apoptotic role by inducing the expression of bcl-2, and in other cases favour apoptosis by inhibition of bcl-2 and bcl-xl [11, 241].

This contradiction might be due to the differential effects of GSE on cancerous cells and normal cells. For example, Bagchi *et al*. showed that grape seed extract is significantly cytotoxic to breast, lung and gastric adenocarcinoma cells *in vitro*, while improving growth and viability of normal non-cancerous cells.

Probably the most beneficial characteristic of GSE is its anti-oxidant capacity. In comparison with other common antioxidant systems, GSE is 20 fold more potent than vitamin C and 50 fold more potent than vitamin E. With GSE supplementation 78-81% of superoxide anions and hydroxyl radicals are scavenged, indicating that it is far more effective than vitamin C and E and β–carotene [11, 241]. It is known to scavenge free oxygen radicals, especially H_2O_2 and inhibit UV radiation induced peroxidation [97].

Oxidative stress is a major effect during ischemia and reperfusion of organs, with a decrease in antioxidant levels. GSE provides cardio-protection against oxidants and improves cardiac recovery during reperfusion conditions. During re-expansion pulmonary oedema, GSE prevents oxidative stress [312].

Another condition contributing to the disturbance of the oxidative balance is during chemotherapy treatment. A chemotherapy agent methotrexate is known to cause a decrease in antioxidant levels, leaving the cells vulnerable to ROS. Damage to fast proliferating cells such as the gastrointestinal mucosa cells and bone marrow cells is a common side effect. GSE has a protective effect on the small intestine and will increase superoxide dismutase and glutathione peroxidase levels [104]. This suggests that it may also have beneficial effects on myoblast proliferation in the injured zone.

Khanna *et al*. investigated dermal wound healing by inflicting dermal excisional wounds on the backs of mice. With topical application of grape seed extract, wound contraction and closure was accelerated with a well-defined hyper-proliferative epithelial region and improved histological architecture. This was probably due to the higher cell density and enhanced deposition of connective tissue at the wound site and increased transcription and expression of VEGF. GSE enhanced the oxidising environment at the wound site and low level production of intracellular NO, contributing to wound repair [136].

GSE induces NO production in a dose-dependent way. As mentioned earlier, very high levels of NO are cytotoxic, thus too high levels of GSE might become detrimental [255]. Vignaud *et al*. found that administration of anti-inflammatory and antioxidant drugs did not significantly affect muscle recovery after myotoxic injury or crush injury. Higher doses of these drugs were lethal and reduced muscle regeneration after the crush injury. However, this is in contradiction with other experiments which showed that administration of anti-inflammatory drugs and other antioxidants improve muscle repair after injury [291].

In a previous study in our laboratory it was found that acute supplementation of GSE after a muscle contusion injury to the *gastrocnemius* caused an earlier activation of satellite cells, and fetal myosin heavy chain, which indicates quicker muscle regeneration. The infiltration of neutrophils was suppressed, while earlier infiltration of macrophages resulted in pro-inflammatory cytokines returning to basal levels much earlier. This could indicate an attenuated secondary damage via the inflammatory response [143].

Both chronic and acute administration of grape seed extract resulted in similar early satellite cell activation and regeneration, with similar effects to the inflammatory cell responses. The oxygen radical absorbance capacity assay (ORAC) revealed that GSE acted as an anti-oxidant in both blood plasma and the skeletal muscle [203]. However, it is not clear from cross-sectional immuno-histochemistry how the satellite cell function was actually affected, since myoblasts go through such a complex and dynamic process during muscle regeneration.

In summary, GSE has a very wide spectrum of biological activity, and plays a protective role in many processes and systems, such as inflammation, oxidative stress, and wound healing in the vascular system and possibly even cancer prevention and treatment. Its role in skeletal muscle injury repair has not been investigated fully.

The general hypothesis was that the previously observed positive effect of GSE on the time course of skeletal muscle recovery post injury is due to a modulation of satellite cell (SC) progression from precursor cells to mature myoblasts. In particular, we hypothesised that myoblasts isolated from injured rats exposed to chronic grape

seed extract supplementation, would show evidence of early differentiation *in vitro* despite incubation in proliferation media, in contrast with myoblasts isolated from placebo supplemented rats.

To test the specific hypothesis, primary myoblasts had to be isolated at different time points after contusion injury in rats, and their properties assessed *in vitro*. Various harvesting protocols were available, but there was no clear indication which technique would be optimal for harvesting myoblasts from injured muscle. We hypothesised that a combination of techniques and a combination of different media would be more effective than following a single protocol, or using only one specific culture medium.

Therefore the aims of the experiments presented in this thesis were i) to optimise the harvesting, isolation and culturing of primary myoblasts from rats by incorporating different techniques into the protocol and ii) to compare the efficiency of different media options to establish such cultures. Thereafter the aims were iv) to use this optimised isolation protocol to harvest primary myoblasts from rat muscle at different time points after a standardised contusion injury and v) to determine the effects of *in vivo* GSE treatment before and during regeneration on the phenotypic progression of these myoblasts compared to those harvested from placebo-supplemented groups.

In the following chapter, the establishment of an optimised protocol for primary rat myoblast isolation and culturing will be presented and discussed. Several modifications were made along the course of the optimisation to improve cell yield and survival. Myoblasts cultured in different media, were compared for myogenic protein expression and proliferation rate. From the findings, a final protocol was compiled to be used to isolate myoblasts from rats, subjected to *in vivo* intervention.

Chapter 3 Establishment of a primary myoblast culture

3.1. INTRODUCTION

When cells are directly isolated from tissue and grown *in vitro*, it is referred to as a primary culture. Although these cells are removed from their niche with all its influences, the possibility to isolate cells from live tissue provides the opportunity to intervene directly with their activity *in vitro* without much ethical complication. When considering primary cultures, one also has the benefit of performing *in vivo* interventions prior to the isolation of the cells, whereafter their properties can be investigated further.

Since the ratio of satellite cells to myonuclei is very low, it is difficult to fully understand the process of muscle regeneration. This has led to the development of many harvesting techniques to isolate satellite cells from skeletal muscle tissue. The first satellite cells to be isolated and grown in culture were obtained from rats by Bischoff [22, 40]. Numerous studies have followed this original one, describing protocols which could be followed for isolation.

Immortal cell lines, such as C2C12 and L6 cells, have been used for decades to model satellite cells. C2C12 has its origin from C3H mice, a strain which was bred for its susceptibility to form spontaneous tumours in the mammary glands. Almost 35 years ago, Yaffe and Saxel isolated the first C2 cells from 2 month old C3H mice, 70 hours after a contusion injury to the thigh muscle [269, 303]. Using a carcinogen this isolated population of cells was growth transformed to become an immortal cell line [64]. The C2C12 cell line was later derived by Blau *et al.* from clonal cultures of the original cell line [26]. L6 cells cannot proliferate for many passages, so C2C12 cells are most commonly used for investigative studies [237, 302].

Isolated satellite cells in a primary culture more closely resemble satellite cells found *in vivo* in skeletal muscle, compared to the more common immortal cell lines [40]. It was found that more than 25% of the genes surveyed and expressed by C2C12

cells, such as FGF-5, -7 and -10, FGFR-2 and cdk-2 and -5, are not expressed by primary satellite cells [68]. Not only does the shape of C2C12 cells differ slightly from primary cells, they also proliferate at a much faster rate. They become confluent more quickly, artificially promoting differentiation. Therefore deductions made from differentiating C2C12 cells may not always be accurate [65]. Furthermore, C2C12 cells are in an activated state, which means the cell line is not very suitable to study quiescence or the transition to activation. Other differences have been found such as their response to electrical stimulation [152] and adhesion protein expression levels (integrin-α3 and -β1 subunits, ADAM 12 and M-cadherin) in different conditions [101].

Nonetheless, C2C12 cells are a stable cell line, easily cultured and useful for proof of concept experiments prior to primary culture experiments. The isolation of primary satellite cells from skeletal muscle poses many challenges that have to be overcome for the establishment of a successful culture. The most common challenges include the maintenance of a sterile environment, provision of adequate nutrients and signalling factors to promote cell growth and perhaps most importantly, the generation of an adequate yield at low passage. Although satellite cells are probably the most abundant tissue specific stem cell, with approximately 50 000 satellite cells per gram muscle in mice [20], satellite cells make up a very small fraction of total muscle mass, which could be a limiting factor for the establishment of a successful primary culture [65].

The low yield in many experiments may be due to the fact that satellite cells *in vivo* are encapsulated between the basal lamina and sarcolemma of the adjacent muscle fibre and connective tissue of the interstitial matrix. When satellite cells are taken out of their niche *in vivo*, they are removed from the factors that play a role in their structure and activity. The low success rate of stem cell therapy of muscle diseases with isolated satellite cells, suggests that satellite cells lose their myogenic capability upon removal from their niche environment [20]. Myofibres release signalling factors such as growth factors which have receptors on satellite cells, and it seems that there is contact-mediated signalling between the myofibre and the satellite cells [279, 195, 49, 145]. Blood supply [58] is essential for the functioning of satellite cells *in vivo* and even when the muscle is damaged, the supply of distant muscle precursor cells and inflammatory cells, hormones and other circulating factors still influence the

satellite cells in their niche. It is almost impossible to mimic the surroundings of the *in vivo* niche in *in vitro* cultures [145].

In order to isolate satellite cells, skeletal muscle tissue is fragmented using scissors, forceps and a scalpel, followed by proteolytic enzyme digestion which disaggregates the tissue into individual cells [41]. Digestive enzymes such as dispase, collagenase and trypsin are very effective in liberating satellite cells from cleaned, minced muscle, as they break down the basal lamina and sarcolemma. Care has to be taken to optimize temperature and duration of exposure to these enzymes, as they could be destructive to satellite cells themselves, damaging the membrane and its associated proteins.

In order to create the optimal *in vitro* environment for primary culture of satellite cells, culture plates are often coated with extracellular matrix proteins. Matrigel®, denatured collagen (PSG) and fibronectin (FN) are commonly used as substrata. Matrigel is a solubilized basement membrane preparation produced from mouse sarcomas and fibronectin is a component of the basement membrane [40]. Rando and Blau (1994) investigated the growth of myoblasts on various substrata and found that collagen and laminin both enhanced the growth of isolated myoblasts [231]. A substratum called E-C-L (entactin-collagen IV-laminin – extracellular matrix proteins), derived from the Engelbreth-Holm-Swarm (EHS) mouse tumor, has been shown to be efficient for myoblasts harvesting [139, 91].

For proper growth of primary cultures, the addition of exogenous medium is essential to provide nutrients, vitamins, minerals, amino acids and inorganic salts. This medium also provides an environment in which waste products from cell metabolism can be released; these waste products are removed upon media change. In addition to medium, serum is included to provide growth factors and other metabolic agents. Fetal bovine serum is most commonly used at 10-20% to promote proliferation, but other sera, such as horse serum, are also beneficial to cell growth. Since serum doesn't have a defined constitution and could contain variable amounts of mitogenic agents, an alternative is to use chemically defined media instead of serum [40].

Rando and Blau compared the growth of isolated myoblasts in several different media. They found that fibroblasts double more rapidly than myoblasts in Dulbecco's Modified Eagle's Medium (DMEM) to become the dominant cell type in the culture.

This was also the case when cells were isolated in Weymouth's media and M199 media. In Ham's F10 media though, myoblasts seemed to have selective growth advantage over other cell types [231]. As a result, many subsequent myoblast isolations were performed with Ham's F10, rather than with DMEM, media. Machida *et al.* used Ham's F10 media for the expansion of primary rat myoblasts [174] and Blanco-Bose *et al.* used the same media for primary mouse myoblasts isolation [25].

Most commercially available culture media contain a number of amino acids as a supplement for muscle protein biosynthesis. One of the most versatile amino acids is glutamine. It is viewed as a conditionally essential amino acid that cannot be substituted in culture media by any other amino acid. *In vitro*, glutamine was shown to stimulate proliferation of many cell types. In skeletal muscle, it is the most abundant free amino acid [150]. Upon muscle injury, there is an efflux of glutamine and a subsequent fall in the intramuscular concentration [176]. This results in an increase of the extracellular glutamine concentration which could play an important role in proliferation of satellite cells *in vivo*.

To establish a successful culture, satellite cells must be separated not only from their tissue, but also from other cell types present in their *in vivo* environment, such as fibroblasts. Differential centrifugation, pre-plating on coated or non-coated dishes with the subsequent differential lifting of lightly attached cells from the culture plates, Percoll gradients and fluorescent activated cell sorting are some of the more common separation techniques used [40]. Many reported protocols use more than one of these methods [52, 53].

In summary, primary isolated myoblasts mimic *in vivo* satellite cells more closely than cell lines such as the C2C12 myoblasts. Many myoblast isolation protocols exist in the literature, and each use varying techniques to successfully isolate and culture primary myoblasts. No gold standard method has yet been developed for all applications. The method preferred in any particular laboratory will most likely be different and will depend on the nature of research to be carried out.

3.2. HYPOTHESES AND AIMS

This has led to our hypothesis that:

1. From the various protocols available, a combination of techniques would be more effective than following a single protocol;
2. A combination of different media would be more beneficial to the success of the primary myoblast culture.

The aims for this section of the thesis are therefore:

1. To optimise the harvesting, isolation and culturing of primary myoblasts from rats for *in vivo* studies;
2. To compare the properties of primary cells to the C2C12 immortal cell line;
3. To investigate the effect of L-glutamine on primary cell proliferation;
4. To compare the effect of two media mentioned in literature on growth and differentiation of primary cells.

3.3. METHOD DEVELOPMENT

3.3.1. Animals

Male Wistar rats of varying age were obtained from the Stellenbosch University Animal Facility where they were bred after ethical approval was granted by the Stellenbosch University Research Ethics Committee: Animals Care and Use (ref: 10NP_MYB01). Animals were weaned at the age of three weeks, whereafter they were divided and housed in groups of four per cage. The cages were supplied with Concorps bedding and rats had access to standard rodent chow and tap water *ad libitum*. The facility runs a 12 hour light/dark cycle with lights on from 6:30 am, ambient temperature is controlled at 21°C and rooms are ventilated with 10 air changes per hour. Rats were handled and weighed daily to ensure they were accustomed to the researcher by the time of sacrifice.

3.3.2. Harvesting protocol

For initial isolations of muscle progenitor cells from rat muscle, a modified protocol from Allen *et al*. [4] was used (personal communication: Prof DuPont-Versteegden, Center for Muscle Biology, University of Kentucky, Lexington, USA).

After rats had been sacrificed, the skin was removed from the right hind limb. All subsequent steps were performed in a biological laminar flow hood (Labgard Class II Biological Safety cabinet, Plymouth, MN, USA). The right *gastrocnemius* muscle was treated with ethanol, removed from the bone and washed twice in warm sterile PBS. Connective tissue, tendons, fascia and as much adipose tissue as possible were trimmed away, whereafter the muscle tissue was mince in a sterile culture dish using a surgical blade (Minora, Johannesburg, South Africa). In a 15 ml conical tube 1 ml of 1.25 mg/ml protease type XIV from *streptomycus griseus* (Sigma Aldrich, Tokyo, Japan, P5147) in PBS was added to the muscle sample. The sample was incubated in a waterbath at 37°C for an hour, shaking every 15 minutes. Then, the sample was centrifuged for 4 minutes at 1500 *g*. The supernatant was discarded and 1.3 ml of warm PBS was added and mixed with the sample for 20 seconds on a vortex. The sample was centrifuged three more times at 500 *g*, for 10 minutes, 8 minutes and 5 minutes respectively. The supernatant was retained each time in a sterile 15 ml tube and the pellet resuspended in 1.5 ml warm PBS. The pooled supernatants were centrifuged at 1500 *g* for 3 minutes to pellet the cells. The pellet was suspended in 1 ml medium (DMEM (Sigma Aldrich, Schnelldorf, Germany, D5671) with 20% Fetal calf serum (Gibco from Life Technologies, Grand Island, NY, USA, 10270106), 1% penicillin/streptomycin/fungizone (Sigma Aldrich, Schnelldorf, Germany, 15240062) and 0.1% gentamicin (Gibco from Life Technologies, Grand Island, NY, USA, 15750037), poured through a sterilized metal cell strainer (tea sieve) which was then rinsed twice with 1 ml media. To obtain a more uniform suspension of single cells from the tissue and any cell clumps, the sample was poured through a cell dissociation sieve (BD Biosciences, Bedford, MA, USA, 100 µm, yellow, 352360) and rinsed twice with 1 ml media. Centrifugation at 1 500 *g* for 3 minutes was repeated and the supernatant discarded. The pellet was re-suspended in 1.5 ml medium, before being pre-plated in a 35 mm uncoated tissue-culture dish and incubated at 37°C. After 2 hours the medium was gently agitated and the cell suspension decanted onto an E-C-L-coated 35 mm dish. Basic human FGF (Promega, Madison,

WI, USA, G5071) was added to a final concentration of 10 ng/ml, before the cell cultures were incubated in a humidified atmosphere of 37°C in 5% CO_2. Medium was replaced daily.

Several modifications were made during subsequent procedures. These included 1 hour additional enzymatic digestion with collagenase I from *Clostridium histolyticum* (Sigma Aldrich, Schnelldorf, Germany, C7657), changing the centrifuge speed to 950 *g* instead of 1 500 *g* and introducing the pre-plate technique. In addition, the DMEM and L-glutamine medium was substituted with Ham's F10 nutrient mix (Gibco from Life technologies, Grand Island, NY, USA, 31550023). See table 3.1 for a comparison between the protocol used initially and the protocol used following modifications.

Table 3.1. Comparison between the initial protocol and after all modifications

Initial protocol	**Modified protocol**
Euthanasia	Euthanasia
Severe leg and submerse in ethanol	***Submerse the whole rat in ethanol***
Gastrocnemius muscle removed and placed in PBS	*Gastrocnemius* muscle removed and placed in PBS
Muscle tissue mince trimmed with a Minora blade	Muscle tissue mince trimmed with a Minora blade
1 h digestion with 1.25 mg/ml protease type XIV from *streptomycus griseus* in a shaking incubator at 37°C	1 h digestion with 1.25 mg/ml protease type XIV from *streptomycus griseus* in a shaking incubator at 37°C
	1 h digestion with 0.2% collagenase I from Clostridium histolyticum in a shaking incubator at 37°C
Centrifugation for 4 minutes at 1500 *g*	Centrifugation of supernatant for ***3 minutes at 950 g***
Supernatant discarded, 1.5 ml PBS added to tissue and vortexed for 20 seconds	Supernatant discarded, 1.5 ml PBS added to tissue and vortexed for 20 seconds
Centrifugation for 10 minutes at 500 *g*	Centrifugation for 10 minutes at 500 *g*

Supernatant transferred to new tube, 1.5 ml PBS added to tissue and vortexed for 20 seconds	Supernatant transferred to new tube, 1.5 ml PBS added to tissue and vortexed for 20 seconds
Centrifugation for 8 minutes at 500 *g*	Centrifugation for 8 minutes at 500 *g*
Supernatant transferred to new tube, 1.5 ml PBS added to tissue and vortexed for 20 seconds	Supernatant transferred to new tube, 1.5 ml PBS added to tissue and vortexed for 20 seconds
Centrifugation for 5 minutes at 500 *g*	Centrifugation for 5 minutes at 500 *g*
Supernatant transferred to new tube, muscle tissue discarded	Supernatant transferred to new tube, muscle tissue discarded
Centrifugation of supernatant for 4 minutes at 1500 *g*	Centrifugation of supernatant for ***3 minutes at 950 g***
Re-suspend pellet in 1 ml DMEM growth media	Re-suspend pellet in 1 ml DMEM growth media
Pour through a metal cell strainer and wash twice with 1 ml DMEM	Pour through a metal cell strainer and wash twice with 1 ml DMEM
Pour through 100µm cell strainer and wash twice with 1 ml DMEM	Pour through 100µm cell strainer and wash twice with 1 ml DMEM
Centrifugation for 3 minutes at 1500 *g*, discard supernatant and re-suspend in 2 ml DMEM media	Centrifugation for ***3 minutes at 950 g***, discard supernatant and re-suspend in 2 ml DMEM media
Pre-plate into an uncoated 35 mm culture dish and incubate for 2 hours	Pre-plate into an uncoated 35 mm culture dish and incubate for 2 hours
Transfer to an E-C-L coated 35 mm culture dish, add bFGF and incubate overnight	***Centrifuge suspension at 950 g for 3 minutes, discard the supernatant and re-suspend in 2 ml Ham's F10 media with bFGF***
	Transfer suspension to the first well of an E-C-L coated 6 well plate and incubate overnight
Discard media, wash with 1 ml PBS and replace with fresh media	***Centrifuge the suspension at 950 g for 3 minutes, re-suspend pellet in 2 ml Ham's F10 and transfer to the next***

	coated well in the 6 well plate.
	Repeat the previous step the following day
	After three such pre-plates, discard the media and replace with fresh media

Abbreviations: bFGF – basic human fibroblast growth factor; DMEM – Dulbecco's modified Eagle's medium; E-C-L – entactin-collagen-laminin; h – hour; PBS – phosphate buffered saline.

For the pre-plate technique, six well-plates were coated with 20 µg/ml E-C-L (Microsep, Temecula, CA, USA, 08-110) and incubated for 1 hour at 37°C. After the 2 hour incubation step of the isolated suspension in a non-coated 35 mm culture dish, the supernatant was centrifuged, the pellet suspended in media and transferred to the first well of the coated plate (now called pre-plate 1 or PP1). After 24 hours, the supernatant was centrifuged again, suspended in media and transferred to the next well of the plate (PP2). Media was replaced in the previous well to sustain cell growth of cells already adhered. These steps were repeated every 24 hours until PP4. Figure 3.1 displays a schematic diagram of the pre-plate technique until PP3.

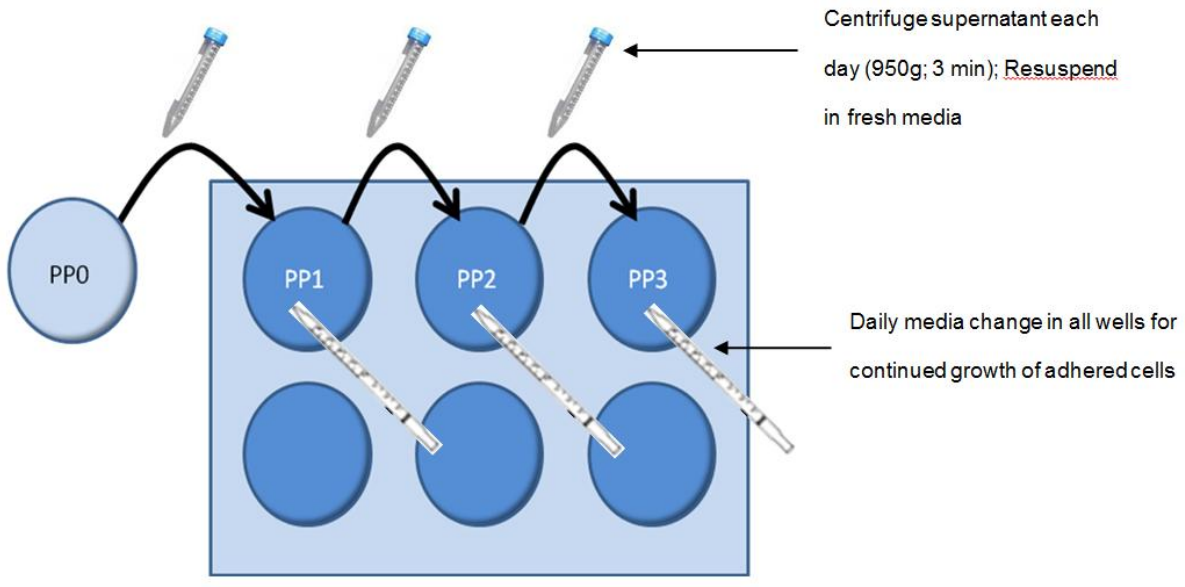


Figure 3.1. A schematic diagram of the pre-plate technique. *The cell suspension is centrifuged after a 2 hour non-coated step, re-suspended and transferred to the first well of an E-C-L-coated 6-well plate (PP1). Every 24 hours the supernatant is centrifuged, suspended in fresh media and transferred to the next well of the plate (PP2 etc.). Media was replaced in all previous wells daily*.

3.3.3. Cell culture and expansion of primary culture

After PP3 or PP4 the supernatant was discarded and the adherent cells were trypsinised and passaged in a T_{25} culture flask for further proliferation. Media was changed every 2 days and when cells reached 70% confluence, they were passaged to the next culture flask. For cryopreservation, cells were suspended in 90% fetal calf serum (FCS) and aliquoted to cryovials with an average of $1x10^6$ cells/cryovial. DMSO (Sigma Aldrich, Schnelldorf, Germany, D2650) was added to a final concentration of 10%. The cryovials were kept at -20^0C for 1 hour, stored overnight in a -80°C freezer, and then transferred to liquid nitrogen for long term storage.

3.3.4. Comparisons

3.3.4. i) Comparison between primary isolated myoblasts and C2C12 cells during proliferation and differentiation

C2C12 cells were compared to primary isolated myoblasts with regards to their proliferation and differentiation capacity. All cells were grown in DMEM proliferation media, supplemented with 20% FCS and 10 ng/ml bFGF, or in DMEM differentiation media supplemented with 2% horse serum. L-glutamine and antibiotics were added to both as explained before. To investigate the effect of L-glutamine another experimental group isolated from *gastrocnemius* muscle grown in DMEM media without L-glutamine, was also included.

3.3.4. ii) Comparison of primary isolated myoblast growth in different media and their differentiation thereafter

The growth and purification of the primary myoblast culture was compared using different media. (See appendix for details). One *gastrocnemius* muscle was used for a culture in DMEM media (A), one *gastrocnemius* muscle for a culture grown in Ham's F10 media (B) and one *gastrocnemius* muscle for a culture grown in a combination of DMEM and Ham's F10 media (1:1) (C). DMEM was supplemented with 4% L-glutamine (Sigma Aldrich, Schnelldorf, Germany, G7513) in all the above groups. All media were supplemented with 20% FCS, antibiotics and bFGF.

3.3.4. iii) Light microscopy

To compare the proliferation ability of cells in the experiments above, cells were transferred to six-well plates (30 000 cells/well; in duplicate) with the appropriate

media. Light microscopy images were taken after the first 4 hours; at 12 hours and then at 12 hour intervals until 96 hours. Media was changed after 48 hours.

For a comparison of differentiation in the experiments above, cells were transferred to six-well plates (200 000 cells/well; in triplicate) with the appropriate media. Cells were allowed to adhere for four hours after which media were changed to differentiation media. Differentiation media for all groups were made up of DMEM, 2% horse serum (Gibco from Life Technologies, Auckland, New Zealand, 16050122), 4% L-glutamine and 1% Penicillin-Streptomycin (Sigma Aldrich, Schnelldorf, Germany, P4333). Media was changed every two days. Light microscopy (Olympus CKX 31, Olympus Corporation, Tokyo, Japan) images were taken (Moticam 2500 camera and Moticam Image Plus 2.0 software, Motic®, Hong Kong, China) at the same time points as for proliferation.

3.3.4. iv) Immunocytochemistry

For immunocytochemistry analysis of cells during proliferation, cells were grown in a 24 well plate on cover slips. An equal number of 20 000 cells were seeded in each well and after 24 hours cells were prepared for staining.

For analysis during differentiation, 50 000 cells/well were seeded in a 24 well plate. Cells were allowed to adhere for four hours after which media was changed to differentiation media. After 7 days cells were prepared for staining.

All cells were fixed with 4% paraformaldehyde (Sigma Aldrich, Schnelldorf, Germany, 158127), followed by permeabilisation with 0.1% Triton x-100 (BDH Laboratory Supplies, Poole, UK, BB306324N), and blocking with 1% bovine serum albumin (BSA) (Roche Applied Science, Mannheim, Germany, 10735094001). Primary antibodies were added and incubated for 2 hours at room temperature. This was followed by incubation in secondary fluorochrome conjugated antibodies for 30 minutes. Nuclear staining was performed with 10 minutes in Hoechst dye (Sigma-Aldrich, Schnelldorf, Germany, 14533, 1:4000). See Table 3.2 for antibody specifications.

Images were obtained with a wide field fluorescent microscope (Olympus IX81, Olympus Corporation, Tokyo, Japan) using a 10x objective and software (Cell^R, Olympus Biosystems GMBH, Hamburg, Germany).

Table 3.2. Antibodies for immunocytochemistry.

Primary antibody	Dilution	Secondary antibody	Dilution
Rabbit anti-Desmin IgG (ab15200[#])	1:100	Goat anti-Rabbit Alexafluor 594 IgG (11012[∞])	1:250
Mouse anti-Pax7 IgG (ab55494[#])	1:200	Goat anti-mouse Alexafluor 488 IgG (a21202[∞])	1:250
Phalloidin-TRITC (P1951*)	1:10 [&]	n.a.	

[#] Abcam, Cambridge, UK
[∞] Molecular Probes, Eugene, OR, USA
[*] Sigma Aldrich, Schnelldorf, Germany
[&] Stock concentration was 50 µg/ml

3.3.4. v) Statistical analysis

For statistical analysis of cell number from phase contrast images, six images from 24 hour time intervals were quantified for each of the media. Data was analysed using factorial analysis of variance (ANOVA) with Bonferroni *post hoc* test. Statistical significance was accepted when $p < 0.05$.

3.4. RESULTS

3.4.1. Satellite cell harvesting, isolation and culturing

The cell yield of the first isolation attempt was very low and some contamination was evident already one day after isolation, which increased until four days after isolation (Figure 3.2.a,b). With protocol modifications (Table 3.1), contamination was eliminated in subsequent isolations and the cell yield increased.

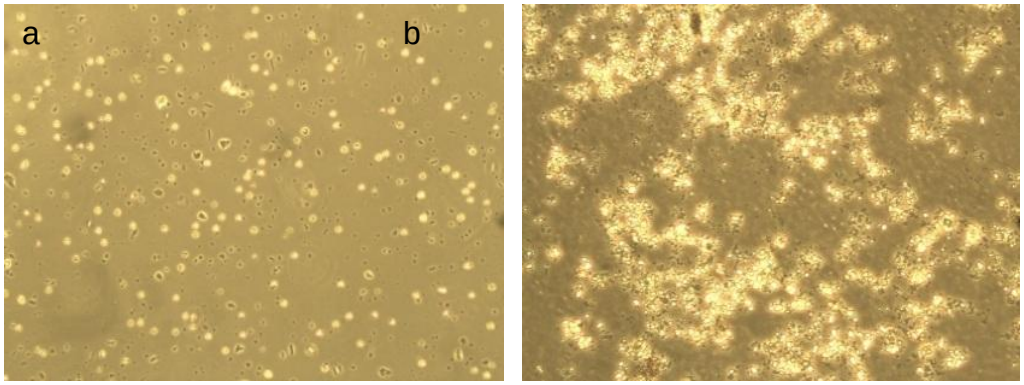


Figure 3.2. Initial isolation. *a) No myogenic like cells, but signs of contamination 1 day after isolation. b) Increased contamination 4 days after isolation with no visible muscle-derived cells*

Cells were stained with desmin to verify the myoblast phenotype of the isolated cells and for qualitative assessment of cell density. As the first cultures were terminated after the first few days due to contamination, they had to be stained very soon after isolation. During later stages of the protocol development, the cultures were established and staining could be done at later time points of culture.

Before the pre-plate technique was introduced, cell yield was low (Figure 3.3.a). After implementing the pre-plate technique, the cell yield increased and all cells in PP1, stained after 10 days in culture expressed desmin (Figure 3.3.b). Cells adhering in pre-plate 4 were also desmin^{+} (Figure 3.3.c).

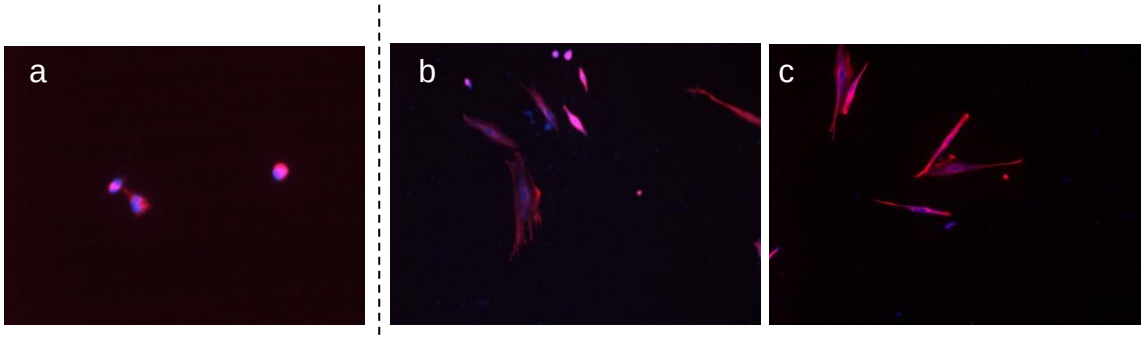


Figure 3.3. Desmin-stained cultures for verification of myoblast phenotype and qualitative assessment of cell density. *(a) Two days in culture with no pre-plating. (b) Cells in pre-plate 1 (PP1), 10 days after isolation. (c) Cells in pre-plate 4 (PP4), 10 days after isolation.*

However, it was not possible to obtain a large enough yield for trypsinisation and further passaging. Following increased enzymatic digestion (Table 3.1), an even

larger cell yield was obtained already in PP1, five days after isolation (Figure 3.4.a). The higher cell yield was sufficient for passaging and further experimentation; all cells stained in passage 2 were desmin^{+} (Figure 3.4.b).

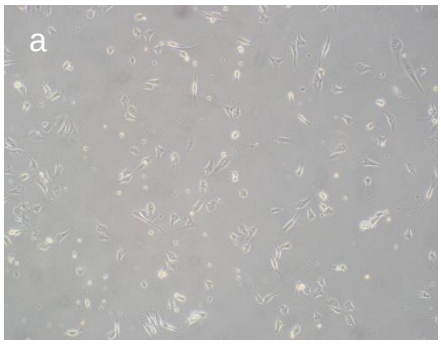


Figure 3.4. ***Effect of increased enzymatic digestion on myoblast yield.*** *a) Cells in PP1 viewed with phase contrast microscopy, 5 days after isolation. b) Cells passaged twice after pre-plating (P2) and stained with desmin (red) and Hoechst (blue).*

The addition of L-glutamine to the media made a significant difference to the yield and subsequent proliferation of isolated primary myoblasts. In PP1 it was clear after 5 days that the addition of L-glutamine was more beneficial to the yield obtained during isolation (Figure 3.5.a,b).

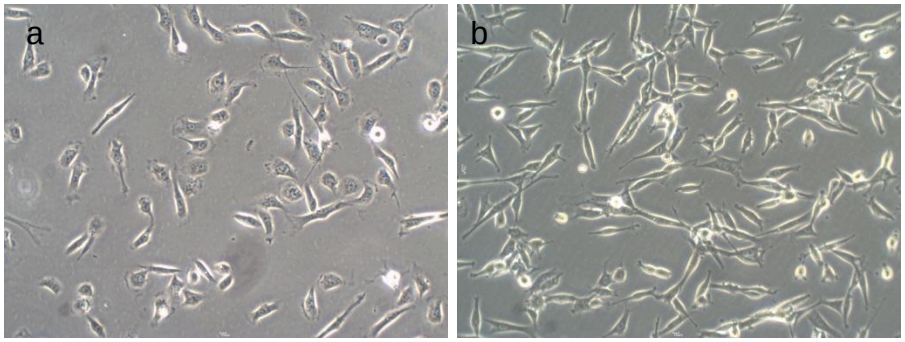


Figure 3.5. ***Effect of L-glutamine on primary rat myoblasts.*** *Cells from PP1, 5 days after isolation in a) DMEM media without L-glutamine and b) in DMEM media with L-glutamine (8 mM).*

When established cultures of primary isolated cells were compared over time, cells in DMEM supplemented with L-glutamine proliferated faster compared to cells in DMEM without L-glutamine (Figure 3.6). When L-glutamine was present, the cell count already increased significantly by 48 hours, but without L-glutamine, a significant increase in cell count was only evident after 72 hours in culture. There

were also significant differences between the two groups at the 72 and 92 hour time points. Significant differences between time points (e.g. 24 and 72 h) were evident within in each group over time, but only the most relevant changes are shown in order to simplify the graph.

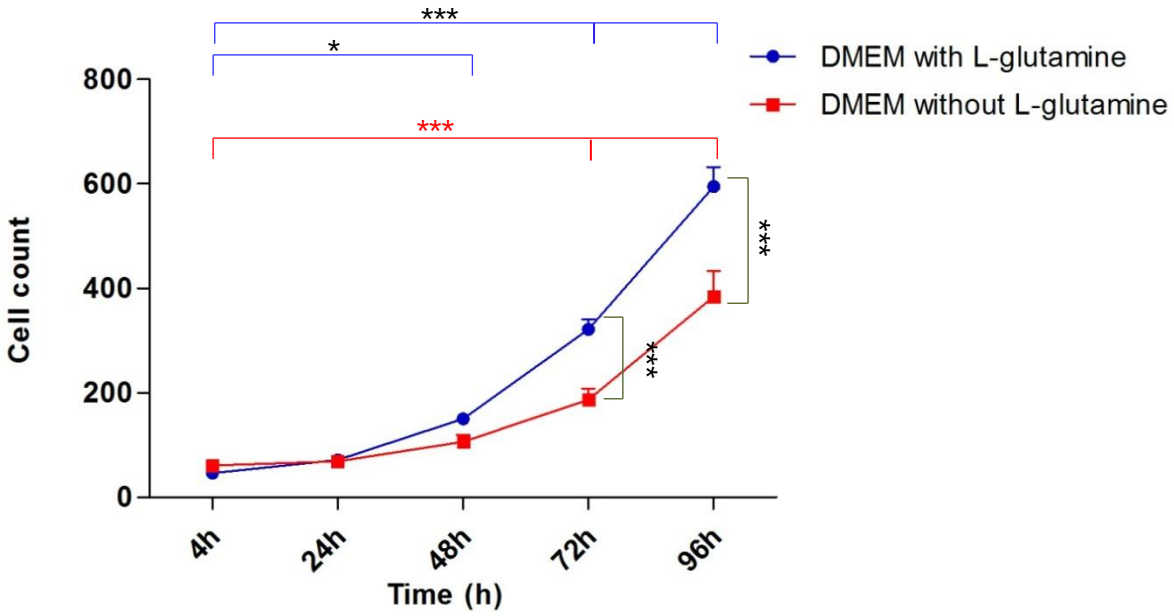


Figure 3.6. Effect of L-glutamine supplementation on the proliferation of primary myoblasts. *Phase contrast images which were taken at 24 hour intervals after seeding, were used for quantification. Data are presented as mean ± SEM. Significance were considered when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$).*

Primary myoblasts grew to 80% confluence within 96 hours after seeding L-glutamine supplemented media, while cells in DMEM without L-glutamine only reached approximately 50% confluency (Figure 3.7).

However, cells grown in DMEM without L-glutamine had a higher percentage of desmin$^+$ cells compared to cells grown in DMEM with L-glutamine (Figure 3.8). Cells stained with desmin and phalloidin after 7 days in differentiation medium did not differ with respect to desmin or actin staining (Figure 3.9).

Figure 3.7. C2C12 cells vs. primary isolated myoblasts cultured with and without L-glutamine in DMEM. *Images were taken at 12 hour time-points after initially seeding equal numbers of cells.*

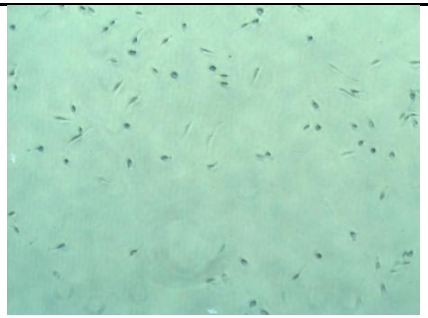
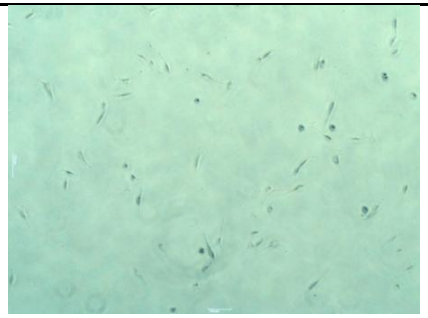
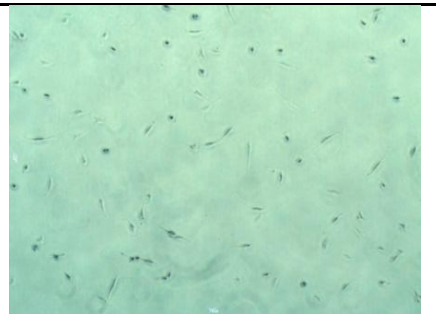
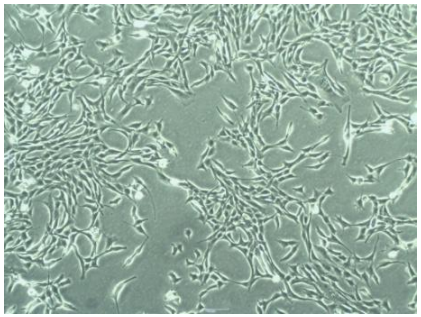
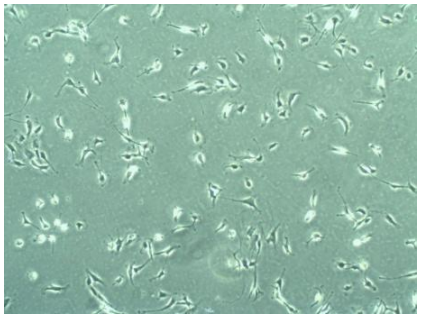
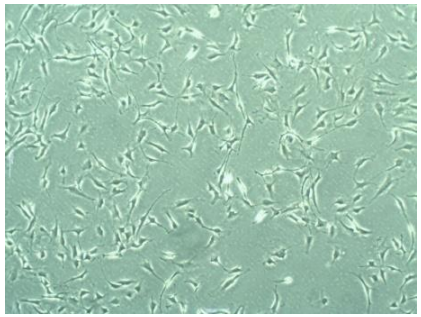
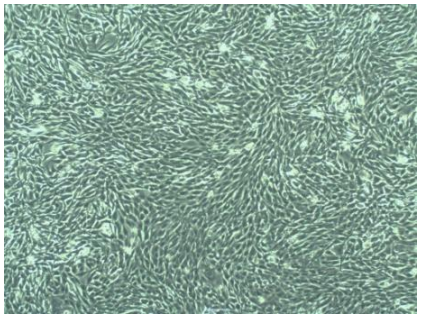
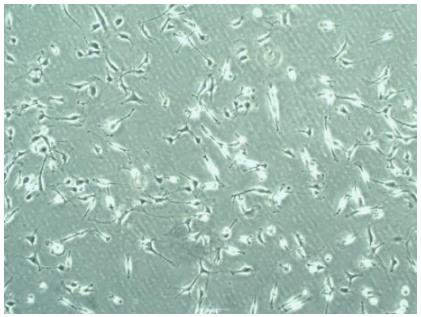
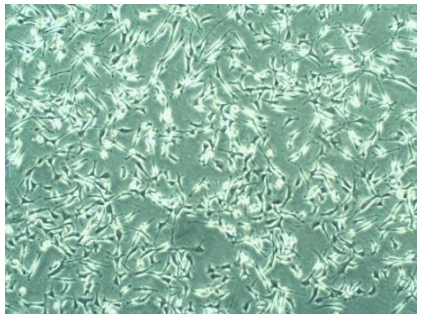
	C2C12 cells (with L-glut)	Primary cells (no L-glut)	Primary cells (with L-glut)
12 h			
72 h			
96 h			

Figure 3.8. C2C12 and primary cells grown in DMEM with and without L-glutamine. *Immunostaining with desmin antibody and Hoechst.*

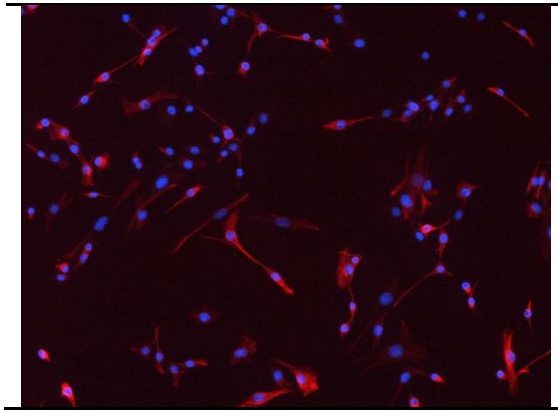
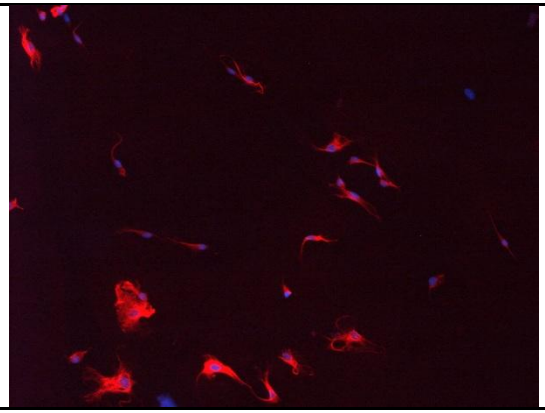
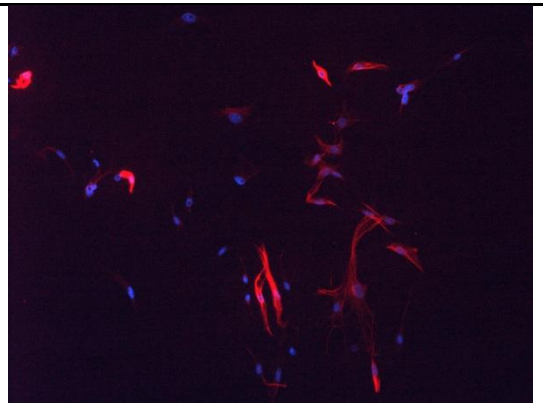
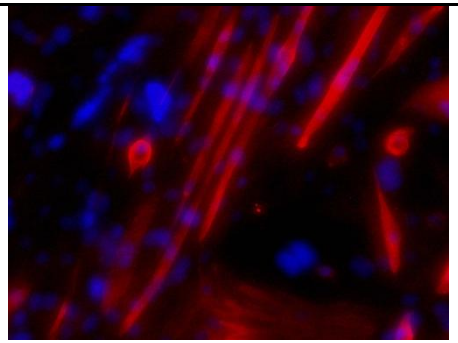
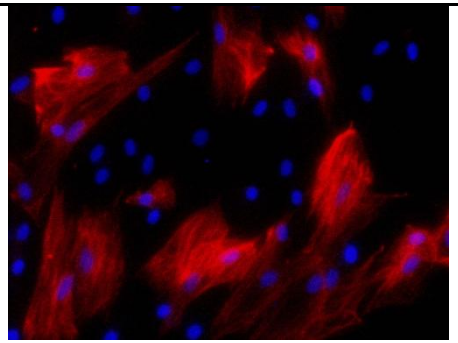
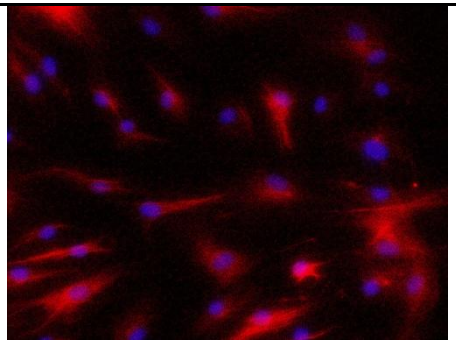
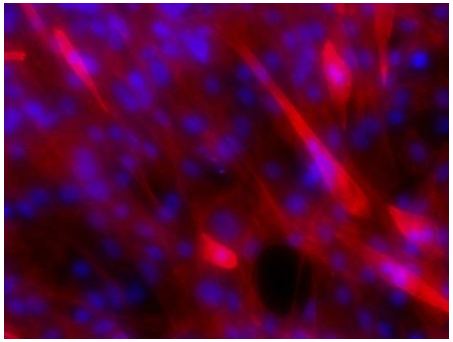
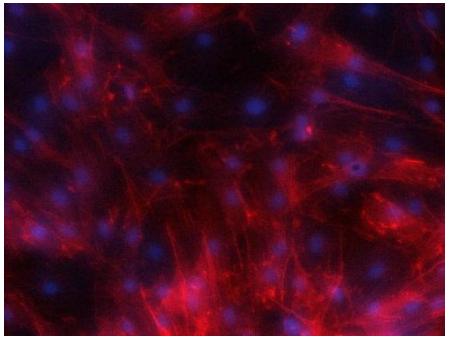
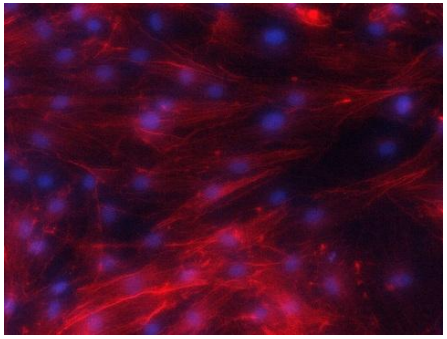
C2C12 cells (with L-glutamine)	Primary cells (no L-glutamine)	Primary cells (with L-glutamine)
		

Figure 3.9. Differentiating C2C12 cells and primary myoblasts. *DMEM proliferation media with and without L-glutamine were substituted after 4 hours with DMEM differentiation media (in all cases including L-glutamine). Immunostaining with desmin antibody and phalloidin, 7 days after differentiation media was introduced.*

	C2C12 (with L-glutamine)	**Primary cells (no L-glutamine)**	**Primary cells (with L-glutamine)**
Desmin			
Phalloidin			

3.4.2. Comparison of primary myoblast culture and C2C12 culture

C2C12 cells proliferated at a much higher rate than primary myoblasts. When cells were seeded in equal quantities in 6 well plates and allowed to proliferate, C2C12 cells were 100% confluent after 96 hours, while primary cells in identical media only reached 80% confluence (Figure 3.7).

As expected all C2C12 cells were desmin$^+$, while the primary cells presented with varying intensity of desmin expression. The shape of C2C12 cells stained desmin$^+$ was more uniform when compared to the shape of the primary myoblasts (Figure 3.8), despite both cell types being cultured in exactly the same proliferation conditions.

After 7 days in differentiation media, C2C12 cell cultures had developed very distinct myotubes, while primary cultures were only vaguely aligning (confirmed by both desmin and phalloidin staining) (Figure 3.9).

3.4.3. Comparison of primary myoblast cultures grown in different media

After plating primary myoblasts into 6-well plates with either DMEM, Ham's F10 or a combination of the two media, phase contrast images were taken at 12 hour intervals. Cell numbers at 24 hour intervals were quantified and are presented in Figure 3.10.

Myoblasts in all three culture media, increased significantly between 4 and 72 hours, while only myoblasts cultured in DMEM and the combined media showed a further significant increase between 72 and 96 hours in culture. The cell count for myoblasts cultured in Ham's F10 plateauted after 72 hours. More significant changes were found within in each group between other time points, but only the most relevant changes are shown for emphasis. Intergroup comparisons indicated that myoblasts cultured in DMEM only, had significantly increased numbers compared to myoblasts cultured in Ham's F10 after 96 hours in culture. However, the cell count of myoblasts cultured in the combination of DMEM and Ham's F10 was not significantly different from either of the two media separately.

Images acquired at the 12 hour, 72 hour and 96 hour time points are presented in Figure 3.11. Primary cells grown in DMEM, including L-glutamine, proliferated fast

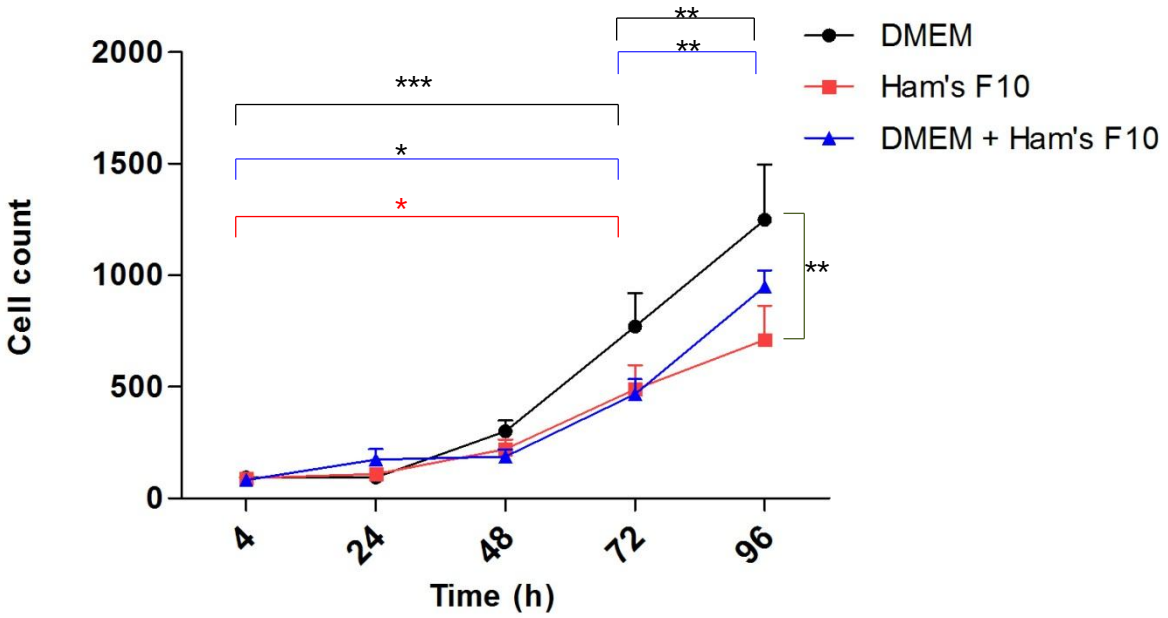


Figure 3.10. Effect of various media on the proliferation rate of primary myoblasts. *Phase contrast images which were taken at 24 hour intervals after seeding, were used for quantification. Data are presented as mean ± SEM. Significance were considered when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$).*

and were almost confluent after 96 hours. Myoblasts grown in Ham's F10 proliferated much slower and were less than 50% confluent after 96 hours, while myoblasts grown in a combination of DMEM and Ham's F10 were 70% confluent at this time point.

For immuno-histochemical analysis, cells were grown on coverslips in a 24 well plate in the specific media and stained using desmin and Pax7 antibodies and secondary antibodies as described earlier. The signal intensity for both desmin and Pax7 was higher in cells grown in Ham's F10 compared to cells grown in DMEM + Ham's F10 and even higher compared to primary cells grown in DMEM alone (Figure 3.12). This suggests that the media used had a greater effect on the expression of specific myogenic proteins than their effect on the cell number.

Media also affected the ability of myoblasts to differentiate. Myoblasts grown in DMEM or Ham's F10 media respectively did not differentiate after 7 days of culture in differentiation media. However, in the cultures grown in a combination of DMEM and Ham's F10 media, some cells aligned and formed very distinct myotubes (Figure 3.13).

Figure 3.11. Images to show the effect of various media on proliferation rate of primary myoblasts. *Images were taken at 12 hour intervals of primary myoblasts cultured under proliferation conditions.*

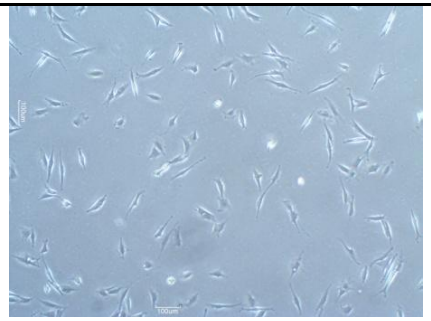
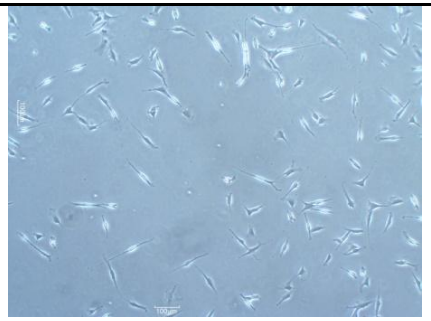
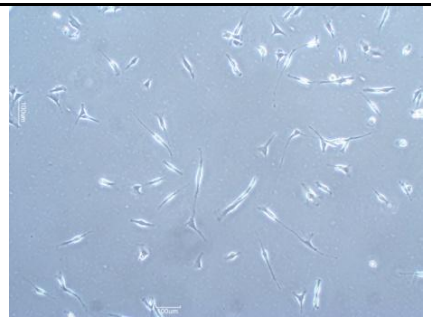
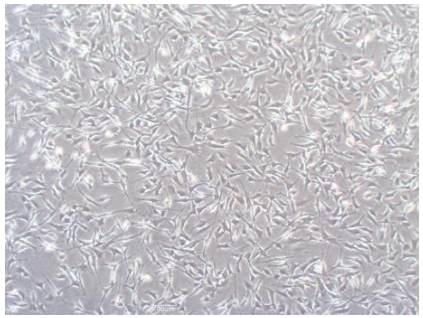
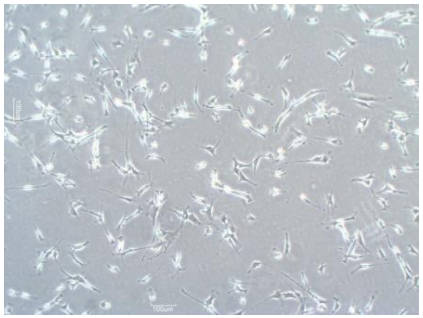
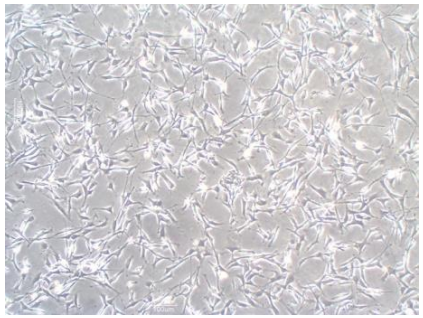
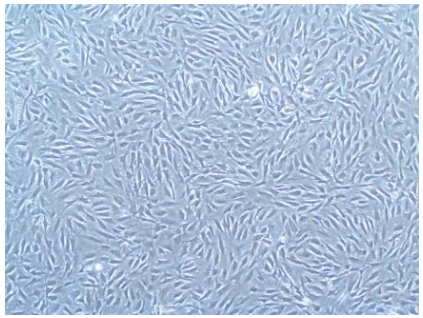
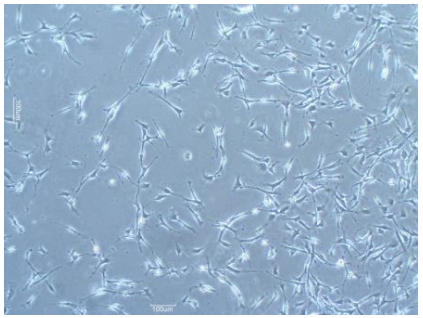
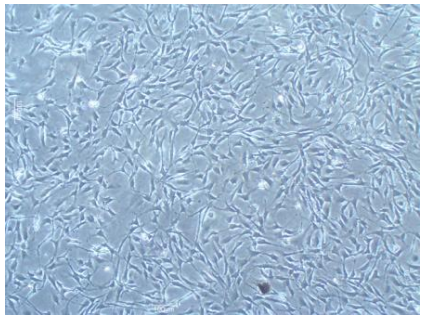
	DMEM	Ham's F10	DMEM+Ham's F10
12 h			
72 h			
96 h			

Figure 3.12. Primary myoblasts in different proliferation media. *Immunostaining with desmin and Pax7 antibodies, 24 hours after initially seeding equal amounts.*

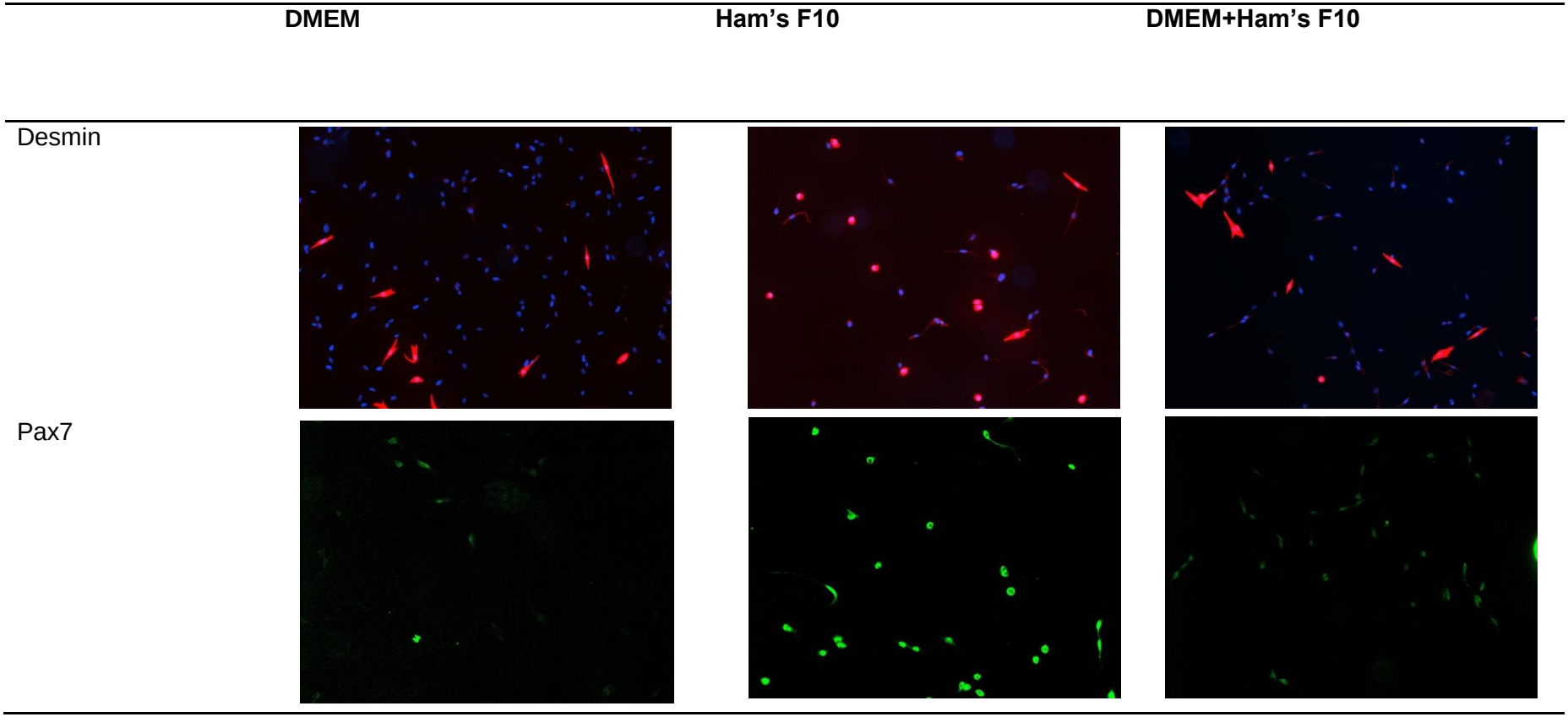
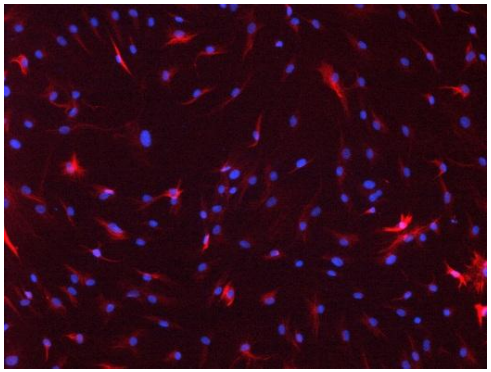
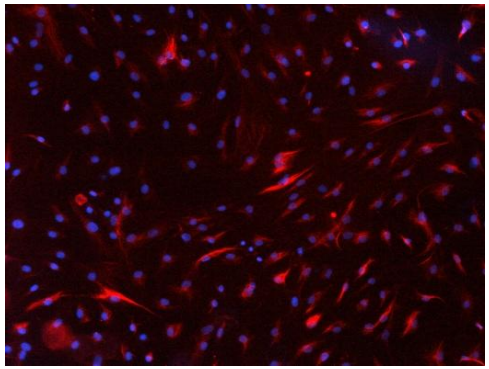
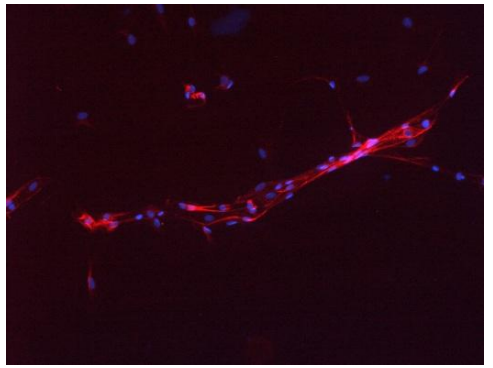
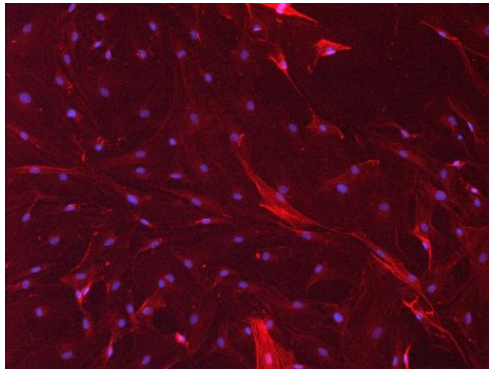
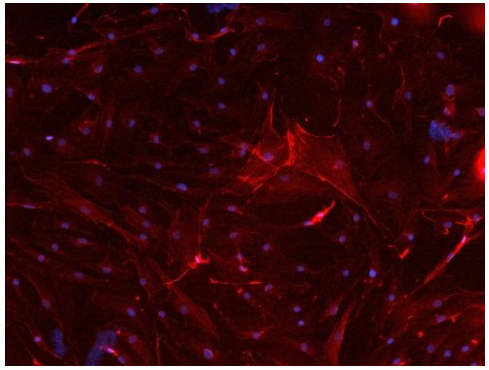
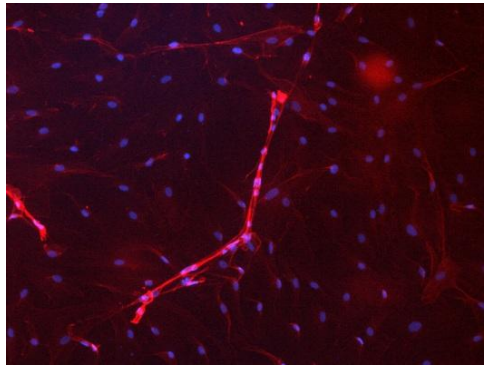


Figure 3.13. Differentiating primary myoblasts, established initially in various media. *Immunostaining with desmin antibody and phalloidin staining, 7 days after differentiation media was introduced.*

	DMEM	**Ham's F10**	**DMEM+Ham's F10**
Desmin			
Phalloidin			

3.5. DISCUSSION

Satellite cells were isolated from rat skeletal muscle and grown in culture for the first time by Bischoff *et al.* in 1974 [22]. Numerous studies have followed, modifying the original protocol, or defining new protocols, for the isolation of rat myoblasts. Various challenges need to be overcome in order to obtain a sufficiently high yield of cells, and to prevent contamination in the culture.

A very successful way to obtain satellite cells is to isolate single fibres and allow the satellite cells to migrate from the fibre onto the culture plate where they would proliferate and fuse to form new fibres under the appropriate culture conditions. This technique has several advantages. Firstly, the result would not only be a pure myoblast culture, but all satellite cells would have originated from the same fibre type, so the satellite cells can be investigated in relation to the fibre type and characteristics of the original fibre [239]. Satellite cells isolated in this way are subjected to the least *ex vivo* manipulation, and should mimic the *in vivo* environment most closely. However, the isolation and subsequent incubation of a single fibre to obtain satellite cells has not yet replaced other isolation methods. This is in part due to the difficulty in isolating single intact muscle fibres from small animal muscle, as muscle fibres are often damaged during this process.

The method refined during this study, would later (Chapter 4) be used to isolate myoblasts from contusion crush injured rat muscle to investigate the characteristics of the myoblasts at different stages of the *in vivo* process of repair. Therefore direct isolation of the myoblasts from the entire injured area was necessary. The benefit of the method is that the progenitors obtained using the extraction method would include more than just the quiescent cells from the satellite niche, but also proliferating myoblasts in the interstitium between injured muscle fibres. Thus, for a model of muscle injury, a myoblast isolation technique would be more appropriate than a fibre explant technique. A variety of different approaches have been reported in literature, but none was found to isolate myoblasts effectively after a crush injury, so it was necessary to optimise a protocol for this purpose.

3.5.1. Optimisation and standardisation

Although published protocols indicated it would be relatively simple to obtain sufficient yield and maintain satellite cells isolated from rats, we found it necessary to modify existing protocols in order to obtain sufficient, contamination-free, myoblasts. Furthermore, to obtain sufficient numbers of satellite cells from rat skeletal muscle, many animals need to be sacrificed and cells pooled, sometimes from a range of different muscles [3, 40]. We needed to isolate satellite cells from a single injured muscle.

A replicable method should therefore eliminate microbial contamination, reduce the presence of non-muscle cells like fibroblasts and result in a good yield of myogenic precursor cells from a single injured muscle.

3.5.1. i) Microbial contamination

To prevent microbial contamination, small laboratory animal surgical dissection kits were autoclaved and all equipment sprayed with 70% ethanol prior to both sacrifice and tissue dissection. A separate set of utensils was used for dissection. The hind leg was cut from the rat after euthanasia, submersed in ethanol and placed in a level 1 laminar flow hood for dissection. However, contamination was frequently evident throughout the initial stages, suggesting that the source of contamination was from an early stage of the protocol (Figure 3.2).

Therefore, to eliminate the contamination seen in cell cultures, several improvements were made during the isolation process. It was found that contamination could be limited if the whole rat was submersed in 70% ethanol before placing it in the laminar flow hood. Other modifications included shaving the rat hindlimb before submersion in the ethanol to limit the amount of hair, which is a possible source of contamination. A routine that further reduced contamination risk was established: The researcher wore a face mask, head cover, surgical gown and surgical gloves at all times. After each use, each piece of dissection equipment was rinsed in ethanol inside the hood before being placed on autoclaved absorbent paper to ensure it remained sterile, even if it was to be used again during the same dissection. Separate sets of gloves were used for injection and sterile dissection and gloves were exchanged between isolations from different rats.

During the culturing stages, modifications were also introduced to prevent further contamination. The 35 mm culture dish was substituted with a 6 well plate as the latter is deeper and better covered to prevent the swilling of media on the plate which was seen during handling of the 35 mm culture dish. Lastly, during the stages of pre-plating, the cell suspension was centrifuged every day, which should separate the heavier cells from the lighter microbial organisms which might have been present in small numbers. Finally, if there were signs of contamination, antibiotics were increased to 4% of the total volume of media.

3.5.1. ii) Presence of non-myoblast cells

To investigate the purity of myoblast cultures, all isolated cells were stained for desmin, a structural protein which is often used in myoblast isolation studies to verify muscle specificity [3, 25, 319]. The most common non-muscle cell type which could contaminate a myoblast culture is the fibroblast [231]. Fibroblasts are similar in size to myoblasts although in culture they have a more elongated shape with multiple long extensions. The many steps of the isolation process would not typically separate these two cell types. Since fibroblasts are known as fast-adhering cells [319, 25] the majority of these cells would adhere to the plate within the initial 2 hour incubation on a non-coated dish. The cells in suspension were used for further culturing, while the uncoated dish with all the adherent cells was discarded.

From the initial isolations very few or no cells were desmin negative. Despite the fact that basic fibroblast growth factor enhances fibroblast proliferation [16], it has also been shown to promote myoblast proliferation [16, 231]. Since all isolated cells were myogenic, this finding allowed us to continue using this growth factor to promote myoblast proliferation. Therefore, all media was supplemented with this growth factor, as many protocols suggest [319, 40, 25, 31].

3.5.1. iii) Cell yield

Many factors can contribute to a low yield of myoblasts, including i) the presence of microbial and fibroblast contamination, ii) the choice to use a single plating step or several steps of pre-plates, iii) efficiency of enzymatic digestion and iv) choice of appropriate media and supplementation.

As mentioned in section 3.5.1.1, several changes in our technique successfully reduced microbial contamination. In addition, because ethanol exposure itself could

also result in cell death during decontamination efforts, we changed the procedure for ethanol-cleansing of the rat from submerging the severed hind limb to submerging the whole rat in ethanol prior to muscle dissection. Cell yield was seen to improve.

Another major potential loss could occur if media is discarded 24 hours after the first plating following the isolation procedure. It is well accepted that myoblasts are slowly adhering cells, compared to other cells, such as fibroblasts [99]. Modifying the method of Allen *et al.* [4] to include several pre-plate steps as described by Gharabeih *et al.* [99], results in re-use of the cell suspension by transferring it daily to a newly coated well. The yield increased dramatically. It was found that three consecutive days of additional pre-plating were sufficient for the majority of myoblasts present in the suspension to adhere to the dishes. The myoblasts were harvested from PP1, PP2 and PP3.

Enzyme digestion is one of the key steps in the procedure to liberate the satellite cells from their niche, where they are embedded between the basal lamina and sarcolemma of the muscle fibre [16, 247]. To break the extracellular matrix structures of the basal lamina, a general protease product was used, consisting of a combination of at least 10 different proteolytic enzymes, including endopeptidases, aminopeptidases and carboxipeptidases [39]. Sufficient cell yield was not obtained with the recommended concentration or incubation period, or even when digestion was increased from 60 to 90 minutes. Due to the possible cytotoxicity of such proteolytic enzymes, the concentration was not increased. Instead, an additional step of digestion with collagenase I for one hour was added. Collagenases are enzymes which break down collagens, the extracellular matrix proteins holding many tissues, including myofibres and their associated satellite cells, together [109]. Due to the more specific digestive action of collagenase, the cell yield increased so much that a successful culture could be established for passaging.

The use of different media could influence the successful establishment of a primary myoblast culture. Two media, Dulbecco's modified Eagle's medium (DMEM) and Ham's F10 nutrient mix, are featured repeatedly in published protocols for myoblast isolations. According to Rando (1994) [231], Ham's F10 nutrient mix is more beneficial to myoblast culture expansion, while DMEM is able to sustain fibroblasts

as well, resulting in a fibroblast enriched culture. Both media are still being used in many protocols, suggesting that there is no consensus on the most efficient choice. Therefore the proliferation and differentiation capacities of primary cells isolated in DMEM and Ham's F10 were compared in this study. Also, a combination of the two media was investigated in the same manner (See section 3.5.2).

3.5.2. A combination of DMEM and Ham's F10 is optimal for primary culture

The immortal satellite cell line, C2C12, is usually grown in DMEM. Although both DMEM and Ham's F10 seem to be adequate to establish a successful myoblast culture, there is controversy about their efficiency to sustain a pure myoblast culture. The combination of the two media has been suggested in method sections of published papers [264, 259, 35, 48], but no direct comparison between the separate media and the combination has been reported to date.

When considering the content of the media, DMEM has much higher amino acid content, while Ham's F10 includes a wider range of amino acids. DMEM also contains more vitamins, higher glucose concentration and a higher concentration of calcium (Table 3.3).

Table 3.3. Contents of DMEM and Ham's F10 nutrient mix

Nutrients (in mg/L)	DMEM	Ham's F10
Amino acids	1021.4 (n=14) (excluding L-glutamine)	549.8 (n=20)
Vitamins	31.6	6.8
Inorganic salts	10906.77	9320.37
D-Glucose (Dextrose)	4500	1100
Calcium chloride	200	44

In the current study, the proliferation rate of primary myoblasts grown in DMEM was much higher than in Ham's F10 nutrient mix. This could be due to the high concentration of amino acids available for protein synthesis and cell growth as well as the high glucose levels as energy source. The difference between the two media in calcium concentrations could also play a major role in cell growth, as it has been shown that influx of extracellular calcium leads to an activation cascade in satellite cell cultures with the upstream release of HGF [108], and the calcium-calmodulin

complex is involved in upstream NOS activation [280]. Here, we showed that proliferation rates were higher in DMEM, the media with substantially higher calcium chloride content. It has also been suggested that calcium influx is reduced during proliferation, and again increased at the onset of differentiation [141]. During differentiation, the calcium dependent protein M-cadherin is responsible for adhesion and the calcium-dependent protease calpain is responsible for destabilisation of the plasma membrane prior to fusion. Here we showed that the combination of media, thus with an intermediate calcium chloride concentration, had similar fusion to DMEM alone. Intracellular calcium is also required for other signalling cascades during fusion [116].

Although the amino acid, glucose and calcium levels of Ham's F10 is much lower than DMEM, it is surprising that myoblasts cultured in this media have a growth advantage above other cells, such as fibroblasts. These myoblasts need to proliferate and fuse to form myotubes and then the musculature, thereby probably representing the tissue with the highest protein content.

However, proliferation and growth are controlled by different factors in different cells. It was not clear from our results here that Ham's F10 sustained a purer myoblast culture. According to literature desmin should be used to determine the myoblast purity of the culture, where desmin negative cells would be identified as fibroblasts [25, 231, 319]. All cultures contained a mix of cells with high and low levels of desmin, but desmin$^-$ cells were rare in all media. However, the percentage of cells with high levels of desmin was greater in Ham's medium compared to DMEM medium. Also, the intensity of Pax7 staining was much higher in myoblasts grown in Ham's F10 than DMEM. Pax7 is usually an indicator of quiescence or early activation, while higher desmin is associated with the differentiation phase where myoblasts fuse to form myotubes. This could mean that Ham's F10 favours the expression of muscle specific proteins due to the wider variety of amino acids, which improves their survival in this medium, even though they are not proliferating at a higher rate. Alanine and glutamic acid, which is present in Ham's F10 and absent from DMEM, are some of the most abundant amino acids found in skeletal muscle [150].

Since both media have important advantages, a combination (1:1) of the two media was also investigated. As could be expected, the proliferation rate was higher than in Ham's F10 and lower than in DMEM. The fluorescent intensity of desmin and Pax7 staining was more comparable with cells grown in DMEM than in Ham's F10. Therefore, for high myoblast yield, DMEM should not be excluded. However, since myoblast differentiation and tube formation must follow, it was important to determine if this compromise in terms of the combined media affected differentiation as well.

To compare the differentiation capacity, the organisation of the cytoskeletal protein actin was evaluated by phalloidin staining, while desmin staining provided a means to evaluate the organisation of sarcomeric proteins. In both DMEM and Ham's F10 media respectively almost no fusion was observed after 7 days in differentiation media although there was some indication that desmin intermediate filaments were aligning in parallel. The cells that were allowed to proliferate in the combined media before differentiation exhibited more signs of protein alignment in both cytoskeletal organisation and sarcomeric protein organisation. In a few cases clear multinucleated myotubes could be observed.

3.5.3. L-glutamine is more than just another amino acid in culture medium

Figure 3.14 is a graphical presentation of all amino acids included in both media, putting into perspective the high concentration of L-glutamine after supplementation in DMEM. In order to find out if it would also be a key ingredient in the media used for isolation, a comparison between DMEM with and without this amino acid was also included in the study.

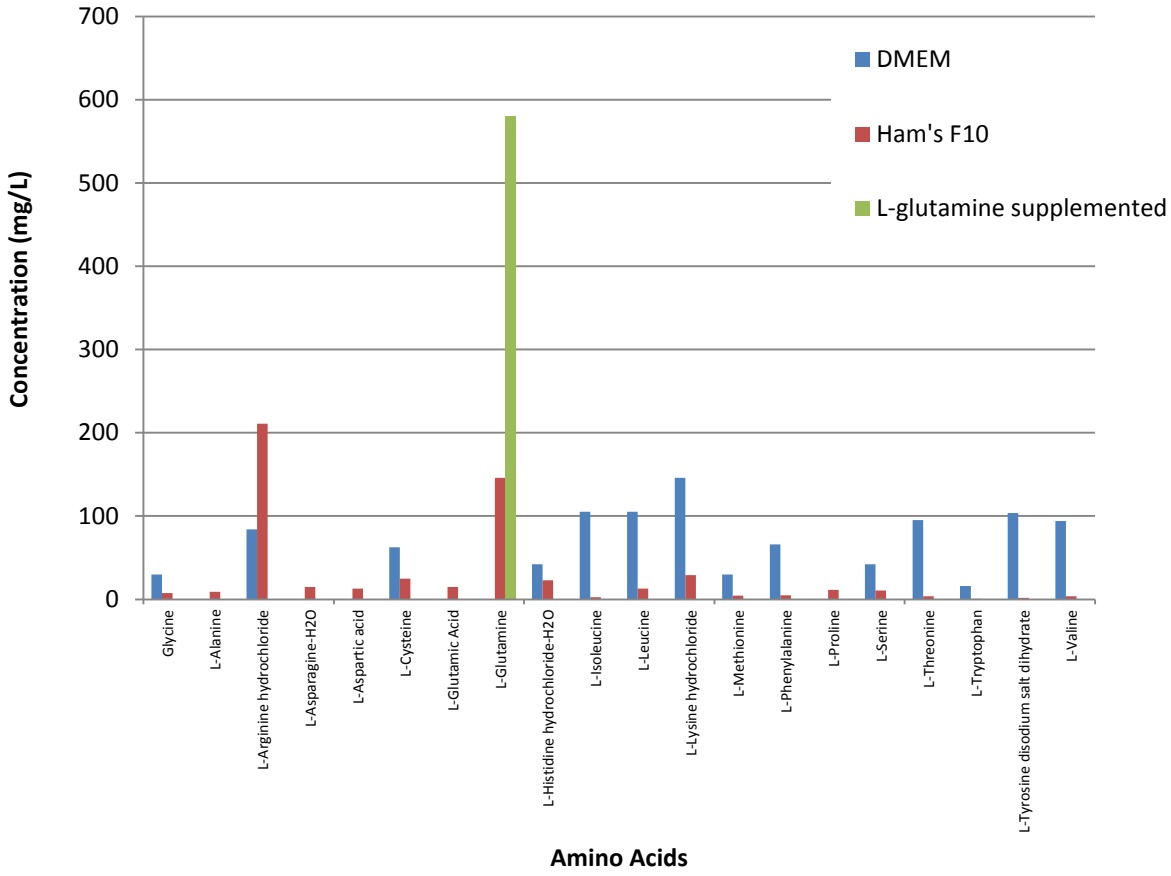


Figure 3.14. Amino acid content of DMEM and Ham's F10 nutrient mix. *The final L-glutamine concentration usually added to DMEM is also included.*

Although primary cultures could be isolated in medium without L-glutamine supplementation, this approach was suboptimal for establishing a viable primary myoblast culture. Myoblasts proliferated at a much higher rate when grown in DMEM with L-glutamine supplementation. This confirms the results found by Tadros *et al.* [271] who also studied the effects of glutamine deprivation on primary rat mononuclear myoblasts and myotubes. They showed that deprivation leads to a reduction in plating efficiency and myotube fusion index. They found that glutamine supplementation leads to the uptake of glutamine while deprivation leads to the release of glutamine by myotubes. Other amino acid transport systems were not affected. Myotubes seemed to adapt to the deprivation by increasing glutamine synthetase activity, although glutaminase activity was not altered.

The immunocytochemical analysis of primary culture myoblasts deprived of L-glutamine in the current study, showed disarrayed organisation of desmin, while no

visible abnormalities were observed in actin (via phalloidin staining) compared to those cultured in L-glutamine-containing media. Although no fusion was visible in primary cultures regardless of whether L-glutamine was present or not, the culture with L-glutamine appeared to be aligning. This indicates that L-glutamine plays an important role in the synthesis of proteins involved during fusion.

Glutamine has been found to improve proliferation in epithelial cell lines through activation of extracellular signal-related kinases (ERKs) and Jun nuclear kinases (JNKs). In turn these kinases phosphorylate and activate nuclear transcription factors. These effects of glutamine are usually associated with the signalling pathway stimulation by epidermal growth factor (EGF) or insulin like growth factor I (IGF I) [236].

3.5.4. SUMMARY AND CONCLUSIONS

The C2C12 cell line has been used in numerous studies as a model of muscle precursor cells. Although L6 cells have also been used as an immortal cell line, their myogenic capability declines with more passages [237, 302]; therefore C2C12 cells are considered a better model to investigate the process of regeneration. However, the quality of a good model is determined by its close resemblance to the actual cells being modelled. We have shown, as others have before, that C2C12 cells differ significantly from muscle precursor cells isolated directly from skeletal muscle.

The proliferation rate of C2C12 cells was much higher than that of isolated primary muscle precursor cells. The C2C12 cells were 100% confluent after 84 hours in culture, while primary myoblasts were not even 100% confluent after 96 hours. When considering the growth rate of myoblasts relative to recovery time after injury, one should use deductions made from C2C12 cells with caution.

In differentiation media, C2C12 cells formed myotubes after 7 days, while primary myoblasts show no evidence of forming myotubes. The high density of nuclei in the C2C12 culture showed that the high density of cell material could have led to faster tube formation. Although both C2C12 and primary myoblasts were seeded at the same density and media was changed to differentiation media only four hours after seeding, C2C12 nuclei were far more abundant than primary myoblast nuclei. This

indicates that C2C12 cells had a better ability to expand even though the media had been changed to differentiation media with much lower serum concentration.

Of note is the fact that the C2C12 line was developed from cells originally isolated from mice, while the primary culture in this study was isolated from rat. This species difference could also have an effect on the results, however the fact that C2C12 cells originated from cells with carcinogenic characteristics is a more likely reason for the continued proliferation and faster differentiation. It is well-known that tumour forming cells have a significantly higher proliferation rate than normal cells. However, tumour cells are less capable of significant differentiation, not a trait shared by C2C12 cells.

The differences pointed out in this study make it clear that the study of C2C12 cells to mimic muscle regeneration could be misleading. Their reaction to various interventions are often used as an indication of the effects of the intervention, but such findings should always be confirmed in a primary myoblast culture, which resembles the myoblasts *in vivo* much more closely.

Finally the culture conditions are not easy to optimise, especially when both proliferation and differentiation are important. Supplementation of L-glutamine is beneficial for primary myoblast proliferation and should be continued throughout the establishment of a primary myoblast culture. Although either DMEM or Ham's F10 nutrient mixes were sufficient to harvest primary myoblasts, a combination of these two media resulted in an enriched myoblast culture. The myoblasts cultured in the combined media expressed muscle specific proteins at higher levels, which resulted in better fusion capacity.

The protocol modified and optimised in this chapter was used to isolate primary myoblasts during the recovery phase after induced muscle damage. The muscle progenitor cells were harvested from injured and non-injured rats which were subjected to anti-oxidant (grape seed extract) or placebo supplementation (Chapter 4). The final modified protocol with pre-platings was used and media choices were made based on the findings of the current chapter.

Chapter 4 Effects of anti-oxidant supplementation on myoblast marker expression after contusion injury

4.1. INTRODUCTION

Stem cell research has risen to prominence over the past few decades, specifically for its potential to delineate how stem cells could be used for treatment purposes of previously untreatable conditions or injuries with inefficient or abnormal repair [229]. The possibility of utilising various stem cells lies in their ability to proliferate, to differentiate into a specific cell type and to be incorporated into existing tissue. The versatility of a specific stem cell population depends on the level of its commitment; this is determined from its existence in a niche, from where it will be recruited. Based on this concept, stem cells can be divided into totipotent, pluripotent and multipotent stem cells [175]. Some stem cell-like cells are already committed to a specific tissue to such an extent that they are rather referred to as precursor cells. The existence of precursor cells gives tissue the ability to replenish and replace old cells and to regenerate damaged areas [248].

Skeletal muscle, with its multinucleated fibre structure, is one of the tissues of the mammalian body with the highest ability to regenerate via the incorporation of precursor cells, called satellite cells [248, 61]. In a dystrophic mouse model, satellite cells from engrafted fibres can repopulate a whole ablated hindlimb muscle [61]. Satellite cells reside in a state of quiescence between the basal lamina and the sarcolemma of the adjacent muscle fibre [313]. Day to day wear and tear on myofibres may result in a small percentage (1-2%) of these cells differentiating and replacing existing myonuclei on a weekly basis [49]. However, inefficient repair following larger injuries often results in fibrotic scarring.

Upon stimulation a large number of satellite cells are activated to exit their growth arrested state. Proliferation, differentiation and fusion, either with existing muscle fibres or with each other to form new skeletal muscle fibres, are the processes required to regenerate damaged tissue [260, 253]. Stimulatory factors could include cytokines, growth factors and reactive oxygen species [268, 278, 281, 89, 87, 73].

Reactive oxygen species (ROS) are produced by contracting muscle and during strenuous exercise; oxidants and free radicals may accumulate in the tissue [234]. Reactive oxygen species, such as superoxide anions, hydrogen peroxide and hydroxyl radicals result in oxidative stress in the muscle resulting in damage to lipids, DNA and proteins [73]. Evidence of oxidative stress has been shown after muscle injuries induced by myotoxins [220] or ischemic contraction [130]. Also, oxidative stress is elevated in conditions such as sarcopenia [266] and amyotrophic lateral sclerosis [107].

Despite the negative connotations, ROS also play an important role in signalling following injury, mainly via nitric oxide systems [242]. Nitric oxide has anti-fibrotic properties [90], contributes to vasodilation [77] and also plays a key role in the activation of satellite cells from growth arrest (G_0) to their entry into the cell cycle [5, 294]. The proliferation of satellite cells, maintenance of the satellite cell pool and fusion in myotubes are all influenced by nitric oxide [77]. However, high levels of nitric oxide lead to the production of peroxynitrite ($ONOO^-$) and peroxynitrous acid (ONOOH), which in turn contribute to the production of highly reactive free radicals ($OH^\bullet$, $NO_2^\bullet$ and $CO_3^{\bullet-}$) and the oxidant NO_2^+ [261].

Clearly, there is a delicate balance between the beneficial and detrimental effects of both reactive oxygen and reactive nitric species. Situations during which this balance could be disrupted include eccentric exercise, muscle trauma and pathology. These are also conditions that activate satellite cells.

The effects of direct muscle injuries, following for instance toxin injection or lacerations, have been used as models of muscle injury [70, 95, 137], but there is still much scope for further investigation of damage and regeneration on the cellular and molecular level. Specifically, the contribution of satellite cells to muscle regeneration following *contusion injury*, which differs substantially from both toxin injury and laceration, has not been investigated extensively.

Contusion, an injury caused by the impact of a hard blunt object to the muscle belly, is common in many contact sports and occurs often in accidents. Characteristic of this type of injury is the formation of a haematoma without any open wounds. Recovery is very rarely complete, with scar tissue remaining permanently in the injured area [18, 19, 126, 202]. In many cases, muscle function and strength is

compromised. To improve the recovery process and to reduce the permanent effects of such injury, a clearer understanding of the regeneration process, its key role players and how these may be influenced, is required.

In response to injury, inflammatory cells infiltrate the injury site to remove necrotic tissue and debris [258]. Simultaneously, the activation of satellite cells, proliferation and differentiation, each occur according to a specific time course [265]. Various treatment options have been investigated to determine whether they can enhance the speed of recovery [137, 191], the complete reversal of damage and removal of scar tissue after injury [158, 94]. Less attention has however been paid to the potential effect of agents that reduce oxidative stress.

Although there are endogenous anti-oxidant enzymes, like superoxide dismutase, catalase and glutathione peroxidase, anti-oxidant capacity may be enhanced by various supplements. Polyphenolic compounds found in black grapes, raspberries and red currants, fall mainly into the categories of anthocyanidins, flavonoids and cinnamic acids [200]. In human subjects, oxidative stress models most often include exercise as the stressor, but few studies have investigated the effects of polyphenols. For example, supplementation with a polyphenol-containing drink prevented protein oxidation after endurance exercise in cyclists, evident from lower carbonyl content in plasma of cyclists. However, no significant difference was found in total antioxidant status [200] indicating that the polyphenols may have additional mechanisms of action. Although this study also found no differences in muscle damage markers (creatine kinase and lactate dehydrogenase activities), the exercise test was non-weight bearing and mechanical muscle damage was likely to be minimal. In an animal model, grape seed extract (GSE) supplementation improved anti-oxidant capacity in plasma and skeletal muscle tissue. This polyphenol reduced the infiltration of neutrophils into the damaged area after contusion injury and was associated with accelerated repair of muscle structure, observed directly from histological analysis. GSE also had an effect on the activity of satellite cells. In GSE-supplemented animals, a significant increase in $Pax7^+$, $CD34^+$ and $CD56^+$ cells was observed under the basal lamina 4 hours after injury. This was in contrast to the placebo group, where a significant increase was only seen at 3 days post-injury. M-cadherin staining did not however show a similar pattern to Pax7, CD34 or CD56; raising many questions with regard to the functional properties of these proteins and

the relation between the time course of their expression and their physiological role *in vivo* [203, 143].

Myogenic progenitor cells are not a homogenous population. In addition, a variety of cells such as pericytes [75], muscle derived stem cells [218] and skeletal muscle side population cells [183] (among others) are also involved in muscle regeneration. The involvement of these cells in myogenesis, and the overlapping expression of some, but not all, myogenic proteins, complicates the current understanding of satellite cell activity during muscle recovery from injury. Also, the relatively low number of satellite cells visible in tissue cross-sections has been a limiting factor. It is therefore necessary to employ other techniques to gain a fuller understanding.

In the following study, a standardised single contusion event was imposed on the rat *gastrocnemius in vivo* and the profile of selected proteins and behaviour of precursor cells post-injury were studied. GSE was administered *in vivo*, so that an integrated physiological effect on the satellite cells could be tested. Isolation of satellite cells from excised muscle allowed for analysis of a much greater proportion of the cells compared to standard histological techniques.

4.2. HYPOTHESIS AND AIMS

We hypothesised firstly that GSE supplementation modulates satellite cell (SC) progression from precursor cell to mature myoblast, and that this could explain the positive effect of GSE on the time course of recovery of skeletal muscle post injury. Secondly, we hypothesised that *in vitro* evidence of early differentiation would be apparent only in myoblasts isolated from injured rats exposed to grape seed extract. To test these hypotheses, primary myoblasts were isolated at different time points after contusion injury in rats, and their properties assessed *in vitro*.

To this end, the aims of the study were:

a) To isolate primary myoblast cultures from rat muscle harvested post-injury in order to reflect the progression of regeneration of injured skeletal muscle

b) To determine effects of *in vivo* GSE treatment on the phenotypic progression of these myoblasts

4.3. METHODS

4.3.1. Animals

Male Wistar rats were bred and housed in the Animal Facility at Stellenbosch University. After weaning, rats were housed together in groups of four. Standard rat chow (Pure harvest mouse and rat feed; Afresh brands, Durbanville, South Africa) and tap water were available *ad libitum*. In the Animal Facility, the ambient temperature was kept at 20ºC and a 12h light/dark cycle was maintained (lights on at 6:30). Rooms were ventilated at 10 changes/hour.

In order to familiarise rats with handling and to monitor growth, rats were weighed daily by the researcher. Two weeks prior to the start of supplementation, rats were orally gavaged with tap water every day, to ensure that rats were accustomed to the protocol at the start of the intervention. Animal care and all experimental procedures were approved by the Stellenbosch University Research Ethics Committee: Animal Care and Use (Ref: 10NP_MYB01).

4.3.2. Interventions

The study involved two interventions, namely the drop-mass contusion injury of the lower hind limb and daily supplementation with either grape seed extract or a placebo, starting two weeks prior to the injury and continuing throughout, until sacrifice.

4.3.2. i) Supplementation

Grape seed extract (GSE, Oxiprovin^{TM},Brenno-Kem, South Africa) was administered fresh each morning. Prior to supplementation, GSE was prepared by dissolving in 0.9% saline, to a final concentration of 20 mg/ml, the concentration which was determined in a previous study [142]. For the placebo, 0.9% saline was used. Rats were orally gavaged with GSE at a concentration of 20 mg/kg body mass per day or with 1 ml/kg 0.9% saline as placebo control.

4.3.2. ii) Mass drop contusion injury

The contusion injury model used in this study was developed previously by Smith *et al*. [258]. In collaboration with the Central Engineering Services of the University of Stellenbosch, an apparatus was designed and built which could create a controlled standardised contusion injury in the desired muscle group. The apparatus consisted

of a vertical plastic tube with centimetre markings on the side and a flat-bottomed mass could be lowered to the desired level (Figure 4.1). A peg held the mass at the desired height and the removal of the peg resulted in the mass dropping at a standardised speed. A large metal base with a smaller cylindrical platform was directly under the plastic tube. The rat could be placed on the metal platform with the slightly extended hind limb positioned on the smaller platform, positioned so that the mass in the tube would fall on the medial surface of the *gastrocnemius* in the central area.

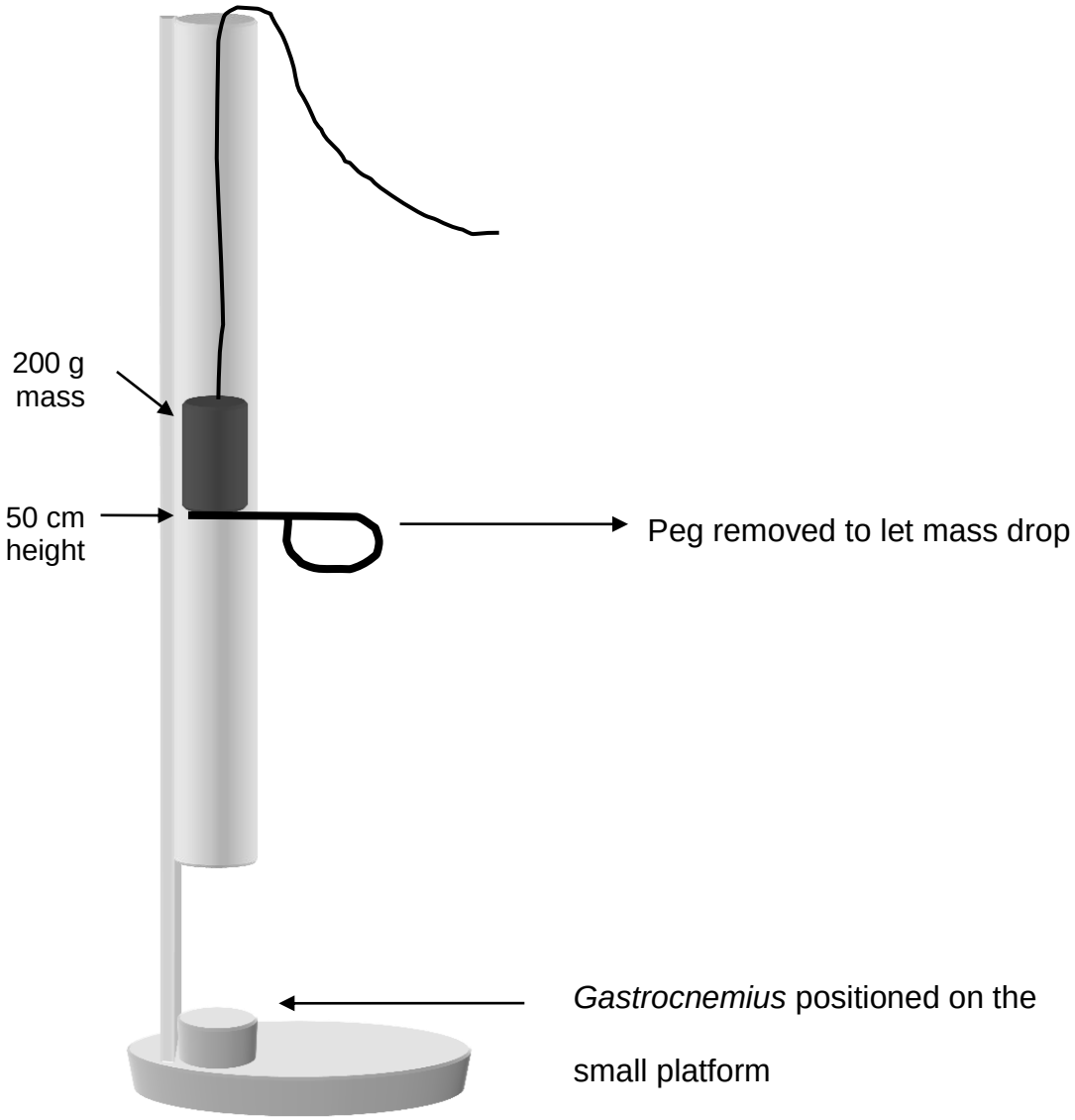


Figure 4.1. Muscle contusion injury jig. *A mass of 200 g was dropped from a height of 50 cm onto a small platform on which the right gastrocnemius muscle of the anaesthetised rat was positioned.*

To perform the injury, animals were anaesthetised via inhalation of oxygen at 1L/min with 5% isofluorane. All injuries were performed between 7 am and 8 am. A mass of 200 g was dropped from 50 cm onto the right *gastrocnemius*, and immediately pulled back with a string to prevent a second rebound injury. The underlying bone was held away from the platform, to prevent damage. After the injury, rats were allowed to recover from the effects of anaesthesia, which usually occurred within 20 minutes.

4.3.2. iii) Study design

Both supplementation groups, GSE and placebo, were subdivided into non-injured (n=3) or injured (n=9). Non-injured rats were euthanised after two weeks of supplementation. Injured rats were euthanised 4 hours after injury (4h), 3 days after injury (3d) or 14 days after injury (14d), with 3 rats per group. Figure 4.2 is a diagrammatic representation of the study design.

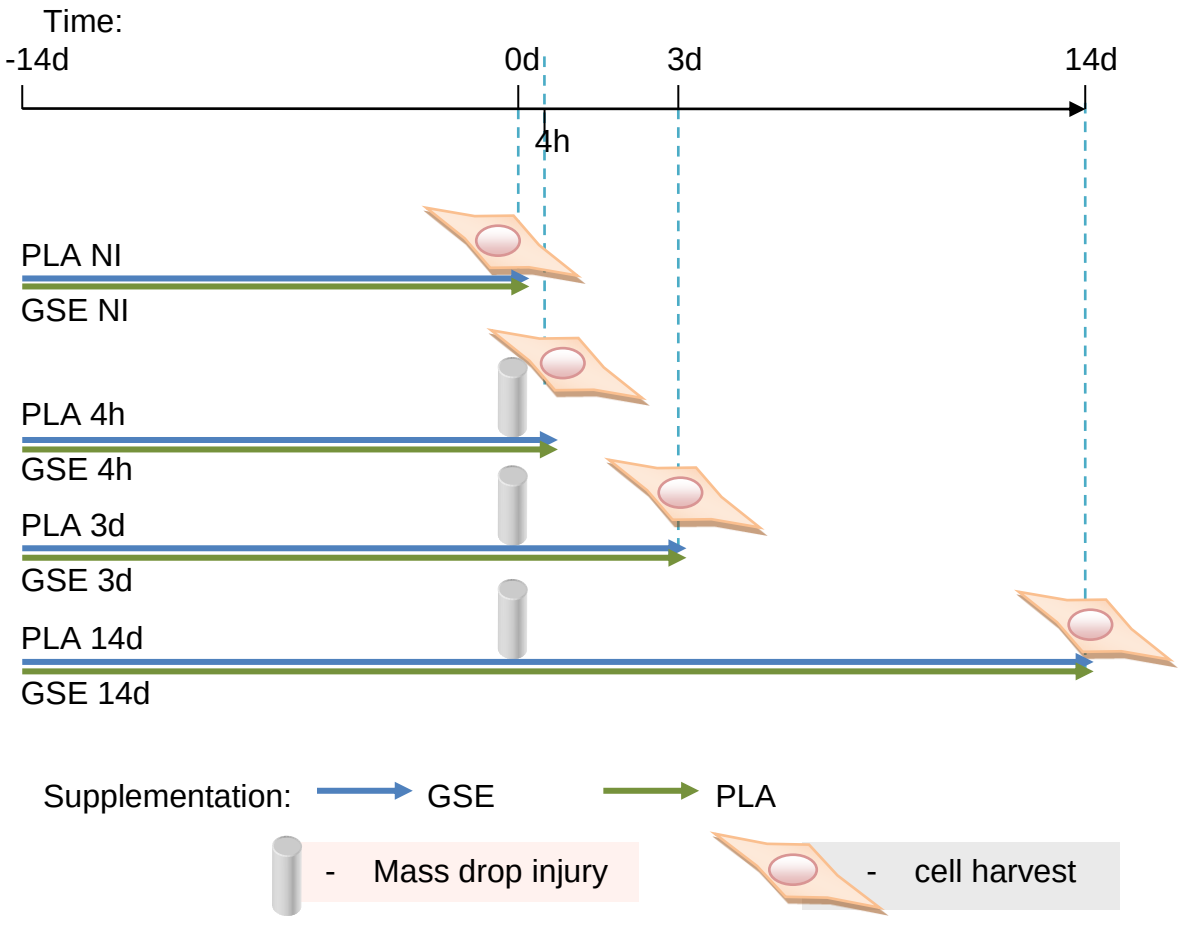


Figure 4.2. Experimental design. *Animals were all supplemented for two weeks with either grape seed extract (GSE) or placebo (PLA). Non-injured (NI) rats were then euthanised and all other groups injured. Injured rats were killed 4 hours, 3 days or 14 days after the injury. Supplementation continued until the end of the experiment.*

4.3.3. Euthanasia and cell harvest

The start of interventions for the different groups was staggered so that no more than three rats were killed on a particular day. With the exception of the 4 hour post-injury group, all rats were euthanised between 8 am and 10 am.

Rats were humanely killed with a sub-peritoneal injection of 200 mg/kg pentobarbitone sodium solution (Euthapent, Kyron Laboratories (Pty) Ltd; Johannesburg, SA,92/2.2/6). As soon as death was confirmed, the right hind leg was shaved with an electrical shaver and the rat was submersed in 70% ethanol for sterilisation. In a sterile Class II Biological safety cabinet (NuAire NU, Plymouth, MN, USA, 437-400), the skin and surrounding connective tissue were removed from the right hind leg to expose the *gastrocnemius* muscle. The muscle was carefully removed from the bone and the central area of the medial part was excised to contain the injured area as well as the border zone around the injured area.

The dissected portion of muscle from three rats was pooled and rinsed in sterile phosphate buffered saline (PBS). Connective tissue, tendons, fascia and as much adipose tissue as possible were trimmed away, whereafter the muscle tissue was mince in a sterile culture dish and harvested as described in Chapter 3 (3.3.2). Briefly, minced muscle was subjected to a 1 hour protease digestion (1.25 mg/ml protease type XIV from *streptomycus griseus*; Sigma Aldrich, Schnelldorf, Germany, P5147) and then a 1 hour collagenase digestion (0.2% collagenase I from *Clostridium histolyticum*; Sigma Aldrich, Schnelldorf, Germany, C7657). This was followed by differential centrifugation steps (500 *g*) where the supernatant was collected each time. The combined supernatant was centrifuged (950 *g*) to form a pellet of isolated cells which was resuspended and filtered through a stainless steel tea sieve and subsequently through a 100 µm cell strainer (BD Biosciences, Bedford, MA, USA, 352360). The strainer and cell strainer were rinsed with 1 ml DMEM media each time. The cell suspension was then plated into a 30 mm culture dish in DMEM media. After two hours, the supernatant was centrifuged and the resulting cell pellet was re-suspended in Hams F10 media. The media consisted of Ham's nutrient mix F10 (Gibco from Life Technologies, Grand Island, NY, USA, 31550-023), 20% fetal calf serum (Gibco from Life Technologies, Grand Island, NY, USA, 10270-106), 1%

antibiotic antimycotic (Gibco from Life Technologies, Grand Island, NY, USA, 15240-062), 0.1% gentamicin (Gibco from Life Technologies, Grand Island, NY, USA, 15750-060) and 10 ng/ml basic human fibroblast growth factor (Promega, Madison, WI, USA, G5071). The cell suspension was plated in the first well of an E-C-L-coated 6 well plate. Pre-plating was continued until PP4 as described in Chapter 3 (3.3.2 and Figure 3.1).

Pre-plates were viewed with an inverted phase contrast microscope at 200x magnification (Olympus CK41, Olympus Corporation, Tokyo, Japan) and phase contrast images were taken using a portable digital camera with microscope lens adapter (Olympus C-5060, Olympus Corporation, Tokyo, Japan).

4.3.4. Cell culture

For further culturing after pre-plating, media was changed to a combination of DMEM and Ham's F10 media (see appendix for details) and cells transferred to non-coated tissue culture flasks. Media was exchanged every two days. Isolated myoblasts were allowed to proliferate until 70% confluence before trypsinization; thereafter they were either passaged to continue proliferation or frozen down as described in Chapter 3 (see section 3.3.3). After trypsinisation during each passage, cultures were plated onto non-coated dishes for 20 minutes to allow any fibroblasts still present to adhere, before the supernatant was transferred to non-coated dishes for further culturing.

4.3.5. Flow cytometric analysis

After several passages to obtain adequate numbers of cells, isolated primary myoblasts were prepared for flow cytometric analysis. Trypsinised cells were fixed for 10 minutes on ice in a 1:1 mixture of acetone (Saarchem-Merck Chemicals, Gauteng, South Africa, UN1090) and methanol (Emsure-Merck Chemicals, Darmstadt, Germany, 603-001-00-x). Immunostaining was performed with different combinations of primary antibodies made up in 1% bovine serum albumin blocking agent (BSA Fraction V; Roche Diagnostics; Mannheim, Germany, 10735094001). Combinations are shown in Table 4.1. Each sample contained approximately 200 000 cells. After 30 minutes incubation with primary antibodies at room temperature, cells were washed in PBS and centrifuged at 950 *g* for 3 minutes; thereafter pellets were suspended in 1% BSA with fluorochrome-conjugated secondary antibodies, and incubated for 30 minutes at room temperature. Secondary antibodies used were

donkey anti-goat IgG-PE (phycoerythrin) (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-3743), donkey anti-mouse Alexafluor 488 (1:200; Molecular Probes, Eugene, OR, USA, A21202) and donkey anti-rabbit IgG-PerCP (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-45095). As a final step before analysis cells were washed in PBS, centrifuged and suspended in 300 µl PBS.

Table 4.1. Primary antibodies used in three different combinations to stain myoblasts. *Co-staining combinations were (1) M-cadherin, CD56 and Pax7, 2) M-cadherin and CD34, and lastly 3) M-cadherin, desmin and MyoD.*

	Primary antibody	Species	Dilution
1	M-Cadherin (sc-6470)*	Goat polyclonal	1:100
	CD56 (sc-8305)*	Rabbit polyclonal	1:100
	Pax7 (ab55494)#	Mouse monoclonal	1:200
2	M-Cadherin(sc-6470)*	Goat polyclonal	1:100
	CD34 (sc-9095)*	Rabbit polyclonal	1:100
3	M-Cadherin(sc-6470)*	Goat polyclonal	1:100
	Desmin (ab15200-1)#	Rabbit polyclonal	1:100
	MyoD (sc-32758)*	Mouse monoclonal	1:100

* Santa Cruz Biotechnology, Santa Cruz, CA, USA
\# Abcam, Cambridge, UK

Analysis was performed on a flow cytometer (BD FACSAria, Becton Dickinson, Biosciences, San Jose, CA, USA), equipped with solid state sapphire, HeNe and violet lasers. Prior to analysis, compensation was set up and calculated using software (BD FACSDiva 6.1) to correct for spectral overlap. Positive signal was defined prior to experimental analysis by using fluorochromes-minus-one (FMO) combinations (see appendix for details). All three fluorochromes, phycoerythrin (PE), PerCP and Alexafluor488 were excited at 488 nm. The fluorescence of PE was detected with a 556 long pass (LP) and a 585/42 band pass (BP) filter, that of PerCP was detected with a 655 LP and a 675/20 BP filter and for Alexa 488, fluorescence was detected with a 502 LP and a 530/30 BP filter. Debris was excluded from the

analysis based on forward and side scatter. The main cell population was gated and 10 000 cells were analysed for each sample.

For co-expression analysis, dot plots were created for two fluorescent markers at a time. Quadrant gates and histograms, determined through the use of FMO's, were used to distinguish between positive and negative expression of the two specific markers.

4.3.6. Sorting

4.3.6. i) Cell preparation for sorting

For sorting purposes the staining protocol was optimised to ensure cells were viable at the time of the sort. Therefore all incubations were performed at 37°C (see appendix for details). Trypsinised cells were incubated for 30 min in 1% BSA to prevent non-specific binding. CD56 primary antibody (1:100; see Table 4.1) was immediately added to the cell pellet and incubated for 45 minutes, followed by an hour incubation in secondary goat anti-rabbit PerCP-conjugated antibody (1:100; see Table 4.1) and CD34 (ICO115) PE (phycoerythrin) conjugated (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-7324). All antibodies were diluted in DMEM medium and cells were kept at 37°C until sorting.

4.3.6. ii) Fluorescence activated cell sorting (FACS)

Warm media was prepared in three collection tubes for each sample. Figure 4.3 shows the quadrant gates used for the sort setup to sort cells according to their expression of CD34 and CD56. The procedure continued until all cells were separated. Sorted samples were centrifuged and seeded in 8 well cover slip chambers (Lab-tek™ chambered cover glass, Rochester, NY, USA, 155411) for immunocytochemistry.

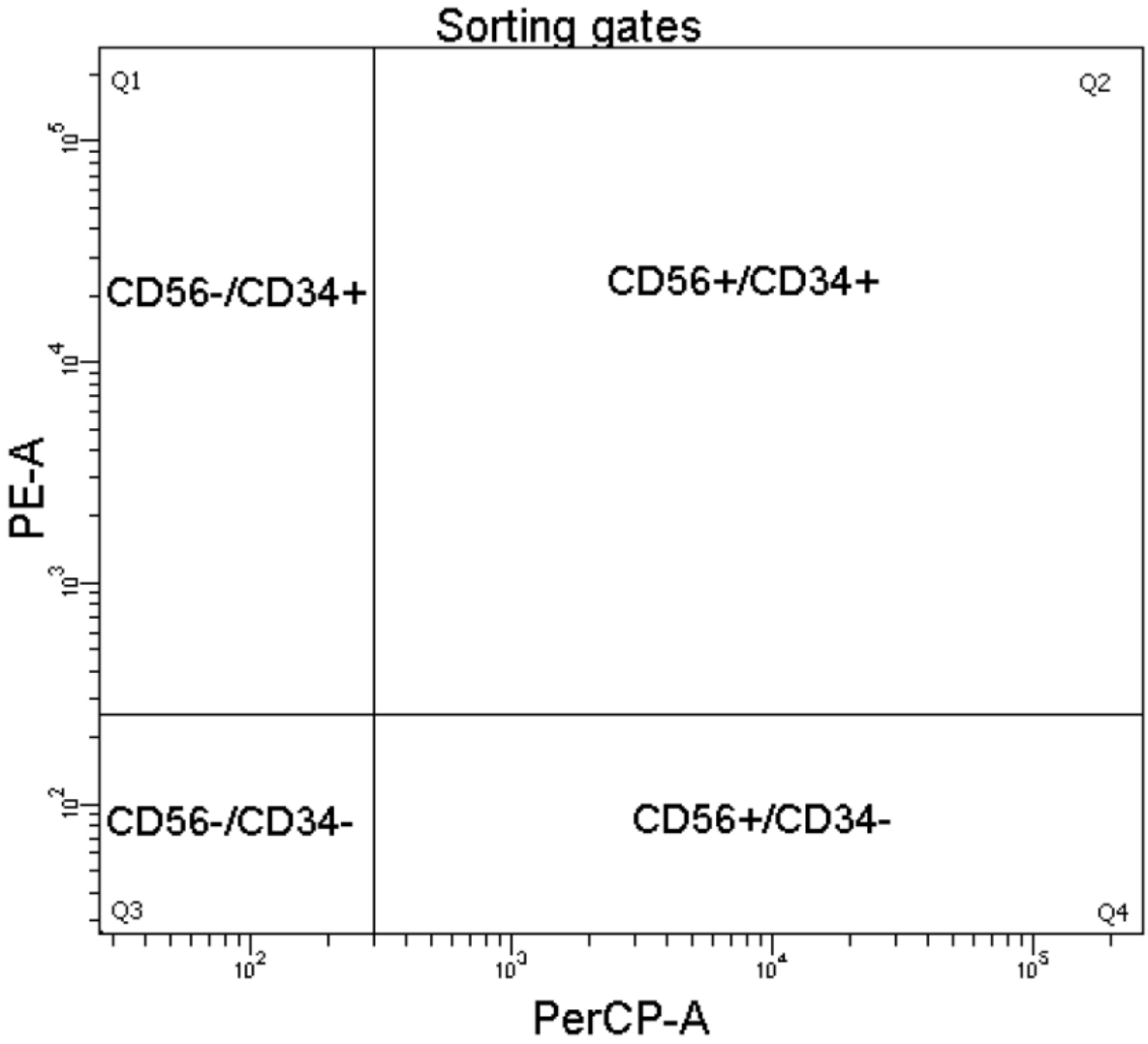


Figure 4.3. Quadrant gates used to sort myoblasts according to CD56 and CD34 expression. *Sub-sets collected were $CD34^-/CD56^-$; $CD34^-/CD56^+$; $CD34^+/CD56^-$ and $CD34^+/CD56^+$ populations. CD56 fluorescence was measured in the PerCP channel, while CD34 fluorescence was measured in the PE channel.*

4.3.7. Immunocytochemistry

Cells were washed with PBS and fixed with 2% paraformaldehyde (Sigma-Aldrich, Schnelldorf, Germany, 158127-25G) for 15 minutes. This was followed by a permeabilisation step using 0.1% Triton X-100 (BDH Laboratory Supplies, Poole, UK, BB306324) for 6 minutes. To prevent non-specific binding of antibodies, cells were blocked with 1% BSA for 30 minutes after which desmin primary antibody (1:100; see Table 4.1) was immediately added and left to incubate for 2 hours at room temperature. Cells were then incubated in secondary goat anti-rabbit

Alexafluor 594 (1:100; Molecular Probes, Eugene, OR, USA, 11012) for 1 hour. This was followed by incubation in mouse anti-myogenin primary antibody (1:100) overnight at 4°C, followed by 1 hour incubation with donkey anti-mouse Alexafluor 488 (1:100; Molecular probes, Eugene, OR, USA,A21202). Prior to imaging, cells were also stained with the nuclear dye Hoechst 33342 (1:500) for 10 minutes. Between all steps cells were washed with PBS, except after blocking with BSA. Cells were kept in PBS in the fridge until imaging.

4.3.7. i) Image analysis

Fluorescent images were taken with a wide field inverted microscope (Olympus IX81, Olympus Corporation, Tokyo, Japan) with an F-view-II cooled CCD camera (Soft imaging Systems, Olympus Corporation, Tokyo, Japan). The microscope was equipped with a Xenon-Arc burner (Olympus Biosystems GMBH, Hamburg, Germany) as light source and 360 nm, 492 nm and 572 nm excitation filters. A UBG triple-band-pass emission filter cube (Chroma Technology Corporation, Bellow Falls, VT, USA) was used to collect emission. Images were acquired with a60x/1.42 oil immersion objective (Olympus Plan APO N, Olympus Corporation, Tokyo, Japan) and software (Olympus Cell^R, Hamburg, Germany). At least three fields of view were imaged per sorted group.

4.3.8. Statistics

For comparisons between all groups the Kruskal Wallis ANOVA for nonparametric data was done with the Dunn's multiple comparisons *post hoc* test. Significant differences were considered when $p < 0.05$.

4.4. RESULTS

4.4.1. Rat age and body mass

At the start of the intervention, the average age of rats was 7.6 ± 0.4 weeks and the average body mass was 277 ± 12 g. This was accepted as the age by when rats reach maturity [141].

There were no significant differences in body mass between groups at the start of the intervention or at the time of injury. Average body mass for all groups throughout the protocol are shown in Figure 4.4.

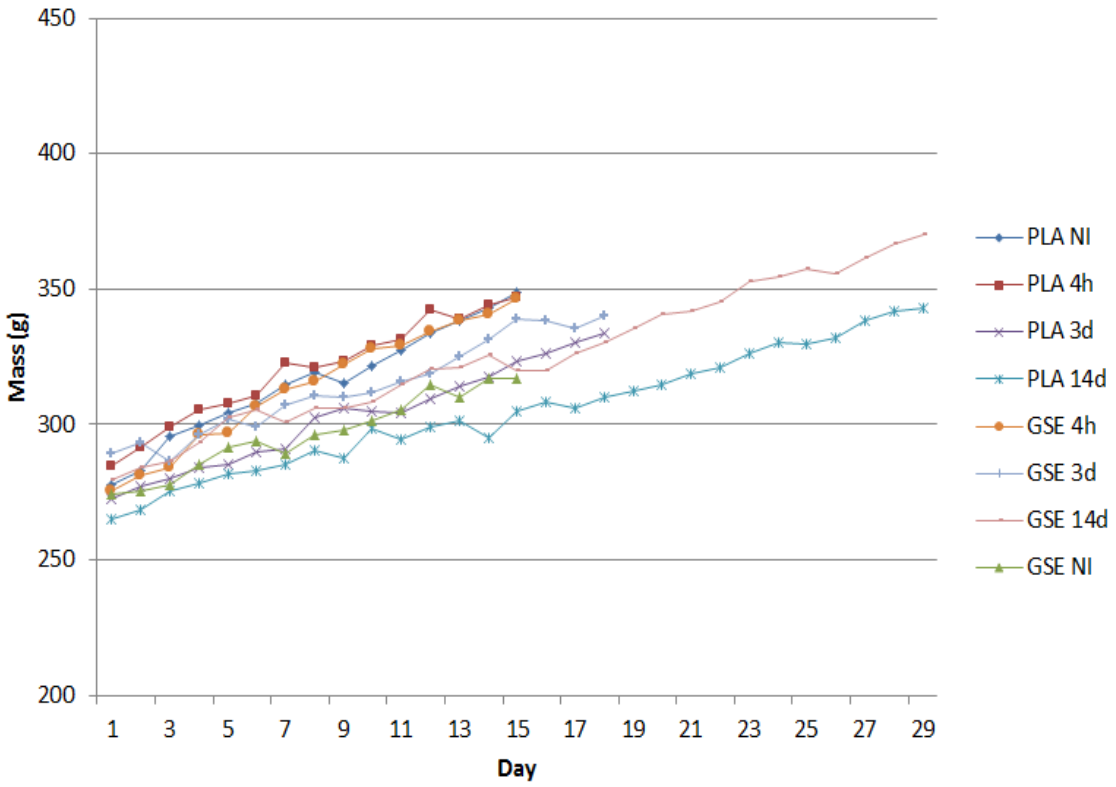


Figure 4.4. Mean daily body mass of each group until day of sacrifice *(n = 3 rats per group).*

The average mass at the time of the injury was 331 ± 21 g. Rats continued to gain weight during the course of the experiment.

Since there were no significant differences in the body mass between any of the groups [141], none of the time-dependent changes or treatment-dependent differences could be ascribed to differences in body mass.

4.4.2. Primary culture observations and cell yield

The various groups of isolated cells behaved very differently in terms of adherence and growth capacity as well as survival after freezing (see appendix for details). Phase contrast images of some of the groups are shown in columns and rows in Figure 4.5.

In both the PLA NI and GSE NI groups cells did not adhere well in PP1. The PLA NI cells adhered well by PP3 and PP4, but GSE NI cells did not adhere well in any of the pre-plates. Therefore the yield of GSE NI cells was very low with only 740 000

cells nine days after harvest. In contrast, the yield of PLA NI cells was much greater and 11 days after harvesting $5.2x10^6$ cells were counted.

At four hours post-injury few cells adhered in PP1 with more cells adhering in the later pre-plates in both groups. Cell counts of $3.2x10^6$ were reached 7 days after harvesting for PLA 4h cells and 8 days after harvesting for GSE 4h cells.

When cells were isolated 3 days post injury, many cells adhered already in PP1, with far fewer cells adhering in the later pre-plates in both treatment groups (see Figure 4.5, column 1, row 1 and 4). Again the yield for cells in the PLA 3d group was much greater compared to the yield of cells in the GSE 3d group ($4.6x10^6$ cells after 7 days vs. 430 000 cells after 11 days in culture).

The cells isolated 14 days post injury, were not adhering as well as at the other post-injury time points and the adherence capacity was more comparable to the non-injured groups (see Table 4.2). No cell counts were available for these cultures.

Table 4.2. Yield of isolated myoblasts. *Cell counts were not done on similar days, due to variable growth and proliferation patterns in different groups (see text).*

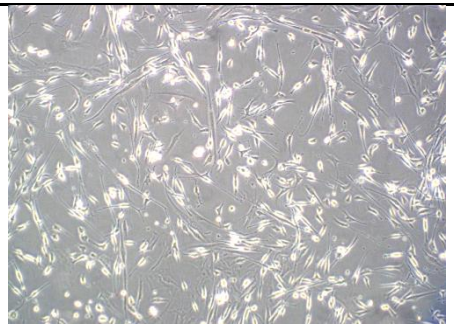
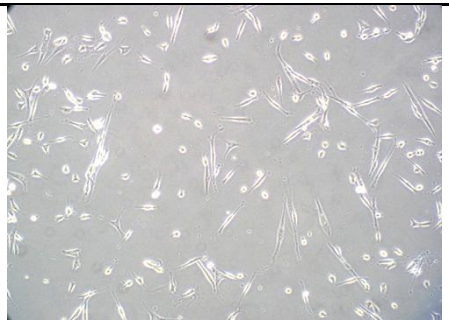
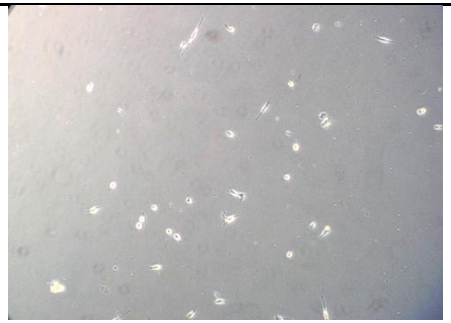
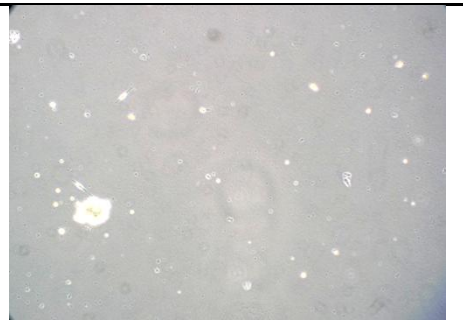
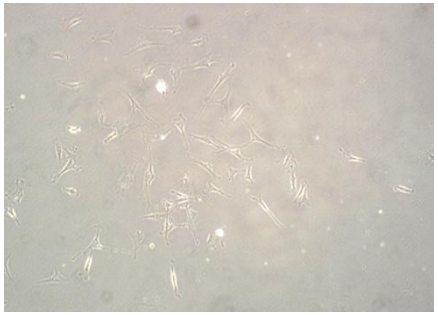
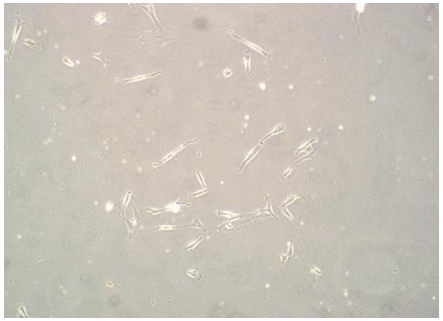
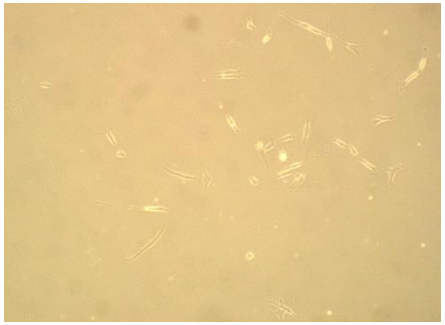
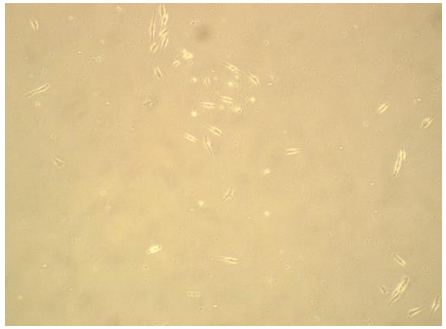
	PLA*	**GSE***
NI	5.2×10^6 in 11 days	0.74×10^6 in 9 days
4h	3.2×10^6 in 7 days	3.2×10^6 in 8 days
3d	4.6×10^6 in 7 days	0.43×10^6 in 11 days

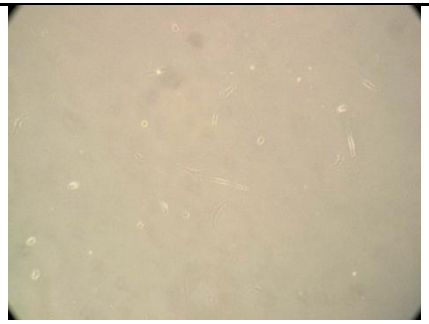
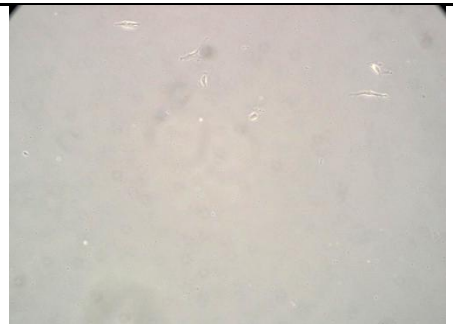
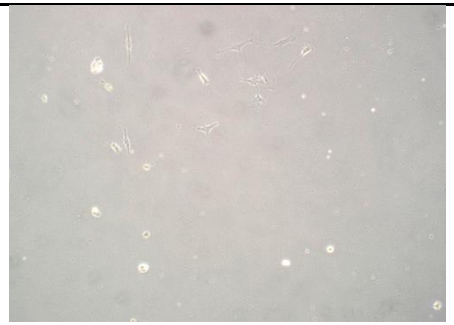
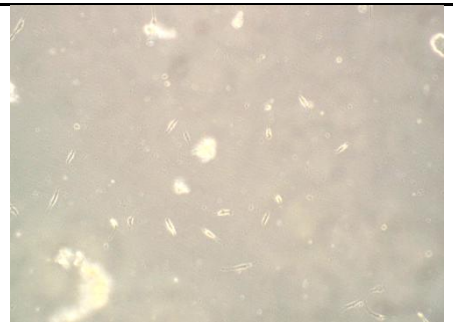
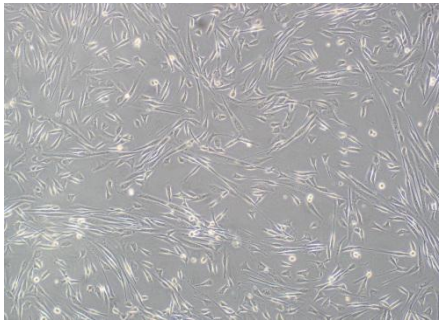
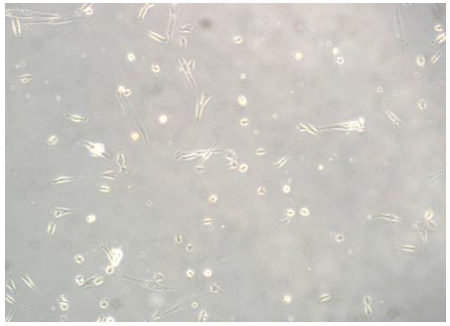
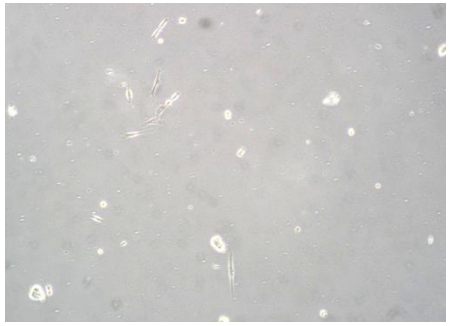
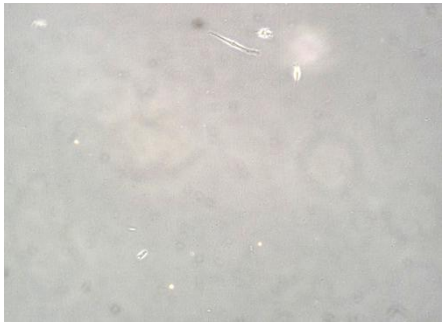
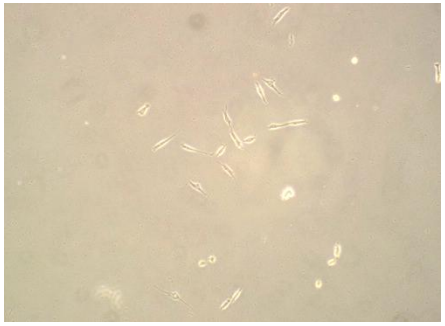
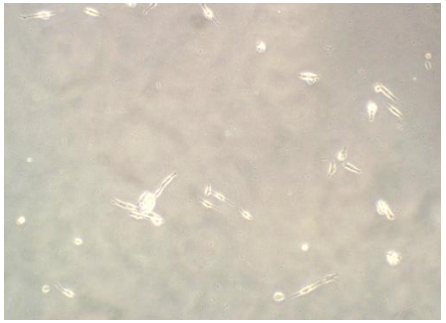
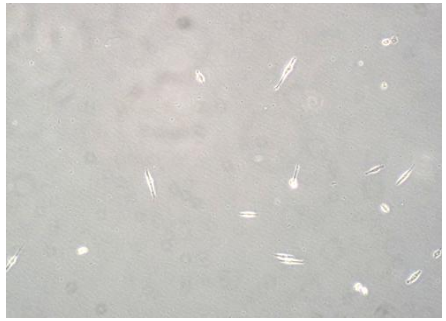
* Total number of trypsinised cells, pooled for each group

Due to the large variance in cell yield from different groups and their different proliferation and survival rates, it was very difficult to analyse all cells in the same passage. Therefore a trial was performed to compare the influence of passage on protein expression in different groups (results not shown). Cells from passage 4 (P4) were compared with cells from passage 10 (P10) for all markers of interest. For this trial experiment, only cells from the PLA NI, the PLA 14d and GSE 14d groups were used due to limited cell numbers in some of the other groups. For most of the markers, namely M-cadherin, Pax7, MyoD, CD34 and CD56, the percentage of

positive cells was very similar in both P4 and P10. The expression of desmin was much more variable amongst the two passages in the PLA NI group and the GSE 14d group. However from the results it was not clear what effect passage played on the expression of desmin, as expression decreased in the higher passage in the placebo group, but increased in passage 10 in the GSE 14d group. Due to limited repeats no statistical analysis was performed.

Figure 4.5. Phase contrast images of pre-plates (PP1-4) taken 5 days after isolation. *Images were taken of cells isolated from the PLA treatment groups, isolated 3d and 14d post-injury. For the GSE treatment groups, images of NI, as well as 3d and 14d post-injury were taken.*

	PP1 cultured for 4 days	PP2 cultured for 3 days	PP3 cultured for 2 days	PP4 cultured for 1 day
PLA3d				
PLA 14d				

	PP1	PP2	PP3	PP4
GSE NI				
GSE 3d				Not available
GSE 14d				

4.4.3. Identification of myoblasts using different proteins

4.4.3. i) Expression of adhesion molecule M-cadherin

There were no significant differences between the GSE and PLA groups at any time point and no significant changes within any treatment group over time for the expression of M-cadherin by isolated cells as determined by number of cells M-cadherin$^+$ (flow cytometry). Almost all cells expressed M-cadherin, although results for the GSE 3d group were more variable. Although different cultures originiated from the same isolation, the subsequent passaging resulted in slightly varying results. For M-cadherin specifically, one passage expressed low levels, which led to a large standard deviation and a lower mean. No cell death or alternative morphology was observed, so there was no reason to exclude this population from the analysis. Table 4.3 presents the percentage of isolated cells which expressed M-cadherin.

Table 4.3. The percentage of total isolated cells which expressed M-cadherin. *Cells were stained with goat anti-M-cadherin and donkey anti-goat PE conjugated IgG. Values are expressed as mean ± standard error of the mean. No significant differences were evident ($p > 0.05$). Cultures were isolated from 3 rats, pooled and analysed in different passages.*

	PLA (%)	GSE (%)
NI	99.6 ± 0.3	98.0 ± 1.7
4h	99.7 ± 0.1	99.8 ± 0.1
3d	99.8 ± 0.1	88.07 ± 10.9
14d	99.7 ± 0.1	99.58 ± 0.2

4.4.3. ii) Expression of transcription factors Pax7 and MyoD

Expression analysis of the satellite cell-specific transcription factor Pax7, confirmed that almost all isolated cells in all groups were indeed satellite cells. Table 4.4 displays the average percentages of cells expressing Pax7 in the GSE and PLA groups for all time points in different passages.

Table 4.4. Percentages of total isolated cells expressing Pax7*. Cells were stained with mouse-anti Pax7 IgG and donkey anti-mouse Alexafluor conjugated IgG. Results are presented as mean percentage ± standard error of the mean. No significant differences were found (p > 0.05). Cultures were isolated from 3 rats, pooled and analysed in different passages.*

	PLA (%)	GSE (%)
NI	99.87 ± 0.04	99.70 ± 0.20
4h	99.96 ± 0.02	99.92 ± 0.03
3d	99.83 ± 0.11	98.53 ± 1.23
14d	99.85 ± 0.06	99.90 ± 0.05

Analysis of MyoD expression revealed very similar results to the expression of Pax7 with ~99% of isolated cells expressing MyoD. Table 4.5 presents the average percentages of cells which were MyoD$^+$ in both the PLA and GSE groups. No significant differences were found amongst the different groups.

Table 4.5. Percentage of total isolated cells expressing MyoD. *Cells were stained with mouse-anti-MyoD IgG and donkey anti-mouse Alexafluor 488 IgG. Results are expressed as mean percentage ± standard error of the mean. There were no significant differences (p > 0.05). Cultures were isolated from 3 rats, pooled and analysed in different passages.*

	PLA	GSE
NI	99.87 ± 0.06	99.87 ± 0.03
4h	99.90 ± 0.10	99.97 ± 0.03
3d	99.90 ± 0.10	98.37 ± 1.48
14d	99.80 ± 0.05	99.90 ± 0.05

4.4.3. iii) Expression of membrane protein CD56

In order to determine co-expression of surface proteins, which would also indicate myoblast maturity, M-cad$^+$ cells were considered for co-expression of CD56. The adhesion molecule CD56 (also called neural cell adhesion molecule, NCAM), was not expessed in as high percentage of isolated cells as seen with the transcription factors Pax7 and MyoD. Values ranged from 4% to 55%. In the GSE group, the

percentage of CD56$^+$ cells peaked at 4 hours after injury and at this time point differed significantly from PLA (see Figure 4.6). The percentage of CD56$^+$ cells in the placebo groups peaked at 3 days after injury. There was a significant increase from 4 hours post injury to 3 days post injury in this group. As almost all cells were expressing M-cadherin, Pax7 and MyoD it was assumed that, despite lower numbers, all cells expressing CD56 were indeed myoblasts.

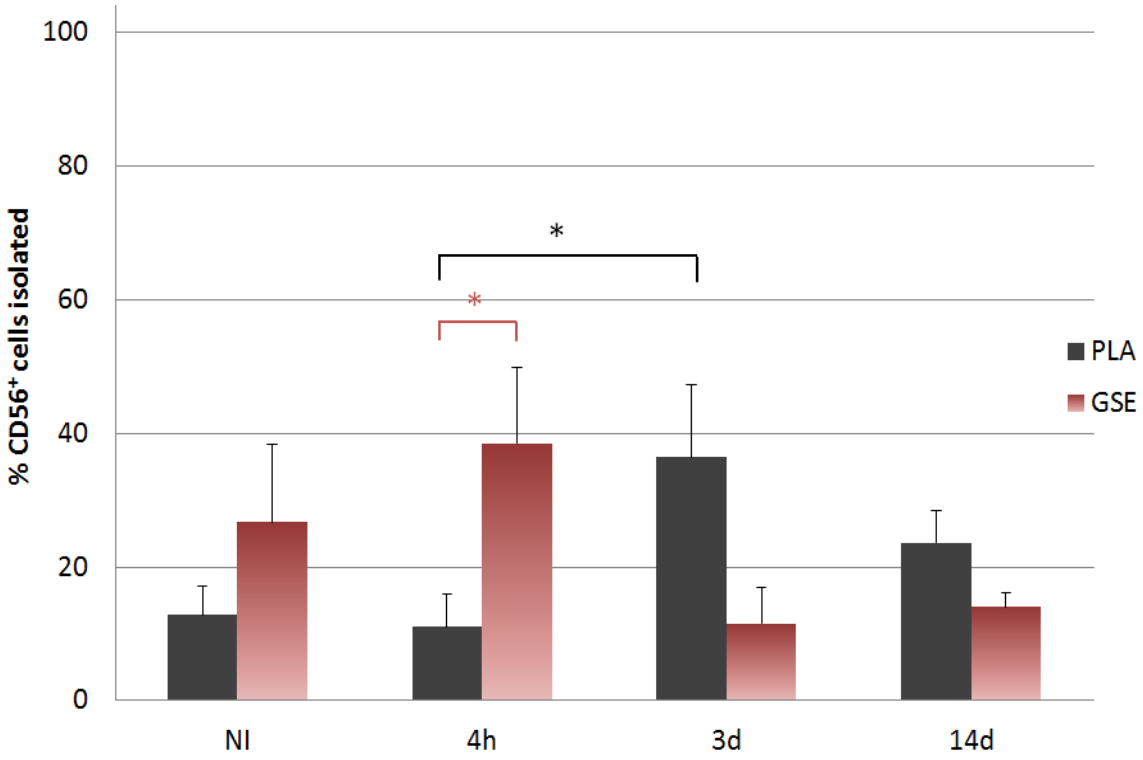


Figure 4.6. Percentage of M-cad$^+$ cells expressing CD56. *Cells in the M-cad$^+$ gate were further analysed for their expression of CD56. Results are represented as mean percentages ± standard error of the mean. Significance was considered as $p < 0.05$. Cultures were isolated from 3 rats, pooled and analysed in different passages.*

4.4.3. iv) Expression of desmin, a structural protein

Desmin, considered a myoblast maturation marker, was expressed in more cells than the membrane proteins CD56 and CD34. Taking all groups into account, average percentages ranged from 66% in the GSE 3d group to 95% in the GSE 14d group. Although desmin was much higher in the GSE 14d group (95.4± 1.3%)

compared to the GSE NI group (83.7 ±4.0%), the difference was not quite significant (p=0.07). No other significant differences were found (see Figure 4.7).

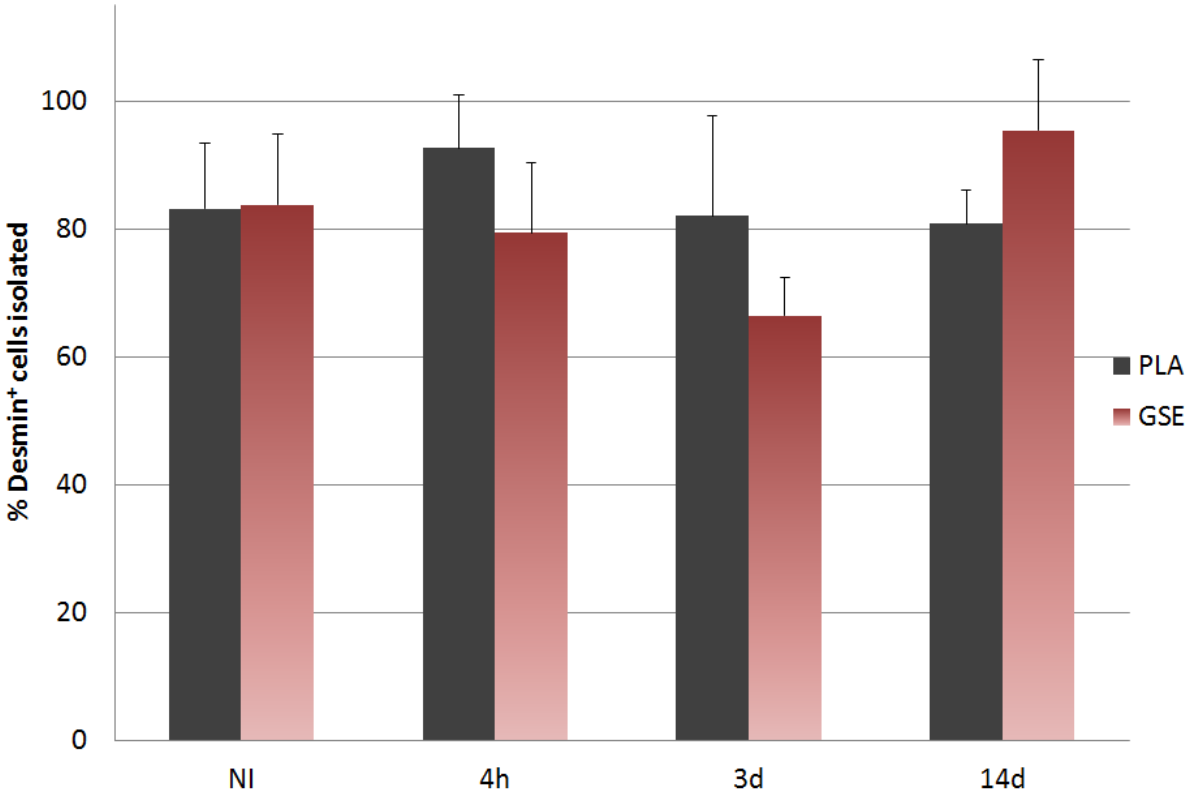
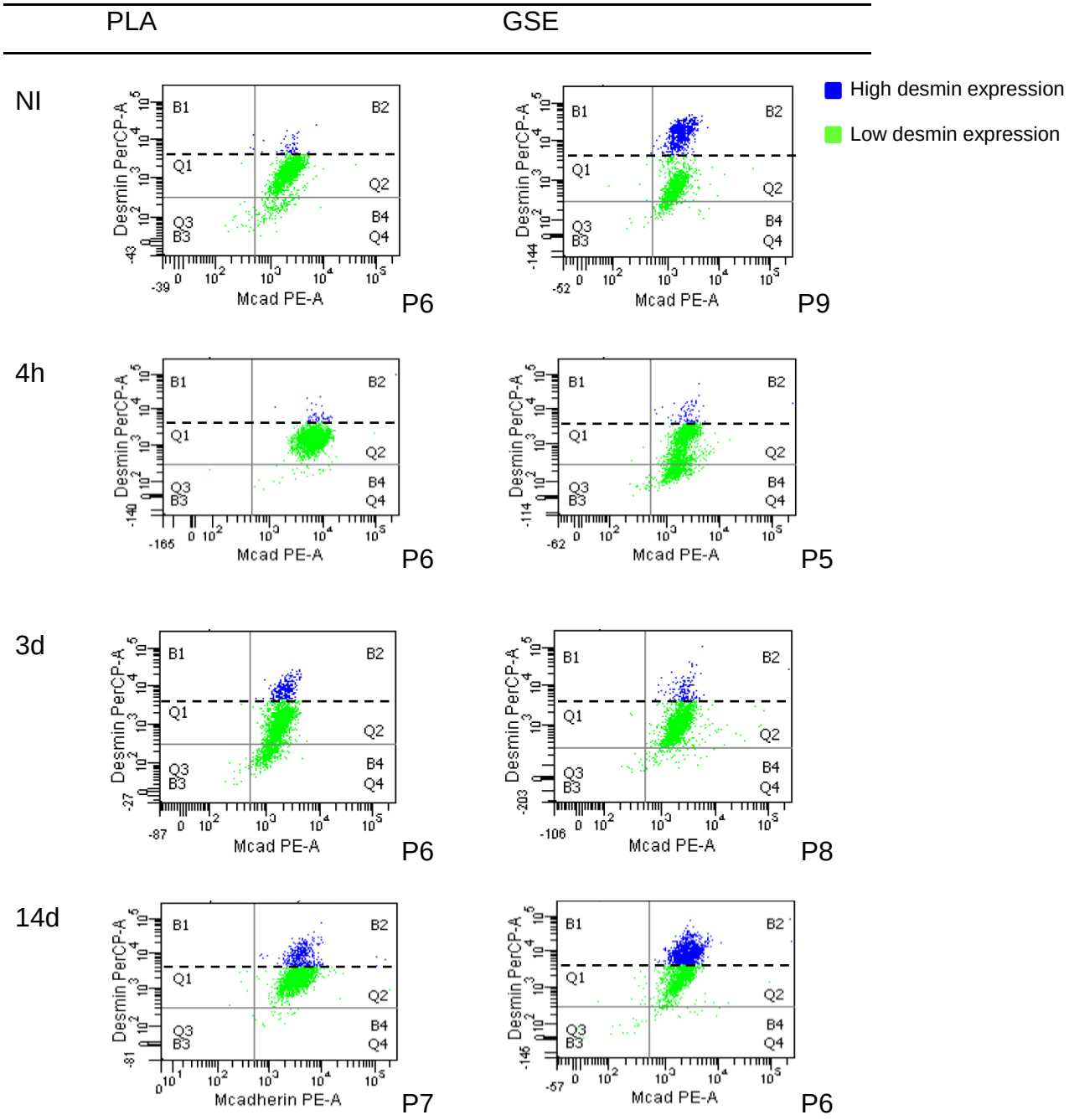


Figure 4.7. Co-expression of M-cad and desmin in isolated myoblasts. *Results are represented as mean percentages ± standard error of the mean. (GSE NI vs. GSE 14 d, p=0.07). Cultures were isolated from 3 rats, pooled and analysed in different passages.*

Also of interest was that desmin$^+$ cells could be divided into those expressing high levels of desmin and those with low expression of desmin. A visual assessment of flow cytometry dot plots indicated that this was mainly evident in the GSE groups in the NI and 4h groups and to a lesser extent in the 3d and 14d groups (see Figure 4.8).

Figure 4.8. Two distinct desmin expressing populations. *The GSE NI gate parameters were used for all groups (see dashed horizontal line).*



Abbreviation: P – passage.

In the placebo groups only a single population could be identified at early time points, whereas a certain level of high and low desmin expression at the same time point emerged at 3d and 14d post injury. Using gating parameters of the GSE NI

group, the number of myoblasts expressing desmin at these high intensities was determined and compared for all groups (see Table 4.6). The GSE NI group had significantly more cells expressing high levels of desmin than the PLA NI group. Figure 4.9 presents the percentages of myoblasts in the NI and 14d which express desmin at high intensities.

Table 4.6. Percentage of myoblasts which expressed desmin at high intensities. *High desmin-expressing cells were determined by a gate distinguishing between two distinct populations of desmin$^+$ cells. Significant differences were accepted as p <0.05. Cultures were isolated from 3 rats, pooled and analysed in different passages.*

	PLA (%)	GSE (%)
NI	3.37 ± 0.89 *	30.87 ± 6.26 *
4h	2.75 ± 0.39	7.53 ± 3.35
3d	9.10 ± 2.82	7.13 ± 0.85
14d	8.75 ± 2.75	29.18 ± 9.20

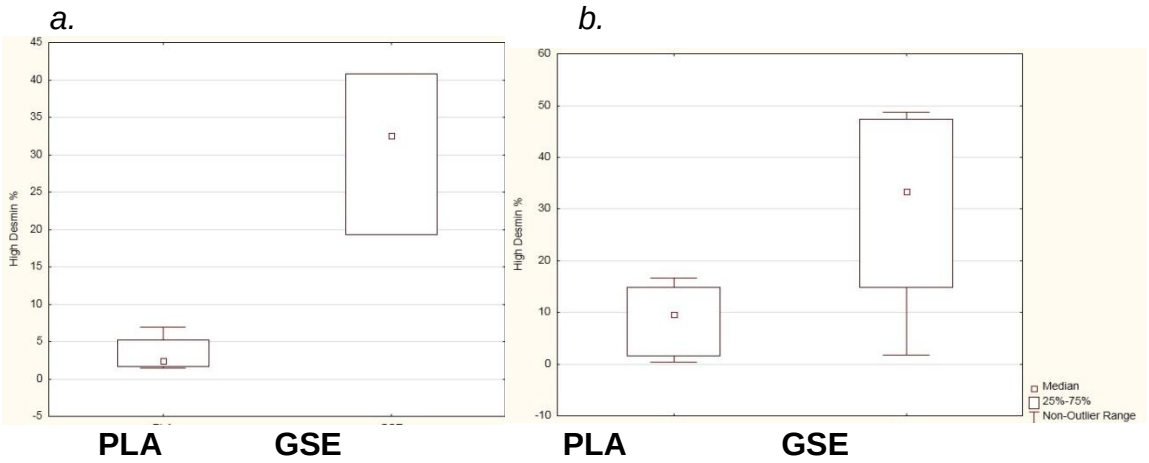


Figure 4.9. Box and whisker plot for proportions of myoblasts expressing high desmin levels. *a) Non-injured and b) 14d post-injury groups.*

4.4.3. v) Expression of progenitor cell marker CD34

On average, the proportional expression of the membrane protein CD34 (see Figure 4.10) was quite similar to expression of CD56, with average percentages ranging

from 16% in the GSE 3d group to 41% in the PLA 3d group. Due to a large variance in most groups, no significant differences were found between the treatment groups or between time points within treatment groups. However, the tendency for the two treatment groups to differ was seen at 4h and 3d which is a similar pattern to that seen for CD56. Lack of significance at these time points are more likely due to the greater proportion of cells expressing CD34 at NI and 14d in both groups compared to CD56.

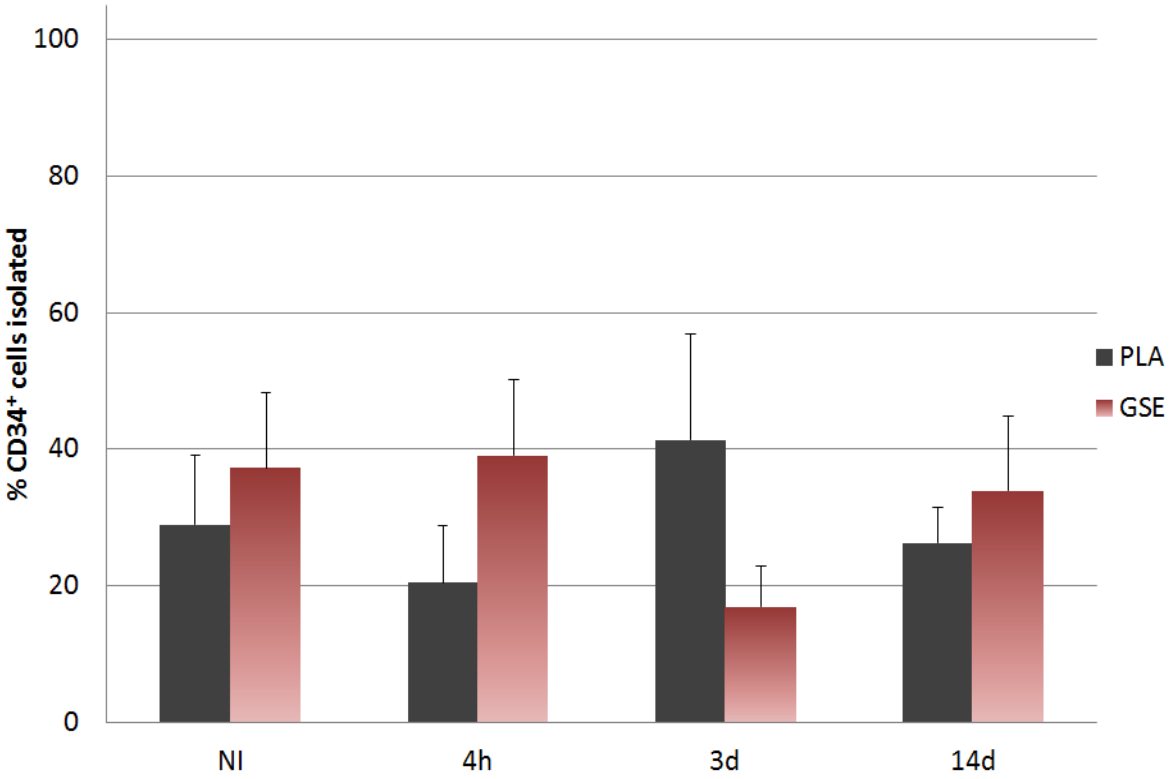
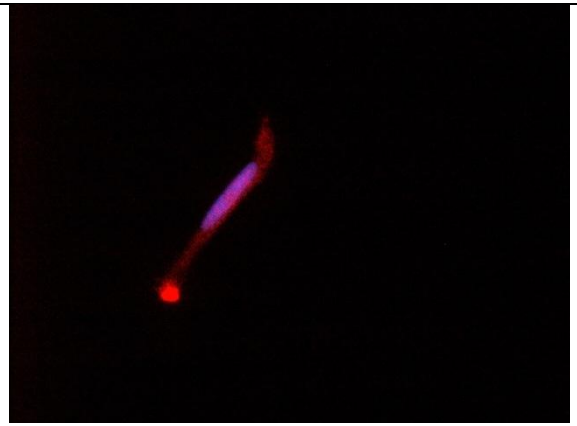
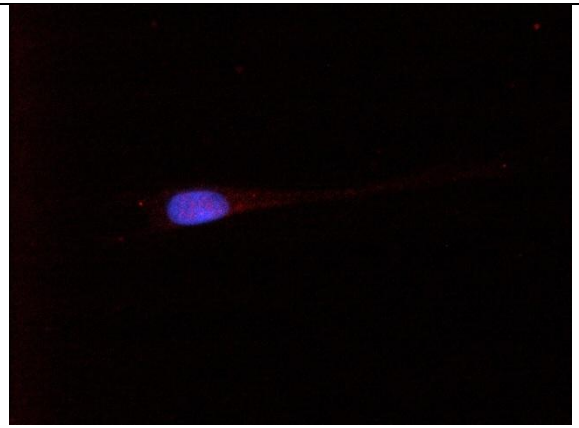
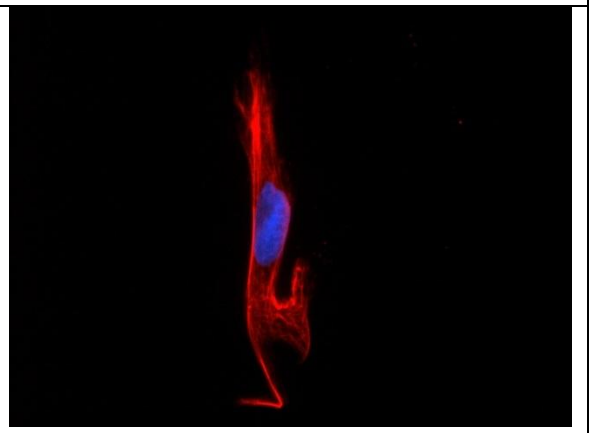
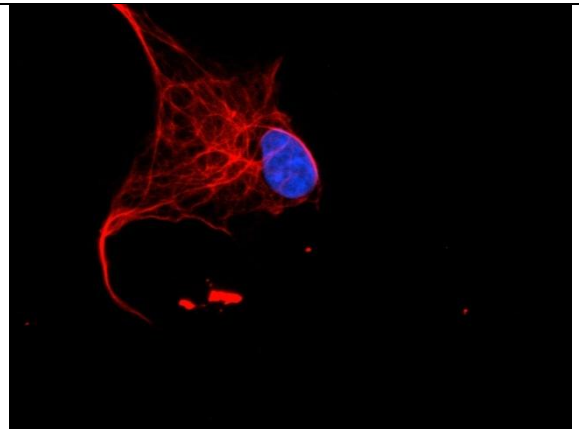
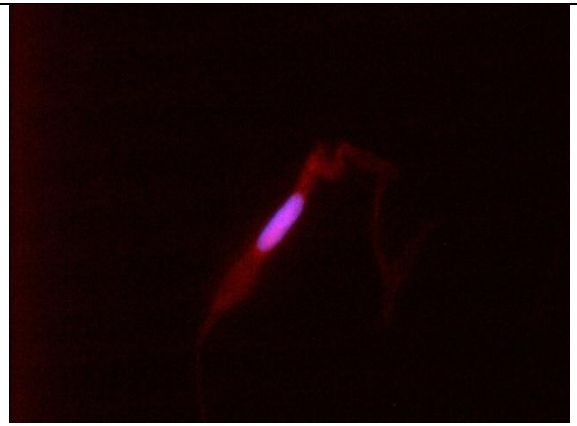
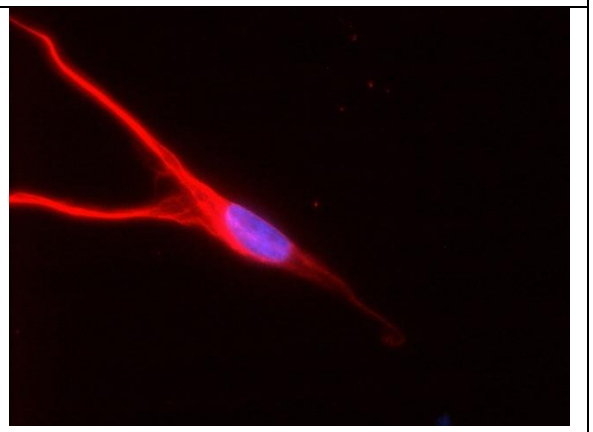


Figure 4.10. Percentage of M-cad$^+$ cells expressing CD34. *Cells in the M-cad$^+$ gate were further analysed for their expression of CD34. Results are represented as mean percentages ± standard error of the mean. There were no significant differences between time points, or groups at each time point ($p > 0.05$). Cultures were isolated from 3 rats, pooled and analysed in different passages.*

Figure 4.11. Desmin expression in the CD56^{+}/CD34^{-}, CD56^{-}/CD34^{+} and CD56^{+}/CD34^{+} sorted populations.

	CD56^{+}/CD34^{-}	**CD56^{-}/CD34^{+}**	**CD56^{+}/CD34^{+}**
PLA			
GSE			

4.4.4. Sorted myoblast populations

Myoblasts from the early time points (4h and 3d) after injury were sorted according to their expression of both membrane proteins CD34 and CD56. Three cell populations for each group were collected, namely i) $CD56^{+}/CD34^{-}$, ii) $CD56^{-}/CD34^{+}$ and iii) $CD56^{+}/CD34^{+}$. These cells were then cultured for 24 hours before being fixed and stained for desmin expression. Representative images of desmin expression in cells sorted according to the three different profiles are depicted in Figure 4.11. These images indicate both a range in morphology and qualitative differences.

Desmin expression was variable in all sorted groups, but more strongly stained desmin cells were observed in $CD56^{+}/CD34^{+}$ and $CD56^{+}/CD34^{-}$ cells than $CD56^{-}/CD34^{+}$ cells, indicating that CD56 and desmin marked more differentiated cells. When cells only expressed CD34, but not CD56, desmin was only expressed in low levels. Since cells were relatively widely separated, immunocytochemistry observations were only considered qualitatively.

4.5. DISCUSSION

In this study, primary myoblasts were successfully isolated and cultured from rats chronically supplemented with placebo or grape seed extract and subjected to a standardised contusion injury to the *gastrocnemius*. Myoblasts were isolated from three different rats per group and pooled, resulting in one initial culture per experimental group that was analysed after subsequent sub-culturing in different passages. Some significant differences were found, but a limitation is that these differences might reflect variations of primary cultures instead of the effects of *in vivo* intervention. However, some of the results that will be discussed that indicated an effect of GSE on primary myoblasts harvested from contusion injured muscle reflect findings of previous studies using an in vivo model, To confirm the results and findings of this study, at least another five to six animals per experimental group should be used in order to get an individual isolation for each.

Flow cytometric analysis confirmed that these isolated cells were expressing known myoblast proteins: Pax7, MyoD and M-cadherin. The main preliminary findings were that: a) Injury favoured adhesion competence of satellite cells during isolation for several days after injury, but injury and polyphenol supplementation reduced myoblast yield. b) A very low proportion of these committed myoblasts expressed the

cell surface protein CD56. c) *In vivo* polyphenol (grape seed extract) supplementation prior to muscle contusion injury significantly altered the time course of CD56 expression with an earlier peak in myoblasts isolated from the supplemented group (4h). d) Although not all myoblasts expressed desmin, the proportion of desmin-expressing cells for most of the groups reached ± 80%, while the intensity of desmin expression indicated different levels of maturity. e) Despite seemingly similar percentages of $CD34^+$ cells compared to $CD56^+$ cells for the groups, this was not indicative of co-expression, since cells could be sorted into three different populations (expressing only one of the transmembrane proteins, or co-expressing both).

4.5.1. Pax7 and MyoD expression confirmed identity of isolated cells

There were three lines of evidence that confirmed the purity of myoblast cultures. The first was the expression of myoblast specific transcription factors, secondly the absence of fibroblasts and thirdly, the expression of muscle specific cadherin.

It is known that injury mobilises satellite cells and if the damage is severe enough one would expect to find activated satellite cells in the interstitium between fibres, released from under the basal lamina [117]. Other non-satellite cells with myogenic capacity have been identified in the interstitium as well, such as side population cells, pericytes and stem cells derived from bone marrow [256, 273]. Myburgh *et al.* reported that numerous mononucleated cells populated the injury zone after contusion, most of which were inflammatory cells, with satellite cells found mainly in the border zone [203]. Pax7, a satellite cell specific paired box transcription factor, was used to confirm identity in tissue sections in a previous study, whilst in the current study primary cells were identified *in vitro*. Almost all cultured cells expressed Pax7, which is known to be expressed by satellite cells, either in a quiescent or activated state [246, 314].

Quiescent satellite cells do not express muscle regulatory factors (MRFs), but when stimulated and activated, satellite cells will enter the cell cycle, proliferate and subsequently express these transcription factors [17]. Almost all the cultured cells expressed MyoD, the earliest MRF, typically already expressed during proliferation, indicating that these cells were activated and most likely proliferating even prior to isolation [21, 68, 265]. Srikuea *et al.* [265] indicated an early increase in $MyoD^+$ cell count post-contusion injury and a significant increase in MyoD mRNA expression

(6h), while Hollnagel *et al.* [115] also found an upregulation of MyoD protein expression within hours after cardiotoxin injury. These results are in correlation with the higher cell yield in both groups in our current study at 4h post-contusion. However, factors other than proliferation influenced yield in the current study.

The proportion of satellite cells isolated in this study is higher than that reported in many other studies [25, 13, 155, 166, 319]. This was probably due the choice of myogenic markers. In many of these studies, desmin expression is analysed and not Pax7, MyoD or M-cadherin. Here we showed the desmin was not expressed in all Pax7+ cells. Another possible reason for differences could have been the media choices, as many of these studies used DMEM media [13, 155], which is not the optimal growth medium to purify myoblast cultures. Also, in this study, cultures were plated on non-coated dishes for a short period of time between passages (P1-P3) to allow any fibroblasts still present to adhere and be eliminated from the continued culture.

The deposition of collagen by infiltrating fibroblasts results in fibrosis and scar tissue *in vivo*. Therefore, in the presence of a contusion injury it is reasonable to expect activation and proliferation of fibroblasts in the injured area [158]. Indeed, many studies report the presence of fibroblasts in primary myoblast cultures [202, 319]. However, fibroblasts have properties distinct from myoblasts, such as *in vivo* adhesion properties. Even *in vitro*, fibroblasts adhere more easily to a range of surfaces and substrates, an inherent property which can be used to advantage *in vitro*. To eliminate possible fibroblast contamination in the myoblast cultures, cells were plated on non-coated culture dishes before pre-plating and between the first few passages (P1-3). The best option to confirm absence of fibroblasts would have been to stain for a fibroblast specific marker. Although a few candidate markers have been identified, such as FSP1 [270], MAb AS02 [244], TE-7 [100] and ER-TR7 [33], none are exclusive markers for fibroblasts in skeletal muscle. Nevertheless, the positive expression of myogenic transcription factors, Pax7 and MyoD in almost all satellite cells, confirmed the purity of these myoblast cultures.

Finally, at the conclusion of isolation, all myoblasts harvested for all time points, also expressed the muscle specific M-cadherin. This finding will be discussed below.

4.5.2. Contusion injury affects adhesion competence and therefore cell yield

Due to the increased satellite cell number reported after muscle injury *in vivo*, the yield of myoblasts isolated from injured skeletal muscle would be expected to be greater compared to non-injured muscle. Of importance is that only adhesion competent cells adhere to the extracellular matrix protein scaffold (entactin-collagen-laminin) on the culture plates in this study. Changes in expression of adhesion molecules would ultimately determine the number of primary myoblasts harvested and kept for further cultures in the current study. The following paragraphs will discuss published findings to support the hypothesis that the changes in expression of adhesion molecules by satellite cells after muscle contusion injury may affect the yield of primary myoblasts at different stages after injury.

Adhesion molecules belonging to the integrin family are possible candidates influencing adherence. Integrin-α7β1 is known to be expressed specifically in skeletal muscle and binds selectively to laminin [262], a main constituent of the basal lamina *in vivo* and also a main ingredient in E-C-L, the coating used on our culture dishes. As a muscle-specific transmembrane protein, this integrin is also expressed by satellite cells [25]. However, Sorokin *et al.* found that only a minor fraction of myoblasts expressed the integrin α7-chain after crush-injury. Rather an increase in integrin β1-chain was associated with myogenic cells in the interstitium [263]. Therefore the current study focussed on a calcium-activated adhesion molecule, cadherin, which has a muscle specific isoform, M-cadherin. *In vivo*, M-cadherin positive cells can increase as much as 5-fold within 4 hours after contusion injury [203]. The extent and timing of this increase suggests that M-cadherin cell count is not influenced by proliferation, but by M-cadherin expression itself.

M-cadherin is usually involved in homophilic binding in cell-cell interactions [115]. Since the culture dishes were coated with E-C-L only, the absence of M-cadherin in the coating makes it highly unlikely that cells adhered to plates due to M-cadherin only. To date there has been no indication of another binding protein for M-cadherin than M-cadherin itself, but Irintchev *et al*. suggested a possible cross reaction of the M-cadherin with other proteins present in the sarcolemma. Although no other literature could be found supporting this suggestion, this possibility cannot be ruled out [120]. Alternatively, the elevated M-cadherin coincides with elevation of several other adhesion proteins influencing both *in vivo* and *in vitro* properties.

In vivo, cells expressing M-cadherin remain above levels seen in non-injured rats for at least four days after contusion injury [142] and this is the most likely explanation for an altered cell yield *in vitro*. However, high adhesion competence played a role in the increased yield mainly in PLA groups.

4.5.3. Adhesion competence is altered by GSE treatment

The influence of grape seed extract (GSE) on satellite cell response to contusion injury has been determined *in vivo* in models of chronic supplementation [203] and acute supplementation [143]. For most time points there was a strong resemblance between cell yield *in vitro* for the current study and M-cadherin expression *in vivo* in the previous studies. GSE significantly reduced the presence of M-cadherin expressing cells in the injury border zone particularly with chronic supplementation [142]. This difference may explain reduced primary myoblast yields in the GSE treatment groups which occurred at all time-points except 4h (see Table 4.2). Even in the NI groups, the cell yield was approximately 7-fold lower in GSE despite the fact that the cells were culture for two additional days.

In contrast with results of the PLA groups, cell yield was greatly reduced by 3d. Such an overriding low yield of myoblasts could be expected to correlate with incomplete recovery of damaged tissue. However, Kruger *et al.* showed with histological analysis that GSE treatment significantly improved muscle recovery after contusion injury. This indicates that lower expression of adhesion molecules may actually be beneficial *in vivo*, possibly due to an effect on maturation or fusion, or both. Fetal myosin heavy chain analysis in the *in vivo* study by Kruger, indicated new myofibres were already present 3 days post-injury, but only in the GSE group. However, the current results suggest a large influence of GSE on adhesion molecule expression on satellite cells irrespective of injury, with an effect of injury overriding the effect of GSE only for a few hours post-injury.

Supporting this theory is the evidence that GSE attenuates expression of adhesion molecules in the vascular system. A component of grape seed extract, activin, was shown to significantly reduce the expression of VCAM-1, ICAM-1 and E-selectin in systemic sclerosis patients [131]. These results were supported by Ma *et al.* [173] who also found a reduction in VCAM-1, correlating with the reduction in von Willebrand factor (an endothelial cell marker). Satellite cells also express VCAM-1

[217], which should be considered in future studies in the context of skeletal muscle injury.

4.5.4. Cell yield could also be influenced by proliferation

When satellite cells are released from their niche under the basal lamina, they are exposed to activation signals and do not remain in a state of quiescence [68, 90]. Severe injury disturbs the basal lamina and activates satellite cells which enter the processes of proliferation and maturation, prior to fusion and subsequent muscle repair. Most studies in rodent models report a significant increase in satellite cell number from 15 hours [251] until 3 [143, 181, 233, 282] or 4 days [118] after contusion injury and increased expression of fetal myosin heavy chain, an indication of newly formed myotubes, approximately a week post-injury [143]. The cell yield in PLA groups in the current study already increased at 4h and increased even further by 3d, possibly indicating an early effect of injury on expression of adhesion molecules and an additive effect of proliferation for the following days *in vivo*. These factors influence the yield during the isolation procedure, while further proliferation is stimulated *in vitro* by the subsequent culturing of myoblasts under proliferation-promoting conditions. One of the questions the current study poses is whether or not GSE supplementation continued to have an effect on myoblast behaviour *in vitro*.

Literature suggests GSE enhances proliferation of precursor cells. Increased proliferation in response to *in vitro* GSE supplementation was evident in primary mouse hair follicle cells [272] and proliferation of mouse neuroblasts in the hippocampal *dentate gyrus* increased *in vivo* in response to chronic supplementation with GSE [309].

One of the effects of GSE is that it reduces the release of TNF-α [11], thus indirectly reducing the upregulation of transforming growth factor-β (TGF-β) and myostatin [205], which are downstream targets of TNF-α. TGF-β and myostatin suppress satellite cell proliferation and differentiation [265]. GSE could relieve inhibitory effects on satellite cells, leading not only to increased proliferation but also to earlier maturation and differentiation.

Before starting the current study, we hypothesised that an investigation of maturity level of isolated myoblasts may provide a better understanding of the effect of GSE on the time course of regeneration. The neural cell adhesion molecule (NCAM or CD56) is involved in cell-cell adhesion during fusion [50], while the structural protein

desmin is important in both sarcomere organisation during adult myogenesis [162, 254, 311], and linking of adjacent sarcomeres at their Z-discs [102]. Therefore, the expression of these two proteins in isolated myoblasts will be discussed in the next section.

4.5.5. Cellular indicators of myoblast commitment and maturity

4.5.5. i) CD56 expression was limited in isolated myoblasts

Neural cell adhesion molecule (NCAM or CD56) is rarely detectable in quiescent satellite cells, but is expressed in myogenic committed cells [120]. It has been used to isolate and purify myoblasts from human craniofacial muscle. However, in our study, its expression was not consistent in all primary myoblasts, with an average expression of CD56 in M-cad$^+$ cells ranging from 11-38% across all groups. This was a rather unexpected result as M-cadherin and CD56 both indicate fusion and differentiation competence in myoblasts. However, Irintchev *et al.* also showed that M-cadherin expression differed significantly from CD56 expression, with M-cadherin, an adhesion molecule expressed already early after activation [120]. Ishido *et al*. proposed that CD56 and M-cadherin expression is induced by different mechanisms. They differ functionally since CD56 is a Ca^+-independent adhesion molecule and M-cadherin is a Ca^+-dependent adhesion molecule. Also, CD56 is a transmembrane protein in myoblasts, whereas it only has an extracellular domain in myofibres. The time-frame during which the different CD56 isoforms were expressed could be responsible for the seemingly variable expression in relation to M-cadherin [122].

Capkovic *et al*. reported CD56 expression in primary myogenic cells committed to differentiation [45]. After differentiation was initiated, CD56 expression increased significantly. The initial percentage of isolated cells in their study was approximately 11%, which is comparable to the percentage CD56$^+$ cells in the PLA NI group in the current study. Myoblasts continued to expand in proliferation media in our study, which could explain why CD56 expression was never elevated above approximately 40%.

As discussed earlier, after contusion injury, myoblasts are activated to proliferate and fuse to form new myofibres. CD56 expression should increase along the time course of muscle repair before the presence of new myofibres. Our results showed a significant increase between PLA 4h and PLA 3d, indicating increased maturity of myoblasts in preparation for fusion. Kruger *et al.* (2012) reported that new myofibres

were evident by day 7 after injury. Also, grape seed extract caused a left shift in expression of satellite cell proteins (CD56, CD34 and Pax7) in comparison with PLA groups, indicating accelerated response to contusion injury. The significant difference between the proportions of $CD56^+$ cells between PLA 4h and GSE 4h in the current study supports the previous *in vivo* results, even though myoblasts were isolated just once and analysed in different passages. The commitment of myoblasts also results in expression of relevant intracellular proteins. For example, Vaittinen *et al.* found intermediate filament proteins to be expressed 6h post contusion injury [288].

4.5.5. ii) Separate desmin expressing populations emerged

Another possible indicator of myoblast maturity is the intermediate filament protein desmin. Vaittinen *et al.* showed that desmin expression increases significantly during myogenesis and remains high in mature myofibres, where it would locate near the Z-discs of sarcomeres to keep myofibrils intact [288]. In the context of muscle injury, myofibres are destroyed. After a single bout of eccentric exercise, desmin decreased, but significantly increased by 3 days post-exercise [15].

In this study, the expression of desmin by $M\text{-}cad^+$ cells was found in a majority, but not all cells. As previously discussed it is highly unlikely that $desmin^-$ cells were fibroblasts, evident from our Pax7, MyoD and M-cadherin results. Due to the activation and maturation expected after contusion injury, the proportion of desmin-expressing myoblasts was expected to increase after contusion injury. However, no significant differences in percentage $desmin^+$ myoblasts were evident between PLA NI and any of the PLA or GSE time points post-injury. Therefore, desmin was probably expressed in the majority of cells early after activation, as reported before [3] and in approximately 80% of adhesion competent cells as reported here.

However, two distinct populations of $desmin^+$ cells could sometimes be distinguished. In the PLA groups, this population was barely noticeable, only to a small extent in the 3d and 14d post-injury groups. At these time points, more maturation was expected. In contrast, a distinct high desmin-expressing population of cells was evident in the GSE NI group as well as the GSE 14d group. Support in literature for the existence of a subpopulation of early maturing myoblasts was the presence of myogenin mRNA as early as 6 hours after a contusion injury and desmin expression in the next 6 hours. This was still before the first doubling of the cell population was evident, which is believed to occur 24 hours post-injury [232, 265]. We have shown a second

population which expresses high levels of desmin for the first time with flow cytometry. In the 4h group this phenomenon was only observed in some samples but not in any samples from the 3d group. The absence of a high-desmin population in the GSE 3d group may indicate that these cells have already been incorporated into existing myofibres or newly formed myofibres *in vivo*, resulting in low harvest myoblasts expressing desmin with high intensity at this time point. By 14 days post-injury the satellite cell niche had been replenished in the GSE group and presence of this second population could be observed again, most likely destined to fuse with the young myofibres rather than with each other or myotubes.

Although the presence of a high-desmin expressing cell population is reduced in the GSE 4h group, the fact that CD56 expression was higher than in PLA indicated that a significant percentage of isolated cells in this group were still more mature compared to PLA. This indicates that desmin expression cannot be accepted as the gold standard evidence of myoblast maturation. A question that remained was whether or not co-expression of desmin and CD56 represented a subpopulation of myoblasts with a more ordered desmin phenotype suggesting a low potential for further proliferation.

It has been shown that some satellite cells are slowly dividing in contrast with a fast dividing sub-population [230]. This could possibly be explained by different origins, since not all satellite cells have been resident in the muscle since birth. Cells which express CD34 can replenish the satellite cell niche in adult skeletal muscle.

4.5.6. Relationship between CD56, desmin and CD34

The relation between CD56 and desmin expression, and possible different origins or localisation of distinct isolated myoblast populations, was investigated by sorting myoblasts from the 4h and 3d post-injury in both PLA and GSE treatment groups for CD56 and CD34 expression and then staining cells with a desmin antibody.

Wei *et al*. showed with flow cytometry that only 5.34% and 4.58% of a $Pax7^+$ human primary cell population isolated from omohyoid muscle expressed CD34 and CD56 respectively [297]. Sinanan *et al*. found that only 5-10% $CD56^+$ cells also expressed CD34 [257]. After sorting in the current study more myoblasts were $CD34^+$ than $CD56^+$. It was perhaps surprising that CD34 expression persisted so long, but this was also observed by Beauchamp *et al.* [17].

The reason for this discrepancy might be that a relatively low number of cells were isolated and the number of myoblasts that survived after sorting was limited. Another possible explanation could be based on the species difference (human vs. rat) as well as the different type of skeletal muscle from which the cells were isolated (*craniofacial* vs. *gastrocnemius*). However, the gating strategies between positive and negative populations for both CD34 and CD56 in the study by Sinanan were different from the current study [257].

The localisation of desmin in sorted cells was also surprising. When desmin was expressed in low levels it was usually found in close proximity to the nucleus, but when high levels of desmin were observed, desmin filaments were spanning from the nucleus through the cytoplasm to the cell periphery. In rare cases a network of filaments were observed, but in cells with a bipolar shape, desmin filaments were mostly in parallel with the direction of the cell. Most cells showed a typical bipolar phenotype as observed by Sorokin *et al.* [263].

Changes in desmin morphology have been reported before in mouse decidual cells, a population of multinucleated cells formed outside the implanted blastocyst in the placenta of pregnant mice. Desmin localised around the nucleus, forming a cap in early formation of these cells, but within a few days of their differentiation, desmin formed a network, radiating from the nucleus to the cytoplasm, whereafter it only concentrated at the cell periphery. The latter suggested that desmin may have been involved in adherence type intercellular junctions [209]. It is clear that the localisation of desmin filaments need to be considered when the maturity level of a cell is determined. Desmin localised in close proximity to nuclei might reflect the production of desmin on ribosomes, with subsequent trafficking to the cytoskeleton.

The co-expression of CD56 and CD34 in a subpopulation of sorted cells, which mostly expressed desmin, was unexpected. However, others have reported CD56 expression in interstitial cells in the skeletal muscle niche, as well as co-expression with CD34 [222, 307].

A better understanding of the cells expressing CD34 and their contribution to myogenesis after injury is required.

4.5.7. CD34 expression by a proportion of primary myoblasts

CD34 is a highly glycosylated transmembrane cell surface molecule [230]. The origin and location of $CD34^+$ cells in skeletal muscle is a field of research of its own, but relevant issues will be discussed below. Without an understanding of these issues, it would be difficult to interpret the current study results regarding the effect of GSE.

4.5.7. i) Location of CD34 expression in skeletal muscle

Literature suggests that CD34 identifies the interstitial myogenic progenitor population. A population of cells upstream of $CD34^+$ cells, which generate $CD34^+$ cells via non-symmetric division, have been found in the muscle interstitium and have the potential to later express Pax7 [274]. In contrast to these findings, CD34 also stains satellite cells under the basal lamina, as shown by Beauchamp *et al.* [17]. Myburgh *et al.* (2011) and Kruger *et al.* (2012) only investigated cells in the traditional satellite cell niche under the basal lamina and found a small percentage of satellite cells to express CD34.

The *in vivo* location of the $CD34^+$ cells isolated in the current study is unclear. Satellite cells are released from their niche during injury, but also through the isolation process. Therefore, interstitial cells are isolated in conjunction with satellite cells. A higher than expected expression of CD34 in all groups indicated the possibility that many myogenic cells from the interstitium were isolated. However, it could be that both interstitial $CD34^+$ cells as well as $CD34^+$ cells from the satellite cell niche have been collectively analysed in this study. The average number of $CD34^+$ cells in the isolated populations ranged from 16%-41% in different groups with more in the GSE group compared to the PLA group, adding to the cell yield in this group.

Two findings from other studies are also important in relation to the current study. It has been shown that isolated cells which were $CD34^+/Pax7^+$ were more adherent than $CD34^+/Pax7^-$ cells [275], a result supported by a similar study by Dupas *et al.* [84]. Due to the fact that only adherent cells were isolated in our protocol, all $CD34^+$ cells isolated also expressed Pax7, which is in agreement with the finding of Tamaki *et al.*

Another possibility for the high numbers of $CD34^+$ myoblasts *in vitro* could be the effect of passage. Machida *et al.* found that only 10% of isolated rat myoblasts express CD34 initially in culture, but this number increased to 30% after the third passage. However, they also found a decrease in Pax7 with higher passages, in

contradiction to our results [174]. An increase in CD34 expression after a few passages might be explained by the presence of stem cell-like cells with higher proliferation capacity [230]. The analysis in this study was performed on cells from passage 4 to 10, but there were no clear indication that passage number influenced CD34 expression.

Although there were no statistically significant differences amongst any of the groups in terms of the number of $CD34^+$ cells, a slightly higher number of $CD34^+$ cells in the GSE muscle could be a result of better access to the interstitium *in vivo* due to treatment. GSE is known to enhance production of nitric oxide, which would cause vasodilation in the injured area, leading to increased blood supply with circulating progenitor cells, like $CD34^+$cells [147, 193, 299]. GSE has been reported to upregulate vascular endothelial growth factor (VEGF) which is responsible for angiogenesis and improvement of membrane permeability, which also suggests circulating progenitor cells may have better access to the injury site [136]. Because of these factors, it would be important to establish the lineage specificity of the isolated myoblasts in future studies. This was not a focus of the current study, thus only a short summary will be provided here.

4.5.7. ii) Lineage specificity of CD34

CD34 is expressed by hematopoietic stem cells, but an increasing number of studies report the existence of a $CD34^+$ cell population with the potential to differentiate into tissues from the mesenchymal cell lineage [84, 147]. To distinguish between haematopoietic origin, classical stem cells and $CD34^+$ muscle progenitors, co-staining with several markers is required.

When CD34 is co-expressed with CD31, an endothelial marker, cells have angiogenic potential with no or little myogenic potential. The more classical stem cells, the $CD34^+/CD31^-/Sca1^+$ cells showed adipogenic potential. $CD34^+/CD45^+$ cells do not exhibit angiogenic, adipogenic or myogenic potential. In contrast $CD34^+/CD31^-/Sca1^-/CD45^-$ cells have high myogenic potential and may express muscle specific transcription factors Pax7, myf5 and MyoD, as well as membrane protein integrin-α7 [84], suggesting hematopoietic origin, but current satellite cell identity.

Many more studies have ruled out the possibility that all $CD34^+$ cells isolated after injury are from the interstitium or circulation. Both LeGrand *et al.* [157] and Sacco *et al.* [245] sorted for $CD34^+$/integrin-$\alpha7^+/CD31^-/CD45^-/CD11b^-/Sca1^-$ and found >95%

of these cells to be $Pax7^+$. Since integrin-α7 is specific to satellite cells [262], this is not surprising. It also does not imply that all integrin-$α7^+$ or $Pax7^+$ will be $CD34^+$.

4.5.7. iii) Time-dependent expression of CD34

A third consideration contributing to the controversy around CD34 expression is the time-dependent expression of this protein *in vivo*. Due to its association with stem cells, it is believed that it is an early marker expressed in progenitor cells of many tissues, including fibroblasts and endothelial cells.

Dreyfus *et al*. showed that GFP^+ tagged bone marrow-derived stem cells will incorporate into the interstitium of skeletal muscle and express CD34 within months after transplantation, and later incorporate into the satellite cell niche expressing myogenic proteins M-cadherin, Pax7 and CD56 [82]. After isolation of adult mouse skeletal muscle satellite cells, Qu-Peterson *et al.* showed that 83% of early pre-plated mouse cells were $CD34^+$ and expressed desmin, while no cells expressed CD45 (haematopoietic origin). MyoD, M-cadherin and myogenin were variably expressed, probably due to their time-dependent expression [230].

Che *et al.* separated cells isolated from rat hind-limb skeletal muscle using a Percoll density gradient system and found $CD34^+$ cells and $Pax7^+$ cells in the less dense fraction compared to M-cad^+ cells, indicating that all myogenic markers are not always simultaneously expressed. Since Sca-1, the stem cell marker, was expressed by cells in all levels of the gradient some of these muscle derived cells were probably infiltrating stem cells with high proliferating capacity [52].

The replenishment of skeletal muscle satellite cells is triggered by muscle damage and mobilisation of $CD34^+$ cells into the blood stream has been shown after strenuous exercise [193]. The mobilisation of $CD34^+$ stem cells with myogenic potential has also been reported in patients with acute myocardial infarction.

The turnover rate of CD34 in already committed tissue might add to many contradictory results. Beauchamp *et al*. found CD34 mRNA was present in undifferentiated myoblasts. CD34 protein expression did not change significantly after the onset of differentiation, but persisted beyond differentiation into myotubes [17]. The continued expression of CD34 in differentiated myotubes indicates that CD34 does not only identify myoblasts destined for proliferation and their role in pre-injured and post-injured muscle still deserves further exploration [17].

4.5.8. Summary and conclusion

The results of this study have shown that all isolated cells were indeed myoblasts, due to their expression of myogenic transcription factors Pax7 and MyoD, muscle specific adhesion molecule M-cadherin, and the skeletal muscle structural protein, desmin.

Contusion injury changed the expression of several proteins in these isolated myoblasts. Due to the nature of the isolation protocol, all adhesion competent myoblasts were harvested, resulting in almost pure M-cadherin^{+} populations. However, cell yields in the various groups differed indicating indirectly that adhesion competence was altered due to contusion injury. Also, the maturation of myoblasts was affected in a time-dependent way after injury, with a significant increase in myogenic committed myoblasts by day 3 after injury.

Firstly, chronic supplementation with grape seed extract altered the effects of contusion injury on myoblasts. Adhesion competence was attenuated by GSE, causing a low yield during isolation of myoblasts from injured skeletal muscle tissue at almost all time points in almost all GSE groups. This was in agreement with the earlier finding that a reduced number of myoblasts *in vivo* expressed M-cadherin, a muscle specific adhesion molecule, in skeletal muscle of GSE supplemented rats. Secondly, myoblasts exposed to GSE *in vivo*, matured earlier than PLA myoblasts, evident from the earlier peak in CD56 expression by 4 hours post-injury. Thirdly, myoblasts could be divided easily into two populations expressing desmin at lower or higher levels. The latter represented {{}}a population of early maturing myoblasts, and these were found in skeletal muscle of GSE treated rats, even if not injured. This indicated that GSE ingestion may have activated progenitor cells and primed them to respond faster to injury and to mature earlier for quicker muscle repair. These results should be confirmed by analysing more isolated myoblast cultures from more animals in each experimental group.

Although all isolated cells were myoblasts, the heterogeneity of the satellite cells raised further questions. Expression of CD34, a marker associated with a more primitive progenitor cell compared to the niche-bound satellite cell, was not significantly different amongst the groups, but the presence of a proportion of CD34^{+} myoblasts in each group, indicated that all satellite cells might not be from the same niche. It is still unclear whether CD34^{+} myoblasts were isolated from the interstitium

or if they were sub-laminal satellite cells still bearing this marker of their original lineage. CD34 is a traditional marker of haematopoietic lineage, but recent publications indicated that mesenchymal stem cells also express CD34, making this marker unreliable to determine cell lineage. Therefore, the co-expression of CD34 with proteins associated with maturation of myogenic cells requires more investigation, particularly in a model of moderate to severe muscle injury when it might be expected that SCs alone would be insufficient or sub-optimal for rapid regeneration.

Chapter 5 Conclusion and future prospects

5.1. SUMMARY OF THESIS FINDINGS

We have successfully established a method to harvest myoblasts from rat skeletal muscle by combining techniques from various available protocols. We found that commercially available growth media, Ham's F10 nutrient mix and DMEM, should be combined for optimal culturing of these primary isolated myoblasts. Also, we have shown that L-glutamine is an essential supplement to growth media, since the expansion of the primary isolated myoblasts was slower when this amino acid was absent from the growth media. Although intervention studies using the immortal cell line C2C12 is a useful tool for proof of concept studies, these cells differ in significant ways from primary myoblasts isolated directly from skeletal muscle. Therefore it is important to investigate regeneration in primary myoblast cultures.

Although the isolation method established in this thesis enabled investigators to harvest adequate numbers of myoblasts from muscle tissue after *in vivo* intervention studies, several challenges were still present (as with many other isolation protocols). These challenges will be discussed in 5.1.2.

The established method to isolate and culture primary myoblasts was then used to isolate primary myoblasts from contusion injured rat hind leg, after chronic *in vivo* supplementation of an anti-oxidant grape seed extract (GSE). From earlier studies it was clear that GSE supplementation modulated satellite cell numbers after contusion injury, as observed by immunohistochemistry. However, how increased numbers of SCs translated to earlier repair was not clear. We hypothesised that GSE had an effect on the progression from precursor cell to mature myoblast, and that this could explain the positive effect of GSE on the time course of recovery of skeletal muscle post injury [143, 203]. To determine the level of maturation of myoblasts *in vitro*, myoblasts were harvested at several time points after contusion injury, either from GSE or placebo (PLA) supplemented rats.

At almost all time points, a lower cell yield was obtained from GSE supplemented rats compared to PLA rats. Due to the fact that the isolation method used here relied on adhesion competence of myoblasts, a low yield indicated attenuation of adhesion competence. Further, an earlier peak in the proportion of CD56 expressing myoblasts indicated that GSE accelerated commitment of myoblasts to fusion. This may have affected yield but to a lesser extent. Although no significant differences were found in the proportion of cells expressing the structural protein desmin, a separate population of myoblasts which expressed desmin in high concentrations was present more frequently in GSE groups than in PLA groups.

On a cellular level we have shown that GSE modulates satellite cell activity during regeneration after a contusion injury. There are a number of possible mechanisms by which GSE could enhance their activity to improve recovery of the injured skeletal muscle. The results of the current study revealed several potential new avenues of investigation to follow. These will be discussed in 5.1.3.

5.2. SEVERAL CHALLENGES OF THE PRE-PLATE HARVESTING METHOD

Despite the various benefits of using primary myoblast cultures to investigate regeneration, these cells are removed from their *in vivo* micro-environment with its specific regulatory systems and influences. While quiescent satellite cells reside between the basal lamina and sarcolemma, primary myoblasts are much more exposed. When the basal lamina is compromised in any way *in vivo*, activation of the satellite cells occurs. Due to the nature of most isolation and culturing processes where the basal lamina is disrupted, the activation of quiescent satellite cells is inevitable [217].

The majority of satellite cells are expected to be quiescent in non-injured muscle, but, as expected, the isolation of satellite cells from this muscle leads to activation and MyoD expression. Also, the change in the micro-environment of satellite cells from the sub-laminal niche *in vivo*, to culture conditions *in vitro* might alter protein expression and influence downstream functions such as adhesion or fusion. Therefore, satellite cells should be investigated directly post-isolation to eliminate as many culture effects as possible.

A weakness of this study was that adequate numbers of myoblasts for analysis could not be obtained directly since the harvested myoblasts from injured muscle were

fewer than required. It was necessary to expand the populations for several passages before analysis, which could have led to some culture effects on the isolated myoblast populations. Nonetheless, despite the possibility that culture conditions could have created a more homogenous group of myoblasts despite *in vivo* differences, this did not appear to be the case. Despite the fact that culture conditions were kept constant, distinct differences were found between treatment groups, indicating that the different *in vivo* responses were still reflected in the isolated myoblasts.

Another weakness of the current study is that *in vitro* cell counts for many of the time points post-injury were done on different days after isolation, due to the differing proliferation rates. Therefore cell yield was not just determined by the original cell number harvested, but also the proliferation rate of harvested myoblasts. To determine cell yield more accurately, cell counts should be performed shortly after isolation to eliminate effect of enhanced proliferation in any of the cultures. We attempted to circumvent this problem by using cells from closely related passages (passage 5 to 8).

No expression of non-myogenic proteins was tested. Especially fibroblasts should be eliminated from the culture, so a specific fibroblast marker should be used to confirm no cells were fibroblasts. However, all myoblasts in this study expressed known myogenic markers. To further validate these results, additional negative control groups could be stained for Pax7, MyoD and M-cadherin. Such controls would include a non-muscle cell type, such as fibroblasts and using different isotypes of the selected antibodies.

5.3. FURTHER INVESTIGATIONS ON THE EFFECTS OF GSE ON SATELLITE CELL ACTIVITY

5.3.1. Adhesion

The altered adhesion competence we observed in the isolated primary myoblasts presents several opportunities for future investigations. The specific adhesion molecules involved in altered adhesion competence in response to GSE supplementation is not clear. It has been shown before that the muscle specific cadherin (M-cadherin) is reduced significantly in injured muscle from GSE

supplemented rats, however no co-staining was done in any of the previous studies. Cadherins are associated with catenins, which are thought to be mediators of cell adhesion linking the cadherins with the actin-based cytoskeleton [206]. Cadherin-catenin complexes can interact with cytoskeletal molecules, such as actin microfilaments or other associated proteins, for example α-catenin which can directly cross-link with the actin filaments and vinculin [135]. The effect of GSE on these interactions or the gene transcription of M-cadherin could be investigated.

However, the contribution of other adhesion molecules should not be excluded from such investigations. Another possible candidate involved in the attenuation of adhesion competence is integrin-α7β1, a muscle specific membrane protein from the integrin family. Integrin chains always act in pairs of alpha and beta chains, but the effect of an injury or intervention might alter the function or expression of these chains separately, as shown by Sorokin *et al.* [263]. Therefore, the effect of GSE on the expression of all candidate integrin chains in injured skeletal muscle could be investigated.

5.3.2. Satellite cell number: migration or proliferation

Supplementation with GSE led to improved recovery of a muscle contusion injury *in vivo* in previous studies. Increased satellite cell numbers were evident in those studies using immunohistochemistry of tissue sections. A possible reason for the increase in satellite cell number in the injured area could have been due to migration of distant satellite cells to the injured area, which may be influenced by the extent, type or cellular location of adhesion molecules expressed or the ability to generate leading edges. Accelerated migration would occur with attenuation of adhesion molecules at the rear of advancing myoblasts, as these adhesion molecules could cause resistance to migration, if too highly expressed. The effect of GSE on the migration of distant satellite cells to the injury site is not clear, leaving some scope for further investigation.

Another possible reason for increased satellite cell number in the injured site, is the proliferation of existing satellite cells. If this was the case, it would be expected that the proliferation rate of primary myoblasts isolated from GSE supplemented rats would have been enhanced. However, the cell yield from GSE rats in the current study did not reflect an increase in satellite cell number. This may not be a biological

finding, but may be due to the effect of GSE on adhesion competence of satellite cells, since only adherent satellite cells were isolated in the current study.

Also, many aspects of *in vitro* conditions influence cell behaviour. Myoblasts release pro-proliferation factors influencing the cells in their near vicinity [47, 250, 179, 267]. If cells in culture are spread widely, such as the myoblasts from GSE groups, the influence they would have on each other, would be attenuated. Therefore, differences in proliferation rate could be affected by both the *in vivo* treatment and *in vitro* culture density. An accurate determination of the proliferation rate of cells from all time points and both treatment groups would require an equal number of seeded cells, stained early after pre-plating with a proliferation marker such as PCNA or Ki67. This may require that more rats are sacrificed at each time point for enhanced yield in the GSE supplemented groups. Nonetheless, potential mechanisms whereby GSE may have an effect on proliferation and adhesion should be explored, including effects of growth factors and cytokines, such as FGF, HGF and interleukins.

5.3.3. Myoblast origin

The expression of CD34 by a proportion of isolated myoblasts in all groups raised some more questions. The origin of these cells is not clear and their contribution to the regeneration process still needs to be investigated further. To determine whether these $CD34^+$ cells were from the haematopoietic lineage or endothelial origin, CD45 and CD31 expression should be determined. $CD34^+$ cells could also be muscle derived stem cells already committed to a myogenic fate, expressing myogenic markers. To verify stem cell properties of the isolated $CD34^+$ cells, cells should be co-stained with the stem cell marker, Sca-1.

5.3.4. Oxidative stress: direct or indirect effects on myoblasts

It has been reported that GSE prevents oxidative stress in the micro-environment of a contusion injury and therefore relieves the muscle progenitor cells from potentially harmful free radicals. Also, the reduction of pro-inflammatory cytokines by GSE prevents their inhibitory effects on the progression of satellite cells. Pro-inflammatory cytokines have been shown to suppress satellite cell activation, proliferation and differentiation. For example, the expression of MyoD is inhibited post-transcriptionally by NF-κB [187] and TGF-β inhibits satellite cell proliferation and differentiation [265].

The effect of growth factors and cytokines on the fusion and differentiation of myoblasts should be investigated. TGF-β increases the expression of HDAC4, a key

inhibitor of myoblast differentiation [298]. Also, TGF-β growth factor binds to a pair of receptors on the cell membrane, which leads to the activation and translocation of SMAD proteins to the nucleus to trigger the expression of target genes [49, 185]. Smad3 is a transducer of TGF-β which leads to the inhibition of differentiation. This intracellular effector of TGF-β signalling represses the MyoD family of transcription factors [165, 167]. However, TGF-β1 through Smad3 inhibits Sca-1, which is an inhibitor of myogenic cell proliferation and differentiation. This might seem contradictory, but Sca-1 enhances turnover of the extracellular matrix and prevention of fibrosis through metalloproteinases (MMPs), therefore opposing the main role of TGF-β [170]. The effect of GSE supplementation on the activity of TGF-β might reveal an indirect mechanism in which GSE enhances myoblast progression towards differentiation if TGF-β can be reduced despite injury.

5.3.5. Growth and maturity

GSE could directly up-regulate structural proteins, like desmin. Desmin deletion resulted in fibrosis [186], thus increased desmin would be anti-fibrotic leading to better wound healing without the formation of scar tissue. A possible venture would be to investigate the mRNA levels of desmin in both satellite cell nuclei as well as myonuclei of mature myofibres in the presence or absence of GSE treatment. To determine whether GSE alters expression of more structural proteins, such as titin, fMHC and dystrophin, these should be investigated too.

The two separate desmin populations identified in this study was one of the main findings. These populations could be isolated to determine the unique characteristics of each population. With confocal microscopy, the morphology of myoblasts and the organisation and localisation of the desmin intermediate filaments can be determined. Super-resolution microscopy can further reveal whether changes in filament structure on the protein level are different in these two populations. The differentiation capacity of each population should then be determined.

The aim of the current study was to investigate satellite cell commitment in the mononuclear stage. To confirm that enhanced maturity in these satellite cells would lead to improved muscle recovery harvested myoblasts should be allowed to fuse under differentiation conditions *in vitro*. Also, myoblasts expressing desmin at higher concentrations could be transplanted into injured, non-supplemented littermates to

determine the efficiency of these primed cells to enhance regeneration of damaged muscle *in vivo*.

In conclusion, grape seed extract have beneficial effects on satellite cells, even in uninjured muscle. There are still many unanswered questions and a large scope for more investigations. The findings here highlight the heterogeneity of the satellite cell population and that the processes involved in their progression through muscle regeneration are complex, especially due to their various responses to their micro-environment. With this in mind, this study emphasises the need for the combined *in vivo* and *in vitro* approach to understanding muscle regeneration.

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Appendix A Cell culture procedures

A.1. CONTENTS OF DIFFERENT MEDIA FOR COMPARISON

Table A.1. DMEM growth media

Product	Concentration	Product code
DMEM	75%	D5671*
L-glutamine	8 mM	G7513*
Foetal calf serum	20%	10270-106#
Antibiotic- Antimycotic	1%	15240-062#
Gentamicin	0.1%	15750-060#
bFGF	10 ng/ml	G5071∞

Table A.2. Ham's F10 growth media

Product	Concentration	Product code
F10 Nutrient mix (Ham's)	79%	31550-023#
Foetal calf serum	20%	10270-106#
Antibiotic- Antimycotic	1%	15240-062#
Gentamicin	0.1%	15750-060#
bFGF	10 ng/ml	G5071∞

Table A.3. DMEM/Ham's F10 growth media combination

Product	Concentration	Product code
F10 Nutrient mix (Ham's)	39.5%	31550-023#
DMEM	37.5%	D5671*
L-glutamine	4 mM	G7513*
Foetal calf serum	20%	10270-106#
Antibiotic- Antimycotic	1%	15240-062#
Gentamicin	0.1%	15750-060#
bFGF	10 ng/ml	G5071∞

* Sigma Adlrich, Schnelldorf, Germany
\# Gibco from Life technologies, Grand Island, NY, USA
∞ Promega, Madison, WI, USA

A.2. COMMON CELL CULTURE PROCEDURES

A.2.1. Thawing cells

- Add 5 ml pre-heated culture medium in a T_{25} culture flask
- Remove vial from liquid nitrogen and place in a waterbath at 37^0C until thawed (± 2 min).
- Transfer contents of cryovial to the T_{25} culture flask and give the flask a good vigorous shake to spread cells.
- Place flask in incubator (37^0C; 5% CO_2) and change medium or passage the following day.

A.2.2. Changing media

- Discard old media with a pipette.
- Rinse flask with 2 ml (T_{25}) or 5 ml (T_{75}) warm PBS.
- Add required warm growth medium:
    - 5 ml for a T_{25} flask
    - 10 ml for a T_{75} flask.
- Incubate 37^0C in 5% CO_2.

A.2.3. Trypsinisation

- Use a flask of cells that is nearly confluent (~80%).
- Discard old medium with a pipette.
- Rinse with 1-5 ml warm PBS. (Rock the flask back and forth, and then discard the PBS by pipetting).
- Add warm trypsin to the flask:
    - 1 ml/well of 6-well plate
    - 2 ml for a T_{25}
    - 4 ml for T_{75} flask
- Incubate for 5 min in 37^0C in the incubated shaker.
- Check under inverted microscope whether cells have lifted. Tap the side of the flask to lift all cells from the surface.
- Add double the amount of warm growth medium to the flask to neutralize the trypsin.
- Now cells can be passaged, frozen or lysed for further procedures

A.2.4. Passaging cells

- Confluency 70-90%.
- Trypsinize cells (see paragraph above).
- Perform a cell count and calculate cells per ml. Add the correct amount of growth medium, to ensure there are 1 000 000 cells/ml.
- Centrifuge for 5 min at 1500 rpm to obtain a pellet of cells/ 3 min at 950 *g* for primary cells.
- Discard growth medium and remove all medium using a pipette and replace with the correct amount of media.
- Distribute cells in suspension to new flasks:
    - Ready for next day use: 200 000 cells per T_{25}
    - Leave over weekend: 40 000 cells per T_{25}
- Add required warm growth medium:
    - 5 ml (T_{25}) or 10 ml (T_{75}).
- Incubate 37^0C in 5% CO_2.

A.2.5. Freezing cells

- Collect suspended cells after trypsinisation in a 15 ml falcon tube. Rinse the culture flask by pipetting in and out a few times.
- Perform a cell count in order to determine how many vials of 10^6 cells can be frozen.
- Centrifuge for 5 min at 1500 rpm to obtain a pellet of cells/ 3 min at 950 *g* for primary cells.
- Discard growth medium and remove all medium using a pipette.
- Add 900 ul of FCS per 10^6 cells and resuspend the pellet.
- Add 100 ul DMSO per 10^6 cells.

  (work very fast, the DMSO will break the cells down)
- Transfer 1 ml of the solution to each separate cryovial.
- Freeze primary cells following these steps:
    - 1 hr in -20°C freezer.
    - Overnight -80°C.
    - Long term in liquid nitrogen.
- C2C12:
    - Store the labelled cryovial in liquid nitrogen.

A.2.6. Cell count

- Clean both the haemocytometer and cover slip with 70% ethanol and let air dry.
- Place cover slip in position on the haemocytometer.
- Use a micropipette, touching the edge of the cover slip, to put 20ul of cell suspension onto each grid.
- With the inverted light microscope, count all cells in the four corner grids on each side.
- To calculate the total amount of cells in the original suspension, use the following formula:
- $$\text{Cell count} = \frac{\text{Amount of cells counted}}{\text{Number of squares}} \times 10000 \times \text{Total volume of cell suspension}$$

A.3. FINAL PROTOCOL FOR PRIMARY MYOBLAST ISOLATION

A.3.1. Preparation

A.3.1.a) Preparing reagents

1. Protease type XIV from *streptomycus griseus* (Sigma Aldrich P5147; 1.25 mg/ml): Dissolve 12.5 mg in 10 ml PBS
   Filter sterilise and store in fridge
2. Collagenase I from *Clostridium histolyticum* (Sigma Aldrich C7657; 0.2%):
   Dissolve 20 mg in 10 ml PBS
   Filter sterilise and store in fridge
3. E-C-L (entactin-collagen-laminin; 20 µg/ml; Microsep 08-110) coated 6 well plates (20 µg/ml): Stock = 1000 µg/ml
   Make up a 50 x dilution in sterile PBS
   Cover wells with diluted E-C-L coating
   Incubate 1 hour at 37°C and remove coating
   Cover in foil and store in fridge
4. Basic recombinant FGF (10 ng/ml; Promega G5071):
   Dilute stock (25 µg) in 250 µl dH2O (=100ng/µl)
   Aliquot into eppindorfs (50 µl each)
   Store in -20°C freezer

A.3.1.b) Equipment for procedure in Cell culture lab:

1. Autoclaved dissection kit – (sharp tweezers, dissection scalpel and blade, Minora blade, large scissors)
2. Autoclaved tea sieve
3. 100 mm culture dish (BD Biosciences; 353003)
4. 15 ml tubes (BD Biosciences; 352096)
5. 50 ml falcon tubes (BD Biosciences; 352070)
6. 35 mm culture dish (BD Biosciences, 353001)
7. 100 µm cell strainers (BD Biosciences, 100 µm, yellow, 352360)
8. Centrifuge
9. Incubating shaker
10. Beaker with warm water
11. Type II laminar flow hood

A.3.1.c) Reagents and equipment for animal house procedures:

1. Pair of gloves for each rat being sacrificed
2. Spray bottle filled with 70% ethanol
3. Large bottle with 70% ethanol – 400 ml per rat
4. Euthanisia agent and injection needles
5. One large (1 L) beaker and two smaller beakers
6. Two 50 ml falcon tubes filled with PBS
7. Autoclaved dissection kit (2 pairs of tweezers, large and small scissors, knife and blade)
8. Autoclaved tissue paper (3 per rat plus extras)
9. Plastic bag
10. Type I/II laminar flow hood

A.3.2. Animal house procedure (for three rats approx 1 hour)

- Spray hood with ethanol and turn on flow
- Put the following into the hood prior to the start:
    a. Autoclaved tissue paper
    b. Dissection kit
    c. Empty small beaker
    d. Small beaker with ethanol to rinse utensils
    e. Falcon tubes with PBS
- Inject rat with pentobarbitone (± 50 cc) and wait for the heart to stop

- Submerse the rat in 400 ml ethanol in the large beaker and put the beaker into the hood
- Drain most of the ethanol from the rat and place onto tissue paper
- Dissect the *gastrocnemius* muscle from the leg
- Rinse twice with PBS from the first falcon tube, over the empty beaker
- Put the muscle into the second falcon tube with PBS

Between rats:

- Swop the tissue paper
- Swop the ethanol in the large beaker
- Rinse the dissection kit in the small beaker with ethanol and let air dry
- Spray hood with ethanol again
- Swop gloves
- Put rat into the plastic bag outside the hood

A.3.3. Cell culture lab procedure

- Dissect each muscle in the 100 mm culture dish to remove all connective tissue, tendons and the fascia (ensure that the sample stay moist by adding a very small amount of PBS to the dish)
- When all samples are pooled in the dish, mince the samples very finely with the surgical blade (Minora)
- Place the minced muscle into a 15 ml falcon tube and add 5 ml protease solution (± 1.5 ml per muscle sample)
- Incubate on the shaker for 1 hour at 37^0C
- Centrifuge at 950 *g* for 5 min, discard the supernatant
- Add 5 ml collagenase and incubate on shaker for 1 hour at 37^0C

- Centrifuge at 950 *g* for 3 min, discard the supernatant
- Add 1.5 ml PBS and vortex 20 seconds
- Centrifuge at 500 *g* for 10 min and transfer supernatant to a new 15 ml tube.
- Add 1.5 ml PBS and vortex 20 seconds
- Centrifuge at 500 *g* for 8 min and transfer supernatant to the other 15 ml tube.
- Add 1.5 ml PBS and vortex 20 seconds
- Centrifuge at 500 *g* for 5 min and transfer supernatant to the other 15 ml tube.
- Centrifuge the pooled supernatants at 950 *g* for 3 min

- Add 1 ml DMEM media to the cell pellet and resuspend
- Pour the suspension through the tea sieve into a 50 ml falcon tube
- Rinse twice with 1 ml DMEM media
- Pour through 100 µm cell strainer and rinse twice with DMEM
- Pour through a second 100 µm cell strainer and rinse twice with DMEM
- Centrifuge at 950 *g* for 3 min and resuspend cell pellet in 2 ml DMEM

- Pre-plate this suspension on a 35 mm culture dish and incubate for 2 hours at 37^0C, 5% CO_2 to allow fibroblasts to adhere
- Rinse all the wells of the E-C-L coated 6 well plate with PBS
- After 2 hours incubation, agitate medium gently and centrifuge at 950 *g* for 3 min.
- Add 2 ml Ham's F10 media to the pellet and plate into first well of the 6 well plate and incubate overnight at 37^0C, 5% CO_2. This will be pre-plate 1 (PP1).

A.3.3. Pre-Plating on each consecutive day

- Transfer media to a 15 ml tube and centrifuge at 950 *g* for 3 min.
- Wash old wells with 1 ml PBS each and add 2 ml Ham's F10 media to each well
- Add 2 ml Ham's F10 media to the cell pellet and transfer to a new well
- Incubate at 37^0C, 5% CO_2
- When PP4 has been incubated for 24 hours, all wells are ready to be trypsinised and cultured in culture flasks.

Appendix B Flow cytometry setup

B.1. STAINING PROTOCOL USED FOR FLOW CYTOMETRY ANALYSIS

- Trypsinise cells and perform a cell count
- Add cold PBS instead of media, to obtain a concentration of $1x10^6$ cells per ml
- Divide cells to have 200 0000-300 000 cells per 15 ml tube.
- Add 1 ml PBS to each tube to wash cells
- Centrifuge cells to form a pellet (950g, 3min)
- Fix/permeabilize in 200 µl cold 1:1 methanol-acetone for 10 min on ice
- Add 1 ml PBS to each tube to wash cells
- Centrifuge (950g; 3 min) and resuspend in 200ul blocking agent (1% BSA)
- Add primary antibody/ies and incubate 30 min at room temperature (RT)
- Add 1 ml PBS to each tube to wash cells
- Centrifuge (950g; 3 min) and resuspend in 200ul blocking agent (1% BSA)
- Add secondary antibody/ies and incubate 30 min in the dark at RT
- Centrifuge (950g; 3 min) and resuspend the pellet in 300 µl cold PBS
- Store on ice until analysis (same day)

Table B.1. Combinations of antibodies used for the triple/double stain for flow cytometry

	1^0 antibody	2^0 antibody
1	• Goat anti-Mcadherin (1:100) • Mouse anti-Pax 7 (1:200) • Rabbit anti-NCAM (1:100)	• Donkey anti-goat IgG-PE (1:100) • Donkey anti-mouse Alexa 488 (1:200) • Donkey anti-rabbit IgG-PerCP (1:100)
2	• Goat anti-Mcadherin (1:100) • Rabbit anti-CD34 (1:100)	• Donkey anti-goat IgG-PE (1:100) • Donkey anti-rabbit IgG-PerCP (1:100)
3	• Goat anti-Mcadherin (1:100) • Mouse anti-MyoD (1:100) • Rabbit anti-Desmin (1:100)	• Donkey anti-goat IgG-PE (1:100) • Donkey anti-mouse Alexa 488 (1:200) • Donkey anti-rabbit IgG-PerCP (1:100)

B.2. SETUP FOR MULTI-COLOUR EXPERIMENT

B.2.1. Compensation

To subtract spillover from different fluorochromes into other detectors of interest compensation values were calculated with software (BD FACSDiva 6.1 software), by using a non-stained sample and a single stained sample for each. Isolated primary myoblasts from non-injured non-supplemented rats were used. The following controls were prepared for compensation setup (Figure B.2.1):

1. Non-stained (addition of antibodies)
2. Mouse anti-Pax7 IgG and donkey anti-mouse Alexafluor 488 IgG
3. Goat anti-M-cadherin IgG and donkey anti-goat PE conjugated IgG
4. Rabbit anti-desmin IgG and donkey anti-rabbit PerCP conjugated IgG

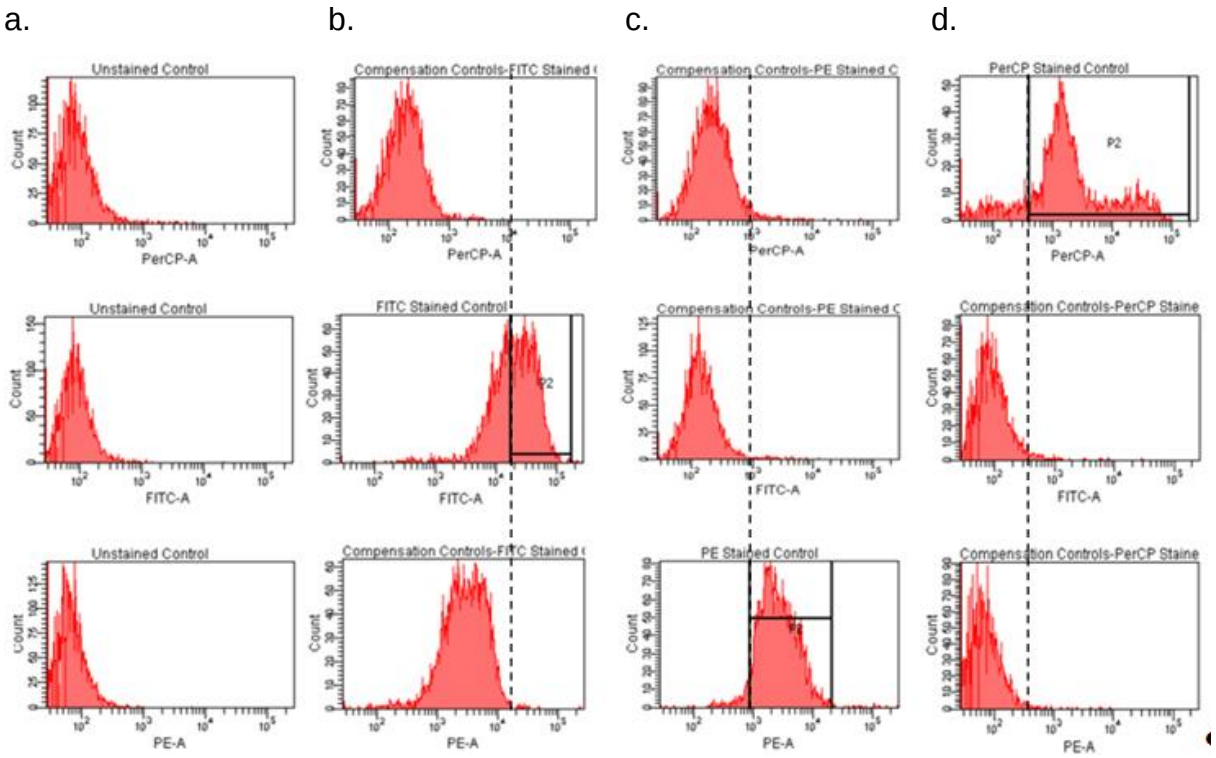

Figure B.2.1. The histogram profiles of each control, indicating the uncompensated spill-over in all detectors of interest. *a) Non-stained control, b) single Alexafluor 488 stain, c) single PE stain and d) single PerCP stain.*

B.2.2. Fluorochrome minus one (FMO) control setup

After compensation was calculated, controls called FMOs were prepared to determine positive and negative separating gates for each fluorochrome. Each

control was prepared to include two of the three fluorochromes which will be used for the experimental samples (see Table B.2)

Table B.2. Fluorochrome minus one (FMO) combinations

Fluorochrome excluded	**Antibodies used**
Alexafluor 488	1. Rabbit anti-Desmin IgG with Donkey anti-rabbit PerCP conjugated IgG 2. Goat anti-M-cadherin IgG with Donkey anti-goat PE-conjugated IgG
PerCP	1. Goat anti-M-cadherin IgG with Donkey anti-goat PE-conjugated IgG 2. Mouse anti-Pax7 IgG with Donkey anti-mouse Alexafluor 488-conjugated IgG
PE	1. Rabbit anti-Desmin IgG with Donkey anti-rabbit PerCP conjugated IgG 2. Mouse anti-Pax7 IgG with Donkey anti-mouse Alexafluor 488-conjugated IgG

B.2.3. Setup for sorting

Care should be taken to keep cells viable throughout preparation and sorting.

- Trypsinise cells and perform a cell count
- Add warm DMEM media, to obtain a concentration of $1x10^6$ cells per ml
- Centrifuge cells to form a pellet (950g, 3min)
- Add 200 µl blocking agent (1% BSA) and incubate for 30 minutes at 37°C
- Add 1 ml PBS to each tube to wash cells and centrifuge (950g; 3 min)
- Resuspend in 200ul DMEM media with rabbit anti-CD56 antibody (1:100)
- Incubate for 45 minutes at 37°C
- Add 1 ml PBS to each tube to wash cells and centrifuge (950g; 3 min)
- Resuspend in 200ul DMEM media with anti-rabbit PerCP conjugated secondary antibody and anti-CD34 PE conjugated antibody
- Incubate 1 h in the dark at 37°C
- Centrifuge (950g; 3 min) and resuspend the pellet in 300 µl warm PBS

After sorting:

- Collect sorted myoblasts in 5 ml tubes half-filled with DMEM and Ham's.
- Centrifuge (950g; 3 min) and resuspend the pellet in 300 µl warm DMEM and Ham's combined media
- Seed suspension in 8 well cover slip chambers (Lab-tek™ chambered cover glass, Rochester, NY, USA, 155411)
- Incubate in 37C with 5% CO2 overnight
- Prepare for immunocytochemistry

Appendix C Immunocytochemistry

C.1. SINGLE COLOUR STAINING OF CELLS

Work in a black box with damp tissue paper in the middle compartment, to prevent bleaching and to prevent samples drying out.

To make up ahead (calculate according to the amount of sections):

- Fixative - acetone:methanol (1:1) or 4% paraformaldehyde (permeabilisation step might be necessary). Keep on ice.
- Permeabilisation – 0.1% Triton X-100, from 0.5% stock made up earlier in PBS
- Blocking – 5 % donkey serum or 1% BSA or 5% goat serum made up in PBS. Keep on ice.
- Primary antibody in PBS
- Secondary antibody in PBS – LIGHT SENSITIVE!
- Hoechst – 1:4000 in PBS – LIGHT SENSITIVE!

Staining cover slips in a 24 well dish:

- Washing x2: Add 250 µl PBS to each well; 5 min
- Fixing: Add 250ul 4% paraformaldehyde per well and leave 10 min;
- Washing x3: Add 250 µl PBS to each well; 5 min
- Permeabilisation: Add 250 µl Triton X-100 (0.1%) to each well and leave 6 min.
- Washing x3: Add 250 µl PBS to each well; 5 min
- Blocking: Add 250 µl of 5% donkey serum to each section for 30 min
- DO NOT WASH NOW!
- Using forceps put each cover slip on a microscope slide on the black box, cells facing up. Use a bent needle to lift the cover slip from the bottom of the well.

- Primary ab: Add 50ul of antibody solution to each cover slip and leave for overnight in fridge
  Remove with tissue paper
- Washing x3: Add 50ul PBS to each section; 5 min
  Remove with tissue paper
- WORK IN THE DARK FROM HERE ONWARDS!
- Secondary ab: Add 50ul antibody to each section and leave 30 min
  Remove with tissue paper
- Washing x3: Add 50ul PBS to each section; 5 min
  Remove with tissue paper
- Hoechst: Add 50ul Hoecsht solution and leave 5 min
  Remove with tissue paper
- Washing x3: Add 50ul PBS to each section; 5 min
  Remove with tissue paper
- Let slides air dry for a few minutes.
- Mount upside down on microscope slides with fluorescent mounting media (Dako; Carpenteria, CA, USA, S3023).
- Air dry slides for at least an hour before freezing.
- Staining cells cultured in 8-well chambered coverslips:
- Repeat all steps as above, but use 100 µl of reagent in each step.
- After staining with Hoechst, add PBS and keep dishes in the fridge until imaging.