Analysis of the remodelling of the satellite cell basal lamina during skeletal muscle regeneration

Thesis presented for the degree of PhD by

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Dedication

To my parents, for everything

In memory of

Sri. Laxmaiah Rayagiri
Sri. Ramanand Gaddam
Dr. Marudvathi Gaddam

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Abstract

Satellite cells (SC) are muscle-specific stem cells involved in muscle growth and repair in adults. The niche of SC consists of basement membrane (basal lamina), muscle fibre and supporting cells. A major component of basement membranes (BM) is laminin, a heterotrimer protein composed of α, β and γ chains. During the transition from embryonic to foetal and to adult, the muscle basement membrane is extensively remodelled. Indeed, the embryonic basement membrane that is associated with the myotome contains Laminin-111 and -511. During foetal stages, Laminin-111 and -511 are progressively replaced by Laminin-211, which is the main Laminin constituent of the adult basement membrane surrounding muscle fibres. As similarities exist between the myogenic programme carried out by satellite cells and that executed by embryonic muscle progenitor cells, I hypothesized that the composition of the basal lamina in adult muscles may be dynamic at the site of satellite cells to support their activation and progression through myogenesis.

In this study, immunofluorescence and quantitative PCR data on *ex-vivo* culture system of mouse extensor digitorum longus (EDL) muscle fibres revealed the expression of Laminin α1 at the site of activated satellite cells, in contrast to previous reports showing that the adult skeletal muscle basal lamina has a uniform Laminin composition (Laminin-211). Laminin α1 association with activated satellite cells was also observed *in vivo* in two distinct models of muscle regeneration, the Dystrophin-deficient *mdx* mouse and in cardiotoxin-injured tibialis anterior (TA) muscles. Laminin α1, secreted by satellite cells, is also expressed at the surface of macrophages *in vivo*. Finally, I provide evidence that Integrin α6β1, the preferred receptor for Laminin α1, is expressed at the surface of activated satellite cells *ex-vivo*. Altogether these results reveal that a remodelling of the basal lamina occurs during skeletal muscle regeneration with the concomitant secretion and deposition of Laminin α1 in the basal lamina overlying activated satellite cells. Laminin α1 may signal through its specific receptor Integrin α6β1 to promote satellite cell progression through myogenesis. Finally, the presence of Laminin α1 may also act as a mediator between activated satellite cells and macrophages, promoting the recruitment of macrophages to the vicinity of satellite cells to support muscle regeneration. Thus, this study provides an insight into a mechanism allowing for the remodelling of the satellite cell niche during muscle regeneration.

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Abbreviations

BMSC: Bone marrow-derived stem cells

CLN: Centrally located nuclei

CMD: Congenital muscular dystrophies

CNS: Central nervous system

CSA: Cross-sectional area

DAPC: Dystrophin associated protein complex

DG: Dystroglycan

DGC: Dystroglycan complex

DKO: Dystrophin double knockout

DMD: Duchenne or Becker muscular dystrophies

ECM: Extracellular matrix components

EDL: Extensor digitorum longus

EHS: Engelbreth-Holm-Swarm

ES: Embryonic stem

FA: Focal adhesion

FAK: Focal adhesion kinases

FAP: Fibrogenic progenitor cells

FDB: Flexor Digitorum Brevis

FGF: Fibroblast Growth Factor

FSHD: Facioscapulohumeral muscular dystrophy

GAG: Glycosaminoglycans

GRMD: Golden retriever dog

HFSC: Hair follicle stem cells

HGF: Hepatocyte Growth Factor

HSPG: Heparan sulfate proteoglycans

HSPG: Heparan sulphate proteoglycans

IBM: Inclusion-body myositis

IFN γ:Interferon-γ

IFSC: Interfollicular epidermal stem cells

IGF: Insulin Growth Factor

IGFR: IGF receptor

IL-1: Interleukine-1

MEF2: Myocytes enhancer factor 2

miRNAs: MicroRNAs

MMP: Matrix metalloproteinase

MPC: Myogenic progenitor cells

MRF: Myogenic regulatory factors

mRNA: ribonucleo-protein

NMJ: Neuromuscular junction

NOS: Nitric oxide synthase

OPMD: Oculopharyngeal muscular dystrophy

PDGF: Platelet Derived Growth Factor

PI3K :Phosphatidylinositol 3-kinase

PRC2: Polycomb repressive complex 2

PSF: Penicillin/streptomycin/fungicide

RSMD1: Rigid spine muscular dystrophy

SAG: Shh agonist

Shh: Sonic Hedgehog

SOL: Slow-twitch soleus

SP: Side population

TA: Tibialis Anterior

TGF: Transforming growth factor

TNFα:Tumor necrosis factor-a

VEFG: Vascular endothelial growth factor

WWS: Walker-Warburg syndrome

Cdo: Cell adhesion molecule-related/down regulated by oncogenes

Cdc42: Cell divison control protein 42

LARGE: Like-acetylglucosaminyltransferase

Chapter 1

Introduction

1.1 Adult skeletal muscle

Skeletal muscles are one of the largest vertebrates tissues. They constitute of around 40% the total body weight (Janssen et al. 2000) and are responsible for skeletal movement, postural behavior and breathing. The development of skeletal muscle starts at the embryonic stages and continues through to adulthood.

1.1.1 Structure of skeletal muscle

Skeletal muscles are made up of muscle fibres, networks of nerves and connective tissue and other cell types in interstitial spaces, blood vessels and extracellular matrix (McComas 1996). At the proximal and distal extremities of muscles, tendons attach muscles to bones. Around 85% of the mass of muscles is due to myofibres and the remaining 15% is mostly connective tissue. Bundles of myofibres (100-150) are organized into fascicles (see Fig 1.1). The overall muscle structure is divided into three levels of organization. The epimysium, the endomysium and the perimysium are made of connective tissue, which is present between the fibres and provides a framework for the muscle structure (McComas 1996). Surrounding each fascicle is another connective tissue, the perimysium. The perimysium is attached externally to the epimysium (see Fig 1.1) through collagen fibres, and extends internally to connect with the endomysium. Arteries and veins run through the endomysisum. Endomysium collagen fibres connect to the perimysium and to the basement membrane, which lies outside the muscle fibre sarcolemma (plasma membrane).

The functional unit of skeletal muscles is the muscle fibre (myofibre), which is a single cylindrical multinucleated muscle cell. A typical muscle fibre in humans is in length about 12cm (McComas 1996). However, the longest myofibre was found in the sartorius muscle, which is up to 60cm in length (Harris et al. 2005). Each myofibre is packed with bundles of myofibrils, which consist of sarcomeres, the contractile units of muscles. The total number of sarcomeres depends on the size and length of the muscle fibre. The sarcomere contains two types of filamentous molecules, the thick filament of Myosin and the thin filament of Actin. Sarcomeres give its striated appearance to myofibres under the light microscope. Interactions between Actin-containing filaments and Myosin-containing filaments form the basic mechanism of

muscle contraction, which operates through a process termed the "sliding filament mechanism" (Huxley 1974).

1.1.2 Muscle activity depends on the fibre type

Myofibre types determine the metabolic and contractile activity of muscles. The two major types of skeletal muscle fibres are slow-twitch fibres and fast-twitch fibres. Slow-twitch-fibres or Type 1, also known as red muscle fibres, contain high levels of myoglobin and oxidative enzymes, are rich in mitochondria, and are well supplied with blood vessels. Muscles containing a high proportion of slow fibres produce relatively less force and are highly resistant to fatigue. They can maintain prolonged, steady ATP production from aerobic metabolism by utilizing reserved Glycogen and fat. In contrast to Type I myofibres, fast-twitch fibres or Type II fibres contain little or no myoglobin and appear white to pink in color. Type II fibres contain fewer mitochondria and blood vessels. The Myosin of these fibres has a high ATPase activity and hence these fibres can produce energy rapidly, but are sensitive to fatigue. In humans, fibres are mainly classified into slow-twitch Type I, and fast-twitch Type IIA and Type IIB. Type I fibres are abundant in the Soleus muscle and Type II in the extensor digitorum longus (EDL) muscle. However, the fibre content varies between muscles within a species, between species and also in response to ageing, exercise and disease (Schiaffino 2010).

1.1.3 Location of adult muscle stem cells: satellite cells

Under normal physiological conditions skeletal muscles are stable and undergo very little turnover. However, upon exercise or injury, new myoblasts are generated that differentiate and fuse to existing fibres. The source of these myoblasts are satellite cells. Satellite cells are muscle-specific stem cells and are classically defined by their position underneath the basal lamina (see Fig 1.1). Alexander Mauro in 1961 observed by electron microscopy a group of mononucleated cells present at the periphery of the adult skeletal myofibre and proposed that satellite cells might represent "dormant myoblasts" (Mauro and Adams 1961). In response to muscle injury they are activated, they proliferate and finally supply new myonuclei to the

myofibre (Bischoff and Heintz 1994). The electron microscopy of satellite cells reveals that they are very closely associated to the basal lamina and have a high nuclear-to-cytoplasmic ratio (Mauro et al., 1961). The number of satellite cells differ depending on myofibre types, muscles, age and species (Schmalbruch and Hellhammer 1976). In mice, they constitute 30% of myonuclei in neonates and then decline to around 4% in adults as in humans (Allbrook et al. 1971). In aged mice the number of satellite cell decreases further to 2% (Bischoff and Heintz 1994; Hawke and Garry 2001). Satellite cells make up to 2-7% of the total nuclei of a single myofibre (Hawke and Garry 2001). The number of satellite cells varies between different muscles. For instance, the fast-twitch EDL muscle contains fewer satellite cells than the slow-twitch soleus (SOL) (P. S. Zammit et al. 2002). In addition, myofibre ends have a higher number of satellite cells than the rest of the fibre (Allouh et al. 2008). The muscle mass of an average man is 20-25kg and it is estimated that there are 2 x 10^5 to 10 x 10^5 satellite cells/g of tissue, which is approximately 1x10^{10} to 2x10^{10} satellite cells per person (Morgan and Partridge 2003).

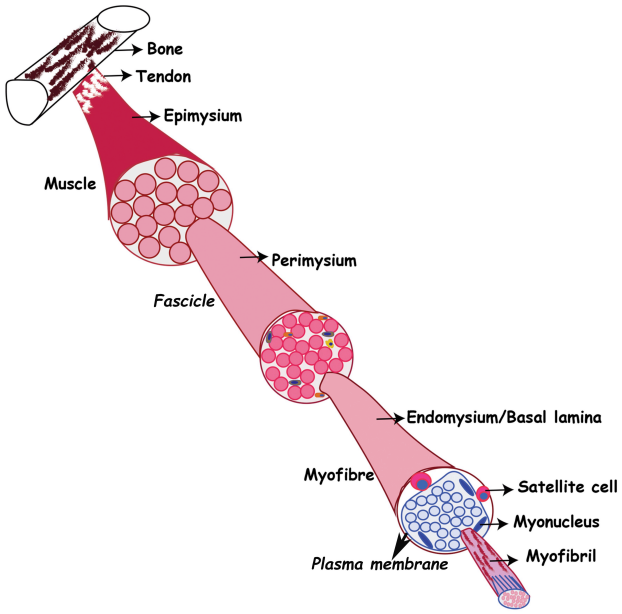


Figure 1.1: ***Schematic representation of skeletal muscle structure:*** Muscles are attached to the bone by tendons. The epimysium covers the whole muscle and individual bundles of myofibres are covered by the perimysium. The endomysium surrounds each myofibre and satellite cells are present underneath the basal lamina and above the plasma membrane of myofibre (redrawn from Otto 2009).

1.1.4 Developmental origin of satellite cells

The majority of trunk skeletal muscles originate from somites, which are transient mesodermal structures (Armand et al. 1983; Christ and Ordahl 1995). Head muscles originate from the unsegmented head mesoderm and from the prechordal mesoderm (Buckingham et al. 2003). Somites mature along the dorso-ventral axis such that the dorsal compartment develops into the dermomyotome, from which striated muscles (myotome) and dermis are formed, whereas the ventral compartment develops into the sclerotome, which is the source for the axial skeleton that includes vertebrae and ribs (Christ and Ordahl 1995). Quail-chick chimera studies demonstrated that all trunk myogenic cells, including satellite cells, originate from the somites (Armand et al. 1983). The origin of satellite cells was further explored using transplantation and genetic approaches. Initially, it was thought that satellite cells arose during late fetal development (Feldman and Stockdale 1992). However, generation of Pax3 and Pax7 knock-in reporter alleles and the use of cre-LoxP based lineage tracing approaches revealed that satellite cells originate from the dermomyotome and remain as proliferating and undifferentiated progenitor cells (Gros et al. 2005; Schienda et al. 2006). As the dermomyotome breaks down, these progenitor cells interspersed with differentiating myogenic cells of the myotome and become the source of fetal muscles (Gros et al. 2005; Relaix et al. 2005). Furthermore, satellite cell progenitors remain in a proliferative state until they are positioned beneath the basal lamina of developing myofibres as quiescent satellite cells. In fact, the first cells with satellite cell characteristics appear at E16.5 in mice (Kassar-Duchossoy et al. 2005).

1.2 Skeletal muscle dystrophies and therapies

Muscular dystrophies are a group of genetic disorders that cause progressive muscle weakness and muscle wasting, and in many cases lead to death. There are over 30 different types of muscular dystrophies, which can be grouped into six major classes affecting different muscles (see Fig 1.2). Duchenne muscular dystrophy is most common and affects 1 in 5000 male worldwide births each year (Mendell et al. 2012). The first muscular dystrophy described was reported by Edward Meryon (Meryon

1852). He described a disease with early age onset and characterized by muscle weakness and wasting that leads to death by late adolescence. Symptoms can appear during childhood or adolescence or during ageing. Dystrophies that affect individuals in early infancy are categorized as congenital muscular dystrophies (CMD).

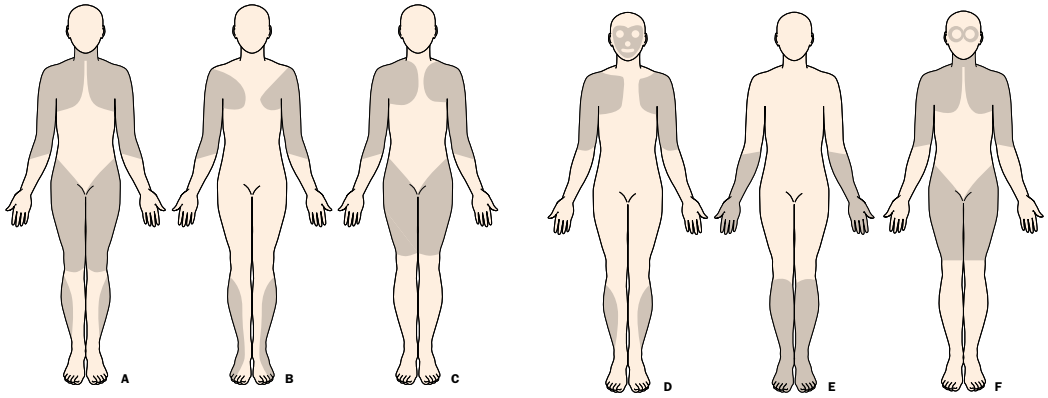


Figure 1.2: ***Distribution of muscle weakness in different dystrophies (taken from Emery 2002):*** The shaded regions indicate the muscles affected in different dystrophies. A: Duchenne-type and Becker-type, B: Emery-Dreifuss, C: Limb-girdle, D: Facioscapulohumeral, E: Distal and F: Oculopharyngeal

1.2.1 Different types of Muscular dystrophies

Different types of muscular dystrophies are due to defects in genes that encode a distinct muscle proteins (see Fig 1.3), which are summarized in Table 1.1. The most common dystrophies are Duchenne or Becker muscular dystrophies (DMD/BMD) and congenital muscular dystrophy (CMD).

1.2.1.1 Duchenne and Becker muscular dystrophies

Duchenne or Becker muscular dystrophies (DMD/BMD) are X-linked disorders (Bulman et al., 1991). They are caused by mutations in Dystrophin, a gene that encodes a protein, which connects the cytoskeleton to the myofibre. In DMD, Dystrophin is completely absent, whereas in BMD the protein is partially functional resulting in a milder form of dystrophy. The onset of both dystrophies is around 3 years of age and skeletal, cardiac and respiratory muscles are affected (Bulman et al. 1991). Death usually occurs in patient in their early 20's as a result of respiratory failure and/or cardiac defects. Eagle and coworkers have shown that better coordinated care has been able to improve the survival rate longer than 20 years

(Bushby et al. 2010; Eagle et al. 2002). The average life expectancy of BMD patients is mid 40's or even more in some patients (Yazaki et al. 1999).

The most widely used mouse model of human Duchenne muscular dystrophy (DMD) is the *mdx* mouse. *Mdx* mice have a point mutation in the *Dystrophin* gene and develop a pathology similar to DMD patients (Carnwath and Shotton 1987; Cavanna et al. 1988; Coulton et al. 1988; Dangain and Vrbova 1984; Muntoni et al. 1993). *Mdx* mice muscles degenerate and regenerate throughout life, although the peak is at 4-8 weeks (Pagel and Partridge 1999). In both DMD and *mdx*, muscle loss of Dystrophin alters the entire dystroglycan complex (DGC) resulting in the destabilization of the sarcolemma (Cullen and Jaros 1988). A golden retriever dog (GRMD) model exhibits a similar phenotype although variability is present amongst littermates (B. J. Cooper and Valentine 1988).

1.2.1.2 Congenital muscular dystrophies

Homozygous mutation in LAMA2 gene that encodes the Laminin α2 subunit causes congenital muscular dystrophy type 1A (MDC1A). Absence of Laminin α2 results in the disruption of the basement membrane and increased apoptosis that results in severe muscle weakness (Hayashi et al. 2001; W. Kuang et al. 1998; W. Kuang et al. 1999). Different murine models for Laminin α2 deficiency have been generated (W. Kuang et al. 1998; W. Kuang et al. 1999; Sunada et al. 1994; Sunada et al. 1995; H. Xu et al. 1994b; H. Xu et al. 1994a). Dy(3k)/dy(3k) are completely deficient for Laminin the α2. Dy/dy mutant mice have been generated with a spontaneous mutation and this mice have small amounts of normal Laminin α2. Dy(W)/dy(W) express further smaller amounts of truncated Laminin α2 that lacks domain VI. All Laminin α2 mutants also lack Laminin α2 in peripheral nerve (L. T. Guo et al. 2003a).

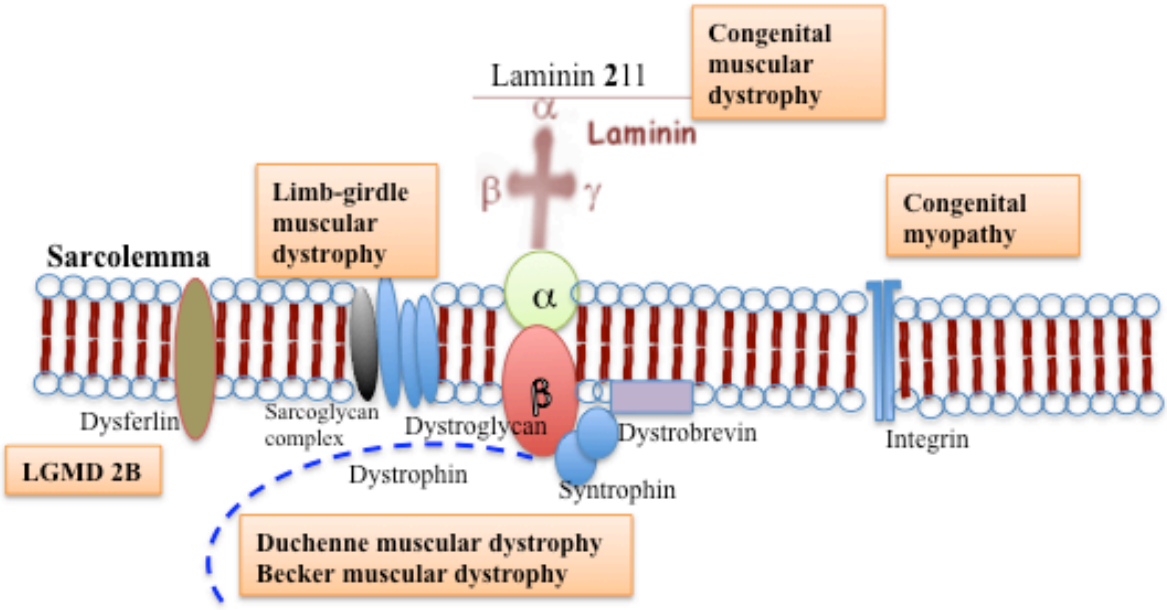


Figure 1.3: ***Proteins targeted by mutation in different dystrophies*****:** Mutations in different plasma membrane proteins, DGC proteins, ECM components and nuclear membrane proteins result in different dystrophies. The interactions between all these proteins is essential for the stability and function of the muscle fibre

Table 1.1 Types of muscular dystrophies

Disease	Genetic mutation / Gene product	Protein function	Muscles affected	Disease onset	Selected references
Duchenen/ Becker muscular dystrophy (DMD/BMD	Dystrophin	Dystrophin connects cytoskeleton to myofibre	Affects the muscles of hips, pelvic area, thighs, shoulder and heart	Early childhood between 3-5 years	Coulton et al., 1988; Dangain and Vrbova, 1984; Moser et al., 1984; Muntoni et al., 1993; Takeshima et al., 2010
Congenital muscular dystrophy (CMD)-MDC1A, MDC1B and MDC1C	Laminin $\alpha 2$	Connects myofibre to basement membrane	Nearly all voluntary muscles like arms and legs	First few months of life	Kuang et., 1998; Guicheney et al., 1997; Sunada et al., 1994; Allamand et al., 1997
Congenital myopathy	Integrin alpha7	Receptor for Laminin	Proximal and distal muscles	Duing birth or early life	Reichmann et al., 1997; Hayashi et a., 1998
Walker-Warburg syndrome (WWS)	Mutation in POMT1 gene	O-mannosyltransferase is required for enzymatic functions	Muscles	During birth	Beltran-Valero de Bernabe et al., 2002; Godfrey et al., 2007
Rigid spine muscular dystrophy (RSMD1)	Selenoprotein 1	Involved in oxidation/reduction reactions	Milder dystrophic phenotype without disruption of basal membrane	Early infacy	Dubowitz et al., 1965; Moghadaszadeh et al., 2001
Limb-girdle muscualr dystrophies (LGMD) 1A	Myotilin	Sarcomeric protein that binds to actin	Muscles of pelvic and shoulder region. Occasioanlly cardiac and respiratory muscles	Between 20-30 years	Lim et al., 1998; Hauser el al., 2000; Reilich et al., 2011
LGMD 1B	Lamin A/C	Components of nuclear lamina			Muchir et al., 2000; Politano et al., 2013
LGMD 1C	Caveolin 3	Sarcolemmal protein			Galbiati et al., 2000; Gazzerro et al., 2011
LGMD 2A	Calpain 3	Sarcomere turnover			Lim et al., 1998; Pathak et al., 2010
LGMD 2B	Dysferlin				Lim et al., 1998; Weiler et al., 1999; van der Kooi et al., 2007
LGMD 2C, 2D, 2E, 2F sarcoglycanopathies	gamma, alpha, beta or delta sarcoglycan	Sarcoglycan complex proteins			Coral-Vazquez et al., 1999; Durbeej et al., 2000; Hack et al., 1998; Piccolo et al., 1996;
LGMD 2I	FKRP (19q13.33)	Fukutin protein adds sugars to Dystroglycan			Kobayashi et al., 1998; Brockington et al., 2001
Facioscapulohumeral muscular dystrophy (FSHD)	Subtelomeric deletion of chromosome 4q	Nuclear and Sarcomeric protein	Retinal vascular system and hearing loss are prominent	Between 20-30 years	Laforet et al., 1998; Tupler and Gabellini, 2004
Oculopharyngeal muscular dystrophy (OPMD)	Increased expansion of GCG that codes for poly (A) binding protein	Poly (A) binding protein transports mRNA from nuclues	Extraocular muscles and upper facial muscles		Brais et al., 1998; Victor et al., 1962; Lim et al., 1992
Emery-Dreifuss muscular dystrophy	*STA* gene encoding emerin	Organises nuclear membrane during cell division	Cervical muscles followed by muscle wasting in upper and distal limbs	Between 10-20 years	Gruenbaum et al., 2000; Fairley et al., 1999

1.2.2 Therapies for muscular dystrophies

There is currently no cure for muscular dystrophies, although research has led to a better understanding of the underlying molecular mechanisms of the disease. However, several therapeutic strategies are being developed, which alone or in combination may help enhancing muscle repair.

1.2.2.1 Myoblast transplantation and cell therapy

One of the first reports of an attempt to counteract muscle dystrophy by cellular therapy involved the injection of mononucleated cells prepared by enzymatic dissociation of normal neonatal mouse muscle into *mdx* mice (Partridge et al. 1989). Following injection, successful fusion of donor muscle precursor cells into host (*mdx*) was demonstrated (Partridge et al. 1989). Human and murine myoblast transplantation in *dy/dy* mice restored moderate muscle integrity (Vilquin et al. 1996; Vilquin et al. 1999). These exciting studies demonstrated the potentials of cell therapy.

This lead to clinical trials in humans. The first transplantation in dystrophic patients occurred in 1990 (Law et al. 1990). Muscle-derived CD133+ cells when transplanted into boys of 7-month old DMD patients, increased ratio of capillary per muscle and a switch from slow to fast fibres was observed (Torrente et al. 2007).

However, cell therapy failed due to the poor survival and limited migratory capacity of injected cells (Cossu and Sampaolesi 2004; Fan et al. 1996; Neumeyer et al. 1998; Skuk et al. 2006). This might be overcome by using blood-born progenitor cells and as expected, it was demonstrated that bone marrow transplantation or systemic administration of bone marrow side population (SP) in *mdx* mice gives rise to dystrophin-positive cells (Ferrari et al. 1998; Gussoni et al. 1999). Moreover, studies from Camargo demonstrated that single progeny of SP cells can reconstitute hematopoietic system and regenerate muscles (Camargo et al. 2003). Another group of cells with the ability to repair muscles in dystrophies are mesoangioblasts (Galvez et al. 2006). Genetically corrected mesoangioblasts when injected into SCID/*mdx* mice intramuscularly, dystrophin production and amelioration of pathological conditions was observed (Tedesco et al. 2011). Intramuscular injection of

mesoangioblasts in *dysferlin* deficient mice, partially restored *dysferlin* and colonized dystrophic fibres (Diaz-Manera et al. 2010). Mesoangioblasts isolated from FSHD upon expansion *in vitro*, were capable of reaching the damaged site within muscles and can limit muscle damage (Morosetti et al. 2007). Embryonic stem (ES) cells have also be considered as a potential source to regenerate muscles. Mouse ES cells co-cultured with myogenic stem cells before transplantation when injected into *mdx* mice contribute to vascularized skeletal muscle formation (Bhagavati and Xu 2005). Also, *in vitro* induction of Pax7-positive satellite like cells from mouse ES cells demonstrated long-term engraftment (Chang et al. 2009). However, the use of ES cell therapy is associated with ethical issues. These issues make the use of induced pluripotent cells a more attractive possibility. The first human iPSC cells were established from skin fibroblast (Takahashi et al. 2007). Recently, protocols have been developed to derive myogenic progenitors from iPSCs (Darabi et al. 2012; Goudenege et al. 2012) Moreover, transplantation of these cells in dystrophic mice restores Dystrophin expression and myoblasts fuse efficiently with existing myofibres (Goudenege et al., 2012). However, there is a risk of tumor formation associated with the use of such approaches. iPS cell lines aberrantly express oncogenic transgenes such as c-Myc, which may cause the development of tumors and also these cells exhibit low reprogramming efficiency (Okita et al. 2007).

1.2.2.2 Other therapies for muscular dystrophies

Another ongoing effort to cure dystrophies involves replacing the mutated or missing protein by viral gene therapy. Adenoviral vectors have been used with some success to transfer mini or micro versions of dystrophin into *mdx* mice (Ragot et al. 1993). Although no immune response was observed in mice, a variable immune response was observed in humans (Gilchrist et al. 2002). To prevent immune responses, "gutted vectors" were developed that do not express viral proteins (DelloRusso et al. 2002). Adenovirus containing a minigene of utrophin, a homolog of Dystrophin was also used and shown to improve the muscle pathology in *Dystrophin/utrophin* double knockout (dko) mice (Gilbert et al. 1998; Wakefield et al. 2000). Adenoviral-mediated gene transfer and restoration of the DGC complex was also demonstrated in

LGMD 2E, 2C and 2D mouse models deficient for β, γ and α-Sarcoglycan deficiency (Allamand et al. 2000; Durbeej et al. 2000).

Pharmacological approaches include using anti-inflammatory molecules. Corticosteroids have been used and found to increase the life span of DMD patients, although side effects such as weight loss and osteoporosis have been reported (Campbell and Jacob 2003; Manzur et al. 2004). Another approach consists in blocking Myostatin, by injecting neutralizing antibodies. This results in a delay in muscle wasting (Bogdanovich et al. 2002). Similarly, overexpression of IGF-1 or Integrin alpha7 can reduce or prevent muscle damage in *dy(W(-)/(-))* mice, a model of MDC1A (Barton et al. 2002; Doe et al. 2011). In addition methods such as exon-skipping have been proposed for discrete mutations. Single administration of AAV vector expressing antisense sequences removed the mutated exon on dystrophic mRNA of *mdx* mice resulting in the production of functional dystrophin (Goyenvalle et al. 2004). some of the mutations in dystrophic phenotype are premature stop codons. It has been demonstrated that administration of gentamycin, an aminoglycoside antibiotic provides protection against muscle injury in *mdx* mice (Barton-Davis et al. 1999). Trials were also carried out to up-regulate or stabilize the utrophin protein in dystrophic muscles, which resulted in reduced dystrophic phenotype (Chakkalakal et al. 2008; J. Tinsley et al. 1998; J. M. Tinsley et al. 2011).

Overall, no single treatment will be sufficient to reverse the progression of the muscle diseases but a combination of treatments may provide an answer to muscular dystrophies.

1.3 Adult muscle repair

Muscle can be damaged by intense exercise, mechanical load, stress or due to innate genetic defects such as muscular dystrophies. Muscle repair process include different phases such as degeneration, inflammation, regeneration and fibrosis (see Fig 1.4).

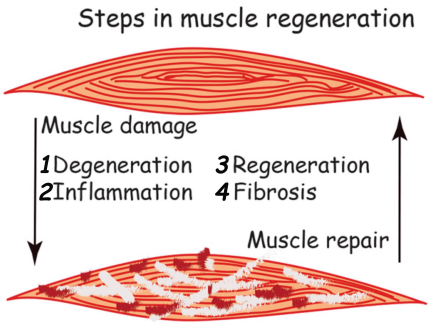


Figure 1.4: ***Steps involved in muscle regeneration:*** Skeletal muscle regeneration involves 4 distinct phases of repair. Degeneration and inflammation are followed by regeneration and ends with fibrosis resulting in a normal intact muscle.

1.3.1 Muscle degeneration occurs as an immediate response to injury

Upon muscle injury, the integrity of the sarcolemma and sarcomere of the myofibre is disrupted and this can be evidenced by the uptake of Evan S-B blue (Brussee et al. 1997; Straub et al. 1998). Loss of sarcolemmal integrity leads to calcium influx and activation of calcium-dependent proteases, such as calpains (Alderton and Steinhardt 2000; Armstrong 1990a). Calpains cleave myofibrils and cytoskeletal proteins, and this leads to necrotic myofibre auto digestion (Belcastro et al. 1998; Ebisui et al. 1995; Mbebi et al. 1999). Myofibres become denervated and myofibre-containing neuromuscular junction gets separated from the myofibre (Rantanen et al. 1995). Calcium influx also disturbs calcium homeostasis, which in turn activates the complement system (Carpenter and Karpati 1989; Orimo et al. 1991). The activation of the complement system induces the chemotactic recruitment of leukocytes, thus initiating the next phase, inflammation.

During degeneration, gaps formed due to the damaged myofibres are filled with haematoma. Haematoma is replaced by the deposition of the extracellular matrix components, such as type III and type I collagen (Kaariainen et al. 2000; Y. Li and Huard 2002). The fibrotic tissue initially provides a scaffold for regenerating myofibres but at later stages inhibits the regenerative growth. TGF-β1 has been shown to play a major role in depositing ECM components and is well documented in Duchenne muscular dystrophies in humans (Amemiya et al. 2000; Yamazaki et al. 1994). IGF-1 has been shown to reduce fibrosis by down-regulating pro-inflammatory cytokines (Husmann et al. 1996; Pelosi et al. 2007). Thus, interaction between IGF

and the inflammatory response is important in determining the progression of muscle injury and repair.

1.3.2 Injury of the muscle initiates an inflammatory response

Inflammation involves the interaction between the immune system and the damaged areas of muscle (see Fig 1.5). Initially, neutrophils infiltrate the damaged site followed by macrophages derived from blood monocytes (Rappolee and Werb 1992; Tidball 1995). Fielding and researchers observed that a damaged muscle is associated with a significant increase in neutrophil number as early as 1-6 hrs after myotoxin or exercise-induced muscle damage (Fielding et al. 1993). Macrophages predominate after 24 hrs post-injury (Lapointe et al. 2002). Originally considered as scavengers involved in the removal of debris, macrophages are now recognized to play a major role in promoting muscle regeneration (Lescaudron et al. 1999).

1.3.2.1 Macrophages are essential for muscle repair

As mentioned above, following the onset of neutrophil invasion, two distinct populations of macrophages have been shown, based on their cell surface markers, to reach the injury site in muscles. The first population to appear are "inflammatory" or "M1" macrophages. They are characterized by the expression of $CD68^{+}$ and reach the highest levels 24 hrs after the injury and then rapidly decline at the end of the phagocytic stage (St Pierre and Tidball 1994). These inflammatory macrophages are responsible for the phagocytosis of debris. Th1-induced immune response results in the secretion of pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNF-α) and Interleukine-1 (IL-1), which act on inflammatory M1 macrophages (Tidball and Villalta 2010). Inflammatory macrophages possess a cytotoxic activity mediated by nitric oxide-dependent and superoxide-independent mechanisms and lyse muscle cells *in vitro* and *in vivo* (Nguyen and Tidball 2003). The cytotoxic activity is enhanced by the presence of neutrophils (Nguyen and Tidball 2003). Consequently, muscle membrane lysis is observed in *mdx* mice and depletion of macrophages decreases muscle lysis *in vivo* (Wehling et al. 2001). A second population, called "anti-inflammatory" or "M2" macrophages, that are CD163+/CD206+ appear soon

after phagocytosis (McLennan 1993). This CD163+/CD206+ M2 population of macrophages is apparently derived from M1 inflammatory macrophages (Tidball and Villalta 2010). Th2 induces the accumulation of M2 macrophages by releasing anti-inflammatory cytokines such as Interleukin-10 (IL-10) and Interleukin-4 (IL-4) (Tidball and Villalta 2010). Several studies have confirmed the role of macrophages in muscle remodelling and repair. For instance, muscle fragment transplanted into monocyte and macrophage-depleted mice do not regenerate muscle efficiently, suggesting a vital role for macrophages in muscle regeneration (Lescaudron et al. 1999).

1.3.2.2 Macrophages promote satellite cell proliferation and differentiation

In vitro analyses showed that conditioned media from macrophage cultures increase the proliferative state of myoblasts by increasing the number of MyoD-positive cells (Cantini and Carraro 1995; Cantini et al. 2002; Merly et al. 1999). It has been also shown that *in vitro* cultures of human myogenic progenitor cells (MPC) attract macrophages, and MPC proliferation is enhanced by cell-cell contact, suggesting a role for macrophages in satellite cell proliferation (Chazaud et al. 2003). Both *in vitro* and *in vivo* data indicate that conditioned media from murine macrophages can increase the proliferative rate of rat and human myoblasts (Cantini et al. 2002). Macrophage co-culture increases proliferation and differentiation of myogenic cells and promote muscle regeneration (Cantini et al., 1995; Lescaudron et al., 1999). Consistent with this, the specific depletion of M2 macrophages results in a reduced number of centrally located nuclei due to an impaired myoblast differentiation (Tidball and Wehling-Henricks 2007). M1 macrophages release the enzyme iNOS that is required to lyse muscle cells in a NO-dependent manner and M2 releases the enzyme Arginase required for L-arginine metabolism (S. Gordon 2003). Both enzymes compete for the same substrate, Arginine. M2 macrophages thus reduce the damage caused by M1 macrophages, and favor regeneration (Villalta et al. 2009). As mentioned above, M2 macrophages also express elevated levels of the cytokines IL-4 and IL-10. IL-10 is known to reduce M1 macrophage-mediated muscle damage (S. Gordon 2003; S. Gordon and Taylor 2005) while IL-4 promotes satellite cell

activation and proliferation (Horsley et al. 2003). In addition, ablation of IFN-γ signaling in *mdx* mice reduces inflammation during the early phase of regeneration and increases activation of M2 macrophages (Villalta et al. 2011a). Transplantation studies also suggest that co-injecting MPC isolated from Tg:CAG-GFP mice (GFP transgene is expressed under the control of promoter, chicken β-actin with cytomegalovirus enhancer) with macrophages enhances survival, expansion and migration of MPC both *in vitro* and *in vivo (Lesault et al. 2012).* All together, these data suggest that macrophages are important for efficient muscle regeneration, as they directly act on satellite cell function.

1.3.2.3 Shift in macrophage phenotype differ in acute and chronic injury

A shift in the macrophage phenotype from M1 to M2 is observed during muscle regeneration, suggesting that macrophages interact with muscle cells and are involved in muscle regeneration (St Pierre and Tidball 1994). Interestingly, the shift from M1 to M2 macrophage populations differs in acute and chronic injury. In acute muscle injury (for instance, upon cardiotoxin injury), the M1 population predominates during the initial phase and is followed by an influx of M2 macrophages (Villalta et al. 2009). In contrast, in the *mdx* model of chronic muscle injury, both M1 and M2 macrophage populations appear simultaneously at 4 weeks of age (Villalta et al. 2009). Thus, shifts in macrophage population is more complex in muscular dystrophies. Collectively, these data suggest that modulating the different macrophage population may influence the course and severity of the dystrophic and injured muscles.

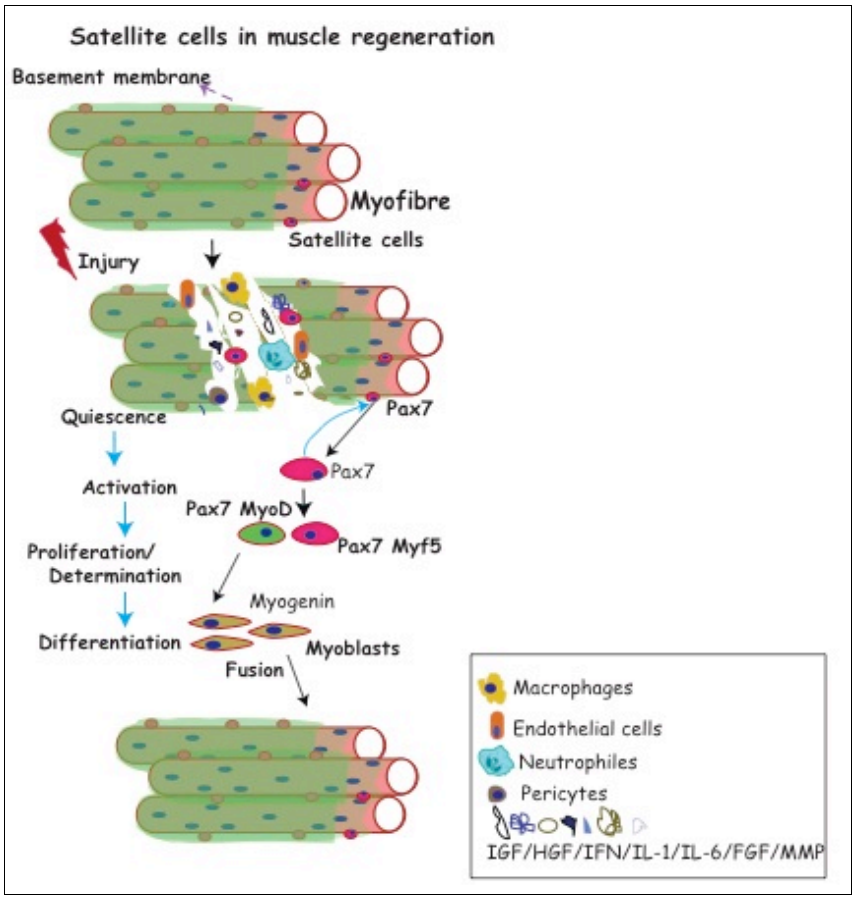


Figure 1.5: ***Skeletal muscle regeneration:*** Satellite cells are required for skeletal muscle regeneration. Upon muscle injury, quiescent Pax7-positive satellite cells enter the cell cycle and expand following the expression of the myogenic markers Myf5 and MyoD. MyoD cells then enter differentiation by expressing Myogenin, allowing the fusion of myoblasts to repair or form new myofibres/muscle. Other non-muscle cells such as neutrophils, macrophages, endothelial cells and pericytes support the regeneration process along with the growth factors and cytokines shown in the picture.

1.3.3 Muscle regeneration is carried out by satellite cells

Adult muscles possess the remarkable ability to regenerate after injury. Functional muscles can be repaired in less than 3 weeks after severe damage (Rosenblatt 1992). It has been shown that skeletal muscles continue to regenerate even after repeated injuries suggesting the existence of stem cells (Luz et al. 2002). Indeed, the capacity to regenerate depends on the activity and contribution of muscle-specific stem cells, called satellite cells (Shi and Garry 2006b). Satellite cells have been identified based on thymidine studies in regenerating muscles (Grounds and Yablonka-Reuveni 1993). Studies in rat muscles showed that satellite cells provide myonuclei to the growing

myofibres (Moss and Leblond 1971). Direct evidence that satellite cells can contribute to growing fibres was demonstrated on isolated myofibres (Bischoff 1986). Transplantation of such single myofibres into muscles showed that indeed satellite cells act as myogenic precursor cells *in vivo* (Collins et al. 2005). Some genetic studies have brought about the definitive proof that muscle regeneration requires satellite cells. Indeed, the depletion of Pax7-positive satellite cells from muscles results in impaired muscle regeneration (Sambasivan et al. 2011). The same approach was used to deplete satellite cells from adult muscles by crossing mice carrying $R26R^{DTA}$ with a $Pax7^{CRE}$ allele. With $Pax7^{CreER}$: $R26R^{DTA}$ or $Pax7^{iCreERT2}$: $R26R^{DTA/+}$ mice almost 90% of satellite cells were ablated, and cardiotoxin injection in such mice resulted in the complete failure of muscle regeneration (Lepper et al. 2011; McCarthy et al. 2011; Murphy et al. 2011). Moreover, grafting Pax7-nGFP satellite cells into cardiotoxin-injured $Pax7^{DTR/+}$ muscles rescued the muscle damage, indicating that satellite cells are required and sufficient for muscle regeneration (Sambasivan et al., 2011). Thus, in the absence of satellite cells, muscles fail to regenerate and other non-muscle cells with regenerative capacity (see 1.3.4) cannot regenerate muscles (Sambasivan et al., 2011).

1.3.3.1 Pax7 function in satellite cell-mediated muscle regeneration

Pax7 requirement in satellite cell function was initially demonstrated in *in vitro* studies (McKinnell et al. 2008). Studies using siRNA-mediated knockdown of Pax7 in satellite cell-derived myoblasts showed that loss of *Pax7* results in the loss of *Myf5* expression and cell growth arrest (McKinnell et al. 2008). Constitutive expression of Pax7 in satellite cells *in vitro* increases their proliferative rate and is required for myogenic cell differentiation to proceed (Collins et al. 2009). Chip-Seq analysis showed that Pax7 regulates distinct genes involved in myoblasts proliferation and inhibition of differentiation (McKinnell et al. 2008). Moreover, Pax7 inhibits myogenic differentiation mainly by down-regulating MyoD and thus Myogenin expression (Olguin and Olwin 2004). Pax7 mutant mice ($Pax7^{-/-}$) exhibit reduced growth, muscle wasting and complete absence of functional satellite cells (S. Kuang et al. 2006). Acute injury in $Pax7^{-/-}$ mice results in a deficit in muscle regeneration,

despite the presence of Pax3 and MyoD-positive interstitial cells. This suggests that Pax7 is required for muscle regeneration (S. Kuang et al. 2006). Supporting this, another recent study shows that the conditional knockout of Pax7 in satellite cells causes an impairment in their self-renewing capacity and causes their precocious differentiation (von Maltzahn et al. 2013). It has been also shown that inactivation of Pax7 in Pax7-expressing cells and in Myf5-expressing satellite cells after muscle damage leads to impaired regeneration (Gunther et al. 2013). This indicates that Pax7 is required for satellite cell-mediated muscle regeneration and Myf5 expression is not sufficient to drive satellite cell activation and muscle repair (Gunther et al. 2013). In addition, differential requirement for Pax7 during post-natal muscle repair was demonstrated. Analysis of conditional Pax7 mutant mice showed that satellite cell dependency on Pax7 was critical prior to P21, but that Pax7 was dispensable for muscle regeneration after juvenile period (Lepper et al. 2009)

Pax3, the paralogue of Pax7, is also expressed in a subset of adult satellite cells (Relaix et al. 2006). To exclude the possibility that Pax3 can compensate for Pax7, compound Pax3;Pax7 mutant mice were injured but muscle regeneration was not impaired (Lepper et al. 2009).

Thus, all these studies demonstrate an essential requirement for Pax7 in satellite cells for skeletal muscle regeneration.

1.3.3.2 Satellite cells recapitulate the embryonic myogenic programme

Many similarities exist between the myogenic programme carried out by muscle progenitor cells in somites and the adult myogenic programme carried out by satellite cells during post-natal development or muscle repair caused by injury (see Fig 1.6). In the embryo, myogenic progenitor cells express Pax3, whereas adult quiescent satellite cells express Pax7 with a minor population expressing Pax3 (Relaix et al. 2006). Once activated, satellite cells up regulate the basic helix-loop-helix (bHLH) transcription factor Myf5 as in embryonic development (R. N. Cooper et al. 1999; Cornelison and Wold 1997; Cornelison et al. 2000; P. S. Zammit et al. 2002). The second myogenic factor MyoD is expressed within 12 h of satellite cell activation, followed by the myogenic differentiation factors Myogenin and MRF4 (Cornelison and Wold 1997;

Cornelison et al. 2000; Ferrari et al. 1998; Grounds et al. 1992; Maley et al. 1994; Megeney et al. 1996; Yablonka-Reuveni and Rivera 1994). The sequential expression of these myogenic factors is reminiscent of the sequence of events taking place in the embryo (R. N. Cooper et al. 1999; Cornelison and Wold 1997; Cornelison et al. 2000; P. S. Zammit et al. 2002). Myogenin-expressing myoblasts then fuse to one another to form nascent myotubes. Additional myoblasts are incorporated into newly-formed myotubes to form the matured myofibres or repair damaged myofibres.

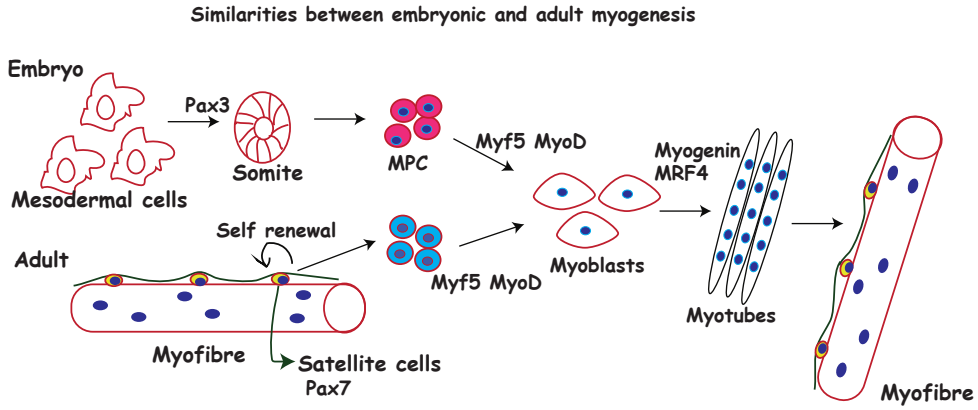


Figure 1.6: ***Similarities between embryonic and adult myogenesis:*** Muscle progenitor cells in the embryo and adult satellite cells follow a similar programme with progenitor cells expressing Pax genes (Pax3 and Pax7). Specification involves the activation of Myf5 and MyoD, while the differentiation programme is initiated following Myogenin and MRF4 expression.

1.3.3.3 Quiescent satellite cells

1.3.3.3.1 Markers expressed during quiescence

Quiescent satellite cells (G0) express the transcription factor Pax7, which is required for satellite cell specification and survival is expressed both in mouse and human (Bosnakovski et al. 2008; Seale et al. 2000; P. S. Zammit et al. 2006b) whereas Pax3 is expressed only in satellite cells of specific muscle groups such as the diaphragm in mouse (Relaix et al. 2006). Pax7 expression is maintained during the progression from quiescence to activation and proliferation (Zammit et al., 2006). Most quiescent satellite cells also express membrane-bound proteins such as M-cadherin (Irintchev et al. 1994), the caveolae-forming protein Caveolin-1 (Gnocchi et al. 2009), Cxcr4

(Ratajczak et al. 2003), Sca-1, Integrin α7 , Integrin β1 (Gnocchi et al. 2009; S. Kuang et al. 2007) are expressed in mouse satellite cells.

In addition, the tyrosine kinase receptor c-Met (R. E. Allen et al. 1995; Cornelison and Wold 1997) and CD34, a sialomucin surface receptor (Beauchamp et al. 2000) (see Table 1.2). These markers are often used in combinations because some proteins are not exclusively expressed by satellite cells. For instance, CD34 is also expressed by non-muscle, hematopoietic cells (Krause et al. 1994).

1.3.3.3.2 Signaling pathways that maintain quiescence

Quiescent satellite cells express the Notch receptor, which binds the Notch ligand, Delta and activates the Notch signaling pathway (Bjornson et al. 2012; Mourikis et al. 2012). Several studies suggest that satellite cell quiescence is maintained through the activation of the Notch pathway (Brack et al. 2008; Brohl et al. 2012). For example, Notch signaling prevents precocious differentiation of satellite cells and in *Notch* mutant mice, satellite cell quiescence but not differentiation is affected, suggesting a role of Notch in maintaining quiescence (Brack et al. 2008; Brohl et al. 2012) (see Fig 1.7). Further, deletion of one of the mediator of Notch, *Rbpj* in Pax7-expressing satellite cells results in spontaneous differentiation and loss of quiescence in satellite cells (Bjornson et al. 2012; Mourikis et al. 2012). In double MyoD:*Rbpj* mutant mice, satellite cells fail to occupy their position under the basal lamina, which suggests that Notch may also be involved in promoting cell adhesion components (Brohl et al., 2012). Likewise, Notch mediator, *Hesr1/3* knockout mice fail to generate undifferentiated quiescent satellite cells (Fukada et al. 2011). Interestingly, a transmembrane protein Megf10 has been identified and is proposed to act upstream of Notch in the regulation of satellite cell quiescence. Indeed, inhibition of Megf10 prevents Notch expression and reduces satellite cell self-renewal, indicating that Notch acts downstream of Megf10 (Holterman et al. 2007). In contrast, following asymmetric divison, Numb inhibits Notch in one of the daughter cells and favours its subsequent differentiation (George et al. 2013). Numb deficient-satellite cells express high levels of Myostatin and p21, suggesting a role in driving the initial steps of satellite cell activation and proliferation (George et al. 2013).

Several growth factors such as insulin growth factor (IGF), fibroblast growth factor (FGF) and platelet derived growth factor (PDGF) impact also on satellite activation and proliferation (deLapeyriere et al. 1993; Dusterhoft and Pette 1999; Floss et al. 1997; Kastner et al. 2000). Satellite cells express the tyrosine kinase receptors of these growth factors. However, Sprouty, an intracellular inhibitor of receptor tyrosine kinase-mediated signalling is expressed and prevents satellite cell activation during quiescence (Fukada et al. 2007). The quiescence state is also atained by inhibiting the cell cycle. *In vivo* studies showed that myostatin, a TGF-β family member induces p21, a cyclin dependent kinase inhibitor to supress satellite cell activation (McCroskery et al. 2003). Consistent with this, *in vitro* and *in vivo* analysis showed an increased number of satellite cells in Myostatin-deficient mice, suggesting a role for Myostatin in maintaining satellite cell quiescence (McCroskery et al. 2003). Thus, satellite cell quiescence is maintained at a molecular level until they receive signals for their activation.

1.3.3.4 Activation of satellite cells in response to injury, exercise and disease

1.3.3.4.1 Markers expressed during satellite cell activation

Satellite cell activation is the process that leads to satellite cell exit from quiescence. Quiescent satellite cells have little cytoplasm, whereas activated satellite cells possess an expanded cytoplasm (J. Anderson and Pilipowicz 2002). Once activated, satellite cells maintain Pax7, caveolin-1 and integrin α7 (Gnocchi et al. 2009) and begin expressing the myogenic regulatory factors Myf5 and MyoD within 12 hours of activation (R. N. Cooper et al. 1999; Yablonka-Reuveni and Rivera 1994) (see Fig 1.7).

1.3.3.4.2 Activation of satellite cells is mediated by different signals

(a) Extracellular signals: Activation of satellite cells occurs when neuronal nitric oxide synthase (NOS) is released from the basal lamina and causes the production nitric oxide (NO). Neuronal nitric oxide synthase (nNOS) is a dystrophin-binding protein localized to the sarcolemma (Brenman et al. 1995). NO released activates matrix metalloproteinases (MMPs), which in turn mediate hepatocyte Growth Factor

(HGF) release from the ECM (Tatsumi et al. 2001; Tatsumi et al. 2002; Yamada et al. 2006). HGF is the first extrinsic signal that activates satellite cells by binding to the c-Met receptor expressed exclusively at the surface of satellite cells (R. E. Allen et al. 1995; Cornelison and Wold 1997; Wozniak et al. 2003). Blocking of HGF with anti-HGF antibodies inhibits satellite cell activity. In contrast, injecting HGF in injured muscle increases satellite cell number and inhibits their differentiation (Tatsumi et al. 1998). Another possible growth factor involved in satellite cell activation is FGF-2. FGF-2 is important for satellite cell activity and is likely to act by binding to the heparan sulfate proteoglycans (HSPGs) syndecan-3 and syndecan-4 expressed at the surface of satellite cells (Cornelison et al. 2001a; Lefaucheur and Sebille 1995a; Tanaka et al. 2009). It has been shown that FGF-2 is released from injured muscles and can enhance satellite cell number (Yablonka-Reuveni and Rivera 1994). Both HGF and FGF-2 transduce their signal through the mitogen-activated kinase (MAPK) pathway (Jones et al. 2005). Later Syndecan-3 and Syndecan-4 signal through different tyrosine kinase receptors to activate satellite cells (Cornelison et al. 2001b). Syndecan-3 and Sydecan-4 are expressed at the surface of satellite cells prior to any detectable expression of MyoD and remain present during satellite cell proliferation (Cornelison et al. 2001b).

(b) Intracellular signals : MicroRNAs (miRNAs) are a class of small non-coding RNAs that inhibit gene expression by targeting the 3' UTRs of target mRNAs (Bartel 2004). Muscle specific miRNA-206 is required for efficient muscle regeneration and the loss of miRNA-206 results in impaired differentiation of satellite cells (N. Liu et al. 2012). Interestingly, it has been shown that Myf5 transcripts present in quiescent satellite cells are regulated by microRNA-31. Activation of satellite cells causes the release of Myf5 transcripts from the messenger ribonucleo-protein (mRNA) granules by inhibiting the suppression of post-transcriptional regulation of Myf5 by microRNA-31 (Crist et al. 2012).

1.3.3.5 Satellite cell proliferation

1.3.3.5.1 Markers expressed during satellite cell proliferation

Activated proliferating satellite cells retain the expression of Pax7, caveolin-1, integrin α7, CD34, Myf5 and MyoD. Importantly, MyoD expression levels are higher at G1 phase in activated and proliferating myoblasts (Cooper et al., 1999). MyoD drives proliferation by controlling entry into the cell cycle and MyoD mutant mice show decreased proliferation with impaired regeneration (Megeney et al. 1996). A study by Olguin et al. suggests that the ratio Pax7/MyoD within activated satellite cells determines whether the cell will progress towards differentiation or will proliferate or self-renew (Olguin et al. 2007). Myf5 and MyoD can compensate for each other during embryogenesis (Rudnicki et al. 1993), but cannot compensate for each other efficiently in adult muscles. Indeed, in the *mdx* background, loss of *Myf5* results in an impaired muscle regeneration due to a defect in satellite cell proliferation, whereas loss of *MyoD* causes an impairment in muscle regeneration due to a failure in satellite cell differentiation replaced by a preferred self-renewal fate (Gayraud-Morel et al. 2007; Megeney et al. 1996; Ustanina et al. 2007). Consistent with this, genetic disruption of Six1, which regulates MyoD expression in the embryo (Y. Liu et al. 2013) leads also to an impaired muscle regeneration as a result of increased satellite cell self-renewal (Le Grand et al. 2012).

1.3.3.5.2 Signals involved in satellite cell proliferation

Proliferation of satellite cells is promoted by IGF-I and IGF-II during muscle regeneration (Machida and Booth 2004). IGF binds to the IGF receptor (IGFR) and modulates the expression of the myogenic regulatory factors (MRFs), which in turn promote proliferation in *vitro* (R. E. Allen and Boxhorn 1989; Coleman et al. 1995) (see Fig 1.7). Binding of IGF to its receptor leads to the activation of phosphatidylinositol 3-kinase (PI3K), which activates the AKT pathway and confers anti-apoptotic effects (Y. H. Song et al. 2005). Conversely, mutations in IGFR, as in IGF1R-/- mice, prevents IGF-I-induced hypertrophy leading to a dystrophic phenotype (J. P. Liu et al. 1993). Likewise, in transgenic mdx mice expressing mIGF-

1, *mdx*:mIGF$^{+/+}$ the up-regulation of the AKT pathway resulted in hypertrophy. This suggests an increase in survival signals promoting regeneration (Barton et al. 2002). Another signal that activates the PI3K/AKT pathway and promotes myoblast proliferation *in vitro* is Sonic hedgehog (Elia et al. 2007). In contrast, TGF-β signaling suppresses myogenic cell activation and proliferation *in vitro* (Olson et al. 1986). Wnt proteins are also key regulators of satellite cell proliferation. Wnt signalling promotes the translocation of β-catenin to the nucleus, which in turn promotes satellite cell proliferation (Otto et al. 2008).

In addition to growth factors, cell surface proteins such as CD34 have been shown to be required for satellite cell proliferation (Alfaro et al. 2011). Impaired satellite cell proliferation was reported in CD34 knockout mice subjected to both acute and chronic injury (Alfaro et al. 2011). Also, Sphingosine-1 phosphate signalling is involved in the induction of proliferation of satellite cells (Nagata et al. 2006a).

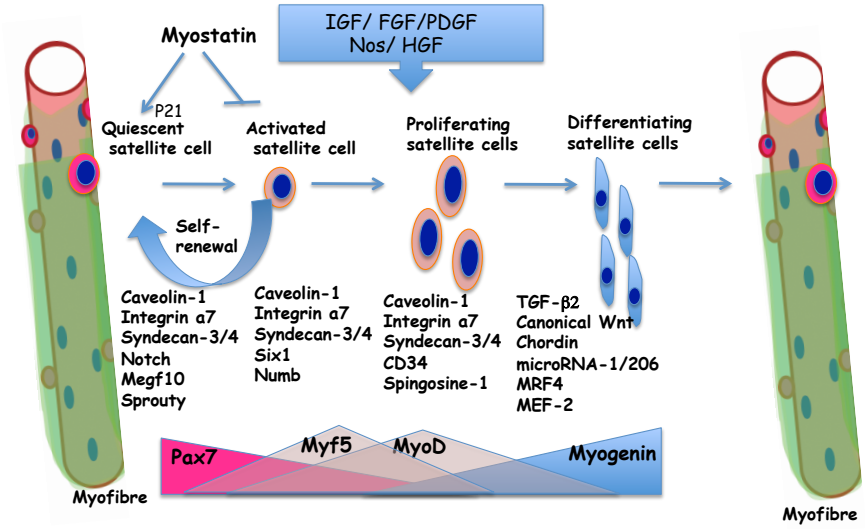


Figure 1.7: ***Schematic of satellite cell myogenesis and expression of different markers:*** At quiescence satellite cells express Pax7. Once activated they express Myf5 followed by MyoD and Myogenin. Different growth factors are involved during activation, proliferation and differentiation which are illustrated in the schematic. Satellite cells also express typical markers at each stage.

1.3.3.6 Satellite cell self-renewal

Satellite cells have self-renewing capacity for the sustainability of muscle homeostasis and regeneration. Satellite cells like other stem cells divide symmetrically or asymmetrically. Two mechanisms were proposed for the self-renewal process, a "stochastic" model and an "asymmetric model" (Dhawan and Rando 2005). In the stochastic model, satellite cells divide symmetrically and proliferate. After a period of proliferation, one cell randomly enters quiescence and the other cell differentiates. In the asymmetric model, asymmetric cell division yields one daughter cell fated for self-renewal and another daughter cell, which undergoes symmetric cell divisions to generate a large number of myoblasts.

The evidence for the self-renewing capability of satellite cells came from a study using single myofibre associated with 7-22 satellite cells transplanted into irradiated *mdx* muscles (Collins and Partridge 2005). The donor fibre gave rise to hundreds of new myofibres with a 10-fold expansion in the number of self-renewing satellite cells (Collins and Partridge 2005). Moreover, satellite cells freshly isolated by FACS (Integrin α7-positive and CD34-positive cells) give rise to a progeny that not only regenerates muscles but also occupies the satellite cell niche when injected into scid-*mdx* mice, suggesting the existence of asymmetric division and self-renewing capability (Sacco et al. 2008). Kuang et al. demonstrated for that satellite cells divide both symmetrically and asymmetrically using Myf5cre;R26R-loxP-stop-loxP-YFP mice (S. Kuang et al. 2007). The decision of satellite cells to undergo symmetric or asymmetric division depends on the orientation of the mitotic spindles. Asymmetric cell division occurs when the mitotic spindles are perpendicular to the myofibre axis with one daughter cell (Pax7+/Myf5-) in contact with the basal lamina and the other daughter cell (Pax7+/Myf5+) usually in contact with the myofibre plasma membrane. The division is also known as apical (myofibre side) and basal (basal lamina side) division. Asymmetric cell division correlates with an asymmetric distribution of Notch signaling components: the notch ligand delta-1 is expressed at higher levels in Pax7+/Myf5+ satellite cells, whereas Notch receptor-3 is expressed at higher levels in Pax7+/Myf5- satellite cells. BrdU-label-retaining studies indicate that Numb is also

distributed asymmetrically and is associated with label-retaining cells, suggesting that inhibition of Notch is important for satellite cell self-renewal (Shinin et al. 2006). Numb inhibits Notch signaling by inhibiting ubiquitination of the Notch intracellular domain (NCID) (Artavanis-Tsakonas et al. 1999). Although, controversial reports suggest that numb expression is detected in daughter cells during asymmetric division (Conboy and Rando 2002).

Recently, another mechanism that regulate asymmetric cell division was reported. Asymmetric localization of the PAR complex (composed of partitioning defective 3, 6 and Protein Kinase C), proteins activates p38α/β MAPK and MyoD in one daughter cell while the other daughter cell enters quiescence in the absence of p38α/β MAPK (Troy et al. 2012). Interestingly, p38α/β regulates also Pax7 via polycomb repressive complex 2 (PRC2) and influences satellite cell differentiation (Palacios et al. 2010). Conditional knockout studies of p38α also suggested that p38α restricts satellite cells and favors timely myoblast differentiation (Brien et al. 2013). Intriguingly, p38α kinase is activated by TNF-α, thus establishing a link between inflammation and satellite cells during muscle regeneration (Palacios et al. 2010). In agreement with this, antibodies that block TNF-α, stimulate satellite cell proliferation.

Satellite cells may favor symmetric cell division during pathological conditions. Notably, it was observed that the percentage of Pax7/Myf5^{-} cells increases from ~10% in physiological conditions up to ~30% in injured muscles (S. Kuang et al. 2007). DNA segregation during satellite cell division is a non-random event and the frequency of segregation of parental DNA into the daughter cells ranges from ~7% to ~50%, depending on the experimental conditions (Shinin et al. 2006). However, the daughter cell inheriting the parental templates retains self-renewing capabilities, whereas the daughter cell inheriting the new template enters differentiation. During this process, MyoD is down-regulated in self-renewing, but not in differentiating cells (Day et al. 2007; Nagata et al. 2006b). Self-renewal of satellite cells is also dependent on Wnt signaling. Wnt7a promotes the symmetric expansion of Pax7-positive Myf5-negative satellite cells through the activation of the non-canonical Wnt pathway (Le Grand et al. 2009). Recently, it has been suggested that Wnt7a signaling is promoted

when the ECM glycoprotein, fibronectin, binds to Syndecan-4 on satellite cells, suggesting that non-canonical Wnt signaling is involved in replenishing the satellite cell pool (Bentzinger et al. 2013b).

1.3.3.7 Satellite cell differentiation

1.3.3.7.1 Markers expressed during satellite cell differentiation

MyoD and Myf5 expression levels remain high at the G1 phase in activated and proliferating satellite cells and determine the myogenic lineage, whereas Myogenin expression is essential for satellite cell differentiation (P. Zammit and Beauchamp 2001; P. S. Zammit et al. 2002). (Olguin et al. 2007). It has been shown that in muscle injury models, *MyoD* and *Myogenin* transcripts are observed within 6 hours post-injury and peak at 24 hours and 48 hours (Grounds et al. 1992). In Myogenin knockout mice, several other differentiation markers such as MRF4 and myosin heavy chain are reduced resulting in undifferentiated myoblasts (Hasty et al. 1993). This suggests that Myogenin drives myoblasts to terminal differentiation. Myogenin expression correlates with the down-regulation of Pax7 expression during differentiation. *Pax7* appears to be the direct target for microRNA-1 and microRNA-206, which are up-regulated during satellite cell differentiation and restrict satellite cell proliferation, favoring differentiation *in vivo* (J. F. Chen et al. 2010). *In vivo* studies using acute and chronic muscle injury models also showed that MyoD-dependent satellite cell differentiation is regulated by MASTR and MRTF-A, myocardin family members (Mokalled et al. 2012). Myogenic regulatory factors interact with serum response factor (SRF), a ubiquitous transcription factor that binds to CArG box DNA element $CC(A/T)_6GG$ to regulate muscle gene expression (Shore and Sharrocks 1995). MASTR and MRTF-A are cofactors of SRF which do not have DNA-binding activity but physically bind to SRF (D. Wang et al. 2001). Deletion of MRTF-A results in aberrant differentiation and excessive proliferation leading to impaired regeneration (Mokalled et al. 2012). Similarly, Myocytes enhancer factor 2 (MEF2), which plays a role during embryonic myogenesis is also expressed in differentiating satellite cells. Recently, it has been shown that conditional deletion of

Mef2 a, b, c and *d* genes resulted in impaired regeneration upon cardiotoxin injury (N. Liu et al. 2014). However, deletion of a single gene at a time did not affect muscle regeneration, indicating the existence of compensatory mechanisms amongst members of the *Mef* gene family (N. Liu et al. 2014). Finally, Caveolin-1, which is expressed in all satellite cells, is down regulated in some of the Myogenin-positive cells (Gnocchi et al. 2009).

1.3.3.7.2 Signals involved in satellite cell differentiation

In vitro and *in vivo* myoblast differentiation correlates with an up-regulation of the canonical Wnt signaling, and inhibition of Wnt interferes with muscle differentiation (Brack et al. 2008) (see Fig 1.7). Wnt signaling also antagonizes Notch signaling and favors differentiation (Brack et al. 2008). Cell cycle exit and terminal differentiation is also regulated by chordin up-regulation, which inhibits BMP signaling in myogenin-positive cells (Friedrichs et al. 2011). Finally, the onset of the expression of myosin heavy chains indicates the completion of the differentiation programme and the fusion of differentiated myoblasts to the damaged fibres or to form new myofibres. TGF-β2 guides the fusion of myoblasts to form myotubes (McLennan and Koishi 2002). Once they reach terminal differentiation, satellite cell-derived myoblasts withdraw from the cell cycle and form small centrally located nuclei (CLN) (Goetsch et al. 2003; P. S. Zammit et al. 2002). Fusion and further growth of the differentiated myoblasts results in restoration of the muscle architecture with nuclei moving to periphery of the myofibre (Snow 1977).

1.3.3.8 Satellite cells are heterogeneous in nature

Increasing evidence show that satellite cells are a heterogeneous population. They express different markers, exhibit different signatures and different functions at different times. Classical experiments such as single muscle fibre isolation and the culture of primary satellite cells identified two distinct populations of cells: differentiated myoblasts and quiescent satellite cells that were Pax7-positive and MyoD-negative or Myogenin-negative (Olguin and Olwin 2004). The Pax7-positive cells can give rise to Sca-1^{+}/$Myf5^{+}$ cells that enter the myogenic programme

(Chapman et al. 2013). Indeed, satellite cells are constituted at 90% of Pax7-positive cells, which are committed myogenic cells and the remaining 10% of non-committed cells (Kuang et al., 2007). However, levels of Pax7 expression varies within the $Pax7^{+}$ satellite cell population. Studies using Tg:Pax7-nGFP mice have identified two subpopulations of Pax7-expressing satellite cells, a Pax7-$nGFP^{Hi}$ population with higher levels of Pax7 expression and a Pax7-$nGFP^{Lo}$ population with lower levels of Pax7 (Rocheteau et al. 2012). Pax7-$nGFP^{Hi}$ cells represent a dormant satellite cell subpopulation with lower metabolic activity that are involved in self-renewal and are less primed to commitment than Pax7-$nGFP^{Lo}$ cell (Rocheteau et al. 2012). Consistent with this observation, previous studies using BrdU labelling and fluorescent dye PKH26 revealed that satellite cells have distinct mitotic rates (Ono et al. 2012). Almost 80% of satellite cells enter the cell cycle upon activation, whereas 20% of satellite cells show a slow mitotic rate (Ono et al., 2012). This population is likely to correspond to the Pax7-$nGFP^{Hi}$ population of satellite cells (Ono et al. 2012; Schultz 1996). In a similar manner, variation in the expression levels of membrane markers have been reported. Immunofluorescence studies revealed that although the majority of satellite cells express CD34, M-cadherin and Myf5, a subpopulation of satellite cells does not express these markers (Beauchamp et al. 2000). However, these differences may also be explained by the variability introduced during sample isolation and processing. To address these challenges, a novel "label-free" method was recently developed (Chapman et al. 2013). In this method, single satellite cells freshly isolated from single fibres are injected into a microfluidic channel. Microfluidic channel is saturated with the target antibody or isotope control. The current impulse of the cell is measured when single cell transit the microchannel. Antibody with high-affinity to a particular epitope on satellite cell leads to longer transit time compared to less-transit time that results from a non-specific interaction such as isotope control (Chapman et al. 2013). Using this method it was shown that individual myofibres of mouse EDL muscles were also heterogeneous for markers such as CXCR4, M-Cadherin, Sca-1, β1-Integrin and Notch-1. Finally, satellite cells of different muscle origins for example head and body muscles, shows distinct

molecular signatures (Harel et al. 2009; Sambasivan et al. 2009). This indicates that the heterogeneity of satellite cells is maintained across different muscles of the body and may be linked to the embryonic origin of these muscles. For instance, head muscles are also heterogeneous in nature with respect to their origin and regulatory mechanisms (Mootoosamy and Dietrich 2002).

Table 1.2: Satellite cell markers (expressed in mouse)

Markers expressed	**Expressed during satellite cell**			**Function**	**Reference**
Membrane markers	Quiescence	Activation	Differentiation		
C-met	Yes	Yes	Yes	HGF receptor	Allen et al., 1995; Cornelison et al., 1997
M-Cadherin	Yes	Yes	Yes	Anchors to myofibre	Irintchev et al., 1994; Cornelison et al., 1997
NCAM	Yes	Yes	Yes	Adhesion molecule	Covault et al., 1986
Integrin-α7	Yes	Yes	Yes	Laminin receptor; signalling	Burkin et al., 1999; Kuang et al., 2007; Gnocchi et al., 2009;
Integrin-β1	Yes	Yes	No	Laminin receptor; signalling	Kuang et al., 2007; Sherwood et al. 2004
Syndecan 3/4	Yes	Yes	No	Maintenance and activation	Tanaka et al., 2009; Cornelison et al., 2001
CD34	Yes	Yes	No	Migration and proliferation	Beauchamp et al., 2000; Alfaro et al.,2011
Caveolin -1	Yes	Yes	Yes/No	Maintains quiescence	Volonte et al., 2005; Gnocchi et al., 2009
CXCR4	Yes	Yes	Yes	Migration	Ratajczak et al., 2003; Sherwood et al., 2004
Notch	Yes	Yes	No	Cell fate determination	Conboy et al., 2002; Kuang et al., 2007
Transcription factor					
Pax7	Yes	Yes	No	Quiescence, muscle repair	Seale et al., 2000; Buckingham et al., 2003
Pax3	Yes	Yes	No	Quiescence, muscle repair	Montarras et al., 2005; Relaix et al., 2003 and 2006
Myf5	No	Yes	Yes	Proliferation, differentiation	Tajbakhsh and Buckingham 1995;
MyoD	No	Yes	Yes	Proliferation, differentiation	Sabourin et al., 2000; Miller et al., 2000; Yablonka-Reuveni et al. 1999a;
MNF	Yes	Yes	No	Involved in muscle repair	Garry et al., 1997
Myogenin	No	No	Yes	Differentiation	Gnocchi et al., 2009
Nuclear Envelope markers					
Emerin	No	Yes	Yes	Differentiation	Gnocchi et al., 2009; Frock et al., 2006
Lamin A/C	No	Yes	Yes	Differentiation	Gnocchi et al., 2009; Frock et al., 2006
Others					
Desmin	No	Yes	Yes	Cytoskeletal marker	Cornelison et al., 1997
Nestin	Yes	Yes	No	Intermediate filament	Day et al., 2007
Numb	No	Yes	No	Cell fate determination	Shinin et al., 2006

1.3.4 Non-muscle cells with muscle regeneration capacity

Several non-muscle cell types have been reported to contribute to the regeneration of muscles. These non-muscle cells include haematopoietic stem cells, mesoangioblasts, pericytes, muscle side population and PW1+ interstitial cells. As they can be expanded *in vitro* without loss of activity, they have been considered suitable candidates for cellular therapeutic applications.

1.3.4.1 Bone marrow-derived stem cells

Cell culture studies have shown that bone marrow-derived stem cells can differentiate and form myotubes (Wakitani et al. 1995). Intramuscular injection of whole bone marrow into injured muscles resulted in the incorporation of bone marrow-derived stem cells (BMSCs) into newly-formed muscles. Yet, the fusion index of myotubes derived from BMSC was lower than that of satellite cells. Indeed, BMSCs express satellite cell markers Myf5, Integrin α7 and c-Met when injected into irradiated mice (LaBarge and Blau 2002). However, in another study, bone marrow-derived myogenic cells expressing Pax3 and Myf5 did not give rise to dystrophin-positive fibres when injected into *mdx* mice, unlike the whole bone marrow (Corti et al. 2002). This suggests that a specific population of bone marrow stem cells has a myogenic potential. To identify this population, studies were performed by injecting single genetically labelled cells into irradiated *mdx* mice (Camargo et al., 2003). When FACS sorted GFP+c-Kit+Lin1-Sca1+ cells, a subpopulation of haematopoietic lineage, injected into irradiated *mdx* mice, incorporated into newly-forming myofibres, and fused directly to form new myofibres (Camargo et al. 2003; Corbel et al. 2003). Interestingly, stromal cells originated from bone marrow are also able to be incorporated into myofibres in injured TA muscles in nude mice (Dezawa et al. 2005). Thus, BMSC have the potential to contribute to muscle regeneration. However, further studies are required to identify the specific population carrying this activity.

1.3.4.2 Skeletal muscle side population

Muscle side population (SP) cells are present in the interstitium of skeletal muscles and differ from bone marrow and satellite cells. SP cells are characterized as a

population of cells of muscle origin that display a different spectrum in FACS analysis using the markers CD45, C-kit and Sca1. They are CD45- C-kit- and Sca-1+ cells. Direct injection of SP cells into regenerating muscles leads to SP cell incorporation into newly-forming fibres (Asakura et al. 2002). Intravenous injection of SP cells into *mdx* mice also restores dystrophin expression. Initially, after 5 weeks of injection, less than 1% of the myofibre expressed dystrophin but by 12 weeks 10% of muscle fibres expressed dystrophin (Asakura et al. 2002; Gussoni et al. 1999; Muskiewicz et al. 2005). The origin of SP cells has been traced back to limb hypaxial somitic Pax3-expressing cells (Schienda et al. 2006). Consistent with this, $Pax7^{-/-}$ mice show reduced levels of satellite cells but contain SP cells. In these mice, although there is extensive muscle wasting and impaired regeneration, a population of Pax3-expressing cells is maintained within SP cells, suggesting a role for Pax3 in SP (Gussoni et al. 1999; S. Kuang et al. 2006; Seale et al. 2000).

1.3.4.3 Mesoangioblasts

A myogenic potential was also been identified in stem cells derived from mesodermal tissue, termed mesoangioblasts (De Angelis et al. 1999b). Mesoangioblasts express satellite cell markers and can give rise to skeletal muscles in addition to cartilage, smooth and cardiac muscle when transplanted into chick embryos (Bladt et al. 1995; De Angelis et al. 1999b; Minasi et al. 2002). Interestingly, mesoangioblasts isolated from α-$Sarcoglycan^{-/-}$ mice and infected with a lentivirus to re-express α–Sarcoglycan are able to re-constitute normal skeletal muscles when injected intra-arterially (Sampaolesi et al. 2003). This suggests that they can migrate efficiently to the site of regeneration in muscles. Mesoangioblasts have also been isolated from human patients suffering of inclusion-body myositis (IBM), an inflammatory myopathy, and shown to restore skeletal muscles when forced to over-express MyoD (Morosetti et al. 2006). Finally, intra-arterial injection of mesoangioblasts into the Golden Retriever dog model of Duchenne muscular dystrophy led to the restoration of Dystrophin expression and muscular function (Sampaolesi et al. 2006). This suggests that mesoangioblasts could be used in therapeutic applications. Although

mesoangioblasts are easy to culture it is difficult to obtain a pure population (Sampaolesi et al. 2003).

1.3.4.4 Pericytes

Pericytes originate from the sclerotome and can give rise to skeletal muscles without expressing satellite cell markers (Dellavalle et al. 2007). Pericytes are known to differentiate into many other cell types such as adipocytes, chondrocytes and osteoblasts demonstrating their multipotent characteristics (Doherty et al. 1998; Farrington-Rock et al. 2004). Purified cultures of human pericytes can differentiate to form skeletal muscles and FACS sorted pericytes that are negative for markers of satellite cells and haematopoietic cells demonstrated myogenic properties (Crisan et al. 2008; Dellavalle et al. 2007). Pericytes only express Myf5 and MyoD after differentiation has been induced and never express Pax7, Myf5 or MyoD during proliferation, suggesting that the myogenic process taking place in pericytes may differ from that taking place in satellite cells (Dellavalle et al. 2007). However, when isolated human pericytes were injected interarterially into *scid-mdx*, they restored Dystrophin expression in regenerating myofibres, indicating a regenerative potential (Dellavalle et al., 2007b). In line with this it was demonstarted that alkaline phosphatase (AP) expressing pericytes enter satellite cell pool and contribute to a significant percentage of muscle fibre growth (Dellavalle et al. 2011). These AP-positive pericytes were able to respond to muscle injury, increases upto five times and upto three times in acute and chronic mucle injury respectively. And thus contribute to myogenenesis. However, pericytes possess differences in regnerative ability which results from different preparations (Dellavalle et al., 2007b).

Mesoangioblats and pericytes are blood-vessel assocaited stem cells. Mesoangioblasts are derived from embryonic stages where as pericytes are associated with postnatal stages (Dellavalle et al., 2007b). Although both are blood-vessel derived stem cell, pericytes are derived only from the skeletal muscle blood vessels (Meng et al. 2011).

1.3.4.5 PW1-positive interstitial cells

In skeletal muscles, PW1 is expressed in satellite cells and in interstitial cells that are $Pax7^-$, $Sca\text{-}1^+$ and $CD34^+$ (Relaix et al. 1996). PW1-positive cells are required for the postnatal growth of skeletal muscles, as indicated by the impaired postnatal, but not embryonic and fetal growth of skeletal muscle development in mice with a dominant negative form of PW1 (Nicolas et al. 2005). This is consistent with *in vitro* studies revealing that PW1-positive cells have myogenic properties. Although, FACS sorted Sca1+/CD34+/CD45- gave rise to satellite cells and interstitial cell when injected into injured muscles (K. J. Mitchell et al. 2010), PW1-positive cells isolated from Pax7-/- mice does not show myogenic potential. This suggests that Pax7 expression is required to confer a myogenic potential to these interstitial cells (K. J. Mitchell et al. 2010). Furthermore, these data indicate that PW1 cells are likely to contribute to the Pax7 muscle phenotype during postnatal development.

Thus, a number of non-muscle cells appear to have myogenic potential and hence can regenerate the muscle. However, satellite cell depletion studies have clearly shown that satellite cells are essential for muscle regeneration (Lepper et al. 2011; Sambasivan et al. 2011; von Maltzahn et al. 2013). This suggests that non-myogenic cells cannot compensate for the loss of satellite cells *in vivo*.

1.4 The adult stem cell niche

1.4.1 Niches regulate stem cell activity

Adult tissue growth and homeostasis is maintained by tissue specific stem cells, which persist for the lifetime of the animal. Stem cells reside in a niche and are engaged in organ formation, in postnatal development and in tissue regeneration. The concept of a niche was originally proposed in haematopoietic stem cells by Schofield in 1978 (Schofield 1978). Niche is “a specific location in a tissue where stem cells can reside for an indefinite period of time and produce progeny cells while self-renewing” (Spradling et al., 2001). Later *in vivo* studies identified that ovary stem cells of invertebrates such as *Drosophila melanogaster* also reside in a niche that support their activity (Yamashita et al. 2005). Niches are complex and dynamic

structures that send signals to stem cells and also receive signals from stem cells (Spradling et al. 2001; Yamashita and Fuller 2005). Stem cells removed from their niche adopt an aberrant behavior, best exemplified by the formation of teratomas when embryonic stem cells are implanted under the skin of nude mice (Fuchs et al. 2004). A hypothetical niche of a mammalian stem cell consists of soluble factors, extracellular matrix, vascular networks, cell adhesion components and stromal cells that improve the functional capacity of stem cells (Spradling et al. 2001; Yamashita and Fuller 2005). Some of the best defined niches in vertebrate tissues are those of hematopoietic stem cells, the intestinal crypt and the skin. For example, the hematopoietic stem cell (HSCs) niche consists of endosteal and perivascular environment (Calvi et al. 2003; Mendez-Ferrer et al. 2010). Endosteum is made up of osteoblasts that support HSCs quiescence through adhesive contacts between N-cadherin and Tie2/Angiopoietin (Arai et al. 2004). The perivascular niche on the other hand is composed of vascular endothelial cells and mesenchymal cells (Mendez-Ferrer et al. 2010). Studies have identified that Nestin GFP-positive cells are a sub-population of mesenchymal cells that possess the ability to form mesenchymal spheres and differentiate into mesenchymal lineages (Mendez-Ferrer et al. 2010). In another tissue with a high turnover, the intestine, intestinal stem cells (ISCs) create the niche by themselves (Sato et al. 2011). In this system, ISCs reside in the crypt of the small intestine and mainly consists of two cell types Lgr5+ cells and Bmi+ cells (Barker et al. 2007). Cells expressing Bmi are quiescent cells located above the epithelium made up of Paneth cells, whereas Lgr5+ cells are present between Paneth cells, which continuously recycle (Barker et al. 2007). Similarly, interfollicular epidermal stem cells (IFSC) found in the basal layer of the epidermis coordinate with hair follicle stem cells (HFSCs) present in the bulge region to form a new hair follicle whereas melanocytes present in their niche pigment the newly formed hair fibre (E. K. Nishimura et al. 2005).

Apart from supporting stem cells, niche components also maintain the stemness of stem cells by repressing differentiating genes. Such components include extrinsic factors, extracellular matrix components (ECM) proteins or crucial intercellular

signaling emanating from matrix-bound growth factors or soluble growth factors or from cell-matrix interactions (Discher et al. 2009). ECM components such as laminins are particularly interesting because they act as ligands and bind specifically to integrin on stem cells, which has been identified as a key regulator of self-renewal and differentiation (Campos et al. 2006; Q. Shen et al. 2008a). For instance, depletion of Integrin β1 in adult epithelial cells alters hedgehog signaling and leads to increased proliferation of epithelial cells (Jones et al., 2006). Another ECM component required for adult stem cell function is tenascin C, a member of the glycoprotein family. Tenascin-C is required for the interaction between stromal cells and hematopoietic progenitor cells, and hematopoietic cell production is affected in Tenascin-C-deficient mice (Ohta et al. 1998). Intriguingly, in the neural stem cell niche, FGF-2 is provided by fractones (ECM) to favor stem cell identity (Kerever et al. 2007). Thus, ECM components and supporting cells of an adult stem cell niche regulate and support the stem cell activity.

1.4.2 The niche components of skeletal muscle satellite cell

Similar to other adult stem cells, satellite cells reside within a niche (Boonen and Post 2008). One obvious difference between satellite cells and other stem cells is that the satellite cell remains quiescent in its niche and becomes activated only upon a stimulus (Bentzinger et al. 2012). In their quiescent state, satellite cells are present between the extracellular matrix and the sarcolemma of the muscle fibre (Mauro and Adams 1961). The niche of the satellite cell supports normal homeostasis during quiescence state (Mounier et al. 2011). Extrinsic signals maintain satellite cell quiescence (as described in paragraph 1.3.3.3). However, the niche of activated satellite cells is very distinct compared to the composition of the niche at quiescence (Bentzinger et al. 2013a; Y. X. Wang and Rudnicki 2012). Activation of satellite cells results in the up-regulation of receptors coupled with changes in gene expression initiated by both intrinsic and extrinsic signals emanating from satellite cells and the surrounding environment, ECM and different cell types (Bentzinger et al. 2013a; Boer et al. 2002; Boonen and Post 2008; Brzoska et al. 2011) (see Fig 1.8). The different components of satellite cell niche are discussed below.

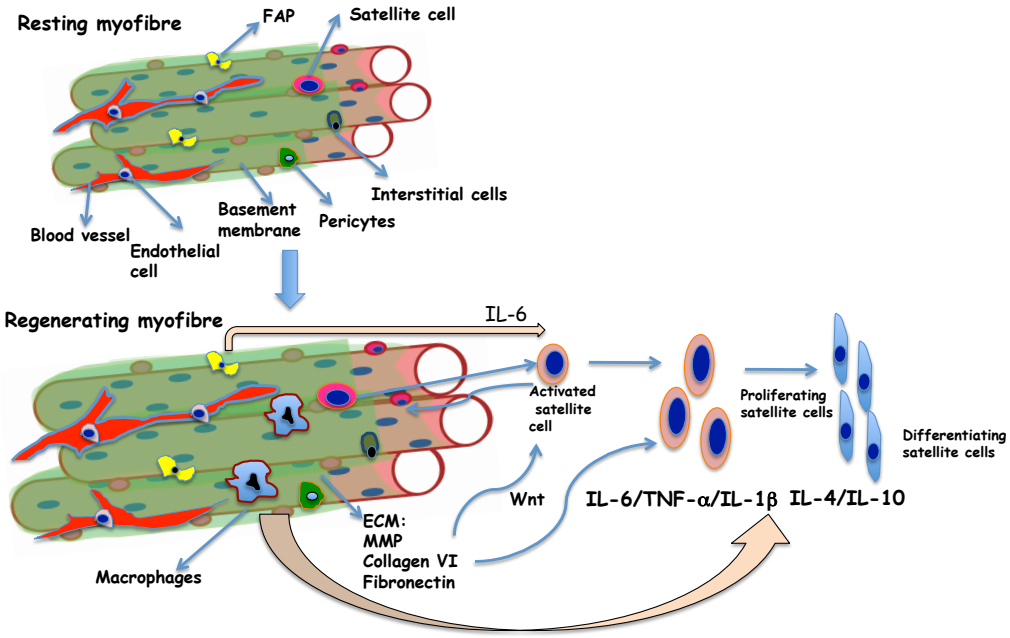


Figure 1.8: ***Satellite cell niche controls satellite cell activity:*** Schematic representation of different external cues influencing satellite cells in its niche. Growth factors and cytokines released from macrophages and FAP; ECM components such as MMPs, Collagen VI and Fibronectin affect satellite cell behavior.

1.4.2.1 Different cell types are involved in the satellite cell niche

The cell types that contribute to the satellite cell niche can be classified into cells that support muscle regeneration such as inflammatory cells, endothelial cells, mesenchymal cells and cells with myogenic potential (see Fig 1.9) (De Angelis et al. 1999a; Ferrari et al. 1998; Mounier et al. 2011).

Endothelial cells: The microvasculature is in close contact with satellite cells and more than 60% of satellite cells are close to capillaries (Christov et al. 2007). It has been suggested that vascular endothelial growth factor (VEFG), secreted by endothelial cells, increase during acute muscle injury and reaches a baseline at 3-day post-injury in CTX injured mice (Ochoa et al. 2007). VEGF is required for the formation of new blood capillaries during muscle repair and in chemokine receptor 2 (CCR2) mutant mice, muscle regeneration is severely delayed as a result of significant reduction in VEGF levels compared to control mice (Ochoa et al. 2007). VEGF acts also directly on satellite cells by promoting their proliferation (Abou-

Khalil et al. 2010). Another evidence that endothelial cells may act as niche cells comes from the finding that binding of Ang1 produced by perivascular cells to its receptor Tie-2 expressed at the surface of satellite cells promotes their quiescence, whereas blocking Tie-2 *in vivo* increases the number of self-renewing cells (Abou-Khalil et al. 2009). Collectively, these data suggest that endothelial cells may contribute to the satellite cell niche and are required to maintain satellite cell quiescence and proliferation during repair.

Inflammatory cells: Inflammatory cells are key effectors in the satellite cell niche, as they guide satellite cells through the regeneration process. As discussed earlier (section 1.3.2.1), they play a major role in satellite cell function (Tidball and Villalta 2010). Briefly, in physiological conditions, very few leukocytes such as mast cells and monocytes are observed in muscles. However, during muscle injury, inflammatory cells increase dramatically and in response to the injury of the muscle they secrete chemokines and cytokines such as TNF-α, IL-4, IL6 and IL-10 (Tidball and Villalta 2010). Initially, neutrophils create a pro-inflammatory environment and remove cellular debris (Dumont et al. 2008). Later two waves of macrophages, M1 and M2, invade the damaged site and participate to the muscle regeneration by supporting satellite cell activation and proliferation (Lesault et al. 2012; Tidball and Wehling-Henricks 2007; Villalta et al. 2009).

Fibroblasts and fibrogenic progenitor cells (FAPs)

Fibroblasts and FAPs are a major source of extracellular matrix components and their interaction with satellite cells is essential during muscle regeneration (Murphy et al. 2011). Quiescent FAPs reside in close contact with blood vessels, but propagate and move to the interstitial space upon muscle injury (Murphy et al. 2011; Pretheeban et al. 2012). FAPs secrete IL-6 cytokine, which is required for satellite cell differentiation (Joe et al. 2010). It was also shown recently that FAPs participate in clearing the debris acquired during muscle damage (Heredia et al. 2013). Ablation of Tcf4-positive fibroblasts cells (similar to FAP) using diphtheria toxin resulted in premature satellite cell differentiation and impaired regeneration (Murphy et al.

2011). This suggests that fibroblasts are involved in satellite cell transient expansion in contrast to FAPs, which aid satellite cell differentiation (Joe et al. 2010).

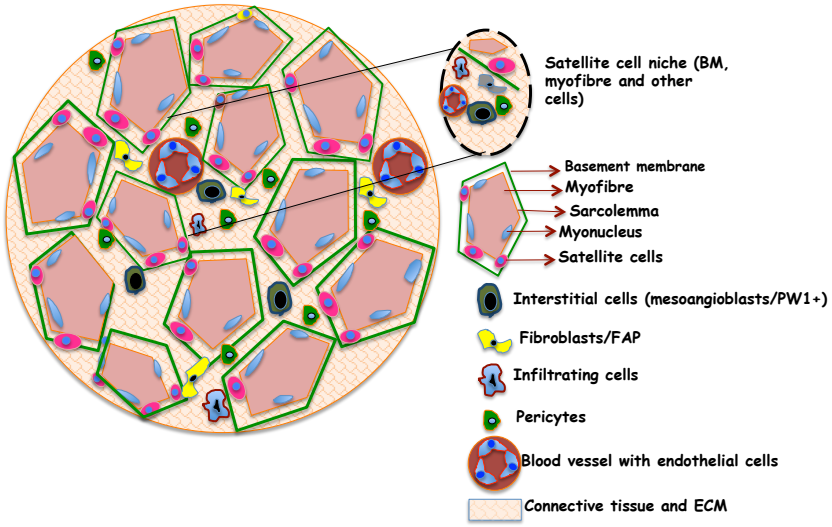


Figure 1.9: ***Schematic representation of a skeletal muscle cross section:*** The image shows the localization of satellite cells in contact with the basal lamina, fibre and other cell types. Typically satellite cells are associated with interstitial cells (mesoangioblasts/PW+), Fibrogenic progenitor cells (FAP), Pericytes, blood vessel and inflammatory cells (during muscle regeneration).

Another population of cell types that occupies the satellite cell niche is interstitial cells and pericytes (Dellavalle et al. 2007; K. J. Mitchell et al. 2010). The function and role of these cells in post-natal muscle growth is described in paragraph 1.3.4.

1.4.2.2 The extracellular matrix may provide signals controlling satellite cell behavior

The role of the ECM in the control of embryonic skeletal muscle formation was investigated before (C. Anderson et al. 2009; Bajanca et al. 2006; Thorsteinsdottir et al. 2011). In the embryo, muscle progenitor cells assemble a basement membrane as they populate the nascent myotome via the up-regulation of the laminin receptors, integrin α6β1 and dystroglycan. Disruption of the ECM itself or of the interaction between ECM and muscle progenitor cells causes aberrant migration and orientation of muscle progenitor cells and disrupts the differentiation process. This indicates that the interaction of the ECM with muscle progenitor cells has several functions in the control of their differentiation, their polarity and their migration.

Study of the role of the ECM within the satellite cell niche is still in its infancy. However, a number of reports suggest that the ECM plays an essential role during muscle regeneration (Boldrin et al. 2012; Brzoska et al. 2011; Pallafacchina et al. 2010; Ross et al. 2012). Recent studies have begun to uncover specific roles played by the ECM on satellite cells (Bentzinger et al. 2013b; Urciuolo et al. 2013). It has been demonstrated that Collagen VI, a major component of ECM is required for maintaining the self-renewing capacity of satellite cells (Urciuolo et al. 2013). The study demonstrated that lack of Collagen VI in *Col6a1*$^{-/-}$ mice causes a reduction in the number of satellite cells, thus affecting muscle regeneration after cardiotoxin injury. Another ECM component, fibronectin binds to Fzd7/Sdc4 a receptor complex expressed at the surface of satellite cells and stimulates Wnt7a signaling required to induce satellite cell expansion (Bentzinger et al. 2013b). Another indication of the role of the ECM in muscle regeneration is given by the effect of matrix-metalloproteinase (MMPs) and their inhibitors during regeneration (Carmeli et al. 2004; X. Chen and Li 2009; T. Nishimura et al. 2008; Yamada et al. 2006). Satellite cells also transcribe a number of genes that encode ECM proteins (Pallafacchina et al. 2010). It has been demonstrated that inhibitors of proteinases, such as *Tfpi2* that prevents ECM remodeling are up-regulated in quiescent satellite cells whereas transcripts for proteinases are up-regulated in activated satellite cells. For instance *Mmp11* transcripts are lower in quiescent satellite cells but increase in activated satellite cells (Pallafacchina et al. 2010). Further transcriptome analyses of quiescent and activated satellite cells purified from *Pax3* GFP/+ and *Pax3*GFP/+:*mdx/mdx* mice revealed a striking difference in the genes encoding cell adhesion and the extracellular matrix. It has been shown that quiescent satellite cells express gene *Fgl2*, which encodes for a fibrinogen-like 2 glycoprotein, involved in cell adhesion and gene *Smoc2*, which encodes a calcium binding protein. In contrast, in activated satellite cells many ECM genes that are responsible to bind with growth factors up-regulated. They include Versican (*Vcan*), Glypicans 1 and 3 (*Gpc1,3*), or Fibrillin 2 (*Fbn2*). This indicates the differential gene expression of ECM proteins and proteinases is required for the breakdown of the niche for efficient regeneration.

Apart from the ECM components, growth factors that are important in regulating satellite cell behavior such as HFG are bound to ECM in an inactive form (Tatsumi et al. 1998). HGF is released by the enzymatic action of matrix metalloproteinases (MMPs) (Yamada et al. 2006). At the site of injury during muscle regeneration MMPs degrade the ECM making room for migrating and dividing satellite cells (X. Chen and Li 2009). Intriguingly, it has been suggested that MMP-9 and MMP-2 are up-regulated at the site of muscle injury (Kherif et al. 1999; Lewis et al. 2000). Overall, global changes in the ECM occur during muscle regeneration some of which may regulate satellite cell behavior during physiological and pathological conditions.

1.4.2.3 Niche is required for satellite cell function

Grafting studies indicate that FACS sorted satellite cells isolated from Pax3GFP+ mice can restore dystrophin expression when grafted into the TA muscle of *mdx* (Montarras et al. 2005). However, when the same cells were cultured *in vitro* prior to grafting, they displayed a reduced regenerative potential, suggesting a loss of stem cell potential upon culturing (Montarras et al. 2005). Consistent with this idea, a single myofibre associated with seven satellite cells isolated from 3F-nLacZ-2E mice, gave rise to hundreds of β-gal+/Dystrophin+ myofibres, when engrafted into irradiated *mdx* mice, indicating that the regenerative potential of satellite cells is powerful when cells remain associated to their fibre even at such low numbers (Collins et al. 2005). It is important to note that satellite cells are associated with their myofibres implying the role of satellite cell niche in maintaining the regenerative potential of satellite cells. Likewise, single myofibres isolated from Myf5nLacZ/+ mice showed a 10-fold increase in regenerative potential, whereas satellite cells isolated by enzymatic digestion were 1000-fold less efficient in forming new myofibres (Sherwood et al. 2004). Further engraftment studies in irradiated *mdx* mice indicated that when host satellite cells are incapacitated by irradiation, donor cells are able to repopulate the niche and efficiently regenerate the muscle. In contrast, if donor cells cannot repopulate the niche, engraftment is poor and thus muscle regeneration is impaired. This suggests that the satellite cell niche is crucial to allow donor cells to engraft into the host (Boldrin et al. 2012). Thus, satellite cell function is supported by

the niche, and this is essential for an efficient repair and regeneration of damaged muscles.

1.5 The skeletal muscle extracellular matrix (ECM)

The extracellular matrix (ECM) is a complex mixture of structural and functional components such as proteins, glycoproteins and proteoglycans arranged in a tissue-specific three-dimensional organization (Hynes 2009). These proteins provide provision for structural support, attachment sites for cell surface receptors and also act as a reservoir for growth and signaling molecules. Thus, they have a major influence on growth, development and phenotype of the tissue (Frantz et al. 2010). The concept of "dynamic reciprocity" between extracellular and intracellular components led to the identification of the role of ECM in cell behavior (Bissell et al. 1982; Boudreau et al. 1995). The deposition of ECM components ultimately forms the basement membrane surrounding the skeletal muscle myofibre. The importance of the ECM was first highlighted in muscle tissue during the conversion of myoblasts to myotubes (Hauschka and Konigsberg 1966). Later McMahan and colleagues demonstrated the presence of ECM proteins at the neuromuscular junction (NMJ) and the basal lamina (McMahan et al. 1978).

1.5.1 Composition of the ECM

The extracellular matrix is categorized into two main matrices: interstitial matrix and pericellular matrix. The interstitial matrix is mostly made of connective tissue containing proteoglycans, glycosaminoglycans and some collagens (Frantz et al. 2010). This matrix is hydrated, highly charged and contributes to the tensile strength of the tissue (Egeblad et al. 2010). Glycosaminoglycans (GAGs) provide gel properties to the ECM. GAG chains are made up of disaccharide units divided into sulfated and non-sulfated moieties (Schaefer and Schaefer 2010). Interstitial matrix is primarily made up of Type I Collagen, which forms collagen fibrils that reside within the interstitial matrix (M. K. Gordon and Hahn 2010). Type I Collagen is porous and also provides gel-like properties to ECM. Collagens regulate cell adhesion, chemotaxis and migration of cells (Rozario and DeSimone 2010). Next to collagen

the second most abundant protein in interstitial matrix is fibronectin. Fibronectin mediates adhesion, migration and differentiation. Integrins bind to specific site of Fibronectin and supports in matrix assembly (Leiss et al. 2008; Mao and Schwarzbauer 2005).

1.5.2 Basement membrane of skeletal muscle

Basement membranes are pericellular structures of the extracellular matrix that surround myofibres. Bowman in 1840 first described the basement membrane in muscles as a "membranous sheath of the most exquisite delicacy" (Bowman 1840). The structural integrity of skeletal muscles is maintained by interactions of the basement membrane with the sarcolemma of myofibres (Engel 1994). Many studies have demonstrated the role of the basement membrane during muscle development, in supporting the integrity of the neuromuscular and the myotendinous junctions and in neuromuscular transmission and reinnervation (Patton 2000; Sanes 2003; H. Xu et al. 1994b). A single basement membrane in a metal impregnated thin section measures from ~50 to ~100nm in thickness (Yurchenco 2011). Overall, the structure of a basement membrane comprises of at least one member of laminin, nidogen, perlecan, heparin sulfate proteoglycans (HSPGs) and type IV Collagen (Yurchenco and Schittny 1990). Basal lamina are further categorized into electron-lucent layer lamina lucida (Laminin, Nidogen, Perlecan, and HSPGs) and reticular lamina comprises of electron-dense layer, lamina densa (type IV Collagen). Transmission electron microscopy initially allowed the identification of type IV Collagen and Laminin networks in placental amnion and EHS tumor cells (Yurchenco et al. 1985, 1986). These molecules assemble to form matrices containing polymers of Laminins and Collagens chained to Nidogens and Perlecans. Laminins bind to the receptors, Integrin and Dystroglycans in the myofibre sarcolemma. The overall process of basement membrane formation is largely self-assembly and interactions to cell receptors.

1.5.2.1 Type IV Collagen anchors the basement membrane to the interstitial matrix

Type IV Collagen is the predominant component of the basement membrane. It anchors the BM to the interstitial matrix and stabilizes the basement membrane. Type IV Collagen is a heterotrimer protein, and six genetically distinct type IV collagens have been identified to date that are distributed in different tissues (Hudson et al. 1993): α1 (IV), α2 (IV), α3 (IV), α4(IV), α5(IV) and α6(IV). The common variant of all these forms is composed of two α1 subunits and one α2 subunit. Each monomer of type IV collagen assembles to form a triple helix with three α–chains. Both the carboxyl and amino terminus of α–chains ends with non-collagenous sequences (NC1). They form self-assembled branched networks by their NC1 domains and by lateral associations (Timpl et al. 1981; Yurchenco and Furthmayr 1984; Yurchenco and Ruben 1987). The composition of Type IV Collagen varies among different muscles with higher concentration in muscles containing slow-twitch fibres than in muscles with fast-twitch fibres (Onofre-Oliveira et al. 2012). Mutational analysis revealed that Type IV Collagen expression is not required at early stages of basement membrane formation but inhibition of Type IV Collagen expression is lethal at later embryonic development (X. D. Guo et al. 1991). Importantly, knockout studies of α1 and α2 demonstrate the lethality at E10.5-E11.5 due to loss of basement membrane in most tissues and also Reichert's membrane (Poschl et al. 2004).

1.5.2.2 Nidogen (Entactin) acts as a linker in basement membranes

Nidogen is a glycoprotein of basement membranes made up of two amino (G1 and G2) and one carboxyl globular domains (G3). These domains are connected by a rod-shaped EGF repeat (Poschl et al. 1994). The major role of this protein is to act as a bridging molecule connecting Laminin and type IV Collagen. On one end the carboxyl-terminal globule binds with the short arm of the Laminin γ subunit and on the other end the remaining two amino globular domains binds to type IV Collagen (Poschl et al. 1994; Takagi et al. 2003). Thus, a non-covalent linkage gets established in between Laminin and type IV Collagen mediated by nidogen forming a stable

basement membrane to support the mechanical stress of the muscle (Poschl et al. 2004). In the mouse, two genes encode for nidogen, *Nid-1* and *Nid-2* (Ries et al. 2001). Nidogen also binds to perlecan and fibulin suggesting multiple interaction within basement membrane (Ries et al. 2001).

1.5.2.3 Perlecan also acts as a linker molecule in basement membranes

Perlecan is a heparan sulfate proteoglycan comprised of three heparan sulfate chains that are linked to a large core protein (~400kda) and five domains (Noonan et al. 1991; Saku and Furthmayr 1989). Domain IV of Perlecan binds with Nidogen whereas domain V binds to its receptors α-Dystroglycan and Integrin α2β1 (Yurchenco and Patton 2009). Perlecan also mediates cell signaling by interacting with receptors and growth factors. The five domains also bind to fibroblast growth factor (FGF), Fibronectin and sonic hedgehog (Palma et al. 2011). Therefore, Perlecan may also be involved in regulating cell growth, adhesion, migration and differentiation. Absence of Perlecan in the mouse is embryonic lethal at the age of E10.5-E19 (Costell et al. 1999). Perlecan mice mutant ($Hspg2^{-/-}$-Tg) had a significantly increased fibre cross-sectional area (CSA) compared to control mice (Z. Xu et al. 2010). Deficiency of Perlecan in this mice reduces Myostatin signaling and causes muscle hypertrophy. In humans, absence of Perlecan causes Schwartz-Jampel syndrome leading to chondroplasia and myotonia (Arikawa-Hirasawa et al. 2002).

1.5.3 Laminins: the major components of the skeletal muscle basement membrane

Laminin is another major component of the basement membrane. The first Laminin that was identified was Laminin-111 from the Engelbreth-Holm-Swarm (EHS) embryonic carcinoma (Timpl et al. 1979). Laminin and Collagen form the bulk of the basement membrane and both are connected via Nidogen (Yurchenco and Patton 2009) (see Fig 1.10). Laminin binds also to cellular membrane-associated Integrins and Dystroglycan. Laminin also binds to other basement membrane components such as Perlecan (see Fig 1.10).

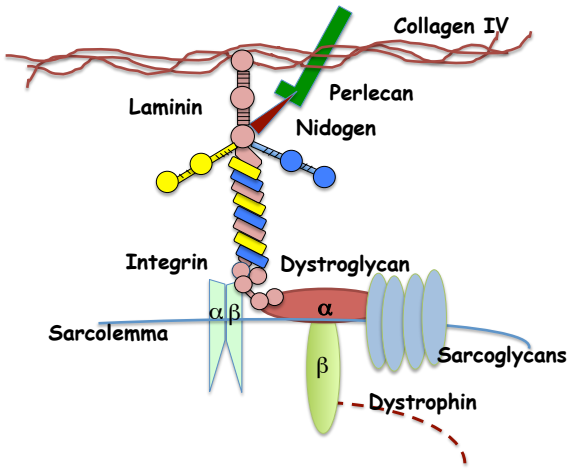


Figure 1.10: ***Schematic representation of skeletal muscle basement membrane and its interaction with the receptors:*** Different components of the basement membrane components interact and form a highly organized sheet like structure. Laminin binding to ECM components and interaction with the receptors is

1.5.3.1 Laminin-111 structure and assembly

Laminins are large heterotrimeric glycoproteins composed of α, β and γ subunits and are conserved among vertebrates and invertebrates. Invertebrates have two Laminin heterotrimers from the combination of two α, one β and one γ (Miner and Yurchenco 2004). In contrast, vertebrates possess at least 18 different Laminin proteins formed through the combination of 5α, 4β and 3γ subunits (Miner and Yurchenco 2004) or as a result of mRNA splicing of α3 and γ2 subunits (Ferrigno et al. 1997). Laminins were first named in the order in which they were discovered. However, the nomenclature was recently revised, and Laminins are now named according to their subunit composition (Aumailley et al. 2005). For example Laminin-111 is composed of Laminin α1, β1 and γ1 subunits.

A typical Laminin consists of one α (440kd), one β (200kd) and one γ (220kd) subunit linked via disulfide bonds to form an asymmetric cross-shaped or T-shaped structure with three short arms and one long arm (Beck et al. 1990). All three subunits possess two or three small globular domains and one globular domain in the short and the long arm respectively (Colognato and Yurchenco 2000; Engel et al. 1981).The longest subunit is the α chain. Although all Laminins have a similar structure, Laminins α3A and α4 are truncated and lack short-arms (Miner et al. 1995).

Laminin subunits assemble into heterotrimers in the Golgi apparatus. Initially, the coiled-coil domains of the β and γ subunits form a dimer, followed by oligomerisation

with the α subunit to form the heterotrimer (Paulsson et al. 1985). The addition of the α subunit is the rate-limiting step for the secretion of the Laminin heterotrimeric molecule (Yurchenco and Cheng 1994; Yurchenco et al. 1997; Yurchenco and Wadsworth 2004). The α chain binds to the Laminin dimer (β and γ) through disulfide bonds and facilitate its secretion (Yurchenco et al. 1997; Yurchenco and Wadsworth 2004). This indicates that α subunits determine the production or not of Laminins.

1.5.3.2 Laminin interaction with other ECM proteins is mediated by different domains

Each Laminin chain is composed of domains I to VI in a C-terminal to N-terminal direction. It also contains coiled-coil rod like domains at the C-terminal and globular domains at the N-terminal (Beck et al. 1990). The C-terminal arm consists of LG 1-5 domains, which bind to cellular receptors, and the N-terminal arm mediates Laminin polymerization, as well as binds to some receptors (Patarroyo et al. 2002). All five LG domains are intact in Laminins α1 and α2, but in other Laminin alpha LG domains such as α3A, α4 and α5 the last two domains are extracellularly cleaved by proteolysis and regulate cell behavior by altering the Integrin-mediated migration of cells (Goldfinger et al. 1998). Coiled-coil repeats of domain I and II contain heptad repeats (Beck et al. 1990). These coiled domains possess binding sites for Agrin and site for motor-neuron attachments (Engvall and Wewer 1996). At the N-terminal of all three subunits are a number of Laminin-type Epidermal factor-like (LE) repeats. LE repeats are present in domain III and V of the short arm. The LE domain of the γ subunit interacts with heparan sulphate proteoglycans (HSPG) and Nidogen, and thus mediates the binding of Laminins to ECM (Poschl et al. 1994). Laminins binds to other ECM components through their globular domain IV. Domain IV is referred as LE domain in β chains and as L4 in γ chains. This domain binds to Fibulin-2 (Utani et al. 1997). The N-terminal domains IV and VI (LN) of α1, α2 and α5 bind to several Integrin α1β1, α2β1, and α3β1 receptors (Colognato et al. 1997; Sasaki and Timpl

2001; Sasaki et al. 2001a; Sasaki et al. 2001b). Thus, Laminins can bind to the cell surface from both their N- and C-terminal domains.

1.5.4 Laminin receptors and signaling

Laminin as a ligand binds to its receptors and mediates numerous cellular signaling, alters the transcription of genes and thus cell behavior. Laminins interact with cells through four trans-membrane receptors: Integrins, dystroglycan, syndecans and Lutheran blood group glycoproteins (Scheele et al. 2007). These receptors bind extracellularly to the ECM and intracellularly to cytoplasmic proteins. The interactions with these receptors link the extracellular environment to the cell and mediate inside-out and outside-in signaling. Integrin binding is mainly mediated via the αLG (1-3) domains and αLN domain. Dystroglycan binding is mediated mainly by αLG (4-5) domains (in Laminin α1 and α5) (Scheele et al. 2007). Syndecan binding has been mapped to the αLG4 domain of all α chains (Miner 2008). Lutheran blood group glycoprotein binding to the α5 chain occurs via the LG3 domain (Miner 2008; Scheele et al. 2007).

1.5.4.1 Integrins are key receptors for Laminins

Integrins mediate cell-cell and cell-matrix interactions in skeletal muscles. They are heterodimeric complexes composed of α and β chains combined by non-covalent linkage. It has been identified that 24 different Integrins are formed from 18α and 8β chains (van der Flier and Sonnenberg 2001). Each Integrin binds to a particular sequence on Laminin. Of all 24 Integrins α1β1, α2β1, α3β1, α6β1, α7β1, α9β1 and αvβ3 are known to bind to Laminins (Nishiuchi et al. 2006).

During muscle development myogenic progenitor cells first encounter ECM in the somite. α1β1 and α6β1 are the prominent Integrins expressed both in avian and mouse somites (Velling et al. 1996). α7β1 is expressed in the somites and intersomitic boundaries. It has been shown that Integrin receptors play a crucial role in sequestering and clustering of Laminin polymers to support their assembly into a basement membrane (Colognato et al. 1999). As MPCs enter the myotome, they begin to express Integrin (Bajanca et al. 2004b; Cachaco et al. 2005). It has been

demonstrated that the formation of the myotomal basement membrane correlates with the expression of Dystroglycan but not Integrin α6β1, which is already expressed by muscle progenitor cells (C. Anderson et al. 2007b; Bajanca et al. 2006). Blocking Laminin binding to Integrin α6 prevents Myf5-positive myogenic cells from populating the myotome and cause disruption of the myotomal basement membrane, ultimately resulting in their premature differentiation within the dermomyotome (Bajanca et al., 2006). This suggests that Integrin α6β1 is required for the assembly of the myotomal basement membrane.

In the adult skeletal muscles, α3β1, α6β1 and α7β1 can recognise all Laminin isoforms (Kikkawa et al. 2000; von der Mark et al. 2002). However, α7β1 is the major Integrin expressed in the adult skeletal muscle and is highly enriched at the myotendinous junction (Bao et al. 1993; Burkin and Kaufman 1999).

As mentioned earlier, Laminins can bind to Integrins through their C-terminal and N-terminal domains, although they mostly interact with the C-terminal LG domains of Laminin α subunits. β and γ Laminin subunits can also bind to Integrins (Patarroyo et al. 2002). Mutational studies showed that loss of the LG3 domain in Laminin-511 results in the loss of Integrin binding (Ido et al. 2004). α1β1 and α2β1 binds to LN domain of Laminin α1 and α2 subunits, whereas αvβ3 binds to domain IVa of Laminin α5 subunit (Colognato et al. 1997). The Laminin α subunit LG domains bind to Integrins with different affinities (Talts et al. 1999). For instance, Laminin α1 affinity for Integrin α6β1 is higher than that of Laminin α2, and both Laminins bind to α7β1 (K. I. Gawlik et al. 2010; Nishiuchi et al. 2006; Talts et al. 1999). However, ligand-binding assays showed that Laminin-111 preferentially binds to α7X2β1, a variant of α7β1, whereas Laminin α2, binds to both variants of α7β1: α7X1β1 and α7X2β1. α7X1β1 is expressed during early regeneration process and in adult muscle the α7X2β1 variant predominates (Ziober et al. 1993).

Laminin-Integrin interaction is one of the major mean of communication between the basement membrane and the cell. In a normal resting state, inactive Integrins are unbound to the ligands. But during physiological or pathological stimulus and during

inflammation, activation from within the cell induces straightening of the Integrin trans-membrane domain exposing the ligand binding site on the extracellular domain (Ratnikov et al. 2005). This process is termed as "inside-out" signalling, which facilitates "outside-in" signalling and affects cell behaviour (Ratnikov et al. 2005). Binding of laminin to integrin activates a series of intracellular secondary messengers such as focal adhesion kinase (FAK), mitogen-activated protein kinases (MAPK), rhoGTPase and phosphatases (Givant-Horwitz et al. 2005; Watt 2002). Binding of laminin to integrin initiates the formation of focal adhesion (FA) complexes, which consists of FAK, Vinculin, Talin and Src signalling molecules. The assembly of theses proteins mediate downstream activation of MAPK, Akt/PI3K and FAK activity that regulates cell survival, proliferation, migration and differentiation (Zaidel-Bar 2013). Integrin/FAK signalling also promotes myoblasts differentiation as it regulates the expression of promyogenic proteins cdo,cdc42 and MyoD (J. W. Han et al. 2011). Furthermore, muscle specific-β1 Integrin binding proteins are expressed before differentiation in myoblasts and down-regulate after differentiation suggesting that this protein is important to decide the fate of precursors for proliferation or differentiation (J. Li et al. 1999). It has been also demonstrated that binding of Laminin-211 to Integrin α7β1 promotes stability and survival of myotubes (Vachon et al. 1997). This is possible because the signaling through Integrin α7β1 up-regulates anti-apoptotic proteins such as Bcl-2 and Bcl-X_2 and down-regulates pro-apoptotic proteins Bax (Laprise et al. 2003; Vachon et al. 1997). Thus, Laminin-Integrins interactions are known to cascade signals for muscle cell survival, proliferation and differentiation.

1.5.4.2 Dystroglycan connects the extracellular matrix to the intracellular cytoskeleton

Dystroglycan (DG) is an essential component of the Dystrophin associated protein complex (DAPC) in skeletal muscles. DG is a trans-membrane receptor composed of two peptide chains, α and β, derived from a common polypeptide encoded by the *Dag-1* gene (Ibraghimov-Beskrovnaya et al. 1993). α and β chains are highly

glycosylated and are attached to each other non-covalently. The α chain contains Laminin binding sites and the β chain binds to the Dystrophin-associated protein complex (DAPC) (Yu and Talts 2003). Components of the DAPC include DG itself, Dystrophin, Dystrobrevin, which associates with the C-terminal of Dystrophin, Syntrophins and the transmembrane proteins Sarcoglycans and Sarcospan (Bredt 1999; Winder 1997). Dystroglycan is expressed throughout muscle development and is present in the subdomains of the myofibre sarcolemma (Durbeej et al. 1998). Mutations that affect DG activity cause dystroglycanopathies (Brancaccio 2005).

DG links the extracellular matrix to the intracellular cytoskeleton and this linkage is essential for muscle growth and maintenance as DG-Laminin interaction facilitates also the deposition of other basement membrane proteins (Henry and Campbell 1998). Laminin domains LG4-5 bind to α-DG (Talts et al. 2000), although Laminin α2 LG1-3 possess additional binding sites for DG, which interact with α-DG with an affinity 4 times higher than the LG4-5 domains (Talts et al. 1999). However, Laminin α1 LG1-3 domains do not bind to DG (Talts et al., 1999). α-DG is heavily glycosylated and contains several N-linked glycans at its N and C terminal domains and O-glycosylation in the central mucin-like domain, which bind to Laminin (Ibraghimov-Beskrovnaya et al. 1993; Tisi et al. 2000). The specificity of ligand binding depends on the glycosylation pattern and Dystroglycan binds to different proteins based on the glycans attached to α–DG and β-DG (Huang et al. 2000).

Alpha-DG not only transduces the signals received from Laminins to the cytoplasm, it is also required for the deposition of Laminin at the surface of myotubes (Montanaro et al. 1999). Reduced levels of α-DG increase apoptosis in myotubes, suggesting a role for α-DG in the maintenance of myotube viability through the deposition of Laminin (Montanaro et al. 1999). As mentioned earlier, glycosylation is important for DG function. The main glycan that binds to α-DG is O-mannosylglycan and aberrant glycosylation of α-DG results in muscular dystrophies (Endo 2005; Michele et al. 2002). In *myd* (myodystrophy), mutations in the *LARGE* gene that encodes a

glycosyltransferase, results in altered post-translational modifications of α-DG leading to impaired interactions with the ECM (Michele et al. 2002). *Myd* is a model for human congenital muscular dystrophy MDC1D (Longman et al. 2003). In *myd* mice, defects in α-DG alter also the satellite cell niche and the regenerative potential of satellite cells (Ross et al. 2012).

Beta-DG interacts with Dystrophin and this interaction is mediated by tyrosine phosphorylation (Ilsley et al. 2001). This interaction initiates the downstream signaling (Langenbach and Rando 2002). Laminin binding to DG activates the PI3K/Akt pathway, which mediates cell survival (Langenbach and Rando 2002). Laminin signaling via Dystroglycan activates a sequence of events involving Dystroglycan-Syntrophin-Grb2-Sos-Rac1-Pak1-Jnk that ultimately results in the phosphorylation of c-Jun mediated by Src interaction (Zhou et al. 2006). Interestingly, in C2C12 muscle cells, lower concentration of Laminin α1 causes proliferation while higher concentration causes apoptosis in myoblasts (Zhou et al. 2012), suggesting a concentration dependent activity of Laminin α1 through DG. RhoA, Rac1 and cdc42, which are downstream signalling molecules of the DGC, are involved in regulating the expression of MyoD and Myogenin (Chockalingam et al. 2002) and down-regulation of these signalling molecules is observed during muscle atrophy (Chockalingam et al. 2002). The cytoplasmic tail of β-DG was also shown to interact with muscle specific Caveolin-3, which competes with Dystrophin, and overexpression of Caveolin-3 leads to muscular dystrophy (Galbiati et al. 2000; Sotgia et al. 2000). Disruption of Dystroglycan also detaches the basal lamina from the sarcolemma and muscles become more sensitive to contraction-induced injuries (R. Han et al. 2009). Thus, Dystroglycan not only provides a structural support but is also involved in muscle cell survival, proliferation, and in regulating differentiation genes and hence acts as one of the key receptors facilitating muscle cell function.

1.5.5 Laminin distribution

Laminins are expressed in a tissue specific manner throughout embryonic development and in adults (see Table 1.3). Laminin α subunits exhibit a more

restricted pattern when compared to Laminin β and γ subunits. In general epithelial tissue basement membranes are composed of α1, α3 and α5, whereas α2 and α4 are mostly associated with tissues derived from mesenchymal and endothelial origin (Tunggal et al. 2000).

The first basement membranes to form during development are the embryonic membrane that separates the epiblast from the embryonic ectoderm and the Reichert's membrane that separate the parietal endoderm from the trophoblasts, and both are made up of Laminin-111 and Laminin-511 (Leivo et al. 1980). Laminin α1 protein is first detected at the morula stage in the form of Laminin-111 (Cooper and MacQueen, 1983) and continues to be expressed along with Laminin α5 in the Reichert's basement membrane and the embryonic basement membrane (Miner et al., 2004). *Lama1* and *Lama5* encode for Laminin α1 and Laminin α5. *Lama1* mRNA is detected very early at the 2-4 cell stage (A. R. Cooper and MacQueen 1983). Thereafter, *Lama5* is expressed prior to gastrulation (Miner et al. 2004). *Lamb1* and *Lamc1* are ubiquitously expressed and thus mutations in these genes result in early embryonic lethality due to the absence of embryonic and Reichert's membranes (Miner et al. 2004). In contrast, *Lama1* null embryos survive until E7 mostly because the embryonic basement membrane forms, suggesting that *Lama5* compensates for the loss of *Lama1* (Miner et al. 2004). In *Lama5* null embryos, the embryonic basement membrane is formed, suggesting that Laminin α1 can compensate for the absence of Laminin α5 (Miner and Yurchenco 2004). However in *Lama5* null embryos, loss of *Lama5* causes defects in vascularization, intestinal smooth muscle differentiation and digit separation, which correlates with the expression patterns of *Lama5* in these tissues (Miner et al. 1998).

Table 1.3: Laminin expression of Laminin isoforms and their receptors

Laminin isoform	Tissue expression	Receptor	Null phenotype	Selected References
Laminin-111, -121	Early embryo, adult kidney, liver, ovary and testis, brain	Integrins α1β1, α2β1, α6β1, α7β1, α9β1, α-DG, syndecans	Deletion of α1: Preimplantation lethality (E7). Deletion of γ1: Peri-implantation lethality with failure of blastocyst differentiation	Yurchenco et al., 2004, Sasaki and Yamada, 1987
Laminin-211	Skeletal muscle, cardiac muscle, heart, nerve	Integrins α1β1, α2β1, α6β1, α7β1, α-DG, syndecans	Deletion of α2 in the adult leads to muscular dystrophy	Miyagoe et al., 1997
Laminin-221	Neuromuscular junction, heart, nerve	Integrins α1β1, α2β1, α6β1, α7β1, α-DG, syndecans	Deletion of α2 in the adult leads to muscular dystrophy	Miyagoe et al., 1997
Laminin-3A32, - 3B32	Placenta, mammary glands, skin, uterus, lung,	Integrin α3β1, α6β1, α6β4, syndecans	Deletion of α3: Neonatal lethality. Deletion of β3: Junctional epidermolysis bullosa	Ryan et al., 1999, Pulkkinen et al., 1995
Laminin-411, -421, -423	Vascular endothelial cells, peripheral nerves, white blood cells	Integrin α6β1, α7β1, syndecans	Deletion of α4: Embryonic and neonatal haemorrhage	Thyboll et al., 2002
Laminin-511, -521, - 523	Mature epithelium and endothelium, smooth muscles, glomerular basement membrane	Integrin α2β1, α3β1, α6β1, α6β4, α7β1, αvβ3, α-DG, syndecans, Lutheran blood group glycoprotein	Deletion of α5: Embryonic lethality at E16.5. Deletion of β1: Embryonic lethality during implantation Deletion in β2: Defects in NMJ and renal glomerulus	Miner et al., 1998 Miner et al., 1995 Hunter et al., 1989 Miner et al., 2004

1.5.5.1 Expression of Laminins in skeletal muscles

In skeletal muscles, different Laminin subunits are expressed during embryonic development and in adult. The expression of Laminins also varies in different functional domains. For instance, synaptic and extrasynaptic regions of the adult myofibre contain different Laminin α subunits (see Table 1.4)

Table 1.4: Laminin expression in mouse skeletal muscles

Laminin subunit	Embryo-Fetal	Adult	Reference
α1	Somites	Not detected	(C. Anderson et al. 2009)Yurchenco et al. 2004; Sztal et al. 2011
α2	Expressed from E11.5	S, MTJ and NMJ	(Patton et al. 1997)
α3	Not detected	-	-
α4	E15: MTJ; Fetal: S, From E15: NMJ	NMJ	(Miner et al. 1997; Sztal et al. 2011)
α5	Somite-Fetal: MTJ; Embryo-fetal: S; Embryo-fetal:NMJ	NMJ	(Anderson et al., 2009; Miner et al. 1997; Yurchenco et al. 2004)
β1	Somites-Fetal:MTJ; E15:NMJ; Embryo-fetal:S	S	(Schuler and Sorokin 1995; Yurchenco et al. 2004; Sztal et al. 2011)
β2	Fetal:MTJ; Embryo-fetal: NMJ	NMJ	(Yurchenco et al. 2004; Sztal et al. 2011)
β3	Not detected	-	-
γ1	E-fetal: MTJ, S and NMJ	S, MTJ, NMJ	(Schuler and Sorokin 1995; Yurchenco et al. 2004; Sztal et al. 2011)
γ2	Not detected	-	-
γ3	Embryo	Adult	(Gersdorff et al. 2005; Sztal et al. 2011)

S: Sarcolemmal basement membrane; MTJ: Myotendinous junction;

NMT: Neuromuscular junction

Laminin expression is dynamic during muscle development. In E9.5-10.5 mouse embryos, *Lama1* is expressed in the sclerotome, ventral neural tube, pre-somitic mesoderm and promesonephros, whereas *Lama5* is expressed in the dermomyotome, notochord and the promesonephros (C. Anderson et al. 2009). Consistent with the pattern of *Lama1* and *Lama5* expression, Laminin-111 and Laminin-511 are detected in the myotomal basement membrane during early development of muscles and then in the basal lamina surrounding muscle fibres until the onset of secondary myogenesis at E11-12 (C. Anderson et al. 2009; Patton et al. 1997) (see Fig 1.11). Coinciding

with the onset of secondary myogenesis, *Lama2* begins to be expressed and Laminin $\alpha 2$ is deposited in the basal lamina, first in the central region of the muscles together with Laminin α5, leaving Laminin α1 confined to the ends of myotubes (Patton et al. 1997). Starting at E11.5, Laminin α4 and β2 levels increase rapidly such that by E15 both Laminins are distributed throughout the myofibre with a higher concentration at the synapses (Patton et al.,1997). Further maturation of the muscle basal lamina occurs peri-natally with a sharp decrease in Laminins α4, α5 and β2 outside the synaptic domain and a restriction at the level of the synapse (Patton et al.,1997) (see Fig 1.11). As muscles reach adult stage, Laminin α2 becomes the major Laminin expressed in the muscle fibre basal lamina (W. Kuang et al. 1998; Patton et al. 1997). Although the non-synaptic region of the myofibre is made of Laminin-211, the basal lamina at the synaptic cleft is rich in Laminin-221, -421 and -521 (Patton et al. 1997). β2 appears at these synaptic site and becomes part of Laminin trimer-211, 421 and 521 (Miner 2008). Laminin α4 is present in Schwann cells and primary cleft, but is absent in basement membrane of junctional folds whereas α5 is absent in Schwann cells (Patton et al. 1999). β1 was present in Schwann cells and extrasynaptic region but absent in synaptic cleft (Sanes et al. 1990). Laminin α4 mutant mice are viable but lack the junctional fold where neurotransmitters are released from presynaptic to postsynaptic membranes (Patton et al. 2001). Likewise, in double knockout mice for Laminin α4 and Laminin α5 presynaptic abnormalities and delay in postsynaptic maturation was observed (Nishimune et al. 2008a). Laminin β2 at the synaptic-cleft is involved in presynaptic differentiation whereas Agrin, a proteoglycan at the NMJ, promotes postsynaptic differentiation during synapse formation (Noakes et al. 1995). Laminin β2 binds directly to calcium channels that are required for the release of neurotransmitters from motor nerve terminals (Nishimune et al. 2004). It has been shown that in Laminin β2 mutant mice, NMJ fails to mature and the mice die at 2 weeks of age (Patton et al. 1998). Thus, the Laminin composition of the synaptic and extrasynaptic basement membranes differs and display a dynamic distribution, which

is essential for the correct function of the neuro-muscular junction in adult skeletal muscles.

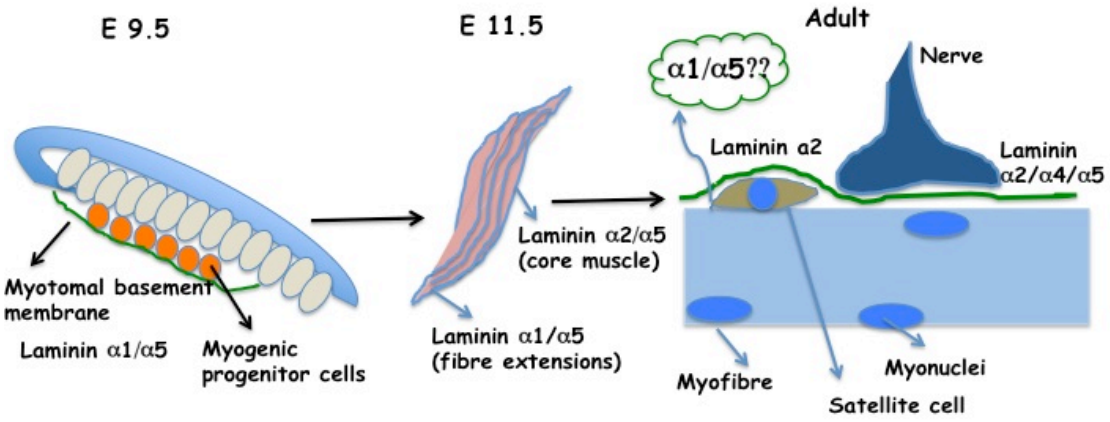


Figure 1.11: ***Expression of Laminin alpha subunits in skeletal muscles:*** Laminin distribution in embryonic and adult muscles showing a Laminin α1 and α5–containing BM at E9.5. At E11.5, progressive replacement of Laminin α1 and α5 by Laminin α2. Laminin α2 is present at the extra-synaptic BM in the adult, whereas the synaptic BM is composed of Laminins α2, α4 and α5.

1.5.6 Laminin function in embryonic muscle development

The first basement membrane formed during myogenesis in the embryo is the myotomal basement membrane (Tosney et al. 1994). Laminin-111 and -511 are the main Laminins in the myotomal basement membrane (C. Anderson et al. 2009; Tiger et al. 1997). Disruption in the myotomal basement membrane assembly such as in Shh-null, *Myf5*-null, *Paraxis*-null or in *Dmrt2*-null embryos leads to aberrant migration and differentiation of myogenic progenitor cells (C. Anderson et al. 2009; Seo et al. 2006; Tajbakhsh et al. 1996). Further studies showed that Shh signalling is required for *Lama1* expression in somites, and exogenously applied Laminin-111 to Shh/Gli3 mutant embryo is sufficient to restore myotomal BM assembly (Anderson et al., 2009). This suggests that the assembly of the myotomal basement membrane requires Laminin-111, which is regulated by Shh, and that in the absence of Laminin-111, Laminin-511 cannot compensate (C. Anderson et al. 2009). Most importantly, Shh also induces the activation of Myf5 in the dermomyotome. Myf5-positive muscle progenitor cells translocate to the myotome and up-regulate the Laminin-111

receptors Integrin α6β1 and Dystroglycan, which in turn cluster Laminin-111 molecules to facilitate their deposition into the myotomal basement membrane (C. Anderson et al. 2009; Bajanca et al. 2004a; Bajanca et al. 2006). Bajanca et al (2006) showed that blocking Laminin interaction with Integrin impairs myotomal BM assembly and results in aberrant differentiation of myogenic progenitor cells.

Thus, Laminin α1 is required for the assembly of the basement membrane associated with the first muscle to form in the embryo, the myotome. As development proceeds, a remodelling of the muscle basal lamina occurs leading to the incorporation of Laminin α2 by the end of primary myogenesis at E11.5 (Patton et al. 1997) and by birth Laminin α1 and α5 are not detected in the extra-synaptic regions of muscles (Patton et al. 1997; Schuler and Sorokin 1995). However, none of the studies have investigated in great details to Laminin composition of the muscle basement membrane during in fetal development. Our previous data show that Laminins involved in the muscle basal lamina are not necessarily produced by muscle cells (C. Anderson et al. 2009) and it should be interesting to re-investigate the expression of *Lama1* and *Lama5* in fetal, neonatal and in adult muscles.

1.5.7 Laminin function in adult muscles

The predominant Laminin in adult skeletal muscles is Laminin α2, which forms Laminin-211 in combination with Laminin β1 and γ1 (Leivo and Engvall 1988). Mutations in the gene encoding Laminin α2 results in congenital muscular dystrophies (CMD). Complete loss of Laminin α2 leads to a severe phenotype, whereas partial deficiency may lead to a milder phenotype (Geranmayeh et al. 2010). Laminin α2 deficiency, which causes congenital muscular dystrophy (MDC1A), was first identified in 1994 by Tome (Tome et al. 1994). Later, the causative recessive mutation in *Lama2* gene was identified (Cohn et al. 1998). The disease mechanism has been studied in *Lama2* mutant mice, known as *dystrophia muscularis* (*dy*) (Michelron 1955) mice (H. Xu et al. 1994b; H. Xu et al. 1994a). Dystrophy in *Lama2* mutants is compounded by defects in myelination and the muscle fibre basal lamina. As Laminin α2 is present in the heart and central nervous system (CNS), these tissues

are also affected (Leivo and Engvall 1988). Further mouse models were generated: dy^{w}/dy^{w}, which expresses small amounts of truncated Laminin α2 and dy^{2J}/dy^{2J}, which carries a mutation in the N-terminal domain (LN) that leads to abnormal splicing and reduced expression of Laminin α2 (Allamand et al. 1997). A null mutant for Laminin α2, dy^{3k}/dy^{3k} was generated which displays severe muscular dystrophy symptoms and growth retardation (Miyagoe et al. 1997).

The receptors that interact with Laminin networks connecting them to cytoskeleton and intracellular signaling are Integrins and Dystroglycans (Ervasti and Campbell 1993; Vachon et al. 1997). The main Integrin in adult skeletal muscles is Integrin α7β1. In mouse and humans, Integrin α7 is present in the form of α7A and α7B (Burkin and Kaufman 1999; W. K. Song et al. 1992). Integrin α7Aβ1 is expressed throughout the myofibre sarcolemma, whereas Integrin α7Bβ1 is expressed at the neuromuscular junction and myotendinous junctions (Mayer 2003). Mutations in *ITGA7* that encodes Integrin α7 have been identified in human patients with muscular dystrophy (Hayashi et al. 1998). Null mice for *Itga7* are not affected during myogenic development but a progressive muscular dystrophy is observed soon after birth (Mayer et al. 1997). This suggests an indispensable role for Integrin α7 in linking muscle fibres and the ECM (Mayer et al. 1997). Further role of Integrin α7 have been identified by generating Integrin α7/Dystrophin double knockout (DKO) mice (C. Guo et al. 2006; Rooney et al. 2006). Muscle fibres from such mice exhibit extensive loss of muscle membrane integrity, greater necrosis and increased muscle degeneration compared to single knockout. Moreover, Integrin α7 regulates Laminin deposition on myoblasts by binding to β1-Integrin binding proteins (J. Li et al. 2003), suggesting that Integrin α7 may also directly promote satellite cell activity. Overall, a direct role for Laminin-211-Integrin α7β1 in maintaining muscle functional integrity of the muscle have been identified from above studies.

Another receptor to which Laminin α2 binds is Dystroglycan. The interaction of Laminin with DG occurs via α–DG (Talts and Timpl 1999). Null mutations in Dystroglycan encoding gene, *Dag1* is lethal at the embryonic development

(Williamson et al. 1997). However, chimeric mice generated with ES cells targeted for DG alleles develop a progressive muscle pathology and disrupted neuromuscular junctions (Cote et al. 1999). Dystroglycan binding to Laminins requires phosphorylation of O-mannosyl Glycans and LARGE is crucial for further modification (Yoshida-Moriguchi et al. 2010). It has been demonstrated that in patients with limb-girdle muscular dystrophy, a missense mutation in *DAG1* interferes with LARGE-dependent maturation of α–DG O-mannosyl Glycans phosphorylation leading to a defect in the binding of Laminin-211 (Yoshida-Moriguchi et al. 2010). Genes that encode for glycosyltransferases are POMT1, POMT2, POMGnT1, LARGE, FKTN and FKRP and mutations of these genes cause also the detachment of Dystroglycan from Laminin-211 and result in secondary dystroglycanopathies (Mercuri et al. 2009). In conditional DG mutant mice that lack Dystroglycan in muscle cells a detachment of the basal lamina from the fibre sarcolemma is observed, which does not occur in Itga7 knockout mice, suggesting a specific role for Dystroglycan in anchoring the BM to the myofibre sarcolemma (R. Han et al. 2009).
In vitro studies show that addition of Laminin α2 to myoblast cell lines promotes myotube stability by preventing apoptosis (Vachon et al. 1996). Laminin-211 is thus important for maintenance and stabilization of muscles (W. Kuang et al. 1998; Vachon et al. 1996). Thus, by interacting with its receptors Integrin and Dystroglycan, Laminin-211 maintains muscle integrity and muscle cell behavior.

1.5.8 Laminin therapies

Injection of Laminin-111 in Integrin α7–null mutant mice after cardiotoxin-mediated muscle injury restores Integrin α7 muscle regeneration suggesting a role for Laminin-111 in muscle repair (Rooney et al. 2009b). The mechanism that underlies Laminin α1–mediated rescue of the dystrophic phenotype remains unknown. However, because Laminin α1 is structurally similar to Laminin α2, it is possible that its incorporation into the muscle basement membrane compensates for the lack of Laminin α2. Overexpression of Laminin α1 chain rescues also dystrophic muscles in dy^{3k}/dy^{3k} mice, suggesting that Laminin α1 can compensate the loss of Laminin α2

and can effectively alleviate muscle damage in dystrophic muscles (K. Gawlik et al. 2004). Nevertheless, there are notable differences between Laminin α1 and Laminin α2 chain. Laminin α2 binds more effectively to Dystroglycan than Laminin α1 (Talts et al. 1999) and myoblasts spread significantly faster on Laminin α2 than on Laminin α1 (Schuler and Sorokin 1995; Talts et al. 1999; Vachon et al. 1996). In spite of these differences, many studies in the last few years have extensively demonstrated rescue of dystrophic phenotype by Laminin-111 (K. I. Gawlik and Durbeej ; K. Gawlik et al. 2004; K. I. Gawlik and Durbeej 2010; Goudenege et al., 2010; Rooney et al. 2009b; Van Ry et al. 2013). Transgenic expression of Laminin α1 in Laminin α2–deficient mice was able to compensate for the loss of Laminin α2 by incorporating into the myofibre basal lamina, resulting in amelioration of the dystrophic phenotype and increased life span (K. Gawlik et al. 2004; K. I. Gawlik and Durbeej 2010). In contrast, transgenic overexpression of Laminin α1 does not rescue the dystrophic phenotype of *mdx* mice, although Laminin α1 was deposited in the muscle basement membrane (K. I. Gawlik et al. 2011). On the other hand, intramuscular-injection of Laminin-111 protein in *mdx* and in Laminin α2-deficient mice up-regulates the expression of Integrin α7 and restores sarcolemmal stability (Rooney et al. 2009a; Rooney et al. 2012; Van Ry et al. 2013). Furthermore, when injected as a co-adjuvant with myoblasts, Laminin-111 increases muscle strength in *mdx* (Goudenege et al. 2010). A possible explanation for the discrepancy between studies is that commercially available Laminin-111 may undergo fragmentation upon injection exposing the epitopes for receptor binding when compared to the full-length Laminin overexpression.

Thus, Laminin α1 is capable of rescuing the dystrophic phenotype in mouse models of CMD, probably by interacting with Integrin receptors. However, none of these studies have identified the mechanism by which Laminin α1 can compensate for loss of Laminin α2 or Integrin α7.

1.5.9 Hypothesis

The essential role of Laminin-111 in myotomal basement membrane assembly (C. Anderson et al. 2009) and its role in adult dystrophic muscles (K. Gawlik et al. 2004; K. I. Gawlik and Durbeej 2010; Rooney et al. 2009a; Rooney et al. 2012) are suggestive of a specific function of Laminin-111 in the control of muscle progenitor cell behavior in the embryo and in adults. The Laminin composition of the basement membrane surrounding embryonic and adult muscles appear to be more dynamic than initially predicted with distinct composition at the neuromuscular junction in adult muscles and during the transitions from embryonic to fetal muscle development (Patton et al. 1997). There is also increasing evidence that basal lamina remodeling takes place in muscular dystrophies. Indeed, in *Lama2*-deficient mice (dy/dy mice), the extra-synaptic BM re-expresses high levels of Laminin α4 and lower levels of Laminin α5 (K. Gawlik et al. 2004; Patton et al. 1997; Sewry et al. 1995). In human CMD patients, up regulation of Laminin α5 was also observed (Tiger et al. 1997). Laminin α1 expression is restricted in adult tissues and is mostly associated with epithelial basement membranes and absent from adult normal or dystrophic skeletal muscles (Falk et al. 1999; Tiger et al. 1997). However, adult muscles appear to contain micro-domains in which the Laminin composition differs (for instance, at the synaptic regions Laminin-211 is replaced by Laminin-221, -421 and -521) (Miner et al. 1997; Patton et al. 1999; Sanes et al. 1990). It is plausible that the distinct Laminin composition at different micro-domains may allow for specific signaling. Consistent with this idea, data suggest that skeletal muscle myonuclei are not equivalent and micro-domains are present within muscle BM which has been identified with distinct gene expression (D. L. Allen et al. 1999).

Thus, one can hypothesize that the basement membrane at the satellite cell niche may also contain distinct Laminins that support the myogenic programme in adult muscles. Here, I hypothesized that the Laminin composition of the basal lamina overlying satellite cells differs from the remaining of the fibre in adult muscles, and that a remodeling takes place to support satellite cell transition from quiescence to activation during muscle regeneration. Thus, this project had three main objectives:

1. To demonstrate that the basement membrane around satellite cells has a distinct Laminin content that modulates satellite cell activity *ex-vivo*.
2. To assess whether basal lamina remodelling occurs during muscle regeneration in acute and chronic injury models *in-vivo.*
3. To examine whether the Laminins expressed during basal lamina remodeling signal through specific receptors.

The expression and requirement of Laminin α1 as one of the key Laminin subunits in the embryo and in dystrophic phenotype rescue experiments in adult mice models, makes it a potential candidate involved in muscle regeneration. Hence, identifying the role of Laminin α1 in the adult muscle both at physiological and pathological level will help to understand the required components of satellite cell niche during muscle regeneration.

Chapter 2

Materials and Methods

2.1 Mouse models used

Animal husbandry was employed at biological services at University of Sheffield. Two mouse strains were used for all the work wild type C57BL/6 and DMD mouse model *mdx*. *Mdx* mice strain was kindly provided by Dr. Gaynor Miller. And this strain was bred on C57BL/6. Male and female mice of both strains are used in all the experiments.

2.2 Isolation, culturing and staining of single EDL muscle fibres

2.2.1 Harvesting Tibialis anterior (TA) and Extensor digitorum longus (EDL) muscles

Tibialis anterior and extensor digitorum longus muscles were harvested using the procedure described below. TA muscles were used for cryosectioning and EDL muscles were used either for cryosectioning or for isolating single myofibres. For the purpose of experiments done on single muscle fibres, EDL muscles were harvested from 6-8 week old C57BL/6 mice. Firstly, the mice were killed by cervical dislocation and 70% ethanol was sprayed on the hind limb. Using a scalpel hair was carefully removed to expose the skin. With the help of the fine scissors the entire length of the skin was cut and peeled off to expose the muscles. The animal was pined (face up) on a thermocol board and visualized under the dissecting microscope. The superficial thin fascia was carefully removed by forceps. EDL muscle was then identified visually just beneath the Tibialis Anterior (TA) muscle. Four thin EDL tendons and one thick TA tendon were located at the paw and now using the forceps the distal tendons of both EDL and TA muscles were exposed and cut carefully by Cohann-Vannas spring scissors. The tendons were carefully looped out from the paw up to the ankle. To further expose the EDL muscle TA muscle was pulled away from the underlying musculature by holding the TA tendon and cutting at the proximal end. The proximal tendons of the EDL muscle were located and cut using the scissors to free the muscle. Carefully holding the distal tendons EDL muscle was gently removed and placed in freshly prepared collagenase for further procedure.

2.2.2 Isolation of single EDL muscle fibres

Ten minutes prior to isolation, 0.2% Collagenase was freshly prepared in DMEM+glutamax media with 1% Penicillin/streptomycin/fungicide (PSF) sterile filtered using 0.2μm filter and kept at 37°C. Four plastic petri dishes (60*15mm) were rinsed with sterile filtered 5% BSA prepared in PBS. One dish was used to dissociate the muscle and the other three for serial washes. 5ml of DMEM media was added to all the four dishes and kept at 37°C in a 5% CO_2 for prior warming. For fibre isolation two Pasteur pipettes were prepared using a diamond pen. One with large bore for muscle handling and dissociation of the muscle and the other with small bore for picking the single myofibres. Both the pipettes were heat polished to smooth pipette's edge.

After harvesting the muscle as described in section 2.3, the EDL muscle was immediately placed in pre warmed collagenase and incubated for 60-90 min at 37°C in a 5% CO_2. After incubation the muscle was carefully placed in the pre warmed media in the petri dish. Using the large bore pipette, the muscle was flushed with warm media to release the single fibres under the dissecting microscope. The separated live single fibres were then gently transferred into the second dish for washing to remove the other non-muscle cells and connective tissue. One fibre at a time was transferred to ensure that fibres are not damaged. The process was repeated with two more dishes to obtain clean single fibres. In the whole process the dishes with the media were not kept at room temperature for more than 10min. The isolated single fibres were immediately fixed in 4% PFA for quiescent satellite cells or cultured *ex-vivo*.

2.2.3. *Ex-vivo* culturing of single EDL myofibres

Prior to culturing the single EDL muscle fibres, three petri dishes were rinsed with 5% BSA and 5ml of complete media was added in each dish. The complete media was prepared by adding 10% horse serum and 0.5% of chick embryo extract to DMEM plain media (DMEM+1% PSF). Single fibres were isolated after wash three as described in section 2.4 and placed in the dish with complete media.

Approximately 25-30 fibres were added to each dish and incubated for 24, 48 and 72hrs at 37°C in 5% CO_2.

2.2.4 Immunohistochemistry on single EDL myofibres

Live single fibres from 0hr or from the *ex-vivo* culture were collected in a 2ml eppendorf tube, the media removed and the fibres were washed with 1X PBS before fixing. They were fixed in pre warmed 4% PFA for 6 minutes and then washed twice with 1X PBS. Fibres were then permeabilized with 0.5% Triton X100 for 8minutes and washed twice (5minutes each) with 1X PBS. Blocking was performed by adding 500µl of 20% horse serum to the fibres for 1hr at room temperature. Appropriate primary antibody (250µl) diluted in PBS was added and incubated overnight at 4°C. To remove the unbound antibody fibres were washed thrice with 0.05% Triton X100 in 1X PBS. Fibres were then incubated in secondary antibody for 1hr 30minutes at room temperature on the shaker. After incubation the fibres were washed twice with 1X PBS and stored in PBS. Using the small bore pipette, the labelled fibres were carefully placed on a glass slide and the excess PBS was removed. Finally mounting media with DAPI (25µl) (VectaSheild) was added on the glass slide at room temperature and coverslip was carefully placed on the slide. The final concentration of DAPI in 25µl is 37.5ng/ml. The slides were then visualized using a fluorescence microscope. Primary and secondary antibodies used are listed in table 2.1.

2.2.5 Matrigel-coated plates

To extract RNA from satellite cells and myofibres (without satellite cells), satellite cells were separated from myofibres on Matrigel. 24 well-plates and glass pipettes were placed in -20^{o}C overnight. Matrigel aliquots were thawed overnight to avoid formation of lumps. Thawed Matrigel aliquots were diluted at a ratio of 1:15 (Matrigel: DMEM plain media) and used at a final concentration of 0.66mg/ml. 500-600µl of Matrigel was placed in each well of 24 well-plate. Matrigel-coated plates were incubated overnight at 4^{o}C or for 1-2 hrs at room temperature. Then, freshly isolated myofibres were slowly placed on the Matrigel-coated plates and incubated at

37°C in 5% CO_2. After 48 hrs, using a glass pipette myofibres were slowly isolated into a sterile eppendorf from the separated satellite cells (there is a possibility that some of the satellite cells may still remain on the fibres) by observing under the microscope and washed twice with cold PBS at 4°C. Pellet of both myofibre and satellite cells was collected for further processing. Finally, satellite cells in Matrigel were collected in a sterile eppendorf and washed twice at 4°c with cold PBS. RNA was extracted from both myofibres (devoid of satellite cells) and satellite cells as described in section 2.4.1.

2.3 Muscle transverse sections

2.3.1 Freezing muscles for cryostat sectioning

Tibialis anterior (TA) and Extensor digitorum longus (EDL) muscles were harvested as described in section 2.2.1. After harvesting the muscles were immediately fixed in either 4% PFA or 2% PFA depending on the antibodies used for labelling the muscles. When 4% PFA was used the muscles were fixed at 4°C for 2hrs and then washed twice with 1X PBS. The muscles were then incubated in a series of sucrose solution. Initially muscles were kept in 20% sucrose for overnight and then 30% sucrose for few hours before freezing. Liquid nitrogen cooled isopentane was used for freezing the muscles. Briefly, the muscles were dipped in OCT(BDH) by holding the muscle tendons to completely cover the whole muscle and then placed in liquid nitrogen cooled isopentane in a beaker. Frozen muscles were stored at -80°C in a cryo vial.

Muscles were mounted on dry-ice chilled chucks using OCT compound (BDH) and 7μm sections were prepared using a cryostat (Bright Instruments). Sections were collected onto superfrost slides (Menzel-Glaser) and were either prepared immediately for immunohistochemistry or kept at -20°C until further use. When 2% PFA with 0.5% triton was used to fix muscles, then muscles were washed all the day

with 1XPBS before incubating in a diluted series of sucrose solution. The rest of the procedure is similar to the one described above for 4%PFA.

2.3.2 Cardiotoxin injections

Cardiotoxin injections were performed on C57BL/6 mice to confer acute local muscle injury. Initially, the mice were anaesthetized using isoflurane. Cardiotoxin (CTX) from *Naja mossambica* (sigma) was prepared at a concentration of 10μM and 50μl was used for intramuscular injections of cardiotoxin or PBS (control) into TA muscle using 30G Hamilton syringes. Injections were performed by Dr.Anne-Gaelle Borycki.

2.3.3 Exercise protocol for *mdx* mice

The wild type, C57BL/6 and *mdx* mice (4 mice per strain) were underwent a 17 day run on a saucer wheel. Briefly, a metal mouse wheel was placed on the cage floor and a sensor of the bicycle pedometer was attached to the back of the cage as shown in the Fig 2.1. The average daily distance ran was noted from the bicycle pedometer. The training protocol started at the mouse age 4 weeks. At the end of 17 days the mice were sacrificed and *in vivo* experiments were started.

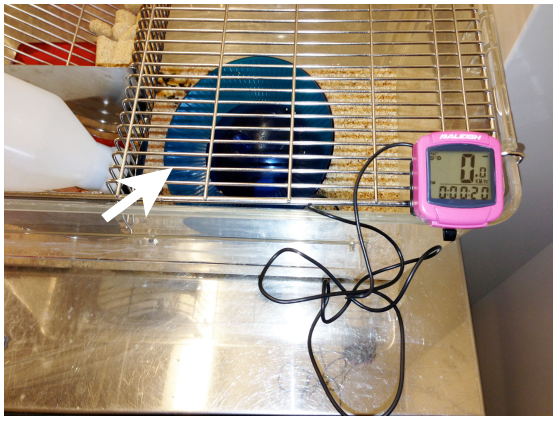


Figure 2.1: Mice exercise on saucer wheel*:* Phtographs and video showing the arrangement of sacucer wheel (free-wheel) in the mice cage for mice exercise activity. (A) Saucer wheel (white arrow) is palced on the floor of the cage and is connected to abicycle prdometer to record the disatnce travelled by the mice. (B) Video of an *mdx* mice runing on the saucer wheel (kindly provided by Dr. Andy Grierson, Departmnet of Neuroscience, University of Sheffield).

2.3.4 Immunohistomchemistry on muscle cryosections

Fresh sections were rehydrated in blocking solution (5% BSA, 2% heat-inactivated goat serum, 2% fetal bovine serum (FBS)+ 0.05% Triton X100 in PBS) at room temperature for 1 hour in a humidified chamber. Frozen sections were dried for an hour and then rehydrated. Appropriate primary antibody diluted in PHT (1% heat inactivated goat serum+ 0.05% Triton X100) was added and incubated overnight at 4°C in a humidified chamber. After incubation the slides were washed thrice (5min each) with PHT to remove excess unbound primary antibody. Secondary antibody diluted in PHT was added and incubated for 1 hour in dark in a humidified chamber. Slides were then washed twice (10min each) with PHT. Finally mounting media with DAPI (VectaSheild) was added on the glass slide and coverslip was carefully placed on the slide. The slides were then visualized using a fluorescence microscope. Primary and secondary antibodies used are listed in table 2.1.

2.3.5 Imaging

Slides were initially observed under Olympus BX51 microscope with X-cite 120 illumination system (EXFO, Quebec, Canada). Images were taken using Zeiss Apotome with Axiovision imaging system. Images were assembled using Photoshop CS version 6. For counting centrally located nuclei in both mdx and CTX-injured muscles and for measuring fibre size of CTX-injured muscles, ImageJ 1.38x software was used. 5 random sections of each muscle were chosen to image and quantitate (making sure to include the areas where degeneration and regeneration of the muscle is evident).

Table : List of antibodies

Name	Source	Catalogue number	Company	Dilution
Primary antibodies				
Caveolin-1	Rabbit polyclonal	sc-894	Santa Cruz	1:400
M-Cadherin	Goat polyclonal	sc-6470	Santa Cruz	1:500
c-Met	Rabbit polyclonal	sc-162	Santa Cruz	1:50
Pax7	Mouse monoclonal	Pax7 supernatant	DSHB	1:20
MyoD	Rabbit polyclonal	sc-304	Santa Cruz	1:1000
Myogenin	Mouse monoclonal	Myogenin	Santa Cruz	1:50
Myf5	Rabbit polyclonal	sc-302	Santa Cruz	1:2000
Laminin alpha 2	Rat	mAb (4H8-2)	Enzo	1:200
Laminin alpha 1	Rat monoclonal	MAB-1903	Chemicon	1:200
Laminin alpha 1	Rat monoclonal	sc-65645	Santa Cruz	1:100
Integrin alpha6	Rat monoclonal	MCA699	AbD SeroTec	1:40
Secondary antibodies				
Alexa 488	Goat anti-rabbit IgG	A11034	Molecular Probes	1:500
Alexa 633	Goat anti-rabbit IgG	A21070	Molecular Probes	1:500
Alexa 488	Donkey anti-rabbit IgG	A21206	Molecular Probes	1:500
Alexa 594	Goat anti-rabbit IgG	A11037	Molecular Probes	1:500
Alexa 594	Donkey anti-goat IgG	A11058	Molecular Probes	1:500
Alexa 488	Donkey anti-mouse IgG	A21202	Molecular Probes	1:500
Alexa 488	Donkey anti-rat IgG	A21208	Molecular Probes	1:500
Alexa 594	Goat anti-mouse IgG	A11005	Molecular Probes	1:500
FITC	Goat anti-rat IgG	112-095-003	Jackson immunoresearch	1:200
Conjugated antibodies				
F4/80	Rat monoclonal	ab105155	Abcam	1:100

2.4 General molecular biological methods

2.4.1 Total RNA isolation from whole muscle and the single fibres

Total RNA was isolated according to the protocol of Invitrogen for Trizol reagent. RNA isolation was performed in sterile and with RNAase free reagents. Whole EDL or TA muscle was homogenized by adding 200µl of Trizol and homogenised using the vortex and small homogenizer. The process was repeated to ensure that the whole muscle was properly homogenized and left for 5min at room temperature. 0.2 ml of Chloroform for 1ml of Trizol was added to the tube containing homogenized muscle, vigorously shaken by hand for 15sec and then incubated for 2-3 min at room temperature. The sample was then centrifuged at 12,000Xg for 15 min at 4°C. Following centrifugation the mixture separates into lower phenol-chloroform phase, an interphase and upper aqueous phase. The upper phase was carefully collected into a clean tube which contains the RNA. Three volumes of Ethanol, 1/20 volume of LiCl and (4M con) of glycogen was added to the sample and incubated overnight at -20°C. The next day the RNA precipitate was clearly observed at the bottom. The sample was centrifuged for 10min at 12,000Xg at 4°C. Supernatant was removed and the RNA was washed in 75% ethanol by centrifuging at 7,500Xg for 5 min at 4°C. The supernatant was removed, RNA pellet was air-dried in a clean chamber and was dissolved in RNase-free DEPC water.

For isolating RNA from single EDL muscle fibres, fibres were collected in a clean 2ml eppendorf tube washed with 1X PBS before adding 200µl of Trizol. The fibres were homogenized using . The rest of the steps followed, were same as described above for whole EDL muscle RNA isolation

2.4.2 cDNA synthesis

mRNA isolated from the above described procedure was used to synthesize cDNA as described in Invitrogen Superscript First Strand Synthesis handbook. 500ng of cDNA was used for PCR reactions and 200ng was used for qPCR.

2.4.3 Primer Design

Primers used were designed using primer premier 5, version 5 (**http://www.premierbiosoft.com/**). The following basic rules were taken into account while designing the primers.

- Generally around 20 base pair long
- Minimum 50% of GC content
- Primers starting and ending nucleotide either guanosine or cytidine
- Tm around 60°C for both the primers and contain no hairpin or dimer structures.

Ensemble BLAST (http://www.ensembl.org/Multi/blastview) and NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast) were used to check the specificity of the primers making sure that the primers do not recognize the other sequences of the genome.

Table 2.2 List of Primers

Gene	Primer (qPCR)	Sequence	Annealing temperature
Lama2	Forward primer	TGTCGTGGGGATTCTGTATGTC	62
	Reverse primer	CAAGAAGGTCCAATCCAACTTT	
Lama3	Forward primer	CAATGTGGACCGAATCCGAG	59
	Reverse primer	TTCTAGGTCATTCGGCAGTCG	
Lama5	Forward primer	ACCCAAGGACCCACCTGTAG	57
	Reverse primer	TCATGTGTGCGTAGCCTCTC	
Lama1	Forward primer	ACCGCAGGACACTCCTGTCAGG	62
	Reverse primer	TTACGCGCCGTCTGGTTC	
GAPDH	Forward primer	ACTCCACTCACGGCAAATTC	58-62
	Reverse primer	GACTCCACGACATACTCAGCACC	

Gene	(For RT-PCR)	Sequence	Annealing temperature
GAPDH	Forward primer	ACTCCACTCACGGCAAATTC	62
	Reverse primer	ACTGTGGTCATGAGCCCTTC	

2.4.5 PCR reaction

The primers designed by above mentioned procedure were amplified by PCR method. The stock concentration of the primers was 100µM. Stock concentration was diluted to 20µM in autoclaved milliQ water. A 20µl reaction was prepared as follows

0.5µl of cDNA

10µl of REDTaq ReadyMix (Sigma)

0.8µl (800nM) of each primer

H_20 upto 20µl*

In some cases 1µ1 DMSO was added to the reaction mix and the volume of water adjusted accordingly. DMSO improves the amplification of the GC rich which increases the amplification of the targeted sequences and thus reduces the amplification of non-specific sequences.

PCR was then performed using Eppendorf® mastercycler gradient PCR machine (Eppendorf). Conditions were optimized by adjusting the annealing temperature using a gradient program. Standard PCR programme was followed which is described below.

1st step: Initial denaturation at 94°C for 5min

2nd step: Denaturation at 94°C for 1min

3rd step: Annealing Tm (specific for each primer) for 1min

4th step: Extension at 72°C for 1min

5th step: Final extension at 72°C for 10min

6th step: Hold at 4°C

The PCR product was run on 10µl a 1% Agarose/TAE gel alongside 3µl GeneRuler™ 1Kb Ladder (Fermentas)

2.4.6 Quantitative real time PCR

Quantitative real time PCR was used to estimate the expression levels of different Laminin alpha subunits in whole muscle and cultured muscle fibres. The aim of the PCR or qPCR was to amplify the target from the starting point. The same temperature at which primers had worked was used for qPCR.

In PCR the product is detected on the gel but in real time PCR DNA amplification combines with the detection of the product in single well using florescent based dyes. This also prevents contamination caused post-amplification as in PCR method. Real time PCR reaction is first an exponential process and then reaches plateau when one of the reagent becomes limited. Over the course of time the instrument plots the accumulation of DNA. Cycle number (ct values) are taken into account, at the point when the signal is higher than the background and the amplification is in exponential phase. The greater the initial concentration of the target DNA, lower the ct values required to amplify the product. Real time PCR measures the increased fluorescence of the dye that intercalates with the double stranded DNA. The dye used in all the experiments was SYBR green. Real-time RT-PCR was performed on iCycler instrument (Biorad). Data was analyzed in iCycler software. To compare the transcript levels ΔΔCt method was used (Livak and Schmittgen, 2001).

Chapter 3

Identification and characterization of satellite cells in the extensor digitorum longus muscle fibres using an *ex-vivo* culture system

3.1 Introduction

Satellite cells are muscle specific stem cells involved in post-natal growth, repair and regeneration of the adult muscle. In 1917, it was noted that myofibres increase in size and in nuclei content in the absence of nuclear division within the myofibre (Lewis and Lewis 1917). Subsequent electron microscopy revealed for the first time that apparently quiescent cells lying at the surface of myofibres beneath the basement membrane are the source of myogenic cells involved in muscle growth. They were termed satellite cells (Mauro and Adams 1961). In the following decades, several molecular markers were characterized that allow reliable identification of satellite cells by conventional and confocal microscopy (Charge and Rudnicki 2004; Yin et al. 2013; P. S. Zammit et al. 2006a). These satellite cell markers have been instrumental in determining the role and behavior of satellite cells during skeletal muscle growth and regeneration. Many different approaches have been used in muscle satellite cell biology to understand the basic mechanism underlying satellite cell function in muscle regeneration. These experimental systems present advantages and disadvantages. I have used an *ex-vivo* system to study satellite cell which is discussed in this chapter.

3.1.1 Aim

The aim of the work described in this chapter was to establish an optimal approach to study satellite cells.

3.1.2 Methods used to study satellite cells

(1) *In vitro* systems

Primary myogenic cell cultures can be prepared from mononucleated cells dissociated from the whole muscle. The steps involved in the process include mincing, enzymatic digestion and trituration of the whole muscle to break the connective tissue around myofibres (Cossu et al. 1980; Naffakh et al. 1993; Nag and Foster 1981; Partridge 1997). For instance, satellite cells have been prepared from extensor digitorum longus (EDL) and Tibialis anterior (TA) muscle using pronase digestion to clean the muscle

for connective tissue and tendons. The resulting cell suspension can then be further purified using double-layered lens paper to remove the debris. Such cell suspensions can be enriched in satellite cells using different approaches. Enrichment of satellite cells has been done using a five-step percoll gradient (Kastner et al. 2000; Morgan 1988). Other methods include plating the cell suspension obtained on uncoated tissue culture plate. In this procedure, satellite cells can be separated from other non-muscle cells based on adhesion characteristics. Indeed, myoblasts cells are less adherent than fibroblasts that constitute the major source of 'contaminating cells' in primary myogenic cells. The cells that do not adhere to the plate are collected after a short period of time and cultured via differential plating (Qu-Petersen et al. 2002; Rando and Blau 1994; Richler and Yaffe 1970). These myogenic cell cultures are used to study the gene expression patterns that underlie the growth, proliferation, differentiation of satellite cells. As many physiological processes are retained, such as myoblast growth, differentiation and fusion, muscle derived cell cultures have been widely used. The establishment of immortalized cell lines from these primary myoblast cultures was key to several advances in the field of myogenesis. The first myogenic cell line developed was the L6 cell line, followed by the L8 cell line from new born rat (Yaffe 1968). Most studies were performed using the L8 cell line. Later the C2 cell line was established from 2-month old C3H mouse injured limb muscles. These cells are understood to be derived from satellite cells. Re-cloning of C2 cells gave rise to the C2C12 cell line (Blau et al. 1985), which differentiates spontaneously in the culture after serum withdrawal. However, the disadvantage of C2C12 is that when transplanted into muscle *in vivo* they form undifferentiated tumors (Morgan et al. 1992). The MM14 cell line was derived from adult mice and allowed to demonstrate the requirement for fibroblast growth factor (FGF) in myoblast proliferation (Linkhart et al. 1980). Another interesting cell line which has proven to be ideal for myogenic research is H2K 2B4 cell line (Muses et al. 2011). H2K 2B4 is a new conditional immortal satellite cell derived cell-line, generated from H-2K^{b}-tsA58 immortomouse (Muses et al. 2011). Unlike other primary myoblasts with

limited proliferative capacity *in vitro* H2K 2B4 cell-line possess extensive mitotic capacity and retains their ability to differentiate *in vitro* and *in vivo* (Muses et al. 2011).

Studies in these cell lines were critical in our understanding of the molecular mechanisms underlying myogenesis. In particular, the role and biochemistry of the myogenic regulatory factors Myf5, MyoD, MRF4 in the determination and differentiation of myoblasts was discovered using satellite cell-derived myoblast cell lines (Megeney et al. 1996).

In recent years, accumulating evidence suggest a link between satellite cells and the myoblasts that appear during muscle regeneration. This has been facilitated by the generation of Pax3 and Pax7 GFP reporter mice (Lepper et al. 2009; Montarras et al. 2005). Satellite cells can now be efficiently separated from other non-muscle cells using FACS. Purified satellite cells have been shown to carry out muscle regeneration (Ieronimakis et al. 2010; Montarras et al. 2005; Sambasivan et al. 2011), although cells from other sources can also contribute to muscle repair (LaBarge and Blau 2002). Nevertheless, in the absence of satellite cells, as in mice depleted of Pax7 expressing cells, no muscle regeneration takes place, demonstrating that satellite cells are essential to the repair process (Sambasivan et al. 2011).

To fulfill the demand for pure satellite cells, fluorochrome labeled antibodies that react with specific satellite cell antigens have been used (Montarras et al. 2005; Sacco et al. 2008). In this method, initially the muscle tissue is subjected to enzymatic digestion using collagenase or collagenase-dispase enzyme, which preserves the surface antigens in contrast to trypsin digestion used in previous methods. In addition to positive selection (Ieronimakis et al. 2010) using CD34 and α7 Integrin, CXCR4, β1 Integrin and Syndecan-4, various labs have developed methods to isolate them by negative selection from a whole population of cells using CD45, CD31 and Sca1 antibodies.

Thus, *in vitro* analyses have provided a wealth of information on the basic properties and behavior of satellite cells. However, it should be noted that the absence of the

niche is likely to have a detrimental impact on satellite cell behavior that includes host fibre, basal lamina and other cells as in *in vivo* system.

(2) *Ex vivo* system

An elegant method to study satellite cells in their niche was designed by Bischoff in 1986 (Bischoff 1986). Bischoff described a protocol to isolate single live myofibres from rat Flexor Digitorum Brevis (FDB) muscle. This method has unique advantages over other methods. First, satellite cells are maintained within part of their natural environment under the basal lamina and are in contact with the myofibre (Shefer et al. 2006; Yablonka-Reuveni and Rivera 1994). Second, it is possible to obtain pure populations of satellite cell (Shefer et al. 2004). Many studies were performed using this *ex-vivo* system to understand satellite cell behavior and their role during regeneration (Collins and Partridge 2005; Lepper et al. 2011; Pasut et al. 2013; Shefer and Yablonka-Reuveni 2005; Urciuolo et al. 2013; P. S. Zammit et al. 2002). It has been also shown that stripping of satellite cell from single EDL muscle fibres is also an efficient way to get enough number of satellite cells required for transplantation studies (Neal et al. 2012). Although studies from 1960's suspected that satellite cells were likely to be the myogenic cells involved in regeneration (Church 1969; Shafiq and Gorycki 1965), clear understanding existed until satellite cells were shown to give rise to a myoblasts using the *ex-vivo* culture of isolated fibres (Bischoff 1975; Konigsberg et al. 1975). Satellite cell activation, proliferation, differentiation and self-renewal in their niche was unambiguously demonstrated using single fibre suspension cultures (Beauchamp et al. 2000; Collins et al. 2007; Day et al. 2007; Gnocchi et al. 2009; P. S. Zammit et al. 2002; P. S. Zammit et al. 2004). It was also demonstrated that single isolated fibres when plated on substrates like Matrigel or Collagen, satellite cells associated with myofibres proliferate, differentiate and later migrate to form terminally differentiated myotubes (Blaveri et al. 1999; Yablonka-Reuveni and Anderson 2006). Myoblasts transplantation studies were done to show their contribution in muscle regeneration. For example, transplantation of normal myoblasts or human myoblasts in dystrophic models convert dystrophin-negative

fibres to dystrophin-positive fibres (Law et al. 1988; Morgan et al. 1990; Partridge et al. 1989; Skuk et al. 2010). Morgan used the *ex-vivo* fibre culture system to understand regeneration (Blaveri et al. 1999). In this study, muscle precursor cells (MPC) injected into a muscle regeneration model *mdx* in nude background (nu/nu) were labeled with LacZ, and subsequently analyzed in isolated single fibres at different time points to establish whether LacZ positive donor MPC had fused with the host fibres.

To further understand the satellite cell population and their behavior, cre based recombination techniques have been applied to the fibre culture system. Using *Myf5-Cre* and *ROSA26-YFP* cre-reporter alleles the existence of heterogeneous populations of satellite cells has been reported based on Myf5 expression levels in satellite cells on single fibres (S. Kuang et al. 2007). This study demonstrated that nearly 10% of Pax7 positive cells are never positive for Myf5, suggesting that these cells are stem cells and not committed progenitors (S. Kuang et al. 2007).

Table 3.1: Comparison of different methods used to study myogenic process in adult skeletal muscle

Advantages		
In vitro	***Ex-vivo***	***In vivo***
Myogenic cells can be cultured over several passages and frozen	Satellite cells are studied within part of their niche (host fibre and basal lamina) closer to *in vivo* system	Global view of all cell types, connective tissue and extracellular matrix involved in adult myogenesis
Consistent with *in vivo* data, the MRF expression pattern is observed in myogenic cell cultures allowing molecular studies of myogenesis	Can be cultures for 72-96hrs and recapitulates myogenic programme following satellite cell activation including self-renewal	More reliable method compared to *in vitro* and *ex-vivo*
Easy to grow and maintain	Easy to grow in suspension after isolating single fibres	-
-	Single satellite cells can be visualized using specific markers	-
-	Symmetric and asymmetric cell division can be visualized on the fibre	-

Disadvantages		
In vitro	***Ex-vivo***	***In vivo***
Lack of physiological conditions and relevance to *in vivo* context. In particular cells are no longer with in their niche and do not completely behave as satellite cells	Fibres can be cultured only for 72-96hrs. After 72hrs, proliferating and differentiating satellite cells begin moving away from the fibre	Very expensive to make and maintain genetically mutated mice to address a specific question
Characteristics of cell cultures changes with increasing number of passages	Does not completely recapitulate *in vivo* model. Specifically lack accessory cells that have been shown to contribute to regeneration (pericytes and macrophages for instance)	Sample size limitation
Contamination with other non-muscle cells is possible during preparation from whole muscle	-	Imaging more challenging

(3) *In vivo*

Satellite cells were first identified *in vivo* through their anatomical distribution on the surface of muscle fibres using electron microscope (Mauro and Adams 1961). However, this technique cannot be used routinely for the study of muscle regeneration as it is very expensive to buy and maintain. Also significant amount of time and training is required to operate the electron microscope. The identification of specific satellite cell markers such as Pax7 (Seale et al. 2000) made possible the detection of satellite cells on muscle cross sections by conventional microscopy. Other markers are available, however some of the markers (discussed in chapter-1) are expressed not only by quiescent satellite cells but also by activated satellite cells. In addition, some of the markers are also expressed by other cell types in the muscle sections. This is the case of instance Syndecan-3 and α7-Integrin (Cornelison et al. 2001a; Gnocchi et al. 2009; Tanaka et al. 2009). The generation of genetically modified mice expressing fluorescence reporter genes under the control of satellite cell specific promoter has greatly facilitated the identification of satellite cells *in vivo* and their study during muscle regeneration. For instance, all satellite cells are labeled in $Myf5^{nLacZ/+}$ mice, which carry the β -galactosidase reporter gene (Bischoff 1975) and *Myf5cre/*+: *RosaYFP/*+ mice have also been used and showed satellite cells were YFP positive,

except for 5-10% of YFP negative satellite cells (S. Kuang et al. 2007). Such studies have been instrumental in identifying distinct populations of satellite cells including cells committed to self-renewal and cells engaged into the myogenic programme.

Reporter lines have been also used to identify the origin of satellite cells *in vivo*. Lineage tracing studies using Pax3cre knock-in mice demonstrated that satellite cells are derived from somites and are specifically from the central dermomyotome (Engleka et al. 2005). Additionally, Pax3 driven cre-dependent reporter lines reveal that some satellite cells of the limb are derived from Pax3 positive cells of hypaxial somatic origin (Schienda et al. 2006). Overall, these studies have highlighted that recombination based technology can be applied to understand the function and role of satellite cells *in vivo.*

To conclude, although studies of satellite cell behavior are preferable using *in vivo* approaches, previous work has demonstrated that the *ex-vivo* culture system provides an invaluable tool, which presents many advantages over all other *in vitro* systems. In this chapter, I describe how I established this system to characterize satellite cells behavior during activation, proliferation and differentiation.

3.2 Results

3.2.1 Enzymatic isolation of single EDL muscle fibres allows for the identification of satellite cells at the quiescent state (0 hrs).

To study satellite cells on single fibres, the extensor digitorum longus (EDL) muscle was harvested from 6-8 week old wild type C57BL/6 mice, digested with collagenase and single fibres were carefully isolated. Isolated fibres were immediately fixed to ensure that satellite cells were in near quiescent state. Besides the criterion of their specific anatomical localization, satellite cells can also be identified by specific markers on single EDL muscle fibres. To identify quiescent satellite cells, I used different antibodies against proteins which are known markers of quiescent satellite cells, including the transcription factor Pax7 (Seale et al. 2000), the cellular receptor c-Met (Cornelison and Wold 1997), the adhesion molecule M-Cadherin (Irintchev et

al. 1994) and the calveolae-forming protein Caveolin-1 (Gnocchi et al. 2009; Volonte et al. 2005). Pax7 expression was specific to satellite cells which were found at the surface of myofibres (Gnocchi et al. 2009; Seale et al. 2000; P. S. Zammit et al. 2002) (Fig 3.1 K). M-Cadherin was also expressed by satellite cells (Fig 3.1 A-C). Similarly, other markers of satellite cells such as Caveolin-1 and c-Met were expressed by satellite cells and not by myofibre myonuclei (Fig 3.1 D-F and G-I respectively). It is well-known that the number of satellite cells decreases between P7 and P21 during postnatal development in mice (Bischoff 1975; White et al. 2010). Subsequently, around five satellite cells per fibre are maintained in adult muscles until the age of 10 months (Beauchamp et al. 2000; Collins et al. 2007; Neal et al. 2012; Shefer et al. 2006). Consistent with the literature, around five satellite cells (see Fig 3.2) were found on single freshly isolated muscle fibres at the quiescent state (0 hrs) (quantification was done using Pax7 antibody alone).

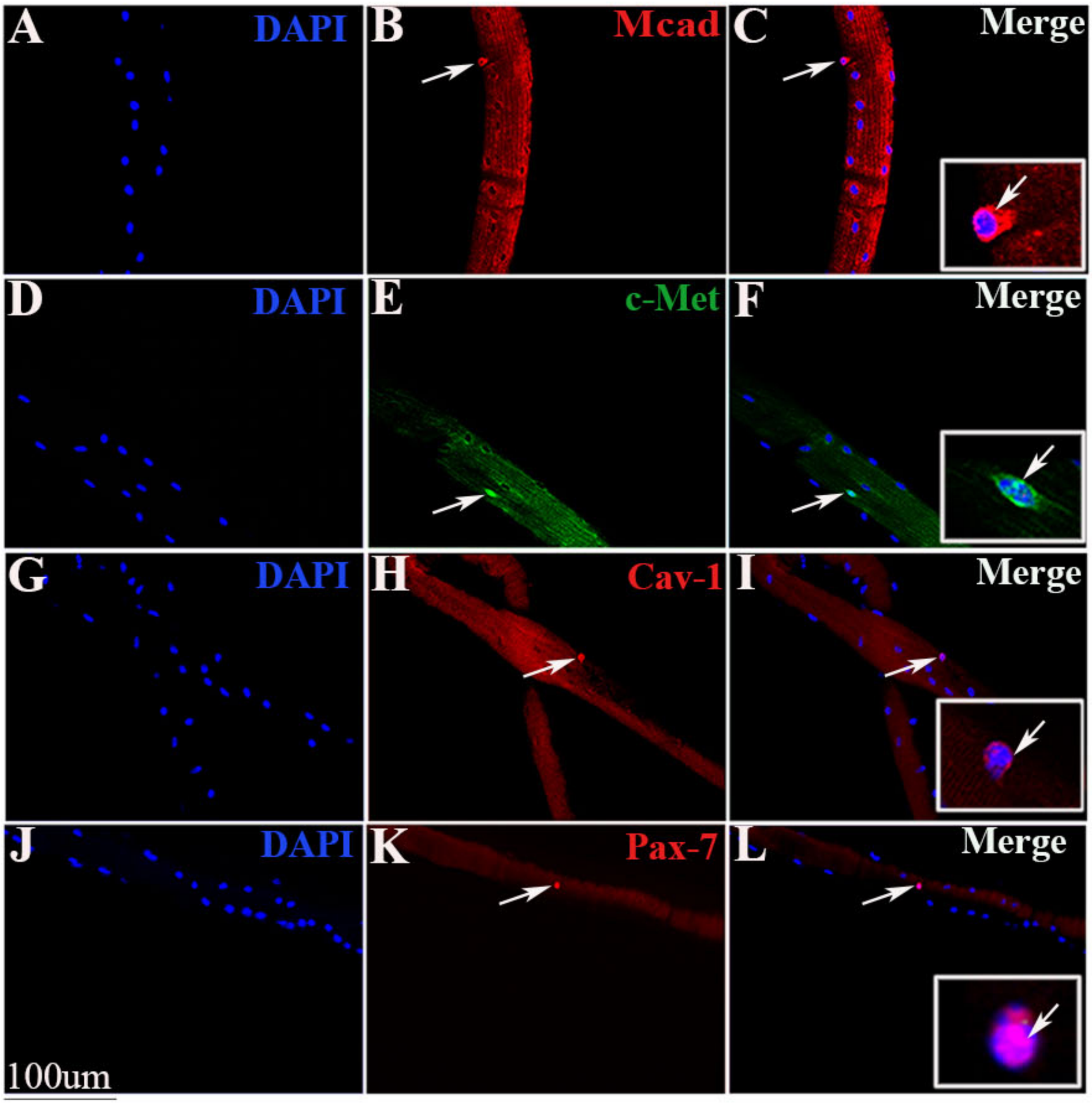


Figure 3.1: Characterization of satellite cells on freshly isolated EDL muscle fibres of 6-8 week old C57BL/6 mice: Immunodetection of M-Cadherin (A-C), c-Met (D-F), Caveolin-1 (G-I) and Pax7 (J-L) on single EDL muscle fibres at time 0 (0 hrs). Freshly isolated EDL muscle fibres were stained for the membrane proteins M-Cadherin, c-Met and Caveolin-1. Nuclei were counterstained with DAPI (A, D, G and J). Membrane markers M-Cadherin (B and C), c-Met (E and F) and Caveolin (H and I) are expressed on satellite cells as shown by white arrows. Pax7 is expressed in the nucleus of satellite cells as shown in K and L. Antibodies used against these markers show that M-Cadherin, c-Met, Caveolin-1 and Pax7 are expressed specifically by satellite stem cells and not by the myonuclei of the myofibre. Merge images are shown in panel C, F, L and I with magnified images in insert panels. Magnification X200.

3.2.2 Satellite cells expand in the *ex-vivo* fibre culture system

Once satellite cells were identified on freshly isolated EDL muscle fibres, my next approach was to culture single EDL muscle fibres and assess satellite cell behavior *ex-vivo*. Thus, freshly isolated EDL fibres from 6-8 week old wild type C57BL/6 mice were cultured for 72 hrs according to the protocol established by (P. S. Zammit et al. 2002). Satellite cells associated with single EDL muscle fibres expanded when cultured *ex-vivo*. Fibres were immunostained using an anti-Pax7 antibody at 0 hrs, 24 hrs, 48 hrs and 72 hrs and the number of Pax7-positive cells was determined.

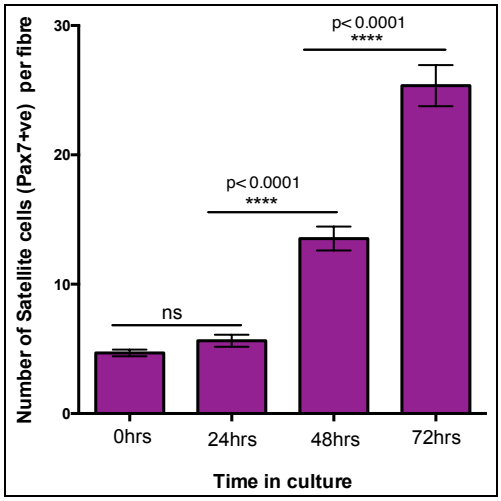


Figure 3.2: Proliferation of satellite cells on single EDL muscle fibres of 6-8 week old C57BL/6 mice: Quantitative representation of the number of Pax7-positive cells per fibre in the *ex-vivo* cultured system. A significant increase in the number of satellite cells is observed between 24 hrs and 48 hrs ($p<0.0001$) and between 48 hrs and 72 hrs ($p<0.0001$). Thus, the proliferation of satellite cells begins between 24 hrs and 48 hrs and continues through to 72 hrs (for each time point, 51 fibres were analyzed from 3 independent experiments). Statistical analyses was performed using Mann-Whitney test.

I observed that the number of Pax7-positive cells increased over time (Fig 3.2). From 0 hrs to 24 hrs, no significant increase in the number of Pax7-positive cells was observed, suggesting that satellite cells are activated during that time (as indicated by Myf5 expression observed at 24 hrs, see Fig 3.3 and 3.4). At 48 hrs, a significant increase in the number of satellite cells was observed, indicating that satellite cells proliferate after 24hrs. Satellite cell expansion continued through 72 hrs, such that by

the end of the culture there was a minimum five fold expansion of the satellite cell population. It should be noted that as Pax7 is down-regulated in satellite cells undergoing terminal differentiation, the number of satellite cells calculated at 72 hrs is likely to be underestimated.

These data suggests that satellite cells are able to become activated and to proliferate when cultured in the single EDL fibre. Furthermore, the profile of satellite cell expansion in culture is consistent with previous publications (Gill et al. 2010; Shinin et al. 2006). In young adult mice, satellite cells initially activate Myf5 at 24 hrs and from 48 hrs, satellite cells proliferate to generate clusters of progeny (Zammit et al., 2004) and I have observed the same with my muscle fibre culture.

3.2.3 Satellite cells are activated and produce transit amplifying myoblasts and differentiate in the *ex-vivo* EDL muscle fibre culture

Previous reports show that self-renewal and myogenesis can be modeled using the *ex-vivo* EDL fibre culture system (Halevy et al. 2004; P. S. Zammit et al. 2004). I previously showed that satellite cells expand in an expected manner in this *ex-vivo* system. However, it remains to be shown that satellite cells were activated and progressed through myogenesis as previously reported. Thus, EDL single muscle fibres were isolated from 6-8 week old C57BL/6 mice and were cultured for 72 hrs to assess their activation and differentiation. Fibres isolated at different time points (0 hrs, 24 hrs, 48 hrs and 72 hrs) were analyzed by immunofluorescence using different antibodies. At 0 hrs, fibres were assayed for Pax7 expression, which marks quiescent as well as activated satellite cells but not terminally differentiated satellite cells (myogenic positive). As expected Pax7 labeled all satellite cells in freshly isolated fibres and on an average, 5-7 satellite cells were present per EDL fibre (Fig 3.3 A-C, Fig 3.4). By 24 hrs satellite cells become activated and began co-expressing Pax7 and Myf5 the earliest myogenic regulatory marker to be induced following satellite cell activation. Around 94% of satellite cells become Pax7 and Myf5 double positive (Fig 3.3 D-F).

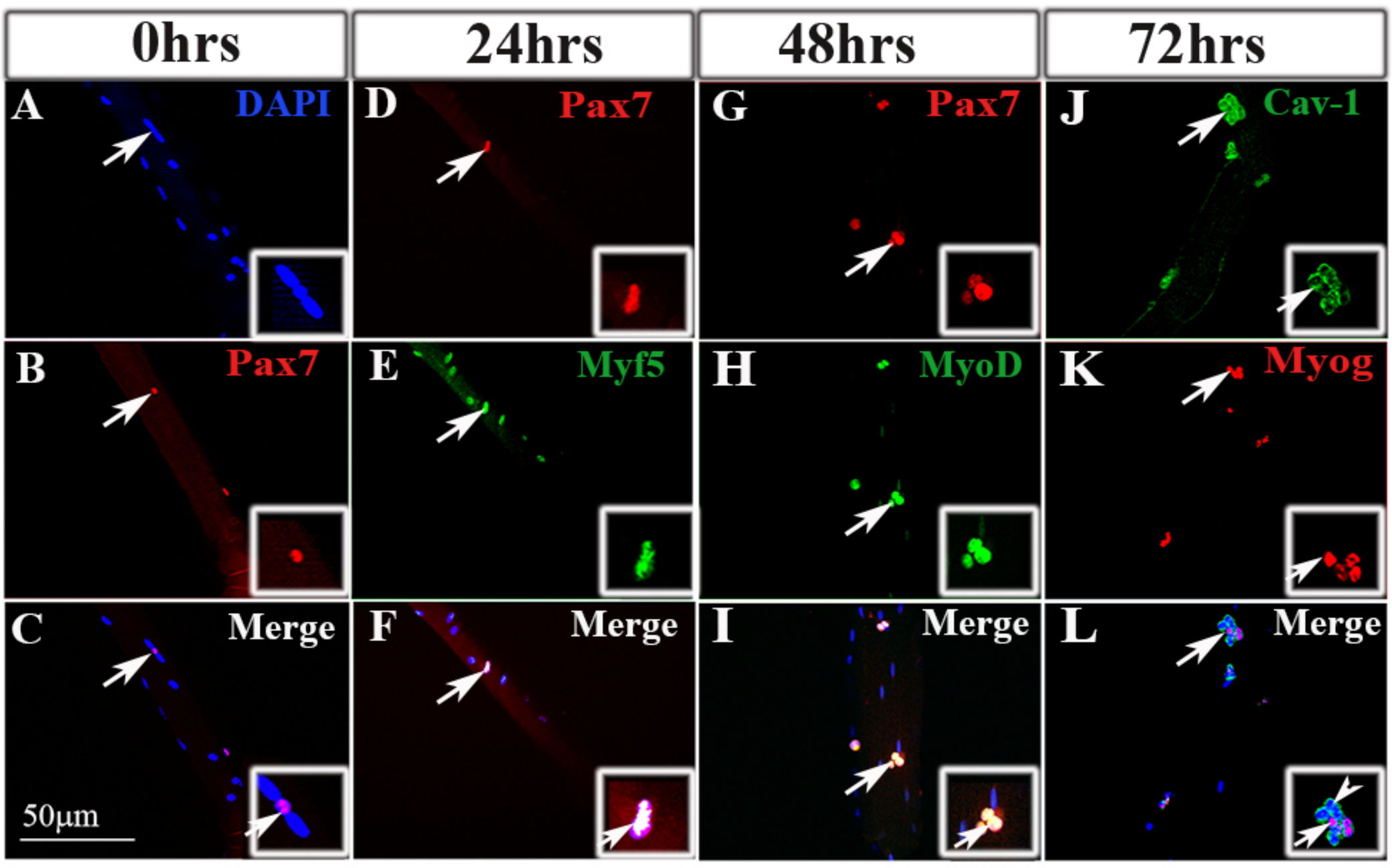


Figure 3.3 : Hierarchical expression of myogenic regulatory factors in *ex-vivo* cultured fibres of 6-8 week-old C57BL/6 mice: Isolated single EDL muscle fibres were cultured for 0, 24, 48 and 72 hrs. Immunodetection of Pax7 (C, F and I), Myf5 (F), MyoD (I), Myogenin (L) and Caveolin-1 (L) was carried out. Nuclei were counterstained with DAPI. *Ex-vivo* cultured EDL muscle fibres recapitulate the embryonic myogenic programme. At quiescent state 0 hrs satellite cells express Pax7. When cultured, they become activated and express Myf5 (E) followed by the expression of MyoD at 48 hrs (H). By 72 hrs, some cells express Myogenin as shown in K indicating that they have entered differentiation. Other cells express Caveolin-1 and may represent a population of cells not yet differentiated (MyoD positive) or a population of self-renewing cells (Pax7 positive) (J) as shown by white arrow. Merge images are shown in C, F, I and L. MagnificatiX200.

Once activated, satellite cells start proliferating and begin expressing MyoD together with Myf5 and Pax7 (Halevy et al. 2004; P. S. Zammit et al. 2004) (Fig 3.4) such that by 48 hrs most of satellite cells are Pax7 and MyoD positive (78%).

Around 22% of satellite cells remain Pax7-positive and do not express MyoD (Fig 3.4). These cells are proposed to be slow-dividing satellite cells that retain "stemness" and generate progeny capable of long term self-renewal (Ono et al. 2012). By 72hrs, most cells down regulate Pax7 and become terminally differentiated cells by expressing Myogenin. These cells can be detected through another satellite cell marker, Caveolin-1. At 72 hrs most satellite cells co-express Myogenin and Caveolin-1 (Fig 3.3 J-L). Other cells (16%) down-regulate MyoD, maintain Pax7 and Caveolin-1 and return to quiescence (Day et al. 2007; Nagata et al. 2006a).

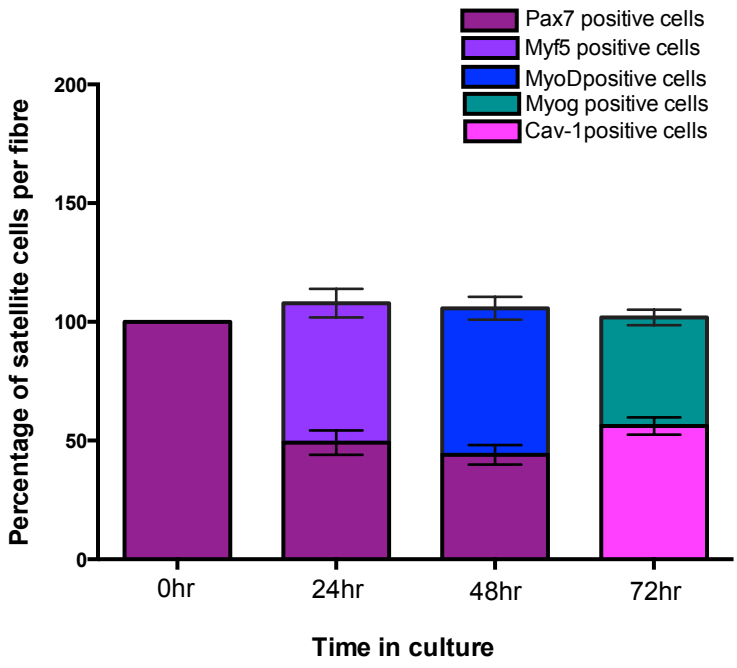


Figure 3.4: Satellite cells recapitulates the embryonic myogenic programme by activating myogenic markers in a sequential manner on single EDL muscle fibres of 6-8 week old C57BL/6 mice: Quantitative representation of the myogenic cells in *ex-vivo* cultured system. Proliferation of satellite cells begins between 24 hrs and 48 hrs and continues through to 72 hrs. n=3 (number of fibres at each time point=31).

3.3 Discussion

3.3.1 The *ex-vivo* system is a valid approach to study satellite cell activation

The *Ex-vivo* myofibre culture system has been widely used since the discovery of the protocol to isolate single myofibres from whole muscles (Bischoff 1975). Although the system does not completely substitute for an *in vivo* system, the proximity of the host fibre and the basal lamina with satellite cells provides a physiologically more relevant system to study satellite cell function. The added advantages of this system over an *in vitro* system is the crucial interaction with the niche, which not only maintains the quiescence of satellite cells but also modulates satellite cell behavior via different signals (Echtermeyer et al. 1996; Stern et al. 2009; Tatsumi et al. 1998). *In vitro* systems using C2C12 cells do not necessarily mimic the adult satellite cell behavior. Moreover, C2C12 cells when used for transplantation studies *in vivo* form undifferentiated tumors (Morgan et al. 1992).

The major disadvantage is that *in vitro* platforms do not maintain the complex cell-cell interactions and cell-matrix interactions which are crucial to satellite cell behavior *in vivo*. Also, cells prepared by enzymatic digestion of whole muscle are likely to contain "contaminating cells" derived from the vasculature. But may reserve cell surface factors of satellite cells. Thus, I have used *ex-vivo* fibre culture system to study the activation, proliferation and differentiation of satellite cells. Using different markers such as Pax7, c-Met, M-Cadherin and Caveolin-1 to label satellite cells on isolated single EDL muscle fibres, I observed approximately five satellite cells per fibre in 6-8 week old EDL muscle (Fig 3.1 and 3.2). Satellite cell number decreases between P7 and P21 this is consistent with previous studies showing that the highest number of satellite cells ~14 per myofibre being detected at P6/7 (White et al. 2010). This number decreases gradually to 9 at P14 and reaches to ~5 satellite cells per fibre by P21 (Neal et al. 2012). DNA labeling studies indicate also that satellite cells (~80%) are mainly proliferating in neonate mouse and rat (Schultz 1996; Shinin et al. 2006), but become quiescent in mature muscles (only 1% of the cells incorporate DNA in 6-8 week old mice) (Grounds and McGeachie 1989; Neal et al. 2012). In this

chapter, I reported that satellite cells recapitulates the embryonic myogenic programme by expressing Myf5, MyoD and Myogenin in a hierarchical manner when cultured on single EDL fibres (Yablonka-Reuveni and Rivera 1994; P. S. Zammit et al. 2002). Furthermore, I have shown that behavior of satellite cells, and their transition from the activation phase (Myf5-positive) to the expansion phase (MyoD-positive) and the differentiation phase (Myogenin-positive) are consistent with the previous reports within the literature (Day et al. 2010; Gnocchi et al. 2009; P. S. Zammit et al. 2002).

3.3.2 The basal lamina contributes to the satellite cell niche in the *ex-vivo* system

It is well established that extracellular matrix components contribute to the regulation of proliferation, differentiation and migration of different cells (Hynes 2009). This is also true for satellite cells. Previous studies have revealed that differentiation of chick myogenic cells requires ECM components such as collagen (Clark et al. 1997; Melo et al. 1996; Osses and Brandan 2002; Stern et al. 2009). However, in *in vitro* systems, key adhesion proteins involved in differentiation are often down-regulated (Grabowska et al. 2011). As this is not the case in the *ex-vivo* system of muscle fibre culture it suggest that important regulators of satellite cells behavior will be maintained.

For instance, the activation of satellite cells results in the dissociation of NO synthase from the membrane, which in turn releases hepatocyte growth factor (HGF) sequestered in the ECM. HGF binds to the c-Met receptor and activates satellite cells (R. E. Allen et al. 1995; Tatsumi et al. 1998). Interestingly, it has been demonstrated that fibronectin or vitronectin also form a complex with HGF/c-Met (Rahman et al. 2005). The role of these soluble factors or growth factors which bind to the ECM, is not taken into account in *in vitro* system, although some *in vitro* studies have demonstrated that proteoglycans can interact with growth factors such as FGF, HGF and TGF-β (Droguett et al. 2006; Zhu et al. 2007). The *ex-vivo* system also provides an excellent model to study the process of regeneration and denervation. During muscle injury, signals from the damaged site in the muscle activate satellite cells

(Tatsumi et al. 1998). The *ex-vivo* cultures mimic to certain extent the *in vivo* system because in this system, satellite cells are in contact with the host fibre and the extracellular matrix. Studies in dystrophic mice (myD) have also shown that disruption of matrix components alters satellite cell function (Ross et al. 2012). Likewise, engraftment studies in *mdx* mice suggests that the preservation of the satellite cell niche promotes muscle regeneration (Boldrin et al. 2012). It has been shown that transplantation of myofibres-associated with satellite cells into irradiated mice or dystrophic mice was more efficient compared to transplantation of isolated cells but these studies were observed for short-term (3-4 weeks) engraftment (Collins et al. 2007). Later, studies have identified the requirement of host environment. Transplantation of myofibre-associated satellite cells into injured TA muscle have shown efficient regeneration upto 31 months (Hall et al. 2010). These data suggest that donor satellite cells are resistant to the aged environment and possess enhanced regenerative capacity when associated with the myofibre. Single myofibre when grafted into *mdx* nude host mice results in hypertrophic effect suggesting a requirement for cross-talk between donor fibre and the host muscle environment (Boldrin et al. 2012).

Altogether, it is clear that the study of satellite cell biology using the *ex-vivo* culture system is more likely to yield physiologically relevant data than in vitro culture systems.

The *ex-vivo* system was therefore chosen to initiate my investigation on the composition of the satellite cell basal lamina.

Chapter 4

Identification of satellite cells in the extensor digitorum longus (EDL) and Tibialis anterior (TA) muscles of normal and regenerating mouse models *in vivo*

4.1 Introduction

Skeletal muscles have a remarkable ability to repair and regenerate in order to maintain a functional skeletal musculature. Muscle regeneration is trigged either during direct trauma such as intense physical activity and injury by toxins or by indirect causes as in dystrophies caused by genetic alterations. Muscle regeneration trigged by any of these causes, is characterized by two important phases: muscle degeneration and muscle regeneration. The first and foremost event in the degeneration phase is necrosis of the myofibres resulting in sarcolemma disruption and increased membrane permeability. Proteins such as creatine kinases, which are usually restricted to the myofibre cytosol are released into the serum as an indication of membrane permeability (Ozawa et al. 1999). Calcium homeostasis is also disturbed resulting in calcium-dependent proteolysis (Alderton and Steinhardt 2000; Armstrong 1990b). Depending on the extent of injury these myofibres then undergo autolysis. The process of degeneration is accompanied by the early inflammatory response. The factors released by the damaged muscle activate inflammatory cells, which provide chemotactic signals to the circulating inflammatory cells (Rappolee and Werb 1992; Tidball 1995). Neutrophils are the first line of cells which invade the injured site, followed by the macrophages to engulf the debris within 48hrs (Fielding et al. 1993; Merly et al. 1999; Robertson et al. 1993; Tidball 1995). Notably, it has been suggested that macrophages are not only involved in removing debris but also involved in satellite cell proliferation and differentiation (Cantini et al. 1994; Cantini et al. 1995; Cantini et al. 2002; Lescaudron et al. 1999; Merly et al. 1999). Thus, the main events in muscle degeneration are muscle fibre necrosis and increased number of nonmuscle cells involved in inflammation. The degeneration phase is rapidly followed by a regeneration phase. The critical event in the regeneration phase is the cellular proliferation of satellite cells (Hawke and Garry 2001). The expansion of these myogenic cells provide a sufficient source for the new myonuclei (Charge and Rudnicki 2004). Satellite cells finally differentiate and fuse to the damaged fibre or fuse to each other to form new myofibres (Grounds et al. 2002; Hawke and Garry 2001; Snow 1977, 1978). It has been shown that as less as seven satellite cells

associated with one myofibre when transplanted into radiation-ablated muscles *in* vivo can generate over 100 new myofibres with thousands of myonuclei (Collins and Partridge, 2005). The fundamental morphological characteristics of muscle regeneration is the appearance of centrally located nuclei in newly formed myofibres (Charge and Rudnicki 2004) (Centrally located nucleation signifies that the muscle had previously undergone regeneration).

Muscle regeneration can be studied using different regeneration models. The kinetics and amplitude of degeneration and regeneration of muscles in these models depends on the extent of the injury, the muscle type and also the animal model used (Lefaucheur and Sebille 1995b; C. A. Mitchell et al. 1992; Pavlath et al. 1998). The intensity of muscle injury varies also in different models. For instance in *mdx* mice, a model for Duchenne muscular dystrophy (DMD), chronic muscle injury is attained due to the complete absence of the Dystophin. Degeneration and regeneration persists throughout the life in *mdx* mice (Pagel and Partridge 1999). In contrast, local injection of toxin confers acute injury to the injected muscle. Cardiotoxin injection causes depolarization and contraction of muscle cells. CTX selectively disrupts muscle fibres without disturbing the extracellular matrix and CTX induced muscle damage is widely used as a classical model to study regeneration (Couteaux et al. 1988; Lepper et al. 2011; Shi and Garry 2006a; Volonte et al. 2005; Yan et al. 2003). Intensive exercise or eccentric exercise also cause muscle damage and henceforth exercise induced muscle damage has been extensively used to exacerbate the dystrophic phenotype. This model also allows to investigate the key characteristics and mechanisms involved in muscle degeneration and repair process (Cerri et al. 2008). However, the impact of exercise on muscle regeneration in *mdx* mice is still controversial. Some studies suggest beneficial effects and some show detrimental effects of exercise in *mdx* mice (Carter et al. 1994; Dupont-Versteegden 1996; Lynch et al. 1996; Sandri et al. 1995). For instance voluntary wheel running of adult *mdx* mice for 48hrs shows a two-fold increase in quadriceps muscles necrosis but not in TA and diaphragm muscles (Radley and Grounds 2006). Likewise, EDL muscles are more affected than soleus muscles (Lynch et al. 1996). It has also been demonstrated

that treadmill exercise increases muscle damage by increasing the number of necrotic fibres and infiltrating mononuclear cells (De Luca et al. 2003). A comparative features of these different models is listed below.

Table 4.1: Comparison between three models of muscle regeneration

	mdx **(Chronic muscle damage)**	**Cardiotoxin induced muscle injury (Acute muscle damage)**	**Exercise induced muscle injury**
Muscle degeneration phase	Extensive muscle degeneration by 3-5 weeks	Induces muscle degeneration leading to infiltration of mononuclear cells within 1 day of CTX injection	Exercise induces muscle damage
	Necrosis and degeneration occur lifelong accompanied by infiltration of mononuclear cells	Inflammatory response and mononuclear cell proliferation is active and rapidly removes dead and damaged myofibres within 1-4 days post injection	DNA damage is observed when inflammation is still in progress
Inflammation phase	Accumulated collagen deposition long term inhibiting myogenic cell activity	Transient collagen deposition short term	Ubiquitin expression increases indicating the apoptosis
	Persistent inflammatory response	Transient inflammatory response	Transient inflammatory response
Myogenesis	Up-regulation of MyoD expression is observed during 2-6 weeks suggesting the activation of the satellite cells	Satellite cell differentiation formation of new fibres observed with in 4-5 days	Satellite cells proliferate with Myf5 expression
Muscle regeneration phase	Active regeneration is observed from 4-8 weeks with the appearance of centrally located nuclei	At about 7-14 days smaller and more centrally located nuclei appear restoring the overall muscle architecture	Normal musculature can be attained in few weeks
	Degeneration and regeneration process occur simultaneously at a lower and constant levels throughout life	Normal musculature can be attained by 3-4 weeks post injection	
	Heterogeneity in fibre size and structure with prolonged regeneration	Homogeneity in fibre size and structure and complete regeneration	

To study satellite cells in muscle regeneration I have used these three models. *Mdx* mice with chronic muscle injury and cardiotoxin with acute muscle injury were used to study the progression of muscle regeneration. Exercised induced muscle injury was

used to understand the altered inflammation in *mdx*. These models will provide a platform to identify and study satellite cells *in vivo* during muscle regeneration. These established models will be further used to characterize the extracellular cellular matrix component Laminin (discussed in chapter 6).

4.1.1 Aim :

The aim of the work presented in this chapter was to establish two skeletal muscle regeneration models and compare the progression of regeneration in chronic (*mdx*) and acute injury (cardiotoxin) models.

4.2 Results

4.2.1 Identification of satellite cells in wild-type C57BL/6 EDL muscles

To locate and study satellite cells *in vivo*, I performed immunofluorescence on EDL muscle transverses using antibodies against satellite cell markers and Laminin. EDL muscles were harvested from 6-8 week old C57BL/6 mice and 6-7μm thick transverse sections were prepared. Muscle sections were analyzed by immunofluorescence for the presence of Laminin and satellite cell markers, c-Met and Pax7. c-Met and Pax7 expression was specifically observed in satellite cells (Fig 4.1 and 4.2). Satellite cells expressing c-Met were present underneath the basal lamina stained for Laminin alpha2 (Fig 4.1 A-C). Pax7, a marker of quiescent satellite cell was also expressed by satellite cells *in vivo* (Fig 4.2 A-C). Positive cells were counted per sections (n=9) from three different animals and reported as cells per square millimeter. I observed an average of 60 Pax7 positive cells and an average of 73 c-Met positive cells per mm^2 in wild-type uninjured muscles. A previous study reported that Tibialis anterior muscle (TA) has an average of around 80 Pax7 positive cells per mm^2 (Le Grand et al. 2012). The number of Pax7 positive cells observed was slightly lower. However, this may be explained by the distinct muscles used. For EDL muscle around 27 cells were reported per mm^2 (Lepper et al. 2011). Notably, the number of c-Met positive cells was slightly higher (121%) compared to the number of Pax7 positive cells.

The slight difference in the number of satellite cell may be due to the different composition of muscle fibre type in TA and EDL muscles. It has been shown that although both TA and EDL muscles predominantly contain type IID and type IIB fibres, EDL muscle also consists of type IIAD, IC and IIA which are completely absent in TA muscle (Augusto et al., 2004). The length of EDL and TA muscles is 12.2mm and 12.9mm respectively. However, the fibre length varies in both these muscles. EDL muscle fibres are 6.2mm in length whereas TA muscle fibres are 7.9mm in length (Burkholder et al. 1994) which also results in the difference in the number of satellite cells per myofibre (Collins et al. 2005).

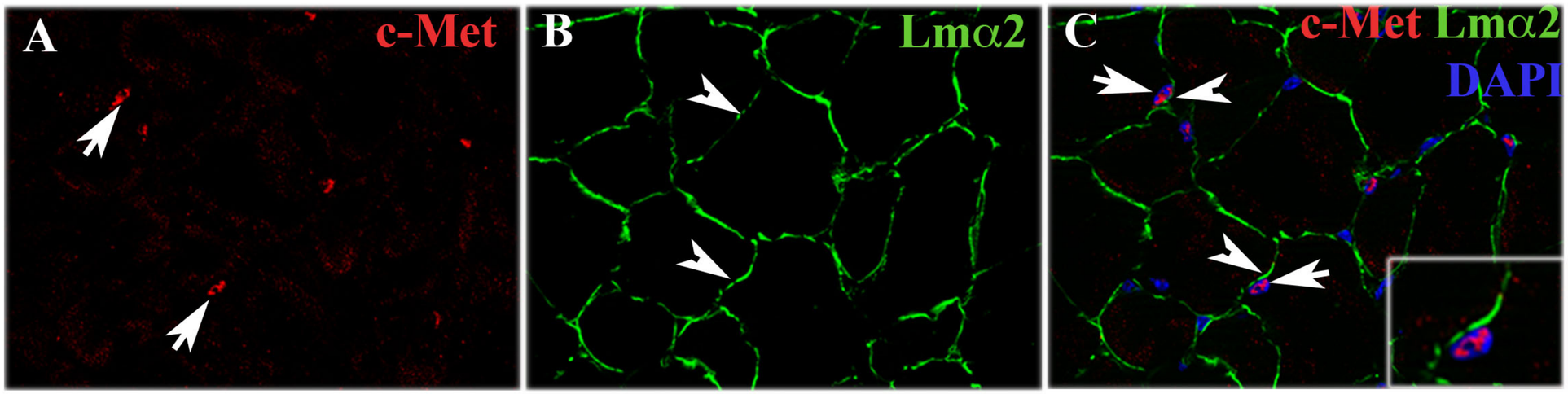


Figure 4.1 Identification of Satellite cells in the 6-8 weeks C57/BL6 EDL muscle cross sections: Representative cross section of EDL muscle of 6-8 weeks C57/BL6 mice.6-8 weeks C57/BL6 EDL muscle sections were stained for c-Met and Laminin alpha2. Satellite cells express c-Met as shown by white arrows (A) and Laminin alpha 2 is expressed in the basal lamina of the myofibres (B). Satellite cells are identified underneath the basal lamina as shown by arrow heads (C). Nuclei are counterstained with DAPI. Magnification X630.

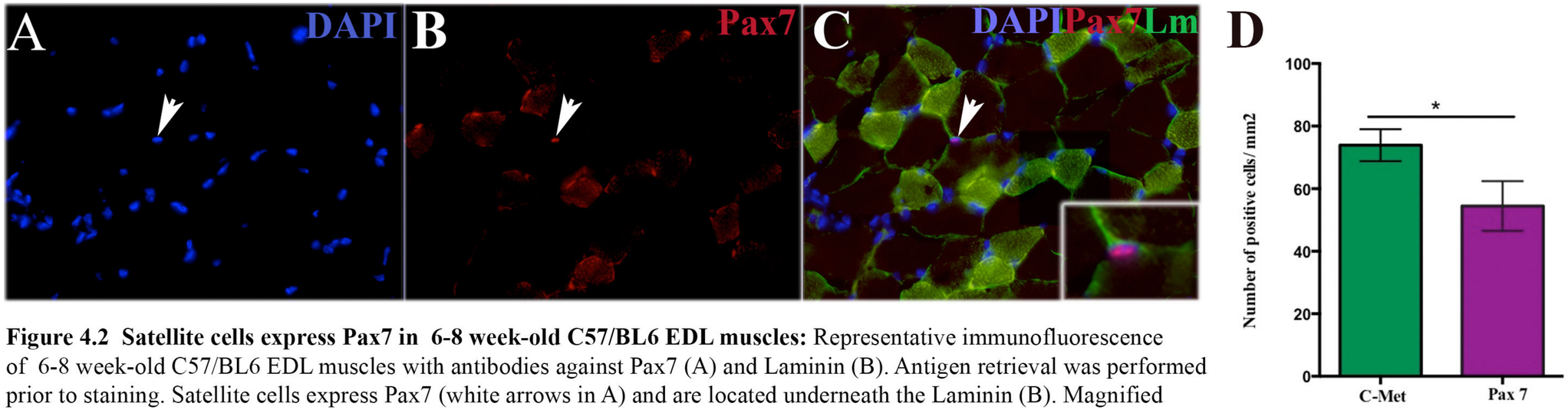


Figure 4.2 Satellite cells express Pax7 in 6-8 week-old C57/BL6 EDL muscles: Representative immunofluorescence of 6-8 week-old C57/BL6 EDL muscles with antibodies against Pax7 (A) and Laminin (B). Antigen retrieval was performed prior to staining. Satellite cells express Pax7 (white arrows in A) and are located underneath the Laminin (B). Magnified image is shown in insert (C). Nuclei are counterstained with DAPI. Magnification X400. (D) Quantitative representation of the number of satellite cells in 6-8 week-old C57/BL6 EDL muscles. The number of c-Met positive and Pax7 positive cells per mm^2 is plotted.

4.2.2 The *mdx* mice is a model of chronic skeletal muscle regeneration

I studied the regeneration of skeletal muscles in the *mdx* mouse, a model of Duchenne muscular dystrophy (DMD). The *mdx* mouse is widely used as a model of the X-linked human muscular dystrophy. Indeed, as in humans *mdx* mice carry a mutation in dystrophin (Hoffman et al. 1987a; Hoffman et al. 1987b). Although humans and *mdx* mice have a similar genetic defect *mdx* mice in contrast to humans are characterized with a successful regeneration process that compensates for the lack of dystrophin (Chamberlain et al. 1987; Dangain and Vrbova 1984; DiMario et al. 1991) with persistent degeneration and regeneration cycles (Pagel and Partridge 1999). Although fibrosis occurs in TA muscles of *mdx* with a reduced fibrosis in heart and diagram muscles, efficient regeneration occurs to maintain the muscle integrity (Boldrin et al. 2009).

In wild type normal skeletal muscles, muscle fibres have a uniform evenly spaced structure (Fig 4.3). In contrast, DMD muscles are characterized by the presence of necrotic fibres (green arrows in Fig 4.3 D and H). Hence, although not perfect this mouse model is considered as a valuable tool to study Duchenne-type muscular dystrophy and muscle regeneration.

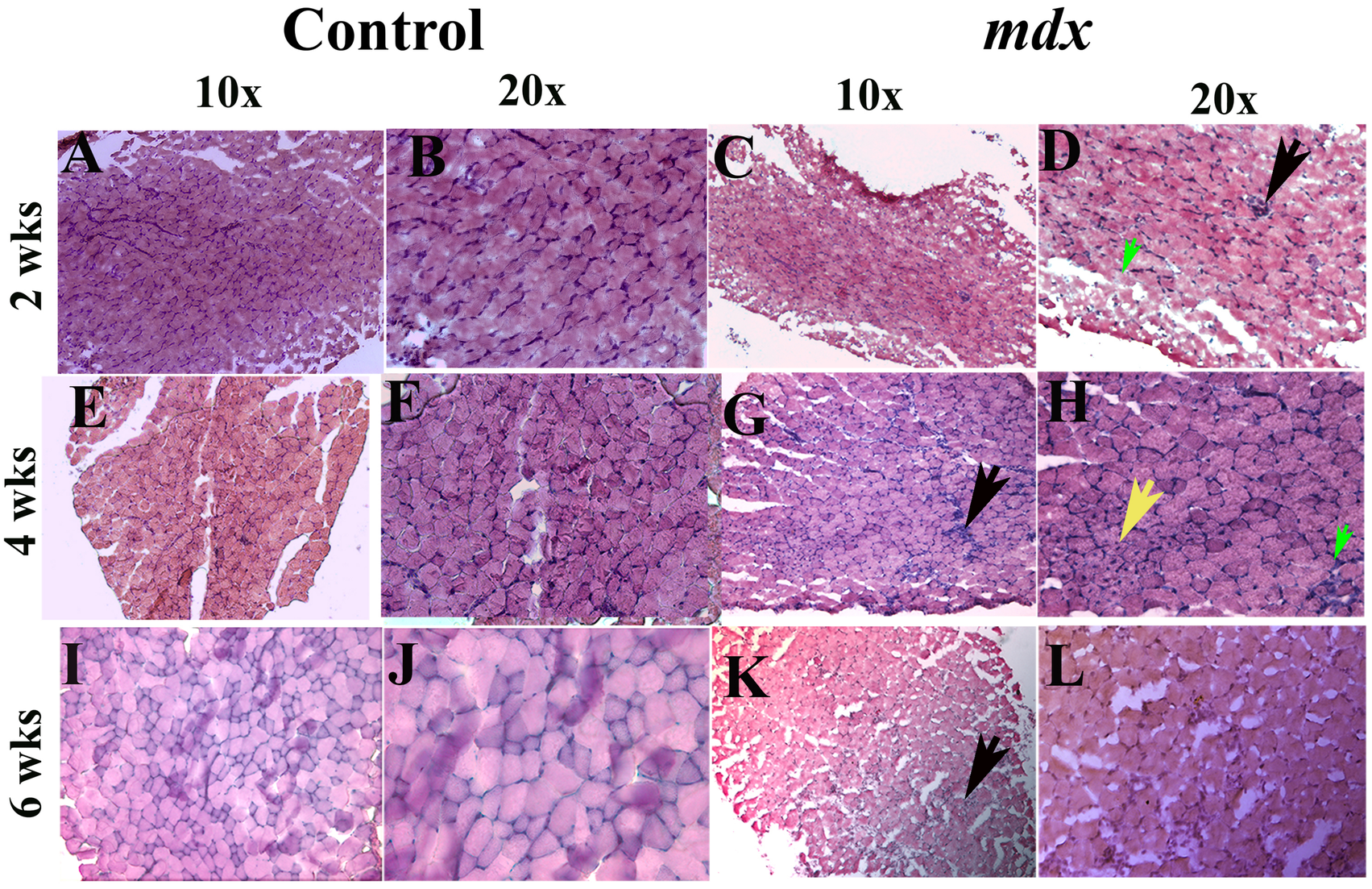


Figure 4.3 : Muscle pathology of *mdx* EDL muscle reveled by histological staining: Hematoxylin and eosin staining of *mdx* and C57BL/6 (control) muscles at 2, 4, 6, 8, 10 and 12 weeks is represented. At 2 and 6 weeks *mdx* mice shows infiltrating cells (white arrow in D and G) while centrally located nuclei are observed from 4 weeks onwards (yellow arrow in G-H). Necrotic fibres are observed at 2 and 4 weeks (green arrows D and H). No infiltrating cells was observed in control muscles (A-B, E-F, I-J)

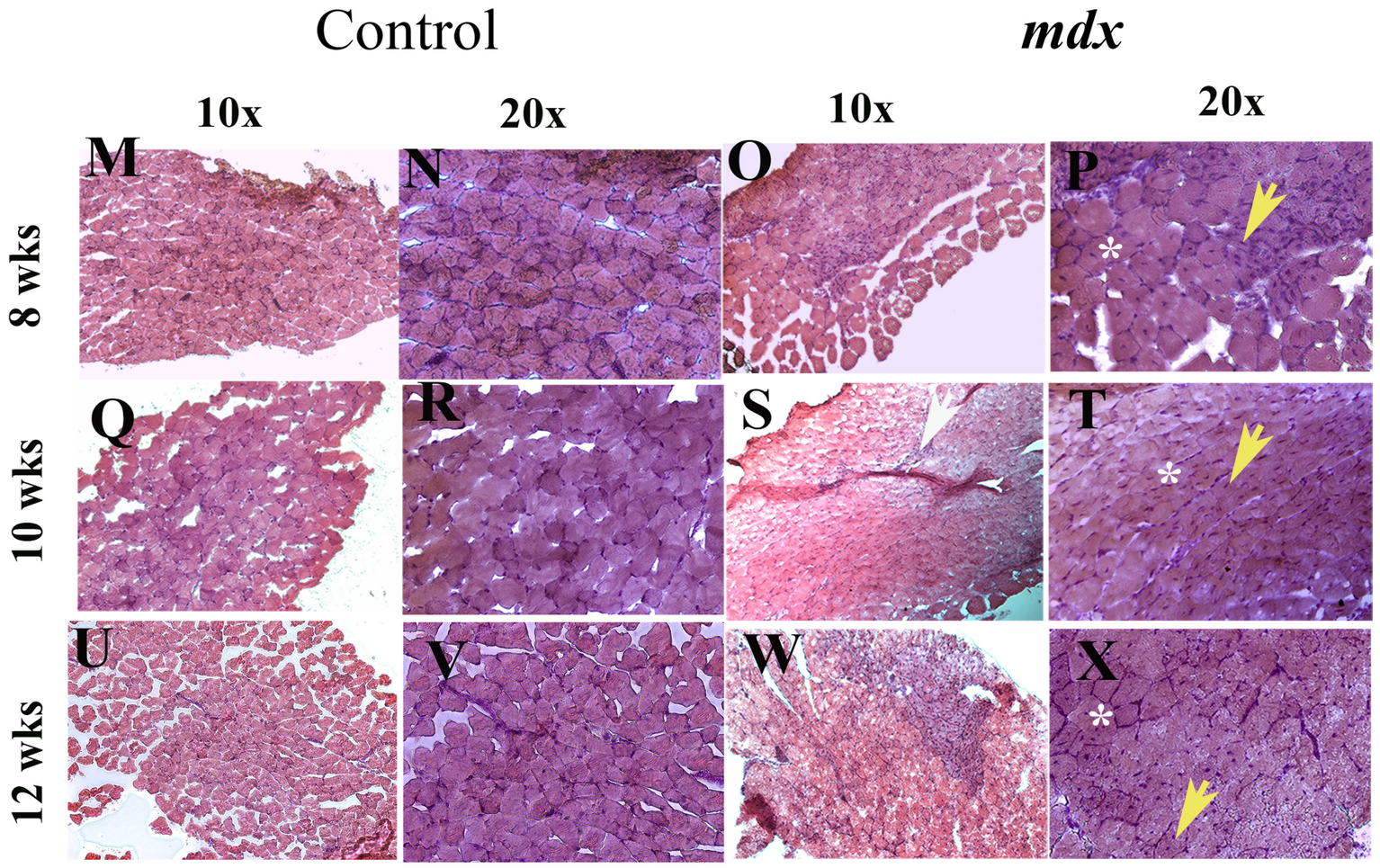


Figure 4.3: ...continued .. From 8 weeks onwards fully regenerated fibres are observed indicated by white asterisks (P, T and X).

4.2.2.1 Histology analysis reveals that degeneration and regeneration takes place during the early post-natal life of *mdx* mice

Histological analyses were carried out on EDL muscles of *mdx* mice from 2, 4, 6, 8, 10 and 12 week-old mice. Muscles were harvested, frozen and 6-7μm transverse sections were prepared. C57BL/6 EDL muscles of matching age were also sectioned as controls. Sections were stained with Hematoxylin (stains nuclei dark violet) and eosin (stains the whole muscle red) dyes, and analyzed under the light microscope. Histological analysis reveals that necrosis and degeneration occurs between 2-6 weeks and regeneration begins at 4 week and continues through to 12 weeks. Severe necrosis and regeneration was observed mainly between 2-6 weeks. Infiltrating cells were mostly observed at 2, 4 and 6 weeks (black arrows in Fig 4.3 D, G and K), although cell infiltration was also noted at 10 weeks (black arrows in Fig 4.3 S). At later postnatal stages, muscles do not degenerate to the same extent (Carnwath and Shotton 1987; Coulton et al. 1988; Dangain and Vrbova 1984). The regeneration process is also noticeable through the heterogeneity in fibre size at 4 and 8 weeks, newly formed fibres are significantly smaller (shown by yellow arrows in Fig 4.3 H and P). Terminally differentiated newly regenerated fibres indicated by asterisks are identified at 8, 10 and 12 weeks by the presence of centrally located nuclei (see Fig 4.3 P, T and X). However, at 2 weeks no fibre with centrally located nuclei was observed suggesting that regeneration process in *mdx* starts between 2 and 4 weeks in mdx mice, following the cycle of necrosis and degeneration as previously reported in the literature (Carnwath and Shotton 1987). To assess the level of regeneration taking place between 2 and 12 weeks post-natally, I determined the percentage of centrally located nuclei. I observed a drastic increase in the number of centrally located nuclei is between 2 and 4 weeks and between 4 and 6 weeks (Fig 4.4 (M)), followed with a gradual increase from 6 weeks onwards (Fig 4.4 M). This suggest that *mdx* mice have an active regeneration process at a young age (Dangain and Vrbova 1984; DiMario et al. 1991; Pastoret and Sebille 1993). Control C57 mice at 2, 4, 6, 8, 10 and 12 weeks showed equally spaced homogeneity in fibre size with peripherally located nuclei as shown in Fig 4.4 (A-C and G-1).

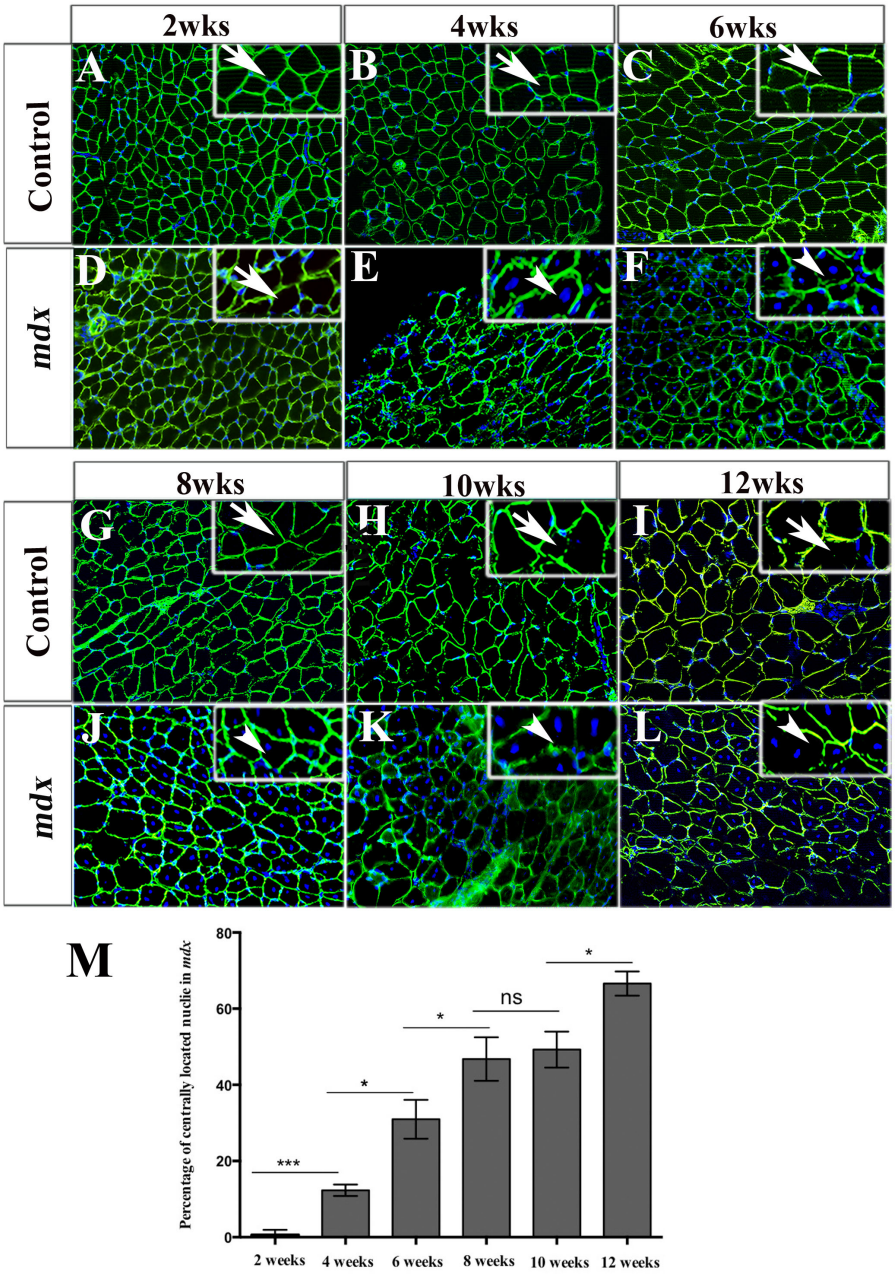


Figure 4.4: Centrally located nuclei indicate muscle regeneration in *mdx* mice:

Figure 4.4: Centrally located nuclei indicate muscle regeneration in *mdx* mice: Transverse sections of control C57/BL6 and *mdx* mice of 2, 4, 6, 8, 10 and 12 week old mice were analyzed by immunofluorescence with antibodies against Laminin alpha2. In both control and *mdx* mice, Laminin α2 delineates the fibres. In *mdx* mice appearance of centrally located nuclei is shown by arrow heads at 4 and 6 weeks (E and F); and at 8, 10 and 12 weeks (G-I) indicating that the fibres under-went regeneration. In contrast no centrally located nuclei were observed in control mice. Magnified images shown in the inserts. Nuclei are counterstained with DAPI. Magnification X200. (3) Quantitative representation of the percentage of centrally located nuclei in *mdx*. Significant difference is observed between 2 and 4 weeks (p=0.0075), 4 and 6 weeks (p=0.0079). n=12 sections analyzed from three different animals. Statistical analyses were performed using Mann-Whitney test.

4.2.2.2 Satellite cells are activated and express MyoD during muscle regeneration in the *mdx* mouse

In wild-type muscles, satellite cells are usually quiescent and only become activated upon injury. In contrast, *mdx* mice satellite cells are continuously activated (J. E. Anderson et al. 1998; Carnwath and Shotton 1987; Sabourin and Rudnicki 2000). One commonly used marker of satellite cells is c-Met, the receptor for hepatocyte growth factor (HGF) released at an early stage of muscle regeneration (Tatsumi et al. 1998). Thus to detect the presence of satellite cells 4 week-old EDL muscle of wild-type and *mdx* mice were harvested, sectioned and analyzed by immunofluorescence for c-Met. As predicted, satellite cells accumulated in *mdx* muscles compared to wild-type muscles (Fig 4.5 C-F) in which only few c-Met positive cells were observed. An almost 8 fold increase in the number of satellite cells was observed in *mdx* compared to wild-type mice (Fig 4.5 G). The presence of numerous small fibres containing c-Met positive cells indicated also that satellite cells had been activated and regeneration was taking place (Fig 4.5 F).

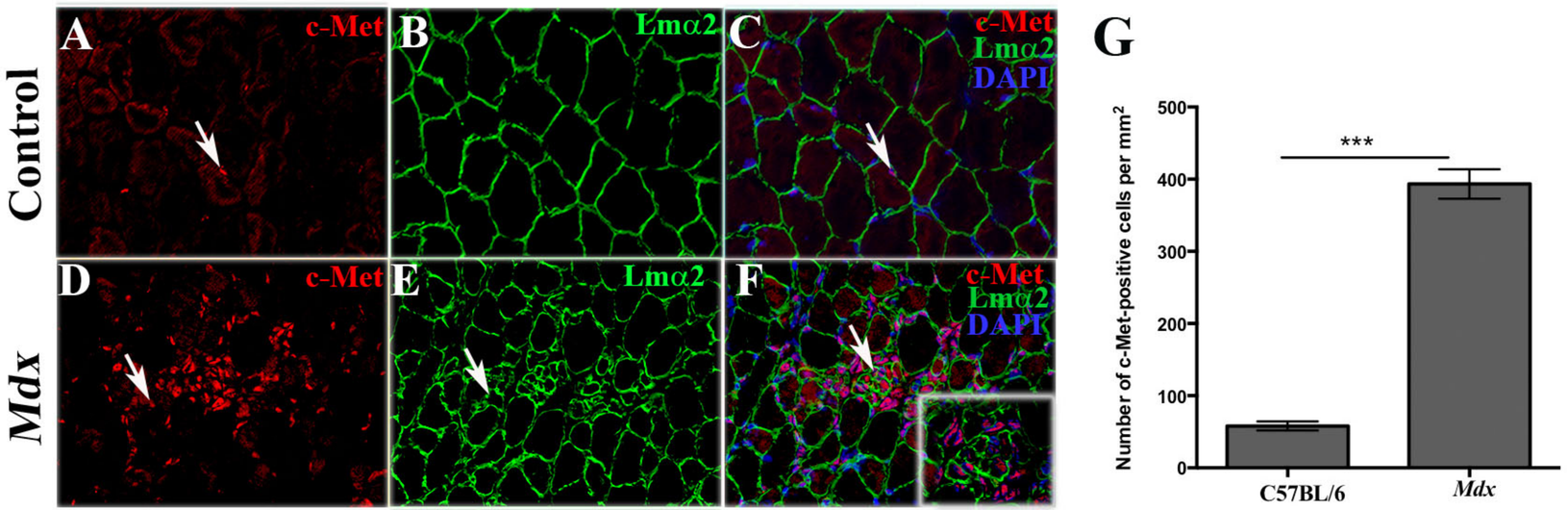


Figure 4.5: Accumulation of satellite cells in 4 weeks-old *mdx* EDL muscle: Immunodetection of c-Met and Laminin alpha2 in 4 week-old *mdx* mice (D-F) and C57BL/6 (A-C) EDL muscles. Satellite cells are detected under the basal lamina labeled by antibodies against Laminin alpha2 (green). (D-F) Satellite cells expressing c-Met (red) accumulate in *mdx* mice (white arrows). Regeneration is visible due to the presence of small fibres with c-Met positive cells. Nuclei are counterstained with DAPI. Magnification X400. (G) Quantitative representation of c-Met positive satellite cells in C57BL/6 and *mdx* mice. Significant difference (p=0.0007) in the number of satellite cells is observed in *mdx* compared to control mice. Statistical analyses is performed using Mann-Whitney test.

To confirm that satellite cells were indeed activated, I tested whether they expresses MyoD. Indeed, MyoD and Myogenin mRNA levels are up-regulated from about 3 weeks and remain high until 6 weeks in *mdx* muscle compared to wild-type muscles (Beilharz et al. 1992). In fact, up-regulation of both these genes is observed also during muscular dystrophy models such as Large ^{myd} and Lama$^{2dy/2J}$ (Onofre-Oliveira et al. 2012). EDL muscles were harvested from C57BL/6 and *mdx* mice at various post-natal ages ranging from 2 weeks to 8 weeks and immunofluorescence was performed to detect MyoD positive cells present underneath the basal lamina stained for Laminin alpha2 (Fig 4.6). MyoD positive cells were observed at all stages tested in *mdx* muscles (Fig 4.6 B-E). In contrast, no MyoD positive cells was observed in wild-type muscles (Fig 4.6 A). Although, it is mentioned in the literature that majority of the activated satellite cells express MyoD at 2 weeks (Neal et al. 2012). The discrepancy may be due to the area of sections analyzed.

However, these data indicates that muscle regeneration is taking place in young *mdx* mice and satellite cells are activated. Activated satellite cells were quantified at all stages (Fig 4.6 M). Interestingly, a significant increase in the number of activated satellite cells was observed in between 2 and 6 weeks (Fig 4.6 M). After 4 weeks a decrease in the number of MyoD positive cells was observed, indicating that differentiation of activated satellite cells might have begun, as evidenced by the presence of an increased number of centrally located nuclei (Fig 4.4) and the presence of small fibres (Fig 4.5 F).

Thus, this data demonstrates the progression of skeletal muscle regeneration from 2-12 weeks in *mdx* mice.

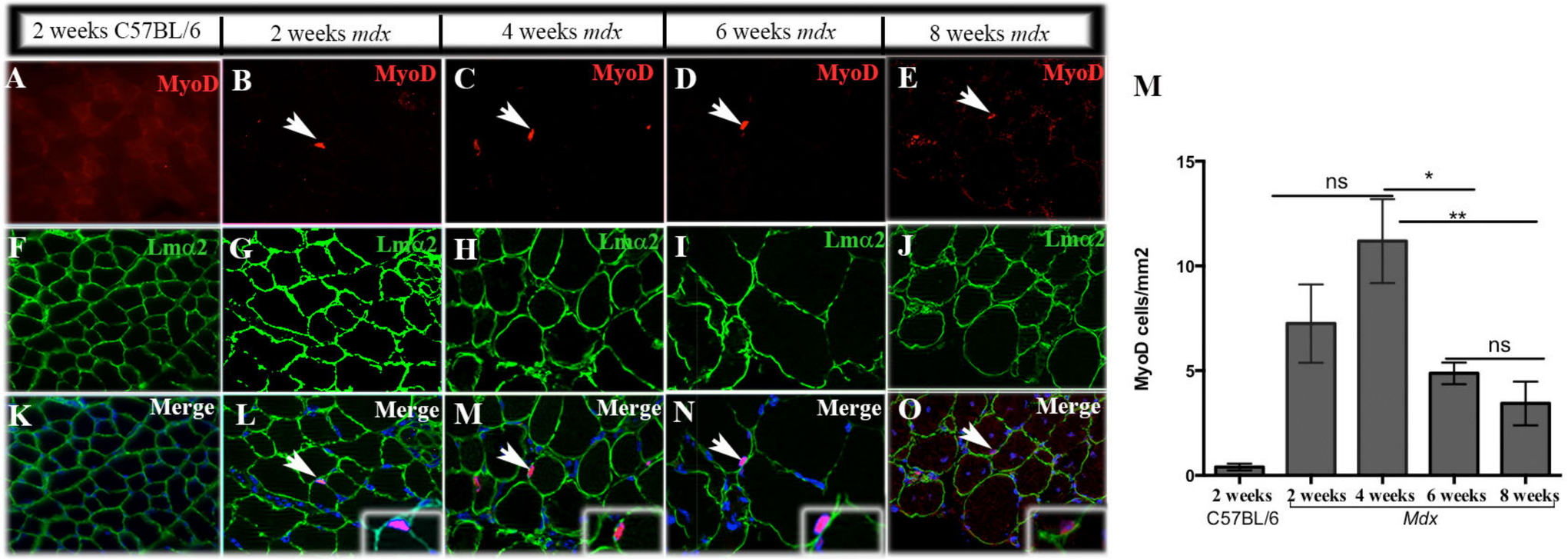


Figure 4.6: Activation of satellite cells during early postnatal weeks in *mdx* mice: Transverse sections of EDL muscles of 2 week-old C57BL/6 mice and 2, 4, 6 and 8 week-old *mdx* mice were analyzed by immunofluorescence for Laminin α2 (F-J) and MyoD (A-E). Activated satellite cells expressing MyoD are observed at 2, 4, 6 and 8 weeks (arrows in B, C, D and E respectively). Merged images are shown in K, L, M and N with magnified images in the inserts. In contrast, no MyoD-positive cells are observed in control C57BL/6 mice (A).Nuclei were counterstained with DAPI. Magnification X400. (M) Quantitative analysis show that the number of MyoD positive cells per mm2. An increase in the number of MyoD positive cells was observed at 2 and 4 weeks indicating activation of satellite cells, followed by decline at 6 weeks. Significant decrease in the number of MyoD positive cells is observed from 4 to 6 ($p=0.0124$) and in between 4 and 8 weeks ($p=0.003$). n=16 sections counted from three different animals. Statistical analyses were performed using Mann-Whitney test.

4.2.2.3 Inflammation in mdx mice

Another characteristic of *mdx* mice is the infiltration of inflammatory cells. The initial breakdown of the basement membrane and the degeneration of the myofibres is associated with this extended inflammatory process (Bridges 1986). I already described that accumulation of cells occurs during the initial post-natal weeks and precedes the formation of new fibres (Fig 4.3). It has been suggested that voluntary wheel running or intense exercise using treadmills can exacerbate the dystrophic phenotype of *mdx* mice (Cerri et al. 2008; De Luca et al. 2003). To assess the severity of damage in exercised *mdx* mice, 4 week-old *mdx* mice (n=4) and wild-type mice (n=4) were trained to voluntary running on a wheel for 17 days. A metal mouse wheel was placed on the cage floor and the average daily distance ran was measured using a bicycle pedometer. The sensor of the pedometer was attached to the back of the cage and the reading was noted every day. Both *mdx* and wild-type mice ran extensively with an average distance of 3.23 km/day ± 0.41 and 4.02 ± 1.21km/day respectively (Fig 4.7). The effect of exercise was assessed by looking at the levels of infiltrating inflammatory cells such as macrophages. EDL muscles from sedentary and exercised animals of *mdx* and wild-type mice were harvested and analyzed by immunofluorescence using F4/80 antibody which labels all macrophage populations. Macrophages were scarcely detected in sedentary wild-type mice (Fig 4.8 A,B). but the number of macrophages increased significantly in exercised mice (Abood and Jones 1991) (Fig 4.8 C,D). As anticipated, *mdx* mice showed increased number of macrophages compared to either sedentary or exercised wild-type mice. Yet the number of macrophages increased further when exercised (See Fig 4.8 E-H). Significant increase in the number of macrophages was observed both in *mdx* and in control animals compared to the sedentary mice as expected. In *mdx* mice 1.2 fold increase in the number of macrophages was observed compared to sedentary *mdx* mice. Surprisingly, in wild-type exercised mice 2.2 fold increase in the number of macrophages was observed compared to sedentary mice.

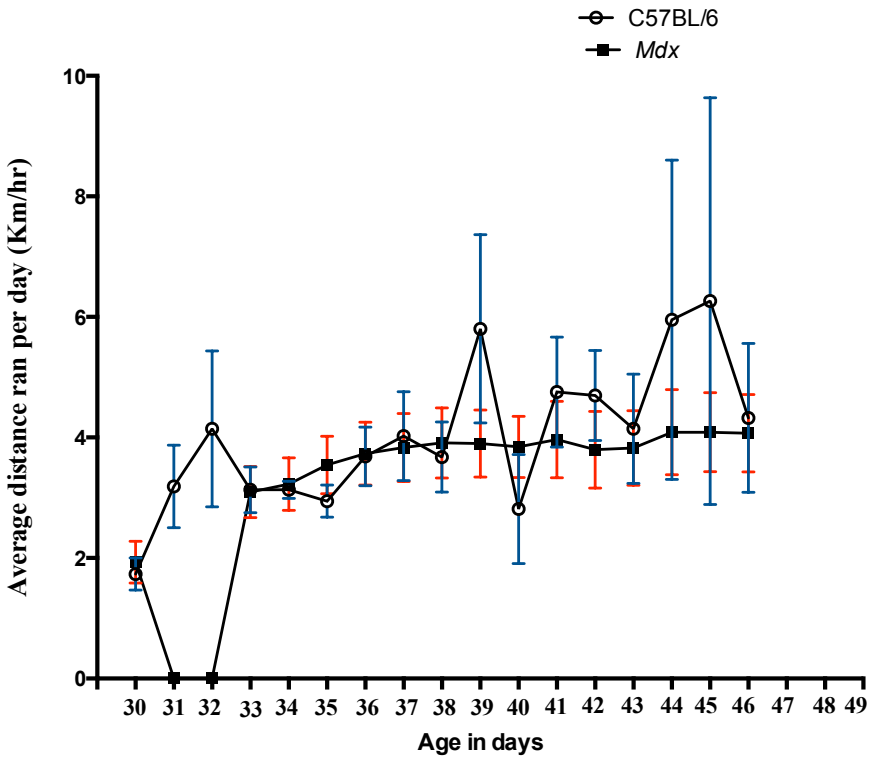


Figure 4.7: Quantification of running activity per day: The average distance (in km) ran per day by C57BL/6 and *mdx* mice was recorded over a period of 17 days. Four week-old mice were offered free wheel running and the average distance ran was recorded using a pedometer attached to the cage. The average distance per day ran by *mdx* and C57BL/6 is 3.23 km/day ± 0.41 and 4.02 ± 1.21, respectively. No significant difference was observed in the average distance both the mice travelled over time. Data is from four different animals for each mice strain (n=4). Note: Error bars represent the standard error of mean.

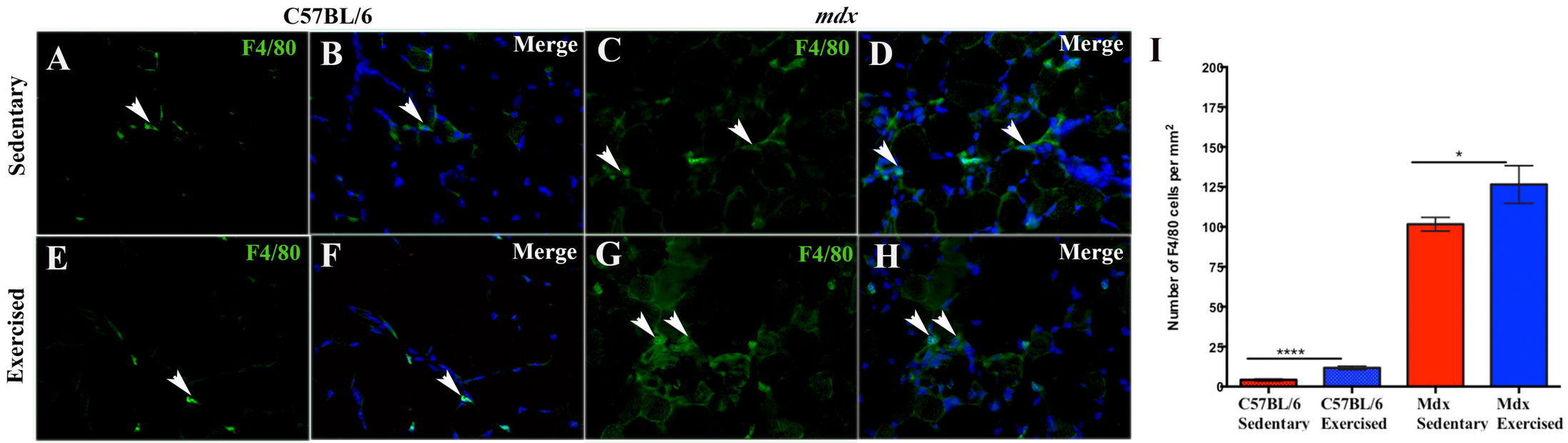


Figure 4.8: Exercise causes an increased infiltration of macrophages in EDL muscles: EDL muscles of sedentary and exercised C57BL/6 and *mdx* were analyzed for the presence of macrophages using an antibody against F4/80. F4/80 positive cells were observed in all muscles (white arrows). Merged images are shown in B, F, D and H. (I) Quantitative representation of the number of positive cells per mm2 of EDL muscle is represented. A significant increase in the number of F4/80 positive cells is observed in exercised mice compared to sedentary mice in C57BL/6 ($p=<0.0001$) and *mdx* ($p=0.0135$). However, macrophages levels were noticeably higher in *mdx* compared to wild-type mice.Statistical analyses was performed using Mann-Whitney test.Note: Error bars represent the standard error of mean.

4.2.3.1 Histology analysis of cardiotoxin-injured muscles

Another model of muscle injury is the cardiotoxin injury model. Cardiotoxin confers acute muscle injury locally. Cardiotoxin (50μl) was injected by intramuscular route into the left Tibialis anterior (TA) muscle of C57/BL6 mice. The right TA muscle injected with the same volume of PBS was used as a control. Injured and control muscles were harvested at 2, 4, 7 and 14 days post-injury in agreement with the literature. To assess the morphological changes taking place following CTX injury, all muscles were stained with hematoxylin and eosin. At two days post-injury, necrosis and infiltration of mononucleated cells are wide-spread at the site of injury (yellow arrows in Fig 4.9 A-C) resulting in the damage of the muscle architecture compared to the non-injured region of the same muscle (green arrowhead Fig 4.9 B). The infiltration of inflammatory cells and satellite cells continues until 4 days (Fig 4.9 D-F). The formation of new myofibres was observed from 7 days post-injury (Fig 4.9 G-O). New myofibres are characterized by the presence of centrally located nuclei at day 7 and 14 (red arrows in Fig 4.9 H-I and K-L). Newly formed fibres were heterogeneous in size. Cross sectional (CSA) analysis reveals that at 2 day approximately 25% of myofibres have a small CSA ($<400mm^2$) and this number increases to 40% at 4 day (Fig 4.10), confirming that regeneration is on-going. In contrast, uninjured muscles have a number of fibres with large CSA $>1000\mu m^2$. The CSA of newly formed fibres increase as the regeneration proceeds between 7 days and 14 days, such that nearly all fibres show a similar profile than uninjured muscles by 14 days (Fig 4.10). This data is consistent with previous reports showing that the majority of myofibres in TA muscles (94%) are fibre type IIDB and IIB with a CSA above $1000\mu m^2$ and the remaining 6% fibres have a CSA inferior to $1000\mu m^2$ (Augusto et al. 2004).

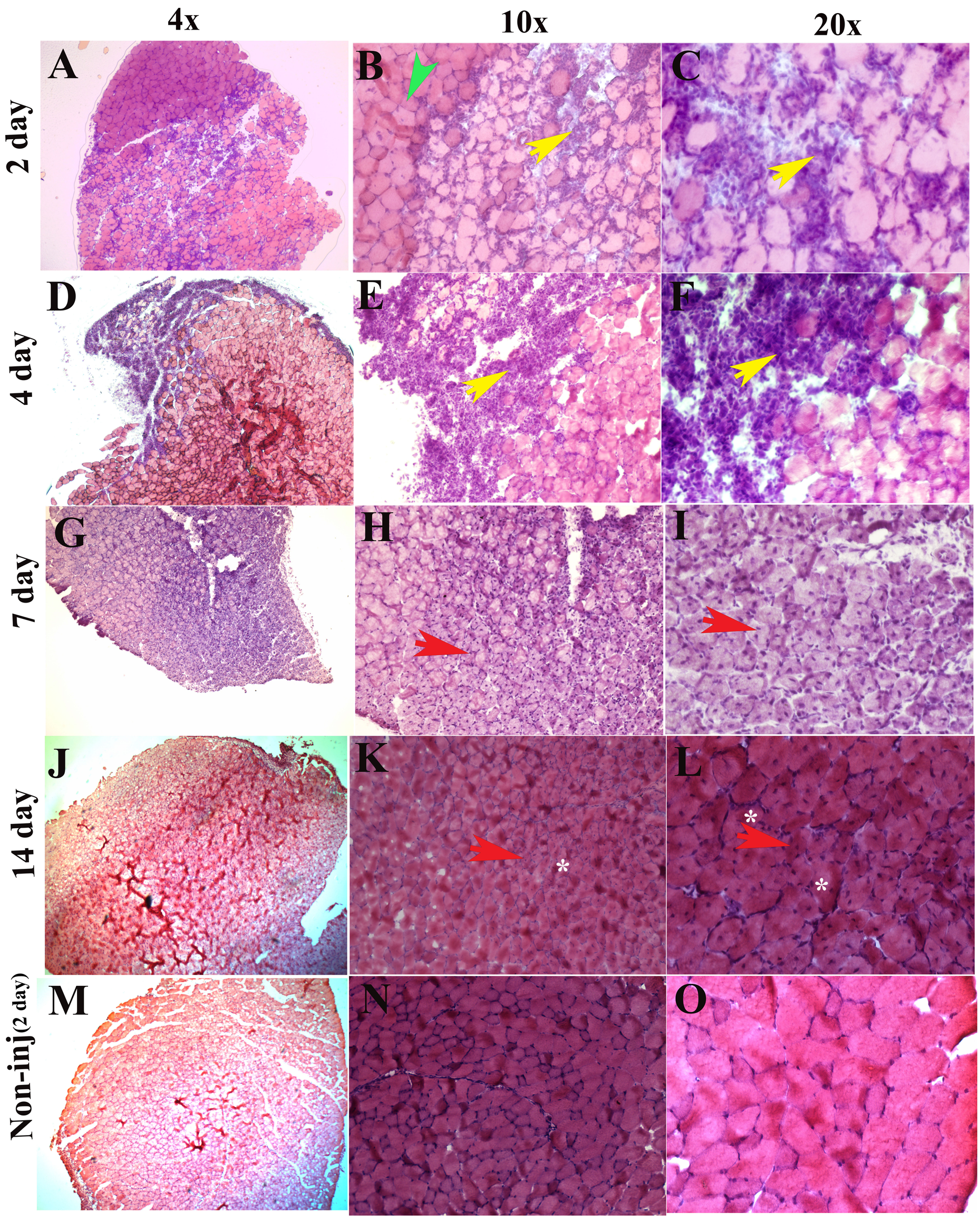
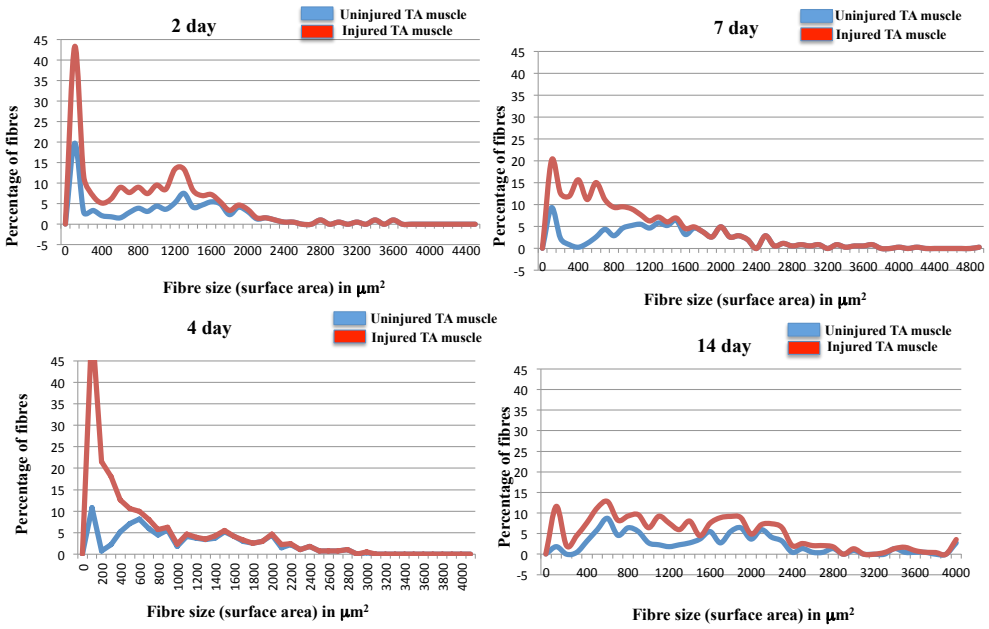


Figure 4.9: Progression of regeneration in cardiotoxin induced muscle injury: Cardiotoxin injured TA muscles from C57BL/6 mice were examined by H and E analysis at 2, 4, 7 and 14 days post-injury. The muscle injury is characterized by the presence of mononucleated cells (satellite cells and inflammatory cells) and necrosis at 2 days (A-C) and 4 days post-injury (D-F). Green arrowheads show non-injured area of the muscle (B). Area with accumulation of infiltrating cells and necrosis at 2 and 4 days is shown by yellow arrows. The presence of regenerated myofibres is observed with the presence of centrally located nuclei (red arrows G-I). Fully regenerated fibres with similar fibre sizes to uninjured muscles (N-O) are observed at 14 days (asterisks in K-L).

A


2 day
Uninjured TA muscle
Injured TA muscle
Percentage of fibres
Fibre size (surface area) in µm²
7 day
4 day
14 day

B

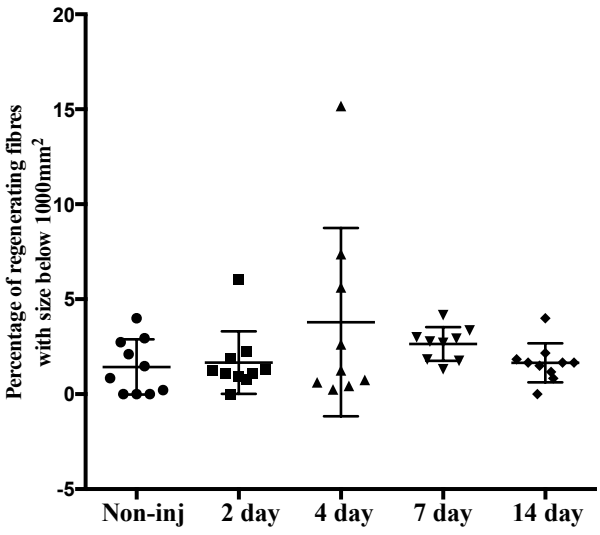

Percentage of regenerating fibres with size below 1000mm²
Non-inj
2 day
4 day
7 day
14 day

Figure 4.10: Muscle regeneration as assessed by the cross-sectional area of myofibres: (A) Cross sectional area of myofibres was analyzed at 2, 4, 7 and 14 days post-injury and compared with that of uninjured muscle. The percentage of fibres is plotted against fibre cross-sectional area in μm^2. A high percentage of small fibres is observed to be high at 2 and 4 days this gradually decreases at 7 days and by 14 days the profile is very much similar to that of uninjured muscles. A clear shift in the increase in fibre cross sectional area is observed in between 12 to 14 days indicating the progression of regeneration. (B) Graph showing the percentage of regenerating fibres smaller than $1000mm^2$ compared to that of uninjured TA muscle fibres confirm that small fibres peak at 4 days before declining at 7 days.

4.2.3.2 Satellite cells are activated during skeletal muscle regeneration following cardiotoxin-mediated injury

To identify satellite cells in cardiotoxin injured muscles, injured and non-injured TA muscles were harvested and cryosectioned to perform immunofluorescence analyses using antibodies against Laminin alpha2 and Pax7. Pax7-positive cells were detected at 2, 4, 7, 14 day post-injury and in control muscles (Fig 4.11 A, C, E, G and I) (white arrows in Fig. 4.11 B, D, F and H). As expected, a rapid 4 fold increase in the number of Pax7 positive cells (Fig 4.11 M) were observed 2 days after injury compared to uninjured muscle. Secondary antibody controls are shown in Fig 4.11 K (Alexa 594) and L (Alexa 488). The population of Pax7 positive cells remained high during the regeneration process and declined only at 14 days when the muscle architecture was considerably restored to normal (Fig 4.9). This data is consistent with reports in the literature (Kojima et al. 2007; Oustanina et al. 2004).

Following their activation, satellite cells proliferate and expand prior to their terminal differentiation (Gnocchi et al. 2009; P. S. Zammit et al. 2002). To detect activated satellite cells, I performed immunofluorescence analyses with antibodies against MyoD. MyoD positive cells were detected at 2, 4, 7 days but not in non-injured muscle (white arrow in Fig 4.12 A, C, E and G). A significant increase in the number of activated satellite cells was observed at 2, 4 and 7 days post-injury ($p=0.0043$, $p=0.0317$ and $p=0.0317$ respectively) (Fig 4.12 I). Interestingly, while the number of Pax7 positive cells remained constant between 2-7 days post-injury (Fig 4.11K), the number of MyoD positive cells continued to increase between 2-7 days post-injury to

reach a total number (300 cells per mm^2) (Fig 4.12I). This is nearly twice as many MyoD positive cells compared to the number of Pax7 positive cells. This suggests that satellite cells are differentiating to form new fibres and thus have down-regulated Pax7. The rise in the percentage of fibres with centrally located nuclei (CLN) between 2 and 14 days post injury (Fig 4.12) is in agreement with this possibility. Newly regenerated fibres are usually small, basophilic and also express neonatal myosin (Ecob-Prince et al. 1986).

To conclude, there is a rapid activation of satellite cells within 2 days following cardiotoxin mediated injury. This activation and expansion is followed by the differentiation of satellite cells starting at 4 days post-injury which culminates at 7 days post-injury with the detection of centrally located nuclei indicating the completion of the regeneration process.

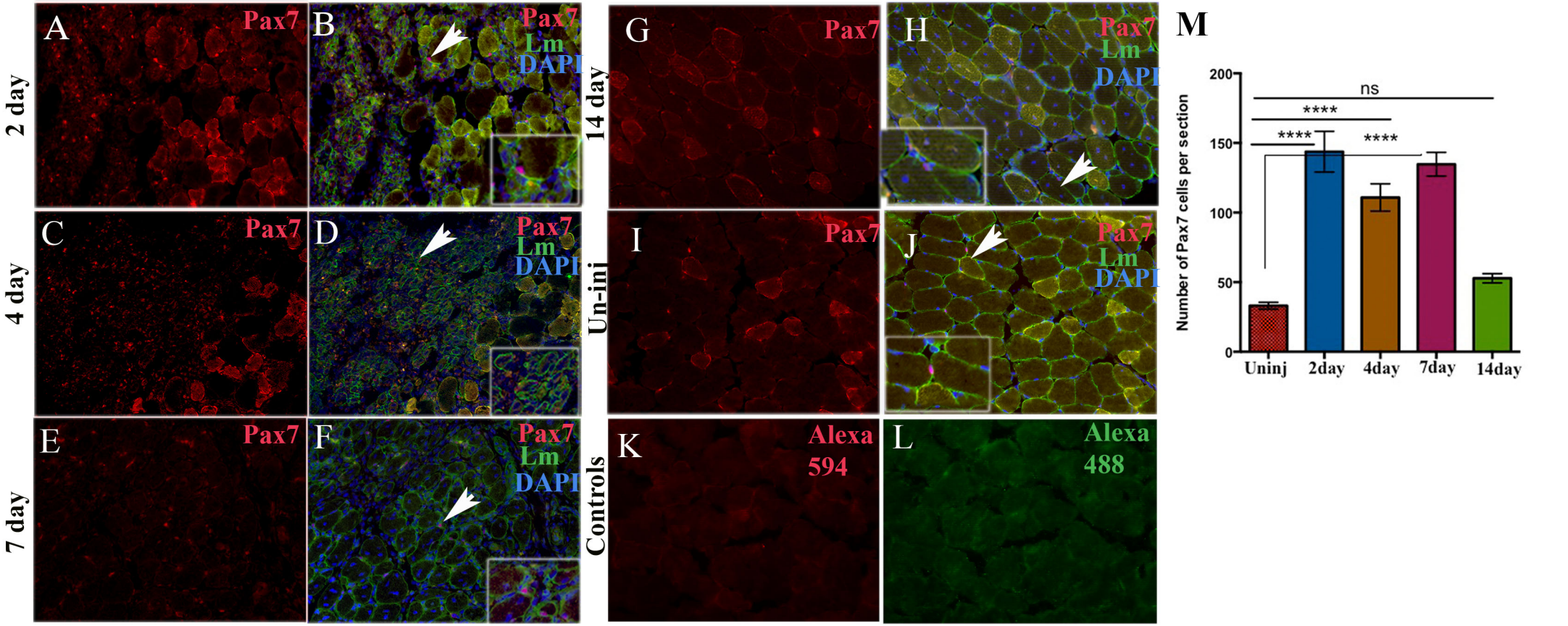


Figure 4.11: Increased number of satellite cells in cardiotoxin injured muscle: Cardiotoxin injured TA muscles were analyzed by immunofluorescence using antibodies against Laminin α2 (green) and Pax7 (red). (A,B) Pax7 cells are detected lying underneath the Laminin (in green) near small degenerating fibres. Pax7 positive cells persisted at 4 and 7 days but decreased by 14 days (C-D, E-F and G-H). (I-J) Pax7 positive cells in uninjured muscle. Merge images with Pax7 in red, Laminin in green and DAPI in blue are shown for injured 2, 4, 7, 14 days and uninjured muscle in B, D, F, H, and J respectively. Secondary antibody controls are shown in K and L for Alexa 594 and Alexa 488 respectively. (M) Quantitative representation of number of Pax7 positive cells per section is represented over different time points after injury. Significant increase in Pax7-positive cells is observed 2, 4 and 7 day post-injury compared to control ($p=<0.0001$). At 14 day no significant difference is observed indicating near completion of the regeneration process post-injury. Statistical analysis is performed using ANOVA.

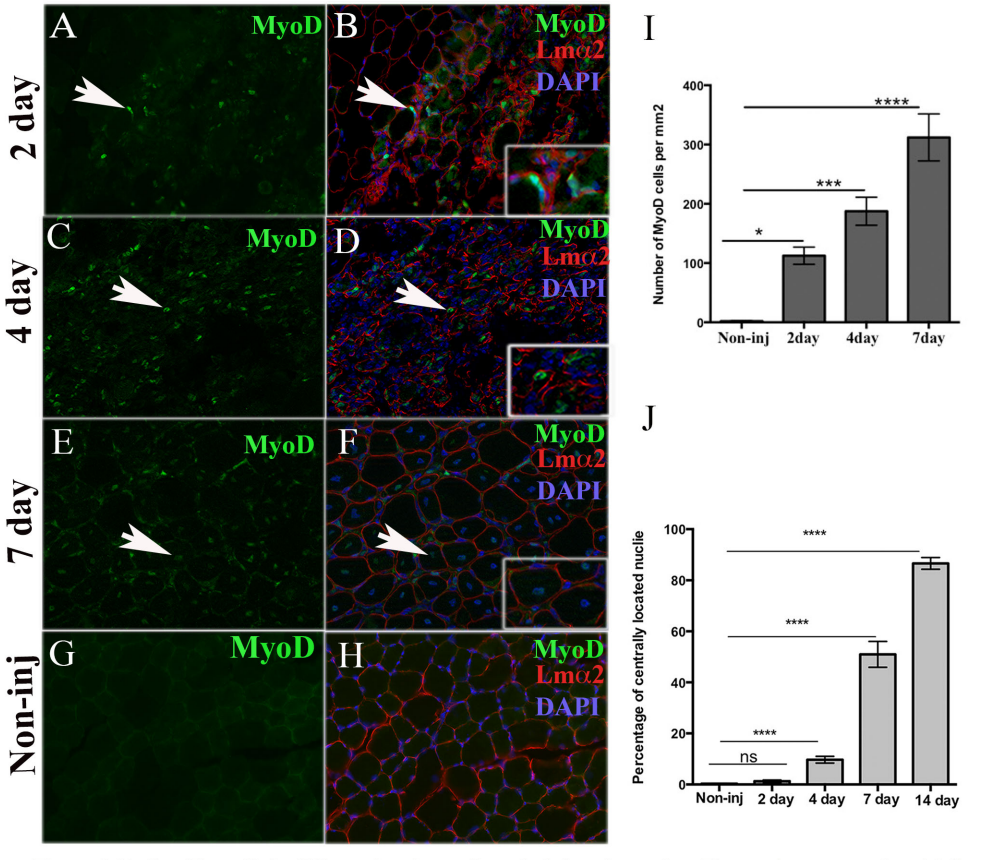


Figure 4.12: Satellite cells in differentiate in cardiotoxin injured muscles: TA muscles were analyzed following CTX mediated injury and the number of activated satellite cells was determined by IF using antibody against MyoD. Activated satellite cells were detected underneath the Laminin alpha2 (red) at 2 (A-B), 4 (C-D), and 7 days (E-F) post-injury (white arrows in green) and compared to non injured TA muscles which shows no expression of MyoD. (I) Quantitative data shows that the number of MyoD positive satellite cells gradually increase upon injury. A significant increase was noted at 2 ($p=0.0138$), 4 ($p=0..0001$) and 7 ($p=<0.0001$) days post injury.
(J) Percentage of centrally located nuclei increase significantly in 4, 7 and 14 day post-injury ($p=<0.0001$) compared to non-injured TA muscle representing regeneration process in the injured TA muscle. Statistical analyses was performed using ANOVA. Error bars indicate standard error of mean.

4.2.3.3 Inflammation in cardiotoxin-injured muscles

Acute muscle injury as a result of cardiotoxin injection into TA muscles causes an inflammatory response by the invasion of neutrophils followed by different populations of macrophages. To assess whether macrophages invaded the injured TA muscle following cardiotoxin-injury muscles were sectioned (6-8μm) and analyzed by immunofluorescence using antibody against F4/80 at 2, 4 and 7 day post-injury. Uninjured TA muscle show very few macrophages (Fig 4.13 A,B). In contrast, a drastic increase in the number of F4/80 positive cells was observed in 2 day post injured muscle (Fig 4.13 C-D).

Macrophages expressing F4/80 persist until 4 days, and then decline at 7days (Fig 4.13 E-I) (Tidball and Villalta 2010; Vidal et al. 2008). The rise in number of F4/80 cells at 2 days post-injury suggests the maximum damage and hence the macrophages invade to remove the debris. Consistently, the number of activated satellite cells increase at 2 and 4 days post-injury (Fig 4.11 and 4.12) suggesting that the rise in macrophage number is necessary for the increase in the number of activated satellite cells. There is existing evidence that intraperitoneal injection of anti-F4/80 antibody prior to injury reduced the number of MyoD-positive cells resulting in impairment of muscle regeneration (Tidball and Wehling-Henricks 2007).

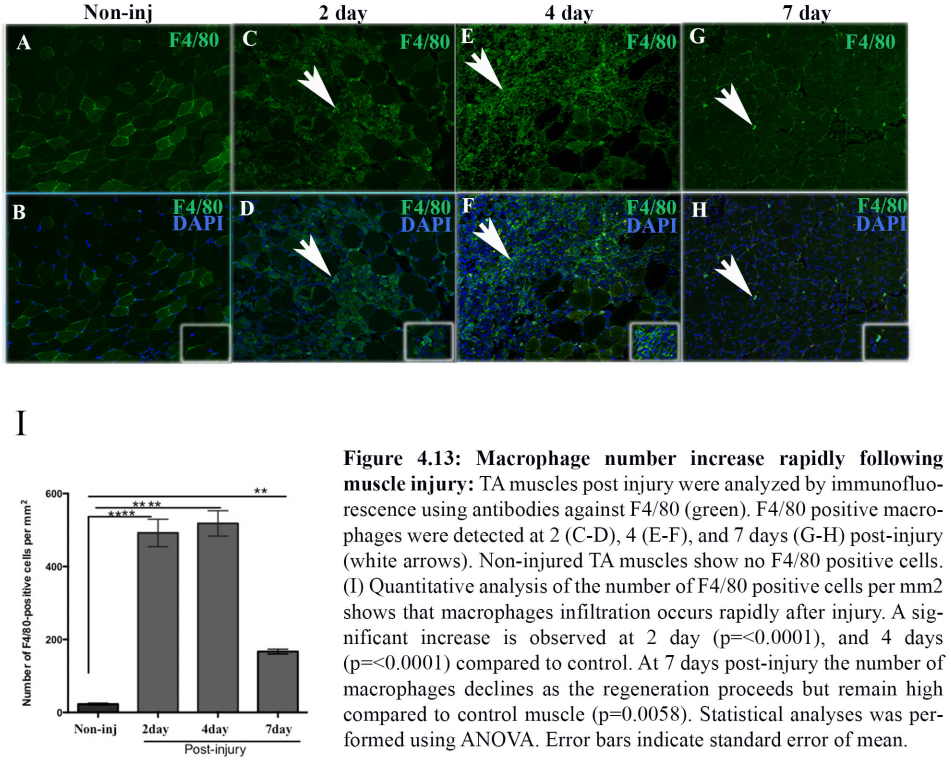


Figure 4.13: Macrophage number increase rapidly following muscle injury: TA muscles post injury were analyzed by immunofluorescence using antibodies against F4/80 (green). F4/80 positive macrophages were detected at 2 (C-D), 4 (E-F), and 7 days (G-H) post-injury (white arrows). Non-injured TA muscles show no F4/80 positive cells. (I) Quantitative analysis of the number of F4/80 positive cells per mm2 shows that macrophages infiltration occurs rapidly after injury. A significant increase is observed at 2 day ($p=<0.0001$), and 4 days ($p=<0.0001$) compared to control. At 7 days post-injury the number of macrophages declines as the regeneration proceeds but remain high compared to control muscle ($p=0.0058$). Statistical analyses was performed using ANOVA. Error bars indicate standard error of mean.

4.3 Discussion

4.3.1 Different models of chronic and acute muscle regeneration to study satellite cell activation

The kinetics and amplitude of degeneration and regeneration of muscles vary depending on the extent of the injury, the muscle types and also the animal models used (J. E. Anderson et al. 1988). The process of muscle regeneration initiates and follows similar steps in acute muscle injury caused by cardiotoxin injection or exercise- induced muscle damage, or in chronic muscle injury such as in the *mdx* mouse model of muscular dystrophy. However, there are dynamic changes at the level of inflammation and interactions among different cell types, expression of cytokines/growth factors, fibrosis and time duration among these models. One of the major difference between cardiotoxin-induced muscle damage (acute) and muscle degeneration in *mdx* mice (chronic) is the deposition of Collagen and other ECM components (Goetsch et al. 2003; Hirata et al. 2003; Kherif et al. 1999). Transient Collagen deposition and inflammation occurs upon cardiotoxin injury, whereas Collagen deposition and persistent inflammation prevails in *mdx* mice (Alexakis et al. 2007).

The time and frequency of satellite cell activation and proliferation vary also between types of muscle injury (R. N. Cooper et al. 1999; Yablonka-Reuveni and Anderson 2006). As reported in the literature, I also observed the difference in the regeneration phases in these two models. In cardiotoxin-injured TA muscles, satellite cells show expansion in the number of Pax7 positive cells (Fig 4.11). I have observed around 239 Pax7-positive cells per mm^2 at 2 days post-injury, declining subsequently to 88 Pax7-positive cells per mm^2 by 14 days post-injury. This is consistent with the literature, that reports around 150 cells at 3 day post-injury (Murphy et al. 2011) and Shea et al. which reports around 300 Pax7-positive cells in cardiotoxin injured TA muscle (Shea et al. 2010).

It has been shown that short term Collagen VI deposition in CTX-injured TA muscle preserves the satellite cell self-renewal pool, making additional satellite cells available for regeneration (Urciuolo et al. 2013). Indeed, I observed an increased activation and

proliferation of satellite cells as evidenced by the number of MyoD-positive cells recorded between 2 and 7 days post-injury. This is consistent with the evidence provided by RT-PCR data which showed that proliferating satellite cells expressing MyoD, are the sole source for muscle regeneration *in vivo* (Yan et al. 2003). I showed that MyoD-positive activated satellite cells appear early after the injury with an increase (around 10 fold) compared to non-injured muscles. The expansion of satellite cells (Fig 4.12) is followed by the appearance of centrally located nuclei indicating that repaired fibres are readily visible at 7 and 14 days post-injury (Fig 4.9). This is a gradual process as the percentage of fibres with a small diameter decreases over time to make room for fibres with large cross-sectional area (Fig 4.10). This is correlated with an increase in the percentage of centrally located nuclei (Fig 4.9 J), as previously reported in the literature (Dentice et al. 2010; Hara et al. 2011; Le Grand et al. 2012).

In contrast, in *mdx* mice the absence of Dystrophin results in muscle degeneration starting at 2 weeks post-natally (P2) with an increase in the number of c-Met positive satellite cells (Fig 4.5) and the activation of satellite cells from 4 weeks onwards. I recorded the highest number of MyoD-positive satellite cells at 4 weeks. This is consistent with previous data showing high levels of MyoD expression at 4 weeks of age in *mdx* mice have (Mokalled et al. 2012). Activated satellite cells persist through to P6. At later stages, the number of MyoD-positive cells decreases and a rise in the number of centrally located nuclei indicating newly regenerated fibres from P4 (Fig 4.6) These observations are consistent with data reported in the literature (Beilharz et al. 1992). It has been shown that in *mdx* mice, MyoD levels significantly rise from about P3 and P6 of post-natal development (Beilharz et al. 1992).

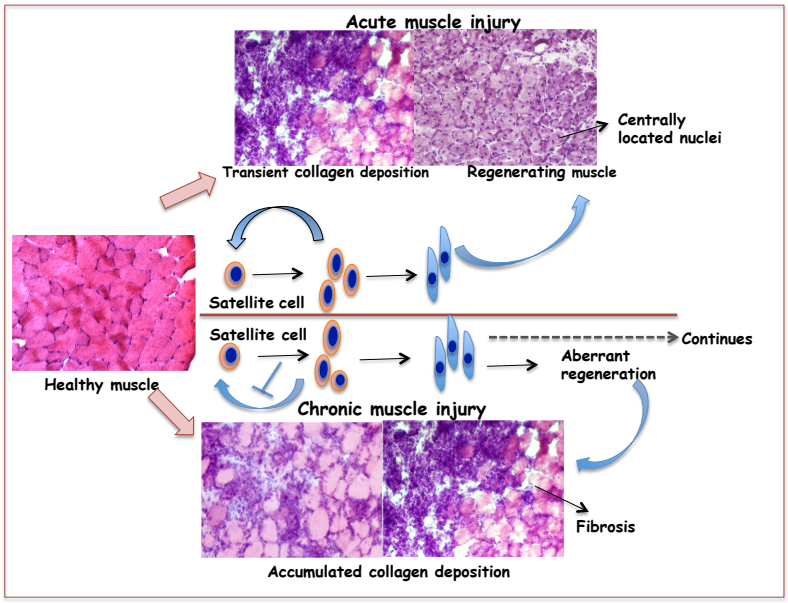


Figure 4.14: Muscle repair during acute and chronic injury: During acute muscle injury controlled inflammation and transient collagen deposition promotes replacement of damaged muscles with the activation of satellite cell at the injury site. In contrast, during chronic muscle injury, excessive accumulation of collagen and other ECM components prevents myogenic repair resulting in aberrant regeneration. Satellite cells divide continuously and the self-renewing pool also gets exhausted in such conditions (in dystrophies).

4.3.2 Inflammation in acute and chronic muscle injury

Although the replacement of damaged muscles is dependent on the activation, proliferation and differentiation of satellite cells, muscle repair is also dependent on interactions between different cell types (Tidball 1995). Indeed, immediately after muscle damage, cytokines and growth factors are released from injured muscles and attract inflammatory cells such as neutrophils and macrophages, to the injured site (Tidball 1995). *In vitro* studies showed that rat L6 muscle cells when co-cultured with either neutrophils or macrophages alone, kill the muscle cells by super-oxide dependent or NO dependent pathways respectively. But the same L6 muscle cells when co-cultured with both neutrophils and macrophages reduced the cytotoxicity suggesting that interactions of different cells modifies the extent of muscle damage (Nguyen and Tidball 2003). Macrophages are one of key cell types that are involved in muscle regeneration. During early phases of acute muscle injury, Th1 inflammatory response results in neutrophil infiltration followed by macrophages. Initially, anti-

inflammatory M1 macrophage population (CD68+) invades the injury site followed by inflammatory M2 macrophage population (CD163+/CD206+) mediated by Th2 inflammatory response (Tidball and Villalta 2010). Both the macrophage populations express F4/80, macrophage specific antigen. I have shown that immediately after muscle damage, the number of F4/80-positive macrophages increases in the cardiotoxin injury model, such that macrophages numbers increase steadily from 2 day post-injury until 4 day before declining rapidly at 7 day (Fig 4.13), at the time when MyoD-positive cells enter differentiation and form new myofibres. Following cardiotoxin-mediated injury, infiltration of M1 macrophages is associated with muscle damage mediated through NO-dependent mechanism and the expression of cytokines such as TNF-α, IL-4 and IL-6 within 1 day post-injury (Deng et al., 2012). Later M2 macrophages express IL-10 mRNA at 3 day post-injury (Novak et al. 2014). Macrophages directly affect muscle regeneration, as it has been shown that intraperitoneal injection of anti-F4/80 antibody in Soleus muscles prior to injury reduced the number of MyoD-positive cells resulting in impairment of muscle regeneration (Tidball and Wehling-Henricks 2007). Because, anti-F4/80 binds with both M1 and M2 macrophages, further depletion studies using diphtheria toxin receptor transgenic mice suggested that transient ablation of M2 macrophages at 2 day post-injury resulted in increased necrotic fibres and small regenerating fibres (H. Wang et al. 2014). This suggest that M2 macrophage population has a role in muscle regeneration during acute injury. In line with this, ablation of IL-10 cytokine increased the expression of pro-inflammatory cytokines such as IL-6 and CCL-2, and also the number of Myogenin-positive cells increased in IL10 mutant mice indicating the early differentiation (Deng et al. 2012). Comparably, I recorded highest number of F4/80 macrophages before 4 day post-injury suggesting that macrophages are essential during the time when satellite cells are active, proliferate and enter differentiation by 7day post-injury.

In contrast in chronic injury such as in *mdx* mice, extensive inflammation occurs as a result of infiltration of M1 macrophages along with M2c, a subpopulation of M2 macrophages (Tidball and Villalta 2010). Role of macrophages during muscle

regeneration was well studied in *mdx* mice. Complete depletion of macrophages at 3-4 weeks using F4/80 antibody resulted in 70-80% reduction in muscle damage (Wehling et al. 2001) suggesting that inflammation in *mdx* promotes further muscle damage. In line with this, *in vivo* ablation of iNOS gene (nitric oxide synthase gene) reduced muscle cell lysis (Villalta et al. 2009). In controversial, recent studies suggests that muscle progenitor cells when co-transplanted with macrophages improve the survival, expansion and migration of the transplanted muscle progenitor cells (Lesault et al. 2012). A balance between both M1 and M2 populations might be required to carry out efficient muscle regeneration in *mdx*. Both M1 and M2a compete for the same arginine substrate and hence enhanced inflammation persists at 4 weeks (Villalta et al. 2009). The cytokines secreted by these macrophage population also affect the regeneration process by directly acting on satellite cells. For instance, suppression of IFN-γ does not reduce the cytotoxicity but increases the number of M2 macrophages and MyoD-positive cells (Villalta et al. 2011a). IL-10 secreted by M2 macrophages has been shown to increase the proliferation of satellite cells (Villalta et al. 2009) while ablation of IL-10 induces early differentiation (Deng et al. 2012). Studies from Chazaud suggests that human myogenic cells attract macrophages which support the survival by cell-cell contacts and growth factors released (Chazaud et al. 2003). In parallel, *in vitro* cultured macrophage cell line, J774 secreted myogenic factors which enhance rat and human myoblasts (Cantini et al. 2002). In addition, in mIGF-1 transgenic mice, increased expression of IL-1 and TNF-α resulted in limited fibrosis with enhanced muscle repair (Pelosi et al. 2007). Blocking the proteins that are required for macrophage production such as G-CSF and M-CSF also resulted in decreased proliferation and differentiation of satellite cells *in vivo* (Hara et al. 2011; Segawa et al. 2008). Overall, macrophages directly affect satellite cell activity mediated by cytokines in *mdx* mice.

Mdx muscle fibres are also vulnerable to exercise (Brussee et al. 1997) and hence EDL muscles analyzed from *mdx* mice exercised voluntarily on a saucer-wheel at 4 weeks of age for around 2 weeks, showed a 1.2 fold increase in the number of macrophages (Fig 4.8). This is in consistent with the data reported in the literature

that single 30-min treadmill exercise in *mdx* can cause an increase in IL-6 mRNA suggesting the activation of pro-inflammatory cytokines (Radley-Crabb et al. 2012). Noticeably control mice, which ran almost the same average distance than *mdx* mice showed a 2.2 fold increase in the number of macrophages. This suggests that genes involved in inflammatory response and extracellular components are altered in *mdx* mice to remodel the ECM and this may result in decrease of the inflammatory response (Boer et al. 2002). Nevertheless, a 2 fold increases in inflammation has been observed in the Quadriceps muscle of *mdx* mice as early as after 48hrs following exercise (Radley and Grounds 2006).

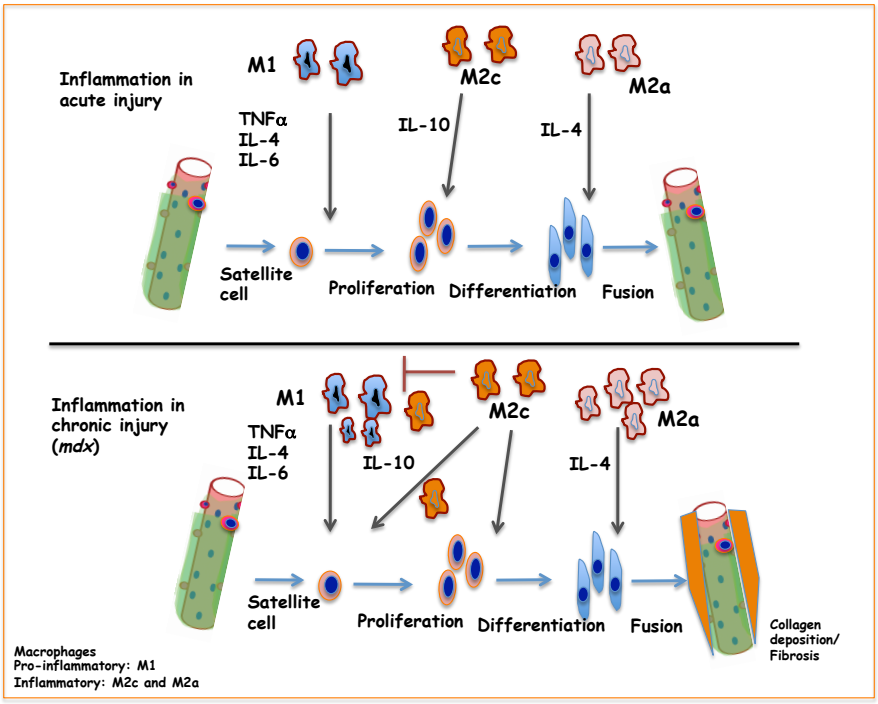


Figure 4.15: Role of macrophages in acute and chronic muscle injury: During acute injury, M1 and M2 macrophages invade the damage site sequentially and promote muscle repair, differentiation and growth by secreting different cytokines. In contrast, during chronic muscle injury both M1 and M2c macrophage population appear simultaneously and M2c prevents M1 macrophage activity. In addition, in chronic injury higher number of macrophages also results in fibrosis leading to aberrant muscle regeneration.

Similar up-regulation of cytokine levels were observed when wistar rats were trained for exercise. Pro-inflammatory cytokines IL-6, and TNF-α concentrations were

higher in exercised rats compared to their controls (Gholamnezhad et al. 2014). Consistently, it has been shown that in humans exercise training results in the increased expression of IL-6 and IL-8 whereas anti-inflammatory cytokines such as IL-10 and IL-4 are slightly up-regulated. Moreover, the changes in cytokine expression level was higher in elderly men compared to young men. This suggest that the inflammation may also have implication in regeneration in the old muscles (Della Gatta et al. 2014). Interestingly, the pro-inflammatory cytokine expression levels was reduced with prolonged exercise indicating the beneficial effects of regular exercise (Ambarish et al. 2012). Altogether, these data suggest that exercise induces inflammation which affects muscle regeneration. Recently, it has been shown that voluntary wheel running exercise activates satellite cells due to accelerated Wnt signaling both in adult and aged mice (Fujimaki et al. 2014) which affects satellite cell proliferation (Otto et al. 2008).

To conclude, in both cardiotoxin injured muscle and in *mdx*, inflammation by infiltrating macrophages and activation of satellite cells is observed as a consequence of muscle injury. And exercise further increases inflammation in *mdx* mice. Thus, I have established both acute and chronic muscle injury models to study muscle regeneration and satellite cell behavior. These *in-vivo* systems established were used to initiate my investigation on the composition of the satellite cell basal lamina (chapter 6).

Chapter 5

Laminin α1 is detected at the site of activated satellite cells and is regulated by Sonic hedgehog signaling in the *ex-vivo* muscle fibre culture system

5.1 Introduction

Tissue specific stem cells reside in a specific microenvironment defined as a niche that provides structural and biochemical cues for the proper functioning of the stem cells. The niche is further defined as "a specific location in a tissue where stem cells can reside for an indefinite period of time and produce progeny cells while self-renewing" (Spradling et al. 2001). Likewise, satellite cells also reside in their own niche that supports satellite cell activity. The physiological and pathological signals from the niche induce satellite cells to exit quiescent state and enter cell cycle. The importance of the niche is demonstrated when satellite cells from old mice were grafted in muscle of young mice and regeneration was successful (Boldrin et al. 2012; Collins et al. 2007). In contrast, when muscles from young mice were grafted into old mice, muscle regeneration was impaired indicating the role of the niche for efficient regeneration (Carlson and Gutmann 1975; Zacks and Sheff 1982). However, it has been shown that in mature adult dystrophic muscle, host environment does not inhibit satellite cell function (Boldrin et al. 2009). Satellite cells freshly isolated from young, normal donor mice when engrafted into young and matured *mdx*-nude mice, donor satellite cells contributed to robust muscle regeneration along with efficient self-renewal in these dystrophic mice (Boldrin et al. 2009). Besides, transplantation studies suggest that reduced regenerative capacity of satellite cells is due to the intrinsic factors of the cell itself and also due to the ageing of the local microenvironment suggesting that ageing of satellite cell niche might have a role in muscle atrophy (Brack and Rando 2007). During aging the proliferative and myogenic potential of satellite cells declines and SCs become susceptible to apoptosis (Brack et al. 2007; Conboy et al. 2003; Schultz and Lipton 1982). It has been shown that alterations in extracellular signals is linked to age-related decline in satellite cell behavior and muscle regeneration drastically declines during aging which in turn is manifested by the loss of functional tissue and reduces satellite cell-mediated myogenesis in response to injury (Brack and Rando 2007; Conboy et al. 2003; Conboy and Rando 2012).

In addition, alterations in SC niche may also cause delayed or reduced response of satellite cells. Changes in the microenvironment alters the expression of TGF-β–inducible genes and Wnt-inducible genes and or in Hepatocyte growth factor/c-Met signaling in aged mice (Barani et al. 2003; Brack et al. 2007). Overall, the proliferation and differentiation during regenerative purpose is tightly regulated by these intrinsic and extrinsic cues to prevent uncontrolled growth. Moreover, the proliferative capacity is reduced when satellite cells are cultured *in vitro* after separating the cells from their niche and then leads to defective regeneration when implanted back in to the muscle (Collins and Partridge 2005; Montarras et al. 2005). Thus the ability of satellite cells is highly dependent on their specific niche.

The satellite cell niche mainly constitutes of extracellular matrix, the muscle fibre and supporting cells. Supporting cells such as neutrophils and macrophages influence the activated satellite cells and the role of these cells during muscle regeneration is beginning to understand (Chazaud et al. 2003; Merly et al. 1999; Nguyen and Tidball 2003; Pelosi et al. 2007; Tidball and Villalta 2010). But the role of extracellular matrix is obscure. Extracellular matrix components such as laminins, fibronectin, hyaluronic acid and tenascin are already known to be altered during muscle regeneration (Calve et al. 2010). *In vitro* studies have also shown a differential response of myoblasts grown on different ECM components (Foster et al. 1987; Girgenrath et al. 2005). Increasing evidence indicates the importance of basement membrane for satellite cell survival and function. It has been shown that satellite cell number declines in *Lama2*-deficient mice that lack a basement membrane (Girgenrath et al. 2005). All these studies indicate the possible role of basement membrane components in satellite cell activity. One of the major BM component is Laminin. Laminin composition is distinct in different domains of the muscle fibre. For instance the myofibre is surrounded by Laminin α2 except at the neuromuscular junction that also constitutes other alpha subunits α4 and α5. The remodelling of basement membrane with incorporation of different Laminin subunits is also evident during embryonic development. Laminin α1 is expressed during early development of muscle and remains expressed until the formation of secondary myogenesis at E11-12

thereafter it becomes confined to the ends of the myotubes during their assembly at E15 (Patton et al. 1997). Expression of Laminin α4 is increased as the myotubes mature at E15. As the muscle reaches adult stage, Laminin α2 becomes the major Laminin expressed in the muscle fibre (W. Kuang et al. 1998; Patton et al. 1997). This indicates that Laminin α2 is required during development whereas Laminin α1 is required in adult muscles. But the basement composition differs at the neuromuscular junction consists of Laminin α4 and α5 (Nishimune et al. 2008b).

Thus, the ECM as a niche remodels during muscle development and confers distinct composition to different domains of the adult myofibre. Henceforth, it becomes crucial to characterize the satellite cell niche in order to understand the key components responsible for controlling satellite cells in particular the basement membrane components. Identifying and understanding the role of niche components will provide better understanding of the function of satellite cells in muscle regeneration and thus provide tools for therapeutic approaches.

Thus, I aimed to characterize the basal lamina composition of satellite cell niche in an *ex-vivo* myofibre culture system.

5.1.1 Hypothesis and aim

The aim of the work carried out and described in this chapter was to characterize the basal lamina composition of the satellite cell niche.

1st hypothesis : The basement membrane around satellite cells is remodeled to support the satellite cell activity.

2nd hypothesis : Laminin α1 becomes incorporated into remodeled basement membrane during adult satellite cell mediated myogenesis.

5.2 Results

5.2.1 Expression profile of Laminin alpha subunits in adult muscles and during satellite cell activation

To assess the Laminin composition in the satellite cell basal lamina an RNA expression profile of genes encoding for different Laminin alpha subunits was

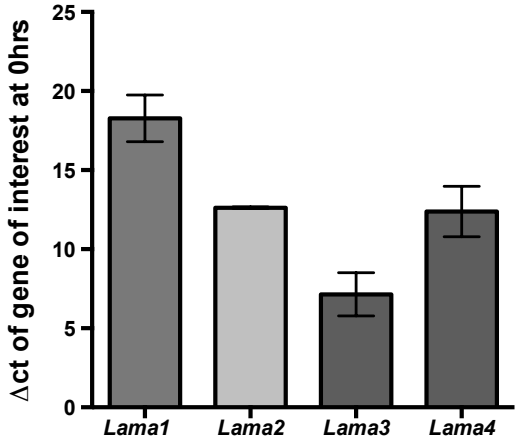
generated by real-time PCR. I choose to evaluate Laminin alpha subunits of satellite cell basal lamina rather than Laminin beta and Laminin gamma subunits because both Laminin beta and Laminin gamma subunits show a less restricted expression pattern and are mostly expressed ubiquitously (Tunggal et al. 2000). cDNA were prepared from 0 hrs, 24 hrs, 48 hrs and 72 hrs fibre cultures, whole EDL muscle, satellite cells separated from fibres following culture on Matrigel and fibres lacking satellite cells (details on the separation of SC and fibres is discussed in chapter 2). For the qPCR reactions, primers complementary to cDNA sequences of *Lama1, Lama2, Lama3* and *Lama5* were used. Ct values obtained for each sample for all genes analyzed were normalized with the corresponding Ct values obtained for the endogenous constitutively expressed housekeeping reference gene *glyceraldehyde-3-phosphate-dehydrogenase* (GAPDH). The Ct values of 0 hrs are normalized with *Gapdh* and plotted for all the samples (Fig 5.1A). Relative gene expression and fold change was calculated using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). For all the genes $2^{-\Delta\Delta C_T}$ values obtained at 0 hrs was used as a baseline and fold change of $2^{-\Delta\Delta C_T}$ values at other time points in respect to 0 hrs was calculated. The graph below shows the fold change for each gene relative to 0 hrs (Fig 5.1 B).

In agreement with previous reports (W. Kuang et al. 1998; Patton et al. 1997), *Lama5* gene expression was not observed at 24hrs although an increase in expression (1 to 3.4 fold) was observed at 48 hrs and 72 hrs, as well as in fibres and in SC. However, no significant difference was observed within these samples as shown in Fig 5.1B.

Likewise, *Lama3* does not appear to be expressed in fibres or muscles (Fig 5.1B), in agreement with previously published data (Gullberg et al. 1999). In contrast, increase in *Lama1* gene expression was observed at 24 hrs, 48 hrs, in fibres and in SC. The highest increase of 11.4 fold was observed in isolated SC, and was significantly different from values measured in the 24 hrs, 72 hrs, fibres and EDL samples. In the fibre cultures, *Lama1* is expressed from 24 hrs and is maintained at 48 hrs but declines rapidly at 72 hrs. cDNA isolated from SCs separated from the fibres suggests that *Lama1* gene is expressed in SCs. However, it should be noted that satellite cells separated on Matrigel are already activated and it would be further interesting to

check *Lama1* expression in quiescent satellite cells (cDNA from near quiescent satellite cells can be prepared by stripping satellite cells from the single fibres). Low levels of *Lama1* expression was found in the muscle, consistent with reports that *Lama1* is not expressed in normal adult muscles (Gullberg et al. 1999; Patton et al. 1997).

A



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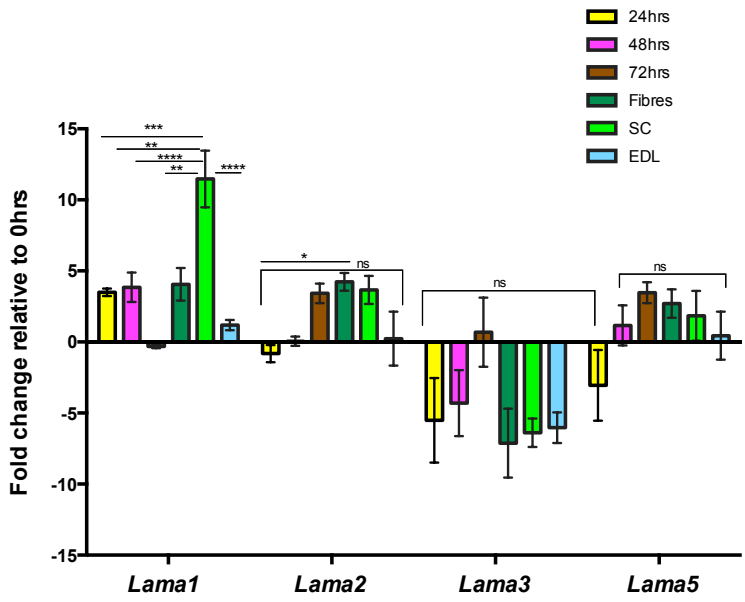


Figure 5.1: Quantitative expression of different Laminin genes by qPCR: (A) Quantitative real time RT-PCR was performed for *Lama1, Lama2, Lama3* and *Lama5* of adult EDL muscle fibres cultured for 0 hrs, 24 hrs, 48 hrs and 72 hrs , fibres (only), SC (only) and whole EDL muscle. The Ct values of 0 hrs normalized with reference (GAPDH) are plotted for each gene. (B) Fold change in the gene expression relative to 0 hrs was calculated and plotted for each gene. *Lama1* gene is highly expressed in SC to 24 hrs, 48 hrs, 72 hrs, fibre and EDL sample. *Lama2* gene expression is higher at 72 hrs, in fibres and in SC. *Lama5* gene is expressed at a very low level with a change of 1 to 3.4 fold for samples 24 hrs, 48 hrs, 72 hrs, fibres and SC. No expression observed at 24 hrs and in EDL muscle. *Lama3* is completely absent in all the samples. For *Lama1*, p=0.008 (between 24 hrs and SC); p=0.0013(between 48 hrs and SC); p=<0.0001 (between 74 hrs and SC and between SC and EDL). Statistical analysis was performed using one-way ANOVA.

In conclusion, consistent with the literature, I observed *Lama2* expression in the adult muscle (Patton et al. 1999). In addition, while and *Lama3* was completely absent from muscles, *Lama5* was found expressed at low levels in 72 hrs cultured fibres and SC. Notably, I found that *Lama1* was up-regulated in activated SC. Specifically, *Lama1* expression increased gradually between 24 hrs and 48 hrs, and declined at 72 hrs. This suggests that *Lama1* is activated at the time satellite cells are activated and expand and is down-regulated as SCs enter differentiation.

5.2.2 Laminin α1 protein is identified at the site of activated satellite cells in adult mouse EDL muscle fibres

To establish that the Laminin α1 transcript detected by qPCR is translated and distributed at the level of satellite cells, I went on to perform immunofluorescence on single EDL muscle cultured fibres.

5.2.2.1 Laminin α2 and β1 accumulate at the site of quiescent satellite cells in freshly isolated EDL muscle fibres

EDL muscles from 6-8 week-old C57BL/6 mice were harvested, treated with collagenase and single EDL muscle fibres were carefully isolated without damaging

the basal lamina to ensure that the Laminin composition is not disturbed during isolation. The fibres associated with satellite cells were immediately fixed analyzed by immunofluorescence. The adult muscle basal lamina is constituted of Laminin-211 (corresponding to α2, β1 and γ1 respectively) (W. Kuang et al. 1998; Patton et al. 1997). To evaluate the distribution of Laminins at the surface of freshly isolated fibres, I first labeled single fibres with antibodies against Laminin α2 and Laminin β1 (Fig 5.2A-B, C-D). Laminin α2 is ubiquitously distributed along the fibre (green arrowhead in Fig 5.2 A). Similarly Laminin β1 was expressed in the basal lamina of the whole fibre (Fig 5.2 B). The expression of these two Laminin subunits is consistent with the literature (Patton et al. 1999). Interestingly, I also observed that Laminin α2 and β1 accumulated at the site of satellite cells (as shown by white arrows in Fig 5.2 B and D). Thus, as expected Laminin α2 and α1 is expressed in the basal lamina of adult muscle fibres. Furthermore, Laminin-211 accumulates at the site of satellite cells, suggesting that the satellite cell basal lamina has a distinct ultrastructure from the rest of the fibre. This may constitute a structural component of the niche.

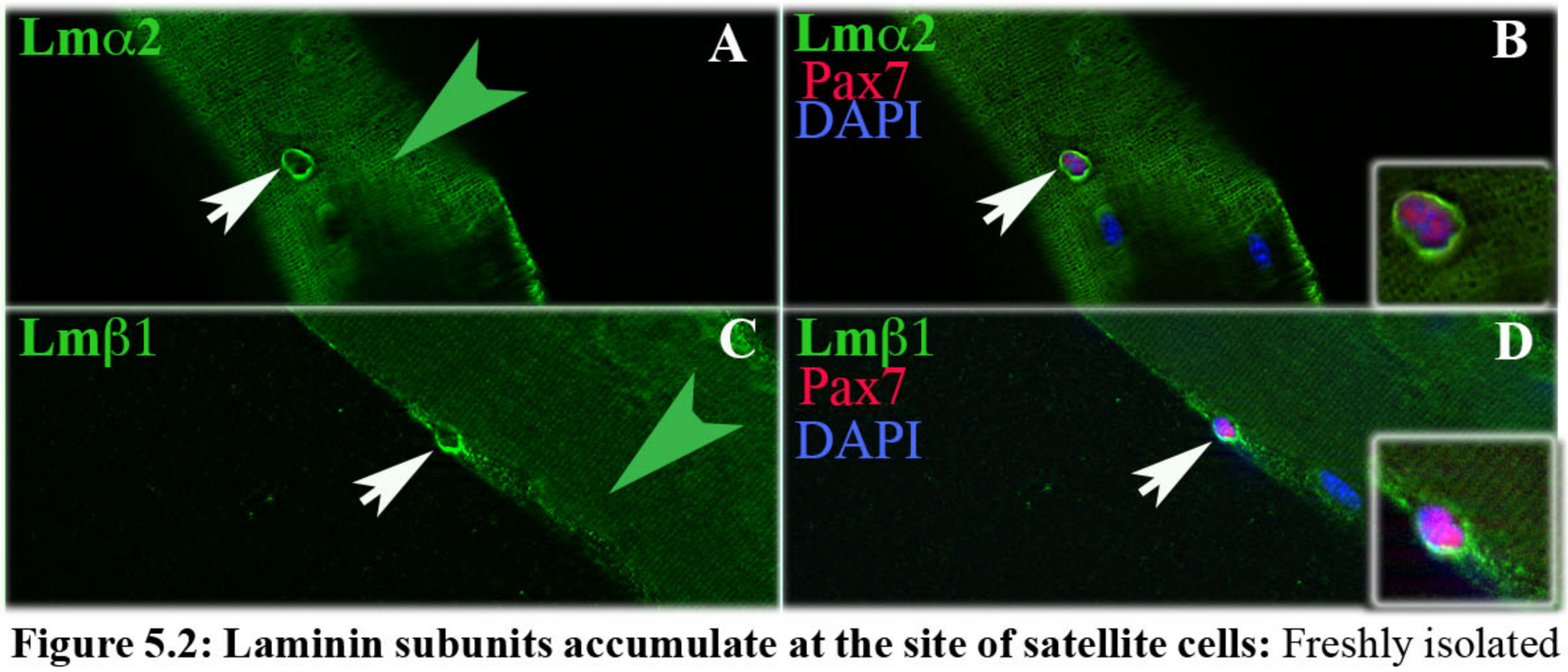
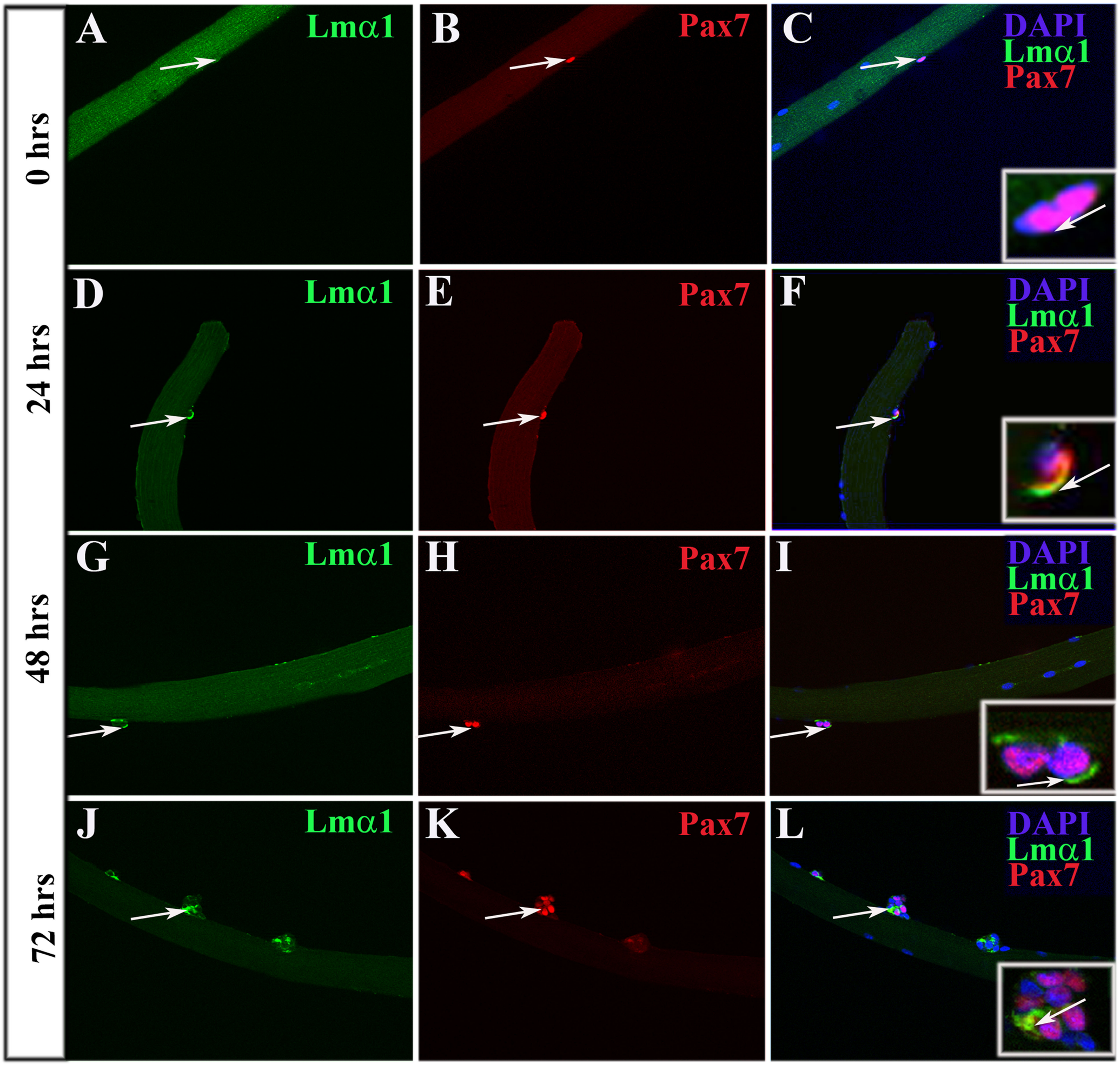


Figure 5.2: Laminin subunits accumulate at the site of satellite cells: Freshly isolated single EDL muscle fibres from 6-8 weekold C57BL/6 mouse were analyzed by immuno-fluroscence using antibodies against the satellite cell marker Pax7 (red) and Laminin α2 and β1 (green). Laminin α2 and β1 are uniformly distributed along the fibre (green arrowheads A and C respectively). In addition, Laminin α2 and β1 accumulate at the site of satellite cells (white arrows B and D). Magnified images are shown in inserts. Nuclei are counterstained with DAPI (blue). Magnification X200.

5.2.2.2 Laminin α1 is detected in the vicinity of activated satellite cells in cultured EDL muscle fibres

To further, assess the composition of the satellite cell basal lamina, single muscle fibres were isolated from EDL muscles of 6-8 week old C57BL/6 mice and cultured for 72 hrs. Freshly isolated fibres were fixed immediately to ensure that satellite cells were in near quiescence (0 hrs) state. Fibres cultured for 24 hrs, 48 hrs and 72 hrs were fixed prior to immunostaining and labeled using antibodies against Pax7 and Laminin α1. At quiescence, satellite cells expressing Pax7 do not have Laminin α1 associated with their basal lamina (white arrow in Fig 5.3 A-C). However, at 24 hrs, 48 hrs and 72 hrs Laminin α1 is detected at the site of activated satellite cells (Fig 5.3 D, G and J, see white arrows pointing to the expression of Laminin α1). The percentage of satellite cells associated with Laminin α1 gradually increases at 24 hrs and 48 hrs, from 50% of cells expressing Laminin α1 at 24hrs to ~90% at 48 hrs (see Fig 5.3 M). By 72hrs, Laminin α1 expression declines with about 60% of satellite cells found associated with a Laminin α1 containing basal lamina.

Thus, although the basement membrane of adult muscle fibres is believed to be uniformly composed of Laminin-211, I have observed that in addition to Laminin α2, Laminin α1 is expressed at the site of satellite cell. This suggests that following their activation different micro-domains exist within the muscle basement membrane that differ in their Laminin composition. It also suggests the existence of a process allowing the remodelling of the muscle basal lamina to incorporate new Laminin subunits.



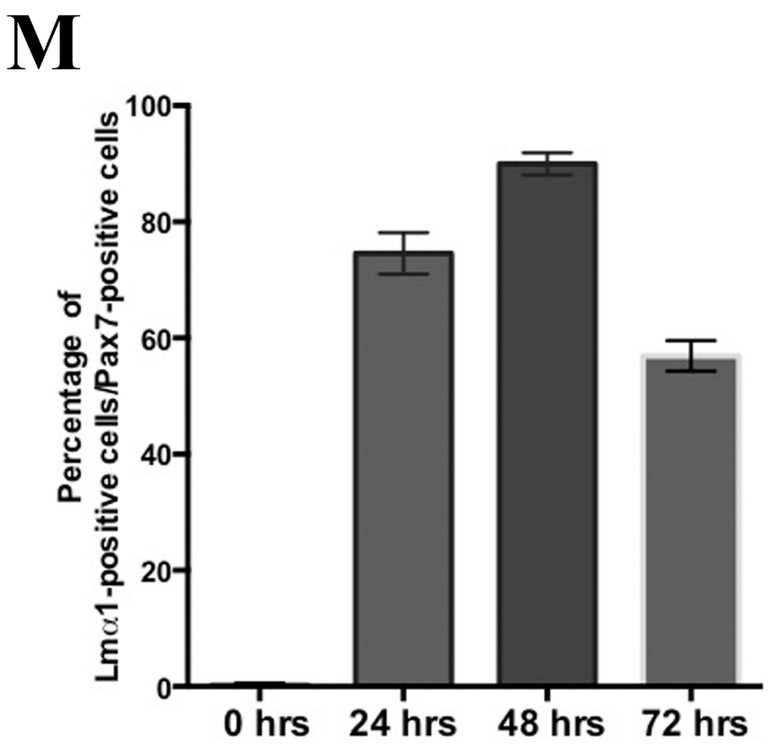


Figure 5.3: Laminin α1 is expressed at the site of activated satellite cells *ex-vivo*: Detection of Laminin α1 in the vicinity of satellite cells. Single EDL muscle fibres were isolated from 6-8 week old C57BL/6 and analyzed by immunofluorescence using antibodies against Pax7 (red) and Laminin α1 (green) at 0 hrs (A-C), 24 hrs (D-F), 48 hrs (G-I) and 72 hrs (J-L). At 0 hrs, Pax7 expressing satellite cells does not show Laminin α1 expression (A-C). *Ex-vivo* cultured fibres show the expression of Laminin α1 at the site of activated satellite cells indicated by Pax7 expression at 24 hrs, 48 hrs and 72 hrs (white arrows in D, G and J respectively). Merged images are shown in C, F, I and L with high magnification images in the insert. Nuclei are counterstained with DAPI. Magnification X200. (M) Graph representing the percentage of Laminin α1 expressing satellite cells at different time points. The maximum percentage of satellite cells with Laminin α1 expression was observed at 48hrs with ~90% cells. The data is from three independent experiments. n=40 to 50 fibres per time point.

5.2.2.3 The distribution of Laminin α1 is confirmed using antibodies from different sources

To further confirm the presence of Laminin α1 in the basal lamina overlying activated satellite cells, I used Laminin α1 antibody obtained from a different source (details in materials and methods). Single muscle fibres were isolated and cultured for 48 hrs. I choose 48hrs as I have previously observed that majority of satellite cells express Laminin α1 at 48hrs. Consistent with my previous observations, I found that Pax7+ve satellite cells express Laminin a1 in their vicinity at 48 hrs (white arrows in Fig 5.4 A-C).

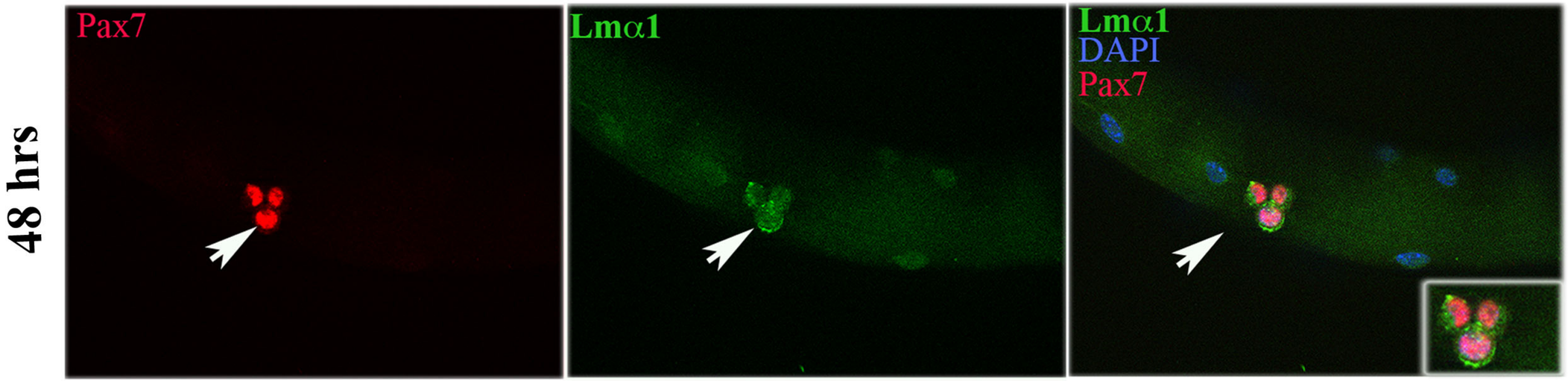


Figure 5.4: Laminin α1 expression detected with antibodies from different sources: Laminin α1 expression is confirmed using antibody from other source (Santa Cruz). EDL muscle fibres isolated from 6-8 week-old C57BL/6 mice were cultured for 48 hrs and analyzed by immunofluorescence using antibodies against Pax7 (red) and Laminin α1 (green). Laminin α1 is expressed at the site of satellite cells (Pax7-positive cells shown by white arrows). Merged image is shown in C with magnified image in the insert. Nuclei are counterstained with DAPI (blue).Magnification X400.

5.2.2.4 Laminin α1 associates preferentially with the basal lamina of satellite cells that enter differentiation

Data obtained so far show that Laminin α1 is associated with Pax7 expressing satellite cells in culture and that Laminin α1 expression begins once the cells become activated, peaks at 48 hrs and then declines (see Fig 5.3). To further assess which population of satellite cells show the expression of Laminin α1 in their vicinity, I immunolabeled cultured fibres for Pax7, MyoD and Laminin α1 to distinguish satellite cells in the expansion phase ($MyoD^{+ve}$) from the satellite cells in the differentiation phase ($Myog^{+ve}$). Indeed, MyoD acts as a gateway through which satellite cells enter the differentiation process and initiates Myogenin expressing satellite cells may also down-regulate MyoD while maintaining Pax7 expression and re-entering quiescence to self-renewal (Day et al. 2007; Nagata et al. 2006a; Neal et al. 2012). Similar to the previous data, Immunofluorescence labeling have shown that Laminin α1 is associated with activated satellite cells ($Pax7^{+ve}$ and $MyoD^{+ve}$) at 24 hrs, 48 hrs and 72 hrs (white arrows in Fig 5.5 F-G, J-K and N-O). Laminin α1 is not expressed at 0 hrs (Fig 5.5 A-D). Quantitative data obtained from three different experiments are plotted and the graphs are shown in Fig 5.5 Q and R.

The following cell populations are expected population to be observed at 24hrs and 48hrs.

1. $Pax7^{+ve}$, $MyoD^{-ve}$, Laminin $\alpha1^{+ve}$

This is a population of satellite cells that have just been activated and would be expected to express Myf5.

2. $Pax7^{+ve}$, $MyoD^{-ve}$, Laminin $\alpha1^{-ve}$

This is most likely a satellite cells population made by either quiescent satellite cells or satellite cells not yet activated.$Pax7^{+ve}$, $MyoD^{+ve}$, Laminin $\alpha1^{+ve}$

This is a satellite cell population which is in the expansion phase prior to differentiation.

3. $Pax7^{+ve}$, $MyoD^{+ve}$, Laminin α^{-ve}

This satellite cell population may either become terminally and thus has down regulated Laminin α1 or may be a satellite cell population that is in the expansion phase and does not express Laminin α1.

4. Pax7^{+ve}, MyoD^{+ve}, Laminin α^{+ve}

This satellite cell population may either enter differentiation phase or can choose to self-renew by down-regulating MyoD.

But at 72hrs two additionally satellite cell populations were observed

5. Pax7^{-ve}, MyoD^{+ve}, Laminin α1^{+ve}

This satellite cell population has entered the differentiation phase and would be expected to be Myogenin positive.

6. Pax7^{-ve} , MyoD $^{+ve}$, Laminin α1^{-ve}

A satellite cell population expected to be Myogenin positive have advanced in the differentiation programme than the population above and therefore in the process of down regulating Laminin α1.

The number of cells expressing Pax7 and MyoD with Laminin α1 increases over time in *ex-vivo* cultured fibres. At 24 hrs and 48 hrs, double-positive cells (Pax7 and MyoD) expressing Laminin α1 in their vicinity remain the major proportion of cells. At 72 hrs, the total number of satellite cells per fibre increases to ~55 cells per fibre. The number of double-positive (Pax7 and MyoD) cells showing Laminin α1 expression remain constant but cells entering differentiation and down-regulating Pax7 are also positive for Laminin α1.

The major population of cells positive for Pax7, MyoD and Laminin α1 is found in 42%, 81% and 34% of satellite cells at 24 hrs, 48 hrs and 72hrs respectively (Fig 5.5.B), indicating that Laminin α1 is expressed when the satellite cells are activated. Activated satellite cells expressing MyoD show sustained Laminin α1 expression at 48 hrs. At 72 hrs the expression declines to 34% when cells enter the differentiation process and down regulate Pax7. Around 24% of cells that express Laminin α1 are negative for Pax7 and positive for MyoD at 72 hrs. Most likely, these cells have the differentiated and express Myogenin. Within this population, not all cells were

positive for Laminin α1. 28% of cells which express Myogenin down regulate both MyoD and Laminin α1. This suggests that once cells are terminally differentiated they down regulate Laminin α1. Very few percentage of cell around 4% were MyoD-positive and negative for Pax7 and Laminin α1 indicating that activated satellite cells that express MyoD are mostly associated with Laminin α1.

During the myogenic process, a small percentage of $Pax7^{+ve}$ cells that were MyoD-negative and Laminin α1−negative were never associated with Laminin α1, suggesting that these cells were set aside as self-renewing cells.

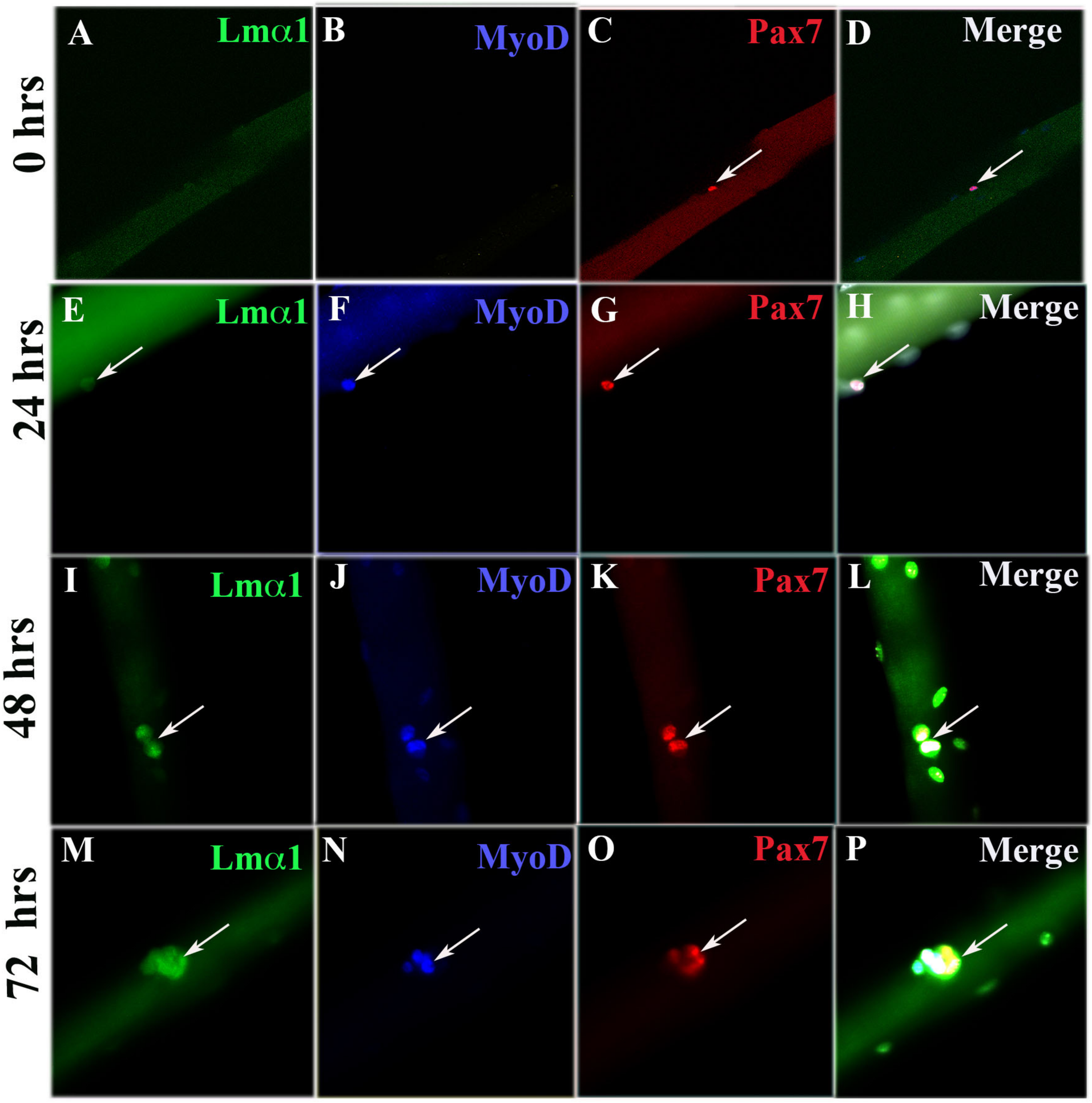
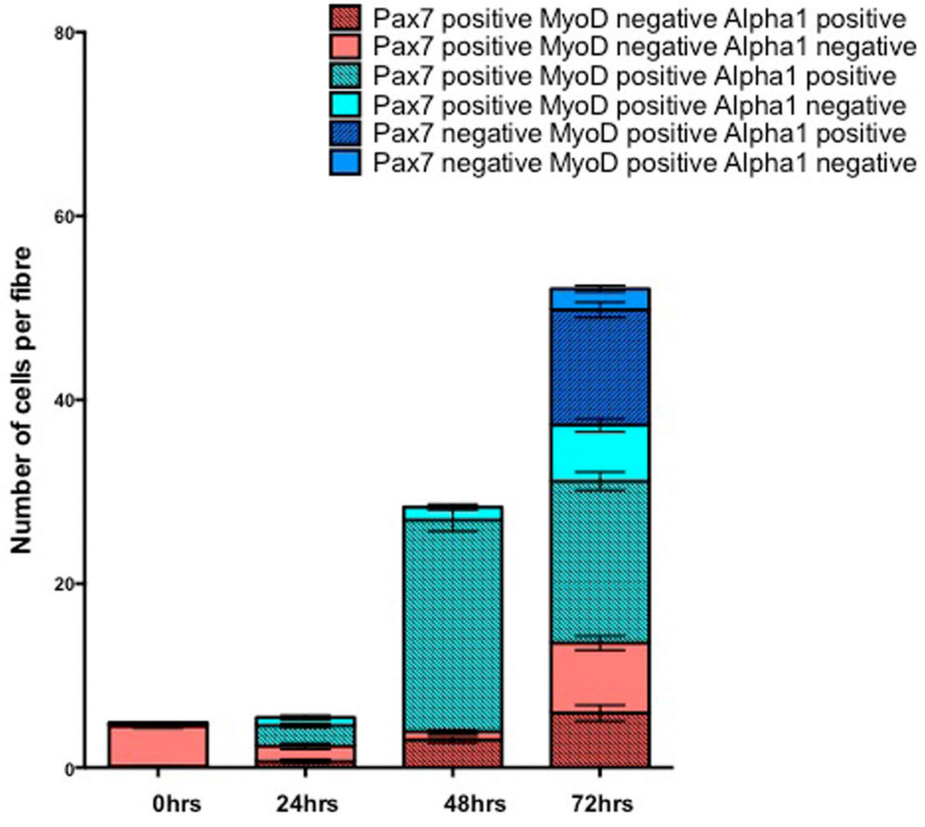


Figure 5.5: Basal lamina remodeling occurs by incorporating Laminin α1 during satellite cell activation and entry into differentiation: (A) Muscle fibres cultured for 0 hrs, 24 hrs, 48 hrs and 72 hrs, were analyzed by immunofluorescence using antibodies against different markers, and the number of satellite cells expressing a given marker and Laminin α1 was determined. Laminin α1 expression associated with satellite cell is shown by white arrows (E-H, I-L and M-P) at 24 hrs, 48 hrs and 72 hrs respectively. In contrast, no MyoD and Laminin α1expression is not observed at 0 hrs (A-D). Merge images are shown in (D, H, L and P) (continued on the next page).



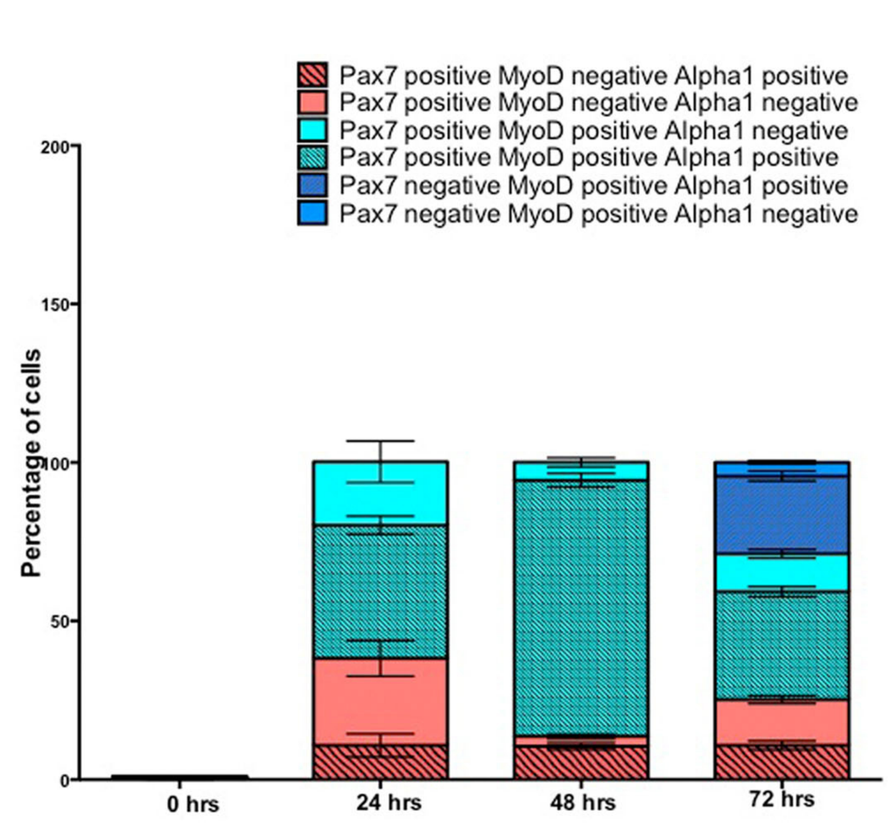


Figure 5.5: Basal lamina remodeling occurs by incorporating Laminin α1 during satellite cell activation and entry into differentiation: (*continued from the previous page).* The graphs shows different population of satellite cells associated (patched pattern) or not associated (plain pattern) with Laminin α1 expression. (Q) and (R) At 24 hrs satellite cells associated with Laminin α1 express Pax7 and MyoD and this population increases by 48hrs. By, 72 hrs Laminin α1 is associated to a novel satellite cell population that down-regulate Pax7 but maintain MyoD. Those cells are suspected to be differentiating satellite cells. Pax7-positive cells that do not express Laminin α1 are suspected to be self-renewing cells. n=26 to 36 fibres per time point.

5.2.3 Laminin α1 receptor Integrin α6 is expressed on the activated satellite cells

The above data show that satellite cells have distinct Laminin content at the level of the basal lamina and that Laminin α1 is expressed at the site of activated satellite cell. During embryogenesis, Integrin α6 is the first Integrin receptor associated with myogenic cells and Integrin α6 interaction with Laminin α1 is required for myotomal basement membrane assembly (Bajanca et al. 2004b). Indeed, blockage of Integrin leads to the dispersion of Myf5-expressing myogenic cells and defects in their differentiation (Bajanca et al. 2006). Although, Laminin α2 and Laminin α1 both bind to the same Integrin receptors (as mentioned in chapter 1), Laminin-111 binds Integrin α6β1 with higher affinity than Laminin-211, which binds preferentially to Integrin α7β1 (Nishiuchi et al. 2006). To check whether Laminin α1-specific receptors are expressed at the surface of satellite cells, I performed immunofluorescence analysis on fibres cultured for 0 hrs, 24 hrs, 48 hrs and 72 hrs using antibodies against Integrin α6 and Pax7. For fibres cultured for 48 hrs antibodies against Integrin α6, Pax7 and MyoD were used. 72 hrs fibres were labeled for Integrin α6, Pax7 and Caveolin-1. I found that Integrin α6 is expressed at the surface of activated satellite cells (white arrows in Fig 5.6) at 24 hrs, 48 hrs and 72 hrs. All the cells were positive for Integrin α6 at 24 hrs, 48 hrs and 72 hrs.

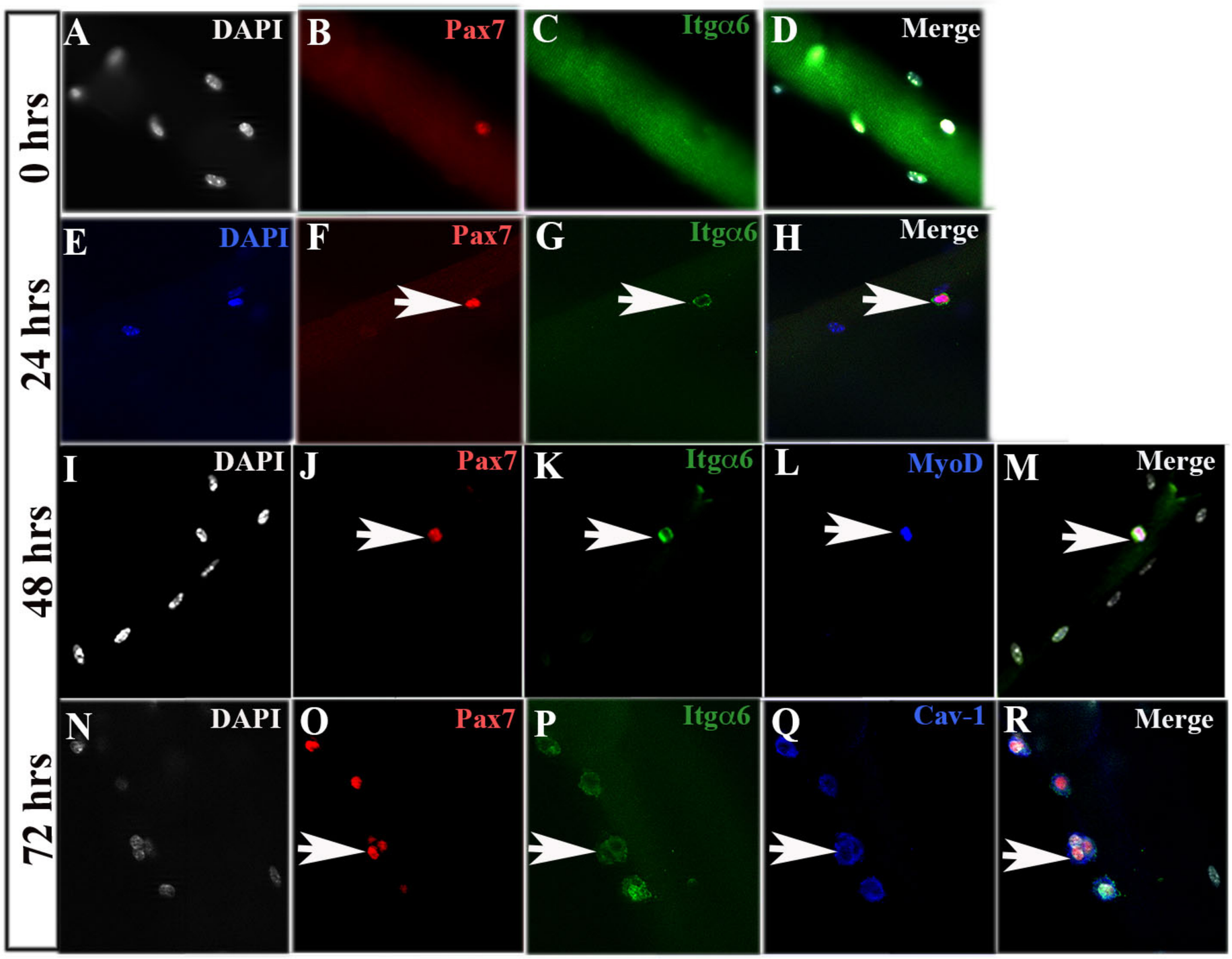


Figure 5.6: The Laminin α1 specific receptor Integrin α6 is expressed at the surface of activated satellite cells: EDL muscle fibres cultured for 0 hrs, 24 hrs, 48 hrs and 72 hrs were analyzed by immunofluorescence by using antibodies against Pax7 and Integrin α6 at 0 hrs (B-C) and 24 hrs (F-G); Pax7, Integrin α6 and MyoD at 48 hrs (J-L); Pax7, Integrin α6 and Caveolin-1 at 72hrs (O-P). At 2h, 48 and 72 hrs, activated satellite cells (white arrows) express Integrin α6 receptor. Merged images are shown in D, H, M and R. Nuclei are counter-stained with DAPI in A, E, I and N. Magnification X200.

5.2.4 Loss and gain of function approaches using *ex-vivo* system reveals that Sonic hedgehog regulates Laminin α1 localization at the site of satellite cells.

Previous work in the lab showed that assembly of the myotomal basement membrane during embryonic development requires Shh-mediated expression of Laminin-111 (Anderson et al., 2009). Indeed, Shh embryos lack Laminin-111 in somites resulting in aberrant migration, orientation and proliferation of myogenic progenitor cells (C. Anderson et al. 2009). Analysis showed that in the absence of Shh signaling, *Lama1* encoding for Laminin α1 is not transcribed.

My data indicate that activated satellite cells incorporate Laminin α1 into their basement membrane, as embryonic muscle progenitor cells, I tested whether Shh signaling is involved in the expression of Laminin α1 in adult muscle fibres.

A preliminary experiment (proof of concept) was performed first to assess whether Sonic hedgehog signaling has an effect on satellite cells. EDL muscle fibres isolated from C57BL/6 mice were cultured in the presence or in the absence of 100nM of the Shh agonist (SAG) (this concentration was chosen based on the dose response curve of SAG activity on NIH 3T3 cells (J. K. Chen et al. 2002). 100nM was shown to be optimum with a higher luciferase expression in Gli dependent luciferase activity in NIH 3T3 cell line). The distribution of Pax7 and Myf5, Pax7 and MyoD, Myogenin and Caveolin-1 was examined by immunofluorescence at 24 hrs, 48 hrs and 72 hrs respectively (Fig 5.7).

Although, no significance was observed, treatment with SAG appeared to cause an increase in the number of $Pax7^{+ve}$ cells (Fig 5.7A). Concomitant with this increase, I observe an increase in the number of satellite cells expressing the myogenic factors Myf5 and MyoD after 24 hrs and 48 hrs treatment as well as an increase in the number of $Myogenin^{+ve}$ cells at 72 hrs in the presence of SAG (Fig 5.7 B,C).

This suggests that Shh can act on satellite cells and control proliferation and differentiation. Thus, I assessed whether Shh signalling can affect Laminin α1 distribution.

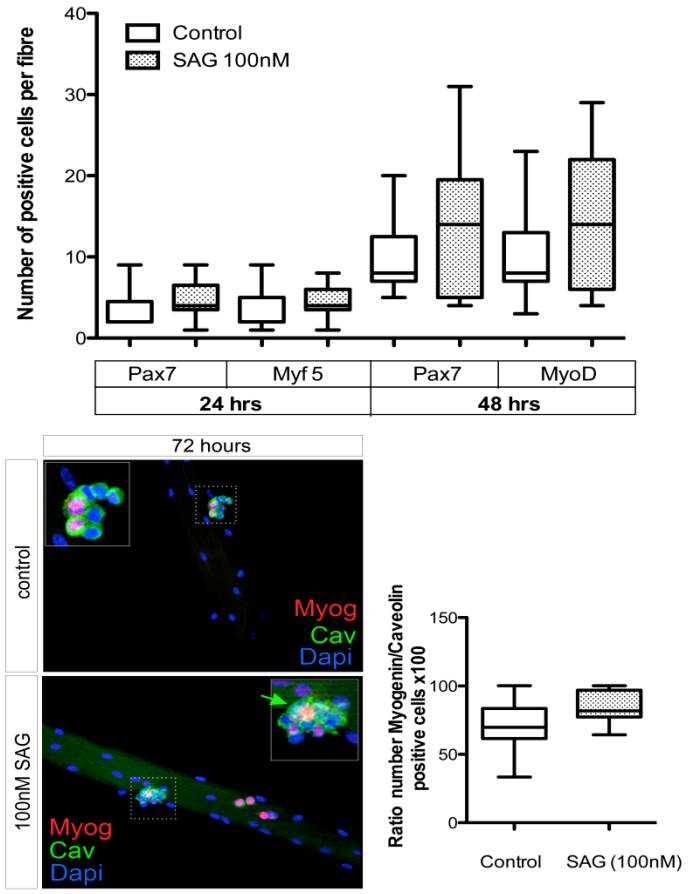


Figure 5.7: Administration of Sonic hedgehog agonist increases the number of satellite cells *ex-vivo*: (A) Quantitative analysis of the number of Pax7, Myf5 and MyoD positive satellite cells per fibre in the presence of 100nMSAG (gray hatched) or in the absence of SAG (white box). (B) Immunodetection of Myogenin (red), Caveolin-1 (green) and DAPI (blue) in fibres cultured for 72 hrs in the presence of 100nM SAG. Graphical representation for 72hrs cultured fibres (C).The data is from three independent experiments. n=22 to 35 fibres for each time point. No significant difference in the ratio of Myogenin/Caveolin positive cells was observed between control and SAG treated fibres.

5.2.3.1 Sonic hedgehog signaling increases the Laminin α1-expressing activated satellite cells in an *ex-vivo* system

I cultured the fibres in the presence or in the absence of 100nM SAG for 24 hrs, 48 hrs and 72 hrs and examined the distribution of Laminin α1 and Pax7 specific antibodies. As shown in Fig 5.8, satellite cells associated with Laminin α1 are observed (white arrows in control (A-C) and in the presence of SAG (D-F). A quantitative analysis shows that the addition of SAG does not change the percentage

of Laminin α1 expressing cells at 24 hrs and 48 hrs (Fig 5.8G). However, a significant difference is observed at 72hrs with ~30% increase in the number of cells positive for Laminin α1. This is consistent with the increased number of differentiated satellite cells at 72 hrs (Fig 5.7C). This suggests that Shh signaling may constitute to the enhancement of Laminin α1 expression after satellite cells are activated.

5.2.3.2 Inhibition of Sonic hedgehog signaling by GANT alters Laminin α1 localization at the site of activated satellite cells

After I observed the effect of SAG, I went on to check whether inhibition of Shh signaling by GANT-61 (Shh signaling inhibitor) has an effect on Laminin α1 expression. I have cultured the fibres for 24 hrs, 48 hrs and 72 hrs in the presence and absence of GANT-61. The cultured fibres were labeled for Laminin α1 and Pax7 and the satellite cells with Laminin α1 expression were counted. In contrast to the control, inhibition of Shh reduced the percentage of cells expressing Laminin α1 both at 24 hrs and 48 hrs. Around 25% and 30% reduction in Laminin α1 positive cells was observed at 24 hrs and 48 hrs, respectively, as shown in Fig 5.9. But no difference was observed at 72 hrs. These data suggest that Shh may regulate the expression of Laminin α1 in the adult satellite cells.

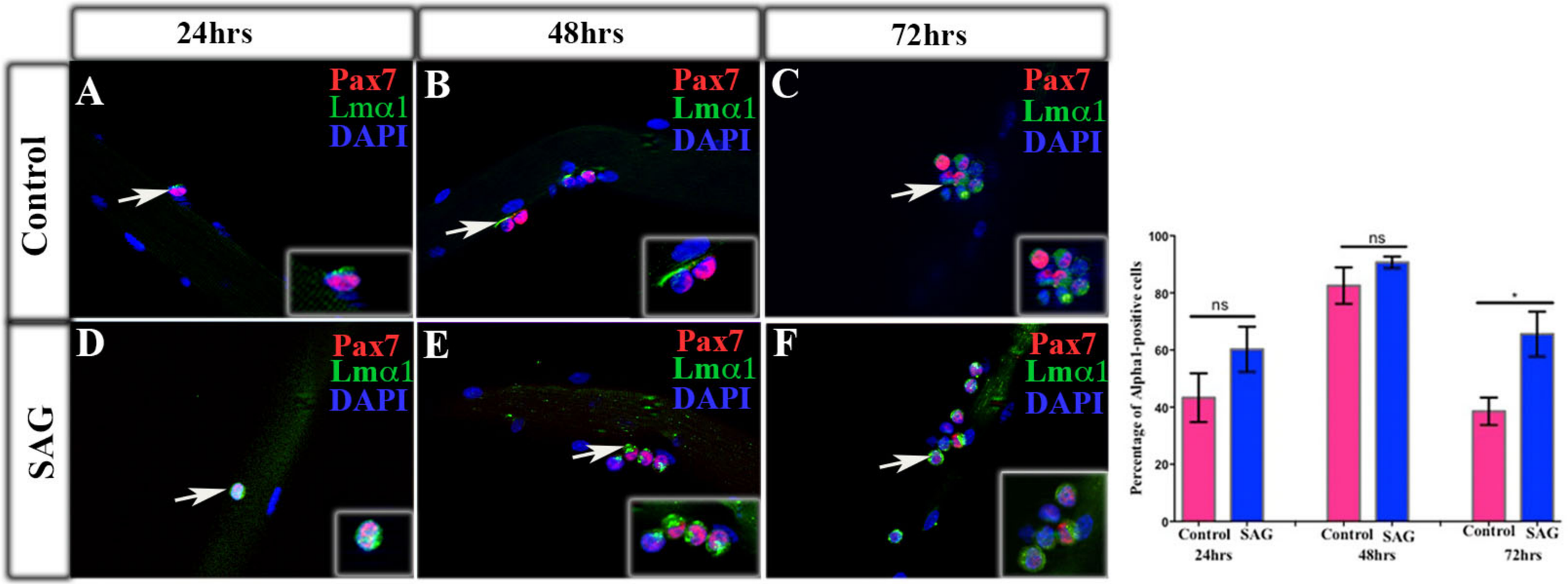


Figure 5.8: Effect of SAG on Laminin α1 expression in the vicinity of satellite cells: EDL muscle fibres cultured for 24 hrs, 48 hrs and 72 hrs in the presence or in the absence of 100nMSAG were cultured and analysed by immunofluorescence for the presence of Laminin α1 (green) Pax7 (red) Nuclei were counterstained with DAPI. Magnification X200. (G) Quantitative representation of percentage of Laminin α1 expressing cells at 24 hrs, 48 hrs and 72 hrs. The addition of 100nM SAG does not affect the percentage of Laminin α1 expressing cells at 24 hrs and 48 hrs, whereas a significant difference (p=0.0382) was observed at 72 hrs . Statistical analyses were performed using t-test (Mann Whitney test). The data is from three independent experiments. n= 15 fibres for each time point.

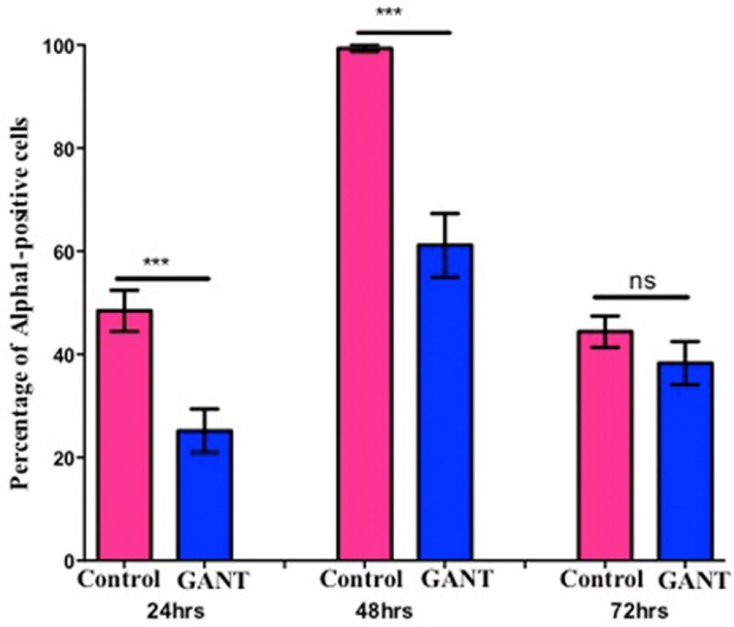


Figure 5.9: Inhibition of Sonic signaling alters Laminin α1 expression in satellite cells cultured *ex-vivo:* Quantitative representation of the percentage of Laminin α1 expressing cells at 24 hrs, 48 hrs and 72 hrs . The addition of 5μM GANT-61 inhibits Shh signaling and causes a reduction in the percentage of Laminin α1 expressing cells at 24 hrs and 48 hrs (p values 0.0007 and <0.0001 respectively). No change in the percentage of Laminin α1 expressing cells was observed at 72 hrs. Statistical analysis was performed by t-test (Mann Whitney test). Data is from three independent experiments. n= 15 fibres for each time point.

5.3 Discussion

5.3.1 Remodelling of the basal lamina occurs at the site of activated satellite cells in *ex-vivo* cultured EDL muscle fibres

The satellite cell niche controls satellite cell activation, proliferation and differentiation. The basement membrane may have essential function within the niche that supports the tissue structurally and functionally. Consistent with this possibility, I have shown the deposition of Laminin α1 in the satellite cell basal lamina following their activation. This suggests that remodelling of the basement membrane overlying satellite cells takes place after satellite cell activation. This remodeling is likely to generate a distinct functional domain within the muscle basement membrane around satellite cell such as what has been described at the neuromuscular junction. Neuromuscular junction is composed of Laminin α4 and α5 , in addition to Laminin α2 (Nishimune et al. 2008a). Both Laminin α4 and α5 are required for postsynaptic maturation in adult muscles (Nishimune et al. 2008a).

This is a novel concept as the adult muscle basal lamina composition was thought to be uniformly composed of Laminin-211. However, it is worth noting that previously it has been shown that a Laminin α1-containing basement membrane supports muscle progenitor cell migration, orientation and differentiation in the embryo (C. Anderson et al. 2009). As, adult myogenesis is often thought to recapitulate the embryonic myogenic programme, it may be expected that ECM components that support the myogenic programme in the embryo would be re-activated in adult muscles. This would suggest that Laminins may play differential roles in satellite cell activity in adult myogenesis.

This is intriguing as both Laminin α1 and Laminin α2 have been reported to promote myoblasts proliferation, fusion and myotubes formation *in vitro* (Schuler and Sorokin 1995; Vachon et al. 1996). They also confer similar adhesion properties to C2C12 cells, albeit myoblasts spread faster on Laminin α2 than Laminin α1 (Vachon et al. 1996). However, Laminin α1 and α2 are known to bind Integrins with distinct

affinities. This may have significant impact on satellite cell behavior. The significance of these findings is discussed below.

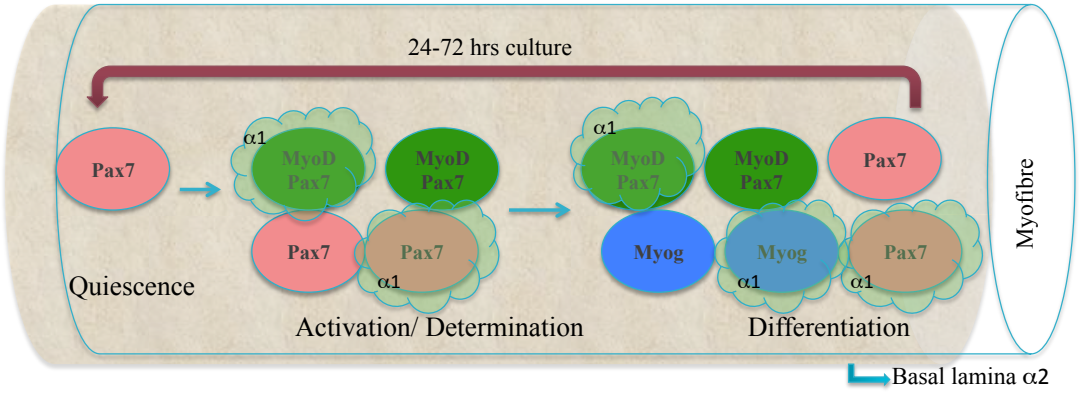


Figure 5.10: Laminin α1 is expressed at the site of activated satellite cells *ex-vivo*: At quiescent state, Pax7 satellite cells do not express Laminin α1. But when satellite cells are activated Laminin α1 becomes expressed in the vicinity of activated satellite cells (indicated by a green cloud around MyoD and Pax7-positive cells in the figure). As satellite cells progress to differentiation, Myogenin-positive cells down regulate Laminin α1 expression. Finally, a minority of satellite cells are never positive for Laminin α1, these are Pax7-positive satellite cells predicted to be self-renewing cells.

During adult myogenesis, satellite cells incorporate distinct Laminins into their niche. Indeed, although at quiescence, the satellite cell basal lamina is composed of Laminin α2 and β1, Laminin α1 appears associated with satellite cells following their activation. Laminin α1 is also found associated with cells entering the differentiation programme at 72 hrs ($Pax7^{-ve}$ and $MyoD^{+ve}$). However, the absence of Laminin α1 in the basement membrane of 72 hrs cultured satellite cells, which down-regulated Pax7 (likely to be Myogenic-positive cells) suggests that terminal differentiation is accompanied by the loss of Laminin α1 from the satellite cell basement membrane. It is thus likely that satellite cell remodel actively their overlying basement membrane

first at the time of their activation and then when they complete the differentiation programme.

Finally the absence of Laminin α1 at all time points in cells positive for Pax7 but negative for MyoD or Myogenin suggests that self-renewing satellite cells do not remodel their basement membrane. The source of Laminin α1 in the *ex-vivo* culture system is likely to be the satellite cell itself. Indeed, my quantitative PCR data confirmed that *Lama1* expression is induced when satellite cells are activated. Conversely, I found significantly less *Lama1* in the fibres depleted of satellite cells. This suggested that *Lama1* is transcribed locally and the protein becomes localized to the vicinity of activated and differentiating satellite cells.

A question remains to be answered is how Laminin α1 produced by satellite cells becomes incorporated solely to the basement membrane overlying satellite cells.

Basement membrane assembly is facilitated by the clustering of Laminin monomers to the cell surface via their binding to the Laminin receptors, Dystroglycan and Integrins (Yurchenco and Patton 2009). In the embryo, the myotomal basement membrane assembles as muscle progenitor cells enter the myotome because they begin to express the Laminin receptors Dystroglycan and Integrin α6β1 (Bajanca et al. 2004a; Cachaco et al. 2005). The expression of the Laminin receptors at the surface of myotomal cells allows the clustering of Laminin-111 and the subsequent deposition of a new myotomal basement membrane at the surface of myotomal cells (C. Anderson et al. 2007a; Bajanca et al. 2004a) showed that blocking Laminin interaction with Integrin β1 impairs the formation of the myotomal BM and the differentiation of myotomal cells (Bajanca et al., 2004), demonstrating that Laminin-111 binding to its receptor Integrin α6β1 facilitate its incorporation into the basement membrane. I found that activated satellite cells express Integrin α6 which binds Laminin α1 with a higher affinity than Laminin α2, whose preferential receptor is Integrin α7 (K. I. Gawlik et al. 2010; Talts et al. 1999) (Fig 5.6). Although not the only mechanism, this suggests that the local expression of Integrin α6β1 at the surface of activated satellite cells allows for the localized incorporation of Laminin-

111 in the satellite cell niche and not in the fibre basement membrane which overlies a sarcolemma niche in Integrin α7β1 for which Laminin-111 has a lower affinity. Supporting, this possibility, Integrin α6 is strongly expressed and localized at the site of muscle regeneration following crush injury in dy/dy mice (which carry a spontaneous mutation in *Lama2* gene leading to reduced levels of Laminin α2 protein) (Sorokin et al. 2000b). However, Integrin α6 was found associated with interstitial cells, endothelial cells and infiltrating cells, as well as with leukocytes and newly formed myotubes (Sorokin et al. 2000b). Recent studies performed in zebrafish suggests that Integrin α6 signaling promotes adhesion of muscle cells to the environment and reduces muscle degeneration by anchoring muscle fibres to the basement membrane (Goody et al. 2012). Primary muscle cells isolated from pig muscles also express Integrin α6 and blocking signaling through this integrin inhibits myoblasts differentiation (Wilschut et al. 2010). This suggest that signaling through Integrin α6 plays an important role in the control of differentiation of satellite cells.

Thus, one may hypothesize that the remodelling of the satellite cell basement membrane at the time of their activation is facilitated by the expression of the Laminin α1 preferred receptor, Integrin α6β1 and serves the purpose to promote signaling through Integrin α6 in order to promote satellite cell differentiation.

5.3.2 Sonic hedgehog signaling is required for the accumulation of Laminin α1 at the site of activated satellite cells.

Satellite cells are known to be associated with a basement membrane and this is also a characteristic of embryonic muscle progenitor cells. The first basement membrane formed during myogenesis in the embryo is the myotomal basement membrane (Solursh and Jensen 1988). Previous work showed that in Shh-deficient embryos, the myotomal BM fails to assemble (C. Anderson et al. 2009). Further studies showed that Shh signaling is required for *Lama1* expression in somites and neural tube, suggesting that Shh signaling control *Lama1* transcription either directly or indirectly. Similar to embryonic myogenic progenitor cells, I have shown that Shh signaling

regulates Laminin α1 deposition in the satellite cell basement membrane in the *ex-vivo* culture system, suggesting the possibility that Shh controls *Lama1* expression in adult muscles. As *Lama1* is produced by satellite cells (my qPCR data) and Shh signaling acts on satellite cells (my own data and Sara Cruz Migoni, personal communication), this raises the possibility that Shh controls *Lama1* expression in satellite cells. To assess this possibility it would be interesting to check *Lama1* expression by qPCR in the fibres cultured with Shh agonist, SAG and Shh antagonist, cyclopamine or direct Gli antagonist GANT-61

Chapter 6

In-vivo analysis of acute and chronic muscle regeneration models reveals Laminin α1 protein localization is associated with activated satellite cells and with non-muscle cells

6.1 Introduction

I have previously shown that Laminin α1 is associated with activated satellite cells using an *ex-vivo* muscle fibre culture system (chapter 5). During adult myogenesis, the process of satellite cell activation and myogenic differentiation involves the remodelling the muscle basal lamina through the incorporation of Laminin α1. Laminin α1 is expressed at the time satellite cells are activated until cells enter differentiation. Notably, upon Myogenin ($Pax7^{+ve}$ and $MyoD^{-ve}$ cells) expression, Laminin α1 is down-regulated. Thus, the deposition of Laminin α1 in the satellite cell basement membrane is transient and accompanies activation, expansion and early differentiation phases of adult myogenesis. Although there are numerous reports showing that the *ex-vivo* system mimics faithfully the *in vivo* context, the additional complex network of ECM molecules and the presence of different cell types *in vivo* is likely to impact directly or indirectly on the muscle regeneration process. Moreover, skeletal muscle regeneration *in vivo* involves also growth factors, cytokines and interactions between different cell types. Thus, although satellite cells are the prerequisite cells for an efficient skeletal muscle regeneration and alterations in the niche of satellite cells impair regeneration process (Carlson and Gutmann 1975; Zacks and Sheff 1982), other cell types play an important role for instance infiltrating inflammatory cells, endothelial cells and fibroblasts are critical for an efficient muscle regeneration to occur (Chazaud et al. 2003; Christov et al. 2007; Lescaudron et al. 1999; Murphy et al. 2011; Tidball et al. 1999). It is therefore important to assess whether basement membrane remodelling takes place *in vivo* and whether it involves the deposition of Laminin α1. The progression of muscle regeneration has been characterized in acute and chronic injury models (chapter 4) and these two models of skeletal muscle regeneration were used to assess the satellite cell basal lamina composition *in vivo* during skeletal muscle regeneration.

6.1.1 Hypothesis

Laminin α1 is associated with activated satellite cells in regenerating muscles *in vivo.*

6.2 Results

6.2.1 The muscle basal Lamina incorporates Laminin α1 at the site of activated satellite cells in *mdx* mice *in vivo*

6.2.1.1 Laminin α1 is associated with the activated satellite cells in *mdx* mice

As discussed in chapter 5, I have identified that basal lamina remodelling occurs during satellite cell activation and leads to the incorporation of Laminin α1 in the single muscle fibre *ex-vivo* culture system (Fig 5.3). To examine whether basal lamina remodeling occurs *in vivo*, I harvested EDL muscles from 2, 4, 6 and 8 week old *mdx* mice, sectioned and analyzed by immunofluorescence. In *mdx* mice necrosis and degeneration occurs between 2-6 weeks and regeneration begins at 4 week and continues through to 12 weeks. Hence I chose to analyze the muscles from 2, 4, 6 and 8 week old *mdx* mice. Previously, in *mdx* mice activated satellite cells (MyoD-positive cells) were observed at 2, 4, 6 and 8 weeks (chapter 4 Fig 4.6).

To investigate whether Laminin α1 is expressed at the site of activated satellite cells, I labeled EDL muscle cross sections from 2, 4, 6 and 8 weeks-old *mdx* mice using antibodies against MyoD and Laminin α1. Consistent with my *ex-vivo* data, Laminin α1 expression was observed at the site of activated satellite cells at 2, 4, 6 and 8 weeks in *mdx* muscles (white arrows Fig 6.1 A-C, D-F, G-I and J-L). This indicates that basal lamina remodelling occurs during *in vivo* muscle regeneration when satellite cells become activated. In contrast, no Laminin α1 expression was observed in the control (C57BL/6) muscles (Fig 6.1 M-O, P-R, S-U and V-X).

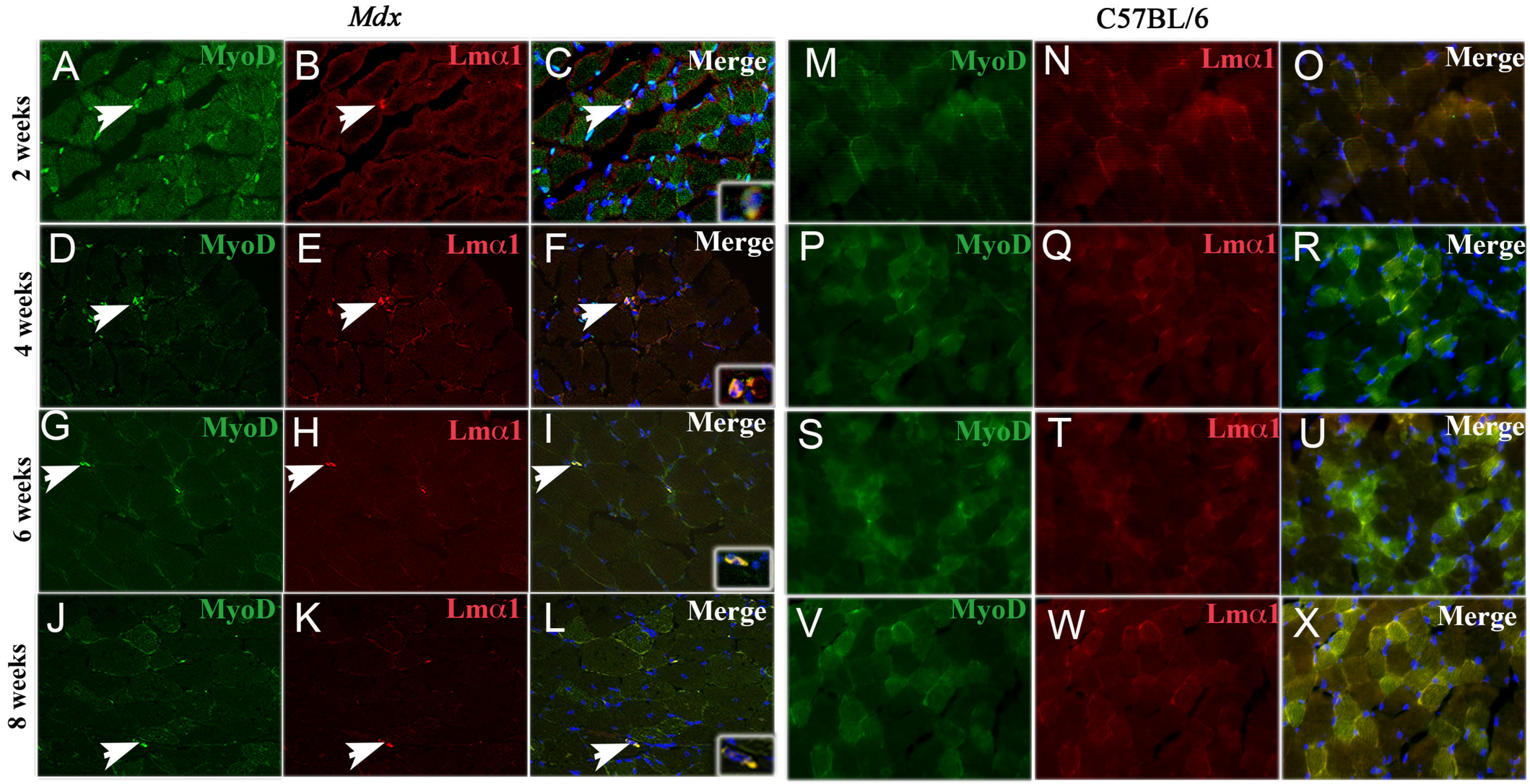


Figure 6.1: Basal lamina remodelling occurs by incorporating Laminin α1 at the site of activated satellite cells in *mdx* EDL muscles

Figure 6.1: Basal lamina remodelling occurs by incorporating Laminin α1 at the site of activated satellite cells in *mdx* EDL muscles: EDL muscles of 2, 4, 6 and 8 week-old *mdx* and C57BL/6 mice were analyzed by immunofluorescence using antibodies against MyoD and Laminin α1. Activated satellite cells expressing MyoD are observed in 2, 4, 6 and 8 weeks-old *mdx* mice (arrows in A, D, G and J). MyoD positive cells are associated with Laminin α1 at 2, 4, 6 and 8 weeks (arrows in B, E, H and K). In contrast no MyoD and Laminin α1 expression was observed at 2, 4, 6 and 8 weeks (M-O, P-R, S-U and V-X) in C57BL/6 mice. Nuclei are counterstained with DAPI. Merged images are shown in C, F, I and L for 2, 4, 6 and 8 weeks, respectively. Magnification 200X.

6.2.1.2Distribution of Laminin α1 in the vicinity of activated satellite cells and also associated with non-myogenic cells in sedentary and exercised *mdx* mice in *vivo*

In parallel to the analysis of Laminin α1 distribution in *mdx* mice, I designed a protocol to increase the severity of the muscle damage in *mdx* mice and assess the effect on activated satellite cells and their association with Laminin α1. To increase the severity of muscle damage, 4 week-old *mdx* mice were trained to voluntary running for 17 days as discussed in chapter 4. Exercised wild type (C57BL/6) were also sectioned and analyzed as a control. Muscles from sedentary and exercised *mdx* mice were harvested, sectioned and analyzed by immunofluorescence using antibodies against MyoD and Laminin α1. Activated satellite cells were observed in both sedentary and exercised mice (white arrows Fig 6.2 A, B and C, D respectively). However, an increased number of MyoD-positive cells were observed in the exercised mice compared to sedentary mice (Fig 6.2 A-C).

In contrast, no activated satellite cells were observed in exercised wild type muscle (see Fig 6.3 A). Laminin α1 was found associated with activated satellite cells in both sedentary and exercised *mdx* mice (white arrows in Fig 6.3 D-F and G-I respectively). However, there appeared more Laminin α1-positive cells in exercised *mdx* mice than in sedentary mice, and interestingly a number of positive cells did not express MyoD (green arrows in Fig 6.3) (It is also important to consider that the half-life of Laminin

α1 and MyoD is not same. Like many other ECM components half-life of Laminins is about 10hrs unlike MyoD (transcription factor) with a half-life of ~30min (Dunsmore et al. 1995; Thayer et al. 1989)).

In contrast, no Laminin α1 immuno labelling was observed in control mice (Fig 6.3 A-C). Thus, consistent with my *ex-vivo* data I observed Laminin α1 expression in the vicinity of activated satellite cells.

To quantify the levels of Laminin α1 expression, I counted cells positive for MyoD for Laminin α1 and for both markers (on the whole muscle sections which include areas of ongoing regeneration and also areas where the muscle is not in the process of regeneration) (Fig 6.3). A significant increase in the number of MyoD-positive cells (approximately 5 fold) was found in exercised *mdx* mice compared to sedentary *mdx* mice (Fig 6.3J). Similarly, a 2-fold increase in the number of activated satellite cells associated with Laminin α1 was observed in exercised *mdx* mice compared to sedentary mice. However, I noticed that the proportion of MyoD-positive cells expressing Laminin α1 is not significantly different between sedentary and exercised mice (Fig 6.3K). In both conditions not all MyoD-positive cells were associated with Laminin α1 suggesting that Laminin α1 is down-regulated when satellite cells enter terminal differentiation and express Myogenin.

Unexpectedly, I observed that Laminin α1 expression is also associated with some MyoD-negative cells (green arrows in Fig 6.3 D-I) that are morphologically different from satellite cells. This suggests that during skeletal muscle regeneration Laminin α1 is not only associated with muscle cells but also with other non-myogenic cells. Surprisingly the number of MyoD-negative cells, which were associated with Laminin α1, showed a 3-fold increase in exercised compared to sedentary *mdx* mice (Fig 6.3 J). The presence of these MyoD-negative and Laminin α1-positive cells in the interstitial space and their close contact with clusters of small rounded cells suggested that these might be cells involved in inflammation. Further investigation was carried out to characterize these non-muscle cells with Laminin α1 expression.

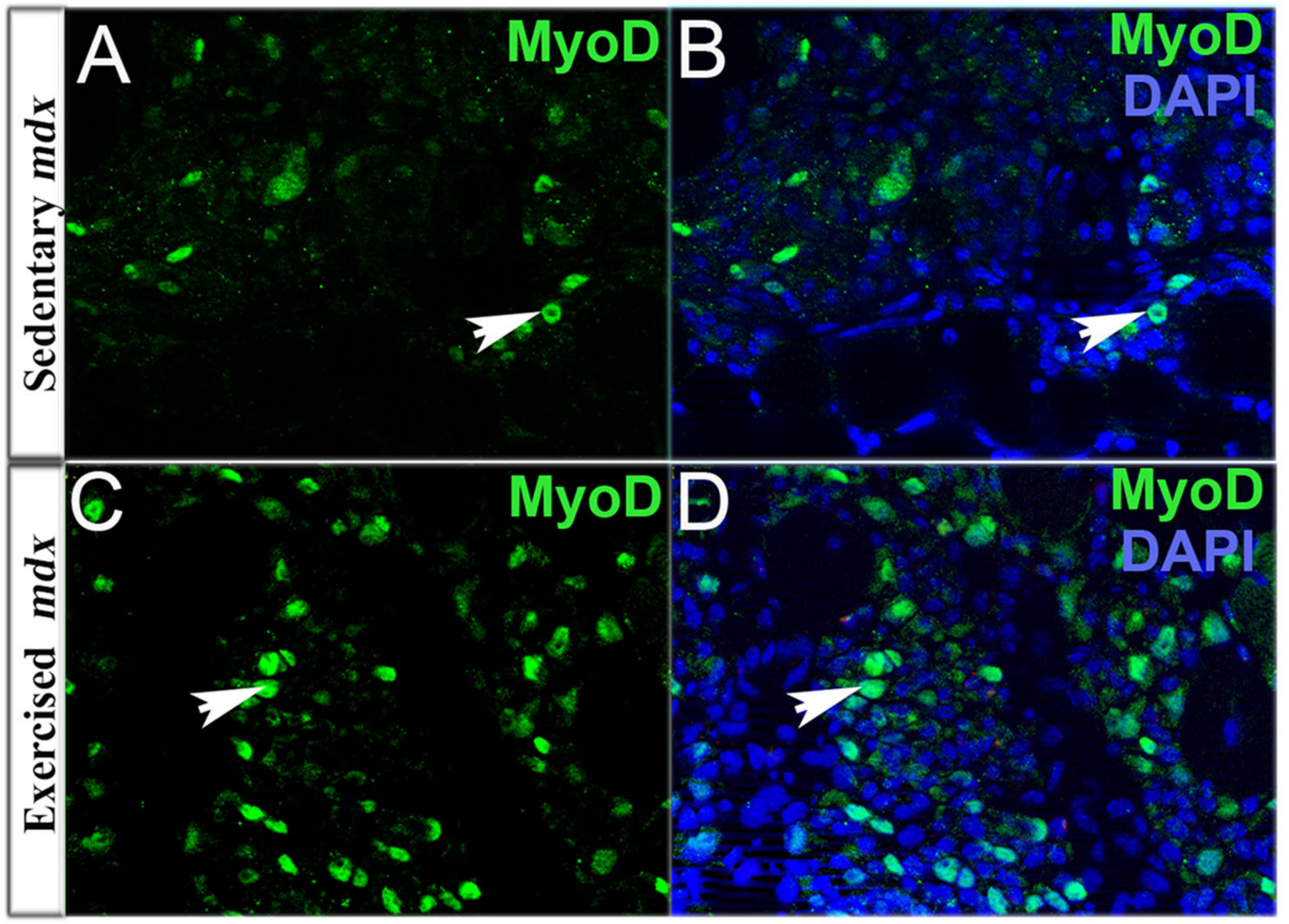


Figure 6.2: Activated satellite cells are more numerous in exercised *mdx* mice than in sedentary *mdx* mice: Immunofluorescence of activated satellite cells in sedentary and exercised 6 week-old mdx mice using antibody against MyoD (green). MyoD-positive satellite cells are indicated by white arrows in sedentary (A-B) and exercised (C-D) *mdx* mice. An increased number of MyoD-positive cells is observed in exercised mice compared to sedentary mice (A). Nuclei are counterstained with DAPI. Magnification 200x.

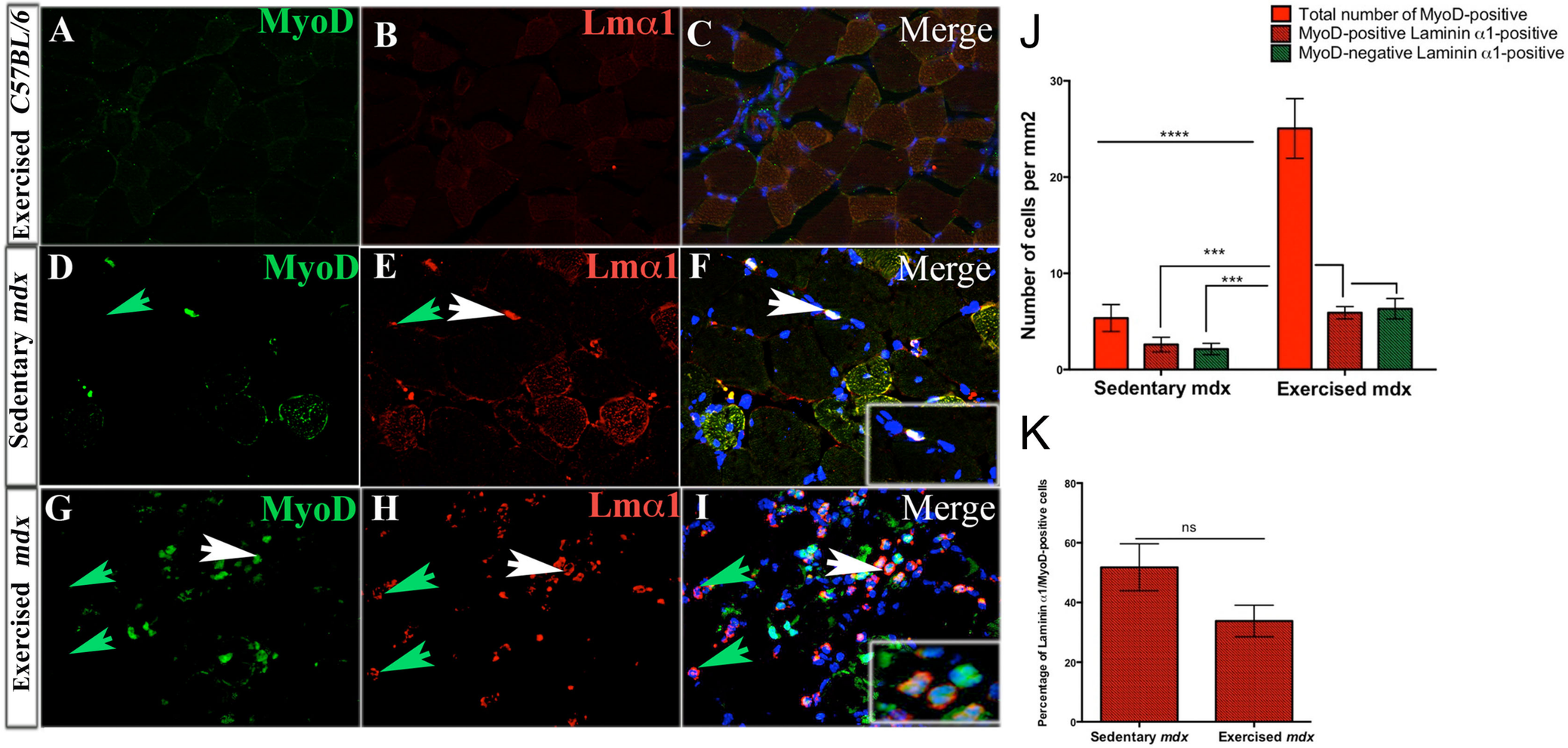
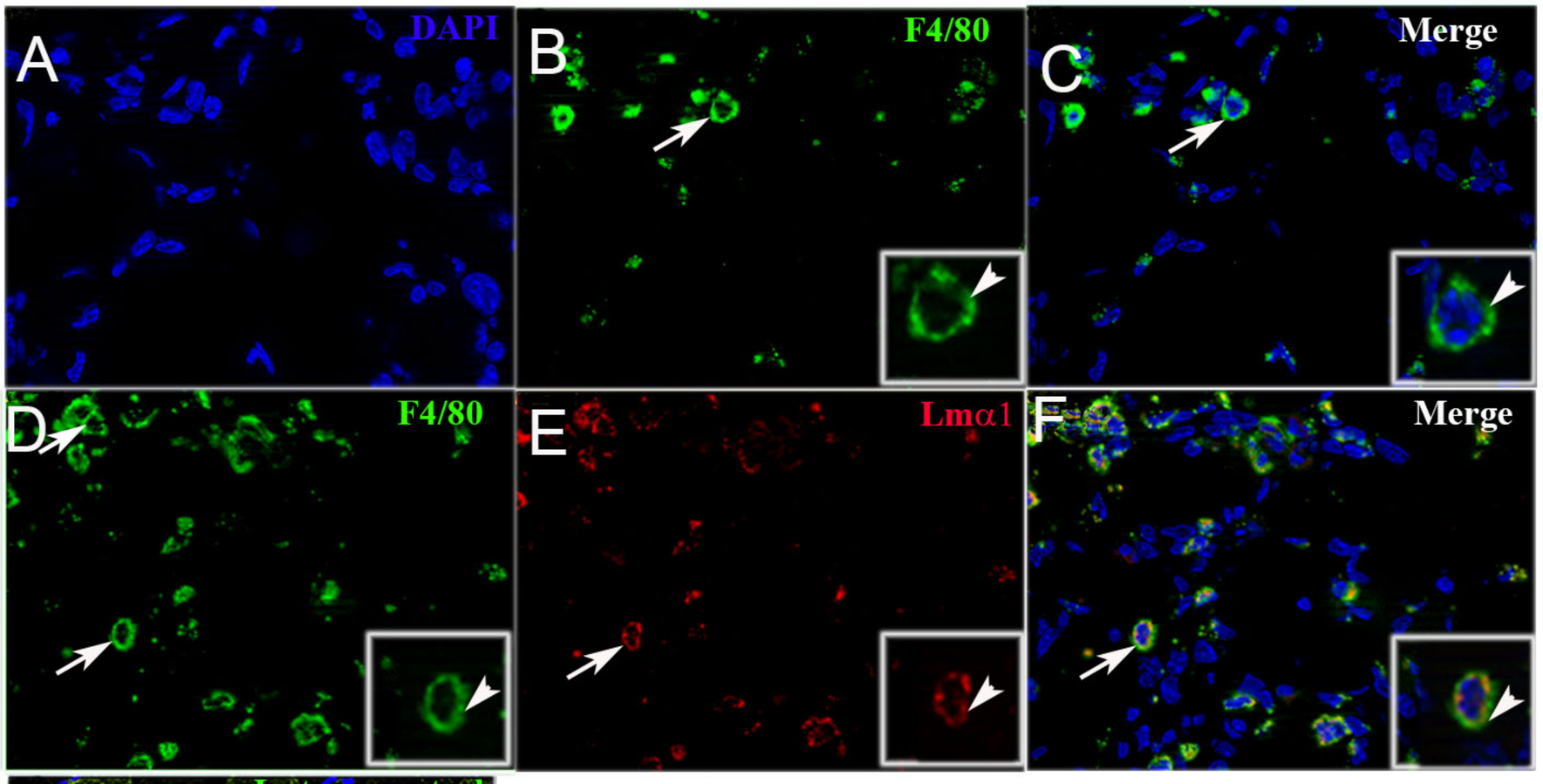


Figure 6.3: Laminin α1 is associated with activated satellite cells in both sedentary and exercised *mdx* mice: (A-C) Immunodetection of MyoD and Laminin α1 in EDL muscles of exercised wild type C57BL/6 mice. (A-C) wild type muscle do not show MyoD and Laminin α1 expression. (D-F) and (G-I) represents EDL muscle transverse sections of sedentary and exercised *mdx* mice respectively labeled for MyoD (green) and Laminin α1 (red). Laminin α1 is associated with MyoD positive satellite cells (white arrows) D-I. Laminin α1 is also associated with non-muscle cells (green arrows) D-I. Nuclei are counterstained with DAPI. Magnification 200x. (J) Quantitative representation of activated satellite cells associated with Laminin α1 and Laminin α1 associated with non-muscle cells. Significant difference is observed between sedentary and exercised *mdx* mice in MyoD-positive cells ($p<0.0001$); MyoD-positive and Laminin α1-positive cells ($p=0.0010$) and in MyoD-negative and Laminin α1-positive cells ($p=0.0006$). (K) Graphical representation of percentage of Laminin α1 associated with activated satellite cells in sedentary and exercised *mdx* mice. Statistical analysis was performed using Mann-Whitney test.

6.2.2 Inflammatory cells macrophages also show the localization of Laminin α1 in non-exercised and exercised *mdx* mice

To investigate the unexpected non-myogenic cell population that were associated with Laminin α1, I harvested EDL muscles from exercised *mdx* mice and 6-7μm sections were prepared and analyzed by immunofluorescence. Because of the morphology of the MyoD-negative cells positive for Laminin α1, I suspected that these cells were macrophages. Thus, I have used the anti F4/80 antibody, which recognizes a glycoprotein expressed at the surface of all the macrophages. As expected, I found that Laminin α1-positive non-muscle cells express F4/80. Fig 6.4 A-C shows that in exercised mice infiltrating inflammatory cells express F4/80, indicating the presence of macrophages during the process of muscle regeneration. Macrophages expressing F4/80 also express Laminin α1 (white arrows in Fig 6.4 D-F). Isotype control for F4/80 antibody confirms the specific binding of F4/80 antibody (Fig 6.4 G) (Isotype is an antibody from the same species as primary antibody and is not raised against any specific antigen). These data indicates that during muscle regeneration Laminin α1 is deposited at the surface of muscle cells and macrophages. This finding may help understand the nature of interaction between these two cell types during muscle regeneration.



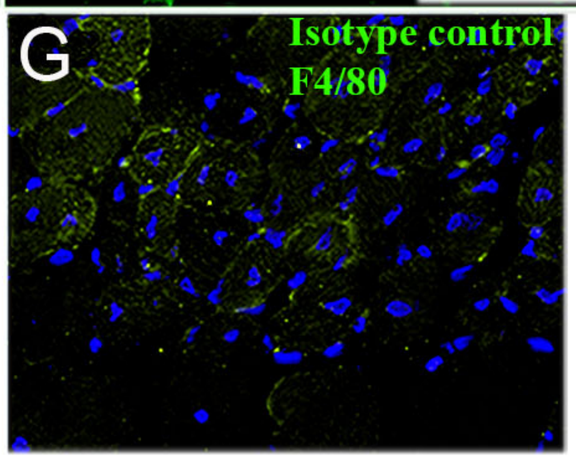


Figure 6.4: Laminin α1 distribution at the surface of macrophages: EDL muscle transverse sections from 6 week-old exercised *mdx* mice were analyzed by immunofluorescence using antibodies against F4/80 (green) to detect macrophage and Laminin α1 (red). (A-C) F4/80 antibody specifically recognizes macrophages (white arrows). Macrophages expressing F4/80 also express Laminin α1 (white arrows D-F). Isotype control shows no specific binding (G). Merged images with inserts are shown in C and F. Nuclei are counterstained with DAPI. Magnification 200x.

To address whether macrophages synthesize Laminin α1 or bind at their surface to a protein synthesized by other cells, total RNA was isolated from Raw 264.7 cells, a mouse leukemic macrophage cell line (kindly provided by Dr. Gaynor Miller) and RT-PCR was performed. Total RNA isolated from mouse embryo E11.5 was used as a positive control. Both samples were also analyzed with GAPDH primers to verify the cDNA preparation from the isolated RNA. No band was observed when laminin α1 primers were used with cDNA prepared from Raw 264.7 cells suggesting that these macrophages do not transcribe *Lama1* (Fig 6.5). In contrast, a band of expected size was observed when the Raw 264.7 cDNA was analyzed with the GAPDH primers and when the E11.5 embryo cDNA was analyzed with *Lama1* primers (Fig 6.5). However, it would be interesting to check whether macrophages isolated from *mdx* express laminin α1.

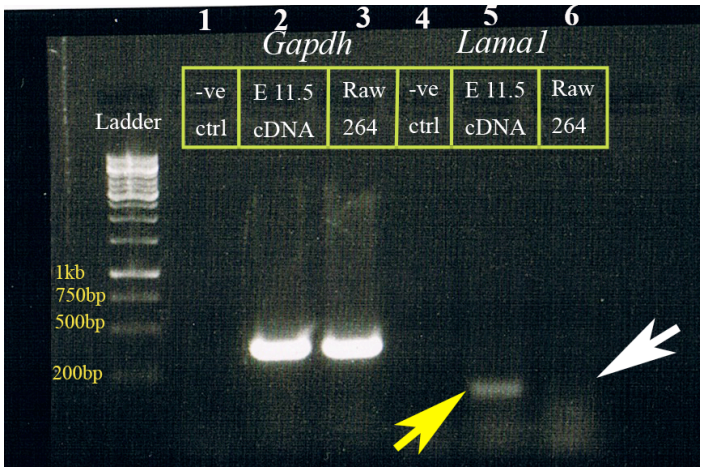


Figure 6.5: **RT-PCR detecting *Gapdh* and *Lama1* in E11.5 (mouse embryo) and Raw 264 cell line.** *Gapdh* (positive control) and is detected at the expected band size of 250bp. *Lama1* is expressed only in E11.5 mouse (band size = 149bp) and not in Raw 264 cell line. Negative controls are shown in Lane 1 and Lane 4 for *Gapdh* and *Lama1* respectively.

6.2.3 Characterization of Laminin α1 distribution in cardiotoxin-injured muscle

As described in Chapter 4 cardiotoxin administration causes acute muscle injury. Muscle regeneration can be visualized in the first 14 days following cardiotoxin injection. As discussed in Chapter 4 following muscle injury satellite cell activation and expansion occurs in between 2-7 days. 7 day post-injury detection of centrally

located nuclei indicated the completion of regeneration process. Henceforth, TA muscles were harvested at different time points 2, 4 and 7 days post-injury and 6-7μm cryosections were prepared. Immunofluorescence was performed to assess the invasion of macrophages and satellite cell activation following cardiotoxin injection (Fig 4.11, 4.12 and 4.13 in chapter 4) and the number of positive cells per mm^2 was counted. The graph presented in Fig 6.6 shows how different cell populations evolve during muscle regeneration. As already reported in the literature (Hirata et al. 2003). F4/80-positive macrophages peak between 2 and 4 day post-injury (green line). Although the number of Pax7-positive satellite cells was higher in injured compared to non-injured muscle, I observed that this cell population remains constant during the regeneration process. In contrast, activated satellite cells (MyoD-positive) peak at 4 days post injury and are maintained at 7 days consistent with the continuous muscle process taking place between 2-7 days post-injury.

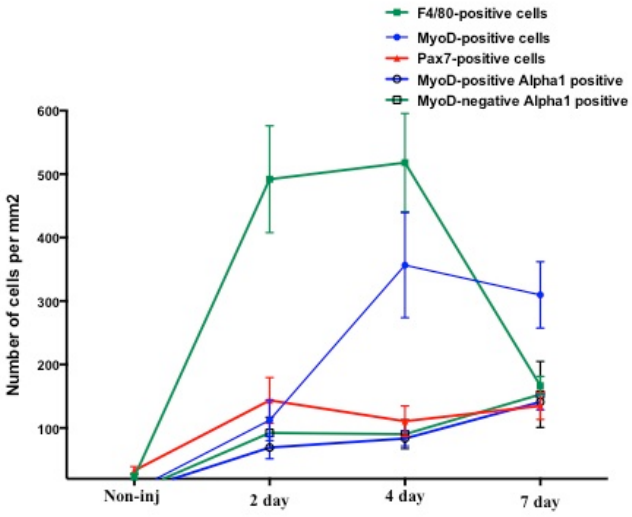


Figure 6.6: Profile of different population during the regeneration process: Cardiotoxin-injured muscles were analyzed by immunofluorescence at 2, 4 and 7 days post-injury and the number of F4/80, MyoD and Pax7-positive cells was determined. The number of F4/80- positive cells peaks at 2 and 4 days post-injury and declines by 7 days. MyoD positive activated satellite cells follow the infiltration of inflammatory cells and peak at 4 days. Pax7-positive cells are maintained at a constant number throughout the first seven days post-injury. However, the three cell populations are increased compared to control muscles.

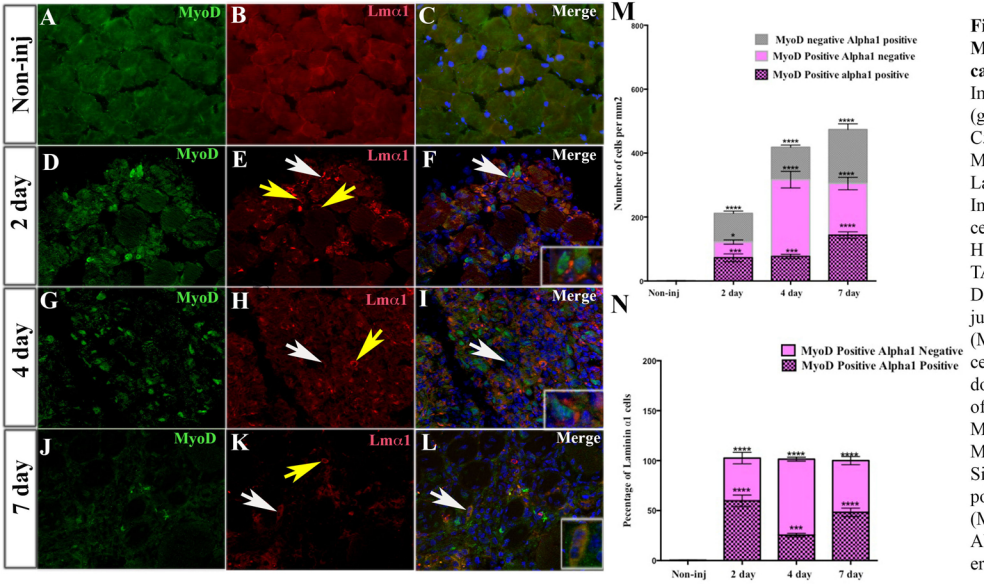


Figure 6.7: The Laminin α1 is associated with MyoD-positive cells and non-muscle cells in cardiotoxin-injured TA muscles of C57BL/6: Immunodetection of Laminin α1 (red) and MyoD (green) in cardiotoxin-injured TA muscles of C57BL/6. At 2 day, 4 day and 7 day post-injury, MyoD-positive cells (green) are associated with Laminin α1 (white arrows in D-F, G-I and J-L). Instead other non-muscle (probably macrophages) cells also express Laminin α1 (yellow arrows in E, H and K). No MyoD was observed in non-injured TA muscle (A-C). Nuclei are counter stained with DAPI. Magnified images for 2 and 4 day post-injury are shown in inserts. Magnification 200x. (M) Quantitative representation of the number of cells positive for either MyoD or Laminin α1 and double-positive cells are represented. The number of cells per mm^2 is plotted. (N) Percentage of MyoD-positive Laminin α1-positive and MyoD-positive Laminin α1-positive are plotted. Significant difference is observed in TA muscles post-injury compared to non-injured TA muscle (M and N). Statistical analysis is performed using ANOVA. Error bars represent standard mean of error.

Cardiotoxin-injured muscles were then analyzed for Laminin α1 expression. Muscles of 2, 4, and 7 day post-injury were harvested, sectioned and analyzed by immunofluorescence using antibodies against MyoD and Laminin α1. Laminin α1 was found associated with the activated satellite cells at 2, 4, and 7 post-day injury muscles (white arrows Fig 6.7 E-F, H-L and K-I respectively). In contrast, no activated satellite cells were observed in exercised control muscles (see Fig 6.7 A-C). Data are from three different mice (total nine sections analyzed).

Quantitative representation shows that total number of MyoD positive cells increases significantly ($p=0.0079$) from 2 day to 4 day post-injury and declines at 7 days but not significantly (see Fig 6.7 M and Fig 6.6) suggesting that a higher number of activated satellite cells are present at 4 and 7 days post-injury. The number of activated satellite cells associated with Laminin α1 expression remains nearly constant at 2 and 4 days with an average of 70 and 84 cells per mm^2, and rises at 7 days to 141 cells per mm^2. However, when I calculate the percentage of activated satellite cells expressing Laminin α1, I noticed that there is a higher proportion of Laminin α1 associated satellite cells at 2 days then at 4 days post-injury (Fig 6.7 N). This suggests that after activation, satellite cells remodel their basal lamina and incorporate Laminin α1. By 4 days post-injury, Laminin α1 expression declines and this correlates with the timing that activated MyoD-positive cells enter differentiation.

Thus, consistent with the data I generated using the *ex-vivo* system, I found that a large proportion of activated, MyoD-positive satellite cells express Laminin α1 in their vicinity during satellite cell activation and during their entry into differentiation. Indeed, in the *ex-vivo* culture system almost 90 percent of satellite cells express Laminin α1 and at 48 hrs (see Chapter 5) approximately 84 percent of satellite cells express Laminin α1 at 2 days post-injury in the cardiotoxin acute injury system, the time at which when most of the satellite cells are activated and enter differentiation.

I also observed MyoD-negative cells which were positive for Laminin α1. These cells may correspond to F4/80-positive cells. As discussed before, F4/80 cells peak at 2 and

4 days post-injury with around 40 fold more number of cells when compared to exercised *mdx* mice. This suggest that during acute injury large influx of infiltrating macrophages occurs locally to support the muscle regeneration.

6.2.4 Could Laminin -111 signals satellite cell through a specific pathway

Laminin-111 (α1, β1 and γ1) and Laminin-211 (α2, β1 and γ1) interact with the trans-membrane receptors Dystroglycan and Integrins (Scheele et al. 2007). Laminin domains LG4-5 bind to α-DG (Talts et al. 2000), although Laminin α2 LG1-3 possess additional binding sites for DG, which interact with α-DG with an affinity 4 times higher than the LG4-5 domains (Talts et al. 1999). However, Laminin α1 LG1-3 domains do not bind to DG (Talts et al., 1999). And also, Laminin α1 affinity for Integrin α6β1 is higher than that of Laminin α2, and both Laminins bind to α7β1 (K. I. Gawlik et al. 2010; Nishiuchi et al. 2006; Talts et al. 1999). These differences in binding affinity to different receptors may confer different signaling.

6.2.4.1 Dystroglycan a receptor for Laminin-111 and Laminin-211 is expressed in *mdx* mice

To assess for the presence of Dystroglycan, I harvested EDL muscles from Wild type C57/BL6 EDL and exercised *mdx* mice and cryosections of 6-7μm transverse sections were prepared and labeled with antibody against β–Dystroglycan and Laminin β1. Laminin β1 is expressed in the basal lamina of both *mdx* and C57BL/6 muscles (Fig 6.8 A-C and E-G respectively). Alpha Dystroglycan is the receptor for Laminin-111 and Laminin-211. Alpha Dystroglycan binds to beta Dystroglycan which is a trans membrane protein. β-Dystroglycan is expressed in wild type C57BL/6 (Fig 6.8 I-K). In exercised *mdx* mice β-DG is receptor is expressed on the myofibres plasma membrane that is in close contact with the MyoD- positive satellite cells (white arrows in Fig 6.8 N-P). Secondary antibody control is shown in 6.8H with merge image in 6.8L. This data shows that Laminin α1 receptor β-DG is expressed in EDL muscles of C57BL/6 and *mdx* mice that binds to Laminins of basal lamina.

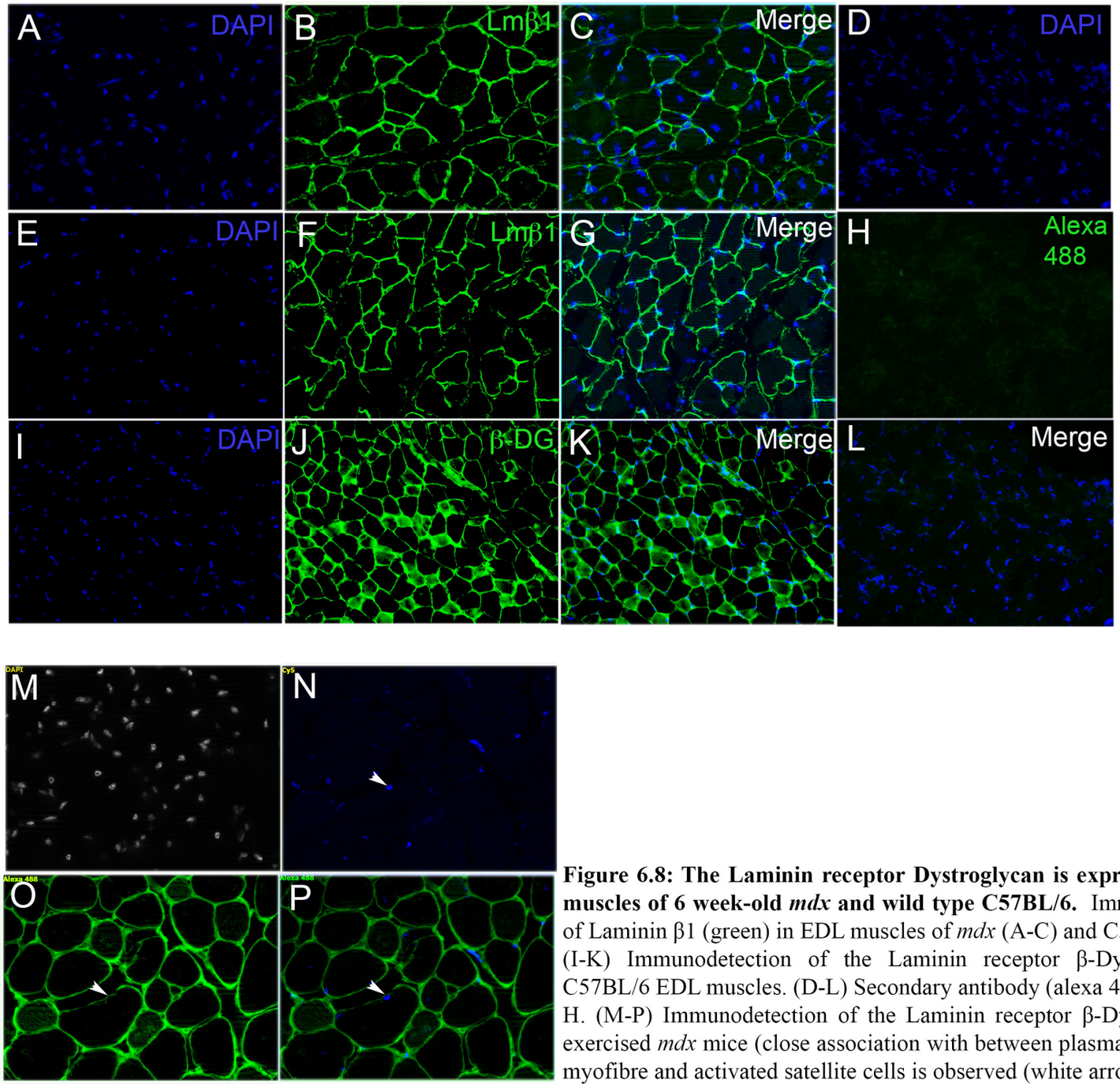


Figure 6.8: The Laminin receptor Dystroglycan is expressed in EDL muscles of 6 week-old *mdx* and wild type C57BL/6. Immunodetection of Laminin β1 (green) in EDL muscles of *mdx* (A-C) and C57BL/6 (E-G). (I-K) Immunodetection of the Laminin receptor β-Dystroglycan in C57BL/6 EDL muscles. (D-L) Secondary antibody (alexa 488) control in H. (M-P) Immunodetection of the Laminin receptor β-Dystroglycan in exercised *mdx* mice (close association with between plasma membrane of myofibre and activated satellite cells is observed (white arrows N-P).

6.2.4.2 The Laminin α1 receptor Integrin alpha6 is expressed on interstitial cells *in vivo*

I have shown that Laminin α1 receptor, Integrin α6 is expressed at the surface of activated satellite cells in *ex-vivo* system (chapter 5). To investigate whether the Laminin α1 receptor Integrin α6 is expressed at the surface of activated satellite cells *in vivo*, I harvested EDL muscles from exercised *mdx* mice and prepared 6-7μm transverse cryosections. Sections were immunolabeled using antibodies against MyoD and Integrin α6. In contrast to the *ex-vivo* system, Integrin α6 was not detected on MyoD-positive cells (white arrowheads Fig 6.9 A and C). Instead, other non-muscle cells express Integrin α6 as shown by arrow heads (Fig 6.9 B-C).

Similarly, Integrin α6 expression is observed in cardiotoxin-injured muscles at 2, 4 and 7 day post-injury (white arrows in Fig 6.10 D-F, G-H and J-L respectively). In contrast, very few cells are positive for Integrin α6 in the control muscle. Integrin α6 expression is not associated with MyoD-positive cells (green arrows in Fig 6.10). Instead, I observed a population of cells with elongated morphology with Integrin α6 expression at 2 and 4 day post-injury (yellow arrows in Fig 6.10 F and I). It is possible that these cells could be myogenic cells (Myogenin-positive cells). Further analysis needs to be done to check this possibility.

The expression of integrin α6 in non-muscle cells is consistent with the literature that suggests that during muscle regeneration following crush injury Integrin α6 is associated with interstitial cells, endothelial cells, infiltrating cells and leukocytes but not with satellite cells (Sorokin et al. 2000b). The failure to detect Integrin α6 in satellite cells may be technical and due to the time point the muscle was analyzed. Indeed, in 6 week-old post-natal muscles a large proportion of satellite cells are MyoD-positive cells are still in activation and proliferation state and not differentiated. As previous *in vivo* data showed that Integrin α6 expression is required for the differentiation of porcine satellite cells (Wilschut et al. 2010), it is possible that the stage analyzed was too early. Further experiments are required to assess whether satellite cells express Integrin α6.

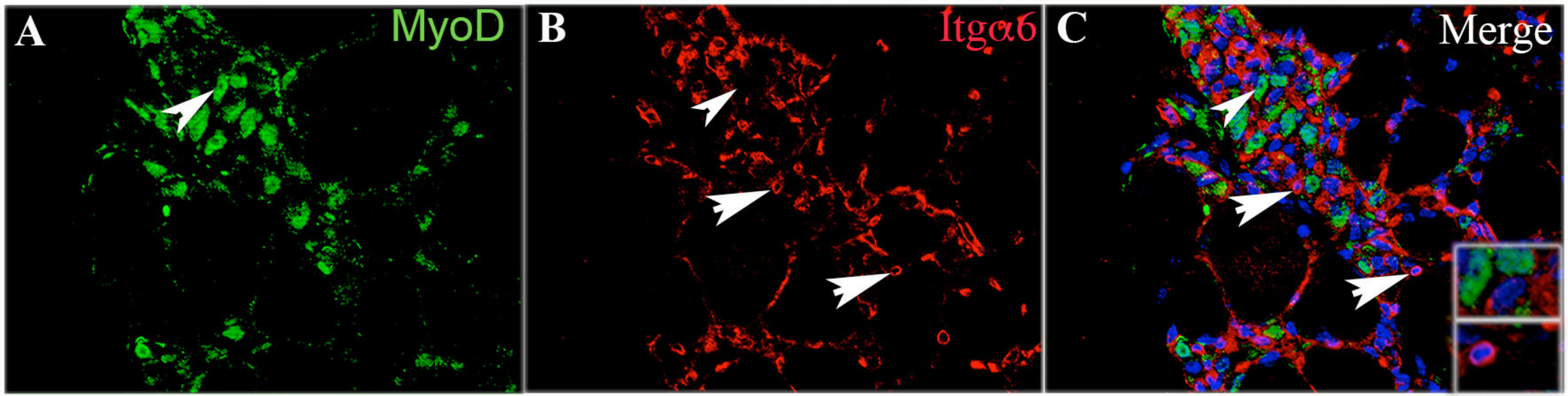


Figure 6.9 :The Laminin α1 receptor Integrin α6 is expressed in non-muscle cells of EDL muscles of 6 week-old exercised *mdx* mice: Immunodetection of Integrin α6 (red) and MyoD (green) in EDL muscles of *mdx* mice. (A-B) MyoD-positive cells do not show the Integrin α6 expression (arrow heads). Instead other non-muscle (interstitial cells) cells express Integrin α6 (white arrows B). Merged images are shown in C. Nuclei are counter stained with DAPI. Magnification 200x.

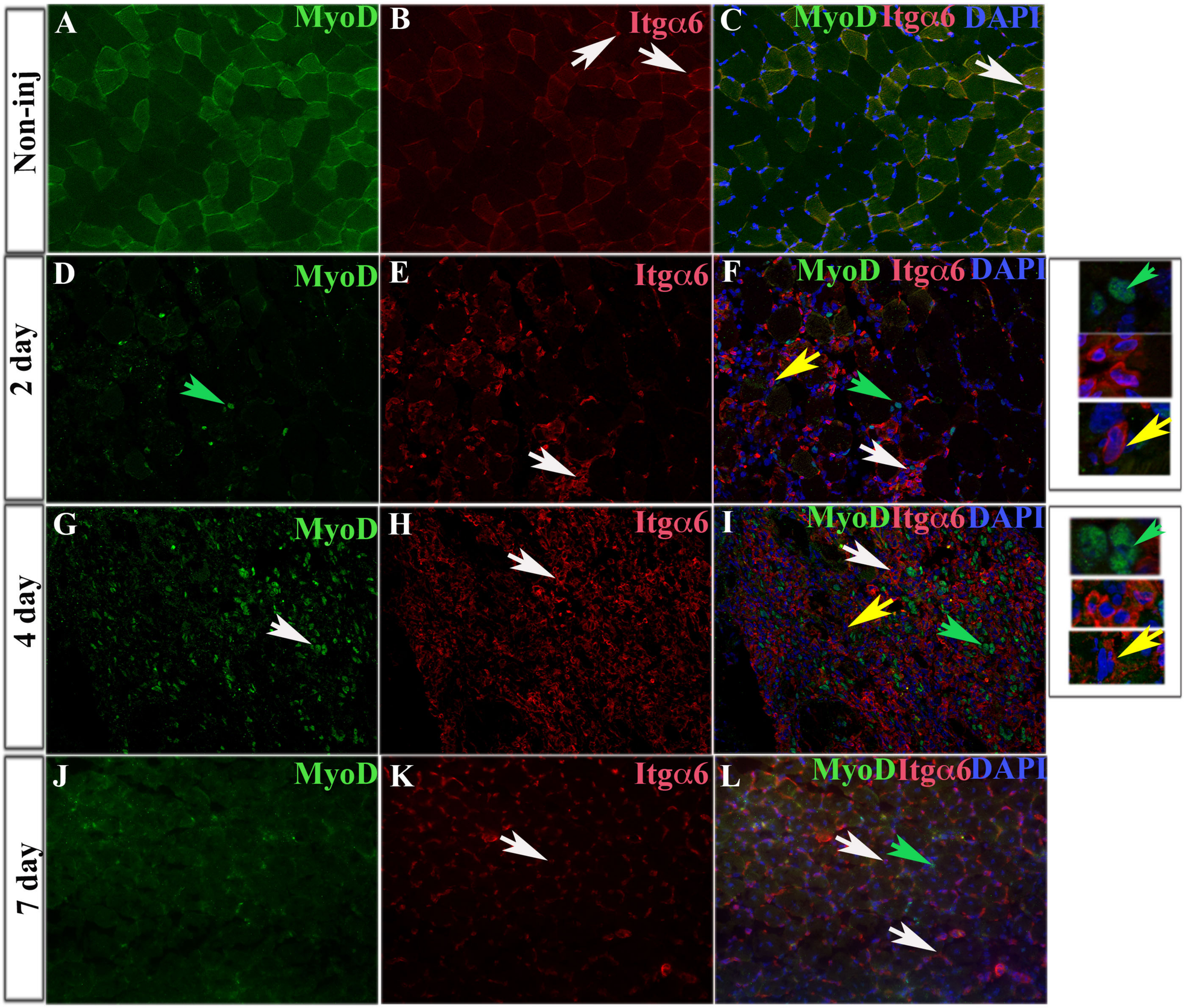


Figure 6.10: The Laminin α1 receptor Integrin α6 is expressed in non-muscle cells in cardiotoxin-injured TA muscles of C57BL/6: Immunodetection of Integrin α6 (red) and MyoD (green) in cardiotoxin-injured TA muscles of C57BL/6. At 2 day, 4 day and 7 day post-injury, MyoD-positive cells (green) are not associated with Integrin α6 (green arrows in D-F, G-I and J-L). Instead other non-muscle (interstitial cells) cells express Integrin α 6 (white arrows in F, L and I). Interestingly, some cells with elongated morphology (probably muscle cells) also express Integrin α6 (yellow arrow in F, I, L). No MyoD was observed in non-injured TA muscle (A-C), but some cells were positive for Integrin α6 (interstitial cells) in white arrows (B and C). Nuclei are counter stained with DAPI. Magnified images for 2 and 4 day post-injury are shown in inserts. Magnification 200x.

6.3 Discussion

6.3.1 Basal lamina remodeling during skeletal muscle regeneration *in vivo*

Muscle regeneration is a complex process mediated primarily by satellite cells. I have shown that Laminin α1, one of the subunits of Laminin-111, is expressed in regenerating mouse muscles. In mouse models of both acute and chronic muscle injury, Laminin α1 is expressed at the surface of activated satellite cells and is down-regulated once cells enter differentiation. In cardiotoxin-injured muscles, the percentage of satellite cells expressing Laminin α1 is higher at 2 day post-injury compared to 7 day-post injury when the regeneration process is almost completed, suggesting that Laminin α1 is required during the phase of satellite cell activation and progression into differentiation. Likewise, Laminin α1 expression is observed associated with satellite cells in *mdx* mice, a chronic muscle injury model. Such dynamic expression and distribution of Laminins has already been observed in several muscular dystrophies. The adult basal lamina is constituted of Laminin-211 and mutations in Laminin α2 causes muscular dystrophies (CMD) in mice and humans (Girgenrath et al. 2005; Guicheney et al. 1997b; L. T. Guo et al. 2003b; Wewer and Engvall 1996). In Laminin-deficient mouse muscles, it was documented that *Lama4* is up-regulated, compensating for the loss of *Lama2* (Patton et al. 1997). *Lama4* is also re-expressed in damaged muscle and is down-regulated after 10 days (Patton et al. 1999), implying dynamic changes in Laminin distribution during regeneration of diseased muscles. In humans, there was a suggestion that Laminin α1 was up-regulated in patients with congenital muscular dystrophy ($Lama2^{-/-}$) (Sewry et al. 1995). However the antibody used at that time is now known to cross-react with Laminin α5 (Tiger et al. 1997). Nevertheless, collectively these data suggest that in *Lama2*-deficent muscles an embryonic program may be re-initiated with the expression of *Lama4*, *Lama5* and perhaps *Lama1*. Supporting this possibility, injection of Laminin-111 was shown to rescue the basement membrane and muscle fibre integrity in a knockout mouse model of CMD (dy^{3k}/dy^{3k}), and transgenic

overexpression of *Lama1* into the congenital muscular dystrophy (MDC1A) model rescues the dystrophic phenotype (K. I. Gawlik et al. 2006; K. I. Gawlik and Durbeej 2010). Intramuscular or systemic administration of Laminin-111 increases the expression of Integrin α7β1 in *mdx* and restores also BM integrity (Rooney et al. 2009a). This rescue is possible because both Laminin α1 and α2 bind to the same receptors Integrin and Dystroglycan.

These studies are crucial as they suggest that in specific circumstances the Laminin composition of the BM can be dynamic, and Laminin-111 can be integrated into the adult muscle basement membrane and perform the functions of Laminin-211. However, none of the studies mentioned above addressed the mechanism of action of Laminin-111 in particular at the level of the satellite cell basal lamina. As Laminin α1 is expressed during muscle development and remains expressed until the onset of secondary myogenesis (Patton et al. 1997), I propose that remodelling of the basal lamina occurs as satellite cells become activated and may have important role in supporting satellite cells as they progress through the myogenic programme. Laminin α1 thus becomes another component of the satellite cell niche (basal lamina) that controls their behavior. As my previous studies (Chapter 5) showed that satellite cell up-regulate *Lama1* expression, it also suggest that activated satellite cells actively remodel their environment to support their own progression through myogenesis.

6.3.2 The Laminin α1 receptor, Integrin α6, is up-regulated during muscle regeneration

Satellite cell activation also leads to the up-regulation of Integrin α6 in the *ex-vivo* fibre culture system, suggesting that the remodelling of basement membrane with the incorporation of Laminin α1 could lead to alterations in the signaling events occurring downstream. This is an attractive idea as there is evidence that signaling via Integrin α6 promotes distinct outcomes than signaling via Integrin α7.

However, I found that Integrin α6 is not associated with MyoD-positive cells in exercised *mdx* mice or in CTX-injured muscle. Integrin α6 was also observed in interstitial and endothelial cells and possibly in macrophages. Further characterization

of the Integrin α6-positive cells is required but one possibility is that *in vivo* Itgα6 is transiently expressed in activated satellite cells.

The concomitant expression of Integrin α6 in the non-muscle cells (which is not observed in the non-injured muscle) suggest also a mechanism by which satellite cells may require other cells necessary for regeneration process. As, Laminin-111 preferably binds to Integrin α6, unlike Laminin-211 which binds to Integrin α7 (Nishiuchi et al., 2006), expression of Integrin α6 in non-muscle cells may provide a mechanism by which satellite cells attract non-muscle cells through the secretion of Laminin-111.

6.3.3 Localization of Laminin α1 in the vicinity of macrophages

I showed that in addition to its association with activated satellite cells, Laminin α1 is also associated with non-muscle cells *in vivo*. Further analyses showed that the these Laminin α1-positive cells detected are predominantly positive for F4/80. F4/80 is a cell surface glycoprotein is expressed by all macrophages (Villalta et al. 2011b). This suggest that Laminin α1 may be one of the cues produced by the satellite cell niche and involved in attracting macrophages to the site of injury. Indeed, skeletal muscle regeneration is always associated with inflammation, although the extent of the inflammatory response depends on the injury type and the extent of the damage (Rappolee and Werb, 1992; Tidball, 1995). Furthermore, infiltrating inflammatory cells appear to be critical to muscle regeneration (Lescaudron et al. 1999; Tidball et al. 1999). Infiltrating cells primarily include neutrophils, which are the first immune cells to invade followed by macrophages. When the muscle is damaged, signals released from degenerating fibres and local microenvironment attract macrophages to the site of injury to remove the debris. In dystrophic muscles, it has been observed that continued degeneration and regeneration cycles are associated with increased inflammation and fibrosis (Wallace and McNally 2009). Interestingly, this prolonged inflammatory response of macrophages is detrimental to muscle regeneration in DMD patients, although macrophage depletion in mice impairs muscle regeneration (Segawa et al. 2008; W. Shen et al. 2008b). This apparent discrepancy can be

explained by the fact that inflammation is highly dynamic process and results in waves of distinct populations of immune cells at each phase of muscle regeneration (Serrano and Munoz-Canoves 2010; Tidball 2005; Tidball and Villalta 2010). In particular, two populations of macrophages, M1 and M2, have been observed during the inflammatory response in injured muscles. M1 and M2 act as pro-inflammatory and anti-inflammatory macrophages respectively (Arnold et al. 2007; Mantovani et al. 2004; Wynn and Barron 2010). Indeed, the initial response to injury is driven by Th1 cytokines, which stimulate the M1 macrophage population with its interferon–γ (IFN γ) and tumor necrosis factor-α (TNFα) responses (Tidball and Villalta 2010; Villalta et al. 2009).

M1 macrophages reach their peak concentrations during initial phase of muscle injury. In contrast, M1 and M2 macrophages derived from Th2 cells mediated by Interleukin 4, 10 and 13 responses, and play an essential role in attenuating the inflammatory response (S. Gordon 2003; Mantovani et al. 2004).

Although all the macrophages express F4/80 (Villalta et al. 2011b), M1 macrophages are labeled with CD68+ , a receptor for low-density lipoproteins that activates phagocytosis by macrophages and aid them to release the required cytokines (Rabinowitz and Gordon 1991), whereas M2 are labeled with CD206+ (Linehan 2005; Villalta et al. 2009). CD206+, is a mannose receptor that binds and internalizes myeloperoxidase (MPO) at the site of inflammation and thus reduces cytotoxicity (Shepherd and Hoidal 1990).

In both acute and chronic injury models, Laminin α1 is associated with inflammatory infiltrating cells that express F4/80. As F4/80 is expressed by both M1 and M2 macrophages, it is not possible at this point to determine which macrophage population is associated with Laminin α1. However, these two macrophage populations appear sequentially after acute muscle injury (cardiotoxin injury). The M1 anti-inflammatory cells are the first to appear as early as 2h after the injury. Their levels peak between 6 and 24h and continue to increase with 2 day post injury (Arnold et al., 2007). Anti-inflammatory M2 macrophages begin appearing at 2 day post injury peak at 4 days and remain until the regeneration is complete (Hirata et al.

2003; Kohno et al. 2011; W. Shen et al. 2008b; Shono et al. 2013). A similar dynamic shift in inflammatory cells takes place in *mdx* mice. Initially M1 macrophages invade the damaged site to remove the debris (Tidball 1995). However, unlike following acute muscle injury, M2 macrophages appear simultaneously with M1 macrophages at 4 weeks of post-natal development (Villalta et al. 2009). As *mdx* mice enter the regenerative phase IL-10 levels increase, resulting in the inactivation of M1 macrophages and the reduction in muscle damage. Simultaneously, the production of anti-proliferative cytokine IL-4 promotes muscle growth (Tidball and Villalta 2010; Villalta et al. 2009).

The timing of Laminin α1 deposition observed at the surface of macrophages following acute muscle injury is consistent with the possibility that Laminin α1 is associated with M2 macrophages, as the M1 population peaks at 24hr and then declines (St Pierre and Tidball 1994). In the chronic muscle injury model (*mdx*), a 2.5 fold increase in the number of cells expressing Laminin α1 was observed in exercised *mdx* mice compared to sedentary *mdx* mice. As exercise induces an increase in the number of inflammatory cells about 1.2 fold compared to non-exercised *mdx* mice (Chapter 4), it is somewhat expected that the levels of Laminin α1 increase. However, further investigation is required to identify the specific population of macrophages that express Laminin α1 upon muscle injury regeneration, and to establish whether macrophage-associated Laminin α1 is synthesized by macrophages or by satellite cells.

6.3.4 Macrophages may be attracted by Laminin α1 to the site of muscle regeneration

To begin addressing whether Laminin α1 is produced by pro-inflammatory or anti-inflammatory macrophages, I performed RT-PCR assay on Raw 264.7, an murine macrophage cell line. Raw 264.7 cells can become M1 or M2 macrophages depending on the source of the cytokine used in the culture system (Coursey et al. 2011). Therefore, the failure to detect *Lama1* mRNA in Raw 264.7 macrophages may simply reflect that *Lama1* transcription is specific to one of the differentiated macrophage

population and not to its ancestors. However, as discussed in the chapter 5, *Lama1* transcripts were observed in both myofibre cultures and in satellite cells suggesting that satellite cells may synthesize Laminin α1, which would be secreted as Laminin-111 that subsequently would bind to macrophages. Such hypothesis is attractive as it would provide a mechanism by which satellite cells would produce an extra-cellular matrix component ultimately leading to the recruitment of cells that contribute to their niche and are required for their own activity. Indeed, several studies explored the role of macrophages beyond their role in phagocytosis during muscle regeneration. *In vitro* studies showed that macrophage-conditioned media can stimulate myogenic cells (Lesault et al., 2012). The transplantation of Pax7-positive cells together with F4/80 macrophages in *mdx* mice showed an improved regeneration capacity by increasing the number of dystrophin positive fibres (Lesault et al. 2012). These data suggest that macrophages somehow support satellite cells. Indeed, satellite cells were found to attract macrophages by chemotaxis and this attraction prevented apoptosis of satellite cells (Cantini et al. 1994; Chazaud et al. 2003). Notably, macrophages differentially affect the proliferation and differentiation of myogenic cells: M1 macrophages stimulate myogenic cell proliferation and inhibit their differentiation (Arnold et al., 2007). In contrast, a decrease in fibre size of newly formed fibres was observed when F4/80 macrophages were depleted at later stages of injury, indicating a role for M2 macrophages to promote myotubes fusion (Arnold et al. 2007; Saclier et al. 2013). Thus, macrophages play a major role in muscle regeneration through their pro and anti-inflammatory functions helping to clear damaged tissues and also through their regulation of proliferation, differentiation and fusion of satellite cells (Arnold et al. 2007; Cantini et al. 1994; Cantini et al. 2002; Lesault et al. 2012; Saclier et al. 2013). This suggests a close interaction between satellite cells and macrophages. Although the exact nature of this relationship is still not fully elucidated, I propose that remodelling of the basal lamina occurring during satellite cell activation results in the production of Laminin-111 by satellite cells and its deposition within the satellite cell basal lamina. The production of Laminin-111 by satellite cells may also contribute to attracting macrophages at the site of injury.

Consistent with this hypothesis, inflammatory macrophages isolated from 3-5 week old C57BL/6 mice express a trypsin-sensitive Laminin receptor to adhere to the vascular system during inflammation (Huard et al. 1986). Macrophages resident in regenerating muscles may also express the Laminin receptors, allowing them to bind to Laminin α1 for better attachment to the vascular basement membrane during muscle regeneration. Laminin-mediated signaling through its receptors also causes the release of granulocyte-colony stimulating factor which in turn activates the macrophages (J. Chen et al. 2003). It is thus conceivable that Laminin α1 enhances macrophage activity in a similar manner.

Thus, Laminin α1 may influence both satellite cells and macrophages during muscle regeneration. It is well documented that Laminin α1 promote myoblasts proliferation *in vitro* (Vachon et al. 1996). This suggests that indeed Laminin α1 is involved in satellite cell activation and early differentiation *ex vivo* and *in vivo.* This may also explain why the dystrophic phenotype was rescued when Laminin-111 was injected into *mdx* mice (Rooney et al. 2009a). Injection of Laminin α1 protein acts on satellite cell activation. Alternatively, Laminin α1 may also promote macrophage recruitment and activation.

To conclude, during muscle regeneration alterations in the satellite cell basal lamina during muscle regeneration may be concomitant with the secretion of Laminin-111 by satellite cells and its binding to the surface of anti-inflammatory macrophages.

This may provide a mechanism for satellite cells and macrophages to support each other and cooperatively regenerate the muscles.

Chapter 7

Discussion

7.1 Introduction

My study explored a poorly understood process, the satellite cell niche composition. It uncovered the existence of a remodelling of the satellite cell basement membrane during satellite cell activation and during muscle regeneration. Specifically, I showed that the adult muscle satellite cell basal lamina exhibits a new composition, distinct from the rest of the myofibre, at the time satellite cells are activated. In particular, this remodeling leads to the deposition of Laminin-α1, a Laminin subunit normally expressed during embryonic development and associated with muscle progenitor cell determination and differentiation. I have also shown that the up-regulation of Laminin-α1 is concomitant with the expression of the preferred Laminin-111 receptor, Integrin α6β1. Finally, I found that Laminin α1 is also expressed at the surface of muscle-resident macrophages, which are known to play essential role during muscle regeneration. Together, these observations, suggest a model (Figure 7.1) whereby the remodelling of the satellite cell basement membrane within the niche accompanies and supports activation and may help recruiting macrophages during adult skeletal muscle regeneration. In this chapter, I discuss the significance of these studies and the future perspectives.

7.2 Model for basement membrane remodeling

Satellite cells are responsible for repairing muscles following an injury or in degenerative diseases such as muscular dystrophies. Although great advances have been made in our understanding of the molecular players and signaling pathways controlling satellite cell activity, one of the outstanding questions in the field is that of the potential role of the extra-cellular matrix as a component of the satellite cell niche. To investigate this, I have re-visited the composition of the basement membrane associated with satellite cell during the various stages of adult myogenesis.

Adult stem cell niches including the basement membrane are known to protect stem cells from depletion and uncontrolled growth (Scadden 2006). This is suspected to be also true for satellite cells (Boonen and Post 2008). There has been recently an

increasing interest in the role of extracellular matrix components (ECM) in satellite cell function. For instance, a recent study showed that collagen VI is expressed by satellite cells and this expression is differentially regulated during satellite cell quiescence and activation of SC in muscle regeneration (Urciuolo et al. 2013). Likewise, Bentzinger and colleagues have shown that committed myogenic satellite cells express elevated levels of fibronectin in their niche following muscle injury (Bentzinger et al. 2013b). They also suggested that in asymmetrically dividing satellite cells, Fibronectin is dynamically and temporally expressed in the daughter cell that has a myogenic potential, and interacts with Syndecan-4 to induce Wnt signaling, which enhances further the symmetric expansion of myogenic satellite cells (Bentzinger et al. 2013b). Together with the data reported in this thesis, these two studies reveal the existence of a remodeling of the satellite cell basement membrane remodeling during satellite cell activation and its importance in supporting satellite cell progression through the myogenic program. Consistent with these studies, I propose a model (Fig 7.1) whereby the Laminin-211-containing basement membrane overlying quiescent satellite cells is remodeled soon after satellite cell activation to incorporate Laminin-111, a Laminin known to be normally associated with the embryonic muscle progenitor cells (C. Anderson et al. 2009; Patton et al. 1999). As disruption in the Laminin-111-containing basement membrane is associated with defects in muscle progenitor cell differentiation and migration in the embryo (C. Anderson et al. 2009; Bajanca et al. 2006), it is highly likely that the re-expression of Laminin-111 in the regenerating adult muscle serves the function of supporting satellite cells during the reiteration of the embryonic myogenic program. In agreement with this, it has been shown that satellite cells maintain Pax7, MyoD and Myogenin expression *in vitro* when cultured in Matrigel, which is mainly made up of Laminin-111 (Grefte et al. 2012). The idea that stem cells may remodel their extra-cellular matrix environment in order to support their activity has already been suggested in other contexts. For instance, a recent study demonstrated that cancer stem cells induce the secretion of Periostin, an extra-cellular matrix protein often found in stem cell niches, by stromal fibroblasts to facilitate their implantation at secondary tumor sites

(Malanchi et al. 2012). In another study, stem cells residing in the hair follicle bulge were shown to remodel their basement membrane to incorporate Nephronectin, which through its signaling via Integrin $\alpha 8\beta 1$, favors the anchoring of arrector pili muscles to the bulge (Fujiwara et al. 2011). However, this is the first study to demonstrate that satellite cells may directly remodel their own basement membrane to facilitate their activation and progression through the myogenic programme. Such process may explain why satellite cells grown on Laminin-111 are more efficient at repairing dystrophic muscles than satellite cells grown on Fibronectin (Ross et al. 2012) and why Laminin-111 therapy has been proposed as a cure for muscular dystrophy (Goudenege et al. 2010).

The mechanism by which this remodeling occurs remains obscure. However, recent data demonstrated that matrix metalloproteases (MMP's), which are enzymes that hydrolyze ECM components are up-regulated upon satellite cell activation (Alexander Raven, unpublished observations). Specifically, MMP-9 is found associated with activated satellite cells in the *ex-vivo* muscle fibre culture system (Alexander Raven, unpublished observations). MMP-9 expression at the time satellite cells are activation and differentiation provides a potential mechanism through myogenesis. Consistent with this possibility, inhibition of MM-9 activity resulted in a delay in satellite cell activation, suggesting that remodeling of the satellite cell basement membrane is essential for satellite cell activation (Alexander Raven, unpublished observations). I thus propose that following satellite cell activation, MMP-9 expression initiates the partial degradation of the ECM overlying satellite cells to facilitate satellite cell migration and the deposition of Laminin-111 into the satellite cell niche, further promoting satellite cell proliferation and differentiation.

Such role of MMPs is consistent with previous studies showing that MMPs facilitate satellite cell migration and differentiation (W. Wang et al. 2009) and that MMP-9 expression increases during myoblast proliferation, whereas MMP-2 is up-regulated during myoblast fusion (Zimowska et al. 2008). Increased activity of MMP-2 and MMP-9 also results in inflammation and necrosis in *mdx* mice (Bani et al. 2008; Fukushima et al. 2007; Kherif et al. 1999). Moreover, emerging data has suggested

that knocking out MMP-10 function in *mdx* is deleterious to muscle regeneration (Bobadilla et al. 2014).

7.3 Satellite cell basement membrane remodeling may be associated with a novel receptor distribution

Remodeling of the basement membrane results in a "new" niche with a distinct Laminin content from that of myofibre basal lamina or that of quiescent satellite cells. In muscular dystrophy mouse models, such as in Laminin-α2 deficient mice, Laminin α4 and α5 are up-regulated but cannot rescue the dystrophic phenotype. In contrast, the dystrophic phenotype is rescued when Laminin-111 is injected before cardiotoxin mediated-injury (Van Ry et al. 2013). Laminin-111 treatment resulted in an increase in Integrin-α7, Pax7 and Myogenin expression at 4 day post-injury, suggesting that in Laminin-α2 deficient mice, Laminin-111 binds to Integrin α7β1 receptor and reduces the pathology (Van Ry et al. 2013). In *mdx* mice, although the dystrophin-associated glycoprotein complex (DGC) is not stabilized due to the absence of Dystrophin, Laminin-111 can rescue the dystrophic pathology by binding to Integrin α7β1 (Nishiuchi et al. 2006). Moreover, transgenic over-expression of Integrin α7 increases satellite cell activity and fusion following a single bout of eccentric exercise (Lueders et al. 2011), suggesting a role for Integrin α7β1 in satellite cell signaling. Further, transplantation studies suggest that myoblasts injected with Laminin-111 restore muscle integrity (Goudenege et al., 2010). Overall, these data suggest a possible role of Laminin-111 in muscle regeneration.

As expected, Integrin-α7 knockout mice exhibit a similar phenotype to Laminin-α2 deficient mice and exhibit reduced satellite cell activation and differentiation (Rooney et al. 2009b). Surprisingly, injection of Laminin-111 protein also rescues the dystrophic phenotype in Integrin-α7 knockout mice. This suggests that Laminin-111 binds to other Integrin receptors in the absence of Integrin α7. Supporting this possibility, I have established that activated satellite cells express Integrin α6β1 in the *ex-vivo* fibre culture system. Thus, Laminin-111 may signal through Integrin α6β1, as

it has been shown that Laminin-111 binds preferentially to Integrin α6β1 receptor over Integrin α7β1 (Nishiuchi et al. 2006). The finding that Laminin-α1 expression is associated Integrin α6β1 expression *ex-vivo* suggests a possible signaling role for the Laminin-111/ Integrin α6β1 axis in controlling satellite cell behavior (chapter 5). Supporting this, Integrin α6 was found to be up-regulated in newly formed fibres in in *dy/dy* mice following crush-injury model (Sorokin et al. 2000a). Other studies reported also that porcine muscle stem cells up-regulate *ITAG6*, which encodes Integrin-α6 when grown on Laminin and this resulted in enhanced Wnt signaling (Wilschut et al. 2010). An analogy may again be drawn between the observations reported here in satellite cells and those previously reported by others in embryonic myogenic progenitor cells. Indeed, α6β1 is the first Laminin receptor to be expressed at the surface of embryonic myogenic progenitor cells (Bajanca et al. 2006) and blocking signaling through this Integrin in mouse embryos results in aberrant differentiation of muscle progenitor cells (Bajanca et al. 2006). This suggests that the incorporation of Laminin-111 into the satellite cell basement membrane may favor signaling through Integrin α6β1 and the progression of satellite cells through the adult myogenic program. Such possibility is reminiscent of a similar observation in the human heart following ischemic cardiomyopathy, which showed that the re-expression of Laminin-111 associated with an increased expression of Integrin α6β1 was involved in protecting cardiomyocytes against apoptosis and in promoting cardiomyocytes migration (Castaldo et al. 2008). Thus, the re-expression of Laminin-111 together with Integrin α6β1 may represent a general mechanism associated with the reiteration of embryonic programs during tissue repair.

7.4 A potential interaction between macrophages and satellite cells via basement membrane components

As mentioned earlier, the normal adult skeletal muscle has a very limited number of macrophages, which mostly located within the epimysium, the connective tissue that surrounds the whole muscle. However, within a few hours of muscle damage,

neutrophils followed by macrophages invade the muscle tissue, most likely through crossing the endothelial barrier of blood vessels. While the role of neutrophils remains uncertain, several studies have demonstrated unequivocally that infiltrating macrophages are essential for muscle regeneration (Cantini et al. 1994; Tidball and Villalta 2010). Furthermore, macrophages have been reported to improve the survival, migration and proliferation of injected myogenic cells in *mdx* mice (Lesault et al. 2012). However, the signals that lead to the recruitment of macrophages to the injured muscle are not fully understood, although previous studies have already suggested that satellite cells and satellite cell-derived myoblasts may secrete factors that stimulate macrophage recruitment (Chazaud et al. 2003). So far, the reciprocal attraction of activated satellite cells and macrophages is thought to involve mainly chemokines and chemokine receptors (Griffin et al. 2010). In this thesis (chapter 6), I have shown a possible alternative mechanism for the reciprocal attraction between satellite cells and macrophages in adult skeletal muscles via the synthesis of extra-cellular matrix components. I showed that Laminin α1 synthesized by satellite cells is deposited at the surface of macrophages in *mdx* mice as well as following cardiotoxin-mediated injury. Given that macrophages appear to express the Laminin-111 receptor Integrin α6β1 but not *Lama1* (see Chapter 6 and (Zhang et al. 2012), this suggests that satellite cells may attract macrophages at the site of injury through their secretion of Laminin-111, which binds to Integrin α6β1 at the surface of macrophages. Thus, upon injury satellite cells synthesize Laminin-111, which attracts macrophages to the site of injury. Satellite cells secrete also MMP-9, which facilitate the incorporation of Laminin-111 into the satellite cell basement membrane, and the progression of satellite cells through the myogenic programme via signaling through Integrin α6β1 expressed at the surface of activated satellite cells. Macrophages also secrete MMP-9 (Carmeli et al. 2004) and therefore can further promote the satellite cell basement membrane remodeling. Thus, the positive feedback loop that operates between macrophages and satellite cells during muscle injury is likely to involve ECM components and enzymes that mediate ECM remodeling.

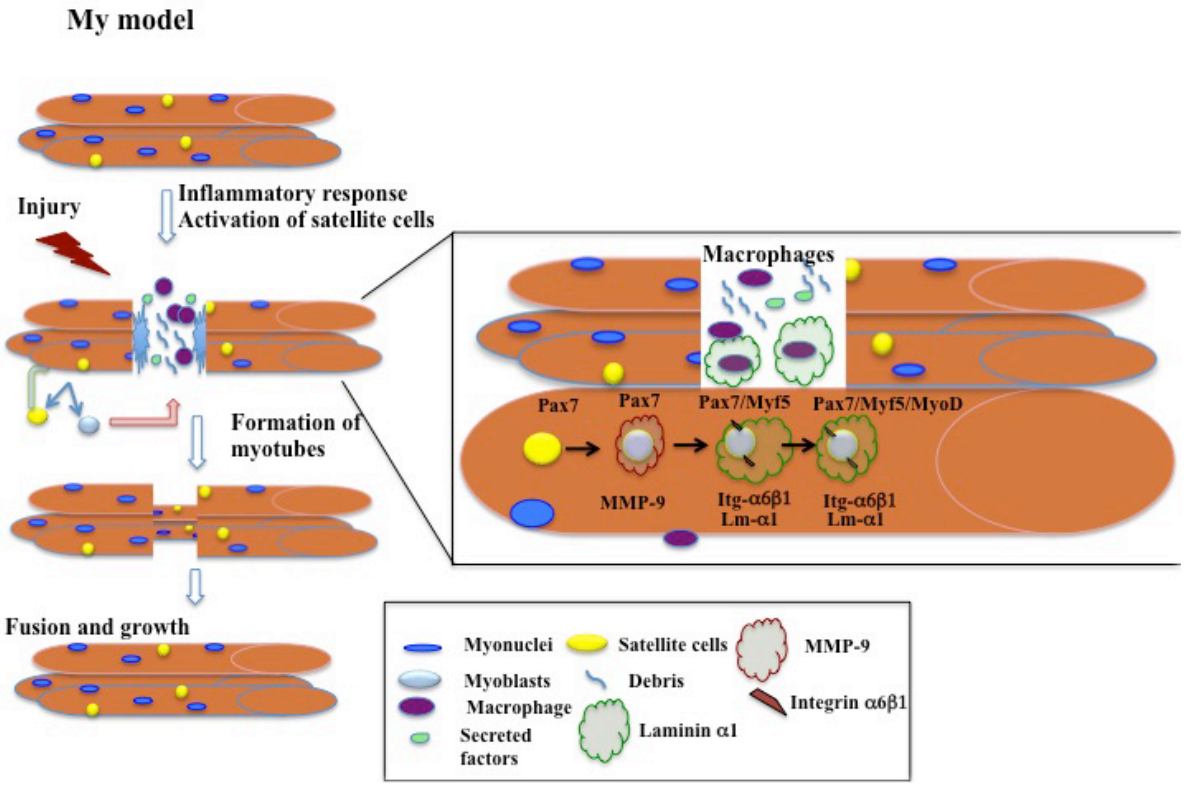


Figure 7.1: ***Basal Lamina remodelling during muscle regeneration:*** Injury to muscle initiates muscle regeneration with the infiltration of macrophages and satellite cell activation. During satellite cell activation basal lamina is remodeled in part through the activity of matrix metalloproteinases MMP-9. This remodelling allows for Laminin-111 incorporation into the satellite cell basement membrane and its signaling to satellite cells via Integrin α6β1. Laminin-111 is also associated with macrophages and this may help the recruitment of macrophages to the site of injury and their support of satellite cell activity.

7.5 Future directions

Data presented in this thesis report the deposition of Laminin-111 in the satellite cell basement membrane at the time of their activation. The requirement of this remodeling for satellite cell activity remains to be demonstrated. It would be interesting to examine the defects in skeletal muscle growth and repair in adult mice lacking Laminin-α1 in their skeletal muscles. This can be achieved by using the conditional knock-out allele of *Lama1* (Alpy et al. 2005). *Lama1*$^{flox/flox}$ mice, can be crossed to a tissue-specific Pax7-Cre(ERT2) line, which express tamoxifen-inducible form of Cre under the control of Pax7 in satellite cells (available through Jackson

Labs). A prediction would be that in the absence of Laminin-111, satellite cells fail to recruit macrophages efficiently to the injury site and are delayed in their activation. Similarly, experiments aiming at blocking Integrin α6–mediated signaling would address the signaling function of Laminin-111 in satellite cells. Finally, the exogenous addition of Laminin-111 protein to myofibres in the ex-vivo culture system would allow me to test whether increasing Laminin-111 levels is sufficient to recruit additional satellite cells to the myogenic programme.

The association of Laminin–111 with macrophages should be further explored. In particular, it is required to identify the specific macrophage population (M1 or M2) that expresses Laminin-α1.

Finally, such study should be further expanded to re-examine the expression of all Laminin subunits during muscle regeneration and to further explore the involvement of matrix metalloproteinases in the remodeling process taking place during muscle regeneration.

7.6 Concluding remarks

The ECM function was until recently thought to mainly provide an anchoring point to stem cells that allow cells to be polarized and undergo asymmetric cell division. Consistent with this idea, asymmetric cell division is disrupted and symmetric cell division is favored when the ECM is abnormal or upon complete loss of ECM (Lu et al. 2012). However, other roles of the ECM in stem cell activity are poorly explored. In particular, an outstanding question that remains in the stem cell field is how ECM components regulate stem cell behavior. In skeletal muscles, it has been shown that preserving the niche significantly enhances transplanted satellite cell ability to repair muscle in irradiated dystrophic mice (Boldrin et al. 2012). Consistent, with this the present study provides novel insights into a possible role for the basement membrane and Laminin-111 in the control of satellite cell activity. Further, this basal lamina protein may mediate an interaction between satellite cells with macrophages. This is an important finding as it suggests that the basement membrane has now not only a

structural function but also regulates stem cell function. This function is mediated by bi-directional signaling following Integrin-mediated cell-cell or cell-matrix contacts. Remodelling of such adult stem cell niche during injury has also been observed in other tissues. For instance, in chronic liver injury, ECM remodelling occurs resulting in fibrosis resolution and Laminin deposition which is likely to play a role in hepatic progenitors expansion and proliferation (Kallis et al. 2011). As, the basement membrane can also selectively bind and release growth factors, the remodeling of the basement membrane may also provide a means to sequester growth factors in the niche in preparation for specific signaling events controlling stem cell behavior. For instance, in *Drosophila* the association of intestinal stem cells with the basement membrane promotes their asymmetric cell fate by maintaining higher levels of BMP signaling in self-renewing intestinal stem cells and not in differentiating daughter cells (Tian and Jiang 2014). Thus, the ECM is an essential component of the adult stem cell niche, and is required not only for structural support but also to instruct and maintain stem cell behavior by modulating a cellular signals in a variety of organ systems.

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