

EXPRESSION PROFILE OF WNT ISOFORMS DURING DIFFERENTIATION OF AGING C2C12 MYOBLAST CELLS

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PREFEACE

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Dr. Carola U. Niesler

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

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ABSTRACT

Satellite cells are known as the definitive muscle stem cells and are responsible for skeletal muscle maintenance and repair. The capacity of these satellite cells to participate in myogenesis decreases with age and as a result, muscle repair and maintenance in an aging organism is characterized by fibrosis, lipid accumulation and atrophy, a process known as sarcopenia. Recent parabiotic studies have shown that satellite cells with reduced myogenic capability in aging muscle can be rejuvenated to undergo effective myogenesis when exposed to a young environment. Further analysis has suggested that the Wnt family of signaling proteins identified in serum is pivotal in regulating cell fate, proliferation and differentiation, during aging. Wnt3a is known to regulate fibrogenesis, Wnt10b adipogenesis and Wnt7 myogenesis. In the current study, we aim to determine the cytosolic and secreted expression profiles of the three Wnt isoforms, Wnt3a, 7 and 10b, during myogenesis of early and late passage C2C12 myoblasts. We then extend our analysis to determine whether conditioned media could improve the myogenic capacity of late passage cells.

Late passage C2C12 cells had elevated Wnt3a cytosolic levels along with reduced differentiation capacity and a rapidly declining Wnt7 levels, in comparison to early passage cells. The elevated Wnt3a suggests an elevated fibrogenic predisposition, whereas the declining Wnt7 cytosolic levels, a decrease in myogenic capacity. Furthermore, analysis of the secreted vs. cytosolic ratio in Wnt7 levels revealed a more rapid decline in late vs. early passage cells during differentiation, supporting the observed decreased myogenic ability. Moreover, late passage cells also showed lower Wnt10b levels compared to early passage cells. This low level of Wnt10b is likely

associated with an increase in adipogenic predisposition. The results obtained in the cross-over experiments indicated that conditioned media from early passage cells did not improve the differentiation of late passage cells by the low levels of Myogenin and MHC. However, early passage cells treated with conditioned media from late passage cells surprisingly showed a marginal increase in both Myogenin and MHC levels. Interestingly, cytosolic Wnt3a and 7 in late passage cells treated with 'young media' were increased compared to control whereas early passage cells treated with 'old' media showed significantly decreased levels of Wnt3a and 7. Furthermore, early passage cells acquired a declining expression when treated with 'young' media whereas late passage cells had an increasing level when treated with 'old' media. This indicates a possible improvement in differentiation in late passage cells.

Taken together, our results support a role for Wnt7 and Wnt10b in promoting myogenesis while Wnt3a may decrease myogenesis. With the increase in passage numbers, the reduced myogenic predisposition is regulated by reduced Wnt10b, 7 and elevated Wnt3a levels, respectively. Moreover, we speculate that the lack of myogenic improvement in the cross-over experiment could be the presence of unknown secreted factors in 'young' media that impedes myogenesis. Finally, cell lines are known to be biologically different to primary myoblasts through the accumulation of mutations which could render the cells less sensitive to growth factors. Therefore, it is imperative that the current study is repeated with primary culture myoblasts.

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ABBREVIATIONS

BM	Bone marrow
BMP	Bone morphogenetic protein
β TrCP	Beta transducin repeat containing protein
ChIP	Chromatin immuno-precipitation
CK-1	Casein Kinase-1
CM	Conditioned media
CTX	Cardio toxin
DKK	Dickkopf
DMEM	Dulbecco's modified eagles medium
Dsh	Disheveled
ECL	Enhance chemiluminescence
ECM	Extracellular matrix
EM	Electron microscopy
EP	Early pre-plate
FACS	Fluorescence activated cell sorting
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GAPDH	Glyeraldehyde 3-phosphate dehydrogenase
GDF	Growth differentiation factor
GSK-3 β	Glycogen snythase kinase-3beta
HGF/SF	Hepatocyte growth factor/Scatter factor
HRP	Horse radish peroxidase

HSC	Hematopoietic stem cell
ICC	Immunocytochemistry
IgG	Immunoglobulin G
kDA	kiloDalton
LP	Late pre-plate
MAPK	Mitogen activated protein kinase
MDSC	Muscle derived stem cells
MHC	Myosin heavy chain
mRNA	messenger Ribonucleic acid
MSC	Mesenchymal stem cell
MRF	Myogenic regulatory factor
Mrf4	Myogenic regulatory factor 4
Myf5	Myogenic factor 5
MyoD	Myogenic differentiation antigen
NCAM	Neural cell adhesion molecule
Pax	Paired-box
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PCNA	Proliferating cell nuclear antigen
PEG	Polyethylene glycol
PKC	Protein kinase C
PLC	Phospholipase C
LRP5/6	Low density lipoprotein receptor-related protein
Rf	Relative mobility

Rock	Rho-associated kinase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SA- β -gal	Senescence-associated beta-galactosidase
SDS	Sodium dodecyl sulfate
sFRP	secreted Frizzled receptor protein
SP	Side population
TCF/LEF	T-cell factor/lymphoid enhancer-binding factor
TGF- β	Transforming growth factor
TTBS	Tween tris-buffered saline
VCAM-1/VLA-4	Vascular cell adhesion molecule-1/Very late antigen-4
WIF	Wnt inhibitory factor
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Maintenance and repair of skeletal muscle is mediated primarily by resident muscle stem cells known as satellite cells and to a lesser extent by other muscle-derived and non-muscle-derived stem cells. Satellite cells, which reside in their niche in a quiescent state, are activated in response to injury or trauma and begin to proliferate (now called myoblasts). Several myoblasts return to quiescence to replenish the satellite cell population, whereas the rest eventually fuse into myotubes thus facilitating maintenance and repair (Kuang and Rudnicki, 2008). This process is highly efficient in a young individual, however, the capacity for maintenance and repair declines with an increase in age, a process known as sarcopenia (Solomon and Bouloux, 2006). Sarcopenic muscles are aging muscles characterized by reduced muscle mass (atrophy) with the accumulation of lipids and fibrosis (Brack et al., 2007; Vertino et al., 2005). This age-related decline in regenerative capacity by satellite cells has generated much debate about the possible cause; specifically the role of extrinsic vs. intrinsic factors remains elusive. The characteristic changes in sarcopenic muscle leads to an altered extrinsic environment which together, directly impact satellite cell function. The altered intrinsic properties of satellite cells from aging muscle include impaired myogenic regulatory factor activities (Degens, 2007). Lastly, quiescent satellite cell numbers have been shown to be reduced in aging muscle (Shefer et al., 2006). This reduction in numbers could be one of the many causes of reduced satellite cell function.

Recent evidence, through parabiotic pairing experiments, suggest that serum factors from the circulatory system of young mice can rejuvenate the regenerative capacity of

satellite cells in old mice (Brack and Rando, 2007; Conboy et al., 2005). Further studies identified high levels of Wnt proteins in the serum of old mice, leading to elevated signaling via β -catenin dependent pathway (Brack et al., 2007). Inhibition of the Wnt signaling pathway through the extensive use of antagonists resulted in successful myogenic regeneration (Brack et al., 2007). Additionally, antagonizing this pathway reduced the tendency of satellite cells to undergo cell fate alteration from a myogenic to a fibrogenic lineage (Brack et al., 2007). Therefore, this study has implicated the possible role of Wnt signaling in regulating satellite cell function in aging muscle.

Wnt proteins act via either the canonical or non-canonical pathways that regulate cellular processes such as migration, apoptosis, differentiation and myogenic specification (Brack et al., 2008; Poleskaya et al., 2003). Although it is well accepted that Wnt proteins control differentiation and myogenic progression, the exact role of the canonical vs. non-canonical pathways is unclear. Myogenic differentiation requires a highly regulated molecular switch of Notch to Wnt signaling, initiated by Wnt3a (Brack et al., 2008). However, an elevated Wnt3a level in aging muscle (which shows an increase in fibrogenic fate) suggests a role for this isoform in fibrogenesis rather than myogenesis (Brack et al., 2007). On the other hand, muscle-derived CD45⁺ cells from uninjured muscles are capable of myogenic potential in the presence of Wnt5a, 5b, 7a and 7b proteins (Poleskaya et al., 2003). Isolated myoblasts from aging mice, which display a lineage change from myogenic to adipogenic lineage, have been shown to have low Wnt10b levels (Taylor-Jones et al., 2002). Similar results were observed in myoblasts of Wnt10b^{-/-} mice where the lack of Wnt10b and absence of Wnt7b compensation, leads to lineage change from myogenic to adipogenic lineage and ultimately lipid accumulation (Vertino et al., 2005). This chapter presents an overview

of the Wnt signal transduction and its role in regulating satellite cell myogenesis in the skeletal muscle aging process.

1.2 Satellite cell characterization

First identified by Mauro (1961) in leg muscles of frogs, satellite cells were primarily identified *in situ* by their morphological characteristics and anatomical location. Subsequent analysis by Muir et al. (1965) revealed the presence of satellite cells in muscles of white mice and fruit bats, in addition, a detailed distribution and cellular structure. Identification by electron microscopy (EM) highlighted their unique niche, located between the basal lamina and sarcolemma, and positioned along the entire length of myofibers (Muir et al., 1965) (Fig. 1.1). Important morphological characteristics include fusiform shape, reduced organelle content, smaller nucleus with increase amount of heterochromatin compared to the surrounding myonuclei and an increase in nuclear-to-cytoplasmic ratio (Charge and Rudnicki, 2004; Mauro, 1961). These characteristics indicate that satellite cells are mitotically quiescent (Charge and Rudnicki, 2004).

Since its discovery, satellite cells were speculated to play a role in muscle repair and regeneration. Indeed, Snow (1978) through trace studies using autoradiography demonstrated that satellite cells contribute to regeneration in muscles of young Sprague-Dawley rats. Furthermore, *in vitro* studies have shown that, when satellite cells are extracted from the niche and maintained in culture, the activated satellite cells readily form myotubes.

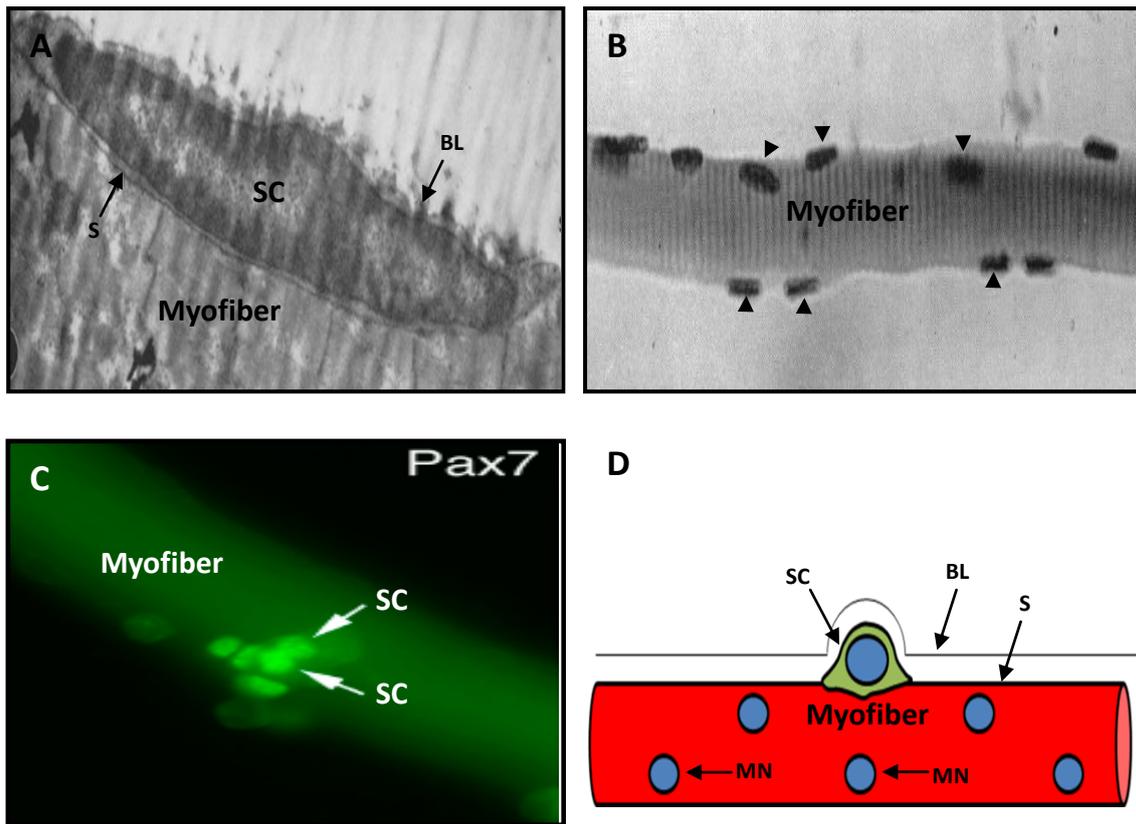


Fig. 1.1: Satellite cell morphology and its unique anatomical niche

Satellite cells (SC) are characterized by their unique niche. Longitudinal view of satellite cells reveals that anatomically, satellite cells are located between the sarcolemma (S) and the basal lamina (BL) along the entire length of myofibers (A and D). Distinction of satellite cells from myonuclei (arrow heads) is difficult under light microscopy as both are situated on the periphery of myofibers (B). The advancement of microscopy and the discovery of molecular markers such as Pax7 allowed the identification of the satellite cells under fluorescence microscopy (C). Images adopted from (A) Mauro (1961), (B) Muir et al. (1965), and (C) Zammit et al (2004).

Subsequently, the transplantation of cultured satellite cells into irradiated mice has demonstrated that the transplanted satellite cells can replenish the depleted satellite cell pool and contributed to new myofiber formation (Blaveri et al., 1999). Moreover, knock-out studies in mice lacking Pax7 ($Pax7^{-/-}$ mice) revealed the lack of muscle satellite cells and hence resulted in compromised muscle regeneration (Buckingham, 2007; Seale et al., 2000). These findings underscore the myogenic potential of satellite cells. Together, these data unraveled the characteristics of satellite cells that lead to

their revised characterization that not only includes anatomical location but molecular marker expression (section 1.3) and myogenic function. Due to these properties, satellite cells are known as the definitive stem cells of skeletal muscle (Brack and Rando, 2007).

1.3 Molecular regulation of satellite cell myogenesis

While the identification of satellite cells is achievable with electron microscopy due to their characteristic ultra-structure, it is impossible to distinguish satellite cells from myonuclei under light microscopy. The advancement in microscopy and discovery of molecular markers allowed further characterization and identification of quiescent satellite cells (Fig. 1.1). Furthermore, knock-out studies of myogenic genes have defined the roles of various myogenic markers such as Pax7 and myogenic regulatory factors (MRF). The molecular markers and their respective functions are summarized in Table 1.1.

1.3.1 Quiescent satellite cell markers and function

M-cadherin, the first molecular marker identified by Irintchev et al. (1994), allowed the first identification of satellite cells under light microscopy. Through the development of *in vitro* intact single fiber (Bischoff, 1989), studies by Seale et al. (2000) identified Pax7 (a paired box transcription factor) as a satellite cell marker. Pax7 plays a pivotal role in myogenic specification, cell fate alteration, anti-apoptosis and prevention of precocious differentiation (Buckingham, 2007; Kuang et al., 2006; Relaix et al., 2006; Seale et al., 2000; Zammit et al., 2006). Like many molecular markers, the role of Pax7 in myogenesis was unraveled in knock-out and transfection studies. Pax7 knock-out (Pax7^{-/-}) mice generated by Seale et al. (2000) showed reduced body mass and size.

Table 1.1: Satellite cell markers in myogenesis

Markers	Expression	Function	References
Cell surface			
c-met	A	Receptor for HGF/SF (Activation)	(Cornelison and Wold, 1997)
VCAM1/VLA4	Q/A	Activation	(Seale et al., 2004a)
NCAM	Q/A/D	Fusion of myoblasts	(Irintchev et al., 1994)
M-cadherin	Q/A/D	Alignment and fusion	(Moore and Walsh, 1993)
CD 34	Q (t)/A (f)	Unknown	(Beauchamp et al., 2000)
SM/C-2.6	Q	Unknown	(Fukada et al., 2004)
Snydecan 3/4	Q	ECM signaling	(Cornelison et al., 2001)
Sphingomyelin	Q	Cell cycle entry	(Nagata et al., 2006)
Transcription factors			
Pax 7	Q, A, D	Myogenic specification, survival	(Seale et al., 2000)
Pax 3	Q	Myogenic specification	(Horst et al., 2006)
PCNA	A,P	Activation, proliferation	(Johnson and Allen, 1993)
MyoD	A	Activation, differentiation	(Cornelison et al., 2000)
Myf 5	Q, A, D	Differentiation	(Ustanina et al., 2007)
Myogenin	D	Differentiation, fusion	(Fuchtbauer and Westphal, 1992)
Mrf 4	D	Differentiation, fusion	(Jin et al., 2007)
Others			
Desmin	A	Cytoskeleton maintenance	(Day et al., 2007)
MHC	A, D	Differentiation	(Bader et al., 1982)

Abbreviations: Q, quiescence; A, activation; P, proliferation; D, differentiation; VCAM-1/VLA-4, Vascular cell adhesion molecule-1/very late antigen-4; NCAM, Neural cell adhesion molecule; t, truncated; f, full; Pax, paired-box; PCNA, proliferating cell nuclear antigen; Myf5, myogenic factor 5; Mrf4, myogenic regulatory factor 4; MHC, myosin heavy chain

Furthermore, Pax7^{-/-} muscle revealed the complete absence of satellite cells which lead to poor myogenesis after cardiotoxin (CTX) injury (Seale et al., 2000). Transfection of non-myogenic, muscle derived CD45⁺:Sca-1⁺ cells isolated from uninjured muscles with Pax7-expressing-retrovirus induced the myogenic program (Seale et al., 2004b). Both studies defined the significant role of Pax7 in myogenesis. Another marker discovered for the identification of quiescent satellite cells is c-met (Cornelison and Wold, 1997).

The tyrosine receptor kinase, c-met, was detected at the mRNA level by single-cell multi-plex RT-PCR in quiescent satellite cells (Cornelison and Wold, 1997). The role of c-met is highlighted by the presence of hepatocyte growth factor/scatter factor (HGF/SF) in crushed muscles whereby HGF/SF ligand binds to the c-met receptor resulting in satellite cell activation (Tatsumi et al., 1998). Further markers include VCAM-1 (Seale et al., 2004a) and sphingomyelin (Nagata et al., 2006). The calcium-dependent cell adhesion molecule, M-cadherin is also detected at the mRNA level, in a subset of quiescent satellite cells, which suggest heterogeneity in the satellite cell population (Cornelison and Wold, 1997). Interestingly, cells that express M-cadherin also express CD34, a hematopoietic stem cell marker (Beauchamp et al., 2000). Therefore, CD34 has the potential to be a useful marker for quiescent detection. Other discovered markers include syndecan 3 and 4 (Cornelison et al., 2001), and Myf5 (Beauchamp et al., 2000; Holterman and Rudnicki, 2005).

Although these mentioned markers are useful to identify quiescent satellite cells, none of the above mentioned markers are specific to the quiescent population. Furthermore, isolation of primary culture satellite cells requires the removal of the cells from their niche which inevitably leads to activation. To overcome the activation dilemma, several methods have been developed – the ‘G0-myoblast’ and myofiber isolation. Utilizing an inhibitor, SB203580, that specifically inhibits the phosphorylation of p38 α / β (a mitogen activated protein kinase, MAPK), the proliferating cultured myoblast exits the cell cycle into a reversible G0-state (Jones et al., 2005). These so called ‘G0-myoblasts’, display molecular characteristics similar to quiescent satellite cells. For the myofiber isolation, individual myofibers are isolated with adherent satellite cells in a pure state and most importantly, activation is delayed (Bischoff, 1989).

1.3.2 Myogenic regulatory factors in myogenesis

In the quiescent state, satellite cells do not display significant expression of MRF at the protein and mRNA level (Dhawan and Rando, 2005; Holterman and Rudnicki, 2005). In the presence of an injury to skeletal muscle, satellite cells are activated from their quiescent state, enter the cell cycle and begin to proliferate. After several cycles, the resulting myogenic progenitors (also called myoblasts) fuse and exit the cell cycle to enter a state of G0 differentiation and complete the repair of injured skeletal muscle (Dhawan and Rando, 2005) (Fig. 1.2). In addition, the myogenic process is accompanied by self-renewal, ensuring a constant population after repeated rounds of repair and regeneration (Zammit et al., 2004). This comprehensive process of myogenesis is mainly regulated by the group of basic helix-loop-helix (bHLH) collectively called the MRF that includes MyoD, Myogenin, Myf5 and Mrf4 (Charge and Rudnicki, 2004). Activation of satellite cells leads to the down-regulation of Pax7 and the immediate sequential up-regulation of MRF, firstly MyoD and Myf5, followed by Myogenin and Mrf4 (Cornelison and Wold, 1997; Fuchtbauer and Westphal, 1992). The expression of any four MRFs leads to an activation of the myogenic program in the C3H10T1/2 fibroblast cell line. This remarkable property of MRF was further demonstrated in differentiated retinal epithelial cells which will activate the myogenic program in the presence of MRFs (Choi et al., 1990). These findings confirm that MRF are indeed the myogenic master switches.

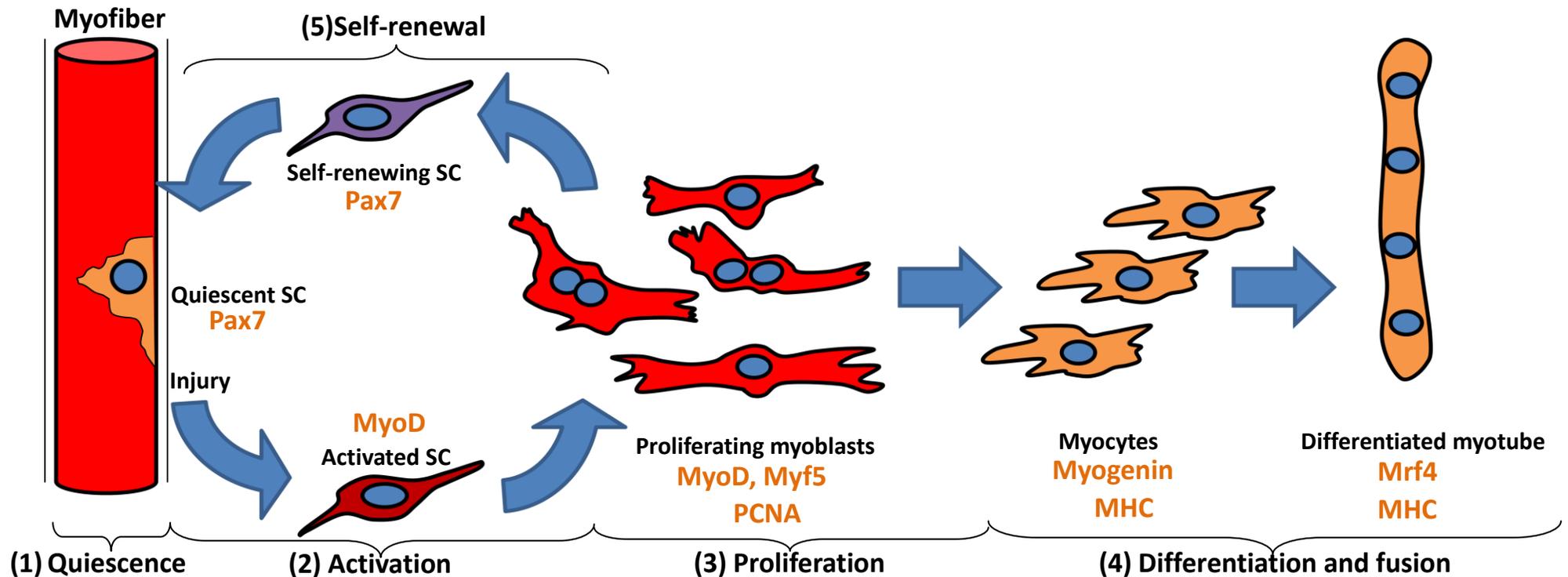


Fig. 1.2: Schematic representation of myogenic lineage progression during skeletal muscle repair and regeneration

Myogenic lineage progression during muscle repair and regeneration is divided into different stages regulated by molecular markers. Satellite cells (SC) reside between the basal lamina and sarcolemma of myofibers in a quiescent state (1). The presence of an injury or trauma in skeletal muscle, activation of satellite cells occurs (2), SC enter the cycle giving rise to proliferating myoblasts. After several cycles of division, myoblasts exit the cell cycle (now called myocytes), align and undergo differentiation and fusion with existing myofibers or form new multinucleated myotubes (4). Repair and regeneration of skeletal muscle are always accompanied by self-renewal (5), therefore, after repeated bouts of repair and regeneration, the quiescent satellite cell population is kept constant. Markers in myogenesis are (1) Pax7, quiescence; (2) MyoD, activation; (3) PCNA, proliferation; (4) Myogenin, Mrf4, MHC, differentiation. MyoD, Myf5, Myogenin and Mrf4 together are known as myogenic regulatory factors (MRFs). Collectively, these transcription factors regulate myogenesis.

1.3.2.1 MyoD and Myf5

Double knock-outs of MyoD and Myf5 indicated a complete absence of skeletal muscle at birth. However, individual knock-outs of MyoD and Myf5 yielded different results and gained insight to the role of individual markers. Cultured MyoD knock-out (MyoD^{-/-}) myoblasts showed a stellate morphology with severely reduced differentiation with continued proliferation even in low-mitogen media (Sabourin et al., 1999). Northern blot analysis of Myogenin and Mrf4 showed a delay in both Myogenin and Mrf4 expression which impeded the MyoD^{-/-} myoblasts from entering the differentiation program (Sabourin et al., 1999). However, differentiation deficit and morphology was restored when MyoD^{-/-} myoblasts were transfected with a MyoD expressing vector (Sabourin et al., 1999). Single fiber cultures with the adherent MyoD^{-/-} quiescent satellite cells by Cornelison et al. (2000) confirmed that the differentiation deficit was indeed associated with the subsequent delay of Myogenin and Mrf4 expression, and additionally M-cadherin which is known to play a role in fusion. The noticeable differentiation and repair defect can be seen by the MyoD^{-/-} satellite cell aggregate and the 'forked' morphology (Cornelison et al., 2000). Genetic profiling performed by Seale et al. (2004a) showed that MyoD^{-/-} myoblasts expressed similar markers to quiescent satellite cells. In all studies, a notable rise in Myf5 was seen which was speculated to be a myogenic compensation effect, however compensation was unsuccessful. Overall, these data have identified that the up-regulation of MyoD is essential to initiate the myogenic program by initiating Myogenin and Mrf4 expressions. Previously, identifying the role of Myf5 in myogenesis has proven to be difficult due to the lack of viable Myf5 knock-out. Recently, viable Myf5 knock-out (Myf5^{-/-}) mice were generated by Ustanina et al. (2007). Likewise to MyoD^{-/-} mice, Myf5^{-/-} mice had a normal muscle development. However, unlike MyoD^{-/-} myoblasts, examination of the ultra-structure with electron

microscopy indicated no difference in satellite cell morphology and no reduction in satellite cell numbers. Interestingly, BrdU incorporation studies on isolated Myf5^{-/-} myoblasts showed a delay in proliferation. Furthermore, CTX induced injury to Myf5^{-/-} mice showed a delay in muscle repair with fibrotic tissue accumulation but eventually repair was completed with no fibrosis (Gayraud-Morel et al., 2007; Ustanina et al., 2007). Analysis of myogenic regulatory genes indicated that Myogenin, Mrf4 and MHC expressions were delayed in Myf5^{-/-} Myoblasts (Gayraud-Morel et al., 2007). In summary, data derived from MyoD and Myf5 knock-out studies seem to suggest that both transcription factors activate different myogenic programs and that MyoD knock-out has a more detrimental effect on myogenesis than Myf5 knock-out. Nevertheless, activation of both transcription factors is essential for effective and rapid myogenesis.

1.3.2.2 Myogenin and Mrf4

Following MyoD and Myf5 expression, Myogenin and Mrf4 are expressed leading to cell cycle exit in differentiating cells (Charge and Rudnicki, 2004). Similar to the difficulty in generating viable Myf5 knock-out mice, Myogenin knock-out mice die at birth. However, Knapp et al. (2006) successfully generated viable Myogenin conditional mutants by mating mice with floxed alleles of Myogenin to a transgenic mice expressing Cre recombinase that deletes Myogenin prior- or post-embryonic muscle development, termed Myog^{floxΔ/floxΔ};Cre-ER⁺ mice. Results from these mutant mice indicated that deletion of Myogenin prior to muscle embryonic development lead to identical muscle deficiencies to Myogenin knock-outs, ultimately leading to death at birth. Post-natal deletions of Myogenin displayed smaller body size in mutant mice but surprisingly, a normal myogenesis and muscle growth (Knapp et al., 2006; Meadows et

al., 2008). Analysis of MyoD, Myf5 and Mrf4 transcript levels in $\text{Myog}^{\text{flox}\Delta/\text{flox}\Delta};\text{Cre-ER}^+$ mice showed insignificant reduced levels compared to wild-type. Furthermore, cultured $\text{Myog}^{\text{flox}\Delta/\text{flox}\Delta};\text{Cre-ER}^+$ myoblasts readily form multinucleated myotubes indicating that low levels of Myogenin do not affect differentiation (Meadows et al., 2008). These results seem to suggest that Myogenin is dispensable during post-natal myogenesis. Mrf4 knock-out ($\text{Mrf4}^{-/-}$) mice are viable, however developed abnormal intercostal muscles, in addition, secondary defects such as abnormal thoracic skeletal development (Vivian et al., 2000). Further studies indicated a possible compensation effect with the up-regulation MyoD, Myf5 and Myogenin in the $\text{Mrf4}^{-/-}$ mice to promote myogenesis, however expression and cellular localization study in wild-type mice indicated the presence and expression of Mrf4 in differentiated muscle fibers and not in satellite cells (Zhou and Bornemann, 2001). Thus, the presence of Mrf4 in myofibers indicated a role in myotube maturation; however its absence in satellite cells seems to suggest that Mrf4 has a distinct myogenic role to MyoD, Myf5 and Myogenin.

In summary, knock-out mice have defined the individual roles of MRF family of proteins. Myf5, Myogenin or Mrf4 knock-outs do not significantly affect proliferation and differentiation, however MyoD knock-out lead to a detrimental effect on differentiation with continuous proliferation. This shows that MyoD plays a more significant role in regulating myogenesis compared to Myf5, Myogenin and Mrf4. Finally, double or triple knock-out of any MRF leads to a more noticeable differentiation impairment than MyoD knock-out (Gayraud-Morel et al., 2007). Thus, the sequential and collective transcription activities of all four MRF are essential for rapid and effective myogenesis.

1.4 Myogenic cell lines

Although myofiber and primary cultures have aided in the discovery of satellite cell markers and increased our understanding of myogenesis, both *in vitro* techniques are hampered by several technical difficulties. These include the difficulty in isolating pure cultures, their maintenance as well as their limited lifespan in culture. Due to these technical difficulties, 'immortalized' myogenic cell lines such as mouse C2C12, rat L6 and mouse MM14 myoblasts are frequently used (Table 1.2). The C2 cell line was isolated from thigh muscles of 2 month old C3H mice, 70 hour post crush injury by Yaffe and Saxel (1977) and from this cell line, Blau et al. (1985) generated a sub-clone called C2C12 cell line by selecting C2 cells that displayed high differentiation capacity. The rat L6 cell line was isolated from thigh muscles of new-born rats (Yaffe, 1968) and MM14 cell line was derived from leg muscle of 2 month old Balb/C male mice (Linkhart et al., 1980). These 'immortal' cell lines such as L6 and C2C12 myoblasts can be serially maintained over several passages and stored in a frozen state (at -80°C) for extended periods (Yaffe, 1968). These advantages make cell lines a popular alternative to primary satellite cell cultures. It should be noted that these 'immortal' cell lines have an altered cell cycle through the accumulation of mutations that allows prolonged maintenance in culture (Cornelison, 2008). The mutations that make cell lines different to primary myoblasts are unclear. For this reason, studies done on cell lines should be repeated on primary cultures for validation purposes. Nevertheless, studies utilizing cell lines have provided an important insight to the process of myogenesis.

Table 1.2: Common myoblast cell lines used to study myogenesis

Cell line	Animal	Injury	References
C2	2 month old C3Hmice	Crush injury	(Yaffe and Saxel, 1977)
C2C12	2 month old C3Hmice	Crush injury	(Blau et al., 1985; Yaffe and Saxel, 1977)
MM14	2 month old Balb/C	-	(Linkhart et al., 1980)
L6	new-born rats	-	(Yaffe, 1968)
L41	new-born rats	-	(Yaffe, 1968)

Studies utilizing ‘satellite cell-like’ myoblasts are often used to study myogenesis due to their ease of use. These myoblast cell lines were generated from primary cultures usually 60 - 70 days post injury and serially selected using the pre-plating technique.

1.5 Myogenic cells from other sources

Tissue-specific stem cells, either muscle-derived or non-muscle-derived, are capable of regeneration of the host tissues in which they reside. However, recent evidence suggests that stem cells from one tissue can differentiate into cell types other than those in the host tissue. For example, hematopoietic stem cells (HSC) found in the bone marrow can differentiate into cells of various lineages such as muscle and neurons (Asakura, 2003; Asakura and Rudnicki, 2002). Similarly, satellite cells under different conditions are capable of differentiating into adipocytes, osteocytes and fibroblasts (Asakura et al., 2001; Brack et al., 2007). Moreover, stem cells isolated from skeletal muscle were shown to have hematopoietic potential and readily differentiate into hematopoietic colonies *in vitro* (Jackson et al., 1999). Taken together, these results suggest that tissue-specific stem cells can differentiate into many cell types depending on the extrinsic signals provided by the host tissue. The identified myogenic stem cells from various sources are listed in Table 1.3, and some will be discussed below.

1.5.1 Muscle derived myogenic cells

Muscle-derived hematopoietic stem cells (CD45, CD34 and Sca-1 positive), display

myogenic potential when reintroduced into mice (Asakura et al., 2002; Bhagavati and Xu, 2004; Seale et al., 2004b). Studies by Qu-Petersen et al. (2002) identified three populations of muscle-derived myogenic cells based on adhesion and proliferation characteristics. Utilizing the pre-plating technique developed by Rando and Blau (1994), the early pre-plate (EP) cells, the late pre-plate (LP) cells and a novel population of muscle-derived stem cells (MDSC) were fractionated and transplanted into Mdx mice to assess the efficiency of muscle regeneration. Although EP and LP cells showed a lack of contribution to regeneration, transplantation of MDSCs enhanced regeneration efficiency, dystrophin delivery and cell survival compared to EP and LP cell transplantation (Qu-Petersen et al., 2002). Further studies by Lee et al. (2000) isolated a population of muscle-derived stem cell called mc13, expressing CD34 and Bcl-2 markers. Importantly, when mc13 cells were injected intravenously into Mdx mice, they facilitated muscle regeneration and partial restoration of dystrophin (Charge and Rudnicki, 2004; Lee et al., 2000). The CD45⁺:Sca-1⁺ cells isolated from uninjured Pax7^{-/-} muscle do not display myogenic capacity whereas CD45⁺:Sca-1⁺ cells from injured muscle gave rise to differentiated myoblasts along with the expression of MRFs such as MyoD and Myf5 (Seale et al., 2004b). However, when non-myogenic CD45⁺:Sca-1⁺ cells from uninjured Pax7^{-/-} muscle were infected with retrovirus that express Pax7, the myogenic program is activated (Seale et al., 2004b). This emphasized that non-myogenic muscle-derived cells can undergo myogenesis following forced expression of transcription factor, Pax7.

1.5.2 Non-muscle-derived myogenic cells

Bone-marrow derived progenitor cells have been shown to display myogenic potential (Ferrari et al., 1998). These non-muscle derived cells have been shown to migrate to

the site of muscle injury and contribute to the repair process. Similarly, bone marrow-derived stem cells were shown both to contribute to muscle regeneration and to replenish the satellite cell pool in irradiation damaged muscle (Ferrari et al., 1998; LaBarge and Blau, 2002).

Lastly, side population (SP) cells derived from muscle and other tissues displayed myogenic potential (Asakura and Rudnicki, 2002; Asakura et al., 2002). SP cells actively exclude Hoechst 33342 dye due to the expression of multi-drug resistance genes (a unique characteristic that permits separation by FACS[®]) and express hematopoietic markers, Sca-1 and CD45, but no detectable satellite cell markers, and readily undergo hematopoietic differentiation and not myogenic differentiation *in vitro* (Goodell et al., 1996). However, when muscle SP cells are co-cultured with primary myoblasts, myogenic specification is induced (Asakura et al., 2002). Interestingly, muscle SP cells from Pax7^{-/-} muscle not only undergo myogenic conversion when transplanted intramuscularly into regenerating muscle, but also give rise to satellite cells and myocytes (Asakura et al., 2002). Taken together, these studies have successfully identified various myogenic cells from different tissues which are molecularly distinct from satellite cells, and are capable of contributing to the repair and regeneration of skeletal muscle.

Table 1.3: Summary of muscle-and non-muscle-derived cells capable of myogenesis

Phenotype/ cell type	Tissue origin	References
Muscle derived		
CD45 ⁺ :Sca-1 ⁺ cells	Muscle (Pax7 ^{-/-} Injured muscle)	(Seale et al., 2004b)
mSP cells	Muscle	(Asakura et al., 2002)
MDSC, EP, LP cells	Muscle	(Qu-Petersen et al., 2002)
MDSC (CD34 ⁺ :Bcl-2 ⁺) cells	Muscle	(Bhagavati and Xu, 2004)
Non-muscle derived		
BM derived cells	Bone marrow	(LaBarge and Blau, 2002)
SP cells	*Diverse adult tissues	(Asakura and Rudnicki, 2002)
BM-MSC	Bone marrow	(Bhagavati and Xu, 2004)
BM progenitor cells	Bone marrow	(Ferrari et al., 1998)

*SP cells were isolated from skeletal muscle, heart, brain, spleen, liver, kidney, lung, and small intestine. Abbreviations: mSP, muscle side-population; MSC, mesenchymal stem cells, MDSC, muscle-derived stem cells; EP, early pre-plate; LP, late pre-plate, BM, bone marrow; MSC, mesenchymal stem cells.

1.6 Regulation of myogenesis by growth factors

Besides the intrinsic molecular regulation of satellite cell myogenesis, growth factors and cytokines from the surrounding local or systemic environment have been shown to regulate myogenesis extrinsically by binding to their specific receptors. Growth factors are known to regulate every phase of myogenesis including activation (Allen et al., 1995), proliferation (Schabert et al., 2009) and differentiation (Brack et al., 2008; Miura et al., 2006) in an autocrine, paracrine and endocrine manner. In this regard, satellite cells are ideally located in close proximity to interstitial cells, capillaries and adjacent myofibers that provide these extrinsic signals (Dhawan and Rando, 2005). The ligand-receptor binding of growth factors activates cellular signal transduction pathways that directly or indirectly control myogenesis. Three families of growth factors, Hepatocyte growth factor (HGF), Transforming growth factor- β (TGF- β) and Fibroblast growth factor (FGF), are discussed.

1.6.1 Hepatocyte growth factor family

Hepatocyte growth factor, also known as scatter factor (SF), was originally purified from rat platelets and known to stimulate DNA synthesis and proliferation in hepatocytes (Nakamura et al., 1984). Furthermore, HGF is known to participate in multiple activities including regeneration (renal and lung), tissue development and wound healing (Nakamura and Mizuno, 2010). HGF functions by binding to the c-met tyrosine kinase receptor and initiates cell intrinsic modifications (Birchmeier and Gherardi, 1998). Likewise, satellite cells also express c-met receptor indicating a possible role in myogenesis (Cornelison and Wold, 1997). Indeed, HGF and crushed muscle extract has been shown to activate quiescent satellite cells in monolayer cultures (Allen et al., 1995). Interestingly, blocking the activity of HGF in crush muscle extract with anti-HGF antibodies prevented quiescent satellite cell activity in mice, therefore activation was indeed induced by HGF (Tatsumi et al., 1998). HGF is expressed in uninjured and regenerating muscle and in the latter; the HGF mRNA transcript and protein levels were shown to increase during the initial stages of myogenesis (Tatsumi et al., 1998). Therefore, these studies show that the expression of c-met indicate that satellite cells is primed to activate in the presence of high HGF levels and that activation are definitely mediated by HGF.

1.6.2 Transforming growth factor- β family

TGF- β superfamily of proteins consists of more than 40 members such as the TGF- β , bone morphogenetic proteins (BMP) and growth differentiation factors (GDF). These members of TGF- β superfamily are known to regulate numerous cellular responses, including cell growth, cell-cycle progression, apoptosis, wound healing and differentiation (Vilar et al., 2006). It is commonly accepted that TGF- β proteins promote

proliferation and suppresses myogenic differentiation. There are three TGF- β members, TGF- β 1, 2 and 3.

TGF- β has been shown to suppress myogenic differentiation in the C2 myoblast cell line (Ewton et al., 1988), and promote proliferation and inhibit myogenic differentiation in C2C12 myoblast cell line (Schabert et al., 2009). Interestingly, TGF- β was shown to have no effect on either proliferation and differentiation in L6-A1 myoblast cell line (Ewton et al., 1988). Most importantly, addition of TGF- β 1 to myofiber cultures indicated a reduction of satellite cells (Bischoff, 1990). These results clearly indicate the suppression of satellite cell proliferation in their natural niche. It is therefore clear that the role of TGF- β in myogenesis is not well defined. However, much of the study of the TGF- β superfamily has shifted towards the recently discovered Myostatin, a potent negative regulator of myogenesis (McCroskery et al., 2003).

1.6.3 Fibroblast growth factor family

Initially FGF was identified based on its ability to promote proliferation in 3T3 fibroblast cell line (Armelin, 1973). Further analysis identified at least 20 FGF members that signal by binding to a dual tyrosine kinase FGF receptor system which activates downstream signals leading to many cellular and physiological processes such as apoptosis, differentiation and wound healing (Barrientos et al., 2008). Satellite cells undergoing myogenesis are shown to express FGF receptors (FGFR), specifically FGFR 1 and 4, which suggest a role of FGF in myogenesis (Armand et al., 2006). In addition, FGF6 levels have been shown to increase exclusively in myogenic cells (Armand et al., 2006). Injection of recombinant FGF6 proteins into CTX injured mice resulted in an accelerated muscle repair (Armand et al., 2003). Together, these results indicate that

FGF does play a role in myogenesis. However, the precise role of this growth factor family in proliferation and myogenic differentiation remains elusive. Analysis of FGF on the C2 myoblast cell line supported proliferation (Rando and Blau, 1994), however, studies with primary satellite cells showed only certain FGF isoforms supported proliferation. Moreover, FGF6^{-/-} knock-out studies generated contradictory results. These results suggest that FGF6 has a dual function in myogenesis by stimulating either proliferation and differentiation in a FGF dose-dependent manner (Armand et al., 2006).

In summary, studies of growth factors on myogenic cell lines, primary satellite cells and myofibers have generated contradictory results. These contradictions are likely from the accumulated mutations in the cell lines and different culturing conditions in the primary and myofibers cultures.

1.7 Age-related changes in skeletal muscle

Myogenesis, mediated mainly by satellite cells, is a highly efficient process. However, aging in skeletal muscle leads to a rapid decline in this process. This decline in myogenesis is likely associated with the intrinsic and extrinsic changes due to an increase in age. Intrinsically, the activity of myogenic specific genes such as MRFs and telomere shortening could to be altered due to aging (Degens, 2007). Extrinsically, vast changes in aging muscle include the alterations in local and systemic environment ranging from the satellite cell niche, basal lamina and the secreted local and systemic factors. Finally, satellite cell numbers have been shown to be reduced in aging muscle (Shefer et al., 2006). Collectively, these age-associated intrinsic and extrinsic changes during aging in skeletal muscle are likely to be the cause of the reduced myogenesis.

1.7.1 Intrinsic changes

Muscle specific genes such as MRF and Pax regulate myogenesis, therefore it was assumed that the reduced myogenesis in aging muscle is associated to the reduced levels and activity of MRF. Indeed, satellite cells isolated from aging muscle had fewer Pax7 expressing cells and when maintained in culture, underwent apoptosis (Collins et al., 2007). However, analysis of MRF levels, specifically the levels of MyoD and Myogenin, were contradictory (Brack et al., 2005; Dedkov et al., 2003). One intrinsic change that occurs in all somatic cells with the increase in age is telomere shortening (Bekaert et al., 2004). Severe shortening of the telomere below critical levels could result in apoptosis or *in vitro* senescence (Campisi, 1997). Studies of telomerase has shown that the telomerase activity is abolished during differentiation rather than aging, refuting the theory that an increase in age results in telomere shortening (O'Connor et al., 2009). Thus, it is not clear from such studies if telomere shortening directly impacts satellite cell function in aging muscle. The reduction of satellite cell numbers with age have also been suggested to contribute to the poor myogenesis in aging muscle. Indeed, quantification of satellite cell numbers have been shown to decrease with age (Renault et al., 2002; Shefer et al., 2006). Interestingly, although the reduction of satellite cells are observed, the myogenic capacity of the residual satellite cells in aging muscle is not altered (Collins et al., 2007). Furthermore, addition of FGF to aging satellite cells promoted efficient myogenesis (Shefer et al., 2006). Together, these data suggest that the inherent myogenic capability of satellite cells may not be altered with age and that changing the extrinsic factors, myogenesis could proceed. Lastly, the discrepancies from studies that aim to quantify the expressions of MRF and satellite cell numbers could stem from the different myofibers analyzed, age of animals, animal species, severity of the induced injury and the lack of quiescent satellite cell-specific

markers (Brack and Rando, 2007).

1.7.2 Extrinsic changes

Aging in skeletal muscle leads to vast changes in the satellite cell niche, local and systemic environment (Fig. 1.3). These changes to aging muscle are known as sarcopenia, a process that is typified by reduced muscle mass (atrophy) and strength with the accumulation of lipids and fibroblasts (Solomon and Bouloux, 2006). Collectively, these age-associated extrinsic changes in sarcopenic muscle ultimately affect the regenerative capacity of satellite cells.

1.7.2.1 Satellite cell niche

Satellite cells in the niche are located in the micro-environment between the overlaying basal lamina and the underlying myofiber. The myofibers serve as the support structure for the satellite cells and provide signals for myogenesis whereas the overlaying basal lamina separates the satellite cells from interstitial cells and fibroblasts to regulate the influx of secreted growth factors from the ECM (Gopinath and Rando, 2008). Secreted signals provided by myofibers are known to maintain satellite cell quiescence until activated, however these signals are yet to be identified. Alterations in the satellite cell niche in aging skeletal muscle results in reduced cross-sectional area with a thicker basal lamina (Brack and Rando, 2007). Generally, a disruption or alteration to the natural niche of stem cells results in the disruption of stem cell activity. Similar studies have shown that aging myofibers have an impaired activation and proliferation of satellite cells through the suppression of Notch signaling (Conboy et al., 2003). In aging muscle, the expression of Notch receptor in satellite cells remains constant; however myofibers fail to express Delta ligand which results in reduced Notch signaling thus

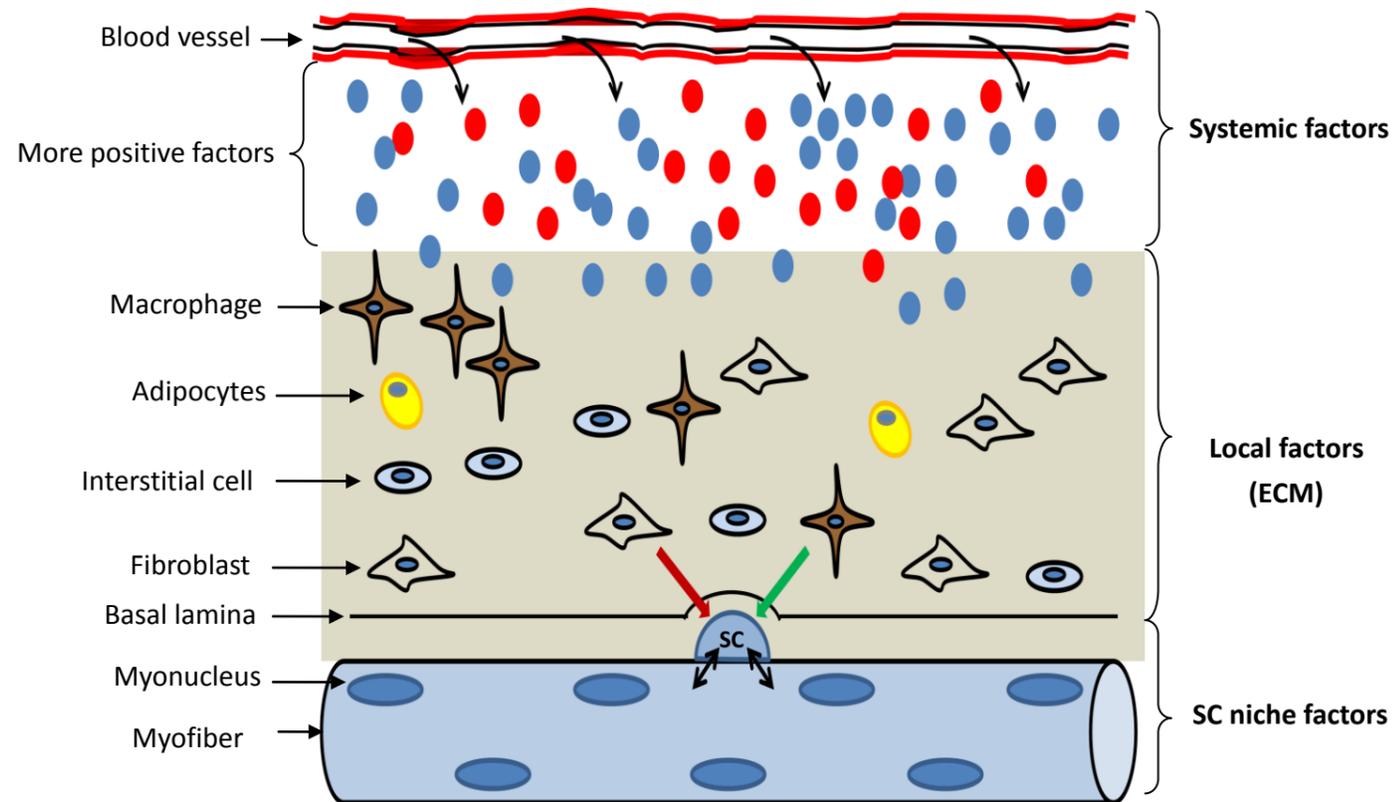
negatively impacting satellite cell myogenesis (Conboy et al., 2003). The basal lamina is composed of collagen type 4, perlecan, laminin, entactin, fibronectin, glycoproteins and proteoglycans (Sanes et al., 1978). Laminin and fibronectin, both components of the basal lamina, have been shown to promote proliferation in MM14 myoblasts (Ocalan et al., 1988). Through the progressive accumulation of toxic-by products and collagen, the basal lamina thickens with the increase in age, in addition, electron microscopic studies have indicated an extra lamina that intrudes between the myofiber-satellite cell interspace (Gopinath and Rando, 2008). The consequence of these changes renders the satellite cell less sensitive to the paracrine factors secreted from the myofibers and cells from the local and systemic environment. Satellite cells are, therefore less responsive to activating signals (HGF), proliferating signals (TGF- β) and differentiating signals (FGF). Thus, changes to the niche impacts negatively on myogenesis.

1.7.2.2 Local and systemic environment

Growth factors secreted by cells in the local and systemic environment regulate every phase of myogenesis (section 1.6). Therefore, changes to these growth factors during aging would likely affect myogenesis. TGF- β , a growth factor that is known to promote fibrosis and impede myogenic differentiation has been shown to be elevated in aging muscles (Carlson et al., 2009; Li and Fan, 2004). This increased level of TGF- β could be a compensation effect because of the less responsive aging satellite cells. Furthermore, HGF and FGF extracts from crushed aging muscle were less capable of activating both young and old myoblasts (Mezzogiorno et al., 1993). These results emphasize that the aging environment of muscles are less supportive of effective myogenesis. Therefore, the failures of satellite cells to interact with these regulatory factors or an imbalance of

negative to positive factors in aging muscles are among the cause of impaired myogenesis. The systemic environment provides another source of myogenesis regulating factors. Evidence for such regulation comes from two kinds of heterochronic studies. Earlier studies extracted whole muscles from young and old rats and grafted these into old and young host rats, respectively (Carlson and Faulkner, 1989). Results indicated that the success of the muscle graft was dependent on the age of the host rat rather than the muscle extract. Following this parabiotic study, where the circulatory system of young and old mice was linked to assess the success of myogenesis with the altered systemic environment was conducted. Indeed, old mice received young serum factors recovered effectively from muscle induced injury, whereas the young mice received the old serum factors had a reduced muscle repair (Conboy et al., 2005). In support of this study, serum from young mice was able to stimulate myogenesis whereas serum from old mice impeded myogenesis, in myoblast cultures (Brack et al., 2007). However, the factors in old serum that negatively impact myogenesis were not identified. In a separate study, both growth factors TGF- β and IGF were identified in serum from old mice and were determined to be elevated (Brack and Rando, 2007; Carlson et al., 2009). Moreover, Wnt proteins were determined to be elevated in serum from old mice which resulted in elevated Wnt signaling, lead to poor myogenesis and increased fibrosis (Brack et al., 2007). Nevertheless, the accumulated data established that satellite cell activity is regulated by local and systemic factors in a paracrine manner and most importantly, the reduced myogenesis by satellite cells in aging muscle is indeed reversible by the exposure to youthful systemic environment.

A. Extrinsic environment in young muscle



B. Extrinsic environment in aging muscle

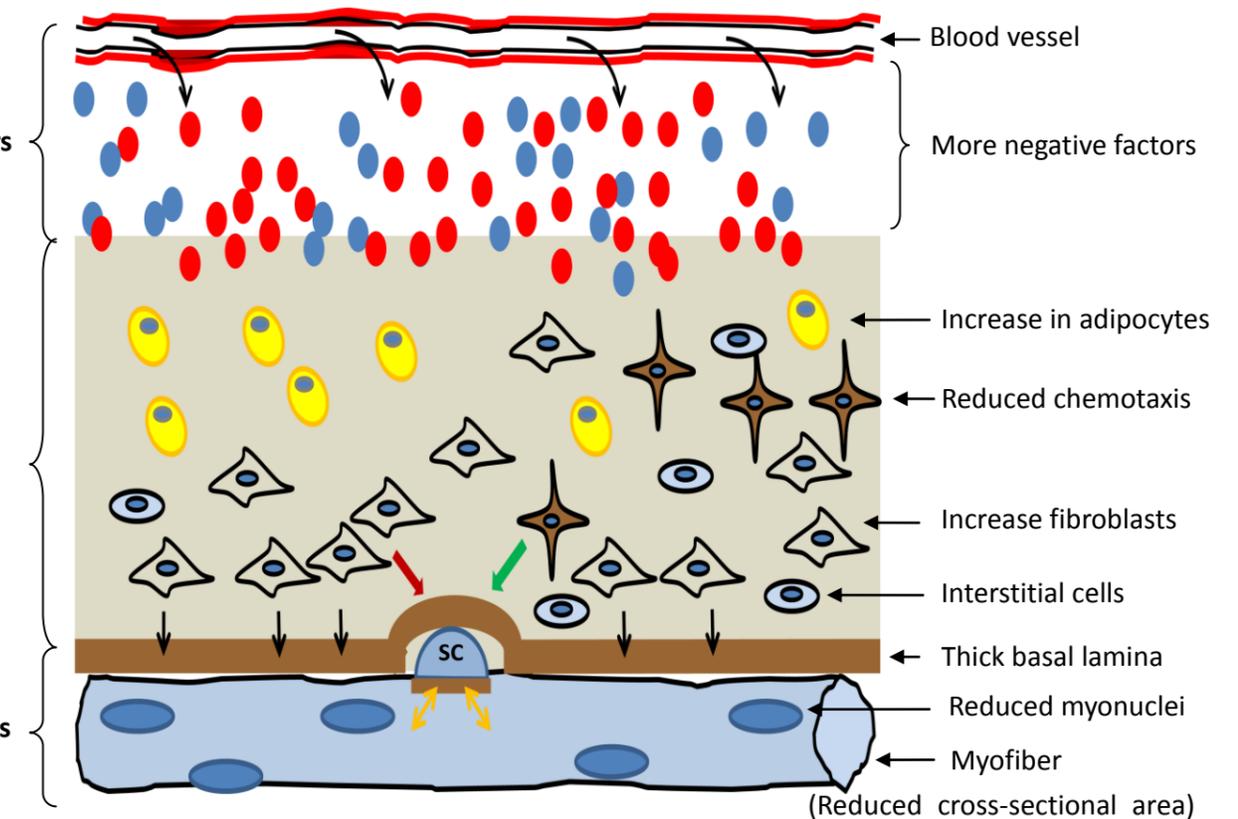


Fig. 1.3: Extrinsic environment of young and aging skeletal muscle

Extrinsic changes from young (A) and aging muscle (B) impacts satellite cell (SC) function. Satellite cells interact intimately with the supporting myofibers and the basal lamina in the satellite cell niche. In young muscle (A), paracrine and autocrine factors (Wnt, HGF, FGF, TGF- β , etc) secreted by fibroblast (red arrow), macrophages (cells of the immune system, green arrow), myofiber (double-headed arrows) and interstitial cells in the ECM modulate satellite cell activity. Furthermore, an influx of positive and negative systemic factors (curved arrows) from the circulatory system (A) further alters the extrinsic environment that impacts satellite cell function. In aging muscle (B), the basal lamina thickens due to collagen deposits from fibroblasts (black arrow). The encapsulating lamina, which envelopes satellite cell, renders satellite cell less susceptible to autocrine and paracrine signals from fibroblasts (red arrow), macrophage (green arrow) and the supporting myofiber (yellow double-headed arrows). Moreover, it has been suggested that high levels of negative factors such as Wnt and TGF- β from the circulatory system in aging muscle contribute to impaired regeneration. Further alterations in aging muscles (B) include an increase in adipocyte number, reduced myonuclei number, increase in fibroblast number and reduced muscle cross-sectional area. Collectively, these changes ultimately modify the properties of satellite cell in aging muscle and are hallmarks of aging sarcopenic muscle.

1.8 The Wnt family of signaling proteins

Wnt proteins are a class of glycosylated, secreted, signaling and in certain isoforms, palmitoylated signaling proteins (Willert et al., 2003). Wnt is coined from the combination of *Drosophila Wingless* (Wg) and mouse Int-1 genes. There are three classes of Wnt signaling: a) the well characterized canonical/ β -catenin dependent pathway and the poorly understood non-canonical/ β -catenin independent pathways namely, b) calcium (Ca^{2+}) and c) planar cell polarity (PCP) pathways that leads to activation of distinct genes (Huelsenken and Behrens, 2002). The outcome of the expressed genes has been shown to play a vital role in a variety of cellular processes such as proliferation, differentiation, apoptosis, orientation, cell senescence and migration (Maiese et al., 2008). More recently, Wnt signaling has been shown to be vital in myogenic differentiation and myogenic lineage specification.

1.8.1 Wnt proteins

Currently, there are at least 21 identified Wnt isoforms with molecular weight of 39-46 kDa (Maiese et al., 2008). Wnt proteins contain a conserved distribution of cysteines and a signal sequence (Kikuchi et al., 2007). Furthermore, these proteins are glycosylated and palmitoylated (or lipidated) and as a result are mostly insoluble (Kikuchi et al., 2007). This high degree of insolubility makes Wnt proteins difficult to purify and therefore difficult to characterize. In addition, the level of secretion of Wnt proteins is known to be very low, therefore it is difficult to obtain sufficient quantities for analysis. The difficulty to obtain purified Wnt proteins leads to alternative methods to assess the effect of Wnts, such as conditioned media and co-culturing. Conditioned media are generated initially by culturing transfected cells (with Wnt containing construct) to secrete the desired Wnts followed by utilizing the Wnt-conditioned media to culture the cells of interest. Co-culturing involves culturing

of transfected cells simultaneously with the cells of interest which offer a constant supply of secreted Wnt. Both methods generate media with high levels of secreted Wnts to monitor the effect on the cells of interest. However, results utilizing these methods have been contradictory to the results generated with purified Wnts. Presently, only Wnt3a and Wnt5 proteins are successfully purified from conditioned media (Mikels and Nusse, 2006; Willert et al., 2003). The effect of the purified Wnt3a was shown to promote hematopoietic stem cell proliferation and suppress differentiation, conversely, Wnt3a-conditioned media from mouse L fibroblast cells promoted differentiation (Willert et al., 2003). Furthermore, analysis of canonical vs. non-canonical pathway initiated by the purified Wnt5a was contradictory to conditioned media (Mikels and Nusse, 2006). These results suggest that other factors in the conditioned media or co-culture secreted by the transfected cells could cause these discrepancies. Therefore, these results highlight the importance of purified proteins to analyze the role of Wnt proteins.

1.8.2 Canonical vs. non-canonical Wnt signaling

The canonical/ β -catenin dependent pathway is activated by binding of Wnt protein to the Frizzled receptor and low-density lipoprotein receptor-related protein-5/6 (LRP5/6) co-receptor. In the absence of Wnts, disheveled protein is inactivated and as a result, a β -catenin destruction complex is formed, comprised of Axin, glycogen synthase kinase-3 β (GSK-3 β), Casein Kinase-1 (CK1) and Adenomatous polyposis coli (APC) (Jin et al., 2008). In this complex, β -catenin is phosphorylated multiple times at serine or threonine residues 41, 37 and 33, firstly by GSK-3 β followed by the phosphorylation by CK1 at serine 45 (Huelsen and Behrens, 2002). Subsequently, the phosphorylated β -catenin is ubiquitinated by β -transducin repeat-containing protein (β TrCP) and degraded by the large protease, proteasome (Fig. 1.4A).

Therefore, no Wnt-related genes are activated. Canonical/ β -catenin dependent pathway is activated with Wnts binding to the Frizzled receptor (Fig. 1.4B). This ligand-receptor interaction activates Dsh and prevents the formation of β -catenin destruction complex. This in turn prevents β -catenin phosphorylation by inhibiting GSK-3 β . Stable cytosolic β -catenin accumulates in the cytoplasm and eventually translocates into the nucleus, complexes with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor and lead to expression of Wnt target genes which include MyoD and Myogenin (Jin et al., 2008). The poorly understood non-canonical/ β -catenin independent pathways include the calcium (Ca^{2+}) and planar cell polarity (PCP) pathways. In the cell polarity pathway, Wnt binds to the receptor without LRP5/6 co-receptor and activates JNK via the activation of GTPase Rho and directs cytoskeletal re-organization. Rearrangement of the cytoskeletal proteins can also be mediated directly by the activation of Rho-associated kinase (ROCK). This pathway has been shown to play a role in migration (Cadigan and Nusse, 1997). The activation of Ca^{2+} pathway leads to the production of intracellular calcium. Binding of Wnt to the Frizzled receptor activates phospholipase C (PLC) and protein kinase C (PKC) possibly through G-protein. Activation of PLC leads to the accumulation of calcium and can activate phosphatase calcineurin which dephosphorylates NF-AT (Huelsen and Behrens, 2002). The de-phosphorylated NF-AT translocates into the nucleus and activates Ca^{2+} pathway genes. It is clear that the non-canonical pathway has only been partially defined; therefore further studies are required to define the interactions of proteins in the non-canonical pathway. Although these studies have partially clarified the mechanism of Wnt signaling and the proteins involved, but due to the lack of purified active Wnt proteins, it has been difficult to verify which Wnt isoform activates which signal pathway.

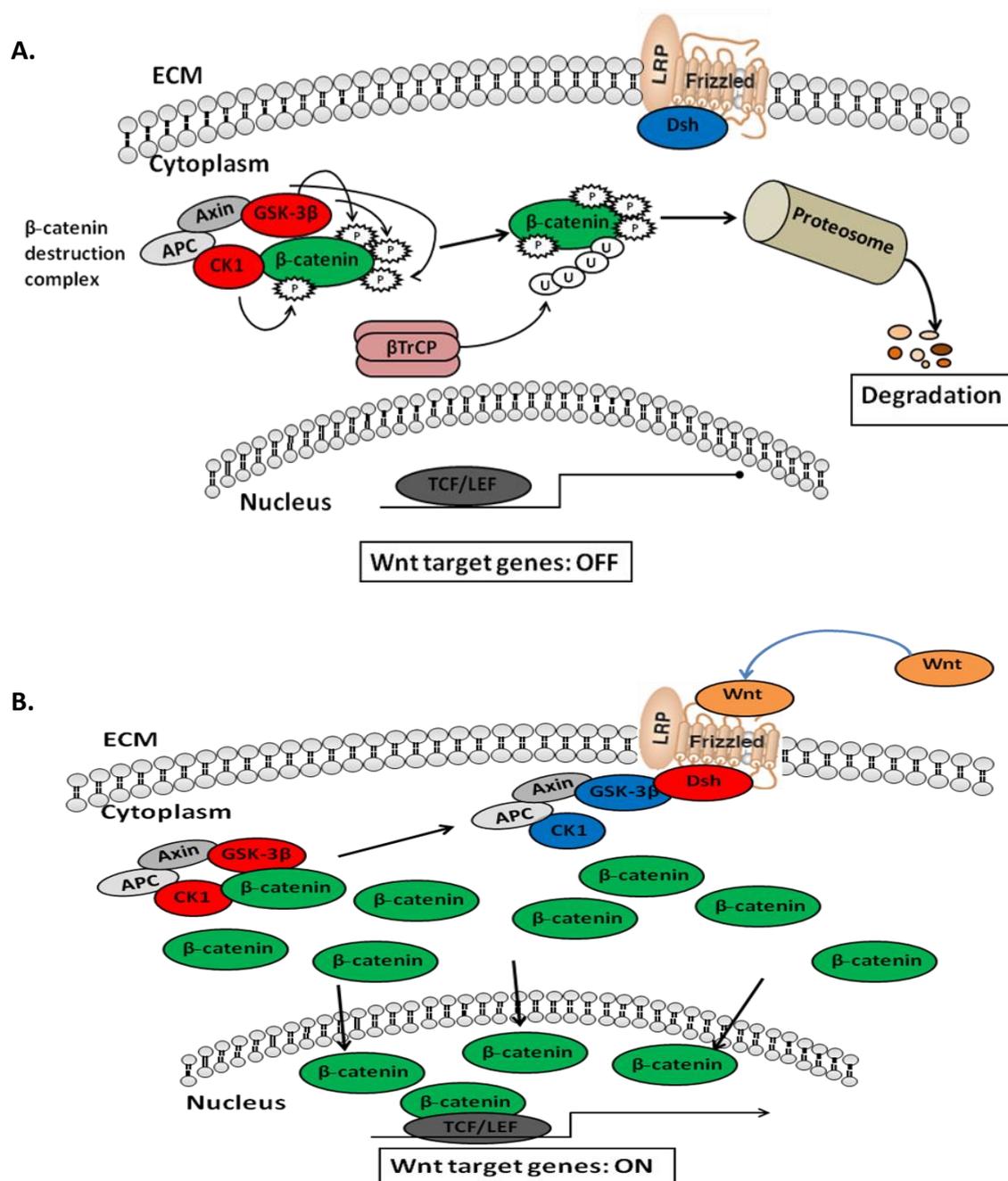


Fig. 1.4: The canonical/ β -catenin dependent Wnt signaling

Activation of canonical Wnt signaling results in the target gene expression via β -catenin. In the absence of Wnt, β -catenin is phosphorylated by GSK-3 β , CK1 in the β -catenin destruction complex, ubiquitinated by β TrCP and actively degraded by the proteasome in the cytoplasm (A). Activation of the canonical Wnt signaling leads to the accumulation of β -catenin (B). Wnt binds to the Frizzled receptor, activates Dsh and prevents the formation of β -catenin destruction complex and accumulates in the cytoplasm. β -catenin translocates into the nucleus, associates with TCF/LEF and activates target genes. Expression of target genes have been shown to play a vital role in numerous cellular processes such as proliferation, differentiation, apoptosis, orientation, cell senescence and migration

The purified Wnt3a has been shown to signal through the canonical pathway, verified by the accumulated cytosolic β -catenin (Willert et al., 2003). Interestingly, Wnt5a was initially classified as a canonical Wnt protein (He et al., 1997), however, recent studies have shown that Wnt5a signals via canonical pathways only in the presence of an appropriate receptor, Frizzled 4 (Mikels and Nusse, 2006). Together, both studies have shown the importance of obtaining pure Wnt proteins to determine its role and the signaling pathway it induces.

1.8.3 Wnt signaling in myogenesis

A wealth of data has been generated documenting the influence of Wnts on embryological development. Wnt5a has been shown to inhibit secondary axis formation in *Xenopus* embryos whereas Wnt1 class induces this formation (Li et al., 2006). Furthermore, Wnts have an effect on myogenesis during embryogenesis. Subsequent studies have led to the understanding of Wnt proteins on adult tissues. In the complicated process of cardiac wound healing in the heart, it has been shown that Wnt10b and Wnt8a are up-regulated while Wnt7b is down-regulated after myocardial infarction (Barandon et al., 2003).

Traditionally, it was accepted that Wnt proteins promote proliferation in a variety of stem cell cultures, however in isolated myoblasts, Wnts were shown to promote myogenic differentiation. Numerous studies have generated adequate evidence that Wnt signaling play a role in myogenic differentiation, specification and lineage progression. Early studies have indicated that Wnt signaling by Wnt3a promotes myogenesis in P19 embryonal carcinoma stem cells by up-regulating MyoD and Myogenin (Ridgeway et al., 2000). Later, β -catenin, a down-stream target of Wnt signaling, was shown to interact directly with MyoD and enhance MyoD

transcriptional activity (Kim et al., 2008; Petropoulos and Skerjanc, 2002). Analysis of myoblast cultures revealed that the induction of myogenic differentiation coincides with the up-regulation of canonical Wnt signaling and by suppressing this pathway with secreted frizzled receptor protein-3 (sFRP3) antagonist, myogenesis was reduced (Brack et al., 2008). This induction of myogenic differentiation requires the correct temporal down-regulation of the proliferation regulating Notch pathway and the up-regulation of Wnt signaling. However, injection of Wnt3a to the regenerating muscles in mice had a detrimental effect on muscle repair which could arise from the early up-regulation of Wnt signaling by the injected Wnt3a that initiated premature differentiation rather than proliferation by the Notch pathway. Furthermore, an increase in Wnt3a mRNA transcript levels were observed which coincide with the up-regulation of Wnt signaling (Brack et al., 2008). Finally, non-myogenic CD45⁺ cells derived from muscles up-regulated Pax7 and MyoD in media containing a Wnt5a, 5b, 7a and 7b (Polesskaya et al., 2003). Collectively, data from these studies indicate that Wnt signaling regulates myogenic differentiation, specification and lineage progression.

1.8.4 Wnt signaling in aging muscle

Aging in skeletal muscle brings about vast changes that alter satellite cell myogenesis (section 1.7). Recently, parabiotic studies indicated that systemic factors impact satellite cell function and following exposure of old mice to a youthful environment, aging satellite cells are rejuvenated and undergo effective myogenesis (Conboy et al., 2005). In support of this hypothesis, muscle sections from heterochronic mice pairs showed a reduced collagen deposition in old muscle with an increase in proliferation, verified by the BrdU incorporation experiment (Brack et al., 2007).

The reduced myogenic capability in aging muscle is accompanied by the increase in fibroblast number. Utilizing a fibroblast marker, ER-TR7, isolated myoblast cultures showed an increase in ER-TR7⁺ cells in aging myoblasts (Brack et al., 2007). Therefore, this age-related decline in satellite cell myogenesis is also accompanied by a myogenic to fibrotic lineage conversion. The effect of serum components were also analyzed for regulating myogenesis on isolated myoblasts from young and old mice. Results showed that young serum promoted myogenesis in old myoblasts, whereas old serum impeded myogenesis in young myoblasts (Brack et al., 2007). It was speculated that the up-regulation of Wnt signaling contributed to the reduced myogenesis. Indeed, analysis of the downstream targets of Wnt signaling such as Axin and β -catenin, in old myoblasts were increased significantly (Brack et al., 2007). Incubation of young myoblasts with serum from old mice showed reduced myogenesis therefore it is likely that Wnt proteins are found in serum. This was verified by Wnt depletion experiment. In the Wnt depletion experiment, two fusion proteins with Frizzled receptor on each were constructed (Brack et al., 2007). The fusion proteins were incubated with the serum from old mice and followed by culturing with myoblasts. The result showed an improvement in myogenesis. *In vitro* and *in vivo* assays showed that the incubation or injection of Wnt3a contributed to this impaired myogenesis. Together, these series of experiments showed that the up-regulation of Wnt signaling by Wnt3a in the serum of the circulatory system was the contributing factor in the reduced myogenesis and the increase in myogenic to fibrotic lineage conversion.

The increase in adipocytes is another hallmark of aging muscle. The balance between myogenic and adipogenic lineage was shown to be controlled by Wnt signaling via Wnt10b, however this balance is lost during aging. Up-regulation of Wnt signaling by

Wnt10b was shown to be crucial to inhibit myogenic to adipogenic lineage conversion by mechanical stretching concurrent with the increase in Wnt10b mRNA transcript levels in C2C12 cells (Akimoto et al., 2005). However, in aging muscle, this increase in Wnt10b mRNA transcript levels was linked to cause a myogenic to adipogenic lineage conversion with age (Taylor-Jones et al., 2002). Interestingly, Wnt10b^{-/-} knock-out mice demonstrated effective myogenesis in young mice (Vertino et al., 2005). Further studies indicated a compensation effect by Wnt7b that contributed to the successful myogenesis, however in aging myoblasts, this compensation was absent and in fact show a down-regulation of Wnt10b and 5b (Vertino et al., 2005). Therefore, results from these studies indicate that regulation of adipogenesis by Wnt signaling via Wnt10b has not been clearly defined and that many discrepancies in data exist. Moreover, it is clear from the knock-out studies that other classes of Wnt proteins do play a role in regulating lineage alteration. However due to the lack of purified Wnt proteins it is difficult to verify which class of Wnts contributes to the reduced myogenesis and the increased lineage alteration with age.

1.9 Objectives of the current study

Canonical Wnt signaling has been demonstrated to gradually increase during myogenesis which corresponds with the gradual rise in mRNA Wnt transcripts, specifically Wnt3a (Brack et al., 2008). However, due to the previous lack of Wnt antibodies, quantitative analysis of relevant Wnt proteins during myogenesis has not been determined. Therefore, the aim of the current study was to: 1) analyze Wnt protein expression, specifically Wnt3a, 7 and 10b isoforms, during proliferation and differentiation in early and late passage C2C12 cells, utilizing the currently available antibodies. Both cytosolic and secreted forms of Wnts were quantified, 2) Determine

the effect of the secreted Wnts in the conditioned media from the early and late passage C2C12 cells on late and early passage C2C12 cells, respectively. We hypothesise that secreted combinations of Wnt3a, 7 and 10b isoforms from early passage C2C12 cells can improve myogenic differentiation in late passage C2C12 cells, and vice versa.

CHAPTER TWO

BASELINE EXPRESSION PROFILE OF WNT ISOFORMS DURING DIFFERENTIATION OF AGING C2C12 MYOBLAST CELLS

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ABSTRACT

Satellite cells are muscle progenitor cells responsible for skeletal muscle maintenance and repair. The capacity of these progenitor cells to differentiate into myotubes decreases with age; as a result, muscle repair in an aging organism is characterized by fibrosis and lipid accumulation rather than myogenesis. Recent studies have suggested that the Wnt family of signaling proteins is pivotal in regulating cell fate, proliferation and differentiation, during aging. Wnt3a is known to induce fibrogenesis and Wnt10b adipogenesis, whereas Wnt7 induces myogenesis.

In the current study, we aim to determine the cytosolic expression and the secreted profiles of the three key Wnt isoforms, Wnt3a, 7 and 10b, during myogenesis of early and late passage C2C12 myoblasts. Late passage myoblasts were seen to have a higher proliferative rate, as well as a higher expression of GAPDH, α -tubulin and surprisingly, MyoD, than early passage cells. However, myogenic capability was reduced in the late passage cells as indicated by both the lack of myotube appearance and Myogenin expression. Analysis of Wnt3a showed higher levels of cytosolic protein in late vs. early passage cells at all-time points analyzed, suggesting an elevated fibrogenic predisposition. Secreted levels of Wnt7 were maintained at higher levels in early vs. late passage cells at day 1 and 3. The rapid decreased

secretion of Wnt7 in late passage cells suggests a possible mechanism for the inefficient myogenesis observed. Lastly, Wnt10b cytosolic levels were constantly elevated in early versus late passage cells. Analysis of the secreted versus cytosolic ratio in Wnt7 levels revealed a more rapid decline in late passage versus early passage cells during differentiation, suggesting a decreased ability to promote myogenesis. Taken together, increased cytosolic production of Wnt3a with decreased secretion of Wnt7 and 10b in late passage cells supports their lack of myogenic potential.

2.1 INTRODUCTION

Satellite cells are resident, quiescent skeletal muscle stem cells located in a niche between the sarcolemma and basal lamina along muscle fibers (Charge and Rudnicki, 2004; Mauro, 1961). Satellite cells are characterized their anatomical location, molecular marker expression and function. In their quiescent state, they express markers such as Pax7, M-cadherin, c-met, SM/C-2.6 and CD34 (Beauchamp et al., 2000; Fukada et al., 2004; Hollnagel et al., 2002; Tatsumi et al., 1998; Zammit et al., 2006). When new myonuclei are required to facilitate hypertrophy or repair, satellite cells exit quiescence, begin to proliferate, differentiate and eventually fuse with each other (or with existing myofibers), to form new myotubes (Burdzinska et al., 2008). The up-regulation of myogenic regulatory factor (MRF) expression such as MyoD, Myf5, Myogenin and Mrf4 is central to this process (Fuchtbauer and Westphal, 1992; Holterman and Rudnicki, 2005). In addition, the repair process is accompanied by satellite cell self-renewal, ensuring that after repeated rounds of regeneration, the stem cell population is maintained (Kuang et al., 2007).

Following a severe injury (such as shearing) the regenerative potential of satellite

cells is compromised (Charge and Rudnicki, 2004). This type of injury causes the complete rupture of the basal lamina, necrosis of myofibers and subsequent collagen deposition that result in scar formation and hampers satellite cell function. On the other hand, aging muscle is characterized by muscle atrophy due to poor maintenance by satellite cells, a condition known as sarcopenia (Solomon and Bouloux, 2006). Numerous studies suggest this age-related lack of regeneration is due to a decline in satellite cell function, most likely associated with the altered intrinsic activities of MRF and low satellite numbers (Conboy et al., 2005; Shefer et al., 2006).

The extrinsic environment is also known to change with age in skeletal muscle. Local (the satellite cell niche and myofiber) and systemic (changes in the circulating serum factors) conditions change which directly impact satellite cell function. With age the satellite cell niche and the myofiber are altered with the thickening of the basal lamina and a further layer of basal lamina between the myofiber-satellite cell interspace. This leads to the loss in sensitivity of satellite cells to paracrine and endocrine factors secreted by the cells of the extracellular matrix (ECM): infiltrating neutrophils, monocyte, macrophages and myofiber itself (Gopinath and Rando, 2008). Essential growth factors such as hepatocyte growth factors (HGF), fibroblast growth factor (FGF) and transforming growth factor-beta (TGF- β) are known to enhance the activation and proliferation of satellite cells (Schabort et al., 2009; Tatsumi et al., 1998; Yablonka-Reuveni et al., 1999). In addition, an increase in adipocyte and fibroblast numbers within aging muscle has been observed.

Systemic changes, specifically serum factors, have been shown to modify satellite cell functions and decrease myogenic lineage progression. Parabiotic studies, where the

circulatory system of old and young mice were linked, revealed that serum factors within the circulatory system of a young mouse were able to rejuvenate the regenerative capacity of the satellite cells in skeletal muscle of the old mouse (Conboy et al., 2005). Results showed that satellite cells in young mice had a reduced myogenic differentiation capability and trans-differentiated to acquire a fibrogenic lineage when exposed to the serum from old mice (Brack et al., 2007). Further analysis revealed that a Wnt protein, specifically Wnt3a, was a pivotal mediator of the observed phenotypic change. This myogenic-to-fibrogenic conversion was reflected by an increased collagen deposition thereby further promoting the fibrotic environment.

TGF- β s are known inhibitors of myogenic differentiation and recently, elevated TGF- β 1 levels were also reported to be found in serum of aging muscle (Carlson et al., 2009). Elevated Wnt and TGF- β levels in serum of aged muscles could both have a deleterious effect on muscle repair and regeneration. Taken together, the parabiotic pairing technique suggests that the extrinsic factors from the local and systemic environment changes with age; this change in turn directly impacts satellite cell function and lineage progression. Interestingly, these results also indicate that the resulting intrinsic changes with age are indeed reversible (Brack et al., 2007).

Wnt signaling is known to play a key role in myogenic differentiation as seen by the fact that a regulated temporal switch from Notch to Wnt signaling is required for myogenesis (Brack et al., 2008). Moreover, a sub-population of CD45⁺ cells (with hematopoietic potential) from injured muscle can adopt a myogenic lineage in the presence of Wnts5a, 5b, 7a, and 7b mix (Polesskaya et al., 2003). Satellite cells are also known to trans-differentiate more readily into an adipogenic lineage with age

which may also be mediated by changes in serum factors and possibly by the presence of elevated Wnt10b levels (Asakura et al., 2001; Vertino et al., 2005). In aging muscle, elevated levels of certain Wnts can activate the Wnt pathway prematurely leading to impaired myogenesis. The expression of Wnt proteins at the mRNA level has been well documented during differentiation of myoblasts; however, due to the previous lack of commercial antibodies, the Wnt protein expression has not been determined to date. Therefore, the aim of his study was to determine the levels of cytosolic and secreted Wnt proteins (specifically Wnt3a, 7 and 10b) during proliferation and differentiation of early and late passage C2C12 cells.

2.2 MATERIALS AND METHOD

2.2.1 Materials

Low passage C2C12 cells were kindly donated by the Cape Heart Centre (University of Cape Town). Specialized reagents to sub-culture, maintain and differentiate C2C12 cells are trypsin-EDTA, horse serum (Sigma), fetal bovine serum (Gibco) and Penicillin-streptomycin (Highveld Biologicals). Dulbecco's Modified Eagles Medium and L-glutamine were from Sigma (South Africa). C2C12 cells were cultured in T75, T25 or 6 well plates from Nunc (Denmark) and maintained in an Innova CO-170 CO₂-incubator (New Brunswick). C2C12 cells were monitored with Moticam 2300 camera (Motic) mounted on Olympus U-MCB Provis microscope.

Table 2.1: Individual reagents for growth and differentiation medium

Reagents	Percentage (%)	Volume
#Fetal calf serum	10	50
*Horse serum	1	5
Penicillin-Streptomycin (Penstrep)	2	10
L-glutamine	2	10
Dulbecco's Modified Eagles Medium (DMEM)	-	430

Growth medium constitutes #fetal calf serum, penicillin-streptomycin, L-glutamine and DMEM. For differentiation medium, fetal calf serum was substituted with *horse serum. The growth and differentiation medium were aliquoted into 50 ml falcon tubes and stored at 4 °C.

General reagents for buffers (Table 2.1) and experiments are NaCl, Potassium ferricyanide, Potassium ferrocyanide, MgCl₂, phosphoric acid, Sodium dodecyl sulfate (SDS), Ammonium persulfate, glycine, glycerol, bromophenol blue, Polyethylene glycol (PEG) 20 000, Potassium chloride (KCl), Na₂HPO₄, KH₂PO₄, gluteraldehyde, Mercaptoethanol and citric acid monohydrate were purchased from Merck. Tris-HCl purchased from Melford, Formaldehyde and methanol from BDH, ethanol from Illovo (South Africa) and Tween 20 from Sigma (South Africa).

Table 2.2: General buffers

Buffers	pH	Application
Phosphate-buffered saline (PBS)	7.4	Cell culture, *ICC
Citrate/phosphate buffer	6	#SA-β-gal assay
Tank buffer	-	SDS-PAGE
Transfer buffer	-	Western blot
Tris-buffered saline (TBS)	7.5	Western blot

*Immunocytochemistry; # Senescence-associated β-galactosidase

Specialized reagents for senescence-associated β-galactosidase (SA-β-gal) assay Bradford assay, SDS-PAGE, western blotting and Immunocytochemistry (ICC) are 5-Bromo-4-Chloro-3-indoyl-β-D-galactopyranoside (X-gal) (Bioline, BIO-37035), Coomassie brilliant blue G-250 (Sigma, 27815), ovalbumin (Roche), dialysis tubes

(Sigma, D9652), acrylamide and bisacrylamide (Sigma), pegGOLD protein marker-V (peqLAB), RIPA buffer (containing: 50 mM Tris-HCl, pH 8.0; 150 mM sodium chloride; 1.0% Igepal; 0.5% sodiumdeoxycholate; 0.1% sodium dodecyl sulfate), protease inhibitor cocktail (containing: AEBSF, Aprontinin, Bestatin hydrochloride, E-64, Leupeptin, Pepstatin A, P8340), nitrocellulose membrane (Hybond-C™ Extra, Amersham), Ponceau S (Fluka, 81460), donkey serum (Sigma, D9663), primary and secondary antibodies (Table 2.3), donkey anti-mouse-FITC, Hoechst (Sigma, B2261), phalloidin-TRITC (Sigma, P1951) and ECL kit (Immun-Star™ WesternC™ Chemiluminescent Kit, BioRad), Biomax light film (Sigma) and fixer and developer (Kodak).

Table 2.3: Dot blot optimized primary and secondary antibodies for western blot analysis

Primary Antibody	Host species	Size (kDa)	Dilution	Clone, company
MHC	Mouse monoclonal	~200	$1/400$	MF 20, Hybridoma Bank
MyoD	Mouse monoclonal	~30	$1/400$	554130, BD Bio (5.8A)
Myogenin	Mouse monoclonal	~36	$1/400$	sc-12732, Santa Cruz (F5D)
Wnt3a	Rat monoclonal	~55	$1/3200$	sc-80457, Santa Cruz (YY-7)
Wnt7a/b	Rabbit polyclonal	~55	$1/800$	sc-32865, Santa Cruz (H-40)
Wnt10b	Rat monoclonal	~55	$1/400$	MAB2110, R&D (254206)
* α -tubulin	Mouse monoclonal	~55	$1/400$	sc-5286, Santa Cruz (B-7)
*GAPDH	Rabbit monoclonal	~36	$1/4000$	2118, Cell Signaling, (14C10)
Secondary antibody			Dilution	Company
Polyclonal rabbit anti-mouse Immunoglobulins/HRP			$1/16000$	P-0260, DAKO
Polyclonal goat anti-rabbit Immunoglobulins/HRP			$1/4000$	P-0448, DAKO
Polyclonal rabbit anti-rat IgG/HRP			$1/40000$	A9542, Sigma

The molecular weights of target antigens were determined via western blotting (section 2.2.7). Primary antibodies were optimized with dot blot analysis prior western blotting (section 2.2.6). *Used as loading control for semi-quantitative western blot-densitometry analysis.

2.2.2 Cell culture

Early (P13) and late passage (P33) murine C2C12 cells were maintained in growth medium containing Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% Fetal bovine serum (Gibco), 2 mM L-glutamine (Sigma) and 1% Penicillin/Streptomycin (Highveld Biological) in an Innova[®] CO-170 CO₂-incubator (New Brunswick) at 37 °C, 5% CO₂. Cells were induced to differentiate when 80% confluent by changing growth medium with differentiation medium containing of Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 1% horse serum (Sigma), 2 mM L-glutamine (Sigma) and 1% Penicillin/Streptomycin (Highveld Biological). Differentiation was monitored and images captured at days 0, 1, 3, 5 and 7 with a Moticam 2300 camera (Motic) mounted on Olympus U-MCB Provis microscope.

2.2.3 Senescence-associated β -galactosidase assay

Early and late passage C2C12 cells were plated into 6 well plates (50 000 cells/well). Upon reaching ~40% confluency, cells were washed with PBS, fixed with 2% formaldehyde: 0.2% gluteraldehyde for 8-10 minutes, washed with PBS and stained with fresh X-gal stain solution: 1 mg/ml X-gal, 40 mM citrate/phosphate buffer, 300 mM NaCl, 5 mM Potassium Ferricyanide, 5 mM Potassium Ferrocyanide, 2 mM MgCl₂ for 16 hours at 37°C. Stained cells were washed with PBS, viewed with an Olympus U-MCB Provis microscope and images captured with a Moticam 2300 camera (Motic). Images were analyzed using Image J software and percentage senescence determined as the number of stained cells divided by the total number of cells in field of view.

2.2.4 Analysis of proliferation

To determine the proliferation potential of early and late passage C2C12 cells, we plated 750 000 cells into T75 flasks containing growth medium (day 0). Cells were trypsinized on days 1 and 2, and counted using a haemocytometer.

2.2.5 C2C12 protein lysate and conditioned media preparation

Early and late passage C2C12 cells were harvested at different time points (day 0, 1, 3, 5 and 7), detached with trypsin-EDTA, lysed in RIPA buffer (Sigma) and protein inhibitor cocktail (Sigma, P8340), and sonicated for 5 seconds in eppendorf tubes submerged in ice. For the conditioned media (CM) preparation, early and late passage cells were plated into T25 flasks in ~10ml growth medium and upon reaching 80% confluency, the growth medium was collected (day 0, control) and cells were induced to differentiate by changing to differentiation medium and subsequently collected at day 1, 3 and 5. All collected CM were stored at -20 °C. To concentrate the diluted CM, CM were transferred into dialysis tubing (Sigma) and concentrated against PEG 20 000 (Merk) to ~500µl. Prior to western blotting, total protein lysates and CM concentrations were done according to Bradford (1976).

Briefly, a standard curve was constructed from an ovalbumin (Roche) standard protein stock with a range of chosen concentration, 0 to 40 µg/ml (Fig. 2.1). A mixture of Bradford dye with the desired ovalbumin concentration for each concentration points was prepared and absorbance reading at 595 nm was taken with a Biomate 3 spectrophotometer (Thermo Scientific). The unknown concentrations of the total lysates and CM were determined with the linear regression formula (Fig. 2.1). The protein lysates were probed for the transcription factors MyoD, Myogenin and the cytosolic Wnt3a, 7 and 10b, whereas the CM was

probed for the secreted form of Wnt3a, 7 and 10b by western blotting.

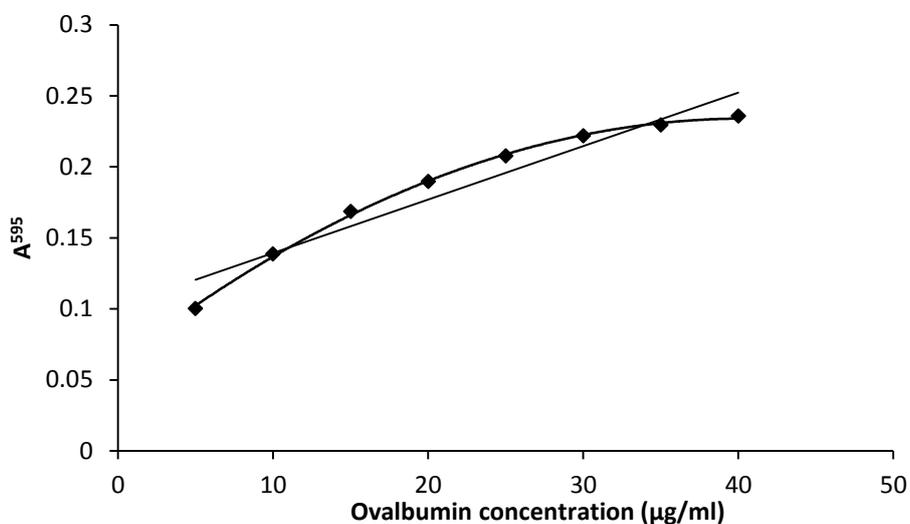


Fig. 2.1: Ovalbumin Bradford standard curve

Ovalbumin standard curve was constructed to determine the unknown concentrations of C2C12 cell lysates and the conditioned media. Ovalbumin protein was made up in d.H₂O to a concentration of 1 µg/ml and subsequently aliquoted at different volumes according to the desired concentrations (0 – 40 µg/ml), Bradford (Coomassie brilliant blue G-250) dye were added and made up to a final volume of 1 ml with d.H₂O in Eppendorfs. The protein-dye mixtures were transferred into plastic curvettes and absorbance readings taken at 595 nm with a spectrophotometer. A linear regression formula was determined ($y = 0.0038x + 0.1017$) with a correlation coefficient of 0.928 using the standard curve and subsequently used to determine the protein concentrations of C2C12 cell lysates and collected conditioned media. To ensure accuracy, quintuplets (n=4) were performed per concentration.

2.2.6 Dot blot optimization of primary antibodies

Due to the sensitivity (at mid-femtomol of protein) of enhanced chemiluminescence (ECL) (Kricka, 1987), protein-protein interactions of lysate proteins with primary antibodies could result in ambiguous 'false-positive' signals. Thus, it is vital to optimize primary antibodies through simple dot blot analysis before proceeding to western blotting. Dot blot analysis provides an indication of a suitable dilution for western blotting and potentially prolongs the use of purchased antibodies (if higher dilutions are ultimately used). Therefore, all purchased primary antibodies were

optimized via dot blot analysis before proceeding to western blotting. Briefly, 1 μ l of cell lysate was dotted onto a nitrocellulose sheet (Hybond-C™ Extra, Amersham), air-dried and blocked with 5% skim milk in TTBS (1 hour). Thereafter, nitrocellulose membrane was cut into single blocks and incubated in individual chambers with a range of chosen diluted primary antibodies, in TTBS (overnight at 4 °C). Starting from the dilution recommended by manufacturer, we increase the dilution series by two-fold (example shown in Fig. 2.2). Four dilution points were usually sufficient to see a variation in signal intensity. After primary incubation, the nitrocellulose membrane was washed and incubated in secondary HRP-linked antibodies (Table 2.3) in TTBS (1 hour) at room temperature. The antigen-antibody complex was visualized using an ECL kit (Immun-Star™ WesternC™ Chemiluminescent Kit, BioRad). The nitrocellulose membranes were exposed onto Biomax light film (Kodak), captured on VersaDoc™ MP imager (BioRad) and visualized with Quantity One software (BioRad). From the signal intensity of the 'dot', the primary antibody dilutions were chosen for western blotting. All dot blot optimized primary antibodies are shown in Table 2.3 with the respective secondary antibodies.

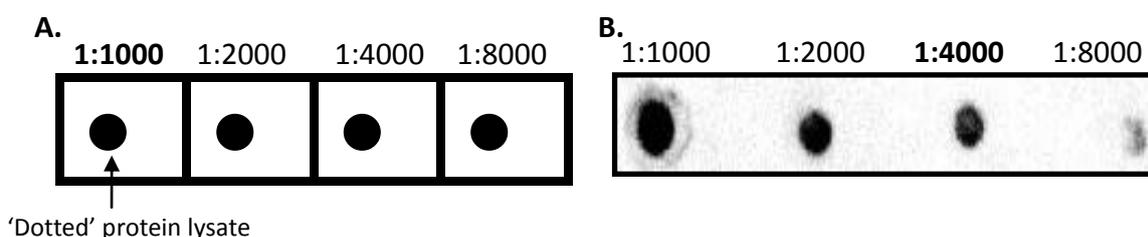


Fig. 2.2: Experimental design of the dot blot analysis for rabbit monoclonal anti-GAPDH antibody

Dot blot analysis was utilized to optimize rabbit monoclonal anti-GAPDH antibody. 1 μ l of cell protein lysates were 'dotted' onto nitrocellulose membrane (A) and incubated in the anti-GAPDH antibody dilutions in TTBS, starting with the manufacture's recommended dilution 1:1000 (**in bold**) to 1:8000. After blocking with 5% skim milk in TTBS and incubated with goat anti-rabbit secondary antibody (DAKO), the antigen-antibody complex was visualized with the addition of ECL reagent (Immun-Star™ WesternC™ Chemiluminescent Kit, BioRad) (B). From the signal intensity, the anti-GAPDH antibody dilution of 1:4000 (**in bold**) was chosen.

2.2.7 Western blotting of C2C12 protein lysates and conditioned media

Western blotting was performed according to Towbin et al. (1992) and used to determine protein expression levels during proliferation and differentiation of C2C12 cells. 30 μ g of total cell lysates or 50 μ g of concentrated CM (protein concentration determined by Bradford assay, section 2.2.5) from each time point were resolved in a reducing 12.5% SDS-polyacrylamide gel at 18 mA/gel (Laemmli, 1970). Following electrophoresis in a Mini-PROTEAN[®] 3 cell apparatus (BioRad), the resolved proteins were transferred with an omniPAGE Electroblotting (Cleaver Scientific) at 400 V (4 hours) onto nitrocellulose membrane (Hybond-C[™] Extra, Amersham), stained with Ponceau S to verify equivalent loading, destained with TTBS and blocked with 5% skim milk in TTBS (1 hour). Thereafter, nitrocellulose membrane was incubated with primary antibodies against MyoD (554130, BD Biosciences), Myogenin (F5D, Santa Cruz), Wnt3a (YY-7, Santa Cruz), Wnt7 (H-40, Santa Cruz), Wnt10b (MAB2110, R&D), and internal controls, α -tubulin (B-7, Santa Cruz) and GAPDH (14C10, Cell Signaling) in TTBS (see Table 2.3 for optimized primary antibody dilutions), overnight at 2 °C. Following primary antibody incubation, the nitrocellulose membrane was washed and incubated in secondary HRP-linked antibodies in TTBS (see Table 2.2 for secondary antibody dilutions) (1 hour), at room temperature. The antigen-antibody complex was visualized using an ECL kit (Immun-Star[™] WesternC[™] Chemiluminescent Kit, BioRad) according to manufacturer's instructions. The nitrocellulose membranes were exposed onto Biomax light film (Kodak) and captured on VersaDoc[™] MP imager (BioRad). The molecular weight of target antigens (Table 2.3) was determined from a standard curve (Fig. 2.3). Densitometry analysis for protein expression levels were quantified with Quantity One software (BioRad).

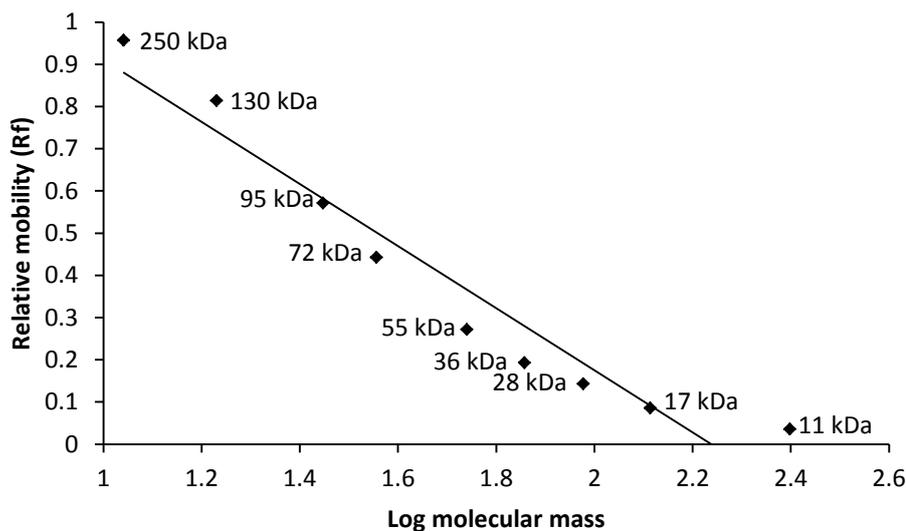


Fig. 2.3: Standard curve of log molecular weight pegGOLD protein marker-V standards relative to the relative mobility in a reducing 12.5% SDS-polyacrylamide gel

The pegGOLD protein marker-V was used to determine the antigens of unknown molecular weight. Recombinant proteins of different size fragments are produced in *E.coli* with nine fragments sizes: 250, 130, 95, 72, 55, 36, 28, 17 and 11 kDa. The linear regression formula was determined ($y = -0.7358x + 1.6464$) with a correlation coefficient of 0.934 using the standard curve and subsequently used to determine the molecular weights of MHC, MyoD, Myogenin, MyoD, Wnt3a, Wnt7, Wnt10b, α -tubulin and GAPDH, in a 12.5% SDS-polyacrylamide gel.

2.2.8 Immunocytochemistry

Early and late passage C2C12 cells were plated onto coverslips in 24 well plates (80 000 cells/well) and induced to differentiate when 80% confluence was reached. At the chosen time points (day 0 and 1), cells were fixed with 4% paraformaldehyde for 10 minutes and non-specific sites were blocked with 5% donkey serum followed by incubation in optimized primary antibody (1:100 MyoD, BD Biosciences, 5.8A) in PBS, overnight at 2 °C. The fixed cells were washed and incubated in secondary antibody (1:4000 donkey anti-mouse FITC, Jackson) for 1 hour, washed and followed by incubation in nuclear stain, hoechst (Sigma) for 5 minutes. Lastly, the fixed cells were washed and coverslips were mounted onto microscope slides with movial and viewed under Carl Zeiss LSM 710 NLO confocor 3 confocal microscopy (Carl Zeiss, Germany) with argon (488 nm) and DPSS lasers (561 nm). Images were analyzed with

Zen software (Carl Zeiss), 2008.

2.2.9 Statistical analysis

For statistical purpose, each experiment were performed a minimum of 3 to 9 replicates (n=3 for MyoD, Myogenin, Wnt3a, 7 and 10b expression analysis; n=5 for analysis of proliferation; n=6 for SA- β -gal assay and α -tubulin expression analysis; n=9 for GAPDH expression analysis). All data are represented as mean \pm standard error of the means (SEM) for each data set shown. The paired Student's *t*-test was performed for parametric data and paired Mann-Whitney *U* test for non-parametric data. All statistical tests were performed using the Genstat 12th edition statistical software (VSN International). Differences in data sets were considered significant if *p* < 0.05.

2.3 RESULTS

2.3.1 Late passage C2C12 cells show an increase in senescence-associated

β -galactosidase staining

Somatic cells enter a state of irreversible growth arrest after several cycles of division, a process termed replicative senescence (Dimri et al., 1995). Therefore, to determine the levels of replicative senescence, we analyzed senescence-associated β -galactosidase (SA- β -gal) expression in passage 13 (i.e. early passage) and passage 33 (i.e. late passage) C2C12 cells. Visibly, we observed more X-gal stain in the late passage than the early passage cells (Fig. 2.4A). Indeed, this was confirmed by the quantification of the X-gal stained images: passage 13 had 75% senescent cells total number of cells in field of view compared to 91% at passage 33, positive for SA- β -gal stain (Fig. 2.4B). This suggests that late passage cells have significantly higher percentage of senescent cells than their early passage counterpart.

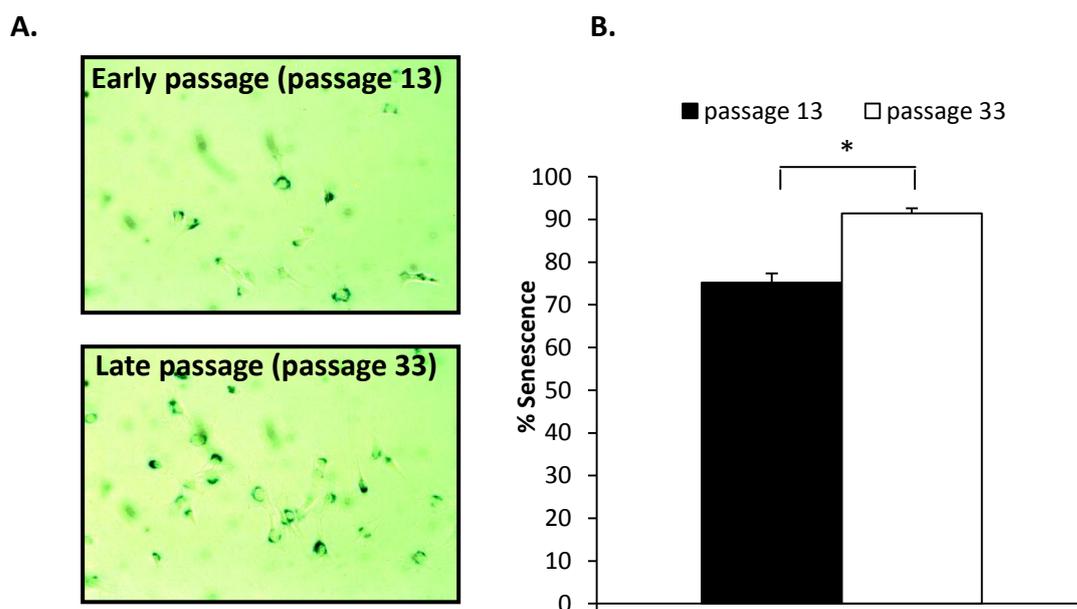


Fig. 2.4: Replicative senescence of passage 13 and passage 33 C2C12 cells

Passage 13 and 33 C2C12 cells were assessed for senescence-associated β -galactosidase (SA- β -gal) activity by staining with X-gal substrate. Cells were plated into growth medium and upon reaching 40% confluency, growth medium was replaced with fresh X-gal staining solution for 16 hours. Brightfield images (A, 10X objective) of SA- β -gal staining indicated that late passage (passage 33) show an increase in percentage senescence compared to early passage (passage 15) cells with a difference of 16% (B). Percentage senescence was determined as number of stained cells divided by the total number of cells per field of view. Experiment repeated, $n=6$, for each passage. ($*p < 0.01$).

2.3.2 Late passage C2C12 cells showed increase rate of proliferation

Proliferation capacity of early and late passage C2C12 cells was assessed. Results indicated that late passage cells were able to significantly achieve confluence (~2.5 million cells) by day 2, whereas, early passage cells were incapable (Fig. 2.5A). The population fold increase was also assessed and revealed population doubling (two-fold increase in population) at 1.7 days (~40.8 hours) for early passage cells, and an earlier doubling at 1.2 days (~28.8 hours) for late passage cells (Fig. 2.6B). Thus late passage cells showed a faster rate of proliferation than early passage cells.

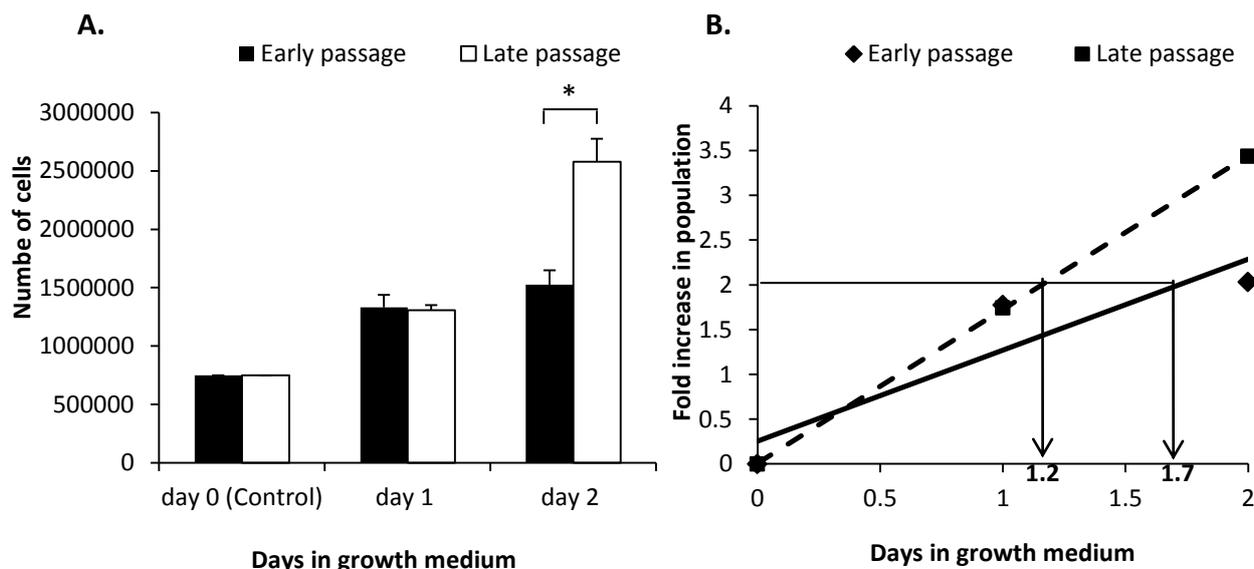


Fig. 2.5: Proliferation of early and late passage C2C12 cells

Early and late passage C2C12 cells were plated into T75 flasks containing growth medium, trypsinized and counted at day 0, 1, and 2 with a hemocytometer. The results showed similar proliferative rates from day 0 to 1 of the two passages, however, the rate of the old passage cells increased significantly from day 1 to 2 (A). The population of early passage cells doubled at 1.7 days (B, arrow) whereas the late passage cells doubled earlier at 1.2 days (B). Experiment repeated, $n=5$, for each passage. (* $p < 0.01$).

2.3.3 Late passage C2C12 cells continued to proliferate even under differentiation inducing medium

The process of myogenic differentiation includes the fusion of individual myoblasts to form multinucleated myotubes. Therefore, we monitored this process by the morphological appearance of multinucleated myotubes under differentiation conditions. Early and late passage C2C12 cells were induced to differentiate and brightfield images were captured at day 0, 1, 5 and 7. Early passage cells readily gave rise to multinucleated myotubes and myotubes were visible as early as day 5 (Fig. 2.6A). However, late passage cells lacked significant myotube formation throughout differentiation, even at day 7 (Fig. 2.6B). This suggests that late passage cells do not differentiate under standard conditions and possibly continue to proliferate. In contrast, differentiation pursued normally in early passage cells.

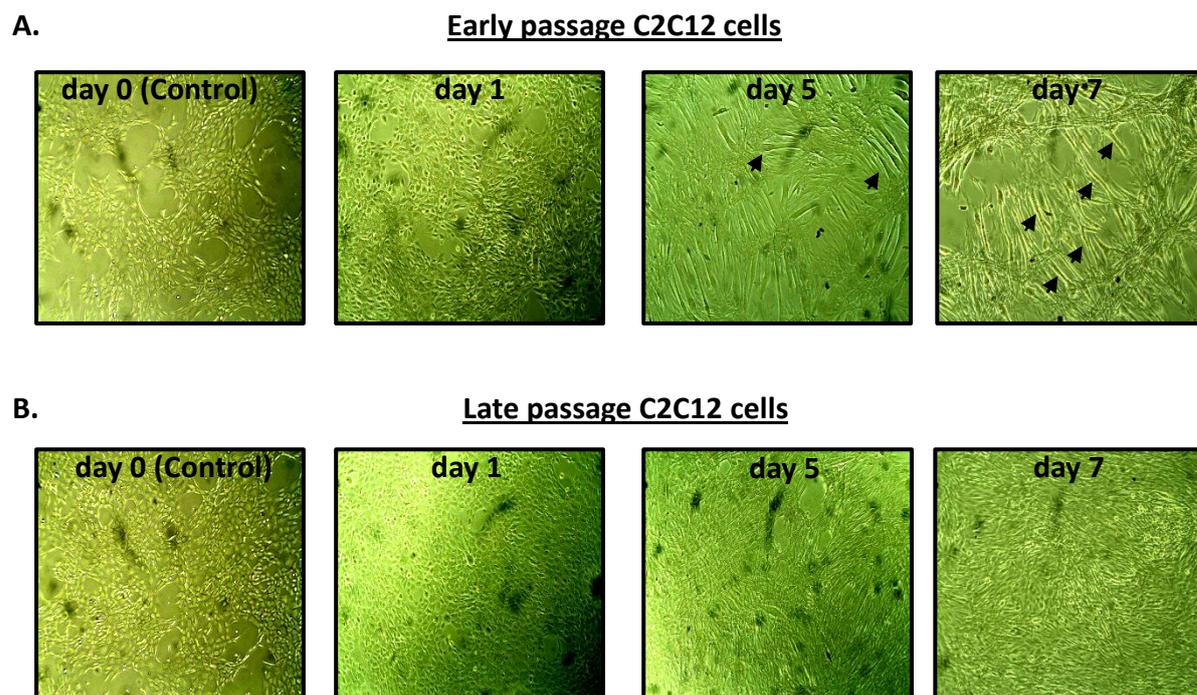


Fig. 2.6: Morphological assessment of differentiation in early and late passage C2C12 cells

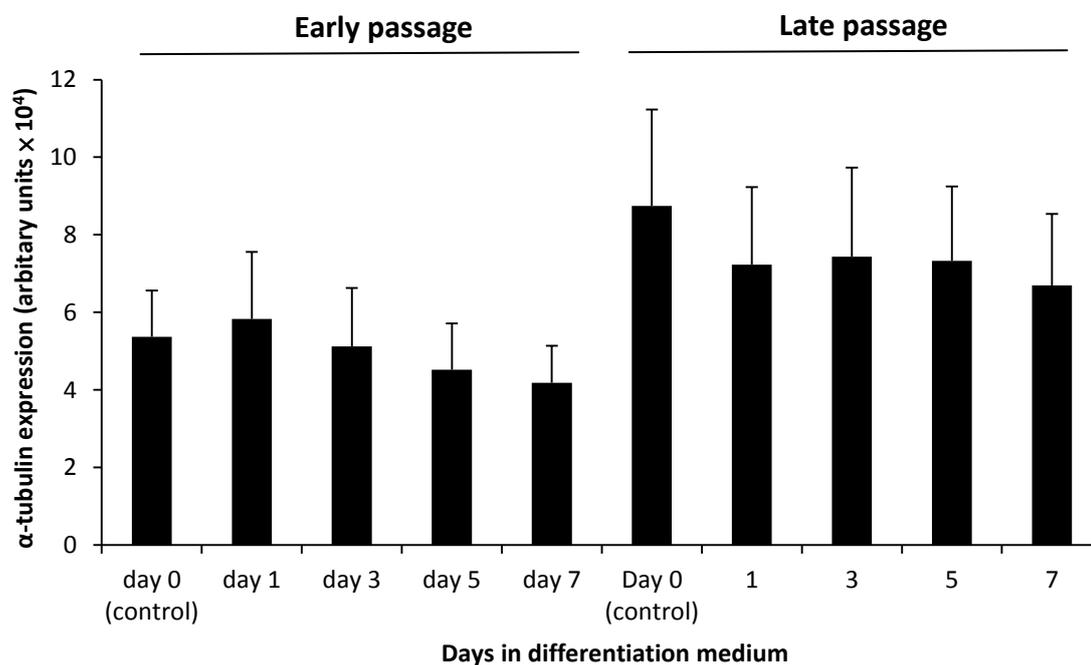
Differentiation of early and late passage C2C12 cells was compared visually by the formation of multinucleated myotubes. Early and late passage cells were plated into 6 well plates containing growth medium and upon reaching 80% confluency were induced to differentiate by replacing growth medium with differentiation medium. Brightfield images were captured at days 0, 1, 5 and 7. Brightfield images (magnification 10 \times) showed that early passage cells (A) readily form multinucleated myotubes (arrow heads), whereas late passage cells (B) displayed no visible myotube formation.

2.3.4 Expression levels of α -tubulin and GAPDH do not change during differentiation within early and late passage C2C12 cells

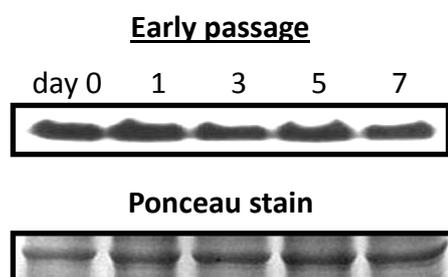
To accurately determine the changes in protein expression, equal loading of cell lysate proteins are essential. Therefore, it is common practice to probe for proteins that are expressed constitutively, regardless of the cellular or culture conditions to correct for protein loading. House-keeping genes such as α -tubulin, β -tubulin, GAPDH and β -actin are often used as loading controls. However, the protein expression of these house-keeping genes have been shown to change under certain conditions such as fluctuations in cell confluency even under equivalent loads of

protein (Greer et al., 2010). For our expression analysis, we intended to use either α -tubulin or GAPDH as internal control proteins but firstly we proceeded to determine whether differentiation affected the expression of the two proteins within early and late passage C2C12 cells. We found no significant change in their expression during differentiation within early and late passage cells (Fig. 2.7 and 2.8). As a result, these internal controls were used to ensure equal protein loading during western blotting. In all subsequent experiments, loading was checked using either α -tubulin or GAPDH. Data was then presented as i) raw data and ii) change in expression relative to day 0 (control). In the latter, the expression levels observed in proliferating cells (day 0, control) were subtracted from the levels observed during differentiation to represent the change in protein expression in response to differentiation medium.

A.



B.



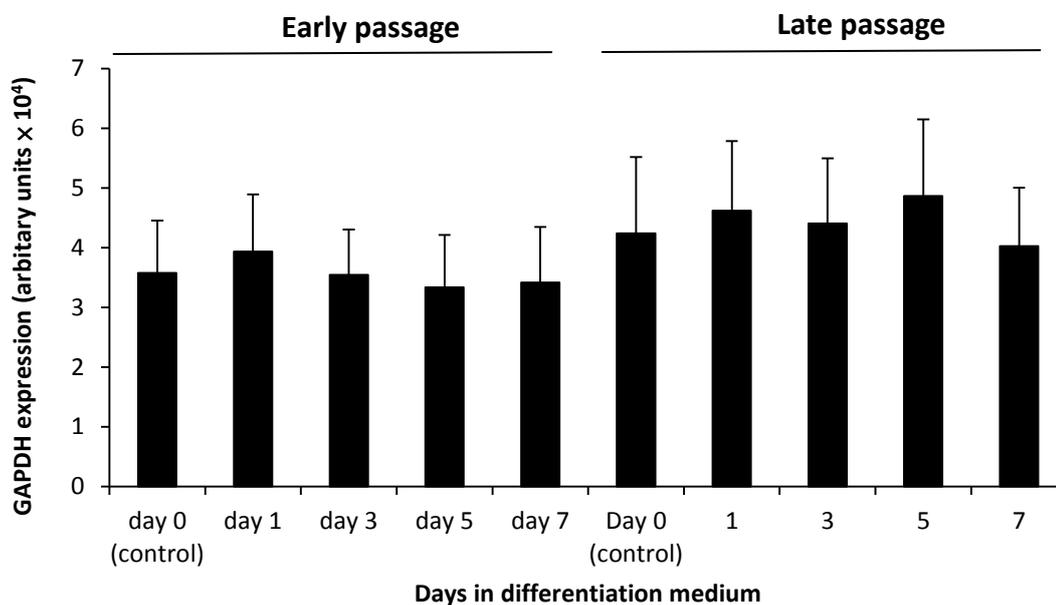
C.



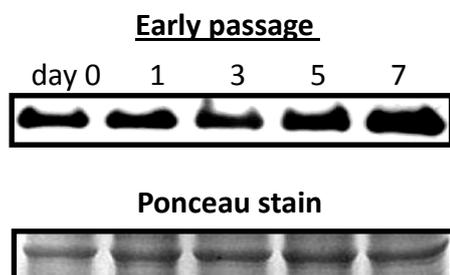
Fig. 2.7: α -tubulin expression levels of differentiating C2C12 cells

The densitometry values of α -tubulin were compared during differentiation in early and late passage C2C12 cells. Early and late passage cells were plated into flasks containing growth medium and upon reaching 80% confluency were induced to differentiate by replacing growth medium with differentiation medium. Cells were harvested at days 0, 1, 3, 5, and 7 of differentiation. The harvested cell lysates were probed for α -tubulin via western blotting, ponceau stained to verify equivalent loading (B and C) and densitometry values determined (A). The values indicated that α -tubulin expression levels did not change significantly during differentiation within early and late passage cells. Experiment repeated, $n=6$, for each passage.

A.



B.



C.



Fig. 2.8: GAPDH expression levels of differentiating C2C12 cells

The densitometry values of GAPDH were compared during differentiation in early and late passage C2C12 cells. Early and late passage cells were plated into flasks containing growth medium and upon reaching 80% confluency were induced to differentiate by replacing growth medium with differentiation medium. Cells were harvested at days 0, 1, 3, 5, and 7 of differentiation. The harvested cell lysates were probed for GAPDH via western blotting, ponceau stained to verify equivalent loading (B and C) and densitometry values determined (A). The values indicated that GAPDH expression levels did not change significantly during differentiation within early and late passage cells. Experiment repeated, $n=9$, for each passage.

2.3.5 Total MyoD but not Myogenin levels are elevated in late passage C2C12 cells

MyoD, Myogenin, Myf5 and Mrf4 are transcription factors, collectively known as the MRFs, play a central role in regulating myogenic activation and differentiation (Holterman and Rudnicki, 2005). In our studies, we chose two of the four MRFs as molecular markers: MyoD, a marker for activation and myogenic differentiation commitment and Myogenin, a marker for terminal differentiation (Holterman and Rudnicki, 2005), to assess differentiation in early and late passage C2C12 cells at the molecular level. Unexpectedly, analysis of total MyoD protein levels as well as the change in MyoD levels in response to differentiation, revealed significantly lower expression in early passage cells (Fig. 2.9A and B). The low MyoD expression level suggests that the differentiation capacity is reduced in early passage cells. However, this is not consistent with the brightfield images of differentiating early passage cells (Fig. 2.6A), which readily differentiate to form multinucleated myotubes. Conversely, the elevated expression in the late passage cells suggests an attempt by the cells to compensate (for the reduced differentiation) via up-regulation of MyoD. Expression levels of Myogenin indicated consistently higher levels in early passage cells during differentiation (Fig. 2.10). Late passage cells showed relatively constant expression levels during differentiation (Fig. 2.10A). However, change in Myogenin expression levels relative to day 0 revealed an increased level at day 1 and a subsequent slower decline in myogenesis in early vs. late passage cells (Fig. 2.10B.). Furthermore, it is clear that Myogenin up-regulation at day 3 is significantly lower in late passage compared to early passage cells (Fig. 2.10B). The low levels of Myogenin seen in late passage cells suggest a lack of commitment to terminal differentiation in late passage cells. The Myogenin analysis agrees with the brightfield images of differentiating early and late passage cells (Fig. 2.6).

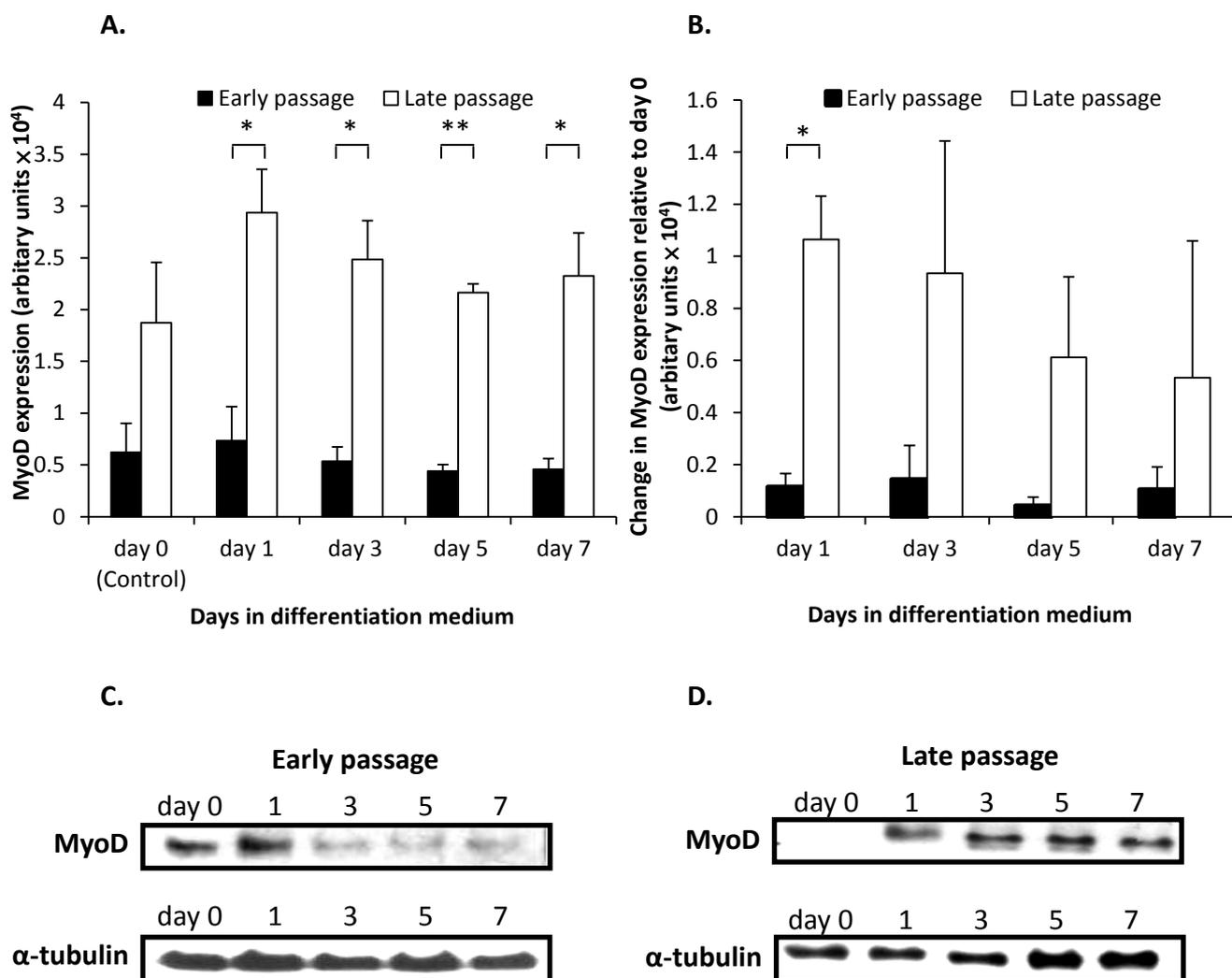


Fig. 2.9: MyoD expression profile of differentiating C2C12 cells

The expression of MyoD from western blotting was compared during differentiation in early and late passage C2C12 cells. Early and late passage cells were plated into flasks containing growth medium and upon reaching 80% confluency were induced to differentiate by replacing growth medium with differentiation medium. Cells were harvested at days 0, 1, 3, 5 and 7 of differentiation. The harvested cell lysates were probed for MyoD and α -tubulin via western blotting (C and D) and densitometry values (A and B) were determined for early and late passage cells. Densitometry data (A and B) indicated that MyoD expression levels were higher in the late passage than the early passage cells. Experiment repeated, $n=3$, for each passage. (* $p < 0.05$, ** $p < 0.005$: early vs. late passage MyoD levels at equivalent time points).

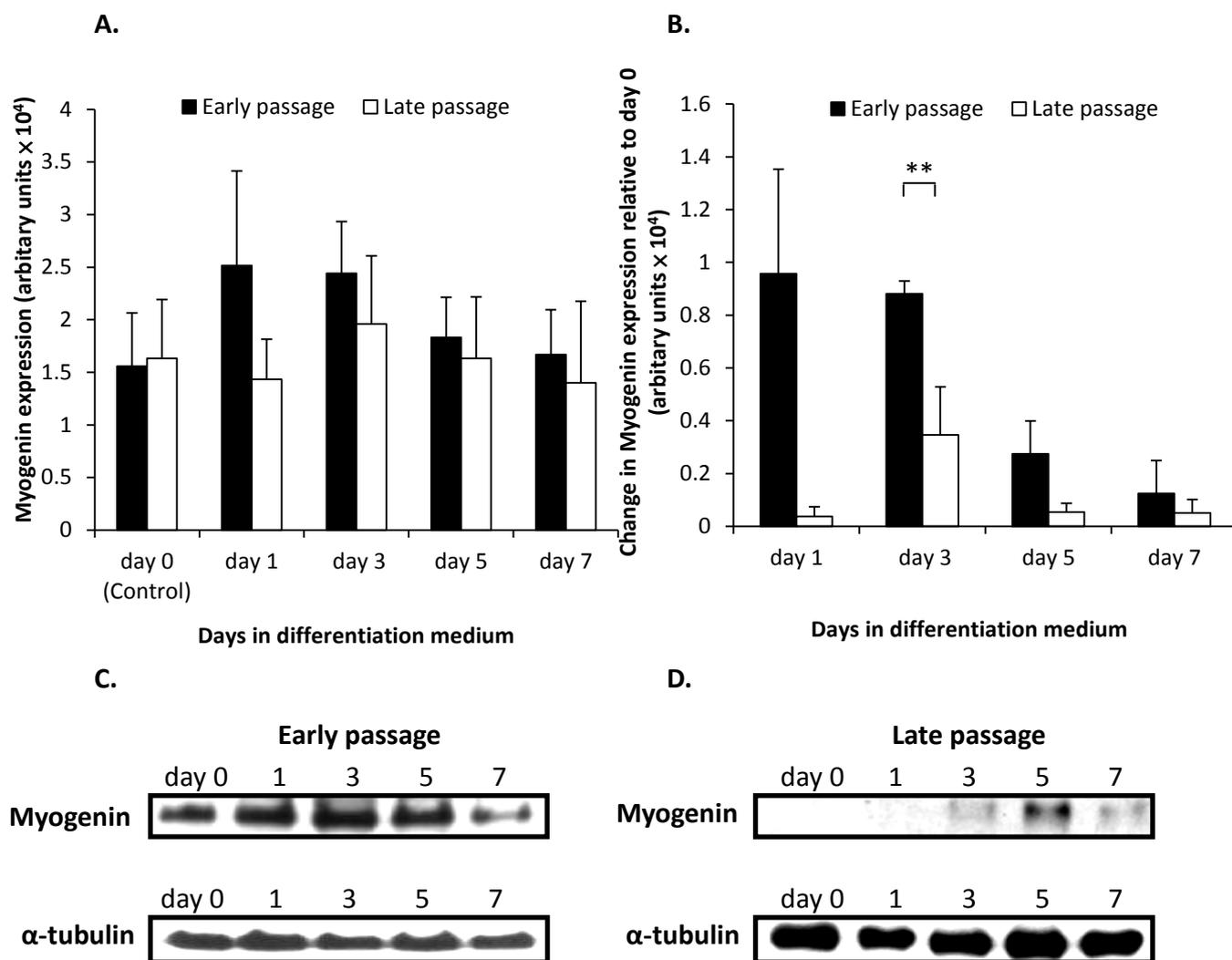


Fig. 2.10: Myogenin expression profile of differentiating C2C12 cells

The expression of Myogenin from western blotting was compared during differentiation in early and late passage C2C12 cells. Early and late passage cells were plated into flasks containing growth medium and upon reaching 80% confluency were induced to differentiate by replacing growth medium with differentiation medium. Cells were harvested at days 0, 1, 3, 5 and 7 of differentiation. The harvested cell lysates were probed for Myogenin and α -tubulin via western blotting (C and D) and densitometry values (A and B) were determined for early and late passage cells. Densitometry data (A and B) indicated that Myogenin expression levels were higher in the early passage than the late passage cells. Experiment repeated, $n=3$, for each passage. (** $p < 0.005$: early vs. late passage Myogenin levels at equivalent time points).

2.3.6 Localization of MyoD does not differ during differentiation of early and late passage C2C12 cells

The total MyoD levels were surprisingly higher in the late passage C2C12 cells; therefore we further determined whether translocation of MyoD to the nucleus was decreased in late passage cells. Confocal analysis of cellular localization revealed more MyoD in the nucleus of C2C12 cells at day 1 of differentiation compared to day 0 within both early and late passage cells, respectively (Fig. 2.11B and D). Furthermore, visually, there was no apparent difference in the levels of nuclear MyoD in early vs. late passage cells at day 1 of differentiation.

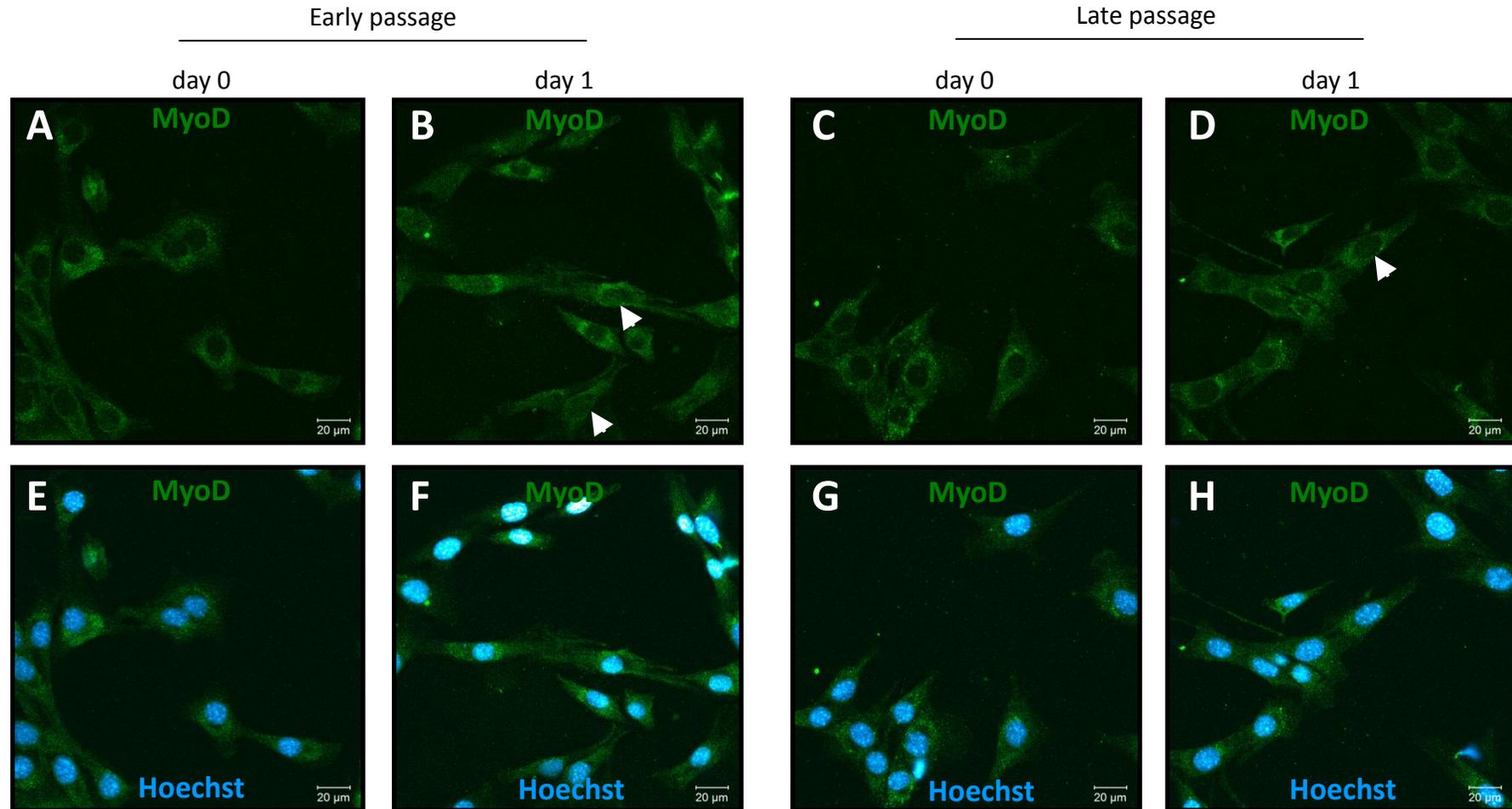


Fig. 2.11: Cellular localization of MyoD in differentiating C2C12 cells

Cellular localization of MyoD was determined in early and late passage C2C12 cells during differentiation. Early and late passage cells were plated into wells with coverslips in growth medium and induced to differentiate by replacing growth medium with differentiation medium. Cells were fixed at day 0 and day 1, and stained for MyoD (FITC, green) and nucleus (hoechst, blue). Arrow heads indicate nuclear localization of MyoD at day 1 for early and late passage cells, respectively.

2.3.7 Analysis of cytosolic and secreted Wnt levels in proliferating and differentiating C2C12 cells

Wnts regulate differentiation through the temporal switch from Notch to Wnt signaling (Conboy et al., 2003). However, in aging muscle, certain Wnt proteins have been shown to specifically regulate myogenesis of satellite cells (Brack et al., 2007; Vertino et al., 2005). Therefore, we aim to determine the cytosolic levels of Wnt3a, 7 and 10b during differentiation of early vs. late passage C2C12 cells. Given that Wnts are secreted proteins, we further analyzed the secreted levels of the three Wnt isoforms in the CM of differentiating early vs. late passage cells.

2.3.7.1 Cytosolic Wnt3a expression is elevated with reduced secretion in late passage C2C12 cells

The absolute expression of cytosolic Wnt3a was found to be consistently higher in the late passage C2C12 cells during differentiation (Fig. 2.12). This supports the premise that high Wnt3a levels lead to a fibrotic lineage conversion with reduced myogenesis in late passage cells (Brack et al., 2007). Indeed, myogenic capability is reduced in late passage cells confirmed by the lack of myotube formation in the brightfield images of differentiating early and late passage cells (Fig. 2.6) and lower levels of Myogenin expression (Fig. 2.10). Contradictory to the cytosolic levels and expression trends, the secreted levels were marginally higher in the early passage cells (Fig. 2.13A). Therefore, the cytosolic Wnt3a levels and trends do not reflect the secreted Wnt3a levels seen in both early and late passage cells. Lastly, analysis of Wnt3a in CM from proliferating cells (day 0-CM) failed to detect Wnt3a but was detected in CM from differentiating cells (day 1- to day 5-CM) (Fig. 2.13B). This agrees with the hypothesis that Wnt signaling by Wnt3a protein plays a role in myogenic differentiation and not proliferation (Brack et al., 2008).

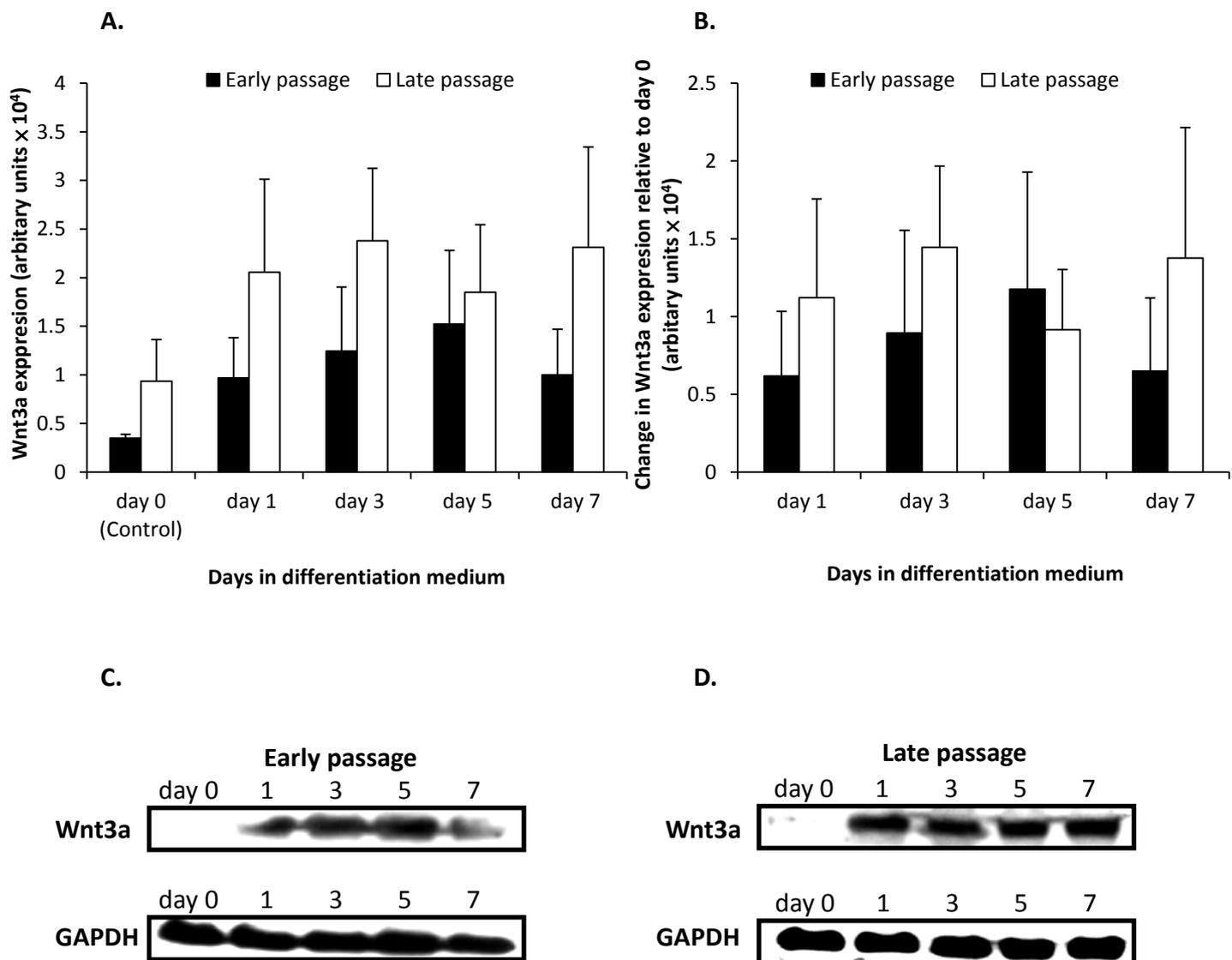


Fig. 2.12: Wnt3a expression profile of differentiating C2C12 cells

The expression of Wnt3a from western blotting was compared during differentiation in early and late passage C2C12 cells. Early and late passage cells were plated into flasks containing growth medium and upon reaching 80% confluency were induced to differentiate by replacing growth medium with differentiation medium. Cells were harvested at days 0, 1, 3, 5 and 7 of differentiation. The harvested cell lysates were probed for Wnt3a and GAPDH via western blotting (C and D) and densitometry values (A and B) were determined for early and late passage cells. Densitometry data (A and B) indicated that Wnt3a expression levels were higher in the late passage than the early passage cells along with a trend of increasing levels during differentiation. Experiment repeated, $n=3$, for each passage.

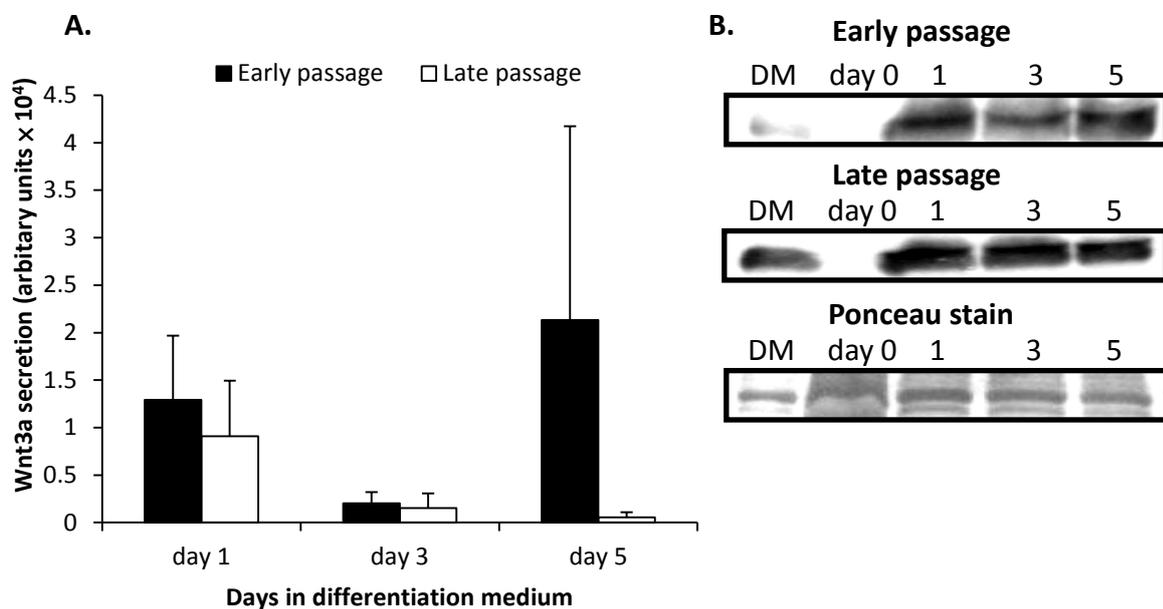


Fig. 2.13: Secreted Wnt3a levels of differentiating C2C12 cells

The levels of secreted Wnt3a were compared during differentiation of early and late passage C2C12 cells. Early and late passage cells were plated into flasks containing growth medium and upon reaching 80% confluency were induced to differentiate by replacing growth medium with differentiation medium. Conditioned media was collected at days 0, 1, 3 and 5 of differentiation, concentrated using PEG 20 000, probed for Wnt3a via western blotting (B) and densitometry values determined (A). The densitometry values (A) generally showed higher Wnt3a secreted levels in the early passage cells. Ponceau stained blots (B) to indicate equivalent loading of conditioned media. Secretion of Wnt3a from each time point were subtracted from the Wnt3a levels present in differentiation media (DM) in order to accurately reflect the increase in secretion by the early and late passage cells. Experiment repeated, $n=3$, for each passage.

2.3.7.2 Cytosolic and secreted Wnt7 levels rapidly declined in differentiating late passage C2C12 cells

Wnt7 isoform has been suggested to play a role in lineage specification and differentiation (Poleskaya et al., 2003). However, levels of Wnt7 in myoblasts from aging muscle have largely been unknown. Hence, we aim to define the role and expression levels of Wnt7 during differentiation in 'aging' C2C12 myoblast. The cytosolic levels of Wnt7 were lower during initial differentiation in early passage cells. Expression in early passage cells peaked at day 3 whereas late passage cells reached a maximum expression at day 1 followed by a rapid decline in levels during differentiation (Fig. 2.14A and B). Similarly, the secreted levels with early passage cells were elevated at a slower rate than late passage cells, the latter showing a rapidly decreased secretion throughout differentiation (Fig. 2.15A). This rapid decline in Wnt7 cytosolic and secreted levels suggest reduced differentiation in late passage cells. This agrees with the premise that signaling via Wnt7 proteins promotes myogenic specification and progression (Poleskaya et al., 2003). Lastly, we failed to detect Wnt7 in CM from proliferating cells but were detected in CM from differentiating cells (Fig. 2.15B) further supporting a role for Wnt7 protein in myogenic differentiation and not proliferation.

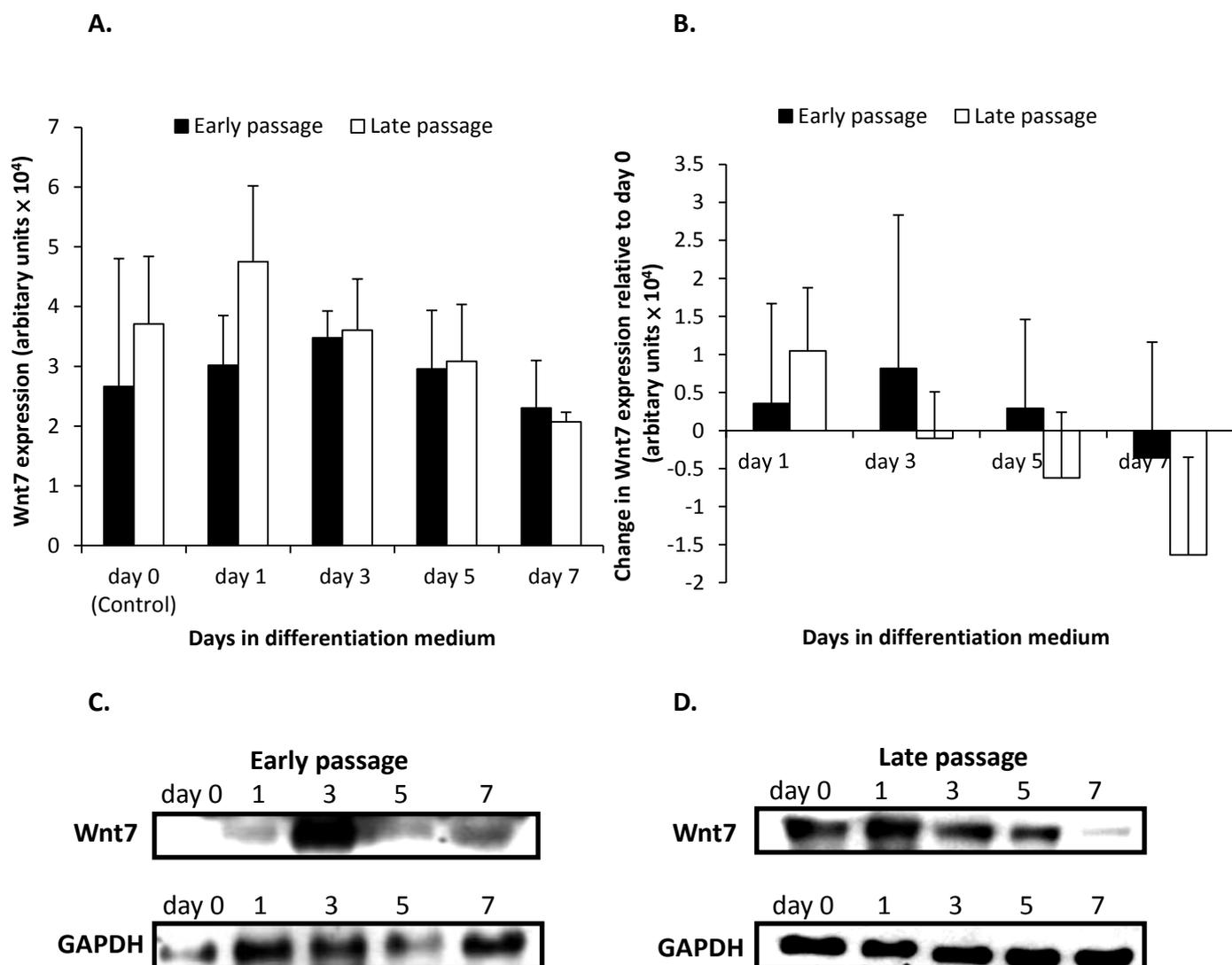


Fig. 2.14: Wnt7 expression profile of differentiating C2C12 cells

The expression of Wnt7 from western blotting was compared during differentiation in early and late C2C12 passage cells. Early and late passage cells were plated into flasks containing growth medium and upon reaching 80% confluency were induced to differentiate by replacing growth medium with differentiation medium. Cells were harvested at days 0, 1, 3, 5 and 7 of differentiation. The harvested cell lysates were probed for Wnt7 and GAPDH via western blotting (C and D) and densitometry values (A and B) were determined for early and late passage cells. Densitometry data (A and B) indicated an increasing trend of Wnt7 expression in early passage cells and conversely, a decreasing trend in late passage cells during differentiation. Experiment repeated, $n=3$, for each passage.

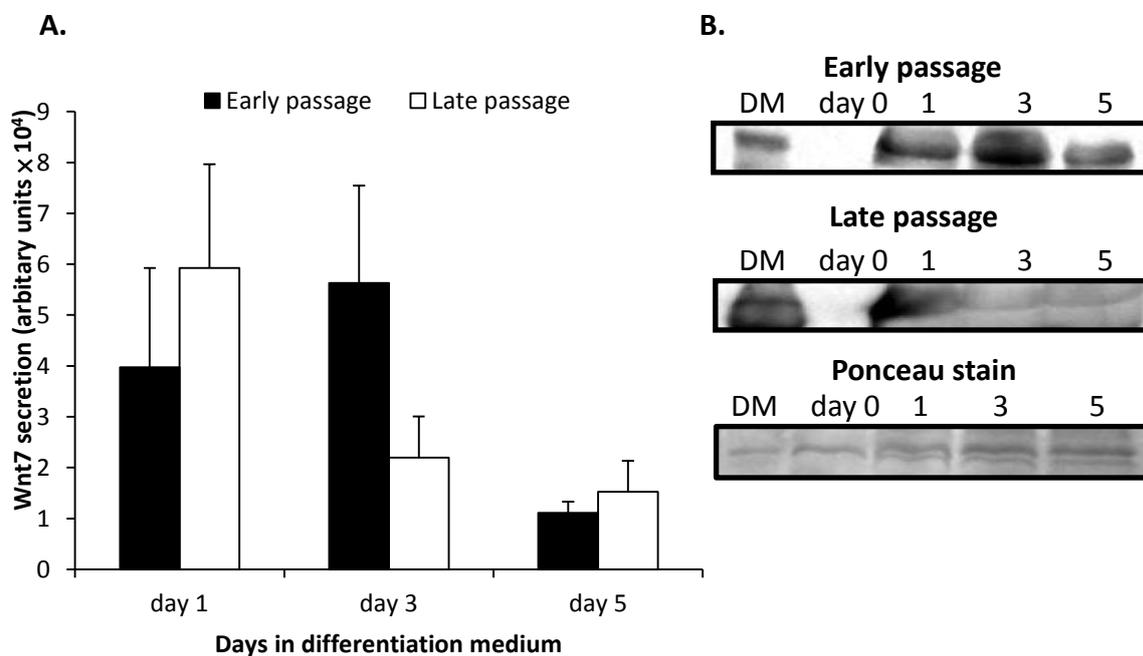


Fig. 2.15: Secreted Wnt7 levels of differentiating C2C12 cells

The levels of secreted Wnt7 were compared during differentiation of early and late passage cells. Early and late passage cells were plated into flasks containing growth medium and upon reaching 80% confluency were induced to differentiate by replacing growth medium with differentiation medium. Conditioned media was collected at days 0, 1, 3 and 5 of differentiation, concentrated using PEG 20 000, probed for Wnt7 via western blotting (B) and densitometry values determined (A). Densitometry data (A and B) indicated an increasing trend of Wnt7 secreted levels in early passage cells and conversely, a decreasing trend in late passage cells during differentiation. Ponceau stained blots (B) to indicate equivalent loading of conditioned media. Secretion of Wnt7 from each time point were subtracted from the Wnt7 levels present in differentiation media (DM) in order to accurately reflect the increase in secretion by the early and late passage cells. Experiment repeated, $n=3$, for each passage.

2.3.7.3. Cytosolic and secreted Wnt10b levels are lower in late passage C2C12 cells

Wnt10b has been implicated in regulating adipogenic conversion of myoblasts in aging muscle (Vertino et al., 2005). Therefore, we set out to analyze the expression levels of Wnt10b during differentiation of 'aging' C2C12 myoblasts. Early passage cells displayed consistently higher cytosolic Wnt10b levels (Fig. 2.16A and B). The late passage cells had lower levels of expression of cytosolic Wnt10b which seem to suggest a loss in myogenic capability. Indeed, late passage cells through Myogenin expression analysis (Fig. 2.10) and brightfield images of differentiating late passage cells (Fig. 2.6) revealed impaired differentiation. This loss of myogenic capability with lower Wnt10b cytosolic levels revealed in late passage cells could likely be associated with lineage conversion, from myogenic to adipogenic or fibrogenic lineage (Brack et al., 2007; Vertino et al., 2005). The secreted levels, similar to Wnt3a secreted levels, do not resemble the cytosolic Wnt10b levels in both passage cells. Unlike the cytosolic levels, the secreted Wnt10b in the late passage cells was higher at day 1 and thereafter, the secreted levels were similar (Fig. 2.17A). Lastly, similar to the secretion analysis of Wnt3a and 7, we failed to detect Wnt10b in CM from proliferating cells but were detected in CM from differentiating cells (Fig. 2.17B). Therefore, Wnt10b not only play a role in regulating a switch from myogenic to adipogenic lineage but also likely to play a role in differentiation which to date has not been shown. The three isoforms Wnt3a, 7 and 10b have their distinct roles but collectively seem to play a role in myogenic differentiation and progression.

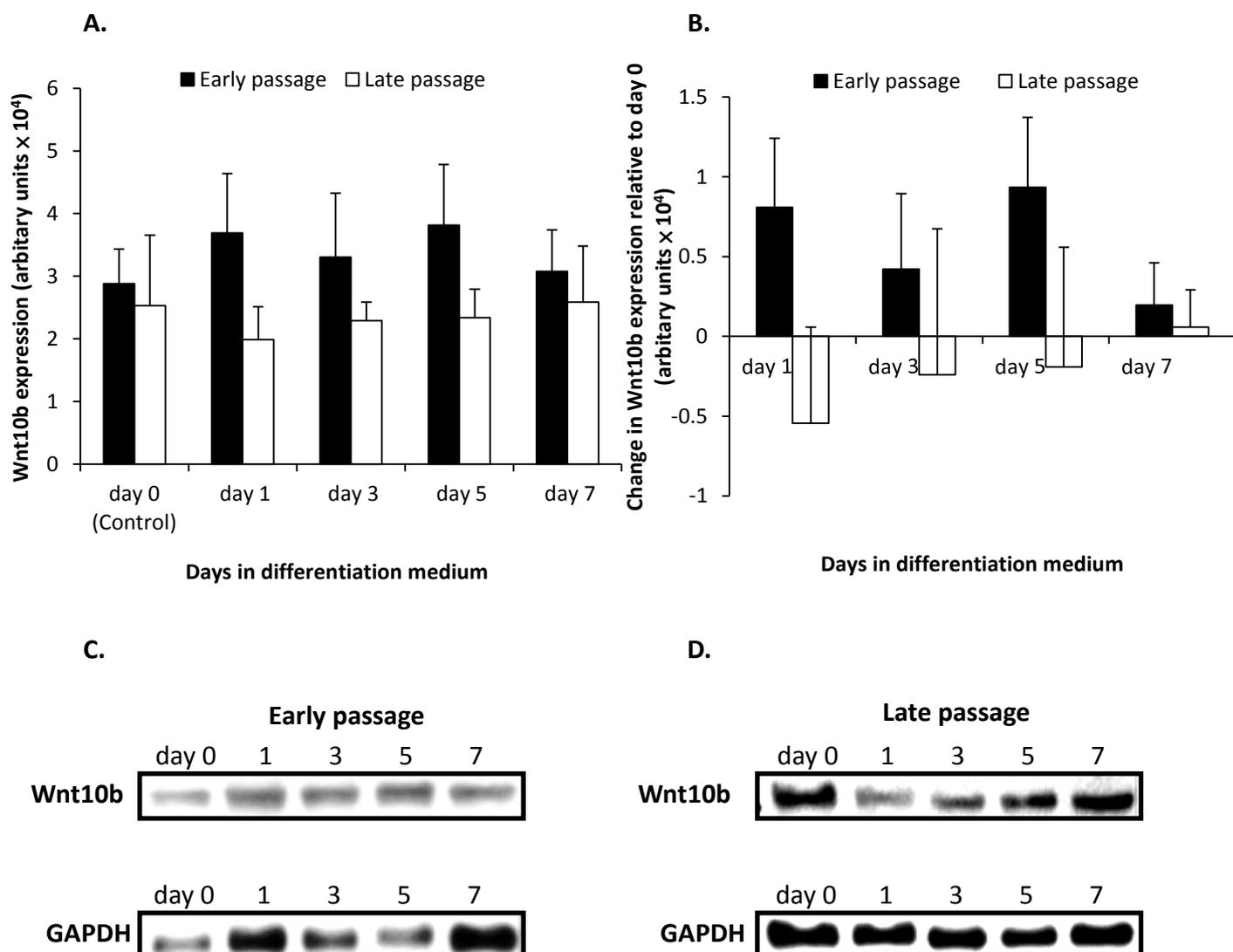


Fig. 2.16: Wnt10b expression profile of differentiating C2C12 cells

The expression of Wnt10b from western blotting was compared during differentiation in early and late C2C12 passage cells. Early and late passage cells were plated into flasks containing growth medium and upon reaching 80% confluency were induced to differentiate by replacing growth medium with differentiation medium. Cells were harvested at days 0, 1, 3, 5 and 7 of differentiation. The harvested cell lysates were probed for Wnt10b and GAPDH via western blotting (C and D) and densitometry values (A and B) were determined for early and late passage cells. Densitometry data (A and B) indicated that Wnt10b expression levels were higher in the early passage than the late passage cells. Experiment repeated, n=3, for each passage.

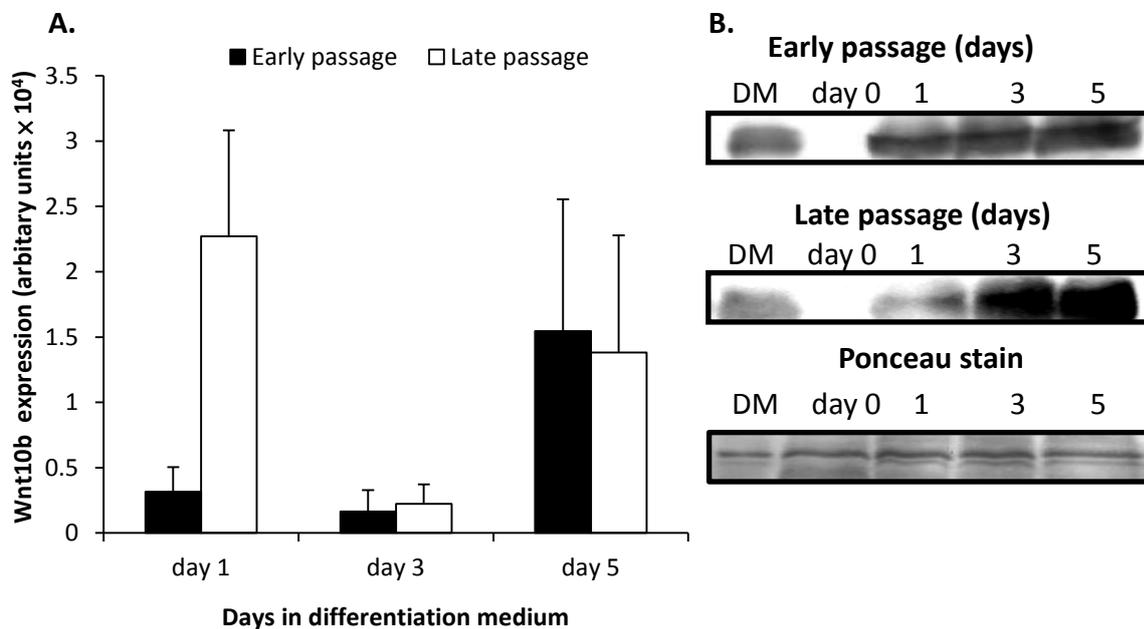


Fig. 2.17: Secreted Wnt10b levels of differentiating C2C12 cells

The levels of secreted Wnt10b were compared during differentiation of early and late passage C2C12 cells. Early and late passage cells were plated into flasks containing growth medium and upon reaching 80% confluency were induced to differentiate by replacing growth medium with differentiation medium. Conditioned media was collected at days 0, 1, 3 and 5 of differentiation, concentrated using PEG 20 000, probed for Wnt3a via western blotting (B) and densitometry values determined (A). Densitometry data (A) indicated that early passage has a high level of Wnt10b secreted level during terminal differentiation and late passage, a higher Wnt10 secreted level during initial stages of differentiation. Ponceau stained blots (B) to indicate equal protein loading of conditioned media. Secretion of Wnt10b from each time point were subtracted from the Wnt10b levels present in differentiation media (DM) in order to accurately reflect the increase in secretion by the early and late passage cells. Experiment repeated, $n=3$, for each passage.

2.4 DISCUSSION

Aging of skeletal muscle ultimately results in extrinsic changes that directly impact satellite cell activity and capacity. These changes have been highlighted and collectively result in poor regeneration and repair compared to that of young muscles. Parabiotic pairing between young and old mice suggest that the exposure of satellite cells to circulatory serum factors of young mice can rejuvenate the satellite cells in muscles of old mice and show effective repair (Conboy et al., 2005). Further studies suggest that these systemic factors in the serum could be Wnt and TGF- β protein factors. Wnt protein isoforms have been shown to act as both activators and inhibitors of myogenic differentiation (Brack et al., 2008; Florini et al., 1986). In our studies, we utilized the C2C12 myoblast cell line (Yaffe and Saxel, 1977), to establish the expression profiles of three Wnt isoforms, Wnt3a, 7 and 10b, in their cytosolic and secreted forms during differentiation. Furthermore, the expression profile of two MRFs, MyoD and Myogenin, were also determined to compare, on a molecular level, the state of differentiation in early vs. late passage C2C12 cells.

To 'age' the C2C12 cells (initial passage 13), we allowed many cycles of cell division with prolonged sub-culturing until passage 33 and performed senescence-associated β -galactosidase (SA- β -gal) staining. β -galactosidase, when detected at pH 6, is relatively specific for SA- β -gal and not acid-lysosomal β -galactosidase (active at pH 4) (Kurz et al., 2000). Experimentally, an increase in passage numbers should correspond to an increase in senescence due to the greater number of cell cycle divisions. Our SA- β -gal staining results did show an increase in percentage senescence in late passage cells. However, it should be noted that C2C12 cells are considered as an 'immortal' cell line with a corrupt cell cycle (Cornelison, 2008). This could explain why both the early and late passage cells have high SA- β -gal staining. It

is also possible that acid lysosomal β -galactosidase was detected in a non-specific manner due to prolonged incubation (post 16 hours). These results should therefore be observed with caution.

We also identified interesting characteristics of late passage C2C12 cells. Firstly, the tendency of late passage cells to undergo differentiation is impaired when exposed to differentiation cues. Instead of differentiating, late passage cells continued to proliferate under differentiation media although this has to be verified with proliferation markers such as Ki-67. Secondly, contrary to our senescence results, the proliferative rate of late passage cells was higher than early passage cells, suggesting that late passage cells undergo division at a faster rate than early passage cells. Proliferation studies using BrdU incorporation by Brack et al. (2007) have shown that satellite cells from aging muscle of mice proliferate poorly which contradicts our results. It should be noted that in their studies, primary culture was used and not a myoblast cell line hence the contradictory results. Therefore, it seems that by maintaining C2C12 cells in growth medium for extended period results in a proliferative phenotypic change whereby cells acquire an enhanced proliferative capability and in the process lose their differentiation potential, as seen in our late passage cells. Contrary to our differentiation study, Conboy et al. (2003) has demonstrated that prolonged maintenance of satellite cells from old muscles which are known to have a defective myogenic differentiation, differentiated as effectively as satellite cells from young muscle. However, it should be noted that in their studies, primary culture was used rather than a cell line. Nonetheless, they have also shown that prolonged maintenance in culture can alter the phenotype of the cells of interest. Collectively, this shows that the surrounding media could possibly change the phenotype of the cultured cells through prolonged culturing (Fig. 2.18).

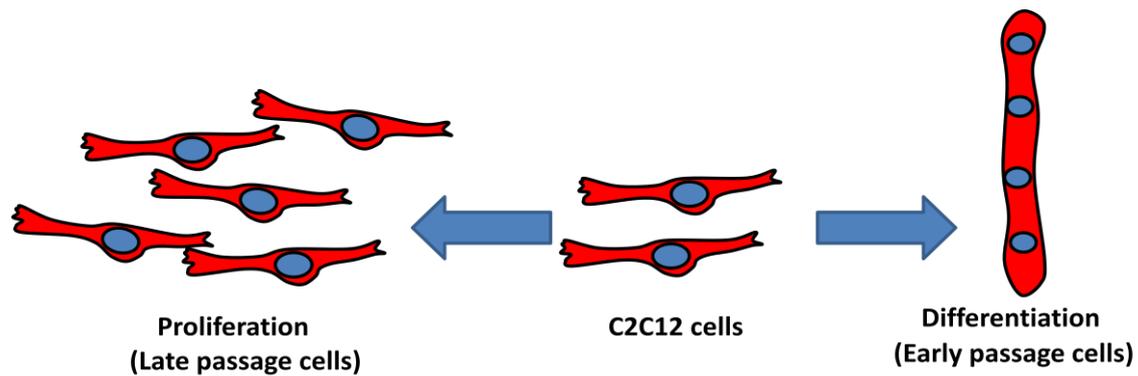


Fig. 2.18: Balance of proliferation and differentiation of early and late C2C12 passage cells

Late passage C2C12 cells through extensive sub-culturing in growth medium lose their ability to differentiate even in the presence of differentiation cues, and rather adopt a proliferative phenotype. On the other hand, early passage C2C12 cells respond effectively to differentiation cues and differentiate into myotubes but do not proliferate as rapidly as late passage cells.

MyoD and Myogenin are transcription factors that regulate myogenic differentiation (Charge and Rudnicki, 2004). The expression levels of both transcription factors give an indication of the state of myogenic determination and differentiation at the molecular level in early and late passage C2C12 cells. As expected, Myogenin levels increased from day 0 to day 1 in early passage but not in late passage cells during differentiation. This agrees with the lack of fusion and myotube formation observed morphologically in the brightfield images of late passage cells.

Unexpectedly, our results indicated higher total MyoD levels in late compared to early passage differentiating cells. These results could be interpreted as a compensation effect in the late passage C2C12 cells due to the fact that differentiation capacity is reduced, the cells up-regulate MyoD in an effort to commit more cells to myogenic differentiation. Previous studies by Schabort et al. (2009) emphasized the importance of MyoD stability during differentiation. In their work,

they demonstrated that TGF- β treated C2C12 myoblasts have reduced differentiation capacity and this correlated with the elevated degradation of MyoD. It is possible that a similar mechanism in the late passage cells could be occurring. Our late passage cells could have a higher rate of degradation of MyoD compared to early passage cells. Given that MyoD is required for the up-regulation of Myogenin (Fuchtbauer and Westphal, 1992), if MyoD is rapidly degraded before it is able to bind the Myogenin promoter, transcription and translation of Myogenin would be compromised which would negatively impact myogenic differentiation. This could be tested by analyzing the half-life of MyoD with the use of cyclohexamide. Because MyoD is a transcription factor, we further determine the presence of nuclear MyoD and visually distinguish the difference in nuclear MyoD between early and late passage cells. Confocal analysis of cellular localization did not reveal a difference in the translocation of MyoD to the nucleus of late vs. early passage cells at day 1 of differentiation, suggesting no difference in the level of potentially active nuclear MyoD, however quantification of the levels of nuclear MyoD would be more reliable. The method by Vindelov et al. (1983) to extract nuclei from various cell lines for flow cytometric DNA analysis could be adopted to extract nuclei of differentiating early and late passage C2C12 cells. These extracted intact nuclei could further be ruptured, probed for nuclear MyoD via western blotting and quantified through densitometry. This method eliminates contaminating cytosolic MyoD and thus only levels of nuclear MyoD is compared between differentiating early vs. late passage cells. The quantification of nuclear MyoD would indicate differentiation state of early and late passage cells.

Besides translocation, total levels and protein stability, protein modifications can affect protein activity. Gillespie et al. (2009) have shown that MyoD modification via

direct phosphorylation by p38 γ results in tighter binding to the Myogenin promoter – this tight binding represses the expression of Myogenin. MyoD in this conformation recruit H3K9 histones which are continuously methylated by KMT1A methyltransferase which also contribute to repression of Myogenin expression. In the absence p38 γ activity, MyoD would bind to the Myogenin promoter and in this conformation would not recruit KMT1A methyltransferase. With the absence of KMT1A methyltransferase, Myogenin is transcribed and expressed, resulting in effective differentiation. In this respect, it would be interesting to compare the expression of total and active p38 γ in early vs. late passage C2C12 cells. Taken together, our data suggests that protein modifications, stability and localization are just as important as total protein levels in controlling myogenic differentiation.

Wnt signaling via Wnt proteins are known to play a role in regulating myogenic differentiation, specification and lineage progression by suppressing Notch pathway (Brack et al., 2008; Poleskaya et al., 2003). Key findings in our results support this hypothesis. Firstly, we failed to detect any secreted Wnts in conditioned media from proliferating cells in both early and late passage cells. However, all three Wnt isoforms were detected in CM from differentiating cells in both passages. This suggests that our investigated Wnts do not play a role in proliferation but myogenic differentiation and lineage progression. Secondly, cytosolic Wnt3a, 7 and 10b expression increased in response to differentiation cues in early passage C2C12 cells. However, only Wnt3a cytosolic expression levels increased in late passage cells. Furthermore, secreted Wnt7 levels decreased in response to differentiation in late passage, but not early passage cells. The increase levels of Wnt proteins during differentiation suggest a possible role of Wnt isoforms in differentiation of early passage cells. Poleskaya et al. (2003) and Brack et al. (2008) reported that Wnt7a

and 7b, and 3a mRNA transcript level increases in regenerating muscle, respectively. The increase in Wnt7a and 7b, and 3a mRNA transcripts would likely be followed by an increase in Wnt3a, 7a and 7b protein levels. These mRNA transcripts are likely to be translated into their respective proteins, undergo post-modification (glycosylation and palmitoylation) and secreted to the external environment. The decrease in myogenesis could be explained by the decreased cytosolic and secreted Wnt7 levels as observed in late passage cells. And lastly, Wnt3a, 7 and 10b isoforms were detected in the differentiation media and not growth media, most likely in the 1% horse serum used to induce differentiation of C2C12 cells. The presence of these Wnts in differentiation media alone suggest their roles in the myogenic determination and differentiation induced by exposure to 1% horse serum.

High levels of Wnt3a are known to impede myogenesis (Brack et al., 2007). Brack et al. (2007) demonstrated that injection of endogenous Wnt3a into young regenerating muscle during the initial stages of regeneration resulted in an increase in connective tissue and poor regeneration. Our results showed higher levels of cytosolic but not secreted Wnt3a in late compared to early passage cells. Therefore, the increase cytosolic Wnt3a seen in late passage cells could explain the decrease myogenesis. However, the low level of secreted Wnt3a by late passage cells is puzzling. This seems to suggest that the cytosolic Wnt3a proteins are stored and not secreted into the media.

Wnt10b has been implicated in directing satellite cells into an adipogenic lineage. Akimoto et al. (2005) demonstrated that cyclic mechanical stretching prevents adipogenesis in C2C12 myoblasts when cultured in adipogenic induction medium. This inhibition is associated with the up-regulation of Wnt10b mRNA transcript.

However, knock-out studies by Vertino et al. (2005) of Wnt10b ($Wnt10b^{-/-}$) in mice revealed no difference in myogenic capacity in relation to the wild-type mice. Subsequently, from mRNA analysis, it was discovered that $Wnt10b^{-/-}$ myoblasts over-expressed Wnt7b to compensate for the lack of Wnt 10b expression, leading to successful myogenesis (Vertino et al., 2005). Further analysis of mRNA transcript levels revealed the absence of this compensation by Wnt7b in aging $Wnt10b^{-/-}$ myoblasts, lead to an adipogenic lineage conversion (Vertino et al., 2005).

This compensation effect is not seen in early passage cells with elevated levels of both Wnt7 and 10b. However, late passage cells initially display this effect with elevated Wnt7 levels from day 0 to 1 but this compensation is lost with progressive decline in Wnt7 levels post-day 1, concurrently with lower levels of Wnt10b during differentiation. The combined decrease in cytosolic and secreted levels of Wnt7 and Wnt10b in late passage cells supports the eventual loss of myogenic capability. It would be interesting to analyze the adipogenesis of the late passage cells with Oil-red O stain to visualize the degree of lipid accumulation.

Secretion of Wnt proteins is essential to exert their effect on neighboring cells, therefore analyzing secretion levels is crucial. We determined the ratio of secretion to cytosolic levels of Wnt3a, 7 and 10b in both early and late passage C2C12 cells (Table 2.4). The ratio indicated substantially higher secretions of Wnt7 and 10b, in the early compared to the late passage cells. This indicates that although cytosolic Wnt7 and 10b were low, high levels of both proteins are immediately secreted by early passage cells. This high secretion is likely associated with promoting differentiation.

Table 2.4: Summary of secreted relative to cytosolic levels of the three key Wnt isoforms

Isoforms	Passage	day 1			day 3			day 5		
		C♦	S*	S/C	C♦	S*	S/C	C♦	S*	S/C
Wnt3a	Early	6175	12938	2.1	8946	2026	0.2	11747	21339	1.8
	Late	11214	9081	0.8	14446	1539	0.1	9145	546	0.1
Wnt7	Early	3569	39753	11.1	8175	56319	6.9	2938	11082	3.8
	Late	10456	59284	5.7	-1009	21989	-21.8	-6242	15231	-2.4
Wnt10b	Early	8090	3160	0.4	4214	1637	0.4	9341	15461	1.7
	Late	-5447	22716	-4.2	-2398	2220	-0.9	-1919	13822	-7.2

♦cytosolic ;*secreted

The ratio of secreted to cytosolic levels was assessed in early and late passage C2C12 cells. Results of the ratio values indicate high Wnt7 and 10b ratios (highlighted in **bold**) which suggest higher secreted levels to cytosolic in early cells compared to late passage cells. This high ratio of secretion to cytosolic levels indicate the role of both Wnt7 and 10b in promoting myogenesis and alternatively, lower ratio, a decrease in myogenesis.

In summary, our studies have for the first time analyzed the profile of cytosolic and secreted Wnt3a, 7 and 10b in early and late passage proliferating and differentiating C2C12 cells. Our results revealed key characteristics of early and late passage cells: early passage cells differentiate effectively whereas late passage cells differentiate poorly. Our results also support the premise that elevated Wnt3a decreases myogenesis, whereas elevated Wnt7 and 10b promotes. Furthermore, Yaffe and Saxel (1977) enhanced the differentiation in their isolated C2 myoblast cell lines by substituting growth medium (containing 20 % fetal calf serum) with differentiation medium (containing 10% horse serum). The factors that enhanced the differentiation have largely been unknown. However, we speculate the presence of Wnt3a, 7 and 10b in differentiation medium could be the crucial factors for the observed enhanced differentiation. However, our analysis of myogenic differentiation have been done with a myoblast cell line which are known have a corrupt cell cycle through the accumulation of mutations. Therefore, it is crucial to repeat the experiments using primary culture myoblasts. Lastly, from our results, we speculate the possible use of

Wnt7 and 10b to promote myogenesis in muscles deficient in myogenesis such as muscular dystrophy muscles.

CHAPTER THREE

MYOGENIC DIFFERENTIATION IN AGING C2C12 MYOBLASTS TREATED WITH MEDIA CONDITIONED BY YOUNG C2C12 MYOBLASTS

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ABSTRACT

Satellite cells are muscle stem cells that play a role in regeneration and repair of injured skeletal muscle. This process of myogenesis by satellite cells declines with age. In support of this hypothesis, our previous analysis of differentiating C2C12 myoblasts revealed that late passage cells lacked differentiation capacity characterized by reduced Myogenin and low Wnt7 expressions. Parabiotic studies have shown that satellite cells with reduced myogenesis can be rejuvenated to undergo effective myogenesis when exposed to young environment. In the current study, we used the media conditioned from early passage cells (conditioned media) which we previously determined to have high levels of differentiation promoting Wnt3a and 7 proteins, and determined whether this media could alter the myogenic capacity of late passage C2C12 cells.

The results from the cross-over experiment indicated that Myogenin and MHC levels were elevated in differentiating late passage cells in response to 'young' media, whereas early passage cells treated with 'old' media, surprisingly showed a slight increase in both proteins. Cytosolic Wnt3a and 7 in late passage cells treated with 'young media' were increased compared to control. Early passage cells treated with 'old' media showed significantly decreased levels of Wnt3a and 7. In summary, both

early and late passage cells showed increased MHC expression in response to their conditioned media and this may be due to changes in Wnt3 and 7 expressions, respectively.

3.1 INTRODUCTION

Satellite cells are quiescent muscle stem cells located between the sarcolemma and basal lamina along myofibers, and play a role in regeneration and repair of injured skeletal muscle (Charge and Rudnicki, 2004; Mauro, 1961). They are characterized by their anatomical location, molecular marker expression and function (Brack and Rando, 2007). Following an injury, satellite cells are activated from their quiescent state, begin to proliferate (now called myoblasts) and after several cycles of proliferation, eventually differentiate and fuse with each other (or existing myofibers), to form new myotubes to complete the repair process. The up-regulation of several transcription factors plays a pivotal role in this process. These include the expression of myogenic regulatory factor (MRF) expression such as MyoD, Myf5, Myogenin and Mrf4 (Fuchtbauer and Westphal, 1992; Gayraud-Morel et al., 2007). The repair process is accompanied by satellite cell self-renewal, which ensures that, after repeated rounds of regeneration, the satellite cell population is maintained.

Sarcopenic muscles are aging muscles typified by an increase in resident adipocytes, fibroblasts and muscle atrophy which results in reduced muscle mass (Gopinath and Rando, 2008; Solomon and Bouloux, 2006). Contrary to young muscle, sarcopenic muscle is hampered by poor regeneration and repair; thus in the presence of a severe injury, fibrosis at the site of injury is prevalent (Brack et al., 2007). This reduced capacity for myogenesis is at least in part due to the altered extrinsic environment (Brack and Rando, 2007). Aging skeletal muscle is characterized by a

thickened basal lamina, reduced myofiber thickness and an altered profile of secreted endocrine and paracrine factors. Collectively, these age-related extrinsic changes directly impact satellite cell function.

Parabiotic studies by Brack et al. (2007) showed improved repair in skeletal muscle of old mice when the circulatory system of old and young mice were linked. Conversely, skeletal muscle of young mice displayed decreased repair with satellite cells trans-differentiating to preferentially acquire a fibrogenic lineage. These results suggested that serum factors in the circulatory system regulate the regenerative capacity of satellite cells in the skeletal muscle in mice. Further analysis revealed that Wnt proteins in the serum of the old mice, specifically Wnt3a, play a pivotal role in the observed phenotypic change (Brack et al., 2007). Taken together, a combination of age-related changes in sarcopenic muscles itself, along with altered systemic factors control satellite cell fate and function. Moreover, parabiotic pairing experiment has demonstrated that the impaired myogenic differentiation capacity of satellite cells in aging muscle is reversible.

The use of the *in vitro* culture system provides a powerful tool to examine the effect of external stimuli on a chosen cell population. The requirements for such study require the purification of these stimuli specifically signaling proteins. However, study and biochemical characterization of Wnt family of signaling proteins are hampered by their purification difficulty. Currently, only Wnt3a and 5a are purified in their active form from conditioned media (Kikuchi et al., 2007; Willert et al., 2003). These purified Wnt proteins allow an accurate *in vitro* assessment on the role of these Wnts on the cells of interest. Muroyama et al. (2004) demonstrated the addition of purified Wnt3a enhanced the differentiation of neural stem cells into

neurons and astroglia. Other studies have shown that the presence of purified Wnt3a in hematopoietic stem cell culture promoted proliferation and reduced differentiation (Willert et al., 2003). Alternatively, other methods are favored to study the effect of Wnt proteins due to their purification difficulty. These include co-culturing with transfected cell line or using conditioned media (CM) which provide an alternative source of Wnt proteins. Co-culturing of non-myogenic CD45⁺ cells with the transfected AtT-20 cell lines that expresses high levels of Wnt5a, 5b, 7a and 7b mix, induces myogenic lineage commitment of CD45⁺ cells (Poleskaya et al., 2003). Willert et al. (2003) utilized mouse L cell fibroblast to generate Wnt3a-CM to examine the effect of Wnt3a on the hematopoietic stem cells and Cha and Heasman (2010), generated Wnt5a- and Wnt11-CM from oocytes to determine the effect of both and individual proteins on Wnt signaling in mouse L cell fibroblasts. Therefore, co-culturing and use of CM provides an alternative source of active proteins. This is particularly important with respect to the Wnt family of proteins which are not readily available commercially and difficult to purify in their active form.

Elevated protein levels of some Wnt isoforms are known to play a role in reduced myogenic progression in aging muscles (Brack et al., 2007). Furthermore, analysis of the CM from our previous studies (Chapter 2) of the three key Wnt isoforms, we have established that early passage C2C12 cells secreted high Wnt7 and 10b levels during differentiation in the CM. This high level of secretion is associated with promoting differentiation in early passage cells. On the contrary, late passage cells displayed defective differentiation coupled with lower levels of Wnt7 and 10b secretion, specifically Wnt10b. From these finding, we hypothesize that the high levels of Wnt7 and 10b in CM from differentiating early passage cells can restore myogenic differentiation of late passage cells. Thus, we expand our studies with the use of

these valuable CM from early and late passage cells. In our current study, we devised an *in vitro* protocol to analyze myogenic differentiation of late passage cells ('old' C2C12 cells) in response to CM collected from early passage C2C12 cells ('young' C2C12 cells), and vice versa. Similar to the *in vivo* parabiotic pairing counterpart, the aim of this study was to restore the differentiation capacity of late passage cells through the use of CM from early passage C2C12 cells.

3.2 MATERIALS AND METHOD

3.2.1 Cell culture

Early (P13) and late passage (P33) murine C2C12 cells were maintained in growth medium containing Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Sigma) and 1% Penicillin/Streptomycin (Highveld Biological) maintained in an Innova[®] CO-170 CO₂-incubator (New Brunswick) at 37 °C, 5% CO₂. Cells were induced to differentiate when 80% confluent by substituting growth medium with differentiation medium containing Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 1% horse serum (Sigma), 2 mM L-glutamine (Sigma) and 1% Penicillin/Streptomycin (Highveld Biological). The CM of early and late passage cells was collected at day 0 (day 0-CM), 1 (day 1-CM), 2 (day 2-CM) and 3 (day 3-CM) during differentiation of early 'young' or late 'old' passage cells, respectively. These collected CM were filtered through 0.2 µm syringe filters to further sterilize and remove non-adherent cells, and stored at -20 °C. The CM are utilized to treat cells in the cross-over experiment.

3.2.2 Cross-over experiment and C2C12 protein lysate preparation

Early 'young' passage C2C12 cells were plated into CM from proliferating late passage cells ('old' day 0-CM), conversely, late 'old' passage C2C12 cells were plated into CM from proliferating early passage cells ('young' day 0-CM), in 6 well plates at 400 000 cells/well. Upon reaching 80% confluency, early and late passage cells were induced to differentiate by changing both 'young' and 'old' day 0-CM with differentiation inducing 'young' and 'old' day 1-CM. To further differentiate the cells, both 'young' and 'old' day 1-CM was substituted with 'young' and 'old' day 2-CM at day 2 of differentiation and at day 3 of differentiation, 'young' and 'old' day 2-CM was replaced with 'young' and 'old' day 3-CM (Fig. 3.1). Cells were harvested at different time points (day 0, 1 and 3), detached with trypsin-EDTA, lysed in RIPA buffer (Sigma) and protein inhibitor cocktail (Sigma, P8340), and sonicated for 5 seconds in eppendorf tubes submerged in ice. For controls, a similar protocol was designed with early and late passage C2C12 cells plated into conventional growth medium and differentiated with conventional differentiation medium and harvested at the same time points of differentiation as the cross-over treated cells.

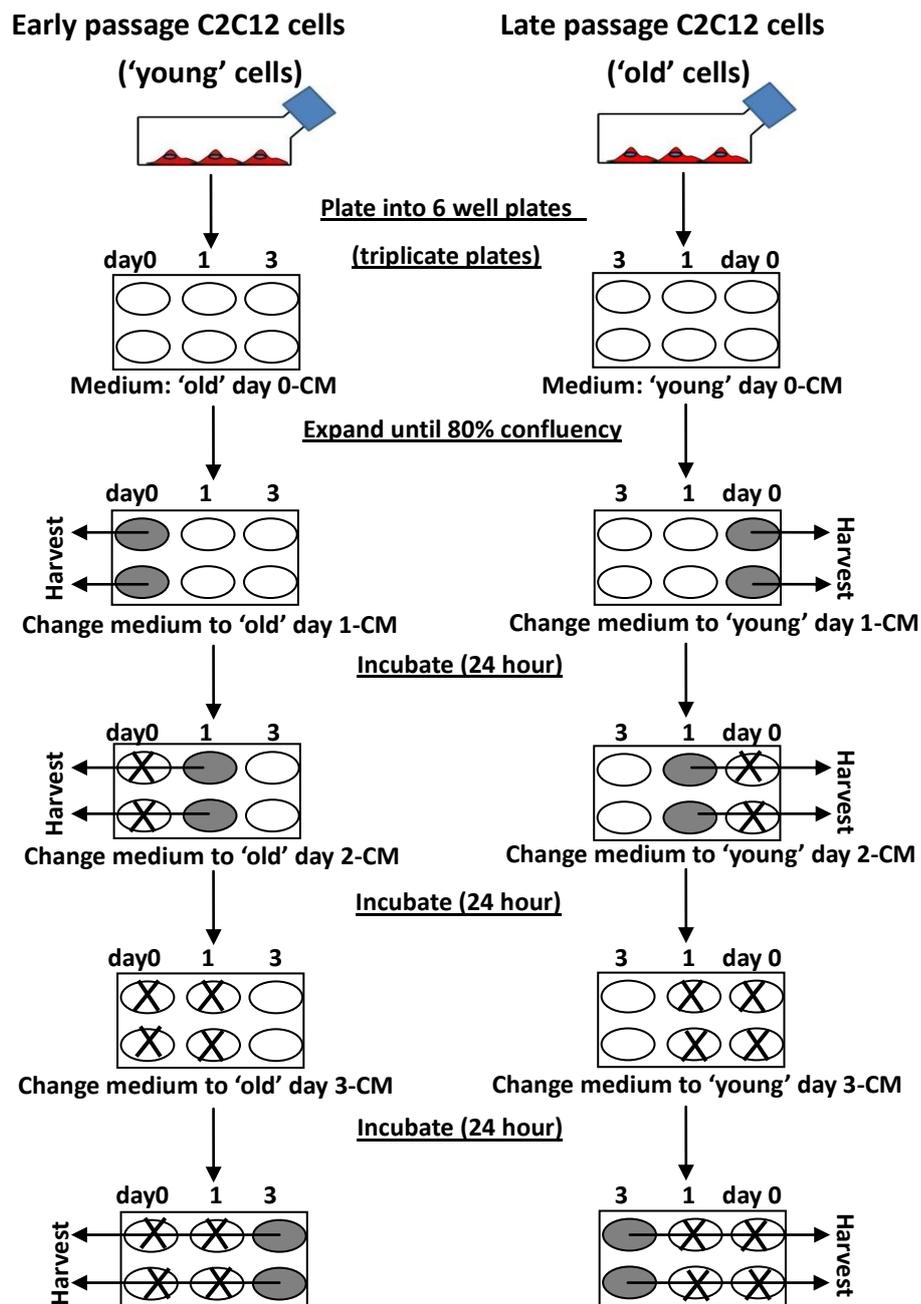


Fig. 3.1: Experimental design of the cross-over conditioned media experiment

Early and late passage C2C12 cells maintained in T75 flasks were plated into 6 well plates at 400 000 cells/well (triplicate plates) into 'old' and 'young' day 0-CM, respectively. Upon reaching 80% confluency, cells were harvested (day 0) and cells in the remaining wells were induced to differentiate with 'young' or 'old' day 1-CM. After 24 hour incubation, day 1 cells were harvested, remaining cells were treated with 'young' or 'old' day 2-CM and further incubated for 24 hours. Thereafter, the media was substituted with 'young' or 'old' day 3-CM, incubated for 24 hours and subsequently harvested at day 3. Harvested cells were lysed and probed for MHC, Myogenin, Wnt3a and 7. Controls cells were treated with the conventional growth and differentiation medium. Experiment were performed in triplicates for each protein (n=3) for the cross-over and control treated cells.

3.2.3 Western blotting of C2C12 protein lysates

Western blotting was used to monitor protein expression levels during proliferation and differentiation of C2C12 cells treated with CM. 30 µg of cross-over and control treated cell lysates (protein concentration determined by Bradford assay, section 2.2.5) from each time point were resolved in a reducing 12.5% SDS-polyacryamide gel at 18 mA/gel (Laemmli, 1970). Thereafter, nitrocellulose membrane was incubated with primary antibodies against MHC (MF 20,Hybridoma Bank), Myogenin (F5D, Santa Cruz), Wnt3a (YY-7, Santa Cruz), Wnt7 (H-40, Santa Cruz), and internal controls, α -tubulin (B-7, Santa Cruz) and GAPDH (14C10, Cell Signaling) in TTBS (see section 2.2.1, Table 2.3, for optimized primary antibody dilutions), overnight at 4 °C. Following primary antibody incubation, the nitrocellulose membrane was washed and incubated in secondary HRP-linked antibodies in TTBS (see section 2.2.1, Table 2.2, for secondary antibody dilutions) (1 hour), at room temperature. The antigen-antibody complex was visualized using an ECL kit (Immun-Star™ WesternC™ Chemiluminescent Kit, BioRad) according to manufacturer's instructions. The nitrocellulose membranes were exposed onto Biomax light film (Kodak) and captured on VersaDoc™ MP imager (BioRad). Densitometry analysis for protein expression levels were quantified with Quantity One software (BioRad) by tracing around the band of interest.

3.2.4. Statistical analysis

For statistical purpose, each experiment were performed a minimum of 3 to 9 replicates (n=3 for MHC, Myogenin, Wnt 3a and 7 expression analysis; n=6 for α -tubulin expression analysis; n=9 for GAPDH expression analysis). All data are represented as mean \pm SEM (standard error of the mean). The paired Student's *t*-test was performed for parametric data and paired Mann-Whitney *U* test for

non-parametric data. All statistical tests were performed using the Genstat 12th edition statistical software (VSN International). Differences in data sets were considered significant if $p < 0.05$.

3.3 RESULTS

3.3.1 Expression levels of α -tubulin and GAPDH in response to conditioned differentiation media

Equal loading of cell lysates is essential to accurately determine changes in protein expression. House-keeping genes such as α -tubulin, β -tubulin, GAPDH and β -actin that are constitutively expressed regardless of different treatments are often probed together with the protein of interest to ensure such equal protein loading. However, the expression of these house-keeping genes has been shown to change under certain conditions such as fluctuations in cell confluency even when equivalent loads of proteins lysates are analyzed (Dittmer and Dittmer, 2006; Greer et al., 2010). In section 2.3.4, we determined that the expression of α -tubulin and GAPDH remained constant during differentiation of early and late passage C2C12 cells. Here, we sought to determine whether CM changed the expression of α -tubulin or GAPDH during differentiation within early and late passage and further compared both proteins between the two passages. As expected, no fluctuations were seen in differentiating cells under control conditions (Fig. 3.2B and 3.3B). The results of the cross-over treated cells indicated that within both differentiating early and late passage cells, α -tubulin and GAPDH expression levels fluctuated but no significant difference were observed. However, late passage cells treated with 'young' media showed a decrease in the expression profile of α -tubulin along with a significant increase in GAPDH compared to early passage expression of both proteins (Fig. 3.2A and 3.3A). Therefore, although treating cells with the conditioned media did not change the

levels of both house-keeping genes (α -tubulin and GAPDH) within each passage, fluctuations were observed. Furthermore, significant differences between passages were seen and due to these fluctuations, ponceau S staining was rather used to ensure equal loading.

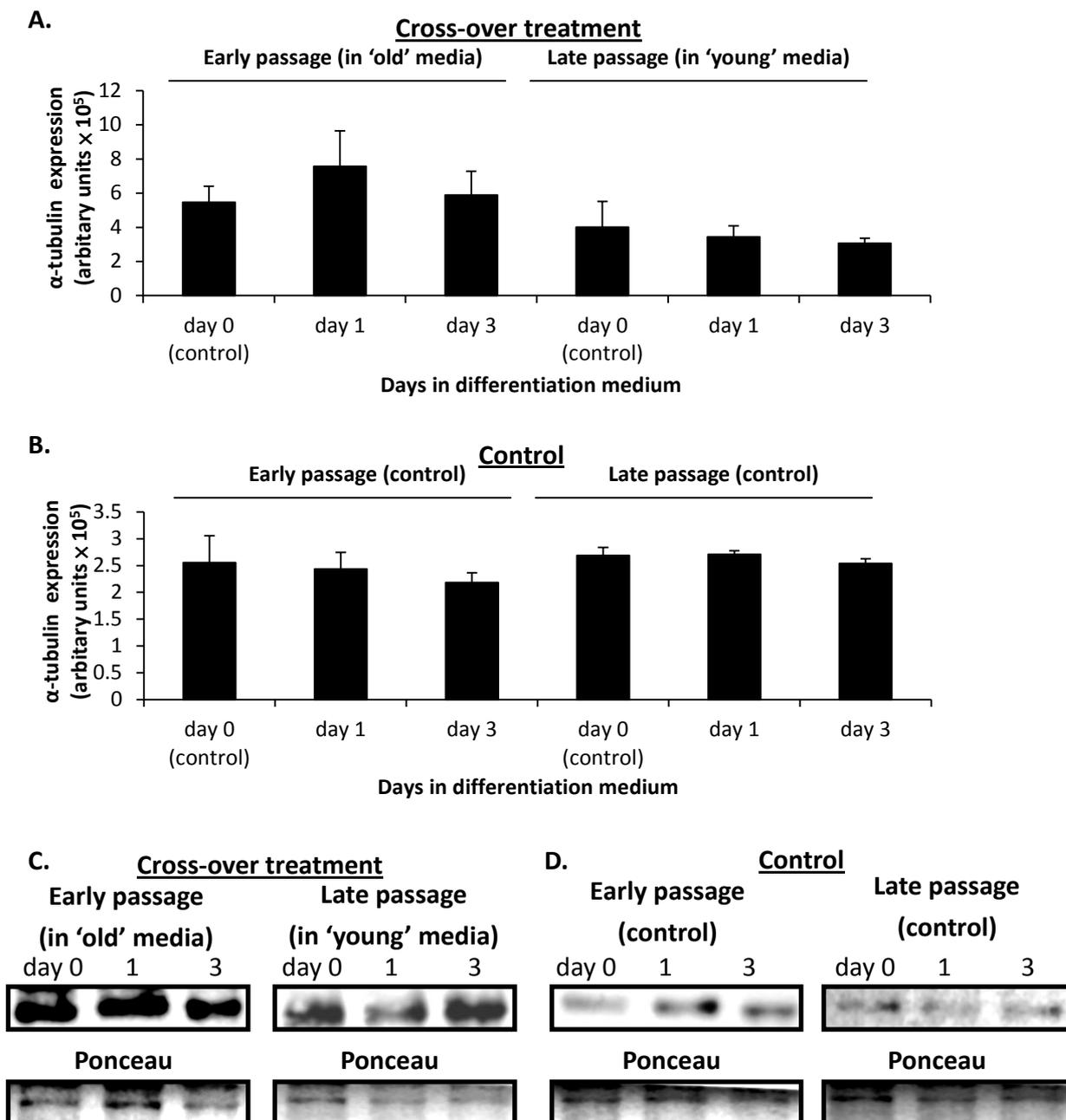


Fig. 3.2: Expression of α -tubulin during C2C12 cell differentiation

The expression of α -tubulin from western blotting was compared during differentiation in cross-over and control treated C2C12 cells. Early and late passage cells were plated into 'old' or 'young' day 0-CM. Upon reaching 80% confluency, cells were induced to differentiate by replacing day 0-CM with 'young' or 'old' day 1-CM and further differentiated with 'young' or 'old' day 2- and day 3-CM. Cells were harvested at days 0, 1 and 3, and probed for α -tubulin via western blotting (C and D). Densitometry values were determined in early and late passage cells treated with CM (A) and controls (B), respectively. Ponceau stain was used to confirm equal loading. Reduced α -tubulin levels in the late passage cells from cross-over treated cells were observed with no difference in α -tubulin levels in control treated cells. Experiment performed in 6 replicates for the cross-over and control treated cells.

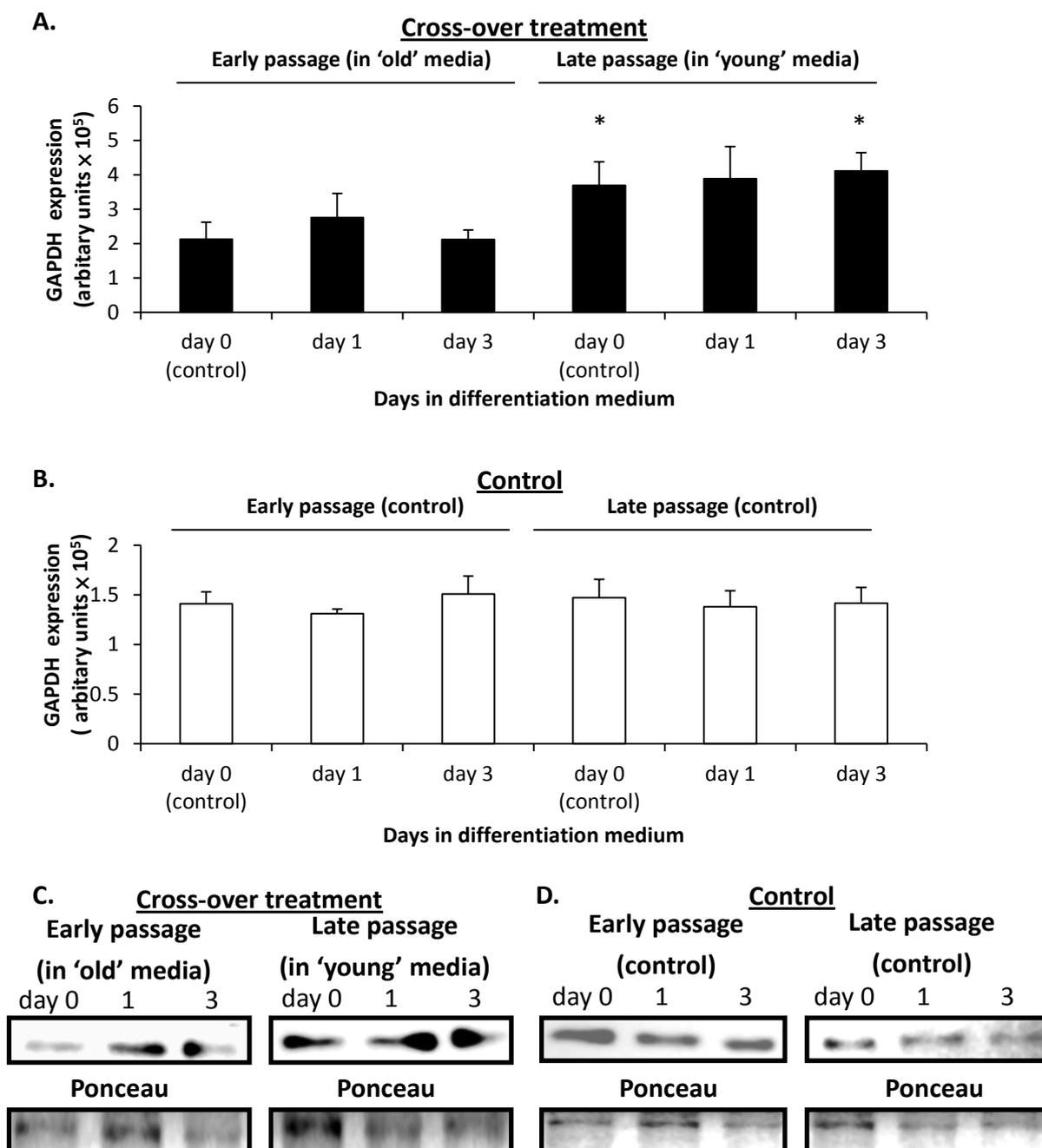


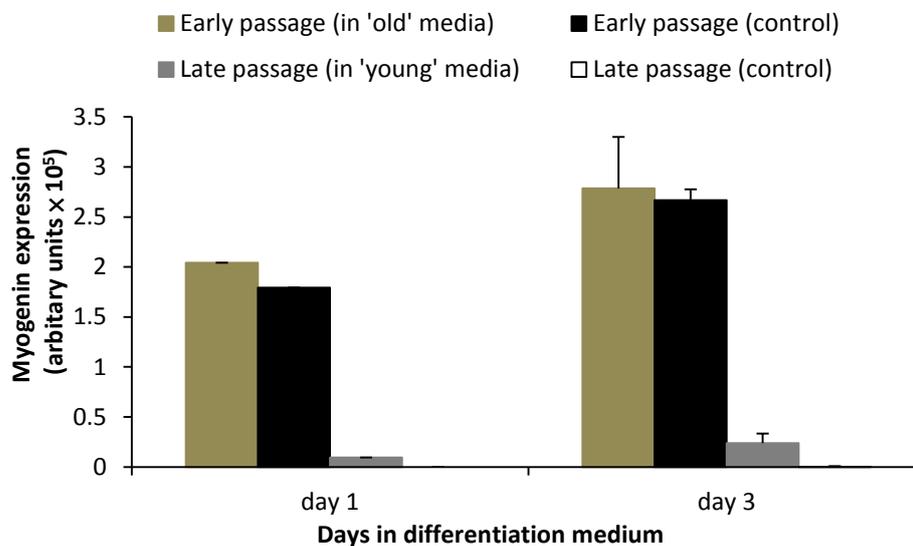
Fig. 3.3: GAPDH expression levels of differentiating C2C12 cells

The expression of GAPDH from western blotting was compared during differentiation in cross-over and control treated C2C12 cells. Early and late passage cells were plated into 'old' or 'young' day 0-CM. Upon reaching 80% confluency, cells were induced to differentiate by replacing day 0-CM with 'young' or 'old' day 1-CM and further differentiated with 'young' or 'old' day 2- and day 3-CM. Cells were harvested at days 0, 1 and 3, and probed for GAPDH via western blotting (C and D). Densitometry values were determined in early and late passage cells treated with CM (A) and controls (B), respectively. Ponceau stain was used to confirm equal loading. Elevated GAPDH levels in the late passage cells from cross-over treated cells were observed. Experiment performed in 9 replicates ($n=9$) for the cross-over and control treated cells. (* $p < 0.05$: early vs. late passage at equivalent time points)

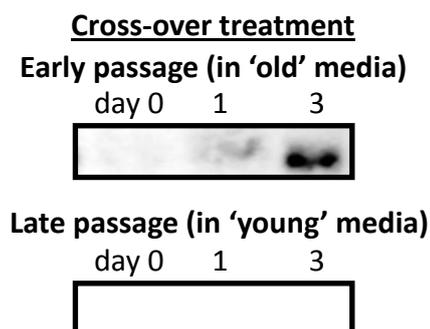
3.3.2 Total Myogenin and MHC levels in response to differentiation conditioned media in C2C12 cells

Previously (Chapter 2), we established that late passage C2C12 cells lack myogenic differentiation capacity reflected in part by reduced Myogenin expression levels (section 2.3.5). In the current study, we utilized Myogenin and Myosin heavy chain, also a marker of myogenic progression (Bader et al., 1982), to analyze the state of myogenic differentiation in differentiating C2C12 cells in the cross-over experiment. Likewise, in this chapter, late passage cells under control conditions displayed poor differentiation capacity with down-regulated Myogenin levels and conversely, early passage cells displayed effective differentiation with up-regulated Myogenin levels (Fig. 3.4A and C). This lack of differentiation capacity in late passage cells was further confirmed by reduced MHC expression profile in the control cells (Fig. 3.5A and C). Treatment of early passage cells with 'old' media did not reduce the level of Myogenin and MHC but surprisingly had elevated the levels of both proteins, along with a significant increase of MHC levels at day 1 (Fig. 3.5A), relative to control levels. Late passage cells with the characteristic impaired differentiation, when cultured in the presence of 'young' media, displayed up-regulated Myogenin and MHC levels, with a significant increase of MHC expression at day 1 and 3 (Fig. 3.5A). Lastly, besides the differences in levels of Myogenin and MHC regardless of the different C2C12 cell treatments, an increasing trend of both proteins was observed with time. This characteristic trend was also prevalent in our previous study (Chapter 2). In summary, secreted factors in 'young' media resulted in higher Myogenin and MHC expression in differentiation poor late passage cells. These elevated levels could indicate that 'young' media could have rejuvenated late passage cells to undergo myogenic differentiation.

A.



B.



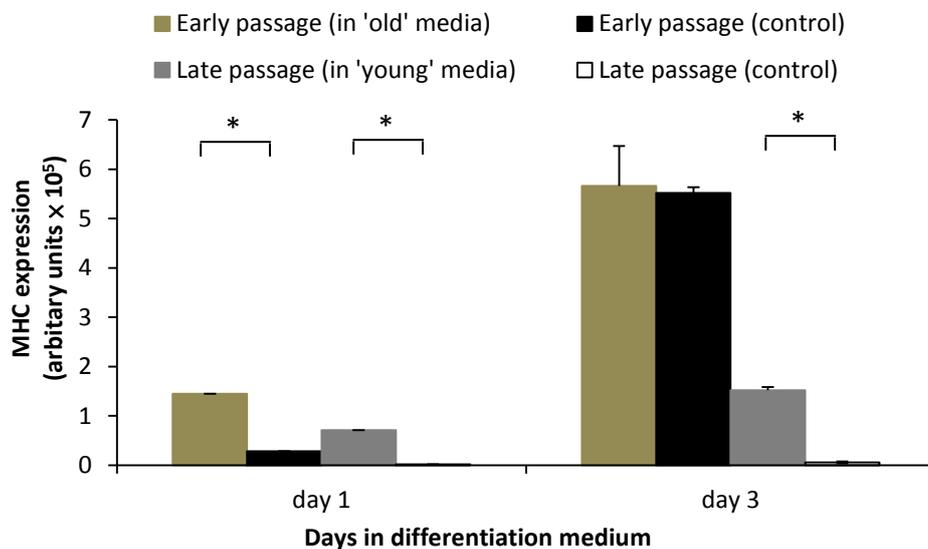
C.



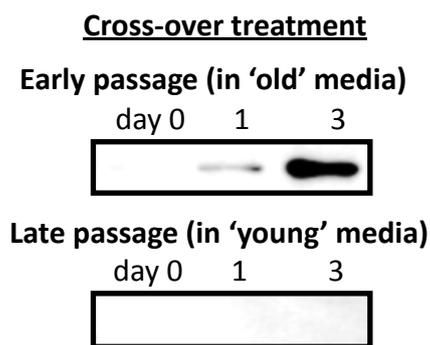
Fig. 3.4: Myogenin expression profile of differentiating C2C12 cells

The expression of Myogenin was compared during differentiation in cross-over and control treated C2C12 cells. Early and late passage cells were plated into 'old' or 'young' day 0-CM. Upon reaching 80% confluency, cells were induced to differentiate by replacing day 0-CM with 'young' or 'old' day 1-CM and further differentiated with 'young' or 'old' day 2- and day 3-CM. Cells were harvested at days 0, 1 and 3, and probed for Myogenin, and densitometry values (A) were determined from western blotting in early and late passage cells treated with CM (B) and the controls (C), respectively. Late passage cells treated with 'young' media indicated a higher expression of Myogenin which suggest myogenic differentiation improvement; however no difference in levels of Myogenin in early passage cells treated with 'old' media was observed when both are compared to the respective controls. Experiment performed in 3 replicates (n=3) for the cross-over and control treated cells.

A.



B.



C.



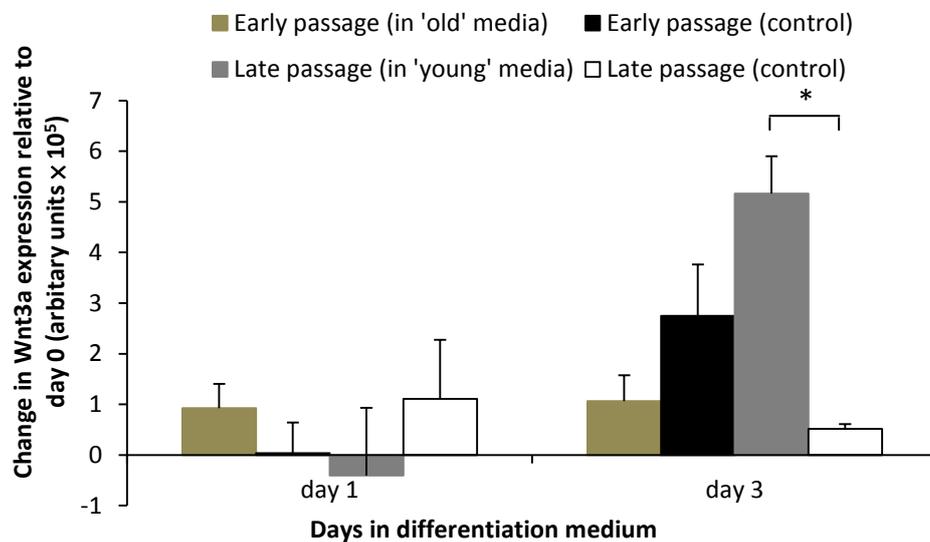
Fig. 3.5: MHC expression profile of differentiating C2C12 cells

The expression of MHC was compared during differentiation in cross-over and control treated C2C12 cells. Early and late passage cells were plated into 'old' or 'young' day 0-CM. Upon reaching 80% confluency, cells were induced to differentiate by replacing day 0-CM with 'young' or 'old' day 1-CM and further differentiated with 'young' or 'old' day 2- and day 3-CM. Cells were harvested at days 0, 1 and 3, and probed for MHC, and densitometry values (A) were determined from western blotting in early and late passage cells treated with CM (B) and the controls (C), respectively. Late passage cells treated with 'young' media has a higher expression of MHC compared to the respective control which suggests myogenic differentiation improvement. However, MHC levels in early passage cells treated with 'old' media had higher expression initially at day 1. Experiment performed in 3 replicates ($n=3$) for the cross-over and control treated cells. ($*p < 0.05$).

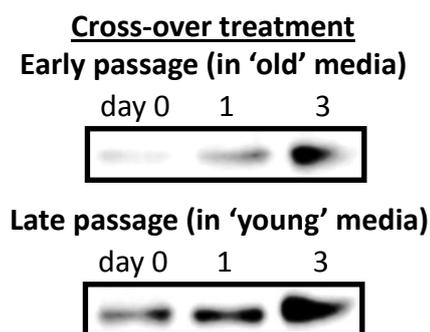
3.3.3 Analysis of cytosolic Wnt levels in response to differentiation conditioned media in C2C12 cells

In Chapter 2, we have established that late passage C2C12 cells displayed high cytosolic Wnt3a expression which supports the elevated myogenic to fibrotic lineage conversion, and a rapid decline in Wnt7 expression. Both characteristic expressions are associated with the lack of myogenic differentiation in late passage cells. To determine the levels of Wnts produced in response to differentiating CM, all results of Wnt expression following addition of differentiating CM were normalized to day 0 by subtracting densitometry data at day 0 from day 1 and 3. Results were presented as the change in expression relative to day 0. Interestingly, late passage cells treated with 'young' media displayed a suppression of Wnt3a levels at day 1; however this suppression is lost at day 3 where Wnt3a levels were significantly higher compared to the control (Fig. 3.6A). Early passage cells treated with 'old' media was expected to increase Wnt3a levels. Indeed, this was observed at day 1 but at day 3, control levels surpassed the cross-over treated early passage cells. Therefore, secreted factors in 'young' and 'old' media initially at day 1 suppressed Wnt3a expression in late passage cells and elevated Wnt3a expression in early passage cells, respectively. However, these expression levels at day 1 did not persist to day 3. The Wnt7 profile altered dramatically in the cross-over treated cells. Late passage cells treated with 'young' media had an elevated expression profile whereas early passage cells treated with 'old' media displayed a reduced level of Wnt7, with a significant reduction at day 3, in comparison to the respective controls (Fig. 3.7A). Therefore, secreted factors in 'young' media altered Wnt7 levels of late passage cells which acquired increasing Wnt7 levels associated with effective myogenic differentiation, whereas early passage cells acquired low and decreasing levels of Wnt7 in the presence of secreted factors in 'old' media.

A.



B.



C.

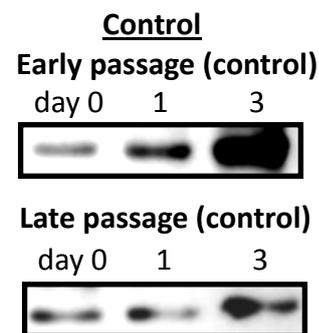
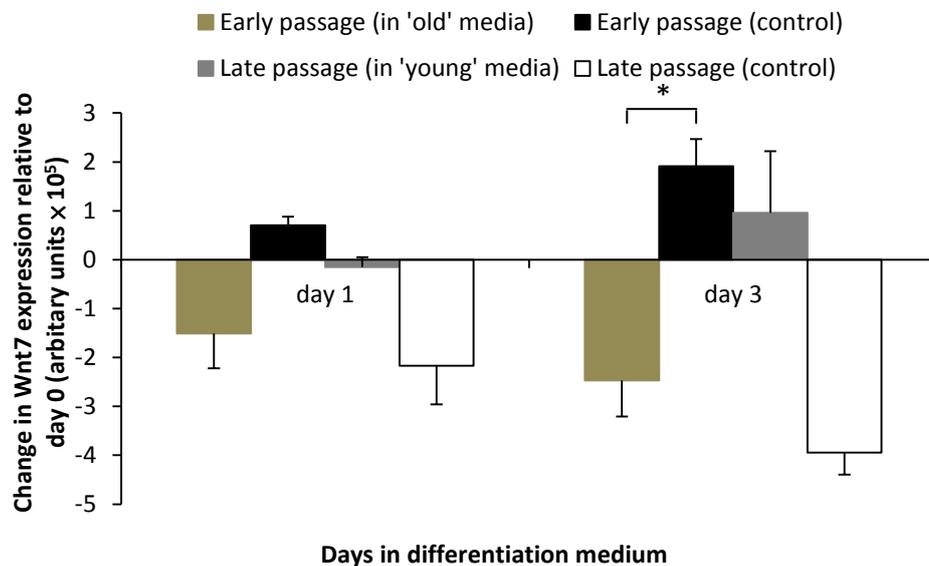


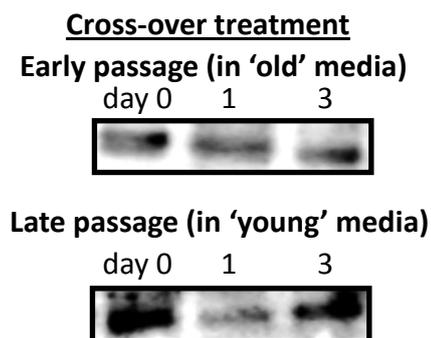
Fig. 3.6: Wnt3a expression profile of C2C12 cells in response to conditioned media

The expression of Wnt3a was compared during differentiation in cross-over and control treated C2C12 cells. Early and late passage cells were plated into 'old' or 'young' day 0-CM. Upon reaching 80% confluency, cells were induced to differentiate by replacing day 0-CM with 'young' or 'old' day 1-CM and further differentiated with 'young' or 'old' day 2- and day 3-CM. Cells were harvested at days 0, 1 and 3, and probed for Wnt3a, and densitometry values (A) were determined from western blotting in early and late passage cells treated with CM (B) and the controls (C), respectively. Compared to the controls, early passage cells treated with 'old' media had higher Wnt3a level at day 1 but lower at day 3; on the other hand, late passage cells treated with 'young' media displayed lower Wnt3a levels at day 1 but higher levels at day 3. The latter seem to indicate suppression of fibrosis by 'young' media on late passage cells at day 1 but this suppression is eventually loss at day 3 of differentiation. Experiment performed in 3 replicates (n=3) for the cross-over and control treated cells. (* $p < 0.05$).

A.



B.



C.

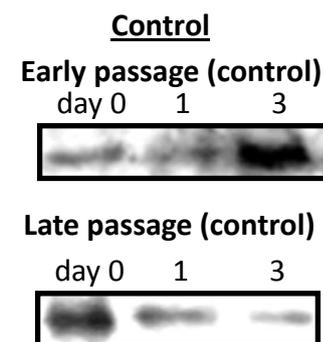


Fig. 3.7: Wnt7 expression profile of C2C12 cells in response to conditioned media

The expression of Wnt7 was compared during differentiation in cross-over and control treated C2C12 cells. Early and late passage cells were plated into 'old' or 'young' day 0-CM. Upon reaching 80% confluency, cells were induced to differentiate by replacing day 0-CM with 'young' or 'old' day 1-CM and further differentiated with 'young' or 'old' day 2- and day 3-CM. Cells were harvested at days 0, 1 and 3, and probed for Wnt7 and densitometry values (A) were determined from western blotting in early and late passage cells treated with CM (B) and the controls (C), respectively. Early passage cells treated with 'old' media had a declining Wnt7 level, whereas late passage cells treated with 'young' media, an increasing Wnt7 level during differentiation. These contrary trends in the cross-over treated cells to the controls seem to indicate an improvement to myogenesis. Experiment performed in 3 replicates ($n=3$) for the cross-over and control treated cells. ($*p < 0.05$).

3.4 DISCUSSION

In Chapter 2, we reported the baseline expression of Wnt3a, 7 and 10b in early and late passage C2C12 cells. The results indicated that early passage cells secreted high levels of Wnt3a and 7 and conversely, low levels of Wnt3a and 7 in the late passage cells. Thus, we hypothesize that the early and late passage C2C12 cells conditioned the surrounding media with the secreted Wnts to either promote or impede differentiation. To test this hypothesis, we harvested the early and late passage C2C12 cells treated with CM and analyzed the myogenic capacity in response to the CM.

Expression of house-keeping genes has been shown to change making them unreliable as internal controls. Dittmer and Dittmer (2006) noted with high protein loading for the detection of low-abundance protein, no difference in signal intensity was observed. Furthermore, Greer et al. (2010) demonstrated that cell confluency affects α -tubulin and GAPDH levels even when equivalent loads of protein are analyzed. Likewise, we observed expression differences in our internal controls when differentiating early passage cells was compared to differentiating late passage cells. This makes normalizing to both internal controls for the expression comparison between early and late C2C12 passage cells for the protein of interest unreliable. However, from our Chapter 2, expressions of both α -tubulin and GAPDH did not change within early and late passage cells in the individual differentiation experiments. Therefore, we utilized both internal controls and ponceau stained blots as an indication of equal loading within early and late passage cells in response to differentiation.

Wnt proteins are known to be difficult to purify in their active form due to their

hydrophobic properties (Kikuchi et al., 2007). Therefore, CM from transfected cells to generate Wnt-CM or normal cell lines to generate CM containing Wnts, provide an alternative approach to study the effect of Wnt secreted proteins on the cells of interest. In our current study, we utilized the latter method to generate media from early passage cells to promote differentiation in late passage C2C12 cells which in chapter 2, we determined to have reduced myogenic capacity. Our results indicated that late passage cells treated with 'young' media had higher Myogenin and MHC levels but surprisingly, a slight increase in levels of both proteins in early passage cells was seen when treated with 'old' media, compared to their respective controls. Contradictorily, secreted factors in 'young' and 'old' media seem to both promote differentiation in late and early passage cells. Studying the effects of CM on the cells of interest is known to yield contradictory results to cells treated with purified proteins. Willert et al. (2003) indicated that Wnt 3a-conditioned media promoted differentiation in hematopoietic stem cell cultures whereas addition of purified Wnt3a protein reduced differentiation and promoted proliferation. They concluded that other factors in the conditioned media promoted differentiation rather than proliferation. Densitometry values indicate an increase in MHC levels in late passage cells treated with 'young' media, it should be noted from the MHC blots that signal bands were very faint; therefore the increase in levels is most likely to be a result of background values and therefore should be taken with caution. To overcome this dilemma, an increase in cell protein lysates loading coupled with an increase in film exposure could possibly result in an elevated signal. Lastly, we noted a possible mobility shift in Myogenin in both early passage cells treated with 'old' media and early passage cells under control conditions. This increase in molecular weight (at day 3) is likely due to the phosphorylation of Myogenin at Threonine-87 (Blagden et al., 2004), and subsequent translocation into the nucleus.

Wnt3a and 7 expression levels were significantly affected by conditioned media. This increase in both Wnt3a and 7 also observed in the Chapter 2 signifies a possible role of both Wnt isoforms in myogenic differentiation. Indeed, Poleskaya et al. (2003) and Brack et al. (2008) reported that Wnt7a and b, and Wnt3a mRNA transcript level increases in regenerating muscle, respectively. However, high elevated Wnt3a has been shown to promote fibrosis (Brack et al., 2007). Therefore, we hypothesized that 'young' media would reduce the Wnt3a levels to suppress fibrosis. Indeed, at day 1 of differentiation, 'young' media reduced the Wnt3a levels in late passage C2C12 cells and 'old' media elevated the Wnt3a levels. However, similar results were not carried to day 3. At day 3, levels of Wnt3a in cross-over treated early passage cells remained at the same level as day 1 but late passage cells had a sudden increase which was significantly higher compared to the control treated cells. Therefore, secreted factors from 'young' media initially suppressed Wnt3a levels in late passage cells and 'old' media elevated Wnt3a levels in early passage cells at day 1, however these effects are lost at day 3. This suggests that Wnt3a alone may not be responsible for the decline in differentiation seen in late passage cells.

Results from Chapter 2 showed that Wnt7 levels decline and increases in differentiating late and early passage C2C12 cells, respectively. These decreasing trends coincided with poor differentiation in late passage cells and vice versa. Furthermore, we determined the importance of Wnt7 in myogenic differentiation and concluded the possible use of Wnt7 proteins to promote myogenesis in myogenic deficient muscle such as muscular dystrophy. Interestingly, Wnt7 expression profile changed in our cross-over treated compared to the control treated cells, with early passage cells treated with 'old' media acquired a reduced Wnt7

expression, whereas late passage treated with 'young' media, acquired an increase of Wnt7. These results suggest that Wnt7 could play a role in altering the differentiation phenotype in late passage cells. Given the importance of Wnt10b (together with Wnt7), it would be interesting to additionally analyze Wnt10b levels in our cross-over treated C2C12 cell lysates.

In our study, we did not extend the differentiation period post-day 3 because the collected CM post-day 3 changed in pH which could have a negative effect on the C2C12 cell differentiation. Therefore, by extending the days of differentiation post-day 3, a more pronounced outcome of Myogenin and MHC levels might be possible. Furthermore, Wnt protein secretion levels are known to be very low (Bradley and Brown, 1995), therefore the failure of 'young' media to dramatically improve the differentiation of late passage cells could also be attributed to the insufficient levels of Wnt proteins in the CM. Alternatively, conditioned media by transfected cell line to over-express Wnts3a and 7 could be used to increase the levels of these particular differentiation-inducing Wnts. Polesskaya et al. (2003) utilized this co-culture method to generate CM containing high levels of Wnts5a, 5b, 7a and 7b by transfecting AtT-20 cell line to over-express these Wnt isoforms. The CM utilized in our studies took a long period to generate and further utilized for the cross-over treatment of C2C12 cells thus could run a risk of protein degradation due to this extended period of use in culture. To prevent protein degradation, co-culture with a transfected cell line could further be utilized to provide a constant supply of paracrine Wnt proteins to the cells of interest.

In summary, Wnt-conditioned media has been used extensively to study the effects of Wnts on cells. However, results are sometimes ambiguous due to the presence of

other secreted factors. Furthermore, we utilized a cell line that has a corrupt cell cycle by accumulated mutations in culture. These mutations could intrinsically alter the cells to be less susceptible to the surrounding secreted factors. Analysis of differentiation in the cross-over experiment should therefore be repeated with primary culture myoblasts. Also it should be noted that the expression profile of Wnt7 changed significantly which could indicate myogenic improvement. Thus, until more Wnt proteins are purified, alternative methods to generate CM with higher concentrations of desired Wnt proteins using transfected cell lines or co-culture should be further investigated.

CHAPTER FOUR

GENERAL DISCUSSION

Satellite cells reside between the sarcolemma and basal lamina of myofibers and are responsible for the maintenance and repair of skeletal muscle (Charge and Rudnicki, 2004). This efficient myogenic process declines with age, typified by the accumulation of adipocytes and fibroblasts, and the accumulation of scar tissue upon injury. The age-related extrinsic and intrinsic changes in aging muscles have collectively been shown to negatively impact satellite cell myogenesis. Parabiotic studies where the circulatory systems of young and old mice are linked implied that satellite cells in old mice can be rejuvenated to undergo effective myogenesis by the exposure to a youthful environment. In addition, the study showed that at least one of the causes of the age-related decline in myogenesis was due to elevated canonical Wnt signaling by serum factors, likely by Wnt3a protein. Although the Wnt mRNA levels during myogenesis have been well documented, the relevant Wnt protein levels had not been quantified. Thus, our study aimed to establish the cytosolic and secreted protein profiles of three chosen Wnts, namely Wnt3a, 7 and 10b, in early and late passage C2C12 cells.

Following satellite cell activation, MyoD is expressed, which in turn facilitates Myogenin expression as the myogenic lineage progresses. MyoD^{-/-} myoblasts from muscles of MyoD knock-out mice continued to proliferate when cultured in differentiation medium, however they fail to differentiate; this lack of differentiation is associated with the suppressed downstream expression of Myogenin (Sabourin et al., 1999). Restoring MyoD with MyoD expressing vector in MyoD^{-/-} myoblasts resulted in effective differentiation and successful Myogenin expression, therefore the expression is dependent on the transcriptional activity of MyoD. The loss in

differentiation capacity of MyoD^{-/-} myoblasts was also observed in our late passage C2C12 cells which continued proliferating in differentiation medium. However, total MyoD levels were higher in the late vs. early passage cells; therefore it is more likely that the failure to differentiate is possibly caused by the repressed activity of MyoD by co-repressors, protein stability or protein modifications, and not due to the total levels.

Indeed, modifications of histones and MyoD were shown to play a central role in this regulation. Mal and Harter (2003) indicated that histone deacetylase (HDAC) complexes with MyoD at the promoter site of Myogenin, and deacetylates H3 histone which subsequently silences Myogenin. Gillespie et al. (2009) indicated that the absence of p38 γ , MyoD is not phosphorylated and in this conformation, KMT1A methyltransferase is not recruited and histone H3K9 methylation is absent which leads to the premature expression of Myogenin. It is possible that both proposed mechanisms are involved in the suppression and activation of MyoD activity. To experimentally verify this in our early and late passage C2C12 cells; chromatin immuno-precipitation (ChIP) assay with the relevant antibodies against HDAC and KMT1A methyltransferase could be assessed. In addition, the p38 γ levels between the two passages could be quantified during differentiation. The molecular repression and activation activity of MyoD can be seen with our early and late passage cells with the expression profile of Myogenin. As Myogenin expression is dependent on MyoD activity, the rise in Myogenin levels suggests that MyoD activity occurred which was seen in our early passage cells. On the other hand, the low levels of Myogenin in late passage cells suggest repression of MyoD. Therefore, these molecular events signify that protein modification and the activity of repressors are just as important as protein levels. It is interesting to note that levels of MyoD and

Myogenin have been shown to be higher in muscles of old senile rats when compared with their younger counterparts which agrees with our MyoD results (Dedkov et al., 2003).

In young muscle, Notch and Wnt signaling have been indicated to regulate proliferation and differentiation, respectively (Brack et al., 2008; Conboy and Rando, 2002). The cross-talk between the two processes is mediated by GSK-3 β ; therefore the onset of differentiation requires the up-regulation of Wnt signaling by inhibiting the activity of GSK-3 β , and in the process down-regulating Notch (Brack et al., 2008) (Fig.4.1).

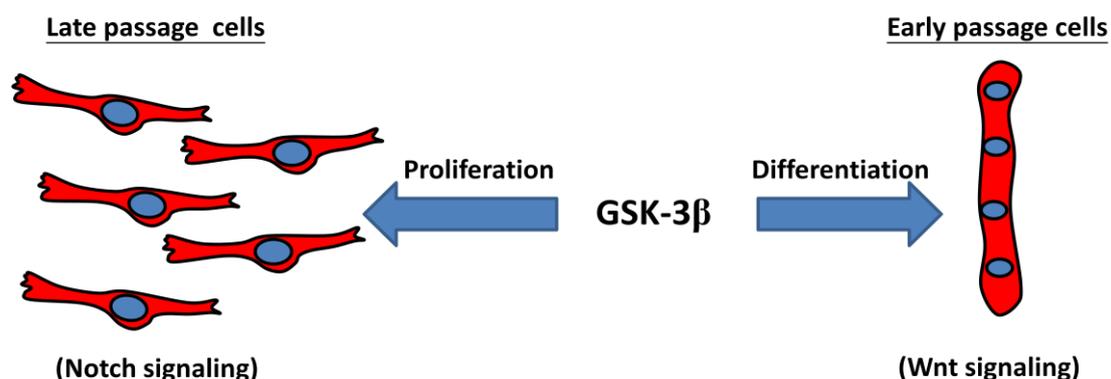


Fig. 4.1: The balance between proliferation and differentiation in early and late passage C2C12 cells

The balance between proliferation and differentiation requires the cross-talk of Wnt with Notch signaling, mediated by the common target protein, GSK-3 β . Early passage C2C12 cells could up-regulate Wnt signaling by inhibiting the activity of GSK-3 β to readily undergo differentiation in differentiation medium and in the process, down-regulate Notch signaling. On the contrary, late passage C2C12 cells could activate GSK-3 β which results in the up-regulation of Notch signaling and down-regulation Wnt signaling, and continued proliferating in differentiation medium. Therefore, we speculate that the increase in passage numbers likely tilted the balance towards Notch signaling even under differentiation conditions and resulted in the continued proliferation seen in our late passage cells.

Indeed, quantification of Wnt3a, 7a and 7b transcripts during muscle regeneration has been shown to increase in regenerating muscle (Brack et al., 2008; Poleskaya et al., 2003). In agreement, Wnt expression profiles of all three Wnt isoforms during differentiation showed an overall increase, specifically with the differentiation competent early passage C2C12 cells. Therefore, the increase in mRNA transcripts would likely reflect the protein levels. To verify this, mRNA transcript levels of our three Wnt isoforms should be further analyzed with RT-PCR. It is interesting to note that the injection of endogenous Wnt3a into regenerating young muscle resulted in poor skeletal muscle repair (Brack et al., 2007). These results could arise from the early onset of differentiation by the premature induction of Wnt signaling by the suppression of Notch signaling. Therefore, the premature up-regulation of Wnt signaling could impede myogenesis. From these accumulated data, the progression of myogenesis requires the temporal balance between Notch and Wnt signaling through the stages of proliferation to differentiation, respectively. It is likely that in aging muscle, this temporal regulation is lost and that the switch from Notch to Wnt signaling does not occur correctly. An evidence of this imbalance was seen in our early vs. late passage cells. Our early passage cells were differentiation competent when provided with differentiation cues whereas the late passage cells were incapable of differentiation, and continued proliferating in the presence of differentiation cues (Fig. 4.1). Therefore, we speculate that our early passage C2C12 cells down-regulate Notch and when induced to differentiate, and up-regulate Wnt signaling. However, in the late passage cells, this switch from Notch to Wnt signaling could be lost. Analysis of our chosen canonical Wnt proteins provided some insight into this imbalance, specifically Wnt7. All three isoforms in the early passage C2C12 cells showed an overall increase in progression of differentiation, however in late passage cells, a rapid decline in Wnt7 and lower Wnt10b levels was observed. This

rapid decline in Wnt7 and low Wnt10b expression is likely associated with the down- and up-regulation of the Wnt and Notch signaling, respectively. Therefore, to verify this imbalance at the molecular level, down-stream target of both pathways such as GSK-3 β can be quantified during proliferation (at day 0) and during differentiation in early and late passage cells, along with markers of proliferation (Ki-67) and differentiation (MyoD and Myogenin). We hypothesize that during proliferation of early passage cells, GSK-3 β levels would be elevated and upon the induction of differentiation, GSK-3 β levels would gradually decline. However, in late passage cells, GSK-3 β levels could remain elevated during proliferation and differentiation. Together, cross-talk between Notch and Wnt signaling is required for effective differentiation and our results indicate that Wnt7 could be a key isoform in regulating this transition.

Analysis of canonical Wnt3a protein during differentiation showed an overall higher and rapid expression in late passage C2C12 cells. These results may seem counterintuitive as Wnt3a also induces canonical Wnt signaling required for effective differentiation. However, seminal work by Brack et al. (2007) demonstrated that elevated Wnt3a signaling accounted for the poor myogenesis observed as well as accumulated tissue fibrosis. However, from the Wnt7 data (early vs. late passage), we derived that a gradual increase in Wnt signaling during differentiation is required. Therefore, the sudden rise in Wnt3a observed in late passage cells could induce Wnt signaling prematurely leading to impaired myogenesis. The lineage alteration suggested by Brack et al. (2007) was not performed in our study. It would therefore be interesting to assay for this lineage change with fibroblast specific marker, ER-TR7. Overall, it may appear that Wnt signaling leads to a disastrous effect on myogenesis from the Wnt3a data, but from the Wnt7 data, it appears that a gradual up-regulation of Wnt signaling is required for successful differentiation.

Wnt10b has been suggested to play a role in a myogenic to adipogenic lineage conversion via the canonical Wnt signaling with age. The Wnt10b mRNA transcript levels were shown to increase in the presence of cyclic mechanical stretching (Akimoto et al., 2005). This mechanical stretch inhibited the myogenic to adipogenic fate in C2C12 cells along with elevated Wnt10b mRNA transcript. Furthermore, the down-regulation of canonical Wnt signaling by inactivating TCF/LEF lead to an adipogenic conversion in C2C12 and G8 myoblast cell lines verified by Oil Red-O staining (Ross et al., 2000). These data seem to suggest that the balance between myogenic and adipogenic potential in myoblasts is controlled Wnt signaling. In aging muscle, this balance seems to be shifted towards adipogenesis by down-regulating Wnt signaling. Comparison of Wnt10b mRNA levels showed higher transcripts in muscles of young mice than old mice (Taylor-Jones et al., 2002). Our Wnt10b cytosolic analysis correlates with the Wnt10b transcript levels with the early passage C2C12 cells showing an overall elevated Wnt10b protein expression. This would likely prevent adipogenesis lineage conversion by up-regulating Wnt signaling. On the contrary, late passage cells showed an overall reduction in Wnt10b expression, thus possibly displaying an adipogenic propensity. The adipogenic propensity was not determined in our cells; however this can be simply determined with Red-O staining. From these data, it seems likely that the up-regulation of Wnt signaling by Wnt10b is required to prevent adipogenic lineage alteration in young muscle. Lastly, knock-out studies of Wnt10b ($Wnt10b^{-/-}$) in mice have provided insight to the complex interactive roles of multiple Wnts in myogenesis. $Wnt10b^{-/-}$ myoblasts from muscle of young knock-out mice differentiated effectively due to the compensation effect of Wnt7, conversely, this compensation action is lost with age in old $Wnt10b^{-/-}$ myoblasts. This compensation by Wnt7 is seen in our study in the early period of differentiation, but is eventually lost. This study agrees with our finding that Wnt7 is

the likely key isoform for myogenic differentiation, Wnt10b for adipogenesis and Wnt3a for fibrogenesis (Fig. 4.2).

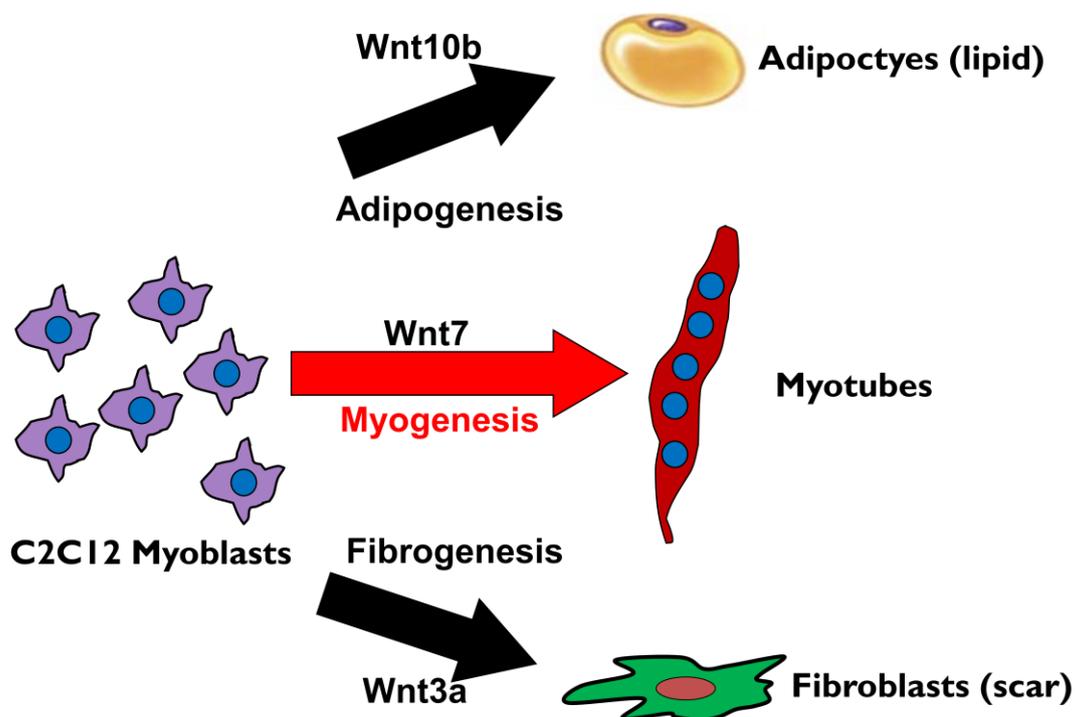


Fig. 4.2: The proposed role of Wnt isoforms in lineage specification of C2C12 myoblasts

C2C12 cells undergo successful myogenesis with the formation of myotubes. However, the increase in passage negatively affects myogenesis with the increase in fibrogenesis and adipogenesis propensity which is likely mediated by Wnt proteins. Early passage cells undergo effective differentiation with the up-regulation of Wnt7 and the down-regulation of Wnt3a. On the other hand, late passage cells showed reduced myogenesis with a declining Wnt7, lower Wnt10b and high Wnt3a levels. The down-regulated Wnt10b and up-regulated Wnt3a levels are likely associated with fibrogenesis and adipogenesis in late passage cells, respectively. Furthermore, the declining levels of Wnt7 in late passage cells reflect the decline in myogenesis.

Next, we quantified the secreted Wnt3a, 7 and 10b levels during differentiation. This novel analysis of the three Wnt isoforms in CM revealed the presence of secreted Wnt proteins only in CM from differentiating, not proliferating C2C12 cells. Overall, we observed a higher secretion of Wnt7 in comparison to Wnt3a and 10b and a characteristic trend in secretion, similar to the cytosolic expression – early passage

C2C12 cells displayed an increase in secretion whereas late passage cells had a rapidly declining secretion. Surprisingly, when we determined the ratio of secreted to cytosolic production of the three Wnts between early and late passage cells, early passage cells displayed a higher ratio in all Wnt isoforms, with the highest seen in Wnt7. These values indicate that early passage cells have a higher tendency to secrete Wnts than late passage cells. The reason for the lack of secretion in late passage cells is unknown. Post-modifications of Wnt proteins involve both palmitoylation and glycosylation. Palmitoylation has been shown to be important for active signaling, but not essential for secretion (Willert et al., 2003), however glycosylation is required for proper protein folding which is a requirement for protein secretion (Kikuchi et al., 2007). Thus, late passage cells could have defective machinery for glycosylation. Nevertheless, it is clear that further characterization of Wnt proteins is required. Lastly, a high Wnt7 ratio of secretion to cytosolic levels was observed in our studies. From the data in the cytosolic expression profiles, we established that Wnt7 play a role in myogenesis. Therefore, high secretion of Wnt7 could reflect the propensity of C2C12 cells, especially early passage cells, to undergo myogenesis.

Following the analysis of the secreted levels, we hypothesized that the secreted Wnt proteins in early passage C2C12 cells would be able to rescue the myogenic capacity of late passage cells. However, in the cross-over treatment of C2C12 cells, we did not observe an improvement in myogenesis in late passage cells when treated with CM from early passage cells. CM contains many secreted factors which may include TGF- β as well as secreted Wnt antagonists. TGF- β 1 has been indicated to be elevated in the serum of aging mice and shown to have an inhibitory effect on myogenesis (Carlson et al., 2009). Wnt antagonists are divided into two classes. One class directly

binds to Wnts and prevents ligand-receptor interaction which includes secreted Frizzled-related protein (sFRP), Wnt inhibitory factor (WIF) and Cerberus (Kawano and Kypta, 2003). And the other binds LRP5/6 co-receptor and prevents the Frizzled-LRP5/6 formation and includes Dickkopf (Dkk) (Kawano and Kypta, 2003). Therefore, the possible presence of these secreted antagonists and TGF- β in CM of early passage cells could potentially antagonize Wnt signaling and suppress myogenesis in late passage cells. Besides the secreted antagonists, it has also been shown that the regulation of Wnt signaling can be receptor dependent (Mikels and Nusse, 2006). Therefore, it is possible that receptors specific to Wnt3a and 7 are not expressed in early passage cells, therefore Wnt signaling is inactivated. Furthermore, Wnt proteins are known to be secreted inefficiently. It is likely that the secretion of Wnt isoforms in our CM was very low. Using CM from transfected cell lines that over-express Wnt proteins could provide a better yield of secreted Wnts.

Interestingly, the expression profile of Wnt7 was dramatically altered with the early passage cells acquiring a declining expression when treated with 'old-CM' whereas the late passage cells acquired an increasing expression treated with 'young-CM'. This change in expression pattern does suggest myogenesis was improved in late passage cells. It is interesting to note that ratio of Wnt10 secretion to cytosolic levels showed a several fold increase in early compared to late passage cells. Therefore, further analysis could include Wnt10b that might yield a different expression of Wnt10b.

Finally, our analysis of protein levels was conducted using C2C12 myoblast cell line. Cell lines by nature are immortal due to the accumulated mutations which could alter growth factor receptors, leading to an altered response to these factors.

Therefore, the experiments of this study should be repeated with primary cultures. In summary, our data has generated insight to the roles of Wnt3a, 7 and 10b isoforms during myogenic differentiation in aging myoblasts.

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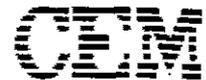
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Oil

Microwave Sample Preparation Note: XprOP-1
Category: Oils

Rev. Date: 6/04

Sample Type: Oil
Application Type: Acid Digestion
Vessel Type: 55 mL
Number of Vessels: 12
Reagents: Nitric Acid (70%)
Method Sample Type: Organic
Sample Weight: 0.5 gram

Step 1:

<u>Acid Type</u>	<u>Volume</u>
Nitric	10 mL

Heating Program: Ramp to Temperature Control

Stage	Max. Power	% Power	Ramp (min.)	Pressure (psi)	Temperature (°C)	Hold (min.)
(1)	1200 W	75	15:00	-	200	15:00

NOTE A: This procedure is a reference point for sample digestion using the CEM Microwave Sample Preparation System and may need to be modified or changed to obtain the required results on your sample.

NOTE B: Manual venting of CEM closed vessels should only be performed when wearing hand, eye and body protection and only when the vessel contents are at or below room temperature to avoid the potential for chemical burns. Always point the vent hole away from the operator and toward the back of a fume hood.

NOTE C: Power should be adjusted up or down with respect to the number of vessels. General guidelines are as follows: 8-12 vessels (50% power), 13-20 vessels (75% power), >20 vessels (100% power).

NOTE D: "Organic Method Sample Type" should be used for most sample types. Choose "Inorganic" for samples with more than 1 gram of solid material remaining at the bottom of the vessel at the end of the digest (ex. leach methods). Choose "Water" for samples that are largely aqueous prior to digestion.